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Chemical constituents and cytotoxicity of the leaves of *Dysoxylum gaudichaudianum* (A. Juss.) Miq.

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ABSTRACT

*The crude dicloromethane leaf extract of Dysoxylum gaudichaudianum (A. Juss.) Miq. was tested for cytotoxicity against breast cancer (MCF-7) and colon cancer (HT-29) cells and exhibited IC_{50} values of 7.35 and 13.19 μg/mL, respectively. Chemical investigation of the dichloromethane leaf extract afforded squalene (**1**), β-sitosterol (**2**), polyprenols (**3**) and triglyceride (**4**). The structures of **1**-**4** were identified by comparison of their ^{1}H NMR data with those reported in the literature. These compounds are known cytotoxic and anticancer compounds.*

Keywords: *Dysoxylum gaudichaudianum* (A. Juss.) Miq., Meliaceae, squalene, β-sitosterol, polyprenols, triglyceride, cytotoxicity, anti-cancer

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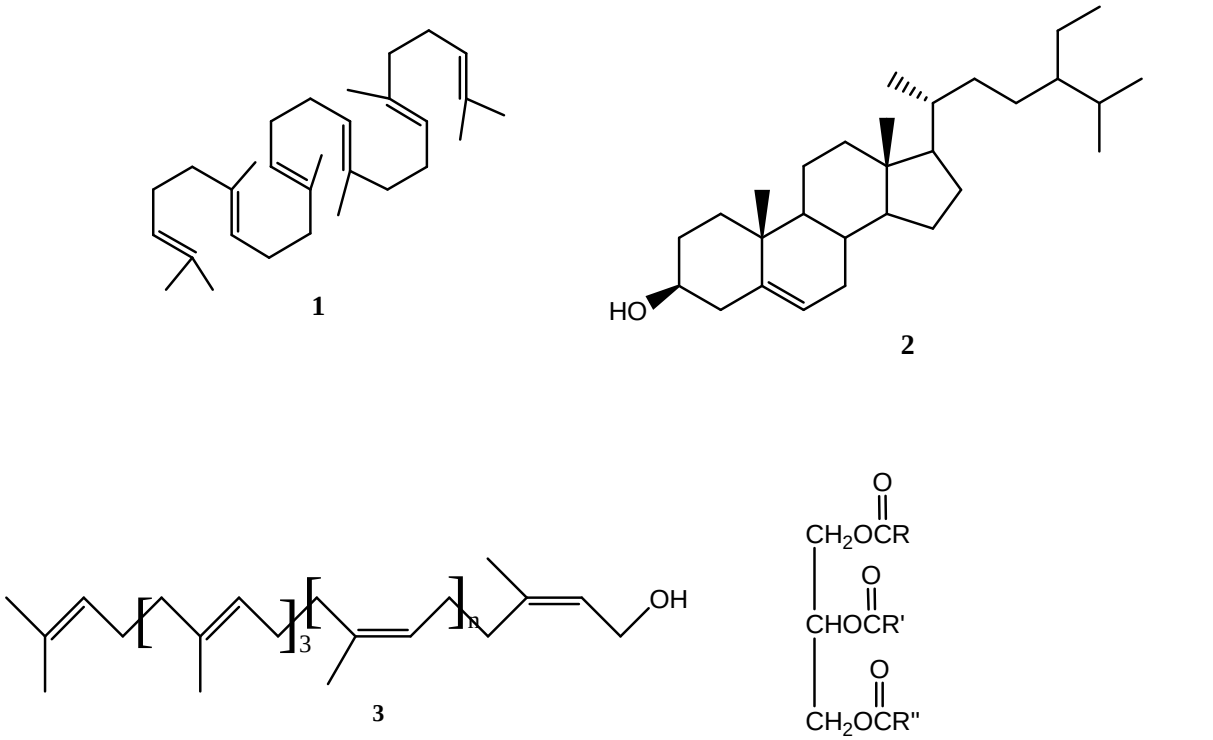
INTRODUCTION

Dysoxylum gaudichaudianum (A. Juss.) Miq. of the family Meliaceae may be found in thickets and forests up to 1,800 m asl in many parts of the Philippines and in other Malesian regions down to Samoa. In folk medicine, different preparations from the leaves and bark are used to treat coughs, skin irritations, sexually transmitted diseases, and are also useful as astringent and emetic agent [1, 2]. A study has reported that the juice prepared from the leaves could be used as an early abortifacient [3]. In another study, the extracts of the plant's bark yielded four new compounds, dysoxylins A−D, belonging to the tetranortriterpenoid family, which showed potent antiviral bioactivity against anti-respiratory syncytial virus (RSV) [4, 5]. Several compounds, *p*-hydroxyacetophenone, β-sitosterol and stigmasterol had been isolated from hexane and chloroform extracts of the plant's stem bark [6]. The methanol extract of the bark of *Dysoxylum gaudichaudianum* afforded a new limonoid, gaudichaudysolin A, which was found inactive 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) cells [7].

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 compounds, 24,25-epoxy-3b,23-dihydroxy-7-tirucallene, squalene, polyprenol, linoleic acid and lutein from the leaves of *Dysoxylum mollissimum* Blume [8]. We report herein the isolation and identification of squalene (**1**), β-sitosterol (**2**), polyprenols (**3**) and triglyceride (**4**) from the leaves of *Dysoxylum gaudichaudianum* (A. Juss.) Miq. (Fig. 1). The cytotoxicities of the dichloromethane leaf extract on breast cancer cells (MCF-7) and colon cancer cells (HT-29) are likewise reported.



1 **2** **3**

4 R = R' = R" = long chain fatty acids

Fig. 1. Chemical constituents of *Dysoxylum gaudichaudianum* (A. Juss.) Miq.: squalene (1), β-sitosterol (2), polyprenols (3) and triglyceride (4)

MATERIALS AND METHODS

1. Cytotoxicity of Crude Leaf Extract

1.1 Sample Collection

Samples of leaves 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 by one of the authors (EHM) and deposited at the De La Salle University Herbarium with voucher specimen #920.

1.2 Preparation of Crude Extract

The *D. gaudichaudianum* leaves were air-dried, ground in a blender, soaked in CH_2Cl_2 for 3 days, then filtered. The solvent was evaporated under vacuum to afford a crude extract which was dissolved in an appropriate concentration of dimethyl sulfoxide to make a 4 mg/mL stock solution.

1.3 Culture of Cell Lines

The bioactivity of the CH_2Cl_2 extract was tested on MCF-7 and HT-29 human cancer cell lines (ATCC, Manassas, Virginia, U.S.A.) which are routinely maintained at the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Science and Ecological Research, De La Salle University. Following standard procedures, cells were grown in DMEM (Gibco®, USA) containing 10% FBS (Gibco®, USA) and 1x antibiotic-antimycotic (Gibco®,

USA) and grown at 37°C with 5% CO_2 in a 98% humidified incubator. Upon reaching 80% confluence, the monolayer cultures were washed with phosphate-buffered saline (PBS pH 7.4), trypsinized using 0.05% Trypsin-EDTA, and resuspended with complete fresh media. Cells were later seeded in 100 µL aliquots into 96-well microtiter plates (Falcon™, USA) in a final inoculation density of 1 x 10^4 cells/well previously counted following standard trypan blue exclusion protocol [9]. The plates were further incubated overnight at 37°C with 5% CO_2 in a 98% humidified incubator until the cells were completely attached.

1.4 PrestoBlue® Assay

After overnight incubation, one hundred microliters of filter-sterilized *D. gaudichaudianum* extract was added to corresponding wells at two-fold serial dilutions to make final treatment concentrations of 50, 25, 12.5, 6.25, 3.125 µg/mL. The treated cells were further incubated for 4 days at 37°C in 5% CO_2 and 98% humidity. Ten microliters of PrestoBlue® (Molecular Probes, Invitrogen, USA) was added to each well and further incubation was done for 1 hr at 37°C in 5% CO_2 and 98% humidity. Wells with cells containing no extract–served as negative controls. Absorbance measurements were carried out with BioTek ELx800 Absorbance Microplate Reader (BioTek® Instruments, Inc.) at 570 nm and normalized to 600 nm values (reference wavelength). Absorbance readings were used to calculate for the half maximal inhibitory concentration, IC_{50} (the concentration of the extract which resulted in a 50% reduction in cell viability). All tests were performed in triplicate and data were shown as mean ± SEM. Nonlinear regression and statistical analyses were conducted using Prism® 6.03 (GraphPad Software, Inc.).

2. Isolation of the Chemical Constituents of the CH_2Cl_2 Leaf Extract

2.1 General Experimental Procedure

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

2.2 General Isolation Procedure

A glass column 20 inches in height and 2.0 inches internal diameter was packed with silica gel. The crude extract from the leaves were fractionated by silica gel chromatography using increasing proportions of acetone in dichloromethane (10% increment) as eluents. One hundred milliliter fractions were collected. All fractions were monitored by thin layer chromatography. Fractions with spots of the same *Rf* values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Five milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

2.3 Isolation

The air-dried leaves of *D. gaudichaudianum* (249.0 g) was ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (16.2 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (3 ×) using 1% EtOAc in petroleum ether to afford **1** (2.02 g). The 20% to 30% acetone in CH_2Cl_2 fractions were combined and rechromatographed by gradient elution in 5% EtOAc in petroleum ether, followed by 7.5% EtOAc in petroleum ether, then finally 10% EtOAc in petroleum ether. The fractions eluted with 5% EtOAc in petroleum ether were combined and rechromatographed (3 ×) in 7.5% EtOAc in petroleum ether to afford **3** (5 mg). The fractions eluted with 7.5% EtOAc in petroleum ether were combined and rechromatographed (4 ×) in the same solvent to afford **4** (6 mg). The 40-50% acetone in CH_2Cl_2 fractions were combined and rechromatographed (5 ×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9) by volume ratio to afford **2** (3 mg) after washing with petroleum ether.

Squalene (**1**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.08-5.13 (6H. =CH), 1.58 (18H, allylic CH_3, *cis*), 1.66(6H, allylic CH_3, *trans*), 1.94-2.07 (20H, allylic CH_2).

β-Sitosterol (**2**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.50 (m, H-3), 2.26, 2.21 (H_2-4), 5.33 (dd, *J* = 5.0, 2.0 Hz, H-6), 0.66 (s, CH_3-18), 0.99 (s, CH_3-19), 0.90 (d, *J* = 7.0 Hz, CH_3-21), 0.79 (d, *J* = 7.0 Hz, CH_3-26), 0.82 (d, *J* = 7.0 Hz, CH_3-27), 0.85 (t, *J* = 7.0 Hz, CH_3-29).

Polyprenol (**3**): ^{1}H NMR (500 MHz, $CDCl_3$): d 4.07 (2H, d, *J* = 7.0 Hz, CH_2OH), 5.43 (1H, =CH), 5.08-5.12 (11H, =CH), 1.95-2.08 (40H, allylic CH_2), 1.73 (3H, allylic CH_3), 1.66 (21H, allylic CH_3), 1.59 (12H, allylic CH_3).

Triglyceride (**4**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.27 (dd. *J* = 4.5, 12.0 Hz), 4.12 (dd, *J* = 6.0, 12.0 Hz, glyceryl CH_2O), 5.32 (glyceryl CHO), 2.30 t (6.6, α-CH_2), 5.33 (olefinic H), 2.77 (double allylic CH_2), 2.03 (allylic, CH_2), 1.23-1.35 (CH_2), 2.79 (d, *J* = 6.5 Hz, double allylic CH_2), 2.77 (d, *J* = 6.5 Hz, double allylic CH_2), 2.75 (d, *J* = 6.5 Hz, double allylic CH_2), 2.74 (d, *J* = 6.5 Hz, double allylic CH_2), 0.95 (t, *J* = 7.5 Hz, CH_3), 0.87 (t, *J* = 7.0 Hz, CH_3), and 0.85 (t, *J* = 7.0 Hz, CH_3).

RESULTS AND DISCUSSION

This study presents preliminary investigations on the cytotoxic activity of the dichloromethane extract of *D. gaudichaudianum* leaves on two human cancer cell lines, breast (MCF-7) and colon (HT-29). Figure 2 shows the cell viability as a function of the logarithmic values of extract concentration. The analyzed data for both cell lines follow the typical sigmoidal curve characteristic of an inhibitory dose-response relationship. It has been reported in literature that crude extracts with IC_{50} of 30 µg/ml or less should be purified further while pure compounds with IC_{50} of 5 µg/ml or less may have some potential for drug development [10]. The extract exhibited high cytotoxic activities with IC_{50} values of 7.35 and 13.19 µg/ml, for breast and colon cancer cells, respectively.

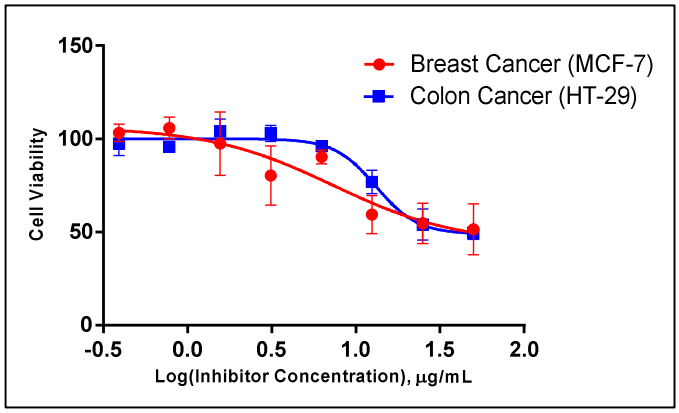


Fig. 2. Dose-response curves showing the cell viability inhibition of the dichloromethane extract of *Dysoxylum gaudichaudianum* leaves against human breast cancer (MCF-7) and human colon cancer (HT-29) cells

Purification of the dichloromethane extract of the leaves of *D. gaudichaudianum* afforded squalene (**1**) [11], β-sitosterol (**2**) [12], polyprenols (**4**) [13] and triglycerides (**3**) [14]. The structures of **1**-**4** were identified by comparison of their ^{1}H NMR data with those reported in the literature [11-14]. The fatty acids esterified to the glycerol in the triglycerides are linolenic acid, linoleic acid and saturated fatty acid. These were deduced from the integration of the resonances at δ 0.95 (t, *J* = 7.5 Hz, CH_3), 2.79 (d, *J* = 6.5 Hz, 2 double allylic CH_2) and 2.77 (d, *J* = 6.5 Hz, 2 double allylic CH_2) for the linolenic acid; δ 0.87 (t, *J* = 7.0 Hz, CH_3), 2.75 (d, *J* = 6.5 Hz, double allylic CH_2) and 2.74 (d, *J* = 6.5 Hz, double allylic CH_2) for the linoleic acid; and δ 0.85 (t, *J* = 7.0 Hz, CH_3) for the saturated fatty acid.

Although no biological activity tests were conducted on the isolated compounds (**1**-**4**), literature search revealed that these have diverse bioactivities.

Squalene (**1**) was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis [15]. It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties [16]. A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 have anti-proliferative effects on breast cancer cells [17]. The preventive and therapeutic potential of squalene containing compounds on tumour promotion and regression have been reported [18]. A recent review on the bioactivities of squalene has been provided [19].

β-Sitosterol (**2**) was reported to exhibit growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells [20]. It was shown to be effective for the treatment of benign prostatic hyperplasia [21]. It attenuated β-catenin and PCNA expression, as welll as quenched radical *in-vitro*, making it a potential anticancer drug for colon carcinogenesis [22]. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells [23]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake [24].

Polyprenols (**3**) act as co-enzymes of membrane active transport systems for polysaccharides, peptidoglycans and carbohydrate containing biopolymers [25]. Polyprenols from *Ginkgo biloba* L showed hepatoprotective effects against CCl_4-induced hepatotoxicity in rats [26] and exhibited antitumor activity [27]. The antidyslipidemic activity of polyprenols from *Coccinia grandis* in high-fat diet-fed hamster model was also reported [28].

Triglycerides (**4**) exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans*, and *T. mentagrophytes* [29]. Another study reported that triglycerides showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation [30]. Linoleic acid which is one of the fatty acids esterified to **4** belongs to the omega-6 fatty acids. It was reported to be a strong anticarcinogen in a number of animal models. It reduces risk of colon and breast cancer [31] and lowers cardiovascular disease risk and inflammations [32]. Linolenic acid which is another fatty acid esterified to **4** belongs to omega-3 fatty acid. A previous study reported that α-linolenic acid (ALA) inhibited the human renal cell carcinoma (RCC) cell proliferation [33]. Another study reported that apoptosis of hepatoma cells was induced by the α-linolenic acidenriched diet which correlated with a decrease in arachidonate content in hepatoma cells and decreased cyclooxygenase-2 expression [34]. γ-Linolenic acid (GLA) and α-linolenic acid (ALA) exhibited greater than 90% cytotoxicity between 500 μM and 1 mM against all but two malignant micro-organ cultures tested in 5-10% serum. GLA and ALA killed tumor at concentrations of 2 mM and above in tests using 30-40% serum [35].

CONCLUSION

The dichloromethane extract of the leaves of *D. gaudichaudianum* exhibited high cytotoxic activities against breast cancer cells (MCF-7) and colon cancer cells (HT-29) with IC_{50} values of 7.35 and 13.19 μg/ml, respectively. Purification of the dichloromethane leaf extract afforded squalene (**1**), β-sitosterol (**2**), polyprenols (**3**) and triglycerides (**4**). These compounds were reported to exhibit cytotoxic and anticancer properties. The high cytotoxicity of the crude dichloromethane leaf extract maybe attributed to the synergistic effects of these compounds, among others which are found in the extract. The high concentration of squalene (**1**) (12.47%) in the extract may also contribute to its high cytotoxicity since squalene was reported to exhibit cytotoxic, antitumor and anticancer properties.

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