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Effects of *Aristotelia chilensis* berry juice on cyclooxygenase 2 expression, NF-κB, NFAT, ERK1/2 and PI3K/Akt activation in colon cancer cells

[Efecto de un concentrado del fruto de *Aristotelia chilensis* sobre la expresión de cicloxigenasa 2, NF-κB, NFAT, ERK1/2 y activación de PI3K/Akt en células de cancer de colon]

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Abstract
Aristotelia chilensis is a native berry from southern Chile with a high content of anthocyanins, compounds that exhibit antioxidant and anti-inflammatory properties. In the present study, we evaluated the effects of *A. chilensis* berry juice on cyclooxygenase (COX)-2 expression, intracellular signaling pathways, and cell viability in colon cancer cells. The treatment of Caco-2 cells with *A. chilensis* diluted juice for 24 h reduced the protein and mRNA expression of COX-2, as well as the TNF-α-induced NF-κB luciferase activity and NFAT activation. In contrast, 4 h after administration, *A. chilensis* transiently reduced the cytoplasmic IκBα levels and increased ERK1/2 and Akt phosphorylation as well as c-fos expression. At concentrations that reduced COX-2 expression, *A. chilensis* did not affected Caco-2 cell viability. Our results suggest a potential anti-carcinogenic and anti-inflammatory effect of *A. chilensis*.

Keywords: *Aristotelia chilensis*, COX-2, NF-κB, colon cancer.

Resumen
Aristotelia chilensis es un berrie originario del sur de Chile, que posee un alto contenido de antocianinas, compuestos con propiedades antioxidantes y anti-inflamatorias. En este estudio, se evaluó los efectos de un concentrado de *A. chilensis* sobre expresión de ciclooxigenasa (COX)-2, vías de señalización y viabilidad en células de cáncer de colon. El tratamiento de células Caco-2 con *A. chilensis* por 24 h redujo la expresión de la proteína y mRNA de COX-2, y disminuyó la actividad luciferasa regulada por NF-κB o NFAT. El tratamiento de células Caco-2 por 4 h con *A. chilensis* redujo transitoriamente los niveles citoplasmáticos de IκBα, aumentó la fosforilación de ERK1/2 y Akt y la expresión de c-fos. *A. chilensis* no afectó la viabilidad celular, a concentraciones que redujo la expresión de COX-2. Los resultados sugieren un potencial efecto anticancerígeno y antiinflamatorio de *A. chilensis*.

Palabras Clave: *Aristotelia chilensis*, COX-2, NF-κB, cáncer de colon.

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INTRODUCTION

Aristotelia chilensis (Maqui) is an endemic Chilean berry. It is a dioecious plant that belongs to the family Elaeocarpaceae that grows in slightly acidic, moderately fertile and well-drained soils. It produces small edible purple/black berries, about 6 mm in diameter, which are eaten fresh or used for juice, jams or wine-making. The leaves and fruits are astringent and have been used in Chilean folk medicine as anti-diarrheic, anti-inflammatory agents, anti-hemorrhagic and febrifuges (Hoffmann *et al.*, 1992). The red color of the aqueous extract of its fruit is due to the presence of anthocyanin pigments.

The average total anthocyanin content is 137.6 ± 0.4 mg/100 g of fresh fruit, with 73% of delphinidin derivatives and 37% derived from cyanidin. The main pigments identified are 3-glucosides, 3,5-diglucosides, 3-sambubiosides and 3- sambubioside-5-glucosides of delphinidin and cyanidin, with delphinidin 3-sambubioside-5-glucoside (34% of total anthocyanins) as the main anthocyanin (Escribano-Bailon *et al.*, 2006). The presence of highly polar and water-soluble polyglycosylated derivatives in *A. chilensis* illustrates that this berry is attractive for extraction and for the potential use in the production of food colorants, as well as for pharmacological uses. Concentrated juice of *A. chilensis* has a high content of phenolic compounds with antioxidant capacity, which protect both LDL from oxidation and endothelial cells from intracellular oxidative stress, suggesting that *A. chilensis* could have antiatherogenic properties (Miranda-Rottmann *et al.*, 2002).

Anthocyanins are known to be powerful antioxidants, and have been demonstrated to inhibit COX-2 expression, an enzyme up-regulated in colon cancer. Anthocyanins inhibit COX-2 and PGE2 production through a nuclear factor-kappaB (NF-κB)-dependent pathway as well as regulate the PI3 kinase/Akt pathway activated by UVB in the human keratinocyte cell line HaCaT (Tsoyi *et al.*, 2008). Delphinidin inhibits COX-2 expression in different cells, such as JB6 promotion-sensitive mouse skin epidermal (JB6 P+) cells (Kang *et al.*, 2008) and TPA-stimulated HT-29 cells (Kim *et al.*, 2008). Cyanidin reduced COX-2 expression and PGE2 production in human prostate cancer cells (Munoz-Espada *et al.*, 2006) and in TPA-stimulated HT-29 cells (Kim *et al.*, 2008). Also, cyanidin-3-O-beta-glucoside inhibits iNOS and COX-2 expression in THP-1 macrophages (Wang *et al.*, 2008). On the other hand, the juice from different small edible berries has been evaluated for its effects on different cancer cell lines, revealing an inhibition of cell proliferation, activation of the nuclear transcription factor NF-κB, and TNF-induced activation of COX-2 expression (Boivin *et al.*, 2007).

All these antecedents suggest a potential anti-inflammatory and anti-carcinogenic effect of *A. chilensis*. In this study, we evaluated the *in vitro* effect of *A. chilensis* on the colon cancer cell line Caco-2, which exhibits a high level of COX-2 expression. We assessed the effect of *A. chilensis* on mRNA and protein COX-2 levels and the signaling pathways involved in COX-2 expression such as NF-κB, NFAT, c-fos, ERK1/2 MAPK and Akt in Caco-2 cells. Our results showed two different effects of *A. chilensis:* the short-term treatment caused a transient stimulation of IκBα, c-fos, Akt and ERK/1/2, whereas at long-term treatment a strong reduction of COX-2 was detected.

MATERIAL AND METHODS

A. chilensis berry juice was kindly provided by Herbal Powers (Bradenton, FL, USA). The content of anthocyanins was determined by HPLC. Concentrated juice *A. chilensis* was diluted in culture medium before each experiment and a concentration equivalent to 50 ng/ml anthocyanins was used in all experiments.

HPLC

Concentrated juice from *A. chilensis* was analyzed according to the analytical method described by Escribano-Bailon (Escribano-Bailon *et al.*, 2006).

Cell Culture

The cell line of colorectal adenocarcinoma Caco-2 (ATCC, Manassas, VA, USA) was grown in Eagle's Minimum Essential Medium medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

Immunoblotting. Caco-2 cells were incubated with *A. chilensis* for different times and total proteins extracts or cytosolic extracts were prepared. Afterwards, 50 μg of proteins were resolved in 12% SDS/PAGE, transferred to a nitrocellulose membrane. The immunoblotting was carried out using antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI, USA), phospho-ERK1/2, phospho-Akt (ser473), total Akt, c-fos, IκBα (Cell Signaling Technology, Boston, MA, USA), total ERK1/2 (Santa Cruz Biotechnology, California, CA, USA), according to the recommended manufacturer's dilution (Hidalgo *et al.*, 2004). Blots were developed with an enhanced chemiluminescence

system. The phosphorylated proteins were normalized against the nonphosphorylated proteins or COX-2 and c-fos against beta-actin (Sigma-Aldrich, St Louis, MO, USA) or alpha-tubulin (Molecular Probes, Eugene, OR, USA). The ImageJ software from NIH was employed to analyze the blots.

Real-time PCR

Caco-2 cells were incubated with *A. chilensis* or solvent for 24 h. Total RNA was isolated with the RNAEasy Kit (Qiagen, Hilden, Germany) and 1 μg was used for cDNA synthesis with oligo dT primers and MMLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed using 2 μl of cDNA, master mix SYBRGreen (Stratagene, La Jolla, CA, USA) and the following primer: COX-2 sense 5'– TGC ATT CTT TGC CCA GCA CT – 3' and antisense 5' – AAA GGC GCA GTT TAC GCT GT – 3'; GAPDH sense 5'– GGC GTG AAC CAC GAG AAG TAT AA – 3' and antisense 5'– CCC TCC ACG ATG CCA AAG T – 3'.

Luciferase activity

Caco-2 cells were transfected with 1 μg of pGL3-NF-kB plasmid or pNFAT-luc and 0.5 μg of pRL-TK plasmid as a control, in FuGene 6 reagent (Roche Diagnostic Corp., Indianapolis, IN, USA) for 24 h, according to the manufacturer's instructions. The cells were treated with *A. chilensis* for 30 min, and then stimulated with 10 ng/ml TNF-α, 100 ng/ml PMA/ 0.5 μM Ionomycin or solvent for 16 h. The cellular extracts were obtained and the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in a luminometer (Luminoskan, Thermo). The luciferase activity was expressed as the ratio of RLU pGL-NF-kB/pRL-TK or pNFAT/pRL.

Cell viability - MTT assay

Caco-2 cells were incubated with different concentrations of *A. chilensis*, solvent or butyrate (10 mM) for 24, 48, 72 or 96 h. Then, the mitochondrial activity was measured by the modified 3-[4,5-dimethylthiazol 2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). This involves determining the mitochondrial dehydrogenase activity in intact cells by incubation for 4 h at 37° C with MTT (10 μl of 5 mg/ml MTT solution per well). The reaction was stopped by addition of cell lysis buffer (50% dimethylformamide and 20% SDS, pH 7.4). ΔA values at 550–650 nm were determined the following day using an microtiter plate reader (Tecan). The results are expressed as a percentage of control.

Flow cytometry

Caco-2 cells were incubated with the diluted juice of *A. chilensis*, solvent or 10 mM butyrate for 24 h. Then, the cells were stained with Annexin V-FITC and propidium iodide (BD) according to the instruction of the manufacturer. The cells were analyzed on a flow cytometer (FACSCanto II, BD).

Statistical analysis

The results were expressed as the ratio or fold of control increase and depicted as the mean ± s.e. An ANOVA was performed and a Dunnet's multiple comparison test was applied using GRAPH PAD V 2.0. A level of significance of 5% was used.

RESULTS

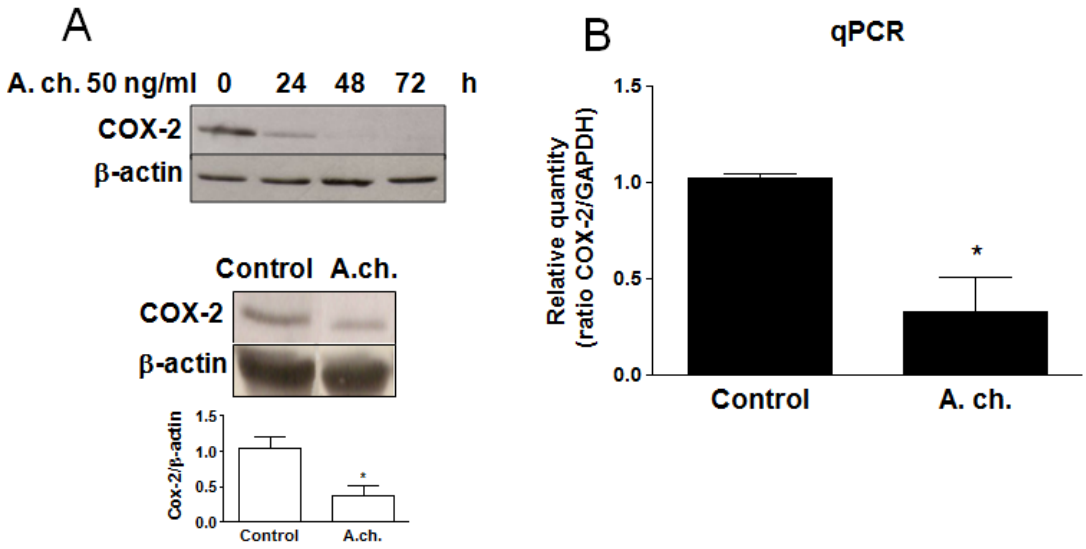
Identification of anthocyanin in A. chilensis concentrate juice

The HPLC analysis showed that the concentrate juice of *A. chilensis* possesses 0.5423% anthocyanins (w/w) / 100 g dry fruit and the following specific anthocyanins species were detected: 0.1359% (w/w) delphinidin 3-*O*-sambubioside-5-o-glucoside, 0.1112 % (w/w) delphinidin 3,5-o-diglucoside, 0.1297% (w/w) cyanidin 3-*O*-sambubioside-5-o-glucoside, 0.0516% (w/w) delphinidin 3-*O*-sambubioside, 0.0735% (w/w) delphinidin 3-o-glucoside, 0.0271% (w/w) cyanidin 3-*O*-sambubioside and 0.0115% (w/w) cyanidin 3-o-glucoside.

***A. chilensis* reduces COX-2 expression in Caco-2 cells**

It has been well established that COX-2 is highly expressed in human colon cancer and Caco-2 cells (Accioly *et al.*, 2008; Eberhart *et al.*, 1994; Kargman *et al.*, 1995; Sano *et al.*, 1995). In this study, Caco-2 cells were incubated with *A. chilensis* for 0, 24, 48 or 72 h and the COX-2 protein was analyzed by immunoblot. The treatment of Caco-2 cells with *A. chilensis* showed a 50% of reduction in the COX-2 protein level from 24 h of incubation compared to the control (Figure 1A). The mRNA expression of COX-2 at 24 h of treatment with *A. chilensis* was analyzed by real-time PCR, and similar to protein expression, a reduction of 50% with respect to the control in the cells treated with *A. chilensis* was observed (Figure 1B). These results indicate that *A. chilensis* interferes with the basal COX-2 expression at the protein and mRNA levels in Caco-2 cells.

Figure 1



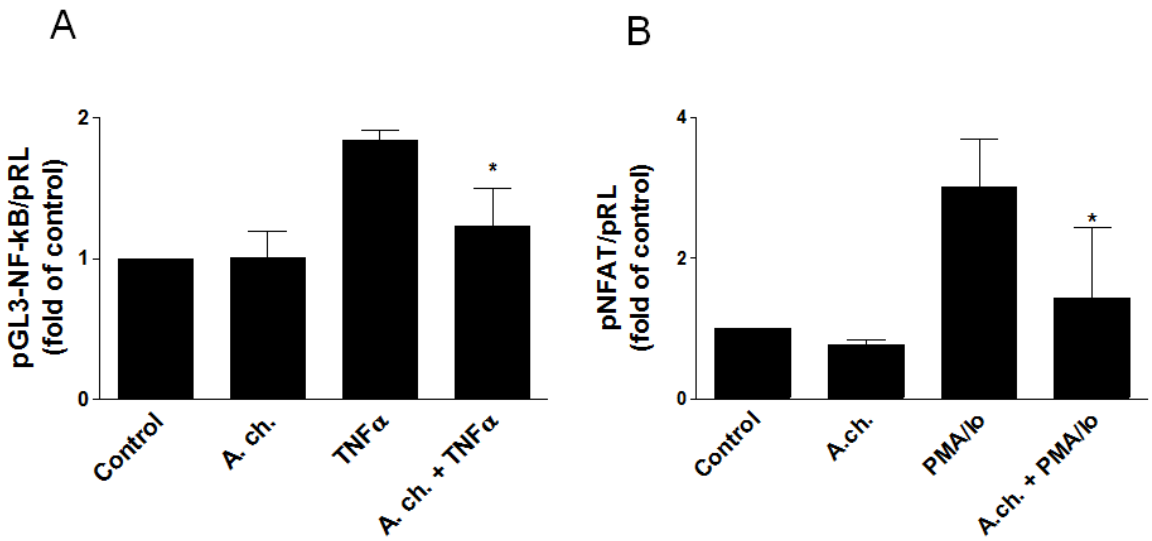
Effect of *A. chilensis* on COX-2 expression. Caco-2 cells were incubated with *A. chilensis* (A.ch.) for 0, 24, 48 or 72 h followed by isolation of total proteins or RNA. (A) The COX-2 protein level was analyzed by immunoblotting using an antibody against COX-2, and the blot was stripped and re-probed with anti-β-actin antibody. (B) The mRNA expression of COX-2 at 24 h of treatment with *A. chilensis* was assessed by reverse transcription and real-time PCR. The graphs show the mean ± S.E. from three independent experiments. *p < 0.05 compared to the control.

NF-κB and NFAT activation are reduced by A. chilensis

NF-κB is a transcription factor activated by pro-inflammatory mediators, which regulates expression of COX-2 (Duque *et al.*, 2006). Caco-2 cells were transfected with the reporter plasmid pNF-κB-luc and pRL-TK, treated with *A. chilensis* or vehicle and incubated with TNF-α or vehicle for 16 h. The treatment with *A. chilensis* itself did not modify the NF-κB-luc activity compared to the control (Figure 2A). Additionally, an increase in the luciferase activity induced by TNF-α was observed, and the pre-treatment of the cells with *A. chilensis* for 30 min and followed by stimulation with TNF-α resulted in an inhibition of TNF-α-induced activation of NF-κB. These results indicate that *A. chilensis* exerts the inhibitory effects on NF-κB only when this pathway was activated by a pro-inflammatory cytokine.

In colon carcinoma cells, the nuclear factor of activated T cells (NFAT) has important implications in the regulation of Cox-2 expression (Corral *et al.*, 2007; Duque *et al.*, 2005). We assessed the effect of *A. chilensis* on NFAT activation in Caco-2 cells transfected with the reporter plasmid pNFAT-luc. PMA/Ionomycin induced the NFAT activation and the pre-treatment with *A. chilensis* statistically decreased this effect, suggesting the participation of NFAT in Cox-2 expression (Fig 2B).

Figure 2



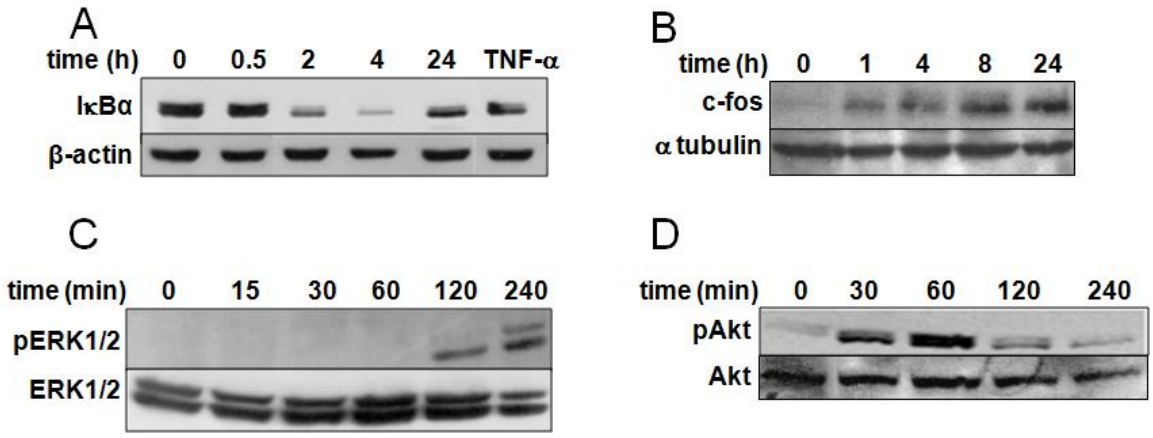
Effect of *A. chilensis* on NF-κB and NFAT activation. Caco-2 cells were transiently transfected with the pGL3-NF-κB (A) or pNFAT (B) and pRL-TK plasmids for 24 h, treated with 50 ng/ml *A. chilensis* or vehicle for 30 min, then TNF-α, PMA/Io or solvent was added for 16 h. Luciferase activity was measured in a luminometer. Each bar represents mean ± S.E., n=3, * $p<0.05$ compared with TNF-α in (A) or PMA/Io in (B).

A. chilensis induces IκBα degradation, c-fos expression, ERK1/2 and Akt phosphorylation

We studied the effect of *A. chilensis* on NF-κB and on c-fos, a component of the AP-1 transcription factor. *A. chilensis* was incubated for different lengths of time (0, 0.5, 1, 2, 4, 8 or 24 h) and the IκBα level and c-fos were evaluated by immunoblot. We assessed the activation of the NF-κB pathway by IκBα degradation, an inhibitory protein of the NF-κB traslocation into the nucleus, which is transcriptionally regulated by the NF-κB pathway. The immunoblotting of IκBα showed that *A. chilensis* transiently reduced the levels of this protein between 2 and 4 h of incubation, suggesting activation of NF-κB. As a positive control, the stimulation with TNF-α was used and a reduction in the level of IκBα was evident (Figure 3A). On the other hand, Caco-2 cells did not express c-fos in the absence of stimuli, but when the cells were treated with *A. chilensis*, an increase in the c-fos expression between 4 and 24 h of treatment was observed (Figure 3B).

Because the transcription factors AP-1 and NF-κB are controlled by ERK1/2 MAPK and Akt pathway, Caco-2 cells were incubated for different times (0 to 240 min) with *A. chilensis*, then ERK1/2 and Akt phosphorylation was analyzed by immunoblot. At time 0, a slight phosphorylation of ERK1/2 was observed, however, the treatment for 2 and 4 h with *A. chilensis* showed an increase in the ERK1/2 phosphorylation, with a maximum effect at 4 h (Figure 3C). On the other hand, *A. chilensis* induced the Akt phosphorylation between 30 and 120 min, with the maximum intensity at 60 min (Figure 3D).

Figure 3



Effect of *A. chilensis* on IκBα degradation, c-fos expression and ERK1/2 and Akt phosphorylation. Caco-2 cells were incubated with 50 ng/ml *A. chilensis* for different times and cytosolic protein extracts were prepared and analyzed by immunoblotting to IκBα detection (A) or total proteins were obtained and used for analysis of c-fos (B), phosphorylation of ERK1/2 (C) and Akt (D) by immunoblotting. The blots were stripped and re-probed with α-tubulin, β-actin or the nonphosphorylated proteins, as a charge control. Picture representative of three independent experiments.

Effect of A. chilensis on cell viability in Caco-2 cells

The effect of *A. chilensis* on cell viability was studied by Annexin V-propidium iodide stain and MTT assay. Caco2-cells were treated with *A. chilensis* for 24 h and apoptosis was assessed by staining of Annexin V-FITC and propidium iodide. Figure 4 (A-C) shows that, at 24 h of treatment, *A. chilensis* did not induce early apoptosis or death of Caco-2 cells (butyrate it was used as a dead control). The effect of *A. chilensis* on viability of Caco-2 cells at different times (24-96 h) was studied by MTT assay. Caco-2 cells treated with *A. chilensis* did not show changes in viability compared to the control at anytime (Figure 4D).

DISCUSSION

The anticancer effects of berries have been mainly attributed to their antioxidant and anti-inflammatory activities. Phenolic compounds, such as anthocyanins, present in different berries show antioxidant activity comparable to commercial antioxidants and cyclooxygenase inhibitory activity (Seeram *et al.*, 2001). In our study, we demonstrated for first time that berry juice from *A. chilensis* reduced the basal expression of COX-2 mRNA and protein in the adenocarcinoma cell line Caco-2. This could be explained by the high content of anthocyanins in the *A. chilensis* juice. Several reports show that anthocyanins reduce COX-2 expression (Kang *et al.*, 2008; Kim *et al.*, 2008; Munoz-Espada *et al.*, 2006; Tsoyi *et al.*, 2008). Additionally, the ability of anthocyanidins to inhibit COX-2 expression in TPA-stimulated HT-29 cells (Kim *et al.*, 2008) and in rats fed with anthocyanin-rich extracts also has been observed (Lala *et al.*, 2006). Moreover, anthocyanin-rich extracts reduced colonic aberrant crypt foci in colon cancer in rats (Lala *et al.*, 2006). Reports suggest an essential role of COX-2 in colon cancer. COX-2 is overexpressed in 40% of human adenomas and in 80% of adenocarcinomas relative to normal mucosa (Eberhart *et al.*, 1994; Kargman *et al.*, 1995; Kutchera *et al.*, 1996). The COX-2 inhibition with nonsteroidal anti-inflammatory drugs (NSAIDs) has been proposed in chemoprevention of cancer (Antonakopoulos *et al.*, 2007; Cha *et al.*, 2007; Harris, 2009). Several studies suggest that either non-selective or selective COX-2 inhibitors produce significant reduction in the risk of colon cancer (Chan *et al.*, 2007; Harris *et al.*, 2008; Huls *et al.*, 2003). A different effect of celecoxib, a selective COX-2 inhibitor, showed a decrease in ICAM-1 and VCAM-1 expression in HT-29 cells, affecting the adhesive properties of the cells (Dianzani *et al.*, 2008; Gallicchio *et al.*, 2008). It has also been described that aspirin and sodium salicylate inhibit COX-2 expression stimulated by VEGF in colon cancer cells (Shtivelband *et al.*, 2003). The inhibitory mechanism of salicylate on COX-2 expression has

been described via inhibition of the binding of CCAAT/enhancer-binding protein beta to the promoter region of COX-2 (Saunders *et al.*, 2001); however previous reports showed that salicylate, at suprapharmacological concentrations, acts via inhibition of the activity of I(kappa)B kinase-beta (Kopp *et al.*, 1994; Yin *et al.*, 1998).

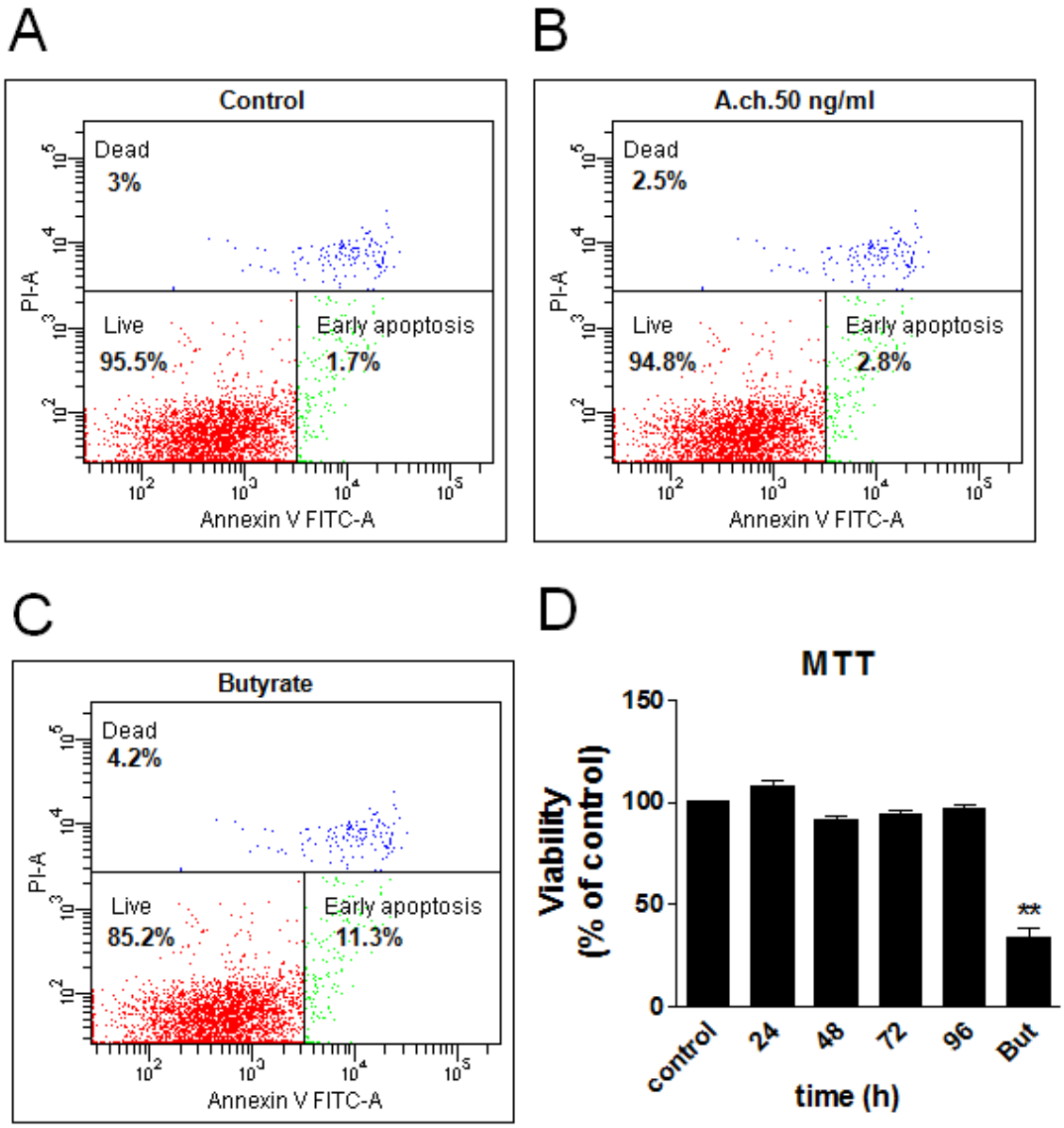


Figure 4

Effect of *A. chilensis* on cell viability. Caco-2 cells were incubated with *A. chilensis* or 10 mM butyrate for 24 h and then stained with AnnexinV-FITC and PI, or analyzed by MTT assay. The AnnexinV-FITC and PI signals were detected by flow cytometry (A-C) and the formazan crystal produced from MTT was registered in a microplate lector (D). Each bar represents the mean ± S.E., n=8. ** $p<0.01$ vs. control.

We assessed the effect of *A. chilensis* on NF-κB activation in Caco-2 cells, and observed that *A. chilensis* did not change the basal levels of NF-κB-luc activity or IκBα degradation at 24 h of treatment. This result and the observations of the effect of *A. chilensis* on the basal COX-2 expression suggest that NF-κB could play a minor role in the basal COX-2 expression in Caco-2 cells. Thus, several potential transcriptional regulatory elements in the 5'-flanking region of the COX-2 gene have been identified, including a

peroxisome proliferator response element (PPRE), two cyclic AMP response elements (CRE), a sterol response element (SRE), two NF-κB sites, an SP1 site, a CAAT enhancer binding protein (C/EBP) motif, two AP-2 sites, an E-box, TATA box and NFAT sites (Corral *et al.*, 2007; Duque *et al.*, 2005; Kang *et al.*, 2007). We observed that NFAT activation induced with PMA/Ionomycin was reduced by *A. chilensis* in Caco-2 cells. Moreover, we did not discard an inhibitory effect of *A. chilensis* on the NF-κB pathway. We showed that *A. chilensis* reduced the TNF-α-induced NF-κB-luc activity, indicating that the NF-κB activation by pro-inflammatory cytokines can be reduced by *A. chilensis*. A previous report showed that juice from different berries significantly inhibited the TNF-α-induced activation NF-κB and COX-2 expression in cancer cell lines of stomach, prostate, intestine and breast (Boivin *et al.*, 2007). Surprisingly, *A. chilensis*, at short time points, induced IκBα degradation, however this effect was transient and returned to the basal level at 24 h.

NF-κB activation is regulated through intracellular signaling pathways such as PI3K/Akt and MAPK cascades (Kyriakis *et al.*, 2001). We observed that *A. chilensis* transiently increased Akt and ERK1/2 phosphorylation after 60 min and 240 min of incubation, respectively. The effect of *A. chilensis* on Akt phosphorylation also displayed a direct relationship with increased IκBα degradation and p65 NF-κB nuclear localization at 4 h of treatment, because it has been extensively demonstrated that the PI3K/Akt pathway can regulate NF-κB activation (Kok *et al.*, 2009). It has also been proposed that the PI3K pathway regulates COX-2 expression. The use of the PI3K inhibitors wortmannin and LY294002 up-regulated the COX-2 expression in HT-29 and Caco-2 cells, and the activation of PI3K with IL-4 and IL-13 down-regulated COX-2 expression, which demonstrate that PI3K negatively regulates COX-2 expression in these cells (Weaver *et al.*, 2001). To assess a role of PI3K in the effect of *A. chilensis* on COX-2 expression, Caco-2 cells were pre-incubated with the LY294002 inhibitor and then stimulated with *A. chilensis* for 24 h, however no differences between the treatments with *A. chilensis* or LY294002 plus *A. chilensis* were observed (data not shown). A known target downstream of the ERK cascade is the immediate early gene c-fos, a component of the transcription factor AP-1, which is expressed and activated via MAPK (Buzzi *et al.*, 2009; Kyriakis *et al.*, 2001). We showed that *A. chilensis* increased c-fos expression from 4 h of treatment, however the role of the increase of c-fos expression induced with *A. chilensis* on Caco-2 cells remain to be studied. On the other hand, ERK1/2 MAPK it has been linked to Caco-2 cell proliferation (Buzzi *et al.*, 2009), therefore we analyzed if the increase in ERK1/2 phosphorylation could be correlated to changes in the cell cycle, however, we did not observe changes by effect of *A. chilensis* (data not shown).

In addition, the treatment with *A. chilensis* at concentrations that reduced COX-2 expression (equivalent to 50 ng/ml anthocyanin) did not modified cell viability of Caco-2 cells. The daily intake of anthocyanins has been estimated to be 12.5 mg/day/person in the United States (Wu *et al.*, 2006). On the contrary, higher concentrations of juice of *A. chilensis* affected the cell viability and induced early apoptosis and death in cancer colon cells (data not shown).

CONCLUSIONS

We showed that berry juice from *A. chilensis* could contains anti-inflammatory activity in colon cancer cells, through reduction in COX-2 expression. Interestingly, *A. chilensis* showed a rapid and transient effect as a cell stimulant. Collectively, these results indicate that *A. chilensis* juice shows diverse and distinctive effects in comparison to other berries (Seeram, 2008), which could be attributable to its composition.

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