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Phenolic profiles and antioxidative effects of hawthorn cell suspensions, fresh fruits, and medicinal dried parts

Thomas Froehlicher [a], Thierry Hennebelle [b], Françoise Martin-Nizard [c,d,e], Patricia Cleenewerck [b], Jean-Louis Hilbert [a], Francis Trotin [a,b], Sébastien Grec [a,*]

[a] UMR 1281, Stress Abiotique et Différenciation des Végétaux Cultivés, USTL, INRA, Université Lille 1 (Université Lille Nord de France), F-59655 Villeneuve d'Ascq, France
[b] EA 1043, Laboratoire de Pharmacognosie, Faculté des Sciences Pharmaceutiques et Biologiques, Université Lille 2 Droit et Santé (Université Lille Nord de France), F-59006 Lille, France
[c] Université Lille 2 Droit et Santé (Université Lille Nord de France), Faculté des Sciences Pharmaceutiques et Biologiques, F-59006 Lille, France
[d] Département d'athérosclérose, Institut Pasteur de Lille, F-59019 Lille, France
[e] INSERM U 545, F-59019 Lille, France

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ABSTRACT

The polyphenolic content of two cell suspension lines (red and yellow) initiated from the ovarian wall of *Crataegus monogyna* flower and their antioxidative potencies against $ABTS^{\cdot+}$, $DPPH^{\cdot}$, and human LDL oxidation were compared to those of red fresh and dry fruits, flower buds and flowering tops. Maximal phenolics and proanthocyanidins contents were found in red suspension extracts displaying high antioxidative effects. In contrast, yellow cell extracts were always the poorest in both phenolics and activity. Flower buds and flowering tops have significant phenolic yields and effects. Both fresh and dried fruits are less active. The amounts in some major phenolic compounds were determined in all tested samples: again, the most antioxidant samples were richer, the red cell line showing particularly high amounts in epicatechin and chlorogenic acid, whilst dried flower buds contained mainly hyperoside and chlorogenic acid. (−)-Epicatechin was confirmed to be more efficient as an antioxidant compound than hyperoside and chlorogenic acid in all assays and more generally, proanthocyanidins were found to be more clearly related to antioxidant activity than other classes of phenolics. The major anthocyanin characterising the red cells of *C. monogyna* was isolated and identified as cyanidin-3-*O*-galactoside.

1. Introduction

The genus *Crataegus* (Rosaceae) is represented by many species that are present in the northern hemisphere. Depending upon their origin (Europe or America) these plants are used in different ways, horticultural, source of edible fruits (e.g. mediterranean *Crataegus azarolus*, american *C. opaca* and mexican *C. pubescens*), whilst some others, often with smaller and more insipid fruits, are mainly restricted to the medicinal use of their flowers, leaves, and fruits (*C. laevigata, C. monogyna*, widespread in Europe). These species, inscribed in the European Pharmacopoeia, as well as the lesser used *C. azarolus, C. nigra*, and *C. pentagyna*, are the most commonly used European and North American phytopharmaceuticals against mild cardiac disorders or nervosity (Chang, Zuo, Harrison, & Chow, 2002). In China, *C. pinnatifida* is widely cultivated for its edible fruits (Cui et al., 2006), and *C. pinnatifida* and *C. cuneata* fruits are used in the treatment of digestive disorders and hyperlipidemia (Pinkas, Peng, Torck, & Trotin, 1996). In this article, the term "hawthorn" is used for the two major European species.

Pharmacological data show that hawthorn and its preparations enhance myocardial contraction and conductivity, protect against ischemia, whilst generally lowering the heart rate (Veveris, Koch, & Chatterjee, 2004). The benefits include enhancement of exercise performance, improvement of coronary blood flow, lowering of blood pressure and clinical tests have confirmed the cardiac interest (Pittler, Guo, & Ernst, 2008; Pittler, Schmidt, & Ernst, 2003). The major components in hawthorn are polyphenols (Svedström, Vuorela, Kostiainen, Laakso, & Hiltunen, 2006): catechins, mainly (−)-epicatechin, oligomeric proanthocyanidins such as the prominent B_2 dimeric procyanidin, and flavonoids such as hyperoside (flowers, fruits) and vitexin-2"-*O*-rhamnoside (leaves). Polyphenols are well-known as antioxidants, veinotonics, and they may be determinant in the interest of fruit and vegetable consumption for prevention of chronic degenerative diseases, especially against atherosclerosis and cancerisation (Kris-Etherton et al., 2002; Scalbert & Williamson, 2000).

Plant cell cultures are a means to study or to produce some active metabolites such as alkaloids, triterpenes, quinones or

* Corresponding author. Present address: UMR 1281, Stress Abiotique et Différenciation des Végétaux Cultivés, USTL, INRA, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France. Tel.: +33 3 20 43 40 19; fax: +33 3 33 72 44.
E-mail address: sebastien.grec@univ-lille1.fr (S. Grec).

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polyphenols (Oksman-Caldentey & Inzé, 2004). The production of these metabolites was studied in *Fagopyrum esculentum* and *C. monogyna* (Bahorun, Trotin, & Vasseur, 1994). A mixed coloured (yellowish-red) callus culture was initiated from flowering buds of the species *C. monogyna* Jacq. This callus culture was shown to produce interesting yields of polyphenols and a derived liquid suspension culture was established. Both callus and cell suspension showed high scavenging activities against H_2O_2 and HOCl *in vitro*, the activity being clearly linked to the total phenolic yield as well as its evolution during growth (Rakotoarison et al., 1997). From the mixed coloured callus culture, a red cell line, as well as a yellow one had been selected.

The aim of this study was to analyse the phenolic contents of these two cell lines, and to compare the antioxidative effect of both, in $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ systems as well as their protective effect against human low density lipoprotein (LDL) oxidation, with those of the common forms of hawthorn consumption (dried fruits, floral buds and flowering tops). The main anthocyanin which distinguished the interesting antioxidant red cell suspension was also isolated and identified.

2. Materials and methods

2.1. Chemicals

Folin–Ciocalteu's reagent, potassium persulphate and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) were obtained from Fluka (Germany); 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and Trolox from Sigma (Germany); natural product standards from Extrasynthese (France). CD_3OD and CF_3COOD were purchased from Euriso-top (France); the HPLC grade organic solvents and TLC silica F_{254} plates from Merck. Column silica used was a TLC silica powder from Merck as well as the RP-18 column Merck Lobar ref 10-625. All other chemicals were of analytical grade purity. Sephadex LH20 was from Pharmacia.

2.2. Plant material

Fresh fruit of *C. monogyna* Jacq. were harvested in Saint-Saulve, near Valenciennes (North of France) in autumn 2006, identified by Prof. F. Trotin and deep-frozen. Commercial dried fruit (batch no. 16239), flowering tops (flowers with young leaves) (batch no. 14595) and flower buds (commercially named "flowers", batch no. 13877) were obtained from Cailleau Herboriste (Chemillé, France).

2.3. Cell cultures

The cell suspensions were derived from two *C. monogyna* callus lines, the one red-coloured, the second yellow, initiated from the ovarian wall of floral buds. Cell suspensions, as for calli, were cultivated on a medium already described (Bahorun et al., 1994). The cells were maintained under 16:8 light/dark conditions at 22 ± 2 °C under rotating agitation (70 rpm), and subcultured every 14 days. They were collected at the 14th day of culture, filtered and deep-frozen.

2.4. General extraction

2.4.1. Total extracts

Crushed plant material was macerated (room temperature in darkness) in $MeOH/Me_2CO/H_2O$ (7/7/3 v/v/v) (Quettier-Deleu et al., 2003) containing 0.2% v/v of AcOH, generally in a ratio 50 g of plant/litre of solvent volume, first for one night, filtered, then macerated twice again, each during 4 h, in the same conditions. The collected filtrates were low-pressure concentrated and taken up in 80% ethanol (1 g of plant /ml). These extracts were further called "*total extracts*".

2.4.2. Ethyl acetate fractionation

One half of the "*total extract*" was added to 150–200 ml distilled water, ethanol eliminated under vacuum, and the remaining water phase partitionned with 5–6 × 100 ml ethyl acetate. The gathered ethyl acetate phase was dried on Na_2SO_4, vacuum evaporated to dryness and taken in 80% ethanol (generally: 1 g of initial plant/ml). These extracts were further called "*ethyl acetate extracts (AcOEts)*". They contain the oligomeric proanthocyanidins, hydroxycinnamic derivatives, and great majority of flavonoids, but no anthocyanins.

2.5. Thin layer chromatography (TLC) analysis

TLC of phenolic extracts was performed on Merck 5554 Silica gel F_{254} aluminium sheets, mostly with toluene/acetone/water: 3/3/1 (v/v). Detection: visible, UV alone, aminoethyl-diphenyl borinate 1% in MeOH with 3% PEG300 (visible and UV), anisaldehyde/H_2SO4 (anisaldehyde 0.2 ml/MeOH 85 ml/AcOH 10 ml/H_2SO4 5 ml) then 3–4 min at 105 °C. Anthocyanins were analysed on Merck 5730 cellulose TLC plates, with "HFW" $HCl/HCOOH/H_2O$: 21/38/41 (v/v) "BAW" *n*-$BuOH/AcOH/H_2O$ 4/1/5 (v/v), "Forestal": AcOH/ conc HCl/H_2O: 30/3/10 (v/v), 60% AcOH.

2.6. Anthocyanin extraction and isolation

The red cell suspension (1.1 kg fresh weight) was macerated thrice in 2 l (20 °C, in darkness) $MeOH/Me_2CO/H_2O$ solvent (7/7/3 v/v/v) containing 0.2% AcOH, first 24 h, then 4 h, then 24 h until extract discolouration. The gathered filtrates were carefully vacuum-concentrated to give a water phase (about 1 l). The major part, 900 ml, was exhaustively partitioned with ethyl acetate (5–6 × 600 ml) to eliminate flavonoids. The remaining red water phase (corresponding to 990 g cells) was carefully concentrated and dissolved in 99 ml of 75% ethanol containing 0.2% acetic acid, concentrated and deposited on a 650 × 32 mm Sephadex LH20 column previously equilibrated with MAW: $MeOH/AcOH/H_2O$ (5/1/14 v/v/v) (Davies & Mazza, 1992). Elution was made at 0.7 ml/min with MAW, (*ca* 180 fractions, 9 ml each) collected and analysed (TLC, HPLC), giving thereafter 8 groups called LH-A to LH-H.

Groups LH, -C, -D, -E were concentrated together and dissolved in MAW then added to a column (500 × 32 mm) of Microcristalline Cellulose Merck ref no. 02331 equilibrated with MAW. Elution (1 ml/min; 9.5 ml/tube) gave 180 fractions, repartitioned after analysis in eight groups called Cel-A to Cel-H. Groups Cel-C and Cel-D were mixed, concentrated and placed on a Merck Lobar Silica Lichroprep RP-18 column equilibrated with 6% acetonitrile in water added of 0.2% AcOH. Elution (0.5 ml/min) was performed with increasing proportions of acetonitrile in water, always with 0.2% AcOH: first 7% MeCN (1700 ml), then 10% MeCN (100 ml), finally 14% MeCN (500 ml). The 115 fractions (*ca* 9.5 ml) could be divided in five groups called RP-A to RP-E.

2.7. Anthocyanin analysis

For comparison of the isolated anthocyanin with authentic samples of idaein and kuromanin, sample solutions were prepared by dissolving 1.0 mg of extract in 10.0 ml MeOH. HPLC analyses were performed with a Shimadzu apparatus (LC-10AS pumps, SCD-10A detector, SCL-10Avp controller, LC solution software). The injection volume was 20 µl. The column was a Supelco Discovery C18, 5 µm, 250 × 4.6 mm. Mobile phase was a mixture of solvent A (0.3% orthophosphoric acid in water) and solvent B (100% acetonitrile) according to a linear gradient changing from 7% to 17% B in 20 min, (flow rate 1 ml/min). The detection was performed at 280 and 525 nm.

Nuclear magnetic resonance (NMR) spectra were recorded in a CD_3OD/CF_3COOD (98:2) mixture (v/v) on a Bruker Avance 500 spectrometer operating at 500 MHz for ^1H- and 125 MHz for ^{13}C NMR and analysed with the software Topspin 1.3, in the Laboratoire d'Application de RMN (LARMN), University of Lille 2. Before a selective monodimensional NOESY experiment was recorded, a specific ^1H experiment was performed on the selected multiplet (δ 3.97, d, J = 3.3 Hz) in order to optimise the integrated area of the spectrum and avoid false correlations.

2.8. Extract contents and activities

2.8.1. Total phenol content

Folin–Ciocalteu's reagent/water (750 µl, 1:14) mixture were added to a 50 µl sample and the reaction was stopped exactly 3 min after by adding 200 µl of 20% Na_2CO_3. The solution was homogenised, heated at 100 °C for 1 min, left in the dark for 30 min. Absorbance was read at 685 nm (each measure in triplicate) and MeOH used as blank (50 µl instead of the extract). Methanolic dilutions of gallic acid were prepared and assayed; total phenol amounts in extracts were expressed in mg gallic acid/100 g dry matter (Singleton & Rossi, 1965).

2.8.2. Individual content in phenolic substances

To calculate the individual phenolic concentration expressed in mg/100 g DW, the area of individual peaks were integrated and compared to their corresponding standard (four points standard curve, each measure in triplicate). HPLC analyses were performed with a Shimadzu apparatus (SIL-20AC autosampler, DGU-20AC degasser, LC-20AD pumps, SPD-M20A diode array detector, CBM 20A controller, LC solution software). The injection volume was 20 µl. The column was a Synergy 4 µm fusion-RP 80A, 4 µm, 150 × 4.6 mm. Mobile phase was a mixture of solvent A (water) and solvent B (acetonitrile, 2% AcOH) according to following sequence of linear gradients: T = 0 min: 8% of solvent B; T = 2 min: 12% of solvent B; T = 16 min: 15% of solvent B; T = 20 min: 30% of solvent B; T = 30 min: 40% solvent B; T = 31 min: 100% of solvent B; T = 35 min: 100% of solvent A.

2.8.3. Flavonoid (sensu stricto) content

In sealed tubes, 1.5 ml of a 2% methanolic solution of $AlCl_3$, $6H_2O$ were added to 0.5 ml sample, then kept in the dark for 10 min. Absorbance was read at 430 nm, methanolic $AlCl_3$ used as blank, each measure in triplicate. A series of methanolic dilutions of rutin was prepared and assayed; flavonoid amounts in extracts were expressed in mg rutin/100 g dry matter (Lamaison & Carnat, 1991).

2.8.4. Proanthocyanidin content

In sealed tubes, 0.5 ml sample was added to a mixture of 0.5 ml MeOH, 6 ml n-BuOH/concentrated HCl (95:5 v/v) and 0.2 ml of a 2% $NH_4Fe(SO_4)_2$, $12H_2O$ solution in 2 M HCl. Absorbance was read at 550 nm before and after heating for 40 min at 95.0 ± 0.2 °C (each measure in triplicate, blank n-BuOH/HCl mixture). A series of dilutions of cyanidin chloride in n-BuOH/HCl was assayed; proanthocyanidin amounts in extracts were expressed in mg cyanidin/100 g dry matter (Porter, Hrstich, & Chan, 1986).

2.8.5. Anthocyanin content

Nine hundred and sixty microlitres of pH 1 (25 ml of 1.49% KCl + 67 ml of 1.7% HCl, pH corrected with HCl) or pH 4.5 (1.64% AcONa, pH corrected with AcOH) buffer solutions were each added to 40 µl of extract. Absorbance was read at 700 and 510 nm against water as blank. Each measure was made in triplicate. The results were expressed in mg cyanidin-3-glucoside/100 g dry matter, according to the formula given by Giusti and Wrolstad (2001).

2.8.6. $ABTS^{\cdot+}$ radical scavenging capacity

One millilitre of an $ABTS^{\cdot+}$ solution (7 mM $ABTS^{\cdot+}$ in H_2O, 2.45 mM potassium persulfate, completed with ethanol to achieve a 0.7 ± 0.02 absorbance at 734 nm) was added to 10 µl sample. After 1 min, absorbance was read at 734 nm, ethanol used as a blank (each measure in triplicate). The values are obtained from the capacity to inhibit the $ABTS^{\cdot+}$ at a defined time point, relative to Trolox and expressed as mM Trolox equivalent per 100 g DW (Re et al., 1999).

2.8.7. $DPPH^{\cdot}$ radical scavenging activity

The $DPPH^{\cdot}$ radical scavenging activity was adapted from the method used by Makris, Boskou, and Andrikopoulos (2007). A 25 µl solution of each extract concentration was added to 975 µl of a 100 µM methanolic solution of $DPPH^{\cdot}$. After 30 min, the optical density was read at 515 nm, methanol used as a blank. Each measure was made in triplicate. Different dilutions of Trolox were used as a standard curve to calculate activities in mM Trolox equivalent/100 g DW.

2.8.8. Inhibition of LDL oxidation

Human LDL were isolated from freshly drawn blood from healthy, normolipidemic, fasting volunteers. Blood was collected into EDTA and the plasma was separated by low-speed centrifugation. LDL were isolated by sequential density gradient ultracentrifugation (Havel, Eder, & Bragdon, 1955). Then, LDL were dialysed against 0.1 M PBS (phosphate-buffered-saline: 0.15 M NaCl, 0.1 M Na-phosphate, pH 7.4) containing 0.01% EDTA, sterilized by filtration through a 0.22 µm pore-filter and stored at +4 °C before use. The protein concentration was determined (Peterson, 1977) and corrected to 125 µg/ml with PBS. Mother solutions of extracts (0.5 g/ml) were used to make 1/100 to 1/100,000 dilution series just before the analyses. LDL oxidation was induced at 30 °C by adding 20 µl of 16.6 µM $CuSO_4$ to 160 µl of LDL (125 µg of protein/ml) and 20 µl of extracts in PBS. Conjugated diene formation was followed by measuring density at 234 nm every 10 min for 8 h in 96-well UV microplaques with a Molecular Device SpectraMaxPlus spectrometer, with the software SoftMaxPro (Molecular Device). ED_{50} was defined as the concentration increasing 1.5 times the lag-phase duration of conjugated diene formation.

3. Results and discussion

3.1. Phenol contents

3.1.1. Total phenols

Total phenol contents measured according to the Folin–Ciocalteu method are shown in Table 1 and Fig. 1, respectively, for total extracts (Total) and ethyl acetate extracts (AcOEts). Expressed in gallic acid equivalents, they are far higher in the case of red cell extracts, as compared to all other tissues. In total extracts were typically observed flavonoids, the global proanthocyanidins (polymeric and oligomeric forms) plus catechins, hydroxycinnamic derivatives, and eventually anthocyanins (if present). Total phenol contents decreased in the following order: red cells > dry flowering tops > dry flowers > dry fruit # fresh fruit > yellow cells. These data roughly divide our materials into three classes: (1) high phenolic content: red cells, dry flowering tops and flowers; (2) medium content: dry and fresh fruit and (3) low content: yellow cells. Ethyl acetate extract typically contained oligomeric proanthocyanidins, hydroxycinnamic derivatives, and nearly all the flavonoids, but

Table 1
Phenolics contents, free radical scavenging, antioxidant activity and inhibition of LDL oxidation of different forms of *Crataegus monogyna*. Amounts in both total (Total) and ethyl acetate extracts (AcOEts) are given as mean values ± standard deviation ($n = 3$).

		Total phenols[a]	Flavo.[b]	Procya.[c]	Anthocy.[d]	ABTS[e]	DPPH·[f]	LDL[g](ED_{50})
Red cells	Total	12333.5 ± 130.4	N.D.	2028.2 ± 55.7	1027.0 ± 0.1	48.5 ± 1.1	85.7 ± 4.4	44.0
	AcOEt	3457.5 ± 136.3	232.2 ± 6.0	365.3 ± 2.3	–	13.0 ± 1.3	26.4 ± 0.9	–
Yellow cells	Total	712.4 ± 36.8	N.D.	114.2 ± 2.3	–	4.7 ± 0.3	3.5 ± 0.2	Undetec.
	AcOEt	141.6 ± 6.6	82.9 ± 7.0	12.7 ± 0.6	–	0.5 ± 0.1	0.5 ± 0.1	–
Fresh fruits	Total	1226.3 ± 33.7	N.D.	108.7 ± 9.2	58.0 ± 0.1	6.6 ± 0.2	5.4 ± 0.4	399.1
	AcOEt	608.9 ± 32.6	147.3 ± 3.3	38.7 ± 1.3	–	3.1 ± 0.2	4.0 ± 0.4	–
Dried fruits	Total	1282.3 ± 76.1	N.D.	96.8 ± 4.8	15.0 ± 0.1	5.4 ± 0.3	6.3 ± 1.1	690.3
	AcOEt	360.3 ± 8.7	103.0 ± 3.4	9.0 ± 0.6	–	1.1 ± 0.1	1.4 ± 0.1	–
D. flowering tops	Total	5629.2 ± 201.7	N.D.	597.3 ± 44.7	–	10.4 ± 0.3	34.8 ± 1.9	139.1
	AcOEt	2077.2 ± 29.8	623.2 ± 3.3	332.2 ± 28.9	–	4.2 ± 0.2	5.7 ± 0.3	–
D. flowers	Total	4931.0 ± 127.1	N.D.	1712.0 ± 75.4	–	14.4 ± 0.7	25.6 ± 2.1	151.3
	AcOEt	1563.0 ± 61.9	1026.6 ± 33.8	82.6 ± 7.2	–	5.0 ± 0.4	9.1 ± 0.7	–

[a] Total phenol: Folin–Ciocalteu, in mg eq. gallic acid/100 g DW.
[b] Flavonoids: $AlCl_3$ method, in mg eq. rutin/100 g DW.
[c] Procyanidins: butanol–HCl methods, in mg eq. cyanidins/100 g DW.
[d] Anthocyanins: direct colorimetry in mg eq. cyanidin-3-*O*-glucoside/100 g DW.
[e,f] Free radical scavenging activity was measured by DPPH· method and antioxidant activity by $ABTS^{\cdot+}$. $ABTS^{\cdot+}$ and DPPH· are expressed in Trolox equivalent mM/100 g DW.
[g] Inhibition of LDL oxidation by different forms of *Crataegus monogyna* (mg DW/ml) are expressed as ED_{50}.

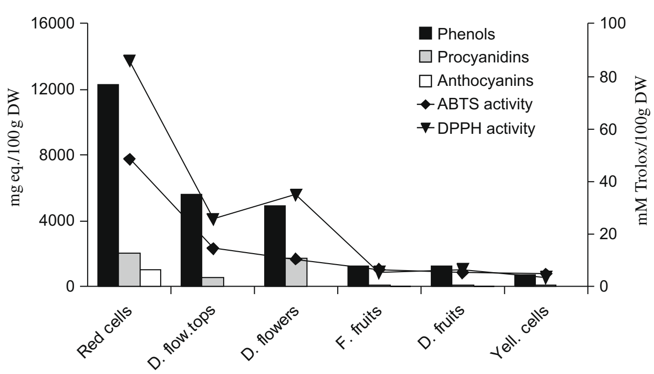


Fig. 1. Phenolic contents, free radical scavenging and antioxidant activities measured in total extracts of different forms of *Crataegus monogyna*. Phenols: Folin–Ciocalteu, in mg eq. gallic acid/100 g DW; Procyanidins: butanol–HCl methods, in mg eq. cyanidin /100 g DW; anthocyanins: direct colorimetry in mg eq. cyanidin-3-*O*-glucoside/100 g DW. $ABTS^{\cdot+}$ and DPPH· are expressed in Trolox equivalent (mM Trolox/100 g DW). Values are given in Table 1.

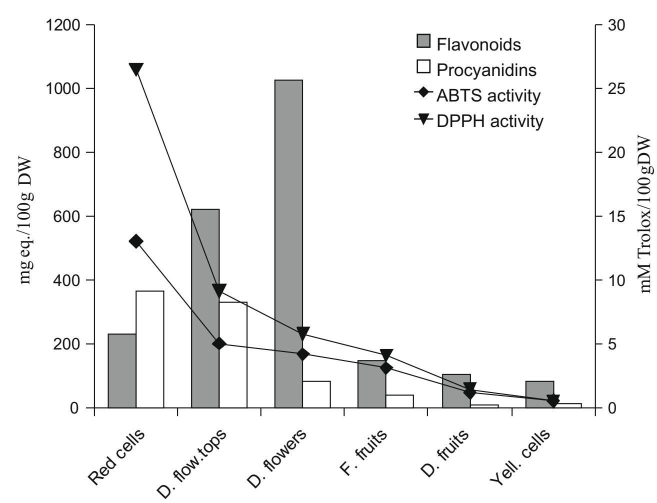


Fig. 2. Flavonoid and oligoprocyanidin contents, free radical scavenging and antioxidant activities measured in ethyl acetate extracts of different forms of *Crataegus monogyna*. Flavonoids: $AlCl_3$ method, in mg eq. rutin/100 g DW; procyanidins: *n*-butanol–HCl methods, in mg eq. cyanidin/100 g DW; $ABTS^{\cdot+}$ and DPPH· results are expressed in Trolox equivalent (mMol Trolox/100 g DW). Values are given in Table 1.

no anthocyanins. As with total extracts the phenolic content of ethyl acetate extracts can be divided into rich, medium and low content classes according to the following order: red cells, dry flowering tops and flowers, fresh and dried fruit, yellow cells.

3.1.2. Proanthocyanidins

Proanthocyanidins are important phenolics having an impact on antioxidant effects. These compound are generally more abundant in total plants extracts, which typically contain both polymeric (polymerisation grade > 6–8 catechin subunits) and oligomeric procyanidins (polymerisation grade: 2 to 6–8 subunits) whilst ethyl acetate fractions mainly contain the oligomeric forms (Porter, 1989; Thompson, Jaques, Haslam, & Tanner, 1972). Total extract procyanidins (Table 1 and Fig. 1) are most abundant in the red cells, then in dried flowers, and a lower level was found in the dried flowering tops. The poorest tissues are yellow cells, fresh fruits and dry fruits, these show similar low values. Oligomeric procyanidin (=oligomeric forms) contents (Table 1 and Fig. 2) are similar in ethyl acetate extracts.

3.1.3. Flavonoids

In order to avoid anthocyanin interference if present in some tissues, flavonoids were only determined in the ethyl acetate fractions. The best yields (Table 1 and Fig. 2) are found in the plant dry parts: flowers and flowering tops. This is consistent with the well-known fact that flavonoids are major components in such parts; values of 0.3–2.5% flavonoids have been cited for dried flowering tops (Wichtl & Anton, 2003). The red cells contain an intermediate level. Fresh fruit, dry fruit and the yellow cells contained the lowest amount of flavonoids. The red cell suspension, characterised by its total phenol and procyanidins contents, is relatively poorer in flavonoids. This fact is clearly confirmed by TLC especially with the DPBAE reagent, where red cell ethyl acetate extracts show a single spot at the level of hyperoside, whilst flowers or flowering tops display a more complex flavonoidic profile (data not shown).

3.1.4. Anthocyanins

Only three samples, the red cells (Table 1 and Fig. 1), the fresh and dried fruits contained anthocyanins. Anthocyanins are visible in the red cell suspensions which are homogeneously coloured; in the fresh fruit they are restricted to the tegument and absent in the pulp and seed. The dried fruit also contained low amounts of anthocyanins. Drying seems to have considerably influenced dried fruit anthocyanin content and their colour is always more brownish than reddish, suggesting anthocyanin modifications.

3.1.5. Analysis of the main individual phenolics

HPLC determination (Table 2) of the main individual phenolics was made in the total extracts. Hyperoside is known as the main flavonol glycoside in the flowering parts of hawthorn (Lamaison & Carnat, 1991) and was previously observed in these callus cultures (Bahorun et al., 2003). The main constitutive flavan-3-ol in hawthorn and in our cells (Bahorun et al., 1994; Thompson et al., 1972) was (−)-epicatechin. The contents in two hydroxycinnamic components, chlorogenic and caffeic acids, were also measured. The red cells are characterised by the highest level in (−)-epicatechin, one of the two most important yields in chlorogenic acid, and a relatively important level in hyperoside. Dried flowers are distinguished by elevated levels of hyperoside and of chlorogenic acid whilst epicatechin is well represented. The dry flowering tops contain predominantly epicatechin and chlorogenic acid. The main individual phenolics of fresh fruit seem to be hyperoside and epicatechin. Dry fruits are generally poor in these substances, except hyperoside (37.65 mg/100 g DW). As previously, the yellow cell suspension was characterised by very low contents.

3.2. Antioxidative effects

3.2.1. Reference substances

The activities of the generally used standards against the $ABTS^{\cdot +}$ radical are given in Table 3 as Trolox-equivalents (mM). The frequently-cited flavonol quercetin, its glycoside rutin, and the dimeric B_2 procyanidin, a well-known active procyanidin oligomer, were included in this list. The most active phenolics include the dimeric B_2 procyanidin, quercetin and the flavan-3-ol epicatechin. A little less potent is the hydroxycinnamic derivative chlorogenic acid, followed in decreasing order by two flavonol glycosides: hyperoside and rutin. This is in accordance with Rice-Evans, Miller, and Paganga (1997), who observed (TEAC test) better activities for flavanols such as epicatechin or catechin gallates than for flavonol glycosides such as rutin or hydroxycinnamic derivatives (caffeic and chlorogenic acids). The same range of activities had previously been observed in the TEAC assay: B_2 procyanidin > epicatechin > chlorogenic acid (Bahorun et al., 2003).

Similar results were obtained with the $DPPH^{\cdot}$ system (Table 3). The most potent phenolics comprise quercetin > B_2 procyanidin > epicatechin. The two flavonol glycosides hyperoside and rutin are a little less efficient and chlorogenic acid is the least potent.

Overall the flavanols epicatechin and procyanidin B_2 appear to have the most effect, followed by flavonols such as quercetin, hyperoside and rutin. The action of chlorogenic acid may vary according to the measuring system. A relatively similar graduation was previously observed for such substances in trapping oxygen reactive species as $O_2^{\cdot -}$, H_2O_2 and HOCl (Quettier-Deleu et al., 2000).

Table 3
Free radical scavenging, antioxidant activity and inhibition of LDL oxidation of reference phenolics.

	$ABTS^{\cdot +}$	$DPPH^{\cdot a}$	LDL^b (ED_{50})
Procyanidin B2	3.2 ± 0.1	6.8 ± 0.6	1.4
Quercetin	3.2 ± 0.3	7.8 ± 0.4	1.7
Rutin	0.7 ± 0.1	3.8 ± 0.3	3.7
Epicatechin	2.6 ± 0.1	6.3 ± 0.7	0.7
Hyperoside	1.4 ± 0.1	4.7 ± 0.3	1.8
Chlorogenic Ac.	1.7 ± 0.1	2.7 ± 0.2	2.5

[a] Free radical scavenging activity of individual phenolic (1 mg/ml) was measured by $DPPH^{\cdot}$ method and antioxidant activity by $ABTS^{\cdot +}$. Both are expressed in mM Trolox equivalent. Values are given as mean values ± standard deviation ($n = 4$).
[b] Inhibition of LDL oxidation by reference phenolics (μM) are expressed as ED_{50}.

3.2.2. Extracts

The comparative antioxidative activities in $ABTS^{\cdot +}$ and $DPPH^{\cdot}$ models are given (Figs. 1 and 2; Table 1) as mM Trolox-equivalents/100 g plant DW. For total extracts (Fig. 1), the decreasing order efficiencies in the $ABTS^{\cdot +}$ system is as follows: red cell suspension > flowers > flowering tops > fresh fruit > dry fruit > yellow cell suspension.

Similar results were obtained in the $DPPH^{\cdot}$ system: red cell > flowering tops > flowers > dry fruit > fresh fruit > yellow cell suspension. Analysis of the ethyl acetate extracts (Fig. 2 and Table 1) shows a rather similar pattern in both $ABTS^{\cdot +}$ and $DPPH^{\cdot}$ systems. The four most efficient samples are: red cells > flowers > flowering tops > fresh fruits. The two least active samples are the dry fruits followed by yellow cells.

3.3. Inhibition of LDL oxidation

3.3.1. Reference substances

Comparative inhibitions by standard phenolics of Cu^{2+}-induced LDL oxidation are shown in Table 3. The best inhibitor is (−)-epicatechin, followed by the B_2 dimer. Among flavonoids, hyperoside (quercetin-*O*-galactoside) and the aglycone quercetin display similar-order actions, better than rutin (quercetin-3-*O*-rhamnoglucoside). The hydroxycinnamic derivative chlorogenic acid is a weaker inhibitor than hyperoside and quercetin, but is here more efficient than rutin. This gradation is in accordance with our previous observations on LDL with such phenolics (Quettier-Deleu et al., 2003).

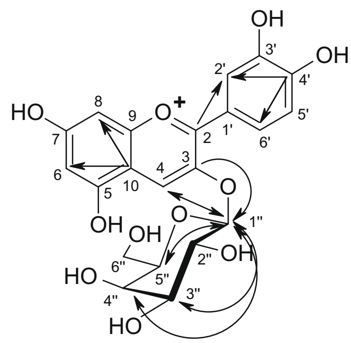
3.3.2. Extracts

The ED_{50} of total extracts are given in Table 1, expressed in mg plant tissue DW/ml. The three best antioxidants are, in decreasing order: red cell suspension > dry flowering tops ⩾ dry flowers. Less effective are the fresh fruit and the dried fruit extract. No antioxidative effect was displayed by the yellow cell extract. This fact, as for $ABTS^{\cdot +}$ and $DPPH^{\cdot}$ reduction, must again be considered in parallel with the phenolic content (especially the total phenol yields) of the extracts.

Table 2
Individual phenolic content measured in total extracts by HPLC. Amounts of compounds in total extracts are given as mean values ± standard deviation ($n = 3$). Integration of area of individual peaks were compared to their corresponding standard (four points standard curve, R^2 presented in the table), to calculate the individual phenolic concentration expressed in mg/100 g DW.

	Chlorogenic Ac. ($R^2 = 0.9997$)	Hyperoside ($R^2 = 0.9996$)	Caffeic Ac. ($R^2 = 0.9999$)	(−)-Epicatechin ($R^2 = 0.9999$)
Red cells	823.4 ± 3.1	105.8 ± 4.8	36.8 ± 0.4	1151.0 ± 97.0
Yellow cells	25.9 ± 0.1	1.3 ± 0.1	8.0 ± 0.1	47.2 ± 0.1
Fresh fruits	13. 8 ± 0.1	57.8 ± 0.8	2.9 ± 0.1	136.6 ± 1.6
Dried fruits	5.4 ± 0.1	37.6 ± 0.1	1.3 ± 0.1	32.4 ± 0.1
Dried flowering tops	636.9 ± 13.6	112.5 ± 0.8	50.9 ± 0.5	237.1 ± 2.9
Dried flowers	828.3 ± 7.1	561.7 ± 4.0	41.1 ± 0.1	135.6 ± 0.9

A



B

	^1H	^{13}C
cyanidin		
2	-	164.4
3	-	145.8
4	9.02	136.9
5	-	159.4
6	6.66 d (*1.9*)	103.4
7	-	170.6
8	6.89 d (*1.9*)	95.2
9	-	157.8
10	-	113.5
1'	-	121.4
2'	8.07 d (*2.3*)	118.5
3'	-	147.6
4'	-	155.9
5'	7.01 d (*8.8*)	117.5
6'	8.27 dd (*8.9, 2.3*)	128.3
galactose		
1''	5.28 d (*7.8*)	104.5
2''	4.02 dd (*9.7, 7.8*)	72.2
3''	3.69 dd (*9.7, 3.4*)	75.0
4''	3.97 d (*3.3*)	70.2
5''	3.83 m	77.9
6'' (a and b)	3.80 m	62.4

Fig. 3. Structure of idaein analysed by NMR techniques. A: structure of idaein and selected correlations of bidimensional NMR experiments (C → H: HMBC; H ↔ H: NOESY). B: ^1H (500 MHz, δ, J in Hz) and ^{13}C (jmod, 125 MHz, δ) NMR data of idaein isolated from red cell suspension culture, recorded in CD_3OD/CF_3COOD (98:2).

3.4. Major anthocyanin of red cell culture

The intense red colouration of the red cells led us to isolate and identify the main anthocyanic component. Similar studies have been recently made on anthocyanins in the fruits of American mayhaw (*C. opaca*) (Trappey, Bawadi, Bansode, & Losso, 2005).

The isolated compound was analysed by mono-(^1H and ^{13}C) and bi-(COSY, HSQC, HMBC, NOESY) dimensional NMR techniques. NMR data are reported in Fig. 3B and the main HMBC and NOESY interactions are shown in Fig. 3A. In the ^1H spectrum, a signal at δ 9.02 (s) was characteristic of H-4 in an anthocyanin derivative. An ABX system (δ 8.27, dd, J = 8.9 and 2.3 Hz, H-6′; δ 8.07, d, J = 2.3 Hz, H-2′; δ 7.01, d, J = 8.8 Hz, H-5′) showed the *ortho*-disubstituted pattern of ring B in the aglycon part. The remaining two signals in the aromatic part of the spectrum were attributed to H-6 (δ 6.66, d, J = 1.9 Hz) and H-8 (δ 6.89, d, J = 1.9 Hz), making it clear that the aglycon moiety of the compound was cyanidin, which was confirmed by the ^1H–^{13}C long-range correlation experiment (HMBC). In the ^{13}C jmod spectrum, six characteristic signals showed the presence of one hexose in the structure of the compound. HMBC (C-3 → H-1'') and NOESY (H-4 ↔ H-1'') correlations proved that it was 3-*O*-substituted. Nevertheless, the coupling constant of the ^1H NMR signal of H-4″ with H-3″ (3.3 Hz) was in favour of galactose (Bjorøy, Fossen, & Andersen, 2007). The NOESY confirmed the nature of the sugar moiety. HPLC analysis and comparison with authentic samples showed that the compound had the same retention time as idaein (cyanidin-3-*O*-galactoside), but not kuromanin (cyanidin-3-*O*-glucoside). Thus, the compound was found to be idaein. This identification was confirmed by comparison with NMR data from authentic idaein, recorded under the same conditions. The fresh-ripe fruits of *C. monogyna* were analysed by HPLC and the major anthocyanin had a retention time that was the same as that of idaein but differed from that of kuromanin. Thus, idaein is likely to be the major anthocyanin in *C. monogyna* fruit. In comparison, Trappey et al. (2005) found in *C. opaca* a majority of kuromanin, besides a lesser proportion of idaein.

4. Conclusion

Comparison of phenolic contents and antioxidant activities of the extracts from *C. monogyna* cell suspensions, fresh fruits, and dried parts shows the positive influence of the yields in total phenols, proanthocyanidins, and flavonoids. The richest extracts were the most efficient in both $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ systems as well as in the protection of human LDL. This is particularly true in the case of total extracts. Several kinds of tissues can be distinguished by a decreasing order of activities: (1) red cell suspension: higher activity and important phenol yields, specially proanthocyanidins; (2) dried hawthorn flower buds and/or flowering tops, generally with similar important activities and important levels of total phenols and flavonoids or proanthocyanidins; (3) fresh and dried fruits, characterised by a lower antioxidative effect and with relatively low yields in total phenols, proanthocyanidins and flavonoids and (4) yellow cell suspension, poor in phenolics and with very weak or non-detectable activity. Very similar results are obtained with the ethyl acetate extracts. Some phenolic classes may be abundant but appear to have less influence on activities e.g. flavonoids in flower buds and flowering tops. These tissues contain flavonoids, especially hyperoside, which, as well as the reference flavonoid rutin, is less antioxidant than flavanols such as epicatechin or B_2 procyanidin. Nevertheless, it is difficult to conclude on the efficiency of one particular phenolic since their global amounts clearly have an effect. The red cell suspension of *C. monogyna* is distinguished by the presence of anthocyanins. Its main anthocyanic component was isolated, identified by NMR as idaein (cyanidin-3-*O*-galactoside), and was shown to also be the major component in the red-coloured fresh fruit of *C. monogyna*. Thus the red cell suspension culture of *C. monogyna* provides a reliable source of a standard for idaein, and has also proven to be a more potent antioxidant than the usual forms of hawthorn consumption.

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