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Chemical constituents of *Cycas aenigma*

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ABSTRACT

Chemical investigation of *Cycas aenigma*, a plant endemic to the Philippines, led to the isolation of a rare neolignan, 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol (**1**), pinoresinol (**2**), and fatty alcohols (**3**) from the leaflets; and triglycerols (**4**), and a mixture of β-sitosterol (**5a**) and stigmasterol (**5b**) from the petiole and rachis. The structure of **1** was elucidated by extensive 1D and 2D NMR spectroscopy, while those of **2-5b** were identified by comparison of their ^{1}H and/or ^{13}C NMR data with literature data.

INTRODUCTION

Cycas resemble palms in morphology and are commonly called sago palm. They are considered as fossil plants though they may have evolved only about 12 million years ago (Nagalingum *et al.*, 2011). They are widely distributed in the Tropics (Donaldson, 2003) where they grow on volcanic, limestone, ultramafic, sandy, or even water-logged soils in grassland and forest habitats (Madulid and Agoo, 2009). The demand of *Cycas* species for domestic and international horticultural trade, grassland and forest fires, and conversion of their natural habitats to settlements and other land uses have threatened to varying degrees the wild populations of the genus (IUCN, 2010). Some of these threatened species are *C. curranii* (Agoo *et al.*, 2010), *C. wadei* (Hill, 2010) and *C. zambalensis* as Critically Endangered (CR) (Agoo *et al.*, 2010), *C. riuminiana* as Endangered (E) (Agoo *et al.*, 2010), and *C. saxatilis* as Vulnerable (V) (Bosenberg JD. 2010). There are no reported chemical and biological activity studies on *C. aenigma*. However, some *Cycas* species have been studied for their chemical constituents and biological activities. Cycasin, a carcinogenic toxin was isolated from the most studied *Cycas* species, *C. revoluta* Thunb. and *C. circinalis* L. (Nishida *et al.*, 1955; Laqueur *et al.*, 1963). Biflavonoids, lignans, flavan-3-ols, flavone-C-glucosides, nor-isoprenoids, and a flavanone were obtained from the methanolic extract of the leaflets of *C. circinalis* L. and the chloroform extract of *C. revoluta* Thunb. Three of the biflavonoids exhibited moderate activity against *S. aureus* and methicillin-resistant *S.* aureus (Moawad *et al.*, 2010). Moreover, the leaves of *C. revoluta* Thunb. and *C. circinalis* L. yielded lariciresinol, naringenin and biflavonoids (Ferreira *et al*, 2009).

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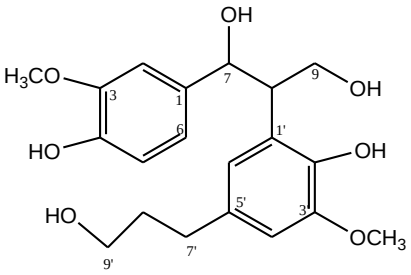
β-Sitosterol β-D-glucoside, stigmasterol β-D-glucoside, β-sitosterol, and stigmasterol were obtained from the seeds of *C. micronesica* K. D. Hill (Marler *et al.,* 2006), while *C. beddomei* afforded a new biflavonoid, along with pinoresinol, hinokiflavone, and amento flavones (Das *et al.,* 2006; Das *et al.,* 2005). The leaves of *C. panzhihuaensis* yielded a new flavone, along with 2,3-dihydrohinokiflavone, a biflavone, vanillic acid, sitosterol and daucosterol (Zhou *et al.,* 2002). Chavicol β-rutinoside, amentoflavone, podocarpusflavone A, a biflavone, β-sitosterol, daucosterol and palmitic acid were isolated from the methanolic extracts of the stems, flowers and seeds of *C. panzhihuaensis* L. (Zhou *et al.,* 1999). This study is part of our research on the chemical constituents of the genus *Cycas*. We earlier 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, and triglycerides, and β-sitosteryl fatty acid esters from the sclerotesta; and β-sitosteryl fatty acid esters from the bark of *Cycas sancti-lasallei* (Ng *et al.,* 2015). We report herein the isolation of 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3 methoxyphenyl) propane-1,3-diol (**1**), pinoresinol (**2**), and fatty alcohol (**3**) from the leaflets; andtriglycerols (**4**), and a mixture of β-sitosterol (**5a**) and stigmasterol (**5b**) from the petiole and rachis. To the best of our knowledge this is the first report on the isolation of these compounds from *Cycas aenigma*.

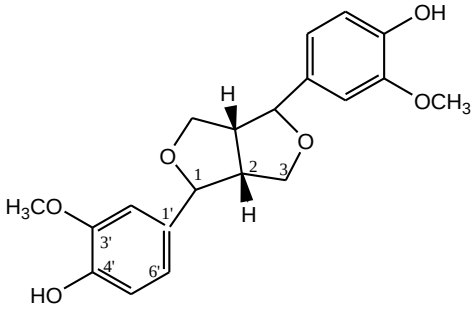
MATERIALS AND METHODS

General experimental procedure

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H 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.



1



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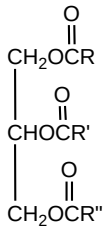
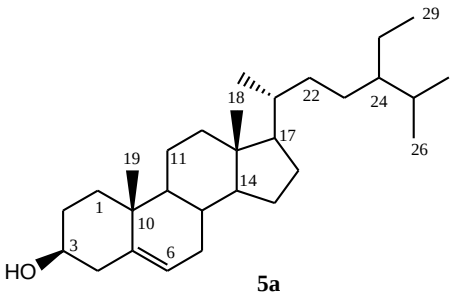
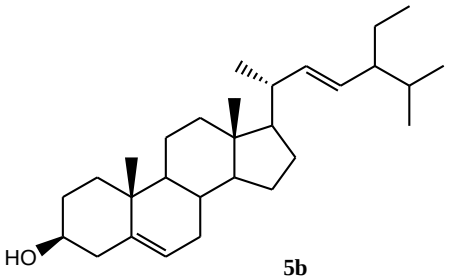


4 R = R' = R" = long chain fatty acids



5a



5b

Plant material

Cycas aenigma leaflets and petiole and rachis were collected in 2013. Voucher specimens were collected and authenticated by one of the authors (EMGA) and deposited in the De La Salle University-Manila Herbarium (DLSUH).

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 leaflets

The air-dried bark of *C. aenigma* (105 g) were 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 (2.3 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 20% increment. The 20% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using 15% EtOAc in petroleum ether to afford **3** (3 mg) after washing with petroleum ether.

The 80% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) in CH_3CN:Et_2O:CH_2Cl_2 (1:1:8 by volume) to yield **2** (9 mg) after trituration with petroleum ether. The 90% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using CH_3CN:Et_2O:CH_2Cl_2 (2.5:2.5:5, v/v) to afford **1** (12 mg) after trituration with petroleum ether.

Isolation of the chemical constituents of the petiole and rachis

The air-dried petiole and rachis of *C. aenigma* (47 g) were 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 (0.7 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 20% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using 7.5% EtOAc in petroleum ether to yield **4** (8 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed using 15% EtOAc in petroleum ether to afford a mixture of **5a** and **5b** in a 1:1 ratio (4 mg) after washing with petroleum ether.

2-[2-Hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane -1,3-diol **(1)**

^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.92 (1H, d, *J* = 1.8 Hz, H-2), 6.86 (1H, d, *J* = 8.5 Hz, H-5), 6.90 (1H, dd, *J* = 7.8, 1.8 Hz, H-6), 5.53 (1H, d, *J* = 7.2 Hz, H-7), 3.59 (1H, m, H-8), 3.89 (1H, dd, *J* = 10.8, 4.8Hz, H-9), 3.95 (1H, dd, *J* = 10.8, 6 Hz, H-9), 6.66 (1H, s, H-4'), 6.67 (1H, s, H-6'), 2.66 (2H, t, *J* = 7.2 Hz, CH_2-7'), 1.88 (2H, m, CH_2-8'), 3.68 (2H, t, *J* = 6.6 Hz, CH_2-9'), 3.85 (3H, s, 3-OCH_3), 3.87 (3H, s, 3'-OCH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 133.08 (C-1), 108.79 (C-2),146.62 (C-3), 145.61 (C-4), 114.25 (C-5), 119.43 (C-6), 87.88 (C-7), 53.79 (C-8), 63.91 (C-9), 127.66 (C-1'), 146.56 (C-2'), 144.20 (C-3'), 115.89 (C-4'), 135.41 (C-5'), 112.43 (C-6'), 32.01 (C-7'), 34.64 (C-8'), 62.32 (C-9'), 55.98, 55.99 (3-OCH_3, 3'-OCH_3).

Pinoresinol **(2)**

^{1}H NMR (600 MHz, $CDCl_3$): δ 4.72 (1H, d, *J* = 4.2 Hz, H-1), 3.08 (1H, dd, *J* = 4.8, 6.6 Hz, H-2), 3.86 (1H, dd, *J* = 3.6, 9.0 Hz, H-3), 4.25 (1H, dd, *J* = 7.2, 9.0 Hz, H-3), 6.89 (1H, d, *J* = 1.8, H-2'), 6.89 (1H, d, *J* = 6.6, H-5'), 6.82 (1H, dd, *J* = 7.8, 1.8 Hz, H-6'), 3.91 (3H, s, -OCH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 85.86 (C-1), 54.16 (C-2), 71.66 (C-3), 132.91 (C-1'), 108.56 (C-2'), 146.68 (C-3'), 145.22 (C-4'), 114.23 (C-5'), 118.96 (C-6'), 55.95 (3'-OCH_3).

Fatty Alcohols **(3)**

^{1}H NMR (600 MHz, $CDCl_3$): δ 3.56 (t, *J* = 7.2 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); ^{13}C NMR (150 MHz, $CDCl_3$): δ 72.04 (C-1), 37.48, 31.92, 31.89, 29.70, 29.68, 29.66, 29.63, 29.62, 29.57, 29.36, 29.32, 25.64, 22.69, 22.68 $(CH_2)_n$, 14.12 (CH_3).

Triacylglycerols **(4)**

^{1}H NMR (600 MHz, $CDCl_3$): δ 4.28 (2H, dd, *J* = 4.2, 12.0 Hz, glyceryl CH_2O), 4.12 (2H, dd, *J* = 6.0, 12.0 Hz, glyceryl CH_2O), 5.32 (1H, m, glyceryl CHO), 2.31 (6H, t, *J* = 7.5Hz, α-CH_2), 5.33 (m, olefinic H), 2.75 (double allylic CH_2), 1.98-2.05 (allylic, CH_2), 1.23-1.35 (CH_2), 0.87 (t, *J* = 6.6 Hz, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 62.09 (glyceryl CH_2), 68.87 (glyceryl CH), 173.26, 173.30 (C = O α), 172.84 (C = O β), 34.02, 34.05, 34.19 (C-2), 24.83, 24.86 (C-3), 29.05, 29.08, 29.12 (C-4), 29.18, 29.20, 29.27 (C-5), 29.48 (C-6), 22.57, 22.69 (C-8), 130.23, 130.01, 129.70 (C-9), 127.89, 128.06, 129.68 (C-10), 25.62, 27.17, 27.19, 27.22, 29.32, 29.34, 29.36, 29.52, 29.62, 29.66, 29.70, 29.76 (CH_2), 31.52, 31.90, 31.92 (CH_2), 14.07, 14.12 (terminal CH_3).

β-Sitosterol **(5a)**

^{13}C NMR (150 MHz, $CDCl_3$): δ 37.2 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9(C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8(C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 12.0 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.0 (C-26), 19.8 (C-27), 23.0 (C-28), 11.9 (C-29).

Stigmasterol **(5b)**

^{13}C NMR (150 MHz, $CDCl_3$): δ 37.2 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-

13), 56.8 (C-14), 24.3 (C-15), 29.1 (C-16), 56.0 (C-17), 12.0 (C-18), 19.4 (C-19), 40.5 (C-20), 21.1 (C-21), 138.3 (C-22), 129.3 (C-23), 51.2 (C-24), 31.9 (C-25), 21.1 (C-26), 19.0 (C-27), 25.4 (C-28), 12.1 (C-29).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extracts of *C. aenigma* yielded **1**-**3** from the leaves; and **4**-**5b** from the petiole and rachis. The structure of **1** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of its NMR data with those reported in the literature for 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol (**1**) (Koyama *et al.*, 2006). Compounds **2**-**5b** were identified by comparison of their NMR data with those reported in the literature for pinoresinol (**2**) (Ragasa *et al.*, 2000), fatty alcohols (**3**) (Ragasa *et al.*, 2014a), triglycerides (**4**) (Ragasa *et al.*, 2015), β-sitosterol (**5a**) (Ragasa *et al.*, 2014b), and stigmasterol (**5b**) (Jamal *et al.*, 2008).

2-[2-Hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol (**1**) was first reported as a constituent of *Taxus yunnanensis* (Koyama *et al.*, 2008). This neolignan was tested for inhibitory effects on induced histamine release from the human basophilic cell line, KU812. Results showed that **1** did not exhibit any antiallergic activity at a 1.5 μg/ml concentration (Koyama *et al.*, 2008). On the other hand, pinoresinol (**2**) was found to have antioxidant and Ca^{2+} antagonist properties (Páska *et al.*, 2002). It was reported to exhibit strong antiinflammatory properties by acting on the NF-κB signaling pathway (During *et al*, 2012). Furthermore, **2** attenuates inflammatory responses of microglia and could be useful in modulation of inflammatory status in brain disorders (Jung *et al.*, 2010). Lignan **2** was shown to possess fungicidal activities and therapeutic potential as an antifungal agent for the treatment of fungal infectious diseases in humans (Hwang *et al.*, 2010). It exhibited inhibitory activity against rat intestinal maltase with an IC_{50} value of 34.3 μM (Wikul *et al.*, 2012). Long-chain fatty alcohols (**3**) were reported to exhibit a protective effect on some mediators involved in the inflammatory damage development (Fernandez-Arche *et al.*, 2015). On the other hand, triacylglycerides (**4**) exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans*, and *T. mentagrophytes* (Ragasa *et al.*, 2013). Another study reported that **4** showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation (Ferruzzi and Blakeslee, 2007). β-Sitosterol (**5a**) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells (Awad *et al.*, 2007). It was shown to be effective for the treatment of benign prostatic hyperplasia (Jayaprakasha *et al.*, 2007). Compound **5a** 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 (Baskar *et al.*, 2010). It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake (Jesch *et al.*, 2009). It was reported to induce apoptosis mediated by the activation of ERK and the down-regulation of Akt in MCA-102 murine fibrosarcoma cells (Moon *et al.*, 2007). On the other hand, stigmasterol (**5b**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions (Ghosh *et al.*, 2011). Compound **5b** 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 (Batta *et al.*, 2006). Other studies reported that **5b** showed cytostatic activity against Hep-2 and McCoy cells (Gómez *et al.*, 2001), markedly inhibited tumour promotion in two stage carcinogenesis experiments (Kasahara *et al.*, 1994), and exhibited antimutagenic (Lim *et al.*, 2005), topical anti-inflammatory (García *et al.*, 1999), antiosteoarthritic (Gabay *et al.*, 2010) and antioxidant (Panda *et al.*, 2009) activities.

CONCLUSION

The dichloromethane extracts of *Cycas aenigma*, a plant endemic to the Philippines with no reported chemical and biological activity studies, afforded a rare neolignan, 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol(**1**), pinoresinol (**2**), fatty alcohols (**3**),triacylglycerols (**4**), β-sitosterol (**5a**), and stigmasterol (**5b**). Compounds **2**-**5b** were reported to exhibit diverse biological activities.

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