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Effect of Extraction Method on the Concentrations of Selected Bioactive Compounds in Mandarin Juice

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A mandarin-type citrus fruit, ponkan (*Citrus reticulata*), was processed by in-line, chopper pulper, and hand-press extractions to investigate the effect of extraction method on the concentrations of bioactive compounds in processed juice. Concentrations of polymethoxylated flavones (tangeretin, nobiletin, and sinensetin) and β-cryptoxanthin in juice, and inhibitory activities against arachidonate cyclooxygenase and lipoxygenases of the juice extract were analyzed. The juice processed by hand-press extraction contained the largest amounts of nobiletin (3.56 mg/100 mL), tangeretin (4.10 mg/100 mL), and sinensetin (0.13 mg/100 mL). Concentrations of β-cryptoxanthin were 0.66, 0.59, 0.55, and 0.50 mg/100 mL in chopper pulper, in-line (5/64 in.), in-line (8/64 in.) and hand-press juices, respectively. Both extracts of in-line juices showed greater inhibitory activity toward platelet 12-lipoxygenase than the others. The inhibitory effect of hand-press juice extract on platelet cyclooxygenase activity was remarkable among juice extracts. All juice extracts effectively inhibited polymorphonuclear 5-lipoxygenase activity at nearly the same rate.

KEYWORDS: Mandarin; ponkan; *Citrus reticulata*; β-cryptoxanthin; polymethoxylated flavone; lipoxygenase; cyclooxygenase

INTRODUCTION

Recently, the production of citrus fruits has been consistently decreasing in Japan. An attempt to identify bioactive phytochemicals in fruits, and then add value in terms of biological activity such as anti-cancer and anti-inflammatory activities, has been made to reverse this trend (*1*, *2*). Ponkan (*Citrus reticulata*) is a mandarin-type fruit belonging to the Citrus-Metacitrus-Acrumen group according to Tanaka's classification (*3*). The yearly production of ponkan in Japan is ∼12000 tons, 20% of which is processed for juice. This fruit contains a wide variety of bioactive compounds such as polymethoxylated flavones, β-cryptoxanthin, and arachidonate cyclooxygenase (COX), and lipoxygenase (LOX) inhibitors. A polymethoxylated flavone, tangeretin, is reported to have suppressive effects on malignant tumor invasion and metastasis (*4*). The effect was explained through correction of the defective function of E-cadherin, a calcium-dependent cell−cell adhesion molecule, and inhibition of invasion by tumor cells. In addition, by examining the morphological features of apoptotic cells and DNA fragmentation, Hirano et al. (*5*) demonstrated that tangeretin efficiently induces apoptosis in HL-60 cells. Nobiletin was reported to effectively down-regulate the production of pro-matrix metalloproteinase and PGE_2 and interfere with the proliferation of synovial fibroblasts, suggesting that it may serve as a drug to maintain articular cartilage and decrease pannus formation in rheumatoid arthritis and osteoarthritis (*6*). Furthermore, nobiletin, tangeretin, and sinensetin showed differentiation-inducing activity toward mouse myeloid leukemia cells (M1), with cells having phagocytic activity (*7*). According to a quantitative analysis of citrus flavonoids in the dried edible parts of fruits, the concentration of tangeretin (9.1 mg/100 g) in ponkan was highest and that of nobiletin (12.8 mg/100 g) second highest among the 66 citrus species investigated (*8*). The presence of sinensetin in the peel of ponkan was reported (*7*), and its concentration was determined as 6.6 mg/100 g in our experiment. β-Cryptoxanthin has been found in citrus plants such as oranges and mandarins (*9*, *10*), and mandarins generally contain it in larger quantities than oranges (*11*). In an in vitro screening experiment for anti-tumor promoters by Tsushima et al. (*12*), β-cryptoxanthin showed one of the strongest inhibitory effects among 51 carotenoids examined, superior to the well-known anti-tumor promoter, β-carotene. Also, β-cryptoxanthin was reported to suppress the incidence of *N*-methylnitrosourea-induced colon carcinogenesis in F344 rats (*13*). The authors suggested that dietary β-cryptoxanthin affects colon carcinogenesis after accumulating in the colonic mucosa, due to absorption from the colon as well as small intestine. Regarding

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the inhibition of arachidonate LOX, species belonging to the Citrus-Metacitrus-Acrumen group, including ponkan, showed a strong inhibitory effect among 45 species tested (*14*). Metabolites in the arachidonic acid cascade play important roles in humans. The COX-mediated production of thromboxane A_2 (TXA_2), an active form of TXB_2, induces platelet aggregation (*15*), and the LOX reaction is also involved in atherosclerotic processes and platelet aggregation (*16*, *17*). 5-LOX catalyzes the initial step in the production of 5-hydroxy-6,8,10,14-eicosatetraenoic acid (5-HETE) and leukotrienes, which mediate allergic and inflammatory responses (*18*, *19*). 12-LOX catalyzes the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), which is involved in atherosclerotic processes (*20*) and tumor metastasis (*21*). Therefore, a specific inhibitor of LOX and COX should be useful as a therapeutic drug for treating or preventing these diseases. So far, 4-*O*-feruloyl-5-*O*-caffeoylquinic acid and its methyl ester derivative have been isolated from ponkan peel as potent inhibitors of LOX (*22*). As mentioned above, ponkan could be thought of as one of the most effective citrus fruits as a source of bioactive compounds among *Citrus* species; however, in many cases, bioactive compounds exist in the peel at a higher concentration than in any other fruit tissue. This situation restricts the exploitation of valuable compounds in citrus juice production, which is the most common form of processing.

There are several methods of processing juice. In-line (IL) extraction is widely used because of its simplicity in quality control and labor-saving advantage. It extracts small amounts of peel compounds because it squeezes peel as peel plugs together with inner tissues in an orifice tube. Chopper pulper (CP) juice contains larger amounts of juice sac-derived components than IL juice but does not extract peel. On the other hand, the juice processed by a pressure extractor such as a hand-press (HP) contains large amounts of compounds from the outer peel (flavedo) such as monoterpene hydrocarbons, aldehydes, alcohols in essential oil (*23*), and coumarins (*24*). Many of these compounds make the juice rich in flavor. As a consequence, each processing method may have its own characteristics in terms of the concentration of bioactive compounds as well as juice quality. This investigation was designed to study the effect of extraction method on the concentrations of polymethoxylated flavones and β-cryptoxanthin in processed juice, the inhibitory effect of juice extracts on platelet COX and 12-LOX, and polymorphonuclear 5-LOX activities using ponkan fruit.

MATERIALS AND METHODS

Materials. The ponkan fruits (*C. reticulata*) used were obtained in mid-January from a commercial grower in Uwajima, Ehime. They were fully ripe and firm. [1-^{14}C]Arachidonic acid (57 mCi/mmol) was obtained from Dupont/NEN (Boston, MA). Authentic 5-HETE was obtained from Cascade Biochem Ltd. (Reading, U.K.). Tangeretin, sinensetin, and β-cryptoxanthin were purchased from Extrasynthese (Genay, France), and nobiletin was from Wako Chemicals (Osaka, Japan). Precoated silica gel sheets were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

Juice Preparation. The processing of juice samples is outlined in **Figure 1**. In the preparation of IL juice, an in-line 291 extractor (FMC Co., Lakeland, FL) with a cup size of $2^3/_8$ in. was used, and two sizes of peel clearance (5/64 and 8/64 in.) were investigated. After extraction, each extract was filtered through a 0.5 mm screen, centrifuged at 5400*g* for 25 s, and pasteurized (90 °C, 30 s). In CP extraction, the peel was scalded (90 °C, 2 min), peeled off, and extracted using a CP-180 extractor (Seikensha Co., Tokyo, Japan). The extract was filtered first through a 1.0 mm and then through a 0.5 mm screen, centrifuged, and pasteurized. In the HP method, fruit was cut into halves, extracted by hand-press extractor (ITO Co., Tokyo, Japan), filtrated through a 1.0 mm screen, and pasteurized. After pasteurization, juice samples were cooled and kept frozen (−20 °C).

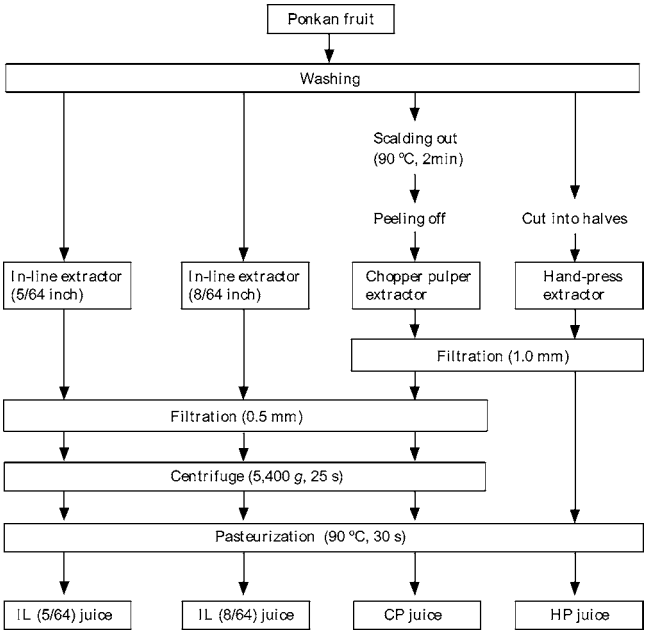

Figure 1. Processing of juice samples from ponkan (*C. reticulata*) fruit.

Analysis of Polymethoxylated Flavones. HPLC samples were prepared by homogenizing the processed juice with a mixer. The homogenate (5 mL) was centrifuged at 3000*g* for 10 min. The supernatant was recovered, and the precipitate was extracted three times with 0.5 mL of methanol/DMSO (1:1, v/v). The combined extract was diluted with water so that the concentration of water amounted to ~90%. To remove polar compounds in the extract, a Sep-Pak C_{18} cartridge (0.36 g) was used. The cartridge was conditioned with 2 mL of methanol followed by 5 mL of water. The extract was transferred onto the cartridge and washed with 5 mL of water, and then the polymethoxylated flavones were eluted with 4 mL of methanol/DMSO (1:1, v/v). After the volume was raised to 5 mL, solutions were filtered through membrane filters (0.5 μm) prior to injection. HPLC analysis of polymethoxylated flavones was performed as described previously (*25*). The HPLC system consisted of a Shimadzu (Kyoto, Japan) liquid chromatography system equipped with LC-10AD pumps, a model SPD-M10AVP photodiode array detector, a model SIL-10AXL auto sampler, and a model CBM-10A communications bus module. A 250 × 4.0 mm i.d., 5 μm, LiChrospher 100 RP-18 analytical column (Merck) was used with acetonitrile/water (40:60, v/v) as the eluent. The detector monitored the eluent at 285 nm and spectra from 200 to 360 nm. The flow rate was 0.6 mL/min. The column was operated at 40 °C. The sample injection volume was 10 μL. Identifications of nobiletin, tangeretin, and sinensetin (**Figure 2**) were made by comparing their retention times and UV spectra with those of standards.

***β*-Cryptoxanthin Preparation.** A 10 g sample of juice was mixed with 1 g of Celite 545 and 10 mL of ethanol, and the mixture was filtered through a medium-porosity Büchner funnel under vacuum. The residue in the Büchner funnel was further extracted with 50 mL of ethanol by mixing with a glass rod. The extract was transferred to a separatory funnel and 60 mL each of diethyl ether and water added. After 30 s of thorough shaking, the aqueous solvent phase was drained off after 5 min. Saponification was carried out according to the method of Goodner et al. (*11*) with some modification. The diethyl ether extract was concentrated to dryness using a rotary evaporator, redissolved in 5 mL of diethyl ether, and placed in a 12-mL culture tube to which was added 5 mL of 10% methanolic KOH. The tube was gently blanketed with nitrogen, then closed, and placed in the dark for 1 h at room temperature. The sample was transferred to a separatory funnel to which were added 20 mL of diethyl ether and 100 mL of distilled water. The diethyl ether layer was washed until free of alkali, and the aqueous phases were drained off. The diethyl ether layer was dried with anhydrous sodium sulfate, filtered through filter paper, and

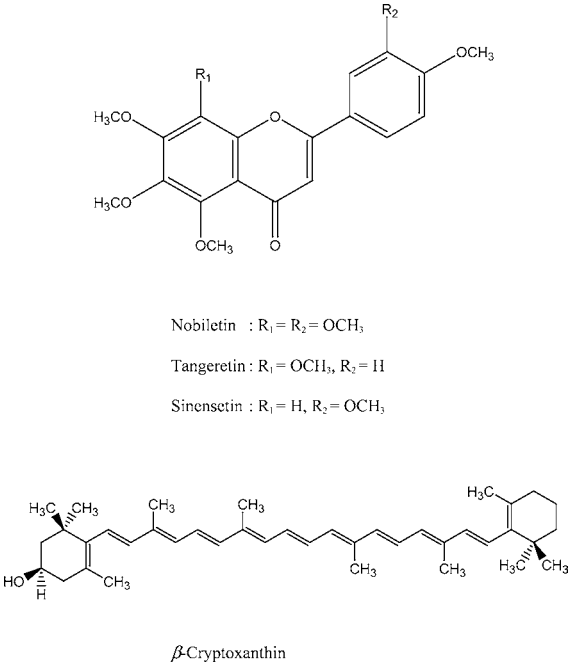

Figure 2. Chemical structures of polymethoxylated flavones and β-cryptoxanthin.

concentrated at low temperature in a rotary evaporator to dryness. The residue was dissolved in 10 mL of HPLC solvent, which consisted of methanol/methyl *tert*-butyl ether (MTBE) (92:8, v/v) and 0.1% ammonium acetate, and filtered through a 0.45 μm membrane filter.

Analysis of β-Cryptoxanthin. β-Cryptoxanthin (**Figure 2**) was analyzed by reversed-phase high-performance liquid chromatography using a stationary mobile phase. Chromatography was carried out with a Shimadzu liquid chromatography system equipped with a model LC-10AD pump, a model SPD-10AV UV−vis detector, a model SIL-10AXL autosampler, and a CBM-10A communications bus module. The mobile phase consisted of methanol/MTBE (92:8, v/v) and 0.1% ammonium acetate. A 150 × 4.6 mm i.d., 5 μm, YMC Carotenoid S5 column (Waters, Milford, MA) including a 23 × 4 mm i.d., 5 μm, guard column was used and kept at 28 °C. The injection volume was 20 μL, and the detection was at 450 nm.

Preparation of Juice Extract for Arachidonate Enzyme Inhibition. Portions (50 mL) of processed juice samples were homogenized by a mixer, and the homogenate was centrifuged at 3000*g* for 10 min. The supernatant was recovered and the solid residue extracted three times with 2 mL of methanol/DMSO (1:1, v/v). The combined extract for each sample was passed through a Sep-Pak C_{18} cartridge (5 g) that had been preconditioned with methanol followed by water. The eluate was discarded, and the cartridge was washed with 30 mL of water. The retained compounds were eluted with 50 mL of methanol and evaporated to dryness. The dried compounds were suspended in a small amount of water, freeze-dried, and stored at −80 °C until use.

Assay for Platelet COX and 12-LOX Activity. Rat platelets were prepared from the blood of Wister−King rats (200−300 g), which were fed normally, as previously described (*26*), and assay reaction conditions were those for the method described by Sekiya et al. (*27*). Briefly, test compounds were incubated with the sonicated platelets (2 mg of protein/mL) for 5 min at 37 °C before the addition of [1-^{14}C]arachidonic acid (4 μM). After metabolites were separated by TLC, the radioactivities of 12-HETE and TXB_2 were measured using a Fujix BAS 1000 (Fuji Photo Film) system.

Assay of PMNL 5-LOX Activity. Peritoneal polymorphonuclear leukocytes (PMNL) were prepared from male Wister rats injected intraperitoneally with 5% glycogen (20 mL/kg). The washed cells were homogenized and centrifuged (100000*g*) at 4 °C for 1 h. The supernatant was used as the crude enzyme (2 mg of protein/mL). Potassium phosphate buffer (50 mM, pH 7.4) was used as the solvent in the reaction. The testing solution (20 μL) was preincubated with 130 μL of the enzyme solution containing 3 mM $CaCl_2$ and 2 mM ATP at 37 °C for 5 min, and then [1-^{14}C]arachidonic acid was added. After incubation for 5 min, the products were subjected to thin-layer chromatography and developed with ether/petroleum ether/acetic acid (50:50:1, v/v) at 4 °C (*28*). The radioactive metabolites were quantified as described above, and 5-LOX activity was measured on the basis of the formation of 5-HETE.

Table 1. Concentrations of Polymethoxylated Flavones in Ponkan (*C. reticulata*) Fruit Tissues[a]

tissue	nobiletin	tangeretin	sinensetin
flavedo	197.8 (3.4)	217.9 (3.7)	11.6 (0.3)
albedo	34.2 (1.9)	41.5 (1.5)	2.2 (0.2)
segment membrane	3.3 (0.2)	2.7 (0.1)	tr
juice sacs	nd	nd	nd
whole fruit	30.3 (1.8)	33.8 (1.8)	1.8 (0.2)

[a] Values are in mg/100 g of fresh weight. Numbers in parentheses are standard deviations. tr, trace amount; nd, not detected.

Table 2. Concentrations of Polymethoxylated Flavones in Processed Juices[a]

extraction method	nobiletin	tangeretin	sinensetin
IL (5/64)	0.6 (0.1)	0.7 (0.1)	tr
IL (8/64)	0.6 (0.1)	0.7 (0.1)	tr
CP	nd	nd	nd
HP	3.6 (0.3)	4.1 (0.4)	0.1

[a] Values are in mg/100 mL. Numbers in parentheses are standard deviations. tr, trace amount; nd, not detected.

RESULTS AND DISCUSSION

The concentrations of polymethoxylated flavones in each ponkan tissue were investigated to assess the efficiency of the extraction methods. A small amount of sinensetin was found in the fruit in addition to the principal two polymethoxylated flavones, nobiletin and tangeretin. The distribution of these compounds in each tissue is shown in **Table 1**. All polymethoxylated flavones were exclusively detected in the flavedo. The weight composition of the flavedo, albedo, segment membrane, and juice sacs of a fruit amounted to 12.6, 14.3, 13.1, and 58.1%, respectively. From this, flavedo contained nobiletin, tangeretin, and sinensetin at 82.4, 81.3, and 82.5%, respectively. Albedo, the white inner layer of the fruit peel, also contained polymethoxylated flavones at 16−18% of the total amount. On the other hand, the concentration of polymethoxylated flavones in segment membrane was <1.5% for each compound and zero in juice sacs. Therefore, the extraction efficiency of flavedo should significantly affect the concentrations of polymethoxylated flavones in juice. As shown in **Table 2**, the concentration of polymethoxylated flavones in HP juice was outstanding compared with other juices: 3.56, 4.10, and 0.13 mg/100 mL for nobiletin, tangeretin, and sinensetin, respectively. This means 11.8, 12.2, and 7.3% of the nobiletin, tangeretin, and sinensetin in a fruit were extracted into the juice. It was reported that the recovery of hesperidin in both Valencia and Hamlin oranges increased with increasing extraction pressure when a hand-press extractor was used (*29*). Although extraction pressure was not defined in HP extraction, a higher pressure may result in higher recoveries of polymethoxylated flavones. On the other hand, concentrations of polymethoxylated flavones were low in IL juices compared to HP juice and zero in CP juice. There was little difference in the concentration of

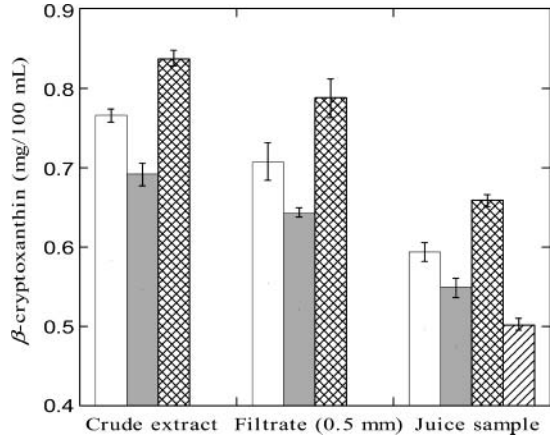

Figure 3. β-Cryptoxanthin content of crude extracts, filtrates (0.5 mm screen), and final juice products of ponkan (*C. reticulata*) fruit: (white bar) IL (5/64); (gray bar) IL (8/64); (cross-hatched bar) CP; (slashed bar) HP.

polymethoxylated flavones between IL (5/64) and IL (8/64) juices, and both contained about 2.0% nobiletin and 2.0% tangeretin of a fruit. From these results, HP extraction is the most effective method for the fortification of polymethoxylated flavones in juice, whereas CP extraction is not suited to this purpose.

In ponkan fruit, β-cryptoxanthin is distributed in the flavedo and juice sacs. The fruit used in this study contained β-cryptoxanthin at 6.3, 1.2, and 1.6 mg/100 g in flavedo, inner tissues (segment membrane and juice sacs), and fruit, respectively. The approximate distribution of β-cryptoxanthin was 54% in the flavedo and 46% in inner tissues. In **Figure 3**, concentrations of β-cryptoxanthin in juices are given for each processing step. CP juice had the highest concentration (0.66 mg/100 mL), followed by IL (5/64) (0.59 mg/100 mL), IL (8/64) (0.55 mg/100 mL), and HP (0.50 mg/100 mL) juices in descending order. The high concentration of β-cryptoxanthin in CP juice was attributed to the effective extraction of juice sacs. It is not clear from which part (flavedo or juice sacs) the β-cryptoxanthin in HP juice is mainly extracted, but the extraction efficiency is low in either case. The higher concentration of IL (5/64) than IL (8/64) was presumably due to the difference in extraction efficiency from juice sacs because this method primarily extracts inner tissues. β-Cryptoxanthin in flavedo did not seem to be effectively extracted with the methods used in this study. The concentrations of β-cryptoxanthin in centrifugate were 77.5, 79.3, and 78.6% of those of crude extract for IL (5/64), IL (8/64), and CP juice, respectively. The loss of β-cryptoxanthin was greater at the centrifugation step (14.5−16.5%) than at the filtration step (6−7.5%). This was the same tendency as in the pulp and oil removal.

The dose-dependent curves of juice extracts for COX activity are shown in **Figure 4A**. The HP extract inhibited COX activity most strongly and retained the effect up to 100 μg/mL. Other extracts had a weaker effect, each showing ∼30% inhibition at 1000 μg/mL, but only slight (IL extracts) or no (CP extract) inhibition at 100 μg/mL. These results suggest that the majority of the inhibitory compounds are derived from the outer peel. In the screening experiment of COX inhibition by vegetable extracts, all materials having inhibitory activity were recovered into the ether layer of a butanol/ether partition system except that extracted from naked barley (*30*). On the assumption that the inhibitory compounds in ponkan are analogous to those in vegetables, they should occur in oil glands in flavedo. As to

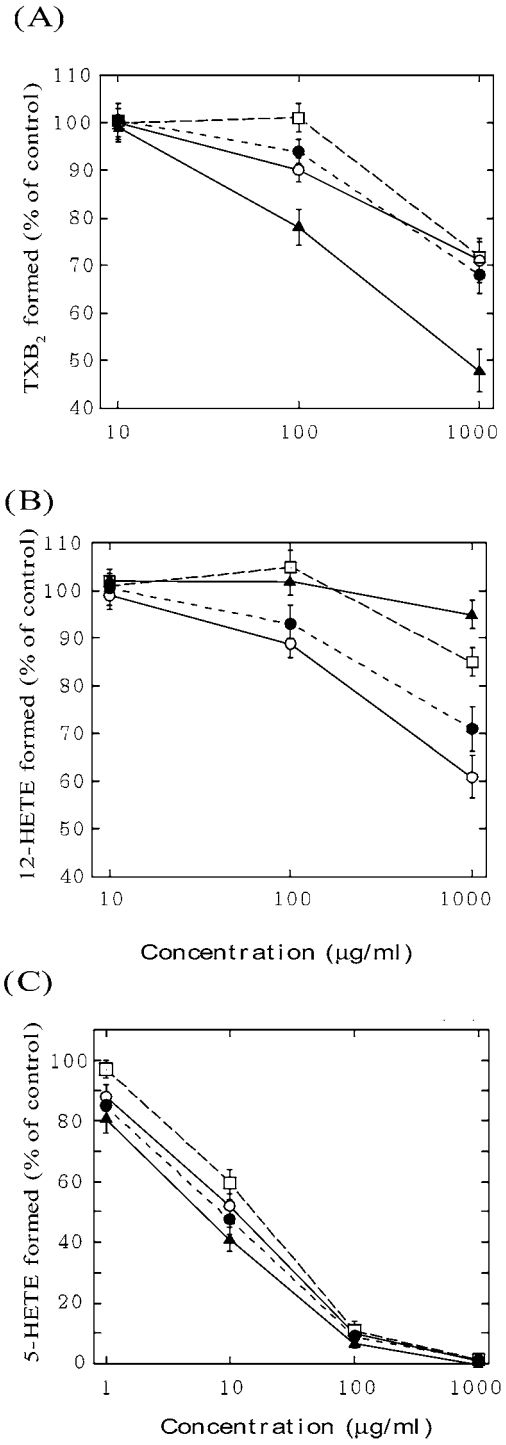

Figure 4. Dose–response curves of the extract of processed juices for (A) cyclooxygenase and (B) 12-lipoxygenase in platelets and for (C) 5-lipoxygenase in PMNL: (○) IL (5/64); (●) IL (8/64); (□) CP; (▲) HP. Platelet cyclooxygenase activity was assayed by measuring the formation of TXB_2 from [1-^{14}C]arachidonic acid. Platelet 12-lipoxygenase activity was assayed by measuring the formation of 12-HETE from [1-^{14}C]arachidonic acid. PMNL 5-lipoxygenase activity was assayed by measuring the formation of 5-HETE from [1-^{14}C]arachidonic acid. Values are the mean ± SE of four replications.

12-LOX inhibition, both IL extracts inhibited the activity, showing 39% (5/64) and 29% (8/64) inhibition at 1000 μg/mL, but the effect of CP was only 15% and that of HP extract was negligible (**Figure 4B**). IL (5/64) extract inhibited a 12-LOX activity slightly greater than than that of the IL (8/64) extract,

which may result from the higher pressure of IL (5/64) in the extraction process as was the case with hesperidin extraction (*29*). Chlorogenic acid derivatives isolated as 12-LOX inhibitors from ponkan fruit were water-soluble and equally distributed in the flavedo and albedo (*14*). Therefore, IL extraction may be effective for the extraction of compounds such as these distributed in whole peel. Concerning 5-LOX inhibition, the 12-LOX inhibitor, 4-*O*-feruloyl-5-*O*-caffeoylquinic acid, is active toward 5-LOX ($IC_{50} = 0.7\ \mu M$) in addition to 12-LOX ($IC_{50} = 0.6\ \mu M$). This implied that IL juice extracts inhibit 5-LOX activity more than CP or HP juice extracts do; however, all extracts had approximately the same dose-dependent curves (**Figure 4C**). Taking into consideration these results, other potent inhibitors, especially of 5-LOX, were suggested to exist in both peel and inner tissues.

In conclusion, we have investigated the most effective squeezing method to fortify selected bioactive compounds in ponkan juice. HP extraction was effective for the extraction of polymethoxylated flavones and inhibitors of COX. This result suggested that HP extraction is effective for the extraction of compounds which occur in the flavedo, especially in oil glands. Conversely, the CP method was most effective for the fortification of β-cryptoxanthin in juice sacs. The IL method extracted both polymethoxylated flavones and β-cryptoxanthin with moderate efficiency compared with the HP or CP method. This method was considered to be suitable for extracting compounds from whole fruit. In general, IL (5/64) was more effective than IL (8/64) for extraction of the compounds investigated. It is not clear if the results of this study are specific for ponkan or apply to other citrus fruits as well because the content and physical properties of peel, segment membrane, and juice sacs differ with citrus species. In this experiment, a general method of preparation after extraction for each squeezing method was adopted. Filtration and centrifugation procedures could be optimized for a certain compound.

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