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Artigo

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Isolamento e Identificação de Substâncias Fenólicas do Caule de *Eryngium glaziovianum* por Cromatografia Contracorrente com Detecção HPLC-DAD Off-line

Resumo: O extrato bruto hidroalcoólico do caule da espécie *Eryngium glaziovianum* (Apiaceae) foi fracionado por Cromatografia Contracorrente (CCC) usando um sistema de solventes bifásico composto por *n*-hexano-acetato de etila-metanol-água (0,5: 6: 0,5: 6, v/v) no modo de eluição normal. As frações obtidas por CCC foram analisadas e parcialmente caracterizadas por HPLC-DAD *off-line* e as principais substâncias foram identificados por ESI-MS/MS e RMN uni e bidimensional. Esta técnica permitiu o isolamento de uma mistura de isômeros dos flavonoides quercetina 3-*O*-β-D-galactopiranosídeo e quercetina 3-*O*-β-D glucopiranosídeo, além dos ácidos fenólicos clorogênico e 3,4-dicafeoilquínico. A CCC provou ser uma técnica eficiente e rápida para a separação e purificação de metabolitos secundários isoméricos e sua combinação com a detecção HPLC-DAD *off-line* permitiu a análise precisa das frações e a caracterização parcial das substâncias fenólicas.

Palavras-chave: *Eryngium glaziovianum*; flavonoides; substâncias fenólicas; cromatografia contracorrente; HPLC-DAD off-line.

Abstract

Crude hydroalcoholic extract from stems of plant species *Eryngium glaziovianum* (Apiaceae) was fractionated by Countercurrent Chromatography (CCC) using a two-phase solvent system composed by *n*-hexane-ethyl acetate-methanol-water (0.5: 6: 0.5: 6, v/v) in normal elution mode. CCC fractions obtained from this purification procedure were analyzed and partially characterized by *off-lin*e HPLC-DAD. It was possible to identify an isomeric mixture of glycosilated flavonoids quercetin 3-*O*-β-D-galactopyranoside and quercetin 3-*O*-β-D-glucopyranoside in addition to phenolic chlorogenic and 3,4-dicaffeoylquinic acids. These compounds had their structures fully elucidated by ESI-MS/MS and 1D/2D NMR experiments. Thus, CCC proved to be an efficient and rapid technique for separation and purification of isomeric secondary metabolites and its combination with *off-line* HPLC-DAD detection permitted the accurate fraction analysis and partial characterization of phenolic compounds.

Keywords: *Eryngium glaziovianum*; flavonoids; phenolic compounds; countercurrent chromatography; off-line HPLC-DAD.

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Isolation and Identification of Phenolic Compounds from *Eryngium glaziovianum* Stem by Countercurrent Chromatography with Off-line HPLC-DAD Detection

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1. Introduction

Eryngium glaziovianum Urb. (Apiaceae) is a plant species popularly known as "gravatá-do-mato", being endemic to Brazil. It occurs in Rio de Janeiro, São Paulo and Minas Gerais states, predominantly in the Atlantic Forest areas.[1,2] A previous study of this plant reports the chemical profile of non-polar extracts of its leaves, inflorescences and stems by GC-MS[3], although in literature, plants of the genus *Eryngium* are known for accumulating flavonoids and coumarins.[4-6]

CCC is a liquid-liquid partition technique which uses only centrifugal force to retain the stationary phase in the equipment.[7-12] Separation is based on the solute distribution between two immiscible liquid phases.[13-15] Use of CCC in isolation and purification of natural products has been increasing in the last years[10] as it is a versatile methodology

which allows total sample recovery, with no degradation or loss of biological activity, low solvents consumption and high sample loading.[16-20]

The use of preparative CCC coupled to analytical HPLC-DAD is indicated to phytochemical researches – when it aims to analyze mixtures of compounds containing chromophores – as it enables isolation and partial characterization of these metabolites. It might be especially helpful in the identification of known phenolic aglycons of glucosides.

Main goal of his work was to use CCC coupled with an *off-line* HPLC-DAD detection for the isolation, analysis and partial identification of major phenolic compounds from the hydroalcoholic stems extract from *Eryngium glaziovianum* which contributes to the knowledge of the secondary metabolism of the plant.

2. Materials and methods

2.1. Reagents

Vetec organic solvents (Analytical Grade) were used for preparation of crude extract and for thin-layer chromatography (TLC). Vetec organic solvents (HPLC grade) and aqueous solutions prepared with pure water produced by Milli-Q water system (18.2MΩ) were used for HPLC analysis and CCC separations.

2.2. Preparation of crude extract

Eryngium glaziovianum stems were collected in the Itatiaia National Park, Rio de Janeiro, Brazil, in February 2013. A voucher specimen is deposited in the herbarium of the Botanical Garden of Rio de Janeiro under the code Trovó 548 (RB). Dried and grounded stems (168 g) were extracted by exhaustive cold maceration using EtOH-H_2O 8:2 (*v/v*). The hydroalcoholic crude extract obtained was concentrated under reduced pressure on a rotary evaporator, yielding 17.5 g of the dried material.

2.3. CCC equipment

HT-Prep Quattro counter-current chromatograph (AECS, Brigend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene multi-layer coils each (26 mL, 1.0 mm i.d.+ 224 mL, 3.2 mm i.d. and 95mL, 2.0 mm i.d.+ 98 mL, 2.0 mm i.d.). The rotation speed is adjustable from 0 to 865 rpm. A 5 mL sample loop was used to inject the sample.

2.4. Selection of the two-phase CCC solvent system

The two-phase solvent system *n*-hexane-ethyl acetate-methanol-water (HEMWat) was tested by changing the volume ratio of the solvents in the system to achieve an optimum composition which provides a suitable partition coefficient (K). The range of proportions tested was 1:1:1:1, 2:6:2:6, 1:6:1:6 to 0.5:6: 0.5:6 (v/v). Small amounts of the sample were dissolved in a small test tube containing the equilibrated two-phase solvent system. The test tubes were shaken and the compounds allowed to partition between the two phases. Equal aliquots volumes of each phase were spotted on silica gel TLC plates (Merck) and developed with the organic phase of the solvent system ethyl acetate-acetone-water 25:15:10 (v/v). Developed plates were visualized under UV light (λ= 254 and 365 nm) and right after 10% sulfuric acid in methanol solution sprayed on it, followed by heating.

2.5. Preparation of the two-phase solvent system and sample solution

The selected solvent system was

thoroughly equilibrated in a separatory funnel at room temperature. The two phases were separated and degassed by sonication for 5 min. The organic upper phase was used as mobile phase while the aqueous lower phase was used as stationary phase in tail-to-head direction, normal elution mode. Sample solutions injected into the chromatograph were prepared by dissolving the sample (550 mg) in 5.5 mL of both aqueous and organic phases (1:1, v/v) of the solvent mixture used for the separation.

2.6. CCC separation procedure

The fractionation of the extract was performed in a 98mL column in normal elution mode. 75 fractions of 4 mL were collected at a 2 $mL.min^{-1}$ flow-rate. Then rotation was turned off and the stationary phase was collected in 25 fractions of 4 mL at a 4 $mL.min^{-1}$ flow-rate. Retention of the stationary phase was 75%.

2.7. HPLC analyses and identification of target compound

Crude extract and combined fractions were analyzed by HPLC-DAD using a 1200 series chromatograph (Agilent, California, USA) equipped with diode array detector (DAD) G4212B, a Poroshell 120 C-18 (100 x 2.1 mm, 2.7 µm) column and a guard-column of same material. Flow-rate of 0.3 $mL.min^{-1}$, elution at room temperature (25°C) and injection volume of 10 µL were used. Mobile phase was A: 1% aqueous acetic acid and B: MeOH in gradient mode: 0% B – 0 min, 30% B – 3 min, 32 % B – 20 min, 40% B – 25 min, 100% B – 30 min, 100% B – 31 min, 0% B – 32 min, 0% B – 37 min. The chromatogram was obtained with UV detection at 254 nm, suitable for phenolic compounds.

^{1}H and ^{13}C NMR data for the phenolic compounds, isolated through CCC experiments, were recorded on a Varian VNMRSYS-500 (California, USA) at 25 °C, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. NMR spectra were recorded in CD_3OD using TMS as internal standard. The spectra were processed using MestReNova® software (version 6.0.2).

Electrospray ionization mass spectrometry (ESI-MS) analyzes were performed using a Thermo Fisher Scientific-USA spectrometer, LCQ FLEET model and ion trap analyzer. The samples were directly injected at a 5.0 $\mu l.min^{-1}$ flow rate in a temperature of 300° C. The drying gas flow-rate (nitrogen) was 4.0 $L.min^{-1}$ and the nebulizer gas (nitrogen) pressure was 0.4 bar. The data were acquired in negative mode and the mass range analyzed was *m/z* 100-1,500. The spectra were processed using Thermo Xcalibur 2.2 SP1.48 software.

3. Results and discussion

Hydroalcoholic crude extract of stems was analyzed by HPLC-DAD and the resultant chromatogram revealed the presence of eight (1-8) major peaks (**Fig. 1**). UV spectra of the signals, highlighted in **Fig. 1**, showed absorptions indicating the presence of flavonoids and phenylpropanoid derivatives. Similar spectra profiles were found in the literature, corroborating this observation[21-24]. The retention time and UV spectra of each peak are described in **Table 1**.

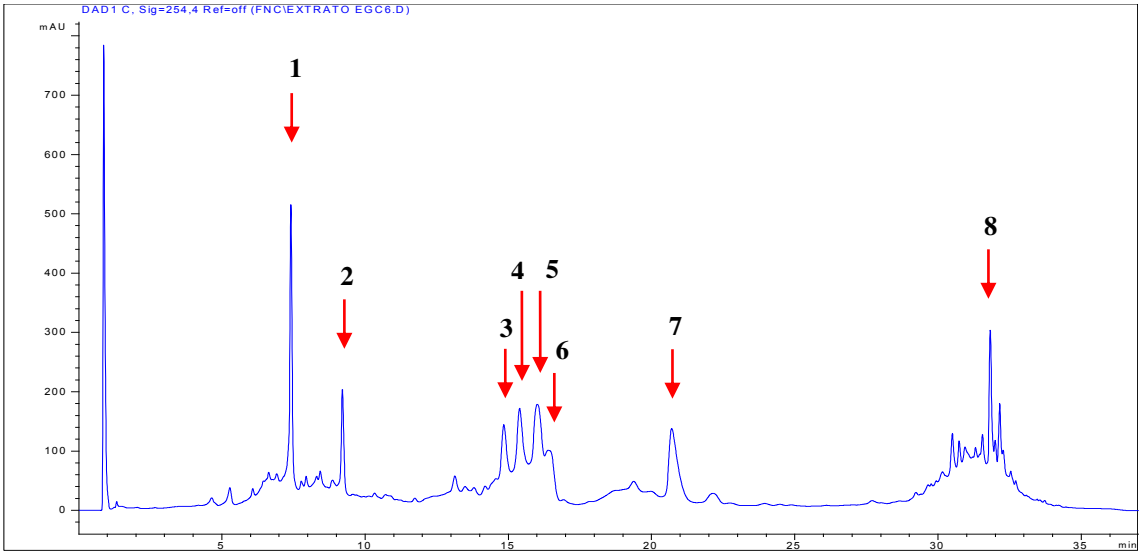

Figure 1. Chromatogram of the hydro-alcoholic stem extract of *Eryngium glaziovianum* showing the major compounds

Table 1. Major peaks observed in the HPLC chromatogram of the hydroalcoholic stems extract of *Eryngium glaziovianum*

Compound	RT[a] (min.)	PA[b] (%)	UV ($\lambda_{máx}$)	Compound Class
1	7.412	13.5378	328	Phenylpropanoid[22-24]
2	9.209	5.0031	270, 335	Flavonoid[21,22]
3	14.842	4.6541	328	Phenylpropanoid[22-24]
4	15.395	6.1137	255, 355	Flavonoid[21,22]
5	16.013	10.5929	255, 355	Flavonoid[21,22]
6	16.384	6.0359	255, 355	Flavonoid[21,22]
7	20.703	12.1470	328	Phenylpropanoid[22-24]
8	31.820	6.1659	315	Phenylpropanoid[22-24]

[a] RT= Retention time

[b] PA= Percentage of area

Phenolics are a large group of important naturally occurring bioactive compounds. Their isolation and purification from natural sources might be a hard work and time consuming when traditional phytochemical techniques are used[25]. In this context, CCC is observed as a technique that offers many advantages due to the lack of a solid support that avoids irreversible adsorption of polar compounds[26].

Choosing a suitable solvent system is an extremely important step for the separation of compounds by CCC, especially in the case of complex mixtures, such as plant extracts[8, 27]. Literature reports *n*-hexane-ethyl acetate-methanol-water (HEMWat) [28-31, 25] solvent system as the most suitable for isolation of flavonoid compounds. Then, this system was selected and tested for the separation of phenolic compounds in hydroalcoholic stems

extract of *Eryngium glaziovianum*. It is proposed to start testing solvent system at ratio 1: 1: 1: 1 (v/v) [9, 20, 32] and change polarity until it achieves partition coefficient (*K*) values close to 1.

This preliminary test showed that most of the compounds of the extract were concentrated in the aqueous stationary phase, when compared to the organic mobile phase. Therefore, solvent system polarity was increased by decreasing ratio of *n*-hexane in relation to ethyl acetate and increasing ratio of water in relation to methanol, maintaining phases proportions equal[24]. After testing 1:1:1:1, 2:6:2:6, 1:6:1:6 to 0.5:6:0.5:6, HEMWat solvent system at ratio 0.5:6:0.5:6 showed a suitable distribution of compounds between the phases, being selected to be used at the preparative separation for 550 mg of the extract by CCC.

TLC detection is the most common way to analyze CCC fractionation due to convenience [33-35] but, sometimes, compounds retardation factor (R_F) are too close from each other, not allowing accurate visualization of the spots, especially in the case of complex plant extracts. Instead of using analytical TLC, *off-line* injections of CCC fractions to HPLC-DAD were chosen to analyse the CCC fractionation. This technique allows a better visualization of the separation profile and it is also able to partially characterize the purified compounds according to their secondary metabolite class through analysis of their UV spectra.

Odd fractions from 11 to 100 were analyzed by HPLC-DAD and the 2D and 3D-graphs (**Fig. 2** and **Fig. 3** respectively) *percentage fraction (%) X fraction X retention time* were constructed to evaluate the separation profile of the existing compounds in the extract.

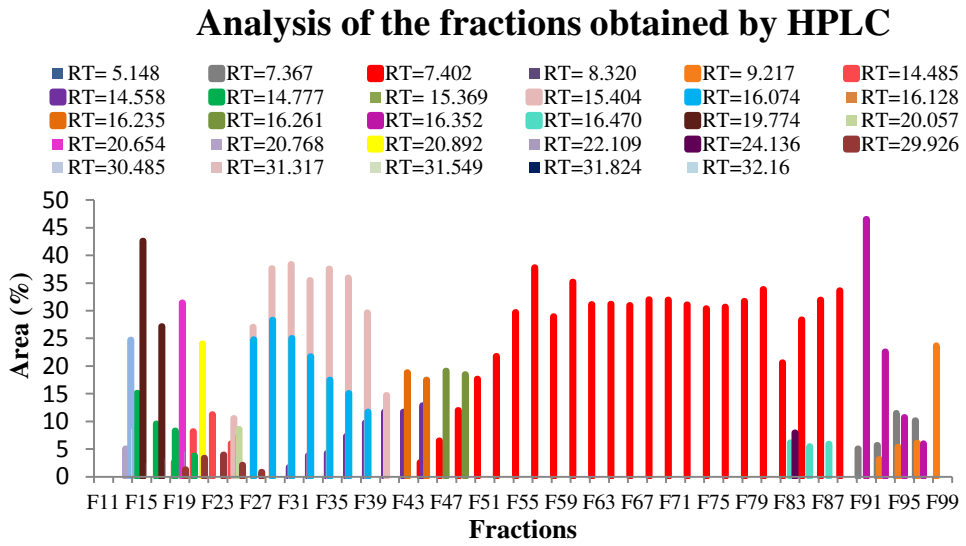

Figure 2. Graph *percentage of area (%) X fraction X retention time* plotted in 2D from analysis by HPLC of fractions 11 to 100 obtained by CCC

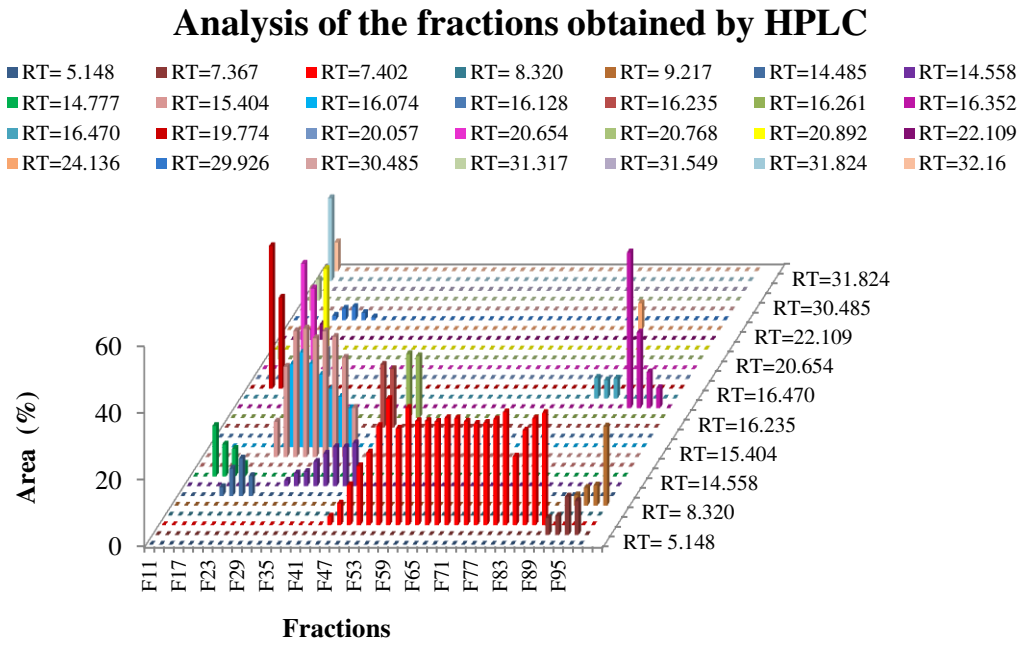

Figure 3. Graph *percentage of area (%) X fraction X retention time* plotted in 3D from analysis by HPLC of fractions 11 to 100 obtained by CCC. RT: Retention Time

Graph shows that the separation process was more efficient between fractions 27-81, where the effective separation of the compounds is observed and the "*window of opportunity*" of the selected solvent system is present - in CCC separations there is a high resolution polarity range, called the "*window of opportunity*" [36], which is directly related to the *K* presented by the substances of the mixture in a given solvent system. Fractions before 27 and after 81 stayed out of the "*window of opportunity*", showing a mixture of high concentrated compounds that needs further steps of purification.

It can be observed in 2D and 3D-graphs that fraction 19 presented a major compound whose UV spectra analysis led to the hypothesis of it as being a phenylpropanoid derivative [22-24] (**Fig. 4**). It also might correspond to compound **7** in the crude extract HPLC preliminary analysis (**Table 1**). Fractions 27 to 39 exhibited a mixture of three different phenolic compounds, from which the two major compounds showed an UV spectra characteristic of flavonol derivatives [21] (**Fig. 4**), corresponding to compounds **4** and **5** in the crude extract (**Table 1**). Fractions 51 to 81 presented one compound with UV spectra characteristic of another phenylpropanoid derivative (**Fig. 4**). It corresponded to compound **1** in the crude material (**Table 1**). CCC experiment led to the isolation of four compounds (**Fig. 5**) out of the eight ones described in **Table 1**, which were fully identified by ^{1}H-NMR, ^{13}C-NMR and comparison to literature[37-39]. Compounds **1**, **4** and **5** were also analyzed by ESI-MS/MS to confirm their chemical structure elucidation.

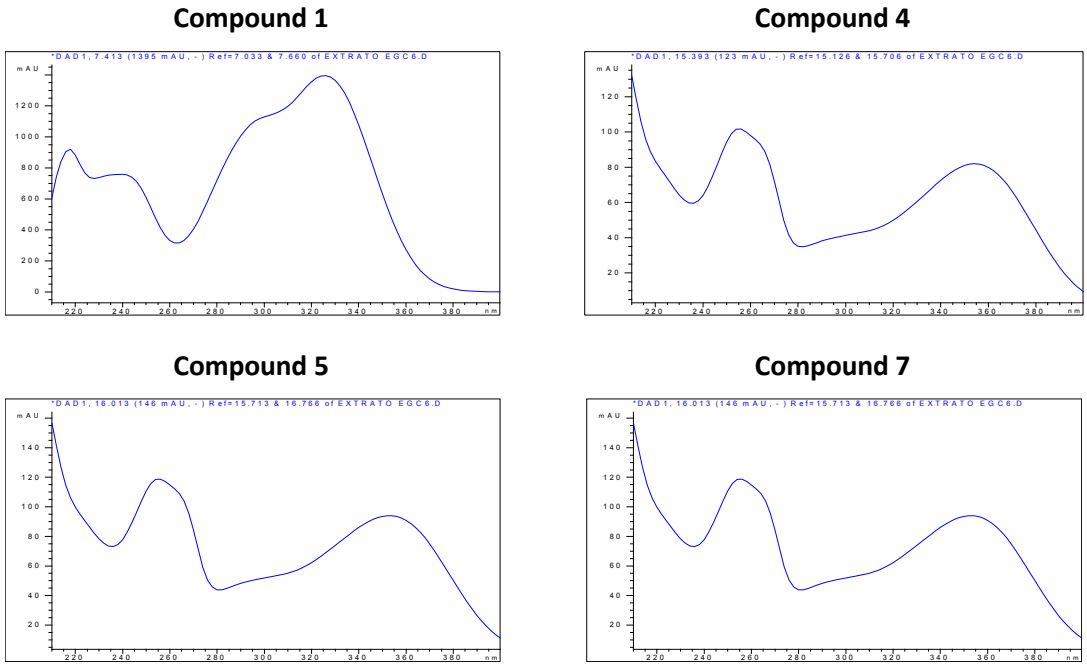

Figure 4. U V spectra of compounds isolated from the hydroalcoholic extract of Eryngium glaziovianum stem. Compound 1 was identified as dicaffeoylquinic acid. Compounds 4 and 5 were identified as a mixture of quercetin 3-O-β-D-galactopyranoside and quercetin 3-O-β-D-glucopyranoside. Compound 7 was identified as chlorogenic acid

Compounds **4** and **5** were identified as a mixture of quercetin 3-*O*-β-D-galactopyranoside and quercetin 3-*O*-β-D-glucopyranoside. Compound 7 was identified as chlorogenic acid.

Compound 1*(dicaffeoylquinic acid):* ^{1}H-NMR (500 MHz, MeOH, TMS) *δ (*ppm): 3.10 (1 H, dd, *J* = 3.4 and 14.5 Hz, H-2ax/eq), 5.11 (1 H, dd, *J* = 3.1 and 9.2 Hz, H-4), 2.94 (1 H, dd, *J* = 9.4 and 14.4 Hz, H-6ax/eq), 7.03 (1 H, d, *J* = 2.0 Hz, H-2'), 6.76 (1 H, d, *J* = 8,0 Hz, H-5'), 6.93 (1 H, dd, *J* = 1.9 and 8.2 Hz, H-6'), 7.51 (1 H, d, *J* =15.9 Hz, H-7'), 6.27 (1 H, d, *J* = 15.9 Hz, H-8'), 6.76 (1 H, d, *J* = 2.0 Hz, H-2''), 6.67 (1 H, d, *J* = 8.0 Hz, H-5''), 6.62 (1 H, dd, *J* = 1.9 and 8.1 Hz, H-6''), 7.51 (1 H, d, *J* =15.9 Hz, H-7''), 6.27 (1 H, d, *J* = 15.9 Hz, H-8''). The results were in agreement with the literature. [37] ESI–MS: *m/z* 515.25 [M-H]$^{-}$. EM/EM (*m/z* 515.25): *m/z* 353; 335; 299; 255; 203; 179 and 173. Fragmentation profile suggests the presence of the 3,4-dicaffeoylquinic acid, as proposed by Clifford *et al.* [40] and intensity of the fragments observed in the MS/MS spectra allows the distinction between different isomers of dicaffeoylquinic acid.

Mixture of compounds **4** and **5** (*quercetin 3-O-β-D-galactopyranoside)*: ^{1}H-NMR (500 MHz, MeOH, TMS) *δ* (ppm): 6.20 (2 H, s, H-6), 6.40 (2 H, s, H-8), 7.84 (1 H, d, *J* = 1.9 Hz, H-2'), 6.86 (2 H, d, *J* = 8.5Hz, H-5'), 7.58 (2 H, dd, *J* = 1.9 and 8.5 Hz, H-6'), 5.16 (1 H, d, *J* = 7.8 Hz, H-1"), 3.81 (m, H-2"), 3.54 (m, H-3"), 3.84 (m, H-4"), 3.47 (m, H-5"), 3.56 (dd, *J* = 2.6 and 6.6 Hz, H-6a"/6b"), 3.64 (dd, *J* = 6.0 and 11.2 Hz, H-6a"/6b"). ^{13}C-NMR (125 MHz, MeOH, TMS) *δ* (ppm): 158.77 (C–2), 135.76 (C–3), 179.53 (C–4), 163.01 (C–5), 99.89 (C–6), 166.07 (C–7), 94.70 (C–8), 158.44 (C–9), 105.62 (C–10), 123.19 (C–1') 117.77 (C–2'), 145.81 (C–3'), 149.95 (C–4'), 116.08 (C–5'), 122.92 (C–6'), 105.38 (C–1"), 73.17 (C–2"), 75.08 (C–3"), 70.02 (C–4"), 77.18 (C–5"), 61.93 (C–6"). *(quercetin 3-O-β-D-glucopyranoside)*: ^{1}H-NMR (500 MHz, MeOH, TMS) *δ* (ppm): 6.20 (2 H, s, H-6), 6.40 (2 H, s,

H-8), 7.84 (1 H, d, *J* = 1.9 Hz, H-2'), 6.86 (2 H, d, *J* = 8.5Hz, H-5'), 7.58 (2 H, dd, *J* = 1.9 and 8.5 Hz, H-6'), 5.25 (1 H, d, *J* = 7.6 Hz, H-1"), 3.48 (m, H-2"), 3.21 (m, H-3"), 3.33 (m, H-4"), 3.41 (m, H-5"), 3.71 (dd, *J* = 2.3 and 12.0 Hz, H-6a"/6b"), 3.92 (m, H-6a"/6b"). ^{13}C-NMR (125 MHz, MeOH, TMS) *δ* (ppm): 158.74 (C–2), 135.61 (C–3), 179.50 (C–4), 163.06 (C–5), 99.89 (C–6), 166.03 (C–7), 94.70 (C–8), 158.46 (C–9), 105.69 (C–10), 123.06 (C–1') 117.54 (C–2'), 145.92 (C–3'), 149.86 (C–4'), 116.00 (C–5'), 122.85 (C–6'), 104.27 (C–1"), 75.72 (C–2"), 78.11 (C–3"), 71.20 (C–4"), 77.84 (C–5"), 62.53 (C–6").) The results were shown in agreement with the literature. [38] ESI-MS: *m/z* 463.28 $[M-H]^-$. MS/MS (*m/z* 463.28): *m/z* 301.02. Two-dimensional NMR analyzes also contributed to confirm the chemical displacements attributed to the carbons and hydrogens of the structures.

Compound **7** *(chlorogenic acid)*: ^{1}H-NMR (500 MHz, MeOH, TMS) *δ* (ppm): 2.25 (1 H, m, H-2ax/eq), 1.58 (1 H, m, H-2ax/eq), 4.12 (1 H, m, H-3), 3.67 (1 H, dd, *J* = 2.3 and 9.0 Hz, H-4), 5.37 (1 H, m, H-5), 2.34 (1 H, m, H-6ax/eq), 1.60 (1 H, m, H-6ax/eq), 7.03 (1 H, d, *J* = 1.8 Hz, H-2'), 6.76 (1 H, d, *J* = 8.2 Hz, H-5'), 6.93 (1 H, dd, *J* =1.8 and 8.2 Hz, H-6'), 7.55 (1 H, d, *J* = 15.8 Hz, H-7'), 6.28 (1 H, d, *J* = 15.8 Hz, H-8'). ^{13}C-NMR (125 MHz, MeOH, TMS) *δ* (ppm): 38.57 (C–2), 72.58 (C–3), 74.53 (C–4), 72.26 (C–5), 39.98 (C–6), 177.30 (C–7), 127.58 (C–1'), 114.78 (C–2'), 146.53 (C–3'), 149.52 (C–4') 116.18 (C–5'), 122,61 (C–6'), 146.50 (C–7'), 115.23 (C–8'), 168.85 (C–9'). The results were in agreement with the literature. [39] Two-dimensional NMR analyzes also contributed to confirm the chemical shifts attributed to the carbons and hydrogens of the structure.

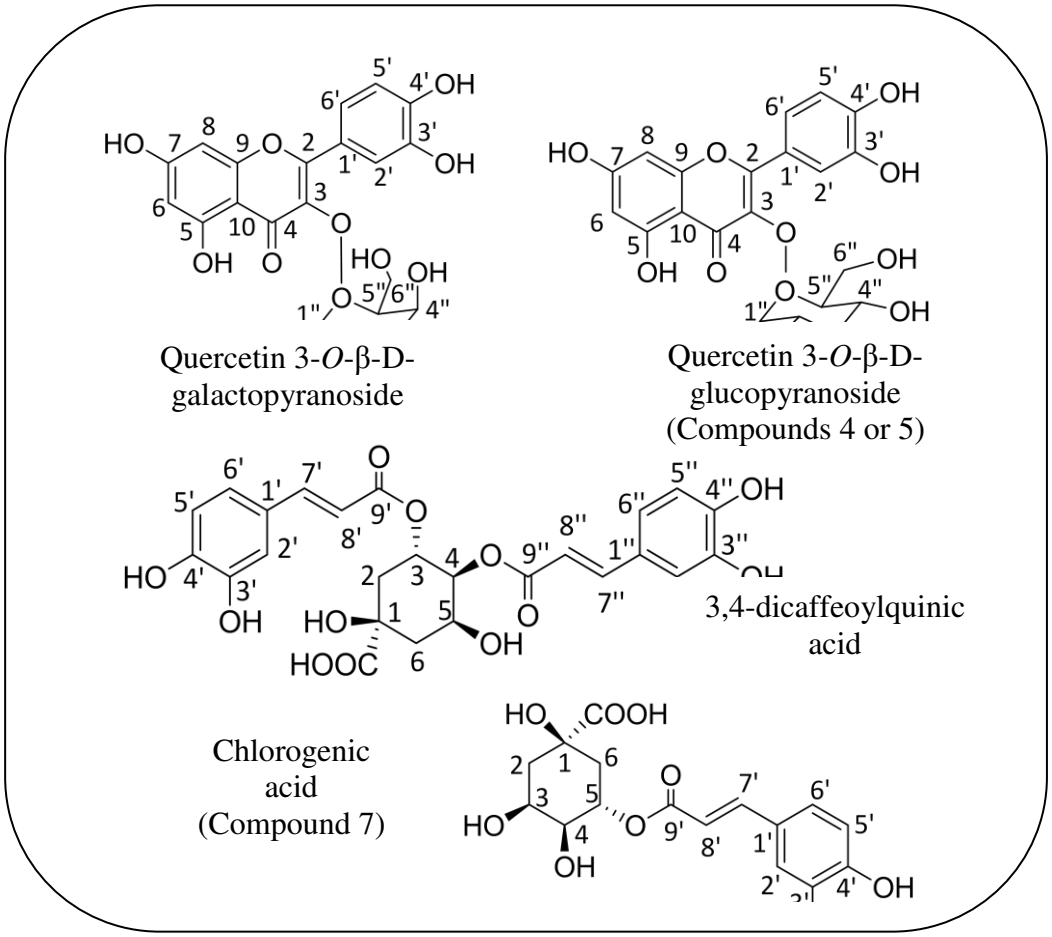

Figure 5. Chemical structure of the phenolic compounds isolated from the hydroalcoholic extract of *Eryngium glaziovianum*

4. Conclusion

The use of CCC allowed the isolation of four phenolic compounds from the hydroalcoholic stems extract of *Eryngium glaziovianum* in a single separation step was possible with no irreversible adsorption of polar compounds. *Off-line* coupling of preparative CCC with analytical HPLC-DAD allowed accurate analysis of each fraction, enabling a preliminary structure characterization and assisting the phytochemical study. From the eight major compounds detected in the crude extract, compound **1** was identified as 3,4-dicaffeoylquinic acid, compounds **4** and **5** were isolated in a mixture and identified with quercetin 3-*O*-β-D-galactopyranoside and quercetin 3-*O*-β-D-glucopyranoside, and compound **7** was identified as chlorogenic acid. This work study brought an important contribution to the phytochemical knowledge of the species *E. glaziovianum*, as there is only a single study on literature[3], so far. This knowledge enables the development of further metabolomic and chemosystematic researchs. The isolated compounds are already known to be present in some plants of the genus *Eryngium* [41-45], but have never been described in *E. glaziovianum.*

Acknowledgements

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