Extraction and identification of bioactive compounds from agarwood leaves

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Abstract. Agarwood commonly known as gaharu, aloeswood or eaglewood have been used as traditional medicine for centuries and its essential oil also being used as perfumery ingredients and aroma enhancers in food products. However, there is least study on the agarwood leaves though it contains large number of biomolecules component that show diverse pharmacological activity. Previous study showed that the extracted compounds from the leaves possess activities like anti-mutagenic, anti-tumor and anti-helminthic. The main objectives of this research were to determine bioactive compounds in agarwood leaves; leaves extract and oil yield obtained from maceration and soxhlet extraction methods respectively. The maceration process was performed at different operating temperature of 25 °C, 50°C and 75°C and different retention time at 30, 60, 90 and 120 minutes. Meanwhile, various solvents were used to extract the oil from agarwood leaves using soxhlet method which are hexane, water, isopropanol and ethanol. The extracted oil from agarwood leaves by soxhlet extraction was analyzed using gas chromatography mass spectrometry. The results showed that the highest extract of 1.53% was obtained when increase the temperature to 75 °C and longest retention time of 120 minutes gave the highest oil yield of 2.10 % by using maceration. This is because at higher temperature enhances the solubility solute and diffusivity coefficient, thus increase the extract yield while longer retention time allow the reaction between solvent and solute occurred more rapidly giving higher extract. Furthermore, the soxhlet extraction using n-hexane as the solvent gave the highest oil yield as compared to other solvent due to the non-polar properties of n-hexane increase the efficiency of oil which is also non-polar to soluble in the solvent. In addition, the results also reported that the oil extracted from agarwood leaves contains bioactive compounds which are phytol, squalene, n-hexadecanoic acid and octadecatrienoic acid. Therefore, oil extracted from agarwood leaves has the potential to be applied in food, pharmaceutical, nutraceutical and cosmetics industries.

1. Introduction

Agarwood (Aquilariasinensis, Aquilariarasna) is widely known as incense in Thailand, Cambodia and Taiwan. The agarwood leaves also being processed as health tea product in some countries such as Taiwan and Thailand. The term “agarwood” refers to resin-impregnated pieces of wood that have been at least partially shaved from the non-impregnated woods [1, 2]. According to Nor Azah [3], this resin impregnated woody tissues produced in the heartwood area mainly found in certain species of Aquilaria which has been a highly priced commodity for more than 2000 years. Agarwood is an evergreen tree that can grow up to 40 m high and 60 cm in diameter while the leaves are 5 to 9 cm long and oblong lanceolate [4]. These trees usually found in low-land tropical forests with optimal sunlight, shade and moisture.

It is also known by many names; in Malaysia and Indonesia, it is called as “gaharu”, “chenhsiang” or “chenxiang” in China; “jin-koh” in Japan; “agar” in India; “chim-hyvang” in Korea; “kristsananoi”
in Thailand; “tram huong” in Vietnam; “bols’dagle”, or “calambour” in French and “oud” in the Middle East [5, 6, 7].

The study of bioactive compounds is very important in discover of therapeutic agent in plant [8]. The most significant bioactive compounds of these plants are alkaloids, tannins, flavonoids and phenolic compounds [9]. Agarwood is used in different of communities and majority of its medicinal uses involved in anti-inflammatory, used to treat rheumatism, arthritis, body pain, asthma and gout [2]. There are some studies revealed that agarwood has remarkable anticancer activity [10]. However, there is least study on the identification of bioactive constituents in agarwood leaves. Therefore, this study aimed to extract and identify the presence of bioactive compounds in agarwood leaves by different extraction method along with gas chromatography-mass spectrometry (GC-MS).

2. Methodology

2.1 Materials
The fresh leaves of agarwood were collected from Masjid Tanah Melaka, Malaysia.

2.2 Preparation of plant materials
The leaves were dried in the oven at the temperature of 60 °C and ground into powder form using POLYMIX® PX-s MFC 90 D grinder and sieved into average particle size of 215µm. The powdered sample was kept in freezer to prevent the growth of microorganisms and protect the sample from light prior to extraction.

2.3 Extraction of agarwood leaves by maceration process
5.0 g of dried powder leaves of Agarwood were placed into macerated in 75 mL of distilled water and placed into shaking bath (Clifton Range®) at constant speed of 220 rpm and retention time of 30 minutes. The shaking maceration process was performed at different temperature of 25 °C, 50 °C and 75 °C. Meanwhile, shaking maceration process was also done at different retention time of 30, 60, 90 and 120 minutes at constant temperature of 75 °C with constant speed of 220 rpm. The crude extracts were freeze dried.

2.4 Extraction of agarwood leaves by soxhlet extraction
Soxhlet extraction method was used to extract the agarwood leaves and the extraction process was performed using different solvents included distilled water, hexane, isopropanol and ethanol. All the solvents used were analytical grade. The extraction process was done for 6 hours at constant temperature of 60 °C. The crude extract of agarwood was evaporated using a rotary evaporator (Heidolph-instruments, Rotavapor, Germany). The weight of all extracts were measured after solvent evaporation and kept into air tight container for analysis purpose.

2.5 Gas chromatography-mass spectrometry (GC-MS) analysis
The extracts from soxhlet extraction were analysed using gas chromatography-mass spectrometry of Hewlett Packard 6890 series Gas Chromatography with Auto-sampler and 5973 N Mass Selective Detector. A HP-5 crosslinked 5% phenyl methyl siloxane fused silica capillary column, (30 m long * 0.25 mm internal diameter) with 0.25 µm film thickness was used. The extracts were dissolved in ethanol and filtered using 0.45 µm pore size whatman polyethersulfone membrane filter. Helium gas was used as carrier gas at a flow rate of 0.9 mL/min.The column temperature was programmed to 50 °C and hold for 2.5 min to 150 °C with the increase of 15°C per min. The temperature of injector and detector were maintained at 250 °C. The temperature of mass spectral ionization was set at 230 °C and operated in the electron impact ionization mode at 70 eV while the detector was performed in scan mode from 20-600 atomic mass unit (amu). Hence, the identification of compounds in the extracts were based on the mass spectral matching with the standard compounds in NIST (National Institute of Standards and Technology) and Wiley libraries. The relative amount of individual components was expressed as percentage of peak area relatives to total peak area.
3. Results and Discussion.

3.1 Effect of operating temperature on overall leaves extract by maceration

Operating temperature and time of extraction are important parameters to be optimized even in order to minimise energy cost of the process [11]. The overall extract obtained from agarwood leaves was increased with the increasing of operating temperature from 25 °C to 75 °C. This is because the increase of operating temperature enhances the solubility of solute and diffusivity coefficient of compounds, thus favours the extraction [12, 13]. In addition, the solubility of leaves extract in the solvent was increased and also the mass transfer was enhanced at higher temperature due to the increase of vapour pressure of solutes with increasing of operating temperature [14]. The solvent viscosity was decreased when increasing the temperature and this allows penetration of solvent into the plant matrix, thus accelerating the whole extraction [15]. Moreover, heating is known to affect the morphological changes in the plant sample matrix and thus increase the mass transfer rate of leaves extracts [16]. Consequently, the effects of higher temperature and mass transfer rates synergistically increase the rate of the extraction from agarwood leaves. The percentage of extracts from Agarwood leaves were shown in Table 1.

Table 1. Percentage of leaves extract obtained at different operating temperature by maceration process

| Operating Temperature, °C | Extract Yield, g | Percentage of leaves extract, % |
|---------------------------|------------------|-------------------------------|
| 25                        | 0.0190           | 0.38                          |
| 50                        | 0.0282           | 0.56                          |
| 75                        | 0.0765           | 1.53                          |

3.2 Effect of retention time on overall leaves extract by maceration

As shown in the Table 2, the percentage of leaves extract increased with the increasing of retention time. By increasing the retention time, prolong the reaction between solvent and matrix interaction, hence favour the extraction process. The swelling of plant cell wall also increase the penetration of the solvent into the plant material, so increase the leaves extract. The percentage of leaves extract at 90 minutes and 120 minutes do not show significant difference due to the extraction process reaching the equilibrium state when all the solute had completely extracted, hence extend the extraction time would not affect the rate of extraction because the extraction process has been completed [17].

Table 2. Percentage of leaves extract obtained at different retention time by maceration process

| Retention time, min | Extract Yield, g | Percentage of leaves extract, % |
|---------------------|------------------|-------------------------------|
| 30                  | 0.0889           | 1.78                          |
| 60                  | 0.0917           | 1.83                          |
| 90                  | 0.1030           | 2.06                          |
| 120                 | 0.1050           | 2.10                          |

3.3 Effect of various solvents used on overall oil yield by soxhlet extraction

Soxhlet extraction traditionally was used in determining the total oil [18]. The extraction yield and extraction activity are strongly dependent on the solvent, thus the selection of extraction solvents is critical for the complex plant samples [15]. According to Wang et al. [19], an extraction solvent system is generally selected based on the purpose of extraction, polarity of the interested compounds, polarity of undesirable components, overall cost, safety and environmental concern. As shown in the Fig.1, the use of hexane as the solvent gives the highest percentage of oil yield in comparison to the other solvents in this order: hexane > isopropanol > ethanol > water. This result is in accordance with polarity of the solvents used in the extraction and solubility of oil from agarwood leaves in solvents [15]. The polarity index of hexane, isopropanol, ethanol and water are 0.1, 3.9, 5.2 and 10.2 respectively. A solid solute tends to dissolve in a liquid solvent when the molecules of both are similar
enough in polarity. Therefore, oil from agarwood leaves is non-polar tend to dissolves in non-polar solvent according to “like dissolves like” adage, thus highest oil yield obtained by using hexane as the solvent.

![Figure 1. Percentage of oil yield obtained by soxhlet extraction with various solvents](image)

3.4 Gas chromatography-mass spectrometry (GC-MS) analysis

The oil yield obtained from soxhlet extraction were analysed using GC-MS. Table 3 shows the results of the GC-MS analysis of the oil yield extracted from Agarwood leaves using isopropanol as the solvent. The analysed results showed that the extracted oil contained squalene, n-hexadecanoic acid, tetramethyl-2-hexadecen-1-1 and octadecatrienoic acid. Squalene also known as 2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18, 22-hexaene is a highly unsaturated hydrocarbon and a chain-like triterpene [20]. Squalene has many physiologic functions, such as promotion of superoxide dismutase activity in vivo, enhancement of the immune responses, anti-aging, anti-fatigue and anti-tumour activities [20]. Besides, n-hexadecanoic is a saturated fatty acid and also known as palmitic acid. Meanwhile, octadecatrienoic acid is C18 polyunsaturated fatty acid. Most of the fatty acids are known to have antibacterial, antifungal properties and also exhibited anti-inflammatory effect [21]. Furthermore, phytol is an acyclic diterpene alcohol and it had been used as the precursor for the production of synthetic form of Vitamin E [22] and Vitamin K1 [23].
Table 3. Results of the GC-MS analysis of the oil yield extracted from agarwood leaves using isopropanol as the solvent

| No | Retention time (min) | Percent of total (%) | Bioactive Compound | Structure |
|----|----------------------|----------------------|--------------------|-----------|
| 1  | 12.07                | 28                   | Tetramethyl-2-Hexadecen-1-ol (Phytol) | ![Structure](image1.png) |
| 2  | 12.32                | 76.3                 | n-hexadecanoic acid, methyl ester | ![Structure](image2.png) |
| 3  | 12.75                | 30.0                 | Octadecatrienoic acid | ![Structure](image3.png) |
| 4  | 14.79                | 32.8                 | Squalene | ![Structure](image4.png) |

4. Conclusion
In this study, maceration process was applied to obtain the leaves extracts from Agarwood leaves and the effects of operating temperature and retention time on the overall leaves extracts were investigated. The results show that leaves extracts increased with increasing of operating temperature and retention time. Meanwhile, soxhlet extraction method with various solvents was used to study the effect of different solvents used on oil yield extracted. Extraction using hexane as solvent gave highest percentage of oil yield. The GC-MS analysis showed that oil of agarwood leaves contains important bioactive compounds of squalene, n-hexadecanoic acid, tetramethyl-2-hexadecen-1-1 and octadecatrienoic acid which very valuable to be applied in pharmaceutical, nutraceutical and cosmetics products.

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