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Non-nutritive functional agents in rattan-shoots, a food consumed by native people in the Philippines

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ABSTRACT

The tender shoots of *Calamus ornatus*, one of the food items consumed by the native people, Kanawan Aytas, in the Bataan region of the Philippines, have not been studied before. A bioassay-guided investigation of its methanolic extract afforded non-nutritive functional agents (NFAs), steroidal saponins **1**–**3**, along with its aglycone (**4**). The NFAs **1**–**4** inhibited cyclooxygenase enzymes, COX-1 and -2, by 47%, 43%, 33%, and 53% and 71%, 75%, 78%, and 73%, respectively, at 28.2, 24.2, 21.2 and 60.4 μM. Treatment of breast (MCF-7), CNS (SF-268), lung (NCI-H460), colon (HCT-116) and gastric (AGS) cancer cell lines with the extract at 100 μg/ml reduced cell proliferation. Similarly, the pure NFAs **2** and **3** reduced the cell viability of breast, CNS, lung, colon and gastric cancer cell lines by 37.5%, 22.4%, 53.3%, 58.2%, 40.3% and 29.8%, 21.3%, 45.6%, 37.1%, 25.0%, respectively, at 24.2 and 21.2 μM. The 50% reduction in cell viability (IC_{50}) concentrations of **2** and **3** against these cancer cell lines were 8.8, 6.1, 7.5, 23.8, 12.1 and 3.8, 7.1, 3.3, 14.3, 12.1 μM, respectively. This is the first report on the isolation of steroidal saponins from *C. ornatus* shoots and their antiinflammatory and tumor cell proliferation inhibitory activities. Therefore, our results suggest that the Kanawan Aytas may yield health benefits from rattan-shoots in their diet.

1. Introduction

The rattan, *Calamus ornatus*, is a member of the *Arecaceae* family grown in the tropical or subtropical climates. Rattans are spiny and climbing palms and comprise about 600 species belonging to 13 genera, concentrated solely in the old world tropics (Dransfield, 1991b). Four genera of rattan with 64 species found in the Philippines are *Calamus*, *Daemonorops*, *Kothalsia* and *Plectocomia*. The largest genus is *Calamus* with 44 species and 23 varieties (Tesoro, 2002). The rattan we have studied, *C. ornatus* Bl. ex. Shult. f. var. Philippinensis Becc., locally known as "limuran" and grown at low and medium altitudes, is endemic to the Philippines (Fernando, Sun, Suh, Kong, & Koh, 2004). In the Bataan region of the Philippines, *C. ornatus* grows at 200–400 m of elevation where the Kanawan Ayta community is located. The Kanawan Aytas forage the forest primarily for food, medicine and firewood. Although the Aytas consume root crops, vegetables and fruits, they also include foodstuffs that are uncommon to the general public in their diet, such as *C. ornatus* shoots (Santos-Acuin, Troy, & Justiniana, 1997).

The acquaintances and discussions with the Kanawan Aytas by some of the authors of this manuscript revealed that consumption of young shoots of *C. ornatus* by the Aytas was not only for nutrition but also for health benefits. Based on the anecdotal reports, we have investigated the antiinflammatory and anticancer activities of non-nutritive functional agents (NFAs) in the edible portion of the *C. ornatus* shoot extract.

The inhibition of prostaglandin and leukotriene production during the metabolic process provides a framework for the treatment of pain and inflammation by non-steroidal antiinflammatory drugs (NSAIDs). Although these agents pose substantial side effects, the selective inhibition of cyclooxygenase-2 (COX-2) enzyme has proven to be beneficial in reducing gastrointestinal toxicity (Vane et al., 1994). Also, the role of COX-2 enzyme in cancer initiation and progression has been reported. For example, COX-2 has been associated with neoplastic transformation, cell growth, angiogenesis, invasiveness and metastasis (Chang, Liu, & Conway, 2004;

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; COSY, correlation spectroscopy; FABMS, fast atom bombardment mass spectroscopy; HMBC, heteronuclear multiple bond coherence; TBHQ, *t*-butylhydroquinone; COX, cyclooxygenase; NSAID, non-steroidal antiinflammatory drug.

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Eberhart et al., 1994; Fujita, Koshida, & Keller, 2002). Therefore, the objective of this study was to isolate the NFAs in *C. ornatus* shoots by employing COX enzyme and human tumor cell proliferation inhibitory assays. This is the first report on the isolation of steroidal saponins with antiinflammatory and tumor cell proliferation inhibitory activities from *C. ornatus* shoots.

2. Materials and methods

2.1. Plant material

The terminal shoots of *C. ornatus* Bl. ex. Shult. f. var. Philippinensis Becc. (*Arecaceae*) were collected from the forest located in Sitio Kanawan, Morong, Bataan, Philippines in May, 2006. The plant material was authenticated by Ulysses F. Ferreras, the Field Botanist and Curatorial Assistant. A voucher specimen (PUH 14579) has been deposited at Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines-Diliman, Quezon City, Philippines.

2.2. General experimental procedures

Melting points were determined on Bristoline and V-Scientific melting point apparatus (MP-1) and are uncorrected. The NMR (^{1}H and ^{13}C) spectra were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. ^{13}C NMR spectra were recorded at 75 and 125 MHz, respectively. The chemical shifts were measured in, DMSO-d_6 or Pyridine-d_5 and are expressed in (ppm). Mass spectra were recorded at the Michigan State University Mass Spectrometry Facility using a JEOL HX-110 double focussing mass spectrometer (Peabody, MA). Merck silica gel (60-mesh size, 35–70 μm) was used for medium pressure liquid chromatography (MPLC). For preparative TLC separation, 500 and 1000 μm (20 × 20 cm) silica gel plates (Analtech Inc., Newark, DE) were used. TLC plates were viewed under UV-light at 254 and 366 nm or sprayed with 10% sulfuric acid solution in methanol. ACS grade solvents were used for isolation and purification. Fetal bovine serum (FBS) and Roswell Park Memorial Institute-1640 (RPMI-1640) medium were obtained from Gibco BRL (Grand Island, NY). Human tumor cell lines used in the assay, breast (MCF-7), central nervous system, CNS (SF-268), and lung (NCI-H460) purchased from the National Cancer Institute (NCI, Bethesda, MD), colon (HCT-116) and gastric (AGS) cell lines purchased from American Type Culture Collection (ATCC, Rockville, MD) were maintained in our laboratory. The COX-1 enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical Research Inc. (Oxford, MI). The COX-2 enzyme was prepared from insect cells cloned with human PGHS-2 enzyme. Aspirin, Celeberex, and Vioxx® were used as positive controls in COX enzyme inhibitory assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and arachidonic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.3. Extraction and isolation

2.3.1. General

Air-dried *C. ornatus* shoots (450 g) were milled to a coarse powder and extracted with hexane (3 × 500 ml), ethyl acetate (3 × 500 ml) followed by methanol (3 × 500 ml) and removal of solvent under vacuum afforded 2.22, 1.77, and 73.2 g of crude extracts, respectively. The methanol extract was syrupy in nature and hence partitioned with water (500 ml) and centrifuged for 15 min at 10,000 rpm. The precipitate and supernatant were lyophilized separately to yield white amorphous powders, 23.72 and 35.69 g, respectively.

An aliquot of the precipitate (3 g) was subjected to medium pressure liquid chromatography (MPLC) using silica gel and $CHCl_3$:MeOH mixtures as eluting solvents. Fractions were collected in 35 ml portions, monitored by TLC and combined to yield fractions I ($CHCl_3$:MeOH, 8:2, 0.42 g), II ($CHCl_3$:MeOH, 7:3, 1.56 g), and III ($CHCl_3$:MeOH, 1:1, 0.90 g). Fractions I and II were found to inhibit COX enzyme and tumor cell proliferation.

Fraction II, showed three spots on TLC with R_f values of 0.3, 0.35 and 0.4, respectively, when developed with $CHCl_3$:MeOH:HOAc (4:1:0.1) as the mobile phase. The bands were UV-inactive and hence were visualized by spraying with 10% H_2SO_4 in methanol. Fraction II (500 mg) was purified further by TLC (500 and 1000 μm) using $CHCl_3$:MeOH:HOAc (4:1:0.05) as the mobile phase. The plates were developed twice in the same solvent system, dried, visualized by spraying with water and the bands eluted separately with methanol. The eluate from band I was concentrated and crystallized from a mixture of $CHCl_3$:MeOH (9:1) to yield pure compound **1** (7 mg). Similarly, crystallization of bands II and band III afforded pure compounds **2** (193.5 mg) and **3** (98 mg), respectively.

Fraction I (0.4 g) was purified by silica gel MPLC using $CHCl_3$:MeOH (9:1, 750 ml) as the mobile phase. A total of 25 fractions were collected in aliquots of 30 ml. Fractions that showed similar TLC profiles were pooled and afforded fractions A (130 mg) and B (190 mg). Fraction A was crystallized from a mixture of $CHCl_3$:MeOH (9.5:0.5) as solvent system to give β-sitosterol glycoside (10 mg), which was identified by NMR spectral data and by comparison with the reported spectral data (Wang & Dubois, 2004). Fraction B was purified further by a MPLC silica gel column by using $CHCl_3$:MeOH (9:1, 500 ml) as mobile phase. In total, 20 fractions were collected in aliquots of 25 ml. Fractions that showed similar TLC profiles were combined and removal of solvent gave a white amorphous solid, compound **4** (21 mg), identified as diosgenin by comparison of its physical and spectral data with the published spectral data of diosgenin (Farid, Haslinger, Kunert, Wegner, & Hamburger, 2002).

2.3.2. Compound *1*

White powder, FABMS (positive mode) *m*/*z*: 907.5 $[M+Na]^+$; ^{1}H NMR (DMSO-d_6): δ 5.32 (1H, brd, J = 7.0 Hz, H-6), 5.05 (1H, s, H-1″), 4.43 (1H, d, J = 7.5 Hz, H-1′), 4.18–4.28 (3H, m, H-16, H-1‴), 1.22 (3H, s, Me-19), 1.07 (3H, d, J = 6.0 Hz, Me-H-6″), 0.95 (3H, s, Me-18), 0.89 (3H, d, J = 6.0 Hz, Me-21), 0.72 (3H, d, J = 6.0 Hz, Me-27). The NMR spectrum of **1** in pyridine-d_5 was not as resolved as in DMSO due to the paucity of the compound isolated. Based on the spectral data, compound **1** was identified as diosgenin-3-*O*-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside (Han et al., 1999; Ohtsuki et al., 2006).

2.3.3. Compound *2*

An amorphous solid, FABMS (positive mode) *m*/*z*: 1053.5 $[M+Na]^+$; ^{1}H NMR (pyridine-d_5): δ 6.17 (1H, brs, H-1″), 5.84 (1H, brs, H-1‴), 5.27 (1H, brd, J = 4.8 Hz, H-6), 5.05 (1H, d, J = 8.0 Hz, H-1⁗), 4.91 (1H, d, J = 7.5 Hz, H-1′), 1.76 (3H, d, J = 6.0 Hz, Me-6″), 1.69 (3H, d, J = 6.0 Hz, Me-6‴), 1.18 (3H, d, J = 4.7 Hz, Me-21), 0.68 (3H, d, J = 5.0 Hz, Me-27), 1.03 (3H, s, Me-19), 0.81 (3H, s, Me-18); ^{13}C NMR (pyridine-d_5): δ 37.5 (C-1), 30.5 (C-2), 77.5 (C-3), 39.3 (C-4), 141.2 (C-5), 122.1 (C-6), 32.7 (C-7), 32.1 (C-8), 50.6 (C-9), 37.8 (C-10), 21.4 (C-11), 40.3 (C-12), 40.8 (C-13), 57.0 (C-14), 32.5 (C-15), 81.4 (C-16), 63.3 (C-17), 16.6 (C-18), 19.7 (C-19), 42.4 (C-20), 15.3 (C-21), 19.6 (C-22), 32.2 (C-23), 30.9 (C-24), 29.6 (C-25), 67.2 (C-26), 17.6 (C-27), 100.4 (C-1′), 78.5 (C-2′), 76.7 (C-3′), 69.8 (C-4′), 78.1 (C-5′), 61.3 (C-6′), 102.0 (C-1″), 72.9 (C-2″), 73.1 (C-3″), 75.5 (C-4″), 70.8 (C-5″), 18.8 (C-6″), 103.1 (C-1‴), 74.3 (C-2‴), 74.4 (C-3‴), 82.1 (C-4‴), 67.2 (C-5‴), 18.9 (C-6‴), 105.4 (C-1⁗), 76.4 (C-2⁗), 78.1 (C-3⁗), 70.8 (C-4⁗), 78.1 (C-5⁗), 62.0 (C-6⁗). Based on the spectral data, compound **2** was identified

as diosgenin-3-*O*-α-L-rhamnopyranosyl-(1–4)-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside (Han et al., 1999; Ohtsuki et al., 2006).

2.3.4. Compound **3**

White amorphous powder, FABMS (positive mode) *m*/*z*: 1053 $[M+Na-146]^+$; ^1H NMR (pyridine-d_5): δ 6.18 (1H, brs, H-1″), 5.78 (1H, s, H), 5.97 (1H, d), 5.90 (1H, d,), 5.28 (1H, brs, H-6), 3.49 (t), 3.26 (m), 1.73 (3H, d, J = 6.5 Hz, Me-6″), 1.63 (3H, d, J = 6.0 Hz, Me-6‴), 1.12 (3H, d, J = 6.5 Hz, Me-21), 0.67 (3H, d, J = 4.5 Hz, Me-27), 1.03 (3H, s, Me-19), 0.82 (3H, s, Me-18). Based on the spectral data, compound **3** was identified as diosgenin-3-*O*-β-D-glucopyranosyl-(1–4)-α-L-rhamnopyranosyl-(1–4)-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside (Han et al., 1999; Ohtsuki et al., 2006).

2.3.5. Compound **4**

An amorphous solid, FABMS (positive mode) *m*/*z*: 415.5 $[M+H]^+$; ^1H NMR (pyridine-d_5): δ 5.41 (1H, brs, H-6), 4.56 (1H, d, J = 7.0 Hz, H-16), 3.51–3.59 (2H, m, H-26), 2.63 (2H, m, H-4), 1.16 (3H, d, J = 6.6 Hz, Me-21), 1.08 (3H, s, Me-19), 0.87 (3H, s, Me-18), 0.70 (3H, d, J = 7.0 Hz, Me-27); ^{13}C NMR (pyridine-d_5): δ 37.4 (C-1), 31.0 (C-2), 71.7 (C-3), 38.2 (C-4), 142.5 (C-5), 121.4 (C-6), 32.6 (C-7), 31.2 (C-8), 50.8 (C-9), 37.4 (C-10), 21.6 (C-11), 40.4 (C-12), 40.9 (C-13), 57.2 (C-14), 32.7 (C-15), 81.5 (C-16), 63.4 (C-17), 16.8 (C-18), 20.0 (C-19), 42.4 (C-20), 15.4 (C-21), 109.7 (C-22), 32.2 (C-23), 29.7 (C-24), 31.0 (C-25), 67.3 (C-26), 17.7 (C-27); FAB mass *m*/*z*: 415 $[M+H]^+$. Based on the spectral data, compound **4** was identified as diosgenin, the aglycone (Farid et al., 2002).

2.4. Acid hydrolysis

Compound **2** (5 mg) in ethanol (3 ml) and 3 M HCl (3 ml) was refluxed for 4 h. The solvent was removed under vacuum and stirred with water. The mixture was then extracted with ethyl acetate. The aqueous portion was concentrated and analyzed by TLC. Sugars were identified as L-rhamnose and D-glucose by comparison with authentic samples. The ethyl acetate extract was concentrated under reduced pressure and the resulting solid was identified as diosgenin (3 mg) based on NMR spectral data. The procedure was repeated for all three compounds to confirm their aglycone and sugar moieties.

2.5. Tumor cell viability assay

Breast, central nervous system, lung, colon and gastric cancer cell lines were grown in RPMI-1640 medium at 37 °C under 5% CO_2 in a humidified incubator. Cells were harvested, counted and transferred into 96-well plates and incubated for 24 h prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in DMSO, followed by dilution with RPMI-1640 medium to yield the final DMSO concentration in the assay well as 0.2%. Stock solutions of compounds **1**–**4** were prepared at 28.2, 24.2, 21.2 and 60.4 μM and diluted to yield desired concentrations. Solutions of test samples (100 μl) at the desired dilutions were added to the wells containing the cells and incubated for 48 h; 25 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (5 mg/1 ml PBS solution) were added to each of the 96 wells (Jayaprakasam, Vanisree, Zhang, Dewitt, & Nair, 2006). The plates were wrapped with aluminium foil and incubated at 37 °C for 3 h. In the solution in each well containing media, unbound MTT and dead cells were removed by suction and 200 μl of DMSO were added to each well. The plates were then shaken and optical density was recorded using a micro plate reader at 575 nm. Adriamycin was used as positive control and DMSO as solvent control. Controls and test compounds were assayed in triplicate for each concentration and replicated three times for each cell line. The cell viability was calculated with respect to DMSO control.

2.6. COX enzyme inhibitory assay

The inhibitory activities of the extract and compounds were carried out against COX-1 and -2 enzymes according to the previously published procedures (Jayaprakasam et al., 2006). The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction, with arachidonic acid as substrate, was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instrument Inc., Yellow Spring, OH). The test compounds, extract and positive controls were dissolved in DMSO separately. An aliquot of 10 μl of DMSO or test compounds or standards in DMSO was added to the reaction chamber containing 0.6 ml of 0.1 M Tris buffer (pH 7), 1 mM phenol and hemoglobin (17 μg). COX-1 or -2 enzymes (10 μl) were added to the chamber and incubated for 3 min. The reaction was initiated by the addition of arachidonic acid (10 μl of 1 mg/ml solution). Instantaneous inhibition was measured by using Quick Log Data acquisition and control computer software (Strawberry Tree Inc., Sunnyvale, CA, USA). The percent inhibition was calculated with respect to DMSO control. Pure compounds **1**–**4** were tested at 28.2, 24.2, 21.2 and 60.4 μM, respectively. Each sample was assayed twice and the standard deviation was calculated for n = 2. Aspirin (60 μM), Celebrex™ (26 nM) and Vioxx® (32 nM) were used as positive controls.

3. Results and discussion

Non-traditional foods and dietary supplements are gaining popularity among consumers. The young shoots of *C. ornatus* have been consumed as an indigenous food by the native Kanawan Aytas in the Philippines. The anecdotal health claims of this non-traditional food prompted us to evaluate the NFAs present in it. In order to isolate both polar and non-polar active agents from dried *C. ornatus* shoots, we have sequentially extracted the plant material with hexane, ethyl acetate and methanol. The percentage yields of the extracts obtained were 2.42, 1.93 and 95.65, respectively, for hexane, ethyl acetate and methanol, on a dry weight basis.

Initially, the hexane, ethyl acetate and methanolic extracts of the shoot were dissolved in DMSO and the resulting solutions were assayed for COX-1 and -2 enzymes at 100 μg/ml and human tumor cell proliferation inhibitory activities at 250 μg/ml. Among the extracts assayed, the methanolic extract was found to be the most active against COX-2 with an inhibition value of 88.8% while inhibiting COX-1 enzyme by 16.9%. In addition, it reduced the viability of breast, CNS, lung, colon and gastric cancer cell lines by 47.7%, 16.6%, 32.9%, 69.8% and 26.8%, respectively, with respect to DMSO control at 100 μg/ml.

The active methanolic extract was therefore stirred with water and centrifuged to separate the supernatant from the precipitate. Bioassay-guided fractionation and purification of an aliquot of the precipitate afforded NFAs **1**–**4**, (Fig. 1) along with the ubiquitous β-sitosterol glycoside.

Compound **2** was obtained as an amorphous solid, and its molecular formula was determined as $C_{63}H_{104}O_{33}Na$, based on positive FABMS data. The ^1H NMR spectrum of compound **2** displayed signals corresponding to an olefinic proton at δ_H 5.27 (1H, d, J = 4.8 Hz, H-6), two secondary methyl groups at δ_H 1.18 (3H, d, J = 4.7 Hz, Me-21) and 0.68 (3H, d, J = 5.0 Hz, Me-27) and two tertiary methyl signals at δ_H 0.81 (3H, s, Me-18) and 1.03 (3H, s, Me-19), which indicated the presence of a steroidal nucleus. It also showed signals at δ_H 1.69 (3H, d, J = 6.0 Hz) and 1.76 (3H, d,

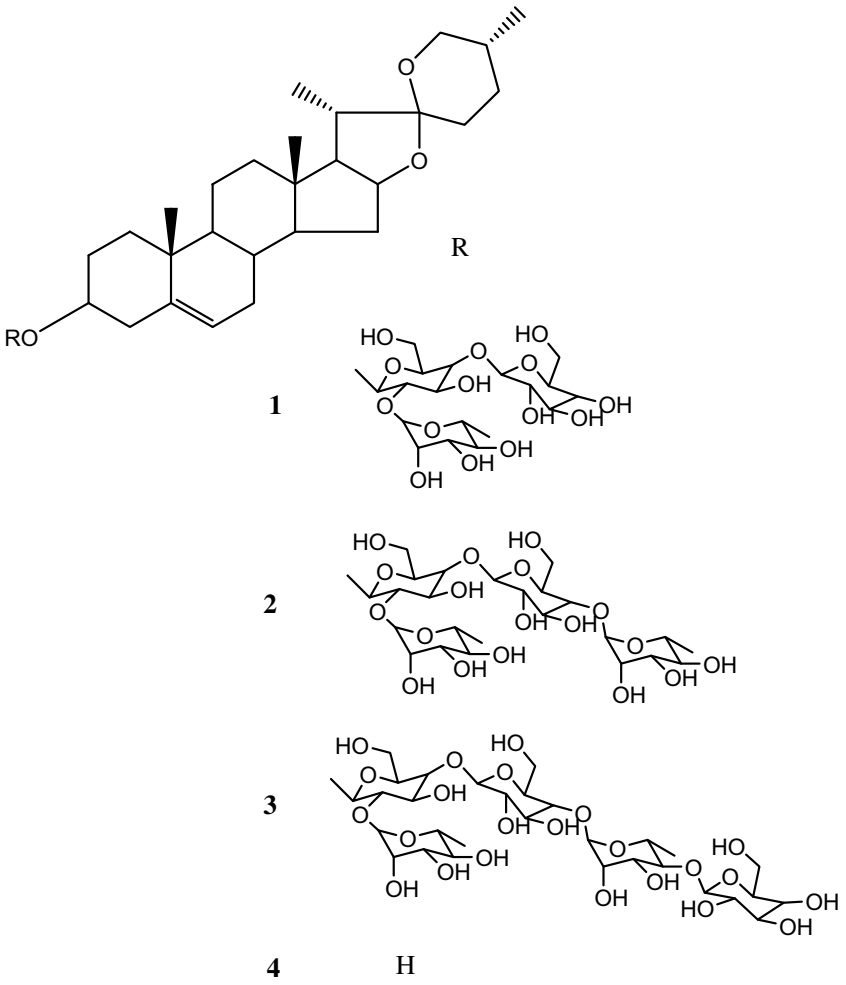

Fig. 1. Structures of NFAs **1**–**4** in *C. ornatus* shoots.

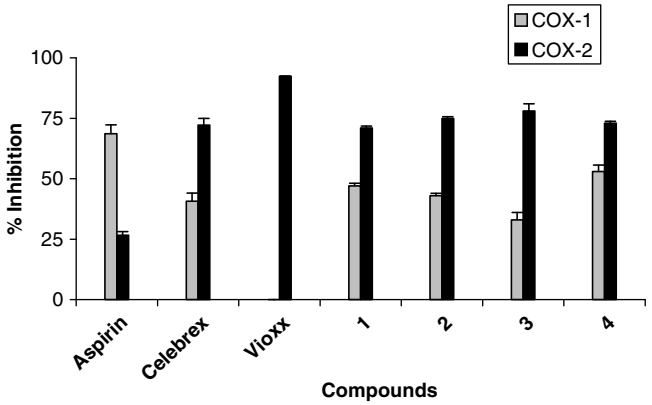

Fig. 2. COX inhibitory activities of compounds **1**–**4** tested at 28.2, 24.2, 21.2 and 60.4 μM. Commercial antiinflammatory drugs, Aspirin, Celebrex and Vioxx were used as positive control and tested at 60 μM, 26 nM, and 32 nM concentrations, respectively. The concentrations of positive controls used were based on their IC_{50}. DMSO was used as solvent control. Percent inhibition was calculated with respect to DMSO control. Vertical bars represent average ($n = 2$) of two experiments ±SD.

J = 6.0 Hz) corresponding to two methyl groups of the 6-deoxyhexapyranose moiety and signals at δ_H 6.17 (1H, brs), 5.84 (1H, brs), 5.05 (1H, d, J = 8.0 Hz) and 4.91 (1H, d, J = 7.5 Hz), corresponding to four anomeric protons. The ^{13}C NMR signals corresponding to an olefinic proton (δ_C 141.2 and 122.1), two secondary methyl groups (δ_C 15.3 and 17.6) and two tertiary methyl groups (δ_C 16.6 and 19.7) further supported the presence of a steroidal moiety in the molecule. It also displayed four anomeric signals at δ_H 104.4, 102.0, 103.1 and 105.5, indicating the presence of a tetrasaccharide moiety in the molecule. The correlations of the four anomeric proton signals at δ_H 6.17, 5.84, 5.05 and 4.91 to the corresponding anomeric carbon signals at δ_H 104.4, 102.0, 103.1, and 105.5, respectively, were established by HMQC spectral experiments. Also, the acid hydrolysis of compound **2** resulted in rhamnose and glucose as the only sugar residues in the molecule, as confirmed by TLC with authentic rhamnose and glucose. The resulting aglycone from the acid hydrolysis was identified as diosgenin by NMR experiments. The identity and the linkage of sugar moieties in the molecule were determined by a combined analysis of ^1H–^1H COSY, HMQC and HMBC spectral data. Therefore, compound **3** was identified as diosgenin-3-*O*-β-D-glucopyranosyl-(1–4)-α-L-rhamnopyranosyl-(1–4)-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside. This was in agreement with the data reported earlier for the same compound by Han et al. (1999) and Ohtsuki et al. (2006).

Compounds **1** and **3** gave similar spectral data except for the sugar residues in the molecule. The structures, diosgenin-3-*O*-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside and diosgenin-3-*O*-β-D-glucopyranosyl-(1–4)-α-L-rhamno- pyranosyl-(1–4)-β-D-glucopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside, were assigned to compounds **1** and **3**, respectively, based on extensive NMR spectral analyses, as in compound **2**. Again, the data were in agreement with the reported values for these

compounds by Han et al. (1999) and Ohtsuki et al. (2006). The aglycone, compound **4,** a product of hydrolysis during the extraction and isolation of compounds, was isolated as colourless solid and identified as diosgenin by comparison of its published proton and carbon NMR spectral data (Farid et al., 2002).

The antiinflammatory activities of compounds **1**–**4** were determined by using COX-1 and -2 enzymes (Hong et al., 2004). Compounds **1**–**3** showed similar trends in activity against COX-1 and -2 enzymes with inhibition values of 47%, 43%, and 33% and 71%, 75% and 78%, respectively, at 28.2, 24.2, and 21.2 μM (Fig. 2). Interestingly, the number of sugar units in the aglycone moiety showed very little impact on the overall COX enzyme inhibitory activity. However, a dose response study revealed that compound **4** significantly inhibited COX-1 and -2 enzymes by 53% and 73%, respectively at 60.4 μM concentration (Fig. 2). The inhibitory concentration at 50% (IC_{50}) of compounds **1**–**4** for COX-2 enzyme were 15.0, 13.9, 10.9 and 37.2 μM, respectively (Fig. 3). The positive controls, Aspirin, Celebrex and Vioxx, were tested at 60 μM, 26 nM, and 32 nM concentrations, respectively. The saponins similar to NFAs **1**–**4** were reported to exhibit a variety of biological and pharmacological activities.

Laboratory and animal studies have shown that NSAIDs and COX-2 specific inhibitors decrease cancer incidence by inhibiting the COX pathway. This results in decreased cell proliferation, increased programmed cell death, reduced formation of blood vessels to feed cancer cells, and enhanced immune responses (Wallace, 2002). Several research reports have described that the antiinflammatory properties of saponins are associated with chemopreventive activities (Kang et al., 2005; Shao et al., 2007). In the present study, we have evaluated compounds **1**–**4** for their inhibitory effects on the proliferation of colon, breast, lung, CNS and gastric cancer cell lines. Compound **3** significantly reduced the viability of all human tumor cell lines tested by 29.8%, 21.3%, 45.6%, 37.1%, and 25.0%, respectively, when tested at 21.2 μM. Whereas compound **2** showed moderate inhibition of tumor cell proliferation, as indicated by their viability at 37.5%, 22.4%, 53.3%, 58.2%, and 40.3%, respectively, at 24.2 μM. The percent cell viability values of compounds **1** and **4** against breast, CNS, lung, colon and gastric tumor cell lines were 83.9%, 58.3%, 75.4%, 65.1%, and 64.1%, and 78.5%, 57.9%, 72.1%, 60.5%, and 61.9%, respectively, at 28.2 and 60.4 μM concentrations. The tumor cell proliferation inhibitory activity of NFAs in *C. ornatus* was enhanced progressively with an increase in the number of sugar units in the aglycone moiety. Compounds **2** and **3** reduced cell viability of human cancer cell lines tested in a concentration-dependent manner

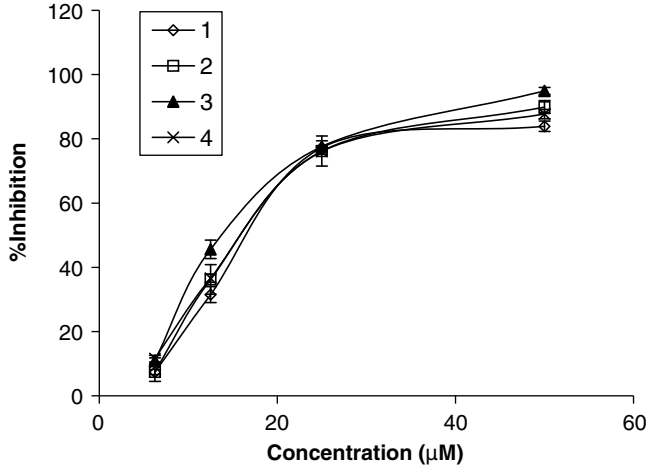

Fig. 3. Dose-response curves for the inhibition of COX-2 enzyme by compounds **1**–**4**. DMSO was used as solvent control. Percent inhibition was calculated with respect to DMSO control and the vertical bars represent averages ($n = 2$) of two experiments ±SD.

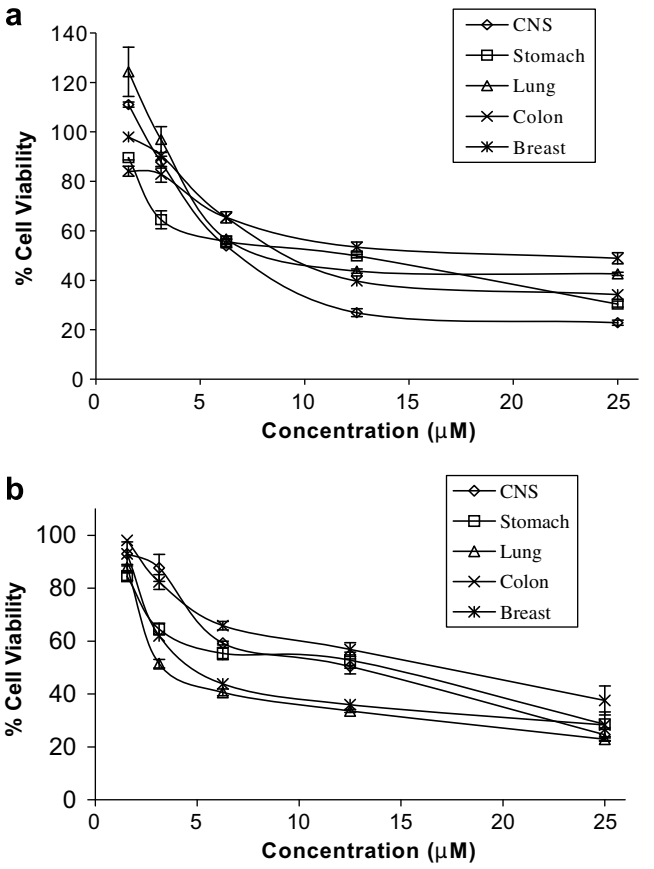

Fig. 4. Dose-response study of compound **2** (a) and **3** (b) on proliferation of HCT-116 (colon), MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS) and AGS (gastric) cancer cell lines, as determined by MTT assay. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. Percentage inhibition is represented as the mean ± SD obtained from three independent experiments performed in duplicate. Vertical bars represent the standard deviation of each data point ($n = 3$).

(Fig. 4a and b). The 50% reduction in cell viability (IC_{50}) concentrations of compounds **2** and **3** against breast, CNS, lung, colon and gastric cancer cell lines were 8.8, 6.1, 7.5, 23.8, 12.1 and 3.8, 7.1, 3.3, 14.3, 12.1 μM, respectively, as shown in Fig. 4a and b.

Saponins are a structurally diverse class of secondary metabolites that occur in plant species. The interest in saponins as a NFA is growing owing to its wide spectrum of biological activities (Miyata, 1992; Nakashima, Kimura, Kimura, & Matsuura, 1993; Niwa, Takeda, & Ishimaru, 1988; Osbourn, 1996; Wu, Chiang, & Fu, 1990). Diosgenyl saponins are abundant among steroidal saponins and they possess a variety of biological functions (Cheung et al., 2005; Zhang et al., 2007). In the present report, diosgenyl steroidal saponins **2** and **3,** from *C. ornatus* shoots, showed strong antitumor activity as well as COX-2 enzyme inhibition. The total saponin content in the fresh shoots of *C. ornatus* is 0.34%. In conclusion, our results suggest that consumption of *C. ornatus* shoots containing these NFAs may prevent inflammation and cancer-related illnesses. Additional research is needed to further substantiate the clinical efficacy and therapeutic dosage of these NFAs for health benefits.

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