POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 205–218, Y. 2022



DOI: 10.31648/pjns.7819

# PROTECTIVE EFFECT OF HONEY AGAINST ALUMINIUM-INDUCED ERYTHROCYTE OSMOTIC FRAGILITY AND HEMOGLOBIN DENATURATION*

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Key words: honey, ascorbic acid, aluminium, human erythrocytes, hemoglobin.

Abstract

The aim of the present study was to investigate the effects of honey against aluminium (Al) and hypotonic pressure-induced hemolysis of human red blood cells (RBCs) and hemoglobin degradation *in vitro*. Human RBCs were pretreated separately with honey samples and ascorbic acid. Four different treatment groups were considered: untreated RBCs (negative control), aluminium treated RBCs (positive control), honey + aluminium treated RBCs, and ascorbic acid + aluminium treated RBCs. Samples were then evaluated by simultaneous measurement of cellular turbidity and hemoglobin (Hb). The results showed that RBCs suspensions treated with honey samples, particularly honey 2 presented highest cell and Hb values compared to that of ascorbic acid and positive control, whereas, ascorbic acid exhibited a prooxidant effect on cell and Hb. The results of this study suggest that honey not only protect cell integrity but also prevent oxidative degradation of Hb. Therefore, the present study demonstrated a protective effect of honey against Al-induced red blood cells hemolysis and hemoglobin degradation.

## Introduction

Aluminium (Al) is the third most abundant element found in the earth's crust (GUPTA et al. 2013). It has become integral part of our modern life and can be found in food (corn, yellow cheese, salt, herbs, spices, tea), water, cosmetics, medicines such as vaccines, deodorants, transport vehicles and electronic appliances (NIU 2018). Al is considered as a non-essential trace element of low toxicity in human beings (SINGLA and DHA-

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* This work was funded by the Algerian Ministry of Higher Education and Scientific Research (PRFU project grant no D01N01UN340120180001).

WAN, 2013), and occurs naturally in the air, water and soil, and across to the body via the respiratory system, skin or gastrointestinal tract which can reach and accumulate in almost all mammalian tissues, including brain, liver, kidneys, heart, blood and bones (JAISHANKAR et al. 2014). The accumulation of Al in tissues and organs leads to their toxicity and dysfunction (VERSTRAETEN et al. 2008, RAHIMZADEH et al. 2022). Several diseases such as dialysis dementia, Alzheimer's disease, Parkinson's disease, osteomalacia and microcytic anemia have been associated with elevated levels of Al (JEFFERY et al. 1996, BONDY 2010, GOULLE and GRANGEOT-KEROS 2020).

Al causes numerous changes in peripheral blood and hemogenic system (OSIŃSKA et al. 2004). It induces hypertension and disturbs the function of erythrocyte membrane (ZHANG et al. 2016). Al bound to transferrin in blood, easily penetrates the erythrocytes, causes alteration of erythrocytes membrane and decreases osmotic resistance (OSIŃSKA et al. 2004). In addition, Al has been reported to generate reactive oxygen species, which causes peroxidative damage to lipids and proteins membrane (SAEED et al. 2021).

In recent decades, much interest has been generated by scientists and epidemiologists for wide ranges of natural antioxidants from food, particularly fruits and vegetables with reports demonstrating their protective effects against a growing list of aging diseases (WIART 2013, LIGUORI et al. 2018). Honey is one of the most used natural products by humankind for the treatment of diseases. It has a great variety of pharmacological activities, particularly antioxidant, antimicrobial and antiviral activity (BERETTA et al. 2005, ISRAILI 2014, BELLIK and SELLES 2017), treatment of wounds, burns (TASHKANDI 2021), skin ulcers (MCLOONE al. 2020) and inflammations (EL SEEDI et al. 2022). The healing property of honey is due to its chemical composition (BERETTA et al. 2005). Honey contains various amounts of polyphenols and flavonoids which confer it good antioxidant properties. The antimicrobial action is due to its acidity, hydrogen peroxide content, osmotic effects, nutritional and antioxidant content (ALZAHRANI et al. 2012).

There are several scientific reports indicating a great variety of pathological effects of Al, however, very little experimental research works have been conducted on the protective effects of natural antioxidants on Al-induced oxidative damage in human red blood cells. RBCs are particularly prone to oxidation because of their high polyunsaturated lipids content, exposure to oxygen and the presence of transition metals, such as iron and copper (KUHN et al. 2017). Furthermore, Hb inside and outside the RBCs undergoes constant transformation from oxyhemoglobin ($Fe^{2+}$-Hb) to met-

hemoglobin ($Fe^{3+}$-Hb). This metHb may cause its own oxidation (autooxidation) or be oxidized by other agents, and so lose its capacity to carry oxygen and become highly reactive and disruptive agent (ALAYASH 2022). Thus, RBCs are considered as a useful model *in vitro* for studying oxidative stress, elucidating the mechanisms involved in metHb reduction (KINOSHITA et al. 2007), and investigating the antioxidant potency of foods (PAIVA-MARTINS et al. 2009). The goal of the current study was to evaluate the protective role of honey on RBCs osmotic fragility and Hb degradation under Al-induced oxidative damage in human erythrocytes.

## Methods

### Chemicals

Folin-Ciocalteu, anhydrous sodium carbonate, aluminium chloride, gallic acid, quercetin, ascorbic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, formalin, were purchased from Sigma Aldrich (Germany) and Biochem Chemopharma (France).

### Honey samples

Two multi-flora honey samples (25 ml for each sample) were used in this study. Honey samples were collected from beekeepers in March 2018 in the regions of Bir Snab and Colla of the city of Bordj Bou Arreridj, located in Northeastern Algeria (latitude 36.3479 and longitude 6.650773). The regional climate is semi-arid, $T_{max}$ ranges between 32 and 37°C, while the $T_{min}$ varies between 0 and 5°C. Average annual rainfall varies between 200 and 500 mm (KOURAT et al. 2022). Honey samples were given numbers prior experiment (Honey 1 for sample collected form Bir Snab and Honey 2 for sample collected from Colla) and were stored in a refrigerator in airtight plastic containers until analysis.

### Preparation of honey extracts

During the sample preparation, 1 g of each pure honey was diluted with phosphate buffered saline and was made up to 10 mL followed by a through mixing. The final concentration of each honey extract was 100 mg/mL (BERETTA et al. 2005).

### Total phenolic content

The total phenolic content in each honey sample was estimated using Folin-Ciocalteu method (SINGLETON and ROSSI 1965). Briefly, aliquots (200 µL) of honey extract or standard solution of gallic acid (0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL), used to establish the calibration curve, were added to 500 µL of Folin-Ciocalteu reagent (10%). The reaction mixture was thoroughly mixed by shaking. The mixture was incubated at room temperature for 5 min, before 1500 µL of $Na_2CO_3$ (7.5%) were added. All the reaction mixtures were then shaken and incubated for 30 min. The absorbance of blue mixtures was recorded at 765 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Total phenolic contents were expressed as milligram of gallic acid equivalent (GAE)/g of honey by using an equation obtained from the standard curve of gallic acid: $y = 9.04x + 0.029$ ($R^2 = 0.998$).

### Total flavonoid assay

The total flavonoid content in each honey sample was measured by colorimetric assay (JAIN et al. 2011), using quercetin for preparing the calibration curve. One mL of honey extract or standard solution of quercetin (0.005, 0.01, 0.02, 0.03 and 0.04 mg/mL) was reacted with 1 mL of aluminium chloride (2%). After incubation at room temperature for 1 hour, the absorbance of the reaction mixture was measured at 420 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Total flavonoid contents were calculated as milligram of quercetin equivalent (QE)/g from the calibration curve: $y = 6.406x + 0.012$ ($R^2 = 0.993$).

### Isolation of human erythrocytes

Peripheral blood was obtained from apparently healthy adult volunteers, with no clinical indications of anemia. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. For each analysis, 4 mL of blood were centrifuged at 3000 rpm for 10 minutes at 4°C using a laboratory centrifuge (Sigma 2-16KHL – Germany). Platelets, leukocytes and plasma were removed by aspiration. RBCs were washed three times and re-suspended in phosphate buffered saline (PBS: 123 mM/L of $NaH_2PO_42H_2O$, 27 mM/L of $Na_2HPO_4$; pH 7.4) to a final hematocrit of 35% (vol/vol).

The samples tested were: negative control (50% RBCs suspension: 50% PBS), positive control (50% RBCs suspension: 50% PBS), ascorbic acid (50% RBCs suspension and 50% ascorbic acid [50 mM/mL]), honey 1

(50% RBCs suspension: 50% honey 1 [100 mg/mL]) and honey 2 (50% RBCs suspension: 50% honey 2 [100 mg/mL]). All samples were incubated with 800 µL $AlCl_3$ for 30 minutes, except the negative control which was treated with an isotonic solution (PBS). All samples were evaluated simultaneous for hemoglobin and cellular turbidity. The latter is the indicative of intact cells, as demonstrated previously (TAKEBAYASHI et al. 2012).

### Cellular turbidity measurement

After incubation for 30 minutes at 37°C, 100 µL from each sample were collected and 200 µL of hypotonic solution (0.7% of NaCl) were added to induce cellular fragility. The mixture was allowed to stand for 15 minutes at room temperature and 100 µL of formalin (4%) were added to fix the cells. An aliquot (100 µL) of the reaction mixture was diluted 20 times with PBS and the optical density was recorded at 620 nm (Shimadzu Corporation, Japan 1601UV) (BELLIK and IGUER-OUADA 2016).

### Hemoglobin measurement

Briefly, RBCs suspensions were diluted 20 times and centrifuged at 3000 rpm for 10 minutes at 4°C. Subsequently, 2 mL of supernatant were recovered and the optical density was measured at 412 nm (BELLIK and IGUER-OUADA 2016).

### Microscopic study

To study morphological changes in RBCs by optical microscopy, 10 µL aliquots of RBCs were taken directly after measurement of cellular turbidity. The samples were mounted on a slide with a cover slip and examined under a microscope at 10×100 magnifications and images were recorded.

### Hemoglobin stability evaluation

Regarding hemoglobin (Hb) stability test, the same procedure was used as for cell turbidity, except the RBCs suspension was replaced by Hb alone. Erythrocytes suspensions, prepared as described above, were lysed in a 999-fold volume of distilled water. Hb stability was recorded spectrophotometrically at 412 nm over a time period of 24 h. The decrease in absorbance at 412 nm represents the degree of hemoglobin breakdown. Briefly, after incubation for 30 minutes, Hb (1 mL of hemolysate) was treated with 800 µL of $AlCl_3$, except the negative control which was treated with an isotonic solution (PBS) and Hb measured for 24 h (BELLIK and IGUER-OUADA 2015).

## Statistical analysis

All experiments were repeated at least three times and the data are shown as means ± S.D. Statistical examination was performed with the analysis of variance (ANOVA) using Statistica Software version 5.5 (Statsoft, France). Values were considered to be significant when $P$ was < 0.05.

## Results and Discussion

### Total phenolic and flavonoids content of honeys

Polyphenols are an important group of compounds that were reported to influence not only the appearance but also the functional properties of honey (Becerril-Sánchez et al. 2021). A significant difference was observed in the phenolic contents of the studied honeys. Honey 2 contained the highest phenolic content with mean value of 1.55 ± 0.04 mg GAE/g while honey 1 showed a concentration of 0.63 ± 0.03 mg GAE/g. Our results are higher than that reported by Akgün et al. (2021), and Habryka et al. (2020), who determined average levels TPC of 0.26±0.07 mg GAE/g and 0.3 mg GAE/ g in multifloral honey, respectively. As with the phenolic content, honey 2 showed the highest levels of flavonoid content (0.17 ± 0.003 mg QE/g) when compared to honey 1 (0.075 ± 0.005 QE/g). The obtained results are in a quite agreement to the range reported by Socha et al. (2016) (5.26 to 14.39 mg QE/100 g of flavonoids) but much higher than that of Habryka et al. (2020) (2.77 mg QE/100 g). Various studies showed that the multi-floral honeys with the highest phenolic compound contents. This high content also confers a high antioxidant capacity.

Table 1

Total phenolic and flavonoid contents of honey samples

| Samples | Phenolic content [mg GAE/g] | Flavonoid content [mg QE/g] |
|---|---|---|
| Honey 1 | $0.63\pm0.03^{a}$ | $0.075\pm0.005^{a}$ |
| Honey 2 | $1.55\pm0.04^{b}$ | $0.17\pm0.003^{b}$ |

### Measurement of erythrocyte and hemoglobin

Figure 1 shows the absorbance values of RBCs control samples (negative control and positive control) and treated samples (ascorbic acid, honey 1 and honey 2). Negative control sample presented the highest values of cells compared to the all other studied samples, the reason is that

negative control sample was not subjected to the oxidation by $AlCl_3$. Suspensions treated with honey samples showed highest cells values compared to that of ascorbic acid and positive control samples.

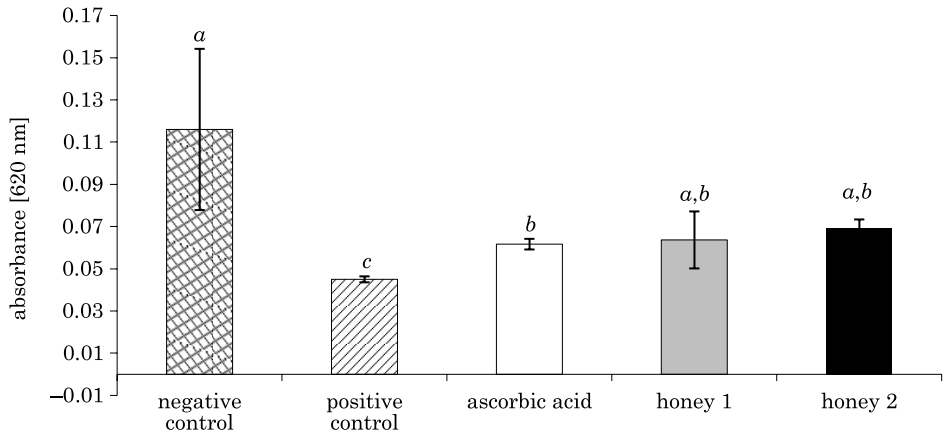


Fig. 1. Cellular turbidity of human erythrocytes when exposed to the hypotonic conditions and oxidation by $AlCl_3$. Values are means ± S.D. of triplicate experiments

Similarly, Hb absorbance was much higher in samples supplemented with honey extracts than in untreated positive control sample (Fig. 2). These seemingly contradicting results suggest honey, particularly honey 2, not only protect cell integrity but also prevent oxidative degradation of Hb.

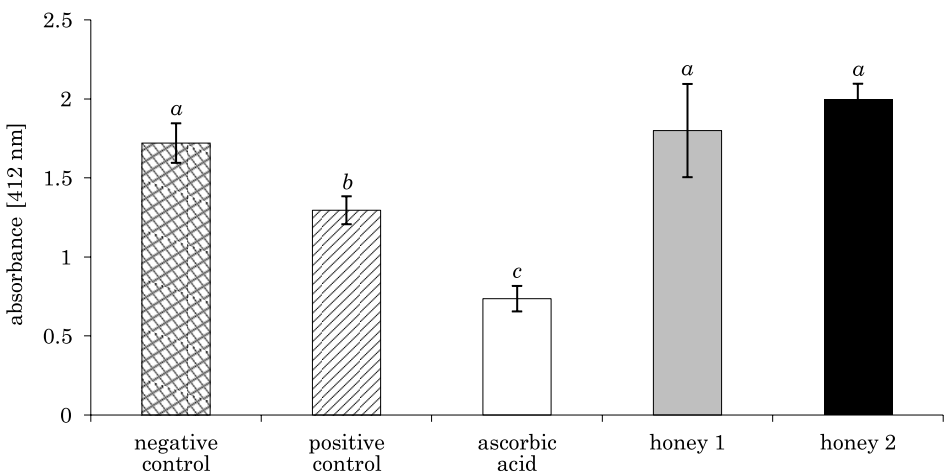


Fig. 2. Hemoglobin levels of human erythrocytes after oxidation by $AlCl_3$. Values are means ± S.D. of triplicate experiments

## Morphological analysis of erythrocytes

With respect to cell morphology, the untreated positive control cells (Fig. 3) as well as suspension cells treated with ascorbic acid showed cytoplasmic membranes abnormalities, whereas negative control cells and erythrocytes treated with honey samples displayed normal cell shape (Fig. 3).

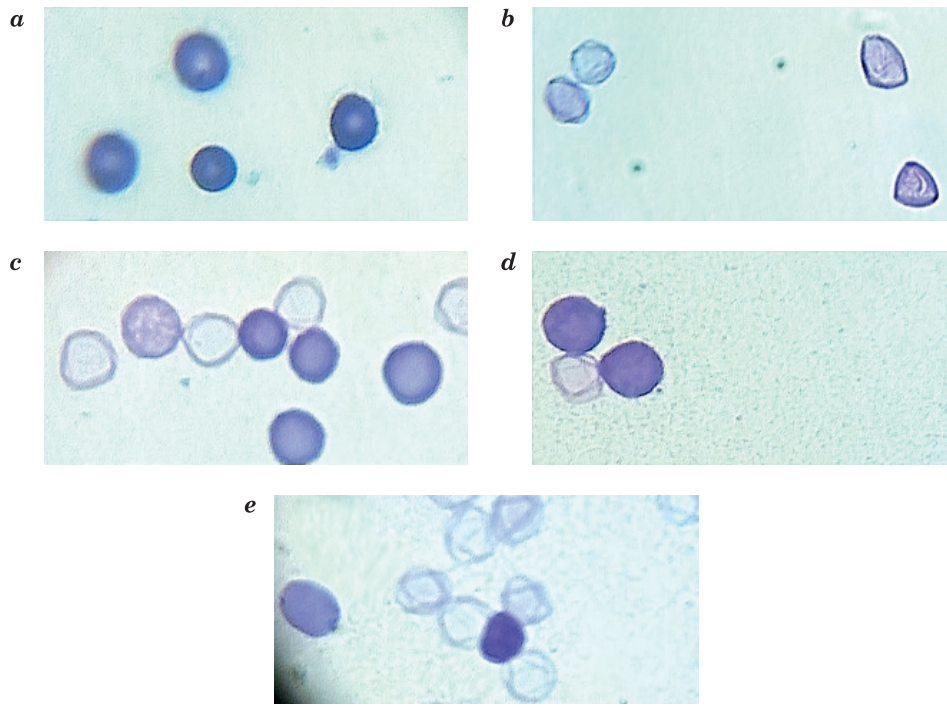


Fig. 3. Microscopic images of erythrocyte morphology under hypotonic conditions and after the completion of oxidation by $AlCl_3$: *a* – negative control; *b* – positive control; *c* – erythrocytes treated with honey 1; *d* – erythrocytes treated with honey 2; *e* – erythrocytes treated with ascorbic acid

## Hemoglobin stability measurement

The kinetic measurement of Hb stability in the presence of honey samples and ascorbic acid was also investigated and the results were shown in Figure 4. A statistically significant decrease in Hb absorbance was observed after 30 minutes of incubation. Nevertheless, the decreases varied according to the studied samples. Honey samples were effective in protecting human Hb against oxidative damage when comparing to the positive control. As shown in Fig. 4, Hb absorbance remained significantly higher in samples treated with honey extracts. However, ascorbic acid appears to

accelerate the denaturation of Hb due to the significant reduction in absorbance from 30 minutes of incubation, and persists until the end of the test (Fig. 4).

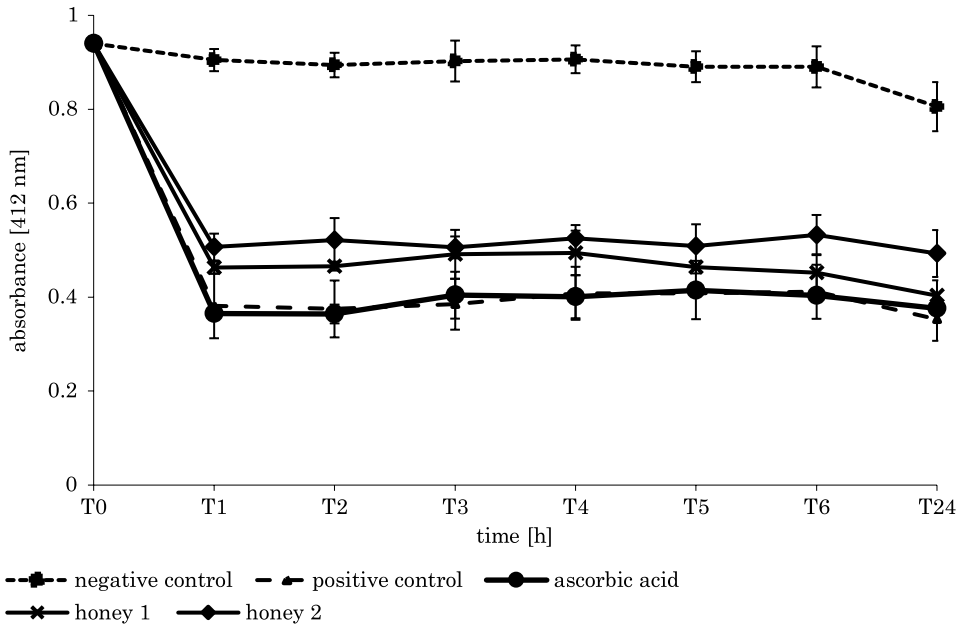


Fig. 4. Kinetics of hemoglobin breakdown exposed to $AlCl_3$

It is worthy noting that negative control sample presented the highest values of Hb compared to the all other studied samples; the reason is that negative control sample was not subjected to the oxidation by $AlCl_3$.

A considerable number of reports suggest that aluminium may present a major threat for humans, animals and plants in causing many diseases (JAISHANKAR et al. 2014, RAHIMZADEH et al. 2022). This critical component can alter membrane phospholipid metabolism either by its direct or indirect interaction and may disturb membrane fluidity and integrity (SINGLA et al. 2013).

In the present study, we demonstrated that honey samples were effective in protecting erythrocytes against hypotonic stress and Al-induced adverse effects with respect to cell absorbance (Fig. 1) and morphology (Fig. 3). This protection against hypotonic stress is most likely due to an increase in membrane fluidity resulting from prior exposure to honey. Several authors outlined the importance of membrane fluidity for the stability of erythrocytes (TSUCHIYA et al. 2002, DUCHNOWICZ et al. 2021). In addition, it is well demonstrated that erythrocytes become more resistant to

hypotonic solution and detergents when membrane fluidity is increased by treatment with tocopherols (SUZUKI et al. 1993). TSUCHIYA et al. (2002) reported that erythrocytes were more resistant to physical stress in conjunction with an increase in membrane fluidity caused by treatment with propofol. Moreover, it has been reported that flavanols and procyanidins interact with membrane phospholipids through hydrogen bonding with the polar groups of phospholipids, these compounds can accumulate at the membranes surface, both inside and the cell, and contribute to maintain membranes integrity by preventing access of deleterious molecules to the hydrophobic region of the membrane (VERSTRAETEN et al. 2015, VILLALAÍN 2022).

The maintenance of the cation gradient by high affinity $Ca^{2+}$-ATPase is fundamentally important in the control of hydration, volume, nutrient uptake, and fluidity of cells (JEWELL et al. 2013, OLURANTI et al. 2021). Low-affinity $Ca^{2+}$-ATPase is considered to be responsible for the shape and deformability of the erythrocytes membrane.

Energy production in the form of ATP is necessary for maintaining RBCs function. As these cells lack mitochondria, energy in RBCs is generated only through glycolysis and pentose phosphate pathway. In this study, hemolysis was attenuated in the presence of honey which is a rich source of glucose. The latter is considered as a main substrate for energetic metabolism of erythrocytes (VISKUPICOVA et al. 2015). Whereas, energy depletion is well known to induce and mediate apoptosis (LANG and LANG 2015). This may be the main reason for the observed low values of RBCs in other untreated samples under oxidative conditions (positive control and ascorbic acid samples).

Ascorbic acid alone has no appreciable effect on osmotic fragility and Al-inducing oxidative damage. The reason why ascorbic acid alone failed to protect erythrocytes may also be due to its prooxidant activity (PODMORE et al. 1998, KAZMIERCZAK-BARANSKA et al. 2020).

Recently, there is growing evidences that cell-free Hb is more susceptible for oxidative modifications than intracellular Hb (XIANG 2013, AGYEMANG et al. 2021, ALAYASH 2022). Hb within red blood cells is protected from oxidative processes by enzymatic and antioxidant molecules as well as the RBCs membrane that provides a physical barrier against oxidation. In the other hand, the redox chemistry of the released Hb is susceptible to toxic reactions due to the interaction with oxidative stress agents such as peroxide, yielding free radicals and highly-oxidizing states at the iron (ferryl, Compound II) (VOLLAARD et al. 2005, AGYEMANG et al. 2021). In addition, Hb amount present in the circulation has been related to hemolysis, vascular diseases, and stroke-related intracerebral hemorrhage (WOOLLARD et al. 2009, SCHAER and BUEHLER 2013).

In agreement with previous cell-free Hb studies (XIANG et al. 2013). The decrease in optical density of Hb corresponds to its inactivation. Inactivation and degradation of Hb are two related processes. In the present work, Hb was found at higher levels when samples were treated with honey, prior to treatment with $AlCl_3$, suggesting a protective effect against inactivation and degradation of Hb. HATHAZI and coworkers (2018) found that caffeic acid slowed down significantly the nitrite-induced oxidation of Hb. This is in line with our previous findings that ginger acts to efficiently reduce the oxidation and degradation of Hb (BELLIK and IGUER-OUADA 2015). However, treatment with ascorbic acid did not protect against Hb degradation. It was found that ascorbic acid exhibited a pro-oxidant effect on both cellular membrane and Hb. SIMONI et al. (2009) reported that ascorbic acid without glutathione failed to protect Hb against hydrogen peroxide-mediated oxidation of Hb and formation of its ferryl intermediate. In addition, It has been demonstrated that Hb breakdown rate is correlated to erythrocytes hemolysis in the presence of ascorbic acid (IBRAHIM et al. 2006, BELLIK and IGUER-OUADA 2016).

According to TIMOSHNIKOV et al. (2020), ascorbic acid could react with oxygen to form the hydrogen peroxide, which could be further react with hemoglobin to form MetHb and superoxide, the superoxide generated in the heme pocket can oxidize the tetrapyrole rings, leading to the degradation of heme and the release of iron. Free heme can react with lipids in cellular membranes, inducing lipid peroxidation and, increase cell permeability leading to hemolysis, which may explain the high level of hemolysis observed in erythrocyte suspensions not treated with honey.

## Conclusion

This study demonstrated that honey protected erythrocytes from hemolysis and Hb from denaturation. It can be assumed that honey has the potential to reverse the aluminium induced adverse effects on membrane and has an important role in preserving cell integrity. As well, our result corroborates the fact that uptake of natural nutrients including honey, fruits and vegetables is more effective at boosting antioxidant activity than ascorbic acid supplementation.

## Conflict of interest

The author declares that there is no conflict of interest.

Accepted for print 5.02.2022

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