RESEARCH ARTICLE

Dicaffeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells

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Scope: The biological functions of caffeoylquinic acid (CQA) derivatives from various plant sources have been partially elucidated. The objectives were to isolate and purify diCQAs from Yerba mate tea leaves and assess their anti-inflammatory and anti-cancer capabilities in vitro and explore their mechanism of action.

Methods and results: Methanol extracts of dried mate leaves were resolved by flash chromatography and further purified resulting in two fractions one containing 3,4- and 3,5-diCQAs and the other 4,5-diCQA with NMR-confirmed structures. Both fractions inhibited LPS-induced RAW 264.7 macrophage inflammation by suppressing nitric oxide/inducible nitric oxide and prostaglandin E_2/cyclooxygenase-2 pathways through inhibiting nucleus translocation of Nuclear factor κB subunits, p50 and p65. The diCQA fractions inhibited Human colon cancer cells CRL-2577 (RKO) and HT-29 cell proliferation by inducing apoptosis in a time- and concentration-dependent manner, but did not affect the protein levels of p21, p27, p53, and Bax:Bcl-2 ratio in RKO cells. In HT-29 cells, however, the diCQA fractions increased Bax:Bcl-2 ratio. The diCQA fractions increased the activation of caspase-8 leading to cleavage of caspase-3 in both RKO and HT-29 colon cancer cells.

Conclusion: The results suggest that diCQAs in Yerba mate could be potential anti-cancer agents and could mitigate other diseases also associated with inflammation.

Keywords:
Apoptosis / Colon cancer / Dicaffeoylquinic acids / Inflammation / Yerba mate

1 Introduction

Caffeoylquinic acid (CQA) derivatives are a class of polyphenolic compounds widely distributed in plants, such as coffee [1], garland (*Chrysanthemum coronarium* L.) [2], and Yerba mate (*Ilex paraguariensis*) tea [3]. The structures of CQAs are composed of one or more caffeic acid molecules connected to one quinic acid molecule through ester bonds (Fig. 1). The potential biological functions of CQA derivatives from various plant sources have been partially elucidated by several investigators, including antioxidant capacity [4–6], antiviral effect [7–9], anti HIV effects [7, 8], neuroprotective [10, 11], and antimutagenic activity [12]. Yerba mate tea has demonstrated potent antioxidant [13–17], anti-inflammatory effects [18–20], chemopreventive effect against mammary [21], and colon carcinogenesis [13, 21]. However, the exact mechanisms of action and the bioactive constituents involved in the protective effects of mate tea against carcinogenesis are not clearly understood. Chloro-

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Abbreviations: CDK, cyclin-dependent kinase; **COSY,** Correlation spectroscopy; **COX-2,** cyclooxygenase-2; **CRC,** colorectal cancer; **CQA,** caffeoylquinic acid; **HMBC,** heteronuclear multiple bond coherence; **iNOS,** inducible nitric oxide; **NF-κB,** nuclear factor κB; **NO,** nitric oxide; **NOE,** Nuclear Overhauser effect; **RKO,** Human colon cancer cells CRL-2577

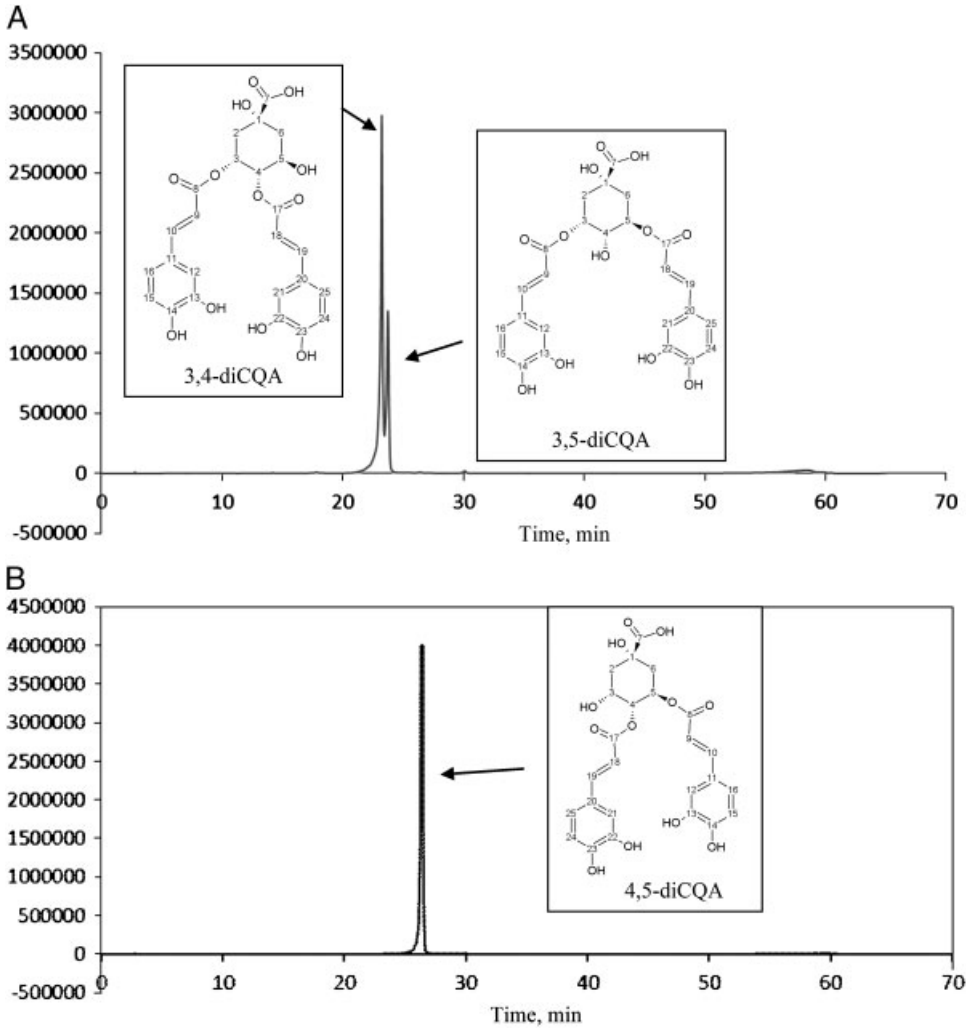

Figure 1. Analytical HPLC chromatograms of (A) 3,4- and 3,5-diCQAs, (B) 4,5-diCQA.

genic acid, 3,4-diCQA, 3,5-diCQAs and 4,5-diCQA are the major CQA derivatives in mate tea [22]. Little is known about the relationship between the biological functions of diCQAs in Yerba mate tea leaves because of the difficulty isolating chemically similar diCQAs and the lack of commercial standards.

Colorectal cancer (CRC) was the third most common cause of cancer deaths in the United States in 2010 [23]. Studies have shown that the risk of developing CRC can be prevented by diet [24]. Chronic inflammation can lead to the development of several types of cancer [25–27] and is considered as a potential risk factor for CRC [28]. Because chronic inflammation generates the production of nitric oxide (NO), a substrate for formation of reactive oxygen species, it stimulates the production of inflammatory cytokines leading to DNA damage and triggers the steps to cancer progression [27]. Nuclear factor κB (NF-κB) is a transcription factor and essential component link between inflammation and cancer. In innate immune pre-neoplastic and malignant cells, NF-κB upregulates the expression of inflammatory cytokines and enzymes including cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS), which are important for the synthesis of inflammatory mediators (prostaglandin E_2; PGE_2 and NO, respectively). The activation of NF-κB in different cancer cell types has been shown to regulate the expression of genes involved in cell growth and proliferation, anti-apoptosis, angiogenesis, and metastasis [29]. Suppressing chronic inflammation by targeting NF-κB has the potential to modulate the risk of developing cancer [30, 31]. Uncontrolled proliferation and inhibition of apoptosis is a hallmark characteristic of all tumor development; hence the control of cell proliferation and induction of apoptosis are targets for therapeutic intervention in all types of cancers [32].

The objectives of this study were to purify the major diCQAs from Yerba mate tea leaves and to evaluate their anti-inflammatory and anti-colon cancer capacities in vitro, to further elucidate their mechanism of action.

2 Materials and methods

2.1 Chemicals

Methanol (99.9%) and acetonitrile were purchased from Fisher Scientific (Hanover Park, IL). The standard compounds chlorogenic acid (≥95%), quinic acid (≥95%), and caffeic acid (≥97%) were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2 Extraction of the diCQAs from mate dried leaves

The diCQAs were extracted and fractionated from mate (*Ilex paraguariensis*) leaves organically grown in Paraguay, following a modification of the general phenolic purification method of Berhow et al. [33]. Briefly, 1 kg of powdered Yerba mate dry leaves was extracted with methanol in a Soxhlet extractor for 72 h. The extracts were reduced by roto-evaporation (at 40°C), then pooled and concentrated by evaporation under the hood (25°C). The dried material was re-suspended in methanol for preparative flash chromatography on a Buchi (Newcastle, DE) Sepacore flash chromatography system with dual C-605 pump modules, a C-615 pump manager, a C-660 fraction collector, and a C-635 UV photometer, with SepacoreRecord chromatography software. A Buchi C-670 Cartridge system was used to load 40 × 150 mm flash columns with approximately 90 g of preparative C_{18} RP, 125 Å, 55–105 μm, bulk packing material (Waters, Milford, MA). The column was equilibrated with 30% acetonitrile and 0.5% acetic acid in water for 5 min at a flow rate of 30 mL per min. After samples (10–15 mL) were injected, the column was developed with a binary gradient to 80% acetonitrile over 30 min. Fractions were pooled based on UV absorbance peaks at 210 nm.

2.3 Isolation of the diCQAs from diCQA-rich extract

The phenolic fractions were further purified using preparative chromatography. Samples of flash fractions (5 mL) were injected on a Shimadzu (Columbia, MD) preparative HPLC system with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, SCL 10Avp system controller all operating under the Shimadzu Class VP operating system, with a Phenomenex (Torrance, CA) Luna C_{18}(2) semi-preparative RP column (10 μ, 100 Å, 250 × 50 mm). The column was pre-equilibrated with a solvent system consisting of 10% MeOH and 90% water (containing 1% HOAc) at a flow rate of 30 mL per min and the eluent was monitored at 210 nm. The column was developed to 100% MeOH over 50 min. Five major sub-fraction peaks were detected at 360 nm and collected. This procedure was repeated to obtain sufficient pooled material that was evaporated under reduced pressure (40°C) and the resulting material was freeze-dried, weighed, and stored at −20°C until use.

2.4 Analytical HPLC methodology

Five semi-purified diCQA fractions were dissolved in methanol at 2 mg/mL. An aliquot was removed from the vial and filtered through a 0.45 μM nylon 66 filter for HPLC analysis. HPLC analysis was conducted with in a Shimadzu (Columbia, MD) HPLC System (two LC20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module) running under the Shimadzu EZStart Version 7.3 software using a column a C_{18} Inertsil RP column (250 mm × 4.6 mm; RP C_{18}, ODS_3, 5 u; with a Metaguard guard column; Varian, Torrance, CA). For diCQA analysis, the initial conditions were 30% acetonitrile and 0.025% acetic acid in water, at a flow rate of 1 mL per min. The effluent was monitored at 360 nm. After injection, the column was developed to 50% acetonitrile and 0.025% acetic acid in a linear gradient over 45 min.

2.5 LC/ESI-MS and NMR analysis for confirmation of the diCQA identity

To confirm the identity of the diCQAs found in the fractions, aliquots were injected on a LC-MS Q-TOF. Samples were run on an Applied Biosystems/MDS Sciex QStar Elite Q-TOF mass spectrometer with a Turbo ion spray electrospray source, and Agilent 1100 series HPLC system all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The data were acquired in the negative ESI-TOF mode. The column used was an Inertsil ODS_3 RP C_{18} column (3 μm, 150 × 3 mm). NMR Analysis ^{1}H, COSY, DEPT, and ^{13}C-NMR spectra were obtained with a Bruker (Billerica, MA) Avance 500 spectrometer equipped with a 5 mm inverse broadband Z-gradient probe (^{13}C NMR, 125 MHz; ^{1}H, 500 MHz). NMR spectra were recorded in methanol-d4, which served as the internal reference (^{13}C, 49.0 ppm, ^{1}H, 3.30 ppm). The data were analyzed using the Advanced Chemistry Development, SpecManager 1D Processor and the HNMR and CNMR Predictor software suite (Toronto, ONT).

2.6 Cell proliferation

Four cell cultures containing mouse macrophage RAW 264.7, normal human colon fibroblast CCD-33Co, human colon cancer cells CRL-2577 (RKO) (CRL-2577), and HT-29 (HTB-38) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate at 37°C in 5% CO_2/95% air. CCD-33Co colon fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum, and 1% penicillin/streptomycin. The cell proliferation assay was conducted using CellTiter 96 Aqueous

One Solution Proliferation assay kit (Promega, WI) as previously indicated [18]. For RAW 264.7, RKO, and HT-29, 5×10^4 cells per well were seeded in a 96-well plate for 24 h. For CCD-33Co, 1×10^3 cells per well were seeded in a 96-well plate and allowed to grow to confluence for 1 wk with replacement of medium every other day. RAW 264.7 cells were treated with different concentrations of the diCQAs (1–200 μM) for 24 h. CCD-33Co, RKO, and HT-29 cells were treated with different concentrations of diCQAs (1–300 μM) for 48 h. After diCQA treatment, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, (MTS) assay as previously reported [18]. The percentage of viable cells was calculated with respect to cells treated with 0.1% DMSO. Solvents used at these concentrations showed no cytotoxicity on cells (>90% cell viability). Each test was conducted for at least two independent trials with three replicates for each trial.

2.7 Nitrite and PGE_2 measurements

RAW 264.7 cells were seeded (2×10^5 cells per well) in a six-well plate for 48 h at 37°C in 5% CO_2/95% air. After 48 h incubation, cells were treated with different concentrations of the diCQAs (1–200 μM) and 1 μg/mL of LPS for 24 h, and then culture supernatant was collected and measured to determine nitrite and PGE_2 as previously described [18].

2.8 Protein expressions in RAW 264.7, RKO and HT-29 cells by Western blot

RAW 264.7, RKO, and HT-29 cells were seeded (2×10^5 cells per well) independently in a six-well plate for 48 h. After 48 h incubation, cells were treated with different concentrations of the diCQA fractions (1–200 μM and 1 μg/mL of LPS for RAW 264.7, 1–100 μM for HT-29 and RKO cells) for 24 h. After treatment, cells were trypsinized and suspended in Bio-Rad's Laemmli sample buffer (Biorad, CA) with 5% β-mercaptoethanol (Sigma-Aldrich, MO). RAW 264.7 cell lysates were used to determine actin, iNOS, COX-2, NF-κB p50, and p65 expressions as previously indicated [18]. Nuclear and cytoplasmic proteins of RAW 264.7 were isolated with a buffer extraction and centrifugation system NE-PER® (Pierce Biotechnology, IL) according to the manufacturer's recommendations. RKO and HT-29 cell lysates were used to determine Bax, Bcl-2, p21, p27, caspase-3 and -8 protein expressions. Equal amount of protein (15 μg) was loaded in 4–20% Tris-HCL ready gels (Biorad, CA). The separated proteins were transferred to PVDF membrane and blocked with 5% non-fat dry milk in 0.1% Tris-buffered saline Tween-20 for 1 h at 4°C. After blocking, the membrane was incubated with actin, iNOS, COX-2, NF-κB p50, NF-κB p65, Bax, Bcl-2, p21, p27, caspase-3, or caspase-8 primary antibodies (Santa Cruz Biotechnology, CA) at 4°C overnight. The membrane was washed and incubated with antimouse IgG conjugated horseradish peroxidase secondary antibody (GE Healthcare, Buckinghamshire, UK) for 3 h at 25°C. The expressions of proteins were visualized using chemiluminescent reagent (GE Healthcare).

2.9 Apoptosis of RKO and HT-29 colon cancer cells

The apoptosis of RKO and HT-29 colon cancer cells was evaluated by flow cytometry using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich). Briefly, 2×10^5 cells per well were seeded in a six-well plate and allowed to grow for 48 h. The cells were then treated with the diCQA fraction (1–100 μM) for 6 and 12 h at 37°C in 5% CO_2/95% air. After treatment, cells were tripsinized and suspended in binding buffer at a concentration of 1×10^6/mL. Five hundred microliters of treated and untreated cells was transferred into a plastic test tube and stained with 5 μL Annexin V-FITC and 10 μL propidium iodide solution for 10 min. Propidium iodide staining was performed concomitantly with Annexin V-FITC staining to determine whether any DNA/nuclei were present in the colon cancer cells. The cells were analyzed immediately by LSR II flow cytometer (BD Biosciences). The analysis was performed at least in duplicate with three replicates for each trial.

2.10 Caspase activities

The activity of caspases-3 and -8 were determined using the caspase colorimetric assay kit (Invitrogen) following manufacturer's protocol. Briefly, RKO and HT-29 cells were seeded (2×10^5 per well) independently in a six-well plate and allowed to grow for 48 h at 37°C in 5% CO2/95% air. Cells were treated with the diCQAs (100 μM) for 0, 2, 4, 6, 8, 12, and 24 h. After treatment, cells were pelleted and resuspended in 50 mL chilled cell lysis buffer and incubated on ice for 10 min. The cells were then centrifuged at $10000 \times g$ for 1 min. Approximately 50 μg of protein was assayed for caspase activity analysis by adding 50 μL reaction buffer containing 10 mM dithiothreitol and 5 μL of the 4 mM corresponding caspase substrates in a 96-well plate. The plate was incubated at 37°C in the dark for 2 h. The absorbance was read at 405 nm using ELx808 microplate reader (Biotek, VT). Analyses were performed in four replicates.

2.11 Statistical analysis

Data are presented as means ± SD for the indicated number of independently performed experiments. Data were analyzed using one-way ANOVA and means were considered to be significantly different at $p < 0.05$ as determined by least significant differences.

3 Results

3.1 Purification and identification of the diCQAs

Figure 1A presents the analytical HPLC profile of diCQA rich fractions identified as a mix of 3,4- and 3,5-diCQAs, while Fig. 1B presents the peak identified as 4,5-diCQA. We confirmed the molecular mass of the diCQAs by conducting LC-MS analysis using negative ESI and absorbance at 360 nm. NMR spectrum analysis confirmed the identity of the first fraction as a mix of 3,4- and 3,5-diCQAs and the second fraction as 4,5-diCQA (Table 1). The key assignment in these compounds lies in the identification of protons 3, 4 and 5. Spectral simulations show that for all three isomers (3,4-, 3,5- and 4,5-dicaffoyl quinic acid), the proton at the unsubstituted position will have a shift of about 4.5 ppm. Both substituted positions will have shifts between 5.0 and 5.5 ppm. From inspection of the proton spectrum it seems that this is one of the asymmetric isomers (either 3,4- or 4,5-dicaffoylquinic acid). The Nuclear Overhauser effect spectroscopy spectrum (data not shown) has a strong Nuclear Overhauser effect (NOE) cross peak that could only occur between protons on the same side of the ring. Since the NOE cross peak indicated proximity of the unsubstituted proton at 4.38 and a substituted one at 5.13 ppm, these must be positions 3 and 4, respectively, of the 4,5-substituted quinic acid. There is an antiphase artifact arising from the strong J-coupling between the protons on opposite sides of the ring (protons 4 and 5, 5.13 and 5.63 ppm, respectively), but no NOE cross peak. The COSY spectrum allows assignment of protons 2 and 6, through their couplings to protons 3 and 5. Proton pairs 9–10 and 18–19 show a coupling of 15.9 Hz, indicating *E* or *trans* configuration of the double bond. Heteronuclear Multiple Bond Coherence (HMBC) spectra showed correlations between protons 4 and 5 to carbonyl carbons 17 and 8, respectively, which allowed the differentiation of the caffoyl moieties. Heteronuclear Single Quantum Coherence (HSQC), HMBC, and COSY spectra were used to complete the assignment. Their purity determined by HPLC with a UV detector using chlorogenic acid as a standard was $>85\%$ (data not shown). The two sub-fractions were then assessed for anti-inflammatory and anti-colon cancer activities.

3.2 Effect of the diCQA fractions on viability of RAW 264.7 cells

Macrophages showed a survival rate of $>90\%$ when incubated with the diCQA fractions at a concentration $\leq 200\,\mu M$

Table 1. NMR spectra of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA[a)]

Position	1H shift			13C shift		
	3,4-diCQA	3,5-diCQA	4,5-diCQA	3,4-diCQA	3,5-diCQA	4,5-diCQA
1	–	–	–	73.8	73.3	74.6
2	2.37 (15.4, 3.7), 2.15 (15.3)	2.33 (13.8, 3.8), 2.18 (13.8, 7.0)	2.31, 2.12	35.6	34.6	37.0
3	5.65 (3.7)	5.45 (3.3)	4.38	68.8	71.1	67.9
4	5.01 (9.1, 3.4)	3.99 (7.5, 3.2)	5.13	75.2	69.2	74.3
5	4.39 (9.8, 4.4)	5.40 (7.9, 4.7)	5.63	64.4	70.1	67.6
6	2.23, 2.10 (13.4, 10.1)	~2.24, ~2.24	2.26, 2.26	40.6	36.2	37.9
7	–	–	–	176.5	175.9	175.3
8	–	–	–	167.2	167.5	166.8
9	6.26[1] (16.0)	6.36 (15.9)	6.20	113.7	114.2	113.3
10	7.56[2] (16.0)	7.63 (15.9)	7.53	146.0[2]	145.6	148.2
11	–	–	–	126.4[6]	126.5[1]	126.3
12	7.05[3] (2.0)	7.07 (1.5)	7.04[1]	113.6	113.9	113.8
13	–	–	–	145.4	145.4	145.4
14	–	–	–	148.1	148.2	148.3
15	6.78[5] (8.2)	6.79 (8.2)	6.77[2]	115.1	115.1	115.1
16	6.94[5] (8.2, 2.1)	6.97 (8.2, 2.0)	6.93[3]	121.8[7]	121.6	121.7
17	–	–	–	167.1	167.0	167.1
18	6.30[1] (16.0)	6.28 (15.9)	6.30	113.7	113.7	113.3
19	7.59[2]	7.59 (15.9)	7.61	145.9[2]	145.9	146.3
20	–	–	–	126.3[6]	126.4[1]	126.3
21	7.03[3] (2.0)	7.07 (1.5)	7.02[1]	113.6	113.9	113.8
22	–	–	–	145.4	145.4	145.4
23	–	–	–	148.1	148.2	148.3
24	6.74[5] (8.2)	6.79 (8.2)	6.75[2]	115.1	115.1	115.1
25	6.88[5] (8.2, 2.1)	6.99 (8.2, 2.0)	6.92[3]	121.7[7]	121.6	121.7

a) ^{1,2,3,4,5,6,7} = may be reversed.

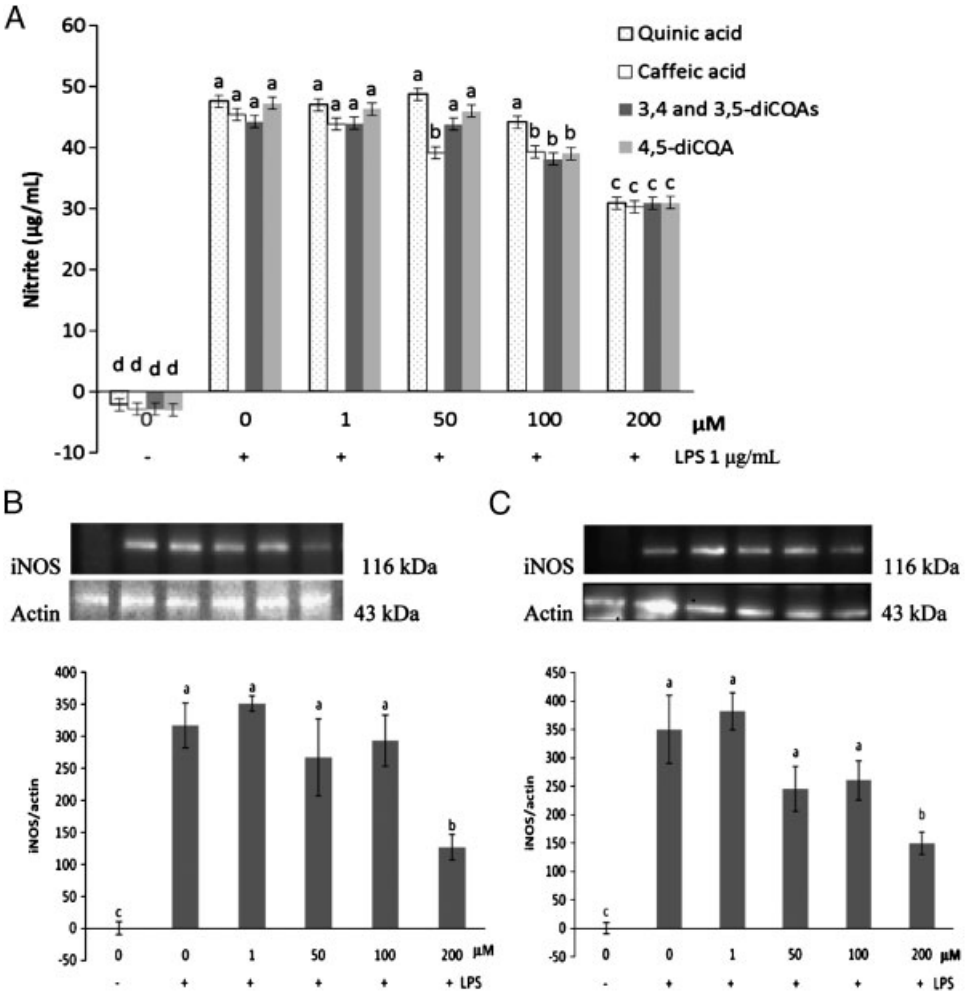

Figure 2. The 3,4- and 3,5-diCQAs and 4,5-diCQA fractions reduced (A) NO production. (B) 3,4- and 3,5-diCQAs fraction, and (C) 4,5-diCQA suppressed iNOS expression of LPS-induced RAW 264.7 cells. The data represent the mean ± SD of a triplicate from two independent experiments. Different letters indicate significant differences, $p < 0.05$.

(data not shown). Therefore, in this study, a concentration of 1–200 μM of the two diCQA sub-fractions was used to treat the cells and thus prevent the compounds from having cytotoxic effect.

3.3 DiCQA reduced NO, PGE_2 production and iNOS, COX-2 protein expression

Figure 2 shows the effect of quinic acid, caffeic acid, 3,4- and 3,5-diCQAs fraction, and 4,5-diCQA on NO production and iNOS protein expression in LPS-induced RAW 264.7. Caffeic acid was the most effective in significantly reducing NO production (50 μM), followed by the 3,4- and 3,5-diCQAs fraction and 4,5-diCQA (100 μM) while quinic acid was the least potent (200 μM) (Fig. 2A). Both 3,4- and 3,5-diCQAs fraction (Fig. 2B), and 4,5-diCQA (Fig. 2C) significantly suppressed iNOS expression at 200 μM.

Figure 3 shows the effect of diCQAs on PGE_2 production and COX-2 protein expression in LPS-induced RAW 264.7. Caffeic acid, quinic acid, and 4,5-diCQA significantly inhibited PGE_2 production at 50 μM while the 3,4- and 3,5-diCQAs fraction significantly inhibited at 100 μM (Fig. 3A). The 3,4- and 3,5-diCQAs fraction significantly inhibited COX-2 protein expression at 50 μM (Fig. 3B); however, 4,5-diCQA significantly suppressed COX-2 at 200 μM (Fig. 3C). Table 2 presents the calculated concentration (μM) of the diCQA fractions that resulted in 50% reduction on pro-inflammatory parameters. Both diCQA fractions inhibited PGE_2/COX-2 pathway more than NO/iNOS pathway.

3.4 The 3,4- and 3,5-diCQAs inhibited nucleus translocation of p50 and p65 NF-κB

The 3,4- and 3,5-diCQAs fraction significantly reduced nucleus protein expressions of NF-κB subunits p65 (Fig. 3D) and p50 (Fig. 3E) at a concentration of 50 μM, suggesting that the 3,4- and 3,5-diCQAs fraction was able to inhibit nucleus translocation of NF-κB subunits. We also found that the 3,4- and 3,5-diCQAs fraction inhibited NF-κB translocation more on the p50 subunit than on the p65 subunit.

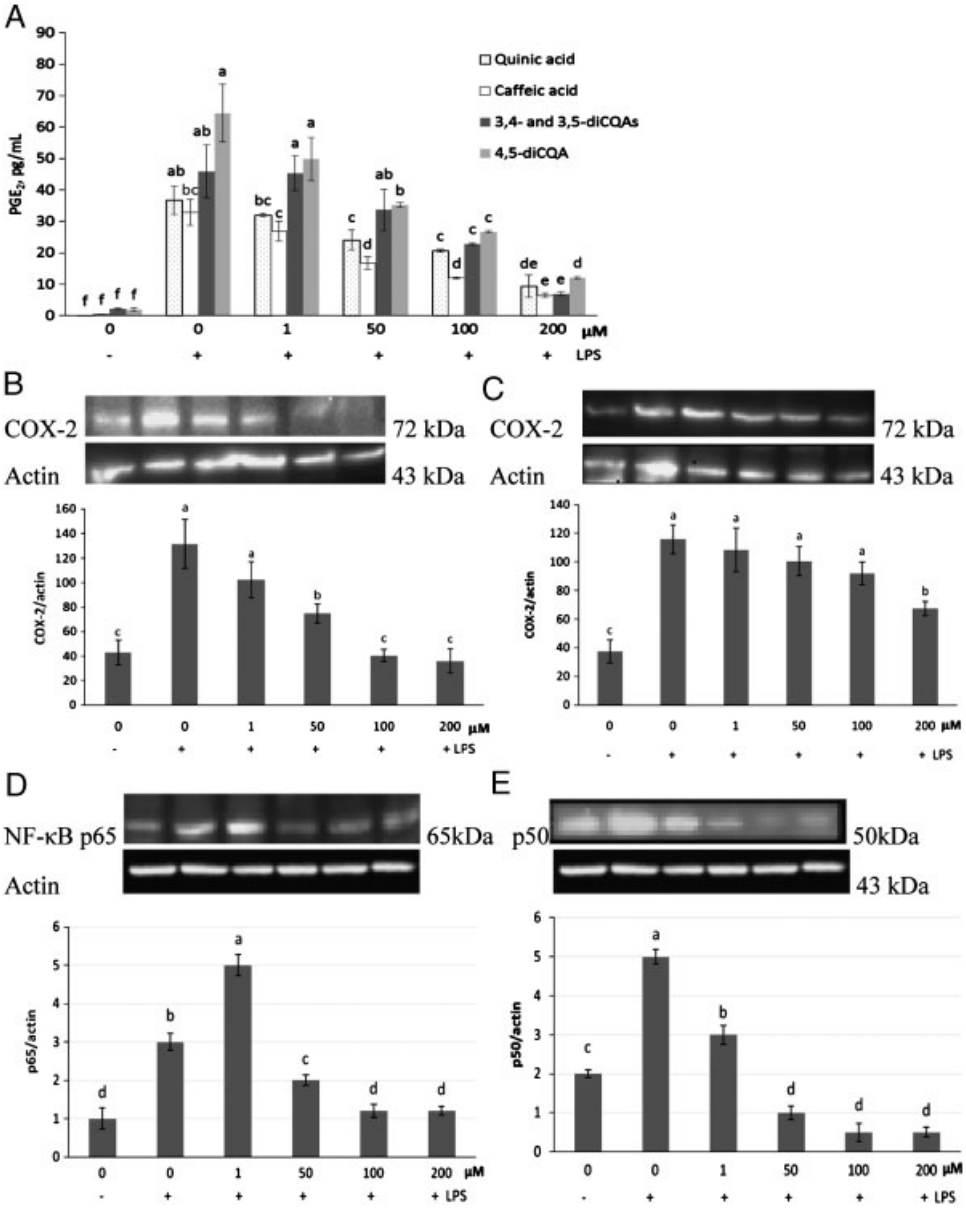

Figure 3. The 3,4- and 3,5-diCQAs and 4,5-diCQA fractions reduced (A) PGE_2 production. (B) 3,4- and 3,5-diCQAs fraction, and (C) 4,5-diCQA reduced COX-2 expression. (D) 3,4- and 3,5-diCQAs fraction inhibited NF-κB subunits p65 and (E) p50 nucleus translocation in LPS-induced RAW 264.7 macrophages. The data represent the mean ± SD of a triplicate from two independent experiments. Different letters indicate significant differences, $p < 0.05$.

3.5 The diCQAs inhibited proliferation of RKO and HT-29 human colon cancer cells

The diCQA fractions did not cause any cytotoxicity to CCD-33co normal colon fibroblasts up to 300 μM (data not shown). As shown in Table 2, the two diCQA fractions inhibited cell proliferation of RKO and HT-29 colon cancer cells. We also found that both fractions have a similar efficacy in inhibiting RKO and HT-29 colon cancer cells. Thus, we selected the 3,4- and 3,5-diCQAs fraction, to further assess their mechanism of action in inhibiting colon cancer cell proliferation.

3.6 The diCQAs promoted apoptosis of RKO and HT-29 colon cancer cells

Figure 4A shows the percentage of RKO and HT-29 colon cancer cells undergoing apoptosis in the presence or absence of the diCQAs after treatment for 6 and 12 h. The diCQAs increased the amount of RKO cells undergoing apoptosis from 0.02% (untreated) to 3.7%, and 4.3% for cells treated with the 10 and 100 μM diCQAs fraction for 6 h, respectively (Fig. 4B). RKO cells significantly increased the amount of apoptotic cells when treated with the diCQAs fraction for 12 h from 2.4% (untreated) to 4.7% and 15.1%

Table 2. IC_{50}[a)] values of diCQA fractions on NO, iNOS, PGE_2, and COX-2 production using RAW 264.7 macrophages and cytotoxicity[b)] of diCQA fractions on human colon cancer cells[c)]

IC_{50} (μM)	3,4- and 3,5-diCQAs	4,5-diCQA
RAW 264.7		
NO	>200	>200
iNOS	145.5	150.0
PGE_2	86.5	102.5
COX-2	80.0	155.2
Cytotoxicity (μM)	3,4- and 3,5-diCQAs	4,5-diCQA
RKO		
IC_{50}	182	190
GI_{50}	118	120
TGI	198	202
LC_{50}	>300	>300
HT-29		
IC_{50}	286	295
GI_{50}	213	227
TGI	274	281
LC_{50}	>300	>300

a) IC_{50} are the concentrations (μM) that resulted in 50% reduction of production/expression of pro-inflammatory responses (mean ± SD, $n = 6$).

b) Cytotoxicity to different human CRC cells was defined by the following parameters based on Monks et al. [50].

c) IC_{50} (inhibitory concentration, 50%): concentration resulting in 50% inhibition of net cell growth = $\%T/C$ (OD of treated cells/OD of control cells) × 100; GI_{50} (growth inhibition, 50%): concentration resulting in 50% reduction of net cell growth in comparison to untreated cells = $100[(T-T0)/C-T0)]$; TGI (total growth inhibition): concentration required to achieve complete halting of treated cell growth = $T0$; LC_{50} (lethal concentration, 50%): concentration lethal to 50% of treated cells = $100[(T-T0)/T0]$. OD, optical density; C, control optical density; T, test optical density; $T0$, optical density at time zero. Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase.

(10 and 100 μM, respectively) (Fig. 4B). The diCQAs increased the amount of HT-29 cells undergoing apoptosis from 7.0% (untreated) to 8.8% and 9.0% for cells treated with 10 and 100 μM diCQAs fraction for 6 h, respectively (Fig. 4C). HT-29 cells significantly increased the amount of apoptotic cells when treated with the diCQAs fraction for 12 h from 8.9% (untreated) to 9.0% and 10.2% (10 and 100 μM, respectively) (Fig. 4C). These results suggest that the diCQAs fraction promoted apoptosis on RKO and HT-29 colon cancer cells depending on time and concentration.

3.7 The 3,4- and 3,5-diCQAs did not affect p21 and p27 expression in RKO and HT-29

In order to investigate the mechanism by which the 3,4- and 3,5-diCQAs fraction inhibits growth of HT-29 and RKO colon cancer cells by affecting cell cycle, we analyzed protein expression of p21 and p27. The 3,4- and 3,5-diCQAs fraction did not affect p21 protein expression in either RKO cells (Fig. 5A) or HT-29 (Fig. 5B). However, we found that the 3,4- and 3,5-diCQAs fraction slightly increased p21 protein expression in HT-29 at low concentration (1 μM) and did not affect p21 at higher concentrations. The 3,4- and 3,5-diCQAs fraction did not significantly affect p27 protein expression in RKO (Fig. 5C) and HT-29 (Fig. 5D), suggesting this fraction did not inhibit cell proliferation by inducing cell cycle arrest of HT-29 and RKO. We also analyzed cell cycle analysis using flow cytometry to confirm that the diCQAs treatment did not affect cell cycle (data not shown).

3.8 The 3,4- and 3,5-diCQAs increased Bax:Bcl-2 expression in HT-29 but not in RKO cells

To determine whether the cell death of HT-29 and RKO are due to apoptosis, we then analyzed the apoptosis induction by assaying the protein expression of apoptosis mediators, Bax and Bcl-2. Figure 5 showed effect of the 3,4- and 3,5-diCQAs fraction on the ratio of Bax:Bcl-2 protein expression. The 3,4- and 3,5-diCQAs fraction at 100 μM did not change in RKO (Fig. 5E), but significantly increased the Bax:Bcl-2 ratio of HT-29 (Fig. 5F).

3.9 The 3,4- and 3,5-diCQAs increased caspase-3 and -8 expression and activity in RKO and HT-29

Figure 6 shows that the diCQAs fraction increased the expression of cleaved caspase-3 and caspase-8 in RKO and HT-29 colon cancer cells. The expression of cleaved caspase-3 significantly increased by 4.9-fold in RKO colon cancer cells when treated with the diCQAs fraction at 50 μM (Fig. 6A). For HT-29 colon cancer cells, the diCQAs fraction significantly increased by 2.0-fold at 100 μM (Fig. 6B). The diCQAs fraction significantly increased the expression of caspase-8 in RKO by 2.1-fold at 50 μM (Fig. 6C) and for HT-29 was significantly increased by 1.4-fold at 100 μM (Fig. 6D). Figure 7 shows the time course of the diCQAs fraction-inducing caspase-3 and -8 activities in RKO and HT-29 colon cancer cells. Caspase-3 and -8 activities began to rise in RKO and HT-29 after 2 h of incubation and increased more than two times after 6 h of incubation. The diCQAs fraction (100 μM) increased by 2.0-fold the activity of caspase-3 and reached its maximum activity at 4 h in RKO (Fig. 7A) and at 6 h in HT-29 (Fig. 7B). The activity of caspase-8 increased to its maximum at 6 h in both RKO and HT-29 colon cancer cells by 2.8-fold and 2.4-fold, respectively (Figs. 7C and D).

4 Discussion

Fractions 3,4-diCQA and 3,5-diCQA, and 4,5-diCQA were isolated from a methanol extract of yerba mate tea leaves powder. The negative ESI mass spectrum of the diCQAs

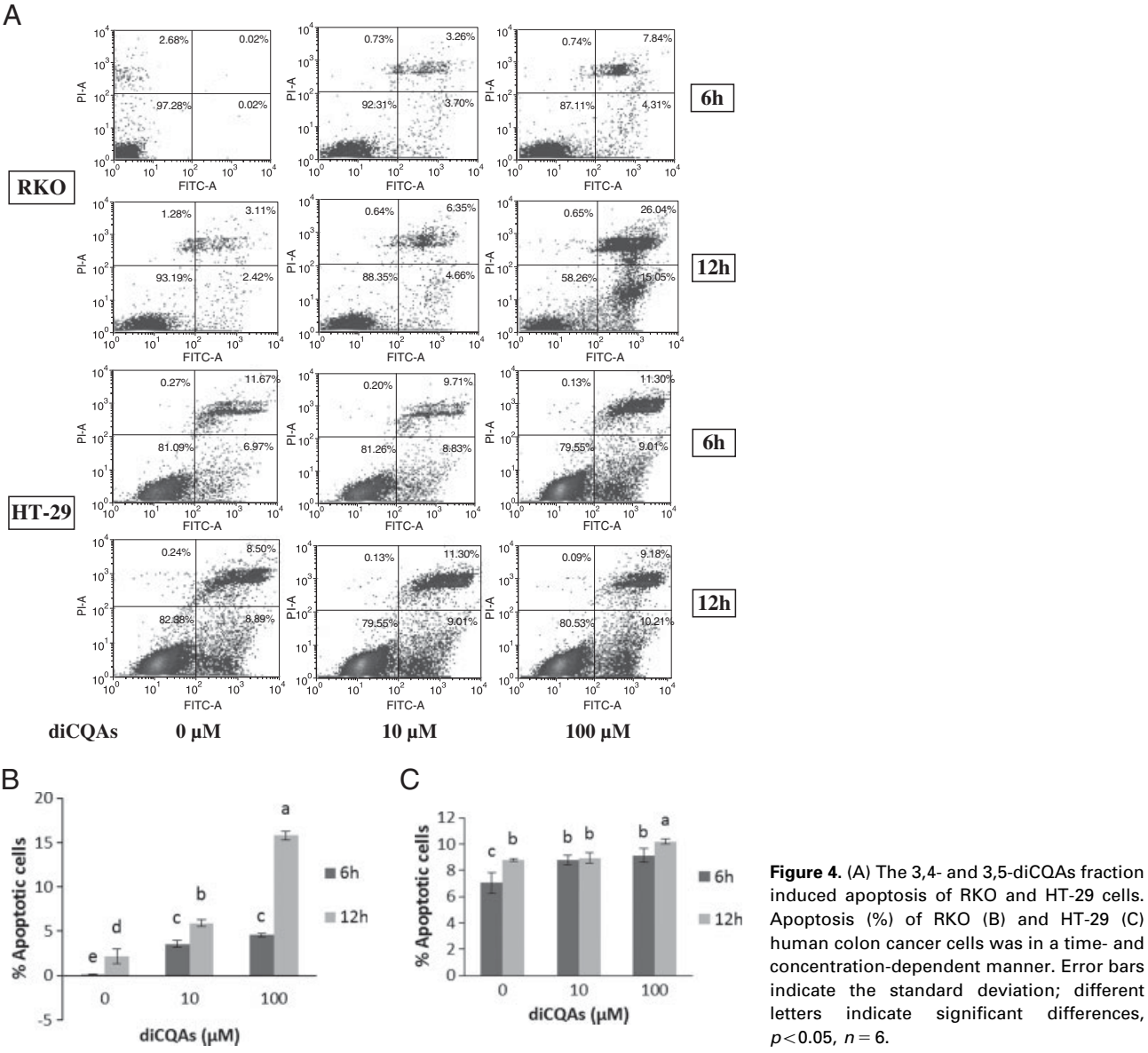

Figure 4. (A) The 3,4- and 3,5-diCQAs fraction induced apoptosis of RKO and HT-29 cells. Apoptosis (%) of RKO (B) and HT-29 (C) human colon cancer cells was in a time- and concentration-dependent manner. Error bars indicate the standard deviation; different letters indicate significant differences, $p < 0.05$, $n = 6$.

showed a molecular ion at *m/z* 515 [M–H]- corresponding to a molecular formula $C_{25}H_{24}O_{12}$. Both 3,4-diCQA and 3,5-diCQA eluted at the same time, which made it difficult to separate these two isomers. We were able to separate 4,5-diCQA from 3,4- and 3,5-diCQAs. Thus, we studied these two sub-fractions anti-inflammatory capacity using LPS-induced RAW 264.7 macrophages and anti-colon cancer using RKO and HT-29 cell lines.

Both the 3,4- and 3,5-diCQAs fraction and the 4,5-diCQA fraction were shown to have anti-inflammatory effect by suppressing the COX-2/PGE_2 and iNOS/NO pathways. The diCQAs fractions reduced NO production ($IC_{50} > 200\,\mu M$ for both fractions) by inhibiting iNOS enzyme ($IC_{50} = 145.5\,\mu M$ for the 3,4-and 3,5-diCQAs fraction, and $150.0\,\mu M$ for the 4,5-diCQA). The capacity in reducing NO production of the diCQAs fractions was comparable to caffeic acid. We found that the two diCQA fractions inhibited pro-inflammatory parameters more potently than chlorogenic acid in RAW 264.7 macrophages [18]. Uncontrolled activation of COX-2/PGE_2 pathway is associated with inflammation and widely spread in human neoplasia. The diCQAs fractions inhibited PGE_2 production with IC_{50} of $86.5\,\mu M$ for the 3,4-and 3,5-diCQAs fraction and $102.5\,\mu M$ for the 4,5-diCQA fraction. The 3,4- and 3,5- diCQAs fraction suppressed COX-2/PGE_2 pathway ($IC_{50} = 86.5\,\mu M$ for PGE_2, and $80.0\,\mu M$ for COX-2) more potently than the 4,5-diCQA fraction ($IC_{50} = 102.5\,\mu M$ for PGE_2, and $155.2\,\mu M$ for COX-2). There were a few studies of the diCQAs on its anti-inflammatory activity and our results showed that the diCQAs fractions suppressed iNOS expression in a dose-dependent manner in agreement with result by Olmos et al. [34], who showed that the diCQAs isolated from *Phagnalon rupestre* (Asteraceae) at

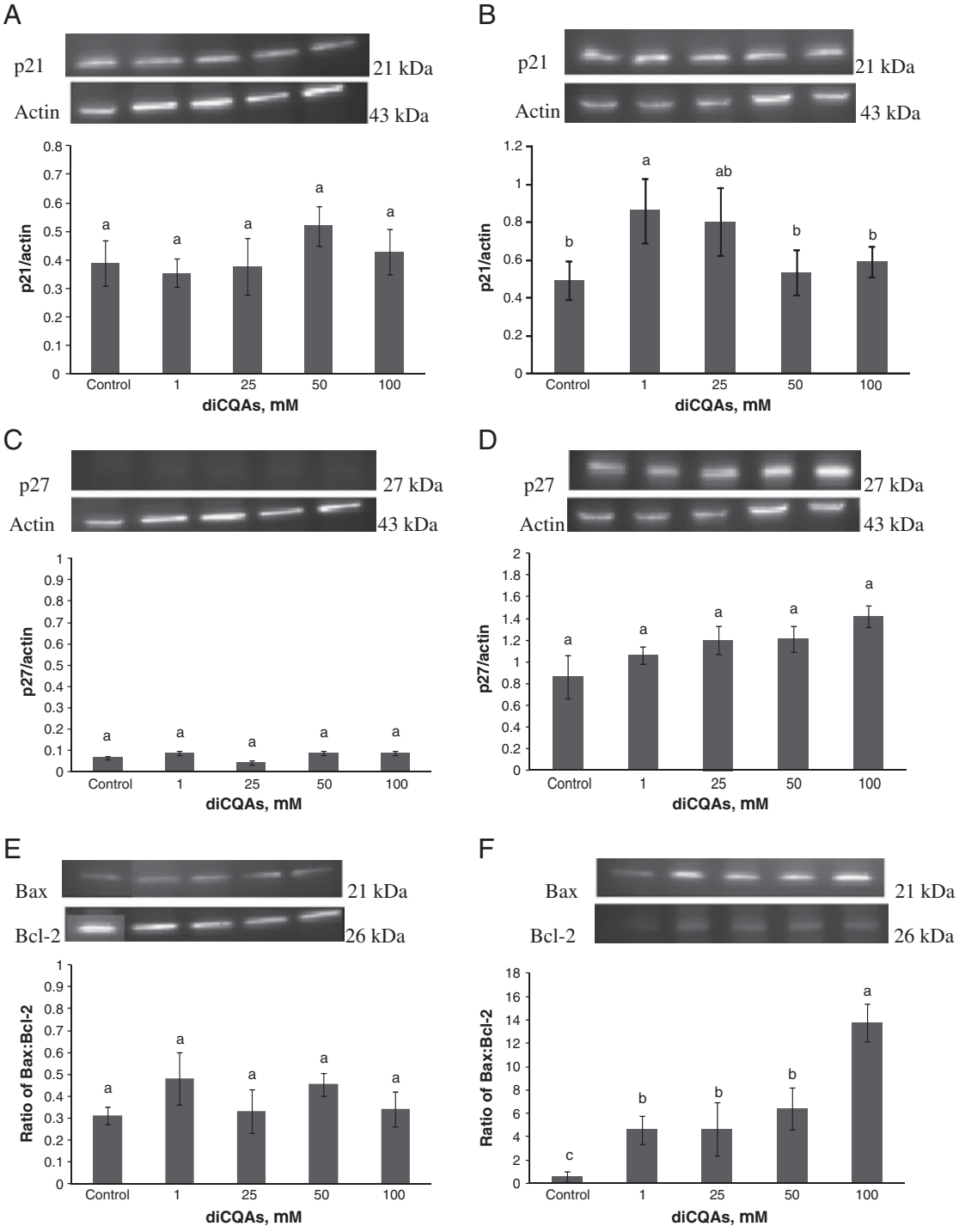

Figure 5. Effect of the 3,4- and 3,5-diCQAs fraction on p21 expression in (A) RKO and (B) HT-29; p27 expression (C) RKO and (D) HT-29; and Bax:Bcl-2 ratio in (E) RKO and (F) HT-29 cells. The data represent the mean ± SD of a triplicate from two independent experiments. Different letters indicate significant differences, $p < 0.05$.

100 μM reduced NO levels and the expression of NOS-2 in LPS-induced RAW 264.7 through inhibiting nucleus translocation of NF-κB [34]. Peluso et al. [35] also found that the 3,4-diCQA and 3,5-diCQA from *Tessaria integrifolia* and *Mikania cordifolia* exhibited anti-inflammatory activity in vitro [35]. Park et al. [36] showed that the 4,5-diCQA, 3,5-diCQA, and 3,5-diCQA methyl ester inhibited expressions of iNOS and COX-2 more than caffeic acid. Shin et al. [37]

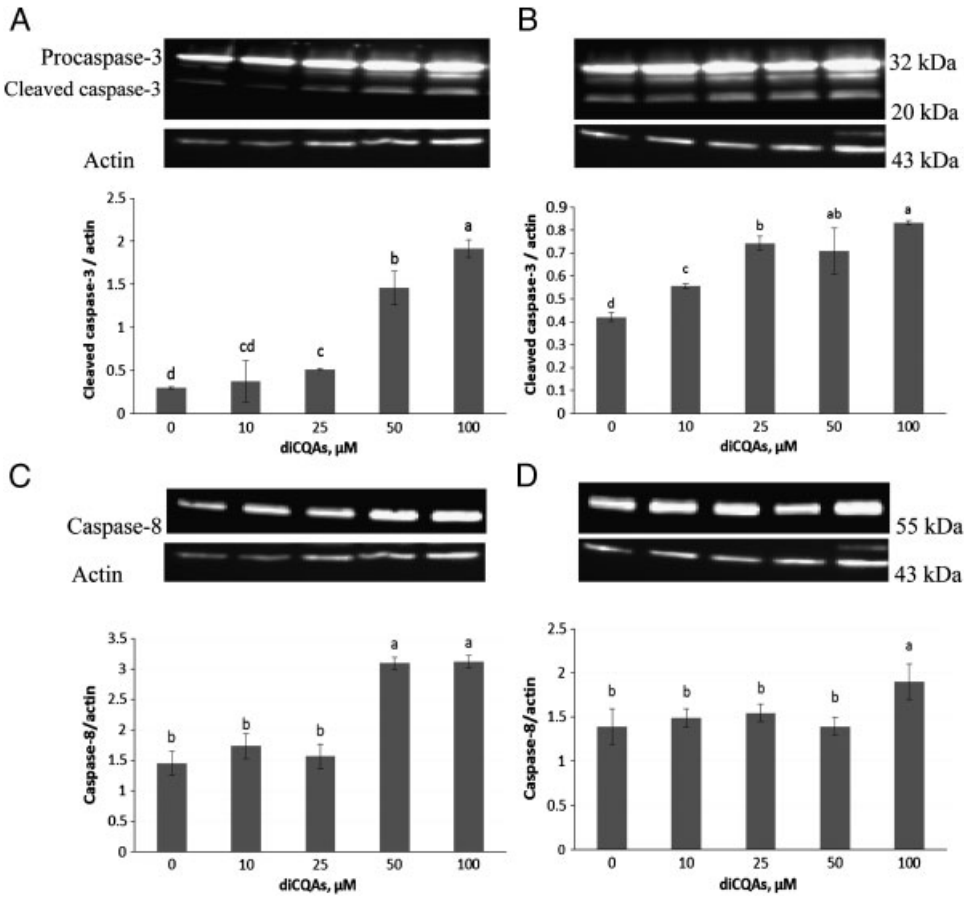

Figure 6. The 3,4- and 3,5-diCQAs fraction increased the activation of caspase-3 cleavage in (A) RKO, (B) HT-29 and increased the expression of caspase-8 in (C) RKO, (D) HT-29 colon cancer cells. Error bars indicate the standard deviation; different letters indicate significant differences, $p<0.05$, $n=6$.

suggested that hydrophobic structures were shown to suppress NF-κB activation more effectively than caffeic acid because of their ability to pass through the cell membrane [37]. Yoshimoto et al. [38] suggested that catechol structure played an important role in the antimutagenicity, increasing number of caffeoyl groups that promoted antimutagenicity [38]. Thus, CQA derivatives effectively inhibited the reverse mutation in proportion to the number of caffeoyl groups bound to quinic acid.

Caffeic and quinic acids have been long known as antioxidant compounds, and the antioxidant mechanism might therefore contribute to their anti-carcinogenic properties [39, 40]. Antioxidants protect against oxidation and free radical scavenging that can lead to cancer [27]. Caffeic acid showed an inhibitory effect of matrix metalloproteinase-9 activity, which is known to be involved in tumor cell invasion and metastasis [41], and anti-inflammatory activities [37]. Murayama et al. [42] identified the 3,5-diCQA, and 4,5-diCQA as antioxidant in edible chrysanthemums. The chemical conformation of the compound determines its degree of antioxidant capacity.

The anti-inflammatory mechanism of the diCQAs fraction was exhibited by the inhibition of the nucleus translocation of NF-κB subunits p65 and p50. This observation suggests that the diCQAs may reduce the binding of NF-κB to its target DNA, thus inhibiting transcription of genes with pro-inflammatory properties, resulting in the decreased expression of both COX-2 and iNOS and the reduced production of PGE_2 and NO. These results suggest that the diCQAs from Yerba mate leaves might play an important role as an anti-inflammatory, and could have potential anti-cancer effects.

Although diCQAs derivatives have been isolated from various plants, there have been few reports on potential anti-cancer effects. Iwai et al. [1] isolated 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA from immature green coffee beans and these diCQAs exhibited more potent tyrosinase inhibitory activities compared to CQAs, arbutin, and ascorbic acid. They also showed these diCQAs exhibited antiproliferative activities in four cancer cell lines; human histiocytic lymphoma (U937), normal human diploid lung fibroblast (WI38), SV40 virally transformed WI38 (WI38VA), human breast carcinoma (MCF-7), and human oral carcinoma (KB) (IC_{50} = 0.10–0.56 mM) [1]. Mishima *et al.* [43] isolated 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA from a water extract of propolis and these diCQAs inhibited human myeloid leukemia (HL-60) cell growth, which is partly attributed to the granulocytic differentiation leading to apoptosis. Kurata et al. [44] isolated

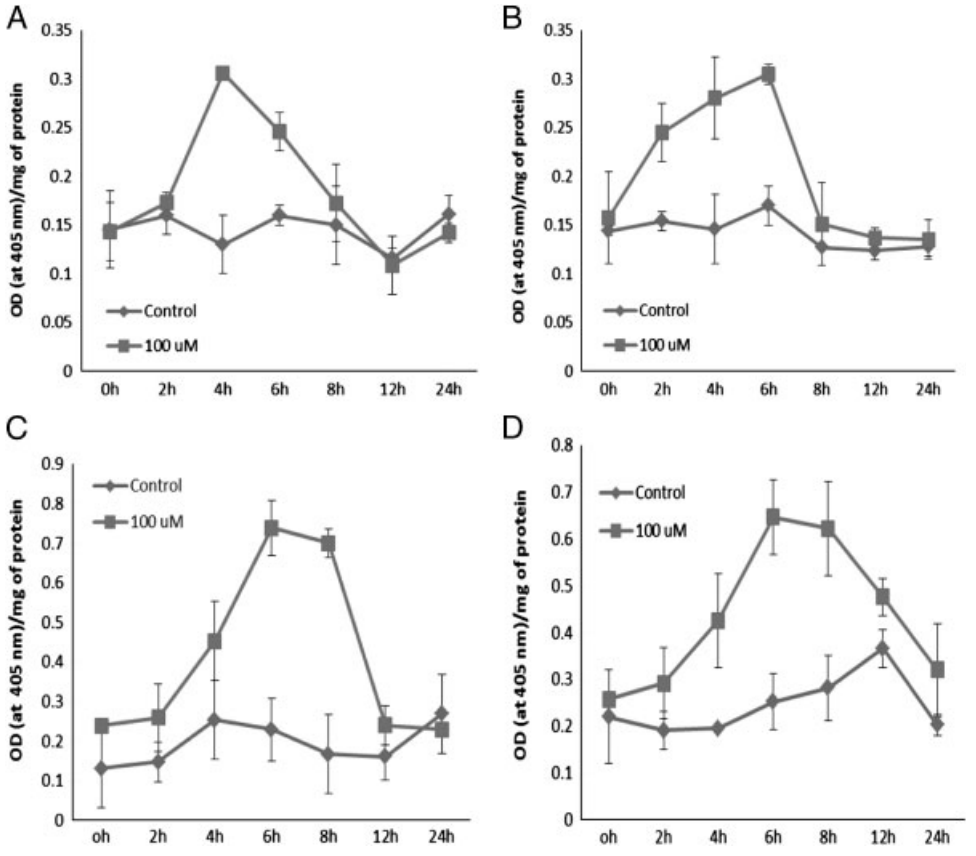

Figure 7. Time course of caspase-3 activity (A) RKO, (B) HT-29 colon cancer cells treated with 100 μM 3,4- and 3,5-diCQAs fraction. Time course of caspase-8 activity in (C) RKO, (D) HT-29 colon cancer cells treated with 100 μM 3,4- and 3,5-diCQAs fraction. Data are represented as mean ± SD, $n = 4$.

diCQAs from sweet potato leaf and diCQAs dose-dependently depressed cancer cell proliferation of human stomach cancer (Kato III), colon cancer (DLD-1), and promyelocytic leukemia (HL-60) cells.

We selected the RKO and HT-29 due to the importance of colon cancer and because there is limited research on these human colon cancer cell lines. The diCQAs fractions inhibited cell proliferation of RKO ($IC_{50} = 182\,\mu M$ for the 3,4-and3,5-diCQAs fraction and $IC_{50} = 190\,\mu M$ for 4,5-diCQA) and HT-29 ($IC_{50} = 286\,\mu M$ for the 3,4-and 3,5-diCQAs fraction, $IC_{50} = 295\,\mu M$ for 4,5-diCQA) human colon cancer cells. We found that the two diCQA fractions were more potent than chlorogenic acid and caffeine in inhibiting cell proliferation of RKO and HT-29 colon cancer cells (data not shown). The diCQA fractions inhibited RKO and HT-29 cell proliferation by inducing apoptosis rather than arresting cell cycle. The diCQA fractions did not affect cell cycle arrest based on the expression of p21 and p27 that did not significantly change. Cell cycle is regulated by the activity of cyclin/cyclin-dependent kinase (CDK). This cyclin–CDK complex is regulated by CDK inhibitors such as p21 and p27 [45]. DiCQAs fraction increased the amount of RKO and HT-29 cells undergoing apoptosis by a time- and concentration-dependent manner.

Apoptosis can be induced either by an extrinsic (death receptors) or by intrinsic (mitochondria) pathways [46]. The death receptor-mediated pathway is initiated by interaction of the ligand with its death receptors, leads to cleavage and activation of initiator caspase-8 and -10, which in turn cleave and activate executioner caspase-3, -6, and -7, terminating in apoptosis. In this study, we found that the diCQAs fraction increased caspase-8 expression and activity in both RKO and HT-29 cells, which also contributes to activation of caspase-3, and indicated the activation of apoptotic extrinsic pathway.

For the mitochondria pathway, the diCQA fractions inhibited HT-29 cell proliferation by inducing apoptosis through induction of the ratio of Bax:Bcl-2 protein expression. Bax controls mitochondrial permeability and cytochrome *c* expression, and increased Bax:Bcl-2 ratio inducing the release of cytochrome *c* from mitochondria to the cytoplasm and contribute to activation of caspase-3. The diCQAs induced the cleavage of procaspase-3 to active caspase-3, which is a key step of apoptosis. Our results suggest that both extrinsic and intrinsic pathways are involved in the diCQAs fraction-mediated regulation of caspase-3 activation in colon cancer cells.

The p53 protein expression was not affected by the diCQAs treatment in RKO cells, which contain wild-type p53 protein (data not shown). In addition, the diCQAs still induced apoptosis in HT-29 cells, which contain mutant p53. These results indicate that wild-type p53 is not involved in the diCQAs-induced apoptosis in colon cancer cells. The induction of apoptosis by the diCQAs was independent of the p53 pathway.

Studies on bioavailability of CQA and diCQA from green coffee extract have shown that these compounds are absorbed and metabolized in humans [47]; they quantified CQA, diCQAs, and phenolics in plasma for up to 8 h after ingestion of 451 μmol total chlorogenic acid. Olthof et al. [48] showed approximately 33% of chlorogenic acid absorbed and 66% potentially reached the colon for further metabolism. The small intestine is a major site for absorption of phenolic acids for coffee metabolism and the colon/microflora play the major role in the absorption and metabolism of chlorogenic acid and phenolic acids from coffee [49]. Thus, the inhibition of inflammation and carcinogenic of the diCQAs may be specific to the colon. The diCQAs from Yerba mate could be a potential anti-cancer agent in colon cancer therapy.

In summary, Yerba mate leaves contain biologically active CQA derivatives that exhibit anti-inflammatory and anticancer properties. The diCQAs from Yerba mate leaves are composed of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA; these compounds possess anti-inflammatory activity through inhibition of NF-κB nucleus translocation, which consequently downregulates the NO/iNOS and PGE_2/COX-2 pathways. The diCQAs inhibit proliferation of colon cancer cells RKO and HT-29 by activating apoptosis through induction of capase-8 and caspase-3, suggesting the possible value of the diCQAs against human colon cancer.

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The authors have declared no conflict of interest.

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