Plasmatic antioxidant capacity as a possible marker of phytodrugs efficacy

A. Müller-Sepúlveda1,2*, I. Saavedra-Saavedra2, M. E. Letelier1

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 Fe3+ 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, EC50 of B. globosa extract in preventing oxidation of microsomal lipid and thiol compounds induced by Cu2+/ascorbate were 3 times lower. These extracts were also able to inhibit microsomal GSH-transferase activity and chelate Cu2+. The oral administration of these extracts to rats provoked an increase in the ability to reduce Fe2+ 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 these compounds exert their redox effect can be additive and/or synergistic [4]. Oxidative stress is associated with 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 preparations however, 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 that the 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 for healing 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, tripyridyltriazine, 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 Laboratorios Ximena Polanco®, 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 Laboratorios Ximena Polanco® (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 °C) 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 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 105 000 g in a XL-90 Beckmann ultracentrifuge for 60 min. Pellets sediments were discarded. Supernatants were then centrifuged at 9000 g in a Suprafuge 22 Heraeus centrifuge for 15 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 mM CuSO4 plus 1 mM sodium ascorbate for 20 min at 37 °C with constant agitation prior to measuring lipid and thiol oxidation.

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. Blank omitted GSH. Appearance of the conjugated formed was continuously recorded at 340 nm for 3 min at 25 °C, in a UV 3 Unicam UV-VIS spectrophotometer. This activity was measured in the presence of B. globosa, P. major, dithiothreitol, N-acetyl cysteine and 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 5.46 and 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 our 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 °C. Plasma obtained was stored at -20 °C 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 Fe³⁺ to Fe²⁺. At low pH, Fe²⁺ forms a coloured complex with tripyridyltriazine, which absorb at 593 nm. The results are expressed as μmol 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 H₃PO₄ 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 μmol 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 vs 7.6 μmol equivalents of catechin/mL of extract and 0.71 vs 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 EC₅₀ 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 globosaHope and Plantago major L extracts

<table>
<thead>
<tr>
<th></th>
<th>Polyphenol</th>
<th>Thiol</th>
<th>EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[μmol of catechin/mL of extract ± SD]</td>
<td>[μmol of GSH/mL of extract ± SD]</td>
<td>Prevention of lipid oxidation</td>
</tr>
<tr>
<td>B. globosa</td>
<td>21.8 ± 1.15*</td>
<td>0.71 ± 0.03*</td>
<td>0.39*</td>
</tr>
<tr>
<td>P. major</td>
<td>7.6 ± 1.48</td>
<td>0.27 ± 0.02</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Polyphenol and thiol values represent the mean of at least 4 independent experiments ± SD. EC₅₀ 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.
As mentioned, there are differences between the antioxidant properties of thiol and polyphenol compounds. The oxidation of polyphenol compounds to quinones cannot be reversed; the conjugation of these metabolites with GSH annuls their electrophilicity 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. In all cell types, this reversibility is the result of concerted enzymatic mechanisms that involve the use of the tripeptide glutathione (GSH), the most abundant non-enzymatic antioxidant in the animal cell [24,25]. To test this property, we incubated microsomes with herbal extracts after adding Cu²⁺/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 of thiol systems 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].

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). Cu²⁺/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.

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 maintaining 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 EC50 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.

EC50 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.
Table 2. Microsomal GSH-transferase activity inhibited by *Buddleja globosa* Hope, *Plantago Major* L, Dithiothreitol, N-acetyl cysteine and cysteine

<table>
<thead>
<tr>
<th>Reducing agents</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. globosa</em></td>
<td>0.01325 ± 0.00066</td>
</tr>
<tr>
<td><em>P. major</em></td>
<td>0.01361 ± 0.00061</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1.510 ± 0.045</td>
</tr>
<tr>
<td>N-acetyl cysteine</td>
<td>2.120 ± 0.064</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.550 ± 0.054</td>
</tr>
</tbody>
</table>

EC50 values represent the concentration of the reducing agents, which inhibits microsomal GSH-transferase activity in 50%. EC50 values were obtained from semi-logarithmic graphs of reducing agent concentration versus the effect on microsomal GSH-transferase activity. EC50 of *B. globosa* and *P. major* are expressed as µmol equivalents of GSH/0.1 mg de microsomal protein. Dithiothreitol, N-acetyl cysteine and cysteine are expressed as µmol/0.1 mg de microsomal protein. Values represent the mean of at least four independent 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 were 1.510, 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.) must be 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. Plasmatic antioxidant capacity in rats.](image)

FRAP (ferric-reducing ability of plasma) and plasmatic MDA (malondialdehyde) were determined as described in Methods. Dose for B. globosa and P. major: 0.546 mg equivalent of catechin/Kg of bodyweight. Each value represents the mean ± SD (n=3). Statistical differences were obtained using 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 phytodrugs to 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
Acknowledgements
The present study was carried out under the CONICYT 2010 scholarship program for the implementation of doctoral thesis

References


