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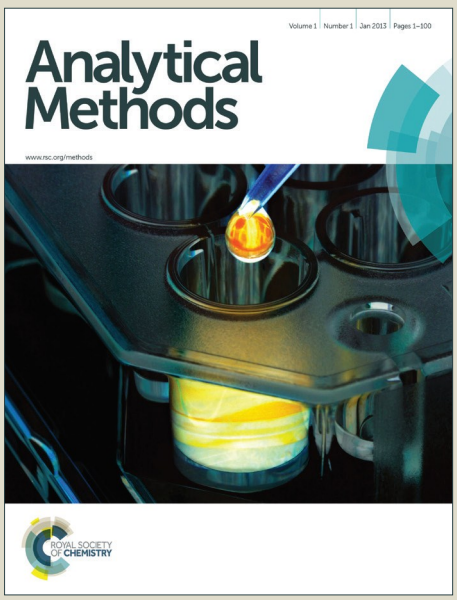

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A useful method based on cell-free hemoglobin analysis for evaluating antioxidant activity.

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Abstract

In the last decades, a vast array of chemical analytical methods have been proposed and applied for the assessment of antioxidant activities. However, there is no simple standard method by which antioxidant capacity in foods and biological samples can be assessed accurately and quantitatively. In the present study a new approach, based on cell-free hemoglobin (Hb) analysis, is proposed to evaluate antioxidant activity. It is based on the spectrophotometric measurement of hemoglobin concentration at specific wavelength (412 nm). Human Hb were pretreated separately with ginger oleoresin, ginger essential oil, and antioxidant standard molecules (BHT, BHA, quercetin, gallic acid and ascorbic acid). Untreated Hb samples served as controls. Oxidative stress was induced by H_2O_2. Samples were then evaluated by the measurement of Hb. The results showed that ginger oleoresin and essential oil or standard molecules exhibited antioxidant activity in dose-dependent manner by protecting Hb against its denaturation and degradation, whereas ascorbic acid exhibited a prooxidant effect on Hb. This simple method is easily accessible and provides biologically relevant data and can be proposed as valuable technique for evaluating antioxidant activity of vegetable and plant extracts.

Key words: antioxidant activity, hemoglobin, oxidative stress, oleoresin, essential oil, standard molecules.

1. Introduction

Medicinal plants have long been, over the years, played a key role in the human health and contributed to the development of modern therapeutic drugs.[1,2] In recent decades, much interest has been generated by scientists and epidemiologists for wide ranges of

phytochemicals with reports demonstrating their protective effects against a growing list of aging diseases.[3,4,5,6] As well, a vast array of chemical analytical methods had been developed to measure the antioxidant activity of vegetable and plant extracts.[7,8,9] However, each method has particular advantages and disadvantages, and it is therefore essential to evaluate antioxidant activities using a combination of several methods.[7,10,11,12,13] Thus, the search for more specific assays, expressing faithfully the *in vivo* action of antioxidant molecules is still pending.

Hemoglobin (Hb) is a major component of blood and a potent mediator of oxidative stress . In the presence of H_2O_2, extracellular Hb is susceptible to heme iron, and protein oxidation associated with pseudoperoxidase activity of Hb. H_2O_2 oxidizes ferrous Hb (Fe^{2+}) to generate the oxo-ferryl ($Fe^{4+} = O^{2-}$) state and, in the case of the reaction with ferric Hb (Fe^{3+}), a protein radical ($Hb^{\cdot+}$ $Fe^{4+} = O^{2-}$) is formed as follows:[14]

(1) Hb (Fe^{2+}) + H_2O_2 ⟶ Hb ($Fe^{4+} = O^{2-}$) + H_2O;

(2) Hb (Fe^{2+}) + Hb ($Fe^{4+} = O^{2-}$) + H+ ⟶ Hb (Fe^{3+}) + HO-;

(3) Hb (Fe^{3+}) + H_2O_2 ⟶ $Hb^{\cdot+}$ $Fe^{4+} = O^{2-}$ + H_2O.

This formed Hb radical presents similar properties compared to the commonly used stable free radicals for evaluating antioxidant activity. However, the advantages of using hemoglobin based-assay are: (1) it is based on a molecule coming from biological origin, this molecule could express accurately the *in vivo* action of antioxidant molecules, (2) it uses H_2O_2 as pro-oxidants and hemoglobin as oxidizable targets so that the results obtained reflect biologically relevant radical-scavenging activity compared to other *in vitro* methods using non-natural radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH),[15] 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$)[16] and non-natural oxidizable targets such as

an oxygen radical absorbance capacity ($ORAC_{FL}$);[17] and (2) it reflects microlocalization of antioxidant effects to the biomolecules (Hb) of erythrocytes. Therefore, the present study aimed to propose a new method based on cell-free hemoglobin analysis as useful tool for evaluating antioxidant activity.

2. Results and discussion

2.1. Kinetic analysis of hemoglobin reaction with hydrogen peroxide

A statistically significant decrease in Hb concentrations was observed after 30 minutes of incubation (Fig. 1 and 2). Nevertheless, the decrease vary according to the studied samples. Ginger oleoresin was effective in protecting human hemoglobin against oxidative damage when comparing to the essential oil (Fig. 1). Likewise BHT, gallic acid and quercetin exhibited an important protective effects compared to the BHA (Fig. 2). However, ascorbic acid appears to accelerate the denaturation of Hb due to the significant reduction in concentration from 30 minutes of incubation, and persists until the end of the test (Fig. 2).

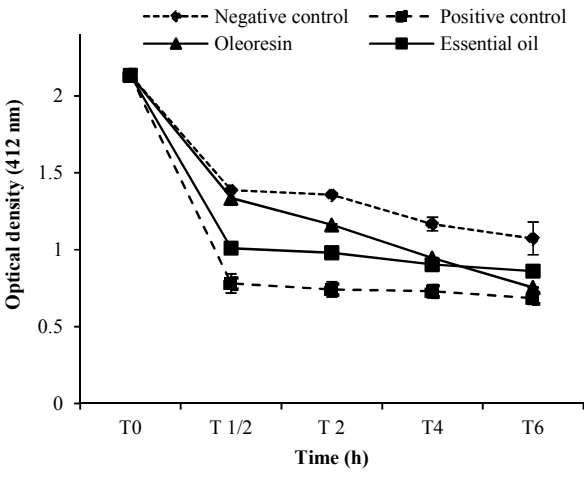

Fig. 1. Kinetics of hemoglobin breakdown exposed to 50 mM H_2O_2 in the the presence of ginger oleoresin and essential oil. Results are means ± SD of five independent experiments.

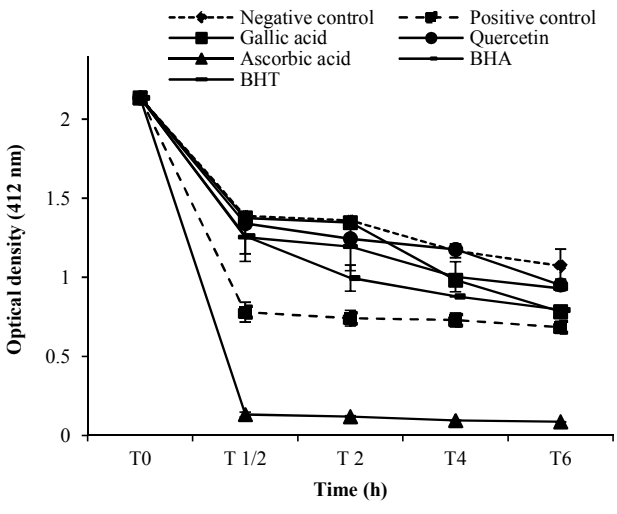

Fig. 2 . Kinetics of hemoglobin breakdown exposed to 50 mM H_2O_2 in the presence of standard molecules. Results are means ± SD of five independent experiments.

2.2. Measurement of antioxidant activity in hemoglobin

To assess antioxidant activity in hemoglobin samples, standard molecules (BHT, BHA, quercetin, gallic acid, and ascorbic acid) were tested separately at different concentrations (10 mM, 20 mM, 30 mM, 40 mM, and 50 mM) at a specific time (30 minutes) as the denaturation of Hb is evident at the first half hour of incubation with hydrogen peroxide (Fig. 1 and 2).

The results showed a dose-dependent protective effect of BHT (panel A), BHA (panel B), and gallic acid (panel C) quercetin (panel D) with the exception of ascorbic acid (panel E), which exhibited, on the contrary, a dose-dependent denaturation effect (Fig. 3).

It can be seen that according to the concentration, the standard molecules afforded a significant protective effect against the denaturion of Hb. This protection is particularly visible for the BHT and BHA at 10 and 20 mM, respectively. For gallic acid and quercetin, it becomes significant only from 30 mM. Ascorbic acid causes a significant reduction in the Hb concentration. There is however a difference in distortion function of the concentration used. Indeed, more the concentration of ascorbic acid is higher more the denaturation of Hb is important.

It is worthnoting that the negative control samples presented the highest values of Hb compared to the all other studied samples, the reason is that negative control samples were not subjected to the oxidation by H_2O_2.

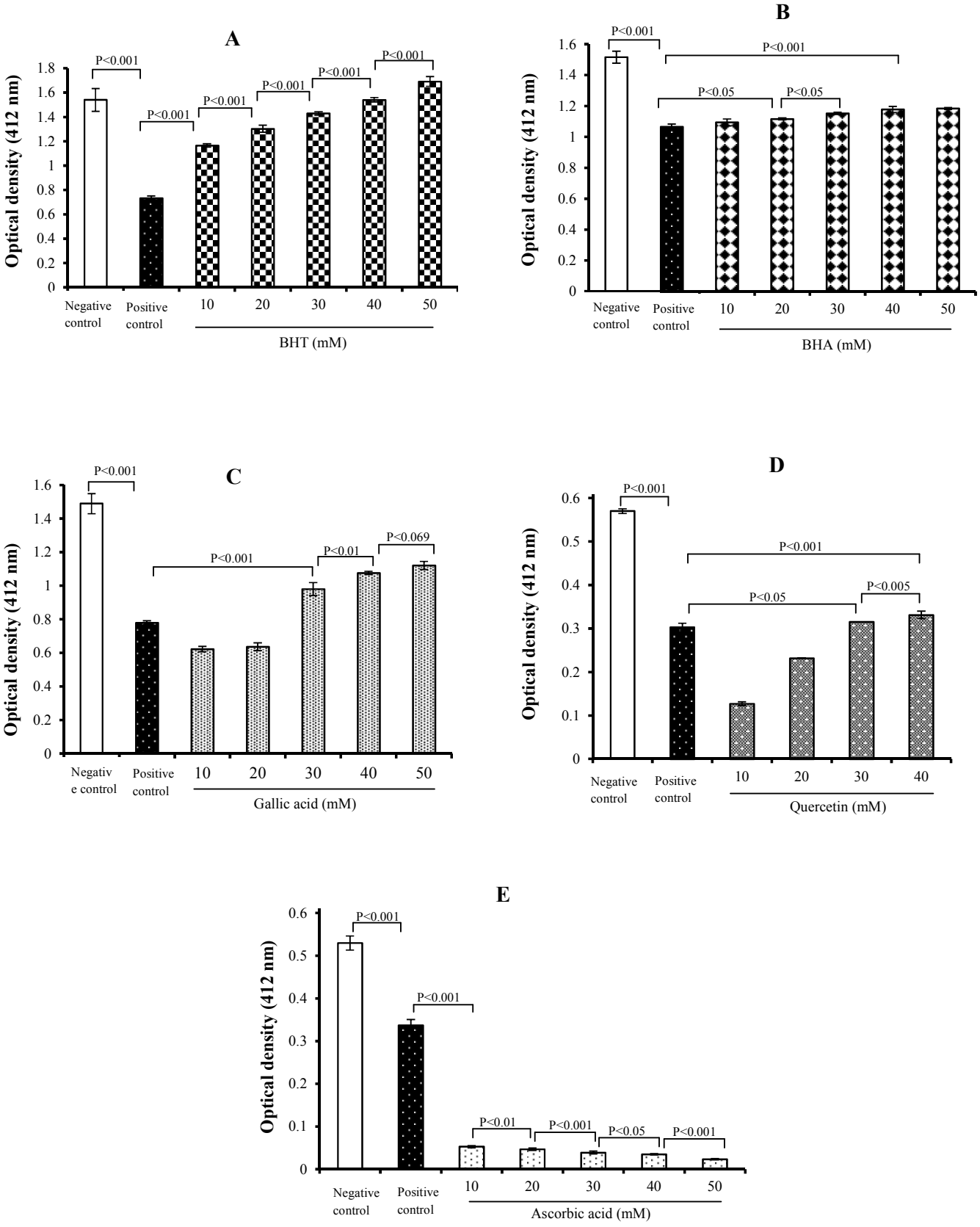

Fig. 3. Dose-dependent effects of BHT(panel A), BHA(panel B), Gallic acid (panel C), quercetin (panel D) and ascorbic acid (panelE) on ROS-scavenging activity in hemoglobin exposed to 50 mM H_2O_2. Results are means ± SD of five independent experiments.

Several methods, based on inhibition of free radicals, have been proposed and applied for the assessment of antioxidant activities.[7,18,19,20] However, there is no simple universal method by which antioxidant capacity of foods and biological samples can be assessed accurately and quantitatively.[19] Therefore, it is essential to use more than one type of antioxidant assay to measure antioxidant activities, and to include at least one assay that has biological relevance. *In vitro* hemolysis assay is one of the spectrophotometric methods that have been frequently used to evaluate the antioxidant properties of plants. It is based on the measurement of the released Hb to express indirectly the rate of cell lysis without cell quantification.[21,22,23,24,25,26] However, the most drawback of this method is the risk of false negative results, where high value of Hb would be considered as important rate of lysis; it had been shown that Hb is protected from degradation in the presence of antioxidant agents.[26] In parallel, it is well established that under oxidative stress, Hb will be oxidized,[27] resulting to Hb denaturation and precipitation.[28] This fact is of high importance and should be considered when working under antioxidant conditions, such as when using plant extracts or other molecules expressing intrinsic antioxidant activities. In such situations, high values of Hb may not be synonymous of cell lysis, but the result of Hb protection against oxidative degradation. Thus the ingenious method described by Takebayashi et al.[20] based on inhibition of AAPH-induced erythrocyte hemolysis by measuring directly cellular turbidity enable correctly the evaluation of large numbers of samples in small quantity at the same time with satisfactory precision and reproducibility.

The hemoglobin based-assay is a useful *in vitro* method for evaluating the biologically relevant antioxidant activities. As hemoglobin is coming from biological origin, this molecule could express accurately the antioxidant statute of the analyzed environment. In this method, isolated Hb is studied under oxidative conditions and in the presence of antioxidants. The

antioxidative activity is determined directly from the concentration of hemoglobin by measuring the optical density at 412 nm. The decline in optical density of Hb corresponds to its inactivation according to the Soret band. The inactivation and degradation of hemoglobin are two correlated processes.[26] It has been demonstrated, more recently, that hydrogen peroxide (H_2O_2 at 500 µM) led to an increase in the relative oxidation levels of purified and unpurified Hb.[27] H_2O_2 can mediate the heme degradation of various hemoproteins.[29] Because of the proximity of the uncoupled electron to the porphyrin ring, any electron transfer from the ferrous state to an extra H_2O_2 moiety would generate a hydroxyl radical, which could, in turn, react with the heme group to produce irreversible inactivation.[26] The reaction of H_2O_2 with Fe^{2+}Hb (oxyHb and deoxyHb) and Fe^{3+}Hb results in the formation of ferrylhemoglobin (ferrylHb) and oxoferrylhemoglobin (oxoferrylHb), respectively;[30] both are strong oxidizing agents. The formation of heme-derived products that are covalently cross-linked to the globin molecule has been reported during the reaction of heme proteins with H_2O_2. In the absence of reducing substrate, oxoferrylHb can react with H_2O_2 to produce metHb and oxygen. Whereas, ferrylHb would oxidize H_2O_2 to produce superoxide and metHb, the superoxide generated in the heme pocket can oxidize the tetrapyrole rings, leading to the degradation of heme and the release of iron.

In the present study, hemoglobin treated with oleoresin, essential oil or antioxidant standard molecules showed higher concentrations following the addition of H_2O_2 suggesting thus a protective effect against its inactivation and degradation. The antioxidant power related with the chelating capacity and free radical-scavenging activity of these antioxidants have been also reported in other experimental models.[31,32,33]

Whereas hemoglobin with ascorbic acid showed an important decline of optical density compared to positive control. This confirms the prooxidant effect of ascorbic acid recognized in the litterature.[34,35,36]

In vitro, ascorbic acid can act as an efficient antioxidant and scavenge a variety of ROS including hydroxyl, peroxyl, thyil, and oxosulphuric radicals. It is also a powerful scavenger of HClO and peroxynitrous acid and can inhibit the peroxidation process. It can react with $^1O_2$ and act synergistically with other antioxidants to regenerate, for example, the tocopherol radical to its reduced form. Indirect evidence has shown that, *in vivo*, ascorbic acid acts directly as an antioxidant,[37] and indirect evidence indicates a decreased peroxidation process and beneficial involvement in disease states associated with ROS.[38]

In fact, ascorbic acid can donate 2 electrons; following donation of 1 electron, it produces the ascorbyl (semidehydroascorbate or ascorbate) radical, which can be further oxidized to produce dehydroascorbate. Because the ascorbyl radical is relatively stable, it makes ascorbate a powerful, and important antioxidant. This radical can lose its electron and be transformed to dehydroascorbic acid or regenerated to the reduced form by obtaining an electron from another reducing agent, such as GSH or NADH, via the mediation of an enzyme. The compound dehydroascorbate is not stable. The chemical nature of enabling it to perform as a reducing agent might imply a deleterious consequence that may occur when ascorbic acid is present in the environs of available transition metals. Cooperation between ascorbic acid and ferric ions may lead to the production of $OH^{\cdot}$ via the Udenfriend reaction.[39] Thus, ascorbic acid under specific conditions may act as a prooxidant and produce radicals that might contribute to oxidative damage.[40,41]

It should be noted also that the product of hemoglobin breakdown, inducing iron release, contributes to ascorbic acid oxidation. This could be the reason of the prooxidant effect observed during incubation of Hb with ascorbic acid.

3. Experiment

3.1. Plant materials

The air-dried ginger roots, of Chinese origin, were purchased from the local spice store in Bejaia, Algeria. Mature and healthy rhizomes were ground using a mortar and a pestle. The essential oil was extracted directly from the mortar and pestle crushed tissues. For the extraction of the oleoresin, these tissues were further ground into a fine powder (500 μm particles) with an electric mill (Ika Labortechnik, Staufen, Germany).

3.2. Extraction of the essential oil and the oleoresin

Essential oil was extracted by hydrodistillation process. The extracted essential oil (0.48 ± 0.19% (wt/wt)) was kept in an air-tight sealed glass vials, covered with aluminum foil, and stored at 4 °C until further studies. Oleoresin was extracted from dry ginger powder using Soxhlet. Briefly, samples (10 g) were wrapped in paper and soaked in methanol at 70 °C for 4−8 hours. The methanol extracts were dried using a rotary evaporator to yield the oleoresin (10.23 ± 1.02% (wt/wt)). Oleoresin sample was reconstituted with 10 ml of methanol and stored in the dark at low temperature (4 °C) until tested.

3.3. Isolation of human erythrocytes

Blood samples were obtained from apparently healthy adult volunteers, who had no clinical indications of anemia. Informed consent was obtained from all study participants. This study was ethically approved by the National Agency for the Development of Health Research

(ANDRS; Agence Nationale pour le Développement de la Recherche en Santé), in accordance with the principles outlined in the Declaration of Helsinki.

For each analysis, a volume of 4 ml of blood was centrifuged at 2500g (4 °C) for 10 minutes. Platelets, leukocytes and plasma were carefully removed by aspiration. The isolated erythrocytes were washed three times then resuspended in phosphate buffered saline (PBS) to a final hematocrit of 35% (vol/vol). Human erythrocyte suspensions were lysed in a 99-fold volume of distilled water. Erythrocyte lysates were centrifuged at 12000g for 20 minutes at 4 °C and the supernatants were collected. These supernatant fractions are designated as cell-free hemoglobin in the text.

3.4. Studied samples

The tested samples were: negative control (50% of cell-free hemoglobin and 50% of PBS), positive control (50% of cell-free hemoglobin and 50% of PBS) standard molecules (50% of cell-free hemoglobin and 50% of gallic acid, ascorbic acid, quercetin, BHT, or BHA at 20 mmol/l), oleoresin (50% of cell-free hemoglobin and 50% of oleoresin at 0.2 mg/ml) and essential oil (50% of cell-free hemoglobin and 50% of essential oil at 20 mg/ml).

All samples were incubated for 30 minutes at 37 °C. The standard molecules were studied for the first time at 20 mmol/l, then at different concentrations of 10, 20, 30, 40 and 50 mmol/l.

3.5. Hemoglobin stability evaluation

Hemoglobin stability was registered spectrophotometrically at 412 nm. The decrease of the absorbance at 412 nm represents the degree of hemoglobin breakdown. Briefly, after incubation with different antioxidants, cell-free hemoglobin suspensions were added of 800 µl of H_2O_2 (50 mmol/l) in PBS (123 mmol/l of $NaH_2PO_42H_2O$, 27 mmol/l of Na_2HPO_4; pH 7.4) for 30 minutes at 37 °C, except the negative control which was treated with an isotonic solution (PBS). The optical density of Hb was measured at 412 nm (Soret band).

3.6. Statistical analysis

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

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