

Cytotoxic Compounds from *Dysoxylum gaudichaudianum* (A. Juss.) Miq.

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ABSTRACT

Squalene (1), polyprenol (2), triglycerides (3), and β -sitosterol (4), isolated from the dichloromethane extract of the leaves of *Dysoxylum gaudichaudianum*, were evaluated for their anti-proliferative 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) using the *in vitro* PrestoBlue[®] cell viability assay. The HT-29 cell line was most susceptible to the compounds tested. Squalene (1) and polyprenol (2) were most cytotoxic against HT-29 (IC₅₀ of 0.84 and 1.059 μ g/mL, respectively), followed by MCF-7 (IC₅₀ of 4.404 and 3.786 μ g/mL, respectively), and HCT-116 (IC₅₀ of 7.859 and 9.258 μ g/mL, respectively). Triglycerides (3) was highly anti-proliferative against HT-29 cells (IC₅₀ of 2.738 μ g/mL) and moderately inhibitory against HCT-116 and MCF-7 cells (IC₅₀ of 13.05 and 16.12 μ g/mL, respectively). β -Sitosterol (4) was most cytotoxic against HT-29 and MCF-7 (IC₅₀ of 1.41 and 2.277 μ g/mL, respectively) and moderately cytotoxic against HCT-116 (IC₅₀ of 11.63 μ g/mL). Comparing the cytotoxic effects of these compounds against the three cancer cell lines, 1-4 were most cytotoxic against HT-29. Compounds 1, 2 and 4 were less inhibitory to MCF-7 and least inhibitory to HCT-116. Comparing the effects of 1-4 on the two colon cancer cell lines, their IC₅₀ values against HT-29 were lower than those of HCT-116. Compounds 1-4 showed moderate cytotoxicity against HDFn cells (11.18 to 17.72 μ g/mL) and were less cytotoxic to the normal cells than the cancer cells.

Keywords: *Dysoxylum gaudichaudianum* (A. Juss.) Miq., squalene, polyprenol, triglycerides, β -sitosterol, cytotoxicity, MCF-7, HCT-116, HT-29, HDFn

INTRODUCTION

Dysoxylum gaudichaudianum (A. Juss.) Miq. of the family Meliaceae may be found in thickets and primary and secondary lowland forests up to 1,800 m asl in many parts of the Philippines where it is locally known as "igyo" and in other Malesian regions down to Samoa¹. Commonly known as ivory mahogany, the mature plant may be used as a general purpose timber². In traditional folk medicine, decoction of chopped leaves and bark are used to treat coughs, skin irritations, body aches, lung hemorrhage, and sexually transmitted diseases and are also used to induce vomiting after food poisoning^{3,4}. In addition, it was reported that the juice extracted from the leaves may be used as an early abortifacient⁵. In another study, the plant's bark yielded four new compounds, dysoxylins A-D, belonging to the tetranortriterpenoid family, which

demonstrated strong bioactivity against anti-respiratory syncytial virus (RSV)^{6,7}. Structure elucidation of isolates from hexane and chloroform extracts of the plant's stem bark yielded *p*-hydroxyacetophenone, β -sitosterol, and stigmasterol⁸. A new limonoid, gaudichaudysolin A, isolated from the plant's bark, was evaluated *in vitro* for its cytotoxic effects against five human cancer cell lines: HL60 (human blood premyelocytic leukemia), RPMI8226 (multiple myeloma), NCI-H226 (non-small cell lung carcinoma), HCT116 (human colon cancer), and MCF7 (human breast adenocarcinoma) cells⁹. This study was conducted as part of our research on the chemical constituents of the genus *Dysoxylum* found in the Philippines. We earlier reported the isolation of new glabretal-type triterpenoids along with the known

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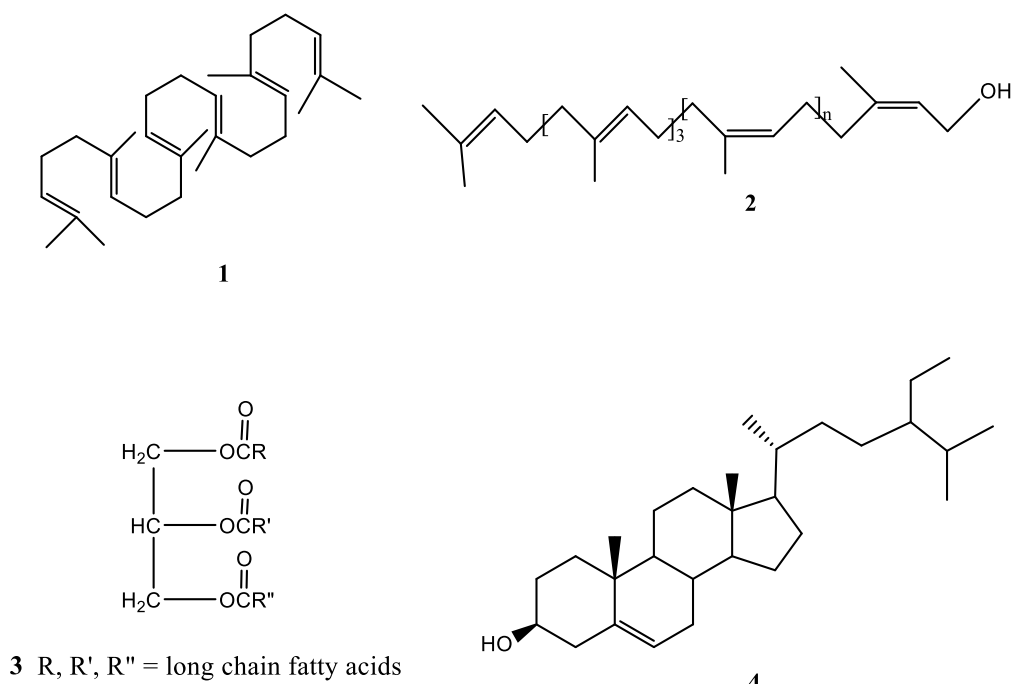


Figure 1: Chemical structures of squalene (1), polyprenols (2), triglycerides (3), and β-Sitosterol (4) from the leaves of *D. gaudichaudianum*.

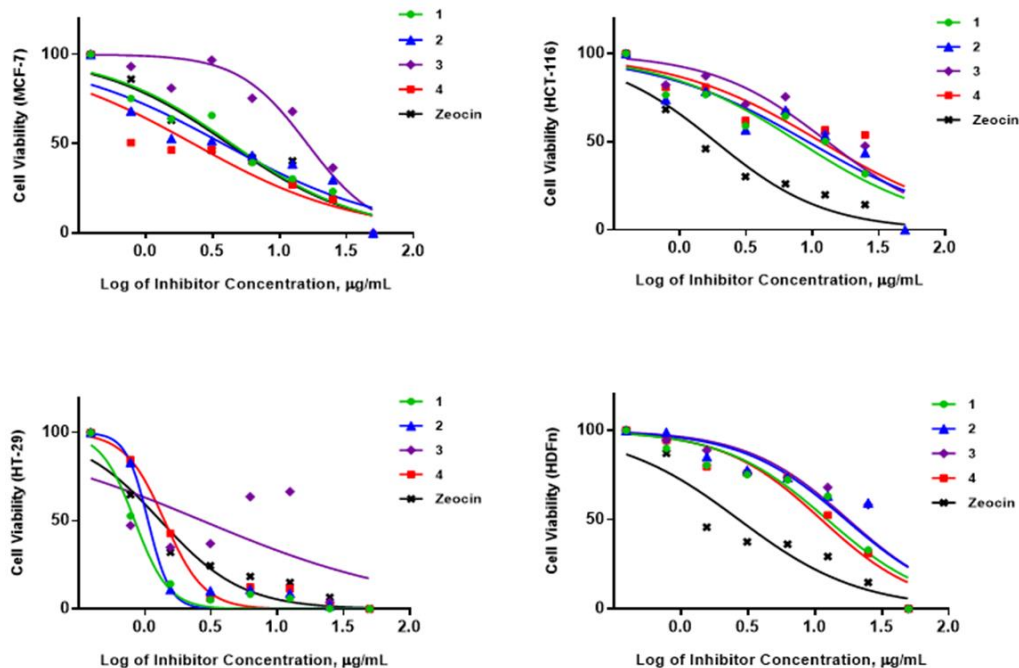


Figure 2: Dose-response curves showing the cytotoxic activities of 1-4 and Zeocin on the cell viability of MCF-7, HCT-116, HT-29 and HDFn. Each plot shows the effect of 1-4 and Zeocin against each cell line. Data shown are means of triplicates. GraphPad Prism 6.07 was used to perform extra sum-of-squares F test to (A) evaluate if the best-fit parameter (half maximal inhibitory concentration) differs among data sets (treatments), and (B) determine the differences among the dose-response curve fits. The results are: MCF-7 (A) $F (DFn, DFd) = F (5, 124) = 3.864$, $p = 0.0027$ and (B) $F (10, 124) = 5.384$, $p < 0.0001$; HCT-116 (A) $F (5, 116) = 9.586$, $p < 0.0001$ and (B) $F (10, 116) = 5.080$, $p < 0.0001$; HT-29 (A) $F (5, 124) = 5.644$, $p = 0.0001$ and (B) $F (10, 124) = 6.194$, $p < 0.0001$; HDFn (A) $F (5, 124) = 11.35$, $p < 0.0001$ and (B) $F (10, 124) = 6.025$, $p < 0.0001$.

compounds, 24,25-epoxy-3 β ,23-dihydroxy-7-tirucallene, squalene, polyprenol, linoleic acid and lutein from the leaves of *Dysoxylum mollissimum* Blume¹⁰. Recently, we reported that the crude dichloromethane leaf extract of *Dysoxylum gaudichaudianum* (A. Juss.) Miq. exhibited

IC₅₀ values of 7.35 and 13.19 μ g/mL against breast cancer (MCF-7) and colon cancer (HT-29) cells, respectively. Chemical investigation of the dichloromethane leaf extract led to the isolation of squalene (1), polyprenols (2), triglycerides (3), and β -sitosterol (4)¹¹. We report herein

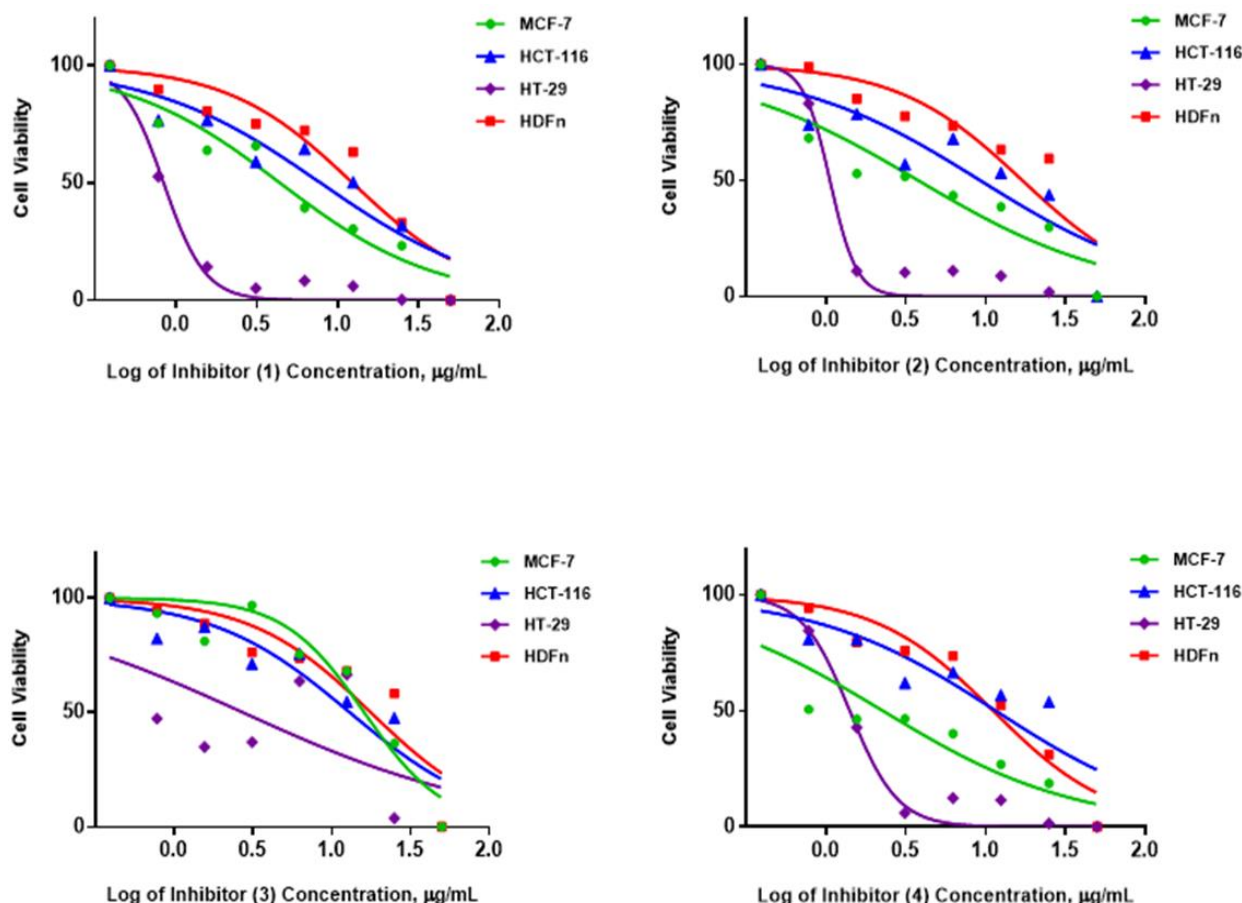


Figure 3: Dose-response curves showing the cytotoxic activities of **1-4** and Zeocin on the cell viability of MCF-7, HCT-116, HT-29 and HDFn. Each plot shows the effect of a compound against all cell lines tested. Data shown are means of triplicates. GraphPad Prism 6.07 was used to perform extra sum-of-squares F test to (A) evaluate if the best-fit parameter (half maximal inhibitory concentration) differs among data sets (treatments), and (B) determine the differences among the dose-response curve fits. The results are: compound **1** (A) $F(DFn, DFn) = F(3, 88) = 39.06$, $p < 0.0001$ and (B) $F(6, 88) = 19.64$, $p < 0.0001$; compound **2** (A) $F(3, 80) = 30.83$, $p < 0.0001$ and (B) $F(6, 80) = 15.61$, $p < 0.0001$; compound **3** (A) $F(3, 88) = 3.711$, $p = 0.0145$ and (B) $F(6, 88) = 3.797$, $p = 0.0021$; compound **4** (A) $F(3, 88) = 10.27$, $p < 0.0001$ and (B) $F(6, 88) = 5.419$, $p < 0.0001$.

the cytotoxicity studies on **1-4** (Figure 1) isolated from the leaves of *D. gaudichaudianum*. To the best of our knowledge this is the first report on the IC_{50} values of **2** and **3** against breast and colon cancers.

MATERIALS AND METHODS

Sample Collection

Samples of leaves and twigs of *Dysoxylum gaudichaudianum* (A. Juss.) Miq. were collected from the De La Salle University – Science and Technology Complex (DLSU-STC) riparian forest in February 2014. The samples were authenticated and deposited at the De La Salle University Herbarium with voucher specimen #920.

Isolation and Structure Elucidation

The isolation and structure elucidation of **1-4** from the leaves of *D. gaudichaudianum* were reported previously¹¹.

Preparation of Compounds for Cytotoxicity Tests

The compounds (**1-4**) from *D. gaudichaudianum* 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 non-toxic DMSO concentration of 0.1%.

Maintenance and Preparation of Cell Lines for Cytotoxicity Tests

The cytotoxicity of **1-4** from the dichloromethane (CH_2Cl_2) extracts from *D. gaudichaudianum* was tested on the following human cell lines: breast cancer (MCF-7), 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^{12,13}, cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) containing 10% fetal bovine serum (FBS, Gibco®, USA) and 1x antibiotic-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%

Table 1: Cytotoxic activities (IC₅₀) of **1-4** and Zeocin against MCF-7, HCT-116, HT-29 and HDFn.

| Compound | IC ₅₀ * (µg/mL) | | | |
|----------|----------------------------|---------|-------|-------|
| | MCF-7 | HCT-116 | HT-29 | HDFn |
| 1 | 4.404 | 7.859 | 0.84 | 12.4 |
| 2 | 3.786 | 9.258 | 1.059 | 17.01 |
| 3 | 16.12 | 13.05 | 2.738 | 17.72 |
| 4 | 2.277 | 11.63 | 1.41 | 11.18 |
| Zeocin | 4.168 | 1.856 | 1.318 | 2.713 |

*IC₅₀ values were extrapolated from dose-response curves generated from nonlinear regression analysis done using GraphPad Prism 6.05. For each cell line, one-way ANOVA was conducted to determine differences among data sets (treatments). The results are: MCF-7, F (5, 118) = 35.74, p < 0.0001; HCT-116, F (5, 110) = 182.4, p < 0.0001; HT-29, F (5, 118) = 107.1, p < 0.0001; HDFn, F (5, 118) = 275.3, p < 0.0001. Results of the Tukey's multiple comparison post hoc test are discussed in this section.

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 96-well microtiter plates (Falcon™, USA) using a final inoculation density of 1 x 10⁴ cells/well. The plates were further incubated overnight (37°C, 5% CO₂, 98% humidity) until complete cell attachment was reached. These cells were used for the cytotoxicity studies as described below.

Cell Viability Assay

The cytotoxicity of the *D. gaudichaudianum* compounds was determined in a cell viability assay using PrestoBlue® (Molecular Probes®, Invitrogen, USA). This test is based on the presence of mitochondrial reductase in viable cells which then reduces the resazurin dye (blue and nonfluorescent) in the reagent to resorufin (red and highly fluorescent). The conversion is proportional to the number of metabolically active cells and is determined quantitatively using 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 0.39 µg/mL. The treated cells were further incubated (37°C, 5% CO₂, 98% humidity) for 4 days. Ten microliters of PrestoBlue® was added to each well. The cells were incubated (37°C, 5% CO₂, 98% humidity) for 2 hr. Wells with no compound added served as negative controls while wells with Zeocin™ (Gibco®, USA) served as positive controls. Wells containing cell culture media only were used to correct for background absorbance. Absorbance measurements were carried out using 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 6.07 (GraphPad Software, Inc.) to extrapolate the half maximal inhibitory concentration, IC₅₀ (the concentration of the compound which resulted in a 50% reduction in cell viability). The cytotoxicity of **1-4**

was expressed as IC₅₀ 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 determine 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 DISCUSSION

This work presents studies on the cytotoxic activities of squalene (**1**), polyprenol (**2**), triglycerides (**3**), and β-sitosterol (**4**), previously isolated from the dichloromethane extracts of *D. gaudichaudianum* leaves¹¹, against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a human normal cell line, human dermal fibroblast, neonatal (HDFn). A known anti-cancer drug, Zeocin, was used as positive control. 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 IC₅₀ values are summarized in Table 1. The breast cancer cell line (MCF-7) is most susceptible to **4**, **2**, and **1** with IC₅₀ values of 2.277, 3.786, and 4.404 µg/mL, respectively, and least susceptible to **3** with an IC₅₀ value of 16.12 µg/mL. One-way ANOVA showed that all treatments are statistically different (p < 0.0001), but Tukey's multiple comparison post hoc test revealed that there are no pairwise differences between **1** and **2**, **1** and Zeocin, **2** and **4**, and **2** and Zeocin (p > 0.05). The colon cancer cell line (HCT-116) is most susceptible to **1** and **2** with IC₅₀ values of 7.859 and 9.258 µg/mL, respectively, followed by **4** and **3** with IC₅₀ values of 11.63, and 13.05 µg/mL, respectively. One-way ANOVA showed that all treatments are statistically different (p < 0.0001), but Tukey's multiple comparison post hoc test showed pairwise differences only between **1** and **4** (p < 0.0001), and **2** and **3** (p < 0.01). The growth of

the other colon cancer cell line (HT-29) exhibited the strongest inhibition at the lowest concentrations of the compounds, with IC_{50} values of 0.84, 1.059, 1.41, 2.738 $\mu\text{g/mL}$ for **1**, **2**, **4** and **3**, respectively. Tukey's multiple comparison post hoc test showed statistical differences between all treatments except for **4** and Zeocin ($p > 0.05$). The normal cell line, HDFn, responded moderately to all the compounds, with IC_{50} values of 11.18, 12.4, 17.01, and 17.72 $\mu\text{g/mL}$ for **4**, **1**, **2**, and **3**, respectively. The pairs of compounds, **1** and **4**, and **2** and **3** are not statistically different ($p > 0.05$). 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 to the compounds tested (IC_{50} value of 0.84 $\mu\text{g/mL}$ for squalene (**1**)). This was followed by MCF-7 cells (IC_{50} values of 2.77 and 3.786 $\mu\text{g/mL}$ for β -sitosterol (**4**) and polyphenol (**2**), respectively). In a preliminary study on the cytotoxic activity of the dichloromethane extract of *D. gaudichaudianum* leaves, the crude extract exhibited cytotoxic activities with IC_{50} values of 13.19 and 7.35 $\mu\text{g/mL}$ for HT-29 and MCF-7 cancer cells, respectively¹¹. In the current study, the isolated compounds from the extract exhibited high anti-proliferative activities against the cell lines used, indicating that these compounds may be responsible for the cytotoxicity of the extract. Among the cancer cell lines tested, HCT-116 showed the least response to the compounds. On the other hand, the normal cell line, HDFn, exhibited moderate susceptibility only. The most cytotoxic effect was seen in HT-29 cells using squalene (**1**), while the least anti-proliferative effect was observed in MCF-7 cells treated with triglycerides (**3**). Overall, **1-4** showed varying, but promising cytotoxic properties. The US National Cancer Institute has defined the active cytotoxic limits of natural products as 20 $\mu\text{g/mL}$ or less for crude extracts and 4 $\mu\text{g/mL}$ or less for pure compounds¹⁴. Pure compounds that exhibit active cytotoxicity may have some potential for drug development¹³. The results showed that **1-4** from the dichloromethane extracts of *D. gaudichaudianum* leaves can be further developed for the treatment of human colorectal type and human breast cancers. The study also showed that the cytotoxic activity of **1-4** was a function of the specific type of cancer cells targeted. When the two colon cancer cell lines are compared, the IC_{50} values of **1-4** for HT-29 were lower than HCT-116, implying that the former could be more responsive to anti-cancer treatments using the compounds tested. It was reported that variances in the expression profiles of several genes associated with drug sensitivity between HCT-116 and HT-29 could influence the cells' response to different inhibitory compounds¹⁵. A related study using four human colon cancer cell lines (HCT-116, HT-29, HCT-15, and KM-12) revealed that gene expression profiling after inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, a known inhibitor of the hsp90 molecular chaperone, could explain how cells respond under different treatment conditions¹⁶. Previous studies

revealed that squalene, polyphenols, triglycerides, and β -sitosterol exhibited cytotoxic and anticancer properties. Squalene (**1**) was reported to exhibit antitumor activities against colon cancer in rodents¹⁷. It was also found to remarkably control colonic aberrant crypt foci (ACF) formation and crypt multiplicity in laboratory rats which supported the hypothesis that it possesses chemopreventive activity against colon carcinogenesis¹⁸. In a study evaluating the anti-proliferative effects of tocotrienols, carotenoids, squalene, and coenzyme Q10 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- κ B) protein in the breast cancer cells exposed briefly to tumor necrosis factor- α (TNF- α)^{19,20}. The protective and therapeutic effects of squalene-containing compounds on the promotion of tumor cells have been reported²¹. Relevant reviews on the bioactive properties of squalene have been provided^{22,23}. Thus, **1** was reported to exhibit cytotoxic properties against colon and breast cancer cells which corroborate our findings that **1** showed high cytotoxicity against colon cancer cells, HT-29 and HCT-116, and breast cancer cells, MCF-7, with IC_{50} values of 0.84, 7.859 and 4.404 $\mu\text{g/mL}$, respectively. Polyphenols (**2**) from *Ginkgo biloba* L. showed hepatoprotective effects against CCl₄-stimulated hepatotoxicity in rats²⁴ and exhibited antitumor activity²⁵. Polyphenols inhibited Heps. S (180) and EC transplanted tumors by at least 50%²⁵. Polyphenol, with concentrations between 10^{-3} - 10^{-4} M, induced apoptosis in multidrug-resistant MCF-7 cells by reducing the resistance marker, P-glycoprotein (Pgp), in the plasma membrane²⁶. In a related study, MCF-7 cells treated with 10^{-4} M of polyphenol resulted in the downregulation of DPAGT1 overexpression which led to the regulation of E-cadherin expression, an important factor in the prevention of tumors and metastasis²⁷. To the best of our knowledge, this is the first report on the cytotoxicity of **2** against colon cancer cells HT-29 and HCT116 and breast cancer cells MCF-7, with IC_{50} values of 1.059, 9.258 and 3.786 $\mu\text{g/mL}$, respectively. Triacylglycerols (**3**) from Tuna (1000 mg/kg) was reported to significantly inhibit the tumor growth in the spleen of mice with intrasplenically-implanted Lewis lung carcinoma²⁸. Triacylglycerols, which account for 12-20% of the total seed weight of pomegranate (*Punica granatum*) seed oil (PSO) extract, was found to significantly decrease cell viability in two breast cancer cell lines, MDA-MB-231 and MCF-7²⁹. To our knowledge, this is the first report on the cytotoxicity of **3** against colon cancer cells, HT-29 and HCT116, and breast cancer cells, MCF-7, with IC_{50} values of 2.738, 13.05, and 16.12 $\mu\text{g/mL}$, respectively. β -Sitosterol (**4**) was found to affect the programmed cell death pathway in human breast cancer cells (MCF-7) and human adenocarcinoma cells (MDA-MB-231), hindering tumor growth by promoting apoptosis³⁰. It also inhibited cell proliferation of human colon cancer cells (HT-29)³¹. β -Sitosterol (**4**) inhibited the growth of HT116 human colon cancer cells by different mechanisms including the activation of caspase-3 and caspase-9, accompanied by proteolytic cleavage of

poly(ADP-ribose)-polymerase and the reduction of the expression of the anti-apoptotic Bcl-2 protein and mRNA and a subsequent increase of the pro-apoptotic Bax protein and mRNA³². *In vitro* studies showed that **4** inhibited the proliferation of human colon cancer cells (COLO 320 DM) with an IC₅₀ of 266.2 µM, inducing apoptosis by scavenging oxidants and attenuating β-catenin and PCNA expression while *in vivo* studies showed that **4** reduced the number of aberrant crypt and crypt multiplicity in DMH-initiated rats in a dose-dependent fashion³³. β-Sitosterol (**4**) significantly decreased the expression of Niemann-Pick C1-like 1 (NPC1L1) in human small intestine epithelial cells (FHs 74 Int), reducing intestinal cholesterol absorption at the cellular level³⁴. It also promoted apoptosis mediated by the activation of ERK and the downregulation of Akt in murine fibrosarcoma cells (MCA-102)³⁵. It was reported that **4** and stigmasterol exhibited anti-proliferative activities against human prostate cancer cells (DU-145) by increasing the expression of p53 protein and inhibiting carcinoma development by decreasing the expression of p21 and p27 proteins³⁶. Thus, **4** was reported to exhibit cytotoxic properties against several cancer cell lines such as colon and breast cancer cell lines which corroborate our findings that **4** showed high cytotoxicity against colon cancer cells, HT-29, and breast cancer cells, MCF-7, with IC₅₀ values of 1.41 and 2.277 µg/mL, respectively.

CONCLUSION

Squalene (**1**), polyphenols (**2**), triglycerides (**3**), and β-sitosterol (**4**), isolated from the dichloromethane extract of *Dysoxylum gaudichaudianum* leaves, 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 IC₅₀ values ranging from 0.84 to 2.738 µg/mL, followed by MCF-7, with IC₅₀ values ranging from 2.277 to 16.12 µg/mL, and HCT-116, with IC₅₀ values ranging from 7.859 to 13.05 µg/mL. All compounds (**1-4**) were moderately cytotoxic against HDFn with IC₅₀ values >10 µg/mL. Overall, **1-4** were more cytotoxic to the breast and colon cancer cell lines than to the normal cell line.

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