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Antioxidant and cytotoxic activities of canadine: Biological effects and structural aspects

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Abstract—The cytotoxic effects of four alkaloids, berberine, canadine, anonaine, and antioquine were evaluated using three different cell cultures, a primary culture (rat hepatocytes) and two cell lines (HepG2 and HeLa). Our results indicate that berberine, anonaine, and antioquine possess a significant the cytotoxic effect. In contrast, canadine does not possess cytotoxic effect at concentrations tested here. A molecular modeling study indicates that the quaternary nitrogen, the aromatic polycyclic and planar structure of berberine could be the pharmacophoric patron to produce the cytotoxic effect. In parallel our results demonstrated that canadine possess a significant antioxidant activity. Stereoelectronic aspects of this alkaloid were found to be closely related to those displayed by α-tocopherol and its water-soluble analogue trolox. The antioxidant activities of canadine, combined with its low-toxic effect, indicated that the potential of this alkaloid as a novel class of antioxidant agent is very interesting and deserves further research.

1. Introduction

Oxidative stress, the consequence of an imbalance of prooxidants and antioxidants in the organism, is gaining recognition as a key phenomenon in chronic illnesses like inflammatory and heart disease, hypertension, and some forms of cancer. Lipid peroxidation can contribute to the etiology of disorders such as various types of cancer, atherosclerosis, liver diseases, and premature aging.[1,2] In addition, several gastrointestinal tract diseases seem to be induced by oxidative stress.[3] Thus, a great deal of research has been conducted to determine the mechanisms of action and their possible use to treat diseases associated with diminished natural antioxidant defenses.[2,4]

Coptisine, berberine, and jatrorrhizine, the main protoberberine alkaloids from *Coptis japonica*, showed strong antiphotooxidative activity in the chlorophyll-sensitized photooxidation of linoleic acid,[5] which was shown to be due to singlet oxygen quenching. These compounds were also shown to exert weak antiperoxidative activity in rat liver mircosomes.[5] Rackova et al. reported antiradical and antioxidant activities of alkaloids (including berberine and jatrorrhizine) isolated from *Mahonia aquifolum*.[6] Previous works reported that different alkaloids are able to interfere with peroxidative processes.[7–10] Martinez et al.[7] reported that canadine and anonaine displayed interesting antioxidant effects; in contrast, berberine displayed only a marginal activity. On the basis of the results of that paper, we focused our attention on the potential antioxidant activities of alkaloids belonging to different structural types. However, it should be noted that there are many articles reporting the cytotoxic effects of berberine and its congeners.[11–18] Orfila et al. reported that berberine (**I**) and other structurally related alkaloids displayed a significant cytotoxic

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activity.[19] The actual reach and limitation of any potential drug depends to a large extent on its toxicity; but in the case of potential antioxidant compounds, this is a determinant requirement. Thus, if we are interested in the potential antioxidant effect of any compound, first it is necessary to test its cytotoxic activity. Therefore, in the first step of our study we tested the cytotoxic effects of four alkaloids with potential antioxidant activity. On the basis of previously reported activities, we chose four compounds with different structural characteristics: Berberine (**I**) (a protoberberine possessing a quaternary nitrogen atom); canadine (**II**) (possessing a non-quaternary nitrogen and two non-aromatic rings); anonaine (**III**) (an aporphine type alkaloid), and antioquine (**IV**) (a bisbenzylisoquinoline compound). The structural features of these alkaloids are shown in Figure 1. In a second step of our study, we tested the antioxidant activity of canadine which was chosen as the best candidate for antioxidant in this series. In the last part of this work, we compare some structural aspects of canadine with those of α-tocopherol (**V**) and its water-soluble analogue trolox.

2. Results and discussion

2.1. Cytotoxic effects of compounds I–IV

We had previously found that **I** isolated from *Berberis heterophylla* displayed an interesting in vitro/in vivo antifungal activity against *Candida albicans*, which is the leading primary agent causing superficial and often-fatal disseminated infections in immunocompromised patients.[20,21] In parallel we tested the acute toxicity of berberine from a toxicity test on fish using a static technique.[20,21] Our results indicated that **I** possessed a lower acute toxicity in comparison to that of ketoconazole, which was used as a reference compound; however, the acute toxicity displayed by berberine was still significant.[20,22] Our previous results, as well as several articles reporting the cytotoxic activity of berberine and structurally related alkaloids, prompted us to evaluate the cytotoxic effects of compounds **I**–**IV** using three different cell cultures; primary cultured rat hepatocytes and two well known cell lines (HepG2 and HeLa). The cytotoxicity of these alkaloids over a relatively wide range of concentrations was investigated after 24 h of treatment.

Figure 2a shows the cytotoxic effects obtained for **I** using the three different cell cultures. Berberine displays a high cytotoxicity against hepatocytes even at relatively low concentrations. The IC_{50} obtained for this alkaloid was 26.5 μg/ml, indicating a significant reduction in cell viability. In contrast, **I** displayed a relatively low-cytotoxic effect against HepG2 and Hela cells; giving IC_{50} values of 146.2 and 138.4 g/ml, respectively. Figure 2b provides the results obtained for canadine (**II**). This alkaloid shows a different behavior with respect to the rest of the compounds tested here (compare Fig. 2b with Fig. 2a, c, and d). Canadine displayed very low cytotoxicity even at relatively high a concentration of 200 μg/ml. Thus, we extended the range of concentration for this compound to 300 μg/ml and high percentages of viability were still maintained for the three types of cells at this concentration.

Anonaine (**III**) shows a significant cytotoxic effect against the three cell cultures tested here (Fig. 2c), giving IC_{50} values of 70.3, 33.5, and 24.8 μg/ml for hepatocytes, HepG2 and HeLa, respectively. Our results indicate that anonaine per se possesses a significant cytotoxic activity.

Figure 2d shows the cytotoxic effects obtained for antioquine (**IV**). This bisbenzylisoquinoline alkaloid is extremely toxic on the hepatocyte cells (note that the

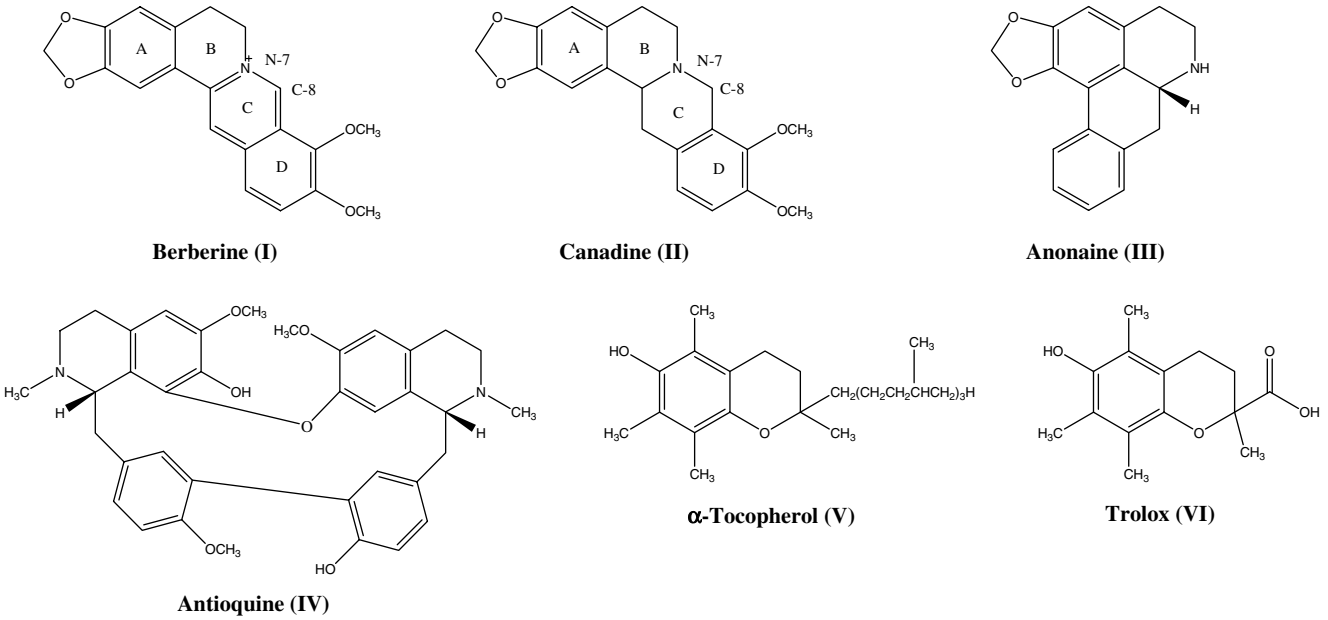

Figure 1. Structures of alkaloids **I**–**IV**, α-tocopherol (**V**), and Trolox (**VI**). Letters assigned to the different rings for **I** and **II** and numbers for N_7 and C_8 atoms are also shown.

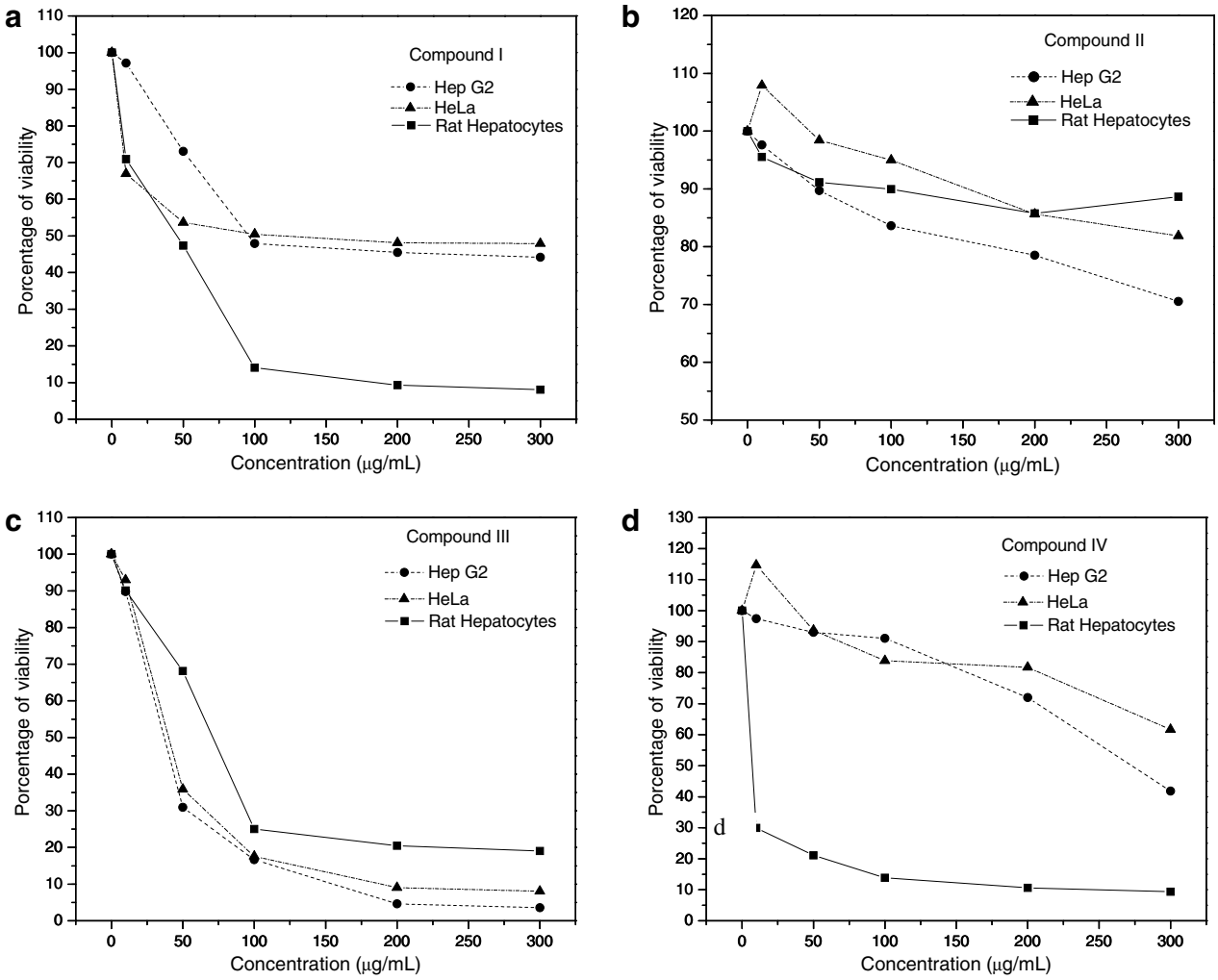

Figure 2. Viability of rat hepatocytes after treatment with alkaloids **I** (a), **II** (b), **III** (c), and **IV** (d). Results are expressed as the percentage of controls (untreated hepatocytes).

IC_{50} obtained for this alkaloid is very low (IC50, 4.06 μg/ml)). However, **IV** does not display cytotoxic effects on the HepG2 and Hela cultures (IC_{50} of 460.0 and 548.5 μg/ml, respectively). These results indicate that the high cytotoxic activity observed in **IV** against hepatocyte cells could be related with some product of its metabolism and not necessarily with antioquine itself. It should be noted that primary cultured rat hepatocytes maintain, at least during 24 h, the metabolic capacity. In contrast, cell lines possess reproductive capacity but not metabolic effects. Thus, the above results suggest that a product of the metabolism of **IV** could be the principal responsible for the cytotoxic effect of antioquine. However, caution is necessary for this assumption and more specific studies are required to confirm these results. Nevertheless, it is clear that the high cytotoxic effect displayed for **IV** against hepatocytes could seriously limit its biological usefulness.

Comparing **I** and **II**, **I** is much more toxic than **II** (compare Fig. 2a and b). These results are in agreement with those previously reported by Wright et al.[23] for the cytotoxic activities of **I** and **II** against KB cells (human carcinoma of the nasopharynx). It is interesting to note that there are few structural differences between **I** and **II**. The nitrogen atom is not quaternary in **II**; moreover, one of the rings (ring C) is not aromatic in this alkaloid (compare structures **I** and **II**).

There are a great number of articles reporting the structural aspects and different bioactivities of berberine and structurally related alkaloids, including canadine. However, compared with these aspects, the action mechanism of these alkaloids, at least at molecular level, has received relatively little attention. Previously, we performed a computer-assisted study reporting the importance of aromatization within the putative bio-medical action mechanism of berberine and related cationic alkaloids with double iso-quinolinoid skeleton.[24] In this putative mechanism of action of berberine, to prevent DNA replication, the first step is aromatization. In contrast to the covalent dehydrogenation, which is endothermic, the aromatization under ionic conditions was found to be exothermic. Our results indicate that in the aromatization process, the ease of hydride ion removal parallels the stabilization energy of the aromatic compounds to be formed. Comparing the nucleophylic additions to the π-systems, the LUMO (lowest unoccu-

pied molecular orbital) energy values suggested a greater accessibility of the N(+) heterocycles in comparison to the polycyclic aromatic hydrocarbons.[24] Thus, it appears that the quaternary nitrogen and the aromatic polycyclic and planar structure of berberine is the pharmacophoric patron to produce the cytotoxic effect.

To understand better our experimental results we performed theoretical calculations on **I** and **II** in order to compare their respective conformational and electronic behaviors. B3LYP/6-31G(d) calculations predict that **I** adopts two almost planar equivalent forms (Fig. 3). In fact, the low-energy conformations of **I** (forms **Ia** and **Ib**) are not completely planar forms since they possess both rings C and D about 30° out of the plane of ring A. DFT calculations predict that the 'switch' of ring B (up or down) crossing by a fully planar first order transition state requires 7.33 kcal/mol. Thus, the fully planar form of **I** is a transition state connecting the two minima (Fig. 3).

Compound **II** possesses at least, two low-energy conformations (**IIa** and **IIb** in Fig. 4), which are non-planar forms. In both conformers, rings B and C are located out of the plane of ring A, and the energy gap between these conformations is 4.11 kcal/mol, the global minimum being **IIa**. It should be noted that the fully planar form of canadine is not a first order transition state, but a higher order transition state structure. Thus, a putative fully planar conformation of **II** would require more than 80 kcal/mol above the global minimum (from DFT calculations) indicating that the spatial ordering adopted by canadine is very different from that obtained for berberine. The different conformations adopted by these alkaloids might be well appreciated in Figure 5.

On the other hand, just comparing the structures of **I** and **II**, there is a clear difference between the electronic distributions of these alkaloids. Whereas **I** possess a positively charged quaternary nitrogen atom, **II** displays a tertiary uncharged nitrogen atom. This electronic difference might be better appreciated from their respective molecular electrostatic potentials shown in Figure 6. In this figure we use the same scale to further appreciate the electronic differences between **I** and **II**.

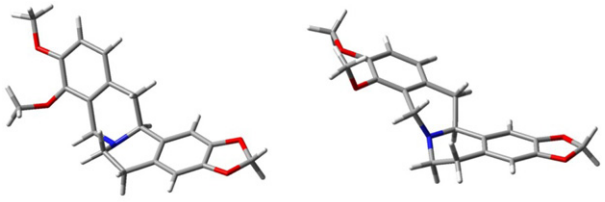

Figure 4. Conformations **IIa** and **IIb** obtained for canadine from B3LYP/6-31G(d) optimizations.

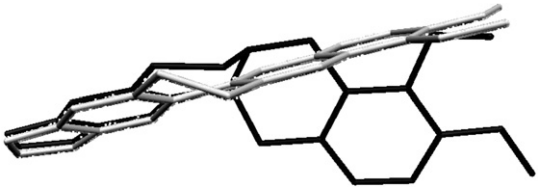

Figure 5. Stereoview of overlapping of conformer **Ia** for berberine (light gray) and conformer **IIa** for canadine (black).

The difference of cytotoxicity between **I** and **II** could be explained from the following chemical structure changes: positively charged quaternary nitrogen atom (in berberine) to unshared electron-bearing tertiary one and saturation of double bond between N-7 and C-8 and consequent change into molecular planarity. Thus, the cytotoxic activities reported here for **I** and **II** provide additional support for the hypothesis suggesting that the quaternary nitrogen and the aromatic polycyclic and planar structure of protoberberines are the minimal structural requirements to produce the biological response. Our results are in a complete agreement with those previously reported for berberine, its 8-hydroxy-7,8-dihydro-derivative and tetrahydroprotoberberine (Thaicanine).[25] It must be pointed out that this pharma-

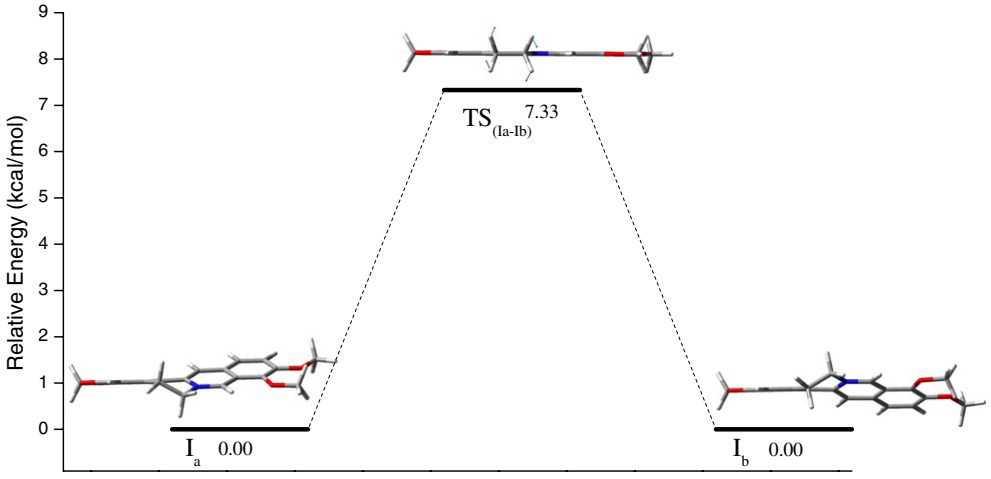

Figure 3. A schematic energy profile for the conformational interconversion between **Ia** and **Ib** forms of berberine. Relative energies at the B3LYP/6-31G(d) level of theory are in kcal/mol.

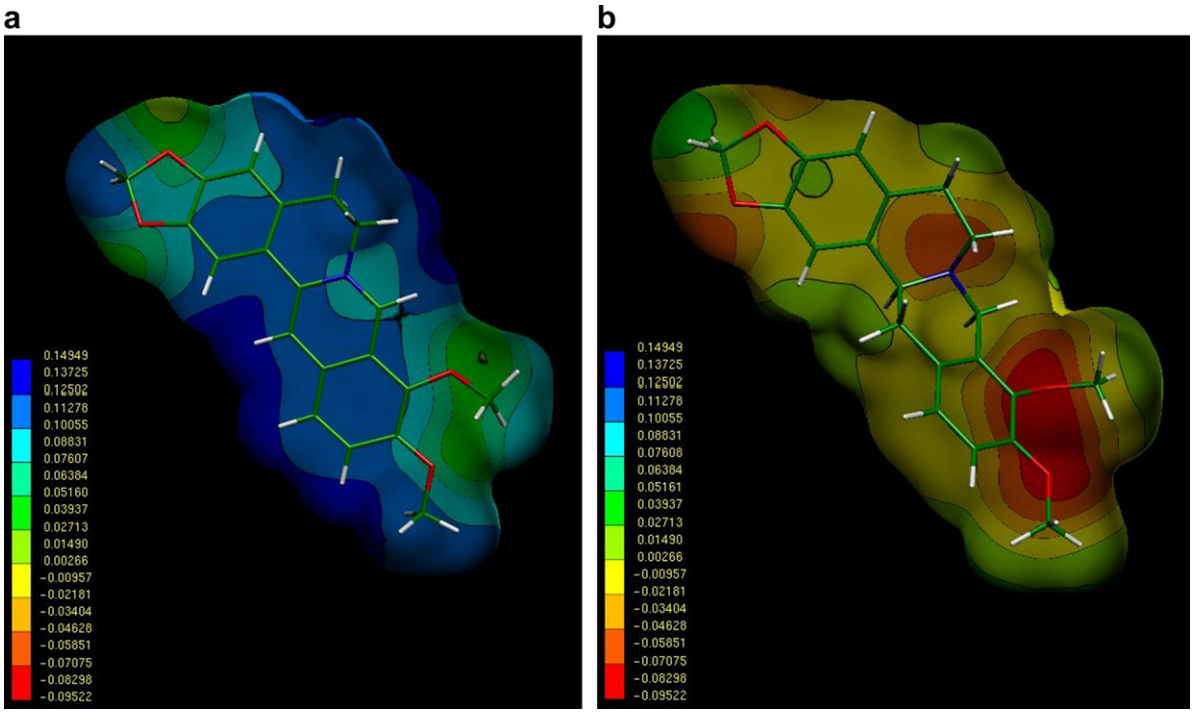

Figure 6. Electrostatic potential-encoded electron density surfaces of the core structures of compounds **I** and **II**. The surfaces were generated with Gaussian 03 after B3LYP minimization with a 6-31G(d) basis set. The coloring represents electrostatic potential with red indicating the strongest attraction to a positive point charge and blue indicating the strongest repulsion. The electrostatic potential is the energy of interaction of the positive point charge with the nuclei and electrons of a molecule. It provides a representative measure of overall molecular charge distribution.

cophoric patron for the cytotoxic activity is not a new concept. Previously Zee-Chang et al.[26,27] argued that antileukemic activity against L1210 and P388 in mice showed changes in the structure of coralyne and related compounds. The structural requirements concerning antitumoral activity are in connection with the planarity and rigidity of molecules.

At this stage of our work, we consider the results obtained for the cytotoxic activities of compounds **I–IV** as certainly significant. The question which arises is: which of these alkaloids is the best candidate to evaluate the antioxidant effect?. To properly answer this question we weigh up two aspects: (i) the previously reported antioxidant effects for compounds **I–IV** and (ii) our own results obtained for the cytotoxic effects of these alkaloids.

Hwang et al.[28] reported on a protective effect of berberine against oxidative stress induced by *tert*-butyl hydroperoxide in rat liver hepatocytes. In addition, Račková et al.[6] reported some antiradical activity for **I**, whereas Martinez et al.[7] reported only marginal effect for this alkaloid. However, the high cytotoxic activity obtained for this compound permits us to discard berberine as a potential antioxidant agent. In the case of anonaine, Martinez et al.[7] reported a significant inhibition of non-enzymic lipid peroxidation (90.3%) by this alkaloid. However, our results indicate that compound **III** possesses a high cytotoxic effect against the three cultures tested here. Thus, the antioxidant activity of **III** should be tested only at sub-cytotoxic concentration, limiting seriously its usefulness. This concept is also valid for antioquine (**IV**). Martinez et al.[7] reported a significant inhibition of non-enzymic lipid peroxidation (93.8%) for canadine. In addition, our results indicate that this alkaloid does not possess cytotoxic activity at concentration tested here. Thus, on the basis of the cytotoxic activities obtained for compounds **I–IV**, and the previously reported antioxidant effects, we chose canadine (**II**) to further evaluate its potential antioxidant activity. Due to the fact that this alkaloid did not display cytotoxic activity at 200 μg/ml and even at higher concentrations, we use sub-cytotoxic concentrations (50 and 100 μg/ml) for the antioxidant assays.

2.2. Antioxidant effects of canadine in rat hepatocytes

Due to the complexity of the oxidation–antioxidation processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample. Thus, we use here two methods widely employed for the evaluation of antioxidant activity, namely intracellular GSH evaluation and lipid peroxidation. Two pathways are considered in the metabolism of *tert*-butyl hydroperoxide in hepatocytes. The first employs the microsomal P450 system leading the ROS production that initiates lipid peroxidation while the second involves the conversion of GSH peroxidase to *tert*-butanol and oxidized glutathione disulfide (GSSG). GSSG is reduced to GSH by GSH reductase, resulting in NAPDH oxidation. Decreased GSH and oxidized NADPH ($NADP^+$) contribute to altered Ca^{2+} homeostasis, which is considered to be a major event in *tert*-butylhydroperoxide toxicity.[29]

In order to evaluate the antioxidant effect of canadine, hepatocytes were pre-treated for 7 h with canadine prior to an induction of oxidative stress with *tert*-butyl hydroperoxide. MDA accumulation in the medium derived from lipid peroxidation was then evaluated after 12 h of the combined exposure to *tert*-butyl hydroperoxide and canadine. Exposure to *tert*-butyl hydroperoxide alone resulted in a dramatic increase in thiobarbituric-reactive substances (approximately twofold over untreated cells for 100 and 250 μM *tert*-butyl hydroperoxide) (Fig. 7a). Preincubation of the cells with canadine (50 or 100 μg/ml) markedly decreased MDA accumulation induced by *tert*-butyl hydroperoxide (>80% of reduction) (Fig. 7a). It should be noted that canadine per se did not show any significant effect against GSH or lipid peroxidation. This is particularly apparent observing their respective controls in Figure 7a and b, respectively. GSH levels were also measured after the 12 h combined exposure to *tert*-butyl hydroperoxide and canadine in cells previously incubated, or not, with canadine for 7 h. Treatment with 100 and 250 μg/ml *tert*-butyl hydroperoxide significantly decreased the intracellular GSH content, whereas preincubation of cells with canadine prevented these effects (Fig. 7b). As seen in Figure 7b, 100 μM *tert*-butyl hydroperoxide produced a significant reduction of GSH levels in relation to untreated cells, which was partially avoided by canadine (50 or 100 μg/ml) pre-treatment. The marked depletion of GSH content (>95% reduction in relation to control) induced by exposure to 250 μM *tert*-butyl hydroperox-

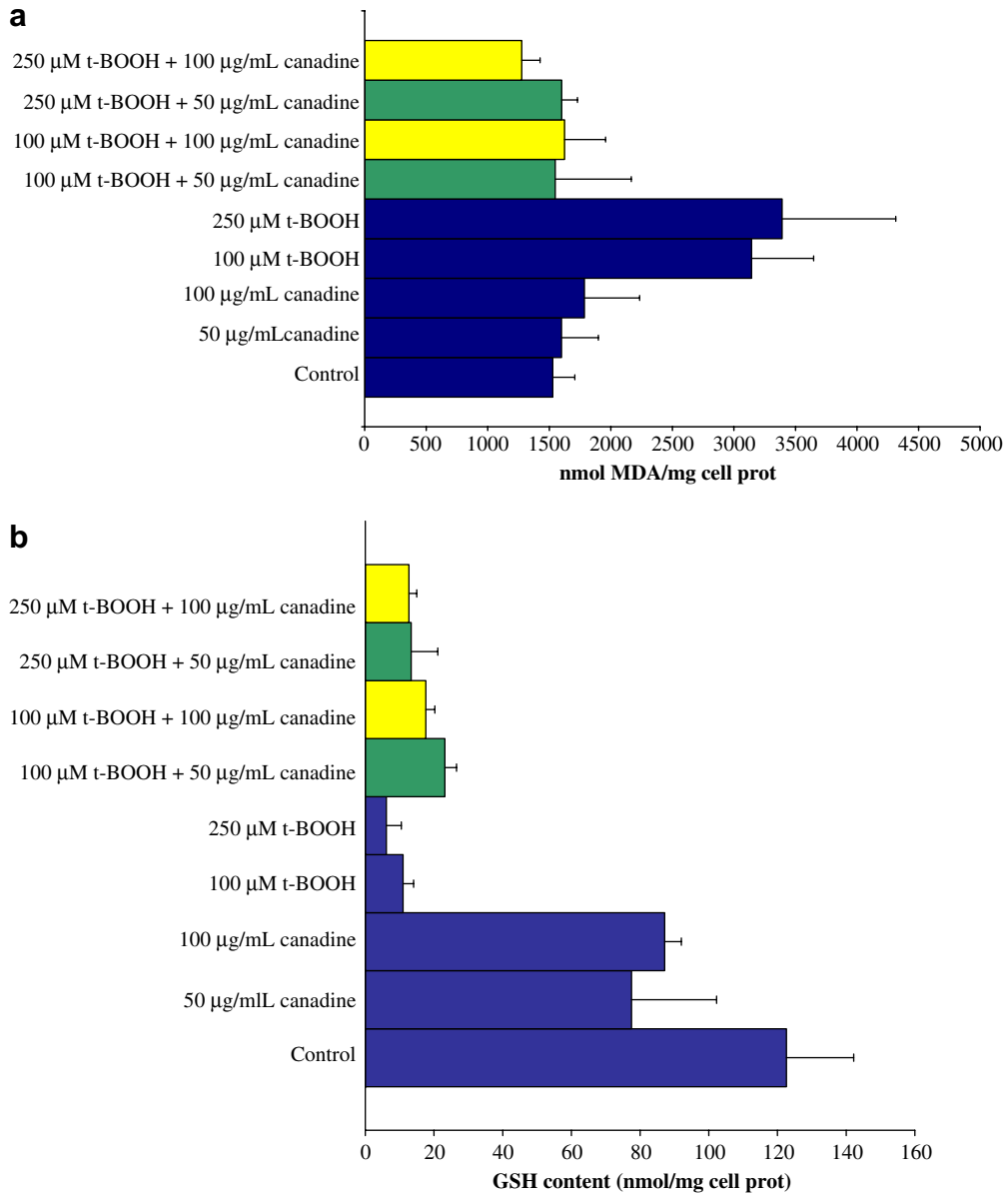

Figure 7. Antioxidant effects of canadine on rat hepatocytes. After 1 h of culture, rat hepatocytes were pre-incubated with increasing concentrations of canadine for 7 h. Then, cells were simultaneously treated with canadine and 100 μM or 250 μM *tert*-butyl hydroperoxide (*t*-BOOH) for 12 h. After treatments, (a) MDA production (b) intracellular GSH content were determined as described in Section 4. Results are expressed as the percentage of the corresponding control (untreated cells). Data are means ± SD.

ide was also significantly prevented by the previous incubation of the hepatocytes with canadine at sub-cytotoxic concentrations.

No cytotoxic effects were produced after 24 h exposure to a quite high concentration of canadine (200 μg/ml), and only a marginal cytotoxicity was observed a higher concentration (300 μg/ml). Our results also show a strong antioxidant capacity of canadine against the oxidative stress induced by *tert*-butylhydroperoxide, a model inducer of endogenous reactive oxygen species (ROS) formation. This biological assay of the protective activity showed that canadine protected microsomial lipid from peroxidation. It is well established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cell and tissues. Microsomes easily produced lipid peroxides and are thought to supply the peroxidation products to other tissues. Thus, our results show the protective effect of canadine against *tert*-butyl hydroperoxide for the first time, which is in accordance with the antioxidant properties obtained for this alkaloid.

The mechanism by which **II** carried out the antioxidant activity has not been elucidated yet, and even though a series of studies have been reported about antioxidant activities of different alkaloids, there are many controversies in relation to the specific antioxidant mechanism due to a lack in a deep knowledge on both the molecular structure of such compounds and the structure–properties relationship. Probably the principal problem encountered when describing antioxidant activity in alkaloids results from the lack of information on the intrinsic reactivity in the entire molecule and in each ring. Thus, in this step of our work we focused our attention in some structural aspects of canadine which might be involved in the antioxidant response. Canadine is a molecule that easily responds to polar and magnetic stimuli, and this is the reason for studying the charge distribution and chemical reactivity of this molecule. The principal objective of calculations performed in this section was to study the molecular properties and chemical reactivity of berberine and canadine and to compare such properties with those of two well known antioxidants, α-tocopherol (**V**) and its water-soluble derivative trolox (compound **VI** in Fig. 1).

Chemical potential properties (Table 1) are defined by different variables tightly related among them: electron affinity (EA), ionization potential (IP), hardness (η), electronegativity (χ), and electrophilicity (ω). Such variables have different meanings; nevertheless, as a group they measure the tendency to give or capture electrons; that is, they are an index of the antioxidant potential[30–32] results from the ability to give electrons.[33] The chemical potential properties are shown in Table 1, indicating that compounds **II**, **V**, and **VI** have close values. In contrast compound I displayed somewhat different results. On the other hand, as can be seen in Table 2, the properties of energy in compounds **II**, **V**, and **VI** are also very similar. The gap values (0.191, 0.178, and 0.182 eV for **II**, **V**, and **VI**, respectively) show practically the same orbital reactivity for these molecules, which are classified as reactive molecules on the basis of these values. In previous studies, it has been found that there is a large energy difference between HOMO (highest occupied molecular orbital) and LUMO, corresponding to stable and little reactive systems, whereas in the opposite case the systems are little stable and highly reactive.[30,34]

Table 1. Chemical potential properties (eV) for compounds **I**, **II**, **V**, and **VI**

Property	I	II	V	VI
Electro affinity ($A = E_{(0)} - E_{(-1)}$)	−0.31	−0.34	−0.16	−0.25
Ionization potential ($I = E_{(+1)} - E_{(0)}$)	4.20	7.01	6.96	7.17
Hardness ($\eta = (I - A)/2$)	2.25	3.67	3.56	3.71
Electro negativity ($\chi = (I + A)/2$)	1.94	3.33	3.26	3.46
Electrophilicity ($\omega = \mu^2/2\eta$)	0.84	2.48	1.49	1.61

It will be useful to examine the highest occupied orbitals and the lowest virtual orbitals for these compounds because the relative ordering of the occupied and virtual orbitals provides a reasonable qualitative indication of the excitation properties.[35] The contour plot of the orbitals for compounds **II**, **V**, and **VI** are depicted in Figures 8 and 9. The electron clouds of the highest occupied molecular orbital are concentrated on the carbon atoms of ring A (Fig. 8). The electronic cloud is mainly composed of the π-orbital ingredient showing the inter-ring bonding character. The LUMO distribution also indicates that the carbon atoms in the ring A are potential sites for nucleophilic attack. In the lower occupied orbitals, the contribution of ring A is also primary (Fig. 9). In this figure, we can observe that the electronic clouds of the lowest unoccupied molecular orbital are concentrated on ring A in **II**, which is mostly composed of the π^* orbitals (inter-ring antibonding character).

Molecular orbital calculations obtained for **II**, which, in addition to providing orbital energies for comparison with **V** and **VI**, furnish a detailed description of orbitals, including spatial characteristics, nodal patterns, and individual atom contributions. It is interesting to note that the HOMO and LUMO contour plot obtained for **II** is closely related to those displayed by compounds **V** and **VI**. Comparing the results obtained for compounds **II** and **V**, it appears that ring A of canadine could mimic the aromatic moiety of **V**, whereas the rest of the molecule could act as the hydrophobic portion of compound **V**. In fact, there are various ways in which **II** may produce its antioxidant activity, on which we can only speculate. In that respect, the similar stereoelectronic behavior observed between **II** and **V** is particularly noteworthy.

3. Conclusions

We tested the cytotoxic effects of four alkaloids possessing different structural features: berberine, canadine, anonaine, and antioquine. Among them, berberine, anonaine, and antioquine displayed a significant cytotoxic effect. In contrast, canadine did not show cytotoxic activity even at relatively high concentrations. Theoret-

Table 2. Properties of the energy for compounds **II**, **V**, and **VI**

Property	**II**	**V**	**VI**
Energy of neutral molecule (0) (Hartree)	−1130.4329980	−1285.7735091	−845.3077392
Energy of charged molecule (+1) (Hartree)	−1130.1750932	−1285.5176494	−845.0440523
Energy of charged molecule (−1) (Hartree)	−1130.4205519	−1285.7676567	−845.2983309
HOMO (eV)	−0.207	−0.195	−0.201
LUMO (eV)	−0.016	−0.017	−0.019
Gap ($\Delta E = E_{LUMO} - E_{HOMO}$) (eV)	0.191	0.178	0.182

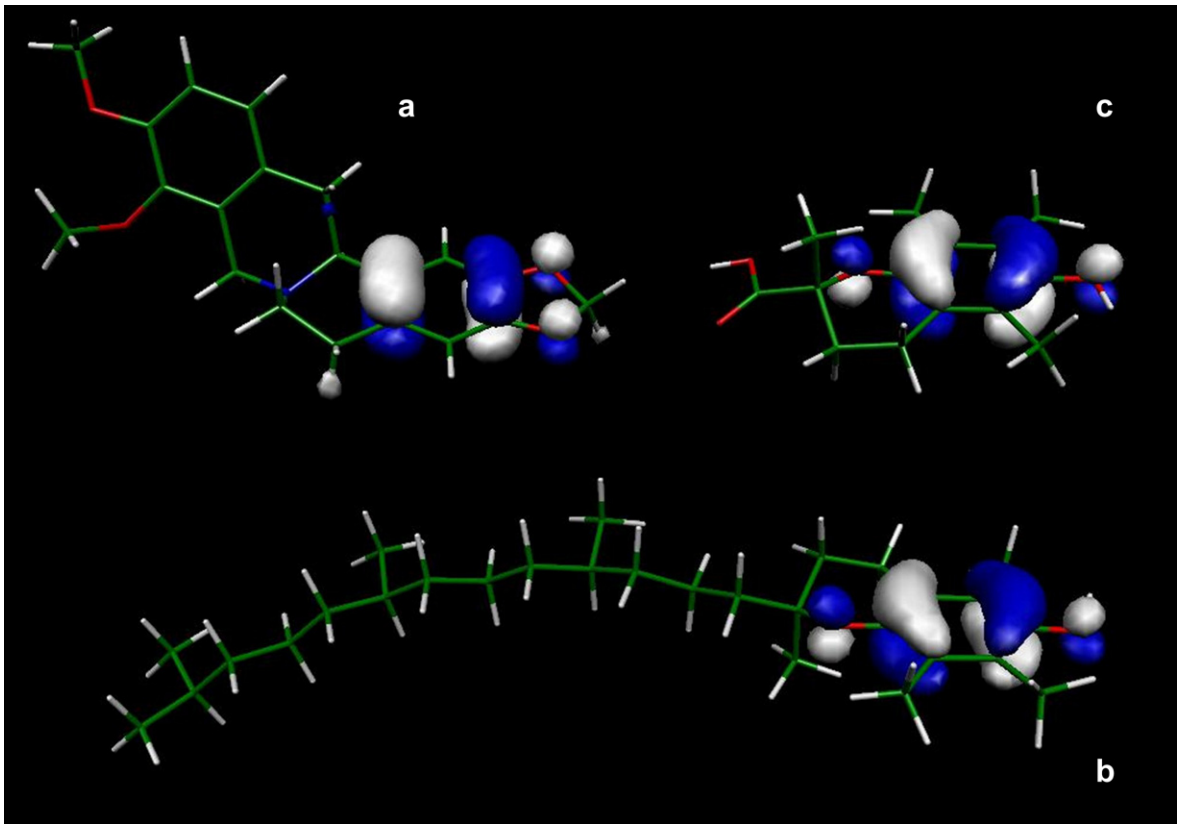

Figure 8. Spatial representation of the frontier molecular orbitals (HOMO) of the compounds **II** (a), **V** (b), and **IV** (c) at the ground state using B3LYP/6-31++G(d,p) calculations.

ical calculations performed on berberine and canadine gives an additional support to a previously reported hypothesis suggesting that the cytotoxic effects of berberine and structurally related alkaloids is directly related to the planarity and rigidity of this molecule. In parallel to the low-cytotoxic activity observed for canadine, our results clearly demonstrated that this alkaloid possesses an interesting antioxidant activity. Furthermore, some structural aspects of canadine were found to be closely related to those of α-tocopherol and trolox.

Given that the ability of a compound to act as antioxidant in vitro does not necessarily mean that it can act in the same manner in vivo, the antioxidant property of canadine was evaluated as protective activity on lipid peroxidation of rat hepatocytes, which is a model system that better simulates the in vivo conditions. The results of the present study demonstrate that canadine was effective in antilipid peroxide production and acts as an antioxidant agent. Thus, the antioxidant activity of canadine combined with the lower cytotoxic effect in comparison with the other alkaloids tested indicate that the potential of canadine as a novel class of antioxidant is particularly interesting and should be investigated more fully.

4. Experimental

4.1. Plant material

Berberis heterophylla Juss, leaves, stems and roots were collected in Comodoro Rivadavia, Chubut, Argentina in June 2003, and were authenticated by Ing. Agr. Mónica Stronati (Department of Biology, National University of Patagonia). A voucher specimen was deposited in the Herbarium of the Natural Sciences Faculty of the National University of Patagonia 'S.J.B.'

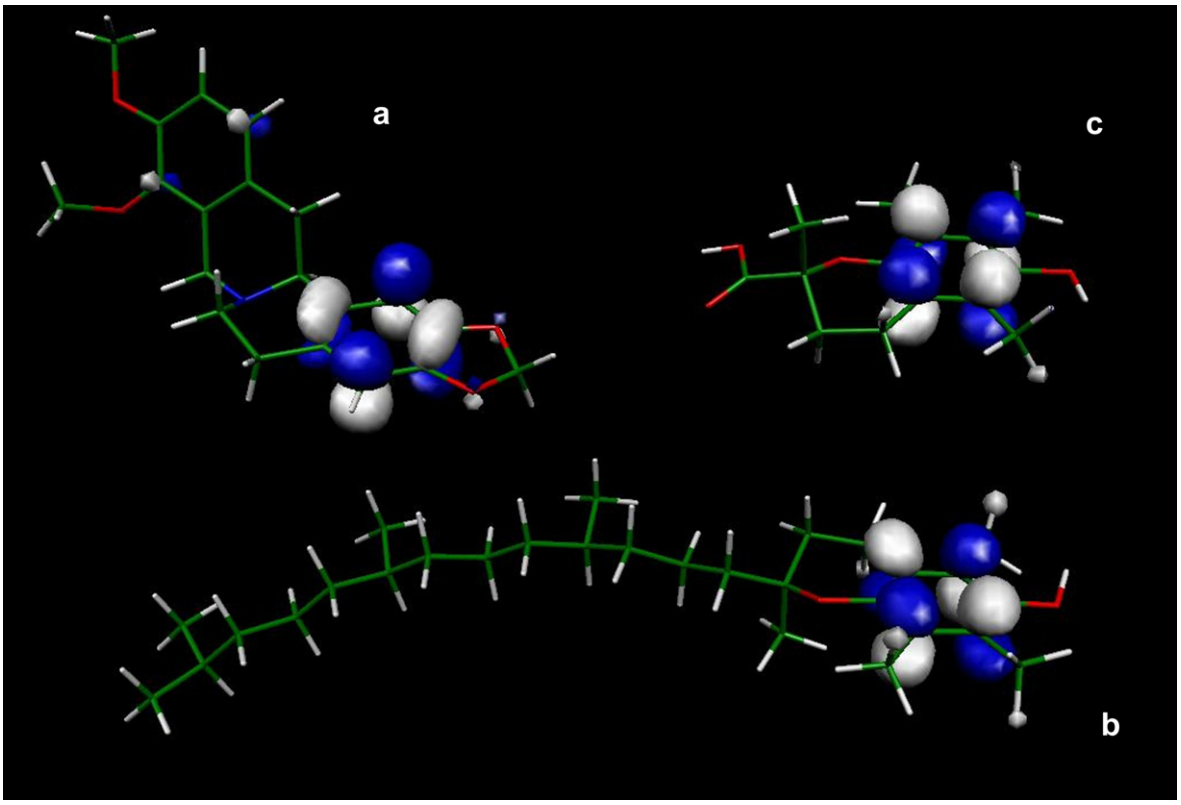

Figure 9. Spatial representation of the frontier molecular orbitals (LUMO) of the compounds **II** (a), **V** (b), and **IV** (c) at the ground state using B3LYP/6-31++G(d,p) calculations.

4.2. Extraction and isolation

Berberine isolated from B. heterophylla: Dried and powdered roots of *B. heterophylla* (730 g) were extracted with methanol at room temperature for 96 h (2 L each) by adding fresh solvent every 24 h and filtered. The combined methanol extracts were concentrated to a dark brown residue (33.5 g). The dry extract was dissolved in 1% aqueous HCl and the solution was extracted with Cl_2CH_2. Next, the aqueous phase was basified to pH 8–9 with 15% NH_3 and extracted with CH_2Cl_2. The precipitate obtained was filtered and then purified by repetitive column chromatography on silica gel (Cl_2CH_2/MeOH, 9:1; 8:2) to produce berberine, as yellow needles (701.4 mg g) which was identified by ^{1}H NMR, ^{13}C NMR analyses and by comparison of its spectral data with literature values.[36,37]

Anonaine: This aporphine alkaloid was isolated from roots of an oceanic annonaceae, *Xylopia papuana* as was previously reported.[38]

Antioquine: This bisbenzylisoquinoline alkaloid was obtained from *Pseudoxandra* aff, *lucida* as was previously reported.[39]

4.3. Chemistry

Canadine was obtained as follow: berberine chloride (105 mg) was dissolved in aqueous acetic acid 66.6% (9 ml), powder Zn was added (10.54 g) with an aqueous HCl solution 35% (23 ml). The solution was refluxed for 2 h at 100 °C, when TLC indicated complete reaction. The reaction mixture was neutralized with an aqueous $OHNH_4$ 30% (20 ml). It was extracted with CH_2Cl_2 (3× 15 ml). The combined CH_2Cl_2 extracts were dried, filtered, and evaporated to yield Canadine (65.4 mg). The Canadine was purified by column chromatography and then identified by ^{1}H NMR, ^{13}C NMR analyses and by comparison of its spectral data with those in the literature and those of the standard substance.

4.4. Bioassays

4.4.1. Isolation and culture of hepatocytes. Hepatocytes were obtained from 200 to 300 g Sprague–Dawley male rats by perfusion of the liver with collagenase as described elsewhere.[40] Cell viability of suspension, assessed by the trypan blue exclusion test, was higher than 85%. Cells were seeded at a density of 8×10^4 viable cells/cm^2 in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with sodium selenite (170 μg/ml), 2% calf serum, 0.2% bovine serum albumin, 50 mU/ml of penicillin, 50 μg/ml of streptomycin and 10 nM insulin and were then incubated at 37 °C in a 5% CO_2 humidified atmosphere. Unattached cells were removed by changing the medium 1 h after plating. Cultures were shifted to serum-free medium supplemented with 10 nM insulin and dexamethasone after the first 24 h. Unless indicated, treatments with alkaloids and usnic acid started 1 h after cell plating, and the medium (with or without extract) was renewed daily. Cells were exposed to the extract

for different periods of time depending on the aim of the experiment.

4.4.2. Culture of HepG2 cells. HepG2 cells were cultured in Ham's F-12/Leibovitz L-15 (1:1, v/v) supplemented with 7% newborn calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml. For sub-culturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37 °C. Cultures were used at 75% confluency.

4.4.3. Culture of HeLa cells. HeLa cells (ECACC No. 85060701) were cultured in DMEM supplemented with 5% fetal calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml. For sub-culturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37°C. Cultures were used at 75% confluency.

4.4.4. MTT assay. Cytotoxicity effects were measured by using the MTT (Tetrazoliun salt) reduction assay after 24 h of exposure to different concentrations of the compound g/ml. The stock solution of the extract or compounds was prepared at a concentration of 1 mg/ml in culture medium and conveniently diluted in culture medium to obtain the final desired concentrations. Control cells (only medium-treated cells) were included in all experiments.

The MTT assay was performed according to the method of Jover et al.[41] Briefly, the medium was removed after exposure and cultures were washed with PBS. Then, 100 μl/well MTT reagent (5 mg/ml in medium) were added to each well and plates were placed in the incubator for a further 3 h. The corresponding supernatants were discarded and cells were washed again. The dye was extracted with DMSO and optical density was read at 490 nm on a Microplate Reader. The percentage of inhibition (%) of the succinic dehydrogenase reduction of MTT was calculated in relation to control cells in each experimental series (control cells were assumed to have 100% viability).

4.4.5. Lipid peroxidation. Lipid peroxidation was determined by measuring the generation of malonyldialdehyde (MDA) in the culture media.[42] After an incubation of 60 min in a boiling bath, the fluorochrome formed was extracted with *n*-butanol, and the fluorescence of the organic phase was measured at 530 ± 25 nm excitation and 590 ± 35 nm emission. Malonyldialdehyde bis (dimethylacetal) was used as standard. To investigate the antioxidant effect of canadine, hepatocytes were incubated with canadine at, 50 and 100 μg/ml for 7 h. Then, cells were treated simultaneously with *tert*-butyl hydroperoxide (100 and 250 μM) and canadine for the following 7 h. At the end of incubation (48 h), MDA production was determined. Data were normalized using cellular protein data.

4.4.6. GSH evaluation. GSH was measured by a fluorimetric reaction with *o*-phthaldialdehyde.[42] Briefly, cells were detached and homogenized by ultrasound. The homogenates were deproteinized and centrifuged. Aliquots of the supernatant were transferred to microtiter plates and allowed to react with *o*-phthaldialdehyde. Fluorescence was measured in a multiwell plate fluorimeter (excitation filter: 355 ± 35 nm; emission filter: 460 ± 25 nm). Known amounts of GSH were used as reference standards. Hepatocytes were pre-incubated with canadine at 50 and 100 μg/ml for 7 h followed by a combined exposure to *tert*-butyl hydroperoxide (100 and 250 μM) and canadine for the following 12 h. At the end, GSH was measured and data were normalized using cellular protein data.

4.4.7. Statistical analysis. Data were expressed as means ± SD using Microsoft Excel. Statistical analysis for processing biological activities was performed by the Student *t*-test. Regression analysis was used to calculate the inhibitory concentration 50 (IC_{50}), defined as the concentration of the extract or compound necessary to produce 50% inhibition. Cytotoxicity and lipid peroxidation results were performed using the *U* Mann–Whitney test. Each experimental procedure was performed in at least three cell preparations. A *P* values less than 0.05 was considered statistically significant.

4.5. Calculation methods

All the computational studies were carried out using density functional theory (DFT) methods implemented in the *Gaussian 03* suite of programs.[43] All the geometries were optimized at B3LYP/6-31 G(d) level of theory. Low-energy conformations as well as transition state geometries were confirmed from a vibrational analysis using B3LYP/6-31G(d) calculations.

The electronic study of berberine and canadine were carried out using molecular electrostatic potentials (MEPs). MEPs have been shown to provide reliable information, both on the interaction sites of molecules with point charges and on the comparative reactivities of these sites.[44–46] These MEPs were calculated by using B3LYP/6-31G(d) wave functions. MEP graphical presentations were created using the Molekel program.[47]

The calculated electronic properties for compounds **II**, **V**, and **VI** were: HOMO–LUMO distributions, HOMO–LUMO gap energies and chemical potential properties (electron affinity, ionization potential, hardness, electronegativity, and electrophilicity). These properties were calculated from B3LYP/63-1++G(d,p) single point calculations using the B3LYP/6-31G(d) geometries. DFT calculations with moderate basis sets and diffuse function can give a reliable outcome,[48] the discussion about compounds **II**, **V**, and **VI** in this paper was based on the B3LYP/6-31++G(d,p) results. All the calculations were performed in gas phase with the purpose of obtaining the intrinsic properties of the alkaloids studies, free of any interaction.

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