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Cyclooxygenase enzyme inhibitory compounds with antioxidant activities from *Piper methysticum* (kava kava) roots

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Summary

Cyclooxygenase enzyme inhibitory assay-guided purification of ethyl acetate extract of *Piper methysticum* (kava kava) roots yielded six biologically active compounds (1–7), which were purified using MPLC, preparative TLC and HPLC methods. These compounds were also evaluated for antioxidant activities. Dihydrokawain (1) and yangonin (6) showed the highest COX-I and COX-II inhibitory activities at 100 µg/ml, respectively. The lipid oxidation assay did not reveal antioxidant activities for demethoxyangonin (2), dihydrokawain (1), kawain (4), dihydromethysticin (5) or methysticin (7) at 50 µg/ml. The antioxidant activities of flavokawain A (3) and yangonin (6) could not be tested in the lipid oxidation assay due to solubility problems. However, yangonin and methysticin showed moderate antioxidant activities in the free radical scavenging assay at 2.5 mg/ml.

Key words: *Piper methysticum*, kawa lactones, chalcones, kava kava, cyclooxygenase, antioxidant

Introduction

P. methysticum Forst (kava kava) is a herb consumed as a drink and used in traditional medicine by many people in the Pacific Islands (Keller et al., 1963). Kava kava is considered as a healthy beverage (Singh, 1992) and also reported as a good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gout-related pain and arthritic conditions (Singh, 1992; Singh and Blumenthal, 1997). Other reported beneficial effects of kava kava include analgesic and diuretic effects, relaxation of muscle tension and alleviation of anxiety (Singh, 1992; Singh and Blumenthal, 1997). Pacific islanders use kava kava extract as an analgesic and as a mouthwash for toothache and canker sores (Singh and Blumenthal, 1997). The rootstock of kava kava is traditionally used for the preparation of kava during by brewing the roots in water at room temperature and as a medicine (Singh, 1992; Singh and Blumenthal, 1997).

Prostaglandin endoperoxide H synthase (PGHS) or cyclooxygenase (COX) enzymes have been used widely to evaluate the anti-inflammatory activities of natural products (Goda et al., 1992). It is hypothesized that one of the mechanisms through which non-steroidal anti-inflammatory drugs (NSAIDs) control inflammation is inhibition of the synthesis of prostaglandin, an inflammatory mediator produced at the site of tissue injury, by inhibiting COX-I and -II enzymes responsible for converting arachidonic acid to prostaglandins.

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Prostaglandins mediate inflammation through which nerves are sensitized to painful stimuli (Winzeler and Rosenstein, 1998). COX enzymes exist in two isoforms, COX-I and COX-II. The COX-II enzyme is considered to be the principal isoform that participates in the inflammatory process in the body (Cryer and Dubois, 1998).

The analgesic effectiveness of dihydrokawain and dihydromethysticin at 120 mg/kg body wt. was reported to be equivalent to that of aspirin at 200 mg/kg body wt. (Lebot et al., 1992). However, no study of the mechanisms by which kava extract or kava lactones work against inflammation in the body has been reported. In order to verify the anecdotal claims that kava extract has numerous phytoceutical benefits, we have investigated kava kava roots for cyclooxygenase inhibitory and antioxidant compounds.

Materials and Methods

General Experimental Procedures

^{1}H-NMR spectra were recorded at 300 and 500 MHz, respectively, and ^{13}C-NMR spectra at 126 MHz in $CDCl_3$, and values are presented in δ (ppm) based on residual δ value of $CHCl_3$ at 7.24 ppm. Coupling constants (J) are in Hz. CD spectra were recorded on a JASCO J710 Spectropolarimeter. Medium pressure lipid chromatography (MPLC) was carried out on silica gel 60 and C_{18} (25–40 µm). Preparative TLC was conducted over silica gel GF glass plates (20 × 20 cm, 250 or 500 µm thickness, Analtech, Newark, Delaware). Bands were viewed under UV light (366 and 254 nm). Positive controls were naproxen, ibuprofen and aspirin for COX enzyme inhibitory assays; tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) for lipid oxidation assays; and 2,2-diphenyl-I-picryhydrazyl radical (DPPH•), vitamin E and vitamin C for free radical scavenging assays, and were purchased from Sigma Chemical Company. All solvents were ACS reagent grade and purchased from Aldrich Chemical Company.

Plant Material

Dried kava kava roots were purchased from Meetex Fiji Ltd., Fiji. The roots were stored at –20 °C in plastic shipping bags prior to extraction. The roots were milled using Thomas-Wiley Laboratory Mill Model 4 (Thomas Scientific, USA, 2 mm filter) prior to extraction.

Cyclooxygenase inhibitory assay

This assay is based on measuring cyclooxygenase enzyme activities by monitoring the rate of O_2 uptake using a YSI Model 5357 oxygen electrode (INSTECH Laboratory, Plymouth Meeting, PA) (Meade et al., 1993; Wang et al., 2000). The assay mixture consisted of 600 µl of 0.1 M Tris-1 mM phenol buffer, 17 µg hemoglobin and 10 µM arachidonic acid. Cyclooxygenase I (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). Cyclooxygenase II (COX-II) enzyme was isolated from PGHS-2 cloned insect cell lysate and diluted with Tris buffer (pH 7.0) until the concentration was 1.5 mg of protein/ml. Reactions were initiated by adding 5–30 µg of microsomal protein in a volume of 10–20 µl of Tris-buffer in the assay mixture contained in a 600-µl microchamber. Instantaneous inhibition of COX enzymes was determined by measuring cyclooxygenase activity at 37 °C, controlled by a circulation bath (Mode-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, USA). Finally, the data were transformed into Microsoft Excel. The assay was conducted in duplicate.

Lipid Oxidation Assay

The lipid oxidation assay was conducted by detecting model liposome oxidation using fluorescence spectroscopy. A mixture containing 5 µmol of 1-stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 5 µl of the fluorescence probe (3-(p-(6-phenyl-)1,3,5-hextrienyl)phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) in $CHCl_3$, was evaporated in vacuo. The resulting lipid film, in 500 µl of solution containing NaCl (0.15 M), EDTA (0.1 mM), and MOPS (0.01 M), was suspended and exposed to ten freeze-thaw cycles using a dry ice/ethanol bath. The liposome in the buffer was then treated with chelating resin (chelex 100) to remove trace metal ions. The lipid-buffer suspension was extruded 29 times through a LiposoFast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce unilamellar liposomes. A 20-µl aliquot of this liposome suspension was diluted to 2 ml with chelex 100-treated HEPES buffer (100 µl, pH 7.0), 1 M NaCl (200 µl), N_2 sparged Millipore water (1.64 ml) and DMSO solution with or without test compounds (20 µl). The liposome was incubated for 5 min at room temperature, and then incubated at 23 °C in a cuvette holder of a Turner Model 450 Digital Fluorometer (Barnstead Thermolyne, Dubuque, IA). Peroxidation was initiated by the addition of 2 mM $FeCl_2$ solution (20 µl) to achieve a final concentration of 20 µM of Fe^{2+} in the absence or presence of test compounds or crude extracts. Controls contained no test compounds.

Fluorescence intensity was measured at an excitation wavelength of 384 nm every 3 min over a period of 21 min. The positive controls BHA, BHT and TBHQ were tested at 10 mM in 2 ml of final solution. The decrease of fluorescence intensity over time indicated the rate of peroxidation (Arora and Strasburg, 1997).

Total free-radical scavenging assay using DPPH•

Total free-radical scavenging capacity of kava kava compounds was determined and compared to those of vitamin E, vitamin C, and BHT according to the procedure reported previously using stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH•) (Blois, 1958; Chen and Ho, 1997). An ethanolic solution of DPPH• (800 µl) was added into a DMSO solution (800 µl) of test compound to start the reaction. Absorbance of each reaction mixture at 517 nm was measured against an ethanol control at 0, 5, 10, 20, 40, and 80 min, respectively. The percent DPPH• radical remaining at each tested time point was calculated using a DPPH• standard curve. A preliminary evaluation of the free radical scavenging assay revealed that only compounds 6 and 7 were active. Therefore, the kinetics of methysticin(6)-DPPH• and yangonin(7)-DPPH• reactions were determined by plotting the percent of unreacted DPPH• against time for each compound tested. The concentration of compounds 6 and 7 in the reaction mixtures was 2.5 mg/ml, while the concentration of DPPH• was 100 µM. The test concentrations for vitamin C, vitamin E, and BHT were 25, 10 and 50 µM, respectively. Reactions were carried out in triplicate for each antioxidant tested.

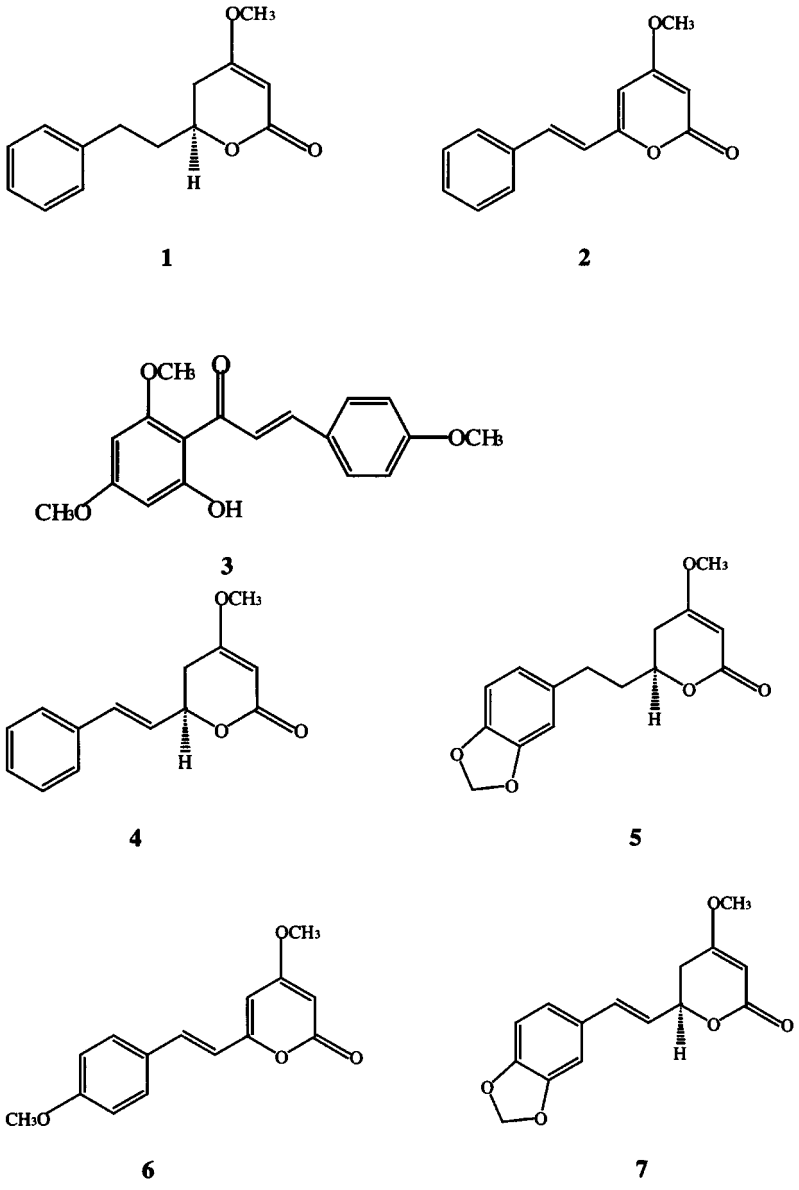

Fig. 1. Cyclooxygenase inhibitory compounds from *P. methysticum* roots. 1 = dihydrokawain; 2 = demethoxyangonin; 3 = flavokawain A; 4 = kawain; 5 = dihydromethysticin; 6 = yangonin; 7 = methysticin.

Extraction of *P. methysticum* Roots

The ground roots (749 g) were extracted sequentially with hexane (3 × 1.96 l, 72 h), ethyl acetate (3 × 1.2 l, 72 h) and methanol (3 × 0.25 l, 72 h). The ethyl acetate extract (1.46 g) showed the highest cyclooxygenase inhibitory activity and was further fractionated by MPLC on silica gel using hexane-acetone (10:1, 110 ml; 6:1, 350 ml; 3:1, 600 ml; 1:1, 400 ml) followed by 100% acetone (140 ml) and MeOH (100 ml). The fractions collected were: hexane-acetone, 3:1; A (75 ml, 5.7 mg), B (210 ml, 18.6 mg), C (180 ml, 6.4 mg), D (120 ml, 22.1 mg), E (75 ml, 0.9 mg); hexane-acetone 1:1; F (195 ml, 317.8 mg), G (60 ml, 458.7 mg), H (30 ml, 109 mg), I (90 ml, 362 mg); 100% acetone, J (30 ml, 15.5 mg), K (75 ml, 20.2 mg), L (30 ml, 8.8 mg) and 100% MeOH, M (100 ml, 84.6 mg). The cyclooxygenase inhibitory assay of fractions A–M revealed that fractions D-I were the most active.

• *Compounds 1–3:* Fraction F (150.9 mg) was purified by preparative TLC (hexane-ethyl acetate 4:1) to yield two major bands, I (36.5 mg, R_f 0.8) and II (84.4 mg, R_f 0.6). Both bands were active when tested for COX-I and COX-II inhibitory activities. Band II was purified further by repeated preparative TLC (hexane-ethyl acetate 75/25) and C_{18} TLC (methanol-water 70/30) followed by crystallization to yield compounds 1 (67 mg, mp 55–56 °C). Spectral analysis confirmed that compound 1 was identical to (+)-dihydrokawain (Spino et al., 1996). Compound 2 (22.1 mg, mp 137–139 °C) was obtained after crystallization of Band I. ^{1}H- and ^{13}C-NMR analyses confirmed that compound 2 was identical to demethoxyangonin (Itokawa et al., 1981). Fraction D yielded pure compound 3 (22.1 mg) and its NMR analysis confirmed that compound 3 was flavokawain A (Shukla et al., 1973; Parmar et al., 1990).

• *Compounds 4–6:* Fraction G (458.7 mg) was dissolved in MeOH to yield MeOH-soluble (415.5 mg) and MeOH-insoluble (43.1 mg) fractions. The MeOH solution was further precipitated using ACN-H_2O (60:40 v/v), centrifuged and the supernatant evaporated under vacuum. The residue from the supernatant (333 mg) was fractionated by C_{18} MPLC using ACN-H_2O gradient and followed with 100% ACN. The fractions collected were: ACN-H_2O, 60:40; 1 (300 ml, 262.2 mg), 2, (60 ml, 7.9 mg), 3 (50 ml, 6.1 mg), 4 (50 ml, 10.6 mg), 5 (30 ml, 14.8 mg); ACN-H_2O, 70:30; 6 (90 ml, 17.7 mg), 10 (50 ml, 2.8 mg), and 11 (300 ml, 2.4 mg). Fraction 1, active in the cyclooxygenase inhibitory assay, was further purified by preparative HPLC, on two Jaigel S-343-15 ODS columns in tandem, under gradient conditions using MeOH-ACH-H_2O (20:20:60) to 100% ACN at a flow rate of 3.5 ml/min and detected at 246 nm. This yielded compound 4 (46.3 mg, R_f 98.27 min, mp 108–110 °C) and compound 5 (3 mg, R_f 94.32 min, mp 116–118 °C). NMR and CD spectral analyses revealed that compounds 4 and 5 were identical to (+)-kawain (Klohs et al., 1959; Abramson and Wormser, 1981) and (+)-dihydromethysticin (Shao et al., 1998; Franca et al., 1973), respectively. Fractions 4 and 5, also active in the cyclooxygenase inhibitory assay, were combined and purified by preparative TLC using $CHCl_3$ as the mobile phase to yield compound 6 (13.19 mg, mp 152–153 °C). NMR data indicated that compound 6 was identical to yangonin (Shao et al., 1998). The ACN-H_2O (60:40)-insoluble portion of fraction G (69.9 mg) and the MeOH-insoluble fraction of fraction G (43.1 mg) were purified by preparative TLC with hexane:ethyl acetate:ether (3:0.5:4) and $CHCl_3$, respectively, followed by crystallization to yield an additional amount of compound 6 (68.1 mg). Fraction H (109 mg) was purified by preparative TLC with $CHCl_3$, $CHCl_3$:ethyl acetate (16:0.5), hexane:ethyl acetate (2:1) and hexane:acetone (4:1), respectively, and the resulting product was crystallized using hexane-acetone to yield an additional amounts of compound 5 (44.8 mg).

• *Compounds 7:* Fraction I (100.6 mg) was purified by preparative TLC with hexane:acetone (4:1) and hexane:ether:acetone (4:12:1). The resulting band (R_f 0.6) was eluted with $CHCl_3$ and the residue was crystallized to yield compound 7 (33.5 mg, mp 138–139 °C). NMR and CD spectral data of compound 7 showed that it was identical to (+)-methysticin (Shao et al., 1998; Dutta and Ray, 1972).

• *CD Spectral analyses of compounds 1, 4, 5 and 7:* Compounds 1, 4, 5 and 7 for CD spectral analysis were dissolved in MeOH at 0.25, 3.4, 0.3 and 0.6 mg/ml, respectively, and measured at 20 °C. The CD spectra of compounds 1, 4, 5 and 7 showed a positive Cotton effect at 262.9, 200.6, 263.6 and 332 nm with CD values of 42.5, 0.64, 12.13 and 1.14 mdeg, respectively.

Results

The kava kava roots purchased from Meetex Fiji, Ltd., were a mixture of fibrous and tap roots. The purification of the ethyl acetate extract using various chromatographic techniques yielded 9.7, 3.2, 1.5, 3.2, 3.5, 4.9 and 2.3% of dihydrokawain, demethoxyangonin, flavokawain A, kawain, dihydromethysticin, yangonin and methysticin, respectively.

Both COX-I and COX-II enzymes were used in the bioassay-directed isolation of potential anti-inflammatory compounds present in kava kava roots. Positive controls used for cyclooxygenase inhibitory assays were ibuprofen, aspirin and naproxen and were dissolved in DMSO at 2.1, 180 and 2.5 µg/ml, respectively. In the COX-I inhibitory assay, ibuprofen, naproxen and aspirin showed 25, 32 and 27% inhibition, respec-

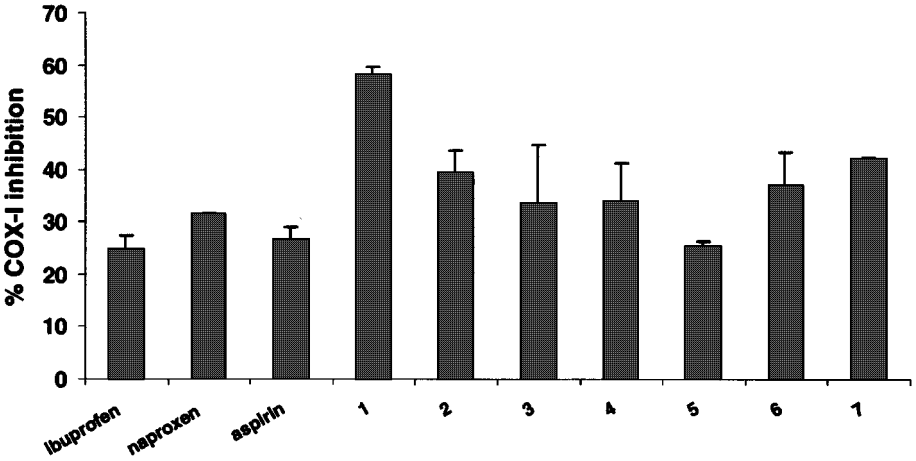

Fig. 2. Percentage COX-I inhibition of compounds from kava kava roots at 100 µg/ml. 1 = dihydrokawain; 2 = demethoxyangonin; 3 = flavokawain A; 4 = kawain; 5 = dihydromethysticin; 6 = yangonin; 7 = methysticin. Ibuprofen, aspirin and naproxen were tested at 2.1, 180 and 2.5 µg/ml, respectively. Vertical bars represent the standard deviation of each data point (n = 2).

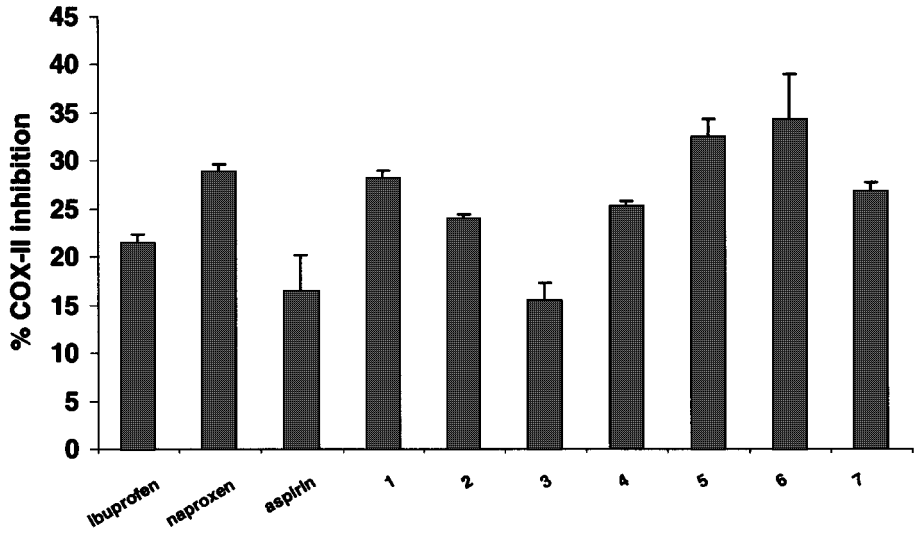

Fig. 3. Percentage COX-II inhibition of compounds from kava kava roots at 100 µg/ml. 1 = dihydrokawain; 2 = demethoxyangonin; 3 = flavokawain A; 4 = kawain; 5 = dihydromethysticin; 6 = yangonin; 7 = methysticin. Ibuprofen, aspirin and naproxen were tested at 2.1, 180 and 2.5 µg/ml, respectively. Vertical bars represent the standard deviation of each data point (n = 2).

tively. Ibuprofen, naproxen and aspirin showed 21, 28 and 16% inhibition, respectively, in COX-II inhibitory assay. The concentrations of each test compound assayed were 100, 50, 25 and 12.5 µg/ml at pH 7.0 for both COX-I and -II inhibitory assays. The percentage inhibition of cyclooxygenase activities for each compound at 100 µg/ml is shown in Figures 2 and 3. Compounds 1–7 showed 58, 39, 33, 34, 25, 36 and 42% inhibition, respectively, in the COX-I inhibitory assay. Similarly, they showed 28, 23, 15, 25, 32, 34 and 26% inhibition, respectively, in the COX-II inhibitory assay.

The lipid oxidation assay did not show antioxidant activities for compounds 1, 2, 4, 5 and 7. The addition of compound 3 to the lipid oxidation assay mixture made the solution opaque and hence the fluorescent data of the solution could not be determined by fluorescence spectroscopy. Compound 6 also precipitated when added to the buffer containing the liposome. However, compounds 6 and 7 showed free radical-scavenging capacity against stable DPPH radicals (Figures 4 and 5). Compounds 6 and 7 showed 64.7 and 64.3% of DPPH• radical scavenging activities at 2.5 mg/ml, compared to 14.3, 8.3 and 31.7% DPPH• radical scavenging activities for vitamin E, vitamin C and BHT at 10, 25 and 50 µM, respectively. The kinetics of yangonin(6)-DPPH and methysticin(7)-DPPH reactions showed an activity profile similar to compounds 6 and 7 at 2.5 mg/ml.

Discussion

In the COX-I assay, compound 1 at 100 µg/ml showed 58% inhibition and was the most active among the kava kava compounds tested. Compound 2 at all four concentrations tested showed better cyclooxygenase enzyme inhibition than ibuprofen, aspirin and naproxen at 2.1, 180 and 2.5 ppm, respectively. All kava kava compounds tested at 100 or 50 µg/ml demonstrated

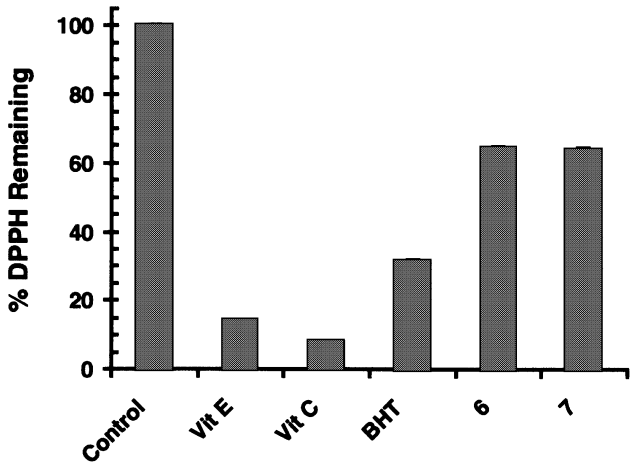

Fig. 4. Comparison of DPPH• radical scavenging activities of compounds 6 and 7 with vitamin E, vitamin C, and BHT. Vit E = vitamin E, Vit C = vitamin C, BHT = butylated hydroxytoluene, Control = solvent control containing no antioxidant. The concentrations of antioxidants in final reaction mixtures were 2.5 and 2.5 mg/ml, 10 µM, 25 µM, and 50 µM for yangonin and methysticin, vitamin E, vitamin C and BHT, respectively. Vertical bars represent the standard deviation of each data point (n = 3).

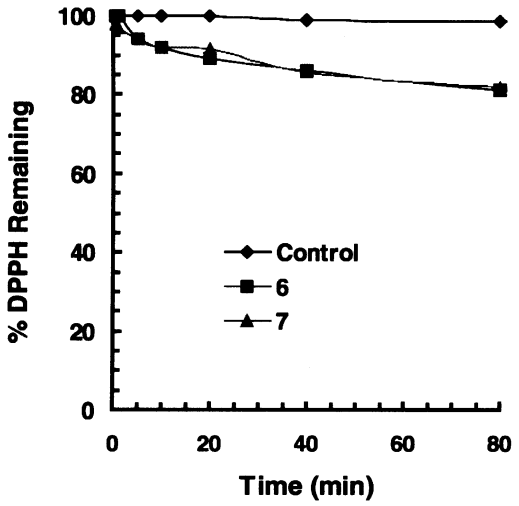

Fig. 5. Kinetics of Yangonin(6)-DPPH• and Methysticin(7)-DPPH• reactions. Control = solvent with no antioxidant. The concentration in final reaction mixtures of compounds 6 and 7 was 2.5 mg/ml.

better or similar COX-I inhibition activities as compared to ibuprofen, aspirin and naproxen. Compounds 1, 2 and 7 displayed 48, 43 and 37% COX-I inhibition, respectively, at 50 µg/ml, better than positive controls. Compounds 1, 2, 4 and 7 at 25 µg/ml concentration showed excellent COX-I inhibitory activities at 34, 41, 21 and 24%, respectively.

The COX-II inhibitory assay exhibited good enzyme inhibition for kava kava compounds 1–7 when tested at 100 and 50 µg/ml. Compound 6 showed the highest percentage inhibition, as shown in Figure 2. Compounds 1, 4 and 5 at 25 µg/ml also showed good COX-II enzyme inhibition, comparable to ibuprofen, aspirin and naproxen at 16, 13 and 20%, respectively.

The antioxidant activities of compounds 3 and 6 in the lipid oxidation assay could not be evaluated due to their poor solubility in DMSO and in the buffer containing the liposome reacted with the fluorescence probe. Compounds 1, 2, 4, 5 and 7 did not have solubility problems but were not active. However, compounds 6 and 7 showed moderate antioxidant activity when compared to vitamin E, vitamin C and BHT in the free radical scavenging assay. The kinetics of yangonin(6)-DPPH and methysticin(7)-DPPH reactions showed an increase in scavenging activities for compounds 6 and 7 with longer reaction times.

The findings of our study provide some scientific basis for the traditional use of kava kava roots for managing inflammatory pains. The compounds isolated from the ethyl acetate extract of kava kava roots exhibited both COX-I and COX-II enzyme inhibitory activities and may therefore be responsible for the folkloric use of kava kava root preparation to alleviate arthritic and gout-related pain. This is the first report of the cyclooxygenase inhibitory activities of dihydrokawain, demethoxyangonin, flavokawain A, kawain, dihydromethysticin, yangonin and methysticin isolated from kava kava roots.

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