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Characterization of *Rubus fruticosus* L. berries growing wild in Morocco: phytochemical screening, antioxidant activity and chromatography analysis

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Abstract

Rubus fruticosus L. is a widespread shrub species which has recently attracted a great attention due a plethora of different pharmacological activities. A comparative study of the volatile profile and polyphenols along with the antioxidant activity of 27 blackberries collected in 3 different locations of the Tangier–Tetouan–Al Hoceima region in Northern Morocco, between June and August 2018, is reported. In terms of antioxidant activity, the highest IC_{50} values were attained for the *Rubus fruticosus* EtOAc extract coming from Beni Messaouar (2.5 mg/mL ± 0.01). Concerning the volatile content of the *Rubus fruticosus n*-hexane extract belonging to Beni Messaouar, a total of 42 compounds were detected and oleic acid turned out to be the most abundant one (14.49%), whereas among the 29 polyphenols detected in the *Rubus fruticosus* EtOAc extract, coming from the same location, quercetin-3-O-glucoside occurred in major concentration (364.58 mg/kg). This is the first report on the physico-chemical and phytochemical properties of Moroccan *Rubus fruticosus* highlighting how these fruits do have a great potential as natural source of antioxidant compounds to be used as a nutraceuticals or functional foods.

Keywords *Rubus fruticosus* L. · Blackberry · Physico-chemical analyses · Antioxidant activity · Volatiles · Polyphenols · Liquid chromatography

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Introduction

Blackberries (*Rubus fruticosus*) are wild fruits that have a delicious taste, pleasant flavor, good appearance and an excellent nutritional profile. These fruits are eaten fresh or processed to make food products such as jam, tea, ice cream, desserts, jellies, and baked goods. The pigments extracted from these berries are employed in many application fields [1].

Rubus fruticosus has a particular importance due to its high nutrient content. Its importance has become greater with the concept of "functional food" which made this fruit more popular [2]. This berry has various health benefits and since ancient time, this species has been recognized for their use in folk medicine: in particular due to its anti-inflammatory properties, it can be advantageously employed for the cure of gums, ulcers of the oral cavity and cough [3]. It is also endowed with anti-diarrheal, diuretic and anti-hemorrhoidal properties [4–6]. Moreover, it has an anxiolytic power by depression of the central nervous system [7]. In addition to these activities, a protective effect against cognitive difficulties has been demonstrated [8]. *R. fruticosus* berries are notable for their high nutritional contents of vitamin C, vitamin B, dietary fiber, α-tocopherol, tocotrienol, calcium, potassium, magnesium, carotenoids, linoleic acid and linolenic acid [7, 9]. Bioactive compounds include phenolic compounds such as ellagic acid and anthocyanins [10–12]. Furthermore, results obtained by GC–MS showed the presence of six major compounds, namely hexadecanoic acid methyl, 9,12-octadecadienoic acid methyl ester, 9,12,15-octadecatrienoic acid methyl ester, phytol, phthalic acid diisooctyl ester and vitamin E, occurring in the leaf extract, whereas four major compounds, namely 2-furancarboxaldehyde, hexadecanoic acid methyl ester, 12-octadecanoic acid methyl ester and phthalic acid diisooctyl ester, were found in each of the stems and the roots extracts [13]. Other results proved that the contents of sugars, total phenolic, total flavonoid, and anthocyanins increase as the fruit development is advanced (ripening) [14].

Considering the high-water content, these fruits do present an irrelevant presence of macromolecules, e.g., proteins lower than 3.5% whereas lipids up to 1%; on the other hand, among minerals, potassium was reported to be the most abundant one [15–17].

The aim of this study was to evaluate the physico-chemical properties and well as the antioxidant activity of 27 blackberries collected from 3 different locations of the Tangier–Tetouan–Al Hoceima region in Northern Morocco, between June and August 2018. The phytochemical profile of the *R. fruticosus* extracts namely ethylacetate (EtOAc) and MeOH:water (80:20 *v/v*), coming from Beni Messaouar (BM), was carried out by HPLC coupled to PDA and MS detection to determine the polyphenolic content. Besides, the *n*-hexane fraction, belonging to the same location, was evaluated in terms of volatile content, investigated by GC–MS.

Materials and methods

Chemicals

Folin–Ciocalteu phenol reagent was obtained from Fluka. Reference materials (gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, vanillin, rutin, kaempferol, quercetin and cyanidin-3-*O*-glucoside) were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Lois, MO). LC–MS grade methanol, acetonitrile, acetic acid, EtOAc, and water were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). All other chemicals were attained from Sigma (St. Louis, MO).

Samples and sample preparation

Twenty-seven wild fruits (*R. fruticosus*) were harvested in Tangier–Tetouan–Al Hoceima region in the extreme northwest of Morocco. Specifically, three fruits, in three different months, viz. June, July and August 2018 were collected in the following locations: Had Gharbia (HG), Ghezaoua (GH), and BM. Specifically, the harvest areas were between the longitudes 5° 55′ 50.7"; 5° 31′ 34"; and 5° 43′ 31.3" and the latitudes 35° 30′ 54.3"; 34° 55′ 43"; and 35° 28′ 41.3" and, respectively, for HG, GH, and BM, respectively. After collection, fruits were stored at − 10 °C at the Laboratory of Valorization of Resources and Chemical Engineering, Abdelmalek Essaadi University, Tangier, Morocco.

5 g of lyophilized powder underwent a defatting step by adding three times 50 mL of *n*-hexane; afterwards, it was dried and homogenized with 50 mL of two solvents with increased polarity, namely EtOAc and MeOH:water (80:20 *v/v*). Each fraction was extracted using an ultrasound bath (130 kHz) for 45 min. After centrifugation at 5000 g for 5 min, the supernatant filtered through a paper filter, dried, reconstituted with MeOH:water, 80:20 (*v/v*), and then, filtered through 0.45 µm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany) prior to HPLC–PDA–ESI/MS analysis [18].

Physico-chemical analyses

Physico-chemical determinations were carried out according to the AOAC International Standard Methods. Parameters

detected were pH, refractive index (RI), total soluble solids (TSS), ratio sugar/acidity (S/A), dry matter content (DM %), ash (%), total sugars (TS%), reduce sugars (RS%), lipid content (mg/g), protein content (mg/g) and vitamin C content (mg/g).

Phytochemical screening

Phytochemical screening was performed according to a previously published work [18].

Test for starch

A small portion of the extract is treated with boiling water for 30 s; afterwards, it is mixed with boiled ethanol for a few minutes. The addition of iodine provides a blue–black color.

Test for saponosides

Roughly, 2 g of the powdered sample is boiled in 20 mL of distilled water in a water bath and filtered. 10 mL of the filtrate is mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing is mixed with 3 drops of olive oil and shaken vigorously, leading to the formation of an emulsion.

Test for flavonoids and anthocyanins

The extract is treated with concentrated sulphuric acid. Appearance of yellowish orange shows the presence of anthocyanins, yellow to orange color shows the presence of flavones, and orange to crimson shows the presence of flavanones.

Test for tannins

Roughly, 0.5 g of the dried powdered sample is boiled in 20 mL of water in a test tube, and then, filtered. A few drops of 0.1% ferric chloride is added and observed for brownish-green or a blue–black coloration.

Test for sterols and steroids

1 mL of concentrated sulphuric acid is added to 1 g of plant extract and allowed to stand for 5 min. After shaking, the formation of golden yellow color in the lower layer indicates the presence of sterols and steroids.

Test for mucilages

Roughly, 5 mg of the extract is dissolved in a vial containing 0.5 mL of deionized water. The vial is heated in a laboratory oven at 100 °C for 5 min and allowed to cool. The liquid is then centrifuged for 1 min and three drops of the supernatant are mixed with 0.5 mL of *o*-toluidine solution. The solution is then heated in an oven for 10 min at 100 °C. Variation of color indicates the presence of mucilages.

Test for coumarins

Roughly, 5 mg of the extract is treated with a solution of dimethylamino-benzaldehyde (5% ethanol), and then, acidified by bubbling gaseous hydrochloric acid. The orange color indicates a positive test.

Test for alkaloids

A small portion of the extract is stirred separately with 1 mL of diluted hydrochloric acid and filtered. The filtrate is treated with Dragendroff's reagent. Appearance of organic precipitate shows the presence of alkaloids.

Test for anthraquinones

A small portion of the extract is boiled with dilute sulphuric acid. Filtered and cooled. The filtrate is extracted with chloroform or benzene and dilute ammonia is added to it. The ammonical layer becomes pink to red due to the presence of anthraquinones.

Test for iridoids

5 g of each extract is mixed in 2 mL of chloroform and concentrated H_2S0_4 (3 mL) are carefully added to form a layer. A reddish-brown coloration of the interface shows positive results for the presence of iridoids.

Test for glycosides

Small quantity of the extract is hydrolyzed with 5 mL of hydrochloric acid for few hours on a water bath and the hydrolysate was subjected to Fehling's test. To 2 mL of Fehling's solution (1 mL of Fehling's A and 1 mL of Fehling's B solution), 2 mL of the extract is added, mixed and boiled. Appearance of yellow or red color precipitate indicates the presence of reducing sugars.

GC–MS analyses

GC analyses of the *n*-hexane fraction of the nine *R. fruticosus* samples, coming from BM, were performed on a GC–MS-QP2020 system (Shimadzu, Kyoto, Japan) equipped with an AOC-20i system auto-injector.

Separations were performed on an SLB-5 ms column (30 m × 0.25 mm I.D.; 0.25 µm; Merck Life Science, Merck KGaA, Darmstadt, Germany). The initial

temperature was set at 50 °C, afterwards increased up to 350 °C (increase rate: 3 °C/min; holding time: 5 min).

GC–MS parameters were as follows: injection temperature, 280 °C; injection volume, 1.0 µL (split ratio: 10:1); pure helium gas (99.9%); linear velocity, 30.0 cm/s; inlet pressure, 26.7 kPa; full scan mode mass range, 40–660 m/*z*; event time, 0.2 s; EI source temperature, 220 °C; interface temperature, 250 °C. Relative quantity of the chemical compounds present in each sample was expressed as percentage based on peak area produced in the GC chromatogram.

Compounds were identified using the FFNSC 4.0 (Shimadzu Europa GmbH, Duisburg, Germany), and "W11N17" (Wiley11-Nist17, Wiley, Hoboken, NJ, USA; Mass Finder 3). Each compound was identified applying a MS similarity match and an LRI filter. Linear retention indices (LRI) were calculated using a C7–C40 saturated alkane reference mixture (49452-U, Merck Life Science, Merck KGaA, Darmstadt, Germany).

HPLC–PDA/ESI–MS analyses

HPLC analyses of the nine *R. fruticosus* samples (EtOAc and MeOH:water, 80:20 *v/v* extracts), were performed on a Shimadzu liquid chromatography system (Kyoto, Japan), consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20A5R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M30A photodiode array detector, and an LCMS-8050 triple quadrupole mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan).

Separations were carried out on 150 × 4.6 mm; 2.7 µm Ascentis Express RP C18 column (Merck Life Science, Merck KGaA, Darmstadt, Germany). The mobile phase was composed of two solvents: water/acetic acid (99.85/0.15 *v/v*, solvent A) and acetonitrile/acetic acid (99.85/0.15 *v/v*, solvent B). The flow rate was set at 1 mL min^{-1} and a gradient elution program was followed: 0–5 min, 5% B, 5–15 min, 10% B, 15–30 min, 20% B, 30–60 min, 50% B, and 60 min, 100% B. PDA range was in 200–400 nm and monitored at λ = 280 nm (sampling frequency: 40.0 Hz, time constant: 0.08 s). MS conditions were as follows: scan range, *m/z* 100–800; scan speed, 2500 µ s^{-1}; event time, 0.3 s; nebulizing gas (N_2) flow rate: 1.5 L min^{-1}; drying gas (N_2) flow rate, 15 L min^{-1}; interface temperature, 350 °C; heat block temperature, 300 °C; DL (desolvation line) temperature: 300 °C; DL voltage, 1 V; interface voltage: − 4.5 kV.

Calibration curves ($R^2 \geq 0.997$) of nine polyphenolic standards were used for the quantification in sample extracts, namely gallic acid, *p*-hydroxybenzoic acid, p-coumaric acid, caffeic acid, vanillin, rutin, kaempferol, quercetin and cyanidin-3-*O*-glucoside. Five concentration levels were investigated in the range from 1 to 500 mg Kg^{-1}.

Determination of the polyphenolic content

Total phenol (TP) content for both EtOAc and MeOH:water (80:20 *v/v*) extracts was estimated using the Folin–Ciocalteu method [18] with some modifications. Gallic acid was used as standard (10, 25, 50, 100, and 200 ppm) and total phenolic content was measured at 755 nm and was expressed as mg of gallic acid equivalents (GAE)/g dry mass (DM). Total flavonoid (TFV) content for both EtOAc and MeOH:water (80:20 *v/v*) extracts was determined according to the method of Zhishen et al. [19]. A known volume of each extract was placed in a 10 mL of volumetric flask and filled with 5 mL of distilled water and 0.3 mL of $NaNO_2$ (1:20). Afterwards, 3 mL of $AlCl_3$ (1:10) and 2 mL of NaOH (1 M) were added. The solution was mixed and the absorbance was measured against a blank at 510 nm. Results were expressed as mg of quercetin equivalents (QE)/g DM. Total anthocyanin (TA) content for both EtOAc and MeOH:water (80:20 *v/v*) extracts was estimated based on the differential pH (pH = 1 and pH = 4.5) with some modifications and expressed as mg of Pg-3-gluc/g DM [20]. Total tannin (TT) content for both EtOAc and MeOH:water (80:20 *v/v*) extracts was determined by the vanillin method [21]; briefly, 0.1–0.5 mL of extracts was taken and put into tubes covered with aluminum foil. Three milliliters of 4% vanillin (w/v) in methanol was added, and the tubes were shaken vigorously with a mixer. Afterwards, 1.5 mL of concentrated HC1 was pipetted and the tubes were shaken again. The absorbances were read at 500 nm after being allowed to stand for 20 min at room temperature and results expressed as mg of catechin equivalents (CE)/g DM.

Determination of the antioxidant activity

Free radical-scavenging $DPPH^{\cdot}$ method for each sample was carried out following a slightly method described by Braca et al. [22]. A rapid TLC-screening method was first performed using a 0.2% DPPH solution in MeOH. The spectrophotometric assay was carried out by adding 30 µL of a methanolic solution containing the single EtOAc and MeOH:water (80:20 *v/v*) extracts to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage of activity was calculated. Butylated hydroxytoluene (BHT) was used as a positive control. IC_{50} was calculated from linear regression (%DPPH remaining radical versus sample concentration).

Statistical analysis

The experiments were carried out in triplicates and the results were expressed as the average of the three measurements ± SD. The comparison of means between groups was performed with one-way analysis of variance

(ANOVA) followed by Tukey test. Differences were considered significant when $p < 0.05$.

Results and discussion

R. fruticosus is a wide species present in the Mediterranean area. In total, 27 wild fruits were harvested in Tangier–Tetouan–Al Hoceima region. Three different locations, namely HG, GH and BM were selected and three fruits, in three different months, viz. June, July and August 2018 were collected.

Determination of phenolic compounds

The mean (n = 9) physico-chemical parameters values obtained for the three different locations are reported in Table S1. The highest values of pH and ash% were found in *R. fruticosus* from the HG location; on the other hand, for TSS, S/A, and DM%, the most prominent values were attained for BM. Statistically, a significant difference was observed between the results of the three regions for all tested parameters ($p < 0.05$). In terms of humidity, the values obtained for the three studied regions are lower than those reported by Hirsch et al. [23] for blackberry cultivars (*Rubus* spp.) distributed in the southern region of Brazil (from 84.8 to 90.3%); the same applies also to ash% values (from 0.27 to 0.49%) and pH values (from 2.8 to 3.1). The DM% contents of *R. fruticosus* are in agreement with another research where a percentage of 36.4% was attained [24]. The S/A ratio obtained complied with an average value obtained by other reports, viz. 3.1 [7]. It has been demonstrated that the variation in sugar content could be attributed to different factors, including stage of ripening, temperature, duration of sun exposure and weather conditions [25]. In terms of TSS, studies on wild blackberries from different regions of Turkey and Serbia showed similar results, e.g., 11.3–13.1 and 12.0–15.6, respectively [26, 27]. The latter also reported TS% and RS% values which are considerably lower than the ones reported in this study from 5.36 to 5.98 and from 1.32 to 1.46, respectively. The differences in such results can be probably due to several factors such as climate and geographic distribution [18]. Concerning the lipid and protein contents, the highest concentrations were obtained for the GH fruits (14.7 mg/g and 1.4 mg/g). Similar values of the same species were previously reported for proteins content, e.g., 1.39 mg/g [7]. With regards to vitamin C, the highest value was attained for the berries of the HG locations (70.0 ± 2.54 mg/g) which is three times over the one already found for the same species [7].

Phytochemical screening

The phytochemical screening of the 27 *R. fruticosus* fruits collected from the HG, GH and BM locations is reported in Table S2. The tested plant showed positive results for variable amounts of saponosides, flavonoids (flavones, isoflavones), tannins, anthocyanins, sterols and steroids, coumarins, mucilages, alkaloids, iridoids and glycosides. Notably, sterols, steroids and mucilages were present in considerable amounts in the samples coming from the three

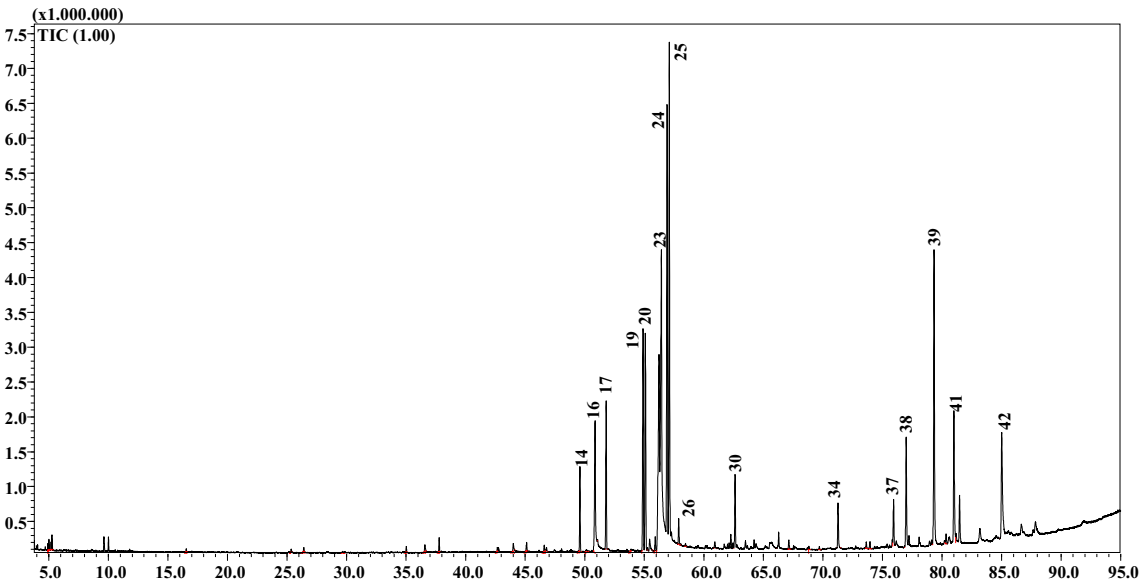

Fig. 1 GC–MS profile of the *n*-hexane fraction of one of the nine *R. fruticosus* samples, coming from BM. Only most abundant peaks are labeled

Table 1 List of compounds identified in all the nine *R. fruticosus* samples from BM by GC–MS

No.	Compound	LRI (lib)	LRI (exp)	Similarity	Area %	Library
1	*n*-Nonanal	1107	1105	91	0.05	FFNSC 4.0
2	(2E,4Z)-Decadienal	1295	1296	93	0.08	W11N17
3	(2E,4E)-Decadienal	1322	1320	93	0.08	FFNSC 4.0
4	Ethyl-decanoate	1399	1394	89	0.03	FFNSC 4.0
5	Vanillin	1394	1398	90	0.05	FFNSC 4.0
6	Methyl-dodecanoate	1527	1524	94	0.12	FFNSC 4.0
7	*n*-Dodecanoic acid	1581	1563	95	0.16	FFNSC 4.0
8	Ethyl-dodecanoate	1598	1593	96	0.31	FFNSC 4.0
9	Methyl-tetradecanoate	1727	1724	91	0.07	FFNSC 4.0
10	*n*-Tetradecanoic acid	1773	1762	84	0.18	FFNSC 4.0
11	Ethyl-tetradecanoate	1794	1793	95	0.18	FFNSC 4.0
12	Neophytadiene	1836	1836	93	0.12	FFNSC 4.0
13	Phytone	1841	1842	93	0.11	FFNSC 4.0
14	Methyl-hexadecanoate	1925	1926	96	1.87	FFNSC 4.0
15	16-Hexadecanolactone	1938	1942	84	0.14	FFNSC 4.0
16	*n*-Hexadecanoic acid	1977	1965	95	4.03	FFNSC 4.0
17	Ethyl-palmitate	1993	1993	97	3.29	FFNSC 4.0
18	Epimanool	2057	2060	87	0.05	FFNSC 4.0
19	Methyl-linoleate	2093	2093	92	5.04	FFNSC 4.0
20	Methyl-oleate	2098	2099	85	5.7	FFNSC 4.0
21	Phytol	2111	2111	91	0.41	FFNSC 4.0
22	Methyl-octadecanoate	2127	2127	92	0.31	FFNSC 4.0
23	Linoleic acid	2144	2138	95	8.95	FFNSC 4.0
24	Oleic acid	2142	2144	89	14.49	FFNSC 4.0
25	Ethyl-linoleate	2164	2161	94	10.89	FFNSC 4.0
26	Ethyl-stearate	2198	2194	95	0.6	FFNSC 4.0
27	(E)-Phytol acetate	2221	2213	91	0.06	FFNSC 4.0
28	*n*-Tricosane	2300	2300	90	0.21	FFNSC 4.0
29	4.8.12.16-Tetramethylheptadecan-4-olide	2364	2349	95	0.33	W11N17
30	(9Z)-Octadecenamide	2375	2362	94	1.82	W11N17
31	*n*-Pentacosane	2500	2499	95	0.42	FFNSC 4.0
32	*n*-Hexacosane	2600	2600	92	0.07	FFNSC 4.0
33	Tetracosanal	2632	2636	91	0.06	W11N17
34	*n*-Heptacosane	2700	2700	95	1.1	FFNSC 4.0
35	*n*-Octacosane	2800	2800	92	0.13	FFNSC 4.0
36	Squalene	2810	2813	88	0.19	FFNSC 4.0
37	*n*-Nonacosane	2900	2900	90	1.11	FFNSC 4.0
38	δ-Tocopherol	2951	2947	94	3.22	W11N17
39	γ-Tocopherol	3055	3054	96	8.88	W11N17
40	*n*-Hentriacontane	3100	3100	95	0.29	FFNSC 4.0
41	Vitamin E	3138	3132	96	4.1	W11N17
42	γ-Sitosterol	3351	3322	92	4.61	W11N17
	TOT. Identified				83.91	
	TOT. not identified				16.09	

Table 2 Polyphenols identified in all the nine *R. fruticosus* EtOAc extracts from BM by HPLC–PDA/ESI–MS

Peak	Tentative identification	t_R (min)	Identification type	λ_{max} (nm)	$[M–H]^-$	Fragments	Quantity (mg kg^{-1})
1	Gallic acid	6.26	PDA/MS	270	169	–	100.30
2	3-*p*-Coumaroylquinic acid	8.86	PDA/MS	258–293	337	–	77.96
3	3-*O*-Caffeoylquinic acid	11.07	PDA/MS	280–321	353	179	39.56
4	*p*-Hydroxybenzoic acid	12.28	PDA/MS	254	137	–	130.81
5	Unknown	13.90	PDA/MS	266	443	265*	–
6	Vanillic acid	15.37	PDA/MS	260–290	167	–	147.24
7	3-Feruloylquinic acid	16.20	PDA/MS	321	367	–	56.18
8	5-*O*-Caffeoylquinic acid	16.58	PDA/MS	321	353	179	70.71
9	Protocatechuic acid	19.71	PDA/MS	291	153	–	190.53
10	*p*-Coumaric acid	21.85	PDA/MS	305	163	–	23.02
11	Ferulic acid	24.82	PDA/MS	230–321	193	–	105.39
12	Vanillic acid derivative	26.21	PDA/MS	281	363	167	61.13
13	Dihydroquercetin	27.07	PDA/MS	284	303	–	309.46
14	p-Coumaroyl tartaric acid glucosidic ester	27.16	PDA/MS	268	475*	–	45.32
15	Cyanidin-*O*-pentosyl (hexoside)	27.83	PDA/MS	281–520	581	449	9.93
16	Unknown	28.15	PDA/MS	349	351	–	–
17	Quercetin-3-*O*-glucoside	28.50	PDA/MS	255–353	463	301	364.58
18	Ellagic acid hexoside	29.10	PDA/MS	352	463	301	–
19	Quercetin-3-O-[6″-*O*-(3-hydroxy-3-methylglutaryl) -β-D-glucopyranoside]	30.96	PDA/MS	353	607	301	56.77
20	Cyanidin-*O*-glucoside	31.76	PDA/MS	284–520	449*	289*	7.07
21	Cyanidin-*O*-hexoside	32.20	PDA/MS	286–520	449*	289*	3.80
22	Cyanidin-*O*-pentoside	33.55	PDA/MS	354–520	449*	289*	6.70
23	Unknown	35.75	PDA/MS	281	359	329	–
24	Ellagic acid pentoside	36.22	PDA/MS	267	433	-	–
25	Unknown	36.56	PDA/MS	261	549	263	–
26	Luteolin	37.97	PDA/MS	287–356	285	–	–
27	Rutin	39.34	PDA/MS	361	609	301	41.61
28	Pelargonidin-succinylarabinoside or Pelargonidin-malonylrhamnoside	42.58	PDA/MS	281–508	503	271	1.27
29	Kaempferol	44.08	PDA/MS	291–359	285	-	6.33

*Acquired in $[M+H]^+$ mode

Table 3 Polyphenols identified in all the nine *R. fruticosus* MeOH/water extracts (80:20, *v/v*) from BM by HPLC–PDA/ESI–MS

Peak	Tentative identification	t_R (min)	Identification type	λ_{max} (nm)	*m/z*	Fragments	Quantity (mg/kg)
3	3-*O*-Caffeoylquinic acid	11.07	PDA/MS	280–321	353	179	30.86
12	Vanillic acid derivative	26.21	PDA/MS	281	363	167	32.90
14	*p*-Coumaroyl tartaric acid glucosidic ester	27.16	PDA/MS	268	475 +	–	21.88
17	Quercetin-3-*O*-glucoside	28.50	PDA/MS	255–353	463	301	48.39
18	Ellagic acid hexoside	29.10	PDA/MS	352	463	301	–
19	Quercetin-3-*O*-[6″-*O*-(3-hydroxy-3-methylglutaryl)-β-D-glucopyranoside]	30.96	PDA/MS	353	607	301	8.96

regions. Notable amounts of catechic tannins occurred in the HG and GH location, whereas gallic tannins only in the HG one. Starch and anthraquinones were not found in any of the samples of *R. fruticosus* investigated. Such findings are in agreement with previously published reports confirming that blackberries are a rich source of flavonoids, tannins, sterols and steroids [7, 28].

GC–MS analyses

The GC–MS analysis of the *n*-hexane fraction of one of the nine *R. fruticosus* samples, coming from BM, is reported in Fig. 1. A total of 42 compounds were detected (Table 1) with a % of similarity ranging from 84 to 97%. Among them, lipids (oleic acid, 14.49%, ethyl-Linoleate, 10.89%, linoleic acid, 8.95%, and methyl-Linoleate, 5.04%), alkanes (n-Nonacosane, 1.11%, n-Heptacosane, 1.1%), tocopherols (γ-tocopherol, 8.88%, δ-tocopherol), sterols (γ-Sitosterol, 4.61%), aldehydes (*n*-Nonanal, 0.05%), etc. were positively identified.

Determination of the polyphenolic content by HPLC–PDA/ESI–MS

Qualitative and quantitative analyses of the *R. fruticosus* EtOAc and MeOH:water (80:20 *v/v*) extracts, coming from BM, were accomplished by HPLC–PDA/ESI–MS. A total of 29 and 6 compounds for EtOAc and MeOH/water (80:20, *v/v*) extracts, respectively (Tables 2 and 3), belonging to phenolic acids, anthocyanins and flavonoids, were positively detected. As an example, Fig. 2 shows the LC-PDA chromatogram of one of the nine *R. fruticosus* samples, coming from BM. Specifically in the EtOAc extract, 14 phenolic acids belonging to hydroxybenzoic and cinnamic acids were found along with 5 anthocyanins and 6 flavonoids, whereas 4 phenolic acids and 2 flavonoids were identified. Among them only rutin was reported as constituent of *R. fruticosus* from Croatia [10], whereas gallic acid, protocatechuic acid, vanillic acid and rutin were reported in *R. fruticosus* pomace from Bosnia and Herzegovina [11]. From a quantitative point of view in the EtOAc extract, quercetin-3-*O*-glucoside

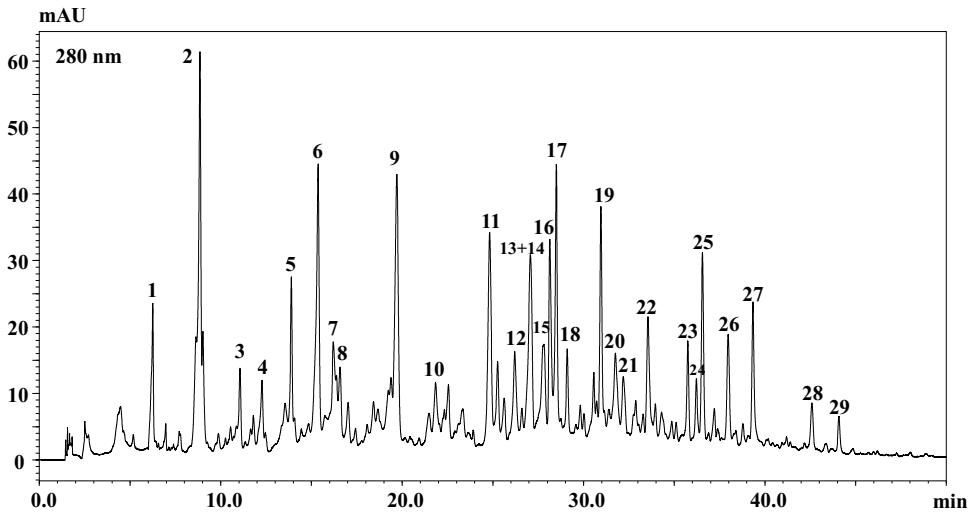

Fig. 2 HPLC–PDA ($\lambda = 280$ nm) fingerprint of the one of the nine *R. fruticosus* samples (EtOAc extract), coming from BM. For peak identification, see Table 2

Table 4 Total phenolic, flavonoid, anthocyanin, tannin contents and antioxidant activity IC_{50} values of the 27 *R. fruticosus* fruits investigated (n = 9)

	Solvent	Samples		
		HG	GH	BM
Total phenolic content (mg GAE/g DM)	EtOAc	$37.4^{b} \pm 3.10$	$50.2^{c} \pm 2.50$	$63.1^{a} \pm 0.20$
	MeOH 80%	$21.2^{c} \pm 0.30$	$23.9^{a} \pm 0.20$	$22.5^{b} \pm 0.50$
Total flavonoid content (mg QE/g DM)	EtOAc	$4.6^{a} \pm 1.55$	$27.1^{c} \pm 0.08$	$49.6^{b} \pm 1.50$
	MeOH 80%	$0.8^{c} \pm 1.05$	$5.8^{a} \pm 0.05$	$3.3^{b} \pm 0.20$
Total anthocyanin content (mg Pg-3-glu/g DM)	EtOAc	$0.3^{b} \pm 0.01$	$0.2^{a} \pm 0.01$	$0.3^{c} \pm 0.00$
	MeOH 80%	$0.0^{b} \pm 0.01$	$0.0^{c} \pm 0.00$	$0.00^{a} \pm 0.01$
Total tannin content (mg CE/g DM)	EtOAc	$103.7^{a} \pm 0.05$	$84.1^{b} \pm 0.04$	$123.3^{a} \pm 0.10$
	MeOH 80%	$13.0^{a} \pm 0.50$	$15.8^{c} \pm 0.05$	$14.4^{d} \pm 0.01$
DPPH-scavenging activity IC_{50} (mg/mL)	EtOAc	$0.3^{a} \pm 0.01$	$1.4^{c} \pm 0.02$	$2.5^{b} \pm 0.01$
	MeOH 80%	$0.1^{a} \pm 0.01$	$0.1^{b} \pm 0.01$	$0.2^{b} \pm 0.01$

The results are expressed as means ± SD (n = 3). In each column, different letters mean significant differences ($p < 0.05$)

Table 5 TSS, IR, TS, proteins, vitamin C, TPP, TFV, TA, TT and IC_{50} values of the 27 *R. fruticosus* fruits investigated

R. fruticosus TSS	IR	TS	Proteins	Vitamin C	TPP	TFV	TA	TT	IC_{50}
TSS 1									
IR 0.999	1								
TS 0.233	0.189	1							
Proteins 0.537	0.549	0.040	1						
Vitamin C 0.151	0.106	0.968	− 0.197	1					
TPP − 0.627	− 0.646	0.137	− 0.887	0.316	1				
TFV 0.276	0.232	0.996	0.038	0.971	0.102	1			
TA 0.232	0.189	0.974	0.129	0.929	0.001	0.975	1		
TT 0.227	0.182	0.796	− 0.114	0.820	0.305	0.778	0.753	1	
IC_{50} − 0.437	− 0.433	− 0.262	0.256	0.302	0.106	− 0.321	0.989	0.046	1

turned out to be the most abundant one (364.58 mg/kg) followed by dihydroquercetin (309.46 mg/kg). Likewise Quercetin-3-O-glucoside was also the most abundant one in the MeOH/water (80:20, *v/v*) extract (48.39 mg/kg).

Antioxidant activity and correlations

It has been reported that the antioxidant activity of phenolic acids depends on the number and the position of hydroxyl groups related to the carboxyl functional group and it increases with hydroxylation degree [29]. Regarding *R. fruticosus* antioxidant activity, a research has shown an IC_{50} equal to 2.14 mg/mL ± 0.12 [30] and it has been demonstrated that antioxidant activity increases at increasing ripening stage with no significant differences in free radical-scavenging activity between immaturity and semi-maturity for all samples [14]. Table 4 reports total phenolic, total flavonoid, total anthocyanin and total tannin contents along with the antioxidant activity (IC_{50} values) of the 27 *R. fruticosus* fruits investigated. The mean IC_{50} of each solvent fraction studied showed that the EtOAc extract showed the highest antioxidant power. Analysis by the ANOVA test showed a very significant difference between the results of the two fractions ($p < 0.01$). Table 5 reports the relationship between the IC_{50} values and TSS, IR, TS, proteins, vitamin C, TP, TFV, TA, TT and of the *R. fruticosus* fruits investigated. It was observed a strong correlation with $R^2 = 0.988$ between IC_{50} and TA, while TT showed the weakest correlation ($R^2 = 0.04$) [27, 30–32]. Several studies on berries and cherries have reported relationship between antioxidant activity and phenolic compounds and anthocyanin content [33–37]. R^2 values of 0.97 were attained for TA and TFV as well as for TA and TS. Proteins were correlated negatively with phenolic compounds ($R^2 = 0.89$), which was explained by their enzymatic interactions during ripening and/or fermentation [38, 39].

It can be assumed that flavonoid compounds, in particular quercetin-3-*O*-glucoside and dihydroquercetin, which were present at higher concentrations in the EtOAc extracts, contribute to some extent to the observed antioxidant effects. However, according to the results attained, the polyphenolic content (particularly flavonoids) were not all consistent with the antioxidant activity. This could be due to the presence of other secondary metabolites responsible for this activity, which could have had potential synergistic or antagonistic effects. This latter could occur in the system based on additional components contained in this fruit, as well as interactions between phenolic compounds and plant matrix physical environment [40]. Some minerals (in particular iron) can complex with phenolic compounds influencing their antioxidant activity [41]. Since several characteristics and reaction mechanisms are likely involved, no single test accurately reflect all antioxidants in a mixed or complex system [42]. The explanation about the absence of correlations between the different parameters and the antioxidant activity (IC_{50}), may be due to some parameters, e.g., solvent, concentration, structure, and size, that can affect the antioxidant capacity of extracts.

Conclusion

Blackberries collected in three different regions of the Northern Morocco were evaluated. A comparative study of volatile and polyphenolic profiles along with the antioxidant properties were evaluated and accomplished by both spectrophotometric and chromatographic approaches. No remarkable differences were noticed for the three different regions investigated, except for the mean IC_{50} of each extraction solvent studied, which showed that the EtOAc extract does possess the highest antioxidant power. The evaluated physico-chemical and phytochemical parameters provided the evidence of an antioxidant potential, which explains some of the medicinal uses and pharmacological properties attributed to this species.

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Author contributions HEC: conceptualization, data curation, investigation, methodology, and writing—original draft. HEB: investigation. GS: investigation. BR: investigation. OEM: data curation and investigation. FA: investigation. JB: conceptualization and supervision; ABA: writing—review and editing. PD: writing—review and editing, and supervision. LM: resources and project administration; FC: conceptualization, methodology, resources, writing—review and editing, and supervision.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Compliance with ethics requirements This study does not contain any studies with human participants or animals performed by any of the authors.

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