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Potential allelochemicals from *Sambucus nigra*

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Abstract

Twenty-four aromatic metabolites belonging to cyanogenins, lignans, flavonoids, and phenolic glycosides were obtained from *Sambucus nigra*. Structures were determined on the basis of their spectroscopic features. Two compounds have been isolated and identified as (2*S*)-2-*O*-β-D-glucopyranosyl-2-hydroxyphenylacetic acid and benzyl 2-*O*-β-D-glucopyranosyl-2,6-dihydroxybenzoate. All the compounds have been assayed on dicotyledons *Lactuca sativa* (lettuce) and *Raphanus sativus* (radish) and monocotyledon *Allium cepa* (onion) to test their stimulatory or inhibitory effects on seed germination and radicle elongation. Cyanogenins have a mainly inhibiting effect while lignans stimulate the growth. Some compounds show different effects on dicotyledons and monocotyledons.

Keywords: *Sambucus nigra*; Adoxaceae; Cyanogenins; Lignans; Flavonoids; Phenolic glycosides; Allelopathic activity

1. Introduction

Weed interference, which consists of the effects of competition and/or allelopathy, represents a serious threat to several crops. *Sambucus nigra* L., a shrub widely found throughout Italy, causes large crops losses, and, in a previous study Jensen and Nielsen (1973) reported the presence of cyanogenic glycosides. These compounds are considered highly phytotoxic since the action of microbes in the rhizosphere produces HCN and benzaldehydes (Conn, 1981). In a study of the detrimental effects of weeds on crops, we have reinvestigated *S. nigra* and, together with the cyanogenic glycosides **1**–**5** (Della Greca et al., 2000a) and three related compounds **6**–**8** (Della Greca et al., 2000b), we have isolated the phenolic glycosides **9**–**13** besides the lignans **14**–**19** and the flavonoids **20**–**24**. All the compounds have been tested for their effects on the seed germination and growth of *Lactuca sativa* L., *Raphanus sativus* L., and *Allium cepa* L.

2. Results and discussion

The air dried leaves of *S. nigra* were sequentially extracted with petrol, EtOAc, and MeOH. A preliminary seed germination test on *R. sativus* evidenced a strong inhibitory activity only of the MeOH extract, which was distributed between water and EtOAc (Scheme 1). The organic fraction was chromatographed on silica gel column to give fractions A–C. Chromatography on Sephadex LH-20 of fraction A, eluted with $CHCl_3$, gave the degraded cyanogenins **6**–**8** (Della Greca et al., 2000b) along with the neolignan (2*R-trans*) 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-5-benzofuranpropanol acetate (**14**) (Popoff and Theander, 1975) and the lignans (+) pinoresinol (**17**) (Pelter et al., 1982), (+) medioresinol (**18**) (Zhuang et al., 1982) and (+) lariciresinol (**19**) (Katayama et al., 1992). Fraction B, eluted with EtOAc, was re-chromatographed on Sephadex LH-20 to give the glucoside **13**, besides a further two neolignans, identified as (2*R-trans*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-5-benzofuran-propanol (**15**) (Toshio et al., 1989) and (2*R-trans*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-

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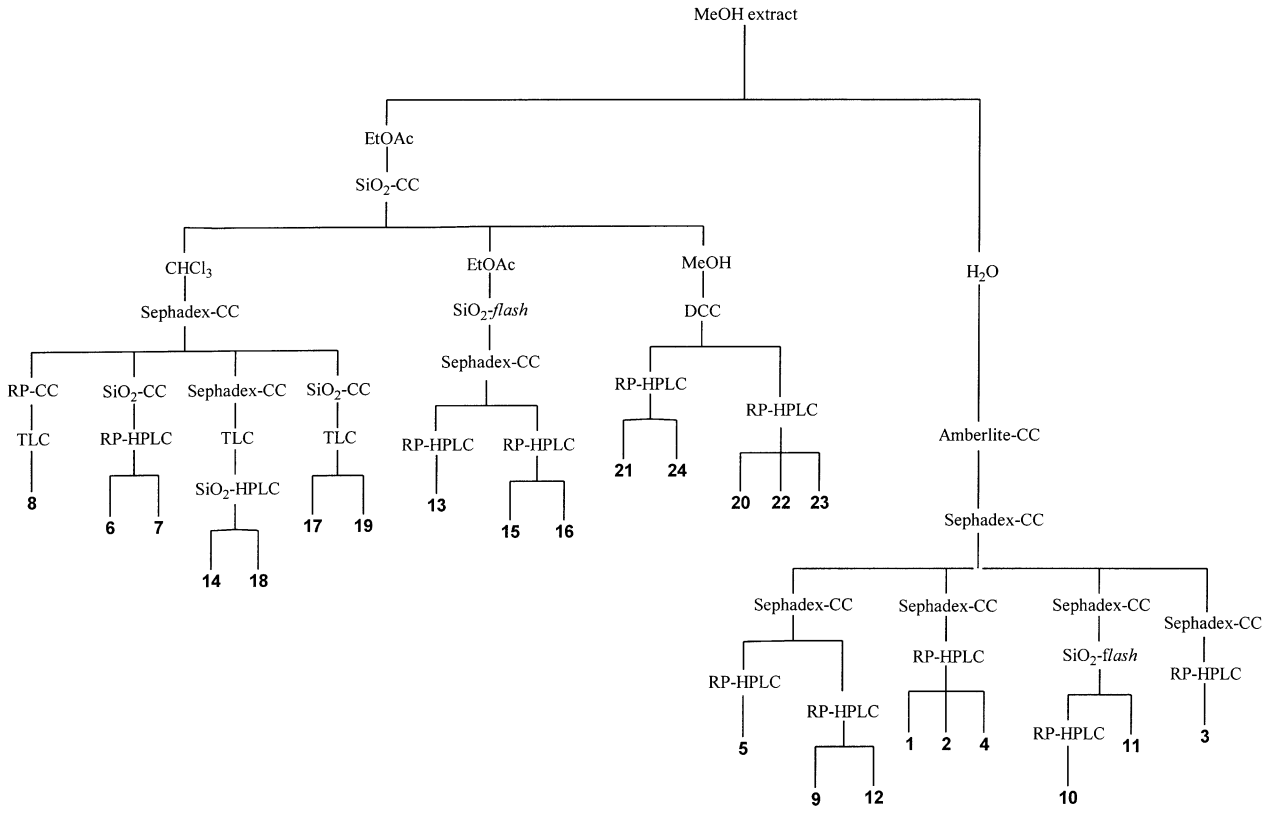


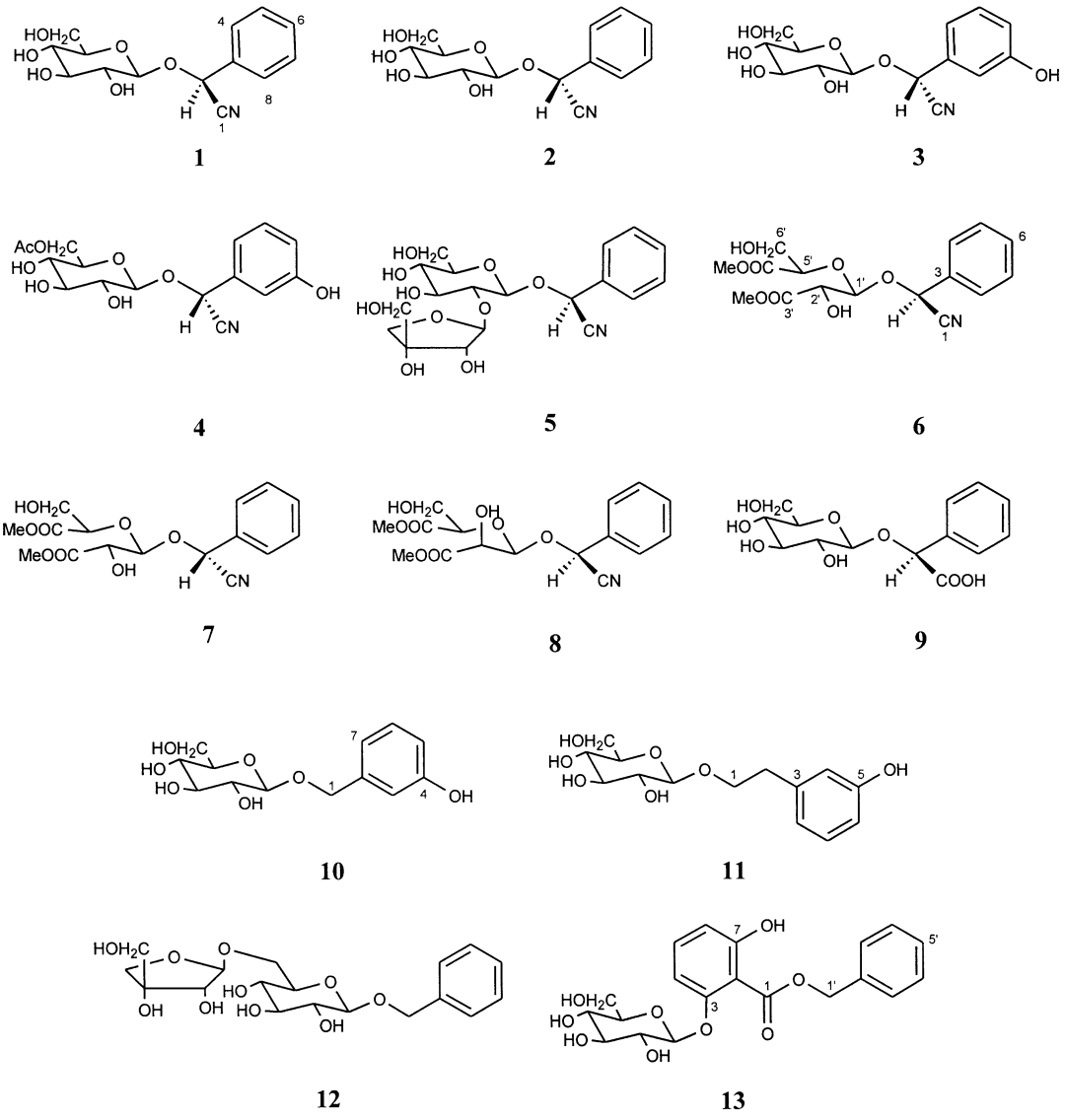
Scheme 1.

hydroxy-5-benzofuran-propanol (**16**) (Popoff and Theander, 1975).

The new compound **13** had the molecular formula $C_{20}H_{22}O_9$ according to the elemental analysis and the ^{13}C NMR data. The EIMS spectrum showed a peak at *m/z* 244 due to the loss of the glucose, besides other peaks at *m/z* 137, 107, 91 and 77. The ^1H NMR spectrum showed three aromatic protons as two double doublets at δ 6.73 and 6.59 and a triplet at δ 7.26, besides five further aromatic protons in the 7.55–7.32 ppm range. A singlet at δ 5.37, two double doublets at δ 3.86 and 3.65, and four overlapping protons in the 3.45–3.35 ppm range were also present in the spectrum. Also on the basis of HMQC and HMBC experiments structure of benzyl 2-*O*-β-D-glucopyranosyl-2,6-dihydroxybenzoate structure. Accordingly enzymatic hydrolysis with β-glucosidase gave verimol K (Sy and Brown, 1998). Fraction C, eluted with MeOH, was chromatographed by DCCC to give the flavone glycosides quercitin 3-glucoside (**20**) and quercitin 3-*O*-neohesperidoside (**24**) (Markham et al., 1978), kampferol 3-*O*-neohesperidoside (**21**) and kampferol 3-glucoside (**22**) (Harborne and Williams, 1988) and rhamnetin 3-glucoside (**23**).

The aqueous fraction was chromatographed on Amberlite XAD-4 and the fraction eluted with MeOH was partitioned on Sephadex LH-20 into fractions A–D. Fraction A, eluted with water, gave the cyanogenin (2*S*)-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranosyl-mandelonitrile (**5**) (Della Greca et al., 2000a) and the glycosides **9** and **12**. The first, $[\alpha]_D$ +6°, has been isolated for the first time and identified as (2*S*)-2-*O*-β-D-glucopyranosyl-2-hydroxy-phenylacetic acid. It had the molecular peak at *m/z* 314 in the EIMS spectrum and showed 14 carbon signals in the ^{13}C NMR spectrum in accordance to the molecular formula $C_{14}H_{18}O_8$. All its physical data were identical to those reported by Kitajima and Tanaka (1993) for (2*R*)-2-*O*-β-D-glucopyranosyl-2-hydroxy-phenylacetic acid, isolated from *Prunus zippeliana*, except for its rotation, thus justifying the *S* configuration at C-2. Accordingly, acid hydrolysis of **9** gave *l*-mandelic acid. We have identified compound **12** as icariside F_2 [benzyl alcohol β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside] by comparison with the NMR data given by Miyase et al. (1989). Fraction B, eluted with MeOH–H_2O (1:3), consisted of the cyanogenins sambunigrin (**1**), prunasin (**2**) and 6-acetyl holocalin (**4**) (Della Greca et al., 2000a). Fraction C, eluted with MeOH–H_2O (1:2), afforded the glucosides **10** and **11**. The first glucoside was obtained by enzymatic synthesis by Trincone et al. (1994) while the latter was reported as a tobacco fragrance-improving agent by Keniji et al. (2000). Fraction D, eluted with MeOH–H_2O (1:1), afforded the cyanogenin holocalin (**3**) (Della Greca et al., 2000a).

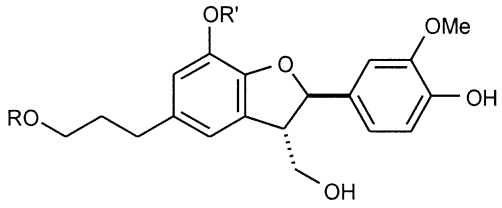
All the compounds have been tested on *L. sativa* (lettuce), *R. sativus* (radish), and *A. cepa* (onion) to evalu-



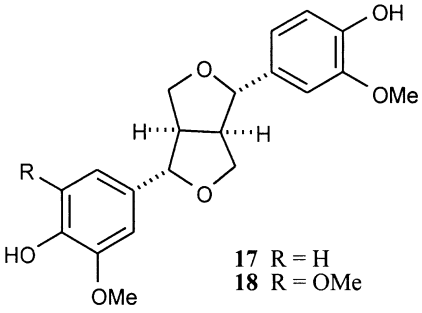
ate their influence on the seed germination and the radicle elongation. The assays have been run according to the procedures optimised by Macías et al. (2000). Flavonoids **20**–**24** show no significant activity, while the effects of the active compounds, presented as percentage differences from the control, are reported in Tables 1 and 2 and in Figs. 1 and 2.

Cyanogenins **1**–**3** inhibit the germination of the test species at the highest concentration. The effects are stronger on dicotyledons *R. sativus* and *L. sativa* than on monocotyledon *A. cepa*. The inhibition decreases with dilution and at 10^{-5} M concentration only the most active holocalin (**3**) conserves its activity while sambunigrin (**1**), and prunasin (**2**) have a slight stimulant effect. A chemical comparison between sambunigrin (**1**) and prunasin (**2**) shows that the different configuration at the C-2 stereocentre is non-influent, while the introduction of the hydroxyl group at the *meta* position of prunasin (**2**) to give holocalin (**3**) enhances the activity. All the modifications of cyanogenins, such as acetylation of holocalin (**3**) to **4**, glycosylation of sambunigrin (**1**) to **5** as well as glucose oxidation, which transforms **1** and **2** into **6** and **7**, reduce the inhibitory activity, and the overall germination effects of the compounds show less than 10% difference from the control. The effects on the radicle elongation of the three species are rather similar to those observed for germination. Cyanogenins **1**–**3** have strong activity at the highest concentrations and an almost total inhibition is observed at 10^{-3} M concentration on *R. sativus*. At the lowest concentrations sambunigrin (**1**) and prunasin (**2**) stimulate the radicle growth of *R. sativus* slightly. The effect of the modification of cyanogenins is less strong on the radicle length. Only acetyl-holocalin (**4**) is practically inactive, while the other cyanohydrins are moderately inhibiting.

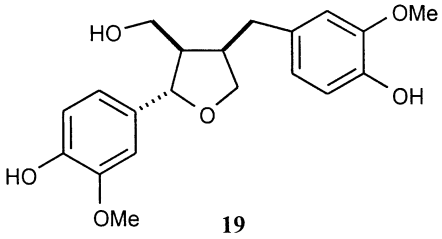
Among glycosides **9**–**13**, **9** and **10** may be considered to derive respectively from sambunigrin (**1**) and holocalin (**3**) by hydrolysis of nitrile or from hydrolysis and subsequent decarboxylation. Both of them affect the



14 R = Ac R' = Me
15 R = H R' = Me
16 R = H R' = H



17 R = H
18 R = OMe



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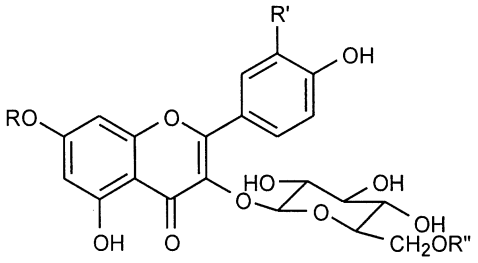

20 R = H R' = OH R" = H
21 R = H R' = H R" = rha
22 R = H R' = H R" = H
23 R = Me R' = OH R" = H
24 R = H R' = OH R" = rha

Table 1
Bioactivity data of compounds **1–19** on the radicle length of *Lactuca sativa*, *Raphanus sativum* and *Allium cepa*[a]

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Lactuca sativa	10^{-3} M	[–]	[–]	[–]	–	–	(–)	(–)	(–)	0	0	(+)	0	+	[+]	[–]	+	[+]	[+]	+
	10^{-4} M	(–)	(–)	(–)	0	–	(–)	(–)	(–)	0	0	(+)	0	+	(+)	0	(+)	[+]	[+]	0
	10^{-5} M	–	–	(–)	0	–	–	(–)	(–)	0	+	0	0	+	(+)	(+)	+	(+)	[+]	(+)
	10^{-6} M	0	0	(–)	0	–	–	–	–	0	+	0	0	0	+	(+)	(+)	[+]	(+)	(+)
Raphanus sativus	10^{-3} M	[–]	[–]	[–]	0	0	–	–	–	–	–	–	0	(–)	–	(+)	–	[+]	+	–
	10^{-4} M	0	–	(–)	0	0	–	–	–	–	–	–	0	–	+	(+)	+	[+]	(+)	–
	10^{-5} M	+	+	–	0	0	–	–	–	–	0	–	0	(+)	[+]	(+)	(+)	[+]	(+)	0
	10^{-6} M	+	+	–	0	0	0	0	0	–	0	0	0	(+)	(+)	[+]	(+)	[+]	+	[+]
Allium cepa	10^{-3} M	(–)	(–)	(–)	0	0	(–)	(–)	–	(–)	–	–	–	–	+	(–)	[–]	+	–	+
	10^{-4} M	(–)	–	0	0	0	(–)	(–)	(–)	–	0	–	–	0	+	+	+	+	0	0
	10^{-5} M	–	(–)	0	–	+	(–)	(–)	(–)	–	0	0	+	(+)	(+)	+	0	0	0	(+)
	10^{-6} M	0	0	0	0	+	0	–	–	–	0	–	+	+	+	(+)	(+)	(+)	–	(+)

[a] 0, Stimulatory or inhibitory values $<10\%$; + and –, stimulatory or inhibitory values between 10 and 30%; (+) and (–), stimulatory or inhibitory values between 31 and 60%; [+] and [–], stimulatory or inhibitory values $>61\%$.

germination of the three species slightly. It is worth noting that the effect is inhibiting on dicotyledons and stimulating on monocotyledon. Further effects are observed on the radicle elongation and a reduction of the length occurs in the test species. The other glycosides **11**–**13** have an analogous behaviour.

The neolignans **14**–**16** have little effect on the germination of the three species, while they stimulate the radicle elongation of lettuce and radish quite well. *A. cepa* is inhibited at its highest concentration. Furofuran lignans **17** and **18** have no effect on the germination of dicotyledons, whereas they do influence onion. It is interesting that compound **17** is stimulating but **18** is inhibiting. The chemical difference between the compounds is the presence of a further methoxyl group in **18**. The radicle elongation test shows that the compounds have strong effects on dicotyledons, while they influence monocotyledon only slightly. The effect is that of a strong increase of the radicle length which is higher with compound **17**. Lignan **19** has little effect on the germination but a good stimulating effect on the radicle elongation.

Table 2
Bioactivity of compounds **1**–**19** on the germination of *Lactuca sativa*, *Raphanus sativum* and *Allium cepa*[a]

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Lactuca sativa	10^{-3} M	(−)	(−)	[−]	0	0	0	0	0	−	0	−	−	0	0	−	0	0	0	0
	10^{-4} M	0	0	(−)	0	0	0	0	0	−	0	−	−	0	0	−	0	0	0	0
	10^{-5} M	0	0	−	0	0	0	0	0	0	0	−	0	0	0	−	0	0	0	0
	10^{-6} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−	0	0	0	0
Raphanus sativus	10^{-3} M	−	−	[−]	0	−	0	0	0	−	0	0	−	0	0	0	0	0	+	0
	10^{-4} M	−	−	−	0	−	0	0	0	−	0	0	−	0	0	0	0	0	+	0
	10^{-5} M	0	0	(−)	0	0	0	0	0	−	0	0	0	0	0	0	0	0	+	0
	10^{-6} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	(+)	0
Allium cepa	10^{-3} M	−	−	−	0	0	0	0	0	+	+	0	+	+	+	+	0	(+)	(−)	0
	10^{-4} M	−	−	−	0	0	0	0	0	+	+	0	+	(+)	+	+	0	(+)	(−)	+
	10^{-5} M	0	+	0	0	0	0	0	0	0	+	+	+	+	+	0	0	(+)	+	+
	10^{-6} M	0	0	−	0	0	0	0	0	0	+	+	+	(+)	+	0	0	(+)	0	+

[a] 0, Stimulatory or inhibitory values <10%; + and −, stimulatory or inhibitory values between 10 and 30%; (+) and (−), stimulatory or inhibitory values between 31 and 60%; [+] and [−], stimulatory or inhibitory values >61%.

In conclusion, *S. nigra* produces phytostimulating and phytoinhibiting metabolites. Lignans belong to the first group, while the cyanogenins and their derivatives belong to the latter.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AC400 spectrometer with 0.05 solns. in $CDCl_3$ or CD_3OD at 37 °C. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^1J_{HC} = 145$ Hz) and HMBC (optimised for $^1J_{HC} = 7$ Hz). Optical rotations were measured on a Perkin–Elmer 343 polarimeter. IR spectra were determined in $CHCl_3$ solns on a FT–IR Perkin–Elmer 1740 spectrometer. UV spectra were obtained on a Perkin–Elmer Lambda 7 spectrophotometer in EtOH solns. EI mass spectra were obtained with a Kratos MS 80 apparatus. FAB mass spectra were recorded on a VG ZAB 2SE apparatus. HPLC UV apparatus consisted of a Beckman pump—System Gold 127, a Beckman System Gold 166 UV detector, and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC were performed using SiO_2 (Maxsil Silica 10 μm, 250×10 mm i.d., Phenomenex), RP-18 (Luna 10 μm, 250×10 mm i.d., Phenomenex), or RP-8 (Luna 10 μm, 250×10 mm i.d., Phenomenex) columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm film thickness. Spots were visualised by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4) followed by heating for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography was performed on Merck Kieselgel 60 (230–400 mesh) at a medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), on Sephadex LH-20® (Pharmacia) or on Amberlite XAD-4 (20-60 mesh, Fluka). DCCC was performed on a D.C.C.-A Tokyo Rikakikai LTD (300 columns, 400×2 mm).

3.2. Plant material

Plants of *S. nigra* L. were collected in Agerola (Naples) in July and identified by professor G. Aliotta. A voucher specimen (HERBNAPG074) was deposited at the Dipartimento di Biologia Vegetale of University Federico II of Naples.

3.3. Extraction and isolation

The air-dried and powdered plant material (4.1 kg) was sequentially and exhaustively extracted with petrol, EtOAc and MeOH. The MeOH extract (370 g) was partitioned between H_2O (2 l) and EtOAc (1 l).

The organic fraction (92.9 g) was chromatographed on SiO_2 to give fractions A–C. Fraction A (57 g), eluted with $CHCl_3$, was chromatographed on Sephadex LH-20 eluting with hexane –$CHCl_3$–MeOH (3:1:1) and collecting volumes of 10 ml. Fractions 15-19 were chromatographed on TLC [$CHCl_3$–AcOEt (4:1)] and purified on RP-8 eluting with MeOH–MeCN–H_2O (3:2:5) to give compound **8** (18 mg). Silica gel chromatography of fractions 20–23 gave the epimeric mixture of **6** and **7** that was resolved on RP-18 HPLC by eluting with MeOH–MeCN–H_2O (1:1:3) (5 and 3 mg, respectively). Fractions 24–30 were re-chromatographed on TLC [$CHCl_3$–MeOH (99:1)] and SiO_2 HPLC [hexane–acetone (13:7)] to give the lignans **14** (15 mg) and **18** (8 mg). Finally, fractions 31–37 were purified first by flash chromatography [$CHCl_3$–MeOH (49:1)] and then by TLC [benzene–MeOH (9:1)], to give the compounds **17** (21 mg) and **19** (19 mg). Fraction B (11 g), eluted with EtOAc, was chromatographed on SiO_2. The fractions so

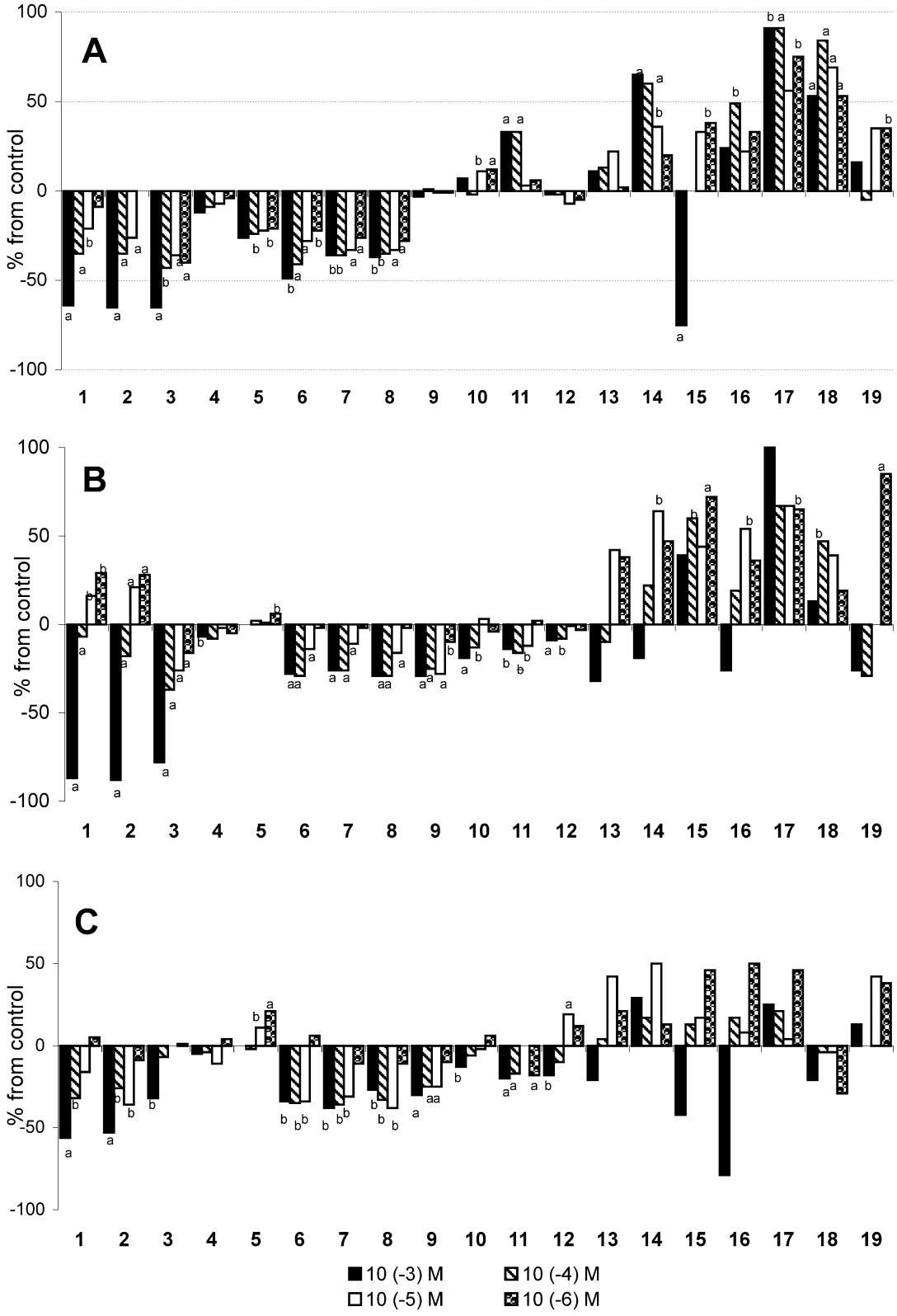

Fig. 1. Effect of compounds **1**–**19** on radical length of *Lactuca sativa* (A), *Raphanus sativus* (B) and *Allium cepa* (C). Values presented as percentage differences from control; $P > 0.05$ for the Welch's test. A, $P < 0.01$; B, $0.01 < P < 0.05$.

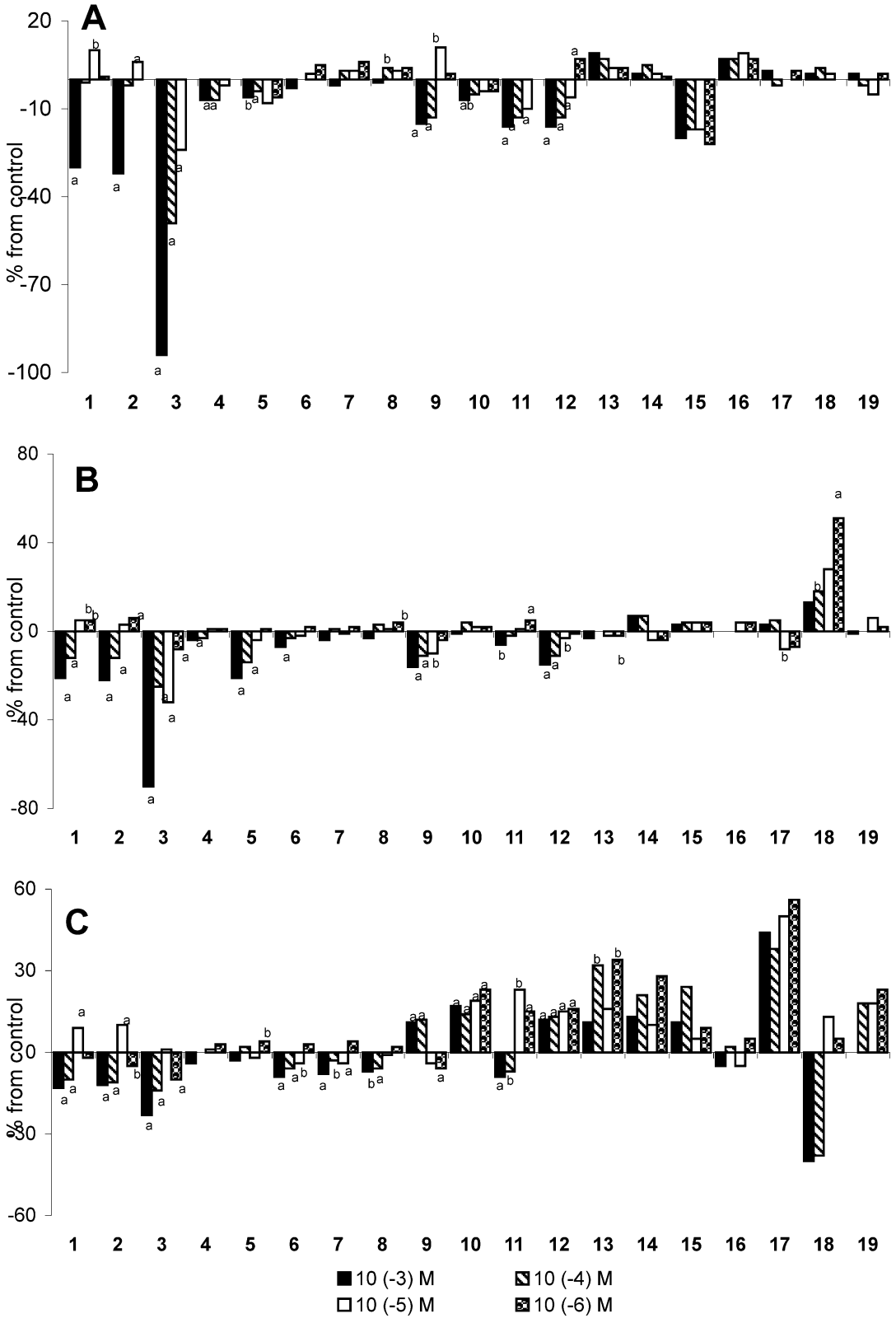

Fig. 2. Effect of compounds **1**–**19** on seed germination of *Lactuca sativa* (A), *Raphanus sativus* (B) and *Allium cepa* (C). Values presented as percentage differences from control; $P > 0.05$ for the Welch's test. A, $P < 0.01$; B, $0.01 < P < 0.05$.

obtained, eluted with $CHCl_3$–MeOH–H_2O (14:5:1), were re-chromatographed by Sephadex LH-20 with MeOH-H_2O (7:3) as eluent; fractions 43–48 were purified by RP-18 HPLC [MeOH–MeCN–H_2O (37:2:11)] to give glucoside **13** (21 mg), while fractions 51-58 were purified by RP-18 HPLC [MeOH–MeCN–H_2O (9:2:9)] to give compounds **15** (11 mg) and **16** (7 mg). Fraction C (10.2 g), eluted with MeOH, was chromatographed on a DCCC apparatus with $CHCl_3$–MeOH–H_2O (13:7:5) in ascending mode, using the upper layer as mobile phase. RP-18 HPLC of the fractions 17–22 [A: 2% AcOH, B: MeOH-2% AcOH (8:2); gradient A:B from 19:1 to 3:2 in 30 min] gave the disaccharide flavonoids **21** (25 mg) and **24** (19 mg); RP-18 HPLC purification of the fractions 75–96 [A: 2% AcOH, B: MeOH-2% AcOH (8:2); gradient A:B from 3:1 to 9:11 in 30 min] gave the flavone glucosides **20** (18 mg), **22** (14 mg) and **23** (6 mg).

The aqueous layer, conc. at 500 ml, was chromatographed on Amberlite XAD-4 and, using chromatography on Sephadex LH-20, the MeOH elute (5.9 g) was partitioned into the fractions A–D. Fraction A (1.9 g), eluted with H_2O, was re-chromatographed on Sephadex LH-20 using MeOH-$CHCl_3$–hexane (4:2:1) as eluent to give crude **5**, which was purified by RP-18 HPLC [MeOH–MeCN–H_2O (17:3:30), 6 mg)] and a mixture of glycosides **9** and **12** that was resolved by RP-18 HPLC (13 and 11 mg, respectively). Fraction B (345 mg), eluted with H_2O–MeOH (3:1) was re-chromatographed on Sephadex LH-20, using MeOH–$CHCl_3$–hexane (4:2:1) as eluent to give a mixture of cyanogenic glycosides **1**, **2** and **4** which was resolved by RP-18 HPLC [A: 2% AcOH, B: MeOH-2% AcOH (8:2); gradient A:B from 17:3 to 7:3 in 25 min; **1**, **2** and **4**: 45, 68 and 8 mg, respectively]. Subsequent purification of fraction C (684 mg), eluted with MeOH–H_2O (2:1), on Sephadex LH-20 and then by flash chromatography [$CHCl_3$–MeOH–H_2O (13:7:3), lower layer] gave the compound **11** (9 mg) and a fraction that, purified by RP-18 HPLC [MeOH–MeCN–H_2O (7:2:4)], gave the glucoside **10** (13 mg). Fraction D (2.0 g), eluted with H_2O–MeOH (1:1) was chromatographed on Sephadex LH-20, using MeOH–$CHCl_3$–hexane (4:2:1) as eluent to give crude **3** (220 mg). An aliquot of the crude product was purified by RP-18 HPLC [A: 2% AcOH, B: MeOH-2% AcOH (8:2); gradient A:B from 4:1 to 3:1 in 10 min].

3.4. Compound characterisation

3.4.1. (2S)-2-O-β-D-Glucopyranosyl-2-hydroxyphenylacetic acid (**9**)

Colorless oil; $[\alpha]_D$ +6°; UV (EtOH) λ_{max} nm: 279; ^{1}H NMR (CD_3OD) δ 7.50 (2H, *m*, H-4 and H-8), 7.32 (3H, *m*, H-5–H-7), 5.27 (1H, *s*, H-2), 4.51 (1H, *d*, J = 8.0 Hz, glc-1), 3.86 (1H, *dd*, J = 11.8 and 1.1 Hz, glc-6), 3.68 (1H, *dd*, J = 11.8 and 5.6 Hz, glc-6), 3.50–3.30 (4H, *m*, glc-2–glc-5). EIMS *m*/*z*: 314 $[M]^+$, 152 $[M-glucose]^+$. (Found: C, 53.3; H, 5.9. $C_{14}H_{18}O_8$ requires: C, 53.5; H, 5.8%.)

3.4.2. 3-Hydroxybenzyl 1-O-β-D-glucopyranoside (**10**)

Colorless oil; UV λ_{max} (EtOH) nm 280; ^{1}H NMR (CD_3OD) δ 7.14 (1H, *dt*, J = 7.7 and 1.0 Hz, H-6), 6.87 (1H, *dd*, J = 7.7 and 1.9 Hz, H-7), 6.86 (1H, *d*, J = 1.8 Hz, H-3), 6.71 (1H, *ddd*, J = 7.7, 1.9 and 1.0 Hz, H-5), 4.85 (1H, *d*, J = 11.8 Hz, H-1), 4.59 (1H, *d*, J = 11.8 Hz, H-1), 4.35 (1H, *d*, J = 7.7 Hz, glc-1), 3.89 (1H, *dd*, J = 12.0 and 2.2 Hz, glc-6), 3.69 (1H, *dd*, J = 12.0 and 5.5 Hz, glc-6), 3.35–3.20 (4H, H-2′–H5′). EIMS *m*/*z*: 286 $[M]^+$, 124 $[M-glucose]^+$, 107 $[M-OH-glucose]^+$. (Found: C, 54.4; H, 6.5. $C_{13}H_{18}O_7$ requires: C, 54.5; H, 6.3%.)

3.4.3. 1-O-β-D-Glucopyranosyl-2-(3-hydroxyphenyl)-ethanol (**11**)

Colorless oil; UV λ_{max} (EtOH) nm: 280; ^{1}H NMR (CD_3OD) δ 7.09–6.78 (4H, *m*, H-4, H-6–H-8), 4.75 (1H, *d*, J = 8.0 Hz, glc-1), 3.92 (1H, *dd*, J = 11.8 and 1.1 Hz, glc-6), 3.73 (1H, overlapped, glc-6), 3.72 (2H, *t*, J = 7.0 Hz, H-1), 3.50–3.30 (4H, *m*, glc-2–glc-5), 2.71 (2H, *t*, J = 7.0 Hz, H-2). EIMS *m*/*z*: 300 $[M]^+$, 138 $[M-glucose]^+$. (Found: C, 59.9; H, 6.8. $C_{14}H_{20}O_7$ requires: C, 56.0, H, 6.7%.)

3.4.4. Benzyl alcohol β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**12**)

Colorless oil; UV λ_{max} (EtOH) nm: 280. ^{1}H NMR (CD_3OD) δ 7.42–7.25 (5H, *m*, H-3–H-7), 5.07 (1H, *d*, J = 2.0 Hz, api-1), 4.91 (1H, *d*, J = 11.9 Hz, H-1), 4.66 (1H, *d*, J = 11.9 Hz, H-1), 4.33 (1H, *d*, J = 7.8 Hz, glc-1), 4.05–3.30 (11H, glc-2–glc-6 and api-2–api-5). FABMS (negative) *m*/*z*: 401 $[M-H]^-$, 269 $[M-api-H]^-$, 107 $[M-api-glc-H]^-$. (Found: C, 53.8; H, 6.4. $C_{18}H_{26}O_{10}$ requires: C, 53.7, H, 6.5%.)

3.4.5. Benzyl 2-O-β-D-glucopyranosyl-2,6-dihydroxybenzoate (**13**)

Colorless oil; IR ν_{max} ($CHCl_3$) cm^{-1}: 1715, 1605. ^{1}H NMR (CD_3OD) δ 7.55–7.32 (5H, *m*, H-3′–H-7′), 7.26 (1H, *t*, J = 8.4 Hz, H-5), 6.73 (1H, *dd*, J = 8.4 and 1.0 Hz, H-4), 6.59 (1H, *dd*, J = 8.4 and 1.0 Hz, H-6), 5.37 (2H, *s*, H-1), 4.93 (1H, *d*, J = 7.2 Hz, glc-1), 3.86 (1H, *dd*, J = 12.0 and 1.2 Hz, glc-6), 3.65 (1H, *dd*, J = 12.0 and 5.9 Hz, glc-6), 3.45–3.35 (4H, *m*, glc-2–glc-5); ^{13}C NMR (CD_3OD) δ 170.3 (C-1), 160.1 (C-3), 158.5 (C-7), 137.6 (C-2′), 134.3 (C-5), 129.8 (C-4′, C-6′), 129.5 (C-3′, C-5′, C-7′), 111.9 (C-6), 108.1 (C-2), 108.0 (C-4), 103.0 (glc-1), 78.6 (glc-5), 78.2 (glc-3), 75.2 (glc-2), 71.5 (glc-4), 68.4 (C-1′), 62.8 (glc-6). EIMS *m*/*z*: 244, 137, 107, 91, 77. (Found: C, 59.0; H, 5.4. $C_{20}H_{22}O_9$ requires C, 59.1, H, 5.5%.)

3.5. Bioassays

Seeds of *R. sativus* L. "Saxa", collected during 2000, were purchased from Improta Co., Naples. Seeds of *L. sativa* and *A. cepa,* collected during 2000, were obtained from Blumen® (Milan, Italy). All undersized or damaged

seeds were discarded and the assay seeds were selected for uniformity.

For the bioassays we used Petri dishes in two sizes (90 and 50 mm diameter) with one sheet of Whatman No. 1 filter paper as support. In three replicate experiments, germination and growth were conducted in aqueous solutions at controlled pH. Test solns. (10^{-3} M) were prepared using MES (2-[*N*-morpholino]ethanesulfonic acid, 10 mM, pH 6) and the rest (10^{-4} –10^{-6} M) were obtained by dilution. Parallel controls were performed. After adding 30 seeds and aqueous solns., Petri dishes were sealed with Parafilm® to ensure closed-system models. Seeds were placed in a growth chamber KBW Binder 240 at 25 °C, with 60% relative humidity, and a 12 h photoperiod, 10,000 lux. Germination percentage was determined daily for four days for radish and five days for onion and lettuce (no more germination occurred after this time). After growth, plants were frozen at −20 °C to avoid subsequent growth until the measurement process.

Data are reported as percentage differences from control in the graphics and tables. Thus, zero represents the control; positive values represent stimulation of the control; positive values represent stimulation of the parameter studied and negative values represent inhibition.

3.5.1. Statistical treatment

Comparison was made between the two blocks assayed by applying Welch's test, a variant of Student's *t* test (Zar, 1984; Martin and Luna, 1990), calculating mean values for every parameter (germination average, and radicle elongation) and their population variance within a Petri dish.

Coefficient of variation (CV) was used to compare dispersion from different populations. This parameter is expressed as a percentage and represents the ratio between the standard deviation and the sample mean.

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