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Demethylzeylasteral from *Costus dubius* (Afzel.) K. Schum Leaves Inhibits Cyclooxygenase (COX-2) *In vitro*

Anabel B. Abulencia,[1,2]* Evangeline C. Amor[2]

[1]*Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Ermita, Manila, 1000*
[2] *Institute of Chemistry, College of Science, University of the Philippines Diliman, Quezon City, Philippines, 1101*

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ABSTRACT

One of the widely studied plant families is the costaceae family also called "Spiral Ginger Family", known to contain bioactive compounds. However, *Costus dubius* (Afzel.) K. Schum., a member of this family, has not yet been explored for its potential bioactivity. Through a series of chromatographic separations and purification, demethylzeylasteral, a potential analgelsic compound, was isolated from the sub-fractions of the hexane extract of *C. dubius* leaves. It inhibited both COX-2 with an IC_{50} of 77.97 ± 14.05 µM and COX-1 with an IC_{50} of 158.90 ± 16.00 µM in a concentration-dependent manner. These results demonstrate that demethylzeylasteral is more selective against COX-2 than COX-1, which is desirable because COX-2 is the enzyme targeted for pain and inflammation while inhibition of COX-1 is associated with maintaining gastrointestinal integrity. This is the first report on the COX-1 and COX-2 inhibitory activity of demethylzeylasteral.

Keywords: Anti-inflammatory, Bioactive, Bioassay-guided, Concentration-dependent, Selective.

Introduction

Natural products from plants have been used for the treatment and prevention of diseases for thousands of years. These compounds can serve as a template for the synthesis of new drugs with higher efficacy and lower toxicity. The use of natural products for disease management and health has been integral throughout history, with renewed interest driven by their potential in treating conditions like cancer, diabetes, and infections, along with their affordability and accessibility.[1] Modern techniques such as combinatorial chemistry, metabolomics, and high-throughput screening are advancing natural product research, underscoring their importance in drug discovery and development, particularly from biodiversity-rich tropical rainforests.[1] It was reported that 61% of the new commercially available drugs introduced between 1981–2002 were inspired by natural products, which include natural products (6%), natural product derivatives (27%), synthetic compounds with natural product-derived pharmacophore (5%) and synthetic compounds designed from a natural product (natural product mimic, 23%).[2,3] Yuan *et al.* reported some natural products with anti-inflammatory and analgesic activity including curcumin, parthenolide, cucurbitacins, 1,8-cineole, pseudopterosins, lyprinol, bromelain, flavonoids, saponins, and Boswellia serrata gum resin.[4] Ali *et al.* reported that the flavonoid-rich extract from *Carica papaya* inhibited enzymes involved in inflammation and prostaglandin production, demonstrating dose- and time-dependent efficacy.[5]

*Corresponding author. E mail: ababulencia@up.edu.ph
Tel: + 63 945 9723985

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The members of the costaceae family are also used in traditional medicine and contains a variety of phytochemicals tested through *in vitro* and *in vivo* assays. A known member of this family is *chamaecostus cuspidatus* (Nees & Mart.) C. D. Specht & D. W. Stev., also known as the 'insulin plant,' due to its anecdotal anti-diabetic activity. This therapeutic potential spurred research on other members of the plant family. A study investigated the hydroethanol leaf extract of *Costus afer* for its anticonvulsant, muscle relaxant, and in-vitro antioxidant activities, showing significant dose-dependent effects in mice.[6] Results suggest that *C. afer* increases seizure latency, decreases seizure duration, improves muscle relaxation, and exhibits radical scavenging properties, supporting its potential use in managing central nervous system disorders.[6] The anxiolytic- and antidepressant-like effects of *C. afer* hydroethanol leaf extract in mice were also investigated, showing that it increased exploratory behavior and reduced immobility of mice in forced swim and tail suspension tests.[7] On the other hand, methanol stem extract and residual aqueous fraction of *C. afer* exhibited significant in-vitro antiplasmodial activity against chloroquine-sensitive, chloroquine-resistant, and artemether-resistant *Plasmodium falciparum* strains.[8] These findings suggest *C. afer* as a potential remedy for resistant malaria or a source of new antimalarial compounds.[8]

Cyclooxygenase (COX) is the enzyme responsible for the production of prostaglandins which mediate the inflammatory response with two isoforms which are COX-1 and COX-2.[9] Inhibition of COX-2 is desirable since its activity is linked to inflammation and pain while inhibition of COX-1 can cause gastrointestinal problems.[10] Thus, selective inhibitors of COX-2 are of interest to researchers. A study found that fatty acid compounds in walnut oil, particularly γ-linolenic acid with the lowest binding energy (-308.0 kJ/mol), exhibit anti-inflammatory properties by inhibiting cyclooxygenase-2 activity.[11] Seven compounds from *Elaeocarpus sphaericus* fruit, including malic acid, benzoic acid, and fumaric acid, were found to be effective COX-2 inhibitors.[12] *Heliotropium indicum* also showed potential for managing dysmenorrhea by inhibiting cyclooxygenase-2 and demonstrating antioxidant activity, with methanol extract exhibiting the strongest anti-inflammatory effects and various bioactive compounds identified through HPLC-DAD analysis.[13] *Echinops erinaceus* extracts, particularly polar and semi-polar ones, demonstrated significant in-vitro antioxidant and anti-inflammatory activities, including inhibition of cyclooxygenases and 15-lipoxygenase.[14] To date, the most widely used

analgesic drugs that may also be anti-inflammatory, are non-steroidal anti-inflammatory drugs (NSAIDs), which have been reported to cause side effects such as gastric ulceration and renal toxicity.[15]In this study, *Costus dubius* (Afzel.) K. Schum is the plant of interest as there are very few studies on it. *C. dubius* may be also a potential source of COX-2 inhibitors.

Materials and Methods

Materials and Equipment

Single-distilled technical grade solvents were used for extraction. Drying was done in a drying oven (Bio-base), Rotary evaporator (Heidolph) and speed vacuum concentrator (VS-802F Centra-Vac System) were used to concentrate extracts *in vacuo*. Isolation procedures were performed using Silica gel 60 G (TLC-grade) (Sigma 107731) for vacuum liquid chromatography (VLC) and Silica gel 60 (35–70 µm) for gravity column chromatography (Sigma 109389). Separation was monitored employing thin layer chromatography (TLC) using plastic-coated TLC Silica gel 60 F254 plates (Sigma 105735). Glass-coated Silica gel 60 for preparative TLC (PTLC) (Sigma Z293024) was used for further purification. The reagents used for the inhibition assay were : Cyclooxygenase-1 from sheep (Sigma C0733), Cyclooxygenase-2 from human (Sigma C0858), Tris HCl (Sigma T5941), Trizma base (Sigma T6066), Indomethacin (Sigma 17378), Ampiflu RedTM (Sigma 92001), Arachidonic acid (Sigma A9673), Hematin porcine (Sigma H3281), Dimethylsulfoxide (AR grade), Ethanol (AR grade), and Sodium hydroxide (AR grade), which were obtained from Belman Laboratories. LC-MS grade solvents (acetonitrile, methanol, water) were used for Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. For the cyclooxygenase inhibition assay, equipment used include pH meter (Thermoscientific), vortex mixer (VM-300), sonicator, Thermo Scientific Multiskan GO Microplate Spectrophotometer, and CLARIOstar (BMG LABTECH).

Instrumentation. The melting point of the isolate was determined using a Fisher-Johns melting point apparatus. The HPLC chromatogram was obtained using Agilent Technologies 1260 Infinity chromatographer equipped with a photodiode array detector. The stationary phase used was an analytical C18 column (Agilent Eclipse Plus C18, 3.5 µm, 4.6 x 100 mm). A gradient HPLC method is used to analyze the isolated compound at 25 ºC at wavelengths of 254 nm and 280 nm. The mobile phase consisted of HPLC pure water (solvent A) and methanol (solvent B) with a flow rate of 2.0 mL•min-1 filtered through a 0.22 µm PTFE filter (Millipore, USA). The initial mobile phase composition is maintained at 50% solvent A (5–10 min), changed linearly to 100% B (10-–20 min), and held for 10 min (20–30 min), then followed by a return to the initial conditions within 10 min (30–40 min). The sample injection volume was 50 µl.

The NMR spectra (1D: ^{1}H and ^{13}C NMR, 2D: DEPT, COSY, HSQC, HMBC, NOESY, ROESY, TOCSY) of the isolated compound in pyridine-d_5 (C_5D_5N) were recorded in 500 MHz (^{1}H NMR) and 126 MHz (^{13}C NMR) Agilent NMR spectrometer equipped with 3-mm One NMR probe and VNMRj ver 2.3 software. The IR spectrum was obtained using IR Prestige Attenuated Total Reflectance (ATR) using an IR Prestige-21 Fourier Transform Infrared Spectrophotometer. The UV spectrum was recorded in AR-grade ethanol using Thermo Scientific MultiSkan Go. UPLC chromatogram and HRMS spectrum were recorded in a UPLC system (Waters Acquity UPLC® connected to a quadrupole time-of-flight high-resolution mass spectrometer (Xevo G2-XS-Qtof) with an electrospray interface (ESI) in positive mode). Pentafluorophenyl (Acquity UPLC® CSH™ Fluoro-phenyl, 1.7 µm, 2.1 x 50 mm) was used as the stationary phase while high purity water (solvent A) and acetonitrile (solvent B) as the mobile phase at a flow rate of 0.3 mL•min^{-1} with a total run time of 6 min. For the elution, the gradient program started at 95% A and after 1 min of an isocratic run; solvent B was increased linearly to 95% at 3 min and kept constant up to 5 min; solvent A was increased back to 95% at 5.5 min and kept constant up to 6 min. The column temperature was maintained at 30 °C and the volume of the sample injected was 1.00 µL. ESI in positive mode with the following operation parameters were used: capillary voltage 3.00 kV, cone voltage of 60 V, the source offset voltage of 82 V, source temperature of 150°C, desolvation temperature of 500°C, cone gas flow of 49 L•h^{-1}, and desolvation gas flow of 800 L•h^{-1}. Two scans were carried out, a full scan (50–1500 Da) and MS/MS using Fast DDA as the acquisition mode with a collision energy ramp that started at 20V and ended at 40V. Data were analyzed using MassLynx 4.1 software.

Plant collection and Identification

The *Costus dubius* (Afzel.) K. Schum rhizomes were obtained from Sto. Tomas, Batangas, Philippines, and planted in the Institute of Biology (IB), College of Science, University of the Philippines – Diliman, Quezon City, Philippines as a constant source of the leaves. The plant was authenticated at the Jose Vera Santos Memorial Herbarium, Institute of Biology, University of the Philippines Diliman, Quezon City, and a voucher specimen (Accession No. 21853) was stored at the same place. *C. dubius* (Afzel.) K. Schum leaves were collected in March 2019.

Preparation of Sample

C. dubius (Afzel.) K. Schum leaves were washed with water to remove possible contaminants. Cleaned leaves were oven-dried at 40°C for 48 hours. Dried leaves were pulverized using an Osterizer Blender (Oster).

Extraction

Dried pulverized leaves (1200 g) were soaked in distilled methanol in 100 g leaves: 1L methanol ratio for 5 days at room temperature. The filtered methanol extract was concentrated *in vacuo* using a rotary evaporator at 40°C followed by storage at 2–8 °C. The resulting methanol extract (74.4 g) was dissolved in water (100 mL) and partitioned exhaustively using 1 part of distilled water and 6 parts of hexane. The hexane layer was collected and concentrated *in vacuo*. The remaining aqueous layer was then partitioned exhaustively using 1 part distilled water and 6 parts ethyl acetate. The ethyl acetate layer was collected and concentrated *in vacuo*. The methanol (74.4 g), hexane (36.58 g), and ethyl acetate (13.62 g) extracts were stored in scintillation vials at 2–8 °C.

Assay-guided isolation of active compound

The hexane and ethyl acetate extracts were further fractionated employing vacuum liquid chromatography (VLC) using Silica gel 60 (70–230 mesh, Merck) with a sample: silica ratio of 1:50 w/w employing gradient elution starting with 100% hexane and increasing 10% increments of ethyl acetate followed by 50% ethyl acetate and 50% methanol and then 100% methanol using a silica: solvent system ratio of 1:3.50 w/v. All fractions were concentrated *in vacuo* and subsequently subjected to thin-layer chromatography (TLC) using Silica gel 60 F254 (Merck) to monitor the separation, developed in hexane: ethyl acetate solvent system (50:50 v/v). The TLC plates were visualized under UV (long- 365 nm; short- 254 nm) and in an iodine chamber. These methods were also employed for fractions that underwent gravity column chromatography (GCC). After TLC, the activity of fractions, together with methanol, hexane, ethyl acetate, and aqueous extracts, were tested in an *in vitro* COX assay.

In vitro Cyclooxygenase Inhibition Assay

The methodology from the Book – 2B of *Tuklas Lunas Protocols for Drug Discovery and Development Manual* was employed to determine the percentage inhibitions of samples against COX-1 and COX-2.[16] This assay indirectly measures the peroxidase (POX) activity. The reduction of PGG2 due to PGH2 is coupled with the oxidation of ADHP (10-acetyl-3,7 dihydroxyphenoxazine), or Ampliflu™ Red, which is subsequently converted to resorufin, a highly fluorescent compound (Supplementary Figure 1). Resorufin fluorescence is analyzed with an excitation wavelength of 530–540 nm and an emission wavelength of 585–595 nm using Thermo Scientific Multiskan GO Microplate Spectrophotometer and CLARIOstar (BMG LABTECH).

Two trials and two replicates each were done for each sample. The enzymes and other reagents used in the assay were mixed in vials while the inhibition reactions were done in a 96-well plate. Due to the sensitivity of the enzymes and coloring reagents to light, the assay

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procedures were done in the dark with a lamp as a source of red light and in a box enclosure filled with nitrogen gas to prevent the enzyme

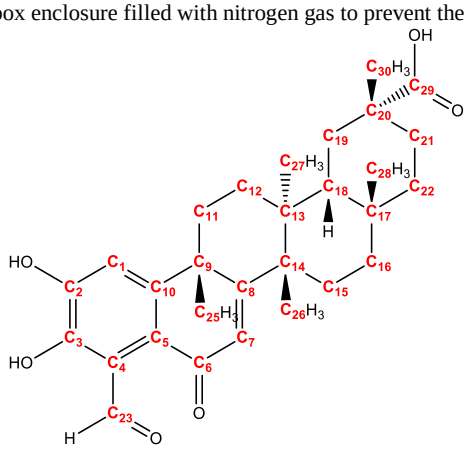

Figure 1. Structure of (9β, 13α, 14β, 20α)-2,3-dihydroxy-6,23-dioxo-D:A-friedo-24-noroleana-l,3,5(10),7-tetraen-29-oic acid or demethylzeylasteral.

from being oxidized. The scintillation vial was cleaned by the addition of 5183 µL of 100 mM tris buffer; separately, 96 µL of 250 U/mL COX-2 or COX-1 enzyme in 2 µM hematin in tris buffer and 480 µL of 20 µM hematin in 0.1 M NaOH were mixed. This mixture called enzyme-cofactor solution was subsequently dispensed into the vial containing the 100 mM Tris buffer. To each test well, 50 µL of the 100 mM Tris buffer and 120 µL of the enzyme-cofactor mixture were added; then, 10 µL of 200 µg/mL of plant extracts in dimethylsulphoxide (DMSO) was added to make a final well concentration of 10 µg/mL. For the positive control, 10 µL of 160 mM indomethacin in DMSO was added instead of the plant extract; and for the negative control, 10 µL of DMSO (3% v/v well concentration) was used. The 96-well plate containing the solutions was then incubated at 25 °C for 15 min using a Multiskan Go spectrophotometer. Ampiflu™ Red assay solution was prepared by adding 900.0 µL Tris buffer to 100 µL of 2000 µM stock solution of AmpifluTM Red in DMSO; and arachidonic acid assay solution was prepared by adding 900.0 µL 100 mM Tris buffer and 50 µL of 0.1 M NaOH to 50 µL stock solution of 40 mM arachidonic acid in ethanol. After incubation, 10 µL of AmpifluTM red assay solution followed by 10 µL of arachidonic acid assay solution were added to initiate the reaction. Nitrogen gas was bubbled into the solution. Thereafter, the fluorescent activity is read every 12 sec for 3 min at 535 nm and 590 nm as the excitation and emission wavelengths, respectively.
The IC_{50} values of the isolated compound against COX-1 and COX-2 were determined employing the same method described above using varying concentrations (2.08–1456.54 µM). The mode of inhibition and K_i were determined by reacting varying concentrations of arachidonic acid (1–500 µM) with 77.97 µM of the isolated compound.

Statistcal Analysis
The percentage inhibition of the samples was calculated using equation 1 where $V_{control}$= reaction velocity of negative control and V_{sample}= reaction velocity of samples. The results were reported as mean ± SD (standard deviation).

$$\%\ Inhibition = \left(\frac{V_{control} - V_{sample}}{V_{control}}\right) \times 100 \qquad \text{(Equation 1)}$$

Samples that exhibit percentage inhibition greater than or equal to 50% and a COX-2-to-COX-1 ratio greater than 1 are considered active. Statistical analyses were performed using SPSS Statistics 17.0 software. One-sample Kolmogorov-Smirnov test was used to determine if the slope is normally distributed with $p>0.05$ considered as normal. Then, Levene's test was used to determine the homogeneity of the variance. If $p>0.05$, data have equal variances, and one-way analysis of variance (ANOVA) followed by Dunnett's test to determine the difference between means of the data. On the other hand, if the result of Levene's test is $p<0.05$, data have unequal variances, and Brown-Forsythe and Welch test followed by Tamhane T2 was used. p-values lower than 0.05 were considered as significantly different for both Dunnett's and Tamhane T2 test. GraphPad Prism 8 was used to calculate the IC_{50} using the template log (inhibitor) vs. variable slope (four parameters), to determine the K_M and V_{max}, and to generate the dose-response curve and Michaelis-Menten plots of the isolated compound. The K_i for competitive inhibition was computed using Equation 2.

$$K_i = \frac{K_M\, x\, [I]}{K_{M\ apparent} - K_M} \qquad \text{(Equation 2)}$$

Results and Discussion

Bioassay-Guided Isolation and Purification
Samples that exhibited percentage inhibition greater than or equal to 50% and COX-2-to-COX-1 ratio greater than 1 were considered active. The methanol extract from *C. dubius* (Afzel.) K. Schum leaves was active at 10 µg/mL COX-1: 74.53 ± 1.58%; COX-2: 76.88 ± 1.35%). Subsequent liquid-liquid extraction yielded hexane (COX-1: 66.91 ± 4.62 %; COX-2: 69.55 ± 1.96%) and ethyl acetate extracts (COX-1: 28.10 ± 2.22%; COX-2: 55.16 ± 5.54%), which are also found to be active at 10 µg/mL. The hexane extract, having a higher yield and activity compared to the ethyl acetate extract, was pursued and fractionated, yielding 10 fractions. The fraction, HV6, eluted out at 40% hexane - 60% ethyl acetate was active (COX-1: 40.63 ± 1.06%; COX-2: 54.95 ± 3.64%). Fraction HV6 resulted in 10 sub-fractions after gravity column chromatography (GCC). Two of these GCC sub-fractions (HV6G5 (COX-1: 55.47 ± 1.24%; COX-2: 72.11 ± 2.36%) and HV6G6 (COX-1: 55.40 ± 3.30; COX-2: 70.40 ± 2.75%) were active at 10 µg/mL, which also yielded yellow solid precipitates. To increase the yield of the yellow solid precipitates, the pooled fractions were subjected to another round of GCC yielding two actives (HV6G5–6.6 (COX-1:41.40 ± 6.03%; COX-2: 52.22 ± 2.58%) and HV6G5–6.7 (COX-1: 67.74 ± 5.10%; COX-2: 73.07 ± 2.96%) among the 10 fractions at 10 µg/mL.
Preparative TLC was employed to isolate the pure yellow crystals (HV6G5–6.6–7ys) with a 50:50 (v/v) hexane: ethyl acetate solvent system. The spot corresponding to the isolate eluted out at an Rf of 0.42. The silica that contains the spot was scraped off from the plate. To isolate the spot, the silica is subjected to filtration with 50:50 (v/v) hexane: ethyl acetate as the solvent system. The filtrate was air-dried resulting in yellow powder. Further purification was done to isolate the pure yellow powder (HV6G5–6.6–7ys) (0.0016 % yield based on dried leaves; 0.0251 % yield based on methanol extract; 0.0511 % yield based on hexane extract), which were also found to be selective for COX-2 (COX-2 IC_{50}: 77.97 ± 14.05 µM; COX-1 IC_{50}: 158.90 ± 16.00 µM). Figure 2 shows the isolation scheme employed. The activities of the extracts and fractions against COX-1 and COX-2 are shown in Figure 3.

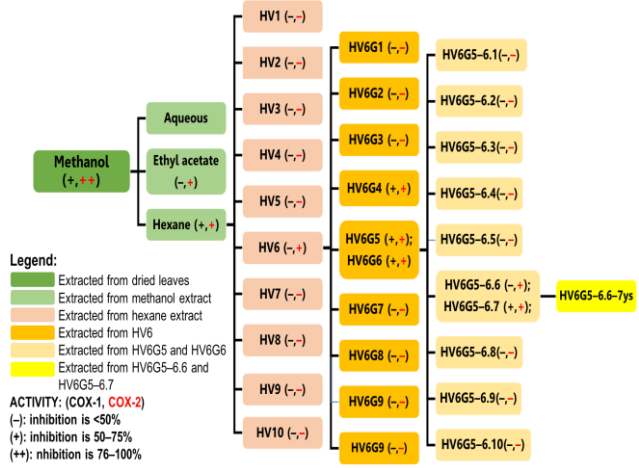

Figure 2. Isolation of demethylzeylasteral (HV6G5–6.6–7ys) from *C. dubius* (Afzel.) K. Schum.

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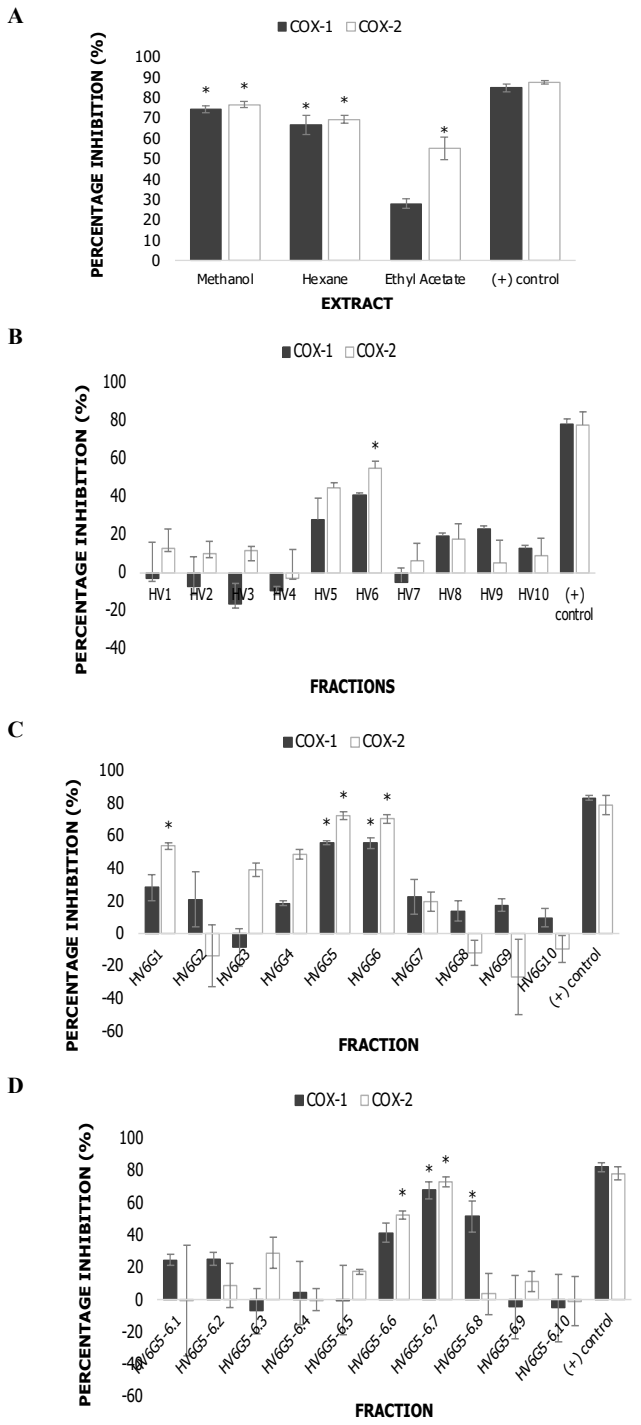

Figure 3. Inhibitory activities of extracts (a), hexane fractions (b), HV6 fractions (c), HV6G65-6 fractions (d) at 10 ppm (*extracts and fractions that are significantly different from negative control at $p<0.05$).

Structure Elucidation

(9β, 13α, 14β, 20α)-2,3-dihydroxy-6,23-dioxo-D:A-friedo-24-noroleana-l,3,5(10),7-tetraen-29-oic acid or demethylzeylasteral (Figure 1): yellow powder; mp 158–160°C; HRMS (positive mode) at 3.00 kV (% relative abundance) $C_{29}H_{36}O_6$ $[M+H]^+$ at 481.2615, calculated 481.2590; UV nm (EtOH, λ_{max}) 247, 269, 307, 370 nm; IR bands (ATR) 3741.90, 2947.23, 2870.08, 2167.99, 2036.83, 1975.11, 1689.64, 1643.35, 1450.97, 1296.16, 1242.16, 1134.14, 1002.98, 941.56, 879.54, 748.38, 617.22 cm^{-1}; ^{1}H-NMR (500, MHz, C_5D_5N, ppm): δ_H 7.45 (1H, s, H-1), 6.50 (1H, s, H-7), 2.19 (1H, d, *J*=12.72 Hz, H-11), 1.90 (1H, m, H-11'), 1.96 (1H, m, H-12), 1.65 (2H, overlapped, t, *J*=12.47(x2) Hz, H-12', H-15), 1.45 (3H, m, overlapped, H-15', H-16', H-21'), 1.82 (1H, m, H-16), 1.59 (1H, d, *J*= 7.83 Hz, H-18), 2.72 (1H, d, *J*=16.14 Hz, H-19), 1.75 (1H, dd, *J*=15.65 Hz, 7.83 Hz, H-19'), 2.53 (1H, d, *J*=13.69(x2) Hz, H-21), 2.36 (1H, t, *J*=13.69(x2) Hz, H-22), 1.00 (1H, d, H-22'), 11.34 (1H, s, H-23), 1.51 (3H, s, H-25), 1.22 (3H, s, H-26), 1.05 (3H, s, H-27), 1.11 (3H, s, H-28), 1.41 (3H, s, H-30). ^{13}C-NMR (126 MHz, C_5D_5N,ppm): δ_C 118.5 (C1), 153.1 (C2, COH), 151.5 (C3, COH), 119.2 (C4), 122.8 (C5), 186.2 (C6, CO), 125.9 (C7), 174.1 (C8), 41.1 (C9), 151.4 (C10), 34.4 (C11), 30.5 (C12), 40.3 (C13), 45.7 (C14), 29.5 (C15), 37.2 (C16), 31.3 (C17), 45.1 (C18), 31.8 (C19), 41.1 (C20), 31.0 (C21), 36.0 (C22), 200.5 (C23, HCCO), 36.7 (C25), 21.1 (C26), 19.3 (C27), 32.2 (C28), 181.6 (C29, COOH), 33.7 (C30). Demethylzeylasteral (DM) was also reported to be isolated from methanol extract of *Tripterygium hypoglaucum* (root bark).[17]

Cyclooxygenase Activity of Demethylzeylasteral

Demethylzeylasteral (DM) inhibited COX-1 and COX-2 in a concentration-dependent manner (Figure 4). The IC_{50} of DM was 77.97 ± 14.05 µM against COX-2 and 158.90 ± 16.00 µM against COX-1. The IC_{50} of DM against COX-2 had a relative standard deviation of about 18.02%. This might be due to errors in pipetting or solutions that are not well-mixed. However, some journals of assay validation reported that an RSD of <20% is acceptable.[18,19] The *in vitro* COX selectivity is described by the ratio of concentrations required to inhibit the activity of COX-1 or COX-2 by 50% (COX-1 IC_{50}/COX-2 IC_{50}).[20] When the ratio is close to 1.0, the inhibitor is nonselective. On the other hand, inhibitors with a selectivity ratio of <1 are considered more selective for COX-1, while inhibitors with selectivity ratios >1 are considered more selective for COX-2. The IC_{50} of DM against COX-1 was almost twice that observed for COX-2. These data indicate that it selectively inhibits COX-2.

Sheep COX-1 is used in several studies involving COX-1 inhibition assay and has provided reproducible and reliable data.[21] It was reported that COX-1 orthologs across vertebrae species are approximately 70-95% identical in terms of amino acid composition.[22] Data obtained from sheep COX-1 is significantly relatable to human COX-1.[22]

Some of the plant metabolites with reported COX-2 activity are eugenol (IC_{50}= 129 µM), pyrogallol (IC_{50}= 144 µM), cinnamaldehyde (IC_{50}= 245 µM), oleanolic acid (IC_{50}= 130 µM), and ursolic acid (IC_{50}= 295 µM).[23] However, these reports of COX-2 activities are obtained through different methods, thus, these cannot be compared with the COX-2 activity obtained for DM. *Triptegyrium wilfordii* Hook. F. was used in China during the 1960s to treat rheumatoid arthritis and inflammation.[24] The extracts from this plant are reported to inhibit the expression of proinflammatory genes of interleukin-2 (IL-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and interferon-gamma (IFN- γ).[25] A study reported that the extract of *T. wilfordii* has COX-1 and COX-2 IC_{50} values of 27 µg•mL^{-1} and 125 µg•mL^{-1}, respectively.[26] However, there is no report on the COX-1 and COX-2 inhibitory activity of DM.

Based on the Michaelis-Menten and Line-weaver Burk plots (Figure 4) generated, DM competitively inhibit both COX-1 (K_M = 202.5 µM) and COX-2 (K_M = 116.0 µM) with increased K_M values and no change in V_{max} values (COX-1: 6.120, COX-2: 3.472) The K_M for arachidonic acid is 57.85 µM for COX-1 (V_{max}: 6.391) and 19.29 µM for COX-2 (V_{max}: 3.712).

In the presence of DM, the K_M increased by four-fold against COX-1 and ten-fold against COX-2, which is indicative of a higher substrate concentration needed to achieve the half-maximal rate of reaction. This shows that DM competes with the arachidonic acid (substrate) for the active site/s of COX-1 and COX-2. However, the mechanism by which it competitively inhibits the enzyme was not determined. One characteristic of a competitive inhibitor is that it is an analog of the substrate. DM is a phenolic triterpenoid while the arachidonic acid, the substrate, is a polyunsaturated fatty acid. These two compounds are not analogs of each other but have similarities such as the alkene double bond, the site of oxidation, and the carboxylic acid group, which may have contributed to the competitive inhibition observed.

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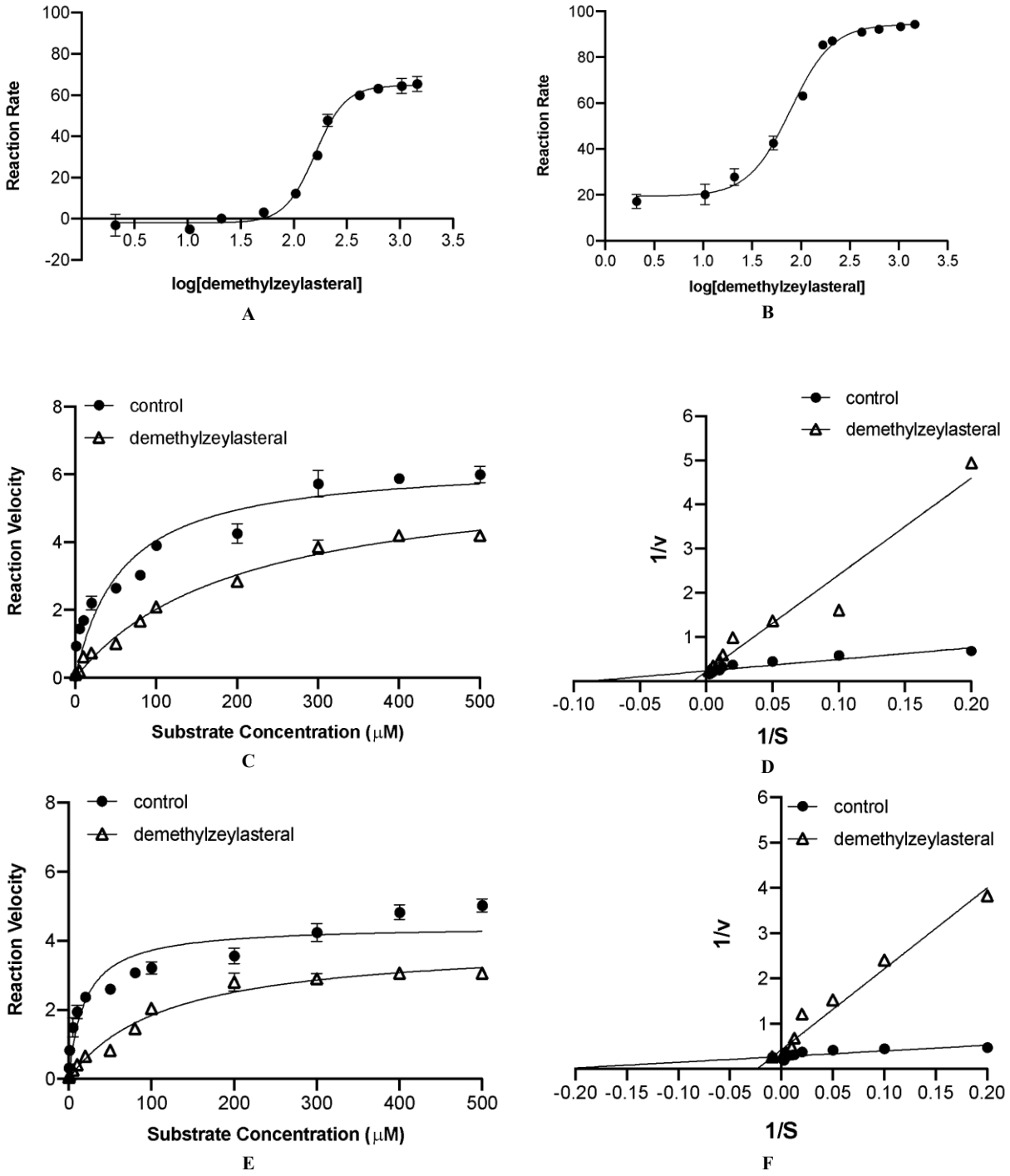

Figure 4. Inhibition kinetics of demethylzeylasteral against (A) COX-1 and (B) COX-2; Michaelis-Menten and Line-weaver Burk plots of the reaction of COX-1 (C, D) and COX-2 (E, F) with varying concentrations of arachidonic acid with and without demethylzeylasteral (control).

Some compounds act as competitive inhibitors binding to the active site of an enzyme even if they do not possess structural similarity with the substrate such as salicylate, which is a competitive inhibitor of alcohol dehydrogenase, and 3-phosphoglycerate kinase.[27]

The K_i of DM was 31.18 μM for COX-1 and 15.15 μM for COX-2. This is evidence that a lower concentration of DM is needed to cause greater inhibition of COX-2 as compared to COX-1. This also indicates that DM binds stronger to the active site of COX-2 than COX-1. This is the first report of the isolation of DM from *C. dubius* and its COX-1 and COX-2 inhibitory activities. DM, a phenolic triterpenoid, was first isolated from *Triptegyrium wilfordii* Hook. F., a member of *Celastraceae* family.[28] It was reported to exhibit anti-tumor property against melanoma cells and was found to inhibit MCL1 which is an anti-apoptotic protein that amplifies multiple human cancers.[29] Zhang and colleagues (2018) reported that DM is a potential agent for malignant glioma therapy.[28] It was also reported to ameliorate the mice lupus nephritis by inhibiting the activation of NF-κB and reducing the downstream pro-inflammatory mediators such as TNF-α, cyclooxygenase-2 (COX-2) and ICAM-1.[30] This is the first report on the COX-1 and COX-2 inhibitory activity of DM. It is also the first study to demonstrate that DM's *in vitro* inhibitory effect against COX-1 and COX-2 is competitive.

Conclusion

A potential selective cyclooxygenase-2 (COX-2) inhibitor was isolated from *Costus dubius* (Afzel.) K. Schum leaves and was identified to be (9β, 13α, 14β, 20α)-2,3-dihydroxy-6,23-dioxo-D:A-friedo-24-noroleana-l,3,5(10),7-tetraen-29-oic acid or demethylzeylasteral (DM). DM moderately inhibited both COX-2 with an IC_{50} of 77.97 ± 14.05 μM and COX-1 with IC_{50} of 158.90 ± 16.00 μM in a concentration-dependent manner. These results showed DM's selective inhibition of COX-2 (IC_{50} (COX-1)/ IC_{50} (COX-2) > 1), which is desirable since COX-2 is the enzyme associated with pain and inflammation while inhibition of COX-1 is attributed to gastrointestinal problems. DM also competitively inhibited both COX-1 (K_i= 31.18 μM) and COX-2 (K_i= 15.15 μM). However, the mechanism by which DM inhibits COX-1 and COX-2 has not been determined. Therefore, it is recommended to conduct a computational molecular dynamics (MD) simulation experiment to identify DM's mechanism of action. Additionally, the structure of DM can be modified to enhance its biological activity, and other potential bioactivities of DM can also be explored.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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