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Toxicology in Vitro 22 (2008) 279–286
Toxicology in Vitro
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# DPPH and oxygen free radicals as pro-oxidant of biomolecules

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Received 27 March 2007; accepted 1 August 2007
Available online 17 August 2007

**Abstract**

Numerous investigations exist about the alterations that oxygen free radicals can provoke on biomolecules; these modifications can be prevented and/or reversed by different antioxidants agents. On the other hand, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), a stable nitrogen synthetic radical, is used to evaluate the antioxidant capacity of medicinal herbal products; however, the structural changes that this radical provoke on the herbal active principles are not clear yet. In this work, we compared the redox reactivity of oxygen free radicals and DPPH radical on phospholipids and protein thiol groups present in rat liver microsomes. $Cu^{2+}$/ascorbate was used as generator system of oxygen free radical and as antioxidant, an extract of *Buddleja globosa*'s leaves. $Cu^{2+}$/ascorbate provoked microsomal lipid peroxidation, microsomal thiols oxidation and oxygen consumption; all of these phenomena were inhibited by *B. globosa* extract. On the other hand, DPPH was bleached in different extension by the herbal extract and phosphatidyl choline; beside, DPPH decreased microsomal thiols content, but this phenomenon were not prevented by the herbal extract. Furthermore, DPPH did not induce oxygen consumption and neither modified the oxygen consumption induced by $Cu^{2+}$/ascorbate. Distinct redox mechanisms may explain the differences between the reactivity of DPPH and oxygen free radicals on biomolecules, which is discussed.


*Keywords:* DPPH and oxygen free radicals; Biomolecules herbal-protection; DPPH bleaching activity; *Buddleja globosa*-antioxidant properties

## 1. Introduction

The balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system. Hyper physiological burden of free radicals causes imbalance in homeostatic phenomena (mechanisms) between oxidants and antioxidants in the body. This imbalance leads to oxidative stress involved in aging and various human diseases like atherosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinsonism (Halliwell, 1994). The majority oxidant species involved in physiological cellular oxidative events are anion superoxide ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot-}$), nitric oxide and peroxinitrite. In stress conditions, these species can initiate further deleterious effects on biomolecules and therefore, cellular damage. These effects can be lipid peroxidation (Jaeschke, 1995), protein oxidation (Neuzil et al., 1993), DNA damage (Satoh et al., 1993), oxidation of the reducing equivalents such as nicotinamides and thiols as glutathione (GSH) and alterations in calcium intracellular homeostasis (Stohs and Bagchi, 1995); all of these phenomena can be prevented and/or reversed, depending of their extension and the biomolecule involved, by the cellular antioxidant capacity, i.e. oxidized

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doi:10.1016/j.tiv.2007.08.002

GSH (GSSG) may be converted to GSH through the enzymatic reaction catalyzed by GSH reductase and NADPH (Tseng et al., 2006).

Research in the recent past has accumulated enormous evidences revealing that natural antioxidants can prevent the onset as well as to treat diseases caused and/or fostered due to oxygen free radicals (Hsu, 2006; Bruckdorfer, 1996; Tiwari, 1999; Pietta, 2000). Different organic functions of biomolecules are blank of pro-oxidant agents that are also present in redox active principles of natural products (thiols, amine, carbon conjugated double bound, etc.).

Diphenyl-picrylhydrazyl radical (DPPH) bleaching is one of the strategies used to evaluate the antioxidant properties of herbal extracts; this method has shown to be rapid and simple and it measures the capacity of herbal extract to bleach the DPPH radical, a nitrogen-centred free radical (Antolovich et al., 2002). The structural changes that this radical provokes on herbal principles as well as the involved mechanism however, are not clear yet (Wang and Zhang, 2003).

In foods, antioxidants have been defined as substances that in small quantity are able to prevent or greatly retard the oxidation of easily oxidable materials such as fats (Chipault, 1962). However, biological antioxidants have a further broad definition, which include systems such as metal transport proteins (e.g. transferrin, albumin, ferritin and ceruloplasmin) to prevent the redox properties expression of metal, antioxidant enzymes and factors involved in vascular homeostasis, signal transduction and gene expression (Frankel and Meyer, 2000). Thus, the cellular antioxidant mechanisms involve suppressing of ROS formation, reducing oxygen free radicals ($O_2^{\cdot-}$, $HO^{\cdot}$, $ROO^{\cdot}$) and $H_2O_2$, sequestering metal ions, scavenging active free radicals, repairing and/or clearing the oxidative damage on biomolecules. Moreover, some antioxidants also induce the biosynthesis of other antioxidants or defence's enzymes. It is necessary to note, that the bioactivity of an antioxidant also depends on factors like its physico-chemical characteristics and *in vivo* radical generating conditions (Tiwari, 2001).

As a manner to compare the reactivity of DPPH radical and oxygen free radicals generated by $Cu^{2+}$/ascorbate on biomolecules, we proved the redox reactivity of these radicals on the phospholipids and protein thiol groups. Beside, we proved the capacity of *Buddleja globosa* leaves extracts to prevent and/or to reverse the redox changes observed. *B. globosa* is a Chilean medicinal plant used for wound healing; the leaves of this medicinal plant are enriched in antioxidant compounds specially polyphenols such as flavonoids, phenolics acids, stilbenes, lignans, lignin and tannins, in comparison with root extracts (Houghton, 1984; Mensah et al., 2001; Houghton and Hikino, 1989; Houghton and Mensah, 1999). Aqueous crude extracts are used by native medicine, so containing polar compounds; similar to water, a mixture of water and ethanol also is a polar solvent, so, the composition of compounds in hydroalcoholic extracts may be similar although no identical to water. To respect, compounds present in the aqueous extracts can be extracted by mixture of butanol/methanol and methanol (Houghton and Hikino, 1989). The protection of cellular membrane has very important for the organism homeostasis. On the other hand, the effective concentration to be reached at the site where the reactive species is being formed is very important to display the antioxidant activity of natural compounds; so, the intermediate polarity of these compounds favours the cellular pharmacological antioxidant activity of them (Tiwari, 2001). The antioxidant activity of phenolics compounds is mainly due to their capacities to act as reducing agents, hydrogen donators and singlet oxygen quenchers; in addition, they act as chelating agents of metal (Larson, 1988; Rice-Evans et al., 1996). In this work, we assay the capacity of a hydroalcoholic extract of *B. globosa* leaves to prevent the lipid peroxidation and the reduction of microsomal thiols provoked by $Cu^{2+}$/ascorbate. As the same manner, we compared the capacity of the herbal extract to prevent the DPPH bleaching activity induced by phosphatidyl choline and microsomal thiol groups.

As expected, $Cu^{2+}$/ascorbate induced microsomal lipid peroxidation, oxidation of microsomal thiols and oxygen consumption; all these phenomena were inhibited by *B. globosa* (crude hydroalcoholic extracts). In this work, DPPH was bleached significantly by *B. globosa* extract and phosphatidyl choline. Similar to $Cu^{2+}$/ascorbate, DPPH decreased microsomal thiols content (~50%), but *B. globosa* extract did not modified this phenomenon. Furthermore, DPPH did not induce oxygen consumption and neither modified the oxygen consumption induced by $Cu^{2+}$/ascorbate. These results show that the redox mechanisms underlying the reactivity of DPPH and oxygen free radicals on biomolecules are different. Moreover, the prevention and/or the reversion mechanisms that natural extracts might exert on the biomolecules redox changes induced by DPPH and oxygen free radicals seem to be also different.

## 2. Material and methods

### 2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB), 2,4-dithiothreitol (DTT), Folin-Ciocalteau-phenol reagent, ascorbic acid, thiobarbituric acid (TBA), bovine albumin fraction IV, phosphatidyl choline, catechin (+) [cianidol-3-(2*R*,3*S*)-*trans*-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol-(+)-*trans*-3,3′,4′,5,7-pentahydroxyflavane] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $CuSO_4 \cdot 5H_2O$ and trichloroacetic acid (TCA) were obtained of Merck-Chile. All other chemicals were of analytical grade.

*B. globosa* hydroalcoholic extracts were donated by Laboratorios Ximena Polanco. The characteristics of these products were: extract (1): 1 g of leaves yielded 3.8 mL of

extract, density 0.916 g/mL to 15 °C, alcoholic grade 59° GL; extract (2): 1 g of leaves yielded 7.18 mL of extract, density 0.935 g/mL to 15 °C, alcoholic grade 50° GL.

### 2.2. Animals

Adult male Sprague Dawley rats (200–250 g), derived from a stock maintained at the University of Chile, were used. They were allowed free access to pelleted food, maintained with controlled temperature (22 °C) and photoperiod (lights on from 07:00 to 19:00 h). Protocols approved by the Institutional Ethical Committee of Chemical and Pharmaceutical Sciences School, University of Chile for all animal procedures were used.

### 2.3. Microsomal preparation

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 °C 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 9000*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 −80 °C until use. Microsomal protein was determined according to Lowry et al. (1951).

### 2.4. Polyphenols determination

As mentioned above, the difference between them was only their concentration: extract (1): 1:7.18 g of vegetable drug and extract (2): 1:3.8 g of vegetable drug. The *B. globosa* hydroalcoholic extracts used in this work were prepared according the same procedure, but polyphenols, compounds highly concentrated in the leaves of this plant, were not evaluated by Laboratorios Ximena Polanco. 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 *B. globosa* extracts was determined essentially by the method described by Price et al. (1989). 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 3950 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 was used as reference standard.

### 2.5. Microsomal lipid peroxidation assay

The extent of microsomal lipid peroxidation following $Cu^{2+}$/ascorbate preincubation was estimated by determining TBARS, according to Letelier et al. (2005). Mixtures (1 mL final volume) contained 1 mg/mL microsomal protein, 25 ηM $CuSO_4$, 1 mM sodium ascorbate, 50 mM phosphate buffer, pH 7.4. Blanks contained all the reagents but microsomal protein. Blanks and samples were incubated for 20 min at 37 °C with constant agitation. Afterwards, 250 μL of TCA 0.24 M (4 °C) were added and all mixtures were centrifuged at 10,000*g* for 10 min at 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. Results are expressed in ηmol of TBARS conjugated/min/mg of microsomal protein using the extinction coefficient 156 $mM^{-1}$ $cm^{-1}$ of malondialdehyde as reference. Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

### 2.6. DPPH bleaching activity

This assay was realized essentially by the method described by Joyeux et al. (1995) and modified by Viturro et al. (1999). In a final volume of 1 mL, the reaction mixture contained 20 μg/mL of DPPH (ethanol solution) and different concentrations of *B. globosa* extract (1), ranged between 0.13 and 0.39 μL/mL. Blanks contained only ethanol and the herbal extract. DPPH bleaching activity of all mixtures was measured continuously at 37 °C for 20 min to 517 nm in a UV3 Unicam UV–VIS Spectrophotometer. Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

There are no data about the capacity of DPPH radical to interact with membrane phospholipid. Phosphatidyl choline is a phospholipid present in the microsomal membrane. Thus, we developed the same proceeding mentioned above to evaluate the capacity of phosphatidyl choline to bleach DPPH. The phosphatidyl choline concentrations ranged between 0.125 and 0.375 mg/mL.

DPPH bleaching activity was expressed as ΔAbsorbance/20 min, which corresponds to the difference between the initial (0 min) and final absorbance (20 min).

### 2.7. Oxygen consumption

To induce oxygen consumption different μM concentrations of $CuSO_4$ and 1 mM sodium ascorbate were used. Oxygen consumption extent in the absence and presence of *B. globosa* extract (2) (13 μL/mL) was continuously determined polarographically during 5 min with a Clark electrode No. 5331 (Yellows Springs instrument) in a Gilson 5/6 oxygraph.

### 2.8. Microsomal thiol content

Microsomal thiols were titrated with DTNB. Microsomes (1 mg/mL total protein) were incubated with 25 ηM $Cu^{2+}$/1 mM ascorbate for 30 min at 37 °C. Afterwards, microsomal thiol content was titrated with DTNB, according to Jaeschke (1995). Thiol concentration was estimated by the equimolar apparition of 5-thio-2-nitrobenzoic acid ($\varepsilon_{410} = 13{,}600\ M^{-1}\ cm^{-1}$). Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

### 2.9. Statistical analysis

Groups of test data (mean ± SD) were comparing using Student's *t*-test for paired observations. Values were considered to differ significantly at the level of $p < 0.05$. Analyses were performed using Origin 7.0 software.

## 3. Results

### 3.1. Extracts polyphenols concentrations

The polyphenols concentration of extract (1) and (2) were 30.6 ± 1.83 and 17.6 ± 1.62 ηmol of catechin/μL of extract, respectively.

### 3.2. Effects of B. globosa on microsomal lipid peroxidation induced by $Cu^{2+}$/ascorbate

Fig. 1 shows the semi-logarithmic curves of the effect concentration–response of *B. globosa* extracts (1) and (2), respectively, on microsomal lipid peroxidation. A concentration-dependent inhibition of the microsomal lipid peroxidation induced by $Cu^{2+}$/ascorbate was observed. The $IC_{50}$ values for the *B. globosa* extracts (1) and (2) obtained of these curves were 0.13 μL and 0.22 μL of extract/mg of microsomal protein, respectively; these values represent the extract concentration that inhibited 50% the microsomal lipid peroxidation induced by $Cu^{2+}$/ascorbate.

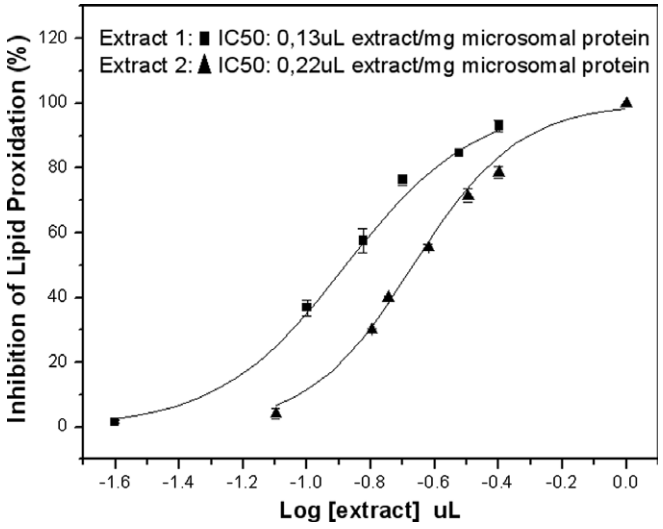


Fig. 1. Effects of *Buddleja globosa* extracts (1) and (2) on microsomal lipid peroxidation induced by $Cu^{2+}$/ascorbate. Microsomes were preincubated with the extracts for 10 min at 37 °C before to induce the lipid peroxidation according to Section 2. Inhibition percentage values were calculated considering as 100% the value of microsomal lipid peroxidation induced by $Cu^{2+}$/ascorbate in the absence of the extracts. The $IC_{50}$ values represent the extracts concentration which inhibited 50% the microsomal lipid peroxidation. Data represent the mean of at least four independent experiments ±SD.

### 3.3. DPPH bleaching activity

*B. globosa* extract (1) and phosphatidyl choline bleached DPPH solution as a concentration–response manner (Figs. 2 and 3a). The DPPH bleaching activity developed by the herbal extract however was significantly higher than that of phosphatidyl choline; thus, the values of ΔAbsorbance/20 min induced by 0.39 μL of herbal extract and 0.375 mg of phosphatidyl choline (the highest concentrations assayed of both agents) were 0.150 and 0.060, respectively.

On the other hand, when methanol was used as dissolvent, the DPPH bleaching activity induced by *B. globosa* extract (1) was 1.5-fold higher than that obtained with ethanol (0.149 vs 0.098) (Fig. 3b). Moreover, the presence of phosphatidyl choline also increased the DPPH bleaching activity induced by *B. globosa* extract (1), but this increasing was significantly higher than that expected of the summatory of DPPH bleaching induced by phosphatidyl choline and *B. globosa* extract separately (Fig. 3).

### 3.4. Oxygen consumption induced by $Cu^{2+}$/ascorbate

$Cu^{2+}$/ascorbate generate the oxygen radicals $O_2^{\cdot-}$ and $HO^{\cdot}$, so diminishing the oxygen concentration in the reaction mixture. When herbal extract (2) and different $Cu^{2+}$ concentration were preincubated for 3 min at 37 °C before to add ascorbate, the oxygen consumption induced by $Cu^{2+}$/ascorbate was inhibited: 64.7% (5 μM $Cu^{2+}$), 82.6%

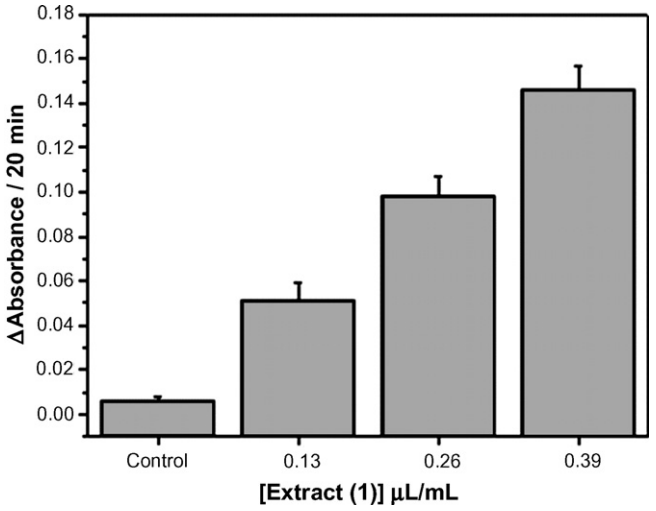


Fig. 2. DPPH bleaching activity of *Buddleja globosa* extracts (1). DPPH bleaching activity was determined according Method. Data represent the mean of at least four independent experiments ±SD.

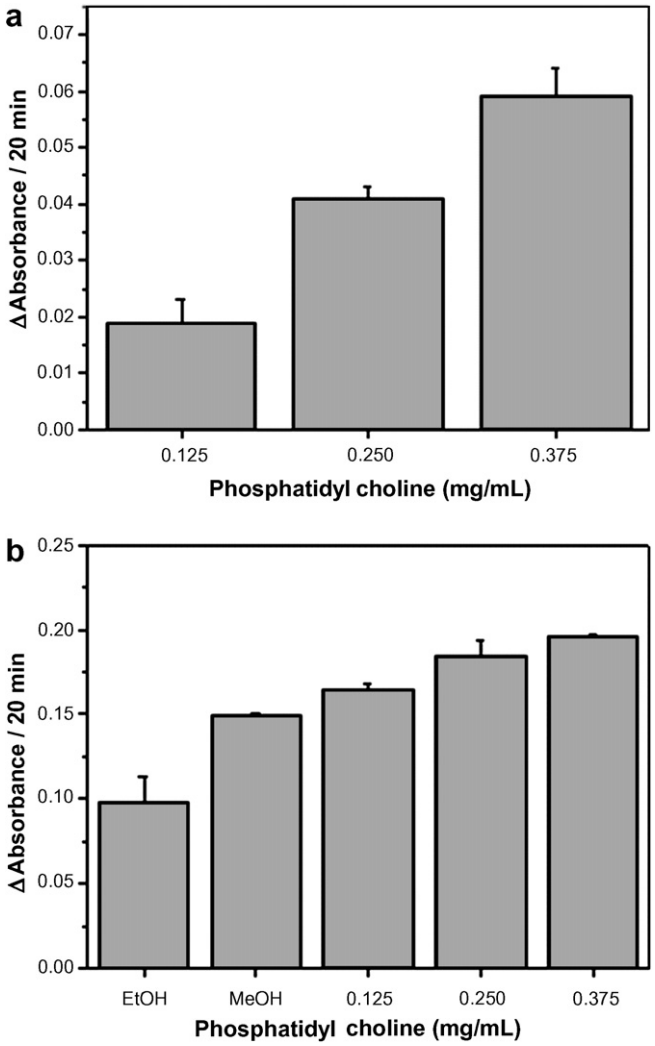


Fig. 3. DPPH bleaching activity of phosphatidyl choline and its effect on DPPH bleaching induced by *Buddleja globosa* extract (1). (a) DPPH bleaching activity of phosphatidyl choline; (b) Effect of phosphatidyl choline on DPPH bleaching induced by *Buddleja globosa*. [Extract]: 0.26 μL/mL. The assays were developed according to Section 2. Data represent the mean of at least four independent experiments ±SD.

(10 μM $Cu^{2+}$) and 85.2% (15 μM $Cu^{2+}$) (Fig. 4a). However, when the extract and ascorbate were preincubated for 3 min at 37 °C before to add $Cu^{2+}$, the inhibition of oxygen consumption observed was significantly lower than that observed in Fig. 4a: 29.4% (5 μM $Cu^{2+}$), 30.4% (10 μM $Cu^{2+}$) and 40.7% (15 μM $Cu^{2+}$) (Fig. 4b). Interestingly, DPPH (10 μg/mL) did not modify the oxygen consumption induced by $Cu^{2+}$/ascorbate independent of the condition assayed: oxygen consumption initiated adding copper ions (condition 1: Fig. 4c) or adding ascorbate (condition 2: Fig. 4c).

### 3.5. Effects of $Cu^{2+}$/ascorbate and DPPH on microsomal thiol content

As shown in Fig. 5, microsomal thiol content was not modified by the presence of *B. globosa* extract (0.13 μL/mg of microsomal protein which corresponds to the $IC_{50}$ of the extract (1) obtained in the lipid peroxidation assays). However, $Cu^{2+}$/ascorbate reduced the microsomal thiol content ~50%. The preincubation of microsomes with *B. globosa* extract before to add $Cu^{2+}$/ascorbate, prevented partially the reduction of microsomal thiol content induced by $Cu^{2+}$/ascorbate (~50%). On the other hand, in the same assay conditions, DPPH also decreased microsomal thiol content ~50%, but the preincubation of DPPH with herbal extract did not modify this phenomenon.

## 4. Discussion

Herbal extracts, especially those prepared of the leaves of medicinal plants, are enriched in antioxidant principles, especially polyphenols. Data exist that demonstrate the different mechanisms through the antioxidant principles can increase the antioxidant capacity in the organism (Larson, 1988; Rice-Evans et al., 1996; Joyeux et al., 1995; Brown et al., 1998; Horie et al., 1999; Hwang et al., 1996). DPPH bleaching activity of herbal extracts is the method generally used to evaluate the herbal extract's antioxidant capacity. Correlation between DPPH bleaching activity of herbal extracts and some antioxidant properties of natural product have been observed (Wright, 2003; Hurd et al., 2005). Even though the DPPH radical is scavenged by herbal antioxidant principles, the structure of the products formed and the mechanisms involved are not clear yet. DPPH is a relative stable radical; however, oxygen free radicals ($O_2^{\cdot-}$ and $HO^{\cdot}$) involved in the cellular oxidative stress are extremely reactive species. Thus, differences in their pro-oxidant activities on biomolecules may be expected. Oxygen free radicals generated by $Cu^{2+}$/ascorbate induce microsomal lipid peroxidation measure by TBARS method; this phenomenon was significantly inhibited by *B. globosa* extracts (Fig. 1). Polyphenols are recognized natural antioxidant principles especially by their capacity to scavenge free radicals and as chelating agents. It is necessary to note that in our assay conditions, 25 ηM copper ion concentration was used; at this copper concentration (in the presence of ascorbate) this metal only display pro-oxidant effects on biomolecules without binding to them (Letelier et al., 2005). Interestingly, the $IC_{50}$ of microsomal lipid peroxidation inhibition of both extracts of *B. globosa* assayed were directly related to its polyphenol concentrations; so, the minor $IC_{50}$ observed corresponded to the herbal extract that contained the highest polyphenol concentration (extract 1).

Phosphatidyl choline, a phospholipid present in the microsomal membrane, bleached poorly the DPPH ethanolic solution (Fig. 3a). Although, methanol is preferred to assay DPPH bleaching activity, we used ethanol as dissolvent because it develops a little toxicity, so it is used in the phytodrugs formulation. Interestingly, the presence of phosphatidyl choline provoked an increasing of the DPPH bleaching activity induced by *B. globosa*, but this increasing was not an additive phenomenon; the value was significantly higher than the summatory of DPPH bleaching

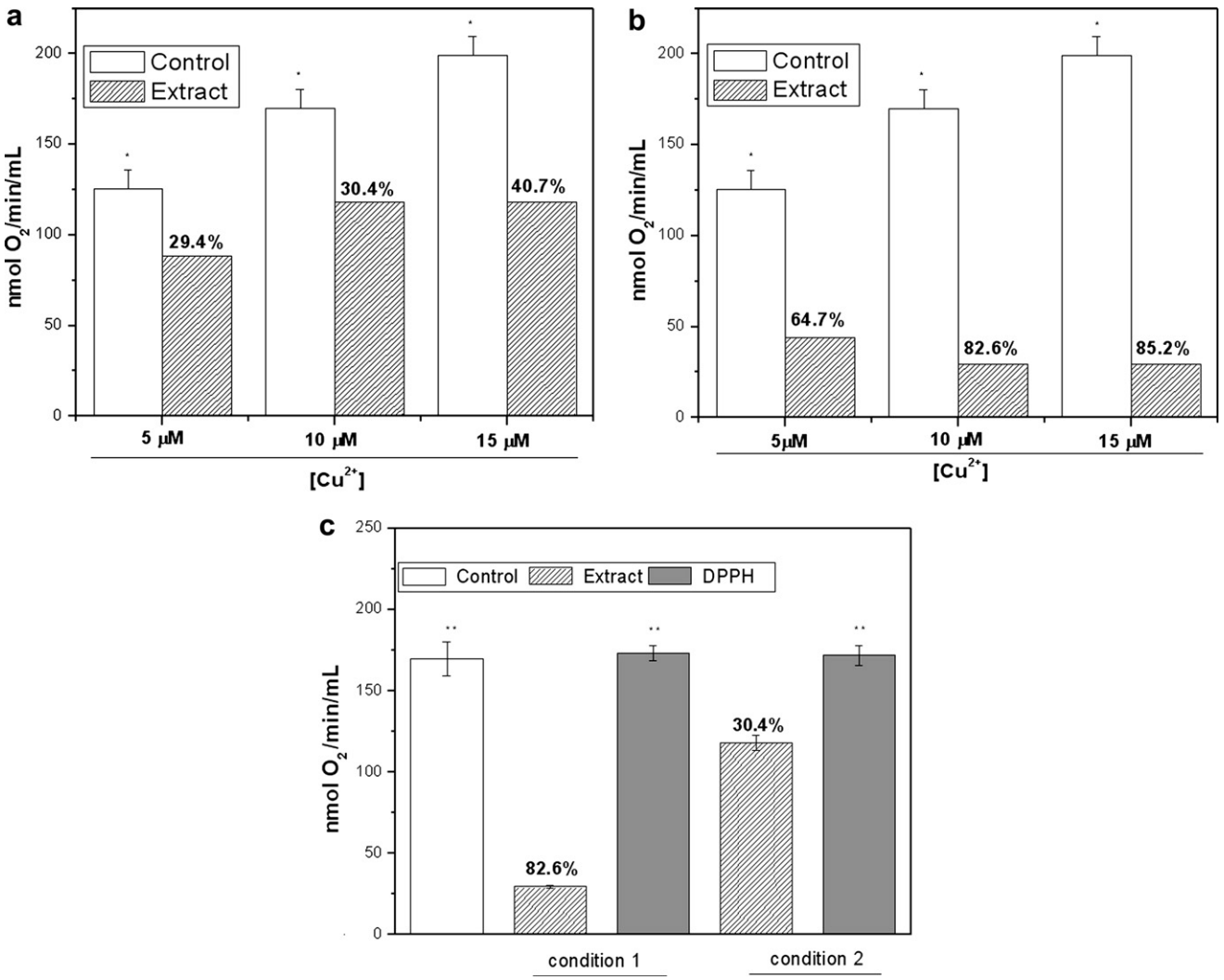


Fig. 4. Effects of *Buddleja globosa* extract and DPPH on oxygen consumption induced by $Cu^{2+}$/ascorbate. (a) *Buddleja globosa* extract (2) [13 μL/mL] and copper ions were incubated during 3 min before to add ascorbate to generate oxygen consumption according to Section 2. (b) *Buddleja globosa* extract and ascorbate were incubated during 3 min before to add copper ions to generate oxygen consumption. (c) [$Cu^{2+}$] 10 μM; [ascorbate] 1 mM; [DPPH]: 10 μg/mL; Condition 1: *Buddleja globosa* extract or DPPH were preincubated during 3 min with $Cu^{2+}$ before to add ascorbate; Condition 2: *Buddleja globosa* extract or DPPH were preincubated during 3 min with ascorbate before to add $Cu^{2+}$ to initiate oxygen consumption. Values on the bars represent the oxygen consumption inhibition percentage; they were calculated considering the control value as 100%. Data represent the mean of at least four independent experiments ±SD. *Values no significantly different ($p > 0.05$).

induced by *B. globosa* and phosphatidyl choline separately ($p < 0.05$) (Fig. 3). Moreover, when ethanol was replaced by methanol as dissolvent in the reaction mixture, a similar phenomenon occurred (Fig. 3c). These data suggest that the reaction mixture's polarity is involved in the extension of the *B. globosa* extract DPPH bleaching activity, but this postulate has to be confirmed.

On the other hand, DPPH radical and oxygen free radical generated by $Cu^{2+}$/ascorbate, reduced the microsomal thiol content in the same extension (~50%); however, the reduction of microsomal thiols provoked by oxygen free radicals was prevented by *B. globosa* extract but not that provoked by DPPH radical (Fig. 5). One of the cellular antioxidant mechanisms is the reversion of –S–S– to –SH groups of proteins, phenomenon catalyzed by various enzymes and small thiol molecules such as GSH. Moreover, the relation between GSH/GSSG is used as a parameter which gives information about the cellular redox state (Hurd et al., 2005). When a thiol groups react with oxygen free radicals, thiyl radicals (RS•, a sulphur-centred radical) are formed, which are stabilized gaining a hydrogen or forming covalent adducts. Herbal extracts antioxidant principles may act as hydrogen donators preventing microsomal lipid peroxidation and protein thiol group's oxidation (Joyeux et al., 1995; Horie et al., 1999). Similar mechanisms have been proposed for the reaction between DPPH radical and herbal antioxidant principles. However, this mechanism seem not be involved in the interaction between DPPH and thiol groups. Probably, DPPH radical and microsomal protein thiol groups form stable covalent adducts, so reducing the microsomal thiols, phenomenon that cannot be prevented and neither reversed by the herbal

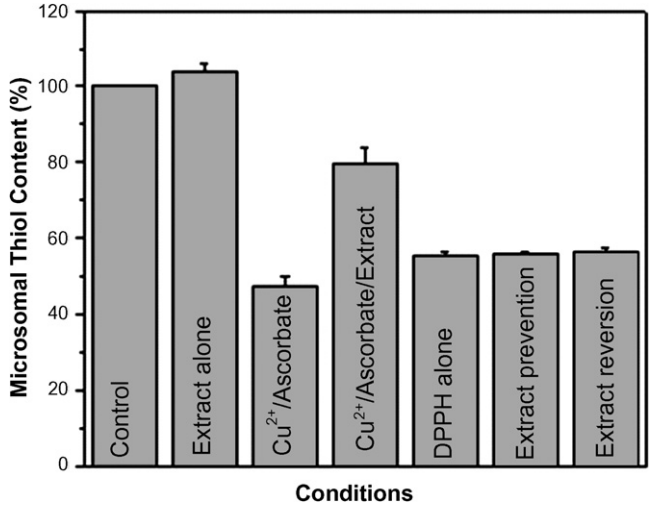


Fig. 5. Comparative effects of *Buddleja globosa* extract and DPPH on microsomal thiol content. [$CuSO_4$]: 25 ηM; [ascorbate]: 1 mM. [Extract alone]: *Buddleja globosa* extract (1) 0.13 μL/mg of microsomal protein. $Cu^{2+}$/ascorbate: microsomes preincubated for 20 min with $Cu^{2+}$/ascorbate to 37 °C before to determine microsomal thiol content. $Cu^{2+}$/ascorbate/extract: microsomes preincubated for 10 min with extract and then 20 min with $Cu^{2+}$/ascorbate. DPPH alone: microsomes incubated only with DPPH (20 μg/mg of microsomal protein). Extract prevention: microsomes preincubated for 10 min with herbal extract (0.26 μL/mg of microsomal protein) and then 20 min with DPPH, before to determine microsomal thiol content. Extract reversion: microsomes preincubated for 20 min with DPPH and then 10 min with herbal extract before to determine the microsomal thiol content. Data represent the mean of at least four independent experiments ±SD.

extract (Fig. 5). New experiments are in progress to confirm this postulate.

*B. globosa* extract inhibited the oxygen consumption induce by $Cu^{2+}$/ascorbate in the absence of microsomes, phenomenon which was $Cu^{2+}$ concentration-dependent (Fig. 4a and b). It is necessary to note, that micromolar copper ion concentrations were used in these experiments and some antioxidant principles of herbal extracts such as phenols, act as chelating agents of transition metals (Pietta, 2000). Interestingly, when the extract was preincubated with $Cu^{2+}$ before to add ascorbate, the inhibition of this consumption was significantly higher than when the extract was preincubated with ascorbate and then $Cu^{2+}$ was added (Fig. 4a and b). Probably, the pre-incubation of herbal extract with $Cu^{2+}$ previously to add ascorbate, favour the sequestering of $Cu^{2+}$ by chelating agents (polyphenols) present in the herbal extract. Free copper ions sequestered cannot take part in the Haber Weiss and/or Fenton reaction to generate oxygen free radicals, so inducing oxygen consumption. Different to $Cu^{2+}$/ascorbate, DPPH radical did not induce oxygen consumption (data not shown) and it neither modified the oxygen consumption induced by $Cu^{2+}$/ascorbate in the absence of microsomes (Fig. 4c). Although the reaction between two free radicals represents one of the mechanisms through free radicals reach stability, these data seem to indicate that DPPH radical not react with oxygen free radicals.

Taken together, DPPH and oxygen free radicals interact with phospholipids and protein thiol groups. The herbal extract considered as a mixture of antioxidant principles prevented the oxidative changes induced by oxygen free radicals; however, it did not prevent and neither reversed the reduction of microsomal thiol content provoked by DPPH radical. By exchange, herbal extract prevents the oxygen free radicals modifications on biomolecules through the similar mechanisms used by the organism, i.e. polyphenols, could act as hydrogen donators to oxygen free radicals, so stabilizing it. The methods based in the prevention of biomolecules oxidation by oxygen free radicals are simple, so they might also be used to screen the antioxidant herbal capacity of herbal preparations oriented to phytodrugs formulation.

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