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Antiradical and antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects

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Abstract—The antioxidant activities of three alkaloids isolated from *Mahonia aquifolium*—berberine, jatrorrhizine, and magnoflorine—were studied with respect to their structural aspects, particularly the presence and the position of –OH groups, steric conditions of unpaired electron delocalization and parameters of lipophilicity and hydration energy. The antiradical activities of the compounds tested were evaluated as the reactivities toward free stable α,α′-diphenyl-β-picrylhydrazyl radical (DPPH). The antioxidant features of the alkaloids tested were investigated in heterogeneous membrane system of DOPC liposomes stressed by peroxidative damage induced by AAPH azoinitiator. Both alkaloids bearing free phenolic groups—jatrorrhizine and magnoflorine—showed better activities in both systems used than berberine not bearing any readily abstractable hydrogen on its skeleton. The former two showed antiperoxidative efficiency in DOPC liposomal membrane comparable to that of an effective scavenger of peroxyl radicals—stobadine–and higher than that of Trolox. We conclude that the favorable antioxidant features of the hydroxylated alkaloids are most probably ensured by the combination of reasonably high antiradical reactivity with high lipophilicity, however, the solvation process was found to markedly interfere with these beneficial effects.

1. Introduction

Studies on structure–activity relationships are vital for understanding the molecular mechanisms of biochemical processes. Such concepts have also been applied to the development of effective biological antioxidants,[1–11] the importance of which is emerging from increasing evidence on the role of oxygen-derived free radicals in many degenerative diseases.[12–14]

Berberine and berberine-type alkaloids representing a structural class of organic cations, produced in numerous plants of the genera *Berberis*, *Mahonia*, and *Coptis*, have been shown to exert a broad spectrum of antimicrobial, anticancerogenic, and antimutagenic activity.[15–20]

Coptisine, berberine, and jatrorrhizine, the main protoberberine alkaloids from *Coptis japonica*, showed strong antiphotooxidative activity in the chlorophyl-sensitized photooxidation of linoleic acid,[21] which was shown to be due to singlet oxygen quenching. These compounds were also shown to exert weak antiperoxidative activity in rat liver microsomes.[21] Antiperoxidative features of other protoberberine and aporphine alkaloids have been also reported.[22–24]

Focusing on the mechanism of action of these alkaloids, we hypothesize that the presence of aromatic –OH group may be responsible for their antioxidant efficiency, similarly to phenolic antioxidants,[25,26] via chain-breaking mechanism by donation of phenolic hydrogen.

The aim of the study was to elucidate antiradical and antioxidant activities of the alkaloids **I**, **II**, **III** (Fig. 1) isolated from *M. aquifolium* with respect to their structural features and physico-chemical properties. The antiradical activity was tested in ethanolic solution as the

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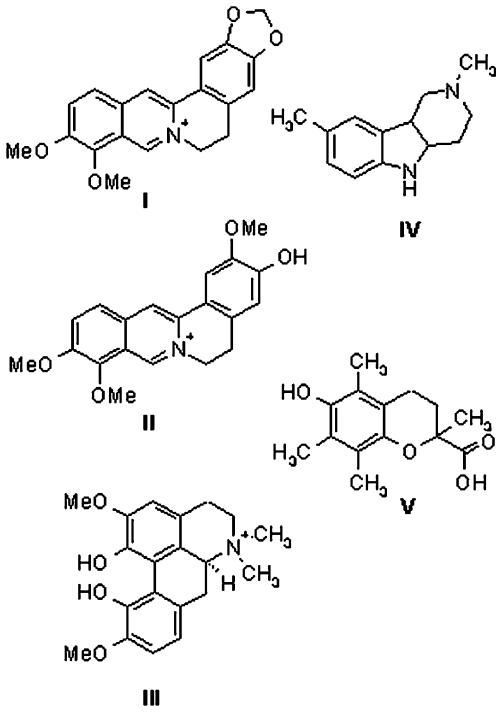

Figure 1. Chemical structure of the compounds tested: berberine (**I**), jatrorrhizine (**II**), magnoflorine (**III**), stobadine (**IV**), and Trolox (**V**).

Table 1. Antioxidant activities of the compounds tested

Compound	DPPH test		DOPC liposomes	
	k ($M s^{-1}$)[a]	n[b]	Lag time[c] (min)	% Inh.[d]
Berberine (**I**)	20.80	0.27	14.7 ± 6.5	34.0 ± 3.7
Jatrorrhizine (**II**)	107.75	0.55	62.0 ± 5.8	54.2 ± 4.1
Magnoflorine (**III**)	439.22	1.1	73.6 ± 5.5	74.2 ± 2.5
Stobadine (**IV**)	493.51	1.56	76.6 ± 3.8	69.0 ± 0.4
Trolox (**V**)	1580.87	2	30.8 ± 2.1	23.6 ± 13.4

[a] Values of the rate constants of the initial consumption of DPPH by the compounds tested (k). The compounds tested (33.3 μM) were incubated in the ethanolic solution of DPPH (60 μM) at ambient temperature.

[b] Values of stoichiometry of the reaction of DPPH with the compounds tested (n), calculated for 5 h reaction time, using the reference value $n = 2$ for Trolox.[27]

[c] Values of inhibition period produced by the compound tested (60 μmol/L) during AAPH-induced peroxidation of DOPC liposomes. DOPC liposomes (0.8 mM) were incubated in the presence of AAPH (10 mM) in phosphate buffer (20 mM; pH 7.4) at 50 °C.

[d] Values of inhibition of AAPH-induced peroxidation of DOPC liposomes at the time interval of 80 min. DOPC liposomes (0.8 mM) were incubated in the presence of AAPH (10 mM) in phosphate buffer (20 mM; pH 7.4) at 50 °C. Each result represents the average of three values calculated as the percentage of the hydroperoxide content in the control samples represented by the complete assay system, but in the absence of the compound tested.

ability to scavenge free α,α′-diphenyl-β-picrylhydrazyl radical (DPPH), while the antioxidant efficiency was evaluated as the ability to inhibit peroxidation of dioleoyl phosphatidylcholine (DOPC) liposomes initiated by thermal degradation of the azoinitiator 2,2′-azobis (2-amidinopropane)hydrochloride (AAPH). As antioxidant standards we used the pyridoindole antioxidant stobadine (**IV**) and the water-soluble analogue of vitamin E, Trolox (**V**).

2. Results

2.1. Radical scavenging activity

In order to assess the radical scavenging potential of the compounds tested, the reactivity toward the stable free radical DPPH was measured at 518 nm by continual absorbance decrease of ethanol solution of DPPH containing the derivative tested. As shown in Figure 2 for jatrorrhizine (**II**), native absorption of the alkaloids did not interfere with the absorption maximum of DPPH at 518 nm. The rate constants of the initial consumption of DPPH (k) by the compounds tested were calculated assuming the 2nd order kinetics (Table 1). The values of stoichiometries (n) were calculated using the absorbance difference ΔA ($A(t=0) - A$(plateau)) for 5 h reaction time, using the reference value of the stoichiometric factor for peroxyl radical trapping for Trolox

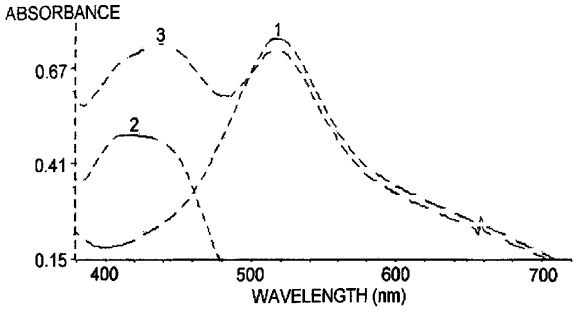

Figure 2. Absorption spectra of the compounds DPPH (1, 62.1 μM), jatrorrhizine (2, 33.3 μM) and of the reaction mixture of DPPH (60 μM), and jatrorrhizine (33.3 μM) in ethanol (3) at 1 min of the reaction time.

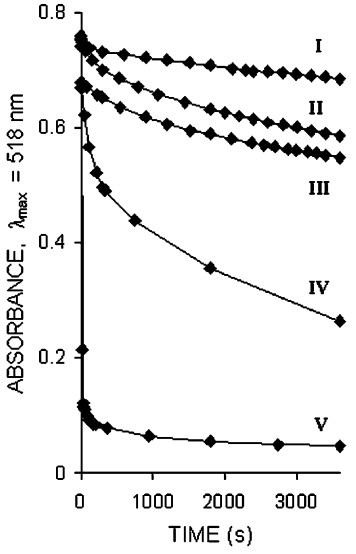

Figure 3. Continual absorbance decrease of ethanolic solution of α,α′-diphenyl-β-picrylhydrazyl (DPPH) radical (60 μmol/L) in the presence of the antioxidants tested (33.3 μmol/L) at λ_{max} = 518 nm.

($n=2$).[27] As shown in Figure 3 and Table 1, the alkaloids tested were less reactive than stobadine (**IV**) and Trolox (**V**). However, magnoflorine (**III**) gave the value of the rate constant close to that of stobadine (**IV**). Magnoflorine (**III**) showed to be a more effective radical scavenger than jatrorrhizine (**II**), while berberine (**I**) showed only marginal activity.

2.2. Inhibition of lipid peroxidation

Unilamellar DOPC liposomes were used to evaluate the antioxidant activity of the alkaloids tested in comparison with that of the standard stobadine (**IV**) and Trolox (**V**). Peroxidation of the liposomal membrane was triggered by thermal decomposition of the hydrophilic azo compound 2,2′-azobis (2-amidinopropane)hydrochloride (AAPH).

Figure 4 shows peroxidation of unilamellar DOPC liposomes induced by the water-soluble initiator AAPH. In a complete reaction system, DOPC liposomes/AAPH/buffer, lipid peroxidation proceeded at a constant rate and an approximately linear time-dependent increase of lipid hydroperoxides was observed without any induction period. No accumulation of hydroperoxides was observed in the absence of AAPH or liposomes. As shown in Figure 4, the alkaloids tested effectively suppressed the oxidation and produced a clear inhibition period.

The length of the inhibition period produced by the compounds tested was taken as a criterion of antioxidant activity. In another strategy, the antioxidant activity was evaluated as the percentage of inhibition of DOPC peroxidation by equimolar (60 μmol/L) amounts of the compounds tested at the fixed time interval of 80 min (see Table 1). Both criteria gave the same order of the antioxidant activities of the alkaloids tested; with

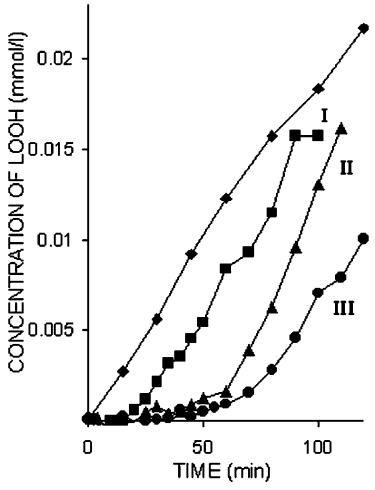

Figure 4. Kinetic curve of AAPH-induced peroxidation of DOPC liposomes (◆) and appearance of inhibition period in the presence of berberine (**I**) (■), jatrorrhizine (**II**) (▲), and magnoflorine (**III**) (●). Concentration of the antioxidants tested was 60 μmol/L. DOPC liposomes (0.8 mM) were incubated in the presence of AAPH (10 mM) and in phosphate buffer (pH 7.4; 20 mM) at 50 °C.

Table 2. Physico-chemical parameters of the compounds tested

Compound	R_M[a]	BDE[b]	E_h[c] (kcal/mol)
Berberine (**I**)	0.87 ± 0.07	—	−7.43
Jatrorrhizine (**II**)	0.70 ± 0.05	89.9	−9.78
Magnoflorine (**III**)	0.34 ± 0.06	88.7	−7.23
Stobadine (**IV**)	0.28 ± 0.03	91.9	−1.12
Trolox (**V**)	—	83.5	−8.35

[a] Values of lipophilicity parameters obtained by the method of reversed-phase thin layer chromatography (RP TLC). The mobile phase consisted of phosphate buffer solution (pH 7.4; 0.1 M) mixture with acetone (20:80, v:v).
[b] Values of the OH bond dissociation energies (BDE) (Eq. 1).
[c] Values of the hydration energy E_h.

berberine (**I**) being the least active compound while magnoflorine (**III**) showed the highest antiperoxidative activity. The antioxidant activity of magnoflorine (**III**) was comparable with that of stobadine (**IV**) and markedly higher than that of Trolox (**V**).

2.3. Physico-chemical properties

Table 2 shows physico-chemical parameters of the compounds tested, that is, lipophilicity parameters represented by R_M values obtained from reversed phase TLC analysis and the values of the –OH bond dissociation energies (BDE) of the compounds **II**–**IV**. For magnoflorine (**III**) only the lower value of BDE is shown. In the case of berberine (**I**), because of lack of any dissociable hydrogen, BDE was not calculated. Table 2 summarizes also the values of the hydration energies E_h calculated by the program HyperChem.[28]

3. Discussion

Berberine and berberine-type alkaloids represent a structural class of organic cations, produced in numerous plants of the genera *Berberis*, *Mahonia*, and *Coptis*. Various parts of these plants have been used for many generations as antibiotics and in treating patients with gastrointestinal disorders. Their extracts and decoctions demonstrated significant antimicrobial activity against organisms including bacteria, fungi, viruses, protozoas, and chlamydia.[15,16] In addition, berberine may possess antitumor promoting properties in colon and bladder cancer cell lines and inhibitory action on the growth of mouse sarcoma cells in culture.[17]

Only recently, berberine and jatrorrhizine were reported to possess significant antimutagenic activity against the chloroplast damaging effect of acridine orange with high potency or with acridine orange-induced mutagenicity in *E. gracilis* assay.[18] Berberine was also shown to exhibit a dual topoisomerase I and II poisoning activity.[19]

It has been shown that active components isolated from methanol extracts of *C. japonica* Makino exerted antioxidant activity toward chlorophyll b-sensitized photooxidation of linoleic acid and their activity was found to be due to singlet oxygen quenching.[21] Moreover, these compounds were found to show also inhibition

activity on rat microsomal peroxidation induced by Fe^{2+}/cysteine[22] and Fe^{2+}/H_2O_2.[23] Hwang et al.[24] reported on a protective effect of berberine against oxidative stress induced by *tert*-butyl hydroperoxide in rat liver hepatocytes.

In this study we evaluated antioxidant properties of three alkaloids isolated from *M. aquifolium* with respect to their structural features and physico-chemical properties.

In the first series of experiments we evaluated the antiradical activity of the compounds tested by using a stable free radical, DPPH, in a homogenous ethanolic solution. DPPH, as a weak hydrogen atom abstractor, is considered a good kinetic model for peroxyl ROO$^{\cdot}$ radicals.[29–31] According to our results, alkaloids bearing the unsubstituted –OH group (**II**, **III**) proved to be able to scavenge free stable DPPH radical, though they showed less activity than the antioxidant standards used (stobadine (**IV**) and Trolox (**V**)) (Fig. 3, Table 1). However, the antioxidant efficiency of the dihydroxylated alkaloid magnoflorine (**III**) was found to be similar to that of stobadine (**IV**). Berberine (**I**) showed only negligible activity, which is in accord with the lack of the group bearing abstractable hydrogen.

The order of antioxidant capacities of the compounds **I**–**IV** in liposomal membranous system was analogous to their antiradical activities in DPPH system: the compounds **II** and **III** bearing OH moieties showed much better LOO$^{\cdot}$ scavenging capacities compared to berberine **I** lacking any group able to donate hydrogen (Table 1, Fig. 4). However, the differences between the antiperoxidative activities of **II** and **III** were not so remarkable as in the case of their antiradical activities in homogeneous solution of DPPH assay. This could be due to a significant contribution of lipophilicity to the apparent antioxidant efficiency of the alkaloids studied in DOPC liposomal membranous system. Membrane favored distribution of less reactive jatrorrhizine (**II**), represented by higher R_M value, ensured probably its better availability in DOPC liposomes in comparison with the more active but less lipophilic magnoflorine **III** (compare Tables 1 and 2).

The high hydrophilicity of Trolox (**V**) ($P = 0.33$ in a two phase system lipid–water),[27] on the other hand, is most likely responsible for its low antioxidant activity observed in DOPC liposomes, a result, which apparently did not correspond to its high intrinsic antiradical activity (Table 1) and the easiness of homolytical cleavage of O–H bond documented by the low BDE value (Table 2). In the system of liposomes stobadine (**IV**) possessing favorable lipophilic features ($P = 3.72$ in a two-phase system octanol–water)[32] may be considered as more relevant antioxidant standard.

The compound **I** exerted some antiradical activity, though not bearing any readily abstractable hydrogen on its skeleton. This polycyclic structure with high lipophilicity may participate in redox reactions by a mechanism analogous to that of isoquinoline alkaloids.[22,33] On the other hand, the photoinitiated prooxidant features of berberine[34] may be responsible for the higher blank observed in lipid peroxidation assays in DOPC liposomes fortified with this compound.

The isosurface 3D maps of the highest occupied molecular orbital (HOMO) developed for the compounds **II** and **III** supported the hypothesis on hydrogen radical abstraction from the free –OH moieties, since they were shown to be more concentrated in the surroundings of these reaction centers (Fig. 5a,b).

Magnoflorine (**III**) showed a lower value of O–H bond dissociation energy (BDE) than jatrorrhizine (**II**) (Table 2), explaining the higher antioxidant efficiency of the former compound. As for magnoflorine (**III**), the calculated value of BDE was lower for –OH moiety attached to C1 (88.7 kcal mol^{-1}) than from hydroxy- group attached to C11 atom (90.9 kcal mol^{-1}), which is indicative of preferable formation of C1-phenoxyl radical of magnoflorine (**III**). As seen in Figure 5b, a similar trend can be observed in the distribution of HOMO density, which is more concentrated in the surroundings of the reaction center in magnoflorine (**III**).

Since both compounds **II** and **III** contain a fused partly saturated ring system, it was useful to investigate whether phenoxyl radicals originated in free radical attack could be stabilized by delocalization of its unpaired electron throughout more than one aromatic

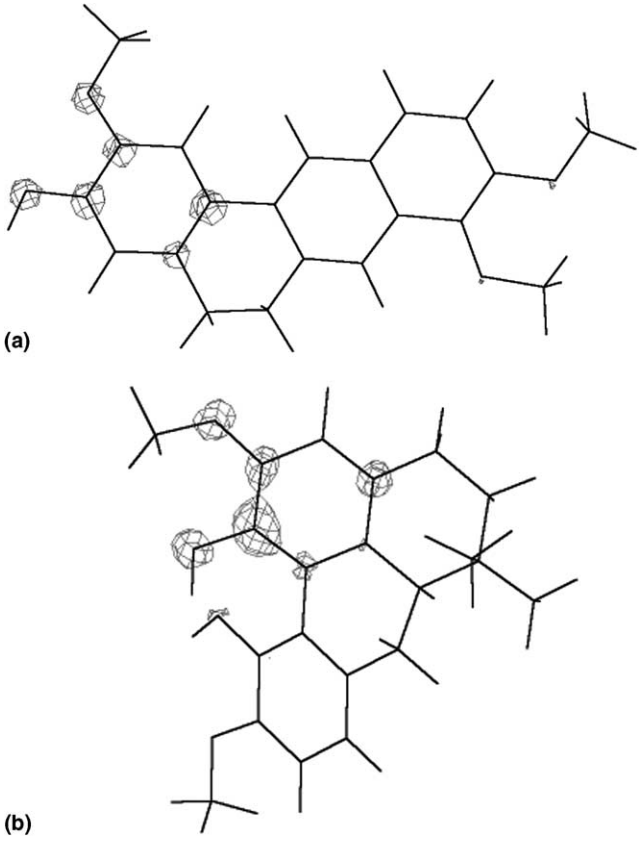

Figure 5. Isosurface 3D maps for the highest occupied molecular orbital (HOMO) density squared with contour value 0.01 of the optimal conformers of the compounds **II** (a) and **III** (b).

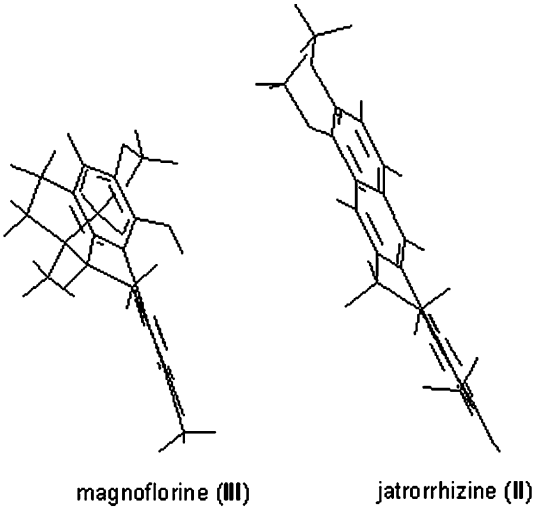

Figure 6. Space distortion from the planar plane of both benzene rings in magnoflorine **III** and of the isoquinoline and benzene ring in the structure of jatrorrhizine **II**.

ring. We found that this type of conjugation did not play a role in phenoxyl radical stabilization, owing to the space distortion from the planar plane (Fig. 6) of both benzene rings in magnoflorine **III** and the isoquinoline and benzene ring in the structure of jatrorrhizine **II**. In addition, the maps of spin densities showed the possible stabilization of the phenoxyl radical formed by delocalization through one aromatic system (Fig. 7a,b).

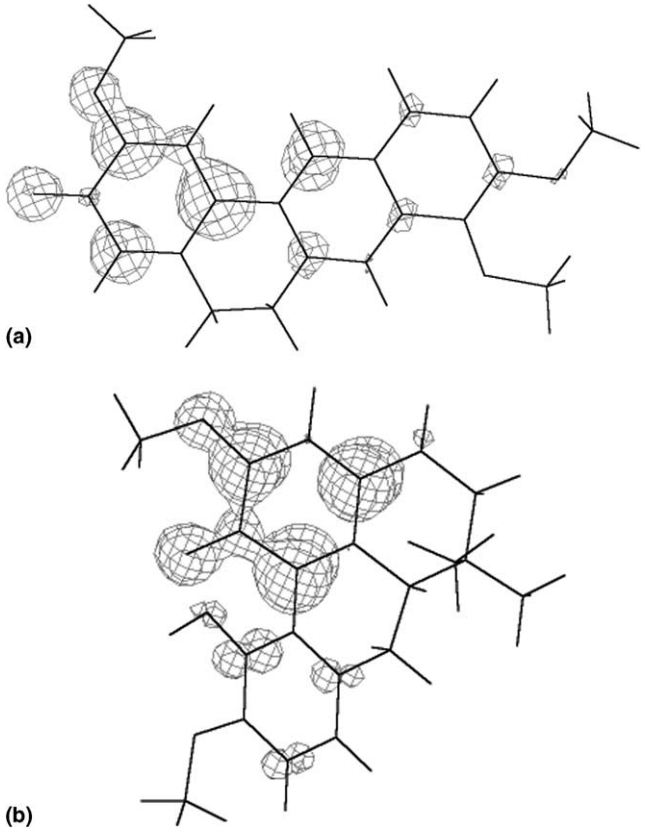

Figure 7. Spin densities isosurface maps with contour value 0.002 of the optimal conformers of the compounds **II** (a) and **III** (b) in the form of radicals.

Magnoflorine (**III**) showed slightly lower antiradical activity in DPPH reaction system than stobadine (**IV**), and in spite of the fact that the BDE value pertinent to stobadine radical formation by abstraction of hydrogen from indole nitrogen was higher (Table 2). The reason of this discrepancy probably consists in the low absolute value of hydration energy for stobadine (**IV**) (Table 2). This could be also the reason of the good antioxidant activity of berberine **I** in liposomal membrane, since this compound showed a relatively low value of hydration energy. At least partial dehydration of molecules of a water-soluble antioxidant is a prerequisite for free radical scavenging to occur in a lipophilic membrane.

4. Conclusion

The hydroxylated alkaloids jatrorrhizine (**II**) and magnoflorine (**III**) isolated from *M. aquifolium* could act as potent scavengers of peroxyl radicals. The presence of free phenolic group on the skeleton was shown to be essential for good intrinsic antioxidant activity. The overall antioxidant efficiency of these compounds in liposomal bilayer, comparable with that of the effective scavenger of peroxyl radicals stobadine **IV**, was ensured by the rather high lipophilicity, and was significantly hindered by hydration of the molecules.

5. Experimental

5.1. Chemicals

Stobadine ((−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]-indole dihydrochloride) (**IV**) was prepared at the Institute of Experimental Pharmacology SAS.[35] Cumene hydroperoxide (~80% in cumene) and 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Egg yolk L-α-phosphatidylcholine dioleoyl (C18:1, [*cis*]-9) (DOPC) (99% grade), 2,6-di-*t*-butyl-*p*-cresol (BHT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel plates G F_{254} were obtained from Merck Other chemicals were purchased from local commercial sources and were of analytical grade quality. All solvents used for lipid peroxidation studies were deareated under nitrogen.

5.2. Plant material

The stem bark of *M. aquifolium* (Pursh) Nutt. (Berberidaceae) was collected in October 1998 in the Arboretum Tesárske Mlyňany, Slovakia. Voucher specimens are deposited at the Herbarium of the Faculty of Pharmacy, Comenius University, Bratislava (No. Ma-108/8). Pure protoberberine alkaloids obtained as the iodide, berberine (**I**) (mp 261 °C), jatrorrhizine (**II**) (mp 212 °C), and one aporphine alkaloid magnoflorine (**III**) (mp 252 °C, $[\alpha]_D^{24} + 201$ (*c* 0.2, $CHCl_3$)) were isolated from dried and powdered stem bark according to the method of Volleková et al.[36] and identified by NMR spectroscopy.

Copies of the original spectra are obtainable from the author of correspondence.

5.3. DPPH test

To investigate antiradical activity of the alkaloids (**I**, **II**, and **III**) in homogeneous solution, 0.1 mL water solution of the antioxidant tested (1 mM) was added to 2.9 mL ethanolic solution of DPPH (62.1 μM) to give the final concentrations 33.3 and 60 μM for the antioxidant and DPPH, respectively. The solution of stobadine (**IV**) was neutralized by solution of NaOH (1 M) before its addition to the reaction mixture. Absorbance decrease of the reaction mixture was continuously recorded at $\lambda_{max} = 518$ nm.

5.4. Liposome preparation and incubation

Due to the high lipophilicity of the alkaloids tested, we used liposomes enriched by these antioxidants prepared as follows:[37] DOPC (15.7 mg) was placed in a round-bottom flask and dissolved in chloroform (5 mL) together with a suitable amount of the alkaloids tested dissolved in methanol. The solvent mixture was subsequently removed under nitrogen and the resulting thin film on the walls was dispersed in phosphate buffer (20 mL, 20 mM, pH 7.4) by vigorous stirring for 2 min followed by sonification for the same period of time. Suspension of unilamellar liposomes (1 mM DOPC) was thus obtained. The liposomes (final concentration 0.8 mM DOPC) enriched by the antioxidants tested (**I**–**III**) (final concentration 60 μM) were incubated in the presence of the water-soluble initiator AAPH (final concentration 10 mM) at 50 °C for different periods of time. The water-soluble antioxidant standards stobadine (**IV**) and Trolox (**V**) were added to prepared liposomal suspension dissolved in phosphate buffer to give the final concentration 60 μmol/L.[32]

5.5. LOOH determination

Aliquots (1 mL) of the incubation mixtures were extracted by 2 mL portions of ice-cold mixture $CHCl_3$/MeOH (2:1, v/v) containing BHT (0.5%). Lipid hydroperoxide content was determined by thiocyanate method according to Mihaljević[38] by sequentially adding $CHCl_3$/MeOH (2:1, v/v) mixture (1.4 mL) and the thiocyanate reagent (0.1 mL). The reagent was prepared by mixing equivalent volumes of methanolic solution of KSCN (3%) and ferrous-ammonium sulfate solution (45 mM in 0.2 mM HCl). After leaving the mixture at ambient temperature for at least 5 min, the absorbance was at 500 nm recorded by Hewlett-Packard Diode Array Spectrophotometer 8452A. The lipid peroxide value was determined by using the calibration curve prepared with standard cumene hydroperoxide.

5.6. Determination of R_M values

The lipophilicity parameters represented by R_M values were measured by reversed-phase thin layer chromatography technique.[39,40] The mobile phase consisted of phosphate buffer solution (pH 7.4; 0.1 M) mixture with acetone (20:80, v:v). The stationary phase was obtained by impregnation of the layer of Silica gel G F_{254} plates with 5% solution of liquid paraffin in ether. The method of impregnating the plates was described elsewhere. The compounds were dissolved in methanol and about 1 μL of the solution was spotted onto the plates. A migration of 10 cm was obtained by spotting the compound on a line 2 cm from the lower edge of the plate. The developed plates were dried and the compounds were detected in UV light at 254 nm. The R_M values were calculated by the formula: $R_M = \log(1/R_F - 1)$.

5.7. Computational methods

The lowest energy molecular conformations of the alkaloids possessing OH group and of the standard antioxidants stobadine and Trolox were calculated using HyperChem molecular modeling software[28] using Austin model 1, Polak–Ribiere conjugate gradient algorithm with 0.01 convergence limit in vacuum. For optimal conformers of antioxidants the total energies E_A were calculated. As the geometric optimization for radicals formed with the abstraction of hydrogen from the phenolic oxygen or indolic nitrogen was performed, corresponding E_F were calculated along with the energy of the hydrogen radical E_H creating thus the theoretical measures of antioxidant activity (1)

$$\mathrm{BDE} = E_F - E_A + E_H \quad (1)$$

The hydration energies (E_h) were also calculated for all the compounds tested.

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