Research Article

Chemical Constituents and Antimicrobial Activity of Essential Oil and Extracts of Heartwood of *Aquilaria crassna* Obtained from Water Distillation and Supercritical Fluid Carbon Dioxide Extraction

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Abstract

Agarwood (*Aquilaria crassna*) is a high value economic plant. Agarwood oil is widely used among various countries as perfumes and ingredient in medical recipes. In this study, the extraction of dried Agarwood powder by water distillation (WD), supercritical fluid carbon dioxide extraction (SFE) and SFE with ethanol as cosolvent (SFE+co) gave 0.2% oil, 0.06% and 0.014% extracts, respectively. The types of chemical constituents of oil and extracts from these 3 extraction methods were quite similar. The major chemical constituents of WD oil, SFE and SFE+co extracts were γ-selinene (13.66%), selina-4,11-dien-14-al (14.25%) and selina-4,11-dien-14-al (13.39%), respectively. The WD oil, SFE and SFE+co extracts had antimicrobial activities against *S. aurues* and *C. albicans*, but were not sensitive to *E. coli* at maximum concentration of the study, 2mg/mL.

Key Words: Agarwood; *Aquilaria crassna*; Distillation; Supercritical fluid carbon dioxide

Introduction

Agarwood, Eagle wood or Aloewood, is a botanical plant in several countries. It is classified under the family Thymelaeaceae. In Thailand, there are four indigenous species of Agarwood, i.e., *Aquilaria subinteqra*, *A. crassna*, *A. malaccaensis* and *A. baillonil*. The *A. crassna* contains good quality Agarwood oil. It is native to Nakhon Nayok, Prachinburi, Sa Kaeo, Nakhon Ratchasima, Buri Ram, Sri Saket, Nan, Chiang Rai, Phrae, Phetchabun, Nakhon Sawan and Kamphaeng Phet provinces (Thitisomboon, 2006). Its heartwood is fine, black or brown in color and fragrant. The aroma of the wood comes from sesquiterpene compounds (Prachakul, 1989). The plant synthesizes these aromatic terpenes when it is injured by insects, physical cuts, bacterial infections and chemical stimulation. The Agarwood is sold in 3 forms as pieces of heartwood, heartwood powder and oil. The heartwood is burned for aromatic vapor in houses and shrines and carved for art and the Holy Spirit image. The powder is used for making incense and medicine. The Agarwood is claimed to possess aphrodisiac, flatulence and diuretic properties. It is one of the ingredients used in the treatment of smallpox, rheumatism, spasm of bronchous and respiration, abdominal cramp, diarrhea,

nausea, vomiting, anxiety, elderly fatigue, pregnancy and post partum illness (Poain and Poain, 2001; Bunyapraphatsara and Chokchaijareonporn, 1996). The Agarwood oil is used in perfumery and incense industries.

The classical method that is currently used in commerce for the Agarwood oil extraction is water distillation (WD). This method consumes 7-10 days and high energy for extraction. The supercritical fluid carbon dioxide extraction (SFE) is known as nonflammable, non-toxic, chemically stable and less energy consumption method. It provides some advantages over the classical method, since supercritical carbon dioxide has low viscosity, high diffusivity, good transport properties and gives faster extraction and high yields (Anklam et al., 1998). Nowadays SFE method has been used to extract volatile components from various kinds of spices and plants for flavor and fragrance ingredients in pharmaceutical and food industries (Abbas, 2008). The aim of this research is to study an alternative method, SFE, for the Agarwood oil extraction. In this study, the chemical constituents of water distillation oil and SFE extracts are also determined.

Material and Methods

Plant

The heartwood of stems and branches of eight years old Agarwood were received from Krissana Panasin Co., Ltd. in January 2008. The Agarwood was cultivated in Chanthaburi, Thrad and Rayong provinces. The tree was injured by hammering nails into its trunk to stimulate oleoresin production. The voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University.

Chemicals

Standard Alkane C8 to C20 and Standard Alkane C21 to C40 were purchased from Fluka, Germany. Carbon Dioxide High Purity grade; Helium Ultra High Purity, 99.999% ; Hydrogen and Nitrogen were purchased from Thai Industrial Gasses Public Co., Ltd., Thailand. Air Zero Grade, High Purity Grade was purchased from Chattakorn Lab Center Co., Ltd., Thailand.

Methods

Water distillation extraction

After 15 kg dried Agarwood powder had undergone water maceration for 10 days, it was water distillated for 7 days. The oil was collected and heated for residual water evaporation by heat of lamp. The water distilled oil was kept in an air tight container and stored in a refrigerator at -4°C until the experiments were done.

Supercritical fluid carbon dioxide extraction (SFE and SFE+co)

The 3.5 kg dried Agarwood powder was extracted by the pilot scale SFE (Guangzhou Masson New Separation Technology, China). The temperature and pressure parameters for extraction of extractor were 90°C and 20 MPa, respectively. The temperature and pressure of separator 1 were controlled at 90°C and 9 MPa and of separator 2 were at 40°C and 6 MPa. The other variables, particle sizes of plant material (60 mesh) and carbon dioxide flow rate (1.0 mL/min) were kept constant. The equilibrium time was 30 mins and the extraction time was 4 hours. When the extraction process was finished, the repeated extraction process was done with 95% ethanol added as co-solvent (calculated as 3% of co-solvent addition). The oleoresin from both extraction methods were separated by the same process as follow. The oleoresin was collected and dissolved in 95% ethanol. The solution was placed in freezer until wax was solidified then filtered. The filtrate was evaporated under vacuum for ethanol elimination. After evaporation the residue was centrifuged and the extract was then isolated. The anhydrous sodium sulphate was added to the extract for trace water elimination and then filtered.

Analysis of chemical compositions of the oil and extracts.

The chemical compositions of the oil and extracts were analyzed by GC (Agilent Technologies GC 6890 gas chromatograph USA) equipped with HP-1 capillary column (25 m x 0.32 mm, film thickness 0.17 μm). The operating parameter for the HP-1 were temperature program 50°C for 5 mins, ramp of 1°C/ min up to 260°C, 260°C for 5 mins; injection temperature 250°C; detector temperature 260°C; detector FID; split ratio 1: 50; Split flow 50 mL/min, carrier gas helium (1 mL/min); hydrogen 40 mL/min, air 400 mL/min, make up gas (nitrogen) 44 mL/min, injection of 2.0 μL. The identification of individual components was performed by comparison of their retention times with some authentic samples and by retention indices (R.I.) relative to the series of n-hydrocarbons. (Adam, 2001; Ishihara and Tsuneya, 1993a and 1993b; and Tamuli et al., 2005).

The GC/MS analyses were performed with Agilent Technologies GC 6890 gas chromatograph (USA) equipped with DB-1MS capillary column (30 m x 0.25 mm, film thickness 0.25 μm). The operating temperature program for GC/MS was the same as that for GC/FID. The detector was Agilent Technologies MSD 5973N mass spectrometer (USA) : EI, 70 eV, mass range 40-550, solvent delayed 3.0 mins.

The identification of individual components was performed by comparison of their retention times with some authentic samples, computer matching against Willey 7n, NIST02 and Pesticide and comparing with the mass spectra reported by Adams (2001) and The Pherobase web site (El-Sayed, 2003)

Microdilution method for antimicrobial assay

Three tested organisms, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Candida albicans* ATCC 17110, were used for the assay. The microorganisms were maintained in Tryptic soy agar (TSA) for bacteria and in Sabouraud dextrose agar (SDA) for yeast at 37.0°C for 18-24 hours. The isolated colony was incubated to broth overnight at 37.0°C. One loop of this cultured broth was transferred to a new tube and resuspended in Tryptic soy broth (TSB) for bacteria and Sabouraud Dextrose Broth (SDB) for yeast, then incubated at 37.0°C for 2-5 hours in a shaking incubator. After 2-5 hours incubation, the microorganism culture was used for the assay. The amounts of *S. aureus*, *E. coli* and *C. albicans* were adjusted by comparing the turbidity to the McFarland suspension No.5 (0.5% $BaCl_2$ in 0.56 N in SO_4 v/v) and further diluted to be 10^6 CFU/ mL using TSB or SDB. To overcome the insolubility of the oils and extracts in the broth, the assay was performed in 0.5-4%v/v DMSO of TSB or of SDB. The 90.0 μL of five-folded dilutions of each oil or extract sample or standard antimicrobial chemicals were prepared in a 96-well plate. The 10.0 μL of each tested organism was added to each well to make a final concentration of approximately 10^5 CFU/mL. In each test, the organism in TSB or SDB was used as the positive growth control, while the oil and extract samples in TSB or SDB were experimental control and the two media alone were used as negative growth control. The plates were then incubated at 37.0°C for 18-48 hours. The minimum inhibitory concentration (MIC) value was defined as the lowest concentration of the oil or extracts that inhibited visible growth by using microplate reader (primary excitation filter position = Abs 550 and primary emission filter position = Diffuser). If the oil or extract could inhibit microbes, a clear solution was found in each well. Each experiment was performed in triplicate. In this experiment, doxycycline and cotrimazole were used as positive reference standards of antibacterial and antifungal chemicals, respectively.

Results

From the water distillation method, the 15 kg dried Agarwood heartwood gave 31 mL (0.2%) of WD oil. In the SFE method, the 3.5 kg dried Agarwood heartwood gave 2 mL (0.06%) of SFE extract and 5 mL (0.14%) of SFE+co extract, SFE with ethanol addition. The WD oil and SFE extract have a yellow-

amber color, while the SFE+co extract has a dark brown color.

The chemical constituents from these three extraction methods were quite similar, but the quantitative compositions of them were different (Table 1). The GC-FID chromatograms of WD oil, SFE and SFE+co extracts were shown in Figures 1, 2 and 3, respectively. The series of 8 major components of WD oil were γ-selinene (13.66%), 10-epi-γ-eudesmol (8.95%), selina-3,11-dien-9-one (8.78%), tetradecanal (8.61%), γ-eudesmol (7.18%), epoxybulnesene (5.25%), valerianol (4.87%) and selina-3,11-dien-14-al (4.00%). The consequence of 8 major components of SFE extract was selina-4,11-dien-14-al (14.25%), octadecanoic acid (3.18%), campesterol (2.93%), oxo-agarospirol (2.90%), γ–sitosterol (2.29%), hexadecanol (2.03%) ,valerianol (2.00%), selina-3,11-dien-9-one (1.85%) and selina-3,11-dien-14-al (1.73%). The series of 8 major components of SFE+co extract were selina-4,11-dien-14-al (13.39%), 3,4-dimethoxyphenol (3.75%), selina-3,11-dien-9-one (3.46%), γ-eudesmol (3.12%), 10-epi-γ-eudesmol (3.09%), tetradecanal (3.03%), oxo-agarospirol (2.56%) and agarospirol (2.14%). The compounds that were found only in oil derived from the water distillation method were β-agarofuran, α-selinene, norketoagarofuran, selina-4,11-dien-14-oic acid and 9,11-eremophiladien-8-one. The components that were found only in extracts from the supercritical carbon dioxide extraction method were p-vinylphenol, benzylacetone, p-vinylguaiacol, 3, 4-dimethoxyphenol, vanillin, guaia-1(10),11-dien-15-oic acid, pentadecanoic

Table 1 The chemical constituents of Agarwood oil from water distillation (WD), supercritical fluid carbon dioxide extraction (SFE) and supercritical fluid carbon dioxide (SFE+co) extraction with cosolvent methods.

Peak No.	Compound	R.I. Ref	R.I. sample	%Area WD	%Area SFE	%Area SFE+co
1	p-methoxyphenol	1198	1197	1.51	0.26	0.23
2	p-vinylphenol	1199	1200	-	-	0.05
3	benzylacetone	1210	1212	-	-	0.12
4	p-vinylguaiacol	1286	1291	-	-	0.15
5	3,4-dimethoxyphenol	1312	1321	-	0.54	3.70
6	vanillin	1367	1389	-	-	0.37
7	γ-selinene	1438	1435	13.66	0.29	1.02
8	humulene	1447	1449	0.23	-	0.14
9	p-methoxybenzylacetone	1459	1458	0.27	-	0.06
10	drima-7,9(11)-diene	1460	1461	0.39	-	0.04
11	β-agarofuran	1474	1473	0.39	-	-
12	α-selinene	1486	1489	0.60	-	-
13	tridecanal	1496	1495	0.49	-	-
14	δ-guaiene	1502	1500	0.33	0.11	0.16
15	α-bulnesene	1502	1504	0.93	0.10	0.41
16	dodecanoic acid	1553	1547	0.75	0.29	0.82
17	norketoagarofuran	1555	1556	1.21	-	-
18	epoxybulnesene	1572	1573	5.25	0.33	1.06
19	guiaol	1584	1585	0.49	0.11	0.32

Table 1 The chemical constituents of Agarwood oil from water distillation (WD), supercritical fluid carbon dioxide extraction (SFE) and supercritical fluid carbon dioxide (SFE+co) extraction with cosolvent methods. (continued)

Peak No.	Compound	R.I. Ref	R.I. sample	%Area WD	%Area SFE	%Area SFE+co
20	tetradecanal	1593	1593	8.61	1.46	3.03
21	10-epi- γ-eudesmol	1599	1599	8.95	1.52	3.09
22	γ-eudesmol	1608	1608	7.18	1.63	3.12
23	1,5-epoxy-nor-ketoguaiene	1614	1619	2.30	0.50	1.05
24	valerianol (kusunol)	1626	1623	4.87	0.69	1.62
25	agarospirol	1631	1628	3.66	1.31	2.14
26	jinkho-eremol	1643	1642	2.31	0.14	0.26
27	tridecanoic acid	1647	1651	1.24	0.23	0.36
28	dehydrojinkoh-eremol	1673	1674	0.73	0.14	0.40
29	selina-3,11-dien-9-one	1687	1684	8.78	1.85	3.46
30	pentadecanal	1695	1697	0.82	0.62	1.00
31	rotundone	1703	1712	1.47	0.79	0.84
32	selina-3,11-dien-9-ol	1721	1720	0.47	0.80	0.82
33	selina-4,11-dien-14-oic acid	1728	1729	0.20	-	-
34	selina-3,11-dien-14-al	1735	1733	4.00	1.73	1.95
35	9,11-eremophiladien-8-one	1740	1741	0.31	-	-
36	tetradecanoic acid	1743	1747	0.49	0.39	0.43
37	guaia-1(10),11-dien-9-one	1752	1752	0.11	1.23	0.91
38	selina-4,11-dien-14-al	1758	1765	0.81	14.25	13.39
39	guaia-1(10),11-dien-15-ol	1770	1772	0.80	0.31	0.36
40	selina-3,11-dien-14-oic acid	1775	1777	0.10	1.23	1.10
41	2-hexadecanone	1782	1785	0.22	0.59	0.59
42	dihydrokaranone	1799	1796	0.21	0.70	0.33
43	guaia-1(10),11-dien-15-oic acid	1811	1811	-	1.03	0.83
44	oxo-agarospirol	1822	1823	0.53	2.90	2.56
45	pentadecanoic acid	1842	1845	-	0.40	0.82
46	hexadecanol	1865	1865	-	2.03	1.26
47	2-hydroxyquia-1(10),11-dien-15-oic acid	1932	1934	0.65	0.83	0.49
48	hexadecanoic acid	1940	Ms	1.36	1.07	0.86
49	1,5-diphenyl-2-pentene	2000	1997		-	0.20
50	oleic acid	2098	2090	-	0.35	0.61
51	octadecanoic acid	2137	2137	-	3.18	1.38
52	2-[2-(4-methoxyphenyl)ethyl]chromone	2545	2545	-	-	0.24
53	6-methoxy-2-(2-(4-methoxyphenyl) ethyl)chromone	2949	2949	-	0.25	0.08
54	campesterol		Ms	-	2.93	1.69
55	stigmasta-5,22-dien-3ol		Ms	-	0.60	0.28
56	γ-sitosterol		Ms	-	2.29	0.89
57	stigmasta-7,22-dien-3-one		Ms	-	0.22	0.21

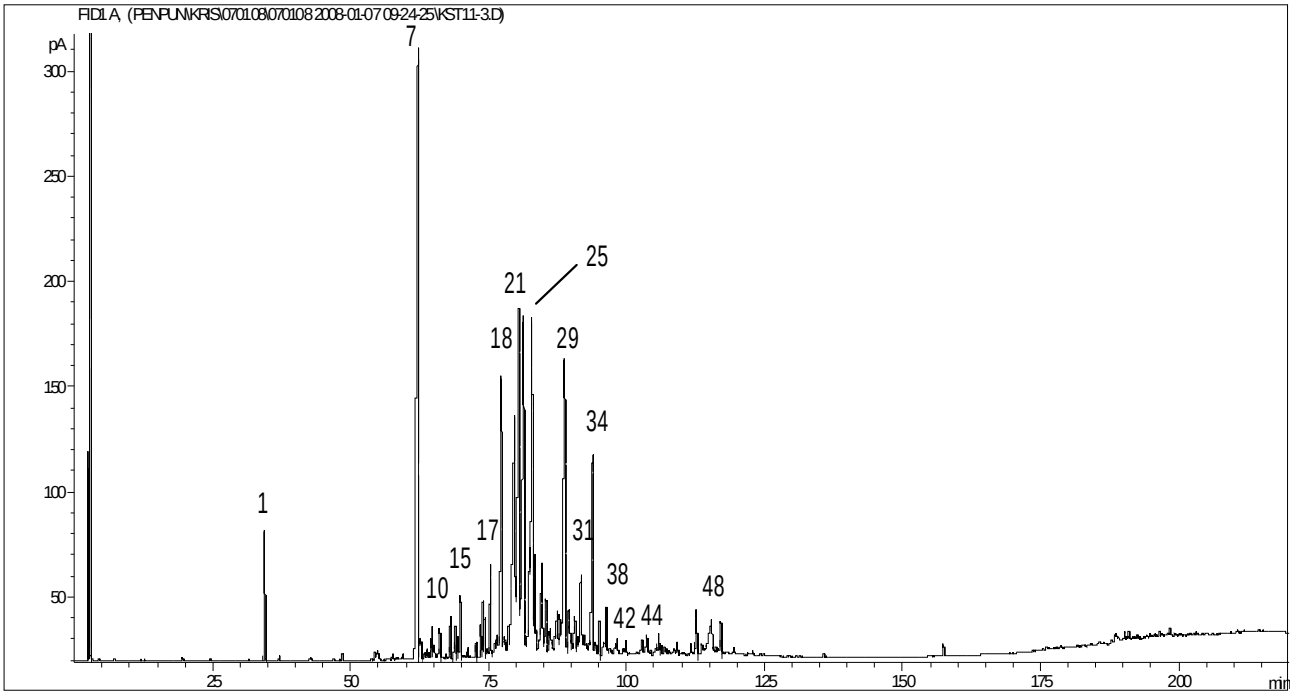

Figure 1 Gas chromatogram of the water distillation of oil

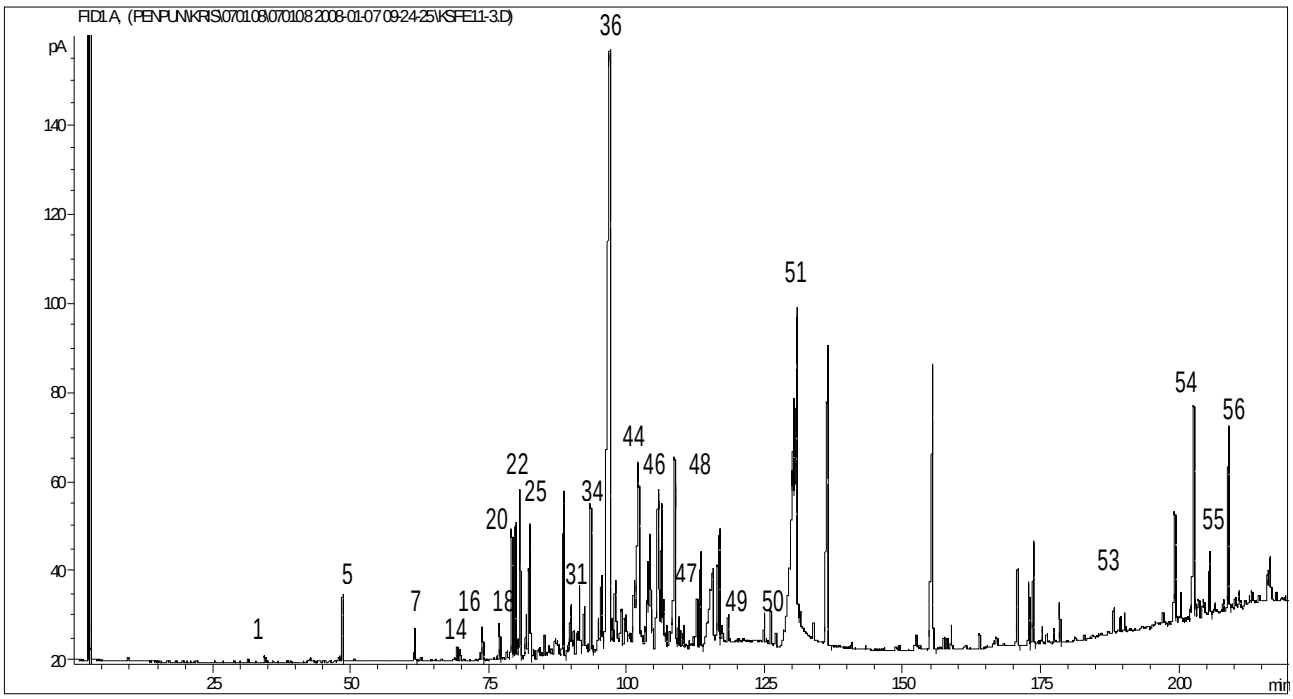

Figure 2 Gas chromatogram of the SFE extract

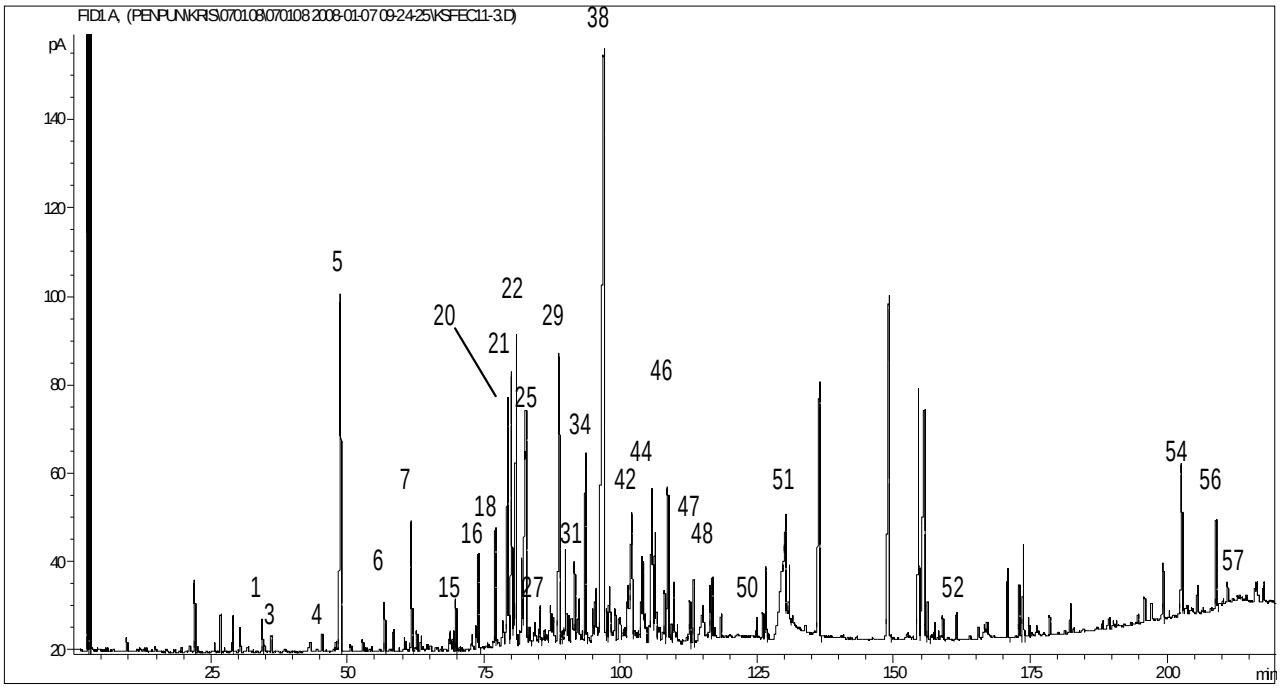

Figure 3 Gas chromatogram of the SFE+co extract

acid, hexadecanol, 1,5-diphenyl-2-pentene, oleic acid, octadecanoic acid, 2-[2-(4-methoxyphenyl)ethyl] chromone, 6-methoxy-2-(2-(4-methoxyphenyl)ethyl) chromone, campesterol, stigmasta-5,22-dien-3ol, γ–sitosterol and stigmasta-7,22-dien-3-one. The constituents that were found in SFE+co extract and did not appear in SFE extract were p-vinylphenol, benzylacetone, p-vinylguaiacol, vanillin, humulene, p-methoxybenzylacetone, drima-7,9(11)-diene, 1,5-diphenyl-2-pentene and 2-[2-(4-methoxy phenyl) ethyl]chromone. The WD oil contained the lowest number of oil constituents while the SFE+co extract had the highest number of compounds in it.

All controlled experiments of the antimicrobial test presented no microbial contamination in the oil, extracts and media. The bacteria and yeast could grow well in both media. The 4%v/v DMSO, the maximum concentration that was used for dissolving oil or extracts, showed no inhibition of the microbial growth. The oil and extracts gave lower antimicrobial activities than the two reference antimicrobial standards. The SFE and SFE+co extracts could inhibit *C. albicans* at the same concentration, 2 mg/mL, but the SFE+co extract inhibited *S. aurues* at 0.5 mg/mL, which was better than that of SFE extract (1 mg/mL), see Table 2. The WD oil exhibited yeast inhibition at 1 mg/mL, while inhibited *S. aurues* equaled that of SFE+co extract. For *E. coli*, the WD oil, SFE and SFE+co extracts showed no inhibition at their maximum concentration, 2 mg/mL, which was used in the experiment.

Conclusion and Discussion

The supercritical fluid carbon dioxide extraction was an economic and fast technique for Agarwood oil extraction. It took only 4 hrs for extraction compared to 7 days for the water distillation method. From Table 1, it was shown that the SFE+co method had strong extractive power, since this method gave the highest number of compounds. Although the supercritical fluid carbon dioxide extraction had good extraction efficiency, it is a low selectivity method since both the SFE and SFE+co methods could extract high-boiling point constituents, with high molecular weights, from the wood. These were confirmed by the analysis data which indicated

Table 2 The antimicrobial activity of Agarwood oil and extracts.

Type of oil, extracts and standard	Minimum inhibitory concentration (MIC)		
	S. aurues	*E. coli*	*C. albicans*
WD	0.5 mg/mL	> 2 mg/mL	1 mg/mL
SFE	1 mg/mL	> 2 mg/mL	2 mg/mL
SFE+co	0.5 mg/mL	> 2 mg/mL	2 mg/mL
Doxycycline	62.5 ng/mL	4 μg/mL	
Clotrimazole	-	-	40 μg/mL

some high-boiling point compounds like fixed oils and plant sterols in the SFE and SFE+co extracts. These high-boiling point constituents were natural contaminants that affected the qualitative characteristics of volatile oils such as specific gravity, refractive index, optical rotation and non-volatile residue values (Giovanni and Angelo, 2002). The SFE with cosolvent technique gave a higher yield of Agarwood extract (0.14%) than that of SFE technique (0.06%). However, the percentage weights of the extracts from these two methods were lower than that of the oil from water distillation method. The water distillation and SFE techniques showed significant difference in the quantity of each composition and in the types of the major components of oil and extracts, (see Table 1). This may be the reason why the fragrance of SFE and SFE+co extracts were different from WD oil.

The antimicrobial activities of oil and extracts were lower in sensitivities than that of doxycycline and cotrimazole. Their activities against gram negative bacteria, *E. coli*, were not present at the maximum concentration, of this experiment, 2 mg/mL. The inhibition of the extracts by supercritical fluid extraction showed equal activity against yeast. However the supercritical fluid extraction technique with cosolvent gave better inhibition against *S. aurues* than that of the technique without cosolvent. Both of supercritical fluid extraction extracts exhibited two times lower activity against yeast than the WD oil, while the result of *S. aurues* sensitivity were the same between WD oil and SFE+co extract. The difference in the antimicrobial results may lie in the variation of chemical constituents and their amount in the oil and extracts. For example, the major constituent in WD oil was γ-selinene which differed from the supercritical fluid extraction extracts which their main constituent was selina-4,11-diene-14-al. The antimicrobial activities of distilled oil were stronger than the supercritical fluid extraction extracts and may be associated with the chemical contamination in SFE and SFE+co extracts which hinders the activities. As the amount of essential oil constituents of the extract was lower than that of WD oil at the same concentration.

From this experiment, although the water distillation is a time and energy consuming method for Agarwood oil extraction, it is still a good method since it gave high yield and pure oil with higher antimicrobial activities than the extracts from supercritical fluid extraction techniques.

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References

Abbas, K. A., Mohamed, A., Abdulamir, A. S., and Abas, H. A. (2008) A Review on Supercritical Fluid Extraction as New Analytical Method. *American Journal of Biochemistry and Biotechnology* 4: 345-353.

Adams, R. P. (2001) *Identification of essential oil components by gas chromatography / quadrupole mass spectroscopy*, Allured Publishing,, Carol Stream. IL.

Anklam, E., Berg, H., Mathiasson, L., Sharman, M., and Ulberth, F. (1998) Supercritical fliud extraction (SFE) in food analysis: A review. *Food Additives and Contaminants* 15: 729-750.

Bunyapraphatsara, N., and Chokchaijareonporn, A. (1996) *Sa-mund-phai Mai-pern-ban*, . Prachachon Co., Bangrak, Bangkok.

El-Sayed, A. M. (2003) The Pherobase. [Online URL:www.pherobase.com/database/compound/compounds-index.php] accessed on September 12, 2008.

Giacomo, A. D., and Giacomo, G. D. (2002) Essential oil production. In *Citrus: The Genus Citrus. Medicinal and Aromatic Plants-industrial Profiles; V. 26* (Giacomo, G. D. and Giacomo, A. D., eds.), pp. 114-147. Taylor & Francis, New York.

Ishihara, M. and Tsuneya, T. (1993a) Components of the Volatile Concentrate of Agarwood. *Journal of Essential Oil Research* 5: 283-289.

Ishihara, M. and Tsuneya, T. (1993b) Components of the Agarwood Smoke on Heating. *Journal of Essential Oil Research* 5: 419-423.

Poain, T. and Poain, N. (2001) *Interested Medicinal Plants*, Offset Press Ltd., Bangkok.

Prachakul, M. (1989) *Histology Characteristic of Normal and Abnormal of Agarwood (Aquilaria crassna* Pierre ex K.Lec.) *Heartwood*, Kasetsart University. Bangkaen.Bangkok.

Thitisomboon, P. (2006) *Cultivated Krisana. Ten thousand Bath per Tree and Million Bath per Rai*, M S H Printing Ltd., Yannawa. Bangkok.

Tamuli, P., Boruah, P., Nath, S. C., and Leclercq, P. (2005) Essential Oil of Eaglewood Tree a Product of Pathogenesis. *Journal of Essential Oil Research* 17: 601-604.