

Chilean crude extract of *Ruta graveolens* generates vasodilatation in rat aorta at cellular subtoxic concentrations

Ignacio Jofré Fernández¹, Patricia Navarrete Gómez^{1,2}, Jorge Parodi^{3*}, Fernando Romero Mejía¹, Raúl Salvatici Salazar¹

¹Center of Neurosciences and Peptides Biology, Faculty of Medicine, Universidad de La Frontera, Temuco, Chile

²Laboratory of Molecular and Cellular Neurobiology, Department of Molecular and Cellular Neurobiology, Neurobiology Institute, Campus Juriquilla-Querétaro, Universidad Nacional Autónoma de México, Mexico City, Mexico

³Laboratorio de Fisiología de la Reproducción, Núcleo de Producción Alimentaria, Escuela de Medicina Veterinaria, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile

Email: *jparodi@uct.cl

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ABSTRACT

In Chile elevated percentage of population have cardiovascular diseases, 70% of this populations is a hypertensive peoples. The *Ruta graveolens* (Ruta) is a medicinal plant used in different parts of the world with different therapeutics properties like dermatologic as far as anti-helminthic properties. We analyzed the vascular action measuring the tension to identify vasodilator effect of Ruta extract in norm-tense rat's aorta incubated and measured in isolated organ bath, and evaluating the toxicity effect in CRL-1730 cell line, through enzymatic assay (MTT), confocal microscopy (propidium iodide stain) and flow cytometry (TUNEL assay), including extracellular reactive oxygen species (ROS) production through luminescence assay. The results show with DE₅₀ 29 ± 0.1 µg/mL evidenced vasodilatation, partially endothelium-depend. The cytotoxicity showed with DE₅₀ 304.6 ± 2 µg/mL in enzymatic assay (MTT) while evidenced membrane permeability in high concentrations (1500 µg/mL), DNA fragmentation in absence of oxidative stress in only observed when high concentrations of Ruta are used over the cell culture. The vasodilatation activity is executed in subtoxic concentration and partially endothelium-depend without permeability effect in the membrane and deterioration of the cells viability suggesting a complex effect of Ruta preparation in the regulation of vascular tone.

Keywords: Vasodilatation; *Ruta graveolens*; Toxicity, Endothelium

1. INTRODUCTION

For most people, a blood pressure above 140/90 is considered as hypertension, the upper number, the systolic pressure, is the highest pressure in the arteries when the heart beats and fills the arteries. The lower number, the diastolic pressure, is the lowest pressure in the arteries when the heart relaxes between beats [1]. In Chile, the hypertension prevalence increased from 18.6% in 1988 to 21.7% in 2004. This occurred in all age groups and in both genders. The state of awareness remained stable: 66.9% in 1988 and 66.6% in 2004. The state of treatment increased from 35.6% (1988) to 59.9% (2004), and the state of hypertension control from 7.5 to 30.7%, respectively [2], between the 2009 and 2010, 11.92% of the population, have a high and 2.5% very high risk of acquire a cardiovascular disease. Hypertension is a life-style-related disease and dietary modifications are effective for its management and prevention.

In the last time, the feeding is essential in the therapy of hypertension. The medicinal plants have been used by indigenous communities since time immemorial. Plants has been used for the control of hypertension or reporting vasodilator effect, like *Nige sativa* seeds [3], *Olea europaea* in patients with stage-1 hypertension [4] and *Ginko biloba* [5], with properties of regulation of arterial pressure. Ruta is a medicinal plant introduce to Chile from Europe, used in the non-traditional medicine. In several reports, Ruta has been described like, anti-inflammatory [6], antibacterial [7], antifungal [8], for eyestrain-induced headache [9]. It has also been used as compound for gastric disorders, stiff neck, dizziness and headache [10], however vascular effects are not described.

*Corresponding author.

Essential components of Ruta, are linear furanocoumarins, psoralen derivatives; bergapten, xanthotoxin, imperatorin and isopimpinelin possessing antiproliferative and photosensitizing, using this property in dermatology and substantially applied in the treatment of leucoderma, psoriasis, vitiligo [11], multiple sclerosis and cutaneous. The more complete molecule obtained from Ruta is the Rutin, these molecule has been reporter like a potassium channels blocker [12], also stimulate a vasorelaxation by nitric oxide pathway [13] and explain systemic in vascular muscles. The Ruta showed positive chronotropic and ionotropic effects on isolated right atria [14]. We proposed effect of the Ruta extract over a vascular tone, mediated by the mixture of the molecules present in the Chilean Ruta, including Rutin and another polyphenols in non toxic condition.

2. MATERIALS AND METHODS

2.1. Extract Preparations

The extract was prepared washing Ruta's leaves with deionizer water (Simplicity 185, Millipore, Germany) and dried to 37°C, later pulverized and extracted with ethanol:water (4:1) for 3 days and filtered in vacuum, concentrated in rotary evaporator and lyophilized to -80°C for 2 days (Chris Alpha1-2, Osterade, Germany).

2.2. Tissue Preparation and Vasodilatation Study

The entire of animals are manager with ethical recommendation from NIH. The Protocol was approved by bioethical commission of the University and present to bioethical national committee in Conicyt and revised by FONDEF committee. Sprague Dawley Rats between 200 to 250 g, were sacrificed through decapitation, and aortas artery was obtained by thoracotomy. The artery was mount in petri dish with solution modified Tyrode balanced salt (NaCl 137 mM, KCl 5.4 mM, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 2.7 mM, $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ 0.5 mM, NaHCO_3 11.9 mM, $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ 0.45 mM and $\text{C}_6\text{H}_{12}\text{O}_6 \times \text{H}_2\text{O}$ 5.55 mM at pH 7.3 and osmolarity 300 mOsm) in constant oxygenation (5% CO_2 - 95% O_2). The aorta was dissect of underlying medium (connective tissue) and separated in rings of 4 mm. For the experiments without endothelium, was realized the separating rubbing with cotton's swabs. The mounting was realized through silver rods and hanging in organ bath (automatic Organ Bath) to isometric transducer (50 g) and suspended in Tyrode solution in constant oxygenation (5% CO_2 - 95% O_2) to 37°C. Later, we stimulated the tissue response performing 3 curves of KCl (60 mM) and maintaining for 10 min, further was washed with Tyrode solution. 1 μM of phenylephrine was added to show maximum

tension, 10 μM of acetylcholine was added to show presence of endothelium (relaxing in presence of endothelium). To evaluated the vasodilator effect mediated by extract, the aortas rings was contracted with phenylephrine (1 $\mu\text{g}/\text{mL}$) for 10 min and realized curves doses-response in doses of 1, 5, 10, 50, 100 and 1000 $\mu\text{g}/\text{mL}$. The acquisition realized through LabChart 6.0 Software. Basal tension was 1 g later to 10 min of stabilization.

2.3. Cell Line and Culture Conditions and Toxicity Study

The CRL1730 endothelial cells, was obtained from ATCC (Manassas, USA), was cultured in FluoroDish for confocal observation and coverglass for morphology study. The cells were cultured in DMEM High Glucose medium (HyClone, South Logan, Utah, USA) supplemented with 10% Fetal Bovine Serum (HyClone, South Logan, Utah, USA) and 1% Antibiotic solution Penicillin-Streptomycin-Amphotericin B, (HyClone, South Logan, Utah, USA). Cell growth was done in 5% CO_2 at 37°C, changing the culture media every three days. The cells separated and seed in the special cover for 48 hours, and were treated with 19 $\mu\text{g}/\text{mL}$ and 1500 $\mu\text{g}/\text{mL}$ of Ruta extract for 30 min. Then are washed with PBS 1X fixed with methanol for 3 min. The cells are exposed to propidium iodide (1.5 mM) for 10 min and washed with PBS 1X. The visualization was realized in Confocal Laser Microscopy (Olympus Fluoview 1000, USA) using 488 of excitation and 617 of emission and stain with Quick Panoptic assay visualized in Optical Microscopy and the photography was acquired with Nikon Camera (DS-Fi1c, USA). The cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Eugene, Oregon, USA). The cells were cultured on microplates (96 wells) and maintained at 37°C in a humidified incubator at 5% CO_2 .

The cells were seeded with a density of 50,000 cell/well. For the cytotoxicity assay, cells were washed two times with PBS 1X (HyClone South Logan, Utah, USA), and 200 μL of DMEM High Glucose media were added. The cells were treated with different concentrations of Ruta extract (0.1, 1, 10, 100, 1000 and 10,000 $\mu\text{g}/\text{mL}$) for 24 h. The media with extract was then discarded and the cells were washed two times with PBS 1X. Aliquots of 100 μL of HBSS free calcium and magnesium (HyClone, South Logan, Utah, USA) and 25 μL of MTT (5 mg/mL) were added. After incubation for 4 h at 37°C and 5% CO_2 , the supernatant was discarding and an aliquot of DMSO (Sigma Aldrich, France) was added. After incubation for 1 h at room temperature, the absorbance value was obtained in a Biotek Synergy HT (Vermont, USA) microplate reader at 570 nm.

2.4. ROS Extracellular Production and TUNEL Assay

The CRL1730 endothelial cells were used for observed production of ROS extracellular. For measured by luminescence assay, the cells were incubated with luminol (5-amino-2, 3 hydro-1, 4-ftalazinediona, Sigma Chemical Co., St. Louis, MO) 200 μ M for 15 min at 37°C in darkness in the presences or absence of Ruta extract and immediately quantifying the luminescence in a luminometer Luminoskan (Thermos Scientifics, China), expressing the results as relative luminescence units (RLU). In each analysis, a control without the addition of Ruta extract and positive control cells treated with H₂O₂ 200 μ M, were added.

The DNA fragmentation was evaluated using In situ cell death detection kit (Roche, Germany). Then the cells were fixed in formaldehyde 4% for 15 min to 4°C and permeabilized with Triton X-100 (0.1% in 0.1% of sodium citrate) for 30 min to room temperature. Later, the cells were washed and incubated with 50 μ L of TdT enzyme and dUTP-FITC conjugate for 1 h to 37°C in darkness and humidity. Finally, the cells were washed and measured in Flow Cytometer (FACs Canto II) to 488 nm. In each assay, one negative control without addition of TdT enzyme and one positive control treated

with DNase I (PROMEGA, USA) were added.

2.5. Statistical Analysis

The data for the different functional parameters evaluated were expressed as mean \pm SEM. The data were analyzed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). The differences between the groups were analyzed using the one way analysis of variance (ANOVA) followed by Turkey multiple comparison tests. P values < 0.05 were considered as significant. For the tension analysis we used LabChart5.0 and data processing with Origin 6 for doses-response.

3. RESULTS

3.1. Ruta Extract Produces Vasodilation in Aortas

We made a hydroalcoholic extraction from Chilean *Ruta graveolens* and we obtained total Ruta extract for evaluation of the physiological effect. We present a scheme for the complete protocol (see **Figure 1** in methods).

Our extract presented a principal component which was Rutin and a second component, in a minor concentration, was Quercetin, observed in a HPLC Ms/Ms (**Figure 1**, methods).

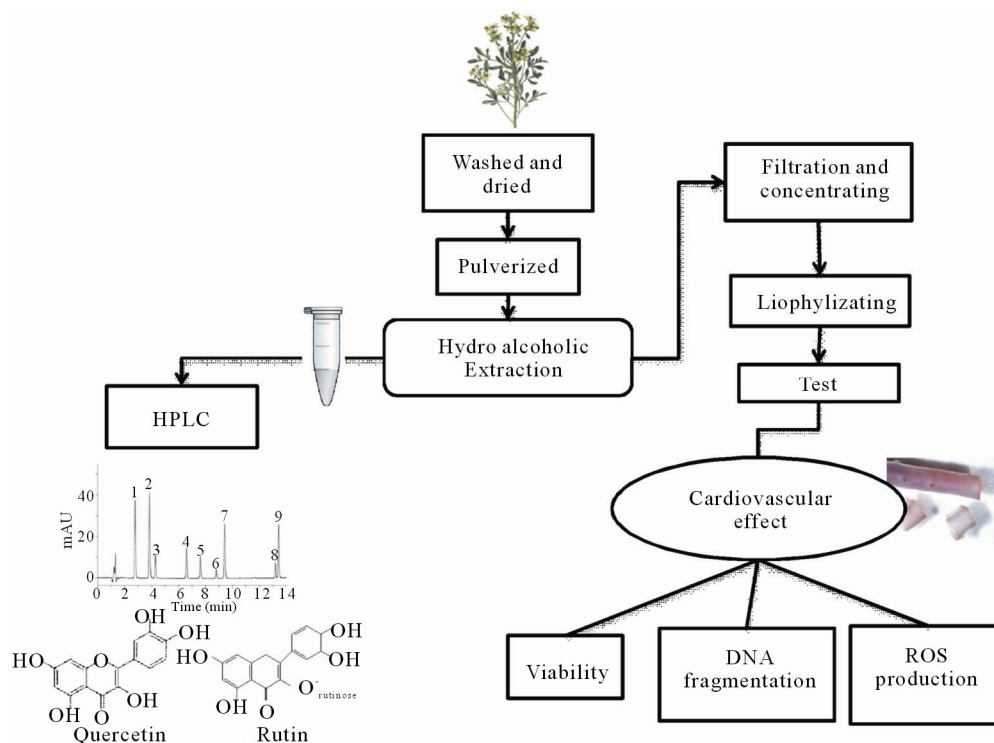


Figure 1. Protocol scheme. Hydroalcoholic extraction was made from fresh Ruta and the contained was observed by HPLC. From these preparations, we separated experiment for study toxicity and function. For test of toxic effect was performed MTT, TUNEL and Luminol dye technique and cardiovascular effect of Ruta extract, was used for functional study.

Was evaluated the vasodilator effect of Ruta extract against rat aortas rings with and without endothelium. The rings are exposed to different concentration of Ruta extract (1 - 50 $\mu\text{g/mL}$) and the tension of the rings are determinate and plotted in a proportional relation with the basal control level (**Figure 2(a)**). The data were plotted in sigmoidal curve (**Figure 2(b)**), with doses effective at 50% (DE_{50}) was observed a value of 29 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ for DE_{50} with and without endothelium, respectively, suggested a relaxation effect non-endothelium dependent. The **Figure 3(A)**, represent example of curves of rings tension experiment, treatment with Ruta extract (50 $\mu\text{g/mL}$) when was contracted previous with KCl 60 mM and phenylephrine. We proposed a change in the rate of the vasodilation when the rings are exposed to Ruta extract, we observed the decay constant TAU (τ) when the rings are exposed to Ruta extract and classical molecules like acetylcholine.

We showed in the **Figure 3(B)** the example of the decay form representative traces in both condition. The bar graph in the **Figure 3(C)**, show the plot of τ rate (seg)

in the experimental condition and we observed that the Ruta's value of τ (300 seg) was minor. The date suggested effect over vascular tone, this regulation is mediated partially-endothelium depend and the value of the τ , different to the acetylcholine, can be explain for a mechanism complex and more indirect.

3.2. Loss Viability with Ruta Extract in CRL1730 Cells

The effect observed over vascular tone indicated a new role of the Ruta extract, however several reports indicated toxic effects of the extract *in vivo* models [15].

We explored the toxic effects of Ruta extract in the endothelial cellular model. We observed the morphology of the cells stain with Quick Panoptic kit (**Figure 4(A)**, upper panel). The cells are exposed 30 min to DE_{50} doses, for vascular effect (29 $\mu\text{g/mL}$, **Figure 4(Ab)**) or to high concentration (1.5 mg/mL, **Figure 4(Ac)**), the microphotography shown alteration of cells when used high concentration of the Ruta extract.

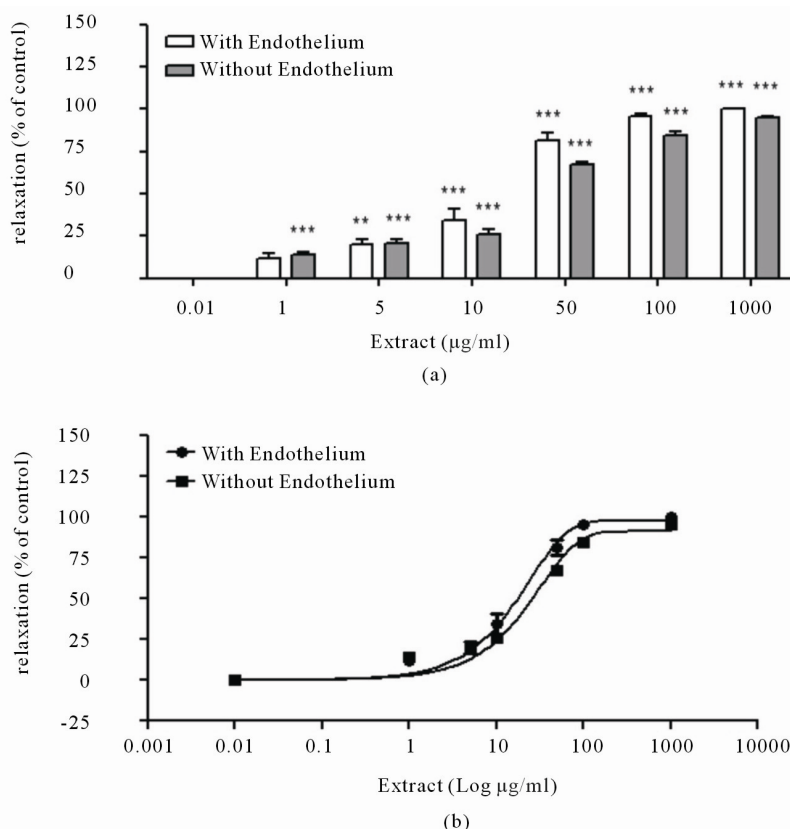


Figure 2. (a) Vasodilator activity of the extract Effect of Ruta extract in concentrations of 1, 5 10, 50, 100 and 1000 $\mu\text{g/mL}$, in conditions of with and without endothelium, with aortas of rats Sprague Dawley and incubated in isolated organ bath; (b) Effect dosis-depend, sigmoidal curve of treatment, DE_{50} 19 $\mu\text{g/mL}$ for the dilatation of aortas. Results shown are means \pm SEM ($n = 8$), significant difference (** $P < 0.01$), (***) $P < 0.001$).

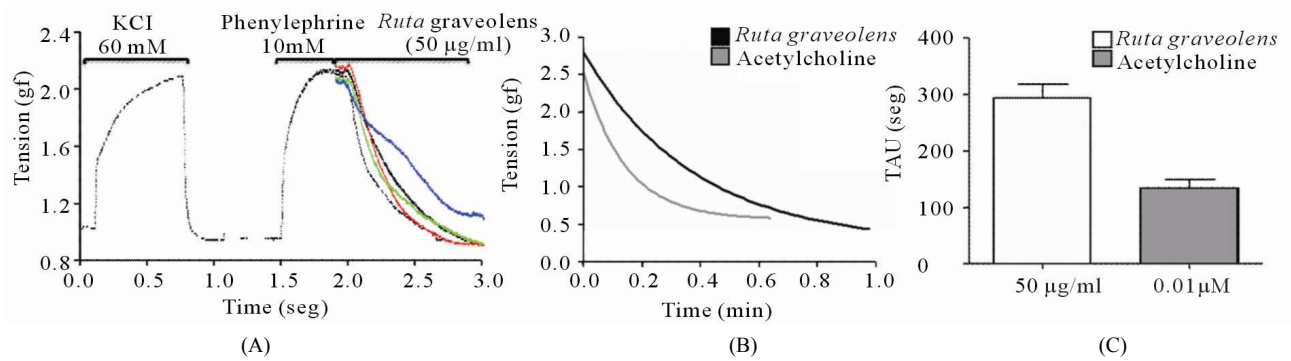


Figure 3. (A) Representative curve of treatments, preincubation of KCl 60 mM, wash and contraction with phenylephrine 10 mM. The effect vasodilator with doses 50 µg/mL of Ruta extract (100% effect); (B) Tension versus time, effect of sigmoidal curve of treatment, DE_{50} 19 µg/mL of Ruta extract for the dilatation of aortas; (C) Time constant (TAU) of the treatments, comparison of vasodilator effect of acetylcholine (0.01 µM) and Ruta extract (50 µg/mL) of the decays time.

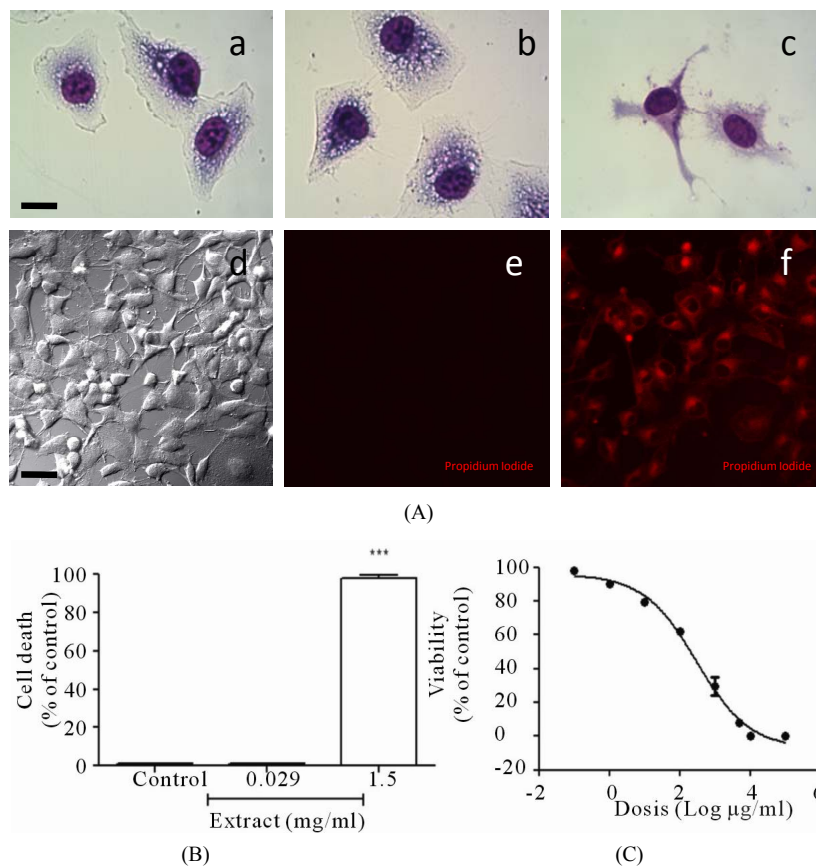


Figure 4. (A) Microphotography of CRL1730 cells treated with Ruta extract. (Aa) correspond to negative control; (Ab) cells treated with 19 µg/mL of Ruta extract for 10 min; (Ac) cells treated with 1500 µg/mL of Ruta extract for 10 min, all of this with Quick Panoptic stain, bars represents 50 µm and Confocal microscopy of cells treated with Ruta extract and dye with propidium iodide; (Ad) transmittance of cells without treatment; (Ae) cells treated with 19 µg/mL of Ruta extract; (Af) cells treated with 1500 µg/mL of Ruta extract, all of this dye with propidium iodide (1 mg/mL), bars represent 100 µm. (B) Counting of cell death, evaluation of the cells dye with propidium iodide treatment with Ruta extract. Percentages represent arbitrary fluorescent units relative to control. (C) Curve doses-response of cytotoxicity effect, activity toxic of Ruta extract in concentration of 0.01 to 100,000 µg/mL, and measured through MTT assay (enzymatic reduction of formazan), and represent DE_{50} 304.6 ± 2 µg/mL. Results show are means \pm SEM (n = 5), Significant differences (***) $P < 0.001$.

When used propidium iodide for see membrane cells permeability (**Figure 4(A)**, lower panel) we observed only effect when the cells are exposed to high concentration of Ruta extract (**Figure 4(Af)**). We can observe not change in the morphology (**Figure 4(Ae)**) or in the permeability when used 29 $\mu\text{g/mL}$ of Ruta extract. We observe the number of the cells death, by count of the propidium positive cells (**Figure 4(B)**) and observed only in the high concentration of Ruta extract a elevated number of death cells. Was evaluated the loss of viability with MTT assay, the results shown loss viability with DE_{50} 304.6 ± 2 $\mu\text{g/mL}$ of extract of Ruta extract. **Figure 4(C)** show the sigmoidal curve of the response with different concentrations of Ruta extract. The date suggested only toxic effect with high concentration of Ruta extract (over 400 $\mu\text{g/mL}$) and moreover we can observe morphological changes. Therefore, the concentrations used for vascular effect not show any toxic effect.

3.3. Extracellular ROS Production with Ruta Extract

The regulation of vascular tone is classic described dependent of nitric oxide production (NO) and this gas induce oxide stress [16,17]. We observed relaxations of aortic rings when are exposed to Ruta, and is effect was independent of endothelium. These suggested another molecule involved in the process. However, the toxic effect in high concentration can be mediated by generation of oxide stress. The toxic effect of oxidative stress can be observed by the DNA fragmentation [18]. We used TUNEL assay and observed by flow cytometer. The **Figure 5(A)** show the dot-plot of the different treatments, and the roman number indicate control non-fluorescent (**Figure 5(AI)**) effect with 29 $\mu\text{g/mL}$ of Ruta extract (**Figure 5(AII)**) and effect with 1.5 mg/mL of extract (**Figure 5(AIII)**), for evaluate the DNA fragmentation when the cultured are exposed to Ruta extract. The

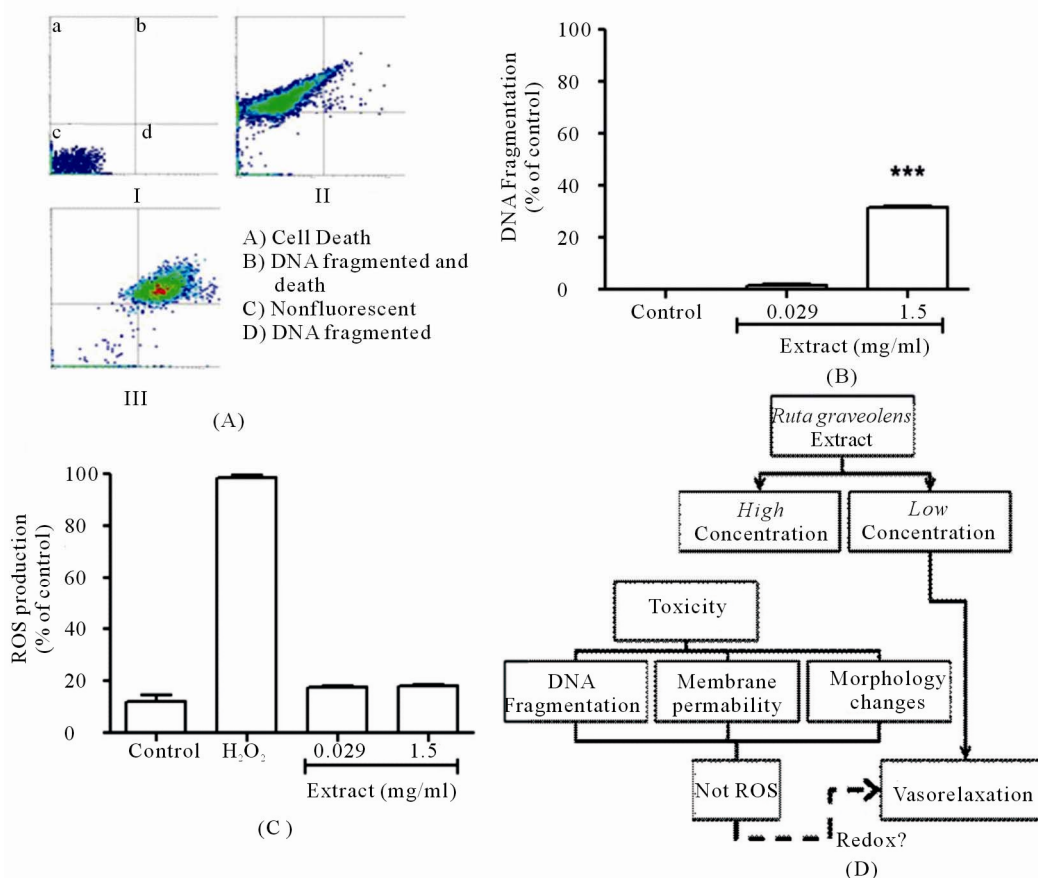


Figure 5. (A) Representative dot-plot of DNA fragmentation, graphic of dispersion of cells population with DNA fragmentation using TUNEL assay. (B) Graphic of DNA fragmentation in cells treated with Ruta extract, 29 and 1500 $\mu\text{g/mL}$, and measured through TUNEL assay in a flow cytometer. (C) Extracellular ROS production, the graphic shown effect of Ruta extract on CRL1730 cells, and measured with luminol probe. (D) Model of effect, the model represent effect of vasodilator activity of Ruta extract in concentrations sub-toxic without DNA fragmentation and extracellular ROS production. Results shown are means \pm SEM ($n = 5$), Significant differences (***) $P < 0.001$.

Figure 5(B) shown a plot when observed the effect of the Ruta extract on DNA fragmentation, only at high concentration (1.5 mg/mL) induces significant changes in the DNA fragmentation (**Figure 5(B)**). Was evaluated the extracellular ROS production in the CRL1730 endothelial cell line at basal level and when the cells are exposed to Ruta extract. Extracellular ROS production was measured by luminescence assays and immediately quantifying luminescence.

These measurements are highly sensitive, allowing the presence of different types of ROS simultaneously, the Ruta extract no produces extracellular ROS production (**Figure 5(C)**) in the low or high concentration, compared to oxidative inductor H_2O_2 (200 μ M).

All the date, suggested a new effect over vascular system, we present overview diagram (**Figure 5(D)**). The effect are observed when used non-toxic concentration of Ruta extract produces oxide stress, suggested a mechanism mediated by stability of redox condition of the rings.

4. DISCUSSION

The plant extract shown specific molecules and direct effect over systemic model, however, the synergism between the different molecules are not completely described [14]. The classic study of preparation of crude extracts and other natural compound, take a total extract and used for describe the physiological effect.

The Ruta plant, contain many molecules like furanocoumarins, psoralen and especially described Rutin. Rutin was described several years ago, as to vasorelaxant via the nitric oxide-guanylyl cyclase pathway and a prostaglandin-mediated mechanism, as well as activation of the ATP-sensitive potassium channel [19] and in addition the traditional medicine in west country used like a abortic and ecboic [20], these result suggested a dual effect of the Ruta extract and the author proposed a complex mechanism for the different effect in our preparation. In Ruta extract we observed the presences of Quercetin, this is a important antioxidant and present several effect in different model, in vascular physiology has been reported like a vasorelaxant mediated by independent nitric oxide pathway [21], the combination of both compound in the crude extract, generates vasodilation in absence of endothelium and present a synergistic effect in the chilean Ruta extract.

We expected a contraction effect, when the rings without endothelium are exposed to the Ruta extract showing a relaxation in any condition in a non-toxic concentration in according to our results, the work of Chiu in 1997, report arterial pressure reduction when mouse model are exposed to Ruta aqueous extract [22].

The Ruta extract induce relaxation in aortic rings, in a

doses-dependent manner, these effect was independent of endothelium cells, suggested a NO independent mechanism and more complex vascular regulation, we proposed a synergistic effect of the different compounds to enhance effect of the Rutin activity and still observed the relaxation in absence of endothelium, we observed a low and high concentration non induce increase of extracellular ROS production and this stabilization of the redox state of the cells are part of the mechanism of the relaxation, in another reports are suggested a relation between redox condition and the vascular tone [23] supporting the idea of indirect effect we find the Ruta extract induce the change in the vascular tone, more slow like a another classic relaxant as acetylcholine, suggested a complex mechanism because the acetylcholine induce a faster relaxation mediated by NO production [24], in our results the more slow response to the Ruta extract are evidence of the more long pathway for generated similar effect. Finally, the Ruta extract a $DE_{50\%}$ concentration can induce change in the vascular tone without toxic effect, however, high concentration induce morphological changes, reduction of viability and increases of permeability of membrane but not induce DNA fragmentation. The DNA fragmentation is recognized tool for explored cellular death in a apoptotic pathway [25], our result suggested membrane effect when the cells are exposed to more high concentration and non apoptotic mechanism of cellular death, however in 1.5 mg/ml of the Ruta extract we can show a significant increases of the DNA fragmentation, is the not more relevant change observed in the cellular death process. We proposed a total extract of the Chilean *Ruta graveolens* (Ruta extract) is a vascular modulator and the summary of the different compound presences in our extract can be used for new approach to development therapeutic against to hypertension diseases.

5. ACKNOWLEDGEMENTS

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