FULL PAPER

In vitro Antioxidant Activity of Ubiquinone and Ubiquinol, Compared to Vitamin E

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Coenzyme Q10 (CoQ10) is the prevalent ubiquinone in human organism, largely present in its reduced form, ubiquinol (QH_2), to which the antioxidant, free radical scavenger activity is ascribed by many authors. However, some studies indicate that also the oxidized form presents some effect in preventing the cellular oxidative stress. In this article four *in vitro* chemical test methods (TEAC, FRAP, DPPH, and BR) were used to assess the free radical scavenging power of CoQ10, QH_2, and vitamin E. The results showed that CoQ10 is almost ineffective, while in three of the tests QH_2 presents a higher antioxidant activity than vitamin E. From these results, it can be concluded that the interconversion CoQ10 $\rightleftharpoons$ QH_2 leading to the prevalence of QH_2 in biological tissues is responsible for the antioxidant action of coenzyme Q10 in living organisms.

Introduction. – Ubiquinones are important physiological compounds, so named by their discoverer [1], just because they are found in many living organisms, animals, and plants (ubi[quitous] quinone – everywhere present quinone). They are also called coenzymes Q because of their participation to the electron transport chain in mitochondria [2]. Ubiquinones are composed by a 1,4-benzoquinone ring with an isoprenoid side chain, a structure similar to that of vitamin E and vitamin K. The coenzyme Q10 (CoQ10: 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, *Fig. 1*) is the most prevalent ubiquinone in humans and mammals, and there is a general consensus that it, together with its reduced form ubiquinol (QH_2: 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-dihydroxybenzene, *Fig. 1*), plays a fundamental role in the mitochondrial respiratory chain and acts as antioxidant free radical scavenger and therefore preventing damages due to oxidative stress [3][4].

Regarding the antioxidant action, it is to be noticed that, for many authors, it is ascribed to the ubiquinol reduced form that is prevalent in several human tissues (from 61% in heart to 95% in liver and intestine [5]); the mean percentage of this form in the whole body is more than 80% of the total ubiquinol + ubiquinone pool [6]. Moreover, it has been reported [7 – 9] that following a dietary supplementation with CoQ10, efficient reduction to ubiquinol QH_2 occurs, either during absorption or rapidly after the appearance of CoQ10 in the blood. However *Maroz et al.* [10] found in an *in vitro* pulse radiolysis study using couples ubquinones/ols (idebenone/ol, mitoquinone/ol) slightly different from CoQ10 and QH_2, that the oxidized forms react rapidly with the superoxide anion radical $O_2^{\bullet -}$, while reduced forms react slowly with $HOO^{\bullet}$, but very quickly with other O- and C-centered radical species. In any case, these authors conclude that the reduced species is the main antioxidant in the prevention of lipid peroxidation. Another finding is that the ubiquinol QH_2 is more efficiently against peroxidation of LDL (low density lipoproteins) than α-tocopherol (vitamin E), which is one of the most potent exogenous antioxidants [11][12]. These effects of ubiquinol are independent of those of exogenous antioxidants, such as vitamin E, although ubiquinol can also potentiate the effect of vitamin E by regenerating it from its oxidized form [13] and prevent the prooxidant effect of vitamin E [14].

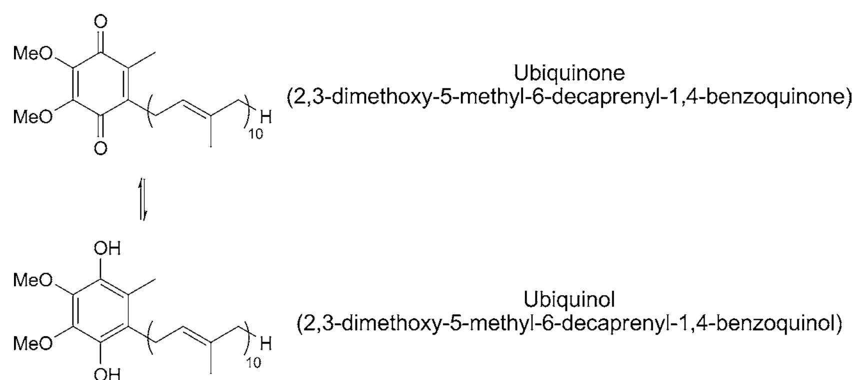

Fig. 1. *Oxidized* (ubiquinone) *and reduced* (ubiquinol) *forms of coenzyme Q10*

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The biosynthesis of CoQ10 is a complicated process. In brief, the benzoquinone portion is synthesized from tyrosine, whereas the isoprene side chain is synthesized from acetyl-CoA through the mevalonate pathway [4]. The interconversion CoQ10/QH_2 in the tissues is also a very complicated process that can be simplified by the overall reaction:

$$NADH + H^+ + CoQ10 \rightleftarrows NAD^+ + QH_2$$

that involves several substrate-enzyme complexes [15].

CoQ10 (MW 863.34 g/mol) is an orange lipophilic powder, colorless and tasteless, quite stable at room temperature, it deteriorates at temperatures of about 46° (US Patent 2005). Until some years ago, the reduced form QH_2 was prepared in MeOH and used immediately [16]. Pure QH_2 (*MW* 865.36) can be prepared by reduction of CoQ10 with sodium hydrosulfite in hexane under an N_2 atmosphere. The crude product is recrystallized from EtOH/petroleum ether. The obtained crystalline white powder is very unstable and must be conserved under vacuum at −20° [17]. The industrial synthesis of ubiquinol is protected (US Patent 2011), today, it is marketed in some stabilized solid pharmaceutical forms (all patented).

Many studies have been conducted on the biochemical [18] and physiological functions of CoQ10 [19] [20], leading to the conclusion that a deficiency of coenzyme Q10 is a (contributory) cause of various mammalian diseases, including several cardiovascular and degenerative neurological and neuromuscular diseases [5]. The redox CoQ10/QH_2 couple was found efficient as DNA protector/repair in human lymphocytes [21]. Recently, the couple CoQ10/QH_2 was found effective in skin protection from serious disease and in reduction of age-related signals [22] [23].

Therefore, it is not surprising if the exogenous (synthetic) ubiquinone CoQ10 and ubiquinol QH_2 were proposed as a therapy for reducing oxidative stress due to an excessive production of free radicals. The compounds can be administered in different pharmaceutical formulations, orally (pills, capsules) or by topical application (creams) [24].

There are many investigations on the *in vitro* biological antioxidant power of ubiquinone and ubiquinol on cell lines using the lipid peroxidation inhibitory assay [25] or forming radicals by pulse radiolysis in a suitable medium, then measuring their quenching after reaction with the couple CoQ10/QH_2 [10]. However, to date, there is a lack of information about the antioxidant capacity with usual chemical *in vitro* tests.

Even if no chemical or biological *in vitro* tests can mimic what happens in the human or other mammalian organism, the results of these tests can give useful information for further biological and clinic investigations. The purpose of this research note is then to determine the relative antioxidant activity of ubiquinone and ubiquinol and comparing it with that of vitamin E, using the TEAC (*Trolox* equivalents antioxidant capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), and BR (*Briggs–Rauscher* reaction) chemical methods. The mechanism of the TEAC, DPPH, and BR assays is a H-atom transfer (HAT) from the antioxidant to the probe that normally is a radical species, while the mechanism of the FRAP is an electron transfer (ET) from the antioxidant to an oxidized species, a ferric complex in this case (see *Exper. Part*).

Results and Discussion. – TEAC, DPPH, and FRAP are colorimetric methods. Absorbances of the assay mixtures are measured after the addition of sample or standard solutions at different concentrations. Suitable comparison of these absorbances give values of the relative antioxidant capacity of the sample (details in the *Exper. Part*). In the BR method, the oscillating behaviour of the electric potential of a *Briggs–Rauscher* reaction is recorded after the injection of sample or standard solutions at different concentrations. Values of relative antioxidant power are then obtained comparing the observed different perturbation effects on the oscillations (details in the *Exper. Part*).

Straight lines $\Delta E6$ *vs.* conc., $\%_{inhib}$ *vs.* conc., Abs. *vs.* conc., and t_{inhib} *vs.* conc. obtained for the examined compounds and the standards using TEAC, DPPH, FRAP, and BR methods, respectively, and used for relative antioxidant activity calculations are reported in *Fig. 2,a – 2,d*).

Relative antioxidant activity values of CoQ10, QH_2, and vitamin E are summarized in the *Table*.

The ubiquinone CoQ10 does not show antioxidant activity with any of the four chemical methods up to a concentration of approximately 4 mM in the mixtures. This is not surprising, since this compound does not contain phenolic OH groups able to subtract radicals *via* HAT and is fully oxidized, hence cannot reduce Fe(III) *via* ET. As mentioned above, the formation of the reduced species ubiquinol-10, QH_2, in non-aqueous solution (*e.g.* MeOH) requires the presence of a strong reducing agent such as sodium borohydride [16], and the reduction of CoQ10 cannot be achieved by any components of the assays.

Remarkable is the detected activity of QH_2 with the three methods TEAC, DPPH, and FRAP. The TEAC value (0.92), although lower than that of known antioxidants such as rosmarinic acid (3.99), cynarin (3.14), and cyanidin-3-*O*-glucopyranoside (1.95) [26a], is much higher than that of substances isolated from *Polygala alpestris* (max. 0.57) and *Polygala vulgaris* (max. 0.20) [26b]. It should also be noted that the TEAC value of ubiquinol-10 is almost twice as that found for vitamin E. The DPPH value (0.91) is higher than about 30% of that found for vitamin E (0.70) and that reported for the vitamin C (0.63). The FRAP value (2.19) is similar to that reported for vitamin C (1.9 – 2.1) [27], and more than double as that found for vitamin E (0.97). This means that QH_2 is a strong reducing agent as all the 1,4-dihydroxy aromatic derivatives that are easily oxidized to quinones. Ubiquinol-10 does not show antioxidant activity by the BR method up to the same concentration as CoQ10. Instead, vitamin E shows a

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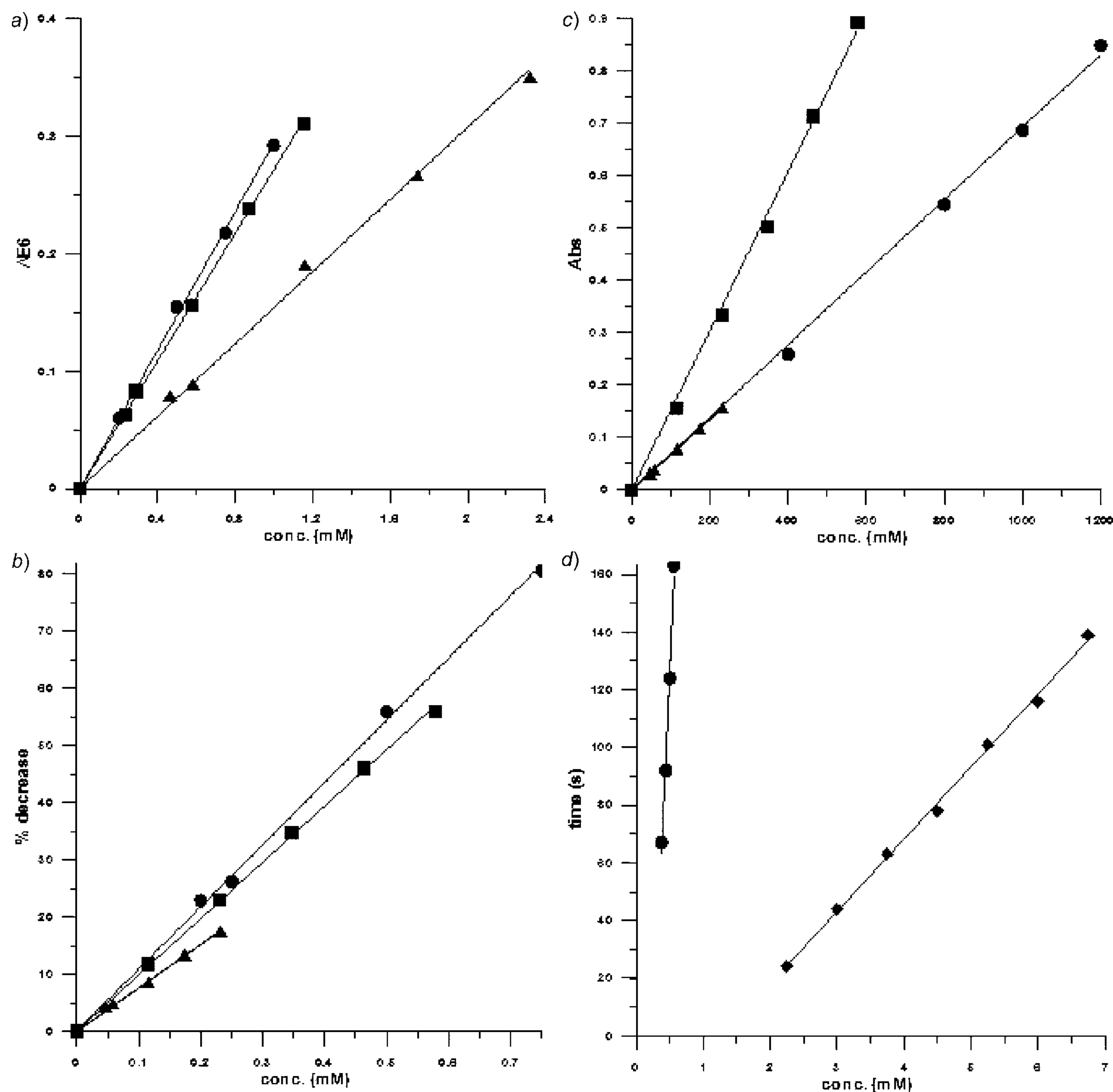

Fig. 2. *a*) *Graphs* $\Delta E6$ vs. *conc. using TEAC method* (■ = ubiquinol, ▲ = vitamin E, ● = standard). *b*) *Graphs %decrease* vs. *conc. using DPPH method* (■ = ubiquinol, ▲ = vitamin E, ● = standard). *c*) *Graphs Abs* vs. *conc. using FRAP method* (■ = ubiquinol, ▲ = vitamin E, ● = standard). *d*) *Graphs* t_{inhib} vs. *conc. using BR method* (■ = vitamin E, ● = standard)

Table. *Summary of Mean Relative Antioxidant Capacities Measured with the Four Chemical* in vitro *Methods*

Compound	TEAC [mM equiv. *Trolox*]	DPPH [mM equiv. *Trolox*]	FRAP [mM equiv. Fe(II)]	BR [r.a.c. mM equiv. 2,6-DHBA]
Coenzyme Q10	none	none	negligible	none
Ubiquinol QH_2	0.92 ± 0.02[a])	0.91 ± 0.02	2.19 ± 0.07	none
Vitamin E	0.52 ± 0.02	0.70 ± 0.02	0.97 ± 0.02	0.10 ± 0.01
Vitamin E acetate	none	none	none	none

[a]) Standard error of the mean.

certain, albeit little, scavenging power towards $HOO^{\bullet}$ radicals in acetonic acidic medium in the concentration range 2 – 7 mM. The rate constants calculated from a BR-system proposed mechanism for the step vit. $E + HOO^{\bullet} \rightarrow R + H_2O_2$ were 7.5×10^2 M^{-1} s^{-1} [28], the value reported in literature for the reaction vit. $E + HOO^{\bullet}$ in ethanolic strongly acidic medium is 2.0×10^5 M^{-1} s^{-1} [29] more than 102 fold that calculated by us. This is an indication that the kinetics of the reaction with the $HOO^{\bullet}$ radicals are much slower in acetonic than in other media and accounts for the

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very poor response of QH_2 in the BR test. Moreover, this result is in line with that reported by *Maroz et al.* [10] on the reactivity of QH_2 towards $HOO^{\bullet}$ radicals. Vitamin E shows radical scavenging activity with all the used methods, even if the TEAC, DPPH, and FRAP values are less than those of ubiquinol-10. In general, vitamin E is present in pharmaceutical forms as tocopheryl acetate. This derivative doesn't show free-radical scavenging activity being the phenolic OH group esterified with an acetate residual (see *Table 1*), but in the human organism, a hydrolysis occurs leading to free vitamin E.

Final Remarks. – In conclusion, the results reported here with four *in vitro* chemical methods show that the reduced form of coenzyme Q10, QH_2, is a strong antioxidant free-radical scavenger and a powerful reducing agent, showing higher activity than vitamin E in three out of four methods. It can be concluded that the continuous interconversion $CoQ10 \rightleftharpoons QH_2$ leading to the prevalence of QH_2 in biological tissues are responsible for the antioxidant action of coenzyme Q10 in living organisms, where QH_2 could also be reconverted to CoQ10 *via* reverse *Fenton*-like reactions.

Experimental Part

Chemicals. Acetone (*Fluka*, reagent grade, > 99.5%), manganese(II)sulphate monohydrate (*Fluka*, r.g., > 99%), $NaIO_3$ (*Merck*, r.g. > 99.5%), 2,6-DHBA (2,6-dihydroxybenzoic acid), *Sigma–Aldrich*, r.g. > 99%), $K_2S_2O_8$ (*Fluka*; r. g. > 99%), ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid diammonium salt, *Fluka*; r.g. > 99%), *Trolox* (6-hydroxy-2,5,8-tetrametylchroman-2-carboxylic acid, *Sigma–Aldrich*, r.g. > 98%), DPPH (2,2-diphenyl-1-picrylhydrazyl, *Sigma–Aldrich*, r.g. ≥ 99%), sodium acetate trihydrate (*Riedel – De Haen*, r.g. 99.5%), $FeSO_4 \cdot 7\,H_2O$ (*Sigma–Aldrich*, r.g. > 99%), $FeCl_3 \cdot 6\,H_2O$ (*Fluka*, 98.0 – 102.1%), TPTZ (2,4,6-tri(pyridin-2-yl)-1,3,5-triazine, *Fluka*, r.g. > 99%), ubiquinone (coenzyme Q10, *Sigma–Aldrich*, > 98%), and ubiquinol (reduced form of CoQ10; *Kaneka Corp.*, Japan), α-tocopherol (Vitamin E, *Aldrich*; 96%) were used without further purification. All stock solns. were prepared with doubly distilled, deionized H_2O. Perchloric acid (*Merck*, 70 – 72%) was analyzed by titration *vs.* a standard 0.1M NaOH soln. (from *Merck*). H_2O_2 (*Merck*, 35 – 36.5%) was standardized daily by manganometric analysis. All measures were performed in triplicate. All other chemicals were reagent grade.

Antioxidant Activity Assay Based on the Acetone-Based Briggs–Rauscher (BR) *Reaction.* The chemical *in vitro* BR method [30] is based on the inhibitory effects of ROS scavengers on the oscillations of the *Briggs–Rauscher* (BR) reaction.

The classic BR system [31] consists of H_2O_2, acidic iodate, malonic acid, Mn(II) as catalyst and works at pH ≈ 2, similar to that of the human gastric juice. For strongly lipophilic compounds, it was found convenient to use acetone instead of malonic acid as substrate [28]. The reaction method is based on the generation of free radicals in the mixture. The generated hydroperoxyl radicals ($HOO^{\bullet}$) are among the main intermediates of the BR system. The mechanism of the action of antioxidants against $HOO^{\bullet}$ radicals in the BR system has been described in detail elsewhere [30][32]. In brief, when antioxidant scavengers of free radicals are added to an active oscillating BR mixture, there is an immediate quenching of the oscillations, an inhibition time (t_{inhib}) that linearly depends on the concentration of the antioxidant added, and a subsequent regeneration of the oscillations. Relative antioxidant activities with respect to a substance chosen as a standard are determined on the basis of the inhibition times. 2,6-DHBA was chosen as standard. One millilitre of suitably diluted samples was added to 30 ml of an active BR mixture (maintained at 25.0 ± 0.1°) after the third oscillation. The oscillatory behaviour was followed potentiometrically by recording the potential of the mixture using a coupled bright Pt-electrode–reference electrode. Electrodes were connected to a multimeter controlled by an IBM-compatible PC. More details about the experimental procedure and relative antioxidant activity (r.a.c) calculation have been reported elsewhere [33]. The r.a.c. is expressed as mM 2,6-DHBA equivalents.

Antioxidant Activity Based on the TEAC (*Trolox* Equivalent Antioxidant Capacity) *Assay.* We used the protocol suggested by *Re et al.* [34]. The green $ABTS^{\bullet +}$ radical cation was prepared by mixing ABTS stock soln. (7 mM in H_2O) with 2.45 mM $K_2S_2O_8$. The mixture was kept in the dark for 12 to 24 h until the reaction was complete and absorbance was stable. For the measurements, the $ABTS^{\bullet +}$ soln. was diluted with PBS (pH = 7.4) or EtOH to an absorbance of 0.800 ± 0.020 at 734 nm. A sample of testing substance was dissolved in acetone. This soln. was suitably diluted. For the photometric assay 3.0 ml of diluted $ABTS^{\bullet +}$ soln. and 30 μl of samples of substance suitably diluted were mixed in a photometric cuvette (1.00 cm optical path length) for 45 s, and the absorbance was measured after exactly 6 min at 734 nm (T = 30.0 ± 0.1°). A blank with acetone was measured in the same way (acetone does not interfer with the TEAC mixture). The difference between the absorbances of the blank and the sample gave $\Delta E6$ ($E6_{blank} - E6_{sample} = \Delta E6$), the value used for further calculations of the *Trolox* equivalents (TEAC) in mM. A stock soln. of *Trolox* 0.25 mg/ml was prepared and diluted to an amount ranging from 0.05 mg/ml to 0.1875 mg/ml. Absorbance was measured using a *Shimadzu UV-1601* spectrophotometer controlled by an IBM-compatible PC. Plots of $\Delta E6$ *vs.* concentration gave rise to straight lines whose slopes could be compared. The relative antioxidant activity is expressed as mM *Trolox* equivalents.

Antioxidant Activity Based on the DPPH (2,2-Diphenyl-1-picrylhydrazyl) *Assay.* In the DPPH assay antioxidants reduce the free radical 2,2-diphenyl-1-picrylhydrazyl, which has an absorption maximum at 515 nm [35]. The radical soln. was prepared by dissolving 2.4 mg $DPPH^{\cdot}$ in 100 ml MeOH. For the photometric assay, 1.95 ml $DPPH^{\cdot}$ sol. and 50 μl antioxidant soln. were mixed. At first, the absorbance of the disposable cuvette with 1.95 ml $DPPH^{\cdot}$ was measured as blank, then the antioxidant soln. in acetone was added and mixed. The absorbance of the mixture was measured after 2/3/4/5/10 min and then in intervals of 5 min until $\Delta E = 0.003\ min^{-1}$. The antioxidant activity was calculated by determining the decrease in absorbance (in %) at different concentrations comparing the slopes of straight lines % decrease *vs.* conc. *Trolox* was chosen as standard, relative antioxidant activity expressed as mM *Trolox* equivalents.

Antioxidant Activity Based on the FRAP (Ferric Reducing Antioxidant Power) *Assay.* This method is based on the reduction of the 2,4,6-tris(2-pyridyl)-1,3,5-triazine-Fe^{3+}, $(TPTZ)_2Fe^{3+}$ complex (colorless) to $(TPTZ)_2Fe^{2+}$ (blue) by antioxidants [27]. Four or five different sample concentrations were tested at 593 nm, and the straight line Abs *vs.* conc. was then compared with that of the standard ($FeSO_4$) to obtain the relative ferric reducing activity, reported as mM equivalent of Fe(II).

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