AAPS PharmSciTech 2006; 7 (1) Article 10 (http://www.aapspharmscitech.org).

Evaluation of Functional Stability of Quercetin as a Raw Material and in Different Topical Formulations by its Antilipoperoxidative Activity

Submitted: April 5, 2005; Accepted: September 17, 2005; Published: February 3, 2006

Rúbia Casagrande,[1,2] Sandra R. Georgetti,[1] Waldiceu A. Verri Jr,[3] José R. Jabor,[1] Antonio C. Santos,[4] and Maria J. V. Fonseca[1]

[1]Department of Pharmaceutical Science, Faculty of Pharmaceutical Sciences of Ribeirao Preto - University of Sao Paulo, Sao Paulo, Brazil
[2]Department of Food and Drugs Technology, Agricultural Sciences Center - Londrina State University, Paraná, Brazil
[3]Department of Pharmacology, Faculty of Medicine of Ribeirao Preto - University of Sao Paulo, Sao Paulo, Brazil
[4]Department of Clinical, Toxicological and Bromatological Analysis, Faculty of Pharmaceutical Sciences of Ribeirao Preto - University of Sao Paulo, Sao Paulo, Brazil

ABSTRACT

The present study evaluates the antioxidant activity of the flavonol quercetin, and its functional stability as a raw material and when added in formulations. The iron-chelating activity was determined using the bathophenanthroline assay, and the functional stability was evaluated with the antilipoperoxidative assay. Raw material presented concentration-dependent antilipoperoxidative and iron-chelating activities. The initial antilipoperoxidative activity of the raw material, cream and gel-cream were 63%, 78%, and 69%, respectively. There was no detectable loss of activity during 182 days (6 months) of storage at all tested temperatures (4°C, room temperature [RT], 37°C, and 45°C) for the raw material. Considering the method variability of 10%, activity loss greater than 10% for nonionic cream was detected after 126 days at 4°C (20.1%), decreasing thereafter to 22.2% after 182 days. At 45°C, the loss of activity started after 182 days (13.2%). For the anionic gel-cream, activity loss started after 84 days (28.4%, 45°C), decreasing after 182 days to 40.3% at 45°C. At 37°C, activity loss was detected after 182 days (12%). In conclusion, the results suggest that the activity of quercetin depends on iron chelation, and its possible usefulness as a topical antioxidant to prevent oxidative stress-induced skin damage depends on maintaining its antilipoperoxidative activity stored at RT, which avoids special storage conditions.

KEYWORDS: antioxidant, flavonoids, quercetin, lipid peroxidation, stability.

INTRODUCTION

The skin is considered to be our biological interface with the surroundings, and it is the first line of defense from toxic external stimuli such as ultraviolet and visible radiation, pro-oxidant chemical compounds, infections, and ionizing radiation.[1-3] Skin exposure to ultraviolet radiation induces the formation of lipid peroxidation products, lipid radicals, melanin radicals, and depletion of endogenous antioxidants.[2] Furthermore, the reactive oxygen species (ROS) formed during the lipid peroxidation can play an important function in skin aging, tumor promotion, auto-immune cutaneous diseases, and phototoxicity/ photosensitivity.[4,5]

Flavonoids, a class of phenolic compounds widely distributed in plants, can protect the organism against ROS and present multiple biological effects, including liver protection, antithrombotic, anticancer, and immunostimulant activities.[6-8] The protective effect of flavonoids against lipoperoxidative damage of membranes is well established and depends on their molecular structure and ability to interact and penetrate the lipid bilayer.[7]

These compounds are chain-breaking inhibitors of the peroxidation process, scavenging intermediary peroxyl and alkoxyl radicals. Moreover, it is suggested that flavonoids possess strong affinity toward iron ions, which catalyze various processes leading to the appearance of free radicals. Thus, the antilipoperoxidative ability of flavonoids can be described as being concomitant with their free radical scavenging and iron-chelating activities.[8-10]

Quercetin is the most abundant and commonly investigated member of plant polyphenolic compounds.[11] Moreover, quercetin exhibits the highest antiradical property toward hydroxyl radical, peroxyl, and superoxide anion compared with other flavonoids.[9] These properties of quercetin are due to the presence of 3 active chemical functional groups in its structure as presented in Figure 1: the o-dihydroxy (catechol) structure of the B ring; the 2,3-double bond in conjugation with a 4-oxo function; and the additional presence of both 3- and 5-hydroxyl groups.[9,10,12,13]

Recently, considerable research has focused on the potential use of flavonoids as free radical scavenger agents to

Corresponding Author: Rúbia Casagrande, Av do Café s/n, CEP 14040-903, Ribeirão Preto, SP, Brazil. Tel: + 55 16 3602 4315; Fax: + 55 16 3633 1941; E-mail: rubiacasa@yahoo.com.br

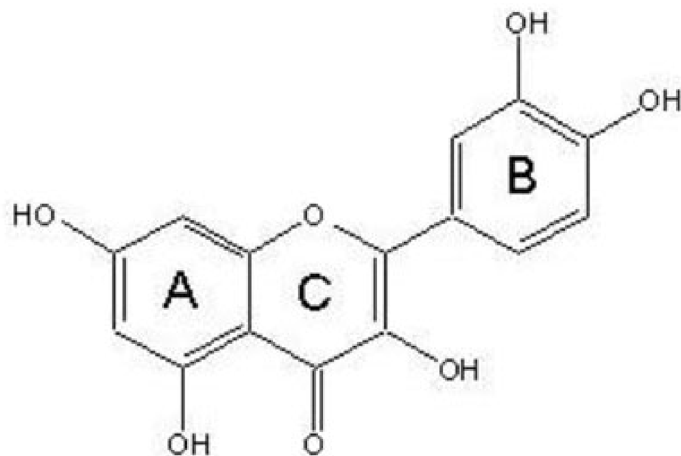

Figure 1. Structure of quercetin.

prevent oxidative damage of the skin. These activities, their additional anti-inflammatory property,[14] and being harmless to the skin[15] explain the rational for the interest in the topical use of flavonoids. Thus, it is very important to evaluate the stability of topical formulations containing flavonoids.[3,6,16]

Therefore, in the present study the functional stability of the raw material quercetin and different topical formulations containing quercetin was evaluated in vitro by measuring the antilipoperoxidative activity using thiobarbituric acid reactive species (TBARS) test. The samples were stored for 182 days (6 months) at different temperatures. Furthermore, the ability of quercetin to inhibit Fe^{2+}-dependent lipid peroxidation and its functional antioxidant Fe^{2+}-chelating activity was also investigated.

MATERIALS AND METHODS

Materials

Quercetin and bathophenanthroline-disulphonic acid were obtained from Acros (Pittsburgh, PA). Thiobarbituric acid was obtained from Sigma Chemical Co (St Louis, MO). Raw materials for formulations were obtained from Galena (Campinas, SP, Brazil) and are presented in the formulation section. All other reagents used were of pharmaceutical grade.

Determination of Iron-chelating Activity of Quercetin Using the Bathophenanthroline Assay

The iron-chelating activity was determined using the bathophenanthroline (BPS) assay.[17] Briefly, $(NH_4)_2Fe(SO_4)_2$ (50 μmol/L), quercetin (1-4 μg/mL), and BPS (0.2 mmol/L) were added to 2 mL of a reaction medium containing 125 mmol/L sucrose, 65 mmol/L KCl, and 10 mmol/L Tris-HCl, pH 7.4 (medium 1). Fe^{2+} and quercetin were left in contact for 0, 5, 10, or 15 minutes in the reaction medium prior to the addition of BPS, and after that all reagents were incubated for 15 minutes at room temperature (RT). Ten μL of stock solutions of $(NH_4)_2Fe(SO_4)_2$, quercetin, and BPS were used to obtain the described final concentrations in the reaction medium. The iron-chelating activity was monitored through the absence of the $Fe_2(BPS)_3$ complex formation. Measurements were performed at 530 to 700 nm. All measurements were made in triplicate.

Isolation of Rat Liver Mitochondria

Mitochondria were used as unsaturated lipid source for the lipid peroxidation assay, and their isolation was done by differential centrifugation.[18] Briefly, male Wistar rats weighing ~200 g were killed by cervical dislocation; their livers (10-15 g) were immediately excised, sliced in 50 mL of a medium containing 250 mmol/L sucrose, 1 mmol/L EGTA, and 10 mmol/L HEPES-KOH, pH 7.4, and homogenized 3 times for 15 seconds at 1-minute intervals in a Potter-Elvehjem homogenizer (Braun, Melsungen, Germany). The homogenate was centrifuged at 770*g* for 5 minutes, and the resulting supernatant was recentrifuged at 9800*g* for 10 minutes. The pellet was suspended in 10 mL of a medium containing 250 mmol/L sucrose, 0.3 mmol/L EGTA, and 10 mmol/L HEPES-KOH, pH 7.4 and centrifuged at 4500*g* for 15 minutes. The final sediment containing the mitochondria was suspended in 1 mL of a medium containing 250 mmol/L of sucrose and 10 mmol/L HEPES-KOH, pH 7.4.[19] The mitochondrial protein content was determined by the biuret reaction.[20]

Fe^{2+}/Citrate-mediated Lipid Peroxidation Assay

A stock solution of quercetin was prepared with the aid of propylene glycol (500 μg/mL) and diluted 125, 25, 10, 5, and 2 times in medium 1 to obtain solutions containing 4, 20, 50, 100, and 250 μg/mL, respectively. Lipid peroxidation was estimated by the formation of malondialdehyde (MDA) based on its first description.[21] This is a widely used method to evaluate antilipoperoxidative activity.[7,22,23] To 1.0 mL of medium 1, were added, respectively, 10 μL of each sample of the above prepared solutions and mitochondria to produce a final concentration of 1 mg of protein, plus 50 μmol/L of $(NH_4)_2Fe(SO_4)_2$ and 2 mmol/L of sodium citrate. The solution was kept for 30 minutes at 37°C. To determine MDA, 1 mL of 1% thiobarbituric acid (TBA), prepared in 50 mmol/L of NaOH, plus 0.1 mL of 10 mol/L NaOH and 0.5 mL of 20% H_3PO_4 were also added, followed

by a 20-minute incubation at 85°C. The MDA-TBA complex was extracted with 2 mL of *n*-butanol, centrifuged at 1660*g* for 10 minutes, and the supernatants read at 535 nm. The following controls were included in the test: one positive control was prepared in the absence of the sample (raw material) and a negative control in the absence of iron. Blanks were prepared from the reaction mixture without mitochondria. All measurements were performed in triplicate.

Formulations

The nonionic cream was prepared with commercially available self-emulsifying wax 10% (Polawax, cetostearyl alcohol and polyoxyethylene derived of a fatty acid ester of sorbitan 20E), macadamia oil 2.5%, squalene 1%, Phenova (a mixture of phenoxyethanol and parabens) 0.4%, and deionized water to complete 100%. The anionic gel-cream was prepared with commercially available self-emulsifying wax 2% (Polawax), anionic hydrophilic colloid (carboxypolymethylene, Carbopol 940) 0.18%, triethanolamine 0.2%, macadamia oil 2.5%, squalene 1%, Phenova 0.4%, and deionized water qs 100%. Quercetin (0.05%) was solubilized in propylene glycol (6%) and then added to the formulations at RT. All concentrations of the ingredients were expressed as percentages wt/wt and indicate the final concentration of each excipient and active in the formulations. The control formulations did not contain flavonoid.

Functional Stability of Raw Material and the Formulations Containing Quercetin Stored at Different Temperatures: Antilipoperoxidative Activity

The raw material and the formulations with or without quercetin were stored at 4°C, RT, 37°C, and 45°C during 182 days (6 months).[24] Samples were collected at predetermined times for the evaluation of the antilipoperoxidative activity as described above (Fe^{2+}/Citrate-mediated Lipid Peroxidation Assay Section). The raw material was diluted in propylene glycol (500 µg/mL). This solution was diluted 5-fold in medium 1, to obtain the quercetin concentration of 100 µg/mL of which 10 µL were used for the reaction (1 µg/mL). Formulations with and without quercetin were diluted in medium 1 to obtain the same concentration (quercetin 1 µg/mL) used for the analysis of the raw material in the reaction medium. The following controls were included in the test: one positive control was prepared in the absence of the sample (raw material) and another by adding the formulations without quercetin (100% of peroxidation), and a negative control in the absence of iron. One positive control was added for each storage condition. Blanks were prepared from the reaction mixture without mitochondria. All measurements were performed in triplicate. There was no difference between incubated control formulations and fresh control formulations in all tests (data not shown).

Statistical Analyses

Data were statistically analyzed by 1-way analysis of variance (ANOVA) followed by Bonferroni's *t* test for iron-chelating activity and evaluation of the formulations' influence in the lipid peroxidation assay. To analyze the influence of time and temperature of storage in the antilipoperoxidative

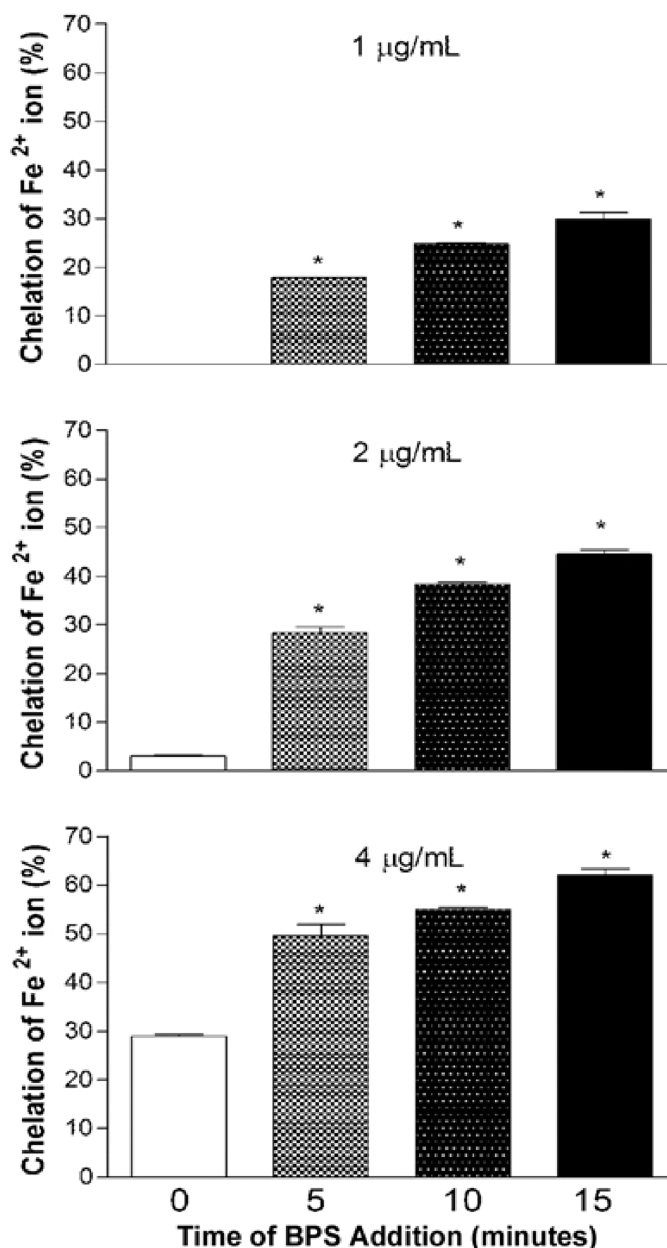

Figure 2. Chelating activity of Fe^{2+} ions by quercetin is concentration- and time-of-contact- dependent. Results are represented by the mean ± SEM. *Significantly different relative to BPS added immediately ($P < .05$).

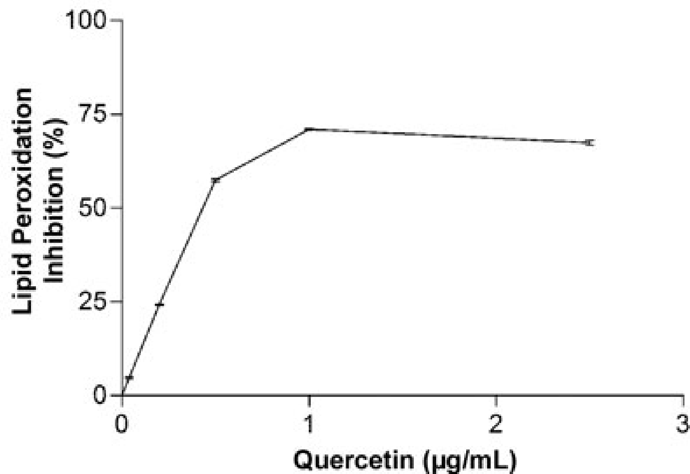

Figure 3. Inhibition by quercetin of lipid peroxidation induced by Fe^{2+}/citrate. Results are represented by mean ± SEM.

activity, 2-way ANOVA test was used. Results were presented as mean ± SEM and considered significantly different when $P < .05$ was obtained.

RESULTS

Iron-chelating Activity by Quercetin Using the Batophenanthroline Assay

The concentration of quercetin (1, 2, and 4 μg/mL) and the time of contact (0, 5, 10, and 15 minutes) between iron and the flavonoid influenced the formation of the iron-quercetin complex prior to the addition of BPS (Figure 2). In fact, with the lower concentration of quercetin tested, there was no detectable iron-chelating activity when quercetin and

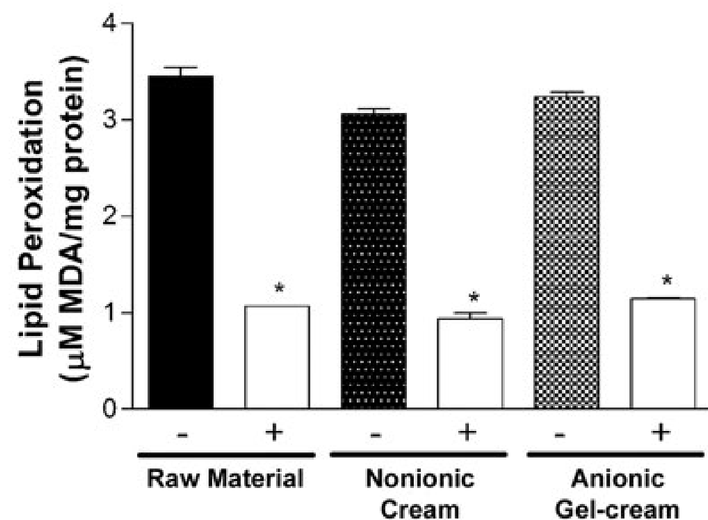

Figure 4. Evaluation of the interference of the formulations containing (+) or not (-) quercetin in the lipid peroxidation. Results are represented as concentration of MDA/mg of protein ± SEM. *Significantly different relative to respective control ($P < .05$).

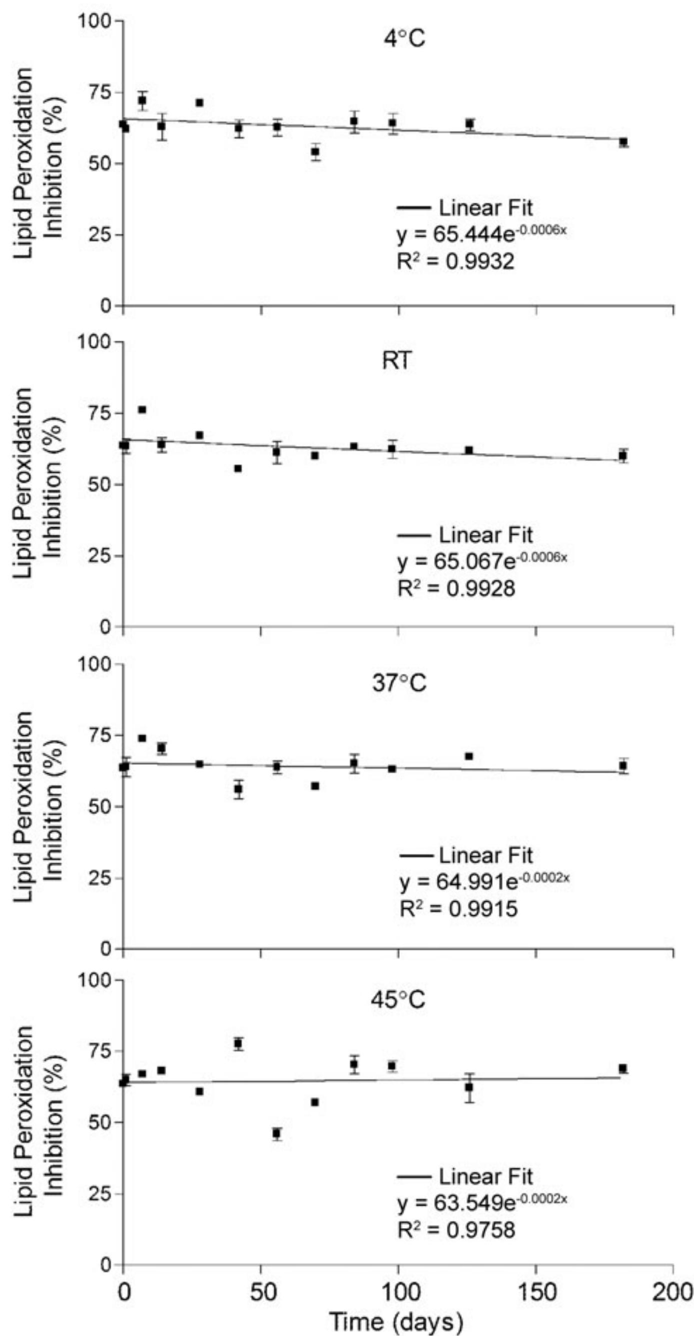

Figure 5. Percentage of antilipoperoxidative activity found in 1 μg/mL quercetin (raw material) following storage at 4°C, RT, 37°C, and 45°C for 182 days (6 months). Results are represented by mean ± SEM.

Fe^{2+} were added at the same time as BPS, and the iron-chelating activity of quercetin increased in a concentration- and time of contact-dependent manners.

Assay of Fe^{2+}/Citrate-mediated Lipid Peroxidation

First, it was investigated whether the lipid peroxidation method was effective to determine quercetin functional activity and what would be the most efficient concentration to

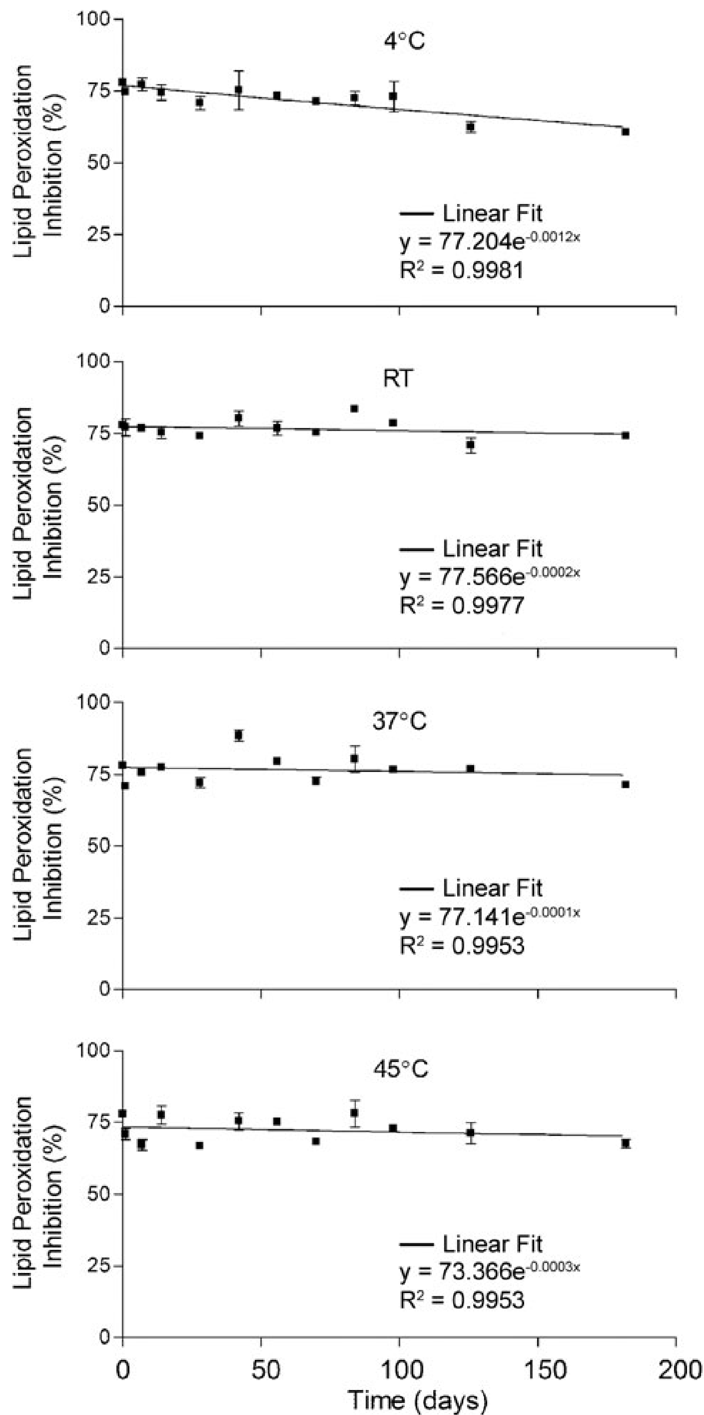

Figure 6. Percentage of antilipoperoxidative activity found in the nonionic cream with 1 μg/mL quercetin following storage at 4°C, RT, 37°C, and 45°C for 182 days (6 months). Results are represented by mean ± SEM.

prevent peroxidation. Quercetin inhibited in a concentration-dependent manner the lipid peroxidation as shown in Figure 3. Quercetin showed significant inhibitory activity of lipid peroxidation compared with the positive control (100% complex MDA-TBA). These results allowed calculation of the IC_{50} (0.34 μg/mL). The maximum percentage of inhibition of MDA formed was obtained using 1 μg/mL of quercetin (Figure 3); at a higher concentration (2.5 μg/mL), a plateau effect was observed. Furthermore, the interassay coefficient of variation over 5 consecutive days using the concentration of 1.0 μg/mL of quercetin was 10%. This variation is acceptable in view of the complexity of the method, since it involves the use of biological material (mitochondria) obtained each day of assay, and a multiple-step reaction involving the hydroxyl radical ($^{\bullet}OH$) production.

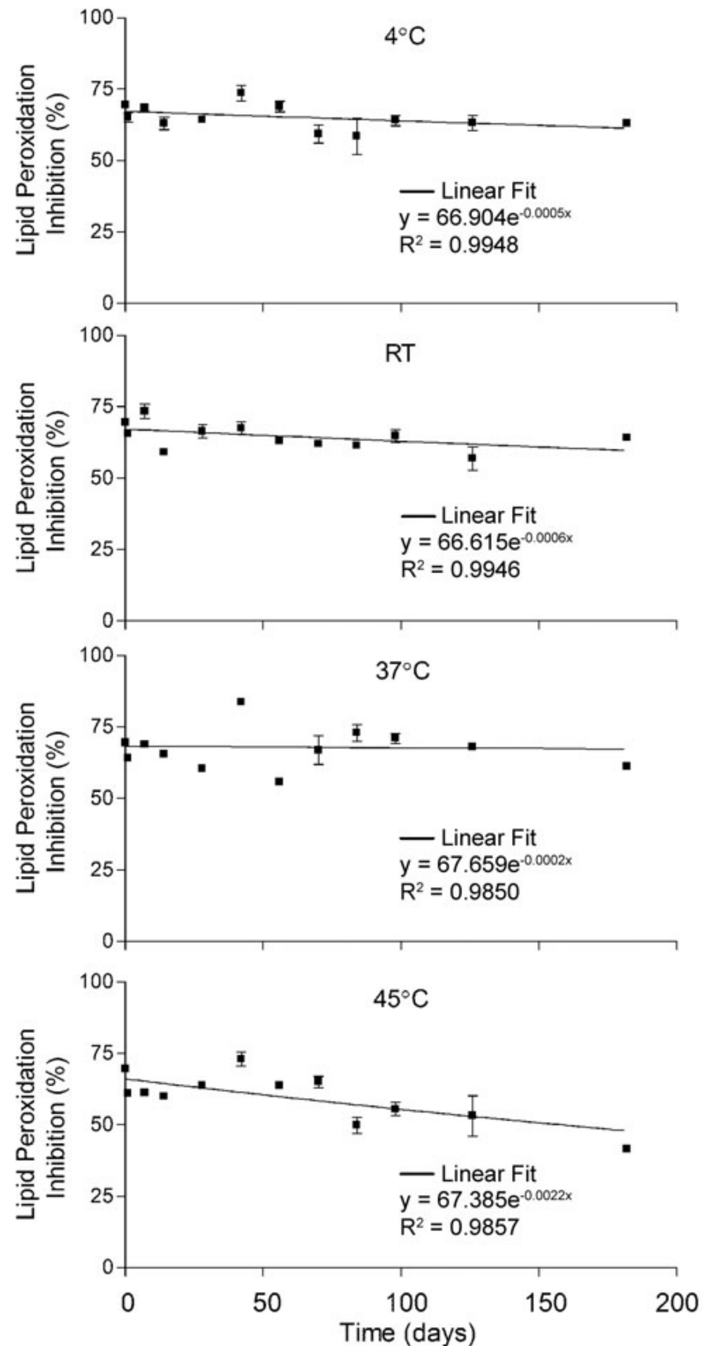

Figure 7. Percentage of antilipoperoxidative activity found in the anionic gel-cream with 1 μg/mL quercetin following storage at 4°C, RT, 37°C, and 45°C for 182 days (6 months). Results are represented by mean ± SEM.

Stability of the Antilipoperoxidative Activity of the Raw Material and of Formulations Containing Quercetin Stored at Different Temperatures

Initially, possible interferences of the formulations components in the lipid peroxidation assay were investigated. The MDA production in the presence or absence of quercetin was compared with the nonionic cream and anionic gel-cream with or without quercetin (Figure 4). MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5 \ M^{-1}$.[22] The results demonstrated no significant influence of the formulations in the MDA production, further confirming the adequacy of the assay to evaluate the quercetin functional stability in those formulations.

The initial antilipoperoxidative activity of the raw material quercetin, the nonionic cream, and the anionic gel-cream containing quercetin were 65.6%, 78%, and 70%, respectively. The results presented in Figures 5, 6, and 7 demonstrate the antilipoperoxidative activity of the raw material and formulations with or without quercetin over 182 days and stored at different temperatures (4°C, RT, 37°C, and 45°C). The best curve fit is presented in each panel, with the respective correlation coefficient value.

Statistical analysis detected significant influences of the temperature and the period of storage in the stability of the raw material and the formulations. Nevertheless, the evaluation of the antilipoperoxidative stability of raw material quercetin did not demonstrate loss of activity during 182 days of storage at all temperatures tested. However, considering the method variability of 10%, greater activity loss than 10% was detected for nonionic cream after 126 days at 4°C (20.1%), decreasing thereafter to 22.2% after 182 days. At 45°C, the loss of activity started after 182 days (13.2%). For the anionic gel-cream, the loss of activity started after 84 days (28.4%, 45°C), decreasing after 182 days (40.3%, 45°C). After 182 days at 37°C, activity loss of 12% was also detected for the anionic gel-cream (Figures 5, 6, and 7).

DISCUSSION

Quercetin is a promising drug to combat free radical diseases such as ischemia, arthritis, cancer, viral infections, and inflammatory conditions.[25] Recently, it has been demonstrated that quercetin from topical formulations does not permeate the pig ear skin, thus not reaching receptor solution in vitro. However, the crucial point is that quercetin is released from the formulations and retained in the skin, reaching viable epidermis. These results strongly suggest its topical use to protect the skin from oxidative stress (R Casagrande, unpublished data, January 2005). The data are further substantiated by preliminary results demonstrating that topical quercetin inhibits UVB skin damages in hairless mice (R Casagrande, unpublished data, March 2005). Corroborating, its antiradical properties are directed against $^{\bullet}OH$ and $O_2^{-\bullet}$, which are species implicated in the initiation of lipid peroxidation. Furthermore, quercetin is a soluble chain-breaking inhibitor of the peroxidation process by scavenging intermediate peroxyl and alkoxyl radicals.[26]

Topical administration of antioxidants is an efficient way to enrich the endogenous cutaneous system of protection, and thus a successful strategy to decrease oxidative damage of the skin by ultraviolet irradiation.[27] The stability evaluation of the antioxidant activity of active principles of formulations stored at different climatic conditions for given times constitutes an important step for the development of new products. It provides information about the shelf-life of pharmaceutical products, as well as about the conditions for their storage.[16,28]

Emulsions must be tested at widely varying temperatures in order to ensure that the product will remain stable throughout all possible environments.[24] For accelerated temperature testing, it is important to remember that temperature stability protocols depend on product category. Studies may range from freezing to very high temperatures and/or cycling between various temperatures. Generally, emulsions are stored at −10°C or 4°C, RT, 37°C, and 45°C or 50°C.[29]

The present study proposes a different approach to evaluate the stability of an active principle, by its activity. First, it was demonstrated that the antilipoperoxidative activity of quercetin depends on iron-chelation. In fact, several lines of evidence indicate that oxygen radical-mediated lipid peroxidation is strictly contingent to the availability of iron.[30] There are 3 possible metal-complexing domains that can interact with metal ions by hydrogen bonding between the 3′, 4'-dihydroxy group localized on the B ring, between the 3-hydroxy and 4-carbonyl group in the C ring and between 5-hydroxy and 4-carbonyl group on the C ring as presented in Figure 1.[31]

Afterwards, the ideal concentration of quercetin to be used in the lipoperoxidative assay was also determined by a concentration-dependence curve. The lipid peroxidation assay is a widely used method to evaluate the involvement of free radicals in cellular damage or antioxidant activity. MDA is one of the end products of peroxidation that has been extensively studied, and flavonoids have been shown to significantly reduce its levels.[32] There is consistent evidence that quercetin presents high antilipoperoxidative activity by scavenging free radicals and chelating metals.[23] Reinforcing these concepts, it was detected that quercetin interferes with the formation of the $Fe_2(BPS)_3$ complex, and additionally, the Fe-flavonoid complex maintains the free-radical scavenging activity.[8,10,33] Due

to these properties of quercetin, the method used in this study was shown to be appropriate.

Since this study evaluates the activity of quercetin added in formulations, possible interferences of the formulations components in the lipid peroxidation assay were investigated. There was no significant difference between the MDA production in the presence or absence of quercetin compared with the nonionic cream and anionic gel-cream with or without quercetin. These results confirm the adequacy of the assay to evaluate the quercetin functional stability in those formulations.

Due to the lipophilic characteristics of quercetin (partition coefficient = 2.80; not shown in figures), it was important for the stability study to test formulations of different lipid content. Lipophilic substances can be solubilized in the lipophilic phase of emulsions. Thus, solubility will increase in proportion to lipid content.[34] In a comparison of the stabilities, quercetin had greater activity loss in the formulation with lower lipid content (anionic gel-cream). Moreover, the lipid environment of solubilization and protection of quercetin structure of the nonionic cream may be disturbed at lower temperatures, since the lipids are more closely positioned, and thus favor the precipitation of quercetin and the loss of its activities. This hypothesis is further substantiated with the data demonstrating a greater than 10% reduction of quercetin activity at 4°C for nonionic cream after 126 days, and no modification of raw material or anionic gel-cream activity at 4°C. Again, it is noteworthy that the lipid content of the nonionic cream and the lipid solubility of quercetin may be responsible for the higher initial antilipoperoxidative activity of this formulation compared with the raw material and anionic gel-cream; disturbing this lipid environment, the solubility and activity of quercetin would also be affected.

As a rule, stability accelerated studies are less suitable for semisolid and heterogeneous formulations (eg, emulsions).[35] Moreover, there are several inherent problems with the estimation of shelf-life. It is applied to linearly degradable drugs, does not consider change in degradation mechanisms above the critical temperatures and does not include the errors associated with the determination of the drug content.[28] In addition, the lipid peroxidation assay reaction order cannot be obtained since it is a multiple-step reaction. Thus, the estimated shelf-life was not calculated.

CONCLUSIONS

The present study demonstrates that quercetin has concentration-dependent antilipoperoxidative and iron-chelating activities. Furthermore, this study suggests that the lipid peroxidation assay is suitable for quality control and stability studies of quercetin, an antioxidant that maintains its activity stored at RT, avoiding special storage conditions.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the statistical analyses of Professor Antônio Caliri. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

1. Fuchs J, Packer L. Photooxidative stress in skin. In: Sies H, ed. *Oxidative Stress: Oxidants and Antioxidants.* London, UK: Academic Press; 1991:559–583.

2. Darr D, Fridovich I. Free radicals in cutaneous biology. *J Invest Dermatol.* 1994;102:671–675.

3. Saija A, Tomaino A, Trombetta D, Giacchi M, De Pasquale A, Bonina F. Influence of different penetration enhancers on in vitro skin permeation and in vivo photoprotective effect of flavonoids. *Int J Pharm.* 1998;175:85–94.

4. Rieger MM. Oxidative reactions in and on skin: mechanism and prevention. *Cosmet Toil.* 1993;108:43–56.

5. Aruoma OI. Free radicals, antioxidants and international nutrition. *Asia Pac J Clin Nutr.* 1999;8:53–63.

6. Bonina F, Lanza M, Montenegro L, et al. Flavonoids as potential protective agents against photo-oxidative skin damage. *Int J Pharm.* 1996;145:87–94.

7. Santos AC, Uyemura SA, Lopes JL, Bazon JN, Mingatto FE, Curti C. Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. *Free Radic Biol Med.* 1998;24:1455–1461.

8. van Acker SABE, van Balen GP, van der Berg D-J, Bast A, van der Vijgh WJF. Influence of iron chelation on the antioxidant activity of flavonoids. *Biochem Pharmacol.* 1998;56:935–943.

9. Morel I, Lescoat G, Cogrel P, et al. Antioxidant and iron-chelation activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem Pharmacol.* 1993;45:13–19.

10. Saija A, Scalese M, Lanza M, Marzullo D, Bonina F, Castelli F. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radic Biol Med.* 1995;19:481–486.

11. Skibola CF, Smith MT. Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med.* 2000;29:375–383.

12. Bors W, Heller W, Michel C, Saran M. Flavonoids as antioxidant: determination of radical-scavenging efficiencies. *Methods Enzymol.* 1990;186:343–355.

13. Lemanska K, Szymusiak H, Tyrakowka B, Zielinski R, Soffers AEMF, Rietjens IMCM. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Radic Biol Med.* 2001;31:869–881.

14. Middleton E Jr, Kandwami C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne JB, ed. *The Flavonoids, Advances in Research Since 1986.* Cambridge, UK: Chapman and Hall; 1994:619–652.

15. Jager A, Wälti M, Neftel K. Side-effects of flavonoids in medical practice. In: Cody V, Middleton E Jr, Harborne JB, Beretz A, eds. *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties.* New York, NY: Alan R. Liss; 1988:379–394.

16. Wessels P, Holz M, Erni F, Krummen K, Ogorka J. Statistical evaluation of stability data of pharmaceutical products for specification setting. *Drug Dev Ind Pharm.* 1997;23:427–439.

17. Bolann BJ, Ulvik RJ. Release of iron from ferritin by xanthine oxidase: role of the superoxide radical. *Biochem J.* 1987;243:55–59.

18. Pedersen PL, Greenawalt JW, Reynafarje B, et al. Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. *Methods Cell Biol.* 1978;20:411–481.

19. Cain K, Skilleter DN. Preparation and use of mitochodria in toxicological research. In: Snell K, Mullock B, eds. *Biochemical Toxicology.* Oxford, UK: IRL Press; 1987:217–254.

20. Gornal AG, Bardill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem.* 1949;177:751.

21. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol.* 1978;52:302–310.

22. Kowaltowski AJ, Castilho RF, Grijalba MT, Bechara EJ, Vercesi AE. Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions: a proposed model for phosphate-stimulated lipid peroxidation. *J Biol Chem.* 1996;271:2929–2934.

23. Rodrigues T, Santos AC, Pigoso AA, Mingatto FE, Uyemura SA, Curti C. Thioridazine interacts with the membrane of mitochondria acquiring antioxidant activity toward apoptosis - potentially implicated mechanisms. *Br J Pharmacol.* 2002;136:136–142.

24. Klein K. Improving emulsion stability. *Cosmet & Toilet.* 1984; 99:121–126.

25. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem.* 1995;43:27–32.

26. Laughton MJ, Halliwell B, Evans PJ, Hoult JR. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem Pharmacol.* 1989;38:2859–2865.

27. Montenegro L, Bonina F, Rigano L, Giogilli S, Sirigu S. Protective effect evaluation of free radical scavengers on UVB induced human cutaneous erythema by skin reflectance spectrophotometry. *Int J Cosmet Sci.* 1995;17:91–103.

28. Singh S. Drug stability testing and shelf-life determination according to international guidelines. *Pharm Technol.* 1999;23:68–88.

29. Idson B. *Stability Testing of Emulsions*. Austin, TX: DCI; 1993: 27–30.

30. Minotti G, Aust S. The role of iron in oxygen radical mediated lipid peroxidation. *Chem Biol Interact.* 1989;71:1–19.

31. Sugihara N, Arakawa T, Ohnishi M, Furuno K. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with α-linolenic acid. *Free Radic Biol Med.* 1999;27:1313–1323.

32. Erden Inal M, Kahraman A. The protective effect of flavonol quercetin against ultraviolet: an induced oxidative stress in rats. *Toxicology.* 2000;154:21–29.

33. Arora A, Nair MG, Strasburg GM. Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radic Biol Med.* 1998;24:1355–1363.

34. Carstensen JT. Disperse and aggregated systems. In: Carstensen JT, ed. *Drugs Stability: Principles and Practices.* New York, NY: Marcel Dekker Inc; 1995:172–175.

35. World Health Organization. *Quality Assurance of Pharmaceuticals: A Compendium of Guidelines and Related Materials*. Geneva, Switzerland: World Health Organization; 1997.