

The catechin flavonoid reduces proliferation and induces apoptosis of murine lymphoma cells LB02 through modulation of antiapoptotic proteins

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Abstract: Flavonoids are products of secondary metabolism of plants. They are present in herbs and trees and also act as natural chemopreventives and anticancer agents. *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh., Loranthaceae, is a hemiparasite species that belongs to Argentine flora. Phytochemical studies have disclosed the presence of quercetin, catechin-4 β -ol and pro-anthocyanidine as polyphenolic compounds in the active extracts. We previously demonstrated that ethyl acetate extract was capable of reducing cell proliferation and inducing apoptotic death of lymphoid tumor cells. The aim of the current study is to determine whether or not catechin, isolated from *L. cuneifolia* extracts can induce leukemia cell death and to determine its effect on the cytoplasmatic proteins that modulate cell survival. Our results show that catechin can reduce proliferation of murine lymphoma cell line LB02. The effect is mediated by apoptosis at concentrations upper to 100 μ g/mL. Cell death is related to the loss of mitochondrial membrane potential ($\Delta\Psi$ m) and a down regulation of survivin and Bcl-2 together with the increase of pro-apoptotic protein Bax. In summary, the current study indicates that catechin present in the extract of *L. cuneifolia* is in part, responsible for the anti-proliferative activity of whole extracts by induction of $\Delta\Psi$ m disruption and modulation of the anti-apoptotic proteins over expressed in tumor cells. These results give new findings into the potential anticancer and chemopreventive activities of *L. cuneifolia*.

Introduction

Flavonoids, polyphenolic compounds present in the plant extracts from the heterogeneous family, are functionally related. Plants containing flavonoid derivatives have been found to be active as a disease preventive and therapeutic agent, present in folk medicine preparations that have been used for thousands of years. They are the main components in a healthy diet rich in fruits and vegetables and are concentrated especially in tea, apples and grapes (Havsteen, 2002; Chung et al., 2003).

As natural products, they contain a wide range of biochemical and pharmacological properties; the most investigated of which being their cancer preventive activities (Middleton et al., 2000; Sporn & Suh, 2002;

Ren et al., 2003; Tsao et al., 2004; Moon et al., 2006). Flavan-3-ols and proanthocyanidins have been shown to have the ability to scavenge free radicals, reduce the rate of LDL oxidation, inhibit lipid peroxidation (Korkina & Afanas'ev, 1997), modify enzymes that activate or detoxify carcinogens (Cavenic Lavier et al., 1996), inhibit the induction of the transcription factors by tumor promoters (Shih et al., 2000) and participate in the modulation of the immune response in several biological systems (Cerdá Zolezzi et al., 2000). In tumor cells, flavonoids have been found to have a variety of effects such as cell growth and kinase activity inhibition among them protein kinase CK2 (Larocca et al., 1996, Hakimuddin et al., 2004; Lolli et al., 2012), apoptosis induction (Cerdá Zolezzi et al., 2005; Jin-Mu et al., 2003) and suppression of matrix metalloproteinases secretion



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(Kim et al., 2008). Furthermore, they are able to modify the invasive behaviour of tumor cells (Hazgui et al., 2008) or impairment of in vivo angiogenesis (Jiang et al., 2000).

Apoptosis is a cell suicide program that has been conserved throughout evolution. It leads to cell death through a tightly regulated process resulting in the removal of damaged tissue. It also plays an important role in the development of various diseases including cancer (Debatin, 2004). Interest has been focused on the manipulation of apoptotic processes in the treatment and prevention of cancer. Thus, efforts being directed to the search for compounds that derivate from herbs that have an influence on apoptosis and their mechanism of action (Yi et al., 2005). The chosen agents include flavonoids as quercetin and flavopiridol, both of which have been shown to modulate haematopoietic cell line growth as acute lymphoblastic leukemia and mieloid leukemia through interaction with type II estrogen binding sites (Larocca et al., 1996).

The hydroxylation pattern of the B ring of the flavones and flavonols, such as catechin or quercetin, appears to have a critical influence on their activities, particularly in the inhibition of protein kinase, which can induce anti-proliferate activities. Interestingly, NF- κ B translocation can be inhibited in cells pre-treated with flavonoids because of their influence in binding NF- κ B to its DNA consensus sequence. This modulatory action can occur in the early stages (regulation of oxidant levels, IKK activation) as well as in the late stages (binding of NF- κ B to DNA) of the NF- κ B activation cascade. As a consequence they can influence the lymphoid response by modulating NF- κ B pathway (Mackenzie et al., 2004). This effect on NF- κ B transcription factor in turn regulates the expression of other proteins that control cell growth as well as cell cycle regulators or anti-apoptotic proteins (Nakanishi & Toi, 2005). In our laboratory, we demonstrated that BAY 11 7082, specifically inhibited the constitutively activated factor NF- κ B, as a consequence, murine leukemia LB cells died by apoptosis (Garcia et al, 2005).

Ligaria cuneifolia (Ruiz & Pav.) Tiegh., Loranthaceae, and *Viscum album* L., Viscaceae, are botanically related hemiparasite species. *V. album* is popular in Europe because of the use of their extracts that produce immunomodulatory effects in cancer treatment. Crude mistletoe preparations have been used in some regions as an adjuvant in non-conventional cancer therapies (Jurin et al., 1993). *L. cuneifolia*, known as the Argentine mistletoe, is used in Argentine folk medicine as a substitute for *V. album* (Domínguez, 1928) and has been well characterised by our group (Wagner et al., 1998). We have established that the whole extract inhibits proliferation of murine mitogen-activated lymphocytes and murine leukemia lymphocytes or breast tumor

cells (Fernández et al., 1998); in addition, when whole extracts were fractionated, we found that the ethyl acetate flavonoid fraction was able to significantly inhibit the proliferation of Con A activated splenocytes and LB02 tumor cells. This Inhibition is mediated by apoptotic cell death (Fernández et al., 1998; Cerdá Zolezzi et al., 2005). Phytochemical studies of the flavonoids have disclosed the presence of quercetin, glycosilated in position 3 of the flavonol skeleton and leucoanthocyanidins, catechin 4 β -ol and proanthocyanidines (Wagner et al., 1998).

The aim of the study was to analyse if catechin, a natural flavonoid isolated from *L. cuneifolia* could produce inhibition of lymphoid tumor cell proliferation and if the effect was mediated by apoptosis induction through the modulation of apoptotic regulatory cytosolic proteins. Our results indicate that catechin can reduce the proliferation of murine lymphoma cells; the effect was mediated by apoptosis at concentrations upper to 100 μ g/mL. It was related with a down regulation of survivin and Bcl-2 together with an increase of Bax protein as a consequence of mitochondrial membrane permeabilization. In summary, the present study indicates that catechin present in the *L. cuneifolia* extract is in part responsible for the anti-proliferative activity of the whole extracts by modulation of anti-apoptotic proteins over-expressed in hematologic malignant cells. These results give new knowledge about the potential anticancer and chemopreventive activities of *L. cuneifolia* extracts.

Materials and Methods

Plant material

Specimens of *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh., Loranthaceae, were collected from different host plants and identification was carried out by a member of the team (MLW). Voucher specimens were kept at the Museo de Farmacobotánica 'Juan A. Domínguez', Facultad de Farmacia y Bioquímica UBA (BAF 9018). Classification of the species was performed by MLW using the key, according to Abbiatti (1946).

Reagents

Cell culture media and reagents were obtained from Invitrogen and fetal calf serum (FCS) was purchased from Natocor (Córdoba, Argentina). Chemical reagents were purchased from Sigma (St. Louis, MO, USA) and Merck (BA, Argentina).

Separation and characterisation of flavonoids

Catechin was isolated by extractive separation followed by a TLC, its purity was confirmed by HPLC. 5.0 mg samples of methanolic extract from *L. cuneifolia*

fraction were dissolved in 10 mL of methanol-water 80% (v/v). These were analysed by microcrystalline cellulose column chromatography. The column was eluted from water to HOAc 60% (v/v). The compounds obtained were eluted in methanol, identified by R_f values of TLC, UV, UV/shifts and the purity was checked by comparison of their NMR and MS spectra against authentic samples and literature data (Mabry, et al. 1970; Waterman & Mole, 1994).

Flavonoid stock solutions

Flavonoid stock solutions (10 mM) were solubilised in 95% methanol and were stored in absence of light. Rutine was purchased from Sigma (St. Louis, MO) and used as a control. Catechin and rutin were added to the cultures in small volumes to keep the methanol concentration below 1%. The same volume of methanol was added to the solvent-only control group.

Cell culture

LB02 tumor cell line was used as in previous studies. It was obtained from murine T-lymphoma in our laboratory and maintained in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 20 mM Hepes Buffer, 10 U/mL penicillin and 10 µg/mL streptomycin and 50 µM 2-mercapto-ethanol, at 37 °C with 5% CO₂ in the air (Fernández et al., 1998).

Cell proliferation assay

In order to determine the effect of each flavonoid on cell proliferation, LB02 cells were re-suspended in RPMI 10% FBS in a concentration of 5x10⁵ cells/mL. The cultures were performed in 96-well round-bottomed microtitre plates in presence of different concentrations of catechin or rutin (10, 50, 100 or 200 µg/mL). After 48 h at 37 °C in a humidified atmosphere with 5% CO₂ in the air, cultures were pulsed with 1 µCi of ³H-thymidine/well (Du Pont, NEN Products, Boston, MA, USA) and maintained for an additional 18 h period prior to a semiautomatic method harvest. Incorporated [³H]TdR was measured in a Liquid Scintillation beta Counter, (Beckman MD, USA). Results were calculated from the mean cpm of [³H]TdR incorporated in triplicate cultures. Each experiment was repeated at least three times, with similar results. Inhibition of growth percentage was calculated as:

$$\% \text{ Growth inhibition} = 100 - \left[\frac{\text{cpm treated cels}}{\text{cpm basal control}} \right] \times 100$$

Untreated cells used as basal control, represented 0% inhibition. We also performed a medium control (RPMI+methanol) in the same experimental conditions as previously mentioned.

Analysis of apoptosis induction

Cellular morphology by acridine orange and ethidium bromide staining

LB02 cells 1x10⁶ cells/mL were cultured in RPMI 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂ in air for 48 h in the presence or absence of each flavonoid fraction (10-200 µg/mL). After treatment, cell suspensions were collected, centrifuged at 190 g for 5 min and mixed with 3 µL of acridine orange and ethidium bromide staining (1 mg/mL). Observation was carried out at 400x using an epi-illumination microscope (Carl Zeiss, Germany) with a filter combination suitable for fluorescein visualisation. At least 200 cells were counted and the number of cells with fragmented nuclei, increased cytoplasm and condensed chromatin, reliably indicate apoptosis. These results were determined as previously described (Caldas Lopes et al., 2001).

Annexin V assay by flow cytometry

LB02 cells, 5x10⁵ cells/mL, were incubated with catechin or rutin for 48 h in the same concentration as previously mentioned. To perform the Annexin assay, the Annexin V-FITC Apoptosis detection kit (Biovision, CA) was used following the manufacturer's specifications. For both assays, basal and medium controls were performed in the same experimental conditions.

Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

For the assessment of $\Delta\Psi_m$, cells were treated with both flavonoids at 50-200 µg/mL at 37 °C in a humidified atmosphere of 5% CO₂ for 24 and 48 h. Cells were harvested and loaded with the $\Delta\Psi_m$ sensitive probe tetramethyl rhodamine ethyl ester [TMRE (Invitrogen)], using a concentration of 0.05 µM in RPMI-1640 medium containing 10% FCS for 20 min at 37 °C in the dark and were analysed in a Partec II flow cytometer. A specific marker was established for the probe of untreated cells and was then used to determine low fluorescent intensity events (FL-2). The depolarising agent m-chlorophenylhydrazon [CCCP] (50 µM) was used as a positive control. The percentage of cells in histograms was calculated using the WinMDI 2.9 research software program as previously described (Bustamante et al., 2004).

Analysis of survival and apoptotic proteins by western blot

Cytoplasmatic extracts were obtained from 1×10^7 cells treated with 100 and 200 $\mu\text{g}/\text{mL}$ of catechin or rutin for 48 h. Cells were washed once in cold PBS and then solubilised at 4 °C in buffer lysis (0.02M Tris pH 8, 0.15 M NaCl, 0.1 M NaF, 1mM PMSF, Glycerol 10%, MP-40 1%, 20 $\mu\text{g}/\text{mL}$ aprotinin, 40 $\mu\text{g}/\text{mL}$ leupeptine). Cells were incubated for 30 min in ice and cytoplasmatic proteins were obtained by centrifugation. Protein concentration in the supernatants was determined by Bradford assay and 50 μg of each extract was boiled in buffer loading, resolved by electrophoresis on a 12% polyacrylamide gel and transferred onto a nitro-cellulose membrane. After blocking the membrane in 3% non-fat dried milk, 2% Gly and PBS 1 X over night at 4 °C, it was washed and incubated with specific antibodies to Bax, Bcl-2, Survivin and actin (Santa Cruz Biotechnology, CA) overnight at 4 °C followed by incubation with a horseradish peroxidase labelled secondary antibody for 1.5 h at 37 °C. The reaction was developed using a chemiluminescent detection system (Western blot Luminol Reagent, Santa Cruz Biotechnology, Inc). Gel images from the gels were obtained with a digital camera and subjected to densitometry analysis (Scion Image, NIH).

Statistical analysis

Statistical significance was compared with each treated group and controlled by a one-way analysis of variance (ANOVA) test using the Graph Pad PRISM™ software (Graph Pad Software Inc., San Diego, CA); p-values <0.05 were considered significant from the control group. Each experiment was performed at least for three times. The results were expressed as mean \pm SD.

Results

Effect of flavonoids on LB02 cell proliferation

To verify the effects on tumor cell growth, LB02 cells were incubated or not in the presence of catechin and rutin at different concentrations. We determined that both flavonoids were able to reduce the proliferation rate in a 97.9 \pm 0.1% for catechin and 61.7 \pm 1.0% for rutin at 200 $\mu\text{g}/\text{mL}$ (p <0.01), while at 100 $\mu\text{g}/\text{mL}$ produced 33.3 \pm 4.8% of inhibitory effect for catechin and 30.9 \pm 2.5% for rutin (p <0.01) (Figure 1). The CI50 result in 0.55mM and 0.27mM for catechin and rutine respectively.

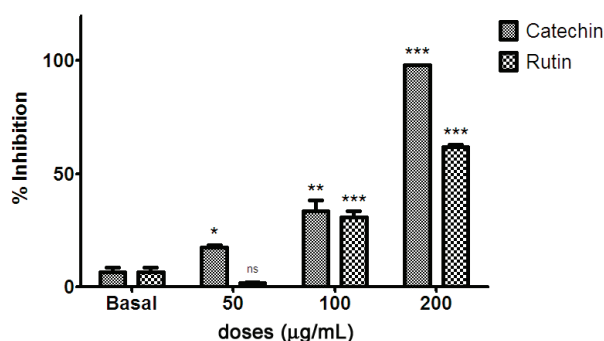


Figure 1. Inhibitory action of catechin on LB02 cells proliferation. Serial dilutions of flavonoids among 0-200 $\mu\text{g}/\text{mL}$ were made in 96-well round-bottomed microtitre plates and cells were added to a final concentration of 5×10^5 cells/mL. The plates were incubated at 37 °C, 5% CO_2 for 48 h and the percentage of inhibition was calculated as:

$$\% \text{ Growth inhibition} = 100 - \left[\frac{\text{cpm treated cels}}{\text{cpm basal control}} \right] \times 100; *p < 0.05; **p < 0.01 \text{ and } ***p < 0.001.$$

Apoptosis induction

To evaluate if the flavonoid isolated from *L. cuneifolia* was able to induce apoptosis, different methods for detection were carried out:

Cellular morphology by fluorescence microscopy

After treatment of LB02 cells with catechin or rutin during 48 h, the morphology of treated cells was studied in order to determine whether or not the inhibition of cell growth was consequence of apoptotic death. It was found that the maximum apoptosis induction was reached at concentrations above 100 $\mu\text{g}/\text{mL}$, being Cat₁₀₀ 36.6 \pm 5.2% (p <0.001), Cat₂₀₀ 61.8 \pm 3.6% (p <0.001), Rut₁₀₀ (17.3 \pm 1.9% (n.s) and Rut₂₀₀ 52.9 \pm 5.2% (p <0.001) vs. 4.9 \pm 1.7% for the untreated cells (Figure 2 A).

Annexin V assay by flow cytometry

We also analysed apoptotic cells by flow cytometry (Annexin V-FITC). This showed a significant apoptosis induction when using 200 $\mu\text{g}/\text{mL}$ of both, catechin and rutin, being 53.6 \pm 5.2% (p <0.05) and 76.5 \pm 3.7% (p <0.01) respectively (Figure 2.B).

Changes on mitochondrial membrane potential ($\Delta\Psi\text{m}$)

Mitochondrial membrane potential changes induced by catechin treatment, in LB02 cells, were

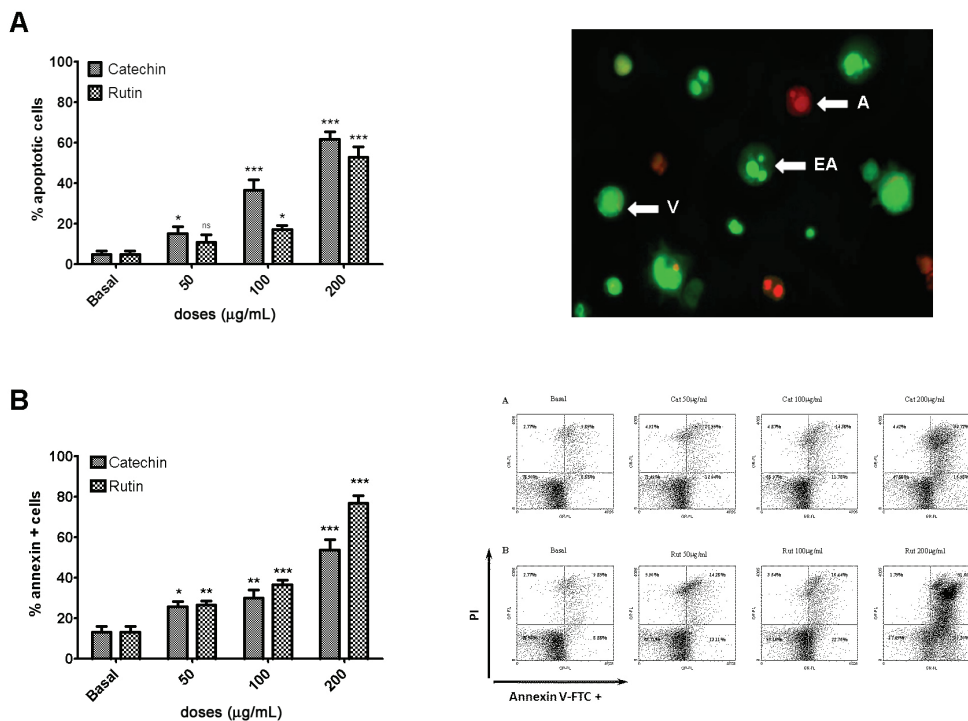


Figure 2. A induction of apoptosis by catechin and rutin. The morphologic alterations were evaluated on cultured cells (1×10^6 cells/mL) after 48 h of treatment using Acridine orange - ethidium propidium by fluorescent microscopy. Experiments were performed three times and each point represents the mean of three assays \pm SD. Apoptotic cells image as marked by arrows; V: viable, EA: early apoptotic, A: apoptotic. B: Bivariate flow cytometry dot plots of FITC-Annexin V (FL1)-stained vs. PI (FL2)-stained LB02 cells treated with catechin and rutin. LB02 cells were stained after 48 h treatment with several concentrations of (A) catechin (0-200 μ g/mL) and (B) rutin (0-200 μ g/mL). The cells were immediately analysed with a flow cytometry equipped with an excitation laser at 488 nm. The FITC- Annexin V (green fluorescence) and the PI (red fluorescence) were collected on a log scale. The lower left quadrant (Annexin V-/PI-) represents viable cells, while the lower right (Annexin V+/PI-) and the upper right (Annexin V+/PI+) quadrants show apoptotic and late apoptotic cells, respectively. Similar results were obtained in three independent experiments. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

assessed using a voltage-sensitive lipophilic cationic probe (TMRE). After 24 h of treatment, the lower dose of catechin was able to induce a 49.6% low mitochondrial membrane potential (depolarised cells), similar to rutin, which induced 53.8% in a dose dependent manner. The effect of the highest concentration of both flavonoids was 77.9 and 61.4% for catechin (Panel B) and rutin (Panel A). Comparable results were obtained after 48 h of treatment, as shown in Figure 3.

Analysis of anti- and pro-apoptotic proteins modulated by flavonoids

To dilucidate the capacity of catechin to modify the relationship between the expression of anti and pro-apoptotic cytosolic proteins, the modulation of Bcl-2, survivin and Bax was analysed by western blot assays. After 48 h of treatment with 100 μ g/mL or 200 μ g/mL of both flavonoids, cytosolic extracts were obtained to determine the levels of apoptotic regulator proteins

(Figure 4). As shown, Bcl-2 and survivin's expression was down regulated by the action of both flavonoids with a significant effect when cells were treated at concentrations of 200 μ g/mL. In contrast, in the same conditions, Bax was increased in a significant manner. Furthermore, the Bax/Bcl-2 ratio showed an increase of between 1.2-5 fold after treatment (Figure 4).

Discussion

Previous publications from our laboratory indicated that extracts of *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh., Loranthaceae, were able to induce tumor cell death. In this report, we show that catechin, isolated from *L. cuneifolia* is able to reduce the proliferation of murine lymphoma cells. This effect was mediated by apoptosis at concentrations upper to 100 μ g/mL. Our results also demonstrate that apoptosis induction is associated with changes in mitochondria membrane potential and a down regulation of survivin and Bcl-2; together with an

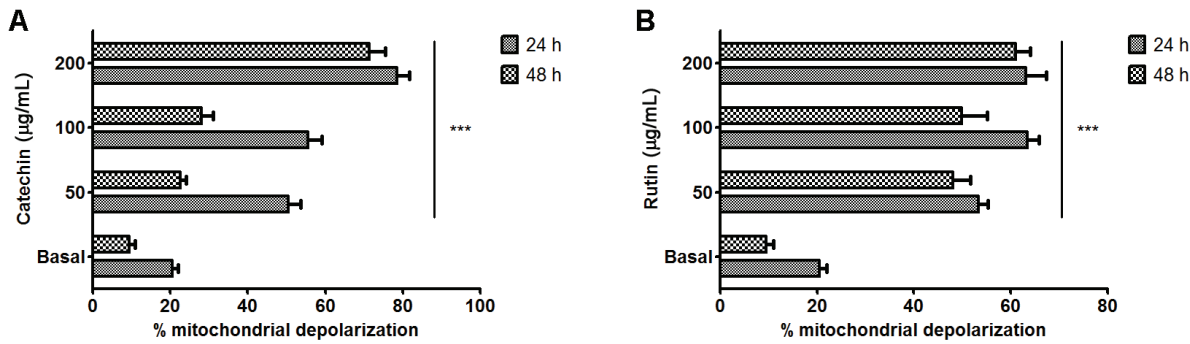
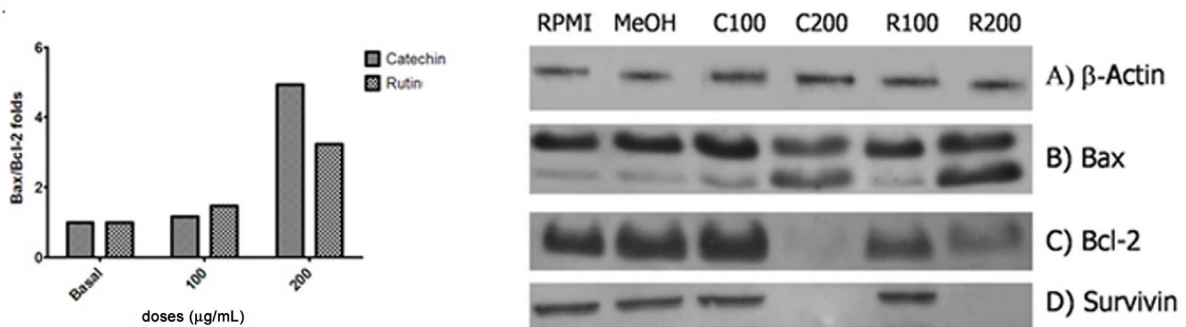


Figure 3. Detection of mitochondria dysfunction during flavonoid mediated apoptosis. LB02 cells were treated with rutin (Panel A) and catechin (Panel B), 50-200 µg/mL, for 24 and 48 h and analysed for mitochondria function by staining with TMRE. Bars represent the percentage of cells with low mitochondrial membrane potential (depolarised cells). Significant differences are indicated *** $p < 0.001$. Data from a single experiment is shown, similar results were obtained in three different experiments.



	Index respect to β-actin			Relationship between Bax/Bcl-2
	Survivin	Bcl-2	Bax	
Basal expression	1.00	1.00	1.00	1.0
Cat 100 µg/mL	0.92	0.90	1.05	1.2
Cat 200 µg/mL	0.18	0.27	1.12	4.9
Rut 100 µg/mL	1.18	0.70	1.09	1.5
Rut 200 µg/mL	0.27	0.59	1.74	3.2

Figure 4. Effect of catequin and rutin on the expression of Bax (B), Bcl-2 (C), Survivin (D); and β-actine (A); catequin 100 µg/mL (C₁₀₀), catequin 200 µg/mL (C₂₀₀), rutin 100 µg/mL (R₁₀₀), rutin 200 µg/mL (R₂₀₀). 50 µg of acellular extracts from treated and non-treated cells were electrophoreaded and transferred onto a nitrocellulose membrane, followed by incubation with primary antibodies and horseradish peroxidase labeled secondary antibody. The reaction was developed using a chemiluminescent detection system. Gel images from gels were obtained with a digital camera, subjected to densitometry analysis. Bars express Bax/ bcl-2 ratio for each treatment.

increase of Bax protein level leads to a 5 fold Bax/Bcl-2 ratio increase. The cell death, which was accompanied by mitochondria membrane potential disruption plus modulation of Bax proapoptotic protein, may indicate mitochondria competency in the death mechanism. This result is critical as the mitochondria potential membrane depolarisation is considered the point of no return in apoptotic death.

Epidemiological studies show a protective effect of flavonoids present in fruits, vegetables and

tea. Chemopreventive agents can be used not only to prevent cancer development but also to treat cancer. Thus, flavonoid mixture of tea origin being proposed as nutritional supplements and as a new way of bio-prevention for some epithelial adenocarcinomas (Hoensch & Kirch, 2005). Furthermore, studies carried out on experimental animal models have indicated that certain dietary flavonoids showed anti-tumor activity and several *in vitro* studies postulating different mechanisms. However, its potential for anti-tumor effects await further

elucidation (Kanadaswami et al., 2005). Advanced knowledge about these bioactive constituents is necessary in order to develop flavonoid-based anticancer strategies.

We previously described that the ethyl acetate flavonoid fraction obtained from *L. cuneifolia*, which represented the bioactive fraction of flavonoid mixture, was able to inhibit tumor as well as mitogen activated normal spleen cell growth. However, the same can not be said for the resting cells; such inhibition was mediated by apoptotic death pathway (Cerdá Zolezzi et al., 2005). Based on these results, we conducted the current study in order to analyse the effect on cell proliferation and the apoptosis induction of catechin isolated from that fraction. As NF- κ B promotes cell survival and proliferation, its down regulation sensitises the cells to apoptosis. We used a murine T Lymphoma cell line, LB02, which constitutively over-expressed transcription factor NF- κ B (Garcia et al 2005); to evidence that the anti-proliferative activity of *L. cuneifolia* flavonid and search the regulatory proteins responsible for the apoptosis induction as a death cell mechanism.

It has been reported that the quercetin flavonoids and flavopiridol have inhibitory effects in either tumor models or in various transformed cell lines (Li et al., 2000). In addition, it has been found that quercetin can arrest cells in the late stages of G1 cell cycle and block signal transduction by inhibiting tyrosine protein kinases and serine/threonine protein kinases (Uddin & Choudhry, 1995). Nevertheless, normal bone marrow was shown to be partially resistant to quercetin actions (Larocca et al., 1996). In the current study, we show that the treatment of LB02 cells by either catechin or rutin can modify the rate of cell proliferation over 90% after 48 h of treatment, similar to that of whole extracts (Wagner et al., 1998; Fernández et al., 1998). The effect was correlated with the induction of apoptosis. Measured by the percentage of cells with the blebbing characteristic of apoptotic cells by exposition of phosphatidylserine on LB02 membrane surfaces and a high number of cells with depolarised mitochondrial membrane potential. Results are also consistent with other reports that showed that apoptosis was induced by flavonoids (Parker et al., 1998; Balan et al., 2007). It was also recently demonstrated that mitochondria appears to be a key target for quercetin, causing a loss of mitochondrial membrane potential (Lugli et al., 2009). In a similar manner, flavonoids present in green tea, epigallocatechin-3 gallate, 6-gingerol and resveratrol can cause a significant suppression of cell proliferation and sensitise cells to apoptosis (Thangapazham et al., 2007; Basu & Haldar, 2009).

NF- κ B factor promotes proliferation and cell survival by enhancing the expression of several genes including Bcl-2, Bcl-XL, cIAP, survivin and TRAF. The proteins coded by these genes function primarily by

blocking the apoptosis pathway. As a consequence, the natural suppression of NF- κ B leads to an abrogation of proliferation which allows cells to come into the death pathways (Nakanishi & Toi, 2005). Most particularly, curcumin has been shown to down regulate the expression of apoptosis suppressor proteins, Bcl-2 and Bcl-XL in several cancer cell lines (Reuter et al., 2008). *Pinus massoniana* bark extract increased the Bax protein expression inducing apoptosis of HeLa cell line (Ma et al., 2008). The apoptotic inhibitory protein, survivin, which regulates cell cycle and death has been shown to increase tumor resistance to apoptotic stimuli in favour of multi-drug resistance. On the contrary, flavonoids can antagonise survivin action. We are currently reporting on the modulation of pro- and anti-apoptotic proteins by treatment of catechin and rutin. In previous studies we determined by EMSA assay that both compounds were able to inhibit NF- κ B nuclear translocation (data not shown), here we show that they can down regulate survivin expression, a critical anti-apoptotic regulator. It may be possible for flavonoids to down regulate cytosolic levels of survivin inhibiting NF- κ B translocation and also enhancing its proteosomal degradation, allowing them to induce apoptotic cell death as previously explained for quercetin (Siegelin et al., 2009).

In addition, the compounds were also able to modify the relationship between the cytosol levels of Bax and Bcl-2, accompanied by mitochondrial permeability transition. Similar results were found by Xie et al. in the MTX- resistant osteosarcoma cells (Xie et al., 2011). When the results are combined, they suggest that catechin could be able to induce apoptosis, most likely acting on intrinsic pathway by alteration of mitochondria permeability potential. An effect that is critical for Bax protein release and apoptosis induction. It has also been demonstrated that flavopiridol, when combined with a histone-deacetylase inhibitor acts on extrinsic pathway. The authors however do not discard that both intrinsic and extrinsic pathway may be implied in breast cancer cell death by flavonoids (Mitcell et al., 2007).

Given that many chemotherapeutic agents act via apoptosis induction, it may be possible for compounds such as catechin to have a beneficial effect as an anti-leukemia agent, resulting in more efficient therapies when combined with a conventional cytotoxic chemotherapy or other apoptosis inducers.

In summary, our results give new findings into the potential of anticancer and chemopreventive activities of *L. cuneifolia*. Thanks to the pharmacological safety of flavonoids, it may now be possible to use them as chemopreventive agents or to combine them with chemotherapeutic agents to either enhance the effect at lower doses or to minimise chemotherapy-induced toxicity.

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Authors contributions

RR and MLW contributed in collecting plant sample and also in the identification and characterization of flavonoids. DLP and AT did most of the laboratory work including cell culture, cell proliferation assays, apoptosis induction determination, western blot and statistical analysis. VC was in charge of cell culture and participated in the apoptotic assays and statistical analysis. SC contributed to cell culture and supervised the laboratory work. EA have designed the experimental work and managed the laboratory work EA and MLW contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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