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Plasmatic antioxidant capacity as a possible marker of phytodrugs efficacy

A. Müller-Sepúlveda[1,2*], I. Saavedra-Saavedra[2], M. E. Letelier[1]

***Corresponding author:**

A.Müller-Sepúlveda

[1] Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Sergio Livingstone Pohlhammer (ex–Olivos) 1007, Independencia, Santiago, Chile
[2]Center of Pharmacological and Toxicological Research, Program of Molecular and Clinical Pharmacology, Faculty of Medicine, University of Chile, Carlos Schatebeck 299, ParqueQuinta Normal , Santiago, Chile

Abstract

A correlation between drug plasma concentration and its therapeutic effect exist. Then we postulate that determination of plasmatic antioxidant capacity could prove phytodrugs efficacy. Therefore, we determined the plasmatic ability to reduce Fe^{3+} and malondialdehyde concentration after orally administration of herbal extracts to rats. For this, we use hydro-alcoholic extracts of *Buddleja globosa* Hope and *Plantago major* L. previously characterized according to their polyphenol and thiol compounds contents, and their capacities to inhibit the oxidation of microsomal lipid and thiol compounds.

The amounts of polyphenol and thiol compounds were three times higher in *B. globosa* than in *P. major* extracts. Moreover, EC_{50} of *B. globosa* extract in preventing oxidation of microsomal lipid and thiol compounds induced by Cu^{2+}/ascorbate were 3 times lower. These extracts were also able to inhibit microsomal GSH-transferase activity and chelate Cu^{2+}. The oral administration of these extracts to rats provoked an increase in the ability to reduce Fe^{3+} and a decrease in malondialdehyde concentration. Since the antioxidant activity of these extracts was reproduced *in vivo*, we believe that the efficacy of phytodrugs used in complementary therapies may be evaluated by measuring the plasma antioxidant capacity.

Keywords: Buddleja globosa Hope Plantago major L. Lipid oxidation Thiol oxidation Ferric reducing ability of plasma

Introduction

In animal cells thiol compounds such as cysteine and GSH, represent the most important non-enzymatic antioxidants. Moreover, when oxidative stress occurs, GSH can reach concentrations up to 10 mM in the liver, where most of oxidative reactions occur[1]. In plant cells, however, there are additional antioxidant compounds, mainly represented by polyphenols [2]. These compounds are more abundant in plant leaves [3]; and the antioxidant mechanisms by which thesecompounds exert their redox effect can be additive and/or synergistic [4].

Oxidative stress is associatedwith inflammation [5]. Inflammation is a physiological phenomenon defence triggered by the activation of cytokines,phenomenon in many cases regulated by cellular redox state[6].It is well known that a relation between oxidative stress and diseases exist. So different commercial herbal preparations have been used as antioxidant-associated therapies to treat different pathologies [7–9].Pharmacological studies of herbal preparationshowever, are scarce.Moreover, the interpretation of these studies become difficult because of the variations in herbal preparations—plant source, part of the plant used, mode of extraction of bioactive compounds, etc. Furthermore, all health agencies require safety and efficacy studies to accept herbal preparations as phytodrugs.

Pharmacokinetic parameters are obtained from AUC curves, which relate time intervals after drug administration with plasmatic concentrations of active principles. But it is difficult to determine plasma concentration of potential active principles of phytodrugs because of their very low concentrations and chemical complexity [2,10,11]. Pharmacokinetic parameters also correlate plasmatic concentration of active principle with the drug´s therapeutic effect. Considering this correlation and the association of oxidative stress to different diseases, we postulate thatthe plasmatic antioxidant capacity could be used as a temporary variable instead of the concentration of active principles to determine pharmacokinetic parameters. Moreover, phytodrugs efficacy could be also studied determining the plasmatic antioxidant capacity.

To test this postulate we use hydro-alcoholic extracts of leaf of *Buddleja globosa*Hope (*B. globosa*) and *Plantago major*L. (*P. major*).Leaves of *B. globosa* are used in folk medicine forhealing wounds and gastric ulcers, because of their analgesic and anti-inflammatory actions [12]. Similarly, *P. major* is used in the treatment of hepatic inflammatory diseases [13]. Results from different methods assayed to characterize the *in vivo* and *in vitro* antioxidant capacity of *B. globosa* and *P. major* leaf extracts are discussed in terms of their pharmacological importance.

Material and Methods

Analytical reagents and chemicals

TRIS-HCl, GSH, Ellman´s reagent: 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine albumin (Fraction IV), catechin, dithiothreitol,

N-acetyl cysteine and cysteine were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, thiobarbituric acid, sodiumascorbate, tripiridyltriazine, malondialdehydeand 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 of analytical grade.

Plant material

Hydro-alcoholic (50-50 v/v) extracts of *B. globosa* (serial number 057201) and *P. major* (serial number 067251)leaves obtained from organic cultures were graciously provided by LaboratoriosXimenaPolanco®, along with proprietary information regarding yield. Botanical properties of *B. globosa* and *P. major* leave as well as physicochemical properties such as the presence of flavonoids, saponins and tannins by TLC, were certified by LaboratoriosXimenaPolanco® (data not shown).

Animals

Adult male Sprague-Dawley rats (approximately 300 g), maintained at the vivarium of the Faculty of Chemical and Pharmaceutical Sciences of the University of Chile, Santiago, Chile) were used. Rats were allowed free access to water and pelleted food, maintained with controlled temperature (22 ºC) and photoperiod (light from 07:00 to 19:00 h). All procedures were performed using protocols approved by the Institutional Ethical Committee of the Faculty of Medicine, University of Chile (CBA protocol # 0486 FMUCH), according to the guidelines of the Guide for the Care and Use of Laboratory Animals (NRC, USA).

Rat liver microsomes

Rats were fasted for 15 h with water *ad libitum*, before being killed. Livers were perfused *in situ* with 4 volumes of 25 mL 0.9% w/v NaCl, excised and maintained in 0.154 M KCl at 4 C. Liver tissue (9-11 g wet weight), freed of connective and vascular tissue, was homogenized with 5 volumes of 0.154 M KCl, with 8 strokes in a Dounce Wheaton B homogenizer. Homogenates were centrifuged at 9000 g in a Suprafuge 22 Heraeus centrifuge for 15 min and sediments were discarded. Supernatants were then centrifuged at 105000 g in a XL-90 Beckmann ultracentrifuge for 60 min. Pellets (microsomes) were stored at -80 ºC until use. Protein determinations were performed according to Lowry et al. [14].

Polyphenol determination

Polyphenols were determined as described in Letelier et al. [15]. Briefly, herbal extracts were mixed with FolinCiocalteau reagent (100 µL), 20% w/v Na_2CO_3 (300 µL) and distilled water to a final volume of 2 mL. Blanks omitted herbal extract. Reaction mixtures were incubated for 2 h protected from light. Absorbance was determined at 760 nm in a UV 3 Unicam UV–VIS spectrophotometer. Catechin was used as standard reference and results were expressed as µmol equivalent of catechin/mL of extract ± SD.

Thiol determination

Thiol groups were titrated with Ellman's reagent. Thiol concentration was estimated by equimolar apparition of 5-thio-2-nitrobenzoic acid at 412 nm (ξ =13600/M/cm). Herbal extract or microsomal proteins were mixed with 2 mM DTNB (50 µL) dissolved in 50 mM sodium acetate (reagent prepared upon use), 1 M TRIS buffer, pH 8 (100 µL) and distilled water to a final volume of 1 mL. Blanks omitted DTNB. Reaction mixtures were incubated for 10 min and then absorbance of mixtures were determined at 412 nm in a UV3 Unicam UV–VIS spectrophotometer. GSH was used as the standard reference and results were expressed as µmol equivalent of GSH/mL of extract ± SD.

Oxidative conditions

Microsomes for lipid oxidation (0,2 mg of microsomal protein) and for thiol oxidation (0,1 mg of microsomal protein) were incubated with 25 nM $CuSO_4$ plus 1mM sodium ascorbate for 20 min at 37 C with constant agitation prior to measuring lipid and thiol oxidation [15].

The extent of microsomal lipid oxidation was estimated assaying thiobarbituric acid - reactive species (TBARS), as previously described in Aracena et al.[17]. Similarly, the extent of microsomal thiol oxidation was determined measuring the residual thiol groups with Ellman's reagent as described before in Thiol determination.

Isobolograms

Isobolograms were made according to Tallarida [18]. For this, EC_{50} values obtained for the oxidation of microsomal lipid and thiol compounds in the presence of *B. globosa* and *P. major* extracts were plotted.

GSH-transferase activity

This enzymatic activity was measured following the method described in Letelier et al. [19]. Briefly, the reaction mixture contained 0.1 mg/mL of microsomal protein, 1 mM 1-chloro-2,4-dinitrobenzene, and 4 mM GSH in 100 mM phosphate buffer, pH 6.5. Blanks omitted GSH. Appearance of the conjugated formed was continuously recorded at 340 nm for 3 min at 25 ºC, in a UV 3 Unicam UV-VIS spectrophotometer. This activity was measured in the presence of*B. globosa*, *P. major*, dithiothreitol, N-acetyl cysteineand cysteine and the results were expressed as EC_{50}, values which represent the concentration of these agents that inhibited half this enzymatic activity.

Chelation of Copper (II)

Modification of the copper (II) sulphate (0.5 mM) spectrum in the absence and presence of herbal extracts at 385 nm was determined in a UV-VIS spectrophotometer model UV3 coupled to a computer.

Treatment of animals used in plasmatic antioxidant capacity

Rats were treated via gastric gavage with herbal extracts. Doses administered to rats were calculated considering that recommended for humans by LaboratoriosXimenaPolanco®. To convert human doses to rat doses, 70 Kg for normal human weight and higher metabolic rate of rats than humans were considered. Doses A for *B. globosa and P. major* were 0.546 mg equivalents of catechin/Kg of body weight; and doses B for *B. globosa and P. major* were 5.46and 2.73 mg equivalents of catechin/Kg of body weight, respectively. Dose B for *P. major*was different to that for *B. globosa*(it was only 5 times higher than dose A) because the volume of *P. major* extract calculated exceeded the maximum volume of ours rat stomach [20]. The control group received only water. Rats were anesthetized with ketamine/xylazine previously to obtained blood samples by cardiac puncture at 0, 2, 4, 6, 8, 10 and 12 h post dosing. All samples were received in EDTA and then centrifuged at 2500 g for 15 min at 4 ºC. Plasma obtained was stored at -20 ºC until use.

Ferric Reducing Ability of Plasma (FRAP)

Determination of the ferric reducing ability of plasma was performed according to Rodrigo et al.[21]. This technique measures the ability of plasma to reduce Fe^{3+} to Fe^{2+}. At low pH, Fe^{2+} forms a coloured complex with tripiridyltriazine, which absorb at 593 nm. The results are expressed as µmols of reduced iron/L of plasma.

Malondialdehyde in plasma

Malondialdehyde (MDA) quantification was developed by fluorimetric detection as described by Young & Trimble [22]. Mixing 250 µL of 1.22 M H_3PO_4 with 450 µL HPLC grade water, 50 µL of the sample and 250 µL of 0.44 M thiobarbituric acid form the reaction. This mixture is incubated for 1 h at 100 C and then cooled in ice. Then, 200 µL of the mixture were treated with 360 µL of HPLC grade methanol and with 40 µL of 1M NaOH. MDA was determined using an HPLC and fluorescence detection system at 532 nm (excitation) and 553 nm (emission). The results are expressed as µmols of MDA/L of plasma.

Statistical analysis

Values presented correspond to the mean of at least 4 independent experiments ± SD. Statistical significance and regression analyses were performed using Graph Pad Prism 5 software. Differences were considered as significant when $p<0.05$.

Results and Discussion

In vitro antioxidant capacity of *B. globosa* and *P. major* extracts

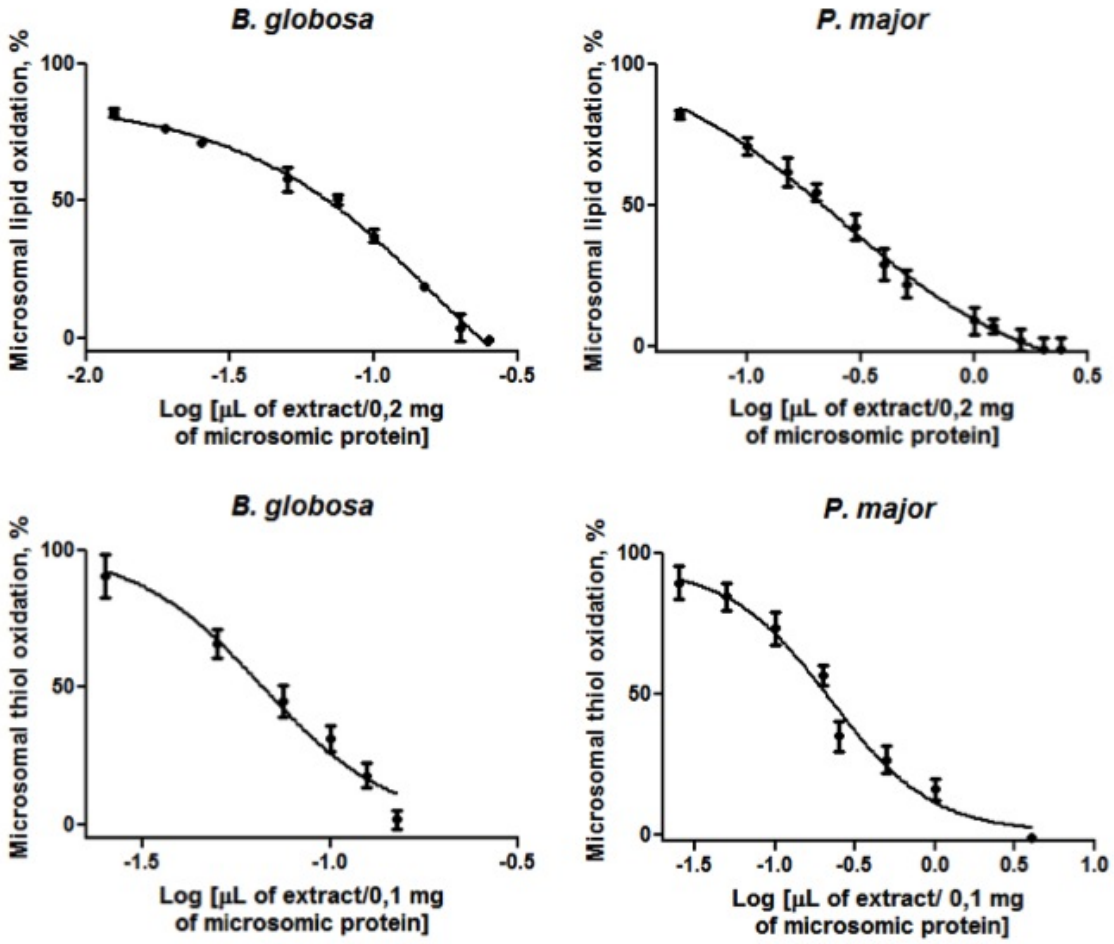
As shown in Table 1, *B. globosa* extract presented approximately three times higher content of polyphenol and thiol compounds than *P. major* extract (21.8 v/s 7.6 µmol equivalents of catechin/mL of extract and 0.71 v/s 0.27 µmol equivalents of GSH/mL of extract). Although polyphenol and thiol compounds act as antioxidant agents, they present differences in some of the mechanism through they exert their antioxidant response. Therefore, different *in vitro* experiments were used to assess the antioxidant profile of these herbal extracts. Both extracts inhibited the oxidation of lipid and thiol compounds (Figure 1), but the EC_{50} values were three times lower for *B. globosa* than *P. major* extract. These results seem to be directly related with the polyphenol and thiol concentration of the extracts. On the other hand, the ratio [polyphenols] / [thiol] for both extract was approximately thirty, suggesting that the antioxidant effects observed could be due mainly to polyphenols.

Table 1. Antioxidant Properties of *Buddleja globosa*Hope and *Plantago major*L extracts

	Polyphenol [µmol of catechin/mL of extract ± SD]	Thiol [µmol of GSH/mL of extract ± SD]	EC_{50}		
			Prevention of lipid oxidation	Prevention of thiol oxidation	Reversion of thiol oxidation
B. globosa	21.8 ± 1.15*	0.71 ± 0.03*	0.39*	0.86*	50*
P. major	7.6 ± 1.48	0.27 ± 0.02	1.25	2.80	140

Polyphenol and thiol values represent the mean of at least 4 independent experiments ± SD. EC_{50} values were obtained of semi-logarithmic graphs and they are expressed as µL of extract/mg microsomal protein. *: Indicates statistically significant differences ($p<0.05$). The assays to determine the antioxidant properties of herbal extracts are described in Methods.

Figure 1.Semi-logarithmic graphs of the effectof *B. globosa*and*P. major* on microsomallipid and thiol oxidation.

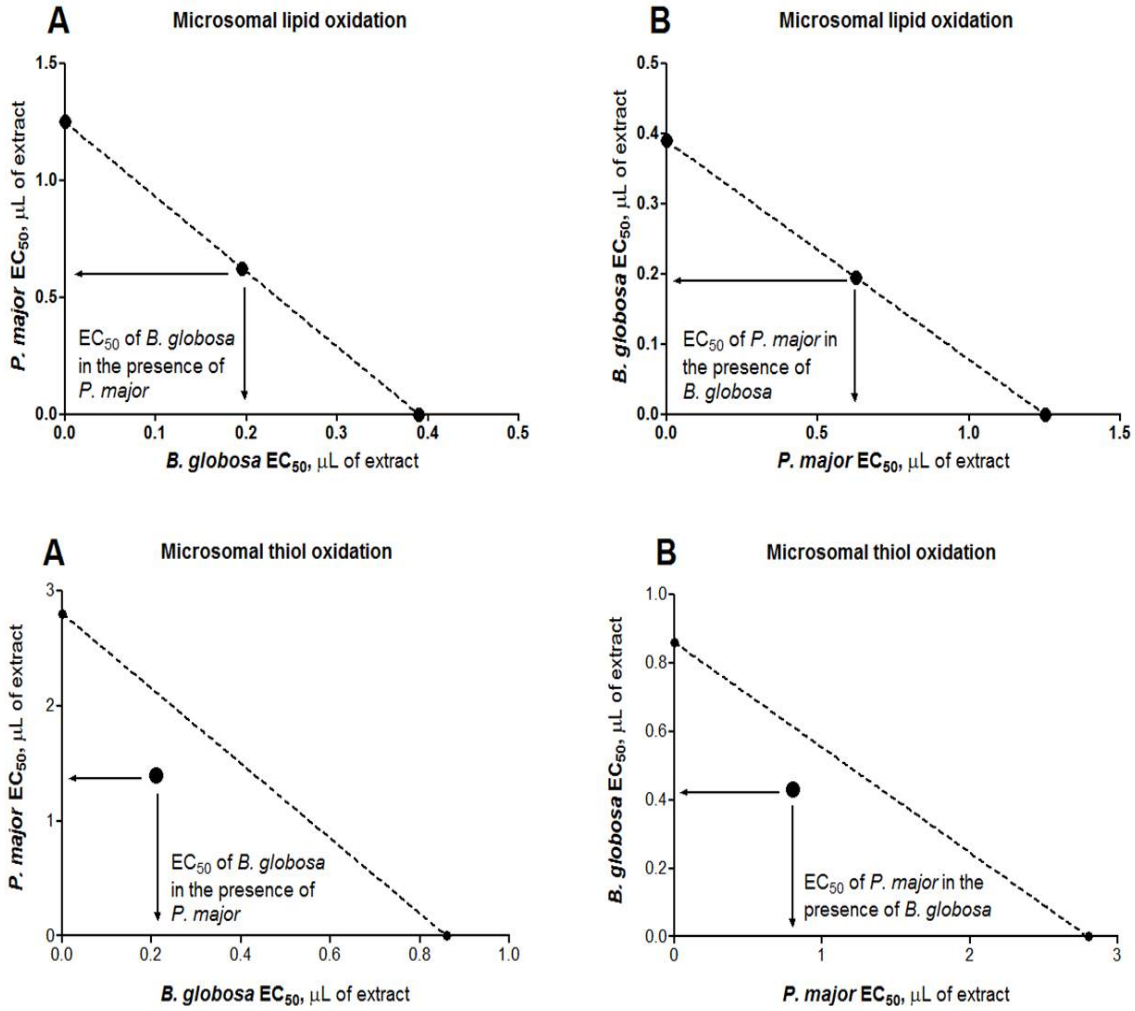


Microsomes were incubated with each of theextractsfor 10 minand then with25 ηMCuSO$_4$and1 mM sodium ascorbatefor20 minat 37 Cwith constant agitation. Determination of lipid and thiol oxidationis describedin Methods.Valuesrepresent the residual %of lipid or thiol oxidation, considering as 100% microsomal lipid or thiol oxidationmeasured in the absence of extracts. Values represent themean of at leastfourindependent experiments± SD.

As mentioned, there are differencesbetweenthe antioxidant properties ofthiol and polyphenol compounds. The oxidation of polyphenol compounds to quinones cannot be reversed; the conjugation of these metabolites with GSH annul theirelectrophilicity and so its toxicity [2,10,23]. Oxidation of thiol compounds, however, can be reversed. This property of thiol compounds allows recovery of the cellular oxidized thiol groups, especially those on proteins. Inall cell types, this reversibilityis the result ofconcertedenzymatic mechanismsthat involve the useof the tripeptideglutathione (GSH), the most abundantnon-enzymaticantioxidantin the animal cell [24,25]. To test this property, we incubated microsomes with herbal extracts after adding Cu^{2+}/ascorbate system. Both herbal extracts reversed the oxidation of microsomal thiol content, being *B. globosa* more effective than *P. major* extract (Table 1). Cellular redox state ofthiolsystems is controlled by thioredoxins, GSH and cysteine[26]. These reactions involve the oxidation of protein thiol as well as the reduction of disulphide bonds formed by oxidation of the thiol groups [25,27]. So, the ability of both extract to reduce disulphide bonds may contribute to maintain cellular redox state ofthiolsystemsin a stable condition.

In order to determine if the antioxidant mechanisms of herbal extracts were similar or different, isobolograms were performed (Figure 2). Microsomal lipid oxidation was inhibited through additive mechanisms by *B. globosa* and *P. major* (Figure 2). Cu^{+2}/ascorbate system, an anion superoxide generator, provokes an oxidative radical chain reaction. Then, free radical trapping could be the mechanism involved; however, more experiments are required to corroborate this postulate.

Figure 2.Isobolograms of the effect of *B. globosa* (A) and *P. major* (B) extracts on microsomal lipid and thiol oxidation.



EC_{50} to prevent microsomal lipid and thiol oxidation in the presence of both extracts were obtained of the semilogarithmic graph. Experimental conditions are described in Methods.

Isobolograms derived from microsomal thiol oxidation are shown in Figure 2. The mixture of both extracts provoked a synergic inhibitory effect on microsomal thiol oxidation (Figure 2). In biological systems, thiol groups are the main cellular antioxidants; they exert their cellular redox activity via different mechanisms, some of which are similar to those exerted by polyphenols (free radical trapping and metal chelating) and others different, such as the capacity to reverse thiol oxidation. Therefore, it is possible that more than one of the mechanisms mentioned could be responsible for the synergic effect observed.

Given the importance of maintain the redox state of cellular thiol circuits, the inhibition of microsomal GSH-transferase (GST) activity was assayed (Table 2). This enzyme is widely distributed in the body; it catalyses the conjugation of electrophilic compound with GSH, avoiding the damage they could cause to biomolecules. Its catalytic active form corresponds to disulphide dimer -S-S- [28].

Both herbal extracts inhibit GSH-transferase activity. When rat liver microsomes were incubated with the same content of thiol compounds of B. globosa and P. major, no differences between them were observed in the inhibition of this enzymatic activity (Table 2). The EC_{50} values for *B. globosa and P. major* extracts were 0.01325 and 0.01361 µmol equivalent of GSH/ 0.1 mg of microsomal protein,respectively. Why the inhibitory effect on GST provoked by both herbal extracts was similar but not that of microsomal –S-S- reversion? Microsomal –S-S- compounds include a variety of molecules, but active GST is a microsomal specific dimer –S-S- which was identified by its enzymatic activity. Redox potentials of microsomal –S-S- compounds are different, but that of active GST is only one. Therefore, the measuring of microsomal –S-S- compounds represents the average effect of all redox agents present in the herbal extracts and in the microsomal preparation, which could explain the difference observed.

Table 2. Microsomal GSH-transferase activity inhibited by *Buddleja globosa* Hope, *Plantago Major* L, Dithiothreitol, N-acetyl cysteine and cysteine

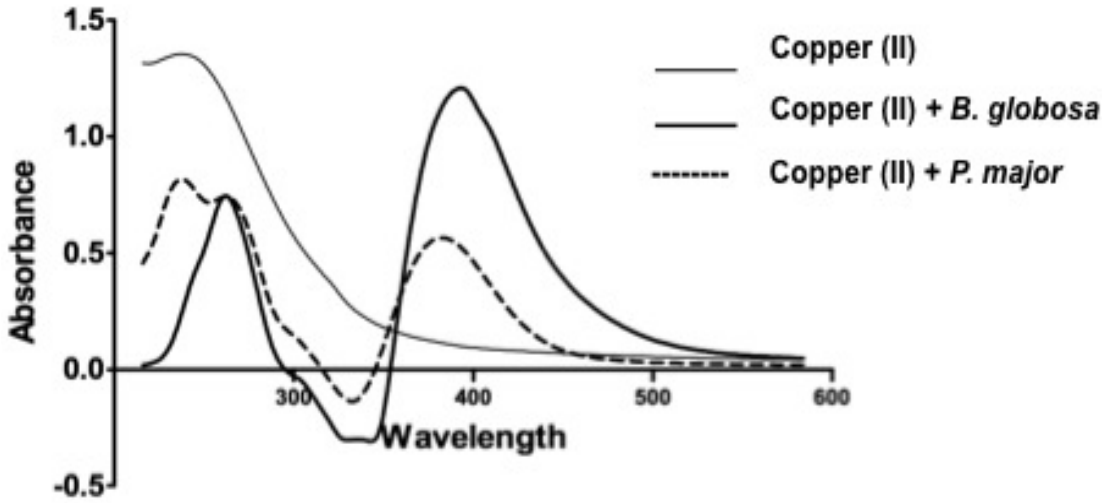
Reducing agents	EC_{50}
B. globosa	0.01325 ± 0.00066
P. major	0.01361 ± 0.00061
Dithiothreitol	1.510 ± 0.045
N-acetyl cysteine	2.120 ± 0.064
Cysteine	1.550 ± 0.054

EC_{50} values represent the concentration of the reducing agents, which inhibits microsomal GSH-transferase activity in 50%. EC_{50} values were obtained from semi-logarithmic graphs of reducing agent concentration versus the effect on microsomal GSH-transferase activity. EC_{50} of *B. globosa* and P. major are expressed as µmol equivalents of GSH/0.1 mg de microsomal protein. Dithiothreitol, N-acetyl cysteine and cysteineare expressed as µmol/0.1 mg de microsomal protein. Values represent the mean of at leastfourindependent experiments± SD. This enzymatic activity was measured according to Methods.

It is noteworthy that a hundred times higher concentration of dithiothreitol, N-acetyl cysteine and cysteine than herbal extracts are need to inhibit half microsomal GST activity (Table 2). The results obtained for dithiothreitol, N-acetyl cysteine and cysteine were1.510, 2.120 and 1.550 µmol/0.1 mg of microsomal protein, respectively. From the pharmacological point of view, these results are significant. If polyvalent herbal extract behave as better antioxidant than synthetic compounds, then lower doses are necessary to achieve the same pharmacological effect. In other words, phytodrugs formulated with these herbal extracts would be safer, their side effects fewer and their therapeutic range wider.

In the cell, another pathway to generate reactive oxygen species (ROS) is through transition metal ions, such as iron and copper. These metals, in their reducing form generate oxygen free radicals through Haber-Weiss and/or Fenton reactions. Therefore, chelation of these free metal ions may contribute to increase cellular antioxidant capacity [11].To evaluate this property, we analysed the spectral changes of copper (II) induced by both herbal extracts (Figure 3). Both extracts changed the spectrum of copper (II). The formation of complexes between molecules of the extract and copper ion may be the reason for the spectral changes observed. Probably, polyphenol and thiol compounds are involved in binding of copper ions.

Figure 3. Chelation of copper (II) by *B. globosa* and *P. major* extracts.



Copper (II) chelating assay are described in Methods.

Summarizing, the *in vitro* assays showed that both herbal extract develop antioxidant effects on biomolecules through additive and synergic mechanisms. Also, synthetic thiol compounds: dithiothreitol, N-acetyl cysteine and cysteine showed a hundred times lower antioxidant effect than the herbal extracts tested. It is important to note that different biological systems (biomolecules, cellular organelles, cellular culture, etc.) mustbe used to characterize the *in vitro*antioxidant capacity, and thus allow a better understanding of what happens *in vivo*.

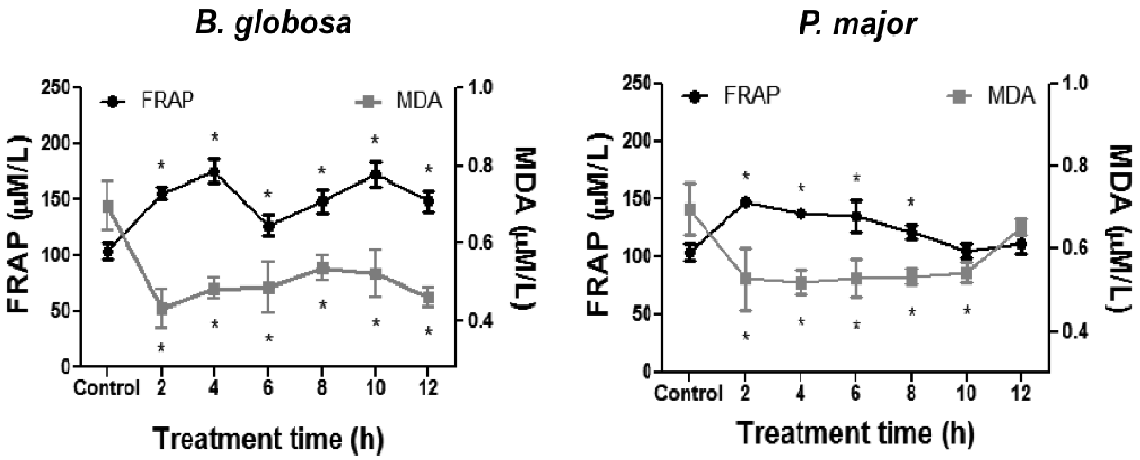
In vivo antioxidant capacity of *B. globosa* and *P. major* extracts

As a form to evaluate*in vivo* antioxidant effects of herbal extracts, the ferric-reducing ability of plasma (FRAP) and malondialdehyde (MDA) concentration were determined after oral administration of the herbal extracts to rats (Figure 4).Dose administered,0.546 mg/Kg of body weight, was obtained of a clinical study realized with this *B. globosa* extract (unpublished data). At 2 h of treatment with

B. globosa and *P. major* extracts the reducing capacity of plasma increased in approximately 72 and 43 µmol of reduced iron/L of plasma compared to control; this represents an increase of 70% and 42%, respectively. Moreover, *B. globosa* showed a second increase of similar magnitude at 10 h, indicating the presence of antioxidant principles that are absorbed more slowly. The antioxidant agents responsible for this second increase could be polyphenols that require biotransformation from gut micro flora[8].Despite the low dose administered, a significant increase in the ferric-reducing ability of plasma was observed.

Another way to assess the antioxidant capacity is determining cellular waste products of cell oxidation. MDA is one of the products of cellular lipid oxidation, so higher plasma antioxidant capacity should contain lower concentration of MDA in plasma. Results of these assays are shown in Figure 4. *B. globosa* showed a decrease of plasmatic MDA. Interestingly, the lowest value of MDA for *B. globosa* appeared at 2 h of treatment, which remained constant until 12 h of treatment. These time values were compatible with the maximum increase on the ferric reducing ability of plasma generated by this herbal extract. *P. major* extract also showed a decrease of plasmatic MDA but this effect remained until 8 h post administration of this extract; at 10 h MDA values were equal to those of control (p>0.05).

Figure 4.Plasmaticantioxidantcapacity in rats.



FRAP (ferricreducingability of plasma) and plasmatic MDA (malondialdehyde) weredetermined as described in Methods. Dosefor*B globosa* and *P. major*: 0.546 mg equivalent of catechin/Kg of bodyweight. Eachvaluerepresentsthe mean ± SD (n=3). Statisticaldifferenceswereobtainedusing Mann-Whitney test. *: p<0.05.

Although polyphenol and thiol concentration of both extracts administered to rats was the same,plasmatic antioxidant capacity of *B. globosa*extract was significantly higher than *P. major* extract (p<0.05). A possible explanation is that antioxidant molecules may act synergistically, as seen in the *in vitro* experiments (Figure 1) and/or other molecules than polyphenols and thiol compounds may be responsible for the antioxidant activity observed.

These results support our postulate indicating that antioxidant capacity of plasma could be use to demonstrate the efficacy of phytodrugsto be used in complementary treatment of different pathologies, especially those associated to oxidative stress, such as cancer, diabetes, neurodegenerative and cardiovascular diseases. Our new objective is to evaluate pharmacokinetic parameters of phytodrugs, so new experiments are underway in our laboratory to complement the results of this work.

Conclusion

Herbal preparation had been mainly used as complementary therapies to treat different pathologies thanks to their redox capacity [2,6,11]. In general, the antioxidant effects of phytodrugs synergistically contribute to the therapeutic effect of synthetic drugs. Our results showed an increase of plasmatic antioxidant capacity of rats after oral administration of herbal extracts, thus demonstrating the antioxidant efficacy of the herbal preparations tested. Considering these results, we could postulate that pharmacokinetic parameters like bioavailability, clearance and half-life of phytodrugs could be determined through the plasmatic antioxidant effect of herbal preparations instead the plasmatic concentration of active principles.

Abbreviations

FRAP: ferric reducing ability of plasma
MDA: malondialdehyde
DTNB: 5,5-dithiobis-(2-nitrobenzoic acid)
B. globosa: Buddleja globosa Hope
P. major: Plantago major L.
GST: GSH-transferase
ROS: reactive oxygen species

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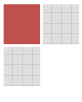

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