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Application of different analytical methods for the determination of phenolics and antioxidant activity in hawthorn (*Crataegus* spp.) bud and sprout herbal extracts

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Summary

Hawthorn (*Crataegus* spp., family: *Rosaceae*) extracts have been used as pharmaceutical preparations owing to positive effects on cardiovascular system. The $AlCl_3$-based official method employed for the determination of pharmacologically active compounds was compared with other techniques such as Folin-Ciocalteau method and HPLC-DAD. Antioxidant activity was determined by ABTS radical cation assay. Methods were applied on extracts from buds and sprouts collected from common hawthorn (*C. monogyna* Jacq., *C. laevigata* (Poir.) DC.) located in Northeastern Italy. Phenolic content determined by $AlCl_3$-based method, Folin-Ciocalteau method, and HPLC-DAD was in the range 23,534-27,728, 75,284-100,616 and 57,317-58,639 mg kg^{-1} of dry matter (DM), respectively, in buds, and 17,280-19,330, 27,653-38,590, and 30,635-32,185 mg kg^{-1} DM, respectively, in sprouts. Antioxidant activity ranged from 119,864 to 174,640 and 31,484 to 52,584 mg Trolox eq. kg^{-1} DM in buds and sprouts, respectively. Phenolic amount and profile were significantly affected by phenological stage and sampling location. Antioxidant activity was related to flavan-3-ol and hydroxycinnamic acid amount, and to non-phenolic substances. $AlCl_3$-based method underestimated total phenolic content owing to lack of selectivity to important phenolic classes whereas Folin-Ciocalteau method was affected by non-phenolic interfering substances. HPLC-DAD proved to be more effective in determining hawthorn phenolics.

Keywords: Antioxidant activity; Hawthorn buds and sprouts; Herbal extracts; HPLC-DAD; Phenolics

Introduction

Hawthorn (*Crataegus* spp., family: *Rosaceae*) is the name of closely related plant species widely distributed in the northern hemisphere and used since a long time as herbal remedy (Liu, 2012). Genus *Crataegus* includes between 150 and 1200 species. Common hawthorn (*C. monogyna* Jacq.), wild hawthorn (*C. laevigata* (Poir.) DC.) and their hybrids are widespread in Middle Europe, including Italy; *C. pentagyna* Waldst. & Kit. ex Willd., *C. nigra* Waldst. & Kit., and *C. azarolus* L. are growing in South and Southeastern Europe, whereas *C. pinnatifida* Bunge and *C. scabrifolia* (Franch.) Rehder are common in China (Prinz et al., 2007). The first three species play an important role as raw materials for pharmaceutical preparations (Prinz et al., 2007; Liu et al., 2011a). Indeed, *C. monogyna*, *C. laevigata*, and their hybrids are allowed by European Pharmacopoeia for the preparation of phytomedicines (Council of Europe, 2004).

The biological effects of hawthorn phenolics have been mainly tested employing extracts obtained from leaves, flowers, and fruits by means of ethanol, methanol, water or mixtures of these solvents. Hawthorn-based herbal products are nowadays marketed as alternative treatments for a number of diseases such as hypertension, angina, arrhythmia, and the early stages of congestive heart failure (Edwards et al., 2012). Several studies have demonstrated that extracts from different plant parts show protective effects on the heart and cardiovascular system, improving coronary circulation and endothelium-dependent vasorelaxation and reducing inflammation (Kim et al., 2000; Schwinger et al., 2000; Pittler et al., 2003; Quettier-Deleu et al., 2003), and possess hypolipidemic effects too (Rajendran et al., 1996; Zhang et al., 2002; Kuo et al., 2009). Hypotensive and radical scavenging properties of *Crataegus* spp. have been also investigated (Walker et al., 2002; Walker et al., 2006; Tadić et al., 2008; Froehlicher et al., 2009; Qiao et al., 2015). The inhibitory effects on human tumor cell growth and a further characterisation of phenolic extracts of hawthorn buds and fruits have been recently carried out (Rodrigues et al., 2012).

Furthermore, the safety of hawthorn fruit and leaf extracts and their suitability for human consumption have been reviewed (Daniele et al., 2006).

Flavone and flavonol glycosides, hydroxycinnamic acids, and B-type procyanidins have been reported as the phenolic compounds exerting the major biological activity (Liu et al., 2011a; Rodrigues et al., 2012). Recent compositional studies of *C. monogyna*, *C. laevigata*, *C. pentagyna*, and *C. pinnatifida* leaves, flowers, and fruits highlighted relevant differences of the phenolic content and profile among species and plant parts (Svedström et al., 2002, 2006; Urbonavičiūtė et al., 2006; Prinz et al., 2007; Liu et al., 2010, 2011a, 2011b).

Among the methods for the determination of hawthorn phenolic compounds, the most widely used are high performance liquid chromatography (HPLC), coupled with diode array detection (DAD) and mass spectrometry (MS) and enabling the determination of individual phenolics, and two spectrophotometric procedures: the Folin-Ciocalteau (FC) and the aluminum chloride ($AlCl_3$)-based methods. FC method is based on an electron-transfer giving rise to the formation of chromogen complexes between specific redox reagents and phenolics with a maximum absorbance at 750 nm (Singleton and Rossi, 1965). $AlCl_3$-based method is adopted by the Official Italian Pharmacopoeia and depends on the capability of $AlCl_3$ of forming, in an acid environment, stable chromogen compounds by binding to flavonoids at the carbonyl located at C-4 and hydroxyl groups sited at C-3 or C-5 of the aglycone moiety (Smirnova and Pervykh, 1998).

A research targeted at comparing the effectiveness of these three analytical methods to detect and quantify hawthorn phenolics is still lacking. The present study was aimed at comparing the method adopted by the Official Italian Pharmacopoeia, FC method and HPLC-DAD in the evaluation of the phenolic content and profile of two common herbal preparations, a glycerinated macerate and a mother tincture, obtained respectively from buds and sprouts of common hawthorn plants. A second objective of this investigation was to verify the effect of the collecting site on the phenolic profile and content of hawthorn buds and sprouts sampled from different locations in the hilly Romagna district, Northeastern Italy. The antioxidant activity of the extracts and its relation to the content of total phenolics and individual phenolic classes were also assessed.

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Materials and methods

Plant collection and extract preparation

The species herein considered belonged to the group of common hawthorn: *C. monogyna* Jacq. and *C. laevigata* (Poir.) DC., with a prevalence of the first one, and many intermediate forms. Species identification was carried out on the basis of Pignatti (1982), where *C. laevigata* is reported as *C. oxyaxantha* L., and confirmed by comparison to vouchers published on Flora Italica Exsiccata.

Hawthorn plants located in Forlì-Cesena and Ravenna provinces were sampled in March-April at two phenological stages: closed buds (0.5-1 cm length) and sprouts (flowers and leaves, 1.5-2 cm length). Buds were collected at Rio dei Cozzi (province: Forlì-Cesena; harvest date: 23 March; 44°18′N 11°92′E, 130 m a.s.l.) and Brisighella (province: Ravenna; harvest date: 24 March; 44°22′N 11°77′E, 107 m a.s.l.). Sprouts were collected at Magliano (province: Forlì-Cesena; harvest date: 24 April; 44°17′N 12°10′E, 35 m a.s.l.), Rio dei Cozzi (harvest date: 24 April), and Santa Sofia (province: Forlì-Cesena; harvest date: 30 April; 43°95′N 11°91′E, 257 m a.s.l.).

Buds and sprouts were harvested during their balsamic period, immediately ground, and then extracted by means of the different maceration processes, commonly adopted in herbal preparation, according to the plant part. In each location buds and sprouts were collected at different sites to allow replication within location. Maceration and extraction were carried out in the laboratories of Cento Fiori s.r.l. (Forlì, Italy).

Buds. Five hundred grams of fresh buds were soaked in the dark for 30 days at room temperature in 1.10 kg of ethanol and 1.10 kg of glycerol, corresponding to 1.39 and 0.88 L of the two solvents, respectively. The amounts of ethanol and glycerol added to the fresh material were chosen in order to have a final 1/20 (w/w) dry matter-to-extracting mixture ratio. Food grade ethanol 96.4% was employed.

Sprouts. Five hundred grams of fresh sprouts were soaked in the dark for 30 days at room temperature in 0.70 kg of ethanol and 0.15 kg of water, corresponding to 0.89 and 0.15 L of the two solvents, respectively. The amounts of ethanol and water added to the fresh material were chosen in order to have a final 1/10 (w/w) dry matter-to-extracting mixture ratio and a 60% (v/v) hydroalcoholic mixture as extracting mean. The amount of water to be added was determined taking into account the moisture content of the fresh material. Food grade ethanol 96.4% was employed.

Dry matter was determined gravimetrically as the mass loss of 10 g of fresh material kept at 105 °C until constant weight. It corresponded to 0.22 and 0.25 g g^{-1} for buds and sprouts, respectively. Extracts were daily shaken for 1 min during the soaking period, pressed, filtered in amber glass bottles and kept at 4 °C until analyses.

Extracts were prepared in duplicate for each sample. Each extract was then analysed separately, performing analytical determinations twice for each replication (n = 4).

Reagents and chemicals

All chemicals and solvents were, unless specified, of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionised water was obtained by an Elix 10 water purification system from Millipore (Bedford, MA, USA). Chlorogenic acid, (-)-epicatechin, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) standards were from Sigma-Aldrich. Vitexin (apigenin-8-*C*-glucoside), vitexin-2″-*O*-rhamnoside, and hyperoside (quercetin-3-*O*-galactoside) standards were bought from Extrasynthese (Genay, France).

Spectrophotometric determination of total flavonoid content (FLAV)

FLAV was evaluated according to the Official Italian Pharmacopoeia (Official Pharmacopoeia of the Italian Republic, 1998) and Smirnova and Pervykh (1998).

A volume corresponding to 0.600 and 0.800 mL of bud and sprout extracts, respectively, was transferred to a 2-mL polypropylene (PP) microtube and adjusted to a 1/1 (v/v) water-to-organic solvent ratio by the addition of 0.425 and 0.190 mL of water, respectively. Extracts were then centrifuged at 21,500 *g* for 5 min at 10 °C. 0.4 mL of the supernatant fraction were further diluted in a second PP microtube adding 1.6 mL of ethanol/water 1/1 (v/v) before spectrophotometric determination. 0.5 mL of diluted extract, to which were added 5.0 mL of 5% (v/v) acetic acid in methanol and 0.25 mL of 2% (w/v) aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) in methanol, were manually shaken in a 10-mL Teflon screw cap glass tube for 10 sec and kept in the dark for 30 min. The absorbance of the solution was then read at 425 nm (25 °C) against methanol in a double beam spectrophotometer (mod. UV-1601) from Shimadzu (Kyoto, Japan). FLAV was quantified by constructing a calibration curve employing hyperoside as a reference compound. From a stock solution (c = 1.872 mg mL^{-1}) in ethanol/dimethyl sulfoxide 9/1 (v/v), diluted solutions were prepared in ethanol/water 1/1 (v/v) in a concentration range of 0.005-0.494 mg mL^{-1} (seven calibration points, $r > 0.99$). Each diluted standard solution was analysed in three replications. Observed absorbance values were corrected subtracting the absorbance of a blank sample prepared employing 0.5 mL of ethanol/water 1/1 (v/v) in place of hawthorn extract.

Spectrophotometric determination of total phenolic content (TPC)

TPC was determined according to the Folin-Ciocalteau spectrophotometric method (Singleton and Rossi, 1965). Before analyses bud and sprout extracts underwent the same dilution procedure formerly described for FLAV determination. 7.5 mL of water, 0.1 mL of the diluted extract and 0.5 mL of Folin-Ciocalteau reagent were then transferred to a 10-mL Teflon screw cap glass tube, manually shaken for 10 sec, added with 2.0 mL of 15% (w/v) sodium carbonate, and finally shaken for further 10 sec. After 2 h in the dark, the absorbance of the solution was read at 750 nm (25 °C) against water in the same double beam spectrophotometer previously described. TPC was assessed by constructing a gallic acid calibration curve. From a stock solution (c = 2.020 mg mL^{-1}) in ethanol, diluted solutions were prepared in ethanol/water 1/1 (v/v) in a concentration range of 0.005-1.010 mg mL^{-1} (eight calibration points, $r > 0.99$). Each solution was analysed in three replications. Observed absorbance values were corrected subtracting the absorbance of a blank sample prepared employing 0.1 mL of ethanol/water 1/1 (v/v) in place of hawthorn extract.

Determination of phenolics by high performance liquid chromatography coupled with diode array detection (HPLC-DAD)

Phenolic extracts were analysed in gradient mode on a HPLC system from Jasco (Tokyo, Japan) equipped with two binary pumps (mod. PU-1580), a diode array UV/Vis detector (mod. MD-1510, quartz flow cell, optical path: 10 mm), and an autosampler (mod. AS-2055 Plus). The following solvent system was employed: mobile phase A: 0.5% (v/v) formic acid in water/acetonitrile/methanol 95/4/1 (v/v/v); mobile phase B: 0.5% (v/v) formic acid in acetonitrile/methanol 4/1 (v/v). Ethanol/water 1/1 (v/v) was used as a cleaning solution for autosampler syringe. Solvents were of chromatographic grade. Mobile phases and cleaning solution were preliminary filtered through a nylon membrane filter (diameter: 47 mm; pore dimension: 0.45 μm) from GVS Filter Technology (Indianapolis, IN, USA), and degassed in an ultrasonic bath for 32 min at room temperature. The gradient program was as follows: 0-35 min, 100 to 74% A; 35-37 min, 74 to 20% A; 37-45 min, 20% A; 45-47 min, 20 to 100% A; 47-57 min,

100% A. The flow rate was 1.0 ml min^{-1} and the injection volume was 5 µL. HPLC-DAD traces were acquired at 280, 330, 350, and 520 nm, whereas absorption spectra were recorded from 200 to 600 nm. A Kinetex 2.6µ C18 100A (75 × 4.6 mm i.d., 2.6 µm particle size) column from Phenomenex (Torrance, CA, USA) was used for compound separation and maintained at 35 °C during analyses. Before injection, 0.600 and 0.800 mL of crude bud and sprout extracts were diluted with 0.425 and 0.190 mL of water, respectively, in a 2-mL PP microtube to reach a 1/1 (v/v) water-to-organic solvent ratio, and centrifuged at 21,500 *g* for 5 min at 10 °C. One mL of the supernatant fraction was then filtered in a HPLC glass vial through a regenerated cellulose (RC) syringe filter (diameter: 13 mm, pore dimension: 0.45 µm) from GVS. Hydroxycinnamic and benzoic acids, flavan-3-ols, and flavonols were quantified by external standard mode at 330, 280, and 350 nm, respectively, using as reference compounds chlorogenic acid, (-)-epicatechin, and hyperoside. Flavones were quantified at 330 or 350 nm in accordance to the absorption spectrum of each compound. Monoglycosyl and unidentified flavones were quantified using vitexin as reference compounds, whereas diglycosyl flavones were quantified by vitexin-2″-*O*-rhamnoside. Stock solutions were prepared at the following concentrations: chlorogenic acid: 2.056 mg mL^{-1} in ethanol; (-)-epicatechin: 2.030 mg mL^{-1} in ethanol; hyperoside: 1.872 mg mL^{-1} in ethanol/dimethyl sulfoxide 9/1 (v/v); vitexin: 1.140 mg mL^{-1} in ethanol/dimethyl sulfoxide 8/2 (v/v); vitexin-2″-*O*-rhamnoside: 2.560 mg mL^{-1} in ethanol. Diluted solutions for calibration curves were prepared in ethanol/water 1/1 and analysed in duplicate each. Concentration ranges of calibration curves were 0.005-1.028 (eight calibration points, $r > 0.99$), 0.005-0.499 (seven calibration points, $r > 0.99$), 0.005-0.494 (seven calibration points, $r > 0.99$), 0.002-0.100 (six calibration points, $r > 0.99$), and 0.005-0.998 mg mL^{-1} (eight calibration point, $r > 0.99$) for chlorogenic acid, (-)-epicatechin, hyperoside, vitexin and vitexin-2″-*O*-rhamnoside, respectively. The limits of detection (LODs) and the limits of quantification (LOQs) of standard compounds were set at 3 × S/N and 7 × S/N, respectively, where S/N is the signal-to-noise ratio. With regard to phenolic determination in hawthorn buds, LODs were at levels of 14, 75, 19, 26, 53, and 27 mg kg^{-1} DM for chlorogenic acid, (-)-epicatechin, vitexin at 330 nm, vitexin at 350 nm, vitexin-2″-*O*-rhamnoside (330 nm), and hyperoside, respectively, whereas for the same compounds LOQs were at level of 33, 175, 44, 60, 123, and 63 mg kg^{-1} DM. With regard to phenolic determination in hawthorn sprouts, LODs were at levels of 5, 26, 6, 9, 18, and 9 mg kg^{-1} DM for chlorogenic acid, (-)-epicatechin, vitexin at 330 nm, vitexin at 350 nm, vitexin-2″-*O*-rhamnoside (330 nm), and hyperoside, whereas for the same compounds LOQs were at levels of 11, 60, 15, 20, 42, and 22 mg kg^{-1} DM.

Phenolic identification

Phenolic identification was carried out comparing peak retention times and UV/Vis absorption spectra with those of standard compounds employed in the construction of calibration curves, and by means of a liquid chromatography system HP 1100 Series equipped with a diode array detector and coupled with a single quadrupole mass spectrometer (1100 MSD Series, mod. G1946A) from Agilent Technologies (Santa Clara, CA, USA). Mass spectra of different peaks were compared to fragmentation patterns and structural information reported in previous works (Liu et al., 2011a, b; Liu, 2012). Other investigations focusing on phenolic determination in green vegetables were useful for a better compound identification and to clarify the order of elution of hydroxycinnamic acids (Clifford et al., 2006; Lin and Harnly, 2009; Rodrigues et al., 2012; Bonta et al., 2017). Compounds reported as unidentified were assigned to the corresponding chemical class on the basis of their UV/Vis spectra. The mass spectrometer operated both in positive and negative atmospheric pressure ionisation-electrospray source (API-ES) under the following conditions: drying gas (nitrogen) temperature: 350 °C; nebuliser pressure: 35 psig; capillary voltage: 3,000 V; fragmentor voltage: 100 V; mass ranges: 100-700 and 700-1,200 *m/z*. Mobile phases were prepared in 0.1 in place of 0.5% (v/v) formic acid to improve the mass detector response.

Spectrophotometric evaluation of the antioxidant activity

Antioxidant activity was evaluated by the disappearance of radical chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), according to the method proposed by Eberhardt et al. (2005), with some modification hereafter reported. Briefly, ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting a 7 mM ABTS stock solution with 70 mM potassium persulphate overnight at 4 °C, as described by Re et al. (1999). $ABTS^{\cdot+}$ ethanolic solution was prepared daily. Bud and sprout extracts underwent the same dilution procedure formerly described for FLAV and TPC determination. Before performing ABTS assay, 0.5 mL of each extracts were transferred to a 2-mL PP microtube and further diluted by adding 1.5 mL of ethanol/water 1/1 (v/v). After the last dilution, 30 µL of solution were added to 3.0 mL of ethanolic $ABTS^{\cdot+}$ and shaken in a plastic cuvette. The absorbance was then read at 734 nm (25 °C) in a double beam spectrophotometer over 6 min. Absorbance correction was performed by a blank sample prepared employing 30 µL of ethanol/water 1/1 (v/v) in place of hawthorn extract. The antioxidant activity was referred to as Trolox equivalent antioxidant capacity (TEAC) by constructing a Trolox calibration curve in a concentration range of 0.005-0.499 mg mL^{-1} (six calibration points, $r > 0.99$). Trolox diluted solutions were prepared in ethanol/water 1/1 (v/v) from a stock ethanolic solution (c = 1.980 mg mL^{-1}). Each solution was analysed in triplicate.

Statistics

Data were preliminary processed by analysis of variance (ANOVA), according to a nested design, including the effects: a) plant part (buds, sprouts), and b) location within the same part, since locations were not exactly coincident for the two harvested plant parts. Fisher's least significance difference test (LSD) was used for multiple comparison. ANOVA and LSD tests were also employed to compare the results obtained by the different analytical methods used to determine total phenolic content. Correlation analysis was used to investigate the relationships between a) the antioxidant activity, phenolics, and individual phenolic classes and b) phenolic amounts obtained by means of different analytical procedures. The SYSYAT© 10.0 Package (Systat Software, Chicago, IL, USA) was used throughout.

Results and discussion

Total flavonoid content (FLAV)

FLAV was significantly affected by phenological stage (Tab. 1). Buds showed a flavonoid content 38% higher than sprouts. Within plant part, the highest flavonoid content was determined in buds from Rio dei Cozzi and sprouts from Magliano. Total flavonoid amounts were higher than values amounting to 6,232 and 10,266 mg kg^{-1} DM and reported by Froehlicher et al. (2009) in commercial *C. monogyna* flowering tops and flowers, respectively.

Total phenolic content (TPC)

On average, TPC was almost three-times higher in buds than in sprouts (+180%), with a different distribution among collection sites, in comparison to total flavonoids (Tab. 1). In fact, TPC was higher in buds from Brisighella than in sample from Rio dei Cozzi, whereas

Tab. 1: Effect of plant part, location and analytical method on flavonoid and phenolic content and antioxidant activity.

Effects[1]	FLAV[2] (mg hyperoside eq. kg^{-1} DM)	TPC[3] (mg gallic acid eq. kg^{-1} DM)	PHENTOT[4] (mg kg^{-1} DM)	TEAC[5] (mg Trolox eq. kg^{-1} DM)
Plant part				
Buds	25,631[c]	87,950[a]	57,978[b]	147,252
Sprouts	18,607[b]	31,439[a]	31,240[a]	38,961
Significance[6]	**	**	**	**
LSD[7]	*420*	*1,356*	*338*	*4,399*
Location (within plant part)				
Buds				
Rio dei Cozzi	27,728[c]	75,284[a]	58,639[b]	119,864
Brisighella	23,534[c]	100,616[a]	57,317[b]	174,640
Significance	**	**	**	**
LSD	*760*	*2,453*	*612*	*7,958*
Sprouts				
Magliano	19,330[b]	27,653[a]	30,901[a]	31,484
Rio dei Cozzi	19,213[b]	28,075[a]	30,635[a]	32,814
Santa Sofia	17,280[c]	38,590[a]	32,185[b]	52,584
Significance	**	*	**	**
LSD	*439*	*1,416*	*353*	*4,595*

[1] Different letters within the same row denote significant difference ($p \leq 0.05$) among FLAV, TPC and PHENTOT values.
[2] FLAV: flavonoid content determined by spectrophotometric $AlCl_3$-based method.
[3] TPC: total phenolic content determined by Folin-Ciocalteau spectrophotometric method.
[4] PHENTOT: total phenolic content determined by HPLC.
[5] TEAC: Trolox equivalent antioxidant capacity.
[6] NS: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$.
[7] LSD: least significant difference ($p = 0.05$), referred to column values.

Santa Sofia sample showed the highest TPC content within sprouts. Buds and sprouts showed higher and lower TPC values, respectively, in comparison to commercial *C. monogyna* flowering tops (56,292 mg gallic acid eq. kg^{-1} DM) and flowers (49,310 mg gallic acid eq. kg^{-1} DM) analysed by Froehlicher et al. (2009).

Phenolic composition determined by HPLC

Thirty phenolic compounds were identified by HPLC-DAD-MS (Tab. 2) and quantified by HPLC-DAD (Tab. 3); five hydroxycinnamic acids, two flavones, and two flavonols were not clearly identified but assigned to the corresponding chemical class by the evaluation of their absorption spectra.

The total phenolic amount determined by HPLC-DAD was significantly affected by phenological stage and location (Tab. 1). Consistently with FLAV and TPC results, buds showed a phenolic content almost twice higher (+86%) than sprouts. Small but significant differences were also noticed between locations, with the highest amounts determined in buds from Rio dei Cozzi and sprouts from Santa Sofia.

The composition of the phenolic fraction was also affected by plant part and location.

(-)-Epicatechin was the only identified flavan-3-ol, representing the fourth most relatively abundant compound in buds (Tab. 4), whereas in sprouts it amounted on average to less than 0.02 mg mg^{-1}. Buds from Brisighella showed the highest (-)-epicatechin fraction. No procyanidins were identified in HPLC-DAD-MS traces, even previously determined in fruits and leaves of *Crataegus* spp. (Liu, 2012). In fact, the absolute and individual amount of these compounds has been reported to vary significantly according to plant part and species (Svedström et al., 2002; Liu et al., 2010).

Hydroxycinnamic acids accounted for about 0.30 mg mg^{-1} of phenolics (Tab. 4); a significantly higher fraction was observed in buds than in sprouts. Within plant part, the relative amount of acids exceeded 0.30 mg mg^{-1} of phenolics in buds from Brisighella and sprouts from Santa Sofia. Chlorogenic acid (AC-6) was prevalent, representing on average the second and the fourth most relatively abundant phenolic compound in buds and sprouts, respectively.

Flavones were the most abundant phenolic compounds in buds and sprouts, accounting for about half of total phenolics (Tab. 5). Significant but not relevant differences were determined within sprouts, whereas higher differences were noticed within buds. Vitexin-2″-*O*-rhamnoside (ON-4) and its acylated form vitexin-2″-*O*-(4‴-*O*-acetyl)-rhamnoside (ON-8) where the dominating compounds, exceeding together 0.40 mg mg^{-1} of total phenolics. Both phenological stage and location affected the relative content of the two main flavones. ON-4 was the most abundant phenolic compound, with a relative content range of 0.23-0.28 mg mg^{-1} of total phenolics. Differences among locations were particularly remarkable for ON-8.

The relative total flavonol amount was highly different between buds and sprouts (Tab. 6). Five out of eight flavonols were either not detected or determined in traces in buds. Location significantly affected flavonol within sprouts. Hyperoside (quercetin-3-*O*-galactoside, OL-2) was the dominant flavonol in sprouts, representing the third most relatively abundant phenolic compound. Another important flavonol was isoquercitrin (quercetin-3-*O*-glucoside, OL-4), accounting for over 0.03 mg mg^{-1} of phenolics in all sprout samples.

HPLC determination of hawthorn phenolics have been carried out on leaves, flowers and fruits of several *Crataegus* species, as reviewed by Edwards et al. (2012). On the whole, buds and sprouts here analysed showed on average a higher total phenolic content in comparison to *C. monogyna* and *C. laevigata* samples surveyed by HPLC-DAD in previous investigations and coming from different plant parts (Prinz et al., 2007; Ringl et al., 2007). In agreement with our results, (-)-epicatechin, chlorogenic acid, vitexin-2″-*O*-rhamnoside, vitexin acetyl rhamnoside, and hyperoside were reported as the five

Tab. 2: Names, abbreviations, retention times, maximum absorption wavelengths and mass spectral data of individual phenolic compounds determined by HPLC in hawthorn bud and sprout extracts.

Compound name	Tag	Peak no.[1]	RT (min)[2]	UV λ_{max} (nm)[3]	MW[4]	Diagnostic fragment ions (*m/z*)	
						Positive (+)[5]	Negative (-)[5]
Hydroxycinnamic acids							
Neochlorogenic acid	AC-1	1	3.85	235, 323	354	163 (100), 377 (44), 393 (10)	179 (14), 191 (20), 353 (100)
Caffeic acid derivative	AC-2	2	4.55	235, 327	-	135 (19), 145 (8), 163 (100)	135 (100), 179 (46)
Unidentified hydroxycinnamic acid	AC-3	3	5.97	235, 327	-	-	-
3-*p*-Coumaroylquinic acid	AC-4	4	6.27	227, 307	338	119 (46), 147 (100), 361 (35), 377 (6)	119 (4), 163 (70), 191 (4), 337 (100)
Caffeic acid	AC-5	5	7.86	235, 323	180	135 (33), 145 (14), 163 (100), 181 (4), 219 (40)	135 (66), 179 (100)
Chlorogenic acid	AC-6	6	8.29	239, 323	354	163 (100), 355 (1), 377 (51), 393 (16)	135 (7), 179 (7), 191 (100), 353 (54)
Unidentified hydroxycinnamic acid	AC-7	7	9.97	235, 323	-	-	-
5-*p*-Coumaroylquinic acid	AC-8	9	10.9	235, 307	338	119 (38), 147 (100), 361 (22)	337 (100)
4-*p*-Coumaroylquinic acid	AC-9	10	11.51	231, 311	338	119 (43), 147 (100), 361 (20)	191 (81), 337 (100)
Feruloylquinic acid[6]	AC-10	11	13.25	231, 307	368	-	191 (100), 367 (18)
Unidentified hydroxycinnamic acid	AC-11	25	22.05	235, 327	-	-	-
Unidentified hydroxycinnamic acid	AC-12	27	24.75	235, 327	-	-	-
Unidentified hydroxycinnamic acid	AC-13	29	26.87	235, 323	-	-	-
Flavan-3-ols							
(-)-Epicatechin	EPI	8	10.45	231, 275	290	291 (21), 313 (100), 329 (16)	289 (100)
Flavones							
Apigenin pentoside hexoside	ON-1	12	15.81	231, 267, 331	564	-	563 (100)
Unidentified flavone	ON-2	13	16.02	231, 267, 339	-	-	-
Vitexin (apigenin-8-*C*-glucoside)	ON-3	14	17.67	235, 267, 335	432	433 (100), 455 (14), 471 (4)	431 (100)
Vitexin-2´´-*O*-rhamnoside	ON-4	15	18.15	235, 267, 339	578	579 (100), 601 (44), 617 (14)	431 (54), 577 (100)
Isovitexin-2´´-*O*-(4´´´-*O*-acetyl)-rhamnoside	ON-5	20	19.93	235, 267, 339	620	621 (100), 643 (58), 659 (46)	619 (100)
Unidentified flavone	ON-6	21	20.75	227, 267, 339	-	-	-
Luteolin hexoside	ON-7	22	21.21	227, 343	448	-	447 (100)
Vitexin-2´´-*O*-(4´´´-*O*-acetyl)-rhamnoside	ON-8	28	25.53	235, 267, 339	620	621 (100), 643 (37), 659 (9)	619 (100)
Flavonols							
Quercetin-3-*O*-rhamnosylgalactoside	OL-1	16	18.52	255, 359	610	-	609 (100)
Hyperoside (quercetin-3-*O*-galactoside)	OL-2	17	18.87	255, 351	464	303 (48), 465 (3), 487 (100), 503 (4)	463 (100)
Rutin (quercetin-3-*O*-rutinoside)	OL-3	18	19.25	255, 351	610	611 (10), 633 (100), 649 (15)	463 (65), 609 (100)
Isoquercitrin (quercetin-3-*O*-glucoside)	OL-4	19	19.53	255, 351	464	303 (39), 465 (5), 487 (100), 503 (7)	463 (100)
8-methoxykaempferol-3-*O*-glucoside	OL-5	23	21.41	263, 347	478	-	477 (100)
Unidentified flavonol	OL-6	24	21.63	255, 351	-	-	-
8-methoxykaempferol-3-*O*-pentoside	OL-7	26	22.69	263, 347	448	471 (100), 487 (4)	447 (100)
Quercetin[6]	OL-8	30	28.97	255, 367	302	303 (100), 325 (1), 341 (13)	301 (100)

[1] Order of elution.
[2] RT: retention time.
[3] λ_{max}: maximum absorption wavelengths.
[4] Molecular weight.
[5] Polarity. For each fragment mass-to-charge (*m/z*) ratio and the relative abundance (in brackets) have been reported.
[6] Co-eluting with unidentified compounds.

Tab. 3: Content ranges of phenolic compounds determined by HPLC.

Compound tag[1]	Content range (mg kg^{-1} DM)[2]	
	Buds	Sprouts
Hydroxycinnamic acids		
AC-1	2,107-3,160	1,106-1,830
AC-2	ND-18	111-156
AC-3	87-105	229-307
AC-4	78-234	284-525
AC-5	ND-TR	TR-34
AC-6	10,248-13,090	3,129-6,198
AC-7	523-760	121-211
AC-8	120-311	151-193
AC-9	202-307	262-702
AC-10	67-138	222-398
AC-11	1,310-1,362	537-911
AC-12	925-1,606	123-173
AC-13	TR	52-383
Subtotal (AC-TOT)	17,279-19,479	7,626-11,144
Flavan-3-ols		
EPI	4,378-11,562	61-878
Flavones		
ON-1	254-290	242-254
ON-2	86-122	72-85
ON-3	1,286-1,930	228-373
ON-4	13,303-15,349	6,658-8,501
ON-5	813-1,824	327-355
ON-6	605-1,129	120-141
ON-7	75-98	48-68
ON-8	6,288-16,813	4,138-6,076
Subtotal (ON-TOT)	24,778-35,486	13,674-14,609
Flavonols		
OL-1	ND	518-921
OL-2	644-736	4,107-5,913
OL-3	TR	138-215
OL-4	333-381	1,097-1,397
OL-5	ND	109-240
OL-6	ND	132-321
OL-7	427-470	223-469
OL-8	ND	5-165
Subtotal (OL-TOT)	1,495-1,497	6,488-9,451
Total phenolic compounds (PHEN-TOT)	57,317-58,639	30,635-32,185

[1] For compound names see Tab. 2.
[2] ND: not detectable; TR: traces.

major phenolic compounds in *C. monogyna* and *C. laevigata* extracts (Svedström et al., 2006; Orhan et al., 2007; Prinz et al., 2007; Ringl et al., 2007; Martino et al., 2008; Froehlicher et al., 2009). As here observed, total phenolic content and, particularly, phenolic profile were significantly affected by plant part and plant location, with fruits showing the lowest amounts, (Orhan et al., 2007; Prinz et al., 2007; Ringl et al., 2007; Froehlicher et al., 2009).

Comparison of analytical methods for phenolic determination

The detected amount of phenolics was significantly affected by the analytical method employed (Tab. 1). TPC content of buds, determined by the Folin-Ciocalteau method, was about three (+243%) and two (+52%) times higher than values obtained by the $AlCl_3$-based method (FLAV) and HPLC-DAD (PHENTOT), respectively. TPC gave the highest values also for sprouts, without any significant difference with respect to PHENTOT.

The lower values obtained by $AlCl_3$-based method in comparison to TPC and PHENTOT were expected, owing to the selectivity of this method towards flavonols and flavones (Smirnova and Pervykh, 1998), and the high relative amounts of other phenolic classes such as flavan-3-ols and hydroxycinnamic acids is hawthorn extracts. In particular, the differences with respect to other methods were higher in buds, where the relative flavonol content was much lower than in sprouts.

On the other side, the higher TPC values determined in buds in comparison to PHENTOT and trends observed within bud extracts, could be related to the presence of non-phenolic substances such as ascorbic acid, aromatic amines and sugars that may interfere with the results of this assay, leading to an overestimation of total phenolic content (Escarpa and González, 2001; Tsao and Yang, 2003). The lack of accuracy of Folin-Ciacalteau method, especially with regard to buds, was confirmed by the negative and significant correlation (r = -0.87, $p < 0.01$) between TPC and FLAVTOT, whereas in sprouts a positive and significant correlation was verified between the two sets of data (r = 0.86, $p < 0.01$). The main drawbacks of the Folin-Ciocalteau procedure and the $AlCl_3$-based method seemed to be their sensitivity to the presence of interfering compounds (proteins, sugars) and to the relative amount of different phenolic classes, respectively. In conclusion, HPLC-DAD proved to be a more reliable and accurate method for total phenolic determination.

Antioxidant activity (TEAC)

TEAC was about four times higher in bud than in sprout extracts (Tab. 1). Within buds, TEAC was 46% higher in sample from Brisighella than in Rio dei Cozzi. Among sprouts, the highest TEAC value was assessed in the sample from Santa Sofia. Froehlicher et al. (2009) assessed lower antioxidant activities in commercial *C. monogyna* flowering tops (26,030 mg Trolox eq. kg^{-1} DM) and flowers (36,042 mg Trolox eq. kg^{-1} DM). The antioxidant capacity of hawthorn extracts needs an in-depth investigation, even if it has not been considered until now as the main biological activity associated with these preparations. Indeed, TEAC observed in bud extracts are comparable to values detected in green tea, a popular antioxidant-rich and healthy perceived herbal preparation (Rusak et al., 2008; Komes et al., 2010).

Correlation of antioxidant activity to total phenolic and phenolic class content

Antioxidant activity showed the highest correlation to TPC for both buds and sprouts (Tab. 7). This result could depend on the effect of the non-phenolic substances, determined by the Folin-Ciocalteau spectrophotometric method, that can also contribute to antioxidant activity (Chun et al., 2003; Kim et al., 2003). A significant but lower correlation was observed in sprouts between TEAC and total phenolics determined by HPLC-DAD, whereas in buds TEAC and PHENTOT were negatively related.

With regard to single phenolic classes, TEAC was positively related to the absolute amount of (-)-epicatechin and hydroxicinnamic acid both in buds and sprouts. A low but still significant positive correlation was determined between sprout TEAC and flavone content, whereas flavone content was negatively related to TEAC in buds. Flavonol content showed no correlation to TEAC both in buds and sprouts.

These findings could partly explain differences in antioxidant activity between locations. Buds from Brisighella and sprouts from Santa Sofia showed the highest TEAC values and also the highest

Tab. 4: Effect of plant part and location on (-)-epicatechin, total and individual hydroxycinnamic acid fractions.

Effects	Relative amount (mg mg^{-1} total phenolics)[1]														
	EPI	AC-TOT	AC-1	AC-2	AC-3	AC-4	AC-5	AC-6	AC-7	AC-8	AC-9	AC-10	AC-11	AC-12	AC-13
Plant part															
Buds	0.138	0.317	0.045	< 0.001	0.002	0.003	TR	0.202	0.011	0.004	0.004	0.002	0.023	0.022	TR
Sprouts	0.011	0.273	0.046	0.004	0.008	0.013	< 0.001	0.135	0.005	0.006	0.014	0.010	0.024	0.005	0.005
Significance[2]	**	**	**	**	**	**	*	**	**	**	**	**	**	**	**
LSD[3]	*0.002*	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*	*0.001*	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*
Location (within plant part)															
Buds															
Rio dei Cozzi	0.075	0.295	0.054	< 0.001	0.002	0.004	ND	0.175	0.009	0.005	0.005	0.002	0.022	0.016	TR
Brisighella	0.202	0.340	0.037	TR	0.002	0.001	TR	0.228	0.013	0.002	0.004	0.001	0.024	0.028	TR
Significance	**	**	**	**	*NS*	**	*NS*	**	**	**	**	**	**	**	*NS*
LSD	*0.003*	*0.003*	*< 0.001*	*< 0.001*	-	*< 0.001*	-	*0.001*	*0.001*	*0.001*	*0.001*	*< 0.001*	*0.001*	*< 0.001*	-
Sprouts															
Magliano	0.002	0.246	0.036	0.004	0.007	0.009	TR	0.123	0.005	0.006	0.013	0.009	0.029	0.005	0.002
Rio dei Cozzi	0.004	0.241	0.057	0.004	0.007	0.017	TR	0.098	0.004	0.006	0.008	0.007	0.026	0.004	0.003
Santa Sofia	0.025	0.333	0.046	0.005	0.009	0.012	0.001	0.182	0.007	0.005	0.021	0.014	0.017	0.006	0.010
Significance	**	**	**	*NS*	**	**	**	**	**	**	**	**	**	**	**
LSD	*0.002*	*0.002*	*< 0.001*	-	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*	*0.001*	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

[1] For compound names see Table 2; ND: not detectable; TR: traces.
[2] NS: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$.
[3] LSD: least significant difference ($p = 0.05$).

Tab. 5: Effect of plant part and location on total and individual flavone fractions.

Effects	Relative amount (mg mg^{-1} total phenolics)[1]								
	ON-TOT	ON-1	ON-2	ON-3	ON-4	ON-5	ON-6	ON-7	ON-8
Plant part									
Buds	0.519	0.005	0.002	0.028	0.247	0.023	0.015	0.001	0.198
Sprouts	0.456	0.008	0.002	0.009	0.253	0.011	0.004	0.002	0.167
Significance[2]	**	**	**	**	**	**	**	**	**
LSD[3]	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*
Location (within plant part)									
Buds									
Rio dei Cozzi	0.605	0.005	0.002	0.033	0.227	0.031	0.019	0.001	0.287
Brisighella	0.432	0.004	0.002	0.022	0.268	0.014	0.011	0.002	0.110
Significance	**	**	**	**	**	**	**	**	**
LSD	*0.002*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*	*0.001*	*< 0.001*	*0.001*
Sprouts									
Magliano	0.447	0.008	0.003	0.007	0.277	0.011	0.005	0.002	0.135
Rio dei Cozzi	0.484	0.008	0.002	0.012	0.257	0.011	0.004	0.002	0.188
Santa Sofia	0.438	0.008	0.002	0.009	0.225	0.011	0.004	0.001	0.178
Significance	**	**	**	**	**	**	**	**	**
LSD	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*

[1] For compound names see Tab. 2; ND: not detectable; TR: traces.
[2] NS: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$.
[3] LSD: least significant difference ($p = 0.05$).

fractions of phenolics such as (-)-epicatechin and hydroxycinnamic acids. Furthermore, TPC data may indicate a higher content of non-phenolic substances in these samples, also contributing to antioxidant activity.

This result partly agreed with FROEHLICHER et al. (2009) that highlighted a higher free radical scavenging property of (-)-epicatechin in comparison to quercetin glycosides such as rutin and hyperoside. Nevertheless, the present investigation pointed out that even hydroxycinnamic acids may positively contribute to the antioxidant capacity of hawthorn preparations.

Tab. 6: Effect of plant part and location on total and individual flavonol fractions.

Effects	Relative amount (mg mg^{-1} total phenolics)[1]								
	OL-TOT	OL-1	OL-2	OL-3	OL-4	OL-5	OL-6	OL-7	OL-8
Plant part									
Buds	0.026	ND	0.012	TR	0.006	ND	ND	0.008	ND
Sprouts	0.260	0.023	0.163	0.006	0.040	0.006	0.008	0.011	0.002
Significance[2]	**	**	**	**	**	**	**	**	**
LSD[3]	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*
Location (within plant part)									
Buds									
Rio dei Cozzi	0.025	ND	0.011	TR	0.006	ND	ND	0.008	ND
Brisighella	0.026	ND	0.013	TR	0.006	ND	ND	0.007	ND
Significance	*NS*	*NS*	**	*NS*	**	*NS*	*NS*	*NS*	*NS*
LSD	-	-	*0.001*	-	*< 0.001*	-	-	-	-
Sprouts									
Magliano	0.305	0.030	0.191	0.007	0.045	0.007	0.010	0.015	< 0.001
Rio dei Cozzi	0.271	0.024	0.169	0.006	0.041	0.007	0.010	0.013	0.001
Santa Sofia	0.203	0.016	0.130	0.004	0.034	0.004	0.004	0.007	0.004
Significance	**	**	**	**	**	**	**	**	**
LSD	*0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*< 0.001*

[1] For compound names see Tab. 2; ND: not detectable; TR: traces.
[2] NS: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$.
[3] LSD: least significant difference ($p = 0.05$).

Tab. 7: Simple correlation coefficients between the absolute amounts of total phenolics and phenolic classes with antioxidant activity, determined in bud and sprout extracts

Variable	Antioxidant activity[1]	
	Buds	Sprouts
FLAV[2]	-0.716*	0.363*
TPC[3]	0.975**	0.980**
PHENTOT[4]	-0.846**	0.795**
(-)-Epicatechin[5]	0.924**	0.944**
Hydroxycinnamic acids[5]	0.884**	0.905**
Flavones[5]	-0.931**	0.352*
Flavonols[5]	0.244	0.009

[1] *: significant for $p \leq 0.05$; **: significant for $p \leq 0.01$.
[2] FLAV: total flavonoid content determined by spectrophotometric $AlCl_3$-based method.
[3] TPC: total phenolic content determined by Folin-Ciocalteau spectrophotometric method.
[4] PHENTOT: total phenolic content determined by HPLC.
[5] Absolute amounts determined by HPLC.

Conclusion

This study focused on the comparison of the official $AlCl_3$-based method with other widespread analytical procedures (Folin-Ciocalteu method and HPLC-DAD) used for the determination of phenolics in extracts obtained from Italian hawthorn buds and sprouts. HPLC-DAD was a more reliable and accurate method for total phenolic determination, in comparison to Folin-Ciocalteau and $AlCl_3$-based spectrophotometric methods, which are known to suffer from the presence of interfering non-phenolic substances and a lack of sensitivity to flavan-3-ols and hydroxycinnamic acids, respectively. Phenological stage and geographical origin significantly influenced total phenolic content and the phenolic profile. Antioxidant activity was positively related to the amount of (-)-epicatechin and hydroxycinnamic acids whereas no positive correlation was assessed with the content of flavones and flavonols.

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Conflict of interest
No potential conflict of interest was reported by the authors.

References

Bonta, R.K., 2017: Application of HPLC and ESI-MS techniques in the analysis of phenolic acids and flavonoids from green leafy vegetables (GLVs). J. Pharm. Anal. 7, 349-364. DOI: 10.1016/j.jpha.2017.06.005

Chun, O.K., Kim, D.O., Moon, H.Y., Kang, H.G., Lee, C.Y., 2003: Contribution of individual polyphenolics to total antioxidant capacity of plums. J. Agr. Food Chem. 51, 7240-7245. DOI: 10.1021/jf0343579

Clifford, M.N., Zheng, W., Kuhnert, N., 2006: Profiling the chlorogenic acids of Aster by HPLC–MS^n. Phytochem. Analysis 17, 384-393. DOI: 10.1002/pca.935

Council of Europe, 2004: European Directorate for the Quality of Medicines & HealthCare (EDQM). European Pharmacopoeia 5th ed. Strasbourg, France, 1712-1715.

Daniele, C., Mazzanti, G., Pittler, M.H., Ernst, E., 2006: Adverse-event profile of *Crataegus* spp. Drug Safety 29, 523-535. DOI: 10.2165/00002018-200629060-00005

Eberhardt, M.V., Kobira, K., Keck, A.S., Juvik, J.A., Jeffery, E.H., 2005: Correlation analyses of phytochemical composition, chemical, and cel-

lular measures of antioxidant activity of Broccoli (*Brassica oleracea* L. var. *italica*). J. Agr. Food Chem. 53, 7421-7431. DOI: 10.1021/jf051495k

Edwards, J.E., Brown, P.N., Talent, N., Dickinson, T.A., Shipley, P.R., 2012: A review of the chemistry of the genus *Crataegus*. Phytochemistry 79, 5-26. DOI: 10.1016/j.phytochem.2012.04.006

Escarpa, A., González, M.C., 2001: Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. Anal Chim. Acta 427, 119-127. DOI: 10.1016/S0003-2670(00)01188-0

Flora Italica Exsiccata - Erbari on line. Vouchers 801.802. Botanical Department – University of Catania, Italy. Retrieved 6th November 2018 at http://www.dipbot.unict.it/herbarium/erbario.aspx.

Froehlicher, T., Hennebelle, T., Martin-Nizard, F., Cleenewerck, P., Hilbert, J.L., Trotin, F., Grec, S., 2009: Phenolic profiles and antioxidative effects of hawthorn cell suspensions, fresh fruits, and medicinal dried parts. Food Chem. 115, 897-903. DOI: 10.1016/j.foodchem.2009.01.004

Kim, D.O., Chun, O.K., Kim, Y.J., Moon, H.Y., Lee, C.Y., 2003: Quantification of polyphenolics and their antioxidant capacity in fresh plums. J. Agr. Food Chem. 51, 6509-6515. DOI: 10.1021/jf0343074

Kim, S.H., Kang, K.W., Kim, K.W., Kim, N.D., 2000: Procyanidins in crataegus extract evoke endothelium-dependent vasorelaxation in rat aorta. Life Sci. 67, 121-131. DOI: 10.1016/S0024-3205(00)00608-1

Komes, D., Horžić, D., Belščak, A., Kovačević Ganić, K., Vulić, I., 2010: Green tea preparation and its influence on the content of bioactive compounds. Food Res. Int. 43, 167-176. DOI: 10.1016/j.foodres.2009.09.022

Kuo, D.H., Yeh, C.H., Shieh, P.C., Cheng, K.C., Chen, F.A., Chen, J.T., 2009: Effect of ShanZha, a Chinese herbal product, on obesity and dyslipidemia in hamsters receiving high-fat diet. J. Ethnopharmacol. 124, 544-550. DOI: 10.1016/j.jep.2009.05.005

Lin, L.Z., Harnly, J.M., 2009: Identification of the phenolic components of collard greens, kale, and Chinese broccoli. J. Agr. Food Chem. 57, 7401-7408. DOI: 10.1021/jf901121v

Liu, P., 2012: Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emplic leafflower (*Phyllanthus emblica*) fruits. PhD Thesis, University of Turku, Finland. 2012. Retrieved 6th November 2018, from https://www.utupub.fi/handle/10024/76646.

Liu, P., Yang, B., Kallio, H., 2010: Characterization of phenolic compounds in Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major*) fruit by high performance liquid chromatography-electrospray ionization mass spectrometry. Food Chem. 121, 1188-1197. DOI: 10.1016/j.foodchem.2010.02.002

Liu, P., Kallio, H., Yang, B., 2011a: Phenolic compounds in hawthorn (*Crataegus grayana*) fruits and leaves and changes during fruit ripening. J. Agr. Food Chem. 59, 11141-11149. DOI: 10.1021/jf202465u

Liu, P., Kallio, H., Lü, D., Zhou, C., Yang, B., 2011b: Quantitative analysis of phenolic compounds in Chinese hawthorn (*Crataegus* spp.) fruits by high performance liquid chromatography-electrospray ionisation mass spectrometry. Food Chem. 127, 1370-1377. DOI: 10.1016/j.foodchem.2011.01.103

Martino, E., Collina, S., Rossi, D., Bazzoni, D., Gaggeri, R., Bracco, F., Azzolina, O., 2008: Influence of the extraction mode on the yield of hyperoside, vitexin and vitexin-2´´-*O*-rhamnoside from *Crataegus monogyna* Jacq. (Hawthorn). Phytochem. Analysis 19, 534-540. DOI: 10.1002/pca.1081

Official Pharmacopoeia of the Italian Republic, 1998: 10th ed. Rome, Italy: Istituto Poligrafico dello Stato.

Orhan, I., Özçelik, B., Kartal, M., Özdeveci, B., Duman, H., 2007: HPLC quantification of vitexine-2´´-*O*-rhamnoside and hyperoside in three *Crataegus* species and their antimicrobial and antiviral activities. Chromatographia 66, 153-157. DOI: 10.1365/s10337-007-0283-x

Pignatti, S., 1982: Flora d'Italia. Bologna, Italy: Edagricole, vol. I, 611-613.

Pittler, M.H., Schmidt, K., Ernst, E., 2003: Hawthorn extract for treating chronic heart failure: meta-analysis of randomized trials. Am. J. Med. 114, 665-674. DOI: 10.1016/S0002-9343(03)00131-1

Prinz, S., Ringl, A., Huefner, A., Pemp, E., Kopp, B., 2007: 4´´´-Acetylvitexin-2´´-*O*-rhamnoside, isoorientin, orientin, and 8-methoxykaempferol-3-*O*-glucoside as markers for the differentiation of *Crataegus monogyna* and *Crataegus pentagyna* from *Crataegus laevigata* (Rosaceae). Chem. Biodivers. 4, 2920-2931. DOI: 10.1002/cbdv.200790241

Qiao, A., Wang, Y., Xiang, L., Zhang, Z., He, X., 2015: Novel triterpenoids isolated from hawthorn berries functioned as antioxidant and antiproliferative activities. J. Funct. Foods 13, 308-313. DOI: 10.1016/j.jff.2014.12.047

Quettier-Deleu, C., Voiselle, G., Fruchart, J.C., Duriez, P., Teissier, E., Bailleul, F., Vasseur, J., Trotin, F., 2003: Hawthorn extracts inhibit LDL oxidation. Pharmazie 58, 577-581.

Rajendran, S., Deepalakshmi, P.D., Parasakthy, K., Devaraj, H., Niranjali Devaraj, S., 1996: Effect of tincture of *Crataegus* on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. Atherosclerosis 123, 235-241. DOI: 10.1016/0021-9150(96)05813-3

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26, 1231-1237. DOI: 10.1016/S0891-5849(98)00315-3

Ringl, A., Prinz, S., Huefner, A., Kurzmann, M., Kopp, B., 2007: Chemosystematic value of flavonoids from *Crataegus* × *macrocarpa* (Rosaceae) with special emphasis on (*R*)- and (*S*)-eriodictyol-7-*O*-glucuronide and luteolin-7-*O*-glucuronide. Chem. Biodivers. 4, 154-162. DOI: 10.1002/cbdv.200790020

Rodrigues, S., Calhelha, R.C., Barreira, J.C.M., Dueñas, M., Carvalho, A.M., Abreu, R.M.V., Santos-Buelga, C. Ferreira, I.C.F.R., 2012: *Crataegus monogyna* buds and fruits phenolic extracts: Growth inhibitory activity on human tumor cell lines and chemical characterization by HPLC–DAD–ESI/MS. Food Res. Int. 49, 516-523. DOI: 10.1016/j.foodres.2012.07.046

Rusak, G., Komes, D., Likić, S., Horžić, D., Kovač, M., 2008: Phenolic content and antioxidant capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chem. 110, 852-858. DOI: 10.1016/j.foodchem.2008.02.072

Schwinger, R.H.G., Pietsch, M., Konrad, F., Brixius, K., 2000: *Crataegus* special extract WS 1442 increases force of contraction in human myocardium cAMP-independently. J. Cardiovasc. Pharm. 35, 700-707.

Singleton, V.L., Rossi, J.A., 1965: Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16, 144-158.

Smirnova, L.P., Pervykh, L.N., 1998: Quantitative determination of the total content of flavonoids in flowers of immortelle *Helichrysum arenarium*. Pharm. Chem. J. 32, 321-324. DOI: 10.1007/BF02580519

Svedström, U., Vuorela, H., Kostiainen, R., Laakso, I., Hiltunen, R., 2006: Fractionation of polyphenols in hawthorn into polymeric procyanidins, phenolic acids and flavonoids prior to high-performance liquid chromatographic analysis. J. Chromatogr. A 1112, 103-111. DOI: 10.1016/j.chroma.2005.12.080

Svedström, U., Vuorela, H., Kostiainen, R., Tuominen, J., Kokkonen, J., Rauha, J.P., Laakso, I., Hiltunen, R., 2002: Isolation and identification of oligomeric procyanidins from *Crataegus* leaves and flowers. Phytochemistry 60, 821-825. DOI: 10.1016/S0031-9422(02)00172-3

Tadić, V.M., Dobrić, S., Marković, G.M., Đorđević, S.M., Arsić, I.A., Menković, N.R., Stević, T., 2008: Anti-inflammatory, gastroprotective, free-radical-scavenging, and antimicrobial activities of hawthorn berries ethanol extract. J. Agr. Food Chem. 56, 7700-7709. DOI: 10.1021/jf801668c

Tsao, R., Yang, R., 2003: Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. J. Chromatogr. A, 1018, 29-40. DOI: 10.1016/j.chroma.2003.08.034

Urbonavičiūtė, A., Jakštas, V., Kornišova, O., Janulis, V., Maruška, A., 2006: Capillary electrophoretic analysis of flavonoids in single-styled

hawthorn (*Crataegus monogyna* Jacq.) ethanolic extracts. J. Chromatogr. A 1112, 339-344. DOI: 10.1016/j.chroma.2006.01.034

WALKER, A.F., MARAKIS, G., MORRIS, A.P., ROBINSON, P.A., 2002: Promising hypotensive effect of hawthorn extract: a randomized double-blind pilot study of mild, essential hypertension. Phytother. Res. 16, 48-54. DOI: 10.1002/ptr.947

WALKER, A.F., MARAKIS, G., SIMPSON, E., HOPE, J.L., ROBINSON, P.A., HASSANEIN, M., SIMPSON, H.C.R., 2006: Hypotensive effects of hawthorn for patients with diabetes taking prescription drugs: a randomised controlled trial. Brit. J. Gen. Pract. 56, 437-443.

ZHANG, Z., HO, W.K.K., HUANG, Y., JAMES, A.E., LAM, L.W., CHEN, Z.H., 2002: Hawthorn fruit is hypolipidemic in rabbits fed a high cholesterol diet. J. Nutr. 132, 5-10. DOI: 10.1093/jn/132.1.5

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