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Antioxidant properties of a dry product from *Vitis vinifera* seeds (Leucoselect®) in rat liver endoplasmic reticulum

[Propiedades antioxidantes de un producto seco de semillas de *Vitis vinifera* (Leucoselect®) en retículo endoplásmico de hígado de rata]

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Abstract

Leucoselect® is a commercial dry product obtained from grape seeds and enriched in procyanidins, which display antioxidant activity in virtue to their ability to scavenge oxygen free radicals and to chelate transition metal ions. The hypoxanthine/xanthine oxidase and Cu^{2+}/ascorbate systems are capable of generating reactive oxygen species; the latter system can also promote non-specific binding of copper ions to proteins. Therefore, we assessed the ability of Leucoselect® to inhibit oxidative phenomena elicited by both oxidative systems on rat liver microsomes: lipid peroxidation, oxidation of protein thiols, and inhibition of the cytochrome P450 system. The antioxidant activity of Leucoselect® was a reflection of its ability to scavenge oxygen free radicals, chelate copper ions, and protect microsomal membranes through direct interaction. These mechanisms were displayed in a dependent manner with the type of biomolecule studied and also with the oxidative system employed, which is an interesting phenomenon to consider when evaluating the antioxidant activity of herbal products.

Keywords: *Vitis vinifera*; microsomes; procyanidins; oxidative stress; copper chelation.

Resumen

Leucoselect® es un producto comercial seco obtenido de semillas de uva y enriquecido en procianidinas, las cuales presentan actividad antioxidante debido a su capacidad para atrapar radicales libres y quelar metales de transición. Los sistemas hipoxantina/xantina oxidasa y Cu^{2+}/ascorbato generan especies reactivas del oxígeno; este último sistema también promueve la unión inespecífica de iones cobre a proteínas. Por lo tanto, evaluamos la capacidad de Leucoselect® para inhibir los fenómenos oxidativos producidos por ambos sistemas oxidantes en microsomas hepáticos de rata: lipoperoxidación, oxidación de tioles proteicos e inhibición de la actividad del sistema citocromo P450. La actividad antioxidante de Leucoselect® fue un reflejo de su capacidad de atrapar radicales libres del oxígeno, quelar iones cobre y proteger membranas microsómicas por interacción directa. Dichos mecanismos se manifestaron en forma dependiente del tipo de biomolécula estudiada y del sistema oxidante empleado, fenómeno interesante de considerar al evaluar la actividad antioxidante de preparados herbales.

Palabras Clave: *Vitis vinifera*; microsomas; procianidinas; estrés oxidativo; quelación de cobre.

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INTRODUCTION

Extracts of *Vitis vinifera*, a native plant of Asia, have been widely used in cardiovascular diseases, cancer, tuberculosis, blood disorders, asthma, diarrhea, ulcers as well as in skin, kidney and liver pathologies (Bombardelli *et al*. 1995). In addition, oligomeric polyphenols have been used in the prophylaxis of chronic degenerative pathologies and skin aging, both diseases associated to oxidative stress (Onyango 2008; Reddy *et al*. 2009; Wells *et al*. 2009). *In vitro* studies using biochemical models have shown that procyanidins display an antioxidant activity, reflected in their oxygen free radicals scavenging ability (Maffei Facino *et al*. 1994). Furthermore, it has been described that these compounds are capable to sequester iron and copper ions, which in their free form can promote generation of reactive oxygen species (ROS) through Fenton/Haber-Weiss reactions (Maffei Facino *et al*. 1994; Bombardelli *et al*. 1995; Maffei Facino *et al*. 1996).

ROS are generated in all aerobic cells and participate in several physiological processes, including the immune response and learning and memory (Droge 2002). Excessive ROS generation, however, promote oxidation of biomolecules other than their physiological targets (Halliwell 2007; Halliwell *et al*. 2007). Cells have evolved antioxidant mechanisms, both enzymatic and non-enzymatic, to protect biomolecules from oxidative damage (Benzie 2000). When generation of ROS overwhelms the cellular antioxidant defense, oxidative stress is ensued (Halliwell 2007; Halliwell *et al*. 2007). Under chronic oxidative stress, damage to lipids, nucleic acids and proteins alter cellular homeostasis and may lead to cell death. The extent of oxidative damage depends on the redox potential of the oxidative agent, cell type, and duration of the oxidative process, all factors that influence the type of cell death (Halliwell 2007; Halliwell *et al*. 2007).

Pharmacokinetic studies of procyanidins from *Vitis vinifera* have reported several metabolites, which include hippuric acid, ethyl-cathecol, and *m*-hydroxyphenyl propionic acid in urine, ethyl-cathecol in feces, and vanillic acid in the bile (Bombardelli *et al*. 1995). Furthermore, distribution studies have shown that connective tissue is the main target for procyanidins from *Vitis vinifera* (Bombardelli *et al*. 1995). Moreover, a study showed that activity of procyanidins is related to their interaction with structural components of the extracellular matrix (*e.g.* collagen and elastin fibers) and of the plasma membrane and cytoskeleton of mesenchymal cells (Groult *et al*. 1991). Stabilization of the extracellular matrix has been associated with procyanidins occurring in herbal extracts, through direct adsorption (Chang and Sung 2008) or regulation of the expression of metalloproteinases (Vayalil *et al*. 2004).

Hepatic endoplasmic reticulum is the main organelle responsible for biotransformation of xenobiotics, including drugs. Enzymatic systems occurring in this organelle include: the cytochrome P450 (CYP450) system, UDP-glucuronyltransferase (UDPGT) and glutathione *S*-transferase (GST) (Kaplowitz 1980; Tephly *et al*. 1990; Hayes *et al*. 1995; Danielson 2002; Kramer *et al*. 2008). Integrity of the lipid membrane of the endoplasmic reticulum appears to be required for appropriate enzymatic function. In fact, detergents or lipid peroxidation has been found to alter these activities in rat liver microsomes (Letelier *et al*. 2005b; Letelier *et al*. 2007; Letelier *et al*. 2009a; Letelier *et al*. 2009b). In addition, cysteine residues occurring in endoplasmic reticulum biotransformation enzymes also appear to be important for their catalytical function. We have shown that thiol oxidation can alter these activities: increase it in the case of microsomal GST and UDPGT activities (Letelier *et al*. 2005b; Letelier *et al*. 2009b), or decrease it in the case of the CYP450 system (Letelier *et al*. 2009a). Furthermore, when using Cu^{2+}/ascorbate as a ROS-generating system *in vitro*, a non-specific Cu^{2+}-binding effect to protein thiol groups can also occur in addition to its pro-oxidant activity (Letelier *et al*. 2005a).

Leucoselect® is a dry product obtained from grape seeds enriched in procyanidins (donated by ARAMA Laboratorios, Santiago, Chile). The relative contribution of different antioxidant mechanisms described for procyanidins from *Vitis vinifera* (*e.g.* oxygen free radical-scavenging activity and their ability to chelate transition metal ions) is yet unclear. It is possible that procyanidins occurring in Leucoselect® may protect rat liver microsomes from oxidative damage through different mechanisms which include free radical scavenging, chelation of transition metal ions, and/or adsorption to the microsomal membrane behaving as a physical barrier. To address these possibilities, we used rat liver microsomes to evaluate the capacity of Leucoselect® to prevent microsomal lipid peroxidation, protein-thiol oxidation, and inhibition of the CYP450 system activity, all phenomena

elicited by the Cu^{2+}/ascorbate system. Using this biological preparation, we can also discriminate among the scavenging and chelating activity of herbal antioxidants. We found that Leucoselect® displayed at least two different antioxidant mechanisms. Thus, this product prevented lipid peroxidation elicited by Cu^{2+}/ascorbate and the inhibition of the CYP450 system activity promoted by the hypoxanthine/xanthine oxidase system, by scavenging oxygen free radicals. Leucoselect® also inhibited oxygen consumption in free solution and prevented the inhibition of the CYP450 system activity in the presence of Cu^{2+} alone by chelating copper ions. In addition, our data suggest that Leucoselect® is also able to directly interact with the microsomal membrane to protect it from oxidative damage. Differential display of antioxidant properties of Leucoselect on microsomal membranes and their pharmacological relevance are discussed.

MATERIALS AND METHODS

Chemicals.

Leucoselect®, a dry standardized product obtained from grape seeds, was donated by ARAMA Laboratorios. Certificated analysis of this product was performed by INDENA, according to the HPLC Method TM/0076: the content of procyanidins of the product was equivalent to 14.6% in catechin (established range of 13.0-19.0%). In addition, polyphenol content was determined according to Letelier *et al*. (Letelier *et al*. 2008): 2.8 ± 0.19 nmoles-eq of catechin/mg of Leucoselect®. Hypoxanthine, xanthine oxidase, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), β-NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, BSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of best available grade.

Animals.

Adult male Sprague Dawley rats (200-250 g), maintained at the vivarium of the School of Chemical and Pharmaceutical Sciences (Universidad de Chile, Santiago, Chile) were used. Rats were allowed free access to pelleted food, maintained with controlled temperature (22ºC) and photoperiod (lights on from 07:00 to 19:00 h). All procedures were performed using protocols approved by the Institutional Ethical Committee of the School of Chemical and Pharmaceutical Sciences, Universidad de Chile, and according to the guidelines of the Guide for the Care and Use of Laboratory Animals (NRC, USA).

Isolation of liver microsomes.

Microsomal fractions were prepared according to Letelier *et al*. (Letelier *et al*. 2005a). Total microsomal protein was determined according to Lowry *et al*. (Lowry *et al*. 1951).

Oxidative conditions.

Microsomes (1mg protein/mL) were incubated with 25nM, 50µM or 100µM $CuSO_4$ in the presence or absence of 1mM sodium ascorbate under different conditions, as detailed in the text. In some experiments, microsomes (1mg protein/mL) were incubated with 0.1mM hypoxanthine in the presence of 0.5U/mL xanthine oxidase, as detailed in the text.

Microsomal lipid peroxidation assay.

Following pre-incubation with Cu^{2+}/ascorbate, the extent of microsomal lipid peroxidation was estimated by determining TBARS, according to Letelier *et al.* (Letelier *et al*. 2005a).

Microsomal thiol content.

Thiol groups were titrated with DTNB as described by Letelier *et al.* (Letelier *et al*. 2005a).

Oxygen consumption.

The extent of oxygen consumption by the Cu^{2+}/ascorbate system from a saturated solution in 50mM phosphate buffer at pH 7.4, in the absence and presence of Leucoselect®, was continuously determined polarographically up to 60s with a Clark electrode No. 5300 (Yellows Springs Instruments Co., Inc., Yellow Springs, OH, USA), coupled with a Windaq transducer, model DATAQ D1-148U. Oxygen concentrations were calculated considering 100% oxygen saturation as 452.5nmoles/mL (at 718mm Hg local atmospheric pressure).

p-Nitroanisole *O*-demethylation.

The *O*-demethylating activity of CYP450 system was determined using *p*-nitroanisole as a substrate, according to Letelier *et al*. (Letelier *et al*. 2009a).

Generation of the CYP450 monooxygenase spectrum.

CYP450 monooxygenase spectrum was obtained according to Omura and Sato (Omura *et al*. 1964) and as described previously (Letelier *et al*. 2009a).

Statistical analysis.

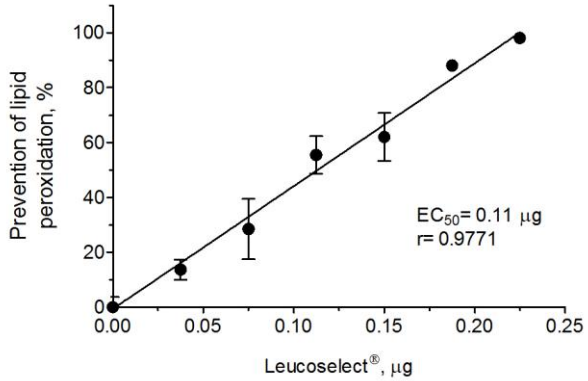
Data presented correspond to the mean of at least four independent experiments ± SD. Statistical significance (ANOVA, followed by Bonferroni post-hoc tests) and regression analyses were performed using Graph Pad Prism 5.0. Differences were considered significant when $p<0.05$.

RESULTS

Effect of Leucoselect® on the oxidative damage promoted by Cu^{2+}/ascorbate on rat liver microsomes

Lipids and proteins occurring in rat liver microsomes can be oxidized by Cu^{2+}/ascorbate (Letelier *et al*. 2005a). To evaluate such oxidation and the protective effect of Leucoselect®, we measured lipid peroxidation and oxidation of thiol groups from microsomal proteins incubated with 25nM Cu^{2+}/ascorbate.

Figure 1. Effect of Leucoselect® on the microsomal lipid peroxidation elicited by Cu^{2+}/ascorbate.

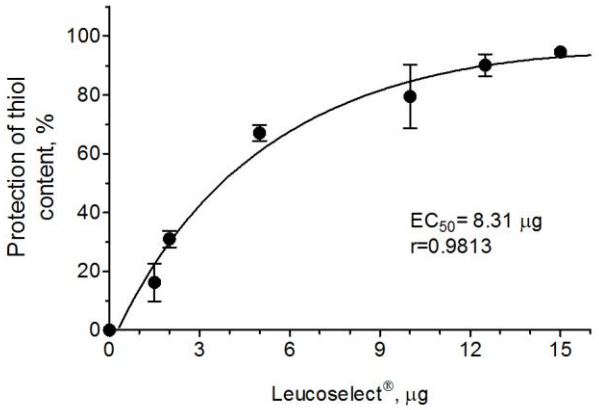


Microsomes (1 mg protein) were incubated with increasing quantities of Leucoselect® (up to 0.225µg) in a 1mL final volume, for 10min at 37°C with constant agitation, prior to incubation with 25nM Cu^{2+} and 1mM ascorbate for 1h at 37°C with constant agitation. Lipid peroxidation was estimated by measuring TBARS, as detailed in Material and Methods. Data corresponds to the % of prevention of lipid peroxidation, considering as 0% prevention the lipid peroxidation measured in the absence of Leucoselect®. Values represent the mean ± SD of at least 4 independent experiments. The solid line represents the linear regression of the data.

As shown in Figure 1, Leucoselect® was able to prevent microsomal lipid peroxidation in a concentration-dependent manner. The EC_{50} for this effect, calculated from the linear regression analysis of the data (n=4 to 8 independent experiments, r=0.9771), was 0.11µg.

In the same experimental conditions, Leucoselect® was also capable to protect the microsomal thiol content from oxidation in a concentration-dependent manner (Figure 2), with an EC_{50} of 8.31µg, calculated from the hyperbolic regression analysis of the data (n=4 to 8 independent experiments, r=0.9813).

Figure 2. Effect of Leucoselect on the loss of microsomal thiol group content elicited by Cu^{2+}/ascorbate.

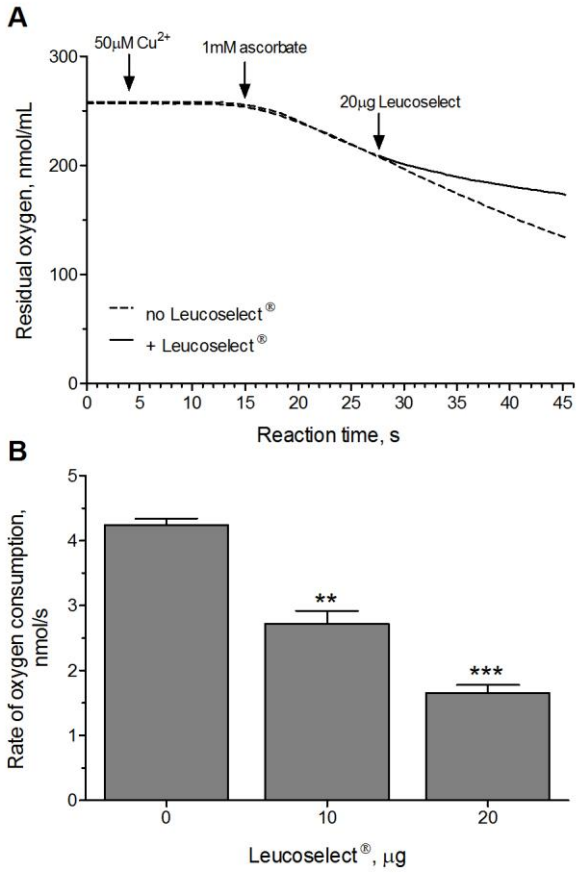


Microsomes (1mg protein) were incubated with increasing quantities of Leucoselect® (up to 15µg) in a 1mL final volume, for 10min at 37°C with constant agitation, prior to incubation with 25nM Cu^{2+} and 1mM ascorbate for 1h at 37°C with constant agitation. Thiol content was titrated as detailed in Material and Methods. Data corresponds to the % of protection of the microsomal thiol content, considering as 0% prevention the loss of thiol groups measured in the absence of Leucoselect®. Values represent the mean ± SD of at least 4 independent experiments. The solid line represents the hyperbolic regression of the data.

Effect of Leucoselect® on oxygen consumption elicited by Cu^{2+}/ascorbate

Figure 3A depicts representative traces of oxygen consumption elicited by 50µM Cu^{2+}/ascorbate in the absence or presence of Leucoselect®. As shown in Figure 3B, this product was able to decrease the slope of oxygen consumption in a concentration dependent manner.

Figure 3. Effect of Leucoselect® on the oxygen consumption elicited by Cu^{2+}/ascorbate.

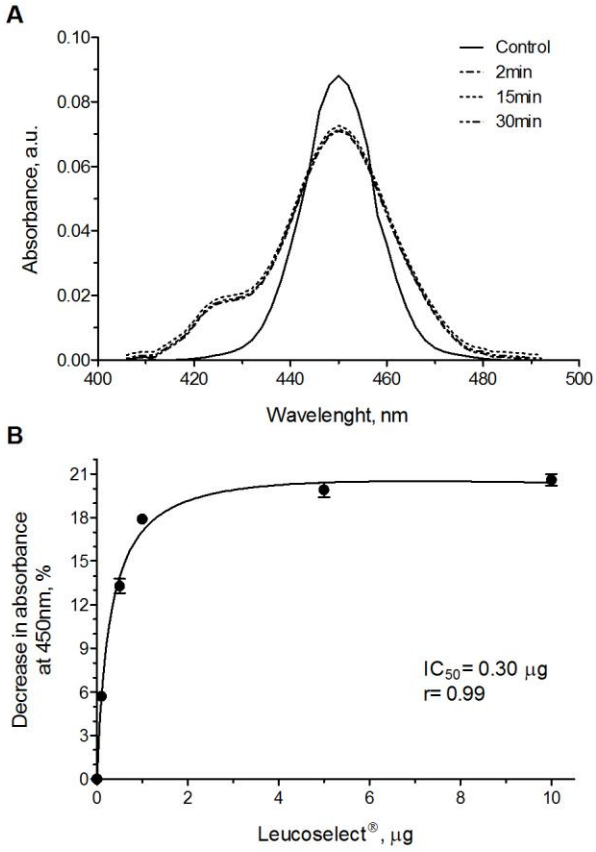


Oxygen consumption elicited by 50µM Cu^{2+} and 1mM ascorbate was determined as detailed in Material and Methods. **(A)** Representative traces of oxygen consumption, in the absence (dotted line) or presence (solid line) of 20µg/mL Leucoselect®. Arrows indicate the addition of each reagent during the continuous recording of the data. **(B)** Rates of oxygen consumption, calculated from linear regression of the data, in the presence of either 10 or 20µg/mL Leucoselect®. Data correspond to the mean of at least 4 independent experiments ± SD. **p<0.01 and ***p<0.001 compared to Control (in the absence of Leucoselect®).

Effect of Leucoselect® on the spectral properties of the CYP450 monooxygenase

The reduced form of microsomal CYP450 monooxygenase can form a complex with CO that displays a characteristic peak at 450nm (Omura *et al.* 1964). This peak can be altered by substrate binding, protein damage or lipid peroxidation. Pre-incubation of microsomal membranes with Leucoselect® prior to reduction of the CYP450 monooxygenase altered the spectral properties of the complex with CO by decreasing the characteristic absorbance peak at 450nm (Figure 4A). This effect was time-independent at the time range used in this study (2-30min). In contrast, this effect was concentration-dependent (Figure 4B), with an EC_{50} of 0.30µg, calculated from the hyperbolic regression analysis of the data (n=4 to 8 independent experiments, r=0.9980).

Figure 4. Effect of Leucoselect® on the spectral properties of the microsomal CYP450 monooxygenase.

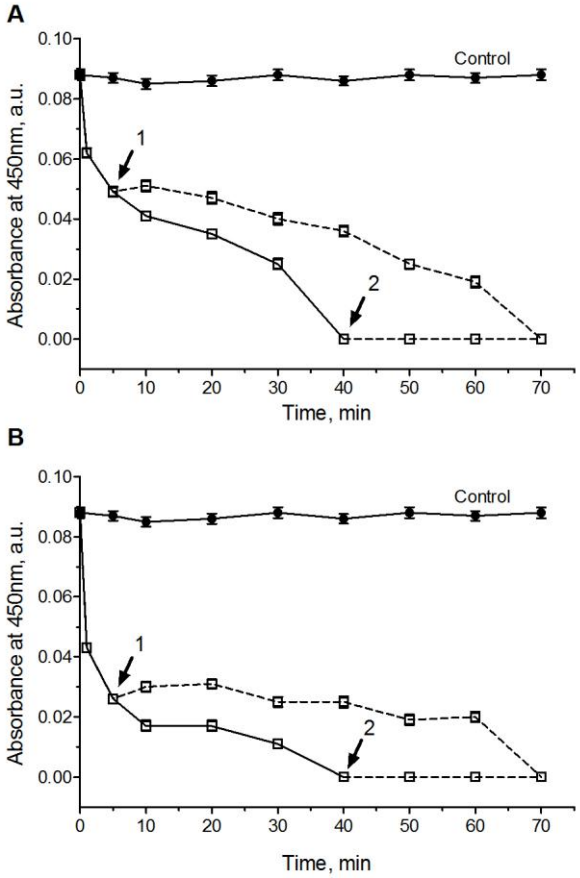


(A) Dependence with time. Microsomes (1mg protein) were incubated with (dotted lines) or without (solid line) 10µg of Leucoselect® in a 1mL final volume, for different periods of time (2-30min) at 25°C, prior to determination of the absorbance spectrum of the CYP450 monooxygenase, as detailed in Material and Methods. Representative traces for each condition are shown. **(B)** Dependence with Leucoselect® concentration. Microsomes (1mg protein) were incubated with different quantities of Leucoselect® (up to 10µg) in a 1mL final volume, for 5min at 25°C, prior to determination of the absorbance spectrum of the CYP450 monooxygenase complex with CO, as detailed in Material and Methods. Data correspond to the absorbance at 450nm from each spectrum and represent the mean ± SD of at least 4 independent experiments. The solid line represents the hyperbolic regression of the data.

Effect of Cu^{2+}/ascorbate on the spectral properties of the microsomal CYP450 monooxygenase: protective effect of Leucoselect®

We have recently shown that Cu^{2+}/ascorbate can elicit damage to the CYP450 system by altering the content of thiol groups of the microsomal CYP450 monooxygenase, both by directly binding Cu^{2+} and through oxidation (Letelier *et al*. 2009a). Therefore, we also tested the ability of Leucoselect® to prevent this damage.

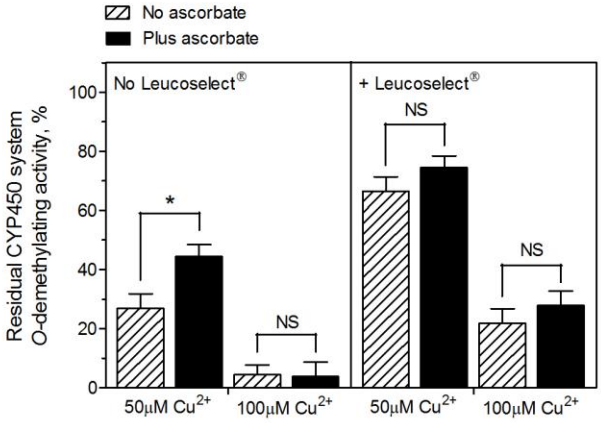
Figure 5. Loss of the absorbance at 450nm of the microsomal CYP450 monooxygenase elicited by Cu^{2+}/ascorbate: Effect of Leucoselect®.



Microsomes (1mg protein) were incubated with (open squares) or without (closed circles) 50µM **(A)** or 100µM **(B)** Cu^{2+} in the presence of 1mM ascorbate for different periods of time (up to 70min), prior to determine the absorbance spectrum of the CYP450 monooxygenase complex with CO, as detailed in Material and Methods. Leucoselect® (10µg) was added at 5min (arrow 1) or 40min (arrow 2) of microsomal incubation with Cu^{2+}/ascorbate. Data correspond to the absorbance at 450nm from each spectrum and represent the mean ± SD of at least 4 independent experiments. Dotted and solid lines connect data from experiments performed in the presence or absence of Leucoselect®, respectively.

As previously described, Cu^{2+}/ascorbate was able to decrease the absorbance of the CYP450 monooxygenase complex with CO at 450nm in a time-dependent manner (Letelier *et al*. 2009a). As shown in Figure 5, the time course of the effect of 50µM and 100µM Cu^{2+}/ascorbate (Figures 5A and 5B, respectively) was biphasic, with a first (fast) decrease stage and a second (slow) decrease stage (squares with solid line). Addition of Leucoselect® at the end of the first stage (arrow 1) inhibited the rate of the second decrease stage at both Cu^{2+} concentrations tested. In contrast, addition of Leucoselect® at the end of the second stage (arrow 2) failed to reverse the loss of absorbance at 450nm.

Figure 6. Inhibition of the O-demethylating activity of the CYP450 system elicited by Cu^{2+}/ascorbate: Effect of Leucoselect®.



Microsomes (1mg protein) were incubated with or without Leucoselect® (10µg) in a 1mL final volume, for 10min at 37°C with constant agitation, prior to incubation with 50µM or 100µM Cu^{2+} in the presence (black bars) or absence (hatched bars) of 1mM ascorbate for 20min at 37°C with constant agitation. *O*-demethylation of *p*-nitroanisole was assayed as detailed in Material and Methods. Data are expressed as % of residual CYP450 system *O*-demethylating activity, considering as 100% the activity measured in the absence of Cu^{2+}/ascorbate or Leucoselect®. Data represent the mean ± SD of at least 4 independent experiments. NS indicates not significant, and *$p<0.05$.

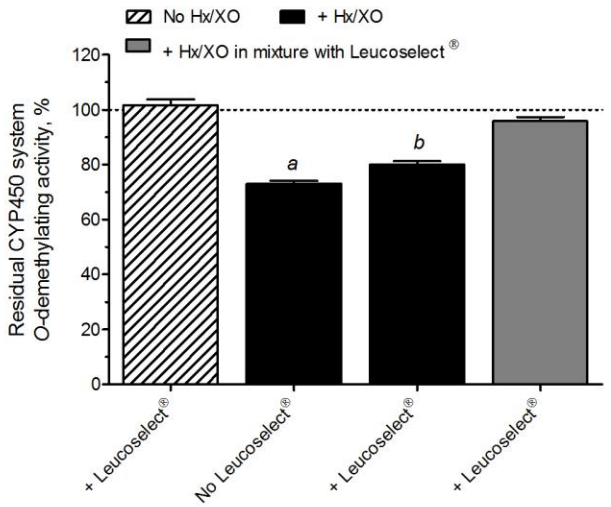
Effect of Cu^{2+}/ascorbate and Cu^{2+} alone on the *O*-demethylating activity of the CYP450 system: protective effect of Leucoselect®

As we have recently reported (Letelier *et al*. 2009a), Cu^{2+}/ascorbate can promote damage to the CYP450 system by decreasing its activity. As shown in Figure 6, incubation of microsomes with 50 or 100µM Cu^{2+}/ascorbate (black bars), decreased the microsomal *p*-nitroanisole *O*-demethylating activity in 42.3 and 96.3%, respectively; similarly, the

incubation of microsomes with 50 or 100μM Cu^{2+} alone, decreased this enzymatic activity to 73.1 and 95.5%, respectively (Figure 6, hatched bars).

Although pre-incubation with Leucoselect® alone did not change this microsomal *O*-demethylating activity, the addition of this product prior to 50 or 100μM Cu^{2+}/ascorbate and 50 or 100μM Cu^{2+} alone decreased the inhibition of this activity to 25.6 and 72.2%, and 33.5 and 78.2%, respectively. Notably, the protection of the *O*-demethylating activity by Leucoselect® was not statistically different, regardless of whether the damage was elicited by Cu^{2+} in the presence or absence of ascorbate.

Figure 7. Inhibition of microsomal *p*-nitroanisole *O*-demethylation elicited by the hypoxanthine/xanthine oxidase system: Effect of Leucoselect®.



Microsomes (1mg protein) were pre-incubated with or without 10μg of Leucoselect in a 1mL final volume, for 10min at 37°C with constant agitation, prior to incubation with (black bars) or without (hatched bar) 0.1mM hypoxanthine plus 0.5U xanthine oxidase (Hx/XO) for 30min at 37°C with constant agitation. The grey bar indicates an experiment in which Hx/XO and Leucoselect were mixed for 10min at 37°C prior to incubation with microsomes. O-demethylation of p-nitroanisole was then assayed as detailed in Material and Methods. Data are expressed as % of residual CYP450 system O-demethylating activity, considering as 100% the activity measured in the absence of Hx/XO or Leucoselect®. Data represent the mean ± SD of at least 4 independent experiments. (a) and (b) $p<0.05$ compared to Control (without Hx/XO) and $p<0.05$ between them.

We also tested whether Leucoselect® was able to protect the *O*-demethylating activity of the CYP450 system from oxidative damage caused by another ROS-generating system that does not contain copper ions, such as the hypoxanthine/xanthine oxidase (Hx/XO) system. As shown in Figure 7, incubation of microsomes with the Hx/XO system decreased 26.9% the microsomal *O*-demethylating activity; pre-incubation with Leucoselect® did not significantly change this effect. In contrast, when microsomes were incubated with a mixture of the Hx/XO system plus Leucoselect®, the oxidative agent failed to significantly decrease the *O*-demethylating activity.

DISCUSSION

It is widely accepted that polyphenolic compounds, such as procyanidins, exert their antioxidant effects by scavenging oxygen free radicals (Maffei Facino *et al*. 1994; Bombardelli *et al*. 1995; Maffei Facino *et al*. 1996). These compounds can also chelate transition metals in their free ionic state, which may generate oxygen free radicals *via* Fenton/Haber-Weiss reactions (Halliwell *et al*. 2007). The wide array and variety of experimental oxidative systems that exert their action through different mechanisms poses a challenge in understanding the antioxidant activity of herbal products rich in polyphenolic compounds. The case of Cu^{2+}/ascorbate is particularly interesting since it promotes damage through both generation of reactive oxygen species (ROS) and non-specific binding of copper ions to proteins; we can experimentally discriminate both mechanisms by changing copper ion concentrations (Letelier *et al*. 2005a; Letelier *et al*. 2009a). Thus, nM concentration of Cu^{2+} in the presence of ascorbate elicits only its pro-oxidant activity; μM concentration of Cu^{2+} in the presence of ascorbate however, can induce damage by pro-oxidant activity and binding of copper ions to biological molecules such as thiol-proteins (Letelier *et al*. 2005a; Letelier *et al*. 2009a).

Therefore, it is reasonable to postulate that the oxidative system employed and the biomolecule studied should be taken into consideration when assessing the antioxidant effect of herbal products. To evaluate this postulate, we used rat liver microsomes as a biological system, which contains the main biotransformation enzymes that metabolize xenobiotics such as herbal compounds. As oxidative systems, we employed Cu^{2+}/ascorbate and hypoxanthine/xanthine oxidase (Hx/XO). Leucoselect®, a herbal product enriched in procyanidins, was used as a source of herbal antioxidants.

Our data show that Leucoselect® prevents both lipid peroxidation (Figure 1) and oxidation of protein thiol groups (Figure 2) elicited by 25nM

Cu^{2+}/ascorbate, an experimental condition in which this system elicits only pro-oxidant effects (Letelier *et al*. 2005a). The EC_{50} value for the protection of microsomal lipids however, was 75-fold lower than that for protection of the microsomal thiol content. In biological membranes, lipids are more exposed to oxidative damage than proteins, which are inserted in the lipid bilayer. This difference may favor protection of lipids over proteins, which may explain the difference found in the EC_{50} values obtained.

We also tested the ability of Leucoselect® to alter the oxygen consumption elicited by 50μM Cu^{2+}/ascorbate, experimental condition in which occurs both pro-oxidant and non-specific binding of copper ions (Letelier *et al*. 2005a). Notably, this product decreased the rate of oxygen consumption promoted by this system. Since polyphenols are capable to scavenge oxygen free radicals, increased oxygen consumption would be expected as a result of reaction displacement towards products. On the other hand, since polyphenols are able to chelate copper ions, a decrease in oxygen consumption would be expected as a consequence of a decrease in copper ions actual concentration (Letelier *et al* 2008). Since Leucoselect® decreased the rate of oxygen consumption (Figure 3), the chelating activity of this product appeared to predominate over its oxygen free radical scavenging activity.

The CYP450 system can be affected by both oxidation and copper binding to protein-thiol groups occurring in the CYP450 monooxygenase, which catalyzes the first and limiting step of the CYP450 system catalytic cycle. We have reported that 50 and 100μM Cu^{2+}/ascorbate lead to inhibition of the catalytic activity of the CYP450 system, mainly through binding of copper ions to the CYP450 monooxygenase (Letelier *et al*. 2009a). Therefore, we evaluated the protection of Leucoselect® on the damage promoted by Cu^{2+}/ascorbate on this enzymatic system. We first evaluated the effect of Leucoselect® by itself on the CYP450 monooxygenase. Leucoselect® (10μg/mg microsomal protein) was able to alter the spectral properties of the CYP450 monooxygenase complex with CO only when added prior to the addition of dithionite (Figure 4A). Since the CYP450 monooxygenase can only bind its substrate in its oxidized ferric form, this result suggests that components present in Leucoselect® may be substrates of the CYP450 system; equal concentrations of this product, however, did not decrease the *O*-demethylation of *p*-nitroanisole, as expected for a substrate of the CYP450 system (Figure 7). Furthermore, the concentration dependence of this effect indicates that 10μg Leucoselect®/mg microsomal protein would completely saturate its binding sites in the microsomal membrane (Figure 4B). We postulate that procyanidins occurring in Leucoselect® may interact with some components of the microsomal membrane, leading to the observed changes in the spectral properties of the CYP450 monooxygenase. This postulate is consistent with the absence of competitive inhibition of the activity of the CYP450 system. Particularly, it has been reported that procyanidins from *Vitis vinifera* have selective distribution in the extracellular matrix, specially its interaction with collagen and elastin fibers, so indicating its affinity towards lipophilic compounds in according with the possible direct interaction of Leucoselect® with microsomal membrane previously postulated (Groult *et al*. 1991).

Furthermore, we have reported that 50 and 100μM Cu^{2+}/ascorbate elicited time-dependent biphasic decreases in the absorbance at 450nm of the CYP450 monooxygenase complex with CO; the biphasic loss of this absorbance is the consequence of both Cu^{2+}-binding to the protein (first stage) and to oxidative damage (second stage) (Letelier *et al*. 2009a). Leucoselect® was able to delay, but not avoid, the second stage of the absorbance loss (Figure 5). These data suggest that components of Leucoselect® may be acting only as oxygen free radical scavengers, since non-specific binding of copper ions to protein-thiol groups is irreversible (Letelier *et al*. 2005a). Noteworthy, Leucoselect® prevented the damage on the *O*-demethylation of *p*-nitroanisole activity elicited by 50 and 100μM Cu^{2+}, regardless of the presence or absence of ascorbate (Figure 6). Thus, the chelating activity of components occurring in Leucoselect® may be a significant contributing factor to its antioxidant capacity when preventing non-specific binding of copper ions to thiol groups of the CYP450 monooxygenase, but not reverse it. To study the protection contributed by Leucoselect® against oxidative damage in the absence of copper ions, we turned to the hypoxanthine/xanthine oxidase (Hx/XO) system, another ROS-generating system. In this case, Leucoselect® was able to partially protect the *O*-demethylating activity of the CYP450 system from damage elicited by Hx/XO (Figure 7). Notably,

mixing Leucoselect® with the Hx/XO system prior to incubation with microsomes completely prevented the damage elicited by this system. This difference may be due to the competition that may occur between oxygen free radicals for microsomal components and Leucoselect®, phenomenon that does not proceed in the absence of microsomal membranes.

CONCLUSIONS

Taken together, our findings suggest that components occurring in Leucoselect® display oxygen free radicals scavenging properties, can chelate copper ions, and potentially protect microsomal membranes through direct interactions. These properties, however, contribute differentially to the protection of biomolecules present in microsomal preparations against oxidative damage. The expression of each protective activity of Leucoselect® components appears to depend on the oxidative system employed, the biological system evaluated and if this product is used to prevent or reverse oxidative damage. Bioavailability of procyanidins is still a matter of discussion. Different formulation approaches, however, may help to improve their bioavailability or avoid depolymerization by host microbiota. This would allow taking advantage of the protection of membranes by adsorption and stabilization of the extracellular membrane components, both actions ascribed to procyanidins. Given the widespread use of herbal compounds as antioxidants in the treatment of several pathologies, it becomes relevant to evaluate their purported antioxidant properties in the specific biological systems for which they are putatively used.

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