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Study of *in vitro* alterations in human blood by aqueous extract of *Bauhinia forficata* leaves commercialized in Argentine

Hermano Mascaro Grosso[1], Maria Teresa Santos[2], Carlota Saldanha[2], Ana Silva-Herdade[2] Bibiana Riquelme[1,3*]

[1]Área Física, Facultad de Cs. Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina

[2]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal

[3]Grupo de Física Biomédica, Instituto de Física Rosario (CONICET-UNR), Argentina

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Abstract: Among the numerous plants of medicinal interest is *Bauhinia forficata* Link, which belongs to the *Fabaceae* family. Its leaves, branches and roots are widely used in Brazil and Argentina in the form of herbal infusions and other phytotherapeutic preparations for the treatment of various diseases, such as diabetes. The glycemic reduction ability of this medicinal plant was demonstrated in several *in vitro* studies, using both rats and human models. Although the composition of this plant was identified, little is known about the pharmacological activity of most of the isolated substances. In this work, we present an evaluation of the rheological alterations in human blood by *in vitro* treatment with aqueous extracts (infusion and maceration) of *B. forficata*. Our results show that, in low concentrations, no hemolysis is observed, erythrocyte deformability and AChE enzyme activity decreased for the extract that received high temperature in their preparation. The analysis of the hemorheologic effects of the two different plant extracts can be considered a fundamental tool to assess their hemocompatibility and the possibility of

using them to reverse some of the alterations observed in vascular processes, particularly in diabetes.

Keywords: *Bauhinia forficata*, aqueous extracts, hemorheology, diabetes

INTRODUCTION

Medicinal plants and their derivatives were the therapeutic basis of pharmacology and currently 25% of the medicines used are of vegetable origin, while 50% are of synthetic origin but are related to the isolated principles. Among the numerous species of plants of medicinal interest is the *Bauhinia forficata* Link[1].

The genus Bauhinia has approximately 300 species distributed in the tropical regions of the world, mainly in America, Africa and Asia[2,3], 200 species being found in South America. *B. forficata* belongs to such genus, subfamily *Caesalpinioideae* and family Fabaceae, is a native species from South America, found in Argentina, Paraguay, Uruguay, Bolivia and Brazil. The subspecies *Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin[3], is mentioned, which is found on the edges of bushes in Paraguay, southern Brazil, Uruguay and Argentina, while *B. forficata* subsp. *forficata*, has only been found in Northeast and Southeast Brazil.

The leaves, branch and root of *B. forficata* species are widely used in Brazil and Argentina in the form of herbal infusions and other phytotherapeutic preparations for treating various diseases, mainly diabetes[2]. Diabetes mellitus is a chronic disease that affects the metabolism of carbohydrates, lipids and proteins, reaching around 250 million people worldwide, and it is estimated that seven million new cases are produced every year[4,5]. This disease results in a state of chronic hyperglycemia and, consequently, in a series of clinical complications, especially cardiovascular and renal ones, demanding a high amount of money from the health system in terms of recovery and treatment of the patients involved[6,7].

The glycemic reduction caused by of *B. forficata* species was shown in several works in which the activity was tested *in vitro*[8], in rats[9,10] and in humans[11]. Although their compounds were identified, little is known about the pharmacological activity of most of the isolated substances of the genus Bauhinia[12].

The aim of this study was to determine the biochemical and rheological blood alterations by the *in vitro* treatment with two different aqueous extracts of *B. forficata*, infusion and maceration. These studies consisted in determining osmotic fragility, acetylcholinesterase (AChE) enzyme activity, erythrocyte deformability, erythrocyte aggregation and nitric oxide (NO) efflux from red blood cells previously treated with infusion and maceration extracts from commercial leaves of *B. forficata*. The hemorheological analysis of the effects of the two different extracts can be considered a fundamental tool to assess its hemocompatibility and the possibility of reversing some of the alterations observed in vascular processes, particularly in diabetes.

MATERIALS

Plant samples: In this study, we used *B. forficata* leaves, which were found with good consistency and healthy appearance, and properly prepared for consumption in the form of tea. The leaves of *B. forficata* were purchased in the city of Rosario, Argentina, brand name PIPERPOL, 50 g package, lot 0197, best before Oct/2019, fractioned by "Laboratorios PIPERPOL S.R.L. Córdoba, Argentina".

Preparation of extractive solutions: The aqueous extracts were prepared at a concentration of 5% by suspending 3 g of commercial leaves of *B. forficata* in 57 mL of saline solution (Mustela lot. 6768143). The following two extractive techniques were used:

I: Infusion of leaves of *B. forficata* in saline solution at 100°C.

M: Maceration of leaves of *B. forficata* in saline solution at room temperature for 12 hours.

The extractive solutions were filtered (100 nm diameter) to remove large particles from the solutions. Finally, the extractive solutions were stored at 4 °C in sterile caramel glass vials. All these steps were performed under standard sterile conditions. Physicochemical properties of extractive solutions[13] were evaluated, pH was adjusted to 7.4 (physiological pH) with a solution of 0.25 M of NaOH and the osmolality was 0.289 mOsm in I and 0.282 mOsm in M.

Human Blood: Blood samples (n=3) were obtained from healthy donors in sterile tubes, anticoagulated with EDTA. Human blood was used under the protocol with Instituto Português do Sangue e Transplantação (IPST) of Lisbon. To carry out the osmotic fragility and AChE assays, blood samples were centrifuged at 2,000 rpm during 5 min at 25 °C. After removing plasma and buffy-coat, RBCs were washed with phosphate buffered saline (pH 8.0; 295 mOsm/kg).

METHODS

Characterization of extracts

Extracts from *B. forficata* obtained by I and M were characterized using a spectrophotometer (Thermo Scientific Genesis 10 UV). Spectrophotometric readings of aqueous extracts were carried out at different wavelengths (340 nm to 540 nm) and concentrations in saline solution (0.5% to 25%).

Erythrocyte alteration analysis

Osmotic fragility: Possible erythrocyte membrane alterations induced by the extracts may be manifested by the greater or lesser degree of hemolysis, reflecting the degree of stability of RBCs[14]. In the study of osmotic fragility, the erythrocytes were suspended in hypotonic medium, which induces an increase in cell volume, producing lysis. The degree of hemolysis was determined by the colorimetric method using saline solutions at 0.32%, 0.45%, 0.6% and 0.9%[15]. Blood samples were centrifuged for 5 min at 2,500 rpm. A volume of plasma (50 µL) was withdrawn and replaced by the bulk of I. In the control sample, the same volume (50 µL) of plasma was replaced by 50 µL of 0.90% NaCl. All samples were incubated at 37°C for 45 min.

Determination of nitric oxide efflux from erythrocyte: Nitric oxide (NO) is an "endothelium derived relaxing factor", which has several cardiovascular functions, particularly as erythrocyte deformability modulator [16]. In this work, a nitric oxide (NO) flow of erythrocytes was measured in the presence of I and M extracts from *B. forficata.* To determine if the NO induced effects on RBC, a technique previously described by Mesquita *et al.* was used[17]. Briefly, 20 µL of plasma were removed from each blood aliquot and replaced by the same volume of SpermineNONOate 10^{-2} M prepared in distilled water (pH 12), for incubation with I and M extracts. Aliquots with SpermineNONOate with the respective control aliquot were incubated at 37°C for 30 minutes.

Cellular membrane integrity: The biochemical properties of the erythrocyte membrane are sensitive to acetylcholinesterase (AChE), then, AChE in erythrocytes can be evidenced as a biomarker of membrane integrity analyzing their relationship with a production of nitric oxide (NO). Mesquita *et*

al. developed a method to evaluate this property[17]. To analyze the behavior of erythrocytes, AChE was carried out under the influence of the different extracts of *B. forficata*. Washed erythrocytes were incubated with the infusion and maceration solutions, I and M respectively. During the washing process, the aspect of the supernatant was observed to confirm the absence of hemolysis. RBCs treated with 0.1 M phosphate buffer solution at pH 8.0 were then incubated with a mixture of quinidine sulfate, acetylcholine, and DTNB (Thermo Scientific Pierce Ellman Reagent), which catalyzes and standardizes as samples for readings with the spectrophotometer.

Erythrocyte aggregation: Erythrocyte aggregation of I and M extracts was evaluated using the MA1 aggregometer from Myrenne (Roetgen, Germany). This instrument consists of a rotational aggregometer that disperses the sample by shear stress ($600/s^{-1}$), and a photometer that determines the extent of aggregation. The intensity of light transmitted through the blood sample was measured using a photodiode.

Erythrocyte deformability: The Rheodyn SSD diffractometer from Myrenne GMBH (Roentgen, Germany) determines RBC deformability simulating the shear stress on the blood flow and vascular walls[18]. RBCs were suspended in a viscous medium and were placed between a rotating optical disk and a stationary disk. A well-defined shear stress was applied to the suspension and the erythrocytes were deformed to ellipsoids aligned with the fluid shear stresses. The diffraction pattern is circular with resting erythrocytes, but becomes elliptical when the erythrocytes are deformed by shear rate. The light intensity of the diffraction pattern is measured at two different points (A and B), equidistant from the center of the image. The erythrocyte elongation index expressed as a percentage (EEI%) is obtained according to the following equation: $EEI\% = (A - B) / (A + B) \times 100$.

Statistical analysis

All measurements were carried out by triplicate and mean values were calculated. Differences between the mean values were evaluated using ANOVA test and were considered statistically significant respect to the control for $p<0.05$.

RESULTS AND DISCUSSION

Table 1 shows the results obtained from spectrophotometric readings at different wavelengths in I and M extracts. The analysis of these results shows that a better light absorption response was found at 400 nm. Consequently, the next spectrophotometric readings in different concentrations of the extracts were made at 400 nm **(Table 2**). These results show that extracts at a concentration of 25% in saline solution render a satisfactory response with the spectrophotometer.

Table 1: Spectrophotometric readings from aqueous extracts of *Bauhinia forficata* at different wavelengths.

λ [nm]	I	M
540	0.080 ± 0.001	0.086 ± 0.001
535	0.093 ± 0.001	0.099 ± 0.001
500	0.112 ± 0.001	0.121 ± 0.001
440	0.191 ± 0.001	0.216 ± 0.001
400	0.481 ± 0.001	0.560 ± 0.001
340	2.429 ± 0.001	2.417 ± 0.001

Table 2: Spectrophotometric readings at 400 nm from different concentrations of *Bauhinia forficata* extracts in saline solution

Cc. extract %	I	M
0.5	0.011 ± 0.001	0.005 ± 0.001
1	0.013 ± 0.001	0.011 ± 0.001
2.5	0.021 ± 0.001	0.020 ± 0.001
5	0.041 ± 0.001	0.043 ± 0.001
25	0.220 ± 0.001	0.223 ± 0.001

Table 3 shows the results of osmotic fragility carried out by the colorimetric method to determine the degree of hemolysis. These results show that no hemolysis occurred at 0.6% NaCl concentration in any of the samples.

Table 3: Osmotic fragility measured with spectrophotometer at 400 nm. Data are presented as mean ± SD.

Cc. NaCl %	Blood	Blood + I
0	1.335 ± 0.014	1.386 ± 0.006
0.32	1.216 ± 0.010	1.305 ± 0.095
0.45	1.153 ± 0.029	1.214 ± 0.008
0.60	0.047 ± 0.001	0.062 ± 0.002

Table 4 shows the amount of nitric oxide (NO) in red blood cells treated with I and M extracts. Results show a lower value of NO in M when compared to the control. In addition, **Table 4** shows the values of red blood cell acetylcholinesterase (AChE) activity in red blood cells treated with I and M extracts. Results show that I extract induce a decreased in AChE enzyme activity. **Table 5** shows the aggregation parameters at 5s and 10s from erythrocytes treated with I and M extracts. Results correspond to mean values and standard deviations and show an increase in the red blood cells aggregation in the M extracts (with 10s).

Table 4: Determinations of nitric oxide (NO) and analysis of AChE and nitric oxide (NO) in red blood cells treated with I and M extracts.

	Optical Density Arbitrary Units		Activity U min^{-1} mg^{-1} Hb	NO nM
	AChE	Hb		
White	0.093 ± 0.004	-	-	-
Control	0.387 ± 0.019	8.0 ± 0.1	319 ± 1	1.8
I	0.335 ± 0.016	8.3 ± 0.1	269 ± 1	0.8
M	0.436 ± 0.021	8.8 ± 0.1	327 ± 1	0.7

Table 5: Aggregation parameters at 5s and 10s of red blood cells treated with I and M extracts. Data are presented as mean ± SD.

	5s	10s
Control	5.3 ± 0.2	8.0 ± 0.4
I	5.4 ± 0.1	8.5 ± 0.3
M	5.6 ± 0.1	7.3 ± 0.2

Figure 1 shows the elongation index of erythrocytes treated with I and M extracts at different shear stress. Data correspond to mean values and experimental errors (ε). As for treatment with infusion extract, erythrocyte deformation response is significantly lower than the control, but is lightly higher than control for maceration.

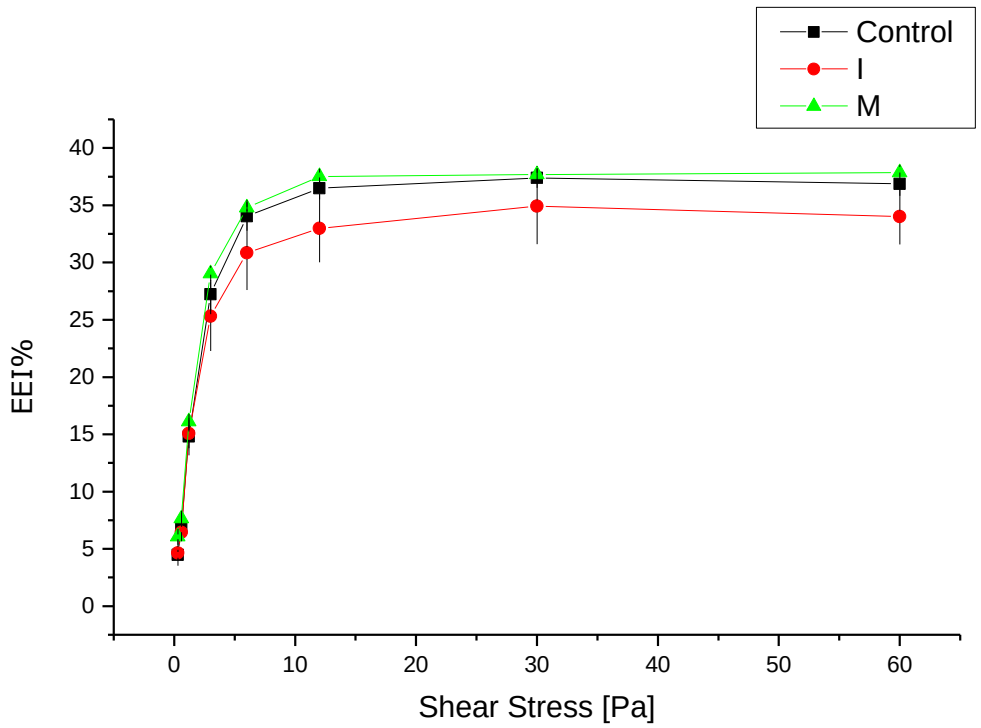

Figure 1: Mean values of elongation index from erythrocytes treated with I and M extracts. Data corresponds to mean ± SD.

CONCLUSIONS

The values of the osmotic fragility at low osmolality medium are higher for the samples with the presence of the leaf infusion extract of *B. forficata*, but very close to the control; it suggests a dilution of the extract then mixture with the erythrocytes directly. Neither infusion nor maceration at those concentrations originated osmotic lysis of the erythrocytes at normal physiological osmolality. The infusion showed a tendency to decrease the erythrocyte deformability while maceration seems to increase it. Infusion extract seems to induce a decreased in AChE enzyme activity probably derived from a non-identified inhibitor in the composition of the infusion. The NO reservoir attributed to glutathione can be influenced by the inactivation of glutathione reductase induced by oxidative stress[19] and strengthened by the antioxidant compounds of *Bauhinia forficata*[20], with a lower value of

the presence of NO to macerate compared to the control. Those very important preliminary results on aqueous extracts of *Bauhinia forficata* leaves must be taken into account for further studies and experimental *in vitro* design in order to obtain significant results to confirm their use in humans.

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Corresponding author: Dra. Bibiana Riquelme

Área Física, FCByF (UNR) Suipacha 531, 2000 Rosario, ARGENTINA
+54 341 4804593 int 282
briquel@fbioyf.unr.edu.ar, riquelme@ifir-conicet.gov.ar