10.1071/FP15204_AC

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Supplementary Material

Hydrogen-rich water-alleviated ultraviolet-B-triggered oxidative damage is partially associated with the manipulation of the metabolism of (iso)flavonoids and antioxidant defence in *Medicago sativa*

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Table S1. The sequences of primers for real-time RT-PCR

Primer name	*M. truncatula* tentative consensus or accession number	Sequences
PAL	X58180	Forward: CTTGATGAGGTGAAGCGTAT Reverse: ACCGTAACTGTCCGTGCC
CHS	AW776018	Forward: TGTTTGTGAATACATGGCACCTT Reverse: TGACTTTGGTTGACCCCATTCT
CHI	KF765782	Forward: TACTTGAGACCCTTGACTT Reverse: GGTGATTGCCTGTAGAAA
FLS	XM_003601032	Forward: CTTGATGAGGTGAAGCGTAT Reverse: ACCGTAACTGTCCGTGCC
IFS	AY167424	Forward: AATGGAGAAATCATAGAGGGCGAGCAG Reverse: GTTGATGAGCTCTGCCAAAGTCCATTC
6IOMT	DQ419913	Forward: ACATGGAAAGCCTATGACTGTTC Reverse: ACACAACTCCAGTCCCACCTG
Cu/Zn-SOD	AF056621	Forward: TAATTGCTGATGCCAACG Reverse: ACCACAGGCTAATC TTCCAC
Mn-SOD	AY145894	Forward: TGTCATCAGCG GCGTA ATCAT Reverse: GGGCTTCCTTTGGTGGTTCA
POD 1A	X90692	Forward: TCAATCGTACGTGGTGTGCT Reverse: TGCACTTTGCTCGCTCACTA
POD 1B	X90693	Forward: AGCTGCATTTGCTGCTCAAG Reverse: TTGGTAAGGTTCGTGCCAGG
POD 1C	X90694	Forward: CCTCGCATGCTTGCTAGTCT Reverse: GGACCTTGTGCCAGAACAGA
POD 2	X90695	Forward: TCCTGCTACCCTTCGTCTCT Reverse: TTCTGCACTGTGGAACAGCA
CAT	TC100988	Forward: TTCTTCTTCTCCACCGTCCTCA Reverse: TCCAAGAGAATTGGACCTCTGG
Actin 2	JQ028730	Forward: AAAAGGATGCCTATGTTGGTG Reverse: AAGTGGAGCCTCAGTTAGAAGTA
GAPDH	GQ398120	Forward: TCATTCCGTGTCCCAACCG Reverse: CCACATCATCTTCAGTGTAACCCA

Table S2. Effects of HRW pretreatment on the relative contents of (iso)flavonoids in the leaves of *Medicago sativa* upon UVB irradiation

14-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ.m^{-2} UVB. After irradiation, seedlings were transferred to the normal growth conditions for another 3 days then sampled. Seedlings without irradiation were set as a control (Con). Relative values of (iso)flavonoids in seedling leaves were determined by UPLC-LTQ-Orbitrap-MS, which were expressed as relative peak areas normalised to the amount of 4-methyumbelliferone added as an internal standard. Data are means ± s.e. from three independent experiments. Different letters within each compound are significantly different at $P < 0.05$ according to Duncan's multiple range test

	No.	Tentative identifications	Relative content in different treatments			
			Con	Con→UVB	HRW→UVB	HRW→Con
Isoflavone	1	Afromosin	0.0065±0.0090c	16.4835±1.9186b	29.9851±2.4090a	0.0056±0.0005c
	2	Afrormosin 7-O-β-D-glucoside-malonate	0.0148±0.0016c	4.8729±0.9170b	6.9882±0.7093a	0.0106±0.0017c
	3	Biochanin A	ND	5.8330±0.9532a	3.8790±0.6862b	0.0030±0.0004c
	4	Daidzein	0.0044±0.0006c	5.6354±1.0341b	11.3043±1.5436a	0.0467±0.0063c
	5	Daidzin	0.0012±0.0002c	0.3483±0.0406b	0.4140±0.0674a	0.0020±0.0004c
	6	Formononetin	ND	0.5423±0.0906a	0.2206±0.0887b	0.0028±0.0004c
	7	Formononetin 7-O-β-D-glucoside-6"-O-malonate	0.0303±0.0039c	10.2994±0.9317b	16.0789±2.0679a	0.0455±0.0065c
	8	Genistein	0.4943±0.0707c	0.8920±0.1753a	0.7027±0.0961b	0.4216±0.0623c
	9	Genistein 7-glucoside	ND	0.6855±0.0906b	1.2425±0.1250a	ND
	10	Irisolidone	ND	9.1137±1.4670a	6.5574±1.1056b	ND
	11	Isoformononetin	ND	5.3424±0.9023a	3.5890±0.4944b	0.0024±0.0002c
	12	Prunetin	ND	0.5995±0.0962b	0.7214±0.1052a	ND
Flavone	13	Apigenin	0.8509±0.1071c	3.2371±0.6387b	4.4512±0.6675a	0.5992±0.0661c
	14	Apigenin 7-galacturonide	11.1845±1.807b	16.4379±2.8824a	16.6542±2.8819a	9.1033±1.0966b
	15	Chrysoeriol	0.0008±0.0001c	4.7487±0.7331a	2.0388±0.2914b	ND
	16	Luteolin	ND	0.8046±0.1324a	0.0695±0.0078b	ND
	17	Millettocalyxin A	ND	1.0408±0.1694b	2.1291±0.3123a	0.0014±0.0001c
	18	Milleyanaflavone	ND	5.2941±0.9279b	6.6593±0.9540a	ND
	19	Ptaeroxylol	0.0018±0.0004c	0.7367±0.1019a	0.3010±0.0273b	0.0133±0.0017c
	20	Syzalterin	0.0026±0.0004c	0.8605±0.1089b	1.0263±0.1382a	ND
	21	2',5,6'-Trihydroxy-7-methoxyflavone	0.0008±0.0001c	5.0220±0.6505a	1.8592±0.1502b	ND
	22	3',4'-Dihydroxyflavone	ND	5.8434±0.9469a	3.7740±0.4849b	0.0026±0.004c
Flavanone	23	Betagarin	ND	0.4590±0.1854a	0.4661±0.1882a	ND
	24	Citronetin	ND	0.0888±0.0142a	0.0562±0.0086b	ND
	25	Garbanzol	ND	4.4301±0.3472b	6.9202±0.8852a	ND
	26	Isosakuranetin	0.0011±0.0002c	0.0207±0.0042b	0.0650±0.0101a	ND

	27	Liquiritigenin	ND	1.5731±0.1540a	1.5468±0.1443a	0.0048±0.0011b
	28	Matteucin	ND	3.1936±0.4958b	6.9565±0.5889a	ND
	29	Naringenin	ND	2.5531±0.4687b	4.4638±0.2971a	0.0065±0.0006c
	30	Pinostrobin	ND	2.2765±0.3445a	1.6271±0.2155b	ND
	31	Sakuranetin	0.0016±0.0002c	0.2468±0.0274b	0.4875±0.0660a	ND
	32	4'-Hydroxy-7-methoxy flavanone	0.0122±0.0023c	0.7838±0.0929a	0.5604±0.0632b	0.0216±0.0044c
Flavonol	33	Kaempferol	ND	0.6437±0.0625a	0.0987±0.0115b	ND
	34	Kaempferol-3-O-β-D-rutinoside	0.0012±0.0002c	0.1777±0.0304b	0.4163±0.0606a	0.0077±0.0012c
	35	3,5-Dihydroxy-4',7-dimethoxyflavone	ND	1.1314±0.1865b	2.1968±0.2467a	ND
	36	3-Hydroxy-3',4'-dimethoxyflavone	ND	0.3163±0.0517b	0.4032±0.0543a	ND
	37	4'-Hydroxy-3,5,7-trimethoxyflavone	ND	0.5851±0.0726b	1.0831±0.1368a	ND
Chalcone	38	Isoliquiritigenin	0.0102±0.0015c	8.3867±1.3805b	10.5248±1.3170a	0.0369±0.0048c
Coumestan	39	Coumestrol	ND	4.1194±1.2824a	2.8179±0.6393b	ND
Pterocarpan	40	Maackian	ND	2.3782±0.4363b	3.9002±0.6101a	ND

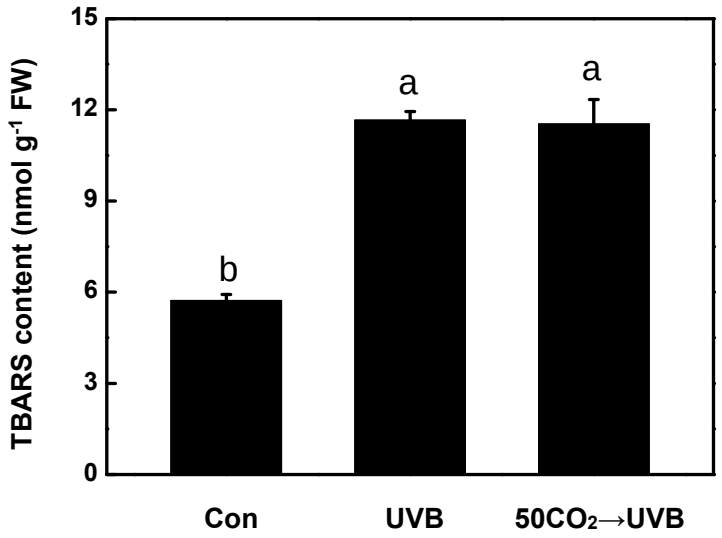
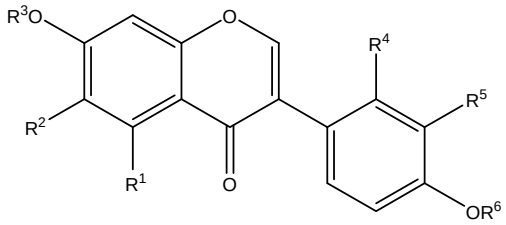
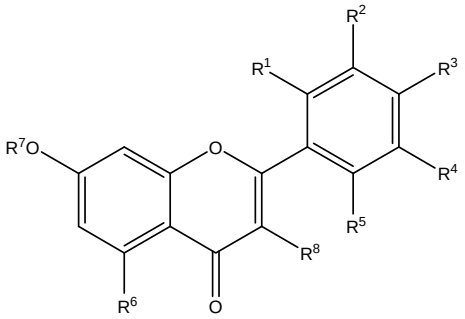


Fig. S1. Effect of CO_2 and UVB irradiation on TBARS content of alfalfa seedlings. 14-day-old seedlings were pretreated with or without 50%-saturated CO_2-rich water ($50CO_2$) for 12 h, and then exposed to 10.8 kJ.m^{-2} UVB. After irritation, seedlings were transferred to the normal growth conditions for another 5 days. Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.



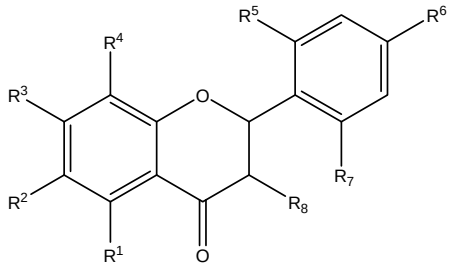
Isoflavone	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
Afromosin	H	OCH_3	H	H	H	CH_3
Afrormosin 7-O-β-D-glucoside-malonate	H	OCH_3	GM	H	H	CH_3
Biochanin A	OH	H	H	H	H	H
Daidzein	H	H	H	H	H	H
Daidzin	G	H	H	H	H	H
Formononetin	H	H	H	H	H	CH_3
Formononetin 7-O-β-D-glucoside-4"-O-malonate	H	H	GM	H	H	CH_3
Genistein	OH	H	H	H	H	H
Genistein 5-glucoside	G	H	H	H	H	H
Irisolidone	OH	OCH_3	H	H	H	CH_3
Isoformononetin	H	H	CH_3	H	H	H
Prunetin	OH	H	CH_3	H	H	H

Fig. S2. Structures of identified isoflavone aglycones from seedling leaves of *Medicago sativa* upon UVB irradiation.



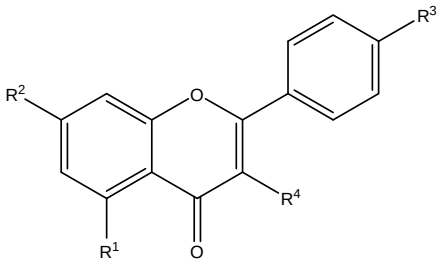
Flavone	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**
Apigenin	H	H	H	H	H	OH	H	H
Apigenin 7-galacturonide	H	H	H	H	H	OH	G	H
Chrysoeriol	H	OCH_3	H	H	H	OH	H	H
Luteolin	H	OH	H	H	H	OH	H	H
Millettocalyxin A	H	-OCH_2O-		H	OCH_3	H	CH_3	H
Milleyanaflavone	H	-OCH_2O-		H	H	H	CH_3	H
Ptaeroxylol	OCH_3	H	H	H	H	OH	H	H
2',5,5'-Trihydroxy-7-methoxyflavone	OH	H	H	OH	H	OH	H	H
3',4'-Dihydroxyflavone	H	OH	OH	H	H	H	H	H
3-Hydroxy-3',4'-dimethoxyflavone	H	H	OCH_3	OCH_3	H	H	H	OH

Fig. S3. Structures of identified flavone aglycones from seedling leaves of *Medicago sativa* upon UVB irradiation.



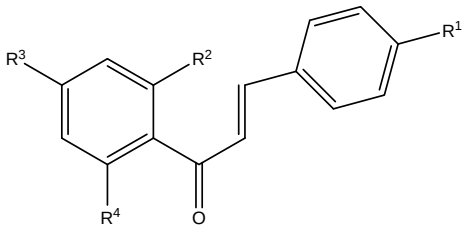
Flavanone	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**
Betagarin	OCH_3	-OCH_2O-		H	OCH_3	H	H	H
Citronetin	OH	H	OH	H	H	H	OCH_3	H
Garbanzol	H	H	OH	H	H	OH	H	OH
Isosakuranetin	OH	H	OH	H	H	OCH_3	H	H
Liquiritigenin	H	H	OH	H	H	OH	H	H
Matteucin	OH	CH_3	OH	CH_3	H	H	H	OH
Naringenin	OH	H	OH	H	H	OH	H	H
Pinostrobin	OH	H	OCH_3	H	H	H	H	H
Sakuranetin	OH	H	OCH3	H	H	OH	H	H
4'-Hydroxy-7-methoxy flavanone	H	H	OCH_3	H	H	OH	H	H

Fig. S4. Structures of identified flavanone aglycones from seedling leaves of *Medicago sativa* upon UVB irradiation.



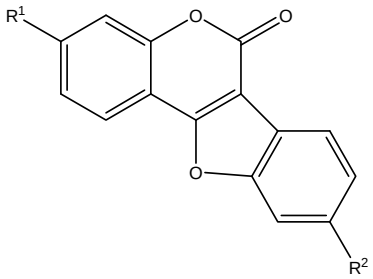
Flavonol	**R1**	**R2**	**R3**	**R4**
Kaempferol	OH	OH	OH	OH
Kaempferol-3-O-β-D-rutinoside	OH	OH	OH	OR
3,5-Dihydroxy-4',7-dimethoxyflavone	OH	OCH_3	OCH_3	OH
4'-Hydroxy-3,5,7-trimethoxyflavone	OCH_3	OCH_3	OH	OCH_3

Fig. S5. Structures of identified flavonol aglycones from seedling leaves of *Medicago sativa* upon UVB irradiation.



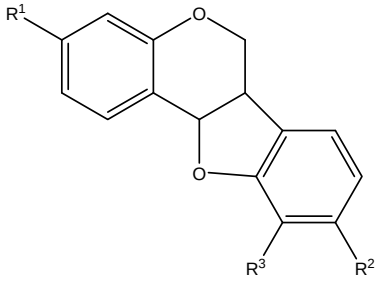
Chalcone	**R1**	**R2**	**R3**	**R4**
Isoliquiritigenin	OH	OH	OH	H

Fig. S6. Structures of identified chalcone from seedling leaves of *Medicago sativa* upon UVB irradiation.



Coumestan	**R1**	**R2**
Coumestrol	OH	OH

Fig. S7. Structures of identified coumestan from seedling leaves of *Medicago sativa* upon UVB irradiation.



Pterocarpan	**R1**	**R2**	**R3**
Maackian	OH	-OCH_2O-	

Fig. S8. Structures of identified pterocarpan from seedling leaves of *Medicago sativa* upon UVB irradiation.

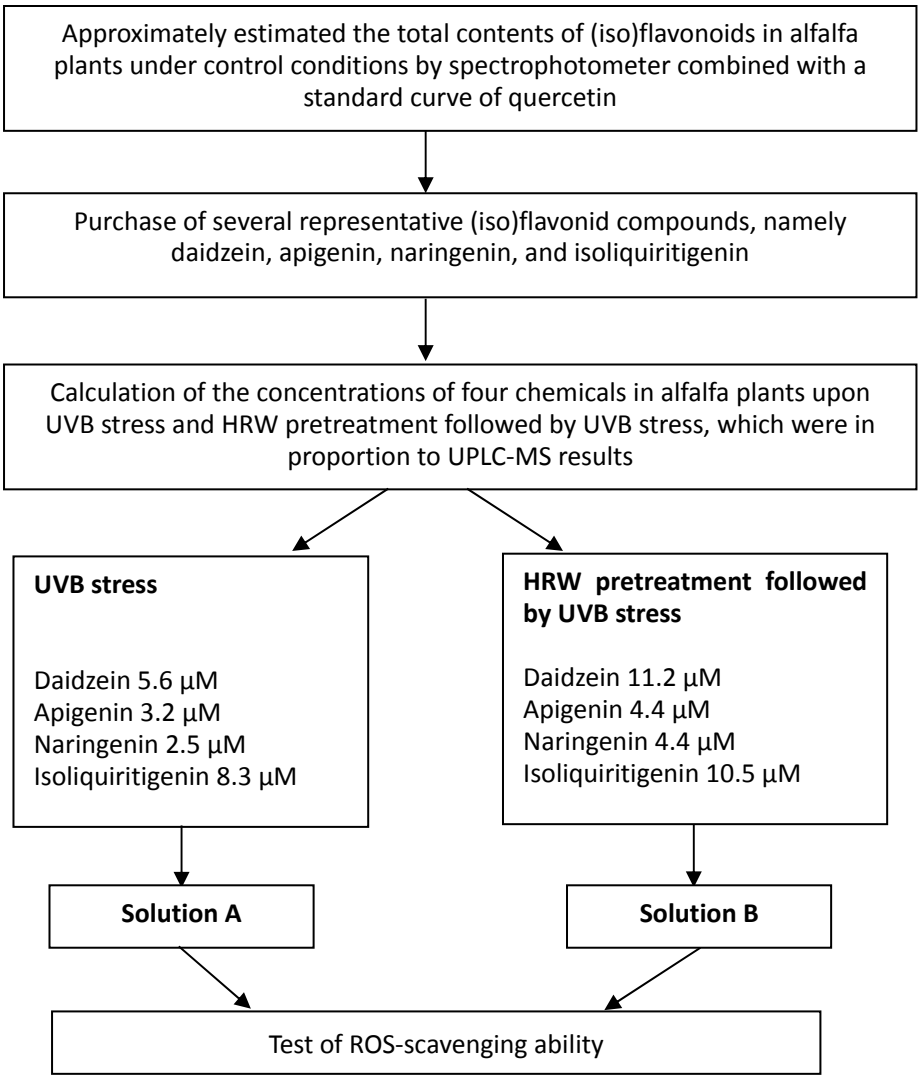


Fig. S9. Workflow for the *in vitro* test of ROS-scavenging ability.

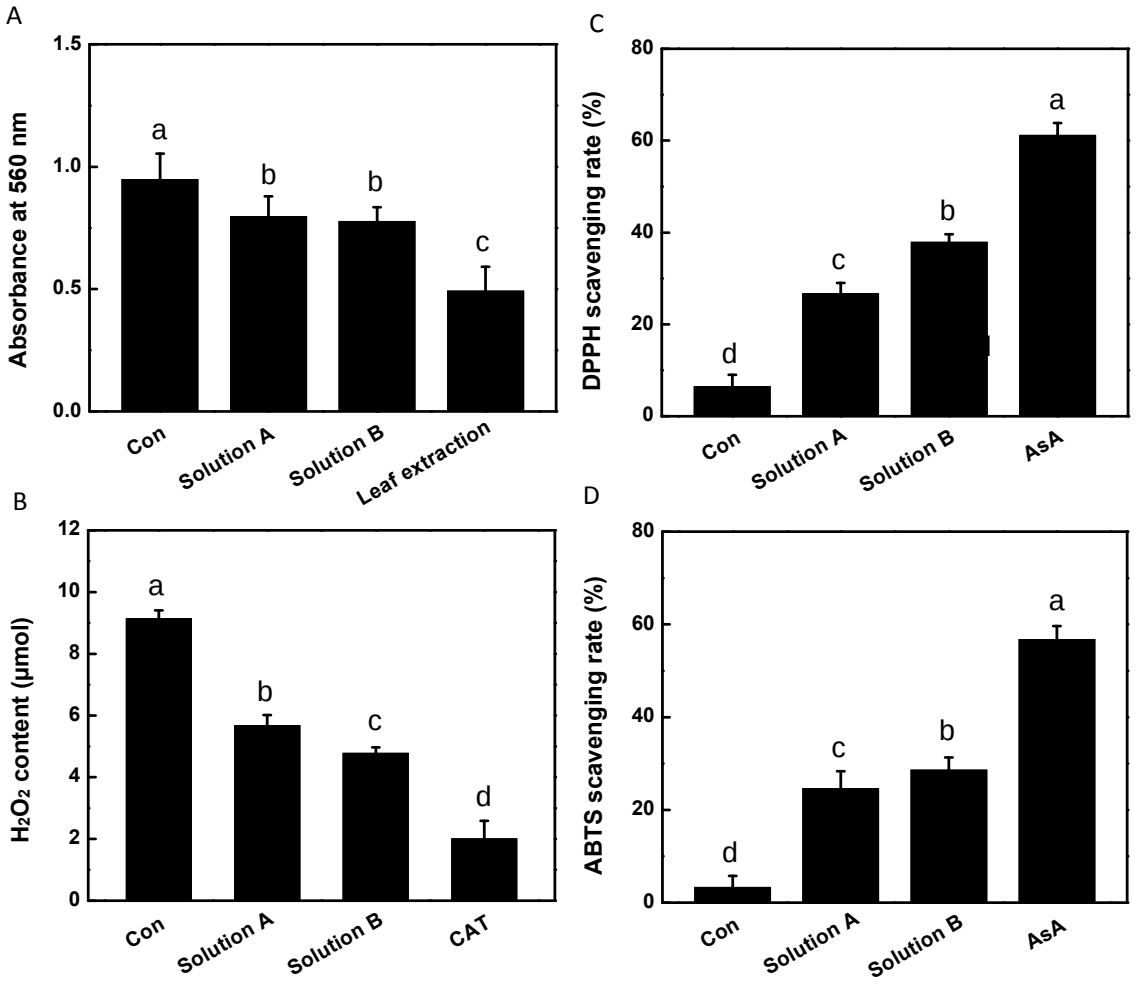


Fig. S10. *In vitro* analysis of ROS-quenching abilities (A and B) and antioxidant activities (C and D). For O_2^--quenching (A), sterilized water was regarded as the control sample (Con). O_2^- was generated by the riboflavin system under illumination, and the photochemical reduction of NBT was monitored (Absorbance at 560 nm) after 5 min of incubation. Crude enzyme extract from alfalfa leaves (leaf extraction from 5-day-old seedlings) was added as a positive control. For H_2O_2-quenching (B), the absorbance of the Fe^{3+}-xylenol orange complex was determined after 45 min of incubation. The specificity of H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture containing catalase alone (CAT; 100 U). *In vitro* antioxidant activities were determined by DPPH free radical-scavenging assay (C) and TEAC assay (D). Sterilized water was regarded as the control sample (Con). The AsA (50 μM) were used as a positive control. Additionally, solution A contains 5.6 μM daidzein, 3.2 μM

apigenin, 2.5 μM naringenin, and 8.3 μM isoliquiritigenin, which could partially mimic (iso)flavonoid profiles of UVB-treated samples. Solution B contains 11.2 μM daidzein, 4.4 μM apigenin, 4.4 μM naringenin, and 10.5 μM isoliquiritigenin, which could partially mimic (iso)flavonoid profiles of samples of HRW pretreatment followed by UVB stress. Data are means ± s.e. from at least three independent experiments. Bars with different letters are significantly different at the $P < 0.05$ level according to Duncan's multiple range test.

Methods and materials

In vitro *ROS-quenching abilities determination*

O_2^- was generated by the riboflavin system under illumination, and the photochemical reduction of NBT was monitored (Absorbance at 560 nm) after 5 min of incubation. Crude enzyme extract from leaves (leaf extraction from 5-day-old *Medicago sativa* seedlings) were added as a positive control. H_2O_2 content was determined by detecting the absorbance of the Fe^{3+}-xylenol orange complex (Jiang *et al*. 1990). Additionally, the specificity of H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture containing catalase (CAT; 100 U) alone. Measurements were performed at least in triplicate.

In vitro *antioxidant activities determination*

The methods of DPPH and TEAC free radical-scavenging assay are based on reaction with electron-donating or hydrogen radicals producing compounds. The DPPH free radical-scavenging activity of samples was determined according to the method described by Liu *et al*. (2009). A 0.1 mM solution of ethanolic DPPH solution was prepared. The initial absorbance of the DPPH in ethanol was measured at 517 nm and did not change throughout the period of assay. Discolorations were measured at 517 nm after the incubation for 30 min at 30 ºC in the dark. In TEAC assay, the ABTS free radical-scavenging activity of each sample was determined according to the method described by Stratil *et al*. (2006). A working solution was diluted to absorbance values between 1.0 and 1.5 AU at 734 nm with phosphate buffer solution. Standards or samples (from 5 to 25 µL according to reaction intensity) were mixed with the working solution (975 µL) and diluted up to 1000 µL with deionized water. A decrease of absorbance was measured at 734 nm after 20 min. Aqueous phosphate buffer solution (1 mL, without $ABTS^{\bullet+}$ solution) was used as a control. Measurements were performed at least in triplicate.

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Stratil P, Klejdus B, Kuban V (2006) Determination of total content of phenolic compounds and their antioxidant activity in vegetables evaluation of spectrophotometric methods. *Journal of Agricultural and Food Chemistry* **54**, 607–616. doi:10.1021/jf052334j