

**IN VITRO EFFECT OF TROFIN<sup>®</sup>, A NATURAL ANTIANEMIC FORMULATION ON IONIC IRON REDOX STATUS**

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**Summary**

The aim of this work was to evaluate the influence of Trofin<sup>®</sup>, a natural antianemic containing heme-iron, on ionic iron redox status in order to propose a rationale for the inclusion of ionic iron into the Trofin<sup>®</sup> formulation. The interactions of Trofin with Fe<sup>2+</sup>/Fe<sup>3+</sup> as well as the influence of pH on such interaction were estimated by spectrophotometric techniques. The oxidative damage of different Trofin<sup>®</sup>-Fe<sup>+2</sup> mixtures on brain homogenate of weaned rat was also studied. Trofin<sup>®</sup> dose dependently caused a diminution of Fe<sup>2+</sup> concentration, an effect that was prevented by ascorbic acid. Trofin<sup>®</sup> also prevented the reductive effect of ascorbic acid on Fe<sup>+3</sup>. In addition, Trofin<sup>®</sup> significantly decreased lipid peroxidation in brain homogenate exposed to Fe<sup>+2</sup>, but the lowest Trofin's concentration contributes to preserve Fe<sup>+2</sup> from iron salt autoxidation. The development of new antianemic formulations containing Trofin<sup>®</sup> plus ferrous iron salts should be observed cautiously and should comprise amounts of Trofin<sup>®</sup> below the proportion 500:1 w/w in relation to iron, with the presence of ascorbic acid. The results also suggest for the first time an antioxidant effect for the Trofin<sup>®</sup> formulation that could has pharmacological potential for the treatment of different pathological conditions associated to an iron-mediated oxidative stress.

**Key words:** heme iron, autoxidation, antioxidant

### **Introduction**

Iron deficiency is the most widespread nutritional disorder in the world, affecting an estimated of 1.2 billion of peoples (1). Iron appears in the nature in two chemical forms that are classified in non heme or ionic iron and heme iron. For the prevention and treatment of the iron deficiency, iron salts as source of ionic iron are broadly used. Nevertheless, these products have a low bioavailability and cause adverse reactions in patients, particularly in children and adolescents (2, 3). The supplementation with heme iron from different natural sources is another alternative that has brought the pharmacist attention. It offers several advantages, such as higher bioavailability and lack of adverse reactions (4).

Trofin<sup>®</sup> is a Cuban antianemic product that contains a mixture of hydrolyzed heme proteins obtained from blood cows, honeybee, and propolis (5). This formulation has been evaluated in different population groups, as antianemic and restorative with a high efficacy and absence of adverse reactions (6, 7). However, there is a possibility to increase its efficacy by incorporating ionic iron, since this formulation contains mainly heme iron. It is well known that iron absorption occurs in the small intestine, and is mediated by two different receptors; DMT1 for the absorption of non-heme iron and HCP1 for the heme iron (8-10). Human nutritional studies have shown that patients supplemented with a mixture of dry bovine haemoglobin and ferrous sulphate had increased iron absorption in relation to the supplementation with heme or ionic iron alone (11). It has also been shown that the ingestion of small quantities of pork meat with a food with high content of phytic acid significantly increased the non-heme iron absorption in humans (12). Thus, the incorporation of ferrous iron to the Trofin<sup>®</sup> formulation could probably improve the bioavailability of total iron.

The above-mentioned scientific evidences led us to propose a new antianemic formulation containing both, dry Trofin<sup>®</sup> as heme iron and an iron ferrous salt as non-heme iron source. This research is the first part of a preformulation study that pretend to evaluate the influence of Trofin<sup>®</sup> on ionic iron status in order to propose a rationale for their mixture into a new antianemic formulation.

### Material and methods

**Reagent and solutions:** Ferrous sulphate 1-hydrate, 1,10 phenantroline, 2-hydroxyethyl-1 piperazineethanesulfonic acid (HEPES), ascorbic acid, and sodium chloride were purchased from Sigma (St. Luis MO, USA). Ferric nitrate was obtained from Merck (Germany). Trofin<sup>®</sup> was obtained from the National Center of Biological Product, Havana, Cuba. Stocks solutions of 1 mM Fe<sup>+2</sup> from Ferrous chloride and 1 mM Fe<sup>+3</sup> from Ferric nitrate were prepared in 50 mM NaCl, pH 7. Stocks solutions of Trofin<sup>®</sup> were prepared at 0.5% (5 mg/ml). All the solutions were daily prepared using double distilled water.

**Effect of Trofin<sup>®</sup> on Fe<sup>+2</sup>:** Four different combination of Trofin<sup>®</sup> plus Fe<sup>+2</sup> were prepared. The w/w Trofin<sup>®</sup>: iron ratios in the mixtures were 500:1, 2 500: 1, 5000:1 and 10 000:1, containing Trofin<sup>®</sup> at 10, 50, 100 or 200 µg/mL respectively + 20 µM Fe<sup>+2</sup> in HEPES buffer 50 mM, pH 6.5. At 0, 5, 10, 15, 20, 25 and 30 minutes 2.94 mL aliquots were removed from the mixture and 1,10 phenantroline (1 mM final concentration) was added to quantify the Fe<sup>+2</sup> remaining at 510 nm (13). In a parallel study, ascorbic acid (1 mM final concentration) was added to the above-mentioned aliquots. Iron concentrations were estimated from a standard curve obtained from different ferrous ions concentrations and 1,10 phenantroline at 510 nm. The same study was performed at pH 2.

**Effect of Trofin<sup>®</sup> on Fe<sup>+3</sup>:** Four different combination of Trofin<sup>®</sup> plus Fe<sup>+3</sup> were prepared. The w/w Trofin<sup>®</sup>: iron ratios in the mixtures were 500:1, 2 500: 1, 5000:1 and 10 000:1, containing Trofin<sup>®</sup> at 10, 50, 100 or 200 µg/ml, respectively plus 20 µM Fe<sup>+3</sup> and 1 mM 1,10 phenantroline in HEPES buffer 50 mM, pH 6.5. At 0, 5, 10, 15, 20, 25 and 30 minutes of incubation, 2.94 ml aliquots were removed from the mixture. The Fe<sup>2+</sup> formation was detected at 510 nm. Control contained 20 µM of Fe<sup>+3</sup> plus 1 mM ascorbic acid.

**Brain homogenate preparation:** Three weaned Sprague Dawley male rats were obtained from The National Center for the Production of Laboratory Animals (CENPALAB), Havana, Cuba. Animals were sacrificed by cervical dislocation. Heads were removed and cerebral hemispheres were quickly dissected and cleaned from blood in 4 °C saline solution. Brain homogenate were prepared in 1.15% KCl ice-cold solution at 20% (w/v) using a potter homogenizer with ice bath. Aliquots of 1ml were dispensed and conserved at -20 °C.

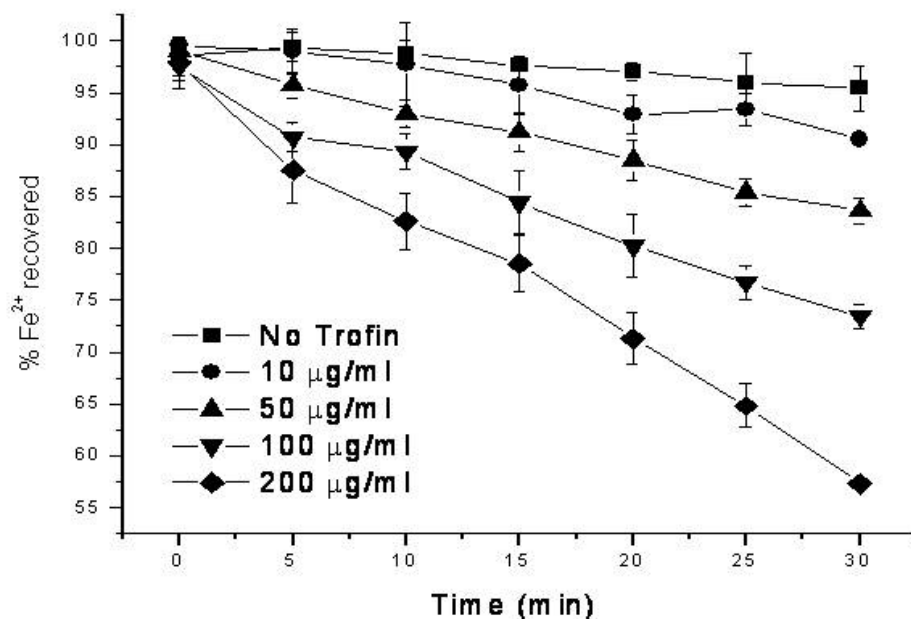
**Lipid peroxidation assay:** To confirm the effects of Trofin<sup>®</sup> on Fe<sup>+2</sup> status in a more biologically relevant system, samples containing 0.5 ml of brain homogenate were incubated with 20  $\mu$ M Fe<sup>+2</sup> or with the mixtures Trofin<sup>®</sup>-iron (20  $\mu$ M Fe<sup>+2</sup>) at 500:1, 5000:1 and 10 000:1 w/w ratio (previously incubated for 30 minutes in 50 mM HEPES buffer, pH 6.5), during 1h at 37 °C in a shaking bath. The reaction was terminated by the addition of 1% trichloroacetic acid solution. Thiobarbituric acid (1% solution prepared in 50 mM NaOH ) was added, and the contents were kept in a boiling water bath for 15 min, cooled and extracted with *n*-butanol. The absorbance of butanol was measured at 535 nm. The concentration of thiobarbituric reactive substances were expressed as malonildialdehyde concentration from  $\varepsilon = 1.56 \text{ M}^{-1}\text{cm}^{-1}$  (14).

**Statistical analyses:** Data from the experiments were analyzed by one-way ANOVA followed by Tukey's post-hoc test performed by Origin 7.5 software (OringinLab Corp., Northampton, MA). When one parameter was compared between two groups, Student's *t*-test was used. Data are presented as mean  $\pm$  SD of at least three experiments conducted with different preparations.

## Results

### Trofin<sup>®</sup> diminished Fe<sup>2+</sup> concentration

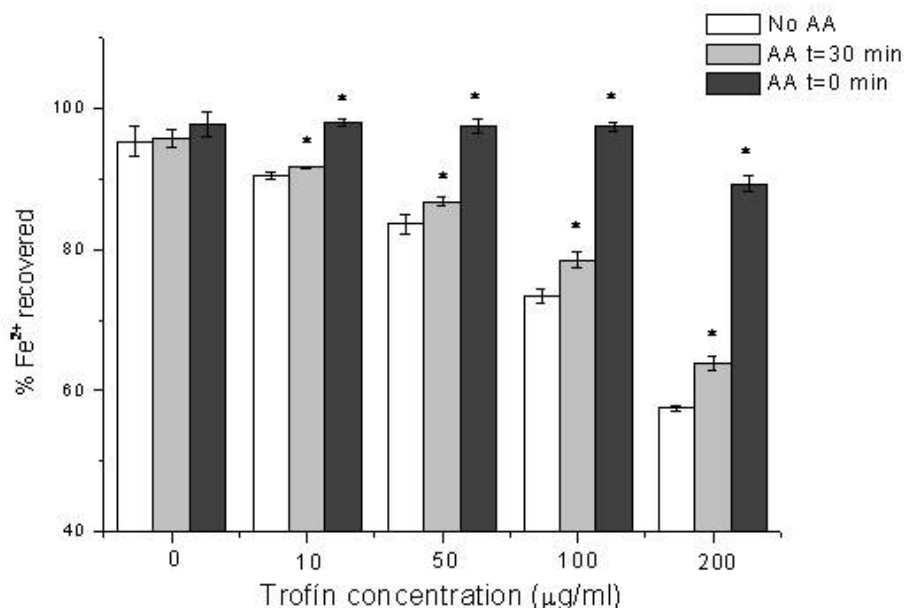
The autoxidation of ferrous ions takes place at a very slow rate in HEPES buffer at pH below 7 (15), that why it was selected as the reaction medium to search for the effect of Trofin<sup>®</sup> on Fe<sup>2+</sup>. In the absence of Trofin<sup>®</sup> only 6% of Fe<sup>2+</sup> is oxidized, that represent around 1 $\mu$ M from the initial 20  $\mu$ M Fe<sup>+2</sup> from ferrous sulphate. Trofin<sup>®</sup> induced a dose-dependent Fe<sup>2+</sup> depletion from the reaction medium, evidenced by a diminution in the absorbance at 510 nm after 5 min of incubation with the metal. After 30 min of incubation it were detected 10, 16, 27 and 43% of Fe<sup>+2</sup> concentration diminution for the mixtures Trofin<sup>®</sup>-iron at 500:1; 2500:1; 5000:1 and 10 000:1, respectively (Figure 1).



**Fig. 1.** Trofin<sup>®</sup> stimulates Fe<sup>2+</sup> autoxidation. The reaction medium contained 50 mM HEPES buffer, pH 6.5. Fe<sup>2+</sup> was added at a final concentration of 20 µM in the absence or presence of different Trofin<sup>®</sup> concentrations. Samples from the mixtures (2.94 ml) were removed at 0, 5, 10, 15, 20, 25 and 30 min. An excess of 1,10 phenanthroline (1 mM final concentration) was added to each sample and after agitation, the absorbance were read at 510 nm. The percent of Fe<sup>2+</sup> concentration remaining were calculated from a standard curve. Experiments were conducted at 22 °C with agitation and were started by the addition of 20 µM Fe<sup>2+</sup>. Values are the average of three determinations and are expressed as mean ± SD.

To assess whether the diminution of Fe<sup>2+</sup> concentration promoted by Trofin<sup>®</sup> was due to a stimulation of Fe<sup>2+</sup> oxidation, an excess of ascorbic acids was added after 30 min of Trofin<sup>®</sup>-iron incubation. Ascorbic acid partly restored the ferrous ions concentrations. It is well known that once Fe<sup>3+</sup> is formed, it is rapidly hydrolyzed giving fully aquated ferric derivatives with solubilities around 10<sup>-17</sup> M.<sup>16</sup> Thus, it is very difficult to obtain 100% Fe<sup>2+</sup> recovering from Fe<sup>3+</sup> aqueous solutions. Then it was designed a set of experiments where ascorbic acid was added to the buffer solutions before the mixture Trofin<sup>®</sup>-iron. We obtained an increase in the percents of Fe<sup>2+</sup>

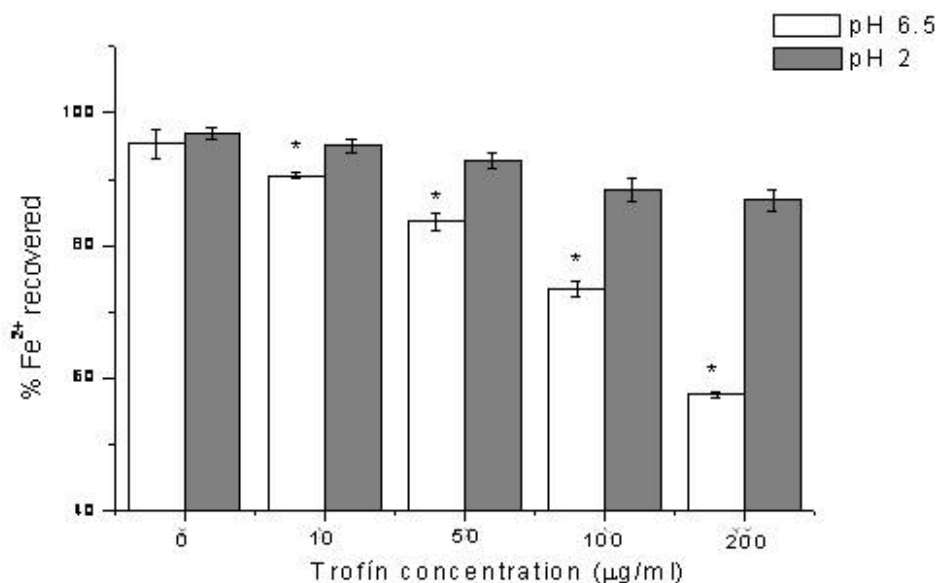
recovered under the above-mentioned condition, which strongly suggests that Trofin<sup>®</sup> stimulates Fe<sup>2+</sup> autoxidation (Figure 2).



**Fig 2.** Ascorbic acid (AA) prevented the stimulation of Fe<sup>2+</sup> autoxidation elicited by Trofin<sup>®</sup>. The reaction medium contained 20 μM Fe<sup>2+</sup>, Trofin<sup>®</sup> (10, 50, 100, 200 μg/ml) and 50 mM HEPES buffer, pH 6.5. Ascorbic acid (1 mM final concentration) was added before (black bars) and after (gray bars) 30 min of Trofin<sup>®</sup> incubation with iron. 1,10 phenanthroline (1 mM final concentration) was added after 30 min of incubation and after agitation, the absorbance were read at 510 nm. The percent of Fe<sup>2+</sup> concentration remaining were calculated from a standard curve. Experiments were conducted at 22 °C with agitation. Bars represent mean ± SD (n=3). \* P < 0.05 *versus* the results in the absence of ascorbic acid (white bars).

The same trends was obtained at pH=2 although Trofin<sup>®</sup> stimulation of Fe<sup>2+</sup> oxidation occurred in a lesser extent than at pH 6.5 probably due to the small concentration of hydroxyl ions at pH=2. It is known that the rate at which Fe<sup>2+</sup> autoxidizes as a function of pH obeys a simplified rate equation where rate is proportional to [Fe<sup>2+</sup>][O<sub>2</sub>][OH<sup>-</sup>]<sup>2</sup> (17). Thus, changes in pH will have a large effect on the rate of Fe<sup>2+</sup> autoxidation. Our results are

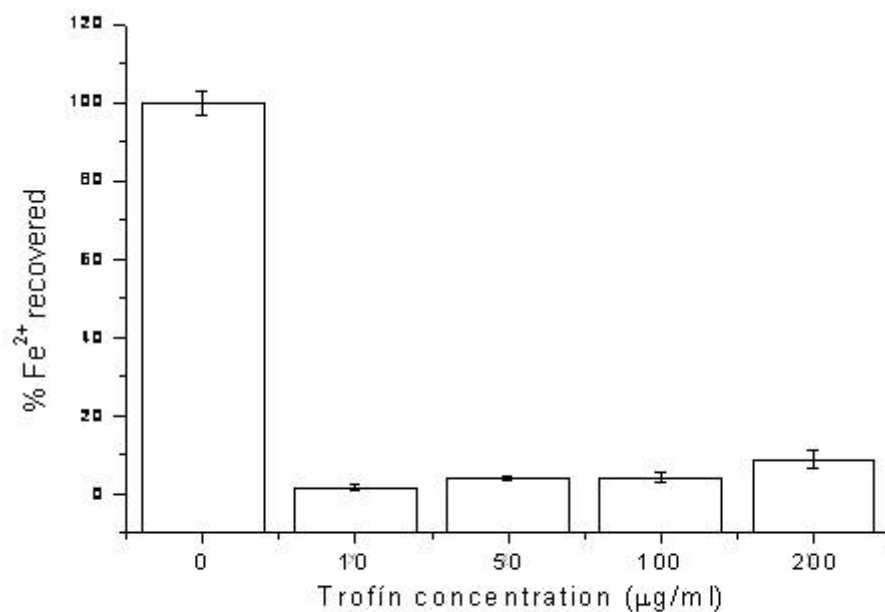
consistent with the rate law described above and support the hypothesis that Trofin<sup>®</sup> stimulates Fe<sup>2+</sup> oxidation (Figure 3).



**Fig. 3.** Influence of pH on Fe<sup>2+</sup> autoxidation elicited by Trofin<sup>®</sup>. The reaction medium contained 50 mM HEPES buffer, pH 2 (gray bars) or pH 6.5 (white bars). Fe<sup>2+</sup> was added at a final concentration of 20 µM in the absence or presence of different concentrations of Trofin<sup>®</sup>. Samples from the mixtures (2.94 ml) were removed at 30 min of incubation. An excess of 1,10 phenantroline (1 mM final concentration) was added to each sample and after agitation, the absorbance were read at 510 nm. The percent of Fe<sup>2+</sup> concentration remaining were calculated from a standard curve. Experiments were conducted at 22 °C with agitation. Bars represent mean ± SD (n=3). \* P < 0.05 *versus* pH 2.

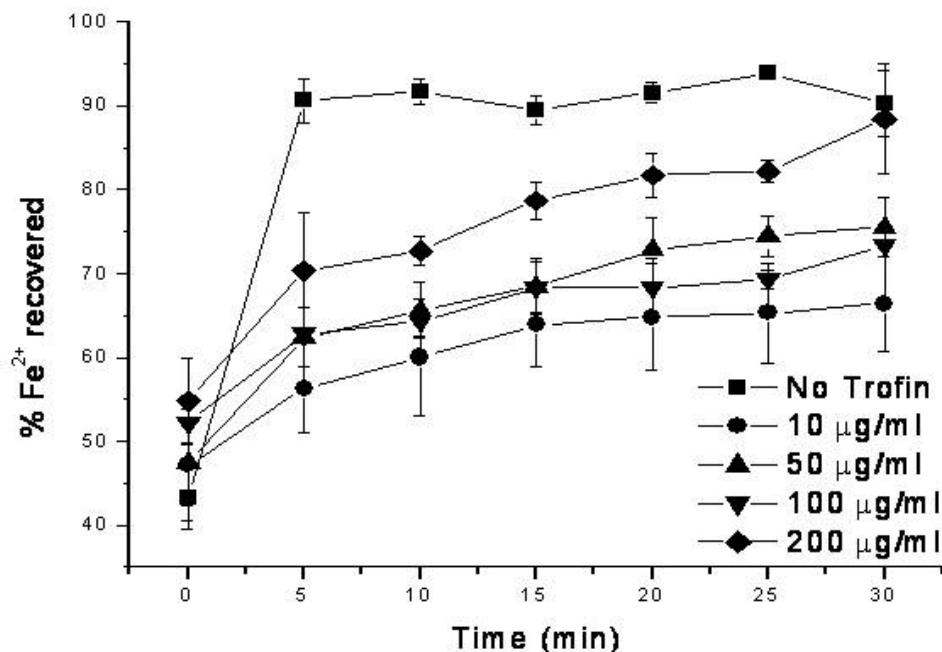
#### Assays of inhibition of Fe<sup>3+</sup> reduction by ascorbate

Trofin<sup>®</sup> has a little ability to reduce Fe<sup>3+</sup>, only the higher proportion of Trofin<sup>®</sup> in the mixture with iron reduced around 9% of the metal after 30 min of incubation, that represent 1.8 µM of the initial 20 µM Fe<sup>3+</sup> concentration (Figure 4). Interesting, this natural product inhibits the reduction of Fe<sup>3+</sup> elicited by ascorbate, reaching the higher level of inhibition at the lower Trofin<sup>®</sup>-iron w : w ratio (500:1) (Figure 5).

**Fi**

**g. 4.** Slight reducing power of Trofin<sup>®</sup> on Fe<sup>3+</sup> ions. The reaction medium contained 1 mM 1,10 phenantroline in 50 mM HEPES buffer pH 6.5. Fe<sup>3+</sup> was added at a final concentration of 20 µM in the absence (ascorbic acid 1 mM) or presence of different concentrations of Trofin<sup>®</sup>. Samples from the mixtures (2.94 ml) were removed at 30 min of incubation and after agitation, the absorbance were read at 510 nm. The percent of Fe<sup>2+</sup> concentration were calculated from a standard curve. Experiments were conducted at 22 °C with agitation. Bars represent mean ± SD (n=3).



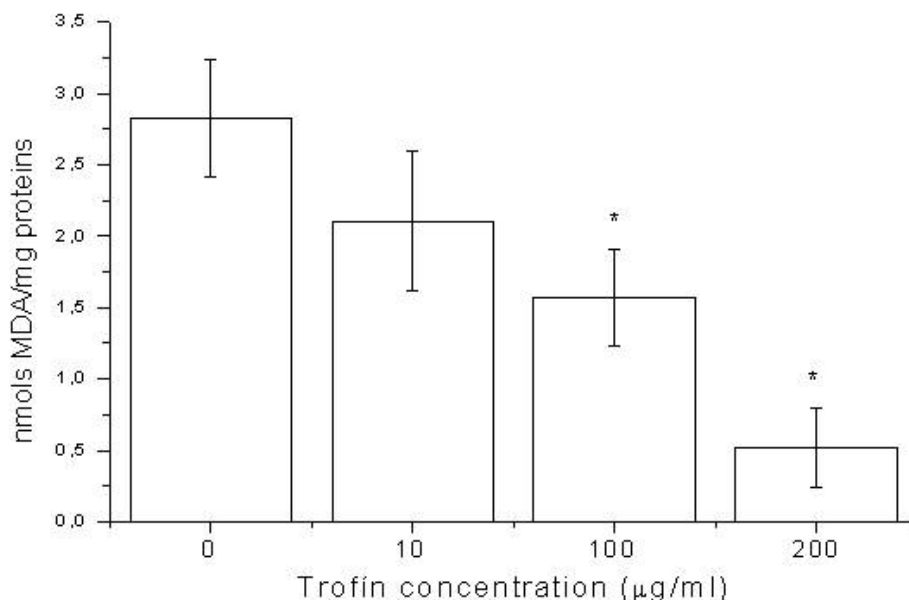


**Fig. 5.** Trofin<sup>®</sup> inhibits Fe<sup>3+</sup>- reduction by ascorbate. The reaction medium contained 20 µM Fe<sup>3+</sup> in 50 mM HEPES buffer, pH 6.5. Ascorbic acid was added at a final concentration of 1 mM in the absence or presence of different concentrations of Trofin<sup>®</sup>. Samples from the mixtures (2.94 ml) were removed at 0, 5, 10, 15, 20, 25 and 30 min. An excess of 1,10 phenantroline (1 mM final concentration) was added to each sample and after agitation, the absorbance were read at 510 nm. The percent of Fe<sup>2+</sup> concentration remaining were calculated from a standard curve. Experiments were conducted at 22 °C with agitation and were started by the addition of ascorbic acids. Values are the average of three determinations and are expressed as mean ± SD.

#### **Trofin<sup>®</sup> inhibits iron-induced lipid peroxidation in brain homogenate**

The ability of Trofin<sup>®</sup> to deplete the reaction medium from Fe<sup>2+</sup> and also to hinder Fe<sup>2+</sup> reloading to the medium from a Fe<sup>3+</sup> solution in the presence of strong reductant is a well documented antioxidant mechanism reported for some polyphenols elsewhere (13, 18-20). Trofin<sup>®</sup> dose dependently inhibits the lipid peroxidation in brain homogenates induced by Fe<sup>2+</sup> (Figure 6).

It is worth to mention that lower concentration of Trofin<sup>®</sup> (10 µg/ml) was unable to prevent the oxidative damage to brain lipids, which is in accordance with the rest of experiments where the lower concentration of the antianemic almost did not affected ferrous iron concentration. This result suggests that Trofin<sup>®</sup> at concentrations above 10 µg/ml, and probably throughout its ability to interact with ionic iron (Figures 1-4), prevents the metal participation in the catalytic formation of free radicals.



**Fig. 6.** Trofin<sup>®</sup> inhibits malondialdehyde- thiobarbituric acid adducts formation induced by 20 µM Fe<sup>2+</sup>. The rat brain homogenate (0.5 ml) were incubated in reaction medium containing 20 µM Fe<sup>2+</sup> in 50 mM HEPES buffer (pH 6.5) with or without Trofin<sup>®</sup> (10, 100 and 200 µg/ml). The experiments were initiated by addition of 20 µM Fe<sup>2+</sup> (except for the control). Incubation period was 1h at 37 °C in a shaking bath. Values are the means ± S.D. (n=3). \* P < 0.05 vs. control.

## Discussion

Autoxidation of Fe<sup>+2</sup> is affected by chelation, and depending upon the chelator, the rate of autoxidation is either increased or decreased. Oxygen,

nitrogen, and sulphur atoms of molecules are most commonly the metal ligands. Chelators in which nitrogen or sulphur atoms serve as the ligand tend to stabilize  $\text{Fe}^{+2}$  and chelators in which oxygen atoms serve as the ligand of iron tend to stabilize  $\text{Fe}^{+3}$ . There are other many factors that affect the reactivity of iron such as the influence of pH, buffers and other heavy metals (15). Since Trofin<sup>®</sup> contains a mixture of hydrolyzed heme proteins obtained from blood cows, honeybee and propolis; it is probable that some of its compounds, mainly aminoacids and sugars, are interacting with iron, stimulating its oxidation. The increase in ferrous iron recovering observed when ascorbate is added after 30 min of Trofin<sup>®</sup>  $\text{Fe}^{2+}$  incubation, the prevention of the iron oxidation when ascorbic acid is added before Trofin<sup>®</sup> iron mixture or the patterns of ferrous iron depletion induced by Trofin<sup>®</sup> at pH 2 and 6, supports such conclusion. Interesting, the results also show that Trofin<sup>®</sup> prevents ferric iron reduction by ascorbate. According to these results, it is plausible that some Trofin<sup>®</sup> compound could form a complex with  $\text{Fe}^{2+}$ , accelerating  $\text{Fe}^{2+}$  oxidation and the formation of more stable  $\text{Fe}^{3+}$ -Trofin<sup>®</sup> complexes, that prevent  $\text{Fe}^{2+}$  reload to the system, which is then unable to participate in Fenton-type reactions and lipoperoxidation propagation phase. Figure 6 shows that Trofin<sup>®</sup> inhibited  $\text{Fe}^{2+}$ -induced lipoperoxidation in rats brain homogenate, a result that strengthens our conclusion in relation to its interaction with ionic iron. This result also suggested that Trofin<sup>®</sup> does not stimulate  $\text{Fe}^{+2}$  autoxidation at low concentration, since lipids peroxidation was not prevented by the antianemic at 10  $\mu\text{g}/\text{ml}$  and supported previous experiments where Trofin<sup>®</sup>, at low proportion in relation to iron (500:1 w/w) almost did not affect ferrous ions status (Figs 1-4). In this regard, it is well known that intestinal absorption of non heme iron by DMT1 occurs in ferrous state (9). The above *in vitro* results suggested that the lowest Trofin<sup>®</sup>'s concentration could stimulate total iron absorption in formulation of Trofin<sup>®</sup> with ferrous iron salt.

In conclusion, these results suggest that the development of new antianemic formulation containing a mixture of Trofin<sup>®</sup> with ferrous salt should be observed cautiously since higher Trofin<sup>®</sup> concentrations in relation to iron may lead to ferrous iron oxidation, and should comprise amounts of Trofin<sup>®</sup> below the proportion 500:1 w/w in relation to iron. An appropriate formulation should also contain ascorbic acid, to prevent or delay the  $\text{Fe}^{2+}$  oxidation stimulated by Trofin<sup>®</sup>. The results also assign an antioxidant potential to the Trofin<sup>®</sup> formulation that could be used for the

treatment of different pathological conditions associated to an iron mediated oxidative stress. In fact it has been recently shown that iron-deficiency anemia enhances red blood cells oxidative stress (21), thus Trofin® as an iron supplier and antioxidant could give a double protection to anemic patients.

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