Studies on Wood and Bark Extractives as Natural Preservatives towards Subterranean Termites

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Studies on Wood and Bark Extractives as Natural Preservatives towards Subterranean Termites

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List of Abbreviations

^{13}C-NMR	Carbon Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D-NMR	Two-dimentional Nuclear Magnetic Resonance
ANOVA	Analysis of variance
C-WS	Water-soluble extract from *A.crassicarpa*
C-WS-AW	Water-soluble Acetone Water extract from *A.crassicarpa*
C.EA	Ethylacetate extract from *A.crassicarpa*
C.EA-AW	Ethylacetate-acetone water extract from *A.crassicarpa*
COSY	Correlation spectroscopy
Da	Dalton (atomic mass unit)
EA-EW	Ethyl Acetate Ethanol Water extract from *A.crassicarpa*
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB-MS	Fast Atomic Bombardment Mass Spectrometry
FAB-MS	Fast Atomic Bombardment Mass Spectroscopy
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared Spectroscopy
GC/MS	Gas Chromatography/Mass Spectrometry
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
He	Helium (gas)
HMQC	Heteronuclear Multiple Quantum Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation Spectroscopy
IPM	Insect Pest Management
IS	Internal Standard (*n*-eicosane)

M-EA	Ethyl Acetate extract from *A.mearnsii*
M-WS	Water-soluble extract from *A.mearnsii*
M-WS-AW	Water-soluble Acetone Water extract from *A.mearnsii*
M.EA	Ethylacetate extract from *A.mearnsii*
M.EA-AW	Ethylacetate-acetone water extract from *A.mearnsii*
MAQ	2-methylanthraquinone
MeOH	Methanol
Min	minute
MS	Mass Spectrometry
MW	Molecular weight
n-Hexane	normal-Hexane
NOESY	Nuclear Overhauser Effect
PD	Paper disc
PLC	Preparative Layer Chromatography
ppm	part per million
Py-GC/MS	Pyrolysis-Gas Chromatography/Mass Spectrometry
TLC	Thin layer chromatography
TMAH	Tetramethylammonium hydroxide
TMS	Tetramethylsilane
UV/Vis	Ultra Violet/Visible

1 General Introduction

1.1 Introduction to Insect Pest Management

In the 1980s, as soil quality declined because of excessive pesticide use, many people realized that insecticide use was not able to efficiently manage plant pests. In the long term, insecticide use will contribute to the evolution of resistant pests and will alter soil microbes resulting in enhanced pesticide degradation and poor control. Farmers and researchers, therefore, began to recognize the issue of an overreliance on insecticide use and the need for a new form of pest control that was sustainable and in synergy with nature. This integrated control system, eventually known as integrated pest management (IPM), aimed to eliminate the issues encountered during the synthetic organic insecticide era.

IPM is defined as the intelligent selection and use of pest-control actions (tactics) that will ensure favorable economic, ecological, and sociological consequences [1]. An IPM program for insect pests (especially termites) differs from one for crops pests because termiticide resistance caused by termiticide use has not previously been encountered. Monitoring the termite population, therefore, is the main step required for a successful termite IPM program [2].

1.2 Termite Biology

Termites, commonly known as “white ants,” play an important role in the environment. They are essential decomposers that help to break down dead trees and plants into micronutrients that increase soil fertility. However, because of their ability to consume cellulose, termites can be a problem when they invade the human sphere, particularly in wooden buildings and cultivated crops. As social insects, termites live together in a colony. A colony of termites is usually marked by tunnels built into a very sophisticated structure called a mound. The nest or mound varies in shape and height depending on the colony size and termite species. Termite nests protect the termites from outside attack and from rainfall and heat.

Every termite colony contains immature termites and three main adult castes: reproductives (queens, kings, and alates), workers, and soldiers [3]. Each caste has a specific function: the king mates with the queen, who lays eggs for the colony; the workers forage for food and water, build and repair the nest, and help in tending the immature termites; and the soldiers protect the colony from external invaders.

In all the termite species worldwide, 183 are listed as causing damage to buildings, with less than half of these listed as causing very significant damage [4]. A large proportion of the damage costs associated with termites is caused by subterranean termites, with the *Coptotermes* (Rhinotermitidae) genus containing the largest number of economically important subterranean termites (28 species). Many tropical and subtropical Asian countries like Indonesia, Malaysia, Thailand, Singapore, Japan, China, India, and the Philippines have focused on the study of subterranean termites. These termites have long been a serious issue for the utilization of wood-based materials and crop plantations. The existence of subterranean termites is marked by shelter tubes and a

nest in the soil, the bottom part of a tree, or a building area with high humidity. Subterranean termite *Coptotermes* spp. are the most important species and account for 70% of all known termite species. Consequently, safer, more effective, and more environmentally friendly methods of termite control are urgently needed. Here, we describe a termite control system that is currently used in Indonesia and Japan.

1.3 Termite Management in Indonesia

Indonesia has extensive biodiversity of both flora and fauna that are widespread across the 17,504 islands [5]. The high amount of rainfall that occurs throughout the year leads to high humidity levels. These conditions are ideal for the termites that range from Sumatera Island in the west to Irian Jaya Island in the east. Termite studies in Indonesia were initiated by Roonwall and Maiti [6] and continued by many researchers who successfully recorded 200 species of termites.

Generally, there are three common termite groups found and categorized as pests by the research community. These are subterranean termites, drywood termites, and macrotermes termites. In most tropical and subtropical countries, subterranean termites have become the major termite pest. These termites not only consume wood-based building materials but can also consume valuable books, documents, and photographs. As subterranean termites are spread by human movement and shipping they occur across a larger area than drywood termites. In Indonesia, *C. gestroi* is the most destructive termite and the most high-cost economic pest. Subterranean termite control is, therefore, needed to protect wood-based structures. Current treatments include wood treatments, soil barrier treatments, and population control using bait systems. An alternative method, where wood is treated pre- or post-construction with a natural extract-based wood preservative, represents an interesting area for further study.

Table 1.1 List of termite species recorded in Indonesia

Family : Kalotermitidae		
Subfamily	Genus	Species
Kalotermitinae	*Neotermes* Holmgren	*Neotermes dulbergiae* alshoven *Neotermes tectonae* (Dammerman)
	Cryptotermes Banks	*Cryptotermes cyanocephalus* Light *Cryptotermes domesticus* Haviland *Cryptotermes dudleyi* Banks
Family : Rhinotermitidae		
Coptotermitinae	*Coptotermes* Wassmann	*Coptotermes curvignatus* Holmgren *Coptotermes kalshoveni* Kemner *Coptotermes travians* Haviland
Rhinotermitinae	*Prorhinotermes* Silvestri	*Prorhinotermes ravani* n. sp.
	Schedorhinotermes Silvestri	*Schedorhinotermes javanicus* Kemner *Schedorhinotermes tarakanensis* (Oshima) *Schedorhinotermes translucens* (Haviland)
Family : Termitidae		
Amitermitinae	*Microcerotermes* Silvestri	*Microcerotermes dammermani* n. sp.
Termininae	*Capritermes* Wasmann	*Capritermes buitenzorgi* Holmgren *Capritermes mohri* Kemner *Capritermes santschii* Silvestri
Macrotermitinae	*Macrotermes* Holmgren	*Macrotermes carbonarius* (Hagen) *Macrotermes gilvus* (Hagen) *Macrotermes malaccensis* (Haviland)
	Odontotermes Holmgren	*Odontotermes grandiceps* Holmgren *Odontotermes javanicus* Holmgren *Odontotermes makassarensis* Kemner
	Microtermes Wasmann	*Microtermes insperatus* Kemner
Nasutitermitinae	*Nasutitermes* Dudley	*Nasutitermes acutus* Holmgren *Nasutitermes matangensis* (Haviland) *Nasutitermes matangensisformis* Holmgren
	Bulbitermes Emerson	*Bulbitermes durianensis* n. sp. *Bulbitermes lakshmani* n. sp.

1.4 Termite Management in Japan

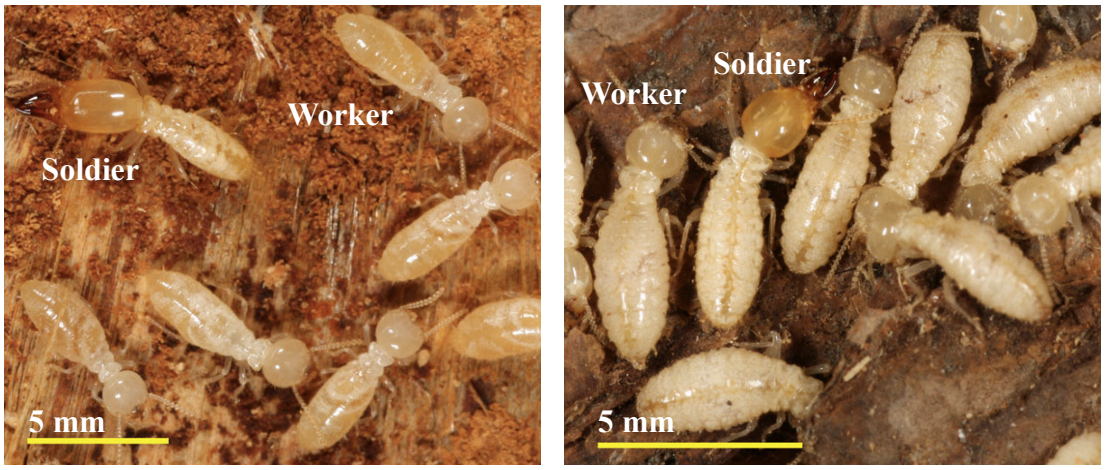
In Japan, as in other subtropical and tropical countries, in Japan, termites have become a serious problem for houses and the construction of wooden buildings. According to the sales figures for termiticides reported in Tsunoda [7], Japan spent at least US $0.8-1.0 billion per year to prevent and control termite infestations.

In Japan, 21 species of termite have been identified [8,9] and are listed in **Table 1.2**. Among these 21 termite species there are two species representing the Rhinotermitidae family of subterranean termite pests, *Reticulitermes speratus* and *C. formosanus* (See **Figure 1.1**), with *C. formosanus* causing 70% more damage than *R. speratus* [10]. Many techniques have been developed and implemented in Japan to reduce the losses associated with termite damage. These include the use of soil treatment chemicals, wood treatment chemicals, baiting programs, physical barriers, biological control, and even construction design. Termiticides have also been applied to houses or buildings to protect cellulosic building material.

Increasing public awareness about the environment has promoted the use of safer termite management techniques, with some studies examining the effectiveness of reduced chemical or even chemical-free treatments compared with the use of traditional synthetic chemicals [11,12,13]. Plant extracts, with bioactive chemicals, have also been used as natural preservatives to protect wood or wood-based materials from termites. Although the use of bio-termiticides is still limited, many studies have developed plant extract-based termiticides that are both effective and environmental-friendly [14,15,16]. Extract-based preservatives can be isolated from many parts of the plant including heartwood [17,18], bark [19,20], leaves, fruit, and seeds [21,22].

Table 1.2 Japanese termites

Family	Species
Kalotermitidae	*Neotermes koshunensis* (Shiraki) *Cryptotermes domesticus* (Haviland) *Incisitermes minor* (Hagen) *Incisitermes immigrans* (Snyder) *Glyptotermes satsumaensis* (Matsumura) *Glyptotermes fucus* (Oshima) *Glyptotermes nakajimai* (Morimoto)
Termopsidae	*Hodotermopsis sjoestedti* Holmgren
Rhinotermitidae	*Reticulitermes speratus* (Kolbe) *Reticulitermes kanmonensis* Takematsu *Reticulitermes amamianus* Morimoto *Reticulitermes miyatakei* Morimoto *Reticulitermes okinawanus* Morimoto *Reticulitermes yaeyamanus* Morimoto *Reticulitermes flaviceps* (Oshima) *Coptotermes guanzhouensis* Ping *Coptotermes formosanus* Shiraki
Termitidae	*Odontotermes formosanus* (Shiraki) *Nasutitermes takasagonensis* (Shiraki) *Pericapritermes nitobei* (Shiraki *Sinocapritermes mushae* (Oshima)



(a) **(b)**

Figure 1.1 *Reticulitermes speratus* or ヤマトシロアリ **(a)** and *Coptotermes formosanus* **or** イエシロアリ **(b)** [23]

1.5 Teakwood

Teak (*Tectona grandis* L. f) is one of the most economically valuable trees worldwide. The increasing annual demand in the market for teakwood has led to the reforestation of teakwood plantations [24,25]. Teak is used for a range of functions including ship building, yacht furnishing, house construction, furniture, decorative building, veneers, and flooring. It has been estimated that this species can be found in 4.35 million different plantation areas distributed across 52 countries [26] . These plantations are mostly located in Asia (83%), followed by Africa and North America.

The durability of teakwood has been studied comprehensively because extracts of teak heartwood have been shown to convey resistance to marine conditions [27], fungal attack [28], and exhibit insecticidal activity [29,30]. Many chemicals have been reported to function in these activities including tectoquinone or 2-methyl anthraquinone [31] as shown in **Figure 1.2**, 3-hydroxy-2-methylanthraquinone [32], 1-hydroxy-2-methylanthraquinone, 2-hydroxymethylanthraquinone, 1,4-dihydroxy2-methylanthraquinone, tectol, dehydrotectol [33], lapachol [34], desoxylapachol [35], and caoutchouc [36]. The chemical structures are closely related to the bio-activity. However, the described activities are also related to the concentration of chemicals in teakwood. In this study, we focused on 2-methylanthraquinone and its termiticidal activity against subterranean termites.

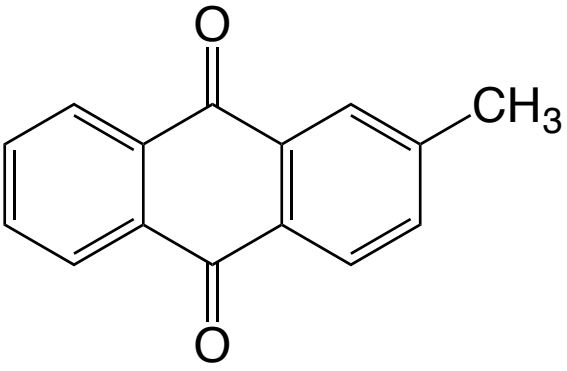


Figure 1.2 The chemical structure of 2-methyl anthraquinone (MAQ)

1.6 The Pulp and Paper Industry in Indonesia

The paper mill industry has been growing significantly for more than 100 years, increasing annually worldwide. It is expected that in the year 2020 this industry will produce almost 500 million tons of paper and paperboard per year [37]. In Indonesia, the pulp and paper industry plays a vital role in supporting the national economy. Improving natural fiber sources for use as raw materials for the pulp and paper industry is, therefore, required as it has been predicted that this would increase production annually to 64 m^2 in 2025. The Ministry of Environment and Forestry of the Republic of Indonesia has promoted 12 new projects for pulp and paper companies to fulfill the demand of an approximately 8-million-ton pulp production capacity [38] (**Figure 1.3**). Almost 75% of total industrial forest in Indonesia supplied raw material to the pulp industry in 2005. They have three scenarios to enlarge forest area in locations with mineral-rich soils such as peatland and mineral soil imperata. Peatland was selected as an alternative to plantation forest as a source of raw material for the pulp and paper industry. In Indonesia, there is a large amount of peatland that has not been properly cultivated. Not all acacia species can grow in peatland, however, as it is nutrient-less with a low pH.

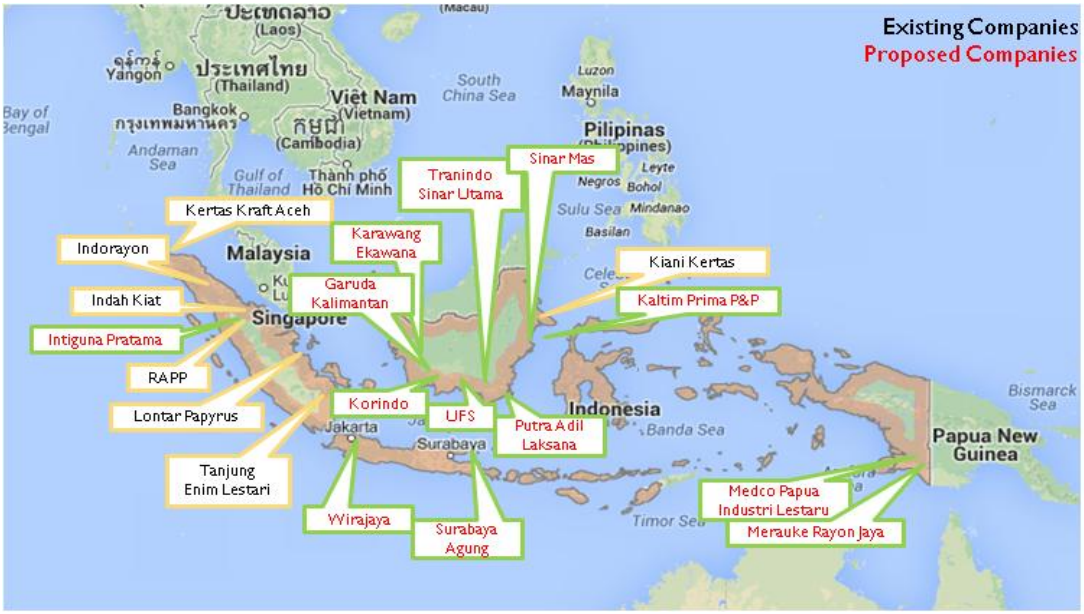


Figure 1.3 Existing and planned pulp and paper mills in Indonesia [38]

1.7 ***Acacia crassicarpa***

Acacia crassicarpa A. Cunn. ex Benth was introduced to Indonesia at the beginning of 1989 [39]. It is a humid/subhumid tropical species of acacia that originated in northeastern Queensland, Australia; Southwestern Papua New guinea, and southeastern Irian Jaya, Indonesia [40, 41]. *A. crassicarpa* is a multipurpose wood [42] found in large plantations in Indonesia that is fast growing and is suitable as a raw material for kraft pulping [43] has. In Indonesia, the large scale production of *A. crassicarpa* is driven by the need for raw material for the 84 pulp and paper mills present in 2013 [44]. The expansion of the pulp and paper industry also represents an environment issue in terms of the large amounts of waste produced in the form of bark byproducts.

The latest data obtained from the Directory for the Pulp and Paper Industry in Indonesia, reported by APKI (Asosiasi Pulp dan Kertas Indonesia or Indonesian Pulp and paper Association), the total annual pulp and paper production using *A. crassicarpa* as the raw material was approximately 350,000 ton and 450,000 ton, respectively, in 2007. Total percentage of bark volume depends on the moisture content and specific gravity of inner and outer bark. Hardwood usually has a bark volume percentage of about 5% of total wood, meaning that all the bark from these trees would be unutilized. As an industry byproduct, this bark is not utilized properly. Conversely, in New Zealand, the bark of South African *Acacia mearnsii* has been utilized as leather and also in adhesive manufacturing [45]. The increasing use of bark waste as a byproduct of the pulp and paper industry is dependent on the chemical or secondary metabolite content of the bark. Unfortunately, few studies have examined the tannin content of *A. crassicarpa* bark, with Pietarinen et al. [46]

reporting only on polyphenolic compounds from knot and stem wood of *A. crassicarpa*. Melacacidin and isomelacacidin were found to be the main compounds and small amounts of the flavanones taxifolin, guercetin, and catechin were identified by GC/MS.

Bark is a natural source of the tannins that are found in seeds, fruits, leaves, roots, and wood [47, 48] leading some researchers to investigate tannin extraction methods or analyze the biological activity of tannins. Table 1 shows the potential biological activity of tannins originating from different plants. To enhance tannin use, efficient tannin extraction methods and structural elucidation techniques are required. Using the waste bark produced by pulp and paper companies for the extraction of tannins would help to eliminate the environmental pollution associated with the unutilized bark byproducts, increasing sustainability.

Table 1.3 Potential biological activities of tannins

Bioassay activity	Source	Chemical constituent	References
Protein precipitation	*Sorghum bicolor* (Lin.) Moench	Vegetable tannin	[49,50]
Insecticide and Herbicide	*Quercus robur* (Leave)	Polyphenol (vegetable tannin)	[47]
Tanning material	Black wattle 'mimosa' bark extract	Condensed tannin	[19]
Wood adhesives	Bark extract from some pine and spruce species	Polyflavonoid tannin	[20]
Wood preservative (from *Trametes versicolor*)	Pinus taeda	Condensed tannin	[51]
Antifeedant activity against *Coptotermes formosanus*	Japanese larch wood	Taxifolin and Aromadendrin	[18]

Figure 1.4 An *Acacia crassicarpa* tree planted at PT. RAPP Pekanbaru, Indonesia (2010)

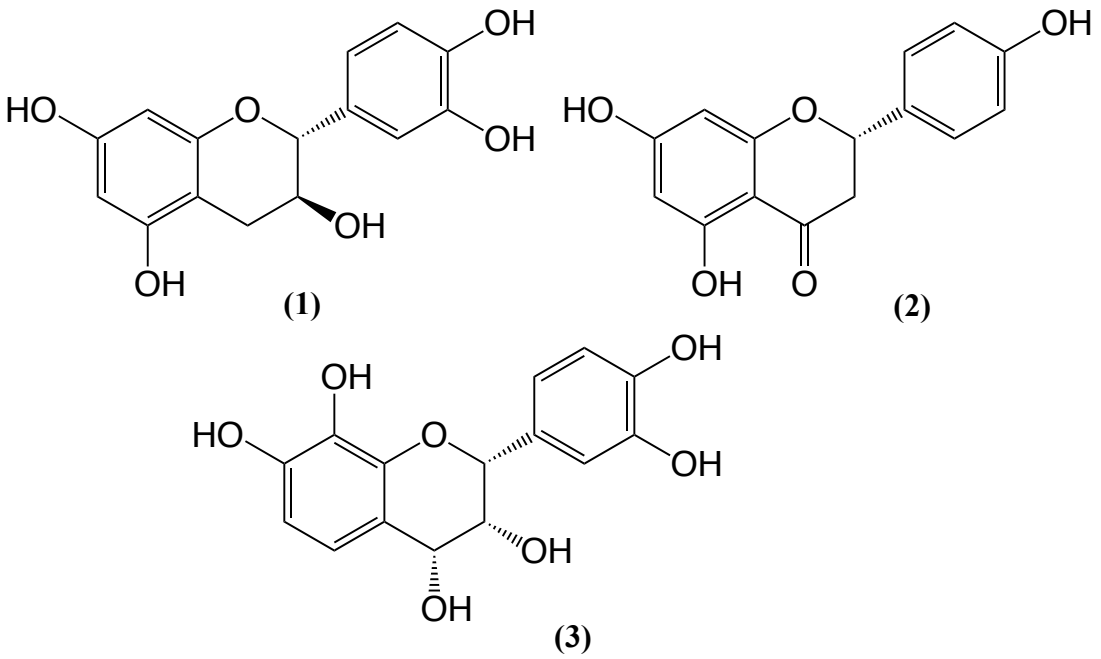


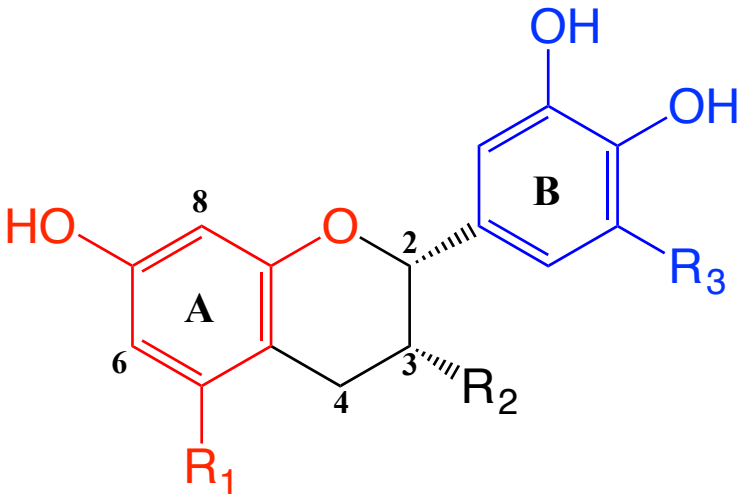
Figure 1.5 Polyphenolic compounds from knot and stem wood of *A. crassicarpa* [46]. Legend: melacacidin (1), isomelacacidin (2), and taxifolin (3)

1.8 Condensed Tannin

Tannin is found worldwide in many different plant families and is the fourth most abundant biochemical substance in vascular plant tissue after cellulose, hemicellulose, and lignin. High concentrations of tannins are found in nearly every part of the plant including bark, wood, leaves, fruit, roots, and seeds. The amounts and types of tannin in trees are affected by species, the tree age, and the growth locations.

Generally, tannin can be divided into two groups, hydrolyzable tannin and condensed tannin. Hydrolyzable tannins are derived from gallic acid (3, 4, 5-trihydroxyl benzoic acid) while condensed tannins are derived from polymer flavonoids. Flavonoids are also secondary metabolites with a heterocyclic ring system derived from phenylalanine (B ring) and polyketide biosynthesis (A ring).

The condensed tannin is formed as a polyhydroxy-flavan-3-ol oligomer and polymer linked by a flavonol subunit that attaches to proteins, metal ions, and polysaccharides [52].



R_1	R_2	R_3	Class
OH	OH	H	Proanthocyanidin
OH	OH	OH	Prodelphinidin
H	OH	H	Profisetinidin
H	OH	OH	Prorobinetinidin
H	H	H	Proluteolinidin

Figure 1.6 Classification of condensed tannins

1.9 Analysis Methods for Identifying Active Compounds in Wood Extractives

1.9.1 Pyrolysis -Gas Chromatography /Mass Spectrometry (Py-GC/MS)

Nowadays, Py-GC/MS is used for polymer analysis where molecules in a sample are broken down by heat at specific temperatures in the absence of oxygen. Advantages of this method include simple sample preparation (drying and milling), rapid analysis time (from min up to 1.5 h), and small sample size (1 to 100 μg). The combination of the pyrolyzer with other instruments is divided into two systems. The "off-line system," when the pyrolysis system is separated from the analytical instrument, is where pyrolysis and analysis are performed in two steps. Conversely, the "on-line system" is when the pyrolysis unit and the analytical instrument are directly coupled, for example with a GC using a detection system based on Flame Ionization Detector (FID), Mass Spectrometry (MS), or Fourier Transform Infrared Spectroscopy (FTIR). Py-GC/MS can also be used to analyze native polymers such as the following:

a. Lignin classification based on the quantities of the three phenylpropanoid units, namely 4-hydroxyphenylpropane (H), guaiacylpropane (G), and syringylpropane (S), formed during pyrolysis of lignocellulosic materials.
b. Microanalysis of lignin in wood cells and cell fragments of different morphological origin
c. Analysis of residual lignin in pulp
d. Fingerprinting and identification of technical lignin from the pulping process [53].

In **Figure 1.7** the Py-GC/MS instrument is shown as an "on-line system" attached to a GC and MS. Also shown is a schema for a simple sample preparation.

A sample is inserted between sheets of thermostable pyrofoil with a standard (*n*-eicosane), wrapped tightly, directly subjected to pyrolyzer, and analyzed by Py-GC/MS.

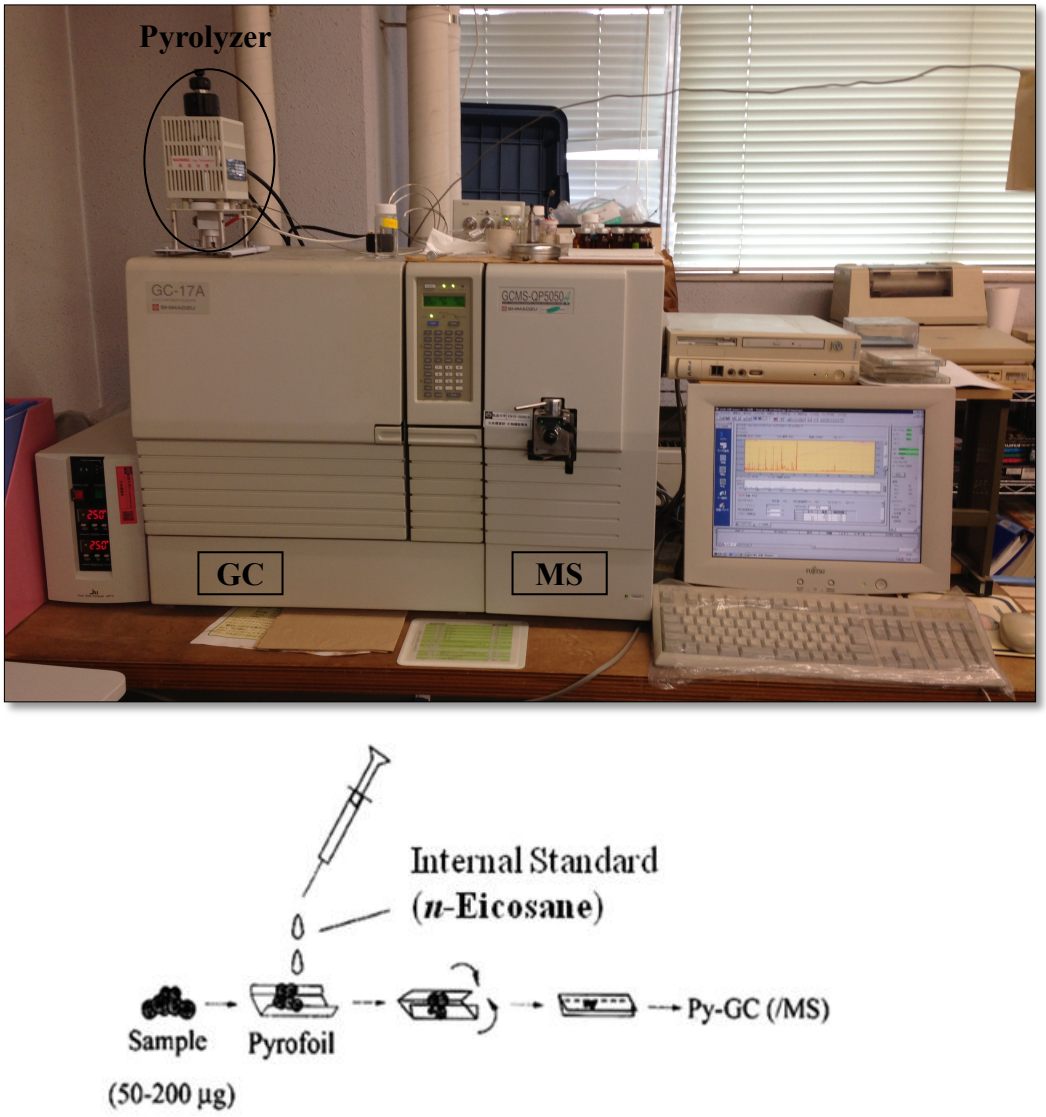


Figure 1.7 Py-GC/MS instrument and sample preparation for Py-GC/MS analysis

1.9.2 FAB-MS

Fast atom bombardment mass spectrometry (FAB-MS) is a popular tool for studying the molecular weight of biopolymers. The molecular weight is an important parameter that can be used to elucidate the structure of tannins. Determining whether condensed tannin occurs as a dimer, trimer, or even oligomer is, however, a difficult task as tannin polymers, which have high molecular weights, are polar and thermally labile. Thus, FAB-MS was conducted at room temperature (called "soft" ionization).

FAB-MS uses a target where the sample is placed and dissolved in a liquid matrix (such as glycerol, thioglycerol, or m-nitrobenzyl alcohol) before the target is bombarded with a fast atom beam (using Xenon atoms) that results in a chemical background that varies with the matrix used [54].

1.9.3 ^{1}H-NMR and ^{13}C-NMR

Nuclear magnetic resonance (NMR) spectroscopy is a technique used to elucidate a compound's unique structure. A sample is placed in a homogeneous magnetic field, irradiated, and a magnetic signal is detected [55]. This technique has been adapted to many areas including chemistry, biology, medicine, material science, and geology. The pioneers of this technique, Felix Bloch and Edward Purcell, were awarded a Nobel Prize in 1952 for their development of new methods for nuclear magnetic precision measurement and the discoveries they made using these techniques [56]

The principle of NMR is related to the spinning of atomic nuclei that generate a magnetic field. Without an externally applied magnetic field, the nuclear spins are random with the nuclei spinning in random directions. When an external magnetic field is present, the nuclei align themselves either with or against the field of the external magnet. As result, we can observe a "chemical shift, ∂" at the position of a signal in an NMR spectrum. This is defined as being the period for which a proton/carbon nucleus is moved from the standard's nucleus to the frequency used. The magnitude of the shift depends on the type of nucleus and surrounding atoms and or molecules.

Figure 1.8 shows a protocol for the use of NMR analysis to elucidate organic chemical structures.

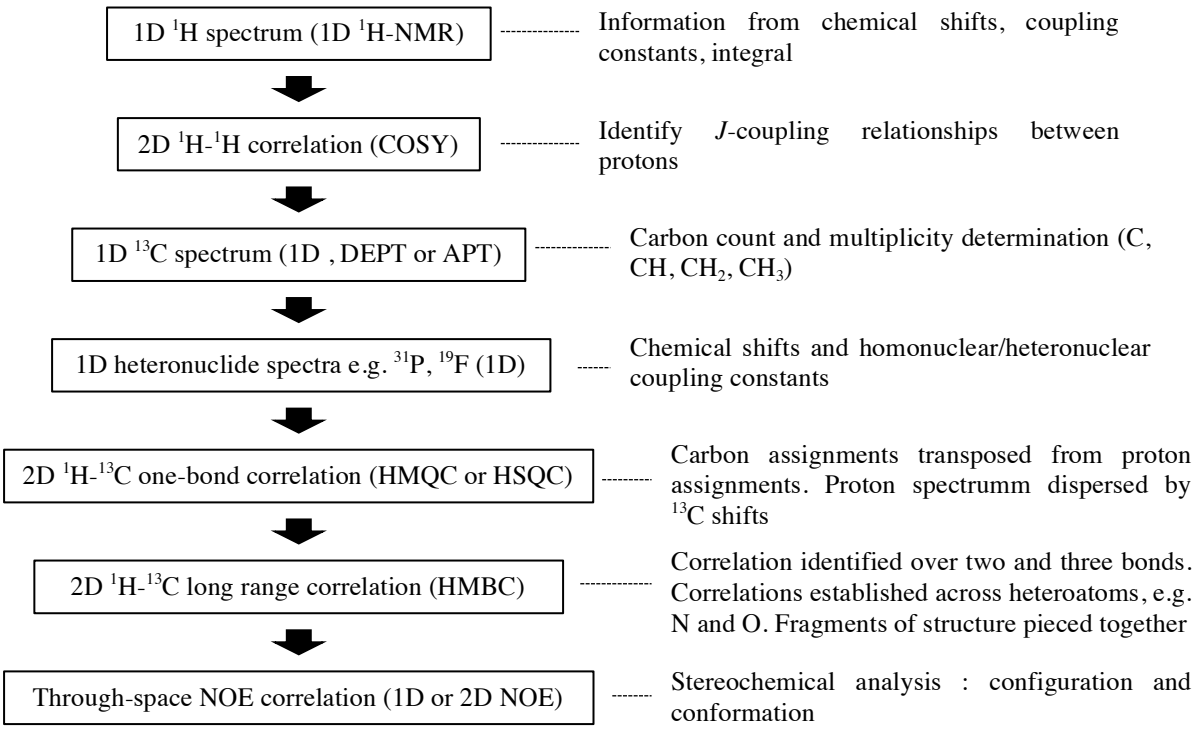


Figure 1.8 A protocol for the structure elucidation of organic material.

1.10 Objective of This Study

In this study, wood extracts that are used as a wood preservative against termites are examined to determine the structure of their active compounds and the termiticidal effects of these compounds against the subterranean termite species *R. speratus* and *C. formosanus*. The aim of this study was to characterize an environmental-friendly termiticide that could replace existing synthetic ones. This was done using extracts from bark waste of acacia and teakwood, which contained 2-methyl anthraquinone (MAQ), and examining their effect on termite mortalities and feeding activities.

The outline of this study is as follows:

In Chapter two, the first objective was to isolate the crude extract from *T. grandis* and fractionate this extract using ethanol, chloroform, and acetone as solvents. The second objective was to determine the MAQ content of crude extracts from *T. grandis* and the fractions derived from these crude extracts. The third objective was to evaluate the toxicity and feeding deterrent effects of MAQ that is found in different amounts in both extracts and fractions.

In Chapter Three, the first objective was to isolate tannin compounds from bark waste of *A. crassicarpa*. The second objective was to elucidate the structure of these tannins using Py-GC/MS, FAB-MS, and NMR. For the third objective, the condensed tannin isolated from *A. crassicarpa* was used in a termiticide bioassay.

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Chapter 2 Toxicity and Feeding Deterrent Effect of 2-Methylanthraquinone from the Wood Extractives of *Tectona grandis* on the Subterranean Termites *Coptotermes formosanus* and *Reticulitermes speratus*

2.1 Introduction

The naturally occurring anthraquinone, 2-methylanthraquinone (MAQ) found in teak (*Tectona grandis*) heartwood is often associated with its high durability [1,2]. According to several studies, teak wood extractives clearly play a role in its resistance against termites [1,2,3] and fungi [1,2,4]. Teak sapwood is generally considered less durable; the recent finding of MAQ in sapwood suggested this compound should not act on the wood durability [5].

Wood preservatives obtained from natural products are of interest with respect to the relationship between wood extractives and wood durability. MAQ has been suspected to be an important compound granting teak wood its durability against termite infestation [4,6,7]. At high concentrations, MAQ is toxic to the termite *Reticulitermes flavipes* and acts as a repellent to drywood termites [8]. In contrast, it is non-toxic to subterranean termites *Coptotermes lacteus* and *Nasutitermes exitiosus*, merely acting as a deterrent [1,2].

Most of these studies, however, have focused on the effects of such compounds within trees, and there is limited information on termite resistance in relation to the amount of MAQ in teak wood extractives. We hypothesize that wood resistance against termites depends on wood compounds and the contents, i.e., certain compounds provide resistance to termites, even at low concentrations. Thus, the MAQ content of teak wood and its extractives was determined in this study; we also

evaluated the activity of MAQ and teak wood extractives against two species of subterranean termites, *Coptotermes formosanus* and *Reticulitermes speratus*.

2.2 Experimental

2.2.1 Material

Debarked disks of *T. grandis* natural wood imported from Myanmar in 2000 was kindly provided by the HOKUSAN Corporation Ibaraki Factory (Ibaraki Prefecture, Japan). A radius of the wood disk was 35 cm, and lengths of sapwood and heartwood parts were 2.5 cm and 32.5 cm, respectively. The number of the annual rings was counted to 210.

2.2.2 Termite

Reticulitermes speratus was collected from the Living Sphere Simulation Field, Research Institute for Sustainable Humanosphere (RISH), Kyoto University (Kagoshima Prefecture, Japan) and maintained at the University of Tsukuba (Ibaraki Prefecture, Japan), under controlled laboratory conditions (28°C and 80% relative humidity). *Coptotermes formosanus* was maintained at the Forestry and Forest Products Research Institute (FFPRI), Ibaraki, Japan.

2.2.3 Extraction and fractionation

Wood powder (2 g, oven-dried weight) was sieved (40–60 mesh size, 180–355 μm opening) and Soxhlet-extracted for 8 h using 150 mL of ethanol, chloroform, or acetone, respectively. All solvents were special grade of Wako Pure Chemical Industries. Three replicates were performed for each extraction and the resulting solvents were evaporated to concentrate and obtain crude extractives. Approximately 100 mg of each crude extractives were dissolved in 0.5 ml of each solvent and fractionated by preparative layer chromatography (PLC) (PLC Silica gel 60 F_{254}

2 mm, 20 × 20 cm, Merck KGaA, Darmstadt, Germany). The mobile phase used for ethanol, chloroform, and acetone crude extractives was *n*-hexane: ethyl acetate (9 mL: 1 mL). Extraction and fractionation are schematically represented in **Figure 2.1**. Each fraction (EB-EF, CA-CE, and AA-AD defined in **Figure 2.1** and **Table 2.2**) was obtained by scraping the silica gel plate based on a retention factor (Rf). The PLC results were visualized with a UV lamp (240-400 nm), and each area spot was categorized as one fraction.

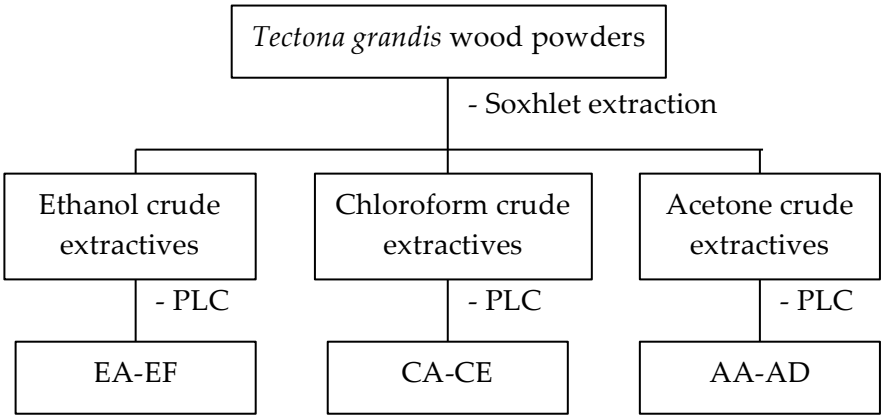

Figure 2.1 Schematic representation of the extraction and fractionation performed for *Tectona grandis* wood extractives.

Notes: PLC = Preparative Layer Chromatography (Silica gel 60 F_{254} 2 mm, 20 × 20 cm); EA-EF = fractions obtained from ethanol crude extractives; CA-CE = fractions obtained from chloroform crude extractives; AA-AD = fractions obtained from acetone crude extractives.

2.2.4 Primary no-choice feeding tests against *R. speratus*

The crude extractives dissolved in each solvent at a 2% (w/v) concentration were used in no-choice feeding tests. An aliquot (30 μL) of each sample was placed onto a paper disc (30 mg, 8 mm in diameter, thick type; ADVANTEC TOYO, Tokyo, Japan), which was dried at 60°C for 24 h to remove the solvent. No-choice feeding tests were performed using 50 workers and 5 soldiers of *R. speratus* in an incubator set at 28°C for 14 days. After this time, paper discs were taken out, cleaned from debris, oven-dried at 60°C for 24 h, and finally weighed to calculate mass loss [9]. As a control, untreated paper discs (PDUs) were dried in the same manner as the discs with the extractives. To measure termiticidal activity, dead termites were counted. This test was carried out using the *R. speratus* maintained at the University of Tsukuba. Three replicates were performed for each sample.

Mass loss (%) = [ODS1-ODS2/ODS1] x 100% .....(1)

ODS1 is oven-dried paper disc before test, while ODS2 is oven-dried paper disc after test

Termite Mortality = [Number of died termite/50] x 100% .....(2)

2.2.5 No-choice feeding tests against *R. speratus* and *C. formosanus* using fractions of teak extractives

A paper disc was impregnated with with 30 μL of each sample containing ethanol, chloroform, or acetone extractives with an equal amount of MAQ (22 μg per paper disc). In addition, a paper disc was impregnated with a fraction for 14 fractions from the three extractives. Disks were then used in no-choice feeding tests, performed as described in section 2.3., with 50 workers and 5 soldiers of *R. speratus*

or *C. formosanus* individuals originating from the colonies maintained at the University of Tsukuba and at FFPRI, respectively.

2.2.6 Statistical analysis

The effects of MAQ on mass loss and termite mortality were examined using an analysis of variance (ANOVA), and samples showing significant differences ($P < 0.05$) were analyzed using Duncan's post hoc test ($\alpha = 0.05$). All analyses were performed using IBM SPSS version 22 (IBM Japan, Tokyo, Japan).

2.2.7 Gas chromatography (GC) and pyrolysis-gas chromatography–mass spectroscopy (Py-GC/MS)

The amounts of MAQ in the extractives (including extractives' fractions) were analyzed using a GC-17A gas chromatograph (Shimadzu, Japan), equipped with a DB-1 column (30 m × 0.25 mm i.d., film thickness, 0.25 μm) and a flame ionization detector using helium as the carrier gas. The injection temperature was 300°C and split ratio was 50:1. The temperature profile for GC analysis was as follows: 5 min at 160°C, 12 min at 160–280°C (10°C/min), and 4 min at 280°C. The MAQ content of the extractives was determined by comparison with a standard prepared from the authentic compound.

The contents of MAQ in raw materials and extracted residues were analyzed by pyrolysis-gas chromatography-mass spectrometry (PyGC/MS). After n-eicosane as an internal standard was added, wood powders or extracted residues (150–200 μg, measured with a micro balance) were wrapped with a pyrofoil and pyrolyzed at 500°C for 4 s using a JHP-5 pyrolyzer (Japan Analytical Industry, Tokyo, Japan), which was interfaced (interface temperature 250°C) with a GC/MS system QP-5050 (Shimadzu, Kyoto, Japan) equipped with an HP-1MS column (30 m × 0.25 mm i.d.

film thickness. 1.0 μm), with electron impact of 70 eV and helium as a carrier gas. The temperature profile for GC was as follows: 1 min at 50°C, 5 min at 50–280°C (5°C/min), and 13 min at 280°C. Products resulting from the pyrolysis were identified by comparing their retention times and mass spectra data with those obtained for authentic compounds and with published data [10].

2.3 Results and discussion

2.3.1 Extraction, fractionation, and determination of MAQ content

Extractives content in teak wood is related to tree age, which is one of the most important factors affecting natural durability of wood [11]. In teak wood, the number of black stripes increases with increasing age and represents a type of defense mechanism that protects teak wood from insects and fungi [2]. In addition to tree age, longitudinal variation, geographical location, environmental conditions, and silvicultural activities can influence heartwood extractives, color properties, and durability [2]. The MAQ content determined in the present study (**Table 2.1**) was similar to previous reports indicating that teak wood extractives contain 0.3–0.9% of MAQ [6,12,13].

The means of crude extractives and MAQ yields of the wood powders of *T. grandis* individually Soxhlet-extracted with ethanol, chloroform, and acetone are shown in **Table 2.1**. MAQ yields in the extractives were determined using GC, while the extracted residues were analyzed by Py-GC/MS. Chloroform produced the highest extractives yield among the three solvents. In the PLC of the crude extractives, R_f values of the main fractions which included MAQ were 0.52–0.56. They were similar to Rf value of the authentic compound of MAQ (**Table 2.2**).

The Py-GC/MS generated a MAQ peak and pyrolysis products (G1-G8, S1-S6, C) peaks corresponding to lignin and carbohydrates (**Figure 2.2**). Chloroform-extraction of the raw material clearly decreased the MAQ peak, while the lignin and carbohydrate pyrolysis product peaks were retained.

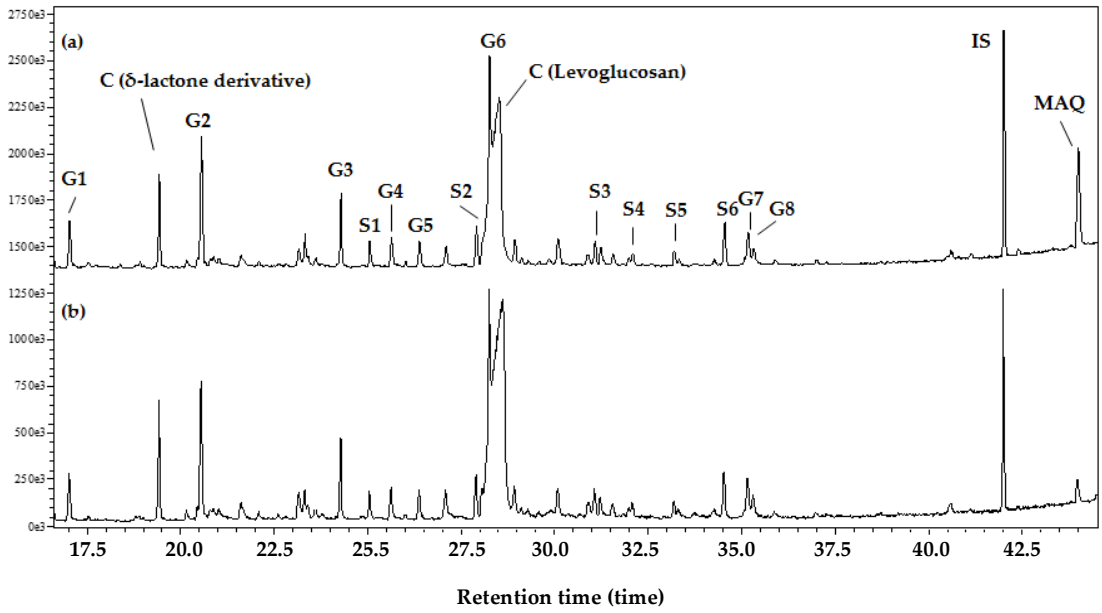

Figure 2.2 Pyrogram obtained in the Py-GC–MS of *Tectona grandis* heartwood (a) and chloroform-extracted residues (b).

Legends: IS = internal standard *(n*-eicosane), G1 = guaiacol, G2 = 4-methylguaiacol, G3 = 4-vinylguaiacol, G4 = eugenol, G5 = vanillin, G6 = *trans*-isoeugenol, G7 = coniferaldehyde, G8 = trans-coniferyl alcohol, S1 = syringol, S2 = 4-methylsyringol, S3 = 4-vinylsyringol, S4 = 4-allylsyringol S5 = syringaldehyde, S6 = *trans*-4-propenylsyringol, G1-G8 = pyrolysis products from guaiacyl lignin unit, S1-S6 = pyrolysis products from syringyl lignin unit, C = pyrolysis products from carbohydrate, and MAQ = 2-methylanthraquinone.

Table 2.1 Content of 2-methylanthraquinone (MAQ) in crude extractives and residues of *Tectona grandis* heartwood and the dosages of crude extractives and MAQ used in no-choice feeding tests

Extraction solvent	Crude extractives recovered (%)[1]	MAQ in crude extractives (%)[1]	MAQ in extracted residue (%)[2]	Dosage in no-choice feeding[3]	
				Crude extractives (µg)	MAQ (µg)
Ethanol	6.5 ± 0.6	0.11 ± 0.01	0.05 ± 0.01	602	10
Chloroform	8.7 ± 0.2	0.10 ± 0.01	0.05 ± 0.02	606	6.8
Acetone	6.6 ± 0.2	0.15 ± 0.01	0.04 ± 0.01	666	15

[1] Mean ± standard deviation analyzed by GC, based on wood

[2] Mean ± standard deviation analyzed by Py-GC/MS, based on residue

[3] No-choice feeding test against *Reticulitermes speratus*

The MAQ peak in the Py-GC/MS analysis was detected in addition to pyrolysis products from lignin and carbohydrates [14]. The MAQ peak detected at the end of the chromatogram was successfully determined, in this study, using a calibration line, indicating a 0.21% yield based on wood weight (Figure 2.2). Carbohydrates are readily fragmented into smaller compounds, making it difficult to determine their origin (cellulose and hemicelluloses), but levoglucosan was detected in the middle of the chromatogram, along with lignin pyrolysis products. This result is consistent with the range of MAQ content (0.2–1.1%) determined in a previous study using a Py-GC/flame ionization detector [15].

The MAQ yield of the remaining residues after wood powder extraction as determined by Py-GC/MS ranged from 0.04–0.05%. The MAQ yield of ethanol extractives was 0.11 %, and the sum of the yields was 0.16%, meaning there was a difference between the content of raw material (0.21%) and the calculated yield (0.16%). This difference might be due to the Py-GC/MS not requiring sample preparation and being more accurate than GC. As MAQ and other anthraquinone derivatives might play a role in teak wood durability, the use of Py-GC/MS is useful for detecting compounds that might have toxicity and feed-deterrent effects.

2.3.2 Primary no-choice feeding test of crude extractives in *R. speratus*

The results of the primary no-choice feeding tests are shown in **Figure 2.3**. The mass loss of paper disks and termite mortality were calculated after 14 days of observations. Mass loss in ethanol, chloroform, and acetone extractives was less than 1% whereas MAQ dosage (10, 6.8, and 15 μg) differed among the three crude extractives (Table 2.1). In addition, mass loss using a 2–10 μg of MAQ was approximately 40% whereas that using 20 μg of MAQ was 20%. Ethanol and

acetone crude extractives presented the highest levels of termite mortality (93% and 90%, respectively), whereas chloroform crude extractives produced a moderate level of mortality (63%). The treatment using 20 µg of MAQ resulted in a lower termite mortality (approximately 10%) than that with the chloroform extractives containing using 6.8 µg of MAQ. Crude extractives had marked effects on no-choice feeding tests but mass loss and mortality were not directly related to the MAQ dosage, in the 2–20 µg range.

ANOVA indicated significant differences ($P = 0.001$) in the mean of mass loss between test and control treatments.

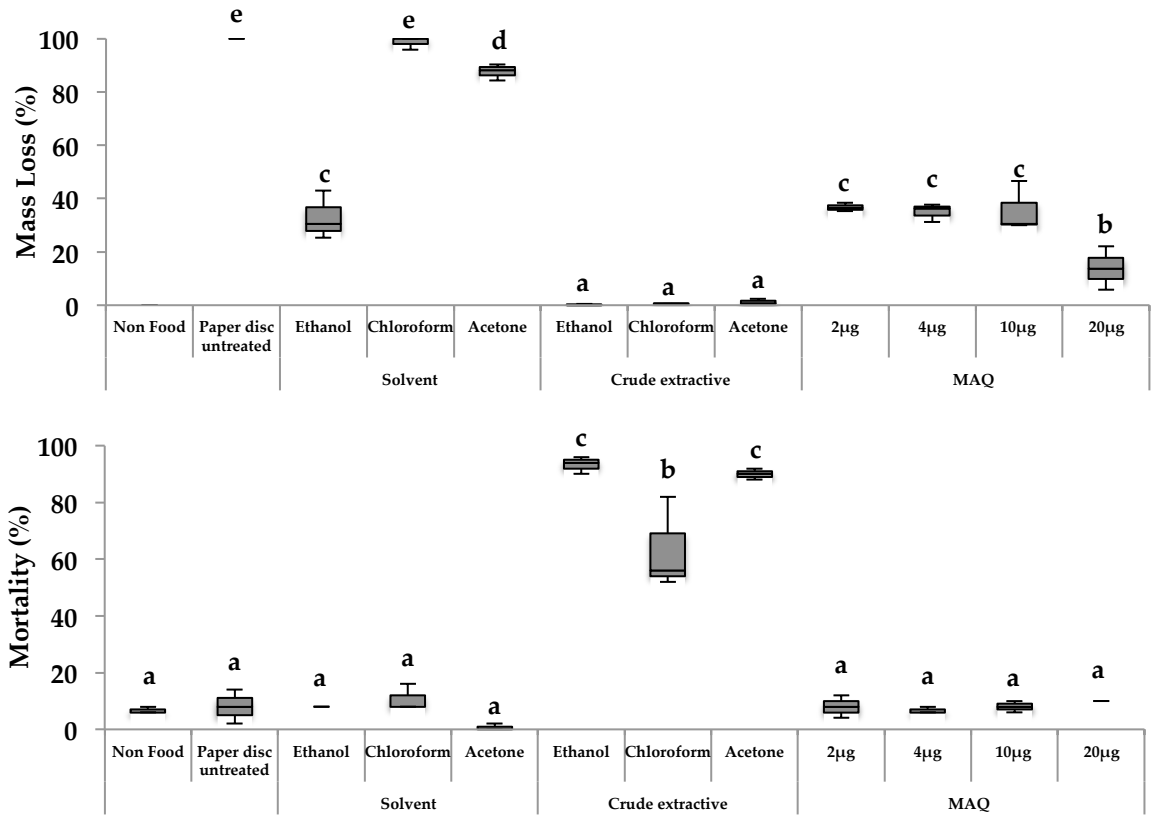

Figure 2.3 Mass loss and mortality of *Reticulitermes speratus* in no-choice feeding tests in *Tectona grandis* crude extractives and MAQ.

Notes: Treatments with the same letters in the same graphic are not statistically different at $p < 0.05$, as determined by Duncan's test; Dosages of the extractives and MAQ are indicated in **Table 2.1**

The primary no-choice feeding tests using *R. speratus* revealed that crude extractives of *T. grandis* clearly displayed a feeding deterrent effect, as indicated by the highly significant difference in the mass loss of paper discs (Figure 2.3). Meanwhile, control tests using a standard MAQ compound indicated that MAQ (2-20 μg) dosage was not directly related to termite feeding activity. Moreover, the higher termite mortalities found in crude extractives tests than in control solvents (blank tests) and MAQ compound tests demonstrated their toxic effect. Termite mortalities caused by ethanol and acetone extractives were high, whereas that caused by chloroform was moderate.

Lukmandaru and Ogiyama [3] suggested that the high termite mortality observed in teak wood extractives might be due to MAQ, which was proposed to have both antifeedant activity and toxicity. However, the effects of MAQ amounts in extractives were not known. Thus, the effect of MAQ dosages on termite resistance should be further investigated. Differences in toxicity might also be related to the species of termites [16] and, therefore, we used two species of subterranean termites, *R. speratus* and *C. formosanus*, which are known to cause extensive damage to wood constructions [16]. In addition, as *C. formosanus* is a native pest, it might provide valuable information regarding the natural durability of teak wood.

2.3.3 No-choice feeding tests of crude extractives in *R. speratus* and *C. formosanus*

2.3.3.1 Mass loss

In order to further clarify the effects of MAQ, no-choice feeding tests were conducted using *R. speratus* and *C. formosanus*. The concentrations of crude extractives were adjusted in order to obtain an equal amount of MAQ (22 μg per

paper disc). In addition, PLC fractions from crude extractives (Table 2.2) were used in no-choice feeding tests. This experiment evaluated the effect of other compounds in the crude extractives on mass loss and termite mortality.

Figure 2.4 shows the mass loss for *R. speratus* and *C. formosanus* after 14 days of observations. A close examination of crude extractives containing 22 μg of MAQ showed a similar mass loss using ethanol, chloroform, and acetone extractives in *R. speratus* tests (2.4%, 4.1% and 5.2%, respectively). Mass losses with *C. formosanus* tests tended to be higher than those with *R. speratus* (18.7%, 5.9% and 27.1% in ethanol, chloroform, and acetone extractives, respectively).

Regarding extractive fractions, low mass losses were obtained for EB (55 μg of MAQ), EC (16 μg of MAQ), CA (0.8 μg of MAQ), AB (162 μg of MAQ), and AC (7.6 μg of MAQ) in R. speratus tests. In contrast, mass losses of these fractions in *C. formosanus* tests tended to be high: notable mass loss values were obtained for EB (6.7%), EC (5.7%), and AB (1.4%).

For comparison, paper discs were also impregnated with a standard MAQ compound (2, 20, 100, and 200 μg per paper disc). Significant differences were observed in both subterranean termites using 100 μg and 200 μg MAQ compound impregnations, with mass losses in the 10–20% range.

The ANOVA revealed significant differences in mass loss among the *R. speratus* tests using MAQ, and ethanol and chloroform extractives (P: 0.078, 0.002, and 0.005, respectively), but not using acetone extractives (P: 0.323). Significant differences in mass loss were found with *C. formosanus* tests for MAQ and ethanol extractives (P: 0.014 and 0.021), but not for chloroform or acetone extractives (P: 0.477 and 0.224).

Table 2.2 Fractions obtained from crude extractives by preparative layer chromatography (PLC)

Fraction[1]	Rf	Extractives weight (µg)	MAQ weight (µg)	Dosage in no-choice feeding test[2]	
				Extractives (µg)	MAQ (µg)
Ethanol crude extractives				1296	22
EB	0.52	5590	468	656	55
EC	0.27	2470	64.4	600	16
ED	0.21	2890	2.55	573	0.5
EE	0.07	10300	2.37	687	0.2
EF	0.00	30400	2.37	779	0.1
Chloroform crude extractives				1960	22
CA	0.55	21900	25.3	683	0.8
CB	0.41	14000	0.81	698	0
CC	0.34	14600	0.12	795	0
CD	0.06	20700	0.35	848	0
CE	0.00	15500	0.46	764	0
Acetone crude extractives				946	22
AA	0.69	2540	3.06	585	0.7
AB	0.56	3730	887	681	162
AC	0.24	7450	68.6	828	7.6
AD	0.00	10120	26.5	707	1.9

Legends: R_f = retention factor

[1] Amounts of ethanol, chloroform, and acetone crude extractives using PLC were 108, 103, and 111 mg, respectively.

[2] No-choice feeding test against *Reticulitermes speratus* and *Coptotermes formosanus*

2.3.3.2 Termite mortality

The Mortality of both *R. speratus* and *C. formosanus* in the no-choice feeding test are summarized in Figure 5. There were significant mortalities in tests of the extractives with 22 μg of MAQ for R. speratus using the ethanol, chloroform and acetone extractives (92.0%, 87.3%, and 91.3%, respectively). In contrast, the extractives had no effect on the mortality against *C. formosanus* (less than 5%).

Notably high mortalities were caused by exposure to CA (0.8 μg of MAQ) or AB (162 μg of MAQ) fractions on *R. speratus* and by CC (none MAQ) on C. formosanus. These results did not appear to be related to MAQ dosages. Meanwhile, the MAQ control test indicated that 200 μg was the only MAQ dosage that significantly affected R. speratus mortality, while none of the dosages affected C. formosanus mortality.

There were no significant differences in termite mortality among tests (MAQ, ethanol, chloroform, and acetone extractives) for both termites (P values for R. speratus: 0.413, 0.574, 0.479, 0.182, respectively; P values for C. formosanus: 0.241, 0.327, 0.160, and 0.674, respectively)

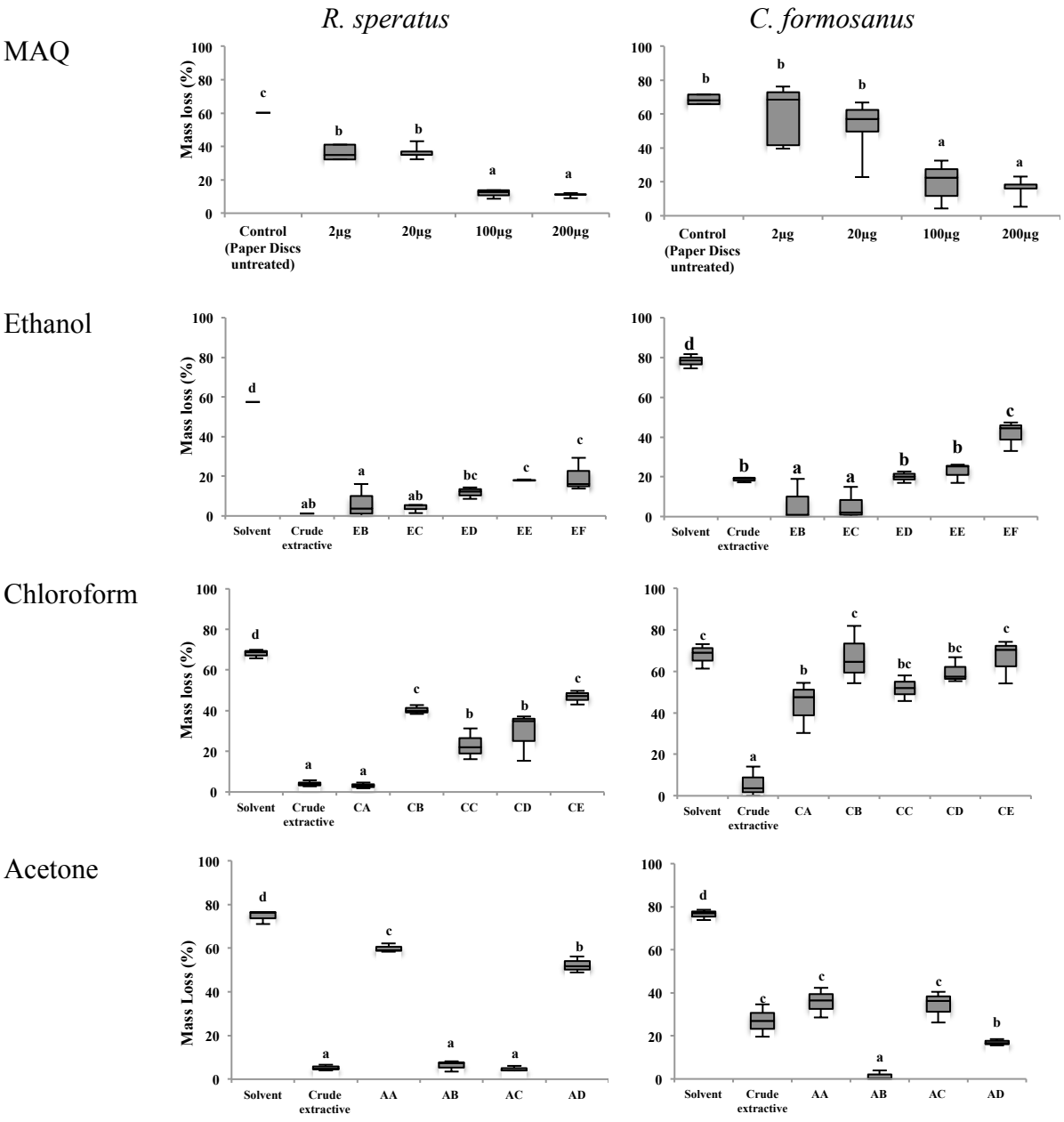

Figure 2.4 Mass loss registered in *R. speratus* and *C. formosanus* no-choice feeding tests using fractions of *Tectona grandis* crude extractives and MAQ.

Notes: Treatments with the same letters in the same graphic are not statistically different at $p < 0.05$, as determined by Duncan's test; EB-EF, CA-CE, and AA-AD are referred to **Figure 2.1** and **Table 2.2**; Dosages of extractives and MAQ are indicated in **Table 2.2**

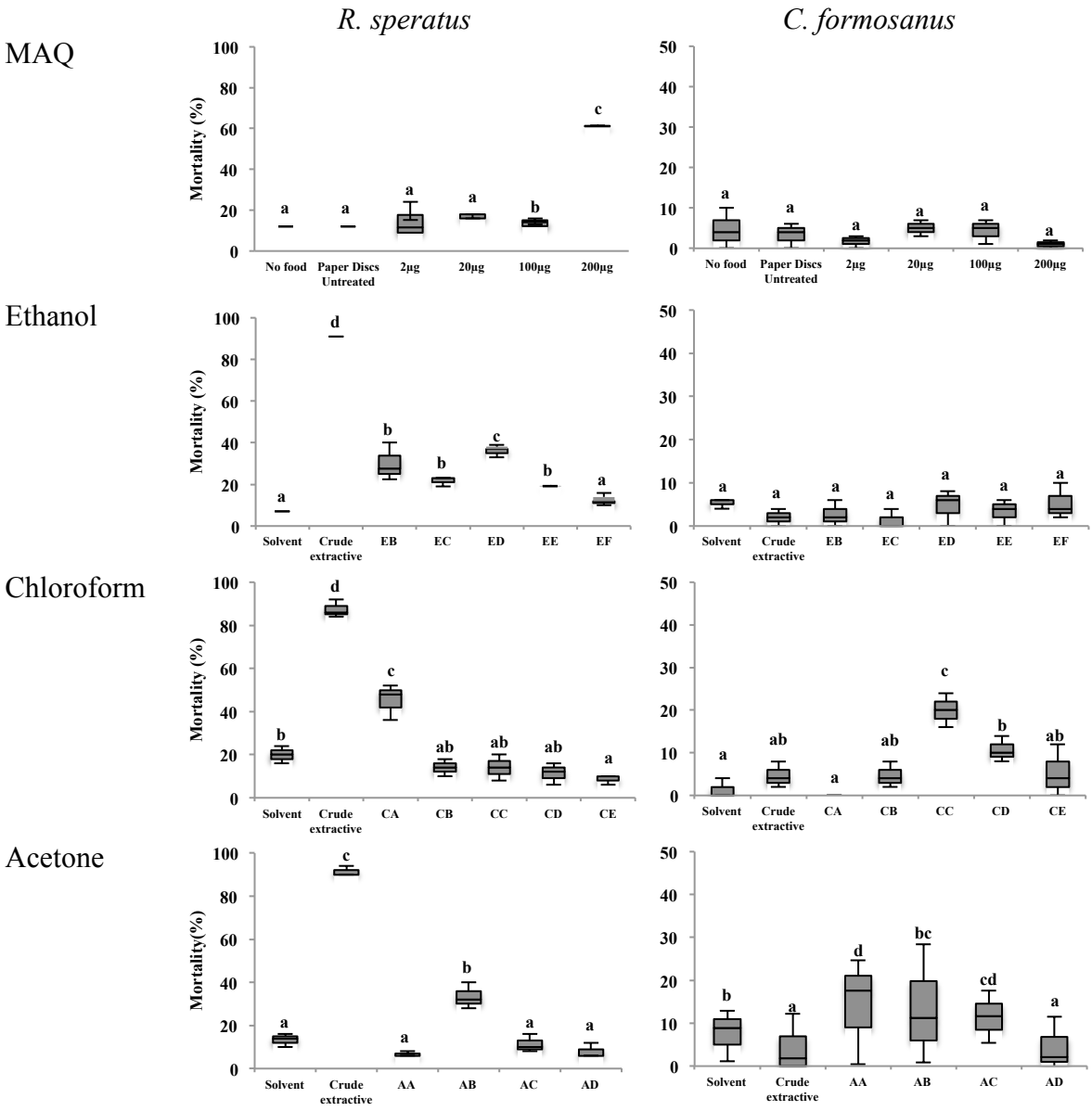

Figure 2.5 Mortality observed for *R. speratus* and *C. formosanus* in no-choice feeding tests using fractions of *Tectona grandis* crude extractives and MAQ.

Notes: Treatments with the same letters in the same graphic are not statistically different at $p < 0.05$, as determined by Duncan's test; EB-EF, CA-CE, and AA-AD are referred to **Figure 2.1** and **Table 2.2**; Dosages of extractives and MAQ are indicated in **Table 2.2**

2.3.4 Toxicity and the feeding deterrent effect of wood extractives (including MAQ) against *R. speratus* and *C. formosanus*

2.3.4.1. Effect of crude extractives

No-choice feeding tests of *T. grandis* crude extractives, including MAQ, clearly displayed feeding deterrent effects and toxicity against *R. speratus* and their feeding deterrent effect against *C. formosanus* (**Table 2.3**). No-choice feeding tests using MAQ against *R. speratus* confirmed its feeding deterrent effects at 100–200 μg dosages and toxicity at 200 μg. C. formosanus, the tests using MAQ also confirmed feeding deterrent effects at 100-200 μg, but not toxicity. The result includes that the amount of MAQ is not directly related to its feeding deterrent effect or toxicity and that *C. formosanus* is more resistant than *R. speratus*. These results are in agreement with previous studies demonstrating that different termite species have different susceptibility and resistance towards wood extractives [16,17,18].

2.3.4.2. Effect of fractions derived from crude extractives

All ethanol extractive fractions (EB-EF) and two of the acetone extractive fractions (AB, AC) clearly displayed feeding deterrent effects against *R. speratus*, similar to that obtained in crude extractives bioassays. EB-EE fractions and AB and AD fractions displayed feeding deterrent effects against *C. formosanus* similar to that of crude extractives. The EC fraction, in particular, showed a higher effect than the crude extractive.

Previous studies [1,2] have suggested that MAQ only affected the palatability of termites referred to wood or merely discouraged termite feeding, and our findings are consistent with this suggestion. Although the effects of extractive compounds on termite resistance are believed to vary with concentration, the no-choice feeding tests

conducted in the present study clearly indicated the opposite. Toxicity and the feed deterrent effect did not depend on MAQ dosages and seem to derive from the coexistence of MAQ and other compounds in teak wood extractives, as MAQ alone did not cause toxicity or feed deterrent effects, except at very high dosages. Thus, the concentrations of other active compounds and the interaction between MAQ and other components in teak wood extractives might lead to the high feeding deterrent effect observed

Table 2.3 Effects of MAQ and *T. grandis* wood extractives on toxicity and feeding deterrence against the subterranean termites *R. speratus* and *C. formosanus*

Fraction	Dosage		*R. speratus*		*C. formosanus*	
	Extractives (µg)	**MAQ (µg)**	**Toxicity**	**Feeding deterrent**	**Toxicity**	**Feeding deterrent**
MAQ	0	200	++	++	-	++
MAQ	0	100	-	++	-	++
MAQ	0	20	-	+	-	-
Ethanol crude extractives	1296	22	++	++	-	++
EB	656	55	+	++	-	++
EC	600	16	+	++	-	++
ED	573	0.5	++	++	-	++
EE	687	0.2	-	++	-	++
EF	779	0.1	-	+	-	+
Chloroform crude extractives	1960	22	++	++	-	++
CA	683	0.8	++	++	-	+
CB	698	0	-	-	-	+
CC	795	0	-	+	-	-
CD	848	0	-	+	-	-
CE	764	0	-	-	-	-
Acetone crude extractives	946	22	++	++	-	+
AA	585	0.7	-	-	-	+
AB	681	162	+	++	-	++
AC	828	7.6	-	++	-	+
AD	707	1.9	-	-	-	++

Legend: Mortality based on control (no food); ++: more than 2 time; +: 1-2 time;
Mass loss based on solvent; ++: less than 30%; +: 30-60%

2.4 Conclusion

Ethanol, chloroform, and acetone extractives of *T. grandis* heartwood had feeding deterrent effects on *R. speratus* and *C. formosanus*. No-choice feeding tests clearly indicated that toxicity and the feed deterrent effects were independent of MAQ dosages, instead deriving from the coexistence of teak wood extractives and small amounts of MAQ. The amount of MAQ in the extractives was not related to toxicity and the feed deterrent effect. *C. formosanus* was more tolerant to the extractives than *R. speratus*, and MAQ was not effective against this species. Interactions between MAQ and other components in teak wood extractives led to a high feeding deterrent effect.

2.5 References

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Chapter 3 Structural Elucidation of Condensed Tannin from Bark Waste of *Acacia crassicarpa* Plantation Wood In Indonesia and Its termiticical activity against *Reticulitermes speratus*

3.1 Introduction

Bark, a by-product of the pulp industry, is a renewable resource at pulp mills in addition to wood wastes and black liquor (the lignin-rich by-product of cellulose fiber extraction) [1]. Indonesia is the third largest pulp and paper manufacturer in Asia with 84 pulp and paper mills [2], and therefore, the country produces a significant amount of bark by-products. Bark contains a high concentration of tannin; as a result, interest in developing methods for utilizing tannin has recently increased. Studies have reported the utilization of tannin as a leathering agent [3], protein precipitate [4,5], insecticide and herbicide [6], wood adhesive [7], and wood preservative [8,9,10]. Tannin is divided into two major classes: condensed tannin and hydrolysable tannin. Condensed tannins consist of flavan-3-ol units with 4→6 and 4 →8 carbon linkages. The biological activity of tannin is closely associated with its structure, functional groups, and stereochemistry [11].

Acacia crassicarpa is a native species of Indonesia [12] that has been developed as a pulp and paper raw material due to its better ability to grow in diverse environments compared to *A. mangium* and *A. auriculiformis* [13]. The increasing amount of bark waste produced by the pulp and paper industries is not yet offset by the utilization of its tannin content. This is partially due to a lack of thorough studies on the tannin in the bark of *A. crassicarpa*; only studies on the polyphenolic

compounds from the knot and stem wood of *A. crassicarpa* have been reported to date [14]. In these studies, melacacidin and isomelacacidin were identified as the main compounds and small amounts of flavanone taxifolin, flavones guercetin, and flavanon catechin were also identified by gas chromatography-mass spectrometry (GC-MS).

Meanwhile, in New Zealand, South Africa, and other countries, *A. mearnsii* has long been planted and its bark extracts utilized as a leather tanning agent. In Japan, *A. mearnsii* is known as Morishima Akashia, and the structure of the condensed tannin contained in its bark has been elucidated [11,15] (**Figure 3.1**).

This study aims to isolate the condensed tannins from *A. crassicarpa*, to elucidate their chemical structures, and to evaluate the termiticidal activity against *R. speratus*.

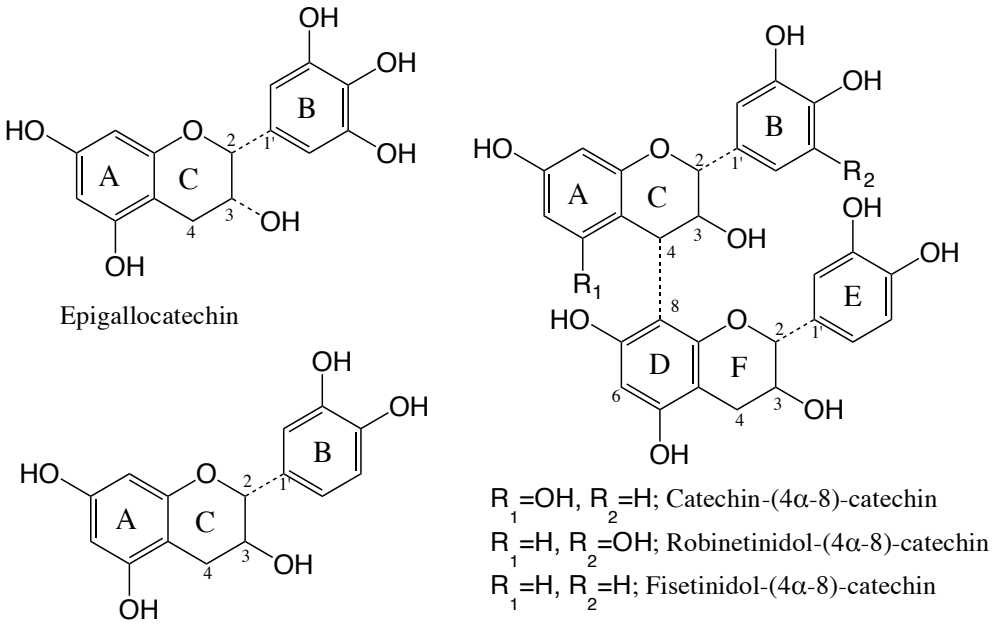


Figure 3.1 Examples of flavan-3-ol units of condensed tannin [11].

3.2 Experimental

3.2.1 Bark sample of *Acacia crassicarpa*

Bark samples of varying dimensions were collected from 4–5 year old *Acacia crassicarpa* trees grown in the Research and Development plantation area of PT Riau Andalan Pulp and Paper located in the Pelalawan area of Riau, Indonesia, in 2011. The samples were dried at room temperature for two weeks. The air-dried bark was then ground in a Wiley mill to obtain a powder with a mesh size of 40–80.

3.2.2 Extraction and fractionation of *A. crassicarpa*

First, 40 g of bark powder (40–80 mesh size) was extracted with 300 mL of a 70% acetone aqueous solution at room temperature for 24 h. Four replicates of the extraction were then conducted. The resulting solvent was evaporated to obtain the 70% acetone extract, which was then diluted in 300 mL of aqueous water and extracted with 300 mL of ethyl acetate three times to yield the ethyl acetate extract (C-EA) and the water-soluble extract (C-WS).

C-EA was purified by column chromatography (length 70 cm and Ø in 2.6 cm) using Sephadex LH-20 (GE Healthcare Bio-sciences AB, Made in Sweden) with ethanol as the eluent, which gave four fractions (C-EA1 to C-EA4). Each fraction was further identified by two-dimensional thin-layer chromatography (2D-TLC) using a mixture of *n*-hexane:ethyl acetate (1:1, v/v) and 0.6% acetic acid as the developing solution. Further separation of C-EA was performed using a 50% acetone aqueous solution to give a C-EA-AW fraction.

Using a similar procedure, C-WS was also purified by column chromatography (Sephadex LH-20) and further separated using a 50% methanol

aqueous solution and 50% acetone, successively, to obtain a C-WS-AW fraction. The fractionation procedure is depicted in **Figure 3.2**.

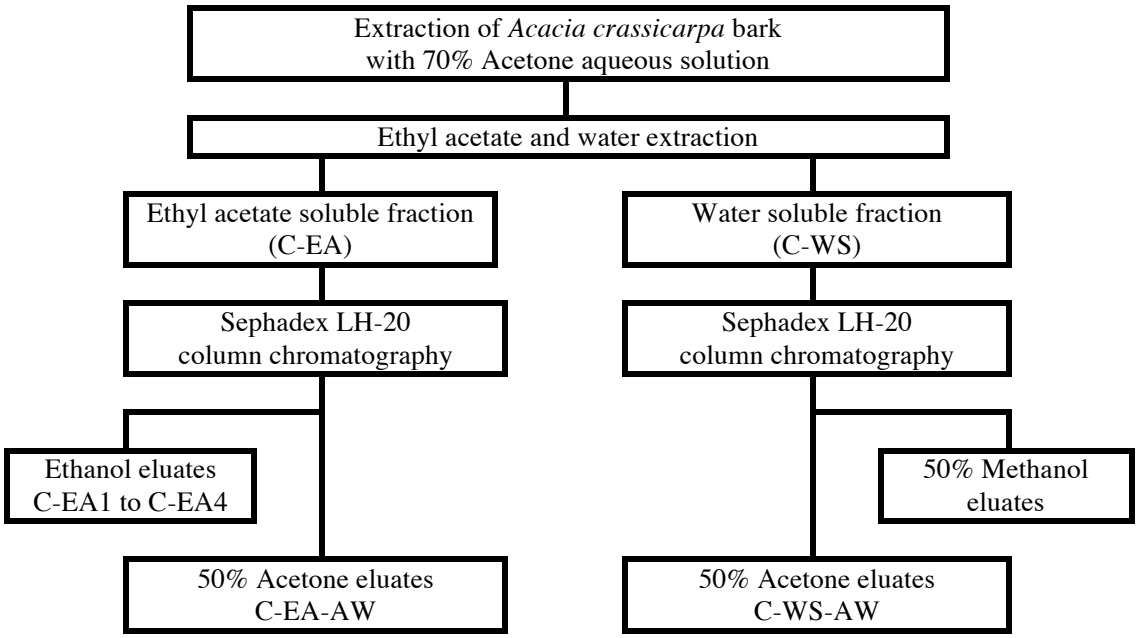


Figure 3.2 Scheme of the extraction and fractionation of bark-waste of *Acacia crassicarpa* bark. Legends: C.EA = ethylacetate extract; C.WS = water-soluble extract; C.EA1-C.EA4 = fractions obtained from C.EA extract eluted by ethanol; C.EA-AW = fraction obtained from C.EA eluted by ethanol and 50% acetone, successively; C.WS-AW = fractions from C.WS eluted by 50% methanol and 50% acetone, successively. The first letter of each abbreviation indicates the origin of bark species, C for *A. crassicarpa*

3.2.3 Pyrolysis-gas chromatography/mass spectrometry

The samples (150–200 μg) and *n*-eicosane as an internal standard were wrapped in a 500°C pyrofoil and pyrolyzed at 500°C for 4 s using a JHP-5 pyrolyzer (Japan Analytical Industry, Japan) that was interfaced (interface temperature 250°C) with a GC-MS (QP-5050, Shimadzu, Japan) equipped with an HP-1MS column (30 m × 0.25 mm i.d., film thickness 1.00 μm), with 70 eV electron impact and He as the carrier gas. The injection port (temperature 280°C) was fit with a split liner (split ratio 50:1). The temperature profile used for the GC analysis is as follows: hold 1 min at 50°C, 5 min at 50°C-280°C (5°C/min), and 13 min at 280°C. The pyrolysis products were identified by comparing their retention times and mass spectral data with those of the authentic samples and with literature data. Catechin and epigallocatechin were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used as model compounds of tannin monomer.

On-line methylated Py-GC/MS was conducted by wrapping the condensed tannin samples in a 500 °C pyrofoil after the addition of 3 μL of a 25% solution of tetramethylammonium hydroxide (TMAH: Tokyo Chemical Industry Co., Ltd.) in methanol. The samples were analyzed using the same procedure described above with Py-GC/MS. Gallic acid and 2,4,6-trimethoxybenzoic acid (Tokyo Chemical Industry Co., Ltd.) were used to identify the pyrolysis product of tannin that had an ion fragmentation m/z 226.

3.2.4 Py-GC/Milli MS analysis

The identification of the pyrolysis product with an m/z value 226 was conducted using catechin, which was analyzed by a JMS-600H spectrometer (JEOL, Japan).

3.2.5 Fast Atomic Bombardment Mass Spectroscopy (FAB-MS)

FAB-MS spectra of the condensed tannin samples were obtained using a JMS-600H spectrometer (JEOL, Japan) with Xe as the primary beam. The FAB-MS measurements were conducted on sample solutions in a glycerol matrix with Ultramax 1621 as the standard.

3.2.6 ^{1}H-NMR and ^{13}C-NMR analysis

The isolated dimer (C-EA1) from *A. crassicarpa* was analyzed using a nuclear magnetic resonance (NMR) spectrometer AVANCE-500 at the Chemical Analysis Division, Research Facility Center for Science and Technology, University of Tsukuba, Japan. The solvent used was ethanol-d6. The ^{1}H-NMR, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT 135) and two-dimensional NMR (2D-NMR) of heteronuclear multiple quantum coherence (HMQC) were measured at 500 Hz.

3.2.7 No-choice feeding tests against *Reticulitermes speratus* using condensed tannin samples from *A. crassicarpa* and *A. mearnsii*

The condensed tannin sample dissolved in each solvent at a 1% (w/v) concentration were used in no-choice feeding tests. An aliquot (30 μL) of each sample was placed onto a paper disc (30 mg, 8 mm in diameter, thick type; ADVANTEC TOYO, Tokyo, Japan), which was dried at 60°C for 24 h to remove the solvent. No-choice feeding tests were performed using 50 workers and 5 soldiers of *R. speratus* in an incubator set at 28°C for 14 days. After this time, paper discs were taken out, cleaned from debris, oven-dried at 60°C for 24 h, and finally weighed to calculate mass loss. As a control, untreated paper discs (PDUs) were

dried in the same manner as the discs with the extractives. To measure termiticidal activity, dead termites were counted. This test was carried out using the *R. speratus* maintained at the University of Tsukuba. Three replicates were performed for each sample.

Mass loss (%) = [ODS1-ODS2/ODS1] x 100% .....(1)

ODS1 is oven-dried paper disc before test, while ODS2 is oven-dried paper disc after test

Termite Mortality = [Number of died termite/50] x 100% .....(2)

3.2.8 Statistical analysis

The effects of MAQ on mass loss and termite mortality were examined using an analysis of variance (ANOVA), and samples showing significant differences ($P < 0.05$) were analyzed using Duncan's post hoc test ($\alpha = 0.05$). All analyses were performed using IBM SPSS version 22 (IBM Japan, Tokyo, Japan).

3.3 Results and discussion

3.3.1 Extraction of condensed tannin

The yield of extract obtained from *A. crassicarpa* (7.0%) using a 70% acetone aqueous solution was one-fifth of that from *A. mearnsii* (Table 3.1). This result is consistent with a previous report that *A. mearnsii* is a good source of tannin [15]. The yield of extract from the bark of *A. crassicarpa* was also lower than that of other acacia species, including *A. mangium*, *A. auriculiformis*, *Rhizophora apiculata*, and *Larix leptolepis*, which gave yields of 37.9%, 28.6%, 20.2%, and 11.0%, respectively [16]. The age of the tree and the location of sampling are also known to affect the tannin yields; the inner part of the bark has a higher amount of tannin because of the higher concentration of the metabolite than outer or whole bark [14].

Table 3.1 Yields of acacia bark extracts containing condensed tannin.

	Acacia crassicarpa (%)	*Acacia mearnsii* (%)
70% Acetone aqueous extracts	7.0	34
Ethyl acetate-soluble fraction	0.8	12
Water-soluble fraction	6.2	22

3.3.2 Structural elucidation of condensed tannin by Py-GC/MS

3.3.2.1 Py-GC/MS of catechin and epigallocatechin

Catechin and epigallocatechin were used as flavanol monomer standards. The main products from pyrolysis are known to be catechol and 4-methylcatechol from the B-ring of catechin or epicatechin, while pyrogallol is the main product from epigallocatechin or gallocatechin. In the current study, the intensity of the catechol peak was higher than that of 4-methylcatechol. This result confirmed that the cleavage of the C1'-C2 bond is easier than the fission of a pyran ring, which has been previously reported [11]. The presence of catechol or pyrogallol in the pyrogram is a very useful marker of the B-ring product that can be used to identify tannin structure. A low-intensity peak for phloroglucinol was also detected as a marker of the A-ring product.

By Py-GC/MS analysis, the A-ring products of pyrolysis are more difficult to detect than the B-ring products because they are highly polar and unstable under the conditions of the technique. To overcome the polarity problem, methylated Py-GC/MS can be used as an alternative method for the analysis of the A-ring pyrolysis products from condensed tannin. In this method, the A-ring products are more easily detected than those from the B-ring. **Figure 3.3** shows the methylated Py-GC/MS pyrogram of catechin and epigallocatechin obtained by using TMAH as the methylating agent. Both the monomer standards, catechin and epigallocatechin, gave the A-ring pyrolysis products, 1,3,5-trimethoxybenzene (d) and 2,4,6-trimethoxytoluene (e). Small amounts of B-ring products, 1,2-dimethoxybenzene (a) and 3,4-dimethoxytoluene (b), were also obtained from catechin, while 1,2,3-trimethoxybenzene (c), 3,4,5-trimethoxybenzaldehyde (f), which had been reported

previously [17], and 3,4,5-trimethoxybenzoic acid methyl ester (g) were obtained from the B-ring of epigallocatechin.

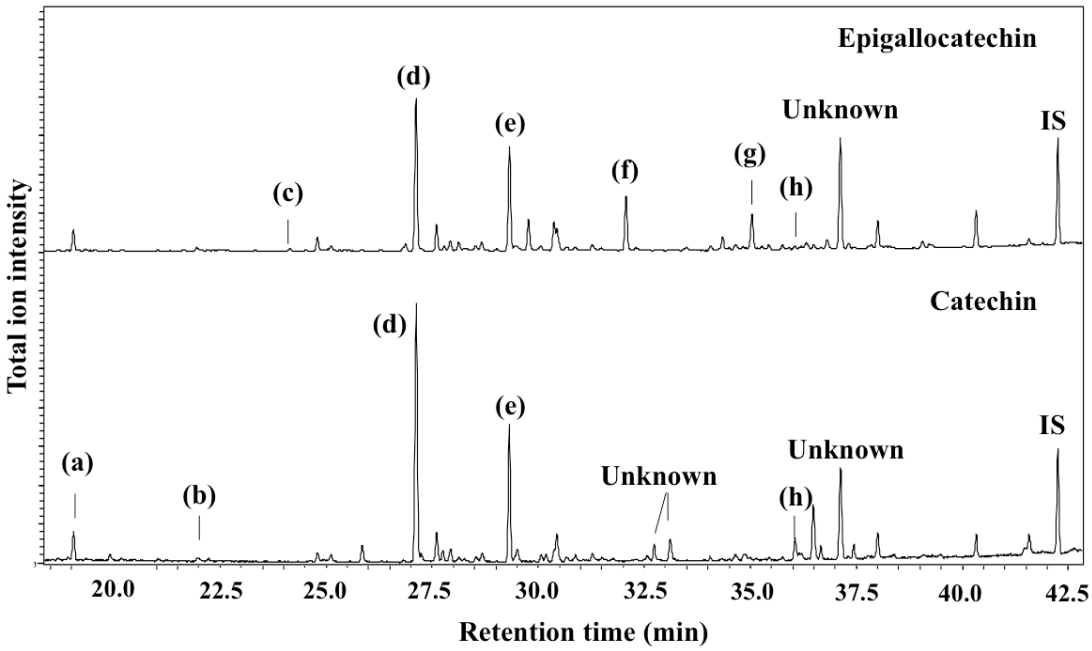
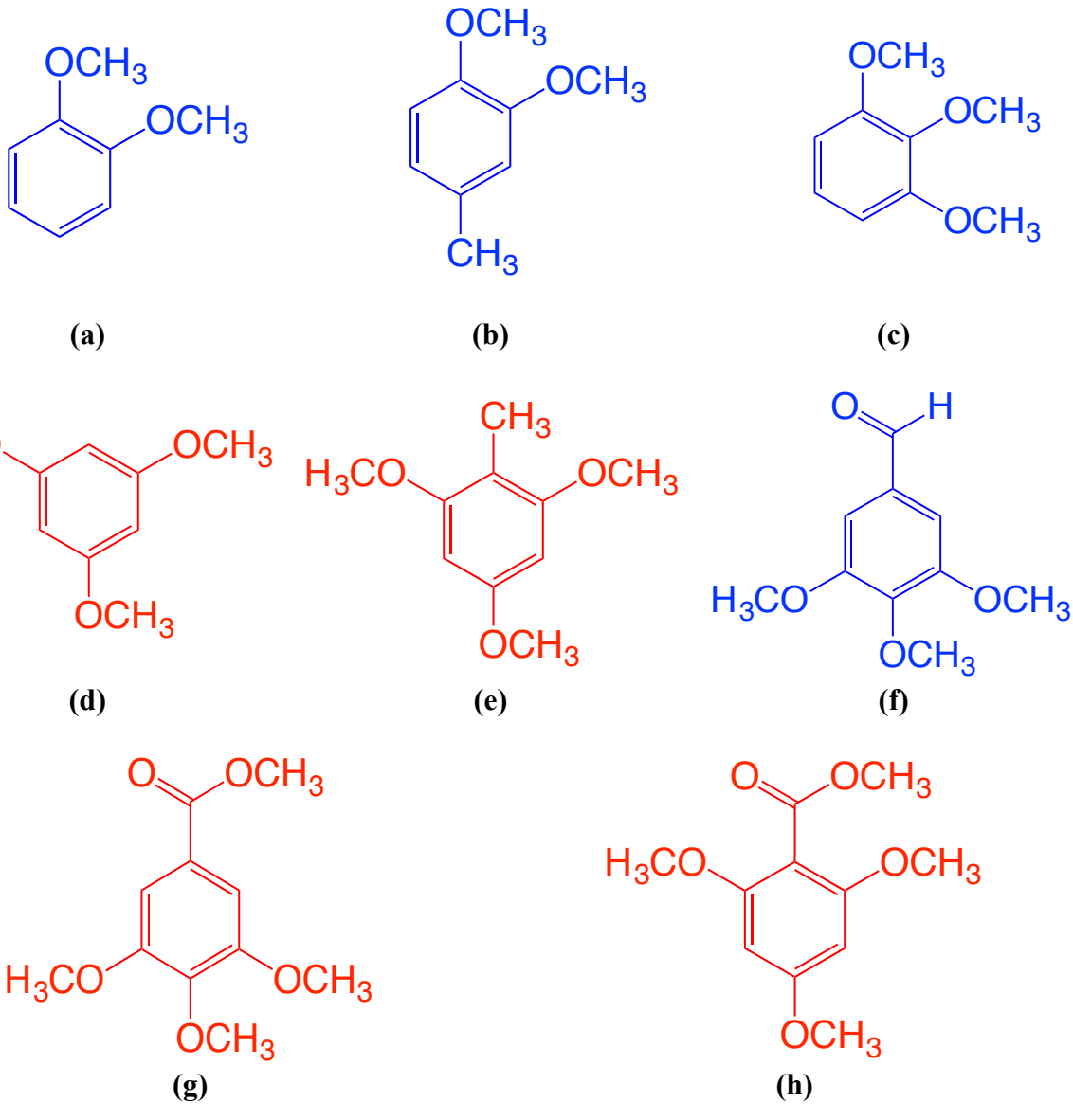


Figure 3.3 Pyrogram of on-line methylated Py-GCMS of epigallocatechin and catechin.

Legend: 1,2-dimethoxybenzene (a); 3,4-dimethoxytoluene (b); 1,2,3-trimethoxybenzene (c); 1,3,5-trimethoxybenzene (d); 2,4,6-trimethoxytoluene (e); 3,4,5-trimethoxybenzaldehyde (f); 3,4,5-trimethoxybenzoic acid methyl ester (g); 2,4,6-trimethoxy benzoic acid methyl ester (h); and *n*-eicosane (internal standard, IS).



Legend :

a = 1,2-dimethylbenzene (M.W 138)
b = 1,3-dimethylbenzene (M.W 138)
c = 1,2,3-trimethoxybenzene (M.W 168)
d = 1,3,5-trimethoxybenzene (M.W 168)
e = 2,4,6-trimethoxytoluene (M.W 182)
f = 3,4,5-trimethoxybenzaldehyde (M.W 196)
g = 3,4,5-trimethoxy benzoic acid methyl ester (M.W 226)
h = 2,4,6-trimethoxy benzoic acid methyl ester (M.W 226)

Figure 3.4 Tannin pyrolysis products by on-line methylated Py-GC/MS;

Legend: M.W = Molecular weight

In the on-line methylated Py-GC/MS pyrogram of catechin and epigallocatechin, an unidentified mass ion (m/z) peak of 226 (h) was observed at a retention time of 36 min. Based on the Py-GC/Milli MS data, this pyrolysis product (h) had a chemical formula of $C_{11}H_{14}O_5$. However, the fragmentation trace and retention time of this peak did not match those of 3,4,5-trimethoxybenzoic acid methyl ester (g), which had been previously reported as a methylated Py-GC/MS product of wine tannin [18,19,20], and the ion fragmentation of 2,4,6-trimethoxybenzoic acid methyl ester (h) has not been reported. The pure compounds 2,4,6-trimethoxybenzoic acid and gallic acid were therefore subjected to methylated Py-GC/MS in order to identify the unknown fragmentation ion, and the results confirmed that the pyrolysis product (h) from catechin and epigalocatechin is 2,4,6-trimethoxybenzoic acid methyl ester (**Figure 3.5**). This product is formed from the cleavage of the pyran ring (C-ring) upon methylation of the tannin monomers, which illustrates that chain cleavage can occur at different locations in condensed tannins; this property can be useful for determining the structures of unidentified condensed tannins.

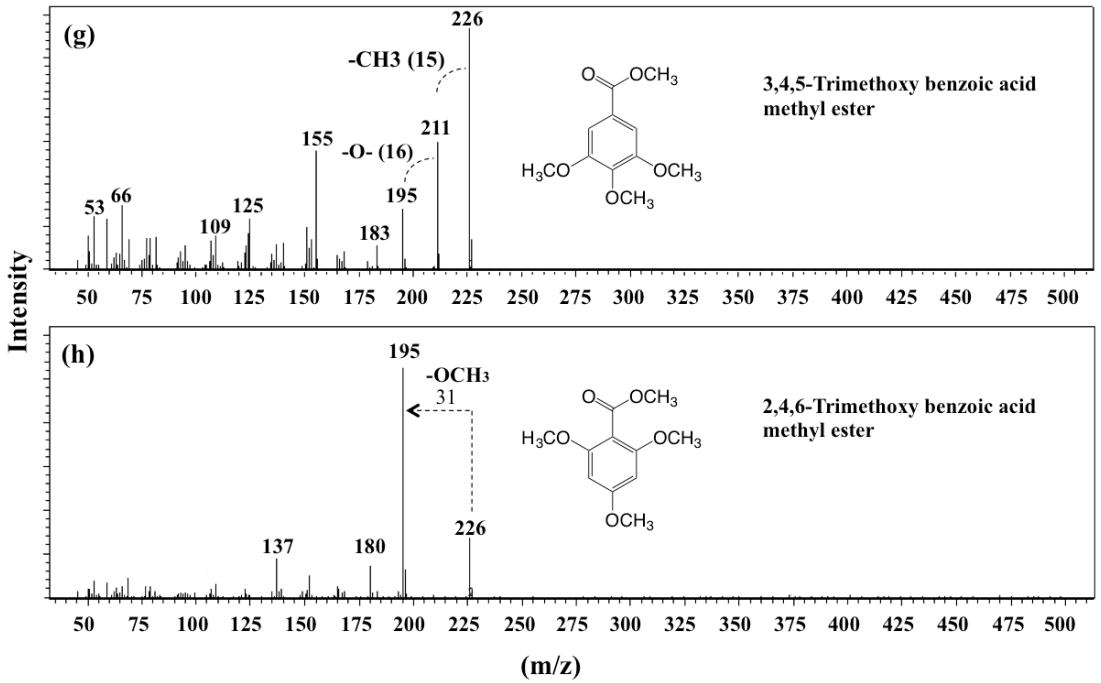


Figure 3.5 Mass spectra from 2,4,6-trimethoxy benzoic acid methyl ester and 3,4,5-trimethoxy benzoic acid methyl ester obtained by on-line methylated Py-GC/MS of 2,4,6-trimethoxy benzoic acid and gallic acid.

3.3.2.2 Py-GC/MS of C-EA1 and tannin polymer fraction from *A. crassicarpa*

Figure 3.5 shows the pyrogram of C-EA1 obtained by Py-GC/MS. Catechol (1) and pyrogallol (4) were detected as the B-ring pyrolysis products, while phloroglucinol (6) was found as an A-ring product, and resorcinol was not observed. The methylated Py-GC/MS pyrogram of C-EA1; 1,3,5-trimethoxybenzene (d), 2,4,6-trimethoxytoluene (e), and 2,4,6-trimethoxybenzoic acid methyl ester (h) were detected as TMAH markers of the A-ring products. Meanwhile, the B-ring pyrolysis products—1,2,3-trimethoxybenzene (c), 3,4,5-trimethoxybenzoic acid methyl ester (g), 1,2-dimethoxybenzene (a), and 3,4-dimethoxytoluene (b)—were observed, the latter two with low intensities. Interestingly, an intense peak for 3,4,5-trimethoxybenzaldehyde (f) was detected as a result of C2-C3 cleavage on the C-ring. Based on these results, we can deduce that C-EA1 is a proanthocyanidin or prodelphinidin, but it cannot be fisetinidol-(4-8)-catechin or robinetinidol-(4-8)-catechin. By Py-GC/MS as a rapid method, we can predict the chemical structure of condensed tannin with timeless consumption and without any sample preparation. It is very useful information to further elucidation analysis using, NMR and FAB-MS.

The purified tannin polymer fraction C-WS-AW contained catechol (1), 4-methylcatechol (3), pyrogallol (4), and 5-methylpyrogallol (6) as the B-ring pyrolysis products in the Py-GC/MS pyrogram. Small amounts of phloroglucinol (6) and resorcinol (2) were also observed from the A-ring (**Figure 3.6**). In the methylated Py-GC/MS pyrogram, C-WS-AW contained 1,3,5-trimethoxybenzene (d), 2,4,6-trimethoxytoluene (e), and 2,4,6-trimethoxybenzoic acid methyl ester (h) as A-ring products (**Figure 3.8**). Together, these results clearly show that the condensed

tannins of *A. crassicarpa* are mostly composed of proanthocyanidin and prodelphinidin.

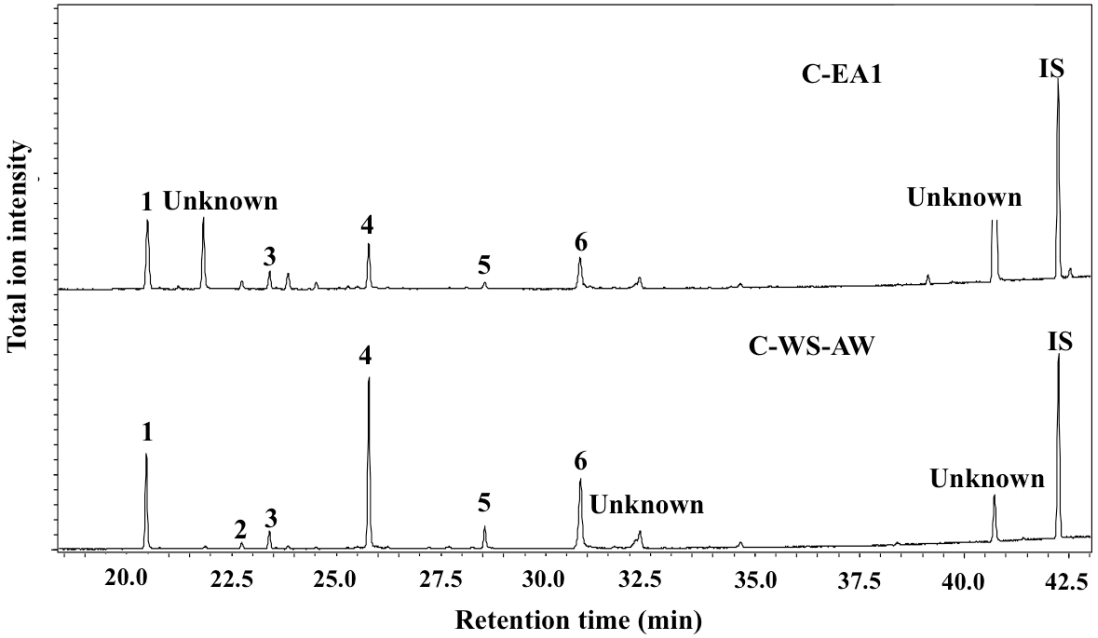
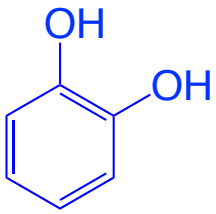


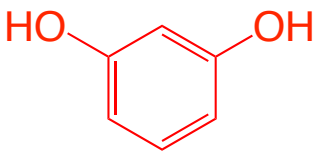
Figure 3.6 Pyrogram of Py-GC/MS of C-EA1 and C-WS-AW from *A. crassicarpa.*

Legend: catechol (1), resorcinol (2), 4-methylcatechol (3), pyrogallol (4), 5-methyl pyrogallol (5), phloroglucinol (6), and *n*-eicosane (internal standard, IS).



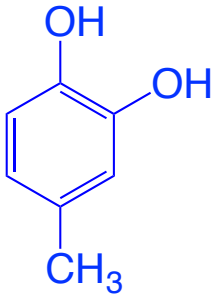
Catechol/M.W 110

(1)



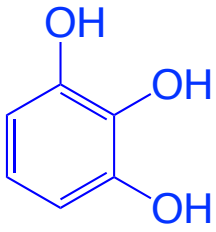
Resorcinol/M.W 110

(2)



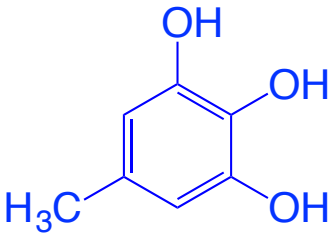
4-methylcatechol/M.W 124

(3)



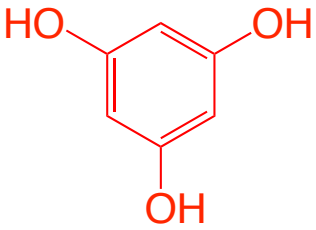
Pyrogallol/M.W 126

(4)



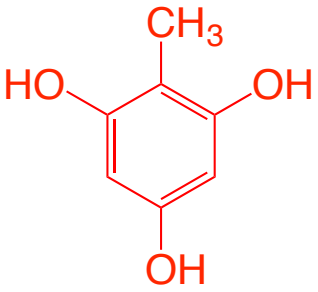
5-methylpyrogallol/M.W 140

(5)



Phloroglucinol/M.W 126

(6)



2-methylphloroglucinol/M.W 140

(7)

Figure 3.7 Tannin pyrolysis products by Py-GC/MS; Legend: M.W = Molecular weight

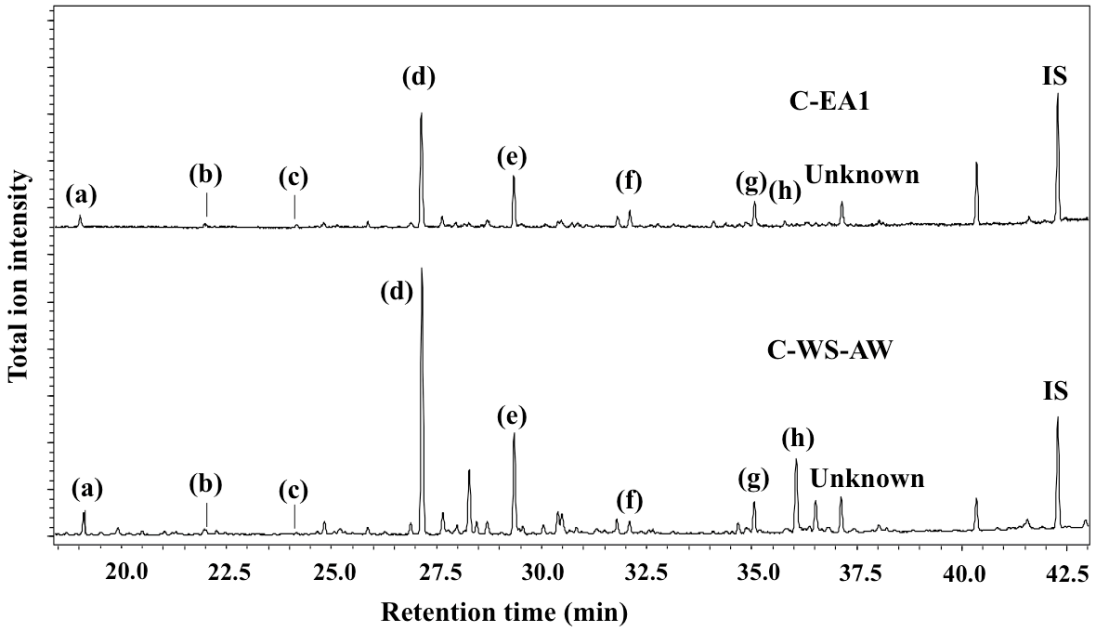


Figure 3.8 Pyrogram of on-line methylated Py-GC/MS of C-EA1 and C-WA-AW from *A. crassicarpa.*

Legend: 1,2-dimethoxybenzene (a); 3,4-dimethoxytoluene (b); 1,2,3-trimethoxybenzene (c); 1,3,5-trimethoxybenzene (d); 2,4,6-trimethoxytoluene (e); 3,4,5-trimethoxybenzaldehyde (f); 3,4,5-trimethoxy benzoic acid methyl ester (g); 2,4,6-trimethoxy benzoic acid methyl ester (h); and *n*-eicosane (internal standard, IS).

3.3.3 Characterization of dimmers, C-EA1 isolated from *A. crassicarpa* by FAB-MS and ^{1}H and ^{13}C-NMR analysis

C-EA1, C-EA2, C-EA3, and C-EA4 were collected from 1 to 64 fractions of the ethyl acetate soluble-fraction of *A. crassicarpa* (C-EA), and reddish brown amorphous powder (92.7 mg) was obtained at Rf values: 0.77 (n-hexane:ethyl acetate, 1:1) and 0.40 (6% acetic acid) of 2D-TLC from C-EA1 (1 to 16 fractions). The FAB-MS spectra of the compound C-EA1 $[M+H]^{+}$ is 579.

Interpretation spectrum of ^{1}H-NMR (TMS, tetramethylsilane): δ 2.40 [dd (double doublet), J (coupling constant) = 2.00 Hz, H-3C(a)], 2.62-2.77 (J = 5.98 Hz, H-4F(a), H-3C(b) and H-4F(b)), 3.84 [q (quartet), J = 2.37 Hz, H-3F], 4.04 [brs (broad singlet), H-4C], 4.41 (dd, J = 2.36Hz, H-2F), 4.63 [s(singlet), J = 2.20, H-2C], 5,80-5.90 [m (multiplet), J = 5.98, H-6A, H-8A, and H-6D], 6.35 (brs, J = 4.02, H-2'B, and H-6'B), 6.42-6.55 (m, J = 3.87 Hz, H-5'E and H-2'E and H-6'E). Fifteen peaks for sixteen protons were observed in the spectrum.

The ^{13}C-NMR (TMS): δ 28.00 (C-3C); 28.80 (C-4F); 67.14 (C-4C); 68.45 (C-3F); 79.51 (C-2C); 82.47 (C-2F); 95.33 (C-8A); 95.69 (C-6A); 96.20 (C-6D); 96.33 (C-8D); 99.62(C-4aD); 100.33 (C-4aA); 106.68 (C-2'B and C-6'B); 106.99 (C-2'E, C-5'E and C-6'E); 130.88 (C-1'E); 131.09 (C-4'B); 133.48 (C-1'B); 146.12 (C-2'E and C-2'E); 146.26 (C-3'B and C-5'B); 156.38 (C-8aD); 156.71 (C-5A); 157.09 (C-7D); 157.16 (C-5D); 157.21 (C-8aA); 157.47 (C-7A). The detail ^{1}H and ^{13}C-NMR spectrum shown in **Figure 3.9** and **Figure 3.10.**

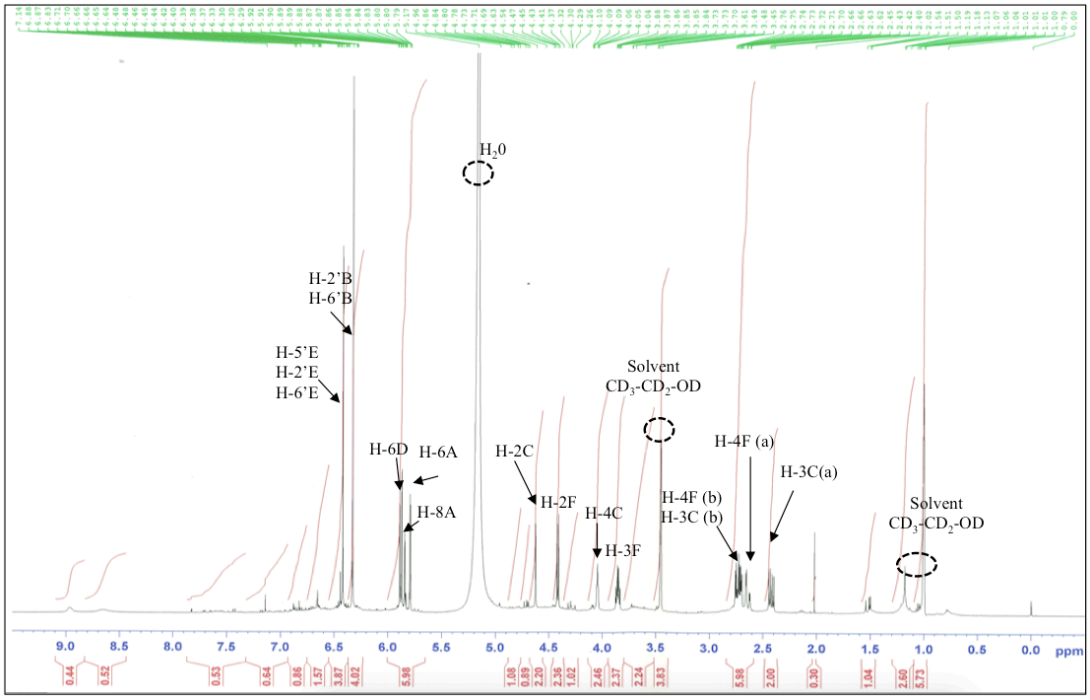


Figure 3.9 ^{1}H-NMR spectrum of the compound purified from C-EA1

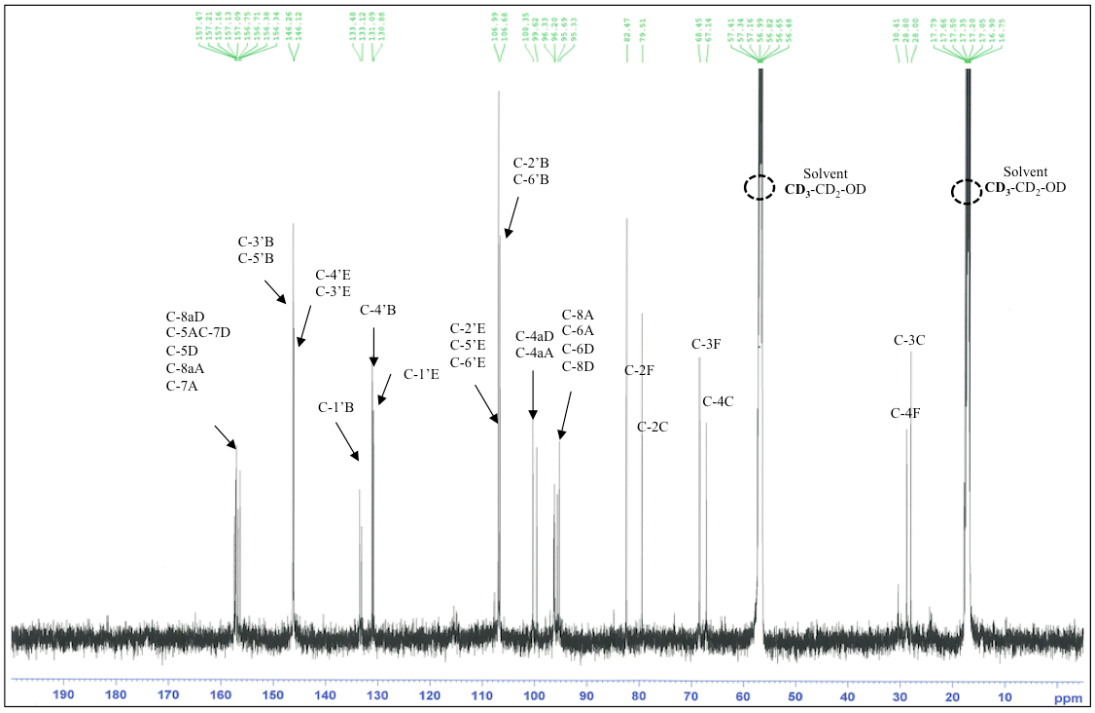


Figure 3.10 ^{13}C-NMR spectrum of the compound purified from C-EA1

The ^{13}C-NMR of DEPT 135 spectrum of C-EA1 exhibited two methylene (-CH2-) carbons with negative values at 28.0 and 28.8 ppm in addition to 12 methine (-CH-) carbons and 16 quaternary carbons (-C-). A total of 30 13C peaks were observed in the spectrum (**Figure 3.11**). Furthermore, analysis of the 1H and 13C-NMR HMQC spectra was used to define the interaction between the protons and carbons in the compound (**Figure 3.12**). This result confirmed the assignments of the C- and F-rings (pyran rings) with the absence of hydroxyl group (-OH). The structures of the B- and E-rings are still uncertain, although the B-ring is assumed to be the pyrogallol type.

Based on the above results, the dimer condensed tannin isolated from the bark of *A. crassicarpa* (C-EA1) is characterized as 2'-(3,4-dihydroxyphenyl)- 2-(3,4,5-trihydroxyphenyl)-3,3,3',4,4',4'-hexahydro-2H,2'H-4,8-'bichromene 3',5,5',7,7'-pentol (**Figure 3.13**), or 5,7,3',4',5'-pentahydroxy-flavan-(4-8)-catechin. This is a novel dimer of condensed tannin that contains a catechin and a flavan structure that lacks an oxygen at C3 of pyran ring. The previously reported dimers fisetinidol-(4-8)-catechin and robinetinidol-(4-8)-catechin [21] were isolated from *A. mearnsii* extracts.

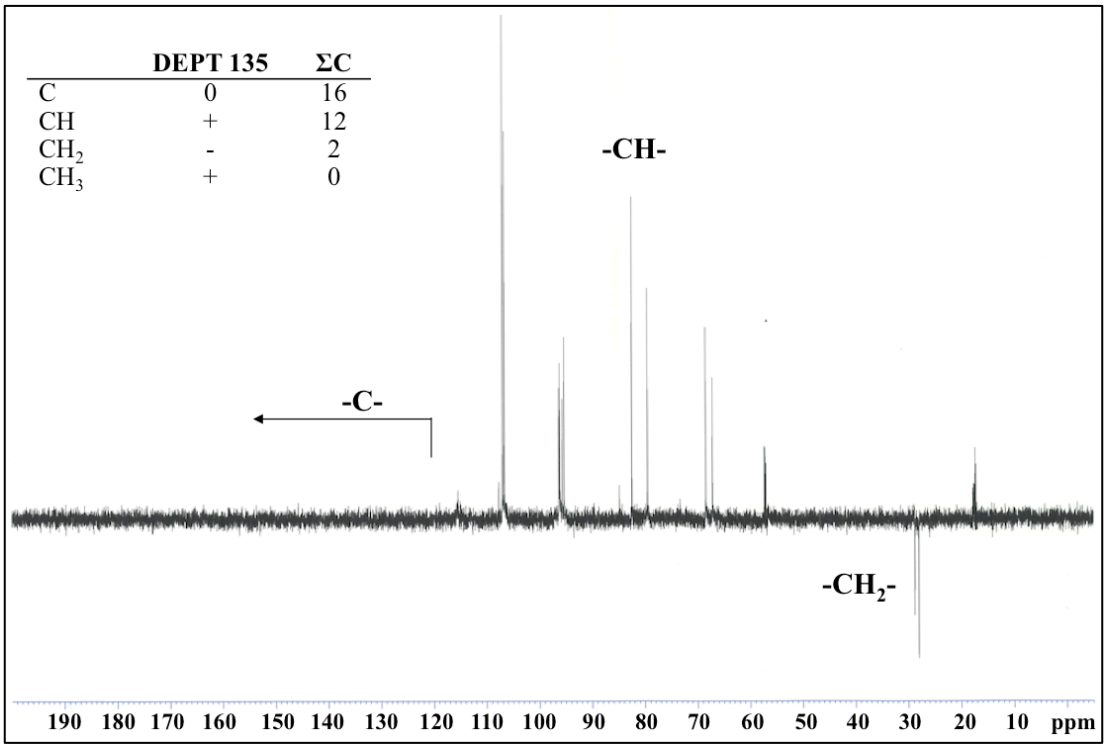


Figure 3.11 ^{13}C-NMR of DEPT 135 spectrum of the compound purified from C-EA1

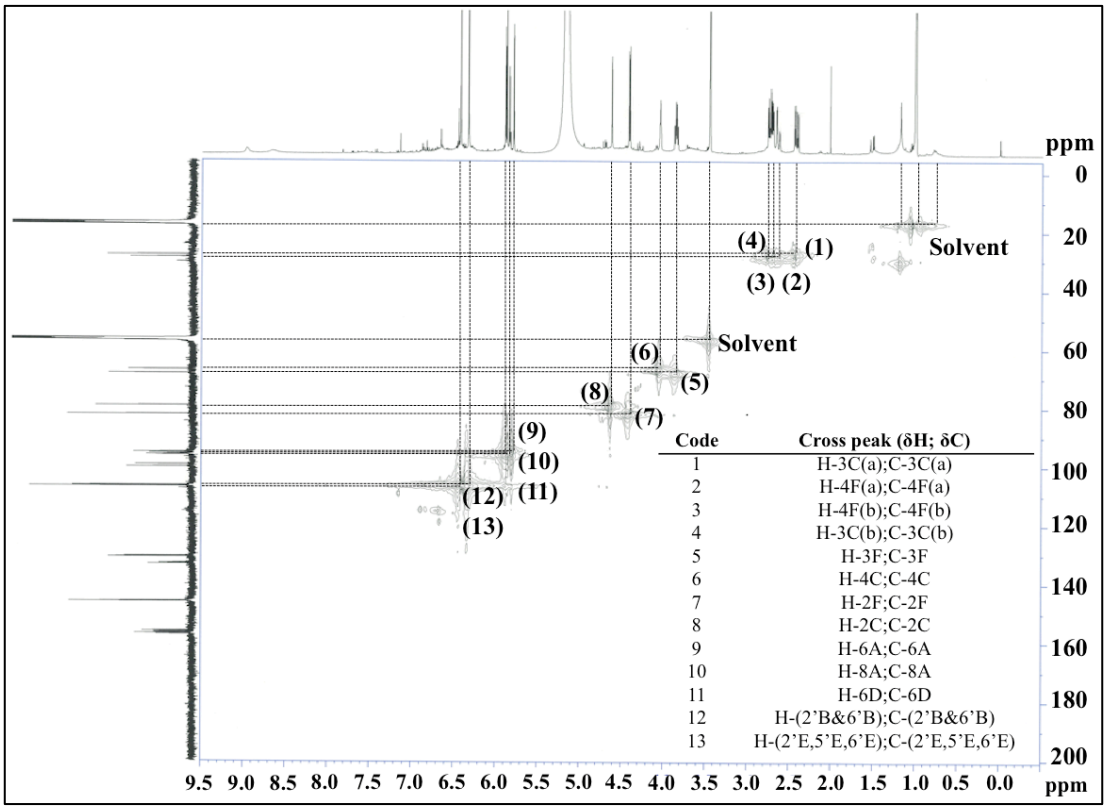


Figure 3.12 ^{1}H and ^{13}C-NMR HMQC spectrum of the compound purified from C-EA1.

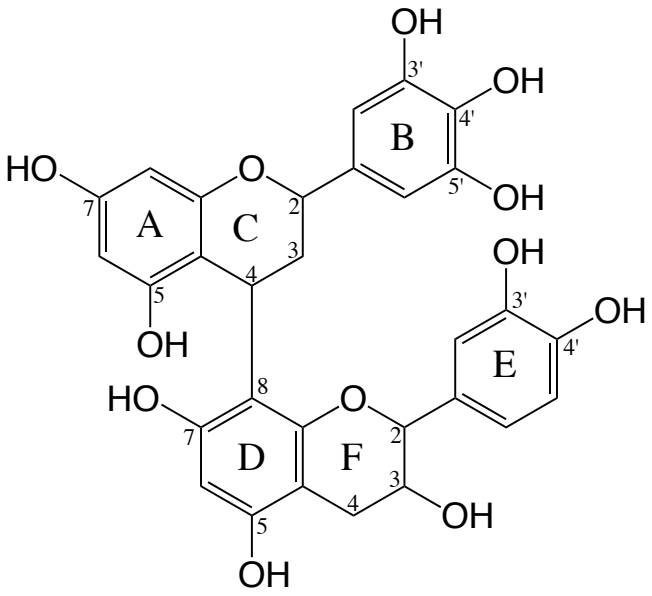


Figure 3.13 A possible structure of condensed tannin dimmer from *A. crassicarpa* (MW: 578, $C_{30}H_{26}O_{12}$).

3.3.4 Characterization of condensed tannin polymer with high-molecular weight by NMR analysis

Polymer tannins are mostly composed of repeating units of flavan-3-ols that are linked through the C4→C6 or C4→C8 bonds [22]. Structural elucidation of condensed tannin polymers is made complicated by their large molecular weight in the range of 2000–5000. This study approached the structural elucidation by analyzing the flavanol units in polymer tannins that contained catechin or epigallocatechin. In the Py-GC/MS pyrogram, the pyrogallol peak was higher than the catechol, which indicates that the ratio of pyrogallol in polymer tannin is higher than catechol.

Analysis of the ^{13}C-NMR spectrum was carried out for the condensed tannin polymers isolated from both *A. mearnsii* and *A. crassicarpa*. The Py-GC/MS data was supported by the presence of high-intensity chemical shifts in 13C-NMR spectrum of WS-AW from *A. mearnsii* corresponding to C2', C6' (B-ring pyrogallol type) and C2', C5' (B-ring catechol type) at 98 ppm and 116 ppm, respectively. In the case of C-WS-AW from *A. crassicarpa*, however, those peaks were slightly different and were detected at 107 ppm and 116 ppm, respectively, in the deshielded area due to the impurities.

3.3.5 Termiticidal activity of condensed tannin of *Acacia crassicarpa* and *Acacia mearnsii*

To evaluate the termiticidal activity of condensed tannin from *A. crassicarpa* and *A. mearnsii*, no-choice tests were conducted using *R. speratus*. The results of mass loss and termite mortality are shown in **Figure 3.14**. After 14 days, mass loss

of dimmer condensed tannin (C-EA1) treated was less than 50%, significantly different with control. In addition, a monomer condensed tannin, catechin was presented the similar result. Meanwhile, the termite mortality of catechin is slightly higher than C-EA1. According to both parameter, the medium level of mass loss and termite mortality, the C-EA1 suggested as antifeedant in mechanism of termiticidal. The feeding activity is decreased after contact with the treated paper disc.

In polymer condensed tannin, the result seems similar to dimmer condensed tannin. Both C-WS-AW and M.WS-AW have a same trend of mass loss and termite mortality. Condensed tannin and hydrolyzable tannin which were contained in the bark effected termite consumption because it caused inactivation of enzyme in termite digestion system [15,23]. In fact, many factors effect termite activity from natural extractives such as compound type, quantity and also extract retention activity. the structure-active relationship of C-EA1 (flavan-3-ols) that lacks an oxygen at C3 of pyran ring is very interesting for further study. Previou study, Quercetin that has a hydroxyl group in the C-5 A-ring showed higher antifeedant activity than fisetin. This indicates that the C-5 and C-7 hydroxyl groups in the A-ring are important for anti-termite activity [10].

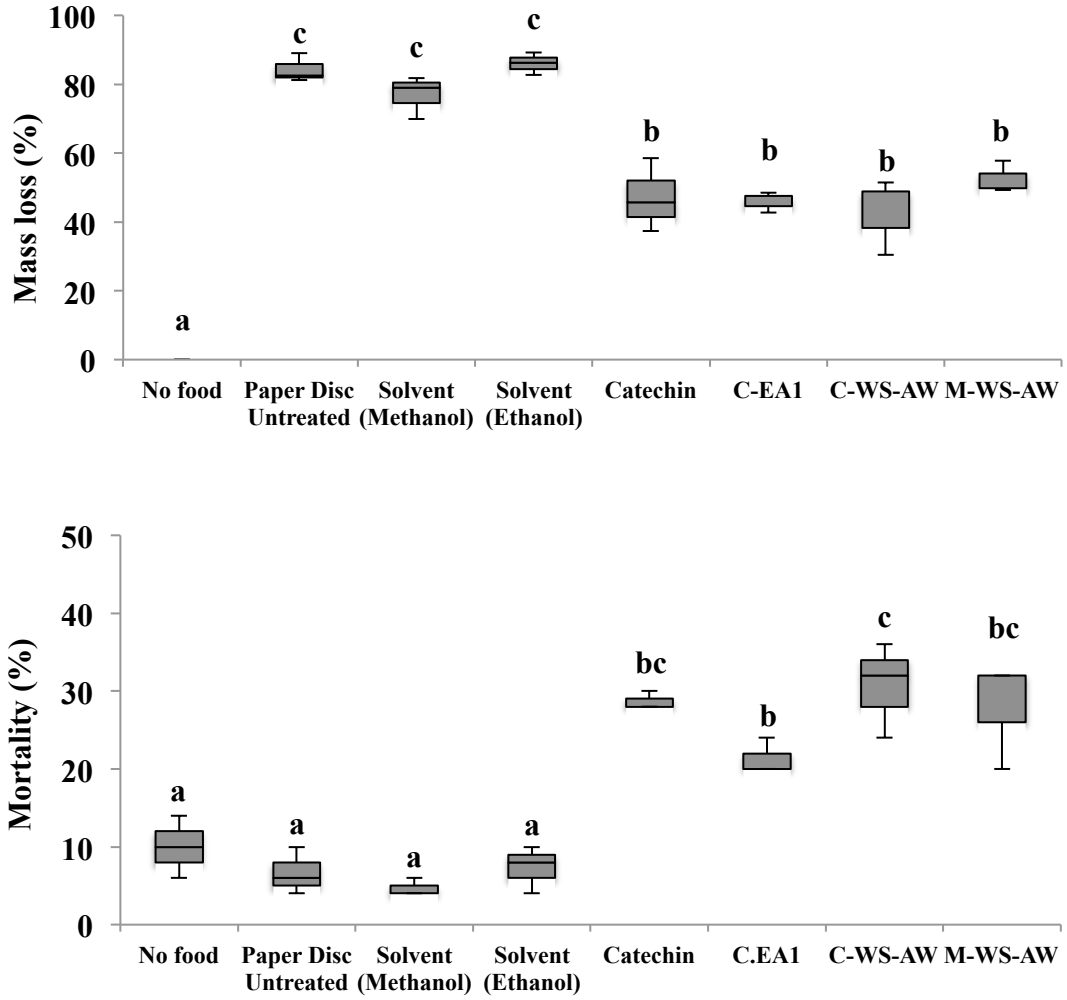


Figure 3.14 Mass loss and mortality of *Reticulitermes speratus* in no-choice feeding tests of condensed tannin from *A. crassicarpa* and *A. mearnsii.*

3.4 Conclusion

This study characterized the structure of condensed tannin in bark wastes of *A. crassicarpa* for the first time. A novel dimer of flavan units that is specific to *A. crassicarpa* was isolated from the bark extracts. The new compound is a gallocatechin-catechin or catechin-gallocatechin flavan dimer that lacks one oxygen at C3 of the pyran ring, which is characterized as 2'-(3,4-dihydroxyphenyl)- 2-(3,4,5-trihydroxyphenyl)-3,3,3',4,4',4'-hexahydro-2H,2'H-4,8-'bichromene-3',5,5',7,7'-pentol, or 5,7,3',4',5'-pentahydroxy-flavan-(4-8)-catechin. The dimmer condensed tannin C-EA1 and polymer condensed tannin from *A. crassicarpa* and *A. mearnsii* are potentially useful as a termite control agent. They act as antifeedant, against *Reticulitermes speratus.*

3.5 References

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Chapter 4 General Conclusion

The present study is about extractive-based preservative which can be an alternative pesticide except synthetic chemical pesticide. By this study, we have focused on the bioactive compound from heartwood of teak (*Tectona grandis*) and bark of *Acacia crassicarpa*.

The toxicity and feeding deterrent effect of 2-methylanthraquinone (MAQ) towards two subterranean termite, *Reticulitermes speratus* and *Coptotermes formosanus* has been conducted using ethanol, chloroform and acetone as solvents. No-choice feeding tests clearly indicated that toxicity and the feed deterrent effect did not depend on MAQ dosages, instead deriving from the coexistence of teak wood extractives and small amounts of MAQ.

The amount of MAQ in the extractives is not related to toxicity and the feed deterrent effect.

As a native pest, *C. formosanus* is more tolerant to the extractives than *R. speratus* and MAQ is not an effective toxic against *C. formosanus*. The interaction between MAQ and other components in teak wood extractives leads to a high feeding deterrent effect

In other study, the characterization structure of condensed tannin in bark wastes of *A. crassicarpa* has been carried out for the first time, using Py-GC/MS, FAB-MS, ^{1}H-NMR and ^{13}C-NMR. A novel dimers of flavan structure which are specific to *A. crassicarpa* were isolated from the bark extracts. The new compound is catechin structures (flavan-3-ol) lacking oxygen at C3 of pyran ring. 2'- (3,4-

dihydroxyphenyl)-2-(3,4,5-trihydroxyphenyl)-3-methyl-3',3',4,4'-tetrahydro-2H,2'H-4,8'-bichromene- 3,5,5',7,7'- pentol.

Beside that, the 2,4,6-trimethoxybenzoic acid methyl ester successfully found as a marker of A-ring product of procyanidin or prodelphinidine by methylated Py-GC/MS using TMAH.

As summary, the finding of this study should contribute to the developing of natural wood preservative from extractive-based. Especially, the study of structure-active relationship (SAR) of teakwood and also the characterization condensed tannin from *A. crassicarpa*, which can improve the utilization of bark-waste from pulp industry.

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