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Oxidative Stress Evaluation in Cancer-Induced Mice Treated with *Ruta graveolens* L. Stem lectins

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Abstract: The production of free radicals is a natural process in aerobic organisms due to the mitochondrial activity. Usually the cells have several mechanisms for scavenging those free radicals, but, in several pathologic processes, such as cancer, those free radicals increase their production, making it impossible to sustain the system stable, generating the condition called oxidative stress. *Ruta graveolens* L. (Rue) is a plant commonly used in traditional medicine, mainly as antiinflamatory; this has been related to some organic components, such as Rutin, but there hasn't been any lectin studies in Rue stem. Lectins are glycoproteins of non-enzymatic and no immune origin, able to bind to simple carbohydrates, which lets them bind selectively to malignant cells against normal cells, killing them via apoptosis and reducing the free radicals level. In this study we intended to characterize rue stem lectins as those weren't reported yet. Also, the anticancer and antioxidant activity of these lectins was evaluated, Rue stem lectins were extracted using a saline solution and semipurified to obtain an enriched extract and administrated to nickel oxide treated mice. Oxidative stress was cuantified using the tiobarbituric acid (TBARS) method to quantify Malondialdehyde (MDA), the Griess method to cuantify Nitrites and enzymatic activity of catalase were cuantified in liver. In this study was found that rue stem lectins are useful as a therapeutic auxiliar, considering that its ratio of antioxidant activity is limited, being a prooxidant agent at high concentrations.

Key words: *Ruta graveolens* L., lectins, oxidative stress, cancer.

1. Introduction

The production of free radicals is a natural process in all aerobic organisms, due to the high production of Reactive Oxygen Species (ROS) during the electron transport chain [1, 2], also having an important role related to the protection against microorganisms and the production of Reactive Nitrogen Species (RNS) as a derivate of Nitric Oxide production as a biological messenger [3]. The overproduction of those compounds would be lethal to any organism, so, there are several mechanisms of defense against them. When the production of free radicals surpasses the scavenging activity over those, it's called oxidative stress, and is highly related to several deceases, such as aging, vascular deceases, diabetes and cancer [4].

Cancer is a complex syndrome characterized for a constant and uncontrollable cellular growth [5], producing a massive amount of free radicals due to its accelerated metabolism, damaging other cell's biomolecules such as lipids, proteins and DNA, generating new malignant cells [6].

Carbohydrates are important in cancer cell recognition as they are usually overexpressed or, not correctly synthesized, being some carbohydrates exposed in the cell membrane [7].

Lectins are glycoproteins with no immune and no enzymatic origin, able to bind to carbohydrates selectively [8], being this process reversible recognizing a malignant cell against a normal cell, also inducing death via apoptosis to the cancerous ones [9]. They are present in any kind of organisms like fungi, bacteria, animals and plants [10]. In plants their functions are considered to be diverse, as storage proteins [11], to bind to nitrogen fixing bacteria [12] and to protect plants against predators, as they are known as toxic when eaten [13]. The richest source for most lectins

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are the seeds or, more generally, the storage organs of plants. These are seeds as in most plants studied so far, but also roots, bark or leaves [11].

Even though Lectins have proven to be a great option for cancer treatment [14-16], there are some evidences of them being toxic for the patient [17], so, it has to be evaluated in several systems and different dosses. Rue lectins hasn't been characterized yet, neither evaluated in a cancer *in vivo* system, even though rue has been used in the traditional medicine as antiinflamatory and recently as an anticancer and antioxidant plant [18-20].

2. Materials and Methods

2.1 Plant Obtention

Rue plants were obtained at the local Xochimilco plants market, leaves and roots were separated and soil was removed. For each extract 100g of stem was weight and then washed with distilled water.

2.2 Lectin Extraction

Saline solution of NaCl 10% W/V was used in a proportion of 3:1 as volume: vegetal material, was macerated and kept in a cold, dry and dark environment for 24 hours, at 4 °C, and then filtrated and centrifugated at 3,500 RPM for 20 minutes and, after collecting the supernatant, centrifugated again at 4,000 RPM for 10 minutes. This was stored at 4 °C and called crude extract, as cited in 2007 by Mendoza *et al* [21].

2.3 Lectin Purification and Characterization

Crude extract was dialyzed using a 3 kDa cellulose membrane in distilled water, changing the dialysis medium every 24 hours and evaluated using Silver nitrate to determine absence of NaCl; and then separated using an affinity column eluting with different sugar solutions at 0.1M, dialyzing again to remove sugar residues, evaluated by dubois method [22] to conclude absence of sugars.

After each step protein concentration was quantified using the biuret method as cited in Burden and Whitney, in 1995 [23], also total sugars with the Dubois method, according to Nielsen 22 in 1998 and reducing sugars using the DNS method as described by Mejía-Giraldo *et al* in 2007 [24]. Hemaglutination was performed in human blood in ABO+ groups as a way to evaluate the possible extracted lectins, as proposed by Rodriguez *et al* in 2004 [25] and the stability of the lectins changing pH to 5, 8 and 11; and temperature at 40, 60, 80 and 100 ºC was evaluated.

2.4 Animals

Female 21 days old mice of the CD-1 train were treated with an intradermal injection of Nickel oxide as cited in Ortiz-Diaz *et al* in 1995 [26]; and kept in vivarium conditions for three months. After this, treated and untreated mice were administrated with rue stem lectins for 10 days, being sacrificed on the 11th day, and liver were obtained and processed to test the different oxidative stress conditions.

Were stablished 9 groups of treatments, being Excipient, 1.5 mg/kg lectin, 1.5 mg/kg lectin + NiO treatment, 1.0 mg/kg lectin, 1.0 mg/kg + NiO treatment, 0.5 mg/kg lectin, 0.5 mg/kg + NiO treatment, Control, which was not treated with NiO nor with lectins, and NiO group.

2.5 Oxidative Stress Evaluation

Tiobarbituic acid (TBARS) assay was performed in order to know the Malondialdehyde (MDA) concentration in liver according to Buege y Aust in 1978 [27]. Nitrites were quantified using the Griess method as cited by Manrique and Ossa in 2010 [28], as an indirect measure of Nitric Oxide production, and Catalase activity was evaluated as proposed by Aebi in 1947 [29]. Protein was quantified in order to compare correctly values obtained as cited previously.

2.6 Statistical Analysis

Statistical analysis of the data was performed using the Tukey test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results and Analysis

3.1 Lectin Characterization

Rue stem total sugar-binding protein added up to 9.29% of total proteins extracted, not being detected reducing sugars. Galactose, sorbitol, saccharose and glucose fractions were found to have hemagglutination activity, and the average pH stability range of these lectins was about 7-8 to be active as it's non-modified equivalent. Temperature range of stability was about 40 °C to 60 °C.

3.2 Malondialdehyde Quantification

Malondialdehyde levels were notably higher in NiO group, being especially low 1.5 mg/kg, 1.5 mg/kg + NiO and 1.0 mg/kg groups against NiO (Fig. 1).This reflects a cronic effect of free radicals in cell membranes, as malondialdehyde is produced as a yproduct of lipidic oxidation.

3.3 Nitrites Quantification

Nitrites levels were significantly low in the 1.0 mg/kg + NiO (Fig. 2). Nitrites are a NO metabolite, so, nitrites production is a reflex of NO production.

3.4 Catalase Activity

Catalase activity in 1.0 mg/kg and 1.0 mg/kg + NiO is higher than the other groups, being 1.5 mg/kg lower than any other group (Fig. 3). This reflects on enzymatic capability to response against a high amount of H_2O_2 as consequence of ROS production.

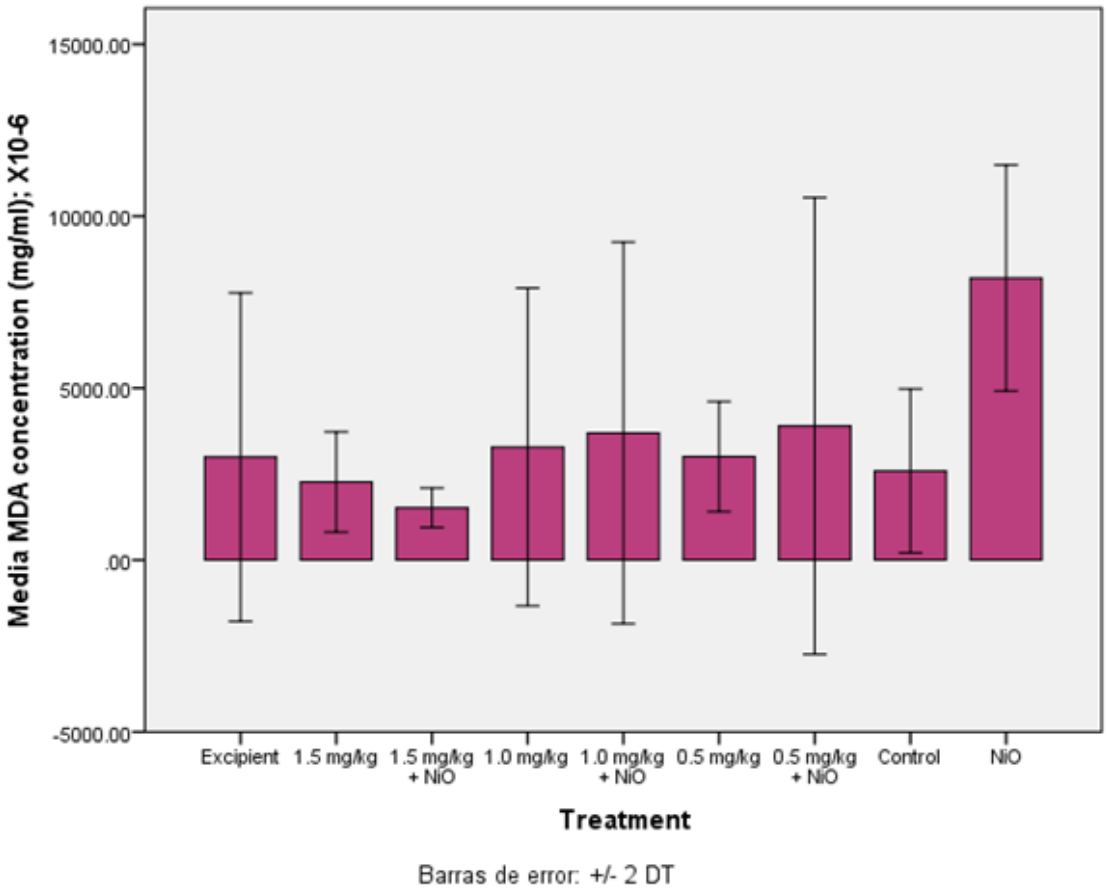

Fig. 1 Malonidialdehyde mean concentration in mice liver. Mice liver was evaluated using TBARS method. Tukey test represents significantly difference in excipient, 1.5 mg/kg, 1.5 mg/kg + NiO, 1.0 mg/kg, 0.5 mg/kg and control groups, $P < 0.05$.

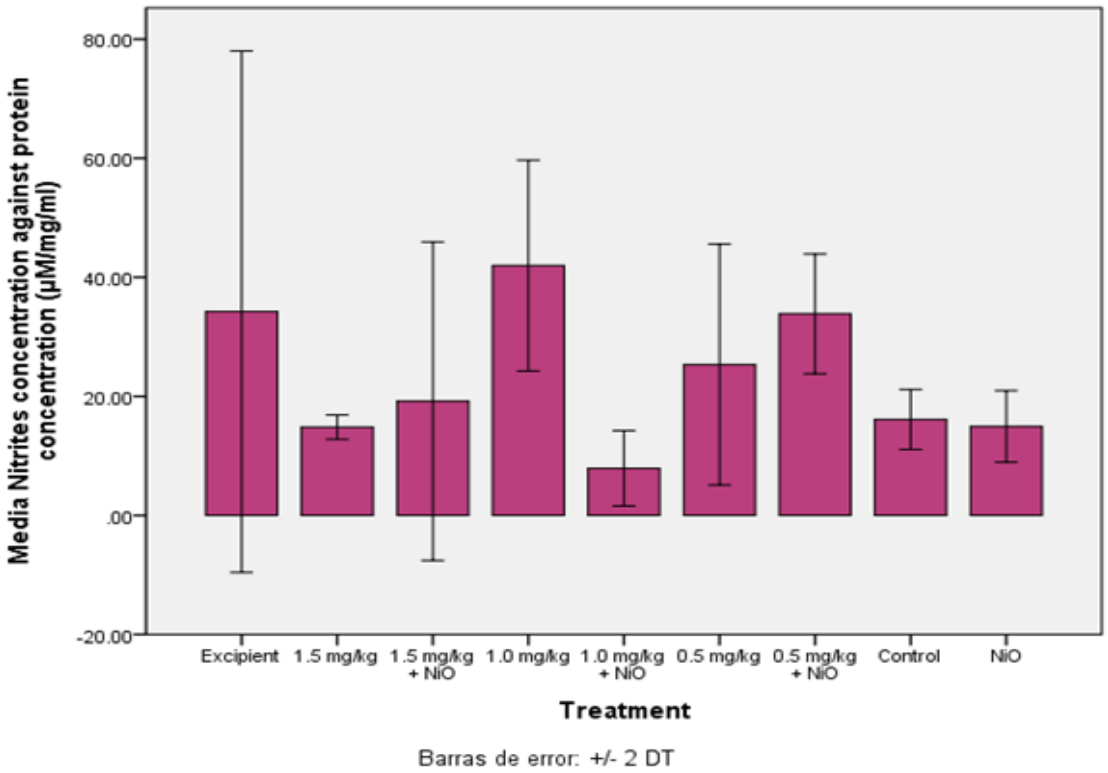

Fig. 2 Nitrites mean concentration against protein concentration in mice liver. Mice liver was evaluated using Griess method. Tukey test represents significantly difference in 1.0 mg/kg and 1.0 mg/kg + NiO groups, $P < 0.05$.

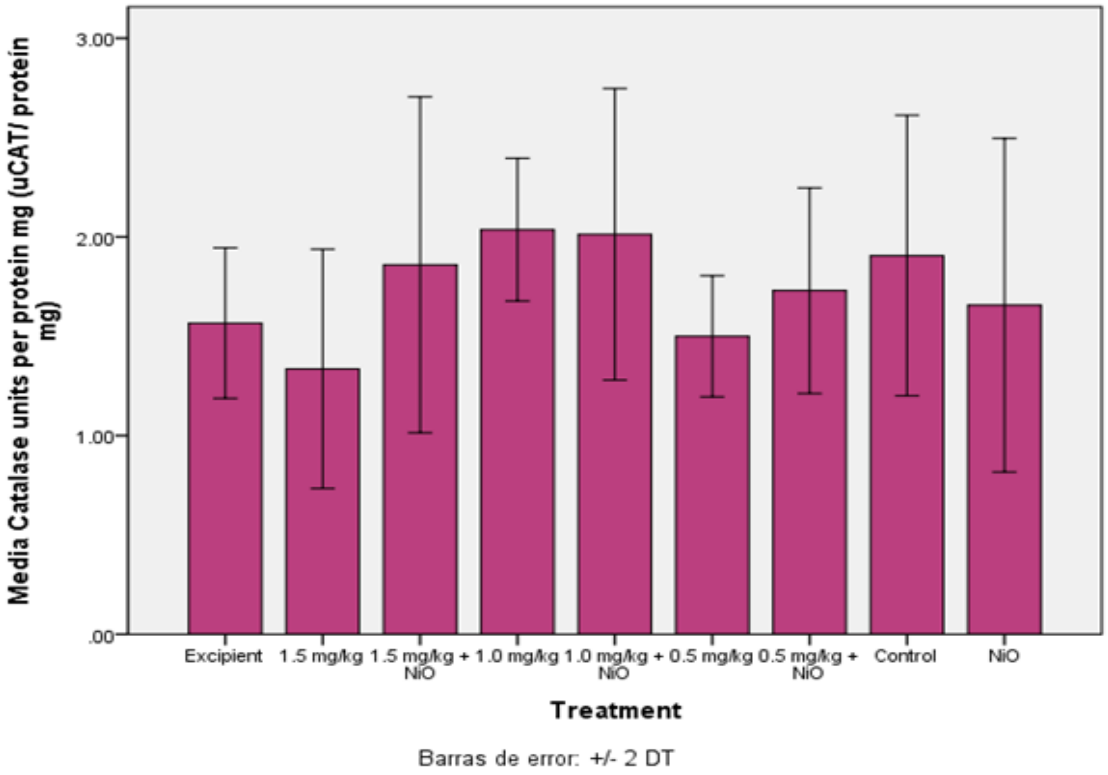

Fig. 3 Mean catalase activity in mice liver. Enzymatic activity was evaluated in mice liver. Tukey test represents significantly difference in 1.0 mg/kg and 1.0 mg/kg + NiO groups, $P < 0.05$.

4. Discussion

The total lectins yield sums up to 9.29% of total proteins present in rue stem, which suits the range from 2 to 10% reported by Hernández-Díaz *et al* in 1999 [30], and can be considered high compared to Concavalin A, which is just 2.2%, according to Ganem-Báez y Martín González in 2000 [31].

Were found more than one hemaglutinant fraction, so it could be said that there are many lectin-type proteins in *Ruta graveolens* stem. The minimum concentration of protein in which hemaglutination was registered was 30 μg/ml, which is notably high compared against *Caesalpinia spinosa* lectin minimum concentration in ABO system, being 7.73 μg/ml for A + y O + blood types according to Mendoza *et al* in 2007 [32], or 1.3 μg/ml for A + y B + blood types as reported by Mendoza-Blanco *et al* in 2012 [33]. Hemaglutinant activity was lost at pH 5 and 11, being stable at pH 8, but not the same as the original activity. This is similar to *Pisum sativum* L. lectin, which has activity in a pH range from 6 to 10, having a total loss of activity at pH 3 and pH 11, and reducing its activity drastically at pH 4 and 5, according to Kabir *et al* in 2013 [34].

MDA production was lower in 1.5 mg/kg and 1.5 mg/kg + NiO and 1.0 mg/kg groups, thus having cell membrane damage reduced against other treatments. Considering lectins as protective against damage in those specific doses, it is being related oxidative stress and malondialdehyde production [35].

Nitrites were notably lower than the rest of groups in the 1.0 mg/kg + NiO, but not in the 1.0 mg/kg dose which was the highest nitrites concentration of all, being probably because these lectins are not suitable for a preventive treatment. Nitrites production is related to Nitric Oxide production by iNOS; related to oxidative stresss [36] and to carcinogenesis, by promoting angiogenesis [37]. This free radical interacts with oxygen molecules and transforms to nitrites and nitrates [38], being much more stable than NO, so decrease of nitrites can be considered a direct reflex of NO production decrease.

Catalase activity in 1.5 mg/kg dose is lowest than any other, being this probably due to a prooxidant effect of lectins at high doses, according to Carrasco-Castilla *et al* in 2012 [39]. Catalase activity is higher at 1.0 mg/kg and 1.0 mg/kg + NiO doses, this could be considered the best dose evaluated in this paper. Catalase activity is normal according to controls at 0.5 mg/kg doses.

Lectin antioxidant activity has been evaluated and determined that it tends to be exposure-response relationship [40], but other authors suggest to consider also the possibility of a synergic activity between lectins and other plant metabolites [41] and even propose that the antioxidant activity is via iron and copper chelation [42], avoiding that those molecules bind to others and maximize oxidative stress; or even that lectins could have a prooxidant effect against other proteins of the same plant [42].

5. Conclusions

Ruta graveolens stem had more than one lectin in its structure, probably being able to bind to more than one kind of cell, being stable at pH 8, in a range of 40-60 ºC. The evaluated lectins were able to regulate oxidative stress in a cancer system, at 1.0 mg/kg dose, generating a prooxidant effect at higher doses. *Ruta graveolens* lectins were not suitable as antioxidant in an unaltered system.

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