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Influence of Vehicle on Antioxidant Activity of Quercetin: A Liquid Crystalline Formulation

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SUMMARY. The effect of a liquid crystalline formulation in the *in vitro* antioxidant activity of quercetin was investigated using different methodologies in order to choose the most suitable method to perform the quality control and stability studies of the antioxidant activity of the liquid crystalline formulation added with quercetin. For all the assays, quercetin showed a dose-dependent activity having an IC_{50} of 0.215 μg/mL in inhibiting lipid peroxidation, 1.125 μg/mL in inhibiting chemiluminescence produced in the H_2O_2/luminol/horseradish peroxidase (HRP) system and 0.834 μg/mL in DPPH$^\bullet$ assay and the antioxidant activity of quercetin in liquid crystalline formulation was successfully evaluated in all the methodologies applied, once no interference by other components of the formulation was found and all the methodologies were sensitive for the quercetin evaluation. Thus, the liquid crystalline formulation containing vitamin E TPGS/ IPM / PG-H_20 (1:1) in the percentage of 63.75/21.25/15 (w/w/w), respectively, might be potential for cosmetic and pharmaceutical use, once it preserved the antioxidant effect of quercetin and all the methodologies applied could be used to perform the quality control and stability studies of the antioxidant activity of the liquid crystalline formulation added with quercetin.

RESUMEN. "Influencia del Vehículo en la Actividad Antioxidante de la Quercetina: la Formulación Líquido Cristalina". El efecto de una formulación líquido cristalina en la actividad antioxidante *in vitro* de la quercetina fue investigada utilizando diferentes metodologías con objetivo de escoger el método más adecuado para hacer el control de calidad y estudios de la estabilidad de la actividad antioxidante de la formulación líquido cristalina adicionada de la quercetina. Para todos los métodos empleados, la quercetina presentó actividad dosis dependiente con IC_{50} de 0.215 μg/mL en la inhibición de la peroxidación lípida, 1.125 μg/mL en la inhibición de la quimioluminiscência generada por el sistema H_2O_2-HRP-luminol y 0.834 μg/mL en el ensayo de DPPH$^\bullet$ y la actividad antioxidante de la quercetina adicionada a la formulación líquido cristalina fue valorada con suceso en todas las metodologías empleadas, ya que no hubo interferencia de los otros componentes da formulación y todas las metodologías se mostraron sensibles para evaluación de la quercetina. Así, la formulación líquido cristalina conteniendo vitamina E TPGS/ MIP / PG-H_20 (1:1) en la porcentaje de 63.75/21.25/15 (p/p/p), respectivamente, pode ser potente para administración cosmética o farmacéutica, una vez que preservó el efecto antioxidante de la quercetina y todas las metodologías empleadas pueden ser utilizadas en el control de calidad y estudios de estabilidad de la actividad antioxidante da formulación líquido cristalina contenido quercetina.

INTRODUCTION

Skin is a biological interface with the environment and functions as the first line of defense against noxious external stimuli such as ultraviolet, visible irradiation, prooxidant chemicals, infection and ionizing radiation [1].

However, by acute or chronic exposure to UV light the skin prooxidant/antioxidant equilibrium can be overwhelmed due to severe decrease of its antioxidant content and to striking formation of reactive oxygen species (ROS). This way the ROS generated in excess can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA. So the deleterious effects of sunlight and particularly UV radiation on the skin can lead to a variety of ravages as inflammation, skin aging, tumour promotion, cutaneous auto-immune disease, and phototoxicity/photosensitivity [1].

Topical administration of antioxidants provides an efficient way to enrich the endogeneous cutaneous protection system and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in

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PALABRAS CLAVE: Antioxidante, Cristales líquidos, DPPH$^\bullet$, Peroxidación lipídica, Quercetina, Quimioluminiscência.

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the skin [2]. In this context, recently work has demonstrated the beneficial effectiveness of topical formulations (non-ionic and anionic emulsions) containing quercetin, a flavonoid which has well-known antioxidant activity; in inhibit the oxidative stress and inflammation induced by UVB irradiation [3].

Considering that the design of new forms that increases the effectiveness of existing drugs is one of new trends observed in pharmaceutical technology in recent years and that liquid crystals have aroused great interest as novel dosage forms, due to their considerable solubilizing capability for both oil and water soluble compounds [4,5], the incorporation of quercetin in a liquid crystalline system in order to improve its activity against ROS come as an important choice.

Lyotropic liquid crystalline systems are characterized by the properties of both liquids and solids, i.e. they exhibit in part a structure typical of fluids and also the structured, crystalline state of solids [6]. Therefore, liquid crystalline phases represent intermediate states and are also called mesophases [7]. They are usually formed from water and one or two surfactants and possibly co surfactants, in the definite proportions of the given components, with low energy input or by means of spontaneous structural organization; their production is therefore relatively simple and energy-saving. They are thermodynamically stable, and can be stored for long periods of time without phase separation [6].

In the presence of a surface-active agent and solvent, different types of aggregate structures can form over a wide range of compositions. Cubic, hexagonal, lamellar and micellar phases can be observed in most cases, which differ from each other in their mechanical properties [4].

Liquid crystalline mesophases have been investigated as modern formulations by many authors [4]. However, since one of most challenging tasks in evaluating topical formulations is to deal with the presence of the formulations compounds that may cause interference if using a non specific method [8], the antioxidant activity of this formulation should be accordingly assessed, using several methodologies, in order to choose the most adequate one.

Therefore, in the present study was investigated the influence of a liquid crystalline formulation in the *in vitro* antioxidant activity of quercetin using different methodologies, in order to verify, if the antioxidant activity of this flavonoid is lost in the presence of formulations compounds or if these compounds interfere with the method of evaluation. Then, it will be possible to choose the most suitable method to perform the quality control and stability studies of the antioxidant activity of the liquid crystalline formulation added with quercetin.

MATERIALS AND METHODS

Chemicals

Quercetin dihydrate 99% ($C_{15}H_{10}O_7.2H_2O$, M_w = 338.26) was purchased from Acros Organics (New Jersey, USA) and vitamin E TPGS from Eastman (Kingsport, Tennessee, USA). Thiobarbituric acid (TBA), luminol, horseradish peroxidase (HRP) and 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); hydrogen peroxide 36% was purchased from Calbiochem (California, USA). All other chemicals were of reagent grade and were used without further purification.

Preparation of the formulation

The lamellar liquid crystalline formulation was prepared by mixing melted vitamin E TPGS (40 °C) and isopropyl myristate (IPM), and immediately thereafter by adding PG : water (1:1) mixture pre-warmed to 40 °C to achieve a vitamin E TPGS/ IPM / PG-water system (63.75/21.25/15 w/w/w). Quercetin was incorporated in this system in the final concentration of 1% w/w.

The resulting formulation was allowed to rest in closed vials for 1 week at room temperature to reach equilibrium and was examined by visual inspection to verify sample homogeneity and through a polarized light microscope (Carl Zeiss, Oberkichen, Germany) to verify the liquid crystalline phase formed.

Preparation of samples

Quercetin was diluted to final concentrations of 4, 20, 50, 100 and 250 µg/ mL; 8.5, 17, 34, 68, and 135 µg/mL; and 5, 25, 50, 75, 250 and 500 µg/mL and the formulation added with 1% of quercetin and quercetin-free formulation were diluted to obtain final concentrations of 20 µg/mL, 105 µg/mL and 50 µg/mL for the following methodologies: inhibition of lipid peroxidation, inhibition of chemiluminescence of H_2O_2/luminol/HRP system and reduction of 2,2-diphenyl-1-picryl-hydrazyl radical ($DPPH^{\bullet}$).

Inhibition of lipid peroxidation induced by Fe^{+2} assay

Ten microlitres of each sample was added to 1.0 mL of a reaction medium which contains

KCl (130 mM) and Tris-HCl (10 mM; pH 7.4), and mitochondria was added to yield a final concentration of 1mg of protein. Then, 50 µM ferrous ammonium sulfate and 2 mM sodium citrate were added and the samples were incubated at 37 °C for 30 min. Mitochondria was isolated by differential centrifugation from livers of male Wistar rats as described by Pedersen [9] and mitochondrial protein content was determined by the biuret reaction [10]. For malondialdehyde (MDA) determination, 1 mL of 1% thiobarbituric acid (TBA) (prepared in 50 mM NaOH), 0.1 mL of NaOH (10 M) and 0.5 mL of H_3PO_4 (20%) were added, followed by incubation for 20 min at 85 °C. The MDA-TBA complex was extracted with 2 mL of *n*-butanol. The samples were then centrifuged at 1660 x *g* for 10 min. The measurement was performed on the supernatant at 535 nm [10]. The amount of TBA-reactive compounds were evaluated as MDA, and was calculated from $\varepsilon = 1.56 \times 10^5/M$.

Inhibition of chemiluminescence of H_2O_2/luminol/HRP system assay

Changes of chemiluminescence intensity of H_2O_2/luminol/HRP system were measured as follows: 10 µL of each sample was mixed with phosphate buffer (0.1 M; pH 7.4), and a 2 M luminol solution in DMSO was added to yield a final concentration of 1.13×10^{-4}M. H_2O_2 was then added to a final concentration of 5×10^{-5} M. The reaction was started by adding HRP at a final concentration of 0.2 IU/mL, yielding final volume of 1 mL of solution [11]. Chemiluminescence was measured for 10 min at 25 °C with an Autolumat LB 953 apparatus. Experiments were conducted in triplicate for each sample.

Antioxidant activity by DPPH• assay

The H-donor ability was evaluated using an ethanol solution of DPPH•, a stable nitrogen-centered free radical. For radical scavenging measurements, 1 mL of 0.1M acetate buffer (pH 5.5), 1 mL of ethanol, 0.5 mL of 250 µM ethanolic solution of DPPH• were mixed, 50 µL of samples were added, then the absorbance was measured after 10 min at 517 nm [12]. Blank was prepared from the reaction mixture without DPPH• solution. All measurements were performed in triplicate.

Statistical analysis

The percentage which caused 50% of inhibition of the systems assessed (IC_{50}) by quercetin was determined using GraphPad Prism® software, version 3.02. Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparisons *t*- test for evaluation of the formulations influence in the antioxidant activity assays. Results were presented as means ± S.E.M (standard error mean) and considered significantly different when $P < 0.05$ was obtained.

RESULTS AND DISCUSSION

The skin, as our major external barrier, is continually exposed to the oxidative injury caused by free radicals [13]. It is considered that topical use of antioxidants from natural products such as quercetin present novel possibilities for the treatment and prevention of oxidative stress-mediated skin diseases [3]. In this context, the development of a new dermal dosage form such as liquid crystalline systems containing the flavonoid quercetin is a good strategy to optimize the topical activity of this well-known natural antioxidant.

Among the main types of lyotropic liquid crystalline systems, mesophases with a lamellar structure that demonstrate the greatest similarity to the intercellular lipid membrane of the skin are primarily recommended for the development of a dermal dosage form [6]. The lamellar phase has a long-range order in one dimension. Its structure consists of a linear arrangement of alternating lipid bilayers and water channels [14]. Water and aqueous solutions can be included in the polar layers, resulting in an increase in layer thickness. Analogously, molecules with appropriate affinity can be included in the nonpolar layers [7].

However, quercetin may lose or decrease its antioxidant activity depending on the formulation into which this flavonoid is incorporated. For this reason, it was evaluated the influence of the vehicle in the free radical scavenging activity of quercetin when incorporated in a lamellar liquid crystalline formulation.

According to Halliwell and Gutteridge [15], mechanisms of antioxidant action can include: (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen species, and (3) upregulating or protecting antioxidant defenses [16]. Therefore, the antioxidant activity of one compound might be evaluated using different tests which involve different mechanisms [17]. In the present study, three analytical methods were used to evaluate the antioxidant activity of such flavonoid alone and incorporated in the lamellar liquid crystalline formulation.

Among the numerous pathological events that are caused by an imbalance between oxidative damage and antioxidant defense systems, lipid peroxidation (LPO) is a well-known example of oxidative damage that affects cell membranes, lipoproteins, and other lipid-containing structures under conditions of oxidative stress [8].

The LPO-inhibiting activity of quercetin was evaluated using the Fe^{2+}- citrate mitochondria system. Iron, either in an inorganic form or as a heme complex, is likely to form an important part of the catalytic system in tissue homogenates and lipid peroxidation in the mitochondrial fraction is stimulated by iron addition [19]. In Fig. 1 the percentage of peroxidation inhibition is plotted against different concentrations of quercetin and the concentration, which caused 50% inhibition, was taken as the IC_{50} value. The same procedure for expressing results was used for all the methodologies employed.

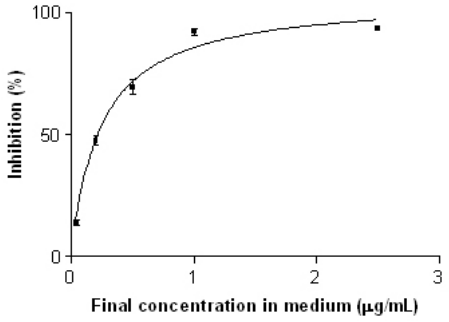

Figure 1. Inhibition by quercetin of lipid peroxidation induced by Fe^{+2}/citrate. Mitochondria were utilized as lipidic material. MDA was determined as described in materials and methods. Results are represented by means ± S.E.M.

The percentage of inhibition was calculated using the equation [**1**],

$$\text{Inhibition (\%)} = 100 - \frac{100 \times A_S}{A_0} \qquad [\mathbf{1}]$$

where A_S is the absorbance observed when experimental samples (quercetin solution or formulation added with quercetin) were added, and A_0 the absorbance of the positive control (quercetin absence or the quercetin-free control formulation).

The IC_{50} for quercetin when LPO method was applied was 0.215 µg/mL, which qualifies the flavonoid quercetin as a good antioxidant according to van Acker's [20] classification that divides flavonoids into three different categories: good (IC_{50} < 45 µM), moderate (45 µM < IC_{50} < 3000 µM) antioxidants and inactive compounds (IC_{50} > 3000 µM). The inhibition of lipid peroxidation was showed in the range from 0.04 to 1.0 µg/mL. Above 1.0 µg/mL, the samples reached a plateau corresponding to the maximum inhibition of lipid peroxidation (approximately 92%). The inhibition of lipid peroxidation may be probably due to the scavenging activity against lipid peroxides, peroxyl and alkoxyl radicals and, in the same extend, to the Fe^{2+} chelating activity. Several studies demonstrated that the high antioxidative potential of quercetin against metal-ion-induced peroxidations is probably a consequence of their combined metal chelating and free radical scavenging abilities [16,21,22].

The antioxidant activity observed in our study was also kept when quercetin was added in the lamellar liquid crystalline system. There was no significant influence of the formulation constituents in the MDA production as determined using the ε=1.56 x 10^5 M^{-1} (Fig. 2). The inhibition of lipid peroxidation by quercetin when analyzed in a final concentration of 0.2 µg/mL was similar to that found for the formulation in which the flavonoid was added (no significant difference), suggesting that this methodology can be used to analyze the inhibition lipid peroxidation capacity of this antioxidant compound incorporated in this topical formulation since formulation components showed no interference.

Among the assays for antioxidant activity, chemiluminescence is advantageous because of its high sensitivity and rapidity. Light emission

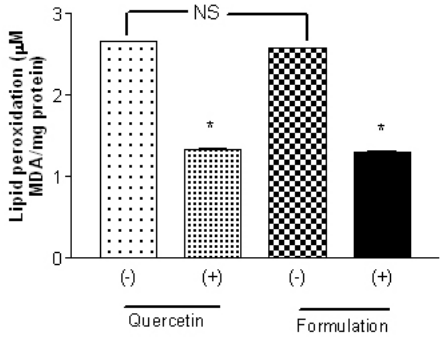

Figure 2. Inhibition of lipid peroxidation induced by Fe^{+2}/citrate by liquid crystalline formulation containing quercetin. Results are represented as concentration of MDA/mg of protein ± S.E.M. (ε=1.56x10^5M^{-1}) of quercetin absence (-), quercetin solution (+), quercetin-free control formulation (-) and formulation added with quercetin (+). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's t- test. *Significantly different compared to respective control (P<0.05).

can be markedly amplified using the HRP/luminol/hydrogen peroxide system, where HRP reacts with hydroxyl peroxide to form an oxidized HRP that reacts with the anion of luminol to form a half-reduced enzyme and a radical of luminol. The enzyme returns to the reduced form (HRP) by reaction with a second molecule of luminol. Addition of compounds to the chemiluminescent solution may lead to an increase or inhibition of light emission. The reduction of light emission can be considered a measure of antioxidant activity [23,24].

The chemiluminescence assays were evaluated based on the measurements of the areas under the time courses of the luminescent emission in the presence of quercetin and the lamellar liquid crystalline system containing this flavonoid. It was estimated the relative inhibitory activity of each sample at different concentrations and in the formulation used.

The percent inhibition caused by each sample was calculated as indicated en Equation [**2**]:

$$\text{Inhibition (\%)} = \frac{100 \text{ x } AUC_1}{AUC_0} \qquad [2]$$

where AUC_0 represents the area under the curve observed for the control (quercetin absence or the quercetin-free control formulation) and AUC_1 (quercetin solution or formulation added with quercetin).

Quercetin inhibited chemiluminescence intensity in a concentrated-related manner, which indicates the antioxidant activity of this compound. A crescent percentage in the inhibition was showed from 0.085 to 1.35 µg/mL (Fig. 3). The IC_{50} calculated in the H_2O_2/luminol/HRP system was 1.125 µg/mL

The chemiluminescence assay using H_2O_2/luminol/HRP system was also performed to assess the formulation activity and the inhibition of chemiluminescence in this system by quercetin, when analyzed in a final concentration of 1.05 µg/mL, was similar to those observed for quercetin solution (data not shown). These results suggest that the formulation components caused no interference with this antioxidant measurement.

2,2-Diphenyl-1-picryl-hydrazyl (DPPH•) is a stable free radical, which can accept an electron or hydrogen radical converting into a stable, diamagnetic molecule. Because of its odd electron, DPPH• has a strong absorption band at 517 nm. As this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. Such

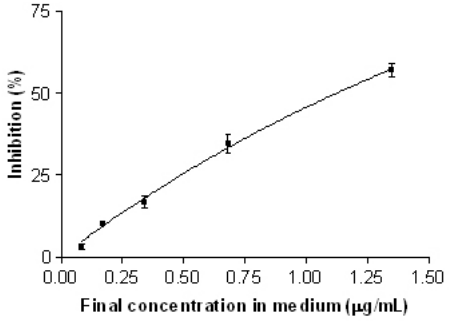

Figure 3. Inhibition of light emission from H_2O_2/luminol/HRP luminescent reactions with luminol found for different concentrations of quercetin. Results are means ± S.E.M. of three measurements run in parallel.

a change in absorbance produced in this reaction has been widely used to test the ability of several molecules to act as free radical scavengers [12].

The quercetin samples tested in the present study for their H-donor ability, measured by the stable free radical DPPH• assay, showed antioxidant activity in the range from 0.1 to 2.0 µg/mL. Above 2.0 µg/mL, the samples reached a plateau corresponding to approximately 90% of absorbency decreasing (Fig. 4). The IC_{50} was 0.834 µg/mL which is very similar with the value founded by Parejo [24] and which demonstrates the high antioxidant activity of this flavonoid. The precision of this method was previously evaluated, showing 1.14% intra-assay precision and 6.43% inter-day repeatability [25]. Fig. 5 shows that the capacity to scavenge DPPH• was maintained in the liquid crystalline formulation containing quercetin when compared with quercetin solution in the same concentration in the reaction medium (1 µg/mL). The result demonstrates that DPPH• assay is able to evaluate the antioxidant activity of the lamellar liquid crystalline system containing the antioxidant quercetin once the formulation components showed no interference.

Therefore, the present work demonstrates that in all the methodologies studied, it was possible to build a dose-response curve, showing that these methodologies are adequate in evaluating the antioxidant activity of the flavonoid quercetin. Furthermore, based on the IC_{50} found it can be concluded that the highest affinity of quercetin is in scavenging hydroxyl; peroxyl and alkoxyl radicals produced in lipid peroxidation and, chelate metals such as Fe^{2+} which participates in the formation of these reactive oxygen species, followed by an affinity of DPPH• radical and luminol one. From the re-

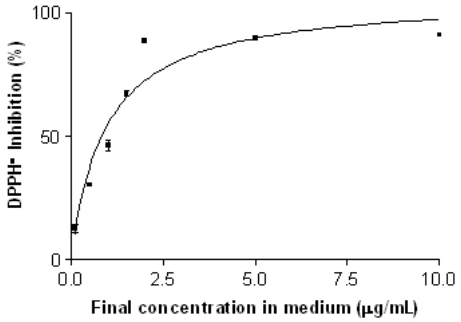

Figure 4. Inhibition of H-donor ability using stable radical DPPH• in 250 µM ethanolic solution found for different concentrations of quercetin. Results are represented by means ± S.E.M. of three measurements run in parallel.

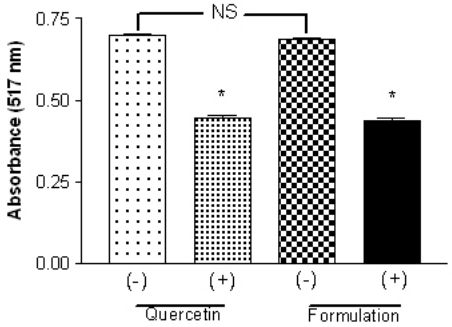

Figure 5. Inhibition of H-donor ability using DPPH• assay by liquid crystalline formulation containing quercetin. Results are represented as absorbance at 517 nm of quercetin absence (-), quercetin solution (+), quercetin-free control formulation (-) and formulation added with quercetin (+). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's t- test. *Significantly different compared to respective control (P<0.05).

sults observed, it can also be deduced that the liquid crystalline formulation containing vitamin E TPGS/ IPM / PG-$H_2$0 (1:1) in the percentage of 63.75/21.25/15 (w/w/w), respectively might be potential for cosmetic and pharmaceutical use, since it preserved the antioxidant effect of quercetin. Besides this, all the three methodologies applied could be used to perform the quality control and stability studies of the antioxidant activity of the liquid crystalline formulation added with quercetin.

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