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Analysis of the effect of an extract of *Cestrum parqui* L´Herit (Solanaceae) on the peroxidation of rat liver microsomes

Javier L. Barberón*, María B.Ventura, Patricio J. Leaden, Pedro A. Zeinsteger, Alejandro Palacios

Cátedra Bioquímica. Facultad de Ciencias Veterinarias. Universidad Nacional de La Plata. 60 y 118 S/N (1900) La Plata, Buenos Aires

*Correspondence to: Professor MV Javier L. Barberón, Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC296, 1900 La Plata, Argentina. E-mail: jbarberon@fcv.unlp.edu.ar

Abstract *Cestrum parqui* L'Herit (CP, Solanaceae) is a plant native to South America that has been used in popular medicine in countries of the region for the treatment of skin diseases, among others. Despite the presence of toxic compounds, other phytochemicals exert antioxidant properties. Present work was carried out to determine the qualitative composition and the effect on the peroxidation of rat hepatic microsomes of a CP methanolic extract. Different chemical reactions were used to determine the presence of phytochemicals, and the concentration of flavonoids. Rat liver microsomes were incubated with different concentrations of CP extract (0.1, 0.2, 0.3 and 0.4 mg) in an *in vitro* non-enzymatic ascorbic acid-Fe^{+2} system in order to determine the oxidative effect on membranes, and to quantify peroxidation level in standardized conditions. Peroxidation was quantified in a liquid scintillation counter Packard 1900 TR by chemiluminescence in cpm (counts per minute). Microsomal membranes without extract were used as controls. Analyzing the results, qualitative phytochemistry demonstrated the presence of flavonoids. Regarding antioxidant activity, it was observed that rat liver microsomes incubated with CP extract were protected against peroxidation when compared to controls.

Keywords *Cestrum parqui*, Antioxidants, Peroxidation, Microsomes

Introduction

Cestrum parqui L'Herit (CP, Solanaceae)is a plant native to South America[1], commonly known as "green cestrum", "duraznillo negro" or "palqui" [2]. It is an annual shrub of 0.80-2.50 m height, branched, with alternate lanceolate to elliptic, 5-13 cm long and 1-4 wide leaves. The flowers are tubular, yellow, about 2 cm long and arranged in axillary clusters of upper and terminal leaves. It has ovoid violet-blackish berries, 4-5 cm long [3].Several compounds with toxicological properties have been identified in extracts prepared from CP(kaurene glycosides, parquin and carboxiparquin), substances synthesized by the plant as defense mechanism against insects and herbivory [4]. When eaten by cattle, the principal lesion caused by the phytotoxins is severe periacinar coagulative necrosis of hepatocytes [5-6], a consequence of the inhibition of mitochondrial respiration and ATP synthesis by means of altering the ADP / ATP carrier system through the membrane of the organelle, blocking the translocation of adenine dinucleotide [7]. Such circumstances take place when food is scarce, particularly during the dry season, when CP is still green and -despite its bad odor and taste- animals feed on it due to extreme hunger [8].

Clinical signs of the intoxication are characterized by behavior alteration (aggression or depression), anorexia, paresis of hind limbs, incoordination, muscle tremors, atony and ruminal pain, dry stools with mucus and blood streaks, sometimes accompanied by tenesmus and moans [9]. Occasionally, the animal remains immobile with its head resting on nearby objects. At necropsy the surface of the liver appears as "nutmeg" (toxic reticulate), edema may be present in organs of the digestive system as well as hard, dry stools inside the intestines. The most important alteration at the histological level is liver necrosis, characterized by coagulative necrosis (evidenced by karyorrhexis, karyolisys and pyknosis), that affects the centrilobular, periportal and intermediate region. There is no effective treatment for this poisoning [10].

Despite its toxicological properties, CP has been used by humans in folk medicine in Latin American countries such as Chile and Argentina. Its use as a medicinal plant is due to the presence of substances such as polyphenols and flavonoids [11]. The latter are micronutrients, some of them related to the reduction in the risk of metabolic diseases such as diabetes, as well as cancer, cardiovascular [12-13], and neurodegenerative disorders [14-16]. Such properties are related to their antioxidant and anti-inflammatory activities, since they act as scavengers of reactive oxygen species, which are known to participate in lipid peroxidation of cell membranes. The latter is a process that involves the stages of initiation, propagation and termination. Lipid peroxidation is a branching chain reaction consisting of four main stages: (1) chain initiation, (2) chain propagation, (3) chain branching and (4) chain termination [17].

$LO_2^* + LO_2^* \ (k) \rightarrow P^* \rightarrow P + \phi h\nu$ (chemiluminescence)

This reaction is particularly interesting since it is accompanied by chemiluminescence, which intensity (*I)* may serve as a measure of peroxide free radical (LO_2^*) concentration, according to the following equation:

$I = K \phi k [LO_2^*]^2$

Where ϕ represents the chemiluminescence quantum yield and *k* the coefficient depending on the net sensitivity of the instrument. LO_2^* is a free radical produced from lipid molecules [18].

Microsomes are interesting organelles for peroxidation studies, being a convenient experimental model for assays regarding the kinetic reaction and peroxidation mechanism. The advantages in the use of liver microsomes include: low costs, simplicity in use, and easy storage. Furthermore, such assays are well characterized as in vitro models for research in drug metabolism [19].

Present study was designed to determine whether microsome membranes from rat liver could be a target for non-enzymatic lipid peroxidation, as well as to establish if there is protection of such structures when incubated with a CP metanolic extract.

Material and methods

Experimental

Female Wistar AH/HOK rats, 7 weeks old, weighing 120-150 g, were used. All rats were fed commercial rat chow and water *ad libitum*. Animals were obtained from Laboratory Animal Facility, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. BSA (fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. L (+) ascorbic acid and methanol were from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma-Aldrich.

Preparation of Microsomes

Rats were euthanized by cervical dislocation and the liver was rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30 % (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mMTris-HCl pH 7.4 using a Potter-Elvejhem homogenizer. The homogenate was spun at 10,000 x g for 10 min. The supernatant (30 ml) was applied to a Sepharose 4B column (1.6 x 12 cm) equilibrated and eluted with 10 mMTris-HCl pH 7.4, 0.01 % NaN_3. The microsomal fraction appearing in the void volume (10-12 ml) was brought to 0.25 M sucrose by adding solid sucrose. All operations were performed at 4 °C and under dim light. The quality of microsomal preparation is similar in composition as regards concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [20].

CP extract preparation

Ten grams of CP dried leaves were milled until gross powder and put in a flask together with 200 mL methanol for extraction, using a magnetic stirrer at 870 rpm and dim light during 12 h. After extraction, CP extract was submitted to vacuum filtration and then concentrated using a rotaevaporator (Senco Ltd.) until total evaporation of the solvent. A residue of 50 mg was obtained, which was re-suspended in 20 mL methanol. From the total methanolic extract, 10 mL were used for the qualitative analysis of phytochemical constituents while the remaining volume was used to perform different assays [20]. For the phytochemical analysis of the extract, the original volume of 10 mL was fractioned in three aliquots, as follows: Fraction A for testing the presence of flavonoids (zinc hydrochloride reduction test, Shinoda's test), tannins (ferric chloride test) and lipids (iodine reaction); Fraction B for the investigation of the presence of steroids (acetic anhydride + concentrated H_2SO_4, Liebermann-Burchard's reaction) and anthraquinones (sodium hydroxide test, Bornträger's test); and Fraction C for the determination of alkaloids (potassium iodide-bismuth nitrated test, Dragendorff's reagent), cardenolides (dinitrobenzoic acid + sodium hydroxide, Kedde's reagent), steroids (Liebermann-Burchard's reaction) and leucoanthocyanins (concentrated HCl + amyl alcohol, Rosenheim's reaction) [21].

Quantification of total flavonoids in CP extract

A modification of Maksimovic *et al.* technique [23] was performed for the determination of total flavonoids in CP. For this, 0.1 mL of CP extract with 1.4 mL deionized water and 0.5 mL flavonoid reagent (133 mg ferric trichloride and 400 mg sodium acetate diluted in 100 mL of a solvent prepared with 140 mL methanol, 50 mL distilled water and 10 mL acetic acid) were incubated at room temperature for 5 minutes. Absorbance was determined at 430 nm. Total flavonoid content was calculated as mg rutin equivalent per g dry weight. Prior to this, a calibration curve was prepared with decreasing concentrations of rutin diluted in methanol (1 mg, 0.500 mg, 0.250 mg, 0.125 mg and 0.0625 mg rutin) [24].

Peroxidation of rat liver microsomes

Chemiluminescence and peroxidation were initiated by adding ascorbate to microsomes [25]. The concentration of microsome protein was determined using BSA as standard [26]. Rat liver microsomes were incubated with the addition of CP extract (0.1, 0.2, 0.3 and 0.4 mg) at 37 °C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, final volume 1 mL The phosphate buffer provides ferrous or ferric iron (final concentration in the incubation mixture was 2.15 µM) for peroxidation of microsome preparations. Microsomal membranes without CP but with ascorbate were used as control. A microsome preparation without CP and ascorbate was tested as well. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as cpm (counts per minute) every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. This assay was performed in a liquid scintillation analyzer Packard 1900 TR equipment with a program for chemiluminescence. Although not all peroxidative processes involve lipids, since there are other peroxidizable molecules, lipid peroxidation is very important in the terms of chemiluminescence.

Statistical analysis

Results are expressed as means ± S.D. of five independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different p-values, which was indicated in each case.

Results

Phytochemical analysis of CP extract

Table 1 shows the results of the phytochemical analysis of CP extract. Qualitative chemical determinations were performed to assess the presence of those constituents that exert antioxidative properties.

Total flavonoids in CP extract

Cestrum parqui extract yielded a total flavonoid content of 52 µg for the plant material used for its preparation (10 g).

Table 1: Qualitative analysis of *Cestrum Parqui* extract.

Determination	Fraction A	Fraction B	Fraction C
Shinoda	+		
Ferric chloride	+		
Iodine	+		+
Liebermann-		+ steroids	
Burchard		- triterpens	
Bornträger		-	
Dragendorff			+
Kedde			-
Rosenheim			-

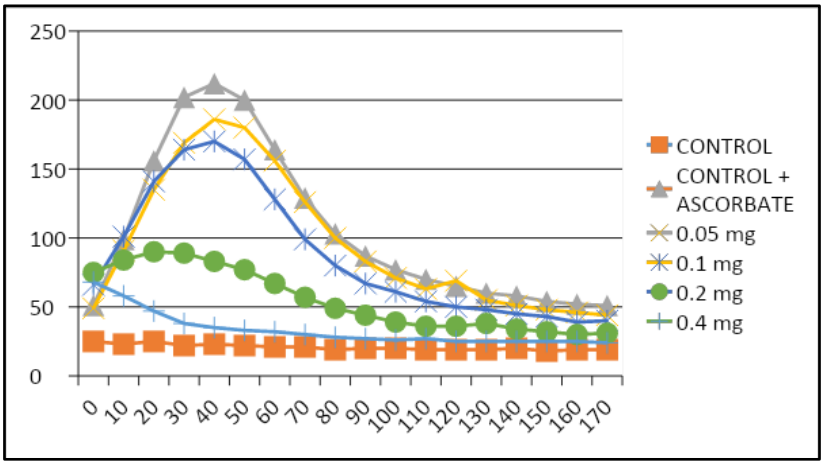

Figure 1: Peroxidation of rat liver microsomes with different concentrations of Cestrum parqui extract

Table 2: Light emission of rat liver microsomes during peroxidation (cpm) with different concentrations of *Cestrum parqui* extract (0.05 mg, 0.1 mg, 0.2 mg, and 0.4 mg).

	Average	Average ± SD
Control	585,67	52,77[1, 2, 3, 4]
Control + Ascorbate	2223,50	88,64[1, 5, 6]
0.05 mg	2130,17	113,89[2, 7, 8]
0.1 mg	1907,67	117,05[3, 9, 10]
0.2 mg	1354,17	63,63[4, 5, 7, 9, 11]
0.4 mg	653,33	33,64[6, 8, 10, 11]

Light emission of rat liver microsomes during peroxidation

The incubation of rat liver microsomes in the presence of ascorbate-Fe^{+2} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of microsomes in an ascorbate-Fe^{+2} system at 37 ºC for 180 minutes, the cpm originated from light emission was lower in the CP group when compared to the control group, this effect was concentration dependent. Figure 1 and Table 2 show the total light emission obtained from CP group and control group, respectively. The values were 2223,5 ± 88,64 cpm in the control group, and 1354,17 ± 63,63 and 653,33 ± 33,64 cpm with the addition of 0.2 mg extract and 0.4 mg / mg protein, respectively.

Data are given as mean ±SD of five experiments. Statistically significant differences in CP concentrations and controls and peroxidized rat liver microsomes are indicated by 1,2,3,4, 6, 8, 10 $p < 0{,}0001$; 7, 11 $p < 0.005$; 5 $p < 0{,}001$ and 9 $p < 0.05$.

Discussion

Cestrum parqui L' Herit (Solanaceae) is a native plant of South America, distributed in tropical and subtropical regions. Despite being classified as poisonous to animals, it also presents medicinal properties [27]. Regarding the toxicological properties, CP has glycosides known as parquin and carboxiparquin and the saponins gitogenin and digitogenin. On the other hand, extracts prepared from this plant has shown to exhibit antioxidant, anti-inflammatory, antipyretic and anticancer properties [28][29][30]. In particular, leaves and bark are used to relief fevers and skin diseases such as infections and sunburns [31][32]. For these reasons, the plant is considered as medicinal, and it is used by means of maceration or decoction of leaves for topical use.

Chemical substances that have been identified in methanol extracts of *C. parqui* include compounds such as phenolic acids, flavonoids, glycosides [33] and other constituents such as sterols, steroids, terpenoids, and free and esterified triterpene alcohols [11]. The phytochemical components present in our CP extract are similar to those found in these previous works. In the present work, rat liver microsomes incubated with CP extract were protected against peroxidation, compared to similar membranes of the control group, as shown by the results of chemiluminescence.

Products of lipid peroxidation are involved in many cellular processes including cellular metabolism, signaling, and cell survival [34]. Lipid molecules, especially polyunsaturated acids (PUFA), undergo oxidation initiated by ROS at varied rates, and in PUFA this initiates a self-propagating chain reaction [35]. The propagation stage of lipid peroxidation involves electron transfer and oxidation of other PUFA in close proximity, starting a chain reaction. The use of medicinal plants is a common practice in many countries, and *Cestrum parqui* is one of the many plants used for this purpose in South America. Such use could be explained by its composition in compounds that act as ROS scavengers, well know to participate in the pathological processes mentioned above.

Conclusion

Our results are consistent with those from other authors, that indicate that the extract of *Cestrum parqui* L' Herit (Solanaceae) may act as a physiological antioxidant *in vitro* in cell membranes. In our case, the latter could be attributable to the flavonoids present in our CP extract which, despite not being identified by quantitative techniques, their presence was confirmed by qualitative methods. More studies are needed regarding the investigation of the antioxidant properties of CP extracts. In particular, chromatographic techniques are needed to determine the exact identity of the compounds that exert such properties, activities that will be performed in next assays with this plant in our laboratory.

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