In vitro enzyme inhibitory and antioxidant properties, cytotoxicity and chemical composition of the halophyte *Malcolmia littorea* (L.) R.Br. (Brassicaceae)

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Abstract

This work reports for the first time the *in vitro* antioxidant (towards DPPH, ABTS, copper and iron), enzymatic inhibitory (on AChE, BuChE, α-glucosidase, α-amylase and tyrosinase), cytotoxicity (towards HepG2 and HEK 293 cells), and metabolomics (by HPLC-MS) of extracts from organs of *Malcolmia littorea* (L.) R.Br. Extracts were constituted mainly by phenolic acids and flavonoids, and main compounds were salycilic acid and luteolin-7-O-glucoside. Samples showed reduced radical scavenging and metal chelating capacity, and only the methanol extracts reduced iron. The root's ethanol and methanol extracts, and the aerial organ's ethanol extract exhibited the highest AChE inhibition. The root's ethanol extract displayed dual anti-cholinesterase activity. Samples showed a low capacity to inhibit α-amylase, but a high α-glucosidase inhibition was obtained with the root's and flower's ethanol extracts, and flower's methanol extract. Overall, samples displayed a high inhibition against tyrosinase, reduced HepG2 cellular viability and were less toxic towards HEK 293 cells.

Keywords: phytochemicals; tyrosinase inhibitors; cholinesterase inhibitors; salt tolerant plants

List of abbreviations

ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AChE: Acetylcholinesterase
BHT: butylated hydroxytoluene
BuChE: Butyrylcholinesterase
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FRAP: ferric reduction activity power
HPLC-MS: High-performance liquid chromatography-mass spectrometry
IC_{50}: Half maximal inhibitory concentration
RSA: radical scavenging activity

1. Experimental

1.1. Chemicals

The following reagents were purchased from Sigma-Aldrich (Germany): 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), terc-butylhydroxytoluene (BHT), standards used for the HPLC analysis, enzymes([electric eel AChE, type-VI-S, EC 3.1.1.7; horse serum BuChE, EC 3.1.1.8; tyrosinase from mushroom, EC1.14.18.1; glucosidase from *Saccharomyces cerevisiae*, EC 3.2.1.20; amylase from porcine pancreas, EC 3.2.1.1), galanthamine, acetyl- and butyrylthiocholine chloride, 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), 4-nitrophenyl dodecanoate (NPD), N-Succinyl-Ala-Ala-Alap-nitroanilide (SANA), N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) and 4- dimethylaminocinnamaldehyde (DMACA). VWR International (Belgium) provided all the additional solvents and chemicals.

1.2. Plant material

Plants (voucher number XBH39) were sampled in Faro Beach, South of Portugal (coordinates: 37°0'0.163" N, -7°9'86.070" W) in July of 2018. Plants were cleaned and divided into aerial vegetative organs (leaves and stems), flowers and roots, dried in an oven (3 days, 50°C), reduced to powder and stored (-20°C).

1.3. Extracts preparation

For the preparation of the extracts, samples were mixed with ethanol, methanol and water (1:40, w/w), and extracted for 30 minutes in an ultrasonic water bath, at room temperature (RT, ca. 20°C). Extracts were filtered (Whatman paper no. 4), and the organic extracts evaporated in a rotary evaporator, at reduced pressure and temperature

(approx. 40 -50°C). Water extracts were freeze-dried. Dried extracts were dissolved in the corresponding solvent at 50 mg/mL and stored (-20°C).

1.4. Identification and quantification of phenolic compounds by HPLC-MS

The phenolic components of the extracts were characterized by an HPLC Agilent 1100 Series with a G1315B diode array detector. A Luna Omega Polar C_{18} analytical column of 150 x 3.0 mm and 5 µm particle size (Phenomenex) with a Polar C_{18} Security Guard cartridge (Phenomenex) of 4 x 3.0 mm were used. The HPLC system was connected to an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics) equipped with an electrospray interface operating in negative mode. Detailed conditions are reported elsewhere (Llorent-Martínez et al., 2018). Compounds identification was carried out based on analytical standards and mass spectra, whereas UV spectra were used for quantification purposes.

1.5. In vitro *antioxidant properties*

1.5.1. Radical-based methods

Radical scavenging activity (RSA) towards the DPPH and ABTS radicals was evaluated by previously described methods (Rodrigues et al., 2019). Samples were tested at concentrations ranging from 0 to 10 mg/mL, assays were performed in 96-well microplates, and absorbances were measured on a multi-plate reader (EZ read 400, Biochrom). Butylated hydroxytoluene (BHT) was used as the standard positive control, at a maximum concentration of 1 mg/mL. Results were calculated in relation to a negative control containing the solvent, expressed as a percentage of RSA inhibition and as IC_{50} values (mg/mL), whenever possible.

1.5.2. Metal-based methods

Copper (CCA) and iron (ICA) chelating capacity was evaluated as described previously (Rodrigues et al., 2016). Tested concentrations and results determination and expression are described in section 1.5.1. Ethylenediaminetetraacetic acid (EDTA) was used as positive control, at a concentration of 1 mg/mL. The ferric reducing activity power (FRAP) of the extracts was also determined according to Rodrigues et al. (2016). The increase in samples' absorbance indicates an increase in the FRAP of the samples, and therefore, results are calculated in relation to the used positive control (BHT, 1 mg/mL) and expressed as percentage of inhibition and as IC_{50} (mg/mL).

1.6. Enzyme inhibitory activities

The inhibitory properties of the extracts towards AChE and BuChE were evaluated as described previously (Zengin 2016). Extracts were tested at 0.5, 1 and 5 mg/mL, galantamine (0.5 - 5 mg/mL) was used as the positive control and results were expressed as the equivalent of galantamine (mg GALAE/g extract).

The α-amylase and α-glucosidase inhibition capacity was evaluated as described by Uysal et al (2017. Samples were tested at concentration ranging from 0.5 and 5 mg/mL and acarbose was used as standard (at the same concentration as the samples). Results were expressed as the equivalent of acarbose (mmol ACAE/g).

The tyrosinase inhibition assay was performed as described by Zengin (2016), on samples at concentrations between 0.5 and 5 mg/mL. Kojic acid was used as standard inhibitor (at the same concentration as the samples) and results were presented as the equivalent of kojic acid (mg KAE/g).

122 *1.7.* In vitro *cytotoxicity*

123 *1.7.1. Cell culture*

124 Human embryonic kidney cells (HEK 293, provided by the Functional Biochemistry
125 and Proteomics group, Centre of Marine Sciences, Portugal) and human
126 hepatocarcinoma cells (HepG2, provided by the Marine Molecular Bioengineering
127 groups, Centre of Marine Sciences, Portugal) were cultured in DMEM culture media,
128 both supplemented with 10% heat-inactivated FBS, 1% L-glutamine (2 mM), and 1%
129 penicillin (50 U/mL) / streptomycin (50 μg/mL) and were kept at 37ºC in moistened
130 atmosphere with 5% CO_2.

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132 *1.7.1. Cellular viability*

133 Exponentially growing HEK 293 and HepG2 cells were seeded in 96-well microplates
134 at a density of 5 x 10^3 cells/well, left to adhere for 24h and treated with the extracts at
135 the concentration of 100 μg/mL, for a period of incubation of 72h. Control cells were
136 treated with culture medium containing the corresponding extract solvent, at the highest
137 concentration used in the treatments (0.2%). Cellular viability was determined by the
138 MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colourimetric
139 assay, as described elsewhere (Rodrigues et al., 2016). Results were expressed as
140 cellular viability (%), in relation to the control cells. The selectivity index (SI) of the
141 extracts was calculated by the equation: SI = VNT/VT, where VNT and VT indicate cell
142 viability on non-tumoral and tumoral cells respectively (Oh et al., 2011).

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144 *2.8. Statistical analyses*

145 Statistical analysis was performed using SPSS Statistics software v.22 (IBM SPSS
146 Statistics for Windows, IBM Corp., USA). Data of all analyses, at least in triplicate, are

expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test ($P < 0.05$) was used to look for statistically significant differences among results. Differences amongst samples were considered significant if *P* values were equal or inferior to 0.05. Half-maximal inhibitory concentration (IC_{50}) values were determined through data sigmoidal fitting in the GraphPad Prism v. 5.0 software.

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Tables

Table S1: HPLC-DAD analysis of the phenolic compounds' contents (mg/g DW) of extracts from *Malcolmia littorea*.

Compound[a]	Water			Ethanol			Methanol		
	Aerial organs	Roots	Flowers	Aerial organs	Roots	Flowers	Aerial organs	Roots	Flowers
Gallic acid	nd	1.78	0.13	< 0.01	1.37	0.30	0.12	5.71	0.34
3,4-Dihydroxybenzoic acid	nd	nd	nd	0.18	0.47	0.28	0.21	0.40	0.36
Neochlorogenic acid	nd	nd	nd	0.12	0.31	nd	0.15	0.45	nd
Gentisic acid	1.45	4.92	10.93	nd	nd	nd	nd	nd	nd
p-hidroxybenzoic acid	nd	nd	nd	nd	nd	0.18	nd	nd	0.44
Catechin hydrate	nd	nd	nd	nd	nd	1.7	nd	nd	nd
4-Hydroxybenzaldehyde	nd	nd	nd	< 0.01	< 0.01		nd	nd	nd
3-Hydroxybenzoic acid	0.15	nd	0.17	0.25	0.14	0.25	0.24	nd	nd
Vanillic acid	0.57	0.29	0.56	0.28	0.15	0.19	0.34	0.21	0.29
Chlorogenic acid	0.09	0.26	0.07	0.20	nd	nd	0.50	0.29	0.21
4-*O*-Caffeoylquinic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cafeic acid	nd	0.70	0.85	nd	0.76	2.59	nd	0.76	2.66
Syringic acid	0.02	0.64	0.20	0.08	0.07	1.12	0.07	0.09	1.39
Epigallocatechin gallate	< 0.01	< 0.01		< 0.01	nd	nd	< 0.01	nd	nd
Epicatechin	1.78	4.89	1.39	1.35	0.79	11.48	0.10	0.05	16.95
Ourateacatechin	0.84	0.56	0.62	< 0.01	0.24	0.66	< 0.01	0.39	1.44
Umbelliferone	0.29	0.23	1.94	0.08	0.13	0.03	0.13	0.47	4.05
Coumaric acid	0.02	< 0.01	0.13	< 0.01	< 0.01	< 0.01	0.25	< 0.01	0.16

Taxifolin	nd	nd	nd	nd	nd	nd	nd	nd	nd
Coumarin	nd	0.1	nd	nd	nd	nd	nd	nd	nd
Ferulic acid	0.53	0.27	1.35	0.37	0.16	0.20	0.78	0.24	0.68
Salicylic acid	3.81	5.11	41.4	3.04	1.62	nd	4.51	3.31	61.72
Naringenin-7-glucoside	< 0.1	nd	0.88	0.12	nd	1.09	0.26	0.09	1.81
Luteolin-7-O-glucoside	0.48	0.06	34.9	1.75	0.03	43.52	2.49	0.06	56.92
Rosmarinic acid	nd	0.11	1.44	nd	nd	1.56	0.01	< 0.1	3.33
Rutin	nd	0.27	0.07	0.01	nd	nd	nd	nd	nd
Ellagic acid	nd	nd	nd	0.03	nd	1.04	0.03	< 0.01	nd
Cinnamic acid	0.20	nd	0.26	0.19	nd	0.17	0.20	0.17	0.18
Quercetin	0.04	nd	0.04	0.07	0.02	0.02	0.08	0.01	0.02
Chrysin	nd	nd	nd	nd	nd	nd	nd	nd	0.05
Total	10.2	20.1	97.3	8.12	6.26	66.3	8.84	12.7	153

175 [a]Identified by comparison of retention parameters with standards and peak purity with UV-vis spectral reference data.

176 nd: not detected.

Table S2

Radical scavenging on DPPH and ABTS, ferric reducing antioxidant power (FRAP), copper (CCA) and iron chelating (ICA) activities of *Malcolmia littorea* extracts. Results expressed as IC_{50} values (mg/mL).

Organs	Extract	ABTS	DPPH	FRAP	CCA	ICA
Roots	Ethanol	3.07 ± 0.59	6.22 ± 0.75	nr	4.99 ± 0.33	nr
	Methanol	2.46 ± 0.48	7.62 ± 1.08	0.04 ± 0.01	2.44 ± 0.16	nr
	Water	5.16 ± 0.62	nr	nr	3.95 ± 0.16	4.89 ± 0.39
Aerial organs	Ethanol	6.84 ± 1.22	8.70 ± 0.73	nr	nr	nr
	Methanol	1.93 ± 0.57	2.88 ± 0.44	0.32 ± 0.03	6.02 ± 0.05	nr
	Water	3.66 ± 0.25	nr	nr	2.39 ± 0.42	3.73 ± 0.26
Flowers	Ethanol	4.55 ± 0.35	4.93 ± 0.63	nr	7.40 ± 0.31	nr
	Methanol	3.39 ± 0.78	4.26 ± 0.17	0.31 ± 0.09	3.67 ± 0.05	nr
	Water	4.13 ± 0.31	3.27 ± 0.44	nr	3.76 ± 0.24	nr
BHT*		0.1 ± 0.02[a]	0.06 ± 0.01[a]			
EDTA*					0.11 ± 0.00[a]	0.07 ± 0.01[a]

Values are mean ± SD, n = 6. In each column, different letters mean significant differences ($P < 0.05$).

nr: IC_{50} not reached

*positive control

Table S3

Enzymatic inhibitory activity of *Malcolmia littorea* extracts. Results expressed as equivalents of the respective positive control.

Organs	Extract	AChE (mg GALAE[1]/g)	BChE (mg GALAE[1]/g)	α-amylase (mmol ACAE[2]/g)	α-glucosidase (mmol ACAE[2]/g)	Tyrosinase (mg KAE[3]/g)
Roots	Ethanol	1.38 ± 0.01[a]	1.23 ± 0.04[a]	0.14 ± 0.01[a]	2.21 ± 0.01[a]	25.32±0.04[a]
	Methanol	1.36 ± 0.04[a]	0.76 ± 0.04[b]	0.14 ± 0.01[a]	0.56 ± 0.04[d]	24.96±0.19[a]
	Water	0.90 ± 0.03[d]	0.62 ± 0.01[c]	0.02 ± 0.01[b]	na	6.28±0.45[b]
Aerial organs	Ethanol	1.46 ± 0.03[a]	0.84 ± 0.02[b]	0.16 ± 0.02[a]	na	25.78±0.18[a]
	Methanol	1.04 ± 0.12[c]	0.37 ± 0.05[e]	0.15 ± 0.01[a]	1.80 ± 0.09[ab]	26.48±0.12[a]
	Water	0.37 ± 0.07[e]	na	0.03 ± 0.01[b]	0.36 ± 0.03[e]	5.32±0.08[b]
Flowers	Ethanol	1.29 ± 0.03[b]	0.46 ± 0.05[d]	0.14 ± 0.01[a]	2.09 ± 0.02[a]	26.56±0.23[a]
	Methanol	1.15 ± 0.22[c]	na	0.15 ± 0.01[a]	1.93 ± 0.12[a]	25.85±0.21[a]
	Water	0.36 ± 0.01[e]	na	0.02 ± 0.01[b]	1.21 ± 0.02[c]	4.33±0.39[b]

[*]Values are means ± SD, n = 3. In each column, different letters mean significant differences ($P < 0.05$). [1]GALAE: Galatamine equivalent; [2]ACAE: Acarbose equivalent; [3]KAE: Kojic acid equivalent;

na: not active.