Journal of Applied Pharmaceutical Science Vol. 5 (Suppl 1), pp. 012-017, May, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.54.S3
ISSN 2231-3354

Chemical Constituents of *Cycas sancti-lasallei*

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ARTICLE INFO

Article history:
Received on: 22/02/2015
Revised on: 16/03/2015
Accepted on: 09/04/2015
Available online: 15/05/2015

Key words:
Cycas sancti-lasallei, Cycadaceae, squalene, β-sitosterol, stigmasterol, triglycerides, phytyl fatty acid esters, β-sitosteryl fatty acid esters.

ABSTRACT

Chemical investigation of the dichloromethane extracts of *Cycas sancti-lasallei*, a plant endemic to the Philippines, led to the isolation of squalene (**1**), β-sitosterol (**2a**), stigmasterol (**2b**), and triglycerides (**3**) from the sarcotesta; **2a**, **2b**, **3**,and phytyl fatty acid esters (**4**) from the endotesta; **2a**, **2b**, **3**,and β-sitosteryl fatty acid esters (**5**) from the sclerotesta; and **3** and **5** from the bark. The structures of **1**-**5** were identified by comparison of their ^{1}H NMR and/or ^{13}C NMR data with those reported in the literature.

INTRODUCTION

Cycas, the only currently known genus of the Family Cycadaceae, are considered as fossil plants though they may have evolved only about 12 million years ago (Nagalingum *et al.*, 2011). The cycads resemble palms in morphology and are commonly called sago palm. These are widely distributed in the Tropics, with species found in Asia, Africa, Southeast Asia, Pacific, and Australia (Donaldson, 2013). They also grow on volcanic, limestone, ultramafic, sandy, or even water-logged soils in grassland and forest habitats (Madulid and Agoo, 2009). In the Philippines, there are eleven cycad species namely, *C. aenigma* K. D. Hill & Lindstrom, *C. curranii* (J. Schust.) K. D. Hill, *C. edentata* de Laub., *C. lacrimans* Lindstrom & K. D. Hill, *C. nitida* K. D. Hill & Lindstrom, *C. riuminiana* Porte ex Regel, *C. saxatilis* K. D. Hill & Lindstrom, *C. sancti-lasallei* Agoo & Madulid, *C. wadei* Merr., *C. vespertilio* Lindstrom & K. D. Hill, and *C. zambalensis* Madulid & Agoo (Madulid and Agoo, 2009; Lindstrom et al., 2008; Agoo and Madulid, 2012). All species, except for *C. edentata*, are endemic to the Philippines (Lindstrom *et al.*, 2008). *C. revoluta*, a widely cultivated species, is an introduced species from Japan and Taiwan. The demand of *Cycas* species for domestic and international horticultural trade, grassland and forest fires of anthropogenic origin, 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 species which have been assessed and evaluated in the 2010 IUCN Red List of 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, 2010). *Cycas sancti-lasallei* (*Cycadaceae*) is a new species from Mindanao, Philippines (Agoo and Madulid, 2012). There are no reported chemical and biological activity studies on *C. sancti-lasallei*. However, some *Cycas* species have been studied for their chemical constituents and biological activities. The most studied *Cycas* are *Cycas revoluta* Thunb. and *C. circinalis* which contain the carcinogenic toxin cycasin (Nishida *et al.*, 1956; Laqueur *et al.*, 1963). The methanolic extract of the leaflets of *C. circinalis* L. and the chloroform extract of *C. revoluta* Thunb. yielded biflavonoids, lignans, flavan-3-ols, flavone-C-glucosides, nor-isoprenoids, and a flavanone. Three of

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the biflavonoids exhibited moderate activity against *S. aureus* and methicillin-resistant *S. aureus* (Moawad *et al*., 2010). Further studies on the chemical constituents of the leaves of *C. revoluta* Thunb. and *C. circinalis* L. afforded lariciresinol, naringenin and biflavonoids which are derivatives of amentoflavone and hinokiflavone (Ferreira *et al*., 2009). Studies on other *Cycas* species have also been conducted. The seeds of *C. micronesica* K. D. Hill yielded β-sitosterol β-D-glucoside, stigmasterol β-D-glucoside, β-sitosterol, and stigmasterol (Marler *et al*., 2006). *C. beddomei* afforded a new biflavonoid, along with pinoresinol, hinokiflavone, and amento flavones (Das *et al*., 2006, 2005). The leaves of *C. panzhihuaensis* afforded a new flavone, along with 2,3-dihydrohinokiflavone, a biflavone, vanillic acid, sitosterol and daucosterol (Zhou *et al*., 2002). The methanolic extracts of the stems, flowers and seeds of *C. panzhihuaensis* L. yielded chavicol β-rutinoside, amentoflavone, podocarpusflavone A, a biflavone, β-sitosterol, daucosterol and palmitic acid (Zhou *et al*., 2009).

We report herein the isolation and identification of squalene (**1**), β-sitosterol (**2a**), stigmasterol (**2b**), and triglycerides (**3**) from the sarcotesta; **2a**, **2b**, **3**, and phytyl fatty acid esters (**4**) from the endotesta; **2a**, **2b**, **3**, and β-sitosteryl fatty acid esters (**5**) from the sclerotesta; and **3** and **5** from the bark (Fig. 1) of *C. sancti-lasallei*. To the best of our knowledge this is the first report on the isolation of these compounds from *C. sancti-lasallei*.

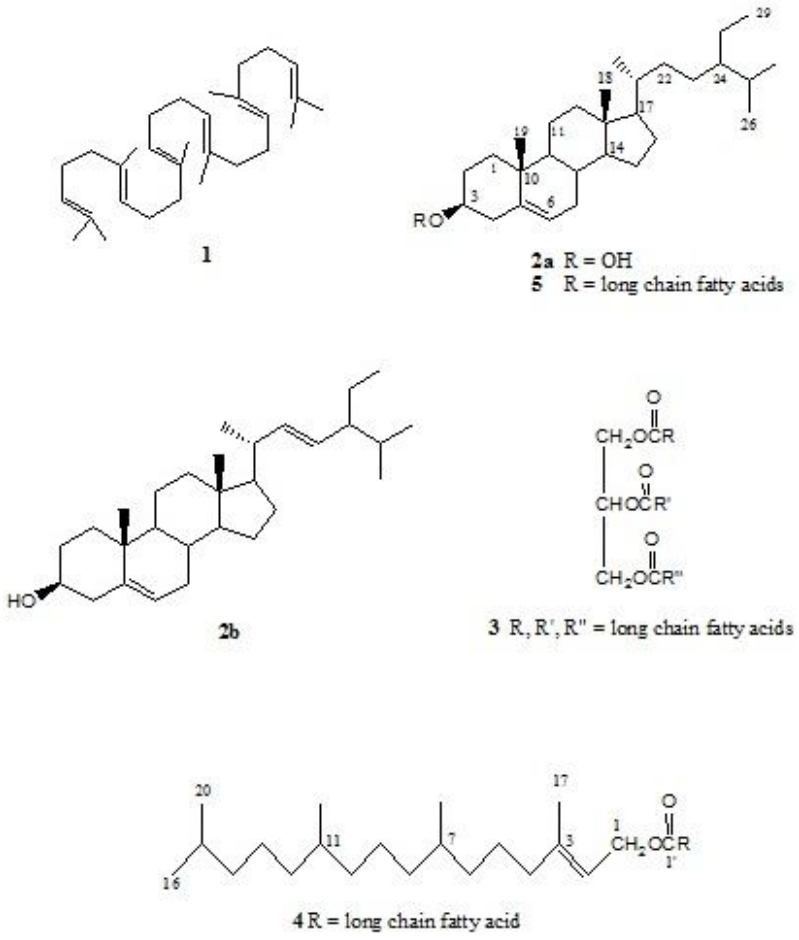

Fig. Chemical constituents of cycas sancti-lasallei: squalene (1), β-sitosterol (2a), stigmasterol (2b), triglycerides (3), phytyl fatty acid esters (4), and β-sitosteryl fatty acid esters (5).

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.

Sample Collection

Cycas sancti-lasallei endotesta, sarcotesta, and bark were collected from Malasag, Cugman, Cagayan de Oro, Misamis Oriental, Philippines on April 25, 2014. Voucher specimens were collected and authenticated by one of the authors (EMGA) and deposited in the De La Salle University-Manila Herbarium (DLSUH 3116).

General Isolation Procedure

A glass column 18 inches in height and 1.0 inch internal diameter was packed with silica gel. The crude extracts were fractionated by silica gel chromatography using increasing proportions of acetone in CH_2Cl_2 (10% increment) as eluents. Fifty 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. Two milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation

The freeze-dried sarcotesta of *C. sancti-lasallei* (218 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 (0.7 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (3 ×) using 1% EtOAc in petroleum ether to afford **1** (2 mg). The 20% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) in 7.5% EtOAc using petroleum ether to afford **3** (7 mg). The 40% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using 20% EtOAc in petroleum ether to afford a mixture of **2a** and **2b** after washing with petroleum ether (5 mg).

The freeze-dried endotesta of *C. sancti-lasallei* (273.5 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 (11.3 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (4 ×) using 2.5% EtOAc in petroleum ether to afford **4** (2mg). The 20% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 7.5% EtOAc in petroleum ether to afford **3** (9mg). The 40% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using 20% EtOAc in petroleum ether to afford a mixture of **2a** and **2b** after washing with petroleum ether (6 mg).

The air-dried sclerotesta of *C. sancti-lasallei* (166 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 (0.2 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (3 ×) using 5% EtOAc in petroleum ether to afford **5** (4 mg). The 20% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using7.5% EtOAc in petroleum ether to afford **3** (7 mg). The 40% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 20% EtOAc in petroleum ether to afford a mixture of **2a** and **2b** (6 mg) after washing with petroleum ether.

The air-dried bark of *C. sancti-lasallei* (98 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 (1.0 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (3 ×) using 2.5% EtOAc in petroleum ether to afford **5** (5 mg). The 20% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using 7.5% EtOAc in petroleum ether to afford **3** (8 mg).

Squalene (**1**)

colorless oil. ^1H NMR (600 MHz, $CDCl_3$): δ 5.08-5.13 (6H, =CH), 1.58 (18H, allylic CH_3, *cis*), 1.66 (6H, allylic CH_3, *trans*), 1.94-2.08 (20H, allylic CH_2).

β-Sitosterol (**2a**)

colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ37.24 (C-1), 31.66 (C-2), 71.81 (C-3), 42.31 (C-4), 140.75 (C-5), 121.72 (C-6), 31.90, 31.89 (C-7, C-8), 50.12 (C-9), 36.14 (C-10), 21.07 (C-11), 39.76 (C-12), 42.31 (C-13), 56.75 (C-14), 24.30 (C-15), 28.24 (C-16), 56.04 (C-17), 11.85 (C-18), 19.39 (C-19), 36.49 (C-20), 19.02 (C-21), 33.93 (C-22), 29.13 (C-23), 45.82 (C-24), 26.04 (C-25), 18.77 (C-26), 19.81 (C-27), 23.05 (C-28), 11.97 (C-29).

Stigmasterol (**2b**)

colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.24 (C-1), 31.66 (C-2), 71.81 (C-3), 42.30 (C-4), 140.75 (C-5), 121.72 (C-6), 31.89, 31.90 (C-7, C-8), 50.12 (C-9), 36.49 (C-10), 21.07 (C-11), 39.67 (C-12), 42.20 (C-13), 56.75 (C-14), 24.35 (C-15), 28.91 (C-16), 55.94 (C-17), 12.04 (C-18), 19.39 (C-19), 40.49 (C-20), 21.07 (C-21), 138.31(C-22), 129.26 (C-23), 51.23 (C-24), 31.89 (C-25), 21.20 (C-26), 18.97 (C-27), 25.40 (C-28), 12.25 (C-29).

Triacylglycerols (**3**)

colorless oil. ^1H NMR (600 MHz, $CDCl_3$): δ 4.27 (dd, J = 4.2, 12.0 Hz, glyceryl CH_2O), 4.12 (dd, J = 6.0, 12.0 Hz, glyceryl CH_2O), 5.24 (m, glyceryl CHO), 2.29 (t, J = 7.2 Hz, α-CH_2), 5.35 (m, olefinic H), 2.75 (t, J = 6.6 Hz, double allylic CH_2), 1.97-2.04 (allylic, CH_2), 1.56-1.60 (β-CH_2), 1.23-1.35 (CH_2), 0.96

(t, *J* = 7.2 Hz, CH_3), 0.86 (t, *J* = 6.6 Hz, CH_3), 0.87 (t, *J* = 6.6 Hz, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ 62.08 (glyceryl CH_2), 68.85 (glyceryl CH), 173.30, 173.26 (C=O α), 172.85 (C=O β), 34.01, 34.04, 34.18 (C-2), 19.20, 19.27, 22.56, 22.67, 24.83, 24.85, 24.87, 25.61, 27.16, 27.19, 27.21, 29.04, 29.08 29.11, 29.12, 29.17, 29.19, 29.27, 29.31, 29.34, 29.35, 29.47, 29.52, 29.60, 29.62, 29.65, 29.70, 29.76, 31.51, 31.90, 31.91 (CH_2), 130.23, 130.02, 130.01, 129.98, 129.71, 129.68, 128.07, 128.06, 127.89, 129.88 (CH=CH), 14.07, 14.11 (terminal CH_3).

Phytyl fatty acid ester (**4**)

colorless oil. δ 4.57 (d, *J* = 6.6 Hz, H_2-1), 5.34 (H-2), 2.00 (H_2-4), 0.87 (d, *J* = 6.6 Hz, CH_3-16), 1.67 (br s, CH_3-17), 0.85 (d, *J* = 6.6 Hz, CH_3-18), 0.83 (d, *J* = 6.6 Hz, CH_3-19), 0.87 (d, *J* = 6.6 Hz, CH_3-20), 2.27 (t, *J* = 7.8 Hz, H_2-2'), 1.60 (H_3-3'), 1.23-1.36 $(CH_2')_n$, 0.87 (t, *J* = 6.6 Hz, CH_3'-terminal).

β-Sitosteryl fatty acid esters (**5**)

colorless solid.^{13}C NMR (150 MHz, $CDCl_3$): δ 36.99 (C-1), 31.52 (C-2), 73.68 (C-3), 42.30 (C-4), 139.71 (C-5), 122.58 (C-6), 32.19, 31.92 (C-7, C-8), 50.01 (C-9), 36.15 (C-10), 21.02 (C-11), 39.71 (C-12), 42.30 (C-13), 56.68 (C-14), 24.29 (C-15), 28.24 (C-16), 56.01 (C-17), 11.84 (C-18), 19.32 (C-19), 36.59 (C-20), 19.02 (C-21), 34.05 (C-22), 29.13 (C-23), 45.82 (C-24), 26.04 (C-25), 18.76 (C-26), 19.81 (C-27), 23.05 (C-28), 11.97 (C-29),173.30 (C-1'), 34.70, 34.05 (C-2'), 29.76, 29.70, 29.65, 29.59, 29.52, 29.48, 29.36, 29.34, 29.32, 29.27, 29.16, 29.11, 29.08, 27.80, 27.21, 27.193, 27.186, 25.62, 25.04, 22.69, 22.57 (CH_2), 130.21 (CH=), 130.06 (CH=), 14.12, 14.07 (terminal CH_3).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extracts of *Cycas sancti-lasallei* led to the isolation of squalene (**1**) (Ragasa *et al*., 2014a), β-sitosterol (**2a**) (Tsai *et al*., 2012), stigmasterol (**2b**) (Ragasa *et al*., 2014b), and triglycerides (**3**) (Ragasa *et al*., 2014c) from the sarcotesta; **2a**, **2b**,**3**, and phytyl fatty acid esters (**4**) (Ragasa *et al*., 2014c) from the endotesta; **2a**, **2b**, **3**, and β-sitosteryl fatty acid esters (**5**) (Julien-David*et al*., 2008) from the sclerotesta; and **3** and **5** from the bark. The structures of **1-5** were identified by comparison of their ^{1}H NMR and/or ^{13}C NMR data with those reported in the literature. The ratios of the mixture of **2a** and **2b** were deduced from the integrations of the ^{1}H NMR resonances for the olefinic protons of **2a** at δ 5.33 (dd, *J* = 1.8, 4.8 Hz, H-6) and **2b** at δ 5.33 (dd, *J* = 1.8, 4.8 Hz, H-6), 5.13 (dd, *J* = 9.0, 15.0 Hz, H-22) and 5.00 (dd, *J* = 9.0, 15.0 Hz, H-23). Based on these integrations, the ratios of **2a** and **2b** in the sarcotesta, endotesta, and sclerotesta are 3:1, 5:1, and 2:1, respectively.

The fatty acids esterified to the glycerol in the triglycerides of endotesta are oleic acid, linoleic acid and saturated fatty acid. These were deduced from the integrations of the resonances at δ 0.87 (t, *J* = 6.6 Hz, CH_3), 1.97-2.06 (allylic CH_2), 2.74 (double allylic CH_2), and 5.30-5.37 (CH=) for the linoleic acid; δ 0.87 (t, *J* = 6.6 Hz, CH_3), 1.97-2.06 (allylic CH_2), and 5.30-5.37 (CH=) for the oleic acid; and δ 0.87 (t, *J* = 6.6 Hz, CH_3) for the saturated fatty acid. A small amount of linolenic acid was detected from the low intensity resonance at δ 0.95 (t, *J* = 7.2 Hz) for the terminal methyl and 2.76 (double allylic CH_2's) of this fatty acid (Human Metabolome, 2013). For the sarcotesta and sclerotesta triglycerides, the same fatty acids esterified to the glycerol were deduced from the integrations in the ^{1}H NMR spectra. However, larger amounts of linolenic acid were detected from the more intense resonances of the terminal methyl at δ 0.95 (t, *J* = 7.2 Hz) and the double allylic methylenes at δ 2.76.

Although bioassays were not conducted on the isolated compounds (**1**-**3**), there were previous studies that reported on their biological activities.

Squalene (**1**) was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis (Rao *et al*., 1998). It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties (Farvin *et al*., 2006). A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 haveanti-proliferative effects on breast cancer cells (Loganathan *et al*., 2013). The preventive and therapeutic potential of squalene containing compounds on tumor promotion and regression have been reported (Desai *et al*., 1996). A recent review on the bioactivities of squalene has been provided (Ronco and De Stéfani, 2013).

β-Sitosterol (**2a**) 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). 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 (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 downregulation of Akt in MCA-102 murine fibrosarcoma cells (Moon *et al.,* 2007). Stigmasterol (**2b**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions (Ghosh *et al*., 2011). 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 (Batta *et al*., 2006). Other studies reported that stigmasterol 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), exhibited antimutagenic (Lim *et al*., 2005), topical anti-inflammatory (Garcia *et al*., 1999), anti-osteoarthritic (Gabay *et al*., 2010) and antioxidant (Panda *et al*., 2009) activities. Triacylglycerides (**3**) exhibited antimicrobial activity against *S. aureus, P. aeruginosa, B. subtilis, C. albicans*, and *T. mentagrophytes* (Ragasa *et al*., 2013). Another study reported that triglycerides showed a direct relationship between

toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation (Ferruzzi and Blakeslee, 2007). The fatty acids esterified to the triglycerides were deduced to be oleic acid, linoleic acid and linolenic acid. Oleic acid has been reported to be responsible for the reduction of blood pressure induced by olive oil (Teres *et al*., 2008). It may hinder the progression of adrenoleukodystrophy, a fatal disease that affects the brain and adrenal glands (Rizzo *et al*., 1986). Oleic acid inhibited cancer cell growth and survival in low metastatic carcinoma cells, such as gastric carcinoma SGC7901 and breast carcinoma MCF-7 cell lines (Li *et al*., 2014). Linoleic acid belongs to the omega-6 fatty acids. It was reported to be a strong anticarcinogen in a number of animal models. It reduces risk of colon and breast cancer (Chan *et al*., 2002) and lowers cardiovascular disease risk and inflammations (Whelan, 2008). Linolenic acid belongs to omega-3 fatty acid. A previous study reported that α-linolenic acid (ALA) inhibited the human renal cell carcinoma (RCC) cell proliferation (Yang *et al*., 2013). Another study reported that apoptosis of hepatoma cells was induced by the α-linolenic acid enriched diet which correlated with a decrease in arachidonate content in hepatoma cells and decreased cyclooxygenase-2 expression (Vecchini *et al*., 2004). γ-Linolenic acid (GLA) and α-linolenic acid (ALA) exhibited greater than 90% cytotoxicity between 500 μM and 1 mM against all but two malignant microorgan cultures tested in 5-10% serum. GLA and ALA killed tumor at concentrations of 2 mM and above in tests using 30-40% serum (Scheim, 2009).

CONCLUSION

The dichloromethane extracts of *Cycas sancti-lasallei*, a plant endemic to the Philippines, afforded squalene (**1**), β-sitosterol (**2a**), stigmasterol (**2b**), triglycerides (**3**), phytyl fatty acid esters (**4**), and β-sitosteryl fatty acid esters (**5**). Compounds **1**-**3** were reported to exhibit diverse biological activities, such as anticancer properties.

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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How to cite this article:

Vincent Antonio Ng, Esperanza Maribel Agoo, Chien-Chang Shen, consolacion Ragasa. Chemical Constituents of Cycas sancti-lasallei. J App Pharm Sci, 2015; 5 (Suppl 1): 012-017.