Identification of anticancer compounds in leaves extracts of agarwood (Aquilaria malaccensis (Lamk.))

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Abstract. Cancer is still become the main causes of death in the world. The leaves of Aquilaria malaccensis have an antioxidant and cytotoxic activity against several cancer cell lines. It can be developed as an alternative medicine. However, comprehensive information about the database of metabolites in A. malaccensis leaves is not yet available. This study aimed to screen the metabolites of chloroform and ethanol extracts of A. malaccensis, which has been reported in the database as an anticancer. Determination of metabolites contained in both chloroform and ethanol extracts were conducted using Gas Chromatography–Mass Spectrometry (GC–MS). GC–MS analysis detected nine metabolites in the chloroform extract while twenty one metabolites in the ethanol extract. The most common compounds observed was fatty acids and terpenoids. Among those detected, metabolites that have potential as anti-cancer in the chloroform extracts were 9–Hexadecanoic acid and Tetracosanoic acid. While in the ethanol extracts include 2,6–Octadien–1–ol, 3,7–dimethyl ; 3,6–Octadecadiynoic acid, 3–Octadecyne, Lauric acid, Myristic acid, Nonadecanoic acid, Oleic Acid, Phytol, Loliolide dan Squalene. Further analysis to confirm which compounds most responsible for the anticancer activity in the A. malaccensis extract is planned as the next steps of this study.

Keywords: Aquilaria malaccensis, metabolites, GC–MS, anticancer, agarwood

1. Introduction

Changes in lifestyle and environment accompanied by fast technological developments significantly affect the people lifestyle in the modern era. One of the effects of changing patterns of people's lives is the emergence of various cases of new diseases, such as cancer. Cancer is one of the most causes of death in the world. In 2012, around 8.2 million deaths were caused by cancer [1]. According to the World Health Ranking, Indonesia ranks 68th in breast cancer patients with a prevalence of 19.33 per 100,000 people [2]. The standard cancer treatment accepted by conventional medical treatment is chemotherapy. However, chemotherapy often gives prolonged side effects on patients, causing people to switch to herbal treatments. The use of potential secondary metabolites as an alternative treatment in Indonesia has been carried out by ancestors for centuries. Herbal medicines have been widely accepted in almost all countries. As many as 80% of the population in African countries use herbal medicines for their primary treatment. WHO recommends the use of traditional medicines, including
herbs in public health maintenance, prevention, and treatment of diseases, especially for chronic diseases, degenerative diseases, and cancer [3].

Agarwood has been traditionally used in many countries as herbal medicine. Agarwood is a non-timber forest product (NTFP) in the form of scented resin. The resin is produced as a result of interaction between the plant with microbial resides the bark. The plants consisted of several species from the Thymeleaceae family. Aquilaria malaccensis (Lamk) is the most cultivated agarwood species in Malaysia and Indonesia. The resins have been used as perfumes, cosmetics, aromatherapy, and traditional medicines with several scientifically proven bioactivities such as anti-asthmatic, antimicrobial [4], and anti-malaria [5]. The leaves of non-infected A. malaccensis are used as an herbal drink in Indonesia [6] Study on antioxidant activity of non-infected leaves extract of A. malaccensis was reported by Hendra [7]. The combined fraction of 1:3 chloroform: methanol from methanol extract showed the highest antioxidant activity with an IC$_{50}$ value of 17.39 ± 1.43 µg/mL. With this high antioxidant activity, leaves extract of A. malaccensis has a tremendous potency to be developed as anticancer. However, no study has been done on the identification of anticancer compound in the leaves extract of A. malaccensis. The metabolite analysis only focused on the metabolites content of the oil of A. malaccensis and the effect of different distillation methods on the content. A study on the metabolite profiling of n-hexane fraction of A. malaccensis leaves reported the presence of trans-squalene compounds (68%), stigmast-4-en-3-one (14.52%), stigmast-5-en-3-ol (5.27%), hexanedioic acid, Bis (2-ethylhexyl) esters (5.01%), and hexadecanoic acid, methyl ester (1.17%) [8].

In this current study, we conducted a metabolite profiling on the leaves extracts of A. malaccensis using GC-MS. Subsequently, we used an online database and references available on the internet to identify the metabolites detected in the extracts which have been reported as anticancer. This study serves as a preliminary study to evaluate the possible potency of the leaves extracts as an anticancer and a guide for the next study on anticancer.

2. Materials and methods

2.1. Materials

The mature leaves of A. malaccensis from the non-infected plant were obtained from the LIPI Botanical Gardens Plant Conservation Center, Bogor, Indonesia.

2.2. Methods

2.2.1. Preparation of Aquilaria malaccensis extract. The leaves were cleaned under running water to remove dirt. Then the leaves were air-dried for 7 days on shading. The leaves were divided into several envelopes and oven at 40 °C until the leaf reached a constant weight. The dried leaf samples are mashed using a blender then allowed to pass a mash to separate the powder and leaf fibers. The powdered samples were extracted using the maceration method in two solvents: chloroform and ethanol with a ratio between samples and solvent at 1:13 (w/v). The maceration was done overnight with periodic stirring.

2.2.2. Gas Chromatography – Mass Spectrometry (GC–MS) analysis. Both chloroform and ethanol extracts were analyzed using GC17A MSQP 5000 SHIMADZU. The GC-MS conditioned was set as follows: injector port temperature 250 °C while oven temperature was programmed at 70 °C for 2 min, 150 °C · min$^{-1}$, up to 260 °C · min$^{-1}$. The interface temperature was set at 250 °C. The DB-35 MS Nonpolar column was used with dimensions of 25 mm OD x 0.25 µm ID x 30 m. Helium was used as a carrier gas at a flow rate of 1 ml · min$^{-1}$. MS was set to scan 50-650 Da. The source was maintained at 200 °C, and the motor vacuum pressure was less than 40 Psi. The ionization energy was programmed at -70eV. When all components were set, an amount of 1 µL of the sample was injected with a split ratio set at 1:50.
2.2.3. *Data analysis.* The chromatogram and MS data were processed further by removing the MS value similar with contaminants. Then each MS profile was checked one by one with MS profile data at NIST to confirm the identification of metabolites suggested by the GC-MS library. The confirmed metabolites were further checked the bioactivities as reported in NCBI as well as references available on the internet.

3. *Results and discussion*

3.1. *Extraction of leaves of Aquilaria malaccensis*

The extraction result using two different polarity solvents showed that the *A. malaccensis* leaves contained a comparable amount of nonpolar and polar metabolites. From 5 g of simplisia, we obtained 0.27 g and 0.28 g for chloroform and ethanol extracts, respectively (table 1). Interestingly, both extracts show almost similar color but differ in the physical character. The ethanol extract was more oily than the chloroform extract indicating different metabolites content. Metabolite compounds contained in a plant organ can be influenced by species, environment, development phase, and environmental stress conditions.

| Solvent       | Simplisia mass (g) | Extract mass (g) | Rendemen (%) | Color and physical properties          |
|---------------|--------------------|-----------------|--------------|----------------------------------------|
| Chloroform    | 5                  | 0.27            | 5.4          | Dark green, concentrated and pasta      |
| Ethanol       | 5                  | 0.28            | 5.6          | Dark green, concentrated, pasta and oily|

3.2. *GC–MS analysis extract agarwood leaves Aquilaria malaccensis*

The chromatograms obtained from GC-MS analysis of both leaves extracts showed that the chloroform extracts contained less number of metabolites compared to the ethanol extract (figure 1). This can be indicated by the number of peaks observed in the chromatogram. Identification of metabolites was made by comparing manually the MS spectra obtained with those of NIST database. Twenty-nine metabolites were confirmed present in the leaves extracts of *A. malaccensis* (table 2).

The metabolites detected in *A. malaccensis* derived from four groups, including fatty acid, fatty alcohol, terpenoid, and aliphatic alcohol. There are 9 secondary metabolites detected in chloroform extract while 21 metabolites were in ethanol extract. The chloroform extract was predominantly with fatty acids and fatty alcohol, while only fatty acids was the major compounds in ethanol extract (figure 2).

The main metabolite compounds of leaves of *A. malaccensis* chloroform extract include 9-octadecen-1-ol, 3-eicosyne, and 1-heptadecyne with a relatives percentage area of 59.94, 20.66, and 18.94%, respectively. The main metabolite compounds in ethanol extract were dodecanoic acid, hexadecanoic acid, and squalene with relatives percentage area of 26.22, 20.72, 9.70%, respectively. Hexadecanoic acids were also reported present in the oil of *Aquilaria* analyzed using GC-MS. Major metabolites that were reported in the oil of *Aquilaria* derived from the resin was not detected in the leaves, such as agarospirol, α agarofuran, dihydroagarofuran, 10–epi–eudesmol, jinkoheremol and jinkohol II [9].

Of the 29 metabolites detected, we enabled to identify 13 metabolites were reported to have anticancer or anti-proliferative activities, including 9-hexadecanoic acid, tetracosanoic acid, calarene, 2,6-octadien-1-ol, 3,7-dimethyl; 3,6-octadecadiynoic acid, 3-octadecene, lauric acid, myristic acid, nonadecanoic acid, oleic acid, phytol, loliolide, and squalene (figure 3).
Hexadecanoic acid extracted from microalgae inhibit the MCF7 breast cancer cell growth in vitro [10]. Tetracosanoic acid showed a high inhibition on the HCT116 colon cancer cell growth, at a concentration of 6.25 µg/mL significantly (P < 0.05) [11]. The fatty acid 3–octadecyne was reported to have a weak cytotoxic activity in the several cell lines including HePG–2, HeLa, PC3, and MCF–7. Nonadecanoic acid significantly reduced the viability of breast cancer cells at 68.7% on MCF–7 and 89% on MDA–MB–231 after 72 hours of exposure [12]. Calarene from Patrinia scabiosaeofolia has anti-proliferation activity on several carcinoma cells including SGC–7901, AGS, HepG2, HT–29, HCT–8, 5–FU/HCT–8, HeLa, and MDA–MB–231 [13]. Interestingly, apoptosis induction activity on BXPC–3 cells was reported for 2,6–Octadien–1–ol, 3,7–dimethyl when detected using flow cytometry [14]. Lauric acid, which predominant in coconut oil, has antiproliferative and proapoptotic activities on breast cancer cells and endometrium by increasing ROS [15]. Lauric acid can induce apoptosis in Caco–2 and IEC–6 cells [16]. Remarkably, the oleic acid at a concentration of 10 µM inhibits the growth of B104 cells in free serum media [17]. Myristic acid reduced the expression of p21 and Bax in response to the destruction of double-stranded DNA, leading to induction of apoptosis [18]. Phytol which abundant in leaves was reported can induce apoptosis of AGS cells [19] while the long fatty acids squalene can inhibit tumor activity [20].

Figure 1. GC–MS chromatogram of *Aquilaria malaccensis*. (a) chloroform extract (b) ethanol extract

Figure 2. Metabolic composition of *Aquilaria malaccensis* leaves: (a) chloroform extract (b) ethanol extract.
are concentrated in the three metabolites that both extracts differ.

Table 2. Metabolites detected in the leaves extract of *Aquilaria malaccensis* analyzed using GC–MS

| No | Retention Time | Name of Compound | Molecular Formula | Molecular Weight | Group          | Ethanol Extract | Chloroform Extract |
|----|----------------|-------------------|-------------------|------------------|---------------|----------------|-------------------|
| 1  | 41.683         | 11–Octadecenoic acid | C_{10}H_{18}O_{2}  | 296             | Fatty Acid    | –              | +                 |
| 2  | 37.182         | 1–Heptadecyne     | C_{17}H_{32}      | 236             | Fatty Acid    | –              | +                 |
| 3  | 12.678         | 3,6–Octadecadiynoic acid | C_{19}H_{30}O_{2} | 290             | Fatty Acid    | +              | –                 |
| 4  | 36.515         | 3–Eicosyne        | C_{20}H_{38}      | 278             | Fatty Acid    | –              | +                 |
| 5  | 37.167         | 3–Octadecynoic acid | C_{18}H_{34}      | 250             | Fatty Acid    | +              | –                 |
| 6  | 41.675         | 4–Nonenoic acid  | C_{10}H_{18}O_{2} | 170             | Fatty Acid    | +              | –                 |
| 7  | 41.677         | 9–Hexadecenoic acid | C_{17}H_{32}O_{2} | 268             | Fatty Acid    | –              | +                 |
| 8  | 28.875         | Decanoic acid     | C_{11}H_{22}O_{2} | 186             | Fatty Acid    | +              | +                 |
| 9  | 39.043         | Dodecanoic acid  | C_{12}H_{26}O_{2} | 200             | Fatty Acid    | +              | –                 |
| 10 | 39.039         | Hexadecanoic acid | C_{16}H_{32}O_{2} | 256             | Fatty Acid    | +              | –                 |
| 11 | 34.593         | Lauric acid       | C_{12}H_{26}O_{2} | 200             | Fatty Acid    | +              | –                 |
| 12 | 37.067         | Myristic acid     | C_{16}H_{32}O_{2} | 256             | Fatty Acid    | +              | –                 |
| 13 | 39.535         | Nonadecanoic acid | C_{21}H_{42}O_{2} | 326             | Fatty Acid    | +              | –                 |
| 14 | 40.909         | Nonanoic acid     | C_{8}H_{18}O_{2}  | 158             | Fatty Acid    | +              | –                 |
| 15 | 42.481         | Oleic Acid        | C_{18}H_{36}O_{2} | 282             | Fatty Acid    | +              | –                 |
| 16 | 39.93          | Tetracosanoic acid | C_{25}H_{50}O_{2} | 382             | Fatty Acid    | –              | +                 |
| 17 | 35.692         | Undecenoic acid  | C_{11}H_{22}O_{2} | 184             | Fatty Acid    | +              | –                 |
| 18 | 33.784         | 1–Dodecanol       | C_{12}H_{26}O     | 186             | Fatty Alcohol | +              | –                 |
| 19 | 28.717         | 1–Octanol        | C_{8}H_{16}O_{2}  | 130             | Fatty Alcohol | +              | –                 |
| 20 | 36.567         | 1–Octanol, 2,7–dimethyl | C_{10}H_{22}O_{2} | 158             | Fatty Alcohol | +              | –                 |
| 21 | 36.246         | 9–Octadecen–1–ol | C_{18}H_{36}O     | 268             | Fatty Alcohol | –              | +                 |
| 22 | 25.098         | Calarene         | C_{14}H_{24}      | 204             | Terpenoid     | –              | +                 |
| 23 | 30.356         | Palustrol        | C_{14}H_{26}      | 222             | Terpenoid     | –              | +                 |
| 24 | 29.304         | iso–geraniol     | C_{10}H_{16}O_{2} | 154             | Terpenoid     | +              | –                 |
| 25 | 35.253         | Loliolide        | C_{11}H_{16}O_{3} | 196             | Terpenoid     | +              | –                 |
| 26 | 38.425         | 2,6–Octadien–1–ol, 3,7–dimethyl | C_{13}H_{22}O_{2} | 210             | Terpenoid     | +              | –                 |
| 27 | 42.084         | Phytol           | C_{20}H_{40}O     | 296             | Terpenoid     | +              | –                 |
| 28 | 45.241         | Squalene         | C_{30}H_{50}      | 410             | Terpenoid     | +              | –                 |
| 29 | 33.574         | 1–Eicosanol      | C_{20}H_{42}O     | 298             | Aliphatic Alcohol | +              | –                 |

*Bold compounds show that it has anticancer activity according to NCBI database*

3.3. *Heatmap analysis of the extract of agarwood Aquilaria malaccensis leaves*

The result of the heatmap analysis comparing the chloroform extract and the ethanol extract shows that both extracts differ in the metabolite profile (figure 4). The metabolites in the chloroform extract are concentrated in the three metabolites 3–eicosyne, 1–heptadecyne, and 3–octadecyne. Interestingly, two of those metabolites, metabolites 3–eicosyne, 1–heptadecyne, were recognized for their anticancer...
activities in breast cancer cell lines MCF7 [10, 12]. The metabolites of ethanol extract more varied than the chloroform extract. Five compounds are constituted the ethanol extract ranging from high to low were dodecanoic acid, 9–hexadecenoic acid, squalene, oleic acid, and 3–octadecyne, respectively. Among these, two compounds were reported to have anticancer, i.e., squalene, and oleic acid. The result of this study emphasis that it is worth to continue the study on the anticancer activity of *A. malaccensis* leaves, especially on breast cancer as indicated above.

**Figure 3.** Molecular structure of metabolites in the leaves extract of *Aquilaria malaccensis* that have potential as anticancer.

**Conclusion**

The metabolites detected in *A. malaccensis* derived from four groups, including fatty acid, fatty alcohol, terpenoid, and aliphatic alcohol. Metabolic compounds that have potential as anticancer are 9-hexadecanoic acid, tetracosanoic acid, calarene, 2,6-octadien-1-ol, 3,7-dimethyl; 3,6-octadecadiynoic acid, 3-octadecyne, lauric acid, myristic acid, nonadecanoic acid, oleic acid, phytol, loliolide, and squalene.
Figure 4. Heatmap analysis of chloroform and ethanol extract *Aquilaria malaccensis*

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