Crataegus monogyna buds and fruits phenolic extracts: growth inhibitory activity on human tumour cell lines and chemical characterization by HPLC-DAD-ESI/MS

Sandra Rodrigues[a], Ricardo C. Calhelha[a,b], João C.M. Barreira[a,c,d], Montserrat Dueñas[c], Ana Maria Carvalho[a], Rui M.V. Abreu[a], Celestino Santos-Buelga[c,*], Isabel C.F.R. Ferreira[a,*]

[a]*CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Apartado 1172, 5301-855 Bragança, Portugal.*

[b]*Centro de Química, Universidade do Minho, Campus de Gualtar 4710-057 Braga, Portugal.*

[c]*GIP-USAL, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

[d]*Centro de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal.*

* Authors to whom correspondence should be addressed (e-mail: iferreira@ipb.pt, telephone +351273303219, fax +351273325405; e-mail: csb@usal.es; telephone +34 923 294537; fax +34 923 294515).

ABSTRACT

Crataegus monogyna has been extensively studied due to its various alleged health benefits. This study aimed to determine the human tumour cells growth inhibitory activity of phenolic extracts of its flower buds and fruits in three phenological stages, and further characterize the extracts by HPLC–DAD–ESI/MS. Flower bud extract showed the highest antiproliferative activity as indicated by the lowest GI_{50} values obtained in all the tested cell lines: MCF-7, breast adenocarcinoma; NCI-H460, non-small cell lung cancer; HeLa, cervical carcinoma; HepG2, hepatocellular carcinoma. Furthermore, porcine liver primary cell culture (PLP2) was used to evaluate toxicity to non-tumour cells. Flavonoids, particularly flavonols and flavones (higher in flower buds) and proanthocyanidins (higher in unripe fruits) were the main classes in the studied samples. Phenolic acids (mainly hydroxycinnamic acid derivatives) were also detected in significant amounts, especially in flower bud extract. Regarding anthocyanins, over ripened fruits gave the highest content. The higher bioactivity observed in flower buds might be related with its higher content in phenolic compounds.

Keywords: Crataegus monogyna; phenolic profiles; human tumour cell lines; antiproliferative activity.

1. Introduction

Cancer is a leading cause of death worldwide and according to World Health Organization (2010) cancer related deaths are projected to increase to over 11 million in 2030.

The vast structural diversity of natural compounds found in plants provides unique opportunities for discovering new drugs. Phenolic compounds, mainly flavonoids, are an example of bioactive compounds with possible beneficial effects on human health, including regulation of proliferation and cell death pathways leading to cancer (López-Lázaro, 2002). *In vitro* studies have concentrated on their direct and indirect actions on tumour cells (Kandaswami et al., 2005), and have found a variety of anticancer effects such as cell growth (Kandaswami et al., 1991) and kinase activity (End et al., 1987) inhibition, apoptosis induction (Lee et al., 2002), suppression of the secretion of matrix metalloproteinases (Kim, 2003) and of tumour invasive behaviour (Parmar et al., 1994). However, it should be taken into account that the *in vivo* bioactive forms of phenolic compounds are not necessarily the natural phytochemical forms, but instead their conjugates and metabolites (Spencer, Mohsen & Rice-Evans, 2004).

Crataegus monogyna Jacq. (common hawthorn) is one of the species that is highly recommended in folk medicine and the "berries" are usually consumed by shepherds, hunters and children, because they are considered to be "healthy" and nutritious (Carvalho, 2010). The nutritional and nutraceutical composition of hawthorn flowers and fruits were previously reported (Barros, Carvalho & Ferreira, 2011). Flowers revealed the highest tocopherols and ascorbic acid contents, as also the best n-6/n-3 fatty acids ratio; over ripened fruits showed the highest levels of carbohydrates, sugars and saturated fatty acids; unripe fruits presented the highest polyunsaturated fatty acids content, as also the most promising antioxidant properties (even higher than the

standard trolox) (Barros et al., 2011). There are some reports on phenolic compounds present in hawthorn flowers and fruits (Froehlicher et al. 2009; Liu, Yang & Kallio, 2010; Barros et al., 2011; Liu, Kallio, Lü, Zhou & Yang, 2011; Barros, Dueñas, Carvalho, Ferreira & Santos-Buelga, 2012). Nevertheless, as far as we know, this is the first report with a systematic comparison of four different hawthorn parts (flower bud, and unripe, ripened and over ripened fruits), evaluating human inhibitory activity on human tumour cell lines (breast, lung, cervical and hepatocellular carcinomas) of their phenolic extracts, that were further chemically characterized by HPLC-DAD-ESI/MS.

2. Materials and methods

2.1. Samples

The material for analysis was gathered in sequence, during 2009 spring, summer and autumn, synchronized with the growth condition of buds and fruits, according to different gathering practices, folk pharmacopoeia and local edible uses reported in the studied area (Bragança, north-eastern Portugal). Six trees exhibiting profuse flower bud development in spring were selected from the outer band of a semi-natural pasture, according to previous ethnobotanical inventory. Four different parts of *C. monogyna* were considered: flower buds with top young leaves (corymbs); unripe fruits corresponding to flower senescence and stand out of the green pomaceous (berry-like) immature fruit; ripened fruits i.e. red pomes in late summer; over ripened fruits i.e. dark red, fleshy, sweet, chewy and coarse-textured pomes in late autumn. All plant parts were gathered from the entire canopy of each selected tree. For each plant part, a final sample for analysis was made with material from the six selected trees. The natural appearance of the used *C. monogyna* parts might be looked up in a previous work dealing with chemical composition and bioactivity of this plant (Barros et al., 2011).

Each sample was lyophilized (Ly-8-FM-ULE, Snijders, Holland) and kept in deep-freezer at -20ºC for subsequent use.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and trifluoroacetic acids were purchased from Prolabo (VWR International, France). The phenolic compounds standards were from Extrasynthese (Genay, France). DMSO (dimethyl sulfoxide) was analytical grade from Fisher Scientific (Paris, France). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Preparation of the phenolic extracts

Each sample (≈1 g) was extracted by stirring with 30 mL of methanol:water 80:20 (*v/v*), at room temperature, 150 rpm, for 1 hour (h). The extract was filtered through Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. The aqueous phase was lyophilized and re-dissolved in a) DMSO at 8 mg/mL for antiproliferative assays, or b) 20% aqueous methanol at 5 mg/mL and filtered through a 0.22-µm disposable LC filter disk for High Performance Liquid Chromatography (HPLC-DAD-MS) analysis.

2.4. Growth inhibition activity in human tumour cell lines

The effects of the extracts on the growth of human tumour cell lines was evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses sulforhodamine B (SRB) assay to assess cell growth inhibition (Skehan et al., 1990; Vichai & Kirtikara, 2006). Four human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7 and NCI-H460) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460, 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. The DMSO concentrations used have no growth inhibitory effect in these cell lines (data not shown).

Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 minutes (min) at 4 ºC. Plates were then washed with deionized water and dried; SRB solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 µL) and the absorbance was measured at 540 nm in a microplate reader (Biotek Elx800). Dose-response curves were obtained for each tested extract and cell line, and the GI_{50} value, corresponding to

the concentration of the extract that inhibited 50% of the net cell growth was calculated (Vichai & Kirtikara, 2006). Ellipticine was used as positive control.

2.5. Growth inhibition activity in non-tumour cells

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Abreu et al., 2011).

2.6. Chemical characterization of the extracts

Analysis of non-anthocyanin phenolic compounds

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over

10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (1-100 μg/mL) of different standards compounds: (+)-catechin ($y = 158.42x - 11.38$; $R^2 = 0.999$); (-)-epicatechin ($y = 160.86x - 6.3472$; $R^2 = 0.999$); caffeic acid ($y = 611.9x - 4.5733$; $R^2 = 0.999$); chlorogenic acid ($y = 313.03x - 58.2$; $R^2 = 0.999$); *p*-coumaric acid ($y = 884.6x -$

184.49; R^2 = 0.999); ferulic acid (y = 505.97x - 64.578; R^2 = 0.999); apigenin-7-*O*-glucoside (y = 159.62x + 7.5025; R^2 = 0.999); quercetin-3-*O*-glucoside (y = 253.52x - 11.615; R^2 = 0.999); kaempferol-3-*O*-glucoside (y = 288.55x - 4.0503; R^2=1.000); *p*-hydroxybenzoic acid (y = 265.74x - 87.777; R^2 = 0.999); protocatechuic acid (y = 291.1x - 6.4558; R^2 = 0.999).

Analysis of anthocyanins

Each sample (1 g) was extracted with 30 mL of methanol containing 0.5% TFA, and filtered through a Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 15 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol/water (80:20, *v/v*) containing 0.1% TFA. The methanolic extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-µm disposable LC filter disk for HPLC analysis.

The extracts were analysed in the HPLC system indicated above using the conditions described by (García-Marino, Hernández-Hierro, Rivas-Gonzalo & Escribano-Bailón, 2010). Separation was achieved on an AQUA® (Phenomenex) reverse phase C_{18} column (5 µm, 150 mm × 4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min and from 30 to 35% for 5 min, at a flow rate of

0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000V in the positive ion mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (50-0.25 μg/mL) of different standards compounds: cyanidin-3-*O*-glucoside (y = 63027x - 153.83; R^2 = 0.9995), pelargonidin-3-*O*-glucoside (y = 268748x - 71.423; R^2 = 1.0000) and peonidin-3-*O*-glucoside (y = 537017x - 71.469; R^2 = 0.9997).

2.7. Statistical analysis

For each type of sample three independent experiments were performed, and in each of them samples were analysed in duplicate. The results were expressed as mean values ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test (homoscedastic distributions) or Tamhane's T2 test

(heteroscedastic distributions) with α = 0.05, coupled with Welch's statistic. All statistical tests were performed with the SPSS v.18.0 software.

3. Results

3.1. Growth inhibitory activity on human tumour cell lines

The effects of the phenolic extracts obtained from hawthorn parts (flower buds and unripe, ripened and over ripened fruits) on the growth of four human tumour cell lines (MCF-7, NCI-H460, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are summarized in **Table 1**. These cell lines were selected because they are well characterized and representative of different tumor cell types, with different tissue origins, being widely used to screen antitumour potential.

The flower buds extract was the most potent in all tested cell lines, presenting GI_{50} values that ranged from 63.55 to 88.45 μg/mL for the HeLa and HepG2 cells, respectively. Nevertheless, none of the samples showed toxicity in the non-tumour tested cells (porcine liver primary cell culture; PLP2), since GI_{50} values were much higher than those corresponding to tumour cell lines (**Table 1**).

Ellipticine, a potent antitumor agent whose mechanism of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II (Stiborová, Bieler, Wiessler, & Frei, 2001), was used as positive control. However it should not be considered as standard because it is a pure and synthetic compound. Furthermore, it shows also high toxicity for non-tumour cells. The obtained results for the most active *C. monogyna* samples were comparable to other natural matrixes also studied by us in the same cell lines (Vaz et al., 2012).

The characterization of the phenolic compounds present in the extracts was performed by HPLC-DAD-MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion,

main fragment ions in MS^2, tentative identification and concentration of phenolic acids, flavonoids and anthocyanins are presented in **Tables 2-4**. As an example, the HPLC phenolic profiles of *C. monogyna* flower bud (A) and unripe fruit (B) recorded at 280 nm can be observed in **Figure 1**. The separation for some minor compounds was not completely effective, hindering their quantification.

3.2. Phenolic acids and derivatives

The found phenolic acids corresponded to hydroxycinnamic acid derivatives, namely chlorogenic acids, a family of esters formed between certain cinnamic acids, most commonly caffeic, *p*-coumaric and ferulic acids and quinic acid (IUPAC, 1976). According to their UV spectra (λ_{max} at 314-330 nm) and pseudo molecular ions $[M-H]^-$ (*m/z* at 353, 337, 367, 515 and 499, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic acid), ten compounds detected in the flower buds (peaks 1, 3, 4, 6, 8, 11, 13, 21, 27 and 28 in Table 2) and three compounds in fruit extracts (peaks 1, 3 and 23 in Table 3) were identified as cinnamoyl-quinic acids containing one or two caffeic acid, *p*-coumaric acid, ferulic acid moieties. Peak assignments of the different hydroxycinnamoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) and the hierarchical keys previously developed by (Clifford, Johnston, Knight & Kuhnert, 2003; Clifford, Knight & Kuhnert 2005). Peak 6, the major phenolic compound found in flower bud, was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard. Peak 1 and 3 in flower buds and fruits, were identified as 3-*O*-caffeoylquinic acid and 3-*p*-coumaroylquinic acid, respectively, due to they yielded deprotonated quinic acid (*m/z* at 191) as base peak and another ion at *m/z* 179 [caffeic acid-H]$^-$ or *m/z* 163 [*p*-coumaric acid-H]$^-$, with

an intensity >50% base peak, a fragmentation pattern characteristic of 3-acylchlorogenic acids (Clifford et al., 2003, 2005).

Peak 4 in flower buds was easily distinguished from its base peak at *m/z* 173 ([quinic acid-H-H_2O]$^-$), accompanied by a secondary fragment ion at *m/z* 179 with approximately 80% abundance, which allowed identifying as 4-*O*-caffeoylquinic acid according to the fragmentation patterns described by (Clifford et al., 2003, 2005). Peaks 8 and 11 were identified as the *cis* and *trans* isomers of 5-*p*-coumaroylquinic acid based on their fragmentation. These two compounds had already been identified by our group in *C. monogyna* flowers (Barros et al., 2012). Similarly, peak 13 was tentatively identified as 5-feruloylquinic acid.

Peak 21 in flower bud and peak 23 in fruits (pseudomolecular ion [M-H]$^-$ at *m/z* 515) were assigned to 3,5-*O*-dicaffeoylquinic acid, based on their fragmentation pattern and relative fragment ion abundances (Clifford et al., 2003, 2005). MS^2 peak at *m/z* 353 was produced by the loss of one of the caffeoyl moieties [M-H-caffeoyl]$^-$, and subsequent fragmentation of this ion yielded the same fragments as a 5-caffeoylquinic acid at *m/z* 191 and 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$ (<50% base peak). Peak 27 and 28 in flower bud presented a similar UV spectra and the same pseudomolecular ion at *m/z* 499 that yielded fragments at *m/z* 353 ([caffeoylquinic acid-H]$^-$, 337 ([*p*-coumaroylquinic acid-H]$^-$), and 179 and 163, corresponding to deprotonated caffeic acid and deprotonated coumaric acid, respectively, which allowed identifying them as two caffeoyl-*p*-coumaroylquinic isomers.

Peaks 2 and 5 (same pseudomolecular ion [M-H]$^-$ at *m/z* 391) in flower buds were assigned to caffeic acid derivatives as they showed UV spectra similar to caffeic acid with λ_{max} at 328 nm and an MS^2 fragment at *m/z* 179 ([caffeic acid-H]$^-$). These

compounds could not be fully identified. One compound with similar characteristics was also identified in fruits (Peaks 4 in **Table 3**).

Peak 7 in flower buds presented UV spectra similar to *p*-coumaroylquinic acid but eluted at a different retention time. No clear signal that could be associated to a pseudomolecular ion could be obtained, although ions at *m/z* 163 (possible deprotonated *p*-coumaric acid) and 119 (further loss of a carboxyl group) were observed at its retention time using ESI detection. Thus, this peak might be tentatively associated to a *p*-coumaric acid derivative.

3.3. Flavonols

In all the studied samples, quercetin derivatives (λ_{max} around 354 nm, and an MS^2 fragment at *m/z* 301) were particularly abundant. Quercetin 3-*O*-rutinoside and quercetin 3-*O*-glucoside were found in flower buds (peaks 16 and 18 in **Table 2**) and fruits (peaks 19 and 20 in **Table 3**). Both were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Other detected quercetin glycosides were peak 20 in flower buds and peak 22 in fruits, which were assigned to a quercetin acetylhexoside (pseudomolecular ion $[M-H]^-$ at *m/z* 505); peak 19 in flower buds and peak 21 in fruits, as quercetin hexosides ($[M-H]^-$ at *m/z* 463); peak 22 in flower buds ($[M-H]^-$ at *m/z* 433), as a quercetin pentoside, and peak 17 ($[M-H]^-$ at *m/z* 609) as a quercetin-rhamnosyl-hexoside. Their identities were assigned based on their pseudomolecular ions and MS^2 spectra, releasing fragments corresponding to the losses of hexosyl (-162 mu), pentosyl (-132 mu), rhamnosyl-hexosyl (-146-162 mu) and the acetyl moieties (-42 mu). In none of them the identity of the sugar and positions of location of the substituents could be established.

Other detected flavonols corresponded to kaempferol and isorhamnetin derivatives. Kaempferol 3-*O*-glucoside (peak 23 in flower buds, **Table 2**) was identified in accordance with its retention, mass spectrum and UV-vis characteristics by comparison with a commercial standard. Peak 25 in flower buds (pseudomolecular ion $[M-H]^-$ at *m/z* 519) was identified as isorhamnetin acetylhexoside from the loss of 204 mu (-162-42 mu, corresponding to hexosyl + acetyl residues) to yield an MS^2 product ion at *m/z* 315 (isorhmanetin).

3.4. Flavones

C-glycosylated flavones were also found in flower buds. Peak 15 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 577, releasing typical MS^2 fragments ions. The loss of 120 mu (ion at *m/z* 457 ($[M-H-120]^-$) is characteristic of *C*-hexosyl flavones (Ferreres, Silva, Andrade, Seabra & Ferreira, 2003), while the loss of 164 mu, releasing the fragment at *m/z* 413 ($[M-H-146-18]^-$) can be associated to an *O*-glycosylation on the hydroxyl group at position 2 of the *C*-glycosylation sugar (Ferreres, Gil-Izquierdo, Andrade, Valentão & Tomás-Barberán, 2007). The remaining ions at *m/z* 341 ($[aglycone + 71)]^-$, *m/z* 311 ($[aglycone + 41)]^-$ and *m/z* 293 ($[aglycone + 41-18]^-$) are usual in mono-*C*-glycosyl derivatives *O*-glycosylated on 2'' position (Ferreres et al., 2007). According to this fragmentation pattern the compound was tentatively identified as 2''-*O*-rhamnosyl-*C*-hexosyl-apigenin. Similar reasoning was applied for the assignment of peaks 24 ($[M-H]^-$ at *m/z* 619), 26 and 29 ($[M-H]^-$ at *m/z* 661) in flower buds with similar MS^2 fragmentation as peak 15, but containing additionally one or two acetyl residues (42 mu). Thus, these peaks were tentatively assigned as 2''-*O*-rhamnosyl-*C*-acetylhexosyl-apigenin (peak 24) and 2''-*O*-acetylrhamnosyl-*C*-acetylhexosyl-apigenin or 2''-*O*-rhamnosyl-*C*-diacetylhexosyl-apigenin (peak 26 and

29), although the fragmentation pattern obtained did not actually allow concluding about the precise location of the acetyl residues.

3.5. Flavan-3-ols

Flavan-3-ols (i.e., catechins and proanthocyanidins) were other relevant flavonoids found in flower bud and, especially, fruit extracts of the *Crataegum* samples. Peak 9 in flower buds and peak 8 in fruits were identified as (-)-epicatechin by comparison of its UV spectra and retention time with a commercial standard. Signals at *m/z* 577, 865 and 1153 in flower buds (peaks 10 and 12) in fruits (peaks 5-7, 10-18) can be respectively associated to B-type procyanidin dimers, trimers and tetramers (*i.e.*, (epi)catechin units linked to C4-C8 or C4-C6 interflavonoid linkages), whereas peak 14 (pseudomolecular ion $[M-H]^-$ at *m/z* 849) in flower buds was coherent with a proanthocyanidin trimer consisting of one (epi)afzelechin unit and two (epi)catechin units. Furthermore, peak 9 in fruit extracts showed a pseudomolecular ion $[M-H]^-$ at *m/z* 863 that could correspond to a procyanidin trimer containing two B-type and one A-type (i.e., C4-C8 or C4-C6 and C2-*O*-C7) interflavonoid linkages.

3.6. Anthocyanins

The anthocyanin profiles obtained for ripened and over ripened fruits were quite similar, consisting of five different compounds, whereas the profile in unripe fruits was simpler and only two anthocyanins were detected. The analytical characteristics, identities and concentrations are presented in **Table 4**. Cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside and peonidin 3-*O*-glucoside were positively identified by comparison with standards. The identity of cyanidin 3-*O*-rutinoside was also confirmed by comparison of its chromatographic and UV and mass spectral characteristics with data in our library.

Peak 4 was assigned as cyanidin pentoside based on its mass spectra, which showed an MS^2 signal at *m/z* 287 (cyanidin; $[M-132]^+$, loss of a pentosyl moiety). Cyanidin-3-*O*-glucoside was the major anthocyanin in all the samples, and over ripened fruits were, by far, the botanical part with the highest anthocyanin concentrations, which was coherent with its higher pigmentation.

4. Discussion

The phenolic compounds found in plants are often related with their bioactivity, mostly resulting from the synergistic or additive influence of the different classes of compounds present in the extract (Ramful et al., 2011). Therefore, the higher antiproliferative activity observed for flower buds extract could be related to its higher concentrations of phenolic compounds, particularly the higher amounts of quercetin derivatives and phenolic acids.

Quercetin was already reported as exerting potent growth inhibitory effects on several malignant tumour cell lines, such as NK/LY ascites tumour cells, HeLa cells, gastric cancer cells (HGC-27, NUGC-2, MKN-7 and MKN-28), colon cancer cells (COLO 320 DM), human breast cancer cells, human squamous and gliosarcoma cells, ovarian cancer cells, human epidermoidal cancer (A431), human liver cancer cells (HepG2) and human pancreatic cancer cells (Kandaswami et al., 2005). In addition, it seems to influence the level of matrix metalloproteinases (MMPs) (Moon et al., 2003).

Also phenolic acids, such as gallic and caffeic acid derivatives had shown antiproliferative effect toward HeLa cervix adenocarcinoma, breast cancer and leukemia cell lines (Gomes et al., 2003). Furthermore, it was demonstrated that treatment of MCF-7 and MAD-MB-231 human breast cancer cells with caffeic acid or chlorogenic

acid partially inhibited the methylation of the promoter region of the RARb gene (De, Baltas & Bedos-Belval, 2011).

Nevertheless, the potential synergistic effect of the different phenolic compounds present in the extracts should not be discarded. Actually, the linear correlations produced among phenolic compounds content and antiproliferative activity GI_{50} values had higher correlation coefficients for grouped flavonoids than for their individual classes (**Table 5**) for all the assayed cell lines, except HepG2.

Conclusions

The extracts obtained from *C. monogyna* parts revealed antiproliferative activity, higher for flower buds, which might be related with the phenolic compounds content found in these extracts. It should be highlighted that the tested samples did not show toxicity for non-tumour cells.

The phenolic profiles of the different parts revealed high predominance of flavonoids, which are compounds that modulate a variety of biological events associated with cancer progression and development, such as cell proliferation, apoptosis, cell differentiation and neovascularization. Therefore, *C. monogyna* may be considered a source of important phytochemicals (flavonoids, phenolic acids and anthocyanins) with bioactive properties to be explored for pharmaceutical applications.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) for financial support to CIMO (strategic project PEst-OE/AGR/UI0690/2011). R.C. Calhelha and J.C.M. Barreira thank to FCT, POPH-QREN and FSE for their grants (SFRH/BPD/68344/2010 and SFRH/BPD/72802/2010, respectively). M. Dueñas thanks

to the *Programa Ramón y Cajal* for a contract. The GIP-USAL is financially supported by the Spanish Government through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD, CSD2007-00063), and *Junta de Castilla y León* (Grupo de Investigación de Excelencia, GR133).

References

Abreu, R.M.V., Ferreira, I.C.F.R., Calhelha, R.C., Lima, R.T., Vasconcelos, M.H., Adega, F., Chaves, R., & Queiroz, M.J.R.P. (2011). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. *European Journal of Medicinal Chemistry*, *46*, 5800-5806.

Barros, L., Carvalho, A.M., & Ferreira, I.C.F.R. (2011). Comparing the composition and bioactivity of *Crataegus monogyna* flowers and fruits used in folk medicine. *Phytochemical Analysis*, *22*, 181-188.

Barros, L., Dueñas, M., Carvalho, A.M., Ferreira, I.C.F.R., & Santos-Buelga, C. (2012). Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. *Food and Chemical Toxicology*, *50*, 1576-1582.

Carvalho, A.M. (2010). Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal. Biblioteca de Ciencias 35. Consejo Superior de Investigaciones Científicas (CSIC), Madrid.

Clifford, M.N., Johnston, K.L., Knight, S., & Kuhnert, N.A. (2003). A hierarachical scheme for LC-MS^n identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry*, *51*, 2900-2911.

Clifford, M.N., Knight, S., & Kuhnert, N.A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *Journal of Agricultural and Food Chemistry*, *53*, 3821-3832.

De, P., Baltas, M., & Bedos-Belval, F. (2011). Cinnamic Acid Derivatives as Anticancer Agents-A Review. *Current Medicinal Chemistry*, *18*, 1672-1703.

End, D.W., Look, R.A., Shaffer, N.L., Balles, E.A., & Persico. F.J. (1987). Non-selective inhibition of mammalian protein kinases by flavonoids *in vitro*. *Research Communications in Chemical Pathology and Pharmacology*, *56*, 75-86.

Ferreres, F., Gil-Izquierdo, A., Andrade, P.B., Valentão, P., & Tomás-Barberán, F.A. (2007). Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1161*, 214-223.

Ferreres, F., Silva, B.M., Andrade, P.B., Seabra, R.M., & Ferreira, M.A. (2003). Approach to the study of *C*-glycosyl flavones by ion trap HPLC-PAD-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). *Phytochemical Analysis*, *14*, 352-359.

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 *Chemistry*, *115*, 897-903.

García-Marino, M., Hernández-Hierro, J.M., Rivas-Gonzalo, J.C., & Escribano-Bailón, M. T. (2010). Colour and pigment composition of red wines obtained from comaceration of Tempranillo and Graciano varieties. *Analytical Chimica Acta*, *660*, 134-142.

Gomes, C.A., Cruz, T.G., Andrade, J.L., Milhazes, N., Borges, F., & Marques, M.P.M. (2003). Anticancer Activity of Phenolic Acids of Natural or Synthetic Origin: A Structure-Activity Study. *Journal of Medicinal Chemistry*, *46,* 5395-5401

IUPAC. (1976) Nomenclature of cyclitols. *Biochemical Journal*, *153*, 23-31.

Kandaswami, C., Lee, L.T., Lee, P.P., Hwang, J.J., Ke, F.C., Huang, Y.T., & Lee, M.T. (2005). The antitumor activities of flavonoids. *In Vivo*, 19, 895-909.

Kandaswami, C., Perkins, E., Soloniuk, D.S., Drzewiecki, G., & Middleton, E.Jr. (1991). Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma *in vitro*. *Cancer Letters*, *56*, 147-152.

Kim, M.H. (2003). Flavonoids inhibit VEGF/bFGF-induced angiogenesis *in vitro* by inhibiting the matrix-degrading proteases. *Journal of Cellular Biochemistry*, *89*, 529-538.

Lee, L.T., Huang, Y.T., Hwang, J.J., Lee, H.P.P., Ke, F.C., Nair, M.P., Kandaswain, C., & Lee, M.T. (2002). Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells. *Anticancer Research*, *22*, 1615-1628.

Liu P., Kallio H., Lü D., Zhou C., & Yang B. (2011). Quantitative analysis of phenolic compounds in Chinese hawthorn (*Crataegus* spp.) fruits by high performance liquid chromatography-electrospray ionisation mass spectrometry. *Food Chemistry*, *127*, 1370-1377.

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 Chemistry*, *121*, 1188-1197.

López-Lázaro, M. (2002). Flavonoids as anticancer agents: Structure-activity relationship study. *Current Medicinal Chemistry - Anti-Cancer Agents*, *2*, 691-714.

Moon, S.K., Cho, G.O., Jung, S.Y., Gal. S.W., Kwon, T.K., Lee, Y.C., Madamanchi, N.R., & Kim, C.H. (2003). Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. *Biochemical and Biophysical Research Communications*, *301(4)*, 1069-1078.

Parmar, V.S., Jain, R., Sharma, S.K., Vardhan, A., Jha, A., Taneja, P., Singh, S., Vyncke, B.M., Bracke, M.E., & Mareel, M.M. (1994). Anti-invasive activity of 3,7-dimethoxyflavone *in vitro*. *Journal of Pharmaceutical Science*, *83*, 1217-1221.

Ramful D., Aumjaud B., Neergheen V.S., Soobrattee M.A., Googoolye K., Aruoma O.I., & Bahorun T. (2011). Polyphenolic content and antioxidant activity of *Eugenia pollicina* leaf extract in vitro and in model emulsion systems. *Food Research International*, *44*, 1190-1196.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., MacMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., & Boyd, M.R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute*, *82*, 1107-1112.

Spencer J.P.E., Mohsen M.M.A.E., & Rice-Evans C. (2004). Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Archives of Biochemistry and Biophysics*, *423*, 148-161.

Stiborová M., Bieler C.A., Wiessler M., & Frei E. (2001). The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. *Biochemical Pharmacology*, *62*, 1675-1684.

Vaz, J.A., Ferreira, I.C.F.R., Tavares, C., Almeida, G.M., Martins, A., & Vasconcelos, M.H. (2012). *Suillus collinitus* methanolic extract increases p53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line. *Food Chemistry*, *135*, 596-602.

Vichai, V., & Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*, *1*, 1112-1116.

World Health Organization. (2010). WHO Library Cataloguing-in-Publication Data World health statistics 2010. 1.Health status indicators. 2.World health. 3.Health services-statistics. 4.Mortality. 5.Morbidity. 6.Life expectancy. 7.Demography. 8.Statistics. I. World Health Organization. ISBN 978 92 4 156398 7 (NLM classification: WA 900.1).

Table 1. Growth inhibitory activity of *Crataegus monogyna* flower buds and fruit extracts on human tumour cell lines and on porcine non-tumour cells.

	Tumour cell lines				Non-tumour cells
Sample	MCF7 (breast carcinoma)	NCI-H460 (non-small lung cancer)	HeLa (cervical carcinoma)	$HepG_2$ (hepatocellular carcinoma)	PLP2 (porcine liver primary culture)
Flower bud	66.96 ± 0.01 b	67.61 ± 4.29 b	63.55 ± 3.56 d	88.45 ± 8.11 b	356.60 ± 2.00
Unripened fruit	82.02 ± 7.73 b	84.18 ± 7.90 b	95.76 ± 6.08 c	297.99 ± 5.79 a	>400
Ripened fruit	223.53 ± 8.24 a	274.94 ± 13.56 a	176.75 ± 9.84 b	318.72 ± 4.87 a	>400
Over ripened fruit	219.44 ± 9.19 a	277.89 ± 9.23 a	228.61 ± 3.54 a	282.00 ± 13.68 a	>400
Ellipticine	1.42 ± 0.18	1.06 ± 0.15	0.81 ± 0.06	1.21 ± 0.20	1.98±0.06

Results are expressed as GI_{50} (concentration of extract in µg/mL that cause 50% of cell growth inhibition), and show means ± SD of 3 independent experiments performed in duplicate. In each column, different letters in tumour cell lines data mean significant differences between results ($p<0.05$).

Table 2. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in *Crataegus monogyna* flower buds.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification* mg/g, dw
				Flower bud		
1	6.5	326	353	191(100), 179(80), 173(15), 161(16), 135(87)	3-*O*-caffeoylquinic acid	1.58±0.07
2	7.5	328	391	217(100), 179(44), 173(16), 135(44)	Caffeic acid derivative	0.45±0.02
3	8.2	312	337	191(100), 173(8), 163(69), 155(3), 119(49)	3-*p*-coumaroylquinic acid	0.07±0.00
4	8.5	330	353	191(58), 179(80), 173(100), 161(7), 135(63)	4-*O*-Caffeoylquinic acid	0.17±0.02
5	8.7	328	391	217(100), 179(61), 173(14), 161(3), 135(37)	Caffeic acid derivative	0.11±0.00
6	9.4	326	353	191(100), 179(5), 173(10), 161(11), 135(2)	5-*O*-caffeoylquinic acid	**5.41±0.01**
7	10.4	314	-	163(30), 119(100)	*p*-Coumaric acid derivative	0.20±0.07
8	11.0	314	337	191(100), 173(47), 163(29)	*cis*-5-*p*-Coumaroylquinic acid	0.02±0.00
9	12.0	280	289	245 (100), 205(62), 151(38), 137(47)	(-)-Epicatechin	**2.32±0.08**
10	13.5	278	865	865(51),739(6), 713(6), 695(15), 577(28), 575(9), 425(33),407(100), 289(48), 287(7)	Procyanidin trimer	0.37±0.01
11	14.0	314	337	191(100), 173(6), 163(12), 119(6)	*trans*-5-*p*-Coumaroylquinic acid	0.12±0.00
12	14.4	278	1153	865(6), 577(6), 575(6), 561(100), 289(53)	Procyanidin tetramer	0.43±0.06
13	15.5	328	367	193(6), 191(100), 173(4), 134(9)	5-Feruloylquinic acid	0.10±0.01
14	16.4	280	849	679(11), 559(36), 289(21), 271(5)	Proanthocyanidin trimer (1(epi)afzelechin+2 (epi)catechin units)	0.13±0.01
15	17.4	338	577	457(2), 413(50), 341(4), 311(19), 293(100)	2''-*O*-Rhamnosyl-*C*-hexosyl-apigenin	**4.33±0.04**
16	19.1	354	609	301(100)	Quercetin 3-*O*-rutinoside	0.33±0.01
17	19.3	356	609	301(100)	Quercetin rhamnosyl-hexoside	0.16±0.00
18	20.3	356	463	301(100)	Quercetin 3-*O*-glucoside	**3.24±0.05**
19	20.7	352	463	301(100)	Quercetin hexoside	1.11±0.00
20	22.0	354	505	463(28), 301(100)	Quercetin acetylhexoside	0.30±0.01
21	22.5	328	515	353(100), 335(6), 191(87), 179(42), 173(12), 135(50)	3,5-*O*-Dicaffeoylquinic acid	1.41±0.05
22	23.1	356	433	301(19)	Quercetin pentoside	0.05±0.01
23	24.3	352	447	285(50)	Kaempferol 3-*O*-glucoside	0.04±0.01
24	24.9	342	619	499(4), 413(69), 293(100)	2''-*O*-Rhamnosyl-*C*-acetylhexosyl-apigenin	0.40±0.04
25	25.3	346	519	315(100), 300(74)	Isorhamnetin acetylhexoside	0.84±0.01
26	26.7	344	661	601(47), 455(45), 395(32), 311(23), 293(100)	2''-*O*-Acetylrhamnosyl-*C*-acetylhexosyl-apigenin or 2''-*O*-rhamnosyl-*C*-diacetylhexosyl-apigenin	0.09±0.05
27	27.1	312	499	353(7), 337(29), 191(19), 173(15), 179(6), 163(100)	Caffeoyl-*p*-coumaroylquinic acid	0.06±0.02
28	27.5	316	499	353(67), 337(15), 191(100), 179(43), 173(13), 163(16)	Caffeoyl-*p*-coumaroylquinic acid	0.07±0.02
29	28.6	340	661	601(19), 455(78), 311(16), 293(100)	2''-*O*-Acetylrhamnosyl-*C*-acetylhexosyl-apigenin or 2''-*O*-rhamnose-*C*-diacetyl-hexoside apigenin	0.86±0.02
					Phenolic acids	**9.76±0.25**
					Flavonoids	**15.02±0.33**
					Phenolic compounds	**23.94±0.59**

*mean ± SD of 3 independent experiments performed in duplicate.

Table 3. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in *Crataegus monogyna* fruit extracts.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification* mg/g, dw		
						Unripened fruit	Ripened fruit	Over ripened fruit
1	6.5	326	353	191(100), 179(82), 173(14), 161(12), 135(82)	3-*O*-Caffeoylquinic acid	1.48±0.13	0.60±0.02	0.49±0.02
2	7.5	328	335	231(17), 217(100), 179(70), 135(24)	Caffeic acid derivative	0.02±0.00	0.01±0.00	0.01±0.00
3	8.2	312	337	191(100), 173(10), 163(69), 155(3), 119(38)	3-*p*-Coumaroylquinic acid	0.32±0.03	0.12±0.00	0.12±0.01
4	8.4	326	391	391(100), 217(57), 179(57), 135(43)	Caffeic acid derivative	0.36±0.03	0.14±0.01	0.38±0.03
5	9.5	278	865	739(8), 713(8), 695(22), 577(35), 575(15), 425(31), 407 (100), 289(42), 287(87)	Procyanidin trimer	0.30±0.04	0.28±0.03	0.29±0.03
6	10.0	280	577	451(38), 425(67), 407(100), 289(76), 287(20)	Procyanidin dimer	1.74±0.13	1.27±0.03	1.71±0.07
7	10.4	278	1153	865(22), 713(4), 577(33), 575(16), 561(20), 289(100)	Procyanidin tetramer	0.19±0.06	0.15±0.01	0.13±0.02
8	12.0	278	289	137(43)	(-)-Epicatechin	5.19±0.59	1.84±0.09	2.98±0.07
9	13.2	278	863	863(100), 711(26), 573(16), 451(18), 411(30), 289(16), 285(12)	Procyanidin trimer with a type-A linkage	0.05±0.00	nd	0.13±0.01
10	13.5	280	865	739(18), 713(18), 695(22), 577(35), 575(22), 425(21), 407 (100), 289(42), 287(87)	Procyanidin trimer	1.44±0.10	0.75±0.02	0.79±0.01
11	14.4	280	1153	865(11), 713(5), 577(30), 575(22), 561(30), 289(100)	Procyanidin tetramer	0.93±0.02	0.38±0.04	0.31±0.03
12	15.3	280	865	739(6), 713(11), 695(11), 577(16), 575(24), 425(11),407(100), 289(8), 287(28)	Procyanidin trimer	0.19±0.02	0.14±0.01	0.12±0.01
13	15.8	280	1153	865(20), 577(27), 575(16), 561(7), 289(100)	Procyanidin tetramer	0.45±0.11	0.14±0.01	0.08±0.00
14	16.2	280	865	577(45), 287(100)	Procyanidin trimer	0.18±0.08	0.11±0.01	0.06±0.00
15	16.8	280	865	739(10), 695(7), 577(42), 575(28), 425(10), 289(58), 287(72)	Procyanidin trimer	0.33±0.07	0.10±0.01	0.04±0.01
16	17.1	280	1153	289(100)	Procyanidin tetramer	0.29±0.05	0.09±0.00	0.09±0.02
17	17.5	280	1153	865(36), 713(7), 577(21), 575(32), 561(7), 289(100)	Procyanidin tetramer	0.13±0.02	0.04±0.00	0.08±0.01
18	18.5	280	577	451(27), 425(57), 407(100), 289(73), 287(15)	Procyanidin dimer	0.32±0.03	0.19±0.05	0.21±0.01
19	19.1	356	609	301(100)	Quercetin 3-*O*-rutinoside	0.05±0.00	0.02±0.00	0.05±0.00
20	20.3	355	463	301(100)	Quercetin 3-O-glucoside	0.59±0.06	0.51±0.02	0.37±0.01
21	20.7	354	463	301(100)	Quercetin hexoside	0.16±0.01	0.21±0.01	0.13±0.01
22	22.0	354	505	463(26), 301(100)	Quercetin acetylhexoside	0.02±0.00	0.03±0.00	0.07±0.00
23	22.5	330	515	353(100), 191(64), 179(37), 173(4), 135(17)	3,5-Dicaffeoylquinic acid	0.05±0.01	nd	nd
					Phenolic acids	**2.25±0.17**	**0.89±0.00**	**1.00±0.05**
					Flavonoids	**12.77±1.24**	**6.19±0.34**	**8.28±0.16**
					Phenolic compounds	**15.02±1.42**	**7.07±0.34**	**9.29±0.22**

*mean ± SD of 3 independent experiments performed in duplicate.

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and concentration of anthocyanins in *Crataegus monogyna* fruit extracts.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (ng/g, dw)		
						Unripened fruit	Ripened fruit	Over ripened fruit
1	20.9	516	449	287	Cyanidin-3-*O*-glucoside	30.32±1.16	483.89±24.61	4052.01±141.71
2	22.7	518	595	449,287	Cyanidin-3-*O*-rutinoside	nd	10.12±0.55	78.72±6.27
3	24.9	502	433	271	Pelargonidin-3-*O*-glucoside	nd	1.03±0.01	5.13±0.26
4	25.6	518	419	287	Cyanidin-3-*O*-pentoside	nd	15.76±0.21	131.55±6.71
5	27.8	516	463	301	Peonidin-3-*O*-glucoside	0.23±0.01	0.48±0.04	7.41±0.22
					Total anthocyanins	30.55±1.17	511.28±25.35	4274.83±155.18

Table 5. Correlations between total phenolics, phenolic acids and flavonoids classes and subclasses with antiproliferative activity GI_{50} values.

Compounds	Equation, R^2			
	MCF7	**NCI-H460**	**HeLa**	**HepG2**
Total phenolic compounds	y = -10.022x + 284.28, 0.8184 F = 9.011; p = 0.095	y = -14.068x + 371.53, 0.8028 F = 8.140; p = 0.104	y = -8.575x + 257.49, 0.7697 F = 6.685; p = 0.123	y = -13.598x + 440.93, 0.8678 F = 13.130, p = 0.068
Flavonoids	y = -21.620x + 367.26, **0.960** F = 48.16; p = 0.020	y = -30.475x + 489.28, **0.9497** F = 37.74; p = 0.025	y = -17.228x + 315.59, **0.7833** F = 7.227; p = 0.115	y = -21.964x + 478.74, 0.5707 F = 2.659; p = 0.245
Phenolic acids	y = -14.923x + 199.62, 0.5563 F = 2.508; p = 0.254	y = -20.844x + 252.34, 0.5404 F = 2.351; p = 0.265	y = -13.813x + 188.67, 0.6124 F = 3.160; p = 0.217	y = -26.302x + 347.01, **0.9955** F = 441.0; p = 0.002
Flavanols	y = -27.065x + 231.34, 0.2229 F = 0.574; p = 0.528	y = -39.968x + 300.23, 0.2301 F = 0.598; p = 0.520	y = -13.427x + 182.22, 0.0705 F = 0.152; p = 0.735	y = 20.000x + 194.37, 0.0701 F = 0.151; p = 0.735
Flavonols	y = -24.312x + 195.69, 0.3896 F = 1.276; p = 0.376	y = -33.737x + 246.41, 0.3735 F = 1.192; p = 0.389	y = -23.146x + 186.29, 0.4536 F = 1.661; p = 0.326	y = -51.057x + 356.20, 0.9896 F = 190.8; p = 0.005
Procyanidins	y = 1.4884x + 142.15, 0.0016 F = 0.003; p = 0.960	y = 1.3634x + 175.18, 0.0007 F = 0.001; p = 0.974	y = 5.7604x + 119.69, 0.0312 F = 0.064; p = 0.823	y = 35.999x + 123.66, 0.5465 F = 2.410; p = 0.261

Figure 1. HPLC chromatogram of the phenolic compounds of *Crataegus monogyna* flower bud (A) and unripe fruit (B) recorded at 280 nm.