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Differences in the phenolic composition and nutraceutical properties of freeze dried and oven-dried wild and domesticated samples of *Sanguisorba minor* Scop

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ABSTRACT

Recent studies have dealt about the phenolic compounds and the nutraceutical properties of *Sanguisorba minor* Scop and about the possibility of their domestication to standardise the plant production. However, it is also known that the storage conditions can affect the bioactive compounds present in plants. Thus, wild (W) and domesticated (F1, F2, F3) *S. minor* samples were exposed to different drying methods (oven-drying at 60 °C until constant weight named OD or freeze-drying until constant weight named FD) and studied for their content in phenolic compounds, antioxidant, antimicrobial, cytotoxic and anti-inflammatory properties. In general, OD samples showed the highest nutraceutical properties and the highest content in phenolic compounds. The most abundant phenolic compounds in both drying methods were kaempferol-3-*O*-glucoside and caffeoyl ester, with some differences between wild and domesticated samples. Wild samples showed higher antimicrobial and cytotoxic activity than domesticated ones, except for the OD F3 and FD F2 samples that reported cytotoxicity against HeLa cells. This study provides important information to choose the most adequate methodology to retain phenolic compounds and nutraceutical properties of *S. minor* species. Further researches are necessary to standardise the domestication of the studied wild species and verify the highest efficiency of the OD method.

1. Introduction

Nowadays, the research of a dietary vegetable variety is increasing, especially in developed countries, to overcome the nutritional deficiencies in the human diet caused by the imposition of using common nutraceutical ingredients derived from the modernisation and globalisation of agriculture (Welch & Graham, 1999). For this reason, the domestication of wild edible herbs has been studied. Different growing techniques have been applied to wild edible species, in order to evaluate their response to domestication through standard farming systems. These results were significant in terms of nutraceutical properties and phenolic compound content as well as in terms of availability of plant material for an yearly marketability (Ceccanti et al., 2019; Finimundy et al., 2020; Karkanis et al., 2019).

Recently, the consumers, as well as the food industry, have started to concern about the quality of foodstuff. They are demanding for high functional formulations, preferentially with natural and nutraceutical ingredients. Since the use of nutraceutical ingredients is continuously increasing, the drying method as a storage method is resulting of extreme importance in the quality of these ingredients and in the quality of the final food product. Indeed, the retention of bioactive molecules and nutraceutical properties during the use of drying methods is the main concept to have new functional ingredients from wild edible species. It has been noted that the drying methods can inhibit the enzymatic activity, which can lead to the senescence of plant material over variable periods and to the loss of phenolic compounds and nutraceutical properties (Roshanak et al., 2016; Ye et al., 2019). The drying method is considered the oldest food storage technique; thus, different drying methods have been developed to assure the retention of the food constituents (Harbourne et al., 2009). In this perspective, scientific research

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has been conducted on different wild edible plants, including green tea (Roshanak et al., 2016), *Anoectochilus roxburghii* (Ye et al., 2019) and *Dendrobium nobile* (Meng et al., 2018), aiming at analysing the effects of different drying methods. The authors concluded that depending on the drying method, the content in phenolic compounds can be compromised as also the bioactive potential (Meng et al., 2018; Roshanak et al., 2016; Ye et al., 2019).

Ye et al. (2019) observed that vacuum and freeze-drying methods maintained the appearance and the nutraceutical properties of the analysed plant.

In contrast, the hot air and microwave drying methods led to higher retention of polysaccharides. Meng et al. (2018) confirmed these results, showing that the hot air dying method maintained the polysaccharide content, whilst the freeze-drying method led to higher retention of bioactive compounds and antioxidant activity than the hot-air drying method. Conversely, Roshanak et al. (2016) reported that the oven drying method at 60 °C maintained the antioxidant activity and the total phenolic and flavonoid content, whilst the freeze-drying method maintained the vitamin C and chlorophyll content.

Sanguisorba minor Scop. is an evergreen perennial species belonging to the *Rosaceae* family, native from Europe, western Asia and Siberia, and northern Africa (Karkanis et al., 2014). During the ancient times, in famine periods, this species was included in the dishes, mainly in mixtures and soups, and more recently, this herb has been introduced in traditional and folk recipes (Guarrera and Savo 2013, 2016). Nowadays, this species is also considered as a nutraceutical and functional species with health-promoting effects to the human health (Ceccanti et al., 2019; Finimundy et al., 2020; Karkanis et al., 2019). Some studies stated that *S. minor* had interesting nutraceutical properties such as anti-inflammatory activity in over-producing ROS (Radical O_2 Species) processes (Kaufmann et al., 2009). This species owned a significant cytotoxic activity against common cancer cell lines, primarily hepatocellular carcinoma line (HepG2), and also antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus* (Karkanis et al., 2019). These nutraceutical properties were directly related to the presence of high amounts in phenolic compounds, responsible for the antioxidant activity and, consequently, for cytotoxic, anti-inflammatory and antibacterial activities showed by *S. minor* species (Ceccanti et al., 2019; Ranfa et al., 2014; Vanzani et al., 2011). Thus, a great variety of different phenolic compounds, especially flavonoids, phenolic acids and phenolic alkaloids, such as avenanthramide 2p and 3,4-diferuloylquinic acid as well as digalloyl-glucoside, catechin, quercetin-*O*-glucuronide were identified in the aerial parts of this species (Ceccanti et al., 2019; Karkanis et al., 2019).

Given the importance that new wild edible species could have in human health and the introduction of a new source of bioactive compounds and nutraceutical properties in the current monotonous diet, the present work aimed at comparing the nutraceutical properties (i.e., inhibition of lipid peroxidation, antibacterial, anti-inflammatory and cytotoxic properties in a range of tumor cell lines) and the phenolic compound's composition of freeze-dried and oven-dried *S. minor* plants collected as wild or domesticated by using three different growing systems as detailed in Table 1 (F1, F2, F3) that were analysed separately. Indeed, the retention of phenolic compounds in *S. minor* resulted in fundamental importance in introducing this species as a new functional ingredient in typical food such as pasta or bread.

2. Material and methods

2.1. Plant material

Wild plant material (W) (about twenty plants) was collected in the Pisa area (Italy, 43°44′39.6″N 10°31′53.39″E) during spring 2019 (April–May), selecting leaves and stems. Regarding the domesticated *S. minor* samples, the plant material was provided by Tirrenofruit S.r.l (Florence, Italy). This local wholesaler selected three local farms (F1, F2, and F3) for providing the material to be used for the present experiment. The *S. minor* plants were domesticated and sampled according to Ceccanti et al. (2020) and as reported in Table 1 and in Supplementary material (Table S1). The selected farms transferred their consolidated growing systems to the cultivation of *S. minor* plants. *S. minor* young aerial parts, selecting only leaves and stems from twenty plants cultivated in five plots (five plants each plot), were randomly collected in each different farm.

Both wild and domesticated samples were well homogeneized and split into two uniform amounts.

2.2. Drying processes

2.2.1. Oven-drying (OD) and freeze-drying (FD)

The OD samples were dried in an electric thermostatic oven (Memmert GmbH + Co. KG Universal Oven UN30, Schwabach, Germany) at 60 °C until it reached a constant weight. The FD samples were dried until it reached the constant weight in a freeze dryer characterised by a standard unheated chamber (Ø 215 mm × 300 mm) (Freeze Dryer Telstar LyoQuest-55, Milan, Italy) through a final condenser temperature of −55 °C.

2.3. Extract preparation

After the applied drying methods, the plant material was ground at 20 μm filters and kept in a desiccator protected from light and humidity. The extraction procedure for the determination of the nutraceutical properties and the phenolic compounds was performed according to Bessada et al. (2016). An amount (1 g) of the dried powder of each drying method was stirred with 30 mL of methanol-water solution (80:20, *v/v*) for 60 min at room temperature. The obtained extracts were filtered through Whatman paper No. 4 filters and evaporated under reduced pressure (Büchi R-210, rotary evaporator, Flawil, Switzerland) at 40 °C and 100 rpm until methanol was entirely removed. Then, the residual aqueous phase of each sample was frozen, lyophilised (Freezone 4.5, Labconco, Kansas City, MO, USA), and stored in a dry room until further analyses.

Table 1
Details of the three domestications (F1, F2, F3).

Domestication method	Growing Area/ Location	Duration Growing Cycle	Soil/Substrate	Pre-treatment of soil/ Fertigation	Average Temperature	Average Relative Humidity	Daily Mean Solar Irradiation
F1	Greenhouse Pisa, Italy	35 days[1]	Peat	Nutritive solution[2]	15.9 °C	79.6%	287.5 W m^{-2}
F2	Greenhouse Siena, Italy	36 days[1]	Peat	Nutritive solution[2]	23.9 °C	52.1%	365 W m^{-2}
F3	Siena, Italy	One month[1]	Mainly silt soil (31% clay, 24% sand, 45% silt, 2% organic matter).	Manure (30 kg ha^{-1}) as pre-treatment of soil,	24.5 °C	46.0%	1050 W m^{-2}

[1,2] Adapted of Ceccanti et al. (2020) and reported in Supplementary material (Table S1).

2.4. Determination of phenolic compounds

The phenolic compounds were determined according to Bessada et al. (2016). The extracted samples were dissolved in methanol-water solution (20:80 *v/v*) to a final concentration of 10 mg/mL and filtered through a 0.22 μm disposable filters. A Dionex Ultimate 3000 (Dionex Ultimate 3000 UPLC and Linear Ion Trap LQT XL, Thermo Scientific, San Jose, CA, USA) ultra-performance liquid chromatographic equipment coupled to a diode array detector and an electrospray ionisation mass spectrometry detector (HPLC-DAD-ESI/MS) was used to analyse the samples. The acquisition and the processing of data were carried out with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA). Authentic standards (Extrasynthése S.A., Genay, France) and data available from the literature were used to identify individual phenolic compounds. The quantification of the detected compounds was based on the calibration curves of authentic standards. The results were presented as mg/g extract.

2.5. Nutraceutical properties

2.5.1. Antioxidant activity

The antioxidant activity was evaluated by the inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS). The obtained extracts were dissolved in a methanol solution (80:20 *v/v*) to obtain a stock solution of 5 mg/mL. Afterward, successive dilutions were made to obtain a range of concentrations of 40 to 0.3 μg/mL. Briefly, porcine (*Sus scrofa*) brains were homogenized in ice-cold Tris–HCl buffer (20 mm/L, pH 7.4). An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 μm/L; 0.1 mL) and ascorbic acid (0.1 mm/L; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. The antioxidant activity resulted from the reduction of TBARS, resulting in the formation of the malondialdehyde-thiobarbituric acid complex (MDA-TBA). The colour intensity, displayed by this complex in the different working concentrations, was measured by its absorbance at 532 nm (UV–Vis Specord 200 spectrophotometer, Analytik Jena). The results were expressed in values of EC_{50}, the necessary concentration of sample able to obtain half of antioxidant activity (Mandim et al., 2020).

2.5.2. Antibacterial activity

The bacterial strains were isolated from patients hospitalised in various departments at the North-eastern local health unit (Bragança, Portugal) and Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria: *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration) and *Morganella morganii* (isolated from urine); and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *Listeria Monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *S. aureus* (MRSA) (isolated from expectoration) were tested. All these microorganisms were incubated at 37 °C in an appropriate fresh medium for 24 h before analysis to achieve the exponential growth phase.

The determination of the Minimal Inhibitory Concentration (MIC) was conducted by the microdilution method and the colourimetric method using *p*-iodonitrotetrazolium chloride (INT) (Panreac Applichem-Barcelona, Spain), according to Pires et al. (2018). Briefly, the extracted samples were dissolved in 5 mL/mL (*v/v*) dimethyl sulfoxide (DMSO) and 95 mL of Tryptic Soy Broth (TSB) to a final concentration of 20 mg/mL. Afterward, successive dilutions were performed in the 96 well microplates, testing a range of concentrations between 20 and 0.15 mg/mL. Finally, 10 μL of inoculum [standardised at 1.5×10^8 Colony Forming Unit (CFU)/mL] were added to all the microplate wells. Three negative controls (one with TSB, another one with the extract, and the third with medium, antibiotics, and bacteria), and one positive control was prepared with Mueller-Hinton broth (MHB), TSB and each inoculum. Ampicillin and imipenem were used for all the Gram-negative bacteria and *L. monocytogenes,* while ampicillin and vancomycin were selected for *E. faecalis* and MRSA. After an incubation period at 37 °C for 24 h, the MIC was detected after the addition of INT and incubation at 37 °C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by the change of the colour from yellow to pink in viable microorganisms. For the determination of the Minimum Bactericidal Concentration (MBC), the lowest concentration allowing to kill the bacterial strains, 10 μL of liquid from each well, that showed no change in colour in the previous test, were plated on solid medium, i.e., blood agar (0.07 mL sheep blood) and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth determined the MBC.

2.5.3. Cytotoxic activity

The cytotoxic activity was evaluated in four different human tumour cell lines obtained from DSMZ- Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen (HeLa: cervical carcinoma; HepG2: hepatocellular carcinoma; MCF-7: breast adenocarcinoma; and NCI–H460: non-small-cell lung cancer) using the sulphorhodamine B (SRB) assay as described by Guimarães et al. (2013). RPMI-1640 medium containing 0.1 mL heat-inactivated FBS and 2 mmol/L glutamine was used to routinely maintain the adherent cell cultures at 37 °C, in a humidified air incubator containing CO_2. Additionally, a primary cellular line from the porcine liver (PLP) was used as a normal cell line to achieve the extract's toxicity. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI–H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Then, cells were treated for 48 h with different concentrations of the extracts in a range of 400 to 1.56 μg/mL.

The adherent cells were fixed with trichloroacetic acid, incubated for 60 min at 4 °C, washed with deionised water and dried. After this process, SRB (0.001 μL in 0.01 μL acetic acid, 100 μL) was added to the microplate wells and incubated for 30 min at room temperature.

The adherent SRB was solubilised with Tris (200 μL), and the absorbance was read at 540 nm in the microplate reader (ELX800 Biotek). The anti-proliferative activity was assessed as the extract concentration providing half of cell growth inhibition (GI_{50}, μg/mL). Ellipticin was used as a positive control.

2.5.4. Anti-inflammatory activity

The anti-inflammatory activity was assessed following a procedure described by Svobodova et al. (2017). The dried extracts were evaluated in mouse lipopolysaccharide (LPS)-stimulated macrophage-like cell line RAW 264.7 in a concentration range from 400 to 1.56 μg/mL. Aliquots of 150,000 cells/well were seeded in 96-well plates, and the cells were left to attach to the plate overnight. Then, cells were treated with prepared concentrations of the sample extracts for 1 h. Further, the cells were stimulated with LPS (1 μg/mL) for 18 h. Dexamethasone (50 μmol/L) was used as a positive control for the experiment. The effect of all analysed samples in the absence of LPS was also evaluated in order to observe if they induced chances in nitric oxide basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in DMEM cell culture medium supplemented with 0.1 mL heat-inactivated fetal bovine serum, glutamine, and antibiotics. For the determination of nitric oxide, a Griess Reagent System kit was used, which contains sulphanilamide, N-(1-naphtyl)ethylenediamine hydrochloride (NED), and nitrite solutions. A reference standard curve of nitrite was prepared in a 96-well plate. The cell culture supernatant was transferred to the plate and mixed with sulphanilamide and NED solutions at room temperature. The production of nitric oxide was determined by measuring the absorbance at 540 nm in the microplate reader (ELX800 Biotek) and by comparison with the standard calibration curve. The results were

expressed as EC_{50} values (μg/mL) (sample concentration providing half of anti-inflammatory activity and, thus, half-inhibition of nitric oxide), and dexamethasone (50 μmol/L) was used as a positive control.

2.6. Statistical analysis

The experimental layout was arranged according to the completely randomized design. The statistical analysis was carried out by using SPSS v. 23.0 software and using the one-way analysis of variance (ANOVA), while means were compared with Tukey's HSD test ($p < 0.05$). Three samples were analysed for each treatment, and all the assays were carried out in triplicate. Data were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Phenolic composition

Data (retention time, λ_{max} in the visible region, molecular ion, and main fragment ions observed in MS^2) obtained by HPLC-DAD-ESI/MS

Table 2
Identification and quantification of phenolic compounds in *S. minor* aerial parts.

Peaks	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (m/z)	Tentative identification	Quantification (mg/g)							
						Oven-dried				Freeze-dried			
						W	F1	F2	F3	W	F1	F2	F3
1	3.87	267	331	271(35), 167 (100)	Gallic acid glucoside[a]	26±2a	0.64 ± 0.02e	1.6846 ± 0.0004d	2.40 ± 0.01cd	3.73 ± 0.02b	0.63 ± 0.02e	2.85 ± 0.05c	3.11 ± 0.01bc
2	4.53	323	353	191(100), 179(44)	3-*O*-Caffeoylquinic acid[b]	22±1a	0.81 ± 0.01 fg	1.48 ± 0.03e	3.689 ± 0.004c	12.01 ± 0.04b	0.69 ± 0.01g	1.329 ± 0.001ef	3.0 ± 0.1d
3	5.29	321	595	297(5), 179 (34), 135 (100)	Caffeoyl ester (isomer 1)[c]	14.8 ± 0.1a	0.87 ± 0.02g	7.6 ± 0.1e	11.0 ± 0.2c	12.38 ± 0.02b	0.5 ± 0.02h	2.91 ± 0.05f	8.9 ± 0.1d
4	6.79	286	483	313(100)	Digalloyl glucoside[d]	22±1a	0.94 ± 0.02 fg	5.0 ± 0.2d	4.8 ± 0.2d	5.6 ± 0.3b	0.365 ± 0.003g	1.49 ± 0.02f	3.5 ± 0.1e
5	8.34	321	595	297(5), 179 (34), 135 (100)	Caffeoyl ester (isomer 2)[c]	5.7 ± 0.2a	0.17 ± 0.03f	1.47 ± 0.01d	3.35 ± 0.02b	1.16 ± 0.01e	0.078 ± 0.004g	1.19 ± 0.02e	2.26 ± 0.01c
6	12.79	228	1401	935(57), 897 (), 633(10)	Lambertianin C[d]	18.6 ± 0.3a	1.76 ± 0.02d	9.7 ± 0.4b	5.3 ± 0.1c	nd	1.401 ± 0.002e	5.31 ± 0.03c	1.87 ± 0.02d
7	13.43	241	934	633(100), 301(56)	Galloyl-bis-HHDP-glucoside[d]	5.5 ± 0.1a	1.8 ± 0.1e	1.93 ± 0.03d	2.03 ± 0.02c	1.89 ± 0.02d	1.46 ± 0.01f	1.54 ± 0.02f	2.75 ± 0.05b
8	15.03	222	1567	1265(100), 1103(8), 933 (16),783(5), 633(77), 301 (65)	Sanguin H-10[d]	18.3 ± 0.4a	1.31 ± 0.01d	3.01 ± 0.06b	3.15 ± 0.01b	nd	1.257 ± 0.001d	2.57 ± 0.03c	nd
9	16.32	256	615	463(100), 301(10)	Quercetin-*O*-hexoside gallate (isomer 1)[d]	4.5 ± 0.1a	0.60 ± 0.04g	1.65 ± 0.04d	2.312 ± 0.001c	0.76 ± 0.01f	0.57 ± 0.004g	1.079 ± 0.002e	2.64 ± 0.02b
10	16.67	346	615	463(15), 301 (100)	Quercetin-*O*-hexoside gallate (isomer 2)[d]	5.3 ± 0.2a	0.784 ± 0.004e	1.13 ± 0.05d	1.81 ± 0.02b	1.27 ± 0.01c	0.562 ± 0.001f	0.82 ± 0.01e	nd
11	17.75	354	477	301(100)	Quercetin-3-*O*-glucuronide[e]	18.0 ± 0.1a	2.61 ± 0.04f	3.9 ± 0.2d	2.30 ± 0.05g	5.48 ± 0.01c	1.23 ± 0.01h	3.00 ± 0.02e	9.42 ± 0.02b
12	20.42	346	447	285(100)	Kaempferol-3-*O*-glucoside[e]	11.3 ± 0.6b	1.49 ± 0.01e	1.995 ± 0.003d	27.6 ± 0.1°	5.13 ± 0.03c	0.30 ± 0.01g	0.62 ± 0.01f	nd
13	21.42	341	609	301(100)	Quercetin-3-*O*-rutinoside[e]	nd	0.53 ± 0.01e	0.43 ± 0.01f	2.99 ± 0.01°	nd	0.58 ± 0.01d	1.047 ± 0.005c	2.21 ± 0.03b
14	21.55	344	447	285(100)	Kaempferol-*O*-hexoside[e]	9.84 ± 0.04a	nd	nd	nd	3.5 ± 0.1b	nd	nd	nd
					Total phenolic acids	22±1a	0.81 ± 0.01 fg	1.48 ± 0.03e	3.689 ± 0.004c	12 ± 0.04b	0.69 ± 0.01g	1.329 ± 0.001ef	3.0 ± 0.1d
					Total hydrolysable tannins	111±1a	7.5 ± 0.1g	30±1c	31.993 ± 0.001b	24.8 ± 0.3d	5.69 ± 0.04h	17.8 ± 0.2f	22.4 ± 0.3e
					Total flavonoids	49.0 ± 0.5a	6 ± 0.1g	9.1 ± 0.2e	36.97 ± 0.03b	16.1 ± 0.2c	3.20 ± 0.03h	6.56 ± 0.05f	14.3 ± 0.1d
					Total phenolic compounds	182±2a	14.3 ± 0.1g	41±1d	72.65 ± 0.02b	52.9 ± 0.3c	9.62 ± 0.1h	25.7 ± 0.3f	39.7 ± 0.4e

nd: not detected. W, F1; F2 and F3 represented samples collected as wild (W) or provided from local farmers (F1, F2, F3). Different letters in the same row show significant difference between oven-dried and freeze-dried samples from wild-collection or provided by different local farmers to Tukey's HSD test ($p < 0.05$).TPA-Total phenolic acids, THT-Total hydrolysable tannins, TF-Total flavonoids, TPC-Total phenolic compounds. Calibration curves: gallic acid ($y = 131538x + 292163$, $R^2 = 0.997$; LOD = 0.80 μg/mL; LOQ = 2.44 μg/mL; peaks 1 and 4); chlorogenic acid ($y = 168823\times - 161172$; $R^2 = 1.000$; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL; peak 2); caffeic acid ($y = 388345x + 406369$, $R^2 = 0.994$; LOD = 0.78 μg/mL; LOQ = 1.97 μg/mL; peak 3 and 5); ellagic acid ($y = 26719\times - 317255$, $R^2 = 0.998$; LOD = 0.41 μg/mL; LOQ = 1.24 μg/mL; peaks 6, 7 and 8); quercetin-3-*O*-glucoside ($y = 34843\times - 160173$; $R^2 = 1.000$; LOD 0.21 μg/mL; LOQ 0.71 μg/mL; peaks 9, 10 and 11); kaempferol-3-*O*-glucoside ($y = 11117x+30861$, $R^2 = 0.9999$; LOD 0.15 μg/mL; LOQ 0.41 μg/mL; peaks 12 and 14), quercetin-3-*O*-rutinoside ($y = 13343x + 76751$; $R^2 = 0.9998$; LOD 0.18 μg/mL; LOQ 0.65 μg/mL; peak 13). References applied for the tentative identification: a – Bunse et al. (2020), b – Clifford et al. (2003), c – Parveen et al. (2008), d – Finimundy et al. (2020), and e − standard, DAD and MS fragmentation pattern.

analysis regarding phenolic compounds identification and individual quantification are presented in Table 2, and a typical chromatogram is presented in Fig. 1. Fourteen phenolic compounds were detected, being one phenolic acid, six flavonoids, and seven hydrolyzable tannins. Compounds 11, 12, and 13 were positively identified as quercetin-3-*O*-glucuronide, kaempferol-3-*O*-glucoside, quercetin-3-*O*-rutinoside, respectively, by comparison with authentic standards and analysis of their MS fragmentation pattern, retention time, and UV–vis characteristics. These compounds were already reported in *S. minor* by other authors (Finimundy et al., 2020; Karkanis et al., 2019). The majority of the identified compounds (peaks 6, 7, 8, and 9) were previously identified by Finimundy et al. (2020) and, therefore, identified in this study considering the previously described molecular ions of the molecules.

Compound 2 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid based on the fragmentation pattern reported by Clifford et al. (2003). Peak 1 ($[M–H]^-$ at *m/z* 331) identified as a gallic acid glucoside was recently reported in *Sanguisorba officinalis* by Bunse et al. (2020). Peaks 3 and 5 presented the same pseudomolecular ion ($[M-H]^-$ at *m/z* 595) and fragmentation patterns; they were identified as two isomers and were assigned to caffeoyl ester based on the literature where they were found in orchardgrass (Parveen et al., 2008). Peak 14 ($[M-H]^-$ at *m/z* 447) presented the same characteristics (DAD and fragmentation pattern) as peak 12; nevertheless, no information about the identity of the sugar moiety and the location on the aglycone could be obtained, so the compound 14 was tentatively identified as kaempferol-*O*-hexoside.

OD samples analysed in the present work exhibited a higher content in total phenolic compounds than FD samples, revealing a higher concentration of phenolic acids, total hydrolyzable tannins, and total flavonoids. As for the qualitative composition, FD samples did not reveal the presence of lambertianin C, sanguiin-H, and quercetin-*O*-hexoside gallate in wild samples. Moreover, both the OD and the FD samples did not present quercetin-*O*-rutinoside in wild plants and kaempferol-3-*O*-glucoside in F3 cultivated plants, showing that not only the drying method can affect the phenolic composition but also the different growing technique and the double effect of drying method and different growing technique. However, in this study, the cultivation parameters (temperature, moisture, solar irradiation, and soil or substrates characteristics) were not standardised during the experiment, which must have the main purpose of explaining how different drying methods affect the phytochemical content and the bioactive properties of *S. minor* species. Nevertheless, we considered useful the domestication of a new species as *S. minor* for the future marketability of a relatively known species.

Most studies reported FD as the most efficient drying method because this method leads to the rupture of cell structure caused by ice crystals formed within plant matrix and, consequently, promotes the extraction efficiency in FD samples, retaining the phenolic content (Meng et al., 2018; Pinela et al., 2012). Conversely, other authors described that 60 °C used in OD is the most indicated strategy in maintaining phytochemical content (Ye et al., 2019; Youssef & Mokhtar, 2014), which followed the results obtained in the present study. Moreover, the obtained results suggested that phenolic compounds deterioration may be affected by many factors other than temperature treatments. These factors may include the activity of polyphenol oxidase (PPO), drying times, and plant moisture (Youssef & Mokhtar, 2014). PPO catalyzes the oxidation of phenols into quinones, which subsequently deteriorate and polymerize into brown pigments (Terefe et al., 2010). Cellular destruction due to extensive drying times promotes phenolic compounds' loss by increasing their polarity in water. In this way, water allows phenolic compounds to dissolve, being dragged to the surface (Alean et al., 2016). Moreover, the higher phenolic content of OD samples compared to FD samples could be linked to more effective extraction of the insoluble phenolic compounds such as phenolic acids or condensed tannins linked to cell wall polysaccharides or, more specifically, proteins (Shonte et al., 2020).

3.2. Antioxidant activity

S. minor extracts with different origins i.e., collected as wild (W) or cultivated in different local farms (F1, F2, F3) were tested for the capacity to inhibit the lipid peroxidation. Fig. 2 reports results obtained by the TBARS assay.

Comparing OD and FD plant material from the same origin, OD *S. minor* plants revealed the highest lipid peroxidation inhibition by TBARS assay (EC_{50} of OD W, F1, F2, F3 samples = 0.005 ± 0.0002; 0.03 ± 0.002; 0.005 ± 0.0002; 0.03 ± 0.0001 μg/mL, respectively). The

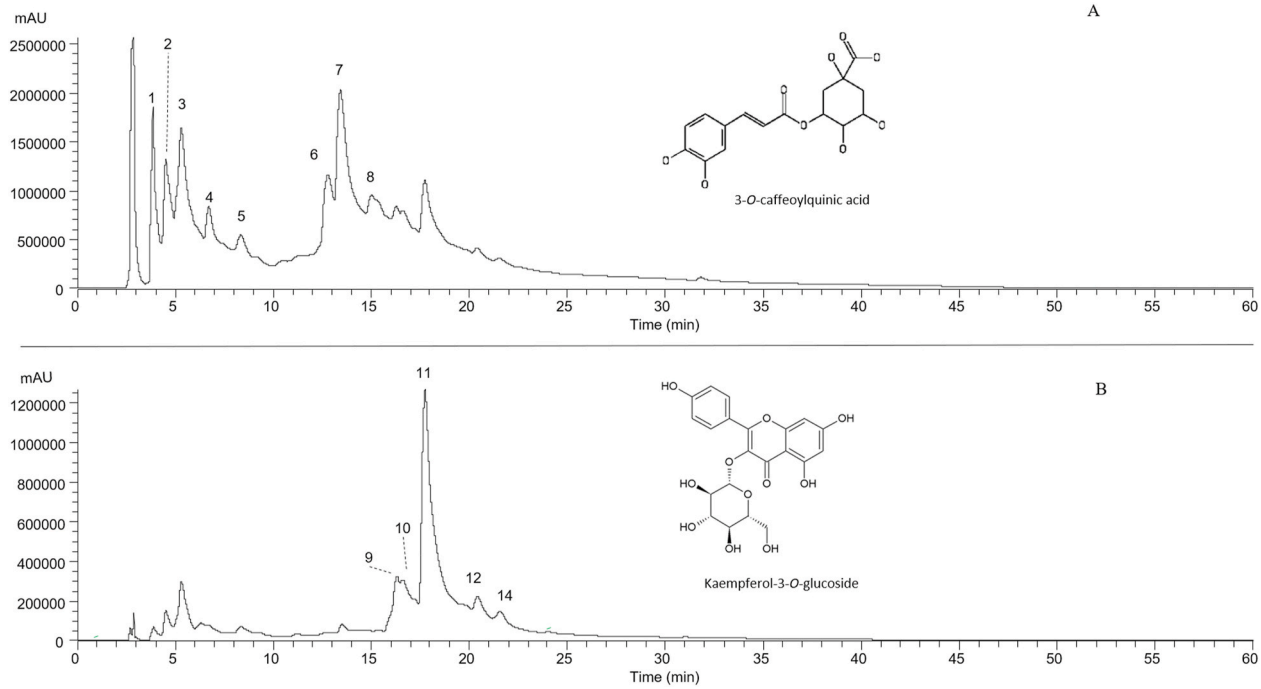

Fig. 1. Phenolic profile of oven-dried *S. minor* plant collected as wild recorded at 280 nm (A) and 320 nm (B). For peak, numbers refer to Table 2.

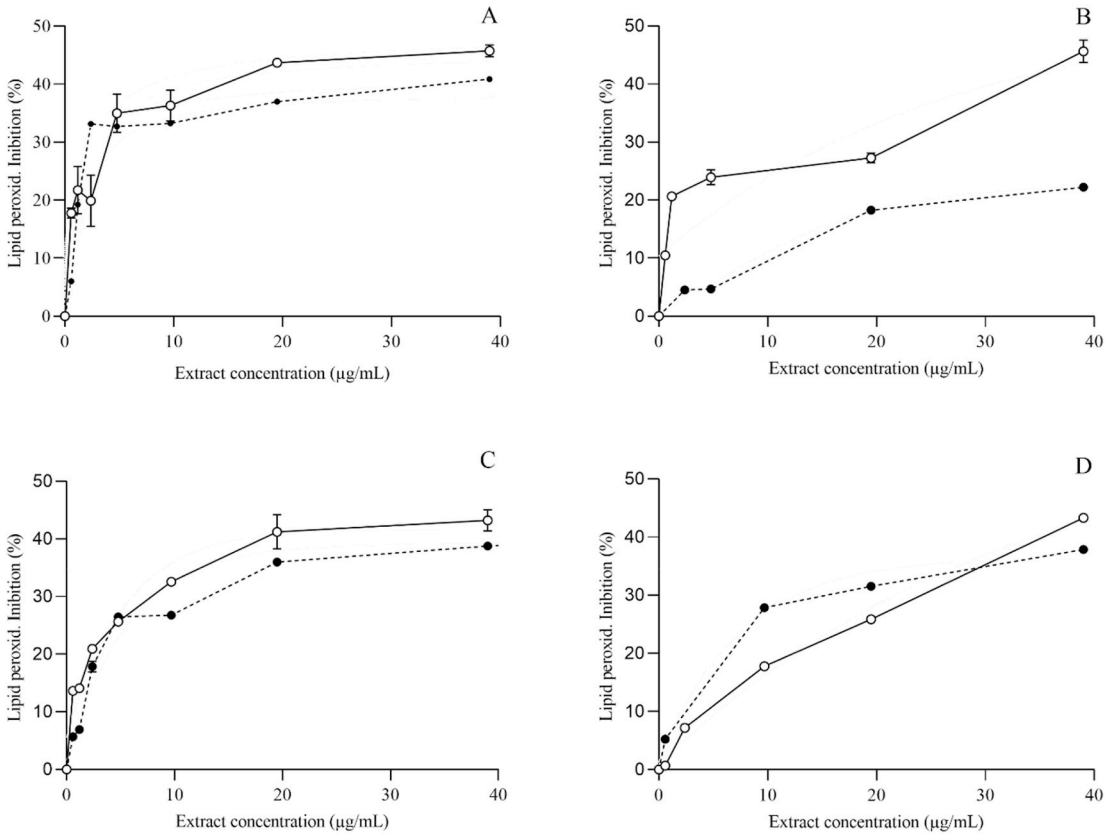

Fig. 2. Percentage of lipid peroxidation inhibition in extracts of oven-dried (–○–) and freeze-dried samples (-●-) of *S. minor* from different origins. EC_{50}: extract concentration corresponding to half of lipid peroxidation inhibition. Trolox standard (EC_{50} values): 0.17 ± 0.01 µg/mL. Oven-dried samples: EC_{50} = 0.005 ± 0.0002; 0.03 ± 0.002; 0.005 ± 0.0002; 0.03 ± 0.0001 µg/mL in plant material collected as wild (A–W) or provided by three different local farms B–F1; C–F2; D-F3), respectively; freeze-dried samples: EC_{50} = 0.04 ± 0.001; 0.06 ± 0.006; 0.02 ± 0.003; 0.05 ± 0.003 µg/mL in plant material collected as wild (A) or provided by three different local farms (B, C, D like F1, F2 and F3), respectively.

weakest activity was observed in FD samples, particularly in samples from F1 and F3 (EC_{50} 0.06 ± 0.006 and 0.05 ± 0.003 µg/mL, respectively). These results were in disagreement with some authors that reported the FD as the most effective method to preserve medicinal and food plants and their nutraceutical properties (Asami et al., 2003; Meng et al., 2018). However, these results agree with recent findings of other authors (Barroso et al., 2018; Roshanak et al., 2016) as well as with the content of phenolic compounds observed in both OD and FD samples in the present study (Table 2). Indeed, Roshanak et al. (2016), comparing seven different drying treatments (sun, shade, oven 60 °C, oven 80 °C, oven 100 °C, microwave, and freeze-drying), observed the maintenance of the antioxidant activity in green tea dried in the oven at 60 °C, whilst the other drying methods reported a decrease of antioxidant activity. Moreover, Barroso et al. (2016) showed higher antioxidant activity in OD *Stevia rabaudiana* Bertoni than in fresh frozen samples.

3.3. Antibacterial activity

The antibacterial activity of OD and FD *S. minor* samples was tested against strains of eight bacteria: *E. coli, K. pneumoniae, M. morganii, P. mirabilis, P. aeruginosa, E. faecalis, L. monocytogenes,* and MRSA and the results were expressed in Table 3. The MIC values varied from 2.5 mg/

Table 3
Antibacterial activity of oven-dried and freeze-dried samples of *S. minor*.

	Oven-dried				Freeze-dried				Ampicilin	Vancomicyn
	W	F1	F2	F3	W	F1	F2	F3		
Antibacterial activity (mg/mL)									Control (20 mg/mL)	
Bacteria	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Gram-negative										
Escherichia coli	0.5	5	5	10	5	5	5	10	<0.15	nt
Klebsiella pneumoniae	5	10	10	10	10	20	20	10	10	nt
Morganella morganii	5	10	10	10	10	10	10	10	20	nt
Proteus mirabilis	10	10	10	20	10	20	>20	20	<0.15	nt
Pseudomonas aeruginosa	10	10	10	10	10	10	>20	10	>20	nt
Gram-positive										
Enterococcus faecalis	5	5	10	10	5	5	5	10	<0.15	<0.0078
Listeria monocytogenes	5	5	10	10	20	10	10	10	<0.15	nt
MRSA	2.5	5	5	20	5	10	5	20	<0.15	0.25

MIC – minimal inhibitory concentration; MRSA-methycilin resistant *S. aureus*; nt: not tested; W–wild-collected samples; F1, F2, F3–samples provided by local farmers.

mL to >20 mg/mL in OD and FD *S. minor* samples. The MBC values were not possible to obtain since the maximum concentration of 20 mg/mL did not present bactericidal activity. All the plant extracts revealed antibacterial activity independently to the growing technique (wild or cultivated), showing higher antibacterial activity in OD samples compared to that of FD samples in most cases. Moreover, each OD wild or cultivated exhibited a higher ability to inhibit the growth of different bacteria if compared with FD samples and, in particular, OD W plants exhibited higher inhibition of *E. coli, K. pneumonia, M. morganii, L. monocytogenes,* and MRSA growth than FD W plants. OD F1 plants showed higher activity against *K. pneumoniae, P. mirabilis, L. monocytogenes,* and MRSA, OD F2 plants against *K. pneumoniae, P. mirabilis* and *P. aeruginosa* and OD F3 plants against *K. pneumoniae, P. mirabilis, P. aeruginosa, E. faecalis* and *L. monocytogenes* than FD F1, F2, F3 samples, respectively. The only exception was the higher antibacterial activity showed by FD F2 plants towards the *E. faecalis* than the OD F2 plants. In general, the more sensitive bacteria to *S. minor* was *E. coli* among Gram-negative and MRSA among Gram-positive bacteria.

In contrast, the most resistant bacteria proved to be *P. mirabilis* and *L. monocytogenes.* The high antibacterial activity of *S. minor* was confirmed by other authors, even though with lower MIC and MBC values (Finimundy et al., 2020; Karkanis et al., 2019). Karkanis et al. (2019) observed MIC and MBC values of domesticated *S. minor* samples, against *Bacillus cereus, S. aureus, L. monocytogenes* and *Salmonella typhimurium* (0.075–0.45 mg/mL and 0.25–0.60 mg/mL, respectively). Beside, Finimundy et al. (2020) reported MIC and MBC values between 2.31 and 0.44 mg/mL; and between 4.61 and 0.88 mg/mL, respectively, against *S. aureus, B. cereus*, *M. flavus, L. monocytogenes, P. aeruginosa, S. typhimurium, E. cloacae,* and *E. coli,* analysing cultivated *S. minor* plants under different fertilization regimes. Despite the achievement of a lower antimicrobial activity than that previously reported by other authors, the present results corroborated *S. minor* species and highlighted its potential as a functional ingredient, especially if subjected to the OD method as the most effective drying technique.

3.4. Cytotoxic and anti-inflammatory activities

The results from the cytotoxic and anti-inflammatory activities of OD and FD *S. minor* samples are presented in Table 4. Regarding the cytotoxic activity, OD W samples exhibited EC_{50} values ranging from 230 ± 1 to 305 ± 7 μg/mL, and FD W samples from 263 ± 6 to 327 ± 8 μg/mL. Both OD and FD W samples presented high activity against the HeLa cell line. Regarding OD samples, the lowest growth inhibition was observed in HepG2 and MCF-7 cell lines that reported 305 ± 7 and 301 ± 7 μg/mL, respectively. On the other hand, the FD samples confirmed the lowest growth inhibition of HepG2 cell line (up to 400 μg/mL), not showing hepatotoxicity to this cell line. However, wild extracts revealed the most potent capacity to inhibit the growth of the studied tumor cell lines. Among the domesticated samples, none of the extracts presented cytotoxic activity, except for the samples from F3 OD and F2 FD that revealed cytotoxicity against HeLa cells in a concentration of 225 ± 10 and 343 ± 5 μg/mL, respectively. The results of this study resulted in agreement with other authors (Finimundy et al., 2020; Karkanis et al., 2019). Karkanis et al. (2019) observed that the extracts from *S. minor* roots showed the highest activity against HeLa (GI_{50} = 75 μg/mL). Similarly, Finimundy et al. (2020) reported the highest activity against HeLa (GI_{50} range from 55 ± 6 to 99 ± 5 μg/mL) from *S. minor* leaves and roots subjected to different fertiliser regimes. Conversely, the weakest activity was verified against NCl–H460 (GI_{50} range from 65 ± 6 to 293 ± 21 μg/mL), confirming our results and suggesting that the growing conditions may affect the cytotoxic activity. Regarding anti-inflammatory activity, to the best of our knowledge, only few reports are available in the literature reporting the activity of *S. minor*. According to the obtained results, the analysed extracts did not present activity at the maximum tested concentration (400 μg/mL) (Table 3). In contrast, Su et al. (2018) suggested that isolated compounds from the *S. officinalis* exhibited anti-inflammatory activity (20 μM), and methanol extract (40 μg/mL) decreased the production of TNF-α in LPS-activated RAW264.7. Arıhan et al. (2015) utilised aqueous *S. minor* (maceration) extracts and obtained anti-inflammatory activity at 25 mg/kg in rats.

4. Conclusions

A renewed interest in *S. minor* species has been showed by scientific literature for its edibility and its nutraceutical properties due to the high content in phenolic compounds. However, appropriate methods must be applied to preserve the nutraceutical properties of this species during the storage. The present study showed that OD samples presented a higher antioxidant and antimicrobial activity and a higher content in phenolic compounds when compared with FD samples, showing that the OD resulted the most indicated drying method to preserve and retain these bioactive compounds. Moreover, some differences were observed between wild and domesticated samples. Wild samples showed higher antimicrobial and cytotoxic activity than domesticated samples. Indeed, the domesticated samples did not report cytotoxic activity against the analysed cell lines, except for the OD F3 and FD F2 samples that revealed cytotoxicity against HeLa cells in a concentration of 225 ± 10 and 343 ± 5 μg/mL, respectively. Further researches are necessary to standardise the domestications and to verify the highest efficiency of OD storage of *S. minor* species.

CRediT authorship contribution statement

Costanza Ceccanti: Methodology, Investigation, Writing – original draft. **Tiane C. Finimundy:** Conceptualization, Methodology, Writing – original draft. **Sandrina A. Heleno:** Conceptualization, Methodology,

Table 4
Anti-inflammatory and cytotoxic activity of oven-dried and freeze-dried samples of *S. minor*.

	Oven-dried				Freeze-dried			
	W	F1	F2	F3	W	F1	F2	F3
Anti-inflammatory activity (EC_{50}, μg/mL)								
RAW 246.7	>400	>400	>400	>400	>400	>400	>400	>400
Cytotoxic activity (GI_{50}, μg/mL)								
MCF-7	301±7[a]	>400	>400	>400	323±9[a]	>400	>400	>400
NCI–H460	252±4[a]	>400	>400	>400	327±8[b]	>400	>400	>400
HeLa	230±1[a]	>400	>400	225 ± 10[a]	263±6[b]	>400	343±5[c]	>400
HepG2	305±7[a]	>400	>400	>400	>400	>400	>400	>400
PLP	>400	>400	>400	>400	>400	>400	>400	>400

EC_{50} values corresponded to the extract concentration that inhibits half of the inflammatory process. Dexamethasone (IC_{50} values): 16 ± 1 μg/mL. GI_{50} values correspond to the concentration that causes half of the inhibition of cell proliferation; MCF-7 - human breast adenocarcinoma; NCI–H460 - human lung carcinoma; HeLa - human cervix adenocarcinoma; HepG2 - hepatocellular carcinoma; PLP - primary culture of non-tumoral pig liver cells. Ellipticine (GI_{50} values): MCF-7: 1.21 ± 0.02; NCI–H460: 0.91 ± 0.11; HeLa: 1.03 ± 0.09; HepG2: 1.1 ± 0.09; PLP: 2.29 ± 0.18 μg/mL. Results expressed in mean values ± standard deviation (SD). Different letters in the line represent significant differences ($p < 0.05$). W–wild-collected samples; F1, F2, F3–samples provided by local farmers.

Writing – review & editing. **Tania C.S.P. Pires:** Methodology, Writing – original draft. **Ricardo C. Calhelha:** Methodology. **Lucia Guidi:** Writing – review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Writing – review & editing. **Lillian Barros:** Conceptualization, Methodology, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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