

## NEW INSIGHTS ON SOME 6-CHLORO-9H-CARBAZOL DERIVATIVES CONCERNING THEIR *IN VITRO* ANTIOXIDANT CAPACITY AND *IN VIVO* CYTOTOXICITY

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### Abstract

According to recent research, both carbazol and 1,3,4-oxadiazol derivatives are known for their antioxidant properties. The aim of our research was the evaluation of antioxidant activity and *in vivo* cytotoxicity of some new heterocyclic compounds, which both contain pharmacophore groups. The antioxidant activity was evaluated by means of scavenger activity towards 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS<sup>•+</sup>) free radicals. *In vivo* cytotoxicity was assessed using *Artemia franciscana* nauplii lethality bioassay. Our results have shown that the analysed compounds can be further used for their antioxidant capacity. For the analysed concentration interval (25 - 1000 μM), the scavenger capacity towards ABTS<sup>•+</sup> free radical was higher compared to the DPPH assay. All tested compounds showed an adequate safety profile. Further research is needed for evaluation of *in vivo* antioxidant effects of tested compounds and furthermore their safety *via* pre-clinical tests.

### Rezumat

Cercetări recente au arătat că atât derivații carbazolului, cât și cei ai 1,3,4-oxadiazolului prezintă proprietăți antioxidante. Scopul acestui studiu a constat în evaluarea activității antioxidante și a citotoxicității unor noi molecule, care conțin ambele grupări farmacofore. Activitatea antioxidantă a fost determinată pe baza capacității de chelatare a radicalilor liberi 2,2'-difeniil-1-picrilhidrazil (DPPH) și 2,2'-azino-bis (acid 3-etilbenzotiazolin-6-sulfonic) (ABTS<sup>•+</sup>). Citotoxicitatea noilor compuși a fost analizată folosind specia marină *Artemia franciscana*. Rezultatele obținute au arătat că derivații analizați prezintă activitate antioxidantă, indiferent de natura radicalului liber, totuși capacitatea de *scavenger* a radicalului ABTS<sup>•+</sup> pe domeniul de concentrații folosit (25 - 1000 μM) a fost superioară valorilor obținute prin metoda DPPH. În ceea ce privește citotoxicitatea, majoritatea compușilor sunt lipsiți de toxicitate sau toxicitatea este extrem de redusă, ceea ce denotă un profil de siguranță ridicat. Sunt necesare cercetări viitoare privind potențialul antioxidant al noilor compuși, prin teste *in vivo*, precum și evaluarea profilului de siguranță în studii pe animale de laborator.

**Keywords:** carbazol derivatives, 1,3,4-oxadiazol derivatives, antioxidant activity, cytotoxic activity

### Introduction

The importance of heterocycles in drug discovery is one of the major areas in medicinal chemistry [2]. The development of heterocyclic compounds with potential antioxidant activity have recently incited the interest of scientists [2]. It is well known that antioxidants, both natural and synthetic, play an important role in many fields. They are used for preserving the quality of foods (antioxidants prevent oxidative deterioration of fats), in the pharmaceutical industry and for maintaining human health being [26]. It is well known that oxidative stress is involved in aging and the development of several diseases (cancer, diabetes, cardiovascular disease, Alzheimers's etc.) [26]. Antioxidants neutralize free radicals and prevent

oxidation of a substrate by various mechanisms: (i) chain breakers, (ii) free radical interceptors, (iii) oxygen scavengers, (iv) metal chelating, (v) decomposition to non-radical species or (vi) absorption of UV radiation [26].

The carbazol nucleus is an important aromatic heterocycle, with a tricyclic structure, containing two benzene rings on other side, and a five-member nitrogen containing ring in the middle [2, 14]. Carbazol and its derivatives are an important class of nitrogen containing heterocyclic compounds, which are widely spread in nature. The carbazol ring is present in a variety of naturally occurring medicinally compounds (such as murrayafoline or carbazomycins) [14]. Series of carbazol derivatives are known for a wide range of pharmacological activities such as antioxidant, anti-

inflammatory, antibacterial or antitumor properties [2, 3, 14]. Recent research has also shown potential antioxidant effects for several 1,3,4-oxadiazol derivatives [3]. Thus, joining these two pharmacophoric fragments in the same molecule may enhance the antioxidant potential.

Taking into consideration the scientific data, the aim of our paper was the evaluation of *in vitro* antioxidant capacity (by means of scavenger activity towards DPPH and ABTS<sup>•+</sup> free radicals) and *in vivo* cytotoxicity (using *Artemia franciscana* nauplii

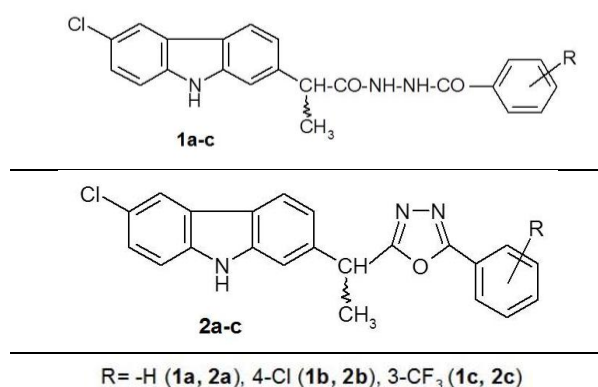
lethality bioassay) of some new 6-chloro-9H-carbazol derivatives.

## Materials and Methods

### Tested compounds

The tested compounds (Figure 1) were three N-[(2*RS*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoyl]-N'-R-substituted-benzoylhydrazine (**1a-c**) and their cyclized (*RS*)-1-(6-chloro-9*H*-carbazol-2-yl)-1-(1,3,4-oxadiazol-2-yl)ethane derivatives (**2a-c**).

The synthesis and physico-chemical properties of these derivatives, along with other biological effects were previously reported [4].



**Figure 1.**

The chemical structure of N-[(2*RS*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoyl]-N'-R-substituted-benzoylhydrazine (**1a-c**) and their cyclized (*RS*)-1-(6-chloro-9*H*-carbazol-2-yl)-1-(1,3,4-oxadiazol-2-yl)ethane derivatives (**2a-c**)

### Reagents and solvents

DPPH (Sigma-Aldrich, Germany), ascorbic acid (Roth, Germany), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, Germany), potassium persulfate (Merck, Germany), ethanol, dimethylsulfoxide (DMSO).

### Antioxidant activity

*Preparation of samples for antioxidant activity evaluation:* the analysed compounds (**1a-c** and **2a-c**) were dissolved in a mixture of 96% ethanol:DMSO = 99:1 (v/v). The concentration of each stock solution was 1000 μM. Successive dilutions were made in order to obtain different concentrations: 25 μM, 50 μM, 75 μM, 100 μM, 250 μM and 500 μM.

### Scavenger activity towards DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical

The antioxidant activity was determined according to Ohnishi M *et al.* [12, 22].

Up to now, scientific literature has not provided a standard work procedure, therefore one can find multiple variants of the assay. For our determination, in order to render soluble the DPPH free radical, we have chosen ethanol as a solvent, although according to the scientific literature hydroethanolic mixtures (above 50%) or methanol can be used [27]. Ethanol was chosen, based on the tested compounds solubility (see preparation of samples). According to the scientific literature, the reaction time between the free radical

and the sample varies between 5 min. [28], 15 min. [30], 30 min. [5], 90 min. [17] or until a plateau is reached [21]. For our analyses we have used a 30 min. reaction time, which was frequently used in our previous studies [8, 9].

Briefly, 0.5 mL of 25 - 1000 μM tested solutions was mixed with 3 mL DPPH ethanolic solution (0.1 mM). The mixture was incubated in the darkness, at room temperature for 30 min. [9]. The absorbance was measured at λ = 515 nm (Jasco V-530 spectrophotometer, Jasco, Japan) [20] against ethanol, that was used as a blank.

The DPPH free radical scavenger activity (I%) was determined according to the following formula [5]:

$$I\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where: A<sub>control</sub> = absorbance of the 0.1 mM DPPH solution in the absence of the tested compounds (1.000 ± 0.02), A<sub>sample</sub> = absorbance of the 0.1 mM DPPH solution in the presence of tested compounds after 30 min.

The antioxidant activity was expressed as ascorbic acid equivalents (mM ascorbic acid/g substance), using a calibration curve (concentration vs. absorbance), obtained in the same experimental conditions (0.05 - 0.4 mg/mL, R<sup>2</sup> = 0.9975, n = 5), as previously described [8]. *Scavenger activity towards ABTS<sup>•+</sup> (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) free radical*

The antioxidant activity was performed according to Re R. and co-workers, as previously described [9, 24]. Briefly, 0.5 mL of 25-1000  $\mu\text{M}$  tested solutions was mixed with 3 mL  $\text{ABTS}^{++}$  ethanolic solution and kept in the dark, at room temperature for 6 min. The absorbance was measured at  $\lambda = 734 \text{ nm}$  (Jasco V-530 spectrophotometer) towards ethanol, used as a blank [24, 29].

$\text{ABTS}^{++}$  free radical scavenger activity (I%) was determined according to the following formula:

$$I\% = \frac{\text{Abs}_{(t=0 \text{ min})} - \text{Abs}_{(t=6 \text{ min})}}{\text{Abs}_{(t=0 \text{ min})}} \times 100,$$

where:  $\text{Abs}_{t=0 \text{ min}}$  =  $\text{ABTS}^{++}$  solution absorbance in the absence of tested compounds ( $0.700 \pm 0.02$ ),  $\text{Abs}_{t=6 \text{ min}}$  =  $\text{ABTS}^{++}$  solution absorbance after 6 min. incubation with analysed compounds.

The antioxidant activity was expressed as ascorbic acid equivalents (mM ascorbic acid/g substance), using a calibration curve (concentration vs. absorbance), obtained in the same experimental conditions (0.01 - 0.1 mg/mL,  $R^2 = 0.9912$ ,  $n = 6$ ) as previously described [8].

#### Statistical analysis

Antioxidant assays were performed in triplicate and results are presented as mean  $\pm$  standard deviation (SD) of three independent determinations. The statistical analysis was performed using Microsoft Office® (Excell 2007) and GraphPad Prism (GraphPad Prism vers. 5 for Windows, Graph Pad, USA). The statistical significance of the difference between the analysed compounds was evaluated using one-way ANOVA followed by Tukey post-test. The correlation between the antioxidant methods was determined using Pearson coefficients. A value of  $p < 0.05$  was considered the threshold for a statistically significant difference.

#### In vivo Artemia franciscana cytotoxicity evaluation

The assessment of toxicity on *Artemia franciscana* Kellog was carried out using the method established by Meyer BM *et al.* [18] and Sam TW [25], with minor adaptations suggested by more novel bibliographic sources [1, 6, 7].

The cysts were purchased from S.K. Trading, having an origin stated as 100% from Great Salt Lake, USA. Artificial seawater obtained from a commercially available salt mixture (Coral Marine, Grotech) was used as a medium. The salts were dissolved in distilled water using an ultrasound bath for 10 minutes, at a concentration of 30 g/L, according to the guidance of the cyst provider. The hatching was carried out at an average temperature of  $26^\circ\text{C}$ , using an air pump for the appropriate aeration of the medium and was initiated about 48 hours before carrying out the testing proper.

The test was performed in a 24-well (6 x 4) plate, in triplicate (three wells for each concentration evaluated). Taking into account the limited solubility of the substances to be tested, these were suspended in artificial seawater using sodium alginate 0.045% to

ensure the stability, the testing being thus made at the level of the solubility limit. For each substance, the following concentrations were used: 100, 50, 25, 12.5 and 6.2  $\mu\text{g/mL}$ . The suspensions were prepared by successive dilutions from the initial one (with a 100  $\mu\text{g/mL}$  concentration). A sodium alginate solution in artificial seawater (0.045%) was employed as a negative control. The hatched nauplii were separated from the cyst residues and concentrated in a well with the help of artificial light, and then transferred in wells using a micropipette. Between 10 and 15 nauplii were transferred in each well, in the testing suspensions (1.5 mL of testing suspension *per* well). All nauplii, dead and alive, were counted at 24 h after their placing into contact with the testing suspensions. The concentration-lethality relationship was modelled logistically with four parameters (4PL), using an implementation with several robust variants of parameter estimation in the R package “dr4pl” [15].

## Results and Discussion

### Antioxidant activity

#### Scavenger activity towards DPPH free radical

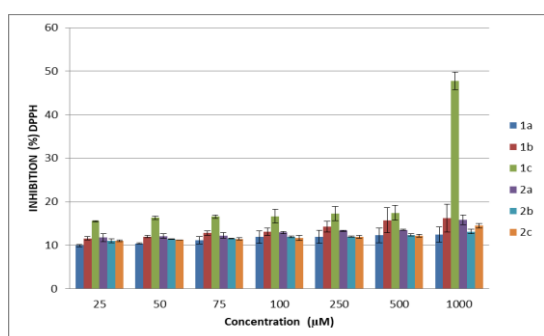
DPPH is a violet colour free radical which is reduced in the presence of an antioxidant to its corresponding hydrazine, which is pale yellow [11, 20]. The method was first described by Brand-Williams W *et al.* [5]. It is a rapid and simple method that is widely used for antioxidant capacity evaluation of different compounds. Moreover, its results are well correlated with other antioxidant assays (such as  $\text{ABTS}^{++}$ ) [10, 16]. The DPPH assay is mainly an electron transfer based method [11], although some authors consider that it has a mixt mechanism (both electron and hydrogen atom transfer); nevertheless, the hydrogen atom transfer is not the prevailing mechanism [23].

Results regarding the antioxidant activity are presented in Tables I and II and Figure 2. Our results pointed out, that for all analysed compounds the absorbance values (Table I) and inhibition (%) (Figure 2), decreased and respectively increased with concentration. The inhibition varied between 9.95% (for **1a** – 25  $\mu\text{M}$ ) and 17.71% (for **1c** – 1000  $\mu\text{M}$ ). Together with compound **1c**, compound **1b** scavenger activity was 16.19% at the highest concentration. Among the analysed compounds, the lowest free radical scavenger activity was observed for compound **1a**, at all tested concentrations. Regarding ascorbic acid equivalents (Table II), the antioxidant activity increased as follows **1a** < **1c** < **2c** < **2a**. For compounds **1b** and **2b** ascorbic acid equivalents were not determined, since the absorbance values were not enclosed in the calibration curve.

**Table I**

Absorbance values of the DPPH solution in the presence of tested compounds

Compound	Concentration ( $\mu\text{M}$ )						
	25 $\mu\text{M}$	50 $\mu\text{M}$	75 $\mu\text{M}$	100 $\mu\text{M}$	250 $\mu\text{M}$	500 $\mu\text{M}$	1000 $\mu\text{M}$
<b>1a</b>	0.6600 $\pm$ 0.0083	0.6179 $\pm$ 0.0398	0.6155 $\pm$ 0.0426	0.6115 $\pm$ 0.0370	0.6092 $\pm$ 0.0376	0.6050 $\pm$ 0.0350	0.5927 $\pm$ 0.0378
<b>1b</b>	0.5035 $\pm$ 0.0073	0.4842 $\pm$ 0.0022	0.4611 $\pm$ 0.0153	0.4510 $\pm$ 0.0132	0.4001 $\pm$ 0.0165	0.3834 $\pm$ 0.0123	0.3435 $\pm$ 0.0163
<b>1c</b>	0.4752 $\pm$ 0.0053	0.4134 $\pm$ 0.0132	0.3943 $\pm$ 0.0051	0.3745 $\pm$ 0.0060	0.2902 $\pm$ 0.0019	0.2252 $\pm$ 0.0079	0.2083 $\pm$ 0.0078
<b>2a</b>	0.5326 $\pm$ 0.0026	0.5318 $\pm$ 0.0018	0.5246 $\pm$ 0.0079	0.5202 $\pm$ 0.0125	0.5138 $\pm$ 0.0037	0.5086 $\pm$ 0.0014	0.4856 $\pm$ 0.0024
<b>2b</b>	0.566 $\pm$ 0.01202	0.5582 $\pm$ 0.0045	0.5562 $\pm$ 0.0031	0.5452 $\pm$ 0.0005	0.5058 $\pm$ 0.0019	0.4685 $\pm$ 0.0026	0.4096 $\pm$ 0.0121
<b>2c</b>	0.5664 $\pm$ 0.0106	0.5526 $\pm$ 0.0088	0.5444 $\pm$ 0.0080	0.5407 $\pm$ 0.0052	0.5129 $\pm$ 0.0025	0.4671 $\pm$ 0.0072	0.4173 $\pm$ 0.0144

Results are presented as mean  $\pm$  SD (n = 3)**Figure 2.**

DPPH free radical scavenger activity (inhibition %) of analysed compounds

ANOVA test didn't reveal significant differences among analysed compounds (**1a**, **1c**, **2a**, **2c**) ( $p = 0.6640 > 0.05$ ).

*Scavenger activity towards ABTS<sup>•+</sup> free radical*

The ABTS<sup>•+</sup> free radical results from the reaction between 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and manganese (II) oxide [19], sodium/potassium persulfate [24], 2,2'-

azobis(2-amidinopropane) dihydrochloride [17] or enzymes (peroxidase) [23]. Regarding the ABTS<sup>•+</sup> assay, the bluish-green radical cation is reduced in the presence of antioxidants (both lipophilic and hydrophilic compounds) [11, 23].

Results regarding the antioxidant activity are presented in Table III and Table IV, and Figure 3. Our results pointed out that the scavenger activity towards ABTS<sup>•+</sup> free radical increased with concentration for all analysed compounds.

**Table II**

Ascorbic acid equivalents - DPPH assay

Compound	Ascorbic acid equivalents (mM ascorbic acid/g substance)
<b>1a</b>	0.7609 $\pm$ 0.6415
<b>1b</b>	nd
<b>1c</b>	1.3593 $\pm$ 1.4007
<b>2a</b>	1.7568 $\pm$ 1.826
<b>2b</b>	nd
<b>2c</b>	1.4260 $\pm$ 9.9823

n.d. = non-detected

Results are presented as mean  $\pm$  SD (n = 3)**Table III**Absorbance values of ABTS<sup>•+</sup> solution in the presence of tested compounds

Compound	Concentration ( $\mu\text{M}$ )						
	25 $\mu\text{M}$	50 $\mu\text{M}$	75 $\mu\text{M}$	100 $\mu\text{M}$	250 $\mu\text{M}$	500 $\mu\text{M}$	1000 $\mu\text{M}$
<b>1a</b>	0.66 $\pm$ 0.0083	0.6179 $\pm$ 0.0398	0.6155 $\pm$ 0.0426	0.6115 $\pm$ 0.0370	0.6092 $\pm$ 0.0376	0.6050 $\pm$ 0.0350	0.5927 $\pm$ 0.0378
<b>1b</b>	0.5035 $\pm$ 0.0073	0.4842 $\pm$ 0.0022	0.4611 $\pm$ 0.0153	0.4580 $\pm$ 0.0132	0.4001 $\pm$ 0.0165	0.3834 $\pm$ 0.0123	0.3435 $\pm$ 0.0163
<b>1c</b>	0.4752 $\pm$ 0.0053	0.4134 $\pm$ 0.0132	0.3943 $\pm$ 0.0051	0.3745 $\pm$ 0.0060	0.2902 $\pm$ 0.0019	0.2252 $\pm$ 0.0079	0.2083 $\pm$ 0.0078
<b>2a</b>	0.5326 $\pm$ 0.0026	0.5318 $\pm$ 0.0018	0.5246 $\pm$ 0.0079	0.5202 $\pm$ 0.0125	0.5138 $\pm$ 0.0037	0.5086 $\pm$ 0.0014	0.4856 $\pm$ 0.0024
<b>2b</b>	0.5605 $\pm$ 0.0101	0.5582 $\pm$ 0.0045	0.5562 $\pm$ 0.0031	0.5452 $\pm$ 0.0056	0.5058 $\pm$ 0.0019	0.4685 $\pm$ 0.0026	0.4096 $\pm$ 0.0121
<b>2c</b>	0.566 $\pm$ 0.0106	0.5526 $\pm$ 0.0088	0.5444 $\pm$ 0.0080	0.5407 $\pm$ 0.0052	0.5129 $\pm$ 0.0025	0.4671 $\pm$ 0.0072	0.4173 $\pm$ 0.0144

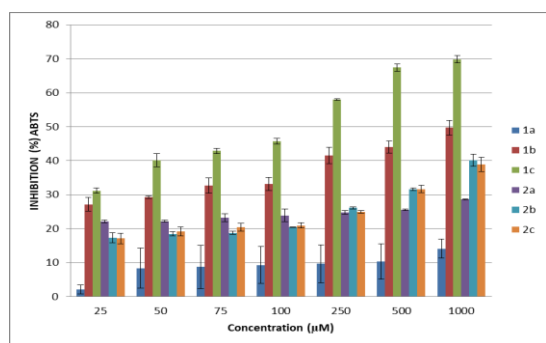
Results are presented as mean  $\pm$  SD (n = 3)

The scavenger activity varied between 2.13 % (for **1a** - 25  $\mu\text{M}$ ) and 69.83 % (for **1c** - 1000  $\mu\text{M}$ ) (Figure 3).

The highest scavenger capacity was observed for compound **1c**, followed by compounds **1b** and **2b**.

Compound **1a** showed the lowest scavenger activity, as previously observed (DPPH assay).

According to our results, the scavenger activity towards ABTS<sup>•+</sup> free radical was higher compared to the DPPH assay. Therefore, we assume that the structural differences between carbazol derivatives have a major impact upon their antioxidant potential. Regarding ascorbic acid equivalents, the antioxidant activity increased as follows: **1a** < **2b** < **2a** < **2c** < **1b** < **1c** (Table IV).



**Figure 3.**

ABTS<sup>•+</sup> scavenger activity (inhibition %) of analysed compounds

**Table IV**  
Ascorbic acid equivalents - ABTS<sup>•+</sup> assay

Compound	Ascorbic acid equivalents (mM ascorbic acid/g substance)
<b>1a</b>	0.4778 ± 0.4102
<b>1b</b>	5.1100 ± 4.5307
<b>1c</b>	6.2838 ± 5.0393
<b>2a</b>	3.3145 ± 2.5135
<b>2b</b>	3.0826 ± 2.7378
<b>2c</b>	4.6844 ± 4.0188

Results are presented as mean ± SD (n = 3)

Our results did not show significant differences among analysed compounds ( $p = 0.2020 > 0.05$ ). The Pearson coefficient (-0.0625) showed a negative, still insignificant correlation between the antioxidant methods ( $p = 0.7565$ ).

#### *In vivo Artemia franciscana cytotoxic capacity*

The cytotoxicity of new compounds can be tested *in vivo*, by using different organisms and methods, from which we have chosen *Artemia franciscana* nauplii lethality bioassay. This method can be used for experiments and applications in ecotoxicity, but also in pharmaco-toxicological screening, to evaluate the effects of chemical, new derivatives or natural compounds, on live organisms. Besides, the method has the advantage of being accessible, inexpensive and rapid [1].

The *in vivo* evaluation of toxicity using *Artemia franciscana* nauplii lethality bioassay was performed on four of the listed compounds, more specifically on derivatives **1a**, **2a**, **2b** and **2c**. Among the four tested derivatives, three (**1a**, **2a** and **2b**) didn't

show any toxicity at the evaluated concentrations, at the solubility limit, in suspension. After 24 hours, all nauplii were alive and were having normal movements. In the case of substance **2c**, a slight toxicity was observed at the maximum concentration evaluated (100 µg/mL), as evidenced by a lethality of 16.67% (10% in one of the three replicas and 20% in the other two replicas). The data were insufficient to allow the computation of IC<sub>50</sub> (possible only by extrapolation), but it may be concluded that this substance has a slightly higher toxicity than the other three, for which no case of toxicity or lethality was registered.

In the scientific literature, it has been suggested that an IC<sub>50</sub> value ranging between 30 and 100 µg/mL for the *Artemia sp.* toxicity corresponds to a modest, weak toxicity [13, 21]. The other evaluated compounds didn't show any toxicity at concentrations up to 100 µg/mL, and thus their toxicity may be considered modest. Still it must be taken into consideration that the solubility limitations did not allow an assessment of toxicity in solution.

## Conclusions

The analysed compounds have shown *in vitro* scavenger activities towards DPPH and ABTS<sup>•+</sup> free radicals. The highest antioxidant activity was observed for compounds **1c** and **1b**. By means of ABTS<sup>•+</sup> assay, promising results were also observed for compound **2b**. Regarding *Artemia franciscana* nauplii lethality bioassay, the results indicate a low level of toxicity for the analysed compounds. Further studies are necessary to confirm the *in vivo* antioxidant capacity of the tested compounds and moreover their safety in pre-clinical studies.

## Conflict of interest

The authors declare no conflict of interest.

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