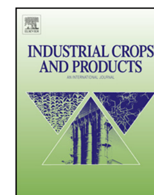
Industrial Crops and Products 87 (2016) 116–123

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop



Improvement of antioxidant activity and polyphenol content of *Hypericum perforatum* and *Achillea millefolium* powders using successive grinding and sieving

Loïc Becker[a,*,1], Ali Zaiter[a,1], Jeremy Petit[b], David Zimmer[b], Marie-Céleste Karam[b], Elie Baudelaire[c], Joël Scher[b], Amadou Dicko[a]

[a] Université de Lorraine, UR-AFPA (Unité de Recherche Animal et Fonctionnalité des Produits Animaux), 1, Boulevard Arago, Metz Cedex 03, 57078, France
[b] Université de Lorraine, LIBio (Laboratoire d'Ingénierie des Biomolécules), 2, Avenue de la Forêt de Haye, TSA 40602, 54518 Vandœuvre-lès-Nancy, France
[c] AGRITECH, 4, Rue Piroux, 54000 Nancy, France

ARTICLE INFO

Article history:
Received 25 January 2016
Received in revised form 21 March 2016
Accepted 14 April 2016
Available online 22 April 2016

Keywords:
Plant powders
Granulometry
Polyphenols
Antioxidant activity
LC-ESI/MS

ABSTRACT

This work aims at evaluating the effect of successive grinding and sieving processes on the polyphenol content of plants. Powders of particle size ranging from 20 to 500 μm and over were produced from aerial parts of *Hypericum perforatum* and *Achillea millefolium*. The evaluation of total phenolic content and antioxidant activity, as well as the identification and quantification of some bioactive compounds by LC-ESI/MS were performed. The highest antioxidant activity was obtained for the 100–180 μm fraction: IC50 of 0.43 and 0.51 mg/mL for *H. perforatum* and *A. millefolium*, respectively. LC-ESI/MS analyses evidenced that two intermediate granulometric classes, 100–180 μm and 180–315 μm, allowed achieving the highest polyphenol content. These results show that fine grinding and sieving lead to a differential distribution of bioactive compounds according to particle size.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Extraction of bioactive compounds from plant materials gains more and more interest in the industry. Recently, there has been much interest in potential health benefits of dietary plant polyphenols, especially as antioxidants (Pandey and Rizvi, 2009). Flavonoids, a subfamily of polyphenols, exhibit several interesting biological activities in addition to their well-known antioxidant power: antibacterial, hepatoprotective, anti-inflammatory, anticancer, and antiviral (Kumar and Pandey, 2013).

Conventional extraction techniques of bioactive compounds mainly consist in maceration, Soxhlet extraction, and hydrodistillation (Azmir et al., 2013). Whereas hydrodistillation is well suited for essential oils, polyphenols extraction is usually performed through maceration in polar solvents. Indeed, Soxhlet extractions are performed at the boiling point of the solvent and hydrodistillation requires heating, inducing the thermal degradation of thermolabile compounds (Luque de Castro and García-Ayuso, 1998). Maceration allows preventing this drawback. Indeed, high recoveries can be achieved at room temperature on condition of long extraction times, usually overnight (Ćujić et al., 2015; Rostagno and Prado, 2013). To enhance selectivity of extraction techniques, preliminary plant grinding into fine particles can be carried out (Sasidharan et al., 2010).

In this context, we applied a method of grinding and sieving processes to concentrate bioactive compounds in some granulometric classes of plant powders. Grinding induces particle size reduction, broadening of particle size distribution, and increases in specific surface area, leading to the improvement of material functionalities (Zhang et al., 2012; Zhao et al., 2009). However, grinding conditions have to be carefully chosen to avoid excessive temperature increase and limit the oxidation of bioactive compounds. Indeed, local temperature increases up to 90 °C in ground materials have been reported (Hu et al., 2012), as 99% of input power is converted into heat during the grinding process (Murthy et al., 1999). Local temperature increase results from the breaking of material chemical bonds, but mostly particle friction during powder flowing in the equipment. This phenomenon induces physicochemical alterations of ground fractions (Maaroufi et al., 2000), such as material browning/darkening (Singh and Goswami, 1999), bioactive

* Corresponding author at: Unité de Recherche Animal et Fonctionnalité des Produits Animaux Université de Lorraine, 1, Boulevard Arago, F-57078 Metz Cedex 03, France.
E-mail address: loic.becker@univ-lorraine.fr (L. Becker).
[1] Loïc Becker and Ali Zaiter are the first authors of this article.

http://dx.doi.org/10.1016/j.indcrop.2016.04.036
0926-6690/© 2016 Elsevier B.V. All rights reserved.

Table 1
Sieved masses, mass fractions, and mean particle sizes of the different granulometric classes and unsieved powders of *H. perforatum* and *A. millefolium*.

Granulometric classes	*H. perforatum*			*A. millefolium*		
	Sieved mass (g)	Sieved mass fractions (%)	Mean particle size D_{50} (μm)	Sieved mass (g)	Sieved mass fractions (%)	Mean particle size D_{50} (μm)
<20 μm	0.7	0.0%	N.D.	0.3	0.0%	N.D.
20–50 μm	12	0.7%	34 ± 0	22	1.3%	45 ± 0
50–100 μm	78	4.5%	83 ± 0	58	3.5%	90 ± 2
100–180 μm	206	12.0%	190 ± 1	102	6.1%	195 ± 5
180–315 μm	314	18.3%	311 ± 0	114	6.9%	349 ± 2
315–500 μm	366	21.3%	520 ± 4	140	8.4%	485 ± 5
> 500 μm	740	43.1%	1040 ± 15	1226	73.8%	1395 ± 5
Unsieved powder (total)	1716.7	100%	438 ± 11	1662.3	100%	1143 ± 102

N.D.: not determined.

compound loss and lower organoleptic properties (Murthy et al., 1999; Singh and Goswami, 1999).

Two plants, namely *Hypericum perforatum* and *Achillea millefolium*, were chosen for their well-known biological activities to assess the suitability of the grinding/sieving procedure. *H. perforatum*, is a plant often used for its antidepressant capabilities in addition to its antioxidant, antiviral, anti-inflammatory, and antimicrobial properties (Barnes et al., 2001). Several antioxidants compounds were identified in *H. perforatum*: hyperoside (Gioti et al., 2009) which exhibit also anti-inflammatory activity (Bernardi et al., 2007), rutin (Nöldner and Schötz, 2002), I3,II8-biapigenin (Colovic and Caccia, 2008), and catechins (Ploss et al., 2001). These latter (Iacopini et al., 2008) may also prevent cancer and reduce the risk of cardiovascular diseases (Higdon and Frei, 2003). Chlorogenic acid was also detected in this plant in significant amounts (Gioti et al., 2009; Tusevski et al., 2013). In addition to its antioxidant activity, anxiolytic and antidepressant properties of chlorogenic acid have been demonstrated in mice (Bouayed et al., 2007).

The antioxidant activity of *A. millefolium*, has been evidenced by Trumbeckaite et al. (2011). Extracts of *A. millefolium* are used in European folk medicine in the treatment of gastro-intestinal, hepatobiliary, and gynecological disorders, against inflammation, and for wound healing (Benedek et al., 2007; Santoro et al., 2007). The main active compounds in *A. millefolium* are chlorogenic acid and its derivatives, namely dicaffeoylquinic acids (Vitalini et al., 2011). These phenolic acids are known for their antioxidant activity (Candan et al., 2003) and protective effects against DNA damage (Xu et al., 2012). Flavonoids have also been reported, like apigenin- and luteolin-glycoside that present antispasmodic (Vitalini et al., 2011) and estrogenic activities (Innocenti et al., 2007).

This article aims at establishing a correlation between particle size and antioxidant activity of *H. perforatum* and *A. millefolium* powders. To this end, total phenolic content was measured using UV-spectrophotometry. Some polyphenols specific to *H. perforatum* and *A. millefolium* were identified and quantified by LC-ESI/MS. Finally, antioxidant activity was evaluated using DPPH radical scavenging assay. These results were compared to the bioactive compound contents of unground plant parts to evaluate the efficiency of the grinding/sieving procedure.

2. Material and methods

2.1. Plants

Dried aerial plant parts of wild and organic *H. perforatum* and *A. millefolium* were provided by Cailleau Herboristerie (Chemillé, France). They were manually harvested in July 2014. For conservation purposes, plants were supplied at about 12% moisture content after natural air drying at ambient temperature.

2.2. Chemicals

HPLC-grade acetonitrile, water, methanol, and formic acid were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Folin-Ciocalteu phenol reagent, gallic acid, 2,2′-Azobis(2-amidino-propane) dihydrochloride (AAPH), standards of chlorogenic acid, 3.4 dicaffeoylquinic acid, 3.5 dicaffeoylquinic acid, 4.5 dicaffeoylquinic acid, (+)-catechin, (−)-epicatechin, hyperoside, luteolin-4-*O*-glucoside, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, I3,II8-biapigenin, and rutin were acquired from Sigma-Aldrich. Sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$) and sodium hydroxide (NaOH) were purchased from Acros Organics (Geel, Belgium).

2.3. Plant grinding

The fine grinding conditions were identical for both plants. Circa 1,7 kg of each dried plant was ground little by little (about 50 g per batch) with the Ultra Centrifugal Mill ZM 200 (Retsch, France), operating by impact and shearing effects. Grinding was performed at 12000 rpm and ambient temperature using a 1 mm sieve with trapezoid holes. This rotor speed was chosen as a compromise between grinding efficiency and bioactive compound retention.

2.4. Plant powder sieving

Ground plant samples were sieved with the vibratory sieve shaker Analysette 3 Spartan (Fritsch, Idar-Oberstein, Germany) at 0.5 mm vibration amplitude for 10 min. Plant powders were sieved in batches of circa 100 g to optimize sieve efficiency. 20 mm diameter sieves (Fritsch) were employed so as to obtain the following granulometric fractions: >500 μm; 315–500 μm; 180–315 μm; 100–180 μm; 50–100 μm; 20–50 μm; <20 μm. For both plants, almost no powder was found under the 20 μm sieve, precluding the analyses of these samples. Granulometric classes were weighted and then stored under vacuum in sealed bags at 10 °C.

2.5. Laser granulometry

The particle size distributions of all granulometric classes and unsieved plant powders were determined at dry state by laser diffraction granulometry (Mastersizer 3000, Malvern Instruments France, Orsay, France) at ambient temperature. The following dispersion conditions were employed to reach about 2% obscuration level:

−100% air pressure, 100% feed rate, 4 mm hopper length for unsieved powders and >500 μm granulometric class of both plants, along with 315–500 μm and 180–315 μm granulometric classes of *A. millefolium;*

−50% air pressure, 50% feed rate, 2 mm hopper length for other powder samples.

Table 2
Chemical composition (moisture, lipids, proteins, ashes, carbohydrates) on wet basis of all granulometric classes except <20 μm, and unground plant parts of *H. perforatum* and *A. millefolium*.

H. perforatum							
Sample	Unground plant	20–50 μm	50–100 μm	100–180 μm	180–315 μm	315–500 μm	> 500 μm
Moisture (%)	11.28 ± 0.39^a	10.96 ± 0.89^a	9.75 ± 0.37^b	10.03 ± 0.40^b	10.19 ± 0.12^b	10.26 ± 0.43^b	9.20 ± 0.17^c
Lipids (%)	2.94 ± 0.44^f	21.22 ± 0.73^a	13.11 ± 0.65^b	11.73 ± 0.65^c	8.32 ± 0.56^d	7.88 ± 1.35^d	4.79 ± 0.92^e
Proteins (%)	7.23 ± 0.44^d	15.67 ± 0.24^b	16.32 ± 0.64^a	17.24 ± 0.55^a	16.86 ± 0.81^a	12.31 ± 0.35^c	7.40 ± 1.18^d
Ashes (%)	3.47 ± 0.16^e	6.37 ± 0.07^a	4.90 ± 0.11^b	4.28 ± 0.08^c	4.42 ± 0.07^c	4.01 ± 0.18^d	2.14 ± 0.16^f
Carbohydrates (%)	75.08 ± 1.43^a	45.78 ± 1.93^e	55.92 ± 1.77^d	56.72 ± 1.68^d	60.21 ± 1.56^c	65.54 ± 2.31^b	76.47 ± 2.43^a
A. millefolium							
Sample	Unground plant	20–50 μm	50–100 μm	100–180 μm	180–315 μm	315–500 μm	> 500 μm
Moisture (%)	11.00 ± 0.31^a	11.20 ± 0.53^a	10.17 ± 0.22^b	9.54 ± 0.27^c	10.10 ± 0.14^b	9.40 ± 0.56^c	9.22 ± 0.07^c
Lipids (%)	4.39 ± 1.19^d	20.73 ± 1.37^a	18.91 ± 0.74^a	15.68 ± 0.88^b	7.72 ± 0.44^c	7.56 ± 0.49^c	4.02 ± 0.41^d
Proteins (%)	7.72 ± 0.58^d	15.61 ± 0.74^a	17.21 ± 1.16^a	13.91 ± 0.85^b	11.56 ± 0.25^c	11.12 ± 0.35^c	8.01 ± 0.34^d
Ashes (%)	5.79 ± 1.56^d	12.73 ± 0.14^a	8.96 ± 0.27^b	8.03 ± 0.25^c	7.70 ± 0.27^c	6.52 ± 0.29^d	5.42 ± 0.35^d
Carbohydrates (%)	71.10 ± 3.64^a	39.73 ± 2.78^e	44.75 ± 2.39^e	52.84 ± 2.25^d	62.92 ± 1.10^c	65.40 ± 1.69^b	73.33 ± 1.17^a

Presented data are mean ± standard deviation of triplicate measures; for each plant, mean values in the same line followed by different letters were statistically different ($p < 0.05$).

Particle sizes were expressed in terms of equivalent spherical diameters in volume. The mean particle size D_{50} was defined as the diameter for which 50% of the volume of particles has a lower diameter.

2.6. Powder composition

Analyses of powder composition (moisture, lipids, proteins, minerals, and carbohydrates) were performed for all granulometric fractions where sufficient amount of powder was available and for the unground plant parts. Water content was measured by weight loss after drying 2 g of powder at 105 °C for 5 h (AFNOR, 1976 AFNOR, 1978). Total protein content was determined by the Kjeldhal method for quantification of nitrogen, using a conversion factor of 6.25 that is suitable for plants (AACC International, 1999a). Fat content was quantified by Folch method (Folch et al., 1957) using 1 (20–50 μm granulometric classes) or 3 g (other samples) of sample. Mineral content was determined by putting 2 g of powder at 500 °C for 5 h until formation of white ashes (ADPI, 2002). Carbohydrate content was calculated by the difference between 100% and the contents in other components; its standard error was deduced by propagation of uncertainties.

2.7. Preparation of extracts

2 g of powder of dried aerial parts were extracted with 20 mL of 70% methanol. Maceration was performed overnight under stirring (300 rpm) in order to extract all extractable compounds and reach the same extraction yield for small and large particles (Ćujić et al., 2015). This soft extraction technique that does not require heating or adding acid was chosen to prevent phenolic compound degradation. Then, hydroalcoholic extracts were centrifuged under 3460*g* for 20 min, the supernatant was filtered, brought to 15 mL with 70% methanol, and stored at 4 °C until analysis.

2.8. UV–vis analyses

2.8.1. Total phenolic content

Total phenolic content (TPC) were measured as described by Wafa et al. (2014). 40 μL of samples was added to test tubes containing 3160 μL distilled water, followed by addition of 200 μL Folin-Ciocalteu reagent (1 N) and 600 μL sodium carbonate (20%). Samples and blank were thoroughly mixed and vortexed. After 40 min of incubation at room temperature, absorbance was measured at 725 nm. Gallic acid (from 1 to 3 mg/mL by 0.5 mg/mL steps) was used for calibration and total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g DM). UV–vis analyses were carried out with the Cary 50 Scan UV–vis spectrophotometer (Agilent, Santa Clara, California, USA).

2.8.2. Antioxidant activity: DPPH assay

Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation (Roginsky and Lissi, 2005). The DPPH radical scavenging activity was evaluated according to the method detailed by Sharififar et al. (2009). 1 mL of 100 μM DPPH solution in methanol was mixed with 1 mL plant extract. The reaction mixture was incubated in the dark for 20 min and its optical density was recorded at 517 nm against the blank. For the control, 1 mL DPPH methanolic solution was mixed with 1 mL methanol. The DPPH radical scavenging activity of phenolic compounds was expressed in terms of IC50 values that refers to the smallest concentration of antioxidants required to scavenge 50% of the DPPH radical.

2.9. LC-ESI/MS analyses

2.9.1. Analytical conditions

LC-ESI/MS analyses were performed with a LC–MS 2020 system (Shimadzu, Tokyo, Japan) associated with an electrospray ionization source. Separation was carried on a Gemini 3 μm C18 130 Å reversed phase column (Phenomenex, Torrance, CA, USA) of 150 mm length and 4.6 mm i.d. The mobile phase consisted in a mixture of (A) 0.5% formic acid in aqueous solution and (B) acetonitrile. The injection volume was 20 μL and the flow rate was set at 0.6 mL/min. The column oven temperature was fixed at 30 °C. The following elution gradient was used: 0–22.5 min: linear gradient from 90:10 (A:B) to 50:50; 22.5–23 min: linear gradient from 50:50 to 10:90; 23–29 min: elution at 10:90; 29–29.5 min: linear gradient from 10:90 to 90:10; 29.5–35 min elution at 90:10 (re-equilibration step). The mass spectrometer settings were identical for all plant extracts: negative mode ESI source, 1.5 L/min nebulization gas flow, 15 L/min drying gas flow, 350 °C heat block temperature, 250 °C desolvation line temperature, −4000 V probe voltage. To avoid contamination of the electrospray source, *H. perforatum* and *A. millefolium* extracts were filtrated, then diluted fifty and thirty times, respectively.

2.9.2. Calibration method

The identification of phenolic compounds in plant extracts was based on standard compound analyses confronting m/z ratio and LC retention time (t_R). For quantitative analysis, a five-level linear calibration curve was obtained by injection of known concentrations (from 10^{-4} to 10^{-2} mg/mL) of standards. Method sensitivity was assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to

Table 3
Quantification of major polyphenols in powder extracts and unground plant part extracts of *H. perforatum* and *A. millefolium*.

Compound	*m*/*z*	Retention time (min)	*H. perforatum*		*M. millefolium*	
			Powders	Unground plant	Powders	Unground plant
epicatechin	289	10.1	3.31 ± 0.12^a (100–180 μm)	2.31 ± 0.09^b	/	/
chlorogenic acid	353	8.7	0.91 ± 0.08^a (180–315 μm)	0.59 ± 0.04^b	2.76 ± 0.05^a (315–500 μm)	2.60 ± 0.01^b
hyperoside	463	13.1	26.94 ± 0.26^a (100–180 μm; 180–315 μm)	15.55 ± 0.38^b	/	/
I3,II8-biapigenin	537	22.51	1.75 ± 0.08^a (100–180 μm)	0.63 ± 0.05^b	/	/
rutin	609	12.4	9.34 ± 0.43^a (100–180 μm; 180–315 μm)	4.81 ± 0.19^b	3.78 ± 0.04^a (100–180 μm)	3.01 ± 0.02^b
apigenin-7-glucoside	431	14.9	/	/	1.73 ± 0.01^a (180–315 μm)	1.53 ± 0.01^b
luteolin-7-glucoside	447	13.1	/	/	0.95 ± 0.01^a (100–180; 180–315 μm)	0.89 ± 0.02^b
3,5 diCQa	515	14.6	/	/	7.97 ± 0.06^a (315–500 μm)	7.63 ± 0.22^b

signal-to-noise (S/N) values of 3 and 10, respectively. The following calibration parameters were obtained: chlorogenic acid ($R^2 = 0.997$; LOD = 2.2 ppm; LOQ = 7.0 ppm); 3.5 dicaffeoylquinic acid ($R^2 = 0.999$; LOD = 1.6 ppm; LOQ = 5.3 ppm); epicatechin ($R^2 = 0.999$; LOD = 1.9 ppm; LOQ = 8.3 ppm); hyperoside ($R^2 = 0.991$; LOD = 0.3 ppm; LOQ = 0.9 ppm); luteolin-7-*O*-glucoside ($R^2 = 0.994$; LOD = 1.3 ppm; LOQ = 4.4 ppm); apigenin-7-*O*-glycoside ($R^2 = 0.997$; LOD = 0.1 ppm; LOQ = 0.3 ppm), I3,II8-biapigenin ($R^2 = 0.995$; LOD = 0.3 ppm; LOQ = 0.9), and rutin ($R^2 = 0.998$; LOD = 0.3 ppm; LOQ = 0.9 ppm). Results were expressed in mg of standard per gram of dry matter.

2.10 Statistical analyses

All experiments were performed in triplicate. Data are expressed as means ± standard deviations. Statistical analysis was conducted using SAS software (v8.2, SAS Institute Inc., Cary, NC, USA). Mean values were compared by using Student's *t* test at $p < 0.05$ significance level.

3. Results and discussion

3.1. Sample granulometry

It was first ensured that the developed grinding/sieving procedure led to well-different granulometric fractions in terms of particle size. Table 1 presents sieving and laser granulometry results. The grinding/sieving procedure was effective in producing enough powder for further analyses in all granulometric classes, except the smallest one (<20 μm). The correct running of the sieving process was confirmed by the fact that mean particle sizes were systematically comprised in or slightly superior to the size range of granulometric classes. All studied powder samples presented monomodal particle size distributions, except the unsieved fraction of *A. millefolium* that was bimodal (first and second modes respectively at 375 and 1527 μm). The mass yield for the 20–500 μm fraction of *H. perforatum* and *A. millefolium* powders reached 56.8% and 26.2%, respectively. A longer grinding time and/or a higher grinding speed could increase mass yield of *A. millefolium*. Using a grinding sieve of smaller mesh can also be considered to fulfill higher mass yields for intermediate size fractions of *A. millefolium* powder.

3.2. Chemical composition

Moisture, lipids, proteins, ashes, and carbohydrate contents of all granulometric classes except the <20 μm one, and unground plant parts of *H. perforatum* and *A. millefolium* were evaluated to investigate the influence of the sieving step (Table 2). The chemical composition of studied plant powders was greatly dependent on granulometric class: when decreasing the particle size, carbohydrate contents decreased, whereas other component (lipids, proteins, and ashes) contents increased. Bioactive compounds are often associated with proteic and fatty fractions, thus the smaller granulometric fractions are expected to have higher levels of bioactive compounds. No clear trend can be drawn from moisture results: all samples had similar moisture contents, showing that the heating effect of grinding (more pronounced for smaller particles) was compensated by the higher specific surface of small particles, facilitating the absorption of surrounding air humidity. Also, all characterized powders were mainly composed of carbohydrates, in agreement with the high fiber content of plants. Fiber-rich materials are expected to be difficult to grind and thus lead to higher particle sizes, which may be the reason why the carbohydrate content fell at lower particle size. However, polyphenols bound to fibers are not accessible through conventional maceration techniques. Indeed, the covalent bonds between phenolic compounds and fiber matrix could be broken only when particle size is reduced down to the nanoscale (Van Craeyveld et al., 2009).

3.3. Total phenolic content

Total phenolic content (TPC) of samples are displayed in Fig. 1. Overall, total phenolic contents of *H. perforatum* samples were more than twice superior to the TPCs of *A. millefolium* samples. The highest TPC was found in the 100–180 μm fraction: 125 and 45 mg GAE/g DM for *H. perforatum* and *A. millefolium*, respectively. The lowest (20–50 μm) and the highest (>500 μm) particle sizes led to lower TPCs than intermediate granulometric classes (50–100 μm, 100–180 μm, 180–315 μm). This phenomenon was expected: on one hand, larger particles contained more carbohydrates and thus less bioactive compounds; on the other hand, smaller particles were subjected to a higher temperature level during grinding, which is detrimental to thermosensitive biomolecules like polyphenols. To limit heating during the process, the grinding could be assisted with airflow cooling systems or even cryogenic grinding could be used. For both plants, TPCs were significantly higher in powders than in unground plant parts, except for particle size superior to 500 μm, showing the interest of successive grinding and sieving processes to optimize bioactive compound content in certain granulometric classes of plant powders.

3.4. LC-ESI/MS analyses

3.4.1. Characterization of phenolic compounds

Plant powder extracts were analyzed by LC-ESI/MS to identify and quantify the main phenolic compounds. Extracted-ion chromatograms of *A. millefolium* and *H. perforatum* extracts are displayed in Fig. 2. The highest signal of phenolic compounds in *A. millefolium* extracts was found at *m*/*z* 515, corresponding to a dicaffeoylquinic acid. Small peaks (4 and 4", Fig. 2) were located at retention times close to the substantial peak of dicaffeoylquinic acid. By comparison with standards, peaks at 14.2, 14.8, and 15.5 min retention times were attributed to 3.4-, 3.5-, and 4.5- dicaffeoylquinic acids, respectively. This elution order of dicaffeoylquinic acids has been reported in several studies (Dias et al., 2013; Vitalini et al., 2011). Chlorogenic acid (3-*O*-caffeoylquinic acid) was detected at *m*/*z* 353 and $t_R = 8.7$ min in

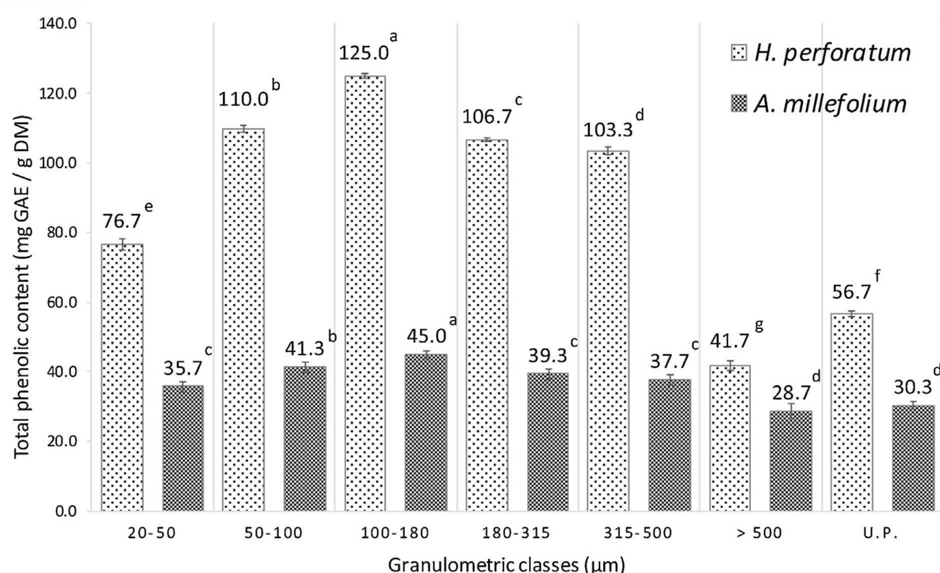


Fig. 1. Total phenolic content and antioxidant activity of *H. perforatum* and *A. millefolium* for the following granulometric classes: 20–50 µm, 50–100 µm, 100–180 µm, 180–315 µm, 315–500 µm, >500 µm and for unground plant "U.P". Presented data are mean ± standard deviation of triplicates (n = 3). Means for a same plant followed by different letters were statistically different ($p < 0.05$).

both *A. millefolium* and *H. perforatum* extracts (peaks 1 and B, Fig. 2). As for flavonoids, epicatechin (t_R = 10.1 min; peak A', Fig. 2), I3,II8-biapigenin (t_R = 22.5 min; peak D, Fig. 2), and hyperoside (t_R = 13.1 min; peak C, Fig. 2) were identified in *H. perforatum*. The latter, a glycosylated quercetin, was the most intense signal recorded for *H. perforatum* extracts. Rutin exhibited intense signals in both plants (t_R = 12.5 min; peaks 5 and D, Fig. 2). Finally, apigenin-7-*O*-glucoside (t_R = 15.1 min) and luteolin-7-*O*-glucoside (t_R = 13.2 min), antiplasmodial compounds, were detected exclusively in *A. millefolium* (peaks 2 and 3′, Fig. 2). The presence of luteolin-4-*O*-glucoside was also confirmed at t_R = 11.1 min (peak 3, Fig. 2).

3.4.2. Quantification of phenolic compounds

The main antioxidant compounds identified in the previous subsection were quantified in the different powder and unground plant extracts (Table 3). In *H. perforatum* extracts, the highest contents in bioactive compounds were found in the 100–180 and 180–315 µm granulometric classes. Hyperoside and I3,II8-biapigenin exhibited the highest content in the 100– 180 fraction: 26.94 and 1.75 mg/g DM, respectively. Rutin content was also noteworthy in intermediate granulometric classes (100–180 µm and 180–315 µm). In *A. millefolium* extracts, phenolic acids were present in important proportions, especially 3.5 dicaffeoylquinic acid (7.97 mg/g DM in the 315–500 µm fraction). Chlorogenic acid content in *A. millefolium* extracts was also higher than in *H. perforatum* extracts. Luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside were the most concentrated in the 100–180 and 180–315 µm granulometric classes: 0.95 and 1.73 mg/g DM, respectively. Rutin was three times more concentrated in *H. perforatum* than in *A. millefolium*.

Overall, polyphenol contents were significantly higher in powder fractions compared to unground plants. Discrepancies between powders and unground plant parts for *A. millefolium* were less important than for *H. perforatum* extracts, but still notable. Finally, these results confirm the trend drawn from the total phenolic content results: the 100–180 µm and 180–315 µm fractions showed the highest contents in bioactive compounds, confirming the efficiency of the grinding and sieving procedure in isolating granulometric classes with higher bioactive compound contents.

Table 4
Antioxidant activity of *H. perforatum* and A. millefolium for the following granulometric classes: 20–50 µm, 50–100 µm, 100–180 µm, 180–315 µm, 315–500 µm, >500 µm and for unground plant parts "U.P".

Granulometric classes (µm)	Antioxidant activity (IC50 mg/mL)	
	H. perforatum	*A. millefolium*
20–50	0.50 ± 0.01[a]	0.76 ± 0.02[a]
50–100	0.44 ± 0.02[b]	0.64 ± 0.04[b]
100–180	0.43 ± 0.02[b]	0.51 ± 0.03[c]
180–315	0.47 ± 0.01[c]	0.58 ± 0.01[d]
315–500	0.51 ± 0.02[a]	0.74 ± 0.03[a]
> 500	0.66 ± 0.03[d]	0.84 ± 0.01[e]
U.P.	0.60 ± 0.01[e]	0.80 ± 0.03[a]

Data were mean ± standard deviation of triplicate (n = 3). Means in the same column followed by different letters were statistically different ($p < 0.05$).

LC-ESI/MS enabled to observe a differential bioactive compound distribution according to particle size. This cannot be attributed to mass transfer limitations according to particle size, as similar extraction yields are expected for large and small particles when using such long maceration times (over 12 h).

3.5. Antioxidant activity

The antioxidant activity of the different powder fractions from *H. perforatum* and *A. millefolium* was evaluated by the DPPH cation radical scavenging assay in terms of IC50 (Table 4). The lower the IC50 value, the more reactive the considered bioactive compound. Maximum antioxidant activities were obtained for the 100–180 µm granulometric classes with IC50 of 0.43 and 0.51 mg/mL for *H. perforatum* and *A. millefolium*, respectively. This confirms the better antioxidant capacity of *H. perforatum* compared to *A. millefolium*. IC50 values were consistent with literature ones: Dias et al. (2013) evaluated the IC50 at 0.50 mg/mL for methanol extract of *A. millefolium*, and the IC50 values determined by Gioti et al. (2009) were comprised between 0.18 and 0.23 mg/mL for *H. perforatum*. Observed differences can be due to intrinsic factors, mainly genetics and plant parts selected for extractions; or extrinsic factors, such as climatic factors, processing conditions (in particular during grinding), and storage conditions (Ghasemnezhad et al., 2011).

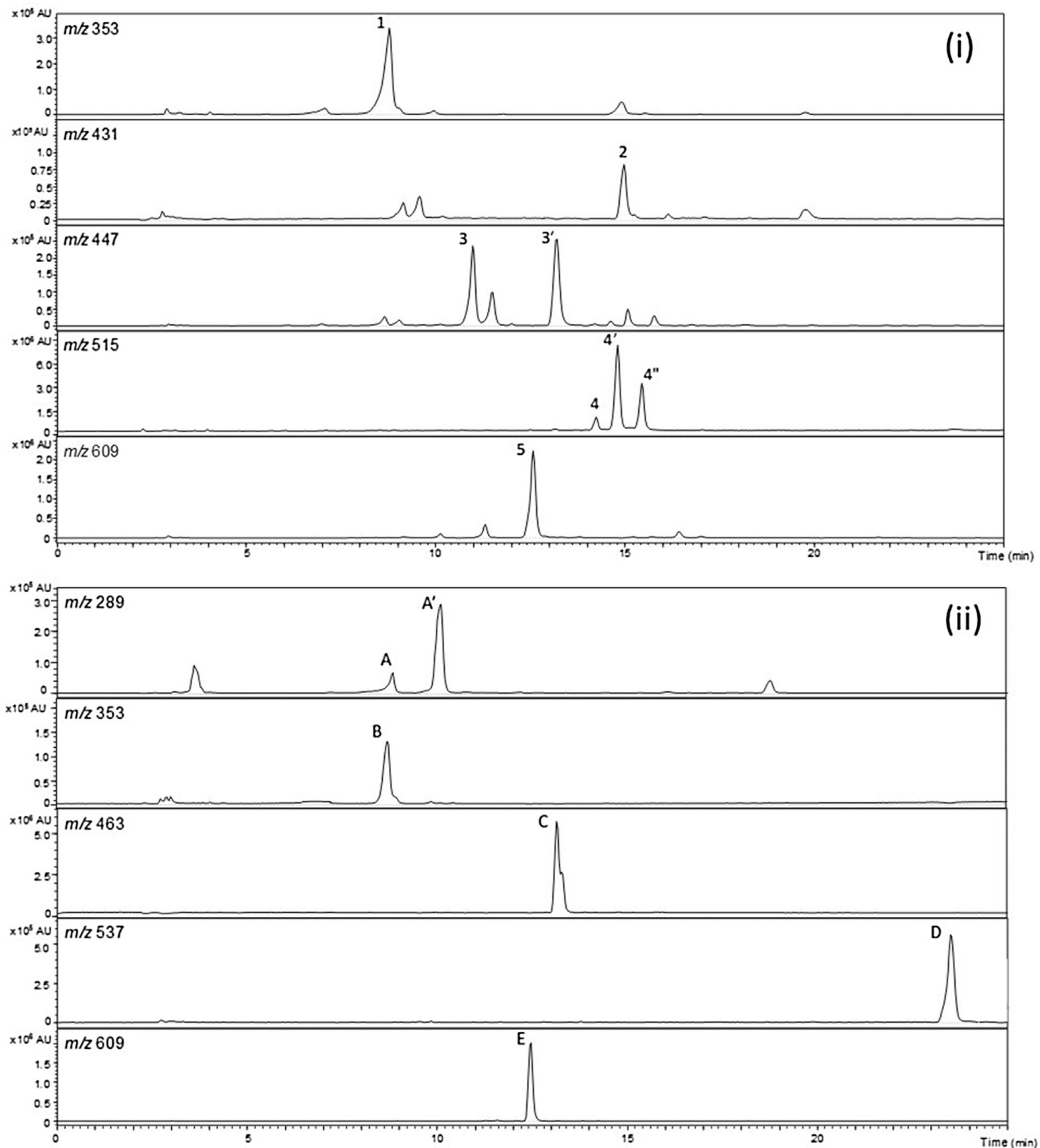


Fig. 2. Extracted-ion chromatograms from LC-ESI/MS analyses of *A. millefolium* (i) and *H. perforatum* (ii) methanolic extracts. Peaks related to *A. millefolium*: chlorogenic acid (1); apigenin-7-*O*-glucoside (2); luteolin-7-*O*-glucoside (3); luteolin-4-*O*-glucoside (3′); 3.4-dicaffeoylquinic acid (4); 3.5-dicaffeoylquinic acid (4′); 4.5-dicaffeoylquinic acid (4"); rutin (5). Peaks related to *H. perforatum*: (+)-catechin (A); (−)-epicatechin (A'); chlorogenic acid (B); hyperoside (C); I3,II8-biapigenin (D); rutin (E).

A similar distribution of antioxidant activity according to granulometric classes was observed for both plants. As previously stated, the observed drop of the antioxidant activity for the smaller particle sizes could be due to the local temperature rise during grinding (causing thermal degradation of thermosensitive bioactive compounds) and to their higher specific area of particles (resulting in higher moisture content, see Table 2). IC50 values for unground plants were significantly higher than IC50 values of powders, except for particle sizes superior to 500 μm, showing that the highest fraction was depleted in bioactive compounds, consistently with its greater carbohydrate (fiber) and moisture contents. The fact that the highest antioxidant activity was obtained from intermediate granulometric classes (50–100 μm, 100–180 μm, and 180–315 μm) confirms the existence of an optimal particle size range for bioactive compound extraction.

Finally, a marked positive correlation was observed between DPPH tests and total phenolic content: the calculated Pearson's correlation coefficient for all investigated samples was equal to 0.87. The increase in the antioxidant activity of studied powders and unground plant parts corresponds to an increase in total phenolic content, both dependent on particle size. This means that antioxidant activity of *H. perforatum* and *A. millefolium* powders mainly derived from polyphenols as described by Orčić et al. (2011).

4. Conclusion

In present work, the effect of successive grinding and sieving processes of *H. perforatum* and *A. millefolium*, plants rich in bioactive compounds, was investigated. Grinding enabled a better accessibility of these compounds towards solvent extraction in resulting powders compared to unground plants. The bioactive compound contents were strongly dependent on particle size, revealing a differential distribution of compounds after the sieving step. DPPH radical scavenging assay, total phenolic content, and LC-ESI/MS quantifications confirmed this conclusion and the existence of optimal granulometric classes for bioactive compound extraction: maximal antioxidant activities and bioactive compound contents were reached for intermediate powder fractions (50–100 μm, 100–180 μm and 180–315 μm) of *H. perforatum* and *A. millefolium*. The lowest fractions led to a loss of polyphenol content owing to the temperature rise during grinding and the increase in the specific area of particles, while fractions over 315 μm are expected to contain more fibers, and so, less bioactive compounds. Thus, this study establishes a link between the antioxidant activity and the particle size of plant powders.

Acknowledgments

The authors thank the "Lorraine Extrapole" project group and the Lorraine region for financial support.

The authors also thank Dr. Florentin Michaux (LIBio) for his expertise about dispersion conditions in laser diffraction analysis, Carole Perroud (LIBio) for her contribution to powder production, and Carole Jeandel and Carole Perroud (LIBio) for their advices about analytical protocols used to determine powder chemical composition.

References

AACC International, 1999a. Method 46-11.02. In: Crude Protein—Improved Kjeldahl Method, Copper Catalyst Modification. Approved Methods of Analysis, 11th ed. AACC International, St. Paul, MN, USA (reapproved November 3 1999).

ADPI (American Dry Product Institute, Elmhurst, IL), 2002. Determination of toal ash in standards for grades of dry milks including methods of analysis. Bulletin 916, 40–41.

AFNOR (Agence Française de NORmalisation), 1976. Détermination de la teneur en eau—méthode par étuvage, No: V04–348.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N., Omar, A.K.M., 2013. Techniques for extraction of bioactive compounds from plant materials: a review. J. Food Eng. 117, 426–436, http://dx.doi.org/10.1016/j.jfoodeng.2013.01.014 (SI: Extraction and Encapsulation).

Barnes, J., Anderson, L.A., Phillipson, J.D., 2001. St John's wort (*Hypericum perforatum* L.): a review of its chemistry: pharmacology and clinical properties. J. Pharm. Pharmacol. 53, 583–600.

Benedek, B., Gjoncaj, N., Saukel, J., Kopp, B., 2007. Distribution of phenolic compounds in middleeuropean taxa of the *Achillea millefolium* L. Aggregate. Chem. Biodivers. 4, 849–857, http://dx.doi.org/10.1002/cbdv.200790072.

Bernardi, A.P.M., López-Alarcón, C., Aspee, A., Rech, S., Von Poser, G.L., Bride, R., Lissp, E., 2007. Antioxidant activity of flavonoides isolated from Hypericum ternum. J. Chil. Chem. Soc. 52, 1326–1329, http://dx.doi.org/10.4067/S0717-97072007000400013.

Bouayed, J., Rammal, H., Dicko, A., Younos, C., Soulimani, R., 2007. Chlorogenic acid, a polyphenol from Prunus domestica (Mirabelle), with coupled anxiolytic and antioxidant effects. J. Neurol. Sci. 262, 77–84, http://dx.doi.org/10.1016/j.jns.2007.06.028, Environmental Neurology Proceedings of the Meetings of the Environmental Neurology Club, held in Paris, France, 2–3 December 2005 and in Metz, France, 7 February 2007, under the aegis of the French Society of Neurology, The University of Metz, and the Environmental Neurology Research Group of the World Federation of Neurology (ENRG-WFN).

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., Ibrić, S., 2015. Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. Food Chem. 194, 135–142, http://dx.doi.org/10.1016/j.foodchem.2015.08.008.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sökmen, A., Akpulat, H.A., 2003. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. millefolium Afan (Asteraceae). J. Ethnopharmacol. 87, 215–220, http://dx.doi.org/10.1016/S0378-8741(03)00149-1.

Colovic, M., Caccia, S., 2008. Liquid chromatography–tandem mass spectrometry of I3 II8-biapigenin, the major biflavone in *Hypericum perforatum* extracts. J. Chromatogr. B 863, 74–79, http://dx.doi.org/10.1016/j.jchromb.2008.01.014.

Dias, M.I., Barros, L., Dueñas, M., Pereira, E., Carvalho, A.M., Alves, R.C., Oliveira, M.B.P.P., Santos-Buelga, C., Ferreira, I.C.F.R., 2013. Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract, infusion and decoction. Food Chem. 141, 4152–4160, http://dx.doi.org/10.1016/j.foodchem.2013.07.018.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Ghasemnezhad, M., Sherafati, M., Payvast, G.A., 2011. Variation in phenolic compounds, ascorbic acid and antioxidant activity of five coloured bell pepper (Capsicum annum) fruits at two different harvest times. J. Funct. Foods 3, 44–49, http://dx.doi.org/10.1016/j.jff.2011.02.002.

Gioti, E.M., Fiamegos, Y.C., Skalkos, D.C., Stalikas, C.D., 2009. Antioxidant activity and bioactive components of the aerial parts of *Hypericum perforatum* L. from Epirus. Greece. Food Chem. 117, 398–404, http://dx.doi.org/10.1016/j.foodchem.2009.04.016.

Higdon, J.V., Frei, B., 2003. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit. Rev. Food Sci. Nutr. 43, 89–143, http://dx.doi.org/10.1080/10408690390826464.

Hu, J., Chen, Y., Ni, D., 2012. Effect of superfine grinding on quality and antioxidant property of fine green tea powders. LWT—Food Sci. Technol. 45, 8–12, http://dx.doi.org/10.1016/j.lwt.2011.08.002.

Iacopini, P., Baldi, M., Storchi, P., Sebastiani, L., 2008. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: content, in vitro antioxidant activity and interactions. J. Food Compos. Anal. 21, 589–598, http://dx.doi.org/10.1016/j.jfca.2008.03.011.

Innocenti, G., Vegeto, E., Dall' Acqua, S., Ciana, P., Giorgetti, M., Agradi, E., Sozzi, A., Fico, G., Tomè, F., 2007. In vitro estrogenic activity of *Achillea millefolium* L. Phytomedicine 14, 147–152, http://dx.doi.org/10.1016/j.phymed.2006.05.005.

Kumar, S., Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. Sci. World J. 2013, e162750, http://dx.doi.org/10.1155/2013/162750.

Luque de Castro, M.D., Garcıía-Ayuso, L.E., 1998. Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Anal. Chim. Acta 369, 1–10, http://dx.doi.org/10.1016/S0003-2670(98)00233-5.

Maaroufi, C., Melcion, J.-P., de Monredon, F., Giboulot, B., Guibert, D., Le Guen, M.-P., 2000. Fractionation of pea flour with pilot scale sieving I. Physical and chemical characteristics of pea seed fractions. Anim. Feed Sci. Technol. 85, 61–78, http://dx.doi.org/10.1016/S0377-8401(00)00127-9.

Murthy, C. t., Rani, M., Rao, P. n. S., 1999. Optimal grinding characteristics of black pepper for essential oil yield. J. Food Process Eng. 22, 161–173, http://dx.doi.org/10.1111/j.1745-4530.1999.tb00478.x.

Nöldner, M., Schötz, K., 2002. Rutin is essential for the antidepressant activity of *Hypericum perforatum* extracts in the forced swimming test. Planta Med. 68, 577–580, http://dx.doi.org/10.1055/s-2002-32908.

Orčić, D.Z., Mimica-Dukić, N.M., Francišković, M.M., Petrović, S.S., Jovin, E.Đ., 2011. Antioxidant activity relationship of phenolic compounds in *Hypericum perforatum* L. Chem. Cent. J. 5, 34, http://dx.doi.org/10.1186/1752-153X-5-34.

Pandey, K.B., Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev. 2, 270–278.

Ploss, O., Petereit, F., Nahrstedt, A., 2001. Procyanidins from the herb of *Hypericum perforatum*. Pharmazie 56, 509–511.

Roginsky, V., Lissi, E.A., 2005. Review of methods to determine chain-breaking antioxidant activity in food. Food Chem. 92, 235–254, http://dx.doi.org/10.1016/j.foodchem.2004.08.004.

Rostagno, M.A., Prado, J.M., 2013. Natural product extraction: principles and applications. R. Soc. Chem.

Santoro, G.F., Cardoso, M.G., Guimarães, L.G.L., Mendonça, L.Z., Soares, M.J., 2007. Trypanosoma cruzi: activity of essential oils from *Achillea millefolium* L., *Syzygium aromaticum* L. and *Ocimum basilicum* L. on epimastigotes and trypomastigotes. Exp. Parasitol. 116, 283–290, http://dx.doi.org/10.1016/j.exppara.2007.01.018.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., Yoga Latha, L., 2010. Extraction: isolation and characterization of bioactive compounds from plants' extracts. Afr. J. Tradit. Complement. Altern. Med. 8, 1–10.

Sharififar, F., Dehghn-Nudeh, G., Mirtajaldini, M., 2009. Major flavonoids with antioxidant activity from *Teucrium polium* L. Food Chem. 112, 885–888.

Singh, K. k., Goswami, T.K., 1999. Studies on cryogenic grinding of cumin seed. J. Food Process Eng. 22, 175–190, http://dx.doi.org/10.1111/j.1745 –4530.1999.tb00479.x.

Trumbeckaite, S., Benetis, R., Bumblauskiene, L., Burdulis, D., Janulis, V., Toleikis, A., Viškelis, P., Jakštas, V., 2011. *Achillea millefolium* L. s.l. herb extract: antioxidant activity and effect on the rat heart mitochondrial functions. Food Chem. 127, 1540–1548, http://dx.doi.org/10.1016/j.foodchem.2011.02.014.

Tusevski, O., Petreska Stanoeva, J., Stefova, M., Simic, S.G., 2013. Phenolic profile of dark-Grown and photoperiod-Exposed *Hypericum perforatum* L. hairy root cultures. Sci. World J. 2013, e602752, http://dx.doi.org/10.1155/2013/602752.

Van Craeyveld, V., Holopainen, U., Selinheimo, E., Poutanen, K., Delcour, J.A., Courtin, C.M., 2009. Extensive dry ball milling of wheat and rye bran leads to in situ production of arabinoxylan oligosaccharides through nanoscale fragmentation. J. Agric. Food Chem. 57, 8467–8473, http://dx.doi.org/10.1021/jf901870r.

Vitalini, S., Beretta, G., Iriti, M., Orsenigo, S., Basilico, N., Dall'Acqua, S., Iorizzi, M., Fico, G., 2011. Phenolic compounds from *Achillea millefolium* L. and their bioactivity. Acta Biochim. Pol. 58, 203–209.

Wafa, G., Amadou, D., Larbi, K.M., Héla, E.F.O., 2014. Larvicidal activity, phytochemical composition, and antioxidant properties of different parts of five populations of *Ricinus communis* L. Ind. Crops Prod. 56, 43–51, http://dx.doi.org/10.1016/j.indcrop.2014.02.036.

Xu, J.-G., Hu, Q.-P., Liu, Y., 2012. Antioxidant and DNA-protective activities of chlorogenic acid isomers. J. Agric. Food Chem. 60, 11625–11630, http://dx.doi.org/10.1021/jf303771s. Zhang, Z., Song, H., Peng, Z., Luo, Q., Ming, J., Zhao, G., 2012. Characterization of stipe and cap powders of mushroom (*Lentinus edodes*) prepared by different grinding methods. J. Food Eng. 109, 406–413, http://dx.doi.org/10.1016/j.jfoodeng.2011.11.007.

Zhao, X., Yang, Z., Gai, G., Yang, Y., 2009. Effect of superfine grinding on properties of ginger powder. J. Food Eng. 91, 217–222, http://dx.doi.org/10.1016/j.jfoodeng.2008.08.024.