© 2009 Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 8 (2), 110 - 120
BLACPMA ISSN 0717 7917

Artículo Original | Original Article

Evaluation of the antioxidant properties and effects on the biotransformation of commercial herbal preparations using rat liver endoplasmic reticulum

[Evaluación de la actividad antioxidante y los efectos sobre biotransformación de productos herbales comerciales en retículo endoplásmico hepático de rata.]

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Abstract

The antioxidant herbal ingredients are xenobiotics for animals, many of them must be biotransformed to be eliminated, especially lipophilic compounds. Biotransformation of such xenobiotics- occurs mainly in the hepatic endoplasmic reticulum, a process that may generate ROS and trigger oxidative stress. Therefore, we used rat liver microsomes to test the antioxidant capacity and the effects on biotransformation enzymes of five commercial herbal preparations containing *Silybum marianum, Tilia cordata, Crataegus oxyacantha, Avena sativa, Melissa officinalis, Valeriana officinalis; Passiflora incarnata, Foeniculum vulgare, Cassia senna, Peumus boldus* and *Opuntia ficus-indica*, as ingredients -alone or in combination-. With the exception of the aqueous preparation of *Opuntia ficus-indica*, all the hydro-alcoholic commercial preparations inhibited the oxidation of microsomal lipids and thiols and prevented the oxidative alterations of several microsomal biotransformation enzymes activities, all phenomena induced by Fe^{3+}/ascorbate. They also inhibited the UDP-glucuronyltransferase (UDPGT), GSH-transferase (GST), and N-demethylating, and Monooxygenase cytochrome P450 activities to a lower extent in the absence of oxidative stress. This last phenomenon might be due to the presence of lipophilic substrates in the herbal preparations. Thus, microsomes could be a good biological system to evaluate both the antioxidant properties and the possible interactions of herbal preparations with the enzymes involved in xenobiotics biotransformation.

Keywords: Microsomes; Polyphenols; Oxidative stress; Biotransformation-enzymes; Herbal-antioxidants; Herbal-ingredient biotransformation.

Resumen

Dependiendo de su lipofilicidad, los principios herbales antioxidantes que son xenobióticos para los organismos animales, deben biotransformarse para ser eliminados. Este proceso ocurre principalmente en el retículo endoplásmico hepático y puede generar ROS induciendo, paradójicamente, estrés oxidativo. En este trabajo hemos utilizado microsomas hepáticos de rata para evaluar la capacidad antioxidante y los efectos sobre la biotransformación de xenobióticos de varios extractos comerciales conteniendo *Silybum marianum, Tilia cordata, Crataegus oxyacantha, Avena sativa, Melissa officinalis, Valeriana officinalis; Passiflora incarnata, Foeniculum vulgare, Cassia senna, Peumus boldus and Opuntia ficus-indica, solos o en combination*-. Excepto el preparado acuoso de *Opuntia ficus-indica*, el resto de extractos comerciales –mayoritariamente hidro-alcohólicos- inhibieron la oxidación de los lípidos y los tioles microsómicos, las modificaciones oxidativas de varias actividades de enzimas microsómicas biotransformantes de xenobióticos, todos estos fenómenos inducidos experimentalmente por Fe^{3+}/ascorbato. En ausencia de estrés oxidativo estas preparaciones también inhibieron las actividades de UDP-glucuroniltransferasa (UDPGT) GSH-transferasa (GST), N-demetilasa y Monooxigenasa citocromo P450, aunque este efecto fue menos marcado que el antioxidante. Este último fenómeno puede deberse a la presencia de sustratos lipofílicos en estas preparaciones herbales. Así, los microsomas hepáticos podrían ser un buen sistema biológico para evaluar simultáneamente las propiedades antioxidantes y las interacciones que sobre la biotransformación de xenobióticos podrían ejercer las preparaciones herbales.

Palabras Clave: Microsomas; polifenoles; estrés oxidativo; enzimas de biotransformación; antioxidantes herbales; biotransformación de principios herbales.

Recibido | Received: October 28, 2008.
Aceptado en Versión Corregida | Accepted in Corrected Version: November 25, 2008.
Publicado en Línea | Published Online: December 17, 2008.
Declaración de Intereses | Declaration of interests: Authors have no competing interests.
Financiación | Funding: This study was partially funded by Laboratorios Ximena Polanco.
This article must be cited as: Maria E. Letelier, Juan F. Cortes, Ana M. Lepe, José A. Jara, Alfredo Molina-Berríos, Carmen Rodriguez, Pablo Iturra-Montecinos, Mario Faúndez. 2009. Evaluation of the antioxidant properties and effects on the biotransformation of commercial herbal preparations using rat liver endoplasmic reticulum. Bol Latinoam Caribe Plant Med Aromat 8(2):110 – 120. {EPub 17 December 2008}.

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INTRODUCTION

Evidence exists for the antioxidant role of herbal products in the prevention of pathologies such as cardiovascular, neurodegenerative diseases and cancer. All these pathologies are especially associated to oxidative stress, a phenomenon triggered when generation of reactive oxygen species (ROS) is excessive and/or its removal deficient (Lee et al., 2003; Leone et al., 2003). In this condition, modifications of biomolecules occur, changing its biological functions, leading to cell death.

Several enzymes which participate in redox processes and are located in different subcellular organelles catalyze the generation of ROS (xantine-oxidase, monoamine oxidase, cytochrome P450 oxidative system) (Drögue 2002). In addition, transition metals such as iron and copper, in its redox-active forms can generate oxygen free radical through Haber-Weiss and/or Fenton reactions (Halliwell and Gutteridge, 1999). All organisms have developed different non-enzymatic and enzymatic antioxidant mechanisms to control ROS actions, accounting for the cellular antioxidant capacity; i.e. GSH and vitamin E are direct scavengers of oxygen free radicals and other ROS and, superoxide-dismutase, catalase and GSH-peroxidase catalyze the reduction of ROS (Drögue, 2002).

Phenolic compounds are the main herbal derivates involved in the antioxidant properties of herbal food and medicinal plants. The expression "phenolic compounds" involves a considerable range of substances whose structures vary from simple molecules (phenolic acids) to highly polymerized compounds (condensed tannins) (Urquiaga and Leighton, 2000). Numerous studies exist about the properties of natural polyphenols antioxidants, which are exercised through several mechanisms; i.e. they scavenge free oxygen radicals, induce superoxide-dismutase, catalase and GSH-peroxidase, and they are metal chelating agents (Halliwell and Gutteridge, 1999; Du et al., 2007). Moreover, *in vitro*, flavonoids inhibit xanthine-oxidase, cyclooxygenase and mitochondrial NADH-oxidase, enzymes that participate in the production of $O_2^{\cdot-}$. Besides, flavonoids induce the GSH-transferase (GST) that catalyzes the conjugation of lipophilic-highly electrophilic compounds with GSH. Although polyphenols act by different antioxidant mechanisms, they are not necessarily complementary, exerting synergistic antioxidant effects (Middleton et al., 2000).

Furthermore, data exist about different effects of herbal products on xenobiotics biotransformation enzymes. Thus, flavones induce epoxyhydrolase, GST, UDPGT and cytochrome P450-dependent mono-oxygenase system. Moreover, flavonoids induce the specific cytochrome P450 monooxygenase iso-enzymes; these enzymes bind the substrate in the first catalytic step of the cytochrome P450 system, which catalyses the biotransformation of lipophilic xeno-biotics (Healy et al., 2002; Siess et al., 1992).

Herbal preparations differ in their biological antioxidant capacity. These differences are due to several factors such as species variety, light, and the extent of ripeness, processing and storage of vegetal drug as well as of the extraction process used. Moreover, the herbal compounds biotransformation depends on their physicochemical properties, especially of their lipophilicity. Thus, in this work, we tested several blind natural commercial preparations with respect to their biological antioxidant properties and their capacity to inhibit UDPGT, GST and cytochrome P450 system catalytic activities, all related to xenobiotics biotransformation pathways. For these purposes, we used rat liver microsomes as biological system and Fe^{3+}/ascorbate as generator of oxygen free radicals. Our results show that a tight relation between the lipophilicity of herbal compounds and its antioxidant properties on microsomal components (lipids and proteins) and the effects on bio-transformation pathways exist. Moreover, polyphenol concentrations of hydro-alcoholic herbal preparations but not the aqueous preparation correlated in an exponential manner with their capacity to inhibit the lipid peroxidation and oxygen consumption. Therefore, microsomes could be a good biological system to evaluate the antioxidant capacity and the possible interactions on the xenobiotics bio-transformation of therapeutic and nutritional herbal products.

MATERIALS AND METHODS

Chemicals

5,5-dithiobis(2-nitrobenzoic acid) (DTNB), GSH, *p*-Nitrophenol (PNP), UDP-glucuronic acid (ammonium salt) (UDPGA), aminopyrine, NADP, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase, Tris-HCl, bovine albumin Fraction IV were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloracetic acid (TCA), thiobarbituric acid (TBA), sodium ascorbate, $FeCl_3$, $MgCl_2$, Folin-

Ciocalteu's reagent were purchased from Merck Co. Chile. 1-chloro-2,4-dinitrobenzene was purchased from ACROS Organics (New Jersey, NJ, USA). Other chemicals were analytical grade. Hydro-alcoholic preparations I-02, I-08, R-01, R-03 and aqueous preparation S-01 were the commercial herbal preparations tested (Table 1), which were donated by Laboratorios Ximena Polanco, Santiago, Chile.

Table 1. Commercial herbal preparations tested in this study.

Blind name*	Commercial name	Herbal Ingredients
I - 02	Cardus Marianus T.M.®	*Silybum marianum* (L.)
I - 08	Melinerv®	*Tilia cordata* Mill.; *Crataegus oxyacantha* (L.); *Avena sativa* (L.); *Melissa officinalis* (L.).
R - 01	Passikit®	*Crataegus oxyacantha* (L.); *Melissa officinalis* (L.); *Valeriana officinalis(* L).; *Passiflora incarnata* (L.)
R - 03	Trique compuesto®	*Foeniculum vulgare* M.; *Cassia senna* (L.); *Peumus boldus* Mol..
S - 01	Naturpep-C®	*Opuntia ficus-indica* (L.).

*The term "blind name" was assigned by the manufacturer to the commercial preparations. The commercial names and vegetal ingredients were provided at the end of this study.

Animals

We used adult male Sprague-Dawley rats (200-250 g) maintained with free access to pellet food, controlled temperature (22 ºC) and photoperiod (lights on from 07:00 to 19:00 h). All procedures were developed according to protocols approved by the Institutional Ethical Committee of the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile.

Liver microsomal fraction

To obtain microsomal fraction we used the method described in Letelier et al. (2005). Animals were fasted for 15 h with water *ad libitum* and sacrificed by decapitation. Livers were perfused in situ with 4 volumes of 25 mL 0.9% w/v NaCl, excised, and placed on ice. All homogenization and fractionation procedures were performed at 4 ºC and all centrifugations were performed using either a Suprafuge 22 Heraeus centrifuge or an XL-90 Beckmann ultracentrifuge. Liver tissue (9–11 g wet weight), devoid of connective and vascular tissue, was homogenized with five volumes of 0.154 M KCl, with eight strokes in a Dounce Wheaton B homogenizer. Homogenates were centrifuged at 9,000*g* for 15 min; sediments were discarded and supernatants were centrifuged at 105,000*g* for 60 min. Sediments (microsomes, enriched in endoplasmic reticulum) were stored at -80ºC until use. Microsomal protein was determined according to Lowry et al. (1951).

Polyphenols Determination

The principal antioxidant property of polyphenols is based in their capacity to scavenge free radicals, phenomenon which will be evaluated in this work. Thus, the total polyphenols concentration of several commercial herbal preparations was determined by the method described in Letelier et al. (2008). In a final volume of 5 mL, herbal extract 50 µL, Folin Ciocalteau reagent 250 µL, 20% w/v sodium carbonate 750 µL and distilled water 3,950 µL. Blanks contained all the reagents with the exception of herbal extract. Then, reaction mixtures were incubated for 2 h under darkness. At the end of this period, the absorbance of the samples was determined at 760 nm in a UV3 Unicam UV–VIS spectrophotometer, using their respective blanks as reference. Catechin, a polyphenol compound was used as reference standard. Results were expressed as nmol of catechin/µL of herbal preparation.

Oxidative stress conditions

The oxidative stress conditions used to evaluate the antioxidant properties of the commercial preparations assayed were 600 µM $FeCl_3$, 1 mM sodium ascorbate and 1 mg of microsomal protein. Iron and copper generate oxygen free radicals through Haber-Weiss and/or Fenton reactions (Halliwell and Gutteridge, 1999).

Microsomal lipid peroxidation assay

The extent of lipid peroxidation following Fe^{3+}/ascorbate microsomal pre-treatment was estimated determining the thiobarbituric acid reactive species (TBARS), according to Letelier et al. (2005). Mixtures (1 mL final volume) contained 1 mg/mL microsomal protein, 600 µM $FeCl_3$, 1 mM sodium ascorbate, 4 mM $MgCl_2$, in 50 mM phosphate buffer, pH 7.4. Blanks contained all the reagents but microsomal protein. Blanks and samples were incubated for 10 min at 37 ºC with constant agitation.

Afterwards, 250 µL of 0.24 M TCA (4 °C) were added and all mixtures were centrifuged at 10,000*g* during 10 min and 4 °C using a Suprafuge 22 Heraeus. Then, mixtures of 500 µL of the supernatants and 500 µL of 35 mM TBA were incubated at 50 °C for 1 h. At the end of this period, the absorbance at 532 nm of samples was measured in a UV3 Unicam UV–vis spectrophotometer, using their respective blanks as reference. To evaluate the antioxidant effects of herbal preparations, microsomes (1 mg of microsomal protein/mL) were incubated 5 min with herbal compounds and then 10 min with Fe^{3+}/ascorbate before determining microsomal lipid peroxidation. Results are expressed in nmols of TBARS conjugated/min/mg of microsomal protein using the extinction coefficient 156 mM/cm of malon-dialdehyde.

Oxygen consumption

The extent of oxygen consumption generated by 50 µM $CuSO_4$ and 1 mM sodium ascorbate was continuously polarographically determined during 10 min with a Clark electrode Nº5331 (Yellows Springs instrument) in a Gilson 5/6 oxygraph. I-02, I-08, R-01, R-03 concentrations used were 1/5 of microsomal lipid peroxidation values. Because the IC_{50} lipid peroxidation of S-01 was not reached, a range between 1 and 20 µL/mL were assayed to determine oxygen consumption IC_{50} value (9 µL/mL). The herbal preparations effects were determined calculating the decrease of Cu^{2+}/ascorbate oxygen consumption slope after adding the herbal preparations.

Microsomal thiol content

Thiol groups were titrated with DTNB as described by Letelier et al. (2005). Microsomes (1 mg/mL total protein) were incubated with 600 µM $FeCl_3$, 1 mM ascorbate, in 50 mM phosphate buffer, pH 7.4. Blanks contained all the reagents but microsomal protein. Blanks and samples were incubated for 30 min at 37◦C with constant agitation. Afterwards, microsomal thiol content was titrated with DTNB. I-02, I-08, R-01, R-03 concentrations used were 1/5 of oxygen consumption IC_{50} values. To evaluate the antioxidant effects of herbal preparations, microsomes (1 mg of microsomal protein/mL) were incubated 5 min with herbal compounds and then 10 min with Fe^{3+}/ascorbate before determining microsomal thiol content. Thiol concentration was estimated by the equimolar apparition of 5-thio-2-nitrobenzoic acid (ε_{410} = 13,600 M/cm).

***p*-Nitrophenol conjugation with UDPGA**

This reaction catalyzed by UDP-glucuronyl-transferase (UDPGT) was measured according to Letelier et al. (2005). I-02, I-08, R-01, R-03 concentrations used were 1/5 of oxygen consumption IC_{50} values. Activity was assayed determining the remaining *p*-nitrophenol after 15 min incubation at the following conditions: 0.5 mM *p*-nitrophenol; 2 mM UDPGA, 100 mM Tris HCl, pH 8.5, 4 mM $MgCl_2$ and 2 mg/ml microsomal protein. Control samples were performed in absence of UDPGA. Reactions were stopped adding trichloroacetic acid (5% final concentration); samples were then centrifuged at 10,000*g* for 10 min. in a Suprafuge 22 Heraeus centrifuge and NaOH was added to the mixture in order to achieve a 0.5 M final concentration. Remaining *p*-nitrophenol was determined at 410 nm using control samples of known *p*-nitrophenol initial concentration as standards. Reaction rates were determined at conditions where product formation were linearly-dependent to time and protein concentration. To evaluate the antioxidant effects of herbal preparations, microsomes (1 mg of microsomal protein/mL) were incubated 5 min with herbal compounds and then 10 min with Fe^{3+}/ascorbate before determining UDPGT activity.

1-Chloro-2,4-dinitrobenzene conjugation with GSH

GSH-transferase (GST) activity to conjugate 1-chloro-2,4-dinitrobenzene was assayed according to Letelier et al., 2006. The reaction mixture contained 0.1 mg/mL microsomal protein, 1mM 1-chloro-2,4-dinitrobenzene, and 4 mM GSH in 100 mM phosphate buffer, pH 6.5. To evaluate the antioxidant effects of herbal preparations, microsomes (1 mg of microsomal protein/mL) were incubated 5 min with herbal compounds and then 10 min with Fe^{3+}/ascorbate before determining GST activity. Conjugated product apparition was recorded continuously for 3 min at 25 °C, at 340 nm (ε_{340} = 9.6×10^{-3} M/cm) in a UV3 Unicam UV–vis spectrophotometer. I-02, I-08, R-01, R-03 concentrations used were 1/5 of oxygen consumption IC_{50} values.

Aminopyrine *N*-demethylation

The cytochrome P450 system ***N***-demethylating activity was determined according to Letelier et al. (1985), measuring the absorbance of the formaldehyde formed in this enzymatic reaction. The reaction mixture contained 1mg of microsomal protein; 5 mM aminopyrine; 35 mM TRIS pH 8.0; 3.5 mM $MgCl_2$;

0.1 M G-6-P; 10 mM NADP; 5 Units of G-6-P dehydrogenase. Blank contained all reagents except G-6-P dehydrogenase. All mixtures were incubated for 15 min at 37 ºC. Reactions were stopped adding trichloroacetic acid (5% final concentration); samples were then centrifuged at 10,000*g* for 10 min. in a Suprafuge 22 Heraeus centrifuge. To develop the colorimetric reaction, 1 mL of supernatant, 0.5 mL of a mixture formed by 0.1 mL of 2,4-pentanodione and 25 mL of 4 M ammonium acetate were incubated for 2 h under darkness. At the end of this period, the absorbance of the samples was determined at 400 nm in a UV3 Unicam UV–VIS spectro-photometer, using their respective blanks as reference. I-02, I-08, R-01, R-03 concentrations used were 1/5 of oxygen consumption IC_{50} values.

Monooxygenase cytochrome P450 spectrum

Monooxygenase cytochrome P450 spectrum was determined according to Omura and Sato (1964). This method uses the ability of the carbon monoxide to coordinate with the monooxygenase; the conjugate formed has a peak of maximum absorbance at 450 nm and its ε is 91 mM/cm. The reaction mixture (blank and sample) contained 1 mg/mL microsomal protein, 5 mM sodium dithionite and 50 mM phosphate buffer, pH 7.4. The spectrum was developed after adding carbon monoxide to the sample. I-02, I-08, R-01, R-03 concentrations used were 1/5 of oxygen consumption IC_{50} values. Interaction of the commercial herbal preparations with Cyt-P450 oxidase was estimated by the decrease in the control absorption at 450 nm of Cyt-P450 monooxygenase measured in their absence.

Statistical analysis

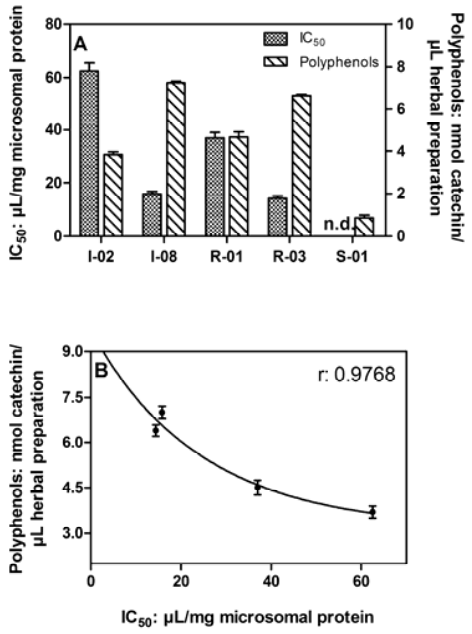
Data presented correspond to the arithmetical mean of at least four independent experiments ± SEM values. GraphPad Prism 5.0 software was used to develop statistical significance (ANOVA) and regression analyses. Differences were considered significant when $p<0.05$.

RESULTS

Antioxidant effects of herbal preparations on microsomal lipid peroxidation and the correlation with its polyphenol concentration

I-02, I-08, R-01 and R-03, hydro-alcoholic preparations, inhibited to different extent the microsomal lipid peroxidation, phenomenon induced by Fe^{3+}/ascorbate. Thus, R-03 and I-08 containing the higher polyphenol concentration had the lowest lipid peroxidation IC_{50} values, 14.4 and 15.8 µL/mg of microsomal protein, respectively. In contrast, R-01 and I-02, which contained the lowest polyphenol concentration, had the highest IC_{50} values, 37.0 and 62.5 µL/mg of microsomal protein, respectively. The polyphenol concentration of aqueous S-01 preparation was very low (0.84 nmol of catechin/µL of herbal preparation). Although quantities higher than 200 µL of this preparation were tested, its IC_{50} value was not obtained (Fig. 1A). Moreover, polyphenol content and lipid peroxidation IC_{50} values of the hydro-alcoholic preparations showed an exponential decay correlation, r = 0.9768 (Fig. 1B).

Figure 1. Antioxidant Effects of herbal preparations on microsomal lipid peroxidation and the correlation with its polyphenol concentration.



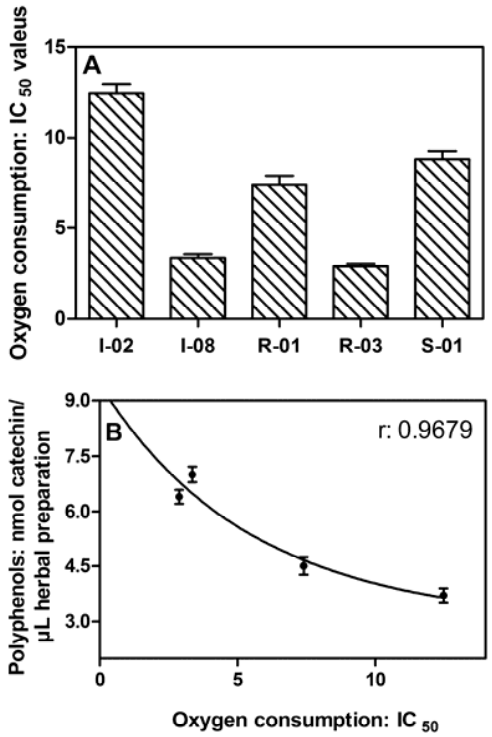
A: Microsomal lipid peroxidation and polyphenol concentrations were determined according to Methods. **B:** Correlation analysis between the polyphenol content and lipid peroxidation IC_{50} values of the hydro-alcoholic preparations. n.d.: IC_{50} value no determined. Each value represents the means of at least four independent experiments ± S.D.

Oxygen consumption developed by Cu^{2+}/ascorbate and effects of herbal preparations

In these assays, Cu^{2+}/ascorbate was used because the slope of oxygen consumption developed by this oxygen free radicals generator system was much greater than that of Fe^{3+}/ascorbate. The hydro-alcoholic preparations caused a great effect on oxygen

consumption; although 1/5 of lipid peroxidation IC_{50} values had to be used to compare their inhibitory effect, all these preparations inhibited 50% of the slope of oxygen consumption. Furthermore, the aqueous preparation S-01 showed an IC_{50} value equivalent to 9 µL (Fig. 2A). Moreover, only polyphenol concentrations of the hydro-alcoholic preparations correlated to oxygen consumption IC_{50} values as an exponential decay manner, r = 0.9679 (Fig. 2B).

Figure 2. Oxygen consumption generated by Cu^{2+}/ascorbate and the effects of herbal preparations.

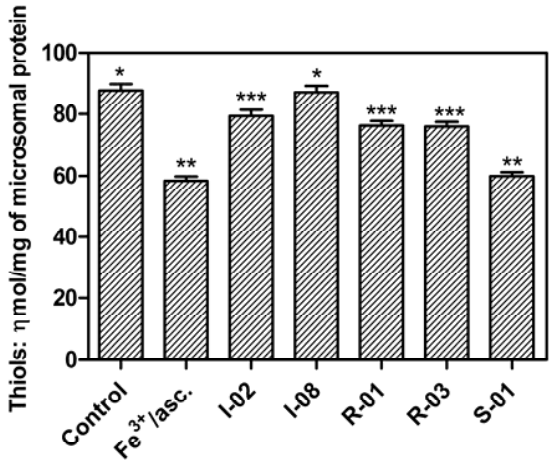


A: Herbal preparations effects on the reduction of the slope of Cu^{2+}/ascorbate oxygen consumption were determined according to Methods. **B:** Correlation analysis between polyphenol content of the hydro-alcoholic preparations and oxygen consumption reduction values. Each value represents the means of at least four independent experiments ± S.D.

Antioxidant effects of herbal preparations on microsomal thiol content

Fe^{3+}/ascorbate decreased microsomal thiol content 33% and the hydro-alcoholic herbal preparations (quantities equivalent to oxygen consumption IC_{50} values) prevented Fe^{3+}/ascorbate-derived microsomal thiol oxidation to different extents. Thus, I-08 totally prevented this phenomenon, I-02, R-01 and R-03 decreased it in the same extent, 11.8% ($p>0.05$) and S-01 had no effect (Fig. 3).

Figure 3. Fe^{3+}/ascorbate-derived oxidative effect on microsomal thiol content and the herbal preparations antioxidant capacity.



Control: microsomes incubated without Fe^{3+}/ascorbate. **Fe^{3+}/Asc.:** microsomes preincubated for 10 min with Fe^{3+}/ascorbate according to Methods. **I-02, I-08, R-01, R-03, S-01:** microsomes preincubated for 5 min with respective herbal preparations and then 10 min with Fe^{3+}/ascorbate before determining microsomal thiol content. Each value represents the means of at least four independent experiments ± S.D. *****: Values not statistically different ($p>0.05$).

Herbal preparations effects on xenobiotics biotransformation activities. UDPGT activity

The hydro-alcoholic preparations (quantities equivalent to oxygen consumption IC_{50} values) inhibited significantly and to different extents the *p*-nitrophenol conjugation with UDPGA, reaction catalyzed by UDPGT; but the aqueous preparation S-01 poorly inhibited this enzymatic activity (12.8%) (Table 2). Fe^{3+}/ascorbate increased the UDPGT activity ~3 fold and all the hydro-alcoholic herbal preparations prevented this phenomenon. In order to estimate the herbal preparations antioxidant effects on Fe^{3+}/ascorbate-derived UDPGT oxidative activation, the ratios between the UDPGT activity measured in the presence of herbal preparation and Fe^{3+}/ascorbate (V_2) and in the presence of herbal preparation alone (V_1), were calculated. All ratios were ~2.00, value significantly lower than that of control ratio 2.91 ($p<0.05$), calculated between UDPGT activity measured in the presence of Fe^{3+}/ascorbate (V_2) and in the absence of this oxidative system (V_1). The exception was the aqueous preparation S-01, whose

ratio was 2.89, value not significantly different to the control ratio ($p>0.05$).

Table 2. Effect of herbal extracts on UDPGT activity in the absence and presence of Fe^{+3}/ascorbate.

	UDPGT activity (nmol of conjugate/min/mg of microsomal protein)		
Conditions	**V_1**	**V_2**	**Activation**
Control	0.94 ± 0.08	2.74 ± 0.13	2.91*
I-08	0.55 ± 0.04	0.94 ± 0.05	1.71
R-01	0.51 ± 0.03	0.96 ± 0.05	1.88
I-02	0.76 ± 0.04	1.45 ± 0.09	1.91
R-03	0.56 ± 0.03	1.13 ± 0.07	2.02
S-01	0.82 ± 0.06	2.37 ± 0.11	2.89*

Control: UDPGT activity measured in the absence of herbal preparations. **V_1**: microsomes preincubated for 5 min with herbal preparations before determining UDPGT activity according to Methods**.** **V_2**: microsomes preincubated for 5 min with herbal preparation and then 10 min with Fe^{+3}/ascorbate before determining UDPGT activity. **Activation:** corresponds to ratios V_2/V_1. Each value represents the means of at least four independent experiments ± S.D. *: Values not statistically different ($p>0.05$).

GSH-transferase activity

The hydro-alcoholic preparations (quantities equivalent to oxygen consumption IC_{50} values) inhibited to different extent the conjugation of 1-chloro-2,4-dinitrobenzene with GSH, reaction catalyzed by microsomal GST, but aqueous preparation S-01 had no effect (Table 3). Fe^{3+}/ascorbate inhibited this enzymatic activity 59.0%. The pre-treatment of microsomes with the hydro-alcoholic preparations and then with Fe^{3+}/ascorbate, significantly increased the GST inhibition in a similar percentage, ~31% (Table 3). However, if the GST activity, measured in the presence of each herbal preparation alone, is considered as the GST activity 100%, the respective inhibition percentages were similar (~39%) and significantly lower than that observed by Fe^{3+}/ascorbate alone, 59% ($p<0.05$). However, S-01 aqueous preparation did not modify the Fe^{3+}/ascorbate-derived GST inhibition (Table 3).

Table 3. Effect of herbal extracts on GSH-transferase activity in the absence and presence of Fe+3/ascorbate.

	GSH-transferase Activity (GST) (nmol of conjugate/min/mg of microsomal protein)		
Conditions	**V_1**	**V_2**	**Inhibition (%)**
Control	132.3 ± 1.7*	54.2 ± 1.05	59.0*
R-03	87.4 ± 0.84	56.2 ± 1.48	35.7
I-02	75.4 ± 1.20	46.5 ± 1.21	38.3
I-08	89.9 ± 0.83	53.1 ± 1.31	40.9
R-01	63.5 ± 1.56	36.5 ± 1.52	42.5
S-01	124.3 ± 5.6*	50.0 ± 1.50	59.8*

Control: GST activity measured in the absence of herbal preparations. **V_1**: microsomes preincubated for 5 min with herbal preparations before determining GSHT activity according to Methods**.** **V_2**: microsomes preincubated for 5 min with herbal preparation and then 10 min with Fe^{+3}/ascorbate before determining GST activity. **Inhibition (%):** percentages calculated considering as 100% the GST activity to V_1 values. Each value represents the means of at least four independent experiments ± S.D. *: Values not statistically different ($p>0.05$).

Cytochrome P450 system

The hydro-alcoholic preparations (quantities equivalent to oxygen consumption IC_{50} values) also inhibited this enzymatic reaction, but S-01 had not effect. I-02 showed the highest inhibition (73.5%) and I-08, the lowest (11.5%). Fe^{3+}/ascorbate inhibited the aminopyrine *N*-demethylating activity of cytochrome P450 system 52.2% and the pre-treatment of microsomes with the hydro-alcoholic preparations and then with Fe^{3+}/ascorbate, significantly increased this inhibition (Table 4). However, if the cytochrome P450 activity, measured in the presence of each herbal preparations alone, is considered as the aminopyrine *N*-demethylating activity 100%, the inhibition percentages were significantly lower than that obtained by Fe^{3+}/ascorbate alone, 52% ($p<0.05$). Moreover, I-02 prevented fully this oxidative phenomenon, I-08, R-01 and R-03 reduced it to ~20% and aqueous preparation S-01 had not effect (Table 4).

Table 4. Effect of herbal extracts on the cytochrome P450 activity in the absence and presence of Fe^{+3}/ascorbate.

	Cytochrome P450 activity (nmol of HCHO/min/mg of microsomal protein)		
Conditions	**V_1**	**V_2**	**Inhibition (%)**
Control	1.13 ± 0.05	0.54 ± 0.03	52.2*
I-02	0.30 ± 0.02**	0.28 ± 0.01**	0
I-08	1.00 ± 0.05	0.80 ± 0.04	20.0
R-01	0.69 ± 0.04	0.54 ± 0.03	21.7
R-03	0.92 ± 0.03	0.71 ± 0.02	22.8
S-01	1.17 ± 0.06	0.55 ± 0.04	52.9*

Control: Aminopyrine *N*-demethylating activity measured in the absence of herbal preparations. **V_1**: microsomes preincubated for 5 min with herbal preparations before determining cytochrome P450 system activity according to Methods**. V_2**: microsomes preincubated for 5 min with herbal preparation and then 10 min with Fe^{+3}/ascorbate before determining cytochrome P450 system activity. **Inhibition (%):** percentages calculated considering as 100% the cytochrome 450 activities V_1 values. Each value represents the means of at least four independent experiments ± S.D. *: Values not statistically different ($p>0.05$).

Effect of herbal preparations on the absorbance spectrum of cytochrome P450 monooxygenase

Cytochrome P450 system catalyzes the biotransformation of lipophilic xenobiotics; the cytochrome P450 monooxygenase in its Fe^{3+}-hemin form binds the substrates in the first step of the catalytical cycle of this system, changing its absorbance spectrum. To evaluate the lipophilicity of herbal compounds present in the commercial preparations and its interaction with this enzyme, its maximum absorbance to 450 nm was determined in two conditions. Thus, microsomes were treated with sodium dithionite (reducing agent for Fe^{2+}-hemin form) after adding herbal preparations (Condition A) and before adding it (Condition B), prior to determining the monooxygenase absorbance to 450 nm according to Methods. The differences between the decreasing percentages obtained in condition A and B allowed estimating the binding to monooxygenase of lipophilic herbal compounds present in the hydro-alcoholic preparations. In both conditions the maximum absorbance of this enzyme decreased, but in condition A this decrease was significantly higher ($p<0.05$); the only exception was observed with the aqueous preparation S-01 whose monooxygenase absorbance values measured in both condition were not significantly different, $p>0.05$ (Table 5). In the condition A, I-02, I-08, R-01, R-03 and S-01 decreased the monooxygenase absorbance 26.2, 25.6, 21.7, 18.4 and 7.7%, respectively; but in the condition B the percentages were 10.4%, 12.8%, 15.0%, 10.0% and 7.1%, respectively. The difference values for I-02, I-03, R-03 and R-01 were 15.8, 12.8, 8.4 and 6.7, respectively (Table 5).

DISCUSSION

Food and medicinal herbal compounds possessing relevant antioxidant properties are xenobiotics for the human being. The hepatic endoplasmic reticulum is the main cellular organelle responsible of the xenobiotics biotransformation and one of the sites in which reactive oxygen species (ROS) are generated. Thus, in this cellular organelle the biotransformation of herbal compounds and the expression of their antioxidant properties may occur. As expected, the commercial herbal preparations assayed inhibited significantly the microsomal lipid peroxidation, phenomenon induced by Fe^{3+}/ascorbate as showed by their IC_{50} values. The only exception was the aqueous herbal preparation S-01; its IC_{50} value could not be obtained although up to 200 µL were assayed (Fig. 1A). Why S-01 microsomal anti-lipoperoxidative effect (IC_{50} value) could not be determined? This may be due to its very low polyphenol concentration (0.84 nmol of catechin/µL of preparation) and the presence of polar antioxidant compounds in this aqueous herbal preparation. Interestingly, polyphenol concentrations and IC_{50} values of hydro-alcoholic preparations correlated as an exponential decay manner (Fig. 1B); the presence of lipophilic compounds in these preparations and the ability of polyphenols to scavenge oxygen free radicals, one of the mechanisms through which they express its antioxidant properties could explain these antioxidant effects.

In addition, all herbal preparations including the aqueous preparation S-01 decreased the oxygen consumption caused by Cu^{2+}/ascorbate (Fig. 2). Interestingly, the effect of hydro-alcoholic preparations was higher than that obtained in the lipid peroxidation assays; only 1/5 IC_{50} value obtained from lipid peroxidation assays were needed to decrease 50% of the oxygen consumption induced by Cu^{2+}/ascorbate. This oxygen consumption depends on free copper ions concentration (Letelier et al., 2008). Polyphenols are chelating agents and hydro-alcoholic preparations showed a high concentration of these compounds.

Table 5. Effects of herbal extracts on the cytochrome P450 monooxygenase spectrum.

Blind name	Condition A		Condition B		Difference A-B
	nmol of monooxygenase/mg of microsomal protein	Decreasing %	nmol of monooxygenase/mg of microsomal protein	Decreasing %	
I-02	0.486 ± 0.023	26.2	0.590 ± 0.029	10.4	15.8
I-08	0.490 ± 0.026	25.6	0.575 ± 0.026	12.8	12.8
R-03	0.538 ± 0.029	18.4	0.593 ± 0.032	10.0	8.4
R-01	0.516 ± 0.024	21.7	0.560 ± 0.029	15.0	6.7
S-01	0.608 ± 0.052*	7.7	0.612 ± 0.034*	7.1	0.6
Control: 0.659 ± 0.032 nmol of monooxygenase/mg of microsomal protein					

Control: nmol of cytochrome P450 monooygenase measured in the absence of herbal preparations. **Condition A**: microsomes preincubated for 5 min with herbal preparations before adding sodium dithionite. **Condition B**: microsomes plus sodium dithionite and then incubated for 5 min with the herbal preparations before measuring the mono-oygenase according to Methods. **Difference A-B**: difference between the decreasing percentages calculated in the conditions A and B. Each value represents the means of at least four independent experiments ± S.D. *: Values not statistically different ($p>0.05$).

Moreover, oxygen consumption IC_{50} values of hydro-alcoholic preparations correlated in an exponential decay manner to polyphenol concentrations indicating that the metal chelating ability of these compounds could be involved in this phenomenon. Likewise, S-01 is a preparation enriched in mucilages (information given by the industry), which are also chelating agents (Wang et al., 2007). Therefore, mucilages chelating ability and in a minor grade, the S-01 polyphenol concentration could explain the decreasing of oxygen consumption caused by this aqueous herbal preparation (Fig. 2).

In contrast to the antioxidant effects on lipid peroxidation and oxygen consumption, the prevention of microsomal thiol oxidation did not correlate to the polyphenol content of herbal preparations. Thus, I-08 completely prevented the microsomal thiol oxidation induced by Fe^{3+}/ascorbate, I-02, R-01 and R-03 partially reduced it to the same extent and S-01 had no effect (Fig. 3). The cysteinyl residues of globular proteins are not equally reactive and the accessibility of herbal ingredients to proteins inserted in the microsomal membrane depends on its lipophilicity. Therefore, both factors could explain the differences in the antioxidant effects on microsomal thiol groups Fe^{3+}/asorbate-derived oxidation.

In addition, the hydro-alcoholic preparations alone inhibited all the enzymatic activities assayed, probably because substrates of these enzymes are present in these preparations (Tables 2, 3 and 4). UDPGT metabolizes alcoholic and phenolic compounds and all herbal preparations inhibited the catalytic activity of this enzyme measured through *p*-nitrophenol conjugation with UDPGA. However, polyphenol concentration of herbal preparations did not correlated to the extent of UDPGT inhibition. In the same way, the hydro-alcoholic preparations inhibited to different extent the GST and cytochrome P450 system activities, but aqueous preparation S-01 had no effect. GST and cytochrome P450 system metabolize lipophilic/electrophilic and lipophilic compounds, respectively, which could explain the absence of inhibitory effect of the aqueous preparation S-01.

In addition, hydro-alcoholic preparations prevented the Fe^{3+}/asorbate-derived oxidative damage on UDPGT, GST and cytochrome P450 system activities, but aqueous preparation S-01 had no effect. UDPGT, GST and cytochrome P450 monooxygenase are thiol proteins and the ROS-derived thiol group's oxidation alters its catalytic activities (Letelier et al*.,* 2005; Alterman et al., 1981; Letelier et al., 2006). Therefore, the antioxidant effects of hydro-alcoholic preparations on Fe^{3+}/ascorbate-derived microsomal thiol oxidation could explain the antioxidant effects observed on UDPGT, GST and cytochrome P450 system activities (Tables 2, 3, and 4). Interestingly, polyphenol concentration of hydro-alcoholic preparation did not correlate to the prevention of Fe^{3+}/ascorbate-derived oxidative damage on the catalytic activities of these biotransformation enzymes. Polyphenol concentrations of hydro-alcoholic preparations neither correlated to its microsomal thiol antioxidant effects (Fig. 3),

confirming that the antioxidant effects on the catalytic activities of these enzymes could relate to the protection of its thiol groups. Besides, the hydro-alcoholic preparation I-02 exerted the highest inhibition of cytochrome P450 system activity, the highest reduction of the monooxygenase absorbance and, totally prevented the Fe^{3+}/ascorbate-derived oxidative damage on the catalytic activity of this system. Instead, the aqueous herbal preparation S-01 had no effect on cytochrome P450 system and GST activities. Besides, it did not alter significantly the absorbance of the monooxygenase and did not prevent the Fe^{3+}/ascorbate-derived oxidative damage on cytochrome P450 system catalytic activity (Table 5). Thus, the lipophilicity of herbal ingredients present in hydro-alcoholic preparations could be another factor involved in the prevention of Fe^{3+}/ascorbate-derived oxidative damage on microsomal enzymes.

Another point to discuss is the biological advantages of total herbal extracts. Very little quantities of different active principles are present in these preparations, which could provoke multiple and synergistic antioxidant effects (Siess et al., 1992). This is evidenced by the ability of hydro-alcoholic preparation to prevent the Fe^{3+}/ascorbate-derived oxidative damage on microsomal membrane components (lipid, thiol groups and xenobiotics bio-transformation enzymes) and the inhibition of oxygen consumption induced by Cu^{2+}/ascorbate. Moreover, all hydro-alcoholic preparations inhibited the microsomal GST activity, which could indicate the presence of lipophilic/electrophilic substrates, physicochemical characteristics of highly reactive metabolites, which can induce oxidative stress or bind covalently to biomolecules. However, all of them showed a high antioxidant activity, so a balance that favours the antioxidant compounds on electrophilic compounds seems to exist in these hydro-alcoholic preparations. We cannot discard other mechanisms that could be involved in the GST activity inhibition; i.e. the binding of some components of herbal extracts to thiol group of microsomal GST, avoiding the formation of its catalytic active dimer may also occur (Aniya et al., 1993)

In general, these results show that in order to evaluate the herbal preparations antioxidant properties, the extraction method used in the manufacture of herbal preparations should be considered. It is necessary to note that ROS are the main biological oxidant species; synthetic nitrogen radicals, different to nitric oxide however, are commonly used as oxidant agents to evaluate antioxidant activity: important physicochemical differences between ROS and those synthetic radicals exist (Letelier et al., 2008). Furthermore, when food and medicinal plants extracts are going to be included in the processed foods or in the formulation of phytomedications, assays that investigate the protection of biomolecules against oxidative damage should be useful. Finally, rat liver microsomes are a good biological target to evaluate not only the biological antioxidant properties of herbal preparations, but also their pharmacokinetic inter-actions with other xenobiotics such as allopathic drugs.

CONCLUSIONS

Biotransformation is one of the main metabolic routes through which herbal active products may generate adverse effects, especially when herbal ingredients are metabolized through cytochrome P450 system. This system catalyzes mainly the bio-transformation of lipophilic compounds, so the extraction method used is very important in the herbal preparations conducing to phytomedications and/or food additives. We hereby put together a battery of tests -all based on the use of liver microsomes- to determine how some commercial herbal preparation can exert antioxidant activities -which are likely part of the mechanisms of their therapeutic activities- without compromising too much the oxidative metabolism of xenobiotics.

Acknowledgement

This study was partially funded by Laboratorios Ximena Polanco.

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