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Protective effect of *Ugni molinae* Turcz against oxidative damage of human erythrocytes

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Abstract

Ugni molinae Turcz, also known as "Murtilla", is a plant that grows in the south of Chile. Infusions of its leaves have long been used in traditional native herbal medicine. The chemical composition of the leaves indicates the presence of polyphenols, which have antioxidant properties. In the present work, the antioxidant properties of *U. molinae* were evaluated in human erythrocytes exposed *in vitro* to oxidative stress induced by HClO. The experiments were carried out by scanning electron microscopy (SEM) and hemolysis measurements. The SEM observations showed that HClO induced a morphological alteration in the red blood cells from a discoid to an echinocytic form. According to the bilayer couple hypothesis, the formation of echinocytes indicates that HClO was inserted in the outer leaflet of the erythrocyte membrane. However, a concentration as low as 10 μM gallic acid equivalents (GAE) *U. molinae* aqueous extract neutralized the shape change effect of HClO applied in a concentration as high as 0.25 mM. The significant protection of *U. molinae* aqueous extract was also shown in the hemolysis experiments. In fact, very low concentrations of the extract considerably reduced the deleterious capacity of HClO to induce hemolysis in red blood cells. It is concluded that the location of the extract 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 can also imply that this restriction could apply to the diffusion of free radicals into cell membranes and the subsequent decrease of the kinetics of free radical reactions.
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Keywords: *Ugni molinae* Turcz; Plant extract; Human erythrocyte; Cell membrane; Hemolysis

1. Introduction

Various medicinal properties have been ascribed to natural herbs (Ivanova et al., 2005). *Ugni molinae* Turcz, also known as "Murtilla", is a plant of the *Myrtaceae* family that grows in the south of Chile. Infusions of *U. molinae* leaves have long been used in traditional native herbal medicine to treat diarrhea and dysenteries (Hoffmann, 1991). Studies of the chemical composition of the leaves indicate the presence of phenolic acids, flavonoids and tannins (Montecinos et al., 1991). These compounds have antioxidant properties with low toxicities (Devany et al., 1997). It has been estimated that about 2% of the oxygen used by normal cells forms reactive oxygen species (ROS) (Chance et al., 1979). When ROS production overcomes the numerous antioxidant barriers of defense, 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 and the hydroxyl OH free

Abbreviations: *U. molinae*, *Ugni molinae* Turcz; GAE, gallic acid equivalents; SEM, scanning electron microscopy; RBC, red blood cells; IUM, isolated unsealed erythrocyte membrane; LUV, large unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine.

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radicals, which react with cell molecules such as lipids, proteins, carbohydrates, DNA and lipoproteins (Sohal and Weindruch, 1996). The antioxidants are molecules that can stop the formation of free radicals in the cells and limit their damage. Thus, the potential use of *U. molinae* extracts as antioxidants has also been considered (Avello, 2000). The molecular mechanisms of the antioxidant action of polyphenols have not yet been 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 membrane fluidity could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions (Arora et al., 2000).

Erythrocytes have been extensively used to study oxidative stress. They have been chosen because they represent a simple cell model. Although lacking protein synthesis machinery and being less specialized than many other cells, their membranes carry on enough functions in common with them such as active and passive transport, and the production of ionic and electric gradients. Oxidants, in fact, produce alterations in the erythrocyte membrane as manifested by a decreased cytoskeletal protein content and production of high molecular weight proteins which can lead to abnormalities in erythrocyte shape and rheological properties (Battistelli et al., 2005). For instance, H_2O_2 and ascorbate/Fe^{2+} induce an echinocytic type of shape alteration, i.e., develop a form characterized by blebs or protuberances over the cell membrane, indicative of oxidative damage (Srour et al., 2000).

Hypochlorous acid (HClO) is a powerful natural oxidant that damages bacteria, endothelial cells, tumor cells and erythrocytes (Tatsumi and Fliss, 1994; Zavodnik et al., 2001). In blood red cells it oxidizes the protein SH^- groups, lysine residues, cholesterol and fatty acids, causes K^+ leaks, membrane deformability, cross-linking of membrane proteins and lysis (Zavodnik et al., 2001; Hawkins and Davies, 1998; Carr et al., 1997; Vissers et al., 1998). In the present work, the antioxidant properties of *U. molinae* were evaluated in human erythrocytes exposed *in vitro* to the oxidative stress induced by HClO. The experiments were carried out by hemolysis measurements and scanning electron microscopy (SEM) observations of the erythrocyte shape changes.

2. Materials and methods

2.1. Plant material

Leaves of *U. molinae* Turcz were collected in the surroundings of Concepción, Chile, in April 2005, and identified by Dr. Max Quezada of the Department of Botany, University of Concepción; a voucher was deposited in the Herbarium under catalogue number- CONC 146511. Leaves of *U. molinae* were washed, air-dried and ground to a fine powder. Extractions were achieved with 100 ml of hot distilled water on 1 g of dry powder. The total polyphenol contents were spectrophotometrically determined at 765 nm by the Folin–Ciocalteu method (Velioglu et al., 1998) using Folin–Ciocalteu reagent (Merck, Darmstadt, Germany). Briefly, aliquots of test samples (0.5 mL) were mixed with 25 mL of 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) (Singleton and Rossi, 1965).

2.2. Hemolysis assays

Red blood cells (RBC) were obtained from healthy, consenting donors. Heparinized blood was centrifuged (Kubota, Japan) at 2500 rpm for 10 min. After removal of plasma and buffy coat, the RBC were washed three times with phosphate buffer (PBS, NaCl (150 mM), NaH_2PO_4 (1.9 mM), 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). RBC (10% v/v) were incubated in a shaking bath for 15 min at 37 °C in PBS in the presence of *U. molinae* aqueous extracts (5×10^{-4}, 0.5 and 1 mM GAE). NaClO (Sigma, Mo, USA), were added as single bolus of a diluted solution in PBS, whose concentrations (0.025, 0.25 and 0.5 mM) were 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 (Vissers and Winterbourn, 1995; Battistelli et al., 2005). After 15 min incubation, an aliquot of RBC suspension was centrifuged (EYDAM, Germany) at 2500 rpm for 10 min. Hemolysis was spectrophotometrically evaluated (Jasco, Japan) at 540 nm as haemoglobin (Hb) released from cells in the supernatant (Beutler, 1975).

2.3. Scanning electron microscopy (SEM) studies on human erythrocytes

Different HClO concentrations were made to interact *in vitro* with erythrocytes by incubating red blood cell suspensions. Subsequently, this incubation was performed adding *U. molinae* aqueous extracts. Blood was obtained from a healthy human donor not receiving any pharmacological treatment, by puncture of the ear lobule. Four to five drops were received in an eppendorff tube containing 10 µl of heparin (5000 UI/ml); 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) were placed in eppendorff tubes to prepare the following samples: (A) control (100 µl saline solution plus 100 µl RBCS); (B) RBCS + *U. molinae* aqueous extract (expressed as GAE) in a range of concentrations (10–50 µM GAE); (C) RBCS + HClO (25 µM, 0.25 mM, 0.5 mM); (D) RBCS + *U. molinae* aqueous extracts + HClO in a range of concentrations. Next, all the samples were incubated in an oven at 37 °C for 1 h. They were then fixed overnight at 4 °C with glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. Finally, previously centrifuged samples were washed in saline solution and drops of each one were placed on Al glass cover stubs, air-dried at room temperature, gold coated and SEM examined in a scanning electron microscope (JEOL JSM-6380LV, Japan).

2.4. Statistical analyses

Statistical analyses were performed using ANOVA one way and Dunnet test. All data were expressed as mean ± S.D of at least three different determinations.

3. Results

3.1. Hemolysis

As may be appreciated from Fig. 1, 0.5 mM (A), 0.25 mM (B), and 0.025 mM (C) HClO induced a 100%

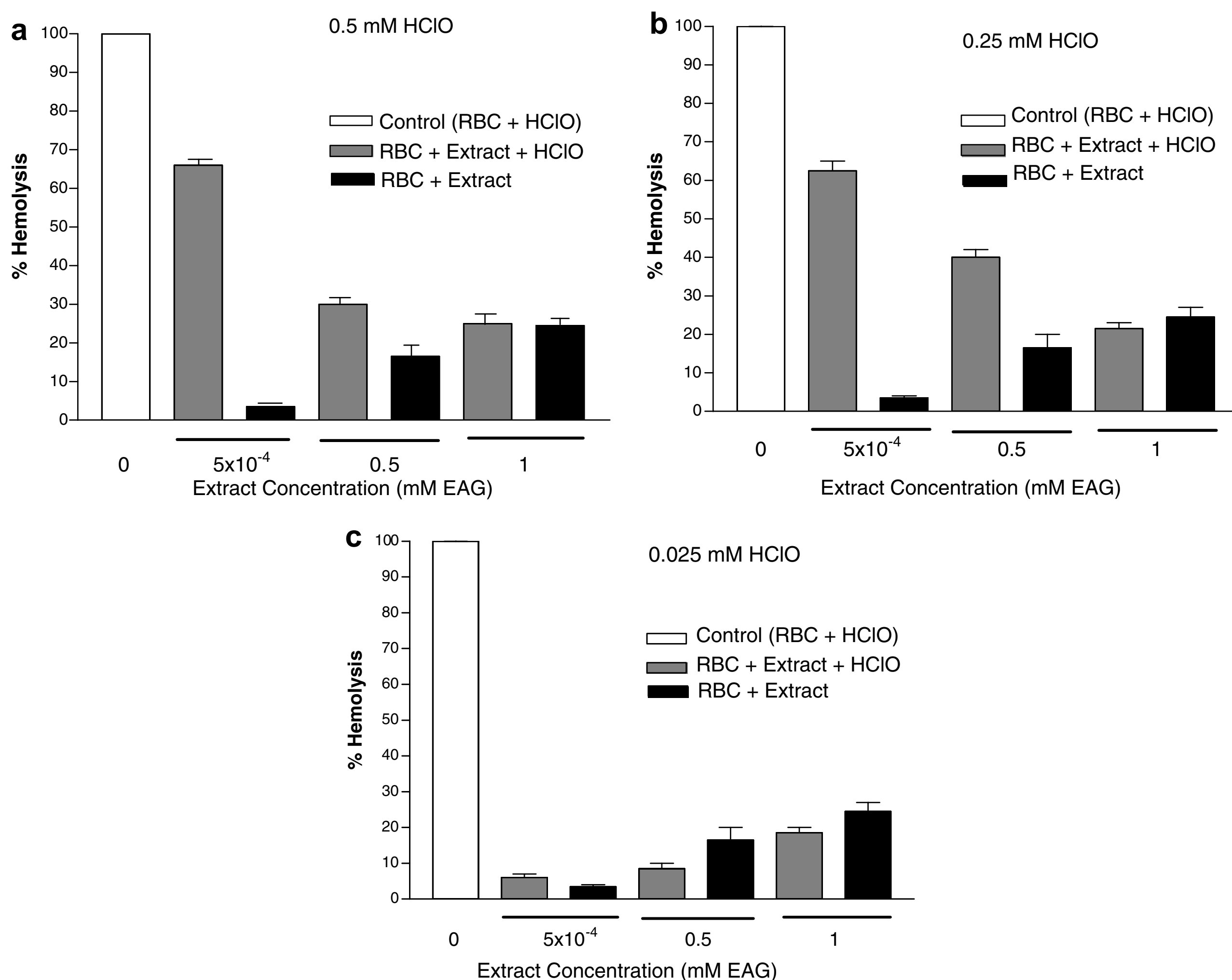

Fig. 1. Percentage of hemolysis of red blood cells (RBC) incubated with different concentrations of HClO and *U. molinae* aqueous extracts. (a) 0.5 mM HClO; (b) 0.25 mM HClO; (c) 0.025 mM HClO. Extract concentration is expressed as gallic acid equivalents (GAE); $n = 3$. Values are the mean ± SD.

hemolysis in human erythrocytes ($A = 2.0$). On the other hand it can also be noticed that the aqueous extract of *U. molinae* produced less than 30% hemolysis at its highest concentrations (0.5 and 1 mM GAE). However, increasing concentrations of the extract reduced the hemolytic effect of HClO, which reached a maximum of about 25% with 0.5 mM GAE extract. These results clearly indicated that *U. molinae* aqueous extract limited the extent of hemolysis induced by HClO.

3.2. Scanning electron microscopy (SEM) observations

The SEM examinations of human erythrocytes incubated with 10 μM GAE *U. molinae* aqueous extract (Fig. 2B) show that only a scanty number of red cells changed their normal biconcave discoid shape (Fig. 2A), while 0.25 mM HClO induced echinocytosis in a considerable number of erythrocytes (Fig. 2C); the extent of these shape changes were dependent on HClO concentration. In that altered condition (echinocytosis), red blood cells lost their normal profile and presented a spiny configuration with blebs in their surfaces, an effect present in all the studied samples. However, this shape alteration of the red blood cells was highly attenuated in samples containing 10 μM GAE of *U. molinae* aqueous extract and 0.25 mM HClO (Fig. 2D). These results demonstrated the protective effect of *U. molinae* against the shape perturbing effect of HClO upon human erythrocytes.

4. Discussion

Potential sources of antioxidant compounds have been searched in many types of plant materials such as fruits, leaves, seeds, etc. (Stanner et al., 2004). In the present study, the protective effects of aqueous extracts of *U. molinae* were evaluated on human erythrocytes exposed to HClO induced oxidative stress. HClO is an extremely toxic biological oxidant generated by neutrophils and monocytes; it is considered one of the most important factors causing tissue injuries in inflammation (Zavodnik et al., 2001). It is directly toxic to bacteria, endothelial cells, tumor cells and red cells. However, because it readily reacts

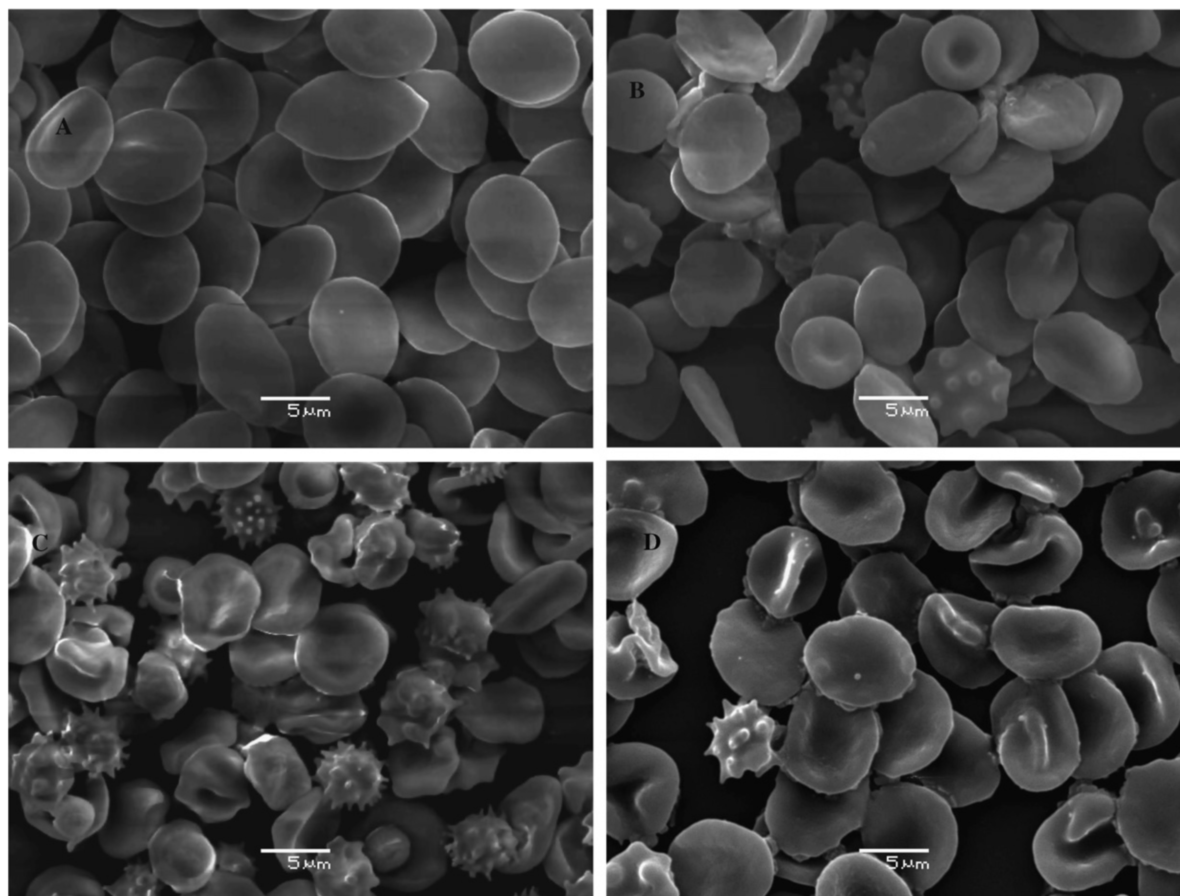

Fig. 2. Effects of *U. molinae* aqueous extracts and HClO on morphology of human erythrocytes. (A) Untreated erythrocytes; (B) erythrocytes incubated with 10 μM GAE *U. molinae* aqueous extract; (C) erythrocytes incubated with 0.25 mM HClO; (D) erythrocytes incubated with 10 μM GAE *U. molinae* aqueous extract and 0.25 mM HClO; 3000X. (GAE, gallic acid equivalents); $n = 3$.

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 model to detect oxidative stress (Battistelli et al., 2005). Their simple internal structure depleted of nucleus and organelles provide an ideal system for this type of study. One major consequence of their exposure to HClO is lysis; although the exact mechanism is not clear the cell membrane is considered the primary site for reaction. In fact, studies have demonstrated that HClO treatment of erythrocyte membrane results in inhibition of Na^+, K^+-, and Mg^{2+}-ATPase activities, oxidation of SH^- groups, tryptophan residues, chloramines formation, changes of membrane fluidity and surface area, and membrane morphological transformations, events that precede cell lysis (Zavodnik et al., 2001; Vissers and Winterbourn, 1995; Vissers et al., 1998).

SEM observations showed that HClO induced morphological alterations to the red cells from a discoid to an echinocytic form. According to the bilayer couple hypothesis (Sheetz and Singer, 1974; Lim et al., 2002) the 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 inserts into the inner monolayer whereas spiculated-shaped echinocytes are produced when it locates into the outer moiety. The finding that HClO induced the formation of echinocytes indicates that it was inserted in the outer leaflet of the erythrocyte membrane. This conclusion is supported by experiments carried out in red cells by fluorescence spectroscopy that showed structural perturbations of the external leaflet attributed to oxidative modifications of the membrane lipids (Zavodnik et al., 2001). These results do not agree with that 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 a concentration as low as 10 μM GAE *U. molinae* aqueous extract almost neutralized the effect of a HClO concentration as high as 0.25 mM demonstrates the protective capacity of the plant extract against the erythrocyte shape change capacity of HClO.

The significant protection of *U. molinae* aqueous extract was also shown in the hemolysis experiments. In fact, very

low concentrations of the extract considerably reduced the deleterious capacity of HClO to induce red blood cell hemolysis. The molecular mechanism by which HClO causes lysis is not known. One hypothesis indicates that lipid modification is not correlated with hemolysis by HClO; however, membrane protein modification, and particularly cross-link formation, might result in clustering of band 3 and other membrane and cytoskeletal proteins to form hemolytic pores (Vissers et al., 1998). We have examined by fluorescence spectroscopy the interaction of *U. molinae* aqueous extract with isolated unsealed human erythrocyte membranes (IUM) and large unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC), a class of phospholipid located in the outer monolayer of the erythrocyte membrane (Suwalsky et al., 2006). Our results indicated that very low concentrations of *U. molinae* extract induced an ordering of the polar and acyl groups of IUM and DMPC bilayers at 37 °C. High-performance liquid chromatography-mass spectrometry analysis of *U. molinae* leaves revealed the presence of polyphenols, basically flavonols and flavanols, myricetin, quercitin and epicatechin (Rubilar et al., 2006). On the other hand, there is no information that the extract contains significant amounts of other compounds which could potentially also interact with the membranes (e.g., alkaloids, glycosides, betalains, pigments and others). Arora et al. (2000) reported that flavonoids and isoflavonoids partition into the hydrophobic core of LUV decreased its fluidity, whereas Nakagawa et al. (2000) indicated that certain flavonoids might stabilize membranes by locating in the lipid and aqueous interphase. It might then be possible that the location of the extract 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 can also imply that this restriction could apply to the diffusion of free radicals into cell membranes and the consequent decrease of the kinetics of free radical reactions. The bioavailability of phenolic compounds has not been fully elucidated. However, it has been indicated that the absorption of polyphenols from plant foods and products might occur mainly in the gastroduodenal region and, in lesser amounts, in the jejunum (Serafíni et al., 1998).

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