Phenolic profiles of *in vivo* and *in vitro* grown *Coriandrum sativum* L.

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Abstract

Coriandrum sativum L. is a source of a variety of polyphenols and other phytochemicals, related to its high antioxidant activity and to its use for indigestion, rheumatism, and prevention of lipid peroxidation damage. Plant cell cultures are a means to study or to produce some active metabolites such as polyphenols. This technique was applied to the investigation of coriander, and a detailed analysis of individual polyphenols *in vivo* and *in vitro* grown samples was performed. The *in vivo* vegetative parts showed quercetin derivatives as the main flavonoids and quercetin-3-*O*-rutinoside (3296 mg/kg dw) was the main polyphenol found in this part of coriander. The fruits revealed only phenolic acids and derivatives, being caffeoyl *N*-tryptophan hexoside (45.33 mg/kg dw) the most abundant phenolic derivative. *In vitro* samples also gave a high diversity of polyphenols, being *C*-glycosylated apigenin (2983 mg/kg dw) the main compound. Anthocyanins were only found in clone A, which was certainly related to its purple pigmentation, and peonidin-3-*O*-feruloylglucoside-5-*O*-glucoside was the major anthocyanin found (1.70 µg/kg dw). *In vitro* culture can be used to explore new industrial, pharmaceutical and medicinal potentialities, such as the production of secondary metabolites like flavonoids.

Keywords: *Coriandrum sativum*; *In vitro* culture; Phenolic acids; Flavonoids; HPLC-DAD-ESI/MS

1. Introduction

The interest in phenolic compounds increased in the last decade due to their structural and protective activity in plants, but also due to their flavour, colour, and antioxidant activity against reactive species of oxygen that cause oxidative stress and consequently, the damage of tissues and biomolecules. Phenolic compounds can act as phytoalexin protective agents against UV light and provide an important role in plant growth and reproduction (Ignat, Volf, & Popa, 2011). Furthermore, in animals they exhibit a wide-range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram, Sundram, & Samman, 2006). Phenolic compounds are one of the largest groups of secondary metabolites consisting of four main groups divided according to the number of phenol rings and the structural elements that bind those rings, including flavonoids (e.g. anthocyanins, flavones, isoflavones), phenolic acids, tannins, and stilbenes and lignans (Balasundram et al., 2006).

Plant cell cultures are a means to study or to produce some active metabolites such as phenolic compounds, alkaloids, triterpenes or quinones (Oksman-Caldentey & Inzé, 2004). *In vitro* culture allows, in a disease and virus-free environment, a means of obtaining numerous copies of plant tissue, through micropropagation, that can be used to explore new industrial, pharmaceutical and medicinal potentialities, such as the mentioned production of secondary metabolites with therapeutic activity (Matkowski, 2008). This technique was applied in our laboratory to the investigation of coriander (Dias, Barros, Sousa, & Ferreira, 2011).

Coriander (*Coriandrum sativum* L.) is a smelling annual herb, belonging to Apiaceae family, widely grown in North Africa and the Middle East, and with increasing interest in Western Europe. It is commonly used for its fresh leaves and dry powder of its fruits

(commonly known as coriander seeds) for its organoleptic and flavouring properties. The coriander plant is mainly used for making sauces and salsas, in other hand the fruits are used in powder to flavouring meat products and fish, in sodas, pickles, bakery, and curry recipes (Bhandari, 1991; Ravi, 2006). Its essential oil is also used in pharmaceutical recipes and as a fragrance in cosmetics (Al-Mofleh et al., 2006; Millam et al., 1997). The reported uses of coriander for indigestion, rheumatism, prevention of lipid peroxidation damage, and its high antioxidant activity have been related to the presence of phytochemicals such as linalool, camphor, geraniol and geranyl acetate (Eguale, Tilahun, Debella, Feleke, & Makonnen, 2007; Samojlik, Lakic, Mimica-Dukic, Dakovic-Svajcer, & Bozin, 2010; Wangensteen, Samuelsen, & Malterud, 2004). Furthermore, according to different authors, this species is also a source of a variety of phenolic compounds (Justesen & Knuthsen, 2001; Meloa, Filho, & Guerra, 2005; Nambiar, Daniel, & Guin, 2010). In a previous study we compared the antioxidant potential of *in vivo* and *in vitro* grown coriander (Dias et al., 2011). Herein, a detailed analysis of individual phenolic compounds was performed, in order to compare their profiles and to explore the potentialities of *in vitro* culture.

2. Material and methods

2.1. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid and trifluoroacetic acid (TFA) were purchased from Prolabo (VWR International, France). The phenolic compounds standards were from Extrasynthese (Genay, France). All other chemicals were of analytical grade and purchased from

chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

In vivo grown samples were vegetative parts and fruits of coriander (**Figure 1A** and **B**) obtained in a local supermarket (Bragança, Portugal). The culture conditions for the *in vitro* grown samples were described previously (Dias et al., 2011): Tmin [16-19] ºC, Tmax [23-26] ºC, photoperiod of 16/8 h (light/dark) supplied by light-bulbs Silvana day light (Phillips, Amsterdam, Netherlands). After 5 weeks, the shoots were excised from the young plants and inoculated in a modified culture medium, MS with 0.1 mg/l IBA and 0.1 mg/l BAP, pH 5.5. They were kept in the same culture conditions and subculture occurred every 4 weeks. After 6 months in culture, two clones were differentiated (with phenotypical stability): clone A showing a notorious purple pigmentation on vegetative parts (leaves and stems), and clone B being just green (**Figure 1C** and **D**). All the samples were lyophilised and reduced to a fine dried powder (20 mesh) for further analyses.

2.3. Analysis of phenolic acids and flavonoids

2.3.1. Extraction procedure

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1h. 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) until complete removal of methanol. The aqueous phase was lyophilized and re-dissolved in 20% aqueous methanol at 5 mg/mL and filtered through a 0.22-µm

disposable LC filter disk for High Performance Liquid Chromatography (HPLC) analysis.

2.3.2. HPLC-DAD-ESI/MS analyses

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 10% B to 15% B over 5 min, 15-25% B over 5 min, 25-35% B over 10 min, isocratic 50% B 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 the 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 show full scan spectra, to give an overview of all 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. EPI mode was further

performed in order to obtain the fragmentation pattern of the parent ion(s) of 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, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: apigenin-6-*C*-glucoside (y=246.05x-309.66; R^2=0.9994); caffeic acid (y=617.91x-691.51; R^2=0.9991); chlorogenic acid (y=600.27x-763.62; R^2=0.9998); cinnamic acid (y=1020.8x-775.68; R^2=0.9944); *p*-coumaric acid (y=447.12x-1580.7; R^2=0.9962); ferulic acid (y=779.11x-869.22; R^2=0.9987); isorhamnetin-3-*O*-glucoside (y=262.31x-9.8958; R^2=1); kaempferol-3-*O*-rutinoside (y=175.02x-43.877; R^2=0.9999); luteolin-6-*C*-glucoside (y=365.93x-17.836; R^2=0.9997); quercetin 3-*O*-glucoside (y=316.48x-2.9142; R^2=1), and quercetin-3-*O*-rutinoside (y=222.79x-243.11; R^2=0.9998). The results were expressed in mg per Kg of dry weight (dw), as mean ± standard deviation of three independent analyses.

2.4. Analysis of anthocyanins

2.4.1. Extraction

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.

2.4.2. Analysis

The extracts were analysed using the HPLC system described above according to Garcia-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón (2010). Separation was achieved on an AQUA® (Phenomenex) reverse phase C18 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 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), malvidin 3-*O*-glucoside (y=477014x–38.376; R^2=0.9991) and peonidin 3-*O*-glucoside (y=537017x–71.469; R^2=0.9997). The results were expressed in μg per Kg of dry weight (dw), as mean ± standard deviation of three independent analyses.

3. Results

3.1. Phenolic acids and flavonoids analysis

Figure 2 shows the phenolic compounds profile of *in vivo* (vegetative parts and fruits) and *in vitro* (clones A and B) grown coriander samples. Data of the retention time, λ_{max} in the visible region, molecular ion, main fragment ions in MS^2, tentative identification and concentration of phenolic acids and flavonoids obtained by HPLC-DAD-MS analysis are presented in **Table 1**. Vegetative parts, fruits and clones presented different profiles. Vegetative parts showed hydroxycinnamic acids derivatives and flavonol derivatives (quercetin and kaempferol derivatives) as main phenolic compounds, while fruits presented only hydroxycinnamic acids derivatives. Clones A and B showed a similar profile in relation to flavones (the main flavonoids) and absence of phenolic acids. Nevertheless, only Clone A revealed anthocyanins in its composition.

Five phenolic acids and four flavonols were found in vegetative parts (**Table 1**). Peaks 1, 2, 3, 4 and 5 showed a UV spectrum similar to hydroxycinnamic acid derivatives. Peak 1 showed λ_{max} at 328 nm and a molecular ion $[M-H]^-$ at *m/z* 369 releasing MS^2

fragments at *m/z* 207 (-162 mass units corresponding to a hexose moiety) that can be associated to a dimethoxycinnamoyl residue, and at *m/z* 189 (-18 amu, further loss of a water molecule), and thus the compound was tentatively identified as a dimethoxycinnamoyl hexoside. This compound was previously identified by Alonso-Salces et al. (*15*) in green coffee beans. Peak 2 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 353 and λ_{max} in the UV spectrum at 324 nm, coherent with a chlorogenic acid, and was identified as 3-*O*-caffeoylquinic acid by comparison with a commercial standard. Peak 3 presented the same mass and UV characteristics and was identified as another caffeoylquinic acid. Peak 4 showed a UV spectrum similar to ferulic acid with λ_{max} at 328 nm, but eluted at a different retention time. It presented a pseudomolecular ion $[M-H]^-$ at *m/z* 355 releasing an MS^2 fragment at *m/z* 193 (loss -162 amu, hexose) attributed to ferulic acid, which allowed tentatively identifying it as a ferulic acid hexoside; its earlier elution (higher polarity) with regard to ferulic acid (17.3 min) was also coherent with this identity. Peak 5 showed a pseudomolecular ion ($[M-H]^-$ at *m/z* 337) and UV spectrum (λ_{max} at 308 nm), similar to *p*-coumaric acid, but eluted at a different retention time. The MS/MS spectrum yielded fragment ions at *m/z* 191 (-146 amu, loss of a *p*-coumaroyl moiety) and at *m/z* 146 (-191 amu, loss of quinic acid), and thus the compound was tentatively identified as a *p*-coumaroylquinic acid.

Peaks 6-9 showed UV spectra characteristic of flavonols. Compounds 6, 7 and 8 were associated to quercetin derivatives; they showed UV spectra similar to quercetin with λ_{max} at 354-356 nm, and presented pseudomolecular ions $[M-H]^-$ at *m/z* 609, 477 and 463, respectively, all of them releasing a unique MS^2 fragment at *m/z* 301 (quercetin). The loss of -308 amu in peak 6 was coherent with a rutinoside moiety, and the compound could be identified as quercetin-3-*O*-rutinoside by comparison with a commercial standard. This compound was the most abundant phenolic in this sample

(3296 mg/kg of dw). Similarly peaks 7 and 8 were positively identified as quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside according to their mass and UV characteristics and comparison with commercial standards. Peak 9 showed a UV spectrum similar to kaempferol, and a pseudomolecular ion $[M-H]^-$ at *m/z* 593, releasing a unique MS^2 fragment at *m/z* 285 (kaempferol); the loss of -308 mass units (rutinoside moiety) and comparison with a commercial standard allowed its identification as kaempferol-3-*O*-rutinoside.

Phenolic acids derivatives were the only compounds found in fruits (**Table 1**), in which peaks 1, 3, 5 and 6 were associated to hydroxycinnamic acid derivatives, corresponding to chlorogenic acid (3-*O*-caffeoylquinic acid), caffeic acid, *p*-coumaric acid and ferulic acid, respectively, identified by comparison of their UV and mass characteristics and retention time with those of commercial standards. Peak 2 presented a UV spectrum similar to ferulic acid, but eluted at a different retention time. No clear signal that could associated to its molecular ion could be obtained for this peak although ions at *m/z* 193 (possible ferulic acid) and 149 (characteristic fragment from ferulic acid cleavage) were observed at its retention time using ESI detection. Since this compound eluted earlier than the ferulic acid standard, it might be tentatively associated to a ferulic acid glucoside as detected in the vegetative parts.

Peaks 4, 7, 9 and 10 were assigned to cinnamoyl-amino acid conjugates. Peak 7 was the major phenolic compound found in this sample (45.33 mg/Kg dw). These compounds showed UV spectra similar to hydroxycinnamic acid with λ_{max} around 327 nm, and all of them presented an MS^2 fragment at *m/z* 203 coherent with the amino acid trytophan; additionally peaks 7 and 10 presented major ion fragments at m/z 365 and 379, respectively, that could be attributed to the loss of a hexose moiety (162 amu). These

characteristics and comparison with the fragmentation patterns and data reported by Alonso-Salces, Guillou, & Berrueta (2009) for conjugated cinnamoyl-amino acid identified in green coffee beans, allowed the tentative identification of the compounds as caffeoyl *N*-tryptophan (peak 4), caffeoyl *N*-tryptophan hexoside (peak 7), feruloyl *N*-tryptophan (peak 9), and feruloyl *N*-tryptophan hexoside (peak 10). Peak 8 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 515 and λ_{max} in the UV spectrum at 326 nm, similar to chlorogenic acid, with a major fragment in MS^2 at *m/z* 353 ($[M-H-162]^-$) corresponding to the loss of a caffeoyl moiety and other fragments (*m/z* at 191, 179, 173 and 135) characteristic of the cleavage of 3-*O*-caffeoylquinic acid (see peak 2 in the vegetative parts), thus the compound was tentatively identified as a di-*O*-caffeoylquinic acid.

Clones A and B, as mentioned above, presented the same flavonoid profile (**Table 1**), but for the presence of anthocyanins in Clone A. Peak 1 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 639 that analysis yielded two fragment ions at *m/z* 477 ($[M-H-162]^-$) and *m/z* 315 ($[M-H-162-162]^-$), corresponding to the successive losses of two hexoside moieties; the ion at *m/z* 315 was coherent with the flavonol isorhamnetin, as well as the UV spectrum of the peak with λ_{max} at 356 nm, which allowed assigning it as an isorhamnetin dihexoside. The position of substitution of the sugar residues could not be definitely concluded, although the successive losses of the two moieties suggested that they were located at different positions on the aglycone. The other seven flavonoid peaks corresponded to flavone derivatives.

Peaks 3, 6 and 7 presented identical UV spectra with λ_{max} at 338 nm, pointing out that they derived from the same aglycone. Peaks 3 and 6 had the same pseudomolecular ion $[M-H]^-$ at *m/z* 563 and yielded a major MS^2 fragment ion at *m/z* 431 (-132 amu, loss of a

pentoside moiety) and minor ones at *m/z* 443 (loss of 120 mass units, characteristic of *C*-hexosyl flavones) (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003), 311 (-132-120 amu) and 269 (-132-162 amu, loss of pentose and hexose moieties; apigenin). The presence of apigenin as aglycone was also supported by the UV spectra of the peaks with λ_{max} at 338 nm. The fragmentation pattern of these peaks suggested that the compounds possessed two glycosyl residues, a hexoside and a pentoside, which were *C*- and *O*-attached respectively on different positions of the aglycone. The fragment [(M-H)-120]$^-$ is more abundant in 6-*C*-glycosyl derivatives than in 8-*C*-glycosyl derivatives; in addition, in 6-*C*-glycosyl flavones another fragment ion ([(M-H)-90]$^-$ is also usually observed (Ferreres, Gil-Izquierdo, Andrade, Valentao, & Tomás-Barberán, 2007). Thus, the absence of this latter ion and the low abundance of the former would indicate that the hexoside was located at position 8-*C*. Regarding the *O*-pentoside residue, the existence of two peaks (3 and 6) with the same mass characteristics at different elution times may suggest substitution on different locations of the aglycone, i.e., the hydroxyl groups at 5- and 7- positions. Since the most probable position of substitution would be hydroxyl group at position 7 (Ferreres et al., 2011), the peak 3 (the majority one) was tentatively identified as apigenin-8-*C*-hexoside-7-*O*-pentoside, and peak 6 as apigenin-8-*C*-hexoside-5-*O*-pentoside. The structural differences between them might also be due to the presence of different sugar residues in each case, although this possibility seems less feasible, as the same substituting sugars are usually found in a given plant sample. Peak 7 presented a pseudomolecular ion [M-H]$^-$ at *m/z* 605, 42 amu greater than compounds 3 and 6 with which it shares similar fragmentation pattern and UV spectrum. The loss of 42 amu may correspond to an acetyl residue and suggests that this compound is an acetyl derivative of peak 3 (rather than of minority peak 6). The fragment at *m/z* 545 (-60 amu) might result from the partial loss of the *C*-hexoside

moiety (Ferreres et al., 2003). The simultaneous loss of the pentoside and acetyl residues, as confirmed from the fragment at *m/z* 431 (-132-42 amu) might suggest that the acetyl residue is located on the pentose rather that in the hexose, but the fragment at *m/z* 311 (associated to acetyl-apigenin) may also suggest that the acetyl might be linked to the aglycone rather than located on the pentose. Therefore, this compound was tentatively identified as an acetylated apigenin-*C*-hexoside-*O*-pentoside.

Peaks 4 and 5 presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 593, that yielded two MS^2 fragment ions at *m/z* 299 and 284; the same two fragments and similar abundance in negative mode were described by Waridel et al. (2001) as characteristic for luteolin-*C*-glycosides. Furthermore, the UV spectra of the peaks (λ_{max} at 342-346 nm) would be coherent with luteolin derivatives. Thus, according to their pseudomolecular ions, peaks 4 and 5 might be tentatively identified as luteolin-*C*-hexoside-rhamnosides, but due to the lack of other fragment ions that may support this identity, it was not possible to assign the sugar position and the glycosilation compounds; therefore, peaks 4 and 5 were tentatively assigned as undefined luteolin hexoside-rhamnosides.

Pseudomolecular ions of peaks 8 and 9 were 42 mass units greater then peaks 4 and 5, and they also shared similar fragmentation characteristics, allowing their tentative identification as the corresponding luteolin acetyl derivatives.

Finally, peak 2 was assigned as a tryptophan hexoside from its MS spectra. This compound was not quantified, as it is not a phenolic compound, but an aromatic amino acid that was simultaneously extracted in the conditions used (Aguilera et al., 2010; Dueñas, Estrella, & Hernández , 2004). Its presence in the clones could be residual from the fruits where the clones germinated from, in which, in the germination process, the endogenous enzymes are activated and the enzymes that are related with the phenolic

compounds (hydrolases and polyphenoloxydases) lead to the release of this aromatic amino acid from phenolic acids found in the fruits (López-Amorós, Estrella, & Hernández, 2006).

3.2. Anthocyanins analysis

Anthocyanins were found only in clone A, which explains its purple pigmentation (**Figure 1c**). Six compounds were detected (**Figure 3a**) that could be assigned to anthocyanins based on their UV-visible spectral shape, although the low levels present in the samples and overlapping in the HPLC run with other majority compounds did not allow obtaining "clean" absorption and mass spectra. The analytical characteristics and tentative identity of these peaks are shown in **Table 2**, as well as their concentrations in the clone A expressed as µg/Kg of dw. All of them showed a shoulder at 316-330 nm in the UV region of their absorption spectra, suggesting that they were anthocyanins acylated with hydroxycinnamic acids. The spectrum of peak 4, the majority detected anthocyanin, is shown as an example in **Figure 3b**. The identities of the compounds were speculated from their molecular ions and the unique MS^2 fragment ion obtained in each case corresponding to the aglycone.

Peaks 1, 2 and 3 were cyanidin-based anthocyanins (fragment ion at *m/z* 287), and their masses matched with compounds bearing two hexose moieties and either a sinapoyl (peak 1), feruloyl (peak 2) or a coumaroyl (peak 3) residue. Chromatographic and spectral characteristics of peak 3 also matched with cyanidin 3-*O*-coumaroylglucoside-5-*O*-glucoside in our data library, which allowed tentatively identifying it as this compound. By analogy with that compound, peaks 1 and 2 were tentatively identified as cyanidin 3-*O*- sinapoyl glucoside-5-*O*-glucoside and cyanidin 3-*O*-feruloylglucoside-5-*O*-glucoside. Similar reasoning can be made for anthocyanins 4, 5 and 6 that were

tentatively identified as peonidin derivatives with the same acylation pattern as in peaks 1, 2 and 3. Peonidin 3-*O*-feruloylglucoside-5-*O*-glucoside was the main anthocyanin found in this sample (1.70 µg/Kg dw). The suggested identities, however, do not totally match with their relative elution orders, as feruloyl derivatives were expected to elute after the equivalent *p*-coumaroyl derivatives, which are theoretically more polar, therefore, the indicated identities should be seen as just tentative.

4. Discussion

Secondary metabolism could respond to oxidative stress and free radicals production, leading to the accumulation of different compounds in samples grown *in vitro* or *in vivo*. In fact, vegetative parts from *in vivo* samples and *in vitro* clones presented different profiles of phenolic compounds, which is certainly related to genetic factors but also to growth environmental conditions that lead to different oxidative stress conditions and therefore, dissimilar response in terms of secondary metabolites production. Furthermore, fruits and vegetative parts showed different phenolic profiles; fruits presented only phenolic acids (and not flavonoids) and in much lower concentration. This is an evidence for a different metabolism in each part of the plant, being secondary metabolism lower in fruits.

In a previous report of our research group (Dias et al., 2011), *in vivo* coriander vegetative parts were described as having the lowest EC_{50} values, corresponding to the highest antioxidant activity and the highest concentration in total phenolics (measured by Folin Ciocalteus colorimetric assay). Those results are in agreement with the highest values of total phenolic compounds (obtained after chromatographic analysis of individual compounds) also found for this sample in the present study. Until now there was no information about phenolic composition of coriander fruits and *in vitro* samples,

although there are a few reports on coriander leaves from Brazil (Meloa et al., 2005), Denmark (Justesen & Knuthsen, 2001) and India (Nambiar et al., 2010), but the authors used different methodologies of extraction and detection, which do not allow a proper comparison of the results. Coriander leaves from India (Nambiar et al., 2010) seemed to be a rich source in quercetin, and the Danish sample (Justesen & Knuthsen, 2001) only presented this compound. In the present work, quercetin derivatives were also the majority compounds found in vegetative parts. However, phenolic acids were reported as majority compounds in Brazilian samples (Meloa et al., 2005), which might be due to the different extraction applied and also to the different techniques used for detection (gas chromatography and thin layer chromatography).

In general, *in vivo* and *in vitro* samples of coriander can be considered a rich source of flavonoids. These phytochemicals have been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), to protect low-density lipoproteins (LDL) from oxidation (which prevents atherosclerotic plaque formation) and to promote relaxation of cardiovascular smooth muscle (antihypertensive, antiarrythmic effects), as well as to have antiviral and anticarcinogenic properties (Middleton, Kandaswami & Theoharides, 2005). Coriander is also reported to have chelating properties and also to be effective as pharmaceutical agents in removing heavy metals (Nambiar et al., 2010). Although the amounts of anthocyanins present in pigmented coriander clones are quite low (concentrations of total antocyanins 6.71 µg/kg dw) compared with other flavonoids, they have also been shown to display a variety of effects on blood vessels, platelets and lipoproteins involved with the risk of coronary heart diseases, as well as a range of other biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities (Mazza, 2007). According to Diederichsen (1996), the synthesis of anthocyanins is strengthened by environmental or physiological

stress like the one related to *in vitro* culture conditions. Thus, the presence of anthocyanins may further increase the phytochemical value of these samples. Overall, *in vitro* culture can be used to explore new industrial, pharmaceutical and medicinal potentialities, such as the production of secondary metabolites like flavones, flavonols and anthocyanins.

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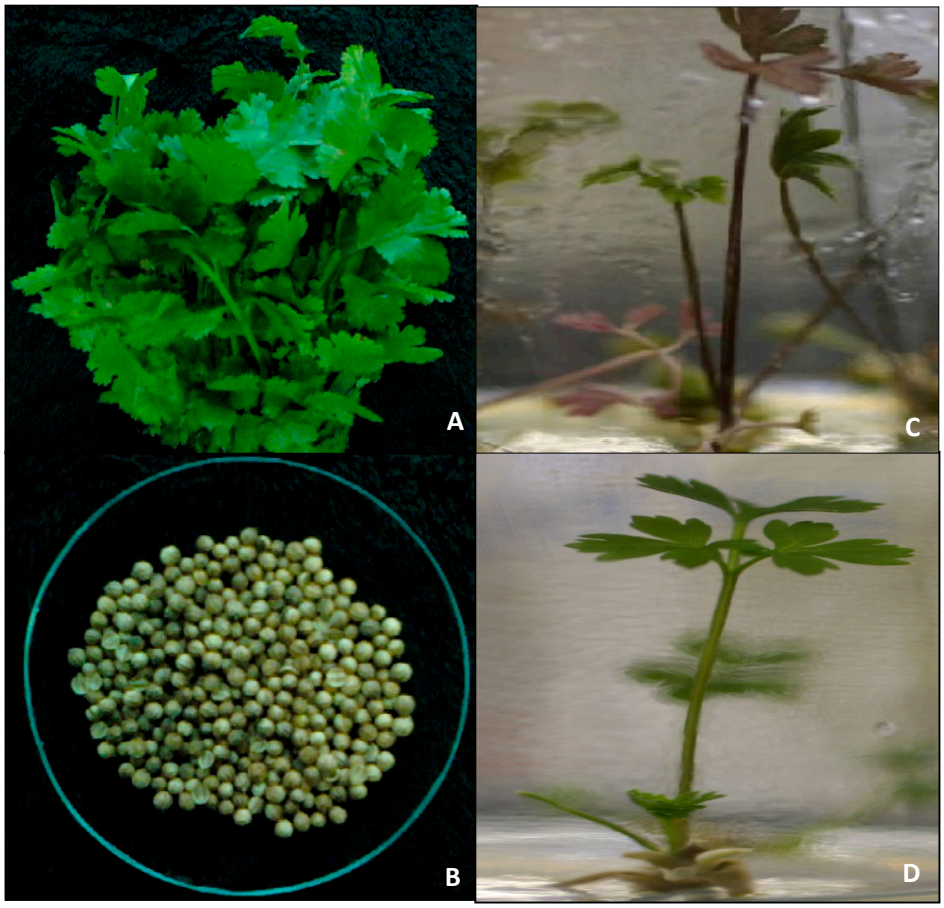
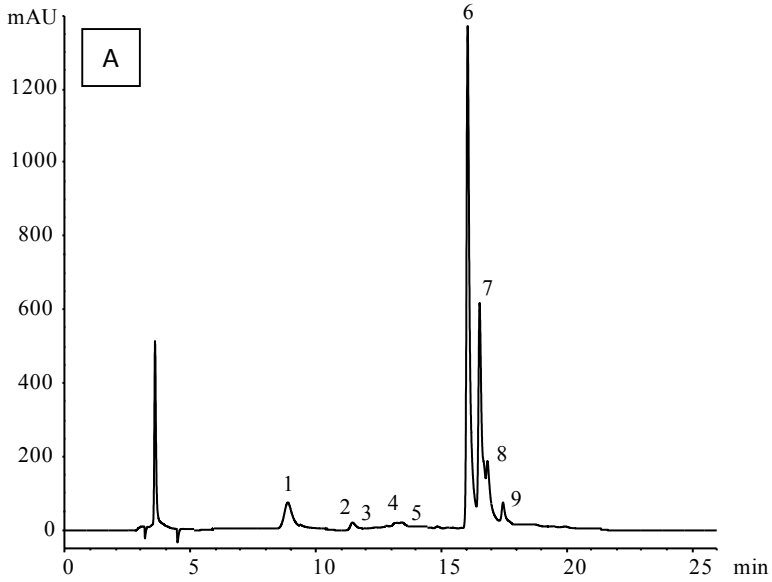


Figure 1. A. *In vivo* grown coriander; B. Fruits of coriander; C. Clone A- *in vitro* grown plants with purple pigmentation; D. Clone B- *in vitro* grown plants completely green.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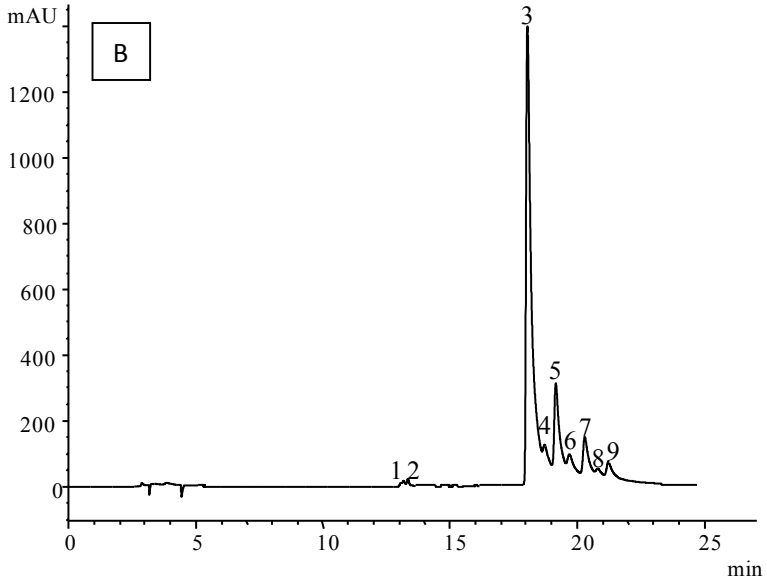
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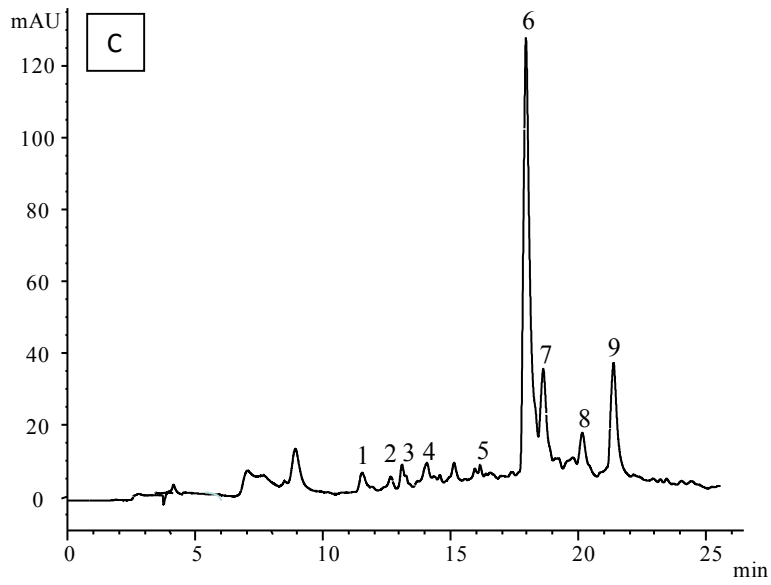

Figure 2. Individual chromatograms of vegetative parts and *in vitro* culture of Coriander. A- Coriander vegetative parts recorded at 370 nm; B- Clone A recorded at 370 nm; C- Coriander fruits recorded at 280 nm.

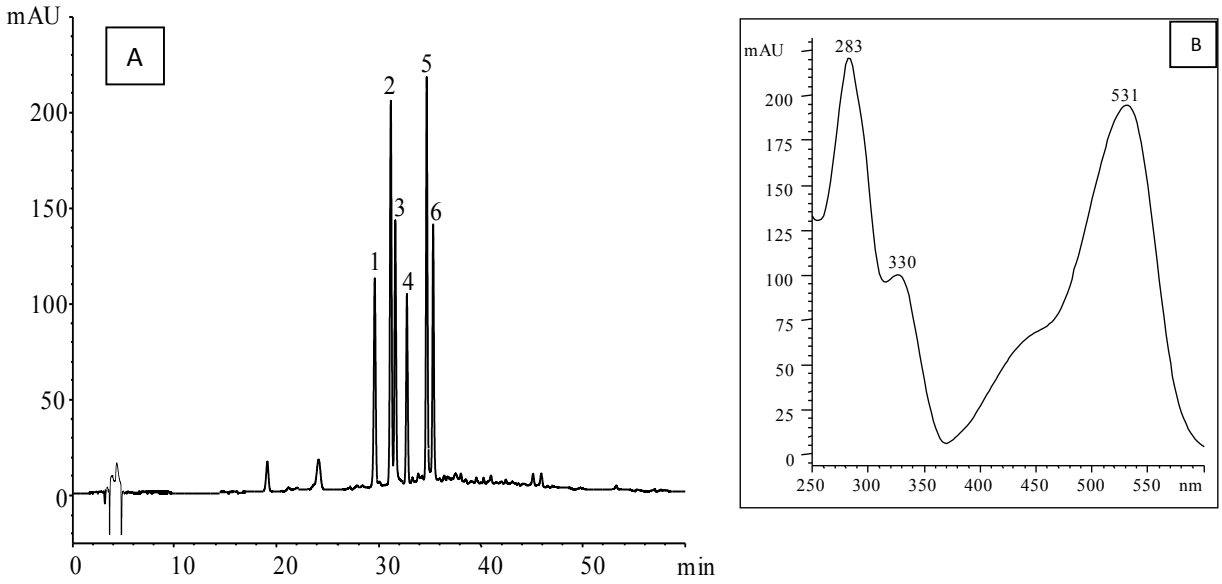

Figure 3. Individual chromatogram profile of anthocyanins in Clone A, recorded at 520 nm (A) and UV spectrum of peak 5 (B).

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in *Coriandrum sativum*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/Kg, dw)
				Vegetative parts		
1	8.90	328	369	207(11), 189(100)	Dimethoxycinnamoyl hexoside	406.39 ± 2.57
2	11.4	324	353	191(100), 179(6), 173(5), 135(4)	3-*O*-caffeoylquinic acid	173.51 ± 11.54
3	11.8	322	353	191(100), 179(45), 173(3), 135(7)	Caffeoylquinic acid	7.92 ± 1.38
4	13.3	328	355	193(100), 149(24)	Ferulic acid glucoside	122.29 ± 12.32
5	14.5	308	337	191(100), 173*, 163*, 146*	*p*-coumaroylquinic acid	303.83 ± 9.27
6	16.0	354	609	301(100)	Quercetin-3-*O*-rutinoside	3296.16 ± 15.50
7	16.5	355	477	301(100)	Quercetin 3-*O*-glucuronide	1237.13 ± 22.72
8	16.8	356	463	301(100)	Quercetin -3-*O*-glucoside	405.36 ± 10.93
9	17.4	354	593	285(100)	Kaempferol-3-*O*-rutinoside	320.86 ± 20.76
					Total phenolic acids	**1013.95 ± 11.24**
					Total flavonoids	**5259.52 ± 69.91**
					Total phenolic compounds	**6273.47 ± 81.16**
				Fruits		

1	11.5	325	353	191(100), 179(6), 173(5), 135(4)	3-*O*-caffeoylquinic acid	1.44 ± 0.27
2	12.7	320	-	193(6), 149(100)	Ferulic acid derivative	7.96 ± 0.34
3	13.1	323	179	135(100)	Caffeic acid	3.07 ± 0.95
4	14.0	326	365	203(100)	Caffeoyl *N*- tryptophan	0.71 ± 0.04
5	16.1	319	163	119(100)	*p*-Coumaric acid	23.81 ± 2.50
6	17.4	324	193	149 (100)	Ferulic acid	8.11 ± 0.74
7	17.9	327	527	365(100), 203(11)	Caffeoly *N*- tryptophan hexoside	45.33 ± 4.36
8	18.6	326	515	353(100), 191(15), 179(50), 173(99), 135(27)	di-*O*-caffeoylquinic acid	9.67 ± 1.62
9	20.2	328	379	203(25), 193(100)	Feruloyl *N*- tryptophan	10.56 ± 1.18
10	21.4	326	541	379(100), 203(5)	Feruloyl *N*- tryptophan hexoside	19.27 ± 1.06
					Total phenolic acids	**129.94 ± 12.42**
				Clone A		
1	13.3	356	639	477(100), 315(62)	Isorhamnetin dihexoside	16.98 ± 2.34
2	14.2	279	365	203(100)	Tryptophan hexoside	nq
3	18.0	338	563	443(6), 431(29), 311(3), 269(100)	Apigenin-8-*C*-hexoside-7-*O*-pentoside	3404.07 ± 19.23
4	18.7	342	593	299(100), 284(7)	Luteolin hexoxyl-rhamnoside	321.18 ± 22.34
5	19.1	346	593	299(100), 284(27)	Luteolin hexoxyl-rhamnoside	820.11 ± 37.50
6	19.73	338	563	443(6), 431(28), 311(17), 269(100)	Apigenin-8-*C*-hexoside-5-*O*-pentoside	272.64 ± 13.18
7	20.33	338	605	545(33), 431(33), 311(27), 269(100)	Acetylated apigenin-*C*-hexoside-*O*-pentoside	354.83 ± 13.56

8	20.84	342	635	299(100), 284(41)	Acetylated luteolin hexoxyl-rhamnoside	117.50 ± 3.72
9	21.26	346	635	299(100), 284(34)	Acetylated luteolin hexoxyl-rhamnoside	201.75 ± 3.42
					Total flavonoids	**5509.07 ± 70.62**
				Clone B		
1	13.09	356	639	477(100), 315(60)	Isorhamnetin dihexoside	22.44 ± 0.66
2	14.19	279	365	203(100)	Tryptophan hexoside	nq
3	18.05	338	563	443(7), 431(27), 311(4), 269(100)	Apigenin-8-*C*-hexoside-7-*O*-pentoside	2982.87 ± 94.89
4	18.70	340	593	299(100), 284(23)	Luteolin hexoxyl-rhamnoside	244.89 ± 10.06
5	19.17	346	593	299(100), 284(30)	Luteolin hexoxyl-rhamnoside	509.10 ± 27.11
6	19.71	338	563	443(6), 431(20), 311(9), 269(100)	Apigenin-8-*C*-hexoside-5-*O*-pentoside	255.95 ± 4.85
7	20.31	338	605	545(18), 431(33), 311(22), 269(100)	Acetylated apigenin-*C*-hexoside-*O*-pentoside	506.43 ± 65.00
8	20.82	342	635	299(100), 284(31)	Acetylated luteolin hexoxyl-rhamnoside	109.52 ± 18.36
9	21.24	346	635	299(100), 284(39)	Acetylated luteolin hexoxyl-rhamnoside	222.60 ± 0.24
					Total flavonoids	**4853.80 ± 98.62**

Figures in brackets after MS^2 fragment ions refer to their relative abundances. *Relative abundance < 2%.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and concentration of anthocyanins in *Coriandrum sativum* Clone A.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/kg of dw)
1	29.3	324sh/534	817	287(100)	Cyanidin-3-*O*-sinapoylglucoside-5-*O*-glucoside	0.78 ± 0.01
2	31.22	330sh/530	787	287(100)	Cyanidin-3-*O*-feruloyl glucoside-5-*O*-glucoside	1.40 ± 0.01
3	31.65	316sh/528	757	287(100)	Cyanidin -3-*O*-*p*-coumaroyl glucoside -5-*O*-glucoside	0.95 ± 0.01
4	32.74	324sh/532	831	301(100)	Peonidin-3-*O*-sinapoylglucoside-5-*O*-glucosid	0.82 ± 0.01
5	34.75	330sh/531	801	301(100)	Peonidin -3-*O*-feruloyl glucoside-5-*O*-glucoside	1.70 ± 0.01
6	35.35	319sh/530	771	301(100)	Peonidin -3-*O*-*p*-coumaroyl glucoside -5-*O*-glucoside	1.08 ± 0.00
					Total anthocyanins compounds	**6.71 ± 0.36**