# Bio-guided studies of Lotus maritimus aerial parts and investigation of their antioxidant, tyrosinase and elastase inhibitory activities

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# Bio-guided studies of *Lotus maritimus* aerial parts and investigation of their antioxidant, tyrosinase and elastase inhibitory activities

Short Title: **antioxidant, elastase and tyrosinase inhibitory activities of *Lotus maritimus* aerial parts**

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**Abstract:**

***Background :*** *Lotus maritimus* L. (Fabaceae) is a perennial herb species with yellow flowers, growing in Europe, Asia, Middle East and Maghreb. Some flavones and flavonols have been identified in their leaves and flowers. Their leaf extract was used as cellulolytic and antiaging cosmetic ingredient.

***Objective :*** The aim of this work was to performed antioxidant, anti-tyrosinase and anti-elastase properties of *L. maritimus* aerial parts and their chemical profile.

***Methods :*** A $^{13}C$ NMR-based dereplication method combined to a bio-guided purification, was used for metabolite identification. Chemical structures were determined by NMR and ESI-MS spectroscopic methods. The antioxidant properties of the fractions and purified compounds was measured using CUPRAC, DPPH and hydroxyl radical scavenging assays. Their inhibitory activities against the fungal tyrosinase and human neutrophil elastase enzymes were also evaluated.

***Results :*** EtOAc and *n*-BF fractions were characterized as the most active parts. Twenty-two compounds were identified from these fractions by using a $^{13}C$ NMR-based dereplication method. This process was completed by the purification of minor compounds in the *n*-BuOH fraction. Eight known compounds were isolated, including many mono-glycosylated flavonoids with variable substitutions on the B-ring, allowing structure-activity relationships.

***Conclusion :*** Twenty-two compounds including phenolic acids, flavonoids and glycoside derivatives, were firstly described in *L. maritimus*. Three quercetin and myricetin-type flavonoids exhibited good antioxidant activities and all flavonoids tested have moderate effects on elastase inhibition.

*Keywords: Lotus maritimus*, Fabaceae, flavonoid, dereplication, antioxidant, tyrosinase, elastase

## 1. INTRODUCTION

Fabaceae, also known as Leguminosae is an economically important family of flowering plants. A few plants from this family have cosmeceutical applications, in skin care or depigmentation formulations [1, 2]. The aerial parts (flowers and leaves) of Fabaceae are a rich source of flavonoids and phenolic compounds, which could be used to slow down skin aging through antioxidant or anti-hyperpigmentation activities [3, 4, 5, 6] but also through extracellular matrix remodeling via an elastase inhibitory effect [7]. The genus *Lotus* (Fabaceae) includes one hundred species, mainly present in the Mediterranean regions, Europe, Africa and Asia. Chemical studies carried out of the genus *Lotus* have revealed flavonoids [8, 10], phenols, isoflavonoids [9], saponins [10] triterpenes and glycosides [11]. *Lotus* species are traditionally used for their contraceptive, anticancer, anti-inflammatory, and antimicrobial properties. Recently, the chemical profiles and biological activities of *Lotus corniculatus* extract were evaluated and reports their antioxidant and anti-tyrosinase activities and that quercetin derivatives geraldol, isorhamnetin and kaempferol-O-coumaroylhexoside-O-deoxyhexoside isomers were dominant in the extracts [10].

In the continuation of our investigation of local plants with anti-aging potential [12, 13], we investigated the chemical composition of *Lotus maritimus* L. also known as *Tetragonolobus siliquosus* L., a perennial herb species in the subfamily Faboideae [14, 15]. *L. maritimus* grows in Europe, Asia, Middle East and Maghreb [16]. Some flavones and flavonols have been identified from leaves and flowers of *L. maritimus* [8], as well as isoflavones and phenols such as benzofuran after fungus inoculation [9]. *L. maritimus* leaf extract was described as active cosmetic ingredient in Canada as antiaging and anticellulite [17] due in part to their antioxidant property but their chemical profile was not study so far.

This work presents the chemical characterization of *L. maritimus* aerial parts using a $^{13}C$ NMR-based dereplication method combined with a bio-guided fractionation and purification process. Antioxidant, tyrosinase and elastase inhibitory activities were evaluated on the crude MeOH extract, fractions and purified compounds.

## 2. MATERIAL AND METHODS

### 2.1. General experimental procedures

$^{1}H$-, $^{13}C$-NMR and 2D-NMR spectra were recorded on a Bruker Avance AVIII-600 spectrometer equipped with a 5 mm TCI cryoprobe (Karlsruhe, Germany). ESI-MS spectra were recorded using a Micromass Q-TOF micro instrument (Manchester, UK).

Centrifugal partition chromatography (CPC) fractionations were performed on a lab-scale FCPE300® apparatus (Rousselet Robatel Kromaton, Annonay, France) equipped with a column of 303 mL capacity and engraved with 231 partition twin-cells (≈ 1 mL per twin cell). The solvents were pumped by a KNAUER Preparative 1800 V7115 pump (Berlin, Germany). The column was coupled on-line with a UVD 170 S detector (Dionex, Sunnivale, CA, USA) monitored at 210, 254, 280 and 366 nm. Fractions of 20 mL were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The column rotation speed was set at 1200 rpm and the flow rate at 20 mL/min.

Preparative HPLC was performed on Armen Instrument apparatus equipped with an AP 250 pump and a Knauer (Merck) detector UV K-2501, and using a manually packed $C_{18}$ column (LiChrospher, 20 × 5 cm, 12 µ). The mobile phase consisted of $H_2O$ acidified with TFA (0.0025%) and $CH_3CN$ with a flow rate of 100 mL/min. The chromatograms were monitored at 205, 254, 300 and 360 nm. Semi-preparative HPLC was performed on a

Dionex apparatus equipped with an ASI-100 autosampler, a STH 585 column oven, a P580 pump, a diode array detector UVD 340S and the Chromeleon® software version 6.8. A prepacked $C_{18}$ column (Interchim, 250 × 10 mm, 5 µ) was exploited for semi-preparative HPLC. The mobile phase consisted of $H_2O$ with TFA (0.0025%) and $CH_3CN$ with a flow rate of 5 mL/min and the chromatograms monitored at 205, 254, 300 and 360 nm.

Analytical HPLC experiments were realized using a Thermofisher Ultimate 3000 (Thermo Fischer Scientific, Villebon-sur-Yvette, France), equipped with a 4 ways pump LPG 3400 SD, an automatic injector WPS 3000 SL, a UV/visible diode array detector 3000 and the Chromeleon® software version 6.8. A prepacked $C_{18}$ column Uptisphere Strategy $C_{18}$ (Interchim, 250×4.6 mm, 5 µ) was selected for analytical HPLC with a mobile phase composed of $H_2O$ with TFA (0.0025% v/v) and $CH_3CN$, and a flow rate of 1 mL/min. The chromatograms were monitored at 205, 254, 300 and 360 nm.

Thin-layer chromatography (TLC) was carried out on silica gel 60 $F_{254}$ pre-coated aluminum plates (0.2 mm, Merck), using the system $CHCl_3$/MeOH/$H_2O$ (70/30/5, v/v/v) as the mobile phase. The spots were visualized under UV light (254 and 366 nm) using high-performance thin-layer chromatography (HPTLC, CAMAG TLC Visualizer 2) and sprayed with 50% $H_2SO_4$ followed by heating.

A FLUOstar Omega spectrophotometer (BMG LABTECH) was used for measuring the absorbance of antioxidant and anti-tyrosinase assays. An Infinite F200 PRO spectrofluorimeter (Tecan, Lyon, France) was used for measuring the fluorescence of anti-elastase assay.

**2.2. Plant material**

The aerial parts of *Lotus maritimus* L. (Fabaceae) were collected in Cormontreuil (Northeastern of France: 49°21′67″N, 4°05′E) in May 2016 and dried at room temperature. A Voucher specimen was deposited under the reference (MA-LM-2016-05) at the Herbarium of the Botanic laboratory of the Faculty of Pharmacy, University of Reims Champagne-Ardenne, after authentication by Dr. Abdulmagid Alabdul Magid.

**2.3. Extraction and isolation**

The dried and powdered *L. maritimus* aerial parts (144 g) were macerated in 80% MeOH (1.5 x 3 L, 24 h) at room temperature, followed by concentration at 40 °C under vacuum to remove the methanol. An aliquot of the resulting aqueous solution (100 mL) was evaporated to dryness to obtain the hydromethanolic extract (HME) (6.3 g). The aqueous solution (1 L) was extracted successively with dichloromethane (3 x 500 mL), ethyl acetate (3 x 500 mL) and *n*-butanol (3 x 300 mL), to give after evaporation under reduced pressure, three extracts: DCMF (2.0 g), EAF (0.7 g), *n*-BF (2.6 g), and the resulting water-soluble part (31.1 g).

**2.4. Centrifugal partition chromatography**

Fraction EAF (680 mg) was dissolved in 10 mL of a mixture of both lower phase (8 mL) and upper phase (2 mL) of the biphasic solvent system composed of M*t*BE/$CH_3CN$/water (3/3/4, v/v/v). The upper phase was pumped for 80 min in the ascending mode at 20 mL/min. Then the column was extruded by pumping the organic phase in the descending mode. The collected fractions were analyzed by TLC and HPLC to give twelve fractions $EAF_{1\text{-}12}$.

Fraction *n*-BF (2.1 g) was dissolved in 25 mL of a mixture of both lower phase (20 mL) and upper phase (5 mL) of the biphasic solvent system composed of M*t*BE/$CH_3CN$/water (4/1/5, v/v/v) and the upper phase was pumped during 120 min before extrusion of the column. Fractions *n*-$BF_{1\text{-}13}$ were obtained after TLC and HPLC analysis.

**2.5. NMR analyses and dereplication of the metabolites**

As a first step of $^{13}C$ NMR-based dereplication [18], structures and names of metabolites already described in the genus *Lotus* (n=14) were collected from the literature. The structures and the predicted $^{13}C$ NMR chemical shifts were stored into a database (NMR Workbook Suite 2012, ACD/Labs, Ontario, Canada). Then, all dry CPC fractions were dissolved in 600 µL of DMSO-$d_6$ ($\delta$ 39.8 ppm) and analyzed by $^{13}C$ NMR. A standard zgpg pulse sequence was used with an acquisition time of 0.9 s, a relaxation delay of 3 s and 1024 scans [12]. All $^{13}C$ NMR signals were automatically collected on each spectrum and the absolute intensities were calculated and stored as a text file [12]. The resulting table was imported into the PermutMatrix version 1.9.3 software (LIRMM, Montpellier, France) and submitted to Hierarchical Clustering Analysis (HCA) for data visualization. Then the chemical shift clusters obtained by HCA were used as search keys in the structure and spectra database to tentatively identify the compounds. To validate the proposed structures, additional $^{1}H$ NMR, HSQC, HMBC, and $^{1}H$-$^{1}H$-COSY spectra were also registered for all fractions.

**2.6. HPLC purification of active fractions of *n*-BF**

Fraction *n*-$BF_3$ (214 mg) was submitted to preparative HPLC eluting with isocratic solvent (25% $CH_3CN$, 45 min) to give compound **1** ($t_R$=2.7 min, 7.9 mg). Subfraction 12-22 (46 mg) was purified by semi-preparative HPLC with an isocratic system (25% $CH_3CN$, 10 min) to give compounds **2** ($t_R$=5.0 min, 12 mg), **3** ($t_R$=5.8 min, 5 mg) and **4** ($t_R$=6.7 min, 17.7 mg). Subfraction 37-45 (20 mg) was purified by semi-preparative HPLC eluting with isocratic solvent (25% $CH_3CN$, 10 min) to yield compounds **4** ($t_R$=6.7 min, 2.4 mg) and **5** ($t_R$=8.9 min, 8.6 mg). Fraction *n*-$BF_5$ (179 mg) was submitted to preparative HPLC eluting with isocratic eluent (25% $CH_3CN$, 45 min) to give compounds **1** ($t_R$=2.7 min, 2.4 mg), **6** ($t_R$=3.9 min, 2.3 mg), **7** ($t_R$=4.9 min, 5 mg) and **8** ($t_R$=6.4 min, 3.8 mg).

**2.7. DPPH radical scavenging activity**

The free radical scavenging capacity was determined by using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical [19]. Hydromethanolic (HEM), dichloromethane (DCMF), ethylacetate (EAF) and *n*-butanolic (*n*-BF**)** extracts, fractions (*n*-$BF_3$-*n*-$BF_6$), and pure compounds **2**-**8** were tested for their DPPH radical scavenging activity using the procedure described in Schmitt et al. [12]. Samples were prepared in triplicate at concentrations ranging from 25 to 200 µg/mL, dissolved in $H_2O$/DMSO (9/1, v/v). The control sample was prepared in EtOH 50%. Ascorbic acid and quercetin were used as positive controls. The $IC_{50}$ values correspond to 50% inhibition.

**2.8. Hydroxyl radical scavenging activity**

The hydroxyl radical scavenging capacities of extracts (HEM, DCMF, EAF, *n*-BF), fractions (*n*-$BF_3$-*n*-$BF_6$), and pure compound **4** were assayed following the procedure described in Schmitt et al. [12]. The absorbance of the hydroxylated salicylate complex was measured at 562 nm, and ascorbic acid and quercetin were used as positive controls. Samples were prepared in triplicate at concentrations of 1330, 665, 332.5, 166.25, 83.12 and 41.56 µg/mL dissolved in $H_2O$/DMSO (9/1, v/v).

**2.9. Power cupric ion reducing (CUPRAC)**

The cupric ion reducing activity (CUPRAC) of extracts, fractions, and pure compounds **1**, **2** and **4**-**8** were assayed following the procedure described in Schmitt et al. [12]. Samples were prepared in triplicate at concentrations of 572, 286, 143, 71.5, 35.75, 17.87, 8.94, 4.47, 2.23, 1.12 and 0.56 µg/mL and dissolved in $H_2O$/DMSO (9/1, v/v). Ascorbic acid, quercetin and trolox were used as positive controls.

**2.10. Tyrosinase enzyme assay**

The inhibitory effects of extracts, fractions, and pure compounds **2**, **4** and **5** on mushroom tyrosinase (Sigma) activity, using L-DOPA as substrate, were determined following the procedure described in Schmitt et al. [13]. All the measurements were conducted in triplicate at concentrations of 1330, 665, 332.5, 166.25 and 83.12 µg/mL dissolved in the mixture phosphate buffer solution (PBS, 20 mM, pH 6.8) and DMSO 9/1 (v/v). Kojic acid was used as a positive control.

**2.11. Elastase enzyme assay**

Elastase inhibition measurement of extract, fractions, and pure compounds **3-5**, **7** and **8** were carried out using human neutrophil elastase also called Human Leucocyte Elastase (HLE) (Merck Biosciences, Ref: 324681). This assay was performed in pre-coated 96-well microplates with 1% Serum Albumin Bovine. HLE (0.8 µM) was incubated for 1 h at 37°C in Tris buffer (50 mM Tris-HCl pH 7.5 containing 500 mM NaCl) containing 0.1 to 1000 µg/mL of tested sample. Sample solvent was used as a control. The assay was initiated by adding HLE fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-AMC ($\lambda_{exc}$ = 380 nm/ $\lambda_{em}$ = 460 nm) at a final concentration of 80 µM. The rate of each substrate cleavage was measured in triplicate for each concentration with one measure per minute for 60 min. HLE activity was calculated according to the following equation: % HLE activity = ($Slope_{sample}$ x 100)/$Slope_{control}$, where $slope_{sample}$ and $slope_{control}$ are the slope of the fluorescence values reported as a function of time. Non-linear regression analysis with Graphpad software (La Jolla, USA) was used to calculate the $IC_{50}$ values.

## 3. RESULTS

### 3.1. Biological screening of crude extract and fractions of *L. maritimus* aerial parts

The water-soluble fraction of a hydro-methanolic extract (HME) of the aerial parts of *L. maritimus* was partitioned successively by using solvents of increasing polarity, into three fractions with dichloromethane (DCMF), ethyl acetate (EAF) and *n*-butanol (*n*-BF). The antioxidant potential of HME and its fractions was estimated by three chemical tests, the measurement of their radical scavenging capacity using the DPPH and hydroxyl radical assays and the evaluation of their cupric reducing capacity with the CUPRAC assay. Their capacity to inhibit human neutrophil elastase and mushroom tyrosinase activity was also measured. As resumed in Table 1, the best antioxidant activities were observed in the order: EAF>*n*-BF>DCMF>HME. The fractions EAF and *n*-BF showed significant cupric ion reducing ability ($IC_{50}$ 0.9 µg/mL and 2.8 µg/mL, respectively), as well as good DPPH radical scavenging activity ($IC_{50}$ 27.8 µg/mL and 42.7 µg/mL, respectively) and a moderate hydroxyl radical scavenging activity ($IC_{50}$ 107.3 µg/mL and 347.5 µg/mL, respectively), compared to the standard used. The fractions EAF and *n*-BF showed a moderate elastase inhibitory potential ($IC_{50}$ 68.7 µg/mL and 144.9 µg/mL, respectively), higher than HME ($IC_{50}$ 578.8 µg/mL). While moderate tyrosinase inhibitory activity was observed for EAF ($IC_{50}$ 132.2 µg/mL) and DCMF ($IC_{50}$ 120.0 µg/mL) fractions, compared to kojic acid used as standard. As, the EAF and *n*-BF fractions showed the most interesting biological activities, we chose to study them chemically through a bioassay-guided isolation strategy and tentatively determine the active constituents.

### 3.2. Chemical profiling of the EtOAc fraction (EAF)

The major compounds of EAF were identified using a dereplication method combining Centrifugal Partition Chromatography (CPC) fractionation with NMR analyses and recognition of metabolite spectral data, without purification of individual compounds [18]. The CPC fractionation of EAF was performed by using the

biphasic solvent mixture M*t*BE/$CH_3CN$/water (3/3/4, v/v/v), to give twelve CPC sub-fractions ($EAF_{1-12}$). After $^{13}C$ NMR analyses of $EAF_{1-12}$, all spectra of the fraction series were processed. After Hierarchical Clustering Analysis (HCA) of $^{13}C$ NMR signals the heat map was obtained and revealed 12 major chemical shift clusters, colored in yellow, corresponding to the major metabolites (Fig. 1).

The search in the locally build database containing predicted $^{13}C$ NMR chemical shift values of natural metabolites related the chemical shifts of cluster 1 in fractions $EAF_{11-12}$ led to the identification of pinitol. This structure was easily confirmed by interpretation of the HSQC, HMBC and COSY spectra of fraction $EAF_{11}$ and by chemical shift comparison with literature data [20]. Using the same strategy, clusters 2 to 9 were identified as: quercetin-3-*O*-β-D-glucopyranoside (compound **2**) [21] (cluster 2; fractions $EAF_{3-6}$), isorhamnetin-3-*O*-β-D-glucopyranoside (compound **4**) [22] (clusters 2, 2' and 5; fractions $EAF_{3-4}$), laricitrin-3-*O*-β-D-glucopyranoside (compound **7**) [23] (cluster 3; fractions $EAF_{4-6}$), myricetin-3-*O*-β-D-glucopyranoside (compound **6**) [24] (cluster 4; fractions $EAF_{4-6}$), kaempferol-3-*O*-β-D-glucopyranoside [25] (clusters 5 and 5'; fraction $EAF_3$), a mixture of *Z*/*E*-*p*-coumaric acid [26], *p*-hydroxybenzoic acid [27] and linoleic acid [28] (cluster 6; fractions $EAF_{1-2}$), a mixture of 3-phenyllactic acid [29] and phenylacetic acid [30] (clusters 7 and 7'; fractions $EAF_{2-3}$), and β-fructose [31] (cluster 8; fraction $EAF_{12}$). For fractions $EAF_{1-2}$ and $EAF_{1-3}$, the database proposed a terpenic unit (cluster 6) and minor phenolic compounds (cluster 9), respectively, which could not be identified unambiguously.

### 3.3. Chemical profiling of the *n*-BuOH fraction (*n*-BF)

The major compounds of *n*-BF were chemically profiled with the same workflow as EAF. The biphasic solvent system M*t*BE/$CH_3CN$/water (4/1/5, v/v/v) was used for the CPC fractionation of *n*-BF to afford thirteen CPC sub-fractions ($n$-$BF_{1-13}$). The resulting HCA heat map, drawn in Fig. 1, revealed 10 major chemical shift clusters (number 10 to 17). Their studies led to the identification of 17 metabolites: pinitol [20] (cluster 10; fractions $n$-$BF_{10-13}$), a mixture of myricetin-3-*O*-β-D-glucopyranoside (compound **6**) [24] and syringetin-3-*O*-β-D-glucopyranoside (compound **8**) [32] (clusters 11 and 11'; fractions $n$-$BF_{4-6}$), laricitrin-3-*O*-β-D-glucopyranoside (compound **7**) [23] (cluster 12; fractions $n$-$BF_{3-5}$), a mixture of isorhamnetin-3-*O*-β-D-(6"-malonylglucopyranoside) (**5**) [33], isorhamnetin-3-*O*-β-D-glucopyranoside (compound **4**) [22] and quercetin-3-*O*-β-D-glucopyranoside (compound **2**) [21] (cluster 13; fractions $n$-$BF_{3-4}$), phenylethyl-β-D-glucopyranoside [34] (clusters 14 and 14'; fraction $n$-$BF_6$), saccharose [20] with β-fructose [31] (cluster 15; fraction $n$-$BF_{11}$), a mixture of *Z*/*E*-*p*-coumaric acid [26] and 3-phenyllactic acid [29] (cluster 16; fraction $n$-$BF_1$), and a mixture of kaempferol-3-*O*-β-D-(6"-malonylglucopyranoside) [33] and monomethyl malonate (cluster 17; fraction $n$-$BF_3$). For fraction $n$-$BF_6$, the database proposed a mixture of two acids, which could not be identified unambiguously (clusters 14 and 14').

The *n*-BF fraction comprises six glycosides newly observed in the genus *Lotus*, against only two for EAF. The fractions $n$-$BF_3$ to $n$-$BF_6$ were therefore evaluated for their biological activity (Table 1). As a result, fractions $n$-$BF_3$ to $n$-$BF_6$ showed good anti-elastase activities with $IC_{50}$ ranging from 17.5 ($n$-$BF_5$) to 37.8 µg/mL ($n$-$BF_3$), better than *n*-BF. These results showed that the *n*-BF fractionation increased the elastase inhibitory activity. In addition, the fraction $n$-$BF_5$ had powerful antioxidant properties and $n$-$BF_3$ had the best capacity to scavenge hydroxyl radicals. Surprisingly, the fractionation of *n*-BF decreases the antioxidant activity, notably in DPPH and CUPRAC assays, suggesting a synergistic effect in *n*-BF.

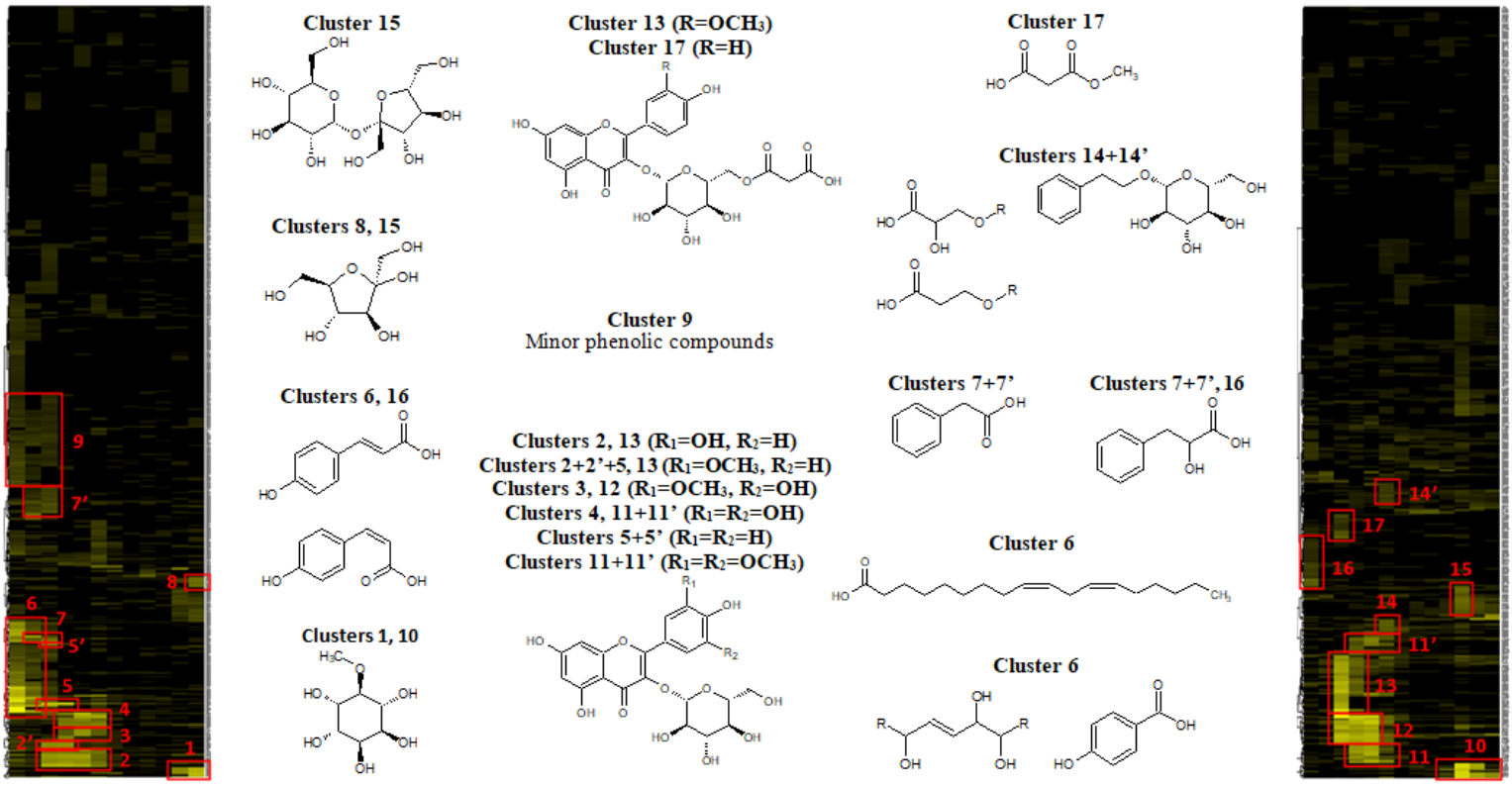


**Fig. 1.** $^{13}$C NMR chemical shift clusters obtained by applying HCA on EAF (left) and *n*-BF (right) CPC fractions from *L. maritimus*.

### 3.4. Purification of the active fractions of *n*-BF

As the *n*-$BF_3$ and *n*-$BF_5$ fractions showed the highest activities, they were purified using preparative and semi-preparative HPLC to give eight known compounds (**1** – **8**). Compounds **1**-**5** were isolated from *n*-$BF_3$ and **6**-**8** from *n*-$BF_5$. The purified compounds were identified based on 2D-NMR and ESI-MS data as succinic acid (**1**) [35], quercetin-3-*O*-β-D-(6"-malonylglucopyranoside) (**3**) [33], and compounds **2**, **4**, **5**, **6**, **7** and **8**, identified during the dereplication process (Fig. 2). These compounds correspond to mono-glycosylated flavonoids, two of which are substituted by malonic acids.

### 3.5. Biological assays on isolated compounds

The isolated compounds were evaluated both for their antioxidant properties and for their tyrosinase and elastase inhibitory activities if the quantities of products were enough (Table 1) and showed that they had lower activities as compared to the standards used. Nevertheless, compounds **2**, **3** and **6** showed the highest antioxidant activities and had the best DPPH radical scavenging activity ($IC_{50}$ 60.8, 91.2 and 59.9 µM, respectively). Compounds **2** and **6** also showed the highest activity in CUPRAC test ($IC_{50}$ 37.7 and 49.8 µM, respectively). Compounds **4** and **5** also exhibited a good cupric ion reducing capacity ($IC_{50}$ 57.7 and 59.9 µM, respectively), and compound **7** showed a moderate potential in DPPH and CUPRAC assays ($IC_{50}$ 104.2 and 91.4 µM, respectively).

In addition, compounds **2**, **4**, and **5** showed no tyrosinase inhibitory activity and compounds **2**, **3**, **4**, **5**, **7** and **8** showed moderate elastase inhibitory properties ($106.2 < IC_{50} < 134.9$ µM) (Table 1).

**Table 1. Antioxidant, tyrosinase and elastase inhibitory activities of crude extracts, fractions, and compounds 1-8 isolated from *L. maritimus aerial parts.***

| | DPPH radical scavenging activity $IC_{50}$ (µg/mL) | OH radical scavenging activity $IC_{50}$ (µg/mL) | Power cupric ion reducing (CUPRAC) $IC_{50}$ (µg/mL) | Mushroom tyrosinase inhibition $IC_{50}$ (µg/mL) | Human neutrophil elastase inhibition $IC_{50}$ (µg/mL) |
|---|---|---|---|---|---|
| **HEM** | 136.3 ± 4.2 | 471.7 ± 5.2 | 11.9 ± 0.5 | 860.0 ± 17.3 | 578.8 ± 1.1 |
| **DCMF** | 77.3 ± 3.2 | 251.3 ± 6.5 | 12.1 ± 1.3 | 120.0 ± 1.5 | n.d. |
| **EAF** | 27.8 ± 0.6 | 107.3 ± 3.1 | 0.9 ± 0 | 132.2 ± 1.8 | 68.7 ± 1.1 |
| ***n*-BF** | 42.7 ± 1.0 | 347.5 ± 2.5 | 2.8 ± 0 | 923.3 ± 16.7 | 144.9 ± 1.3 |
| ***n*-$BF_3$** | 181.3 ± 4.3* | 83.0 ± 3.0 | 22.4 ± 0.6 | (< 5%)[a] | 37.8 ± 1.1* |
| ***n*-$BF_4$** | 142.3 ± 9.4 | 278.3 ± 25.2 | 22.9 ± 0.3 | (< 5%)[a] | 24.5 ± 1.2** |
| ***n*-$BF_5$** | 140.5 ± 1.8 | 511.7 ± 2.9 | 12.5 ± 0 | (15%)[a] | 17.5 ± 1.1** |
| ***n*-$BF_6$** | 174.7 ± 1.0* | 630.0 ± 15.0 | 22.3 ± 0.6 | (16%)[a] | 33.5 ± 1.2* |
| **Quercetin[c]** | 5.4 ± 0.2 | 52.2 ± 4.8 | 13.6 ± 1.3 | 15.1 ± 0[45] | 6.0 ± 0[44] |
| **Ascorbic acid[c]** | 2.3 ± 0.4 | 229.2 ± 2.3 | 13.3 ± 0.5 | | |
| **Kojic acid[c]** | | | | 8.5 ± 0.5 | |
| **Trolox[c]** | | | 5.4 ± 0.3 | | |
| | **$IC_{50}$ (µM)** | **$IC_{50}$ (µM)** | **$IC_{50}$ (µM)** | **$IC_{50}$ (µM)** | **$IC_{50}$ (µM)** |
| **1** | n.d. | n.d. | 1332.0 ± 152.4 | n.d. | n.d. |
| **2** | 60.8 ± 1.1 | n.d. | 37.7 ± 1.1 | (< 5%)[a] | > 100[7] |
| **3** | 91.2 ± 0.7 | n.d. | n.d. | n.d. | 108.6 ± 1.9 |
| **4** | (47%)[b] | 2339.4 ± 0 | 57.7 ± 5.6 | (< 5%)[a] | 106.2 ± 2.3 |
| **5** | 318.4 ± 6.2 | n.d. | 59.9 ± 4.6 | (13%)[a] | 122.4 ± 1.9 |
| **6** | 59.9 ± 0.4 | n.d. | 49.8 ± 0.6 | n.d. | n.d. |
| **7** | 104.2 ± 3.0 | n.d. | 91.4 ± 2.2 | n.d. | 115.9 ± 2.2 |
| **8** | (49%)[b] | n.d. | 257.7 ± 21.2 | n.d. | 134.9 ± 2.2 |
| **Quercetin[c]** | 17.9 ± 0.7 | 172.7 ± 15.9 | 45.0 ± 4.3 | 50.0 ± 0[45] | 20.0 ± 0[44] |
| **Ascorbic acid[c]** | 13.1 ± 2.3 | 1301.5 ± 13.1 | 75.5 ± 2.8 | | |
| **Kojic acid[c]** | | | | 59.8 ± 3.5 | |
| **Trolox[c]** | | | 21.6 ± 1.2 | | |

[a] % inhibition at 1330 µg/mL, [b] % Inhibition at 200 µg/mL, [c] used as positive control, n.d. not done., statistically significant data *( $p < 0.005$), **( $p < 0.001$)

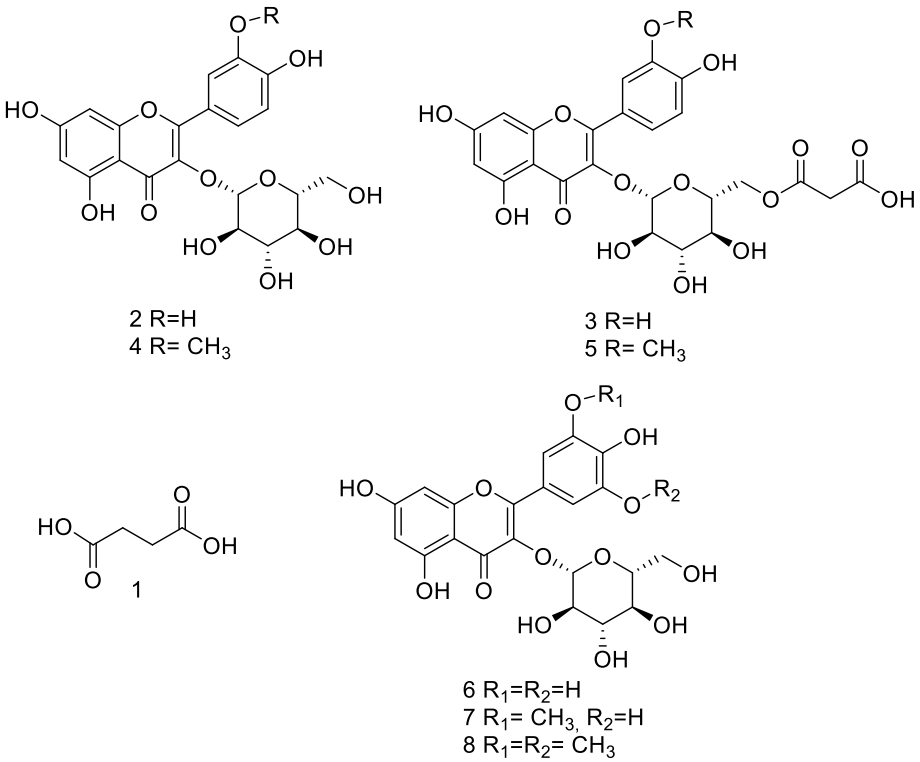


**Fig. 2.** Chemical structures of compounds **1**-**8** isolated from *L. maritimus* aerial parts.

## 4. DISCUSSION

The biological screening of an 80% MeOH extract of the aerial parts of *Lotus maritimus* and these fractions obtained using solvents of increasing polarity allowed us to identify that the EtOAc and *n*-BuOH soluble fractions were the most active. The chemical profiling of these fractions resulted in the identification of twenty-two compounds including flavonoids, phenolic acids and glycoside derivatives (Figure 1).

From a chemotaxonomic viewpoint, eight of the identified compounds were known in *Lotus* genus: pinitol [36], saccharose [37], quercetin-3-*O*-β-D-glucopyranoside (compound **2**) [38], isorhamnetin-3-*O*-β-D-glucopyranoside (compound **4**) [10], kaempferol-3-*O*-β-D-glucopyranoside [39], *p*-coumaric acid [40], *p*-hydroxybenzoic acid [40], and linoleic acid [41]. The other compounds are described here for the first time in the genus *Lotus*. All compounds are newly described in the species *L. maritimus*.

In addition, the originality of the characterized structures lies in the presence of flavonoids with variable substitutions on the B-ring: from one to three substitutions, free OH or *O*-$CH_3$ groups. The flavonols were previously described in their aglycone form in the first study of this species [8].

Compounds **1** – **8** were evaluated for their antioxidant activity, tyrosinase and elastase inhibitory properties and showed that they had lower activities as compared to the standards used (Table 1). The quercetin glycosides **2** and **3** showed better antioxidant activities than the isorhamnetin glycosides **4** and **5**. It is known that quercetin derivatives have better antioxidant capacity than isorhamnetin and kaempferol based on the presence of two free OH group at 3' and 4' positions of the B-ring [42]. Similarly, flavonoids presenting a substitution at position 5' must have a free OH group to keep high capacity, like myricetin derivatives [43]. The presence of *O*-$CH_3$ groups in the B-ring reduces the antioxidant potential. Indeed, the activity of tri-substituted flavonoids in positions 3', 4'

and 5' on the B-ring decreases in the following order: compound **6** > compound **7** > compound **8**. The most active compounds **2**, **3** and **6** are quercetin and myricetin-type flavonoids, which confirm this structure-activity relationship.

Concerning the elastase inhibitory activity, all glycosylated flavonoids tested **3**, **4**, **5**, **7** and **8** showed moderate potential (106.2 < $IC_{50}$ < 134.9 µM). In Sartor et al [44] different flavonoids were evaluated for their potential in blocking leucocyte elastase and their results have deduced that a crucial role in inhibition might be played by a galloyl moiety or hydroxyl group at position 3, three hydroxyl groups at B ring, one hydroxyl group at position 4′, and a 2,3-double bond. Our results support that the presence of sugars linked to hydroxyl group at position 3 of the aglycone reduce the anti-elastase activity, compared to quercetin ($IC_{50}$ of 20mM) or myricetin ($IC_{50}$ of 4mM), possessing a free hydroxyl group at position 3 and two of three hydroxyl groups at B ring, respectively [7, 44].

## CONCLUSION

Twenty-two compounds were identified for the first time in *Lotus maritimus* aerial parts. Among the eight purified compounds, quercetin and myricetin-type flavonoids **2**, **3** and **6** showed a good antioxidant activity and all flavonoids tested showed a moderate elastase inhibitory activity. This study demonstrates that the aerial parts of *L. maritimus* as well as the EAF, *n*-BF fractions and their glycosylated flavonoids of quercetin and myricetin type could be valorized as active antioxidant in dermo-cosmetics. The *n*-BF fraction and their flavonoids could also be valued for their anti-elastase activity.

## LIST OF ABBREVIATIONS

*n*BF = *n*-butanol fraction

COSY = Correlated Spectroscopy

CUPRAC = cupric ion reducing activity

CPC = Centrifugal partition chromatography

DCMF = dichloromethane fraction

DPPH = Diphényl Picryl Hydrazyl

EAF = ethyl acetate fraction

ESI-MS = ElectroSpray Ionisation- Mass Spectrometer

HLE = Human Leukocyte Elastase

HPLC = High-performance liquid chromatography

HME = Hydromethanolic extract

HSQC = Heteronuclear Single Quantum Coherence

HMBC = Heterocnuclear Multiple Bond Correlation

NMR = Nuclear Magnetic Resonance

TLC =Thin-layer chromatography

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No humans and animals were used in experiments that were the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The raw data supporting the conclusion of this study will be made available by the author,


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## CONFLICT OF INTEREST

The authors declare no conflict of interest.


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