Research article

Secondary metabolites with ecologic and medicinal implications in *Anthemis cretica* subsp. *petraea* from Majella National Park

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Abstract: *Anthemis cretica* subsp. *petraea* (Ten.) Greuter is a plant belonging to the Asteraceae family and endemic of central Italy. In this paper, the first analysis of the ethanolic fraction of samples collected in the Majella National Park is reported. Seven compounds were isolated and identified namely **parthenolide** (**1**), **9α-acetoxyparthenolide** (**2**), **tamarixetin** (**3**), **7-hydroxycoumarin** (**4**), **4'-hydroxyacetophenone** (**5**), **leucanthemitol (conduritol F)** (**6**), and *proto*-**quercitol** (**7**). Isolation of the compounds was achieved by means of column chromatography (CC), while their identification was achieved through spectroscopic and spectrometric techniques. The presence of these compounds is of great relevance. Compounds **1** and **2** are chemosystematic markers of the family, thus confirming the correct botanical classification of the species. Conversely, compounds **3**, **5,** and **7** were identified for the first time in the species and, instead, confirm the tendency of endemic entities to develop characteristic metabolite patterns in respect to cosmopolite species. Moreover, the presence of compounds **6** and **7** has ecologic implications and may be linked to this taxon's adaption to dry environments. The production of these osmolytes may, in fact, represent the reason why this species is able to survive in extreme conditions of aridity. Lastly, from a medicinal standpoint, the isolated compounds are endowed with interesting biological activities and may justify, on a molecular base, the widespread traditional uses of the *Anthemis* species, as well as a basis for the use of the subspecies *petraea*.

Keywords: adaptation; secondary metabolites; diterpenoids; flavonoids; cyclitols; abiotic stress; traditional uses; chemotaxonomy

1. Introduction

Plants belonging to the *Anthemis* genus are well known to have several biological activities and are widely used in folk medicine. In fact, they are able not only to treat gastrointestinal disorders, haemorrhoids, cough, stomach aches, and liver failure [1-4] but are also able to soothe pains and irritations and to clean wounds [5]. Additionally, they are also utilized as herb teas, in cosmetics, and in the pharmaceutical industry [6].

In literature, there are several works regarding the *Anthemis* genus, but they focus mainly on essential oil composition [7,8] and the non-volatile components, thus confirming the presence of terpenoids and flavonoids [4,9]. Few literary works are focused on the species *cretica* showing the presence of coumarins and terpenoids [10,11].

Anthemis cretica subsp. *petraea* is an aromatic plant that was described by Tenore [12] as an autonomous species. It is endemic of the Central Apennines, especially of the Abruzzo region [13], where it is scarcely present and reported only on Majella, Morrone and Gran Sasso mountains [14]. The present research group has a series of ongoing projects for the phytochemical study of endemic and uncommon species growing in the Majella National Park, a region considered to be a hot spot of biodiversity. In previous years, several species belonging to Lamiaceae [15], Plantaginaceae [16], and Asteraceae [17,18] families have been examined and, in all cases, peculiar metabolite patterns were evidenced.

Another reason justifying this study rests on the fact that there are no previous studies recorded concerning the phytochemistry of this subspecies. Therefore, in this paper, we report the first analysis of the secondary metabolites produced by this subspecies.

2. Materials and Method

2.1. Botanical Description and Plant material

Anthemis cretica subsp. *petraea* (Ten.) Greuter is a perennial herbaceous species belonging to the Anthemideae tribe of the Asteraceae family. It is also known as *Anthemis montana* subsp. *petraea* (Ten.) Briq. & Cavill. or with the Italian name of "Camomilla Montana" (Mountain Chamomile).

The name of the species derives from the union of the Greek words "ανθεμον" (ánthemon), meaning "flower," and "Κρήτη" (Krētē), meaning "coming from the island of Crete." Furthermore, the name of the subspecies derives from the Latin "*petraea*" meaning "scree", highlighting the fact that it grows in stony habitats. This is one of the several *A. cretica* subspecies present in Italy [14].

The plant is glabrous with several ascending stems, scarcely branched, and widely naked upward. The leaves are 2-pinnatifid, fleshy, and dotted with glands. The capitula are 3 cm large, with black-bordered involucral bracts; the receptacle has scales, with the outer 3-teethed or lacerated on the top. The achens are generally smooth, with a short oblique crown. It grows on stony pastures between 1100 and 2600 m a.s.l. and blooms from June to August.

A sample of *A. cretica* subsp. *petraea* was collected in the territory near the town of S. Eufemia a Majella inside the Majella National Park in the Abruzzo Region (Central Italy; GPS coordinates: 42°11'65" N; 14°07'82" E). The sample was identified by the botanists of the Park (Dr. Mirella Di

Cecco and Dr. Giampiero Ciaschetti) through comparison with available literature [12-14]. The plant was dried in a desiccator at room temperature after harvesting. A voucher specimen of the studied plant is stored for further reference and registered under the accession number AC19062013.

2.2. Chemicals

The following chemicals were utilized: 96% ethanol, *n*-butanol, distilled water, chloroform, methanol, *n*-hexane, ethyl-acetate, silica gel (granulometry 40–63 µm), and deuterated solvents such as D_2O, CD_3OD, and $CDCl_3$. All the solvents had RPE purity grade, if not differently specified, and were purchased from Sigma Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy), while silica gel 60 (70-230 mesh ASTM) was purchased from Fluka Analytical.

2.3. Instruments

NMR spectra were recorded on a Varian Mercury 300 MHz instrument and/or on a Bruker Avance III 400 MHz instrument. The chemical shifts were expressed in ppm from TMS for spectra in $CDCl_3$; the signal of HDO (*s*) at 4.78 ppm was used as the reference for spectra in D_2O, while the internal solvent signal (*m*5) at 3.31 ppm was the reference for spectra recorded in CD_3OD.

Mass spectrometry (MS) spectra were performed on a Q-TOF MICRO spectrometer (Waters; Manchester, UK) equipped with an ESI source operating in the negative or positive ion mode. All samples were solubilized in methanol having RS purity grade before injection. The flow rate of the sample infusion was 10 µL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

2.4. Extraction of polar compounds

Dried plant material, consisting of 85.9 g of exsiccated aerial parts, was subjected to three consecutive extractions with 96% ethanol (300 mL each, 48 h of infusion) for an exhaustive extraction of the plant material. The extracts were collected together and then filtered. The ethanol was eliminated at reduced pressure until a water suspension was obtained. Throughout the previously described step, the pH of the solution was measured and emerged a value near of pH 8. This check was necessary to verify that pH was not acidic (meaning not under the value of 5.5) because extreme acidity in the solution may cause secondary unwanted reactions on the constituents, such as hydrolysis of glycoside or ester groups. The water suspension was then frozen at −20 °C and lyophilized at the same temperature to preserve temperature-sensitive compounds which could might have been present. The dried crude extract had a final weight of 10.2 g.

2.5. Isolation and identification of the secondary metabolites

A 2.1 g aliquot of the crude extract was separated using a silica gel (60.0 g) chromatographic column using *n*-butanol saturated with distilled water (82:18, v/v) as eluting system. From this step, the following compounds were identified: **parthenolide** (**1**) (Fr.6A) (73.3 mg) [19] and **leucanthemitol** (**conduritol F**) (**6**) (Fr.28-30A) (22.9 mg) [20].

The grouping of fractions from 3 to 9A, with a weight of 522.1 mg, was further separated on

silica gel (16.0 g) using an eluting system of $CHCl_3$/MeOH, starting from the concentration of 98:2 v/v. During the chromatographic run, polarity was gradually raised to a solvent concentration of 95:5 v/v. From this separation step, the following compounds were identified: **9α-acetoxyparthenolide** (**2**) [21,22] and **parthenolide** (**1**) in mixture (2:1) (Fr.7-8B) (125.6 mg), **parthenolide** (**1**) (Fr.11B) (15.6 mg), and **tamarixetin** (**3**) (3.2 mg) (Fr.52-53B) [23].

Also, the grouping of fractions from 63 to 73A, with the weight of 288.9 mg, were further purified on silica gel CC (10.0 g) and eluted with $CHCl_3$/MeOH. The elution system concentration was initially 8:2 v/v but was modified by gradually raising the polarity to 7:3 v/v during the chromatographic run. Fractions 58-61C (34.3 mg) reported the presence of ***proto*-quercitol** (**7**) [24].

The fractions 9-10B (89.2 mg) from the second chromatographic run were separated again on silica gel CC (3.1 g) but a mixture of *n*-hexane/ethyl-acetate was used as eluting system instead. The initial concentration of the two solvents were 8:2 v/v, but was increased 6:4 v/v. From this procedure, the compounds **4'-hydroxyacetophenone** (**5**) (Fr.12-15D) (1.2 mg) [25] and **7-hydroxycoumarin** (**4**) [26] (Fr.20-24D) (2.6 mg) were isolated and identified within a mixture of other compounds.

The assignment of the structures was performed through consideration of the signals in the obtained spectra and through comparison with literature data. In the fraction 6A, several proton signals lead the researchers to hypothesize the presence of a terpenoid compound. Four diagnostic signals were of interest: the two doublets present at δ 6.29 (J = 3.6) and 5.60 (J = 3.6), which were clear signs of a esocyclic terminal alkene; the broad doublet at 5.18 (J = 10.7), typical of the presence of an olefinic proton near a methylene group; the signal at 3.84 ppm revealing the presence of a proton geminal to an oxygenated substituent and, per its multiplicity (t, J = 8.6), also adjacent to two methine groups.

Other signals of interest included two singlets, each integrating for three protons. The first was at 1.68 ppm and revealed the presence of a methyl group linked to a double bond. In contrast, the second was at 1.27 ppm and was deshielded by the presence of an electronegative nucleus, such as oxygen. The ^{13}C spectrum revealed a set of twenty peaks, which clearly evidenced the presence of a diterpene derivative: the signal at 169.45 ppm was typical of a carboxylic group; the signals at 139.26, 134.67, 125.29, 121.40 ppm resulted from two double bonds; lastly, the signals at 82.55, 66.44 and 61.72 ppm marked the presence of three oxygenated carbons and the most deshielded carbon (82.55 ppm) was also indication for the presence of an ester.

In the end, the experimental data was found to be in accordance with those available in literature for the compound parthenolide (**1**). This was also confirmed through recognition of the compound's pseudomolecular ions with mass spectroscopy. These ions were three in total: the first was a monomolecular adduct with sodium (*m/z* 271.25), the second was a monomolecular adduct with potassium (*m/z* 287.28), and the last was a bimolecular adduct with sodium (*m/z* 519.52).

The signals observed in the fractions 7-8B, were due to the presence of two compounds in mixture. One of them represented the minor component and showed the same peaks of parthenolide (**1**). Due to this, the signals were not taken into account.

The remaining signals gave evidence for the presence of some interesting functional groups as well. There was a terminal double bond recognized by the two coupled peaks at 6.26 and 5.64 ppm having the same multiplicity (d, J = 3.0). There was also an olefin proton due to the presence of a peak at 5.56 ppm. Moreover, a signal at 5.19 ppm (dd, J = 5.2, 2.0) and two other singlets at 1.83 and 1.39 ppm, both integrating for three protons, were present. Lastly, a singlet integrating for three protons were present at 2.07 ppm, gave indication of the presence of an acetyl group.

From the carbon spectrum, two sets of signals were seen. The first set referred to the minor component (parthenolide (**1**)) and were not considered again. The remaining 22 signal peaks indicated the presence of an acetylated diterpenic compound. This compound had two carboxyl groups (169.9 and 169.4 ppm), four olefin carbons (139.2, 134.6, 124.0 and 121.3 ppm), and two secondary alcoholic carbons in the ester form. The first ester was confirmed by the presence of the signal at 82.5 ppm, while the second ester was confirmed by the presence of the signal at 72.9 ppm. The peaks at 66.4 and 61.6 ppm led to consideration of an oxirane moiety.

In conclusion, the obtained spectroscopic data of this compound were similar to those of compound (**1**). The main difference was due to the presence of the acetyl group. Further comparison with literature data and observation of its pseudomolecular ion (*m/z* 329.13) as an adduct with sodium through mass spectrometry confirmed the presence of compound (**2**).

The presence of tamarixetin (**3**) in fractions 52-53B was first hypothesized by the observation of several NMR signals compatible with the structure of a flavonoidic aglycone. In particular, these signals included the broad doublet at 7.39 ppm (br d, *J* = 8.0), the doublet at 6.91 ppm (d, *J* = 8.0), and the broad singlet at 7.38 ppm. Each signal integrated for one proton and was clear indication for the presence of one 1,3,4-tri-substituted benzene, which is the form of the tri-substituted B-ring of a flavonoid. Further evidence for the presence of the flavonoidic aglycone came after viewing the other signals present in the spectrum. The signal at 6.56 ppm (possibly two overlapped signals) integrated for two protons that were assigned as protons in the positions 6 and 8 of the A-ring. They were shielded because of their contemporaneous *ortho/para* orientation towards the oxygenated substituents present on the A-ring in the positions 5, 7, and 9. The singlet, which integrated for three protons at 3.88 ppm, is typical of the presence of a methoxy substituent and, per its delta value, was linked in 4' position. In comparison, one methoxy group linked in the positions of 3, 3', 5, or 7 would have created a peak more deshielded than 3.90 ppm. The pseudomolecular ion of (**3**) was recognized through mass spectrometry experiments in both negative (*m/z* 315.16) and positive ionization modes (*m/z* 339.16 and *m/z* 355.19) as adducts with sodium and potassium, respectively.

In the ^{1}H NMR spectrum of fractions 12-15D, a doublet at 7.63 ppm (*J* = 9.5 Hz) was identified coupled to a doublet at 6.25 ppm (*J* = 9.5 Hz), both of which are typical of olefin protons in β and α positions, respectively, to a carbonyl group. In practice, these two signals corresponded to the protons in the position 4 and 3, respectively. The signal of the former peak was more deshielded due to the presence of the carbonyl moiety and by resonance effects. A set of three signals were also present at 7.35 ppm (d, *J* = 8.4), 6.81 ppm (d, *J* = 2.4) and 6.78 ppm (dd, *J* = 8.4, 2.4), with coupling amongst the three. This revealed the presence of one 1,2,4-tri-substituted benzene ring. Among these signals, those which presented a chemical shift lower than 7.0 ppm, were directly influenced by the presence of oxygen substituents in the *ortho* and *para* positions. In contrast, the one peak which was more deshielded was not influenced by oxygen substituents and was likely to be in the *meta* position in respect to these oxygenated functions. These data were compatible with those of one hydroxy-substituted coumarin and were also in accordance with literature data. The pseudomolecular ion corresponding to 7-hydroxycoumarin (**4**) was recognized in the mass spectrum at *m/z* 161.08 in the negative ionization mode. Moreover, this hypothesis was also confirmed by the presence of both the adducts with proton and sodium in the positive ionization mode which are at *m/z* 163.06 and *m/z* 185.05, respectively.

From the grouping of fractions 12-15D, 4'-hydroxy-acetophenone (**5**) was identified by the presence of doublets at 7.90 ppm (d, *J* = 8.8) and 6.87 ppm (d, *J* = 8.8); both integrated for two

protons. This situation is typical of a *para*-substituted benzene ring showing an A2B2 spin system. Also of significance was the singlet that integrated for three protons at 2.55 ppm. This singlet was assigned to a methyl group linked to a carbonyl carbon. Also in this case, the *para* substituent was likely a hydroxyl group due to the evident shielding effect on the adjacent *ortho*-orientated protons. The pseudomolecular ion corresponding to the hypothesized structure of 4'-hydroxyacetophenone (**5**) was recognized in the negative ionization mode at the value of *m/z* 135.09.

The proton spectrum of the fractions 28-30A showed two signals compatible with the presence of olefin protons at δ 5.90 and 5.81. In addition to this, signals which could be assigned to protons in geminal with hydroxyl substituents were present. These signals were at 4.30, 4.10, 3.64 and 3.62 ppm. This supposition was confirmed by the observed chemical shifts recorded on the carbon spectrum: sp^2 carbons peaks were at δ 132.27 and 126.60 and secondary alcoholic carbon peaks were at 72.32, 72.27, 70.66 and 66.52 ppm.

The study of the observed coupling constants recorded in the proton spectrum and the heteronuclear correlations visualized by two-dimensional experiments (see the supplementary materials) gave final evidence for the presence of a cycloexene tetraol structure. This structure was further confirmed after recognition of the pseudomolecular ions in mass spectrometry in both the negative (*m/z* 144.97) and positive (*m/z* 168.91) ionization modes. Literature survey permitted the assignment of the name leucanthemitol (conduritol F) to compound (**6**).

The assembly of fractions 58-61C revealed a proton spectrum very similar to that of compound (**6**). The main differences were the absence of olefin protons and the presence of an AB spin system due to geminal protons of aliphatic nature. These peaks were clearly seen at 2.14–2.02 ppm (1H, m) and 1.47 ppm (1H, ddd, *J* = 14.1, 11.9, 2.5). The other signals at 3.98, 3.82–3.71, 3.56, 3.37 and 3.17 ppm were also compatible with protons belonging to secondary alcoholic carbons. This was collaborated on the carbon spectrum with the presence of signals at δ 79.30, 75.68, 74.32, 69.81, and 69.67. The presence of the sp^3 carbon was identified by the peak at 37.07 ppm. In the two-dimensional NMR experiment, this last signal showed a contemporaneous correlation with the proton signals both at 2.14–2.02 and 1.47 ppm, thus confirming the presence of the AB spin system.

In this case, the study of the coupling constants, two-dimensional NMR spectra, and literature data aided in the naming of the compound as *proto*-quercitol (**7**). Additional confirmation was obtained by the mass spectrum. Here, the pseudomolecular ion was recognized as an adduct of the compound with sodium having *m/z* 187.07.

The following lists specific NMR and MS data associated with each experimental compound:

Parthenolide (1): ^{1}H-NMR (300 MHz, CDCl$_3$) δ: ^{1}H NMR (300 MHz, CDC$_3$) δ: 6.29 (1H, d, *J* = 3.6 Hz, H13a), 5.60 (1H, d, *J* = 3.6 Hz, H-13b), 5.18 (1H, br d, *J* = 10.7 Hz, H-1), 3.84 (1H, t, *J* = 8.6 Hz, H-6), 2.76 (2H, overlapped signals, H-5, H-7), 2.33 (1H, d, *J* = 13.0 Hz, H-2a), 2.16 (1H, d, *J* = 13.1 Hz, H-2b), 1.68 (3H, s, H-14), and 1.27 (3H, s, H-15); other proton signals overlapped.
^{13}C NMR (75 MHz, CDC$_3$) δ: 169.45 (C-12), 139.26 (C-11), 134.67 (C-10), 125.29 (C-1), 121.40 (C-13), 82.55 (C-6), 66.44 (C-5), 61.72 (C-4), 47.68 (C-7), 41.24 (C-9), 36.38 (C-3), 30.68 (C-8), 24.19 (C-2), 17.33 (C-15), and 17.01 (C-14).
ESI-MS: *m/z* 271.25 [M+Na]$^+$; *m/z* 287.28 [M+K]$^+$; *m/z* 519.52 [2M+Na]$^+$.

9α-acetoxyparthenolide (**2**): ^{1}H-NMR (300 MHz, CDCl$_3$) δ: 6.26 (1H, d, *J* = 3.0 Hz, H-13a), 5.64 (1H, d, *J* = 3.0 Hz, H-13b), 5.56 (1H, m, H-1), 5.19 (1H, dd, *J* = 5.2, 2.0 Hz, H-9), 3.82 (1H, dd, *J* =

8.6, 8.4 Hz H-6), 2.79–2.73 (2H, m, H-5, H-7), 2.07 (3H, s, (CH$_3$CO), 1.83 (3H, br s, H-14), and 1.39 (3H, s, H-15); other proton signals overlapped.

^{13}C NMR (75 MHz, CDCl$_3$) δ:169.9 (CH$_3$_CO_), 169.4 (C.12), 139.2 (C-11), 134.6 (C-10), 124.0 (C-1), 121.3 (C-13), 82.5 (C-6), 72.9 (C-9), 66.4 (C-5), 61.6 (C-4), 40.3 (C-7), 36.3 (C-8), 34.0 (C-3), 23.0 (C-2), 21.2 (C-2'), 17.3 (C-15), and 16.6 (C-14).

ESI-MS: *m/z* 329.13 [M+Na]$^+$.

Tamarixetin (**3**): ^{1}H NMR (400 MHz, MeOD) δ: 7.39 (1H, br d, *J* = 8.0 Hz, H-6'), 7.38 (1H, br s, H-2'), 6.91 (1H, d, *J* = 8.0 Hz, H-5'), 6.56 (2H, overlapped signals, H-6; H-8), and 3.88 (3H, s, 4'-O_CH$_3$_).

ESI-MS: *m/z* 315.16 [M-H]$^-$; *m/z* 339.16 [M+Na]$^+$; *m/z* 355.19 [M+K]$^+$.

7-hydroxycoumarin (**4**):^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.63 (1H, d, *J* = 9.5 Hz, H-4), 7.35 (1H, d, *J* = 8.4 Hz, H-5), 6.81 (1H, d, *J* = 2.4 Hz, H-8), 6.78 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), and 6.25 (1H, d, *J* = 9.5 Hz, H-3).

ESI-MS: *m/z* 161.08 [M-H]$^-$; *m/z* 163.06 [M+H]$^+$; *m/z* 185.05 [M+Na]$^+$.

4'-hydroxyacetophenone (**5**): ^{1}H NMR (400 MHz, CDCl$_3$) δ:7.90 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 6.87 (2H, d, *J* = 8.8 Hz, H-3'/H-5'), and 2.55 (3H, s, CH$_3$CO).

ESI-MS: *m/z* 135.09 [M-H]$^-$.

Leucanthemitol (**6**): ^{1}H NMR (400 MHz, D$_2$O) δ: 5.90 (1H, ddd, *J* = 10.0, 5.0, 2.0 Hz, H-5), 5.81 (1H, dd, *J* = 10.0, 2.0 Hz, H-6), 4.30 (1H, dd, *J* = 6.5, 2.0 Hz, H-1), 4.10 (1H, dd, *J* = 5.0, 3.9 Hz, H-4), 3.64 (1H, d, *J* = 6.5 Hz, H-2), and 3.62 (1H, d, *J* = 3.9 Hz, H-3).

^{13}C NMR (100 MHz, D$_2$O) δ: 132.27 (C-5), 126.60 (C-6), 72.32 (C-3), 72.27 (C-4), 70.66 (C-2), and 66.52 (C-1).

ESI-MS: *m/z* 168.91 [M+Na]$^+$; *m/z* 144.97 [M-H]$^-$.

***Proto*-quercitol** (**7**): ^{1}H NMR (400 MHz, CD$_3$OD) δ: 3.98 (1H, dd, *J* = 5.9, 3.0 Hz, H-5), 3.82–3.71 (1H, m, H-1), 3.56 (1H, br t, *J* = 9.4 Hz, H-2), 3.37 (1H, dd, *J* = 9.4, 3.1 Hz, H-4), 3.17 (1H, br t, *J* = 9.2 Hz, H-3), 2.14–2.02 (1H, m, H-6a), and 1.47 (1H, ddd, *J* = 14.1, 11.9, 2.5 Hz, H-6b).

^{13}C NMR (100 MHz, CD$_3$OD) δ: 79.30 (C-3), 75.68 (C-4), 74.32 (C-2), 69.81 (C-1), 69.67 (C-5), and 37.07 (C-6).

ESI-MS: *m/z* 187.07 [M+Na]$^+$.

3. Results

The study of the ethanolic extract, obtained from the aerial parts of *Anthemis cretica* subsp. *petraea*, led to the isolation and identification of seven compounds having chemotaxonomic, medicinal, and ecologic relevance. In particular, these were: **parthenolide** (**1**), **9α-acetoxyparthenolide** (**2**), **tamarixetin** (**3**), **7-hydroxycoumarin** (**4**), **4'-hydroxyacetophenone** (**5**), **leucanthemitol** (**conduritol F**) (**6**), and *proto*-quercitol (**7**) (Figure 1). These compounds belong to five major classes of natural organic compounds: **1** and **2** are sesquiterpene lactones, **3** is a flavonoid, **4** is a coumarin, **5** is a acetophenone derivative, and **6** and **7** are cyclitols.

Beside the chemosystematic markers of the Asteraceae family (**1** and **2**), many of the isolated compounds were recognized in this species for the first time during this study and/or found to be uncommon compounds in Asteraceae. The presence of the unusual compounds **3** and **5** are interesting from a systematic standpoint and may be related to the peculiar metabolic features developed by this endemic subspecies in contrast to the nominal species.

In fact, the presence of unusual and characteristic metabolites is often recognized in the molecular pattern of endemic species and could be related to the evolution and adaption of the endemic species to the colonized environment [27]. Just like many natural products, most of the isolated compounds are endowed with interesting biological activities and may also play an important role in the adaption of this species to its habitat.
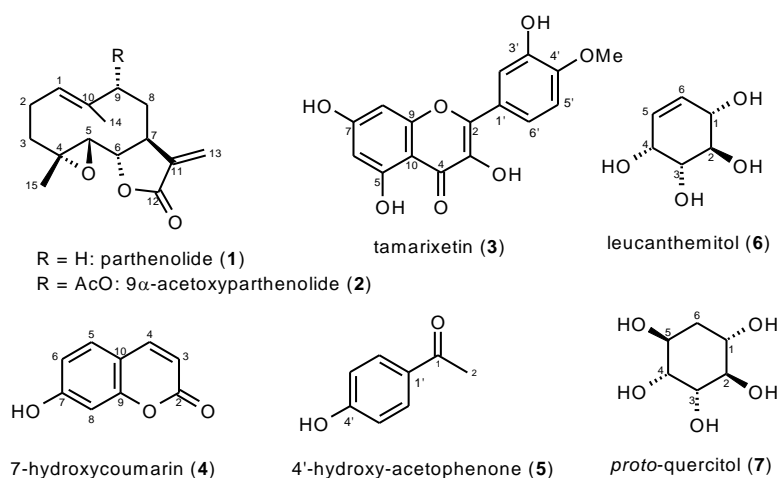

R = H: parthenolide (**1**)
R = AcO: 9α-acetoxyparthenolide (**2**)

tamarixetin (**3**)

leucanthemitol (**6**)

7-hydroxycoumarin (**4**)

4'-hydroxy-acetophenone (**5**)

proto-quercitol (**7**)

Figure 1. Structures of the compounds found in *Anthemis cretica* subsp. *petraea* (Ten.) Greuter.

4. Discussion

4.1. Chemotaxonomic implications of the isolated compounds

The presence of the sesquiterpene lactones (**1**, **2**) reported in the less polar fractions of the extract is important, mainly from a chemotaxonomic standpoint. In fact, diterpene lactones having a germacrane and/or guaiane skeleton are considered to be taxonomic markers of the genus *Anthemis* [28]. These kinds of compounds have already been identified in species belonging to the Sect. Hiorthia (DC.) R. Fernandes, where *A. cretica* is included [11,22].

In particular, **parthenolide** (**1**) and **9α-acetoxyparthenolide** (**2**) have been already recognized in *Anthemis cupaniana* [22], *Anthemis melampodina* [29], as well as in other Asteraceae species which are in close chemotaxonomic proximity, such as *Matricaria suffructicosa* var. *leptoloba* [21], *Tanacetum parthenium* (L.) Sch.Bip. [30], and *Parthenium integrifolium* L. [31]. This provides a chemotaxonomic rationale for the botanical classification of the studied species, as well as confirming its systematic proximity with related taxa of Asteraceae.

The presence of the flavonoid **3**, found in the middle polar fractions, is interesting for both chemosystematics and bioactivity. Considering the former, it may be mentioned that **tamarixetin** (**3**) has never been reported before as a constituent of *Anthemis* and represents another uncommon

compound isolated from the studied sample. To the best of our knowledge, the only Asteraceae taxa which showed the presence of the flavonoid **3** are a few species in the *Baccharis* genus [32] and *Eremanthus elaeagnus* Schultz-Bip [33].

7-hydroxycoumarin (**4**) has been already recognized in several species of the Asteraceae family, such as *Saussurea katochaete* [34], *Achillea millefolium* L. [35], and *Artemisia ramosa* Buch [36]. Furthermore, this family is well known to include species which are able to biosynthesize coumarins. In contrast, **4'-hydroxyacetophenone (5)**, also known by the trivial name of piceol, represents another uncommon secondary metabolite in the Asteraceae family since it has only recognized in only two species, i.e. *Farfugium japonicum* var. *formosanum* [37] and *Ligularia vellerea* (Franch.) Hand-Mazz [38].

From the polar fraction two interesting compounds were isolated: a cyclohexenetetraol, **leucanthemitol** (**conduritol F**) (**6**), previously recognized in *Chrysanthemum leucanthemum* L., *C. maximum* and *C. corimbosum* (Asteraceae) [39] and, more recently, in *Marsdenia tomentosa* (Apocynaceae) [40]. A cyclohexanepentol, called ***proto*-quercitol** (**7**), has never been reported before as a constituent of *Anthemis* spp.. From literature data, compound (**7**) has been recently detected in *Eucalyptus* spp. [41]. Considering the stereochemistry of the chiral centers from a biogenetic standpoint, these last two compounds seem to be related and one might represent the precursor of the other. The presence of **6** and **7** may also bear relevance when considering the physiology and the ecology of this species.

4.2. Ecologic implications of the isolated cyclitols

In a recent study on *Eucalyptus* species, it was hypothesized, and then confirmed that the presence of cyclitols in many species of this genus was linked to their evolution and particularly to their adaption to dry environments. In the study by Merchant and co-workers [41], it was shown in more detail that the presence of these cyclitols was directly linked to the species' adaptation to aridity. It was found that these compounds were bio-synthesized to face drought and excess salinity in the soil. Moreover, they were mainly evidenced in *Eucalyptus* species adapted to a dry environment. It also seems that the higher the soil salinity, the larger the quantity of cyclitols produced, particularly of the *proto*-quercitol kind [42]. The explanation of this aspect lies in the chemical and physical properties of these compounds. Both contribute a surprising osmotic ability which was tested and gave positive results [42]. In our opinion, the physiologic explanation of the presence of these compounds in *Anthemis cretica* subsp. *petraea* is very much in accordance with that observed in *Eucalyptus* spp. by Merchant and co-workers [41]. *A. cretica* subsp. *petraea*, is a species that grows in the rocky fields of mountainous environments, where slopes and soil texture do not allow for maintenance of adequate humidity in the substrate. Also, *A. cretica* subsp. *petraea* may have developed a similar molecular system, based on the presence of cyclitols, in order to adapt itself to these environmental conditions. Furthermore, cyclitols may aid in extracting water from the small amount of moisture present in the soil and to prevent water loss, due to their osmotic properties. ***Proto*-quercitol** (**7**) and **leucanthemitol** (**6**) may play a very important role in the survival of *A. cretica* subsp. *petraea* in the extreme environmental conditions of its colonized habitat.

Beside this, compounds **6** and **7** may also be involved in another interesting ecological aspect: interaction with pollinators. In fact, it was shown that the presence of cyclitols represent a positive stimulus to oviposition in the chestnut tiger butterfly (*Parantica sita* Kollar) [43]. Considering this, it is

quite likely that the recognized cyclitols have also a similar function in *A. cretica* subsp. *petraea*. In any case, other studies and a direct observation of this phenomenon are necessary to confirm this function.

4.3. Bioactivities of isolated compounds

Compound **1** is endowed with interesting bioactivities such as anti-cancerous [30,31,44,45], neuroprotective [46], anti-nociceptive, vasodilatory [47], and anti-inflammatory [48]. **Tamarixetin** (**3**) showed proapoptotic activity towards human leukemia cells [49], as well as an antiplasmodial action [50] and a modulating activity on steroidal hormone receptors (human androstane receptor [51] and pregnane X receptor [52]). Similar to other coumarins, compound **4** is endowed with antioxidant activity [53] and showed a protective action in dyslipidemia and cardiac hypertrophy in rats [54]. Moreover, it was responsible of the activation of caspase-3 in A549 cancer cells, leading to apoptosis [55].

4'-hydroxyacetophenone (**5**) was shown to have an anti-inflammatory activity in mice [56] with a potency comparable with those of phenylbutazone. Phenylbutazone is a well-known NSAID (nonsteroidal anti-inflammatory drug) no longer used in humans due to its severe adverse effects. On the contrary, it is still largely employed in veterinary field as an analgesic and antipyretic agent. Cyclitols are known to act as α-glucosidase inhibitors, particularly **leucanthemitol** (conduritol F) (**7**), which showed a potency five times higher than acarbose, a standard antidiabetic drug [20]. For this reason, there is increasing interest on polyols, which may be used as starting materials for the synthesis of new antidiabetics and insulin-release modulators [57].

5. Conclusion

In this first analysis of the endemic *A. cretica* subsp. *petraea*, several compounds were isolated. Some were considered as specific chemotaxonomic markers (**1** and **2**), others (**3** and **7**) which were recognized for the first time in this genus, and one (**5**) which is an uncommon compound in Asteraceae.

The identification of these constituents gives experimental evidence of the diversity of the secondary metabolite pattern in species which colonize restricted areas when compared with taxonomically related and widespread entities [27]. This fact also refers to endemic species. Moreover, in the most polar fraction, compounds **6** and **7** were recognized. These are two cyclitols possessing osmotic properties which, in our opinion, may be directly related to the metabolic adaption of this species to the arid environment where it normally lives. These cyclitols may have also another ecologic role as pollinators attractants, but further studies are necessary to confirm this last aspect.

From the ethno-medicinal point of view, all the isolated compounds are responsible of interesting biological activities (antioxidant, neuroprotective, anti-inflammatory, antileukemic, proapoptotic, and antidiabetic) which may substantiate the large use of *Anthemis* species in the traditional medicine, even on a molecular base. This also provides the evidence that the endemic species *A. cretica* subsp. *petraea* may be used as such at its own time.

Conflict of Interest

The authors declare no conflict of interest.

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