Food and Chemical Toxicology 48 (2010) 306–313

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Effects of *Areca catechu* L. containing procyanidins on cyclooxygenase-2 expression *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 6 July 2009
Accepted 11 October 2009

Keywords:
Areca catechu L.
Procyanidin
COX-2
Antioxidant

ABSTRACT

Polyphenols are widely distributed in plants and known for antioxidant and anti-inflammatory properties. Areca nut, rich in polyphenols, is the major component of betel quid and we have previously shown that the extract of areca nut can induce oxidative stress *in vitro*. In this study, we have further pinpointed that areca nut extract (ANE) contains catechin based procyanidins which range from dimers to decamers and polymers; this was carried out by HPLC and electrospray ionization/mass spectrometry (ESI/MS). To quantify their antioxidant potential, oligomeric and polymeric procyanidins of ANE were separated and evaluated using the Trolox equivalent antioxidant capacity (TEAC) assay. The results clearly demonstrated that the antioxidant capacity of the ANE procyanidins increased with the degree of polymerization. The anti-inflammatory potential of ANE was also tested using 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated human oral cancer SAS cells. ANE inhibited TPA-induced cyclooxygenase-2 (COX-2) protein expression at low doses, which correlated with the inhibition of ERK phosphorylation in the SAS cells. Furthermore, feeding rats with ANE at 1 and 10 mg/kg/day for 5 days significantly repressed carrageenan-induced inflammatory exudates and PGE_2 formation. In conclusion, ANE, which contains catechins based oligomeric and polymeric procyanidins, regulates COX-2 expression *in vitro* and possess anti-inflammatory potential *in vivo*.

1. Introduction

In plant kingdom, procyanidins are widely distributed polyphenolic compounds and are known with their pharmacological effects. Procyanidins provide natural defense from pathogens and herbivores due to their high protein-binding affinity, which also is responsible for the astringency they induce in food. In the nomenclature, procyanidins are categorized as a proanthocyanidin (condensed tannin), which are conjugation products with two or more flavan-3-ol subunits, catechin and epicatechin (Fig. 1). Generally, most procyanidins are conjugated with C_4–C_8 or C_4–C_6 single-bond linkages (B type) and a few are conjugated with C_4–C_8/C_2–O_7 double-bond linkages (A type) (Xie and Dixon, 2005). The different linkage types, subunits and chain length of the proanthocyanidins give rise to variable structures that offer different biological activities (Pierini et al., 2008).

Procyanidins have excellent antioxidant and anti-inflammatory capacity against various types of oxidative stress-induced damage. Grape seed procyanidins have been found to protect LLC-PK_1 tubule cells from high glucose-induced oxidative stress and cytotoxicity (Fujii et al., 2006). In addition, procyanidins have also been reported to induce cellular catalase, superoxide dismutase (SOD) and glutathione *S*-transferase (GST) activity and protect cardiac cells from oxidative stress (Du et al., 2007). One previous study has indicated that, in Raw 264.7 macrophages, procyanidins inhibit interferon-γ plus lipopolysaccharide (LPS)-induced inflammatory effects, including nitric oxide production, the induction of nitric oxide synthase (iNOS) protein expression and prostaglandin E_2 (PGE_2) synthesis (Terra et al., 2007). Therefore, the antioxidant and anti-inflammatory capacity of procyanidins might be based on an ability to reduce oxidative stress and upregulate endogenous antioxidant enzymes.

In Taiwan and South-Eastern Asia, betel quid (BQ) chewing has been associated with the development of oral squamous cell carcinoma (OSCC) through epidemiological studies. BQ, which comprises areca nut, lime and *Piper betle* inflorescence or leaf, has been classified as a human carcinogen by the IARC (2004). According to the *in vitro* results of a chemiluminescence assay, areca nut extract (ANE) reacts with the lime and this generates reactive oxy-

Abbreviations: ANE, areca nut extract; BQ, betel quid; COX-2, cyclooxygenase-2; DP, degree of polymerization; EGCG, epigallocatechin-3-gallate; TEAC, trolox equivalent antioxidant capacity; TPA, 12-*O*-tetradecanoylphorbol-13-acetate..

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doi:10.1016/j.fct.2009.10.014

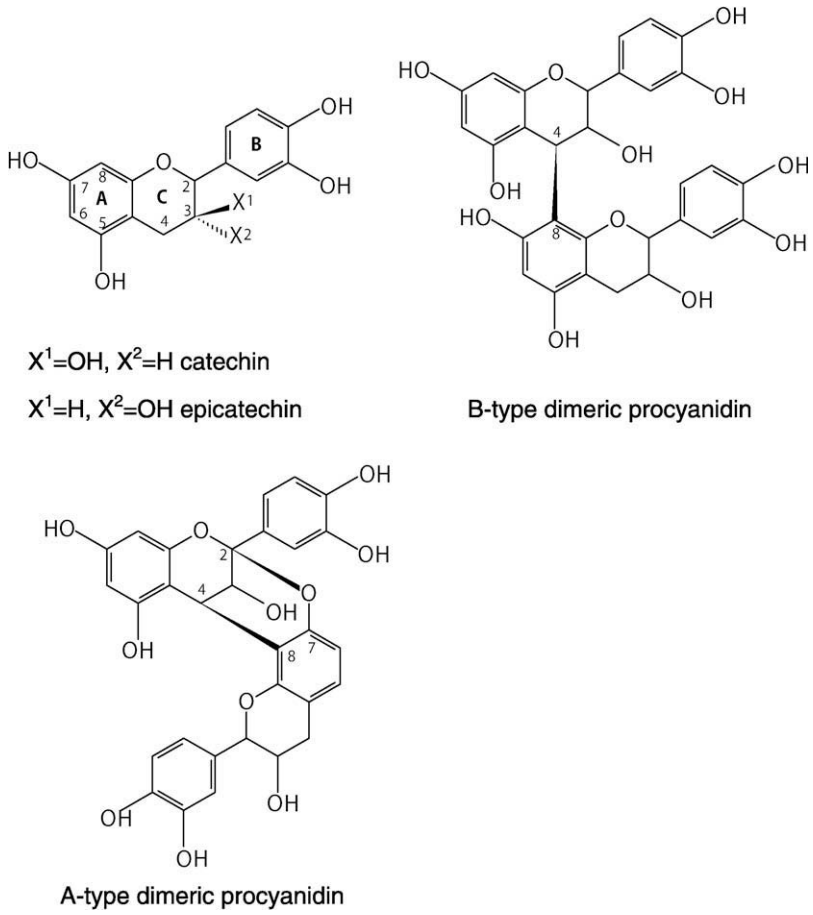

Fig. 1. Structures of catechins and dimeric procyanidins.

gen species (ROS) including superoxide anion radicals and hydrogen peroxide. Continuous painting of such prepared ANE combined with lime on hamster cheek pouches for 5 days significantly increased the frequency of micronucleate cells formation compared to the controls (Nair et al., 1992). We have also demonstrated that continuous painting of ANE for 14 days significantly induces the oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in hamster check pouches (Chen et al., 2002). The above evidence indicates that BQ induced oral carcinogenesis might correlate with areca nut containing polyphenol generated oxidative stress under alkaline condition.

It has long been known that areca nut contains dimeric, trimeric and tetrameric procyanidins (Nonaka et al., 1981). However, the exact nature of the procyanidins in areca nut is still unclear. Moreover, procyanidins are known to have antioxidant and anti-inflammatory potential, and yet ANE induces oxidative stress *in vitro* and *in vivo*. In this study, we have isolated procyanidins from ANE and evaluated their antioxidant capacity using fractionated procyanidins with different degrees of polymerization (DP). We also tested the anti-inflammatory effects of ANE on TPA-induced COX-2 expression in oral cancer cells and studied its anti-inflammatory responses *in vivo*.

2. Materials and methods

2.1. Chemical

Catechin, epicatechin, epigallocatechin-3-gallate (EGCG), 2,2′-azino (bis-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dimethyl sulfoxide (DMSO), TPA, PD98059, indomethacin and λ-carrageenan were purchased from Sigma (St. Louis, MO). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchased from Aldrich (Steinheim, Germany). Potassium persulfate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The HPLC grade solvents were purchased from J.T. Baker (Phillipsburg, NJ).

2.2. Preparation of areca nut extract

The extraction was modified from our previous study (Liu et al., 1996). Fresh tender areca nuts, the fruit of *Areca catechu* L., were purchased from local shops in Taipei, Taiwan. After removing the husks, the nuts were extracted three times with 80% acetone (1:10 w/v). The acetone was then removed using a vacuum evaporator (Büchi RE111, Switzerland) under reduced pressure. The aqueous residue was subsequently partitioned with *n*-hexane and ethyl ether to remove lipid materials. After lyophilizing the aqueous layer, the resulting light-yellow powder was used as ANE and kept at −20°C under argon.

2.3. Separation and purification ANE procyanidins by normal-phase HPLC

ANE, dissolved in 30% methanol, was separated using an Agilent 1100 HPLC system with Luna silica 250 × 4.6 mm column (Phenomenex, Torrance.CA) according to the published method (Gu et al., 2002). The mobile phase consisted of (A) dichloromethane: methanol: 50% acetic acid = 82: 14: 4 and (B) methanol: 50% acetic acid = 96:4. The gradient was programmed at: 0–20 min, 0–12% B;

20–50 min, 12–26% B; 50–55 min, 26–88% B; 55–65 min, 88% B; 65–70 min, 88–0% B. The flow rate was 1 mL/min and monitored at 280 nm with UV detector. Each fraction was collected according to the UV signal with an Agilent fraction collector.

2.4. ESI/mass analysis

ANE (1 mg/mL in 30% methanol) was analyzed using a LichroCART C_{18} 250 × 4.6 mm column (Merck & Co., Inc. Germany). The mobile phase consisted of (A) 0.1% acetic acid and (B) 0.1% acetic acid in acetonitrile. The gradient was as follows: 0–8 min, 8% B; 8–40 min, 8–24% B; 40–50 min, 24–80% B; 50–55 min, 80–8% B and the flow rate was 1 mL/min (Gu et al., 2002). All the quantitative and qualitative analyses were conducted using an Agilent 1100 HPLC system. MS analysis were carried out using a LCQ Deca XP plus (Thermo Electron, San Jose, CA) with electrospray ionization (ESI) in the negative ion mode under the following optimized conditions: source voltage, 5.2 kV; capillary voltage, −36 V; capillary temperature, 300 °C; nitrogen shear gas flow, 60 arb (arbitrary units); nitrogen auxiliary gas flow, 10 arb. Full scan MS spectra (m/z 200–2000) were first recorded during the chromatographic run and the pseudomolecular ions of each catechins and procyanidins were identified. MS–MS spectra were recorded using a data dependent scan. The collision-induced-dissociation energy (CID) required in this process was set to 35%. The dataset was collected and analyzed with Xcalibur software.

2.5. Thiolysis of procyanidins

The fractionated procyanidins were dried with Maxi Dry Plus vacuum concentrator (Heto, Wettenberg, Germany) and subjected to thiolysis according to the published method (Gu et al., 2002). In brief, 50 μL of fractionated ANE (2 mg/mL in 30% methanol) reacted with 50 μL HCl acidified methanol (3.3% HCl, v/v), and 100 μL benzyl mercaptan (5% v/v in methanol) at 40 °C for 30 min then was stored at −20 °C until analysis. Each sample was analyzed using a LichroCART C_{18} column with the same conditions as used in the ESI/MS analysis, and detected at 280 nm with UV detector. The mean DP (mDP) in each fraction was calculated using the equation: total area of catechin benzylthioether and epicatechin benzlthioether/total area of catechin and epicatechin + 1.

2.6. Total antioxidant potential determination

The $ABTS^{\cdot+}$ stock solution was generated from 7 mM ABTS reacting with 2.45 mM potassium persulfate for 16 h in the dark at room temperature. ANE oligomeric procyanidin fractions and polyphenolic antioxidants were dissolved in DMSO. Each polyphenolic compound (3 μL, with series dilution) was reacted with ddH_2O diluted $ABTS^{\cdot+}$ solution (0.3 mL, 0.1 mM) for 6 min and detected absorbance at 734 nm with a SpectraMax M5 spectrophotometer (Molecular Devices, USA). The reduced absorbance of the $ABTS^{\cdot+}$ solution was recalibrated against a Trolox standard curve and expressed as a TEAC value (mM of Trolox/mM polyphenolic compound) (Re et al., 1999).

2.7. Cell culture and treatment

Oral cavity squamous cancer SAS cells were maintained and subcultured as described previously (Lin et al., 2005). The SAS cells were deprived of serum for 18 h before all treatments. After 1 h treatment with ANE, the cells were treated with 10 nM TPA for another 30 min to evaluate phosphorylation of ERK, JNK and Akt proteins. The same procedure, but after pretreatment with PD98059 for 1 h or not before the TPA treatment for 4 h, was used to evaluate COX-2 protein expression.

2.8. Western blot analysis

Proteins were harvested and lysed with M-PER lysis buffer (Pierce, Rockford, IL). The protein concentration was then determined using a protein assay kit (BioRad Laboratories, CA). In total, 50 μg of protein was resolved on a 10% SDS–PAGE gel and then transferred onto a nitrocellulose membrane (PerkinElmer, MA). These nitrocellulose membranes were blocked with non-fat milk and then incubated with primary antibodies overnight at 4 °C. Primary antibodies against COX-2, p-ERK (Thr202 and Tyr204), ERK, p-Akt (Ser473), Akt, p-JNK (Thr183 and Tyr185) and JNK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibody against actin and secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody were purchased from Chemicon (Temecula, CA). The signals were detected using Western Lightening chemiluminescence reagent (PerkinElmer, MA). Signal intensities were measured with NIH Image-J software and normalized against actin as the protein loading control. All experiments were repeated at least three times independently.

2.9. Air pouch model

Seven weeks old male Sprague Dawley rats were obtained from BioLASCO Co. (Taiwan) and housed under standard condition (12:12 h light/dark cycle) with food and water *ad libitum*. All animals were randomly divided into 5 groups: control (3 animals), carrageenan alone (5 animals), ANE 1 mg/kg with carrageenan (5 animals), ANE 10 mg/kg with carrageenan (4 animals), and indomethacin with carrageenan (5 animals, as a positive control group). All procedures for the animal experiments were approved by the institutional animal care and use committee of Taipei Veteran General Hospital, Taipei, Taiwan. After one week for adaptation, the rats received ANE (1 and 10 mg/kg/day) and saline (control groups) intragastrically for 5 days. On the 2nd day, all animals received 0.2 μm filtrated 20 mL air subcutaneously along the dorsal surface to form an air pouch; they were re-injected with 10 mL filtered air on the 4th day to maintain the pouch (Kuo et al., 2004). On the 5th day, indomethacin (2 mg/kg, i.p.) was given 1 h before carrageenan (1% in saline, 2 mL) was injected into the pouches of all treatment groups; the control group was injected with saline (2 mL). After 6 h stimulation with carrageenan, the animals were sacrificed and the pouch exudates were harvested. All collected exudates had indomethacin added at a final concentration of 10 μM to stop PGE_2 formation. The PGE_2 level in the exudates was measured using a PGE_2 EIA kit (Cayman Chemical Co., MI) and normalized against the protein concentration.

2.10. Statistical analysis

All results were expressed as mean ± standard deviation from at least three independent experiments. Student's-t-test was used to determine the statistical significance between the means of two groups. Pearson's correlation was used to determine the correlation between two parameters in the TEAC assay. In this study, p values less than 0.05 are considered to be statistically significant.

3. Results

3.1. Characterization of procyanidins in ANE

The areca nut procyanidins were analyzed by normal-phase HPLC and characterized with ESI/MS. After separating ANE with a

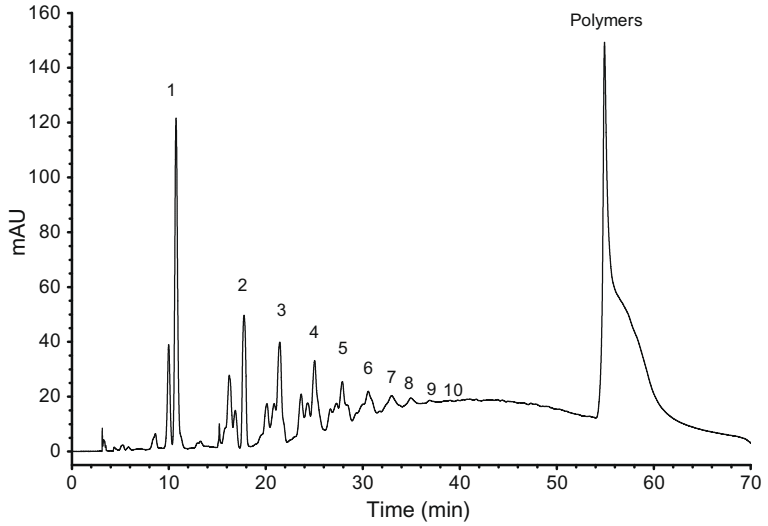

Fig. 2. Chromatographic distribution of ANE procyanidins in normal-phase HPLC. ANE was separated according to DP and detected by a UV detector. The labeled peaks in the chromatograph show the DP of the procyanidins. mAU, mili Absorbance unit.

well documented method, each group of peaks was sequentially eluted depending on the DP (Gu et al., 2002). Peaks designated from 1 to 10 are monomers (catechin and epicatechin, designated as 1), oligomeric procyanidins (2–10 as dimers to decamers) and polymeric procyanidins (hendecamers and above) (Fig. 2). The chromatographic distribution of the monomeric fraction was confirmed with authentic standards, catechin and epicatechin. The mDP was verified with thiolysis, and the mDP of the dimeric fraction, of the polymeric fraction and of ANE were shown to be 2.0 ± 0.1, 17.5 ± 0.4 and 8.7 ± 0.5, respectively.

To confirm the polyphenolic composition, ANE was separated and analyzed with HPLC–ESI/MS. The total ion spectrum of ANE exhibited $[M-H]^-$ ions at *m*/*z* 289, 577, 865, 1153, 1441, 1729 and $[M-2H]^{2-}$ ions (designated with *) at *m*/*z* 576, 720, 864, 1008, 1152, 1296, 1440 in the negative mode (Fig. 3a). The $[M-H]^-$ ions increased from *m*/*z* 289 to 1729, which is indicative of B-type procyanidins from monomers to hexamers. The higher oligomeric procyanidins ($\geqslant$ tetramers) tend to lose more than one proton and form ions with multiple charges during electrospray ionization (Guyot et al., 1997). The ions at *m*/*z* 576, 720, 864, 1008, 1152, 1296 and 1440 corresponded to doubly charged tetramers, pentamers, hexamers, heptamers, octamers, nonamers and decamers. All identified ions from monomeric to decameric procyanidins are summarized in Table 1. The monomeric to hexameric procyanidins of ANE were further identified by extracted-ion mass chromatograms (Fig. 3b) and each oligomers was found to consist of several isomers. For example, after extracting monomeric procyanidins at *m*/*z* 289, two peaks at 14.2 and 21.0 min were identified as catechin and epicatechin using authentic standards.

3.2. Antioxidant capacity of ANE

To study whether DP will influence the antioxidant capacity of procyanidins, ANE was separated into oligomeric and polymeric procyanidin fractions with normal phase-HPLC. All purified procyanidins fractions were evaluated for their antioxidant scavenging capacity using stable $ABTS^{\cdot+}$ radical and equated with Trolox to give TEAC values. The molecular weights of each fraction are proportional to their DP, therefore, the monomers to decamers, polymers and ANE were set as having molecular weights of 290, 578, 866, 1154, 1442, 1730, 2018, 2308, 2594, 2882, 5186 and 2594, respectively. Using this approach, the TEAC values of other polyphenolic antioxidants from ANE (catechin, epigallocatechin-3-gallate and quercetin), and the monomeric fraction, which contains catechins, were equivalent to the valid standard catechin. Interestingly, the higher DP fractions exhibited proportionately higher TEAC values, meaning that they had relatively greater antioxidant capacity (Fig. 4). The correlation between the TEAC values and the DP of the oligomeric and polymeric fractions were analyzed by Pearson's analysis, and the correlation coefficients demonstrated the DP was highly correlates with the antioxidant capacity ($p = 0.0036$).

3.3. Effect of ANE on COX-2 expression

We investigated the relationship of ANE and the inflammatory responses in SAS cells as assessed by COX-2 and related ERK, Akt and JNK expression. TPA, a COX-2 protein inducer and skin tumorgenesis promoter, induced COX-2 protein expression in a dose- and time-dependent manner (Fig. 5a). Pretreatment with ANE for 1 h modulated 10 nM TPA-induced COX-2 protein expressions in two different ways. As shown in Fig. 5b, pretreatment with a lower concentrations of ANE (0.1 and 1 μg/mL) abrogated TPA-induced COX-2 protein expression in the SAS cells, but this was absent at higher concentrations (2 and 5 μg/mL). Furthermore, ANE alone induced COX-2 expression at higher concentrations (2 and 5 μg/mL) in SAS cells. The COX-2 expression of each treatment was normalized against actin and this is shown in Fig. 5c. We next examined the effect of the above treatments on the proteins that might be involved. Specifically, TPA-induced ERK phosphorylation was significantly inhibited by ANE pretreatment (Fig. 6a). However, Akt and JNK in the SAS cells were not phosphorylated in the presence of ANE or TPA (Fig. 6a and b). The correlation between TPA-induced COX-2 protein expression and ERK phosphorylation were explored using PD98059 (ERK inhibitor). Pretreatment with a series of increasing concentrations of PD98059 for 1 h suppressed TPA-induced COX-2 protein expression (Fig. 6c). Therefore, ANE attenuated TPA-induce COX-2 expression through suppressing ERK phosphorylation at 0.1 and 1 μg/mL, but this effect was absent at higher concentrations.

3.4. ANE inhibited inflammation in vivo

The anti-inflammatory potential of ANE was estimated using a carrageenan-treated acute inflammation animal model. There was no exudates formation after injecting 2 mL saline into the

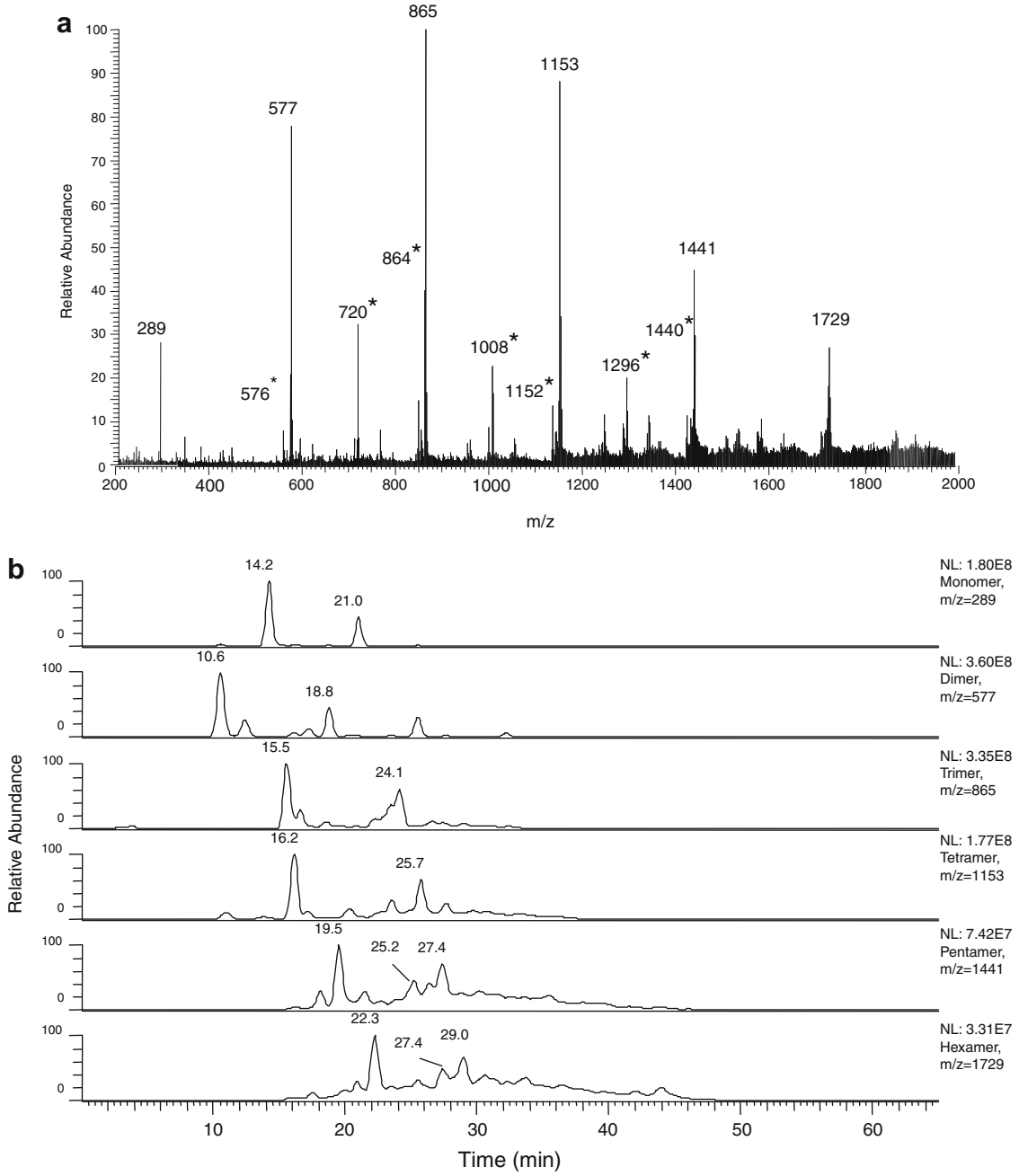

Fig. 3. ESI/mass profiles of ANE procyanidins: (a) total ion spectrum and (b) extracted ion chromatography of monomeric to hexameric procyanidin from *m/z* 289 to 1729. *, doublely charged ion.

prepared rat air pouch, and approximate 3 mL exudates was induced by carrageenan. Pretreating with 10 mg/kg/day ANE for 5 days reduced the exudate volume by 30%. The exudates volume also decreased after pretreating with indomethacin (2 mg/kg) (Fig. 7a). In addition, one major enzymatic product of the COX-2 protein, PGE_2, was measured to evaluate the level of inflammation. In the ANE pretreatment groups, 1 and 10 mg/kg ANE reduced PGE_2 formation about 20% and 70%, respectively (Fig. 7b). As the result of the reduction in exudate volume, indomethacin further inhibited PGE_2 formation significantly (Fig. 7b) .

Table 1
Identification of procyanidin oligomers by ESI/MS in negative mode.

Procyanidins	$[M-H^-]^-$	$[M-2H^-]^{-2}$
Monomers	289	
Dimers	577	
Trimers	865	
Tetramers	1153	576
Pentamers	1441	720
Hexamers	1729	864
Heptamers		1008
Octamers		1152
Nonamers		1296
Decamers		1440

4. Discussion

The results of this study have demonstrated that polyphenols in areca nut are classified as catechin based B-type procyanidins. The ESI/MS data (Fig. 3) also indicates that procyanidins in areca nut lacking the gallate moiety, which is consistent with a recent

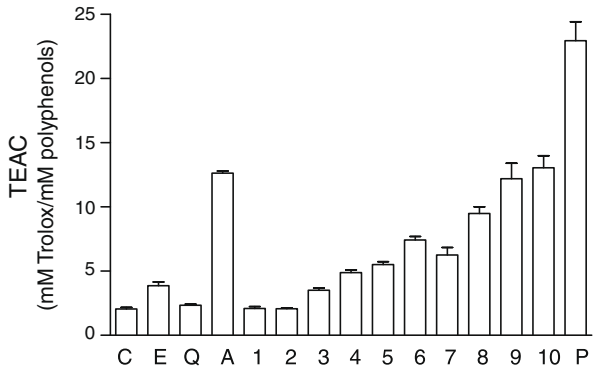

Fig. 4. The relative potency of ANE and polyphenolic antioxidants in terms of TEAC values. ANE (A), ANE oligomeric (1–10, monomers to decamers) and polymeric (P) procyanidins fractions and polyphenolic antioxidants (catechin, C; epigallocatechin-3-gallate, E; quercetin, Q) were analyzed using a series of concentrations and recalibrated against a Trolox standard curve to give an equivalent in terms of mM Trolox/mM polyphenols. All results are presented as the mean of three independent determinations ± SD (standard deviation).

publication (Wu et al., 2007). The polymeric procyanidins fractions (>10 mer) constitute 37% of ANE with a mDP at 17.5, which is higher than that in grape seed (6.4–7.3), cocoa (13.9) and brown sorghum bran (13.5), but less than grape skin (33.8–85.7) and lowbush blueberry (38.8) (Gu et al., 2002; Monagas et al., 2003). According to our TEAC results, the antioxidant capacity of procyanidins is highly correlated with the DP (Fig. 4). Nevertheless, Steinberg et al. (2002) speculated that oligomeric procyanidins that prolong LDL oxidation do not show an association with DP if assessed in terms of monomeric catechin units. Our results showed the same pattern when the TEAC values were converted into equal weights rather than molar concentrations (data not shown). Interestingly, when assayed by equivalent weight, the higher DP apple procyanidins have been found to be more potent than lower ones when inhibiting pancreatic lipase activity *in vivo* (Sugiyama et al., 2007) and inducing oesophageal carcinoma cells apoptosis (Pierini et al., 2008). This difference may be a result of the interaction between the procyanidins and the reacting species. Generally, procyanidins retard lipid oxidation by chelating metals, trapping radicals and reacting with oxidants. When procyanidins interact with lipophilic oxidants (2,2′-azobis (2,4-dimethylvaleronitrile), AMVN) or the polar head groups of phospholipids bilayers, the higher DP procyanidins seem to have a better antioxidant capacity than the lower ones (Lotito et al., 2000; Verstraeten et al., 2003). In the TEAC assay, each molecule of procyanidins donates hydrogen to reduce the $ABTS^{\cdot+}$ radical cation, and the higher DP procyanidins offer more potent radical scavenging capacity. Therefore, the above evidence indicates that the DP of procyanidins affects their specific role in terms of their antioxidant and biological activities.

Our previous study showed that ANE has pro-oxidative properties, as shown by an increase in the level of the oxidative stress marker 8-OH-dG in CHO-K1 cells (Liu et al., 1996). Recently, ANE has been shown to induce COX-2 expression through the activation of the mitogen-activated protein kinase (MAPK) and NF-κB signal transduction pathways (Lin et al., 2005). This study also proved that high dose (5 μg/ml) ANE-induced COX-2 expression correlated with NF-κB translocation (data not shown). However, the results of this study indicate that ANE blocked TPA-induced COX-2 expression and ERK phosphorylation at low doses (0.1 and 1 μg/ml) but fails to do this at higher doses (Fig. 6). The results with low ANE doses on TPA-induced ERK and COX-2 expression in SAS cells are consistent with those for cocoa procyanidins in JB6 P^{+} mouse epidermal cells (Kang et al., 2008). Accumulating evidence suggests that inhibition of COX-2 activity exerts its anti-tumor promoting effects due to a blocking of the transcriptional activities of AP-1

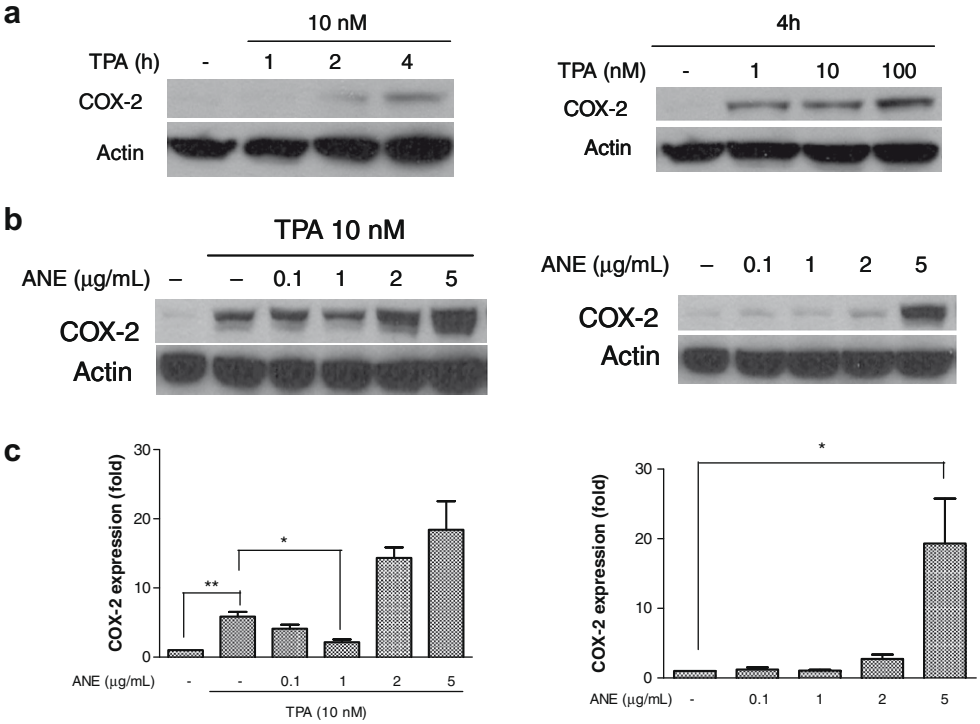

Fig. 5. Effects of ANE on TPA-induced COX-2 expression in SAS cells: (a) TPA induced COX-2 protein expression as a function of time and dose and (b) ANE modulated TPA-induced COX-2 expression. SAS cells were pretreated with ANE for 1 h and then exposed to 10 nM TPA for 4 h or with ANE alone for 5 h at 37 °C. (c) Quantified COX-2 expression was normalized against actin and is presented as the fold of induction as compared to the control using the mean of five independent determinations ± SD (*, $p < 0.05$, **, $p < 0.001$).

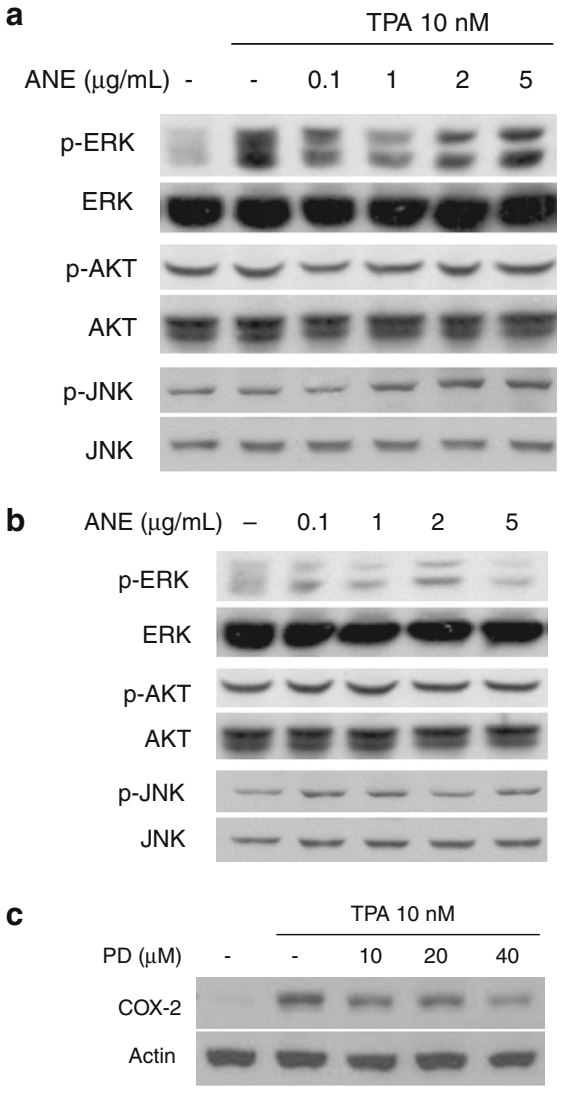

Fig. 6. Effects of ANE on TPA-induced ERK, Akt and JNK expression in SAS cells: (a) SAS cells were pretreated with different concentrations of ANE for 1 h then treated with 10 nM TPA. Thirty minutes later, the cells were harvested and ERK, Akt and JNK phosphorylation were detected using immunoblot analysis, (b) effects of ANE alone on ERK, Akt and JNK expression in SAS cells and (c) pretreatment with series of PD98059 concentrations for 1 h, which down-regulated TPA-induced COX-2 expression.

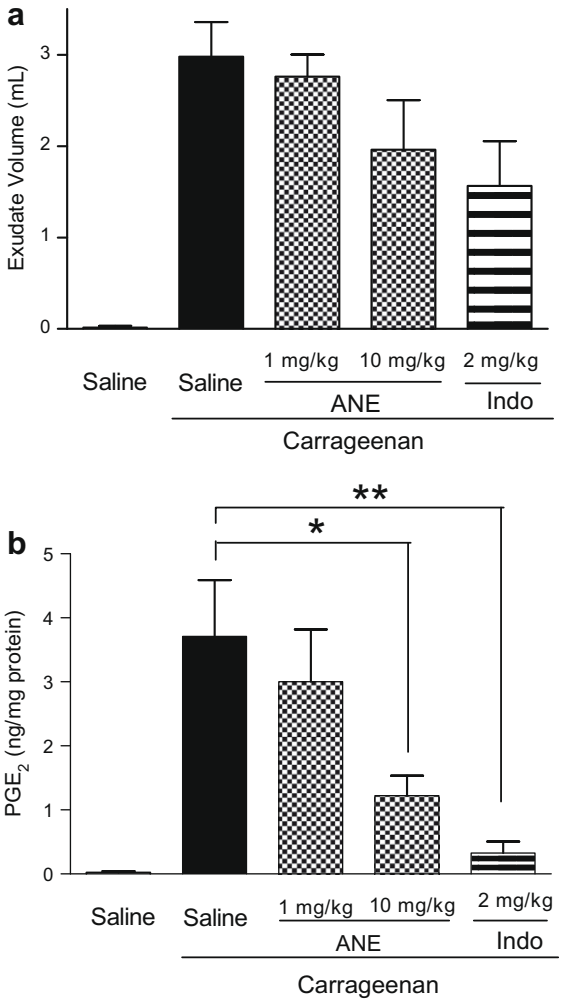

Fig. 7. ANE treatment blocked carrageenan-induced inflammatory response in the rat air pouch model: (a) ANE pretreatment reduced the amount of carrageenan-induced exudate. Rats were treated with ANE at 1 and 10 mg/kg (p.o.) for 5 days, and the air pouch exudates (mL) were measured 6 h after carrageenan challenge. Indomethacin (Indo) served as positive control and was given 1 h before carrageenan stimulation and (b) ANE treatment diminished carrageenan-induced PGE_2 formation (ng/mg protein). (*, $p < 0.05$, **, $p < 0.001$).

and NF-κB (Kang et al., 2008). The dual effects of ANE on COX-2 expression in this report are similar to the effects of EGCG on human lymphocytes; EGCG induces DNA strand breakage at higher concentrations, but protected lymphocytes from bleomycin induced damage at lower concentrations (Kanadzu et al., 2006). The anti-oxidant/pro-oxidant effects of ANE and EGCG may be interpreted as being characteristics of polyphenols. Due to the structures associated free radical quenching reactivity, polyphenols are easily and rapidly auto-oxidized to form phenolic dimers or epimers and this can result in hydrogen peroxide formation (Sang et al., 2005). Thus, low doses of polyphenols that generated some peroxide might tip the redox balance and induce the antioxidant defense system, which consequently will protect the cells from further lethal damages. This is considered to be the cellular redox adaptive response, which comes about by regulation of the phase II enzymes though a transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf-2) that activates antioxidant–response elements (ARE). However, when an overload of peroxide is presented, this actives NF-κB and induces the inflammatory cytokines (Rahman et al., 2006). In conclusion, the pro-oxidant/anti-oxidant roles of ANE are dependent on the concentration of ANE and show a biphasic dose–response relationship.

The anti-inflammatory potential of ANE was further investigated with carrageenan-induced acute inflammation in SD rats. The carrageenan-induced acute inflammatory animal model provides a pleural cavity for exudates accumulation. After carrageenan irritating the subpleural tissues, the secreting inflammatory related cytokines and mobilized cells (neutrophils and polymorphonuclear cells) were accumulated in the exudates. Quantify the contents of exudates proving useful information of chemicals on inflammatory effects (Vinegar et al., 1982). Continuously feeding 10 mg/kg/day ANE for five days prevented carrageenan-induced inflammatory exudates formation. This should be compared with a previous study where Wistar rats were intraperitoneal injected with 30 mg/kg *Ribes nigrum* L. proanthocyanidins and this inhibited carrageenan-induced polymorphonuclear cells infiltrate and cytokines release (Garbacki et al., 2004). This shows that intake of proanthocyanidins either intraperitoneal or intragastical is effective at reducing the carrageenan-induced acute inflammatory responses in rats. Nevertheless, the complex structures and huge molecular weights of procyanidins may affect their metabolism

and bioavailability. Deprez et al. (2000) found that polymeric procyanidins are degraded into the low-molecular weight phenolic acids, 3-(*m*-hydroxyl)-propionic acid, by human colonic microflora *ex vivo*. In normal human ileostomy fluid, the mDP of procyanidin was degraded from 5.7 to 3.4 (Kahle et al., 2007). In an isolated rat jejunum, catechin and epicatechin were glucuronidated, *O*-methylated and *O*-methyl-glucuronidated through catecho-*O*-methyl transferase (COMT) and metabolized into 3′-and 4′-methyl epicatechin; both of these chemicals are good inhibitors of the NADPH oxidase in HUVEC cells (Kuhnle et al., 2000; Steffen et al., 2008). 3,4-Dihydroxyphenylacetic acid, a metabolite of epicatechin that is also found in gut microflora, suppressed endogenous α-tocopherol consumption and conjugated diene hydroperoxide formation in a rat plasma oxidation assay (Raneva et al., 2001). Even so, the non-specific protein-binding affinity and large molecular sizes of the procyanidins limit absorption and permeability in cultured cells. In intestinal epithelial Caco-2 cells, oligomeric procyanidins (DP > 3) find it difficult to permeate the cell membrane compared with monomers or dimers (Deprez et al., 2001). However, procyanidins maintain cellular membrane integrity and cell viability during bile acid-induced damage due to their strong protein-binding affinity, which occurs in a multidentate fashion manner (Baxter et al., 1997; Erlejman et al., 2006). Taken together, cellular absorption and adsorption of procyanidins is an important issue and needs to be explored in future studies. Higher DP procyanidins may not be easily absorbed by cultured cells; nonetheless they seem to be digested into smaller fragments and metabolized in the mammalian gastrointestinal tract and have various biological activities.

In this study, the procyanidins of areca nut are classified into catechins based procyanidins with 8.7 polymerization units on average. Based on the antioxidant and anti-inflammatory potential of ANE, areca nut could be alternative source of procyanidins in further investigations. The pro-oxidant properties of ANE also provide information on the cause and effect of oral carcinogenesis among BQ chewers. Therefore, an exploration of the pharmacological and nutritional roles of areca nut is very important.

Conflict of Interest

None.

Acknowledgments

This study was supported by grants from the National Science Council (NSC95-2320-B-010-070-MY2) and Taipei Veterans General Hospital (V97C1-128).

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