UNIVERSITI PUTRA MALAYSIA

COMPARATIVE ANALYSIS OF DIFFERENT GRADES OF GAHARU USING NMR- AND GCMS- BASED METABOLOMICS APPROACH

SITI NAZIRAH ISMAIL

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman: **Professor Khozirah Shaari, PhD**
Faculty: **Institute of Bioscience**

Gaharu, also referred to as agarwood, kalambak, aloeswood or eaglewood, is the resinous heartwood of infected *Aquilaria* sp. where infection may occur due to the attack by fungi, insects or other external injuries. In gaharu trade, gaharu wood is graded by experts using organoleptic methods because there is no other scientific method to determine the grades. Normally they are graded according to observable traits such as odor upon burning, color and wood density which reflects the resin content of the woods. Basically price of gaharu follow the grades with highest grade being the most expensive. The aim of this study was to carry out a comparative analysis of the different grades of gaharu by making use of their multicomponent metabolite profiles in order to ascertain whether they can be used to differentiate between one grade to another. To achieve this aim, ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and Gas Chromatography Mass Spectrometry Spectrometry (GCMS) combined with multivariate data analysis were used to analyze the variation between the metabolite profiles of the different grades of gaharu samples.

Gaharu woods finely milled into fine powder, then extracted by using distilled methanol and dried using Genevac. The gaharu crudes were proceed for ^{1}H NMR analysis, by dilution with deuterated methanol. Visual inspection on ^{1}H-NMR gaharu spectra shows that the grades have similar profile but different concentration of various constituents. Major constituents identified are aromatics (δ6.0-7.5), sugar and glycosides (δ2.5-5.0), fatty acids/aliphatics (δ0.5-2.0) and aldehydic compound (δ9.32). However, since the difference among the grades is not clear from visual analysis, the analysis were carried out using multivariate analysis. Principal Component Analysis (PCA) model overview of 4 batches of all grades of gaharu does not provide useful information, thus further analysis was carried out on consistency of individual grade for each batch of gaharu to see which batch and grades are suitable as class model. According to their chemical constituents, the grades reclassified into 5 new groups (A, B, C, D and E). Group A more significant in sugar constituents (4.24-4.28 ppm) and possibly terpenoids (1.12, 1.16, 1.32, and 2.04 ppm), while group C and E are different

from the rest due to higher aliphatics (3.0, 4.0, 4.52 and 7.2 ppm). On the other hand, group B and D different from other grades by possibly terpenoidal constituents as well (0.80, 0.84, 0.88, 1.24, and 1.28 ppm).

In order to identify the chemical markers that contributed to the differentiation of the gaharu, NMR experiments, comprising 1D and 2D as well as *J*-resolve, were carried out on selected fractions of the gaharu extract. The highest grade gaharu was selected for the fractionation *via* solid phase extraction (SPE). An ethylphenylchromone type compound was identified as on of the major constituents in the 50% SPE fractions of gaharu grade 1. Further analysis by Liquid Chromatography-Mass Spectrometry (LCMS) resulted in the identification of 6-hydroxyethylphenylchromone and dihydroxy-2-[(2-phenyl)-ethyl]chromones, two fatty acids which were (*9Z,12Z*)-15,16-dihydroxyoctadeca-9,12-octadecadienoic acid and (*11Z,14Z*)-17,18-dihydroxyicosa-11,14-dienoic acid as well as sesquiterpenes which were aquilarone B, aquilarone D or E, and aquilarone F.

Gaharu smoke was captured on the glass fiber filter using a simple lab setup comprising an incense burner, connected to a glass tube containing the glass fiber. To ensure absorption of smoke, the setup was linked to a vacuum line. The fiber filter containing smoke were directly analyzed by headspace GCMS analysis. Volatile constituents detected in the gaharu smoke included aldehydes (benzaldehyde and cuminaldehyde) and aromatics (toluene, 2-methylanisole, *p*-cresol, creosol, anisole, *o*-cresol and acetophenone). Other constituents detected were furfuryl alcohol and benzylideneacetone. Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) of the gaharu smoke profiles can be differentiated into a terpene rich and a chromone rich groups. The terpene rich group consisted of the higher grades gaharu samples, while the chromone rich group consisted of the lower grade samples.
Further treatment of the variables using Variable Importance in Projection (VIP) for independent variables resulted in the identification of *p*-cresol (m/z 107.05), 2-methylanisole (m/z 122.10), toluene (m/z 91.05) and *p*-anisaldehyde (m/z 135.10), as some of the discriminating chemical markers present in the high grade gaharu smoke. Meanwhile furfuryl alcohol (m/z 98.05) and 5-methylfurfural (m/z 110.05) were some of the markers present in lower grades. On the overall, this study has shown that spectrometry-based metabolomics could offer a better approach in grading of gaharu qualities.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

ANALISIS PERBEZAAN ANTARA GRED GAHARU MENGGUNAKAN PENDEKATAN METABOLOMIK BERASASKAN RESONAN MAGNETIK NUKLEUS DAN KROMATOGRAFI GAS-SPEKTROSKOPI JISIM

Oleh

SITI NAZIRAH ISMAIL

Jun 2015

Pengerusi: **Profesor Khozirah Shaari, PhD**
Fakulti: **Institut Biosains**

Gaharu,yang turut dikenali sebagai agarwood, kalambak, aloeswood atau eaglewood adalah bahagian berdamar pada kayu teras bagi kayu dari spesis *Aquilaria* yang dijangkiti oleh jangkitan yang mungkin berpunca oleh serangan fungus, serangga atau kecederaan luaran. Dalam perdagangan gaharu, kayu gaharu digredkan oleh pakar menggunakan cara organoleptik kerana tiada cara saintifik untuk menentukan gred gaharu. Kebiasaannya, gaharu digredkan berdasarkan ciri-ciri yang dapat dilihat seperti bau ketika pembakaran, warna dan densiti kayu yang memaparkan kandungan damar pada kayu. Pada asasnya, harga gaharu adalah mengikut gred dimana gred tertinggi adalah yang paling mahal. Matlamat penyelidikan ini adalah untuk menganalisis perbezaan antara gred gaharu dengan menggunapakai profil komponen pelbagai metabolit dalam memastikan samada ia boleh digunakan untuk memebezakan antara gred. Untuk mencapai objektif tersebut, ^{1}H Nuclear Resonan Magnetik Nukleus Proton (^{1}H NMR) dan Spektrometri Jisim Kromatografi Gas (GCMS) digabungkan dengan analisis data multivariat digunakan untuk menganalisis variasi antara profil metabolit bagi gred gaharu yang berbeza.

Kayu gaharu dikisar menjadi serbuk halus, kemudian diekstrak menggunakan metanol dan dikeringkan menggunakan Genevac. Kemudian, pati gaharu digunakan untuk analisis ^{1}H NMR dengan mencairkannya bersama metanol terdeuterad. Analisis secara visual menunjukkan bahawa kesemua gred mempunyai profil yang sama tetapi berbeza dari segi kepekatan pelbagai komponen. Komponen utama yang dikenalpasti adalah aromatik (δ6.0-7.5), gula dan glikosida (δ2.5-5.0), asid lemak/alifatik (δ0.5-2.0) dan sebatian aldehida (δ9.32). Walaubagaimanapun, memandangkan beza antara gred tidak jelas melalui analisis secara visual, analisis diteruskan menggunakan analisis data multivariat. Model gambaran keseluruhan bagi analisis komponen utama (PCA) untuk keempat-empat kumpulan bagi kesemua gred gaharu tidak memberikan maklumat penuh, jadi analisis multivariat dijalankan untuk melihat kekonsistenan bagi setiap gred bagi memilih gred yang sesuai digunakan sebagai model kelas. Berdasarkan komponen

kimia, gred-gred tersebut diklasifikasi semula kepada 5 kumpulan (A, B, C, D and E). Kumpulan-kumpulan tersebut mempunyai metabolit berbeza dan taburan metabolit yang berbeza. Kumpulan A lebih signifikan dalam kandungan gula (4.24-4.28 ppm) dan mungkin terpenoid (1.12, 1.16, 1.32, and 2.04 ppm), manakala kumpulan C dan E berbeza dari kumpulan lain disebabkan oleh kandungan alifatik (3.0, 4.0, 4.52 and 7.2 ppm). Sementara itu kumpulan B dan D berbeza dari kumpulan lain mungkin disebabkan oleh terpenoid juga (0.80, 0.84, 0.88, 1.24, and 1.28 ppm).

Analisis fitokimia oleh 1D NMR, 2D NMR dan *J*-resolve membenarkan pengenalpastian sebatian jenis etilfenilkromon sebagai komponen utama dalam gaharu gred 1. Analisis selanjutnya oleh Spektrometri Jisim-Kromatografi Cecair (LCMS) menunjukkan pengesanan 6-hidroksietilfenilkromon dan dihidroksi-2-[(2-fenil)-etil]kromon, serta dua asid lemak iaitu asid (*9Z,12Z*)-15,16-dihidroksioktadeka-9,12-oktadekadienoik dan asid (*11Z,14Z*)-17,18-dihidroksiikosa-11,14-dienoik dan seskuiterpen iaitu akuilaron F, akuilaron D atau E, dan akuilaron B.

Asap gaharu dikumpul dalam penapis fiber kaca menggunakan persediaan makmal secara ringkas, kemudian disambungkan ke tiub kaca. Untuk memastikan penyerapan asap, ia disambungkan ke vakum. Penapis fiber kaca dianalisis terus menggunakan analisis headspace GCMS. Komponen meruap yang dikesan dalam asap gaharu adalah aldehida, (benzaldehida dan kuminaldehida) dan aromatik (toluena, 2-metilylanisol, *p*-kresol, kreosol, anisol, *o*-kresol and asetofenon). Komponen lain yang dikesan dalam asap gaharu adalah alkohol furfuril dan benzilidinaaseton.

Analisis Diskriminan-Ortogon Persegi Separa Kurangnya (OPLS-DA) pada profil asap boleh dibahagikan kepada sampel kaya terpen dan sampel kaya kromon. Kumpulan kaya terpen mempunyai gred gaharu yang tinggi, manakala kumpulan kaya kromon memiliki sampel gred rendah Seterusnya, analisis variabel diteruskan menggunakan Pembolehubah Penting dalam Unjuran (VIP) kepada *p*-kresol (m/z 107.05), 2-metilanisol (m/z 122.1), toluena (m/z 91.05)and *p*-anisaldehida (m/z 135.1) sebagai sebahagian daripada penanda pembezaan bahan kimia yang wujud dalam gaharu gred tinggi sementara itu alkohol furfuril (m/z 98.05) and 5-metilfurfural (m/z 110.05) adalah beberapa penanda yang wujud dalam gred rendah. Secara keseluruhannya, kajian ini menunjukkan bahawa metabolimik berdasarkan-spektrometri mampu menawarkan pendekatan yang lebih baik dalam penggredan kualiti gaharu.

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All gratification is referred to Allah S.W.T."

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I certify that a Thesis Examination Committee has met on 25 June 2015 to conduct the final examination of Siti Nazirah binti Ismail on her thesis entitled "Comparative Analysis Of Different Grades Of Gaharu Using Nmr- And Gcms- Based Metabolomics Approach" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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δ	-	Chemical shift in ppm
ppm	-	Parts per million
°C	-	Degree in Celsius
µg	-	Microgram
µL	-	Microliter
µm	-	Micrometer
^{1}H	-	Proton NMR
HMBC	-	Heteronuclear multiple bond correlation
HSQC	-	Heteronuclear single quantum coherence
Hz	-	Hertz
MHz	-	Megahertz
Psi	-	Pounds per square inch
J	-	Coupling constant in hertz
m	-	Multiplet
kg	-	Kilogram
g	-	Gram
mm	-	Milimiter
m	-	Meter
mL	-	Mililiter

mg	-	Miligram
min	-	Minute
MTIB	-	Malaysian Timber Industry Board
NMR	-	Nuclear Magnetic Resonance
PCA	-	Principal component analysis
FID	-	Free induction decay
PC	-	Principle component
s	-	Singlet
SIMCA-P	-	Soft Independent Modeling Class Analogy-P
PLS-DA	-	Partial Least Square-Discriminant Analysis
VIP	-	Variable Importance in Projection for Independent Variables
RT	-	Retention time
OPLS-DA	-	Orthogonal Partial Least Square-Discriminant Analysis
CITES	-	Convention on International Trade in Endangered Species of Wild Fauna and Flora
TRAFFIC	-	The Wildlife Trade Monitoring Network
Eds	-	Editors
IUCN	-	World Conservation Union
SPE	-	Solid phase extraction
UHPLC	-	Ultra High Performance Liquid Chromatography
ANOVA	-	Analysis of Variance

w/w	-	Weight over weight
GCMS	-	Gas Chromatography Mass Spectrometry
NIST	-	National Institute of Standards and Technology
FFNSC	-	Flavour and Fragrance Natural and Synthetic Compounds
kV	-	Kilovolt
rpm	-	Revolutions per minute
TMS	-	Tetramethylsilane
FTIR	-	Fourier transform infrared spectrometer

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Gaharu is the resinous heartwood of infected *Aquilara* species, traded for perfumery, aesthetic and religious purposes. Depending on the country that it is traded in, gaharu is also known as eaglewood, aloeswood, agarwood, kalamabak, *jin-koh* (Japanese), oudh (Arabic), *tram huong* (Vietnamese), *chen xiang* (Chinese) (Persoon, 2008). It is also well known as 'gold of the forest' due to its high price which varies indiscriminately, very much dependent on demand and availability. The supply of this valuable material is slowly, but certainly, depleting due to over collection from its natural stand (Persoon, 2008).

Gaharu producing trees grow typically in a mixed forest habitat and usually grow up to 30 m tall with 1 m maximum diameter at breast height (Persoon, 2008). Among the gaharu-producing species, *Aquilaria malaccensis* grows up to 75 m above sea level, thus it is easier to harvest the resinous wood in comparison to *Aquilaria hirta*. However, *Aquilaria hirta* which grows 300 - 600 meters above sea level, produces better grade of gaharu which are usually very hard in texture and dark in color indicating its high quality.

Gaharu are usually obtained from Orang Asli communities, via middleman or traders and resold at higher price. Different countries import different types of gaharu depending on the final intended use or application (Persoon, 2007). For instance, Japan buys gaharu in the form of wood blocks to make religious sculptures and other items of worship and aesthetic purposes, while countries in the Middle East prefer wood chunks, for burning as incense in aromatherapy, and powdered agarwood, for hydrodistillation and perfumery (Chakrabarty et al., 1994).

Recently, there has been an increase in products on the market which incorporate gaharu as one of their main active ingredients, for example gaharu tea, gaharu coffee, skincare and cosmeticeutical products. These development has more or less contributed significantly in the increase in demand for gaharu (Persoon, 2008). In order to address the supply and demand issue, plantations of *A. malaccensis* and several other gaharu producing species were established and artificially induced to produce gaharu (MTIB, 2010).

Gaharu is largely believed to be the product of fungal infection of the producing tree, sustained through injury on trunks, branches and roots of the life tree. Artificial induction of gaharu has been the subject of research carried out in various countries including Thailand and Malaysia. Researchers and planters have tried wounding plantation trees and mimicking fungal infection using fungal or chemical based inoculants (Persoon, 2008; MTIB, 2010). Although some gaharu was successfully induced in the planted trees, the amount of gaharu produced was low and of undefined quality in comparison to gaharu produced in natural stands. In some of the artificial induction trials, some trees even died during the wounding and inoculation processes if it is not done carefully (Turjaman, 2011). Theoretically, trees that grow in a natural forest environment are expected to be more profilic and efficient producer of gaharu since these species have to compete for nutrients and combat a whole range of external predators and diseases. It is still unclear how much more efficient the plantation species will be in comparison to the wild species. The whole process of gaharu formation is very slow and thus, the returns promised by artificial production of gaharu may take many years to realize.

The main chemical components of gaharu such as phenolic compounds and sesquiterpenes *viz.* agarofuran, jinkohol, jinkoh-eremol and eudesmol, are believed to play a very important role in plant defense (disease resistance) (Marinova et al., 2005). The presence of these chemical components are therefore related to gaharu production since an attack by pathogens or in response to changes in environmental condition or external perturbation will affect their biosynthetic production. Usually, inoculated planted trees are expected to only produce better quality of gaharu after 5 years (Turjaman, 2011). As shown by the plantation and induction trials, often times the quality of gaharu produced is very low (commonly grade C) and only suitable for oil extraction. At most, the amount of gaharu that can be produced by a tree is approximately 2 kg per tree. Thus, in terms of monetary returns, the most that a planter can obtain is an estimated RM4000.00 per tree (MTIB, 2010).

In trade, the quality of gaharu depends largely on the traders and buyers, who decide which grade is the best grade. The grader grades according to several criteria of the wood that he has collected at a time. For example, if the grader was given a kilogram of gaharu wood, he will classify all the wood pieces according to shape, color, density and odor (usually upon burning) (MTIB, 2010). It is easiest to determine the best grade but it is rather difficult to differentiate between the rest of the grades. The Malaysia Timber Industry Board (MTIB) grades gaharu as Super King, Double Super, Super grade A series (A1 to A10), Grade B series (B1 to B5), Grade C series (C1 to C10) and lastly, the oil grade gaharu.

1.2 Problem statement

The classification of gaharu grades differ according to the product in trade and in which country the trade is taking place. The type and number of gaharu grades may vary widely between the countries. Different species have been shown to contain different chemical components (Ishihara et al,. 1991 & 1993), but this may not necessarily be the criteria that is important to traders and consumers who largely selects the country of origin and wood quality as criteria for grading the gaharu products. The scenario as a whole paints a very confusing system of grading which seems to rely heavily on subjective preferences and thus making it very difficult to clearly delineate between the various grades. Currently, there is no proper scientific method of determining the grades of gaharu, and more research are needed in finding feasible methods for grading gaharu. To date, some methods developed based on electronic measurements have met with some success, but more confirmatory data is needed to assess whether the classification or grading is accurate and reproducible. A rapid and practical method to differentiate between various grades of gaharu is needed. On this basis, the current study was undertaken to address the following objectives:

1. To differentiate various grades of gaharu by applying 1H NMR-based metabolomics on gaharu extracts.

2. To identify chemical markers responsible for the differentiation of gaharu grades.

3. To differentiate various grades of gaharu by applying GCMS-based metabolomics on gaharu smoke.

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