Nutrición Hospitalaria

Nutr Hosp. 2014;29(5):1116-1122
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Original / *Alimentos funcionales*

Antioxidant responses of damiana *(Turnera diffusa* Willd) to exposure to artificial ultraviolet (UV) radiation in an *in vitro* model; part II; UV-B radiation

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Abstract

Introduction: **Ultraviolet type B (UV-B) radiation effects on medicinal plants have been recently investigated in the context of climate change, but the modifications generated by UV-B radiation might be used to increase the content of antioxidants, including phenolic compounds.**

Objective: **To generate information on the effect of exposure to artificial UV-B radiation at different high-doses in the antioxidant content of damiana plants in an *in vitro* model.**

Methods: **Damiana plantlets (tissue cultures in Murashige-Skoog medium) were irradiated with artificial UV-B at 3 different doses (1) 0.5 ± 0.1 mW cm^{-2} (high) for 2 h daily, (2) 1 ± 0,1 mW cm^{-2} (severe) for 2 h daily, or (3) 1 ± 0.1 mW cm^{-2} for 4 h daily during 3 weeks. The concentration of photosynthetic pigments (chlorophylls *a* and *b*, carotenoids), vitamins (C and E) and total phenolic compounds, the enzymatic activity of superoxide dismutase (SOD, EC 1.15.1.1) and total peroxidases (POX, EC 1.11.1), as well as total antioxidant capacity and lipid peroxidation levels were quantified to assess the effect of high artificial UV-B radiation in the antioxidant content of *in vitro* damiana plants.**

Results: **Severe and high doses of artificial UV-B radiation modified the antioxidant content by increasing the content of vitamin C and decreased the phenolic compound content, as well as modified the oxidative damage of damiana plants in an *in vitro* model.**

Conclusion: **UV-B radiation modified the antioxidant content in damiana plants in an *in vitro* model, depending on the intensity and duration of the exposure.**

(*Nutr Hosp.* 2014;29:1116-1122)

DOI:10.3305/nh.2014.29.5.7092

Key words: *Antioxidants. Antioxidant capacity. Phenolic compounds.* Turnera diffusa. *UV-B radiation. Vitamin C.*

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Recibido: 1-XI-2013.
1.ª Revisión: 18-XII-2013.
2.ª Revisión: 23-I-2014.
Aceptado: 15-II-2014.

RESPUESTA ANTIOXIDANTE EN DAMIANA *(TURNERA DIFFUSA* WILLD) EXPUESTA A RADIACIÓN ULTRAVIOLETA (UV) ARTIFICIAL EN UN MODELO *IN VITRO;* PARTE II; RADIACIÓN UV-B

Resumen

Introducción: **Los efectos de la radiación ultravioleta tipo B (UV-B) sobre las plantas medicinales se han investigado recientemente en el contexto del cambio climático, pero las modificaciones que genera la radiación UV-B podrían emplearse para modificar el contenido de compuestos antioxidantes, incluyendo los compuestos fenólicos.**

Objetivo: **Generar información sobre el efecto de una alta exposición a UV-B artificial en el contenido antioxidante de damiana *(Turnera diffusa,* Willd) en un modelo *in vitro.***

Método: **Plántulas de damiana en cultivo de tejidos (medio Murashige-Skoog) fueron irradiadas con UV-B artificial en 3 diferentes dosis: (1) 0,5 ± 0,1 mW cm^{-2} (alto) por 2 h diarias, (2) 1 ± 0,1 mW cm^{-2} (severa) por 2 h diarias, o (3) 1 ± 0,1 mW cm^{-2} durante 4 horas diarias por 3 semanas. Se cuantificó la concentración de pigmentos fotosintéticos (clorofilas *a* y *b,* carotenoides), vitaminas (C y E) y compuestos fenólicos totales, la actividad enzimática de la superóxido dismutasa (SOD, EC 1.15.1.1) y las peroxidasas totales (POX, EC 1.11.1), así como la capacidad antioxidante total y la peroxidación de lípidos para evaluar el efecto de la alta radiación UV-B artificial en el contenido antioxidante de damiana *in vitro.***

Resultados: **Dosis altas y severas de radiación UV-B artificial modificaron el contenido antioxidante incrementando el contenido de vitamina C y disminuyendo el contenido de compuestos fenólicos totales, además de modificar el daño oxidativo de plantas de damiana en un modelo *in vitro.***

Conclusión: **La radiación UV-B modifica el contenido antioxidante en damiana en un modelo *in vitro,* dependiendo de la intensidad y el tiempo de exposición.**

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Palabras clave: *Antioxidantes. Capacidad antioxidante. Compuestos fenólicos. Radiación UV-B.* Turnera diffusa. *Vitamina C.*

Abbreviations

DPPH: 2,2-diphenyl-2-picrylhidrazyl.
BCS: Baja California Sur.
GAEAC: Gallic acid equivalent antioxidant capacity.
HPLC: High-performance liquid chromatography.
ROS: Reactive oxygen species.
SOD: Superoxide dismutase.
TBARS: Thiobarbituric acid-reactive substances.
UV: Ultraviolet radiation.
UV-B: Ultraviolet type B.

Introduction

Plants are naturally exposed to all components of sunlight, including ultraviolet (UV) radiation. UV radiation is divided, based on wavelength range, in UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). Climate changes due to natural and anthropogenic causes increase UV-B radiation levels on Earth's surface[1]. UV-B radiation possesses high energy levels and induces a wide variety of physiological and morphological responses, most of which are only occasionally beneficial, in terrestrial organisms[2,3]. In plants, UV-B radiation has different effects on growth and development, metabolic processes such as photosynthesis, morphology and biochemistry (concentrations of secondary metabolites and other compounds)[2-6]. In addition, UV radiation generates an increase in the production of reactive oxygen species (ROS) potentially causing oxidative stress[7] and, likely, decreasing the yield and quality of plants[8]. As a mechanism to prevent oxidative damage due to increased ROS production induced by UV-B radiation the plant's antioxidant defenses change; depending on the species, exposure to a small to moderate UV-B dose may increase, while larger UV-B doses may overwhelm the antioxidant defenses[9,10]. This is an especially interesting alternative for medicinal plants because they constitute an important source of molecules with pharmaceutical and economic value[1,11-12], as reflected in the growing number of studies addressing UV-B radiation effects in the production of secondary compounds in wild medicinal plants and herbs[10-11,13-15]. Phenolic compounds, such as flavonoids, are expected to increase in response to UV-B radiation given their effectiveness in absorbing UV-B radiation and antioxidant properties[6]. Recently, Kumari and Prassad[1] suggested the importance of UV-B treatment on medicinal plants to increase natural compounds as an alternative to pharmacological manufacture.

Damiana (*Turnera diffusa*, Willd) plants are used for medicinal purposes and in the elaboration of diverse dietary products[16]. These shrubs grow wild in arid and semiarid climates, such as those prevalent in Baja California Sur (BCS), Mexico [16], where plants are subject to elevated intensity of solar radiation (maximum 7 kWh m^{-2} day^{-1} in Summer)[17]. Therefore, damiana is exposed naturally to high UV radiation levels; elevated antioxidant compound content was observed recently in damiana plants from BCS[18,19], and previous results suggest differences in antioxidant content between damiana plants grown in different regions with similar environmental conditions but different natural solar radiation levels[19]. This medicinal plant has a large antioxidant potential, perhaps due to the environmental conditions and exposure to naturally elevated UV-B radiation[19]. Studies on the effect of UV-B radiation suggest that exposing plants to artificial UV radiation can be an alternative to increase phytochemicals of interest. Therefore, the objective of this study was to analyze the effect of exposure to artificial UV-B radiation at different high-doses in the antioxidant content of damiana plants in an *in vitro* model.

Materials and methods

Plant material and experimental setup

Damiana (*T. diffusa*) plantlets were grown aseptically in tissue culture in Murashige-Skoog (MS) medium following the method described by Alcaraz-Melendez *et al.*[20]. Damiana plants were grown for 4 weeks (to reach 7.5 cm of size) prior to being irradiated with artificial UV-B (UV-B Lamp F875, 315 nm, BioRad, Hercules. CA, USA). Lamps were placed 25 cm above the top of the plants maintaining this distance constant throughout the experiment. Aluminum strips were placed on top of the lamps and plants were placed inside a box, fully covered with aluminum foil, to increase UV-B reflection. Treatments were as follows; 1) control, plants grown in white light (fluorescent light to 100 µE m^{-2} sec^{-1}) at 25 ± 2°C without exposure to UV-B; 2) plants exposed to 0.5 ± 0.1 mW UV-B cm^{-2} for 2 h per day and 22 h of white light at 25 ± 2°C; 3) plants exposed to 1 ± 0,1 mW UV-B cm^{-2} for 2 h per day and 22 h of white light at 25 ± 2°C; 4) plants exposed to 1 ± 0,1 mW UV-B cm^{-2} for 4 h per day and 20 h of white light at 25 ± 2°C. A radiometer (ultraviolet intensity meter, General Tools and Instruments, NY, USA) was used to quantify UV-B radiation below and above the tops of the experimental damiana plants within each container. Samples were collected after the first irradiation treatment and subsequently every week, during 3 weeks. Plants collected at the onset of the experiment were labeled as day zero and newly irradiated as day one. Leaves were separated from stems, samples were immersed in liquid nitrogen, and stored at -80°C until analyzed.

Chlorophylls a and b, and total carotenoids

Prior to pigment quantification using the method of Lichtenthaler and Wellburn[21], damiana leaves (250 mg) were extracted with water:methanol:acetone (2:3:5) at 4°C in the dark. Pigment content in each sample was

determined at 666, 653 and 470 nm by using a microplate reader (BioRad™ 550, Japan) as previously described[18-19]. Chlorophyll *a*, chlorophyll *b*, and total carotenoid content are expressed in μg g^{-1} of fresh weight. The variability coefficients calculated for the methodologies employed to quantify chlorophyll a, chlorophyll *b*, and total carotenoid content were 4.8, 5.8, and 21.8, respectively.

Superoxide dismutase activity

Damiana leaves were homogenized in phosphate buffer ($KHPO_4$, 0.1 M, pH 6.8) with polyvinylpyrrolidone (PVP), and centrifuged at 3,000 × g for 20 min at 4°C. Supernatant was used immediately to assess total superoxide dismutase (SOD, EC 1.15.1.1) activity following the method of Suzuki[22] at 560 nm (Beckman Coulter DU-800, CA, USA) as previously described[18-19] Results are expressed in units of SOD activity mg^{-1} protein. One unit of SOD activity is defined as the amount of enzyme necessary to cause 50% inhibition of the maximum rate of NBT reduction at 560 nm. The variability coefficient for this method was 14.2.

Peroxidase activity

Leaf samples (250 mg) were homogenized in phosphate buffer ($KHPO_4$, 0.1 M, pH 6.8) with PVP, centrifuged at 3,000 × g for 20 min at 4°C. Reaction mixture containing hydrogen peroxide (H_2O_2, 50 μM), pyrogallol (50 μM), and plant extract was incubated for 1 min at 25°C. Reaction was stopped by adding 0.5 mL of H_2SO_4 (5%, v/v), following the method described by Kar and Mishra[23] as previously described[18-19]. Total peroxidases (POX, EC 1.11.1) activity was detected spectrophotometrically at 420 nm in a Beckman Coulter DU 800 (USA) spectrophotometer. Results are expressed in units of POX activity mg^{-1} protein. One unit of POX activity is defined as the amount of POX enzyme required to increase the absorbance by 0.1. The coefficient of variability was 11.7 for this methodology.

Vitamin C

Vitamin C (L-ascorbic acid) content was quantified using high-performance liquid chromatography (HPLC, Waters, Milford, MA, USA). Conditions and methods used to assess vitamin C content in damiana leaves were previously described[18]. Leaves (0.2 g), metaphosphoric acid (3% w/v), acetic acid (8% w/v), and EDTA (0.01 M) were mixed and incubated for 40 min at 4°C in the dark. The homogenate was centrifuged at 23,895 × g for 15 min, and filtered with cellulose acetate filters. Samples (10 μL) were injected into an HPLC and two mobile phases were employed, acidified deionized water (pH 2.4 with H_2SO_4) and acetonitrile (HPLC grade 100%) in a BDS hypersyl C8 column as previously described[18-19]. The internal standard method and a calibration curve of L-ascorbic acid (5-200 ng μL^{-1}) at 245 nm were used to calculate vitamin C content in damiana leaves. Results are expressed as mg vitamin C g^{-1} fresh weight; the variability coefficient was 0.3.

Total phenolic compound content and antioxidant capacity

Leaf extracts were obtained using acetone-methanol-water (5:3:2 respectively, v/v) and were used for determinations of total phenolic compound content and total antioxidant capacity. Total phenolic compound content in leaf samples was determined following the method of Singleton and Rossi[24] in a microplate reader (BioRad™ 180 550, CA, USA) at 750 nm and was calculated from a standard curve of gallic acid (0-350 μg mL^{-1}) as previously described[18-19]. Briefly, samples (10 μL) were mixed with Folin-Ciocalteu reagent (1:1, v/v diluted with water) and incubated for 1 min with sodium carbonate (2% v/v); then sodium hydroxide (0.4% w/v) was added and the mixture was allowed to incubate for another 20 min at room temperature in the dark. Results are expressed as mg gallic acid equivalents (GAE) g^{-1} fresh weight. The coefficient of variability was calculated to be 9.9.

Total antioxidant capacity of damiana leaves was quantified in a microplate reader (BioRad™ 550, CA, USA) at 515 nm by using the method of Brand-Williams *et al.*[25] and using the commercial free radical 2,2-diphenyl-2-picrylhidrazyl (DPPH) as previously described[18,19]. A standard curve of gallic acid (50-300 μg mL^{-1}) was employed to calculate total antioxidant capacity in damiana leaves. Results are expressed as μM gallic acid equivalent antioxidant capacity (GAEAC) g^{-1} fresh weight. The coefficient of variability for this method was 7.5.

Lipid peroxidation

The concentration of thiobarbituric acid-reactive substances (TBARS) was quantified as an indicator of lipid peroxidation, at 535 nm with correction at 600 nm (Beckman Coulter DU 800), following the method of Persky *et al.*[26]. Samples were homogenized with phosphate buffer (K_2HPO_4, 50 mM, pH 6.8) and centrifuged at 2,200 × g 20 min at 4°C. The homogenate was incubated with trichloroacetic acid (20% w/v in HCl 1 M), and thiobarbituric acid (TBA, 1 % w/v) at 90°C for 30 min. The reaction was stopped on an ice-bath and centrifuged at 3,000 × g for 15 min at 4°C as previously described[18-19]. Samples and standard curve (1-bis tetraetoxipropane TEP, 0-20 nmoles mL^{-1}) were quantified spectrophotometrically. TBARS levels are expressed as nmol of TBARS mg^{-1} protein; the variability coefficient for this method was 10.5.

Total protein content

To standardize the results for antioxidant enzyme activities and lipid peroxidation levels, total soluble protein content was determined following the method of Bradford[27], modified for use with a microplate reader (BioRad™), and using a standard curve of bovine serum albumin (BSA, 5-400 µg mL^{-1}) as previously described[18-19]. Results are expressed as mg protein g^{-1} fresh weight.

Statistical analyses

All samples (the pool of all leaves from 5 plantlets in each treatment, n = 5) were analyzed in triplicate, and data are expressed as mean ± standard error. Data were tested for normality and homoscedasticity of variance. Analysis of variance (ANOVA) and post-hoc tests (Tukey) were used to determine significant differences between treatments. Statistical significance was considered when $p < 0.05$. All statistical analyses were run with Statistica 6 software (StatSoft, Inc., 2001).

Results and discussion

Chlorophyll *a* and *b* content in damiana (*T. diffusa*) leaves increased significantly during the first day of treatment with high and severe UV-B doses compared to control plants ($p = 0.001$, table I). Chlorophyll *a* and *b* levels remained constant in control plants throughout the experimental period (table I). In contrast, from experimental day 7, chlorophyll *a* and *b* levels decreased in damiana plants irradiated with UV-B in comparison with control plants ($p < 0.001$, $p = 0.022$, respectively, table I). Carotenoid content was not affected by UV-B treatment ($p = 0.322$, table I). The amount of UV radiation used may be too severe and may produce loss of photosynthetic pigments in damiana (*T. diffusa*) plants in a dose-dependent manner (table I). Similar decrease in the chlorophyll content was reported by Mohammed and Tarpley[28] in rice leaves as a result of high UV-B levels, which generate chlorophyll degradation. Katerova *et al.*[29] suggest that exposure to UV-B and UV-C radiation damages photosynthetic pigments due to the fact that UV radiation has enough energy to destroy chemical bonding of biomolecules. Hideg[9] proposed that exposure to UV-B increases light sensor pigments, but excessive radiation can generate photoinhibition as well as structural and functional damage to photosystems and photosynthetic pigments since they are sensitive to UV-B radiation[30,31]. UV-B radiation can modify chlorophyll content directly or indirectly by binding proteins to chlorophylls or affecting the chlorophyll biosynthesis pathways[2]. Therefore, it is possible that UV-B radiation affects damiana plants either via photoinhibition or the synthesis of photosynthetic pigments.

In the present study, damiana SOD (EC 1.15.1.1) activity increased following exposure to UV-B radiation, mainly at severe dose treatments (UV-B severe 2 h and UV-B severe 4 h) ($p < 0.05$, table I). The response appears to be immediate, since plants showed a significant increase in SOD activity when compared to non-irradiated controls after the first exposure to severe UV-B doses for 2 h or 4 h ($p < 0.05$, table I). No significant differences were observed in POX (EC 1.11.1) activity between control damiana plants and those exposed to UV-B radiation ($p = 0.547$, table I). A similar increase in SOD activity after exposure to supplementary UV-B doses was observed in sweeflag, *Acorus calamus* L.[32]; Ravindran[13] observed increased SOD and POX activity in true indigo (*Indigofera tinctoria*, L.) seedlings, and Hassan *et al.*[6] in bean (*Vicia faba* L.) plants exposed to supplementary UV-B radiation. Rybus-Zajac and Kubis[33] reported that UV-B radiation induces increased SOD, guaiacol peroxidase and syringaldazine peroxidase activities in cucumber seedlings. Hagh *et al.*[34] described an increase in guaiacol peroxidase and ascorbate peroxidase activities in roots and shoots of sunflowers plants. Recently, Ramya and Balakrishnan[15] observed increased POX activity (84%) in sola pith (*Aeschynomene aspera*) plants irradiated with different doses of UV-B radiation for 9 days, this increase along with the other antioxidant enzymes generate protection to the plant during the oxidative stress produced by increased UV-B radiation. The increase in SOD activity in damiana (*T. diffusa*) plants after exposure to UV-B radiation may be due to increased ROS production. UV-B radiation increases ROS production, which activates both enzymatic and non-enzymatic antioxidant responses in plants[9].

Vitamin C content was 0.8 ± 0.18 mg ascorbic acid g^{-1} FW in control damiana (*T. diffusa*) plants (table I). UV-B radiation increased vitamin C levels in damiana leaves to 1.4 ± 0.04 mg ascorbic acid g^{-1} FW at 0.5 mW cm^{-2} 2 h of UV-B radiation treatment for 21 days (table I). The content of vitamin C, translated to the same units as those reported in the present study, is in the range of 25 to 46.76 mg ascorbic acid g^{-1} in acerola (*Malpighia glabra*) fruits, 0.506 mg ascorbic acid g^{-1} in orange pulp[35], and 0.135 to 0.149 mg ascorbic acid g^{-1} in tomato fruits[36]. Therefore, the content of vitamin C in damiana (*T. diffusa*) is within the range reported for fruits and vegetables considered rich in vitamin C. Furhter, exposure to UV-B radiation increased the vitamin C content in damiana. Nasibi and Kalantari[37] reported increased vitamin C content in plants of *Brassica napus* in response to UV-B radiation (5.8 Wm^{-2}). However, Kumari *et al.*[32] reported the lack of an effect of supplementary UV-B radiation (1.8 kJ m^{-2} d^{-1} and 3.6 kJ m^{-2} d^{-1}) on vitamin C levels in *A. calamus* plants. It is possible that increasing levels of ascorbic acid, perhaps via the glutathione-ascorbate cycle, aids damiana plants to counteract the increased ROS production presumably induced by UV-B radiation.

Table I

Enzymatic and non-enzymatic antioxidant content in damiana (Turnera diffusa Willd) plantlets exposed to UV-B radiation (0 mW cm^{-2} (control), 0.5 mW cm^{-2} 2 h, 1 mW cm^{-2} 2 h, and 1 mW cm^{-2} 4 h)

Treatment	*Clorophyll a (µg g^{-1} FW)*					*Chlorophyll b (µg g^{-1} FW)*					*Total carotenoids (µg g^{-1} FW)*					*POX (U mg^{-1} protein)*					*SOD (U mg^{-1} Protein)*					*Vitamin C (mg ascorbic acid g^{-1} FW)*				
Days	*0*	*1*	*7*	*14*	*21*	*0*	*1*	*7*	*14*	*21*	*0*	*1*	*7*	*14*	*21*	*0*	*1*	*7*	*14*	*21*	*0*	*1*	*7*	*14*	*21*	*0*	*1*	*7*	*14*	*21*
Control	432.6 ±6.9a*	432.6±6.9b*	403.8±3.7a*	379.1 ±2.4a*	414.1±10.3a*	395.2±10.7a*	395.2 ±10.7a*	435.9 ±47.4a*	335.2±3.1a*	356.8±7.3a*	11.5 ±1.0a	11.5±1.0a	2.1 ±0.8b	11.7 ±1.2a	7.4±0.8b	0.279±0.01a	0.279±0.01b	0.106±0.01a	0.051±0.001b	0.048±0.002a	4.1±0.05a**	4.1±0.05b**	3.4±0.27c**	1.8±0.16b**	4.3±0.51a**	0.8±0.18a**	0.8±0.18b**	0.93±0.06a**	0.62 ±0.2c**	0.8±0.04b**
0.5 mWcm^{-2} 2 h	432.6 ±6.9a**	493.3±4.9a**	307.4 ±5.4b**	227.2 ±1.3c**	268.8±4.5b**	395.2±10.7a**	423.3 ±7.8a**	305.7±9.8b**	238.0±1.1c**	262.9 ±1.8b**	11.5 ±1.0a	14.7±1.6a	2.5 ±0.2b	2.9 ±0.2b	5.8±0.3b	0.279±0.01a	0.252±0.02b	0.057 ±0.003bc	0.022±0.001c	0.037±0.002b	4.1±0.05a***	1.6±0.23c***	3.6±0.11c***	1.5±0.4b***	1.4±0.22b***	0.8±0.18a*,**	0.8±0.05ab*,**	0.8±0.03a*,**	1.07±0.05b*,**	1.4±0.04a*,**
1 mWcm^{-2} 2 h	432.6 ±6.9a**	433.3±7.4b**	267.9±4.9c**	251.1 ±8.5b**	151.4±2.9c**	395.2±10.7a**	401.5 ±8.0a**	260.1±3.4b**	297.0±9.3b**	196.9±3.7c**	11.5 ±1.0a	6.2±0.8b	9.9±0.05a	3.3 ±0.3b	22.7 ±0.7a	0.279±0.01a	0.486±0.04a	0.072±0.005b	0.052±0.003b	0.050±0.002a	4.124±0.05a*	5.7±0.09a*	6.6±0.09a*	4.0±0.36a*	4.4±0.41a*	0.8±0.18a*,**	0.9±0.08ab*,**	0.9±0.08a*,**	1.15±0.01ab*,**	0.77±0.04b*,**
1 mWcm^{-2} 4 h	432.6 ±6.9a**	485.8±7.8a**	275.2±0.9c**	209.8 ±3.8c**	ND	395.2±10.7a**	425.8 ±11.5a**	284.4±1.4b**	235.4±6.6c**	ND	11.5 ±1.0a	2.4±0.4b	11.6±0.4a	6.5 ±0.8b	ND	0.279±0.01a	0.205±0.005b	0.033±0.003c	0.067±0.005a	ND	4.1±0.05a*	6.0±0.44a*	4.7±0.13b*	3.5±0.10a*	ND	0.8±0.18a*	1.1±0.03a*	1.0±0.09a*	1.27±0.05a*	ND
• p (day)	—	<0.01	<0.01	<0.01	<0.01	—	0.13	0.003	<0.01	<0.01	—	<0.01	<0.01	<0.01	<0.01	—	<0.001	<0.001	<0.001	0.006	—	<0.01	<0.01	<0.01	0.002	—	0.028	0.165	<0.01	<0.01
• p (treatment)			0.015					0.022					0.321					0.547					<0.001						0.007		

POX = peroxidases; SOD = superoxide dismutase.
Data are shown as mean ± standard error of a sample of n = 5; all analyses were run in triplicate.
• ANOVA and Tukey's post-hoc tests, were applied to probe for differences between treatments.
Different letters denote significant differences between days within each treatment; *denotes significant differences between treatments; p < 0.05. ND = Not detected.

Table II

Phenolic compound content, total antioxidant capacity and oxidative damage (lipid peroxidation) in damiana (Turnera diffusa Willd) plantlets exposed to UV-B radiation (0 mW cm^{-2} (control), 0.5 mW cm^{-2} 2 h, 1 mW cm^{-2} 2 h, and 1 mW cm^{-2} 4 h)

Treatment	*Phenolic compound contents (mg gallic acid g^{-1} FW)*					*Total antioxidant capacity (mM gallic acid g^{-1} FW)*					*Lipid peroxidation (nM TBARS g^{-1} protein)*				
Day	*0*	*1*	*4*	*7*	*10*	*0*	*1*	*4*	*7*	*10*	*0*	*1*	*4*	*7*	*10*
Control	25.497 ±0.953a*	25.497 ±0.953ab*	26.141 ±0.564a*	23.519±0.462a*	29.681 ±0.630a*	901.03±22.85a*,**	901.03 ±22.85b*,**	928.63 ±53.66b*,**	1243.8 ±30.84a*,**	984.45 ±24.91a*,**	19.66±0.17a**	19.66±0.17b**	24.31±2.11c**	20.91±1.74a**	7.53±0.26c**
0.5 mWcm^{-2} 2 h	25.497 ±0.953a**	22.147 ±0.449c**	25.051 ±0.549ab**	22.967 ±0.541a**	22.233 ±0.261b**	901.03 ±22.85a*	1567.4 ±17.47a*	1348.2 ±42.12a*	918.57 ±55.60b*	776.78 ±28.67b*	19.66±0.17a**	19.15 ±2.798b**	30.59±0.38b**	12.96±1.84b**	14.12±0.04b**
1 mWcm^{-2} 2 h	25.497 ±0.953a**	26.257 ±0.469a**	25.002 ±1.062ab**	23.137 ±0.400a**	22.242 ±0.604b**	901.03 ±22.85a**	292.06 ±13.49c**	946.79 ±39.51b**	909.96 ±52.75b**	769.71 ±26.81b**	19.66±0.17a*	35.49±3.81a*	38.61±0.32a*	20.82 ±0.497a*	41.57±1.69a*
1 mWcm^{-2} 4 h	25.497 ±0.953a**	22.948 ±0.496bc**	22.365 ±0.280b**	22.904 ±0.464a**	ND	901.03 ±22.85a**	881.32 ±22.56b**	769.67 ±36.49b**	798.16 ±12.85b**	ND	19.66±0.17a**	13.52±0.44b**	32.58±0.56b**	15.02±0.25ab**	ND
• p (day)	—	0.001	0.01	0.79	<0.001	—	<0.001	<0.001	<0.001	<0.001	—	0.001	<0.001	0.004	<0.001
• p (treatment)			<0.001					<0.001					0.005		

TBARS = thiobarbituric acid reactive substances, determined as indicator of lipid peroxidation.
Data are shown as mean ± standard error of a sample of n = 5; all analyses were run in triplicate.
• ANOVA and Tukey's post-hoc tests were applied to probe for differences between treatments.
Different letters denote significant differences between days within treatments; * denotes significant differences between treatments; p < 0.05. ND = Not detected.

Kumari and Prasad[1] suggested the relevance of studying the effects of UV-B radiation as an alternative to increase the content of molecules that are important to the plant s metabolism, or have nutraceutical properties and, thus, commercial value, such as phenolic compounds. Levels of phenolic compounds, including flavonoids, increase in response to UV-B radiation[38]. In this study, the phenolic compound content in damiana plants decreased significantly following exposure to UV-B radiation in all treatments in comparison to control plants ($p < 0.05$, table II); such decrease could be due to reduction in photo-assimilation at high UV-B radiation doses. Similar decrease in phenolic compound content was reported by Mohammed and Tarpley[8] in rice leaves exposed to 10 kJ UV-B radiation. Thus, exposure to moderate or severe UV-B radiation could be an alternative mechanism to increase the content of phytochemical compounds of interest in damiana.

In *T. diffusa*, carotenoid and phenolic compound content decreased in response to high UV-B doses, suggesting that either this plant is sensitive to UV-B radiation or that the amount of UV-B radiation supplied was beyond the tolerance limit of damiana plants. The treatments used in this study only considered UV-B radiation without photosynthetically active radiation. Exposure of plants directly to UV-B radiation without visible-light may not provide the energy needed for the plant to synthesize the response-elements to protect itself. Caldwell[39] mentioned that the photoreactivation mechanism is initiated by UV-A radiation and visible-light (blue spectrum), but plants irradiated with monochromatic radiation (UV-B radiation only) cannot induce repair mechanisms.

At high UV-B doses (UV-B high 2 h), total antioxidant capacity of damiana (*T. diffusa*) plants increased significantly compared to non-irradiated control plants ($p < 0.05$, table II) during the first 7 days of treatment; at severe UV-B doses (UV-B severe 2 h and 4 h) total antioxidant capacity of damiana plants decreased (table II) during 7 days of irradiation. Oxidative damage, quantified as lipid peroxidation levels (TBARS), increased in damiana plants irradiated with UV-B as compared to non-irradiated plants ($p < 0.05$, table II). Hassan *et al.*[6] reported an increase of 40% in bean (*Vicia faba* L.) plants in response to UV-B radiation. UV-B radiation increases ROS production, which can cause oxidative damage to lipids, proteins and DNA[10,40]. Plants vary in their ability to withstand UV radiation based on the increase in ROS production[30] and their capacity to synthesize compounds that prevent or reduce oxidative damage[37].

Damiana plants irradiated with UV-B showed dose-dependent changes in both enzymatic and non-enzymatic antioxidants. The loss of phenolic compounds (table II) and photosynthetic pigments (table I) in response to UV-B exposure suggest that either antioxidant defenses in damiana plants are not sufficient to provide protection against artificial UV-B irradiation, that damiana in BCS may be near its maximal capacity to induce antioxidant defenses, or that the UV-B dosage used in this study exceeds the tolerance capacity of this plant. Ramya and Balakrishnan[15] mentioned that plants have different sensibility to UV-B radiation, depending on treatments and intensity of radiation. In a previous study[18], the antioxidant content in wild and cultivated damiana plants from BCS, naturally exposed to high solar radiation, was reported. In wild and cultivated damiana plants the phenolic compound levels are lower than in *in vitro* damiana irradiated with UV-B in this study. Yet, wild and cultivated damiana had lower lipid peroxidation levels than damiana irradiated *in vitro* with UV-B. The results from this study suggest that artificial UV-B radiation decreases the defense capacity, decreases the antioxidant content, and increases oxidative damage in leaves of damiana plants. Even so, photosynthetic pigments and vitamin C content increased in damiana irradiated *in vitro* with UV-B.

Conclusion

Antioxidant defense mechanisms, such as SOD activity and vitamin C, are activated by artificial UV-B radiation to protect damiana plants *in vitro*. At high UV-B doses (UV-B high 2 h), total antioxidant capacity of damiana plants increased while at severe UV-B doses (UV-B severe 2 h and 4 h) total antioxidant capacity of damiana plants decreased significantly compared to non-irradiated control plants. At severe doses, UV-B exposure increased SOD activity, photosynthetic pigments (chlorophylls), vitamin C content and lipid peroxidation. Phenolic compound content in damiana plants decreased significantly following exposure to UV-B radiation in all treatments.

Acknowledgments

Authors wish to thank M.G. Angulo-Villavicencio, O. Lugo-Lugo, N.O. Olguín-Monroy, S. Real-Cosío, and M. Rodríguez-Álvarez for technical support in experimental setup and sample analyses. This study was supported by grants from CIBNOR (PC2.0, PC0.10, PC0.5, PC0.9), UAM-Iztapalapa and CONAFOR (CONAFOR-PE09.02). LlAASM is the recipient of a CONACyT scholarship (205249). The manuscript was prepared following the Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org.

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