Cytotoxic Compounds from *Kibatalia gitingensis* (Elm.) Woodson

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ABSTRACT

The cytotoxic activities of ursolic acid (1), squalene (2), a mixture of α-amyrin acetate (3a) and lupeol acetate (3b), and isoscopoletin (4), which were extracted from the dichloromethane extracts of Kibatalia gitingensis's leaves and twigs, were tested against three human cancer cell lines: MCF-7 for breast cancer, HT-29 and HCT-116 for colon cancer, and HDFn, a normal cell line, using the in vitro PrestoBlue® cell viability assay. The IC50 values for compounds 1-4 ranged from 0.6931 to 1.083 µg/mL, indicating high cytotoxic effects against HT-29 cells. In addition, the IC50 values for 1-4 ranged from 4.065 to 11.09 µg/mL, indicating significant cytotoxicity against HCT-116 cells.

The MCF-7 cells were least affected by these substances, with IC50 values varying between 8.642 and 25.87 µg/mL. On the one hand, 2, 4 and 1, respectively, are the most cytotoxic to HT-29 cells, HCT-116 cells, and MCF-7 cells.

Key words: Subfamily Apocynaceae, Kibatalia gitingensis Cytotoxicity, MCF-7, HCT-116, HT-29, HDFn, ursolic acid, squalene, α-amyrin acetate, Lupeol acetate, isoscopoletin, and PrestoBlue® cell viability test.

INTRODUCTION

Local names for the Apocynaceae family member Kibatalia gitingensis (Elm.) Woodson include "laniti" and "laneteng-gubat" in the Philippines. According to the International Union for the Conservation of Nature's Red List of Threatened Species, it is considered susceptible to extinction.The alkaloid concentration gives it therapeutic benefits, and it is also widely utilized as a construction material and for ornamental carvings.gitingensine, a steroidal alkaloid isolated from K. gitingensis leaves, has ataraxic effects (the ability to relax smooth muscles) and antispasmodic activity (the ability to widen the arteries of skeletal muscles and the splanchnic area).5 Kibataline6,7 and 20-(epi-N-methyl) paravallarine were found in K. gitingensis leaves in other research.8 The azasteroidal alkaloid found in the plant eliminated serotonin-induced spasms and stimulated spontaneous movement in canines and mice.Paravallarine, N-methylparavallarine, and 20-epiparavallarine are among the several alkaloids extracted from K. gitingensis bark.Not only that, but lanitine (2α-hydroxy- N-methylparavallarine) and its 2β-isomer were found in the plant's stem bark, according to reports.11

Our ongoing investigation into the bioactivities and chemical compositions of native and indigenous Philippine plants includes this study. In a previous paper, we detailed the procedure for extracting and identifying ursolic acid (1), squalene (2), a combination of α-amyrin acetate (3a) and lupeol acetate (3b) from the leaves, and 1-3 and isoscopoletin.

MATERIALS AND METHODS

Sample Collection

Samples of leaves and twigs of *Kibatalia gitingensis*(Elm.) Woodson were collected from the De La Salle University–Science and Technology Complex (DLSU-STC) reforested area in February 2014. The samples were authenticated and deposited at the De La Salle University Herbarium with voucher specimen#908.

Isolation and Structure Elucidation

The isolation and structure elucidation of **1-4** from the leaves and twigs of *K. gitingensis* were reported previously.[12]

Preparation of Compounds forCytotoxicity Tests

The compounds (**1-4**) from *K. gitingensis* were dissolved in dimethyl sulfoxide (DMSO) to makea 4 mg/mL stock solution. Working solutions were prepared in complete growth medium to a final non-toxic DMSO concentration of 0.1%.

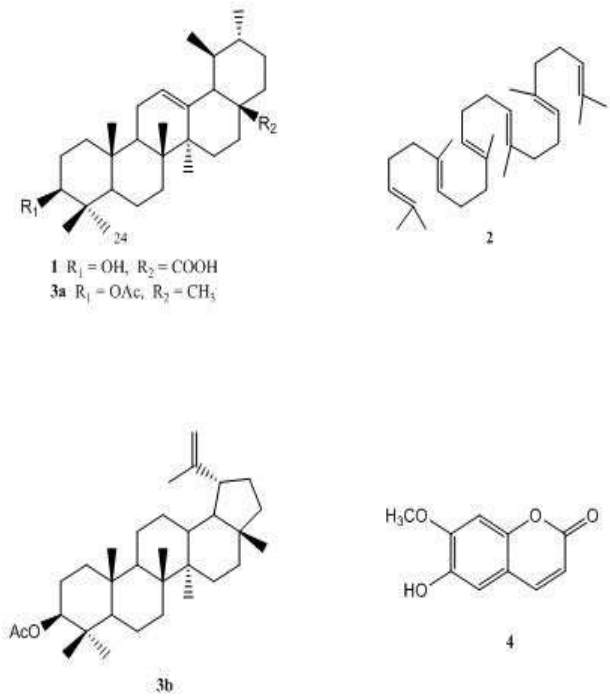

Figure 1: Chemical structures of ursolic acid (**1**), squalene (**2**), α-amyrin ace-tate (**3a**), lupeol acetate (**3b**), and isoscopoletin (**4**) from *Kibatalia gitingensis*.

Maintenance and Preparation of Cells for Cytotoxicity Tests

The cytotoxicity of **1-4** from the dichloromethane (CH_2Cl_2) extracts of *K. gitingensis* was tested on the following human cell lines: breast cancer (MCF-7) and colon cancer (HCT-116 and HT-29) (ATCC, Manassas, Virginia, U.S.A.), and human dermal fibroblast-neonatal (HDFn; Invitrogen Life Technologies, U.S.A.), 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,[13,14] cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) containing 10% fetal bovine serum (FBS, Gibco®, USA) and 1x anti- biotic-antimycotic (Gibco®, USA) and kept in an incubator (37°C, 5% CO_2, 98% humidity). At 80% confluence, the monolayers were washed with phosphate-buffered saline (PBS, 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 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 cells/well. The plates were further incubated overnight (37°C, 5% CO_2, 98% humidity) until complete cell attachment was reached. These cells were used for the cytotoxicity studies as described below.

Cell Viability Assay

The breast cancer cell line (MCF-7) is only moderately susceptible to 0.39 µg/mL. Wells with no compound 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 incubated (37°C, 5% CO_2, 98% humidity) for 2 hr. Absorbance measurements were carried out using the BioTek ELx800 Absorbance Microplate Reader (BioTek® Instruments, Inc., U.S.A.) at 570 nm and normalized to 600 nm values (reference wavelength). Absorbance readings were used to calculate for the cell viability for each sample concentration following the equation below.

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

Nonlinear regression and statistical analyses were done using GraphPad Prism 7.00 (GraphPad Software, Inc.) 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 of **1-4** was expressed as IC_{50} values. All tests were performed in triplicates and data were shown as means. 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 deter- mine the differences among dose-response curve fits according to the software's recommended approach. One-way ANOVA ($p<0.05$) was also used to determine significant differences among treatments, followed by Tukey's multiple comparison post hoc test ($p<0.05$), to compare different pairs of data sets. Results were considered significant at $p<0.05$.

RESULTS AND DISCUSION

Ursolic acid (**1**), squalene (**2**), a mixture of α-amyrin acetate (**3a**) and lupeol acetate (**3b**), and isoscopoletin (**4**), isolated from the dichloro- methane extracts of the leaves and twigs of *K. gitingensis*, were evalu- ated for their anti-proliferative activities against three human cancer celllines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast-neonatal (HDFn), using the *in vitro* PrestoBlue® cell viability assay.

The % cell viability as a function of the logarithmic values of compound concentration is shown in Figures 2 and 3. Most plots nearly follow the typical sigmoidal curve which is characteristic of an inhibitory dose- response relationship between treatments and cell viability. Figure 2 compares the anti-proliferative effects per cell line, while Figure 3 compares the effects per compound. The corresponding IC_{50} values are summarized in Table 1.

The cytotoxicity of the *K. gitingensis* compounds was determined in an *in vitro* cell viability assay using PrestoBlue® (Molecular Probes®, Invit- rogen, USA). This test is based on the principle that the enzyme, mito- chondrial reductase, present in viable cells, can reduce the nonfluores- cent, blue resazurin dye in the reagent, converting it to resorufin which is red and highly fluorescent. Hence, only viable cells are able to cause color change. The conversion is proportional to the number of metaboli- cally active cells and is correlated to absorbance measurements. To the monolayers in the microtiter plate, 100 µL of filter-sterilized **1**-**4** 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

1 and **3a** and **3b**, with IC_{50} values of 8.642 and 11.13 µg/mL, respectively, and least susceptible to **2** and **4** with IC_{50} values of 25.87 and 23.35 µg/mL. One-way ANOVA showed statistical difference between treatments ($p<0.0001$), but Tukey's multiple comparison post hoc test revealed thatthere are no pairwise differences between **1** and **3a** and **3b**, and **2** and **4**($p>0.05$).

The colon cancer cell line (HCT-116) is most susceptible to **4** with IC_{50} values of 4.065 µg/mL, but showed moderate susceptibility only to **1**, **2**, and **3a** and **3b**, with IC_{50} values of 7.225, 9.226, and 11.09 µg/mL, respec- tively. One-way ANOVA showed that all treatments are statistically different ($p<0.0001$), but Tukey's multiple comparison post hoc test showed no pairwise differences between **1** and **2**, and **2** and **3a** and **3b** ($p>0.05$). The growth of the other colon cancer cell line (HT-29) exhibited

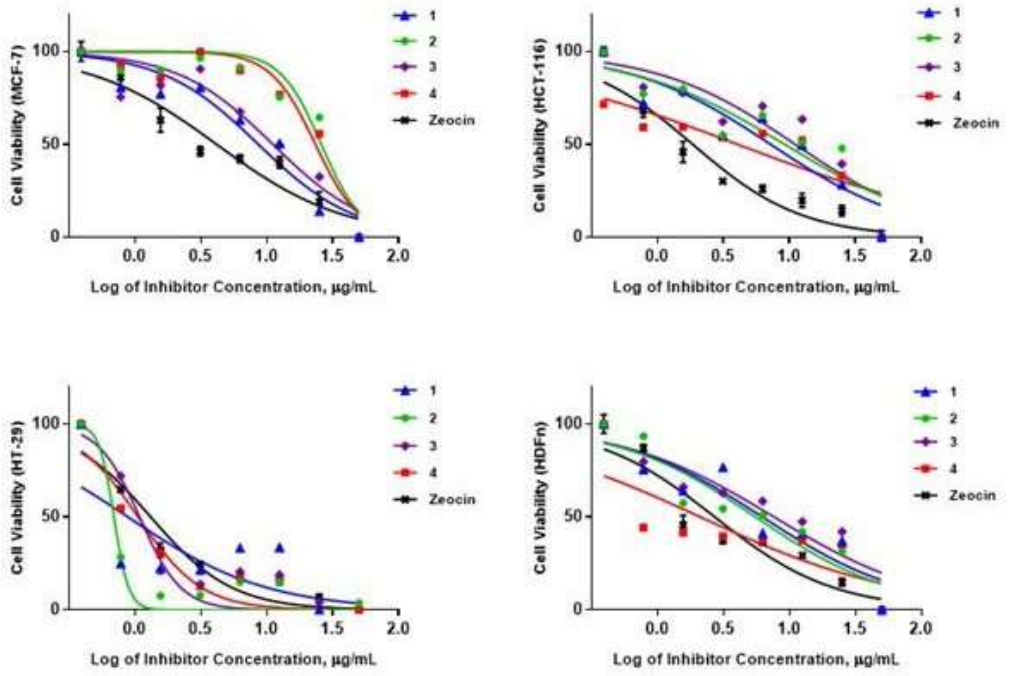

Figure 2: Cytotoxic activities of **1-4** and Zeocin (per cell line). Extra sum-of-squares F test was performed to evaluate differences in: (A) best-fit parameters (IC_{50})among treatments, (B) dose-response curve fits. Results: MCF-7 (A) F(DFn, DFd) = F(5,124) = 4.398, p=0.0010 and (B) F(10,124) = 8.142, p<0.0001; HCT-116 (A) F(5,123) = 4.477, p=0.0009 and (B) F(10,123) = 2.513, p=0.0087; HT-29 (A) F(5,124) = 3.419, p=0.0063 and (B) F(10,124) = 2.221, p=0.0205; HDFn (A) F(5,124) = 2.62, p=0.0274 and (B) F(10,124) = 1.888, p=0.0528.

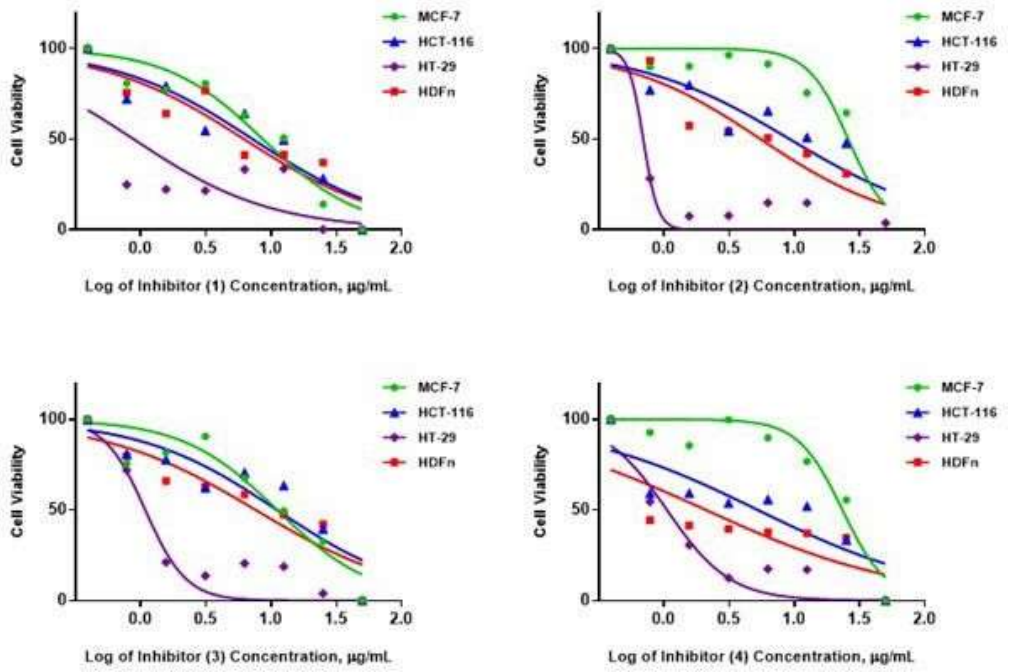

Figure 3: Cytotoxic activities of **1-4** (per compound). Extra sum-of-squares F test was performed to evaluate differences in: (A) best-fit parameters (IC_{50}) amongtreatments, (B) dose-response curve fits. Results: **1** (A) F(DFn,DFd) = F(3,88) = 5.473, p=0.0017, (B) F(6,88) = 3.085, p=0.0087; **2** (A) F(3,88) = 25.03, p<0.0001, (B) F(6,88) = 14.24, p<0.0001; **3a** and **3b** (A) F(3,88) = 8.594, p<0.0001, (B) F(6,88) = 4.419, p=0.0006; **4** (A) F(3,88) = 15.19, p<0.0001, (B) F(6,88) = 7.728, p<0.0001.

Table 1: Cytotoxic activities (IC_{50}) of 1-4 and Zeocin against MCF-7,HCT-116, HT-29 and HDFn

Sample	IC_{50}* (µg/mL)			
	MCF-7	HCT-116	HT-29	HDFn
1	8.642	7.225	0.8836	6.218
2	25.87	9.226	0.6931	5.519
3a and 3b	11.13	11.09	1.083	7.628
4	23.35	4.065	1.054	2.106
Zeocin	4.168	1.856	1.318	2.713

*IC50 values were extrapolated from dose-response curves generated from non- linear regression analysis done using GraphPad Prism 7.00. For each cell line, one-way ANOVA was conducted to determine differences between data sets (treatments). The results are: MCF-7, F(5, 118) = 31.17, p < 0.0001; HCT-116, F(5, 117) = 84.93, p < 0.0001; HT-29, F(5, 118) = 65.87, p < 0.0001; HDFn, F(5, 118) = 51.73, p < 0.0001. Results of the Tukey's multiple comparison post hoctest are discussed in this section.

the strongest inhibition at the lowest concentrations of the compounds, with IC_{50} values of 0.6931, 0.8836, 1.054, and 1.083 µg/mL for **2**, **1**, **4**, and **3a** and **3b**, respectively. Tukey's multiple comparison post hoc test showed statistical differences between **2** and all other samples except **1**(p>0.05).

The normal cell line, HDFn, responded to all the compounds, with IC_{50}values of 2.106, 5.519, 6.218, and 7.628 µg/mL for **4**, **2**, **1**, and **3a** and **3b**, respectively. The pairs of compounds, **1** and **2**, and **1** and **3a** and **3b** are not statistically different (p>0.05). All the cell lines are susceptible to Zeocin. Data analysis showed statistical differences in the best-fit parameter (half maximal inhibitory concentration) among treatments and among the dose-response curve fits (Figures 2 and 3).

Overall, comparing the three human cancer cell lines, HT-29 showed the most cytotoxic response with comparable IC_{50} values for all the

Previous studies revealed that ursolic acid (**1**), squalene (**2**), α-amyrin acetate (**3a**) lupeol acetate (**3b**), and isoscopoletin (**4**) exhibited cytotoxicproperties.

Ursolic acid (**1**) was reported to promote apoptosis in tumor cells by activation of caspases and modulation of pathways influencing cell proliferation and migration.[20] It also decreased growth and induced apoptosis in gastric

cancer cell line BGC-803 and hepatocellular cancer cell H22 xenograft, both *in vivo* and *in vitro* studies.[21] Other works showed that **1** exhibited anti-tumor activity against human colon carcinoma HCT15 cells,[22] and inhibited the colon cancer-initiating cells by targeting STAT3.[23] Triterpene **1** and betulinic acid were found useful as therapeutic agents against estrogen-dependent tumors.[24] Furthermore, the anti- proliferative and apoptotic effects of **1** was found to have potential therapeutic use against prostate cancer.[25] A recent study reported that **1** suppressed the proliferation of Jurkat leukemic T-cells, inhibiting PMA/PHA induced IL-2 and TNF-α production in a concentration- and time-dependent manner.[26] Another study using cervical cancer TC-1 cells reported that ursolic acid-activated autophagy induced cytotoxicity and reduced tumor growth in a concentration-dependent manner as well.[27] The anti-tumor activities of **1** against U87MG brain cancer cells were evaluated and it was found that both G1-phase arrest and autophagy were induced by the compound.[28] In a study evaluating the anti-cancer properties of ursolic acid and three flavonoids, daidzein, baicalein, and hesperidin, it was found that **1** and baicalein inhibited the proliferation of MCF-7 breast cancer cells induced by PhIP, a food-derived carcinogen exhibiting estrogenic activities.[29] The anti-cancer potential of **1** present in different berries has been reviewed.[20] Thus, ursolic acid (**1**) was reported to exhibit cytotoxic properties against different cancer cells including colon and breast cancer cell lines which corroborate our findings that **1** showed high cytotoxicity against colon cancer cells with the lowest IC_{50} values of 0.8836 µg/mL obtained for HT-29, 7.225 µg/mL for HCT-116, and 8.642 µg/mL for MCF-7.

Squalene (**2**) was reported to exhibit anti-tumor activities against colon cancer in rodents.[30] It also reduced colonic aberrant crypt foci (ACF) formation and crypt multiplicity in laboratory rats, indicating potential chemopreventive activities against colon carcinogenesis.[31] In a study evaluating the anti-proliferative effects of squalene and other compounds from palm oil against two human breast cancer cell lines, MDA-MB-231 and MCF-7, it was found that there was a suppression of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) in breast cancer cells exposed briefly to tumor necrosis factor-alpha (TNF-α),[32,33] hence affecting the mechanisms of apoptosis and carcinogenesis. The protective and therapeutic effects of squalene-containing compounds on skin tumor cells in laboratory mice have been reported as well.[34] Relevant reviews on the bioactive properties of squalene have also been provided.[35,36] Thus, **2** was reported to exhibit cytotoxic properties against colon and breast cancer cells which corroborate our findings that **2** showed high to moderate cytotoxicities against colon cancer cells, HT-29 and HCT-116, and breast cancer cells, MCF-7, with IC_{50} values of 0.6931, 9.226, and 25.87 µg/mL, respectively.

α-Amyrin acetate (**3a**) was mostly studied for its various potential medicinal applications. At a concentration of 100 mg/kg, **3a** isolated from *Alstonia boonei* showed significant ($p<0.05$) inhibition of egg albumen-induced paw edema in mice.[37] The same study showed that it promoted 60.3% reduction in total leucocyte count and significant ($p<0.05$) suppression (47.9%) of neutrophil infiltration. Lupeol, lupeol acetate and α-amyrin acetate exhibited significant anti-tyrosinase activity against the mushroom tyrosinase enzyme, with percent inhibi- tions of 67.7%, 66.2% and 62.2%, respectively,[38] indicating potential melanin biosynthesis inhibitory properties. Both α-amyrin acetate and β-amyrin acetate were also reported to exhibit sedative,

affected by **4** with an IC_{50} value of 4.065. Among the cancer cell lines tested, MCF-7 showed the least response to the compounds. The compounds exhibited cytotoxic activities against the normal cell line, HDFn. The known anti-cancer drug, Zeocin, showed anti-proliferative activities as expected. Overall, **1-4** showed varying, but promising cytotoxic properties, especially for the treatment of HT-29 type of colon cancer cells. 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.[15] Pure compounds that exhibit active cytotoxicity may have some potential for drug development.[14] The results showed that **1-4** from the dichloromethane extracts of *K. gitingensis* leaves and twigs can be further evaluated for the treatment especially of human colorectal type of cancer.

The study also revealed that the cytotoxic activities of **1-4** were a function of the specific type of cancer cells targeted. When the two colon cancer cell lines were compared, the IC_{50} values of **1-4** for HT-29 were lower than HCT-116, implying that the former could be more susceptible to anti-cancer treatments using the compounds tested. A difference in treatment responses between two colon cancer cell lines was also seen in previous studies.[16,17] It was reported that changes in the expression profiles of genes associated with drug sensitivity between HCT-116 and HT-29 could influence how the cells react to different inhibitory compounds.[18] A similar study using four human colon cancer cell lines (HCT-116, HT-29, HCT-15, and KM-12) showed that gene expression profiling after inhibition of signal transduction by 17-allylamino-17-demetho-xygeldanamycin, a known inhibitor of the hsp90 molecular chaperone, could explain the cells' response under different treatment parameters.[19]

anxiolytic

and anti-convulsant properties.[39] Very few studies have been made on the cytotoxic properties of **3a**. 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 anti-proliferative activities against the human cancer cells, lung adenocarcinoma epithelial (A549), stomach adenocarcinoma (AGS), prostate (PC3), and colon adenocarcinoma (HT29).[40] Lupeol acetate (**3b**) was reported to exhibit cytotoxic activity against breast cancer cell (MCF7) with an IC_{50} value of 26 µg/mL.[41] Triterpenes, germincol, lupeol, α-amyrin, β-amyrin, olean-18-ene, and lupeol acetate, were isolated from the methanol extract of leaves and stems of *Lactuca steriolla* and showed varying cytotoxic activities against non-small cell lung adenocarcinoma cells (A549), human hepatocellular liver carcinoma cells (HepG2), human breast carcinoma cells (MCF7) and human colon carcinoma cells (HCT116).[42]

The chloroform extract of the leaves of *Acokanthera oblongifolia*, con- taining mixtures of isolated triterpenes, α-amyrin, lupeol acetate, lupeol, betulinaldehyde, and betulinic acid, showed some cytotoxic activities against human cancer cell lines, hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7) and colorectal (HCT116), with IC_{50} val-ues of 37.6, 65.4 and 66.8 µg/ml, respectively.[43] Thus, **3a** and **3b** were reported to exhibit cytotoxic properties against colon and breast can- cer cells which corroborate our findings that the mixture of **3a** and **3b** showed high to moderate cytotoxic properties against colon cancer cells, HT-29 and HCT-116, and breast cancer cells, MCF-7, with IC_{50} values of 1.083, 11.09, and 11.13 µg/mL, respectively. It is hypothesized that the synergistic effects of both compounds could have caused the observed anti-proliferative effects against the cancer cells studied.

Isoscopoletin (**4**) showed substantial inhibition in a cell proliferation assay using human CCRF-CEM leukaemia cells with an IC_{50} value of

4.0 µM.[44] Another study reported that **4** exhibited cytotoxic activities against human lung cancer (A549), human breast cancer cell (MCF7) and human liver cancer (HepG2) with IC_{50} values of 5.25, 8.58 and 4.76 µM, respectively.[45] Moreover, **4** showed cytotoxicity against colon cancer

(HCT116) cells with an IC_{50} value of 10% at 100 ppm.[46] Compound **4** from *Artemisia argyi*, artemisinin from *Artemisia annua*, and the latter's semi-synthethic derivative, artesunate, showed the greatest activity in

in vitro cytotoxicity tests against HCT116 colon adenocarcinoma cell line, with IC_{50} values ranging in concentration from micromolar to millimolar amounts.[47] It was hypothesized that isoscopoletin enhanced its anti-cancer property by influencing the activity of p53 tumor protein which is a genetically important process in cancer progression. Thus, **4** was reported to exhibit cytotoxic properties against several cancer cell lines such as colon and breast which corroborate our findings that **4** showed varying cytotoxic activities against colon cancer cells, HT-29 and HCT-116, and breast cancer cells, MCF-7, with IC_{50} values of 1.054, 4.065, and 23.35 µg/mL, respectively. Compound **4** also exhibited the highest cytotoxicity against the human dermal fibroblast-neonatal (HDFn) normal cell line, with an IC_{50} value of 2.106 µg/mL. More stud- ies are needed to fully examine and understand the effects of **4** on normal cells.

CONCLUSION

Ursolic acid (**1**), squalene (**2**), a mixture of α-amyrin acetate (**3a**) and lupeol acetate (**3b**), and isoscopoletin (**4**) from the dichloromethane extracts of *Kibatalia gitingensis* 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). The anti-proliferative activities of **1**-**4** were highest against HT-29, with IC_{50} values ranging from 0.6931 to 1.083 µg/mL, followed by HCT-116, with IC_{50} values ranging from 4.065 to 11.09 µg/mL, and

MCF-7, with IC_{50} values ranging from 8.642 to 25.87 µg/mL. Com- pounds **1**-**4** were also cytotoxic against HDFn with IC_{50} values ranging from 2.106 to 7.628 µg/mL.

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CONFLICT OF INTEREST

There is no conflict of interest.

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