Toxicity of Dichloromethane and Methanol-soluble Extractives from *Eusideroxylon zwageri* and *Potoxylon melagangai* heartwoods

Ismail Jusoh[#], Sim Shiang Ping, Zaini Assim

Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan
#Corresponding author. Email: jismail@frst.unimas.my; Tel: +6082-582990; Fax +6082-583160

ABSTRACT Natural durability of *Eusideroxylon zwageri* and *Potoxylon melagangai* are known to be very high. One of the reasons for high wood durability is the presence of extractives. The objectives of this study were firstly to determine the amount of dichloromethane (DCM) and methanol (MeOH) crude extracts from *E. zwageri* and *P. melagangai,* secondly to assess antifungal activitiey of DCM and MeOH extracts and thirdly to identify the chemical constituents of DCM and MeOH extracts. Sequential solvent extraction using DCM followed by MeOH were carried out. Toxicity or antifungal activities of extractives soluble in DCM and MeOH were determined using agar dilution method. The selected fungi used were *Trametes versicolor*, *Gloeophyllum trabeum* and *Chaetomium globosum* representing white-rot, brown-rot and soft-rot, respectively. Gas chromatography–mass spectrometry techniques were used to identify the chemical constituents and compositions of DCM and MeOH crude extract fractions from *E. zwageri* and *P. melagangai*. The total DCM crude extracts obtained from *P. melagangai* (3.30%) was higher than that of *E. zwageri* (0.60%). Crude extracts of MeOH extracted from *E. zwageri* (8.37%) was higher than *P. melagangai* (4.81%). There were 46 compounds detected in DCM crude extract of *E. zwageri* and the major compounds were 1,2,3-trimethoxy-5-[(1E)-1-propenyl]benzene (16.8%), cadina-3,9-diene, 4-methoxy-6-(2-propenyl)-1,3-benzodioxole, 4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene. For *P. melagangai* DCM crude extract, 29 compounds were identified and the major compounds were cadalene (21.8%), n-dotriacontane and γ-muurolene. There were 76 compounds identified in MeOH extract of *E. zwageri* and the major ones were 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane, methyl octacosanoate, tetratetracontane and methyl elaidate. Out of 40 compounds detected in MeOH extract from *P. melagangai,* 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane, 2,4-di-tert-butylphenol and diisooctyl phthalate were the major compounds. Compounds that were found in both *E. zwageri* and *P. melagangai* include γ-muurolene, heneicosane and tetratetracontane. MeOH and DCM crude extracts from *E. zwageri* and *P. melagangai* were toxic to *Trametes versicolor*, *Gloeophyllum trabeum* and *Chaetomium globosum*. Hexanedeconic acids, 2-4-di-ter-butylphenol, methyl hexadecanoate, methyl octadeconate, γ-muurolene, α-cadinol and myristicin might be responsible to the natural durability of *E. zwageri* and *P. melagangai* extractives.

KEYWORDS: *Eusideroxylon zwageri*; *Potoxylon melagangai*; extractives; antifungal activities; γ-muurolene; myristicin

INTRODUCTION

Antifungal compounds in wood can be extracted using different solvents. Dichloromethane (DCM) is a semi-polar solvent that can extract semi-polar compounds. Basically DCM solvent can extract aliphatic compounds, aromatic compounds, alicyclic compounds, non-ionic polymers and lignin. While, MeOH is a polar solvent, it usually used to extract polar chemical compounds from wood which include alcohols, ketones, carboxylic acids, phenols, carbohydrates and fatty acids. Extractive compounds have significant impact on properties of wood which are durability, strength, odour, taste, inflammability, toxicity, density, economic value and factory uses (Negi, 1997; Shmulsky & Jones, 2011; Rastaon & Tuah, 2016). Wood extractives include waxes, tannins, resins gums, fatty acids, resin acids, and terpenes. They are classified as phenolic, aliphatic, alicyclic compounds, or other lesser compounds. In addition, 97-99% of over-dry wood substances by weight are cellulose, hemicellulose and lignin form the primary substances of wood (Smith *et al.*, 2003).

Since *Eusideroxylon zwageri* Teijsm. & Binnend (Belian) and *Potoxylon melagangai* Kosterm (Malagangai) are very durable timbers due to their extraneous substances, thus it is important to

identify the compounds responsible for the decay resistance in *E. zwageri* and *P. melagangai* heartwoods. Currently chemical wood preservatives are used to treat non-durable timbers. Chemical-based preservatives lead to a number of environmental concerns, thus the potential of natural wood preservatives as effective replacement has gained interest of many researches. The discovery of these environmental-friendly compounds may replace the role of toxic chemical, which currently used as wood preservatives. The advantages of using natural wood preservatives to treat wood are, as natural product they are usually harmless to human and environment. The potential development of wood extractives as natural wood extractives not only important to provide alternative treatment for wood preservation industry but also may be useful for therapeutic and cosmetic industries.

Wood extractive consists of different kinds of chemical compounds. It is a non-structural wood component and represents a small fraction in wood. Heartwood has higher extractives compare to sapwood (Fengel and Wegener, 1989; Sjostrom, 1993). The amount of extractive varies with species, growth conditions, age of the tree and locality. It can contain up to 40% but normally range between 2-10% of dry wood weight. The roles of heartwood extractives for natural durability and antioxidant activity have been demonstrated (Ismail & Ipor, 2004; Singh & Singh 2012; Kirker *et al.*, 2013; Vun-Sang *et al.*, 2017; Latiff *et al.*, 2017). Wood with high amount of extractive is more resistant to decay (Zabel & Morrell, 1992; Schultz & Nicholas, 2000; Ismail & Ipor, 2004). The quantity of extractable heartwood extractive varies from the solvent that is used for extraction (Gierlinger, 2003). The colour of extractive can be yellow, red and brown and colourless, to which they impart colour to wood. Currently there are limited information on the chemical compounds from *E. zwageri* and *P. melagangai*. The objectives of this study were firstly to determine the amount of DCM and MeOH crude extracts obtained from *E. zwageri* and *P. melagangai* heartwoods. Secondly, to assess antifungal properties of DCM and MeOH extracts. Thirdly, to identify the chemical constituents of DCM and MeOH extracts from *E. zwageri* and *P. melagangai* heartwoods.

METHODOLOGY

Preparation of Wood Samples
Wood samples of *E. zwageri* was obtained from Kakus, Bintulu, Sarawak. While, wood samples of *P. melagangai* was obtained from Limbang, Sarawak. Heartwood samples of both species were ground by using a grinder to produce wood meal. The wood meals were then air-dried for four weeks before storing in closed container.

Extraction of Wood Meal
Extraction of wood meal was carried out using solvent extraction method according to procedure described by Solis *et al*. (2004) with slight modifications. *Eusideroxylon zwageri* and *P. melagangai* wood meals were extracted sequentially using DCM followed by MeOH. A total of 1300 g of wood meal was immersed in two liter DCM in separator funnel in room temperature. After three days, the DCM-soluble extract was drained and collected into a round flask. Subsequently, MeOH was added into the separator funnel containing DCM-extracted wood meal. The MeOH-soluble extract was collected into another round bottom flask. The solvents containing extracts were weighed and evaporated into dryness using vacuum rotary evaporator at 35°C to obtain pure crude DCM and MeOH extracts. The extractions using DCM and MeOH were repeated two more times. All dried crude extracts were weighed. The amount of dried crude extract was determined based on the weight of solidify crude extract divided by weight of wood meal expressed as percentages.

Antifungal Assay

Antifungal assays were evaluated using the agar dilution method according to procedure described by Yen *et al*. (2008) and Chang *et al.* (1999, 2000) with slight modifications. Crude extracts were dissolved in DCM and MeOH separately. Solution of DCM extract was added into sterilized malt extract agar (MEA) when the agar was still in liquid form to yield the final concentrations of 10, 5 and 2.5 mg/mL. Methanol crude extracts were mixed with MEA to become concentrations of 150, 50, 25, 10, 5 and 2.5 mg/mL. The mixtures were poured into 9 cm Petri dish. Agar media mixed with only DCM and MeOH were used as negative control. Antifungal assays were carried out in triplicate.

Fungal strains used in this study obtained from Forest Research Institute Malaysia (FRIM) were *Trametes versicolor*, *Gloeophylum trabeum* and *Chaetomium globosum* representing white rot, brown rot and soft rot, respectively. Fungal plugs from the edge of actively growing cultures were transferred onto the centre of the Petri dishes and incubated in dark at 27°C and 70% relative humidity for 7 days. The cultures diameter was measured daily. Antifungal indexes were calculated when the mycelium fungi reached the edges of control dishes. The diameter in all experimental dishes was measured and antifungal index (AI) expressed as % inhibition was calculated by the following; $AI\% = [(D_b - D_a)/D_b] \times 100$, where D_a is the mean diameter of growth zone in experimental dish with extract (cm) and D_b is the mean diameter of growth zone in control dish (cm).

Column Chromatography Fractionation

Fractionation was carried out to reduce number complexity of compounds present in crude extract. The fractionation of crude DCM extract was performed according to procedure described by Buco *et al*., (2004) with slight modifications. The glass column (30 cm × 1 cm i.d.) was packed with 8 g of 70–230 mesh silica gel and deactivated with 5 % of distilled water. An aliquot of the DCM crude extracts (30 mg in 1 ml hexane) was placed on the top of the column and then fractionated into aliphatic hydrocarbons (F1), aromatic hydrocarbons (F2) and polar compounds (F3). All fractions were collected in pear-shaped flasks. F1 was eluted with 30 ml n-hexane (F1), while F2 was eluted with 20 ml *n*-hexane–dichloromethane (90:10; v/v) and 40 ml *n*-hexane–dichloromethane (80:20; v/v). Finally, F3 was eluted with 40 ml dichloromethane–methanol (80:20; v/v). All the fractions were evaporated to near dryness using rotary vacuum evaporator. The fractions were redissolved with 2-5 ml dichloromethane and transfer to a 10 ml-capacity vial using Pasteur pipette. The fractions were evaporated to dryness under gentle nitrogen stream and were stored in dark places until further analysis. Prior to gas chromatography-mass spectrometry (GC-MS) analysis, the fractions were diluted with 200 uL of dichloromethane.

The crude MeOH extract was subjected to silica gel column chromatography for fractionation process according to Alet (2011). Methanol crude extract was purified on the chromatography column (4.0 cm i.d x 45 cm length) packed with 100 g silica gel. The crude extract was eluted with increasing polarity solvent system. A total of 100 mL for each solvent were used to fractionate MeOH crude extract. Fractions of 25 mL each was collected in test tube then were subjected to Gas Chromatograph-Mass Spectrometry analysis.

Gas Chromatograph-Mass Spectrometry (GC-MS) Analysis

Gas chromatographic analyses of the samples were carried out on model Shimadzu (QP 2010) plus mass spectrometer (GC-MS) fitted with BPX-5 capillary column. Approximately, 1 µL sample was injected on splitless mode. The GC oven was maintained at 50°C for 2 minutes, ramped to 280°C at ramp rate 6°C/min. Temperature for injector and interface were maintained at 280°C and 300 °C, respectively. The mass spectra were obtained by scanning at range of 35 to 450 a.m.u at the rate of 2

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scan/min. The compounds were identified by comparing the mass spectrum of obtained from the analysis with those Spectral Library in the data system.

RESULTS AND DISCUSSION

Crude Dichloromethane and Methanol Extractives

The results in Table 1 show that total amount of crude extract isolated with DCM were 0.61% and 3.30 % for *E. zwageri* and *P. melagangai*, respectively. Results also showed that amount of the MeOH extract of *E. zwageri* was more than that of *P. melagangai* at 8.37% and 4.81%, respectively. This study showed that MeOH extract contain more extractive than DCM which suggests that *E. zwageri* and *P. melagangai* contain more polar extracts than semi-polar extracts. The amount of extractives is influenced by several species, growth conditions, age of the tree (Gierlinger, 2003) and locality (Fengel and Wegener, 1989; Negi, 1997; Gierlinger, 2003).

Table 1: Percentage of crude DCM and MeOH extract from *E. zwageri* and *P. melagangai.*

Wood meal sample (g)	Solvent (L)	Crude extract (%)
Eusideroxylon zwageri	Dichloromethane	0.61
	Methanol	8.37
Potoxylon melagangai	Dichloromethane	3.30
	Methanol	4.81

Antifungal Activities of DCM and MeOHl Crude Extracts of E. zwageri and P. melagangai

The results of antifungal activities of crude extracts obtained from *E. zwageri and P. melagangai* heartwoods are shown in Tables 2 and 3. The extracts showed the inhibition effects on the growth of *T. versicolor*, *G. trabeum* and *C. globosum*. Antifungal index of DCM crude extract of *P. melagangai* was higher compare to *E. zwageri* (Table 2). This indicates that DCM soluble extract from *P. melagangai* has higher inhibitory ability on *Trametes versicolor* and *Gloeophyllum trabeum* than *E. zwageri*. However, crude DCM of *E. zwageri* have higher antifungal index than *P. melagangai* against *Chaetomium globosum.* Thus, this means that crude DCM of *E. zwageri* has higher inhibitory ability on *Chaetomium globosum* than *P. melagangai.* Concentration of 2.5 mg/mL *E. zwageri* and *P. melagangai* DCM crude extract recorded antifungal index against *Trametes versicolor* of 57% and 74%, respectively (Table 2). However, at the same concentration, DCM crude extract of *E. zwageri* and *P. melagangai* have lower antifungal index against *Chaetomium globosum* which were 36% and 34%, respectively. Thus, DCM crude extracts of *E. zwageri* and *P. melagangai* were more effective against *Trametes versicolor* than *Gloeophyllum trabeum* and *Chaetomium globosum.* Crude extract of DCM of *E. zwageri* and *P. melagangai* able to inhibit fungal grow. The higher concentration of crude extract, the higher the antifungal index. These suggest that DCM soluble extracts of these two species contain antifungal compounds that inhibit the growth of *Trametes versicolor, Gloeophyllum trabeum* and *Chaetomium globosum*.

Antifungal index increases as the concentration of MeOH extract of *E. zwageri* and *P. melagangai* increases (Table 3). At the concentration of 10 mg/mL, the antifungal index was more than 70%. Methanol extract of *E. zwageri* and *P. melagangai* markedly inhibited the growth of *Trametes versicolor*, *Chaetomium globosum* and *Gloeophyllum trabeum* at 50 mg/mL. The results suggest that MeOH crude extract of *E. zwageri* and *P. melagangai* have the ability to resist wood decay due to *Trametes versicolor*, *Chaetomium globosum* and *Gloeophyllum trabeum.*

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Table 2. Antifungal index of different DCM crude extract concentrations from *E. zwageri* and *P. melagangai* against *T. versicolor*, *G. trabeum* and *C. globosum*.

Concentration (mg/mL)	*T. versicolor*		*G. trabeum*		*C. globosum*	
	E. zwageri	*P. melagangai*	*E. zwageri*	*P. melagangai*	*E. zwageri*	*P. melagangai*
0*	0	1	0	2	1	1
2.5	57	74	42	50	36	34
5	66	79	57	60	52	48
10	79	80	77	93	80	73

*Negative control, agar mixed with DCM only

Table 3. Antifungal index of different methanol crude extract concentrations from *E. zwageri* and *P. melagangai* against *T. versicolor*, *G. trabeum* and *C. globosum*.

Concentration (mg/mL)	*T. versicolor*		*G. Trabeum*		*C. globosum*	
	E. zwageri	*P. melagangai*	*E. zwageri*	*P. melagangai*	*E. zwageri*	*P. melagangai*
0*	1	0	0	0	1	1
2.5	64	46	32	32	56	49
5	81	77	45	45	71	56
10	83	83	90	79	90	73
25	90	93	93	81	92	82
50	92	93	93	93	93	93
150	93	93	93	93	93	93

*Negative control, agar mixed with MeOH only

Chemical Constituents and Compositions DCM Extracts of E. zwageri and P. melagangai

A total of 46 compounds were identified in DCM crude extract of *E. zwageri*. The major compounds and their relative composition detected in DCM extracts of *E. zwageri* and *P. melagangai* are listed in Table 4 arranged according to increasing retention time. In DCM crude extract of *E. zwageri*, 1,2,3-trimethoxy-5-[(1E)-1-propenyl]benzene recorded the highest compound with composition of 16.8%. Compound cadina-3,9-diene was the second major compound with composition of 11.9%. The third and fourth major compounds were 4-methoxy-6-(2-propenyl)-1,3-benzodioxole and 4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene with composition of 10.2% and 7.9%, respectively. The compound, 4-methoxy-6-(2-propenyl)-1,3-benzodioxole which is also known as myristicin belonging to apiole group can efficiently killed fourth instar larvae of lepidopterous insect pest *Spilarctia obliqua* after 24 hours (Srivastava *et al.*, 2001). A study done by Fang *et al*. (2011) stated that myristicin was the major composition in volatile oil from the nutlets of *Clausena anisum-olenas*. This volatile oil had strong inhibitory effect against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Bacillus cereus*.

For *P. melagangai* DCM crude extract, there were 29 compounds detected and the highest percentage compound detected were cadalene with composition of 21.8% followed by n-dotriacontane and γ-muurolene with the composition of 10.9% and 10.8%, respectively (Table 4). Essential oil of *Teucrium montanum* contains chemical composition that has antibacterial properties as well as antifungal effect and one of the compounds detected from the essential oil was cadalene with 4.91% (Vukovic *et al.*, 2007). The sesquiterpenes hydrocarbon compound, γ-muurolene, found in both *E. zwageri* and *P. melagangai*, was one of the major compounds in the essential oil of Japanese cedar (*Cryptomeria japonica*) heartwood that possesses excellent antifungal activities (Cheng *et al.*, 2005).

Chemical Constituents and Compositions MeOH Extracts from E. zwageri and P. melagangai

There were 76 compounds identified in MeOH extract of *E. zwageri*. The major compound detected in MeOH extract of *E. zwageri* was 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane (43.5%) (Table 5). Methyl octacosanoate was the second major compound with 10.0% followed by tetratetracontane (8.3%), methyl elaidate (4.4%) and methyl hexadecanoate (3.2%).

Table 4. Major chemical constituents and compositions of dichloromethane extracts identified in both *E. zwageri* and *P. melagangai*.

Constituent	Retention time (min)	Peak area in GCMS (Relative %)	
		E. zwageri	*P. melagangai*
4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene	17.6	7.9	0
γ-muurolene	18.8	2.3	10.8
1-methyl-4-(5-methyl-1-methylene-4-hexenyl)cyclohexene	19.4	0	7.7
Cadina-3,9-diene	19.6	11.9	0
α-Panasinsen	19.7	7.1	0
Cadina-1,3,5-triene	19.8	0	7.5
α-calacorene	20.6	0	4.3
Cadalene	22.9	1.0	21.8
n-Dotriacontane	36.5	0	10.9
1,2,3-Trimethoxy-5-[(1E)-1-propenyl]benzene	40.1	16.8	0
4-methoxy-6-(2-propenyl)-1,3-Benzodioxole (Myristicin)	41.0	10.2	0
Other compounds combined	-	42.8	37

Table 5. Chemical constituents and compositions of methanol extracts identified in both *E. zwageri* and *P. melagangai*.

Constituent	Retention time (min)	Peak area in GCMS (Relative %)	
		E. zwageri	*P. melagangai*
1,3-Bis(1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane	10.3	43.5	41.3
Tert-butyl-[2-oxyethoxy]dimethylsilane	16.1	0	3.5
2,4-di-tert-butylphenol	19.4	0	18.0
Methyl hexadecanoate	26.9	3.2	0
1-Nonadecene	28.0	0	2.6
Methyl elaidate	29.8	4.4	0
Diisooctyl phthalate	35.8	1.7	14.1
Tetratetracontane	41.1	8.3	0
Methyl octacosanoate	43.2	10.0	0
Other compounds combined	-	28.9	20.5

In *P. melagangai* MeOH extract, 40 compounds were detected. The major compounds identified was also 1,3-bis(1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane with similar composition as in *E. zwageri* (41.5%). The second major compound was 2,4-di-tert-butylphenol (18.0%) followed by diisooctyl phthalate (14.1%), tert-butyl-[2-oxyethoxy]dimethylsilane (3.5%) and 1-nonadecene (2.6%). Other significant compounds found in *E. zwageri* and *P. melagangai* MeOH extracts with small quantities include tetratetracontane, hexanedecanoic acid, eicosane and heneicosane. Oil from bulb of *Crinum ornatum* contain large amount of tetratetracontane (10.5%), hexanedecanoic acid (13.1%),

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heneicosane (13.1 %), %). These compounds were toxic to brine shrimp (Oloyede *et al.*, 2010). Hexane extract of *Temnopleurus alexandri* had antibacterial activities that contain eicosane and heneicosane as the major compounds (Uma & Parvathavarthini, 2010). In Australia, diisooctyl phthalate is used to manufacture rubber compounds for automotive hoses and parts. Besides, diisooctyl phthalate had showed low acute oral and dermal toxicity in laboratory animals. Phthalate are used as used as ingredients of insect repellents, as solvents in lacquer and pesticides (Maag *et al.*, 2010).

Xavier *et al.* (2011) found that *Caryocar villosum* possess major compounds namely methyl hexadecanoate (32%), methyl octadeconate (29%) and methyl (E)-octadecanoate (29%) that have anti-inflammatory activity. In our study, methyl hexadecanoate can be found in both *E. zwageri* and *P. melagangai*. *Taiwania cryptomerioides* is an excellent durable species in Taiwan. The chemical α-cadinol was one of the strong antifungal compounds found in *Taiwaina cryptomerioides* that make it naturally durable. Compound α-cadinol able to inhibit the fungal growth at 100 ppm and it also possesses antitumor property (He *et al.*, 1997). Compound α-cadinol was found to be active against human tumor cell, HT-29 colon adenocarcinoma (Chang *et al.*, 2000). In this study, α-cadinol compound can be found in *E. zwageri* in DCM and MeOH extracts with 0.4% and 0.2%, respectively.

CONCLUSIONS

Total *P. melagangai* DCM extract was higher than *E. zwageri*, however *E. zwageri* MeOH extract was higher in *E. zwageri* than that of *P. melagangai.* Antifungal index revealed that at concentration of 10 mg/mL inhibited the growth of *Trametes versicolor*, *Gloeophyllum trabeum* and *Chaetomium globosum.* Dichloromethane crude extract have higher inhibitory ability on *Trametes versicolor* followed by *Gloeophyllum trabeum* and *Chaetomium globosum*. As for MeOH crude extract higher inhibitory ability was observed on of *Trametes versicolor* followed by *Chaetomium globosum* and *Gloeophyllum trabeum.* This study had shown that DCM-soluble and MeOH-soluble extractives from *E. zwageri* and *P. melagangai* contain compounds that are toxic to *Trametes versicolor* (white rot), *Gloeophyllum trabeum* (brown rot) and *Chaetomium globosum* (soft rot). Based on GC-MS analyses and literature, hexanedeconic acids, 2-4-di-ter-butylphenol, methyl hexadecanoate, methyl octadeconate, γ-muurolene, α-cadinol and myristicin might be responsible for the antifungal activities in *E. zwageri* and *P. melagangai* heartwoods.

ACKNOWLEDGEMENTS

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