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ANTIFUNGAL ACTIVITY OF HEARTWOOD EXTRACTS AND THEIR CONSTITUENS FROM CULTIVATED *TECTONA GRANDIS* AGAINST *PHANEROCHAETE CHRYSOSPORIUM*

Irshad Ul Haq Bhat, H.P.S. Abdul Khalil
University Sains Malaysia, School of Industrial Technology
Penang, Malaysia

Nor Shifa Shuib, Ahmad Mohd Noor
University Sains Malaysia, School of Chemical Sciences
Penang, Malaysia

ABSTRACT

Heartwood extracts obtained from *Tectona grandis* by successive extractions with dichloromethane, ethanol and ethanol-toluene (1 : 2 v/v) were tested for their biological activity against fungus *Phanerochaete chrysosporium*. The major bioactive compounds obtained from heartwood include 2-methyl anthraquinone, 1,4-naphthoquinone and lapachol, which were analyzed by GC/MS and HPLC. The results revealed that dichloromethane extract exhibit higher antifungal activity as compared to ethanol and ethanol-toluene (1 : 2 v/v) extracts.

KEY WORDS: *Tectona grandis, Phanerochaete chrysosporium*, dichloromethane extractives, antifungal activity, GC/MS, HPLC

INTRODUCTION

Teak (*Tectona grandis*) occupies two areas of native range: the western portion includes most of peninsular India and the eastern portion includes parts of Burma, Laos, and Thailand (Weaver 1993). It has been cultivated since ancient times in Asia and today the species is planted in much of the moist tropics. *Tectona grandis* has naturalized in at least the Philippines, Java (Little and Wadsworth 1964), and Puerto Rico (Francis and Liogier 1991). The heartwood of teak contains toxic substances that render it resistance to attack by decay fungi. The isolation of these extractives, determination of their toxicity to fungi and identification of their chemical structures may lead to development of new, environmentally safe wood preservatives. The need for such investigation is reflected by the concern of environmental groups and governmental agencies over the potential

health and ecological hazards associated with some of the wood preservative in current use. Teak has worldwide reputation as a quality lumber, resistant to fungal infections (Tee 1995) and termite attacks (Sandermann and Simatupang 1960). (*Tectona grandis* L.f) has long been known to possess compounds having a quinone as the core system, which have promising biological activity e.g. anthraquinones and tectoquinones (Pahup et al. 1989; Yamamoto et al. 1998; Simatupang and Yamamoto 1999; Haluk et al. 2001). Various studies on biologically active extractive compounds such as 2-methyl anthraquinone and 2-hydroxymethyl anthraquinone, lapachol and deoxylapachol against brown rot fungi were reported (Myo 1988, Haupt et al. 2003, Windeisen et al. 2003). Recently Breger et al. (2007) identified lapachol as being an antifungal agent using the Candida elegans pathogenicity assay.

However, the relative role of total extractive content of the teak wood and its individual chemical components in imparting the decay resistance against white-rot fungi *Phanerochaete chrysosporium* has not been studied. The purpose of the present study is to determine the relative importance of total extractive content and major chemical components, 2-methyl anthraquinone, 1,4-naphthoquinone and lapachol in determining the white-rot fungi decay resistance of teak with respect to different stem height and longitudinal positions. (TIH = Top inner heartwood, TOH = Top outer heartwood, BIH = Bottom inner heartwood, BOH = Bottom outer heartwood).

MATERIAL AND METHODS

Teak was obtained from cultivated tree of 20 years age from Changlun, northern part of Peninsular Malaysia. Dichloromethane 95 %, ethanol 95 %, toluene 95 %, acetonitrile (HPLC grade), methanol (HPLC grade), hexane (HPLC grade), chloroform (Analytical grade) were obtained from System ChemAR. Potato Dextrose agar (PDA) and Silica gel 60-200 mesh size was procured from Merck. 2-methyl anthraquinone and 1,4-Naphthoquinone standards were obtained from Merck, lapachol standard was purchased from Extrasynthese.

Identification of extractives and fractions.

Extracts and fractions were identified using Agilent 5890 Gas chromatography Mass Spectroscopy (GCMS), Agilent. Quinone derivatives from extracts were quantitated using Shimadzu LC-10ATVP High Performance Liquid Chromatography (HPLC) coupled with UV detector. HPLC condition: mobile phase Methanol-acetic acid (67 : 33, v/v), HPLC column C-18 Shiseido 250 mm x 4.6 mm. Oven temperature 40 °C. UV wavelength 254 nm and 276 nm.

Sample preparation

The cultivated trees had diameter at breast height (DBH) ranging from 0.4 to 0.6 m and were approximately 6.5 m tall. The wood was cut 5 feet above the ground and was divided into three sections (top, middle and bottom). Every section was 4 feet long. Top and bottom heartwood sections were used in this study. The specimens were taken from inner heartwood corresponding to growth rings 3 - 4 (near the pith) and outer heartwood corresponding to growth rings 11 (periphery). The samples were prepared in cube (20 x 20 x 5 mm) and in powder forms, respectively. The samples were dried in oven for 48 hours, weighed and kept in desiccators. The dried samples were placed in extraction thimbles (43 mm × 123 mm) and successive extractions were performed in a Soxhlet apparatus with dichloromethane, ethanol and ethanol-toluene (1 : 2) each step for 8 hours. The crude extract was obtained after solvent evaporating the solvent and extractive content was determined. The extractive content of the samples were determined based on oven dried weight.

Antifungal test:

The method was based on study by Wang et al. (2005). The petri dishes containing sterilized potato dextrose agar (PDA) was inoculated with *Phanerochaete chrysosporium*. A 5 mm hole was made on the center of the petri dish. Then 100 ppm of extractive (make up in original extraction solvent) from powder form in different level of concentration, 100 ppm, 500 ppm and 2000 ppm was injected into the hole.

Compared to the one with extractive another four parallel experimental groups were assigned, each was injected with dichloromethane, ethanol and ethanol-toluene (1 : 2) and the other was blank. Antifungal activity for standards of 2-methyl anthraquinone, 1,4-naphthoquinone and lapachol at various concentrations (100 ppm, 500 ppm and 1000 ppm) were carried out in the same manner. The antifungal activity was expressed as the percentage of growth diameter calculated and based on the diameter growth of controls. The antifungal activities were determined by measuring the radius of the fungal colony after subtracting the diameter of the inner hole containing extractives after seven days. The antifungal index was calculated by using equation 1.

$$\text{Antifungal index} = (1\text{-}Da/Db) \times 100$$

Where Da: the diameter of growth zone in the test plate, Db: Diameter of growth zone in the control plate.

Extract fractionation.

The crude BIH and TIH dichloromethane extract which exhibited higher antifungal activity was fractionated using chromatographic technique. BIH (1 gram) and TIH (1 gram) dichloromethane extract were chromatographed on silica gel as packing material (30 g, silica gel 60-200 mesh, column size 20 x 750 mm). A gradient of dichloromethane and hexane was used as eluent (Each eluent was prepared as 200 ml) from 20 : 80, 50 : 50, 80 : 20 and finally methanol 100 %. The composition of each fraction was examined by plating eluent on silica gel TLC plates with hexane/ethyl acetate (3 : 1 (v/v)) as the developing solvents. The plates were then placed on UV for the visualization of the components of each fraction and further confirmation by High performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Extractive content

It was observed that outer heartwood in powder form (12.24 %) contain higher total extractive amount compare to inner heartwood (9.69 %). The same trend also happens to top section which outer heartwood having 13.12 % of extractive amount compare to inner heartwood having 9.34 %. These results reflected with paler color of inner heartwood and darker color of heartwood. According to Bhat et al. (2005), the paler color of teak heartwood is associated with lower extractive content. They studied on 35-years old teak wood from wet and dry site home garden teak wood and plantation in Kerala, India. They found that dry site teak wood having higher extractive content with darker colored wood than wet site teak wood which having lower extractive content with paler color wood.

Among all extraction solvent, ethanol removes the most extractive content in the heartwood follow by dichloromethane and finally ethanol: toluene (1 : 2, v/v). Therefore, polar compound are the main extractive component in the heartwood follow by non-polar compound and finally intermediate polar compound.

Overall, total extractives content or amount being removing from the heartwood is ranging from 9.34 % until 13.12 %. The mean values of extractive content are comparable against reported values on teak wood from different sources. For instance, Puri (1962) reported around 10.3 % of outer heartwood total extractives from the ring number 91 from the pith of Nilambur Teak. Windeisen et al. (2003) recorded a total extractive content of 8.8 % to 9.4 % in two plantation sites from Panama. They also reported a total extractive content of 14.1 % for durable teak in Myanmar.
The mean extract yields after successive treatment for each heartwood section in powder and cube form (20 x 20 x 5 mm), respectively with various solvents (based on oven dry wood) is given in Tab. 1.

Tab. 1: The mean extract yields

Heartwood	Dichloromethane (%)		Ethanol (%)		Ethanol-Toluene (1:2, v/v) (%)		Total (%)	
	Powder	Cube	Powder	Cube	Powder	Cube	Powder	Cube
BIH	2.18	0.76	6.03	1.41	1.49	1.60	9.70	3.77
TIH	2.32	1.03	6.95	1.53	0.08	1.75	9.35	4.31
BOH	4.92	1.90	6.13	2.74	1.20	2.40	12.25	7.04
TOH	3.60	0.58	5.54	0.87	3.10	1.37	12.24	2.82

Antifungal Test:

The dichloromethane extractives in BIH extractives possess higher antifungal activity as compared to the other extractives followed by ethanol: toluene (1 : 2 v/v) extractives in BIH (Tab. 2). The dichloromethane extractives of BIH show antifungal activity starting from 100 ppm to 2000 ppm in which inhibition effect were larger than the other extractives. However inhibition effect for dichloromethane extractives of TIH was the highest as the inhibition zone was the largest among all extractives follow by BIH, TOH and finally BOH. Other extractives also show antifungal activity but at higher concentration (2000 ppm). Ethanol extractives in BOH, BIH, TOH and dichloromethane extractives in TIH exhibited antifungal activity at 2000 ppm only.

Tests were carried out to discover which of the main constituents possessed antifungal activity, therefore, biologically active compound, 1,4-naphthoquinone, 2-methyl anthraquinone and lapachol were used for standard references. 1,4 Naphtoquinone shows antifungal activity against *Phanerochaete chrysosporium* at 100 ppm to 2000 ppm. Lapachol shows antifungal activities at a concentration higher than 2000 ppm only and 2-methyl anthraquinone did not exhibit any antifungal activity at any concentration.

Tab.2: Antifungal results using agar plate method on heartwood extract

	Concentration, ppm		
Sample	**100**	**500**	**2000**
	Antifungal index (100 %)		
TIH DCM	-	10	12
TIH ethanol	-	-	-
TIH ethanol:toluene(1:2)	-	-	12
TOH DCM	-	10	12
TOH ethanol	-	-	12
TOH ethanol:toluene(1:2)	-	-	-
BIH DCM	8.9	10	12.3
BIH ethanol	-	-	8.9
BIH ethanol:toluene(1:2)	-	6	10
BOH DCM	-	-	-
BOH ethanol	-	-	8.9
BOH ethanol:toluene(1:2)	-	-	-
Control solvent Dichloromethane	-	-	-
Control solvent ethanol	-	-	-
Control solvent ethanol:toluene	-	-	-
Std 2-methyl anthraquinone	-	-	-
Std 1,4- naphtoquinone	5.56	8.9	20
Std Lapachol	-	-	-

GCMS and HPLC analysis analysis for heartwood extractives

All extractives with concentration at 200 000 ppm were run through GCMS analysis and compared against 2 standards, 1,4-naphthoquinone, 2-Methyl anthraquinone and lapachol in order to understand the chemical extractives that impart inhibition on white rot fungi, *Phanerochaete chrysosporium*. Retention time and ion abundance of main peaks of standards are given in Tabs. 3 and 4. The relative percentage of 2-methyl anthraquinone, lapachol and 1,4- naphthoquinone is shown in Tab. 5.

The components 2-methyl anthraquinone and lapachol were found in dichloromethane, ethanol and ethanol : toluene (1 : 2) extractives at inner and outer heartwood of top portion. However 1,4-naphtoquinone was not detected in the extractives which probably in trace level. Both biologically active compounds were also detected in all extractive solvents at inner heartwood bottom portion. However at outer heartwood of bottom portion, lapachol was not detected for dichloromethane and ethanol extractives. 2-methyl anthraquinone was detected for all extractives solvent. Relatively

extractives having ion abundance of lapachol above 200000 showed antifungal activity. For example ethanol : toluene (1 : 2 v/v) extractives having ion abundance of 1,4-naphtalenedione 296001 was the second highest having antifungal activity.

Tab. 3: Retention time of standards used in GCMS analysis.

Retention time/min	Compounds	Match quality
13.325	2-methyl Anthraquinone	92 %
8.2	1,4-Naphtoquinone	92 %
12.817	Lapachol	92 %

Tab. 4: The ion abundance of main peaks for the teak heartwood extractives in 2000 µg.ml^{-1} sample solution

	Ion abundance			
	TIH	**TOH**	**BIH**	**BOH**
Dichloromethane				
2-methyl anthraquinone	74317	410193	351745	59211
Lapachol	77652	445324	540727	-
Squalene	270162	1294265	1357283	896351
Ethanol				
2-methyl anthraquinone	79416	3869	108609	58423
Lapachol	149507	15288	100661	65963
Ethanol-tolune (1 : 2 v/v)				
2-methyl anthraquinone	17274	7486	316128	26547
Lapachol	47136	168917	296001	101266

Tab. 5: Percentage of 2-methyl anthraquinone (1), 1,4 Naphtoquinone (2), Lapachol (3) and from teak heartwood extract using HPLC analysis in 500 µg.ml-1 sample solution (Amount from 1ml was divided by dry sample weight)

	Dichloromethane %			Ethanol %			Ethanol:toluene %		
	1	2	3	1	2	3	1	2	3
TIH	5.82	1.90	10.71	0.22	0.17	0.02	0.20	-	0.03
TOH	4.64	1.85	7.36	0.12	0.14	0.03	2.02	0.28	3.41
BIH	3.80	1.82	5.24	0.58	0.58	0.03	0.18	0.15	-
BOH	2.56	1.1	3.26	0.07	0.03	0.07	0.38	-	-

The compounds 2-methyl anthraquinone, 1,4-naphthoquinone and lapachol were detected at retention time 13.3, 8.2 and minute 12.8 minutes, respectively. Ion abundance for 1,4 naphtoquinone and lapachol was the highest among all extractives and 2-methyl anthraquinone was the second highest among all extractives. Besides it was earlier reported that squalene is the main component in dichloromethane extractives of BIH, based on the HPLC analysis with base ion peak 69.10 and retention time at 17.2 minutes.

However, previous studies on teak wood extractives have not confirmed squalene to be responsible in teak wood durability. Squalene was found as major compound in petroleum ether extract in teak heartwood. They suggested squalene may contribute towards durability in the form of a hydrophobic barrier or as a precursor to toxic triterpene compounds (Windeisen et al., 2003). As no evidence yet to prove squalene as compound to inhibit *Phanerochaete chrysosporium* growth only 1,4-naphtoquinone and lapachol remain as the only possible compound to impart antifungal activity.

CONLUSIONS

Different quinones were found to occur in extracts obtained from heartwood of *Tectonia grandis* and their presence was supported by GCMS and HPLC. The results shows the importance of the composition of extracts against the white rot fungi, particularly 1,4-Napthoquinone was found as most bioactive compound for the inhibition of *Phanerochaete chrysosporium*.

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Irshad Ul Haq Bhat
University Sains Malaysia
School of Industrial Technology
Paper and Coating Division
Bio-Resources
Penang 11800
Malaysia
E-mail: akhalilhps@gmail.com

H.P.S. Abdul Khalil
University Sains Malaysia
School of Industrial Technology
Paper and Coating Division
Bio-Resources
Penang 11800
Malaysia

Nor Shifa Shuib
University Sains Malaysia
School of Chemical Sciences
Penang 11800
Malaysia

Ahmad Mohd Noor
University Sains Malaysia
School of Chemical Sciences
Penang 11800
Malaysia