Journal of Thermal Biology 81 (2019) 73–81

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Thermoprotective properties of *Opuntia ficus-indica* f. *inermis* cladodes and mesocarps on sheep lymphocytes

Imen Belhadj Slimen[a,b,*], Hanène Chabaane[c], Mohamed Chniter[a], Mahmoud Mabrouk[d], Adbeljelil Ghram[e], Khaled Miled[e], Imen Behi[e], Manef Abderrabba[b], Taha Najar[a,b]

[a] Department of Animal Sciences, Food and Halieutic Resources, National Institute of Agronomy of Tunisia, Tunis, Tunisia
[b] Laboratory of Materials, Molecules and Application, Preparatory Institute for Scientific and Technical Studies, Tunis, Tunisia
[c] Laboratory of Bioagressors and Integrated Protection in Agriculture, National Institute of Agronomy of Tunisia, Tunis, Tunisia
[d] Central Laboratory, Arid Lands Institute, Medenine, Tunisia
[e] Laboratory of Epidemiology and Veterinary Microbiology, Pasteur Institute, Tunis, Tunisia

ARTICLE INFO

Keywords:
Heat stress
Opuntia ficus-indica
Lymphocytes
Betalains
H_2O_2

ABSTRACT

This study aims to investigate the thermoprotective properties of *Opuntia ficus-indica* f. *inermis*. Extracts were prepared from cladodes (CE) and mesocarps (ME), then subjected to a spectrophotometric and LC-MS analyses. Lymphocytes were isolated from peripheral blood of non-stressed sheep, supplemented with CE, ME, betanin or α-tocopherol, and subjected to two thermal treatments: 40 and 41 °C, for 6 h. Viable lymphocytes and H_2O_2 production were evaluated.

The antioxidant activity of ME was 3.43 folds higher than CE. The LC-MS analysis of CE and ME allowed identifying 11 phenolic acids, 2 flavanones, 6 flavones, 3 flavonols and 1 betanin type betacyanin.

Lymphocytes mortality increased linearly as function of the severity and the duration of heat stress. This mortality was correlated with H_2O_2 production. At 41 °C, only ME allowed maintaining lymphocytes viability. Moreover, ME was more efficient than CE in reducing H_2O_2 production. This thermoprotection was ensured by betaxanthin and betacyanin pigments. Interestingly, betanin was more efficient than α-tocopherol in preventing hyperthermia-induced lymphocytes' mortality.

We report here for the first time the thermoprotective properties of cladodes and mesocarps of *Opuntia ficus-indica* f. *inermis*. Betanin was able to maintain lymphocyte viability through reducing H_2O_2 production, and therefore the oxidative-induced heat stress.

1. Introduction

Opuntia ficus-indica, often called prickly pear, or cactus pear, belongs to the dicotyledonous angiosperm Cactaceae family. *Opuntia* is an old vegetation of Mexico and much of Latin America wherein it is regarded as a typical symbol of people identity. Between the end of the Pleistocene (ca. 100 000 years B.P.) and the beginning of the fifth millennium AD, prickly pear was the basic foodstuff of the prehistoric indigenous groups of the semi-arid basins and valleys of the states of Hidalgo, Mexico, Morelos, Guerrero, Puebla and Oaxaca (Flannery, 1985). Nowadays, cactus pear is widespread in semi-arid and arid regions, the South Africa, and the Mediterranean region. *Opuntia ficus-indica* is believed to have been introduced to the South Africa at least 250 years ago (Zimmerman and Moran, 1991), giving this country the oldest record of *Opuntia* introduction as a fodder crop.

de Sahagún (1997) reported that native Americans lived for many years and were "healthy and strong." He justified their vitality by the type of their diet which included prickly pear leaves, fruits and, roots, as well as mesquite pods. The prickly pear has been used in traditional medicine for the treatment of digestive problems, edema, and for burn and wound care (Shetty et al., 2012). Moreover, this plant has been used as a diuretic and fever reducer. Mashed pads historically were used to relieve inflammation. Furthermore, medicinal uses include urinary problems, tumors, ulcers, bronchitis, and human immunodeficiency virus (Kaur et al., 2012). Analytic chemistry revealed that *Opuntia ficus-indica* due its medicinal properties to a myriad of phytochemicals having antioxidant and biological activities such as vitamins, phenolic compounds, flavonoids, carotenoids and betalains (Núñez-Gastelum et al., 2018; Belhadj Slimen et al., 2017a, 2017b, 2016).

* Corresponding author at: Department of Animal Sciences, National Institute of Agronomy of Tunisia, Tunis, Tunisia.
E-mail address: belhadj_slimen_imen@yahoo.fr (I. Belhadj Slimen).

https://doi.org/10.1016/j.jtherbio.2019.02.018
Received 10 September 2018; Received in revised form 8 December 2018; Accepted 25 February 2019
Available online 27 February 2019
0306-4565/

Nowadays, heat stress is with a major concern since it jeopardizes farm animals' production performances and disturbs their immunity system. These effects could be accentuated in the future with climate change. *In vivo* studies reported a decrease of lymphocytes count in heat stressed sheep, compared to those reared under thermal neutral conditions (Liu et al., 2016; de Alvarenga et al., 2013). Our previous in vitro investigations confirmed a hyperthermia-induced lymphocyte death (Belhadj Slimen et al., 2015) and were corroborated by the reports of Romero et al. (2013) and Kamwanja et al. (1994).

In this study, we hypothesized an ROS-mediated pathway of cell death and we suggested that supplementation with natural antioxidants extracted from *Opuntia ficus-indica* cladodes and mesocarps may confer thermoprotection to heat stressed lymphocytes.

2. Materials and methods

2.1. Extracts preparation

Young cladodes (age < 30 days) and fruits of *Opuntia ficus-indica* f. *inermis* were harvested from the improved pasture managed by the Office of Livestock and Pasture, and located in Sawwaf, Zaghouan governorate (North of Tunisia). The peel was separated manually from the pulp. Samples were washed, chopped into small pieces, homogenized with an automatic press and extracted over a night using distilled water for cladodes and hydroethanol 50% (v:v) for mesocarps. Cladode (CE) and mesocarp extracts (ME) were centrifuged at 4500*g* at 4 °C for 15 min. The supernatants were collected and stored at −20 °C until use.

2.2. Determination of total phenolics

Total phenolic content was estimated according to Ainsworth and Gillespie (2007). Briefly, 600 µL of 10% (v/v) Folin Ciocalteu reagent was added to 300 µL of each extract, standard or 95% (v/v) methanol blank and vortexed thoroughly. Then, 2.4 mL of 700 mM Na_2CO_3 (in water) was added and the assay tubes were incubated at room temperature for 2 h. Absorbance was determined at 765 nm using a Lasany uv–vis spectrophotometer. A calibration curve was calculated from the blank-corrected *A765* of the gallic acid standards (gallic acid solutions of 50 mg/L – 400 mg/L (in 95% methanol)). Total phenolics were then calculated as gallic acid equivalents (GAE) using the regression equation between gallic acid standards and *A765*.

2.3. Determination of total flavonoids

Flavonoid content was determined as described by Zhishen et al. (1999). 250 µL of the extract was placed in a 15 mL volumetric flask. 75 µL NaNO2 (1:20 w:v) was added, followed by 150 µL $AlCl_3$ (1:10) 5 min later. After incubation for 6 min, 500 mL of NaOH (1 molL-1) was added and then solution was adjusted with distilled water until the total volume reached 2.5 mL. The solution was vortexed thoroughly and absorbance was measured against a blank (ethanol) at 510 nm with a Lasany UV–Vis spectrophotometer. Quercetin was used as a standard. The results were expressed as Quercetin equivalents (QE).

2.4. Determination of ascorbic acid

Ascorbic acid content was determined according to Dürüst et al. (1997). Briefly, 0.10 mL of each extract (a dilution factor in oxalic acid at 0.4% was considered for each sample) was added to 0.100 mL of acetate buffer. Then, 0.800 mL of DCPI (2,6-dichloroindophenol sodium) was added. The absorbance of the mixture was measured after 15 s at 520 nm with a Lasany UV–Vis spectrophotometer. Ascorbic acid was used as a standard.

2.5. Determination of betalains

As described by Prakash and Manikandan (2012), 10 mL of each extract was mixed with 10 mL of 50% aqueous methanol solution and the mixture was agitated at 250 rpm for 30 mn. Then, the samples were centrifuged at 4731*g* for 15 min at 4 °C.

Betaxanthin and betacyanin were assessed spectrophotometrically (Lasany UV–Vis spectrophotometer) as indicaxanthin and betanin equivalents at 480 and 536 nm, respectively. Betalain content was evaluated as reported by Stintzing et al. (2005) using Eq. (1).

$$BC\left(\frac{mg}{L}\right) = \frac{A \times DF \times MW \times 1000}{\varepsilon \times l} \quad (1)$$

where: BC (mg/L) is betacyanin or betaxanthin content, *A* is the absorption value of the sample, MW is the molecular weight (indicaxanthin = 308 g/mol and betanin = 550 g/mol), ε is the molar extinction coefficient (indicaxanthin = 48.000 L mol^{-1} cm^{-1} at 480 nm and betanin = 65.000 L mol^{-1} cm^{-1} at 536 nm), and 1 cm is the path length of the cuvette.

2.6. Free radicals scavenging activity

Antiradical activity was measured with DPPH. (1,1-diphenyl-2-picrylhydrazyl radical) as described by Brand-Williams et al. (1995) and modified by Thaipong et al. (2006). The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and stored at −20 °C until use. The working solution was obtained by mixing 10 mL of the stock solution with 45 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. To evaluate the antiradical activity, 150 µL of each extract was allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. The absorbance was taken at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid) was used as a standard.

2.7. LC-MS analysis

Chromatography was performed on Shimadzu Ultra Fast Liquid system (Shimadzu prominence UFLC XR, Japon). HPLC separations were performed on an AQUASIL thermo C18 (3 × 150 mm, 3 µm) at 40 °C. An AQUASIL thermo C18 (3 × 10 mm, 3 µm) guard column was used. Chromatographic separation conditions for phenolic acids and flavonoids were as follows: The mobile phase consisted of water + 0.1% formic acid (A) and methanol + 0.1% formic acid (B). The separation lasted 55 min, followed by 5 min equilibrium time. The binary gradient elution was as follows: 0–45 min, 10% B; 45–55 min, 100% B; 55–55.1 min, 10% B; 55.1–60 min, 10% B. The flow rate was 0.4 mL/min, and the injection volume was 5 µL for analysis.

Betanin separation lasted 25 min, with an additional 5 min equilibrium time. The mobile phase consisted of water + 2% formic acid (A) and methanol (B). The binary gradient elution was planned as follows: 0.01–15 min, 5% (B); 15–20 min, 15% (B); 20–22 min, 70% (B); 22–25 min, 100% (B); 25–25.01 min, 100% (B); 25.01–30 min, 5% B. Quantitative determination was carried out using calibration curves of standards.

A Shimadzu 2020 (Japon) Quadrupole mass spectrometer equipped with a positive/negative ESI source was used as a detector. Mass spectrometer was operated in the negative selected ion monitoring (SIM) with capillary voltage at 1.2 V for phenolic compounds identification, and in a positive SIM for betanin. The conditions of MS analysis were designed as follows: the spray voltage was −3.5 V, the dissolving line temperature was 250 °C, the nebulizer gas flow was 1.5 L/min, the heat block was set at 400 °C, the drying gas flow was 12.00 and 15.00 respectively for phenolics and betanin. Finally the detector voltage was 1.2 V.

Table 1
Antioxidant activity and phytochemical contents of *Opuntia ficus-indica* peels and cladodes.

	Antioxidant activity (mg TE/g)	Polyphenols (mg GAE/g)	Flavonoïds (mg QE/g)	Ascorbic acid (mg/g)	Betacyanins (μg/g)	Betaxanthins (μg/g)	Total betalains (μg/g)
Peels	1.23 ± 0.03	1.45 ± 0.01	0.5 ± 0.01	0.33 ± 0.08	40 ± 0.00	20 ± 0.00	60 ± 0.00
Cladodes	0.36 ± 0.02	0.91 ± 0.02	0.35 ± 0.0	0.16 ± 0.01	–	–	–

TE: Trolox equivalents, GAE: Gallic acid equivalents, QE: Quercetin equivalents.

2.8. Peripheral blood lymphocytes

Sheep lymphocytes were isolated as described by Niwano (1991) with some modifications. A volume of 10 mL of sheep EDTA venous blood was mixed with 10 mL of RPMI 1640 supplemented with 1% of antibiotics. 10 mL of the blood-medium mixture were then carefully layered over 5 mL of Histopaque gradient solution. After centrifugation at 1990 rpm for 30 min, sheep peripheral blood lymphocytes appeared as a buffy coat between the plasma-medium mixture and the Histopaque cushion. The white band of lymphocytes was collected, transferred to a sterile plastic tube containing 5 mL of RPMI 1640, and centrifuged at 2455 rpm for 15 min. This washing procedure was repeated 3 times. Lymphocytes were then collected, counted, suspended in RPMI containing 10% of FBS (fetal beef serum), into a 15 mL flask at a concentration of 5×10^6 viable cells/mL, in 5% CO_2 atmosphere at 37 °C.

2.9. MTT cytotoxicity assay

Cytotoxicity of CE and ME was assessed by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as previously described (Mosmann, 1983). In brief, lymphocytes were supplemented with different doses of each extract varying from 0.1% to 5% and incubated for 24 h at 37 °C. The cells were further incubated with the MTT reagent (0.5 mg/mL) for 4 h at 37 °C with 5% CO_2. Then, a volume of 100 μL of DMSO was added to dissolve the farmazan crystals and the absorbance was recorded at 490 nm by using an ELISA plate reader.

2.10. Thermoprotection assay

Viable lymphocytes were isolated as described above then cultured in presence of CE or ME (0.1% and 5%) for one night at 37 °C in 5% CO_2 atmosphere. Therefore, the concentration was adjusted at 3×10^6 viable cells/mL and the lymphocytes were subjected to 2 thermal treatments (40 and 41 °C) for 6 h using a water bath as previously described (Belhadj Slimen et al., 2015). Viable lymphocytes were counted each 2 h interval using a Neubauer cell counter.

2.11. Amplex red assay

Lymphocytes (3×10^6 cells/mL) were supplemented with different doses of *Opuntia* extracts then subjected to thermal stress as described above. The production and the release of H_2O_2 into the extracellular medium were investigated using the fluorescent probe Amplex Red, as previously described (Zhou et al., 1997). Heat stressed lymphocytes (100 μL) were incubated at room temperature with a reaction mixture containing 100 μL of 50 μM Amplex Red and horseradish peroxidase (1.0 U/mL). After incubation for 60 min at 37 C in a 5% CO_2 atmosphere, the fluorescence in each cell was measured in a fluorescence microplate reader using an excitation of 530 nm and an emission detection of 590 nm.

2.12. Betanin and vitamin E assay

Cells were cultured in RPMI 1640 medium in presence of 10, 25, 50 and 100 μg/mL of betanin or α-tocopherol (Sigma Aldrich) over night. They were subjected thereafter to 6 h of thermal stress at 40 and 41 °C. Viable cells were counted at the end of the heat exposure.

2.13. Statistical analysis

Statistical analyses were performed using the SAS software (9.1.3). Data were analyzed using General Linear Model (GLM) fitting cell viability and hydrogen peroxide production as fixed effects. When statistical significance was detected, mean comparisons were performed with Duncan's multiple-range test. The relationships between variables were determined by calculating Sperman's correlation coefficients. The results are expressed as the mean ± S.D. Significance level was set at $\alpha = 0.05$.

3. Results and discussion

3.1. Phytochemicals and antioxidant activity

The radical scavenging activities of ME and CE were evaluated based on their ability to quench the synthetic DPPH radicals. Interestingly, this assay gives information about the capacity of the studied samples to scavenge free radicals independently from any enzymatic activity (Mahakunakorn et al., 2004). The results showed that the antioxidant activity of mesocarps is 3.41 folds higher than that of cladodes (Table 1).

It is well known that many phenolic acids and flavonoids have important antioxidant properties. Our results showed that phenolics and flavonoids are respectively 1.6 and 1.43 folds higher in mesocarps than in cladodes. Similarly, ascorbic acid content is 2 folds greater in mesocarps. In addition to these compounds, *Opuntia* peels contain considerable amounts of betalains (Table 1). Several studies strongly confirmed the high radical scavenging activity of these latter pigments. Many authors reported that the antiradical activity of betalains is much higher than that of Trolox, ascorbic acid and rutin (Cai et al., 2003), as well as α-tocopherol (Kanner et al., 2001). Interestingly, Stintzing et al. (2005) and Fernández-López et al. (2010) reported that ascorbic acid did not contribute to the radical scavenging activity of cactus pear fruits.

3.2. Bioactive phenolics and betanin

The phytochemical analysis of *Opuntia ficus-indica* peels and cladodes allowed identifying 11 phenolic acids (1–11), 2 flavanones (12–13), 6 flavones (14–19), 3 flavonols (20–22) and 1 betanin type betacyanin (23) (Fig. 1(a,b), Table 2).

To the best of our knowledge naringin (12), naringenin (13), quercetrin (22), hyperoside (21), acacetin (18) and cirsiliol (19) are new compounds reported here for the first time in *Opuntia* peels and cladodes.

The identified benzoic acid derivates are syringic acid (6) and protocatechuic acid (9). Cinnamic acid derivates include quinic acid (1), chlorogenic acid (2), 4-*O*-caffeoylquinic acid (3), cafeic acid (4), 1,3-di-*O*-caffeoylquinic acid (5), p-coumaric acid (7), ferulic acid (8) and salvianolic acid (10).

Both benzoic and cinnamic acid derivates possess radical

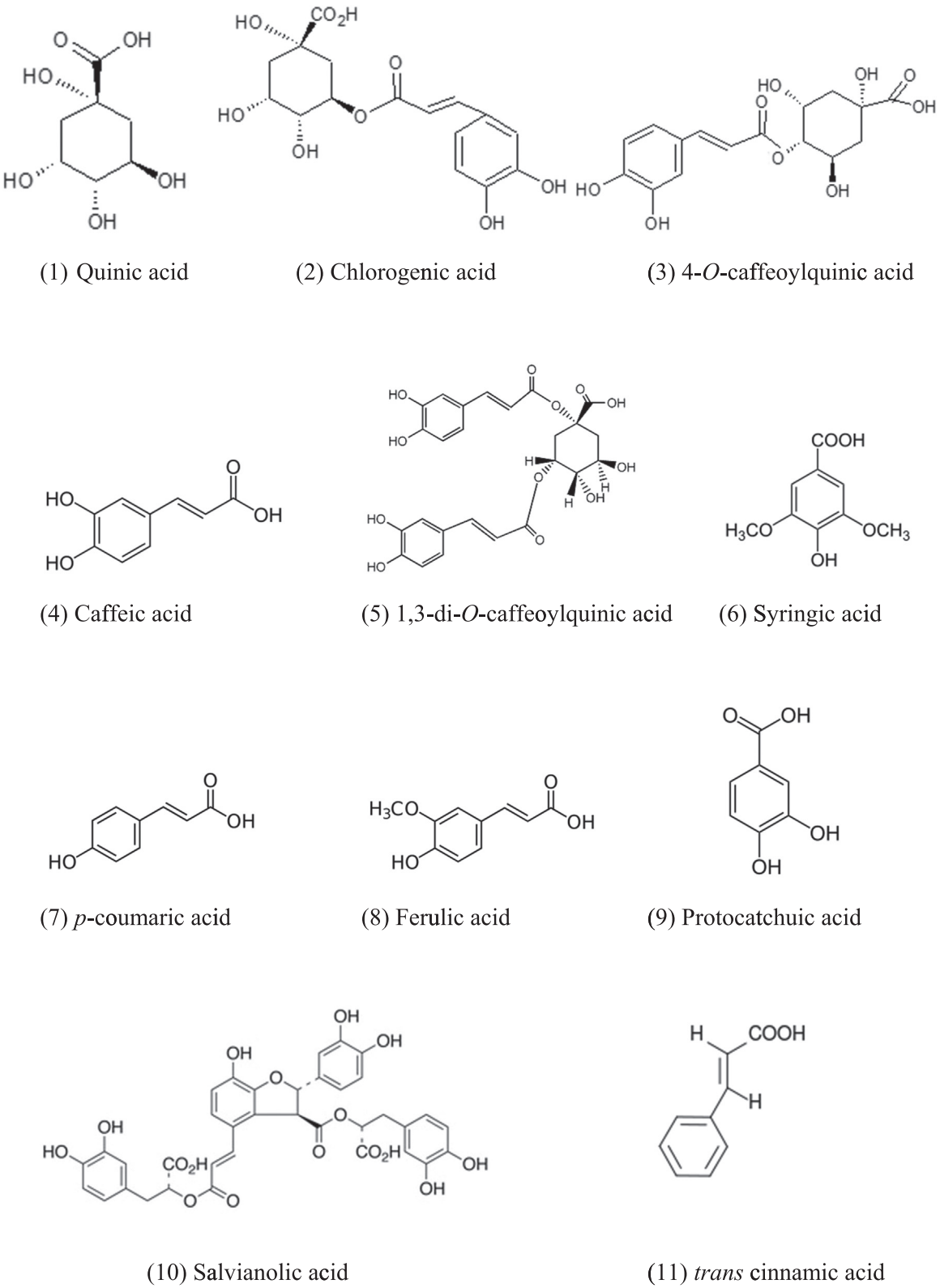
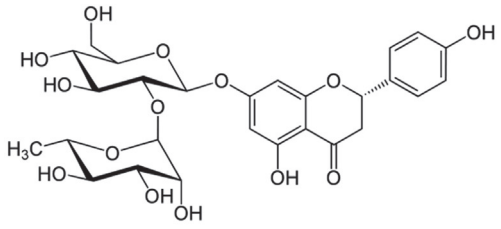
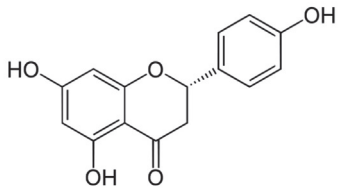


Fig. 1. Phenolic acids (1–11), Flavonoids (12–22) and Betanin (23) identified from *Opunttia ficus-indica* peels and cladodes.

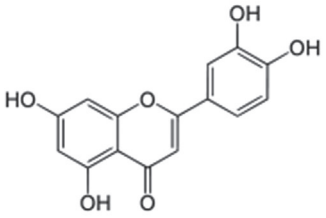
scavenging activity (Pontiki et al., 2014). Merkl et al. (2010) reported that *p*-hydroxybenzoic acid, gentisic acid, ferulic acid, isoferulic acid, gallic acid, salicylic acid, sinapic acid, coumaric acid, vanillic acid, protocatechuic acid and syringic acid possess good antioxidant effect as reveratol and trolox. Interestingly, gallic acid has the highest antioxidant effect. The antioxidant activity of phenolic acids is generally ascribed to the reaction with oxidants to form resonance stabilized phenoxyl radicals (Baum and Perun, 1962). The presence of a second hydroxyl group, as in caffeic and protocatechuic acids, strengthens this activity through the formation of an intramolecular hydrogen bond. With regard to the aromatic substitution, the increase of the number of the methoxy substitutions or the catechol structures increases the



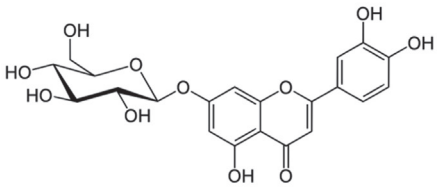
(12) Naringin



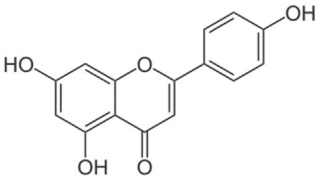
(13) Naringenin



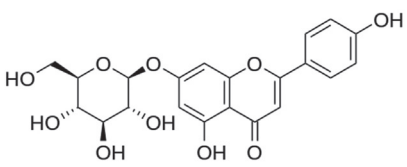
(14) Luteolin



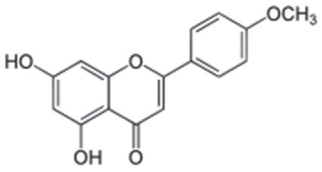
(15) Luteolin-7-*O*-glucoside



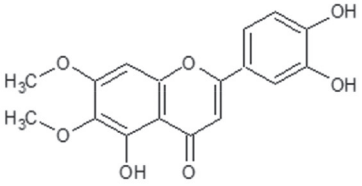
(16) Apigenin



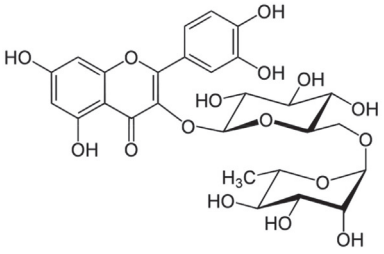
(17) Apigenin-7-*O*-glucoside



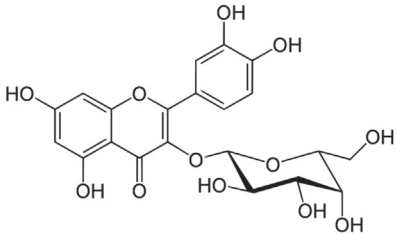
(18) Acacetin



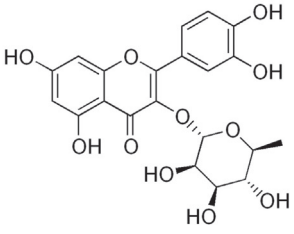
(19) Cirsiliol



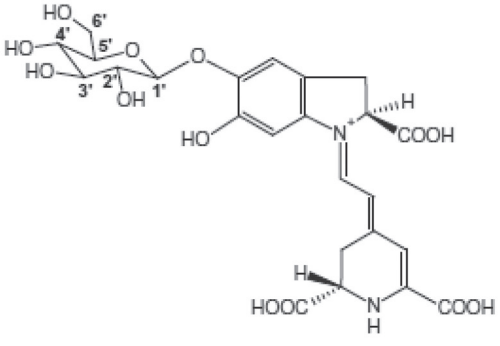
(20) Rutin



(21) Quercetin-3-*O*-galactoside



(22) Quercetrin



(23) Betanin

Fig. 1. (*continued*)

Table 2
Quantification of betanin and some phenolic compounds in *Opuntia* cladodes and mesocarps.

Compounds	Phytochemical class	Molecular formula	*m/z* peak	Retention time (min)		Quantification (mg/Kg)	
				C	M	C	M
Quinic acid	phenolic acid	$C_7H_{12}O_6$	191.00	3.022	2.879	429.827	1450.709
Chlorogenic acid	phenolic acid	$C_{16}H_{18}O_9$	353.00	11.391	–	1.634	ND
4-*O*-caffeoylquinic acid	phenolic acid	$C_{16}H_{18}O_9$	353.00	12.817	13.083	0.07	0.505
Caffeic acid	phenolic acid	$C_9H_8O_4$	179.00	12.673	12.866	0.089	0.175
1,3-di-*O*-caffeoylquinic acid	phenolic acid	$C_{25}H_{24}O_{12}$	515.00	13.075	13.765	0.174	0.798
Syringic acid	phenolic acid	$C_9H_{10}O_5$	197.00	13.672	–	3.016	ND
p-coumaric acid	phenolic acid	$C_9H_8O_3$	163.00	16.138	16.120	0.09	0.39
trans ferulic acid	phenolic acid	$C_{10}H_{10}O_4$	193.00	17.122	17.144	1.103	3.772
Naringin	flavanone	$C_{27}H_{32}O_{14}$	579.00	19.248	19.247	0.33	1.7
Luteolin-7-*O*-glucoside	flavone	$C_{21}H_{20}O_{11}$	447.00	20.754	20.758	0.141	0.074
Hyperoside (quercetin-3-*O*-galactoside)	flavonol	$C_{21}H_{20}O_{12}$	463.00	20.895	20.691	0.05	1.676
Rutin	flavonol	$C_{27}H_{30}O_{16}$	609.00	20.781	20.819	31.927	51.667
Salvianolic acid	phenolic acid	$C_{36}H_{30}O_{16}$	717.00	20.698	21.036	0.242	0.169
Apigenin-7-*O*-glucoside	flavone	$C_{21}H_{20}O_{10}$	431.00	22.507	22.427	0.001	0.029
Cirsiliol	flavone	$C_{17}H_{14}O_7$	329.00	30.294	30.309	15.024	6.581
Apigenin	flavone	$C_{15}H_{10}O_5$	269.00	30.325	30.158	0.004	0.002
Acacetin	flavone	$C_{16}H_{12}O_5$	283.00	36.391	36.416	7.342	6.942
Gallic acid	phenolic acid	$C_7H_6O_5$	–	–	–	ND	ND
Protocatchuic acid	phenolic acid	$C_7H_6O_4$	153.00	–	7.364	ND	0.153
Catechin (+)	flavan-3-ol	$C_{15}H_{14}O_6$	–	–	–	ND	ND
Epicatechin	flavan-3-ol	$C_{15}H_{14}O_6$	–	–	–	ND	ND
3,4-di-*O*-caffeoyquinic acid	phenolic acid	$C_{25}H_{24}O_{12}$	–	–	–	ND	ND
O-coumaric acid	phenolic acid	$C_9H_8O_3$	–	–	–	ND	ND
Rosmarinic acid	phenolic acid	$C_{18}H_{16}O_8$	–	–	–	ND	ND
4,5-di-*O*-caffeoyquinic acid	phenolic acid	$C_{25}H_{24}O_{12}$	–	–	–	ND	ND
Quercetrin	flavonol	$C_{21}H_{20}O_{11}$	447.00	–	22.539	ND	0.303
trans cinnamic acid	phenolic acid	$C_9H_8O_2$	147.00	–	23.517	ND	8.644
Myricitin	flavonol	$C_{15}H_{10}O_8$	–	–	–	ND	ND
Naringenin	flavanone	$C_{15}H_{12}O_5$	271.00	–	24.385	ND	0.377
Silymarin	flavon-lignan	$C_{25}H_{22}O_{10}$	–	–	–	ND	ND
Luteolin	flavone	$C_{15}H_{10}O_6$	285.00	–	27.709	ND	0.005
Quercetin	flavonol	$C_{15}H_{10}O_7$	–	–	–	ND	ND
Kaempferol	flavonol	$C_{15}H_{10}O_6$	–	–	–	ND	ND
Cirsilineol	flavone	$C_{18}H_{16}O_7$	–	–	–	ND	ND
Isorahmethin	flavonol	$C_{16}H_{12}O_7$	–	–	–	ND	ND
Aucubin	iridoid glycoside	$C_{15}H_{22}O_9$	–	–	–	ND	ND
Betanin	Betacyanin	$C_{24}H_{27}N_2O_{13}$	551.00	–	1.649	–	2473

C: cladodes, M: mesocarps, ND: Not detected peak.

antioxidant activity (Shahidi and Wanasundara, 1992). Hence, sinapic acid is 6 times as efficient as trolox, followed by caffeic (4 times), syringic (1.3 times), ferulic (0.9 times), and protocatechuic acid (0.79 times). *p*-Coumaric, *p*-hydroxybenzoic, and vanillic acids are hardly effective.

Quercetin is a flavonol known for its proven health promotive properties. It is one of the most potent antioxidants among polyphenols (Materska, 2008). Rutin is the glycoside combining the flavonol quercetin and the disaccharide rutinose. This molecule is known for its strong antioxidant properties.

Both naringin and naringenin are strong antioxidants. However, the latter has the highest activity because the sugar moiety in naringin causes steric hindrance of the scavenging group (Renugadevi and Prabu, 2009). Cirsiliol has been previously isolated from Salvia species. Acacetin is a 5,7-dihydroxy-4′-methoxyflavone, and is responsible for many plant color shades It was reported to inhibit glutathione reductase and cytochrome P450 (Doostdar et al., 2000; Zhang et al., 1997).

Betanin (23), a betanidin 5-O-ß-glucoside, was identified for the first time from red beet. The EC_{50} measured by the DPPH. assay showed that batanin antiradical activity is higher than ascorbic acid, rutin, (+)-catechin and ferulic acid (Cai et al., 2005, 2003; Kanner et al., 2001). This activity is structurally explained by the presence of phenolic hydroxy groups allowing stabilization by resonance and hydrogen bonding as well as the resonance shared between the imino and the tetrahydropyridine groups (Belhadj Slimen et al., 2017a).

3.3. Cytotoxicity assay

Lymphocytes were supplemented with different doses of ME and CE extracts ranging from 0.1% to 5%. No significant absorbance difference was noted after using the MTT colorimetric assay for living cells. Hence, all of the tested doses are safe.

The MTT colorimetric assay utilizes a colorless substrate that is modified to a colored product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, and therefore the reaction occurs only in living cells. CE and ME extracts were therefore non-toxic to lymphocytes, as well as distilled water and aqueous ethanol that were used as positive controls.

3.4. Thermoprotection test

As compared to the controls, both doses of each extract succeeded to ensure thermoprotection of lymphocytes after 6 h of heat stress at 40 °C (Fig. 2a). At 41 °C, only ME ensured heat-protection of lymphocytes. Cladodes extract ensured partial thermoprotection up to 3 h of thermal stress. Thereafter, lymphocytes viability declined. After 6 h of exposure at 41 °C, viable lymphocytes were $3.12 \pm 0.17 \times 10^6$ cell/mL vs $1.50 \pm 0.12 \times 10^6$ cell/mL, respectively for ME and CE (Fig. 2b).

Cell viability was affected significantly by the extract ($p < 0.0001$), the exposure temperature ($p = 0.0002$) and the duration of heat stress ($p < 0.0001$), and this was also evident for the interaction effect

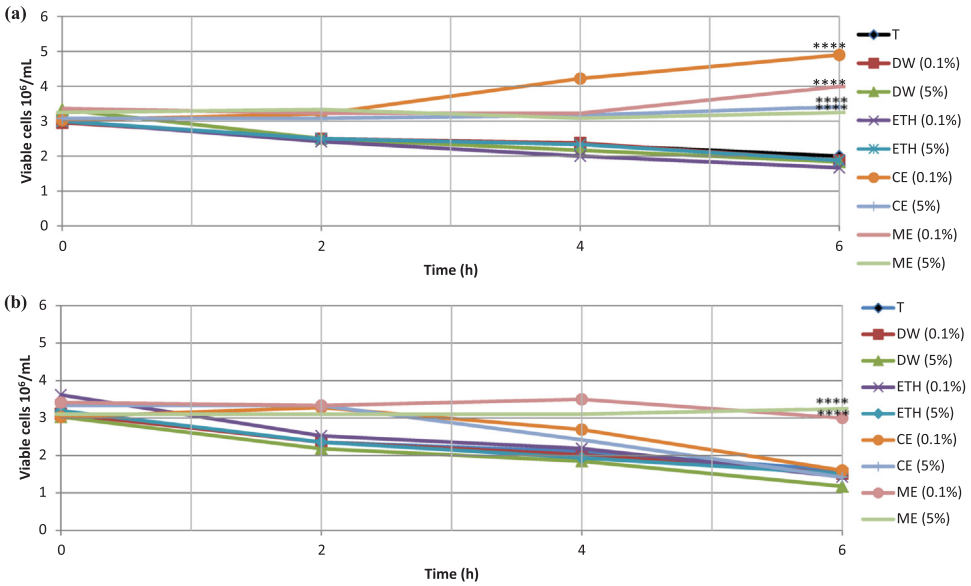


Fig. 2. Kinetics of lymphocytes viability (a) at 40 °C (b) at 41 °C, T: negative control, DW: distilled water, ETH: aqueous ethanol, CE: cladodes extract, ME: mesocarps extract.

between the extract and the exposure temperature ($p = 0.0273$). The applied dose didn't affect significantly the cell viability.

Our previous reports, as well as those of several authors, confirmed a hyperthermia-induced lymphocyte death (Belhadj Slimen et al., 2015; Romero et al., 2013; Kamwanja et al., 1994). In this work, we report for the first time the thermoprotection properties of *Opuntia* peels and cladodes. At high physiologic temperature (41 °C), ME is more efficient than CE in protecting and maintaining lymphocytes viability. This may be associated with the higher antioxidant activity of ME and its composition containing betacyanins and betaxanthins.

3.5. Hydrogen peroxide production

As shown in Fig. 3, after heat exposure, H_2O_2 production was the highest in non-treated cells (22.8 nL ± 2.95), followed by cells treated with CE (14.02 nL ± 4.01), and finally those treated with ME (7.19 nL ± 2.7). Interestingly, H_2O_2 production increased in the same trend as the exposure temperature (25.11 nL ± 1.88 and 9.73 nL ± 1.68 at 41 and 40 °C, respectively).

H_2O_2 production was significantly affected by the extract ($p < 0.0001$) and the exposure temperature ($p < 0.0001$).

Correlation analysis showed that the different phytochemicals of the extracts contributed to the decrease of the hydrogen peroxide production under heat stress conditions. However, only betacyanins and betaxanthins were positively correlated to cell viability at 41 °C. Noteworthy, cell viability was strongly and negatively correlated to the level of H_2O_2 (Table 3).

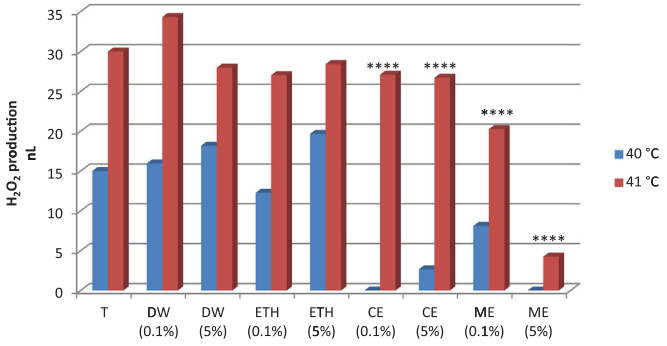


Fig. 3. Hydrogen peroxide production by heat stressed lymphocytes, T: negative control, DW: distilled water, ETH: aqueous ethanol, CE: cladodes extract, ME: mesocarps extract. Signification levels: $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***, $p < 0.0001$: ****.

Our results suggest a heat-induced ROS-mediated pathway of lymphocytes mortality. The measurement of H_2O_2 was performed using Ample Red, a specific fluorescent probe for hydrogen peroxide, in the extracellular medium. This colorless and non- fluorescent derivative of resorufin allows the detection of as little as 5 pmoL H_2O_2 per 100-mL sample (50 nM) in a 96-well fluorescence microplate assay (Zhou et al., 1997).

H_2O_2 is one of the most important ROSs and the second key player in mitochondrial ROS production. The initial product of NADPH oxidase-mediated oxygen reduction is primarily superoxide anion. Subsequently, superoxide is converted to H_2O_2 either spontaneously or by superoxide dismutase. H_2O_2 is further reduced to the extremely reactive hydroxyl radical via the Fenton reaction, producing the extremely reactive hydroxyl radical (OH.). Due to its small size and relatively benign reactivity, H_2O_2 can diffuse freely across cell membranes and is able to mediate toxic effects far from its production site (Liochev and Fridovich, 1999). Heat stress was shown to increase H_2O_2 formation (Belhadj Slimen et al., 2014; Kikusato and Toyomizu, 2013). Nowadays, there is evidence that heat stress induces apoptotic cell death by a mitochondria-induced pathway (Katschinski et al., 2000). When the ROS production increases above the steady state of the physiological concentrations, oxidative damages accumulate. ROS help the permeabilization of the outer mitochondrial membrane and mediate the release of cytochrome C and Bax to the cytosol (Zhao et al., 2006). Once released, cytochrome C binds to apoptotic protease activating factor 1 (Apaf-1) and forms the Apaf-1/procaspase-9 apoptosome, thereby activating the initiator caspase-9, which in turn activates the downstream effectors, caspases-3 and 7. Finally, apoptotic cell death occurs (Li and Oberley, 1997).

On the basis of the assumption that the radical scavenging components of *Opuntia* may be responsible for reducing oxidative damage, many studies have focused on fruits and extracts from different parts of this plant. In this trend, Tesoriere et al. (2004) reported that supplementation of healthy humans with cactus pears decreases malondialdehyde and LDL hydroperoxides. Furthermore, Brahmi et al. (2011) as well as Akacha et al. (2015) described the ability of cladode extracts to reduce oxidative stress induced by various oxidizing agents. Our findings showed that hydrogen peroxide production by

Table 3
Correlation matrix between the extract phytochemical classes, lymphocyte viability and hydrogen peroxide production.

	Antioxidant activity	Total phenols	Flavonoïds	Ascorbic acid	Betacyanins	Betaxanthins	H_2O_2 production	Viability
Antioxidant activity	1							
Total phenols	0.906****	1						
Flavonoïds	0.871****	0.997****	1					
Ascorbic acid	0.925****	0.960****	0.943****	1				
Betacyanins	0.900****	0.636*	0.574*	0.718**	1			
Betaxanthins	0.903****	0.638*	0.576*	0.723**	0.997****	1		
H_2O_2 production	− 0.814****	− 0.560*	− 0.501.	− 0.658**	− 0.908****	− 0.917****	1	
Viability	0.483.	0.192	0.137	0.295	0.683**	0.684**	− 0.832****	1

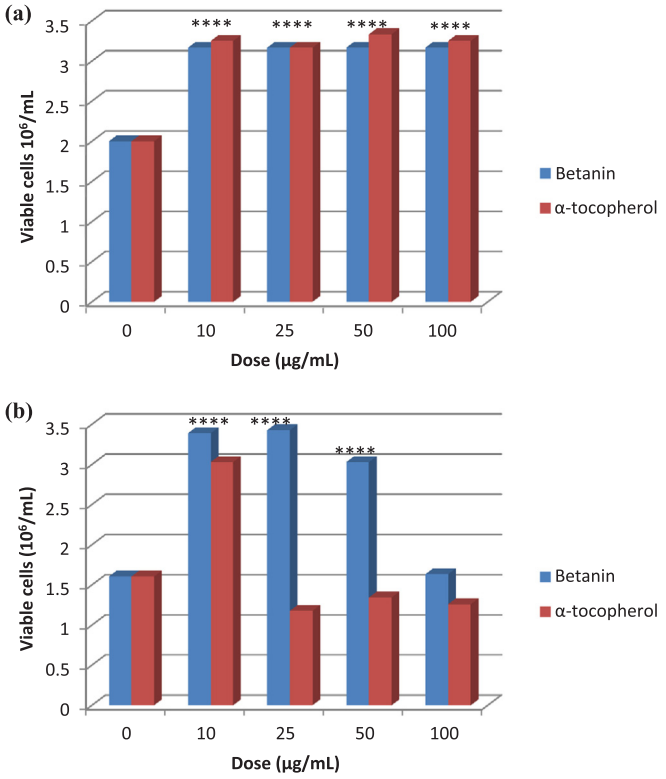


Fig. 4. Lymphocytes viability treated by different betanin and α-tocopherol doses after 6 h of heat exposure (a) at 40 °C, (b) at 41 °C.

hyperthermic lymphocytes treated with *Opuntia* extracts is 2.13 folds lower than control groups. Moreover, this production is 1.98 folds lower in lymphocytes treated with ME, as compared to those treated with CE. These results allowed suggesting that the thermoprotective properties of *Opuntia* extracts may be explained, among other factors, by their ability to scavenge H_2O_2 and therefore reduce oxidative stress damage. Here, we report for the first time the ability of peel and cladode extracts to reduce H_2O_2 production in heat-treated lymphocytes. Interestingly, Kamwanja et al. (1994) concluded that neither exogenous glutathione nor thioredoxin did overcome the effects of heat shock on lymphocytes.

Furthermore, our data suggested that some bioactive molecules in the cactus pear extracts, other than phenolic acids, flavonoïdes and vitamin C, helps protecting lymphocytes from heat-induced mortality. Although H_2O_2 production was negatively correlated to the contents of polyphenols, flavonoids and ascorbic acid recorded in the extracts, lymphocyte viability was correlated with only to betalain pigments (betacyanins and betaxanthins). Due to their amphiphilic character ensured by their hydrophilic groups (imino and alcohol groups) and their lipophilic moieties (hydrocarbon chain), betalains are able to bind to membranes and to scavenge free radicals from the water phase and the propagating lipoperoxyls from the polar phospholipidic bilayer. Thus, betalains are able to maintain cell membranes stability, neutralize intra and extracellular radicals, and protect cell membranes from peroxidation (Turco-Liveri et al., 2009).

3.6. Betanin and α-tocopherol effects

The different tested doses of betanin and α-tocopherol showed thermoprotection properties after 6 h of heat exposure at 40 °C. However, this thermoprotection declined at 41 °C and was dose-dependant. Whereas the highest dose of betanin (100 µg/mL) failed to maintain lymphocytes' viability, only the lowest dose (10 µg/mL) of α-tocopherol showed thermoprotection properties (Fig. 4(a) and (b)). This thermoprotection depended on the antioxidant molecule ($p < 0.0001$), its applied dose ($p < 0.0001$) and the stress temperature ($p < 0.0001$). Interestingly, both interaction effects antioxidant*exposure temperature and dose*exposure temperature were significant ($p < 0.0001$).

Betanin and α-tocopherol showed a dose dependant thermoprotection properties. At 41 °C, all applied betanin doses, except the highest one (100 µg/mL), maintained lymphocytes' viability during 6 h of heat exposure. However, only the lowest dose (10 µg/mL) of α-tocopherol ensured thermoprotection (Fig. 4). This temperature-dose interaction leads to suggest that betanin is more stable than α-tocopherol upon electron abstraction. Several studies strongly confirmed the high radical scavenging activity of betalains. Many authors reported that the antiradical activity of betalains is much higher than that of Trolox, a water soluble derivate of vitamin E (Gandía-Herrero et al., 2009), ascorbic acid (Cai et al., 2003), rutin (Cai et al., 2003) and α-tocopherol (Kanner et al., 2001). In that way, various doses of betanin, a betacyanin-type pigment, and α-tocopherol were tested for their thermoprotective effects. Betalain stability during oxidoreduction reactions is due to their chemical structure involving the phenolic hydroxyl-, the imino- and the tetrahydropyridine groups. Hence, betalains structure allows not only both resonance and hydrogen bonding stabilization of neutralized free radicals, but also remain stable after oxido-reduction reaction (Belhadj Slimen et al., 2017a). However, the prooxidant effects of betanin and α-tocopherol need to be investigated.

Acknowledgments

The authors thank Pr. Touhami Khorchani in Livestock and Wildlife Laboratory, Arid Lands Institute (I.R.A), Médenine and Dr. Makrem Essafi in Pasteur Institute Tunis for their contribution in samples' analysis.

Conflict of interest

The authors declare no competing financial interest.

References

Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nat. Protoc. 2, 875–877.

Akacha, A., Rebai, T., Zourgui, L., Amr, M., 2015. Preventive effect of ethanolic extract of cactus (*Opuntia ficus indica*) Cladodes on methotrexate induced oxidative damage of the small intestine in Wistar rats. Arch. Med. 7, 1–7.
de Alvarenga, A.B.B., Dallago, B.S.L., Mc Manus, C., Ramos, A.F., de Menezes, A.M., de Almeida, A.M.B., Bernal, F.E.M., 2013. Physiological parameters in different breeds of rams as a measure of adaptation to environmental conditions in the Federal district-Brazil. Int. J. Anim. Vet. Adv. 5, 256–263.
Baum, B.O., Perun, A.L., 1962. Antioxidant efficiency versus structure. Polym. Eng. Sci. 2, 250–259.
Belhadj Slimen, I., Najar, T., Ghram, A., Dabbebi, H., Ben Mrad, M., Abdrabbah, M., 2014. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. Int. J. Hyperth. 30, 513–523.
Belhadj Slimen, I., Ghram, A., Najar, T., Abderrabba, M., Ben M'Rad, M., 2015. Heat stress induces mortality of Barbary sheep lymphocytes. Bull. De. Soc. Zool. Fr. 140, 109–116.
Belhadj Slimen, I., Najar, T., Abderrabba, M., 2016. *Opuntia ficus-indica* as a source of bioactive and nutritional phytochemicals. J. Food Nutr. Sci. 4, 162–169.
Belhadj Slimen, I., Najar, T., Abderrabba, M., 2017a. Chemical and Antioxidant Properties of Betalains. J. Agric. Food Chem. 65, 675–689.
Belhadj Slimen, I., Mahmoud, M., Chaabane, H., Najar, T., Abderrabba, M., 2017b. LC-MS analysis of phenolic acids, flavonoids and betanin from spineless *Opuntia ficus-indica* Fruits. Cell Biol. 5, 17–28.
Brahmi, D., Bouaziz, C., Ayed, Y., Ben Mansour, H., Zourgui, L., Bacha, H., 2011. Chemopreventive effect of cactus *Opuntia ficus indica* on oxidative stress and genotoxicity of aflatoxin B1. Nutr. Metab. (Lond.) 8, 73.
Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. Food Sci. Technol. 28, 25–30.
Cai, Y., Sun, M., Corke, H., 2003. Antioxidant activity of betalains from plants of the amaranthaceae. J. Agric. Food Chem. 51, 2288–2294.
Cai, Y.Z., Sun, M., Corke, H., 2005. Characterization and application of betalain pigments from plants of the Amaranthaceae. Trends Food Sci. Technol. 16, 370–376.
Doostdar, H., Burke, M.D., Mayer, R.T., 2000. Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology 144, 31–38.
Dürüst, N., Sümengen, D., Dürüst, Y., 1997. Ascorbic acid and element contents of foods of Trabzon (Turkey). J. Agric. Food Chem. 45, 2085–2087.
Fernández-López, J.A., Almela, L., Obón, J.M., Castellar, R., 2010. Determination of antioxidant constituents in cactus pear fruits. Plant Foods Hum. Nutr. 65, 253–259.
Flannery, R.V., 1985. Los orígenes de la agricultura en México: las teorías y las evidencias. In: Rojas, T., Sanders, W. (Eds.), Historia de la Agricutura Epoca Prehispánica Siglo XVI. INAH, México, pp. 7–266.
Gandía-Herrero, F., Escribano, J., García-Carmona, F., 2009. The role of Phenolic hydroxy groups in the free radical scavenging activity of betalains. J. Nat. Prod. 72, 1142–1146.
Kamwanja, L.A., Chase, C.C., Gutierrez, J.A., Guerriero, V., Olson, T.A., Hammond, A.C., Hansen, P.J., 1994. Responses of bovine lymphocytes to heat shock as modified by breed and antioxidant status. J. Anim. Sci. 72, 438–444.
Kanner, J., Harel, S., Granit, R., 2001. Betalains–a new class of dietary antioxidants. J. Agric. Food Chem. 49, 5178–5185.
Katschinski, D.M., Boos, K., Schindler, S.G., Fandrey, J., 2000. Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. J. Biol. Chem. 275, 21094–21098.
Kaur, M., Kaur, A., Sharma, R., 2012. Pharmacological actions of Opuntia ficus indica: a review. J. Appl. Pharmacol. Sci. 2, 15–18.
Kikusato, M., Toyomizu, M., 2013. Crucial role of membrane potential in heat stress-induced overproduction of reactive oxygen species in avian skeletal muscle mitochondria. PLoS One 8, e64412.
Li, J.J., Oberley, L.W., 1997. Overexpression of manganese-containing superoxide dismutase confers resistance to the cytotoxicity of tumor necrosis factor alpha and/or hyperthermia. Cancer Res. 57, 1991–1998.
Liochev, S.I., Fridovich, I., 1999. Superoxide and iron: partners in crime. IUBMB Life 48, 157–161.
Liu, H., Li, K., Mingbin, L., Zhao, J., Xiong, B., 2016. Effects of chestnut tannins on the meat quality, welfare, and antioxidant status of heat-stressed lambs. Meat Sci. 116, 236–242.
Mahakunakorn, P., Tohda, M., Murakami, Y., Matsumoto, K., Watanabe, H., 2004. Antioxidant and free radical-scavenging activity of Choto-san and its related constituents. Biol. Pharm. Bull. 27, 38–46.
Materska, M., 2008. Quercetin and its derivates: chemical structure and bioactivity- a review. Pol. J. Food Nutr. Sci. 58, 407–413.
Merkl, R., Hradkova, I., Filip, V., Smidrkal, J., 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech J. Food Sci. 28, 275–279.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. lmmunol. Methods 65, 55–63.
Niwano, Y., 1991. Studies of the Immune Status Demonstrated by Lymphocyte Function in Domestic Animals Exposed to Environmental Stressors (Thesis). Thesis Kyoto University.
Núñez-Gastelum, J.A., González-Fernández, R., HernándezHerrera, A., Campas-Baypoli, O.N., Rodríguez-Ramírez, R., Lobo-Galo, N., Valero-Galván, J., 2018. Morphological characteristics, chemical composition and antioxidant activity of seeds by four wild Opuntia species from North of Mexico. J. Prof. Assoc. Cactus Dev. 20, 23–33.
Pontiki, E., Hadjipavlou-Litina, D., Litinas, K., Geromichalos, G., 2014. Novel cinnamic acid derivatives as antioxidant and anticancer agents: design, synthesis and modeling studies. Molecules 19, 9655–9674.
Prakash, M.J., Manikandan, S., 2012. Response surface modeling and optimization of process parameters for aqueous extraction of pigments from prickly pear (*Opuntia ficus-indica)* fruit. Dyes Pigments 95, 465–472.
Renugadevi, J., Prabu, S.M., 2009. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. Toxicology 256, 128–134.
Romero, R.D., Montero Pardo, A., Montaldo, H.H., Rodríguez, A.D., Cerón, J.H., 2013. Differences in body temperature, cell viability, and HSP-70 concentrations between Pelibuey and Suffolk sheep under heat stress. Trop. Anim. Health Prod. 45, 1691–1696.
de Sahagún, F.B., 1997. Historia General de las Cosas de la Nueva España. Porrúa, México.
Shahidi, F., Wanasundara, P.K.J., 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. 32, 67–103.
Shetty, A.A., Rana, M.K., Preetham, S.P., 2012. Cactus: a medicinal food. J. Food Sci. Technol. 49, 530–536.
Stintzing, F.C., Herback, K.M., Mosshammer, M.R., Carle, R., Yi, W., Sellappan, S., Akoh, C.C., Bunch, R., Felker, P., 2005. Colour, betalain pattern, and antioxidant 23 properties of cactus pear (Opuntia spp.) clones. J. Agric. Food Chem. 53, 442–451.
Tesoriere, L., Butera, D., Pintaudi, A.M., Allegra, M., Livrea, M.A., 2004. Supplementation with cactus pear (*Opuntia ficus-indica*) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C. Am. J. Clin. Nutr. 80, 391–395.
Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H., 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal. 19, 669–675.
Turco-Liveri, M.L., Sciascia, L., Allegra, M., Tesoriere, L., Livrea, M.A., 2009. Partition of indicaxanthin in membrane biomimetic systems. A kinetic and modeling approach. J. Agric. Food Chem. 57, 10959–10963.
Zhang, K., Yang, E.B., Tang, W.Y., Wang, K.P., Mack, P., 1997. Inhibition of glutathione reductase by plant polyphenols. Biochem. Pharmacol. 54, 1047–1053.
Zhao, Q.L., Fujiwara, Y., Kondo, T., 2006. Mechanism of cell death induction by nitroxide and hyperthermia. Free Radic. Biol. Med. 40, 1131–1143.
Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559.
Zhou, M., Diwu, Z., Panchuk-Voloshina, N., Haugland, R.P., 1997. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Ann. Biochem. 253, 162–168.
Zimmerman, H.G., Moran, V.C., 1991. Biological control of prickly pear, *Opuntia ficus-indica* (Cactaceae) in South Africa. Agric. Ecosyst. Environ. 37, 29–35.