Title: Agarotetrol in agarwood its use in evaluation agarwood quality

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Abstract
Agarwood, which is used as medicine and incense, contains sesquiterpenes and chromones. Agarotetrol is a chromone derivative found in high concentrations in the water extract fraction of agarwood and thus may be present in pharmaceutical products made from decoctions of agarwood. Agarotetrol has been reported to be present at the early stages of cell death in calli. We therefore examined the presence of agarotetrol in medical- and incense-grade agarwood, in agarwood source plants lacking resin deposits, and in artificially made agarwood. Agarotetrol appeared as a large peak in the HPLC chromatograms of all samples of medical-grade and artificially made agarwood, and in most incense-grade agarwood samples. In contrast, agarwood samples lacking resin deposits did not contain agarotetrol. These results show that agarotetrol is characteristic of resin formation. Agarotetrol was also detected in decoctions of agarwood. A newly developed TLC method for the detection of agarotetrol in agarwood is described.

Keywords
agarwood, chromone derivatives, quality evaluation, agarotetrol

Introduction

The Japanese Standards for Non-pharmacopoeial Crude Drugs 2018 (Non-JPS 2018) [1] define agarwood as a resin deposited by some species of the genus *Aquiralia*. Agarwood is used as incense, and in Kampo formula such as chokoshiteito and zenshikunshito and household medicines such as rokushingan and kiohgan.

Generally, the agarwood used in incense and traditional medicine is distributed via different routes. Agarwood used as incense is more valuable than that used as medicine. Chromone derivatives, sesquiterpenes, and low molecular weight aromatic compounds are the principle ingredients in agarwood resin and give agarwood its scent [2,3,4]. Chromone derivatives do not readily volatilize and have no scent of their own, but sesquiterpenes are volatile and thus fragrant. Low molecular weight aromatic compounds such as benzylacetone are volatile and can be detected when agarwood is heated [5]. Agarotetrol (Fig.1) is a chromone and is involved in the production of low molecular weight aromatic compounds such as benzylacetone when agarwood is heated [6]. Agarotetrol is not believed to be present in the non-resinous part of the agarwood plant because low molecular weight aromatic compounds such as benzylacetone are not detectable when agarwood plants that lack resin deposits are heated [6]. In addition, agarotetrol and other chromones are not detected in healthy agarwood trees [3,6]. Studies of agarwood calli have shown that agarotetrol can be detected in the early stages of cell death in calli [7]. The presence of agarotetrol may indicate whether or not an agarwood plant contains deposited resin because it is absent in healthy agarwood plants and is likely produced during cell death. Agarotetrol is detected in large quantities in hot water extracts of

agarwood [6], suggesting that agarotetrol may be present in decoctions of Kampo prescriptions such as chokoshiteito and zenshikunshito. As noted above, the presence of agarotetrol can been used to determine the presence of agarwood resin, and agarotetrol is expected to be included in decoctions of agarwood. Accordingly, in this study we discuss the application of agarotetrol in component analysis and the quality evaluation of agarwood.

Materials and methods

Materials

Eight samples of medical-grade agarwood (M-1–M-8) which were distributed for medical use and five for use as incense (collected from Indonesia (I-1), Papua new Guinea (I-2), Bangladesh (I-3), Vietnam (I-4), India (I-5)) which were distributed for incense were prepared for analysis. Two samples of artificially made agarwood from cultivated trees were obtained from a market in Vietnam (samples A, B). A non-resinous sample was collected from an agarwood tree (*Aquilaria sinensis*) growing at the Experimental Station for Medicinal Plants at the Graduate School of Pharmaceutical Science, Kyoto University, and cut into small pieces to dry.

Methods

Each sample of agarwood was cut into small pieces and crushed using a mortar and pestle with liquid nitrogen. The powdered samples (about 50 mg each) were transferred to screw cap tubes and volatile components were extracted with 5 mL diethyl ether for 24 h on a tube rotator. The diethyl ether extract was filtered through a filter paper (ADVANTEC 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to remove the residue, dehydrated with anhydrous sodium sulfate, and concentrated for GC and GC-MS analyses. The residue was extracted twice with 5 mL ethyl acetate for 24 h to extract non-volatile, poorly volatile, and non-polar compounds. The paper-filtered extracts were combined, concentrated to dryness, dissolved in MeOH (1 mg/ml) and filtered again (Cromatodisc 13P, 0.45 μm; GL Sciences, Tokyo, Japan), and then subjected to HPLC analysis. The residue was further extracted twice with 5 mL of distilled water for 24 h to extract non-volatile polar compounds. Following paper filtration, these extracts were combined, freeze-dried, dissolved in 20% MeOH (1 mg/ml) and filtered again, and then analyzed by HPLC. A decoction of medical-grade agarwood was prepared as follows. Sample was cut into small pieces and crushed using a mortar and pestle with liquid nitrogen. The powdered sample (1.50 g) were added to 600 ml of water in a covered glass jar and heated on an electric decoction apparatus (microcomputer decoction pot 2 HMJ-1000N (Hario, Tokyo, Japan) for 1 h. The decoction was filtered through a filter paper, freeze-dried, dissolved in 20% MeOH (1 mg/ml), filtered again, and subjected to HPLC analysis.

GC analysis

Samples were analyzed using a G-5000 GC instrument (Hitachi, Tokyo, Japan) equipped with an InertCap-WAX column (60 m×0.25 mm, film thickness 0.25 μm; GL Sciences, Tokyo, Japan). The split ratio was 99:1. The conditions for GC analysis were as follows: carrier gas, helium; flow rate, 1 ml/min; injector temperature, 230°C; flame ionization detector (FID) temperature, 250°C; column oven program, 100°C initially, then increased by 4°C per min to 180°C, maintained at 180°C for 30 min, then increased by 5°C per min to 240°C, then maintained for 18 min; Injection volume, 3 μl.

GC-MS analysis

Samples were analyzed on a GC-MS instrument (6850GC with 5975MDS, Agilent Technologies, Santa Clara, CA) equipped with a DB-WAX column (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies). The conditions for GC-MS analyses were as follows: carrier gas, helium; flow rate, 1 ml/min; injector temperature, 230°C; column oven program 100°C initially, then increased by 4°C per min to 180°C, and maintained at 180°C for 30 min. The temperature was then increased by 5°C per min to 240°C and maintained for 18 min; injection volume, 1 μl. Peaks were identified by comparing the retention times and mass fragmentation patterns of the samples with the National Institute of Standards and Technology database.

HPLC analysis

The HPLC system consisted of an L-7100 pump, L-7300 column oven, and an L-7420 UV detector (Hitachi, Ltd.). The conditions were as follows: $_5C_{18}$MS-II (Cosmosil, Nacalai Tesque Inc. Kyoto, Japan) column, 4.6 mm I.D. × 250 mm; solvent, MeOH-water: 20% (0 min) → 30% (60 min) → 45% (80 min) → 64% (140 min) MeOH; flow rate, 1ml/min, injection volume, 10 μl; detection at 254 nm. Agarotetrol in the samples was identified by comparing peak retention times with the retention time of agarotetrol purified from agarwood.

Isolation and quantification of agarotetrol

Freeze-dried hot water extract of agarwood in our previous paper [6] was also used in this report. Freeze-dried powder (61.52 mg) was dissolved in 20% MeOH (15 mg/ml) for fractionation. Conditions for fractionation by HPLC were as follows: $_5C_{18}$MS-II (Cosmosil, Nacalai Tesque Inc.) column, 20 mm I.D. × 250 mm; solvent, MeOH-water; gradient from 20% (0 min) to 30% (120 min) MeOH; flow rate, 8 ml/min; detection at 254 nm. The amount of target compound obtained was 17.36 mg. ^{1}H- and ^{13}C-NMR spectra of the isolated compound was measured on a 500 MHz spectrometer (JNMECA 500KP, Jeol Ltd., Tokyo, Japan). Compound was identified by comparing the data with those in the published literature [8].

The concentration of agarotetrol in each sample was calculated according to the peak area of agarotetrol on the HPLC chromatogram based on a calibration curve constructed using 1-, 10-, 100- and 1,000-μg/ml solutions of agarotetrol standard.

TLC

Medical-grade agarwood (60 mg) was extracted with 2 mL MeOH by ultrasonication for 10 min and the extract was separated by filtration. Agarotetrol standard was dissolved in MeOH. Sample solutions were spotted on a TLC plate (TLC silica gel 60 RP-18 F_{254}S; Merck Ltd., Tokyo, Japan) using a glass capillary, the plate was developed with a mixture of MeOH and water (1:1) to a distance of about 5 cm, and air-dried. The spot corresponding to agarotetrol was examined under ultra-violet light (main wavelength: 254 nm).

Results

GC and GC-MS analysis

Table 1 shows the results of GC and GC-MS analyses of diethyl ether extracts of medical-grade and incense-grade agarwood. Sesquiterpenes reported previously as components of agarwood were detected in the diethyl ether extracts of agarwood for use as incense. No sesquiterpenes were detected in the diethyl ether extracts of medical-grade agarwood whereas compounds such as cinnamaldehyde and eugenol were detected. These compounds were likely contaminants from residual Cinnamon Bark and cloves stored together with the agarwood samples prior to our purchase of the samples [9,10].

Weights of the extracts and HPLC patterns of each sample

Table 2 shows the weights of the ethyl acetate and water extracts of medical-grade agarwood, incense-grade agarwood, samples generated by treatment, and of a non-resinous sample. The average amount of extract detected in each solvent was generally higher in the incense-grade samples than in the medical-grade samples. However, the amount of extract from several medical-grade samples was higher than that from incense-grade samples, suggesting that the amount of extract is not always relevant to the grade of the sample.

HPLC chromatograms of the extracts are shown in Fig. 2. All water extracts of medical-grade agarwood provided a large agarotetrol peak and agarotetrol was also detected in water extracts of incense-grade agarwood, but the chromatogram patterns differed, and some peaks were larger than the agarotetrol peak. Both samples of artificially made agarwood provided large agarotetrol peaks whereas both the ethyl acetate and water extracts of the non-resinous sample provided few peaks upon HPLC analysis.

HPLC analysis of a decoction of medical-grade agarwood

Agarotetrol was detected in a decoction of medical-grade agarwood (Fig. 3). Extraction of 1.50 g of medical-grade agarwood provided 63.17 mg extract which contained 805.4 μg of agarotetrol.

Detection of agarotetrol on TLC

One spot with Rf = 0.46 was observed. This Rf value is the same as that of agarotetrol standard (Fig. 4).

Discussion

Resin deposition occurs in species of *Aquilaria* such as *A. mallaccensis*, in several Thymelaeaceae such as *A. sinensis* and *A. crassna*, and in *Gyrinops* species [11-15]. It was previously shown that the number of chromone derivatives increases as resin deposition is enhanced [3].

Previous studies using agarwood-calli showed that the amount of chromone derivatives increases as time course [16], and agarotetrol in particular was detected at early stages of cell death [7]. In the present study, agarotetrol was detected as a main peak in the HPLC profiles of commercial medical-grade agarwood. Given that sesquiterpenes were not detected in such agarwood, and fewer HPLC peaks were observed in its ethyl acetate and water extracts compared with incense-grade agarwood, this medical-grade agarwood might be at an early stage of resin deposition.

GC, GC-MS, and HPLC analyses showed the presence of sesquiterpenes and the absence of agarotetrol in Papua New Guinea sample I-2. In addition, the amounts of water-soluble and ethyl-acetate-soluble components were smaller than those from other agarwood samples. To our knowledge, there are no reports that only agarwood from Papua New Guinea has the above characteristics, although there are reports of differences in composition patterns in agarwood collected from different locations. Also, as described above, the pattern of chromone derivative components can change depending on the degree of resin deposition. Furthermore, various factors can be considered, such as the possibility that processed agarwood can be permeated with extract from other agarwood samples under pressure. Further discussion regarding the composition pattern of agarwood will require samples with traceable backgrounds, such as the original plant source and the number of years of resinification.

Cinnamaldehyde and eugenol were detected in diethyl ether extracts of medical-grade agarwood residue. These are likely contaminants transferred into the samples during lengthy storage in the same warehouse as crude drugs containing many volatile aromatic compounds, such as those found in cinnamon and clove [9,10].

Comparison of agarwood samples of various qualities obtained from markets requires knowledge of the composition of medical-grade agarwood. Shimada et al. reported a quantitative analysis of compounds in an acetone extract of agarwood [17]. They observed a rough correlation between the amount of acetone extract and the agarwood grade and a higher correlation between the amount of agarotetrol + isoagarotetrol and the grade of each agarwood sample. In the present study, we measured the amounts of ethyl acetate and water extracts of incense-grade and medical-grade agarwood and found that the amount of extract tended to be higher for incense-grade agarwood. However, we also observed variations between samples, with some medical-grade agarwood samples producing more extract than incense-grade samples. Additionally, the amount of water extract obtained from fresh plants lacking resin formation was very large, consistent with a previous report [18]. This result indicates that the weight of ethyl acetate or water extract alone is insufficient for quality evaluation.

The definition of agarwood in Non-JPS 2018 [1] includes the phrase "emits an aroma when heated". Low molecular weight aromatic compounds such as benzylacetone and benzaldehyde are detected when agarotetrol is heated [6]. Agarotetrol is thus a characteristic of agarwood and plays an important role in the quality evaluation of agarwood. Medical-grade agarwood is consumed orally in the form of a decoction when prescribed in Kampo medicines such as chokoshiteito and zenshikunshito. We prepared and analyzed a decoction of 1.50 g agarwood with 600 mL of water and found 805.4 μg of agarotetrol in the decoction. The pharmacological ingredients in agarwood decoctions remain unknown but agarotetrol, which is water-soluble, is likely one component.

TLC is a common identification method used in the Japanese Pharmacopoeia, Seventeenth Edition (JP17) [19] and Non-JPS 2018 to confirm the quality of crude drugs. Although the target analyte in the TLC test is currently unspecified in the section on the quality evaluation of agarwood, agarotetrol

is likely a useful indicator of quality because it was present at high concentration in all samples of medical-grade agarwood analyzed in this study and may be present in agarwood decoctions. TLC conditions previously used to separate agarotetrol in agarwood extract used chloroform [17,18,20]. However, there is a global trend away from solvents containing halogens due to environmental concerns, and the Japanese Pharmacopoeia also recommends avoiding solvents containing halogens as much as possible. The present study demonstrated the separation of agarotetrol without using chloroform.

The amount of wild agarwood is decreasing. Three genera reported to produce agarwood were listed in Appendix II of the Convention on International Trade in Endangered Species of World Fauna and Flora (CITES) in 2005 [21]. Consequently, farmers in agarwood-producing countries plant agarwood trees and artificially damage them to promote resin deposition. Previous studies reported the detection of chromone derivatives in artificial agarwood [22]. In this report, we also observed the deposition of agarotetrol, suggesting that the same reactions occur in both natural and artificial agarwood.

Conclusion

Agarotetrol was present at high concentrations in all medical-grade samples tested and in agarwood-decoction, but was absent in agarwood plants that lacked resin deposits. We also developed an easy, halogen-free TLC method to detect agarotetrol. Our findings show that agarotetrol is likely characteristic of medical-grade agarwood and can be used as an easily applicable index in quality evaluations of agarwood.

Acknowledgements

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References

1. The Japanese standards for non-Pharmacopoeial crude drugs 2018, The Ministry of Hearth Labour and Welfare, Japan
2. Neaf R (2011). The volatile and semi-volatile constituents of agarwood, the infected heartwood of Aquilaria species: A review. Flavor Fragr. J. 26:73-89
3. Yagura T, Shibayama N, Ito M, Kiuchi F, Honda G (2005). Three novel diepoxy tetrahydrochromones from agarwood artificially produced by intentional wounding. Tetrahedron Letters, 46:4395-4398
4. Kishida K, Ito M (2009). Components of Agarwood Oils and Their Effects on Spontaneous Motor Activity of Mice. Shoyakugaku Zasshi, 63 (2):39-45
5. Ishihara M, Tsuneya T (1993). Compounds of the Agarwood Smoke on heating. J. Essent. Oil Res., 5:419-423
6. Takamatsu S, Ito M (2018). Agarotetrol: a source compound for low molecular weight aromatic compounds from agarwood heating. Journal of Natural medicines 72:537-541
7. Okudera Y, Ito M (2009). Production of agarwood fragrant constituents in Aquilaria calli and cell suspension cultures. Plant Biotechnology 26:307-315
8. Shimada Y, Konishi T, Kiyosawa S, Nishi M, Miyahara K, Kawasaki T (1986). Studies on the Agarwood (Jinko). IV. Structures of 2-(2-Phenylethyl)chromone Derivatives, Agarotetrol and Isoagarotetrol. Chem. Pharm. Bull. 34 (7):2766-2773
9. Kanbe T, Ikuta-Shimadzu N, Hirano T (2005). Difference in the constituents of green tea leaves by storage conditions. Memoirs of Beppu University, 46:91-100
10. Sato Y (2015). Food preparation and off-flavor. Journal of Cookery Science of Japan, 48, No.5:333-341
11. Yagura T, Ito M, Kiuchi F, Honda G, Shimada Y (2003). Four New 2-(2-phenylethyl)chromone Derivatives from Withered Wood of *Aquilaria sinensis*. Chem.Pharm.Bull, 51(5):560-564
12. Yang DL, Mei WL, Zeng YB, Guo KZ, Zhao YX, Wang H, Zuo WJ, Dong WH, Wang QH, Dai

HF (2013). 2-(2-phenylethyl)chromone Derivatives in Chinese Agarwood “Qi-Nan” from *Aquilaria sinensis*. Planta Med, 79:1329-1334
13. Yang JL, Dong WH, Kong FD, Liao G, Wang J, Li W, Mei WL, Dai HF (2016). Characterization and Analysis of 2-(2-Phenylethyl)-chromone Derivatives from Agarwood (Aquilaria crassna) by Artificial Holding for Different Times. Molecules, 21: 911
14. Shao H, Mei WL, Dong WH, Gai CJ, Li W, Zhu GP, Dai HF (2016). 2-(2-phenylethyl)chromone Derivatives of Agarwood Originating from Gyrinops salicifolia. Molecules, 21:1313
15. S.M.C.U.P. Subasinghe, D.S Heharacterisation(2015). Characterisation of agarwood type resin of *Gyrinops walla* Gaertn growing in selected populations in Sri Lanka. Industrial Crops and Products, 69:76-79
16. Shu YQ, Meng LH, Li DL, Chuan HZ, Lan JH, Hui ZZ (2005). Production of 2-(2-phenylethyl) chromones in cell suspension cultures of *Aquilaria sinensis*. Plant Cell, Tissue and Organ Culture, 83:217-221
17. Shimada Y, Tominaga T, Kiyosawa S (1986) Studies on the Agalwood. (Jinko). IV. Correlation between the Grading of Agalwood on the Market and the Chromone Derivatives. YAKUGAKU ZASSHI, 106 (5):391-397
18. Yagura T (2004) Doctoral dissertation, Kyoto University
19. Japanese Pharmacopoeia, Seventeenth Edition, The Ministry of Hearth Labour and Welfare, Japan
20. Konishi T, Okutani Y, Iwagoe K, Kiyosawa S, Shimada, Y (1989). Studies of the Agarwood “Jinko” (IX)[1)] Quantitative Analysis of 2-(2-phenylethyl)chromone derivatives (Supplement). Shoyakugaku Zasshi, 43 (1):1-6
21. ANNUAL REPORT OF THE SECRETARIAT 2004, Convention on International Trade in Endangered Species of Wild Fauna and Flora
22. Huang JQ, Liao YC, Chen HJ, Zhang Z (2017). Chemical solution is an efficient method to induce the formation of 2-(2-phenylethyl) chromone derivatives in *Aquilaria sinensis*. Phytochemistry Letters,19:64-70

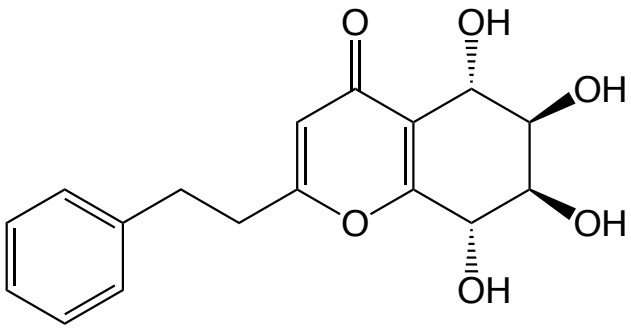
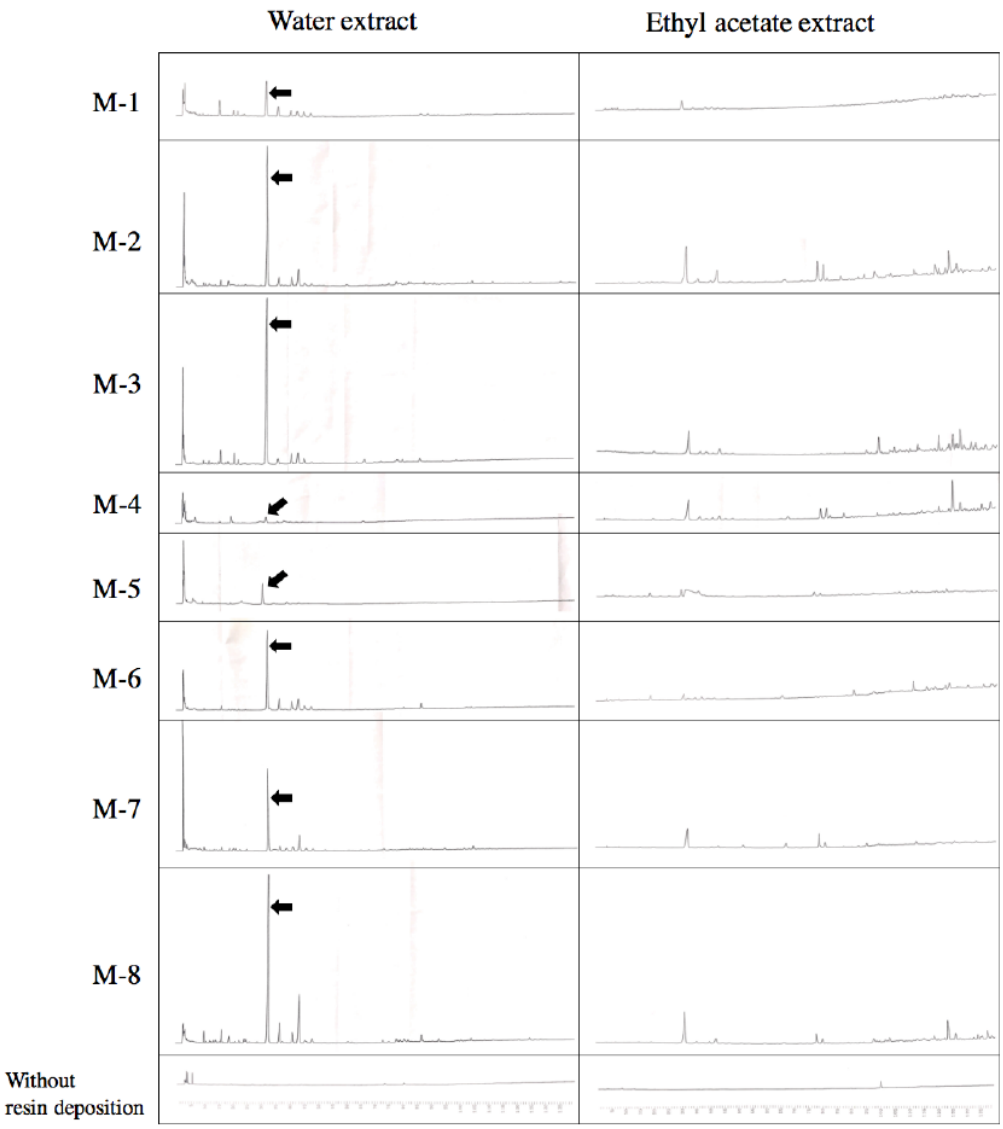


Fig.1 Structure of agarotetrol


Water extract
Ethyl acetate extract
M-1
M-2
M-3
M-4
M-5
M-6
M-7
M-8
Without
resin deposition

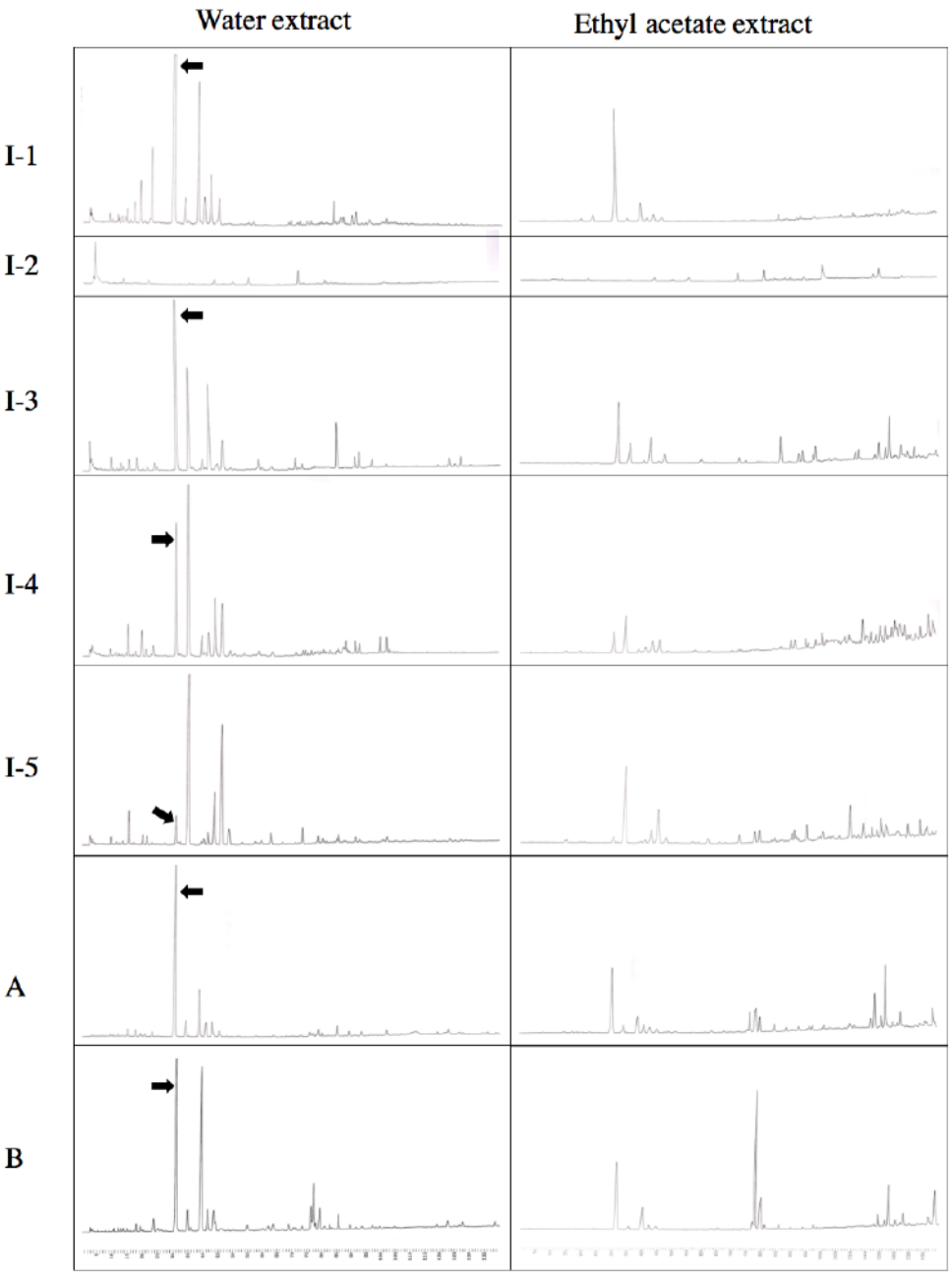


Fig. 2 HPLC chromatograms of water and ethyl ether extracts of agarwood.
Black arrow: Agarotetrol peak (in the water extract chromatogram)

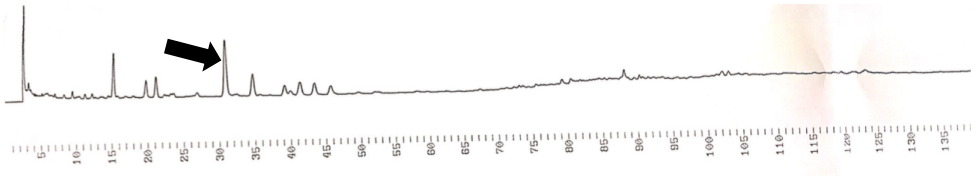


Fig.3 HPLC chromatogram of a decoction of medical-grade agarwood
Black arrow: Agarotetrol peak

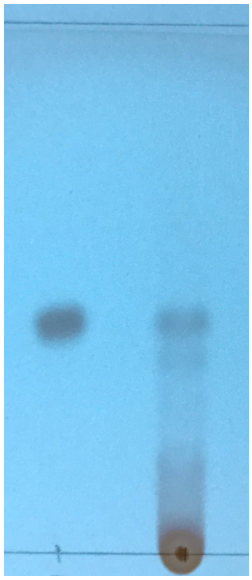


Fig. 4 Detection of agarotetrol by TLC

Table 1 Compounds detected in diethyl ether extracts of agarwood

		Medical-grade agarwood								Incense-grade agarwood					Artificial		Original agarwood lacking resin depositions
	RI	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	I-1	I-2	I-3	I-4	I-5	A	B	
Benzaldehyde	1469	-	-	-	-	-	-	-	-	-	-	0.23	-	-	-	-	-
Valencene	1669	-	-	-	-	-	-	-	-	-	0.08	-	-	-	-	-	-
(-)-α-Panasinsen	1693	-	-	-	-	-	-	-	-	-	2.26	-	-	-	-	-	-
Anethole	1815	-	-	-	0.01	-	0.18	-	-	-	-	-	-	-	-	-	-
Benzylacetone	1830	-	-	-	-	-	-	-	-	-	-	0.28	-	-	3.24	2.44	-
(E)-Cinnamaldehyde	2018	-	10.54	9.25	5.99	11.71	8.49	8.50	6.48	-	-	-	-	-	-	-	-
8-epi-γ-Eudesmol	2051	-	-	-	-	-	-	-	-	-	-	7.79	-	-	-	-	-
Eugenol	2076	-	11.85	23.77	12.10	19.08	37.90	-	6.10	-	-	-	-	-	-	-	-
γ-Eudesmol	2082	-	-	-	-	-	-	-	-	-	-	1.05	-	-	-	-	-
Hinesol	2090	-	-	-	-		-	-	-	-	-	0.07	-	-	-	-	-
α-Eudesmol	2208	-	-	-	-		-	-	-	-	-	1.20	-	-	-	-	-
β-Eudesmol	2212	-	-	-	-		-	-	-	-	2.89	2.88	-	-	3.57	-	-

*RI= retention index on a DB-WAX column

‘-‘ signifies not detected

Numbers are peak area% quantified by their FID peak area

Table 2　Weight% of agarwood ethyl acetate and water extracts

Samples	Ethyl acetate extract of agarwood	Average (M-1~8 and I-1~5)	Water extract of agarwood	Average (M-1~8 and I-1~5)
M-1	0.80		1.63	
M-2	9.29		2.75	
M-3	7.11		2.76	
M-4	7.40	5.66	2.14	2.26
M-5	1.19		2.80	
M-6	5.16		2.35	
M-7	6.24		1.81	
M-8	8.07		1.86	
I-1	7.65		3.65	
I-2	11.19		4.20	
I-3	13.27	8.54	3.42	3.09
I-4	5.03		2.16	
I-5	5.54		2.04	
Artificial agarwood A	11.18		7.38	
Artificial agarwood B	13.76		4.38	
Without resin deposition	2.40		7.99	

Values expressed as a weight percentage of agarwood powder