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Research paper

Hemocompatibility and hemorheological activity of aqueous extracts from *Phyllanthus sellowianus*: effects on *in vitro* glycated erythrocytes

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ABSTRACT

Introduction: Phyllanthus sellowianus (Klotzsch) Müll Arg. is an Argentinian and Brazilian native plant used to treat diabetes. In vascular diseases like diabetes, erythrocytes form anomalous aggregates resistant to dissociation, associated with high glucose levels. This work aims to evaluate the hemocompatibility and hemorheological activity of aqueous extracts from *P. sellowianus* and its anti-diabetic activity using *in vitro* glycated erythrocytes as occur by hyperglycemia.

Methods: For this work, *P. sellowianus* specimens were collected on the Nogoyá River coast, Argentina, and morphoanatomical identified. Infusion, maceration, digestion, and cooking extracts were prepared with collected leaves and bark using saline as solvent. The oOsmolality and pH of the extracts were adjusted to an adequate interaction with human cells. Red blood cells (RBCs) from healthy donors (n = 3) were used and incubated with glucose solution for *in vitro* simulation of the hyperglycemia effects. RBCs were incubated with the aqueous extracts to assess their hemocompatibility. Also, glycated RBCs were incubated with the extracts to analyze their anti-diabetic properties. Erythrocyte Rheometer and Optical Chip aggregometer were used for the hemorheological evaluation.

Results: Assays with the different *P. sellowianus* extracts modified the viscoelasticity and aggregation of RBCs. Moreover, in some cases, *P. sellowianus* extracts reversed the *in vitro* glycation effect and hemorheological parameters got nearer control values.

Conclusion: These results would be helpful for the development of pharmaceutical formulations using this species or its phytocomponents. They also give relevant information to understand the action mechanisms through which these extracts or their components can be used as anti-diabetics in Phytomedicine.

Introduction

Many species of plants exist in the world of flora with therapeutic properties. For this reason, medicinal plants and their derivatives have long been the therapeutic basis of medicines (Hamburguer and Hostettmann, 1991). The use of medicinal plants as natural therapeutic agents is a growing interest because they are complex mixtures of biologically active products, many of which can serve as models for synthesizing many drugs (Toledo et al., 2003). The sarandí or white sarandí (*Phyllanthus sellowianus*) is a hydrophilic shrub of the phyllantaceae family. The species *P. sellowianus* (Klotzsch) Müll. Arg. is native to southern Brazil, northeastern Argentina, and the coastal region of Uruguay. It grows on the banks of streams and rivers, often forming "sarandisales" together with other species of the genus, such as red sarandí and black sarandí, leaning its branches over the water (Buszniez et al., 2014). The species *P. sellowianus* (Klotzsch) Müll. Arg. is popularly known as "rompe-piedra", "hierba-paloma", "arrebate-piedra", "filanto", "sarandí-blanco", "sarandí leño", a shrub that flowers in spring and bears fruit in summer and is used in popular medicine in Brazil, Argentina, Paraguay, and Uruguay (Calixto et al., 1998). In particular, the aqueous extract of the bark, the leaves, and the whole plant is recommended as a diuretic, and hypoglycemic agent (Smith et al.,

Abbreviations: A.u., arbitrary units; Abs, absorbance; AI, erythrocyte aggregation index; Amp, amplitude; C, cooking or decoction; D, digestion; DI, erythrocyte deformability index; I, infusion; M, maceration; PBS, phosphate buffered saline; RBC, red blood cells; $T_{1/2}$, half time; ρ, density; η, viscosity; η_m, erythrocyte membrane surface viscosity; μ, erythrocyte elastic modulus

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1988; Hnatyszyn and Ferraro, 1999; Mascaro, et al., 2023). Also, herbalists and pharmacies sell the bark and leaves as an industrial product for consumption in the form of tea in Argentina and Brazil (Mascaro, et al., 2017). Extracts from various botanical sources have shown to be promising resources for obtaining new isolated metabolites, constituting sources of mixtures of compounds with differential and synergistic effects at biochemical, cellular, and physiological levels. Analysis of plant polyphenols, namely flavonoids, has provided valuable information, demonstrating that multi-drug combinations of traditional medicine often outperform trends toward modified single constituents observed in contemporary medical literature and practice (Wagner, 2011). Moreover, the scientific literature describes studies on the antioxidant property of the Phyllanthus genus associated with the pronounced presence of flavonoids, a representative group of plant antioxidants (Mao, et al., 2016; Navarro et al., 2017). For example, the extract of *Phyllanthus emblica* fruits had significantly high antioxidant activities (Yongyu et al., 2019). Also, Kumar et al. (2017) have identified 51 compounds in ethanolic extracts of *P. emblica*, *P. fraternus*, *P. amarus* and *P. niruri*.

Diabetes mellitus is a chronic disease that affects the metabolism of carbohydrates, lipids, and proteins. Conceptually, diabetes mellitus is a heterogeneous syndrome caused by genetic-environmental interaction and characterized by chronic hyperglycemia as a consequence of a deficiency in insulin secretion or action (Alvin, 2016). Consequently, diabetes causes a series of clinical complications, demanding a large amount of money from the health system for treatment, recovery, and maintenance of the patients involved. In addition, several studies in diabetes have found hemorheological abnormalities, such as increased blood viscosity and alterations in erythrocyte viscoelasticity (Riquelme et al., 2003, 2006,), and the parameters of erythrocyte aggregation observing that erythrocytes form abnormal aggregates (clusters) resistant to dissociation (Lebensohn et al., 2009; Delannoy et al., 2015; Riquelme et al., 2001, 2005, 2022). The high blood glucose levels that occur in diabetes are known as hyperglycemia. This phenomenon produces glycation of red blood cells (RBC), altering their mechanical and aggregation properties (Riquelme et al., 2001, 2022). Currently, there are different and effective pharmacological alternatives for the control of diabetic patients. However, there is a population group in which the usual pharmacological therapies are ineffective due to drug resistance, lack of access to therapy due to availability problems, or the high cost of treatment (Dariya and Nagaraju, 2020; Negri, 2005). The use of *P. sellowianus* to support the treatment of diabetes is listed in the Argentine Pharmacopoeia (Farmacopea Argentina, 2013), being of current interest in the study of its mechanism of action and its hemocompatibility for future therapeutic uses (Mascaro et al., 2023).

This work aimed to evaluate the *in vitro* hemocompatibility and hemorheological action of different aqueous extracts from *P. sellowianus* on human red blood cells. Previously, red blood cells were incubated in glucose solution to have the glycation by the hyperglycemia in diabetes (Batista Da Silva et al., 2022). Then, the results would provide information on the hemorheological anti-diabetic activity of the *P. sellowianus* aqueous extracts.

Materials

Vegetal material

Leaves and branches of *P. sellowianus* were recollected in the sarandisal on the coast of the Nogoyá River (Nogoyá city, Entre Ríos, Argentina, 32°23'43.3"S59°45'39.3"W-32.395351,-59.760908) (Buszniez et al., 2014). The collection was carried out in the summer (02/03/2019). Figure 1 shows a photograph of the sarandisal and the collected specimens. Voucher specimens are preserved at the Argentina National Herbarium, which are cited according to the abbreviations listed in the Index Herbarium (Holmgren et al., 1990) as follows: ARGENTINA.

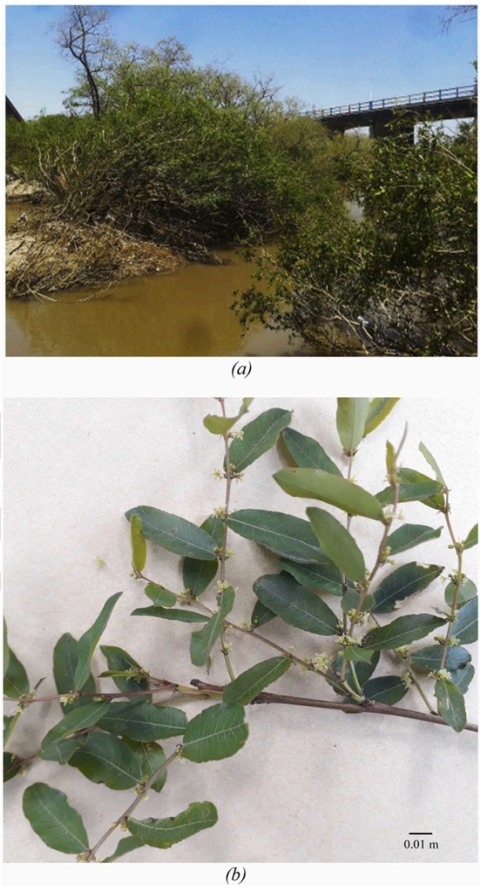


Fig. 1. (a) Sarandisal in Nogoyá River; (b) leaves of *Phyllanthus sellowianus*.

Provincia de Misiones. Dto. Gral. Belgrano, 15-III-2002, Múlgura M.E., 3371 (SI). Dto. Cainguás, 21-IX-1999, Biganzoli F., 546 (SI). Provincia de Buenos Aires. 15-XII-2003, Hurrell J.A., 5512 (MU, SI).

The selected leaves and branches (clean and not damaged by insects and animals) were stored in the dark opened paper bags to allow air circulation and dry desired. The vegetal material reached ideal drying after eight days (dry enough to prevent fungal growth) and was crushed before preparing the extracts.

Characterization and identification of the collected vegetal material

Problems in the genus *Phyllanthus* identification are frequent due to morphological similarities between species belonging to the same genus (García, 2004). Therefore, the epidermal and morphoanatomical characteristics of the leaf have a high diagnostic value at the specific and family level for taxonomic identification.

Figure 1b shows the leaves of the specimen collected in Nogoyá. These leaves are simple, alternate, deciduous, glabrous, 0.015–0.050 m long, x 0.005–0.010 m; blades discoid, elliptic or obovate-elliptic, with entire or gently wavy edge, acute, mucronate apex, attenuated base; spirally inserted, replaced by cataphylls only at shoot tips; stipules 3–4.5 mm long, triangular-subulate, with a scarious edge, finely fimbriated. In addition, they have a glabrous short petiole up to 2–3 mm long. These characteristics coincide with those described by Govaerts et al. (2000) for some genera of Phyllanthaceae, which before were included in Euphorbiaceae (Di Sapio, 2015). Consequently, according to what was established by these authors, the specimens collected correspond to *P. sellowianus*.

Also, complementary microscopic images were obtained from leaves of collected *P. sellowianus* (Figs. 2 and 3) using an optical microscope (Carl Zeiss whit a camera Axion Cam ERc 5s Zeiss). The fresh leaves were previously dipped in a solution of acetic acid (10 ml), formaldehyde (20 ml), distilled water (70 ml), and ethanol (100 ml). Then, the material was dehydrated with alcohol rising from 70 to 100°. The diaphanized epidermis and the foliar venation analysis were carried out using the Dizeo De Strittmatter technique (Strittmatter, 1973). Sections were made using a Minot rotary microtome at 20 μm. The stains used were cresyl violet, 1% safranin fast Green. Subsequently, the specimens were mounted in gelatin-glycerin.

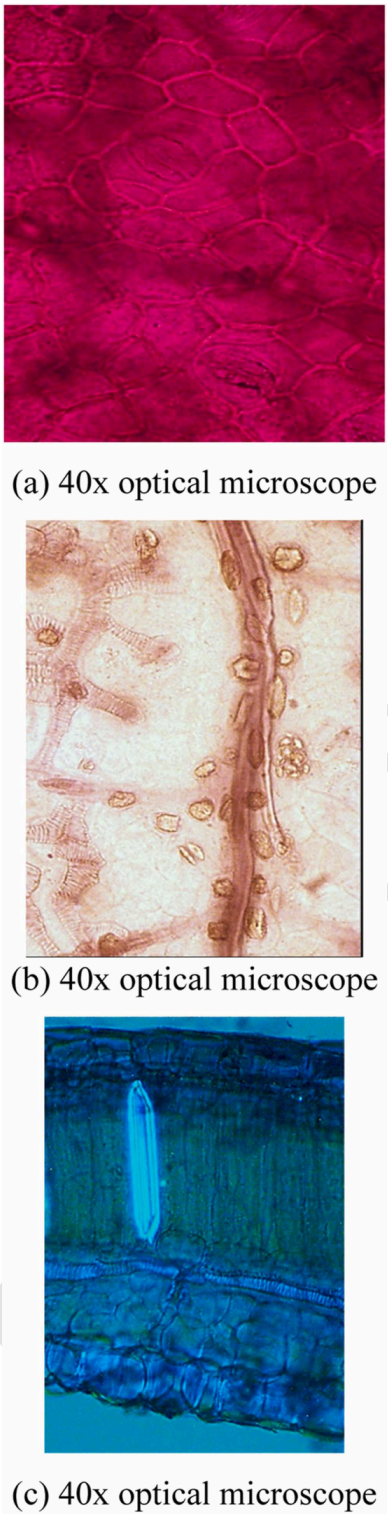


Fig. 2. (a) Abaxial epidermis and stomas; (b) druses, styloids and prismatic crystals of calcium oxalate; (c) heterogeneous mesophyll with an elongated prismatic crystal of calcium oxalate. Microscopic images obtained with objective 40x.

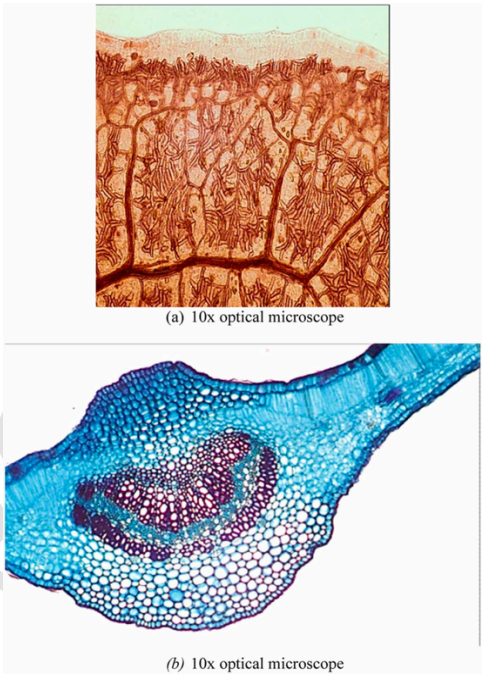


Fig. 3. Foliar architecture: (a) Image of the leaf, pinnate, camptodroma, and brochidodroma; (b) sheet cross section. Microscopic images obtained with objective 10x.

The morphoanatomical characteristics observed by optical microscopy are the following: the leaf presents the adaxial and abaxial epidermis, which is made of polygonal cells with straight to wavy contours and slightly thickened anticlinal walls with simple intercellular connections (Fig. 2a). Both epidermises are unistrata and have large, rectangular cells with convex radial and tangential walls and a thin cuticle. Paracytic stomata are located in both epidermises, being more numerous in the abaxial face. Large-diameter helical vessels form the vascular system. Drusen, styloids, and prismatic calcium oxalate crystals are present along the different nerves. The mesophyll is heterogeneous, formed by a single layer of palisade parenchyma, very continuous, with few intercellular spaces. Elongated prismatic calcium oxalate crystals (styloid) are abundant. The spongy parenchyma presents 3–4 rows of cells with intercellular spaces (Figs. 2b and c).

Figure 3a shows that foliar architecture is pinnate, camptodromous, and brochidromous, and the marginal venation is incomplete. The areolas are quadrangular or rectangular polygonal with simple or branched, straight, or curved vascular endings. The cross-section of the leaf has a contour with a single prominence towards the abaxial surface in the area of the main vein. The vascular bundles of the median nerve are arranged in an arc, the secondary and tertiary bundles are immersed in the mesophyll, which are collateral. Adjacent to the conduction elements, with abundant xylem of medium-sized vessels and almost surrounded, caps of sclerenchyma fibers are observed surrounding the phloem. In the perivascular parenchyma, a regular amount of druses and rhombic and prismatic calcium oxalate crystals are observed (Fig. 3b).

Aqueous extracts

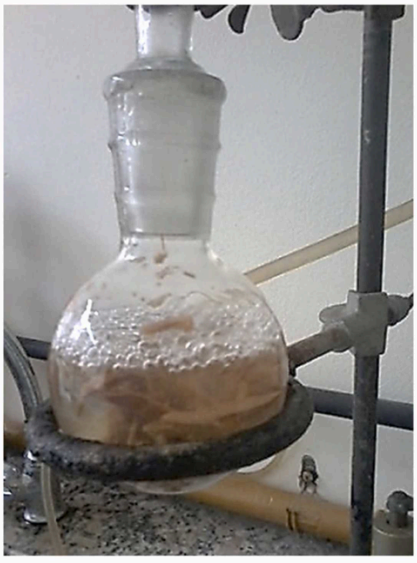
The aqueous extracts were prepared at 5% (w/v) by suspending 3 g of leaves and branches of *P. sellowianus* in 57 ml of saline solution (B. Braun Medical S.A, pH 7.4, and 300 mOsm/L). Saline was used as a solvent to achieve suitable pH and osmolality for subsequent incubation with human erythrocytes (Buszniez et al., 2017). An example of the material used is shown in Figure 4.

Maceration (M): the dried leaves and bark in contact with the solvent, acting for 12 hours at room temperature and protected from light.

Infusion (I): the leaves and bark dried in contact with boiling solvent until reaching room temperature.

Digestion at controlled temperature (D): the leaves and bark dried in contact with the solvent in a water bath until reaching a temperature of 40ºC, which was maintained for 20 min.

(a)



(b)

Fig. 4. Example of the material used: (a) crushed leaves and branches; (b) container showing the process to obtaining the infusion.

Cooking or decoction (C): the dried leaves and bark were introduced into the solvent, and the mixture was brought to 100 °C for 5 minutes. Distilled water was added to compensate for evaporative loss and to maintain constant volume and osmolality.

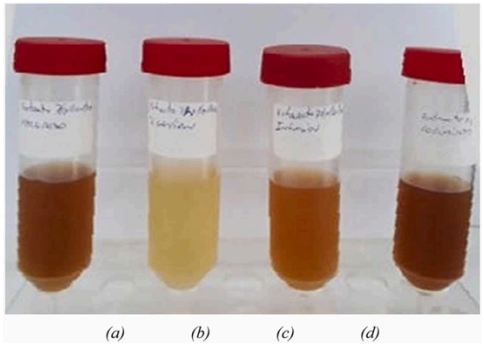
The filtration of the extracts was carried out using a syringe filter (25 mm Acrodisc brand filters), which allows the elimination of particles with diameters greater than 0.2 μm. The filtration step proceeded in sterile tubes under laminar flow according to standard protocols. The solutions were then fractionated into labeled containers and stored at 4 °C (Fig. 5).

Red blood cells

The fresh blood sample was collected from healthy donors (n = 3) by venipuncture in sterile vials containing anticoagulant (EDTA-K3). The study was approved by the Bioethics Committee of the Facultad de Ciencias Bioquímicas y Farmacéuticas of the Universidad Nacional de Rosario (Res. No. 735/2015 on October 30th, 2015), and all donors signed the informed consent. Healthy donors were between 25 and 35 years old, non-alcoholic, non-smokers, and not on medication. Complete clinical history and physical examination, including standard laboratory tests, were performed for each donor. Sample collection and processing were carried out within 4 hours from extraction time. All procedures were performed according to the Hemorheological Laboratory Techniques (Baskurt et al., 2009). Then, the RBC were separated by centrifugation (1.500 g, 5 minutes at room temperature) and washed with Phosphate Buffered Saline solution (PBS; pH 7.4, and 300 mOsm/L).

RBC glycated by in vitro hyperglycemia model

Hyperglycemia was *in vitro* modeled by incubating equal volumes of washed RBC and glucose solution according to the previously tested and published technique (Buszniez et al., 2017; Batista Da Silva et al., 2022). The incubation medium was prepared by solving glucose (dextrose Biopack, batch 16882015, Bat: 16882015) in PBS at a concentration of 0.4 g/dl. The RBCs were incubated at equal volume for 2 hours at 36.5 °C under stirring at 15 rpm (BOECO Mini-rocker Shaker MR-1). The glucose measurement (Accu-Chek Smart Pix) corresponds to a glycaemia of (220 ± 20) mg/dl in the samples incubated for 2 hours to obtain the glycated red blood cells (gRBC). Another aliquot of the washed RBC was incubated under the same conditions only with PBS (pH 7.4, 300 mOsm/L) as the *Control*. After incubation, the cells were washed with PBS by gentle centrifugation (1.500 g, 5 minutes at room temperature) before the subsequent treatment with plant extracts.



(a) (b) (c) (d)

Fig. 5. Tubes containing the aqueous extractive solutions of *Phyllanthus sellowianus*: (a) maceration, (b) digestion; (c) infusion; (d) cooking. The image shows the color variations due to the different substances extracted in each technique.

Methods

Characterizations of Phyllanthus sellowianus extracts

Previously, the physical and physicochemical characterizations were carried out in the different extractive aqueous solutions from the leaves and bark of *P. sellowianus*. The evaluation and control of the pH and osmolality of the prepared extracts is fundamental to maintaining the *in vitro* red blood cells (non-glycated and glycated red blood cells) closer to the *in vivo* conditions (Mascaro, et al., 2023; Buszniez, et al., 2017; Laura and Leela, 2022; Batista Da Silva et al., 2022). Also, the density, absorbance, and viscosity can change according to the different phytochemicals extracted, which can give qualitative information about their concentration (Mascaro, et al., 2017, 2023; Buszniez, et al., 2017).

The following parameters were determined at room temperature:

- *pH*: measured with a pH-meter (model pHS-2F).
- *osmolality*: measured using a cryoscopic osmometer (Osmomat 030 Gonotec).
- ρ: density measured by pycnometer technique.
- Abs^{405}: absorbance at a wavelength of 405 nm measured with a photocolorimeter (ANDALI). The 405 nm wavelength is frequently employed to measure the presence of different phytochemicals in plant extracts (Rodrigues et al., 2021; Buszniez et al., 2017).
- η: viscosity measured using a cone/plate rotational viscometer (Brookfield DV-II) at 25 °C and a shear rate of 115.2 s^{-1}.

The *M*, *I*, and *C* extracts were diluted at 50% in saline to measure the light absorbance with the photocolorimeter because they were too cloudy.

Incubation of RBC with Phyllanthus sellowianus extracts

RBCs from healthy donors were incubated with prepared extracts to evaluate the hemorheological activity, particularly the effects on erythrocyte viscoelasticity and aggregation (Mascaro, et al., 2018). Briefly, the RBC samples and the gRBC were incubated in equal volumes with each plant extract for 1 hour at 37 °C under stirring at 15 rpm (BOECO Mini-rocker Shaker MR-1). Then, the erythrocyte samples (*Control, RBC + M, RBC + D, RBC + I, RBC + C* and *gRBC, gRBC + M, gRBC + D, gRBC + I, gRBC +* C) were washed with PBS (pH 7.4, 300 mOsm) and suspended at 40% in autologous plasma.

Hemorheological techniques and instruments developed in the Biomedical Physics Group of the Rosario Physics Institute (CONICET-UNR) were employed.

Optical chip erythrocyte aggregometer

The optical chip aggregometer was used to evaluate the extract effect on erythrocyte aggregation kinetics. This instrument is based on a laser transmission technique using only 15 µl of sample in the chip hole (Toderi et al., 2015, 2017; Riquelme et al., 2022). The following parameters were obtained from the syllectograms of 400 seconds:

- *Amp* (Amplitude): light intensity at 400 seconds indicating the amount of RBC aggregates.
- $t_{1/2}$ (half time): the time required to reach the light intensity Amp/2, indicating the characteristic time constant for the average level of aggregation calculated at 400 seconds.
- *AI* (Aggregation Index): the ratio of the area below the syllectogram and the total area at 400 seconds, indicating the normalized amount of accumulated RBC aggregates.

Viscoelasticity of erythrocytes

Measurements were carried out in quintuplicate using the Erythrocyte Rheometer (Invention Patent: AR 091467 B1) (Riquelme et al., 2013), which was developed and patented in our laboratory (IFIR, CONICET-UNR). This instrument is based on the laser diffractometry technique and measures the time deformation of RBC subjected to stationary and oscillatory shear stresses at the frequency of 0.5, 1, and 1.5 Hz corresponding to 30, 60, and 90 cycles/Min. The following viscoelastic parameters were determined to evaluate the *P. sellowianus* extracts on the erythrocytes (Castellini and Riquelme, 2018; Riquelme et al., 2003, 2018, 2022):

- μ: elastic modulus.
- η_m: membrane surface viscosity.
- *DI*: deformability index.

For these determinations, the hematocrit of each sample was adjusted to 40% by suspending the RBC in autologous plasma. Then, 100 µl of each sample (control or treated) were poured into 4.5 ml of a PVP solution. This solution was prepared with polyvinyl-pyrrolidone (PVP360, Sigma) at 5% (w/v) in phosphate buffer solution (PBS, pH 7.4, 295 mOsmol/L) and had a viscosity of 22 cp at 25 °C.

Statistical analysis

For each sample, measurements were performed with the Erythrocyte Rheometer in quintuplicate, and with the Optical Chip Aggregometer in triplicate. A categorical variable was previously defined to label each group of data obtained to analyze the significant differences between the *P. sellowianus* extract treatments and the respective control. Then, the normality of the data was verified with the Shapiro-Wilk test, taking as the null hypothesis "the data have a normal probability distribution with given deviation and mathematical expectation". Within a significance level of 5%, those batches that met this premise were accepted. The EXCEL software was used to calculate the mean values, standard deviations, and *P*-values against control. Differences from the respective control were assumed to be statistically significant for $P < 0.05$.

Results

The Table 1 shows the values obtained for the absorbance at a wavelength of 405 nm (Abs^{405}), *pH*, density (ρ), and viscosity (η) of the maceration (*M*), digestion (*D*), infusion (*I*) and cooking (*C*) extracts from leaves and bark of *P. sellowianus*. The variations in these parameters are related to the different temperatures and times used during the extractions. Particularly, significant absorbency and viscosity changes were observed in maceration and digestion extractions, which could be due to the obtained phytochemicals (for example, flavonoids) with this extraction method at room temperature. The extraction method I uses

Table 1
Measured parameters of extractive solutions prepared from leaves and bark of *Phyllanthus sellowianus*. Mean ± standard deviations from each extractive solution measured by triplicate.

Solution	Abs^{405}	pH	ρ 10^3 kg/m^3	η cp
Saline solution	0.075 ± 0.001	7.4 ± 0.1	1.01 ± 0.01	1.02 ± 0.02
M	0.206 ± 0.001[a]	6.00 ± 0.01[a]	1.04 ± 0.04	1.20 ± 0.02[a]
D	0.193 ± 0.001[a]	6.45 ± 0.01[a]	1.04 ± 0.04	1.02 ± 0.04
I	0.197 ± 0.001[a]	6.42 ± 0.01[a]	1.01 ± 0.02	0.94 ± 0.02[a]
C	0.214 ± 0.001[a]	6.70 ± 0.01[a]	1.02 ± 0.04	1.02 ± 0.04

Abs, absorbance; C, cooking or decoction; D, digestion; ρ, density; I, infusion; M, maceration; η, viscosity.
[a] $P < 0.001$.

boiling temperature and these parameters decrease probably due to the degradation of the phytochemicals.

Table 1 shows that the lowest absorbance values were obtained for *D* and *I*, and the highest for *M* and *C*. Also, the extracts obtained from the collected bark and leaves of *P. sellowianus* have acid characteristic, and only the *C* extract presented a *pH* close to the physiological range. Consequently, the *pH* was adjusted by the NaOH addition to attain the pH of 7.4 necessary for the RBC incubation. The higher viscosity was presented by *M* and the lower for *I* extract. No significant differences were observed between the densities of the extracts and saline solution.

Table 2 shows the initial and adjusted osmolality of the extracts obtained from the leaves and bark of *P. sellowianus*. Results were different from the physiological osmolality required of 300 mOsm/L. Consequently, the values were adjusted by NaCl addition for the *M* and *D* extracts, and distilled water for *I* and *C* to achieve physiological osmolality. The osmolality is related to the extraction method of the phytochemical solutions being the lowest value for *D* extract, which was made at a temperature lower than 40 ˚C.

Results from the hemorheological evaluation are presented in Table 3 showing the stationary viscoelastic parameters of the erythrocytes (*RBC* and *gRBC*) incubated with the different *P. sellowianus* extracts. Table 3 shows no significant differences in the *DI* and the μ for any treated samples. It is observed that the *gRBC* sample presented a higher value than the control for η_m, followed by the *RBC + D* sample. The *gRBC + I* sample presented a significant decrease in η_m ($P < 0.01$), being lower than the control. These variations in the hemorheological activity observed between the different extracts could be due to the phytochemicals extracted in each method according to the observation in the physicochemical parameter variations (Table 1).

Table 4 shows the results obtained with the optical chip aggregometer to evaluate the aggregation kinetics of *RBC* and *gRBC* treated with the different *P. sellowianus* extracts. Table 4 shows that the sample previously incubated with the glucose solution (*gRBC*) presented a significant increase in *Amp* and *AI*, and a decrease in $t_{1/2}$, compared to the control. Glycation of RBC produced a significant increase in the rate and magnitude of erythrocyte aggregation, evidenced by less $t_{1/2}$ and higher *Amp* and *AI* with respect control samples. This effect is reverted in *gRBC* by the extract treatment. In particular, the aggregation shows a slightly significant increase with respect to the controls only for *gRBC* treated with *M*. Moreover, the *gRBC* samples treated with *I* and *C* extracts show a significant decrease concerning the aggregation of control.

Table 2
Initial and adjusted osmolality values of the extracts obtained from the leaves and bark of *Phyllanthus sellowianus*. Mean ± standard deviations from each extractive solution measured by triplicate.

Sample	Initial mOsm/L	Adjusted mOsm/L
Saline	305 ± 1	-
M	290 ± 1[a]	315 ± 1[a]
D	209 ± 1[a]	294 ± 1[a]
I	345 ± 1[a]	310 ± 1[a]
C	436 ± 1[a]	312 ± 1[a]

C, cooking or decoction; D, digestion; I, infusion; M, maceration.
[a] $P < 0.001$.

Table 3
Stationary viscoelastic parameters of *RBC* and *gRBC* treated with the aqueous extracts of *Phyllanthus sellowianus*. Mean ± standard deviations from three samples measured by quintuplicate.

Sample	DI	η_m 10^{-7} N.s/m	μ 10^{-6} N/m
Control	0.62 ± 0.02	1.8 ± 0.2	4.8 ± 0.3
RBC + M	0.63 ± 0.01	2.0 ± 0.3	4.8 ± 0.2
RBC + D	0.63 ± 0.01	2.2 ± 0.1[a]	4.8 ± 0.2
RBC + I	0.63 ± 0.02	2.1 ± 0.2	4.8 ± 0.1
RBC + C	0.62 ± 0.02	2.1 ± 0.7	4.8 ± 0.5
gRBC	0.63 ± 0.02	2.4 ± 0.2[b]	4.7 ± 0.3
gRBC + M	0.64 ± 0.01	1.8 ± 0.4	4.7 ± 0.1
gRBC + D	0.60 ± 0.02	2.1 ± 0.2[a]	4.8 ± 0.5
gRBC + I	0.61 ± 0.02	1.6 ± 0.2[b]	4.8 ± 0.5
gRBC + C	0.62 ± 0.02	2.1 ± 0.3	4.8 ± 0.5

C, cooking or decoction; D, digestion; DI, erythrocyte deformability index; gRBC, glycated red blood cells; I, infusion; M, maceration; RBC, red blood cell; η, viscosity.
[a] $P < 0.05$;
[b] $P < 0.01$.

Table 4
Aggregation kinetics parameters of the *RBC* and *gRBC* incubated with the different extractive solutions *Phyllanthus sellowianus*. Mean ± standard deviation from three samples measured by triplicate.

Sample	Amp a.u.	$t_{1/2}$ s	AI a.u.
Control	92.1 ± 0.6	48 ± 2	0.81 ± 0.03
RBC + M	95 ± 1[a]	49 ± 1	0.82 ± 0.01
RBC + D	95.9 ± 0.4[a]	36.6 ± 0.2[a]	0.85 ± 0.01[a]
RBC + I	95 ± 1[a]	45.0 ± 0.4[b]	0.82 ± 0.01
RBC + C	75 ± 4[a]	38 ± 3[a]	0.9 ± 0.3
gRBC	96.1 ± 0.2[a]	32.3 ± 0.2[a]	0.86 ± 0.03[b]
gRBC + M	95.3 ± 0.7[a]	43.0 ± 0.1[a]	0.83 ± 0.01
gRBC + D	93 ± 2	54.2 ± 0.6[a]	0.80 ± 0.01
gRBC + I	80 ± 1[a]	20.5 ± 0.1[a]	0.79 ± 0.01
gRBC + C	71 ± 5[b]	97 ± 5[a]	0.64 ± 0.30[c]

AI, erythrocyte aggregation index; Amp, amplitude; C, cooking or decoction; D, digestion; gRBC, glycated red blood cells; I, infusion; M, maceration; RBC, red blood cell.
[a] $P < 0.001$;
[b] $P < 0.01$;
[c] $P < 0.05$.

Discussion

The *P. sellowianus* extracts presented particular organoleptic characteristics. These characteristics have different intensities of color and smell for the distinct extractive methods used (Fig. 5). The extracts presented acid characteristics and osmolality outside the physiological range. Consequently, the pH and osmolality were corrected for direct contact with the red blood cells (Buszniez et al., 2017; Mascaro, et al., 2017, 2018, 2023). The extracts prepared with lower temperatures (M and D) show the lowest osmolality values. The extracts I and C present higher osmolality values, which was possible because it has been prepared at high temperatures, at evaporation was not negligible (Rodrigues et al., 2021; Buszniez et al., 2017; Mascaro et al., 2018).

The hemorheological activity of the extracts on RBC was evaluated using rheological and optical techniques developed and tested in the Biomedical Physics Group of the Rosario Institute of Physics (CONICET-UNR). Results show that the extracts do not influence the erythrocyte viscoelastic parameters. Differently, the erythrocyte aggregation parameters are modified concerning the control. Mainly, RBC treated with the M, D, and I extracts show a significant increase in the erythrocyte aggregation. While, the *C* extract show a decrease in the erythrocyte aggregation. Therefore, the extracts modify the erythrocyte aggregation when incubated with RBC from healthy donors, regardless of the method used. It will be necessary to continue the studies to significantly differentiate the response obtained using the cooking method by varying the concentration and the extraction time.

The same hemorheological tests were carried out on *in vitro* glycated erythrocytes simulating what occurs in diabetes. Results in the gRBC sample show an increase of η_m with respect to the control. Aggregation parameters are fully modified, showing an increase in the rate and magnitude of erythrocyte aggregation. This behavior is similar to that observed in the RBC of diabetic patients (Batista Da Silva et al., 2022;

Buszniez et al., 2017; Delannoy et al., 2015). The greater rate and increase in erythrocyte aggregation produced by glycation would be associated with a decrease in electrostatic repulsion between gRBC by the surface electrical charge decrease (Riquelme et al., 2001, 2022). Results from gRBC incubated with the different extracts indicate that the D, I, and C treatments modify the hemorheological parameters. Moreover, $gRBC + D$ and $gRBC + I$ significantly reverse the surface viscosity of the membrane modified by glycation approaching the value of the control sample.

The *gRBC* treated with *I* and *C* samples present a decrease in aggregation that could be due to their ability to disarm the cell agglomerates formed by glycation, which are very difficult to dissociate. Likewise, the most relevant results are those obtained with these treatments of *gRBC* since the *P. sellowianus* extract also reverses the glycation process by decreasing the erythrocyte aggregation.

The utilization of saline (pH 7.4 and osmolality 295 mOsmol/L) as a solvent and subsequent correction of the pH and osmolality of the prepared extracts is essential to maintain *in vitro* RBCs closer to *in vivo* conditions (Mascaro et al., 2023; 2022).

The density, viscosity, and absorbance analysis can give information about the quality of the different extracts prepared with the same vegetal drug (Mascaro et al., 2017, 2023; Buszniez, et al., 2017). However, more specific studies (Ortíz Fernández et al., 2016; Valenzuela Bustamante, 2015) are necessary to determine the chemical composition and concentration of phytochemicals extracted from *P. sellowianus* collected from Nogoyá River (Argentina). In particular, among the chemical compounds described in the literature, flavonoids have gained considerable attention in recent decades (Tariq, et al., 2023). Several studies have demonstrated the beneficial activity of flavonoids in different pathologies and their potentiality in industrial applications (Fadilah, et al., 2024).

Conclusions

The analysis of the hemorheological activity of the herbal extracts is considered a fundamental tool to evaluate their hemocompatibility and the possibility of reversing some of the alterations observed in vascular diseases, particularly in diabetes and arterial hypertension. The results show that the different *P. sellowianus* aqueous extracts can affect the erythrocyte aggregation and viscoelasticity in different ways. When evaluating the effect on glycated red blood cells (previously treated with glucose at 0.4 g/dl), the effect of glycation was reversed in some cases, obtaining the hemorheological parameters closer to the control values, according to the methods of extraction used. Consequently, these results provide information of great importance for studies on the hemocompatibility of the different extracts and the understanding of the mechanisms of action by which these extracts, or their chemical components, are used as anti-diabetics in phytomedicine. Moreover, the *P. sellowianus* hemorheological activity could help to treat the microvascular alterations in diabetes, arterial hypertension and other vascular pathologies.

Also, the study of the phytochemicals from the Phyllanthus genus is presented as a promising window into the medical research field (Mao, et al., 2016). Consequently, the hemocompatibility analysis of the different phytochemicals obtained from *P. sellowianus* extracts could interest the pharmaceutical industry in developing new drugs and pharmaceutical formulations that attenuate the clinical complications of diabetes in the injectable form.

Ethics approval

The study was approved by the Bioethics Committee of the Facultad de Ciencias Bioquímicas y Farmacéuticas of the Universidad Nacional de Rosario (Res. No. 735/2015 on October 30th, 2015), and all donors signed the informed consent. Healthy donors were between 25 and 35 years old, non-alcoholic, non-smokers, and not on medication. Complete clinical history and physical examination, including standard laboratory tests, were performed for each donor.

Author contributions

Bibiana D. Riquelme: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Patricia Buszniez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Hermano Mascaaro Grosso:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Uncited reference

Lebensohn et al., 2009.

Declaration of Competing Interest

Author have not conflict of interests.

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