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PROTECTIVE EFFECT OF *PHILESIA MAGELLANICA* (COICOPIHUE) FROM CHILEAN PATAGONIA AGAINST OXIDATIVE DAMAGE

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ABSTRACT

Philesia magellanica (P. magellanica) is a plant collected in the Chilean Patagonia. Its antioxidant properties were assessed in human erythrocytes exposed *in vitro* to oxidative stress induced by HClO. Scanning electron microscopy (SEM) observations showed that HClO induced a morphological alteration in the red blood cells from a normal discoid to a spherocytic form, and cells of unequal size. However, a concentration as low as 1 µM gallic acid equivalents (GAE) of *P. magellanica* aqueous extract neutralized the change effects of 50 µM HClO. On the other hand, 20 µM (GAE) of the extract considerably reduced the deleterious capacity of 0.25 mM HClO to induce hemolysis in red blood cells. In addition, X-ray diffraction experiments were performed in molecular models of the human erythrocyte membrane. These consisted in multilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of lipids preferentially located in the outer and inner monolayers, respectively of the human erythrocyte membrane. It was observed that *P. magellanica* only interacted with DMPC affecting its multilayer structure. It was also observed that 0.1 µM (GAE) of *P. magellanica* neutralized the structural perturbation induced by 0.05 and 0.5 mM HClO. These experiments confirmed the antioxidant properties of *P. magellanica* aqueous extracts.

Keywords*:* *Philesia magellanica*; Coicopihue; Plant extract; Antioxidant; Human erythrocyte; Cell membrane.

Abbreviations: *P. Magellanica, Philesia Magellanica*; GAE, gallic acid equivalents; SEM, scanning electron microscopy; RBCS, red blood cell suspension; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatisylethanolamine, ROS, reactive oxygen species.

1. INTRODUCTION

Patagonia is the southernmost region of Argentina and Chile. The Eastern side of the mountains is covered by broad steppe with low shrubs and small perennials.[1] We report here our study on the native bush *Philesia magellanica* J.F. Gmel. *Philesiaceae* (Coicopihue) (*P. magellanica*) (Fig. 1).

Fig 1. *Philesia magellanica* J.F. Gmel. *Philesiaceae* (Coicopihue) (*P. magellanica*) (www.chileflora.com)

The lack of knowledge about the potential use of *P. magellanica* in alternative medicine as an antioxidant and its toxicity as well as the particular interest of a plant growing in extreme environmental conditions lead us to investigate the plant's effects on cell membranes. Analyses of the plant extracts showed that contained flavonoids, of which their antioxidant properties have been extensively explored.[2-3] It has been estimated that about 2% of the oxygen used by cells forms reactive oxygen species (ROS).[4] When the ROS production overcomes the antioxidant defense barriers, damage 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.[5] The main ROS are the superoxide anion ($O_2^{\bullet}$), the hydroxyl ($OH^{\bullet}$) free radicals and singlet oxygen ($^1O_2$) which may damage cell molecules such as lipids, proteins, carbohydrates, DNA and lipoproteins.[6] The antioxidants are molecules that scavenge ROS and also can stop their formation in the cells, thus limiting their harmful effects. The molecular mechanisms of the antioxidant action of flavonoids have not been fully elucidated. 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 their reactions.[7-8] On the other hand, the toxic side effects of plant extracts are increasingly considered.[9-10]

This article describes the interaction of aqueous extracts of *P. magellanica* with human erythrocytes and 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 their membranes are 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. Oxidants 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, rheological properties and release of hemoglobin.[11-12] To better understand the molecular interactions of *P. magellanica* with the erythrocyte membrane we utilized molecular models consisting in multibilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipids classes located in the outer and inner monolayers of the human erythrocyte, respectively.[13-14] The capacity of *P. magellanica* to perturb the bilayer structure of DMPC and DMPE was evaluated by X-ray diffraction.

These systems and techniques have also been used in our laboratories to determine the effects of *Ugni molinae* (Murtilla) leaves and fruit,[15-17] *Aristotelia chilensis* (Maqui) leaves,[18] and *Balbisia peduncularis* (Amancay) stems[19] infuses on human erythrocytes.

Hypochlorous acid (HClO) is a powerful natural oxidant that damages bacteria, endothelial cells, tumor cells and erythrocytes.[20-21] In this work, the antioxidant properties of *P. magellanica* were evaluated in human erythrocytes

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exposed *in vitro* to the oxidative stress induced by HClO. The experiments were carried out by means of scanning electron microscopy (SEM), hemolysis measurements and X-ray diffraction.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium hypochlorite (NaClO) (Sigma, MO, USA); at pH 7.4 NaClO exists as HClO and ClO^- in an approximately equimolar ratio,[11,24] and is referred to hereafter as HClO; its concentration was spectrophotometrically determined at 292 nm (e = 350 $M^{-1}cm^{-1}$);[25] Folin-Ciocalteu reagent (Merck, Germany); dimyristoylphosphatidylcholine (DMPC, lot 140PC-224, MW 677.9) and dimyristoylphosphatidylethanolamine (DMPE, lot 140PE-54, MW 635.9) (Avanti Polar Lipids,ALA, USA); composition of phosphate buffered saline (PBS) was 150 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4; saline solution (0.9% NaCl, pH 7.4).

2.2. Plant material

Leaves of *P.magellanica* were collected from natural stands in the Chilean part of Patagonia (Puyuhuapi, 44º21'S, 72º34'W) during 2008 Southern Hemisphere summer. Specimens were identified by Dr. Roberto Rodríguez at the Department of Botany, University of Concepción, Chile, and a voucher specimen was deposited at its herbarium (CONC 177959).

2.3. Qualitative and quantitative analysis of extract species

2.3.1. Estimation of total phenolic content

A reducing capacity of the plant material (total phenolic content) was spectrophotometrically determined (Bausch & Lomb SP2000UV, USA) at 765 nm by the Folin-Ciocalteu method using Folin-Ciocalteu reagent.[26] 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 were expressed as gallic acid equivalents (GAE).[27] Solutions containing a range of polyphenol concentration for the different assays (0.1-10 mM GAE) were attained by dissolving calculated amounts of plant powder in the adequate volumes of distilled water.

2.3.2. Chromatographic analysis of phenolic acids

A portion of dry, pulverized plant material (0.5 g) was suspended in 50 mL of water and heated under reflux until boiling. After filtration, the water extract was concentrated *in vacuo* to obtain 10 mL of condensed solution. The solution was further filtered through the membrane filter (pore size 0.2 mm) and an aliquot (5 mL) of the filtrate was injected into an HPLC system to analyze simple phenolics in unhydrolysed extract. The remaining filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 10 mL of 2M HCl and heated under reflux for 30 min to make the hydrolysis as complete as possible.[28] After cooling, the hydrolysate was extracted three times with 5 mL of EtOAc. The organic extracts were combined and evapored under reduced pressure. The dry residue was redissolved in 70 % MeOH (1 mL) and centrifuged (11,340*g*, 5 min) prior to HPLC analysis. Analytical RP-HPLC separations of the samples were performed using an Agilent 1200 Series HPLC system (Agilent Technologies, USA), equipped with a Rheodyne manual sample injector, quaternary pump, degasser, column oven and a diode array detector. Chromatographic separations of phenolics were carried out at 25 ºC, on a Zorbax Eclipse XDB-C18 column, 4.6 x 150 mm (Agilent Technologies, USA), as it was described earlier.[29] The compounds were semi-quantified using two point calibration curves, based on peak areas measured at 325 nm (caffeic acid, ferulic acid), 305 nm (p-coumaric acid) and 250 nm (p-hydroxybenzoic acid) (concentration range 0.02 – 0.40 mg mL^{-1}). The phenolic acids were identified by comparison of their retention times and UV spectra with those of authentic samples and co-chromatography with standards.

2.3.3. Isolation of catechin

Dried and pulverized plant leaves (0.5 g) were extracted with hot 50 % MeOH, for 30 min, under reflux. The extract, after evaporation under reduced pressure, yielded 117 mg of a dry residue. The residue was suspended in MeOH (1.5 mL) and separated by paper chromatoghraphy (filter paper - Whatman 3MM). Solvent system (15 % AcOH) was applied in descending mode. After chromatographic development, bands showing fluorescence in UV light were excised and separately eluted with MeOH. The band which showed a blue fluorescence and Rf value of 0.53, after elution with MeOH and evaporation of the solvent, yielded yellowish residue (4.8 mg).

2.4. X-ray diffraction studies of phospholipid multilayers

Synthetic DMPC and DMPE were used without further purification. About 2 mg of each phospholipid were introduced into 1.2 mm diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany), which were filled with 150 mL of (a) distilled water and (b) aqueous extracts of *P. magellanica* in a range of concentrations (10 µM-10 mM GAE). The specimens were incubated for 1 h at 30 ºC and 60 ºC with DMPC and DMPE, respectively**,** centrifuged for 10 min at 3500 rpm and X-ray diffracted with Ni-filtered CuKα from a Bruker Kristalloflex 760 generator (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 a 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. The same procedure was used to study the protective capacity of *P. magellanica* aqueous extracts (0.1, 1.0 and 10 µM GAE) against the oxidant property of HClO (0.05 mM and 0.5 mM). In this case, the assays were performed only in DMPC.

2.5. Scanning electron microscopy (SEM) studies of human erythrocytes

A range of *P. magellanica* aqueous extract concentrations were made to interact *in vitro* with red blood cells with and without HClO. With this aim, two blood drops from a human healthy donor not receiving any pharmacological treatment were obtained by puncture of the ear lobule and received in an Eppendorff tube containing 100 µL of heparin (5000 UI/ml) in 900 µL of saline solution; after centrifuged, (1000 rpm x 10 min) the tube 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) in saline were placed in seven Eppendorff tubes and added to each one different concentrations of the plant aqueous extract and HClO; these concentrations were attained diluting the extract in saline to a final volume of 500 µL. The aqueous extract solutions used for the preparation of these samples was previously sonicated for 15 min, placed on a thermo regulated bath at 37 ºC and filtered. All the samples were then incubated at 37 ºC for 1 h, period in line with the larger effects induced by compounds on red cell shape.[30-31] They were fixed overnight at 4 ºC with 400 µL 2.5% glutaraldehyde in distilled water, reaching a final fixation concentration of about 2.4%. Samples were centrifuged (1000 rpm x 10 min.), glutaraldehyde was discarded and replaced (three times) with 400 µL of distilled water; about 20 µL of each sample was placed on Al glass cover stubs, air-dried at room temperature, gold coated and examined in scanning electron microscopes (Etec Autoscan, Etec Corp., Hayward, CA, USA, and JEOL JSM-6380LV, Japan).

2.6. 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 at room temperature, and resuspended in PBS four times its volume for subsequent analyses.[32] RBC (10 % v/v) were incubated in a shaking bath for 15 min. at 37 ºC in PBS in the presence of *P. magellanica* aqueous extracts (0.02, 0.1, 0.3 and 0.5 mM GAE). 0.25 mM NaClO was added to the extract as single bolus of a diluted solution in PBS. 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.[33]

2.7. Statistical analysis

Statistical analysis 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

1.1 Phenolic constituents of P. magellanica

3.1.1. Total phenolic contents

Total reducing capacity of the plant material (total phenolic content) was 48.7 mg/g dry weight (expressed as GAE equivalents), determined by the Folin-Ciocalteau method. This content of phenolics is close to that found in chicory leaves [34], which are excellent dietary source of antioxidants.

3.1.2. Phenolic acid analysis

Chromatographic separations of aqueous extract from *P. magellanica* leaves revealed that free phenolic acids are absent from the analysed solution. However, the UV spectra of major peaks present in the chromatogram (Fig. 2) were identical with those of caffeic and ferulic acid. After acidic hydrolysis of the water extract, the following phenolic acids were found: ferulic acid (Rt – 15.5 min, l_{max}- 318 nm) – 33 ± 0.80 mg/100 g of dry tissue, caffeic acid (Rt – 8.6 min, l_{max}- 316 nm) – 12 ± 0.38 mg/100 g, *p*-coumaric acid (Rt – 12.9 min, l_{max}- 305 nm) – 5 ± 0.40 mg/100 g and *p*-hydroxybenzoic acid (Rt – 12.9 min, l_{max}- 250 nm) – 10 mg ± 0.04 mg/100 g (Fig. 2). Limits of detection (LOD) and

quantification (LOQ) for hydroxycinnamates, under the given chromatographic conditions, were 0.3 mg/mL and 0.8/mg mL, respectively.

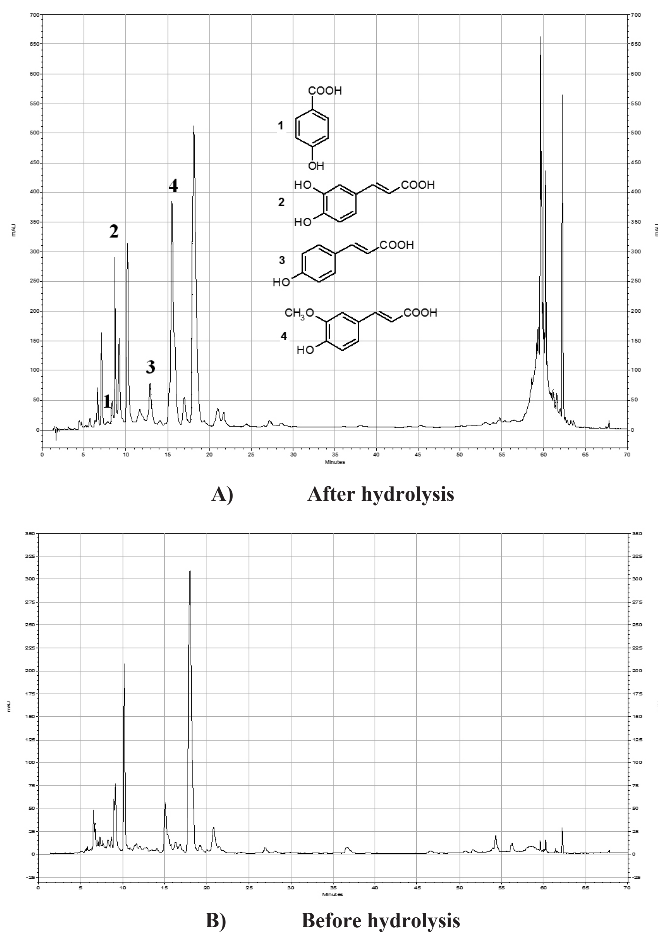

Fig. 2: Chromatograms of phenolic acids after (A) and before (B) hydrolysis. 1 - (*p*-hydroxybenzoic acid, Rt - 7,48 min (the peak is low because absorption maximum of this compound is at 250 nm and the chromatograms were monitored at 320 nm); 2 – caffeic acid, Rt - 8,68 min; 3 - *p*-coumaric acid, Rt - 12,90 min; 4 – ferulic acid, Rt - 15,53 min).

3.1.3. Isolation of catechin

Fractionation of hydroalcoholic extract from the plant leaves yielded catechin - 3',4',5,7-tetrahydroxyflavan-3-ol (4.8 mg, isolation yield – 0.96 %), which is a well known plant antioxidant and a building block of condensed tannins. The compound was identified by comparison of its spectral data (UV, ^{1}H NMR) to those found in the literature.[35] Although catechin is less soluble in water than in water-alcohol mixtures, the compound very likely contribute to the antioxidant potential of *P. magellanica* aqueous extract.

3.2. X-ray diffraction studies of phospholipid multilayers

Fig. 3A exhibits the results obtained by incubating DMPC with water and aqueous extracts of *P. magellanica*. 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 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.[36]

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 *P. magellanica* in the range 10 µM - 10 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. 3B. 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 are indicative of the gel state reached by DMPE in water after heating and cooling it. Increasing concentrations of the plant extract in the range 10 µM - 10 mM GAE only induced a slight gradual increase of the low-angle reflection intensities. Thus, the extract induced a very moderate molecular order of DMPE bilayers. Figures 3C and 3D show the protective effect of *P. magellanica* against the oxidative action of HClO. As it can be appreciated in Fig. 3C, 0.05 mM HClO induced a significant reduction of DMPC low- and wide-angle reflection intensities indicating a structural perturbation of DMPC bilayers. However, a concentration as low as 0.1 µM (GAE) *P. magellanica* neutralized the deleterious effect of HClO. Fig. 3D shows the same protective effect of 0.1 µM (GAE) *P. magellanica* extract against 0.50 mM HClO.

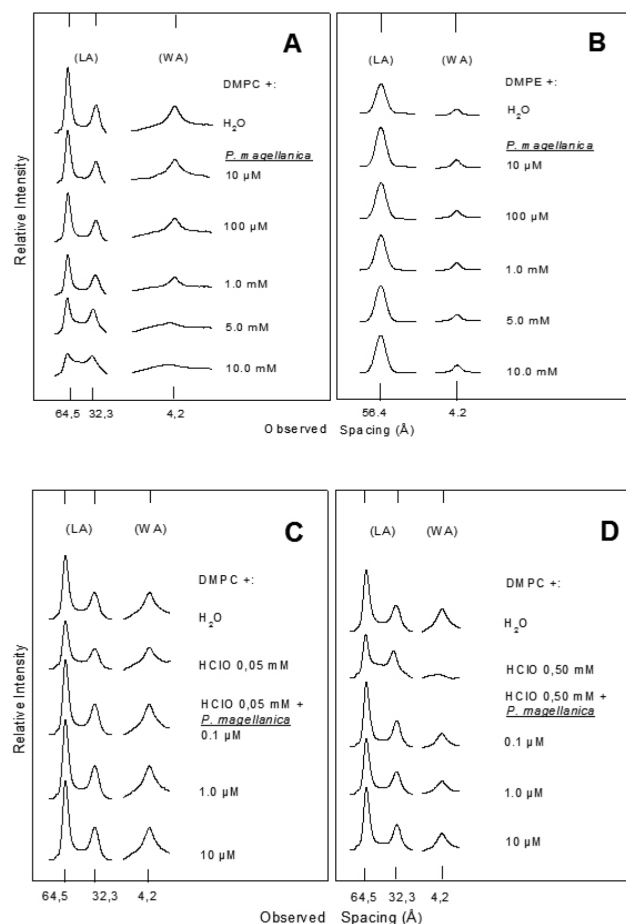

Fig. 3 Microdensitograms from X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous extracts of *P. magellanica*; (C) DMPC + 0.05 mM HClO + aqueous extracts of *P. magellanica*; (D) DMPC + 0.5 mM HClO + aqueous extracts of *P. magellanica*. (LA) low-angle and (WA) wide-angle reflections. Extract concentrations are expressed as gallic acid equivalents (GAE).

3.3. Scanning electron microscopy (SEM) studies of human erythrocytes

Human erythrocytes incubated with 50 µM HClO induced spherocytosis (sphere-shaped rather than bi-concave disk shaped) and anisocytosis (red blood cells of abnormal size) in a considerable number of erythrocytes (Fig. 4B). However, this shape alteration of the normal red blood cells (Fig. 4A) was highly attenuated in samples containing 50 µM HClO and 1 µM GAE of *P. magellanica* (Fig. 4C). These results demonstrated the highly protective effect of *P. magellanica* against the shape and size perturbing effect of HClO upon human erythrocytes.

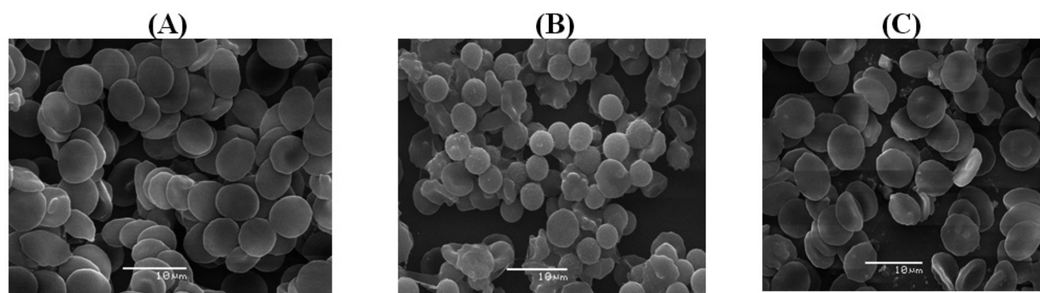

Fig. 4 Effects of *P. magellanica* aqueous extracts and HClO on morphology of human erythrocytes. (A) Untreated erythrocytes; (B) erythrocytes incubated with 50 μM HClO; (C) erythrocytes incubated with 1 μM (GAE) *P. magellanica* aqueous extract and 50 μM HClO. Extract concentrations are expressed as gallic acid equivalents (GAE).

3.4. Hemolysis assays

Fig. 5 shows that 0.25 mM HClO induced a 70% hemolysis in human erythrocytes. On the other hand 0.02 mM (GAE) and increasing concentrations of the plant extract considerably reduced the hemolytic effect of HClO.

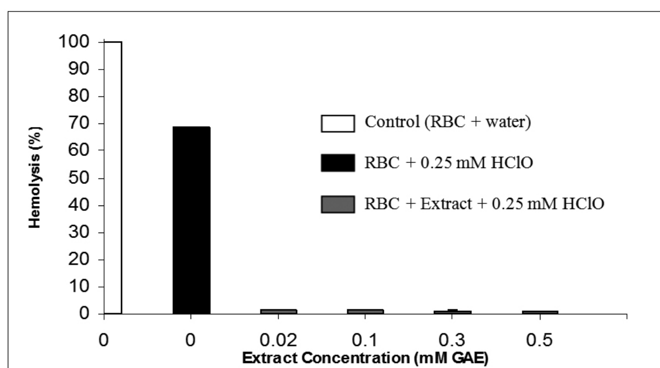

Fig. 5 Percentage of hemolysis of red blood cells (RBC) incubated with 0.25 mM HClO and different concentrations of *P. magellanica* aqueous extracts. Extract concentrations are expressed as gallic acid equivalents (GAE); n=3. Values are the mean ± SD.

4. DISCUSSION

The protective effects of aqueous extracts of *P. Magellanica* were evaluated on human erythrocytes and molecular models of the erythrocyte membrane exposed to HClO-induced oxidative stress. HClO is an extremely toxic biological oxidant generated by neutrophils and monocytes.[21] 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.[37] Human erythrocytes are a reliable and easily obtainable mechanism to detect oxidative stress.[11] 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.[21,31,37] Our SEM observations showed that 50 μM HClO induced morphological alterations to the red cells, from a discoid to a spherocytic form. According to the bilayer couple hypothesis shape changes induced in erythrocytes by foreign molecules are due to differential expansion of the two monolayers of the red cell membrane.[38-39] Thus, stomatocytes are formed when the compound is inserted into the inner monolayer whereas spiculated-shaped echinocytes are produced when is situated into the outer moiety. Battistelli et al. reported that 0.5 mM HClO induced the formation of echinocytes in most of the observed erythrocytes.[11] This effect might be due to the interaction of HClO with phosphatidylcholines located in the outer monolayer of the red cell membrane as we observed in our X-ray experiments (Fig. 2 D). This result does not agree with that reported by Vissers and Winterbourn who indicated that HClO penetrates into the red cells passing through the hydrophobic lipid bilayer without the membrane acting as a major barrier.[24] Our finding that the formation of spherocytes with 50 μM HClO might be due to the interaction of HClO with membrane cytoskeleton.[37,40] The result that a concentration as low as 1 μM GAE *P. magellanica* practically neutralized the effect of a 50-fold higher HClO concentration (50 μM) demonstrated the protective capacity of the plant extract against the erythrocyte shape change capacity of HClO. On the other hand, very low concentrations of the extract considerably reduced the deleterious capacity of HClO to induce red blood cell hemolysis.

We also examined by X-ray diffraction the interaction of *P. magellanica* with DMPC and DMPE. Results showed that 10 μM (GAE) and higher extract concentrations disordered the polar and acyl chain regions of DMPC bilayers whereas DMPE bilayers were not significantly affected by *P. magellanica* 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 allows the incorporation of polyphenols into DMPC bilayers and their consequent interaction by hydrogen-bonding with the lipid polar head groups. Our experimental results showed that 0.1 μM GAE *P. magellanica* neutralized the deleterious effects of 0.05 and 0.5 mM HClO. Thus, molecules present in *P. magellanica* aqueous extract would act by blocking access of oxidants into the lipid bilayer contributing to preserve the structure and functions of biological membranes. In conclusion, the experiments carried out on human erythrocytes and molecular models of the red cell membranes demonstrated the antioxidant properties of *P. magellanica* aqueous extracts.

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