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An In Vitro Study of the Antioxidant and Antihemolytic Properties of *Buddleja globosa* (Matico)

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Abstract The antioxidant and antihemolytic properties contained in the leaves of *Buddleja globosa* (*B. globosa*), also known as "Matico," were determined. Aqueous extracts of leaves were assayed in human erythrocytes and molecular models of its membrane. The latter were bilayers built-up of lipids located in the outer and inner leaflets of the erythrocyte membrane. Observations by scanning electron microscopy showed that the extract altered the morphology of erythrocytes inducing the formation of crenated echinocytes. This result implied that the extract components were inserted into the outer leaflet of the cell membrane. This conclusion was confirmed by experiments carried out by fluorescence spectroscopy of red cell membranes and vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) and by X-ray diffraction of DMPC and dimyristoylphosphatidylethanolamine bilayers. Human erythrocytes were in vitro exposed to HClO, which is a natural powerful oxidant. Results demonstrated that low concentrations of *B. globosa* aqueous extract neutralized the harmful capacity of HClO. Hemolysis experiments also showed that the extract in very low concentrations reduced hemolysis induced by HClO.

Keywords *Buddleja globosa* · Antioxidant · Human erythrocyte · Cell membrane · Plant extract

Abbreviations

Buddleja globosa	*B. globosa*
DMPC	Dimyristoylphosphatidylcholine
DMPE	Dimyristoylphosphatidylethanolamine
SEM	Scanning electron microscopy
GAE	Gallic acid equivalents
RBCS	Red blood cell suspension
ORAC	Oxygen radical absorbance capacity
IUM	Isolated unsealed human erythrocytes
LUV	Large unilamellar vesicles
GP	Generalized polarization
r	Fluorescence anisotropy
AAPH	2,2′-Azobis(2-amidino-propane)dihydrochloride

Introduction

Buddleja globosa (*B. globosa*), commonly called Matico, belongs to the *Buddlejaceae* family, and it grows in the forests of Chile, Argentina, Bolivia and Peru. Infusions of its leaves have been used in traditional folk medicine to heal ulcers, washing wounds, treating chronic dysentery, hemorrhoids, hepatitis and catarrh (Houghton et al. 2005). Much attention has been devoted to its chemistry and pharmacological properties (Houghton et al. 2005; Mensah et al. 2000, Backhouse et al. 2008; Xiao et al. 2016); however, little attention has been paid to its antioxidant

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properties and effects on human erythrocytes. Extracts of the leaves of this plant are an interesting source of flavonoids, terpenoids, phenylethanoids and iridoids. The presence of these compounds ensures biological activity, including antioxidant properties (Houghton et al. 2005). When cells use oxygen to generate energy, free radicals are created as a consequence of ATP production by the mitochondria. These by-products known as free radicals are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS). These free radicals are beneficial in moderate levels but at higher concentrations can damage tissues by oxidative stress, which play a major part in the development of chronic and degenerative ailments. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ or externally supplied through foods. In particular, the presence of polyphenol compounds such as flavonoids have been shown to possess radical scavenging/antioxidant activity (Martínez et al. 2014; Rao et al. 2011; Pham-Huy et al. 2008).

The present research was essentially aimed to determine whether polyphenols present in *B. globosa* aqueous extract of its leaves were able to interact with human erythrocytes and molecular models of its membrane, affect their shape and inhibit oxidative damage. The erythrocyte membrane is constantly exposed to oxidative stress given its oxygen transport function and the high unsaturated lipid content. Intact human erythrocytes incubated with aqueous extracts of *B. globosa* leaves were examined by scanning electron microscopy (SEM), while isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy. The capacity of *B. globosa* to interact with the bilayer structures of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) was evaluated by X-ray diffraction, while large unilamellar vesicles (LUV) of DMPC were studied by fluorescence spectroscopy. DMPC and DMPE are lipids representing those usually found in the outer and inner monolayers of the human erythrocyte membrane, respectively (Boon and Smith 2002; Devaux and Zachawsky 1994). The antioxidant properties of *B. globosa* were determined in human erythrocytes in vitro exposed to the oxidative stress induced by hypochlorous acid (HClO), which is a powerful natural oxidant (Tatsumi and Fliss 1994; Zavodnik et al. 2001; Hawkins and Davies 1998; Carr et al. 1997; Vissers and Winterbourn 1995). Similar studies have also been carried out in our labs on *Ugni molinae* leaves (Murtilla) (Suwalsky et al. 2006, 2007), *Aristotelia chilensis* leaves (Maqui) (Suwalsky et al. 2008), *Balbisia peduncularis* stems (Amancay) (Suwalsky et al. 2009), *Philesia magellanica* (Coicopihue) Suwalsky et al. 2015 and *Solanum crispum* (Natre) (Suwalsky et al. 2016).

Materials and Methods

Plant Material

Leaves of *B. globosa* were collected in the surroundings of Concepcion, Chile, and identified in the Laboratory of Pharmacognosy of the Faculty of Chemistry and Pharmacy, University of Concepcion, and a voucher was deposited in its corresponding herbarium. They were washed, air-dried and ground to a fine powder. Extraction of 13.2 g of dry powder in 250 mL of hot distilled water was performed in a soxhlet. The extract was lyophilized at −40 °C and 2.5 pa (Syclon-10N, MRC, Israel), and the total polyphenol content was determined by molecular spectrophotometry at 765 nm using the Folin-Ciocalteu reagent (Merk, Darmstadt, Germany) and method (Vegliolu et al. 1998). The total flavonoids were determined by the Chang et al. (2002) method. Gallic acid was used as the standard for the calibration curve, and the total polyphenol content was expressed as gallic acid equivalents (GAE) (Singleton and Rossi 1965).

HPLC Analysis

High performance liquid chromatography (HPLC) was employed for identifying polyphenols, where the analysis followed the protocol established by Liang et al. (2013). Phenolic compounds were detected at 280, 320, 365 and 525 nm on the diode array detector and, at the same time, spectrum scans were performed from 210 to 600 nm. Both sample and standards were diluted in methanol at 9.5 and 1 mg/mL, respectively. The lyophilized extract was resuspended in water and tested to identify the presence of chemical species by classical phytochemical reactions. Finally, all tannins and flavonoids were identified in the aqueous extract.

Scanning Electron Microscopy (SEM) Studies of Human Erythrocytes

The experimental procedure to perform the SEM observation has already been described in some of our previous publications; e.g., Suwalsky et al. (2015, 2016). Red blood cell suspensions (RBCSs) were incubated for 1 h at 37 °C with different concentrations of the plant aqueous extract (0.01, 0.05, 0.5 and 1 mM GAE); these concentrations were attained diluting the extract in saline solution to a final volume of 1 mL. In order to test the antioxidant capacity of *B. globosa*, 0.25 mM NaClO (Sigma, Mo, USA) was added to *B. globosa* solutions and RBCSs following the same procedure.

Hemolysis Assays

RBCSs (10% v/v) were incubated in a shaking bath for 15 min at 37 °C in PBS in the presence of *B. globosa*

aqueous extracts (0.01, 0.05, 0.5 and 1 mM GAE). NaClO was added as single bolus of a diluted solution in PBS, whose concentrations (0.05, 0.25 and 0.5 mM) were spectrophotometrically determined at 292 nm ($e = 350\ M^{-1}\ cm^{-1}$) (Morris 1966). At pH 7.4, NaClO exists as HClO and ClO^- in an approximately equimolar ratio (Vissers and Winterbourn 1995; Battistelli et al. 2005). After 15-min incubation, an aliquot of RBCS was centrifuged (EYDAM, Germany) at 2500 rpm for 10 min. Hemolysis was spectrophotometrically evaluated (Jasco, Japan) at 540 nm as hemoglobin (Hb) released from cells in the supernatant (Beutler 1975).

Oxygen Radical Absorbance Capacity (ORAC)

The method proposed by Cao (Cao et al. 1993, 1995) was selected because of its advantages in biological systems (Cao and Prior 1998; Prior and Cao 1999). On the other hand, with fluorescein as the fluorescent probe in the ORAC assay ($ORAC_{FL}$), the hydrophilic and lipophilic fractions could be measured. The reproduced protocol was that by Wu et al. (2004), where *B. globosa* extract was diluted in ultrapure distilled water. The oxidant agent 2,2′-azobis(2-amidino-propane)dihydrochloride (AAPH) was used as peroxyl generator and Trolox® as a standard. Data were expressed as micromoles of Trolox equivalents per gr of sample (μmol of TE/g).

Fluorescence Spectroscopy of Isolated Unsealed Human Erythrocyte Membranes (IUM) and DMPC Large Unilamellar Vesicles (LUV)

The influence of *B. globosa* extract on the physical properties of human erythrocyte membranes was analyzed by fluorescence spectroscopy on IUM and DMPC LUV. The measurement of the fluorescence parameters anisotropy (r) and generalized polarization (GP) was achieved using two different fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan), respectively. According to the previously described method (Suwalsky et al. 2009), *B. globosa* extracts were incorporated into IUM and LUV suspensions by addition of adequate aliquots in order to obtain different concentrations. These samples were then incubated for 10–15 min. Blanks were prepared using samples without probes. Data presented in the figures represent mean values and standard error of 15 measurements in two independent samples.

X-ray Diffraction of DMPC and DMPE Multibilayers

Synthetic DMPC (lot. 140PC-251, MW 677.9) and DMPE (lot. 140PE-59, MW 635.9) from Sigma (St. Louis, MO) were used without further purification. About 2 mg of each phospholipid was introduced into 1.5-mm-diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) and then suspended in 160 μL of (a) distilled water and (b) aqueous extracts of *B. globosa* in a range of concentrations (1 μM–1 mM). The experiments, whose technical details have been previously described (Suwalsky et al. 2016), were performed at 18 ± 1 °C, which is below the main phase transition temperature of both phospholipids. Each experiment was performed at least in triplicate.

Results

Yield

Total polyphenol content of *B. globosa* leaves was 19.3 mM (GAE). The total flavonoid represents 20.3% of total polyphenols. Results were obtained after dissolving 101.2 mg of lyophilized plant powder in 50 mL of distilled water.

HPLC Analysis

Phenolic acids and flavonoids were identified from their retention times relative to the internal standard. Linking together the retention times with the spectral profiles of the individual peaks from the chromatograms (Fig. 1), the specific individual phenolic compounds identified in the lyophilized extract of *B. globosa* were the flavonol

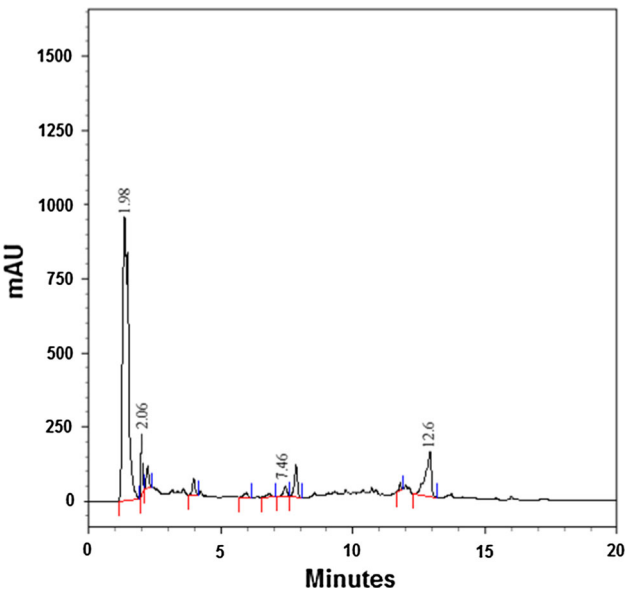

Fig. 1 RP-HPLC chromatogram; retention times of standards identified: gallic acid (RT = 2.06), ferulic acid (RT = 7.46), luteolin (RT = 12.6) and the flavonol quercetin (RT = 1.98)

quercetin (RT = 1.98), gallic acid (RT = 2.06), ferulic acid (RT = 7.46) and luteolin (RT = 12.6).

Scanning Electron Microscopy (SEM) of Human Erythrocytes

SEM observation of human erythrocytes incubated with *B. globosa* extract in the range of 50 μM–1 mM (GAE) revealed changes in the normal biconcave shape of the erythrocytes (Fig. 2). The morphological analysis revealed that a few of the cells treated with 50 μM (Fig. 2b) presented echinocytosis, an altered condition in which the erythrocytes show a spiny configuration with protuberances in their surfaces, and in a number of the rest stomatocytosis (a cup-shaped form with evagination of one surface and a deep invagination of the opposite face; 0.5 mM induced the formation of stomatocytes, a few echinocytes and knizocytes (triconcave erythrocytes,) (Fig. 2c); 1 mM produced a large number of echinocytes and a few stomatocytes (Fig. 2d). Figure 3 shows the antioxidant capacity of *B. globosa* to neutralize the effects of 0.25 mM HClO. In fact, 0.25 mM HClO (Fig. 3c) induced an intense echinocytosis with the prevalence of microcytes. As shown in Fig. 3d, 50 μM aqueous extract of *B. globosa* (GAE) neutralized the deleterious effect induced by HClO completely restoring the erythrocytes to their normal shape.

Hemolysis Assays

As it may be appreciated from Fig. 4, 0.5 mM HClO induced 75% hemolysis in human erythrocytes in comparison with control with distilled water (100%). On the other hand, increasing *B. globosa* concentrations from 10 μM up to 0.5 mM GAE produced a concentration dependent hemolysis effect, which reached about 20% hemolysis with the highest assayed extract concentration. However, 50 μM and 0.5 mM extract (GAE) considerably neutralized the hemolytic effect of HClO.

Oxygen Radical Absorbance Capacity (ORAC) Assays

The measurements of total antioxidant capacity (TAC) for *B. globosa* extracts were from 3720 μmol of TE/g sample. These results are comparable with the high antioxidant capacity revealed by the hemolysis assays.

Fluorescence Spectroscopy of Isolated Unsealed Human Erythrocyte Membranes (IUM) and DMPC Large Unilamellar Vesicles (LUV)

The effect of *B. globosa* extract on DPH anisotropy (Fig. 5a) shows a large effect on erythrocyte membranes (IUM) at 37 °C and DMPC vesicles (LUV) at 18 °C. However, no

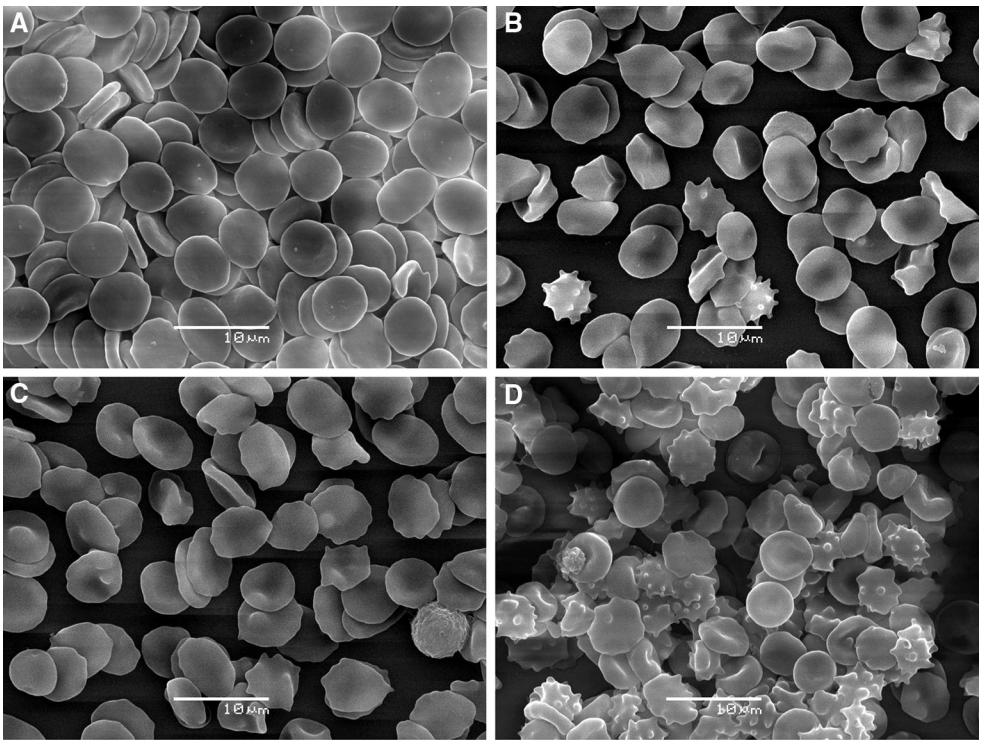

Fig. 2 Effects of *B. globosa* aqueous extracts on the morphology of human erythrocytes. Scanning electron microscopy (SEM) images of control (**a**), and incubated with 50 μM (**b**), 0.5 mM (**c**), and 1 mM (**d**) expressed as gallic acid equivalents (GAE); ×3000; $n = 3$

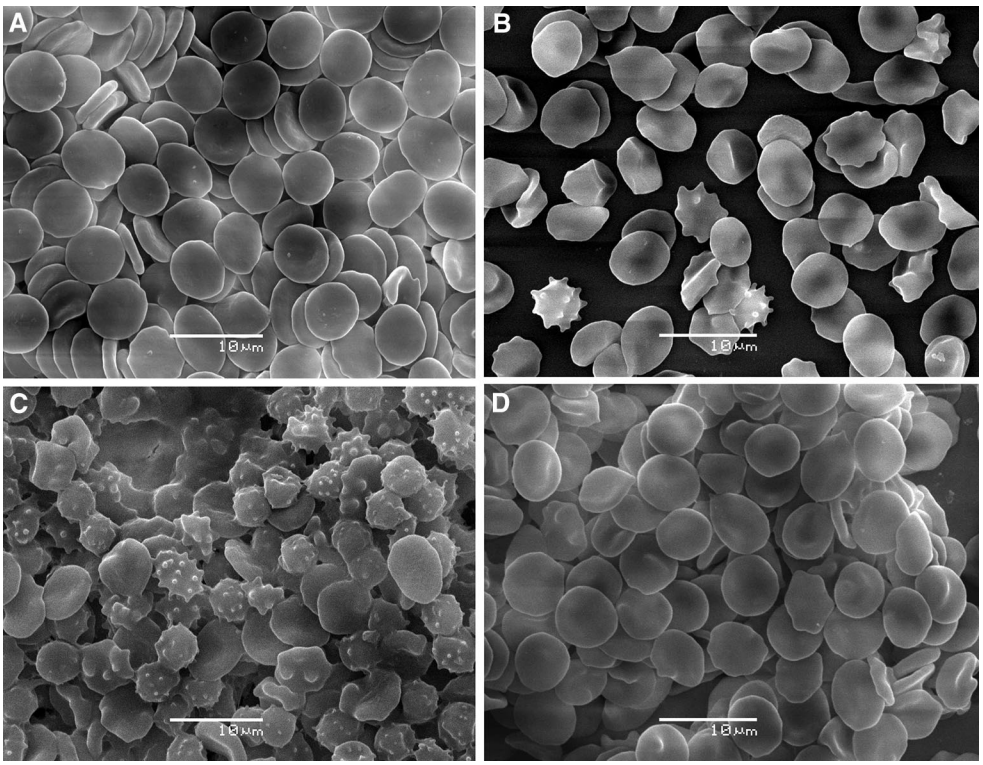

Fig. 3 Protective effect of *B. globosa* aqueous extracts against oxidative capacity of HClO. Scanning electron microscopy (SEM) images of control (**a**); erythrocytes incubated with: **b** 50 µM *B. globosa*; **c** 0.25 mM HClO, and **d** 0.25 mM HClO with 50 µM *B. globosa*; aqueous extract expressed as gallic acid equivalents (GAE); ×3000; $n = 3$

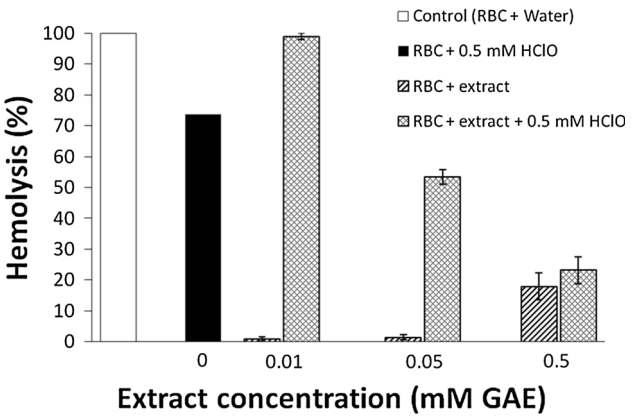

Fig. 4 Percentage of hemolysis of red blood cells (RBC) incubated with *B. globosa* aqueous extracts and 0.5 mM HClO. Extract concentration is expressed as gallic acid equivalents (GAE); $n = 2$. Values are the mean ± SD

significant effect was observed on DMPC large unilamellar vesicles at 37 °C. This result can be explained because the fluidity of DMPC vesicles at 37 °C is high, and thus, small changes in the degree of order are not evidenced by fluorescence anisotropy. The effect on IUM at 37 °C and DMPC at 18 °C showed a steep slope down to 30 µM *B. globosa*, indicative of the structural perturbation of their hydrophobic acyl regions. However, not such effect is observed in DMPC at 37 °C as at this temperature the lipid is in a fluid state. The spectral shift of the laurdan probe (Fig. 5b) is related to changes on the hydration at the lipids polar heads or on water molecules dynamics. The effect of *B. globosa* on the GP of laurdan shows a small but significant change in IUM at 37 °C, a slight change in DMPC LUV at 18 °C and no significant change in DMPC LUV at 37 °C. The fluorescence lifetime is very sensitive to dielectric constant change in the environment of the probe, mainly due to hydration changes in lipid lamellas. Laurdan probe is located in the hydrophobic–hydrophilic interface, and DPH is located at the hydrophobic deep level of the lipids acyl chains. Figure 5c shows that *B. globosa* produced a large effect on the hydration of IUM acyl chains and weaker effects on those of DMPC vesicles at 18 and 37 °C. On the other hand, the results of the lifetime of laurdan probe (Fig. 5d) show only very slight changes in the hydration at the hydrophobic–hydrophilic interface in erythrocyte membranes and DMPC LUVs at 18 and 37 °C. From these results, it can be concluded that *B. globosa* higher effects were induced in the hydrophobic acyl regions of both IUM and DMPC.

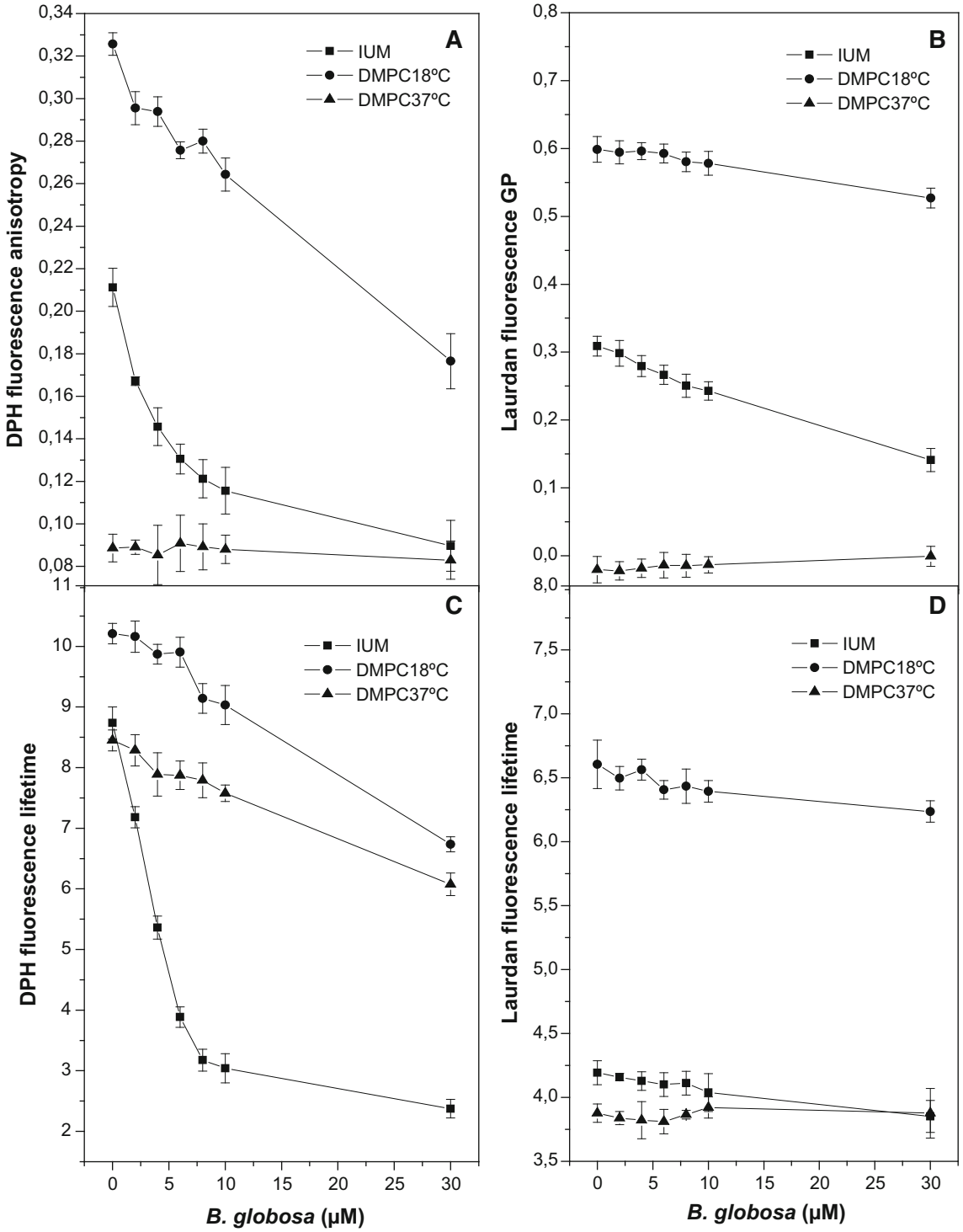

Fig. 5 Fluorescence anisotropy of DPH (**a**) and laurdan GP (**b**); fluorescence lifetime of DPH (**c**) and laurdan (**d**) in isolated unsealed human erythrocyte membranes (IUM) at 37 °C, and large unilamellar vesicles (LUV) of DMPC at 18 and 37 °C

X-ray Diffraction of DMPC and DMPE Multibilayers

In Fig. 6a, the results after incubating DMPC with water and *B. globosa* aqueous extracts are presented. Water affected DMPC structure as its bilayer repeat (bilayer width plus the width of the water layer between bilayers) increased from about 55 Å in its dry crystalline form (Suwalsky 1996) to 64.5 Å when immersed in water, and its low-angle reflections (LA in the figure), which

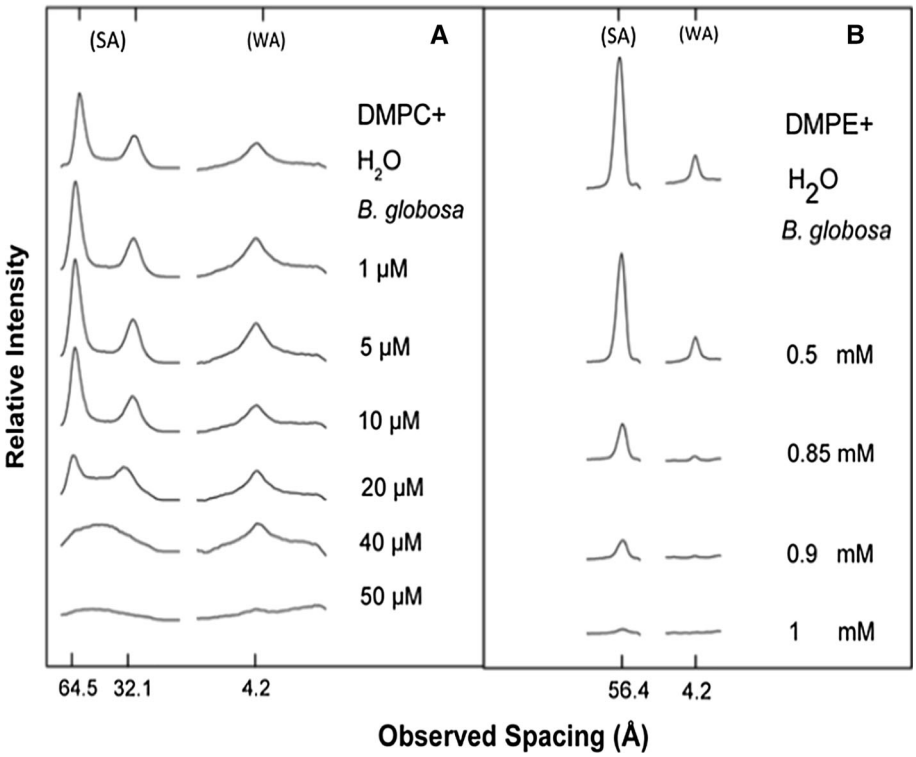

Fig. 6 Microdensitograms from X-ray diffraction patterns of **a** dimyristoylphosphatidylcholine (DMPC) and **b** dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous extracts of *B. globosa*; *SA* small-angle and *WA* wide-angle reflections. Concentrations are expressed as gallic acid equivalents (GAE)

correspond to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer repeat. On the other hand, in the wide-angle region (WA in the figure), only one strong reflection of 4.2 Å showed up which corresponds to the average distance between fully extended and hexagonally packed acyl chains. After being exposed to 20 μM GAE *B. globosa* aqueous extract, there was a strong weakening of the low-angle lipid reflection intensities, whereas no reflections were observed with 50 μM extract, implying a complete perturbation of the bilayer structure at this concentration. Figure 6b presents the results obtained with DMPE. The only reflection of 56.4 Å in the low-angle region and that of 4.2 Å in the wide-angle region are indicative of the gel state reached by DMPE in water after heating and cooling. The plant extract in the 0.5–1 mM range of concentrations induced a gradual reduction of both reflection intensities, until both were absent at the maximum assayed concentration. Although this complete perturbation of DMPE bilayer structure was attained with 1 mM GAE concentration, a similar effect was induced in DMPC but with a concentration 20 times lower (50 μM GAE). These results imply that *B. globosa* extract has a higher degree of interaction with DMPC than with DMPE bilayers.

Discussion

In the literature, there are several studies dealing with chemical and biological properties of *B. globosa* (Houghton et al. 2005; Mensah et al. 2000, Backhouse et al. 2008; Xiao et al. 2016). However, there is a lack of reports dealing with its antioxidant properties and effects on human erythrocytes. Phytochemical studies of the profile of *B. globosa* indicate the presence of phenolic acids and flavonoids; specific individual phenolic compounds were gallic acid, ferulic acid, luteolin and the flavonol quercetin. In addition, phenylethanoid esters and terpenoids including iridoids, sesquiterpenes, diterpenes and saponins have been reported (Houghton et al. 2003). The disruption of lipids forming the cell membrane caused by the free radical peroxidation process is one of the main causes to the development of pathological events (Abuja and Alvertini 2001). Flavonoids present several biological properties which may be related to their antioxidant and free radical scavenging ability (Saija et al. 1995). The molecular mechanisms of flavonoid antioxidant action are poorly understood. However, it has been proposed that their antioxidant capacity might be attributed to their insertion into cell membranes modifying the lipid bilayer

arrangement and fluidity (Arora et al. 1998, 2000; Nakagawa et al. 2000; Chaudhuri et al. 2007). The interaction between flavonoids and the lipid bilayer results in either the binding at the lipid–water interface or the distribution in the hydrophobic core of the membrane. The different location of these molecules was determined by their chemical properties (Oteiza et al. 2005). It has been suggested that flavonoids may be concentrated near the membrane surface suitable for scavenging aqueous oxygen radicals; for instance, Terao et al. (1994) and Pawlikowska-Pawlega et al. (2003) determined that quercetin was localized in the polar head region of a lipid multilamellar liposomes. On the other hand, Arora et al. (2000) suggested that flavonoids and isoflavonoids locate into the hydrophobic region of phospholipids. Ollila et al. (2002) related these interactions to their respective hydrophobicities. The embedding of quercetin in bilayers depends on the pH of the media; at acidic pH, quercetin is deeply embedded in planar bilayers (Movileanu et al. 2000), whereas at physiological pH it interacts with the polar head groups at the water–lipid interface (Pawlikowska-Pawlega et al. 2003). Tsuchiya (2001) reported that epicatechin, being more hydrophobic than its isomer catechin, has a greater degree of interaction with membrane lipids.

Results of the present study indicate that aqueous extracts of *B. globosa* interacted with the human erythrocyte membrane inducing morphological alterations of the normal discoid shape of erythrocytes (Fig. 2). According to the bilayer couple hypothesis (Sheetz and Singer 1974; Lim et al. 2002), the fact that low concentrations of *B. globosa* extracts produced echinocytes proves that the flavonoid molecules located mainly in the outer monolayer of the red cell membrane. This conclusion was confirmed by fluorescence (Fig. 5) and X-ray diffraction (Fig. 6) performed on DMPC, class of lipid preferentially located in the outer monolayer of human erythrocytes (Boon and Smith 2002; Devaux and Zachawsky 1994). In fact, at 18 °C *B. globosa* aqueous extracts induced structural perturbations to both the polar group and acyl chain regions of DMPC, being this effect more pronounced in the lipophilic polar groups. On the basis of these results, it might be postulated that cell membrane lipid bilayers are in general potential targets for *B. globosa* polyphenols. Thus, the morphological alterations of the human erythrocytes can be explained by the insertion and consequent functional perturbation of cell membrane lipid bilayers. This conclusion agrees with others reached with polyphenols extracted from various plants and assayed by different methods (Saija et al. 1995; Arora et al. 1998, 2000; Nakagawa et al. 2000; Kajiya et al. 2002).

As shown in Fig. 3, HClO changed the morphology of the red cells from their normal shape to echinocytes. This result indicates HClO inserted into the outer monolayer of the red cell membrane. This conclusion is supported by experiments carried out in red cells by fluorescence spectroscopy that showed structural perturbations of the external monolayer due to alterations of the membrane lipid packing arrangement (Zavodnik et al. 2001). These results are not in line with those reported by Vissers and Winterbourn (1995) which indicated that HClO penetrates into the red cells passing through the hydrophobic lipid bilayer without the membrane acting as a major barrier. On the other hand, Schraufstatter et al. (1990) also reported that low HClO concentration (10–20 μM) induced functional disturbances to the plasma membrane of tumor cells. Our finding that 50 μM *B. globosa* aqueous extract annulled the effect of 0.25 mM HClO demonstrates the plant extract power to neutralize HClO capacity to change the shape of erythrocytes. The significant protection of *B. globosa* aqueous extract was also shown in the hemolysis experiments (Fig. 4). The molecular mechanism by which HClO causes lysis is unknown. Our X-ray experiments showed that HClO up to 0.25 mM did not interact with DMPC neither with DMPE (Fig. S1); however, modification of membrane proteins, cross-links formation might result in the cluster of band 3 and other membrane proteins to form hemolytic pores (Vissers et al. 1998). Arora et al. (2000) reported that flavonoids and isoflavonoids locate in LUV hydrophobic region, whereas Nakagawa et al. (2000) indicated that certain flavonoids locate in the lipid and aqueous interphase. It might then be possible that the insertion of the extract components into the membrane bilayer hinders the penetration of HClO and avoids its consequent damage. It might also imply that this restriction can be applied to the diffusion of free radicals into cell membranes and avoid the consequent deleterious effects.

HPLC analysis of *B. globosa* revealed the presence of phenolic acids and flavonols. It has been suggested that other compounds characterized in *B. globosa* can also interact with the cell membrane causing alterations like iridoids (Garg et al. 1994), saponins (Nakamura et al. 1979) and terpenoids (Havaux 1998). However, phenolics compounds present in the extract are the only one responsible for the protective effect observed, since they actually interact directly with reactive oxygen species (Arora et al. 1998; Rice-Evans et al. 1997). The high antioxidant capacity of these compounds might explain the high ORAC value of *B. globosa* extract. The total antioxidant capacity (TAC) of the extract (3720 μmol of TE/g) is comparable to extracts with antioxidant properties like grapes (2016 μmol of TE/g) and strawberries (5938 μmol of TE/g), considering their lipophilic and hydrophilic ORAC values (Wu et al. 2004; Sak 2014).

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