

Protective Effect of *Aloe vera* (*Aloe barbadensis* Miller) on Erythrocytes Anion Transporter and Oxidative Change

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ABSTRACT

The purpose of this study was to evaluate the ability of aqueous extract of *Aloe barbadensis* Miller (*Aloe vera*) on oxidative damage and Anion Exchanger 1 (AE1, also known as Band 3) expression in human erythrocytes exposed to the water soluble free radical initiator 2,2'-azobis-2-amidinopropano dihydrochloride (AAPH). In addition, total phenolic compounds in the extracts were determined as catechin equivalent and the various antioxidant activities were compared to natural and synthetic standard antioxidants such as BHA and ascorbic acid. Since *Aloe vera* extract did not cause a consumption of the cytosolic antioxidant, glutathione (GSH) when it was direct incubated with GSH in basic aerated aqueous solution, this indicates that *Aloe vera* extract does not proceed auto oxidation at this experimental condition. Furthermore, *Aloe vera* extract prevent the consumption of GSH, in radical treated RBCs. It also inhibit consumption of GSH when it was direct incubated with AAPH. *Aloe vera* gel extract inhibits the generation of diphenyl-2-picrylhydrazyl (DPPH) and the scavenging activity was increased in a dose dependent manner. *Aloe vera* extract was shown the similar reducing power than standards BHT and ascorbic acid. Biochemical analysis by SDS-PAGE and western blotting showed that AAPH-induced oxidative stress increased the susceptibility of AE1 to proteolytic degradation. Of note, our data evidenced that *Aloe vera* treatment was able to partially restore the normal RBC membrane protein profiles in a dose-dependent manner. These results clearly demonstrate the antioxidative activity of *Aloe vera* gel extract that might be ascribed to a synergistic action of the bioactive compounds contained therein.

Keywords: AE1 Transporter; *In Vitro* Oxidative Damage; *Aloe barbadensis* Miller; Antioxidant; Red Blood Cells Membrane Protein; Free Radicals

1. Introduction

Aloe vera (L.) BURM. fil. (synonym *A. barbadensis* MILLER) (Liliaceae), is a perennial succulent plant belonging to the Aloeaceae family, a sub-family of the Asphodelaceae [1]. The plant is composed of turgid green leaves joined at the stem in a rosette pattern. Each leaf consists of an outer green rind (skin) and an inner clear pulp (gel) contained water soluble and fat-soluble vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids [2]. Studies have reported that *Aloe vera* gel has many activity including anti-inflammatory [3], antibacterial, antioxidant [4], hypoglycemic and hypolipidemic properties [5,6], and also exert diverse pharmacological and therapeutic activities [7]. As source of synergistic bioactive compounds, it includes anthraquinones, glycoproteins, polysaccharides, vitamins and enzymes [8]. In addition several works reported on the effect of consumption of *Aloe vera* gel on gastric ulcer,

gastric microcirculatory changes, hepatoprotective and clinical treatment of sepsis [9,10]. The *Aloe vera* gel could be useful to neutralize free radicals for maintaining the integrity of the antioxidant status. According to this potential activity, the aim of this work was to study the properties of *Aloe vera* as radical scavenger protecting Red Blood Cell Membrane (RBCM) from 2,2'-Azobis (2-methylpropionamide) dihydrochloride (AAPH) induced oxidative injury and preserving the RBC Anion Exchanger 1 (AE1), also called Band 3. This transporter is 95 kDa protein is composed of two functionally distinct domains. The 43 Kda N-terminal cytoplasmic domain of band 3 provides an anchor for the membrane skeleton via interaction with ankyrin, protein 4.1, and protein 4.2. The 53 kDa C-terminal membrane domain that is thought to traverse the bilayer 12 times and exchanges Cl⁻ for HCO₃⁻ across the plasma membrane during the gas transporter in the blood and also mediates removal of senescent RBCs from circulation. Since quan-

titative changes in this protein are important in maintaining cell morphology [11], we evaluated the RBCM modification as manifested by a decreasing cytoskeletal protein content, which can lead to abnormalities in RBC shape and disturbances in the microcirculation [12,13].

2. Material and Methods

2.1. Chemicals and Materials

The following chemicals were used: Diphenyl-1-picrylhydrazyl (DPPH), Potassium phosphate, Catechin hydrate, Butylated hydroxytoluene (BHT), Sodium phosphate dibasic, Sodium phosphate monobasic, Potassium ferricyanide(III), Trichloroacetic acid (TCA), Iron(III) chloride, L-Ascorbic Acid, L-Glutathione reduced (GSH), Sodium tetraborate decahydrate, Boric Acid, 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), Phosphate buffered saline (PBS), Methanol, Sodium carbonate, meta-Phosphoric acid, Ethylenediaminetetraacetic acid (EDTA), Phenylmethanesulfonyl fluoride (PMSF), Acrylamide, N,N'-Methylenebis (acrylamide), (hydroxymethyl) aminomethane (Tris), Sodium dodecyl sulfate (SDS), Ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), Glycine, Red Ponceau, Bovin Serum Albumins (BSA), Tween 20, Whatman® 3 MM paper, Protran® nitrocellulose membranes 0,45 µm, were purchased from Sigma Chemical Co. (St.Louis, MO). Potassium ferricyanide, Ferric chloride, Sodium Chloride, and Folin-Ciocalteu phenol reagent were acquired from Merck (Milan, Italy). Mouse monoclonal anti-Band 3 and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Biotinylated anti-horse IgG and Avidin-biotin HRP visualization systems (Vecstain® ABC kit PK-6100) were procured Vector Laboratories, Inc (California, USA). 7315 UV/V spectrophotometer (Jenway, Staffordshire, United Kingdom) was used for spectrophotometric assay. Centrifuge centric 200/R (Technica, Železniki, Slovenia) was used for centrifugations.

2.2. Plant Material

Phenolic substances, known to be responsible for the antioxidant activity of plant extracts, are mostly extracted by organic solvents but as plants are commonly consumed as water extracts by people, the aqueous extract of *Aloe vera* leaves were preferably used in our investigation. Mature *Aloe vera* leaves was collected from Drogo farm, Rocca Imperiale, Cosenza (Italy).

2.3. *Aloe vera* Leaf Gel Extract

The gel (100 g = 0.75 g dry matter) was homogenized in a electric blender, then diluted with an equal volume of

PBS and homogenized for a second time. The extract was kept at 4°C overnight, then filtered through cloth. The clear filtrate was kept at -20°C in aliquots.

2.4. Total Phenols Content (TPC)

Aliquot of 0.1 ml of *Aloe vera* gel extract was made up to 4.6 ml with distilled water in a tube. After addition of 0.1 ml Folin-Ciocalteu reagent (previously diluted 3-fold with distilled water) and 0.3 ml 2% aqueous sodium carbonate solution, tubes were vortexed and the absorbance of the blue color that developed in each assay mixture was recorded after 2 h at 760 nm, against a blank containing 0.1 ml of the extraction solvent [14]. Catechin hydrate (0.1 - 0.8 mg/g) was used for calibration of a standard curve. The results were expressed as grams Catechin Equivalents (CEs)/L of gel extract. The data were presented as the average of triplicate analyses.

2.5. DPPH Free Radical Scavenging

Free radical scavenging activity was determined using DPPH assay [15]. When DPPH reacts with an antioxidant compound which can donate hydrogen, it is reduced. The change in color from deep violet to golden/light yellow can be measured at 517 nm. Briefly, 1 mL of 0.3 mM of DPPH solution was added to 1 mL of *Aloe vera* gel extract (5 - 30 µM CE), and incubated in the dark at room temperature for 30 min. Inhibition of free radical DPPH in percent (%) was calculated from the absorption according to the following equation:

$$\text{Scavenging effect [\%]} = \left(\frac{1 - \text{Abs sample}_{517\text{nm}}}{\text{Abs control}_{517}} \right) \times 100 \quad (1)$$

Ten independent experiments were carried out and results were expressed as mean values ± standard error of the mean (S.E.M.). The extract concentration providing 50% inhibition (EC50%) was calculated from the graph of scavenging effect percentage against the extract concentration. Ascorbic acid and BHT were used as standards.

2.6. Reducing Power

Aloe vera gel extract (5 - 30 µM CE) were mixed with 200 mM PBS (pH 6.6) and 1% K₄[Fe(CN)₆]. The mixture was incubated at 50°C for 20 min and 10% TCA was then added to the mixture and centrifuged 650 g for 10 min. The upper layer of solution was mixed with distilled water and 0.1% FeCl₃. The increase in absorbance at 700 nm of the reaction mixture indicated reducing power [16]. BHT and Ascorbic Acid were used as standards.

2.7. Autooxidation

Aloe vera gel (50 - 500 μM CEs) was incubated with GSH (100 μM) in a borate buffer (50 mM, pH 9.25). An appropriate volume of the reaction mixture was removed at intervals and added to an aqueous solution of DTNB (0.2 mM, pH 9.0). After 3 min incubation, absorbance at 412 nm was measured and thio concentrations calculated by using [17]:

$$\varepsilon_{412 \text{ nm}} \times 13,600 \text{ cm}^{-1} \cdot \text{M}^{-1} \quad (2)$$

2.8. Preparation of RBCs Suspension

Blood was obtained from donor volunteers (10 men aged 47 ± 11 years) by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The samples were centrifuged at 1100 g/min for 10 minutes; plasma and buffy coat were carefully removed and discarded. RBCs were washed three times with PBS pH 7.4. RBCs used on the day were pre-incubated with AAPH at final concentration of 50 mM. In all sets of experiments incubations of RBCs were carried out at 37°C for 180 minutes in the presence of AAPH with and without *Aloe vera* gel under gentle shaking. Untreated RBCs has been used as control.

2.9. Glutathione Content in RBCs

Blood GSH level was measured spectrophotometrically using Ellmans reagent (DTNB) as a coloring reagent as the method described by Beutler *et al.* [18]. Briefly, 0.2 ml blood was mixed with 1.8 ml double distilled water and glacial meta-phosphoric acid (a precipitating solution) was added, centrifuged and supernatant was collected. Lastly, the supernatant was mixed with 300 mM disodium hydrogen sulphate and DTNB reagent and was allowed to stand for 2 min at room temperature. The absorbance was read at 412 nm. GSH values were determined using the molar extinction coefficient of $13,600 \text{ cm}^{-1} \cdot \text{M}^{-1}$.

2.10. Preparation of RBCs Membrane

Performed RBCs were lysed in 20 volumes of lysis buffer (5 mM sodium phosphate, 1 mM EDTA, pH 7.9), in presence of the protease inhibitor phenylmethylsulfonyl fluoride (final concentration of 0.1 mM) were centrifuged (20 min at 4°C , 13500 g). To removal from the button granulocyte debris, the membrane pellet was re-suspended in the above buffer and centrifuged once more. The ghosts were washed 5 - 7 times in a similar manner until white [19]. The protein concentration of the RBC membrane suspensions was determined by Bradford method [20].

2.11. Western Blotting

Lysates (25 μg) were equal loaded for 5 min with β -actin and then subjected to 10% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in low-fat (1%) milk diluted 1:20 into TBST (10 mM Tris HCl [pH 7.5], 50 mM NaCl, 0.1% Triton X-100) for 30 min, followed by incubation for 1.5 h with the anti-Band 3 antibody. Membranes then were washed extensively with TBST and immuneoreactive protein was detected by incubation with biotinylated anti-horse IgG. Protein was visualized using Vecstain[®] ABC kit.

2.12. Statistical Analysis

Statistical analyses of the results were performed using one and two-way ANOVA followed by Bonferroni's test. P value < 0.05 was considered statistically significant.

3. Results

3.1. Total Phenols Content

The antioxidant activity of plant materials closely correlated with the content of their phenolic compounds [21]. The *Aloe vera* gel extract was shown to contain 1.34 ± 0.087 grams of CEs/L.

3.2. Reducing Power

The presence of reductants in the samples tested would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700 nm. **Figure 1** shows the reducing power of *Aloe vera* gel and the reference compounds, BHT and Ascorbic Acid. Here, *Aloe Vera* gel (10 μM CEs) was shown the same reducing power than standards.

3.3. DPPH Radical Scavenging Activity

Aloe vera gel extract inhibits the generation of DPPH radical in a dose dependent manner and its IC_{50} value was found to be $2.9 \pm 0.1 \mu\text{g/ml}$ (10 μM CEs), which is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color). A lower value of IC_{50} indicates the greater antioxidant activity of a test substance. **Figure 2** shows the radical scavenging ability of *Aloe vera* gel and the reference compounds.

3.4. Autooxidation of *A. vera* Gel Extract

AAPH induce a decrease in sulfhydryl groups after incubation with glutathione in basic aerated aqueous solution (borate buffer, pH 9.25). *Aloe vera* gel extract did not cause a consumption of GSH when it was direct incubation with AAPH (**Figure 3**).

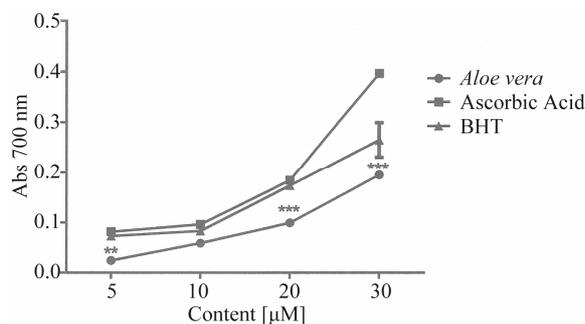


Figure 1. Reducing power of *Aloe vera* gel vs BHT and ascorbic acid. Values are mean \pm S.E.M. of ten independent determinations. Two-way ANOVA, significant different from ascorbic acid and BHT: “***” $P < 0.01$; “****” $p < 0.001$.

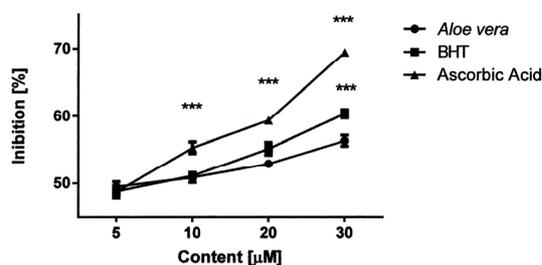


Figure 2. Antioxidant ability of *Aloe vera* gel extract vs BHT and ascorbic acid. Value are mean \pm S.E.M. of ten independent determinations. Two-way ANOVA, significant different from *A. Vera* gel: “****” $p < 0.001$.

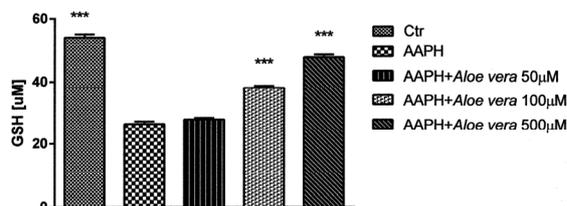


Figure 3. Autooxidation of *Aloe vera* gel extract and consumption of GSH by AAPH. Value are mean \pm S.E.M. of ten independent determinations. One-way ANOVA, significant different from AAPH “****” $p < 0.001$.

3.5. Effects of *A. vera* Gel Extract on GSH Content

The GSH content of untreated RBCs at 37°C after 180 min was $0.37 \pm 0.029 \mu\text{M}$. AAPH (50 mM) induced a rapid consumption of cytosolic GSH ($0.25 \pm 0.015 \mu\text{M}$). Addition of different concentrations *Aloe vera* gel extract (5, 10, 20 and 30 μM CEs), prevented the GSH consumption induced by AAPH, (Figure 4).

3.6. Effects of *A. vera* Gel Extract on AAPH-Induced Changes in RBCM Proteins

Western Blot analysis of RBCM isolated after AAPH in-

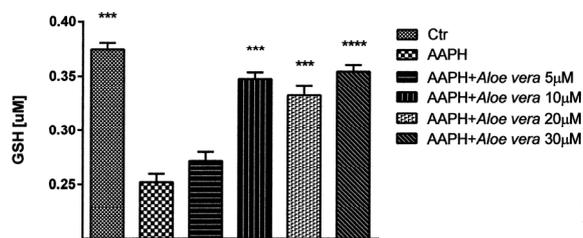


Figure 4. Effects of *Aloe vera* gel extract on AAPH-induced depletion of intracellular GSH content in RBCs. Values are mean \pm S.E.M. of ten independent determinations. One-way ANOVA, significant different from AAPH “****” $p < 0.001$.

cubation with and without *Aloe vera* gel extract (5 - 30 μM CEs) were performed with mouse monoclonal antibody anti-Band 3 (Figure 5). Our dates showed: (a) an increased susceptibility to proteolysis breakdown of AE1 transporter during oxidative damage (lane 2) compared with the intact Band 3 in untreated, control sample (lane 1); (b) a reduction of Band-3 degradation after incubation with *Aloe vera* gel extract in a dose dependent manner (lanes 3-6); β -actin was used as loading control.

4. Discussion

DPPH scavenging activity showed the effectiveness of the plant extract in donating hydrogen proton to the lone pair electron of the radical. Since *Aloe vera* gel extract inhibits the generation of DPPH radical in a dose dependent manner, it could be assumed that gel extract contains compounds capable of donating protons to the free radicals thus demonstrating its reducing power. This property is generally associated with the presence of reductones which have been reported react with certain precursors of peroxide, thus preventing their formation [22]. Much of the evidence on the beneficial effects of dietary polyphenols is derived from experiments performed *in vitro* or in animal models, and by using concentrations much higher than those generally contained in the human diet. For instance, the range of concentrations required for an effect *in vitro* varies from 0.1 μM to 100 μM while physiologic concentrations do not exceed 10 μM [23]. In accordance with these previous results, our data demonstrate that *Aloe vera* extract (and its bioactive compounds) at a concentration of 5 - 10 μM of catechin equivalent is capable to prevent consumption of GSH and the protein breakdown induced by AAPH at the experimental condition. Radical attack could be the result of GSH oxidation that occur indirectly through the reduction of reactive thiol groups in oxidized membrane proteins. Antiradical properties of *Aloe vera* gel extract could be implicated in the regeneration of GSSG in its active reduced form. These results could explain why *Aloe vera* gel extract did not proceed AAPH-induced depletion of intracellular

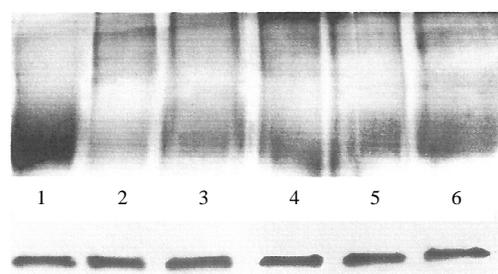


Figure 5. Western blotting analysis of RBCM prepared from the reaction mixture. Lane 1, intact RBC membrane proteins from control sample. Lane 2, RBC exposed to AAPH (50 mM) at 37°C for 3 h. Lane 3 - 6, RBC exposed to AAPH in the presence of 5, 10, 20 and 30 μ M *Aloe vera* gel extract. The amount of protein loaded was 25 μ g in each case, β -actin serves as a loading control.

GSH content in RBCs. It is well known that AAPH can exert formation of High Molecular Weight (HMW) proteins with a concomitant decrease of Low Molecular Weight (LMW) proteins [24]. In accordance, our Western Blotting analysis showed that *Aloe vera* gel extract inhibited AAPH-induced changes in RBC membrane proteins in a concentration dependent manner. AAPH-induced proteolysis of AE1 is thought to play a pivotal role in the assembly of the membrane skeleton which is crucial for the mechanical integrity of RBCs [25,26]. A loss of interaction between band 3 and the cytoskeleton may be responsible, at least in part, for an increased susceptibility of band-3 to crosslinking which, in turn, could lead to membrane vesiculation. Vesicle release *in vivo* is considered an integral part of the physiological aging process, enabling the removal of damaged membrane patches from otherwise functional erythrocytes [26].

5. Conclusion

The present study demonstrates that *Aloe Vera* gel extract induces beneficial effects in terms of protection against reduction of GSH content, RBCM protein breakdown and band-3 degradation caused by AAPH. Our results also underline the important role of *Aloe vera* gel extract in the maintenance of the antioxidant status and antioxidant defense in RBCs. This scavenging activity might be due to the synergistic actions of bioactive compounds present in the plant extracts. We suggest that dietary *Aloe vera* supplementation can help to prevent oxidative stress and might be also useful for the treatment of oxidative stress-related human disorders by virtue of its antioxidant activity.

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