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Article

Virginicin, a New Naphthalene from *Kosteletzkya virginica* (Malvaceae)

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A new naphthalene, 8-methoxy-2,7-dihydroxyl-4-(1'-hydroxyl-isopropyl)-6-methyl-1-naphthaldehyde, together with twelve known compounds were isolated from the tubers of *Kosteletzkya virginica.* Their structures were elucidated by spectroscopic methods, including 1D-, 2D-nuclear magnetic resonance (NMR) and high-resolution time of flight electrospray ionization mass spectrometry (HRTOFESIMS). Some compounds were evaluated for their potential in scavenging diphenyl-picryl hydrazyl radical (DPPH$^{\bullet}$), inhibition of nitric oxide (NO) induced by lipopolysaccharide (LPS), and cytotoxic activity. The new compound showed activities against DPPH$^{\bullet}$, NO, human acute promyelocytic leukemia (HL-60) and human colorectal adenocarcinoma (LOVO), with IC_{50} of 34.6, 12.5, 40.5, 31.7 μmol L^{-1} respectively.

Keywords: naphthalene, *Kosteletzkya virginica,* DPPH$^{\bullet}$, nitric oxide, cytotoxic activity

Introduction

Kosteletzkya virginica (L.) Presl. (Malvaceae) is a perennial root herb native to the saline tidal marshes in the United States east coasts from Texas to the Delaware, and was first introduced to China in 1992-1993.[1] As a halophytic species with potential for agroecotechnology in Jiangsu Province, China,[2] *K. virginica* produces a relatively high yield of seeds. The hulled seeds having a high protein and fat content (25-35% protein, 20-30% oil composed largely of unsaturated fatty acids, high potassium and low sodium),[3] whereas the oil can be used as an edible oil or biodiesel.[4] Mucilage from seed is suitable for industrial use as candy or gum. Considering these properties, *K. virginica* has a great potential for being developed to food (feed) or oil crops, and has been served as a candidate species of the current development and utilization of saline flats in the east of China such as Liaoning, Jiangsu, and Shandong Provinces. In addition, the fleshy root of this plant is used in American Indian traditional medicine for the treatment of the upper respiratory tract inflammation.[5]

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In our study, we have isolated and identified the structures of a new naphthalene (**1**) and twelve known compounds (**2-13**). The antioxidant, cytotoxic and anti-inflammatory activities of compounds **1-7** were also evaluated.

Experimental

General comments

Melting points were measured using a XT-4 Boetius micro-melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded on a Nexus 870 FT-IR spectrometer. HR-ESI-MS spectra were measured on Agilent 6530 UPLC/Q-TOF/MS spectrometer. NMR data were acquired on a Bruker DRX500 or 300 NMR spectrometer with ^{1}H and ^{13}C NMR observed at 500 or 300 and 125 or 75 MHz, with chemical shifts (δ) given in ppm with reference to the solvent, and coupling constants (J) in Hz. Silica gel (200-300 mesh) and Sephadex LH-20 for column chromatography were purchased from Qingdao Marine Chemical Factory, and Pharmacia Biotech, respectively. HPLC analyses were performed with a YMC ODS-5 μm (150 × 4.6 mm) column, an Agilent

pump 1100 and a ELSD detector Alltech 500. All other chemicals used in this study were of analytical grade. TLC analyses were carried out on silica gel 60 F_{254} (Merck) plates. The compounds were monitored by spraying 1% vanillin-H_2SO_4 reagent, followed by heating at 105°C for 1-2 min. Bioassays were performed with an Infinite M200 microplate reader which was purchased from TECAN.

Plant material

In this study, tubers of *K. virginica* were collected at Yancheng, Jiangsu province, China, in May, 2010, and identified by Prof Yuan Changqi. A voucher specimen (20101001) was deposited in Jiangsu for Research and Development of Medicinal Plants, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences.

Extraction and isolation

Fresh tubers of *K. virginica* (13 kg) were continuously reflux extracted with 95%, 85%, and 70% ethanol at 80 °C (each extraction for 2 h), yielding 912 g of crude extract. The extract was partitioned with petroleum ether and ethyl acetate (EtOAc) to produce a petroleum ether-soluble fraction (PE fraction), an EtOAc-soluble fraction (EA fraction) and a water fraction, respectively. The EA fraction (119 g) was subjected to silica gel column chromatography (CC). The material was eluted successively with a petroleum ether/EtOAc gradient (100:0 to 0:100) to obtain 11 fractions (fr. EA 1-11). Fr. EA 3 (3 g) was subjected to silica gel CC (petroleum ether/EtOAc, 25:1), followed by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1), to yield (-)-syringaresinol (**10**, 33 mg). Fr. EA 4 (4.2 g) was eluted with a petroleum ether/EtOAc gradient (10:1 to 0:100), concentrated and rechromatographed by silica gel CC using CH_2Cl_2/MeOH (18:1). The crude coumarinolignan elute was purified by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1) to produce jatrocin B (**3**, 311 mg) and cleomiscosin A (**4**, 53 mg). Fr. EA 5 (3.8 g), eluted with CH_2Cl_2/MeOH (50:1), was concentrated and rechromatographed with silica gel CC using CH_2Cl_2 with increasing amounts of MeOH. The coumarin elute was purified by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1), followed by ODS gel CC with 50% aqueous MeOH to yield virginicin (**1,** 37 mg). Fr. EA 6 (3.2 g) was eluted with a petroleum ether/EtOAc gradient (5:1 to 0:100), concentrated, and rechromatographed with silica gel CC using EtOAc/MeOH (25:1) to yield cleomiscosin C (**5**, 196 mg) and (-)-syringaresinol-4-*O*-β-D-glucopyranoside (**11**, 324 mg). Fr. EA 8 (1.2 g), eluted with a petroleum ether/EtOAc gradient (1:1), was concentrated, and the crude coumarinolignan eluted was then purified by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1) to obtain cleomiscosin B (**6**, 73 mg) and cleomiscosin D (**7**, 7 mg). The PE fraction (45 g) was subjected to silica gel CC and eluted successively with a n-hexane/CH_2Cl_2 gradient (100:0 to 0:100) to obtain five fractions (fr. PE 1–5). Fr. PE 4 (6.2 g) was subjected to silica gel CC (petroleum ether/EtOAc, 50:1), followed by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1), to give scopoletin (**12**, 37 mg) and isoscopoletin (**13**, 24 mg). Fr. PE 3 (13.2 g), eluted with petroleum ether/EtOAc (75:1), was concentrated and rechromatographed by silica gel CC using n-hexane with increasing amounts of CH_2Cl_2, followed by Sephadex LH-20 CC ($CHCl_3$/MeOH, 2:1), to give syriacusin A (**2**, 23 mg), chrysoeriol (**8**, 13 mg), and isorhamnetin-3-*O*-rutinoside (**9**, 20 mg).

Virginicin (**1**)

Yellow needles; mp 273-274 °C; IR (KBr) *v*/cm^{-1} 3210, 2843, 2742, 1672, 1660, 1595, 1580, 1510, 1456, 1362, 1269, 1206, 1147, 1014, 943, 859, 789, 765, 629, 565; ^{1}H NMR and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$), (Table 1); HRTOFESIMS $[M+H]^+$ 291.1235 (calcd for $C_{16}H_{19}O_5$, *m/z* : 291.1232).

DPPH assays

A solution of DPPH$^{\bullet}$ radical (0.16 mmol L^{-1}) in absolute ethanol was prepared. Compounds **1-7** were dissolved at a concentration of 5 mmol L^{-1} in DMSO and diluted into several concentrations. 100 μL of DPPH$^{\bullet}$ solution, 20 μL of samples and 80 μL distilled water were added into wells of a 96-well plate, making the final concentrations of 500, 250, 125, 62.5 and 31.25 μmol L^{-1}. The absorbance (A_i) was measured at 517 nm after the 96-well plate was heated at 25 °C for 15 min. The absorbance of 20 μL of DMSO and 100 μL of absolute ethanol were measured respectively as A_0 and A_j. All experiments were conducted in triplicate and Vitamin C (VC) was used as the standard antioxidant. The radical scavenging activity of each sample was calculated by the DPPH$^{\bullet}$ inhibition percentage (%I_{DPPH}) according to the following equation:[6-7]

$$\%I_{DPPH} = [A_0-(A_i-A_j)]/A_0*100 \quad (1)$$

The antioxidant activities of the tested compounds were expressed as IC_{50}, defined as the concentration corresponding to %I_{DPPH} equal to 50%.

Measurement of NO production and cell inhibition in LPS-activated murine macrophages cells (RAW264.7)

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). In order to measuring NO production, the cells were dispensed in wells of a 96-well plate (2.5×10^5 cells/well). 24 h later, different concentrations of compounds **1-7** were added to the cells for 30 min. Then the cells were stimulated with LPS (0.1 μg mL^{-1}) for 24 h under the conditions of 37 °C and 5% CO_2. 50 μL of the supernatant was harvested and mixed with 50 μL of Griess reagent. After culturing 15 min at room temperature, the absorbance was then read at 540 nm. Then, a [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] (MTT) assay can be used for measuring cell inhibition.[8] Indomethacin was tested as a positive control.

Cytotoxicity assays

HL-60 was maintained in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% heat-inactivated bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Lovo were grown under the same conditions described above, except for the basal medium (DMEM medium instead of RPMI-1640). The media were changed every two day. All the above cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The effect of compounds **1-7** on the viability of tumor cells was determined with the MTT assay. The cells were plated at 10,000 cells *per* well in 100 μL of complete culture medium and treated with various concentrations of the compounds in 96-well microtiter plates. Each concentration of the compound was repeated in six wells. After incubation for 72 h at 37 °C in a humidified incubator, cell viability was determined. MTT (5 mg mL^{-1}) was added to each well and incubated for 4 h. Then 100 μL of the solubilization solution (10% sodium dodecyl sulfate (SDS) in 0.012 M HCl) were added into each well, and the plates were standed overnight in the incubator. Absorbance was recorded on a microplate reader at a wavelength of 570 nm (reference wavelength: 690 nm). The 50% inhibitory concentration (IC_{50}) was determined by interpolation from dose-response curves. *Cis*-platin was tested as a positive control.

Results and Discussion

Fresh tubers of *K. virginica* (13 kg) were continuously reflux extracted with 95%, 85% and 70% of ethanol. Then the extract was partitioned with petroleum ether and ethyl acetate (EtOAc) to produce a petroleum ether-soluble fraction (PE fraction) and an EtOAc-soluble fraction (EA fraction). The EA fraction was subjected repeatedly to column chromatography on silica gel, Sephadex LH-20, ODS and semi-preparative reversed phase high performance liquid chromatography (RP-HPLC) to afford one new naphthalene (**1**), together with twelve known compounds which were identified (Figure 1) by comparison of their spectral data with those reported in literature as syriacusin A (**2**),[9] jatrocin B (**3**),[10] cleomiscosin A (**4**),[11] cleomiscosin C (**5**),[12] cleomiscosin B (**6**),[13] cleomiscosin D (**7**),[14] chrysoeriol (**8**),[15] isorhamnetin-3-*O*-rutinoside (**9**),[16] (-)-syringaresinol (**10**),[17] (-)-syringaresinol-4-*O*-β-D-glucopyranoside (**11**),[18] scopoletin (**12**),[19] and isoscopoletin (**13**).[20]

Compound **1** was isolated in the form of yellow needles, and the molecular formula was determined as $C_{16}H_{18}O_5$ by HRTOFESIMS at *m/z* 291.1235 $[M+H]^+$ (calcd. *m/z* 291.1232), corresponding to eight degrees of unsaturation. The IR spectrum showed the occurrence of hydroxyl (3210 cm^{-1}), carbonyl (2843, 2742 and 1672 cm^{-1}) and benzene rings (1600-1450 cm^{-1}). The ^1H NMR showed one phenolic hydroxyl group at δ_H 13.63 (s, 1H), one aldehyde group at δ_H 10.99 (s, 1H), two aromatic hydrogens at δ_H 8.43 (s, 1H) and 7.10 (s, 1H), a methoxy group at δ_H 3.74 (s, 3H), a methyl group of aromatic ring at δ_H 2.43 (s, 3H) and two methyl groups at δ_H 1.82 (s, 6H). The ^{13}C NMR spectrum combined with DEPT spectrum exhibited 16 carbons, including three methines carbons (δ_C 197.5, 126.5 and 114.8), four methyl carbons (δ_C 59.7, 31.6,31.6 and 16.4), as well as nine quaternary carbons (δ_C 164.7, 154.9, 148.3, 140.5, 126.1, 123.0, 121.1, 111.1 and 74.1). Combining with eight degrees of unsaturation, the presence of ten carbons in the range of δ_C 111.1-164.7 was consistent with the pattern of a bicyclic naphthalene carbon skeleton. The heteronuclear single quantum coherence (HSQC) spectrum showed the correlations H-9 (δ_H 10.99)/C-9 (δ_C 197.5), H-12 (δ_H 3.74)/C-12 (δ_C 59.7), H-11 (δ_H 2.43)/C-11 (δ_C 16.4), H-10a, H-10b (δ_H 1.82)/C-10a, C-10b (δ_C 31.6), H-5 (δ_H 8.43)/C-5 (δ_C 126.5), and H-3 (δ_H 7.10)/C-3 (δ_C 114.8), which confirmed the existence of an aldehyde group, a methoxy group, an aromatic methyl, two methyls and two aromatic hydrogens. In the heteronuclear multiple-bond correlation (HMBC) spectrum, the correlations from 2-OH (δ_H 13.63) to C-1 (δ_C 111.1), C-2 (δ_C 164.7), C-3 (δ_C 114.8) and C-4 (δ_C 154.9), correlations from H-9 (δ_H 10.99) to C-1 (δ_C 111.1), C-2 (δ_C 164.7) and C-3 (δ_C 114.8), allowed to assign the location of a phenolic hydroxyl group and an aldehyde group at C-2 and C-1, respectively. Similarly, the correlations from H-11 (δ_H 2.43) to C-5 (δ_C 126.5) and C-7 (δ_C 148.3), correlations

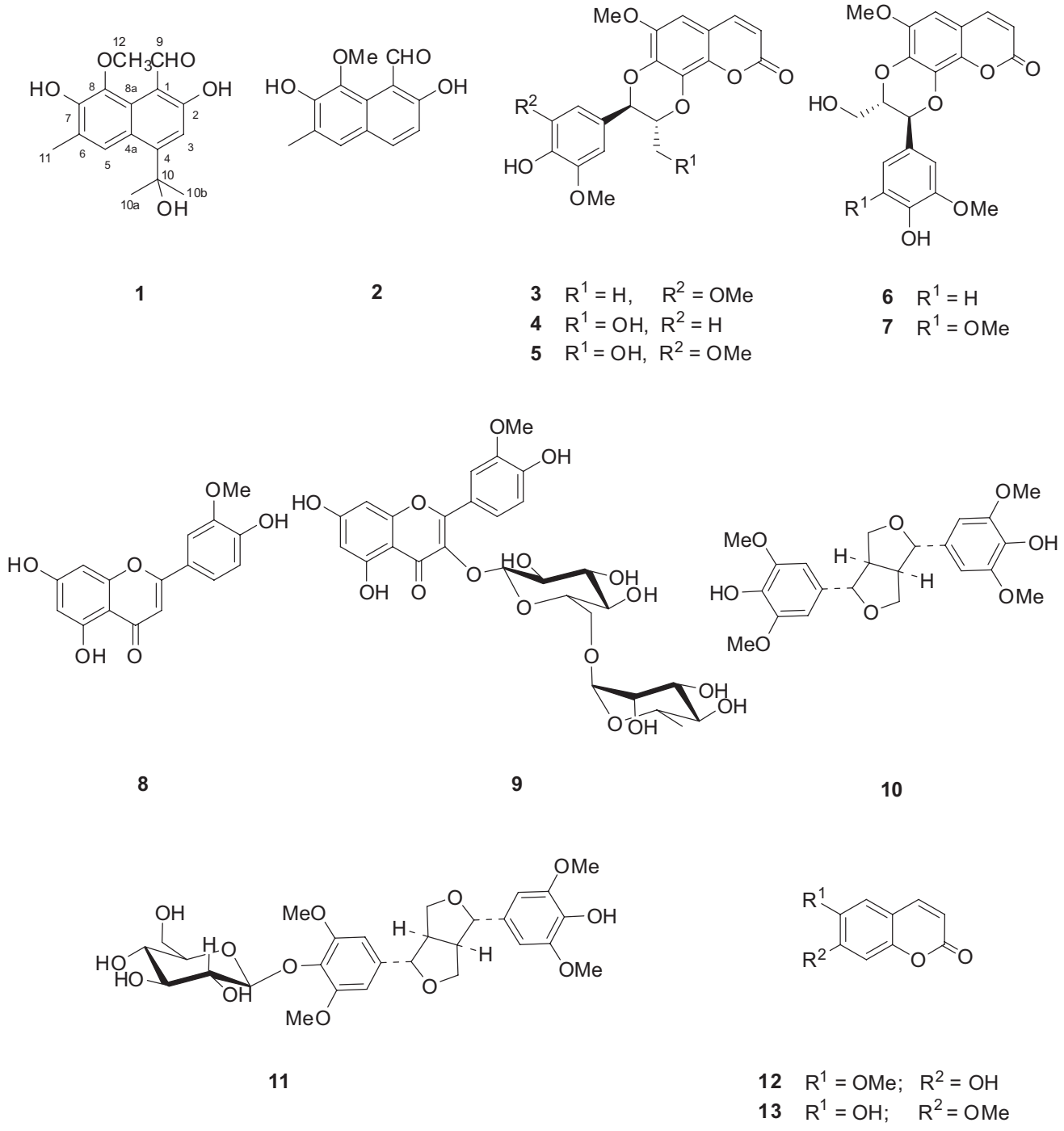

Figure 1. Structure of compounds **1**-**13** isolated from *Kosteletzkya virginica*.

from H-5 (δ_H 8.43) to C-4 (δ_C 154.9), C-7 (δ_C 148.3), C-6 (δ_C 126.1) and C-11 (δ_C 16.4), allowed to assign a phenolic hydroxyl group at C-7 and confirmed the location of a methyl group and a hydrogen at C-6 and C-5, respectively. A methoxy group was positioned at C-8, from H-12 (δ_H 3.74) to C-8 (δ_C 140.5). The HMBC correlations from H-10a, H-10b (δ_H 1.82) to C-10 (δ_C 74.1), C-3 (δ_C 114.8) and C-4 (δ_C 154.9), H-3 (δ_H 7.10) to C-10 (δ_C 74.1), as well as the data of ^{1}H NMR, ^{13}C NMR, HSQC and molecular weight, revealed an isopropanol group connected to C-4. In addition, the rotating-frame Overhauser spectroscopy (ROESY) correlations H-3 (δ_H 7.10)/H-10a (δ_H 1.82), H-3 (δ_H 7.10)/H-10b (δ_H 1.82), H-5 (δ_H 8.43)/H-10a (δ_H 1.82) and H-5 (δ_H 8.43)/H-10b (δ_H 1.82) also evidenced the substitution at C-4. The above findings support the structure of **1** as 8-methoxy-2,7-dihydroxyl-4-(1'-hydroxyl-isopropyl)-6-methyl-1-naphthaldehyde, named as virginicin (Figure 2) (Table 1).

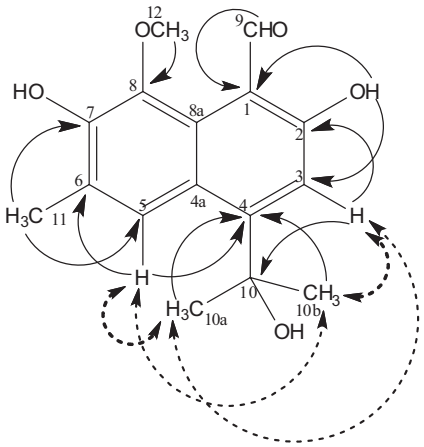

Figure 2. Key HMBC (H ⟶ C) and ROESY (H ⟷ H) correlations of compound **1**.

Table.1 ^{1}H NMR, ^{13}C NMR (500 and 125 MHz, respectively) and relevant HMBC correlations for compound **1** in $CDCl_3$

Position	δ_H / ppm	δ_C / ppm	HMBC
1		111.1	
2		164.7	
3	7.10 (s, 1H)	114.8	C-1,C-2,C-4a,C-10
4		154.9	
4a		121.1	
5	8.43 (s, 1H)	126.5	C-4,C-6,C-7,C-11
6		126.1	
7		148.3	
8		140.5	
8a		123.0	
9	10.99 (s, 1H)	197.5	C-1,C-2,C-3
2-OH	13.63 (s, 1H)		C-1,C-2,C-3,C-4
10		74.1	
10a, 10b	1.82 (s, 6H)	31.6	C-3,C-4,C-10
11	2.43 (s, 3H)	16.4	C-5,C-7
12	3.74 (s, 3H)	59.7	C-8

Compounds **1-7** were evaluated for their antioxidant, anti-inflammatory and antitumor activities (Table 2). Since these compounds have aromatic hydroxyl groups, most of them exhibited DPPH$^{\bullet}$ scavenging activity, especially compounds **1** and **2**, which exhibited IC_{50} values of 58.1 and 34.6 μmol L^{-1}, respectively. Furthermore, compounds **1** and **2** demonstrated to inhibit the LPS-induced production of NO, indicating their anti-inflammatory effects, which corroborates the traditional use of *K. virginica* in the treatment of the upper respiratory tract inflammation.[5] Results of *in vitro* antitumor assay showed that compound **1** had cytotoxicity against HL-60 and Lovo cell lines with IC_{50} values of 40.5 and 31.7 μmol L^{-1}, respectively, whereas compound **2** had selective cytotoxicity to HL-60 with an IC_{50} value of 87.6 μmol L^{-1}. Compound **3-7** were found to be inactive (IC_{50} > 100 μmol L^{-1}).

Table 2. Antioxidant, anti-inflammatory and antitumor activities (IC_{50} / μmol L^{-1}) of compounds **1-7**

Compound	Antioxidant activity	Anti-inflammatory activity	Antitumor activity	
	DPPH$^{\bullet}$	LPS-induced NO	Lovo	HL-60
1	58.1	12.5	31.7	40.5
2	34.6	17.7	87.6	> 100
3	> 500	> 100	> 100	> 100
4	> 500	> 100	> 100	> 100
5	103.1	> 100	> 100	> 100
6	191.5	> 100	> 100	> 100
7	86.6	> 100	> 100	> 100
Vit C	18.7	–	–	–
Indomethacin	–	0.2	–	–
Cis-platin	–	–	9.6	2.2

In addition, it was reported that cleomiscosin A (**4**), cleomiscosin B (**6**) and cleomiscosin C (**5**) have certain protective effect on liver injury.[21] Since *K. virginica* is enriched with these compounds, its hepatoprotective effect worth detailed studying.

Conclusions

The first phytochemical investigation on the tubers of *Kosteletzkya virginica,* here described, resulted in the isolation of a new naphthalene (**1**) named as virginicin, along with a known naphthalene (**2**), two flavones (**8**, **9**), two lignans (**10**, **11**) and seven coumarins (**3**, **4**, **5**, **6**, **7**, **12**, **13**). Compounds **6**, **8**, **9** and **13** are being reported from Malvaceae family for the first time. Compound **1** showed potent activities in scavenging DPPH$^{\bullet}$, inhibition of nitric oxide (NO) induced by LPS, and cytotoxicity towards LOVO and HL-60 cell lines. These promising results, in addition to the agricultural potential and traditional medicinal use of this species,[2,3,5] justify its further research and development.

Supplementary Information

Supplementary data associated with this paper are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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