Analysis of Odor-active components, Volatile Constituents, Antioxidant Activity and Bioactivity of *Melodorum fruticosum* flower

By

Dr. Patcharee Pripdeevech

School of Science

Mae fah Luang University

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Dr. Patcharee Pripdeevech

บทคัดย่อ

องค์ประกอบที่มีกลิ่นในดอกลำดวนถูกสกัดด้วยเทคนิคการสกัดด้วยวัฏภาคของแข็งในระดับจุลภาคโดยใช้ไฟเบอร์ที่แตกต่างกัน 3 ชนิดคือ โพลีไดเมธิลไซลอกเซน (พีดีเอ็มเอส), โพลีไดเมธิลไซลอกเซน-ไดไวนิลเบนซีน (พีดีเอ็มเอส-ดีวีบี) และคาร์บอกเซน-โพลีไดเมธิลไซลอกเซน (ซีเออาร์-พีดีเอ็มเอส) หลังจากนั้นวิเคราะห์องค์ประกอบที่มีกลิ่นที่สกัดได้ด้วยเครื่องแก๊สโครมาโทกราฟี-แมสสเปกโทเมตรี (จีซี-เอ็มเอส) พบองค์ประกอบที่มีกลิ่นซึ่งทราบโครงสร้างแล้วจำนวน 55 องค์ประกอบ เมื่อใช้ไฟเบอร์ที่แตกต่างกัน จากผลการทดลองพบว่าไฟเบอร์พีดีเอ็มเอส-ดีวีบีมีประสิทธิภาพในการสกัดองค์ประกอบที่มีกลิ่นได้ดีที่สุด โดยพบองค์ประกอบหลักที่มีกลิ่นในปริมาณสูงได้แก่ β-phellandrene, *Z*-β-ocimene, linalool, δ-cadinene และ *p*-methylanisole ตามลำดับ หลังจากนั้นสกัดองค์ประกอบทางเคมีในดอกลำดวนด้วยเทคนิคการกลั่นด้วยไอน้ำและการแช่ในตัวทำละลายต่างๆ ได้แก่ เฮกเซน, ไดคลอโรมีเทน และเมทานอล หลังจากนั้นวิเคราะห์องค์ประกอบสารระเหยดอกลำดวนที่ได้จากการสกัดต่างๆด้วยเครื่อง จีซี-เอ็มเอส พบองค์ประกอบที่ทราบโครงสร้างแล้วจำนวน 88 องค์ประกอบ พบองค์ประกอบคือ phenyl butanone, linalool, benzyl alcohol, α-cadinol, globulol และ viridiflorol พบว่าสารสกัดที่ได้จากการแช่ดอกลำดวนในตัวทำละลายไดคลอโรมีเทนนั้นมีฤทธิ์ในการฆ่าเชื้อราต่างๆได้อย่างมีประสิทธิภาพมากกว่าสารสกัดที่ได้จากการแช่ดอกลำดวนในตัวทำละลายเฮกเซน, น้ำมันหอมระเหยดอกลำดวน และจากการแช่ดอกลำดวนในตัวทำละลายเมทานอล ตามลำดับ นอกจากนี้พบว่าสารสกัดที่ได้จากการแช่ดอกลำดวนในตัวทำละลายไดคลอโรมีเทนนั้นมีค่าไอซีฟิบตี้ที่ต่ำคือ 87.6 ไมโครกรัม/มิลลิลิตร ในขณะที่สารสกัดอื่นๆ มีค่าไอซีฟิบตี้ที่สูงซึ่งอยู่ในช่วง 100.13 ถึง 194.50 ไมโครกรัม/มิลลิลิตร

Abstract

The odor volatilesof *M. fruticosum* flowers were extracted by solid-phase microextraction (SPME) with three different fibers, polydimethylsiloxane (PDMS), polydimethylsiloxane-divinylbenzene (PDMS-DVB) and carboxen-polydimethylsiloxane (CAR-PDMS). The extracted volatiles were identified by using gas chromatography-mass spectrometry (GC-MS). Fifty-five odor components were identified amongst the SPME extraction with three different fibers. PDMS-DVB was evaluated to be the most efficient fiber in trapping the key scents. The results indicated that β-phellandrene *Z*-β-ocimene, linalool, δ-cadinene and *p*-methylanisole were of high levels in the SPME extract. The chemical compositions of *M. fruticosum* flowers were then extracted by hydrodistillation and maceration with hexane, dichloromethane and methanol, separately. The chemical compositions of all extracts were investigated by GC–MS. Eighty-eight volatile components were identified. Phenyl butanone, linalool, benzyl alcohol, α-cadinol, globulol and viridiflorol were found to be the major components, respectively. The dichloromethane extract of *M. fruticosum* flowers played major role as the remarkable fungicide according to their inhibition action against all tested pathogens followed by hexane extract, essential oil and methanol extract, respectively. Besides, the dichloromethane extract were also evaluated to be superior to all extracts tested with an IC_{50} value of 87.6 µg/ml whereas other extracts showed their IC_{50} values ranged from 100.13 to 194.50 µg/ml.

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ABBREVIATIONS AND SYMBOLS

C_8	=	octane
C_{22}	=	docosane
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
ml	=	milliliter
h	=	hour
°C	=	degree celsius
v/v	=	volume/volume
GC-MS	=	Gas chromatography-Mass Spectrometry
m	=	meter
mm	=	millimeter
min	=	minute
eV	=	electron volt
m/z	=	mass/charge
UV/vis	=	UV/visible
µg	=	microgramme
IC_{50}	=	50% Inhibition concentration
w/w	=	weight/weight

CHAPTER 1
INTRODUCTION

1.1 Introduction and literature reviews

Melodorum fruticosum belongs to the family of Annonaceae, commonly known as Devil Tree, White Cheesewood and Lamduan (Thai) (Rujjanawate et al., 2008). This is widely distributed throughout Indo-China and Thailand containing mixed deciduous forests and dry evergreen forests in central and eastern parts of the country. *M. fruticosum* flowers are solitary, pale yellow and scented. Flowers bloom in April for approximately two months whereby the flowers produce their finely scented perfume, spreading it over great distances. The essential oil of *M. fruticosum* flowers was used in the aromatherapy and as traditional medicine in Thailand (Rujjanawate et al., 2008). *M. fruticosum* is a medium-size tree, tall 5-20 meters. The stem is brown shell split rough and arranges alternate edge parallel shape, 2.5-4.0 centimeter width, 5.0-11.5 centimeters length. Its fruit is sweet and keep sour with green color when ripe is black color. Its flower consists of six petals, three petals spread outer and three petals are inner. The flowers have 0.6 centimeter diameter and its character are sphere. However, there is no report describing the antifungal and antioxidant activities of *M. fruticosum* flowers.

Odor components are one of the essential properties emitted from flowers. They are often pleasant to the sensory system of humans. The relative contributions of specific odor volatile compounds to the flavor of flowers have been studied by many researchers. Studies of odor volatiles have led to the synthesis of new odor components used in fragrance applications. The identification of these components play a role in aromatic characteristics and flavors of the flowers while also serve as a significant starting point. Gas chromatography-mass spectrometry (GC-MS) is a useful technique that has been employed to investigate the volatile components of flowers (Wang et al., 2009; Li et al., 2006; Bertrand et al., 2006; Flamini et al., 2007). Recently, solid-phase microextraction (SPME), introduced by Pawliszyn et al. (Arthur, 1990) is a fast, efficient, solvent-free alternative to conventional sample extraction techniques. The analytes establish

equilibrium among the sample matrix, the headspace above the sample, and a polymer-coated fused fiber. These are then desorbed from the fiber to an injection port of gas chromatograph for analysis whereby the number of extracted components onto the fiber depends on the partition of analytes from a sample into a SPME fiber. Due to its sensitivity, reproducibility and high concentration capability, SPME has been widely used for extracting the volatile components from flowers and other parts of plants (Wang et al., 2009; Barboni et al., 2009; Pinho et al., 2009).

Most diseases in plant are caused from various pathogens including fungi, nematodes, bacteria, and viruses (Montesinos, 2003). Fungi are the major pathogen and source of many diseases of plants. Pathogenic fungi also could decrease products in numerous economically important crops (Fletcher et al., 2006). Synthetic fungicides are the main solutions for fungal pathogenic diseases. Conversely, resistance of fungicides by various plant pathogenic fungi is the critical causes of poor disease control of agriculture (Steffens et al., 1996; Aguin et al., 2006; Ishii, 2006). Moreover, the use of synthetic fungicides could be dangerous to humans and environment. Nowadays, tendency of using the synthetic fungicides is reduced continuously. Thus the substitutions of their materials by natural products have been interested significantly in natural product researches because they are safe for humans and the environment (Duke, 1993; Daayf et al., 1995). Alternative natural pesticides are need for development in the control of pathogenic fungal diseases in plants. Essential oils and many extracts from various parts of plants are one of the most promising groups of natural compounds which may be developed for using as natural fungicides substituted the synthetic pesticides due to presences of terpene constituents with different functional groups in the oils. There are many evidences that reported the essential oils and various extracts of plants were employed as fungicides (Chang et al., 2008; Manohar et al., 2001; Yen et al., 2008). Moreover, they were applied as antimicrobial (Xianfei et al., 2007; Ünlü et al., 2002; Shunying et al., 2005) and antioxidant compounds (Politeo et al., 2007; Tepe et al., 2007; Kelen et al., 2008).

1.2 Scope of study

The purpose of this present study was to identify the volatile constituents released from fresh *Melodorum fruticosum* flowers which were obtained by the extraction of SPME with three different fibers. The chemical compositions of *M. fruticosum* flowers essential oil were also extracted by hydrodistillation and maceration with various solvents. All constituents in all extracts were identified by GC-MS. The antifungal and antioxidant activities of essential oil and various extracts of *M. fruticosum* flowers were then investigated and discussed.

1.3 Expected output

Value knowledge consisting odor chemical constituents, bioactivity and antioxidant activity of *M. fruticosum* obtained from various extracts as well as international publication. These results may be applied as alternative natural pesticide for using to screen and develop the natural fungicides in the biocontrol of many agricultural plant pathogens.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material and chemicals

Aerial portions of *M. fruticosum* flowers at the flowering stage were collected from Mae Fah Luang University, Chiang Rai Province located in the northern part of Thailand in April 2009. Voucher herbarium specimens (QBG No.41461) of the plant were identified and deposited at the Queen Sirikit Botanical Garden, Mae Rim, Chiang Mai, Thailand.

2.2 Solid-phase microextraction (SPME)

The SPME apparatus with a SPME fiber assembly holding 1.0 cm fused-silica fibers was purchased from Supelco, Bellefonte, PA, USA. A 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB) and 75 μm carboxen-polydimethylsiloxane (CAR-PDMS) fiber were selected to extract the odor volatiles of *M. fruticosum* flowers in this study. All fibers were mounted in the manual SPME holder and preconditioned for 2 h in a GC injection port set at 250 °C. For each extraction, 25 g of fresh *M. fruticosum* flowers were picked and immediately placed into a 250 ml headspace bottle sealed with a silicone septum and a Teflon cap. The sample bottle was equilibrated at room temperature around 25 °C for 30 min. By insertion through the septum of the sample bottle, the fiber was then exposed to the sample headspace for 30 min prior to desorption of the volatiles into the splitless injection port of the GC-MS instrument for 30 min. Extraction of each fiber was performed in triplicate.

2.3 Gas chromatography-mass Spectrometry (GC-MS)

The volatile constituents of fresh *M. fruticosum* flowers obtained from the SPME extracts with three fibers were analyzed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA). It was equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm i.d., film

thickness 0.25 μm; Agilent Technologies, USA) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 50 °C and then increased by 2 °C/min to 180 °C. The injector and detector temperatures were 250 and 280 °C, respectively. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. EI mass spectra were collected at 70 eV ionization voltages over the range of *m/z* 29-300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Identification of volatile components was performed by comparison of their Kovát retention indices, relative to C_8-C_{22} *n*-alkanes, and comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST 98 databases. Component relative quantity of all samples was determined directly from the GC peak areas.

2.4 Extraction of essential oil

The extraction was carried out in a modified Likens-Nickerson apparatus for 5 h to obtain the essential oil. After extraction, the distillate in a conical flask was dried over anhydrous Na_2SO_4 and concentrated using vacuum rotary evaporator. The oil obtained was kept in a sealed vial until required with a yield of 0.59% (w/w).

2.5 Preparation of the crude extracts

The dried flowers of *M. fruticosum* were blended to powder and then 50 g of these samples was macerated individually with 200 ml of hexane, dichloromethane and methanol. Each extraction was performed at room temperature for 10 days. All extracts were filtered through filter paper and concentrated under vacuum using a rotary evaporator. All crudes extracted were stored at 4°C for further analysis. The extracts obtained yields 0.91%, 4.83% and 3.14% for hexane, dichloromethane and methanol extracts, respectively.

2.6 Analysis of antifungal activity of all extracts

The plant pathogenic fungi used in this study were *Collectotrichum asianum* CaMBK, *Collectotrichum siamense* Cach4LB, *Collectotrichum gloeosporioides* Cglg1CM, *Collectotrichum fruiticola* Cap4CM, *Collectotrichum acutatum* BRIP28519, *Collectotrichum acutatum* CaM1VT, *Collectotrichum* sp. CaRA6VT, *Tricoderma reesei* TISTR3080 and *Lasiodiplodia theobromae* MFU. The antifungal activity of all extracts was determined by using agar disc diffusion method. Each sample was serially diluted with dimethyl sulfoxide (DMSO) by using the twofold dilution method (Murray et al., 1995) to final concentrations of 1000, 500, 250, 125 and 62.5µg/ml, respectively. The pathogenic fungi were culture on potato dextrose agar (PDA) media and incubated at 30 °C for 1 week. The pathogenic fungi were cut with diameter of 5 mm from 1-week-old cultures on PDA media and placed on the centre surface of the sterilized plates and incubated at 30 °C for a week. The plates were then impregnated with various concentration of all samples and placed on the surface of the agar plate after that incubated at 30 °C for a week. The growth inhibition of each fungal strain was calculated as the percentage of inhibition of a radical growth relative to the control following formula: Percentage of inhibition (%) = 100 × [(1- radical growth of treatment (mm)/radical growth of control (mm)]. All experiments were performed in triplicate.

2.7 Antioxidant activity

2.7.1 DPPH radical scavenging assay

The radical scavenging abilities of *M. fruticosum* flowers oil and various crude extracts were analyzed comparing to a standard butyl hydroxyl toluene (BHT) and α-tocopherol based on the reaction with 2,2-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$). This method was evaluated by a spectrophotometric method followed the modified method of Blois (1958). One milliliter of various concentrations of the each sample in methanol was added to 1 ml of a 0.003% methanol solution of DPPH and the reaction mixture was shaken vigorously. The tubes were allowed to stand at room temperature for 30 min. Each reaction mixture was then placed in the cuvette holder of the Perkin Elmer-

Lamda 25 UV/Vis spectrophotometer and monitored at 517 nm against blank which used methanol as the baseline correction. The scavenging ability was calculated as follows: Scavenging ability (%) = 100 × [Absorbance of control – Absorbance of sample/Absorbance of control]. The antioxidant activity of all samples was expressed as IC_{50} which was defined as the concentration (in µg/ml) of oil required to inhibit the formation of DPPH radicals by 50%. The experiment was carried out in triplicate.

2.7.2 Determination of total phenolic contents

Total phenolic content of all samples obtained from *M. fruticosum* flowers was determined using the Folin–Ciocalteu reagent according to the modified method of Singleton and Rossi (1965) using gallic acid as standard. The oil solution (0.2 ml) was mixed with 1.0 ml of Folin–Ciocalteu reagent, 1.0 ml of an aqueous solution of 7% Na_2CO_3 and 5.0 ml of distillated water, respectively. Then, the mixture was vortexed vigorously. The reaction mixtures were allowed to stand for 30 min before absorbance at 765 nm was measured. The same procedure was also applied to the standard solutions of gallic acid. The calibration equation for gallic acid was $y = 0.00515x-0.00400$ ($R^2 = 0.999$) where y is the absorbance and x is the concentration of gallic acid in mg/ml.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Volatile odor compounds analysis by SPME-GC-MS

The different odor volatiles were detected among three SPME fibers with the SPME method. The affinity of SPME fibers extracting the analytes was based on the "like dissolve like" concept and thickness of selected fibers. Three different SPME fibers of PDMS, PDMS-DVB and CAR-PDMS were selected to extract the odor constituents of *M. fruticosum* flowers. The results are shown in Table 1. Using PDMS fiber, 37 odor constituents were identified. The majority of the constituents, approximately 85.23%, were represented with the dominant components of β-phellandrene (8.98%), *p*-methylanisole (7.02%), δ-cadinene (4.18%), germacrene B (3.81%) and bicyclogermacrene (3.62%). Forty-six odor constituents representing 83.59% were identified by PDMS-DVB fiber. The principal scent volatiles were found to be β-phellandrene (17.19%), *Z*-β-ocimene (9.07%), linalool (7.38%), δ-cadinene (4.15%) and *p*-methylanisole (3.01%). Using the CAR-PDMS fiber, 26 components (80.91%) were identified with the major scents being, *p*-methylanisole (18.04%), *Z*-β-ocimene (10.42%), β-phellandrene (7.24%), linalool (4.52%) and α-phellandrene (4.09%). The volatiles extracted by the SPME method with PDMS-DVB and CAR-PDMS fibers showed higher amounts of monoterpenes than sesquiterpenes compared to those extracted by PDMS fiber. As can be observed, the PDMS-DVB fiber has much better extraction efficiency than PDMS and CAR-PDMS fibers, respectively. Most constituents extracted by PDMS-DVB were superior to the other fibers under the same conditions due to the intermediate polarity of PDMS-DVB fiber. It demonstrated the best technique for trapping the key scent constituents of *M. fruticosum* flowers with different polarities whereby most components were a group of hydrocarbon and oxygenated monoterpenes. PDMS fiber is recommended for extraction of only non polar components which showed better extraction efficiency than the CAR-PDMS fiber which extracted both polar and non polar compounds due to a mutual potential effect of adsorption and distribution to the

stationary phase (Pawliszyn, 1999; Kataoka and Pawliszyn, 2000). The different fibers have significant effects on percentage composition of the odor volatiles in fresh *M. fruticosum* flowers obtained by SPME methods. As can be seen, *p*-methylanisole showed the higher content by the SPME extract with CAR-PDMS fiber than those obtained from both PDMS and PDMS-DVB fiber whereas β-phellandrene provided the conversed results. It is noted that the SPME technique is more sensitive for volatile components which played a significant role as the key scent in *M. fruticosum* flower.

Table 1 Chemical constituent of *Melodorum fruticosum* flowers with the percentage of content extracted by SPME with three different fibers.

Constituents	*LTRI*	Content (%)		
		PDMS	PDMS-DVB	CAR-PDMS
Ethyl acetate	800			1.72 ± 0.01
Methoxymethylvinyl ether	811			1.12 ± 0.01
Ethylbutyrate	885			1.41 ± 0.02
Ethyl crotonate	890			1.86 ± 0.06
Ethylisovalerate	900			1.76 ± 0.04
alpha-Thujene	935			1.18 ± 0.02
alpha-Pinene	940	1.44 ± 0.02	0.98 ± 0.01	1.52 ± 0.02
Sabinene	981	1.65 ± 0.06	1.58 ± 0.02	1.89 ± 0.02
beta-Pinene	979	1.24 ± 0.04	0.74 ± 0.02	0.98 ± 0.06
Myrcene	995	1.88 ± 0.05	2.09 ± 0.01	2.57 ± 0.04
delta-2-Carene	1000	1.20 ± 0.01	0.62 ± 0.03	1.76 ± 0.02
alpha-Phellandrene	1006	2.18 ± 0.02	2.05 ± 0.04	4.09 ± 0.03
alpha-Terpinene	1019		0.47 ± 0.02	1.45 ± 0.02
p-Methylanisole	1025	7.02 ± 0.02	3.01 ± 0.03	18.04 ± 0.16
p-Cymene	1032		1.15 ± 0.02	
beta-Phellandrene	1040	8.98 ± 0.03	17.19 ± 0.02	7.24 ± 0.04
delta-3-Carene	1047		0.67 ± 0.01	
Z-beta-Ocimene	1050	2.43 ± 0.06	9.07 ± 0.01	10.42 ± 0.02
gamma-Terpinene	1065	1.29 ± 0.04	0.59 ± 0.02	

Table 1 (cont.)

Constituents	*LTRI*	SPME fiber		
		PDMS	PDMS-DVB	CAR-PDMS
Methyl benzoate	1086		0.56 ± 0.02	
Linalool	1100	2.93 ± 0.04	7.38 ± 0.02	4.52 ± 0.03
allo-Ocimene	1142			2.85 ± 0.02
neo-allo-Ocimene	1150			2.37 ± 0.03
alpha-Humulene	1460	1.34 ± 0.08		
2-methoxy-*p*-Cresol	1196	1.35 ± 0.02	1.23 ± 0.03	
E-Piperitol	1205		0.41 ± 0.04	
Pulegone	1240	1.42 ± 0.03	0.64 ± 0.02	3.04 ± 0.02
Methyl geranate	1330	1.21 ± 0.01	0.73 ± 0.02	
delta-Elemene	1339	1.55 ± 0.02	0.43 ± 0.01	
alpha-Cubebene	1354		0.75 ± 0.03	
Neryl acetate	1360	1.27 ± 0.03	0.77 ± 0.04	
alpha-Copaene	1384	1.42 ± 0.05	0.70 ±0.01	2.95 ± 0.01
beta-Elemene	1395	2.90 ± 0.01	1.85 ± 0.01	0.95 ± 0.02
Z-Caryophyllene	1412	1.80 ± 0.03	0.14 ± 0.01	1.38 ± 0.02
gamma-Elemene	1438	2.93 ± 0.02	1.06 ± 0.02	
2-Methyl butyl benzoate	1440	2.02 ± 0.02	1.74 ± 0.02	
E-Muurola-3,5-diene	1457		0.89 ± 0.04	
E-Cadina-1(6),4-diene	1482	1.42 ± 0.02	0.69 ± 0.02	
gamma-Gurjunene	1489		0.89 ± 0.03	1.47 ± 0.02
Germacrene D	1495	2.69 ± 0.01	0.77 ± 0.02	
beta-Selinene	1499		0.68 ± 0.02	
E-Muurola-4(14),5-diene	1504	1.31 ± 0.03	0.73 ± 0.01	
Bicyclogermacrene	1507	3.62 ± 0.04	2.38 ± 0.01	1.23 ± 0.04
alpha-Muurolene	1509	1.49 ± 0.08	0.90 ± 0.04	
gamma-Cadinene	1515	1.64 ± 0.04	1.40 ± 0.12	
delta-Cadinene	1531	4.18 ± 0.05	4.15 ± 0.05	1.14 ± 0.02
E-Cadina-1(2),4-diene	1536	1.30 ± 0.06	0.68 ± 0.02	
alpha-Cadinene	1542	1.42 ± 0.01	0.74 ± 0.03	

Table 1 (cont.)

Constituents	*LTRI*	SPME fiber		
		PDMS	PDMS-DVB	CAR-PDMS
Z-Sesquisabinene hydrate	1547		0.68 ± 0.02	
Elemol	1552	1.41 ± 0.02	0.75 ± 0.03	
Germacrene B	1568	3.81 ± 0.03	1.60 ± 0.04	
epi-alpha-Cadinol	1652	2.13 ± 0.01	2.69 ± 0.05	
epi-alpha-Muurolol	1660	2.52 ± 0.02	2.89 ± 0.02	
Z-Methyl jasmonate	1669	1.73 ± 0.02	0.57 ± 0.02	
Benzyl benzoate	1764	1.63 ± 0.03	1.09 ± 0.05	
Total number of volatile components		37	46	26

LTPRI: linear temperature program retention index

3.2 GC-MS analysis of M. fruticosum flowers essential oil

The essential oil of *M. fruticosum* flowers was extracted using a modified Likens-Nickerson apparatus appeared as yellow viscous liquids with a percentage yield of 0.59 (w/w). The essential oil obtained was subjected to detailed GC-MS analysis in order to investigate the volatile constituents. The volatile components identified by GC-MS, their relative area percentages and their retention indices are summarized in Table 2. The essential oil of *M. fruticosum* flowers contained high percentages of the group of monoterpenes and sesquiterpene. In this study, 88 constituents representing 89.08% of the *M. fruticosum* flowers oil were identified. The dominant components were 1-phenyl butanone (20.52%), linalool (9.27%), benzyl alcohol (8.75%), α-cadinol (5.04%), globulol (4.25%) and viridiflorol (3.49%). Pulegone (3.29%), epi-α-cadinol (3.05%), terpinen-4-ol (2.64%), germacrene A (2.28%) and *para*-methyl anisole (2.09%) were also found to be the minor components of the *M. fruticosum* flowers oil.

Table 2 Chemical constituents of *M. fruticosum* flowers oil with the percentage of content obtained by hydrodistillation.

No.	compound	*LRI*	%
1	Furfural	829	0.34
2	α-Thujene	919	0.15
3	α-Pinene	925	0.23
4	Camphene	940	0.11
5	Sabinene	964	0.14
6	β-Pinene	968	t
7	Myrcene	982	0.10
8	Mesitylene	988	0.12
9	α-Phellandrene	999	t
10	δ-2-Carene	1004	t
11	α-Terpinene	1010	0.17
12	*para*-methyl Anisole	1016	2.09
13	β-Phellandrene	1021	0.35
14	δ-3-Carene	1028	0.13
15	*Z*-β-Ocimene	1039	0.46
16	Benzyl alcohol	1042	8.75
17	γ-Terpinene	1050	0.11
18	Acetophenone	1061	0.19
19	*Z*-Sabinene hydrate	1062	0.12
20	*Z*-linalol oxide (furanoid)	1066	0.23
21	meta-Cymenene	1077	0.13
22	α-Terpinolene	1079	0.17
23	methyl Benzoate	1088	0.12
24	Linalool	1098	9.27
25	1,3,8-para-Menthatriene	1109	0.35
26	1,3,8-ortho-Menthatriene	1113	0.59
27	Phenylethyl Alcohol	1115	0.16
28	*Z*-para-Menth-2-en-1-ol	1118	0.80
29	1-Terpineol	1136	0.83
30	Ethyl benzoate	1163	0.26
31	Terpinen-4-ol	1171	2.64
32	Cryptone	1178	0.11
33	Geraniol	1183	0.60

Table 2 (cont.)

No.	compound	*LRI*	%
34	2-methoxy-para-Cresol	1184	0.13
35	α-Terpineol	1188	1.21
36	*Z*-Piperitol	1191	0.14
37	*E*-Piperitol	1203	0.33
38	Pulegone	1235	3.29
39	δ-Elemene	1326	0.15
40	α-Cubebene	1337	t
41	Isoledene	1361	0.52
42	α-Copaene	1366	0.24
43	β-Bournonen	1372	0.32
44	β-Cubebene	1378	0.20
45	β-Elemene	1382	0.15
46	α-Gurjunene	1397	0.25
47	*Z*-Caryophyllene	1411	0.84
48	β-Duprezianene	1416	0.28
49	α-Guaiene	1424	0.16
50	γ-Elemene	1425	0.29
51	Aromadendrene	1430	0.67
52	1-phenyl Butanone	1438	20.52
53	α-Humulene	1443	0.18
54	allo-Aromadendrene	1448	0.49
55	*E*-Cadina-1(6),4-diene	1464	0.17
56	Germacrene D	1472	0.58
57	*Z*-Eudesma-6,11-diene	1473	0.13
58	β-Selinene	1477	0.21
59	*E*-Muurola-4(14),5-diene	1478	0.12
60	Bicyclogermacrene	1483	0.53
61	Viridiflorene	1485	0.15
62	Germacrene A	1491	2.28
63	*E*-β-Guaiene	1495	0.53
64	γ-Cadinene	1504	0.10
65	6-methyl-α-Ionone	1506	t
66	δ-Cadinene	1512	0.13
67	β-Sesquiphellandrene	1518	0.17
68	Elemol	1532	0.11

Table 2 (cont.)

No.	compound	*LRI*	%
69	Germacrene B	1548	0.33
70	Ledol	1562	1.44
71	Germacrene D-4-ol	1564	0.16
72	Spathulenol	1575	0.51
73	Globulol	1581	4.25
74	Viridiflorol	1588	3.49
75	5-epi-7-epi-α-Eudesmol	1596	0.10
76	Sesquithuriferol	1607	0.17
77	1,10-di-epi-Cubenol	1615	1.39
78	1-epi-Cubenol	1620	0.15
79	10-epi-γ-Eudesmol	1621	0.21
80	Eremoligenol	1625	0.10
81	epi-α-Cadinol	1634	3.05
82	epi-α-Muurolol	1635	0.12
83	α-Cadinol	1649	5.04
84	*Z*-methyl Epijasmonate	1671	0.21
85	Acorenone B	1675	0.34
86	Eudesm-7(11)-en-4-ol	1685	0.36
87	*Z*-α-Bisabolene epoxide	1733	0.77
88	Benzyl benzoate	1755	1.55
	Total		89.08

LRI, linear temperature program retention index on DB-5 column

t, trace amount < 0.1%

3.3 Antifungal activity of essential oil and crude extracts of *M. fruticosum* flowers

The results of antifungal activities of essential oil of *M. fruticosum* flowers are shown in Table 3. The essential oil of *M. fruticosum* flowers demonstrated a moderate antifungal activity against the plant pathogenic fungi tested. As the results, the essential oil at the concentration of 1000 µg/ml (5 µL) showed the inhibition effects on the growth of *C. asianum* CaMBK (35.1%), *C. siamense* Cach4LB (32.9%), *C. gloeosporioides* Cglg1CM (38.3%), *C. fruiticola* Cap4CM (44.1%) and *C. acutatum* BRIP28519 (30.2%).

Conversely, the flowers oil did not have antifungal activity against 4 fungal strains including *C. acutatum* CaM1VT, *Collectotrichum* sp. CaRA6VT, *T. reesei* TISTR3080and *L. theobromae* MFU. The flower extracts obtained by dichloromethane at the concentration of 1000 µg/ml (5 µL) exhibited the strongest antifungal properties against all the plant pathogens tested ranging from 22.2% to 52.9%. Hexane extracts was found to be the fungicide significantly against plant pathogens of *Collectotrichum* genus ranging from 28.1% to 39.5% whereas no inhibition effect was resulted in *T. reesei* TISTR3080 and *L. theobromae* MFU fungi. Low antifungal activity was observed in the methanol extract against 5 fungi consisting *C. asianum* CaMBK (24.5%), *C. siamense* Cach4LB (30.2%), *C. gloeosporioides* Cglg1CM (37.8%), *C. fruiticola* Cap4CM (25.5%) and *Collectotrichum sp.* CaRA6VT (29.8%). The minimum inhibitory concentrations (MICs) described as the lowest concentrations of each extract that provided the complete growth inhibition of all the plant pathogens are also depicted in Table 4. The MICs of *M. fruticosum* essential oil were found to be 1000, 500, 500, 250 and 500 µg/ml, respectively. As can be observed, *C. fruiticola* Cap4CM was found to be the weakest fungal strain to the essential oil of *M. fruticosum* flowers followed by *C. gloeosporioides* Cglg1CM, *C. siamense* Cach4LB (32.9%), *C. acutatum* BRIP28519 and *C. asianum* CaMBK, respectively. The antifungal action of the essential oil could be attributed to the occurrence of high proportion of monoterpenes and sesquiterpenes in the oil as study of Cakir et al. (2004). Moreover, Gudzic et al. (2002) and Cakir et al. (2004) have reported that most terpene compounds would seem reasonable that their antimicrobial or antifungal properties of action might be related to these compounds which have high potential to strongly inhibit microorganism pathogens. The present components were tentative to play important as antifungal components were linalool, α-cadinol, globulol and viridiflorol, pulegone, epi-α-cadinol, terpinen-4-ol, germacrene A and *para*-methyl anisole according to high proportion of their constituents. On the other hand, Cakir et al. (2004) also reported that there was no significant correlation between the activity and the percentage of the identified components. MIC values of dichloromethane, hexane and methanol extract ranged from 125 to 250, 250 to 500 and 500 to 1000 µg/ml,

respectively. It appeared that the flower extracts of dichloromethane, hexane and methanol exhibited border antifungal activity than the flower oil. The results indicated that the dichloromethane extract was found to be the remarkable fungicide in this study according to their inhibition action against all tested pathogenic fungi followed by hexane extract, essential oil and methanol extract. This was reasoned from the intermediary polarity of dichloromethane which can extract higher number of the intermediary polarity compounds with different polarity which may involve the fungal inhibition in the sample than hexane and methanol which can extract only non polar and polar components, separately. In addition, decomposition of components could be achieved during distillation process according to using high temperature. As the result, the antifungal property may drop slightly comparing to dichloromethane extract.

Table 3 Percentage of growth inhibition of fungal pathogens by each extract of *M. fruticosum* flowers (5 µL corresponding to concentration of 1000 µg/ml)

Fungi	Radical growth inhibition (%)			
	Essential oil	Hexane extract	Dichloromethane extract	Methanol extract
Collectotrichum asianum CaMBK	35.1 ± 1.3	39.5 ± 6.3	42.6 ± 4.4	24.5 ± 4.5
Collectotrichum siamense Cach4LB	32.9 ± 2.8	35.1 ± 4.9	38.4 ± 3.7	30.2 ± 1.5
Collectotrichum gloeosporioides Cglg1CM	38.3 ± 3.7	36.1 ± 3.2	40.9 ± 2.0	37.8 ± 4.6
Collectotrichum fruiticola Cap4CM	44.1 ± 1.3	38.6 ± 5.8	49.7 ± 3.3	25.5 ± 5.9
Collectotrichum acutatum BRIP28519	30.2 ± 0.2	31.5 ± 2.5	35.5 ± 6.1	-
Collectotrichum acutatum CaM1VT	-	28.1 ± 4.0	36.4 ± 5.8	-
Collectotrichum sp. CaRA6VT	-	32.0 ± 8.7	46.7 ± 3.2	29.8 ± 3.3
Tricoderma reesei TISTR3080	-		22.2 ± 3.8	-
Lasiodiplodia theobromae MFU	-		52.9 ± 5.1	-

-, Antifungal activity not detected

Table 4 MIC value assay by each extract of *M. fruticosum* flowers (5 µL corresponding to concentration of 1000 µg/ml)

Fungi	MIC (µg/ml)			
	Essential oil	Hexane extract	Dichloromethane extract	Methanol extract
Collectotrichum asianum CaMBK	1000	500	250	1000
Collectotrichum siamense Cach4LB	500	250	250	500
Collectotrichum gloeosporioides Cglg1CM	500	250	250	500
Collectotrichum fruiticola Cap4CM	250	250	125	1000
Collectotrichum acutatum BRIP28519	500	500	250	-
Collectotrichum acutatum CaM1VT	-	500	250	-
Collectotrichum sp. CaRA6VT	-	500	125	1000
Tricoderma reesei TISTR3080	-	-	250	-
Lasiodiplodia theobromae MFU	-	-	250	-

-, Antifungal activity not detected

3.4 Antioxidant activity

According to various extracts, the antioxidants properties may consider to be different. Antioxidant activities of the all extracts were tested by the DPPH radical scavenging. The violet color of the radical disappeared when a mixed with the substances in the sample solution that can donate a hydrogen atom. Antioxidant activities of all samples, standard BHT and α-tocopherol are presented in Fig. 1 in which lower IC_{50} values indicates higher antioxidant activity. The flower oil, dichloromethane, hexane and methanol extracts were able to reduce the stable free radical DPPH with an IC_{50} of 100.13, 87.60, 118.00 and 194.50 µg/ml whereas IC_{50} of standard BHT and α-tocopherol were 30.53 and 50.23 µg/ml, respectively. In this study, the dichloromethane extract exhibited the greater antioxidant activity than other extracts. As noticed, essential oil contained high level of monoterpenes and sesquiterpenes showed the moderate antioxidant activity due to higher proportion of linalool as Jilisni and Simon (2002) reported that the better antioxidant activity was found in the essential oil containing

linalool with high amount. Besides, Tepe et al. (2007) reported that the essential oils which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have greater antioxidant properties. More evidences were reported by the work of Ruberto and Baratta (2000). The monoterpene hydrocarbons had a significant protective effect with several variants due to the different functional groups. The amounts of total phenolic compounds in all extracts were also investigated spectrometrically according to the Folin–Ciocalteu procedure, calculated as gallic acid equivalents shown in Fig. 2. The total phenols of all extracts ranged from 52.20 to 86.40 µg/ml. The highest phenolic concentration was observed in the dichloromethane extract followed by the essential oil, hexane and methanol extract, respectively. Similar results were obtained from the study of total phenolic content and free radical scavenging activity. The measurements of phenols in various *M. fruticosum* flowers extracts may be related to their antioxidant properties as reported by Frankel et al. (1995).

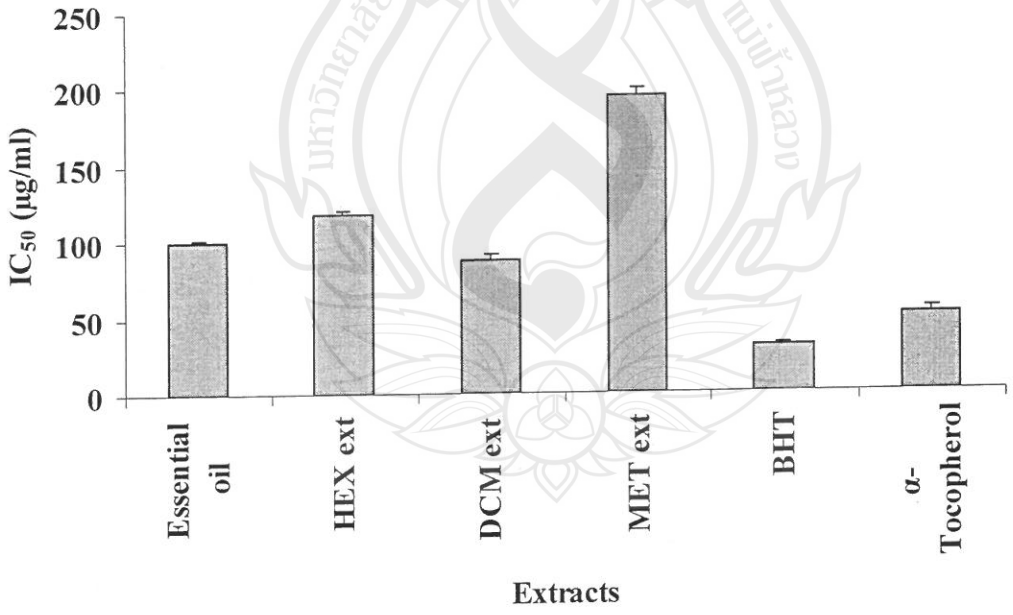

Fig. 1 Antioxidant activities (IC_{50}) of different *M. fruticosum* flowers extracts. Values represent averages ± standard deviations for triplicate experiments. HEX ext; hexane extract, DCM ext; dichloromethane extract and MET ext; methanol extract.

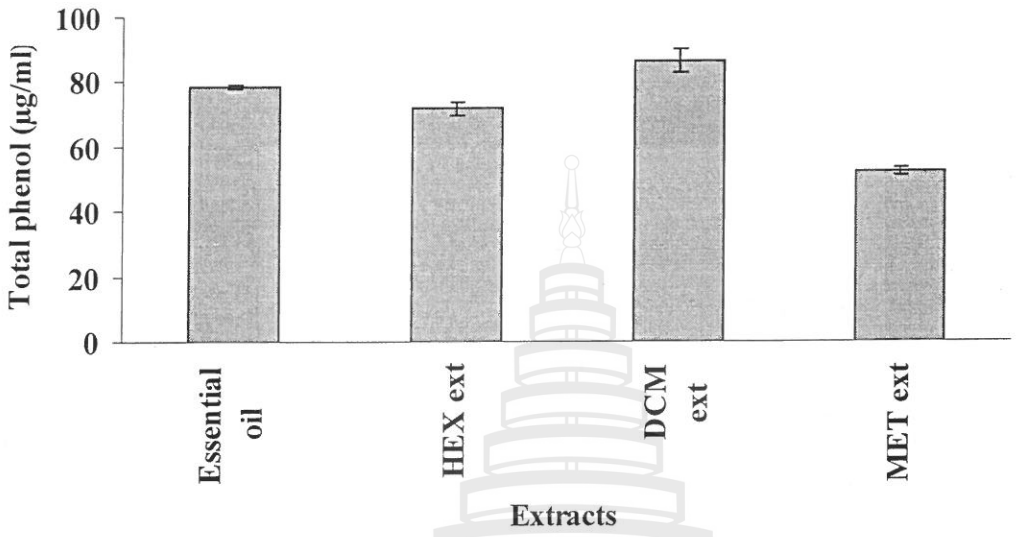

Fig. 2 Total phenols of different *M. fruticosum* flowers extracts. Values represent averages ± standard deviations for triplicate experiments. HEX ext; hexane extract, DCM ext; dichloromethane extract and MET ext; methanol extract.

CHAPTER 4
CONCLUSION

It demonstrated the best technique for trapping the key scent constituents of *M. fruticosum* flowers with different polarities whereby most components were a group of hydrocarbon and oxygenated monoterpenes. PDMS fiber is recommended for extraction of only non polar components which showed better extraction efficiency than the CAR-PDMS fiber which extracted both polar and non polar compounds due to a mutual potential effect of adsorption and distribution to the stationary phase [9, 10]. The different fibers have significant effects on percentage composition of the odor volatiles in fresh *M. fruticosum* flowers obtained by SPME methods. As can be seen, *p*-methylanisole showed the higher content by the SPME extract with CAR-PDMS fiber than those obtained from both PDMS and PDMS-DVB fiber whereas β-phellandrene provided the conversed results. It is noted that the SPME technique is more sensitive for volatile components which played a significant role as the key scent in *M. fruticosum* flower. Various extracts of *M. fruticosum* flowers exhibited the border antifungal and antioxidant activities. The antifungal and antioxidant activity may be related to the presence of the terpene components especially monoterpenes. These activities may be attributed to the presence of linalool, α-cadinol, globulol and viridiflorol, pulegone, epi-α-cadinol, terpinen-4-ol, germacrene A and *para*-methyl anisole. It can be concluded that the dichloromethane extract and the essential oil of *M. fruticosum* flowers could be considered as alternative natural fungicides for using to screen and develop the natural fungicides in the biocontrol of many agricultural plant pathogens.

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