Chalcone Derivatives with Cyclooxygenase Inhibiting Activity

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ABSTRACT

Dimethylcardamonin (DMC) or (*E*)-2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl chalcone was isolated from *Syzygium samarangense* (Blume) Merr. leaves using vacuum liquid chromatography and normal phase silica-gel column chromatography. DMC was purified from the fraction that eluted out of 9:1 (v/v) hexane: ethyl acetate to 7:3 (v/v) hexane: ethyl acetate. Using a modified method from Geissman (1948), DMC was derivatized via alkaline peroxidation from which compounds A and B were obtained. Compound A was identified to be (*2S*)-7-hydroxy-5-methoxy-6,8-dimethyl flavanone ($C_{18}H_{18}O_4$) while B had a methoxy group on its 4' position instead of a hydroxyl group with respect to DMC. The flavanone derivative may have been formed due to substituent effects on the ring.

DMC, A, and B were tested for their inhibitory activity against cyclooxygenase enzymes at 10 and 100 ppm. A and B gave at least 50% inhibitory activity at 100 ppm and were found to be COX-2 selective inhibitors. DMC was inactive at both 10 and 100 ppm.

Keywords: dimethylchalcone, flavanone, *Syzygium samarangense*, alkaline peroxidation, cyclooxygenase inhibition

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INTRODUCTION

The earliest remedy for inflammation is the extract of willow bark (*Salix* sp.), which dates back to 1862 (Cavaillon 2017; Desborough and Keeling 2017). Willow bark extract is found to contain salicylic acid (2-hydroxybenzoic acid), which was derivatized in 1897 by acetylation to produce aspirin (2-acetyloxybenzoic acid) (Jeffreys 2004; Desborough and Keeling 2017). Aspirin was commercialized in 1905 and since then has been used to relieve pain, inflammation, and fever (Desborough and Keeling 2017). In 1963, a new drug called indomethacin was developed for people experiencing pain and inflammation due to rheumatoid arthritis (Shen 1982). Aspirin and indomethacin are examples of non-steroidal anti-inflammatory drugs (NSAIDs). However, these drugs are gastrotoxic and cause ulceration (Marnett and Kalgutkar 1998; Marnett 2002; Pasa et al. 2009). This side effect was related to the ability of NSAIDs to inhibit both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2)—enzymes mediating inflammation. Thus, a new class of anti-inflammatory drugs called coxibs was developed. These drugs selectively inhibit COX-2 which means that they do not possess the gastric toxicity of NSAIDs (Patrono and Rocca 2009). However, it was later found that coxibs are hepato- and cardiotoxic (Patrono and Rocca 2009; Badri et al. 2016). Many coxibs including celecoxib, rofecoxib, and valdecoxib were banned from use (Sun et al. 2007; Hawboldt 2008; Thomas et al. 2017). This led to a search for a new class of COX-2 selective inhibitors.

Among the interesting derivatized products from chalcones are the aurones and flavones, which are products of the oxidative cyclization of 2'-hydroxychalcones (Masesane 2015). Biologically, aurones are synthesized from chalcones with the aid of aureusidin synthase (Nakayama et al. 2000; Nakayama 2002; Davies et al. 2006). However, not all plants have this enzyme to synthesize this subclass of phytochemicals. Some researchers were able to synthesize aurones from chalcones in vitro and synthetic routes have been explored to hasten the study of aurones. One of the developed methods is the oxidative cyclization of 2'-hydroxychalcone using the acetate salt of metals at high oxidation states (e.g., mercury (II) acetate) in an appropriate organic solvent such as dimethylsulfoxide (DMSO) (Detsi et al. 2002; Agrawal and Soni 2006), pyridine (Agrawal and Soni 2006), or acetic acid (Sekizaki 1988). Modifications to this process had been done, such as the use of copper (II) bromide in DMSO (Detsi et al. 2002) and the use of gold (I) catalyst (Harkat et al. 2008). Another possible method is through the alkaline peroxidation of 2'-methoxy chalcones (Geissman 1948).

The three chalcones (Figure 1) isolated from *S. samarangense* (Blume) Merr. are known to inhibit the lipopolysaccharide (LPS)-induced cellular production of nitric oxide (NO) and prostaglandin E2 (PGE2) (Kim et al. 2010). However, it has not been confirmed whether DMC, the most abundant chalcone isolated from *S. samarangense* by Amor et al. (2007), could also inhibit the production of prostaglandins through the direct inhibition of cyclooxygenases. This study re-isolated DMC from *S. samarangense* and derivatized it to determine the cyclooxygenase-inhibiting activity of the derivatives.

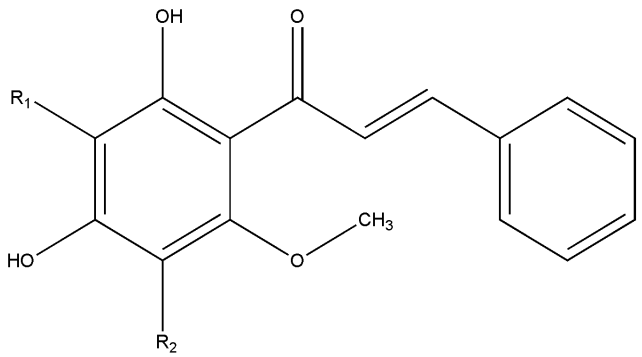

Figure 1. Structure of cardamonin (R_1, R_2 = H), stercurensin (R_1 = CH_3, R_2 = H), and dimethylcardamonin (R_1, R_2 = CH_3).

MATERIALS AND METHODS

Plant Collection, Extraction, and Solvent Partitioning

S. samarangense leaves were collected from Diliman, Quezon City. The samples were authenticated, and a voucher specimen was deposited at the Jose Vera Herbarium of the University of the Philippines (UP) Diliman Institute of Biology with Accession No. 14258.

Ground *S. samarangense* leaves (541 g) were soaked in single distilled technical grade (TG) methanol (~1.5 L) for 3-5 days done six times. After extraction, the resulting filtrate was evaporated in vacuo with a rotary evaporator (IKA RV-10) at 40°C to obtain the crude methanol extract (46 g).

The concentrated methanol extract (15 g) was suspended in distilled water (100 mL) and was extracted successively with TG hexane (600 mL) up to six times to obtain the hexane extract.

Fractionation, Isolation, and Purification

All solvents (methanol, hexane, and ethyl acetate) used for fractionation, isolation, and purification are of analytical grade except for those used for vacuum liquid chromatography (VLC) where TG solvents were used. The hexane extract (4.1 g) was fractionated using vacuum liquid chromatography (silica gel 60 G, thin-layer chromatography grade, Merck) employing gradient elution from 100% hexane to 100% ethyl acetate at 10% (v/v) increments, and then 100% ethyl acetate to 100% methanol at 50% (v/v) increments. A total of twelve VLC fractions were collected, concentrated, and dried in vacuo at 40°C using a rotary evaporator. Separation was monitored by thin layer chromatography (TLC) using silica gel 60 GF_{254} (TLC grade, Merck 105554). TLC plates (4.5 in x 4.5 in) were viewed under an ultraviolet (UV) lamp set at 254 nm (far UV) and 365 nm (near UV) and exposed to iodine crystals for visualization.

Yellow-orange crystals formed from the VLC fraction that eluted from 9:1 (v/v) hexane: ethyl acetate. This fraction was dissolved in 9:1 (v/v) hexane: ethyl acetate and was subjected to normal phase silica-gel column chromatography (npcc) using a wet-packed stationary phase (silica gel 60, column chromatography grade, Merck 1.07734.1000). Gradient elution was performed from 9:1 (v/v) hexane: ethyl acetate to 100% ethyl acetate at 10% (v/v) increments. Subfractions were collected at 5-mL portions monitored with TLC. A pure compound was obtained from the subfraction which eluted at 7:3 (v/v) hexane: ethyl acetate.

(*E*)-2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl chalcone (DMC): yellow-orange crystals; ESI-MS positive mode $C_{18}H_{18}O_4$ $[M+H]^+$ found m/z 299.1293 (calcd 299.1283, error 3.34 ppm). ^{1}H-NMR (500 MHz, $CDCl_3$, ppm) δ 2.15 (s, 3'-CH_3), 2.17 (s, 5'-CH_3), 3.67 (s, 6'-OCH_3), 5.42 (s, 4'-OH), 7.43 (m, H-3), 7.43 (m, H-4), 7.43 (m, H-5), 7.66 (dd, H-2), 7.66 (dd, H-6), 7.86 (d, β-H), 8.01 (d, α-H), 13.62 (s, 2'-OH); ^{13}C-NMR (126 MHz, $CDCl_3$, ppm) [DEPT] δ 7.57 (C-1) [C], 8.24 (3'-CH_3) [CH_3], 62.38 (6'-OCH_3) [CH_3], 106.54 (C-1') [C], 108.85 (C-3') [C], 109.07 (C-5') [C], 126.71 (C-α) [CH], 128.42 (C-3) [CH], 128.42 (C-5) [CH], 128.93 (C-2) [CH], 128.93 (C-6) [CH], 130.2 (C-4) [CH], 135.36 (C-1) [C], 142.9 (C-β) [CH], 158.86 (C-6') [C], 159.18 (C-4') [C], 162.04 (C-2') [C], 193.38 (C=O) [C].

Alkaline Peroxidation of Chalcone

The alkaline peroxidation procedure was adopted from Geissman and Fukushima (1948) with slight modifications. A total of 50 mg of the chalcone was dissolved in 2.5 mL cold methanol. This solution was kept in an ice bath (~5°C). Subsequently, 0.75 mL of 16% cold aqueous NaOH (18 equiv.) and 0.5 mL of cold 20% H_2O_2

(25 equiv.) were added. The mixture was left to stand overnight at 5°C. If any precipitation was observed, 3N HCl was added to neutralize the mixture. The resulting solution was then extracted with 13 mL ethyl acetate. Afterward, another set of peroxidation was done using 10 mL cold methanol, 3.0 mL of cold aqueous 16% NaOH (72 equiv.), and 2.0 mL of cold 20% H_2O_2 (102 equiv.), which was followed by extraction using 50 mL ethyl acetate.

The resulting ethyl acetate extracts were then dried, and the products were purified using dropper column chromatography. The first peroxidation procedure yielded compound A (8.0% yield), while the second produced compound B (18% yield).

Compound A ((*2S*)-7-hydroxy-5-methoxy-6,8-dimethyl flavanone): yellow crystals. ^{1}H-NMR (500 MHz, $CDCl_3$, ppm) δ 2.15 (s, 6-CH_3), 2.15 (s, 8-CH_3), 2.84 (dd, Ha-3), 2.98 (dd, Hb-3), 3.82 (s, 5-OCH_3), 5.42 (dd, H-2), 5.43 (s, 7'-OH), 7.42 (m, H-2'), 7.42 (m, H-3'), 7.42 (m, H-4'), 7.42 (m, H-5'), 7.42 (m, H-6').

Compound B ((*E*)-2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl chalcone): faint yellow crystals. ^{1}H-NMR (500 MHz, $CDCl_3$, ppm) δ 2.12 (s, 3'-CH_3), 2.14 (s, 5'-CH_3), 3.48 (s, 4'-OCH_3), 3.65 (s, 6'-OCH_3), 7.40 (d, H-3), 7.40 (d, H-4), 7.40 (d, H-5), 7.64 (dd, H-2), 7.64 (dd, H-6), 7.83 (d, β-H), 7.98 (d, α-H), 13.62 (s, 2'-OH).

Cyclooxygenase Inhibition Assay

The reagents used in the cyclooxygenase inhibition assay include 100 mM Tris buffer (pH 8.0), 1 mM Hematin cofactor in 0.1 M NaOH, Ampliflu™ Red or ADPH (10-acetyl-3,7-dihydroxyphenoxazine): 2 mM in DMSO, 40 mM arachidonic acid in ethanol, indomethacin (Sigma) positive control, cyclooxygenase-1 from sheep (Sigma) and recombinant cyclooxygenase-2 from human (Sigma).

DMC and its derivatives were screened for their cyclooxygenase inhibiting activity at 10 ppm and 100 ppm using the method developed by the Terrestrial Natural Products Laboratory at the Institute of Chemistry, UP Diliman (Opog et al. 2019). A 10,000 ppm stock sample solution was prepared in a microcentrifuge tube by dissolving 3 mg of the sample in 300 µL of DMSO. Working stock solutions with concentrations of 200 ppm and 20 ppm were prepared in microcentrifuge tubes from the 10,000 ppm stock. The assay was done in a closed chamber flushed with nitrogen gas, using indomethacin (Sigma) with an effective concentration of 1,500 ppm as the positive control. From the working stock, 10 µL of the test sample was added to a mixture of 50 µL of Tris buffer and 120 µL of COX-Hematin solution. After incubation for 15 minutes at 25°C, 10 µL Amplex Red reagent and 10 µL arachidonic acid were added. The fluorescence was then monitored every 12 seconds for

3 minutes at 535 nm and 590 nm as the wavelengths of excitation and emission, respectively using a Clariostar® microplate reader.

Each assay was done in two trials with two replicates per trial. The percent inhibition was calculated using the equation:

$$\%\text{Inhibition} = \frac{V_{negative} - V_{sample}}{V_{negative}} \times 100\% \qquad (1)$$

where $V_{negative}$ = reaction velocity of the negative control, and

V_{sample} = reaction velocity of the sample

A sample is considered active when the COX-2 inhibition is greater than 50% and the ratio of the COX-2 and COX-1 inhibition is greater than 1. The assay data collected were subjected to several statistical methods, including the one-sample Kolmogorov-Smirnov test, Levene's test, ANOVA, Welch's t-test, Brown-Forsythe test, and the post-Hoc test (Dunnett T3 or Tamhane's T2).

Liquid Chromatography-Mass Spectrometry (LC-MS)

The samples were analyzed using MassLynx™ Software v4.1 by Waters and MZmine v2.6. The parameters used for the analysis are summarized in Table 1.

Table 1. LC-MS parameters and conditions for sample analysis

Parameter	Condition
Unit	Xevo G2-XS QTOF, ACQUITY UPLC® System
Column	ACQUITY UPLC® CSH™ Fluoro-Phenyl, 1.7µm (2.1 x 50mm), T=30°C
Mobile Phase	Gradient of 100% ACN-95% H_2O-100% ACN
Flow Rate	0.3 mL/min
Data Processing	MassLynx™ v4.1

Nuclear Magnetic Resonance (NMR)

Isolates with enough amount for elucidation were subjected to ^{1}H-NMR and ^{13}C-NMR spectroscopy using 500-MHz Agilent NMR spectrometer at the Analytical Services Laboratory of the Institute of Chemistry, UP Diliman. The solvent used was deuterated chloroform ($CDCl_3$). Data were processed using MestReNova™ ver 6.1© MestreLab Research S.L.

RESULTS AND DISCUSSION

Isolation of Chalcone

Approximately 200 mg of yellow-orange needle-like crystals were collected from the VLC fraction that eluted out from 9:1 (v/v) hexane: ethyl acetate and purified using npcc with 7:3 (v/v) hexane: ethyl acetate. It has an Rf value of 0.65 in 7:3 (v/v) hexane: ethyl acetate solution and is visible under UV (254 nm and 365 nm). The compound was identified to be (*E*)-2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) and its structure (Figure 2) was elucidated using NMR and LC-HRMS data. The relative configuration was determined to be (*E*)- based on the coupling constant of the α- and β-hydrogens (15.7 Hz).

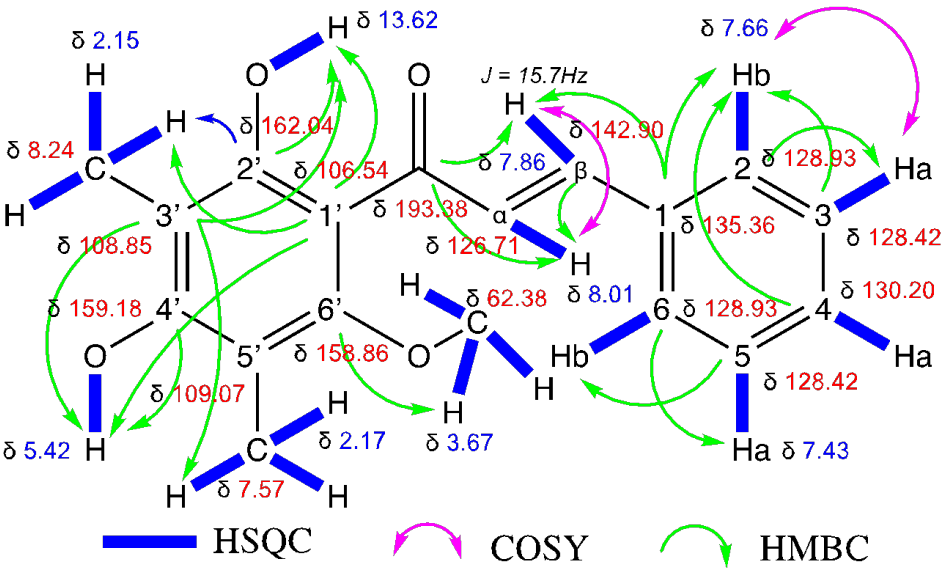

Figure 2. DMC or (*E*)-2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl chalcone elucidated using 1D-NMR, 2D-NMR, DEPT, and LC-HRMS spectra. ^{13}C-NMR shifts are indicated in red while ^{1}H-NMR chemical shifts are indicated in blue. Drawn using ChemDraw.

Derivatization of Chalcones

DMC was subjected to alkaline peroxidation and a red-orange solution was formed. Two sets of derivatizations were performed for the oxidation of DMC-producing compounds A and B (Figure 3).

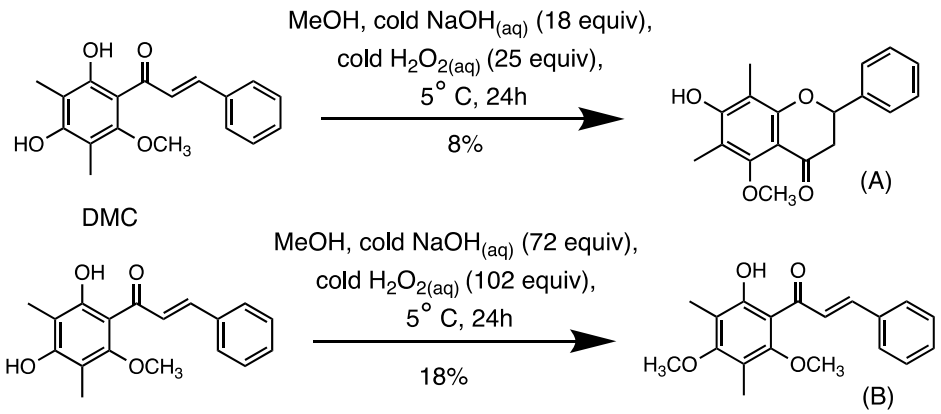

Figure 3. Reaction schemes for the alkaline peroxidation of DMC yielding compounds A and B.

From the first peroxidation procedure, four milligrams of yellow needle-like crystals (A) with an Rf value of 0.53 in 7:3 (v/v) hexane: ethyl acetate was collected after purification. This was identified to be (*2S*)-7-hydroxy-5-methoxy-6,8-dimethyl flavanone (Figure 4) and is visible under UV (254 nm) with a fluorescence observed at 365 nm.

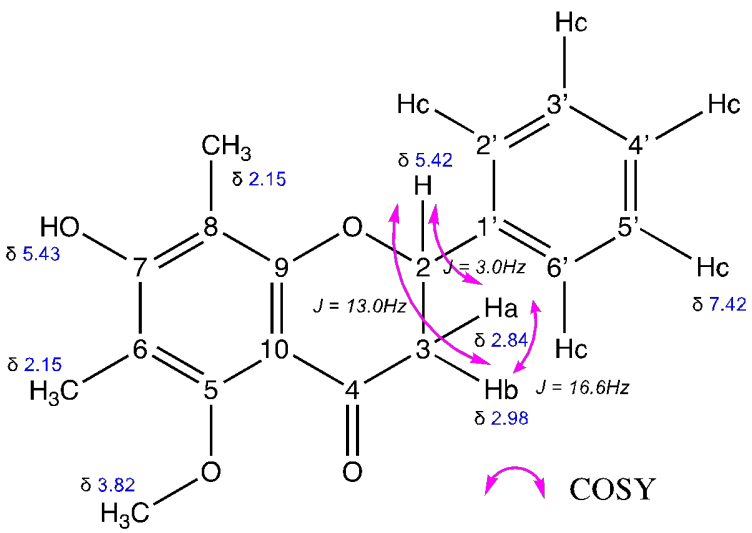

Figure 4. (*2S*)-7-hydroxy-5-methoxy-6,8-dimethyl flavanone structure elucidated using ^{1}H-NMR and COSY spectra. ^{1}H-NMR chemical shifts are indicated in blue. Drawn using ChemDraw.

Peroxidation of DMC was monitored through ^{1}H-NMR. The doublet of doublet signals at δ 2.84, 2.98, and 5.42 is indicative of a flavanone skeleton. This was confirmed to be 7-hydroxy-5-methoxy-6,8-dimethyl flavanone when chemical shifts obtained were compared with published literature values (Marinho et al. 2008; Memon et al. 2015) and has an (*S*)- relative configuration based on the coupling constants (3.0 and 12.9 Hz). The flavanone derivative of DMC was obtained by direct nucleophilic attack by the anionic oxygen atom on the β-carbon atom of the oxide intermediate (Geissman and Fukushima 1948). The substituents of the ring did not provide substantial inhibition to the resonance in the system that would have led to the formation of the aurone.

Another set of alkaline peroxidation of DMC was carried out using excess reagents. Nine milligrams of compound **B** (Figure 5) were collected.

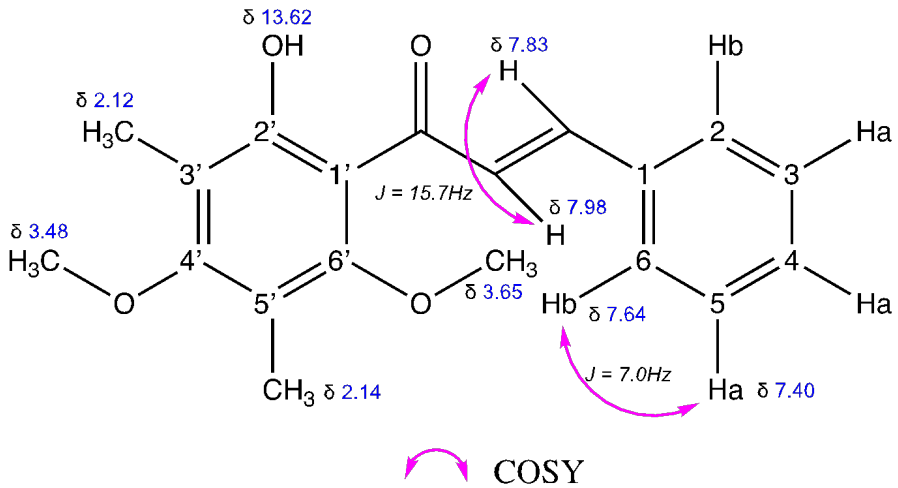

Figure 5. (*E*)-2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl chalcone structure elucidated using ^{1}H-NMR and COSY spectra. ^{1}H-NMR chemical shifts are indicated in blue. Drawn using ChemDraw.

The collected compound **B** has an Rf value of 0.6 in 7:3 (v/v) hexane: ethyl acetate and is visible under UV (254 nm). The ^{1}H-NMR spectrum of **B** shows two signals for a methyl group (-CH_3) at δ 2.13 and δ 2.16, two methoxy (-OCH_3) groups at δ 3.49 and δ 3.66, aromatic protons (Ar-H) at δ 7.34 – 8.00 [δ 8.00 (d, *J* = 15.7 Hz, 1H), δ 7.84 (d, *J* = 15.7 Hz, 1H), δ 7.66 (dd, *J* = 8.4, 6.3 Hz, 2H)], and a signal at δ 13.63 for the hydroxyl (-OH) proton. Compound **B** has a methoxy group instead of a hydroxyl group at the 4' position with respect to DMC.

Validation of the Cyclooxygenase Inhibition Assay

The cyclooxygenase inhibition assay is based on the action of the inhibitor on the cyclooxygenase and its substrate, arachidonic acid. To confirm that the assay is working and the data collected from it are valid, the activity of the enzyme and substrate was monitored by determining if the Michaelis-Menten constant (K_M) and V_{max} are constant and within the acceptable range. Acceptable range means the parameters collected are either in line with theoretical data based on previous studies or within a constant range of values established historically in the laboratory. The responses of varied concentrations of arachidonic acid against a constant amount of enzyme were recorded and plotted in Figure 6.

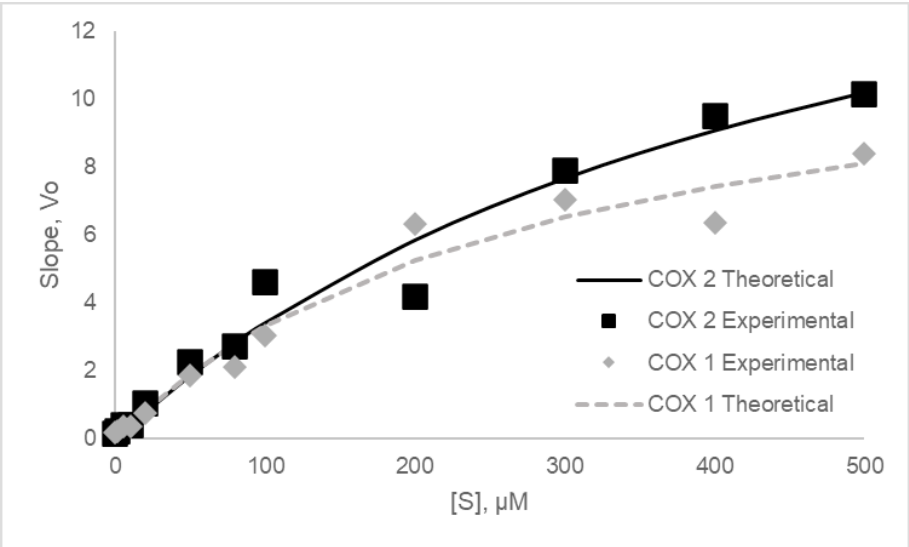

Figure 6. Michaelis-Menten plot of COX-1 and COX-2 enzymes with arachidonic acid substrate.

The K_M and V_{max} values obtained for COX-1 were 279.4 µM and 12.6 Fi/s, respectively, while 488.0 µM and 20.2 Fi/s, respectively, were calculated for COX-2. These values are consistent with the historically established data range (COX-1: K_M = 200 - 550 µM; COX-2: K_M = 200 – 600 µM) for the assay parameters (Opog et al. 2019).

The assay results for the cyclooxygenase inhibiting activity of DMC and its derivatives are shown in Figure 7.

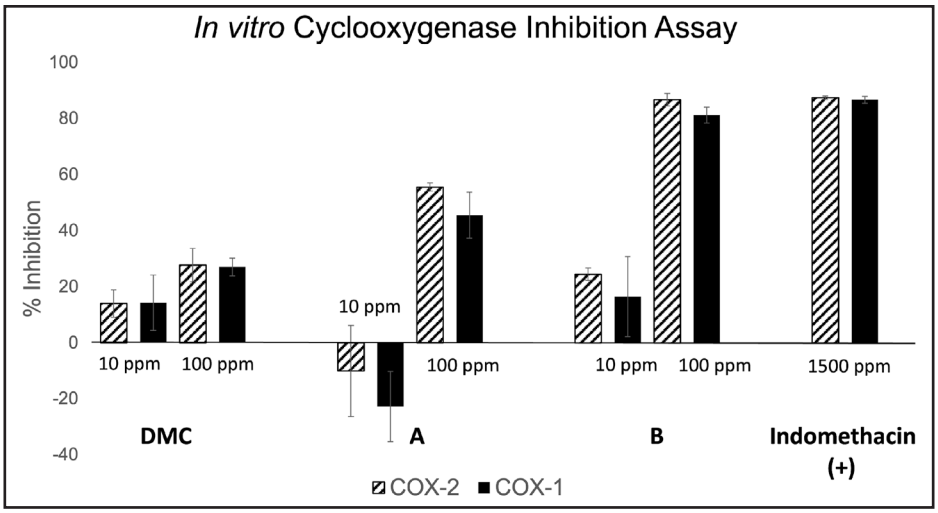

Figure 7. COX inhibition summary for DMC and its derivatives.

Compound **A** gave 55.57 ± 1.43% inhibitory activity against COX-2 at 100 ppm and 45.54 ± 8.19% against COX-1. Compound **B** gave 86.70 ± 2.25% inhibitory activity against COX-2 at 100 ppm and 81.22 ± 2.84% against COX-1. Compounds **A** and **B** were found to be COX-2 selective inhibitors with COX-2 to COX-1 inhibition ratios of 1.22 and 1.07, respectively.

CONCLUSION

Alkaline peroxidation of (*E*)-2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl chalcone from the leaf extracts of *Syzygium samarangense* (Blume) Merr. yielded two products, 7-hydroxy-5-methoxy-6,8-dimethyl flavanone and 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl chalcone. This is the first report of the peroxidation of DMC producing these flavonoids.

Flavonoids are plant-derived natural products that have long been studied for their anti-inflammatory properties (Read 1995; Zhang et al. 2006). Some flavanones have been reported to exhibit *in silico* COX-2 selective inhibiting activity such as eriodictyol (3',4',5,7-tetrahydroxyflavan-4-one), hesperetin (3',5,7-trihydroxy-4'-methoxyflavan-4-one), and naringenin (4'-5,7-trihydroxyflavan-4-one) (Akinloye et al. 2019). On the other hand, synthetic chalcone derivatives have also been reported as COX-2 selective inhibitors in several studies (Razmi et al. 2013; Jantan et al. 2014).

DMC, A, and B were tested for their anti-inflammatory activity using an in vitro cyclooxygenase inhibition assay. Both derivatives show selective inhibition of cyclooxygenase-2 at 100 ppm. This is the first report of the selective cyclooxygenase-2 inhibiting activity of 7-hydroxy-5-methoxy-6,8-dimethyl flavanone and 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl chalcone. These flavonoids could potentially be lead candidates in the development of new anti-inflammatory drugs.

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