HPLC Quantification of Flavonoids and Biflavonoids in Cupressaceae Leaves

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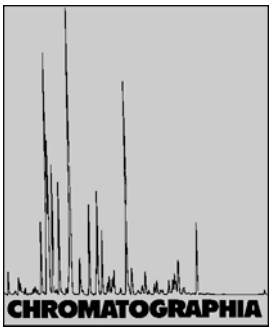

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Key Words

Column liquid chromatography – mass spectrometry
LC-diode array detection
Flavonols
Biapigenin
Cupressaceae leaves

Summary

The aim of this investigation was to obtain qualitative and quantitative profiles of the flavonoid and biflavonoid composition of six cypress species – *Cupressus funebris* L., *Cupressus sempervirens* L., *Cupressus glabra* L., *Cupressus arizonica* L., *Cupressus goveniana* L., and *Cupressus lusitanica* L. HPLC-diode-array detection (DAD), HPLC-MS, and HPTLC were used to identify the individual compounds. A chromatographic method was optimized for identification and quantification of the main flavonoid glycosides and biflavonoids. The flavonoids identified and calibrated were: rutin, quercetin glucoside, quercetin rhamnoside, and kaempferol 3-*O*-rhamnoside. The biflavonoids identified and calibrated were: cupressuflavone, amentoflavone, robustaflavone, hinokiflavone, methylrobustaflavone, methylamentoflavone, and dimethylcupressuflavone.

Introduction

The *Cupressaceae* family comprises several genera and many species. Cypress trees are found in the northern hemisphere only and are divided into three geographical groups – Afromediterranean, American, and Asiatic. The best known and most widespread species in Italy is *Cupressus sempervirens* L., an evergreen conifer with small, scale-like leaves which can grow as high as 30 m. Cypress is usually cultivated as an ornamental tree to beautify parks and cemeteries; in many areas, Tuscany in particular, it constitutes an unmistakable element of the landscape [1, 2].

The aerial parts of the plant have been widely used in folk medicine. Nowadays they are sometimes used for suffumigations, as antitussives, and in solution for washing and bandages for treatment of circulatory diseases [3]. As reported by Hegnauer [4], the main chemotaxonomic chemical components of the *Cupressus* genus are the biflavonoids. Since the first biflavone, gingetin, was isolated in 1929 more than one hundred biflavonoids have been identified in plants [5]. A wide variety of biological activity has been ascribed to these molecules, e.g. peripheral vasodilatation, hypoglycemic, antimicrobial, and antidiabetic effects, and inhibitory effects on lipid peroxidation [6–8]. Other more specific activity has also been reported, e.g. stimulation of RNA synthesis in rat epatocyte suspensions, cytotoxicity, inhibition of the expression of EBV virus gene, and anti-spasmogenic, antibradykinin, and hepatoprotective activity [9, 10]. Recent studies have found evidence that the molecules have antifungal and anti-inflammatory activity [11–13], and especially remarkable antiviral activity against HIV, adenovirus, HSV, HCMV, varicella zooster virus, and hepatitis B [14, 15].

Despite these properties, biflavones are a class of phenolic compounds which have rarely been studied. Several studies have been performed on the biflavonoid content of species such as *Ginkgo biloba* and *Hypericum perforatum*, which are also important in the pharmaceutical industry [16–20], but few data are available about their occurrence in the genus *Cupressus*. The chemical structures of the main biflavones present in cypress species are reported in Figure 1.

Gadek and Quinn [21] first reported the presence of amentoflavone and cupressuflavone in the leaves of *Cupressus sempervirens* L., *Cupressus lusitanica* L., and *Cupressus glabra* L. Heimler and Pieroni [22] separated and identified flavonoid glycosides and biflavonoids from *Cupressus sempervirens* L. by use of two different thin-layer chromatographic methods. Although a variety of biflavonoids has been reported to be present in cypress tissues,

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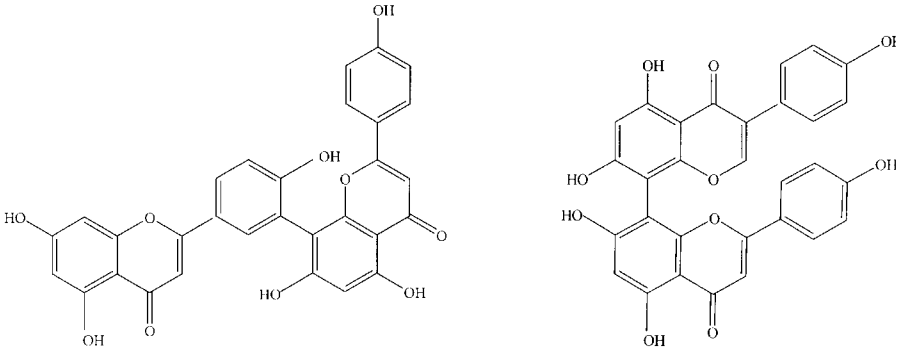
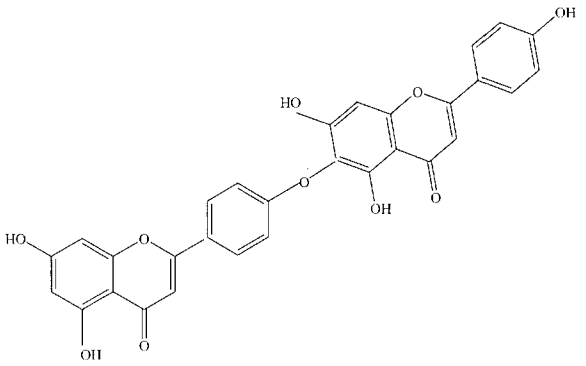


Amentoflavone

Cupressuflavone



Hinokiflavone

Figure 1. Structural formulae of the main biflavonoids detected in *Cupressus* leaves.

Table I. Linear solvent gradient used for analytical HPLC-DAD and HPLC-MS analysis.

H_2O (%)	CH_3CN (%)	Time (min)
93.0	7.0	0.1
50.0	50.0	5.0
25.0	75.0	10.0
25.0	75.0	13.0
0.0	0.0	15.0
0.0	0.0	20.0

the identity of the compounds was usually inconclusive and quantification was not attempted [21, 23].

In the investigation reported here the flavonoid and biflavonoid content of hydroalcoholic extracts of six cypress species – *C. funebris* L. (Asiatic group), *C. sempervirens* L. (Afromediterranean group), *C. glabra* L., *C. arizonica* L., *C. goveniana* L., and *C. lusitanica* L. (American group) – was determined by HPLC-DAD, HPLC-MS, and HPTLC. A chromatographic method was optimized for identification and quantification of the main flavonoid glycosides and, in particular, biflavonoids. To the best of our knowledge this is the first report on quantification of the main individual polyphenols in *Cupressus* leaf tissues.

Experimental

Sample Preparation and Extraction of Polyphenols

Green leaves of *C. sempervirens*, *C. funebris*, *C. glabra*, *C. goveniana*, *C. lusitanica*, and *C. arizonica* were analysed; all the samples were collected in October 2001. Leaf laminae were frozen rapidly in liquid nitrogen and stored at –80 °C until analysis. Frozen leaf tissue was then ground under liquid nitrogen by use of a pestle and mortar. Fresh tissue (1 g) was extracted with 4 × 30 mL 70:30 (% *v/v*) EtOH-water adjusted to pH 2.0 by addition of HCOOH. The raw ethanolic extract was then evaporated to dryness under vacuum (Rotavapor 144 R; Büchi, Switzerland) at room temperature and then dissolved in 100 mL water at pH 2.0 (adjusted by addition of HCOOH). This solution was then defatted, by extraction with 4 × 50 mL *n*-hexane, and the ethanolic extract was concentrated under reduced pressure and finally dissolved in pH 2 ethanol to a final volume of 5 mL. A sample (6 µL) of this solution was analysed by HPLC with diode-array detection (DAD) and HPLC-MS for qualitative and quantitative evaluation.

Identification and Quantification of Individual Flavonoids and Biflavonoids

Identification of individual polyphenols was achieved by use of their retention times and both spectroscopic and spectrometric data. Authentic standards of quercetin 3-*O*-glucoside (isoquercitrin), quercetin 3-*O*-rhamnoside (quercitrin), quercetin 3-*O*-rutinoside (rutin), hinokiflavone, and amentoflavone were purchased from Extrasynthèse (Lyon, Nord-Genay, France).

Individual polyphenols were quantified by use of a five-point regression curve ($r^2 \geq 0.999$) in the range 0–30 µg on the basis of authentic standards; calibration was performed directly by HPLC-DAD at the wavelength of maximum absorbance (350 nm).

Calibration for isoquercitrin, quercitrin, rutin, amentoflavone, and hinokiflavone was performed by use of the appropriate pure standards. Calibration for cupressuflavone was performed by use of amentoflavone as reference compound. Calibration for kaempferol 3-*O*-glucoside was performed by use of kaempferol as reference compound after correcting for the specific molecular weight.

Analytical Techniques and Equipment

HPLC-DAD Analysis

Analysis was performed by use of an HP 1100L liquid chromatograph equipped with a DAD (Agilent Technologies). The polyphenol compounds were separated at 26 °C on a 150 mm × 3.0 mm i. d., 5-µm particle, Luna C18 (2) column (Chemtek analytica, Bologna) equipped with a 4 mm length × 3.0 mm i. d. ODS (C_{18}) precolumn. The mobile phase was a four-step linear gradient prepared from water (adjusted to pH 3.2 by addition of H_3PO_4) and acetonitrile. The starting composition

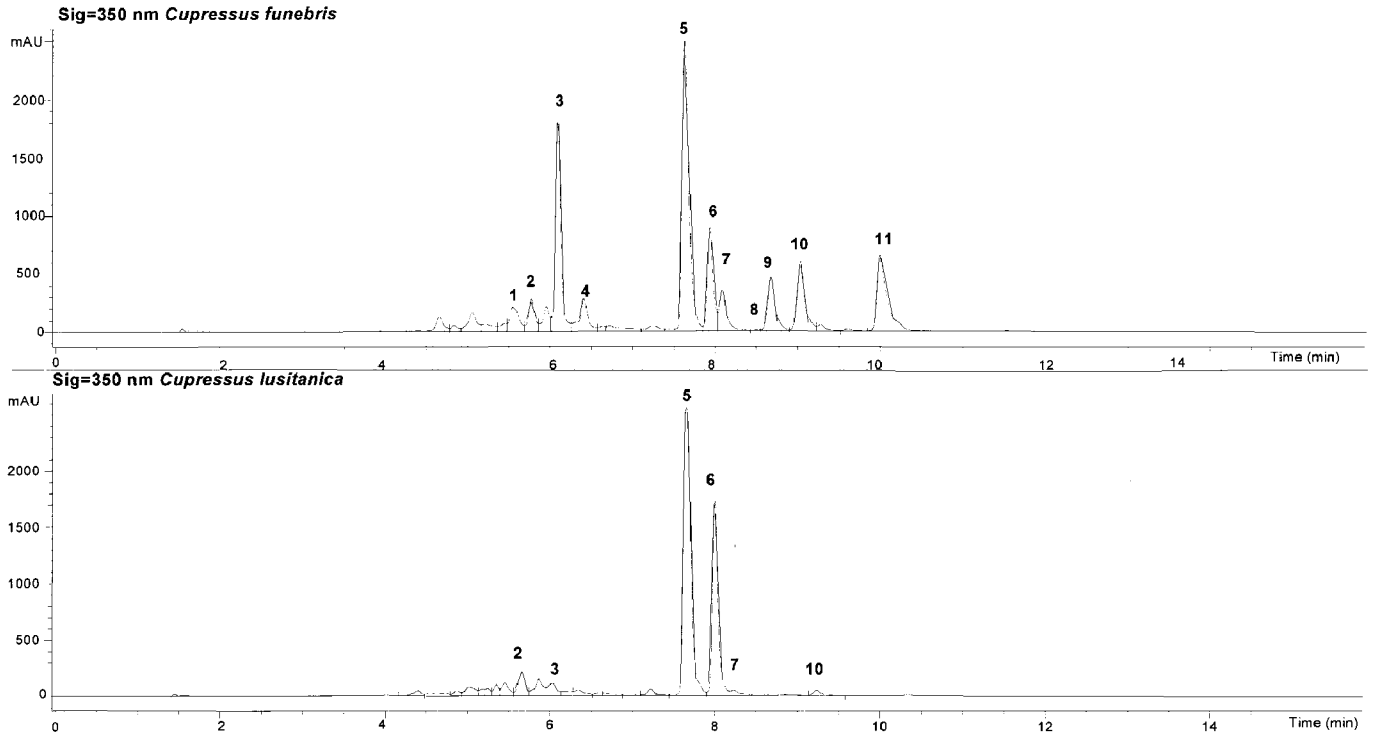


Figure 2. Chromatographic profile acquired by use of HPLC-DAD at 350 nm from ethanolic extracts of *Cupressus funebris* and *Cupressus lusitanica*. Peaks: **1** = rutin; **2** = quercetin 3-*O*-glucoside; **3** = quercetin 3-*O*-rhamnoside; **4** = kaempferol 3-*O*- rhamnoside; **5** = cupressuflavone; **6** = amentoflavone; **7** = robustaflavone; **8** = hinokiflavone; **9** = methylrobustaflavone; **10** = methylamentoflavone; **11** = dimethylcupressuflavone.

was 93:7 (% *v/v*) H_2O–CH_3CN and the CH_3CN content was increased to 75% over a 13-min period. The composition of the gradient is reported in Table I. The mobile phase flow rate was 0.6 mL min^{-1}. UV-visible spectra were recorded in the range 190–450 nm and chromatograms were acquired at 240, 280, 330, and 350 nm.

HPLC-MS Analysis

HPLC-MS analysis was performed, as described elsewhere [24], by use of an HP 1100 MSD API-electrospray coupled to an HP 1100L liquid chromatograph equipped with a DAD (Agilent Technologies). Positioning of the nebulizer orthogonal to the capillary inlet enabled the use of the same conditions as for HPLC-DAD analysis, although in this analysis the water was adjusted to pH 3.2 by addition of HCOOH.

HPTLC Analysis

Two-dimensional HPTLC was performed on 5 cm × 5 cm silica gel $60F_{254}$ plates (Merck) in a Desaga (Carlo Erba, Milan, Italy) chromatography chamber for horizontal development comprising a solvent-proof body (Teflon) with a tray for mobile phase and a tight-fitting glass lid. The mobile phase was transferred from the tray to the layer by means of an exchangeable sintered-glass plate. The mobile phase for both runs was toluene-pyridine-formic acid, 100:20:7 [22]. After development the plates were dried and sprayed with a 1% methanolic solution of the complex of diphenylboric acid with ethanolamine, followed by 5% ethanolic poly(ethylene glycol) 4000. Spots were identified by virtue of their fluorescence at 365 nm.

Results and Discussion

Identification of Individual Flavonoids and Biflavonoids

The aim of this work was to develop a rapid HPLC method for identification and quantification of the main flavonoids and biflavonoids present in the leaf tissue of six species of *Cupressaceae*. As examples the chromatographic profiles obtained from *C. funebris* and *C. lusitanica* extracts, recorded at 350 nm, are presented in Figure 2. The figure reveals both the qualitative composition of the cypress leaves and the efficiency of the chromatographic method used. The flavonoids rutin, isoquercitrin, quercitrin, and kaempferol 3-*O*-rhamnoside, with retention times between 0.0 and 6.5 min, were identified, as were the biflavonoids amentoflavone, cupressuflavone, robustaflavone, hinokiflavone, and other biflavonoids, with retention times between 7.0 and 11.0 min.

For better characterization of both flavonoid glycosides and biflavonoids, HPLC-DAD was combined with HPLC-MS operating in the negative ion mode with modulated fragmentation patterns. Isoquercitrin, quercitrin, rutin, and amentoflavone were identified by comparison of retention times and UV-visible spectra of leaf extracts with those from authentic standards.

Kaempferol-3-*O*-rhamoside was detected by HPLC-MS; the fragmentation pattern contains signals at *m/z* 431 and 285, corresponding, respectively, to the quasi-molecular ion $[M - H]^-$ and to the fragment obtained by loss of rhamnose $[M - 146]^-$. The position of the substituent was confirmed by comparing results from the cypress extract with those from a grape extract in which this compound had previously been detected [23].

The mass spectrum and chemical structure of cupressuflavone, the most representative biflavone in all the samples analysed, are shown in Figure 3. The most important peaks are those at *m/z* 537 and 375, which correspond to the quasi-molecular ion $[M - H]^-$ and to the fragment $[M - 162]^-$.

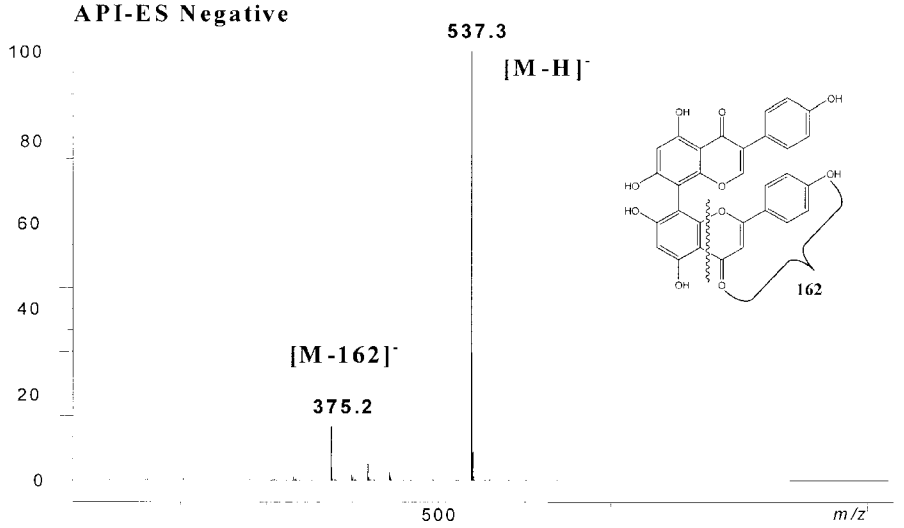


Figure 3. Negative-ion mass spectrum of cupressuflavone, and the corresponding chemical structure. The spectrum was acquired by use of API-electrospray HPLC-MS analysis at a negative fragmentor potential of 180 V.

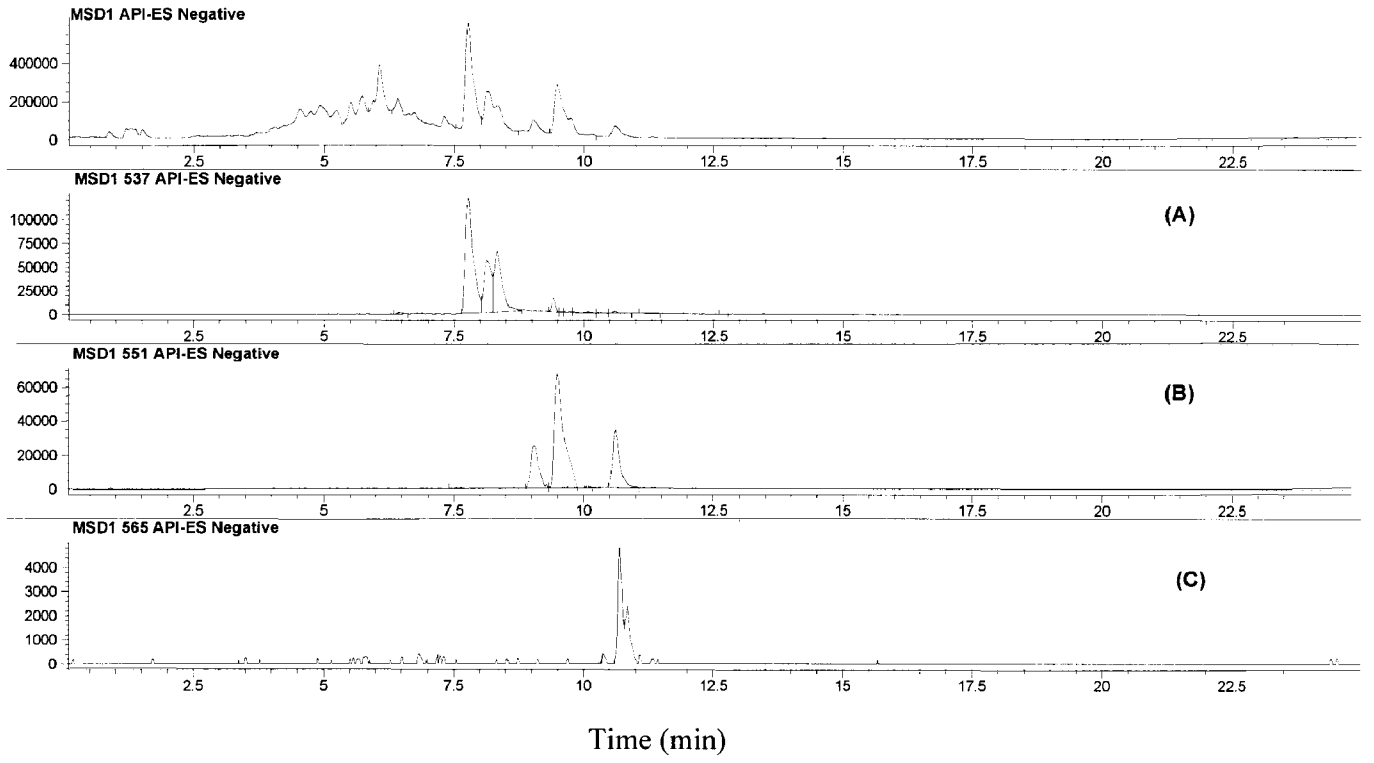


Figure 4. Extracted ion-current chromatograms obtained from the hydroalcoholic extract of *Cupressus funebris* leaves. The spectrum was acquired by use of API-electrospray HPLC-MS analysis operating in negative ionization mode at 180 eV. Chromatograms were recorded at *m/z* 537, 551, and 565, which correspond to the *m/z* of cupressuflavone, amentoflavone, hinokiflavone, and robustaflavone ([M – H] = 537) (**A**); their methyl derivatives ([M – H] = 551) (**B**), and their dimethyl derivatives ([M – H] = 565) (**C**), respectively.

Gadek and Quinn [21] reported the occurrence of variable amounts of hinokiflavone and its derivatives in *Cupressus* species. Two-dimensional HPTLC was used to verify the presence of these molecules in the cypress samples analysed. The accuracy and reproducibility of HPTLC method are good when compared with those of HPLC. The fluorescence characteristics (at 365 nm) and R_F of components of the cypress extracts were compared with those of authentic standards of amentoflavone and hinokiflavone. This revealed the presence of trace amounts of hinokiflavone in two species – *C. funebris* and *C. arizonica*. This technique was used because fluorescence detection is more than two orders of magnitude more sensitive than HPLC-DAD, as reported elsewhere [25].

Krauze-Baranowska et al. isolated the biflavonoids 4′-*O*-methylcupressuflavone, 7′-*O*-methylamentoflavone, and 4‴-*O*-methylamentoflavone from the leaves of *Cupressocyparis leylandii*, a member of the subfamily *Cupressaceae*, and characterized the compounds by use of both mass spectrometry and NMR [26]. It has previously been suggested that methylamentoflavone, methylhinokiflavone, and

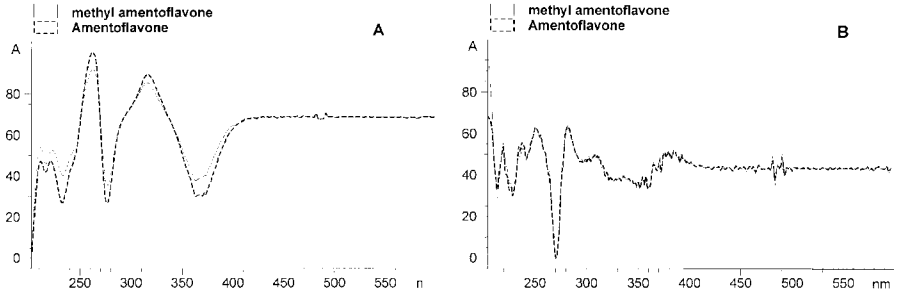


Figure 5 A. First derivative spectra of amentoflavone and methylamentoflavone. **B**. Second derivative spectra of amentoflavone and methylamentoflavone.

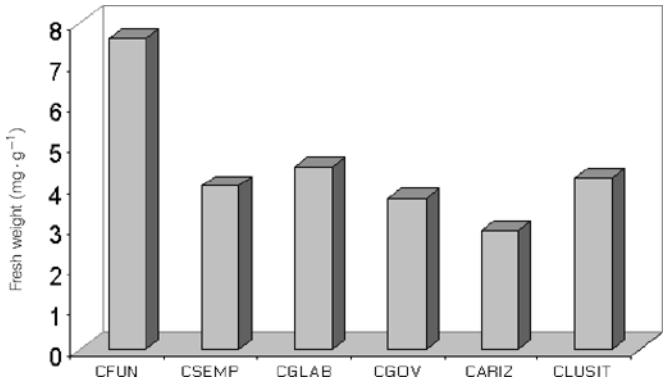


Figure 6. Total polyphenol content of the hydroalcoholic extracts of leaf tissue from six cypress species (CFUN = *Cupressus funebris*; CSEMP = *Cupressus sempervirens*; CGLAB = *Cupressus glabra*; CGOV = *Cupressus goveniana*; CARIZ = *Cupressus arizonica*; CLUSIT = *Cupressus lusitanica*). Quantitative data are expressed as mg g^{-1} fresh weight.

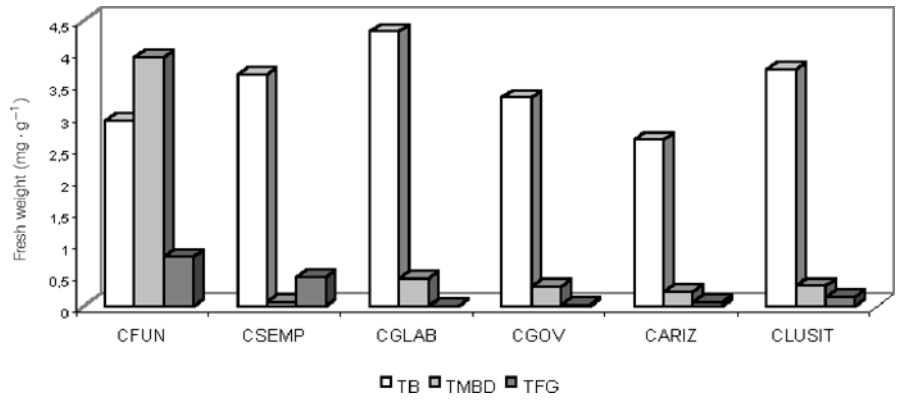


Figure 7. Different polyphenol subclasses present in the hydroalcoholic extracts of leaf tissue from six *Cupressus* species. (CFUN = *Cupressus funebris*; CSEMP = *Cupressus sempervirens*; CGLAB = *Cupressus glabra*; CGOV = *Cupressus goveniana*; CARIZ = *Cupressus arizonica*; CLUSIT = *Cupressus lusitanica*; TB = total biflavonoids; TMBD = total methyl biflavonoid derivatives; TFG = total flavonoid glycosides). Data are expressed as mg g^{-1} fresh weight.

a methylrobustaflavone occur in cypress leaves, although their chemical structure was not fully clarified [21, 27]. Although no data are available on the occurrence of more highly methylated biflavonoids in these species, the presence of such derivatives was assessed by investigation of HPLC-MS extracted-ion profiles and the corresponding mass spectra. As an example, Figure 4 shows the extracted ion chromatograms obtained from the extract of the leaf tissue of *C. funebris*. These chromatograms were recorded in the negative-ion mode at the *m/z* of biflavonoids (*m/z* 537), the *m/z* of biflavonoid methyl derivatives (*m/z* 551), and the *m/z* of dimethyl derivatives (*m/z* 565). This furnished evidence for the presence of four biflavonoids, two methyl derivatives and a dimethyl derivative in this species.

Methyl derivatives were identified in all the species analysed, but a dimethyl derivative was found in *C. funebris* and *C. goveniana* only. Because the fragmentation pattern, which shows the quasi-molecular ion $[M - H]^-$ and fragments indicative of the loss of one or two methyl units, is similar for these molecules, their chemical structures are not easily determined. More detailed information about these molecules was obtained by application of the derivative function to UV-visible spectra – derivative spectra reveal more specific details than the original spectra when different compounds are being compared. Small differences between spectra are much more obvious, and easier to identify visually. Overlaid derivative spectra of amentoflavone and its methyl derivative are reported in Figure 5. The small difference between the spectra was indicative of correlation between them, and so the presence of a methyl derivative of amentoflavone was confirmed. When the same mathematical function was applied to the compound spectra of all the leaf extracts a methyl derivative of robustaflavone and a dimethyl derivative of cupressuflavone were identified. Our findings are in agreement with previous results showing the occurrence of biflavonoid monomethyl derivatives as minor constituents of *Cupressaceae* [26, 27]; this is, however, the first report of the presence of the more highly methylated biflavones.

Quantification of Flavonoids and Biflavonoids in Cupressus Leaf Tissues

The amounts of polyphenols determined in the samples, expressed in mg g^{-1} fresh weight, are listed in Table II; all the data are averages from three analyses; standard deviations were < 2%. The total amount of polyphenols varies from 2.91 to 7.63 mg g^{-1} fresh weight, as shown in form of a histogram in Figure 6. The largest amount was found in *C. funebris* and the lowest in *C. arizonica*. The polyphenol content of the other species was similar, ranging from 3.69 to 4.48 mg g^{-1} fresh weight. The results reported in Table II indicate that the biflavonoids are the most representative compounds in the extracts, and they seem to be the only polyphenols present in *C. glabra*.

Cupressuflavone was the most abundant biflavonoid, levels ranged from 2.02 to 2.55 mg g^{-1} fresh weight and accounted for between 43 and 66% of total biflavonoids except for *C. arizonica*, in which it

Table II. Qualitative and quantitative results from HPLC-DAD analysis of flavonoids and biflavonoids in different *Cupressaceae* leaves. Data are averages from three analyses and are expressed as mg g^{-1} fresh weight.

	CFUN	CSEMP	CGLAB	CGOV	CARIZ	CLUSIT
Rutin	0.11 ± 1.910^{3}	0.01 ± 1.910^{4}	nd	0.01 ± 1.810^{4}	nd	nd
Quercetin glucoside	0.09 ± 1.610^{3}	0.18 ± 3.210^{3}	nd	0.01 ± 1.910^{4}	nd	0.09 ± 1.510^{3}
Quercetin rhamnoside	0.46 ± 7.210^{3}	0.15 ± 2.810^{3}	Trace	0.01 ± 2.010^{4}	0.06 ± 9.010^{4}	0.05 ± 1.010^{3}
Kaempferol 3-*O*-rhamnoside	0.12 ± 1.810^{3}	Trace	nd	nd	nd	Trace
Cupressuflavone	2.02 ± 2.210^{2}	2.12 ± 2.410^{2}	2.55 ± 2.710^{2}	2.38 ± 2.510^{2}	2.38 ± 2.410^{2}	2.39 ± 2.610^{2}
Amentoflavone	0.64 ± 9.710^{3}	1.50 ± 1.710^{2}	1.72 ± 1.910^{2}	0.86 ± 9.210^{3}	0.18 ± 3.110^{3}	1.33 ± 1.510^{2}
Robustaflavone	0.23 ± 3.510^{3}	0.03 ± 5.810^{4}	0.05 ± 8.910^{4}	0.04 ± 7.510^{4}	0.07 ± 1.210^{3}	0.01 ± 1.910^{4}
Hinokiflavone	0.04 ± 8.910^{4}	nd	nd	nd	0.05 ± 9.310^{4}	nd
Methylrobustaflavone	0.42 ± 6.910^{3}	nd	0.16 ± 2.210^{3}	0.19 ± 3.210^{3}	0.17 ± 2.910^{3}	nd
Methylamentoflavone	2.74 ± 3.210^{2}	0.01 ± 1.910^{4}	0.00	0.13 ± 2.010^{3}	nd	0.31 ± 5.610^{3}
Dimethylcupressuflavone	0.76 ± 1.210^{3}	nd	Trace	0.06 ± 1.110^{3}	nd	nd

CFUN = *Cupressus funebris* L.; CSEMP = *Cupressus sempervirens* L.; CGLAB = *Cupressus glabra* L.; CGOV = *Cupressus goveniana* L.; CARIZ = *Cupressus arizonica* L.; CLUSIT = *Cupressus lusitanica* L. nd = not detected.

accounted for 81.8% of total biflavonoids. Another important compound is amentoflavone; amounts of this compound were in the range 0.18–1.72 mg g^{-1} fresh weight. Robustaflavone was <4% of total biflavones, and hinokiflavone, present in *C. funebris* and *C. arizonica* only, accounted for approximately 2% of total biflavones.

These data show that *C. funebris* is different from the other species analysed, because of its higher methyl and dimethyl biflavonoid content – in this species they account for 51.37% of total biflavones whereas the amounts in the other species vary from 0.25% (*C. sempervirens*) to 10.3% (*C. goveniana*).

These data show that the amounts of flavonoids are very low – the amounts of these compounds in the samples analysed vary from 0 to 10.2%, with the *C. funebris* extract being richest in flavonols. The most commonly occurring flavonoid is quercitrin, except in *C. lusitanica*.

The main polyphenol subclasses present in cypress leaf extracts are shown in Figure 7. The data reveal the peculiar quantitative composition of *C. funebris*. This sample contains more of all the polyphenol subclasses, in particular methyl and dimethyl derivatives, than the other species, in which the most abundant compounds are the biapigenins. Flavonoids are present as minor amounts relative to the other sub-classes, although the amounts present in *C. funebris* and *C. sempervirens* are quite high. Because biflavonoid patterns have been regarded as valuable characteristics in the taxonomy of some species [26], these findings might provide a basis for the hypothesis that *Cupressus funebris* belongs to the genus *Chamaecyparis* rather than the genus *Cupressus*, in agreement with literature data [28–30].

The results of our work have revealed significant differences between the biflavonoid content of these species, for example the large amounts of methyl and dimethyl derivatives in *C. funebris* (Asiatic group) and the presence of hinokiflavone in C. *funebris* and C. *arizonica* only. The biflavonoid content of the Afromediterranean group (*C. sempervirens*) and the American group (*C. goveniana, C. arizonica, C. glabra, C. lusitanica*) are not significantly different.

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