Chemical composition and anticancer activities of methanol-extracted agarwood (Gyrinops verstegii [Gilg.] Domke)

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Abstract. This research aimed to study about chemical composition and anti-cancer activities of natural agarwood and cultivated agarwood (Gyrinops verstegii [Gilg.] Domke). Agarwood used in the research was of lowest qualities, which comprised agarwood with natural kemedangan type (A), with cultivated kemedangan-I type (B1), and with cultivated kemedangan-II type (B2), all after methanol extraction. Chemical composition was examined using GC-MS instrument, meanwhile tests on lungs associated anticancer activities (A549’s cancer cells) were performed using MTT method. Chemical composition in low-quality agarwoods was predominantly sesquiterpene compounds, comprising among others guaiacol, cumene, aromadendrene, alpha-humulene, velleral, etc; and conservely did not contain chromone derivative compounds which are compounds characterizing quality agarwood. Low-quality agarwood extracts afforded efficacious potency as anticancer actions against A549’s lungs-attacking cancer cells with IC₅₀ values at 144.92 µg mL⁻¹ (A); IC₅₀ at 206.88 µg mL⁻¹ (B1), and IC₅₀ 187.97 µg mL⁻¹ (B2).

1. Introduction

Agarwood typifies as one commodity of Non-Wood Forest Products (NWFP). Agarwood commodity is very valuable, due to the presence of sweet-smelling resins. Agarwood resins are found in particular portions of several plant species (e.g., trunks, stems, twigs, and roots) of the genus Aquilaria, Gyrinops, Aetoxylon, Wikstroemia, Enkleia, and Gonystylus, under the family Thymelaeaceae [1,2]. Currently, agarwood commodity is beneficial as perfumery items, drugs, aroma therapy, incenses, religious rituals, coughing medicine, antimicrobe, antitumor, and anticancer items [3-6].

Agarwoods of the genera Aquilaria and Gyrinops signify as the genera that can produce best agarwood commodity in Indonesia, which covers A. malaccensis, A. filaria, and G. verstegii species [7]. Those agarwoods typify as endemic species in Indonesia, which have spread out in particular Indonesia’s regions, among others Lombok, Sumbawa, Sumba barat, Alor, Flores even until Sulawesi, Maluku, and Papua [8,9]. G. verstegii population nowadays tends to decrease, particularly those in West and East Nusa Tenggara provinces [10,11]. The main cause for their decreasing population in that region is brought about by over exploitation to harvest agarwood commodity (wood resins). Such exploitation occurs frequently by cutting off agarwood’s host tree stands [11]. One effort to increase the population of G. verstegii species is by replanting actions, which have been performed intensively in West Java, Central Java, East Java, West Nusa Tenggara, East Nusa Tenggara, Gorontalo, and Papua [12].
Information about genera *Gyrinops* is still relatively only a little or meager, compared to the information about genera *Aquilaria*; and even according to the agarwood traders, they are in fact more familiar with agarwoods of the genera *Aquilaria* than those with other genera. Relevantly, this research has been conducted to provide information about chemical composition and efficacy of anticancer activities of *G. versstegii*’s agarwoods, particularly the activities of lowest-quality *G. versstegii*. This is because the presence of low-quality *G. versstegii* species is in fact more numerous, compared to the presence of high-quality species. The related details are forthcoming.

2. Materials and Methods

2.1. Materials

Agarwood as main materials used for this research was of *G. versstegii* species, indigenous from Lombok, West Nusa Tenggara, Indonesia. In characteristics, the selected *G. versstegii* were agarwoods with lowest quality, which comprised natural kemedangan (A), cultivated-kemedangan 1 (after 6 months of inoculation) (B1), and cultivated-kemedangan 2 (after 9 months of inoculation) (B2) qualities. Those low-quality agarwoods were visually presented in Figure 1.

![Figure 1. Agarwood with “kemedangan” or lowest quality (A, B1, and B2 types).](image)

2.2. Extraction of agarwoods

The initially, agarwood sample was shaped into powder form. The agarwood powder was then filtered through a screen having the holes of 80-mesh to obtain the filtered agarwood powder with uniform size. The resulting filtered powder was further ready for extraction using methanol solvent in a continuous hot extractor of the Soxhlet apparatus. As such, as much as 10 g of filtered agarwood powder was taken, and then packed into an extraction thimble (equipped with fritted-glass disk). Afterwards, the agarwood-filled thimble was inserted into the Soxhlet extractor apparatus, wherein the agarwood extraction was performed using as much 100 mL of methanol as extracting solvent. The heater mantle for the hot extractor was equipped with the thermostat for constant heating of the Soxhlet flask (inside which the methanol solvent was placed). The thermostat was set to maintain constant temperature at 65-70°C (which approximately corresponded to the boiling point of methanol). As such, the methanol extraction lasted for 8 hours. The obtained agarwood’s methanol extract (liquid form) was then collected into a conical flask, while the extracted agarwood powder that remained in the extraction thimble was discarded. Further, the collected agarwood’s methanol extract was sieved using filter paper, and then repeatedly washed with distilled water (under suction). The washing was terminated when the residual washing water passing out through the sieving media (filter-paper) looked apparently clear or colorless as indication that the agarwood extract became free of impurities; and in this way the obtained sieved extract was called as agarwood’s crude extract. Afterwards, the impurity-free crude extract was stored temporarily in the cool dark room for further investigation (particularly for its possible activity as anticancer agent).

2.3. Analysis on chemical compounds in agarwood extract

Agarwood extract was analyzed for its chemical compounds using pyrolysis-GCMS device (GC/MS-QP2010 SHIMADZU). Related to the analysis, the oven temperature was maintained at 50°C for 2 min and then elevated from 50°C to 280°C at a rate of 2°C/min. The mass spectrometer was operated in
an electron impact (EI) mode at 70 eV. The compounds were then identified by comparing the results with values in the Wiley 7n computer library.

2.4. Tests on anti-cancer activities of agarwood extracts [13]
Cancer cells used in the study were A549’s lungs-attacking cancer cells (ATCC CCL 185) obtained from the Primate Study Center, Bogor Agriculture University in Indonesia. Cancer cells as much as 100 mL (5×10^4 cells/wells) were inoculated in Dubecoo’s Modified Eagle’s Medium (DMEM) for 24 hours. A total of 100 mL with a concentration of active agarwood extracts (regarded as anti-cancer agents) at 0 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL was added to the inoculant then incubated in CO₂ incubator at 37°C for 24 hours. Further, MTT (3 - [4,5-dimethylthiazol-2-yl] 2,5-difeniltetrazolium bromide) as much as 100 μL was added in each well and incubated for 4 hours at 37°C. The absorbances of formazan were measured by ELISA reader at 595 nm wavelength. The inhibition (in percentage) in the growth of lung cancer cells (due to anti-cancer activities by presumably agarwood extracts) was calculated using the following formula:

\[
\text{% Inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100
\]

3. Results and Discussion

3.1. Chemical composition in agarwood extracts
Results of the analysis on chemical composition are presented in Table 1. Chemical composition of agarwood extracts was characterized by mainly the compounds of sesquiterpene group and of chromone derivative group [5,14]. The presence of sesquiterpene compounds as contained in agarwood resins could bring about their sweet-smelling smell aroma. Further, it turned out that chemical composition in those three low-quality kemedangan-type agarwoods (A, B1, B2) of G. versteegii species (after methanol extraction) only contained sesquiterpene (guaiacol, cumene, humulene, celerene, aromadendrene compounds, etc) compounds; and conversely did not contain chromone derivative compounds. Based on Indonesia’s National Standard (SNI 7631) [15], the agarwood samples are classified as kemedangan quality. Accordingly, kemedangan-type agarwoods could be regarded as still not yet perfectly developed; and consequently, the community/traders categorized kemedangan-type agarwoods as the lowest-quality agarwood. Chromone compounds possibly have not yet been formed in kemedangan-type agarwood. Conversely in the cultivated agarwoods of G. versteegii species (i.e., after being inoculated by fungi), sesquiterpene and chromone derivative compounds were already formed 2 months after the inoculation [16].

Further, aromadendrene compounds were only present or detected in B1-type and B2-type agarwoods (Table 1), wherein those compounds typified as one of sesquiterpene compounds, which could serve as identifier (marker) compounds for agarwoods, besides chromone derivative compounds that could also perform as identifier [17].

In addition to sesquiterpene compounds that could induce specific smelling aroma at agarwoods, there were aromatic compounds called as benzylacetone and anisylacetone that could also impart specific aroma (Table 1). Those compounds are present-detected in the three low-quality agarwood samples (A-type, B1-type, B2-type). Further, there were also present lauric acids in B1-type and B2-type kemedangan agarwoods. Lauric acids were inherently one of aromatic fatty acids, which therefore could add the specific smelling aroma as well to agarwoods [18].

Several agarwood compounds function as medicines: guaiacol (anticancer, antiimflammatory, antiseptic, antioxidants) [22,23]; linalool (antiinflammatory, peripheral analgesic activities) [24-26]; benzylacetone (antiepileptic, antiseptic, antihypertension) [27,28]; cumene (bacteriostatic, antioxidants) [29,30]; globulol (antioxidants, antimutagenic) [31-33]; aromadendrene (anticancer, antitumor, antimicrobial) [34,35]; hydrocinnamic acid (antityrosinase, anticollagenase) [36]; humulane (antioxidants, antiinflammatory) [37], etc.
Anticancer activities of agarwood extracts

Activities of agarwoods (as methanol extracts) with kemedangan or lowest qualities (A, B1, and B2), at 0 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, and 800 µg/mL concentrations on inhibiting the growth of lung cancer cells (A549) are disclosed in Figure 2. It revealed that the higher the extract concentrations, then the more effective would be those extracts in non-activating or killing the lung cancer cells, as implied by the lower cell-growth inhibition.

Further, the correlation of cell death and extract concentration was then analyzed by using a line regression test, then it could be determined the IC50 values. According to the National Cancer Institute (NCI), IC50 value can be defined in this regard (i.e. anticancer action) as the concentration of agarwood extracts to inhibit cancer cell growth until 50% [19]. Further, scrutinizing the relation curve, the IC50 value for natural kemedangan agarwood extracts was determined at 144.92 µg/mL (A-type); IC50 for cultivated-kemedangan agarwood extracts at 206.88 µg/mL (B1-type); and IC50 for cultivated-kemedangan agarwood extracts at 187.97 µg/mL (B2-types). IC50 value is less than 1000 µg/mL, the material or extract is an anticancer [20]. The most active extract with lowest IC50 value (144.92 µg/mL) was natural agarwood extract (A-type). Thus, the methanol extracts of agarwood (A-type, B1-type and B2-type) are useful as an anticancer because it contains sesquiterpene compound which are useful as anticancer [21]. This occurrence implied that kemedangan agarwoods could afford high or efficacious potency as lung anticancer agents. The minimum concentration (IC50 values) of those three types of kemedangan agarwood extracts were in fact higher than the concentration of agarwood essential oil (Aquilaria malaccensis) for inhibiting the growth of breast cancer cell (MCF-7) at IC50 44 µg/mL [38]; the concentration of agarwood methanol extracts (Gyrinops versteegii) for inhibiting the growth of breast-cancer cell (MCF-7) at IC50 8.32 µg/mL [6]; and for inhibiting prostate cancer cells (PC3) at 110.2 µg/mL [39].
Concentration (μg/mL)

Remarks: A = natural agarwood; B1= cultivated agarwoods, B2 = cultivated agarwoods (all of kemedangan-type or low quality agarwoods)

**Figure 2.** Anticancer activity of methanol-extracted agarwoods, expressed as the concentration of agarwood methanol-extracts against the growth inhibition of lung cancer cells.

4. Conclusions

Agarwoods of kemedangan-type (natural and cultivation results) after methanol extraction only contained sesquiterpene compounds; and conversely did not contain chromone derivative compounds. Accordingly, it makes sense that kemedangan-type agarwoods were regarded as lowest quality agarwoods, because they did not meet the criteria requirements for high-quality agarwoods. High-quality agarwoods contain both sesquiterpene and chromone derivative compounds.

Despite those deficiencies, agarwood of low quality (kemedangan type) indicatively afforded effective or efficacious potency as anticancer agents, with the IC₅₀ values at 144.92 μg/mL (for natural agarwoods, A-type), IC₅₀ at 206.88 μg/mL (cultivated agarwoods, B1-type), and IC₅₀ at 187.97 μg/mL (cultivated agarwoods, B2-type).

**References**

[1] Sidiyasa K and Suharti M 1986 Jenis-jenis gaharu di Indonesia Jurnal Penelitian dan Pengembangan Kehutanan 2 7-16

[2] Subasinghe S M C U P, Hettiarachchi D S and Rathnamalala E 2012 Agarwood-type resin from *Gyrinops walla* Gaertn: A new discovery *J. Trop. Forest Environ.* 2(2) 43-8

[3] Mei W L, Yang D L, Wang H, Yang J L, Zeng Y B, Guo Z K, Dong W H, Li W, and Dai H F 2013 Characterization and determination of 2-(2-phenylethyl) chromones in agarwood by GC–MS *Molecules* 18 12324-45

[4] Eurlings M C M, Heuveling Van Beek H, and Gravendeel B 2010 Polymorphic microsatellites for forensic identification of agarwood (*Aquilaria crassna*) *Forensic Sci. Int.* 197 30-4

[5] Naef R 2011 The Volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: A review *Flavour Frag. J.* 26 73-89

[6] Waluyo T K and Pasaribu G 2017 Screening of anticancer activities from agarwood’s methanol extract (*Gyrinops vertegi* [Gilg.] Domke) *Int. J. Pharma Bio. Sci.* 8(3) 67-72

[7] Mashur 2009 Peluang pasar gaharu budidaya *Seminar Nasional 1 Gaharu*
[8] Susilo A, Kalima T, and Santoso E 2014 **Panduan Lapangan Pengenalan Jenis Pohon Penghasil Gaharu Gyrinops** spp. (Bogor: IPB Press)

[9] Mulyaningsih T, Yamada I 2007 Notes on some species of agarwood in Nusa Tenggara, Celebes and West Papua **Natural resource management and socio-economic transformation under the decentralization in Indonesia: Toward Sulawesi area studies** pp. 365-72

[10] Mulyaningsih T, Marsono D, Sumardi, and Yamada I 2017 The presence of eaglewood Gyrinops versteegii in the natural forest of West Lombok Island, Indonesia **Ecol. Environ. Conserv.** 23(2) 723-9

[11] Fiqa A P, Budiharta S, Siahaan F A, and Rindyastuti R 2020 Population structure of Gyrinops versteegii within floristic community in Nggalak Protection Forest, Flores Island, Indonesia **Biodiversitas** 21(4) 1561-8

[12] Turjaman M and Hidayat A 2017 Agarwood-planted tree inventory in Indonesia **IOP Conf. Ser.: Earth Environ. Sci.** 54 012062

[13] Thakur A N, Thakur N L, Indap M M, Pandit R A, Datar V V, and Muller W E G 2005 Antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria **Mar. Biotechnol.** 7 245-52

[14] Chen H Q, Wei J H, Yang J S, Zhang Z, Yang Y, Gao Z H, Sui C, and Gong B 2012 Chemical constituents of agarwood originating from the endemic genus Aquilaria **Plants Chem. Biodiversity** 9 236-50

[15] [BSN] Badan Standardisasi Nasional 2018 **Gaharu: SNI 7631** (Jakarta)

[16] Nasution A A, Siregar U J, Miftahudin, and Turjaman M 2019 Identification of chemical compounds in agarwood-producing species Aquilaria malaccensis and Gyrinops versteegii. **J. For. Res.** 31(4) 1371-80

[17] Pasaribu G T, Waluyo T K, and Pari G 2015 Analysis of chemical compounds distiguisher f agarwood qualities **Indonesia J. Forest Res.** 2(1) 1-7

[18] Fazila K N and Halim K H K 2012 Effects of soaking on yield and quality of agarwood oil **J. Trop. For. Sci.** 24(4) 557-64

[19] Boyd M R, Paull K D, and Rubinstein L R 1992 Data Display and Analysis Strategies for The NCI Disease-Oriented In Vitro Antitumor Drug Screen In Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development (Boston MA: Springer) pp. 11-34

[20] Arifanti L, Sukardiman H S, and Rakhmawati L M 2014 Uji aktivitas ekstrak biji sirsak (Annona muricata L.) terhadap sel kanker mamalia secara in vitro **Jurnal Farmasi dan Ilmu Kefarmasian Indonesia** 1(2) 63-6

[21] Breitmaier E 2006 **Terpenes: Flavors, Fragrances, Pharmaca, Pheromones** (John Wiley & Sons)

[22] Widiyarti G, Abbas J, and Anita Y 2014 Biotransformation and cytotoxic activity of guaiacol dimer **Indo. J. Chem.** 14(2) 179-84

[23] Feng P, Wang H, Lin H, and Zheng Y 2019 Selective production of guaiacol from black liquor: Effect of solvents **Carbon Resources Conversion** 2(1) 1-2

[24] Kamatou G P P and Viljoen A M 2008 Linalool—A review of a biologically active compound of commercial importance **Natural Product Communications** 3(7) 1183-92

[25] Peana A T and Moretti M D L 2008 Linalool in essential plant oils: Pharmacological effects **Bot. Med. Chap.** 79 716-24

[26] Ghelardini C, Galeotti N, Salvatore G, and Mazzanti G 1999 Local anaesthetic activity of the essential oil of Lavandula angustifolia **Planta Medica** 65 700-3

[27] Norul A S, Nazira, M, and Tajuddin S N 2015 Biotransformation of benzyl acetone from aquilaria malaccensis using microorganisms **Aust. J. Basic & Appl. Sci.** 9(8) 155-9

[28] Kessler D, and Baldwin T 2006 Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of Nicotiana attenuate **The Plant Journal** 49 840-54
[29] Habe H, Kasuga K, Nojiri H, Yamane H, and Omori T 1996 Analysis of Cumene (isopropylbenzene) degradation genes from Pseudomonas fluorescens IP01 Appl. Environ. Microbiol. 62(12) 4471-7

[30] Aneta B, Cedrowski J, Olchowik-Grabarek E, Ratkiewicz A and Witkowski S 2019 Synthesis, DFT calculations, and in vitro antioxidant study on novel carba-analogs of vitamin E Antioxidants 8(589) 1-17

[31] Tan M L, Zhou L, Huang Y, Wang Y, Hao X J, and Wang J 2008 Antimicrobial activity of globulol isolated from the fruits of Eucalyptus globulus Labill Nat. Prod. Res. 22(7) 569-75

[32] Salleh W H H and Ahmad F 2016 Antioxidant and anti-inflammatory activities of essential oils of Actinodaphne macrophylla and A. pruinose (Lauraceae) Natural Product Communications 11(6) 853-5

[33] Rodriguez S, Sueiro R A, Murray A P, and Leiro J M 2019 Bioactive sesquiterpenes obtained from Schinus areira L. (Anacardiaceae) essential oil Multidisciplinary Digital Publishing Institute Proceedings 41(1) 85

[34] Sawant S S, Youssef D T A, Sylvester P W, Walia V, and El Sayed K A 2007 Antiproliferative sesquiterpenes from the red sea soft coral Sarcophyton glaucum Natural Product Communications 2(2) 117-9

[35] Hoque M N, Mondal M F, and Khan M M H 2018 Chemical composition and antimicrobial activity of the essential oils from Aquillaria malaccensis in Bangladesh Saudi J. Life Sci. 3(10) 600-8

[36] Taofiq O, González-Paramás A M, Barreiro M F, and Ferreira I C F R 2017 Hydroxycinnamic acids and their derivatives: cosmeceutical significance, challenges and future perspectives, a review Molecules 22(281) 1-24

[37] Yeo D, Hwang S J, Song Y S, and Lee H J 2012. Humulene inhibits acute gastric mucosal injury by enhancing mucosal integrity Antioxidants 10 7621

[38] Hashim Y Z H Y, Phirdaous A, and Azura A 2014 Screening of anticancer activity from agarwood essential oil Phcog. Res. 6(3) 191-4

[39] Dahham S S, Tabana Y M, Sandai D, Ahmed M A, and Majid A M S A 2016 In vitro anticancer and antiangiogenic activity of essential oils extracts from agarwood (Aquilaria crassna) Med. Aromat. Plants 5(4) 1-10

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