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Phenolic Compounds from *Atriplex littoralis* and Their Radiation-Mitigating Activity

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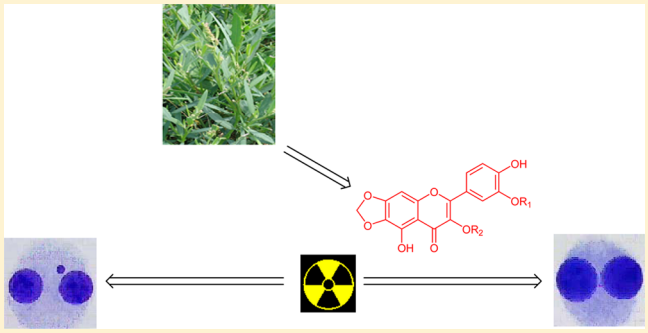
Supporting Information

ABSTRACT: From the aerial parts of *Atriplex littoralis*, three new flavonoid glycosides named atriplexins I–III (**1**–**3**), a known flavonoid glycoside, spinacetin 3-*O*-*β*-D-glucopyranoside (**4**), arbutin (**5**), and 4-hydroxybenzyl-*β*-D-glucopyranoside (**6**) were isolated. Their structures were elucidated on the basis of detailed spectroscopic analysis, including 1D and 2D NMR (COSY, NOESY, TOCSY, HSQC, HMBC) and HRESITOF MS data. The compounds were tested for in vitro protective effects on chromosome aberrations in peripheral human lymphocytes using a cytochalasin-B-blocked micronucleus (MN) assay in a concentration range of 0.8–7.4 *μ*M of final culture solution. Chromosomal damage was induced by 2 Gy of *γ*-radiation on binucleated human lymphocytes, and the effects of the compounds were tested 2 to 19 h after irradiation. The frequency of micronuclei (MNi) was scored in binucleated cells, and the nuclear proliferation index was calculated. The highest prevention of in vitro biochemical and cytogenetic damage of human lymphocytes induced by *γ*-radiation was exhibited by **3** (reduction of MN frequency by 31%), followed by **4** and **6**.



Atriplex littoralis L. is an annual, unisexual, dioecious herb that typically grows in coastal and saline habitats throughout Europe, North Africa, Asia, and the United States. The species is known to be a typical salt-tolerant plant, i.e., an obligate halophyte confined to coastal habitats, frequently found in silt at the mouths of estuaries and in sand on fairly sheltered beaches as well as a constituent of both inland and coastal saltmarsh habitats.[1]

Ionizing radiation is a well-known inducer of chromosome aberrations and micronuclei (MNi) formation, and the frequency of chromosome aberrations in lymphocytes is scored by a common biological radiation dosimetry.[2] DNA damage is one of the first consequences of the irradiation, which is related to hydroxy radical-induced chromosome breaks and formation of an increased number of MNi in cells under division.[3,4] Radiation exposure-induced chromosomal aberrations such as dicentrics, rings, and translocations all appear as a result of double-strand breaks and misrepaired damage.[5]

Biological dosimetry (also biodosimetry), based on the analysis of micronuclei in the cytokinesis-block micronucleus (CBMN) assay, could be an alternative method for scoring dicentric chromosomes in the studies of radiation protection. Biological dosimetry, in addition to physical dosimetry, is mostly performed to assess the individual dose. It is known that the number of radiation-induced MNi is strongly correlated with dose and quality of radiation.[6,7]

Micronuclei are found in the cytoplasm outside the main nucleus, resembling it in shape, structure, and staining properties. MNi arise from acentric fragments that fail to incorporate into the daughter nuclei during cell division due to the lack of a centromere.[8] MNi can also be formed by entire chromosomes that lag behind during mitosis because of a failure of the mitotic spindle[9] or by complex chromosomal configurations that pose problems during the anaphase. Thus, formation of MNi can be induced by both physical agents, such as ionizing radiation and mitotic inhibitors. It was estimated that approximately 80% of MNi in *γ*-irradiated human lymphocytes have a DNA content of 6% or less compared to the interphase nucleus, which implicates an origin from acentric fragments.[10] The remaining MNi, with a DNA content of 6–20% of the main nucleus, are assumed to arise mainly as a result of spindle defects. Most MNi are able to synthesize DNA during the S phase,[11] and at least some of them do so when the main nucleus itself is not in the S phase.[12]

Thiol-based synthetic compounds such as amifostine are recognized to be efficient radioprotectants. Amifostine is known as a strong radioprotective compound compared to other agents[13] but has limited use in a clinical setting because of its side effects and toxicity. The search for less toxic radiation

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Table 1. ^{13}C and ^{1}H NMR Data of Compounds 1−3 (125.80 MHz for ^{13}C and 500.26 MHz for ^{1}H, Methanol-d_4

	1		2		3	
C/H	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	159.1		158.8		159.3	
3	134.8		134.6		135.6	
4	179.9		179.9		180	
5	142.7		142.7		142.5	
6	131.1		131.2		131.4	
7	155.9		155.9		156.2	
8	90.3	6.64 s	90.3	6.67 s	90.7	6.68 s
9	153.9		153.9		154	
10	109		109		108.8	
11	104.3	6.08 s	104.4	6.01 s	104.5	6.08 s
12			57.1	3.97 s	56.9	3.94 s
1′	123.4		123.4		122.9	
2′	117.4	7.63 d (2.0)	114.6	7.97 d (2.0)	114.6	7.94 s
3′	146.2		148.6		148.5	
4′	149.8		150.9		151.1	
5′	116.2	6.87 d (8.3)	116.2	6.91 d (8.4)	116.3	6.89 d (8.1)
6′	123.4	7.61 dd (8.3, 2.0)	123.8	7.57 dd (8.4, 2.1)	124.2	7.64 dd (7.8)
1″	100.4	5.77 d (7.6)	100.3	5.89 d (7.3)	104.3	5.28 d (7.3)
2″	80.2	3.67 dd (9.2, 7.6)	80.4	3.64 dd (7.7, 9.2)	76.1	3.47 m
3″	79.1	3.58 t (9.2)	79	3.61 m	78.3	3.45 m
4″	71.8	3.35 t (9.3)	72	3.31 m	71.9	3.23 m
5″	78.4	3.26 m	78.5	3.30 m	77.5	3.38 m
6″a	62.7	3.55 m	62.6	3.57 dd (4.0,11.7)	68.8	3.82 brd (11.1)
6″b		3.74 dd (12.0, 2.3)		3.77 m		3.42 m
1‴	102.8	5.23 d (1.2)	102.9	5.19 d (1.6)	102.7	4.52 d (1.5)
2‴	72.6	4.02 dd (3.4, 1.2)	72.5	4.00 m	72.2	3.58 dd (3.0, 1.5)
3‴	72.5	3.79 dd (9.6, 3.4)	72.5	3.76 m	72.4	3.45 dd (3.0, 9.5)
4‴	74.2	3.35 t (9.6)	74.04	3.31 m	73.9	3.23 m
5‴	70.2	4.05 dd (6.2, 9.6)	70.1	4.00 m	69.9	3.39 m
6‴	17.6	0.97 d (6.2)	17.6	0.88 d (6.2)	18	1.08 d (6.2)

protectors has spurred an interest in the recognition of suitable natural product candidates able to prevent the harmful effects of ionizing irradiation because they are nontoxic and have some proven therapeutic benefits.[14] These candidates have only recently started to receive attention as possible modifiers of harmful radiation effects.[15] In this regard, herbal extracts with high flavonoid concentrations have been shown to be effective.[16]

There are many studies of the antimicrobial, antiviral, antiallergenic, vasodilatory, and anti-inflammatory actions of plant flavonoids. The antioxidant activity of flavonoids, linked to their ability for free radical scavenging and reduction of their formation, is also acknowledged.[17] Halophytic species such as *A. littoralis* L. have recently been noted to be an interesting source of various secondary metabolites, exhibiting various biological effects such as antimicrobial,[18] antioxidant,[19,20] antiviral, anticancer, and other biological activities.[21] Among the halophytic species of the genus *Atriplex* the best studied is *A. halimus*, which is known to contain tannins, flavonoids, saponins, alkaloids, and resins.[22] This species has been evaluated in terms of the antioxidant properties of its principal secondary metabolites. Some of the leaf flavonoids showed significant DPPH radical scavenging activity.[23] The flavonols quercetin, kaempferol, and isorhamnetin, occasionally spinacetin, patuletin, and tricin were identified in the leaves of eight *Atriplex* species, excluding *A. littoralis* L.[24] In the latter, a new acylated patuletin-diglycoside and the known patuletin 3-*O*-*β*-D-glucopyranoside were present.[25] The MN assay was used to evaluate the protective effects of six phenolic compounds isolated from the halophyte *A. littoralis* on DNA damage. This is the first report of radiation-mitigating effects of secondary metabolites isolated from a halophytic species and the first report of the new flavonoid glycoside atriplexins isolated from *A. littoralis*.

RESULTS AND DISCUSSION

Semipreparative reversed-phase HPLC separation of the *n*-butanol extract of the aerial parts of *A. littoralis* L. revealed six phenolic compounds. The isolated compounds were identified according to their 1D and 2D NMR, UV, IR, and MS data. Three of the compounds are new methylenedioxy flavonol glycosides, named atriplexins I−III (**1**−**3**). Two known phenolic compounds, arbutin (**5**)[26] and 4-hydroxybenzyl-*β*-D-glucopyranoside (**6**),[27] and the flavonol spinacetin 3-*O*-*β*-D-glucopyranoside (**4**)[28] were also isolated.

The UV spectrum of compound **1**, obtained as a yellow powder, exhibited a λ_{max} value at 348 nm, indicating the presence of a highly conjugated system in accordance with the presence of aromatic ring absorption bands at 1565, 1478, and 828 cm^{-1} in the IR spectrum. Moreover, IR absorptions at 1680 and 3367 cm^{-1} indicated carbonyl and hydroxyl groups, respectively. Compound **1** showed a deprotonated molecular ion in the negative HRESIMS data at m/z 637.1445 $[M - H]^-$, which, in conjunction with the ^{13}C NMR data, was consistent with a molecular formula of $C_{28}H_{30}O_{17}$. The ^{1}H NMR data of **1** (Table 1) revealed the presence of four proton signals in the

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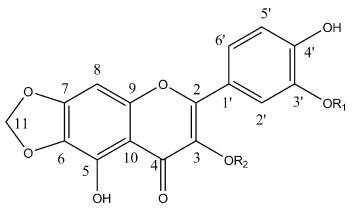
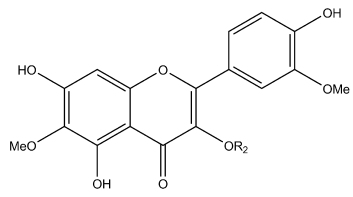
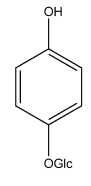
aromatic region: an ABX system, characteristic of catechol-containing B-ring flavonoids, and a singlet at δ_H 6.64. The ^{13}C NMR spectrum revealed the presence of 15 carbon signals in the aromatic region, with chemical shifts characteristic for a quercetagetin nucleus.[29] The presence of a catechol moiety was confirmed by the 22 nm bathochromic shift observed in the UV spectrum for band I upon treatment with a methanolic $NaOAc/H_3BO_3$ solution. The bathochromic shift observed for band I (40 nm) and band II (18 nm) upon treatment with a methanolic $AlCl_3/HCl$ solution was indicative of a free 5-OH group. The proton singlet at δ_H 6.08 (2H) indicated the presence of a methylenedioxy group. Its position was unambiguously assigned to C-6/C-7, based on the HMBC correlations of H-11/C-6, C-7.



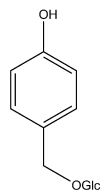
1 R_1 = H, R_2 = Rha(1-2)Glc-, atriplexin I
2 R_1 = Me, R_2 = Rha(1-2)Glc-, atriplexin II
3 R_1 = Me, R_2 = Rha(1-6)Glc-, atriplexin III



4 spinacetin 3-O-β-D-glucopyranoside



5 arbutin



6 4-hydroxybenzyl β-D-glucopyranoside

Hydrolysis of compound **1** afforded D-glucose and L-rhamnose, which were identified by HPLC comparison of the retention times of the sugar moieties with those of authentic standards. The presence of two monosaccharide moieties was deduced from the presence of two anomeric proton signals at δ_H 5.77 (d, J = 7.6 Hz) and 5.23 (d, J = 1.2 Hz), which were attached to carbons resonating at δ_C 100.4 and 102.8, respectively. The remaining ring protons of the monosaccharide residues were assigned by means of COSY, TOCSY, NOESY, HSQC, and HMBC experiments, starting from the anomeric protons. Moreover, the relative configurations and conformations of the monosaccharide units were deduced by means of NOESY correlations and vicinal couplings. Thus, the values of the vicinal interproton couplings of $J_{1'',2''}$ = 7.6, $J_{2'',3''}$ = 9.2, $J_{3'',4''}$ = 9.3, and $J_{4'',5''}$ = 9.3 Hz revealed the *trans*-diaxial H-1″/H-2″, H-2″/H-3″, H-3″/H-4″, and H-4″/H-5″ relationships, corresponding to a β-D-glucopyranosyl moiety in a $^4C_1$-conformation, which was also confirmed by strong NOESY correlations of H-1″/H-3″, H-1″/H-5″, H-3″/H-5″, and H-2″/H-4″. The vicinal couplings of $J_{1''',2'''}$ = 1.2, $J_{2''',3'''}$ = 3.4, $J_{3''',4'''}$ = 9.6, and $J_{4''',5'''}$ = 9.6 Hz, together with a NOESY correlation of H-3‴/H-5‴, suggested an anomeric α-configuration of the L-rhamnopyranosyl moiety in a $^1C_4$ conformation.

Determination of the interglycosidic and sugar−aglycone linkages was performed by HMBC and NOESY spectra. The HMBC correlations H-1″/C-3, H-2″/C-1‴, and H-1‴/C-2″ indicated the position of an α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl disaccharide moiety at C-3. This linkage was also confirmed by the NOESY correlation of H-2″/H-1‴. On the basis of the above spectroscopic data, the structure of compound **1** (atriplexin I) was unambiguously established as quercetagetin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

Compound **2**, obtained as a yellow powder, showed a deprotonated molecular ion in the negative HRESIMS spectrum at m/z 651.1615 $[M - H]^-$, suggesting the molecular formula $C_{29}H_{32}O_{17}$. The ^1H and ^{13}C NMR data of compound **2** (Table 1) were similar to those of compound **1**. Compound **2** possessed an additional *O*-methyl group, whose position was unambiguously assigned to C-3′, based on the HMBC correlation of H-OCH_3/C-3′ together with the NOESY correlation of H-OCH_3/H-3′. The ^{13}C NMR spectrum of compound **2** revealed the presence of 15 carbon signals in the aromatic region, with chemical shifts similar to compound **1**, characteristic of a quercetagetin nucleus.

The presence of a free 4′-OH group was confirmed by the 46 nm bathochromic shift observed for band I, in the UV spectrum, without decrease in intensity upon treating a methanolic solution of **2** with $NaOCH_3$. The bathochromic shift observed for band I (38 nm) and band II (18 nm) in the UV spectrum obtained upon the addition of $AlCl_3$, which showed no changes after treatment with HCl, confirmed a free 5-OH group and the absence of an *ortho*-dihydroxy functionality. Following hydrolysis of compound **2**, D-glucose and L-rhamnose were identified by HPLC. The protons of the sugar moiety were assigned by means of COSY, TOCSY, NOESY, HSQC, and HMBC experiments. The NOESY correlations of H-1″/H-3″, H-1″/H-5″, and H-3″/H-5″ revealed the presence of a β-D-glucopyranosyl moiety in a $^4C_1$-conformation, whereas the vicinal coupling of $J_{1'''-2'''}$ = 1.6 Hz and a strong NOESY correlation of H-3‴/H-5‴ suggested an anomeric α-configuration of the L-rhamnopyranosyl moiety in a $^1C_4$ conformation. The HMBC correlations of H-1″/C-3, H-2″/C-1‴, and H-1‴/C-2″, together with the NOESY correlation of H-2″/H-1‴, indicated the attachment of an α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl moiety at C-3. Based on the above spectroscopic data, the structure of compound **2** (atriplexin II) was unambiguously established as 3′-*O*-methylquercetagetin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

According to the HRESIMS data showing a deprotonated molecular ion at m/z 651.1606 $[M - H]^-$ (molecular formula $C_{29}H_{32}O_{17}$), similar UV features and aromatic regions of the ^1H and ^{13}C NMR spectra of compound **3** compared to compound **2**, it was concluded that compound **3** is an isomer of compound **2**. Hydrolysis of compound **3** again afforded D-glucose and L-rhamnose as identified by HPLC.

The NOESY correlations of sugar protons indicated that the β-D-glucopyranosyl moiety was in a $^4C_1$-conformation, whereas the α-L-rhamnopyranosyl moiety adopted a $^1C_4$ conformation. The HMBC correlations of H-1″/C-3, H-6″a/C-1‴, and H-6″b/C-1‴, together with the NOESY correlation of H-6″b/H-1‴, indicated the attachment of the α-L-rhamnopyranosyl(1→6)-β-D-glucopyranosyl moiety at C-3. On the basis of the above physical data, the structure of compound **3** (atriplexin III) was

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Table 2. Incidence of MN, CBPI, Distribution of MN per Cell, and Frequency of MN Measurement in Cell Cultures of Irradiated Human Lymphocytes Treated with Investigated Compounds

cmpd (μM)	MN/BN cells[a] (mean ± SE)	CBPI[b] (mean ± SE)	% BN cells with MN[c] (mean ± SE)	MN/1000 BN cells[d] (mean ± SE)	frequency of MN[e]	distribution of MN 0	1	2	3	4	5
controls	1.2 ± 0.03	1.6 ± 0.1	15.5 ± 0.6	183.0 ± 9.6	100.0%	1050.5	160.5	28.8	3.0	0.3	0
mifos. (4.7)	1.2 ± 0.02	1.6 ± 0.1	13.9 ± 0.7	166.0 ± 0.7	90.7%	1013.3	135.5	23.5	4.0	0.3	0
MMC (3.0)	1.3 ± 0.02	1.6 ± 0.1	17.9 ± 0.2[f,g]	226.1 ± 3[f,g]	123.5%	1029.3	177.8	35.8	7.5	2.3	0.5
spinacetin glc (1.0)	1.2 ± 0.03	1.6 ± 0.1	12.1 ± 0.5[f,h]	141.8 ± 5.3[f,h]	77.5%	1017.3	120.5	19.8	2.0	0	0
spinacetin glc (2.0)	1.2 ± 0.01	1.6 ± 0.1	11.7 ± 0.7[f,h]	139.3 ± 7.9[h]	76.1%	1018.3	112.5	20.8	2.3	0	0
spinacetin glc (4.0)	1.2 ± 0.02	1.6 ± 0.1	12.3 ± 0.6[f,h]	148.0 ± 9.1[h]	80.9%	1004	115.3	22.5	2.5	0.5	0
atriplexin I (0.8)	1.2 ± 0.03	1.7 ± 0.1	15.1 ± 0.5[h]	181 ± 9.9[h]	98.9%	1011.3	146.5	31	1.8	0.5	0
atriplexin I (1.5)	1.3 ± 0.02	1.7 ± 0.1	14.1 ± 0.6[h]	178.8 ± 10.9[h]	97.7%	986.3	126	25.3	3.5	0.5	0.3
atriplexin I(3.1)	1.2 ± 0.02	1.7 ± 0.1	15.3 ± 0.8	187.0 ± 8.3[h]	102.2%	993	146	28.3	4.5	1	0
atriplexin II (0.8)	1.2 ± 0.03	1.7 ± 0.1	14.4 ± 0.4[h]	172.0 ± 6.9[h]	94.0%	1013.8	108	20	2.8	0.5	0
atriplexin II (1.5)	1.2 ± 0.02	1.7 ± 0.1	14.1 ± 0.5[h]	170.9 ± 6.8[h]	93.4%	999.3	127.3	28	3.8	0.3	0
atriplexin II (3.1)	1.2 ± 0.03	1.8 ± 0.1	14.3 ± 0.4[h]	175.3 ± 7.8[h]	95.8%	993	133.5	28.3	3.8	0.5	0
atriplexin III (0.8)	1.2 ± 0.01	1.6 ± 0.1	11.2 ± 0.4[f,h]	126.6 ± 4.8[f,g,h]	69.2%	791	82.5	15	1.8	0	0
atriplexin III(1.5)	1.2 ± 0.03	1.6 ± 0.1	10.8 ± 0.7[f,h]	131.3 ± 9.2[f,h]	71.7%	983	99.8	19.3	2.8	0.5	0
atriplexin III(3.1)	1.2 ± 0.02	1.6 ± 0.1	11.7 ± 0.6[f,h]	141.4 ± 6.5[h]	77.3%	1015	108.5	22.5	3	0.3	0
4-hydroxybenzyl glc (1.8)	1.2 ± 0.04	1.7 ± 0.1	13.1 ± 0.7[h]	156.4 ± 4.1[h]	85.5%	1013	128.8	21.3	2.3	0.5	0.5
4-hydroxybenzyl glc (3.5)	1.2 ± 0.02	1.8 ± 0.1	13.1 ± 0.4[h]	155.7 ± 4.6[h]	85.1%	1012.5	128	21	3.5	0.3	0
4-Hydroxybenzyl glc (7.0)	1.2 ± 0.02	1.7 ± 0.1	13.6 ± 0.4[h]	159.2 ± 5.3[h]	87.0%	1015.3	137.3	19	3	0.8	0
arbutin (1.8)	1.3 ± 0.02	1.7 ± 0.1	15.1 ± 0.1[h]	191.2 ± 3.4[h]	104.5%	991.3	137	34	5.3	0.8	0
arbutin (3.7)	1.3 ± 0.02	1.8 ± 0.1	15.0 ± 0.1[h]	189.3 ± 4.4[h]	103.4%	970	130.5	36.3	3.5	0.8	0
arbutin (7.4)	1.2 ± 0.02	1.6 ± 0.1	15.8 ± 0.7	197.2 ± 6.6[h]	107.8%	1026.3	151.3	36.8	4.5	0.5	0

[a]MN/Bn cells: incidence of micronuclei in binucleated cells. [b]CBPI: cytokinesis-block proliferation index. [c]% BN cells with micronuclei. [d]MN/1000 BN cells: incidence of micronuclei in 1000 binucleated cells. [e]The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $p < 0.01$. [f]Compared with control groups, statistically significant difference $p < 0.01$. [g]Compared with amifostine WR 2721, statistically significant difference $p < 0.01$. [h]Compared with mitomycin C, statistically significant difference $p < 0.01$.

unambiguously established as 3′-*O*-methylquercetagetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

Effects of Flavonoids on Irradiated Lymphocytes. Treatment of irradiated lymphocytes with amifostine WR-2721 (positive control) at a concentration of 4.7 μM caused reduction in the MN frequency by 9% (166.0 ± 0.7 MN/1000 BN cells), compared to irradiated control cell cultures (183.0 ± 9.6) (Table 2). Irradiated lymphocytes treated with 0.8, 1.5, or 3.1 μM atriplexin III caused a significant ($p < 0.01$) decline in the MN frequency by 31% (126.6 ± 4.8), 28% (131.3 ± 9.2), and 23% (141.4 ± 6.5), respectively, compared to irradiated cell cultures (183.0 ± 9.6) not treated with the isolated compounds (Table 2) and showed the best protecting activity among the investigated compounds. Treatment of irradiated lymphocytes with 1.0, 2.0, or 4.0 μM spinacetin 3-*O*-β-D-glucopyranoside resulted in a significant ($p < 0.01$) decrease in the frequency of MN by 22.5% (141.8 ± 5.3), 24% (139.3 ± 7.9), and 19% (148.0 ± 9.1), respectively, compared to irradiated cell cultures (183.0 ± 9.6) not treated with investigated compounds. The same concentration of 4-hydroxybenzyl-β-D-glucopyranoside exhibited a slightly lower effect [14.5% (156.4 ± 4.1), 15% (155.7 ± 4.6), and 13% (159.2 ± 5.3), respectively]. It was found that atriplexins I and II exhibited effects that were similar to or weaker than the effect of irradiated control cell cultures. Arbutin exhibited weaker effects in comparison with irradiated control cell cultures (Table 2).

The results show that the protecting potential of arbutin and atriplexins I and II is not significantly different than that of amifostine WR-2721. The effect of the isolated compounds on cell proliferation was investigated by determining the CBPI. The compounds did not exhibit a significant decrease in CBPI, which is regularly used to determine the cytotoxicity of compounds in the in vitro micronucleus (MN) test.[30] Because MN expression is dependent on cell division, quantification of cell proliferation and cell death should be assessed to obtain cell kinetics and MN frequencies. Assessing toxicity as measured by mitotic index is a suboptimal choice, as mitotic values may result from mitotic block (Table 2). The present study verified the mitigation activity of the isolated compounds from *A. littoralis*, synthetic compounds (amifostine WR-2721), and alkylating agent (MMC) in cell cultures of irradiated human lymphocytes.

There is insufficient information about the radioprotective and mitigation effects of herbal extracts against ionizing radiation-induced chromosomal abnormalities in human lymphocytes. Although many compounds of plant origin and herbal preparations have been reported as radioprotective, i.e., mitigation agents in animals, there are few experiments demonstrating the efficacy of these extracts in humans.[31] In fact, "radioprotectors" should be comprehended in terms of protection (prophylaxis or mitigation) and treatment when describing the use of drugs to potentially modify radiation injury.[32] In addition to the ROS-scavenging properties of

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flavonoids, their protecting role against oxygen species-induced DNA damage was also reported.[33] Because the investigated compounds were added after irradiation in our experiment, it may be assumed that the protective activity could be ascribed similarly to their DNA repair potential, as it was deduced for amifostine WR-2721.[34] However, the mechanisms of these flavonoids' mitigating activities are not fully comprehended and should be further studied.

Natural products, including plant flavonoids, are suitable candidates for preventing harmful effects of ionizing irradiation because they are nontoxic and have some proven therapeutic benefits.[14] Herbal medicines have only recently begun to receive attention as potential modifiers of the radiation response.[15] Herbal extracts of high flavonoid content have been evaluated for strong anticlastogenic (upon reduction in radiation-induced micronuclei in blood reticulocytes) and antioxidant (upon thiobarbituric acid assay to assess lipid peroxidation) activities.[16] However, the practical applicability of the majority of synthetic compounds is limited owing to their toxicity at their optimum protective dose. To reduce the toxic effects of synthetic compounds such as amifostine WR-2721, there is a need to explore compounds of natural origin that could be less toxic and highly effective at nontoxic doses. Thus, it could be expected that more attention will be paid to studies of plant products as alternative radioprotectors and radiation mitigators. However, the use of plant products as radioprotectors and radiation-mitigating drugs requires scientific evaluation and validation to verify that the natural radioprotectors/mitigators are more successful than synthetic chemicals.[17] With respect to side effects induced by ionizing radiation in patients undergoing radiotherapy or people exposed to radiation at their workplace, radioprotectors should play an important role in health preservation. Today, development of effective radiation mitigators or modifiers is an important and rapidly growing field. Most available radioprotectors are expensive and toxic at high doses or with recurrent usage, including one of the main radioprotective thiol-based synthetic compounds, amifostine WR-2721. Therefore, the development of effective radioprotectants or modifiers on the basis of plant secondary metabolites and other natural products is very important.

Thus, the recent demand for new pharmaceuticals and new sources of bioactive compounds has facilitated phytochemical and pharmacological research on secondary metabolites in a range of understudied plants, including salt-tolerant plants. Halophytic species are known for their ability to tolerate an excess of sodium salts in their growing media due to a very complex set of adaptation mechanisms, including oxygen radical scavenging, cytoplasmic osmoregulation, stress signaling, and related synthesis of different classes of plant metabolites. Our study on the halophyte *A. littoralis* confirmed bioactivity of flavonol glycosides in terms of their radiation-mitigating activity. Among the four studied flavonol glycosides, three were new and named atriplexins I–III and the other is spinacetin 3-*O*-β-D-glucopyranoside. The isolated compounds reduced the frequency of micronuclei in γ-radiation-induced cytogenetic damage of the human lymphocytes treated in vitro.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical AUTOPOL IV automatic polarimeter. UV spectra were recorded using a GBC Cintra 40 UV/vis spectrometer. IR spectra were recorded on a ThermoScientific Nicolet 6700 FT-IR spectrometer using a capillary film technique. All NMR spectra were recorded on a Bruker Avance III 500 spectrometer at 500.26 for ^{1}H and 125.80 MHz for ^{13}C, with methanol-d_4 as solvent and TMS as reference. HRESIMS data were obtained on an Agilent Technologies 6210 time-of-flight LC/MS system. Analytical TLC was carried out on silica gel 60 GF_{254} 20 × 20 cm plates, with a layer thickness of 0.25 mm (Merck). Semipreparative HPLC was performed on an Agilent 1100 series instrument equipped with a DAD. The column used was a Zorbax Eclipse XDB C_{18} column (i.d. 9.4 mm × 250 mm, particle size 5 μm). D-Glucose in hydrolysates was determined on a Dionex ICS 3000 DP liquid chromatography system equipped with a quaternary gradient pump with an electrochemical detector (gold as working and Ag/AgCl as reference electrode). The anion-exchange column was a Carbo PacPA100 pellicular column (i.d. 4 mm × 250 mm). Solvents for HPLC analysis were of chromatographic grade. Standards of D-glucose and L-rhamnose were purchased from Tokyo Chemical Industry, TCI (Belgium). All aqueous solutions were prepared using Ultrapure TKA deionized water.

Plant Material. The plant material was collected in August 2012 from a saltmarsh situated in northern Serbia, near the village of Melenci (N 45.29449, E 20.298898). Plant material authentication and deposition of a herbarium voucher were performed by the Department of Agricultural Botany, Faculty of Agriculture, University of Belgrade, Republic of Serbia.

Extraction and Isolation. The air-dried aerial parts (125.0 g) were powdered and extracted with MeOH (2 × 380 mL, 1 h) at room temperature with the use of an ultrasonic bath. The extract was filtered and concentrated under reduced pressure to give 12.0 g of residue, which was suspended in H_2O (75 mL) and washed with CH_2Cl_2 (75 mL). The water layer was extracted with *n*-BuOH (3 × 75 mL). The butanol extract residue (2.0 g), obtained after evaporation of the solvent under vacuum, was dissolved in MeOH and fractionated by semipreparative HPLC-DAD on an ODS column into pure compounds using gradient elution with a flow rate of 4 mL/min. Solvent A was 0.2% HCOOH in H_2O (v/v); solvent B was MeCN acidified with 0.2% HCOOH (v/v). Gradient program: 0–15 min 20–36% B, 15–18 min 36–70% B, 18–20 min 70–100% B. The detection wavelength was 280 nm. After semipreparative HPLC fractionation, 31 mg of atriplexin I (**1**), 33 mg of atriplexin II (**2**), 18 mg of atriplexin III (**3**), 21 mg of spinacetin 3-*O*-β-D-glucopyranoside I (**4**), 12 mg of arbutin (**5**), and 15 mg of 4-hydroxybenzyl-β-D-glucopyranoside (**6**) were obtained.

Acid Hydrolysis of Compounds. Compounds **1**–**3** (1.0 mg each) were individually hydrolyzed with 2 M HCl (1.0 mL) for 30 min at 100 °C. After cooling, each mixture was neutralized to pH 7 with solid $NaHCO_3$, filtered through an HPLC 0.45 μm filter, and analyzed on a Dionex ICS 3000 DP LC system. Standard solutions of D-glucose and L-rhamnose were prepared in ultrapure H_2O at concentrations of 20 μg/mL with the addition of 70 mg of NaCl (because the same quantity of NaCl remained in the samples after neutralization). The specialized program used for analysis of monosaccharides was as follows: 0–5.0 min, isocratic elution 15% A, 85% C; 5.0–5.1 min, gradient to 15% A, 2% B, 83% C; 5.1–12.0 min, isocratic elution 15% A, 2% B, 83% C; 12.0–12.1 min, gradient to 15% A, 4% B, 81% C; 12.1–20.0 min, isocratic elution 15% A, 4% B, 81% C; 20.0–20.1 min, gradient to 20% A, 20% B, 60% C; 20.1–30.0 min, isocratic elution 20% A, 20% B, 60% C, where A is 600 mM NaOH, B is 600 mM NaOAc, and C is ultrapure H_2O. The flow rate was 0.7 mL/min. Before analysis, the system was preconditioned at 15% A, 85% C for 30 min.

Subjects. Venous blood samples were collected from five healthy male volunteers. Two 5 mL aliquots of blood were taken from each subject, according to Serbian health and ethical regulations and code of ethics of the World Medical Association (Helsinki Declaration, 1964, revised in 2002).

Irradiation. Collected blood samples were irradiated using a ^{60}Co γ-ray source. The blood samples were placed in a Plexiglas container and placed on a pedestal within the radiation field. The radiation dose was 2 Gy (therapeutic dose); the dose rate was 0.45 Gy/min. Blood samples were irradiated at room temperature and set up in cell cultures

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2 h after irradiation; isolated plant compounds were added at the same time.

Micronucleus Analysis. The blood lymphocytes were set up in RPMI-1640 medium, supplemented with calf serum and phytohemagglutinin (Invitrogen−Gibco-BRL, Vienna, Austria). A level of 2×10^6 of human lymphocytes was chosen because it corresponds to 0.5 mL of human blood. The concentration of each compound (six isolated phenolic compounds and the positive and negative controls) added to the cell cultures was adjusted to correspond to the total blood amount of an average adult, estimated as approximately 5 L. The isolated compounds at a concentration range of 0.8−7.4 μM were added to the irradiated samples and were used as usual therapeutic doses. The concentration of amifostine of 4.7 μM was determined to be optimal in the in vitro experiment; higher concentrations were assumed to be toxic.

One cell culture served as a control, not containing compounds isolated from *A. littoralis*. The cell culture with added synthetic radioprotector—amifostine WR-2721 (98%, S2[3-aminopropylamino]-ethylphosphothioic acid; obtained from Marlingen-Biosciences, USA) at a concentration of 4.7 μM—served as the positive control for comparison with isolated plant phenolic compounds, as reported.[35] Because the radioprotective mechanisms of amifostine, including interactions and effects on alkaline phosphatase activity, have been reported, especially when radioprotectivity of amifostine was analyzed in vivo and by the comet test, they were of no interest here and thus were not analyzed. One cell culture included mitomycin C (MMC) (3.0 μM, in phosphate buffer), an alkylating agent that served as the negative control.

All cultures were incubated at 37 °C. Treatment with flavonoids lasted for 19 h. All cultures (including amifostine WR-2721 and MMC) were rinsed with pure medium, transferred to fresh RPMI 1640 medium, and incubated for a duration of 72 h.

DNA damage was measured using the cytokinesis-block micronucleus assay, enabling determination of MN in cells that had completed nuclear division and thus were not influenced by variations in cell division kinetics.[36]

The incidence of radiation-induced MN in control samples was determined as previously described.[37] At least 1000 binuclear (BN) cells per sample were scored, registering MN according to the criteria of Countryman and Heddle.[37] The cytokinesis-block proliferation index (CBPI) was calculated as suggested by Surralles et al.[38] and as previously reported.[35]

$$\mathrm{CBPI} = [(M\mathrm{I} + 2M\mathrm{II} + 3(M\mathrm{III} + M\mathrm{IV})]/N$$

where *M*I−IV represent the number of cells with 1 to 4 nuclei, respectively, and *N* is the number of cells scored. The criteria for selection of binuclear cells and identification of MN given on the HUMAN project Web site (http://www.humn.org) were followed. The number of binuclear cells with 1, 2, 3, or more MN was then tabulated.

Statistics and Index Calculations. The data were processed using the Origin software package, version 7.0. The differences between data pairs were evaluated by analysis of variance (one-way ANOVA), followed by the Tukey test. The results are presented as the percent of change compared to the control.

Atriplexin I (1): yellow powder; $[\alpha]_D^{22}$ −87 (*c* 1, MeOH); UV λ_{max}, (MeOH) 260, 274, 350; (NaOMe), 276, 388, ($AlCl_3$), 280, 300sh, 428, ($AlCl_3$ + HCl), 278, 390, (NaOAc), 266, 274, 350; (NaOAc + H_3BO_3), 264, 290sh, 372; IR ν_{max} 3367, 2936, 1680, 1622, 1565, 1511, 1478, 1395, 1346, 1257, 1217, 1128, 1101, 1072, 1019, 828 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 637.1445 [M − H]$^-$ (calcd for $C_{28}H_{30}O_{17}$−H 637.1410).

Atriplexin II (2): yellow powder; $[\alpha]_D^{22}$ −82 (*c* 1, MeOH); UV λ_{max}, (MeOH) 240, 270, 346 nm, 270, 392 (NaOMe), 244, 268, 286, 382 ($AlCl_3$), 244, 268, 286, 378 ($AlCl_3$ + HCl), 260, 278, 346 (NaOAc), 260, 278, 348 (NaOAc + H_3BO_3); IR ν_{max} 3428, 2936, 1681, 1621, 1566, 1514, 1478, 1392, 1346, 1257, 1217, 1128, 1101, 1072, 1019, 828, 799 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z*: 651.1615 [M − H]$^-$ (calcd for $C_{29}H_{32}O_{17}$−H 651.1567).

Atriplexin III (3): yellow powder; $[\alpha]_D^{22}$ −22 (*c* 1 MeOH); UV λ_{max}, (MeOH) 240, 270, 346 nm, 270, 394 (NaOMe), 246, 270, 286, 384 ($AlCl_3$); 246, 270, 286, 384 ($AlCl_3$ + HCl), 260, 280, 348 (NaOAc), 260, 280, 348 (NaOAc + H_3BO_3); IR ν_{max} 3428, 2936, 1680, 1621, 1565, 1514, 1478, 1392, 1346, 1256, 1216, 1130, 1101, 1072, 1020, 828 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 651.1606 [M − H]$^-$ (calcd for $C_{29}H_{32}O_{17}$−H 651.1567).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Spectroscopic data of the isolated compounds (PDF)

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Notes

The authors declare no competing financial interest.

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