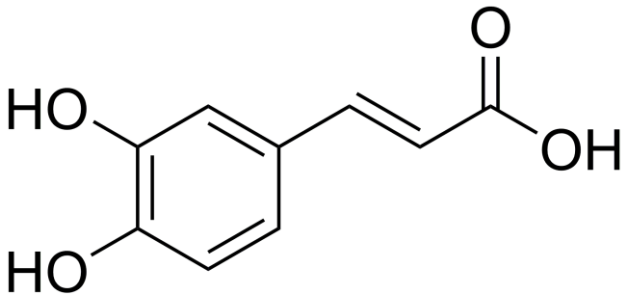

Effects of phenolipids on the human erythrocyte oxidative damage

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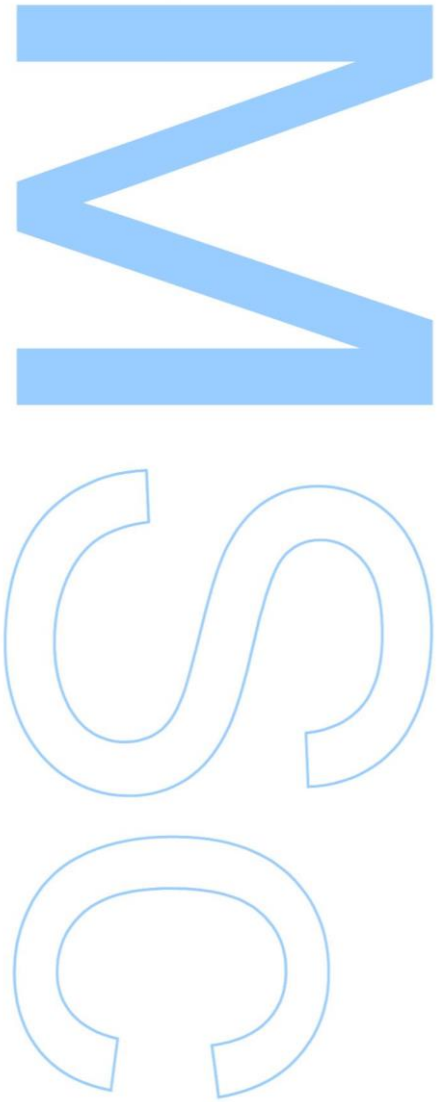

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Resumo

Os eritrócitos possuem funções vitais no organismo humano, de salientar o transporte e a capacidade/defesa antioxidante. Os eritrócitos estão expostos constantemente ao oxigénio, ocorrendo a formação de espécies reativas de oxigénio (ROS), e estão por isso mais suscetíveis de sofrer e/ou acumular lesões oxidativas. Em condições normais, o eritrócito possui sistemas de defesa antioxidante que mantêm um equilíbrio entre a produção e a destruição de ROS. Quando esse equilíbrio deixa de existir, a célula fica sob "stress oxidativo" e acaba por sofrer danos que podem levar à sua hemólise ou à sua remoção precoce da corrente sanguínea pelo sistema fagocitário.

Vários compostos fenólicos têm mostrado uma importante ação de proteção contra o stress oxidativo. Desta família de compostos faz parte o ácido cafeico, e crê-se que a sua estrutura aromática hidroxilada seja a responsável pelas propriedades antioxidantes. Porém, a natureza hidrofílica da molécula acaba por limitar a sua capacidade protetora em meios lipofílicos, como as membranas dos eritrócitos. Um dos métodos usados para melhorar a capacidade antioxidante é a lipofilização, que consiste em introduzir grupos lipofílicos na estrutura do fenol. A molécula passa a assumir a designação de fenolípido.

Neste trabalho, o ácido cafeico foi usado como composto parental. Efetuou-se a esterificação deste com álcoois de cadeia linear com diferentes grupos alquilo ($-CH_3$, $-CH_2CH_3$, $-CH_2(CH_2)_2CH_3$, $-CH_2(CH_2)_4CH_3$, $-CH_2(CH_2)_6CH_3$, $-CH_2(CH_2)_{14}CH_3$), obtendo-se os respetivos cafeatos. Assim, pretende-se determinar qual a molécula que apresentará melhor atividade antioxidante, uma vez que o tamanho da cadeia alquilo adicionada é um fator determinante para a sua localização nos sistemas celulares e, por conseguinte, para a sua bioatividade. Deste modo, o objetivo deste trabalho consiste em avaliar a capacidade protetora do ácido cafeico e dos diferentes cafeatos, frente ao stress oxidativo induzido nos eritrócitos usando o 2,2'-azobis(2-amidinopropano) dihidrocloreto (AAPH), um gerador de radicais livres, e que pode desencadear a hemólise.

Verificou-se que todos os compostos, a todas as concentrações testadas, protegeram os eritrócitos da hemólise oxidativa induzida pelo AAPH após 4 h de incubação, com exceção do ácido cafeico. No caso dos cafeatos mais hidrofílicos (C1-C4), esta proteção foi dependente da dose mas no caso dos cafeatos mais lipofílicos, a atividade mostrou-se praticamente independente da concentração. Assim, a 2,5 µM e a 5 µM, a atividade protetora dos compostos aumentou com o aumento do tamanho da cadeia alquilo mas a 50 µM, os cafeatos C1-C4 mostraram melhor atividade que os cafeatos C6-C16. Os resultados obtidos nos estudos de hemólise mostraram-se em acordo com os resultados observados na análise da densidade celular efetuada por microscópio ótico.

A análise de proteínas da membrana não mostrou diferenças significativas na presença de AAPH e dos compostos, mas verificou-se uma pequena interação entre os compostos mais lipofílicos e a espectrina. Também se observou um aumento da percentagem de hemoglobina ligada à membrana na presença de AAPH e dos compostos. No entanto, a análise efetuada à hemoglobina mostrou que os cafeatos conseguiam inibir a oxidação da hemoglobina, pelo que o aumento da hemoglobina ligada à membrana não se deve exclusivamente à oxidação desta.

Após adição do AAPH aos eritrócitos, foi observado um aumento significativo nos níveis da peroxidação lipídica mas este aumento, apesar de não ser significativo, foi ligeiramente atenuado pela presença de todos os cafeatos. Por outro lado, a atividade da catalase no citosol também diminuiu significativamente na presença de AAPH e, novamente, foi observada uma proteção não-significativa da atividade da catalase na presença dos cafeatos. Como o número de ensaios foi muito limitado, estes resultados que mostram alguma proteção dos eritrócitos pelos cafeatos precisam de ser confirmados no futuro.

Palavras-chave: Eritrócitos; Stress Oxidativo; Espécies Reativas de Oxigénio; Membranas dos eritrócitos; Compostos Fenólicos; Ácido Cafeico; Cafeatos; Lipofilização.

Abstract

Erythrocytes have vital functions in the human organism, emphasizing the transport and the antioxidant capacity/defense. Erythrocytes are constantly exposed to oxygen, occurring the formation of reactive oxygen species (ROS), and therefore they are more susceptible to suffer and/or accumulate oxidative damages. Under normal conditions, erythrocytes have antioxidant defense systems which maintain a balance between the production and destruction of ROS. When this balance is disrupted, cells undergo "oxidative stress" and suffer damages that may lead to their hemolysis or their early removal from the bloodstream by the phagocytic system.

Several phenolic compounds have shown an important protective action against oxidative stress. Caffeic acid belongs to this family of compounds, and it is believed that its hydroxylated aromatic structure is the responsible for the antioxidant properties. However, the hydrophilic nature of the molecule limits its protective ability on lipophilic mediums, such as erythrocyte membranes. One of the methods used to improve the antioxidant capacity is lipophilization, which consists of introducing lipophilic groups into the phenol structure. The molecule assumes the designation of phenolipid.

In this work, caffeic acid was used as parental compound. It was esterified with linear chain alcohols with different alkyl groups (-CH_3, -CH_2CH_3, -$CH_2(CH_2)_2CH_3$, -$CH_2(CH_2)_4CH_3$, -$CH_2(CH_2)_6CH_3$, -$CH_2(CH_2)_{14}CH_3$) to obtain the respective caffeates. So, it is pretended to determine which molecule will present the best antioxidant activity, since the size of the alkyl chain added is a determining factor for its location in cellular systems and, therefore, for its bioactivity. Thus, the aim of this work is to evaluate the protective capacity of caffeic acid and the different caffeates against the oxidative stress in erythrocytes induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical generator, and that can trigger hemolysis.

All compounds at all tested concentrations were found to protect erythrocytes from AAPH-induced oxidative hemolysis after 4 h incubation, except for caffeic acid. In the case of the more hydrophilic (C1-C4) caffeates, this protection was dose-dependent but in the case of more lipophilic caffeates, the activity was almost independent of the concentration. Thus, at 2.5 µM and 5 µM, the protective activity of compounds raised with the increase of the alkyl chain size but at 50 µM, the C1-C4 caffeates showed better activity than the C6-C16 caffeates. The results obtained in the hemolysis studies were in agreement with the results observed in the analysis of cellular density performed by optical microscope.

Membrane protein analysis did not show significant differences in the presence of AAPH and the compounds, but there was a small interaction between the more lipophilic

compounds and the spectrin. An increase in the percentage of membrane-bound hemoglobin was also observed in the presence of AAPH and the compounds. However, the hemoglobin analysis showed that the caffeates were able to inhibit the oxidation of hemoglobin, so the increase in hemoglobin bound to the membrane is not exclusively due to its oxidation.

After addition of AAPH to erythrocytes, a significant increase in lipid peroxidation values were observed but this increase, besides not being significant, was slightly attenuated by the presence of all caffeates. On the other hand, the catalase activity in the cytosol also decreased significantly in the presence of AAPH and again, a non-significant protection of the catalase activity was shown in the presence of caffeates. Because the number of experiments was very limited, these results showing some protection given by caffeates to erythrocytes need to be confirmed in the future.

Keywords: Erythrocytes; Oxidative stress; Reactive Oxygen Species; Erythrocyte Membranes; Phenolic Compounds; Caffeic Acid; Caffeates; Lipophilization.

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Abbreviations

2,3-DPG	2,3-Diphosphoglycerate
AAPH	2,2′-Azobis(2-amidinopropane) dihydrochloride
ADP	Adenosine Diphosphate
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
CA	Caffeic Acid
CAT	Catalase
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
G3PD	Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT-1	Glucose Transporter
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione Dissulfide
Hb	Hemoglobin
Ht	Hematocrit
LPO	Lipid Peroxidation
MBH	Membrane-Bound Hemoglobin
MDA	Malonaldehyde
MetHb	Methemoglobin
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance

oxyHb	Oxygenated Hemoglobin
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
Prx2	Peroxiredoxin-2
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAR	Structure-Activity Relationship
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TEMED	*N,N,N',N'*-tetramethylenediamine
Trx	Thioredoxin

I. Introduction

1. Red Blood Cell

1.1. Composition of Red Blood Cells

The human red blood cell (RBC), also called erythrocyte, is a non-nucleated cell of the circulatory system, coated externally with a unique layer of plasma membrane and does not contain inner membranes and organelles [1, 2]. The cytosol is mostly occupied by hemoglobin (Hb), about 95 to 97% of the cytosolic proteins [3], and few intracellular proteins [1]. Besides Hb, the cytosol also contains potassium ions (K^+) and sodium ions (Na^+) (with intracellular $[K^+]>[Na^+]$), glucose, intermediate products of glucose metabolism and enzymes [2].

Normal human RBCs display a biconcave disc shape, with approximately 7.5 to 8.7 µm in diameter, 1.7 to 2.2 µm in thickness [4], 135 μm^2 in surface area and 90 fL in volume. This shape gives a high surface area-to-volume (S/V) ratio to cell, allowing reversible and large deformations of the cell [4, 5].

Human RBCs are formed from pluripotent stem cells of the bone marrow. These cells can proliferate and differentiate into mature RBCs, white cells and platelets. Mature RBC results from reticulocytes which had retained some organelles, such as mitochondria and ribosomes, and ribosomal RNA for hemoglobin synthesis (about 20-30% of the total Hb) and proteins but had ejected their nucleus before being released into the circulation. In circulation, reticulocytes loss mitochondria, endoplasmic reticulum and ribosomes and are transformed into mature RBCs [3]. The lack of intracellular organelles limits the metabolic capacity of the RBC and thereby the energy for metabolic processes is produced almost exclusively by anaerobic glycolysis. A mature RBC can exist in blood circulation for an average of 120 days [2].

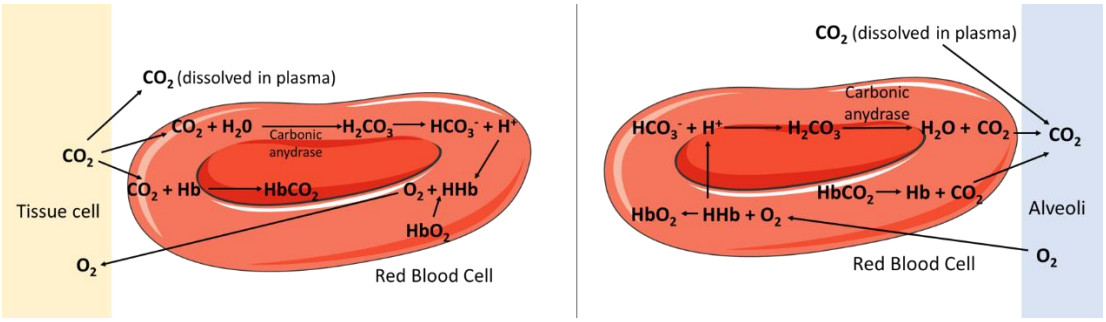

Figure 1. Exchange of oxygen and carbon dioxide in RBC. Carbon dioxide (CO_2) is produced in tissues and can diffuse to plasma, combine with hemoglobin (Hb) to produce carbamino-hemoglobin ($HbCO_2$) or combine with water to produce carbonic acid (H_2CO_3). Carbonic acid dissociates into bicarbonate (HCO_3^-) and hydrogen ion (H^+). Hydrogen conjugates with Hb and, together with O_2, it is formed oxygenated hemoglobin (HbO_2).

RBCs has as primary physiological function the transport of oxygen (O_2) from lungs to tissues by binding to Hb [6], whereas carbon dioxide (CO_2) is transported from tissues back to lungs and then exhaled [1] (**Figure 1**).

In order to perform an adequate gas transport, RBCs must constantly move through narrow capillaries of smaller caliber under a wide range of flow conditions. As such, these cells must be able to deform repeatedly [1, 6, 7] and withstand to the different incident forces from blood vessels and visceral wall, without losing its structural integrity and avoiding fragmentation [1, 7].

The deformability of RBCs is very important to their function and have been identified 3 primary constitutive features responsible for deformability, which are the cell geometry, the cytoplasmic viscosity regulated by intracellular Hb concentration, and the rheologic properties of the cell membrane [5, 7].

Other factors can also affect the shape and deformability of RBCs, such as osmotic concentrations in the medium (RBC assumes a spherocyte form in a hypotonic medium, and an echinocyte form in a hypertonic medium); intracellular calcium ion (Ca^{2+}) concentration, which rise results in an increased cellular rigidity and decreased deformability; nitric oxide (NO); temperature; change of membrane proteins and/or lipids; adenosine triphosphate (ATP) levels; and cell aging [5].

1.2. RBC Membrane

The RBC membrane is composed by 39.5% of proteins, 35.1% of lipids, 19.5% (w/w) of water and 5.8% of carbohydrates [8].

The lipid bilayer consists of phospholipids and cholesterol, and exhibits a characteristic asymmetric distribution of phospholipids with functional implications to the cell. Phospholipids are asymmetrically disposed between the two lipid bilayers: phosphatidylcholine and sphingomyelin predominantly in the outer monolayer, and phosphatidylethanolamine and phosphatidylserine in the inner monolayer. For example, the exposure of phosphatidylserine in the outer monolayer leads to the phagocytosis of the RBC [7].

The phospholipid bilayer is anchored to a peripheral elastic network of skeletal proteins through vertical interactions, between cytoplasmic domains of integral membrane proteins with peripheral proteins or, additionally, by direct interaction between membrane lipids and the peripheral protein network [4, 7]. Also, can exist horizontal protein interactions, that can be involved in the formation of the peripheral protein network [4] or multiprotein complexes in the membrane. Any disruption of the interactions results in misshapen RBCs (for example, spherocytes, elliptocytes or ovalocytes) [9] (**Figure 2**).

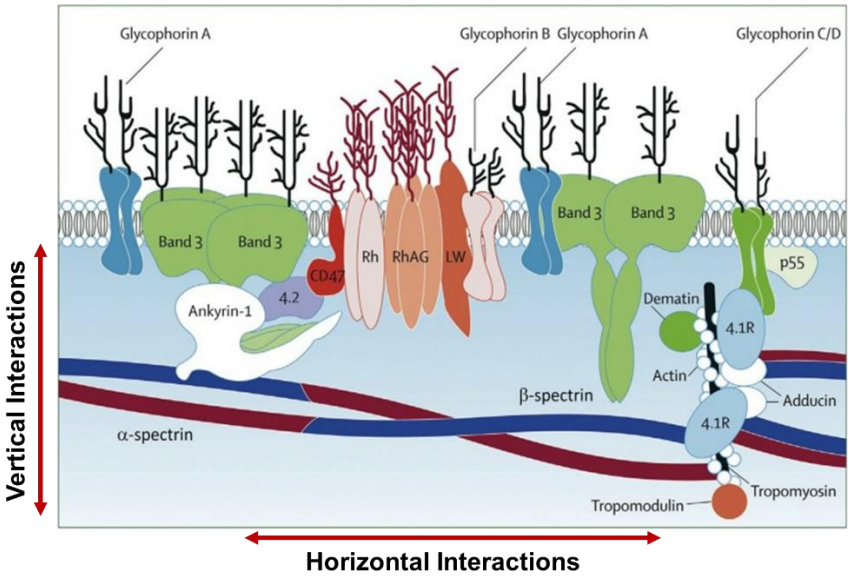

Figure 2. A schematic representation of RBC membrane components and their interactions. Abbreviations: 4.1R, protein 4.1R; 4.2, protein 4.2; p55, 55 kDa palmitoylated protein; Rh, rhesus polypeptide; RhAG, Rh-associated glycoprotein; LW, LW glycoprotein recently named Intercellular Adhesion Molecule-4. Adapted from Christensen et al. [10].

The lipid bilayer contains an amount of integral membrane proteins with diverse functional heterogeneity. A large fraction of them carries blood group antigens, whereas the others may function as transporters or pumps, signaling receptors, adhesive proteins, and other still undefined activities. These proteins are organized into multiprotein complexes, one ankyrin-based and other protein 4.1R-based, which are very important to structural integrity of the cell membrane [7].

Band 3, or anion exchanger-1, is the main integral membrane protein of RBCs and anchors the membrane to the skeletal protein network through specific attachments to ankyrin, protein 4.1R and protein 4.2 [1]. This binding is responsible for maintaining the cell shape [3]. Moreover, it also has binds to glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (G3PD), hemoglobin and hemichromes [1, 11].

The skeletal protein network is formed by most of peripheral membrane proteins and represents the membrane skeleton. The composition of the membrane skeleton consists mainly of spectrin, actin, ankyrin and protein 4.1R [11]. Spectrin is responsible for maintaining the RBC shape, regulates the horizontal motion of integral membrane proteins and supports the lipid bilayer [1].

The protein network underlying the lipid bilayer is vital for cell function and maintenance of the cell shape, and regulates the protein organization and the mechanical properties of the RBC membrane. It allows the reversible deformation of the membrane, maintaining a suitable membrane surface area and without loss its functionality and structural integrity under high fluid stresses in blood circulation [7, 9, 12].

1.3. Metabolic Pathways in RBCs

RBCs must perform a set of metabolic functions for its own survival, including generation of metabolic energy, reducing agents and 2-3-diphosphoglycerate (2,3-DPG), and further must maintain the ionic and concentration gradients across the membrane [13].

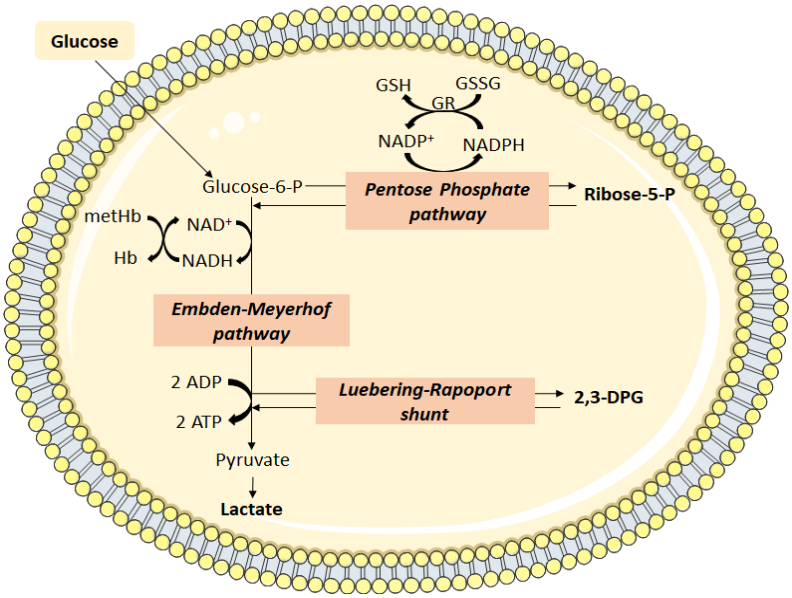

Figure 3. RBC metabolism main pathways: Embden-Meyerhof pathway, Luebering-Rapoport shunt, and Pentose Phosphate pathway. Abbreviations: Glucose-6-P, glucose-6-phosphate; Hb, hemoglobin; metHb, methemoglobin; NAD, NADH, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NADP, NADPH, nicotinamide adenine dinucleotide phosphate; GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; Ribose-5-P, ribose-5-phosphate; 2,3-DPG, 2,3-diphosphoglycerate. Adapted from Turgeon [2].

Since RBCs lack nucleus and intracellular organelles, such as mitochondria, they have a limited metabolic machinery and are unable to synthesize proteins and lipids, and to perform oxidative phosphorylation to obtain energy [14, 15]. Instead, RBCs take up plasma glucose via the insulin-independent glucose transporter (GLUT-1), whereas insulin is responsible for the regulation of glycolysis in RBCs and cell functions [13]. RBCs metabolize glucose by anaerobic glycolysis, the *Embden-Meyerhof pathway* (**Figure 3**), for generate and storage energy (ATP) [16]. Energy is essential to vital functions in RBCs, including maintains glycolysis, the electrolyte balance through the membrane, the integrity and plasticity of cell membrane, modulates constituents in an active form (enzymes, hemoglobin, etc), and protects against oxidative stress and others [15, 16].

The Embden-Meyerhof pathway represents about 90% of the glucose metabolism under physiologic conditions and is the unique energy source of RBCs. Glucose is catabolized anaerobically and it is produced pyruvate/lactate, ATP and NADH [14]. The final

energy gain are two moles of ATP for each mole of glucose metabolized. In addition, it is generated a reductive potential by the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH catalyzed by G3PD. NADH can be used as cofactor of NADH-cytochrome b5 reductase (also known as methemoglobin reductase) to reduce oxidized hemoglobin (methemoglobin) to hemoglobin, and the final product of glycolysis will be pyruvate. Otherwise, NADH is used by lactate dehydrogenase (LDH) to reduce pyruvate to lactate, regenerating NAD^+ [16].

The Embden-Meyerhof pathway could present a bypass, the *Luebering-Rapoport shunt* (**Figure 3**), unique to RBCs [14]. In this shunt is produced 2,3-DPG, that prevents excessive ATP production [17], and together with ATP regulates the affinity of Hb to oxygen [14]. The increase of 2,3-DPG levels stabilizes the deoxygenated state of Hb and facilitates the release of oxygen into tissues due to decreasing Hb oxygen affinity [17].

Under physiological conditions, the remaining 10% of the glucose metabolism occurs via aerobic glycolysis, the *pentose phosphate pathway* [14] (**Figure 3**). It synthesizes ribose as sugar components of nucleotides and provides a redox potential in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), necessary to glutathione system protect the cell against oxidative stress. This pathway controls and maintains the redox equilibrium [14, 15].

Thus, the RBC metabolism needs ATP, NADH and NADPH to perform its metabolic functions [15].

1.4. Oxidative Stress in RBCs

RBC is very susceptible for reactive oxygen species (ROS) formation due to its physiological function as oxygen carrier and to the high content in Hb and heme iron [18]. ROS are highly reactive species with one or more unpaired electrons, formed from molecular oxygen (O_2), which acts as an electron acceptor. These molecules can be non-radicals, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), or radicals superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^\bullet$), peroxyl radical ($ROO^\bullet$) and nitric oxide ($NO^\bullet$) [19].

Under physiological conditions, RBCs are equipped with antioxidant systems, including non-enzymatic (reduced glutathione, ascorbic acid, α-tocopherol, etc) and enzymatic antioxidants (catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxin-2, etc) to protect against ROS damage [8]. The production of ROS, outside or within the RBC, is balanced with the antioxidant system. The disruption of this balance by depletion of antioxidants and/or excess accumulation of ROS leads to an “oxidative stress” condition, with deleterious effects on cell at functional and structural level, which may ultimately result in hemolysis [8, 20, 21].

During their life-span, RBCs are continuously exposed to oxidative damages and accumulate injuries that impair its function and, ultimately, RBCs are removed from blood circulation by the reticuloendothelial system, which comprises the mononuclear phagocytic cells in the spleen, liver and lymph nodes [22].

Oxidative stress has been implicated in the ageing process and in the development and progression of several human pathologies, including neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, and other chronic and degenerative diseases, such as cancer, atherosclerosis, diabetes mellitus, rheumatoid arthritis and cardiovascular diseases [19, 23]. It also has been associated with different hematological pathologies, including sickle cell anemia, β-thalassemia and glucose-6-phosphate dehydrogenase deficiency [24].

Besides oxidative stress, RBCs are also continuously exposed to a series of redox reactions involving reactive nitrogen species (RNS), which include nitric oxide (NO) and nitrite (NO_2^-), yielding to nitrosative stress that, similar to oxidative stress, also gives a diverse array of products that can damage cellular constituents [3].

Hemoglobin is the major factor that determinates oxidative stress in RBCs [3]. Physiologically, Hb reversible binds oxygen when its heme irons are in the reduced or ferrous state (Fe^{2+}), forming oxygenated hemoglobin (oxyHb) (**Figure 4**). Although oxyHb is considered a relatively stable molecule, a small amount undergoes autoxidation at a rate of ~0.5–3% daily, producing methemoglobin (metHb), unable to bind oxygen, and reactive oxygen species ($O_2^{\bullet-}$) [25-27]. $O_2^{\bullet-}$ is rapidly dismutated to H_2O_2, spontaneously or by superoxide dismutase, which can promote the oxidation of Hb [27], whereas metHb can be reduced back to functional Hb [24, 26]. Under oxidative stress, metHb formation increases [18] and further oxidation forms hemichromes (denaturated Hb precipitates) [24].

ROS generated inside RBCs are almost entirely derived from Hb autoxidation [27, 28]. But, RBCs can be also exposed to oxidative injury by extracellular oxidants that can be taken up by the cell [3] or even ROS released from neutrophils, macrophages and endothelial cells into the plasma [22].

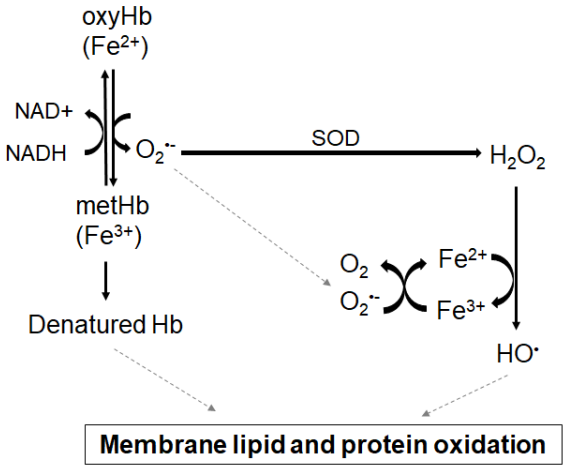

Figure 4. Hemoglobin and pathways of oxidative stress in RBC membrane. Under physiological circumstances, oxygenated hemoglobin undergoes oxidation, generating methemoglobin and reactive oxygen species (ROS). ROS can be neutralized into less reactive molecules or, in case of intense oxidative stress, can accumulate and react with other molecules. Abbreviations: Hb, hemoglobin; oxyHb, oxygenated hemoglobin; metHb, methemoglobin; NADH, nicotinamide adenine dinucleotide; SOD, superoxide dismutase. Adapted from Yoshida and Shevkoplyas [6].

H_2O_2 is not a reactive molecule but it can cross RBC membranes and be converted into more harmful products [20]. In the presence of transition metals ions (Fe^{2+} or Cu^{+}), H_2O_2 is converted into the highly reactive $HO^{\bullet}$ in the Fenton reaction [19]. $HO^{\bullet}$ cannot be neutralized enzymatically and can, therefore, induce a higher oxidative damage in RBCs [27] (**Figure 4**).

1.4.1. Membrane damages

Oxidized Hb and/or denaturated products of Hb have a higher affinity for the RBC membrane. These forms and exogenous reactive species or formed by the redox reactions can damage RBC membrane lipids, proteins, and the cytoskeleton, essential elements to maintain the cell shape and deformability. Since RBCs are anucleate and have no intracellular organelles, the cells cannot replace damaged proteins and have no ability to *de novo* synthesize lipids [3].

RBC membranes are rich in polyunsaturated fatty acids (PUFAs) with reactive methylene groups, which make them susceptible to oxidation [29]. Lipid peroxidation (LPO) is a free radical chain process. In the first step, a hydrogen atom is abstracted from a methylene group in the lipid by a reactive oxygen specie, forming a fatty acid radical; in presence of sufficient oxygen, it reacts with the fatty acid radical and forms $ROO^{\bullet}$; these

radicals (ROO•) abstract another hydrogen atom from another fatty acid molecule, and become a lipid hydroperoxide that can further decompose to an aldehyde or to form other products, whereas the other fatty acid molecule becomes a radical [30]. LPO is a cycle that ends with the reaction of two radicals, resulting in cross-linked lipids [6]. One of the products of LPO is malonaldehyde (MDA) and its levels can be measured [31] and used as an index of LPO, an indicator of oxidative stress [32]. MDA is a highly reactive molecule that cross-links phospholipids and proteins in the RBC membrane. This alters the structure and function of the membrane and eventually induces RBC death (hemolysis) [33]. The increase of lipid peroxidation has been implicated in pathologies associated with oxidative stress (vascular inflammation, diabetes, neurodegenerative diseases and others) [34].

Additionally, the loss of membrane lipid organization by oxidative processes results in the exposure of phosphatidylserine in the outer membrane. Phosphatidylserine-exposed RBCs are recognized by macrophages and removed from blood circulation [27].

An adequate protein organization is essential for the shape, deformability and stability of the RBC membrane [25].

Under oxidative stress, Hb binds to the cytoplasmic domain of band 3 protein in the RBC membrane, induces the band 3 cluster formation and the disruption of ankyrin binding, leading to dissociation of band 3 from membrane skeleton [27]. It is also induced the binding of natural anti-band 3 antibodies to the clusters and the complement activation on the RBC surface, triggering the removal of senescent or damaged RBCs from circulation by macrophages of the reticuloendothelial system [20, 27, 35], as reported in hemolytic disorders, such as sickle cell disease [36] and β-thalassemia [27, 37], and in the clearance of senescent RBCs [27].

The increased affinity of oxidized/denaturated Hb to the RBC membrane [3] allows the quantification of membrane-bound hemoglobin (MBH) as oxidative stress marker [24].

ROS can also attack other proteins in RBCs through oxidation of specific amino acid, formation of cross-links between proteins, oxidation of the protein backbone [8], degradation and fragmentation [8, 30]. The amino acid oxidation is considered tremendously harmful because if it occurs at the enzyme active site, it can result in enzyme inactivation. Changes in RBC proteins have been reported in aging [21] and some pathologic conditions, such as diabetes and neurodegenerative diseases [8].

The skeletal protein network can be also affected under oxidative stress, impairing their interactions or with proteins that link the membrane and the cytoskeleton [27].

Changes in RBC membrane impair membrane stability and deformability, alter the shape and increase senescence signaling, which consequently reduces the RBC functionality and viability [25].

1.5. RBCs Antioxidant Defenses

In order to protect against the deleterious effects of oxidative stress, RBCs have a diversity of antioxidant endogenous defenses, ranging from non-enzymatic (e.g. glutathione, ascorbic acid and α-tocopherol) to enzymatic antioxidants (e.g. superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin 2).

1.5.1. Non-enzymatic antioxidant defenses

Endogenous non-enzymatic antioxidants can be grouped according to their phase solubility: lipophilic (e.g. α-tocopherol, carotenoids, ubiquinon) or hydrophilic (e.g. glutathione, ascorbic acid, ceruloplasmin, transferrin, haptoglobin) [33].

Glutathione is the most important non-enzymatic antioxidant responsible for regulating the intracellular redox homeostasis in all cells. It contains an active thiol that can undergo reversible oxidation during redox reactions. Thus, glutathione can exist in a reduced (GSH) or oxidized (GSSG) form [33].

RBCs can take up α-tocopherol (vitamin E) and ascorbic acid (vitamin C) to protect membranes against ROS-induced lipid peroxidation [26, 28], while ascorbic acid also participates in the reducing of metHb [28].

1.5.2. Enzymatic antioxidant defenses

The main endogenous enzymatic antioxidants directly scavenge superoxide radicals and H_2O_2 and convert them into less reactive species (**Figure 5**). These enzymatic antioxidants have been reported with altered activity under oxidative stress circumstances, which allows to use them as reliable markers of oxidative stress [8].

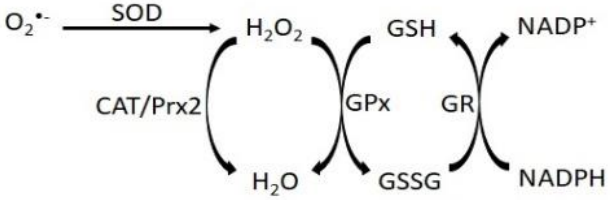

Figure 5. Enzymatic antioxidant defenses. The dismutation of $O_2^{\bullet-}$ by SOD yields H_2O_2, which is converted into H_2O by CAT, Prx2 or GPx. GPx requires GSH and the formed GSSG is reduced back to GSH by GR, using NADPH. Abbreviations: SOD, superoxide dismutase; CAT, catalase; Prx2, peroxiredoxin 2; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; NADPH, nicotinamide adenine dinucleotide phosphate. Adapted from Wojcik et al. [19].

Catalase (CAT) is an important enzyme in protecting the cells from oxidative stress and can be found in all aerobic organisms [21]. The human CAT can be found in every organ but its activity is higher in RBCs, liver and kidney [38]. It is a tetrameric enzyme with four heme-porphyrin groups [39] and neutralizes H_2O_2 to water and O_2 without consuming endogenous reducing equivalents. CAT has a dominant "catalytic" activity (decomposition of H_2O_2) but in the presence of small substrates (methanol, hydroperoxides, etc), the enzyme decomposes them by a peroxidatic mode. In RBCs, the oxygen transport generates high amounts of H_2O_2 and CAT is able to neutralize more than 50% of H_2O_2 [38]. The enzyme is very important to detoxify the H_2O_2 in cells, and a decrease in its activity may promote the accumulation of H_2O_2 and the damage of several tissues [39].

$$2\,H_2O_2 \xrightarrow{\boldsymbol{CAT}} 2\,H_2O + O_2$$

Superoxide dismutase (SOD) is a family of enzymes (CuSOD, ZnSOD, MnSOD, and extracellular SOD) responsible for protecting RBCs from $O_2^{\bullet-}$ [33] (**Figure 5**). The enzyme readily catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 [8, 33]. Since RBCs lack mitochondria, RBCs depend in more extent of the activity of cytosolic Cu,Zn–SOD to scavenge free radicals and preventing metHb formation [33].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\boldsymbol{SOD}} H_2O_2 + O_2$$

Glutathione peroxidase (GPx) is a selenoprotein that detoxifies H_2O_2 from the RBC by reducing H_2O_2 and oxidizing GSH, yielding water, oxygen and GSSG [8, 24] (**Figure 5**). GSSG is reduced back to GSH by glutathione reductase (GR) in the presence of NADPH [24]. GPx also catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols [40].

$$2GSH + H_2O_2 \xrightarrow{\boldsymbol{GPx}} 2H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow{\boldsymbol{GR}} 2GSH + NADP^+$$

Peroxiredoxin 2 (Prx-2) is a cytosolic protein highly abundant in RBCs [18]. Prx-2 scavenges H_2O_2 (**Figure 5**) in a thiol-dependent manner and regulated by the thioredoxin (Trx)/Trx reductase/NADPH reducing system [18, 24]. Prx-2 was described as being important in maintaining low levels of H_2O_2 mainly derived from Hb autoxidation [18, 41].

Overall, Prx-2 and GPx reduce low concentrations of H_2O_2 (usually endogenous from Hb autoxidation), hydroperoxides and peroxynitrites. Whereas CAT plays a major role in protecting RBCs against exogenous and/or high concentrations of H_2O_2 [24].

2. Phenolic Compounds

2.1. Chemistry and Biosynthesis

Phenolic compounds are nowadays attracting a growing interest due to their antioxidant properties against oxidative damage and potential health benefits [42]. They are secondary metabolites widely distributed in plant kingdom, produced by plants to defend themselves from other organisms [43] and climatic conditions, and are present abundantly in human diet, in vegetables, fruits, and beverages such as tea, coffee and wine. Phenolic compounds have diverse physiological properties, including antioxidant, anti-allergic, anti-inflammatory, antimicrobial, cardioprotective and vasodilatory effects [44]. Indeed, the high consumption of food rich in phenolic compounds has been reported to have a favorable impact in human health, preventing and lowering the development and risks of many chronic diseases, such as diabetes, cancer, cardiovascular disease and degenerative diseases, many of them related to oxidative stress [43, 45].

Phenolic compounds comprise a class of chemical compounds having at least one hydroxyl group (-OH) covalently bonded to at least one aromatic ring [46], and present a wide range of molecules ranging from simple molecules to highly polymerized compounds [44].

In plants, phenolic compounds are biosynthesized by the pentose phosphate and shikimate pathways through phenylpropanoid metabolization [47]. The shikimate pathway uses phosphoenolpyruvate and erythrose 4-phosphate from glycolysis and pentose phosphate pathway, respectively, to give rise the three aromatic amino acid (phenylalanine, tyrosine and tryptophan) [47, 48]. From this point, the phenylpropanoid metabolism generates the different phenolic compounds, such as phenolic acids. Highlighting phenolic acids synthesis, it initiates with the deamination of phenylalanine and/or tyrosine, giving cinnamic and/or *p*-coumaric acids, respectively. These intermediates are then modified mainly by hydroxylation and methylation to originate different phenolic acids [49].

According to the nature and complexity of their carbon skeleton; to the degree of skeletal modification; and to the conjugation of the phenolic group(s) with other molecules or with other secondary metabolites [50], phenolic compounds can be subdivided into various classes. The major representatives are phenolic acids (e.g. caffeic acid), flavonoids (e.g. quercetin, catechin) and tannins [44, 46].

The phenolic acid family can be divided into two main groups, 1) benzoic acids derivatives (e.g. gallic acid, ellagic acid) derived from a general structure of seven carbon atoms (C6-C1), and 2) cinnamic acids derivatives (e.g. caffeic, coumaric, ferulic, chlorogenic acid) derived from a general structure of nine carbon atoms (C6-C3) directly

from cinnamic acid (**Figure 6**). These compounds exist as hydroxybenzoic and hydroxycinnamic acids, respectively, and can be found in their free or conjugated form in plants [51].

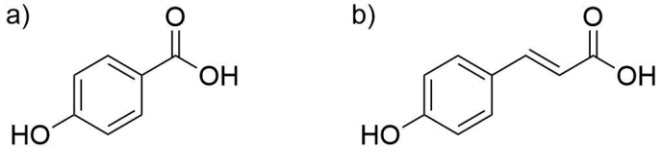

Figure 6. Chemical structures of phenolic acids: a) hydroxybenzoic acid and b) hydroxycinnamic acid.

The free radical scavenging activity of these molecules is normally increased by the presence of a second hydroxyl group in *ortho*- or *para*-position relatively to the first hydroxyl group.

Coffee is a beverage highly consumed in the world, and its high content in hydroxycinnamic acids has been related to their various biological and pharmacological activities, which include antioxidative activity [52].

2.2. Structure – Activity Relationship (SAR)

Phenolic compounds have shown remarkable antioxidant properties, mainly due to their free-radical scavenging ability, by breaking the reaction chain triggered by free radicals; donating electron or hydrogen atom; chelating transition metals, such as Cu and Fe, that catalyze oxidative reactions; inhibiting enzymes involved in oxidative stress (xanthine oxidase) and increasing endogenous antioxidants concentrations (such as glutathione peroxidase, catalase and superoxide dismutase) [29, 43, 51, 53].

Antioxidant properties depend upon the structural features of the molecules, existing a structure-activity relationship (SAR). The ability of phenolic compounds, including hydroxycinnamic acids, to reduce free radicals is due to the presence of at least one phenolic hydroxyl group. In fact, the hydroxylation of the aromatic ring leads to a greater reducing activity when a higher number of hydroxyl groups exists in the aromatic ring. This is justified by the higher ability of the phenoxyl radical formed, after oxidation by ROS, be stabilized by the adjacent electron-donating hydroxyl groups. On the other hand, the *ortho*-position between phenolic hydroxyl groups is also determinant because it allows the establishment of an intramolecular H-bond between the phenolic hydroxyl groups that stabilizes the phenoxyl radical. Furthermore, the presence of some phenolic ring substituents or carboxylic group substituents may also change properties compared to parent compounds. Finally and regarding hydroxycinnamic acids, the presence of a double

bond on the side chain (CH=CH-COOH) is also important because it stabilizes the phenoxyl radical due to the increased electron delocalization [46, 51].

2.3. Lipophilization

The interactions of phenolic acids with lipid membranes may possible include the adsorption onto the membrane surface through hydrogen bonding of hydrophilic parts of the compounds with the polar head groups of phospholipids at the water-lipid interface of the membranes, and the position of the hydrophobic parts of the compounds into the hydrophobic fraction of the membrane. These interactions may contribute to the antioxidant capacity of phenolic acids, as well can protect the integrity of the lipid bilayer [52].

Phenolic compounds are effective antioxidants under their native form, but their hydrophilic nature may limit its antioxidant efficacy due to the low solubility or bioavailability in lipophilic matrices, such as the lipid bilayer of cell membranes [54]. A highly reactive antioxidant should be able to insert into cell membranes in a proper position and should be able to move and diffuse easily to the site of action. However, diffusion and location are modulated by the hydrophobicity of compounds [55].

Typically, one strategy to improve the antioxidant activity of hydrophilic antioxidants, such as phenolic acids, is to incorporate a properly positioned lipophilic moiety on parental molecule, without affecting the functional groups responsible for its radical scavenging activity. It has been, therefore, designed more surface-active molecules, generating a new class of molecules named “phenolipids” [54, 55]. This approach, “lipophilization”, adjusts the hydrophilic/lipophilic balance of hydrophilic antioxidants through grafting a lipophilic moiety(ies) [54] by different reactions (etherification, esterification, transesterification and amidation) performed chemically and/or enzymatically, according to the structure and the reactivity of parental molecules [56]. A large number of antioxidants, such as phenolic acids, flavonoids, and tocopherols, have already been lipophilized with lipophilic moieties [54, 55] including fatty alcohols, alkyl chains [57], diacylglycerol [58] or phospholipids [59].

A molecule can affect the membrane structure, changing their molecular and lipid arrangement. According to Durand et al. [54], the phenolic structure in phenolipids seems to position near phospholipids head groups, while the lipophilic tail positions parallel to the phospholipid’s tails. Therefore, phenolipids can change the lipid packing characteristics in the hydrophilic and hydrophobic areas of membranes, and the nature of the grafted chain on the hydrophilic phenolic head can affect the phenolipid location on membrane [54]. The higher hydrophobicity allows a more internalization of the antioxidant, so it is expected a more efficient protective activity against ROS within membranes [58].

It is accepted that the intrinsic structural properties of the molecules determine their antioxidant activity. However, their efficacy is also modulated by their physico-chemical properties, such as hydrophobicity, polarizability, etc. The hydrophobicity of antioxidants is highly relevant to their activity [56] and was first approached by Porter's group, who postulated the concept of "polar paradox" [60]. This hypothesizes that nonpolar antioxidants tend to be more active than their polar homologues in emulsions and membranes. The hypothesis assumes that antioxidants orient themselves according to their affinity toward the different phases. So, it would be expected that the higher efficiency of nonpolar antioxidants on membranes would be justified by their affinity to the interfacial membrane. It could implicate that the antioxidant activity would have a linear relationship with the antioxidant hydrophobicity [46, 55, 61]. However, a non-linear relationship between antioxidant activity and hydrophobicity of a homologous series of alkyl esters of chlorogenic acid in emulsified systems has been observed [62]: the effectiveness of antioxidants increases with the rise of hydrophobicity (corresponding to the alkyl chain length - C_n) until a threshold is reached, the so-called critical chain length (CCL). Beyond that threshold, any additional extension of the chain length results in a decrease of the antioxidant efficacy [46, 55]. This behavior is the so-called "cut-off effect" [62].

Durand et al. [54] studied the relationship between phenolipid's alkyl chain length from rosmarinic acid and interaction/affinity and penetration depth in lipid membranes. Both parameters showed a nonlinear behavior with the increase of the hydrophobicity of the phenolipid, which consequently affected the antioxidant bioavailability and, therefore, the bioefficacy. Thus, an easier penetration and transfer of the phenolipid through membranes occur by adding a suitable alkyl chain length on compounds, resulting in an increased bioavailability and bioefficacy. This suggest a direct correlation between the phenolipid's activity and their membrane interaction/affinity [54].

Phenolipids display diverse physico-chemical and biological properties, such as antioxidant, emulsifying, anti-inflammatory, anti-carcinogenic, among others [56]. Phenolipids conjugate the biological attributes of the parental phenolic compounds with enhanced pharmacokinetic conferred by lipophilic moieties [63]. Thus, lipophilization can be an interesting approach to the development of molecules with better pharmaceutical properties, by using them in drug delivery systems, as well as in food and cosmetics formulations [55, 58].

2.4. Caffeic Acid (CA)

2.4.1. Structure and Bioactivity

The main representative of hydroxycinnamic acids is caffeic acid (3,4-dihydroxycinnamic acid). In foods, it is commonly found chlorogenic acid (5-caffeoylquinic acid), mostly in coffee, that results from the esterification of caffeic acid with quinic acid [64]. Caffeic acid has three ionizable hydroxyl groups, a carboxylic group (pKa = 4.8), a *para*-hydroxyl (pKa = 8.6) and a *meta*-hydroxyl group (pKa = 11.2) [65] (**Figure 7**).

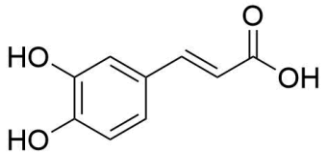

Figure 7. Structure of caffeic acid (CA).

Caffeic acid has demonstrated a strong intracellular antioxidant ability by scavenging ROS; preventing hemolysis; attenuating the excessive formation of malonaldehyde, a product of lipid peroxidation; contributing to maintenance of the structural integrity of the membrane; decreasing the need for high activity of antioxidant enzymes and reducing the effects of oxidative stress on enzymes and non-enzymatic compounds [42].

2.4.2. Bioavailability

When evaluating the health benefits of phenolic compounds, it is important to consider their bioavailability (absorption and metabolism), which indicates the fraction of the ingested compound able to reach the systemic circulation and then a specific target and exert its action on it. *In vivo,* the ability of the compound to reach the target tissue is a crucial factor for its biological properties [66]. The chemical structure, metabolism and biological activity of the compound are determinant features to the bioavailability [52].

Phenolic acids are absorbed from the gastrointestinal tract and, during absorption they undergo extensive modifications in their initial structure in the small intestine and later in the liver by conjugation (*methylation* catalyzed by catechol-O-methyltransferase; *sulfation* catalyzed by sulfotransferases; or *glucoronidation* catalyzed by UDP-glucoronosyltransferase). After metabolization, the molecules circulate in human plasma in their conjugated forms, displaying higher or lower bioactivities according to the type and position of conjugation. The conjugation is a very important process in detoxification and to increase the compounds hydrophilicity to facilitate their transport in the blood and their elimination by the biliary or the urinary route [49].

As discussed by Wang et al. [42], one research group [64] determined the absorption of caffeic acid in ileostomyzed patients and showed that after 24 h intake of 500 mg of caffeic acid, 95% of the ingested caffeic acid was absorbed in the small intestine, after which it seems to be metabolized and entered the bloodstream. Olthof et al. [64] demonstrated that caffeic acid is stable in human gastric juice or duodenal fluid and can be taken orally [42]; and also suggested that the absorbed fraction of caffeic acid and its metabolites may induce biological effects in the blood circulation [64].

According to “Lipinski rule of 5” [67], the probability of compounds to be absorbed, in this case into the bloodstream, can be related to their lipophilicity, which may be calculated by the partition coefficients in octanol:water mixtures (as log *P*) [52]. Caffeic acid has relatively lower partition coefficient and solubility in octanol due to its polarity (when compared with p-coumaric and ferulic acid). It could reflect a more difficult entry in lipid bilayers and a reduced bioavailability of CA [52]. In the light of this, it would be expected an easy absorption of more lipophilic caffeates.

2.4.3. Synthesis of phenolipid derived from caffeic acid

Phenolic acids are usually lipophilized through chemical and/or enzymatic esterification of the carboxylic group with fatty alcohols or the phenolic hydroxyl group with fatty acids. Chemical esterification is a simple process that needs drastic conditions of temperature and pH. Due to the susceptibility of phenolic acids to oxidation in alkali media, the reaction is usually performed with strong acidic catalysts: homogeneous (hydrochloric, sulfuric or *para*-toluene sulfonic acids) or heterogeneous (sulfonic resins). Some of the disadvantages of these processes can be avoided by enzymatic catalysis, that is a highly selective and more environmentally friendly process. However, this kind of synthesis is also not devoid of problems such as the higher costs and reaction time as well issues with the enzyme activity. An intermediate process is the chemo-enzymatic lipophilization, that chemically converts the phenolic acid into its corresponding methyl ester, which is then transesterified by enzymatic catalysis with a fatty alcohol originating the phenolipid [56].

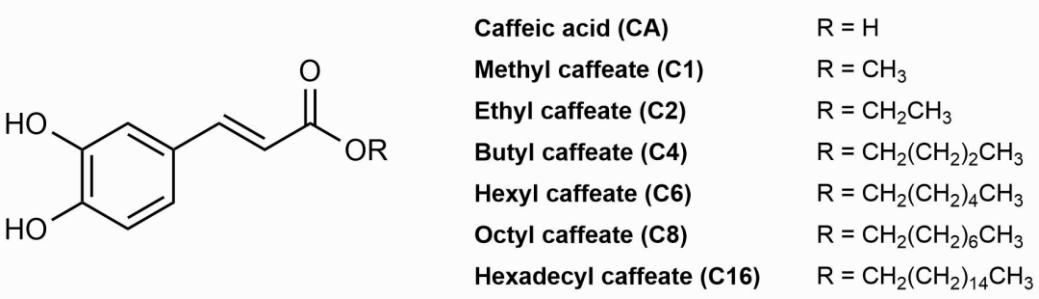

Figure 8. Molecular structure of caffeic acid and synthesized alkyl caffeates (methyl, ethyl, butyl, hexyl, octyl and hexadecyl).

In this study, the parent compound caffeic acid was modified by adding an alkyl chain through an ester bond, originating the different caffeates (**Figure 8**). In this specific case, however, the esters of caffeic acid will not be obtained by direct esterification of caffeic acid. Instead, a Verley-Doebner modification of Knoevenagel condensation between a linear monomalonate and the 3,4-dihydroxybenzaldehyde will be used, according to the **Figure 9.**

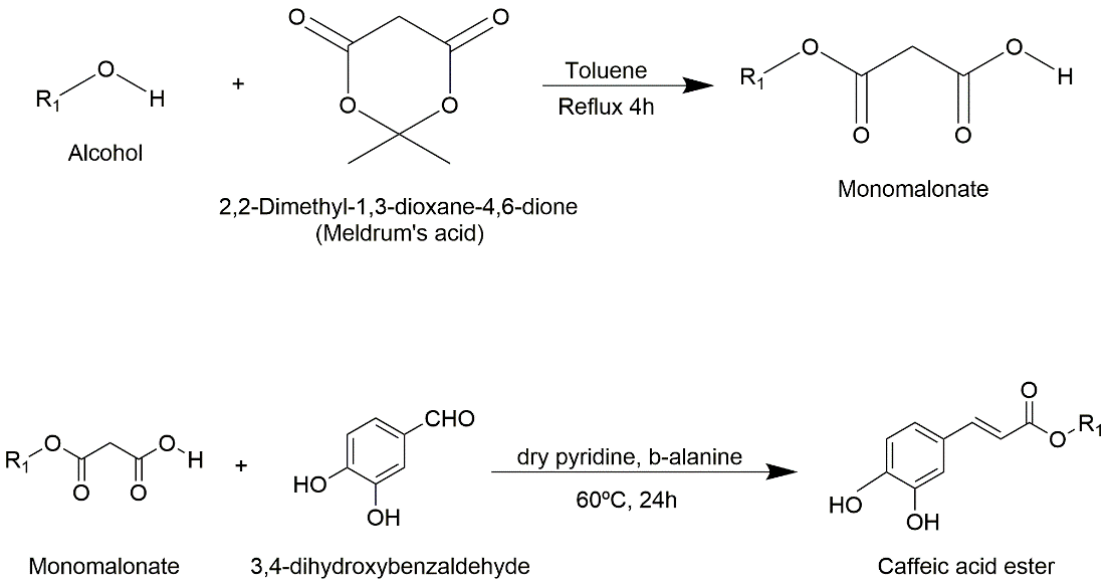

Figure 9. Synthetic strategy pursued to obtain caffeic acid esters derivatives [68].

Aim

Phenolic compounds have been highlighted by their prevention and action against a range of pathologies associated to oxidative stress. In fact, their antioxidant activity has shown a great impact on human health.

Caffeic acid is a phenolic compound that can be easily found in the human diet and its antioxidant activity depends on its molecular structure. However, its hydrophilic character may be limiting for its effective action in some mediums, such as RBC membranes. The synthesis of caffeic acid alkyl esters (phenolipids) intends to increase the bioavailability and antioxidant efficiency of caffeic acid, however, is necessary to elucidate the influence of the alkyl chain length in their activity and interaction with the membrane bilayer.

The aim of the study is to evaluate the protective role of caffeic acid and its esters derivatives against the oxidative stress in normal human RBCs, induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), a generator of peroxyl radicals that attack the RBC membrane. The RBC is a simple metabolic cell and highly susceptible to oxidative damage, so on is regularly used as a cellular model in oxidative studies. Several parameters were evaluated on RBCs, including the capacity of hemolysis inhibition, cell's morphology, lipid peroxidation, membrane-bound hemoglobin, membrane protein profile and catalase activity.

II. Materials and Methods

Materials and Methods

1. Phenolic compounds

In this work, it was evaluated the protection of caffeic acid (CA) and six caffeates derivatives (methyl, ethyl, butyl, hexyl, octyl and hexadecyl) (**Figure 10**) against the oxidative injury of RBCs.

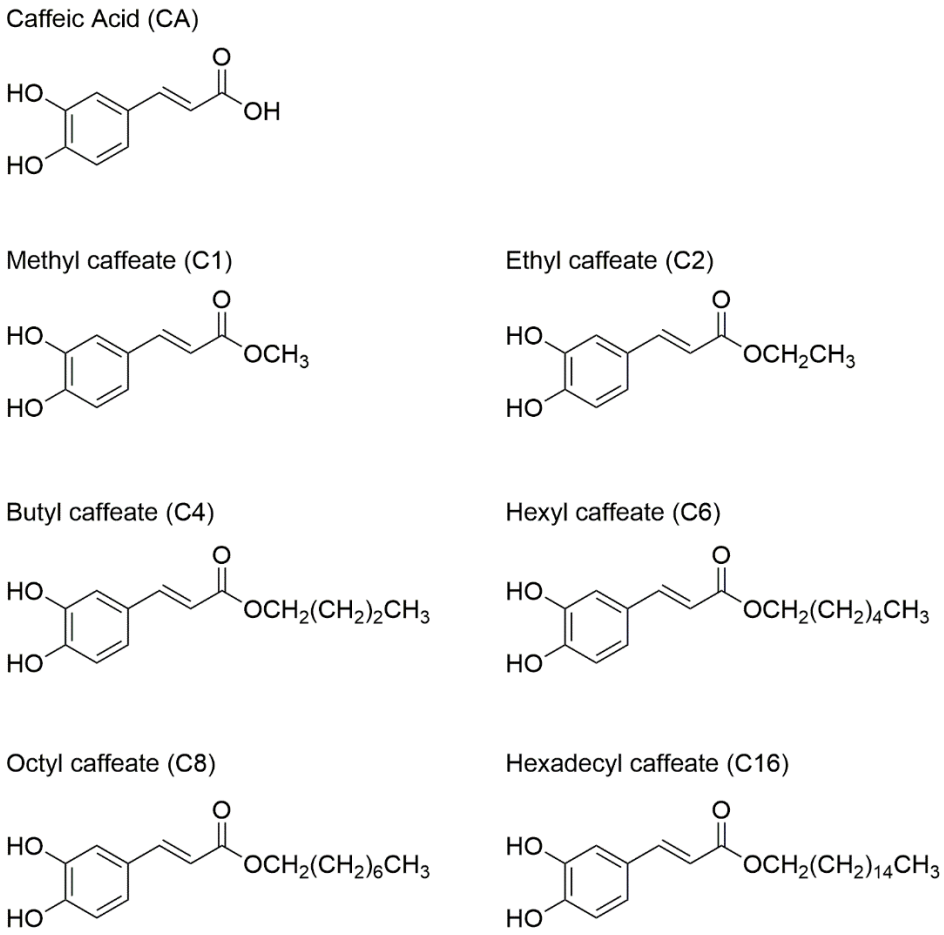

Figure 10. Chemical structures of caffeic acid and its alkyl esters. Caffeic acid was esterified by different alkyl chain (C1-methyl, C2-ethyl, C4-butyl, C6-hexyl, C8-octyl, C16-hexadecyl).

1.1. Preparation of caffeate esters and their precursors

Figure 9 summarizes the synthetic route for the preparation of the caffeic acid esters.

1.1.1. Synthesis of butyl and octyl monomalonates

Meldrum's acid was converted to monomalonates by reaction with alcohols of chain length of 4 and 8 carbons, following a published procedure [68, 69], according to **Figure 11**.

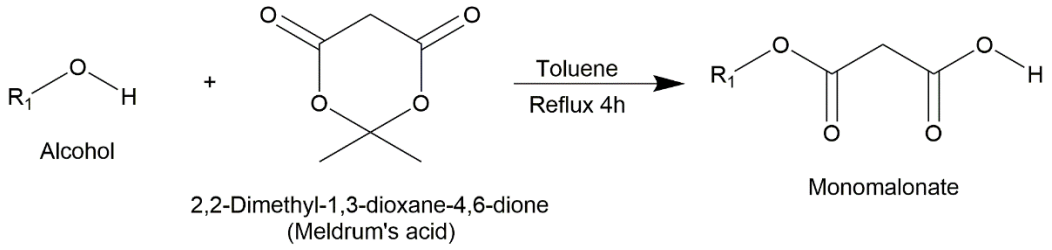

Figure 11. Reaction between the alcohol with the desired length chain and the Meldrum's acid to alkyl monomalonate.

Equimolar amount (10 mmol) of the appropriate alcohol and Meldrum's acid were refluxed in toluene (5 mL) for 4 hours. The reaction was cooled to room temperature and then extracted with saturated aqueous $NaHCO_3$. The bicarbonate layer was neutralized with concentrated HCl to release the half esters of malonic acid, that were extracted with ethyl ether, washed with water, dried over anhydrous Na_2SO_4 and evaporated to yield pure half esters of malonic acid. The malonates were identified and their purity checked by ^{1}H and ^{13}C NMR. The results were in good agreement with previously published results [68].

1.1.2. Synthesis of alkyl caffeates C4 and C8

Each monomalonate reacted with 3,4-dihydroxybenzaldehyde by the Verley-Doebner modification of Knoevenagel condensation, according to **Figure 12** [68, 69].

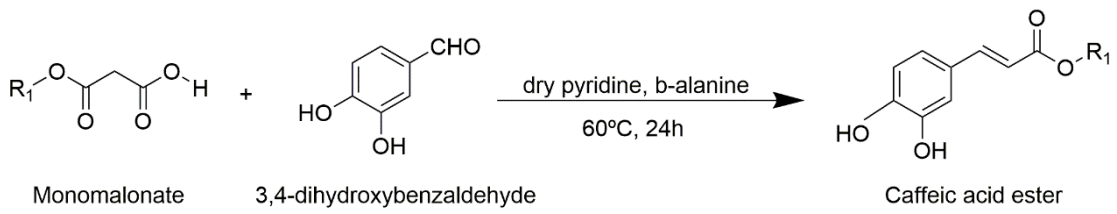

Figure 12. Reaction between the monomalonate and 3,4-dihydroxybenzaldehyde to caffeic acid ester.

The reaction was carried out at 60°C for 24 hours. Final yields were ≥75% for both caffeates. Equimolar amounts (2 mmol) of the appropriate half ester of malonic acid and 3,4-dihydroxybenzaldehyde were mixed with dry pyridine (1.0 mL) and β-alanine (15 mg) at

60°C for 24 hours. After cooling the reaction mixture in an ice bath, concentrated HCl (1.0 mL) was added. The mixture was extracted with Et_2O to obtain the crude caffeates, that were further purified by column chromatography over silica gel using ethyl acetate / petroleum ether 40-60 (1:1) as eluent. ^{1}H and ^{13}C NMR data for the butyl (C4) and octyl (C8) caffeates were in good agreement with the results reported previously [68].

Caffeic acid was purchased from Sigma-Aldrich and methyl (C1), ethyl (C2), hexyl (C6) and hexadecyl (C16) caffeates used in the present study were previously synthesized at the FCUP laboratories.

1.2. Phenolic compounds solutions

Caffeic acid and its esters C1, C2, C4 and C6 stock solutions were prepared at 800 μM in DMSO at 18.1% and an aliquot of each phenolic solution was added to the RBC suspensions in order to achieve the final concentration of compounds of 2.5, 5, 20 and 50 μM (final DMSO concentration of 1.13%). In the case of C8 and C16, solutions were prepared in DMSO and then an aliquot was added to the RBCs suspension in order to achieve the same concentrations above indicated and a DMSO final concentration of 1.13%.

2. Hemolysis assay

2.1. Preparation of RBC suspension

Blood was obtained from healthy volunteers by venipuncture. Blood samples (about 9 mL each) were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Blood samples were centrifuged (Gyrozen Multi-Purpose High Speed Centrifuge1580R) at 4°C, 700 g for 10 min; plasma and buffy coat were carefully removed by aspiration and discarded. RBCs were washed three times with phosphate-buffered saline (PBS) pH 7.4 and centrifuged at 4°C, 700 g for 7 minutes. Supernatant was carefully removed after each wash. RBCs were resuspended in PBS pH 7.4 after the final wash to obtain 2.6% hematocrit (Ht).

2.2. AAPH-induced hemolysis

In order to evaluate the protection of the different phenolic compounds (CA and its alkyl esters) against the AAPH-induced hemolysis of RBCs, in vitro assays (*n≥6*) were prepared according to **Table 1** and **Table 2**.

Table 1. Assay conditions in the study of AAPH-induced hemolysis in the presence of CA and alkyl esters C1, C2, C4 and C6.

		PBS pH 7.4 (µL)	DMSO 18.1% (µL)	Compound 800 µM (µL)	RBCs suspension Ht=2.6% (µL)	AAPH 600 mM (µL)
Positive control	Without DMSO	85	-	-	500	65
	DMSO 1.13%	44.4	40.6	-	500	65
Negative control	Without DMSO	150	-	-	500	-
	DMSO 1.13%	109.4	40.6	-	500	-
CA, C1, C2, C4, C6	2.5 µM	44.4	38.6	2.0	500	65
	5 µM	44.4	36.5	4.1	500	65
	20 µM	44.4	24.4	16.2	500	65
	50 µM	44.4	-	40.6	500	65

Positive control, RBCs incubated with AAPH; Negative control, only RBCs; CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate.

Table 2. Assay conditions in the study of AAPH-induced hemolysis in the presence of CA and alkyl esters C8 and C16.

		RBCs suspension Ht=2.6% (µL)	Compound in DMSO (µL)	PBS pH 7.4 (µL)	AAPH 600 mM (µL)
C8, C16	2.5 µM	500	7.3	77.7	65
	5 µM	500	7.3	77.7	65
	20 µM	500	7.3	77.7	65
	50 µM	500	7.3	77.7	65

C8, octyl caffeate; C16, hexadecyl caffeate.

RBCs suspensions were prepared at a final hematocrit of 2%, and the assays were performed using phenolic compounds at final concentrations of 2.5, 5, 20 and 50 µM, and AAPH (Sigma Aldrich Chemistry) at final concentration of 60 mM (chosen according to previous work [35]). Sample tests were run in duplicate. Negative controls (RBCs in PBS, with and without DMSO 1.13%) and positive controls (RBCs in PBS with AAPH, and with

or without DMSO 1.13%) were run in triplicate. RBCs suspensions were incubated at 37°C for 10 min with each phenolic compound before addition of AAPH. After that, AAPH was added and the incubations of RBCs suspensions were carried out at 37°C for 4 hours, under gentle shaking. Tubes were shaken by inversion at the end of each hour.

Hemolysis was determined spectrophotometrically according to the method described by Ko et al. [70]. After 2 and 4 hours of incubation, an aliquot of each tube was diluted with PBS and an aliquot with same volume was diluted with distilled water to yield complete hemolysis. All eppendorfs were centrifuged (Heraeus™ Fresco™ 21 Centrifuge, Thermo Scientific) at room temperature, 700 g for 10 minutes. The supernatant (300 µL) from each eppendorf was collected and the absorbance was read at 540 nm (ref. 690 nm) (Synergy HTX multi-mode reader, Biotek). The percentage of hemolysis was calculated by the following formula:

$$\%\, Hemolysis = \frac{A}{B} \times 100$$

A represents the absorption of the supernatant from the aliquot diluted in PBS, while B represents the absorption of the supernatant from the aliquot diluted in distilled water.

Spectral scans (450 – 650 nm) were performed to evaluate the concentration of oxy-hemoglobin in hemolysates [35].

2.3. AAPH-induced morphological changes

The morphological changes of RBCs suspensions were studied by optical microscopy (Nikon Eclipse *Ci*, Nikon Corporation Japan). At the end of 2 h and 4 h of incubation, aliquots (50 µL) were collected from controls and tubes with phenolic compounds at 5 µM (with AAPH) and 50 µM (with and without AAPH) and mounted on a slide with a cover slip. The samples were visualized on the 400x lens and the morphological differences were compared in the presence and absence of AAPH, as well the relative concentration of intact RBCs.

3. RBC membrane protein/lipid profiles study

3.1. Preparation of RBC suspension

Blood was obtained from healthy volunteers by venipuncture and collected 9 mL (for the procedure described in the point 3.2) and 22 mL (for the procedure described in the point 3.3) into tubes containing EDTA. Plasma and leukocytes were separated from RBCs after

centrifugation (Gyrozen Multi-Purpose High Speed Centrifuge1580R) on a double density gradient (Histopaque 1.077 and 1.119, Sigma-Aldrich) at 25°C, 700 g for 30 min and discarded. RBCs were washed twice in PBS pH 7.4 and centrifuged at 4°C, 700 g for 7 minutes. The supernatant was removed to obtain approximately a 100% hematocrit.

3.2. AAPH-induced RBC membrane changes

In order to study the effect of the radical initiator AAPH and to choose its more suitable concentration to induce the oxidative stress, preliminary assays were performed with a wide range of concentrations. The assay to study the RBC membrane protein changes in the presence of AAPH was performed according to **Table 3**.

Table 3. Assay to study the RBC membrane protein changes induced by AAPH.

		RBCs suspension Ht=100% (µL)	PBS pH 7.4 (µL)	DMSO 18.1% (µL)	AAPH 600 mM (µL)
Negative Control	Without DMSO	500	4500	-	-
AAPH	0 mM	500	4187.8	312.2	-
	30 mM	500	3937.8	312.2	250
	60 mM	500	3687.8	312.2	500
	120 mM	500	3187.8	312.2	1000
	240 mM	500	2187.8	312.2	2000
	300 mM	500	1687.8	312.2	2500

Negative control, only RBCs.

RBCs suspensions were prepared at a final hematocrit of 10% to obtain a significant volume of erythrocytic membranes. RBC suspensions containing PBS pH 7.4 and DMSO at the final concentration of 1.13% were incubated at 37°C for 10 min before the addition of AAPH. After the addition of increasing concentrations (0, 30, 60, 120, 240 and 300 mM) of AAPH, incubations were performed at 37°C for 4 hours under gentle shaking. The negative control had no DMSO neither AAPH. Each tube was shaken by inversion at the end of each hour.

After incubation, suspensions were transferred to centrifuge tubes (Nalgene) and RBCs were lysed according to Dodge et al. [71] in hypotonic solutions: 5P8/Dodge buffer (phosphate buffer 5 mM pH 8.0) supplemented with 0.1 mM phenylmethylsulfonyl fluoride

(PMSF) as a protease inhibitor. The tubes were placed on ice for 10 min and then centrifuged at 4°C, 20000 g, for 10 min (Sigma 3K18 Centrifuge). The supernatant (cytosolic fraction) was collected and stored at -80°C for further studies. Membranes were washed one more time with Dodge buffer with 0.1 mM PMSF, placed on ice for 10 min and then centrifuged at 4°C and at 20000 g for 10 min. The last wash was only with Dodge buffer and centrifuged at 4°C and at 20000 g for 10 min. The supernatant was rejected as much as possible and the membranes were transferred to eppendorfs and centrifuged at 4°C again at 20000 g for 10 min (Heraeus™ Fresco™ 21 Centrifuge, Thermo Scientific). The concentrated suspension of RBC membranes obtained was aliquoted and stored at -80°C.

3.3. Protective effect of phenolic compounds against AAPH-induced RBC membrane changes

The protective effect of phenolic compounds against AAPH-induced membrane oxidative injury was also studied. The assays to study the RBC membrane changes in the presence of AAPH and phenolic compounds were performed (*n=3*) according to **Table 4** and **Table 5**.

Table 4. Assay to study the RBC membrane changes induced by AAPH in the presence of CA and alkyl esters C1, C2, C4 and C6.

		RBCs suspension Ht=100% (µL)	Compound 800 µM (µL)	PBS pH 7.4 (µL)	DMSO 18.1% (µL)	AAPH 600 mM (µL)
Positive control	Without DMSO	500	-	4000	-	500
	DMSO 1.13%	500	-	3687.8	312.2	500
Negative control	Without DMSO	500	-	4500	-	-
	DMSO 1.13%	500	-	4187.8	312.2	-
CA, C1, C2, C4, C6	25 µM	500	156.2	3687.9	155.9	500
		500	156.2	4187.9	155.9	-

Positive control, RBCs incubated with AAPH; Negative control, only RBCs; CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate.

Table 5. Assay to study the RBC membrane changes induced by AAPH in the presence of CA and alkyl esters C8 and C16.

		RBCs suspension Ht=100% (µL)	Compound 10000 µM in DMSO (µL)	PBS pH 7.4 (µL)	AAPH 600 mM (µL)
C8, C16	25 µM	500	56.5	3943.5	500
		500	56.5	4443.5	-

C8, octyl caffeate; C16, hexadecyl caffeate.

RBCs suspensions with a final hematocrit of 10% were incubated with 25 µM of each phenolic compound at 37°C for 10 min before addition of AAPH at final concentration of 60 mM. Afterwards, the incubation was performed at 37°C for 4 hours under gentle shaking. Tubes were shaken by inversion at the end of each hour. The protocol was carried out as described before in point 3.2.

3.4. Quantification of protein concentration in RBC membrane suspension and cytosol

The total protein concentration in membrane and cytosol samples was determined by spectrophotometry according to Bradford's method. This method is based on the change of the absorbance by the binding of the *Coomassie Brilliant Blue G-250* dye to proteins, with a maximum absorption at 595 nm [72]. Briefly, 0.1 mg/mL of bovine serum albumin (BSA) standard solutions (0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL) were prepared to obtain a calibration curve. To BSA standard solutions, membrane and cytosol samples was added Bradford's reagent (100 mg of *Coomassie Brilliant Blue G-250*, Sigma; 50 mL 95% ethanol, Merck; 100 mL of 85% phosphoric acid, Merck; and dH_2O). Standards and samples were run in triplicate.

Absorbance was read at 595 nm (ref. 690 nm) (Synergy HTX multi-mode reader, Biotek) and the standard curve was plotted according to the equation *abs = f(BSA concentration)*. The total protein concentration (µg/µL) for each sample was determined by the following formula:

$$[total\ proteins](\mu g/\mu L) = \frac{[proteins](\mu g)}{V_{membrane\ suspension}(\mu L)}$$

3.5. Samples preparation to electrophoresis

Membrane suspensions were prepared with a final protein concentration of 1 µg/µL. Samples were treated with an equal volume of a solubilization buffer (0.125 M tris-Cl, pH 6.8; 4% sodium dodecyl sulphate (SDS); 20% glycerol; 10% 2-mercaptoethanol, 0.1% bromophenol blue) and the final volume (50 µL) was completed with 5P8 solution. Proteins were denaturated by heat at 100°C for 5 minutes.

3.6. Linear gradient polyacrylamide gel electrophoresis by Laemmli method

RBC membranes were submitted to polyacrylamide gel electrophoresis (SDS-PAGE) (8 µg protein/lane). Electrophoresis was carried out in a discontinuous system using a 5-15% linear acrylamide gradient gel, according to Laemmli method [73]. Afterwards, proteins were stained with Coomassie brilliant blue and scanned (Bio-Rad ChemiDoc™ Touch Imaging System).

This system comprises two gels with different porosity. The stacking gel has larger pore sizes than the running gel, and allows proteins to move freely and concentrates, or stacks, into a sharp band before it enters the running gel. In this specific system, the running gel is a gradient gel with 5% acrylamide at the top and varying up to 15% acrylamide at the bottom of the gel. Thus, the top of the gel has a large pore size and as the sample moves down through the gel, the pore size slowly decreases [74].

To obtain the running gel with a concentration in gradient 5-15%, it was used a gradient mixer where it was applied dH_2O, Acril./Bisacril. Solution (30:0.8), Tris buffer 1.5 M pH 8.8, APS 10% and TEMED. The addition of ammonium persulphate (APS) and the base *N,N,N',N'*-tetramethylenediamine (TEMED) initiates the polymerization of acrylamide [74]. After filling the support with the running gel, n-butanol was added on the gel surface and the gel was let to polymerize. Afterwards, the stacking gel (dH_2O, Acril./Bisacril. Solution (30:0.8), Tris buffer 1.5 M pH 6.8, APS 10%, TEMED) was added and, after polymerization, the system was immersed in the migration buffer (0.0177 M tris-Cl, 0.1% SDS, 0.192 M glycine, pH 8.3). The system was connected to an energy source and a pre-migration for 10 minutes at 100 V was made in order to homogenize the gel. Samples were applied and run at 100 Volts, until they reach the running gel and then the run was increased to 200 Volts.

At the end of the electrophoresis, the gel was placed in a dye solution (0.125% *Coomassie Brilliant Blue R-250*; 50% methanol; 10% acetic acid) under gentle shaking until proteins bands were visible. The gel was decolorized with decoloring solution I (50% methanol; 10% acetic acid), then with decoloring solution II (5% methanol; 7% acetic acid)

and finally, scanned (Bio-Rad ChemiDoc™ Touch Imaging System). The relative amount of each major proteins (as a percentage of the total) was quantified by densitometry (*Bio1D++ version 99*, Vilber Lourmat). This technique allows the quantification of the major protein constituents of RBC membrane (α-spectrin, β-spectrin, ankyrin, band 3, protein 4.1, protein 4.2, actin, G3PD and stomatin/tropomyosin) [75].

3.7. Membrane-Bound Hemoglobin (MBH) determination

The membrane-bound hemoglobin percentage (% MBH) is determined by a spectrophotometric process. MBH was measured at 415 and 700 nm (Synergy HTX multi-mode reader, Biotek), after dissociation of membrane proteins with Triton X-100 (Sigma-Aldrich) (5% w/v in Dodge buffer) [24].

The absorbance at 415 nm (maximum absorption of hemoglobin) was corrected by subtracting the absorbance at 700 nm (background absorbance). The % MBH was calculated according to the following formula:

$$\%\,MBH = \frac{A415 - A700}{[Total\ proteins\ (\mu g/\mu L)]} \times 100\ \times \left(\frac{1}{optical\ correction\ factor}\right)$$

3.8. Thiobarbituric Acid Reactive Substances (TBARS) determination

The lipid peroxidation in RBC membrane was determined by measuring thiobarbituric acid reactive substances (TBARS) based on the procedure of Uchiyama et al. [31]. This method consists in reacting thiobarbituric acid (TBA) with the malonaldehyde (MDA) present in samples, producing a pink fraction that is measured spectrophotometrically and that is proportional to the lipid peroxidation level (**Figure 13**).

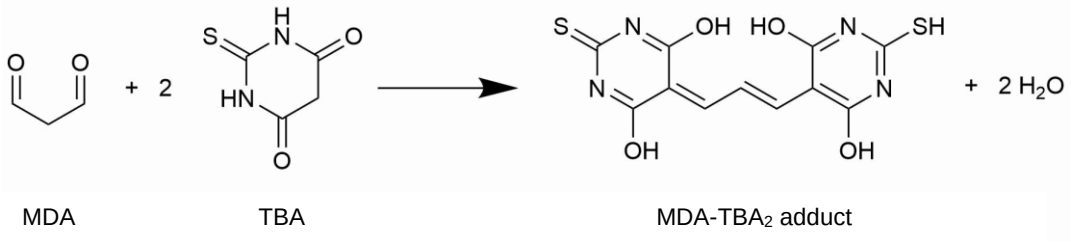

Figure 13. Thiobarbituric acid reaction test. MDA, malonaldehyde; TBA, thiobarbituric acid.

Membrane suspensions (30 µL) were mixed with Triton X-100, H_3PO_4 (1% v/v, Merck) (v/v) and TBA (0.6% w/v, Sigma-Aldrich) and then incubated at 100ºC for 45 minutes. After cooling in ice for 10 minutes, n-butanol was added and samples were centrifuged at 1000

g for 2 minutes (Heraeus™ Fresco™ 21 Centrifuge, Thermo Scientific). The absorbance of the pink supernatant was read at 535 and 520 nm (Synergy HTX multi-mode reader, Biotek). The LPO of each RBC membrane sample was calculated by the formula:

$$LPO = (\frac{A535 - A520}{optical\ correction\ factor})/([Total\ proteins\ (\mu g/\mu L)] \times\ 0.03)$$

3.9. Quantification of Catalase (CAT) activity

The quantification of catalase activity is performed by a spectrophotometric method (adapted from Johansson and Borg [76]) based on the peroxidatic activity of the enzyme. In the presence of catalase and H_2O_2, it is initially formed an intermediate compound that via the peroxidatic activity of the enzyme reacts with hydrogen donors, such as methanol (CH_3OH). In this procedure, it was used CH_3OH as the hydrogen donor and the production of formaldehyde (CH_2O) was measured by reading the absorbance at 540 nm (Synergy HTX multi-mode reader, Biotek) after the formation of a colored compound, obtained by the reaction with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). Purpald changes from colorless to a magenta or purple color when oxidized in the presence of aldehydes [76] (**Figure 14**).

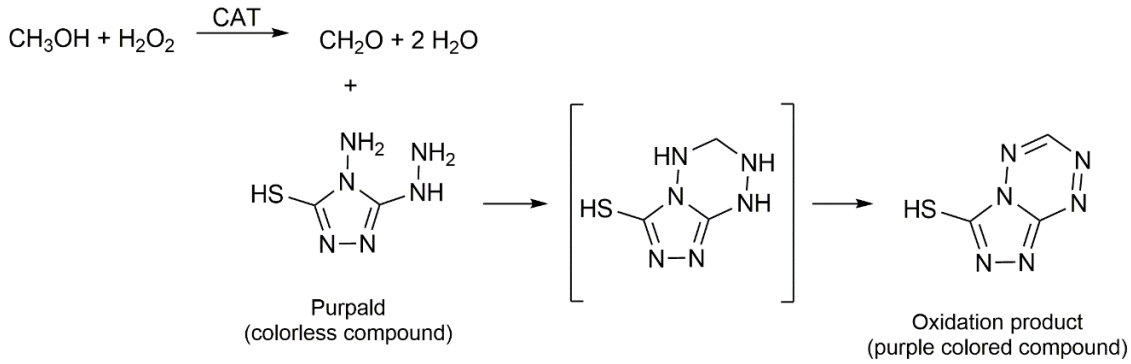

Figure 14. Purpald reaction test. CAT, catalase.

To obtain a calibration curve, formaldehyde (Merck) standard solutions were prepared at the final concentrations of 0, 5, 15, 30, 45, 60 and 75 µM within phosphate buffer 100 mM pH 7.0. To formaldehyde standards solutions, membrane and cytosol samples was added phosphate buffer 100 mM pH 7.0, methanol, and H_2O_2 35.0 mM (Sigma-Aldrich) to initiate the reaction. After an incubation of 20 minutes at room temperature, the reaction was stopped with KOH 10.0 M, and purpald 70.0 mM (Sigma-Aldrich) was added and the solutions were incubated for 10 min at room temperature. Then, it was added KIO_4 300 mM and the solutions were incubated more 5 minutes at room temperature.

The absorbance was read at 540 nm (ref. 690 nm) (Synergy HTX multi-mode reader, Biotek) and the standard curve was plotted according to the equation *abs = f(formaldehyde concentration)*. The catalase activity of each sample was quantified by interpolation in the standard curve and using the formula:

$$Cat\ act.\left(\frac{nmol\ min^{-1}}{mL}\right) = \frac{[Formaldehyde\ (\mu M/\mu L)] \times (\frac{0.17}{0.02})}{20\ min} \times Dilution\ Factor\ (\mu L)$$

The value of catalase activity was corrected to the total protein concentration (µg/µL) of each sample.

4. Statistical analysis

For the statistical analysis was used the IBM SPSS Statistics 25 for Windows (SPSS Inc.). The results obtained for the experiments are expressed mean ± SEM (standard error of the mean). Statistical comparisons of means were performed by one-way ANOVA with *post hoc* testing using Duncan's test. A p value of <0.05 was assumed as statistically significant.

III. Results

Results

1. Phenolic compounds synthesis

The structures and synthetic strategy to obtain the caffeic acid esters derivatives (methyl, ethyl, butyl, hexyl, octyl and hexadecyl) are depicted in **Figure 10** and **Figures 11** and **12**, respectively. In the present study, butyl and octyl caffeates were synthesized and obtained in a yield of 85% and 75%, respectively. These compounds have a surfactant structure (containing a hydrophilic moiety and hydrophobic tail) giving to these compounds some emulsifier ability. In fact, the bigger alkyl chain length of C8 derivative facilitates the emulsion of the compound during the purification processes by extraction, justifying the minor yield of octyl caffeate compared to the one of butyl caffeate.

2. AAPH-induced hemolysis

In this assay, the protection of CA and its alkyl esters was studied at different concentrations (2.5, 5, 20 and 50 µM) against AAPH-induced hemolysis in human RBCs after 4 hours of incubation at 37°C. For this purpose, it was evaluated the percentage of inhibition of hemolysis where increase reflects a higher protection by the compound in study. The percentage of inhibition of hemolysis was measured by comparing the spectrophotometric absorbance values of aliquots of RBC suspensions supernatant in PBS pH 7.4 at 540 nm and other aliquots in water. Thus, it was possible to conclude which compounds, and the respective concentration, confer more protection against hemolysis.

Initially, hemolysis assays were performed after 2 and 4 hours of RBCs incubation (**Figure 15**). However, 2h was not time enough to cause noteworthy AAPH-induced hemolysis, as seen by the similar results of negative (Cr -) and positive (Cr +) controls. After 4h of incubation was observed an increase of hemolysis in positive control. Thus, 4h were chosen as the ideal time of incubation to perform the hemolysis assays.

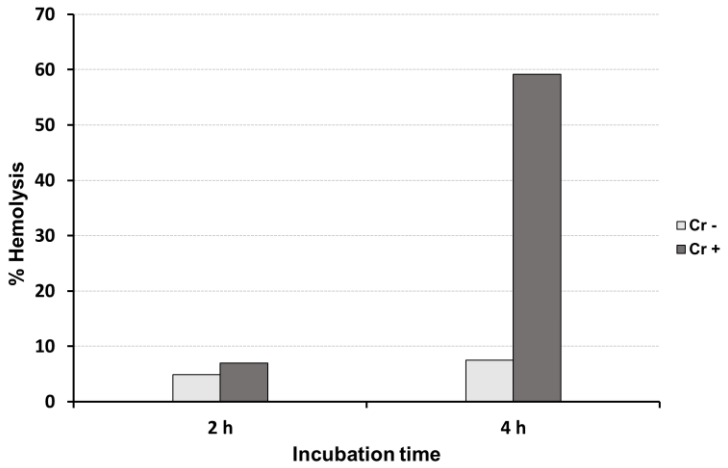

Figure 15. Representative percentage of hemolysis of RBCs at 2% hematocrit after 2 and 4 hours of incubation at 37°C. Cr -, negative control (only RBCs); Cr +, positive control (RBCs incubated with AAPH 60 mM).

In the presence of AAPH (**Figure 16**), all caffeates significantly protected RBCs from oxidative-induced hemolysis and their activity was higher than the observed for caffeic acid, which almost had no protective activity (<12%) at all concentrations. C1, C2, and C4 significantly protected RBCs from hemolysis in a concentration-dependent manner, i.e., the larger the caffeate concentration, the larger the inhibition of hemolysis. On the other hand, C6, C8 and C16 had an identical protection capacity at all tested concentrations.

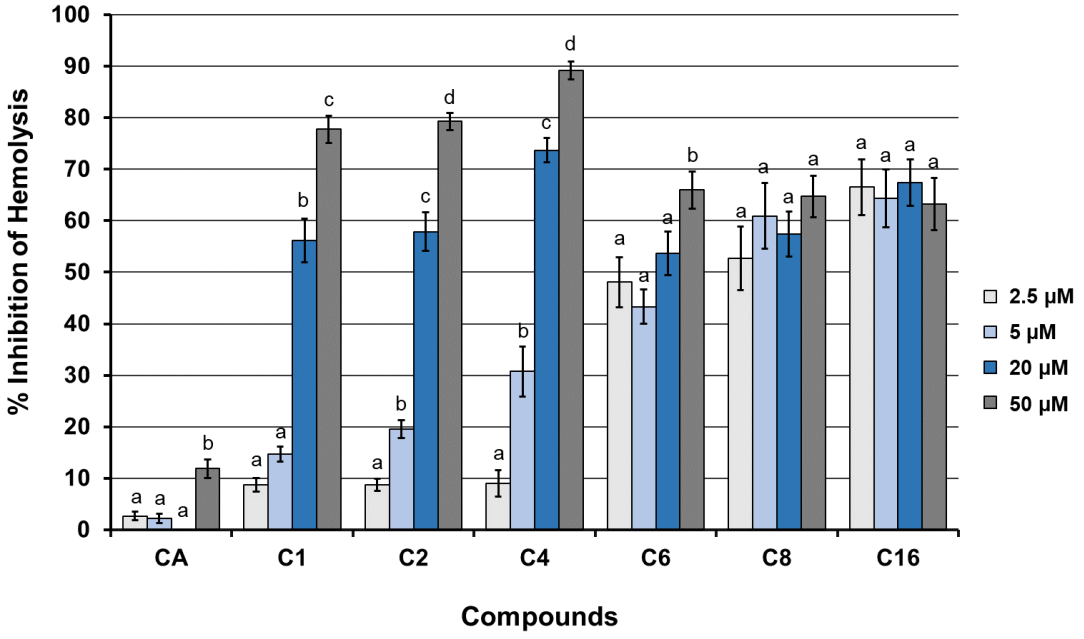

Figure 16. Percentage of inhibition of lysis of RBCs (mean ± SEM) at 2% hematocrit incubated with phenolic compounds (2.5, 5, 20 and 50 µM) and AAPH (60 mM) for 4 hours at 37°C. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Different letters in the same compound represent significant differences ($p < 0.05$) between the mean values of each concentration. Error bars indicate the standard error of the mean ($n \geq 6$).

In general, the inhibition of hemolysis raised with the increase of the alkyl chain length for the lower concentrations used. At 2.5 μM, the activity order was CA < C1 ≈ C2 ≈ C4 < C6 ≈ C8 < C16, and at 5 μM the activity order was CA < C1 ≈ C2 < C4 < C6 < C8 ≈ C16 (**Figure 17**).

At the lowest concentration (2.5 μM), C6-C16 exhibited a much better protective effect (inhibition of hemolysis between 48-70%) than derivatives C1-C4 (<10%) (**Figure 17**). Increasing the concentration to 5 μM, the protective effect of the derivatives C1-C4 was increased but not of the derivatives C6-C16. The same was observed for the concentrations of 20 μM and 50 μM (**Figure 17**). Therefore, at 50 μM the derivatives C1-C4 showed a better protective activity than derivatives C6-C16. In fact, a clear dose dependent effect was only observed for the derivatives C1-C4 (**Figure 16**).

The individual analysis of each phenolic compound and concentration leads to the conclusion that the better antioxidant against hemolysis was the C4 at 50 μM.

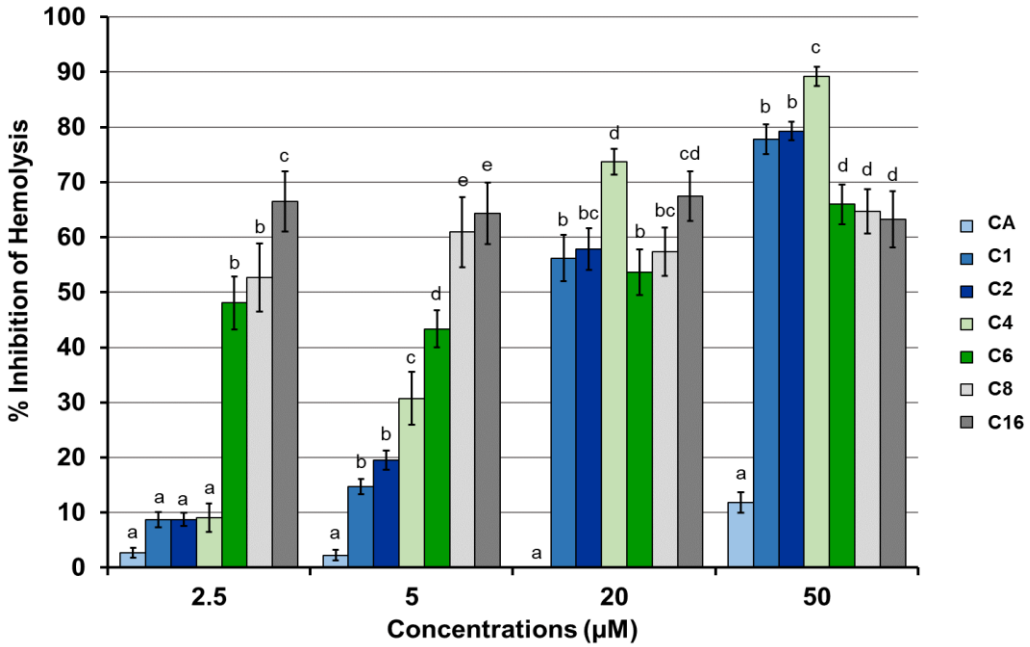

Figure 17. Percentage of inhibition of lysis of RBCs (mean ± SEM) at 2% hematocrit incubated with different concentrations (2.5, 5, 20 and 50 μM) of phenolic compounds and AAPH (60 mM) for 4 hours at 37°C. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Different letters in the same concentration represent significant differences ($p < 0.05$) between the mean values of each compound. Error bars indicate the standard error of the mean ($n \geq 6$).

3. AAPH-induced morphological changes

The effects of AAPH at 60 mM and the phenolic compounds at 5 or 50 µM on the RBC morphology and density were evaluated by optical microscopy and are illustrated in **Figure 18**.

In the absence of AAPH, RBCs suspensions displayed a large number of cells with an echinocytic (crenated) form and, similar result was also visualized in the presence of each phenolic compound at 50 µM (BCA, BC1, BC2, BC4, BC6, BC8 and BC16). In the presence of AAPH, the echinocytes density was substantially fewer, which may be related to the overall decrease of the cellular density.

Relatively to cellular density, comparisons between RBCs with AAPH (positive control) and only RBCs (negative control), or RBCs with phenolic compounds (50 µM) and RBCs with phenolic compounds (50 µM) plus AAPH showed that, in general, AAPH reduced the number of cells and increased the number of lysed cells (*ghosts*). But such reduction was attenuated by the presence of the phenolic compounds, as seen with the RBCs incubated with the compounds plus AAPH. On the other hand, the compounds did not affect, in relevant extent, the cellular density under conditions free of oxidative stress (without AAPH). Regarding the effects at 5 µM, an increase in the density of cells from the caffeic acid to the C16 derivative was observed. These observations are in line with the hemolysis results obtained with the concentration of 5 µM. Regarding the effects at 50 µM, a small difference between caffeates were observed.

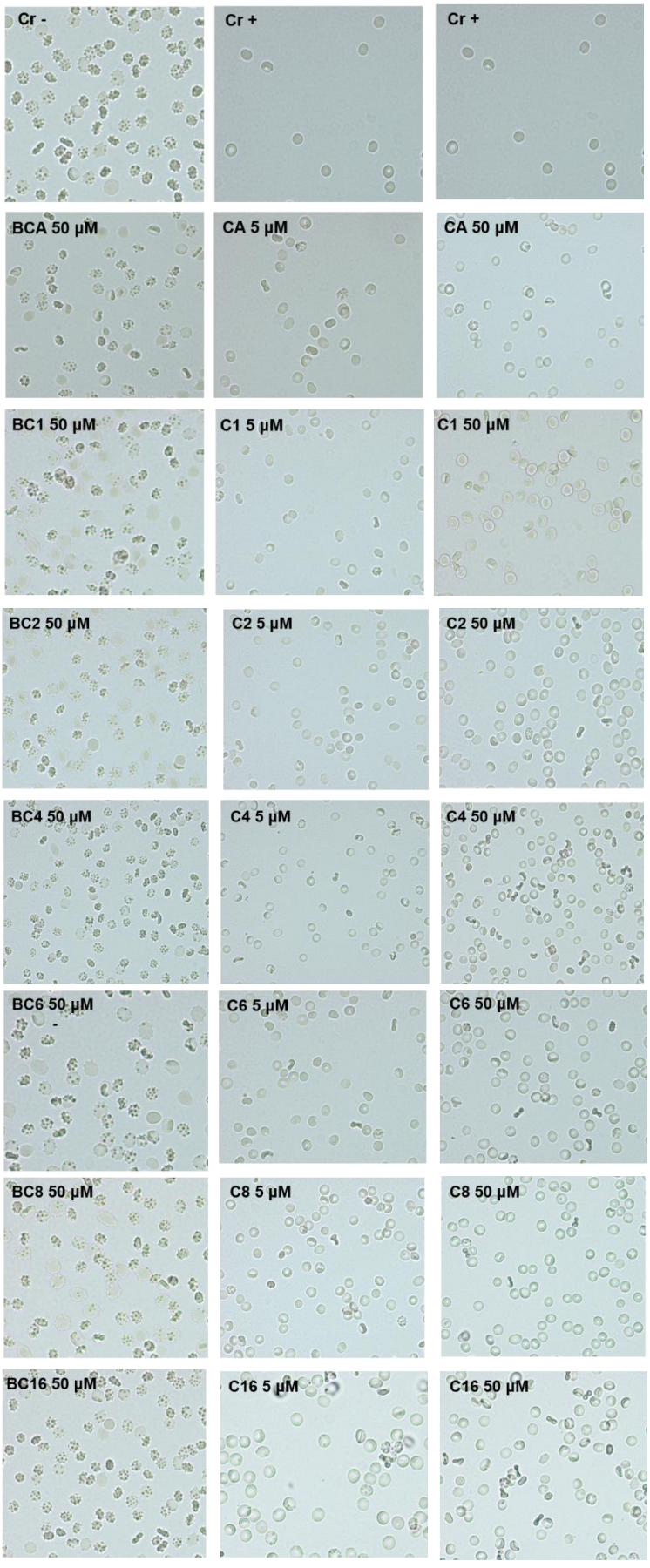

Figure 18. Optical microscopic evaluation of the effect of AAPH and phenolic compounds on RBC morphology (400x magnification). Cr-, negative control (only RBCs); Cr+, positive control (RBCs incubated with AAPH); BC_n 50 µM (n=A, 1, 2, 4, 6, 8, 16), RBCs incubated with phenolic compounds at 50 µM; C_n 50 µM (n=A, 1, 2, 4, 6, 8, 16), RBCs incubated with phenolic compounds at 50 µM and AAPH; C_n 5 µM (n=A, 1, 2, 4, 6, 8, 16), RBCs incubated with phenolic compounds at 5 µM and AAPH.

4. Spectral scans of lysed RBC suspensions

Spectral scans (450-650 nm) of lysed RBC suspensions (**Figure 19** and **Figure 20**) were performed to evaluate the nature of Hb in the hemolysate of samples containing phenolic compounds and AAPH, after 4 hours of incubation. The spectral scans performed in the presence of phenolic compounds (without AAPH) did not show any change in the oxy-hemoglobin peaks (540 and 578 nm) or in its concentration, when compared with the negative control. The presence of AAPH decreased the intensity of oxy-Hb peaks. They partially recovered with the addition of phenolic compounds at any concentration, in a concentration-dependent manner.

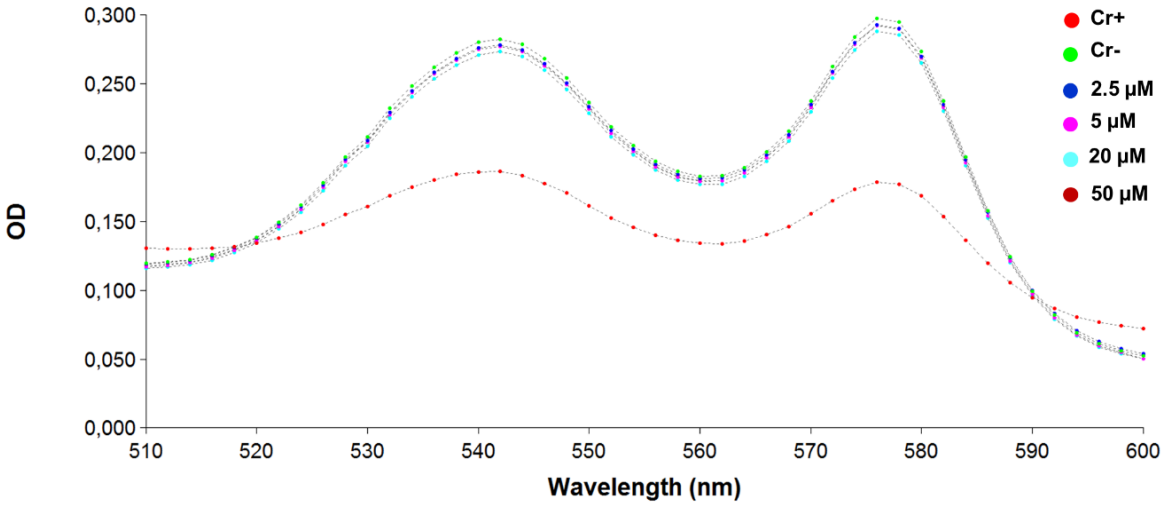

Figure 19. Representative spectral scans (510-600 nm) of lysed RBC suspensions after 4 hours of incubation with phenolic compounds at different concentrations (2.5, 5, 20 and 50 µM). Dots: Red, positive control; Green, negative control; Blue, 2.5 µM; Pink, 5 µM; Light blue, 20 µM; Dark red, 50 µM.

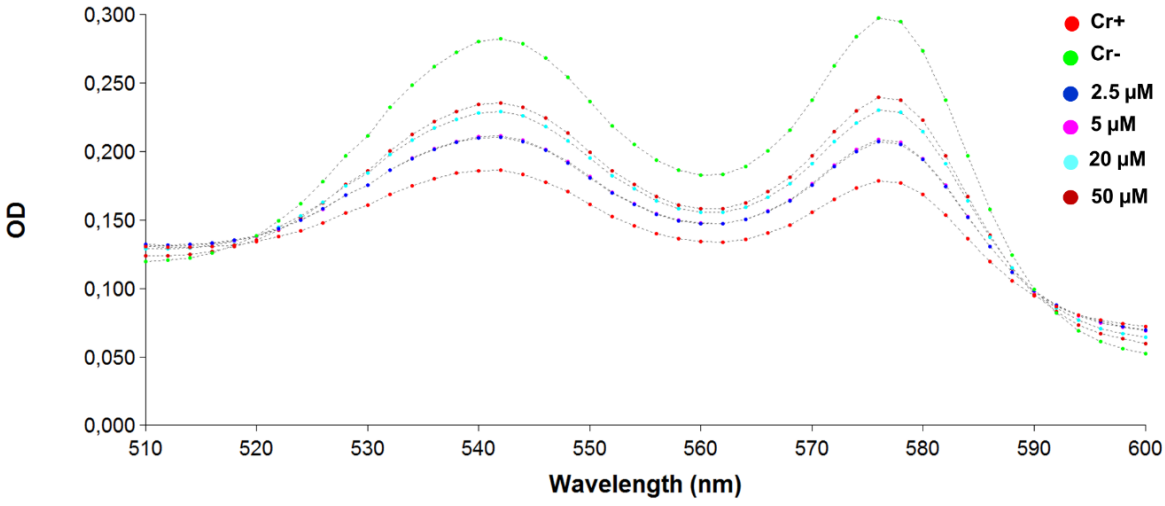

Figure 20. Representative spectral scans (510-600 nm) of lysed RBC suspensions after 4 hours of incubation with phenolic compounds at different concentrations (2.5, 5, 20 and 50 µM) and AAPH (60 mM). Dots: Red, positive control; Green, negative control; Blue, 2.5 µM; Pink, 5 µM; Light blue, 20 µM; Dark red, 50 µM.

5. AAPH-induced RBC membrane changes

In order to choose the ideal concentration of AAPH to be used along the assays, preliminary tests were performed with AAPH at concentrations of 0, 30, 60 and 120 mM (**Figure 21**). The concentration of 60 mM was selected as the more adequate to be used because it was enough to produce modifications in RBC membrane proteins. As described by Niki et al. [77] and Sato et al. [78], AAPH induces hemolysis concentration dependently. At 0 and 30 mM of AAPH, none or very slight changes were observed, whereas at 120 mM the changes were too strong with the production of an erythroid pellet, from where it was difficult to obtain RBC membranes.

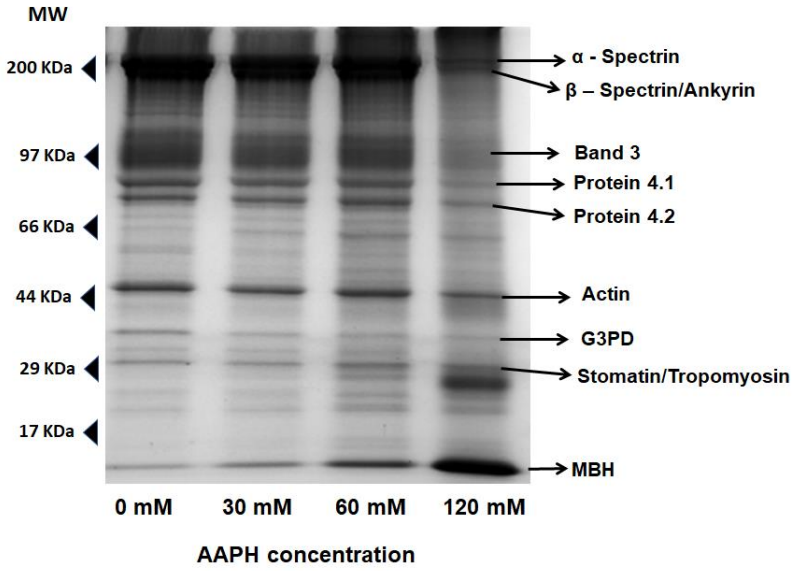

Figure 21. SDS-polyacrylamide gel electrophoresis (linear gradient gel) of RBC membrane proteins, with band identification and their molecular weight (MW), after incubation of RBC at 10% hematocrit with different concentrations of AAPH (0, 30, 60 and 120 mM) for 4 hours.

To address whether caffeic acid and its derivatives treatment have a protective activity against AAPH-induced damage to RBC membrane proteins, SDS-PAGE experiments were performed to assess possible changes in membrane proteins. The electrophoretic profile of membrane proteins is represented in **Figure 22** and the relative percentage of each protein in the RBC membrane in **Figure 23** and **Figure 24**.

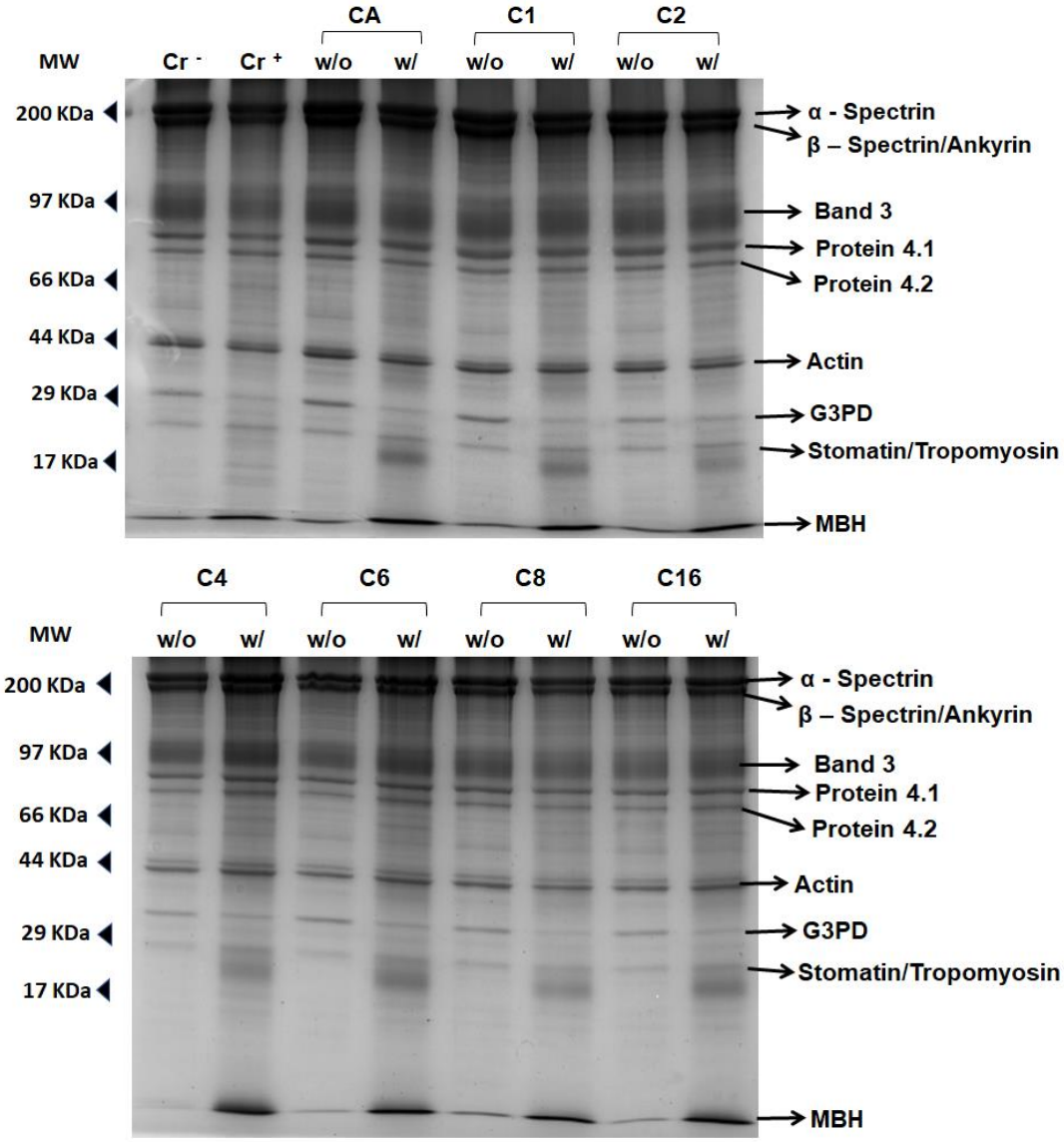

Figure 22. Representative SDS-polyacrylamide gels electrophoresis (linear gradient gel) of RBC membrane proteins, with band identification and their molecular weight (MW). RBC suspension at 10% hematocrit was incubated with phenolic compounds for 4 hours. Lanes: Cr -, negative control (only RBCs); Cr +, positive control (RBCs incubated with AAPH); w/o, RBCs only with phenolic compounds at 25 µM; w/, RBCs with phenolic compounds at 25 µM and AAPH at 60 mM. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate.

The membrane proteins analysis (**Figure 23** and **Figure 24**) performed without AAPH showed that phenolic compounds did not significantly affect the membrane proteins. In the presence of AAPH, no significant differences between membrane proteins were observed. However, the relative percentage of each protein in the membrane of RBC without AAPH showed significantly higher values for G3PD protein and lower values for stomatin/tropomyosin proteins when compared with RBC with AAPH. Despite the absence of statistical difference, spectrin ($p=0.260$) and band 3 ($p=0.099$) also showed some differences between compounds.

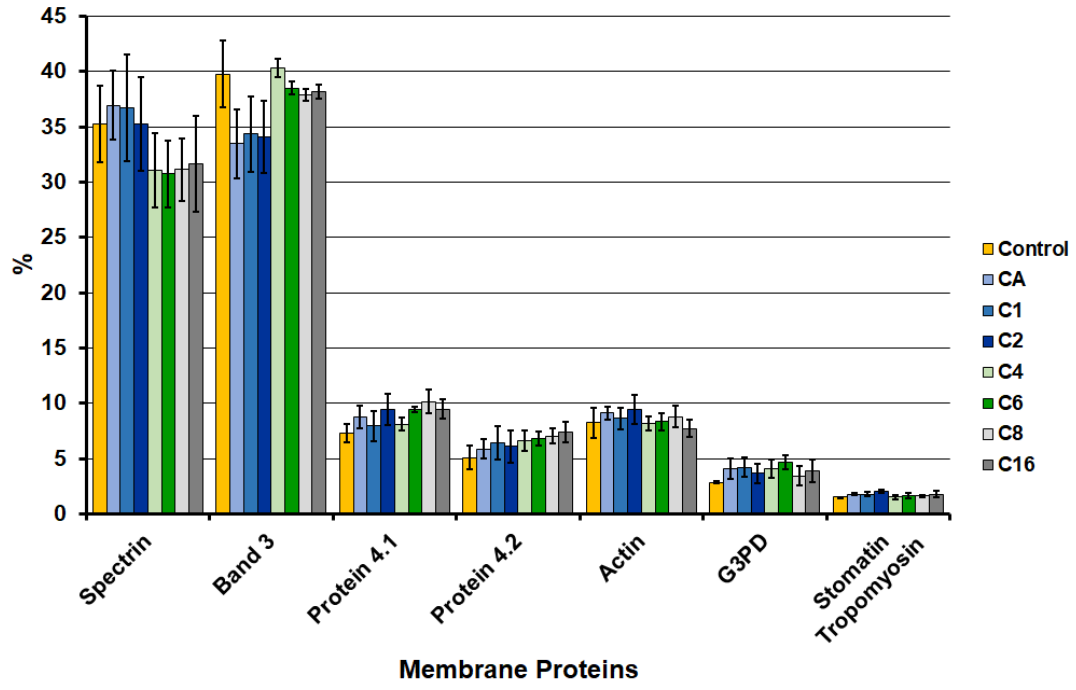

Figure 23. Effects of phenolic compounds in RBC membrane proteins (Spectrin (α and β/Ank), Band 3, Protein 4.1, Protein 4.2, Actin, G3PD, Stomatin/Tropomyosin) by densitometric analysis (mean ± SEM). Control, only RBCs (without AAPH); CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Error bars indicate the standard error of the mean (n = 3).

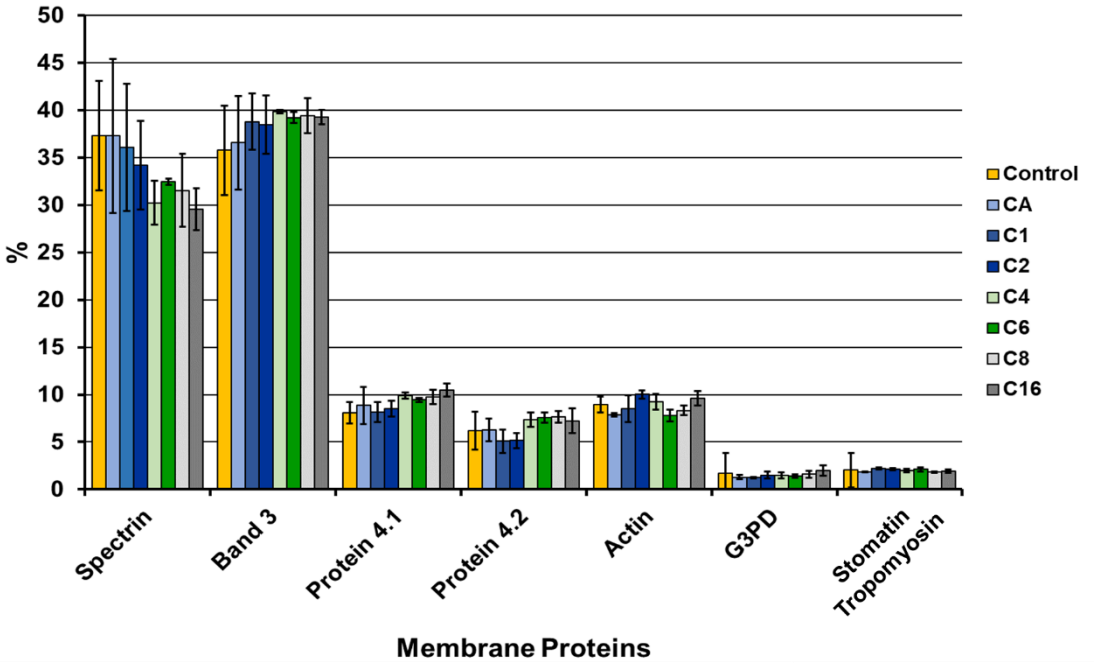

Figure 24. Effects of phenolic compounds on AAPH-induced changes in RBC membrane proteins (Spectrin (α and β/Ank), Band 3, Protein 4.1, Protein 4.2, Actin, G3PD, Stomatin/Tropomyosin) by densitometric analysis (mean ± SEM). Control, RBCs incubated with AAPH; CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Error bars indicate the standard error of the mean (n = 3).

6. Biomarkers of oxidative stress

In order to evaluate the RBCs status under oxidative stress and the effect of the phenolic compounds, different biomarkers were quantified, the MBH and LPO.

Under oxidative stress, Hb links to the membrane, thus membrane-bound hemoglobin (MBH) is used as a biomarker of oxidative damage [18, 24, 32, 75].

In the assays performed without AAPH, the incubation of RBCs with C8 and C16 significantly affected the percentage of MBH, as illustrated in **Figure 25**. The addition of AAPH significantly increased the MBH values. To address whether phenolic compounds were able to attenuate AAPH-induced MBH increase, RBCs were also incubated with phenolic compounds and it was observed a significant increase in the percentage of MBH. However, no significant differences were observed between compounds. In fact, the MBH value showed a tendency to rise with the increase of the alkyl chain length of caffeates in the absence of AAPH but this was not observed in the presence of AAPH.

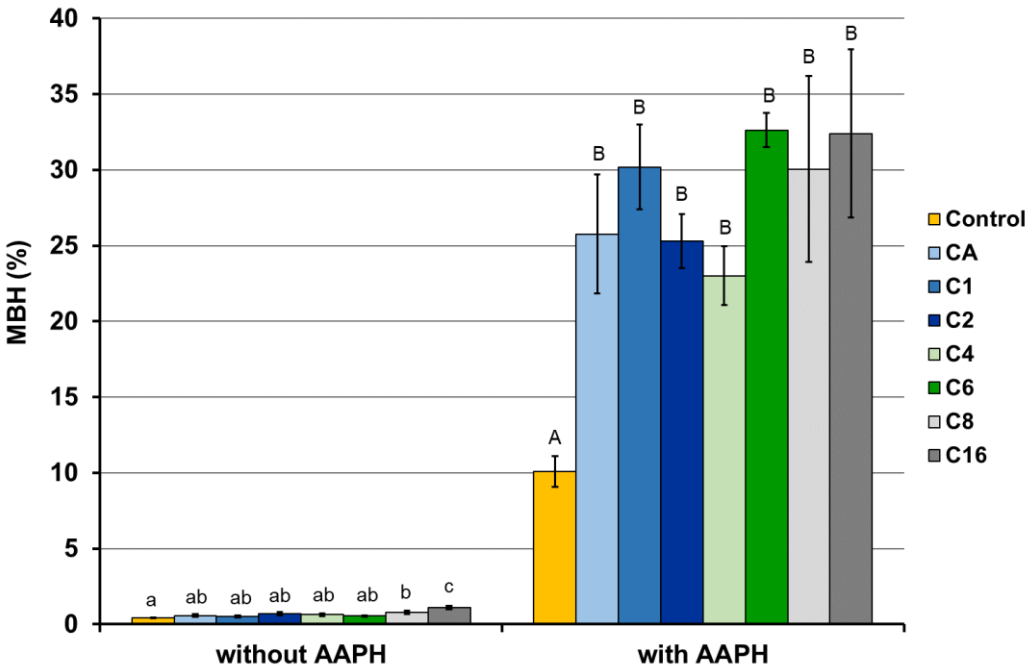

Figure 25. Percentage of RBC membrane-bound hemoglobin (MBH) (mean ± SEM) after incubation of RBC suspension at 10% hematocrit with phenolic compounds (25 µM) and without or with AAPH (60 mM) for 4 hours. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Mean values between compounds with different lowercase letters in the absence of AAPH are significantly different ($p < 0.05$). Mean values between compounds with different uppercase letters in the presence of AAPH are significantly different ($p < 0.05$). Error bars indicate the standard error of the mean ($n = 3$).

The lipid peroxidation in RBC membrane is a commonly studied biomarker of oxidative stress, in which MDA is a major end-product of this reaction [31, 32]. In the absence of AAPH, phenolic compounds increased LPO levels but this increase was not significative (**Figure 26**). After addition of AAPH to RBCs, a significant increase in LPO values was observed, for positive control and for all compounds. No significant differences were observed between compounds and the positive control. Therefore, it was not verified any dependence between the LPO values and the alkyl chain length of compounds. Eventhough, it was observed a trend (p=0.132) to lower values in LPO with all compounds when compared to positive control.

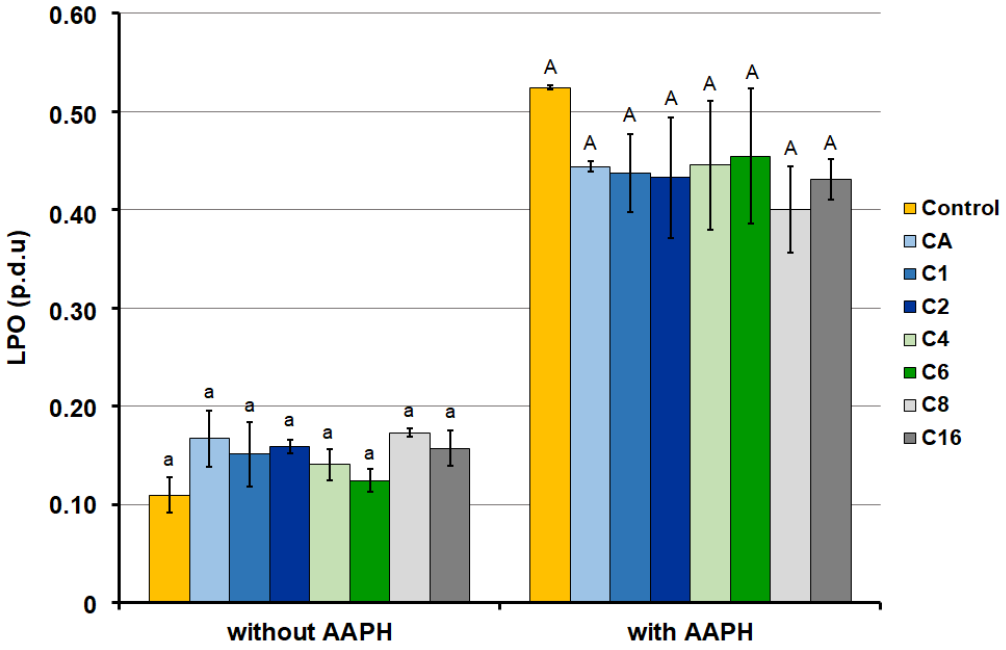

Figure 26. Effects of phenolic compounds on AAPH-induced lipid peroxidation (mean ± SEM) in RBCs. RBCs suspension at 10% hematocrit was incubated with phenolic compounds (25 µM) and with or without AAPH (60 mM) for 4 hours. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean; p.d.u, procedure defined unit. Mean values of each compound with the letter “a” in the absence of AAPH are not significantly different ($p > 0.05$). Mean values of each compound with the letter “A” in the presence of AAPH are not significantly different ($p > 0.05$). Error bars indicate the standard error of the mean ($n = 3$).

7. Catalase activity (cytosol and membrane)

Catalase (CAT) is one of the main enzymatic antioxidant defenses in human RBCs [21] and its activity was evaluated in RBC cytosol (**Figure 27**) and membrane (**Figure 28**).

In the absence of AAPH (**Figure 27**), the enzyme activity in the cytosol decreased in the presence of compounds and this decrease was more significant with the CA. The enzyme activity was similar with all caffeates. In the presence of AAPH, the CAT activity decreased and it was similar with all compounds.

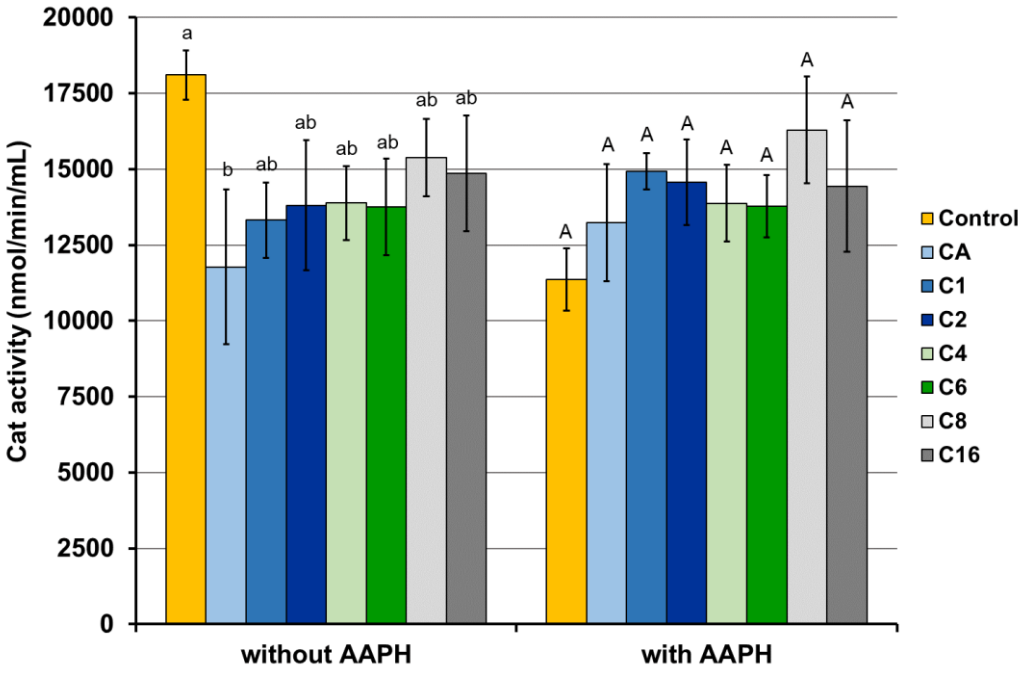

Figure 27. Quantification of catalase activity in the cytosol (mean ± SEM) after incubation of RBC suspension at 10% hematocrit with phenolic compounds (25 µM) and with or without AAPH (60 mM) for 4 hours. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Mean values between compounds with different lowercase letters in the absence of AAPH are significantly different ($p < 0.05$). Mean values of each compound with the letter "A" in the presence of AAPH are not significantly different ($p > 0.05$). Error bars indicate the standard error of the mean ($n = 3$).

In the absence of AAPH (**Figure 28**), the CAT activity in the RBC membrane was similar in the presence of all phenolic compounds (comparing to negative control). In the presence of AAPH, the CAT activity significantly increased. The CAT activity was similar in the presence of all compounds, except for the C16 derivative. The CAT activity in RBC membrane was increased in the presence of compounds, when compared with the positive control.

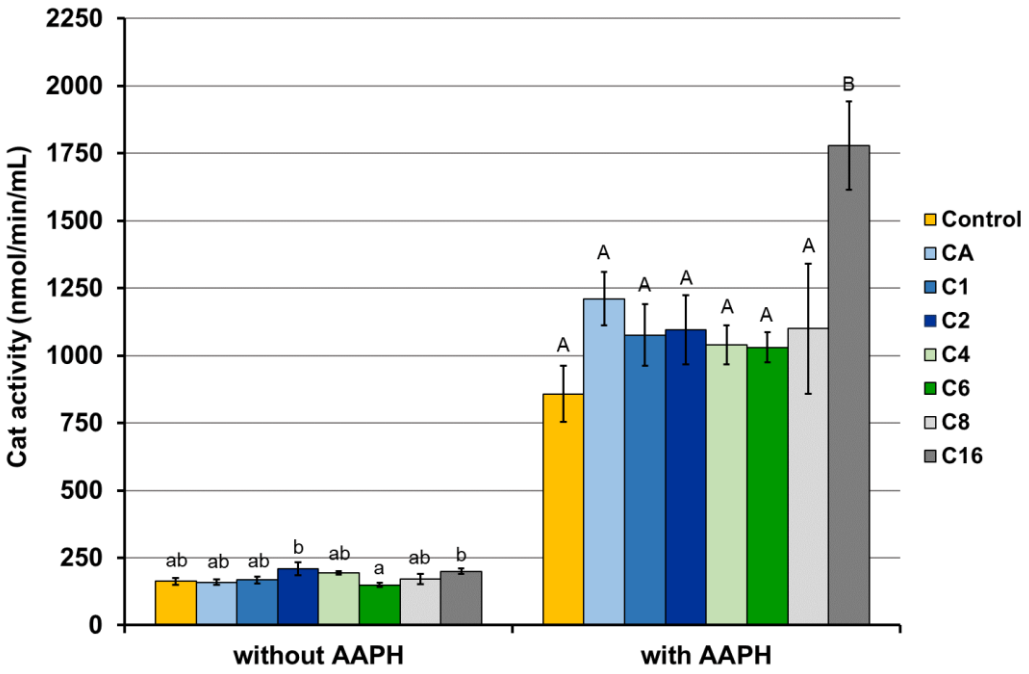

Figure 28. Quantification of catalase activity in the membrane (mean ± SEM) after incubation of RBC suspension at 10% hematocrit with phenolic compounds (25 µM) and with or without AAPH (60 mM) for 4 hours. CA, caffeic acid; C1, methyl caffeate; C2, ethyl caffeate; C4, butyl caffeate; C6, hexyl caffeate; C8, octyl caffeate; C16, hexadecyl caffeate; SEM, standard error of the mean. Mean values between compounds with different lowercase letters in the absence of AAPH are significantly different ($p < 0.05$). Mean values between compounds with different uppercase letters in the presence of AAPH are significantly different ($p < 0.05$). Error bars indicate the standard error of the mean (n = 3).

IV. Discussion

Discussion

Phenolic compounds have been receiving remarkable attention due to their antioxidant properties [79]. They are effective antioxidants, but their hydrophilicity may be, in certain circumstances, a limiting factor for their antioxidant efficiency. Therefore, phenolic compounds bioavailability may be improved by lipophilization through covalent association with a lipophilic moiety, to design compounds known as phenolipids [54, 58]. In this work, we tried to increase the protective activity against oxidative stress of a natural polyphenol, the caffeic acid, by grafting an alkyl chain to the molecule. According to the literature, this modification in the caffeic acid molecule does not interfere with its reactivity against radicals [68] but may affect absorption, distribution and metabolism of caffeic acid and, therefore, its bioactivity. Caffeic acid is a phenolic compound with a wide range of physiological and pharmacological activities [79, 80].

In this study, a serie of caffeic acid esters derivatives were synthesized with variable alkyl chain length in order to investigate their antihemolytic and antioxidant abilities against the AAPH-induced oxidative stress in human RBCs. The RBC is commonly used as an *in vitro* model due to its susceptibility to free radical-mediated peroxidation, to its structural simplicity and to be representative of the plasma membrane in general [81, 82].

AAPH is an azo compound that generates peroxyl radicals at constant rate in the aqueous phase by thermal decomposition and reaction with O_2 at 37°C. These peroxyl radicals attack the RBC membranes from the external medium to induce the chain oxidation of lipids and proteins [77, 83, 84], and eventually to cause hemolysis [77]. AAPH has a half-life of about 175 hours and produces radicals at a rate of 1.3×10^{-6} [AAPH] per second [83] (**Figure 29**).

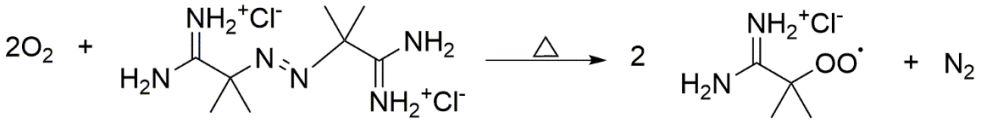

Figure 29. AAPH thermal decomposition generates nitrogen and two peroxyl radicals that attack the RBC membranes.

In this work, different parameters were evaluated: inhibition of hemolysis, RBC morphology, membrane protein profile, biomarkers of oxidative stress and catalase activity. In general, the AAPH exposure notably induced a substantial damage to RBC.

Hemolysis of human RBCs is considered a good model system for study the oxidative damage in membranes induced by free radicals and to evaluate the antioxidant/pro-oxidant activity of compounds [81, 83]. Concerning this, it was evaluated the protective activity of caffeic acid and its alkyl esters derivatives (caffeates) against AAPH-induced hemolysis.

This evaluation was done without interferences of exogenous antioxidant defenses, that were removed by washing RBCs prior to incubation with the phenolic compounds and AAPH.

RBC hemolysis was induced by AAPH (**Figure 15**), which had been described as an efficient oxidative hemolysis inductor [77, 78, 83, 85], after 4 h of incubation. Hemolysis results from the disruption of the cellular membrane and it is released Hb [77]. Moreover, the lower hemolysis after 2 h of incubation of RBCs with AAPH may be explained by the presence of endogenous antioxidants capable of trap AAPH-induced free radicals, whereas after 4 h, they must be very reduced and the hemolysis rate is higher [86].

RBCs treated with caffeates without AAPH showed an inhibition of hemolysis nearly equal to negative control. Thus, caffeates by themselves did not demonstrate a pro-hemolytic effect (assay not shown). In the presence of AAPH, all caffeates were capable to inhibit AAPH-induced hemolysis in more extent than CA (<12%) (**Figure 16**), demonstrating a protective activity. In contrast to the obtained results, Wang et al. [42] reported a significantly inhibition in the AAPH-induced hemolysis with the increment in CA concentrations (0, 3, 6, 12, 24, or 48 μg/ml) [42].

In the present study, the lower activity of CA at all concentrations than caffeates may be justified by the carboxylic moiety be deprotonated at neutral pH, that prevents the diffusion of CA through the plasma membrane [87]. With this in mind, the lipophilization of CA on the carboxylic moiety by esterification with different alkyl chain length could be an advantageous strategy to increase the bioavailability of the molecule and thus its antioxidant activity. Indeed, more lipophilic compounds can cross phospholipid bilayers easier thanks to their membrane-like feature, but it may not necessarily imply a better antioxidant activity (“cut-off” effect) [88].

C1, C2, and C4 derivatives protected RBCs from the oxidative hemolysis in a concentration-dependent manner with statistical significance (**Figure 16**). Nevertheless, the modification of the side chain length above a butyl group reduced the protective effect of caffeates at the highest concentration (50 μM) (**Figure 16**).

The present study pretends to clarify of whether lengthening the alkyl chain leads to more efficient antioxidant activity against oxidative hemolysis. At 2.5 μM and 5 μM (**Figure 17**), the increase of the alkyl chain significantly increased the protective activity of caffeates and the “cut-off effect” is not observed. At 20 μM and 50 μM (**Figure 17**), all compounds showed a high antihemolytic activity. At 50 μM (**Figure 17**), it is clear a cut-off effect, with the maximum of activity at the C4 derivative. The lower activity of the more lipophilic derivatives (C6, C8 and C16), when compared with the C1-C4 derivatives, may be justified by the destabilization that these concentrations combined with the larger size of the

caffeates may induce in the membrane, independently of their antioxidant action, and consequently may cause hemolysis. At 2.5 µM and 5 µM, they may have a better location in the membrane and ability to protect without destabilizing it.

According to Abdallah et al. [79], phenolic compounds can bind at the lipid-water interface of the lipid bilayer or to be included in the bilayer interior, depending on their chemical properties that determine their location and distribution. Durand et al. [54] studied that the addition of rosmarinate esters affected the membrane structure, and the disordering effects varied with the phenolipid chain length.

The hemolysis results were corroborated by the cellular density of RBCs (**Figure 18**). The analysis of the cellular density was performed qualitatively by optical microscopy. In conditions of maximal oxidative stress (positive control), the number of intact cells decreased and were visible some “ghosts” (lysed cells). The addition of caffeates (5 or 50 µM) avoided the reduction of the cellular density, in accordance with their ability in inhibit hemolysis. Moreover, caffeates by themselves did not seem to decrease the cellular density (negative control vs BC_n 50 µM). These results are in accordance with the study of hydroxytyrosol and its glucoronides in the same system [89].

Considering the concentrations used and the hemolysis assay, it would be expected a higher cellular density at 50 µM than at 5 µM in the case of C1-C4 derivatives. The cellular density seems to be in agreement with the expected.

RBCs are usually described by displaying a discocyte form and, the treatment with AAPH (50 mM) was reported to induce a morphological change to echinocyte or acanthocyte [81]. Baccarin et al. also uses the bilayer couple hypothesis [90] to explain that echinocytes are produced by the insertion of compounds into the outer lipid monolayer, expanding the surface [81].

In the present study, it was observed a high number of echinocytes in the absence of AAPH (negative control), even in the presence of phenolic compounds (BC_n 50 µM) (**Figure 18**). Whereas the addition of AAPH reduced in large extent the presence of echinocytes. Some studies [42, 81, 84] are not in accordance with this morphological analysis. In fact, Wang et al. [42] visualized that AAPH induced the formation of spheroechinocytes and rounded bubble-like erythrocytes, and the treatment of RBCs with 48 µg/ml CA maintained its morphological features largely normal. Mazzula et al. [84] reported a RBC morphological change to acanthocytes with AAPH (50 mM).

The oxidative damage caused in RBCs have been associated with an increase in membrane-bound hemoglobin (MBH) [32]. In the present study, it was observed a significant increase of MBH with AAPH (negative control vs positive control) (**Figure 25**). CA and its derivatives significantly affected the RBC membrane. Thus, it is possible that

compounds somehow are able to affect the membrane and, in the presence of AAPH, the linkage of Hb to the RBC membrane is promoted. In fact, the compounds in the absence of the oxidant seem to induce the linkage of Hb to the RBC membrane in slight extent (BC_n vs negative control) and according to the size of their alkyl side chain. These observations could suggest that CA and caffeates may favor the binding of Hb to the membrane without AAPH in the medium, being the binding enhanced under oxidative stress (in the presence of AAPH). Similar results can be confirmed in a published study [35], in which AAPH increased MBH values but some of the tested compounds further increased MBH values.

Considering the hemolysis assay, phenolic compounds should reduce the MBH levels, avoiding membrane damages and posterior hemolysis. It is important to highlight that the compounds concentration in membrane assays is equivalent to 5 µM and so, comparations with the hemolysis assay should be made at this concentration. The results showed that a lower hemolysis rate does not imply a lower MBH content, despite of the relatively high protection given by the compounds against hemolysis.

The obtained results were in agreement with SDS-PAGE gels (**Figure 22**), where it was visible a high intensity band corresponding to MBH (not quantified).

To further understand the increase in the Hb linked to the RBC membrane values, it was also important to investigate the nature of Hb by performing spectral scans (450-650 nm) of lysed RBC suspensions (**Figure 19** and **Figure 20**). Usually, oxy-hemoglobin have two peaks at 540 and 578 nm [89, 91, 92]. It was observed a decrease in peaks intensity in the presence of AAPH (negative control vs positive control). In the absence of AAPH (**Figure 19**), it was not observed a decrease in peaks intensity in the presence of the compounds (negative control vs BC_n); whereas, in the presence of AAPH, the peaks intensity increased with the presence of the phenolic compounds and in a concentration-dependent manner (**Figure 20**). These results suggest that most of the porphyrinic ring of the Hb linked to the RBC membrane was not in the oxidized form. Similar results have also been observed with other polyphenols [35, 89].

The increased RBC hemolysis has been associated with higher oxidative modification on membranes and decayed antioxidant capacity [86].

The protein organization network underlying the inner membrane layer maintains the RBC membrane shape and stability [81]. It is well known that AAPH alters the membrane protein profile by reducing cytoskeletal LMW (low-molecular-weight) protein content and increasing the production of HMW (high-molecular-weight) proteins [70, 85].

Spectrin is essential for RBC shape, and membrane integrity and stability [93]. Thus, it would be expected some change by AAPH in spectrin bands and consequently in other proteins that interact with spectrin. Celedón et al. [94] had found a preferential

modification/loss of band 3 protein instead of spectrin by radicals generated in the extracellular medium, due to localization of band 3 in the membrane lipid bilayer whereas spectrin is extrinsically localized in the membrane cytosolic side. The protein loss was suggested to result from the fragmentation of the polypeptide chain caused by free radicals and/or by proteinases [94]. These authors [94] further confirmed that membrane-bound proteinases act/degrade the oxidatively modified proteins and that this was prevented by proteinases inhibitors (e.g. PMSF). Moreover, Celedón et al. [95] observed the decrease of band 3 protein in oxidized cells, whereas spectrin band was not modified and LMW peptides (lower than 45 kD) bands were decreased.

In the present work, SDS-PAGE gels showed changes in G3PD and stomatin/tropomyosin in the presence of AAPH (**Figure 23** and **Figure 24**). Although without statistical significance, spectrin ($p=0.260$) and band 3 ($p=0.099$) also showed some differences between compounds. In the absence of AAPH, it was observed interactions between the compounds and spectrin, and between the compounds and band 3. Apparently, the more lipophilic compounds could interact with spectrin, located at the inner surface of the membrane. On the other hand, the more hydrophilic compounds seemed to interact with band 3, a transmembrane protein. In both cases, the percentage of membrane proteins decreased. In the presence of AAPH, similar interactions were observed between spectrins and the more lipophilic compounds with a decrease in their percentage. In contrast, all compounds tend to decrease the changes in band 3 protein, in particular, the more lipophilic compounds, rising the percentage above the value found for the positive control. The interactions of the more lipophilic compounds (C6-C16) with spectrins seem to protect RBCs from hemolysis.

Proteins can also undergo oxidative modification by aldehydes from lipid peroxidation, such as malonaldehyde (MDA) and 4-hydroxy-2-nonenal (HNE) [96], which production was induced by AAPH. AAPH decomposes into peroxyl radicals that induce oxidation of PUFAs in RBC membranes, initiating the lipoperoxidation (LPO) process [83] and the consequent formation of TBARs [84], such as malonaldehyde (MDA). MDA is a highly reactive molecule that can cross-link RBC phospholipids and proteins. This impairs a variety of membrane-related functions and ultimately leads to hemolysis, releasing Hb [33, 86].

It has been previously referred that AAPH radicals do not induce significant lipid peroxidation of RBCs before the onset of hemolysis, which suggested that peroxyl radicals do not reach PUFAS in the inner surface of the RBC membrane [94]. Nevertheless, the incubation of RBCs with AAPH significantly increased LPO levels (negative control vs positive control) (**Figure 26**), which was already observed by Wang et al. [42]. In this work, we also observed a very significant increase in the LPO values in the presence of AAPH.

In the presence of compounds, the values of LPO were slightly lower but not considered significant. Wang et al. [42] had observed an inhibition of MDA formation by CA, and related this result with a maintenance of the structural integrity of RBC membranes and a protection from oxidative damage. Yang et al. [86] also reported an increase in LPO induced by AAPH and a reduction with RBCs also treated with pearl powder. Salini et al. [85] also suggested that the prevention of LPO could inhibit the AAPH-induced hemolysis.

The results suggest that caffeates lessen membrane peroxidation and protect RBCs from oxidative injuries and consequent hemolysis.

The RBC oxidative stress increases protein oxidation and membrane LPO and decreases the endogenous defenses. These alterations may contribute to the architectural damage of RBCs [86].

Catalase is one of the major antioxidant systems in human RBCs used to protect against the toxicity of H_2O_2 [96]. In the present work, the CAT activity was significantly decreased in the presence of AAPH in the cytosol (**Figure 27**), but increased in the membrane (**Figure 28**). In the presence of compounds, a different behavior was observed in the absence and in the presence of AAPH. In the absence of AAPH, the CAT activity was reduced in the presence of compounds but in the presence of AAPH, the CAT activity tended to increase ($p=0.065$) in the presence of compounds in the cytosol. In the absence of AAPH, phenolic compounds may scavenge radicals in the extracellular medium, so their levels in cytosol will be low and CAT will not be activated in full extent. Mayo et al. [96] reported a rapid enzyme inactivation by the alkyl-peroxyl radicals formed by AAPH at 40 mM. They found that alkyl-peroxyl radicals modify the structure and function of CAT [96]. In fact, our results (negative control vs positive control) are in agreement with Mayo et al. [96]. On the other hand, the CAT activity reduced under AAPH-induced oxidative stress (negative control vs positive control) but the CAT activity increased in the presence of compounds.

In membranes, the obtained results for the CAT activity in the presence of compounds also show similar results: the activity of CAT increased in the presence of AAPH. It is known that CAT can also interact with the inner side of RBC membranes [97]. However, the CAT functioning is not clear. According to literature [98], under oxidative stress, CAT binds to the RBC membrane possibly to protect it. Indeed, it was observed a higher CAT activity on membranes under oxidative stress (negative control vs positive control). Although the CAT activity is higher in the presence of compounds, the increase has not statistical significance, except with C16. So, it is possible that C16 promotes the CAT binding to RBC membrane.

V. Conclusion

Conclusion

Caffeic acid is a phenolic compound abundantly found in the human diet and has demonstrated a strong intracellular antioxidant ability. However, its hydrophilicity may limit its action in lipophilic mediums, such as RBC membranes. A strategy to increase the bioavailability and antioxidant efficiency of caffeic acid consists in synthesizing caffeic acid alkyl esters (phenolipids) by adding different alkyl chains and evaluate their effectiveness in more lipophilic mediums against AAPH-induced oxidative stress.

In this work, it was observed that AAPH was an efficient inductor of oxidative stress in human RBCs by inducing hemolysis, and producing damages detected in the protein profile of RBC membranes, in oxidative stress biomarkers and in the catalase activity.

All caffeates tested showed to be more protective than the parent compound (CA) against the AAPH-induced oxidative damage and capable of preventing it. At 2.5 and 5 µM, the more lipophilic compounds (C6-C16) showed a remarkable activity against hemolysis. However, at 20 and 50 µM, the more hydrophilic compounds (C1-C4) were able to present a better activity than the more lipophilic ones (C6-C16), and we could observe a clear “cut-off effect” with the maximum activity at the C4 at 50 µM. The hemolysis results were in accordance with the analysis of the cellular density performed by optical microscopy.

The membrane proteins analysis did not show clear changes in the protein profiles but some interactions between spectrin and the more lipophilic compounds were observed. Moreover, the % MBH apparently increased in the presence of compounds but in the absence of AAPH, no oxidation of the porphyrinic moiety of hemoglobin was observed and could actually be reduced by compounds when in the presence of AAPH, showing that these compounds interact with hemoglobin besides their antioxidant activity. These interactions between caffeates and proteins seem to protect the RBC from hemolysis.

As future work, these results should be confirmed with a higher number of samples.

VI. References

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