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Anti-inflammatory and antimicrobial constituents from the leaves of *Villaria odorata*

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Three iridoids (**1–3**) and a sesquiterpenoid (**4**) were isolated from the leaves of *Villaria odorata*, a Philippine endemic Rubiaceae species. The structures were elucidated based on NMR and MS and in comparison with the literature. The anti-inflammatory activity of **1–4** was evaluated against COX-II, while the MIC and MBC of **3** and **4** were determined using the microtiter plate dilution assay.

Keywords: Rubiaceae, Villaria, antiinflammatory, antimicrobial, MIC, MBC, COX-II

Introduction

The family Rubiaceae is considered to be the fourth largest angiosperm family in the world. With more than 13,000 species distributed to 611 genera [1], this coffee family is significant source of interesting and biologically-active secondary metabolites. Diverse structures of natural products from Rubiaceae include the alkaloids, terpenoids, fatty acids, flavonoids, iridoids, steroids, coumarins, and quinones. Pharmacological activities displayed by these compounds range from cytotoxic, anti-infectives, hepatoprotective, antioxidants, anti-inflammatory, anti-Alzheimer, and analgesic activities among others.

One of the Rubiaceae genera endemic in the Philippines is the *Villaria* Rolfe. Belonging to the tribe Hypobathreae, it is distributed in the coastal areas and comprises of six endemic species which are either shrubs or small trees [2, 3]. These include *Villaria acutifolia*, *V. fasciculiflora*, *V. glomerata*, *V. leyetensis*, *V. odorata*, and *V. uniflora*. To date, only β-sitosterol, 4-hydroxybenzaldehyde, and villarinol have been identified from the genus *Villaria* [4]. In our continuing effort on the study of endemic and indigenous Philippine plants [5–7] including the family Rubiaceae [8, 9] with interesting biological activities, we have embarked on the isolation and biological evaluation of iridoids and a norsesquiterpenoid from the leaves of *V. odorata*. The COX-II inhibition and the antimicrobial activity of the isolated compounds are reported herein.

Materials and methods

Plant material. Fresh leaves of *V. odorata* were collected from Cagsiay Uno, Mauban, Quezon, Philippines in April 2011. The identity of the plant

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was confirmed by one of the authors (GJDA) and a voucher specimen (USTH 5033) was deposited at the UST Herbarium, Research Center for the Natural and Applied Sciences, University of Santo Tomas.

Extraction and isolation. Air-dried and ground leaves of *V. odorata* (1.18 kg) were soaked thrice for a total of 7.6 L dist. MeOH at room temperature and filtered. The combined filtrates were concentrated under reduced pressure to obtain the crude extract (70 g). The crude extract was suspended in water and partitioned sequentially with hexane (3 × 100 mL), CH_2Cl_2 (4 × 100 mL) and *n*-BuOH (3 × 50 mL) to afford the semi-crude extracts. The CH_2Cl_2 extract was subjected to silica gel gravity column chromatography (CC) using gradient of CH_2Cl_2/MeOH to yield four pooled sub-fractions (V_A, 139.8 mg; V_B, 84.8 mg; V_C, 307.3 mg; V_D, 374 mg). Fraction V_A was subjected to silica gel gravity CC to give fractions V_A1–V_A3. Fraction V_A1 was purified using silica gel flash CC (90% EtOAc in hexane) to afford compound **1** (10 mg, colorless oil). Fraction V_C was subjected to silica gel gravity CC (30% $CHCl_3$ in EtOAc) obtaining compound **2** (15 mg, colorless oil) and sub-fractions V_C1–V_C5. Fraction V_C5 was purified using silica gel flash CC (2% $CHCl_3$ in EtOAc) to afford compound **4** (6.5 mg, colorless oil). Fraction V_D was subjected to silica gel gravity CC using gradient of $CHCl_3$/MeOH to obtain sub-fractions V_D1–V_D4. Fraction $V_D1$was purified using silica gel flash CC (5% MeOH in EtOAc) to afford compound **2** (5 mg, colorless oil) and compound **3** (8 mg, colorless oil).

Determination of antimicrobial activity. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the different compounds were determined using microtiter plate dilution assay. Extracts were diluted to a concentration of 1 mg/mL, placed in microwells, then serially diluted (1:2) into eight wells to a final volume of 100 µL for each test organism. Three bacteria were used for the assay: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. A 100 µL of bacterial suspension (1.5×10^8 CFU/mL) was added to each well and incubated at 37°C for 24 h. The concentration in the last well with no growth after 24 h was reported as the MIC. All wells with no growth were then sub-cultured into nutrient agar (NA) plates to determine the MBC. The lowest concentration of extract which did not show bacterial growth in the NA plates after 24 h was reported as the MBC. All setups were done in triplicate for each of the compounds.

Determination of the COX percentage inhibition. The COX-II percentage inhibition of the compounds was determined using the microtiter plate colorimetric COX inhibitor screening assay kit. For the blank wells, 160 µL assay buffer, 10 µL heme, and 10 µL AR grade MeOH (used to dissolve the compound) were added. For the 100% initial activity wells, 150 µL assay buffer, 10 µL heme, 10 µL COX-II enzyme, and 10 µL AR grade MeOH were added. For the inhibitor wells, 150 µL assay buffer, 10 µL heme, 10 µL COX-II enzyme, and 10 µL of the compound dissolved in AR grade MeOH were added. The plate was shaken for 10 s and incubated for 5 min at 25°C. Then 20 µL colorimetric substrate solution and 20 µL arachidonic acid were added to the wells. The plate was shaken and incubated for precisely 2 min at 25°C before the absorbance was read at 590 nm.

RESULTS AND DISCUSSION

Structures of compounds 1–4. Chromatographic purification of the *V. odorata* extract led to the isolation and identification of the three iridoids morindolide (**1**), hydrophylin A (**2**), and hydrophylin B (**3**), and a norsesquiterpenoid, vomifoliol (**4**). The structures (Fig. 1) were elucidated based on 1D and 2D NMR, MS and by comparison with literature data.

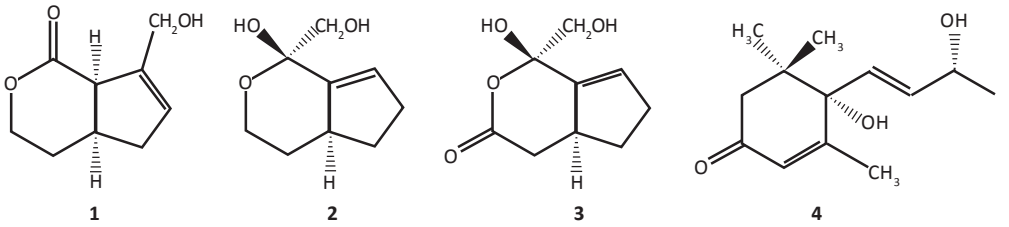


Figure 1. Isolated compounds from *V. odorata*

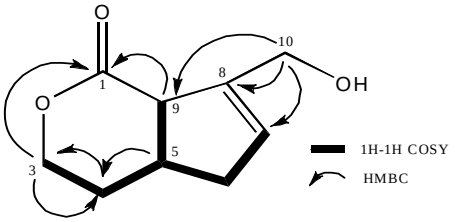
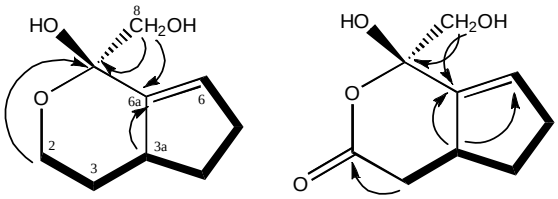


Figure 2. COSY and selected HMBC (H→C) correlations of **1**



Hydrophyllin A (2) Hydrophyllin B (3)

Figure 3. COSY and selected HMBC (H→C) in compounds **2** and **3**

Morindolide (**1**) was isolated as colorless oil. The ^{1}H- and ^{13}C-NMR (in $CDCl_3$) spectra showed the presence of an olefinic group (5.78, 1H, br s, H-7; 129.1, CH, C-7; 140.0, C, C-8), a lactone carbonyl (173.0, C-1), and two oxygenated methylenes (4.39, 1H, m, H-3a, 4.28, 1H, m, H-3b, 67.2, CH_2, C-3; 4.28, m, H_2-10, 60.6, CH_2, C-10). The ^{1}H-^{1}H COSY spectrum indicates a continuous spin system from H_3-H_4-H_5-H_6-H_7, and H_5-H_9 (Fig. 2). Significant HMBC correlations include H-3 (δ 4.39) with C-1 (δ 173.0); H-9 (δ 3.76) with C-1, C-7 (δ 129.1), and C-8 (δ 140.0); H-10 with C-7, C-8, and C-9 (Fig. 2). Thus, the structure was elucidated as shown in **1** and identified as morindolide [10].

Hydrophylin A (**2**) was isolated as colorless oil. The ^{1}H and ^{13}C NMR (in CD_3OD) indicated the presence of an olefinic group (δ 5.83, 1H, br s, H-6; δ 131.4, C-6; δ 143.9, C-6a), an acetal quaternary carbon (δ 100.0, C-7), and two oxygenated methylenes (δ 3.65, 1H, d, J=11.5, H-8a; δ 3.61, 1H, d, J=11.5, H-8b; δ 66.7, C-8) and (δ 3.88, 1H, m, H-2a; δ 3.59, 1H, m, H-2b; δ 67.8, C-2). Structure elucidation of **2** was based on 2D-NMR (Fig. 3). A continuous spin system in the COSY was observed from H_2-H_3-H_{3a}-H_4-H_5-H_6. Important HMBC correlations in **2** were observed as follows: H-2→C-7; H-6→C-7, C-6a; H-6→C-6a; H-5→C-6, C-6a. Hence, the structure of **2** was identified as hydrophylin A and was in exact match with the literature data [11].

Hydrophylin B (**3**) showed the presence of a carbonyl lactone (δ 179.8, C-2), an acetal carbon (δ 102.4, C-7), and an olefinic group (δ 5.99, 1H, br s, C-6; δ 133.5, C-6, δ 143.4, C-6a). 1H-1H COSY elaborated the H-3→H-3a→H-4→H-5→H-6 spin system. Significant HMBC correlations in **2** are shown in Fig. 3 as follows: H3→C-2, H3a→C-6, C-6a, H-8C-7, C-6a. Thus, **3** was depicted as hydrophylin B [11].

Compound **4** exhibited an NMR spectrum which indicated two olefinic groups, one coupled with a methyl (allylic coupling) (δ 5.91, 1H, br s, H-4; δ 127.0, C-4; δ 162.5, C-5; δ 1.90, 3H, d, J=1.5, H-

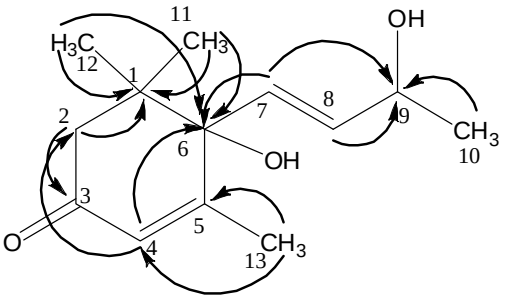


Figure 4. HMBC correlations (H→C) in compound **4**

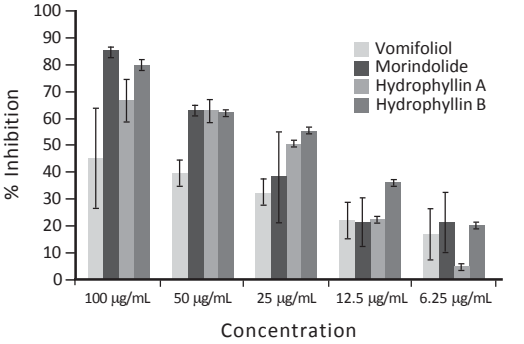


Figure 5. Anti-inflammatory activity of the isolated compounds

Table 1: Percentage inhibition of compounds 1-4 against COX-II

	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL
1	84.94 ± 0.88	62.73 ± 1.76	37.96 ± 16.84	20.98 ± 8.84	20.77 ± 11.00
2	66.25 ± 7.53	62.32 ± 1.76	50.09 ± 1.40	21.79 ± 0.71	4.28 ± 1.30
3	79.54 ± 1.16	62.16 ± 0.58	54.90 ± 0.85	35.67 ± 1.28	19.88 ± 0.58
4	44.58 ± 18.45	39.17 ± 4.52	31.77 ± 5.00	21.59 ± 6.70	16.40 ± 9.60

Table 2. Antimicrobial assay of Hydrophyllin A (3) and Hydrophyllin B (4)

Compound	MIC (µg/mL)			MBC (µg/mL)		
	E. coli	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*
3	125	250	250	500	>500	500
4	125	250	250	500	500	500

13; δ 18.9, C-13); and the other having a *trans* orientation (δ 5.79, 1H, d, J=15.7, H-7; δ 5.86, 1H, dd, J=15.7, 5.1, H-8; δ 135.7, C-7; δ 129.0, C-8). A lactone carbonyl was observed at d 197.9 (C-3), an oxygenated methine at δ 4.42, 1H, m, H-9; δ 68.1, C-9; while methyl groups were observed at δ 1.01 (3H, s, H-11), δ 1.09 (3H, s, H-12), and δ 1.31 (3H, d, J=6.3, H-10). Analysis of the COSY spectrum showed a correlation of H-7→H-8→H-9→H-10 protons. HMBC correlations (Fig. 4) were deduced as follows: H-12 and H-11 with C-1 (δ 41.1), C-6 (δ 79.0); the H-2 geminal protons (δ 2.25, 1H, d, J=16.8 and 2.45, 1H, d, J=16.8) with C-1, C-3, C-4; H-4 with C-6. Hydroxyl groups were assigned to C-9 and C-6 as evidenced by the low-resolution electron-impact mass spectra which exhibited M^+ at *m/z* 224, the base peak at *m/z* 83 (100%), and $[M-H_2O]^+$ at *m/z* 206 (1.5%) and the carbon signals at **d** 68.1 (CH unit) and 79.0 (C unit). Thus, the structure of **4** was elucidated as vomifoliol [12].

Biological evaluation on the isolated compounds. The family Rubiaceae is known to contain iridoids and its glycosides with significant anti-inflammatory activity [13]. The isolated metabolites in this study were tested for their inhibition against COX-II, an enzyme which is responsible for pain and inflammation (Fig. 5, Table 1). Results showed (*n*=3) that increasing the concentration also increases their inhibition. At 100 µg/mL, morindolide (**1**) showed

the highest inhibition at 84.94% ± 0.88 followed by hydrophylin B (**3**) with 79.54% ± 1.16 inhibition. The synthetic compound DuP-697 was used as the positive control which showed a 94.38% ± 3.15 inhibition at 50 μg/mL.

The MIC and MBC (Table 2) for **3** and **4** were also determined using the microtiter plate dilution assay. The MIC refers to the lowest concentration of a compound that will inhibit the visible growth of a microorganism after overnight incubation while MBC refers to the lowest concentration of a compound that will prevent the growth of an organism after sub-culture in an antibiotic-free media [14]. Results revealed a moderate activity for both compounds **3** and **4** against the three organisms as indicated by their MIC values. Our previous report on **4** also showed a moderate antibacterial activity against *Klebsiella oxytoca* [9].

These results showed that *V. odorata*, an endemic Philippine plant, to be a promising source of biologically-active secondary metabolites. These may serve as lead compounds which can be used in synthesis-structure activity relationship studies.

Conclusion

Phytochemical investigation of *V. odorata*, a Philippine endemic Rubiaceae species, led to the identification of three iridoids, morindolide (**1**), hydrophylin A (**2**), and hydrophylin B (**3**) and a norsesquiterpenoid, vomifoliol (**4**). All compounds were identified for the first time from *V. odorata*. Evaluation of the anti-inflammatory and antimicrobial activities of compounds **1–4** showed the promising potential of *V. odorata* as new sources of biologically-active natural products.

Acknowledgment

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