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IRON-QUERCETIN COMPLEX REDUCES LIPID AND PROTEIN OXIDATION IN STREPTOZOTOCIN DIABETIC RATS COMPLICATIONS INDEPENDENTLY TO GLUCOSE LOWERING.

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ABSTRACT

Chronic hyperglycemia of diabetes mellitus causes toxic effects on organs and leads to diabetes complications. Quercetin, a polyphenolic compound ubiquitously distributed in vegetables, is capable to complex pro-oxidant metal cations like iron (II/III) and subsequently enhances its antioxidant potencies to scavenge free radicals. The antioxidant and multitarget therapeutic potencies of these metallic complexes have not been exactly elucidated *in vivo* yet. Herein, we investigate the possible anti-redox effect of ferrous/ferric quercetin complexes on streptozotocin (STZ) diabetic rats during eight weeks of experiment. Eight groups of Wistar rats weighing 200-280g were used. Control groups C, CQ, CFe and CX received by gavage, 1 ml/day of solvent, 25 mg/kg/day of quercetin, 2.5 mg/kg/day of iron and iron-quercetin complex, respectively. Diabetic groups D, DQ, DFe, DX received the same treatment like control groups in addition to a single injection of 45 mg/kg of STZ *ip*. The results suggest that iron-quercetin gavage enhanced serum total protein, transaminase (AST, ALT) activities and catalase activity in kidney and erythrocyte. However, iron-quercetin complex reduced serum albumin, triglyceride and glutathione in erythrocyte. Attenuation of TBARS and carbonyl proteins levels in erythrocytes and organs (liver, kidney, adipose tissue,) were also found in treated

rats. Oral administration of complex decreased the catalase activity in adipose tissues and plasma vitamin C. to sum-up, iron-quercetin complex decreased TBARS and carbonyl proteins levels in organs and erythrocytes but not serum glucose levels. The antioxidant mechanism of iron-quercetin complex is independent from glucose lowering levels in diabetic animals.

KEYWORDS: Experimental diabetes, Streptozotocin, Redox stress, Iron-quercetin complex, Stoichiometry.

ABBREVIATIONS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCYTB, transmembrane ferrireductase called duodenal cytochrome *b;* DMSO, dimethyl sulfoxide; DTM1, divalent metal transporter 1; ESI-MS, electrospray ionization mass spectrometry; Fe(II), ferrous iron; Fe(III), ferric iron; GLUT, glucose transporters; GSH, reduced glutathione; HNE, 4-hydroxynonenal; LD_{50}, lethal dose; LOD, limit of detection; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; ONAB; national office of cattle food; STZ, streptozotocin; TBARs, thiobarbituric acid reactive substances; ZIP14, subfamily of metal-ion transport proteins.

INTRODUCTION

Diabetes mellitus (DM) is one of the undivided common metabolic disorders that shares the phenotype of hyperglycemia[1] and also causes a dysfunction in protein and lipid metabolism.[2] It is a complex chronic world-wide disease that requires lifelong treatment.[2] The newest estimation of the International Diabetes Federation revealed that there are 381.8 million diabetic people in 2013 with a predictable increase of 591.9 million by 2035.[3] About 95% of diabetic patients suffer from type-2 diabetes. It is caused by a combination of insulin resistance, impaired insulin secretion as well as by alterations of receptor and/or post-receptor insulin signalling pathway.[4] It is now well recognized that, in DM state, the increase of oxidative stress has a close relation with the development, the progress of the pathogenesis and its subsequent complications.[5,6] This latter could be caused by non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation and decrease of ascorbic acid levels.[7]

Despite significant progresses during the last decade in the treatment of diabetes and management of its risk factors, the complications of this disease still exist and are less

controlled with the actual arsenal treatment. The finding of alternative therapeutic strategies is one of the most important challenging tasks that face health care professionals today.[5] Toward this achievement, numerous studies have been focused on the beneficial activity of phytochemical compounds present in the human diet and its ability to prevent the occurrence of degenerative diseases, cancer, cardiovascular pathologies[8,9] and diabetes.[7,10]

Quercetin (Figure 1) belongs to one of these heterogeneous classes of flavonoids (flavanols) that are related to the well-known vast polyphenols family. It can be found in significant amounts in fruits, vegetables, beverages and in many dietary supplements and herbal remedies.[5,9] It has been reported to display pleiotropic health benefit[11] and wide pharmacological properties that include anticancer, antidiabetic, antimutagenic, anti-inflammatory, antithrombotic, vasodilatory, anti-atherosclerotic, antiviral activities,[12] and capacity to reduce oxidative stress in STZ induced experimental diabetes in rat's model.[13]

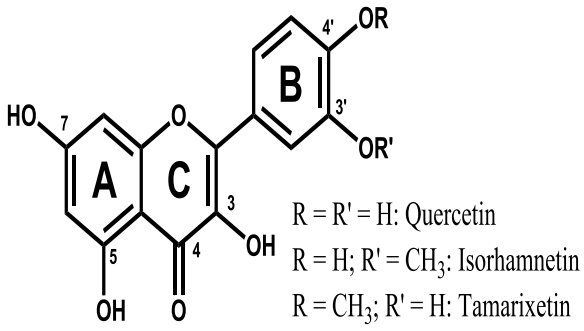

Figure 1. Chemical structures of quercetin (3,5,7,3',4'-pentahydroxyflavone) and some of its metabolites.

At present, it is recognized that the various pharmacological and physiological activities of flavonoids can be attributed to some extent to the nature and character of their interactions with other chemical species like metal ions[12] and their ability to form stable complexes[14] through their multiple OH groups and the carbonyl moiety, when ever present.[15] A wide variety of metallopharmaceutical agents have been proposed and some of them have been clinically used since the 20th century like vanadium complexes in diabetes therapy.[16] On the other hand, complexation of metal cations by quercetin has been already reported for a large number of metal ions such as Mo(VI), Fe(II)/Fe(III), Cu(II), Zn(II), Al(III), Tb(III), Pb(II), Co(II).[17]

Iron is an essential nutrient for all living cells, whether they are microbial, vegetal or animal. The only known exceptions are strains of bacterium *Lactobacillus*, where iron is replaced by manganese and cobalt.[18] Iron is found in a wide variety of cellular enzymes, with functions ranging from electron and oxygen transport to free radical induced coupling reactions.[19,20] Healthy individuals have in average 4-5 grams of iron in their bodies that is tightly regulated.[21] In the human body, Fe(III) is mainly present at neutral pH (pH = 7.4). Therefore, to ensure its solubility under these physiological conditions, iron is constantly (and mostly) bound to proteins such as hemoglobin (~2.5 g) and transferrin (iron carrier), to ferritin complexes (~2 g in adult men) that are present in all cells, as well as to low molecular weight moderate chelators such as citrates.

In mammals, and particularly in humans, both low and high levels of iron can cause serious problems.[22] Iron deficiency results in anemia as its most common consequence. There is increasing evidence that iron deficiency affects other metabolic processes, including electron transport, DNA synthesis, catecholamine metabolism and several enzyme systems.[23] Adverse effects on work performance, neurological function, immune response and epithelial tissues have also been attributed to iron deficiency.[23] Increased levels of iron in the human organism (known as iron overload) have a negative effect on the liver, pancreas, heart and other tissues. It is believed that iron-derived oxidative stress is the cause of these effects. Indeed, iron, even in tiny amounts, catalyses hydroxyl radical production by the redox cycling known as Fenton cycle.[24] In consequence, organisms have developed not only antioxidant defensive systems, but also repair systems that prevent the accumulation of oxidatively damaged molecules. However, hydroxyl radical, being more reactive than physiological reactive oxygen species (hydrogen peroxide or superoxide anion), can cause all sorts of oxidative damage to proteins, lipids and nucleic acid.[24]

Quercetin is able to chelate this iron transition metal and reduce its toxicity both *in vitro* and *in vivo* conditions. It has been proposed to display greater affinity for Fe(II) than Fe(III), confirming that the binding efficiency of flavonoids with metal ions is closely related to the nature and state of the transition metal ions.[12] Even though flavonoids are partially bioavailable due to their poor water solubility, complexation with transition metal ions, however, makes them more hydrophilic and water-soluble than the corresponding ligands.[25] This metallic complexe thus possess higher scavenger potencies toward superoxide than the parent flavonoids and may act as superoxide dismutase mimics.[12,25] Further insights on the

precise antioxidant mechanisms require thorough studies of their reactions with the biological molecular targets.[15]

Studying the interaction between flavonoids such as the broadly spread quercetin and transition metals, such as iron, in biological milieu becomes an interesting research prospect in life sciences and chemistry to explore the mechanisms of action and the influence of the structure of the corresponding complexes in biological activities and therapeutic significance. In addition, the antioxidant and multitarget therapeutic potency of these complexes *in vivo* have not been exactly elucidated so far and only scarce data are reported up to date that describes the metabolism and the therapeutic potency of these complexes in biological system. For all these reasons, the aim of the following work was to evaluate the anti-oxidative stress properties and antidiabetic potency of synthetic complexes between iron (used as its Fe(II) state for the sake of solubility), the most wide spread transition metal in the human body, and quercetin that was shown to display a strong antioxidant efficacy in diabetic STZ rats.

MATERIALS AND METHODS

Animals

A total of 64 male Wistar rats, provided by Pasteur's institute, Algeria, weighing 200-280g, were housedin spacious stainless steel cages. The rats were maintained under standard laboratory conditions (12:12±1h light-dark cycle), temperature (20±2°C) and relative humidity (50±15%). Animals had a free access to food (standard diet for rats ONAB) and water *ad libitum*. The study was conducted in accordance with the nationalguidelines for the care and use of laboratory animals. Allthe experimental protocols were approved by the Regional Ethical Committee.

Experimental design

Animals were housed five per cage and submitted to a period of 15 days of adaptation. Diabetes was induced by a single intraperitoneal injection (*i.p.*) injection of STZ (45 mg/kg.b.w/rat; Sigma Chemical) in freshly prepared 0.1 mol/L sodium citrate at pH 4.5. Control groups received just *i.p.* injection of the same amount of citrate buffer.

STZ-treated rats received 5% (w/v) of sucrose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycemic shock. Diabetes was confirmed after

the third day of STZ injection by estimation of blood glucose using an autoanalyzer Blood Glucose Meter (On Call Plus, USA).

Only the rats showing a fasting glucose of more than 127 mg/dl were considered diabetic, and used for the current study.

The rats were randomly divided into eight groups:

- Control group I (C: *n* =8); normal rats treated orally every two days with buffer solution;
- Control Quercetin group II (CQ: *n* =8); normal rats treated orally every two days with quercetin (25 mg/kg.b.w/d) dissolved in buffer solution;
- Control Iron group III (CFe: *n* =8); normal rats treated orally every two days with iron sulfate (2.5 mg/kg.b.w/day)dissolved in buffer solution;
- Control complex group IV (CX: *n* =8); normal rats treated orally every two days with quercetin-iron complex as described below;
- Diabetes control group V (CD: *n* =8); diabetic rats treated orally every two days with buffer solution;
- Diabetes Quercetin group VI (DQ: *n* =8); diabetic rats treated orally every two days with quercetin (25 mg/kg.b.w/day) dissolved in buffer solution;
- Diabetes Iron group VII (DFe: *n* =8); diabetic rats treated orally every two days with iron sulfate(2.5 mg/kg.b.w/day) dissolved in buffer solution;
- Diabetes complex group VIII (DX: *n* =8); diabetic rats treated orally every two days with quercetin-iron complex as described below.

Complex Synthesis

The Fe(II)-quercetin complex was synthesized according to a modified method.[25,26] Iron (II) was chosen due to its higher aqueous solubility. The complex was synthesized every two days according to the evolution of rats' weight throughout the experimentation. The doses used in this study of quercetin.$2H_2O$ and iron sulfate ($FeSO_4.7H_2O$) are 25mg/kg.b.w/rat (far from the LD50 = 237 mg/kg.b.w/rat oral) and 2.5mg/kg.b.w/rat, respectively. We calculated the adequate amount of quercetin according to the rat's weight evolution. We added the adequate amount of quercetin in 20 ml of buffer solution (DMSO 5% (v/v) in NaCl 0.9% (w/v) pH=8 in a 50 mL necked round-bottomed flask equipped with an electromagnetic stirrer and thermometer. The solution was stirred until solid quercetin was completely dissolved within 15 min. The colour of the solution was light yellow. The adequate calculated

amount of iron (II) sulfate was then added quickly in the reaction mixture. The colour of the solution spontaneously turned brownish yellow. After 1h of stirring at room temperature, the brown yellowish complexes were immediately administered orally to experimental rats.

Blood samples and erythrocyte lysate preparation

After eight weeks of experiment, the animals were fasted overnight and sacrificed. They were anaesthetized with *i.p.* of chloral 10% (w/v) (3mL/kg.b.w). Blood samples were collected from abdominal aorta in a vacutainer with and without anticoagulant for plasma and serum separation. Plasma was separated, and the packed cell volume (PCV) was washed three times with 0.9% sodium chloride (normal saline). Erythrocytes were lysed with cold distilled water (1/10), stored at -4°C for 15 min and the cell debris were removed by centrifugation (2000 g for 15 min). The separated water layer was used as erythrocyte lysate for estimation of oxidant/ antioxidant markers.

Organs homogenate preparation

Liver, kidney and abdominal white adipose tissues were removed, and thoroughly washed with ice-cooled 0.1 mol/L phosphate buffered saline (PBS), quickly blotted and weighted. An aliquot of each tissue was homogenized with an Ultra-Turrax homogenizer (Bioblock Scientific, Illkirch, France) in 10 volumes of ice-cold 10 mmol/L phosphate-buffered saline (pH 7.4) containing 1.15% KCl. The homogenate was subjected to 6000 g centrifugation at 4°C for 15 min. The supernatant fractions were collected and used for evaluation of redox stress status.

Biochemical analysis

The levels of serum iron, glucose, total proteins, albumin, triglycerides, uric acid and aspartate aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2) activities were determined spectrophotometrically using Chronolab Systems kits, Spain.

Catalase activity (CAT, EC 1.11.1.6) evaluation

This enzymatic activity was measured in erythrocyte lysate and organs homogenate by spectrophotometric analysis of the rate of decomposition of hydrogen peroxide.[27] In the presence of catalase, the decomposition of hydrogen peroxide leads to a decrease of the absorption of the solution of H_2O_2 over time. The reaction medium contains the erythrocyte lysate or organs homogenates, H_2O_2, and phosphate buffer (50 mmol/L, pH 7.0). After

incubation, the staining reagent, titanium oxide sulfate ($TiOSO_4$) (prepared in 2N H_2SO_4), was added. Absorbance was measured at λ = 240 nm.

Reduced Glutathione estimation

Reduced glutathione (GSH) in lysate or organs homogenates were assayed by the method of Ellman[28] in which the reduction of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) by a thiol group produces 2-nitro-5-mercaptobenzoic acid, an intensely yellow compound whose absorbance was measured at λ = 412 nm.

Carbonyl proteins determination

The carbonyl protein in plasma and organs homogenates were measured by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method of Levine et *al.*[29] Briefly, 50 μL of plasma and organs homogenate were incubated for 1h at room temperature in the presence of 1 mL of 2 g/L DNPH in 2 mol/L of HCl as control blank. Then, proteins were precipitated with 200 μL of 500 g/L trichloroacetic acid (TCA) and washed three times with 1:1 (v/v) ethanol: ethyl acetate and three times with 100 g/L TCA. The final precipitate was dissolved in 6 mol/L of guanidine and the absorption spectrum of DNPH versus HCl controls was measured from 350 to 375 nm.

TBARS determination

Thiobarbituric acid reactive substances (TBARS) was the most used lipid peroxidation marker. This assay was performed according to the method of Draper and Hadley[30] by a hot acid treatment using thiobarbituric acid (TBA). The plasma and organshomogenate were incubated for 20 minutes at 100°C with TBA and TCA. After incubation, cooling and centrifugation at 4000 rpm for 10 min, the absorption reading, that was measured at λ = 532 nm, was performed on the supernatant which contains the TBARs.

Vitamin C determination

Plasma vitamin C was assayed according to the method of Jacota and Dani[31] using the Folin-Ciocalteau reagent and ascorbic acid as reference range. After precipitation of plasma proteins by TCA and centrifugation, the supernatant was incubated in the presence of thediluted Folin-Ciocalteau reagent. Vitamin C present in the plasma reduces the Folin-Ciocalteau reagent givingrise to a yellow coloration. The absorption was measured at λ = 769 nm (maximum of absorption of the Folin-Ciocalteau). The concentration was determined from the standard curve obtained with a know solution of ascorbic acid.

Statistical analysis

Results are expressed as means ± SD. The results were tested for normal distribution using the Shapiro–Wilk test. Data not normally distributed were logarithmically transformed. Data were analysed using a two-way analysis of variance to determine differences between controls and diabetics, the various treatments (without, iron, quercetin, and iron-quercetin complex) with a level of significance of $p < 0.05$. When significant changes were observed in ANOVA tests, Tukey multiple range test was applied to identify the specific significant differences between each pair. These calculations were performed using STATISTICA version 4.1 (STATSOFT).

Analysis of the total metal contents in the standard dietsfor rats ONAB

About 0.5 g of the standard diet was introduced in a mixture of 8 ml of high purity concentrated nitric acid (HNO_3, Sigma-Aldrich >69.5%, for trace analysis) and 1 ml of hydrochloric acid for 60 minutes to ensure complete dissolution of the samples. The samples were then diluted by a factor of about 40 with deionised water. The diluted digests were analysed for concentrations of iron, copper and zinc on a Varian Inductively Coupled Plasma Optical Emission Spectrometer 735-ES. The iron/zinc contents in the standard diet were found to be 90±1/66±1 ppm. The copper level was found to be lower than the LOD and was not taken into account.

Acido-basic and Fe(III) complexation properties of quercetin

For the sake of solubility, quercetin was dissolved in a mixed solvent made of 80% of methanol (Merck) and 20% of purified distilled water by weight. All the stock solutions were prepared by weighing solid products using an AG 245 Mettler Toledo analytical balance (precision 0.01 mg). The ionic strength was maintained at 0.1 mol/L with n-tetrabutylammonium perchlorate (NEt_4ClO_4, Fluka, puriss), and all measurements were carried out at 25.0(2)°C.

Stock solutions of quercetin were first preparedin methanol. The flavonol solutions were then diluted in CH_3OH/H_2O solvent (80/20 by weight) containing the supporting electrolyte at 0.1 mol/L(NEt_4ClO_4, Fluka, puriss). The potentiometric titrations of quercetin (~1.1×10^{-3}mol/L) and its ferric complexes ($[Q]_{tot}/[Fe]_{tot}$~3) were performed using an automatic titrator system 794 Basic Titrino (METROHM) with a combined glass electrode (METROHM 6.0234.500, Long

Life) filled with 0.1 mol/L NaCl in water and connected to a microcomputer (TIAMO light 1.2 program for the acquisition of the potentiometric data).

The combined glass electrode was calibrated as a hydrogen concentration probe by titrating known amounts of perchloric acid (~ 7×10^{-2}mol/L from $HClO_4$, Prolabo, normapur, 70% min) with CO_2-freetetraethylammonium hydroxide solution (~ 10^{-1}mol/L from Aldrich, purum, ~ 40 % in water).[32] The $HClO_4$ and NEt_4OH solutions were freshly prepared just before use in CH_3OH/H_2O solvent. The cell was thermostated at 25.0 ± 0.2°C by the flow of a LAUDA E200 thermostat. A stream of argon, pre-saturated with water vapor, was passed over the surface of the solution. The GLEE program[32] was applied for the glass electrode calibration (K_w = -14.42, standard electrode potential E_0/mV and slope of the electrode/mV pH^{-1}) and to check carbonate levels of the NaOH solutions used (< 5%). The potentiometric data of quercetin and its ferric complexes (about 300 points collected over the pH range 2.5-12.6) were refined with the HYPERQUAD 2000[33] program which uses non-linear least-squares methods.

At least three titrations were treated as single sets or as separated entities, for each system, without significant variation in the values of the determined constants. The successive protonation constants were calculated from the cumulative constants determined with the program. The uncertainties in the log *K* values correspond to the added standard deviations in the cumulative constants. The distribution curves of the protonated species of quercetin and its ferric complexes as a function of pH were calculated using the Hyss program.[34]

Characterization of the Fe(III) complexes with quercetin by ESI-MS

Electrospray mass spectra of ferric complexes with quercetin were obtained with an AGILENT TECHNOLOGIES 6120 quadripole equipped with an electrospray (ESI) interface. Solutions of 5.0×10^{-5}mol/L Fe(III) with 3 equivalents of quercetin ($[quercetin]_{tot}/[Fe]_{tot}$~ 3) have been prepared in methanol/water (80/20) in the absence of any background salt. The sample solutions were continuously introduced into the spectrometer source with a syringe pump (KD SCIENTIFIC) with a flow rate of 300 µL.h^{-1}. For electrospray ionization, the drying gas was heated at 250°C and its flow was set at 6 L.min^{-1}. The capillary exit voltage was fixed at 5 kV and the skimmer voltage was varied from 150 to 400 V in order to optimize the signal responses. Scanning was performed from m/z = 100 to 1400.

RESULTS

Acido-basic properties of quercetin

Quercetin possesses five ionizable deprotonation sites (namely, the 3-OH, 5-OH, 7-OH, 3'-OH and 4'-OH units). As this polyphenolic compound is sparingly soluble in pure water (*ca.* 10^{-5} - 10^{-4}mol/L), its protolytic properties have been investigated in a mixed solvent composed of 80% of spectroscopic grade CH_3OH and 20% of water (w/w).

Figure **2** depicts atypical potentiometric titration of quercetin that was recorded under these experimentalconditions (I = 0.1 mol/L NEt_4ClO_4, T= 25°C).

The potentiometric data sets (at least three independent titrations processed simultaneously) were processed using statistical tools[34] and allowed calculating three protonation constants (pK_{a1} = 8.3(2), pK_{a2} = 9.9(1) and pK_{a3} = 11.04(8)) among the five existing ones for quercetin. It is noteworthy that the protonation constants of the 5-OH and 3'-OH groups were not reachable under our experimental conditions.

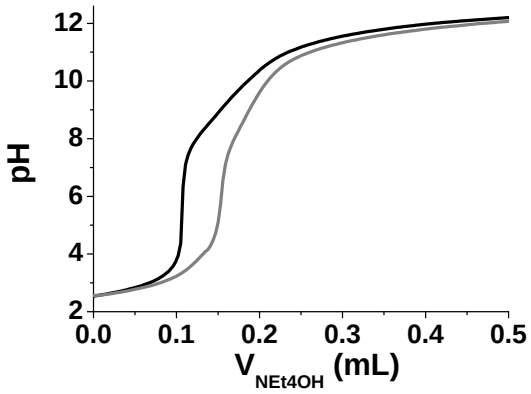

Figure 2. Potentiometric titration curve of quercetin (black) and of its ferric complexes (grey). Solvent: CH_3OH/H_2O (80/20 *w/w*); I = 0.1 mol/L (NEt_4ClO_4); T = 25.0 ± 0.2°C; [quercetin] = 1.05× 10^{-3}mol/L; [Fe(III)]/[quercetin] = 2.98.

On the basis of the 5-hydroxy-flavone (pK_a = 11.44 in dioxane/water 50/50 *w/w* and pK_a = 11.34 in CH_3OH/water 50/50 *w/w*), the chrysin flavone (pK_{a1} = 7.90 and pK_{a2} = 11.40 in CH_3OH/water 50/50 *w/w*[35]), the flavon-3-ol (pK_a = 9.99 in CH_3OH/water 50/50 *w/w*[35]), the 3',4'-dihydroxyflavone (pK_{a1} = 8.39 and pK_{a2} = 13.43 in CH_3OH/water 50/50 *w/w*[35]) and the catechol compound (pK_{a1} = 9.65 and pK_{a2} = 11.70 in C_2H_5OH/water 40/60 *w/w*[36]) the pK_a values of the 5-OH and 3'-OH of quercetin were indeed estimated to be >> 12 under our experimental conditions (CH_3OH/water 80/20 *w/w*) due to strong intramolecular hydrogen bonds with the β-carbonyl and the 4'OH groups, respectively.[37] The p*K*a values reported in

the literature for analogous flavones such as the flavon-3-ol (pK_a = 10.34 in dioxane/water 50:50 *w/w*[37] and pK_a = 9.99 in CH_3OH/water 50/50 *w/w*[35]) or the 7-hydroxyflavone (pK_a = 8.48 in dioxane/water 50:50 *w/w*[37]) allowed us to attribute the pK_{a1} and pK_{a3} values to the 7-OH and 3-OH of quercetin, respectively, while pK_{a2} could be related to the 4'-OH ionizable site. Irrespective of the experimental conditions, the fully protonated and neutral quercetin is the predominant species at physiological pH (7.4, Figure 3).

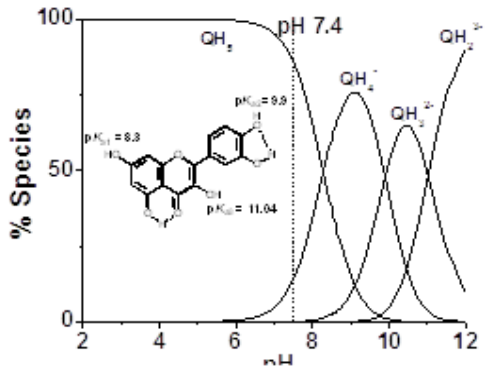

Figure 3. Distribution diagrams of the protonated species of quercetin. Solvent: CH_3OH/H_2O (80/20 *w/w*); I = 0.1 mol/L (NEt_4ClO_4); *T* = 25.0 ± 0.2°C; [quercetin] = 1.05 × 10^{-3}mol/L.Q stands herein for the fully deprotonated quercetin. Fe(III) complexation by quercetin.

The stoichiometries of the ferric complexes formed with quercetin have been assessed by electrospray mass spectrometry (ESI-MS) in the positive mode. ESI-MS mass spectra were recorded for aqueous methanolic solutions containing 1 equivalent of Fe(III) and 3 equivalents of quercetin (Figure 4).

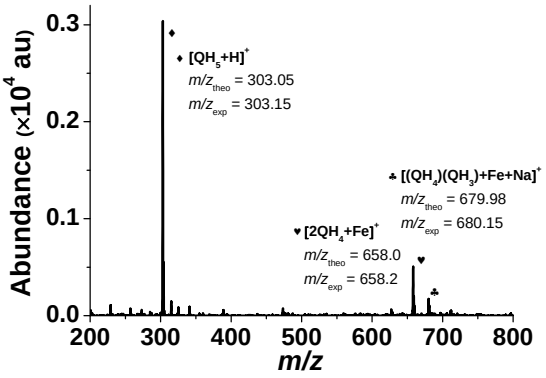

Figure 4. ESI mass spectra of the ferric complexes formed with quercetin. Solvent: H_2O:CH_3OH; positive mode; Vc = 300 V $[quercetin]_{tot}$ = 1.5 × 10^{-4}mol/L; $[quercetin]_{tot}/[Fe(III)]_{tot}$ = 3. Q stands for the fully deprotonated quercetin.

Monoferricbischelates were clearly detected which suggests that two quercetin ligands are able to strongly bind Fe(III)to one of its potential bidentate binding sites (α-hydroxy-carbonyl, β-hydroxy-carbonylor catechol). Ionization of the ferric complexes mainly took place by deprotonation of quercetin and/or addition of sodium ions. Under these experimental conditions, no Monoferric monochelates were observed likely due to the large solvation of the corresponding species. On the other hand, no Monoferric trischelate was also evidenced emphasizing the inability of a third quercetin ligand to complete the first coordination shell of Fe(III). The pseudo-molecular ions of the different species as well as their corresponding isotopic profiles were found to be in good agreement with the simulated ones.

The thermodynamic stability constants for the formation of ferric complexes with quercetin have been assessed by potentiometry (

Figure **2**) for solutions containing Fe(III) and quercetin taken in excess (~ 10^{-3}mol/L) using the values of the protonation constants determined before. The values of the hydrolysis constants of Fe^{3+}(log $K_{Fe(OH)}$ = −1.37(7) in CH_3OH/H_2O (80/20 *w/w*) were taken from literature.[38] The statistical process of the potentiometric data allowed characterizing monoferricmonochelate FeQ^* and bischelates FeQ^*_2 (Q^* stands for the monoprotonated quercetin species being protonated on the 5-position) under different protonation states ($[FeQ^*H_2]^+$: $\log\beta_{FeQ^*H2}$ = 38.52(4); $[FeQ^*_2]^{5-}$: $\log\beta_{FeQ^*2}$ = 24.1(2); $[FeQ^*_2H_2]^{3-}$: $\log\beta_{FeQ^*2H2}$ = 47.1(1); $[FeQ^*_2H_4]^-$: $\log\beta_{FeQ^*2H2}$ = 68.2(1). These data are in excellent agreement with the ESI-MS investigation which also revealed the formation of these two ferric species (Figure 5).

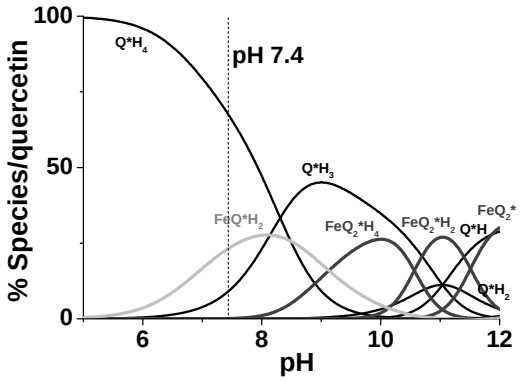

Figure 5. Distribution diagrams of the ferric complexes of quercetin.Solvent: CH_3OH/H_2O (80/20 *w/w*); I = 0.1 mol/L (NEt_4ClO_4); *T* = 25.0 ± 0.2°C; [quercetin] = 1× 10^{-3}mol/L; $[Fe]_{tot}$ = 3.33 × 10^{-4}mol/L.Q* stands herein for the monoprotonated quercetin being protonated on its 5-position.

Interestingly but also intricately, quercetin displays three potential bidentate sites for metal complexation, namely the 3',4'-dihydroxy, the 3-hydroxy-4-carbonyl and the 5-hydroxy-4-carbonyl. The Fe(III) binding properties of valuable models such as chrysin, flavon-3-ol and 3',4'-dihydroxy-flavone were previously established.[39] In order to evaluate and comparethe respective affinities of these three different flavonoids towards iron(III), pFe value were calculated at physiological conditions (pFe = -log[Fe^{3+}] with a total ligand concentration of 10^{-5}mol/L and total Fe(III) concentration of 10^{-6}mol/L, p[H] = 7.40).[39] The Fe(III) binding sequence (pFe = 14.76 for 3',4'-dihydroxy-flavone >> pFe = 12.20 for flavon-3-ol ~ pFe = 12.00 for chrysin) strongly suggests that Fe(III) complexation by quercetin preferentially occurred through its catecholate site (pFe = 12.04).

Weight gain and serum biochemical evaluation of the investigated rats

Table1 lists several characteristics of the rats in each group that were measured. As expected, iron sulfate administration significantly increased the serum iron levelsin both groups (CFe and DFe) of rats when compared to untreated rats (C and D). Administration of quercetin (CQ and DQ) has weak to no effect on serum iron levels when compared to untreated rats in control and diabetic rats. Even though quercetin has seemingly no effect on iron serum levels when used alone (CQ and DQ), its combination as metal complex with iron (CX and DX) significantly decreased the serum iron levels with respect to iron supplemented rats (CFe and DFe).

With respect to glucose levels in the investigated rats, administration of STZ systematically induced a significant increase of glucose levelsforuntreated rats (D 1.43 g/L *versus* C 0.74 g/L), quercetin (DQ 1.1 g/L *versus* CQ 0.69 g/L), iron (DFe 1.9 g/L *versus* CFe 1.7 g/L) and iron-quercetin complex (DX 1.52 g/L *versus*CX 1.18 g/L) supplemented rats. Quercetin gavagemarkedly diminished blood glucose concentration in diabetic rats (DQ) compared to diabetic control (D), the effect being weaker for control rats (CQ 0.69 g/L versus C 0.74 g/L). A significant increase of serum albumin levels was observed for quercetin CQ, iron CFe and complex CX control groups with respect to the control rats C. For diabetic rats, a decrease of serum albumin concentrations were observed for iron DFe and DX diabetic groups with respect to untreated diabetic rats, while the albumin levels increase when quercetin was used alone (DQ).

Iron and/or quercetin gavageis related to a relatively higher amount of total proteins in the control and diabetic rats. The striking point is the significant increase of total proteins

inquercetin treated diabetic rats DQ with respect to control rats CQ, while no significant variation of the total proteins can be observed between diabetic and control rats in the other conditions.

The triglycerides level was significantly augmented for diabetic rats when compared to homologous controls whatever the gavage used. However, quercetin used alone or in combination with iron reduces the triglyceride either for diabetic rats or control rats, while iron administration markedly increases the triglycerides concentrations in both control and diabetic animals.

Quercetin, iron and complex gavages were found to increase notably the serum uric acid levels in the control and diabetic groups. A noticeable exception is the diabetic rats fed with iron-quercetin whose serum uric acid concentrations are comparable to D diabetic STZ or C control groups.

Lastly, the biochemical blood analysis of serum transaminase activities (AST, ALT) showed an increase forfree iron and iron-quercetin complex treated control rats. Only theiron-quercetin complexgavage significantly enhances serum AST and ALT activities in diabetic animals. In this latter, AST transaminase activity was significantly reduced by iron treatment (DFe) when compared control (D).

Table 1.Serum biochemical characteristics of the investigated rats.

	Serum iron (uM)	Glucose (g/L)	Albumin (g/L)	Total protein (g/L)	Triglycerides (g/L)	Uric acid (mg/L)	AST (IU/L)	ALT (IU/L)
Group	**Control rats**							
C	31.48±3.04^{b}	0.74±0.05^{e}	28.3±1.16^{c}	54.2±1.09^{b}	0.98±0.02^{e}	73±4.9^{d}	20.25±0.5^{d}	16.9±0.92^{d}
CQ	30.84±2.56^{b}	0.69±0.04^{e}	35.61±1.14^{b}	54.3±1.3^{b}	0.87±0.01^{f}	100±15.82^{c}	18.89±0.9^{d}	15.25±0.5^{d}
CFe	43.5±2.52^{a}	1.7±0.05^{b}	36.11±1.23^{b}	70.1±2.24^{a}	1.33±0.02^{c}	182±14.37^{a}	31±1.5^{b}	28.48±1.02^{b}
CX	35.45±2.82^{b}	1.18±0.03^{d}	41.28±1.23^{a}	58.7±2.25^{b}	0.85±0.03^{f}	104.33±17.36^{c}	31.5±3.21^{b}	21±1.01^{b}
Diabetic rats								
D	20.87±2^{c}	1.43±0.05^{c}	38.89±0.4^{a}	55.6±3.91^{b}	1.57±0.03^{b}	70.54±2.8^{d}	34±1.35^{b}	28.9±1.03^{b}
DQ	19.59±2.36^{c}	1.1±0.05^{d}	43.55±1.06^{a}	71.14±0.52^{a}	1.42±0.04^{c}	95.82±2.26^{c}	38±0.62^{b}	26.32±0.54^{b}
DFe	41.53±2.74^{a}	1.9±0.05^{a}	31.97±1.22^{b}	67.14±5.66^{a}	1.73±0.04^{a}	153.26±2.84^{b}	24.63±1.72^{c}	27.15 ±0.84^{b}
DX	34.29±2.21^{b}	1.52±0.03^{c}	33.47±1.86^{b}	61.63±2.68^{a}	1.2±0.04^{d}	73.68±2.59^{d}	52.75±2.15^{a}	37.5±0.68^{a}
P (ANOVA)								
P	0.0004	0.0004	0.008	0.006	0.001	0.001	0.005	0.01
P'	0.0001	0.0001	0.01	0.01	0.003	0.005	0.006	0.01

Values are presented as means±standard deviations (SD). Data were tested by two-way ANOVA and Tukey post hoc tests. Values with different superscript letters (a, b, c, d,) are significantly different according Tukey test at $P < 0.05$ (P: diabetes, P': complex administration).

Redox stress evaluation in liver, kidney and adipose tissue of the investigated rats

In the followingstudy (Table. 2), TBARS, carbonyl proteins andcatalase activitiesin liver, kidney and adipose tissues were measured as biomarkers of the oxidative stress of the different rats groups. The TBARs levels in liver and kidney were markedly reduced byquercetin-iron complex gavage in both control and diabetic animals, whereasthe same feature wasonly observed on adipose tissues for the diabetic DX group. No significant difference of TBARs was indeed observedon adipose tissues of control groups CX.

As a general rule, free iron supplementation significantly increases the carbonyl proteins whatever the organ considered. Oral administration of iron-quercetin complex(DX) compensate this deleterious effect and significantly reduced carbonyl proteins to remarkable low levels in liver, kidney and adipose tissue in diabetic rats, while the carbonyl proteins similarly decrease upon gavage of control rats by quercetin alone (CQ).

Lastly, the catalase activity of the liver and kidney tissues is significantly reducedfor the iron control group (CFe) and for the fourdiabetic rats groups (D, DQ, DFe and DX) when compared to control animals (C, CQ or CX). The catalase activity of the adipose tissues stands in an interesting contrast with a much higher catalase activity in all the diabetic groups when compared to the corresponding control groups.

Table 2.Organs redox stress evaluation in the studied rats.

	Liver			Kidney			Adipose tissue		
Group	**TBARs (nM/g of tissue)**	**Carbonyl proteins (nM/g of tissue)**	**Catalase activity (U/g of proteins)**	**TBARs (nM/g of tissue)**	**Carbonyl proteins (nM/g of tissue)**	**Catalase activity (U/g of proteins)**	**TBARs (nM/g of tissue)**	**Carbonyl proteins (nM/g of tissue)**	**Catalase activity (U/g of proteins)**
Control Rats									
C	1.71 ± 0.06^{a}	1.55 ± 0.04^{c}	67.14 ± 2.9^{a}	3.16 ± 0.05^{b}	2.06 ± 0.22^{b}	16.55 ± 0.15^{a}	1.66 ± 0.04^{c}	0.17 ± 0.03^{a}	8.11 ± 0.26^{c}
CQ	1.19 ± 0.04^{d}	1.51 ± 0.03^{c}	69.97 ± 2.41^{a}	2.48 ± 0.06^{c}	1.08 ± 0.03^{c}	17.35 ± 0.38^{a}	1.39 ± 0.03^{c}	0.13 ± 0.02^{b}	8.29 ± 0.48^{c}
CFe	2.48 ± 0.05^{b}	2.23 ± 0.15^{a}	46.67 ± 1.2^{c}	3.88 ± 0.06^{a}	2.78 ± 0.11^{a}	7.31 ± 0.45^{c}	1.49 ± 0.05^{c}	0.13 ± 0.02^{b}	9.22 ± 0.41^{c}
CX	1.18 ± 0.03^{d}	1.63 ± 0.08^{c}	68.39 ± 0.96^{a}	1.51 ± 0.03^{d}	1.15 ± 0.06^{c}	17 ± 0.33^{a}	1.43 ± 0.03^{c}	0.14 ± 0.02^{b}	9.77 ± 0.37^{c}
Diabetic Rats									
D	3.15 ± 0.1^{a}	2.32 ± 0.04^{a}	56.66 ± 1.34^{b}	4.28 ± 0.05^{a}	2.87 ± 0.16^{a}	8.85 ± 0.48^{c}	2.42 ± 0.21^{a}	0.19 ± 0.01^{a}	14.69 ± 0.31^{a}
DQ	2.81 ± 0.15^{a}	1.93 ± 0.07^{b}	55.65 ± 1.52^{b}	4.78 ± 0.14^{a}	2.91 ± 0.09^{a}	11.5 ± 0.44^{b}	2.43 ± 0.11^{a}	0.17 ± 0.01^{a}	11.96 ± 0.3^{b}
DFe	2.86 ± 0.19^{a}	2.66 ± 0.1^{a}	57.52 ± 1.1^{b}	2.91 ± 0.09^{a}	3.09 ± 0.07^{a}	9.76 ± 0.27^{c}	1.9 ± 0.13^{b}	0.19 ± 0.02^{a}	15.89 ± 0.45^{a}
DX	1.75 ± 0.08^{c}	1.5 ± 0.06^{c}	49.33 ± 0.8^{b}	1.15 ± 0.05^{c}	1.15 ± 0.05^{c}	10.5 ± 0.16^{b}	1.48 ± 0.05^{c}	0.13 ± 0.02^{b}	12.59 ± 0.32^{b}
P (ANOVA)									
P	0.001	0.001	0.003	0.004	0.007	0.005	0.003	0.005	0.003
P'	0.002	0.0005	0.0001	0.0001	0.01	0.0004	0.004	0,002	0.0006

Values are presented as means±standard deviations (SD). Data were tested by two-way ANOVA and Tukey post hoc tests. Values with different superscript letters (a, b, c, d,) are significantly different according Tukey test at $P < 0.05$ (P: diabetes, P': complex administration).

Redox stress evaluation in plasma and erythrocyte lysate of the studied rats

The TBARs and reduced glutathione were measured in plasma and erythrocyte lysate (Figure. 6). TBARs erythrocyte lysate levels were significantly enhancedby free ironsulfate in control group (CFe). Irrespective of the supplementation of the rats, the TBARs erythrocyte lysate levels are significantly increased in diabetic rats by STZ injection with respect to untreated groups. More specifically, quercetin and ironprovided separately by gavage todiabetic rats markedly induced an increase of the TBARs erythrocyte lysate levels with respect to D group, while the combination of both (DX) normalized this lipids peroxidation marker compared to the control group (C, CQ or CX, Figure. 6). No significant difference of reduced glutathione (GSH) in erythrocyte lysate was observed in all the non STZ treated groups (C, CQ, CFe and CX). However, the levels of erythrocyte GSH were reduced in diabetic groups that were subjected to STZ with or without iron gavage (D or DFe), while theaddition of quercetin either restore (DQ) or markedly increases the GSH levels by comparison with the control animals (Figure. 6).

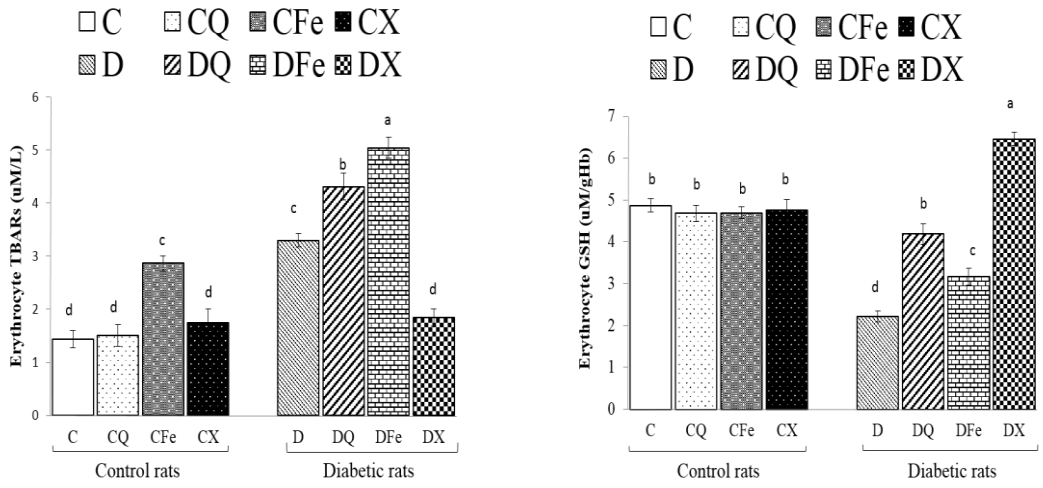

Figure 6. TBARs and GSH levels in erythrocyte lysate during eight weeks of study in normal and diabetic rats.

Values are presented as means±standard deviations (SD). Data were tested by two-way ANOVA and Tukey post hoc tests. Values with different superscript letters (a, b, c, d,) are significantly different according Tukey test at $P < 0.05$.

Figure.7 depicts the catalase activity and carbonyl proteins of erythrocyte lysate. For the diabetics STZ groups, the administration of iron (DFe), quercetin (DQ) and complex (DX) enhanced the erythrocyte catalase activity. STZ administration with (D) or without (DFe) also

significantly increased the erythrocyte carbonyl proteins level; however, this latter was normalized by quercetin (DQ) or iron-quercetin complex (DX) gavagein diabetic ratsgroups compared to controls.

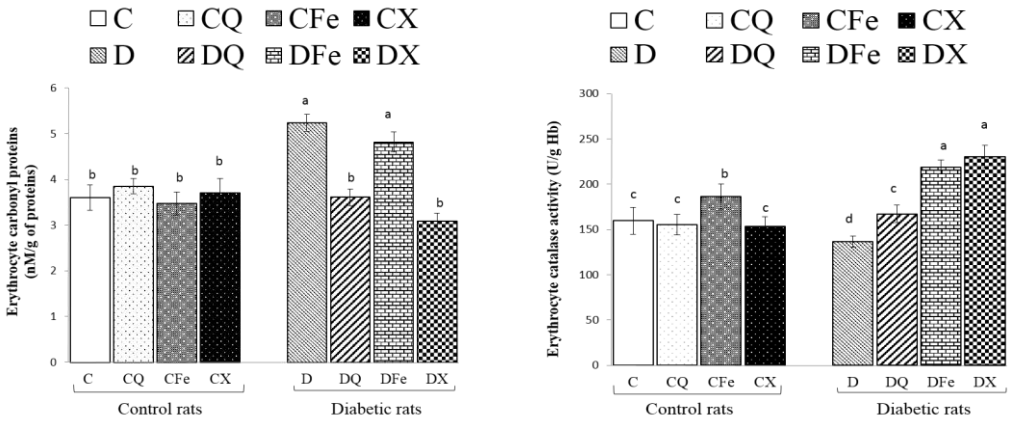

Figure 7. Carbonyl proteins and catalase activity levels in erythrocyte lysate during eight weeks of study in normal and diabetic rats.

Values are presented as means±standard deviations (SD). Data were tested by two-way ANOVA and Tukey post hoc tests. Values with different superscript letters (a, b, c, d,) are significantly different according Tukey test at $P < 0.05$.

To sum up, the analysis of the erythrocyte redox stress status clearly demonstrated that the STZ injection in rats increases TBARs and diminishes reduced glutathione levels in erythrocyte lysate. Nonetheless, administration of iron-quercetin complex decreased TBARs and carbonyl proteins concentration and enhanced the reduced glutathionelevel and catalase activity in erythrocyte lysate of diabetic animals.

We then turned our attention to the plasma vitamin C levels (Figure. 8). The plasma vitamin C level was significantly increased following a supplementation with iron-quercetin complex in controlgroup (DX) as well as after STZ injection (D). These levels were, however, significantlydecreased to values close to those measured for the control groups (C, CQ and CFe) after treatment of the diabetic rats with quercetin (DQ), iron (DFe) or complex (DQ).

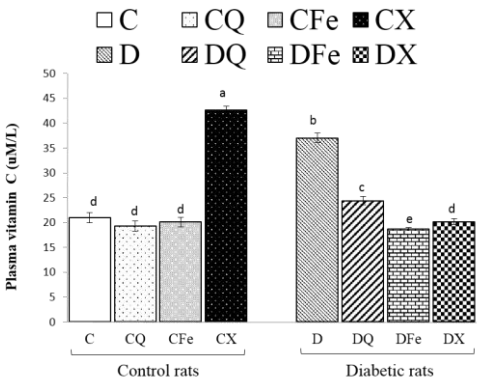

Figure 8. Plasma vitamin C levels during eight weeks of study in normal and diabetic rats.

Values are presented as means±standard deviations (SD). Data were tested by two-way ANOVA and Tukey post hoc tests. Values with different superscript letters (a, b, c, d,) are significantly different according Tukey test at $P < 0.05$.

DISCUSSION

As being mentioned above, quercetin constitutes a common flavonoid widely distributed in foods and well knownfor its anti-diabetic and antioxidant properties *in vitro* and *in vivo*[40] to cite a few. This flavonoid was shown to complex, among other, a wide range of transition metals to catalyse electron transport and promote free radical capture. Radical scavenging and/or iron-chelation[41] are indeed common pathways that account for the global antioxidant property of polyphenols. With respect to the iron-chelation pathway (*i.e.* chelation of the pro-oxidant Fe(II) metal cation) and depending on the stability of the corresponding ferrous complexes, polyphenols can either strongly bind to Fe(II), thus preventing the pro-oxidant cation from reacting with hydrogen peroxide(*i.e.* Fenton reaction), or promotes the oxidation of the less stable ferrous complexesinto more stableferricones (*i.e.* auto-oxidation) that cannot contributeany longerto the Fenton reaction. The metal chelation by flavonoids wasrecently the focus of a renewed interest given the fact that experimental data have shownthat, upon metal complexation, flavonoids might exhibit a much greater antioxidant activity than flavonoids used alone.[42]

In the present study, we then investigated the Fe(III) binding properties of quercetin and examined in details the antioxidant properties of the iron-quercetin complex in STZinduced diabetic rats. For the sake of solubility and bioavailability, quercetin was associated to Fe(II) sulfate[43] for the experiments on rats while the physico-chemical investigations were

conducted with Fe(III) perchlorate in CH_3OH/H_2O. Ferrous salts such Fe(II) sulfate, Fe(II) gluconate, and Fe(II) fumarate are indeed the most widely iron preparation recommended by the WHO.[44] On the other hand, it has been clearly shown that polyphenols, that are capable of increasing the rate of Fe(II) oxidation, are generally the more potent antioxidants. Quercetin-Fe(II) complex was shown to auto-oxidize into a ferric complex with a first order rate constant of 1.52 min^{-1}($t_{1/2}$ ~ 30 s) for a [quercetin]/[Fe^{2+}] = 3. The auto-oxidation rate constants of the ferrous complexes were indeed demonstrated to be intimately related to the anti-oxidant properties of the polyphenolic compounds.[45] Furthermore, strong Fe(III) binding by polyphenols also significantly lowers the Fe(II)/Fe(III) redox potentialand thus increases the rate of iron(II) oxidation.

On the other hand, it is noteworthy that to be properly absorbed in the duodenum by enterocytes of the duodenal lining, Fe^{3+} has to be reduced into its Fe^{2+} state by a ferric reductase DCYTB (transmembrane ferrireductase called duodenal cytochrome *b*) and thentransported by the DTM1 protein (divalent metal transporter 1) into the cells.[46] All these features demonstrate the key role of polyphenol in iron redox biochemistry and homeostasis.

In the present study, we have shown that quercetin is able to firmly bind Fe(III) through its catecholate bidentate binding site. Monoferric monochelate and bischelate were evidenced and their occurrence depend on the acidity of the medium (Figure 5). At pH 7.4, free quercetin predominates under its neutral and fully protonated state and is able to lead in the presence of Fe(III) to a mixture a Monoferric monochelate and bischelate. The stability constants of Fe(III)with catecholatebased ligands are usually rather high (pFe^{III} = 12.0 for quercetin, pFe^{III} = 14.76 for 3',4'-dihydroxy-flavone, and pFe^{III} = 15.66 for catechol (Avdeef et al., 1978).[47] Stability constants for Fe(II) catecholatescomplexes are, however, muchweaker as exemplified by the lower pFe^{II} = 9.85 measured for catechol (Dubey and Nepal, 1986).[48]

Administration to healthy and STZ-induced diabetic rats of 2.5 mg/kg/day of Fe(II) sulfate via oral route for eight weeks clearly increased the levels of serum iron and glucose (Table.1) in both groups of controls (CFe) and diabetics rats(DFe). Serum iron levels are, however, diminished in STZ diabetic animal (D) when compared to the group of controls (C). These observations are in agreement with the results of Li et *al.* on STZ diabetic rats overloaded by 300 mg of elemental iron/kg.i.p of body weight. There are strong evidences that iron overload is involved in the pathogenesis of diabetes and its complications.[49] Moreover, it was

suggested that iron overload influenced glucose metabolism and insulin action and is intimately linked to oxidative stress. Upon its ferrous state in the presence of H_2O_2, It also participates to the formation of highly toxic free radicals, such as the hydroxyl radical, through the Fenton reaction. It is well known that this hydroxyl radical $HO^{\bullet}$ affects insulin physiologic properties and makes it more hydrophobic leading to impairment in the interaction of insulin with its receptor inducing peripheral hyperinsulinemia.[50] Vantyghem and co-workers had reported that peripheral organs extraction and metabolism of insulin werereduced with increasing iron stores affecting the main organs effectors of insulin action like liver and skeletal muscle leading to insulin resistance and hyperglycemia.[51]

Our resultsstrikingly demonstrate that quercetin has different responseson the levels of serum iron and glucose in non-diabetics (CQ) and diabetic rats (DQ). Thus, the serum iron and glucose lowering effects of quercetin were shown only in the diabetic group (DQ) and not in control group (Table.1, Figure. 9). These data agree with those from Vessal et *al.* that investigated the antidiabetic effects of quercetin in STZ diabetic rats (Vessal et al., 2003).[52] The mechanism of glucose lowering activity of quercetin is questionable. Some mechanisms of action were proposed by Aguirre et *al*. where quercetin improved glycemic control by either the reduction of intestinal glucose absorption in glucose transporters GLUT2 *in vitro* and the increase of glucokinase activity or the prevention of the capital number of pancreatic β-cells islet from degeneration *in vivo* studies.[53] In other studies, quercetin was reported to possess α-glucosidase inhibitory activity *in vitro*. This enzyme reduces the rate of digestion of carbohydrates. The acute consumption of quercetin was found to be effective in controlling postprandial blood glucose in STZ and alloxan induced diabetic animals.[54]

The combination of iron with quercetin (CX and DX) markedlyreducesthe serum iron and glucose levels (Table.1) compared to both groups treated with iron alone (CFe, DFe). Even though a beneficial effect of iron-quercetin complexes can be proposed, the measuredserum iron and glucose levelsremain still higher compared to control animals (C and D). On the other hand, quercetin used alone has weak to no effect on serum iron and glucose levels of control groups (CQ *versus* C) while it appreciably affects the glucose level of diabetic rats (DQ *versus* D) without altering the corresponding serum iron concentrations. Thecatechol group of the quercetin B ringhas been shown to be mainlyinvolved in Fe(II)/Fe(III)chelation. Quercetin also display two other potential bidentate binding sites such as the α-hydroxy-carbonyl (C ring) and the β-hydroxy-4-carbonyl (A and C rings) that can also participate in

metal chelation (Figure. 1).[55] The influence of quercetin on iron absorption and homeostasis cannot therefore be put aside because this complexation is potentially able to restrict the bioavailability of free iron on target organs.[56] This would explain to some extentthereduction of iron and blood glucose concentrations in control (CX) and diabetic (DX) groups compared to CFe and DFe groups (Table. 1).

Serum albumin levels werealso found to be significantly increased by quercetin, iron, and complextreatments in control groups (CQ, CFe, CX, Table. 1). This serum albumin level was decreased notably by iron and complexgavages in diabetic animals (DFe and DX *versus* D, Table. 1).

With respect to the total proteins, their concentrations were significantly increased by iron gavagein both control and diabetic groups of rats (CFe and DFe *versus* C and D, respectively, Table. 1). On the other hand, quercetin and quercetin-iron complex gavages of diabetic groups also induced a notable increase of serum total protein concentrationsthat was not observed in the control groups. It is noteworthy that albumin, that accounts for ~60% of total serum protein,[57] is implicated in metals and flavonoids transport.[55] Recent studies also demonstrated that the level of ZIP14, a metal transporter protein, increased in the liver of rats fed with high iron diet. This iron loading and glycosylation impaired Ubiquitin proteasome function implicated in protein metabolism.[58] Moreover, an enhancement of iron-quercetin complex metabolism by liver might lead to free iron and quercetin metabolites, that could likely disturb the most important two ways of proteolytic systemsorchestrated by lysosome and proteasome.[59] Last but not the least, binding of flavonoids to proteins lowers plasma concentrations of unbound bioactive flavonoids. These latter and iron binding with albumin are more stable against oxidative degradation whichendure their biological availability and further extend their plasma half-life.[60] These biochemical interactions between iron, quercetin and proteinswould protect target tissues from high leveluptake of quercetin and ironwhich might produce a toxic reaction in cells.

The management of hypertriglyceridaemia is also important feature in prevention from diabetes complications.[61] In this work, we have shown that quercetin and quercetin-iron complex gavage significantly reduced serum triglycerides in control (CQ and CX *versus* C) and diabetic (DQ and DX *versus* D) rats (Table.1). However, iron gavage induced an increase of triglycerides levels in both animals control (CFe *versus* C) and diabetic (DFe *versus* D). Our findings agree with those of Vessal *et al.* that reported that quercetin increased

glucokinase activity of the liver and reduced plasma triglycerides in diabetic animals.[52] Simcox and McClaindemonstrated that high dietary levels of iron augmented fatty acid oxidation and also stimulated lipogenesis, so the net effects of iron on lipid metabolism are complicated.[62] However, there is a clear association between iron stores, triglycerides andglucose factors that are often negatively impacted by diabetes.[63] The precise mechanisms by which iron-quercetin complex exerted its effects *in vivo* are intricate and remain not fully understood due to the lack of information reported in this field.

Quercetin, iron and its complex was shown to increase notably serum uric acid level in control groups (CQ, CFe, CX) as well as in quercetin and iron diabetic groups (DQ, DFe, Table. 1). No significant change was noted in complex diabetic (DX) group compared to control (C) and diabetic STZ rats (D).Uric acid is the end-product of the purine metabolism.[64] Its serum levels are closely associated with the health of the kidney and its ability to excrete, reabsorb, and secrete uric acid. Most cases of hyperuricemia are due to impaired excretion,[65] and directly associated with iron metabolism and diabetes.[66] Recently, Wang *et al.* have demonstrated that quercetin regulates renal urate transport related proteins to reduce hyperuricemia.[67] Controversially, Procházková et *al.*, indicated that the consumption of flavonoid-rich foods may increase plasma urate, which is a major contributor in enhancing plasma total antioxidant capacity, but theunderlying mechanism still remains unclear.[68] The insignificant change in uric acid levels observed in STZ group (D) with respect to control group (C) could be explained by the low doses of STZ injection used in this study. Moreover, it was noticed that administration of iron-quercetin complexis less hyperurecemic than administration of iron alone in control groups. Taking into account our experimental data, the iron complexes with quercetinseemingly play an important role in limiting metal bioavailability, suppressing metal toxicity, and enhancing its bioactivity.[69]

The biochemical blood analysis of serum transaminase activities (AST and ALT) was then investigated to evaluate liver dysfunctions. Iron and quercetin-iron complex gavages significantly increase those parameters in the control (CFe and CX) and diabetic (DX) groups compared to respective controls C and D (Table.1 and Figure.10) with a marked effect being observed in diabetic animals fed with iron-quercetin complex (DX). For STZ-treated rats, AST transaminase was significantly reduced by iron (DFe) compared to control (D).As observed in our study, liver enzymes levels are usually raised in acute hepatoxicity, but tend to decrease with prolonged intoxication due to damage of liver.[70] It was also suggested that kidney impairment led to an increase of AST and ALT activity. These markers were found to

be significantly increased by quercetin supplementation[54] in contradiction with our findings (CQ *versus* C and DQ *versus* D). Increased and prolonged redox stress can cause acute tissue damages leading to development of diabetic complications. The liver, kidneyadipose tissue indeed plays a critical role in maintaining glucose homeostasis in diabetes affected by hyperglycemia.[54] This last affects the oxidant /antioxidant balance in these organs leading to its impairment.[13]

Oral administration of iron-quercetin complex was, however, shown to reduce significantly TBARs and carbonylproteins levels in kidney, liver and adipose tissuesof diabetic rats compared to control (Table. 2).

It was reported that lipid peroxidation correlated well with the iron concentration in liver and kidney of iron-overloaded animals.[71] Quercetin was demonstrated to be an effective lipid peroxidation inhibitor.[72] In addition, the process of lipid peroxidation generates numerous cytotoxic degradation products such as MDA and HNE, which can form covalent adducts with proteins in liver rats iron overloaded.[73] This high formation of these MDA-macromolecules adducts could be a potential mechanism involved in diabetes oxidative stress and organs injury. The inhibition of lipid and protein oxidation by iron-quercetin complex could be one of the signs of organs protection injury under oxidative stress conditions. These lipids and proteins oxidation markers were found to be significantly neutralized by iron-quercetin complex gavage. Therefore, the alterations in antioxidant enzyme activity could be primarily due to the diabetic state.[49]

Our results demonstrate that iron gavage reduce catalase activity in liver and kidney homogenates of control groups (CFe versus C). However, this activity was also reduced in the same organs of all the diabetic groups (D, DQ, DFe, DX) compared to control animals (C). Additionally, a high catalase activity was observed in adipose tissue of all the diabetic groups, whereas no significant difference was noted in control groups (Table. 2). Marcel et *al*. also noticed a decrease in catalase activity of kidney and liver in the diabetic groups, but no such reduction was noted in liver of any gavage treatments when the diabetic rats were subjected to 5, 25 and 50 mg/kg of quercetin. They rationalized the reduction in catalase activity in diabetic rats to a direct glycation of enzyme protein.[13] De Boer et *al*. found that the tissues distribution of quercetin and its metabolites (isorhamnetin and tamarixetin, Figure 1), in rats fed with 0.1 or 1% quercetin diet, had intermediate quercetin concentrations in the major metabolizing organs (liver and kidney) and low concentrations in white fat.[74] These

results suggest probably that the tissues uptake and metabolism of iron-quercetin complex was different in tissues with respect to quercetin alone leading to a difference of antioxidant response in organs. Altogether, our data indicate an improving effect of quercetin-iron complex treatment on organ redox stress that has been shown by measuring the alterations in levels of TBARs, carbonyl proteins, and catalase in diabetic groups.

A further insight was provided by the determination of the redox markers of erythrocyte lysate of the control and diabetic groups (Figure. 6 and Figure. 7). The redox stress status analyses of erythrocytes first showed that the STZ injection in rats (D) increased TBARs and diminished GSH levels in erythrocyte lysate with respect to the control group (C). Orally administered treatments of iron-quercetin complex, however, decreased TBARs and carbonyl proteins, and increased GSH level and catalase activity in erythrocyte lysate of diabetic animals (DX). It was reported that erythrocytes contain high concentration of polyunsaturated fatty acids, ferrous ions and molecular oxygen which makes it vulnerable to oxidative stress.[75] Catalase activity is an important agent in the enzymatic defence against oxidative stress induced in diabetes situation. However, Likidlilid et *al*. explained that the controversy results, reported by literature concerning catalase activity (increased, decreased or unchanged), might be caused by glycation or fragmentation by autoxidation of this enzyme and made them impaired.[76] These alterations of catalase activity due to diabetes are probably normalized by treatment with iron-quercetin complex. Antioxidant defence mechanisms involve both enzymatic and non-enzymatic strategies. Common antioxidants include among other: catalase, GSH and vitamin C. Plasma vitamin C levels (Figure. 8) was significantly increased after treatment with iron-quercetin complex in control groups (CX) and STZ injection (D). Nonetheless, quercetin, iron and combination of both have been shown to decrease the vitamin C levels in diabetic groups DQ, DFe, DX (Figure. 8), respectively. It is not known how ascorbic acid is transported out of epithelial cells and into the plasma, although it has been suggested that it diffuses through the Na^+-dependent Transporter in basolateral membrane. On the other hand, according to the literature, the oxidized form of vitamin C (dehydroascorbic acid) is transported by glucose transporters (GLUT1, GLUT3, GLUT4). Insulin is important for cellular uptake of dehydroascorbic acid. The competition of the uptake between this latter and the physiological levels of glucose with respect to transporters can inhibit dehydroascorbic acid uptake in several cell types like adipocytes, erythrocytes, and smooth muscle cells, explaining the high plasma level of vitamin C. In fact, it is thought that the impaired cellular uptake of vitamin C and glucose could be one of the

causes of pathology in diabetes.[77] Besides this, ascorbic acid under physiological conditions can reduce free iron(III) to its ferrous stateleading to vitamin C radical.[78] Once in the cell, vitamin C radical is converted back to vitamin C by GSH. This latter may contribute to antioxidant defence by networking with other antioxidants,[79] and acting with them in synergy to quench reactive species.[80] Quercetin has been shown to penetrate erythrocytes and prevent glutathione depletion. This impairment was induced by dehydroascorbic acid and decreased reduction of oxidized glutathione catalysed by glutathione reductase as a consequence of NADPH depletion due to elevated polyol pathway.[81] Pandey and Rizvi demonstrated that quercetin rich diets may reduce the erythrocyte damage of biomolecules such as lipids and proteins in various degenerating diseases including diabetes.[82]

CONCLUSION

In conclusion, ESI-MS data provide direct evidence for the formation of iron-quercetin complexes in the solution as monoferric bischelates which quercetin ligands are able to strongly bind Fe(III) to one of its potential bidentate binding sites (α-hydroxy-carbonyl, β-hydroxy-carbonyl or catechol). There are overwhelming evidences that illustrated the damaging consequences of oxidative stress and its role in experimental diabetes caused by over production of free radicals and/or lack in antioxidant defense. As outlined above, iron supplementation was observed to exacerbate diabetes state and oxidative stress. However, its complexation with quercetin ameliorates these diabetes disorders. Therefore, oral iron-quercetin complex administration during eight weeks was shown to have a pleiotropic health effects *in vivo* between control and diabetic animals which could be useful in treatment of diabetes. These features were evidently observed in reduction of TBARs, carbonyl proteins levels and in modulation of catalase activity in kidney, liver, adipose tissues and erythrocyte lysate of diabetic rats compared to control. Besides this, iron-quercetin complex was observed to reduce also serum triglycerides level and responsible to rise the serum uric acid amount in diabetic groups. Altogether, the antioxidant mechanism and health benefits of iron-quercetin complex observed in this work are tangible and independent from its blood glucose lowering in streptozotocin diabetic animals.Thus, we can concludethat this complex possibly will became a promising adjuvant agent in diabetes mellitus therapy in association with hypoglycemic drugs.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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