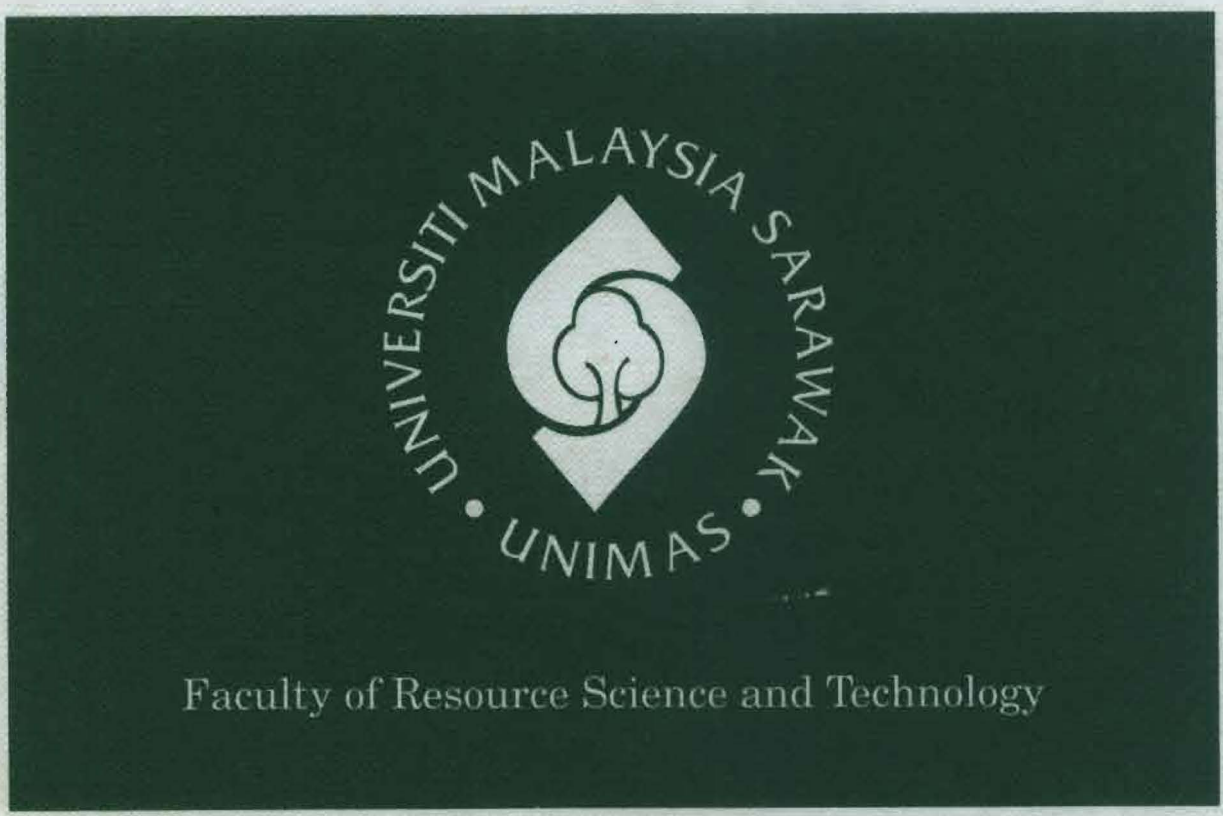

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF ESSENTIAL OILS AND EXTRACTS FROM *AGATHIS BORNEENSIS*

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This dissertation is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours in Resource Chemistry

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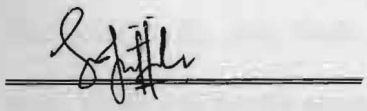
DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.



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ABSTRACT

Essential oils from bark, heartwood, and resin of *Agathis borneensis* obtained by hydrodistillation method were analysed by Gas Chromatography/Flame Ionization Detector (GC/FID) and Gas Chromatography/Mass Spectrometry (GC/MS). Resin contents highest essential oils (3.4%, v/w), followed by bark and heartwood with 0.25% and 0.1%, respectively. Bark and heartwood of *A. borneensis* were extracted with methanol using Soxhlet extractor. The crude extract yield for bark and heartwood are 14.31% and 10.45%, respectively. The crude extracts were then subjected to fractionation on silica gel column chromatography. The five fractions were then analysed on GC/MS. The major compounds identified in bark of *A. borneensis* are α-copaene, β-caryophyllene, 1S cis-calamenene, benzaldehyde and pimaric acid. Meanwhile the majors compound identified in heartwood are ethyl benzene, benzaldehyde, hexadioic acid dioctyl ester, bis 1,2-benzenedicarboxylic acid and stigmast-5-en-3-ol (3 beta,24S). Toxicity testing on *Artemia salina* for essential oils and antifungal for crude extracts were carried out. This show that bark oils has a significant toxicity toward *A. salina*. Antifungal test for crude extracts showed both bark and heartwood have a potential to decrease the fungi growth rate.

Key words: *Agathis borneensis*, essential oils, crude extracts, gas chromatography, antifungal

ABSTRAK

Minyak pati pada bahagian kulit, teras dan resin dari kayu Agathis borneensis telah diperolehi melalui kaedah penyulingan stim. Minyak pati ini dianalisis dengan menggunakan Kromatografi Gas/Pegesan Ion Nyalaan dan Kromatografi Gas/Spektrometri Jisim (KG/SJ). Resin mengandungi minyak pati yang paling tinggi (3.4% mengikut berat), diikuti oleh kulit dan teras kayu iaitu sebanyak 0.25% dan 0.1%, masing-masingnya. Kulit dan teras kayu A. borneensis diekstrak dengan menggunakan pelarut metanol pada pengekstrak Soxhlet. Hasil ekstrak kasar bagi kulit dan teras kayu adalah sebanyak 14.31% dan 10.45%, masing-masingnya. Ekstrak kasar tersebut dipisahkan melalui pemfraksian pada turus kromatografi gel silika. Lima fraksi yang diperoleh kemudian dianalisis dengan menggunakan KG/SJ. Sebatian yang paling banyak dikesan dalam kulit kayu A. borneensis ialah α-kopaena, β-kariofilena, 1S cis-kalamenena, benzaldehida dan asid pimarik. Sebatian yang paling banyak dikenal pasti dalam teras kayu ialah etil benzena, benzaldehida, asid heksadioik dioktil ester, asid bis 1,2-benzenedikarbosilik dan stigmast-5-en-3-ol (3 beta,24S). Ujian ketoksikan bagi minyak pati ke atas A. salina dan ujian anti-kulat bagi ekstrak kasar telah dijalankan. Hasil kajian menunjukkan minyak dari kulit mempunyai kesan toksik terhadap A. salina. Ujian anti-kulat pula menunjukkan kedua-dua bahagian kulit dan teras kayu berupaya untuk mengurangkan kadar pertumbuhan kulat.

Kata kunci: Agathis borneensis, minyak pati, ekstrak kasar, kromatografi gas, anti-kulat

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Agathis borneensis locally known as bindang in Sarawak, mengilan or tambunan in Sabah, dammar minyak or tulong in Peninsular Malaysia and Borneo kauri or Malayan kauri in Europe belongs to the family of Araucariaceae. It is synonym with *A. dammara*, *Pinus dammara*, *Dammara alba*, and *A. alba* (Soerianegara and Lemmens, 1994). *A. borneensis* is large softwood of South-east Asia and extending from Malaysia through Papua New Guinea and Philippines (Bootle, 1983). The tree is often tapped to obtain a copal resin used in the manufacture of varnishes and lacquers. *A. borneensis* is a tall monopodial tree with bole dipped and grey-brown. The inner bark is brown streaked red. It has a milky white or pale white sticky sap appear fast on cutting the bark. The sapwood of it was yellowish or whitish in color (Smith, 1999). The heartwood is pale yellowish brown, sometimes with a pinkish tinge. The heartwood not sufficiently durable for external use, but can be impregnated with preservatives. This tree timber is widely used as plywood, patternmaking, kitchen utensils, matches, joinery, vat, sounding boards for musical instruments, artificial limb and others (Bootle, 1983).

There are two major chemical constituents of wood which are carbohydrate (65-75%) and lignin (18-35%). Carbohydrate contains cellulose and hemicelluloses. Extractives in wood are components instead of cellulose, hemicelluloses and lignin (Petterson, 1994).

According to Zabel and Morrel (1992), extractives are mostly low molecular-weight compounds that are readily extracted from the wood by solvent such as water, alcohol, benzene or ether. They represent many classes of compounds including a large number that are species specific. A few examples are carbohydrates such as starch, glucose, fructose and sucrose; phenolic compound such as stilbenes, tannins, phlobaphenes, flavanoids and lignans; oils and waxes; esters of organic acids; alkaloids and tropolones.

According to Umezawa (2001), specific fragrances of different woods are usually due to the composition of monoterpenes and volatile sesquiterpenes. They can be easily separated from wood by steam distillation, and the oily substance obtained is called "essential oils". Turpentine, essential oils from *Pinus* spp., is obtained by steam distillation of exudates from pine tree (oleoresin); the residue is gum resin.

Essential oils are considered the most widely used natural products in many areas such as preservatives for flour, fragrance and cosmetic product, for prevention of the progression of various disease states and etc. Essential oils are composed of many kinds of classes of molecules including terpenoids, phenolics, aromatics, cyclic and acyclic compounds, acetonides, and sulfur- and nitrogen- containing compounds, depending on the plant and the extraction method. Terpenoids comprise the largest organic chemical group, not only in essential oils but also in natural products. The need for alternatives to toxic and non-biodegradable synthetic pesticides or fungicides is a strong incentive for developing new product that employs the natural biological activities of essential oils (Nakatsu *et al.* 2000).

1.2 Objectives of the Project

The chemistry of *A. borneensis* has not been studied in detail. There has no report in the analysis of essential oils and crude extract from *A. borneensis*. Thus, the aim of the present study was (a) to determine the chemical constituent of essential oils from heartwood, bark, and resin of *A. borneensis*; (b) to determine the chemical composition of extracts from heartwood and bark of *A. borneensis*; and (c) to know and study about biological activities of essential oils and extracts from *A. borneensis* in order to examine the biological and antifungal activities of a number of plant compounds from natural product which is harmless to human and environment.

CHAPTER 2

LITERATURE REVIEW

2.1 *Agathis* species

Approximately 20 species of *Agathis* have been described, occurring from Malaysia to New Zealand. Some of them are very useful trees, economically important for their region. Throughout its range *Agathis* occurs mainly as scattered small groups of single trees. The timber is more valuable than most other species' in the same regions. It is pale, light but strong and knot free, and useful for all kind of building purpose (Vidakovic, 1991). *Agathis* is the most tropical of all conifers. They are enormously important economically since wood is widely used for furniture and other constructions and they are one of the main sources of pulp for paper. They are used for ornaments and many are resinous, with the resin widely used in gums and varnishes. Turpentine is derived from pine resin (Lack and Evans, 2001).

Agathis produces a high class, much valued, utility timber and it is grown widely as a timber tree. In Malaysia, it is the most important commercial softwood, and it is also widely planted in Indonesia. The copal-yielding species are very tall trees, up to 60 m high, often with a near-cylindrical bole. However, there can be some variation in the characters of the living tree, as well as the ecological conditions under which it occurs. The term copal applies to a large group of resins characterized by their hardness and relatively high melting point. They are soluble in alcohol. They were among the best of

natural resins for use in varnish and paint manufacture, and traded in very large volumes. In the oil-soluble form they were also used in the manufacture of linoleum. Copal has been produced from a large number of different tree species from many parts of the world - Africa, Asia and South America. Today, most copal of commerce originates from *Agathis* species of Southeast Asia (FAO, 1995).

2.2 Essential oils

According to Nakatsu *et al.* 2000, essential oils are known as aromatic substances produced by specific plant species. Most of these oils have been used as fragrance raw materials and flavoring agents since ancient times. They are called essential because it was once thought that the oil represented the essence of the original plant. From this standpoint, essential oils are considered the most widely used natural products in many areas because many traditional folk medicines are based mainly on plant materials.

From the past research 1,8-cineole, found in the essential oils of *Ocimum kenyense* (Ayobangira), is active against stored product beetles. The essential oils of *Cymbopogan winterianus* root oil consisting of α-eudesmol, β-eudesmol, and elemol, have insecticidal activity against the rice weevil *Sitophyllus oryzae.* Terpenes and other oxygenated constituents of *Acorus calamus* (calamus) oil, including terpineol, farnesol, cineole, citral, capric acid, lauric acid, and carvone, demonstrate an insecticidal activity toward the pyllid *H. cubana* (Nakatsu *et al.* 2000).

The methods of extraction of essential oils from plants significantly affect the chemical constituent and composition of essential oils. Steam distillation under normal pressure is the basic isolation method to obtain essential oils. However, it has the disadvantage of alteration of the components as the sample is maintained under severe condition (Kobayashi and Kawakami, 1991).

Mellani *et al.* (2004) state that the composition of a new chemotype of *Elsholtzia strobilifera Benth* has been identified based on Gas Chromatography and Gas Chromatography-Mass Spectroscopy indicated the presence of neral and geranial which found to be the major compound of its essential oils.

2.3 Extractives from wood

According to Baeza and Freer (2001), the extraneous components are nonpolymeric (except pectins and condensed tannins) and may be separated from the insoluble cell wall materials by their solubility in water or organic solvents. Because most of the extraneous compounds are commonly isolated from wood by solvent extraction, they are called extractives.

There is a minor amount of extraneous materials (2-10%) in wood, mostly in the form of extractives such as tannins, lignans, flavanoids, stilbenes, terpenoid, starch, lipids, pectins, alkaloids, protein, fat and waxes and as well as trace amount of inorganic minerals (0.1-1.0%) (Preston and Jin, 1991). According to Umezawa (2001), extractives

are the wood constituents which can be extracted with neutral solvents. They are obtained by extracting wood meal with organic solvents or water or by steam distillation, and some are obtained as exudates from wounded tree.

Although the extractives are low molecular in concentration compared with those of cell wall polymers, this fraction characterizes each wood species chemically. Most components of wood extractives are classified as secondary metabolites, and the distribution of specific compounds is restricted in certain wood species. This feature provides the basis of chemotaxonomy of woody plants. Extractives are the predominant contributors to wood color, fragrance, and durability. Extractives also influence the pulping, drying, adhesion, adhesion, hygroscopicity, and acoustic properties of wood. Many extractives have specific biological activities, and various woods have been used as crude drugs and medicines for centuries (Umezawa, 2001).

The composition and the amount of the extractives are dependent on the wood species, within and among trees, tree age, and the environmental conditions under which they grow. The extractives sometimes characterized into chemical classes such as saponifiables (fatty acids, resin acids, some steryl esters, and glycerides) which form soluble soaps under alkaline condition and unsaponifiables (waxes, some steryl ester, diterpene alcohol and aldehyde, sterols, triterpene alcohols and fatty alcohols) which do not form soap (Baeza and Freer, 2001).

Wood dissolvation in several of solvent indicates the composition of extractives. There is no solvent able to extract or isolate all the extractives. Ether is a non-polar solvent which can extracts fat, resin, oils, sterol and terpene. Ethanol or benzene is a polar solvent which ether dissolves substance and organic substance which not dissolved in water. Hot water extracts several inorganic salt and low molecular weight polysaccharides include gum and starch (Petterson, 1994).

Baeza and Freer (2001) state that the techniques of analysis of extractives involve the isolation of components (extraction, distillation of volatiles, chemical or chromatographic separation) and analysis (GC, LC, GC/MS, NMR, IR etc.). A large variety of analytical technique are used in the analysis of extractives, but the method very depending, among other things, on the type of information required from the analysis.

2.4 Fungi

Fungi are heterotrophs, and because of this, they play several distinctive roles in ecosystems as saprotrophs, as parasites of plants and animals, as mutualistic symbionts of many phototrophic organisms. There are an enormous number of fungi. Hawksworth (1991) has estimated that there are may be as many as 1.5 million species. They exist in a wide range of habitats such as in fresh water and the sea, in soil, litter, decaying living plants and animals, in dung, and in living plants and animals. In order to grow, fungi require source of carbon and nitrogen, a supply of energy and certain essential nutrients such as potassium and phosphorus.

To grow on wood, fungi also need to able to tolerate certain chemical and physical stresses associated with wood. Typically high levels of tannins, phenols and other antifungal aromatics are present in wood. Concentrations are particularly high in heartwood. The presence of these substances is believed to be a major factor accounting for the selectivity of wood decay fungi for certain wood species (Dix and Webster, 1995).

Dix and Webster (1995) stated that specific and non-specific biologically active substrates that have the potential to inhibit fungi occur commonly as constituents of plant tissues. Antifungal compounds are particularly widespread in woody plant species, e.g. *Betula pendula, Populus tremula, Acer platanoides, Fagus sylvatica* and conifers.

The antifungal activity of bark components has been known and the activity has been traditionally utilized in fermentation of Philippine sugarcane wine. A polyphenol component obtained from an aqueous extract of samac (*Macharanga grandifolia*) bark, which gave catechin, cyaniding, delphinidin, and sugars upon acid hydrolysis, inhibited the growth of lactic acid bacteria which caused deterioration of the fermentation mixture (Sakai, 2001).

Kusuma *et al.* (2004) reported that an antifungal compound was isolated from the n-hexane solubles in methanolic extracts of amboyna wood by bioassay using a wood-rotting fungus. The compound showed 21% of the antifungal activity of the methanolic extract as control.

CHAPTER 3

MATERIAL AND METHOD

3.1 Sample collection

Agathis samples were collected from Bario Highland in Miri Division of Sarawak. The wood samples were cut into pieces and then ground to obtain wood meals. The meal of each sample was kept in plastic bag and air-dried for one or two days to balance the moisture of sample with the air moisture.

3.2 Isolation and Fractionation of Chemical Components

This study was focused on essential oils and extractives from *A. borneensis*. The trunk of *A. borneensis* was sampled and separated into heartwood and bark. Resin from *A. borneensis* tree was also obtained.

3.2.1 Extraction of essential oils

Essential oils were extracted from *A. borneensis* according to Lee and Ogg method as described by Datta (1987). Samples of *A. borneensis* were extracted using a Clevenger type modified hydrodistillation apparatus. 100 g of wood meals were weighed and transferred to 2 L flat bottom flask. It was then mixed with 1.5 L of distilled water. The flask was assembled to the Clevenger trap and connected to the condenser.

Hydrodistillation was carried out for 8 hours. Flask was heated to maintain the distillation rates of 2 drops per second. After 8 hours, the oil trap in the Clevenger was cooled at the room temperature. The oily layers were then separated and dried over anhydrous sodium sulphate and stored at 4-5 °C. The hydrodistillation process was repeated for three times and the average percentage of the oils was calculated.

3.2.2 Methanol extracts

Meal samples of *A. borneensis* was extracted with methanol based on method used by Simatupang *et al.* (1992) using Soxhlet extractor. 20 g wood meal samples were placed in extraction thimble. 50 mL of methanol and several antibumping chips were then placed in 500 mL round flask. The Soxhlet extractor was assembled and the wood meals were put in carefully. 300 mL methanol was drained through the thimble. The extract was carried out for 8 hours. The extracts were transferred to pear-shaped flask. The extract was then evaporating using vacuum rotary evaporator until dryness. The pre-weighed pear-shaped flask together with the dried extract was then weighed. The extract was dilute again with 3 mL methanol and then transferred into 5 mL vial. The extract in vial was then evaporated using gently stream of pure nitrogen gas. The dried extract was kept until further analysis.

3.3 Silica Gel Column Chromatography Fractionation

A column chromatography was prepared by packing a 50 mL buret with 7.5 g silica gel. The packing of silica gel in the column was done using the aid of n-hexane as a medium. The sample was then dissolved with 1 mL hexane and placed at the top of silica gel column. Five fractions (F1-F5) were collected in fine clean 50 ml pear-shaped flask by eluting the column with a solvent or a mixture of solvent. The fraction and the elution solvents are presented in Table 1. The fractions were then concentrated by rotary evaporator until dryness and subsequently dissolved with 1 mL dichloromethyl before transferred into vial.

Table 1: Fraction and elution solvent used in column chromatography

Fraction	Elution solvent
F1	n-hexane
F2	25% toluene in hexane
F3	a) 50% toluene in hexane b) 5% EtOAc in hexane
F4	10% EtOAc in hexane
F5	20% EtOAc in hexane

3.4 Gas Chromatographic Analysis

Analysis of essential oils and column chromatography fraction from methanol extracts were performed on capillary Gas Chromatography/Flame ionization detector (GC/FID) at Resource Chemistry Laboratory, UNIMAS and capillary Gas Chromatography/Mass

spectrometry (GC/MS) at Analytical laboratory, Pepper Marketing Board (PMB), Kuching.

3.4.1 Gas Chromatography / Flame Ion Detector (GC/FID)

The essential oils were analyzed on Hewlett Packard 6890 Series GC system which is equipped with a Flame Ionization Detector (FID) using a fused silica capillary column DB-5 (25 m x 0.22 mm internal diameter). Prior to GC analysis, 1 µL essential oils were diluted with 200 µL n-hexane. Nitrogen gas was used as a carrier gas with velocity of 2 mL/min. The initial temperature was programmed at 50 °C and hold for 2 minutes. The temperature was then increased to 250 °C at a rate of 6.5 °C/min. The final temperature was held for 7 minutes. The temperature for the injector and detector was set at 280°C and 320 °C respectively. 1 µL of diluted sample was then injected into GC/FID.

3.4.2 Gas Chromatography / Mass Spectrometry (GC/MS)

The essential oil and the fraction were analyzed with GC-MS model GC 17 A Shimadzu QP-5000 with capillary column 25 X 0.3 mm X 0.25 µm. 1 µL diluted sample in section in section 3.4.1 was injected in split less mode. Helium was used as carrier gas. The oven temperature was maintained at 50 °C for 2 min and increased to 300 °C at rate 6.5 °C/min. Maintained the temperature in isothermal at 300 °C for 10 min. The temperature for the injector and detector was set at 280 °C and 320 °C respectively. The mass spectrum obtained for particular peaks in chromatogram was then compared to the mass

spectra stored in Wiley Incorporated spectral database. Only the mass spectrum with similarity more than 85% compared to mass spectrum in the Wiley Library is accepted and authenticated as the individual component.

3.5 Qualitative Analysis

3.5.1 Percentages of essential oils

The percentage of the essential oil obtained from the *A. borneensis* was calculated and the yields were average over triplicate experiments.

$$\% \text{ essential oils} = (V/W) \times 100$$

where,

V: volume of oil

W: weight of sample

3.5.2 Percentage of crude extract

The percentage of extractives was obtained by this equation:

$$\% \text{ crude extract} = \frac{\text{sample}_0 - \text{sample}_1}{\text{sample}_0} \times 100\%$$

where,

sample_0: sample weight before extract

sample_1: sample weight after extract