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Bioactivity, proximate, mineral and volatile profiles along the flowering stages of *Opuntia microdasys* (Lehm.): defining potential applications

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Opuntia spp. flowers have been traditionally used for medical purposes, mostly because of their diversity in bioactive molecules with health promoting properties. The proximate, mineral and volatile compound profiles, together with the cytotoxic and antimicrobial properties were characterized in *O. microdasys* flowers at different maturity stages, revealing several statistically significant differences. *O. microdasys* stood out mainly for its high contents of dietary fiber, potassium and camphor, and its high activities against HCT15 cells, *Staphylococcus aureus*, *Aspergillus versicolor* and *Penicillium funiculosum*. The vegetative stage showed the highest cytotoxic and antifungal activities, whilst the full flowering stage was particularly active against bacterial species. The complete dataset has been classified by principal component analysis, achieving clearly identifiable groups for each flowering stage, elucidating also the most distinctive features, and comprehensively profiling each of the assayed stages. The results might be useful to define the best flowering stage considering practical application purposes.

Introduction

Among angiosperms, the Cactaceae is one of the most distinctive and successful families of plants of the New World, comprising more than 1600 species.[1] Plants in the genus *Opuntia* are members of the Cactaceae family, being widely distributed in semi-arid countries throughout the world, especially in the Mediterranean area and Central America.[2,3] *Opuntia* spp. flowers have been traditionally used for medical purposes for a long time. The dried flowers of prickly pear are usually sold in the popular Tunisian markets, being commonly used as infusions in traditional Tunisian and in Sicilian medicine for their diuretic activity, capacity to help in renal calculus expulsion and to cure ulcer.[4,5] In fact, different parts of *Opuntia* sp. are being increasingly used in nutritional and pharmacological applications (including at the industrial level). Nevertheless, the number of reports characterizing the chemical profiles of their flowers is still scarce, especially throughout their ripening, which motivated our present investigation.

The methanolic extracts from flowers of *Opuntia microdasys* (Lehm.) have been recently reported as containing high quantities of polyphenols (especially flavonoids) and a strong antioxidant activity.[6] The interest in plant materials containing phenolic compounds is increasing due to their high antioxidant potency, which may offer protection against different diseases, such as cancer through the inhibition of oxidative damage (known for being a potential cause of mutation).[7] Furthermore, there is a rising awareness regarding the use of volatiles in both the food and the pharmaceutical industries, justifying a systematic examination of plant extracts for these compounds.[8]

Despite only a few reports describe the volatile composition of *Opuntia* spp. flowers, it is possible to point out tetradecanoic acid, hexadecanoic acid, octadecadienoic acid and camphor as the main volatile compounds.[9–11] *Opuntia* flowers have also been reported to contain high levels of minerals (*e.g.*, K, Ca, Mg) and fiber.[12] Likewise, there are some published studies describing their antibacterial activity.[11,12]

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However, the effect of the phenological stage in which the components are evaluated was not studied for this species. This is an important research topic, since the amounts and nature of compounds may vary along the flowering stage, suggesting changes in the secondary metabolism of flowers.

Overall, this study seeks to contribute to the knowledge of the nutritional value and biological properties of *O. microdasys* flowers, characterizing their potential use as a functional food. Furthermore, the flowers' volatile composition at different flowering stages has never been reported before in this species.

Experimental

Samples

Opuntia microdasys (Lehm.) flowers were collected in 2013 from the cliff of Monastir (Tunisia) at three phenological stages: (F1) the vegetative stage, with green closed petal flowers (harvested in the beginning of June); (F2) the full flowering stage: stamens are separated around the style, the flowers are fully opened and the nectar production starts (harvested after the first fortnight of June); (F3) the post flowering stage: the flowers are dried and close to senescence (obtained in the last week of June). Samples for analysis (50 g for each flowering stage) were dried in the shade, ground using a Warring blender (Phillips, France), reduced to a fine dried powder (20 mesh) and mixed to obtain a homogeneous sample.

Standards and reagents

Micro (Fe, Cu, Mn and Zn) and macroelement (Ca, Mg, Na and K) standards (>99% purity), as well as $LaCl_2$ and CsCl (>99% purity) were purchased from Merck (Darmstadt, Germany). Mueller–Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Acetic acid, ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA), Tris, streptomycin (Sigma-Aldrich S6501) and ampicillin (Sigma-Aldrich A9393) were purchased from Sigma (St Louis, MO, USA). Bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia) were used as the reference fungicides.

Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediamine tetraacetic acid), nonessential amino acid solution (2 mM), penicillin/streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Dimethylsulfoxide (DMSO), (Merck KGaA, Darmstadt, Germany) was used as a solvent. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of analytical grade and purchased from common sources.

Proximate analysis and fiber composition

The samples were analyzed for chemical composition (moisture, proteins, fat and carbohydrates) using the AOAC procedures.[13] The crude protein content (AOAC 928.08) of the samples was estimated by the macro-Kjeldahl method (N × 6.25); the crude fat (AOAC 991.36) was determined by extracting a known weight of the powdered sample with petroleum ether in a Soxhlet apparatus; the ash content was determined by incineration at 550 ± 15 °C. Total carbohydrates were calculated by subtracting the amounts of protein, ash and fat, considering 100 g of dried sample.

Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were determined according to the AOAC enzymatic–gravimetric method (993.19 and 991.42).[14] Freeze-dried samples were treated with alpha-amylase (heat-stable), protease and amyloglucosidase. The soluble and insoluble fractions were separated by vacuum filtration. Waste from the digests was dried at 100 °C. Total dietary fiber (TDF) was the sum of SDF and IDF; both were expressed as g per 100 g of dry weight.

The energy value was calculated according to the following equation: Energy (kcal per 100 g dw) = 4 × (g protein) + 4 × (g carbohydrate − g TDF) + 2 × (TDF) + 9 × (g fat).

Ash content and mineral composition

The method 930.05 of AOAC was used. A sample of 500 mg was incinerated under high pressure in a microwave oven (Muffle Furnace mLs1200, Thermo Scientific, Madrid, Spain) for 24 h at 550 °C, and ashes were gravimetrically quantified. The residue of incineration was extracted with HCl (50%, v/v) and HNO_3 (50%, v/v) and made up to an appropriate volume with distilled water, where Fe, Cu, Mn and Zn were directly measured. An additional 1/10 (v/v) dilution of the sample extracts and standards was performed to avoid interferences between different elements in the atomic absorption spectroscopy: for Ca and Mg analysis in 1.16% La_2O_3/HCl (leading to $LaCl_2$); and for Na and K analysis in 0.2% CsCl.[15] All measurements were performed using atomic absorption spectroscopy (AAS) by using Analyst 200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with 99.9% purity analytical standard solutions for AAS made with $Fe(NO_3)_3$, $Cu(NO_3)_2$, $Mn(NO_3)_2$, $Zn(NO_3)_2$, NaCl, KCl, $CaCO_3$ and the Mg band, supplied by Merck (Darmstadt, Germany) and Panreac Química (Barcelona, Spain).

Volatile compound analyses

Supelco (Bellefonte, PA) SPME devices coated with poly-dimethylsiloxane (PDMS, 100 μm) were used to sample the headspace of a dry flower inserted into a 5 mL vial and allowed to equilibrate for 30 min. SPME sampling was performed using the same new fiber, preconditioned according to the manufacturer's instructions, for all the analyses. Sampling was accomplished in an air-conditioned room (22 ± 1 °C) to guarantee a stable temperature. After the equilibration time, the fiber was exposed to the headspace for 50 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of the GC-MS system. All the SPME sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each first SPME extraction and randomly repeated during each series. Quantitative comparisons of rela-

tive peak areas were performed between the same chemicals in the different samples.

GC-Electron Impact Mass Spectrometry (EIMS) analyses were performed with a Varian (Palo Alto, CA) CP3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, Santa Clara, CA, USA) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperatures were 220 and 240 °C, respectively; the oven temperature was programmed from 60 to 240 °C at 3 °C min^{-1}; the carrier gas was helium at 1 mL min^{-1}; splitless injection. The identification of the constituents was based on a comparison of the retention times with those of authentic standards, which comprise all the compounds indicated in Table 3, except iso-pentyl acetate, valerolactone, 2-pentyl furan, (*E*,*Z*)-3,5-octadien-2-one, (*E*,*E*)-3,5-octadien-2-one, 1-nonen-3-ol, β-selinene and dihydroactinidiolide, which were identified according to their MS spectra and linear retention indices (LRI), and on computer matching against commercial and home-made library mass spectra and data, specifically NIST 2000 and ADAMS 2007.[16]

Cytotoxicity assays

General. Each sample (~1 g of freeze-dried powder) was extracted by stirring with 40 mL of methanol at 25 °C for 1 h and filtered through Whatman no. 4 filter paper. The residue was then extracted with an additional 40 mL portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland), re-dissolved in water at a concentration of 8 mg mL^{-1}, and stored at 4 °C until determination of GI_{50} values (concentration that inhibited 50% of the net cell growth; expressed in μg mL^{-1}). Ellipticine was used as a positive control.

Evaluation of cytotoxicity in human tumor cell lines. Four human tumor cell lines were used: HCT15 (colon carcinoma), HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7 and HCT15) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U per mL penicillin and 100 mg per mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells per well for MCF-7 and HCT15 or 1.0×10^4 cells per well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilized with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA).[17]

Evaluation of cytotoxicity in a porcine liver primary cell culture. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U per mL penicillin, 100 μg per mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM non-essential amino acids and 100 U per mL penicillin, 100 mg per mL streptomycin and incubated at 37 °C under a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U per mL penicillin and 100 μg per mL streptomycin.[17]

Evaluation of the antimicrobial activity

General. The methanolic extracts were re-dissolved in a 5% solution of DMSO in distilled water at 100 mg mL^{-1}. Successive dilutions were made from the stock solution and subjected to antibacterial and antifungal assays. Bacterial and fungal organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Sinisa Stanković", University of Belgrade, Serbia. DMSO (5%) was used as a negative control.

Antibacterial activity. The following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC10240), and *Listeria monocytogenes* (NCTC7973) and Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), and *Enterobacter cloacae* (ATCC 35030) were used.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method. Briefly, a fresh overnight culture of bacteria was adjusted using a spectrophotometer to a concentration of 1×10^5 CFU mL^{-1}. The requested CFU mL^{-1} corresponded to a bacterial suspension determined using a spectrophotometer at 625 nm (OD_{625}). Dilutions of inocula were cultured on a solid medium to verify the absence of contamination and check the validity of the inoculum. Different solvent dilutions of the methanolic extract/fractions were placed in the wells containing 100 μL of tryptic soy broth (TSB) and afterwards 10 μL of inoculum was added. The microplates were incubated for 24 h at 37 °C.

The minimum inhibitory concentration (MIC) of each extract was detected following the addition of 40 μL of iodonitrotetrazolium chloride (INT) (0.2 mg mL^{-1}) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bac-

teria in comparison with the positive control was identified as the MIC. The minimum bactericidal concentration (MBC) was determined by serial subculture of 10 μL into microplates containing 100 μL of TSB. The lowest concentration not showing growth after this subculturing was read as the MBC. Standard drugs, namely, streptomycin and ampicillin, were used as positive controls. DMSO (5%) was used as a negative control.

Antifungal activity. For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (ATCC1022), *Aspergillus ochraceus* (ATCC12066), *Aspergillus versicolor* (ATCC11730), *Aspergillus niger* (ATCC6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC9112), *Penicillium verrucosum* var. *cyclopium* (food isolate), and *Trichoderma viride* (IAM 5061). The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and subcultured once a month.[18] The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline ($\approx 1.0 \times 10^3$ μL^{-1} per well). The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

MIC determination was performed by a serial dilution technique using 96-well microtitre plates. The extracts were dissolved in a 5% solution of DMSO and added to a broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentration (MFC) was determined by serial subculture of 2 μL in microtitre plates containing 100 μL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. Bifonazole and ketoconazole were used as positive controls.[19]

Statistical analysis. For all the experiments three samples (n = 3) were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD), except for antimicrobial assays. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

The differences between the flowering stages were analyzed using one-way analysis of variance (ANOVA). The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk and the Levene tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Principal component analysis (PCA) was applied as a pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

Results and discussion

Proximate composition and dietary fiber

The results for the proximate composition value are shown in Table 1. At the first two flowering stages, water was the major component (more than 80 g per 100 g fw), but it drastically decreases at the post-flowering stage (19.6 g per 100 g fw). On a dry weight (dw) basis, carbohydrates were the most abundant macronutrients (more than 80 g per 100 g dw) at all flowering stages, showing slightly higher values at the full flowering stage. Most (around 70%) of these carbohydrates were present as total dietary fiber (TDF), especially in insoluble forms. In

Table 1 Nutritional and dietary fiber composition (g per 100 g dw; mean ± SD, n = 9) of the three flowering stages of *Opuntia microdasys* (Lehm.)

				p-Value	
	F1	F2	F3	Homoscedasticity[a]	1-Way ANOVA[b]
Moisture (g per 100 g fw)	82.3 ± 0.2 b	86.5 ± 0.3 a	19.6 ± 0.2 c	0.363	<0.001
Fat (g per 100 g dw)	2.0 ± 0.2 b	1.6 ± 0.1 c	2.2 ± 0.1 a	<0.001	<0.001
Proteins (g per 100 g dw)	6.3 ± 0.1 a	6.0 ± 0.1 c	6.2 ± 0.1 b	0.003	<0.001
Ash (g per 100 g dw)	9.6 ± 0.1 a	9.4 ± 0.1 b	9.1 ± 0.1 c	<0.001	<0.001
Total carbohydrates (g per 100 g dw)	82.1 ± 0.3 c	83.1 ± 0.1 a	82.5 ± 0.1 b	<0.001	<0.001
IDF (g per 100 g dw)	44 ± 1 a	42 ± 1 b	44 ± 1 a	0.181	<0.001
SDF (g per 100 g dw)	20 ± 1 a	16 ± 1 b	14 ± 1 c	0.166	<0.001
TDF (g per 100 g dw)	64 ± 1 a	58 ± 1 b	58 ± 1 b	0.909	<0.001
Energy (kcal per 100 g dw)	243 ± 2 c	254 ± 2 b	259 ± 2 a	0.912	<0.001

dw: dry weight; fw: fresh weight; F1: vegetative stage, F2: full flowering stage, F3: post-flowering stage. IDF: insoluble dietary fiber; SDF: soluble dietary fiber; TDF: total dietary fiber. [a] Homoscedasticity among the flowering stages was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [b] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one flowering stage differs from the others (in this case multiple comparison tests were performed). For each stage, means within a row with different letters differ significantly ($p < 0.05$).

comparison with other species of the genus *Opuntia*, the TDF values were higher than those reported by Ammar *et al.*[20] for *O. ficus-indica* and *O. stricta*. Furthermore, it is recommended that a third of TDF of the diet (approximately 20 g in the present case) should be soluble dietary fiber, and the distribution of dietary fiber in the three flowering stages is in agreement with the nutritional recommendations.[21] In fact, these results are noticeable, even when compared to the dietary fiber contents detected in some cereal sources,[22] raising the possibility of using *O. microdasys* flowers as a potential source of dietary fiber and/or to be added as a food ingredient to other food products to improve the fiber intake of the population.

In addition, *O. microdasys* flowers showed significant ash contents (higher than 9 g per 100 g dw) and proteins (higher than 6 g per 100 g dw), both components with minor differences among the flowering stages. Fat was the least abundant component (around 2 g per 100 g dw), with slightly higher values at the post flowering stage. In general, the moisture content in F1 and F2 was similar to the one quantified in the cladodes (92 g per 100 g fw) and pulps (87 g per 100 g fw) of this species. Fat levels in all flowering stages were close to the ones detected in the pulp (2.46 g per 100 g dw), while protein contents were comparable to those measured in the cladodes (4.25 g per 100 g dw). Ash contents were lower than the ones quantified in the cladodes (16.4 g per 100 g dw) and pulps (16.4 g per 100 g dw), whilst carbohydrates were nearly the same.[23] The flowers' nutritional profiles resulted in energetic values close to 250 kcal per 100 g dw, with the highest values being detected at the full flowering stage. These values are similar to those reported for *O. ficus-indica* and *O. stricta* flowers.[20]

Mineral composition

The content of mineral elements is one of the most important aspects influencing the use of edible flowers in human nutrition.[24] The mineral profile was composed of 4 major elements (K, Ca, Mg and Na) and 4 trace elements (Fe, Mn, Zn and Cu), as shown in Table 2. Indeed, the mineral composition for *O. microdasys* during flowering stages shows that K was the predominant component followed by Ca > Mg > Na > Fe > Mn > Zn > Cu. Except for the iron content, some significant differences were found among the assayed flowering stages (probably due to the modifications related to the ripening of flowers), but the quantities of each mineral element maintain their relative proportions throughout these stages.

The low sodium content (1.2–1.5 mg per 100 g dw) is certainly noteworthy, considering the deleterious effect of this mineral element for cardiovascular diseases.[25] On the other hand, potassium (the most abundant element in *O. microdasys*) is one of the most important intracellular ions and is essential for the homeostatic balance of body fluids, besides controlling muscle contraction, particularly of the myocardium.[26] The consumption of food products rich in potassium is also recommended for the prevention of oncogenic diseases.[27] Moreover, calcium, the second major element, is well-known for being one of the essential minerals needed for building the bones and teeth in animals and humans.

Despite the lower concentrations of microelements, when compared to those reported in other *Opuntia* species,[20] the detected elements are also relevant: Cu (0.008–0.016 mg per 100 g dw), Mn (0.15–0.21 mg per 100 g dw) and Fe (0.19–0.21 mg per 100 g dw) play an important role in redox processes, besides acting as cofactors of different enzymes;[28] Zn (0.05–0.10 mg per 100 g dw) is, for instance, recognized as an essential element against prostate pain.[29] Accordingly, *O. microdasys* flowers showed high potential as alternative sources of these mineral elements (despite the low bioavailability that characterizes some of the identified minerals) and might have applications as food supplements in meals and drinks.

Aroma volatiles of flower at three stages of maturity

Table 3 lists the volatile compounds identified in each of the flowering stages of *O. microdasys*. A total of 53 volatile compounds were detected (29 in F1, 30, in F2 and 28 in F3), but

Table 2 Micro and macroelements (mean ± SD, n = 9) of the three flowering stages of *Opuntia microdasys* (Lehm.)

				p-Value	
	F1	F2	F3	Homoscedasticity[a]	1-Way ANOVA[b]
Macroelements (mg per 100 g dw)					
Ca	21 ± 3 a	17 ± 2 b	23 ± 2 a	0.595	<0.001
Mg	13 ± 1 a	12 ± 1 b	10 ± 1 c	0.157	<0.001
Na	1.3 ± 0.1 ab	1.2 ± 0.1 b	1.5 ± 0.3 a	<0.001	0.025
K	2909 ± 68 b	3711 ± 229 a	2564 ± 521 b	<0.001	<0.001
Microelements (mg per 100 g dw)					
Cu	0.016 ± 0.001 a	0.013 ± 0.002 b	0.008 ± 0.001 c	0.141	<0.001
Fe	0.21 ± 0.01	0.19 ± 0.03	0.20 ± 0.02	0.001	0.385
Mn	0.21 ± 0.02 a	0.15 ± 0.01 b	0.15 ± 0.01 b	0.002	<0.001
Zn	0.06 ± 0.01 b	0.10 ± 0.01 a	0.05 ± 0.01 c	0.002	<0.001

dw: dry weight; fw: fresh weight; F1: vegetative stage, F2: full flowering stage, F3: post-flowering stage. [a] Homoscedasticity among the flowering stages was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [b] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one flowering stage differs from the others (in this case multiple comparison tests were performed). For each stage, means within a row with different letters differ significantly ($p < 0.05$).

Table 3 Comparative percentages of compounds in the volatile oils of the three flowering stages of *Opuntia microdasys* (Lehm.)

					p-Value	
Compound	LRI	F1	F2	F3	Homoscedasticity[a]	1-Way ANOVA[b]
Hexanal	803	0.27 ± 0.05	nd	nd	0.003	—
Furfural	833	nd	nd	0.42 ± 0.05	<0.001	—
Furfuryl alcohol	856	nd	0.18 ± 0.05	nd	<0.001	—
1-Hexanol	870	2.2 ± 0.1 b	0.8 ± 0.1 c	3.2 ± 0.2 a	0.029	<0.001
iso-Pentyl acetate	878	nd	0.62 ± 0.05	nd	<0.001	—
Ethyl pentanoate	900	0.60 ± 0.05 a	0.22 ± 0.04 c	0.40 ± 0.05 b	0.166	<0.001
Methyl hexanoate	929	5.6 ± 0.4 a	2.8 ± 0.1 c	3.1 ± 0.1 b	0.001	<0.001
Valerolactone	952	0.18 ± 0.03 b	0.20 ± 0.05 b	0.68 ± 0.05 a	0.371	<0.001
Benzaldehyde	963	nd	nd	0.48 ± 0.05	<0.001	—
1-Heptanol	971	0.29 ± 0.04 b	0.15 ± 0.03 c	0.39 ± 0.05 a	0.671	<0.001
Hexanoic acid	979	0.40 ± 0.05 b	0.19 ± 0.05 c	0.82 ± 0.05 a	0.980	<0.001
6-Methyl-5-hepten-2-one	897	nd	nd	0.54 ± 0.04	0.001	—
2-Pentyl furan	993	nd	0.61 ± 0.05	nd	<0.001	—
Ethyl hexanoate	998	4.8 ± 0.3 b	1.9 ± 0.1 c	6.1 ± 0.3 a	0.003	<0.001
1-Hexyl acetate	1010	nd	0.40 ± 0.04	0.61 ± 0.05	0.001	<0.001
Methyl heptanoate	1028	0.7 ± 0.1	nd	0.5 ± 0.1	<0.001	<0.001
p-Cymene	1028	nd	0.40 ± 0.04	nd	<0.001	—
Limonene	1032	2.2 ± 0.2 b	7.0 ± 0.3 a	2.3 ± 0.2 b	0.724	<0.001
iso-Octanol	1058	0.52 ± 0.05	nd	nd	0.002	—
γ-Terpinene	1063	nd	0.9 ± 0.1	nd	<0.001	—
(*E*)-2-Octenal	1063	nd	nd	0.50 ± 0.05	<0.001	—
(*E*,*Z*)-3,5-Octadien-2-one	1070	nd	nd	1.6 ± 0.1	<0.001	—
1-Octanol	1072	0.7 ± 0.1	nd	nd	<0.001	—
cis-Linalool oxide (Furanoid)	1076	nd	0.40 ± 0.05	nd	<0.001	—
(*E*,*E*)-3,5-Octadien-2-one	1079	nd	nd	0.9 ± 0.1	<0.001	—
1-Nonen-3-ol	1080	0.21 ± 0.03	nd	nd	<0.001	—
o-Guaiacol	1088	0.36 ± 0.05	nd	0.40 ± 0.05	0.001	<0.001
Ethyl heptanoate	1097	4.1 ± 02	nd	nd	<0.001	—
Linalool	1101	nd	7.0 ± 0.4	2.7 ± 0.2	<0.001	<0.001
Nonanal	1104	nd	nd	5.6 ± 0.3	<0.001	—
Phenylethyl alcohol	1111	1.0 ± 0.1	nd	nd	0.001	—
Methyl octanoate	1128	2.1 ± 0.2	nd	nd	<0.001	—
Camphor	1146	40 ± 1 c	48 ± 1 a	46 ± 1 b	0.605	<0.001
(*E*)-2-Nonenal	1164	nd	nd	0.62 ± 0.05	<0.001	—
1-Nonanol	1172	0.9 ± 0.1 b	0.7 ± 0.1 c	1.1 ± 0.1 a	0.067	<0.001
4-Terpineol	1179	0.30 ± 0.05	nd	nd	<0.001	—
2-Decanone	1194	nd	nd	0.9 ± 0.1	<0.001	—
cis-Dihydrocarvone	1194	0.70 ± 0.05	nd	nd	<0.001	—
Ethyl octanoate	1197	5.5 ± 0.4	nd	nd	<0.001	—
n-Dodecane	1200	nd	2.1 ± 0.1	2.3 ± 0.1	0.002	<0.001
Methyl nonanoate	1227	4.0 ± 0.3 a	0.7 ± 0.1 c	2.5 ± 0.1 b	0.014	<0.001
Cumin aldehyde	1241	nd	0.56 ± 0.05	nd	<0.001	—
Carvone	1244	3.5 ± 0.3 b	15.8 ± 0.3 a	3.1 ± 0.2 c	0.522	<0.001
2-Undecanone	1293	nd	0.5 ± 0.1	1.6 ± 0.1	<0.001	<0.001
Ethyl nonanoate	1298	9.1 ± 0.4 a	2.1 ± 0.1 c	5.1 ± 0.4 b	0.054	<0.001
n-Tridecane	1300	nd	1.2 ± 0.1	nd	<0.001	—
1-Nonyl acetate	1313	nd	0.21 ± 0.04	nd	<0.001	—
α-Copaene	1377	0.37 ± 0.05	0.10 ± 0.01	nd	<0.001	<0.001
Ethyl decanoate	1397	0.28 ± 0.05	0.20 ± 0.03	nd	<0.001	<0.001
β-Caryophyllene	1419	nd	0.18 ± 0.03	nd	<0.001	—
Aromadendrene	1440	0.41 ± 0.04	nd	nd	<0.001	—
β-Selinene	1486	nd	0.10 ± 0.01	nd	<0.001	—
Dihydroactiniodiolide	1530	0.47 ± 0.05	nd	nd	<0.001	—
Percentage of identified compounds	94.8%	96.5%	92.3%	—		

F1: vegetative stage, F2: full flowering stage, F3: post-flowering stage; LRI: linear retention indices. [a] Homoscedasticity among the flowering stages was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [b] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one flowering stage differs from the others (in this case multiple comparison tests were performed). For each stage, means within a row with different letters differ significantly ($p < 0.05$).

only 12 were common to all the three stages. The identified compounds accounted for 92.3–96.5% of the total aroma. In general, each volatile compound is characterized by an odor threshold (varying from a few ppb to several ppm), so even if the qualitative composition of different fruits is almost the same, the aroma may vary when the relative proportions are different[30] or even when minor constituents with a low odor threshold are present.[31]

In general, oxygenated monoterpenes were found to be the most important groups of volatiles during the maturation of flower, probably being the main contributors to the aroma in *O. microdasys* flowers. These compounds are widely used as fragrances and flavors in the cosmetic, perfume, drug and food industries. Another important group of volatiles was represented by ester compounds, particularly methyl and ethyl esters of hexanoic, heptanoic, octanoic and nonanoic acids. Among the alcohols, 1-hexanol, known for conferring a fresh-mowed grass scent, was the main compound, whilst nonanal (which provides a soapy-fruity flavor) was the aldehyde detected in highest amounts, despite being only detected at the post flowering stage.[32]

The major compound at all flowering stages was camphor (40% in F1, 48% in F2 and 46% in F3), followed by ethyl nonanoate (9.1%) in F1, carvone (15.8% in F2) and ethyl hexanoate (6.1%) in F3. Camphor (2-bornanone) derives naturally from the bark of the *Cinnamomum camphora* tree, but is also a major essential oil constituent in aromatic plants, such as the Greek sage (*Salvia fruticosa*), Spanish sage (*Salvia lavandulifolia*), Lavender cotton (*Santolina insularis*), and sweet wormwood (*Artemisia annua*).[33] Camphor is known for its biological properties and industrial applications, despite being limited to a threshold value of 11% in medical products.[34,35]

When compared to other *Opuntia* species, the volatile composition of *O. microdasys* is quite dissimilar, specifically due to the absence of camphor. In *O. ficus-indica*, for instance, 1-hexanol and germacrene D were the major volatile compounds.[10] These differences might be understood as an indicator of the high species-specificity of these types of compounds.

Cytotoxic activity

Results regarding the effects of the three flowering stages on four human tumor cell lines (MCF-7, HCT-15, HeLa and HepG2) are presented in Table 4. None of the tested extracts showed an inhibitory effect against the MCF7 cell line. In contrast, they were effective against all the remaining tumor cell lines. *O. microdasys* flowers were particularly active against the HCT15 cell line; among the tested flowering stages, the vegetative stage (F1) was the most effective in all the tested cell lines, as indicated by its lower GI_{50} values (97–185 μg mL^{-1}).

Despite its cytotoxic activity on human tumor cell lines, the extracts of *O. microdasys* flowers did not show any hepatotoxicity in normal cells (PLP2), since the maximum assayed concentration (400 μg mL^{-1}) had no significant inhibitory effect.

Antimicrobial activity

Results of the antibacterial activity towards pathogenic bacteria (evaluated by the microdilution method), are presented in Table 5. The three flowering stages exhibited significant levels of antibacterial activity, but the full flowering stage (F2) turned out to be the most effective antibacterial agent, either considering its bacteriostatic (MIC varying from 0.312 mg mL^{-1} to 1.25 mg mL^{-1}) or its bactericidal (MBC varying from 0.625 to 2.50 mg mL^{-1}) effects. Regarding bacterial sensitivity, *Staphylococcus aureus* was the most susceptible species, whilst *Listeria monocytogenes* and *Enterobacter cloacae* stood out as the species with the highest resistance against the *O. microdasys* flower extracts. Comparing the results with those of the standard drugs ampicillin and streptomycin, F2 exhibited higher activity than ampicillin against *Pseudomonas aeruginosa* and *Salmonella typhimurium*.

On the other hand, the extracts obtained from the vegetative stage (F1) were the most active in inhibiting the fungal growth (MIC varying from 0.95 mg mL^{-1} to 5 mg mL^{-1}) and exerting fungicidal activity (MFC varying from 1.25 mg mL^{-1} to 10 mg mL^{-1}) (Table 5), somehow reflecting the compositional differences highlighted in Tables 2 and 3. *Aspergillus versicolor* and *Penicillium funiculosum* were the most susceptible fungal species, whereas *Penicillium ochrochloron* showed the highest resistance against the *O. microdasys* extracts. The tested standards (bifonazole and ketoconazole) had lower MIC and MFC for all bacterial species.

The detected antimicrobial activity may be provided by the phenolic compounds present in the methanolic extracts. In fact, phenolic compounds may interact with the microorganism's cell membrane or cell wall through hydrogen bonds involving their hydroxyl groups, thereby causing changes in membrane permeability and cell destruction.[36,37] In fact, active natural compounds have been compared with representative antibacterial active ingredients commonly employed in medicine (*e.g.*, chlorhexidine and Triclosan), to determine their effectiveness.[38] Considering some pre-set criteria from the relevant literature, agents with MIC values of isolated phytochemicals below 20 mg mL^{-1} may be considered useful for therapeutic applications,[39] which classifies *O. microdasys* flowers as potential sources of compounds for these uses.

Table 4 Cytotoxic activity GI_{50} (μg mL^{-1}) of methanolic extracts obtained from the three flowering stages of *Opuntia microdasys* (Lehm.)

				p-Value	
Cell lines	F1	F2	F3	Homoscedasticity[a]	1-Way ANOVA[b]
In human tumor cell lines					
MCF7	>400	>400	>400	—	—
HCT15	97 ± 1 c	185 ± 1 a	126 ± 8 b	<0.001	<0.001
HeLa	117 ± 4 c	232 ± 1 a	129 ± 2 b	<0.001	<0.001
HepG2	238 ± 5 c	350 ± 5 a	278 ± 5 b	0.665	<0.001
In non-tumor cells					
PLP2	>400	>400	>400	—	—

F1: vegetative stage, F2: full flowering stage, F3: post-flowering stage. [a] Homoscedasticity among the flowering stages was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [b] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one flowering stage differs from the others (in this case multiple comparison tests were performed). For each stage, means within a row with different letters differ significantly ($p < 0.05$).

Table 5 Antimicrobial activity (MIC, MBC and MFC in mg mL^{-1}) of the three flowering stages of *Opuntia microdasys* (Lehm.)

Species	F1 MIC MBC	F2 MIC MBC	F3 MIC MBC	Streptomycin MIC MBC	Ampicillin MIC MBC
Bacteria					
Staphylococcus aureus	0.450	0.450	0.312	0.04	0.25
	0.625	0.625	0.625	0.10	0.40
Bacillus cereus	0.312	0.95	0.450	0.10	0.25
	0.625	1.25	0.625	0.20	0.40
Micrococcus flavus	1.25	1.25	1.25	0.20	0.25
	2.50	2.50	2.50	0.30	0.40
Listeria monocytogenes	3.75	1.25	2.50	0.20	0.40
	5.00	2.50	5.00	0.30	0.50
Pseudomonas aeruginosa	0.95	0.450	0.95	0.20	0.75
	1.25	0.625	1.25	0.30	1.20
Escherichia coli	0.95	0.95	0.95	0.20	0.40
	1.25	1.25	1.25	0.30	0.50
Enterobacter cloacae	3.75	1.25	2.50	0.20	0.25
	5.00	2.50	5.00	0.30	0.50
Salmonella typhimurium	0.625	0.312	0.625	0.25	0.40
	2.50	0.625	2.50	0.50	0.75
Fungi					
Aspergillus fumigatus	2.50	5.00	1.25	0.15	0.20
	5.00	10.0	5.00	0.20	0.50
Aspergillus versicolor	0.95	1.25	0.95	0.10	0.20
	1.25	2.50	2.50	0.20	0.50
Aspergillus ochraceus	1.85	10.0	5.00	0.15	1.50
	2.50	12.5	10.0	0.20	2.00
Aspergillus niger	1.25	10.0	2.50	0.15	0.20
	5.00	12.5	10.0	0.20	0.50
Trichoderma viride	2.50	5.00	2.50	0.15	1.00
	5.00	10.0	5.00	0.20	1.00
Penicillium funiculosum	0.95	1.25	1.25	0.20	0.20
	1.25	2.50	2.50	0.25	0.50
Penicillium ochrochloron	5.00	5.00	5.00	0.20	2.50
	10.0	10.0	10.0	0.25	3.50
Penicillium verrucosum	5.00	5.00	3.75	0.10	0.20
	10.0	10.0	5.00	0.20	0.30

F1: vegetative stage, F2: full flowering stage, F3: post-flowering stage; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration.

Principal component analysis (PCA)

In the former section, the differences induced by the flowering stage were compared considering each parameter individually. As explained, several significant differences were found, but the parameter levels which best characterize each flowering stage could not be determined. Accordingly, in the present section, the results were evaluated considering data for all parameters (except for antimicrobial activity indicators) simultaneously, by applying principal component analysis (PCA).

The plot of object scores and component loadings (Fig. 1) indicated that the first two dimensions (first: Cronbach's α, 0.987; eigenvalue, 37.166; second: Cronbach's α, 0.978; eigenvalue, 28.570) account for most of the variance (90.0%) of all quantified variables (50.9% and 39.1%, respectively). Groups corresponding to each flowering stage (F1, F2 and F3) were completely individualized (objects corresponding to each stage were highlighted to facilitate the visualization), and the biplot also allows concluding which of the assayed parameters characterize better the assayed flowering stages. The vegetative stage (F1) is clearly typified by low levels of *n*-dodecane, camphor and 1-hexyl acetate, while presenting high quantities of fiber, manganese, methyl hexanoate, 1-octanol, hexanal and isooctanol. Likewise, the full flowering stage (F2) is mainly characterized by low levels of fat, calcium, 1-hexanol, 1-heptanol, 1-nonanol, hexanoic acid and ethyl hexanoate, whereas it presents high contents of potassium, zinc, furfuryl alcohol, isopentyl acetate, 2-pentylfurane and *p*-cymene. Finally, the post-flowering stage (F3) stood out for having low quantities of water, ash, SDF, copper, magnesium, ethyl decanoate and α-copaene, simultaneously presenting raised levels of hexanoic acid, (*E*)-2-octenal, furfural, benzaldehyde, (*E*,*Z*)-3,5-octadien-2-one, (*E*,*E*)-3,5-octadien-2-one, valerolactone and 6-methyl-5-hepten-2-one.

Conclusion

The *O. microdasys* flowers in different ripening stages showed statistically significant differences in the proximate, mineral and volatile compound profiles, observed also in cytotoxic and antimicrobial properties. Even so, some overall con-

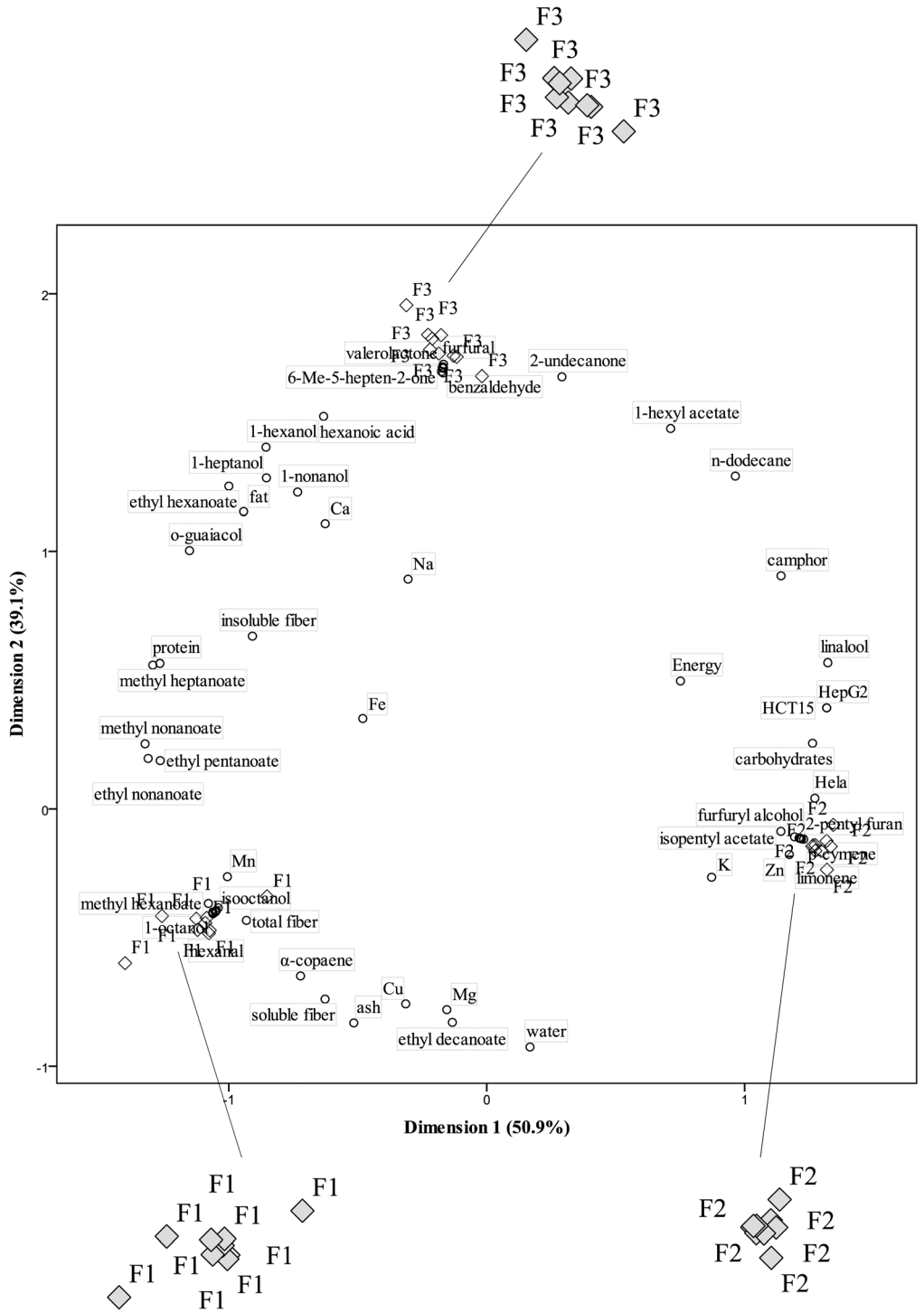

Fig. 1 Plots of object scores (flowering stages) and component loadings (selected parameters). Object scores were highlighted for a better visualization.

clusions might be drawn: *O. microdasys* presented high contents of dietary fiber, potassium and camphor; regarding the bioactivity, the performances against HCT15 cells, *Staphylococcus aureus*, *Aspergillus versicolor* and *Penicillium funiculosum* deserve special attention. The vegetative stage (F2) showed the highest levels of cytotoxicity and antifungal activity, whilst the full flowering stage (F3) gave the best results for antibacterial activity. By analysing all the results simultaneously through principal component analysis, it was possible to characterize the chemical and bioactive profiles, which better characterize each of the flowering stages, while identifying their most distinctive features. These results

could be useful to select the optimum flowering stage for a determined application.

Competing interests

The authors declare no competing financial interests.

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