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Modulatory Effect of an *Urera Aurantiaca* Extract on Immune and Tumoral Cells During Inflammation

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There is a well known link between inflammation and cancer during initiation, propagation and metastasis. *Urera aurantiaca* (UA) Wedd. (Urticaceae) is a medicinal plant used in traditional medicine to treat inflammatory processes with proven *in vivo* antiinflammatory and antinociceptive effects. The effects of a methanolic extract (UA) and a purified fraction (PF) on the proliferation of normal and tumoral lymphocytes under the effect of prostaglandin E_2 (PGE_2) and on nitric oxide production by lipopolysaccharide-stimulated macrophages was evaluated. Both UA and PF stimulated normal lymphocytes but, in presence of PGE_2, a modulatory effect was observed. The normal lymphocyte proliferation induced by PGE_2 was driven by pathways involving both PKC and H_2O_2. In macrophages, UA and PF did not modify cell viability and abrogated the synthesis of nitric oxide induced by lipopolysaccharide. In tumoral lymphocytes, the UA exerted a biphasic effect: at low concentrations it increased cell proliferation, while at high concentrations, it displayed an antiproliferative effect. UA and PF were capable of reverting the proliferative action of PGE_2. The tumoral cell proliferation induced by PGE_2 is related to PKC, ERK 1/2 and MAP Kinase P38 pathways. The observed effects could be attributed to polyphenols, flavonoids and tannins. This work demonstrates the modulatory effects of the UA on different cell types during inflammatory conditions, which reinforces its antiinflammatory action.

Keywords: inflammation; cancer; *Urera aurantiaca*.

INTRODUCTION

Inflammatory processes, exerted as a body response to tissue damage caused by mechanical, chemical or microbial stimuli, play a central role in the pathogenesis of many diseases. Once triggered, this process may be short-lived or become a chronic phenomenon. In the case of acute inflammation, the main cells involved are polymorphonuclear leukocytes, macrophages and endothelial cells, whereas, monocytes/macrophages and lymphocytes are involved mainly in the chronic stage. When these cells become activated, especially monocytes/macrophages, they aggregate and infiltrate tissues where they undergo a respiratory burst, increasing their oxygen consumption and production of cytokines, prostaglandins, reactive oxygen species and other inflammation mediatiors. These events can initiate and also perpetuate inflammatory cascades and cause subsequent tissue damage (Kaplan *et al*., 2007).

Frequently, in the context of a chronic inflammation that leads to autoimmune diseases, T lymphocytes entering the inflamed tissue can activate either macrophages or B lymphocytes to synthesize cytokines and antibodies, respectively. The latter scenario then, worsens the autoimmune disease.

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The role of inflammation in tumorigenesis has now been demonstrated. An inflammatory microenvironment is an essential component of all tumours. Inflammatory responses play decisive roles at different stages of tumour development, including initiation, promotion, malignant conversion, invasion and metastasis. Inflammation also affects immune surveillance and responses to therapy. The tumour microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells and natural killer cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes and mesenchymal cells). These diverse cells communicate with each other by means of direct contact or by cytokine and chemokine production and act in autocrine and paracrine manners to control and shape tumour growth (Grivennikov *et al*., 2010).

Therefore, in the fight against cancer, the isolation and/or synthesis of drugs capable of acting on different cell type, modulating their proliferative rate, is of paramount importance. Medicinal plants are sources of drugs to reach these objectives.

Urera aurantiaca (UA) Wedd. (Urticaceae), commonly known as 'ortiga colorada', 'pino guasú' and 'pica pica', is an Argentinean native herb that is also distributed in Paraguay, Uruguay, Bolivia, and Brazil (Martínez Crovetto, 1981; Rondina *et al*., 2003). UA is used in folk medicine to treat rheumatic pain, varicose veins, furuncles, bruises and inflammation (Martínez

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Crovetto, 1981). This plant species is also used for tooth pain, dermal diseases and trauma (Rondina *et al*., 2003).

The *in vivo* antiinflammatory and antinociceptive effects of the methanolic extract of UA have been previously reported. The UA has proved to have antiinflammatory activity in the ear oedema test in mice (34.3% inhibition); it caused a reduction of myeloperoxidase activity (49.6–68.5%); a reduction of intense dermal oedema and reduction of the intense cellular infiltration by inflammatory cells of the ear tissue; inflammation inhibition (45.5%, 5 h after the treatment) in the carrageenan-induced hind paw oedema test in rats and a decrease in myeloperoxidase activity (maximum inhibition: 71.7%). The UA also caused a significant and dose-dependent inhibitory effect on the increased vascular permeability induced by acetic acid; significant inhibition on nociception induced by acetic acid administered intraperitoneally and orally (ED50: 8.7 mg/kg, i.p.); and presented antioxidant activity in both 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azinobis 3-ethylbenzothiazoline 6-sulfonic acid tests (Riedel *et al*., 2015).

Taking into account the popular medicinal use of UA, its antiinflammatory activity in *in vivo* models and its antioxidant effect, the objectives of this work were as follows: to analyse the effect of the UA on the proliferation of normal and tumoral lymphocytes under the effect of an inflammatory agent such as prostaglandin E_2 (PGE_2), and to evaluate the effect of the extract on nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated macrophages.

MATERIALS AND METHODS

Plant material. *Urera aurantiaca* Wedd. (Urticaceae) was collected in Parque Nacional Cerro Corá, Naranjahai (22° 38′S; 58° 36′W, 288-m elevation, 2.5-m alt.) Departamento Amambay, Paraguay, in March 2008 and identified by Dr R. H. Fortunato. A voucher specimen (no. 9339) is deposited at Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

Extraction. The dried aerial parts of UA were ground into a fine powder and extracted sequentially by maceration with solvent of different polarity. Briefly, the plant powder was first extracted with dichloromethane, then with ethyl acetate, and finally, the marc was extracted with methanol. Solvents were put in contact with the plant material overnight in order to obtain an extract rich in polyphenolic compounds. Secondly, a purified fraction (PF) was obtained from the methanolic extract by means of a preparative paper chromatography performed on Whatman no. 3 using n-BuOH:HOAc:H_2O (4:1:1) as mobile phase. The major band was isolated (PF). After preparation, the PF and the UA (yield: 1.81%) were lyophilized.

Phytochemical analysis. *Thin Layer Chromatography (TLC) analysis*. To screen for the presence of flavonoids, the UA was analysed by TLC on SigmaCell type 100 cellulose with a 254 nm fluorescent indicator on polyester (Sigma) and employing 40% OHAc as mobile phase. The Natural Product reagent was employed for development of the plate. After drying, the spots were visualized under visible and 254 and 366 nm ultraviolet light.

Total phenol content. Total phenol content in the UA was determined by the Folin–Ciocalteu colorimetric method as described by Singleton et al. (1999). The absorbance was measured at 760 nm and compared with a gallic acid calibration curve. Results were expressed as mg of gallic acid equivalents per gram of extract (GAE/g).

Flavonoids determination. Total flavonoids content on the UA was determined by aluminium chloride colorimetric method as described by Chang *et al*. (2002). Briefly, the diluted standard solutions or the extract (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Dilutions of quercetin in ethanol were used to generate the calibration curve (125, 250, 500 and 1000 μg/mL).

Tannins determination. The tannin level was calculated as the difference between the total phenol level and the non-complex residual phenol level (Santos and Mello, 2004), once the tannins are removed from the medium through complexation with casein as described by Amorim *et al*., 2008 with slight modifications. Briefly, 500 mg of casein were transferred into a 25 mL Erlenmeyer flask before adding 5 mL of the extract and 5 mL of distilled water. After 2 hours (time required for the complexing of the tannins to the total protein), the extract was filtered into a 10 mL volumetric flask and its volume was adjusted to 10 mL with distilled water. Tannin levels were expressed as the percentage of the total phenols that were complexed with casein.

Cell suspensions and culture conditions. The RAW264.7 murine macrophage cell line American Type Culture Collection (ATCC) (5×10^5 cells/mL) was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum (FBS) and kept in a humidified incubator at 5% CO_2 and 37°C. Normal lymphocytes were obtained from lymph nodes as described previously (Anesini *et al*., 1996). Cells (1×10^6/mL) were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% FBS (Sigma, St Louis, MO, USA), 2 mM glutamine and 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma, St Louis, MO, USA). The tumoral EL-4 murine lymphocytic leukaemia cell line (ATCC) generated by induction with 9,10-dimethyl-1,2-benzanthracene in C57BL/6 N mice was cultured at the optimal concentration of 5×10^5 cells/mL in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% (FBS), 2 mM glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma, St Louis, MO, USA).

Proliferation assays. Normal lymphocytes were incubated without any treatment (basal) and with PGE_2

(0.01, 0.1 and 1 nM) or the UA (0.1, 1 and 10 μg/mL) alone during 24 h or in the presence of 2 μg/mL Concanavalin A (ConA), during 48 h. Cells were also incubated with 1 nM PGE_2 in the presence of the inhibitors PD 98059 (5 μM), staurosporine (ST) (10^{-9} M), sodium azide (SA) (2 mM), and in the presence of different concentrations of the UA (0.1, 1 and 10 μg/mL). Tumoral cells were incubated without any treatment (basal) or with PGE_2 (0.1, 1 and 10 nM) or the UA (1, 10, 100 and 1000 μg/mL) or with 0.1 nM PGE_2 in the presence of different inhibitors: SB 203580 (10 mM), PD 98059 (5 μM), ST (10^{-9} M), SA (2 mM), and in the presence of different concentrations of the UA (1, 10 and 100 μg/mL) for 24 h. Besides, PF (1 μg/mL) was studied on normal and tumoral lymphocytes proliferation in the absence or in the presence of PGE_2.

The normal lymphocyte proliferation was evaluated by the tritiated thymidine ([^{3}H]TdR Sigma, St Louis, MO, USA) uptake assay. Results were expressed as cpm. Cells were pulsed with [^{3}H]TdR (20 Ci/mmol) for the last 6 h of incubation. Tumoral proliferation was determined by MTT assay and expressed as absorbance (Martino *et al.*, 2013). When cell proliferation was determined by the MTT method, results were expressed as fold change with respect to basal values (assigned value of 1). Results represent mean ± SEM of at least three determinations performed in triplicate

Cell viability measurement and nitric oxide determination. To determine cell viability and NO production, macrophages were incubated during 24 h without any treatment (basal) or with LPS (1 μg/mL) alone or in the presence of different concentrations of the (UA 0.1, 1, 10 and 100 μg/mL). Cells were then centrifuged at 900 *x g* during 10 min. After centrifugation, the supernatant was separated to determine the NO levels and the cells in the pellet were used to determine viability. Cell viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, cells (1×10^6/mL, were incubated in 100 μl of RPMI 1640 containing 10 μl of 5 mg/mL MTT (Sigma, St Louis, MO, USA). After incubation, the formazan formed was dissolved in acidified isopropanol (0.04 N HCl in isopropanol) and read at 570 nm. Results were expressed as fold change respect to basal values (assigned value of 1) (Davicino *et al.*, 2010). The nitrite accumulated in the culture medium, as an indicator of NO production, was measured by the Griess reaction (Becherel *et al.*, 1997). Briefly, 100 μl of each supernatant were mixed with 50 μl of a 1% sulfanilamide solution in 5% phosphoric acid and 50 μl of a 0.1% naphthylene diamine dihydrochloride solution. The mixture was then incubated at room temperature for 10 min, and the absorbance was read at 550 nm. As standard curve, serial dilutions (0–100 μM) of a $NaNO_2$ solution were employed. Levels of NO were expressed as nM/10^6 cells. Moreover the effect of PF at 1 μg/mL was studied on the viability and NO production in macrophages in the absence and the presence of 1 μg/mL LPS.

Results were expressed as mean ± SEM of three determinations performed in triplicate.

Statistical analysis. Data were expressed as the average of triplicate values of three independent experiments. To compare two values, the Student's *t*-test was used. Multiple comparisons were performed by an analysis of variance (ANOVA) and the Dunnett's test. A $p \leq 0.05$ was considered statistically significant.

RESULTS

As a first step, the methanolic extract was standardized in polyphenols, flavonoids and tannins (Table 1). A TLC analysis was done in order to determine the chemical groups present in the extract. The chromatographic system and spray reagent employed are specific for polyphenols. Chromatograms revealed that the UA is made up of at least four compounds; whereas the PF is made up of a major compound (yellow spot, flavonoid) and at least one more polar compound (Fig. 1).

The effect of PGE_2 on normal lymphocytes proliferation was studied. PGE_2 induced cell proliferation in concentration dependent fashion (Fig. 2A). However, the prostaglandin was not able to induce cell proliferation in the presence of the polyclonal mitogen ConA. In order to study the mechanism by which PGE_2 stimulated cell proliferation, specific inhibitors were employed to analyse the intracellular signals involved. Under basal conditions, bothe PD 98059 (an ERK kinase ½ inhibitor) and ST (a PKC inhibitor) increased cell proliferation, while SA (a peroxidise inhibitor) did not induce any change in basal values (Fig. 2B). The co-incubation of PD and ST did not allow cells to proliferate (Fig. 2B).

However, both ST and sodium azide prevented cells from proliferating under the stimulus of PGE_2 (Fig. 2B). Moreover, the UA (1 μg/mL) increased basal and ConA-driven cell proliferation (Fig. 2C). At all the concentrations employed, the UA abrogated the lymphocytes proliferation induced by PGE_2 (1 μg/mL, Fig. 2D). Furthermore, the PF increased normal lymphocyte proliferation and inhibited the PGE_2-induced cell proliferation (Table inserted in Fig. 2).

In an attempt to emulate an inflammation status, the effect of the UA on NO production and viability of macrophages previously stimulated with LPS was studied. LPS decreased cell viability and increased NO production (Fig. 3A and B). The UA alone did not affect cell viability at any concentration assayed; however, in the presence of LPS, the UA employed at the highest concentration (100 μg/mL) reverted the deleterious effects of LPS on cell viability (Fig. 3A). The UA caused a significant decrease in the basal production of NO (Fig. 3A and B). Besides, the UA, employed at 70 and 100 μg/mL, prevented the increase

Table 1. Determination of polyphenols, flavonoids and tannins in the UA

Flavonoids (mg/100 mg)	3.21
Polyphenols (mg GAE/g)	35.4
Polyphenols/tannins ratio	23.9/76.1%

Flavonoids were expressed as mg of quercetin per 100 mg of extract, and polyphenols were expressed as GAE mg per g of extract.

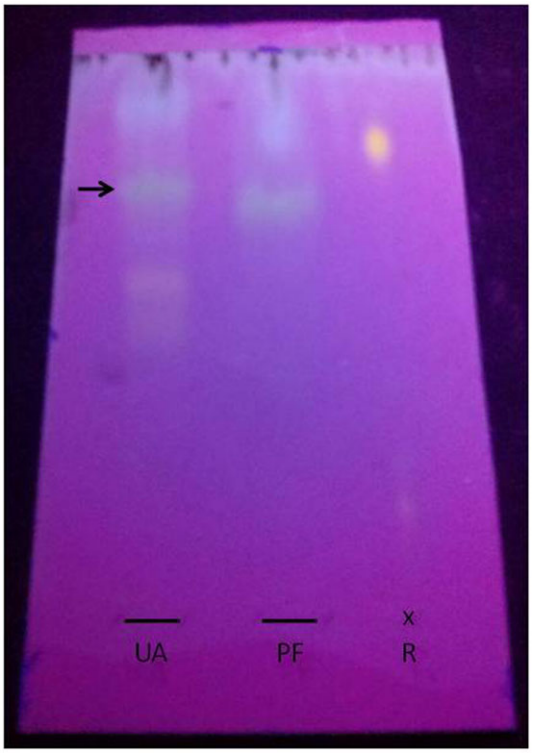


Figure 1. TLC analysis of the *Urera aurantiaca* (UA). The TLC analysis of the UA, purified fraction (PF) and rutin was performed with cellulose as the stationary phase; 40% acetic acid as the mobile phase; natural product as developing reagent. Flavonoids were evidenced by presence of yellow and orange spots. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

of NO production induced by LPS (Fig. 3B). The PF did not affect cell viability but reverted the viability decrease caused by LPS. Besides, the PF alone did not cause any effect on NO production but it blunted the increase induced by LPS (Table inserted in Fig. 3).

Finally, the effect on tumoral lymphocytes proliferation was studied. PGE_2 induced a significant increase in the cell proliferation rate when employed at 0.1 nM at 24 and 48 h (Fig. 4A). When the inhibitors PD 98059, SB 203580 (a MAP kinase 38 inhibitor) and ST were co-incubated with PGE_2, a reversion of the effect caused by the inflammatory agent was observed (Fig. 4B). The UA had a biphasic effect, that is, at low concentrations, it stimulated basal cell proliferation while at high concentrations and at 24 h of cell culture, an inhibition was observed (Fig. 4C). However, in the presence of 0.1 nM PGE_2, and at all concentration assayed, the UA was able to revert the proliferative effect exerted by PGE_2 at 24 and 48 h of cell culture (Fig. 4D). The PF did not modify tumoral lymphocyte proliferation but it inhibited the increase exerted by PGE_2 (Table inserted in Fig. 4).

DISCUSSION

In this work the modulatory activity of an extract of UA on immune cells, such as lymphocytes and macrophages, and on tumoral lymphocytes was demonstrated.

The effect of the UA was studied on lymphocytes and macrophages because these cells participate in the acute and chronic stages of inflammation, in which they collaborate with one another and worsen the process to drive into a chronic state of tissue damage. In order to determine the capacity of the extract to modulate the cell proliferative rate, it was crucial not only to study the effect of the UA on basal conditions but also on cells previously stimulated with inflammatory agents that are usually secreted during the inflammatory process, such as PGE_2.

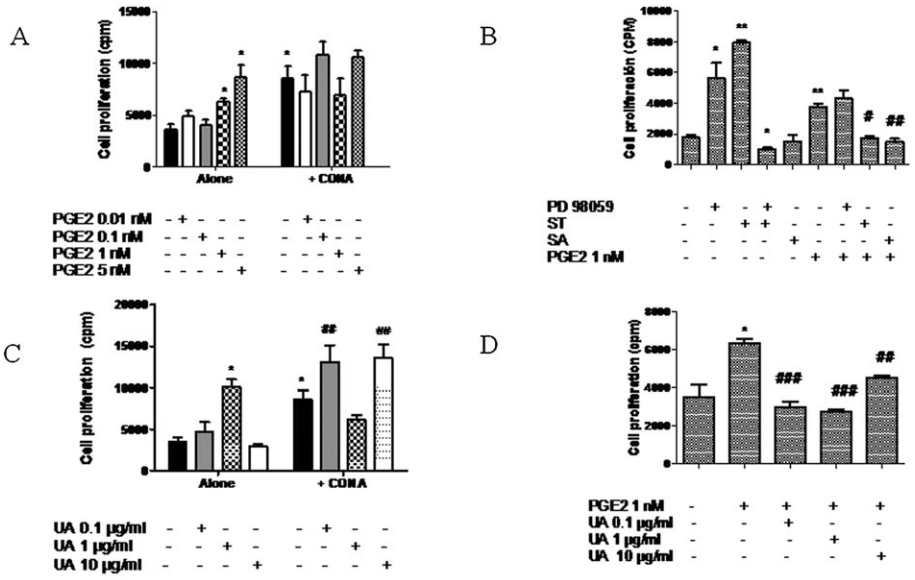
Effect on normal lymphocytes

The proliferation of normal lymphocytes stimulated or not with a polyclonal mitogen such as ConA and under the influence of different concentrations of PGE_2 was studied. Under basal conditions, PGE_2 was capable of inducing T and B cells proliferation in a concentration-dependent manner. This effect was only observed in the absence of ConA, suggesting that the stimulatory effect of PGE_2 was probably masked by the effect exerted by ConA.

It is known that prostaglandins have a regulatory role during inflammatory processes and in the course of the immune response. PGE_2 is known to upregulate the expression of receptors on lymphocytes; to promote immunoglobulin synthesis; and to inhibit interleukin production, cytotoxicity and lymphocyte proliferation. Cellular responses are modulated through the activation of multiple pathways. Pauli *et al.* (1999) have demonstrated that PGE_2 is involved in the increase of lymphocyte proliferation induced by a cell culture supernatant of *Actinobacillus actinomycetemcomitants* (Pauli *et al.*, 1999). Although an inhibitory effect of PGE_2 on T lymphocyte proliferation has been demonstrated, Phipps *et al.* (1991) have suggested that PGE_2 does not only have an immunosuppressive action but also to be pleiotropic and to have stimulatory properties. Other authors have suggested that PGE_2 has a stimulatory effect and an inhibitory action on B and T lymphocytes, respectively (Rojo *et al.*, 1982). Under normal conditions, in presence of ConA, PGE_2 did not increase cell proliferation. It is known that ConA is a polyclonal mitogen that stimulates T lymphocytes, which suggests that the stimulatory effect of PGE_2 on lymphocytes is related to an increase in the number of B and not T lymphocytes. To investigate the mechanism involved in cell proliferation, specific inhibitors that block the proliferation pathways were tested. The inhibition of ERK 1/2 by PD 98059 stimulated basal cell proliferation, indicating that, under this condition, cells could be using other proliferative pathway such as that involving PKC. When cells were treated with ST, a PKC inhibitor, an increase in cell proliferation was observed, indicating the probable activation of the ERK 1/2 pathway. When cells were incubated with the two inhibitors together, cell proliferation was blunted suggesting that the two pathways were involved in cell proliferation and that they can be activated alternatively to accomplish cell proliferation.

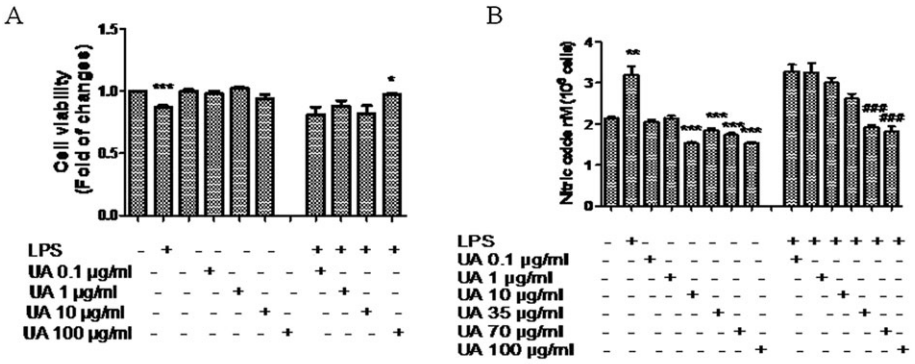
Finally, sodium azide did not modify cell proliferation indicating that under basal conditions this process was not mediated by H_2O_2.

On the other hand, the stimulating effect of PGE_2 on cell proliferation was completely reverted by SA and ST, indicating the participation of PKC and H_2O_2 in



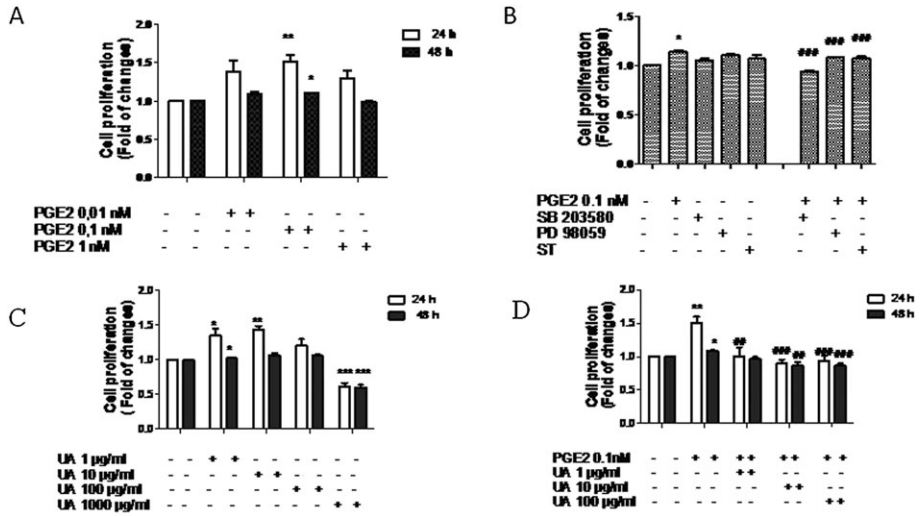
Treatments	Lymphocytes cell proliferation (cpm)
Basal	3500 ± 624
PF 1 µg/ml	8900 ± 890 *
PGE2 1nM	6300 ± 274 *
PF 1 µg/ml+ PGE2 1nM	2500 ± 200 ##

Figure 2. Effect of PGE_2 and the *Urera aurantiaca* (UA) on normal lymphocyte proliferation. (A) Effect of different concentrations of PGE_2 on normal lymphocyte proliferation alone and in the presence of ConA. Cells were incubated for 24 and 48 h in presence of ConA. (B) Effect of cell signal inhibitors (PD 98059, staurosporine (ST), and sodium azide (SA)) alone and on the stimulatory activity exerted by PGE_2 on normal lymphocyte proliferation. Cells were incubated for 24 h. (C) Effect of different concentrations of the UA on normal lymphocyte proliferation alone and in the presence of ConA. Cells were incubated for 24 and 48 h in the presence of ConA. (D) Effect of different concentrations of the UA on normal lymphocyte proliferation in the presence of PGE_2. Cells were incubated for 24 h. Statistical differences with respect to the control groups were determined by ANOVA + Dunnett's test (*$p < 0.05$, **$p < 0.01$, vs. control group without treatment; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. control group with PGE_2 or ConA. Table inserted shows the effect of purified fraction (PF) (1 µg/mL) on normal lymphocyte proliferation, alone or in presence of 1 nM PGE_2, for 24 h. *Significant difference from basal, $p < 0.05$, ANOVA + Dunnett's test; # Significat difference from 1 nM PGE2, $p < 0.001$, Student's *t*-test.



Treatments	Macrophages viability (Fold of changes)	Nitric oxide (nMx 10^6 cells
Basal	1	2.14 ± 0.029
PF 1 µg/ml	0.98 ± 0,010	2.15 ± 0.06
LPS 1 µg/ml	0.87± 0.013 *	3.19 ± 0.21 *
PF 1 µg/ml+ LPS 1µg/ml	0.94 ± 0.0062 ##	1.5 ± 0.10 ##

Figure 3. Effect of the *Urera aurantiaca* (UA) on RAW264.7 macrophages. Cells were incubated for 24 h in the presence of the UA alone or with lipopolysaccharide (LPS). (A) Effect of different concentrations of the UA on macrophages viability alone and in the presence of LPS. (B) Effect of different concentrations of the UA on nitric oxide production alone and in the presence of LPS. Statistical differences from the control group were determined by ANOVA + Dunnett's test (**$p < 0.01$, ***$p < 0.001$ vs. control group without LPS; ###$p < 0.001$ vs. control group with 1 µg/mL LPS. Table inserted shows the effect of 1 µg/mL purified fraction (PF) on macrophages viability and nitric oxide production alone or in the presence of 1 µg/mL LPS for 24 h. *Significant difference from basal $p < 0.05$ ANOVA + Dunnett's test; # Significant difference from 1 nM PGE_2, $p < 0.001$, Student's *t*-test.



Treatments	Tumoral cell proliferation (Fold of changes)
Basal	1
PF 1 µg/ml	1.025 ± 0.10
PGE2 1nM	1.53 ± 0.10 *
PF 1 µg/ml+ PGE2 1nM	0.77 ± 0.06 ##

Figure 4. Effect of PGE_2 and the *Urera aurantiaca* (UA) on EL-4 tumoral lymphocyte proliferation. (A) Effect of different concentrations of PGE_2 on tumoral lymphocyte proliferation. Cells were incubated for 24 and 48 h. (B) Effect of cell signal inhibitors (SB 203580, PD 98059, staurosporine (ST)) alone and on the stimulatory effect exerted by PGE_2 on tumoral lymphocyte proliferation. (C) Effect of different concentrations of the UA on tumoral lymphocyte proliferation. Cells were incubated during 24 and 48 h. D: Effect of different concentrations of the UA on the stimulatory effect exerted by PGE_2 on tumoral lymphocyte proliferation at 24 and 48 h. Statistical differences from the control group were determined by ANOVA + Dunnett's test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. control group without PGE_2; $\#\#\#p < 0.001$ vs. control group with 0.1 nM PGE_2. Table inserted shows the effect of 1 µg/mL purified fraction (PF) on tumoral lymphocyte proliferation, alone or in presence of 1 nM PGE2, for 24 h. *Significant differences from basal conditions, $p < 0.05$, ANOVA + Dunnett's test; # Significant differences from 1 nM PGE_2, $p < 0.001$ Student's *t*-test.

the control of cell proliferation induced by PGE_2. In fact, PKC appeared to be involved in cell proliferation; meanwhile, H_2O_2 seemed to block this process.

It has previously been demonstrated that PGE_2 acting on the PKC pathway induces cell proliferation in many cells types: in human oesophageal squamous cell carcinomas, which display a high proliferation rate, COX-2 is frequently overexpressed producing high levels of PGE_2. In this case, PGE_2 increases c-Myc expression through activation of EP_2 receptor, which is responsible, at least in part, for the mitogenic action of PGE_2. PGE_2 upregulates c-Myc via the EP2/PKC/ERK pathway to stimulate cell proliferation (Yu *et al*., 2009); Moreover, PGE_2, exerts its facilitating activity on agonist-induced platelet activation by priming PKC to activation by other agonists (Vezza *et al*., 1993). Moreover, PGE_2 has proved to be involved in cell invasion in hepatocellular carcinoma via increased β1-integrin expression and cell migration, activating the PKC/NF-kB signaling pathway (Bai *et al*., 2014).

Azide anions bind to trivalent iron in porphyrin complexes, inhibiting the activity of catalase and peroxidases, enzymes that are involved in H_2O_2 breakdown. The azide-mediated blockade induces an increase in the levels of H_2O_2. High levels of H_2O_2 are known to be related to the inhibition of cell proliferation. H_2O_2 is a second messenger acting via the oxidation of proteins that can modulate cell proliferation. Davicino *et al*. (2009) have demonstrated that at low concentrations, H_2O_2 can increase normal lymphocytes proliferation, by modulating ERK and P38 proteins; however, at high concentrations, an opposite effect is observed. The latter observations could explain the reversion of the PGE_2-induced cell proliferation by sodium azide. A relationship between PGE_2 and H_2O_2 can also be established, for example, PGE_2 increases peroxidase secretion in rat submandibular glands (Anesini and Ferraro, 2006) decreasing H_2O_2 levels; besides, PGE_2 seems to have a cytoprotective effect by stimulating the activities of SOD and catalase in the gallbladder muscle of Guinea pigs (Xiao *et al*., 2004). *In vivo* and *in vitro* exposure to PGE_2 alters the extracellular release of H_2O_2 by stimulated murine peritoneal macrophages (McLeish *et al*., 1987). Therefore, it can be speculated that PGE_2 induces cell proliferation by maintaining low levels of H_2O_2 through peroxidase activation.

Finally, the UA was capable of increasing lymphocyte proliferation, with this effect being maximum when the extract was employed at 1 µg/mL. Although the UA did not stimulate lymphocyte proliferation at 0.1 and 10 µg/mL, in presence of ConA, a stimulatory effect was observed at these concentrations, probably facilitating ConA effect.

Under inflammatory conditions emulated by PGE_2, the UA blunted its effect, demonstrating a modulatory effect at all the concentrations analysed. To our knowledge, this is the first report on the effect of the UA on lymphocyte proliferation. It is important to noteworthy that the PF, which contained the major compound (a flavonoid) and other polar components at a lower concentration, mimicked the effect of the whole extract on cell proliferation and blunted the effect of PGE_2, suggesting that one or more

flavonoids are implicated in the effects exerted by the extract.

Effect on macrophages

In order to emulate an inflammatory status, macrophages were stimulated with LPS. LPS increased the levels of NO and decreased cell viability. This effect has already been described by other authors who demonstrated that in response to an infectious challenge, bacterial components such as LPS induce monocyte differentiation into classically activated macrophages or M1; once activated, these macrophages are able to kill pathogens through phagocytosis, production of reactive oxygen species, NO enzymes and inflammatory cytokines (Bueno-Silva *et al*., 2015); LPS-induced toxicity of macrophages may be related to the high levels of $NO_2^- + NO_3^-$, which are known to be responsible for tissue damage (Li *et al*., 2015).

Under basal conditions, the UA did not modify cell viability but it was capable to revert the decrease in cell viability induced by LPS only at the highest concentration. Moreover, the UA decreased NO production when employed at high concentrations. The UA also caused a complete reversion of the NO increased induced by LPS at the same concentrations required to revert the decrease in cell viability exerted by LPS. In previous studies, other species belonging to the Urticaceae family, such as *Urtica circularis*, have proved to have an antiinflammatory effect on macrophages stimulated with LPS. The latter effect was accomplished by inducing a decrease of NO levels (Marrassini *et al*., 2011). Even though the antiinflammatory effect of the UA has previously been demonstrated *in vivo* (Riedel *et al*., 2015), this is the first time that the antiinflammatory activity of UA has been demonstrated in macrophages, reinforcing the results obtained *in vivo*. Also, the PF mimicked the effect of the whole extract. These results support the hypothesis that the active compounds are flavonoids.

Effect on tumoral lymphocytes

As many authors support the idea of the existence of a connection between inflammation and tumour development (Coussens and Werb, 2002; Rakoff-Nahoum, 2006), the effect of PGE_2 on a tumoral cell line proliferation was assessed. PGE_2 increased cell proliferation at 0.1 nM at 24 and 48 h of cell culture, and this effect was partially reverted by ST, PD 98059 and SB 203580, indicating that PKC, ERK 1/2 and MAP Kinase P38, respectively, were involved in the proliferation induced by PGE_2.

The tumour immune microenvironment is featured by a remarkably complex interplay between cells and mediators that can drive a tumour towards limitless growth or imminent destruction. Among these, PGE_2 has proved to be a mediator that not only impacts classical oncogenic signaling pathways in tumour cells, but it also contributes to shifting the tumour microenvironment towards immune suppression and evasion, promoting tumorigenesis. For example, the over expression of COX-2 and the subsequent rise of PGE_2 levels are involved in human oesophageal squamous cell carcinoma proliferation. The later effect is due to an increase in the mRNA and protein expression of c-Myc via PKC/ERK and its association with the binding partner Max. In these tumoral cells, ERK activation by PGE_2 was completely abolished by PKC inhibitors (Yu *et al*., 2009). Moreover, overexpression of COX-2 has been reported to occur in human ovarian cancer and to be associated with poor prognosis. COX-2-derived PGE_2 promotes human ovarian cancer cell invasion (Qiu *et al*., 2014). In colonic malignant cells, PGE_2 increased mTORC1 activity, which induces cell proliferation. The activity of mTORC1 is classically regulated by a complex of two proteins, TSC1 and TSC2 that link extracellular stimuli to mTORC1. Both TSC1 and TSC2 are phosphorylated by multiple kinases including AKT, ERK and GSK3. Because PGE_2 has been demonstrated to influence the activity of these three kinases, the activation of mTORC1 by PGE_2 might result from the coordination of multiple signals (Dufour *et al*., 2014). In addition PGE_2 can promote hepatocellular carcinoma cell invasion, and gastric cancer development.

Collectively, our findings suggest that PGE_2 would upregulate c-Myc via the EP2/PKC/ERK pathway. Thus, the use of antiinflammatory drugs could be beneficial because they would delay the cancer progression and would decrease the recurrence probability (Ruan and So, 2014).

The UA exerted a biphasic effect on cell proliferation: at low concentrations, it increased cell proliferation at 24 h of cell culture, but at higher doses, an antiproliferative activity was observed. Despite its low antiproliferative effect on tumoral cells, the UA completely reverted the proliferative action of PGE_2 on these cells at 24 and 48 h. Results in other studies have demonstrated an antiproliferative effect of other species belonging to the Urticaceae family, for example, an aqueous extract of *Urtica dioica* was found to have a significant growth inhibitory effect on the MCF-7 human breast cancer cell line in a dose-dependent manner; a methanolic extract of *U. dioica* exhibit cytotoxic activity on prostate cancer cells (Fattahi *et al*., 2013); a crude polysaccharide fraction of *Urtica fissa* was found to have a significant inhibitory effect on animal models of prostatic hyperplasia (Zhang *et al*., 2008).

This is the first report on the effect of the UA on the proliferative rate of normal and tumoral lymphocytes, as well as on NO production by macrophages under inflammatory conditions.

In summary, T and B lymphocytes can worsen the inflammatory status in tissues by collaborating with macrophages for the secretion of mediators of inflammation, which in turn, initiate tumoral cell proliferation. The UA was able to decrease the proliferation of lymphocytes and the production of NO by macrophages, and to modulate the proliferation induced by inflammatory agents in tumoral cells; this phenomenon could be crucial in the treatment of diseases that have an inflammatory component.

Finally, polyphenols constitute one of the most numerous and ubiquitously distributed groups of plant secondary metabolites. They are known to be strong antioxidants, preventing oxidative damage and reducing inflammation. The mechanisms by which flavonoids exert their antioxidant and antiinflammatory effects

are generally considered to arise from their ability to scavengr free radicals, to restore the activities of antioxidant enzymes and to regulate cytokine-induced inflammation (Zhang and Tsao, 2016). Polyphenols are a wide class of substances, which include flavonoids and tannins. Tannins have been demonstrated to possess beneficial effects such as antioxidant, antitumor, cardioprotective, antiinflammatory and antimicrobial (Macáková *et al*., 2014). Flavonoids were found to have antiinflammatory activity *in vitro* and *in vivo*. The mechanisms of the antiinflammatory action include the antioxidant activity, the capacity to inhibit the eicosanoid enzymes involved in eicosanoid synthesis, the ability to decrease the production of proinflammatory molecules as well as the modulatory activity on proinflammatory genes expression. They are also able to modulate the function of inflammatory cells such as lymphocytes, natural killer cells, monocytes, neutrophils, mast cells and macrophages (Romano *et al*., 2013). Our results are in agreement with the effects previously mentioned.

To conclude, our results demonstrated that the UA exerted a modulatory activity under inflammatory conditions. This effect was probably accomplished by the flavonoids present in the plant preparations, which would reinforce the *in vivo* observations. These results also support the potential use of the extract as an antiinflammatory drug.

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