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Original Article

Cytoprotective activity of extracts from *Adelia ricinella* L. aerial parts

[Actividad citoprotectora de extractos de las partes aéreas de *Adelia ricinella* L.]

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Abstract

Context: Several factors can affect the erythrocyte cell membrane integrity targeting important hematological alterations that can be avoided by the cytoprotective effect offered by some plant extracts.

Aims: To evaluate the cytoprotective activity of *Adelia ricinella* L. extracts on red blood cells (RBCs) membrane after hypotonic and oxidative treatments.

Methods: Total phenols and flavonoid contents were spectrophotometrically determined in three extracts: AR1 (ethanol 95%), AR2 (ethanol 50%), and AR3 (aqueous extract). Luteolin and apigenin were quantified using HPLC-DAD techniques. Hypotonic erythrocyte membrane stabilizing activity of plant extracts, their antioxidant protective effect on H_2O_2-induced cell damage, and cytotoxicity on Vero cells were explored. Control cells were treated with sodium diclofenac or ascorbic acid.

Results: AR2 extract showed the highest values of total phenols/flavonoids, as well as, for luteolin and apigenin with 207.5 and 1.86 µg/mL respectively. The extracts did not exert spontaneous hemolysis following the INVITOX protocol, presumably by the protective effect of high flavonoid content. A concentration-dependent pattern was observed on the hypotonic erythrocyte membrane stabilizing assay, in which both ethanol extracts but mainly AR1 (IC_{50} = 16.46 µg/mL) showed a significant activity with lower IC_{50} values than diclofenac-control group. On the other hand, AR2 (IC_{50} = 17.49 µg/mL) displayed the most potent cytoprotective effect on RBCs after H_2O_2-induced cell damage. *Adelia ricinella* extracts were not cytotoxic to mammalian Vero cells (IC_{50} > 256 µg/mL).

Conclusions: The study suggests that *Adelia ricinella* extracts can promote erythrocyte cytoprotection by protecting both membrane layers, thus preventing potential hematological alterations induced by oxidizing damage and probably, in inflammation-related diseases.

Keywords: *Adelia ricinella*; antioxidant; cytoprotection; flavonoids; red blood cells.

Resumen

Contexto: Varios factores afectan la integridad de la membrana eritrocitiaria, provocando alteraciones hematológicas, que pueden evitarse mediante el efecto citoprotector de extractos vegetales.

Objetivos: Evaluar la actividad citoprotectora de extractos de *Adelia ricinella* L. sobre la membrana eritrocitaria luego de tratamientos hipotónicos y oxidativos.

Métodos: El contenido de fenoles y flavonoides totales se determinó espectrofotométricamente en tres extractos: AR1 (etanol 95%), AR2 (etanol 50%) y AR3 (acuoso); la luteolina y apigenina se estimaron mediante HPLC-DAD. Se evaluó el efecto estabilizador de los extractos en membranas sometidas a tratamiento hipotónico, la actividad antioxidante ante el daño inducido por H_2O_2, y la citotoxicidad en células Vero. El diclofenaco de sodio y el ácido ascórbico se emplearon como controles.

Resultados: AR2 mostró valores superiores de fenoles totales/flavonoides, y de luteolina y apigenina con 207,5 y 1,86 µg/mL, respectivamente. Los extractos no causaron hemólisis espontánea en el protocolo INVITOX, probablemente debido al efecto protector de los flavonoides. Se observó un comportamiento dependiente de la concentración en el ensayo de estabilización de la membrana en solución hipotónica, en el que ambos extractos etanólicos (principalmente AR1, IC_{50} = 16,46 µg/mL), evidenciaron una actividad significativa con valores de IC_{50} menores al control con diclofenaco. AR2 (IC_{50} = 17,49 µg/mL) mostró el efecto citoprotector más potente frente al daño inducido por H_2O_2. Los extractos no resultaron citotóxicos en células Vero (IC_{50} > 256 µg/mL).

Conclusiones: Los extractos de *Adelia ricinella* L. pueden promover la citoprotección eritrocitaria en ambas superficies, y así prevenir posibles alteraciones hematológicas inducidas por daño oxidativo y presumiblemente, por enfermedades inflamatorias.

Palabras Clave: *Adelia ricinella*; antioxidante; citoprotección; flavonoides; glóbulos rojos.

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INTRODUCTION

Several factors can affect cell integrity, thus leading to important pathological conditions in humans. In general, ionizing radiations, chemotherapeutic drugs and exposition to a wide range of toxicants may generate cell disturbances through the impairment of key biomolecules with the subsequent loss of functionality, e.g.: disruption of erythrocyte cells membrane (Botta et al., 2014; Casares et al., 2019). After these damaging factors, hematological alterations are common adverse effects that normally compromise the patient quality of life. In addition to bone marrow failure, mechanisms involved in erythrocyte damage have been related to the oxidative stress and its accompanying deterioration of lipid and membrane proteins (Mohanty et al., 2014; Spengler et al., 2014; Tibaldi et al., 2020). Therefore, increasing the cytoprotection by erythrocyte membrane stabilization and functionality has become an interesting approach reducing the risk of abnormal hematological changes.

Plant-based complementary therapies have gained an upsurge of interest because of their promising healing potential, cytocompatibility and safety (Anwar et al., 2018). Many phytochemicals reveal powerful pharmacological activities mitigating the harmful effect in cellular functions produced by chemotherapeutic drugs and/or other factors (Osman et al., 2016). The cytoprotective effect of some plant compounds have been mainly associated to polyphenol-like metabolites, which are recognized as good antioxidants (Sazwi et al., 2013; Valentová, 2020). Natural antioxidants (such as flavonoid types) have been suggested to exert beneficial and protective effects on red blood cells. They act primarily by altering the surface charges of cells after scavenging cations, thus preventing the breakdown of the lipid membrane and increasing resistance to hemolysis (Oyedapo et al., 2010; Vijayraja and Jeyaprakash, 2019).

Erythrocytes are critical targets for natural products and many other drugs. Indeed, human erythrocytes have been used as a model for studies of biological effects of reactive oxygen species induced oxidant stress for several reasons. They are continuously exposed to high oxygen tensions, the membrane lipids are composed partly of polyunsaturated fatty acid side chains that are vulnerable to peroxidation, and a substantial part of metabolism is conserved, including antioxidant systems. Moreover, they are structurally simple and easily obtained (Konyalioglu and Karamenderes, 2005). In this context, several studies have demonstrated the usefulness of the *in vitro* human red blood cell membrane stability method as an excellent strategy to explore the cytoprotective role of natural products. Some investigations comprised a flavonoid and steroid-enriched methanolic extracts of *Millettia pachycarpa* leaves (Chowdhury et al., 2013), different extracts obtained from *Sargassum muticum* and *Ulva lactuca* algae and *Castanea sativa* plant (Botta et al., 2014), and a methanolic extract from the marine brown alga *Turbinata ornata* (Vijayraja and Jeyaprakash, 2019).

Adelia ricinella L. (*Euphorbiaceae*) is a tree that grows in the Caribbean area and empirically used by Cuban inhabitants, but scarcely studied in their medicinal and safeness evidence based properties. It was recently reported that aqueous and ethanol preparations from the aerial parts consist of a rich matrix of active compounds identified as polyphenols and mainly flavonoids, which were probably responsible of the scavenging effect against DPPH and ABTS radicals found in cell-free assays (Berenguer et al., 2018; 2021). However, their biological properties are not completely elucidated, including the antioxidant-mediated cytoprotective mechanisms. That is why this work is aimed to evaluate the cytoprotective potential of *Adelia ricinella* L. extracts on red blood cell membrane stability after treatment with a hypotonic solution, or hydrogen peroxide. In this sense, *Adelia ricinella* extracts may prove their capacity of reducing the hematological alterations and cell toxicity by participating in the prevention of oxidizing damage and inflammation-related processes, thus supporting its safeness traditional use.

MATERIAL AND METHODS

Plant material and extract preparation

The plant parts (leaves) of *Adelia ricinella* L. were collected in the Siboney-Juticí Ecological Reserve, in the periphery of Santiago de Cuba city (Latitude: 19.9603 and Longitude: -75.7081). Taxonomy identification was performed by specialists of the Eastern Center for Ecosystems and Biodiversity (BIOECO, Santiago de Cuba). A vegetal sample was stored at the botanic herbarium of this institution with code 14780. The extracts were obtained following the methodology previously reported by Berenguer et al. (2021). Briefly, 10 g of shadow-dried leaves were extracted with a Soxhlet apparatus. Extraction was done using 95% ethanol (commercial grade, for AR1 extract), a mixture of ethanol/water (50%) (for AR2 extract) and water (for AR3 extract) as solvents, and stopped after four hours of refluxing. The extracts were filtered, evaporated to dryness in a rotary evaporator at 45ºC (Kika Werke RV 05, Germany), and then stored at -20ºC until use.

Determination of total phenols and flavonoids content

Total phenols

The total phenolic content was determined by the Folin-Ciocalteu's colorimetric method according to Escalona et al. (2011) measuring the absorbance at 765 nm on a Genesys UV/VIS spectrophotometer (Switzerland). Briefly, samples consisting of 50 µL (0.1 mg/mL solution-based total extractable substances) were mixed with 100 µL of 50 % Folin-Ciocalteu's reagent (Sigma-Aldrich, St. Louis, MO, USA) and incubated in darkness at room temperature. Subsequently, 200 µL of saturated sodium carbonate solution (Riedel-de Haën, 99.5 % pure, Munich, Germany) were added. The solutions were incubated under the same conditions for an additional hour. The samples were measured and expressed as gallic acid (Sigma-Aldrich, St. Louis, MO, USA) using a calibration curve with seven points (from 9.7 to 625 µg/mL). Results were calculated using equation [1] and expressed as the average value of the three replicates.

$$y = 0.018x - 0.012 \qquad R^2 = 0.996 \qquad [1]$$

Total flavonoid content

Flavonoids were measured by reaction with aluminum trichloride ($AlCl_3 \times 6\ H_2O$, Riedel-de Haën, 99.9 % pure, Munich, Germany) according to Llauradó et al. (2020). Samples consisting of 50 µL (0.1 mg/mL solution-based total extractable substances) were mixed with 15 µL of 5% $NaNO_2$ solution (abcr GmbH, Karlsruhe, Germany) and 250 µL of distilled water. After vortex homogenization and five minutes repose, 30 µL of $AlCl_3 \times 6\ H_2O$ at 10% ethanol were added and incubated for another six minutes. Subsequently, 100 µL of 1 mol/L NaOH and 55 µL of distilled water were added, and the absorbance was measured at 510 nm in a Genesys UV/VIS spectrophotometer (Switzerland). Results were expressed as quercetin (95% pure, Sigma, USA) using a calibration curve with seven points (from 6.25 to 100 µg/mL) using equation [2] and expressed as the average value of the three replicates.

$$y = 0.008x - 0.067 \qquad R^2 = 0.988 \qquad [2]$$

HPLC-DAD quantification of luteolin and apigenin

HPLC-DAD quantification of luteolin and apigenin was carried out according to Berenguer et al. (2021). Briefly, 20 µL of each sample was injected onto a Phenomenex Luna C18 column (250 × 10 mm × 5 µm, Utrecht, Netherland) in an Agilent 1200 system coupled with a DAD detector (Agilent Technologies, Santa Clara, CA, USA). An aqueous formic acid (0.1%, v/v) solution was used as mobile phase A, while HPLC grade acetonitrile (Fisher Chemical, UK) was used as mobile phase B. The gradient program was: 5% B (0–5 min), 5–95% B (5–25 min), 100% B (25–30 min) at a flow rate of 1 mL/min. The column temperature was maintained at 26°C while the DAD signal was monitored and recorded at 210, 235, 254, 260, 270, 280 and 366 nm. Samples (AR1, AR2 and AR3) were prepared at a concentration of 1 mg/mL in methanol 50% enriched with a standard solution consistent in 20 µg/mL of both standards (Sigma Aldrich, St. Louis, MO, USA).

Cytoprotection and antioxidant assessment

Erythrocyte suspension

Fresh blood samples (12-15 mL) were collected from healthy human volunteers who didn't consume any anti-inflammatory drugs in the previous 15 days. Samples were added to tests tubes with sodium citrate (1:10), and plasma was separated by centrifugation (2 500 rpm × 15 min) and then discarded. Afterward, erythrocyte suspension was washed with a sterile phosphate buffer solution (PBS: 0.2 g of K_2HPO_4, 1.8 g of glucose, and 9 g of NaCl, pH 7.4) four times till the supernatant was colorless and the pellet was reconstituted (1:40 v/v, 8 × 10^9 cells/mL). The erythrocyte suspensions (RBC: Red Blood Cells) were always prepared prior to the *in vitro* assessment.

Hemolysis assay

The hemolysis assay was performed following the INVITOX protocol No. 37 (Pape, 1992). Plant extract concentrations (8–256 µg/mL) were chosen considering previously determined pharmacological activities (Berenguer et al., 2021). Samples were diluted in PBS, added to 25 µL of RBC, and incubated at room temperature with stirring (100 rpm) for 10 min. The suspension was centrifuged (3 500 rpm × 10 min) and the supernatant (hemoglobin content) was measured at 530 nm (Genesys UV/VIS spectrophotometer, Switzerland). Hemolysis value of 100% was settled by measuring the absorbance of RBC incubated with distilled water, while the 0% value was defined by determining the spontaneous hemolysis of RBC incubated with PBS. All the samples were prepared in triplicate.

Hypotonic erythrocyte membrane stabilizing activity

The human red blood cell membrane stabilization *in vitro* assay has often been considered a technique to evaluate the *in vitro* anti-inflammatory effect of phytochemicals (Tantary et al., 2017). Briefly, 1 mL of each plant extract (ranging 8-256 µg/mL) or positive control (sodium diclofenac, 98%, abcr GmbH, Karlsruhe, Germany, 8-256 µg/mL) were mixed with 0.5 mL of erythrocytes suspension in PBS (1 mL of phosphate buffer and 2 mL of hypotonic NaCl solution 0.45%). Hypotonic NaCl 0.45% (1 mL) was used as negative control instead of plant samples. After incubation (37°C for 30 min), samples were centrifuged at 3 000 rpm, 10 min. Then, hemoglobin concentrations were recorded spectrophotometrically at 560 nm (Genesys UV/VIS spectrophotometer, Switzerland). The percentage of hemolysis was assessed by assuming the hemolysis produced in control as 100%. The erythrocyte stabilization membrane on a hypotonic medium was estimated by equation [3]. The concentration in which extracts protect 50% of the RBC membrane integrity (HMP_{50}) was estimated when possible using a linear regression approach. All the samples were prepared in triplicate.

$$HMP\ (\%) = (100 - (A1\text{-}A2/A1)) \times 100 \quad [3]$$

where: HMP (%) = Hypotonic Medium Protection percentage, A1 = Absorbance of negative control and A2 = Absorbance of test sample in hypotonic saline solution.

Antioxidant erythrocyte membrane stabilizing activity

The assay was performed by the hemolysis oxidative inhibition induced by hydrogen peroxide (H_2O_2) in RBC, following the method reported by Escalona-Arranz et al. (2014) with a modification. Erythrocyte suspensions were treated with PBS, H_2O_2 (10 mM) (negative control), ascorbic acid (positive control), and plant extracts (AR1, AR2 and AR3) ranging 8-256 µg/mL. All experimental groups were incubated at 37°C during 1 h and stirring with a vortex each 10 min (Heidolph REAX 2000, Germany). Afterward, the samples were centrifuged, the supernatants were collected, and then absorbances were measured at 530 nm (Genesys UV/VIS spectrophotometer, Switzerland). The percentage of hemolysis was calculated assuming 100% of hemolysis in the positive control (H_2O_2). The erythrocyte stabilization membrane facing the oxidant agent was estimated by equation [4]. The concentration in which extracts protect 50% of the RBC membrane integrity (AP_{50}) was estimated when possible using the logarithm of the concentration using a linear regression approach. All the samples were prepared in triplicate.

$$AP\ (\%) = (100 - (A1\text{-}A2/A1)) \times 100 \quad [4]$$

where: AP (%) = Antioxidant Protection percentage, A1 = Optical density of negative control and A2 = Optical density of test sample in the oxidant medium.

Erythrocytes morphological changes

To determine the nature in which *A. ricinella* extracts protect the erythrocyte membrane, the morphological changes induced in the experiments with H_2O_2 as an oxidizing agent were observed in RBCs. A volume of 50 µL of RBC from the pellets of each experiment was added to a Neubauer chamber and visualized in a Transmitted Light Microscope (Leica DM 1000, Switzerland) coupled with a digital camera (Leica MC 170 HD, 400×, 2592 × 1944 pixels, Switzerland). Membrane integrity; erythrocyte form; presence/absence of deformations such as spherocytes, equinocytes, and other morphological alterations were considered for all test samples and controls (Selvaraj et al., 2015).

Cytotoxicity of plant extracts in Vero cells

The resazurin dye reduction test evaluated the potential cytotoxic effect of plant extracts on Vero cells. Vero cell line from ATCC (American Type Culture Collection, USA) was maintained at 37°C, 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 2% L-glutamine and 4.5 g/L D-glucose. A cell suspension (200 µL, 1 × 10^6 cells/mL) was seeded in a 96-well plate and incubated at the abovementioned conditions. After 48 h, the cells were washed twice with 200 µL of DPBS (Dulbecco's phosphate-buffered saline) and fresh DMEM without FCS was added. Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and fetal calf serum (FCS) were purchased from Gibco® (New York, NY, USA). The plant extract (ranging 8-256 µg/mL) were added into each well and incubated 24 h at 37°C, 5% CO_2. Subsequently, 50 µL of Resazurin solution (2.2 µg/mL) was added and the fluorescence was recorded after 4 h (λ excitation 550 nm, λ emission 590 nm) using a TECAN GENios microplate reader (Männedorf, Switzerland). Two independent experiments were performed and the samples were tested by triplicate in each one. Tamoxifen was included as a reference drug for cytotoxicity.

Statistical analysis

Statistical analysis was performed using the statistical software package GraphPad Prism 7 (Windows, V. 7.04, 2017). All results obtained were statistically analyzed and expressed as the arithmetic means ± standard deviation (SD). One-way analysis of variance by ranks of Kruskal-Wallis followed by the *post hoc* Dunn's test was applied to determine the significance of differences between groups. Differences at $p \leq 0.05$ were accepted as significant.

RESULTS

Total phenols and flavonoids contents quantification in *Adelia ricinella* extracts

The total phenol and flavonoid content in the three extracts are presented in Table 1. Extract prepared with ethanol 50% (AR2) reached the highest values of both metabolites, followed by aqueous (AR3) and ethanol 95% (AR1) (p= 0.0273). Similar results were reported by Berenguer et al. (2018).

Table 1. Total phenols and flavonoid concentrations in *Adelia ricinella* extracts.

Extract	Total phenols (mg/g)*	p-value	Total flavonoids (mg/g)**	p-value
AR1	395.67 ± 12.66^c		10.24 ± 0.25^c	
AR2	667.67 ± 24.83^a	0.0273	19.02 ± 0.21^a	0.0273
AR3	495.00 ± 16.37^b		16.83 ± 0.23^b	

All values are expressed as the arithmetic mean ± SD (n = 3). Different letters mean statistical differences between extracts (Kruskal-Wallis coupled to Dunn's test). * Expressed as gallic acid equivalents, ** Expressed as quercetin equivalents. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

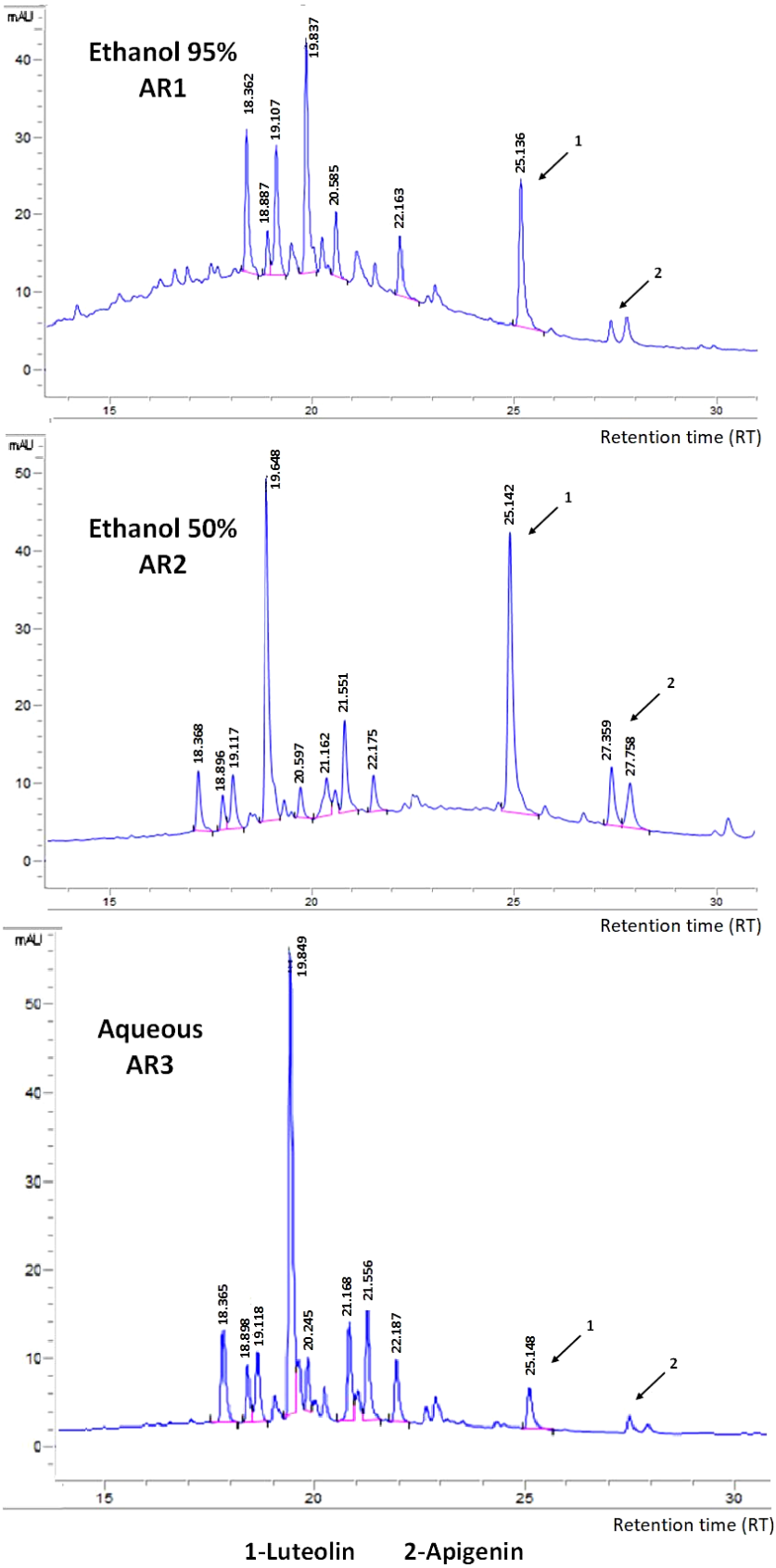

Figure 1. Chromatograms at 366 nm of *Adelia ricinella* extracts obtained by High Performance Liquid Chromatography with Diode Array Detector.

Samples (AR1, AR2 and AR3) were prepared at a concentration of 1 mg/mL in methanol 50%. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

HPLC-DAD quantification of luteolin and apigenin

Chromatograms at 366 nm of *A. ricinella* extracts are shown in Fig. 1. Peaks with retention times from 25.136 to 25.148 min and signed as (1) correspond to luteolin, while peaks with retention times from 27.347 to 27.361 min and signed as (2) belong to apigenin. The concentration of those compounds on each extract was performed using internal standards, and the values estimated for luteolin (µg/mL) were: 22.6, 207.5 and traces; while for apigenin (µg/mL) were: traces, 1.86 and traces in AR1, AR2 and AR3 extracts, respectively.

Cytoprotection and antioxidant assessment of *Adelia ricinella* extracts

Hemolysis assay

Firstly, the potential cytotoxic/destabilizing effect of *A. ricinella* aqueous and ethanol extracts at different concentrations on RBCs membrane was evaluated. Results revealed that the extracts acted as protectors against direct hemolysis when the concentration increases (Fig. 2). Some degree of hemolysis was observed only for the lower concentration in the three extracts (8 µg/mL) with no statistical differences between them. In the same way, AR1 extract (at 16 µg/mL) caused a hemolysis ratio of 19.21%, in contrast with the others two extracts (AR2 and AR3) that reached 2 and 4%. Higher concentrations did not produce hemolysis, suggesting any erythrocyte membrane rupture.

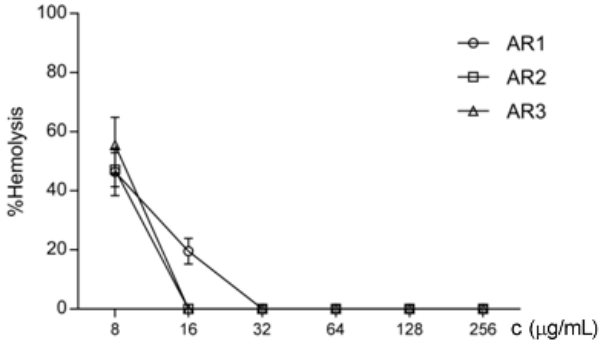

Figure 2. Hemolysis percentage induced by different extract concentrations of *Adelia ricinella* (8–256 µg/mL).

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

Hypotonic erythrocyte membrane stabilizing activity

In general, *A. ricinella* extracts were able to protect the RBCs membrane from disruption caused by a NaCl hypotonic solution. Ethanol extracts at higher concentrations significantly demonstrated a stabilizing activity on red blood cells membrane with values of HMP over 70%. This activity slightly exceeds the protective effect exerted by diclofenac as a positive control (Fig. 3). In the three test samples and in the diclofenac group, a concentration-dependent pattern was observed, allowing the estimation of the HMP_{50}. Results revealed the followed behavior: AR1>AR2>diclofenac>AR3 in their protective capacity with HMP_{50} values of 16.46, 30.05, 37.10 and 47.40 µg/mL, respectively. Significant statistical differences were found between all experimental groups.

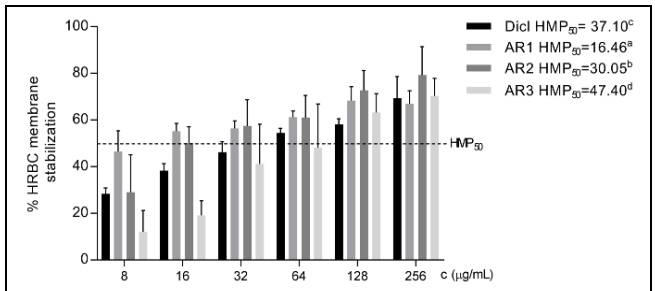

Figure 3. Red blood cell membrane stabilization induced by different extract concentrations of *Adelia ricinella* under a NaCl hypotonic medium.

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Diclofenac (Dicl) was used as a positive control. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

Antioxidant erythrocyte membrane stabilizing activity

The evaluation of the cytoprotective effect of plant extracts on erythrocyte membranes was also explored using H_2O_2 as an oxidant agent. Many studies promote RBC-related models to assess the action of antioxidant substances on membrane protection because they are highly susceptible to oxidative damage. AR2 extract showed a statistically significant protecting effect (>90% at 64 µg/mL) in erythrocyte membranes after H_2O_2 exposure in comparison with AR1 and AR3 extracts, which were less actives (Fig. 4). The effect exerted by AR2 was similar to that of ascorbic acid (control

group) on RBCs cytoprotection. As a result, the statistical analysis reveals significant differences between the experimental groups favoring AR2 (AP_{50} = 17.493 µg/mL) as the most active extract followed by AR1 and AR3 with AP_{50} = 37.937 and 57.720 µg/mL, respectively. None of the extracts was more active than the ascorbic acid control (AP_{50} = 10.358 µg/mL).

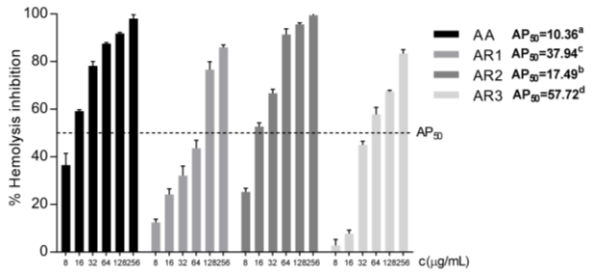

Figure 4. Cytoprotection exerted by different extract concentrations of *Adelia ricinella* on red blood cells treated with hydrogen peroxide as an oxidant agent.

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Ascorbic acid (AA) was used as a positive control. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

Erythrocytes morphological changes

Normally, erythrocytes are biconcave, non-nucleated, and discoid structures containing hemoglobin. The erythrocyte susceptibility to chemicals, osmotic media, and magnetic/electric radiation is well known, expressed in its shape deformation and, subsequently, the loss of functionality. Therefore, monitoring the external morphology can be considered a valuable tool for measuring the damage degree of such harmful agents. At the same time, substances acting as erythrocyte protective agents can prevent such deformations. The erythrocyte membrane stabilizing activity of three *A. ricinella* extracts (AR1-AR3) was explored in the experiments with H_2O_2 as an oxidizing agent. The results were in agreement with the spectrophotometric determinations, indicating that AR2 was the most active extract. Main deformations in both experiments were associated with the presence of spheroechinocytes, echinocytes, and spherocyte forms (Fig. 5).

Cytotoxicity of A. ricinella extracts in Vero cells

Vero cells are a helpful cell system to explore the cytotoxic potential of toxicants, synthetic and natural agents, like phytochemicals (Kwan et al., 2016; Safriana et al., 2018). All the tested *A. ricinella* extracts were no cytotoxic to mammalian Vero cells (Fig. 6). Based on this finding, the estimated IC_{50} values of the three *A. ricinella* extracts were higher 256 µg/mL, classified as non-toxic at the concentrations assessed in this study. It is also noticed that all extracts at lower concentrations, slightly promoted the metabolic activity of Vero cells, as shown in the resazurin dye reduction test.

DISCUSSION

Several factors can strongly damage the cell membrane integrity by the impairment of key biomolecules like lipids and proteins (Spengler et al., 2014). Red blood cells (RBC) possess a very sensitive cell membrane that is susceptible to abnormal changes, leading to important hematological alterations. For this reason, RBC membranes with their mosaic of proteins distributed in lipid bilayers serve as an excellent *in vitro* tool for understanding membrane destabilization/toxicity induced by synthetic or natural products. In this sense, some authors suggest the RBC model as an alternative but a reliable assay to explore the cytoprotective effect of antioxidants and anti-inflammatory natural drugs and/or synthetic analogous on lysosomal organelle, because both membrane components are structurally similar (Bag et al., 2013; Przystupski et al., 2019).

From the structural point of view, it is well recognized that while the outer side of the erythrocyte membrane contains high quantities of phosphatidyl choline and sphingomyelin, the inner side holds the charged phospholipids phosphatidyl ethanolamine and phosphatidylserine. This membrane configuration with an asymmetric distribution of molecules across the lipid bilayer determines not only their curved shape but also the way in which interact with the external medium.

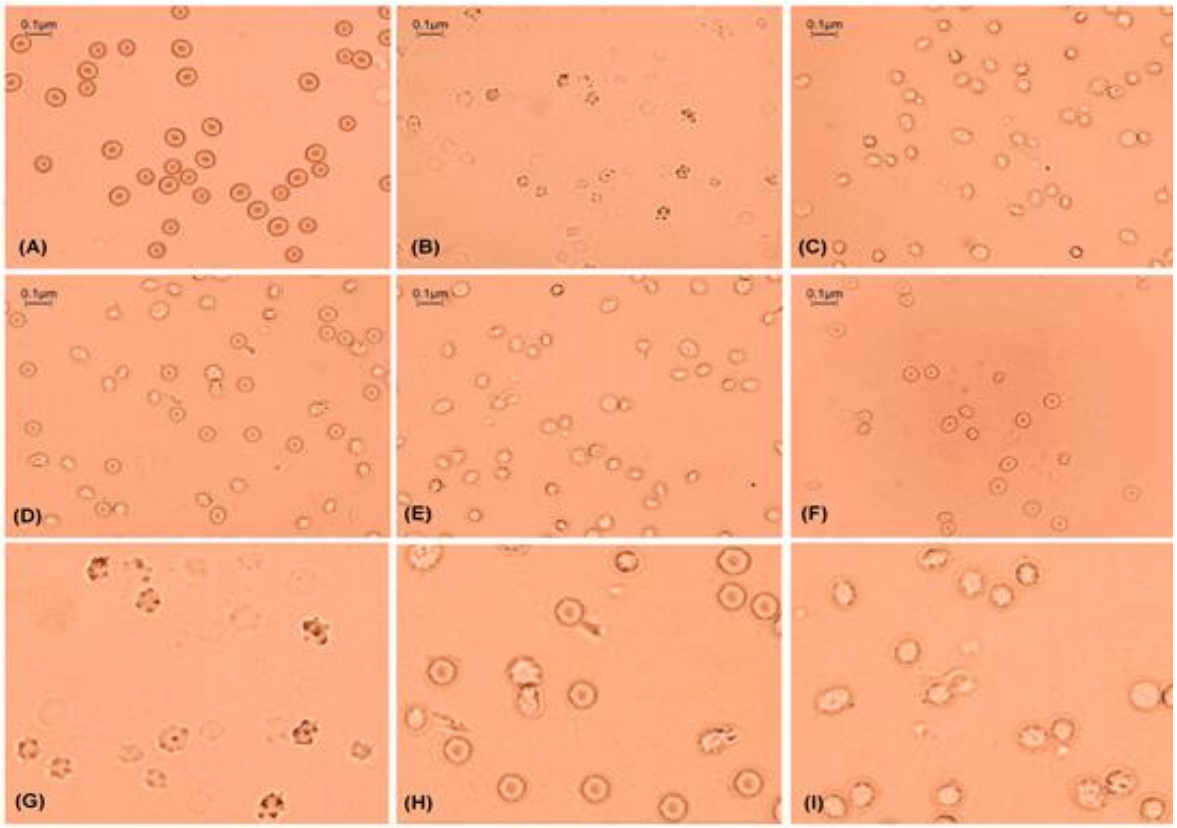

Figure 5. Optical micrographs (400×) showing erythrocyte protection by *A. ricinella* extracts at 32 µg/mL to H_2O_2-induced oxidative damage.

(A) Normal cells, **(B)** Negative control (H_2O_2), **(C)** AR1, **(D)** AR2, **(E)** AR3, **(F)** Positive control (ascorbic acid, 32 µg/mL). Pictures **G**, **H** e **I** are digital magnifications (zoom 3×) to highlight membrane changes: **(G)** Negative control (H_2O_2), **(H)** AR2, **(I)** AR3. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

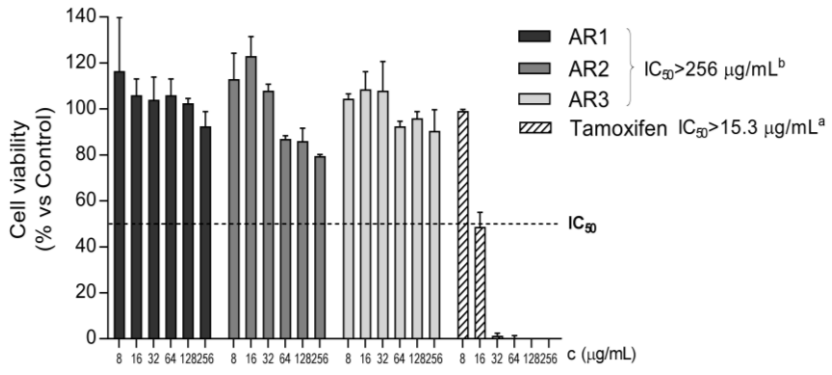

Figure 6. Cell viability of Vero cells incubated with different concentrations of *Adelia ricinella* extracts.

The potential cytotoxic effect of plant extracts on Vero cells was evaluated by the Resazurin dye reduction test. Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

Thus, drugs/extracts interacting with the outer side generate different morphological types (mainly echinocyte type) compared with inner leaflet (Sheetz and Singer, 1974). In this interaction, the diameter, height, and density of the echinocyte-type spicules are proportional to the number of molecules penetrating and interacting with the outer leaflet. On the other hand, it is also accepted that substances that interact with the membrane inner side generates shape changes leading to oval or elliptical erythrocyte forms by "affecting" the cytoskeletal network (Turgeon, 2020).

The use of plant-derived products emerges as an interesting and, in many cases, a safer approach to prevent cellular membrane disruption. Flavonoid-type compounds and other polyphenols are probably the most explored plant metabolites in the last decade due to their wide range of medicinal activities primarily associated with the antioxidant capacity (Ambriz et al., 2016; Vijayraja and Jeyaprakash, 2019). In this context, the results showed in this paper highlights that: i) flavonoid-enriched extracts derived from aerial parts of *A. ricinella* exhibited a cytoprotective action on red blood cell by membrane stabilization when exposed to hypotonic and oxidative environments, ii) they were not cytotoxic to RBC and Vero cells.

The presence of 13 flavonoid derivates from luteolin and apigenin aglicones, being orientin and luteolin the most abundant, was reported in a previous study with the same *A. ricinella* extracts explored in this paper (AR1-AR3) (Berenguer et al., 2021). Based on the signal intensity found in Fig. 1, it can be noticed that for AR1 and AR2 extracts, the peak corresponding to luteolin was the second most abundant, in agreement with such report. According to microphotographs, H_2O_2 oxidative damage must have impaired both erythrocyte layers, because most of the "modified erythrocytes" are echinocyte or spheroechinocyte types (Fig. 5B and 5G). On the other hand, *A. ricinella* extract components are able to protect both sides of the erythrocyte membrane against the damage (Fig. 5C –AR1, 5E and 5I -AR3, and mainly 5D and 5H – AR2). For this, compounds present in *A. ricinella* extracts must be able to penetrate across the membrane, also acting in their inner side.

The transport of flavonoids across the membranes is not yet fully understood. However, some evidence have already accepted, such as: i) the relatively planar structure of flavones and flavonols boost the penetration degree through biological membranes by increasing their affinity towards the lipid membranes (Murota et al., 2002), ii) glycosidic substitutions with more than one carbohydrate monomer unit increases molar volume and decreases hydrophobicity, affecting the flavonoid membrane penetration (Selvaraj et al., 2015). Considering that the 13 flavonoids identified in the extracts of *A. ricinella* are mono- or disaccharides of luteolin and apigenin, we hold the hypothesis that they could interact on both sides of the erythrocyte membrane, exerting their cytoprotective activity in such layers.

However, a prerequisite to exert their effect at that level is that flavonoids and other polyphenols (mainly glycosides) need to be bioavailable to reach the experimental concentrations in blood. The absorption and metabolism of polyphenolic compounds are determined primarily by their physicochemical characteristics. For example, molecular size, basic structure, degree of polymerization or glycosylation, solubility, and conjugation with other phenolics can be considered critical (Di Lorenzo et al., 2021). As mentioned before, the physicochemical characteristics of flavonoids identified in *A. ricinella* extracts derived from luteolin and apigenin (planar structure of flavones and low degree of glycosylation) could favor their absorption and bioavailability.

Until now, *in vivo* investigations of the absorption, bioavailability, and metabolism of these flavonoids are limited (Liu et al., 2019). In agreement with structural features favoring flavones bioaccessibility, Yasuda et al. (2015) reported that after oral administration of *Chrysanthemum morifolium* extract (1.7 g/kg body weight, equivalent to 22.8 and 58.3 μmol/kg of luteolin and luteolin-7-O-glucoside, respectively) to rats, luteolin and its glycosides were detected in the plasma. Their levels were highest at one hour after administration

(0.76 ± 0.27 μM). These compounds were also detected in media on the basolateral side from Caco-2 cells treated with the *C. morifolium* extract. These results suggest that luteolin and luteolin monoglucoside are rapidly absorbed after administration of *C. morifolium* flower extract. The concentrations of these compounds in plasma also showed a secondary increase, meaning that the *C. morifolium* extract intake led to the retention of these compounds in the circulatory system for a long time.

Thus, we can hypothesize that major flavone-type flavonoids presented in *A. ricinella* extracts may also exert the observed beneficial effects *in vivo*. Erythrocytes-based assays are one step further in extrapolating to living models. Nevertheless, the combination of different assays, including some *in vitro* analytical methods for determining bioaccessibility and bioavailability of bioactive compounds (e.g., pre-digestion simulations), cell models "closer" to what could happen *in vivo*, and studies with animal models (Carbonell-Capella et al., 2014) is a major recommendation to bring out the medicinal value of *A. ricinella* extracts.

On the other hand, previous reports on *A. ricinella* extracts revealed a high antioxidant capacity to neutralize radicals such as ABTS and DPPH. The AR2 extract showed a lower IC_{50} in the ABTS assay than the ascorbic acid and higher levels of antioxidant metabolites (total phenols and flavonoids) compared to its analogous AR1 and AR3 extracts, similar that in the present study (Table 1). It is fully accepted that the harmful action of reactive oxygen species on the erythrocyte membrane reduces their cytoskeletal protein content producing high molecular weight proteins aggregates. These changes triggered abnormalities in RBC structure and in consequence to their shape, membrane-related functions, rheological properties, and ultimately leading to diminished erythrocytes survival (Khairy et al., 2008). Hence, *A. ricinella* extracts can protect RBC membrane by scavenging oxidant compounds.

The relationship between the content of phenols/flavonoids and the cytoprotective action of plant extracts has been associated with the antioxidant activity of these metabolites. For example, Sazwi et al. (2013) reported the cytoprotective capacity of *Piper betle*, *Areca catechu*, and *Uncaria gambir* in human gingival fibroblasts after inducing H_2O_2 damage, result associated with a high concentration of polyphenols. Moreover, the safety of *A. ricinella* extracts was confirmed on Vero cells, without impairment in cell viability and the metabolic activity.

CONCLUSIONS

Adelia ricinella flavonoids-enriched extracts, particularly the ethanol AR1 and AR2 extracts, were able to promote the erythrocyte cytoprotection by stabilization of both membrane layers in an oxidative environment. Thus, *A. ricinella* extracts prove their capacity of reducing the hematological alterations and cell toxicity by participating in the prevention of oxidizing damage. The plant therefore could be regarded as a natural source of membrane stabilizers, and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders and diseases.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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Contribution	Berenguer CA	Fong O	Escalona JC	de la Vega J	Arro DJ	Guisado F	Llauradó G	Morris HJ
Concepts or ideas	x	x	x				x	x
Design	x	x	x				x	x
Definition of intellectual content	x		x				x	x
Literature search	x	x	x	x			x	
Experimental studies	x	x		x	x	x		
Data acquisition	x	x		x	x	x		
Data analysis	x		x				x	x
Statistical analysis	x		x				x	
Manuscript preparation	x	x	x				x	x
Manuscript editing	x		x				x	x
Manuscript review	x	x	x	x	x	x	x	x

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