Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2017, **8**, 3111

Detailed phytochemical characterization and bioactive properties of *Myrtus nivelii* Batt & Trab

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Received 19th May 2017,
Accepted 10th July 2017

DOI: 10.1039/c7fo00744b

rsc.li/food-function

The endemic Algerian myrtle, *Myrtus nivelii* Batt & Trab., was evaluated for its bioactive properties, such as antioxidant, anti-inflammatory, cytotoxic and antibacterial activities, and correlated with the individual phenolic compounds identified in its crude aqueous extract and subsequent organic fractions (ethyl acetate and butanol). Flavonols, such as myricetin, kaempferol and quercetin glycoside derivatives, were the major phenolic compounds found, along with the presence of ellagitannins. The ethyl acetate fraction contained the highest amount of phenolic compounds, followed by the butanol fraction and, finally, the crude aqueous extract. In general, all samples exhibited an excellent bioactivity, namely the ethyl acetate fraction that presented strong antioxidant activity, when compared to the standard trolox, strong cytotoxicity and antibacterial activity, especially against MRSA and MSSA. The present study revealed that the ethyl acetate fraction of *M. nivelii* could be used as a source of bioactive compounds in the food and pharmaceutical industries.

1. Introduction

Plants are able to produce a great number of secondary metabolites, among them are terpenoids, alkaloids and phenolic compounds. These last molecules, mainly flavonoids, phenolic acids, lignans, tannins and stilbenes, are very common in different plant parts, and widely exploited and used in the food, cosmetic and pharmaceutical industries. Phenolic compounds are not only present in the most commonly consumed products, such as fruits and vegetables, but they are also present in medicinal herbs.[1] Polyphenols are widely studied in the medicinal field, where they are recognized for their bioactivities, such as antioxidant, anti-inflammatory[2] and antifungal[3] activities. They also have positive effects on obesity[4] and cancer.[5] In particular, these molecules are strong inhibitors of oxidative stress, hence they contribute to reactive oxygen species (ROS) scavenging,[6] preventing oxidation effects by activating the endogenous antioxidant defense system,[7] and also protecting against the lipid oxidation phenomenon, which is responsible for the development of oxidative diseases.[6] Moreover, phenolic compounds, such as phenolic acids, flavonoids and tannins, have been isolated from different plant sources and have inhibited various pathogenic bacteria[8] and fungi.[3]

Myrtaceae is a widespread plant family in the Mediterranean area, especially in Algeria, where a great number of species from the genus of this family can be found. In this family, there are numerous endemic species that are used in traditional therapy, in order to cure or prevent a diverse number of pathologies.[2,9] These bioactivities have been correlated with a wide variety of secondary metabolites, such as hydroxybenzoic acid derivatives and flavonoids.[9]

The *Myrtus* herba commonly named Myrtle is considered the most famous genus used to treat diabetes, hypertension and respiratory tract diseases.[10] This is due to the presence of bioactive compounds with innumerous biological activities reported in the literature such as antioxidant, hypotensive, antimicrobial, anti-inflammatory, hypoglycemic, and anticancer activities.[11] The most abundant compounds present in

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these species, mostly in the leaves, are phenolic compounds, such as flavonoids (myrecitin and quercetin glycoside derivatives, and flavan-3-ol derivatives) and phenolic acids (gallic, caffeic and ellagic acids).[10]

In Algeria, there are two characteristic species of Myrtle: *Myrtle communis* L., the most known and common species in the Mediterranean area, being more abundant in the north of Algeria; and *Myrtus nivelii* Batt. & Trab., which is found in the desert, in the south of Algeria, and is much less explored. The common name of this species is Saharan myrtle (Rihan Esahraa in Algerian), an endemic plant found in the mountains (in altitude above 1500 to 2000 m), in the central Sahara, being widespread in the Hoggar and Tassili N'Ajjer mountains in Algeria, the Tibesti mountains in Chad and Lybia. This plant species is well known in southern Algeria for its therapeutic properties; its leaves are collected throughout the year and prepared as decoctions that are taken orally for the treatment of liver problems, gastrointestinal disorders, diarrhea, fever, diabetes, respiratory infections, rheumatism, allergies, and fungal infections,[12] while leaf infusions also used against blennorrhea. The antioxidant, antimicrobial and anti-inflammatory activities of aqueous and methanol extracts obtained from *M. nivelii* were previously reported,[13–15] but only correlated with total compounds,[15] without a clear identification of individual bioactive molecules.

The aim of the present work was to investigate the individual phenolic compounds of *Myrtus nivelii* Batt. & Trab. (crude aqueous extract, and acetyl acetate and butanol fractions), as well as to evaluate its biological activities (antioxidant, cytotoxic, anti-inflammatory and antibacterial).

2. Materials and methods

2.1. Plant material

The leaves of *Myrtus nivelii* Batt & Trab. (Saharan myrtle; local name: "Targui": tafeltest and "arabe": rihan Sahraa) were collected at Tamanrasset (south of Algeria) in November 2013. The taxonomic identification of the plant material was confirmed by Dr Rabéa Sahki, researcher in the National Institute for Forest Research of Tamanrasset, Algeria.

The plant material was dried under air circulation conditions (oven-dried at 40 °C for 48 h) and then ground to a fine powder (~40 mesh).

2.2. Standards and reagents

HPLC-grade acetonitrile and ethyl acetate (99.8%) were from Fisher Scientific (Lisbon, Portugal). Butanol (99%) was from Lab-Scan analytical sciences (Gliwice, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma-Aldrich (St Louis, MO, USA). Phenolic compound standards were purchased from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). The culture media Muller Hinton Broth (MHB) and Tryptic Soy Broth (TSB) were obtained from Biomerieux (Marcy l'Etoile, France), as well as the blood agar with 7% sheep blood and Mac Conkey agar plates. The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St Louis, MO, USA) and it was used as a microbial growth indicator. The water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Extraction procedure

The crude aqueous extract of *M. nivelii* was obtained under heat reflux extraction with distilled water 10% (w/v) for 30 min, three times. Subsequently, each extract was filtered and lyophilized (Gardiner, NY, USA) to dryness. The obtained lyophilized extracts were solubilized in distilled water (100 mL) and successive partitions were performed by liquid–liquid extraction by using increasing polarity solvents (chloroform, ethyl acetate and *n*-butanol). The crude aqueous extract and its ethyl acetate and butanol fractions were used in the subsequent assays. The crude aqueous extract and fractions were dissolved in water and water/methanol (80 : 20, v : v), respectively, at 5 mg mL^{-1}, in order to obtain the phenolic compounds profile; water and methanol, respectively (5 mg mL^{-1}), for antioxidant activity evaluation; in the medium (20 mg mL^{-1}) for antibacterial assays; and in water (8 mg mL^{-1}) for anti-inflammatory and cytotoxicity tests. For bioactivity evaluation assays, the stock solutions were further diluted and tested.

2.4. Phenolic compounds' characterization

The phenolic compounds profile was determined by using an LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), as previously described by Bessada *et al.*[16] For the double online detection, 280 and 370 nm were used as the preferred wavelengths for DAD and in a mass spectrometer (MS). MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed using standard compounds, when available, by comparing their retention times, UV-vis and mass spectra; and also, comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg g^{-1} of the extract.

2.5. *In vitro* antioxidant activity assays

The antioxidant activity was assessed by using four different methodologies, DPPH radical-scavenging, reducing power,

inhibition of β-carotene bleaching and TBARS inhibition assays.[17] The results were expressed as EC_{50} values (μg mL^{-1}), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as the positive control.

2.6. *In vitro* anti-inflammatory assays

The evaluation of the anti-inflammatory properties was performed according to the previously described method by Sobral *et al.*[18] For the determination of nitric oxide, a Griess Reagent System kit was used, which contains sulfanilamide, NED and nitrite solutions. The nitrite produced was determined by measuring the optical density at 515 nm, using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) and was compared to the standard calibration curve. The results were expressed in percentages of inhibition of NO production in comparison with the negative control (100%) and EC_{50} values (sample concentration providing 50% of inhibition of NO production) were also estimated. Dexamethasone was used as the positive control.

2.7. Cytotoxicity assays

Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCIH460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Sulforhodamine B assay was performed according to a procedure previously described by the authors, Barros *et al.*[17] For evaluation of the cytotoxicity in non-tumor cells, a cell culture (assigned as PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors, Abreu *et al.*;[19] ellipticine was used as the positive control and the results were expressed in GI_{50} values (concentration that inhibited 50% of the net cell growth).

2.8. Antibacterial activity

The antibacterial activity was evaluated against clinical isolates obtained from patients hospitalized in the Local Health Unit of Bragança and Hospital Centre of Trás-os-Montes and Alto-Douro-Vila Real, Northeast of Portugal. The bacterial strains studied were six Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL, *Klebsiella pneumoniae* and *K. pneumonia* ESBL, *Morganella morganii*, and *Pseudomonas aeruginosa*), and four Gram-positive bacteria (MRSA – methicillin-resistant *Staphylococcus aureus*, MSSA – methicillin-susceptible *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*). MIC determinations were performed by the microdilution method using the *p*-iodonitrotetrazolium chloride (INT) colorimetric assay according to Dias *et al.*[20] MBC concentrations were calculated by adding 10 μL of the MIC and higher concentrations to fresh culture medium to see if the bacteria were able to grow. After 24 h of incubation at 37 °C, the bactericidal concentrations were registered. The antibiotic susceptibility profile of Gram-negative and Gram-positive bacteria was previously described by Dias *et al.*[20]

2.9. Statistical analysis

Three crude extracts and three of each one of the fractions were used and all assays were performed in triplicate. The results were expressed as mean values and standard deviations (SD). The results obtained were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. In the case of phenolic composition, a Student's *t*-test was also used to determine significant differences when the number of cases for a determined condition was less than 3, with $\alpha = 0.05$. This treatment was carried out using the SPSS v. 23.0 program (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Phenolic compounds

The characterization of phenolic compounds in *M. nivelii* crude aqueous extract and acetyl acetate and butanol fractions is shown in Table 1, and an example of the profile is presented in Fig. 1. To the authors' best knowledge, this is the first report on the identification and quantification of individual phenolic compounds present in *M. nivelii*. Twenty-seven different compounds were identified, comprising hydrolysable tannins (gallotannin and ellagitannin derivatives), flavonoids (myricetin, quercetin, kaempferol and catechin derivatives) and a phenolic acid (ellagic acid). The ethyl acetate fraction revealed the presence of 25 compounds (Fig. 1), while the butanol fraction and the crude aqueous extract revealed only 19 and 17 compounds, respectively.

Peaks 1, 2, 4, 5, 7, 9, 10, 14, 16 and 18 showed UV spectra coherent with galloyl hexahydroxydiphenoyl (HHDP) derivatives (peaks 1, 2, 4, 7, 10 and 14) or gallotannins composed of trimeric and tetrameric galloyl moieties linked to glucose (peaks 5, 9, 16 and 18). According to the literature, the main characteristic in the mass spectra of these compounds is the deprotonated molecule $[M - H]^-$ and the loss of one or more gallic acid (170 mu) and/or galloyl groups (152 mu).[21] Mass characteristics of peaks 1 and 2 ($[M - H]^-$ at *m*/*z* 633; fragment ions at *m*/*z* 463 and 301) coincided with a galloyl-HHDP-glucose, whereas peaks 4, 7 and 10 ($[M - H]^-$ at *m*/*z* 785; fragment ions at *m*/*z* 633, 615, 483, 419, 301) correspond to digalloyl-HHDP-glucoside, while peak 14 ($[M - H]^-$ at *m*/*z* 937; fragment ions at *m*/*z* 767, 635, 465 and 301) was coherent with a trigalloyl-HHDP-glucose.[22] Compounds 5 and 9 (the $[M - H]^-$ ion at *m*/*z* 635) revealed a typical fragmentation pattern of a trigalloylglucoside, while compounds 16 and 18 (the $[M - H]^-$ ion at *m*/*z* 787) presented a fragmentation pattern characteristic of tetragalloylglucoside.[21]

Peak 3 ($[M - H]^-$ at *m*/*z* 495) was identified as digalloylquinic acid, releasing three MS^2 fragment ions at *m*/*z* 343 (release of the first galloyl group $[M - 152\text{-}H]^-$), *m*/*z* 191 (release of the second galloyl group $[M - 152\text{-}H]^-$) and *m*/*z* 169 (release of the quinic acid with the formation of the deprotonated gallic acid).[23] Similarly, peak 8 ($[M - H]^-$ at *m*/*z* 647) was identified as trigalloylquinic acid. Peak 12 ($[M - H]^-$ at *m*/*z* 469) was identified as a valoneic acid dilactone, taking into account the

Table 1 Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in *M. nivelii* leaves (mean ± SD)

Peak	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1} extract)			Student's *t*-test
						Crude aqueous extract	Ethyl acetate fraction	Butanol fraction	
1	4.43	267	633	463(100), 301(62)	Galloyl-HHDP-glucoside[1]	nd	nd	10.1 ± 0.4	—
2	5.09	266	633	463(100), 301(51)	Galloyl-HHDP-glucoside[1]	29.0 ± 0.5	nd	17.4 ± 0.2	<0.001
3	5.32	273	495	343(00), 191(86), 169(60)	Digalloylquinic acid[2]	nd	6.41 ± 0.07	14.3 ± 0.2	<0.001
4	5.63	274	785	633(38), 615(23), 483(93), 419(21), 301(100)	Digalloyl-HHDP-glucoside[1]	14.5 ± 0.2^c	19.28 ± 0.08b	26.7 ± 0.3^a	—
5	6.5	269	635	483(19), 465(100), 421(80), 313(52), 295(28), 169(32)	Trigalloylglucoside[2]	3.0 ± 0.2^c	7.1 ± 0.8^a	6.18 ± 0.05^b	—
6	6.85	256	953	909(5), 785(50), 765(15), 633(100), 615(3), 483(53), 419(10), 301(20)	Rugosin B[1]	5.4 ± 0.1^c	7.0 ± 0.7^b	9.4 ± 0.2^a	—
7	7.77	265	785	633(21), 615(8), 483(100), 419(12), 301(72)	Digalloyl-HHDP-glucoside[1]	8.9 ± 0.1^c	20.72 ± 0.02^a	16.9 ± 0.4^b	—
8	8.67	276	647	495(100), 477(18), 343(80), 325(43), 191(20), 173(13), 169(47)	Trigalloylquinic acid[2]	11.2 ± 0.2^c	22.2 ± 0.3^b	31.3 ± 0.2^a	—
9	9.9	276	635	483(18), 465(100), 421(6), 313(80), 295(12), 169(24)	Trigalloylglucoside[2]	nd	12.3 ± 0.1	12.2 ± 0.3	0.589
10	10.23	270	785	633(17), 615(12), 483(99), 419(18), 301(100)	Digalloyl-HHDP-glucoside[2]	7.22 ± 0.02^b	11.1 ± 0.3^a	6.6 ± 0.2^c	—
11	10.61	273	915	457(100), 331(58), 305(26), 169(98)	Gallocatechin-gallate-dimer[3]	10.9 ± 0.2^c	35.6 ± 0.4^a	13.2 ± 0.2^b	—
12	12.6	258 364	469	425(100), 301(75)	Valoneic acid dilactone[1]	nd	9.5 ± 0.2	8.47 ± 0.01	0.005
13	14.04	356	631	479(100), 317(63)	Myricetin-hexosyl-gallate[4]	13.4 ± 0.2^c	37.0 ± 0.5^a	17.8 ± 0.1^b	—
14	14.78	276	937	767(100), 635(17), 465(56), 301(37)	Trigalloyl-HHDP-glucoside[1]	4.63 ± 0.05	17.1 ± 0.4	nd	<0.001
15	15.15	357	479	317(100)	Myricetin-3-*O*-glucoside[4]	8.48 ± 0.09^c	19.88 ± 0.05^b	23.6 ± 0.3^a	—
16	15.5	273	787	635(27), 617(100), 301(15)	Tetragalloylglucose[1]	nd	16.3 ± 0.1	nd	—
17	16.01	260	1105	1061(100), 937(87), 785(29), 767(84), 635(14), 465(61), 419(21), 301(41)	Rugosin A[1]	4.9 ± 0.2^c	9.7 ± 0.1^a	7.8 ± 0.2^b	—
18	16.4	277	787	635(15), 617(100), 301(25)	Tetragalloylglucose[1]	4.02 ± 0.02	13.2 ± 0.5	nd	<0.001
19	17.13	352	615	463(100), 301(66)	Quercetin-hexosyl-gallate[5]	1.88 ± 0.08^c	10.54 ± 0.06^a	1.99 ± 0.04^b	—
20	17.83	356	463	317(100)	Myricetin-3-*O*-rhamnoside[4]	11.3 ± 0.4^c	85.75 ± 0.03^a	12.4 ± 0.1^b	—
21	18.75	354	463	301(100)	Quercetin-3-*O*-glucoside[5]	1.64 ± 0.02^c	3.1 ± 0.2^a	2.18 ± 0.02^b	—
22	19.17	251 366	301	284(15), 245(20), 185(12), 173(5), 157(3)	Ellagic acid[1]	nd	27.1 ± 0.1	6.4 ± 0.4	<0.001
23	19.61	356	599	447(100), 285(28)	Kaempferol-hexosyl-gallate[6]	4.3 ± 0.4	8.34 ± 0.07	nd	<0.001
24	21.52	351	447	285(100)	Kaempferol-3-*O*-glucoside[6]	nd	3.3 ± 0.1	nd	—
25	22.8	348	447	301(100)	Quercetin-3-*O*-rhamnoside[5]	nd	3.2 ± 0.2	nd	—
26	25.05	370	317	299(18), 289(29), 273(12), 245(14)	Myricetin[4]	nd	5.5 ± 0.1	nd	—
27	27.93	316	625	479(100), 317(32)	Myricetin-coumaroylhexoside[4]	nd	2.12 ± 0.04	nd	—
					Total hydrosable tannins	93 ± 1^c	172 ± 1^a	167.5 ± 0.4^b	—
					Total phenolic acids	—	27.08 ± 0.08	6.4 ± 0.4	<0.001
					Total flavonoids	45 ± 1^c	200 ± 2^a	62.9 ± 0.7^b	—
					Total phenolic compounds	138 ± 3^c	398 ± 3^a	236.8 ± 0.1^b	—

nd – not detected. Standard calibration curves: (1) ellagic acid ($y = 26719x - 317\,255$, $R^2 = 0.9986$); (2) gallic acid ($y = 208604x + 173\,056$, $R^2 = 0.9999$); (3) catechin ($y = 84950x - 23\,200$, $R^2 = 0.9999$); (4) myricetin-3-*O*-glucoside ($y = 23287x - 581\,708$, $R^2 = 0.9988$); (5) quercetin-3-*O*-glucoside ($y = 34843x - 160\,173$, $R^2 = 0.9998$); (6) kaempferol-3-*O*-glucoside ($y = 11117x + 30\,861$, $R^2 = 0.9999$). In each row, different letters mean significant differences between *M. nivelii* extract and fractions ($p < 0.05$). When only two samples were present a Student's *t*-test was used to determine the significant difference between two different samples, with $\alpha = 0.05$.

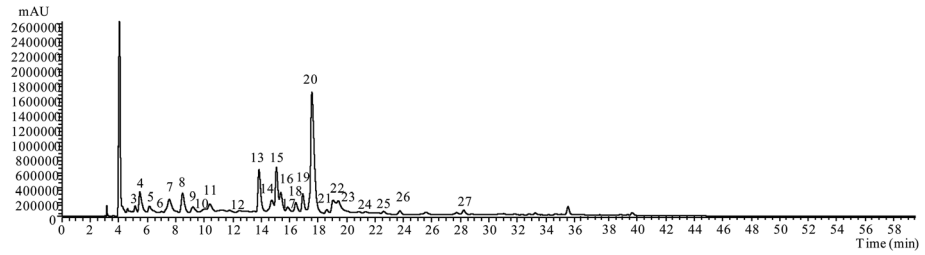

Fig. 1 Phenolic compounds profile of *M. nivelii* ethyl acetate fraction, recorded at 280 nm. Peak numbering corresponds to the molecules identified in Table 1.

fragmentation pattern and UV-Vis spectra presented in ref. 23. Similarly, peaks 6 ($[M - H]^-$ at *m*/*z* 953) and 17 ($[M - H]^-$ at *m*/*z* 1105) were identified as rugosins A and B, respectively, taking into account all the MS characteristics discussed by the author Fecka.[24]

Compound 22 (ellagic acid) was positively identified according to its retention, mass and UV-vis characteristics by comparison with a commercial standard.

The remaining compounds correspond to flavonols (peaks 13, 15, 19–21, 23–27) and to one flavan-3-ol (peak 11). This last peak ($[M - H]^-$ at *m*/*z* 915) was tentatively identified as a dimer of gallocatechin-gallate, presenting a fragment pattern with the fragments at *m*/*z* 457 (gallocatechin-gallate) and *m*/*z* 169 (gallate), previously reported by Fracassetti *et al.*[25] The detected flavonols were derived from quercetin (λ_{max} around 350 nm, MS^2 fragment at *m*/*z* 301), kaempferol (λ_{max} around 348 nm, MS^2 fragment at *m*/*z* 285) and myricetin (λ_{max} around 354 nm, MS^2 fragment at *m*/*z* 317) (Table 1). Myricetin-3-*O*-glucoside (peak 15), quercetin-3-*O*-glucoside (peak 21), kaempferol-3-*O*-glucoside (peak 24) and myricetin (peak 26) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. The flavonoid myricetin-3-*O*-glucoside has been previously reported in the leaves of *Myrtus communis* L.[11,26] Compounds 20 ($[M - H]^-$ at *m*/*z* 463) and 25 ($[M - H]^-$ at *m*/*z* 447) presented an MS^2 fragment at *m*/*z* 317 (myricetin) and *m*/*z* 301 (quercetin) corresponding to the loss of a rhamnosyl (−146 mu) moiety. These compounds were assumed as myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside, due to their previous identification in the leaves of *M. communis* by Romani *et al.*[27] and Messaoud *et al.*[11] Furthermore, peaks 13, 19 and 23 were identified as hexosyl derivatives of myricetin, quercetin and kaempferol that also appeared to be attached to a gallic acid moiety, yielding deprotonated ions at *m*/*z* 631 (peak 13), consistent with myricetin-hexosyl-gallate, *m*/*z* 615 (peak 19) with quercetin-hexosyl-gallate, and *m*/*z* 599 (peak 23) with kaempferol-hexosyl-gallate. Finally, peak 27 ($[M - H]^-$ at *m*/*z* 625) was identified as a myricetin-coumaroylhexoside, due to the fragmentation of the product ion at *m*/*z* 479 (loss of the *p*-coumaroyl residue, −146 u) and *m*/*z* 317 (loss of the hexosyl moiety). This peak was identified as a flavonol glycoside linked to a phenolic acid due to its late elution time, since the presence of the hydroxycinnamoyl residue implies a decrease in polarity, as also due to its UV-Vis spectra (λ_{max} 316 nm).

The highest phenolic content was found in the ethyl acetate fraction (398 mg g^{-1} of extract, Table 1), myricetin glycoside derivatives being the most abundant compounds (total of 136 mg g^{-1}), especially myricetin-3-*O*-rhamnoside and myricetin-hexosyl-gallate, respectively. These derivatives accounted for 34% of the total phenolic compounds in this fraction and were three fold higher than that in the butanol fraction (46 mg g^{-1}) and four fold higher than that in the crude extract (26 mg g^{-1}). The compound gallocatechin gallate was also mainly present in the ethyl acetate fraction (35.6 mg g^{-1}), being three times higher than the one in the crude aqueous extract (10.9 mg g^{-1}) and in its butanol fraction (13.2 mg g^{-1}).

Hydrolysable tannins were also relevant constituents in all samples, although presenting a higher concentration in the ethyl acetate fraction (172 mg g^{-1}, Table 1). Nevertheless, this fraction did not reveal the presence of galloyl-HHDP-glucosides (peaks 1 and 2), which were the most abundant compounds in the crude aqueous extract, while trigalloylquinic acid (peak 8) was the most abundant compound in the butanol fraction and the most abundant hydrolysable tannin in the ethyl acetate fraction.

The results obtained in this study showed that there are two main groups of polyphenolic compounds in the studied Algerian desert myrtle extracts: flavonols and hydrolysable tannins. This is in agreement with previous studies on this genus, especially on the leaves of *M. communis*, in which the main phenolic compounds are also flavonols (myricetin 3-*O*-galactoside, myricetin-3-*O*-rhamnoside and quercetin-3-*O*-glucoside), flavan-3-ols (catechin, gallocatechin and epigallocatechin) and hydrolysable tannins.[26,27]

3.2. Antioxidant activity

The antioxidant activity was evaluated by using four methodologies, representing different mechanisms, such as DPPH radical scavenging activity, reducing power, β-carotene bleaching inhibition and inhibition of lipid peroxidation through the TBARS assay; the results are presented in Table 2. This genus has been shown to have interesting antioxidant capacity, which can be related to its antiproliferative properties, particularly associated with the presence of flavonoids and hydrolysable tannins.[27]

The very low EC_{50} value presented by the ethyl acetate fraction (3.27 μg mL^{-1}) indicates the highest DPPH scavenging activity, when compared to the butanol fraction and crude

Table 2 Antioxidant activity and NO formation inhibition capacity of *M. nivelii* leaves

	Crude aqueous extract	Ethyl acetate fraction	Butanol fraction	Positive control[a]
Antioxidant activity(EC_{50}values, μg mL^{-1})				
DPPH scavenging activity	7.1 ± 0.1[a]	3.27 ± 0.05[c]	4.6 ± 0.2[b]	42 ± 1
Reducing power	6.23 ± 0.04[a]	3.15 ± 0.01[c]	3.93 ± 0.03[b]	41 ± 1
β-Carotene bleaching inhibition	112 ± 3[a]	82 ± 2[c]	92.9 ± 0.2[b]	18 ± 1
TBARS inhibition	0.87 ± 0.02[a]	0.46 ± 0.02[c]	0.74 ± 0.06[b]	23 ± 1
Anti-inflammatory activity (EC_{50} values, μg mL^{-1})				
Nitric oxide (NO) production	149 ± 8[a]	104 ± 6[c]	127 ± 12[b]	16 ± 1

[a] Trolox and dexamethasone for antioxidant and anti-inflammatory activities, respectively. The antioxidant activity was expressed as EC_{50} values (mean ± SD), which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. The results of anti-inflammatory activity are expressed in EC_{50} values: sample concentration providing 50% of inhibition of nitric oxide (NO) production. In each row, different letters mean significant differences between *M. nivelii* extract and fractions ($p < 0.05$).

aqueous extract (EC_{50} values of 4.6 and 7.1 μg mL^{-1}, respectively). The reducing power methodology measures the ability of an antioxidant to donate electrons to Fe(III) resulting in the transformation of the Fe^{3+}/ferricyanide complex to the Fe^{2+} complex. In fact, the extracts presented a great reducing power, ranging from 3.14 to 6.22 μg mL^{-1}. Once again, ethyl acetate revealed the highest ability to reduce Fe(III) followed by the butanol fraction and the crude aqueous extract. The β-carotene bleaching inhibition method is based on the discoloration of β-carotene due to its reaction with radicals, which are formed by linoleic acid oxidation in an emulsion. The bleaching inhibition of β-carotene can be delayed in the presence of antioxidants which have the ability to neutralize free radicals and inhibit the lipid peroxidation. All the extracts demonstrated strong antioxidant properties, displaying that the ethyl acetate fraction has the greatest inhibition effect (3.30 μg mL^{-1}), followed by the butanol fraction (3.72 μg mL^{-1}) and the crude aqueous extract (4.49 μg mL^{-1}). The capacity of the extracts to inhibit the formation of the complex MDA-TBA (malondialdehyde-thiobarbituric acid) was measured using porcine brain tissue homogenates (Table 2). All samples showed high capacity against lipid peroxidation, the ethyl acetate fraction again being the most efficient when compared to the butanol fraction and crude aqueous extract (EC_{50} values of 0.46, 0.74 and 0.87 μg mL^{-1}, respectively).

Overall, the ethyl acetate fraction exhibited the highest antioxidant activity, followed by the butanol fraction and the crude aqueous extract in all the methods (Table 2). With the exception of β-carotene bleaching inhibition, all samples presented higher antioxidant activity than the standard trolox (used as a reference compound).

Moreover, the antioxidant activity presented by the two fractions and by the crude aqueous extract could be correlated with the phenolic composition, especially with flavonol and hydrolysable tannin contents. These phenolic groups have been previously reported to show a high antioxidant activity.[28] The considerable higher levels of myricetin-hexosyl-gallate, myricetin-3-*O*-rhamnoside, gallocatechin-gallate-dimer, digalloyl and trigaloyl-HHDP-glucoside, tetragalloylglucoside, and of the quercetin and kaempferol derivatives, in the ethyle acetate fraction (Table 1), may be considered the main factor contributing to its higher antioxidant capacity. This is also in accordance with the findings reported by several authors.[29,30] Pereira *et al.*[31] showed that myricetin-3-*O*-galactoside and myricetin-3-*O*-rhamnoside isolated from *M. communis* demonstrated a strong antioxidant activity. These results could also confirm the highest antioxidant activity of the ethyl acetate in comparison with the other fraction and with the crude extract.

In fact, many reports indicate the antioxidant effect of polyphenolic components including myricetin, quercetin, kaempferol, catechin and their derivatives.[32] Similarly, the relationship between the antioxidant activity and phenolic content of *M. nivelii* could also be established in this study.

3.3. Anti-inflammatory activity

Murine macrophage-like RAW 264.7 cells are commonly used in anti-inflammatory response studies. Nitric oxide (NO) is an important oxidative and inflammatory mediator produced by macrophages and used as an indicator of inflammation induced by LPS (lipopolysaccharide). Table 2 presents the results obtained for the anti-inflammatory activity of the ethyl and butanol fractions and of the crude aqueous extract. The results reveal that all extracts suppressed the production of NO with EC_{50} = 104, 127 and 149 μg mL^{-1}, for ethyl acetate and butanol fractions and, finally, the crude aqueous extract, respectively. The ethyl acetate fraction was the most active in the suppression of NO, whereas the butanol fraction and the crude aqueous extract reduced less the NO production. These results were in agreement with Touaibia & Chaouch,[14] who demonstrated the anti-inflammatory effectiveness, by the inhibition of paw edema, of the methanol extract of *M. nivelii* by using an *in vivo* test with carrageenan paw edema from male Swiss mouse.

The NO inhibition potential of the extracts could be attributed to the presence of bioactive compounds like flavonols, ellagitanins and phenolic acids, the main molecules found in this species, which are known for their *in vivo* and *in vitro* anti-inflammatory effect.[33] The differences between the samples

could be attributed to the different biomolecules' distribution in fractions and crude extract.

3.4. Cytotoxic properties

The results of cytotoxicity of the crude extract and its fractions are summarized in Table 3. All tested samples showed promising cytotoxic properties towards the selected tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2). The ethyl acetate fraction showed a significant higher potential against all the studied cell lines: MCF-7 (16.12 μg mL^{-1}); NCI-H460 (49.01 μg mL^{-1}); HeLa (15.39 μg mL^{-1}) and HepG2 (17.67 μg mL^{-1}), followed by the butanol fraction and the crude aqueous extract. However, all samples also presented toxicity effects for non-tumor cells, although the ethyl acetate presented the lowest toxicity.

It has been previously reported that certain phenolic compounds can contribute to the protection of cancer disease, such as ellagic acid, ellagitannins and quercetin and its derivatives.[34–37] These compounds have exhibited antimutagenic and anticarcinogenic activity *in vitro* and *in situ* in various human tumor cell lines, such as growth inhibition of lung tumorigenesis in mice,[37] anticarcinogenic effects against the colon tumor cell line HCT-116[35] and antiproliferative effects of cervical carcinoma (Caski, HeLa and Siha cell lines).[36] Liberal *et al.*[34] confirmed the cytotoxic potential of ellagitannins on human hepatocellular carcinoma cells.

Therefore, the inhibition effects exhibited by the studied extracts are related to their contents in bioactive molecules, which can be considered promising cytotoxic phytochemicals.

3.5. Antibacterial activity

The antibacterial activity of the crude aqueous extract and of its two fractions is shown in Table 4. All extracts exhibited antibacterial activity against the ten tested bacteria strains, with minimal inhibitory concentrations (MICs) ranging between 0.078 and 10 mg mL^{-1}. All extracts revealed an excellent antibacterial activity and in a similar order to the other bioactivities mentioned above, ethyl acetate fraction > butanol fraction > crude aqueous extract. MRSA and MSSA were the most susceptible Gram-positive bacteria, while *E. coli* and ESBL *E. coli* were the most sensitive Gram-negative bacteria, for all tested samples. It is important to highlight that all the samples presented strong bactericidal concentrations, especially against MRSA and MSSA.

Nevertheless, the inhibition growth was higher with the ethyl acetate fraction in comparison with the other samples and this activity can be associated with the highest levels of the majority of phenolic compounds present in this fraction. Similarly, Mert *et al.*[38] also tested the antibacterial potential of *M. communis*, against *E. coli*, *S. aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* by the disc diffusion method. In this study, the ethyl acetate fraction also gave the strongest activity against *S. aureus*. Quercetin-3-*O*-rhamnoside, isolated from the ethyl acetate fraction of *Anacardium occidentale* L. leaves, also revealed a good antimicrobial activity against all studied strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *P. fluorescence* NCIB 3756, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* NCIB 3610, *Klebsiella pneumonia* (clinical strain) and *Clostridium sporogens* NCIB 532).[39] The combination between two isolated compounds, quercetin-3-*O*-rutinoside and quercetin-3-*O*-rhamnoside, also revealed a higher antimicrobial activity than all extracts, which confirms

Table 4 Antibacterial activity of *M. nivelii* leaves

Bacterial strains	Crude aqueous extract	Ethyl acetate fraction	Butanol fraction
Gram-positive		MIC/MBC (mg mL^{-1})	
Enterococcus faecalis	5/20	1.25/2.5	5/10
Listeria monocytogenes	10/20	5/10	10/20
MSSA	0.625/1.25	0.078/0.156	0.078/0.156
MRSA	0.625/1.25	0.078/0.156	0.078/0.156
Gram-negative		MIC/MBC (mg mL^{-1})	
Escherichia coli	0.625/2.5	0.3125/0.625	0.625/1.25
E. coli ESBL	0.625/2.5	0.3125/0.625	0.625/1.25
Klebsiella pneumoniae	1.25/5	1.25/2.5	1.25/2.5
K. pneumoniae ESBL	1.25/5	1.25/2.5	1.25/2.5
Morganella morganii	1.25/5	0.625/1.25	0.625/1.25
Pseudomonas aeruginosa	10/20	2.5/5	5/10

MRSA – methicillin resistant *Staphylococcus aureus*; MSSA – methicillin susceptible *S. aureus*; MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; ESBL – spectrum extended producer of β-lactamases.

Table 3 Cytotoxic properties of *M. nivelii* leaves in human tumor cell lines and non-tumor liver primary cells

	Crude aqueous extract	Ethyl acetate fraction	Butanol fraction	Ellipticine
Human tumor cell lines (GI_{50} values. μg mL^{-1})				
MCF-7 (breast carcinoma)	29 ± 2^{a}	16 ± 1^{b}	30 ± 2^{a}	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer)	72 ± 4^{b}	49 ± 3^{c}	89 ± 7^{a}	1.03 ± 0.09
HeLa (cervical carcinoma)	61 ± 5^{a}	15 ± 1^{c}	37 ± 3^{b}	1.91 ± 0.06
HepG2 (hepatocellular carcinoma)	223 ± 19^{c}	18 ± 1^{b}	27 ± 2^{b}	1.1 ± 0.2
Non-tumor cells (GI_{50} values. μg mL^{-1})				
PLP2 (porcine liver primary cells)	97 ± 6^{b}	112 ± 3^{a}	71 ± 5^{c}	3.2 ± 0.7

GI_{50} values (mean ± SD) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row, different letters mean significant differences between *M. nivelii* extract and fractions ($p < 0.05$).

the synergistic effect of these two compounds.[39] Ellagitannins have been also reported to present high antimicrobial activity,[40] which can explain the high antibacterial potential of the herein studied samples.

Overall, the differences found in the bioactive properties of the aqueous extract and the organic fractions can be related to the different profiles and quantities of phenolic compounds present in each sample. To the authors' best knowledge, the current study is the first report on the individual phenolic profile of *M. nivelii* leaves and evaluation of antioxidant, anti-inflammatory, cytotoxic and antibacterial activities. The present study also confirms the traditional medicinal use of *M. nivelii*, highlighting the bioactive potential of ellagitannin and flavonoid derivatives.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2013), L. Barros contract and S. Heleno (SFRH/BPD/101413/2014) grant. We greatly acknowledge the MESRS of Algeria for the financial support that allowed us to complete the internship period at the IPB in the PNE program. The authors are also grateful to the Interreg España-Portugal for financial support through the project 0377_Iberphenol_6_E).

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