Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2015; 7(4); 727-731

ISSN: 0975-4873

Research Article

Secondary Metabolites from the Male Cone of *Cycas vespertilio*

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Available Online: 29th June, 2015

ABSTRACT

Chemical investigation of *Cycas vespertilio* male cone led to the isolation of pinoresinol (**1**), lariciresinol (**2**), mixtures of α-amyrin acetate (**3a**) and lupeol acetate (**3b**) in a 2.5:1 ratio and β-sitosterol (**4a**) and stigmasterol (**4b**) in a 2:1 ratio, triglycerides (**5**), and fatty alcohols (**6**). The structures of **1** and **2** were elucidated by extensive 1D and 2D NMR spectroscopy, while those of **3a-6** were identified by comparison of their ^{1}H and/or ^{13}C NMR spectra with those reported in the literature.

Keywords: *Cycas vespertilio* male cone, Cycadaceae, pinoresinol, lariciresinol, α-amyrin acetate, lupeol acetate, β-sitosterol, stigmasterol, triglycerides, fatty alcohols

INTRODUCTION

Cycas, the only currently known genus of the Family Cycadaceae, are considered as fossil plants[1]. Their long existence and persistence through time have sparked special interest in their biology and evolution. The cycads resemble palms in morphology, thus are commonly called sago palm. These are widely distributed in the Tropics, with species found in Asia, Africa, Southeast Asia, Pacific, and Australia[2]. They also grow on volcanic, limestone, ultramafic, sandy, or even water-logged soils in grassland and forest habitats[3]. Of the eleven cycad species in the Philippines, ten are endemic to the country[4]. Except for our recent paper on the chemical constituents of *Cycas sancti-lasallei*[5], there are no reported studies on the chemical constituents of these endemic cycad species.

This study is part of our research on the chemical constituents of *Cycas* species endemic to the Philippines. In an earlier study, we reported the isolation of squalene, β-sitosterol, stigmasterol, and triglycerides from the sarcotesta; β-sitosterol, stigmasterol, triglycerides and phytyl fatty acid esters from the endotesta; β-sitosterol, stigmasterol, triglycerides, and β-sitosteryl fatty acid esters from the sclerotesta; and triglycerides and β-sitosteryl fatty acid esters from the bark of *Cycas sancti-lasallei*[5]. We report herein the isolation of pinoresinol (**1**), lariciresinol (**2**), triglycerides (**5**), and fatty alcohols (**6**) from the dichloromethane extract of *Cycas vespertilio* male cone. Mixtures of α-amyrin acetate (**3a**) and lupeol acetate (**3b**); and β-sitosterol (**4a**) and stigmasterol (**4b**)were also obtained from the dichloromethane extract.

MATERIALS AND METHODS

General experimental procedure

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Plant material

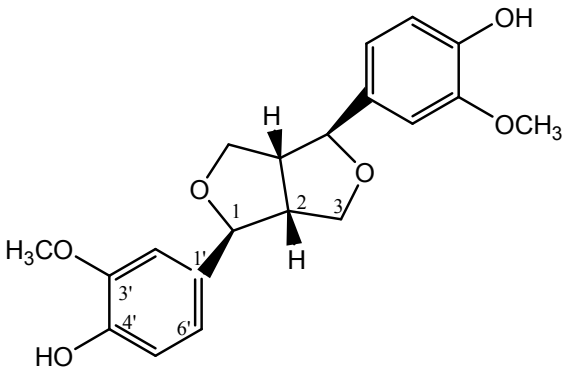
Cycas vespertilio A. Lindstr. & K. D. Hill male cone were collected from Iloilo, Panay Island, Philippines in April 2013. Voucher specimens were collected and authenticated by one of the authors (EMGA) and deposited in the De La Salle University-Manila Herbarium (DLSUH 3112).

General isolation procedure

A glass column 18 inches in height and 1.0 inch internal diameter was used for the chromatography of the crude extracts. Twenty milliliter fractions were collected. All fractions were monitored by thin layer chromatography. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography of smaller fractions from the first column. Five milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation of the chemical constituents of the male cone

**Author for Correspondence*



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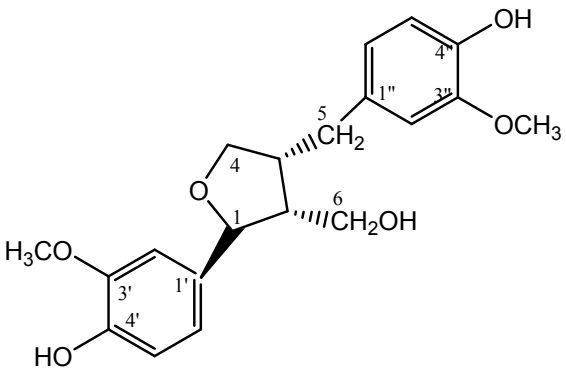

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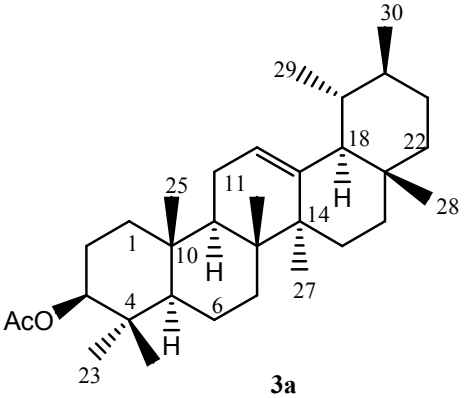
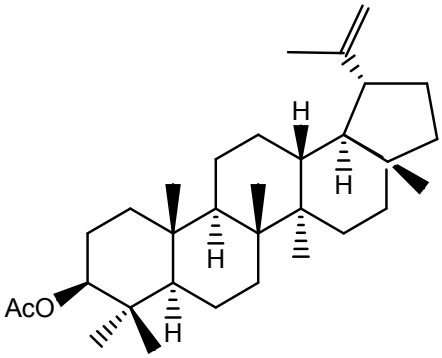
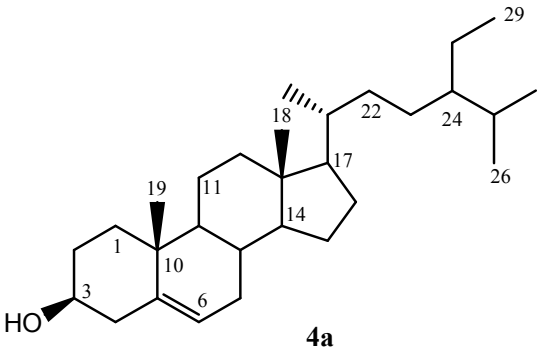


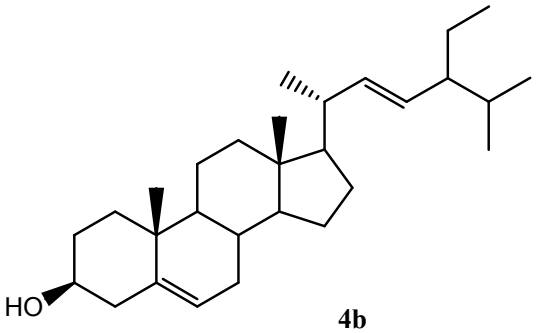
3a



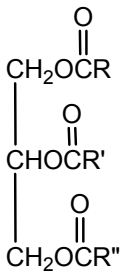
3b



4a



4b



5 R = R' = R" = long chain fatty acids

The air-dried *C. vespertilio* male cone (85.0 g) was ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (0.8 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 10% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) in 7.5% EtOAc in petroleum ether to yield **5**(5 mg). The 20% acetone in CH_2Cl_2 fraction

was rechromatographed in 5% EtOAc in petroleum ether. The less polar fractions were combined and rechromatographed (3 ×) in 5% EtOAc in petroleum ether to afford a mixture of **3a** and **3b**(4 mg) after washing with petroleum ether. The more polar fractions were combined and rechromatographed (2 ×) in 10% EtOAc in petroleum ether to afford **6**(4 mg) after washing with petroleum ether. The 40% acetone in CH_2Cl_2 fraction was rechromatographed in 20% EtOAc in petroleum ether to yield a mixture of **4a** and **4b** (5 mg) after washing with petroleum ether. The 80% acetone in CH_2Cl_2fraction was rechromatographed (4 ×) in CH_3CN:Et_2O:CH_2Cl_2 (1:1:8 by volume) to yield **1**(9 mg) after trituration with petroleum ether. The acetone fraction was rechromatographed (5×) in CH_3CN:Et_2O:CH_2Cl_2 (2:2:6, v/v) to yield **2** (8 mg) after trituration with petroleum ether.

Pinoresinol (**1**):^{13}C NMR (150 MHz, $CDCl_3$): δ 85.86 (C-1), 54.15 (C-2), 71.66 (C-3), 132.90 (C-1'), 108.56 (C-2'), 146.68 (C-3'), 145.22 (C-4'), 114.24 (C-5'), 118.95 (C-6'), 55.94 (3'-OCH_3).

Lariciresinol (**2**):^{13}C NMR (150 MHz, $CDCl_3$): δ 82.81 (C-1), 52.61 (C-2), 42.41 (C-3), 72.90 (C-4), 33.35 (C-5), 60.96 (C-6), 134.77 (C-1'), 108.25 (C-2'), 146.61 (C-3'), 145.02 (C-4'), 114.14 (C-5'), 118.75 (C-6'), 132.27(C-1"), 121.19 (C-2"), 146.49 (C-3"), 143.98 (C-4"), 114.38 (C-5"), 111.16 (C-6"), 55.93, 55.91 (2 × OCH_3).

α-Amyrin acetate(**3a**):^{1}H NMR (600 MHz, $CDCl_3$): δ ^{1}H NMR d: 0.80 (6H, s), 0.83 (3H, s), 0.89 (6H, s), 0.90(3H, s), 0.99 (3H, s), 1.00 (3H, s), 2.03 (3H, s), 4.49 (1H, m, H-3), 5.10 (1H, t, *J*=3.6 Hz, H-12).

Lupeol acetate **(3b):** ^{1}H NMR ($CDCl_3$, 600 MHz):δ4.67 (1H, brs, H-29b), 4.55 (1H,brs, H-29a), 4.45 (1H,dd, *J*= 5, 13 Hz, H-3), 2.02 (3H, s, H-2 of OAc), 1.69 (3H, s, H-30), 1.05 (3H, s, H-25) 0.94 (3H, s, H-28), 0.86 (3H, s, H-23),0.85 (3H, s, H-24), 0.83 (3H, s, H-26), 0.78 (3H, s, H-27).

β-Sitosterol (**4a**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.51 (1H, m, H-3), 2.26, 2.21 (2H, H-4), 5.33 (1H, dd, *J*=5.4, 1.8 Hz, H-6), 0.66 (3H, s, H-18), 0.99 (3H, s, H-19), 0.90 (3H, d, *J*=6.6 Hz, H-21), 0.79 (3H, d, *J*=6.6 Hz, H-26), 0.82 (3H, d, *J*=7.2 Hz, H-27), 0.85 (3H, t, *J*=7.2 Hz, H-29).

Stigmasterol (**4b**): ^{1}H NMR ($CDCl_3$, 600 MHz): δ 0.68, 0.79, 0.82, 0.86, 0.91, 1.01 (each 3H, s, Me × 6), 3.51 (m, H-3), 5.33 (dd, *J* = 5.4, 1.8 Hz, H-6), 5.14 (dd, *J*=9.0, 15.0 Hz, H-22), 5.01 (dd, *J*=9.0 Hz, 15.0 Hz, H-23).

Triacylglycerols (**5**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 62.09 (glyceryl CH_2), 68.86 (glyceryl CH), 173.30 (C=O β), 172.85, 172.84 (C=O α), 34.02, 34.05, 34.19 (C-2), 24.83, 24.86 (C-3), 27.17, 27.19, 27.22 (allylic CH_2), 25.62, 25.65, 29.05, 29.08, 29.12, 29.13, 29.17, 29.20, 29.27, 29.32, 29.34, 29.35, 29.36, 29.48, 29.52, 29.57, 29.62, 29.63, 29.66, 29.70, 29.76, 31.52, 31.90, 31.92$(CH_2)_n$, 127.88–130.23 (127.88, 127.89, 128.06, 128.07, 129.68, 129.98, 130.01, 130.02, 130.23, CH=), 14.07, 14.12 (terminal CH_3).

Fatty alcohols (**6**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.32 (t, *J* = 4.8 Hz, =CH), 3.62 (t, *J*=6.6 Hz, terminal CH_2OH), 2.00 (allylic CH_2), 1.56 (m, α-CH_2), 1.23-1.34 (br s, CH_2), 0.86 (t, *J*=7.2 Hz, terminal CH_3).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of *Cycas vespertilio* male cone afforded **1-6**. The structures of **1** and **2** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{13}C NMR data with those reported in the literature for pinoresinol (**1**)[6] and lariciresinol (**2**)[6]. The structures of **3a-6** were identified by comparison of their NMR data with those reported in the literature for α-amyrin acetate (**3a**)[7], lupeol acetate (**3b**)[8], β-sitosterol (**4a**)[9],stigmasterol (**4b**)[8], triglycerides (**5**)[10], and fatty alcohols (**6**)[11]. The 2.5:1 ratio for the mixture of **3a** and **3b** was deduced from the ^{1}H NMR integrations for the olefinic protons at δ 5.10 for **3a** and δ 4.67 and 4.55 for **3b**. The 2:1 ratio for the mixture of **4a** and **4b** was deduced from the ^{1}H NMR integrations for the olefinic protons at δ 5.33 for **4a** and δ 5.33, 5.14 and 5.01 for **4b**.

Although no biological activity tests were conducted on the isolated compounds (**1-6**), a literature search of these compounds revealed thatthese have diverse bioactivities. Pinoresinol (**1**) was found to have antioxidant and Ca^{2+} antagonist properties[12]. It was reported to exhibit strong antiinflammatory properties by acting on the NF-κB signaling pathway[13]. Furthermore, **1** attenuates inflammatory responses of microglia and could be useful in modulation of inflammatory status in brain disorders[14].Lignan **1**was shown to possess fungicidal activities and therapeutic potential as an antifungal agent for the treatment of fungal infectious diseases in humans[15]. It exhibited inhibitory activity against rat intestinal maltase with an IC_{50} value of 34.3 μM[16].

Lariciresinol (**2**) inhibited the tumor growth and tumor angiogenesis. In MCF-7 xenografts, **2** enhanced tumor cell apoptosis and increased estrogen receptor beta expression, indicating the importance of **2** in theinhibition of breast cancer development[17]. Furthermore, **2** inhibited lipid peroxidation and is a good scavenger of superoxide radicals[18]. Lignan **2** was also reported to possess fungicidal activities by disrupting the fungal plasma membrane and therapeutic potential as a novel antifungal agent for the treatment of fungal infectious diseases in humans[19].

The oral administration of α-amyrin acetate (**3a**)significantly improved the diabetic condition in streptozotocin-induced diabetic rats and in model type 2 diabetic mice at 50 mg/kg^{-1} dose level[20]. Another study reported that **3a** showed sedative, anxiolytic and anticonvulsant properties[21]. On the other hand lupeol acetate (**3b**) was reported to exhibit a strong antimicrobial effect against gram positive organisms, but no activity against gram negative bacteria and fungi[22,23].

β-Sitosterol (**4a**) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells[24]. It was shown to be effective for the treatment of benign prostatic hyperplasia[25]. It was also reported to attenuate β-catenin and PCNA expression, as well as quench radical *in vitro*, making it a potential anticancer drug for colon carcinogenesis[26]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake[27]. It was reported to induce apoptosis mediated by the activation of ERK and the

downregulation of Akt in MCA-102 murine fibrosarcoma cells[28]. Stigmasterol (**4b**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions[29]. It lowers plasma cholesterol levels, inhibits intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Winstar as well as WKY rats[30]. Other studies reported that stigmasterol showed cytostatic activity against Hep-2 and McCoy cells[31], markedly inhibited tumour promotion in two stage carcinogenesis experiments[32], exhibited antimutagenic[33], topical anti-inflammatory[34], antiosteoarthritic[35] and antioxidant[36] activities.

Triacylglycerides (**5**) exhibited antimicrobial activity against *S. aureus, P. aeruginosa, B. subtilis, C. albicans,*and *T. mentagrophytes*[37]. Another study reported that triglycerides showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation[38].On the other hand, long-chain fatty alcohols(**6**)was reported to exhibit a protective effect on some mediators involved in the inflammatory damage development[39].

CONCLUSION

The dichloromethane extracts of *Cycas vespertilio*, a plant endemic to the Philippines, affordedpinoresinol (**1**), lariciresinol (**2**), α-amyrin acetate (**3a**), lupeol acetate (**3b**), β-sitosterol (**4a**), stigmasterol (**4b**), triglycerides (**5**), and fatty alcohols (**6**). Compounds **1-6** were reported to exhibit diverse biological activities.

ACKNOWLEDGEMENT

A research grant from the Commission on Higher Education –Philippine Higher Education Research Network (CHED–PHERNet) of the Philippines is gratefully acknowledged.

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