The microelement Calcium enhances the reactivity of Rutin towards singlet oxygen

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SUMMARY

Flavonoids deactivate efficiently singlet oxygen and could be valuable antioxidants in systems under oxidative stress, in particular if a flavonoid rich diet was previously consumed. Furthermore, in the presence of metal ions, the flavonoid reactivity towards singlet oxygen is enhanced. In this work we report the results of singlet oxygen quenching by the complex formed from the interaction between the glycoside rutin and Ca^{+2}, the most abundant macroelement in the human body. The results obtained, indicates that the glycoside Rutin forms a stable association complex with Ca^{+2} ions in both, MeOH and water as solvents with stability constants equal to 5.5 M^{-1} and 10.3 M^{-1}. The complex reacts with moderate to high rates with singlet oxygen with total rate constants ($k_T = k_r + k_q$) of (5.82 ± 0.47) x 10^5 M^{-1} s^{-1} and (509.7 ± 40.8) x 10^5 M^{-1} s^{-1} and chemical reaction constants of (1.10 ± 0.08) x 10^5 M^{-1} s^{-1} and (8.99 ± 0.91) x 10^5 M^{-1} s^{-1} in methanol and water, respectively. The high value of the physical quenching rate constant in water, suggest that the association complex Rutin – Ca^{+2} could be a more efficient antioxidant in biological media than the glycoside. Furthermore, the ratio k_r/k_T for the complex equal to 0.022 indicates that the physical quenching of $^1O_2$ predominates largely over the chemical reaction, then, the quenching effect of Rutin - Ca^{+2} complex take place practically without antioxidant loss, a very wanted characteristic of molecules with capabilities to protect biological systems under oxidative stress conditions.

Keywords: Flavonoids; flavonoid-metal ions complexes; singlet oxygen.

RESUMEN

Los flavonoides desactivan eficientemente al oxígeno molecular singulete y se ha propuesto que son valiosos antioxidantes en sistemas bajo estrés oxidativo, en particular si previamente se ha consumido una dieta rica en flavonoides. Además, en presencia de iones metálicos, aumenta la reactividad de los flavonoides hacia el oxígeno excitado. En este trabajo informamos los resultados de la desactivación de oxígeno singulete por el complejo formado a partir de la interacción de glucósido Rutina con Ca^{+2}, el macroelemento más abundante en el cuerpo humano. Los resultados obtenidos indican que la Rutina forma un complejo de asociación estable con iones Ca^{+2} tanto en MeOH como en agua, con constantes de estabilidad iguales a 5,5 M^{-1} y 10,3 M^{-1}. El complejo reacciona a velocidades moderadas a altas con oxígeno singulete, con constantes de velocidad total ($k_T = k_r + k_q$) iguales (5,82 ± 0,47) x 10^5 M^{-1} s^{-1} y (509,7 ± 40,8) x 10^5 M^{-1} s^{-1} y constantes reactivas de (1.10 ± 0.08) x 10^5 M^{-1} s^{-1} y (8.99 ± 0.91) x 10^5 M^{-1} s^{-1}, en metanol y agua, respectivamente. El elevado valor de la constante de velocidad de desactivación física en el agua sugiere que el complejo de asociación Rutina - Ca^{+2} podría ser un antioxidante más eficaz en medios biológicos que el glucósido. Además, la relación k_r/k_T para el complejo igual a 0,022 indica que la desactivación física de $^1O_2$ predomina en gran extensión por sobre la reacción química, luego, el efecto desactivante del complejo Rutina- Ca^{+2} ocurre prácticamente sin pérdida de antioxidante, una característica muy deseada en molé-

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culas con capacidad para proteger sistemas biológicos sometidos a condiciones de estrés oxidativo.

Palabras clave: Flavonoides; complejos flavonoides-iones metálicos; oxígeno singulete.

RESUM

Els flavonoides desactiven eficientment a l'oxigen molecular singulet i s'han proposat com valuosos antioxidants en sistemes sota estrès oxidatiu, en particular si prèviament s'ha consumit una dieta rica en flavonoides. A més, en presència d'ions metàl·lics, augmenta la reactivitat dels flavonoids cap al oxigen excitat. En aquest treball informem sobre els resultats de la desactivació de l'oxigen singlet pel complex format a partir de la interacció del glucòsid Rutina amb Ca^{+2}, el macroelement més abundant en el cos humà. Els resultats obtinguts indiquen que la Rutina forma un complex d'associació estable amb ions Ca^{+2}, tant en MeOH com en aigua, amb constants d'estabilitat iguals a 5,5 M^{-1} i 10,3 M^{-1}. El complex reacciona a velocitats entre moderades i altes amb l'oxigen singlet, amb constants de velocitat total ($k_T = k_r + k_q$) iguals (5,82 ± 0,47) x 10^5 M^{-1} s^{-1} i (509,7 ± 40, 8) x 10^5 M^{-1} s^{-1} i constants reactives de (1.10 ± 0.08) x 10^5 M^{-1} s^{-1} i (8.99 ± 0.91) x 10^5 M^{-1} s^{-1}, en metanol i aigua, respectivament. L'elevat valor de la constant de velocitat de desactivació física a l'aigua suggereix que el complex d'associació Rutina - Ca^{+2}, podria ser un antioxidant més eficaç en mitjans biològics que el glucòsid. A més, la relació k_r / k_T igual a 0,022 en cas del complex indica que la desactivació física de $^1O_2$ predomina en gran extensió per sobre la reacció química. Consequentment , l'efecte desactivant del complex Rutina- Ca^{+2}, es manifesta pràcticament sense pèrdua d'antioxidant, un característica molt desitjada en molècules amb capacitat per protegir sistemes biològics sotmesos a condicions d'estrès oxidatiu.

Paraules clau: Flavonoides; complexos flavonoides-ions metàl·lics; oxigen singlet.

INTRODUCTION

An increasing number of studies has revealed the relationship between reactive oxygen species (ROS) present in systems that suffer oxidative stress, and a significant number of physiopathologies in humans, including many multifactorial diseases, especially cancers[1] cardiovascular disease,[2] and inflammatory disorders.[3] These studies have promoted research involving various types of exogenous antioxidants and the factors that affect their behaviour in biological systems. The main non-enzymatic antioxidants present in the human organism are glutathione, bilirubin, a-tocopherol, estrogenic hormones, coenzyme Q, melanin, melatonin, uric and lipoic acids. Moreover, many studies have now confirmed that exogenic antioxidants, mainly those supplied by foods, are vital for reducing oxidative stress. These antioxidants mainly come from vegetables and foods made from these, in the form of phenolic compounds such flavonoids, flavonoid glycosides, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols, ascorbic acid and carotenoids.

Flavonoids pharmacological effects are typically connected to their antioxidant activity due to their ability to scavenge free radicals[4,5] and to their interaction with several enzymes.[6] Also, synergistic effects with other antioxidants[7] improve its capacity to trap radicals. Another antioxidant mechanism may result from the flavonoid interactions with transition metal ions to produce complexes that prevent the participation of metal ions in free radical generating reactions such as Fenton and Haber–Weiss reactions.[8,9] Unsaturated lipid substrates are key targets of oxidation in biological environments. Mechanism of lipid oxidations are complex but most frequently involves the initiation by oxygen in the presence of heat, free radicals, light, photosensitizers and metal ions. Main accepted reaction pathways are: i) chain autoxidation mediated by free radicals; ii) nonradical photooxidation; and iii) enzymatic oxidation. The first two types of oxidation includes a large number of reactions with involvement of triplet oxygen, $^3O_2$, and singlet oxygen, $^1O_2$, the first excited state of the oxygen molecule. There are several sources of $^1O_2$ but its presence is frequently due to the absorption of UV-VIS light by photosensitizers. In addition, singlet oxygen in biological systems can also be formed by a chemical, rather than photochemical, process in neutrophils during the oxidative burns response.[10] Singlet molecular oxygen reactions are important in biological systems, where it can play deleterious (damaging valuable biomolecules) or beneficial roles such as in photodynamic therapy.[11,12]

We recently showed that flavonoids are good quenchers of singlet oxygen and could be valuable antioxidants in systems under oxidative stress, in particular if a flavonoid rich diet was previously consumed.[13] In the course of these studies, we surprisingly found that several metallic ions, Cu^{+2}, Ca^{+2} and Mg^{+2} increase the reactivity of the flavonoid Quercetin towards singlet molecular oxygen by approximately one order of magnitude and that this effect could be ascribed to the formation of an association complex between the flavonoid and the metal ion. Also, García et al. studied the behavior of 3,3'-dihydroxyflavone and chrysin and its complexes with La^{3+} and Cu^{2+}, respectively,[14,15] as quenchers of reactive oxygen species such as singlet molecular oxygen $^1O_2$ and superoxide radical anion ($O_2^{\cdot-}$) photogenerated by riboflavin. However, not further studies have been published. Considering these facts, the importance of singlet molecular oxygen as deleterious species in systems suffering oxidative stress and the widespread use of flavonoids rich diets to prevent the harmful effects of active oxygen species, we plan a systematic study related to the effect of selected macro and microelements on reactivity of flavonoids towards singlet molecular oxygen in the

presence of metal ions. In this work we report the results of singlet oxygen quenching by the complex formed from the interaction between Rutin (Fig 1), the glycoside resulting of the binding between the flavonol quercetin and the disaccharide rutinose, with Ca^{2+}, the most abundant macroelement in the human body. Rutin is a citrus flavonoid present in several plans species including *Carpobrotus Edulis, Olive (black), Fagopyrum esculentum, Asparagus, blackberries, etc.*

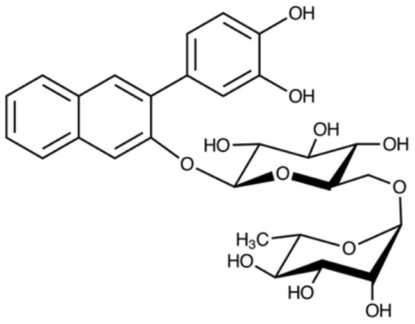


Figure 1. *Molecular structure of Rutin.*

MATERIALS AND METHODS.

Materials.

Rutin (Sigma-Aldrich), Bengal rose (RB, Sigma-Aldrich), 9,10-dimethylanthracene (Sigma-Aldrich), anhydrous calcium chloride (Merck) were used without previous purification. All solvents used were spectroscopic or HPLC grade.

Methods

Absorption spectra were recorded in a Perkin Elmer Lambda 262 spectrophotometer using 1 cm quartz cells. Data were processed with UV-Vis software.

Singlet oxygen measurements.

The phosphorescence of $^1O_2$ was detected by means of a customized PicoQuant Fluotime 200 system. A diode-pumped pulsed Nd:YAG laser (FTSS355-Q3, Crystal Laser, Berlin, Germany) working at 1 kHz repetition rate (2 μJ per pulse) was used for excitation of Rose Bengal at 532 nm. The luminescence exiting from the side of the sample was filtered by a 1100 nm cut-off filter (Edmund Optics) and a narrow bandpass filter at 1270 nm (NB-1270-010, Spectrogon) to remove any scattered laser radiation. A plane-convex lens (23 mm diam. X 75 mm) was used to focus the light emitted onto the photomultiplier window. A near-IR sensitive photomultiplier tube assembly (H1033A-45; Hamamatsu Photonics) was used as detector. The rate constant for $^1O_2$ quenching by flavonoid ($k_T = k_q + k_r$, where k_q and k_r stand for the rate constant for physical and chemical quenching path, respectively) was determined by measuring the $^1O_2$ lifetime as a function of the flavonoid concentration. $^1O_2$ was generated by photoexcitation of a 50 μM RB solution at 532 nm and the concentration of the flavonoid was varied in the range (5 – 60 mM). A plot of the reciprocal lifetime, $1/\tau_\Delta$, vs. the concentration of the flavonoid afforded k_r as the slope of the linear fit, equation (1),

$$\frac{1}{\tau_\Delta} = \frac{1}{\tau_\Delta^0} + k_T\left[Flavonoid\right] \qquad (1)$$

where τ^0_Δ is the $^1O_2$ lifetime in the neat solvent.

Solution preparation

The flavonoid stock solutions were prepared by dissolving an appropriate amount of Rutin in methanol. The standard solutions of Ca^{2+} in MeOH or H_2O were prepared with concentrations in the range 0.1-1.0 M.

Inclusion complex preparation

Typically a set of 8 – 10 solutions were prepared containing a constant concentration of Rutin near to 0.04 mM and variable concentrations of $CaCl_2$. The solutions were homogenized in an ultrasonic bath at room temperature by 2 min and finally measured in a spectrophotometer

RESULTS AND DISCUSSION

Rutin –Ca^{2+} association complex formation.

The absorption spectra of Rutin in MeOH, shows two characteristic absorption bands, as typically observed in flavonoids, with maxima at 258 and 357 nm, corresponding to the absorption of the 2-(3,4-dihydroxyphenyl) substituent and the 5,7-dihydroxy-3-[oxy]-4*H*-chromen-4-one, respectively. Addition of calcium chloride to a 4 x 10^{-5} M of Rutin in methanol gives rise to a meaningful change in the UV-Vis spectra of Rutin as shown in Fig 2A.

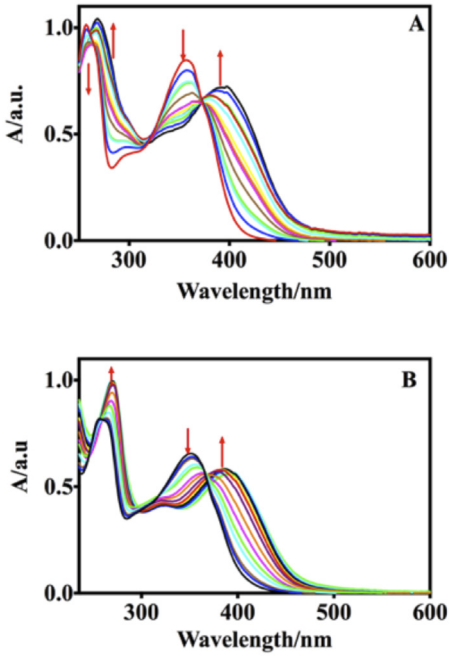


Figure 2. *UV-Vis spectra of Rutin in the absence and the presence of increasing Ca^{2+} concentrations. A: MeOH as the solvent; B: water as the solvent. Red arrows indicate the changes observed with added Ca^{2+}.*

As can be seen in Fig 2A, addition of Ca^{2+} results in a decrease of the absorbance at both maxima, although the band at larger wavelength is affected to a greater extent. Concomitantly the onset of two new absorption bands at 268 and 397 nm is observed, whose intensity increases with the concentration of Ca^{2+}. Moreover, two isosbestic points can be observed at 375 and 322 nm. A very similar behaviour was observed when water was the solvent (Fig 2B). These results suggest the formation of an association complex between Rutin and Ca^{2+}, possibly due to the interaction of the alkaline earth metallic ion with the 4-keto and 5-hydroxy groups of the chromen-4-one ring. To verify the one-to-one association complex formation and to determine the stoichiometry and the association constant, we employed the Benessi-Hildebrand –Scott method.[16]

The association complex equilibrium between Rutin and Ca^{2+} in the presence of an excess of Ca^{2+}, can be expressed as:

$$Ru + Ca^{2+} \rightleftharpoons Ru - Ca^{2+} \qquad (2)$$

Then, the equilibrium constant is:

$$K_{association} = \frac{\left[Ru - Ca^{2+}\right]}{\left[Ru\right]\left[Ca^{2+}\right]} \qquad 3)$$

The change in Rutin absorbance (ΔA) is experimentally measured, with A_0 being the absorbance before addition of Ca^{2+} and A being the absorbance at any point of the reaction.

$$\Delta A = A - A_0 \qquad (4)$$

Using the Lambert-Beer law, it can be shown that:

$$\left[Ru - Ca^{2+}\right] = \frac{\Delta A}{\Delta \varepsilon} \qquad (5)$$

where $\Delta\varepsilon$ is the difference in the molar absorption coefficients between the associated and free Rutin and the optical path length is 1 cm. If, in a set of experiments, Rutin concentration is constant and equal to $[Ru]_0$ and the free-Rutin concentration at any reaction time is expressed in terms of $[Ru]_0$ and the association complex concentrations, then the association constant can be rewritten as:

$$K_{association} = \frac{\frac{\Delta A}{\Delta \varepsilon}}{\left[Ca^{2+}\right]\left(\left[Ru\right]_0 - \frac{\Delta A}{\Delta \varepsilon}\right)} \qquad (6)$$

Equation (6) is an example of an association isotherm, namely the dependence of the concentration of one component with the concentration of another component at constant temperature. Rearranging equation (6), the dependence of ΔA with Ca^{2+} concentration can be expressed as:

$$\Delta A = \frac{\Delta\varepsilon\left[Ru\right]_0 K_{association}\left[Ca^{2+}\right]}{\left(1 + K_{association}\left[Ca^{2+}\right]\right)} = \frac{ab\left[Ca^{2+}\right]}{1 + b\left[Ca^{2+}\right]} \qquad (7)$$

where a and b are adjustable parameters with $a = \Delta\varepsilon [Ru]_0$ and $b = K_{association}$

The dependence in the absorbance change of Rutin with the added Ca^{2+} in MeOH and H_2O as solvents is shown in Fig 3. A very good fit of the data was obtained by using equation (7) assuming a 1:1 stoichiometry of the association complex. From the adjusted parameters, the association equilibrium constant is found to be 5.5 M^{-1} and 10.3 M^{-1} in MeOH and H_2O, respectively.

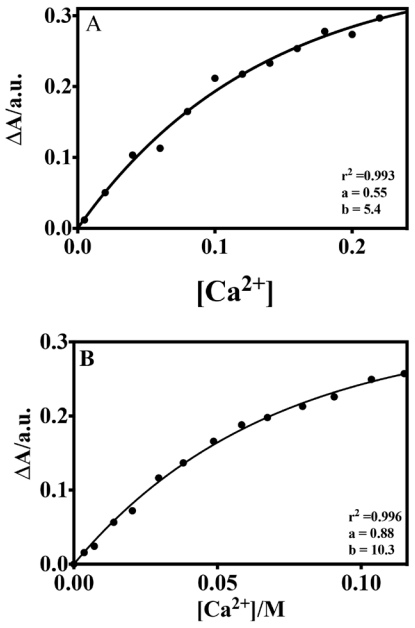
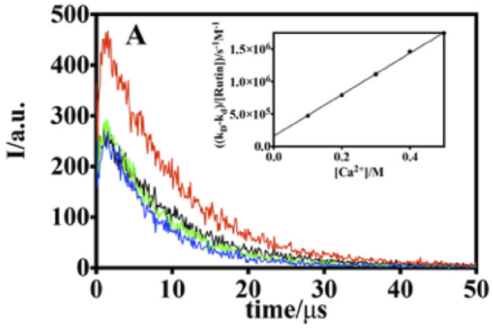


Figure 3. *Benessi-Hildebrand treatment for the association complex generated by the interaction between Rutin and Ca^{2+} in MeOH (A) and H_2O (B) as solvents.*

Reactions of Rutin and the Rutin-Ca^{2+} association complex with singlet oxygen.

The ability of Rutin and the Rutin-Ca^{2+} association complex to react with $^1O_2$ was assessed in methanol and water by means of steady-state and time-resolved methods. Rose Bengal was used as sensitizer to photogenerate $^1O_2$. Fig 4A, shows representative luminescence decays of $^1O_2$ in MeOH as solvent in the presence of a constant concentration of Rutin and increasing Ca^{2+} concentrations. Fig 4B illustrates similar experiments performed in water as solvent.



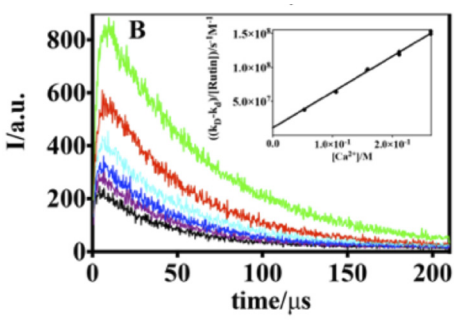


Figure 4. *Singlet oxygen near IR luminescence decays in the presence of fixed Ru concentration (4,17 x 10^{-4}M) with different Ca^{+2} concentrations, RB as sensitizer. Inset shows the data plotted according to equation (9). A: MeOH as solvent; B: H_2O as solvent*

Given that in these experiments Rutin and Ca^{2+} are added to the measurement cell, the equilibrium described by equation (2) is established and $^1O_2$ reacts simultaneously with both Rutin and the Ru-Ca^{2+} complex. The measured rate constant for the $^1O_2$ luminescence decay can be expressed according to equation (8):

$$k_D = k_d + k_T^{Ru}\left[Rutin\right] + k_T^{Ru-Ca^{2+}}\left[Ru-Ca^{2+}\right] \qquad (8)$$

where k_D is the pseudo first-order rate constant for the observed $^1O_2$ luminescence decay, k_d is the rate constant for singlet oxygen decay in the media, k_q^{Ru} is the rate constant for singlet oxygen quenching by Rutin and $k_q^{Ru\text{-}Ca2+}$ is the rate constant for singlet oxygen quenching by the Ru-Ca^{2+} association complex.

Rearranging equation (8), it can be easily demonstrated that:

$$\frac{k_D - k_d}{\left[Rutin\right]} = k_T^{Ru} + k_T^{Ru-Ca^{2+}} K_{association}\left[Ca^{2+}\right] \qquad (9)$$

Equation (9) shows that the experimental kinetic parameters correlate linearly with the concentration of Ca^{2+}. Then, from the intercept and slope of the plots shown in Fig 4, the corresponding rate constants for $^1O_2$ quenching by Rutin and the Ru-Ca^{2+} association complex can be extracted. The results are collected in Table 1.

Table 1. *Kinetic parameters for the reaction of Rutin and Rutin - Ca^{+2} complex with $^1O_2$. Values in brackets were obtained in independent experiments observing the luminescence decay of singlet oxygen as a function of Rutin concentration.*

	Solvent	
	MeOH	H_2O
k_T^{Ru} / 10^5 M^{-1} s^{-1}	1.59 ± 0.10 (2.75 ± 0.14)	112.6 ± 12.4 (152.9 ± 6.1)
$k_T^{Ru\text{-}Ca^{2+}}$ / 10^5 M^{-1} s^{-1}	5.82 ± 0.47	509.7 ± 40.8
k_r^{Ru} / 10^5 M^{-1} s^{-1}	1.10 ± 0.08[a]	8.99 ± 0.91[b]
$k_r^{Ru\text{-}Ca^{2+}}$ / 10^5 M^{-1} s^{-1}	2.37 ± 0.03[a]	11.2 ± 1.6[b]
$k_q^{Ru\text{-}Ca^{2+}}$ / k_q^{Ru}	6.3	4.8
$K_{association}$ / M^{-1}	5.5 ± 0.22	10.3 ± 0.38
$k_r^{Ru\text{-}Ca^{2+}}$ / $k_T^{Ru\text{-}Ca^{2+}}$	0.40	0.022

[a] 9.10-dimetylanthracene as reference, k_r = 6.3 x 10^7 M^{-1} s^{-1}.[17]
[b] 9,10-anthracene dipropionic acid as reference, k_r = 8.2 x 10^7 M^{-1} s^{-1}.[18]

Likewise, the reactive rate constants k_r were obtained by comparing the rate of consumption of Rutin and of its association complex with Ca^{2+} to that of the reference compounds 9,10-dimethylanthracene in MeOH and 9,10-anthracene dipropionic acid in water. The results obtained are also collected in Table 1. Values of k_T^{Ru} in MeOH and H_2O obtained from the intercept of equation (9) are similar to those determined in independent experiments observing the luminescence decay of singlet oxygen as a function of Rutin concentration (values in parenthesis) and close to previous reported data,[13] although, more relevant are the values measured for $k_T^{Ru\text{-}Ca^{2+}}$. As can be observed in Table 1, the total rate constant for reaction of Rutin with $^1O_2$, increases 3.7- and 4.6-fold, in MeOH and water, respectively, when it forms an association complex with Ca^{2+}. Furthermore, the reactive rate constant increases only 2.1- and 1.25-fold in MeOH and water, respectively, which implies that the physical quenching path is more favorable when Rutin is associated to Ca^{2+}.

In conclusion, our results indicate that the glycoside Rutin forms a stable association complex with Ca^{+2} ions both in MeOH and water as solvents. Although the complex has small stability constants, it reacts with moderate-to-high rates with singlet oxygen. The high value of the physical quenching rate constant in water suggests that the association complex Rutin – Ca^{+2} could be a more efficient antioxidant in biological media. Furthermore, the very low ratio k_r/k_T for the complex, 0.022, indicates that physical quenching of $^1O_2$ predominates largely over the chemical reaction, hence the quenching effect of the Rutin-Ca^{+2} complex occurs with very little antioxidant loss, a very wanted characteristic of molecules with capabilities to protect biological systems under oxidative stress conditions.

ACKNOWLEDGEMENTS

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