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Terpenoids and Sterols from *Hoya multiflora* Blume

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ABSTRACT

Chemical investigation of the dichloromethane extracts of *Hoya multiflora* Blume led to the isolation of lupeol (**1a**), α-amyrin (**1b**), β-amyrin (**1c**), lupeol acetate (**2a**), α-amyrin acetate (**2b**), and β-amyrin acetate (**2c**) from the stems; and **1b**, bauerenol (**3**), squalene (**4**), lutein (**5**), β-sitosterol (**6a**), and stigmasterol (**6b**)from the leaves. The structures of **1-6** were identified by comparison of their ^{1}H and/or^{13}C NMR data with those reported in the literature.

INTRODUCTION

Hoya plants are also called wax plants due to the waxy appearance of their leaves or flowers. There are at least 109 species of *Hoya* found in the Philippines, 88 of these are endemic to the country (Aurigue, 2013). The *Hoya multiflora*, also called shooting-star hoya is indigenous to the Philippines. This plant was called multiflora due to its multiple flowers (about 40) in the convex umbel (Aurigue, 2013). There are no reported chemical studies and biological activities on *H. multiflora*. However, congeners of the plant have been studied for their chemical constituents. Gas chromatographic analysis on the chemical constituents of *Hoya naumanii* led to the detection of the triterpenes β-amyrin, lupeol and α-amyrin and their 3, 4-*seco*-3-oic acid methyl esters (Baas and Van Berkel, 1991). The isolation of pentacyclic triterpenols δ-amyrin, β-amyrin, lupeol and α-amyrin and their 3, 4-*seco*-3-*nor*-2-ol derivatives (australinols A–D) from the leaf wax, of, *Hoya australis*, have been reported (Baas et al., 1992). Moreover, the β-amyrin derivative 5-isopropyl-10 (2-methoxycarbonylethyl)des-A-olean-12-en and the taraxerol derivative 5-isopropyl-10 (2-methoxycarbonylethyl)des-A-olean-14-en were isolated from *Hoya lacunose (*Baas, 1983). The oligosaccharides 6-deoxy-3-, *O*-methyl-β-allopyranosyl (1→4)- β-cymaropyranosyl (1→4)-β-cymaronic acid δ-lactone and 6-deoxy-3-*O*-methyl-β-allopyranosyl (1→4)-β-oleandropyranosyl (1→4)-β-cymaropyranosyl (1→4)-β-cymaronic acid δ-lactone and its sodium salt were isolated from *Hoya carnosa (*Yoshikawa *et al*., 2000). A review on the chemical and pharmacological aspects of *Hoya* species has been provided (Pandey *et al*., 2006). *Hoya* species yielded pregnanes, lipids, sterols, flavanols, triterpenes, sesquiterpenes and disaccharides. They were reported to exhibit antinematodal activity, hypo sensitization, immunological properties and phytotoxicity; used for the treatment of occupational asthma and sea-squirt asthma and allergies; and employed as antigens and insecticides (Pandey et al., 2006).

This study was conducted as part of our research on the chemical constituents of the genus *Hoya*. We earlier, reported the isolation of lupenone and lupeol from the roots; lupeol, squalene and β-sitosterol from the leaves; and betulin from, the stems of *Hoya mindorensis* Schlechter (Ebajo *et al*., 2014).

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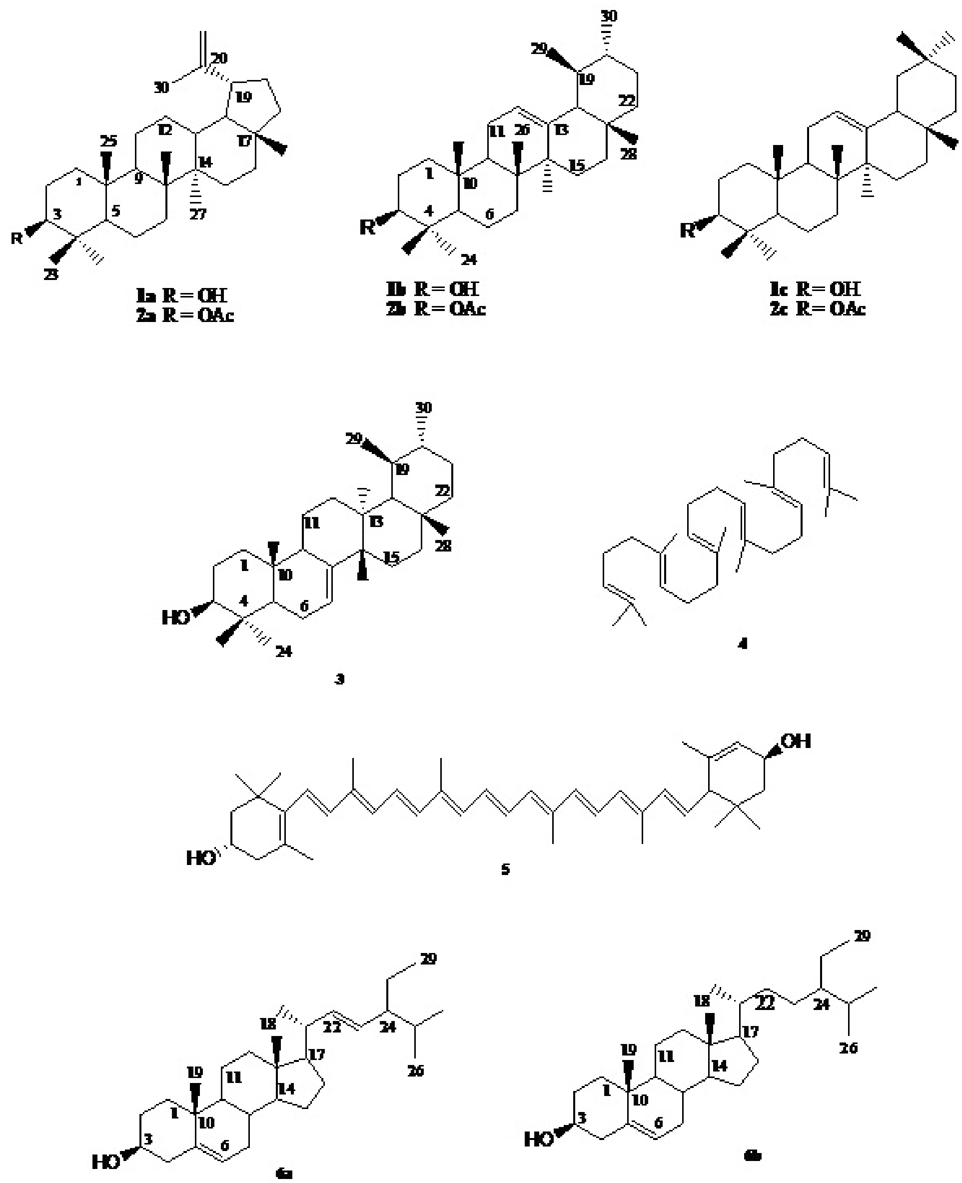

Fig. 1: Chemical Constituents of *Hoya multiflora* lupeno (1a), lupeol acetate (2a), α-amyrin (1b), α-amyrin acetate (2b), β-amyrin (1c), β-amyrin acetate (2a), bauerenol (3), squalene (4), lutein (5), β-sitosterol (6a), and stig masterol (6b).

We report herein the isolation of lupeol (**1a**), α-amyrin (**1b**), β-amyrin (**1c**), lupeol acetate (**2a**), α-amyrin acetate (**2b**), and β-amyrin acetate (**2c**) from the stems; and **1b**, bauerenol (**3**), squalene (**4**), lutein (**5**), β-sitosterol (**6a**), and stigmasterol (**6b**) from the leaves of *Hoya multiflora* Blume. To the best of our knowledge this is the first report on the isolation of these compounds from *H. multiflora.*

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

Hoya multiflora Blume was collected from a garden in Pangasinan, Philippines in September 2013. Voucher specimens were authenticated at the Botany Division of the Philippine National Museum.

General Isolation Procedure

The air-dried leaves (23.0 g), and stems (86.5 g) of *H. multiflora* were ground in a blender, soaked in CH_2Cl_2 for three days and then filtered. The filtrates were concentrated under vacuum to afford crude extracts of leaves (2.0 g), and stems (1.0 g) which were each chromatographed by gradient elution with CH_2Cl_2, followed by increasing amounts of acetone at 10% increment by volume as eluents. A glass column 12 inches in height and 0.5 inch internal diameter was used for the fractionation of crude extracts. Two milliliter fractions were collected. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. Rechromatography and final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation of Chemical Constituents of the Stems

The CH_2Cl_2 fraction from the chromatography of the crude extract was rechromatographed using 1% EtOAc in petroleum ether to afford a mixture of **2a-2c (**9 mg) after washing with petroleum ether. The 20% acetone in CH_2Cl_2 fraction was rechromatographed using 5% EtOAc in petroleum ether, to afford a mixture of **1a-1c (**4 mg) after washing with petroleum ether.

Isolation of Chemical Constituents of the Leaves

The CH_2Cl_2 fraction from the chromatography of the crude extract was rechromatographed (3 ×) using petroleum ether to afford **4 (**5 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed (4×) using 20% EtOAc in petroleum ether to afford a mixture of **1b** and **3 (**15 mg) after washing with petroleum ether. The 40% acetone in CH_2Cl_2 fraction was rechromatographed (2×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9, v/v) afford a mixture of **6a** and **6b (**4 mg) after washing with petroleum ether. The 60% acetone in CH_2Cl_2 fraction was rechromatographed using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) afford **5** (3 mg) after washing with Et_2O.

***Lupeol (*1a**)

Colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.66 (d, *J* = 2.4 Hz, H-29b), 4.55 (d, *J* = 2.4 Hz, H-29a), 3.18 (H-3), 0.98 (s, H_3-23), 0.78 (s, H_3-24), 0.83 (s, H_3-25), 0.95 (s, H_3-26), 1.05 (s, H_3-27), 0.90 (s, H_3-28), 1.66 (s, H_3-30).

***α-Amyrin (*1b**)

Colorless solid.^{1}H NMR (600 MHz, $CDCl_3$): δ 3.15 (H-3), 0.67 (H-5), 5.12 (H-12), 0.95 (s, H_3-23), 0.76 (s, H_3-24), 0.75 (s, H_3-25), 0.89 (s, H_3-26), 1.01 (s, H_3-27), 0.95 (s, H_3-28), 0.85 (d, *J* = 6.0 Hz, H_3-29), 0.75 (d, *J* =7.0 Hz, H_3-30).

***β-Amyrin (*1c**)

Colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.15 (H-3), 0.67 (H-5), 5.16 (H-12), 0.77 (s, H_3-23), 0.90 (s, H_3-24), 0.74 (s, H_3-25), 0.93 (s, H_3-26), 1.16 (s, H_3-27), 1.07 (s, H_3-28), 0.86 (s, H_3-29), 0.79 (s, H_3-30).

***Lupeol acetate (*2a**)

Colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.44 (C-1), 27.42 (C-2), 80.94 (C-3), 38.44 (C-4), 55.36 (C-5), 17.99 (C-6), 34.19 (C-7), 55.41 (C-8), 50.32 (C-9), 37.06 (C-10), 20.92 (C-11), 25.07 (C-12), 38.02 (C-13), 42.05 (C-14), 27.42 (C-15), 35.55 (C-16), 47.63 (C-17), 48.27 (C-18), 47.99 (C-19), 150.96 (C-20), 29.81 (C-21), 40.01 (C-22), 28.07 (C-23), 15.72 (C-24), 15.96 (C-25), 16.17 (C-26), 14.49 (C-27), 18.22, 18.25 (C-28, C-30), 109.34 (C-29), 171.01, 21.39 (OAc).

***α-Amyrin acetate (*2b**)

Colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.44 (C-1), 27.59 (C-2), 80.96 (C-3), 37.69C-4), 55.24 (C-5), 18.22 (C-6), 32.85 (C-7), 40.01 (C-8), 47.63 (C-9), 36.77 (C-10), 23.35 (C-11), 124.30 (C-12), 139.61 (C-13), 42.05 (C-14), 26.58 (C-15), 28.05 (C-16), 33.73 (C-17), 59.04 (C-18), 39.63 (C-19), 39.59 (C-20), 31.23 (C-21), 41.52 (C-22), 28.73 (C-23), 16.85 (C-24), 15.72 (C-25), 16.73 (C-26), 23.21 (C-27), 28.73 (C-28), 17.50 (C-29), 21.39 (C-30), 171.01, 21.32 (OAc).

***β-Amyrin acetate (*2c**)

Colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$), δ 39.59 (C-1), 27.93 (C-2), 80.8 (C-3), 39.59 (C-4), 55.24 (C-5), 18.19 (C-6), 33.73 (C-7), 38.37 (C-8), 47.4 (C-9), 35.55 (C-10), 23.59 (C-11), 121.62 (C-12), 145.20 (C-13), 42.05 (C-14), 28.73 (C-15), 27.93 (C-16), 32.85 (C-17), 59.0 (C-18), 40.01 (C-19), 41.52 (C-20), 31.23 (C-21), 42.01 (C-22), 29.69 (C-23), 15.72 (C-24), 15.72 (C-25), 16.84 (C-26), 23.59 (C-27), 28.73 (C-28), 17.50 (C-29), 21.39 (C-30), 170.8, 21.32 (OAc).

***Bauerenol* (3)**

Colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): 36.87 (C-1), 27.69 (C-2), 79.05 (C-3), 38.88 (C-4), 50.41 (C-5), 24.15 (C-6), 116.43 (C-7), 145.35 (C-8), 48.22 (C-9), 35.33 (C-10), 16.85 (C-11), 32.42 (C-12), 37.69 (C-13), 41.52 (C-14), 28.87 (C-15), 37.69 (C-16), 32.04 (C-17), 54.88 (C-18), 35.20 (C-19), 32.04 (C-20), 29.67 (C-21), 31.52 (C-22), 27.53 (C-23), 14.66 (C-24), 12.98 (C-25), 23.65 (C-26), 22.66 (C-27), 39.65 (C-28), 25.63 (C-29), 22.55 (C-30).

***Squalene (*4**)

Colorless oil. ^{13}C NMR (150 MHz, $CDCl_3$): δ 25.69 (C-1), 131.24 (C-2), 124.31 (C-3), 26.66 (C-4), 39.74 (C-5), 134.89 (C-6), 124.41 (C-7), 26.77 (C-8), 39.76 (C-9), 135.10 (C-10), 124.31 (C-11), 28.28 (C-12), 17.68 (C-13), 16.04 (C-14), 16.00 (C-15).

***Lutein (*5**)

Orange crystals. ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.05 (s, 2 ring A CH_3), 0.83 (s, ring B CH_3), 0.98 (s, ring B CH_3), 1.60 (allylic CH_3), 1.71 (allylic CH_3), 1.89 (allylic CH_3), 1.951 (allylic CH_3), 1.94 (2 allylic CH_3), 1.45, 1.75 (CH_2), 1.35, 1.85 (CH_2), 2.35, 2.00 (allylic CH_2), 2.38 (allylic CH), 4.23 (br s, CHOH), 3.98 (m, CHOH), 5.52 (br s, =CH), 5.41 (dd, *J* = 9.6, 15.0 Hz, =CH), 6.56-6.65, 6.33 (dd, *J* = 15.0, 3.0 Hz), 6.23 (br d, *J* = 9.6 Hz), 6.09-6.14 (=CH).

***β*-Sitosterol (6a)**

Colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.50 (m, H-3), 2.26, 2.21 (H_2-4), 5.33 (dd, *J* = 1.8, 4.8 Hz, H-6), 0.66 (s, CH_3-18), 0.99 (s, CH_3-19), 0.90 (d, *J* = 6.6 Hz, CH_3-21), 0.79 (d, *J* = 6.6 Hz, CH_3-26), 0.82 (d, *J* = 6.6 Hz, CH_3-27), 0.86 (t, *J* = 7.2 Hz, CH_3-29).

***Stigmasterol (*6b)**

Colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.50 (m, H-3), 5.33 (dd, *J* = 1.8, 4.8 Hz, H-6), 0.68 (s, CH_3-18), 0.99 (s, CH_3-19), 1.01 (d, *J* =6.6 Hz, CH_3-21), 5.13 (dd, *J* =8.4, 15.0 Hz, H-22), 5.00 (dd, *J* =9.0, 15.0 Hz, H-23), 0.84 (d, *J* =6.6 Hz, CH_3-26), 0.83 (d, *J* = 6.6 Hz, CH_3-27), 0.80 (t, *J* = 6.6 Hz, CH_3-29).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extracts of *Hoya multiflora* Blume afforded a mixture of lupeol (**1a**) (Ragasa *et al*., 2014a), α-amyrin (**1b**) (Ragasa *et al*., 2014a) and β-amyrin (**1c**) (Ragasa *et al*., 2014a) in about 2:1:0.3 ratio and another mixture of lupeol acetate (**2a**) (Tsai *et al*., 2012), α-amyrin acetate (**2b**) (Ragasa *et al*., 2014b) and β-amyrin acetate (**2c**) (Feleke and Brehane, 2005) in about 3:1:0.3 ratio from the stems; and a mixture of **1b** and bauerenol (**3**) (Raga *et al*., 2013a) in about 1:2.5 ratio, squalene (**4**) (Ragasa *et al*., 2014c), lutein (**5**) (Ragasa *et al*., 2014d), and another mixture of β-sitosterol (**6a**) (Ragasa *et al*., 2014e) and stigmasterol (**6b**) (Ragasa et al., 2014e) in about 2:1 ratio from the leaves. The ratio of about 2:1:0.3 for the mixture of **1a**, **1b** and **1c** was deduced from integrations of the ^{1}H NMR resonances for the olefinic protons of **1a** at δ 4.55 (d, *J* = 2.4 Hz) and 4.66 (d, *J* = 2.4 Hz), **1b** at δ 5.10 (t, *J* = 3.6 Hz) and **1c** at δ 5.16 (t, *J* = 3.6 Hz). The integrations of the ^{1}H NMR resonances for the olefinic protons of **2a** at δ 4.55 (d, *J* = 2.4Hz) and 4.66 (d, *J* = 2.4 Hz), **2b** at δ 5.11 (t, *J* =3.6 Hz) and **2c** at δ 5.16 (t, *J* =3.6 Hz) indicated that the ratio of **2a**, **2b** and **2c** is about 3:1:0.3. The 1:2.5 ratio of the mixture of **1b** and **3** was determined from the integrations of the ^{1}H NMR resonances for the olefinic protons of **1b** at δ 5.15 (t, *J* = 3.6 Hz) and **3** at δ 5.39 (dd, *J* = 3.0, 7.2 Hz, H-7). The integrations of the ^{1}H NMR resonances for the olefinic protons of **6a** at δ 5.33 (H-6) and **6b** at δ 5.33 (H-6), 5.13 (dd, *J* = 8.4, 15.0 Hz, H-22) and 5.00 (dd, *J* = 8.4, 15.0 Hz, H-23) suggested that the ratio of **6a** and **6b** is about 2:1. The structures of **1**-**6** were identified by comparison of their ^{1}H and/or^{13}C NMR data with literature data.

Although no biological activity tests were conducted on the isolated compounds (**1–6**), literature search revealed that these have diverse bioactivities as follows., Lupeol (**1a**) exhibited antiurolithiatic and diuretic activity (Vidya *et al.,* 2002). It prevented the formation ofvesical calculi and reduced the size of the preformed stones in rats (Anand *et al*., 1994). It also showed antifungal activity against *Fusarium oxysporum* and *Penicillium notatum (*Manzano *et al.,* 2013). Lupeol significantly reduced the 451Lu tumor growth in athymic nude mice (Saleem*et al*., 2008), inhibited the proliferation of MDA-MB-231 human breast cancer cells in a dose dependent manner (Lambertini *et al*., 2005), and induced growth inhibition and apoptosis in hepatocellular carcinoma SMMC7721 cells by down-regulation of the death receptor 3 (DR3) expression (Zhang *et al*., 2009). Lupeol and lupeol acetate (**2a**) have shown hypotensive activity (Saleem *et al*., 2003), while **1a** also exhibited antidyslipidemic activity in hamster at 100 mg/Kg body weight (Reddy *et al.,* 2009). It exhibited potent anti-inflammatory activity in an allergic airway inflammation model by a significant reduction in eosinophils infiltration and in Th2-associated cytokines levels that trigger the immune responses in asthma (Vasconcelos *et al*., 2008). A review on the biological activities of lupeol has been provided (Gallo and Sarachine, 2009). β-Amyrin (**1c**) and α-amyrin (**1b**) were reported to possess anti-inflammatory (Recio *et al*., 1995; Madeiros *et al*., 2007; Okoye *et al*., 2014) and analgesic (Otuki *et al*., 2005; Soldi *et al*., 2008) properties. β-Amyrin showed antifungal activity against *A. rabiei* with an MIC value of 0.0156 mg/mL (Jabeen *et al*., 2011)., α-Amyrin was proposed as a possible biomarker for the fungal resistance of grape-vine leaves (*Vitis vinifera*) (Batovska *et al*., 2008). The mixture of **1b** and **1c** effectively reduced the elevated plasma glucose levels during the oral glucose tolerance test (OGTT). Furthermore, the mixture of **1b** and **1c** at 100 mg/kg significantly decreased the VLDL and LDL cholesterol and increased the HDL cholesterol (Santos *et al.,* 2012). A review on the sources and biological activities of **1b** and **1c** has been provided (Vasquez *et al.,* 2012). The anti-inflammatory effect of lupeol acetate (**2a**) involves the opioid system, as indicated by the complete blockade of the opioid antagonist naloxone (Lucetti *et al*., 2010). α-Amyrin acetate (**2b**) at 100 mg/kg showed significant ($p < 0.05$) inhibition of egg albumen-induced paw edema with 40 % inhibition at the 5th hour. β-Amyrin acetate (**2c**) and **2b** isolated from the *Alstonia boonei* stem bark exhibited profound anti-inflammatory activity (Okoye *et al*., 2014). Triterpenes **2b** and **2c** were also reported to exhibit sedative, anxiolytic and anticonvulsant properties (Aragao *et al*., 2009). A mixture of bauerenol (**3**), **1b** and **1c** obtained from *Ardisia* species exhibited angio-suppressive effects on duck chorioallantoic membrane (CAM) (Raga *et al.,* 2013b); restricted inter-capillary length and reduced branch point with 100% CAM viability and embryo survivability and promoted intense expression of the von Willebrand factor (F8) (Raga *et al.,* 2013c); was found toxic to *A. salina* nauplii after 48h of exposure and showed teratologic manisfestations on *Danio rerio* embryos (Raga *et al.,* 2014a); and exhibited analgesic property in the acetic acid writhing test and hot plate assay (Raga *et al.,* 2014b). Another study reported that a mixture of bauerenol, α-amyrin and β-amyrin from *Carmona retusa* exhibited 51% analgesic activity and showed 20% anti-inflammatory activity at dosage of 100 mg/kg mouse, while of 250 mg/kg mouse showed a 29% anti-diarrheal activity (Villasenor *et al.,* 2004). Squalene (**4**) was reported to significantly suppress colonic ACF formation and crypt multiplicity**y 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 have anti-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).

Dietary lutein (**5**), especially at 0.002%, inhibited tumor growth by selectively modulating apoptosis, and by inhibiting angiogenesis (Chew *et al.,* 2003). Another study reported that the chemopreventive properties of all-*trans* retinoic acid and lutein may be attributed to their differential effects on apoptosis pathways in normal *versus* transformed mammary cells (Sumantran *et al.,* 2000). Moreover, very low amounts of dietary lutein (0.002%) can efficiently decrease mammary tumor development and growth in mice (Park *et al.,* 1998). Another study reported that lutein and zeaxanthine reduces the risk of age related macular degeneration (SanGiovanni *et al*., 2007).

β-Sitosterol (**6a**) 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 down regulation of Akt in MCA-102 murine fibrosarcoma cells (Moon *et al.,* 2007).

Stigmasterol (**6b**) 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.

CONCLUSION

Hoya multiflora is a Philippine indigenous ornamental plant with no reported chemical studies and biological activities. This study reports on the terpenoids and sterols with known diverse biological activities which were isolated from the leaves and stems of the plant. Most of these compounds (**1a-1c, 3-6**) were reported to exhibit cytotoxic and anticancer properties, while **2a-2c** were reported to possess anti-inflammatory activity.

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