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Two Antifungal Xanthones from the Heartwood of *Calophyllum Symingtonianum*

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Abstract

The antifungal constituents from the heartwood of *Calophyllum symingtonianum* (bintangor bukit; Clusiaceae) were investigated. Methanol extracts, *n*-hexane-solubles, ethyl acetate-solubles and ethyl acetate-insolubles obtained from the heartwood of *C. symingtonianum* were subjected to antifungal assay against the brown-rot fungus, *Gloeophyllum trabeum*, and the white-rot fungus, *Pycnoporus sanguineus*. *n*-Hexane-solubles and methanol extracts showed the highest activity against *G. trabeum*, followed by ethyl acetate-solubles. *n*-Hexane-solubles showed the highest activity against *P. sanguineus*, followed by methanol extracts and ethyl acetate-solubles. Two major xanthones, 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone (**1**) and 6-desoxyjacareubin (**2**) were isolated from ethyl acetate-solubles and *n*-hexane-solubles, respectively, and the structures of these compounds were determined. They were isolated for the first time from *C. symingtonianum*. Xanthones **1** and **2** were active against the both fungi and showed significantly high activity against *G. trabeum* and *P. sanguineus*, respectively. The activities of these antifungal xanthones were comparable to or higher than those of the positive control, glycyrrhizic acid dipotassium salt. The results suggested the significant potential of the heartwood of *C. symingtonianum* as a source of fungistats.

Discipline: Forestry and forest products
Additional key words: Malaysian timber, basidiomycete, *Gloeophyllum trabeum*, *Pycnoporus sanguineus*, bintangor bukit

Introduction

Calophyllum symingtonianum M.R.Hend. & Wyatt-Sm. (common Malaysian local name, bintangor bukit; Clusiaceae) is an evergreen broad-leaved tree distributed in the Malay Peninsula. It usually grows in hill forests, at an altitude of 100–150 m[20]. *Calophyllum* spp. (bintangor) often produces rather decorative figures on flat-sawn boards, and the distinctive colors of the timber make it attractive for decorative purposes, such as furniture, parquet flooring, solid door construction, and veneer and plywood. The poisonous latex from the bark of several species is also used to numb fish and mixed with rice to kill rats. A decoction of the bark and the latex of some species (e.g. *C. inophyllum*) is used medicinally, internally against diarrhea and after childbirth, externally against skin and eye diseases and rheumatism; while the leaves, flowers and seeds are sometimes also used in local medicine[19]. The constituents of *C. inophyllum* are known to show various bioactivities such as anti-HIV[3, 19], anticancer[7], antifungal[15], antibacterial[18], cytotoxic[5], piscicidal[12], and trypanocidal[1] activities.

In our previous paper, extracts from the heartwood, sapwood and bark of 11 Malaysian timbers were antifungal assayed against *Gloeophyllum trabeum* (a brown-rot fungus) and *Pycnoporus sanguineus* (a white-rot fungus)[9]. In the results of the antifungal assay, only the methanol extracts from the heartwood of *C. symingtonianum* showed very high activity against both fungi. In the present study, therefore, we investigated the compounds contained in the heartwood extracts of *C. symingtonia-*

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num, which are responsible for antifungal activity. Two major xanthones were isolated from the methanol extracts of the heartwood, and structurally determined. The antifungal activities of these compounds were investigated, and the potential of constituents and extracts from the heartwood of *C. symingtonianum* as sources of fungistats was evaluated.

Materials and methods

^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JNM-LA400 (400 MHz) spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a JEOL JMS-DX303HF mass spectrometer. High-performance liquid chromatography (HPLC) was performed with a system of Shimadzu LC-20AD pumps, SPD-20A UV/VIS detector, and CTO-20AC column oven, using columns (Nihon Waters, μ Bondasphere 5 μ Silica 100Å, μ Bondasphere 5 μ C_{18} 100Å, 150 × 3.9 mm i.d. for analytical and 150 × 19.0 mm i.d. for preparative purposes respectively, Tokyo, Japan). The solvents used for the HPLC were HPLC gradient grade (Sigma-Aldrich Inc., St. Louis, MO, USA).

1. Plant material

Approximately 50-year-old *Calophyllum symingtonianum* in Kedah, Malaysia was sampled in 2007. The heartwood of *C. symingtonianum* was ground in a Wiley mill (Retsch, cutting mill SM 1) into meal (<1 mm). The completely processed sample meals were then placed into a freezer at –20°C. A voucher specimen is deposited in the Division of Bio-resource, Paper and Coatings Technology, Universiti Sains Malaysia.

2. Extraction and fractionation

Air-dried sample meals (252.0 g, moisture content: 10.1%) were extracted under reflux with 350 mL of methanol (MeOH) four times (extraction time: 5 min, 30 min, 1 h, 4 h). The extracted solution was filtered and the solvent removed *in vacuo* (30°C) to give residual MeOH extracts (3.77 g). The MeOH extracts were successively partitioned between *n*-hexane/water (H_2O) and ethyl acetate (EtOAc)/H_2O to give *n*-hexane-solubles (0.189g), EtOAc-solubles (3.073 g) and EtOAc-insolubles (0.493 g) after removing solvents, respectively.

3. Antifungal assay

Antifungal assays were performed based on our previous papers[9, 10, 11]. The fungal strains used were *Gloeophyllum trabeum* MI-102 from the School of Biology, Universiti Sains Malaysia, and *Pycnoporus sanguineus* KUM 70097 from the Forest Research Institute Malaysia (FRIM). These fungi were selected for antifungal assays in this work, because *P. sanguineus* is a local fungus in Malaysia, and the optimum temperature for the growth of *G. trabeum* is high[22]. The fungi were incubated for 10 days in a liquid medium based on the malt extract. After incubation, the hyphae were homogenized for 2 min at 10,000 rpm, the liquid medium was removed by centrifuging, and the homogenized hyphae were washed with physiological saline. The hyphae (1 mL) was added to 12 mL of sterilized potato dextrose agar medium and mixed, whereupon the mixture was poured into 9-cm Petri dishes. Sterilized paper discs (diameter 6 mm, Advantec Toyo Inc.) were permeated with 10 μL of the methanol solutions (0.5, 1, 2.5, 5, 10, 20, 50, 100 μg/μL) containing each of the methanol extracts, fractions, isolated compounds (xanthones) or positive control, glycyrrhizic acid dipotassium salt (GADS). The discs were allowed to dry in air for 15 min, and then placed on the agar surface in each dish. Discs without any constituents were used as negative controls. The width of the inhibition zone around each disc was measured after 3 days at 26 °C, and the minimum inhibitory concentration (MIC, μg/disc) of the fungal growth was determined. Tests were carried out in triplicate.

4. Isolation of antifungal xanthones by preparative HPLC

EtOAc-solubles (150.0 mg) were subjected to reverse-phase preparative HPLC (flow rate: 4.0 mL/min, detection: UV 228 nm, eluent: MeOH:H_2O=92:8, v/v) to elute fraction (Fr.) 1 and Fr. 2. Fr. 1 (112.0 mg) was further subjected to reverse-phase preparative HPLC (flow rate: 4.0 mL/min, detection: UV 228 nm, eluent: MeOH:H_2O=85:15, v/v) to elute Fr. 1-1– Fr. 1-3. 1,3,5-Trihydroxy-2-(3-methylbut-2-enyl)xanthone (**1**) (9.5 mg) was obtained from Fr. 1-3. *n*-Hexane-solubles (170.0 mg) were subjected to normal-phase preparative HPLC (flow rate: 4.0 mL/min, detection: UV 228 nm, eluent: *n*-hexane–$CHCl_3$–MeOH, 80:12:8, v/v/v) to elute Fr. 1–Fr. 3. 6-Desoxyjacareubin (**2**) (23.0 mg) was obtained from Fr. 3.

1,3,5-Trihydroxy-2-(3-methylbut-2-enyl)xanthone (**1**)

Pale yellow amorphous powders. ^{1}H-NMR spectral data were identical with those of the literature[6]. ^{13}C-NMR spectral data were not shown in the literature[6]. ^{13}C-NMR (acetone-d_6)(int. std., TMS): δ 17.86 (C-5'), 21.93 (C-1'), 25.85 (C-4'), 94.27 (C-4), 103.52 (C-1a), 111.53 (C-2), 116.29 (C-8), 121.15 (C-6), 122.23 (C-9a), 123.19 (C-2'), 124.65 (C-7), 131.63 (C-3'), 145.93 (C-10a), 146.83 (C-5), 156.36 (C-4a), 161.48 (C-1), 164.19 (C-3), and 181.55 (C-9). EIMS *m/z* [rel. int. (%)]: 312 (60, M^+),

297 (35), 295 (29), 269 (55), and 257 (100).

6-Desoxyjacareubin (**2**)

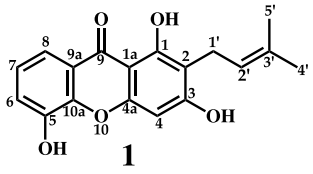
Yellow amorphous powders. ^{1}H-NMR spectral data were identical with those of the literature[8]. ^{13}C-NMR spectral data were not shown in the literature[8]. ^{13}C-NMR ($CDCl_3$-CD_3OD)(int. std., TMS): δ 28.43 (C-4',5'), 78.54 (C-3'), 95.47 (C-4), 103.73 (C-1a), 104.78 (C-2), 115.44 (C-1'), 115.87 (C-8), 120.64 (C-6), 121.49 (C-9a), 124.11 (C-7), 127.92 (C-2'), 145.43 (C-10a), 145.81 (C-5), 157.09 (C-4a), 157.45 (C-3), 161.04 (C-1), and 181.43 (C-9). EIMS *m*/*z* [rel. int. (%)]: 310 (27, M^+), and 295 (100).

Results and discussion

The antifungal activity of extracts and constituents from the heartwood of *Calophyllum symingtonianum* was investigated. Fractions were obtained from methanol extracts of the heartwood of *C. symingtonianum* by fractionation using solvents. The results of the antifungal assay of methanol extracts and fractions against the brown-rot fungus, *Gloeophyllum trabeum* and the white-rot fungus, *Pycnoporus sanguineus* are summarized in Table 1. The yield of ethyl acetate (EtOAc)-solubles was the highest, while that of *n*-hexane-solubles was approximately 1/16 as much as EtOAc-solubles. *n*-Hexane-solubles showed the highest antifungal activity against *G. trabeum* (MIC, 25 μg/disk) and *P. sanguineus* (MIC, 10 μg/disk), which was higher than that of the positive control, glycyrrhizic acid dipotassium salt (GADS). *n*-Hexane-solubles showed higher activity against *P. sanguineus* than against *G. trabeum*, and EtOAc-solubles showed higher activity against *G. trabeum* than against *P. sanguineus.* EtOAc-insolubles showed no activity against both fungi at concentrations of 1,000 μg/disk.

Two major xanthones were isolated from ethyl acetate-solubles and *n*-hexane-solubles of methanol extracts from the heartwood of *C. symingtonianum.* These xanthones were identified by comparison of the ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra and electron impact mass (EIMS) spectra with the literature data. 1,3,5-Trihydroxy-2-(3-methylbut-2-enyl)xanthone (**1**) and 6-desoxyjacareubin (**2**) were identified as major xanthones in the heartwood of *C. symingtonianum*. These two xanthones **1** and **2** were isolated for the first time from *C. symingtonianum*, although **1** and **2** had been isolated from the wood of *C. cuneifolium*[6] and the heartwood of *C. scriblitifolium*[8], respectively. Compound **1** can be considered the putative biogenetic precursor of compound **2**[14]. As for other biological activity, compound **2** was reported to show high trypanocidal activity against epimastigotes of *Trypanosoma cruzi*[1].

Compounds **1** and **2** were subjected to antifungal assay against *G. trabeum* and *P. sanguineus.* The results of the antifungal assay and the yields of these constituents based on oven dried plant material are summarized in Table 2. The yield of compound **1** was approximately eight-fold that of compound **2**. Against brown-rot fungus, *G. trabeum*, compound **1** showed high activity and com-



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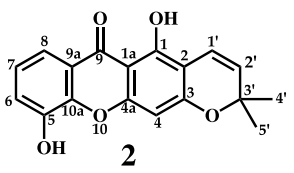

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Table 1. Antifungal activity [a] of the MeOH extract, fractions from the heartwood of *C. symingtonianum* and their yields

Fractions	Minimum inhibitory concentrations (MIC, μg/disc)		Yields (%)[d]
	G. trabeum[b]	*P. sanguineus* [c]	
MeOH extract	25	50	1.664
n-Hexane-solubles	25	10	0.083
EtOAc-solubles	50	100	1.356
EtOAc-insolubles	>1000	>1000	0.218
GADS [e]	200	100	–

[a] According to the assay using a medium in which homogenized hyphae were dispersed, negative controls (solvents only) showed no activity.
[b] Test fungus: *Gloeophyllum trabeum* MI-102.
[c] Test fungus: *Pycnoporus sanguineus* KUM 70097.
[d] Percentages based on oven-dried heartwood of *C. symingtonianum.*
[e] Positive control, glycyrrhizic acid dipotassium salt (GADS).

pound **2** showed moderate activity. In the experiment of the constituents from the heartwood of *C. brasiliensis* by Reyes-Chilpa et al., 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone (**1**) showed activity against brown-rot fungus, *Postia placenta*, whereas 6-desoxyjacareubin (**2**) did not[15]. These results were similar to ours. The difference in the intensity of activity was probably due to the difference in the antifungal assay method used or the species of the fungus[10, 22]. In the present work, the antifungal assay of compounds **1** and **2** against white-rot fungus was performed for the first time. Compound **2** showed higher activity against a white-rot fungus, *P. sanguineus* than compound **1** in contrast to a brown-rot fungus, *G. trabeum*. The activities of two antifungal xanthones isolated from *C. symingtonianum* were comparable to or exceeded those of the positive control.

The activity of extracts often decreases in the processes of fractionation or isolation due to the loss of synergism[10]. However, in the present work, the antifungal activity of *n*-hexane-solubles against *P. sanguineus* was improved in comparison with the methanol extracts reported in our previous paper (Table 1)[9]. Compounds **1** and **2** showed activities of MIC, 25 μg/disk against *G. trabeum* and MIC, 50 μg/disk against *P. sanguineus*, respectively, and the activities were the same as methanol extracts (Tables 1 and 2).

The tendencies of antifungal activities of compounds **1** and **2** against the brown-rot fungus, *G. trabeum* and the white-rot fungus, *P. sanguineus* differed (Table 2). The factors of brown-rot are brought up as cellulase[16], nonenzymatic degradation of cellulose and hemicellulose caused by Fenton reaction[2], biosynthesized oxalic acid[4, 17], and lignin-degrading enzyme[17]. The white-rot fungi possess higher activities of lignin-degrading enzymes (lignin peroxidase, manganese peroxidase and laccase) than brown-rot fungi[13]. These enzymes possess high oxidizability, and have low substrate specificities. The difference in terms of the antifungal active tendencies of compounds **1** and **2** could be due to the difference of function on the wood decay of each fungus.

In conclusion, it was proved that the heartwood of *C. symingtonianum* has great potential as a source of fungistats due to the isolation of the major antifungal xanthones. When using constituents having remained in the mixture, *n*-hexane-solubles, which show high activity against *P. sanguineus*, can be easily prepared by the *n*-hexane treatment of methanol extracts.

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Table 2. Antifungal activity[a] of xanthones isolated from the heartwood of *C. symingtonianum* and their yields

Compounds	Minimum inhibitory concentrations (MIC, μg/disc) [MIC, μM/disc]		Yields (%)[d]
	G. trabeum[b]	*P. sanguineus*[c]	
1	25 [0.080]	100 [0.32]	0.086
2	100 [0.32]	50 [0.16]	0.011
GADS[e]	200 [0.22]	100 [0.11]	–

[a-e] Same as in Table 1.

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