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Original Article

Triterpenes and Sterols from Leaves of *Hoya meliflua* Merr.

Vincent Antonio S. Ng[1,*], Raymond S. Malabed[2], Fernando B. Aurigue[3], Consolacion Y. Ragasa[1,4]

ABSTRACT

Introduction: *Hoya* plants are also called wax plants due to the waxy appearance of their leaves and flowers. Most species are cultivated as ornamental plants. In the Philippines, there are at least 109 species of *Hoya*; 88 of these are endemic to the country. One of the endemic species is *Hoya meliflua* often confused with *H. diversifolia*, which can also be found in other countries. This study is part of our research on the chemical constituents of Philippine native *Hoyas*. **Methods:** The compounds were isolated by silica gel chromatography and identified by NMR spectroscopy. **Results:** Chemical investigation of the dichloromethane extract from the leaves of *Hoya meliflua* afforded squalene and mixtures of β-amyrin (**1a**), α-amyrin (**1b**) and lupeol (**1c**) in about 1:1:0.25 ratio; oleanone (**2a**), ursenone (**2b**) and lupenone (**2c**) in about 1:1:0.3 ratio; β-amyrin cinnamate (**3a**), α-amyrin cinnamate (**3b**) and lupenyl cinnamate (**3c**) in about 0.5:0.3:1 ratio; and β-sitosterol and stigmasterol in about 5:1 ratio. **Conclusion:** The results of our study indicate that *Hoya meliflua* shares similar chemical characteristics with other members of the genus *Hoya*. The triterpenes and sterols obtained from *H. meliflua* were also identified from other *Hoya* species. It is interesting to note that although most *Hoya* plants have no known biological activity and medicinal property, the compounds isolated from *H. meliflua* possess diverse bioactivities.

Key words: *Hoya meliflua*, Apocynaceae, Squalene, β-amyrin, α-amyrin, Lupeol, Oleanone, Ursenone, Lupenone, β-amyrin Cinnamate, α-amyrin Cinnamate, Lupenyl Cinnamate, β-sitosterol, Stigmasterol.

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INTRODUCTION

Hoya Br. is considered the largest genus of flowering plants in the family Apocynaceae. Commonly called wax plants due to the waxy appearance of their leaves and flowers, most species have ornamental value. A few have been reported to have medicinal properties or used in traditional medicine. In the Philippines, there are at least 109 species of *Hoya*; 88 of these are endemic to the country. One such endemic species is *Hoya meliflua* Merr. found in Apayao, Bataan, Laguna, La Union and Rizal in Luzon Island and in Leyte, Mindoro, Negros, Palawan and Panay islands in Central Philippines all at low altitudes.[1] New records of occurrence in other provinces, such as Laguna, have not been published yet. The Plant List.[2] states that *H. meliflua* is an unresolved name because some data suggest that it is synonymous with *H. diversifolia* Blume which is also found in other countries. This may be attributed to the confusion on the identity of the two Philippine native species.

This study is part of our research on the chemical constituents of Philippine native hoyas. We earlier reported the isolation of lupenone (**I**) and lupeol (**II**) from the roots; **II**, squalene (**III**) and β-sitosterol (**IV**) from the leaves; and betulin (**V**) from the stems of *H. mindorensis* Schlechter.[3] In another study, we reported the isolation of **II**, α-amyrin (**VI**), β-amyrin (**VII**), lupeol acetate (**VIII**), α-amyrin acetate (**IX**) and β-amyrin acetate (**X**) from the stems; and **III**, **IV**, **VI**, bauerenol (**XI**), lutein (**XII**) and stigmasterol (**XIII**) from the leaves of *H. multiflora* Blume.[4] Furthermore, the isolation of β-amyrin cinnamate (**XIV**) and taraxerol (**XV**) from the stems; and **IV**, **XIII**, **XV**, triglycerides (**XVI**) and chlorophyll a (**XVII**) from the leaves of *H. wayetii* Kloppenb has been reported.[5] Moreover, the isolation of **IV**, **XIII**, **XIV**, **XV**, taraxerone (**XVIII**) and α-amyrin cinnamate (**XIX**) from the stems; **IV**, **XV** and **XVIII** from the roots; **XIV** and **XIX** from the flowers; and **III**, **IV** and saturated hydrocarbons (**XX**) from the leaves of *H. buotii* has been reported.[6]

We also reported the isolation of **II**, **III**, **IV**, **VI**, **VII**, **XIV** and **XX** from the leaves; and **III**, **IV**, **XIII**, **XV** and lupeol cinnamate (**XXI**) from the stems of *H. diversifolia* Blume.[7] Chemical investigation of the dichloromethane extracts of *H. cumingiana* Decne. yielded **II**, **IV**, **VI**, **VII**, **XIII** and bauerenol (**XXII**) from the leaves; and **XV** from the stems.[8] Recently, we reported the isolation of **IV**, **XIII** and **XV** from the stems[9] and **I**, **IV**, **XIII**, 2-hydroxyethylbenzoate (**XXIII**) and fatty acid methyl esters (**XXIV**) from the leaves[10] of *Hoya pubicalyx*. In another study, the dichloromethane extracts of *H. paziae* Kloppenb. yielded **IX**, **X**, **XV** and taraxeryl acetate (**XXV**) from the stems[11] and **II**, **VI**, **VII**, lupeol fatty acid esters

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(**XXVI**), α-amyrin fatty acid esters (**XXVII**) and β-amyrin fatty acid esters (**XXVIII**) from the leaves.[12] Furthermore, the dichloromethane extracts of *H. cagayanensis* afforded **I**, **II**, **IV**, **XIII**, **XX** and dihydrocanaric acid (**XXIX**), from the stems; and 2-hydroxyethyl benzoate (**XXX**) from the leaves.[13] Recently, the dichloromethane extracts of *H. madulidii* yielded **II**, **IV**, **XIII**, **XIV**, **XIX**, **XX** and **XXI** from the stems and **III**, **IV**, **XIII**, **XVII** and **XX** from the leaves.[14]

In this study, the dichloromethane extract of the leaves of *Hoya meliflua* yielded squalene, β-amyrin (**1a**), α-amyrin (**1b**), lupeol (**1c**), oleanone (**2a**), ursenone (**2b**), lupenone (**2c**), β-amyrin cinnamate (**3a**), α-amyrin cinnamate (**3b**), lupenyl cinnamate (**3c**), β-sitosterol and stigmasterol (Figure 1).

MATERIALS AND METHODS

General Experimental Procedure

Sample spectra were obtained on a JEOL ECS 400 spectrometer with $CDCl_3$ as solvent. 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 meliflua leaves and stems were collected from healthy vines of a clone that has been cultivated for more than 10 years. Samples labeled as PNRI-H.CA were provided under Material Transfer Agreement No. 2016-004 dated June 24, 2016. The original plant came from Quezon Province, Philippines and authenticated by one of the authors (FBA).

General Isolation Procedure

A glass column 12 inches in height with 0.5 inch internal diameter was used for the fractionation of the crude extract. Fractions of 10 mL volumes were collected and monitored by thin layer chromatography. Fractions containing spots with similar *Rf* values were combined and rechromatographed using the appropriate solvent. Final purification was carried out using Pasteur pipette as the column, collecting 1 mL fractions. TLC-pure isolates were combined and after evaporation of the solvent, were subjected to NMR analysis.

Isolation of Chemical Constituents of the Leaves

The air-dried leaves (252.8 g) were ground in a blender, soaked in CH_2Cl_2 for three days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (7.8 g) which was chromatographed by gradient elution with CH_2Cl_2, followed by increasing amounts of acetone at 10% increment by volume as eluents.

The CH_2Cl_2 fraction was rechromatographed (2 ×) using petroleum ether to afford squalene (12.8 mg). The 10% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 1% EtOAc in petroleum ether to yield a mixture of **3a**, **3b** and **3c** (5.0 mg), after washing with petroleum ether. The 20% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 2.5% EtOAc in petroleum ether to yield a mixture of **2a**, **2b** and **2c** (10.3 mg), after washing with petroleum ether. The 30% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using 7.5% EtOAc in petroleum ether to afford a mixture of **1a**, **1b** and **1c** (10.6 mg) after washing with petroleum ether. The 40% acetone in CH_2Cl_2 fractions was rechromatographed (3 ×) using 10% EtOAc in petroleum ether to yield a mixture of β-sitosterol and stigmasterol (21.0 mg) after washing with petroleum ether.

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract from the leaves of *Hoya meliflua* afforded squalene and mixtures of β-amyrin (**1a**), α-amyrin (**1b**) and lupeol (**1c**) in about 1:1:0.25 ratio; oleanone (**2a**), ursenone (**2b**) and lupenone (**2c**) in about 1:1:0.3 ratio; β-amyrin cinnamate (**3a**), α-amyrin cinnamate (**3b**) and lupenyl cinnamate (**3c**) in about 0.5:0.3:1 ratio; and β-sitosterol and stigmasterol in about 5:1 ratio. The structures of these compounds were identified by comparison of their NMR data with those reported in the literature.

The NMR spectra of **1a** are in accordance with data reported in the literature for *β*-amyrin,[4] **1b** for *α*-amyrin,[4] **1c** for lupeol,[4] **2a** for oleanone,[15] **2b** for ursenone,[15] **2c** for lupenone,[16] **3a** for β-amyrin cinnamate,[6,17] **3b** for α-amyrin cinnamate,[6,17] **3c** for lupeol cinnamate,[17] squalene,[18] *β*-sitosterol[18] and stigmasterol.[18]

The ratio of about 1:1:0.25 for the mixture of β-amyrin (**1a**), α-amyrin (**1b**) and lupeol (**1c**) was deduced from the intensities and integrations of the ^{1}H NMR resonances for the olefinic protons of **1a** at δ 5.16 (brs),[7,12] **1b** at δ 5.11 (brs)[7,12] and **1c** at δ 4.68 (brs) and 4.56 (brs).[7,12,18] The ratio of about 1:1:0.30 for the mixture of oleanone (**2a**), ursenone (**2b**) and lupenone (**2c**) was deduced from the intensities and integrations of the ^{1}H NMR resonances for the olefinic protons of **2a** at δ 5.19 (t, *J* = 3.2 Hz),[15] **2b** at δ 5.14 (t, *J* = 3.2 Hz)[15] and **2c** at δ 4.67 (brs) and 4.55 (brs).[19] The ratio of about 0.5:0.3:1 for the mixture of β-amyrin cinnamate (**3a**), α-amyrin cinnamate (**3b**) and lupenyl cinnamate (**3c**) was deduced from the intensities and integrations of the ^{1}H NMR resonances for the olefinic protons of **3a** δ 5.18 (brs),[6,17] **3b** at δ 5.13 (brs);[6,17] and **3c** at δ 4.68 (br s) and 4.56 (br s).[16] The ratio of about 5:1 for the mixture of β-sitosterol and stigmasterol was deduced from the intensities and integrations of the ^{1}H NMR resonances for olefinic protons at δ 5.33 (brs, H-5) and methyl protons at δ 0.66 (s) for β-sitoterol and olefinic protons at δ 5.33 (brs, H-5), 5.13 (dd, J = 8.4, 15.2 Hz) and 5.00 (dd, J = 8.4, 15.2 Hz) and the methyl protons at δ 0.68 (s) for stigmasterol.[18]

H. meliflua is cultivated as an ornamental plant in the Philippines. There is no reported biological activity and medicinal property for the plant, but Blanco[20] mentioned as early as 1845 a record that *H. meliflua* causes "stoppage of birth," or perhaps as an abortifacient. However, literature search revealed that the compounds isolated from the plant possess diverse activities.

β-Amyrin (**1a**) and α-amyrin (**1b**) were reported to possess anti-inflammatory.[21-23] and analgesic[24-25] properties

Triterpene **1a** showed antifungal activity against *A. rabiei* with an MIC value of 0.0156 mg/mL,[26] while **1b** was proposed as a possible biomarker for the fungal resistance of grape-vine leaves (*Vitis vinifera*).[27] The mixture of **1a** and **1b** effectively reduced the elevated plasma glucose levels during the oral glucose tolerance test (OGTT). Furthermore, the mixture of **1a** and **1b** at 100 mg/kg significantly decreased the VLDL and LDL cholesterol and increased the HDL cholesterol.[28] A review on the sources and biological activities of **1a** and **1b** has been provided.[29]

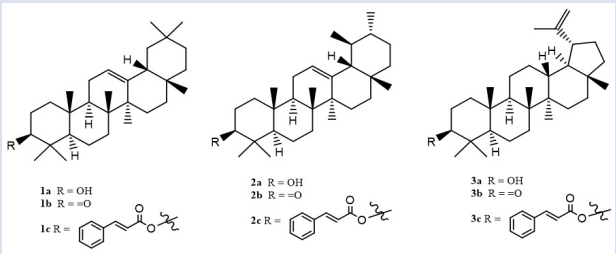

Figure 1: Chemical structures of β-amyrin (1a), α-amyrin (1b), lupeol (1c), oleanone (2a), ursenone (2b), lupenone (2c), β-amyrin cinnamate (3a), α-amyrin cinnamate (3b) and lupenyl cinnamate (3c).

On the other hand, lupeol (**1c**) exhibited antiurolithiatic and diuretic activity.[30] It prevented the formation of vesical calculi and reduced the size of the preformed stones in rats.[31] It also showed antifungal activity against *Fusarium oxysporum* and *Penicillium notatum*.[32] Triterpene **1c** significantly reduced the 451Lu tumor growth in athymic nude mice,[33] inhibited the proliferation of MDA-MB-231 human breast cancer cells in a dose dependent manner[34] and induced growth inhibition and apoptosis in hepatocellular carcinoma SMMC7721 cells by down- regulation of the death receptor 3 (DR3) expression.[35] 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.[36] Another study reported that **1c** and lupeol acetate have shown hypotensive activity.[37] A review on the biological activities of lupeol has been provided.[38]

Lupenone (**2c**) inhibited adipocyte differentiation by suppressing PPARγ and C/EBPα protein levels.[39] It also increased the tyrosinase enzyme expression via mitogen-activated protein kinase phosphorylated extracellular signal regulated kinases 1 and 2 phosphorylation inhibition which results to stimulation of melanogenesis. This suggests that **2c** could be a possible treatment for hypopigmentation.[40]

β-Amyrin cinnamate (**3a**) was reported to inhibit the TPAS-induced inflammation (ID_{50} 0.27 µmol/ear; CI 95% 0.23–0.33 µmol/ear) which is more inhibitory than the positive control, indomethacin (ID_{50} 0.91 µmol/ear ;CI 95% 0.23–0.33 µmol/ear).[19] α-Amyrin cinnamate (**3b**), lupeol cinnamate (**3c**) and **3a** exhibited marked anti-inflammatory activity against TPA-induced inflammation (ID_{50} = 0.15–0.75 µmol/ear). Triterpene **3c** showed the highest activity with ID_{50} of 0.15 µmol/ear.[12] This triterpene at a dose of 10 mg/kg also exhibited anti-inflammatory activity on rat hind paw edema induced by carrageenan.[12] Furthermore, **3c** exhibited inhibitory effect on skin tumor promotion in an *in vivo* two-stage carcinogenesis test using DMBA as an initiator and TPA as a promoter.[12] Triterpenes **3a–3c** showed moderate inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) in Raji cells as a primary screening test for inhibitors of tumor promoters.[12]

Squalene was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis.[41] It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties.[42] A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 have anti-proliferative effects on breast cancer cells.[43] The preventive and therapeutic potential of squalene containing compounds on tumor promotion and regression have been reported.[44] A recent review on the bioactivities of squalene has been provided.[45]

β-Sitosterol was observed to have growth inhibitory effects on human breast MCF-7 and MDA- MB-231 adenocarcinoma cells.[46] It was shown to be effective for the treatment of benign prostatic hyperplasia.[47] 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.[48] It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake.[49] It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-t102 murine fibrosarcoma cells.[50]

Stigmasterol showed therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions.[51] It lowered plasma cholesterol levels, inhibited intestinal cholesterol and plant sterol absorption and suppressed hepatic cholesterol and classic bile acid synthesis in Winstar as well as WKY rats.[52] Other studies reported that stigmasterol showed cytostatic activity against Hep-2 and McCoy cells,[53] markedly inhibited tumour promotion in two stage carcinogenesis experiments,[54] exhibited antimutagenic,[55] topical anti-inflammatory,[56] antiosteoarthritic[57] and antioxidant[58] activities.

CONCLUSION

The results of our study indicate that *Hoya meliflua* shares similar chemical characteristics with other members of the genus *Hoya*: *H. multiflora*,[4] *H. diversifolia*,[7] *H. cumingiana*,[8] and *H. paziae*[12] which yielded β-amyrin (**1a**) and α-amyrin (**1b**); *H. mindorensis*,[3] *H. multiflora*,[4] *H. diversifolia*,[7] *H. cumingiana*,[8] *H. paziae*,[12] *H. cagayanensis*,[13] and *H. madulidii*[14] which contained lupeol (**1c**); *H. mindorensis*,[3] *H. pubicalyx*,[12] and *H. cagayanensis*[15] which afforded lupenone (**2c**); *H. wayetii*,[5] *H. buotii*,[6] and *H. madulidii*[14] which yielded β-amyrin cinnamate (**3a**); *H. buotii*,[6] and *H. madulidii*[14] which afforded α-amyrin cinnamate (**3b**); *H. diversifolia*[7] and *H. madulidii*[14] which provided lupenyl cinnamate (**3c**); *H. mindorensis*,[3] *H. multiflora*,[4] *H. wayetii*,[5] *H. buotii*,[6] *H. diversifolia*,[7] *H. cumingiana*,[8] *H. pubicalyx*,[9,10] *H. cagayanensis*,[13] and *H. madulidii*[14] which contained β-sitosterol and stigmasterol; and *H. mindorensis*,[3] *H. buotii*,[6] *H. diversifolia*[7] and *H. madulidii*[14] which contained squalene. The most common constituents of the eleven native *Hoya* species studied are β-sitosterol and stigmasterol which are found in ten species. Of the twelve compounds isolated from *H. meliflua*, lupeol is found in eight species, squalene, β-amyrin and α-amyrin in five species, lupenone and β-amyrin cinnamate in four species, α-amyrin cinnamate and lupenyl cinnamate in three species. Oleanone and ursenone are reported for the first time from Philippine *Hoya* species.

It is interesting to note that although most *Hoya* plants have no known biological activity and medicinal property, the compounds isolated from *H. meliflua* possess diverse bioactivities.

Lastly, the difference in results of phytochemical analysis of *H. meliflua* is a proof that it is an entirely different and distinct species from *H. diversifolia*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CH_2Cl_2: Dichloromethane; **EtOAc:** Ethyl acetate; **Et_2O:** Diethyl ether.

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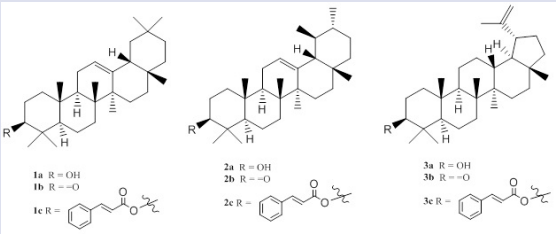
GRAPHICAL ABSTRACT



SUMMARY

- Chemical investigation of the dichloromethane extract from the leaves of *Hoya meliflua* afforded squalene and mixtures of β-amyrin (1a), α-amyrin (1b) and lupeol (1c) in about 1:1:0.25 ratio; oleanone (2a), ursenone (2b) and lupenone (2c) in about 1:1:0.3 ratio; β-amyrin cinnamate (3a), α-amyrin cinnamate (3b) and lupenyl cinnamate (3c) in about 0.5:0.3:1 ratio; and β-sitosterol and stigmasterol in about 5:1 ratio. The structures of these compounds were identified by comparison of their NMR data with those reported in the literature.

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