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ORIGINAL ARTICLE

Cytotoxic Compounds from *Wrightia pubescens* (R.Br.)

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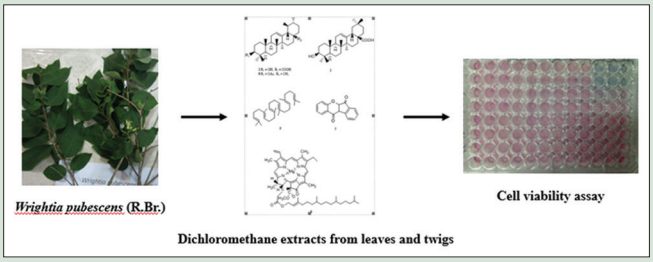
ABSTRACT

Background: Mixtures of ursolic acid (**1**) and oleanolic acid (**2**) (1:1 and 1:2), oleanolic acid (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**) were isolated from the dichloromethane (CH_2Cl_2) extracts of the leaves and twigs of *Wrightia pubescens* (R.Br.). **Objectives:** To test for the cytotoxicity potentials of **1**–**6**. **Materials and Methods:** The antiproliferative activities of **1**–**6** against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast neonatal (HDFn), were evaluated using the PrestoBlue® cell viability assay. **Results:** Compounds **4**, **1** and **2** (1:2), **2**, **1** and **2** (1:1), and **5** exhibited the most cytotoxic effects against HT-29 with half maximal inhibitory concentration (IC_{50}) values of 0.68, 0.74, 0.89, 1.70, and 4.07 μg/mL, respectively. Comparing **2** with its 1:1 mixture with **1** (IC_{50} = 1.70 and 7.18 μg/mL for HT-29 and HCT-116, respectively) and 1:2 mixture with **1** (0.74 and 3.46 μg/mL for HT-29 and HCT-116, respectively), **2** also showed strong cytotoxic potential against HT-29 and HCT-116 (0.89 and 2.33 μg/mL, respectively). Unlike the mixtures which exhibited low effects on MCF-7 (IC_{50} = 20.75 and 30.06 μg/mL for 1:1 and 1:2, respectively), **2** showed moderate activity against MCF-7 (10.99 μg/mL). Compound **6** showed the highest cytotoxicity against HCT-116 (IC_{50} = 4.07 μg/mL). **Conclusion:** Mixtures of **1** and **2** (1:1 and 1:2), **2**, **3**, **4**, **5**, and **6** from the CH_2Cl_2 extracts of the leaves and twigs of *W. pubescens* (R.Br.) exhibited varying cytotoxic activities. All the compounds except **6** exhibited the strongest cytotoxic effects against HT-29. On the other hand, **6** was most cytotoxic against HCT-116. Overall, the toxicities of **1**–**6** were highest against HT-29, followed by HCT-116 and MCF-7. All the compounds showed varying activities against HDFn (IC_{50} <30 μg/mL).

Key words: Apocynaceae, chlorophyll a, cytotoxicity, half maximal inhibitory concentration, HCT-116, HT-29, HDFn, MCF-7, oleanolic acid, PrestoBlue® cell viability assay, squalene, ursolic acid, *Wrightia pubescens* R. Brown, wrightiadione, α-amyrin acetate

SUMMARY

Mixtures of ursolic acid (**1**) and oleanolic acid (**2**) (1:1 and 1:2), oleanolic acid (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**), isolated from the dichloromethane extracts of the leaves and twigs of *Wrightia pubescens* (R.Br.), showed varying cytotoxic activities against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast-neonatal (HDFn), as evaluated using the PrestoBlue® cell viability assay.



Abbreviation Used: IC_{50}: Half maximal inhibitory concentration.

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INTRODUCTION

Wrightia pubescens (R.Br.), of the family Apocynaceae, is one of the eight known species of *Wrightia* in Malaysia.[1] Locally found in the Philippines where it is known as "lanete," it is also abundant in mainland China, India, and Australia. *W. pubescens* is a medium-sized to fairly-large tree which can grow up to 35-m tall in deciduous lowland thickets and forests.[2,3] The wood is normally used to make furniture, paper, pencil, and musical instruments.[4] In traditional medicine, the root and bark extracts from the tree are employed to treat scrofula and rheumatic arthralgia,[3] and the latex is used against dysentery.[5] In Chinese medicine, preparations containing *W. pubescens* are used to treat acute upper respiratory infection in children,[6] intractable hiccups,[7,8] and osteoarthritis.[9] The plant's latex has been shown to exhibit inhibitory activities on prostaglandin E2 production and cyclooxygenase 2 protein expression in RAW 264.7 mouse macrophages, and these were associated to the anti-inflammatory and antinociceptive properties of the plant.[10]

This study is part of our research on the chemical constituents and bioactivities of trees found at the riparian forest and reforested area of De La Salle University Laguna Campus, Laguna, Philippines. The other trees studied included *Calophyllum inophyllum* Linn.,[11] *Cordia dichotoma* G. Forst,[12] *Dysoxylum gaudichaudianum* (A. Juss.) Miq.,[13,14] *Kibatalia gitingensis* (Elm.) Woodson,[15,16] and *Pipturus arborescens* (Link) C. B. Rob.[17,18] Studies on the chemical constituents and cytotoxic properties of compounds isolated from the dichloromethane (CH_2Cl_2) extracts of these plants have been reported previously.[11-18] In an earlier study on *W. pubescens* from the same site, the isolation and identification of ursolic acid, oleanolic acid, squalene, β-sitosterol, and chlorophyll a from the leaves; and ursolic acid, oleanolic acid, α-amyrin acetate, and wrightiadione from the twigs were reported [Figure 1].[19,20] We report herein the results of the cytotoxicity studies on the following compounds

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from the leaves and twigs of *W. pubescens*: ursolic acid (**1**) and oleanolic acid (**2**) in a 1:1 ratio, and **1** and **2** in a **1**:**2** ratio, (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**).

MATERIALS AND METHODS

Sample collection

Samples of leaves and twigs of *W. pubescens* (R.Br.) were collected from the DLSU Laguna Campus riparian forest in February 2014. The samples were authenticated previously and deposited at DLSU with collection number #915.

Isolation and structure elucidation

The isolation and structure elucidation of **1**–**6** from the leaves and twigs of *W. pubescens* were reported previously.[19,20]

Preparation of compounds for cytotoxicity tests

The compounds (**1**–**6**) from *W. pubescens* were dissolved in dimethyl sulfoxide (DMSO) to make a 4 mg/mL stock solution. Working solutions were prepared in complete growth medium to a final nontoxic DMSO concentration of 0.1%.

Maintenance and preparation of cell lines for cytotoxicity tests

The effects on the cell proliferation of **1**–**6** from the dichloromethane extracts of *W. pubescens* were tested on the following human cell lines: breast cancer (MCF-7) and colon cancer (HCT-116 and HT-29) (ATCC, Manassas, Virginia, USA), and human dermal fibroblast-neonatal (HDFn; Invitrogen Life Technologies, USA), which are routinely maintained at the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Science and Environmental Research, De La Salle University, Manila, Philippines. Following standard procedures,[21,22] cells were grown in Dulbecco's Modified Eagle Medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1X antibiotic-antimycotic (Gibco, USA) and kept in an incubator (37°C, 5% CO_2, 98% humidity). At about 80% confluence, the monolayers were prepared for cell counting and inoculation. The cells were washed with phosphate-buffered saline (pH 7.4, Gibco, USA), trypsinized with 0.05% Trypsin-EDTA (Gibco, USA), and resuspended with fresh complete media. Cells were counted following standard trypan blue exclusion method[21] using 0.4% Trypan Blue Solution (Gibco, USA). Cells were seeded in 100 µL aliquots into a 96-well microtiter plate (Falcon, USA) using a final inoculation density of 1×10^4 viable cells/well. The plates were further incubated overnight (37°C, 5% CO_2, 98% humidity) until cell attachment was achieved. These monolayer cultures were used for the cytotoxicity studies as described below.

Cell viability assay

The cytotoxicity of the *W. pubescens* compounds was determined in an *in vitro* cell viability test using PrestoBlue (Molecular Probes, Invitrogen, USA). This bioassay is based on the ability of viable cells with active enzymes, mitochondrial reductases of the electron transport chain, to convert the resazurin dye (blue and nonfluorescent) to resorufin (red and highly fluorescent). The conversion is proportional to the number of metabolically active cells and is determined quantitatively using absorbance or fluorescence measurements. To the monolayers in the microtiter plate, 100 µL of filter – sterilized **1**–**6** were added to corresponding wells at two-fold serial dilutions to make final screening concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 µg/mL, respectively.[22] Wells with no compound added served as negative controls, wells with Zeocin™ (Gibco, USA) served as positive controls, and wells containing only cell culture media were used to correct for background color. The cells were further incubated (37°C, 5% CO_2, 98% humidity) for 4 days. Ten microliters of PrestoBlue® was added to each well. The cells were further incubated (37°C, 5% CO_2, 98% humidity) for 2 h. Absorbance was measured using BioTek ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., USA) at 570 nm and normalized to 600 nm values (reference wavelength). Absorbance readings were used to calculate for the cell viability for each compound concentration following the equation below.

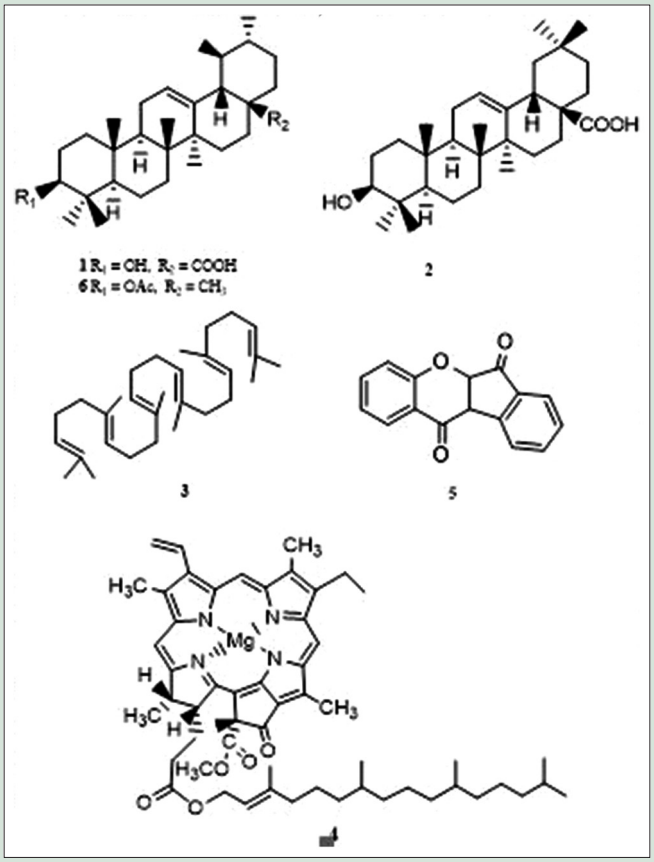

Figure 1: Chemical structures of ursolic acid (**1**), oleanolic acid (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**) from *Wrightia pubescens*

$$\text{Cell viability}\,(\%) = \frac{(\text{Absorbance of Treated Sample} - \text{Absorbance of Blank})}{(\text{Absorbance of Negative Control} - \text{Absorbance of Blank})} \times 100$$

Nonlinear regression and statistical analyses were done using GraphPad Prism 7.02 (GraphPad Software, Inc., USA) to extrapolate the half maximal inhibitory concentration (IC_{50}), the concentration of the compound which resulted in a 50% reduction in cell viability. The cytotoxicity (antiproliferative potential) of **1**–**6** was expressed as IC_{50} values. All tests were performed in triplicates, and data were shown as means ± standard error of mean. The extra sum-of-squares *F*-test was used to evaluate the differences in the best-fit parameter (half maximal inhibitory concentration) among data sets (treatments) and to determine the differences among dose-response curve fits following the software's manual. One-way ANOVA was used to determine differences in IC_{50}

under different treatments, followed by Tukey's multiple comparison *post hoc* test to further evaluate differences between pairs of data. The results were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Ursolic acid (**1**) and oleanolic acid (**2**) (1:1), **1** and **2** (1:2), **2**, squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**), isolated from the dichloromethane extracts of the leaves and twigs of *W. pubescens*[19,20] were evaluated for their antiproliferative activities against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a human normal cell line, HDFn, using the *in vitro* PrestoBlue cell viability assay. Zeocin, a known anticancer drug, was used as positive control. The percentage cell viability as a function of the logarithmic values of compound concentration is shown in Figures 2 and 3. Most of the curves follow the typical sigmoidal curve which is characteristic of an inhibitory dose-response relationship between treatments and cell viability. Figure 2 shows the antiproliferative effects per cell line, whereas Figure 3 shows the effects per treatment used. The IC_{50} values are summarized in Table 1.

The 1:1 mixture of **1** and **2** is strongly effective against HT-29 ($IC_{50} = 1.70$ μg/mL), but only moderately effective against HCT-116 (IC_{50} value = 7.18 μg/mL). The mixture exhibited low toxicity against MCF-7 ($IC_{50} = 20.75$ μg/mL). One-way ANOVA showed statistical differences in IC_{50} values among the four cell lines ($P < 0.0001$). Tukey's multiple comparison *post hoc* test revealed significant differences between all pairs of cell lines with $P < 0.0001$ except between HT-29 and HDFn with $P < 0.01$. The same trend is seen for the 1:2 mixture of **1** and **2** which is also strongly effective against HT-29 ($IC_{50} = 0.74$ μg/mL) and HCT-116 ($IC_{50} = 3.46$ μg/mL) but not as effective against MCF-7 ($IC_{50} = 30.06$ μg/mL). One-way ANOVA showed statistical differences in IC_{50} values among the four cell lines ($P = 0.0040$). However, Tukey's *post hoc* test showed that there is only significant difference between MCF-7 and HT-29 ($P < 0.01$). Comparing the overall cytotoxic effects of 1:1 and 1:2 mixtures of **1** and **2** against the cancer cell lines, the data showed that 1:2 is more effective except for MCF-7 cells. Comparing the bioactivities of the two mixtures against **2**, the latter exhibited the same trend, with the strongest antiproliferative effect against HT-29 ($IC_{50} = 0.89$ μg/mL) and HCT-116 ($IC_{50} = 2.33$ μg/mL). Compound **2**, however, is more effective against MCF-7 ($IC_{50} = 10.99$ μg/mL) compared to its 1:1 and 1:2 mixtures with **1**. One-way ANOVA showed statistical differences in IC_{50} values among the four cell lines ($P < 0.0001$). Tukey's *post hoc* test consistently showed significant differences between all pairs of cell lines ($P < 0.0001$).

Squalene (**3**) seemed to show the same trend in bioactivity against the cancer cell lines (Table 1). However, it failed the test for significance ($P = 0.8483$) after ANOVA. None of the pair-wise data comparing the cell lines is significant. This implies that though squalene may still be considered bioactive as shown in the previous studies[16] and the magnitude of IC_{50} values obtained in this work, the significance of this parameter (per cell line) cannot be established statistically. Chlorophyll a (**4**) exhibited the strongest potential against HT-29 ($IC_{50} = 0.68$ μg/mL), but, unlike **1**–**3**, it showed the lowest toxicity against HCT-116 ($IC_{50} = 15.45$ μg/mL). One-way ANOVA showed statistical difference between treatments ($P < 0.0001$), but Tukey's multiple comparison *post hoc* test revealed that there is no pair-wise difference between MCF-7 and HDFn ($P > 0.05$). Wrightiadione (**5**) showed the same trend as **4**, exhibiting a strong antiproliferative effect against HT-29 cells ($IC_{50} = 4.07$ μg/mL) and MCF-7 cells ($IC_{50} = 5.69$ μg/mL), but low toxicity against HCT-116 ($IC_{50} = 25.11$ μg/mL). One-way ANOVA showed statistical difference between the treatments ($P < 0.0001$), but Tukey's multiple comparison *post hoc* test revealed that there is no pairwise difference between HCT-116 and HDFn ($P > 0.05$). α-Amyrin acetate (**6**) is strongly potent for HCT-116 ($ic_{50} = 4.07$ μg/ml) and moderately toxic against HT-29 ($IC_{50} = 7.97$ μg/mL) and MCF-7 ($IC_{50} = 13.81$ μg/mL). One-way ANOVA showed statistical difference between treatments ($P < 0.0001$), but Tukey's multiple comparison *post hoc* test revealed that there is no pairwise difference between HT-29 and HDFn ($P > 0.05$).

When the two colon cancer cell lines (HCT-116 and HT-29) are compared, the IC_{50} values of **1**–**6** for HT-29 were generally lower, implying that this cell line is more responsive to anticancer treatments using the samples tested. Differences in treatment response between the same colon cancer cell lines were also seen in previous studies.[14,16,18] It was reported that differences in the expression profiles of genes associated with drug sensitivity in HCT-116 and HT-29 cells could be a contributory factor influencing how the cells respond to inhibitory

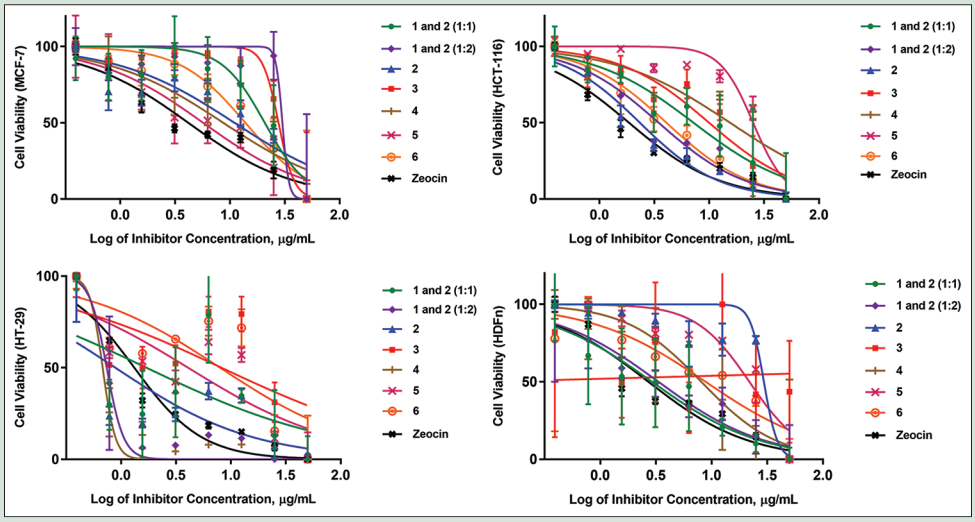

Figure 2: Cytotoxicity of **1**–**6** (per cell line). Extra sum-of-squares *F*-test was performed to evaluate differences in (a) best-fit parameter (half maximal inhibitory concentration) among treatments, and (b) dose-response curve fits. Results: MCF-7 (a) F (DFn, DFd) = F (8, 190) = 5.522 ($P < 0.0001$), (b) F (16, 190) = 6.688 ($P < 0.0001$); HCT-116 (a) F (8, 182) = 21.81 ($P < 0.0001$), (b) F (16, 182) = 11.29 ($P < 0.0001$); HT-29 (a) F (8, 190) = 7.503 ($P < 0.0001$), (b) F (16, 190) = 6.09 ($P < 0.0001$); HDFn (a) F (8, 190) = 3.745 ($P = 0.0004$), (b) F (16, 190) = 2.721 ($P = 0.0006$)

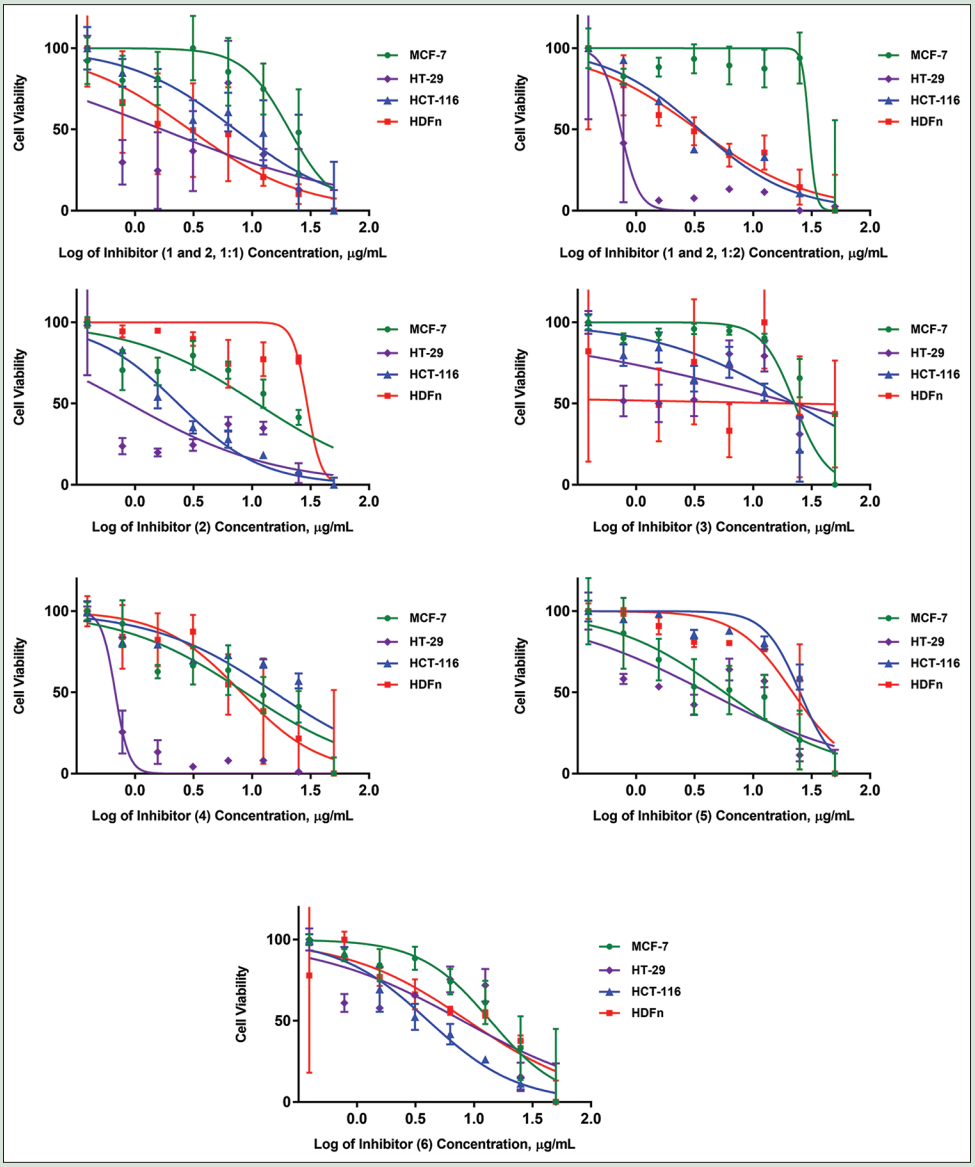

Figure 3: Cytotoxicity of **1**–**6** (per sample). Extra sum-of-squares *F* test for (a) best-fit parameters (half maximal inhibitory concentration) and (b) dose-response curve fits. Results: **1** and **2** (1:1) (a) F (DFn, DFd) = F (3, 80) = 5.96 (P = 0.0010), (b) F (6, 80) = 3.364 (P = 0.0052); **1** and **2** (1:2) (a) F (3, 88) = 23.5 (P < 0.0001), (b) F (6, 88) = 13.38 (P < 0.0001); **2** (a) F (3, 88) =19.03 (P < 0.0001), (b) F (6, 88) =10.22 (P < 0.0001); **3** (a) F (3, 88) =1.465 (P = 0.2297), (b) F (6, 88) = 2.843 (P = 0.0141); **4** (a) F (3, 88) = 23.08 (P < 0.0001), (b) F (6, 88) =12.48 (P < 0.0001); **5** (a) F (3, 88) =11.08 (P < 0.0001), (b) F (6, 88) = 7.632 (P < 0.0001); **6** (a) F (3, 88) = 2.929 (P = 0.0380), (b) F (6, 88) = 1.746 (P = 0.1198)

compounds.[23] In another work, variations in the sensitivity of HCT-116 and HT-29 cells against two known metabolic stressor compounds, ribavirin and metformin, were attributed to the genetic and metabolic activities of the cell lines, suggesting differences in the use of nutrients and the metabolic pathways taken which then influence *in vitro* survival under stressors.[24] Another study which evaluated four human colon cancer cells (HCT-116, HT-29, HCT-15, and KM-12) showed that gene expression profiling following the inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, an inhibitor of the hsp90 molecular chaperone, could explain why cellular response to similar treatment conditions varied.[25]

For all the samples tested, strong to low antiproliferative activities against the normal cell line, HDFn, was seen. As discussed above, the IC_{50} value of 0.10 µg/mL for **3** cannot be claimed as determined statistically. Hence, from the remaining data, the strongest inhibition was seen in the 1:1 mixture of **1** and **2** with an IC_{50} value of 2.92 µg/mL. Zeocin, as expected, showed strong cytotoxicity against all the cell lines used (strongest against HT-29 with IC_{50} = 1.32 µg/mL). ANOVA and Tukey's *post hoc* analyses consistently showed that the IC_{50} values are significant (P < 0.0001).

Overall, **1** and **2** (1:1), **1** and **2** (1:2), **2**, **3**, **4**, **5** and **6** exhibited the strongest antiproliferative effects against the HT-29 cells, followed by HCT-116. Both are colon cancer cell lines. Among the cancer cell lines tested, MCF-7 showed the least response to the samples. The samples also exhibited cytotoxic activities against the normal cell line, HDFn. The known anticancer drug, Zeocin, showed antiproliferative activities as expected. In general, **1**–**6** showed varying but promising cytotoxic properties. The US National Cancer Institute has defined the active cytotoxic limits of natural products as 20 µg/mL or less for crude extracts and 4 µg/mL or less for pure compounds.[26] Pure compounds that exhibit active cytotoxicity may have some potential for further

Table 1: Cytotoxic activities (half maximal inhibitory concentration) of **1**-**6** and Zeocin against MCF-7, HT-29, HCT-116, and HDFn cells

Compound	IC_{50} (μg/mL)*			
	MCF-7	HCT-116	HT-29	HDFn
1 and 2 (1:1)	20.75	7.18	1.70	2.92
1 and 2 (1:2)	30.06	3.46	0.74	3.33
2	10.99	2.33	0.89	29.49
3	27.70	9.96	8.20	0.10
4	8.69	15.45	0.68	8.09
5	5.69	25.11	4.07	21.83
6	13.81	4.07	7.97	8.84
Zeocin	4.17	1.86	1.32	2.71

*IC_{50} values were extrapolated from dose-response curves generated from nonlinear regression analysis performed using GraphPad Prism 7.02. For each compound/mixture, one-way ANOVA was conducted to determine differences between data sets (cell lines). The results are **1** and **2** (1:1), F (3, 80)=102.3, P<0.0001; **1** and **2** (1:2), F (3, 88)=4.774, P=0.0040; **2**, F (3, 88)=491.5, P<0.0001; **3**, F (3, 88)=0.268, P=8483; **4**, F (3, 88)=536.3, P<0.0001; **5**, F (3, 88)=303. **6**, P<0.0001; **6**, F (3, 88)=50.72, P<0.0001. IC_{50}: Half maximal inhibitory concentration; HDFn: Human dermal fibroblast-neonatal; HCT-116 and HT-29: colon; MCF-7: breast

drug development.[22] The results showed that **1**–**6**, isolated from the dichloromethane extracts of the leaves and twigs of *W. pubescens*, can be further evaluated for the treatment especially of the human colorectal types of cancer.

Previous studies revealed that ursolic acid (**1**), oleanolic acid (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**) exhibited cytotoxic activities.

Ursolic acid (**1**) was reported to cause apoptosis in tumor cells by activating the enzyme, caspase, which is involved in programmed cell death, and by modulating pathways relevant to cell proliferation and migration.[27] This compound also decreased growth and promoted apoptosis in gastric cancer cell line BGC-803 and hepatocellular cancer cell H22 xenograft, under both *in vivo* and *in vitro* studies.[28] Other studies showed that **1** exhibited antitumor activity against human colon carcinoma HCT-15 cells[29] and inhibited colon-cancer-initiating cells by targeting the gene, STAT3, essential in chemical signaling pathways within cells.[30] The triterpenoids **1** and betulinic acid, were found important as therapeutic agents against estrogen-dependent tumors.[31] The cytotoxic activities of **1** against prostate cancer have been reported.[32,33] Another study showed that **1** suppressed the proliferation of Jurkat leukemic T-cells, inhibiting phorbol myristate acetate/phytohemagglutinin-induced IL-2 and tumor necrosis factor-alpha (TNF-α) in a concentration and time-dependent manner.[34] Another study using TC-1 cervical cancer cells reported that ursolic acid-activated autophagy induced cytotoxicity and reduced tumor growth in a concentration-dependent manner as well.[35] The antitumor activities of **1** against U87MG brain cancer cells were attributed to the G1-phase arrest and autophagy that were both induced by the compound.[36] In a study evaluating the anticancer properties of ursolic acid and the three flavonoids, daidzein, baicalein, and hesperidin, it was found that the mixture of **1** and baicalein inhibited the growth of MCF-7 breast cancer cells which was induced by 2-amino -1-methyl-6-phenylimidazo[4,5-b] pyridine, a food-derived carcinogen, exhibiting estrogenic activities.[37] Thus, ursolic acid (**1**) was reported to exhibit cytotoxic properties against different cancer cells which corroborate our findings that **1**, in a 1:1 or 1:2 mixture with oleanolic acid (**2**), showed high cytotoxic activities against colon cancer cells, with the lowest IC_{50} values obtained for HT-29 (IC_{50} = 1.70 and 0.74 μg/mL, for the 1:1 and 1:2 ratios of **1** and **2**, respectively).

Oleanolic acid (**2**) was found to be antimutagenic and antitumor, inhibiting the proliferation of gastric, colon, and liver cancer cells by inducing apoptosis and necrosis.[38] Triterpene **2** inhibited mouse skin tumor[39] and exhibited significant antitumor activity against human colon carcinoma cell line HCT-15.[29] Another study identified **2** as an antitumor compound, suppressing aerobic glycolysis in MCF-7 breast cancer cells by promoting a metabolic switch in the PKM2 to PKM1 ratio, an important event in cancer development.[40] An extensive review of the cytotoxic nature of oleanolic acid and other triterpenes has been presented.[41] Thus, oleanolic acid (**2**) was reported to exhibit cytotoxic properties against different cancer cells which corroborate our findings that pure **2** or in combination with ursolic acid (**1**) (1:1 or 1:2), showed high cytotoxic activities against colon cancer cells, with the lowest IC_{50} values obtained for HT-29 (IC_{50} = 0.89 μg/mL) and HCT-116 (IC_{50} = 2.33 μg/mL).

Squalene (**3**) was shown to exhibit antitumor activities against colon cancer found in rodents.[42] It also reduced colonic aberrant crypt foci formation and crypt multiplicity in laboratory mice, demonstrating chemopreventive activities against colon carcinogenesis.[43] In a study using compounds extracted from palm oil, squalene and other isolates were found to have antiproliferative effects against two human breast cancer cell lines, MDA-MB-231, and MCF-7, resulting from the suppression of nuclear factor kappa-light-chain-enhancer of activated B-cells in breast cancer cells exposed briefly to TNF-α, hence, affecting apoptosis and carcinogenesis.[44,45] The protective and therapeutic effects of squalene-containing compounds on skin tumor cells in laboratory mice have been reported as well.[46] Relevant review papers on the bioactivities of squalene and its derivatives have been provided.[41,47] Thus, **3** was reported to exhibit cytotoxic properties against colon and breast cancer cells which corroborate our findings that **3** generally showed potential antiproliferative activities especially against the colon cancer cells used in the study (IC_{50} = 8.20 μg/mL for HT-29 cells).

Chlorophyll a (**4**) and its derivatives are popularly used in the traditional medicine for its various therapeutic applications.[48] Natural chlorophyll and its derivatives have been evaluated for wound healing,[49] anti-inflammatory properties,[50] control of calcium oxalate crystals,[51] anticancer activities,[52-54] and chemopreventive effects in humans.[55,56] A review on the digestion, absorption, and cancer preventive activities of dietary chlorophyll has been presented.[57] In a recent study evaluating the cytotoxic activities of chlorophyll a and its derivatives against human cell lines, it was found that the compounds exhibited photoinduced cytotoxic activities *in vitro*.[58] Thus, **4** was reported to exhibit anticancer properties which corroborate our findings that **4** showed antiproliferative activities which was very strong against HT-29 colon cancer cells (IC_{50} = 0.68 μg/mL) and moderately effective against MCF-7 breast cancer cells (IC_{50} = 8.69 μg/mL).

Wrightiadione (**5**), isolated from *Wrightia tomentosa*, was reported to exhibit cytotoxic activity against the murine P-388 lymphocytic leukemia cell line.[59] The activities of **5** were compared with wrightiamines a and b and all were found cytotoxic against the same vincristine-resistant murine leukemia P-388 cells.[60] There are limited reports on the cytotoxic properties of **5**. In this study, **5** was found to exhibit antiproliferative activities which was strongest against HT-29 colon cancer cells (IC_{50} = 4.07 μg/mL) and MCF-7 breast cancer cells (IC_{50} = 5.69 μg/mL).

α-Amyrin acetate (**6**) were mostly studied for its various potential medicinal applications. Compound **6**, isolated from *Alstonia boonei*, showed inhibition of egg albumen-induced paw edema in laboratory mice.[61] The same study showed that it promoted reduction in total leukocyte count and suppression of neutrophil infiltration. Lupeol, lupeol acetate, and α-amyrin acetate exhibited anti-tyrosinase activity, indicating potential melanin biosynthesis inhibitory properties.[62] Both α-amyrin acetate and β-amyrin acetate were reported to exhibit sedative, anxiolytic, and anticonvulsant properties.[63] Limited studies have been conducted

evaluating the cytotoxic properties of **6** against human cancer cells. The dichloromethane extract of *Ficus odorata* (Blanco) Merr., containing α-amyrin acetate, 1-sitosteryl-3-β-glucopyranoside-6'-*O*-palmitate, squalene, lutein, lupeol acetate, and β-carotene, exhibited antiproliferative activities against the human cancer cell lines, lung adenocarcinoma epithelial (A549), stomach adenocarcinoma (AGS), prostate (PC3), and colon adenocarcinoma (HT-29).[64] Thus, **6** was reported to exhibit cytotoxic properties which corroborate our findings that **6** showed antiproliferative activities which was strongest against the two colon cancer cell lines, HCT-116 (IC_{50} = 4.07 μg/mL) and HT-29 (IC_{50} = 7.97 μg/mL).

It remains to be explored if other parts of the plant, such as stem bark and roots, will be able to afford the same compounds and exhibit other bioactivities such as antibacterial, anti-inflammatory, and antioxidative, similar to other studies.[65-68]

CONCLUSION

Mixtures of ursolic acid (**1**) and oleanolic acid (**2**) (1:1 and 1:2), oleanolic acid (**2**), squalene (**3**), chlorophyll a (**4**), wrightiadione (**5**), and α-amyrin acetate (**6**) from the dichloromethane extracts of the leaves and twigs of *W. pubescens* (R.Br.) exhibited varying cytotoxic activities against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast - neonatal (HDFn) Compounds **4**, **1** and **2** (1:2), **2**, and **1** and **2** (1:1) exhibited the strongest cytotoxic effects against HT-29 with IC_{50} values of 0.68, 0.74, 0.89, and 1.70 μg/mL, respectively. The two colon cancer cell lines responded well under all treatments, with HCT-116 generally less susceptible to the treatments. When **2** was compared with its 1:1 mixture with **1** (IC_{50} = 1.70 and 7.18 μg/mL for HT-29 and HCT-116, respectively) and 1:2 mixture with **1** (IC_{50} = 0.74 and 3.46 μg/mL for HT-29 and HCT-116, respectively), the data for **2** also showed strong antiproliferative potential against HT-29 (IC_{50} = 0.89 μg/mL) and HCT-116 (IC_{50} = 2.33 μg/mL). However, unlike the two mixtures which both exhibited low antiproliferative effects on MCF-7 (IC_{50} = 20.75 and 30.06 μg/mL for 1:1 and 1:2, respectively), **2** exhibited moderate activity against MCF-7 (IC_{50} = 10.99 μg/mL). Overall, the activities of **1**–**6** were highest against HT-29, followed by HCT-116 and MCF-7. Compounds **1**–**6** also showed varying toxicities against HDFn (IC_{50} <30 μg/mL).

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Conflicts of interest

There are no conflicts of interest.

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