

# *In vitro* protective effect of *Rhodiola rosea* extract against hypochlorous acid-induced oxidative damage in human erythrocytes

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**Abstract.** *Rhodiola rosea* L. (Crassulaceae) is a plant living at high altitudes in Europe and Asia. Its roots have long been used in the traditional medical system of these geographical areas to increase the organism resistance to physical stress; today, it has become an important component of many dietary supplements. In this study we investigate the antioxidant capacity of the *R. rosea* aqueous extract evaluating its ability to counteract some of the main damages induced by hypochlorous acid (HOCl), a powerful oxidant generated by activated phagocytes, to human erythrocytes. Ascorbic acid was used as a reference substance because of its physiological HOCl-scavenging ability. Our study demonstrates that *R. rosea* is able to significantly protect, in a dose-dependent manner, human RBC from glutathione (GSH) depletion, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inactivation and hemolysis induced by the oxidant. Furthermore, we demonstrate that *R. rosea* aqueous extract acts from the inside of the erythrocyte suggesting a probable involving of cell components. The protection on GSH afforded by the *R. rosea* extract with respect to ascorbic acid, occurred also if added 2 or 5 min. later than the oxidant, suggesting a more rapid or powerful effect.

**Keywords:** Antioxidant activity, hypochlorous acid, human erythrocyte, *Rhodiola rosea* L., glutathione, glyceraldehyde-3-phosphate dehydrogenase

## 1. Introduction

*Rhodiola rosea* L. (Crassulaceae), also known as “arctic root” or “golden root”, is a plant living at high altitudes in Europe and Asia. Roots and rhizomes have long been used in the traditional medical system of these geographical areas to increase the organism resistance to different chemical, biological and physical stress [13]; today this plant has become an important component of many dietary supplements. In addition to *R. rosea*, over 200 different species of *Rhodiola* have been identified. The chemical composition and physiological properties of *Rhodiola* species are to a degree species-dependent, although some overlap

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in constituents and physiological properties does exist. Twenty-eight compounds have been isolated from the roots and above ground-parts of *R. rosea* and among these, numerous antioxidant substances: p-tyrosol, organic acids (gallic acid, caffeic acid, and chlorogenic acid) and flavonoids (catechins and proanthocyanidins) [17]. Salidroside and cinammoyl-glycosides (rosin, rosavin and rosarin, distinctive of the *rosea* species) along with p-tyrosol are responsible for high therapeutic action [17]; it has been shown that alcoholic and/or aqueous extracts of different *Rhodiola* species act as free radical scavengers against DPPH (1,1-diphenyl-2-picrylhydrazyl) and hydroxyl radical [16,17,21]. The p-tyrosol is readily and dose-dependently absorbed after an oral dose and appears to show a significant antioxidant activity *in vivo* [7,29]. It is also demonstrated that the administration of *R. rosea* extracts to rats and mice treated with antitumoral drugs such as cisplatin and cyclophosphamide, makes these drugs more efficient and less cytotoxic [24,32].

The aim of the present study was to investigate the possible protective effect of *R. rosea* aqueous extract in an *in vitro* model system represented by human erythrocytes exposed to hypochlorous acid (HOCl). HOCl is a powerful oxidant generated by activated phagocytes (neutrophils and monocytes) and it is produced by the myeloperoxidase (MPO)-catalyzed oxidation of chloride by hydrogen peroxide [1, 38]. Because of the high reactivity toward a large number of biological molecules, HOCl is considered to be a major cause of tissue injury in inflammation [1,12,38]. Several studies have reported HOCl damages on bacteria, endothelial cells, tumor cells and erythrocytes [6,10,14,15,26,30,37]. Erythrocytes have been used extensively as a model system for investigating mechanisms of neutrophil-mediated cell damage since these cells, lacking protein synthesis machinery, represent a simplified model [4,34–36] and moreover they are one of the main targets for neutrophil-derived oxidants, including HOCl, during inflammation states [28].

In red blood cells, it is reported that oxidation of GSH and of -SH groups of membrane proteins occurs at low concentration of hypochlorous acid [34,38]; the reaction between HOCl and protein lysine residues yields chloramine species which subsequently decompose giving nitrogen-centered protein radicals [11,12]; cholesterol and fatty acids are modified by HOCl yielding  $\alpha$ - and  $\beta$ -chlorohydrins [3, 4]; inactivation of some important enzymes such as glyceraldehyde-3-phosphate dehydrogenase and glutathione-S-transferase are also documented in different model systems [22,27,33]. Moreover, some authors have demonstrated that in human erythrocytes HOCl causes increase of  $K^+$ -leak, alteration of membrane deformability, cross-linking of membrane proteins and extensive disruption of the membrane inducing lysis [35,36].

Since numerous are the examples of protective effects afforded by plant extracts on damages induced by an oxidative stress [20,25,31], the aim of the present study was to investigate the possible protective effect of *R. rosea* extract on GSH depletion, GAPDH inactivation and hemolysis induced *in vitro* by HOCl to human erythrocytes, in comparison with ascorbic acid (AA) effect. Ascorbic acid is, in fact, one of the main physiological scavenger of HOCl [5,9,22].

## 2. Materials and methods

### 2.1. Materials

*Rhodiola rosea* roots and rhizomes were kindly provided by Alpine Botanic Garden “Chanousia” (Aosta, Italy). Sodium hypochlorite (NaOCl) was purchased by Sigma-Aldrich (Italy). At pH 7.4 this reagent contains approximately 1:1 ratio of HOCl and OCl<sup>-</sup> and is subsequently referred to as HOCl [35]. All reagents used were produced by Sigma-Aldrich (Italy) and ICN (Italy). Composition of phosphate buffered saline (PBS) was 0.15 M NaCl, 1.9 mM NaH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4.

## 2.2. Preparation of *R. rosea* aqueous extract

The *R. rosea* roots and rhizomes were air-dried and powdered. Aqueous extract was obtained by a threefold extraction of the plant material with phosphate buffered saline (PBS) at room temperature (12 mg/2.4 ml final volume). The filtered aqueous phase was tested for antioxidant activity as described below.

## 2.3. Determination of antioxidant activity

A spectrophotometric method based on the formation of a phosphomolybdenum complex was used to analyze the antioxidant activity of the *R. rosea* extract as reducing ability [23].

Different doses of the extract (10–50  $\mu$ l) were incubated at 95°C with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After 90 min. of incubation, the absorbance at 695 nm was measured against a blank. Results were referred to concentrations of ascorbic acid which was chosen as a reference substance.

## 2.4. Blood samples

Red blood cells (RBC) were obtained from healthy consenting donors. Heparinized blood was centrifuged at 3,000 rpm for 10 min. at 4°C. After removal of plasma and buffy coat, the RBC were washed three times with cold PBS and processed for subsequent analyses.

## 2.5. Incubation of red blood cells with *R. rosea* extract and HOCl

RBC (10% v/v) were incubated in a shaking bath for 15 min. at 37°C in PBS in the presence of the *R. rosea* extract (150  $\mu$ l/100  $\mu$ l packed RBC). HOCl was added as a single bolus of a diluted solution in PBS, which concentration was determined spectrophotometrically at 292 nm ( $\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$ ) [19].

In some experiments *R. rosea* extract was added to the RBC suspensions 2 or 5 min. after HOCl. After 15 min. of incubation, RBC suspensions were centrifuged at 3,000 rpm for 10 min. at 4°C and washed one time with PBS. Then RBC were lysed with 19 volumes of cold distilled water and after 10 min. on ice, the lysates were used for determination of glutathione (GSH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hemoglobin (Hb).

## 2.6. Pre-incubation of red blood cells with *R. rosea* extract

RBC (10% v/v) were pre-incubated for 10 min. with *R. rosea* extract (150  $\mu$ l/100  $\mu$ l packed RBC) in PBS, in a shaking bath at 37°C. After centrifugation at 3,000 rpm for 10 min. at 4°C, the supernatant was discarded and packed RBC were resuspended (10% v/v) in PBS with 0.5 mM HOCl. The incubation was carried on for 15 min. as previously described.

## 2.7. Incubation of lysed red blood cells with *R. rosea* extract

Washed RBC were lysed with 1 volume of cold water and maintained at 4°C for 10 min. The lysates were resuspended with 4 volumes of PBS and 0.5 mM HOCl was added in the presence or absence of *R. rosea* extract (150  $\mu$ l/100  $\mu$ l lysed RBC). After 15 min. of incubation in a shaking bath at 37°C, the lysates were used for determination of GSH and GAPDH as described below.

### 2.8. GSH and GAPDH evaluation

Glutathione and glyceraldehyde-3-phosphate dehydrogenase were measured spectrophotometrically as described by Beutler [2].

### 2.9. Evaluation of hemolysis

The hemolysis was evaluated as hemoglobin (Hb) released from cells in the supernatant. Hb was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler [2].

### 2.10. Statistical analysis

Statistical analyses were performed using Kruskal-Wallis test. All data are expressed as mean  $\pm$  S.D. of at least five different determinations.

## 3. Results

### 3.1. *R. rosea* extract antioxidant activity

Total antioxidant activity of *R. rosea* aqueous extract (12 mg/2.4 ml PBS) measured as reducing ability was assessed with the spectrophotometric method described by Prieto et al. [23] based on the reduction of Mo(VI) to Mo(V) and on the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. This method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid or  $\alpha$ -tocopherol. In the present study ascorbic acid was chosen as a reference substance because of its known ability as HOCl scavenger [5,9,22]. The Fig. 1 shows a linear dose-response curve that was obtained till 60  $\mu$ l of *R. rosea* extract; absorbance results were therefore correlated to equivalents of ascorbic acid. It should be noted that 25  $\mu$ l of *R. rosea* extract have the same reducing ability as 230  $\mu$ M ascorbic acid.

This assay, repeated every 5 days on the extract maintained at 4°C for at least one month, revealed that the *R. rosea* extract antioxidant activity did not show any decrease. Moreover, besides antioxidant compounds are usually light-sensitive, there were no differences in antioxidant activity between *R. rosea* extracts kept in the dark or by the light (data not shown).

### 3.2. Dose-dependent effect of *R. rosea* extract on HOCl damages

Antioxidant activity of *R. rosea* extract was then tested in a known *in vitro* model system of oxidative stress represented by human erythrocytes exposed to hypochlorous acid (HOCl) [34–36,38]. Glutathione (GSH) depletion, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) inactivation and hemolysis are some important damages induced by HOCl to human erythrocytes [34–36,38]. In order to verify a possible protective effect, increasing doses of *R. rosea* extract were added to RBC suspensions at the same time as HOCl. As evidenced in Fig. 2, the presence of 25  $\mu$ l *R. rosea* extract/100  $\mu$ l packed RBC resulted in a significant protection on GSH, GAPDH and hemolysis; doses of the extract equal to or above 150  $\mu$ l/100  $\mu$ l packed RBC were able to provide a complete protection on these effects. For this reason in subsequent experiments the selected dose was 150  $\mu$ l *R. rosea* extract/100  $\mu$ l RBC.

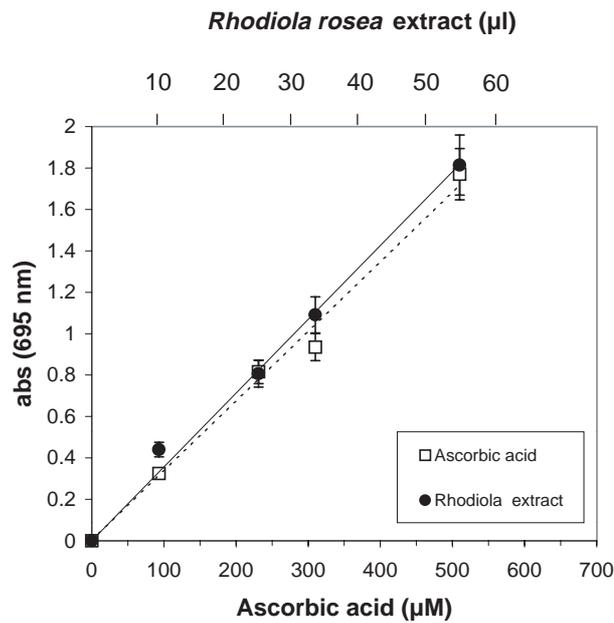


Fig. 1. Dose-response curve of *R. rosea* extract and ascorbic acid according to the Prieto's method. The spectrophotometric method described by Prieto et al. [23] was applied to *R. rosea* extract to evaluate its total antioxidant activity. Different doses of *R. rosea* extract were mixed with the reagent solution as reported in Materials and Methods. A calibration curve with ascorbic acid was used as reference (- -□- -). 25  $\mu$ l of *R. rosea* extract are equivalent, as reducing ability, to 230  $\mu$ M ascorbic acid. Data are the mean  $\pm$  S.D. of at least 5 experiments.

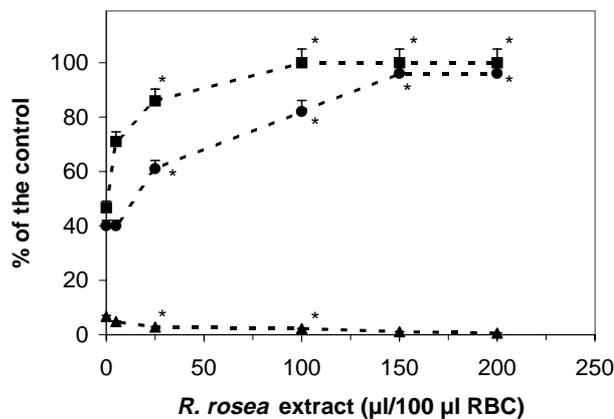


Fig. 2. Dose-dependent protective effect of *R. rosea* extract on HOCl damages in intact red blood cells. Human RBC (10% v/v) were incubated with 0.5 mM HOCl at 37°C for 15 min. in the presence of increasing doses of *R. rosea* extract (10–25–100–150 and 200  $\mu$ l). GSH levels (●), GAPDH activity (■) and hemolysis (▲) were measured as reported in Materials and Methods. \* $P < 0.05$  vs. HOCl. Data are the mean  $\pm$  S.D. of at least 5 experiments.

### 3.3. Effect of *R. rosea* extract on the GSH depletion induced by HOCl in human RBC

The protective effect observed with 150  $\mu$ l *R. rosea* extract on the GSH depletion induced by HOCl is reported in Fig. 3. The results were compared with the effect of an equivalent ascorbic acid concentration (1.3 mM), measured as reducing ability. If erythrocytes are incubated for 15 min. with 0.5 mM HOCl,

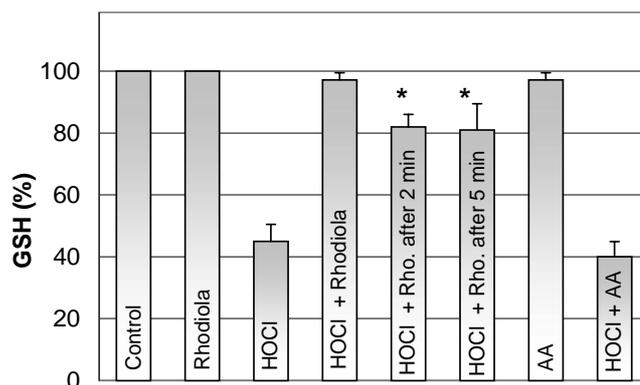


Fig. 3. Effect of *R. rosea* extract on HOCl-induced oxidation of GSH in intact red blood cells. Human RBC (10% v/v) were incubated with 0.5 mM HOCl at 37°C for 15 min. in different conditions. 150  $\mu$ l aliquot of *R. rosea* extract was added at the time of HOCl or after 2 and 5 min. 1.3 mM ascorbic acid (AA) was used as a reference substance for its equivalent reducing ability. GSH levels were measured as in Materials and Methods and expressed as % of the control. Data are the mean  $\pm$  S.D. of at least 5 different determinations. \* $P < 0.05$  vs. HOCl + *R. rosea* added at the same time.

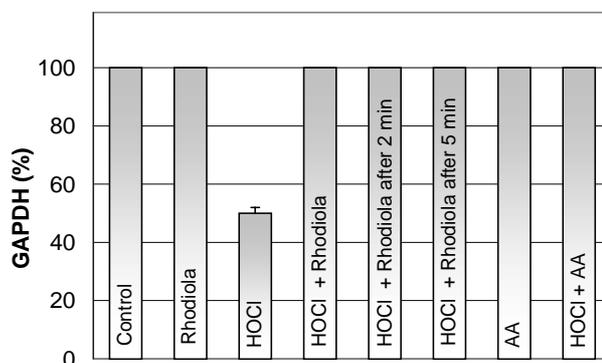


Fig. 4. Effect of *R. rosea* extract on HOCl-induced inactivation of glyceraldehyde-3-phosphate dehydrogenase in intact red blood cells. Human RBC (10% v/v) were incubated with 0.5 mM HOCl at 37°C for 15 min. in different conditions: 150  $\mu$ l of *R. rosea* extract were added at the same time as HOCl or after 2 and 5 min. 1.3 mM ascorbic acid (AA) was used as reference substance for its equivalent reducing ability. GAPDH activity was measured as in Materials and Methods and expressed as % of the control. Data are the mean  $\pm$  S.D. of at least 5 different determinations.

it is possible to observe a 55% GSH loss. As clearly evidenced by the Figure, when *R. rosea* extract is added at the same time of the oxidant it gave a complete protection on GSH, whereas ascorbic acid added in the same experimental conditions, did not. In experiments in which *R. rosea* extract was added to RBC suspensions 2 or 5 min. after HOCl, a significant but not complete protection was observed.

### 3.4. Effect of *R. rosea* extract on the GAPDH inactivation induced by HOCl in human RBC

The Fig. 4 shows that 0.5 mM HOCl caused to human erythrocytes a 50% loss of GAPDH activity after 15 min. of incubation. 150  $\mu$ l *R. rosea* extract added at the same time as HOCl or after 2 and 5 min., afforded a complete protection to the enzyme activity. 1.3 mM ascorbic acid (with the same reducing ability as 150  $\mu$ l *R. rosea* extract), added at the time of HOCl, showed the same protective effect.

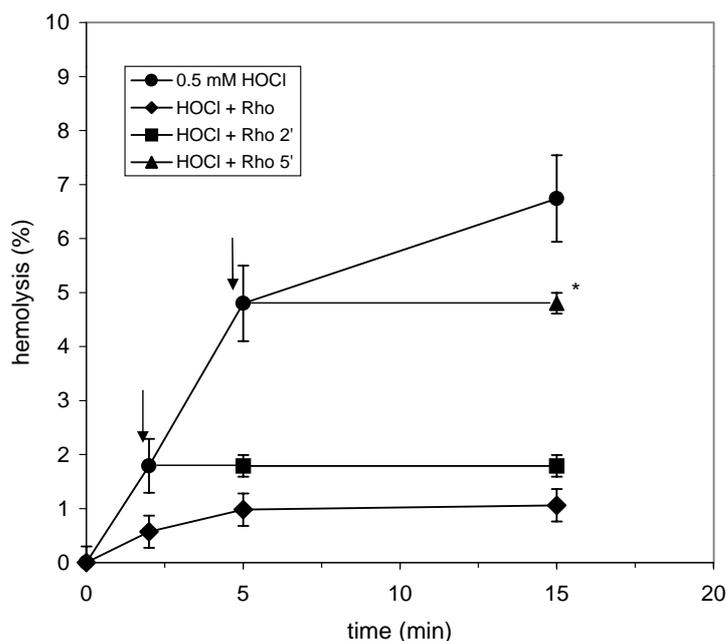


Fig. 5. Effect of *R. rosea* extract on HOCl-induced hemolysis. RBC (10% v/v) were incubated in PBS with 0.5 mM HOCl (●) and the *Rhodiola* extract was added immediately before HOCl (◆), after 2 min. (■) and after 5 min. (▲) as indicated by arrows. Hemolysis was determined as reported in Materials and Methods. Data are the mean  $\pm$  S.D. of at least 5 different determinations. \* $P < 0.05$  vs. HOCl.

### 3.5. Effect of *R. rosea* extract on hemolysis induced by HOCl

Red cell lysis caused by HOCl is well documented [34–36,38]. Figure 5 shows that RBC incubation with 0.5 mM HOCl induced a  $6.74 \pm 1.18\%$  hemolysis. The addition of *R. rosea* extract (150  $\mu$ l/100  $\mu$ l RBC) at the same time as HOCl or 2 min. later, significantly protected RBC from lysis (1.06% and 1.79% hemolysis respectively). *R. rosea* extract added 5 min. after HOCl was able to stop the hemolysis at the reached level: 4.80% was in fact the hemolysis induced by HOCl to RBC after 5 min. of incubation. Our data confirm that ascorbic acid is able to protect erythrocytes from HOCl-induced hemolysis only if added before the oxidant and not if added after 2 or 5 minutes as already demonstrated by Vissers et al. [36] (data not shown).

### 3.6. Effect of the pre-incubation of RBC with *R. rosea* extract

In order to understand if *R. rosea* protective effect was the result of an external scavenging of HOCl, a 10 min. pre-incubation of RBC with *R. rosea* extract (150  $\mu$ l/ 100  $\mu$ l RBC) was performed; after centrifugation and discarding of the supernatant, RBC were re-incubated with 0.5 mM HOCl for 15 min. As shown in Fig. 6(A), the *R. rosea* extract gave the same protective effect on GSH as in experiments where *R. rosea* was co-incubated with HOCl (Fig. 3). Figure 6(B) shows that the pre-incubation with *R. rosea* extract was also effective in preserving GAPDH activity.

Pre-incubation with 1.3 mM ascorbic acid did not protect GSH from oxidation (Fig. 6(A)) confirming the results obtained when ascorbic acid and HOCl were co-incubated (Fig. 3), whereas a significant protection was observed for GAPDH activity (Fig. 6(B)). 1.3 mM ascorbic acid corresponds to 150  $\mu$ l *R. rosea* extract as reducing ability.

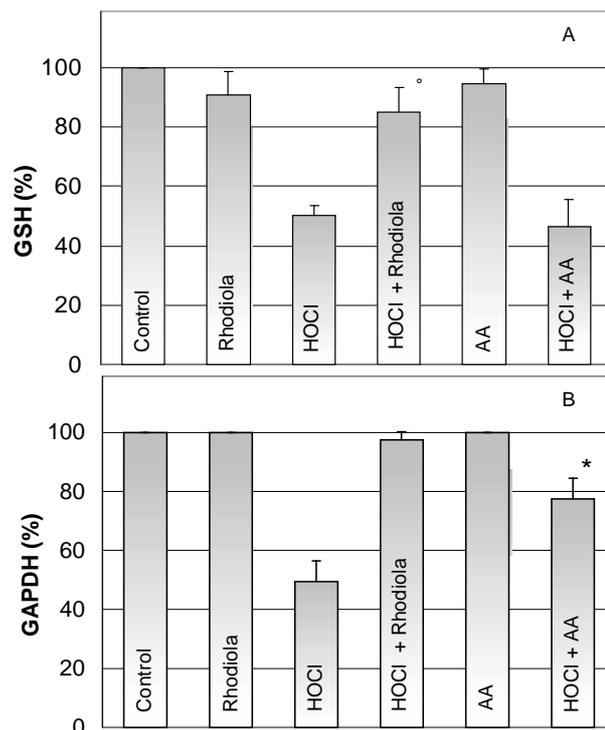


Fig. 6. RBC pre-incubation with *R. rosea* extract: Protective effect on GSH and GAPDH. RBC (10% v/v) were pre-incubated for 10 min. with 150  $\mu$ l of *R. rosea* extract or with 1.3 mM ascorbic acid (AA) used as reference substance for its equivalent reducing ability. After centrifugation, packed RBC were incubated (10% v/v) for 15 min. at 37°C with 0.5 mM HOCl. Figure 6(A) shows the GSH levels and Fig. 6(B) the GAPDH levels. GSH and GAPDH were measured as in Materials and Methods and expressed as % of the control. Data are the mean  $\pm$  S.D. of at least 5 different determinations.  $^{\circ}$   $P > 0.05$  vs. control; \* $P < 0.05$  vs. HOCl.

### 3.7. Effect of *R. rosea* extract on the GSH loss and GAPDH inactivation in hemolysates exposed to HOCl

It has been demonstrated that erythrocyte membrane provides only a partial barrier to HOCl and as a consequence the GSH is oxidated in a similar way both in intact cells and in hemolysates [34]. In Fig. 7(A) it should be noted that in RBC lysates the adding of 0.5 mM HOCl caused a GSH loss of about 60%. Samples with *R. rosea* extract (150  $\mu$ l/100  $\mu$ l lysed RBC) in the presence of HOCl showed a complete protection on GSH levels. This protection was already evident in the first 5 min. of incubation (data not shown). 1.3 mM ascorbic acid in the same experimental conditions provided similar results as *R. rosea* extract, in contrast with data shown for intact cells. Concerning GAPDH (Fig. 7(B)), HOCl induced a 55% loss of enzyme activity which was not observed if *R. rosea* extract was added to the medium. Ascorbic acid also showed a complete protection on GAPDH activity. 1.3 mM ascorbic acid corresponds to 150  $\mu$ l *R. rosea* extract as reducing ability.

## 4. Discussion

Besides the few international literature confirming the antioxidant activity of *Rhodiola rosea*, this plant has recently received attention as the main component of many dietary supplements. The aim of the

present study was to investigate the effect of a *Rhodiola rosea* aqueous extract in an “*in vitro*” model of oxidative stress such as human erythrocytes exposed to hypochlorous acid (HOCl).

Initially the antioxidant activity, as reducing ability, of *R. rosea* aqueous extract (12 mg/2.4 ml PBS) was tested with the spectrophotometric method described by Prieto for the determination of antioxidant capacity of plant and seed extracts [23]. This method revealed a good antioxidant capacity of *R. rosea* extract as evidenced by the comparison with ascorbic acid antioxidant activity (Fig. 1). The fact that this ability persisted for at least a month, even if the extract was maintained by the light, represents an important property.

Experiments of incubation of human erythrocytes with HOCl demonstrated that the presence of *R. rosea* aqueous extract was able to significantly protect, in a dose-dependent manner, human erythrocytes from GSH depletion, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inactivation and hemolysis induced by the oxidant (Fig. 2). We have compared the *R. rosea* effect with the equivalent concentration, as reducing ability, of ascorbic acid, considering its scavenging ability vs. HOCl [5,9,22]; 150  $\mu$ l of *R. rosea* extract are equivalent to 1.3 mM ascorbic acid.

The GSH depletion observed in RBC incubated with 0.5 mM HOCl was totally prevented by the presence in the medium of at least 150  $\mu$ l *R. rosea* extract (12 mg/2.4 ml PBS). Adding *R. rosea* extract 2 or 5 min. after HOCl (Fig. 3), it was possible to evidence a significant but not complete protection on GSH, as was expected since GSH oxidation occurs in less than 2 min. after HOCl addition [34].

The adding of 1.3 mM ascorbic acid to the medium, equivalent to 150  $\mu$ l of *R. rosea* extract as reducing ability, failed to provide any protection on GSH when intact RBC were exposed to 0.5 mM HOCl. This result suggests that the unique extracellular scavenging effect was not sufficient to counteract the reaction between HOCl and intracellular GSH. Ascorbic acid uptake is in fact a slow process [5, 18] and a concentration insufficient to afford a protection on GSH was achieved inside the cell after only 15 min. of incubation. The hypothesis of a unique external scavenging mechanism by *R. rosea* extract was discarded also by pre-incubation experiments in which erythrocytes were firstly incubated with *R. rosea* extract, and, after elimination of the medium, re-incubated with HOCl, to avoid the direct interaction between *R. rosea* and HOCl outside the cells. In these experimental conditions, the same protective effect on GSH depletion as in contemporaneous incubations was observed (Fig. 6(A)). These experiments provided us indirect information about the uptake of *R. rosea* extract by the cell firstly, and demonstrated that the protective effect was probably sustained by a cell-mediated mechanism. This latter hypothesis was also confirmed by chemical reaction experiments in which the incubation at 37 °C of the mixture of HOCl and GSH in PBS in the presence of *R. rosea* extract, did not reveal any protection on GSH loss induced by the oxidant (data not shown).

Since almost all of the GSH loss after treatment of human erythrocytes with HOCl was accounted for as GSSG [34], experiments with hemolysates were carried on to verify if the effect of *R. rosea* extract on GSH was the result of the reduction of GSSG; it is reported that the exposure of lysed or intact RBC to the action of HOCl induced similar decline in GSH levels [34]. In the present study we demonstrated that *R. rosea* extract added to hemolysates at the same time as HOCl was able to afford a complete protection on GSH (Fig. 7(A)) even in the first 5 min. of incubation (data not shown), suggesting that *R. rosea* could inhibit HOCl damage to GSH rather than stimulate GSSG reduction. In confirmation of this hypothesis, a direct GSSG reduction was discarded after chemical reaction experiments in which GSSG was incubated with increasing concentrations of *R. rosea* extract and no GSH was detected (data not shown). A recovery of GSH from GSSG was previously reported by Vissers et al. for RBC treated with HOCl and re-incubated with glucose, but it needed 30 min. of incubation [34]. Moreover, as we reported in a previous work, quercetin, a common flavonoid of many fruits and vegetables, was indeed

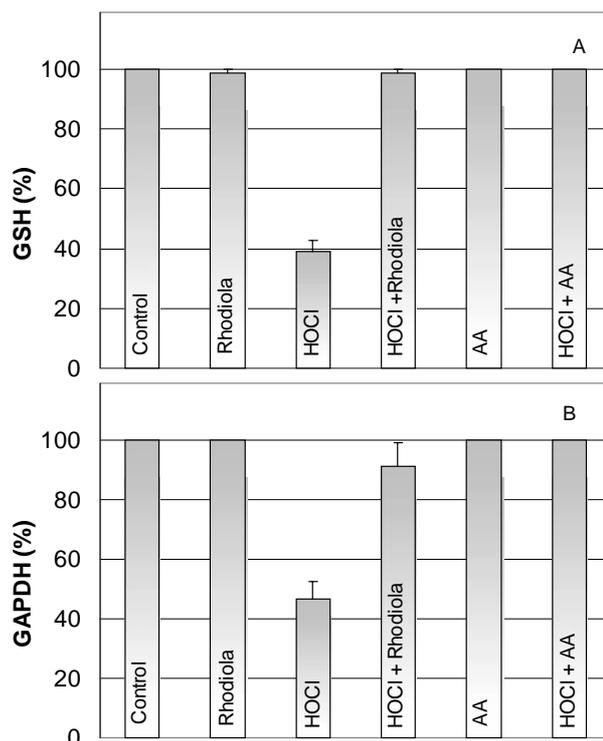


Fig. 7. Effect of *R. rosea* extract on HOCl-induced oxidation of GSH and GAPDH in lysed RBC. Human RBC were lysed with 1 volume of H<sub>2</sub>O. The hemolysate was then diluted with PBS and incubated with 0.5 mM HOCl at 37°C for 15 min. in the presence of 150  $\mu$ l of *R. rosea* aqueous extract or in the presence of 1.3 mM ascorbic acid (AA) used as reference substance for its equivalent reducing ability. Figure 7(A) reports the GSH levels and Fig. 7(B) the GAPDH levels. GSH and GAPDH were measured as in Materials and Methods and expressed as % of the control. Data are the mean  $\pm$  S.D. of at least 5 different determinations.

able to induce an enzymatic recovery of GSSG when rabbit RBC were exposed to dehydroascorbic acid (DHA), but the loss of GSH observed in the first 5 min. of incubation of rabbit RBC lysates with DHA, was followed by a time-dependent (120 min.) recovery of GSH if quercetin was present [8].

The complete protection of GSH observed in the presence of ascorbic acid in hemolysates (Fig. 7(A)), also in the first 5 min. of incubation, is consistent with the ability of this substance to inhibit HOCl damages by a scavenging mechanism [5,9,22,36]. The apparent difference of the ascorbic acid effect in intact and lysed cells is probably the consequence of the slow uptake of this molecule by the cell [5,18].

Taken together, these results suggest that the ability of *R. rosea* aqueous extract to prevent GSH loss induced *in vitro* by HOCl to human erythrocytes is based on a more complex mechanism in comparison with the scavenging mechanism demonstrated for ascorbic acid [5,9,22,36]. For the *R. rosea* extract it could be excluded the single scavenging mechanism of HOCl in the medium. In fact, the component/s responsible for the GSH protection act, as also supported by the chemical experiments, not from the outside of the cell, but mainly from the inside as clearly demonstrated by pre-incubation experiments (Fig. 6(A)). The cell membrane do not represent a strong limiting barrier since *R. rosea* extract is effective more rapidly than ascorbic acid which cell uptake occurs by a slow process [5,18] as demonstrated by experiments on intact RBC (Fig. 3). The hypothesis of a protective effect on the GSH loss and not a GSH recovery from GSSG, was mainly sustained by chemical experiments and also by experiments on intact RBC (Fig. 3) where if *R. rosea* was added after 2 or 5 min. gave not the same complete protection

on GSH (Fig. 3). GSH is, in fact, oxidized by HOCl in less than 2 min. supporting the hypothesis of a GSH protection more than a GSH recovery from GSSG. For all these reasons it should be expected that this protective effect on GSH is probably sustained by a cell-mediated mechanism that not requires the functional character of the intact erythrocyte, in view of the fact that the same protective effect is also afforded in experiments with lysed RBC (Fig. 7(A)).

In the present study, we also demonstrated that *R. rosea* extract was able to give a complete protection on GAPDH activity in a dose-dependent manner (Fig. 2). This protective effect was observed in all experimental conditions: intact RBC (Fig. 4), RBC pre-incubated with *R. rosea* (Fig. 6(B)) and hemolysates (Fig. 7(B)). The prompt protection afforded by *R. rosea* extract and by ascorbic acid to this enzyme is probably the consequence of the different location of this molecule with respect to GSH; that is, GAPDH, a membrane hydrophilic enzyme of the cytoplasmic surface, may be more readily protect from HOCl damage than intracellular GSH.

Between HOCl damages in erythrocytes, hemolysis was also described [34–36,38]. Vissers et al. have demonstrated that HOCl causes significant membrane changes associated with erythrocyte lysis consisting in alteration of membrane deformability, increase of K<sup>+</sup>-leak, cross-linking of membrane proteins and extensive disruption of the membrane [35,36]. It has been suggested that HOCl-mediated damage to erythrocyte membrane proteins or to lipid bilayer comprises an initial damaging event that sets the cell on a path toward lysis [35,36]. Experimental data reported in Fig. 5 suggested that *R. rosea* extract was able to counteract the HOCl damages on RBC leading to lysis even if added 5 min. after HOCl. On the contrary, it is described by other authors that the addition of HOCl scavengers as ascorbic acid, methionine or taurine, inhibited hemolysis only if they were present before HOCl treatment [36]. These results are in agree with our data, confirming that ascorbic acid was not able to block the hemolysis if added 2 or 5 min. after HOCl (data not shown). The same authors showed that the presence of glucose did not prevent the lysis caused by HOCl.

In conclusion, the results obtained in the present study demonstrate that the *R. rosea* aqueous extract was able to protect human erythrocytes against some of the main damages induced *in vitro* by HOCl, with a mechanism different from that proposed for ascorbic acid, suggesting an important antioxidant activity, as already demonstrated for other plant extracts [25,31].

Further studies are in progress in our lab to investigate the mechanisms of the observed effects and to verify if they arise from the action of a single component or from a synergistic action of more compounds present in the *R. rosea* extract.

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