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Antioxidant and radical scavenging activities of some norcantharidin and bridged perhydroisoindole derivatives

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Abstract: A series of norcantharidin and bridged perhydroisoindole derivatives were evaluated for their antioxidant and radical scavenging activities. Different *in vitro* methodologies, such as total reducing power, 1,1-diphenyl-2-picryl-hydrazil (DPPH$^{\bullet}$) free radical scavenging, superoxide anion radical scavenging and metal chelating activities were used. Among the 11 tested compounds, 7 compounds showed potent reducing power activity and 7 compounds showed potent superoxide anion radical scavenging activity. All the tested compounds exhibited potent free radical scavenging ability. The results showed that the synthesized compounds have effective antioxidant power.

Keywords: norcantharidin; bridged perhydroisoindole; reducing power; superoxide anion radical.

INTRODUCTION

The imide moiety is an integral structural part of various important bioactive molecules, such as fumaramidmycin, granulatimide, isogranulatimide and rebeccamycin. These molecules are reported to exhibit antitumour, anti-inflammatory and antimicrobial activities.[1–3] A literature search revealed that certain compounds with antitumour activity, and in particular molecules able to interact with DNA, are characterized by the presence of both an extended π-system and an imide function. In addition, *N*-substituted imides, such as maleimides,[4] isohematinic acids[5] and especially bicyclic and tricyclic derivatives such as tandospirone derivatives[3,6] are known for their broad spectrum of pharmacological properties, thus showing antibiotic, fungicidal, analgesic, anxiolytic and cytostatic effects.

On the other hand, derivatives of the tricyclic anhydride *exo*-5,6-dehydronorcantharidin (**2**, Fig. 1) are also pharmacologically active.[7] This compound shows

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a comparable activity to that of cantharidin (**1**, Fig. 1), which is the major effective ingredient in pharmaceuticals for the treatment of certain malignant tumours in China. Norcantharidin is a synthetic demethylated analogue of cantharidin, a traditional Chinese herb, now being worldwide used as an antitumour agent for its safety without myelosuppression in patients and effectiveness against cells with a multidrug resistance phenotype. It was reported that norcantharidin could induce cell apoptosis and inhibit the proliferation of a variety of human tumour cell lines *in vitro*, including colorectal cancer, oral cancer, cervical cancer, breast cancer, hepatoma, leukaemia, melanoma and gall bladder carcinoma, and it was proved to block tumour invasion, metastasis and angiogenesis.[8] Norcantharidin and *exo*-5,6-dehydro-norcantharidin (**2**) have been widely employed in clinical practice, as they are less toxic and much easier to synthesize.[9,10] Perhydroisoindoles are also selective sigma receptor antagonists and have a low potential for movement disorder side effects associated with typical antipsychotic agents.[11,12]

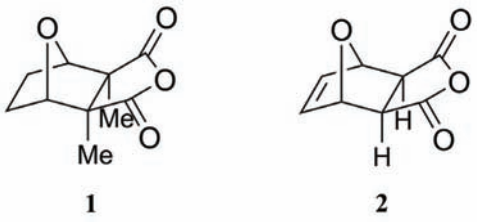
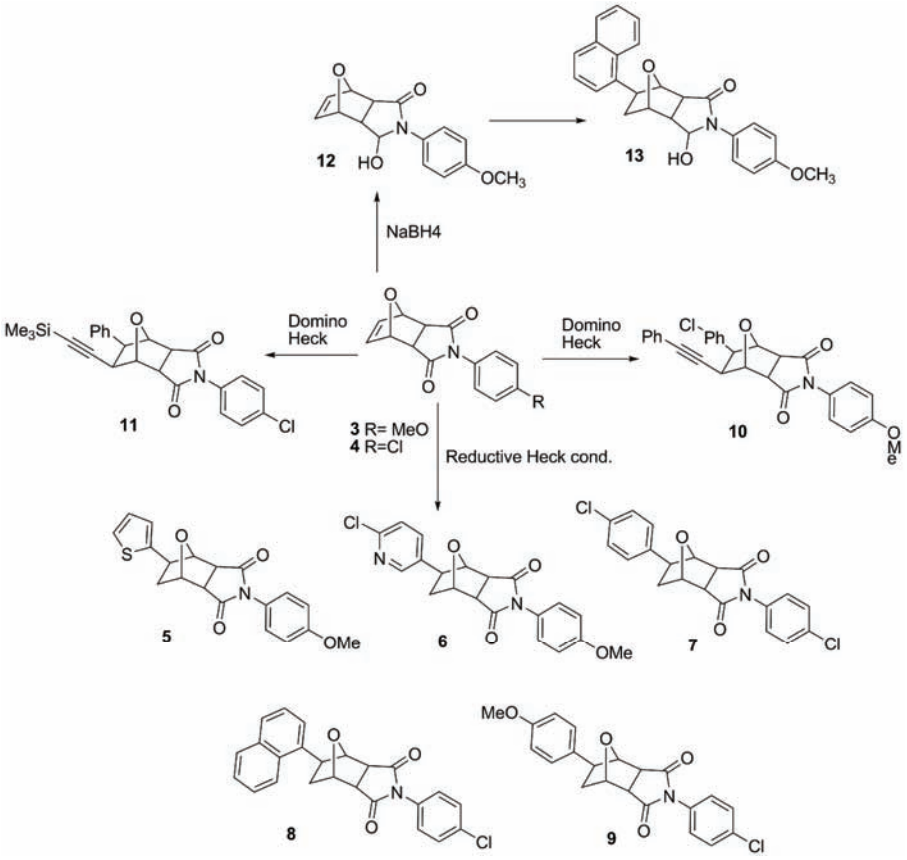


Fig. 1. The structures of cantharidin (**1**) and *exo*-5,6-dehydronorcantharidin (**2**).

Therefore, the synthesis of bioactive norcantharidin analogues, that represent aryl-modified bicyclic imide systems, was also considered to be of interest. Thus, *N*-(4-methoxyphenyl)-7-oxabicyclo[2.2.1]hept-5-ene-2-*exo*-3-*exo*-dicarboximide (**3**) and the *p*-chlorophenyl derivative **4** were prepared as the starting compounds in good yields (70 and 72 %, respectively).[9,13] Then, their hydroarylation reactions with aryl- and heteroaryl iodides in the presence of triphenylarsine (Scheme 1) and subsequent reduction by $NaBH_4$ were investigated to open new access to perhydroisoindole derivatives.[14] The synthesized compounds were evaluated for antioxidant and free radical scavenging activities.

Reactive oxygen species (ROS) are chemical entities that include oxygen free radicals. They can be generated in metabolic pathways within body tissues, and can also be introduced from external sources, such as drugs, food, UV radiation and environmental pollution. *In vivo*, such species are securely coupled at their site of generation or are detoxified by endogenous anti-oxidative defences so as to preserve the optimal cellular function. In pathological conditions, however, the detoxifying mechanisms are often inadequate as excessive quantities of ROS can be generated. This resulting pro-oxidant shift, a process known as oxidative stress, can result in the degradation of cellular components such as DNA, carbohydrates, polyunsaturated lipids and proteins, or precipitate enzyme inacti-

vation, irreversible cellular dysfunction and ultimately cell death if the pro-oxidant–antioxidant balance is not restored.[15,16] Thus, antioxidants are important inhibitors against oxidative damage.[17–19] Antioxidants interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers. Therefore, the preparation of more effective new antioxidants is a very important area of research.



Scheme 1. Synthesis of bioactive norcantharidin analogues and the perhydroisoindole derivatives used in this work. *N*-(4-Methoxyphenyl)-7-oxabicyclo[2.2.1]hept-5-ene-2-*exo*--3-*exo*-dicarboximide (**3**) and the *p*-chlorophenyl derivative **4** were synthesized as the starting compounds.

The aim of this study was to investigate the reducing power, free radical scavenging, superoxide anion radical scavenging and metal chelating activities of some norcantharidin and bridged perhydroisoindole derivatives. The results were

compared to commercial and standard antioxidants, such as Trolox, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

EXPERIMENTAL

Chemicals

Reduced nicotinamide adenine dinucleotide (NADH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picrylhydrazil (DPPH$^{\bullet}$) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Germany). Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were provided by Fluka (Buchs, Switzerland). All other employed chemicals were of analytical grade and obtained from either Sigma-Aldrich, Fluka or Merck.

Norcantharidin and the bridged perhydroisoindole derivatives were prepared according to previously reported procedures and characterized by comparing their spectral data to those reported earlier.[14]

Evaluation of antioxidant and radical scavenging activities

Reducing power assay. The reducing power capacity of norcantharidin and the bridged perhydroisoindole derivatives was measured according to the method of Oyaizu.[20] Various amounts of the samples (10–50 μg) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide, $K_3[Fe(CN)_6]$ (1 %, w/v), and the mixture was incubated at 50 °C for 30 min. Then, after addition of 2.5 mL of trichloroacetic acid (10 %, w/v), the mixture was centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the upper-layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1 %, w/v) and the absorbance was measured at 700 nm. α-Tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

Free radical scavenging activity assay. The free radical scavenging activity of norcantharidin and the bridged perhydroisoindole derivatives was measured with DPPH$^{\bullet}$ using a slightly modified method of Brand-Williams *et al.*[21] Briefly, 20 mg/L DPPH$^{\bullet}$ solution in methanol was prepared and 1.5 mL of this solution was added to 0.75 mL of the sample, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox or ascorbic acid (5–20 μg mL^{-1}). The mixture was shaken vigorously and the decrease in absorbance at 517 nm was measured after 30 min. Water (0.75 mL) instead of the sample was used as the control. The percent inhibition activity was calculated using the following equation:

$$\text{Free radical scavenging activity (\%)} = 100(A_0 - A_1)/A_0 \quad (1)$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of a sample solution. The radical scavenging activity was expressed as the IC_{50} value, the concentration required for a 50 % reduction in the DPPH$^{\bullet}$ concentration, which was determined from a calibration curve for each compound.

Superoxide anion scavenging activity assay. Measurement of superoxide anion scavenging activity of norcantharidin and the bridged perhydroisoindole derivatives were based on the method described by Liu *et al.*[22] Superoxide anions were generated in a non-enzymatic phenazine methosulphate–nicotinamide adenine dinucleotide (PMS–NADH) system by oxidation of NADH and assayed by reduction of NBT. In this experiment, the superoxide anion was generated in 3 mL of tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution and 100 μg mL^{-1} concentration of a sample solution. The reaction was started by adding 1 mL of PMS solution (10 μM) to the mixture. The reac-

tion mixture was incubated at 25 °C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. BHA, BHT, and Trolox were used as standard samples (100 μg mL^{-1}). The inhibition of superoxide anion radical generation (%) was calculated using Eq. (1).

Metal chelating activity assay. The chelating activity of norcantharidin and the bridged perhydroisoindole derivatives on ferrous ions was measured according to the method of Decker and Welch.[23] Aliquots of 1 mL of different concentrations (25, 50, 75 and 100 μg mL^{-1}) of the samples were mixed with 3.7 mL of deionised water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 mL) for 30 min. After incubation, the reaction was initiated by addition of ferrozine (5 mM and 0.2 mL) and after 10 min at room temperature, the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of the extract on Fe^{2+} was compared with that of EDTA at same concentrations. Chelating activity was calculated using the following formula:

$$\text{Metal chelating activity (\%)} = 100(1 - A_S/A_{EDTA}) \quad (2)$$

where A_S and A_{EDTA} are the absorbances in the presence of a sample and EDTA, respectively.

A control test was performed without addition of a sample.

RESULTS AND DISCUSSION

Three 5,6-dehydronorcantharidin, eight norcantharidin and bridged perhydroisoindole compounds (Fig. 2, Table I) were investigated for their antioxidant and radical scavenging activities. Reducing power is used as one of the indicators of antioxidant capability. In the reducing power assay, the presence of reductants (antioxidants) in the tested samples resulted in a reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The amount of Fe^{2+} complex can therefore be monitored by measuring the formation of the Perls' Prussian Blue at 700 nm. The reducing power of norcantharidin and the bridged perhydroisoindole derivatives and standard antioxidants are given in Table II. The results show that the reducing power of the samples was not concentration dependent. Based on a comparison of the absorbance at 700 nm, compounds **7**, **11**–**13** showed the lowest reducing power. Compounds **8** and **9** exhibited moderate reducing power. Compounds **4**–**6** and **10** gave similar results. A higher activity was found for compound **3**. The tested compounds **3**–**6** and **10** exhibited higher activity than BHA, BHT and α-tocopherol at a 10 μg mL^{-1} concentration. A correlation was found between the reducing capabilities and the substituents. The reason for the higher reducing power capacity of the compounds can be explained by considering the structure of the compounds. The presence of a carbonyl group and an alkene or alkyne on the ring system seems to increase the reductive capacity of the com-

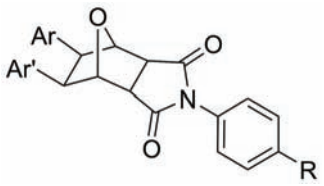


Fig. 2. Structure of the bridged perhydroisoindole derivatives used in this study.

pounds. These results revealed that these 5,6-dehydronorcantharidin, norcantharidin and the bridged perhydroisoindole derivatives were good electron and hydrogen donors and could terminate a radical chain reaction, converting the free radicals to more stable products.

TABLE I. The three 5,6-dehydronorcantharidins (**3**,**4** and **12**) and eight norcantharidins and bridged perhydroisoindole derivatives investigated in this study

Compound	R	Ar	*Ar'*
3	OMe	–	–
4	Cl	–	–
5	OMe	(2-thienyl)	–
6	OMe	(6-chloropyridin-3-yl)	–
7	Cl	(*p*-chlorophenyl)	–
8	Cl	(1-napthyl)	–
9	Cl	(*p*-methoxyphenyl)	–
10	OMe	(*p*-chlorophenyl)	(phenylethynyl)
11	Cl	(phenyl)	(trimethylsilyl)ethynyl
12	OMe	–	–
13	OMe	(1-napthyl)	–

TABLE II. Reducing power (absorbance at 700 nm) of different concentrations (10, 20, 30, 40 and 50 μg mL^{-1}) of norcantharidin and the bridged perhydroisoindole derivatives. BHA, BHT and α-tocopherol were used as reference antioxidants. The given values are means ± *SD* (*n* = 3). A higher absorbance indicates a greater reducing power

Compound	*c* / μg mL^{-1}				
	10	20	30	40	50
3	0.14±0.01	0.14±0.01	0.15±0.01	0.15±0.00	0.15±0.01
4	0.10±0.01	0.10±0.01	0.11±0.01	0.11±0.01	0.11±0.01
5	0.10±0.01	0.10±0.00	0.10±0.01	0.11±0.01	0.11±0.01
6	0.10±0.01	0.10±0.01	0.10±0.00	0.10±0.00	0.11±0.01
7	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
8	0.03±0.00	0.04±0.01	0.04±0.01	0.04±0.00	0.04±0.00
9	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00
10	0.09±0.01	0.09±0.01	0.10±0.00	0.10±0.01	0.11±0.01
11	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
12	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
13	0.01±0.00	0.01± 0.00	0.01±0.00	0.01±0.00	0.01±0.00
BHA	0.09±0.02	0.17±0.02	0.25±0.01	0.33±0.03	0.41±0.02
BHT	0.03±0.00	0.06±0.00	0.09±0.01	0.14±0.01	0.16±0.01
α-Tocopherol	0.04±0.00	0.07±0.01	0.12±0.01	0.12±0.01	0.18±0.01

Hydrogen-donating ability is an index of primary antioxidants. These antioxidants donate a hydrogen to a free radical, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation. 1,1-Diphenyl--2-picrylhydrazil (DPPH$^{\bullet}$) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH· has been used as

a free radical to evaluate the anti-oxidative activity of some natural and synthetic sources. The DPPH radical scavenging effects of norcantharidin and the bridged perhydroisoindole derivatives are presented in Table III. Their comparable scavenging activities were also expressed as IC_{50} values (Table IV). All the tested compounds showed lower free radical scavenging activities when compared to BHA, BHT, Trolox and ascorbic acid. Compounds **5**–**8** exhibited similar activity to BHT at 5 and 10 μg mL^{-1} concentrations. The DPPH free radical scavenging activity was improved by the presence of N, S and Cl on the heterocyclic ring and alkene groups. From these results, it could be stated that the tested compounds have a moderate ability to scavenge free radicals and could serve as free radical inhibitors or scavengers according to the synthetic antioxidants. From the inhibition concentration IC_{50} values of the compounds, it was seen that compounds **4**, **5**, **7**, **8**, **12** and **13** had the highest activities, as shown by the lowest values of IC_{50}, followed by compound **6**, **9** and **10**, while compound **3** and **11** had the lowest activities. A higher DPPH radical scavenging activity is associated with a lower IC_{50} value. It was evident that the compounds did show hydrogen-donating ability to act as antioxidants.

TABLE III. Free radical scavenging activity (%) of different concentrations (5, 10, 15 and 20 μg mL^{-1}) of norcantharidin and the bridged perhydroisoindole derivatives. BHA, BHT, Trolox and ascorbic acid were used as reference antioxidants. The given values are means ± *SD* (*n* = 3)

Compound	*c* / μg mL^{-1}			
	5	10	15	20
3	17.9±0.3	18.7±0.6	18.7±0.9	18.7±0.1
4	16.3±0.5	16.7±1.4	17.5±0.1	19.9±0.1
5	30.1±2.2	31.7±1.4	31.7±0.8	32.1±1.2
6	30.9±1.2	30.9±0.6	31.7±0.9	32.1±1.0
7	30.5±0.4	30.9±0.1	31.7±1.4	32.1±1.2
8	30.1±0.1	31.7±0.5	31.7±0.5	32.1±0.5
9	31.3±0.8	31.7±1.0	32.1±1.2	32.5±0.8
10	17.9±0.9	17.9±0.9	18.3±0.8	20.7±0.4
11	18.3±0.8	18.7±0.7	19.1±0.7	19.1±0.7
12	16.7±0.6	16.7±0.6	18.3±0.8	20.7±0.4
13	15.9±0.1	18.3±0.7	18.3±0.8	19.5±0.6
BHA	68.3±2.7	78.9±2.5	88.2±2.1	92.7±1.5
BHT	31.7±2.0	36.6±1.5	42.3±0.6	45.5±2.7
Trolox	95.5±1.7	95.9±0.6	96.8±0.6	97.2±1.7
Ascorbic acid	95.9±1.7	96.3±1.0	96.3±0.6	96.3±0.6

The superoxide anion radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species, such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA. In a biological system, its toxic role can be eliminated by superoxide dismutase.[14] In the PMS–NADH–NBT system, the superoxide an-

ion derived from dissolved oxygen by the PMS–NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with antioxidants indicates the consumption of the superoxide anion in the reaction mixture. The superoxide radical scavenging activities by 100 μg mL^{-1} of norcantharidin and the bridged perhydroisoindole derivatives in comparison to the same amount of BHA, BHT and Trolox are given in Table V. Compounds **5**–**7** and **10** showed lower superoxide radical scavenging activity than BHA, BHT and Trolox, while compounds **3**, **4**, **11**–**13** showed higher activity than BHT. It is important to mention that compounds **8** and **9** exhibited more potent activity when compared to those of all the tested standard antioxidants, *i.e.*, BHA, BHT and Trolox at the same concentration. These results clearly indicate that the Cl atom present in the structure and the alkene group seems to induce the activity of the compounds and they act as good superoxide radical scavengers.

TABLE IV. Free radical scavenging activity (IC_{50} / μg mL^{-1}) of norcantharidin and the bridged perhydroisoindole derivatives. BHA, BHT and Trolox were used as reference antioxidants. A higher DPPH radical scavenging activity is associated with a lower IC_{50} value

Compound	IC_{50} / μg mL^{-1}
3	130.6±8.2
4	29.9±5.1
5	33.1±3.9
6	44.1±2.5
7	35.3±4.5
8	33.1±5.2
9	46.9±2.6
10	37.4±5.1
11	112.8±6.8
12	25.6±1.3
13	31.7±2.3
BHA	2.3±0.1
BHT	4.8±0.3
Trolox	4.5±0.3

TABLE V. Superoxide anion radical scavenging activity of norcantharidin and the bridged perhydroisoindole derivatives at a concentration of 100 μg mL^{-1}. BHA, BHT and Trolox acid were used as the reference antioxidants. The given values are the means ± *SD* (n = 3)

Compound	Superoxide anion radical scavenging activity, %
3	75±0.8
4	72±0.5
5	36±2.9
6	25±0.5
7	26±1.3
8	95±1.3
9	91±0.8
10	57±1.2

TABLE V. Continued

Compound	Superoxide anion radical scavenging activity, %
11	75±1.1
12	74±1.7
13	63±1.1
BHA	78±0.8
BHT	59±2.8
Trolox	81±1.7

Chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. Iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. Especially Fe^{2+} is the most powerful pro-oxidant among the various species of metal ions. The ferrous ion chelating effects of norcantharidin and the bridged perhydroisoindole derivatives are presented in Table VI. Compounds **3**–**7** exhibited no chelating activity on ferrous ions at any of the tested concentrations. Compound **8**–**13** showed moderate chelating activity on ferrous ions after an incubation time of 30 min. The results were compared with EDTA at the same concentrations. At a concentration of 100 μg mL^{-1}, EDTA had a 96 % chelating effect on ferrous ions after an incubation time of 30 min. The data obtained from this study revealed that compounds **8**–**13** had a slightly effective capacity for iron binding, suggesting that their action as antioxidants might be related to their iron binding capacity. It is known that compounds with structures containing two or more functional groups, such as OH, SH, COOH, N, S and O, can show metal chelating activity.

TABLE VI. Metal chelating activity (%) of different concentrations (25, 50, 75 and 100 μg mL^{-1}) of norcantharidin and the bridged perhydroisoindole derivatives. EDTA was used as a standard compound. The reported values are the mean ± *SD* (*n* = 3)

Compound	*c* / μg mL^{-1}			
	25	50	75	100
3	0	0	0.6±0.0	1.8±0.0
4	0	0	0	0
5	0	0	0	0
6	0	0	1.1 ± 0.0	1.9±0.1
7	0	0	0	1.1±0.0
8	10.7±0.2	14.3±0.1	15.5±0.2	19.9±0.1
9	13.9±0.2	14.9±0.3	17.3±0.1	23.5±0.6
10	4.2±0.2	6.3±0.1	11.3±0.2	15.2±0.7
11	16.7±0.6	16.7±0.2	17.9±0.3	22.6±2.3
12	16.1±0.0	16.4±0.1	16.9±0.1	19.1±0.1
13	9.8±0.4	11.9±0.2	14.3±0.0	14.9±0.1
EDTA	85.0±1.2	95.0±2.7	96.0±2.1	96.0±1.2

CONCLUSIONS

A series of norcantharidin and bridged perhydroisoindole derivatives (**3**–**13**) were synthesized and their antioxidant properties were evaluated. The results showed that most of the synthesized derivatives exhibited significant antioxidant and radical scavenging activities *in vitro*. According to the obtained results, a correlation exists between the radical scavenging and antioxidant activities of the compounds and the substituents. Among the synthesized compounds, compounds **3**–**6** and **8**–**10** were found to be the most active reducing agents. Compound **3**, **4**, **8**, **9** and **11**–**13** were determined to have the highest superoxide anion radical scavenging ability in addition to being potent antioxidants and thus they represent a new class of antioxidant and antiradical agents.

ИЗВОД

АНТИОКСИДАТИВНА АКТИВНОСТ И СПОСОБНОСТ ХВАТАЊА СЛОБОДНИХ РАДИКАЛА НЕКИХ НОРКАНТАРИДИНСКИХ И ПЕРХИДРОИЗОИНДОЛНИХ ДЕРИВАТА СА МОСТОМ

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Утврђивана је антиоксидативна активност и способност хватања слободних радикала серије норкантаридинских и перхидроизоиндолних деривата са мостом. Одређиван је укупни редукујући капацитет, способности хватања радикала методом DPPH, способност хватања супероксидног анјона и способност хелатирања метала. Од 11 анализираних једињења, 7 је испољило велики редукујући потенцијал, а 8 способност хватања супероксидног анјона. Сва тестирана једињења су могла везивати слободне радикале, потврђујући њихову антиоксидативну активност.

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