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Novel Compounds from *Piper methysticum* Forst (Kava Kava) Roots and Their Effect on Cyclooxygenase Enzyme

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Milled *Piper methysticum* roots were extracted sequentially with hot water and methanol. Cyclooxygenase (COX) enzyme inhibitory assay directed purification of the methanol extract yielded bornyl esters of 3,4-methylenedioxy cinnamic acid (**1**) and cinnamic acid (**2**), pinostrobin (**3**), flavokawain B (**4**), and 5,7-dimethoxyflavanone (**5**). The structures of compounds **1**−**5** were accomplished by spectral experiments. The aqueous extract contained previously reported kava lactones, as confirmed by TLC analysis. Compounds **3** and **5** were isolated for the first time from kava kava roots. Compound **4** showed the highest COX-I inhibitory activity at 100 μg/mL. All the compounds tested gave good COX-I and moderate COX-II enzyme inhibitory activities at 100 μg/mL. This is the first report of COX-I and -II inhibitory activities for compounds **1**−**5**.

KEYWORDS: ***Piper methysticum*; kava kava; cyclooxygenase; bornyl ester; cinnamic acid; pinostrobin; flavokawain**

INTRODUCTION

Piper methysticum Forst (kava kava) is a perennial herb of Piperaceae family grown widely in the Pacific Islands (*1*). The rootstock of kava kava is commonly used to prepare a beverage for ceremonial activities by the native Pacific Islanders. Also, it is used in traditional herbal medicine for treating gonorrhea, menstrual pain, tuberculosis, respiratory tract infections, and chronic pain related to gout and arthritic conditions (*2*, *3*). Pacific Islanders routinely apply kava kava root extract as an analgesic and as a mouthwash for toothache and canker sores (*4*, *5*). In Europe, kava kava was used for the treatment of chronic inflammations of the urinary tract at the beginning of the twentieth century (*6*, *7*).

The antiinflammatory activities of natural products are determined mainly by evaluating their ability to inhibit cyclooxygenase (COX) enzymes (*8*). One of the mechanisms by which antiinflammatory agents control inflammation is by inhibiting the synthesis of prostaglandins. Prostaglandins, inflammation-causing hormones, are formed by the conversion of membrane lipid catalyzed by COX and other enzymes. Two isoforms of the COX enzymes responsible for the prostaglandins formation are cyclooxygenase-I (COX-I) and cyclooxygenase-II (COX-II) (*9*, *10*). However, they differ in their distribution in the body by location and in their physiological activities. COX-I enzyme is a constitutive form and expressed in most cells. It performs "housekeeping" functions such as maintaining gastrointestinal mucosa, protecting renal blood flow function, and influencing platelet aggregation. COX-II enzyme, on the other hand, is an inducible form of the enzyme which is expressed in response to inflammatory and other physiological stimuli. The prostaglandins produced by COX-II enzyme mediate pain and the inflammatory process. Over-the-counter pain relief agents such as aspirin, ibuprofen, and naproxen inhibit both COX-I and COX-II enzymes at therapeutic doses (*10*).

Kavain, one of the six major kavalactones present in kava kava roots, was determined to have antithrombotic action on human platelets (*11*). It was also shown to prevent the formation of prostaglandin E_2 and thromboxane A_2 by inhibiting the action of COX and thromboxane synthase enzymes (*11*). To evaluate the antiinflammatory effect of the constituents in kava kava roots, we have investigated compounds other than kava lactones present in the MeOH extract of kava kava roots. In this paper, we report the compounds isolated for the first time from kava kava roots (**Figure 1**) with COX-I and COX-II inhibitory activities.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H NMR spectra were recorded at 300 or 500 MHz, and ^{13}C NMR spectra were recorded at 75 or 126 MHz (INOVA 300 and VXR 500, Varian Inc., Palo Alto, CA). All compounds were dissolved in $CDCl_3$, and ^{1}H- and ^{13}C NMR shift values were presented in δ (ppm) based on the residual δ value of $CHCl_3$ at 7.24 and 77.0 ppm, respectively. Electron ionization mass spectra (EIMS) were obtained on a JEOL AX-505H double-focusing mass spectrometer at 70 ev. IR spectra analysis were conducted on a Galaxy Series FTIR 5020 (Thermo Mattson Co., Madison, WI). Medium-pressure liquid chromatography (MPLC) was carried out on silica gel

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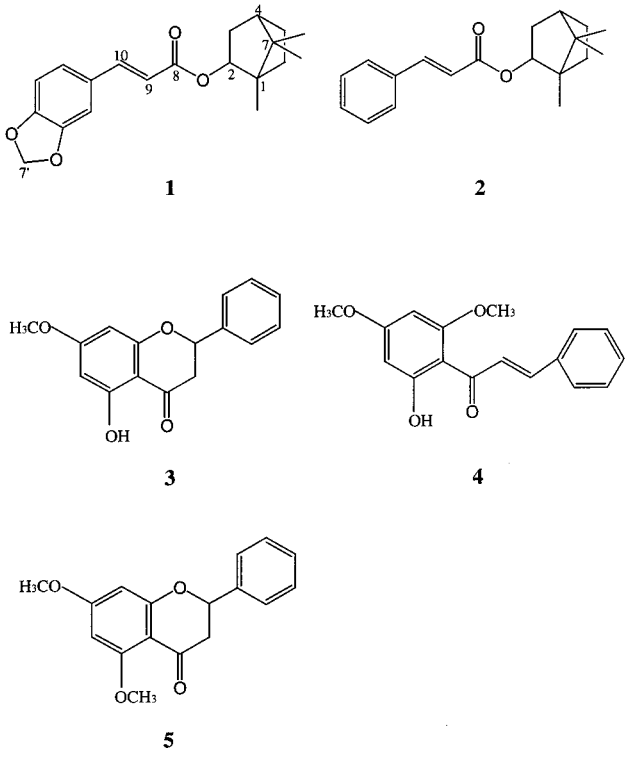

Figure 1. Cyclooxygenase inhibitory compounds from *P. methysticum* roots. **1**, bornyl ester of 3,4-methylene dioxy cinnamic acid; **2**, cinnamic acid bornyl ester; **3**, pinostrobin; **4**, flavokawain B; **5**, 5,7-dimethoxyflavanone.

60, and preparative TLC was done with silica gel GF glass plates (20 × 20 cm, 250 or 500 μm thickness, Analtech, Newark, DE). Bands were viewed under UV light at 254 and 366 nm. Preparative HPLC was conducted on an LC-20 (Japan Analytical Industry Co., Tokyo) with a JAIGEL-ODS column (A-343-10, 250 mm × 20 mm i.d., 10 μm, Dychrom, Santa Clara, CA). A UV detector was used at 246 nm and peaks were recorded by a model D-2500 Chromato-integrator (Hitachi Co., Tokyo). Positive controls, naproxen, ibuprofen, and aspirin for COX enzyme inhibitory assays were purchased from Sigma Chemical Company (St. Louis, MO). Celebrex capsules and Vioxx tablets, as physician's professional samples, were provided by Dr. Subash Gupta, Sparrow Pain Center, East Lansing, MI. All solvents were ACS reagent grade and purchased from Aldrich Chemical Co.

Plant Materials. Dried kava kava roots were purchased from Meetex Fiji Ltd., Fiji. The roots were stored at −20 °C in plastic shipping bags until extraction. The roots were milled using a Thomas-Wiley Laboratory Mill model 4 (Thomas Scientific, Swedesboro, NJ, 2-mm filter) prior to extraction.

Extraction of *Piper methysticum* Roots. The ground roots (200 g) were extracted twice with stirring with water (3L for 3h and 1L for 1h) at 60 °C. The water extracts were combined, cooled to room temperature, and centrifuged, and the supernatant was lyophilized. The precipitant from the aqueous extract mainly contained very fine particles of plant material and therefore was combined with the residue prior to the MeOH extraction. The residue, plant material after aqueous extraction, was extracted with MeOH (3 L for 24 h) at the room temperature and evaporated. The MeOH extract residue (8 g) showed the highest COX inhibitory activity when compared to the water extracts. Therefore, the MeOH extract was used for this study and fractionated by MPLC on silica gel using hexane/acetone (10:1, 750 mL; 7:3, 400 mL; 1:1, 200 mL; 3:7, 200 mL) followed by 100% acetone (1.05 L) and MeOH (2.3 L). The fractions obtained were as follows: A (0.505 g, hexane/acetone 10:1 and 7:3, 1.15 L), B (3.038 g, hexane/acetone 1:1, 250 mL), C (1.798 g, hexane/acetone 3:7, 250 mL), D (1.279 g, 100% acetone, 250 mL), E (0.161 mg, 100% acetone, 910 mL),and F (0.674 g, 100% MeOH, 2 L). The COX inhibitory assay revealed that fractions A−D were the most active. Fraction A was further purified by preparative TLC with hexane/acetone (4:1) to yield eight bands. Bands I (101.5 mg, R_f 0.7), II (33.7 mg, R_f 0.5), IV (77.1 mg, R_f 0.4), V (212 mg, R_f 0.3), and VII (63.5 mg, R_f 0.2) were active in COX-I and COX-II enzyme inhibitory assays.

Isolation of Compounds 1 and 2. Compound **1** (5.1 mg, 0.003% yield) was obtained from the crystallization of band II from MeOH. Band II from fraction A was further purified by TLC with hexane/acetone (8:1) to yield compound **2** (60.3 mg, 0.03% yield, R_f 0.8).

Compound 1. White needlelike crystals; mp 138−140 °C. IR (KBr), 2954, 1700, 1635, 1520, 1444, 1255, 1180, 931, 807, 755 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz), δ 7.03 (1H, s), 6.9 (2H, dd, J 8.1, 2.1 Hz), 6.88 (2H, dd, J 15.9 Hz), 5.98 (2H, s), 5.00 (1H, m), 2.40 (1H, m), 2.02 (1H, m), 1.75 (1H, m), 1.68 (1H, t), 1.36 (1H, m), 1.26 (1H, m), 1.03 (1H, dd, J 13.8, 1.8 Hz), 0.92/0.89 (2 × 3H, 2s), 0.85 (3H, s). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 167.5, 149.5, 148.3, 143.9, 128.9, 124.4, 124.3, 124.3, 116.7, 101.6, 79.8, 44.9, 48.9/47.8, 36.8, 28.0, 27.2, 19.7/18.8, 13.5. HREIMS (% rel intensity), m/z 328.1673 (calc. 328.1685, MF $C_{20}H_{24}O_4$) [M^+] (100), 192 (50), 175 (100), 145 (80) (*12*).

Compound 2. Pale yellow oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.03 (5H, m, aromatic H), 6.91 (2H, dd, J 15.9 Hz, H-9/H-10), 5.01 (1H, m, H-2e), 2.41 (1H, m, H-3e), 2.04 (1H, m, H-6a), 1.77 (1H, m, H-5e), 1.70 (1H, t, H-4e), 1.36 (1H, m, H-6e), 1.29 (1H, m, H-5a), 1.05 (1H, dd, J = 13.8, 1.8 Hz, H-3a), 0.93/0.89 (2 × 3H, 2s, $2CH_3-C_7$), δ 0.87 (3H, s, CH_3-C_1). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 167.3 (C-8), 144.2, 134.5, 130.1, 128.8, 128.8, 128.0 (C-1′-C-6′), 128.0 (C-10), 118.0 (C-9), 79.9 (C-2), 44.9 (C-4), 48.9/47.8 (C-1/C-7), 36.8 (C-3), 28.0 (C-5), 27.2 (C-6), 19.7/18.8 ($2CH_3-C_7$), 13.5 (CH_3-C_2). EIMS (% rel intensity), m/z 284 [M^+][25], 153 (72), 131 (100), 109 (30), 103 (30). Compound **2** was identified as cinnamic acid bornyl ester. The spectral data of **2** were in agreement with the published values (*12*, *13*) (**Figure 1**).

Isolation of Compounds 3−5. Band IV from fraction A was further purified by repeated preparative TLC with hexane/acetone (4:1, × 2) to yield one major band (66 mg, R_f 0.7). This band, inhibitory to both COX-I and COX-II, was purified by preparative TLC with toluene/ethyl acetate (13:1) and hexane/acetone (8:1, × 4) followed by crystallization from hexane/acetone to afford compound **3** (14.5 mg, 0.007% yield). Compound **3** was identified as pinostrobin on the basis of the published chemical shift values for pinostrobin (*14*). Band V from fraction A was crystallized from hexanes/ether to afford compound **4** (73.1 mg, 0.04% yield) and confirmed as flavokawain B. The NMR data of **4** were identical to the published values of flavokawain B (*15*, *16*). The mother liquor (76.3 mg) after the crystallization of compound **4** was further purified by preparative TLC using toluene/ethyl acetate (8:1) as the mobile phase to afford five bands. Band 1 (49.1 mg, R_f 0.8) was further purified by preparative TLC using hexanes/ethyl acetate (8:3) to yield compound **3** (10.7 mg, 0.005% yield) and an additional supply of compound **4** (39.0 mg, 0.02% yield).

Fraction B from the MeOH extract was further fractionated by silica MPLC using hexane/acetone (10:1, 550 mL; 6:1, 560 mL; 4:1, 150 mL; 3:1, 160 mL; 2:1, 420 mL; 1:1, 200 mL). Six fractions were collected: hexane/actone 6:1; 1 (48.2 mg, 300 mL), 2 (24.5 mg, 100 mL); hexane/acetone 6:1 and 4:1, 3 (111.6 mg, 300 mL); hexane/acetone 3:1 and 2:1; 4 (489.2 mg, 300 mL), 5 (636.7 mg, 200 mL); hexane/acetone 1:1; 6 (1.35 g, 300 mL). Fraction 6 was active in COX enzyme inhibitory assays, and 500 mg of it was further purified by preparative HPLC on a Jaigel-ODS column under isocratic conditions using MeOH/H_2O (60:40) at a flow rate of 3 mL/min and detected at 210 nm. This yielded compound **5** (7 mg, 0.004% yield, R_t 174.7 min), identified as 5,7-dimethoxyflavanone (**Figure 1**). The NMR data of **5** were in agreement with the published data (*17*, *18*).

Cyclooxygenase Enzyme Inhibitory Assay. This assay is based on measuring COX enzyme activities by monitoring the rate of O_2 uptake using an oxygen electrode (YSI model 5357, INSTECH Laboratory, Plymouth Meeting, PA) (*19*−*21*). The assay mixture consisted of 600 μL of 0.1 M Tris/1 mM phenol buffer and 17 μg hemoglobin. COX-I enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical Research, Inc., Oxford, MI. COX-I enzyme solution was prepared by dissolving 0.46 mg of protein/mL in 30 mM Tris buffer (pH 7.0). COX-II enzyme was isolated from prostaglandin endoperoxide H synthases-2 cloned insect cell lysate and

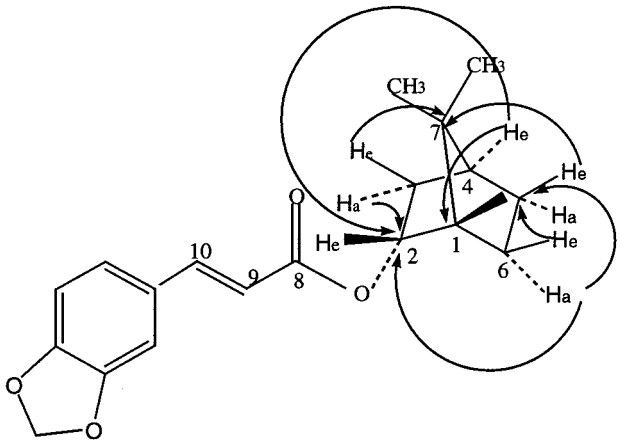

Figure 2. Significant HMBC correlations observed in compound **1**.

diluted with Tris buffer (pH 7.0) to a concentration of 1.5 mg of protein/mL. Reactions were initiated by adding 10 μL of arachidonic acid (0.25 mg/0.5 mL Tris buffer) in the assay mixture. The test samples or controls as DMSO solutions and 5−25 μg of COX enzymes in 10−20 μL were preincubated for 10 min prior to injection of 10 μL of arachidonic acid in a 600 μL microchamber. Instantaneous inhibition of COX enzymes was determined by measuring the COX enzyme activity at 37 °C controlled by a circulation bath (model-1166, VWR Scientific Products, Chicago, IL). The enzyme activity was monitored by a biological oxygen monitor, and the data were collected using Quicklog data acquisition and computer software (Strawberry Tree Inc., Sunnyvale, CA). Finally, the data were transformed into Microsoft Excel. The assay was conducted in triplicate.

RESULTS AND DISCUSSION

Compounds **1**, **2**, **3**, and **5** were isolated for the first time from kava kava roots. The structure of compound **1** was deduced by using ^{1}H- and ^{13}C NMR, one and two-dimensional spectroscopy, and IR and MS spectrometric techniques. The ^{1}H NMR spectrum of **1** gave a singlet at δ 7.03, and a doublet of doublet at δ 6.91 suggested a trisubstituted aromatic moiety. The singlet at δ 5.98, integrated for two protons, and a corresponding carbon chemical shift at δ 101.6 suggested a methylene dioxy group in the molecule. A doublet of doublet signal, integrated for two protons, at δ 6.88 with a coupling constant of 15.9 Hz, corresponded to a trans olefinic group. A singlet at δ 5.00 was determined to be a proton connected to an oxygenated carbon. Three singlets at δ 0.92, 0.89, and 0.85, each integrated for three protons, were indicative of three CH_3 groups. The ^{13}C NMR spectrum of compound **1** revealed 19 peaks. They were assigned to one carbonyl carbon (δ 167.3), six aromatic carbons (δ 149.5, 148.3, 143.9,128.9, 124.4, 124.3), two olefinic carbons (δ 124.3, 116.7), two quaternary carbons (δ 48.9, 47.8), two CH (δ 79.8, 44.9), four CH_2 (δ 101.6, 36.8, 28.0, 27.2), and three CH_3 (δ 19.7, 18.8, and 13.5) as confirmed by the DEPT spectrum. DQF−COSY, HMQC, and HMBC spectral data provided additional evidence to confirm the structure of compound **1**. The important connectivities in DQF−COSY spectrum of **1** were those observed for H-3 and H-4, H-2 and H-3, and H-5 and H-6. The decoupling spectrum of compound **1** revealed long-range couplings between H-3e and H-5e, as well as between H-2e and H-6e, and also indicated that dihedral angles ϕ between H-4e and H-5a, as well as between H-4e and H-3a, were almost 90° (*22*). The HMBC spectrum of compound **1** (**Figure 2**) showed significant correlations for C-1 and H-4. The signals for C-4 correlated to H-3, H-5, and H-6a, whereas C-1 displayed cross signals to H-3e, H-4e, H-5a, and H-6. Also, the correlation of C-5 to H-3 and H-6, as well as the correlation of C-2 to H-3, H-4e, and H-6, supported the proposed structure. One of the methyl groups at δ 0.85 correlated to C-2. The presence of a carbonyl group in **1** was suggested by a strong peak at 1700 cm^{-1} in its IR spectrum. Another strong peak at 1635 cm^{-1} was assigned to C═C and indicated conjugation to a carbonyl group. Peaks at 1255 and 1180 cm^{-1} corresponded to C−O functionality. The MS analysis of **1** gave a strong M^+ ion at *m*/*z* 328.1673 (100%) which suggested the molecular formula of **1** as $C_{20}H_{24}O_4$. The suggested fragmentation pattern of **1** under EIMS conditions is shown in **Figure 3**. The EIMS fragments at *m*/*z* 192, 175, and 145 supported the proposed structure for compound **1**. Based on all the spectral analyses, compound **1** was deduced to be bornyl ester of 3,4-methylenedioxy cinnamic acid.

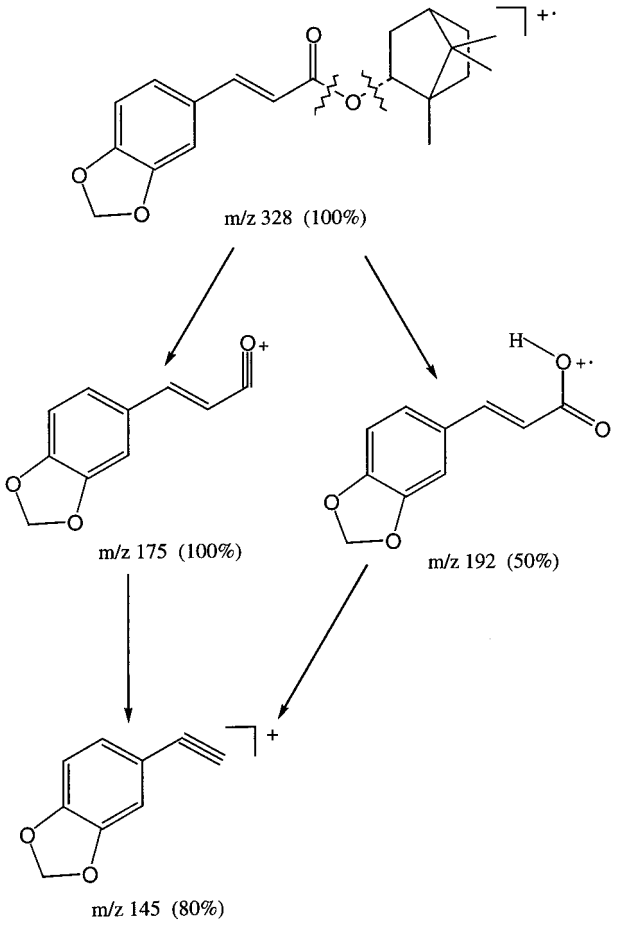

Figure 3. Suggested fragmentation pattern of compound **1** under EIMS conditions.

The structure of compound **2** was deduced from ^{1}H and ^{13}C NMR and MS spectrometric data. The ^{1}H- and ^{13}C NMR spectra of compound **2** were similar to those of compound **1** except for minor changes in the aromatic moiety. The multiplet at δ 7.03, integrated for five protons, was assigned to a monosubstituted aromatic moiety. On the basis of ^{1}H- and ^{13}C NMR spectra of **2**, it was proposed to be cinnamic acid bornyl ester, and the results of HMQC and HMBC experiments supported this structure. The MS analysis of **2** gave a M^+ ion at *m*/*z* 284. The EIMS fragments at *m*/*z* 153, 131, 109, and 103 provided further evidence to confirm the structure of **2**. On the basis of all the spectroscopic data, compound **2** was assigned as cinnamic acid bornyl ester.

COX-I and COX-II enzyme inhibitory assays were used in the bioassay-directed isolation of potential antiinflammatory compounds present in kava kava roots. Ibuprofen, naproxen,

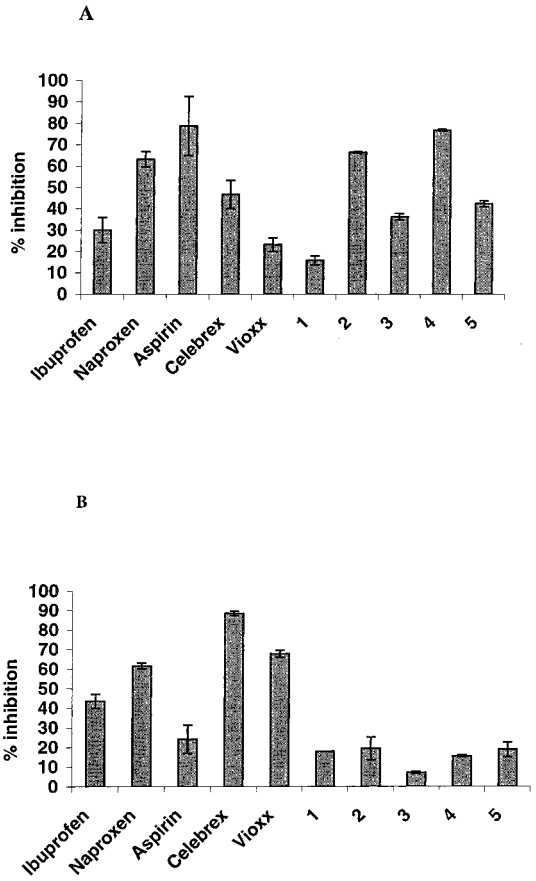

Figure 4. Percentage COX-I inhibition (A) and COX-II inhibition (B) of compounds from kava kava roots at 100 μg/mL. **1**, bornyl ester of 3,4-methylene dioxy cinnamic acid; **2**, cinnamic acid bornyl ester; **3**, pinostrobin; **4**, flavokawain B; **5**, 5,7-dimethoxyflavanone. Ibuprofen, naproxen and aspirin were tested at 2.1, 2.5, and 180 μg/mL, respectively. Celebrex and Vioxx were tested at 1.67 μg/mL. Vertical bars represent the standard deviation of each data point ($n = 3$).

aspirin, Celebrex, and Vioxx were used as positive controls in these assays, and they were dissolved in DMSO at 2.1, 2.5, 180, 1.67, and 1.67 μg/mL, respectively. In the COX-I enzyme inhibitory assay, ibuprofen, naproxen, aspirin, Celebrex, and Vioxx demonstrated 30, 63, 78, 47, and 23% inhibition, respectively. Similarly, in the COX-II enzyme inhibitory assay ibuprofen, naproxen, aspirin, Celebrex, and Vioxx showed 44, 61, 24, 88, 67% of inhibition, respectively. All test compounds were assayed at 100 μg/mL and pH 7.0 in both COX-I and COX-II enzyme inhibitory assays. The percentage inhibitions of COX enzymes activities for test compounds at 100 μg/mL are shown in **Figures 4A** and **4B**. Compounds **1**−**5** showed 16, 66, 36, 77, and 42% inhibition, respectively, in the COX-I enzyme inhibitory assay while they gave 18, 19, 7, 16, and 19% inhibition, respectively, in the COX-II enzyme inhibitory assay. Because compound **4** was the most COX-I active, it was further tested at 50, 25, 12.5, and 6.25 μg/mL and revealed 79, 64, 55, and 20% inhibition, respectively.

In the COX-I inhibitory assay, compound **4** exhibited 77% inhibition; it was the most active among the compounds tested at 100 μg/mL. It also showed good COX-I enzyme inhibitory activities at 6.25 μg/mL. However, it demonstrated only 16% inhibition of COX-II at 100 μg/mL. This indicated that compound **4** is not a preferred antiinflammatory agent. Compounds **2**, **3**, and **5** also displayed higher COX-I inhibition at 100 μg/mL than over-the-counter and prescribed NSAIDS. However, compounds **1**−**5** showed a relatively low percentage of COX-II inhibition at 100 μg/mL.

Bornyl cinnamate derivatives, (−)-bornyl ferulate, (−)-bornyl p-coumarate, and (−)-bornyl caffeate exhibited antiinflammatory activities in assays using the 5-lipoxygenase enzyme (*23*). The inhibitory activities of these compounds on 5-lipoxygenase enzyme was due to their antioxidant properties as shown in both in-vitro and ex-vivo assays (*23*). Two of the bornyl cinnamate derivatives isolated in our laboratory also revealed antiinflammatory properties by inhibiting COX-I and COX-II enzymes.

The results of our study provided some scientific support for the anecdotal claims on the traditional use of kava kava roots by Pacific Islanders for controlling inflammatory pain. The compounds obtained from the MeOH extract of kava kava roots after extraction with hot water demonstrated substantial COX-I and moderate COX-II inhibitory activities. Therefore, a combination of kava lactones and compounds **1**−**5** may account for the traditional use of kava kava roots to alleviate arthritic and gout related pain. This is the first report of cyclooxygenase enzyme inhibitory activities for compounds **1**−**5**.

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