

Effects of proanthocyanidin enriched extract from *Ligaria cuneifolia* on plasma cholesterol and hemorheological parameters. *In vivo* and *In vitro* studies

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Abstract. It was demonstrated that *Ligaria cuneifolia* (*Lc*) crude extract increased blood viscosity and decreased plasma cholesterol in rats. In the present study, we analyzed the *Lc* proanthocyanidin enriched fraction (*PLc*) to determine if it is capable of altering the hemorheological parameters while diminishing the plasma cholesterol. *In vivo* studies in adult male Wistar rats, randomized in three groups ($n=6$ each one) were performed: 1. Control: saline intraperitoneal (i.p.); 2. *PLc* 0.6 mg/100 g body weight (b.w.) i.p. and 3. *PLc* 3 mg/100 g b.w. i.p., every 24 hours during 3 days. *In vitro* studies: with blood obtained by cardiac puncture, separated in aliquots and incubated with: 1. Saline solution (Control); 2. *PLc* 0.1 mg/mL, and 3. *PLc* 1.0 mg/mL, equivalent to doses *in vivo* experiments. The results demonstrated that *in vivo* *PLc* 0.6 and *PLc* 3 reduced plasma cholesterol (Cho) and LDL-Cho. Neither blood nor plasma viscosity was altered. Decrease of plasma cholesterol could be due to an increase of cholesterol and bile salts excretion leading to an increase of bile flow. *In vitro* experiments showed a direct interaction of *PLc*, at high concentration, with the erythrocyte membrane, inducing a switch from discocyte to stomatocyte. Only, *PLc* without hepatic metabolism produces hemorheological changes. Thus, *PLc in vivo* might be a pharmacological agent capable of decreasing plasma cholesterol.

Keywords: *Ligaria cuneifolia*, proanthocyanidin, blood viscosity, plasma cholesterol, erythrocyte deformability, biliary excretion

1. Introduction

Ligaria cuneifolia (R. et P.) Tiegh. (Loranthaceae) (*Lc*), popularly known as “Argentine mistletoe”, is a widely distributed hemiparasite plant from the Northern and Central regions of Argentina [1]. *Lc* is popular

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30 due to its medicinal properties. It has been used traditionally in the forms of crude extracts, infusions, to
31 decrease blood pressure and to increase blood fluidity, lowering the excess of cholesterol (Cho) [26, 29].
32 Pharmacological and phytochemical studies of the Argentine mistletoe showed the presence of tyramine,
33 which has sympathicomimetic activity [12]. Thus, the administration of the extract of this plant could
34 be able to induce an increase in blood pressure [23]. A review of the literature revealed that studies on the
35 potential hemorheological effects of this plant are lacking. In this regard, our group was the first to begin
36 such studies. In this connection, earlier studies in our laboratories revealed that the treatment with the
37 crude extract of *Lc* by via intraperitoneal (i.p.) to the adult male Wistar rats produced an increase in blood
38 viscosity as a consequence of an augmentation of erythrocyte rigidity, and also a diminution of plasma
39 Cho levels. Moreover, we suggest that the diminution of plasma Cho could be caused by an increase of the
40 biliary excretion rate of Cho and bile salts (products of hepatic metabolism of Cho) which was obtained
41 in our studies in the treated rats [16]. Advancing in the analysis of different extracts of *Lc*, we studied
42 in rats, the effect of intraperitoneal treatment with the methanolic fraction (FMLc). In these studies we
43 observed that treatment with FMLc produced similar effects to those observed for the crude extract of
44 *Lc*, such as the diminution of plasma Cho level and the increased erythrocyte rigidity which caused the
45 augmentation of blood viscosity [10]. The micromolecular study of the flavonoid composition from *Lc*
46 extracts, disclosed the presence of free-quercetin, glycosylated-quercetin, catechin and proanthocyanidins
47 corresponding to cyanidin monomer [9]. Based on this knowledge of the composition of the extract of
48 *Lc* we also investigated the effect of some fractions extracted from *Lc*, rich in various flavonoids. In
49 this regard, we analyzed the effect of both fractions extracted from *Lc*, one enriched in catechin and the
50 other, in free-quercetin. These studies (both *in vivo* and *in vitro*) showed that the two fractions produced
51 changes in the shape of the erythrocytes from discocytes to stomatocytes, and also produced an increase
52 of the erythrocyte rigidity leading to an increase in blood viscosity. Contrary to the observed for the crude
53 extract of *Lc* none of the two fractions led to a diminution in the plasma Cho levels, thus suggesting that
54 another compound from *Lc* would be responsible for the decrease in plasma Cho [8].

55 In the present work, we studied the effect of *Ligaria cuneifolia* proanthocyanidin enriched extract on
56 plasma Cho and its bile excretion, and the hemorheological parameters such as erythrocyte shape and
57 deformability.

58 2. Materials and methods

59 2.1. Plant material

60 Samples of *Lc* growing on *Geoffroea decorticans* (Hook. & Arn.) Burkart (Fabaceae) were collected
61 from Córdoba a Mediterranean Argentine province. Classification of the specie was performed by means
62 of the key according to Abbiatti [1]. Voucher specimen is kept at the Museum of Pharmacobotanics “Juan
63 A. Dominguez”, School of Pharmacy and Biochemistry, National University of Buenos Aires.

64 2.2. Preparation of plant extracts

65 To obtain crude extract (CE) 20 g of *Lc* air-dried leaves were grounded in a rotary blade mill and
66 extracted with 200 mL 80% methanol (v/v) for 48 hours at room temperature. The CE was filtered and
67 the solution was dried and evaporated under reduced pressure at 40°C and the residue was successively
68 extracted with 50 mL of ethyl acetate (EAF). The EAF was concentrated and dried by evaporation under

69 reduced pressure, and the residue was stored in absence of light at -20°C to obtain the *Ligaria cuneifolia*
70 proanthocyanidin enriched fraction (PLc).

71 2.3. Animals

72 Adult male Wistar rats weighing 360 to 410 g were housed two per cage and maintained under a 12 h
73 light/dark cycle. The animals were fed with a normal standard pellet diet (Cargill, type C, product Senasa
74 no 21/A. Pilar, Córdoba, Argentina: 25 g% protein, 3.5% lipid, 43% carbohydrate and 6 g% fibre) and
75 water *ad libitum*. Animal care and treatments were conducted according with Institutional guidelines
76 (Expedient 6109/012 E.C. Resolution 267/02) in compliance with National and International laws and
77 policies.

78 2.3.1. In vivo experiments

79 The animals were divided in three experimental groups ($n = 6$ each one). Basal values for microhema-
80 tocrit and Cho were determined in tail blood samples (0.5 ml).

81 Afterwards, the animals of each experimental group were injected every 24 hours during 3 days, via
82 i.p. with: 1. Control: saline solution; 2. PLc 0.6: *Ligaria cuneifolia* proanthocyanidin enriched extract
83 (0.6 mg/100 g b.w.); 3. PLc 3: *Ligaria cuneifolia* proanthocyanidin enriched extract (3 mg/100 g b.w.).
84 Doses were calculated considering their relative concentrations in the aqueous extract of *Lc*, as in previous
85 studies [16].

86 2.3.1.1. *Experimental procedures.* Twenty four hours after the experimental procedure above described,
87 the animals were weighed and anaesthetized with sodium pentobarbital solution (50 mg/kg b.w., i.p.).
88 The bile duct was cannulated and bile was collected on ice-cold pre-weighed tubes, every 15 min for
89 60 min. The animals were maintained anaesthetized during bile collection and the rectal temperature was
90 kept at $38 \pm 0.5^{\circ}\text{C}$ with a heating lamp [20].

91 At the end of bile collection, 10 to 12 ml of blood was obtained by heart puncture and the liver was
92 removed, washed and weighed.

93 2.3.1.2. *Determination of bile flow and biliary excretion of bile components.* The bile flow (BF) was
94 estimated by gravimetry, assuming a bile density of 1.0 g/mL; BF was expressed as $\mu\text{l}/\text{min}$ per g of liver.
95 Bile salts concentration was determined by Talalay's method modified by Berthelot [3, 24]. Bile Cho
96 concentration was determined by the enzymatic esterase-oxidase method [14]. The bile output of each
97 component was calculated as the product of BF by their bile concentration.

98 2.3.1.3. *Hemorheological assays.* The rheological measurements were performed according to the guide-
99 lines of Committee for Standardization in Haematology. All measurements were performed at room
100 temperature ($24 \pm 1^{\circ}\text{C}$) within 4 hours after blood collection anticoagulated with EDTA (ethylenedi-
101 aminetetraacetic acid, 1 mg/ml blood) (Wiener Lab) in order to obtain plasma. Whole blood viscosity
102 and plasma viscosity measurements were performed in a Wells-Brookfield LVT-CP viscometer at 230 s^{-1}
103 shear rate (γ). The shear rate selected was assuming that the rheological behaviour of blood is Newto-
104 nian [2]. Therefore, relative viscosity of blood at a standard hematocrit (Htc) of 45% was calculated,
105 thus avoiding the influence of both plasma viscosity and percentage of red blood cells on viscosity
measurements.

106 2.3.1.4. *Rigidity index (RI)*. Erythrocyte filtration was performed in a computerized instrument using
107 the Reid et al. technique [18]. Briefly, a 10% suspension of washed erythrocytes was passed through a
108 polycarbonate filter, 5 μ m pore size (Nucleopore Corp. U.S.A), using a negative filtration pressure of
109 10 cm H₂O. The flow time required for 1 ml of RBC suspension to pass through the filter was measured.
110 Results were expressed as the rigidity index (RI) that is an estimation of erythrocyte rigidity (inverse of
111 erythrocyte deformability), defined as: $RI = (T_b - T_s)/(T_s) \times 100/Htc$

112 Where: T_b : is time of passage of the cell suspension through the filter; T_s : time of passage of an equal
113 volume of PBS; Htc : is the microhematocrit of 10% of RBC suspension.

114 Red blood cells were counted using a Neubauer chamber. Mean corpuscular volume (MCV) was
115 calculated using microhematocrit and red blood cells count. Cell shape was assessed by direct light
116 microscope (World Precision Instruments Inc, Mod H602T-240, USA, magnification 40 \times) of the whole
117 blood sample (150 cells per aliquot), assuming an index according to Bessis classification. Morphological
118 Index (MI) was calculated as follows: $MI = \Sigma (\text{shape index} \times \text{cell number}/\text{total cell number})$ [4].

119 2.3.1.5. *Plasma Cho concentration*. The heparinized blood samples were used to determine plasma Cho
120 concentration by the enzymatic esterase-oxidase method and also of LDL-Cho (low-density lipoprotein)
121 and the HDL-Cho (high-density lipoprotein) [14].

122 2.3.1.6. *Histology*. Liver pieces were fixed in 10% formaldehyde; dehydrated, 24 hours later they were
123 embedded in paraffin, cut and stained with hematoxylin-eosin and trichromica Masson-Alcian blue. Light
124 microscopic analysis was performed in Olympus BX40, U-MDOB model (magnification 400 \times).

125 2.3.2. *In vitro experiments*

126 In a second set of experiments, adult male Wistar rats ($n=6$) were housed two per cage and main-
127 tained under a 12 h light/dark cycle. Animals were fed *ad libitum* with a normal standard diet and water.
128 The animals weighing 360 to 410 g were anaesthetized with sodium pentobarbital (50 mg/kg b.w. i.p.).
129 Between 12 to 15 ml of blood was obtained by heart puncture and divided into three aliquots incubated
130 as follows: 1. Control: saline solution, 2. *PLc* 0.1, *Ligaria cuneifolia* proanthocyanidin enriched extract
131 (0.1 mg/ml) and 3. *PLc* 1.0, *Ligaria cuneifolia* proanthocyanidin enriched extract (1.0 mg/ml). All frac-
132 tions were incubated at 37 $^{\circ}$ C for 30 min. The *in vitro* concentrations were equivalent to 0.60 mg/100 g
133 b.w. and 3 mg/100 g b.w., respectively, calculated considering rat blood volume (4.5 ± 1.6 ml/100 g b.w.).
134 At the end of the incubation, the following assays were carried out: 1) Rigidity index (RI) to estimate
135 erythrocyte deformability and the other hematological indices were determined by the above mentioned
136 methods (see section "Hemorheological assay" for *in vivo* experiments). 2) Osmotic fragility: Red blood
137 cells samples were incubated for 30 min in NaCl solutions ranging from 0 to 290mOsm/kg, measuring
138 hemolysis percentage by photocolorimetry to obtain the parameter X_{50} (NaCl concentration (mM) yield-
139 ing 50% of hemolysis) [6, 7]. 3) Cell shape was assessed by Bessis' classification, as was mentioned
140 above in "Hemorheological assays" for *in vivo* experiments.

141 2.4. *Statistical analysis*

142 Both *in vivo* and *in vitro* experiments comparisons were performed with one-factorial ANOVA, followed
143 by Newman-Keul's test. Results were expressed as mean \pm SE. SPSS 11.0 statistical package was used
for processing data. Differences were considered as statistically significant when $p < 0.05$.

3. Results

3.1. *In vivo* experiments

There was no change in body weight neither in Control nor in treated groups after three days, as follows: I. *Basal*: I. Control: 386.0 ± 7.8 g; 2. *PLc* 0.6: 385.4 ± 6.5 g; and 3. *PLc* 3: 386.5 ± 5.4 g. II. *After treatment*: 1. Control: 386.0 ± 8.5 g; 2. *PLc* 0.6: 382.5 ± 5.5 g; and 3. *PLc* 3: 390.5 ± 7.4 g.

3.1.1. Hemorheological parameters

No significant differences were observed in relative blood viscosity values in both *PLc*-treated groups compared with Control group respectively, as follows: 1. Control: 4.26 ± 0.39 ; 2. *PLc* 0.6: 4.39 ± 0.11 ; and 3. *PLc* 3: 4.45 ± 0.41 .

No significant differences were observed in RI values, expressed as percentage in both *PLc*-treated groups compared with Control group respectively, as follows: 1. Control: 7.50 ± 0.40 ; 2. *PLc* 0.6: 7.61 ± 0.36 ; and 3. *PLc* 3: 7.78 ± 0.47 .

3.1.2. Plasma Cholesterol (Cho), LDL-Cho and HDL-Cho

Table 1 shows the values for Plasma Cholesterol (Cho), LDL-Cho and HDL-Cho for the experimental groups: Control, *PLc* 0.6 and *PLc* 3. Both doses of *PLc* produced a significant decrease in plasma Cho compared to the Control ($p < 0.05$).

Both *PLc* fractions induced a decrease of LDL-Cho in comparison with Control group, in case of *PLc* 0.6 was 17%, while for *PLc* 3 was 37% ($p < 0.05$).

The plasma HDL-Cho showed no significant difference in both experimental groups when compared with the Control group (see Table 1).

3.1.3. Bile parameters

Table 2 shows the values for the biliary excretion of Cho, bile salts, and also the bile flow for the experimental groups: Control, *PLc* 0.6 and *PLc* 3. Both doses of *PLc* produced a significant increase in

Table 1
Plasma Cholesterol (Cho), LDL-Cho and HDL-Cho

	Control (n=6)	<i>PLc</i> 0.6 (n=6)	<i>PLc</i> 3 (n=6)
Cholesterol (Cho) (mg/dL)	90.3 ± 4.2	$72.2 \pm 2.3^*$	$71.4 \pm 2.6^*$
LDL-Cho (mg/dL)	19.5 ± 2.4	$16.2 \pm 1.5^*$	$12.5 \pm 0.5^*$
HDL-Cho (mg/dL)	38.5 ± 1.0	37.5 ± 1.5	39.6 ± 3.2

Values are expressed as mean \pm SE. *Significantly different from Control group ($p < 0.05$).

Table 2
Biliary excretion of Cholesterol and Bile Salts

	Control (n=6)	<i>PLc</i> 0.6 (n=6)	<i>PLc</i> 3 (n=6)
Biliary excretion of Cholesterol (nmol/min \times g of liver)	3.0 ± 0.1	$4.8 \pm 0.5^*$	$3.9 \pm 0.6^*$
Biliary excretion of Bile Salts (nmol/min \times g of liver)	48.3 ± 2.1	$58.0 \pm 3.2^*$	$72.5 \pm 2.6^*$
Bile Flow (μ L/min \times g of liver)	1.90 ± 0.10	$2.68 \pm 0.14^*$	$2.90 \pm 0.25^*$

Values are expressed as mean \pm SE. *Significantly different from Control group ($p < 0.05$).

Table 3
In vitro effect of *PLc*: Osmotic fragility

	Control (n=6)	<i>PLc</i> 0.1 (n=6)	<i>PLc</i> 1 (n=6)
X ₅₀ (mM)	0.45 ± 0.01	0.44 ± 0.02	0.50 ± 0.01*

X₅₀: concentration of NaCl which produces 50% of hemolysis. Values are expressed as mean ± SE. *Significantly different from Control group ($p < 0.05$).

Table 4
In vitro effect of *PLc*: Morphological index (MI) and percentage of stomatocytes type III

	Control (n=6)	<i>PLc</i> 0.1 (n=6)	<i>PLc</i> 1 (n=6)
MI	-0.50 ± 0.10	-0.64 ± 0.08	-2.20 ± 0.01*
Stomatocytes type III (%)	2.62 ± 0.95	3.34 ± 0.82	6.66 ± 0.73*

Values are expressed as mean ± SE. *Significantly different from Control group ($p < 0.05$).

biliary excretion of Cho compared to the Control ($p < 0.05$). Both doses of *PLc* produced a significant increase in biliary excretion of bile salts compared to the Control ($p < 0.05$). The bile flow showed increase in both experimental groups when compared with the Control group ($p < 0.05$).

3.1.4. Histology

Histological assessment of several liver sections of the three experimental groups (Control and of the two *PLc*-treated ones) were performed. Hepatic lobular architecture was preserved in the three groups. The liver did not show any sign of either inflammation or injury of hepatocyte with intraperitoneal treatment.

3.2. *In vitro* experiments

3.2.1. Hemorheological parameters

No significant differences were observed in RI values in the blood aliquots incubated with *PLc* 0.1 in comparison with the Control, as follows: Control: 7.67 ± 1.19 ; *PLc* 0.1: 7.98 ± 0.34 . However, when blood aliquots were incubated with *PLc* 1, they showed a significant increase in the RI *PLc* 1: 9.98 ± 0.25 in comparison with Control ($p < 0.05$).

Table 3 shows the values for X₅₀ as an estimation of the osmotic fragility. The incubation with *PLc* 0.1 yielded unchanged values for osmotic fragility, whilst the incubation with *PLc* 1 produced a significant increase regarding the Control ($p < 0.05$). A higher X₅₀ points out to less resistant erythrocytes to hypotonic concentrations [22].

The MI did not show any change *in vitro* when blood was incubated with *PLc* 0.1. In contrast, incubation with *PLc* 1 yielded a significant decrease in MI due to presence of type III stomatocytes [4] (Table 4).

4. Discussion

In previous works it was demonstrated in normocholesterolemic rats a decrease of plasma cholesterol associated with a decrease of blood fluidity, due to an increase in blood viscosity as well as a lower

erythrocyte deformability, using the following *Lc* extracts: crude, methanolic fraction, catequine, and quercetine enriched fractions [8, 10, 16].

The *Ligaria cuneifolia* proanthocyanidin enriched extract, which is a normally present flavonoide in *Lc*, was administered intraperitoneally during three days to normocholesterolemic rats producing a significant decrease in plasma cholesterol, LDL-Cho and HDL-Cho without alterations in blood viscosity. Besides, the intraperitoneal administration of *PLc* did not produce any hepatic damage, confirmed by the absence of hepatotoxicity through histological examination.

The liver plays a main role in the regulation of plasma cholesterol. It is known that two major output pathways responsible for excretion of cholesterol and homeostasis maintenance are involved in the chemical transformation of cholesterol to bile acids, i.e., their biliary excretion, and canalicular cholesterol secretion [5, 20, 21, 28]. Moreover, it is admitted that the increase of the biliary secretion of bile acids provides the primary stimulus for bile flow leading to its augmentation [25], which was demonstrated by our results. The bile acids excretion increase, could be due to the inductive effect of the Cho 7- α -hydroxylase enzyme, through the *PLc* administration that might partially decrease the plasma cholesterol in the experimental animals. Furthermore, the *PLc* produced a decrease of plasma cholesterol and also of both HDL-Cho and LDL-Cho, without impairment of plasma viscosity.

It has been demonstrated that certain compounds intraperitoneally administered are transformed when they enter the liver through the portal vein to be metabolized, thus, converting them into hydrosoluble ones to be secreted either to the bile or the plasma [15]. So far, these products could alter the bile salts synthesis in the hepatocyte, and/or their biliary secretion as well as the cholesterol one [15]. Similarly, the *PLc* intraperitoneally administered could mimic the latter mechanism inducing changes in the bile acids and the bile cholesterol secretions.

To explain the absence of hemorheological effect of *PLc* *in vivo*, *in vitro* studies that avoid the liver metabolism were carried on.

In vitro effect of *PLc* induced a discocyte-to-stomatocyte III switch decreasing the osmotic resistance at a higher concentration. Other authors described the interaction of several compounds on erythrocytes leading to alterations on their membrane curvature [13, 17].

On the other hand, *PLc* is a flavonoid composed by a common skeleton of diphenyl-pyranes with two phenyl rings and OH groups in positions 1, 3 and 6 of the phenyl rings [13]. There are authors that described drugs with a similar structure that in contact with normal red blood cell leads to the formation of cup-shaped cells (stomatocytes) [11, 19]. Taking into account the above issues we analyzed, in the present study, the impairment of the erythrocyte shape produced by the incubation with *PLc* 1 that yielded a more negative MI, therefore, suggesting that the *PLc* could be able to produce alterations in the cell membrane curvature of erythrocyte by a direct interaction.

Nevertheless, there are authors that considered that the discocyte-to-stomatocyte switch points out a membrane stress [27], consequently, an alteration of osmotic fragility, as the one observed by us regarding the *PLc* interaction with the plasma membrane. We also observed a lowering of osmotic resistance in treated erythrocytes (higher X_{50}) at a concentration of 1 mg/mL.

5. Conclusion

The *PLc* *in vivo* studies lead to a decrease of plasma cholesterol and LDL-Cho, in part due to an increase of biliary excretion of bile acids and cholesterol without impairment of hemorheological parameters.

The *PLc in vitro* studies showed a direct interaction of *PLc* with the erythrocyte membrane, which induced changes in the erythrocyte shape from discocyte to stomatocyte, and a decrease of osmotic resistance at a high concentration.

The *in vivo* results demonstrate that *PLc* might be a potential pharmacological agent to decrease plasma cholesterol, therefore, to be considered an important issue in cardiovascular disease prevention.

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Conflict to disclose

None.

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