

## Evaluation of the Antioxidant Activity of Different Flavonoids by the Chemiluminescence Method

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### ABSTRACT

The objective of the present investigation was to study the antioxidant action of different flavonoids (quercetin, glabridin, red clover, and Isoflavin Beta, an isoflavones mixture) in order to determine if they could be added to a topical formulation used to treat damage caused by free radicals. Samples of 10  $\mu$ L of the test compounds at different concentrations were mixed with 0.1 M phosphate buffer, pH 7.4, and a luminol solution was added to yield a final concentration of 0.113 mM. Hydrogen peroxide was then added at a final concentration of 0.05 mM. The reaction was started by introducing the horseradish peroxidase enzyme at a final concentration of 0.2 IU/mL, in a final volume of 1.0 mL. Chemiluminescence was measured for 10 minutes at room temperature, and dimethylsulfoxide (DMSO) was used as a control. All samples showed marked inhibition of oxidative stress, with a concentration-dependent action for quercetin and Isoflavin Beta. The highest inhibition was observed with glabridin and the dry red clover extract. All flavonoids proved to be adequate for addition to topical formulations because of their high antioxidant activity.

**KEYWORDS:** chemiluminescence, luminol, antioxidants, flavonoids, peroxidase

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### INTRODUCTION

Ultraviolet radiation (UVR)-induced skin damage includes acute reactions such as erythema and edema, as well as premature skin aging (photoaging) and carcinogenesis largely determined by chronic exposure. The important role of reactive oxygen species (ROS) in UVR-induced skin damage is well documented. ROS cause injury by reacting with biomolecules such as lipids, proteins, and nucleic acids, as well as by depleting the skin of endogenous enzymatic and/or nonenzymatic antioxidants. Antioxidants may intervene at different levels in the oxidative process (eg, by scavenging for free radicals and lipid peroxyl radicals, removing oxidatively damaged biomolecules, and having other types of action).<sup>1-5</sup>

Recent reviews have shown the wide use of the chemiluminometric method using the horseradish peroxidase-luminol-hydrogen peroxide system (HRP-luminol-H<sub>2</sub>O<sub>2</sub>) as a sensitive assay for monitoring free radicals and reactive metabolites from cell-free, enzyme, cell, or organ systems and for screening antioxidant activity.<sup>6-9</sup> The peroxidase-catalyzed chemiluminescent oxidation of luminol involves the formation of a complex between oxidant (H<sub>2</sub>O<sub>2</sub>) and peroxidase to produce a luminol radical. Luminol radicals then undergo further reaction resulting in the formation of an endoperoxide, which decomposes to yield an electronically excited 3-aminophthalate dianion emitting light on return to its ground state.<sup>10</sup>

Flavonoids, a group of plant polyphenolic compounds possessing broad biological properties have been demonstrated to exert beneficial effects on some diseases involving uncontrolled lipid peroxidation. The capability to interact with protein phosphorylation and the antioxidant, iron-chelating, and free radical scavenging activity may account for the wide pharmacological profile of flavonoids.<sup>11-13</sup> Furthermore, flavonoids are known to possess good anti-inflammatory activity both in humans and animals, and recently their topical application has met with

considerable interest. Thus, topical administration of flavonoids might be effective in the prevention of UVR-induced skin damage.<sup>14</sup>

The aim of this study was to determine the ability of quercetin, glabridin, Isoflavin Beta, and a dry red clover extract to inhibit free radicals using the chemiluminescence method.

## MATERIALS AND METHODS

### Drugs and Chemicals

Quercetin was purchased from Acros Organics (New Jersey), Glabridina (Aqua Licorice extract PT), the commercial isoflavonoids mixture (Isoflavin Beta), and the dry red clover extract were a generous gift from Galena (Campinas, São Paulo, Brazil). Luminol and horseradish peroxidase (HRP) were purchased from Sigma (St Louis, MO). Hydrogen peroxide 36% was purchased from Calbiochem EMD Biosciences Inc (San Diego, CA). All other chemicals were of reagent grade and were used without further purification.

### Samples Preparation

A stock solution of quercetin (676 µg/mL) was prepared using dimethylsulfoxide (DMSO). This solution was further diluted 80, 40, 20, 10, and 5 times in DMSO to obtain concentrations of 8.5, 17.0, 34.0, 68.0, and 135 µg/mL. The Aqua Licorice Extract PT (ALE) was diluted in 0.1 M phosphate buffer, pH 7.4, 4000, 2000, 1000, 500, and 200 times to obtain final concentrations of 0.25, 0.5, 1.0, 2.0, and 5.0 µL/mL. DMSO was also used to prepare both stock solutions of Isoflavin Beta (125 µg/mL) and dry red clover extract (125 µg/mL). The Isoflavin Beta solution was diluted 6, 4, 3, and 2 times in DMSO to obtain concentrations of 21.0, 31.0, 41.5, and 62.5 µg/mL. The red clover solution was diluted 2.5, 1.9, 1.6, and 1.3 times in DMSO to obtain concentrations of 50, 65, 80, and 100 µg/mL.

### Chemiluminescence in the Presence of HRP

Samples of 10 µL of each test compound or of 10 µL DMSO as control for quercetin, Isoflavin Beta, and dry red clover extract and 0.1 M phosphate buffer pH 7.4, as control for ALE, were mixed with 0.1 M phosphate buffer, and luminol solution (2 mg/mL in DMSO) was added to yield a final concentration of  $1.13 \times 10^{-4}$  M. H<sub>2</sub>O<sub>2</sub> was then added to a final concentration of  $5 \times 10^{-5}$  M. The reaction was started by adding HRP at a final concentration of 0.2 IU/mL, yielding a final volume of

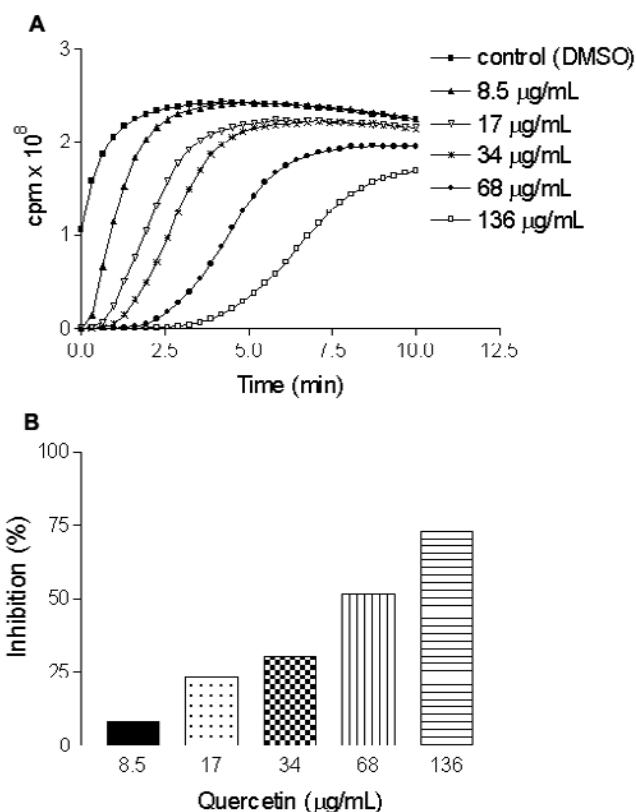
1.0 mL.<sup>15</sup> Chemiluminescence was measured for 10 minutes at 25°C with an Autolumat LB953 apparatus (EG & G Berthold, Gaithersburg, MD).

## RESULTS

Based on the measurement of the area under the time-course of the chemiluminescence curves for quercetin (**Figure 1A**), ALE (**Figure 2A**), Isoflavin Beta (**Figure 3A**), and red clover (**Figure 4A**), we estimated the relative inhibitory activity of each flavonoid tested at different concentrations. The inhibition ratio (%) of each sample was calculated as follows:

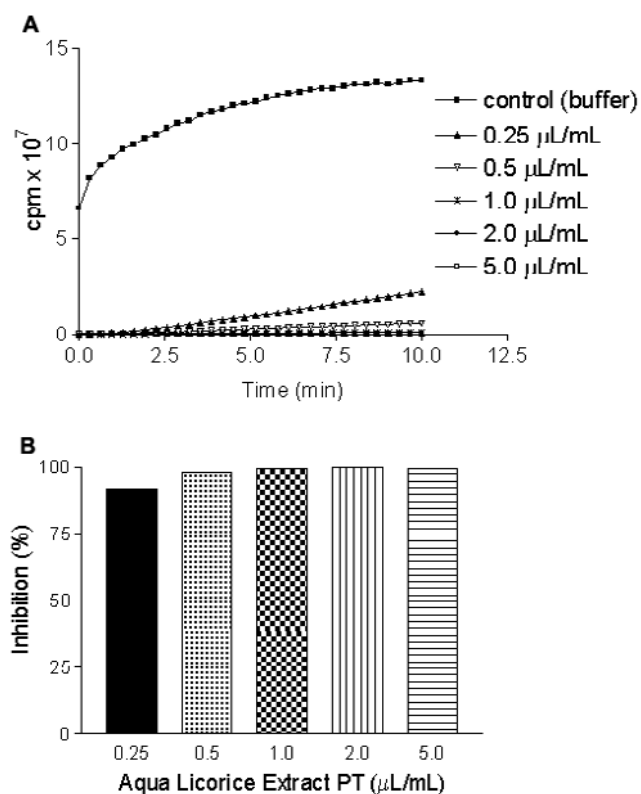
$$\text{Inhibition}(\%) = \frac{100 \times \text{AUC}_1}{\text{AUC}_0} \quad (1)$$

where AUC<sub>0</sub> and AUC<sub>1</sub> represent the area under the curve observed for the control and in the presence of the sample solution, respectively.



**Figure 1.** Chemiluminescence of quercetin (A) intensity of light emission vs time; (B) inhibition (%) of light emission from the HRP-catalyzed luminescent reactions with luminol.

The rate of inhibition (%) found for quercetin at different concentrations is shown in **Figure 1B**, the rate for ALE in **Figure 2B**, and the rates for Isoflavin Beta and red clover in **Figures 3B** and **4B**, respectively.



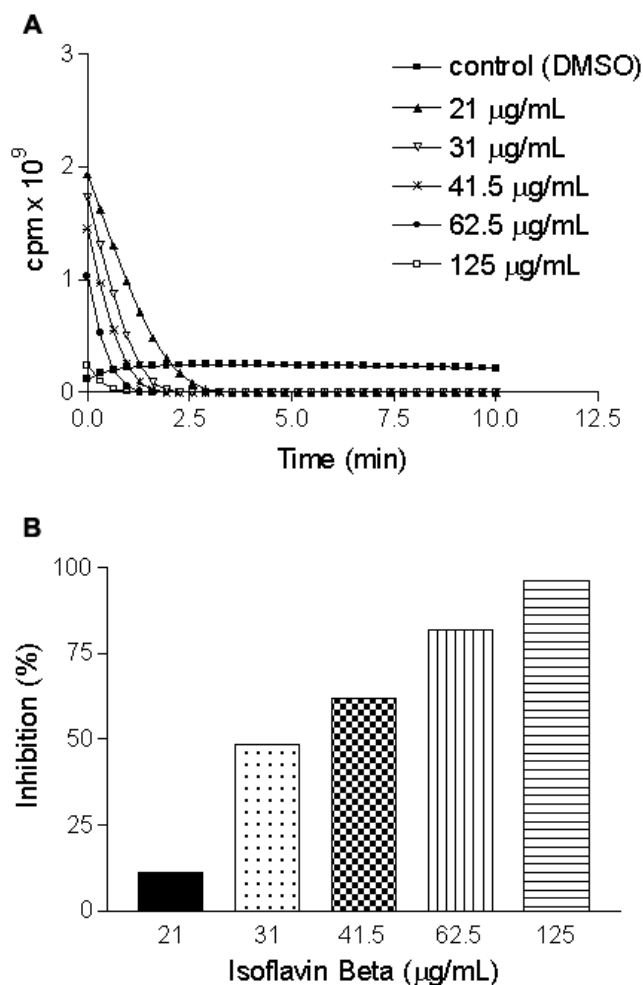
**Figure 2.** Chemiluminescence of Aqua Licorice Extract PT: (A) intensity of light emission vs time; (B) inhibition (%) of light emission from the HRP-catalyzed luminescent reactions with luminol.

## DISCUSSION

Among the assays for antioxidant activity, chemiluminescence is advantageous because of its high sensitivity and rapidity.<sup>16</sup> Light emission 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.<sup>17</sup>

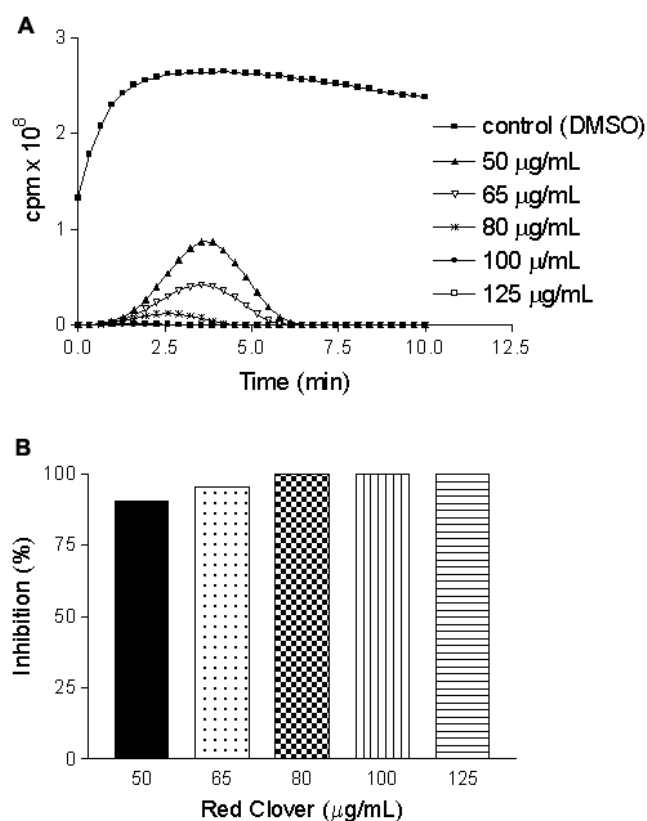
Addition of compounds to the chemiluminescent solution may lead to an increase or inhibition of light emission.<sup>18</sup> The reduction of light emission can be considered a measure of antioxidant activity.

To determine the antioxidant activity of the commercial vegetal extracts Isoflavin Beta, red clover, and ALE and the synthetic raw material quercetin, by the chemiluminescence method, we varied the hydrogen peroxide, luminol, and peroxidase concentrations in order to obtain maximum chemiluminescence intensity (data not shown). Quercetin is commonly used as a standard for flavonoid studies, and since the time course of its reaction was 10 minutes, this period was used as a standard for the analysis of the other raw materials.



**Figure 3.** Chemiluminescence of Isoflavin Beta: (A) intensity of light emission vs time; (B) inhibition (%) of light emission from the HRP-catalyzed luminescent reactions with luminol.

The commercial vegetal extracts ALE, Isoflavin Beta, and red clover did not follow the quercetin time course profile. Moreover, they showed time course profiles that differed from those of quercetin as well as from each other. Such differences are probably because of the different structures and different grades of purity of these flavonoids.



**Figure 4.** Chemiluminescence of red clover extract: (A) intensity of light emission vs time; (B) inhibition (%) of light emission from the HRP-catalyzed luminescent reactions with luminol.

The Isoflavin Beta time course showed an increase and a quick decay of chemiluminescence. This profile was not observed with the other compounds tested. The observed increase may be because of the presence of enhancers, such as some heterocyclic compounds with hydroxyl or amino groups or of phenol derivatives.<sup>19</sup> This chemiluminescence enhancement can be related to the acceleration of one or more steps in the peroxidase-catalyzed reaction prior to the light emission reaction.<sup>20</sup> On the other hand, the observed quick decay suggests the antioxidant action of this raw material. This antioxidant activity was confirmed by other methodologies such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay,<sup>21</sup> metal ion chelating activity,<sup>22</sup> and iron-induced lipid peroxidation<sup>23</sup> (data not shown).

Among the extracts studied, the ALE, which has glabridin as its active compound, showed a large chemiluminescence inhibition at zero time. This result suggests that the extract may inhibit luminol radical formation by HRP enzyme inhibition, by H<sub>2</sub>O<sub>2</sub> scavenging, or by reducing the oxidized HRP enzyme rather than luminol. The ALE antioxidant activity was con-

firmed by the DPPH free radical assay<sup>21</sup> and iron-induced lipid peroxidation<sup>23</sup> (data not shown).

Analyzing the percent results of chemiluminescence inhibition versus concentration, we observed that it was not possible to match the dose responses with the different raw materials employed as these samples contained different flavonoids, which can show varying antioxidant activity resulting from different grades of purity as well as from possible chemiluminescence enhancers or inhibitors.

Chemiluminescence is a quick, sensitive, and widely used method to measure antioxidant activity of several compounds. Nevertheless, since it follows several reaction steps, and the vegetal extracts are mixtures of different compounds, antioxidant activity measurements by chemiluminescence should be confirmed by other suitable methodologies.

If all samples studied prove to be inhibitors of the chemiluminescence generated by an enzymatic system, and their antioxidant activity is confirmed by other methods, they could be used in topical formulations for the treatment of diseases caused by free radicals. Topical formulations are being tested for the effects of the addition of these flavonoids.

Topical administration of antioxidants could provide an efficient way to enrich the endogenous cutaneous protection system and thus may be a successful strategy for diminishing UVR-mediated damage to the skin.<sup>12</sup>

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