Journal of Applied Botany and Food Quality 91, 145 - 154 (2018), DOI:10.5073/JABFQ.2018.091.020

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Comparison of terpene and phenolic profiles of three wild species of *Echeveria* (Crassulaceae)

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(Submitted: September 8, 2017; Accepted: May 11, 2018)

Summary

Echeveria species (Crassulaceae) are used in traditional medicine and some of their biological activities are demonstrated (*e.g.* antimicrobial, anti-inflammatory, anticancer). However, their chemical composition has been scarcely studied. The methanol extracts (ME) of three *Echeveria* species (*E. craigiana, E. kimnachii* and *E. subrigida*) from Mexico were analyzed for the sterol (GC-MS) and phenolic (HPLC-DAD-ESI-MS^n) composition. Eleven sterols were identified, *E. kimnachii* showed the highest total content (7.87 mg/g ME), and the main constituents were γ-sitosterol in *E. craigiana* (33.9%) and *E. subrigida* (54.4%), and lupenone in *E. kimnachii* (28.9%). The phenolic analysis showed differences among the *Echeveria* species, which contained flavonoids derivatives and tannins as the main components. The main flavonoids in *E. craigiana* were hexoside derivatives of quercetin and isorhamnetin, both with a 3-hydroxy-3-methylglutaroyl substituent; in *E. subrigida* hexosides of isorhamnetin, quercetin and kaempferol; and *E. kimnachii* showed the greatest diversity including proanthocyanidins and less common flavonoid derivatives of kaempferol *O,O*-disubstituted by acyl derivatives. The characteristic phytochemicals of each studied *Echeveria* species could be responsible of its specific biological activities and useful as chemotaxonomic markers. The kaempferol derivatives in *E. kimnachii* are rare in nature and they will be isolated and characterized.

Keywords: *Echeveria*, Crassulaceae, terpenes, GC-MS, phenolics, flavonoids, HPLC-DAD-ESI-MS^n.

Abbreviations
GC-MS: gas chromatography mass spectrometry; HPLC-DAD-ESI-MS^n: high performance liquid chromatography with diode array detector coupled with electrospray ionization mass spectrometry; EC: *Echeveria craigiana*; EK: *Echeveria kimnachii*; ES: *Echeveria subrigida*; masl: meters above sea level; BHT: butylated hydroxytoluene; ME: Methanol extracts; EI: Electronic Impact; ESI: Electrospray ionization; E: Equivalents; MS: Mass spectrum; UV: Ultraviolet-visible.

Introduction

The genus *Echeveria* is distributed worldwide, but it is predominant in the Americas (155 species). Mexico has the greatest diversity with 140 species and 85% of them are endemic. *Echeveria* plants are well adapted to environmental stresses (e.g., hydric, high CO_2), and they usually live in semiarid and rocky habitats. These plants are grown mainly for ornamental purposes, and they are appreciated by horticulturists and gardeners worldwide (CONACYT Agencia Informativa, 2016). Remarkably, some *Echeveria* species have been used in traditional medicine to treat symptoms/diseases such as pain, oral herpes, inflammation, and stomach infections (Instituto Nacional Indigenista, 2009). Some of the biological activities that have been demonstrated for the *Echeveria* species include the contraceptive (*Echeveria gibbiflora*) (Reyes et al., 2005); antibacterial, antiparasitary, and cytotoxic (*Echeveria leucotricha*) (Martinez-Ruiz et al., 2012); anticancer (*Echeveria peacockii*) (Huang, 2012); as well as antioxidant, antibacterial, inhibitory of glucosidase, and antimutagenic (*Echeveria craigiana*, *Echeveria kimnachii*, and *Echeveria subrigida*) (López-Angulo et al., 2014; López-Angulo et al., 2016). Regarding the chemical composition of these species, qualitative phytochemical analyses showed the presence of triterpenes, sterols, glycosides, flavonoids, tannins, saponins, coumarins, alkaloids, free anthracenics, and lactones (López-Angulo et al., 2014; López-Angulo et al., 2016; Martinez-Ruiz et al., 2012; Stevens et al., 1995). Moreover, specific components have been identified in *Echeveria* species such as proanthocyanidins in *E. peacockii* (Huang, 2012) and organic acids (i.e., quinic, citric, and tartaric), oleanane, lupane and taraxerane in *E. lilacina* (Jovanovic et al., 2016; Stojanovic et al., 2015). In general, plants of other Crassulaceae genera (e.g., *Rhodiola, Kalanchoe, Sedum, Sempervivum*) contain terpenes (e.g., amyrin, taraxerane, germanicol), sterols (e.g., sitosterol, stigmasterol, isofucosterol, campesterol), phenolic acids (e.g., gallic, caffeic, ferulic), proanthocyanidins, and flavonoid glycosides (e.g., glycosides of quercetin, kaempferol, myricetin, and isorhamnetin) that are mainly 3,7-diglycosides of glucose and rhamnose. Interestingly, Crassulaceae plants of importance in traditional medicine contain some compounds characteristic of the genus such as bryophyllin B and bufadienolides in *Kalanchoe* and flavonoids (e.g., rhodiolin) and phenyl propanoids in *Rhodiola* (Alberti-Dér, 2013; Booker et al., 2016; Jin et al., 2009; Jovanovic et al., 2016; Milad et al., 2014; Stojanovic et al., 2015).

Terpenes and phenolics are important plant secondary metabolites that show multiple and relevant functions, as well as biological activities for the ecosystems and the human being (Pérez-Urria and Ávalos-García, 2009); moreover, they are used as chemotaxonomic markers due to their common presence in plants and diversity of chemical structures (Almaraz-Abarca et al., 2013; Jovanovic et al., 2016; Stojanovic et al., 2015). Molecular studies classify the *Echeveria* genus and some Mexican species of the *Sedum* genus in an American clade that distinguishes these plants from those of other Crassulaceae genera (Mort et al., 2001), suggesting also differences among their chemical composition. Up to date, the terpene and phenolic profiles of the *Echeveria* spp. plants have not been reported, and these compounds could be contributing with their reported biological activities, as well as prospecting new ones. This research shows the qualitative and quantitative analyses of terpenes and phenolics in methanol extracts of three *Echeveria* species (*E. craigiana*, *E. kimnachii*, and *E. subrigida*) by gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography with diode array detector coupled with electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS^n), which represents the first report of this type for the *Echeveria* genus.

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Materials and methods

Plant material

Echeveria plants were collected from the state of Sinaloa, Mexico, during September to November; for each species description, we show the coordinates, the name of the collector, and the assigned number in the herbarium of the Faculty of Agronomy, Autonomous University of Sinaloa (UAS), in parenthesis. *Echeveria craigiana* E. Walther (EC) is distributed in the states of Chihuahua, Sonora and Sinaloa; it was collected in "El Zapote" community, Choix, Sinaloa (1050 meters above sea level, masl; 26°46´03´´ N, 108°08´34´´ W; Vega-Aviña R.; 10816). *Echeveria kimnachii* J. Meyrán & R. Vega (EK) is endemic of Sinaloa, specifically of the municipality of Culiacan; it was collected from the South of the "Estancia de los García", Culiacan, Sinaloa (450 masl; 24°21´45´´ N, 107°01´05´´ W; Vega-Aviña R.; 9206). *Echeveria subrigida* (B. L. Rob. & Seaton) Rose (ES) is distributed in Mexico State, Guanajuato, Queretaro and Sinaloa; it was collected near "El Palmito" town, Concordia, Sinaloa (2000 masl; 23°34´06´´ N, 105°50´53´´ W; Vega-Aviña R.; 11742) (López-Angulo et al., 2014).

Leaves were freeze dried (VirTis 25EL, VirTis Co. USA) and milled to get a fine flour that passed through a 40 mesh (sample moisture (%) of 95.38 ± 0.01 for EC; 96.50 ± 0.74, EK; and 93.62 ± 0.09, ES) (López-Angulo et al., 2014). Dried leaf flours were stored at –20 °C in darkness until their use.

Reagents and solvents

The reagents purchased from Sigma/Aldrich (St. Louis, MO, USA) were α-cholestane, butylated hydroxytoluene (BHT), gallic acid, quercetin, isorhamnetin and kaempferol. HPLC and spectrometry grade organic reagents were from Baker Inc. (Phillipsburg, NJ, USA). All reagents were of analytical grade.

Preparation of the methanol extracts

Methanol extracts (ME) of the three *Echeveria* species were obtained by maceration. Leaf flours of *Echeveria* (10 g) were extracted with methanol (1:20 w/v) by shaking at 150 rpm/ darkness/ room temperature for three days; the solvent was exchanged every 24 h and the methanol phases were mixed. The solvent was eliminated under vacuum (40 °C) with a rotary evaporator (BÜCHI Labortechnick AG, Switzerland), followed by removal of any residual solvent in a vacuum oven at 40 °C. The ME obtained were stored at –20 °C in darkness until their use.

Terpene and sterol profiles

Terpenes and sterols were quantified as described by Conforti et al. (2008). The ME (50 mg) were dissolved in 5 mL of MeOH:H_2O (9:1 v/v), mixed with 20 µL of the internal standard α-cholestane (3 mg/mL), and 15 µL of the antioxidant BHT (1 mg/mL). The mixture was homogenized and partitioned with hexane (3 × 5 mL); the hexane phases were combined and evaporated. The residue was redissolved in 1 mL of hexane and filtered through a PVDF syringe filter (17 mm × 0.45 µm, TITAN) (Thermo Scientific Inc., USA) prior to GC-MS analysis.

GC-MS analysis

Samples were analyzed by Gas Chromatography Mass Spectrometry with the HP 6890 GC Instrument, 5973 Network (Agilent Technologies, USA). The separation was carried out on a capillary column QUADREX 007 CARBOWAX 20M (30 m × 0.25 mm i.d., film thickness 0.25 µm) (Quadrex Corporation, USA) using helium as carrier gas (0.9 mL/min). The operation temperatures were: injector, 250 °C; oven, initial 60 °C, kept for 1 min, 5 °C/min to 200 °C, 10 °C/min to 275 °C, and held at 275 °C to the end of the analysis; ion source, 245 °C; and quadrupole, 150 °C. The sample was injected (5 µL) without flow division. MS detection was performed in Electron Impact mode (EI) at 70 eV ionization energy, and operating in full-scan mode in the 50-800 amu range; the sample components were identified by comparison with the mass spectra in the National Institute of Standards and Technology library (NIST08.LIB), and they were quantified using the response factor method as follows:

$$Cs = As \times Cis/Ais$$

Where: ***Cs*** and ***As*** are the concentration and peak area for the terpene/sterol, respectively; ***Cis*** and ***Ais*** are the concentration and peak area for the internal standard.

Phenolics profile

The methanol extract of each species (50 mg of EC and ES; 100 mg of EK previously deffated with hexane) was dissolved in 5 mL of H_2O:MeOH (9:1 v/v) and partitioned with ethyl acetate (3 × 5 mL). The organic phase was recovered, and the solvent was evaporated. The residue was dissolved in methanol (10 mg/mL, EC and ES; 20 mg /mL, EK), filtered through a PVDF membrane (17 mm × 0.45 µm, TITAN) (Thermo Scientific Inc., USA), and analyzed by HPLC-DAD-ESI-MS^n.

HPLC-DAD-ESI-MS^n analysis

The HPLC system consisted of an ACCELA instrument (Thermo Scientific, USA) with quaternary pumps and diode array detector. Separation was carried out with an ACE EXCEL C18-Amide column (150 × 30 mm × 3 µm) (Advanced Chromatography Technologies, UK). The mobile phase was 1% formic acid (A) and acetonitrile (B); the chromatographic elution started with 0.5% B, lineal gradient to 30% B in 10 min, 30% B isocratic during 10 min, lineal gradient to 60% B in 10 min, and 60% B isocratic during 5 min. The flow rate was 0.4 mL/min and the injection volume was 15 µL. The main components were detected at 250, 320, and 325 nm.

For peak identification, the above described HPLC system was hyphenated with an ESI source and coupled with a lineal trap LTQ-XL mass spectrometer (Thermo Scientific, USA). Mass spectra were acquired in negative mode over the range *m/z* 200-1500, resolution of 30000. The MS worked at 300 °C and 35 V in the capillary tube, source voltage at 5 kV, and tube lens voltage at 110 V. The gas flows (units) were sheat 25, auxiliary 15 and sweep 0. For tandem mass spectrometry analysis (MS^n), the energy for the collision-induced dissociation (CID) was adjusted between 15 and 25%, ultrahigh-purity helium was used as the collision gas. Data were acquired and processed by using the Xcalibur 2.2 software. Peaks were identified by their fragmentation patterns and by comparison with MS data of both available commercial standards (i.e., gallic acid, quercetin, isorhamnetin, and kaempferol) and published in the literature.

Compound quantification was carried out with calibration curves of the commercial standards. According to the plot of the peak-area ratio (y) vs. concentration (x, µg/mL), the regression equations of standards and their correlation coefficients were as follows: gallic acid, y = 47.407x + 11.38 (R^2 = 0.9991); quercetin, y = 30.589x + 94.024 (R^2 = 0.9989); isorhamnetin, y = 32.664 + 175.8 (R^2 = 0.9984) and kaempferol, y = 35.059x + 81.8 (R^2 = 0.9988). The results were calculated as milligrams equivalents of phenolic compounds per gram of methanol extract (mg E/g ME).

Results

Terpene and sterol profiles

Eleven compounds were tentatively identified in the studied *Echeveria* species, and qualitative differences were found among their profiles (Fig. 1). The three species showed campesterol, sito-

sterol, and amyrin. *Echeveria kimnachii* had the highest content of terpenes/sterols (7.87 mg/g ME), highlighting the presence of lupenone, sitosterol, and hopenone B; moreover, hopenone B and friedelin were only found in this species, whereas germanicol was absent in EK but present in the other two *Echeveria* species. *Echeveria craigiana* and ES showed high contents of γ-sitosterol and amyrin, whereas EC was distinguished by the presence of simiarenol. Remarkably, none of the species showed stigmasterol (Fig. 1 and Tab. 1), which has been identified in plants of some genera of the Crassulaceae family.

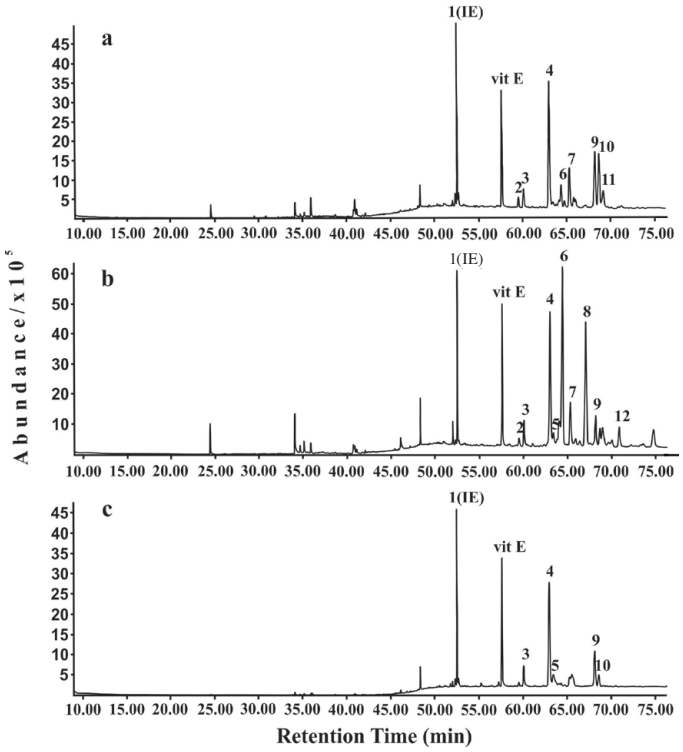

Fig. 1: Comparative GC-MS chromatograms of terpenes/sterols in *Echeveria* species. (IS internal standard). (a) *E. craigiana*, (b) *E. kimnachii* and (c) *E. subrigida*. The identification of the constituents is shown in Tab. 1.

Phenolic profiles

The HPLC-DAD-ESI-MS^n analyses showed differences in the type and content of phenolics among the studied *Echeveria* species (Fig. 2 and Tab. 2). Twelve main compounds were tentatively identified, including flavonoid derivatives of quercetin (**1C**, **4K**, and **10S**),

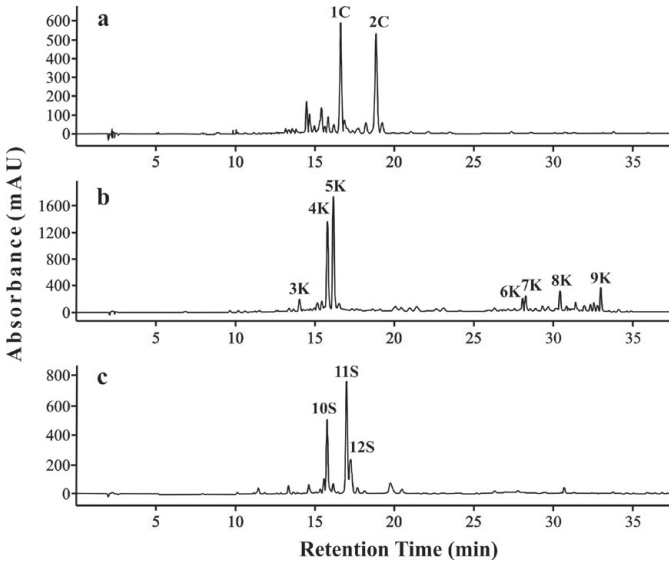

Fig. 2: Comparative HPLC-DAD chromatograms at 320 nm of phenolic profiles in *Echeveria* species. (a) *E. craigiana*, (b) *E. kimnachii* and (c) *E. subrigida*. The tentative identification of the constituents is shown in Tab. 2.

Tab. 1: Terpenes/sterols profile (GC-MS) of *Echeveria* methanol extracts (ME)

Peak	Tentative identity[a]	RT[b] (min)	Content[c] (mg/g ME) (Match)		
			E. craigiana	*E. kimnachii*	*E. subrigida*
1	Cholestane (IS)	52.59			
2	Cholestan-6-one, 3-(acetyloxy)-(3á5á)-	59.52	0.093 (585)	0.097 (550)	___
3	Campesterol	60.11	0.210 (890)	0.273 (864)	0.187 (890)
4	γ-Sitosterol	63.07	1.601 (920)	1.853 (922)	1.093 (920)
5	Fucosterol	63.45	___	0.187 (772)	0.197 (884)
6	Lupenone	64.47	0.262 (856)	2.276 (878)	___
7	Lupeol	65.34	0.553 (883)	0.582 (844)	___
8	Hopenone B	67.11	___	1.874 (916)	___
9	Amyrin	68.21	0.881 (832)	0.413 (803)	0.409 (822)
10	Germanicol	68.55	0.797 (809)	___	0.126 (690)
11	Simiarenol	69.13	0.325 (696)	___	___
12	Friedelin	70.86	___	0.317 (596)	___
	Total content		4.723	7.871	2.012

[a] Compounds are listed in order of elution, see Fig. 1.
[b] RT stands for retention time.
[c] Values are given in mg/g of methanol extract.

isorhamnetin (**2C** and **11S**), and kaempferol (**6** - **9K** and **12S**); as well as tannin-like compounds (**3K** and **5K**). Considering the quantity of phenolics (Tab. 2), clear differences were found among the *Echeveria* species. *Echeveria subrigida* showed the highest flavonoid content with isorhamnetin-3-*O*-hexoside (**11S**) (4.6 mg EI/g ME) and quercetin-7-*O*-hexoside (**10S**) (2.6 mg QE/g ME) as the main compounds. *Echeveria craigiana* showed the same quantity (2.3 mg) of its two main flavonoids measured as mg EQ/g ME (**1C**) and mg EI/g ME (**2C**). *Echeveria kimnachii* showed the highest diversity of components but in lower quantities, its content of flavonoids ranged from 0.2 to 0.3 mg E/g ME.

The compound identification was carried out by comparison of the UV spectra and MS^n data, fragmentation in negative mode, with literature data. The nomenclature to denote fragment ions was assigned as proposed by Domon and Costello (1988).

Quercetin derivatives

The fragmentation of the main ion $[M - H]^-$ of all quercetin derivatives (i.e.; **1C**, 607; **4K**, 463; and **10S**, 463) produced the same ion at *m/z* 301, which corresponded to the deprotonated aglycone Y_0^- (Fig. 3). This data suggested that quercetin derivatives are 7-*O*-monosubstituted compounds (Alberti-Dér, 2013; March et al., 2006). The MS^n experiments of ion at *m/z* 301 (Y_0^-) gave fragment ions at *m/z* 257, 179, and 151, consistent with the quercetin aglycone (Koolen et al., 2013; Ye et al., 2012). The compounds **1C**, **4K**, and **10S** showed similar retention times (15.6 - 15.8 min), but their ion fragmentations (MS^n) indicated the presence of different substituent groups (Fig. 3). Compound **1C** exhibited $[M - H]^-$ ion at *m/z* 607 that yielded fragment ions at *m/z* 463 $[(M - H) - 144]^-$ (Y_1^-), 545 $[(M - H) - 18 - 44]^-$, and 505 $[(M - H) - 18 - 44 - 40]^-$, associated with the release of the characteristic fragments of acyl substituent;

Tab. 2: HPLC-DAD-ESI-MS^n data and content of phenolic of *Echeveria* methanol extracts (ME)

Peak[a]	RT (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	Fragmentation MS^n $[M-H]^-$, *m/z* (relative abundance)	Tentative identification[b]	mg E/g ME[c]
1C	15.8	252, 355	607	MS^2 $[607]^-$→ 607 (20), 545 (10), 505 (23), 463(100) MS^3 $[463]^-$→ 463 (56), 301 (100)	Quercetin-7-*O*-[3-hydroxy-3-methylglutaroyl]hexoside	2.3±0.26
2C	17.0	254, 354	621	MS^2 $[621]^-$→ 621 (3), 559 (30), 519 (51), 477 (100), 315 (9) MS^3 $[477]^-$→ 315 (100), 301 (1)	Isorhamnetin 7-*O*-[3-hydroxy-3-methylglutaroyl]hexoside	2.3±0.07
3K	13.9	236, 272	457	MS^2 $[457]^-$→ 413 (11), 331 (100), 305 (39), 169 (75)	(epi)gallochatequin gallate (GCG/EGCG)	0.8±0.04
4K	15.6	314	463	MS^2 $[463]^-$→ 463 (5), 445 (39), 419 (73), 401 (36), 301 (100) MS^3 $[301]^-$→ 301 (100), 257 (14), 179 (44), 151(33)	Quercetin-7-*O*-caffeoyl ester	0.2±0.01
5K	15.9	232, 277	881	MS^2 $[881]^-$→ 845 (65), 843 (100), 729 (86), 711 (19), 559 (10), 407 (7), 289 (2)	Digalloylated procyanidin (P2G2)	0.3±0.02
6K	27.9	263, 347	863	MS^2 $[863]^-$→ 803 (26), 717 (33), 574 (100), 532 (44), 419 (41), 283 (38) MS^3 $[574]^-$→ 514 (13), 283 (100), 255 (13)	Kaempferol derivative	0.2±0.02
7K	28.1	263, 344	761	MS^2 $[761]^-$→ 719 (7), 701 (16), 615 (43), 430 (100), 283 (22) MS^3 $[430]^-$→ 430 (19), 283 (100), 255 (1)	Kaempferol derivative	0.2±0.01
8K	30.3	264, 346	819	MS2 $[819]^-$→ 818 (49), 759 (4), 573 (7), 562 (100), 537 (69), 531 (7) MS3 [562] $^-$→ 562 (31), 283 (8), 225 (100)	Kaempferol derivative	0.3±0.02
9K	32.9	263, 344	861	MS2 $[861]^-$→ 824 (10), 819 (10), 801 (39), 615 (63), 573 (41), 572 (100), 530 (56), 283 (13) MS3 $[572]^-$→ 572 (6), 283 (100), 255 (16)	Kaempferol derivative	0.2±0.02
10S	15.8	253, 354	463	MS2 $[463]^-$→ 301 (100) MS3 $[301]^-$→ 301 (23), 273 (16), 257 (16), 179 (100), 151 (64)	Quercetin-7-*O*-hexoside	2.9±0.06
11S	17.0	252, 354	477	MS2 $[477]^-$→ 357 (26), 315 (49), 314 (100), 285 (5) MS3 $[314]^-$→ 299 (16), 286 (61), 285 (100), 271 (75), 243 (20)	Isorhamnetin-3-*O*-hexoside	4.6±0.15
12S	17.2	262, 348	447	MS2 $[447]^-$→ 447 (47), 357 (3), 327 (25), 285 (84), 284 (100), 255 (9) MS3 $[284]^-$→ 256 (25), 255 (100), 227 (10), 211 (1)	Kaempferol-3-*O*-hexoside	1.9±0.10

[a] Letters accompanying the peak numbers identify the associated *Echeveria* species: *E. craigiana* (**C**), *E. kimnachii* (**K**), and *E. subrigida* (**S**).

[b] Compound identification was based on data reported in literature.

[c] Values are the mean ± standard deviation of at least three measurements and are calculated as equivalents of quercetin (peaks **1, 4,** and **10**), isorhamnetin (peaks **2** and **11**), gallic acid (peaks **3** and **5**), and kaempferol (peaks **6-9** and **12**).

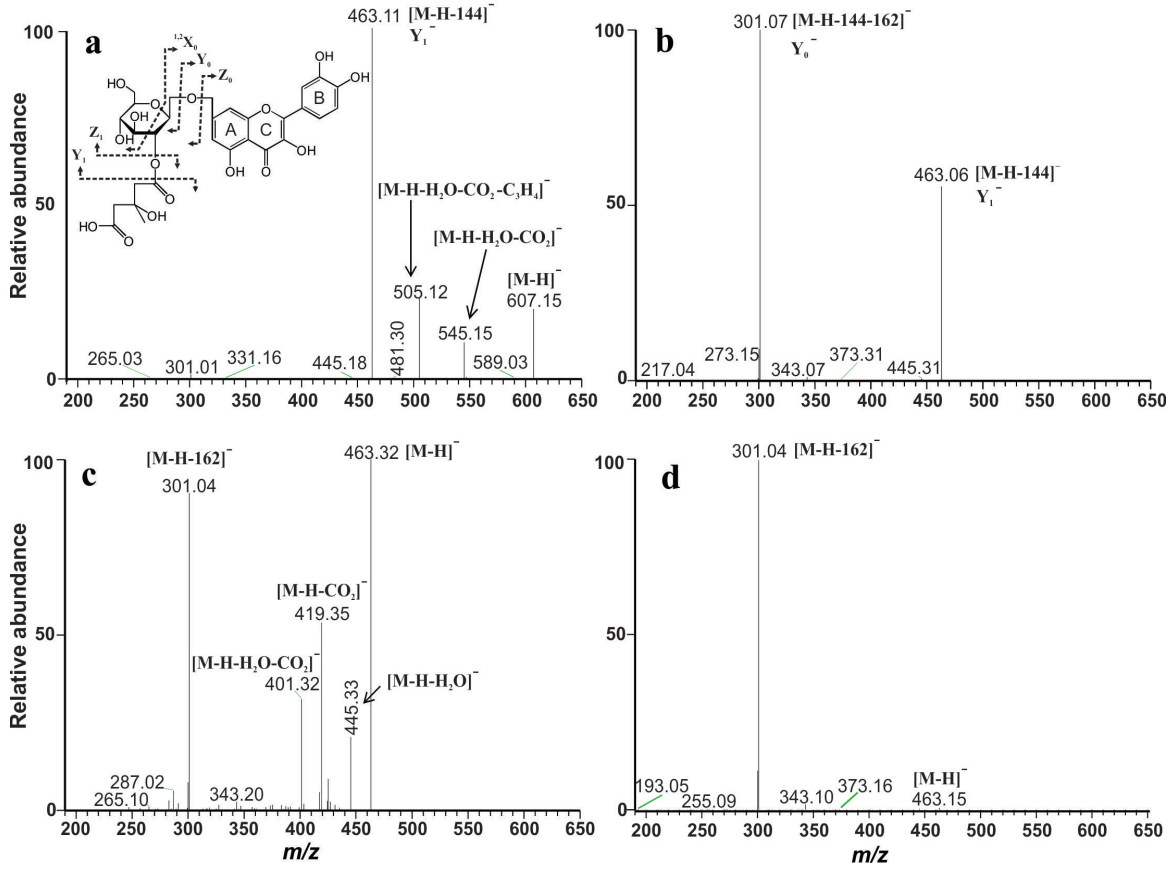

Fig. 3: ESI-MS/MS spectra of quercetin derivatives. (a) MS^2 of $[M - H]^-$ at m/z 607 and (b) MS^3 (607→463) of compound **1C**. MS^2 of $[M - H]^-$ at m/z 463 of compounds **4K** (c) and **10S** (d).

i.e., H_2O, CO_2, and $[C_3H_4]^+$, respectively (Fig. 3a). The MS^3 (607 → 463) gave the ion at *m/z* 301 $[(M - H) - 144 - 162]^-$ of the quercetin aglycone. A neutral loss of 162 u corresponds to a hexose, but the loss of 144 u is less common and it was assigned to 3-hydroxy-3-methylglutaroyl as reported previously (Liu et al., 2015; Song et al., 2010; Tahir et al., 2012); thus, compound **1C** was identified as quercetin-7-*O*-[3-hydroxy-3-methylglutaroyl] hexoside (Fig. 3a).

Compounds **4K** and **10S** showed the $[M - H]^-$ ion at *m/z* 463 and fragment ion for the quercetin aglycone at *m/z* 301 $[(M - H) - 162]^-$, but they differed in their MS and UV spectra (Tab. 2). For compound **10S**, the fragmentation of the $[M - H]^-$ ion produced the ion at *m/z* 301 $[(M - H) - 162]^-$ by the loss of a hexose residue (Fig. 3d). For compound **4K**, the MS^2 of $[M - H]^-$ ion at *m/z* 463 gave other ions in addition to that of *m/z* 301 $[(M - H) - 162]^-$; they were at *m/z* 445 $[(M - H) - 18]^-$, 419 $[(M - H) - 44]^-$, and 401 u $[(M - H) - 18 - 44]^-$ (Fig. 3c), whose MS^3 (463 → 445), (463 → 419) and (463 → 401) did not produce the ion at *m/z* 301, suggesting that the fragments observed could be derived of losses of H_2O (ring B) and CO_2 (ring C) of the aglycone structure, respectively (Koolen et al., 2013). Moreover, compound **4K** gave an UV spectrum characteristic of hydroxycinnamic acids and the loss of 162 u could be associated to caffeic acid (Ye et al., 2005). Based on this information, the compounds were tentatively identified as quercetin-7-*O*-caffeoyl ester (**4K**) and quercetin-7-*O*-hexoside (**10S**).

Isorhamnetin derivatives

Fragmentation of the pseudomolecular ions of compounds **2C** (*m/z* 621 $[M - H]^-$) and **11S** (*m/z* 477 $[M - H]^-$) agreed with that reported for the isorhamnetin aglycone (i.e., *m/z* 315, 314, 301, 300, and 285 u) (Fig. 4) (Ye et al., 2005). For compound **2C**, fragmentation of the $[M - H]^-$ ion at *m/z* 621 showed similar losses than compound **1C**, which corresponded to acyl groups in the structure, and the produced ions were at *m/z* 559 $[(M - H) - 18 - 44]^-$, 519 $[(M - H) - 18 - 44 - 40]^-$, a principal ion at *m/z* 477 $[(M - H) - 144]^-$ (Y_1^-), and that of 315 $[(M - H) - 144 - 162]^-$ for the aglycone (Y_0^-). Based on the described ions and the peak intensity of Y_0^- indicating the preferential loss of acylhexoside at the 7-*O*-position (March et al., 2006), these information allowed us to do the tentative assignment as isorhamnetin-7-*O*-[3-hydroxy-3-methylglutaroyl] hexoside (**2C**).

Compound **11S** showed a $[M - H]^-$ ion at *m/z* 477, and its MS^2 fragmentation yielded ions at *m/z* 387 $[(M - H) - 90]^-$ and 357 $[(M - H) - 120]^-$ (Fig. 4c). This pattern has been associated to *C*-glycosides (Alberti-Dér, 2013; Benayad et al., 2014), but the presence of ions at *m/z* 314 $[(M - H) - 162 - H]^-$ $(Y_0 - H)^-$ and 315 $[(M - H) - 162]^-$ (Y_0^-) of less intensity (49%) suggested that **11S** was a 3-*O*-glycosylated compound (Alberti-Dér, 2013; March et al., 2006) that was tentatively identified as isorhamnetin-3-*O*-hexoside (**11S**).

Kaempferol derivatives

Compounds **6** - **9K** and **12S** were identified as kaempferol derivatives by the registered fragment ions at *m/z* 285 (Y_0^-) and 283 ($[Y_0 - 2H]^-$) (Fig. 5), whose additional fragmentation yielded ions (*m/z* 255 and 227) characteristic of kaempferol aglycone (Francescato et al., 2013). For the MS spectra of compounds **6** - **9K** (Fig. 5a - d), we did not find similar spectra in literature. However, their MS spectra showed an ion at *m/z* 283 suggesting that the compounds are *O*,*O*-disubstituted (Alberti-Dér, 2013). For compound

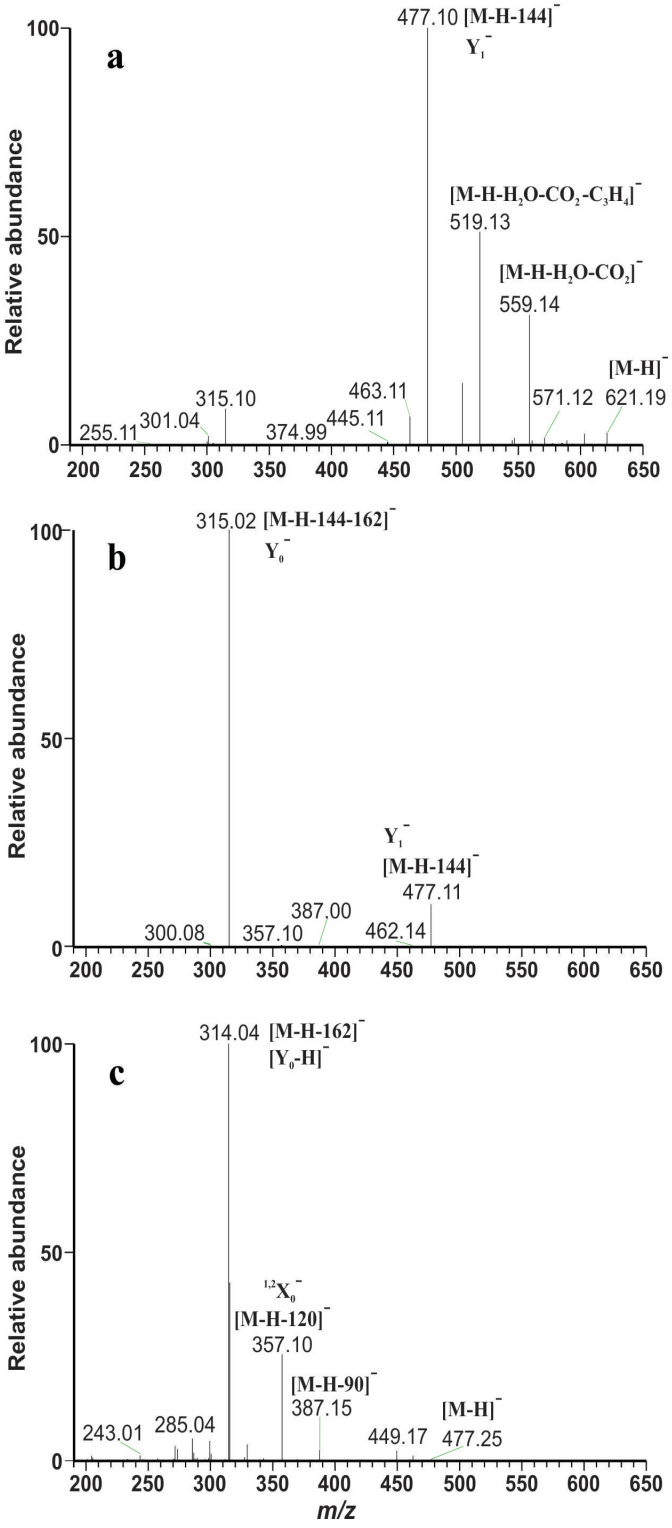

Fig. 4: ESI-MS/MS spectrum of isorhamnetin derivatives. (a) MS^2 of $[M - H]^-$ at m/z 621 and (b) MS^3 (621→477) of compound **2C**. (c) MS^2 of $[M - H]^-$ at m/z 477 of compound **11S**.

6K, the MS^2 of $[M - H]^-$ ion at *m/z* 863 gave ions *at m/z* 826 $[(M - H) - 38]^-$ and 803 $[(M - H) - 18 - 42]^-$, indicating the loss of H_2O and C_2H_2O, which could correspond to acyl derivatives; the major fragment at *m/z* 717 $[(M - H) - 146]^-$ and fragment ion at *m/z* 711 $[(M - H) - 152]^-$ with low intensity (3%), which are probably associated with the loss of terminal molecules of deoxyhexose and of a gallic acid residue, respectively; and the ion of intermediate intensity at *m/z* 574 $[(M - H) - 152 - 137]^-$ by the additional elimination of 3,4-dihydroxybenzoic acid residue. The fragmentation of ion 574 generated the ion at *m/z* 283 $[(M - H) - 146 - 144]^-$ of the corresponding aglycone, which resulted of the release of the deoxyhexose and 3-hydroxy-3-methylglutaroyl fragments. The proposed structure of compound **6K** corresponded to kaempferol-*O*-(deoxyhexosyl)-3-hydroxy-3-methylglutaroyl-*O*-(galloyl)-dihydroxybenzoyl, but it must be confirmed by further experiments.

The fragmentation of peak **7K** suggested that it is an *O*-*O*-disubstituted compound $[Y_0 - 2H]^-$. The MS spectrum of the $[M - H]^-$ ion at *m/z* 761 was complex (Fig. 5b). It showed fragments by losses of H_2O (743 $[(M - H) - 18]^-$); C_2H_2O (701 $[(M - H) - 18 - 42]^-$); deoxyhexose (615 $[(M - H) - 146]^-$); and of acyl derivatives of phenolic acids, i.e., fragment of gallic acid 609 $[(M - H) - 152]^-$ and fragment of dihydroxyferulic acid 430 u $[(M - H) - 152 - (178 + H)]^-$. The MS^3 fragmentation of the ion at *m/z* 430 produced only one fragment at *m/z* 283 ($Y_0 - 2H$) by the loss of a protonated deoxyhexose (146 + H), which suggested as possible kaempferol substituents the galloyl-dihydroferulic in one position and deoxyhexose in the other.

The pseudomolecular ion of compound **8K** was at *m/z* 819 $[M - H]^-$, and its MS spectrum was not enough to identify the substituent groups (Fig. 5c). The aglycone ion at *m/z* 285 (Y_0^-) suggested that **8K** is a kaempferol *O*-monosubstituted compound.

The MS spectrum of compound **9K** (Fig. 5d) was similar to that of compound **6K** (Fig. 5a) but with differences in the *m/z* of the pseudomolecular ion, 2 u lower with $[M - H]^-$ ion at *m/z* 861, and of the main fragments. It was identified as a disubstituted compound considering the aglycone ion at *m/z* 283 $[Y_0 - 2H]^-$. The MS^n data (861 → 572 → 283) showed the loss of identical fragments of 289 u, which could be due to the loss of two 3-hydroxy-3-methylglutaroyl units (144 + (144 + H)). The compound **9K** was tentatively identified as kaempferol-3,7-*O*-di(3-hydroxy-3-methylglutaroyl).

Compound **12S** (Fig. 5e) showed $[M - H]^-$ ion at *m/z* 447 and fragment ions at *m/z* 357 $[(M - H) - 90]^-$, 327 $[(M - H) - 120]^-$, and 284 $[(M - H) - 162 - H]^-$ ($Y_0 - H$). As discussed for **11S**, the peak intensity of fragment ion at *m/z* 284 (100%) indicated a 3-*O*-glycoside; **12S** was tentatively identified as kaempferol-3-*O*-hexoside.

Tannins

The UV spectra of compounds **3K** and **5K** were characteristics of derivatives of hydroxybenzoic acids. The $[M - H]^-$ molecular ion of compound **3K** at *m/z* 457 was fragmented as reported for gallocatechin gallate or its isomer epigallocatechin gallate (Tab. 2) (Lin et al., 2008; Sun et al., 2007). The breakdown of the ester bond produced fragment ions at *m/z* 169 and 305, which were assigned to gallic acid and (epi)gallocatechin, respectively. As reported by Sun et al. (2007), we also observed a fragment ion at *m/z* 331, which could be derived of the breakdown of ring C ($^{1,2}A$). The UV spectrum of peak **5K** showed two absorption maxima (232, 277 nm), which were similar to those of proanthocyanidins (Kajdžanoska et al., 2010). The MS^n of $[M - H]^-$ ion at *m/z* 881 showed fragment ions assigned to the loss of water and gallic acid moiety at *m/z* 843 $[(M - H) - 2H_2O]^-$, 729 $[(M - H) - (\text{gallic acid} - H_2O)]^-$, 559 $[(M - H) - 2(\text{gallic acid} - H_2O) - H_2O]^-$, and 407 $[(M - H) - 3(\text{gallic acid} - H_2O) - H_2O]^-$ (Tab. 2). Moreover, the fragment ion at *m/z* 289 confirmed the presence of (epi)catechin. After contrasting with the spectrum reported in the literature, compound **5K** was identified as digalloylated procyanidin (Russo et al., 2013).

Discussion

The analysis of the terpene/sterol and phenolic composition showed clear differences among the studied *Echeveria* species. Studies of

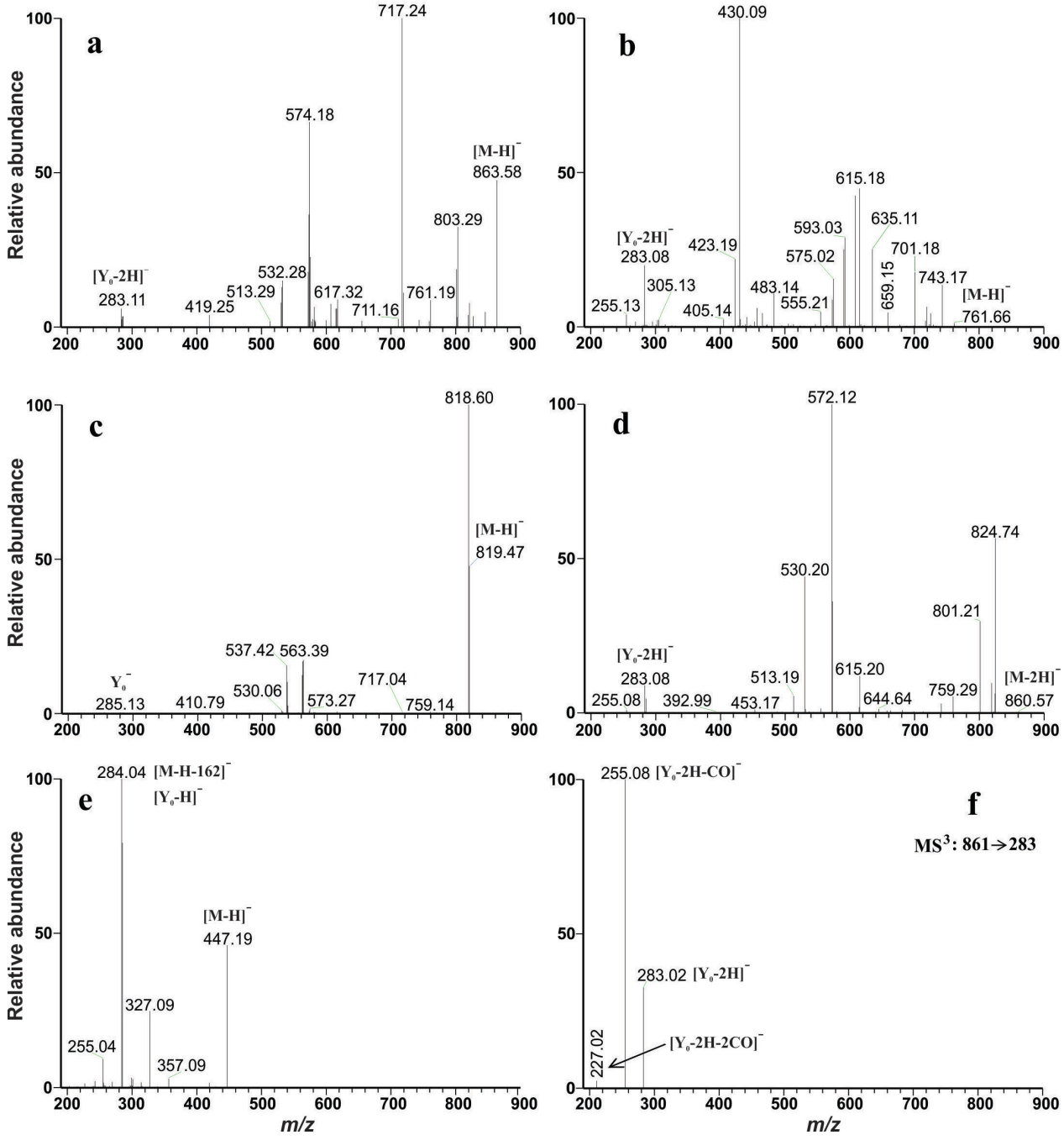

Fig. 5: ESI-MS/MS spectrum of kaempferol derivatives. MS^2 of the $[M - H]^-$ ion of compounds: (a) **6K**, (b) **7K**, (c) **8K**, (d) **9K** of *E. kimnachii* and (e) **12S** of *E. subrigida*; (f) MS^3 (861→283) of compound **9K**.

these compounds in *Echeveria* are scarce; only phenolics and terpenes have been reported in *Echeveria lilacina*, plant included in a chemotaxonomic study of Crassulaceae. The compounds quantified in *E. lilacina* were the quinic, tartaric, citric, and protocatechuic acids (STOJANOVIC et al., 2015); as well as the terpenes oleanane and taraxerane in wax (JOVANOVIC et al., 2016). On the other hand, several reports have shown terpene and phenolic profiles of other genera of the Crassulaceae family (*e.g. Rhodiola*, *Kalanchoe*, *Sedum*, and *Sempervivum*).

One of the main sterols found in the studied *Echeveria* species was γ-sitosterol (23.55-54.35% out of the total terpenes/sterols content). Compared with the γ-sitosterol content of these species, sitosterol, stigmasterol, and campesterol are the most common sterols in higher plants and β-sitosterol is the principal (AKIHISA et al., 1991). Eighteen sterols have been reported for *Kalanchoe pinnata*, including 24-ethyldesmosterol, sitosterol, clerosterol, and fucosterol (AKIHISA et al., 1991). Also, sterol analysis by GC-MS in *Rhodiola sachalinensis* shows 18 compounds (e.g., β-sitosterol, stigmasterol, and cicloartenol). An interesting datum of our study was that unlike the presence of stigmasterol in the Crassulaceae *Rhodiola* and *Kalanchoe* (AKIHISA et al., 1991; JIN et al., 2009), this sterol was absent in the *Echeveria* species used in the present study, but its derivative fucosterol was found in EK (2.38%) and ES (9.78%). *Kalanchoe pinnata* also showed fucosterol (AKIHISA et al., 1991; KAUR et al., 2011). All studied *Echeveria* species had campesterol, which was identified previously in *K. pinnata* and *R. imbricata*

(Akihisa et al., 1991; Tayade et al., 2013).

Considering the triterpene composition of Crassulaceae, *Rhodiola* shows monoterpene alcohols (e.g., geraniol, linalool) and *p*-cymene (Dascaliuc et al., 2008; Jin et al., 2009); *Kalanchoe* has glutinol, friedelin, taraxerone, and α- and β-amyrin (Siddiqui et al., 1989; van Maarseveen and Jetter, 2009); and *Sedum* contains oleanane, lupane, and taraxerone (Jovanovic et al., 2016). Amyrin was the only triterpene found in the three studied *Echeveria* plants, which is commonly found in Crassulaceae plants, and it was one of the most abundant triterpenes of EC (18.66%) and ES (20.31%), whereas it was found only in small amounts in EK, as in *Kalanchoe daigremontiana* (van Maarseveen and Jetter, 2009). Germanicol was identified in EC and ES, whereas friedelin was characteristic of EK, both components have been reported in *K. daigremontiana* (van Maarseveen and Jetter, 2009). Lupenone was abundant in EK, altogether with lupeol were found in both EK and EC; these compounds have been reported in leaves of *Aeonium lindleyi* Webb & Berthel (Crassulaceae) (Kennedy, 2012). Considering the hopenone B (A'-Neogammacer-22(29)-en-3-one) content, it was one of the abundant terpenes in EK, but it has not been reported in other Crassulaceae.

Regarding phenolic compounds in Crassulaceae, plants of the genera *Rhodiola, Sempervivum, Kalanchoe*, and *Sedum* contain proanthocyanidins; phenolic acids (e.g., gallic, caffeic, and ferulic); flavonoids, mainly quercetin, kaempferol, isorhamnetin, and myricetin, as well as flavonoid glycosides (Alberti-Dér, 2013; Ertas et al., 2014; Ming et al., 2005; Singab et al., 2011; Tatsimo et al., 2012), which are commonly 3,7-*O*-disubstituted glycosides of rhamnose and glucose (Alberti-Dér, 2013; Milad et al., 2014; Singab et al., 2011). Every studied *Echeveria* species showed a specific profile of phenolics; most Crassulaceae plants contain derivatives of the flavonoid aglycones quercetin, isorhamnetin, and kaempferol. Moreover, this pattern of flavonoids has been also reported for some Cactaceae plants that show CAM metabolism, as the *Echeveria* plants (Cai et al., 2008). The main flavonoids in EC and ES were monosubstituted quercetin and isorhamnetin glycosides, the hexose could be glucose. *Echeveria subrigida* also showed a kaempferol glycoside, and this species was the only with the three flavonoid aglycones reported commonly in Crassulaceae. Regarding to this and comparing to the EC flavonoids, a hexose substituted with hydroxy-3-methyl-glutaroyl group has been previously reported mainly in quercetin derivatives (Liu et al., 2015; Oszmianski et al., 2015), and there is only one report in a isorhamnetin derivative (Sommella et al., 2015). All of these reports indicate that flavonoids are substituted in the 3-*O* position, but our results suggested that substitution in EC flavonoids is in 7-*O* position. As it is well known, Crassulaceae produces high levels of organic acids, supporting the presence of such substituent (Alberti-Dér, 2013). In this regard, glucoside derivatives of quercetin and kaempferol presenting a 3-hydroxy-3-methylglutaroyl substituent have been isolated from *Graptopetalum paraguayense* E. Walther (Crassulaceae) (Liu et al., 2015).

The highest diversity of phenolics was found in EK, the only studied species with tannins such as digalloylated procyanidin (P2G2) and (epi)gallocatechin gallate, the last compound has been reported in *Rhodiola heterodonta* (Yousef et al., 2006). Considering the chemical data of the EK flavonoids, the UV spectra of **4K** (quercetin-7-*O*-caffeoyl ester) corresponded to a hydroxycinnamic acid, but the mass spectra suggested a flavonoid *O*-substituted; Francescato et al. (2013) reported similar results with the compounds quercetin-3-*O*-caffeoyl-glucoside of *Equisetum giganteum* L. In general, the EK flavonoids were not common since they are esterified with acids (i.e., gallic, dihydroxybenzoic, hydroferulic, and 3-hydroxy-3-methylglutaric). Some Crassulaceae have shown this type of compounds such as 11-*O*-(4´-*O*-methylgalloyl)-bergenin in *Crassula capitella* (El-Hawary et al., 2016) and myricetin-3-*O*-(3´´-galloyl-rhamnoside) in *Sedum sediforme* (Winekenstadde et al., 2015). Flavonoids with more than three substituents are rare and most of them are substituted with carbohydrates, the kaempferol derivatives in EK showed more than two acyl substituents that it is an unusual characteristic. Llorach et al. (2003) reported high molecular weight flavonoids, mainly kaempferol-triglucosides substituted with different hydroxycinnamic acids, in the industrial byproducts of *Brassica oleracea* L. var. Botrys.

Considering the terpene and phenolic profiles of the three studied *Echeveria* species and their geographical origin, *E. kimnachii* grows in an area of higher temperatures, where a larger number and greater diversity of herbivore insects is found (DeLucia et al., 2012). The high content of terpenes in this species could be a defense mechanism against pests (Paré and Tumlinson, 1999). On the other hand, *E. subrigida* is from a mountainous area characterized by lower temperatures and high sunlight irradiation; this species showed the lowest terpene content and the highest phenolic content, which corresponds with previous studies demonstrating an increased phenolics content in plants exposed to high sunlight irradiation (Bachereau et al., 1998).

Conclusions

The terpene and phenolic profiles of every studied *Echeveria* species result from the combination of genetic and environmental factors. Many of these secondary metabolites are produced as a plant defense mechanism against biotic and abiotic agents (Almaraz-Abarca et al., 2013; Wink, 2003), and they are important to preserve their corresponding ecosystems. Remarkably, several of the compounds identified in the present study have shown biological activities of importance for the human beings; e.g., γ-sitosterol (antidiabetic) (Balamurugan et al., 2011), lupeol (antioxidant, hepatoprotective), amyrin (anti-inflammatory, analgesic, antioxidant) (Dzubak et al., 2006), and phenolics (antioxidant, antimutagenic, antithrombotic, antidiabetic, antibacterial) (Kumar and Pandey, 2013), and the studied *Echeveria* species have potential for the prevention/treatment of infectious and chronic degenerative diseases (e.g. cancer and diabetes) (Ahumada-Santos et al., 2016; López-Angulo et al., 2014; López-Angulo et al., 2016). This study improves the knowledge of *E. craigiana, E. kimnachii*, and *E. subrigida*; and based on the chemical composition, it supports their potential use in the food and pharmaceutical industries, and the development of strategies for their sustainable preservation.

Authors' contributions

GLA, SPDC and FDV conceived and designed the experiments. GLA and JMA did the chemical analysis. GLA, JMA and JALV analyzed the data and prepared the manuscript. RVA and FDV carried out the field work and plant authentication. FDV supervised the study, reviewed and edited the work. All authors read and approved the manuscript.

Acknowledgments

Authors acknowledge the financial support of CONACYT and PROFAPI - "Universidad Autónoma de Sinaloa", as well as to Nancy Yareli Salazar-Salas who helped with the LC-DAD-ESI-MS^n analysis.

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