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Antioxidant Properties and Effects of Aporphine Alkaloids and Their Phenanthrene Seco-Isomers on Acetylcholinesterase Activity

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Abstract—Model phenanthrene seco-alkaloids (seco-glaucine and seco-boldine) obtained in the medium of subcritical water (SBW) from plant aporphine alkaloids have been studied for the first time as antioxidants and inhibitors of acetylcholinesterase (AChE). Antioxidant activities (in vitro) of model aporphine and phenanthrene alkaloids: boldine, seco-boldine, glaucine and seco-glaucine (BD, s-BD, GL and s-GL) were studied in the reaction with a stable free radical DPPH (1,1-diphenyl-2-picrylhydrazyl). In vivo, antioxidant activity was determined in a bioluminescent test system using genetically modified *E. coli* strains. In the experiments in vitro (DPPH test) and in vivo (biotest), phenanthrene alkaloids s-GL and s-BD demonstrate the higher antioxidant activity than their aporphine precursors GL and BD. The anticholinesterase activity of alkaloids and their phenanthrene seco-isomers was studied (in vitro) using Ellman's method with minor modifications. The data on the inhibitory activity of the AChE enzyme with aporphine and phenanthrene alkaloids expressed as IC_{50} values obtained from dose–response curves demonstrate that the inhibitory activity for seco-boldine (IC_{50} = 0.21 mM) and seco-glaucine (IC_{50} = 0.04 mM) is higher than for the initial aporphine alkaloids boldine (IC_{50} = 0.29 mM) and glaucine (IC_{50} = 0.44 mM), respectively. Thus, it has been shown that phenanthrene alkaloids obtained in SBW exhibit the higher antioxidant activity and the better inhibitory AChE activity than their aporphine precursors.

Keywords: subcritical water, aporphine alkaloids, glaucine, boldine, antioxidant activity, anticholinesterase activity, phenanthrene alkaloids, seco-glaucine, seco-boldine, acetylcholinesterase, Alzheimer's disease

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INTRODUCTION

In the past decade there has been an avalanche-like increase in the number of works aimed at synthesizing novel pharmaceutical substances on the basis of plant metabolites successfully used in folk medicine. Aporphine alkaloids are one of the promising groups of plant metabolites. Aporphine alkaloids **I** (Fig. 1) demonstrate a wide range of biological and antioxidant activity [1, 2]. It has been shown [3] that plant and semisynthetic aporphine **I**-related phenanthrene derivatives **II** (seco-alkaloids) often exhibit higher antioxidant activity and can demonstrate new therapeutic applications different from those of the original aporphine substances. In addition, it is known that phenanthrene seco-alkaloids **II** obtained from plant aporphines **I** can be used as initial substances for the synthesis of tetrahydronaphtho-[2,1-f] isoquinoline alkaloids **III** (so-called litebamines) with a broad range of biological activities. According to the published data, litebamine is able to inhibit acetylcholinesterase [4], which makes it, as well as this class of compounds, a promising agent for treating Alzheimer's disease [5–7].

Previously, it has been shown that litebamine and its derivatives can be obtained from the aporphine alkaloid glaucine through stepwise recycling via its phenanthrene analog, seco-glaucine [8]. In general, the development and investigation of novel pharmaceutical substances such as, e.g., litebamine and its derivatives **III**, starting with plant aporphines, seems to be relevant from both theoretical and practical viewpoints.

Recent works have demonstrated the production of phenanthrene seco-alkaloids from aporphines in the subcritical water (SCW) medium by the example of glaucine and boldine (Fig. 2) [9–12].

Glaucine-(S)-*N*-methyl-1,2,7,8-tetramethoxydibenzo [de, g] octahydro-quinoline isolated from yellow horned poppy has a broad range of biological activities [13, 14]. The availability of glaucine makes it a convenient model for chemical modifications. Boldine-[(S)-2,9-dihydroxy-1,10-dimethoxyaporphine] is widely known as a powerful plant antioxidant and demonstrates anti-inflammatory, antitumor, antidiabetic and cytoprotective effects. The studies aimed at

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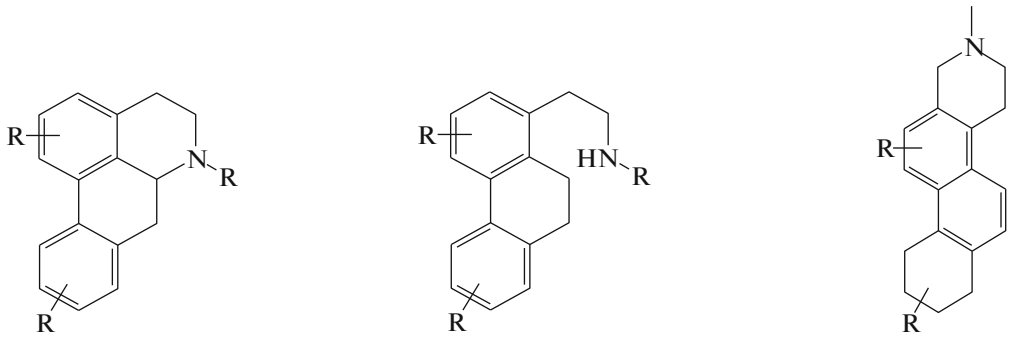
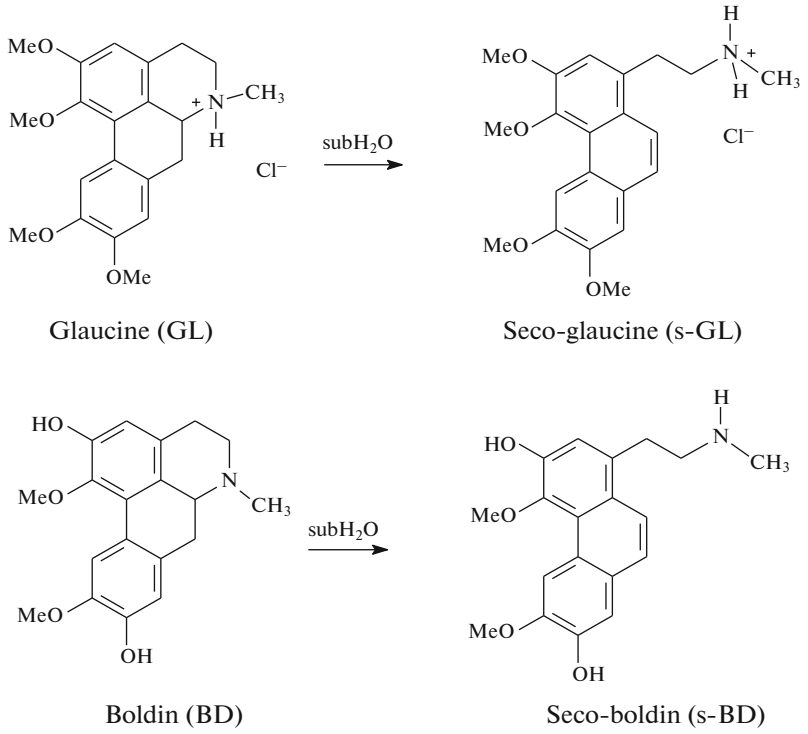


I
Aporphine alkaloids

II
Phenanthrene alkaloids (secoderivatives)

III
Litebamines (tetrahydronaphtho-[2,1-f isoquinolines).

Fig. 1. Structural formulas of typical plant alkaloids and their derivatives.



Glaucine (GL)

Seco-glaucine (s-GL)

Boldin (BD)

Seco-boldin (s-BD)

Fig. 2. Structural formulas of compounds under study and the scheme of transformation in the SCW medium: glaucine into seco-glausine, boldine into seco-boldine.

the elucidation of how the antioxidative effect of boldine works have shown that the boldine molecule acts as an effective absorber of hydroxyl radicals (HO). According to literature data, boldine and seco-boldine are able to inhibit ROS generated in endothelial cells by angiotensin II [15]. It is important that semisynthetic seco-alkaloids are able to prevent lipid peroxidation and protein damage and/or to absorb free radicals [16].

It is known that the generalized inflammatory response is related to pathogenesis of different neurodegenerative diseases, e.g., atherosclerosis and Alzheimer's disease, which are now believed to have an inflammatory basis, and this can lead to construction of new therapeutic strategies for controlling these diseases [17]. One of such strategies is the search of plant metabolites exerting a neuroprotective effect through inhibition of AChE or oxidative stress. Such a promis-

ing group of plant metabolites can be aporphine alkaloids (glaucine, boldine) and phenanthrene alkaloids on their basis (seco-glaucine and seco-boldine). The analysis of the published data has shown that only a few research works are devoted to investigation of the inhibitory activity of these compounds [18, 19].

The work [20] was devoted to the study of activity of the alkaloids boldine (IC_{50} = 8.6 μM) and seco-boldine (IC_{50} = 10 μM) obtained from methanol extracts of the plants *B. alloiophylla* and *B. kunstleri,* the genus *Beilschmiedia*, the family *Lauraceae*. Another research team has studied the inhibitory properties of boldine against AChE and butyrylcholinesterase (BuChE) and shown that it can inhibit both enzymes, demonstrating a noncompetitive mechanism of inhibition, which is the most widespread inhibitory mechanism for choline esterases [18]. Recently [21, 22], the experiments in vivo have shown that oral administration of boldine for 12 weeks to APP/PS1 mice (the mouse model of Alzheimer's disease) has an anti-inflammatory effect on hippocampal neurons. In addition to the reduction of oxidative stress markers and the area of infarct, mice demonstrate the increased exploratory activity along with the reduction of deficit in spatial object recognition and working memory deficit. Learning and memory were considerably improved after intraperitoneal administration of boldine over seven consecutive days to both young and old mice, without affecting locomotion (the movement of animals in space). It attenuated oxidative stress in the brain, reduced the levels of malondialdehyde and nitrite, increased the level of glutathione, and suppressed the acetylcholinesterase activity [23]. Thus, aporphine alkaloids (glaucine, boldine) and their phenanthrene derivatives (seco-glaucine, seco-boldine) may be a new interesting pharmacological tool for attenuating the progression of pathologics in Alzheimer's disease.

In this context, the present work was aimed at studying and comparing the antioxidant and anticholinesterase properties of aporphine and phenanthrene alkaloids with the purpose of searching for new AChE inhibitors based on plant metabolites, which are aimed at treating Alzheimer's disease.

EXPERIMENTAL

Chemical compounds (reagents): acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) (AChE, type VI-S,3.1.1.7, 200–1000 units/mg protein), acetylcholine iodide (AChI) (≥98%, United States), 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) (99%, United States, ReagentPlus®: ReagentPlus is the registered trademark of Sigma-Aldrich Co. LLC), boldine (analytical standard, United States), 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Glaucine hydrochloride (analytically pure) was produced at the Shymkent Chemical-Pharmaceutical Factory (Kazakhstan) and is a racemic mixture.

Determination of antioxidant activity (AOA) of alkaloids by DPPH assay (in vitro) and bioluminescence test (in vivo). The antioxidant activities (in vitro) of aporphine and phenanthrene alkaloids: boldine, seco-boldine, glaucine and seco-glaucine (BD, s-BD, GL and s-GL), were studied in the reaction with a stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical [24, 25] as described [26].

In vivo antioxidant activity of alkaloids studied under the conditions of H_2O_2-induced oxidative stress was determined in a bioluminescent test system [27, 28] with a genetically modified *E. coli* strain (MG1655 pKatG-lux) obtained at the State Research Institute of Genetics and Selection of Industrial Microorganisms (Moscow) [27]. Hydrogen peroxide was used as a suitable source of hydroxyl radicals, which are the most reactive free radicals of oxygen. Hydrogen peroxide was added at a concentration of 10^{-2} M. Bioluminescence intensity in the cultures containing analyzed compounds and in the control cultures was measured for 2–3 h with a LM-01A 96-well microplate luminometer (Immunotech, Czechia).

The AOA of glaucine and seco-glaucine has been studied previously using the above test systems in the work [26], while the data on the antioxidant activity of boldine and seco-boldine obtained from the biotest and DPPH assay are presented for the first time.

Determination of the anticholinesterase properties of alkaloids. The anticholinesterase activity was studied (in vitro) by the Ellman method [29] with minor modifications as described [30]. The kinetic studies were performed with the commercial enzyme AChE from electric eel (lyophilized powder containing Tris buffer salts) from Sigma.

Working solution of the enzyme (2 U/mL) was prepared in phosphate buffer at pH 7.0 (0.02 M). Enzyme solutions and reagents were stabilized with phosphate buffers, pH 7.0 and 7.4 (0.1 M). The working solutions of DTNB (C = 0.25 mM) and AChI (C = 1.88 mM) were prepared in phosphate buffer, pH 7.4. The main alkaloid solutions (2 mM) were prepared in ethanol and then diluted to working concentrations with phosphate buffer (pH 7.4).

The activity of AChE inhibition by compounds under study was determined using a series of alkaloid solutions of different concentrations (from 0.1 to 1.7 mM). The reaction system was prepared at a room temperature of 25°C in a 1-cm quartz cuvette. The reaction was initiated by adding the enzyme. The reaction system consisted of 0.6 mL of alkaloid solution, 0.36 mL of acetylcholine iodide substrate (1.88 mM) and 1.44 mL of the Ellman–DTNB reagent (0.25 mM), followed by the addition of 0.12 mL of AChE solution (2 U/mL) after 5-min incubation. The blank sample (control) consisted of all chemical substances except for the inhibitor. The mixture was stirred, and the opti-

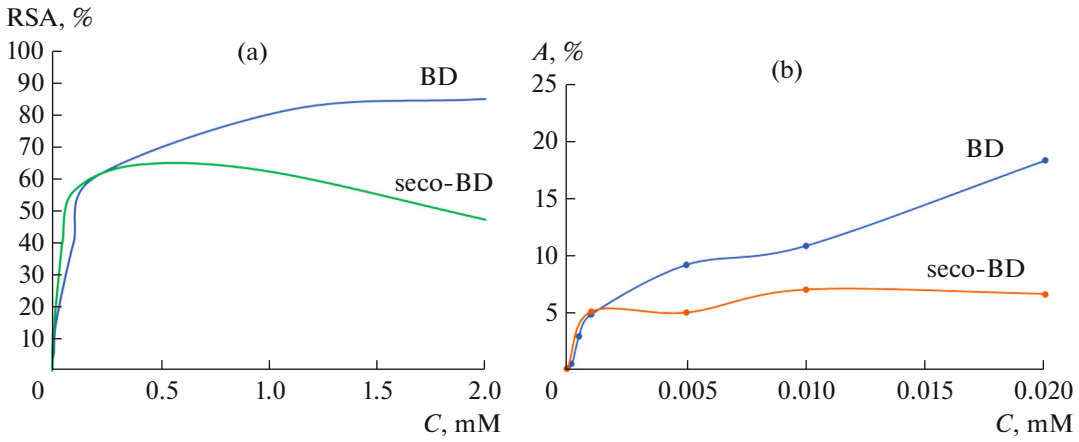


Fig. 3. Dependence of antioxidant activity AOA (RSA) on concentrations of alkaloids BD and s-BD (C = 0.001–2 mM) in the DPPH assay (a) and AOA values determined in the test in vivo with the strain *E. coli* MG1655 (pKatG-lux) under oxidative stress conditions (b).

cal density was measured at a wavelength of 412 nm for 6 min since the beginning of the reaction (after addition of the enzyme) with a SPEKS SSP 705 spectrophotometer (UV-Vid, 190–1100 nm) (Spectroscopy Systems, Russian Federation). The hydrolysis of acetylcholine iodide was controlled by the formation of yellow anion, 5-thio-2-nitrobenzic acid, as a result of reaction between DTNB and thiocholines, which is catalyzed by AChE. The measurements and calculations were assessed using the UV-VISanalyst software. The analysis was performed in triplicate (n = 3). The results are presented as the mean. The percentage of inhibition is calculated by the formula:

$$\%\ \text{of inhibition} = 1 - \left[\frac{\text{Test sample absorption at 412 nm}}{\text{Control absorption at 412 nm}}\right] \times 100.$$

The results were expressed as IC_{50} values calculated as a concentration of alkaloids that leads to 50% inhibition of the acetylcholinesterase activity.

RESULTS AND DISCUSSION

In accordance with the objectives of the present work, the AOAs of boldine (BD) and seco-boldine (s-BD) were studied at the initial stage for direct comparison of antioxidant activities of aporphine and phenanthrene alkaloids by the example of BD and s-BD.

Figure 3 shows the dependence between the antioxidant activity (AOA) on the concentration of alkaloids in the DPPH assay (Fig. 3a) and bioluminescence test (biotest) (Fig. 3b) for BD and s-BD.

As follows from the results (Fig. 3a), BD, in contrast to s-BD, shows an increase in antioxidant activity along with the increase in concentration. In case of phenanthrene s-BD, the decrease in AOA (RSA) at higher concentrations (above 0.1 mM) may be due to the low solubility of this alkaloid in ethanol. In the low concentration range, BD and s-BD demonstrate high antioxidant activities in the neutralization of radical states. Similar to the DPPH assay, the biotest in vivo showed the higher AOA values (in %) for BD solutions compared to s-BD at increasing concentrations of alkaloids.

The antioxidant activities (RSA) of four alkaloids under study at a concentration of 7×10^{-4} M were compared at the next stage. The results show the maximum activity of the phenanthrene alkaloid seco-glaucine in the DPPH assay and in the test with biosensor strains (Fig. 4).

At the next stage, the characteristics of inhibitory activity of the selected model compounds were studied.

Figure 5 shows the typical histograms of inhibitory activity for 1-mM glaucine and boldine solutions.

As follows from Fig. 5, the percentage of inhibition of glaucine and boldine solutions reaches the maximum values on minute 3 of the enzyme reaction. Therefore, dose–effect curves were based on the values of inhibition by alkaloids (%) obtained on minute 3 of the reaction with AChE (Fig. 6).

The results of studying the AChE inhibitory activity of aporphine and phenanthrene alkaloids expressed as IC_{50} values obtained from the dose–response curves demonstrate that the inhibitory activities of seco-boldine (IC_{50} = 0.21 mM) and seco-glaucine (IC_{50} = 0.04 mM) are higher compared to those of boldine (IC_{50} = 0.29 mM) and glaucine (IC_{50} = 0.44 mM), respectively.

The dose–effect curves for seco-glaucine and seco-boldine were also based on the values of inhibition by alkaloids (in %) obtained on minute 3 of the reaction with AChE (Fig. 6).

The analysis of literature data shows that aporphine alkaloids (glaucine, boldine) and their phenanthrene

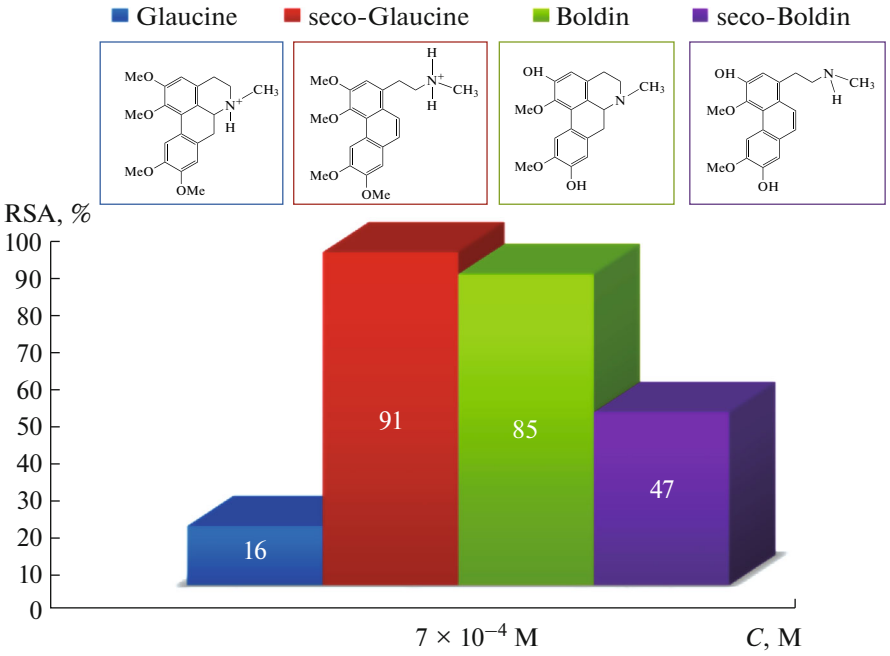


Fig. 4. Comparison of antioxidant activities of GL, s-GL, BD and s-BD in the DPPH assay.

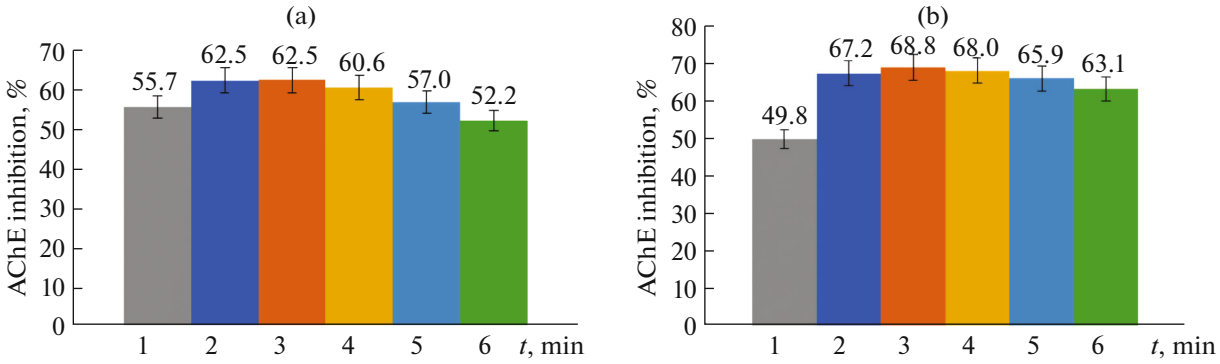


Fig. 5. AChE inhibition % values for 1-mM solutions of glaucine (a) and boldine (b) depending on the time of reaction with the enzyme.

derivatives (seco-glaucine, seco-boldine) can be a new interesting pharmacological tool for attenuating the progression of pathologies in Alzheimer's disease. In this context, the present work is devoted to the study and comparison of antioxidant and anticholinesterase activities of the aporphine alkaloids boldine and glaucine and their phenanthrene seco-derivatives. The results are given in the table.

Thus, it has been shown that phenanthrene seco-alkaloids obtained in SCW exhibit a higher inhibitory activity against acetylcholinesterase (AChE) compared to the initial aporphine alkaloids.

The dependences obtained made it possible to determine the "effective content" of alkaloid (EC_{50}), which is necessary to reduce the number of DPPH free

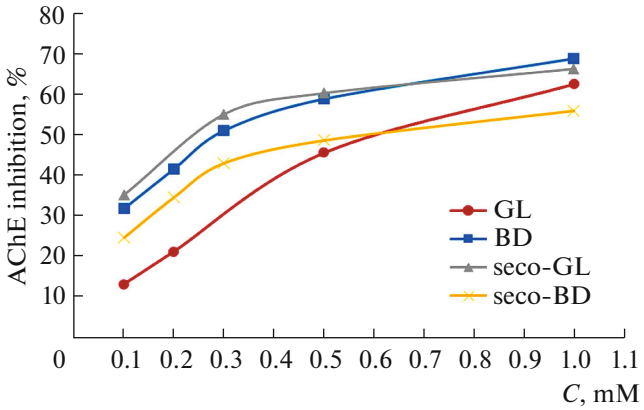


Fig. 6. AChE inhibition % curves (dose–effect) for the solutions of alkaloids (*C*, 0.1–1 mM).

Table 1. Anticholinesterase (anti-AChE) and antioxidant activity (AOA) values for aporphine and phenanthrene alkaloids obtained in the present work and literature data on bioactivity of these compounds

Chemical compound	IC_{50} (mM) for AChE inhibition			Effective concentrations EC_{50} (mM) for AOA of alkaloids	
				DPPH assay (in vitro)	antioxidant activity in bioluminescence test (in vivo)
data source	results of the present work	[20]	[18]	results of the present work	results of the present work
Boldine	0.29	0.0086	0.37	0.035	–
Seco-boldine	0.21	0.01	–	0.012	–
Data source		[19]		[26]	[26]
Glaucine	0.44	0.089		5.3	0.9
Seco-glausine	0.04	–	–	0.3	0.05

*The IC_{50} and EC_{50} values of alkaloids were determined by dose–response curves.

radicals two times (Table 1). The EC_{50} value was 0.3 mM for s-GL and 5.3 mM for GL, as described previously [26]. The data presented in the Table show that seco-boldine has the most effective concentration (EC_{50} = 0.012 mM), i.e., the 50% protective effect is achieved at a very low concentration of seco-boldine.

Here is should be noted that, according to the previously published data [18], the values of effective concentration (IC_{50}) for the phenanthrene alkaloid seco-boldine were less than for the aporphine alkaloid boldine tested for AOA in the systems with generation of reactive oxygen species ROS (oxidative stress), which is in agreement with our results.

The table also shows the 50% effective concentrations (EC_{50}) of alkaloids, i.e., the concentrations when ROS production by hydrogen peroxide decreased two times in the bioluminescence test. As is described [26], the EC_{50} values of the GL and s-Gl antioxidant activities in the bioluminescent test system (in vivo) were six times lower than in the DPPH assay, indicating higher sensitivity of the biotest.

For boldine and seco-boldine, it was impossible to determine EC_{50} values in the range of concentrations under study, because the 50% protective effect was not achieved for these compounds in the bioluminescence test.

Thus, the experiments in vitro (DPPH assay) and in vivo (biotest), as well as Ellman test, have shown that phenanthrene alkaloids have better antioxidant and anticholinesterase properties compared to their aporphine analogs.

CONCLUSIONS

(1) The antioxidant activities (in vitro) of model aporphine and phenanthrene alkaloids BD, s-BD, GL and s-GL were studied in the reaction with a stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical. In vivo antioxidant activity was determined in a bioluminescent test system with genetically modified *E. coli* strains. In the experiments in vitro (DPPH assay) and in vivo (biotest), phenanthrene alkaloids s-GL and s-BD demonstrate higher antioxidant activities compared to their aporphine precursors GL and BD.

(2) The anticholinesterase activity of the alkaloids and their phenanthrene seco-isomers was analyzed (in vitro) using Ellman method with minor modifications. The data on the activity of AChE inhibition by aporphine and phenanthrene alkaloids expressed as IC_{50} values obtained from dose–response curves demonstrate that the inhibitory activities of seco-boldine (IC_{50} = 0.21 mM) and seco-glaucine (IC_{50} = 0.04 mM) are higher compared to the initial aporphine alkaloids boldine (IC_{50} = 0.29 mM) and glaucine (IC_{50} = 0.44 mM), respectively.

(3) Thus, it has been shown that the phenanthrene alkaloids obtained in SCW exhibit the higher antioxidant activity and better AChE-inhibiting activity compared to their aporphine precursors. The results will be a basis for the search of new AChE inhibitors based on plant metabolites for treating Alzheimer's disease.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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