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Contributed Paper

Supercritical CO_2 Extraction and Characterization of Agarwood Extract Derived-from Vietnamese *Aquilaria crassna* Woodchips

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ABSTRACT

The agarwood extract was extracted from woodchips of cultivated non-inoculated agarwood trees *Aquilaria crassna* Pierre ex Lecomte by means of supercritical carbon dioxide (SCO_2) extraction. Response surface methodology (RSM) was used to optimize the parameters of the SCO_2 extraction. The yield of SCO_2 extraction was chosen to be response variable, and independent variables were temperature (30, 45 and 60°C), pressure (100, 115 and 130 bar), and extraction time (1.0, 3.5 and 6.0 h). The optimal factors were observed at 37°C for extraction temperature, 107 bar for pressure and 5.7 h for extraction time and maximum extraction yield was 0.317 % (w/w). The volatile compounds of the agarwood extract were analyzed by gas chromatography–mass spectrometry (GC-MS). Besides, agarwood SCO_2 extract was separated repeatedly by silica gel column chromatography. Two compounds were successfully purified, and their structures were interpreted by spectroscopic methods. One of them, 4-methoxylonchocarpin has been found in the SCO_2 extract of *A. crassna* for the first time.

Keywords: *Aquilaria crassna*, agarwood extract, β-sitosterol, 4-methoxylonchocarpin, SCO_2 extraction

1. INTRODUCTION

Aquilaria genus is an archaic tropical tree widely distributed in South and Southeast Asia. *Aquilaria crassna* Pierre ex Lecomte is principally occurred in Indochina area including Vietnam [1–3]. Agarwood from *Aquilaria* spp. is considered to be a pathological product produced by fungi naturally or artificially inoculation methods and is known as famous incense. It is used as a sedative, analgesic, digestive, cardiatonic and carminative medicine in traditional medicine [2]. Agarwood oil is also used widely in the cosmetic and pharmaceutical industries [1, 3]. Previous studies have found that agarwood are rich in sesquiterpenes of spirovetivane-, eudesmane-, guaine-, and eremophilane-derivatives and chromone derivatives [3–8]. Agarwood oil and

other essential oils have been produced by using of various techniques such as hydro-distillation, steam-distillation, solvent extraction and supercritical fluid extraction [2, 9–12].

Supercritical carbon dioxide (SCO_2) extraction is a processing technique for the extraction of flavor, fragrance and bioactive compounds from natural resources. Recently, this has been regarded as a promising alternative method for the extraction and isolation of natural products [10, 11]. The SCO_2 is a solvent with low critical temperature and pressure (Tc = 31.1°C; Pc = 72 bar), hence it is considered to an excellent non-polar solvent for extractions with some advantages such as: i) higher diffusion and improved mass-transfer rates than liquid solvents, ii) lower viscosity than liquid solvent, iii) higher vapour pressure than liquid solvent, iv) higher density compared to gases, v) low polarity of carbon dioxide can be modified with co-solvents, and vi) solubility and selectivity can be operated by changing the pressure and temperature [9–12]. In addition, the extraction with SCO_2 is able to prevent labile compounds from thermal degradation and solvent residual contamination [9, 11].

The application of SCO_2 to industrial scale including food, cosmetic, pharmaceutical and chemical industry is now extended [9, 11]. Those applications are not only economic, but also safe to handle and physiologically sound to the very low residual levels in its products because CO_2 is non-toxic and non-flammable, non-corrosive, low-cost, readily available at high purity, and easy to be recovered and reused. There are many applications and studies involving the SCO_2 extraction such as extraction of essential oils and valuable products from ginger, jasmine, vetiver, and grapefruit flower concrete [10, 12–15]. However, the optimization of SCO_2 extraction of *A. crassna* and identification of phytochemicals in the extract have not yet investigated and reported.

The main objectives of this study were to determine the effects of pressure, temperature and extraction time on SCO_2 extraction of Vietnamese agarwood (*A. crassna*) and to find out the optimum conditions by response surface methodology (RSM). In addition, its volatile constituents and non-volatile compounds was analyzed and characterized by chromatographic and spectroscopic methods.

2. MATERIALS AND METHODS

2.1 Materials

The agarwood chips of *A. crassna* collected in Da Nang province of Vietnam was provided by Secoin group (Hanoi, Vietnam). The agarwood plants used in the SCO_2 extraction experiments were determined to be cultivated non-inoculated *A. crassna*. The carbon dioxide uses for the SCO_2 extraction was 99.98 wt % pure and purchased from Viet Anh Co. Ltd (Hanoi, Vietnam).

2.2 Experiment Apparatus and Procedure

SFT-250 SFE processing system used in the extracting experiment was manufactured by Supercritical fluid technologies Inc, USA (Supplementary Figures S12-S13). Liquid carbon dioxide from a cylinder passed through a cooling bath (Julabo F12) with temperature of about 0 - 1°C, and then was compressed to the desire pressure by a diaphragm compressor (INGERSOLL-Rand T30) with a maximum rating of 10,000 psi (689.5 bar) into a extraction vessel (2000 ml) with a heating tape by a high pressure pump. The pressure in the extractor was measured by a digital pressure transducer and maintained by a back-pressure regulator therefore the pressure in the system is monitored at the pump and the back-pressure regulator. The temperature was monitored continuously by a thermocouple. The mixture of SCO_2 and extract went through a restrictor valve in which the pressure reduced to the atmosphere level and the extract is collected in a 400 ml collection vessel with a heat coat. The temperature of collection vessel was set at 35-40°C.

A 100 grams ground sample with the moisture of 13.203% was loaded into the 2000 ml-extraction vessel in each experiment. The extraction was then

conducted at varied parameters of temperature (°C), pressure (bar) and extraction time (h) based on the Box-Behnken design (Table 1).

2.3 Experiment Design

On the basis of single - factor experiment for the SCO_2 extraction of agarwood, proper ranges of extraction temperature (30, 45 and 60°C), pressure (100, 115 and 130 bar) and extraction time (1.0, 3.5 and 6.0 h) were preliminarily determined. The process parameters were studied for optimization of agarwood SCO_2 extraction on the basis of response surface methodology (RSM) following a Box–Behnken design [12, 16, 17]. The Box–Behnken design with 3 factors, three levels and 15 runs and the experimental points used according to this design are shown in Table 1. For the convenience of solving for coefficients in the design, the independent variables were coded as -1, 0, +1. The second-order regression equation obtained from this design was applied to express the SCO_2 extract yield as a function of the independent variables as follows:

$$\begin{aligned} Y = \; & b_0 + b_1 \times T + b_2 \times P + b_3 \times h \\ & + b_{12} \times T \times P + b_{13} \times T \times h \\ & + b_{23} \times P \times h + b_{11} \times T \times T \\ & + b_{22} \times P \times P + b_{33} \times h \times h \end{aligned} \quad (1)$$

Where Y is the predicted response, b_0 is constant, and bi, bij, and bii are the linear, cross, quadratic factor interactive coefficients, respectively. The independent variables are temperature (T), pressure (P) and extraction time (h). The coefficients of the equation were calculated by using Design Expert software version 10.0.8 (Stat-Ease, Inc, USA). The significance of each coefficient was verified by the P-value at 95% confidence level [13, 17]. The P-values of less than 0.05 were considered to be statistically significant. Three-dimensional surface response plots were generated by varying two variables while the experiment range and

Table 1. Box-Behnken design of experiments and experimental data.[1]

No	T	P	t	Observed	Predicted	Obs - Pred	Conf. int(±)
1	-1	-1	0	0.178	0.176	0.002	0.026
2	1	-1	0	0.110	0.108	0.002	0.026
3	-1	1	0	0.202	0.204	-0.002	0.026
4	1	1	0	0.104	0.106	-0.002	0.026
5	-1	0	-1	0.111	0.119	-0.008	0.026
6	1	0	-1	0.131	0.140	-0.009	0.026
7	-1	0	1	0.297	0.288	0.009	0.026
8	1	0	1	0.109	0.101	0.008	0.026
9	0	-1	-1	0.226	0.220	0.007	0.026
10	0	1	-1	0.264	0.253	0.011	0.026
11	0	-1	1	0.294	0.305	-0.011	0.026
12	0	1	1	0.291	0.298	-0.007	0.026
13	0	0	0	0.284	0.287	-0.003	0.017
14	0	0	0	0.287	0.287	0.000	0.017
15	0	0	0	0.291	0.287	0.004	0.017

[1] T: temperature (°C), P: pressure (bar), and t: extraction time (hour), Observed: yield observed (%), Predicted: yield predicted (%) and Conf.int: Confident interval.

holding the other variable.

2.4 GC-MS Analysis of Volatile Constituents

The volatile compounds in SCO_2 extract was analyzed by using GC-MS (HP GC-MS 5890B SERIES II; Hewlett- Packard Ltd., Bracknell, United Kingdom). The analytical conditions were as follows: column, HP-5 column (30 m × 0.32 mm, 0.25 μm film thickness); column temperature, 60°C for 2 min and then increased to 280°C at 4°C min^{-1} ; injector temperatures, 250°C; ion source temperature, 280°C; ionizing voltage, 70 eV.

2.5 Isolation and Structural Analysis of non-Volatile Compounds from SCO_2 Extract

A 500-mg of SCO_2 extract was subjected to a silica gel column (63-100 mesh) using mixtures of *n*-hexane/EtOAc with increasing polarity (99/1 to 80/2, gradient 5%) as the mobile phase. This afforded fractions A1 to A10. Fraction A3 was chromatographed over a silica gel (63-100 mesh) column with a solvent system of *n*-hexane/EtOAc = 95/5 to afford 11 fractions (A31-A311). Fraction A310 was further re-chromatographed on a silica gel column (63-100 mesh, 6 x 230 mm) using *n*-hexane/DCM = 7/3 as eluent to yield AF3 (R_f =0.2, *n*-hexane/DCM = 7/3). AF3 was purified by recrystallisation method to obtain 3 mg of **AF3**, yellow needles.

Fraction A7 was separated on a silica gel column with a mixture of *n*-hexane/DCM/AC = 50/50/0.5 as eluent to afford 7 fractions (A71-A76). Fraction A75 was re-chromatographed by FC method on a silica gel column (6 × 120 mm) using *n*-hexane/DCM/AC = 50/50/0.1 as eluent to obtain 5 mg **AF75**, white needles, after being recrystallized.

TLC analysis of sub-fractions was performed on precoated silica gel F254 aluminum plates (Merck, 0.25 mm) and visualized by spraying with a mixture of methanol/ acetic acid/ sulfuric acid /anisaldehyde (85/10/5/0.5) followed by heating for 4-6 min.

Chemical structures of the pure compounds were determined by spectroscopic methods. The 1D and 2D NMR spectra of non-volatile compounds were recorded on a Bruker AV500 NMR spectrometer (^{1}H-500 MHz; ^{13}C 125 MHz), using TMS as internal standard and $CDCl_3$ as solvent. Mass spectrometry (MS) spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JMS-DX303; Jeol Ltd., Tokyo, Japan).

3. Results and Discussion

3.1 Optimization of the Experimental Conditions for Agarwood SCO_2 Extraction

The optimization of the experimental conditions for the SCO_2 extraction was studied by RSM following with three independent variables of the pressure, temperature and extraction time. Box–Behnken design was planed with the aid of Design Expert software version 10.0.8 to determine the settings of factors that would result in the optimum value of the response. The P-value was used to check the significance of each coefficient, which indicates the pattern of the interactions between the variables. The linear coefficients (T, h), quadratic term coefficients (T^2, P^2) and cross product coefficients (T×h) were significant, with very small P-values ($P < 0.05$). The other coefficients were not significant ($P > 0.05$) (data not shown). The model adequacy was checked by an F-test and the determination coefficient R^2. The analysis of variance showed that this regression model was highly significant ($P < 0.01$) with F-value of 76.32. The parameter "model validity" (which is a measure of the lack of fit) has been considered good, because the F-value of 17.07 for lack of fit with $P > 0.05$ is over 0.25 implied that it is not significant comparing to the pure error. Furthermore, the fitness of the model was confirmed by a satisfactory value of determination coefficient $R^2 = 0.993$), indicating that 99.30% of variability in the response could be predicted by the model (Figure 1). It was considered reasonable to use the regression model

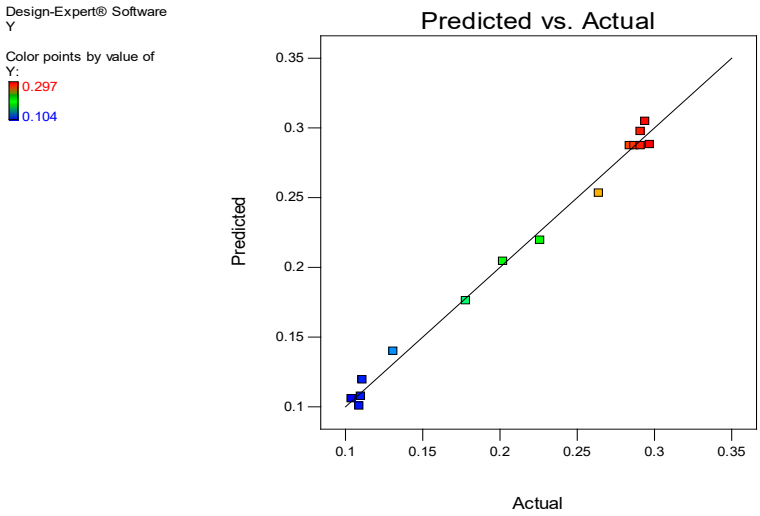

Figure 1. Model (predicted) vs. experimental values process parameters on the extraction yield of agarwood SCO_2 extract.

to analyze trends in the responses. The value of the adjusted determination coefficient (Adj R^2 = 0.980) also confirmed that the model was highly significant. The yield of extract has been found to fit a second order equation as follows:

Final equation in terms of coded factors:

$$Y = 0.2873 - 0.0417\times T + 0.0323\times h - 0.1228\times T^2 - 0.016\times P^2 - 0.052\times T\times h \quad (2)$$

Final equation in terms of actual factors

$$Y = -2.23745 + 0.055\times T + 0.0192\times h - 5.457\times T^2 - 7.1296\times P^2 - 1.1386\times T\times h \quad (3)$$

The effects of pressure, temperature and extraction time on the yield were presented in the response surface plots. The zone of optimum values of the response surface was shown on three-dimensional surface response plots (Figure 2).

The optimization result for maximum extraction yield was predicted as follows.

Y_{max} = 0.320 % at T_{opt} = 36.6 °C ;

P_{opt} = 106.86 bar; t_{opt} = 5.762 h

The recognition of the optimal points was achieved by performing validation experiments under the optimal predicted parameters. The experiment was repeated twice and the validation showed that the maximum yield of 0.317 % (w/w) was repeated at 37°C, 107 bar and 5.7 h.

In previous reports, the yields by SCO_2 extraction are found to be higher than those by hydro-distillation since the SCO_2 extracts contain non-volatile compounds [9]. The SCO_2 extraction of vetiver roots produced a vetiver extract with a yield of 0.625% (w/w) at 60°C, 116.8 bar and ca. 7 h, while hydro-distillation experiment just archived a yield of 0.5% (w/w) [15]. About 1.5 - 3% yield of ginger essential oil obtained by the hydro-distillation of ginger roots [9]; however, in a previous study, we extracted oleoresin of ginger by SCO_2 extraction with a yield of 6.22% (w/w) and the main constituents in SCO_2 extract were identified as (6)-, (8)- and (10)-gingerols [14].

In the present study, compared with the yield of essential oils obtained by hydro-distillation (0.061%) [18], the extraction yield of agarwood

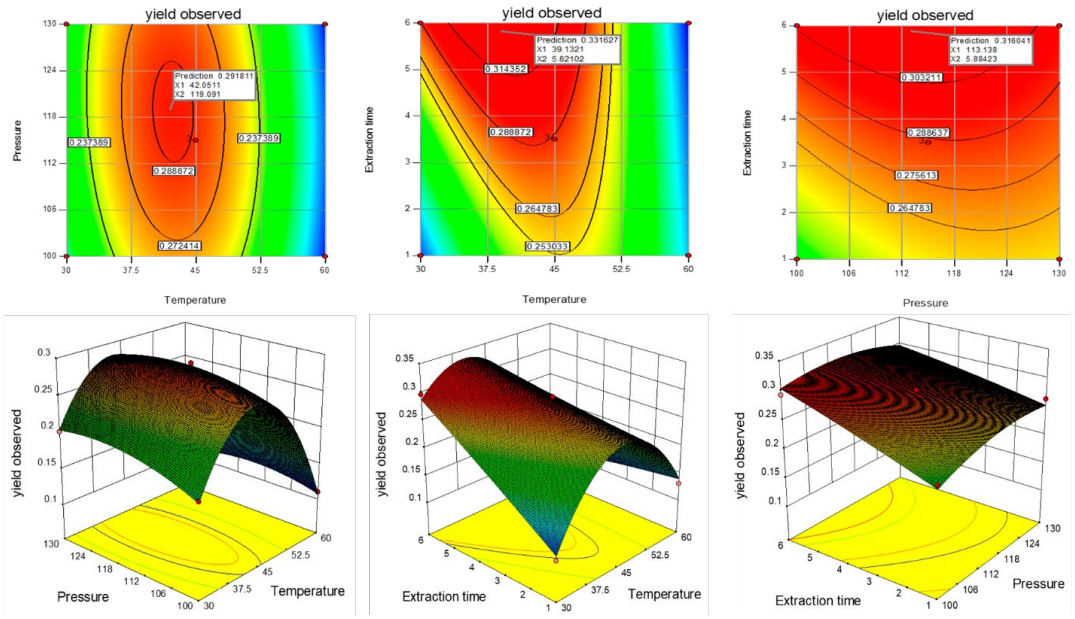

Figure 2. 3D response surface plot and contour diagram of three-dimensional response surface plots representing the effects of process parameters on the extraction yield of agarwood SCO_2 extract. (A) The effect of pressure and temperature on the yield of SCO_2 extract at extraction time of 3.5 h; (B) The effect of temperature and extraction time on the yield of SCO_2 extract at pressure of 115 bar; (C) The effect of pressure and extraction time on the yield of SCO_2 extract at temperature of 45°C.

SCO_2 extract at the optimal conditions (0.317 % (w/w)) is much higher. In the facts of commercial productions and our experiments, the hydro-distillation took time more than 48 h for reaching to the exhausting stage, and thus the SCO_2 extraction time of 5.7 h is an extreme advantage for recovering the product.

3.2. Volatile Constituents in Agarwood SCO_2 Extract

The yield of SCO_2 extract at the optimal conditions was 0.317 % (w/w) during extraction time of 5.7 h. The volatile composition of SCO_2 extract was also listed in Table 2. A total 29 compounds have been determined in the SCO_2 extract. The composition of SCO_2 extract is more abundant and its individual quantities of oxygenated sesquiterpenes were significant. Some oxygenated sesquiterpenes, which make odor of product more valuable, such as 3-thujanol; nootkatone; 1,3-elemadien-11-ol; 10-aromadendranol and 1(10),11-spirovetivadien-2-one were detected in the SCO_2 extract. Besides, some heavy hydrocarbons, waxes (C14, C18, C20, C32) were extracted by SCO_2, making effects on the density and melting point of the extract. However, it also showed that SCO_2 extraction was both selective and partly efficient in the isolation of sesquiterpenes and hydrocarbon from the agarwood sample.

The volatile constituents of the SCO_2 extract was compared with those of agarwood oils from high grade agarwoods, which were published in the literatures [2–4, 16, 18]. It was found that noticeable components including 10-epi-γ-eudesmol, α(β)-eudesmol were absent, whereas 2-bornanol cinnamate and cinnamyl cinnamate were found in the SCO_2 extract. This is similar to the Tamuli's claims of the occurrences of the

Table 2. The volatile constituents of agarwood SCO_2 extract analyzed by GC-MS.

No	Composition	CAS	RT	(%)
1	2,7(14),10-Bisabolatriene	495-61-4	24.50	0.54
2	α -Agarofuran	5956-12-7	26.16	0.81
3	3-Thujanol	21653-20-3	27.30	2.04
4	3-Cedranol	77-53-2	27.92	2.94
5	Santolina alcohol	35671-15-9	28.35	0.41
6	3-Thujopsene	470-40-6	28.47	1.02
7	Benzophenone	119-61-9	28.63	0.94
8	1(10)-Spirovetiven-11-ol (Hinesol)	23811-08-7	28.92	3.00
9	1(5),11-Guaiadiene	3691-12-1	29.16	1.84
10	1(5)-Guaien-11-ol	489-86-1	29.40	2.60
11	6,7-Epoxy-3(15)-caryophyllene	1139-30-6	30.52	0.81
12	Vitrenal	77283-84-2	33.54	0.99
13	1(10),11-Eremophiladien-2-one (Nootkatone)	4674-50-4	34.59	2.05
14	1,3-Elemadien-11-ol	639-99-6	35.97	0.92
15	2,6,10-Bisabolatriene	13062-00-5	36.95	1.12
16	10-Aromadendranol (Globulol)	489-41-8	37.11	1.52
17	1(10),11-Spirovetivadien-2-one	54878-25-0	37.69	2.20
18	Limonene diepoxide	96-08-2	39.83	0.95
19	Tetradecane	629-59-4	37.81	2.11
20	Octadecane	593-45-3		4.55
21	Eicosane	112-95-8	38.53	3.31
22	Triacontane	638-68-6	43.59	3.10
23	Isobomyl cinnamate	41755-67-3	44.51	10.97
24	Cinnamyl cinnamate	122-69-0	44.88	2.31
25	3-Phenyl propanitrile	645-59-0	47.68	1.18
26	Dotriacontane	544-85-4	48.54	2.58
27	Nonaconsane	630-03-5	50.22	1.26
28	Octaconsane	630-02-4	52.10	1.63
29	Heneicosane (9CI)	629-94-7	54.30	2.01

RT: Retention time

components in the oil of healthy and cultivated non-inoculated agarwood plants [3].

3.3 Analysis of non-Volatile Components in Agarwood SCO_2 Extract

Compound **AF3** (Figure 3A) was isolated and crystallized as yellow needles (m.p 129-130°C). The EI-MS spectrum of **AF3** indicated the presence of a molecular peak at m/z 336[M^+] together with ^{1}H- and ^{13}C –NMR spectral data (Table 3, Supplementary Figures S1-S2 and S7) suggested that compound **AF3** had the molecular formula of $C_{21}H_{20}O_4$. The ^{13}C-NMR and DEPT data showed that the structure of **AF3** included two methyls, ten methines, a quaternary carbon, a ketone, and two alkenyl carbons (Supplementary Figures S2-S3). The chemical shift of carbon signal at δ 192.0 ppm in the ^{13}C-NMR spectrum and the highly deshielded signal at δ 13.77 ppm (*s*) in the ^{1}H-NMR spectrum were noted as evidence for the presence of β-hydroxyl and conjugated carbonyl moiety. Two of the proton signals formed an AB system at δ 7.43 and 7.84 ppm (1H, *d*, J = 16.0 Hz), where the large coupling constant indicated the *trans* geometry of a double bond. The ring B was monosubstituted at C-4 due to the presence of an AA'BB' system at δ 6.95 ppm (2H, *d*, J = 9.0 Hz) and 7.61 ppm (2H, *d*, J = 9.0 Hz). An AX system at δ 6.37 and 7.71 ppm (1H, *d*, J = 9.0 Hz) were assigned to H-5' and H-6' together with the presence of a singlet at δ 1.47 (6H, *s*) integrating for 2 methyl groups, suggested the presence of a cyclized dehydrogenated prenyl side chain. Two proton signals at δ 5.59 and 6.75 (1H, d, J = 10.0 Hz) were observed in the HSQC and ^{1}H-^{1}H COSY experiments indicated the *cis* geometry of a double bond between C-3" and C-4" (Figure 3B). Heteronuclear correlations between the proton signal at δ 3.86 ($OC\underline{H}_3$, *s*) and the carbon signal at δ 161.8 (C-4) in the HMBC experiment further confirmed the presence of a methoxy (OCH_3) group at C-4 position in AF3 (Figure 3B). Therefore, on the basis of the foregoing spectroscopic data, compound **AF3** was identified as 4-methoxylonchocarpin (Figure 3A). The spectroscopic data attributed to **AF3** (Table 3) were fully in agreement with the literature data reported [19].

Compound **AF75** is white needles (m.p140-141°C). The EI-MS spectrum of **AF75** indicated the presence of a molecular peak at m/z 414 [M^+] and fragments at m/z 396, 381, 329, 303, 289, 273, 255, 213, 159, 145, 119, 95, 81, 69, 55; this suggested the molecular formula of $C_{29}H_{50}O$ and fragmentation of a stigma sterol. ^{1}H- and ^{13}C –NMR data of protons and carbons for AF75 was assigned on the basis of 1D-NMR spectra and resembled to those of β-sitosterol (stigmast-5en-3β-ol) (Figure 3C, Supplementary Figures S8-S10) that published previously [20].

Out of the two isolates, β-sitosterol is a common phytosterol that usually occurs in vegetable oils such as soybean, peanut and avocado oils. The sterol is considered to agent lowers serum

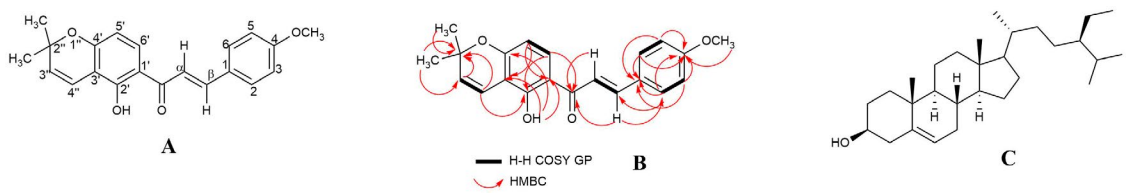

Figure 3. Chemical structure of 4-methoxylonchocarpin (A), ^{1}H-^{1}H DQF COSY and HMBC correlations of 4-methoxylonchocarpin (B) and β-sitosterol (C).

Table 3. NMR data for 4-methoxylonchocarpin isolated from the SCO_2 extract of *A. crassna.*

C	^{1}H NMR[a]	^{13}C NMR[b]	HMBC (H to C)[c]
1	-	127.6 (*s*)	
2	7.61 (1 H, *d, J*=9 Hz)	130.3 (*d*)	C-4,1, α
3	6.95 (1 H, *d, J*=9 Hz)	114.5 (*d*)	C-1, 4
4	-	161.8 (*s*)	
5	6.95 (1 H, *d, J*=9 Hz)	114.5 (*d*)	C-1, 4
6	7.61 (1 H, *d, J*=9 Hz)	130.3 (*d*)	C-4,1, α
β	7.84 (1 H, *d, J*=16)	144.1 (*d*)	C-2,6, β'
α	7.43 (1 H, *d, J*=16)	117.9 (*d*)	C- β'
CO	-	192.0 (*s*)	
1'	-	109.5 (*s*)	
2'	-	159.7 (*s*)	
3'	-	114.1 (*s*)	
4'	-	160.9 (*s*)	
5'	6.37 (1H, *d, J*=9 Hz)	108.2 (*d*)	C-1', 3'
6'	7.71 (1H, *d, J*=9 Hz)	130.6 (*d*)	C-2',4', β'
2"	-	77.79 (*s*)	
3"	5.59 (1H, *d, J*=10 Hz)	128.1 (*d*)	C-2"
4"	6.75 (1H, *d, J*=10 Hz)	115.9 (*d*)	C-2", 2'
2Me	1.47 (6H, *s*)	28.39 (*q*)	C-2", 3"
-OH	13.77 (*s*)		C-1'.3'.4'
-OMe	3.86 *(s)*	55.45 *(q)*	C-4

[a]500 MHz, [b]125 MHz, [c]100 MHz, optimized for JCH = 8.3 Hz.

cholesterol and can be used as neutraceutical foods. β-Sitosterol and analogous sterols were found to be extracted with SCO_2 from plants and other bio-resources [9]. **AF3**, also known as 4-methoxylonchocarpin (Figure 3A), was isolated from some medicinal plants of genus *Dorstenia* and seed of insecticidal plant *Millettia pachycarpa* [19, 21]. The chemical structure of 4-methoxylonchocarpin is chalcone's type and seems to be analogs of chromone derivatives that were isolated from *A. agallocha, A. crassna* and *A. sinensis* [8, 22]. However, this paper is the first report on occurrence of 4-methoxylonchocarpin in *A. crassna.* This compound was reported recently that it possesses valuable bioactivities such as antioxidant, antitumour, antimalarial and antimicrobial activities [21, 23]. Mbaveng et al., (2008) also showed that 4-methoxylonchocarpin has a wide-spectrum and good antimicrobial activity against pathogenic bacteria and fungi. This was found to be active on gram-positive strains *Streptococcus faecalis* (MIC 4.9 μg/mL), *Staphylococcus aureus* (MIC 4.9 μg/mL); *Bacillus cereus* (MIC 4.9 μg/mL), *Bacillus megaterium* (MIC 1.2 μg/mL), *Bacillus stearothermophilus* (MIC 1.2 μg/mL), *Bacillus subtilis* (MIC 4.9 μg/mL), gram-negative strains *Enterobacter cloacae* (MIC 1.2 μg/mL), *Morganella morganii* (MIC 1.2 μg/mL); *Shigella flexneri* (MIC 4.9 μg/mL) and fungi such as *Candida albicans,Candida gabrata, Trichophyton rubrum* [23]. This supports the traditional use of

agarwood products from *A. crassna* and together with such antimicrobial activity, our results indirectly suggested to conduct further studies on the role of 4-methoxylonchocarpin in co-relationships of pathological products as agarwood and the mechanism of pathogenic resistance for the *A. crassna* trees.

4. Conclusions

Response surface methodology was successfully applied for the optimization of the SCO_2 extraction of agarwood extract from *A. crassna*. The fitted second-order regression equation and the optimal conditions for the extraction were determined. The yield of agarwood SCO_2 extract (0.317%) was much higher than that of essential oil; besides, the composition of the oxygenated sesquiterpenes in SCO_2 extract was more abundant and the terpene hydrocarbons which decrease the quality of the product were lower.

Non-volatile compounds in SCO_2 extract were identified as 4-methoxylonchocarpin and β-sitosterol. Particularly, 4-methoxylonchocarpin was found in the SCO_2 extract of *A. crassna* and *Aquilaria* genus for the first time.

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Supplementary materials

Supplementary materials. Spectra of AF3 (1D and 2D-NMR and MS), Spectra of AF75 (1D-NMR), Gas chromatogram of agarwood SCO_2 extract and Photos of the SFT-250 SFE processing system.

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