Full Length Research Paper

Isolation and characterization of antimicrobial, anti-inflammatory and chemopreventive flavones from *Premna odorata* Blanco

Lunesa C. Pinzon[1]*, Mylene M. Uy[1], Kung Hong Sze[2], Mingfu Wang[3] and Ivan Keung Chu[2]

[1]Department of Chemistry, College of Science and Mathematics, MSU-Iligan Institute of Technology, Iligan City, Philippines.
[2]Department of Chemistry, Faculty of Science, University of Hong Kong, Hong Kong SAR.
[3]School of Biological Sciences, Faculty of Science, University of Hong Kong, Hong Kong SAR.

Premna odorata Blanco (*Verbenaceae*) is a native tree of the Philippines where its leaves are used traditionally for vaginal irrigation and tuberculosis. It is one of the seven components of a commercialized Philippine herbal preparation called "Pito-Pito". Its medicinal uses, however, have not been scientifically validated. This tree is not commonly cultivated and thrive in the less accessible limestone forests of the Philippines. Solvent partitioning and fractionation of the ethanolic crude extract of the leaves isolated two yellow amorphous powders. The identities of these compounds were determined by LC/MS/MS and NMR spectroscopic analyses, and their spectra were compared with literature data. The isolates were flavone aglycones which were the widespread acacetin and the non-widespread diosmetin. These flavones were isolated from the *P. odorata* for the first time ever. They had been reported by earlier studies to exhibit medicinal properties as antimicrobial, anti-inflammatory and chemopreventive. Thus, the current study has provided a scientific evidence of the medicinal properties of the leaves of *P. odorata* that could become the popular basis for the plant's sustainable use, conservation and cultivation.

Key words: *Premna odorata* Blanco (*Verbenaceae*), "pito-pito", "alagau", antimicrobial, anti-inflammatory, chemopreventive, flavones, diosmetin, acacetin.

INTRODUCTION

Premna odorata Blanco (*Verbenaceae*) is a native of temperate and tropical Asia which includes the Philippines. It is also known by a few other scientific names such as, *P. curranii* H. Lam., *P. oblongata* Miq. var. *puberula* H. Lam., *P. pubescens* Blume. var. *odorata* H. Lam., *P. serratifolia* Blanco and *P. vestita* Schauer. It has many Philippine names, but is more popularly known as "alagau" and "agbau". In the Philippines, the decoction of the leaves is used for vaginal irrigation and tuberculosis (Quisumbing, 1978). It is one of the seven components of a commercialized Philippine herbal preparation called "Pito-Pito". The ethnomedicinal uses of *P. odorata*, however, have not been scientifically validated. Apart from its uncommonly known ethnomedicinal uses, this tree does not have much economic value and is generally cut and replaced with plants that are perceived to be more profitable. For this reason, even though it is a native plant, it is not generally cultivated and not commonly found in populated areas, but thrive in the less accessible secondary limestone forests of the Philippines. The isolation of bioactive and medicinal compounds from the leaves of *P. odorata* would provide a scientific evidence of the medicinal

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*Corresponding author. E-mail: lunesa.pinzon@g.msuiit.edu.ph
Tel:/Fax: (63)-(63)-221-4068.

properties of the plant. The validated medicinal properties, in turn, could become the popular motivation for the plant's sustainable use, conservation and cultivation. The current study was able to isolate two flavone aglycones, diosmetin and acacetin, from the crude EtOH extract of the leaves.

Diosmetin or 5,7,3'-trihydroxy-4'-methoxyflavone was classified as a non-widespread flavone (Valant-Vetschera and Wollenweber, 2006). Many earlier studies have reported its bioactivities. It inhibited 3H-dopamine uptake in control and differentiated neuroblastoma cells and in small-cell lung carcinoma cells. It also inhibited 3H-serotonin uptake in both cell types. These inhibitory effects could be responsible for the increased vascular tone observed *in vivo* after treatment with diosmetin and its glycoside, diosmin, as vasotonic agents (Sher et al., 1992). As an antimicrobial agent, diosmetin exhibited the MIC at 25 µg/mL against *B. subtilis* and the MIC at 50 µg/mL against the fungus *Trichophyton rubrum*, the most common cause of athlete's foot (Meng et al., 2000). It down-regulated the enzyme cyclooxygenase-2 and so, could be an anti-inflammatory agent (Lopez-Posadas et al., 2008). It increased osteoblast differentiation and so, could be a potential agent for treating osteoporosis (Hsu and Kuo, 2008). At 60 µM, it exhibited 55% inhibition of the enzyme α-□phosphatidylinositol-3-kinase (Agullo et al., 1997). At 6 µM, it exhibited 70% inhibition of the enzyme 17-β □hydroxysteroid dehydrogenase type 1. As a chemopreventive compound, diosmetin directly inhibited the activity of the enzyme cytochrome P450 1A1 (Ciolino et al., 1998). It blocked apoptosis that was induced by dimethylbenz(a)anthracene (Ciolino et al., 2002). It exerted cytostatic effects on cell cycle progression and proliferation of breast cancer cells (Androutsopoulos et al., 2009).

Acacetin or 5,7-dihydroxy-4'-methoxy-flavone was classified as a widespread flavone (Valant-Vetschera and Wollenweber, 2006). Several bioactivites of acacetin have been reported by earlier studies. As an antifungal agent, acacetin had a comparable potency with the antifungal drug Amphotericin B against *Candida glabrata* KCTC 7219, *Candida tropicalis* KCTC 7725 and *C. tropicalis* KCTC 7212 (Rahman and Monn, 2007). It was reported to be a promising agent for the treatment of atrial fibrillation (Li et al., 2008).

As a chemopreventive agent, acacetin had antiproliferative effect on human liver cancer cell line HepG2 (Hsu et al., 2004a) and in human nonsmall cell lung cancer A549 cells (Hsu et al., 2004b). It was capable of preventing inflammation-associated tumorigenesis (Pan et al., 2006). It induced apoptosis of human breast cancer MCF-7 (Shim et al., 2007). It inhibited the invasion and migration of human prostate cancer DU145 (Shen et al., 2009). It inhibited TPA-induced MMP-2 and u-PA expressions of human lung cancer cells (Fong et al., 2010).

MATERIALS AND METHODS

Plant material

Green, healthy and mature leaves of *P. odorata* were collected during the pre-flowering phase of a single tree in Luinab, Iligan City, Philippines; with geographical coordinates and elevation as: 8° 14' 29.73" N; 124° 16' 03.87" E; 36 m elevation. The identification of the plant was done by Prof. Carmelita Garcia-Hansel, a botanist from the Mindanao State University, Marawi City, Philippines. A voucher specimen (Voucher No. 1051) is deposited at the Natural Science Museum of MSU-Iligan Institute of Technology, Iligan City, Philippines. Figure 1 shows the leaves of *P. odorata* at its pre-flowering phase.

Chemicals

Except for the technical grade 95% EtOH, the various brands of solvents used were of analytical or HPLC grade.

Extraction and isolation

Air-dried leaves, weighing 5 k, were soaked in absolute ethanol for 48 h. The crude ethanolic extract was filtered, concentrated with a rotary evaporator at temperatures below 55°C, suspended in water, and solvent-partitioned sequentially using Hex, DCM and EtOAc. Isolation of the two flavones was done by two rounds of gravity open-column chromatography using Amberlite XAD16 and silica gel (200 to 300 mesh). With Amberlite XAD16, chromatography was by gradient elution using mixtures of 95% EtOH and water; starting with EtOH:H$_2$O (10:90), increasing EtOH by 10%, and ending with 95% EtOH. When silica gel was used, gradient elution was done with Hex, DCM, EtOAc and their mixtures; starting with Hex-DCM (50:1). Thin layer chromatography (TLC) was done to monitor the fractionation. The developed chromatograms were visualized with 5% sulfuric acid in ethanol, with which the isolated flavonoids were yellow.

Purification of the Isolates

Chromatographic fractions that showed yellow spots on their TLC plates were pooled together, evaporated dry and resuspended in MeOH to yield an orange colloidal mixture. The resulting colloidal mixture was suction filtered, using MeOH to wash down the non-targeted plant components, thus leaving behind the purified pale yellow isolate. The purification process was repeated until the resulting colloidal mixture was no longer orange, but dark brown. The collected filtrate was evaporated for subsequent chromatography. The purified isolate was also collected, dried in a vaccuum dessicator, weighed and spectroscopically analyzed.

HPLC

HPLC was used to assess the purity of isolates. The analytical HPLC system used consisted of Shimadzu LC-20AT series pumping system, SIL-20A automatic injector, SPD-M20A UV visible detector set at 285 nm and Class-Vp chromatography data station software with the analytical column, Ultimate XB-C18 column (250 × 4.6 mm, 5 µm) which was purchased from Welch Materials, Inc. of Shanghai, People's Republic of China. The analyses were carried out on an Ultimate XB-C18 column (250 × 4.6 mm, 5 µm). The mobile phase was 0.2% formic acid (solvent A) and acetonitrile

Figure 1. Leaves of *P. odorata.*

(solvent B). The gradient elution was as follows: 20% B for 0 to 10 min, 20 to 40% B for 10 to 20 min, 40 to 60% B for 20 to 30 min, 60 to 80% B for 30 to 40 min, 80 to 95% B. The post-running time was 10 min. Flow-rate was set at 1.0 mL/min. The UV-Vis detector was set at 254 nm. The column was maintained at room temperature.

LC/MS/MS analysis

1100 LC system with one well-plate auto sampler. Samples were separated on a 150 x 4.60 mm, 5 micron, Jupiter C18 column (Phenomenex). The mobile phase consisted of 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B). Separations were effected by a gradient using a flow rate of 0.2 mL/min as follows: Starting with 5% B, 0 to 5 min; 5 to 50% B, 5 to 15 min; 50 to 80% B, 15 to 25 min; 80% B, 25 to 35 min.

Reversed-phase liquid chromatography was performed on agilent column eluent was directed into a QTrap hybrid triple-quadrupole mass spectrometer equipped with a turbo ionspray source (QTrap 2000, Applied Biosystems/MDS Sciex). Samples were run in positive ion mode using optimized parameters as follows: ion spray voltage is 5 kV, source temperature was 450°C, nebulizer gas was 25 (arbitrary units), curtain gas was 10 (arbitrary units), collision gas was 35 (arbitrary units). CID spectra were acquired using information-dependent acquisition (IDA); a full scan over a mass range of m/z 100 to 1000 as a survey scan, followed by two MS/MS scans of the most abundant peaks over a mass range of m/z 50 to 1000. Data were acquired using the Analyst 1.4.1® software.

NMR Spectroscopy

The purified isolates were dissolved in DMSO-d_6. The ^{1}H (500 MHz), ^{13}C (125 MHz), DEPT-135, and 2 D-NMR spectra (1H-1H COSY, 1H-1H TOCSY, 1H-1H NOESY and HETCOR) were acquired on a Bruker DRX500 NMR spectrometer equipped with a BBIz 5mm probe. The 2D-HMBC spectra were acquired on a Bruker AVANCE 600 instrument with cyroprobe. TOCSY spectra were recorded with spin-locking times of 70 ms. The NOESY spectra were recorded using mixing times of 300 m. A total of 2048 complex data points with 512 complex increments were collected for each 2D-NMR experiment. The spectra were processed within the Xwin-NMR software, where HETCOR and HMBC raw data were zero filled to 4 K x 2 K prior to Fourier transformation. Spectra were plotted with the Xwin-plot software.

Fluorescence

The purified isolates were dissolved in DMSO and analyzed for

Table 1. Various spectroscopic data on the isolates.

Spectroscopic analyses	Isolate ID1	Isolate ID2
ESI MS		
(Positive ion mode); (M+H),	Most abundant peak at m/z 301.242.	Most abundant peak at m/z 285.0697.
Fluorescence (in DMSO):	544.0 nm (excitation) 574.0 nm (relaxation)	404.0 nm (excitation) 468.0 nm (relaxation)
^{1}H-nmr (in DMSO-d$_6$, 500 MHz)	δ 12.935 (1 H, s), 9.563 (1 H, broad s), 7.559 (1 H, d, J = 8.0 Hz), 7.441 (1 H, s), 7.110 (1 H, d, J = 8.5 Hz),), 6.762 (1 H, s), 6.498 (1 H, s), 6.224 (1 H, s), 3.884 (3 H, s)	δ12.91 (1 H, s), 8.03 (2 H, d, J = 2.1 Hz), 7.00 (2 H, d, J = 2.1 Hz), 6.85 (1 H, s), 6.49 (1 H, d, J = 1 Hz), 6.19 (1 H, d, J = 1 Hz), 3.71 (3 H, s)
^{13}C-nmr (in DMSO-d$_6$, 125 MHz)	δ 181.777, 164.301, 163.639, 161.520, 157.417, 123.067, 118.812, 112.99, 112.266, 103.821, 103.580, 98.981, 94.028, 55.852.	δ 181.717, 164.470, 163.242, 162.282, 161.430, 157.352, 128.290, 122.845, 114.567, 103.647, 103.516, 98.942, 94.048, 55.538.
DEPT-135 (in CDCl$_3$, 500 MHz)	(+ ; for CH- and C-types) δ118.564, 112.729, 112.005, 103.324, 98.729, 93.783, 55.599.	(+ ; for CH- and C-types) δ128.033, 114.308, 103.260, 98.654, 93.774, 55.280.

fluorescence with Jasco FP-777 Spectrofluorometer.

RESULTS

Solvent partitioning of EtOH crude extract of the leaves (5 k) resulted in four fractions weighing 73 g of the Hex semicrude extract, 92 g of the DCM semicrude extract, 24 g of the EtOAc semicrude extract, and 125 g of the aqueous semicrude extract. On the first round of adsorption chromatography of the DCM semicrude extract with Amberlite XAD16, more retained fractions showed a yellow spot on their TLC plates. These fractions were pooled together and through the subsequent purification process, a total of 500 mg of a yellow powder was collected. This powder was designated as isolate ID1 and was later spectroscopically identified as the flavone aglycone diosmetin. Table 1 shows various spectroscopic data on isolate ID1. The R$_f$ of isolate ID1 with EtOAc was 0.75. The collected filtrate resulting from the purification of isolate ID1 was dried and chromatographed for the second time by normal-phase partition chromatography. The more retained fractions showed a yellow spot on their TLC plates. These fractions were pooled together and through the subsequent purification process, a total of 50 mg of a yellow powder was collected. The collected yellow powder weighed 50 mg and was designated as isolate ID2. It was later spectroscopically identified as the flavone aglycone acacetin.

(Table 1) shows various spectroscopic data on the isolates. The R$_f$ value of isolate ID2 with EtOAc was 0.86.

DISCUSSION

The ESI mass spectra (positive ion mode) of isolate ID1 showed that the most abundant peak was at m/z 301.35 (M+H), so the molecular ion must be 300 and was calculated for the molecular formula of $C_{16}H_{12}O_6$. Two likely flavonoidal structures were initially suggested; a flavone and a flavonol. The ^{1}H-nmr spectra of ID1 was not much of a help to distinguish between a flavone or a flavonol. Its ^{13}C-nmr spectra however, clearly distinguished between the two types of flavonoids. The ^{13}C-nmr spectra of ID1 showed no peaks around δ136 to 139.0 ppm that might correspond to C-3 in flavonols, nor peaks at δ172 to 177 for C-4. On the other hand, characteristic peaks of flavones for the C-3 at δ103.0 to 111.8 and C-4 at δ177.3 to 184.0 (Bohm, 1998) were shown on the spectra of isolate ID1. Literature search showed that the NMR spectra of isolate ID1 were basically the same as that of diosmetin. Only one peak (Table 2) was observed for the two hydroxyl protons at C-5 and C-7. This was because, in general, when a compound contained several hydroxyl protons, only one signal at the average position was observed due to rapid exchange. However, a separate peak for the hydroxyl proton at C-3' was detected. This could be because the C-3' hydroxyl proton was far enough from those at C-5 and C-7 and, as in most cases with DMSO, the exchange could be slow (Pretsch et al., 2000). Table 3 shows the carbon-proton correlations in the isolate ID1 based on its HMBC spectra and Figure 2 shows the long-range couplings of C-4 and C-4' with various protons. The ESI mass spectra (positive ion mode) of isolate ID2 showed

Table 2. Carbon and proton chemical shifts of isolate ID1 (Diosmetin) in DMSO-d_6.

Carbon shifts; 125 MHz (δ ppm)	Carbon position	Proton shifts; 500 MHz (δ ppm)	Number of proton (s)	Proton position
181.777	C-4	*12.935; s	1 H	5-OH
164.301	C-2		1 H	7-OH
163.639	C-7	9.563; br s	1 H	3'-OH
161.520	C-5	7.559; d, (J = 8 Hz)	1 H	H-6'
157.417	C-9	7.441; br s	1 H	H-2'
151.257	C-4'	7.110; d, (J = 8.54 Hz)	1 H	H-5'
146.869	C-3'	6.762; s	1 H	H-3
123.067	C-1'	6.498; s	1 H	H-8
118.812	C-6'	6.224; s	1 H	H-6
112.992	C-2'	3.884; s	3 H	4'-OCH$_3$
112.266	C-5'			
103.821	C-10			

*only one peak at the average position was observed for the two hydroxyl protons at C-5 and C-7 due to rapid exchange.

Table 3. The carbons and protons of the isolate ID1 (DIOSMETIN) with long-range couplings or HMBC correlations.

Carbon position	HMBC correlations (600 MHz)
C-4	OH-5 ; H-3 ; H-8 ; H-6
C-5	OH-5 ; H-6
C-9	H-8
C-4'	H-6' ; H-2' ; H-5'
C-3'	H-6' ; H-2' ; H-5'
C-1'	H-2' ; H-5' ; H-3
C-6'	H-2'
C-2'	H-6'
C-5'	H-6'
C-10	OH-5 ; H-8 ; H-6
C-3	OH-5 ; H-3
C-6	OH-5 ; H-8 ; H-6
C-8	H-8 ; H-6

that the most abundant peak was at m/z 285.07 (M+H), so the molecular ion must be 284 and was calculated for the molecular formula of $C_{16}H_{12}O_5$. The ^{13}C-nmr spectra also showed the characteristic carbon chemical shifts for C-3 and C-4 of a flavone (Bohm, 1998). Library search showed that isolate ID2 had basically the same spectra with acacetin. Only one peak (Table 4) was observed for the two hydroxyl protons at C-5 and C-7 due to rapid exchange. The structure of diosmetin (Figure 3) contains one more hydroxyl group than that of acacetin. This extra hydroxyl group at C-3' position makes diosmetin more polar than acacetin. The greater polarity of diosmetin over acacetin is manifested by its lower R_f value with EtOAc of 0.75, while that of acacetin is 0.86.

The scientifically validated therapeutic properties of both Diosmetin and Acacetin, as reported by other earlier studies, are not directly related to the traditional use of

the leaves of *P. odorata* as a vaginal wash and for the treatment of tuberculosis. The medicinal compounds that might be directly related to the traditional use of *P. odorata* could be present in the other semicrude extracts and in the other fractions from the DCM semicrude extract. Finding Diosmetin and Acacetin in the leaves makes it reasonable to report that *P. odorata* is indeed a medicinal plant. Diosmetin, in particular, has been commercially available for years as the glycoside diosmin. It is indicated as a vasotonic agent for the treatment of varicose veins, hemmorrhoids and other venous diseases. It is marketed under several brandnames. Veno-active drugs (VAD) based on diosmetin were a subject of an international consensus statement among medical specialists on hemorheology and microcirculation. The final resolution was that the VAD are safe and effective and may be applied in chronic
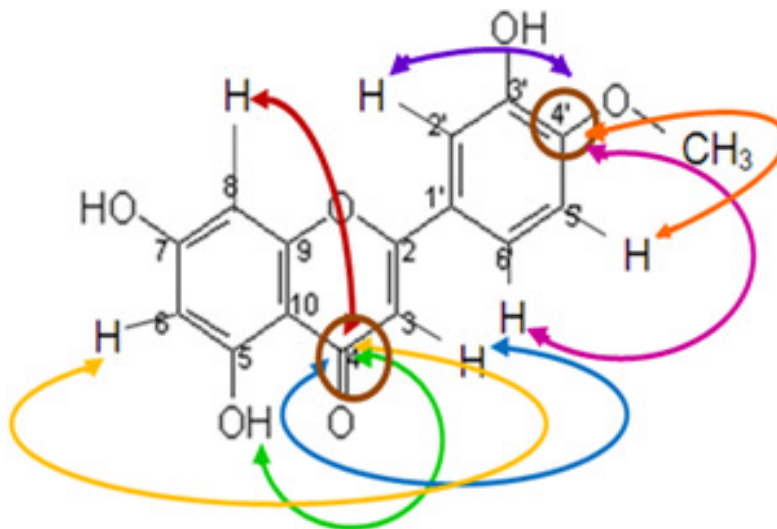
Figure 2. Long-range couplings of C-4 and C-4' with various protons based on the HMBC spectra of the isolate ID1 (Diosmetin).

Table 4. Carbon and proton chemical shifts of isolate ID2 (Acacetin) in DMSO-d_6.

Carbon shifts; 125 MHz (δ ppm)	Number of carbon(s)	Carbon position	Proton shifts; 500 MHz (δ ppm)	Number of proton(s)	Proton position
181.717	1C	C-4	*12.91; s	1 H	5-OH
164.470	1C	C-7		1 H	7-OH
163.242	1C	C-2	8.03; d,(J = 2.1 Hz)	2 H	H-2',6'
162.282	1C	C-4'	7.11; d,(J = 2.1 Hz)	2 H	H-3',5'
161.430	1C	C-5	6.85; s	1 H	H-3
157.352	1C	C-9	6.49; d,(J = 1 Hz)	1 H	H-8
128.290	2C	C-2', C-6'	6.19; d, (J = 1 Hz)	1 H	H-6
122.845	1C	C-1'	3.71; s	3 H	4'-OCH$_3$
114.567	2C	C-3', C-5'			
103.647	1C	C-10			
103.516	1C	C-3			
98.942	1C	C-6			
94.048	1C	C-8			
55.538	1C	4'-OCH3			

*only one peak at the average position was observed for the two hydroxyl protons at C-5 and C-7 due to rapid exchange.

venous disease, or chronic venous insufficiency, when symptomatic (Ramelet *et al.*, 2005). Diosmetin, being not widespread in nature and commercially available as a nutraceutical, renders the plant too valuable to ignore medicinally and agriculturally. The plant itself should be extensively promoted as a medicinal plant, so that it would be sustainably used, conserved and cultivated.

the leaves supports the use of *P. odorata* as a medicinal plant. Diosmetin, Acacetin and the unidentified compounds in the other fractions could be investigated for significant bioactivities, particularly in relation to the plant's ethnomedicinal use. Moreover, an efficient and healthy method of extracting and purifying Diosmetin or Diosmin from the leaves of *P. odorata* could be developed.

CONCLUSION AND RECOMMENDATIONS

The presence of Diosmetin and Acacetin, which are antimicrobial, anti-inflammatory and chemopreventive, in
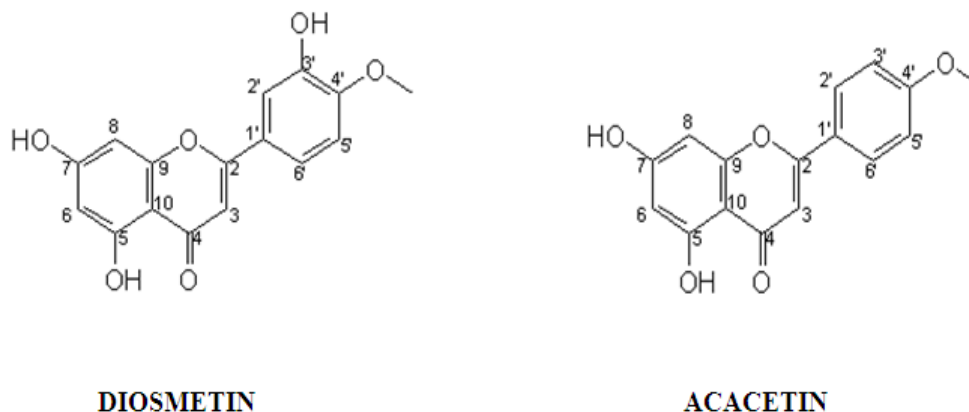
Figure 3. Structures of diosmetin and acacetin.

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