J Membrane Biol (2014) 247:703–712
DOI 10.1007/s00232-014-9692-6

# Antioxidant Capacity of *Ugni molinae* Fruit Extract on Human Erythrocytes: An In Vitro Study

**Mario Suwalsky · Marcia Avello**

Received: 27 March 2014 / Accepted: 22 May 2014 / Published online: 14 June 2014


**Abstract** *Ugni molinae* is an important source of molecules with strong antioxidant activity widely used as a medicinal plant in Southern Chile–Argentina. Total phenol concentration from its fruit extract was 10.64 ± 0.04 mM gallic acid equivalents. Analysis by means of HPLC/MS indicated the presence of the anthocyanins cyanidin and peonidin, and the flavonol quercitin, all in glycosylated forms. Its antioxidant properties were assessed in human erythrocytes in vitro exposed to HClO oxidative stress. Scanning electron microscopy showed that HClO induced an alteration in erythrocytes from a normal shape to echinocytes; however, this change was highly attenuated in samples containing *U. molinae* extracts. It also had a tendency in order to reduce the hemolytic effect of HClO. In addition, X-ray diffraction experiments were performed in dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine bilayers, classes of lipids preferentially located in the outer and inner monolayers, respectively, of the human erythrocyte membrane. It was observed that *U. molinae* only interacted with DMPC. Results by fluorescence spectroscopy on DMPC large unilamellar vesicles and isolated unsealed human erythrocyte membranes also showed that it interacted with the erythrocyte membrane and DMPC. It is possible that the location of *U. molinae* components into the membrane outer monolayer might hinder the diffusion of HClO and of free radicals into cell membranes and the consequent decrease of the kinetics of free radical reactions.

**Keywords** *Ugni molinae* · Fruit extract · Antioxidant · Human erythrocyte membrane

**Abbreviations**

| | |
|---|---|
| SEM | Scanning electron microscopy |
| RBCS | Red blood cell suspension |
| DMPC | Dimyristoylphosphatidylcholine |
| DMPE | Dimyristoylphosphatidylethanolamine |
| ROS | Reactive oxygen species |
| GAE | Gallic acid equivalents |
| LUV | Large unilamellar vesicles |
| IUM | Isolated unsealed human erythrocyte membranes |

## Introduction

*Ugni molinae* Turcz. *Myrtaceae* (*U. molinae*) also known as Murtilla, Murta, Murtillo, Uñi is a perennial wild plant that grows in the south of Chile and Juan Fernandez islands (Medel 1979; Hoffmann 1991; Gomes et al. 2009; Scheuermann et al. 2008; Fuenzalida 2008). Its fruits are considered useful to relieve circulation disorders and to increase visual acuity, especially at night. Furthermore, mastication of fresh fruit, devoid of skin is useful to treat conditions of the mouth such as thrush, and stomatitis (Rozzi 1984). Fruits are also consumed fresh due to its organoleptic characteristics, and they are also used for the preparation of jams, syrups, desserts, and liqueurs (Hoffmann 1991). The fruit is a small globular berry containing many fleshy, sweet, and aromatic seeds (Hoffmann 1991; Scheuermann et al. 2008; Torres et al. 1999); it contains

M. Suwalsky (✉)
Faculty of Chemical Sciences, University of Concepción, Concepción, Chile
e-mail: msuwalsk@udec.cl

M. Avello
Faculty of Pharmacy, University of Concepción, Concepción, Chile

antioxidants such as phenolic compounds (www.murtillachile.cl), mainly flavonols, flavan-3-ols, and anthocyanins, whose concentrations vary depending on the geographic location where the plant grows (Ruiz 2008). It presents two anthocyanins, peonidin-3-glucoside and the most abundant cyanidin-3-glucoside, being the total concentrations about 1 μmol/g of dry matter (Ruiz 2008). Phenolic compounds act as exogenous antioxidants systems, composed of molecules mainly from the diet, and are proposed to protect against oxidative damage by oxygen-derived free radicals and reactive species (ROS) (Lambert et al. 2007). ROS are involved in the mechanism that contributes to endothelial dysfunction and other metabolic disorders (Foncea et al. 2000); therefore, the antioxidant activity of natural phenolic compounds could reverse these damages. The molecular mechanisms of the antioxidant property of phenols and flavonoids have not yet 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 ROS and thereby decrease the kinetics of free radical reactions (Arora et al. 2000).

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 chemical species. With the aim to better understand the molecular mechanisms of the interaction of *U. molinae* fruit aqueous extracts with cell membranes, we have utilized human erythrocytes and molecular models of RBC membranes. Human erythrocytes were chosen because of their only one membrane, and no internal organelles are an ideal cell system for studying chemical compound–biomembrane interactions. On the other hand, although less specialized than many other cell membranes, they carry on enough functions in common with them such as active and passive transport as well as the production of ionic and electric gradients to be considered representative of the plasma membrane in general. Molecular models of the erythrocyte membrane consisted of bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of cell membranes, particularly of the human erythrocyte, respectively (Boon and Smith 2002; Devaux and Zachowsky 1994). The capacity of *U. molinae* fruit aqueous extracts to interact with membrane bilayers was evaluated by X-ray diffraction on DMPC and DMPE; DMPC large unilamellar vesicles (LUV) and isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy, and intact human erythrocytes were observed by scanning electron microscopy (SEM). These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other plant aqueous extracts (Suwalsky et al. 2006, 2008, 2009).

## Materials and Methods

### Plant Material

Fruits of *U. molinae* were collected in February 2009 in the main campus of the University of Concepción, Chile. These were identified in the Department of Botany, Faculty of Natural and Oceanography Sciences of the same university (CONC 146 511).

### Preparation of Lyophilized Extracts

800 g of fresh fruit was grounded using a mortar and a blender (Waring®); subsequently, they were lyophilized (Labconco Corp., USA), packed, and stored at −20 °C until use. The lyophilization process consisted of two steps: first freezing plant material for 48 h; the second step involved the sublimation of water contained in the plant sample, without the use of high temperatures (Bornschein and Voigt 1982).

### Standardization

Total polyphenols were determined by means of the Folin–Ciocalteu method in triplicate, expressed as gallic acid equivalents per g of dry matter (GAE/g); gallic acid (Merck, Germany) was used as standard (Velioglu et al. 1998). All assays were performed in triplicate.

### HPLC/MS Characterization of Anthocyanins

Analyses of fruits extracts from *U. molinae* (3 mg/mL) were carried out according to Cho et al. (2004). 100 mg of lyophilized extract was dissolved in 5 mL of 95 % ethanol and 1.5 M HCl in a 85:15 v/v ratio, and centrifuged at 2,500 rpm for 10 min in order to sediment solid particles (Vargas-Simón et al. 2002; Jiménez et al. 2004). For the analysis, a LC–MS system (Agilent Technologies Inc., CA, USA) was used. This system was equipped with binary pumps, an online degasifier, automatic injector, and a UV–Vis detector. Separation of phenolic compounds was carried out by means of a Zorbax Eclipse column XDB-C18 150 × 4.6 mm, 5 μm and 80 Å (Agilent Technologies Inc., CA, USA). Injection volume was 20 μL, with a flow of 1.0 mL/min. Solvent system was composed of the solvent A (double-distilled water containing 0.1 % formic acid v/v) and the solvent B (acetonitrile containing 0.1 % formic acid). The gradient system was as follows: 0–5 min,

5 % B; 5–50 min, 100 % B; 50–55 min, 100 % B; 55–57.5 min, 100–5 % B; and 57.5–60 min, 5 % B. LC/MS detection was carried out immediately after UV–Vis measurements. Analyses were carried out by means of Bruker Esquire 4000 (Bruker Daltonik, GmbH, Germany) ions trap ESI-IT mass spectrophotometer, operating under the following ion optics: capillary temperature, 225 °C; capillary voltage, 5.7 kV; cone voltage, 35 V; and voltage spray 2.8 kV. Nitrogen was used as nebulizer gas (pressure: 30 psi, temperature: 325 °C) and drying gas (10 L/min). Products from mass spectra were recorded in the range of *m*/*x* 50–1,500 in both positive and negative mode. Data were collected by means of the Esquire Control 5.2 software and processed by means of Data Analysis 3.2 software (Bruker Daltonics Esquire 5.2, Bruker Daltonik GmbH, Germany). Instrument parameters were optimized in a routine prior to the analysis of extract.

### X-Ray Diffraction of Phospholipids Multilayers

The capacity of the extract of *U. molinae* to interact with DMPC and DMPE multilayers was evaluated by X-ray diffraction. Synthetic DMPC (lot 140PC-224, MW 677.9) and DMPE (lot 140PC-230, MW 635.9) (from Avanti Polar Lipids, AL, USA) 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), which were then filled with 200 µL of (a) distilled water and (b) aqueous solutions of *U. molinae* fruit extract in a range of concentrations (0.2–1.0 mM in the case of DMPC, and up to 10 mM for DMPE). Specimens were X-ray diffracted after 1 h incubation at 37 and 60 °C with DMPC and DMPE, respectively. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered Cu Kα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray generator was used. The relative reflection intensities were obtained in a MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. 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.

### Fluorescence Measurements of DMPC LUV and IUM

The influence of *U. molinae* fruit extract on the physical properties of cell membranes was also determined in DMPC LUV and human erythrocyte membranes (IUM), used as membrane models. In these systems, the parameters anisotropy (*r*) and generalized polarization (GP) were measured by fluorescence spectroscopy. This was achieved using two different fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylamino-naphthalene (laurdan). DMPC LUV suspended in distilled water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions, reaching a final concentration of 0.4 mM through two polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure. Measurements of LUV suspensions were performed at 18 °C, and of IUM at 37 °C. IUM were prepared with human blood obtained from healthy male donors according to the method of Dodge et al. (1963). Briefly, by means of isotonic and hypotonic phosphate buffered saline (PBS), erythrocytes were separated from plasma, lysed, and washed in order to obtain the unsealed membranes, which were stored in isotonic solution at 20 °C. Both IUM and LUV were incubated with DPH and Laurdan by addition of smalls aliquots of concentrated solution of the probe in tetrahydrofuran and ethanol, respectively, at 37 °C for about one h. Fluorescent spectra and anisotropy measurements of IUM and LUV were performed on a K2 steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA) interfaced to computer, using the corresponding ISS software. Temperature was monitored before and after each measurement by a digital thermometer (Omega Engineering Inc., Stanford, CT, USA). Samples were measured using 5 mm path-length square quartz cuvettes. Anisotropy measurements were made in the L configuration using Glan–Thompson prism polarizers in both exciting and emitting beams. Using an excitation wavelength of 360 nm, the emission was measured with a WG-420 Schott high-pass filter (Schott, Mainz, Germany) with negligible fluorescence. DPH fluorescence anisotropy (*r*) was calculated according to the definition $r = (I_{\parallel} - I_{\perp})/(I_{\mathrm{l}} - 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light (Lakowicz 1999). Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (Parasassi and Gratton 1995), which is defined by the expression $GP = (I_{\mathrm{b}} - I_{\mathrm{r}})/(I_{\mathrm{b}} + I_{\mathrm{r}})$, where $I_{\mathrm{b}}$ and $I_{\mathrm{r}}$ are the emission intensities at the blue and red edges of the emission spectrum, respectively. With excitation at 360 nm, 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). *U. molinae* extracts were incorporated in IUM and LUV suspensions by the addition of adequate aliquots in order to obtain different concentrations in the 0–1.5 mM GAE range. These samples were then incubated for 10–15 min at 37 °C. 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.

### SEM of Human Erythrocytes

In vitro interaction of *U. molinae* fruit extract with erythrocytes was attained by incubating red blood cell suspensions (RBCs) derived from a healthy human male donor not receiving any pharmacological treatment. Blood samples (0.1 mL) were obtained by puncture of the ear lobule and by aspiration into a tuberculin syringe without a needle containing 5,000 U/mL heparin in 0.9 mL PBS, pH 7.4. RBCs were then centrifuged for 10 min, washed three times in PBS, resuspended in PBS containing *U. molinae* extract in a range of concentrations and incubated for 1 h at 37 °C. Controls were cells resuspended in PBS without extract. Specimens were then fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 mL of 2.5 % glutaraldehyde, washed twice in distilled water, placed on siliconized Al stubs, air dried at 37 °C for 30 min, and gold coated for 3 min at 13.3 pa in a sputter device (Edwards S 150, Sussex, England). Specimens were examined in a JEOL (JEM 6380 LB, Japan) SEM. Data were expressed as mean ± standard deviation of 50 cell counts. The same procedure was used to study the protective capacity of *U. molinae* aqueous extracts against the oxidant property of HClO.

### Hemolysis Assays

RBCs were obtained from healthy consenting donors through venipuncture and received in heparin-containing Eppendorf tubes (165 µL/mg). Heparinized blood was centrifuged (Kubota, Japan) at 2,500 rpm for 10 min. After removal of plasma and buffy coat, RBC samples were washed three times with phosphate buffer (PBS, NaCl (150 mM), $NaH_2PO_4$ (1.9 mM), and $Na_2HPO_4$ (8.1 mM), pH 7.4) at room temperature, and resuspended in PBS four times its volume for subsequent analyses (Vives et al. 1999). RBCs (10 % v/v) were incubated in a shaking bath for 15 min at 37 °C in PBS in the presence of *U. molinae* extract in a range of concentrations. NaClO (Sigma, Mo, USA) was added as single bolus of a diluted solution in PBS, whose concentration (5 mM) was spectrophotometrically determined at 292 nm ($\varepsilon = 350\ M^{-1}\ cm^{-1}$) (Morris 1966). At pH 7.4, NaClO exists as HClO and $ClO^-$ in an approximately equimolar ratio (Battistelli et al. 2005; Vissers and Winterbourn 1995). After 15 min of incubation, an aliquot of RBC suspension was centrifuged at 2,500 rpm for 10 min. Hemolysis was spectrophotometrically evaluated (Jasco, Japan) at 540 nm as hemoglobin released from cells in the supernatant. The results were expressed as inhibition percentage of hemolysis. All samples were assayed in triplicate and the mean value calculated.

### Statistical Analyses

Statistical analyses were performed through the Dunnet test. All data were expressed as mean ± SD of at least three different determinations.

## Results

### Analysis of Extract Species

The total phenols concentration obtained through the Folin–Ciocalteu method was 10.64 ± 0.04 mM GAE. Identification of *U. molinae* main phenolic compounds was carried out by means of HPLC–ESI–MS. Analyses were performed at two wavelengths due to anthocyanin two absorption bands, one between 260 and 280 nm and the other between 490 and 550 nm. The assignment of peaks was carried out by fragmentation pattern analysis, by comparison with mass spectra data from standards and literature. Both retention time and *m/z* in positive polarity of the respective extracts are presented in Table 1 and the chromatogram in Fig. 1. Spectral characteristics were obtained from signals considered intense and pure. Positive mode was also used for the confirmation of some compounds. Results were the following:

(a) peak 1 (tR = 10.9 min): in negative polarity, it was assigned to the pseudomolecular $[MH]^-$ ion of *m/z* 447, although the absence of fragmentation would not confirm its identity. In positive polarity, it was observed the pseudomolecular $[MH]^+$ ion of *m/z* 625 whose fragmentation *m/z* 605, *m/z* 301, and *m/z* 463 corresponds to peonidin-di-glucopyranoside.

(b) peak 2 (tR = 11.5 min): in negative polarity, it was assigned to the pseudomolecular $[MH]^-$ ion of *m/z* 447, which provided a fragment of *m/z* 285 that would indicate the presence of cyanidin glucoside.

(c) peak 3 (tR = 12.5 min): it was assigned to the pseudomolecular $[MH]^-$ ion of *m/z* 461 whose

**Table 1** HPLC–MS analysis of *U. molinae* fruit extract

| Peak | tR (min) | $\lambda_{max}$ (nm) | $[M–H]^-$ | Ions MS/MS | Tentative identification |
|---|---|---|---|---|---|
| 1 | 10.9 | 520 | 447 | 447 | Peonidin-di-glucopyranoside |
| 2 | 11.5 | 520 | 447 | 285 | Cyanidin glucoside |
| 3 | 12.5 | 520 | 461 | 299, 284 | Peonidin glucopyranoside |
| 4 | 15.0 | 520 | 463 | 300 | Quercetin glucoside |

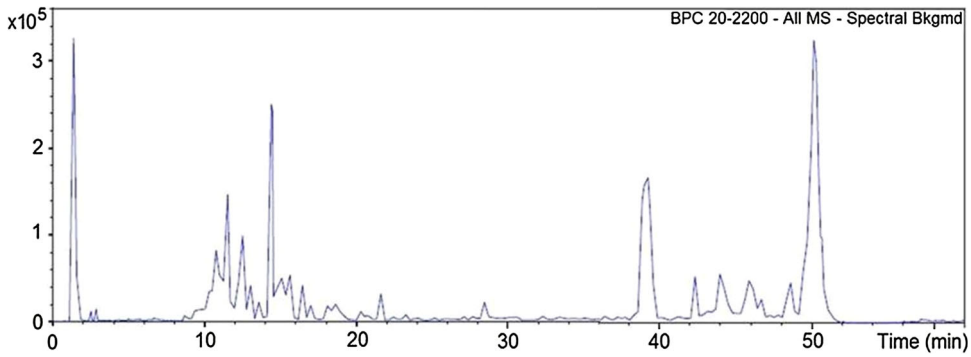



**Fig. 1** HPLC chromatogram of *U. molinae* fruit extract

fragmentation gave *m/z* 299 and *m/z* 284, indicating the presence of peonidin-glucopyranoside.

(d) peak 4 (tR = 15.0 min): in negative polarity, it was assigned to the pseudomolecular $[MH]^-$ ion of *m/z* 463, which according to their fragmentation in *m/z* 300 indicated the presence of quercetin glycoside.

## X-Ray Diffraction of Phospholipids Multilayers

Figure 2a exhibits the results obtained by incubating DMPC with water and fruit extracts of *U. molinae*. 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 immersed in water, and its low-angle reflections (indicated as LA) 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 (indicated a WA), which corresponds to the average lateral distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. The figure discloses that after exposure to *U. molinae* in the range 0.4–1.0 mM GAE, there was a gradual weakening of the low- and wide-angle lipid reflection intensities. From these results, it can be concluded that the extract produced a structural perturbation of the polar and acyl regions of DMPC bilayers. Results from similar experiments with DMPE are presented in Fig. 2b. The fact that only one strong reflection of 56.4 Å is observed in the low-angle region, and the presence of the 4.2 Å in the wide-angle region is indicative of the gel state reached by DMPE in water after heating and cooling it. Increasing concentrations of the fruit extract in the range 0.5–10 mM GAE practically did not induce any significant structural perturbation to DMPE bilayers.

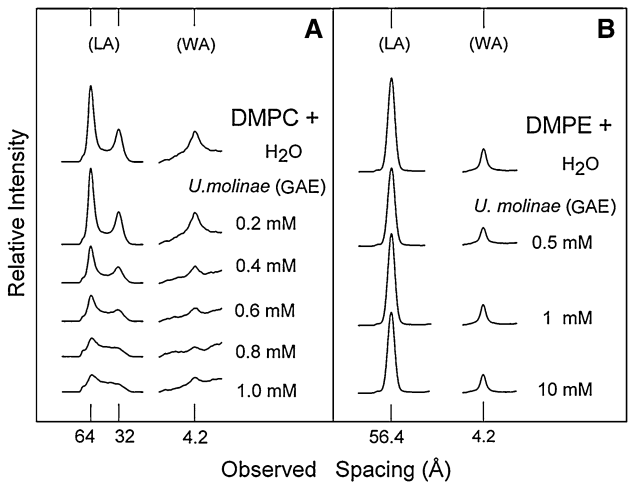



**Fig. 2** X-ray diffraction patterns of **a** DMPC and **b** DMPE in water and with aqueous extracts of *U. molinae* fruits; (LA) low-angle and (WA) wide-angle reflections

## Fluorescence Measurements of DMPC LUV and IUM

*Ugni molinae* concentration-dependent effects on DMPC LUV and IUM 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*). Figure 3 shows that increasing concentrations of *U. molinae* (starting at about 50 μM GAE) sharply decreased both *r* values of DMPC LUV at 18 and 37 °C. These results imply that the fruit extract induced structural perturbations in the acyl chain packing arrangement of the lipid bilayer. Figure 4 shows somewhat similar although more moderate effect in the GP at both 18 and 37 °C. This result can be interpreted as a disordering effect induced by *U. molinae* extract on DMPC polar groups. Figures 5 and 6 show very sharp decreases of the anisotropy and GP of IUM at 37 °C. These results imply strong perturbation of the acyl chains and polar group packing arrangement of the erythrocyte membrane lipid bilayer.

## SEM of Human Erythrocytes

SEM examinations of human erythrocytes incubated with *U. molinae* in the range 0.2–0.8 mM GAE indicated that its fruit aqueous extract induced echinocytosis. In that altered

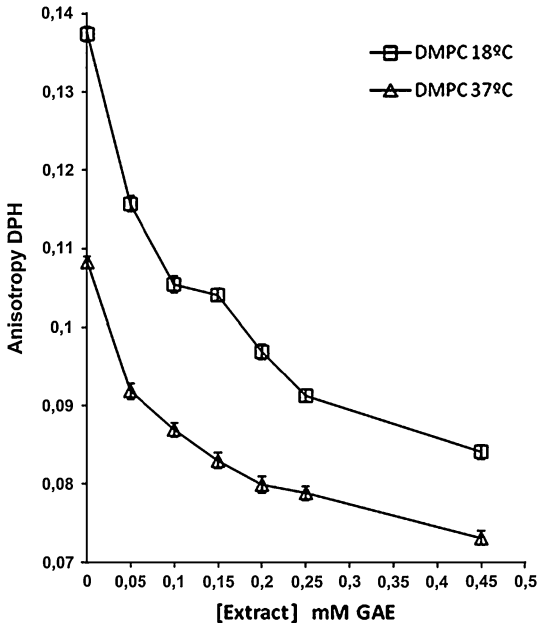



**Fig. 3** Effect of aqueous extracts of *U. molinae* fruits on the anisotropy (*r*) of DPH embedded in DMPC LUV at 18 and 37 °C

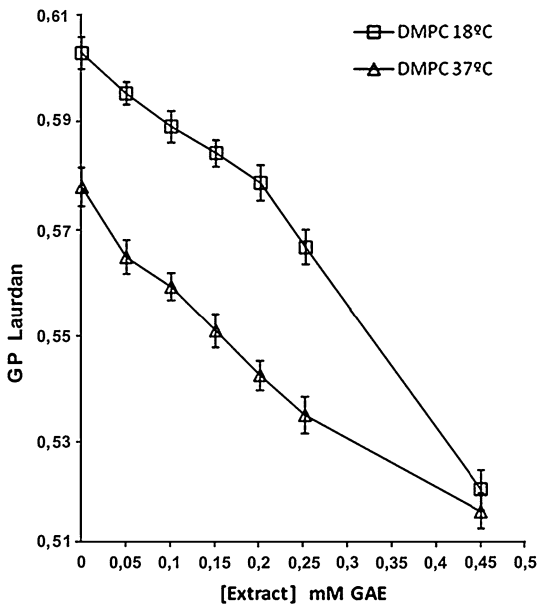



**Fig. 4** Effect of aqueous extracts of *U. molinae* fruits on the GP of laurdan embedded in DMPC LUV at 18 and 37 °C

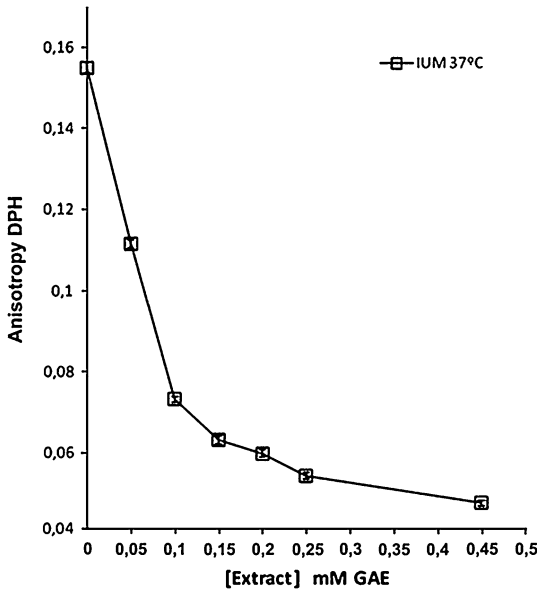



**Fig. 5** Effect of aqueous extracts of *U. molinae* fruits on the anisotropy (*r*) of DPH embedded in IUM at 37 °C

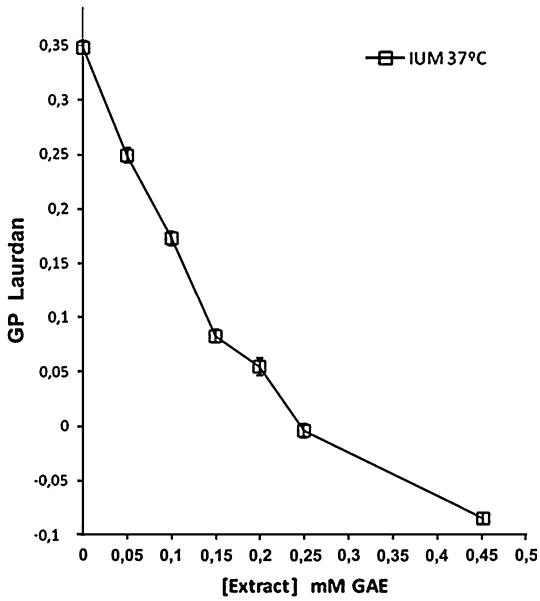
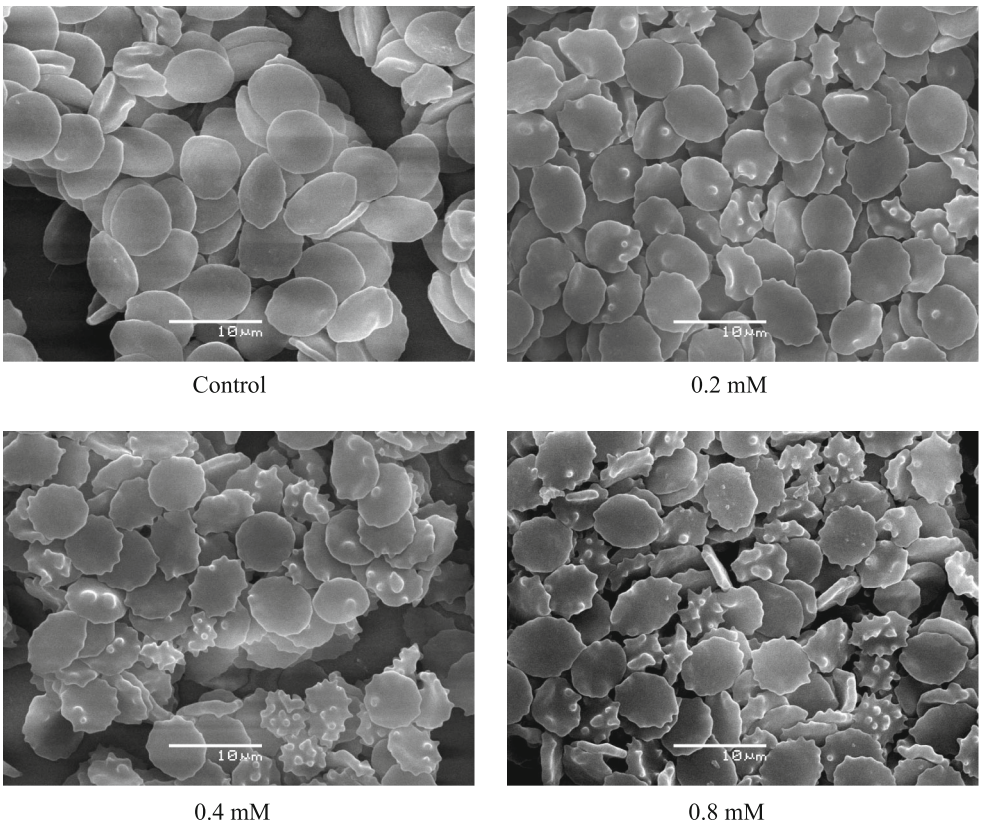



**Fig. 6** Effect of aqueous extracts of *U. molinae* fruits on the GP of laurdan embedded in IUM at 37 °C

condition, RBCs lost their normal biconcave profile and presented a spiny configuration with blebs in their surfaces (Fig. 7). The extent of these shape changes was dependent on the extract concentration (expressed as GAE). Figure 8 shows that 2.5 mM HClO induced echinocytosis in a considerable number of erythrocytes. However, this shape alteration of the human RBCs was highly attenuated in samples containing 1 mM GAE of *U. molinae* extract and 2.5 mM HClO. These results demonstrated the protective effect of *U. molinae* against the shape perturbing effect of HClO upon human erythrocytes.

## Hemolisis Assays

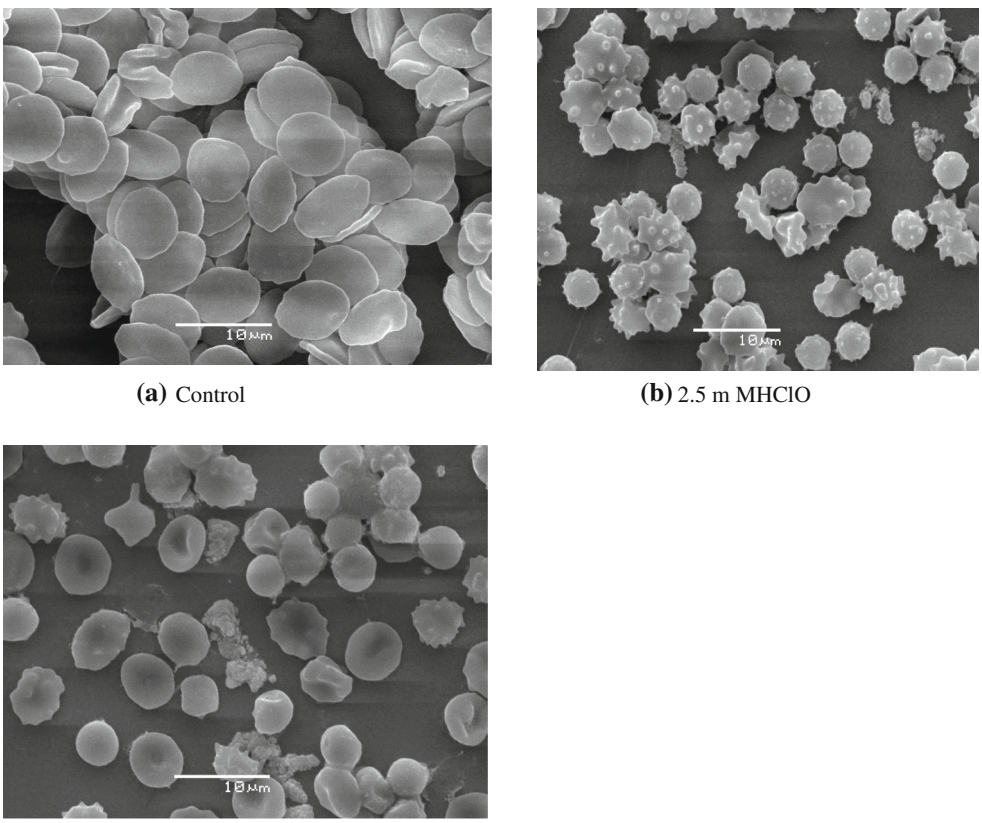
Figure 9 shows the protective effect of *U. molinae* on human erythrocytes against the oxidative capacity of 5 mM HClO. As it can be observed, there is a clear tendency of




Control 0.2 mM

0.4 mM 0.8 mM

**Fig. 7** Effect of aqueous extracts of *U. molinae* fruits on morphology of human erythrocytes. SEM images of control, and incubated with 0.2, 0.4, and 0.8 mM aqueous extracts of *U. molinae*. Extract concentrations are expressed as GAE




**(a)** Control **(b)** 2.5 m MHClO

**(c)** 2.5 mM HClO + 1,0 mM *U. molinae*.

**Fig. 8** Effect of aqueous extracts of *U. molinae* fruits and HClO on morphology of human erythrocytes. SEM images of **a** control, and incubated with **b** 2.5 mM HClO, and **c** 2.5 mM HClO plus 1.0 mM aqueous extracts of *U. molinae*. Extract concentration is expressed as GAE

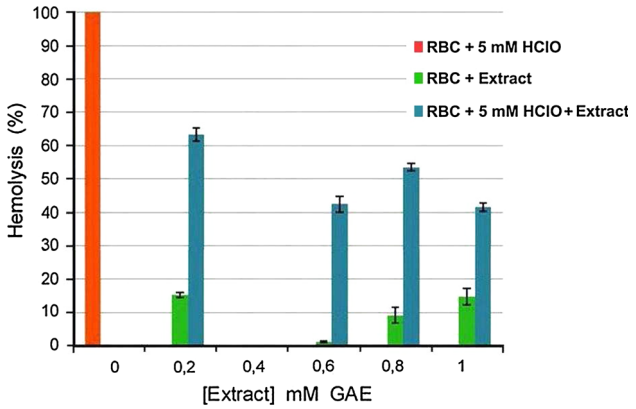



**Fig. 9** Percentage of hemolysis of red blood cells (RBC) incubated with 2.5 mM HClO and different concentrations of *U. molinae* aqueous extracts. Extract concentrations are expressed as GAE; $n = 3$. Values are the mean ± SD

increasing fruit extract concentrations to reduce the hemolytic effect of HClO, which at the highest extract concentrations show an about 55 % hemolysis reduction.

## Discussion

Phenolic compounds are widely distributed in plants, and they are of great nutritional interest as well as for maintenance of human health. In fact, since 1990, several international organizations recommend a daily intake of at least five servings of fruits and/or vegetables to ensure adequate intake of antioxidants and prevent diseases related to oxidative stress (Martínez-Navarrete et al. 2008). The total phenol concentration in a 2 % aqueous extract of *U. molinae*, fruit, determined by the Folin–Ciocalteu method was 10.64 ± 0.04 mM GAE, whereas in leaves is 1.22 mM GAE in a 1 % aqueous extract (Orellana 2005). The analysis by HPLC/MS indicated the presence of the anthocyanins cyanidin and peonidin, both in the glycosylated form, result that agrees with those of Ruiz et al. (2010). It was also detected the flavonol quercetin in the glycosylated form.

The protective effects of extracts of *U. molinae* fruits were evaluated on human erythrocytes exposed to HClO-induced oxidative stress. HClO is an extremely toxic biological oxidant generated by neutrophils and monocytes (Zavodnik et al. 2001). However, because it readily reacts with a range of biological targets, it has been difficult to identify which reactions are critical for its cytotoxic effects (Vissers et al. 1998). Human erythrocytes are a reliable and easily obtainable mechanism to detect oxidative stress (Battistelli et al. 2005). Although the exact mechanism is not clear, the cell membrane is considered the primary site for reaction. HClO treatment of erythrocyte membrane results in changes of membrane fluidity, surface area, and morphological transformations, events that precede cell lysis (Zavodnik et al. 2001; Vissers and Winterbourn 1995; Vissers et al. 1998). Our SEM observation that 2.5 mM HClO induced morphological alterations to the red cells, from the normal discoid shape to an echinocytic form, (Fig. 8) certainly agrees with Battistelli et al. (2005) report that HClO induced the formation of echinocytes in most of the observed erythrocytes. According to the bilayer couple hypothesis (Sheetz and Singer 1974; Lim et al. 2002), shape changes induced in erythrocytes by foreign molecules are due to differential expansion of the two monolayers of the red cell membrane. Thus, stomatocytes are formed when the compound is inserted into the inner monolayer, whereas spiculated-shaped echinocytes are produced when it is situated into the outer membrane moiety. The observed echinocytosis might be then due to the insertion of HClO in the outer monolayer of the red cell membrane. This result does not agree with that reported by Vissers and Winterbourn (1995) who indicated that HClO penetrates into the red cells passing through the hydrophobic lipid bilayer without the membrane acting as a major barrier. The result that 1 mM GAE extract concentration practically neutralized the effect of 2.5 mM HClO concentration as shown in SEM observations (Fig. 8) demonstrated the protective capacity of *U. molinae* fruit extract against the erythrocyte shape change capacity of HClO.

The main consequence that suffers erythrocytes exposed to HClO is cell lysis. The mechanism by which this effect occurs is unclear. Some studies have shown that exposure of red cell membranes to HClO produces inhibition of the activity of $Na^+$, $K^+$ and $Mg^{++}$ ATPase, oxidation of SH groups, proteins, lysine and tryptophan residues, cholesterol and fatty acids, and formation of chloramines. Also, there are changes in the membrane fluidity, increased $K^+$ efflux, crosslinked proteins, and extensive separation membrane. All these interactions precede cell lysis (Hawkins and Davies 1998; Carr et al. 1997; Nakagawa et al. 2000). The protective effect of *U. molinae* on human erythrocytes against the hemolytic capacity of HClO was studied by measuring hemolysis induced by HClO, which was measured by quantification of hemoglobin released. *U. molinae* fruit extract alone produced hemolysis; however, levels were considerably inferior to those produced by HClO. As shown in Fig. 9, the extract was able to neutralize to some extent the deleterious capacity of HClO.

We also examined by X-ray diffraction the interaction of *U. molinae* fruit extract with DMPC and DMPE bilayers. Results showed that 0.4 mM (GAE) and higher extract concentrations disordered the polar and acyl chain regions of DMPC, whereas DMPE bilayers were not significantly affected even at the highest assayed concentration (10 mM GAE). DMPC and DMPE differ only in their terminal amino

groups, these being $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE. DMPE molecules pack tighter than those of DMPC due to their smaller polar groups and higher effective charge, resulting in a very stable bilayer system. However, the hydration of DMPC results in water filling the highly polar interbilayer spaces with the resulting increase of their width. This phenomenon might allow the incorporation of the hydrophilic anthocyanins into DMPC bilayers and their consequent interaction by hydrogen bonding with the lipid polar head groups. Results by fluorescence spectroscopy on DMPC LUV and IUM also showed the interaction of *U. molinae* fruit extract with the erythrocyte membrane and DMPC. These results agree with those reported by Arora et al. (2000) who reported that flavonoids and isoflavonoids partition into the hydrophobic core of liposomal membranes decreasing their fluidity, whereas Nakagawa et al. (2000) indicated that flavonoids stabilize membranes by locating in the lipid and aqueous interphase. It is then possible that the location of *U. molinae* components into the membrane bilayer and the resulting restriction on its fluidity might hinder the diffusion of HClO and its consequent damaging effects. This conclusion may also imply that this restriction can be applied to the diffusion of free radicals into cell membranes and the consequent decrease of the kinetics of free radical reactions.

## Conclusions

Protective effects of *U. molinae* fruit aqueous extracts were evaluated on human erythrocytes exposed to HClO-induced oxidative stress. Our SEM observation indicated that HClO induced morphological alterations to the red cells, from the normal discoid shape to an echinocytic form. However, 1 mM GAE extract practically neutralized the effect of 2.5 mM HClO. The protective effect was also demonstrated in hemolysis assays as the extract was able to neutralize to some extent the deleterious capacity of HClO. The explanation of *U. molinae* protection property might be explained by the insertion of flavonoids in cell membrane bilayer hindering diffusion of free radicals. X-ray and fluorescence experiments support this conclusion as they showed that molecules present in the aqueous extract interacted with a class of lipid present in the outer monolayer of the human erythrocyte membrane.

**Acknowledgments** To FONDECYT (Project 1130043) and to P. Osorio and Dr. C. P. Sotomayor for valuable technical assistance.

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