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Human erythrocytes are affected in vitro by flavonoids of *Aristotelia chilensis* (Maqui) leaves

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ABSTRACT

Aristotelia chilensis (Mol.) Stuntz (*A. chilensis*), also known as maqui, is a plant of the Elaeocarpaceae family that grows in central and southern Chile as well as southwestern Argentina. Infusions of its leaves have long been used in the traditional native herbal medicine to treat different ailments. Phytochemical studies of the plant's chemical composition of the plant indicate the presence of indolic alkaloids, flavonoids, cianidine glucosides, delfidine, malvidine, petunidine, cumarines and triterpenes. These compounds, particularly the flavonoids, have antioxidant properties. In order to evaluate the mechanisms of its toxicity and their antioxidant properties, the leaves' aqueous extracts were induced to interact with human red cells, their isolated unsealed membranes (IUM), and molecular models of the human erythrocyte membrane. These consisted of multibilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipids classes located in the outer and inner monolayers of the human erythrocyte membrane, and large unilamellar vesicles (LUV) of DMPC. The capacity of *A. chilensis* aqueous extracts to perturb the bilayer structure of DMPC and DMPE was evaluated by X-ray diffraction, DMPC LUV and IUM were studied by fluorescence spectroscopy, and intact human erythrocytes were observed by scanning electron microscopy (SEM). Results of the present study indicate that aqueous extracts of *A. chilensis* induced an alteration of human erythrocyte morphology from the normal discoid shape to an echinocytic form, changes that are explained in terms of the extract interaction with the membrane's outer phospholipid monolayer.

1. Introduction

Aristotelia chilensis (Mol.) Stuntz (*A. chilensis*), also known as maqui, clon, queldron, koelon, is a plant of the Elaeocarpaceae family that grows in central and southern Chile as well as southwestern Argentina. This species is a rich source of phenolics with antioxidant capacity (Miranda-Rottmann et al., 2002). Infusions of its leaves have long been used in the traditional native herbal medicine to treat sore throats, kidney pains, stomach ulcers, diverse digestive ailments (tumors and ulcers), fever, scarring injuries, and as an anti-inflammatory agent (Céspedes et al., 2008; Silva and Bittner, 1992). It has also been reported to be used as an astringent, to treat hemorrhoids, and to facilitate childbirth (Montes and Wilkomirsky, 1977). The fruits are eatable, with a relatively high anthocyanin content (Escribano-Bailón et al., 2006), which are often used against diarrhea (Silva and Bittner, 1992). Phytochemical studies of the chemical composition of the plant indicate the presence of indolic alkaloids, flavonoids, cianidine glucosides, delfidine, malvidine, petunidine, cumarines and triterpenes (Silva and Bittner, 1992). These compounds, particularly the flavonoids, have antioxidant properties with low toxicities (Hou et al., 2004). It has been estimated that about 2% of the oxygen used by normal cells forms reactive oxygen species (ROS) (Chance et al., 1979). When the ROS production overcomes the numerous antioxidant defense barriers, damage of a range of cellular structures and functions is produced. This process, known as oxidative stress, leads to pathologies such as atherosclerosis and cancer, and ultimately to cell death (McCall and Frei, 1999). The main ROS are the superoxide anion ($O_2^{\bullet}$) and the hydroxyl ($OH^{\bullet}$) free radicals, which react

Abbreviations: SEM, scanning electron microscopy; IUM, isolated unsealed erythrocyte membrane; RBCS, red blood cells suspensions; LUV, large unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; *r*, fluorescence anisotropy; GP, generalized polarization; DPH, 1,6-diphenyl-1,3,5-hexatriene; laurdan, 6-dodecanoyl-2-dimethylaminonaphtalene; GAE, gallic acid equivalents.

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with cell molecules such as lipids, proteins, carbohydrates, DNA and lipoproteins (Sohal and Weindruch, 1996). The antioxidants are molecules that scavenge free radicals and also can stop their formation in the cells, thus limiting their damage. Thus, the potential use of *A. chilensis* extracts as antioxidants has also been considered (Valladares, 2007). The molecular mechanisms of the antioxidant action of flavonoids have yet to be fully elucidated and are still a matter of considerable debate. However, it has been suggested that the ability of these compounds to partition in cell membranes and the resulting restriction on their fluidity could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions (Arora et al., 1998, 2000).

The toxic side effects of plant extracts are increasingly considered (Barbosa-Ferreira et al., 2005; Lu et al., 2005). In the course of in vitro systems search for the toxicity screening of chemicals, different cellular models have been applied to examine their adverse effects in isolated organs. This article describes the interaction of aqueous extracts of *A. chilensis* with human erythrocytes as well as with molecular models of the erythrocyte membrane. The cell membrane is a diffusion barrier which protects the cell interior. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with exogenous species. Erythrocytes were chosen because although less specialized than many other cell membranes they carry on enough functions in common with them such as active and passive transport, and the production of ionic and electric gradients, to be considered representative of the plasma membrane in general. Intact human erythrocytes incubated with aqueous extracts of *A. chilensis* were observed by scanning electron microscopy (SEM) while isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy. With the aim to better understand the molecular interactions of *A. chilensis* with the erythrocyte membrane we have utilized molecular models, consisting in multibilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipids classes located in the outer and inner monolayers of many cell membranes including that of the human erythrocyte (Boon and Smith, 2002; Devaux and Zachowsky, 1994), and large unilamellar vesicles (LUV) of DMPC. The capacity of *A. chilensis* aqueous extracts to perturb the bilayer structure of DMPC and DMPE was evaluated by X-ray diffraction while DMPC LUV was studied by fluorescence spectroscopy. These systems have also been used to determine the interaction with, and perturbing effects on human erythrocytes by extracts of the *Ugni molinae* leaves (Murtilla) (Suwalsky et al., 2006, 2007).

2. Materials and methods

2.1. Plant material

Leaves of *A. chilensis* were collected in the main campus of the University of Concepción, Chile, in April 2007. The plant was identified by the Department of Botany of the Faculty of Natural and Oceanographic Sciences, University of Concepción, and a voucher specimen was deposited at its Herbarium. The leaves were washed, air-dried and ground to a fine powder. An extraction was achieved with 50 mL of hot distilled water on 0.5 g of dry powder. The total polyphenol content of the aqueous extract was spectrophotometrically determined (Bausch & Lomb SP2000UV, USA) at 765 nm by the Folin–Ciocalteu method (Vegliolu et al., 1998) using Folin–Ciocalteu reagent (Merck, Germany). Briefly, aliquots of test samples (0.5 mL of 1% extract) were mixed with 25 mL of distilled water, 2.5 mL Folin–Ciocalteu reagent, 10 mL 20% Na_2CO_3, and completed to 50 mL with water, shaken for 30 min and allowed to react for 30 min. Gallic acid was used as the standard for a calibration curve and the total polyphenol contents expressed as gallic acid equivalents (GAE).

2.2. Scanning electron microscope (SEM) studies of human erythrocytes

A range of *A. chilensis* aqueous extract concentrations were made to interact in vitro with red blood cells by incubating erythrocyte suspensions obtained from a human healthy donor not receiving any pharmacological treatment. Blood was obtained by puncture of the ear lobule; 100 μL were received in an eppendorff tube containing 10 μL of heparin (5000 UI/5ml). 900 μL of saline solution (NaCl 0.9%, pH 7.4) was added. The tube was centrifuged (1000 rpm × 10 min), the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. Fractions of this stock of red blood cells suspension (RBCS) and saline solution were placed in each one of five eppendorff tubes to prepare (A) the control, by mixing 100 μL saline solution plus 100 μL RBCS, and (B) a range of concentrations of aqueous extracts (30 μM–10 mM GAE) by mixing 100 μL of RBCS in saline with 150 μL of adequate extract concentrations. All the samples were then incubated in an oven at 37 °C for 1 h, period in line with the larger effects of studied compounds on red cell shape (Zimmermann and Soumpasis, 1985; Malheiros et al., 2000). Afterwards, they were fixed for 12 h at 4 °C with 500 μL 2.5% glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. Finally, previously centrifuged samples at 1000 rpm were washed in saline solution, resuspended in 200 μL of saline; 1 drop of each one was placed on Al glass cover stubs, air-dried at room temperature, gold coated and examined in a scanning electron microscope (JEOL JSM-6380LV, Japan).

2.3. Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The influence of the *A. chilensis* extracts on the physical properties of DMPC LUV and IUM was examined by fluorescence spectroscopy using 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphtalene (laurdan) fluorescent probes (Molecular Probe, Eugene, OR, USA). DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.4 mM) through two stacked polycarbonate filters of 400 nm pore size (Sterlich Corp., WA, USA) under nitrogen pressure at 10 °C above the lipid phase transition temperature. Human erythrocyte membranes were prepared with blood obtained from healthy volunteers according to the method of Dodge et al. (1963). DPH and laurdan were incorporated into DMPC LUV and IUM suspensions by addition of 1 μL/ml aliquots of ca. 1 mM solutions of the probe in tetrahydrofuran and ethanol respectively in order to obtain final analytical concentrations of 1 mM and incubating them at 37 °C for 45 min. Fluorescence spectra and anisotropy measurements were performed on a K-2 steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA) interfaced to computers, using the corresponding ISS software for data collection and analysis. Measurements of LUV suspensions were made at 18 and 37 °C and IUM suspensions at 37 °C using 5 mm pathlength square quartz cuvettes. Anisotropy measurements were made in the L configuration using Glan Thompson prism polarizers (I.S.S.) in both exciting and emitting beams. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence. DPH fluorescence anisotropy (r) was calculated according to the definition:

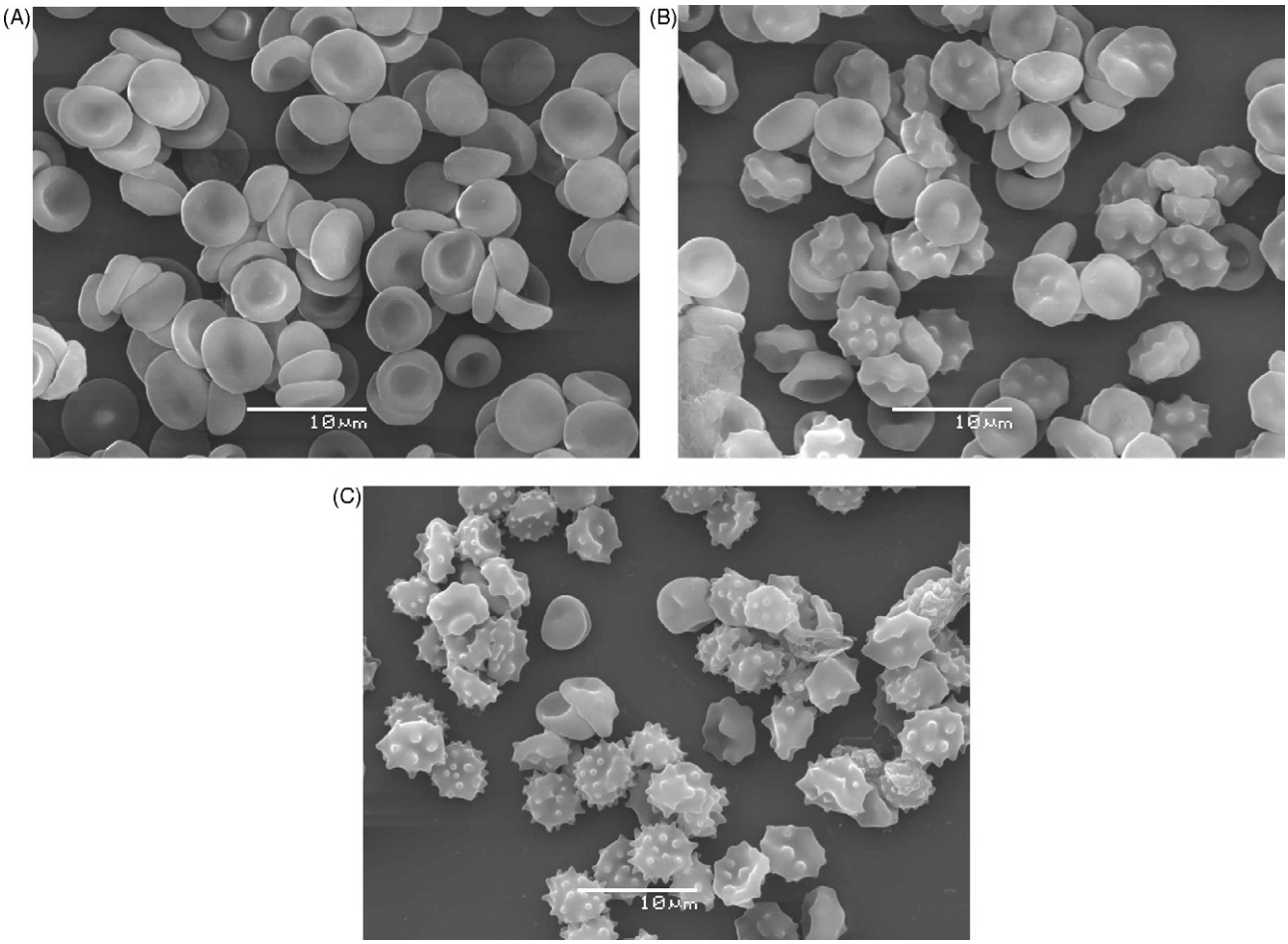

Fig. 1. Effects of aqueous extracts of *A. chilensis* on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 1 mM; (C) 3 mM (expressed as gallic acid equivalents, GAE).

$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the corresponding vertical and horizontal emission fluorescence intensities with respect to the vertically polarized excitation light (Lakowicz, 1999). Laurdan fluorescence spectral shifts were quantitatively evaluated using the generalized polarization (GP) concept (Parasassi and Gratton, 1995), which is defined by the expression $GP = (I_b - I_r)/(I_b + I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively (Parasassi et al., 1990). *A. chilensis* extracts were incorporated in LUV and IUM suspensions by addition of adequate aliquots in order to obtain the different concentrations used in this work (1–3 mM GAE). The samples thus prepared were then incubated for ca. 15 min. Blank subtraction was performed in all measurements using unlabeled samples without probes. The data presented in the figures represent mean values and standard error of ten measurements in two independent samples. Unpaired Student's *t*-test was used for statistical calculations.

2.4. X-ray diffraction studies of phospholipid multilayers

The capacity of *A. chilensis* aqueous extract to perturb the structures of DMPC and DMPE multilayers was determined by X-ray diffraction. Synthetic DMPC (lot 140PC-224, MW 677.9) and DMPE (lot 140PE-54, MW 635.9) from Avanti Polar Lipids

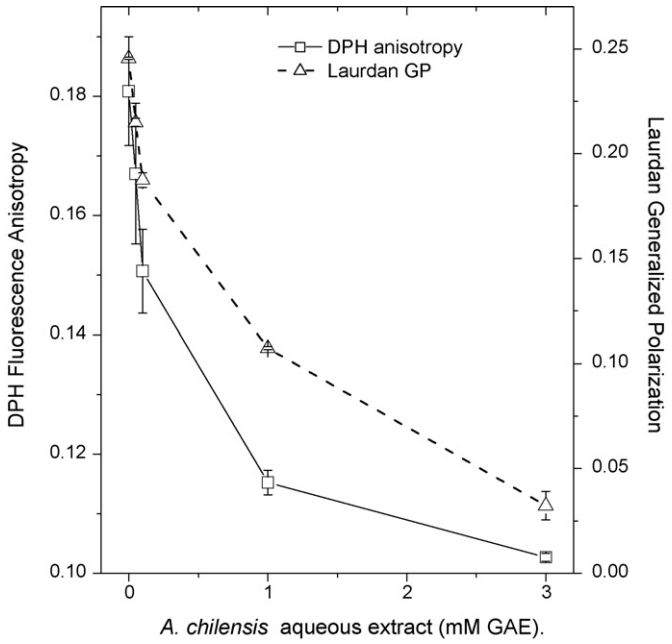

Fig. 2. Effects of aqueous extracts of *A. chilensis* on the anisotropy of DPH and on the general polarization (GP) of laurdan embedded in isolated unsealed human erythrocyte membranes (IUM) at 18 °C and 37 °C.

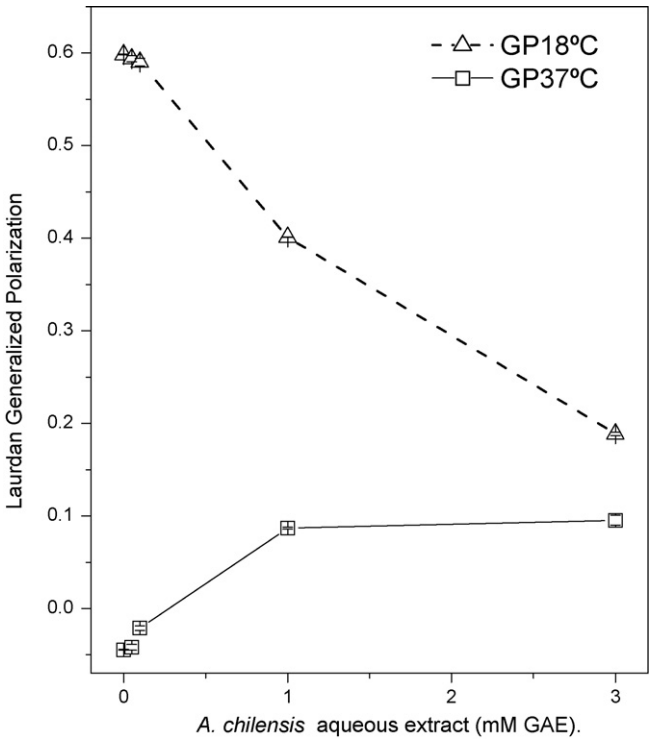

Fig. 3. Effects of aqueous extracts of *A. chilensis* on the generalized polarization (GP) of laurdan embedded in DMPC large unilamellar vesicles (LUV) at 18 °C and 37 °C.

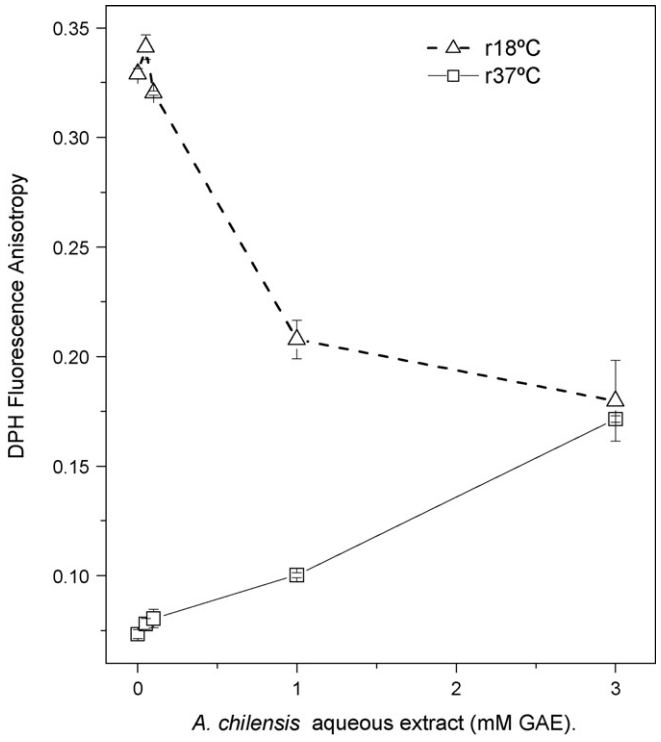

Fig. 4. Effects of aqueous extracts of *A. chilensis* on the anisotropy of DPH embedded in DMPC large unilamellar vesicles (LUV) at 18 °C and 37 °C.

(ALA, USA) were used without further purification. About 2 mg of each phospholipid were introduced into 2 mm diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany), which were then filled with 150 μL of (a) distilled water and (b) aqueous extracts of *A. chilensis* in a range of concentrations (1–10 mM GAE). The specimens were incubated for 1 h at 30 °C and 60 °C with DMPC and DMPE, respectively, centrifuged for 10 min at 2000 rpm and X-ray diffracted with Ni-filtered CuKα from a Bruker Kristalloflex 760 (Karlsruhe, Germany). Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. The relative reflection intensities were obtained in an MBraun PSD-50M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 19 ± 1 °C, which is below the main phase transition temperature of both DMPC and DMPE. Each experiment was repeated three times, and additional experiments were carried out when there were doubts.

3. Results

3.1. Scanning electron microscope (SEM) studies of human erythrocytes

The SEM examinations of human erythrocytes incubated with *A. chilensis* aqueous extracts indicated that 1 mM GAE and higher concentrations induced echinocytosis. Intact red blood cells (Fig. 1A) lost their normal profile and presented a spiny configuration with blebs in their surfaces (Fig. 1B and C). Furthermore, the extent of these shape changes was dependent on the extract concentrations. As it can be observed in Fig. 1B (1 mM GAE), about 90% of cells were affected, while with 3 mM (EAG) practically all the cells showed echinocytosis (Fig. 1C).

3.2. Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

A. chilensis extract concentration-dependent effects on IUM and DMPC LUV were explored at two different depths of the lipid bilayer: at the hydrophilic/hydrophobic interface level, estimated from the laurdan fluorescence spectral shift through the GP parameter, and in the deep hydrophobic core, determined by the DPH steady-state fluorescence anisotropy (r). Fig. 2 shows that the *A. chilensis* extract induced a decrease of the generalized polarization of erythrocyte IUM at 37 °C, implying an increase in the environmental molecular dynamics and/or water penetration at the glycerol backbone level, a result that can be attributed to a disordering effect on the membrane bilayer polar groups. Fig. 2 also shows that the *A. chilensis* extract induced a decrease of the anisotropy (r), result that implies a structural perturbation in the acyl chain packing arrangement of the erythrocyte membrane lipid bilayer. The incorporation of *A. chilensis* extracts to DMPC LUV at increasing concentrations showed a decrease of the laurdan GP (Fig. 3) and DPH anisotropy (Fig. 4) values at 18 °C and increases at 37 °C. These results imply that *A. chilensis* extract induced the following effects: (a) an increase in the molecular dynamics and/or water penetration at the glycerol backbone level as well as a decrease in the phospholipid acyl chain packing order when the bilayer is in the gel-crystalline state at 18 °C, and (b) the opposite effect at 37 °C when the bilayer is in the much more fluid liquid-crystalline state.

3.3. X-ray diffraction studies of phospholipid multilayers

Fig. 5 exhibits the results obtained by incubating DMPC with water and aqueous extracts of *A. chilensis*. As expected, water altered the DMPC structure: 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

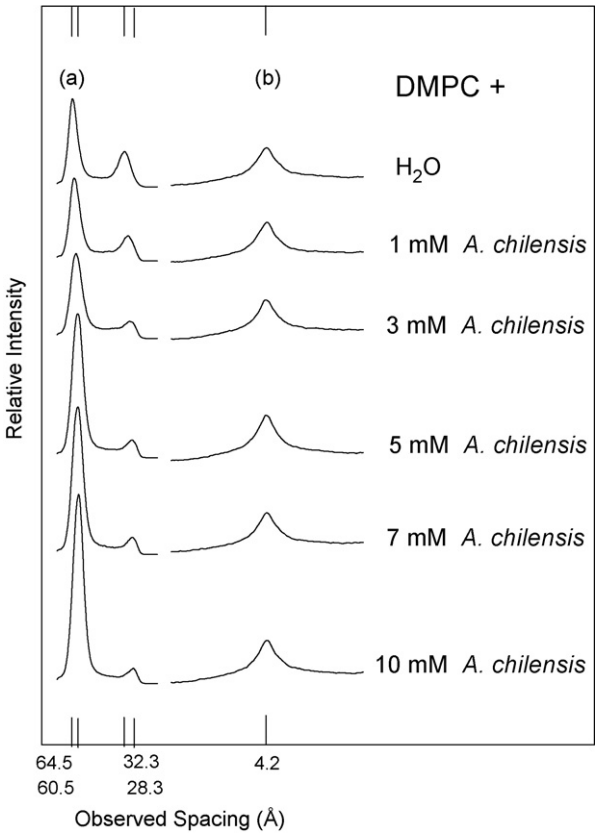

Fig. 5. Microdensitograms from X-ray diffraction patterns of dimyristoylphosphatidylcholine (DMPC) in water and aqueous extracts of *A. chilensis*; (a) low-angle and (b) wide-angle reflections. Concentrations are expressed as gallic acid equivalents (GAE).

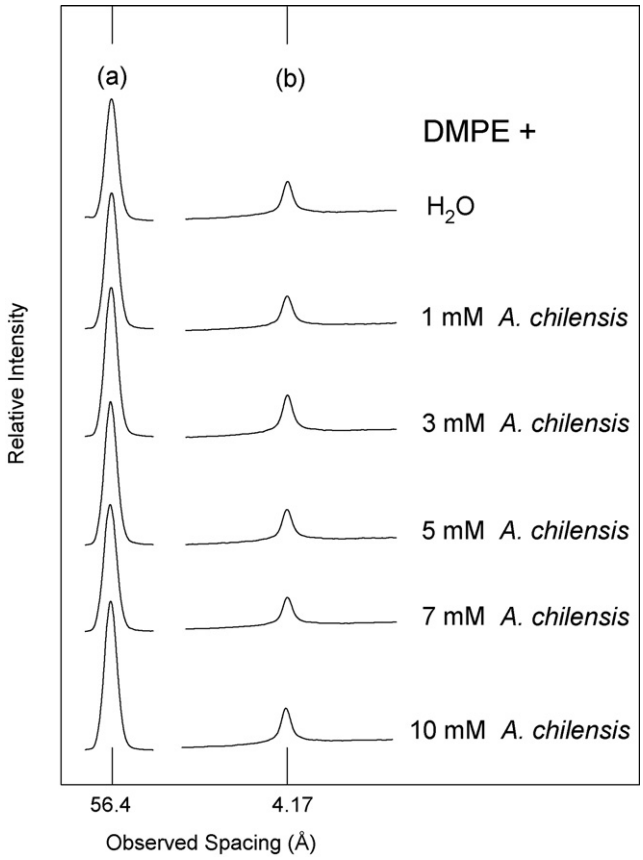

Fig. 6. Microdensitograms from X-ray diffraction patterns of dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous extracts of *A. chilensis*; (a) low-angle and (b) wide-angle reflections. Concentrations are expressed as gallic acid equivalents (GAE).

immersed in water and its low-angle reflections, which correspond to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer repeat. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region, which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. The figure discloses that after exposure to 1 mM GAE and higher concentrations of the aqueous extract there was a weakening of the low- and wide-angle lipid reflection intensities (indicated as (a) and (b) in the figure, respectively). From these results it can be concluded that the extract produced a structural perturbation of DMPC bilayers. However, the 64.5 Å reflection intensity increased with 5 mM GAE and higher extract concentrations to a value about 50% higher of that shown by DMPC in pure water. At the same time, the low-angle reflection spacings decreased from 64.5 Å and 32.3 Å to 59.1 Å and 29.6 Å, respectively. These effects might be due to the location of extract molecules in the lipid polar region inducing an ordering of DMPC amino terminal groups, and displacement of water molecules in the interbilayer space. On the other hand, Fig. 6 shows that the extract did not induce any significant change to DMPE, even at the highest assayed concentration (10 mM GAE).

4. Discussion

In cell membranes, the free radical peroxidation of lipids disrupts their structural and protective functions; as a consequence, this oxidation might imply several pathological events (Abuja and Albertini, 2001). On the other hand, the molecular mechanisms of polyphenol antioxidant action are poorly understood. However, their lipophilicities make lipid rich membranes important targets of their interaction. In fact, it has been suggested that the antioxidant property of these compounds might lie in their ability to insert into cell membranes and modify the lipid packing order and fluidity (Arora et al., 1998, 2000; Nakagawa et al., 2000). Phytochemical studies of the chemical composition of *A. chilensis* indicate the presence of indolic alkaloids, flavonoids, cianidine glucosides, delfidine, malvidine, petunidine, cumarines and triterpenes (Silva and Bittner, 1992). These compounds, particularly the flavonoids, have antioxidant properties with low toxicities (Hou et al., 2004). There are no pharmacokinetics studies reported on leaf extract of *A. chilensis*. Information about the absorption, metabolism and excretion of individual flavonoids in humans is rather scarce (Cook and Samman, 1996). According to Mazza et al. (2002) flavonoids are absorbed from the intestinal tract of humans in glycosolated and possible acylated form and are excreted either unchanged or as flavonoid metabolites in the urine or feces.

In biological and in model membranes, the interaction between some 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 being determined by their chemical properties (Oteiza et al., 2005). Ollila et al. (2002) showed an inverse correlation between the capacity of quercetin and myricetin to induce membrane permeabilization and their retention in a phosphatidylcholine coated column. The authors reported that this correlation was related to the relative hydrophobicity of the compounds. 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). In addition, Tsuchiya (2001) demonstrated that epicatechin, which is more hydrophobic than its geometrical isomer catechin, has a higher degree of interaction with membrane lipids.

Results of the present study indicate that aqueous extracts of *A. chilensis* induced an alteration of human erythrocyte morphology from the normal discoid shape to an echinocytic form. According to the bilayer couple hypothesis (Sheetz and Singer, 1974; Lim et al., 2002), the fact that *A. chilensis* extracts induced the formation of echinocytes indicates that polyphenol molecules located mainly in the outer monolayer of the red cell membrane. These results were confirmed by the fluorescence experiments performed in IUM. In fact, they concluded that the extract induced structural perturbations in the phospholipid acyl chain and polar group packing arrangements of the erythrocyte membrane lipid bilayer, disordering them as the extract concentration increased. Fluorescence spectroscopy performed in DMPC LUV, a lipid mainly located in the outer monolayer of red cells (Boon and Smith, 2002), confirmed these results. In fact, at 18 °C *A. chilensis* aqueous extracts induced structural perturbations to both the polar group and acyl chain regions of DMPC. In contrast, similar measurements performed at 37 °C, when the lipid is in a fluid state, showed increases in GP and the anisotropy, implying a partial ordering of DMPC polar and hydrophobic groups. The X-ray diffraction results also support these conclusions since they showed that *A. chilensis* aqueous extracts interacted with DMPC and not with DMPE, class of lipid located in the inner monolayer of the human erythrocyte membrane. On the basis of these results it might be postulated that cell membrane lipid bilayers are in general potential targets for *A. chilensis* polyphenols. Therefore, their molecular mechanism of action and toxicity can be attributed to functional perturbation of cell membrane lipid bilayers. This conclusion agrees with others reached with polyphenols extracted from various plants and assayed by different methods (Arora et al., 1998, 2000; Nakagawa et al., 2000; Kajiya et al., 2002).

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