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NMR 800Hz characterization, mass spectrometry discussion and anti-proliferative effects of flavonoids, senecic acid and caffeic acid isolated from *Senecio leucanthemifolius poiret* growing in Morocco

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ABSTRACT

The present study consists at the chemical characterization, the structure elucidation and the in-vitro cytotoxic bioassay of some natural compounds isolated from Senecio leucanthemifolius poiret flowers growing in the Moroccan Western Middle Atlas mountains called Atlas of Beni- Mellal. The extraction by Soxhlet was carried out successively using the Ethyl Acetate and Methanol 90%. Each extract was fractionated using different chromatographic columns. The fractions and compounds obtained were purified by using HPLC semi-prevarative then analyzed by using HPLC, Mass Spectrometry (LC/MS; HRMS) and NMR 800Hz spectrometer. The isolated compounds were further subjected to antiprofliferative test on MCF7, HT1080, K562 and HL60 tumor cell lines. Four flavonoids were isolated and characterized: Isorhamnetin -3-O-ß- glucose (7.0 mg) (Compound 1), Isorhamnetin, 3, 7-O- ß-glucopyranoside (2.5 mg) (Compound 2); Quercitin-3-O- glucopyranoside (1.4mg) (Compound 3); Quercitin-7-O- ß- glucopyranoside (1.2 mg) (Compound 4). Moreover, we could isolate one Caffeic acid (5.0 mg) (Compound 5) and two stereoisomers senecic acids (Compound 6) (Compound 7) respectively isolated with amounts of 1.4 mg and 1.0 mg. With the tested concentrations from 0.625 to 20 µM, compounds 1 and 2 highlighted a relative inhibition of the cell growth. However, compounds 5 and 6 showed a very moderate antiproliferative effect. These secondary metabolites are isolated and descripted for the first time from Senecio leucanthemifolius Poiret. According to the literature, it has been proved that there was no previous concerning their cytotoxic activity.

Keywords: Senecio, HRMS, NMR 800Hz , Flavonoids, cytotoxicy.

INTRODUCTION

Plants have been used as the essential element of traditional medicine systems to serve people all over the world for thousands of years (Samuelsson et al., 2004). An evaluation of the World Health Organization validated that these ethical medical systems continue to play an important role in the primary health care of about 80% of the world's residents (Farnsworth et al., 1985). Globally, at least 119 compounds derived from 90 plant species can be considered as important drugs. 74% of these substances were found by the chemical studies through the isolation of the bioactive compounds from plants used in traditional medicine (Newman et al., 2000).

Senecio leucanthemifolius Poiret is a medicinal plant known by many traditional therapeutic applications. This species belongs to the *Asteraceae* family. More than 1500 species throughout the world were indexed (Loizzo et al., 2005; Nordestam et al., 2009). In the Moroccan and occidental traditional medicine, *Senecio* is used as an emmenagogue, anti-inflammatory, vasodilatator, and hypoglycemic drug (Bellakhdar, 1997; Loizzo et al., 2005; Tundis et al., 2007). Many works showed that Senecio genus extracts had, antibacterial, and cytotoxic activity on various cellular lines (Barbetti et al., 1986; Christov et al., 2002; Ouchbanin et al., 2015). Recently we proved the antiproliferative effect of *Senecio leucanthemifolius* poiret essential oil on human cervix uteri cancerous cellular line: HeLa (ATCC - American Standard Culture Collection) carrying the papilloma virus human type 18 considered as highly oncogenes (Ouchbani et al., 2011). This plant is known by the presence of pyrrolizidin alkaloids such as senecionine, integerrimine and jacaranone. Moreover, the n-hexane extract highlighting the presence of terpenoids. Alkaloids were detected in dichloromethane extract. The ethyl acetate and butanol extracts the presence of flavonoids (Tundis et al., 2007). The originality of this present research work is based on many parameters. Firstly the lake of studies concerning the characterization and biological activity of *Senecio leucanhtemfolius* poiret secondary metabolites. Moreover the analytical techniques used in this study are very advanced and sensitive and not usually used in the plant secondary metabolites characterization especially the NMR 800 Hz. In fact some research groups isolated and characterized the *Senecio leucanthemifolius* Poiret pyrrolizidin alkaloids but until now, we don't find any study concerning the isolation and characterization of flavonoids, senecic acid or cafeic acid from this species. For this reason, we were interested in the characterization and the elucidation of some Quercitin glucosides, Isorhamnetin glucosides, senecic acid and caffeic acid by using very advanced analytical techniques which are not usually used such as the NMR 800Hz. Moreover, the characterized compounds are isolated for the first time from the Moroccan *Senecio leucanthemifolius* poiret. It is also important to mention that the evaluation of their anti-proliferative activity was also achieved for the first time.

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MATERIAL AND METHODS

Materiel plant:

The whole plant was collected in April 2007 during the flowering season (root, stem, leaves and flowers). The collection was carried out in the Western Middle Atlas Mountains (called Atlas of Beni- Mellal) (Ouchbani, 1996). The voucher specimen was identified by Prof. S.OUCHBANI from the Scientific Institute, University Mohamed V, Rabat, Morocco.

The Moroccan species is a plant of 10-30 cm, very polymorphic, radical leaves, obovate, petiolate, dentate, sinuate-dentate or pinnatifid leaves. Inflorescence is present in modest capitulum with an involucre from 5 to 6 mm length. The flowers are yellows and the calycule is variable. Bracts of the involucre are considered after the achenes dispersal, which are often hairy, and never glandulate (Quezel et al., 1963).

Extraction, isolation and purification material:

Different chromatographic techniques were used for the isolation of natural compounds like TLC, Chromatographic Columns, or semi-preparative HPLC. The extraction by Soxhlet was carried out successively using the Ethyl Acetate and Methanol 90%. Each extract was fractionated using an ion exchange column (Amberlit XAD. Diaion hp 20, Dowex MCI Gel Merck) prepared with 80%MeOH and 20% H2O; Silica Gel column (60, 40−63 μm mesh size Merck) or a Size-exclusion chromatography column Sephadex Gel (LH 20, 25−100 μm mesh size Merck) prepared with 100%MeOH. The fractions obtained were purified using Pre-coated TLC plates (Aluminium, Silica Gel 60 F254, layer thickness 0.2mm) and HPLC semi-preparative (Eurospher 100-C18, Knauer (250 × 4.6 mm, ID and 250 × 21.4 mm, ID; Merck/Hitachi).

HPLC Analysis:

The identification of the compounds isolated from S. *leucanthemifolius* extracts was made by HPLC Dionex :

- Pump: P 580A LPG Dionex
- Autosampler: ASI-100T (injection volume = 20 μl) Dionex

- Detector: UVD 340S (Photodiode array detector) Dionex
- Column oven: STH 585 Dionex
- Column: Eurospher 100-C18, [5 μm; 125 mm × 4 mm] Knauer
- Pre-column: Vertex column, Eurospher 100-5 C18 [5-4 mm] Knauer
- Software: Chromeleon (V. 6.30)

The gradient program used in HPLC analysis was detailed in Table 1.

Table-1. The gradient program used in HPLC analysis

Time (minutes)	% Méthanol	% Water
0	10	90
5	10	90
35	100	0
45	100	0
46	10	90
60	10	90

LC/MS and HRMS-ESI Analysis:

The LC/MS analysis was developed using:

- Analytical HPLC: Agilent 1100 series (Photodiode array detector) Agilent
- MS: Finigan LCQ-DECA Thermoquest
- Ionizer: ESI and APCI Thermoquest
- Vacuum pump: Edwards 30 BOC
- Column: Eurospher 100-C18, [5 μm; 227 mm × 2 mm] Knauer
- Pre-column: Vertex column, Eurospher 100-5 C18 [5−4 mm] Knauer

The gradient program is the same used in HPLC analysis. The High resolution mass (HRMS)-ESI spectra were registered by the mass spectrometer (Waters) in positive and negative modes using the SYNAPT G2 HDMS Quadripole, QqToF-MS/MS. The ESI was performed by using the “turbo Ion spray” as a source operational at 3KV and in a temperature between 80 and 120°C. The mass spectra In ESI mode was registered at 40 eV.

NMR Analysis:

Almost NMR experiences were registered on Brucker Advance 800 MHz spectrometer equipped with cryosondes (TXI mm) in Methanol- *d4* (δ_H centered at 3.3 ppm and δ_c centered at 49.0 ppm). The spectra registered at 298K, included mono-dimensional spectra (1D) of proton 1 H / carbon ^{13}C and di-dimensional spectra 2D such as HMQC, HMBC, NOESY, COSY and/or COSY-DQF. The chemical shifts are represented by particles per million (ppm), the signals multiplicity are designed by the notation: s: singulet, d: doublet, dd: doublet of doublet and t: triplet. The coupling Constance (J) are calculated in Hz as a unity of frequency.

Anti-proliferative activity bioassay:

Preparation of the products to be tested:

The compounds (1), (2), (5) and (6) were solubilized in dimethyl sulfoxide (DMSO). The final concentration of the DMSO in the solution must be lower than 0.01%. A series of dilutions with the culture medium was carried out in order to obtain solutions with various concentrations.

Cellular lines and culture conditions:

In this study, we used four different Human Cancer cell lines:

MCF7: breast cancer cell line
K562 : Human erythromyeloblastoid leukemia cell line
HT1080 : human fibrosarcoma
HL60 : Human promyelocytic leukemia cells

Each cell line was cultured in the appropriate medium supplemented with 10 % fetal bovine serum; 1 % L-Glutamine, 1 % mixture antibiotics PSN (Penicillin Streptomycin- Neomycin). The cell lines were maintained at 37°C in a 5 % CO2 wet atmosphere.

Evaluation of the cytotoxicity activity:

The cells were seeded in 24-well microliter plates, each well is corresponding to a concentration of 20X10^3 cells/ml and 2.10 ^{4} cells. After 24h of incubation, various concentrations prepared from100µl of the solutions containing the product to be tested, were deposited in the wells. After 24, 48, 72 and 96 hours of incubation, the cells were washed with PBS (phosphate buffered saline), than recovered thanks to trypsine effect. Finally, the cells were treated by trypan blue. The survival cells were counted under microscope using the Kova microscope slides. The cellular viability was determined with the Trypan blue test. The percentage of the cell growth is obtained by the CellTiter 96® AQueous test.

RESULTS AND DISCUSSION

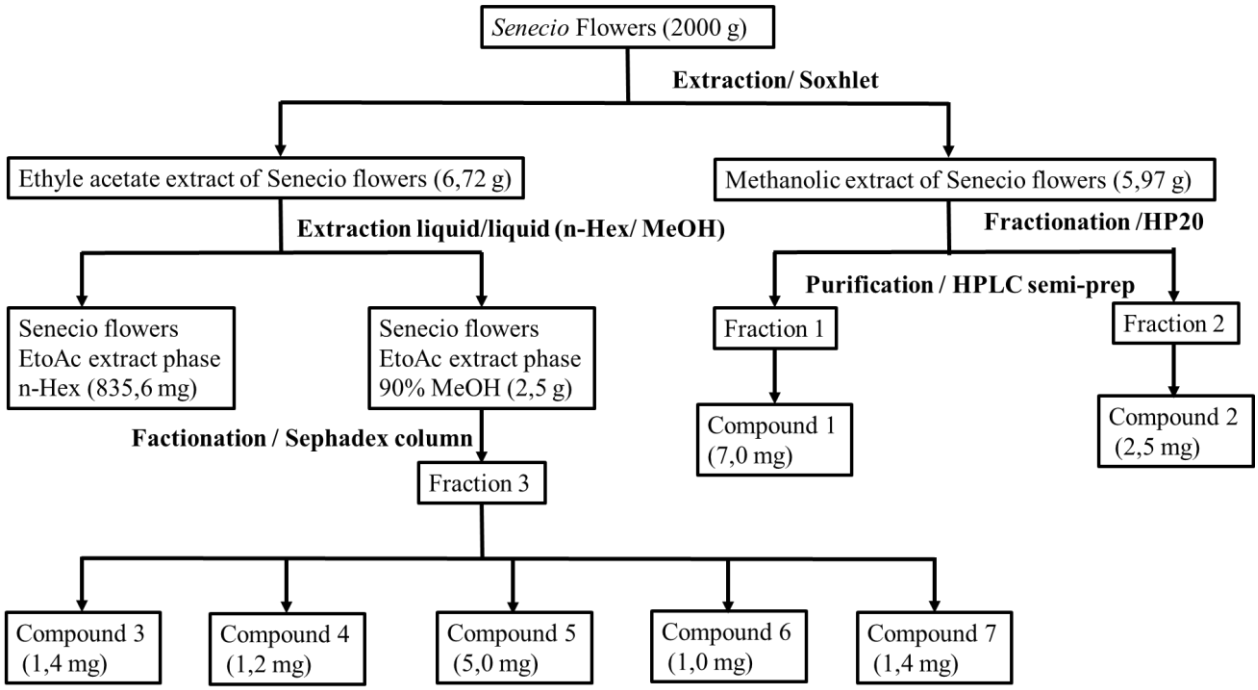
Phytochemical study:

Compounds isolation and purification:

In this study, we start with 2000g of flowers to obtain an Ethyl acetate (EtoAc) extract (6.72 g) and a Methanolic (MeOH) extract (5.97g). After series of fractionation by Diaion Hp20 and Sephadex chromatographic columns, the recuperated fractions were injected in the semi-preparative HPLC. After the purification protocol, we recuperated the Compounds (1) (7.0 mg) and (2) (2.5 mg) from the methanolic extract and the Compounds (3) (1.4 mg), (4) (1.2 gm), (5) (5 mg), (6) (1.0mg) and (7) (1.4 mg) from the

methanolic fraction of *Senecio leucanthemifolius* poiret flowers Ethyl acetate extract (Figure-1).

Figure-1. Sheme of extracts fractionation and secondary metabolism isolation.



***Chemical analysis and compounds characterization*:**

Compound 1 was obtained as a yellow powder from the *Senecio leucanthemifolius* poiret methonolic extract. The UV spectra showed a maximal absorbance λmax (MeOH) at 254 and 354 nm. This kind of spectra is characteristic of flavonoids. The Liquid chromatography coupled to the Mass spectrometry (LC/MS) analysis in the negative mode showed the formation of an ion $[M-H]^-$ at *m/z* = 476.9 and in the positive mode a protoned molecular ion $[M+H]^+$ detected at *m/z* = 478.8 which is confirmed by the presence of a pic *m/z* = 987.9 belonging to the molecular ion $[2M+Na]^+$. The LC/MS spectra let also a pic in the positive mode immerge at *m/z* = 317.2 which could be derived from the protoned molecular ion *m/z* = 478.8 after the departure of the fragment corresponding to the mass162 g/mol ($C_6H_{10}O_5$). According to the literature, this fragment could correspond to a hexose. Finally, the pic *m/z* = 317.2 could represent the Flavonoid part ($C_{16}H_{11}O_7$). This hypothesis drawn from the LC/MS spectra was confirmed by High Resolution Mass Spectra (HRMS) which registered on positive mode a pic corresponding to the ion $[2M+Na]^+$ at *m/z* 979.228 and another pic at *m/z* 317.058. We can conclude that the compound 1 has a molecular weight of 478g/mol corresponding to the Molecular formula $C_{22}H_{22}O_{12}$ which could correspond to a flavonoid glycoside. This result was confirmed by NMR 1D and 2D analysis which were in accordance with the literature data.

The Proton NMR and COSY (H-H; L, R) spectra revealed the presence of two aromatic rings. The first one with 3 protons: (1H; 7.94ppm; d; 2Hz; $H_{2'}$); (1H;7.6ppm, dd, (2 Hz; 8.38Hz); $H_{6'}$) and (1H;6.92ppm; d; 8.38Hz; $H_{5'}$).

The second one displayed two aromatic protons (1H; 6.42ppm; d; 2Hz; H_6); (1H; 6.22ppm; d; 2Hz; H_8) in Meta position. The NMR proton spectra integration expressed also a singular signal (s) corresponding to 3 protons with a chemical shift □ □ □ □ = 4.2 ppm which is corresponding to a methoxy group ($-OCH_3$). The coupling constants and NMR COSY correlations confirmed that the compound contain a hexose part which is a β-glucose with an anomeric proton (1H; 5.42ppm; d; 7.48 Hz; $H_{1''}$) (Table 2). The HMBC spectra displayed a spot correlation between the carbon C_3 □ □ □ □ □ ppm and the anomeric proton $_1$H□ □ $H_{1''}$) □ □ □ □ □ □ □ □ ppm. This indication confirmed the exact attached position between the glucose and the flavonoid part (Table-2; Figure-2). From the UV data, LC/MS, HRMS, NMR 1D and 2D, we can conclude that the compound 1 is the Isorhamnetin-3-O-β-glucopyranoside (Figure 2). This result is confirmed by the literature (Bedir et al., 2000; Cui et al., 2003). But this is the first isolation of Compound 1 from the species *Senecio leucanthemifolius* poiret.

Compound 2 was isolated as a yellow powder from the *leucanthemifolius* Poiret flower methanolic extract. The UV spectrum showed a maximal absorbance at λmax (MeOH) = 254 and 354 nm, characteristic of flavonoids. The LC/MS spectra showed in the positive mode a molecular ion $[M+Na]^+$ at *m/z* = 663.1 and a signal at *m/z* = 478.9 which correspond to a flavonoid mono glycoside. The HRMS gave the molecular weight and the molecular formula of the compound 2, which

are respectively 640g/mol and $C_{28}H_{31}O_{17}$. In comparison with the mass spectra and the molecular weight of the compound 1, we found a difference of 162, which could correspond to a second hexose. That is to say, that compound 2 could be flavonoid bi-glycosides. In fact, we conclude from the NMR 1D and 2D data that compound 2 is an Isorhamnetin with two glucoses. Their exact positions were identified thanks to the HMBC experience. The anomeric proton of the first glucose (1H; 5.48ppm; d; 7.23Hz; $H_{1''}$) in β position correlate with the carbone □ □ □ C□ □ □ □ □ □ □ □ □ ppm. Whereas the second glucose anomeric proton □ □ □ □ □ □ .07 ppm; d; 7.28Hz; $_{1'''}$H in β position, correlate with the Carbone □ □ □ C□ □ □ □ □ □ □ □ □ ppm (figure 2 Table 2). From UV, LC/MS, HRMS, NMR 1D and 2D data, we deduce then, compound 2 is the Isorhamnetin-3, 7-O-β-di-glucopyranoside. This result is also compatible with the literature data (Lawrence et al. 2004; Wang et al., 2012). But this is the first isolation of Compound 2 from the species *Senecio leucanthemifolius* poiret.

Compounds 3 and 4:

The UV spectra is characteristic of flavonoids λmax = (MeOH) 254 and 354 nm. The LC/MS spectra indicated in the negative mode, the presence of a molecular ion $[M-H]^-$ *m/z* = 463.0 and a pic at *m/z*= 926.9 corresponding to $[2M-H]^-$. In the positive mode, we found two protonated molecular ions $[M+H]^+$ at *m/z* 464.9 and $[2M+Na]^+$ at *m/z* 950.7. In the same positive mode, we detected a signal at *m/z* 303.2, which is due to the departure of one Hexose (162).

The NMR ^{1}H spectra presents five aromatic protons: (1H; 7.94ppm; d; $H_{2'}$), (1H; 6.92ppm; d; $H_{5'}$), (1H; 7.6ppm; dd; $H_{6'}$), (1H; 6.42ppm; d; H_6), (1H; 6.22ppm; d; H_8) and 4 aliphatic protons with chemical shifts values ranging between 3.0 and 4.0 ppm. By the same elucidation method as the compounds 1 and 2 we therefore deduced that compounds 3 and 4 are Quercitin β- glucopyranoside. The HMBC spectra showed that in the compound 3, the anomeric proton (1H; 5.22ppm; d; 7.5Hz; $H_{1''}$) correlates with the carbon □ □ □ C3)□ □ 133.1 ppm. However, in the compound 4, the anomeric proton $H_{1''}$ correlate with the carbon □ □ □ C□ □ □ □ 165.0 ppmWe conclude that Compound 3 and 4 are respectively Quercitin-3-O- β-glucopyranoside and Quercitin-7-O- β-glucopyranoside. (Figure 3 and 4, Table 2). These results are confirmed with the literature data (Hassan et al., 2012; Mabry et al., 1970; Markham, 1982). But this is the first isolation of compounds 3 and 4 from *Senecio leucanthemifolius* poiret.

Compound 5 was obtained as a white powder from the methanolic fraction of *Senecio leucanthemifolius* Poiret flower ethyl acetate extract. The UV spectrum presents a maximal absorbance λmax (MeOH) at 220.0, 238.0 and 322.0 nm. The mass spectra in the positive mode highlighted the presence of the molecular ion $[2M+Na]^+$ at *m/z* = 268.4 and the ion $[M-H2O]^+$ detected at *m/z* = 162.9. The High resolution mass spectra (HRMS/ESI) showed in the negative mode a pic at à *m/z*= 179.010 corresponding to the molecular ion $[M-H]^-$. Similarly, it confirmed the molecular weight and the molecular formula which are respectively 180.16 g/mol and $C_9H_8O_4$.

The NMR ^{1}H spectra integration revealed the presence of five protons including 3 aromatic protons. The NMR ^{13}C spectra confirms that the molecule is made up of 9 carbons. One of them was detected at a chemical shift □ □ □175.71 ppm. This Carbone could correspond to an unsaturated acid function. This information was later confirmed by the HMBC experience. The NMR (2D) COSY and HMQC confirmed that the structure has an aromatic ring with 6 carbons and 3 protons. The aromatic proton H_6 data (1H; 6.93ppm; dd; (2.0; 8.0Hz)) explain the Ortho and Meta positions on the aromatic ring. (Figure 5, Table 3). The acid function is outside the aromatic ring. They could be linked together by a lateral olifenic system constituted by 2 carbons: $C_{2'}$ □ □149.71ppm and $C_{1'}$□ □116.37ppm. Each carbon of them has its own proton: $H_{2'}$ (1H; 7.51ppm; d; 16.0Hz) for $C_{2'}$ and $H_{1'}$ (1H; 6.22ppm; d; 16.0Hz) for $C_{1'}$. The double band between these two (-CH) groups has a Trans configuration (*E*). From the NMR 1H, 13C, COSY, HSQC and HMBC data we deduce that compound 5 is a Trans (E) Caffeic acid. This is the first isolation of compounds 3 and 4 from *Senecio leucanthemifolius* poiret.

Compounds 6 and 7 were obtained as a white power and isolated from the methanolic fraction of *Senecio leucanthemifolius* Poiret flowers ethyle acetate extract. The UV spectra showed an optimal absorbance λmax (MeOH) at 220 nm. The mass spectra in the positive mode revealed the presence of an ion $[2M+Na]^+$ detected at *m/z*= 454.8. The molecular weight is 216.23 g/mol corresponding to the molecular formula $C_{10}H_{16}O_5$.

The NMR ^{13}C experience indicated the presence of 10 carbons including the function acid carbon which was detected at □ □C_1) =180.03ppm (Table 4). The integration of NMR ^{1}H spectrum displayed the presence of the aliphatic protons only: Three methyl groups (-CH3) (3H; 1.34ppm; s; H_2'); (3H; 0.86ppm; d; 6.01Hz; $H_{3'}$) and (3H; 1.92ppm; dd; (0.91, 7.01Hz); H_7), one methylene group (-CH2) (2H; 1.99ppm; large (d); 7.29 Hz; H_4) and two methine groups (–CH) (1H; 2.47ppm; q;(9.19 ; 18.01Hz); H_3) and H_6 (1H; 5.96; ;q ;(7.05 ; 14.1Hz)) (Figure 6 and 7, Table 4). The NMR 2D COSY, HSQC and HMBC spectra explain respectively the correlations between protons, and the correlations between proton and carbons. Finally, the NMR 1D and 2D data and their correlations were sufficient to elucidate the chemical structure of the stereoisomers 6 and 7 which are Senecic acids. The difference between the two Senecic acids 6 and 7 is from one side the absolute configuration due to the presence of two asymmetric carbons C_2 and C_3. From the other side the configuration Cis *(Z)* and Trans (*E*) due to the double

Table-2. NMR data of Compounds 2; 1; 3 and 4

Position	Compounds							
	2		1		3		4	
	δ (^{1}H) (ppm); n ; m; J(Hz)	δ (^{13}C) (ppm)	δ (^{1}H) (ppm); n; m; J(Hz)	δ (^{13}C) (ppm)	δ (^{1}H) (ppm) ;n; m; J(Hz)	δ (^{13}C) (ppm)	δ (^{1}H(ppm);n; m; J(Hz)	δ (^{13}C) (ppm)
2	-	159.8	-	158	-	158.0	-	158.0
3	-	135.5	-	133	-	133.1	-	134.8
4	-	149	-	178	-	178.1	-	178.1
5	-	95.9	-	157	-	157.0	-	157.0
6	6.80; 1; d; 2.1	93.5	6.42; 1; d; 2	93	6.42 ; 1 ; d	93.3	6.42 ; 1; d	93.3
7	-	164.2	-	165	-	165.0	-	165.0
8	6.51; 1; d; 2.09	98.5	6.22 ; 1 ; d ; 2	98	6.22 ; 1 ; d	98.1	6.22 ; 1 ; d	98.1
9	-	163	-	162	-	162.2	-	162.2
10	-	107.5	-	105	-	105.0	-	105.0
1'	-	124	-	122	-	122.1	-	122.1
2'	7.95; 1; d; 1.99	178.6	7.94 ; 1 ; d ; 2	113	7.94 ; 1 ; d	113.2	7.94 ; 1 ; d	113.2
3'	-	148	-	147	-	147.0	-	147.0
4'	-	149.7	-	150	-	150.0	-	150.0
5'	6.92 ; 1 ; d ; 8.31	113.7	6.92 ;1 ;d ; 8.38	115	6.92 ; 1 ; d	115.1	6.92 ; 1 ; d	115.1
6'	7.63;1 ; dd ; (8.32 ; 2.0)	164.2	7.6 ;1 ;dd ; (2 ; 8.38)	124	7.6 ; 1 ; dd	123.9	7.6 ; 1 ; dd	123.9
1"	5.48 ; 1 ; d ; 7.23	100.8	5.42 ;1 ; d ;7.48	103	5.22 ; 1 ; d	103.0	5.22 ; 1 ; d	107.0
2"	3.47 ; 1 ; dd	74.2	3.45;1;m	74	3.47 ; 1 ; m	75.7	3.47 ; 1 ; m	75.7
3"	3.54;1 ; q	77.03	3.47;1;m	77	3.51 ; 1 ; m	77.0	3.51 ; 1 ; m	77.0
4"	3.40;1 ; m	69.76	3.31;1; m	70	3.35 ; 1 ; m	71.1	3.35 ; 1 ; m	71.1
5"	3.30 ; 1 ; m	70.1	3.25;1;m	78	3.25 ; 1 ; m	78.2	3.25 ; 1 ; m	78.2
6"a	3.91 ; 1 ; dd ; (2.33 ; 12.21)	59.9	3.73;1;dd; (2.24 ;11.93)	61	3.85 ; 1 ;dd	61.1	3.85 ; 1 ;dd	61.1
6"b	3.70; 1 ; dd ; (5.95 ; 12.26)	59.7	3.57; 1 ; dd ; (5.59 ; 11.97)	61	3.6 ; 1 ; dd	61.1	3.6 ; 1 ; dd	61.1
-O-CH_3	3.95 ; 3 ; s	54.8	4.2 ; 3 ; s	55				
1"'	5.07 ; 1 ; d ; 7.28	99						
2"'	3.45 ; 1 ; m	76.39						
3"'	3.49 ; 1 ; dd	76.28						
4"'	3.36 ; 1 ; m	69.6						
5"'	3.25 ; 1 ; m	77						
6"'a	3.73 ; 1 ; dd ; (3.25 ; 11.98)	60.3						
6"'b	3.56 ; 1 ; dd ;(5.59 ; 12.03)	60.1						

δ (^{1}H) (ppm): Proton chemical shift; n: Number of ^{1}H; m: multiplicity ; J(Hz): coupling constant; δ (^{13}C) (ppm): Carbon chemical shift

Table-3. NMR data of Compound 5

	1H				
Position	**Number of ^{1}H**	**^{1}H (ppm)**	**Multiplicity (m)**	**J (Hz)**	**(^{13}C (ppm)**
1	-	-	-	-	128.23
2	1	7.03	d	2.0	115.36
3	-	-	-	-	147.14
4	-	-	-	-	147.00
5	1	6.78	d	8.0	116.81
6	1	6.93	dd	2.0 ; 8.0	123.11
1'	1	6.22	d	16.0	116.37
2'	1	7.51	d	16.0	149.71
3'	-	-	-	-	175.71

Table 4 NMR data of Compounds 6 and 7

	^{1}H				
Position	**^{1}H (ppm)**	**Number of ^{1}H**	**Multiplicity (m)**	**J (Hz)**	**(^{13}C (ppm)**
1	-	-	-	-	180.03
2	-	-	-	-	78.04
2'	1.34	3	s	-	24.37
3	2.47	1	q	9.19 ; 18.01	38.32
3'	0.86	3	d	6.01	13.22
4	1.99	2	Large (d)	7.29	41.45
5	-	-	-	-	134.06
5'	-	-	-	-	172.02
6	5.96	1	q	7.05 ; 14.1	138.39
7	1.92	3	dd	0.91 ; 7.01	16.16

bond. This later was studied thanks to the NMR NOESY experience. Indeed, the compound 6 NOESY spectra revealed a correlation between the methylene group protons (-CH2) (1.99ppm; large (d); 7.29 Hz; H_4) and the methyl group protons (1.92ppm; dd; 0.91, 7.01Hz; H_7). This outcome demonstrates that compound 6 is a Senecic acid with a configuration Trans (*E*) (Figure 6). The NOESY spectra of compound 7 showed a correlation between the methylene group protons (-CH2) (2H; 1.99ppm; large (d); 7.29 Hz; H_4) and the (-CH) methine group proton (1H; 5.96ppm; q ;(7.05; 14.1Hz); H_6). From this observation we can conclude that compound 7 is a Cis (*Z*) Senecic acid (Figure 6). The Senecic acid is isolated for the first time from *Senecio leucanthemifolius* poiret.

Evaluation of the cytotoxicity activity:

Isorhamnetin, 3-O- β- glucopyranoside (Compound1), Isorhamnetin, 3, 7-O- β- glucopyranoside (Compound 2), Caffeic Acid (Compound5) and Senecic Acid (Compound 6) are the isolated and purified secondary metabolites tested at well determined concentrations on the MFC7 cell lines (Table-5).

Table-5. The isolated and purified secondary metabolites tested with the MFC7 cell lines at determinate concentrations

	Compound 1	**Compound 2**	**Compound 6**	**Compound 5**
The Initial Solution (mM)	15	12	32	38
The tested Concentration (µM)	15	12	32	38

The curves below represent the variations of the cells viability according to the treatment period (Fig.7, 8, 9, 10). The cellular viability was determined by the

Trypan blue test. The percentage of the cell growth was obtained by the CellTiter 96® AQueous test.

Figure-2. HMBC Correlations of Isorhamnetin- 3-O-β- glucopyranoside (compound 1)

Figure-3. HMBC Correlations of Isorhamnetin- 3,7-O- β-di- glucopyranoside (compound 2)

Figure-4. HMBC Correlations of Quercitin-3-O-β-glucopyranoside (compound 3)

Treatment, in comparison with untreated cells (Fig-8. and 9). This Inhibition is proportionate to the treatment period. The number of viable cells represents it after the treatments by the isolated compounds. However, the compounds 5 and 6 showed a very moderate inhibition of the MCF7 cell growth. After this selective result, only compounds 1 and 2 were chosen to continue the Cell growth inhibition test on the K562 HT1080 and HL60 cancer cell lines. The the cytotoxic activity in-vitro evaluation manifested a modest antiproliferative activity with the tested concentrations from 0.625 to 20 µM (Fig.12 and 13).

Figure-5. Chemical structure of Quercitin-7-O- β-glucopyranoside (Compound 4)

Figure-6. Chemical structure of Caffeic acid (Compound 5)

Figure-7. NOESY Correlations of Compounds 6 and 7: Compound 6: Trans (*E*) Senecic acid

Compound 7: Cis (*Z*) Senecic acid

Before starting the antiproliferative test, we referred to the literature data to choose the adequate compounds, which will be tested as antiproliferative agents. Compounds 1; 2; 5 and 6 were chosen as original molecules. According to the literature, it has

been proved there was no previous concerning their cytotoxic activity. The published literature data describe the important antiproliferative activity similar molecules of compounds 1 and 2.

Figure-8. Effect of Isorhamnetin, 3-O- β-glucose (Compound 1) at a determinate concentration of 15 µM on the MFC7 cell line viability according to the treatment period (1,2,3 and 4 days).

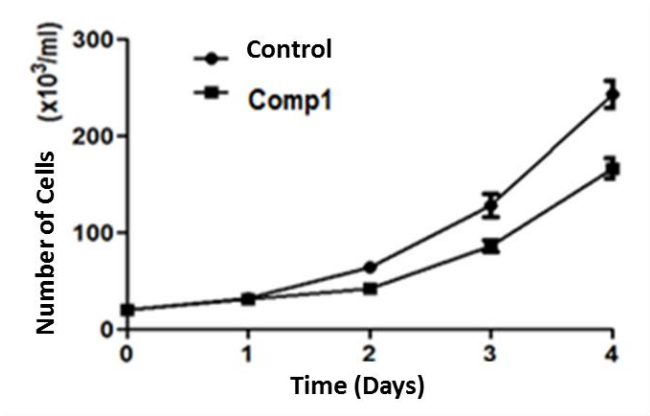
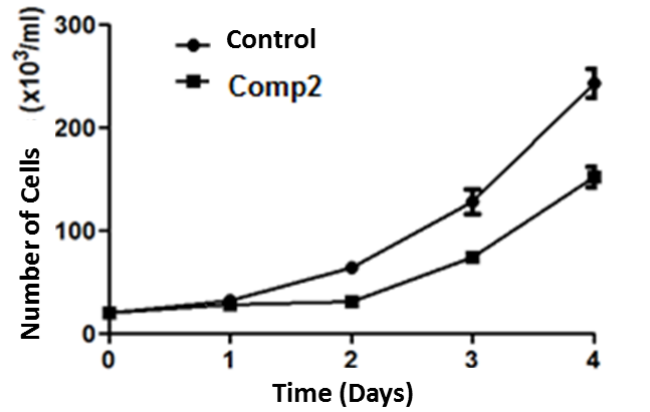


Figure-9. Effect of Isorhamnetin, 3,7-O- β-di-glucose (Compound 2) at a determinate concentration of 12 µM on the MFC7 cell line viability according to the treatment period (1,2,3 and 4 days).



In fact Isorhamnetin without glucopyranoside part can induce an antiproliferative effect of Skin epithelium carcinoma A431 cells with an IC_{50} = 10 µg/ml. Other publications explain the Isorhamnetin cytotoxic and apoptosis activities with an IC_{50} close to 50 µg/ml (Lee et al., 2008; Ma et al., 2007; Teng et al., 2006). In our antiproliferative test, we have noticed that during 96 h and up to a maximal tested concentration of 20 µM, compounds 1 and 2 didn't have any significance cytotoxicity activity on HL60, K562, MCF7 and HT1080 cell lines. This observation is different from what Lee and al. have obtained from the test of Isorhamnetin (without glucopyranoside part) on A431 cell lines (Kim et al., 2011). However, Seo and al. discovered the compound 1 inhibition effect on the matricial metalloproteinase activation (Kong et al., 2008).

Figure-10. Effect of Cafeic acid (Compound 5) at a determinate concentration of 38 µM on the MFC7 cell line viability according to the treatment period (1,2,3 and 4 days).

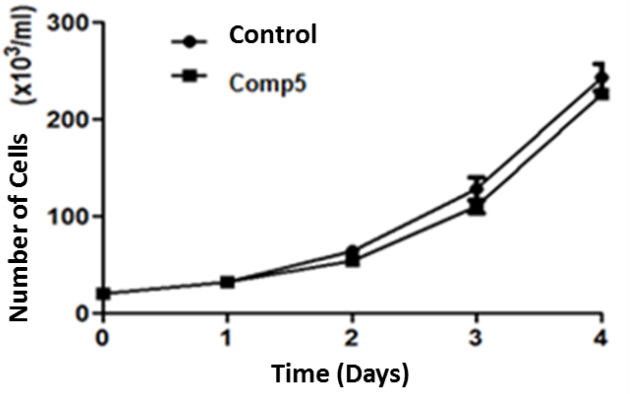


Figure-11. Effect of Senecic acid (Compound 6) at a determinate concentration of 32 µM on the MFC7 cell line viability according to the treatment period (1,2,3 and 4 days).

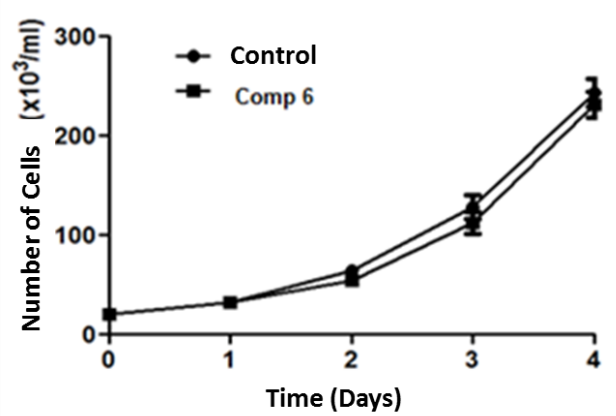
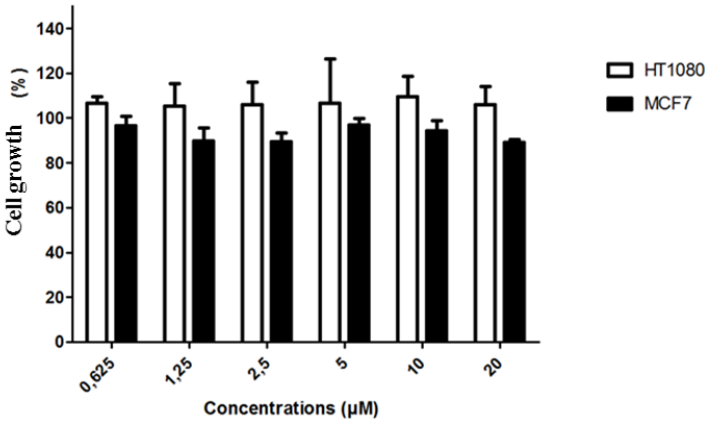


Figure 12. Effect of the Isorhamnetin, 3-O- β-glucose (Compound1) in different concentrations on the cell growth of HL60; K562 (A) and HT1080; MCF7 (B) cell lines. The data are obtained with the CellTiter 96® AOueous test.



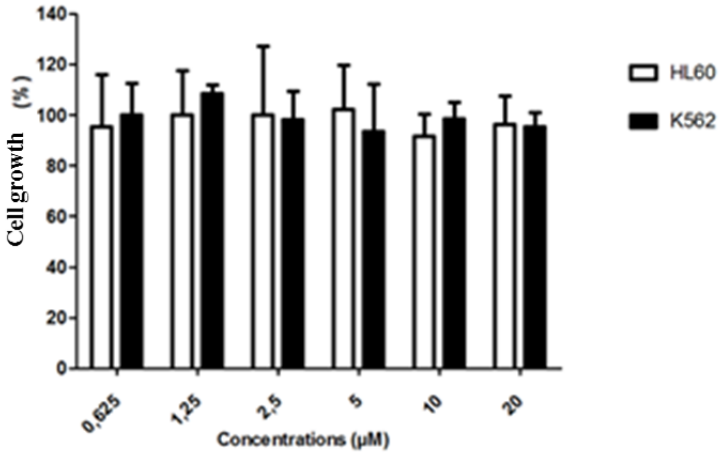
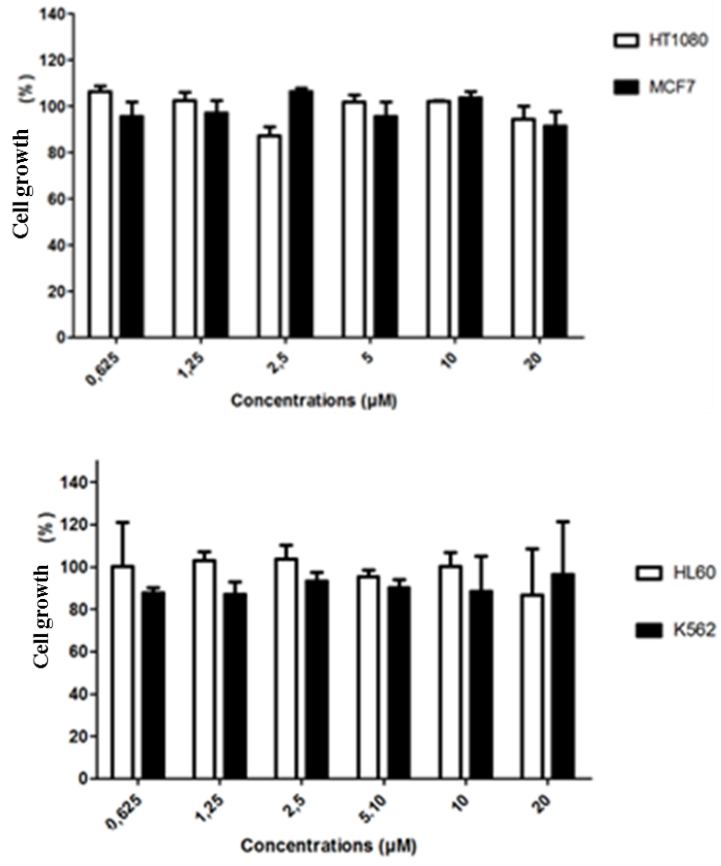


Figure-13. Effect of Isorhamnetin, 3,7 -O- β-di-glucose (Compound 2) in different concentrations on the cell growth of HL60 ; K562 (A) and HT1080 ; MCF7 (B) cell lines. The data are obtained with the CellTiter 96® AQueous test.



Other publications explain that some Quercitin derivatives such as Quercitin- rhamnose or rhutinose yielded a good inhibition effect on the tumorale increase by blocking the matricial metalloproteinase activation with respect to the MCF7 cell lines (Lin et al., 2008).

Conclusion

Is seems that Quercitin glucopyranoside and isorhamnetin glucopyranoside are the principal flavonoids produced by the *Senecio leucanthemifolius* poiret Species growing in Morocco, in addition of caffeic acid and senecic acid which is characteristic of the *Senecio* Genus. These secondary metabolites are isolated and descripted for the first time from *Senecio leucanthemifolius* Poiret. To sum up their cytotoxic activity on MCF7; K562; HT1080; HL60 cell lines, we can justify that the lack of efficiency of compounds 1 and 2 on the grounds of the inability to put an end to the proliferation of these cell lines categories. Moreover, the presence of glucopyranoside part in our molecules is likely not really an incentive to get a good antiproliferative activity. Knowing that basically, a lot of research groups describe the Isorhamnetin cytotoxic and antiproliferative activities without containing the glucopyranoside part.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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