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Effects of diterpene alkaloids on lipid peroxidation in mitochondria

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Abstract

Antioxidant activity of the alkaloids songorine, napelline, and 1-O-benzoylnapelline has been studied in rat liver mitochondria by lipid peroxidation. Songorine, napelline, and 1-O-benzoylnapelline alkaloids had a protective effect on mitochondria, reduced the damaging effect of Fe^{2+}/ascorbate and releasing of malondialdehyde (MDA) into the secondary products of peroxidation. The effect of alkaloids songorine, napelline, and 1-O-benzoylnapelline on the processes of MDA formation in rat liver mitochondria *in vitro* has been studied. The alkaloids napelline and songorine at 200 μM of concentration inhibited the formation of MDA by 54 and 44 %, respectively, and 1-O-benzoylnapelline at this concentration inhibited by 95 %. Obtained data revealed that 1-O-benzoylnapelline more strongly inhibited the formation of MDA compared to songorine and napelline.

Introduction

Mitochondria play a central role in the energy metabolism of a cell. Oxidative disbalance in mitochondria develops against the background of oncogenic, neurodegenerative, cardiovascular, and other diseases (Figueira *et al.* 2013). One of the mechanisms of mitochondrial disorders is the intensification of lipid peroxidation (LPO). Activation of LPO is often one of the trigger mechanisms of a number of diseases and is also an aggravating factor in many pathological conditions (Lobo *et al.* 2010). The LPO process leads to damage of the structural organization of cell membranes, changes in membrane permeability, decreasing of membrane potential, uncoupling of oxidative phosphorylation and hydrolysis of ATP, decreasing in the rate of electron transfer along the respiratory chain. Free radical chain oxidation of unsaturated fatty acids is known to play an important role in the normal functioning of cells as well as in pathological processes and in the pathogenesis of various types of liver damage (Lobo *et al.* 2010).

Lipid peroxidation product (MDA) characterizes the state of the antioxidant system in the organism. The study of the role of LPO in the regulation of the most important cell functions is of interest for a number of reasons. The effect of lipid peroxidation on mitochondrion functions is carried both at the level of the direct effect of LPO products on the

lipid matrix of membranes and at the level of various indirect effects. Antioxidants are able to neutralize the activity of free radicals, protect the phospholipids of cell membranes from oxidation (Kiplimo *et al.* 2011). The mechanisms of action and antioxidant properties of biologically active substances isolated from plants are extensively studied in order to inhibit the process of LPO caused by oxidative stress, to correct membrane disorders. It is known that plant compounds are the main source of biological material in the production of drugs with antioxidant properties (Almeida *et al.* 2006). The antioxidant properties of aporphine alkaloids and flavonoid have been studied (Kiplimo *et al.* 2011; Vetrova *et al.* 2017). Diterpene alkaloids are a promising class of plant substances with a wide spectrum of pharmacological activity. They have antiarrhythmic (Dzhakhangirov *et al.* 1997; Shakhidoyatova *et al.* 2001), antispasmodic (Dzhakhangirov *et al.* 2013), antioxidant (Khan *et al.* 2018), antidepressant (Nesterova *et al.* 2011), antimetastatic (Kruczynski *et al.* 2006), anti-inflammatory (Nesterova *et al.* 2013; Marya and Khan 2017), and other effects. Diterpene alkaloids are preferred over narcotic and non-narcotic analgesics. They affect the accumulation of nerve impulses that are not enough to induce strong arousal, such as morphine, without the side effects of drug dependence, and can be used both for acute (e.g. postoperative) pain and for pathologically debilitating pain. The studied diterpenoid alkaloids and their derivatives have advantages over popular drugs and non-narcotic analgesics. In addition to their effect on central and visceral pain, these alkaloids have myotropic and antispasmodic effects (Dzhakhangirov *et al.* 2013). Since the mechanisms of action of diterpene alkaloids on mitochondria have not been previously studied, we aimed to study the effect of these alkaloids on the LPO process in mitochondria.

Experimental

Extraction and separation of alkaloids

The homogeneity of the substances was tested on plates with silica gel on TLC (Fluka Analytical, Germany) brand in the benzene-ethanol system in ratios of 9 : 1, 20 : 1, the chloroform-methanol system in ratios of 9 : 1 and plates with aluminum oxide in benzene-ethanol 9 : 1. For column chromatography, silica gel of the TLC brand and deactivated aluminum oxide of the "For Chromatography" brand were used. IR spectra were taken on a Perkin-Elmer-2000 instrument; ^{1}H NMR and ^{13}C spectra were taken on a JEOL instrument (400 and 600 MHz Japan) in a CDCl3 solution.

Extraction of alkaloids of the Aconitum karakolicum plant

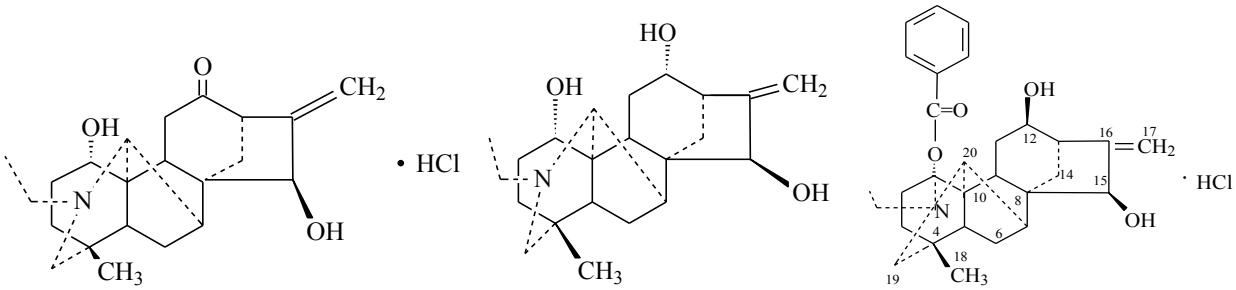
1.84 kg of air-dry crushed aboveground *Aconitum karakolicum* was exhaustively extracted with 80 % ethanol. The extracts were combined and condensed. The aqueous residue was alkalized with NaOH to pH 12 and exhaustively extracted with chloroform. After distilling the solvent, 9.18 g of the total alkaloids were obtained, which was 0.49 % of the plumb line of the dry plant.

Isolation of alkaloids from Aconitum karakolicum

The amount of alkaloids was divided on a column with aluminum oxide (300 g). The alkaloids were eluted with cyclohexane, cyclohexane-acetone (1 : 1), chloroform-methanol (5 : 1), (1 : 1), and a 5 % solution of sulfuric acid. The acidic solution was alkalized with NaOH to pH 7, 8, 9, 10, 11, 12, respectively, and according to the basicity strength they were exhaustively extracted into 6 fractions with chloroform. From the fraction 4, 0.14 g of songorine was separated using methanol. Fractions 3, 5, and uterine solution 4 fractions were separated on the silica gel column, eluding with chloroform, gradually adding methanol, and 0.13 g of songorine, and 0.12 g of napelline were isolated (Sultankhodzhaev *et al.* 1978). Alkaloid songorine was also isolated from *Aconitum monticola* by the above methods (Nezhevenko *et al.* 1975). The 1-O-benzoylnapelline was obtained by synthesis from these alkaloids (Dzhakhangirov *et al.* 2013; Sultankhodzhaev *et al.* 2017).
The structural formulas of alkaloids were drawn by the ChemOffice 2002, ChemDraw Ultra 7.0 software (Fig. 1).

Animal treatment

In the experiments, white outbred male rats, weighing 180–220 g, were used (obtained from the vivarium of the Pharmacology Department



Songorine Napelline 1-O-benzoylnapelline

Fig. 1. Structural formulas of diterpene alkaloids studied in this work.

of the Institute of Bioorganic Chemistry of the Academy of Sciences of the Republic of Uzbekistan, Tashkent) in accordance with the ethical rules for working with laboratory animals, according to the "Regulations on the bioethics of the use of laboratory animals in scientific research" of Institute of Biophysics and Biochemistry at the National university of Uzbekistan named after Mirzo Ulugbek (Protocol dated 22. 02. 2019). The rats were housed under standard laboratory conditions (20–24 °C; natural light regime of sunlight; 65 % humidity, food and water available *ad libitum*), immobilized with light ether anesthesia and decapitated.

Isolation of mitochondria

Mitochondria were isolated from livers by conventional differential centrifugation described by Schneider and Hageboom (1951). Rat liver was homogenized in a medium containing 250 mM sucrose, 10 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 1 mM ethylenediamine tetraacetic acid Na_2-salt (EDTA), pH 7.4 centrifuged at 1,500 × g for 7 min (-2 ° C, -4 °C). Mitochondria were sedimented by centrifugation of supernatant at 6,000 × g for 15 min (-2° C, -4°C). The final mitochondrial pellet was suspended in a small volume of medium containing 250 mM sucrose, 10 mM Tris-HCl, was kept on ice prior to experiments. The mitochondrial protein content was determined by the Lowry method modified by Peterson (1977).

Lipid peroxidation as measured by Fe^{2+}/ascorbate

LPO was recorded by inhibition of Fe^{2+}/ascorbate-dependent liver mitochondrial swelling by photometric method in incubation medium contained 125 mM potassium chloride (KCl), 10 mM Tris-HCl, pH 7.4, the final amount of protein in the incubation medium was 0.4 $mg.mL^{-1}$ (Schneider *et al.* 1948). 10 μM ferrous sulfate heptahydrate ($FeSO_4$) and 200 μM ascorbic acid were added to induce mitochondria swelling. All experiments were conducted at 24–26 °C so that the integrity of the mitochondria was maintained during incubation. The antioxidant activity of the tested compounds was measured by inhibition of Fe^{2+}/ascorbate-dependent swelling of rat liver mitochondria at 540 nm. The choice of such a methodological approach is due to the fact that a linear correlation relationship between the intensification of LPO processes, induced by the Fe^{2+}/ascorbic acid system, and swelling of mitochondria was previously established. LPO in the Fe^{2+}/ascorbate system on the mitochondrial membrane was also assessed by other authors by measuring the swelling of mitochondria under conditions of LPO activation (Almeida *et al.* 2006) which indicated the suitability of using this model as a test system for studying the antioxidant properties of various substances.

The intensity of lipid peroxidation in mitochondrial membranes is determined by measuring the concentration of the final product, malondialdehyde (MDA). Peroxides of unsaturated fatty acids with 2-3 diene bonds, formed during LPO, are

ultimately converted into MDA, which interacts with thiobarbituric acid. Induction of non-enzymatic Fe^{2+}/ascorbate-dependent LPO was performed by adding 10 µM $FeSO_4$ and 200 µM ascorbic acid to the incubation medium containing 125 mM KCl, 10 mM Tris-HCl, pH 7.4. The separation of LPO products was carried out in the presence of thiobarbituric acid (TBA). The reaction was stopped by adding 0.220 mL of 70 % (w/v) trichloroacetic acid to incubation medium. Thereafter, the mitochondrial suspension was centrifuged at 1,500 × g for 15 min. Then, 2 mL of supernatant was taken and poured in 1 mL of 75 % TBA (w/v). 2 mL of H_2O and 1 mL of TBA were added to the control solution. The mixture was incubated for 30 min in a water bath. After cooling, a change in optical density was detected at a wavelength of 540 nm (Δ_{540}). The amount of formed MDA was calculated using the molar extinction coefficient (e = 1.56 × 10^5 $M^{-1}.cm^{-1}$) (Devienne *et al.* 2007) according to the following formula: nmol MDA/mg protein = D/1.56 × 30. The amount of mitochondrial protein was 0.3–0.4 mg per 1 mL of incubation medium.

Drugs and chemicals

The following chemical reagents were used: EDTA (Sandoz, Switzerland), Tris-HCl (Serva, Germany), sucrose, $FeSO_4$, ascorbic acid, KCl, TBA, trichloroacetic acid (Chemreaktivsnab, Russia). All reagents were p.a. grade.

Data analysis

The results were analysed statistically using the Origin Pro 7.5 (Microsoft, USA). The data were evaluated using parametric Student's t-test and are expressed as M ± m. The results that were deemed significant are indicated as follows: * $P<0.05$ and ** $P<0.01$.

Results

A number of properties of diterpenoid alkaloids have been identified by our colleagues. They investigated the vasorelaxant effect of these alkaloids (1-O-benzoylnapelline, songorine, zeravshanizine, l-O-benzoylkarakoline, 14-O-benzoyltalatisamine) when inhibiting the entry of Ca^{2+} ions through Ca^{2+} L and Ca^{2+} R-channels of the plasma membrane and their release from the sarcoplasmatic reticulum. The effect of these alkaloids on K_{ATP} channels in smooth muscle cells were also studied, while they protect the rat aorta from damage as a result of hypoxia, showing a vasoprotective effect (Mirzayeva *et al.* 2016; Yesimbetov *et al.* 2019). It was found that the diterpenoid alkaloid songorine (Muratova *et al.* 2021) and 1-O-benzoylnapelline (Muratova *et al.* 2020), similar to diazoxide, activate the mitoK_{ATP}-channel. Napellin-type diterpene alkaloids and their aromatic ester-free derivatives exhibit weak spasmolytic activity, while its derivative 1-O-benzoylnapelline has spasmolytic activity (Dzhakhangirov *et al.* 2013). The high antioxidant/antiradical activity of polyphenol compounds has also been studied (Gayibov *et al.* 2019).

Here was studied the effect of 1-O-benzoylnapelline, napelline, and songorine on LPO processes in the membrane mitochondria *in vitro* experiments. Fe^{2+}/ascorbate was used as the LPO inducer. As a result of the studies, it was found that the addition of Fe^{2+}/ascorbate to the incubation medium increased the rate of swelling of mitochondria by (t–5 min, ΔA_{540} = 0.330 ± 0.012) 100 % compared to the control. Experiments have shown that the addition of Fe^{2+}/ascorbate to the incubation medium causes swelling of mitochondria in comparison with the control, which indicates LPO and permeabilization of mitochondrial membranes. A study of the effect of 1-O-benzoylnapelline on Fe^{2+}/ascorbate induced mitochondrial swelling showed a concentration of 0.1 µM inhibited mitochondrial swelling by 19.7 ± 2.9 %. Experiments have shown that 1-O-benzoylnapelline had a concentration-dependent inhibitory effect on mitochondrial swelling (Fig. 2). Adding 1-O-benzoylnapelline to the incubation medium at a concentration of 0.5 µM for 39.8 ± 2.3 %, 1 µM for 64.9 ± 1.9 %, 5 µM for 79.5 ± 1.7 %, and 10 µM for 96.5 ± 1.2 % prevented the effect of Fe^{2+}/ascorbate on LPO. Experiments have shown that the maximum efficiency was observed at a concentration of 10 µM and the half-maximum inhibitory concentration (IC_{50}) of the fraction on mitochondrial swelling is 0.79 µM. Also, similar results were obtained with the action of napelline

on Fe^{2+}/ascorbate-induced swelling of mitochondria. Napelline also inhibits Fe^{2+}/ascorbate-induced mitochondrial swelling. Adding napelline to the incubation medium at a concentration of 10 µM at 12.4 ± 2.4 %, 25 µM at 30.1 ± 2.4 %, 50 µM at 47.3 ± 2.6 %, 75 µM at 64.5 ± 2.1 %, and 100 µM at 77.1 ± 1.8 prevented the effect of Fe^{2+}/ascorbate on lipid peroxidation (Fig. 3).

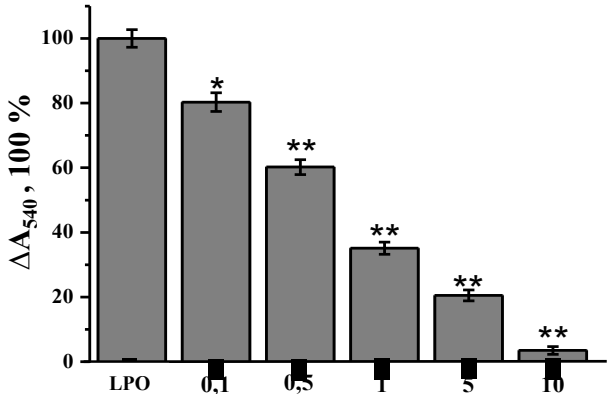


Fig. 2. Effect of 1-O-benzoylnapelline on mitochondrial swelling induced by lipid peroxidation. The *y-axis* shows the swelling of mitochondria by Fe^{2+}/ascorbate and the *x-axis* shows the concentration of 1-O-benzoylnapelline (µM) (*$P<0.05$; **$P<0.01$; n = 6, error bars indicate ± SE of the mean).

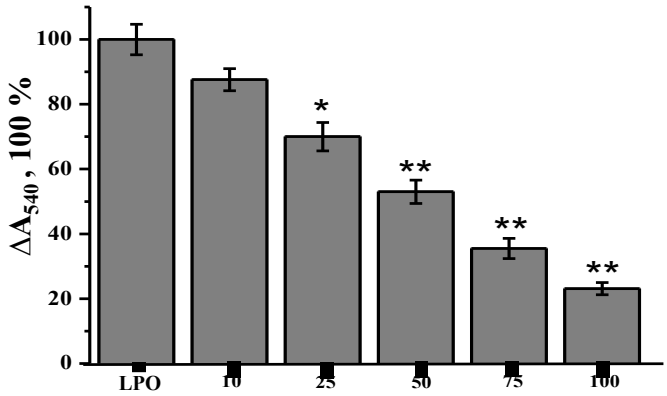


Fig. 3. Effect of napelline on mitochondrial swelling induced by lipid peroxidation. The *y*-axis shows the swelling of mitochondria by Fe^{2+}/ascorbate and the *x*-axis shows the concentration of napelline (µM) (*$P<0.05$; **$P<0.01$; n = 6, error bars indicate ± SE of the mean).

Subsequently, the effect of songorine on the LPO system induced by Fe^{2+}/ascorbate has been studied (Fig. 4). Songorine also prevented the effect of Fe^{2+}/ascorbate on the swelling of rat liver mitochondria. Thus, songorine at concentrations of 10, 25, 50, 75, and 100 µM, respectively, inhibited mitochondrial swelling by 10.8 ± 1.3 %, 23.6 ± 2.5 %, 44.8 ± 2.4 %, 57.5 ± 1.9 %, and 78.0 ± 1.5 % compared with control. When investigating the alkaloid songorine at concentrations of 10, 25, 50, 75, and 100 µM, its inhibitory action on Fe^{2+}/ascorbate-induced swelling of mitochondria turned out to be concentration-dependent. Experiments have shown that the maximum efficiency was observed at a concentration of 100 µM songorine. The experiments showed that the addition of the Fe^{2+}/ascorbate system to the incubation medium increased the accumulation of MDA in mitochondrial membranes (3.15 nmol.MDA.mg.protein^{-1}) 100 % relative control. The addition of 1-O-benzoylnapelline at a concentration of 50 µM prevented the formation of MDA on LPO membranes by 33 ± 3.2 %.

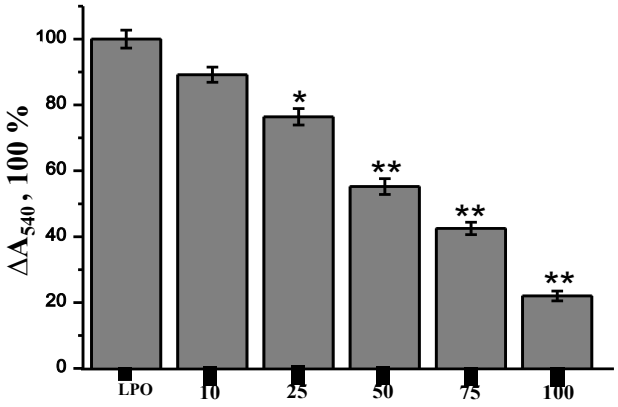


Fig. 4. Effect of songorine on mitochondrial swelling induced by lipid peroxidation. The *y*-axis shows the swelling of mitochondria by Fe^{2+}/ascorbate and the *x*-axis shows the concentration of songorine (µM) (*$P<0.05$; **$P<0.01$; n = 6, error bars indicate ± SE of the mean).

Inhibition of the LPO process was also observed under the action of other concentrations of 1-O-benzoylnapelline. Thus, the alkaloid 1-O-benzoylnapelline at a concentration of 100 µM reduced the accumulation of MDA to 60 ± 2.9 %, 150 µM by 79 ± 2.8 %, 200 µM by 95 ± 2.3 % (Fig. 5). In the next series of experiments, the effect of napelline on the LPO system induced by Fe^{2+}/ascorbate (Fig. 5) was studied by adding 50, 100, 150, and 200 µM of napelline alkaloid to the incubation medium. These concentrations of alkaloids reduced the accumulation of MDA to 12 ± 3.5 %, 32 ± 3.3 %, 45 ± 3.1 %, and 54 ± 2.7 %. Under these conditions, the effect of songorine on the LPO system induced by Fe^{2+}/ascorbate was

studied (Fig. 5). The addition of an alkaloid at a concentration of 50 µM by 4 ± 2.6 % and 100 µM by 20 ± 3.6 % prevented the formation of MDA on LPO membranes. Alkaloid songorine at a concentration of 150 µM reduced the accumulation of MDA to 34 ± 2.7 % and 200 µM by 44 ± 2.9 %.

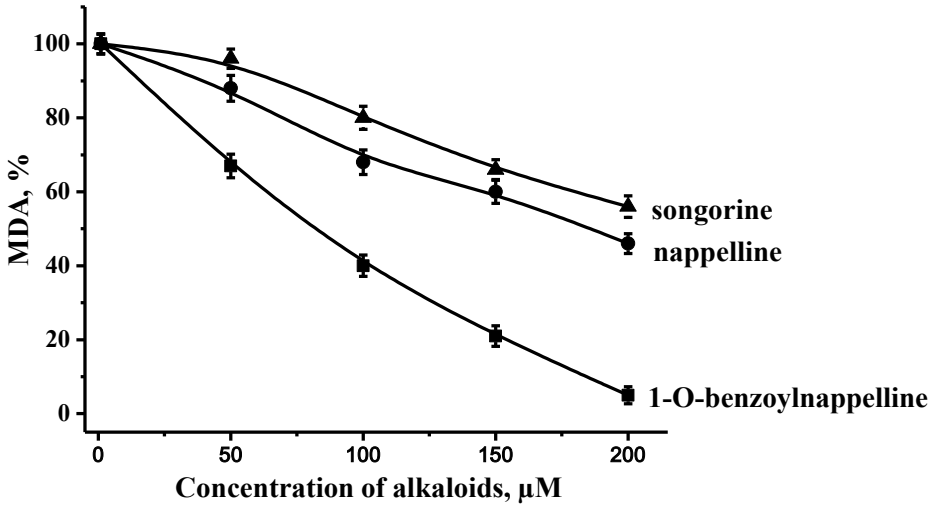


Fig. 5. The influence of different concentrations of alkaloids, MDA accumulation on LPO in mitochondria induced by Fe^{2+}/ascorbate (n = 5, error bars indicate ± SE of the mean).

Discussion

The pharmacological properties of diterpene alkaloids and their derivatives and structural activity relationships have shown that many alkaloids and their derivatives have clear antiarrhythmic activity. The analgesic and anti-inflammatory properties of 1-O-benzoylnapelline (Sultankhodzhaev *et al.* 2017) and the increase in the rate of recovery of granulocytic hematopoiesis of napellin have also been studied (Zyuz'kov *et al.* 2013).

Thus, we have established that the alkaloid 1-O-benzoylnapelline acts more effectively on LPO in the mitochondrial membrane than other alkaloids. We found that 1-O-benzoylnapelline acts more effectively than napelline on the mitoK_{ATP} channel of rat liver mitochondria (Muratova *et al.* 2020). Because, 1-O-benzoylnapelline effectively reduced the damaging effect of Fe^{2+}/ascorbate and the releasing of MDA, due to the introduction of a benzoyl group into the napelline structure at positions C(1), respectively. It is known that LPO led to a change in the permeability of biomembranes, decreasing in membrane potential, uncoupling of oxidation-phosphorylation, and hydrolysis of ATP in mitochondria. The effect of LPO on mitochondria function was realized both at the level of the direct effect of LPO products on the lipid matrix of membranes and various indirect effects. Literature data have been shown that alkaloids significantly improve neurobiological outcomes and reduce oxidative stress and neuronal apoptosis. This is associated with a significant decrease in lipid peroxidation, an increase in superoxide dismutase, and a decrease in glutathione levels (Ishii *et al.* 2018; Singh *et al.* 2019).

Conclusions

Our results show that the doses used in the LPO study ranged from 495 µg.L^{-1} to 4.95 mg.L^{-1} for 1-O-benzoylnapelline, from 3.95 mg.L^{-1} to 39.5 mg.L^{-1} for the alkaloid songorine, and from 3.59 mg.L^{-1} to 35.9 mg.L^{-1} for the alkaloid napelline. It can be concluded that the doses we have studied are close to therapeutic doses. Experiments on mitochondria showed that alkaloids prevented mitochondrial swelling caused by Fe^{2+}/ascorbate and inhibited MDA accumulation in membranes. These alkaloids have a protective effect on mitochondrial membranes by reducing the damaging effect of the Fe^{2+}/ascorbate system.

Conflict of Interest

The authors declare that they have no conflict of interest.

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