ISSN 1615-9306 · JSSCCJ 45 (17) 2022 · Vol. 45 · No. 17 · September 2022

Journal of SEPARATION SCIENCE

17|2022

www.jss-journal.com

Methods
Chromatography · Electroseparation

Applications
Biomedicine · Foods · Environment

WILEY-VCH

Received: 21 March 2022 | Revised: 16 May 2022 | Accepted: 29 May 2022

DOI: 10.1002/jssc.202200235

JOURNAL OF SEPARATION SCIENCE

RESEARCH ARTICLE

Determination of the polyphenolic content of *Ammodaucus leucotrichus* Cosson and Durieu by liquid chromatography coupled with mass spectrometry and evaluation of the antioxidant and antiglycation properties

Ginane Selama[1] | **Hafssa El Cadi**[2] | **Btissam Ramdan**[3] | **Yassine Oulad El Majdoub**[4] | **Paola Dugo**[4,5] | **Luigi Mondello**[4,5,6] | **Francesco Cacciola**[7] | **Mohamed Nhiri**[1]

[1]Laboratory of Biochemistry and Molecular Genetics, Abdelmalek Essaadi University, Tangier, Morocco

[2]Department of Chemistry, Laboratory of Valorization of Resources and Chemical Engineering, Abdelmalek Essaadi University, Tangier, Morocco

[3]Department of Biology, Laboratory of Biotechnology and Valorization of Natural Resources, Faculty of Science, Ibn Zohr University, Agadir, Morocco

[4]Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences, University of Messina, Messina, Italy

[5]Chromaleont s.r.l., Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

[6]Department of Sciences and Technologies for Human and Environment, University Campus Bio-Medico of Rome, Rome, Italy

[7]Department of Biomedical, Dental, Morphological, and Functional Imaging Sciences, University of Messina, Messina, Italy

Correspondence
Francesco Cacciola, Department of Biomedical, Dental, Morphological and Functional Imaging Sciences, University of Messina, Messina, Italy.
Email: cacciolaf@unime.it

[Correction added on 15 July 2022, after first online publication: CRUI-CARE funding statement has been added.]

Ammodaucus leucotrichus Cosson and Durieu, known as Sahara cumin, is a plant belonging to the Apiaceae family with a very strong smell of anise growing in the maritime sands in the countries of North Africa. The present work aims to study the polyphenolic profile of its seeds hydroalcoholic extract along with the determination of the antioxidant and antiglycation properties. The phytochemical screening revealed the presence of 16 compounds, out of which 15 have been detected in this extract for the first time. Luteolin-glucoside turned out to be the most abundant one (281.32 ± 0.34 mg/kg), followed by apigenin-hexoside (235.06 ± 0.29 mg/kg) and luteolin (202.41 ± 0.40 mg/kg). In terms of antioxidant activity, a half-maximal inhibitory concentration value as high as 0.39 ± 0.003 mg AAE/ml (w/v) was attained. Further, the antiglycation activity was determined to yield interesting results: at a concentration of 1.5 mg/ml, the extract showed an antiglycation activity (%I) of 61.86 compared to metformin as a positive control (%I = 84.01); on the other hand, increasing the concentration to 10 mg/ml,

Article Related Abbreviations: AGEs, advanced glycation end products; DAD, diode array detection; DW, dry weight; MG, methylglyoxal; PAGE-NATIVE, native polyacrylamide gel electrophoresis; RS, reducing sugars; TPC, total phenolic content; TTC, total tannin content.

the inhibition activity switched to advanced glycation end products formation activation (%I = 41.71).

KEYWORDS
Ammodaucus leucotrichus, antiglycation activity, antioxidant activity, liquid chromatography, polyphenols

1 | INTRODUCTION

Various food and natural products present extraordinary chemical structures with potentially significant biological activities [1, 2]. In this context, due to the ever-higher incidence of diabetic diseases, natural remedies are currently used, because of their accessibility and affordability, in low-income countries to treat this illness: for instance, antidiabetic agents based on herbal medicines became a normal daily routine of many urban and rural Moroccans lives. However, both chemical composition and ethnopharmacological knowledge of some regions in Morocco is still limited. Several changes in the body can affect the onset of diabetes: the oxidative stress-activated by free radicals production when the amount of antioxidants in the body is decreased, thus leading to cell damage, apoptosis, and activation of the inflammatory process, all of which might contribute to very severe health problems [3]. Despite a large array of chemical medications being commercially available to restore blood glucose levels, adverse effects, for example, hypoglycemia, obesity, hepatopathy, and others might occur thus leading the researcher's focus to look into new and safer alternatives [4].

In this context, *Ammodaucus leucotrichus* Cosson and Durieu (*A. leucotrichus*), known as Sahara cumin, is a plant belonging to the Apiaceae family with a very strong smell of anise. It grows in the maritime sands of North and tropical Africa [5]. In Morocco, where it is known locally as "kammûn es-sofi or akâman", the powder is used by the local population to treat gastric-intestinal pains, gastralgias, otitis, hypotensive, indigestion, cold, fever, pulmonary diseases, labor pains, anorexia and cardiac diseases [6]. Recently, the functional properties of such a species with regards to antioxidant, antibacterial, antifungal, antidiabetic, anti-inflammatory, anticholinesterase and cytotoxicity activities have been highlighted [7, 8].

Interestingly, some studies have recently been focused on the antiglycation activity of plant extracts [9, 10]. Glycoxidation is a toxic complicated reaction that undergoes a binding process between aldehydes such as methylglyoxal (MG) free or protein-bound, which induces free radicals and other reactive intermediates (e.g., H_2O_2 and other peroxides), and as a consequence contributes to advanced glycation end products (AGEs) formation [11, 12]. Clinical results showed that the accumulation of AGEs may be reduced by the consumption of natural bioactive compounds with the antioxidant ability [9]. Therefore, it is very important to study the effect of antioxidant supplementations found in natural products that inhibit protein modifications and as results inhibit AGEs formation that causes diabetic complications [10].

Due to its various utilization, the present study aims to characterize the seeds hydroalcoholic extract of *A. leucotrichus* by the determination of their polyphenolic content by HPLC coupled to photodiode array and ESI/MS detection, followed by the evaluation of the antioxidant and antiglycation activities.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

All chemicals (ACN, water, ethanol, hexane, BSA, Na_2CO_3, $AlCl_3$, CH_3CO_2K, $As_2H_6Mo_3O_{12}$, trichloroanisole, Iron(II) chloride, Iron(III) chloride ferrozine, 2,2-diphenyl-1-picrylhydrazyl, caffeic acid, *p*-coumaric acid, naringin, quercetin, luteolin, apigenin, isorhamnetin-3-*O*-glucoside, gallic acid, ascorbic acid, tannic acid, Folin Ciocalteu's reagent) unless otherwise stated, were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany).

2.2 | Sample and sample preparation

A. leucotrichus fruit was harvested in the Marrakech-Tensift-El Hawz region in western Morocco in May 2019 (latitude 31.562808 and longitude −7.959286). After collection, the fruit was stored at −10°C under a vacuum at the laboratory of Biochemistry and Molecular Genetics, University AbdelMalek Essaadi, Tangier, Morocco. Dried seeds were crushed into a fine powder, sieved, then vacuum sealed in a plastic bag and stored at −20°C until use. All samples were spiked prior to the extraction with 50 µl of

vanillin (1000 ppm). Binary solvent extraction was carried out according to Waszkowiak and Gliszczynska-Swigło [13]. Briefly, seeds were defatted by using hexane, extracted with an ethanol-water solvent, and placed in an ultrasound bath (130 kHz) for 45 min. Notably, on the basis of previous results by Zhang et al. five extraction solvents of various ethanol-to-water ratios were applied (60:40, 65:35, 70:30, 80:20 90:10; v/v) [14]. After 10 min of centrifugation, the supernatants from all extractions were combined and filtered through a Whatman nylon filter (Merck Life Science, Merck KGaA) and evaporated by a rotary evaporator. The extraction method was repeated three times. The obtained extract was then stored at 4°C until use.

2.3 | Determination of total phenolic, tannin, flavonoid, and reducing sugars contents

Total phenolic content (TPC) was determined by the Folin-Ciocalteu method [15], with minor modifications. Gallic acid was used as a standard by using five concentration levels (10–200 mg/ml). TPC content was measured at 725 nm and was expressed as milligrams of gallic acid equivalent to GAE/g of dry weight (DW).

Total tannin content (TTC) was executed [15], with minor modifications. Tannic acid was used as a standard by using five concentration levels (10–200 mg/ml). TTC content was measured at 760 nm and was expressed as milligrams of tannic acid equivalent to TAE/g of DW.

Total flavonoid content was carried out by using an aluminum trichloride ($AlCl_3$) colorimetric method and described in the literature with some modifications [16]. The standard range was prepared from solutions of quercetin in the range of 10–200 mg/ml. The absorbance was read at 415 nm and was expressed as milligrams of quercetin equivalent to QE/g of DW.

Reducing sugars (RS) content was determined according to the Nelson-Somogyi method with small modifications [17, 18]. Absorbance was measured at 620 nm and glucose was used as standard in a range of 0–100 mg/ml. The results were expressed as milligrams of glucose equivalent per gram of DW.

2.4 | HPLC analyses

LC analyses were performed using a Shimadzu HPLC system (Kyoto, Japan) equipped with a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps, a DGU-20A5R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M20A photodiode array detector, and an LCMS-2020 single quadrupole mass spectrometer, through an ESI source operated in negative ionization mode (Shimadzu). Data acquisition was performed by Shimadzu LabSolution software ver. 5.91.

Chromatographic separations were carried out on 150 ×4.6 mm^2; 2.7 μm Ascentis Express RP C18 columns (Merck Life Science, Merck KGaA). The mobile phase was composed of two solvents: water/formic acid (99.90/0.10 v/v, solvent A) and ACN/formic acid (99.90/0.10 v/v, solvent B). The flow rate was set at 1 ml/min, and was split to 0.2 ml/min prior to ESI-MS detection, under gradient elution 0–20 min, 20% B, 30 min, 35% B, 40 min, 80% B, 45 min, 100% B, 60 min, 100% B. The injection volume was 5 μl. Diode array detection (DAD) was applied in the range of 200–400 nm and monitored at a wavelength of 330 nm (sampling frequency: 40.0 Hz, time constant: 0.08 s). MS conditions were as follows: scan range and the scan speed were set at a mass-to-charge ratio (m/z) 100–800 and 2500 amu/s, respectively, event time: 0.3 s, nebulizing gas (N_2) flow rate: 1.5 L/min, drying gas (N_2) flow rate: 15 L/min, interface temperature: 350°C, heat block temperature: 300°C, desolvation line temperature: 300°C, desolvation line voltage: 1 V, interface voltage: −4.5 kV.

Calibration curves of nine polyphenolic standards ($R^2 > 0.9968$) were used for the quantification of the polyphenolic content in sample extract: the concentration range was 10–500 mg/L and five different concentration levels were considered. Each analysis was performed in triplicate.

2.5 | Radical scavenging activity

The anti-free radical activity was determined using the 2,2-diphenyl-1-picrylhydrazyl method according to a method earlier described [19–22]. Briefly, different concentrations of extracts (25 μl) were added to 2 ml of 2,2-diphenyl-1-picrylhydrazyl (6.25×10^{-5} M) solution. After gentle mixing and 30 min of standing at room temperature, the absorbance of the resulting solutions was measured at 517 nm. Ascorbic acid was used as the positive control.

2.6 | In-vitro glycation assay

2.6.1 | Sample preparation

BSA-MG model was performed in this study on the seeds hydroethanolic extract following the method described in the literature with small modifications [23]. Briefly, BSA (5 mg/ml) was incubated with MG (100 mM) and phosphate buffer (100 mM, pH 7.4) containing 0.002% of sodium azide, in the presence of the extract at

different concentrations (10, 2.5, and 1.5 mg/ml) at 37°C for 7 days. Three controls were prepared in parallel; a blank (BSA with phosphate buffer), a negative control BSA with MG and phosphate buffer) and the last positive control (BSA with MG and phosphate buffer in the presence of metformin).

2.6.2 | Identification of glycated albumin

In order to reveal the glycated albumin, a Native-PAGE polyacrylamide gel electrophoresis system was used according to literature data [24]. In a micro-well plate, the reaction mixture was added to the charge buffer, and 8 µl of the sample was deposited in each well. The migration ran for 2 h at 80V and 30 mA at 4°C. After the migration was completed, the gel was stained in 0.1% of Coomassie brilliant blue for 1% at 37°C and then washed several times in 7% of acetic acid.

The percentage inhibition related to the formation of the Amadori products was calculated using the following equation:

$$\%I = [1 - (F_i/F_0)] \times 100$$

F_i is the migration distance of the reaction mixture (BSA-MG-Extract)

F_0 is the migration distance of the reaction mixture without extract (BSA-MG).

2.6.3 | Fluorescence measurement

AGEs are fluorescent products due to AGE-protein binding [24]. In order to follow the fluorescent AGEs formed in the system BSA-MG, the fluorescence measurements were done by a spectrofluorometer (Jasco FP-830) (excitation at 370 nm and emission at 423 nm). The percentage of inhibition of AGEs glycation was calculated using the same equation reported in the previous section.

3 | RESULTS AND DISCUSSION

3.1 | Polyphenolic content

The seeds hydroalcoholic extract of *A. leucotrichus* yielded TPC and total flavonoid content values of 83.07 ± 0.37 mg GAE/g DW (w/w) and 67.53 ± 0.34 mg QE/g DW (w/w) respectively. Such values are higher than those obtained by Mouderas et al., 45.69 ± 0.4 mg GAE/g DW (w/w) and 18.94 ± 0.29 mg QE/g DW (w/w), respectively, for the aerial parts of such a spice [25]. Likewise, Halla et al. revealed an even lower value of TPC 10.32 ± 0.373 mg GAE/g DW (w/w) with regard to *A. leucotrichus* fruits extract [26]. On the other hand, such values are lower than the ones reported by Hellal et al. [27] and Louail et al. [28], who reported TPC values as high as 124.98 mg GAE/g extract and 146.18 ± 5.82 mg GAE/g DW for the aerial parts and fruits, respectively. This difference might be due to the period of harvesting, growing region, or different plant parts studied and environmental characteristics [29]. An RS content estimated in 12.31 ± 0.24 mg GE/g DW (w/w) was attained, whereas a TTC of 28.61 ± 0.39 mg TAE/g DW was attained (w/w) respectively. The occurrence of RS and tannins has been also reported in the hydroalcoholic extract of *A. leucotrichus* from Algerian Sahara, despite no quantification data being reported [26].

3.2 | Polyphenolic profile by HPLC coupled with photodiode array and ESI-MS detection

As an internal standard, vanillin, an aromatic aldehyde not occurring in *A. leucotrichus* seeds, was employed; for all polyphenolic compounds identified in the sample the highest recovery value (87%) was attained for the 70:30, v/v ethanol/water extract. Figure 1 reports the polyphenolic profile of the seeds hydroalcoholic extract of *A. leucotrichus* carried out by HPLC-DAD-ESI/MS analysis. A total of 16 compounds were positively detected and 15 out of them were identified for the first time in such extract on the basis of their retention times, co-standard injection, MS data, and literature survey (Table 1). Interestingly, almost the totality of the compounds belongs to the flavonoids class, mainly flavones and flavanols, with the exception of peak no. 5 *p*-Coumaroyl-hexoside and peak no. 9, which are phenolic acids derivatives. 10 out of them were previously reported among the polyphenolic content of *Apiaceae* plants [7, 29–35], and only one, for example, peak no. 4, luteolin-glucoside in edible *A. leucotrichus* from Algerian Sahara. Four apigenin derivatives were found in the extract: peaks no. 2 and 3 (λ = 330 nm; $[M\text{-}H]^-$ *m/z* 473) were inferred to be apigenin-(acetyl)hexoside, confirmed by the presence of a fragment at *m/z* 269, corresponding to apigenin aglycone with the loss of 42 amu (acetyl moiety). These findings are consistent with the same compounds occurring in other *Apiaceae* species from Turkey and from Madeira Archipelago [29, 30]. Concerning the other two apigenin derivatives, the one detected at t_R of 26.30 min (peak no. 7, apigenin-hexoside) was never reported before with the exception of the other detected at t_R of 31.54 min (peak no. 12) which was reported in parsley (*Petroselinum crispum* (Mill.) Nym ex A. W. Hill) and marjoram (*Origanum majorana* L.) products [32].

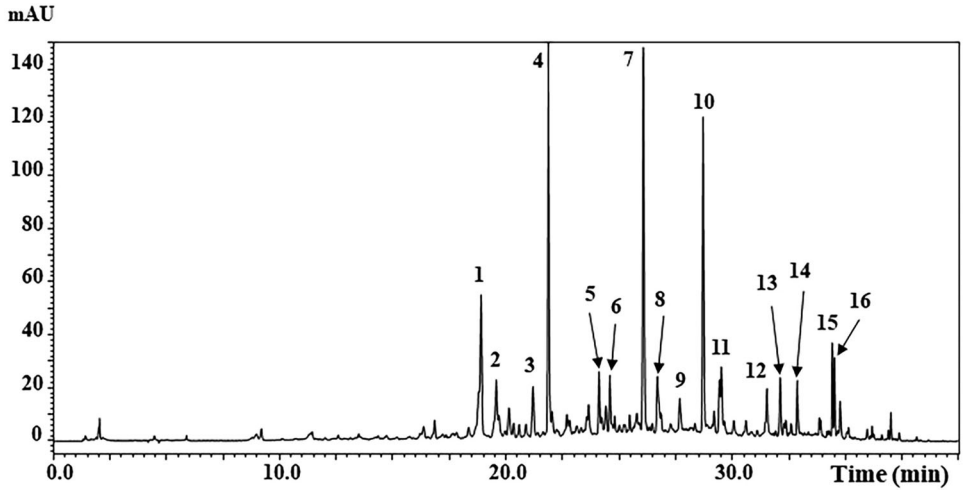

FIGURE 1 HPLC-DAD (λ = 330 nm) chromatogram of *A. leucotrichus* seeds hydroalcoholic extract obtained on a C18 column, 15 × 4.6 mm; 2.7 mm particles. For peak identification, see Table 2

TABLE 1 List of polyphenolic compounds identified in the seeds hydroethanolic extract of A. leucotrichus through HPLC coupled to photodiode array and ESI-MS detection. Values represent averages of polyphenols in the tested extracts, expressed in mg/kg ± SD of triplicate measurements

Peak no.	Compound	t_R (min)	UV_{max} (nm)	$[M-H]^-$	Quantity	Ref.
1	Naringenin-hexoside	18.92	290	443, 271*	84.51 ± 0.21	–
2	Apigenin-(acetyl)hexoside	19.60	330	473, 269*	27.61 ± 0.25	[7, 29, 30]
3	Apigenin-(acetyl)hexoside is.	21.13	331	473, 269*	25.41 ± 0.29	[29, 30]
4	Luteolin-hexoside	21.89	347	447, 285*	281.32 ± 0.34	[7]
5	*p*-Coumaroyl-hexoside	24.02	306	325	49.50 ± 0.32	[31]
6	Isorhamnetin-(malonyl)glucoside	24.56	340	563, 315*	45.01 ± 0.41	–
7	Apigenin-hexoside	26.01	334	431, 269*	235.06 ± 0.29	–
8	Luteolin-glucuronide	26.30	344	461, 285*	35.74 ± 0.19	[32]
9	Caftaric acid	27.21	320	311	28.21 ± 0.25	[33]
10	Luteolin	28.22	345	285	202.41 ± 0.40	[34]**
11	Naringenin	29.44	289	271	81.21 ± 0.40	–
12	Apigenin	31.54	337	269	50.33 ± 0.67	[32]**
13	Chrysoeriol	32.13	344	299	35.69 ± 0.46	[35]
14	Rhamnazin	32.88	365	329	53.74 ± 0.33	[35]
15	Naringenin methyl ether	34.42	303	285, 271*	65.61 ± 0.22	–
16	Unknown	34.52	315	227	nq	–

*In-source fragments observed; nq: not quantified; ** Identified by standard co-injection.

Luteolin derivates were three, namely, peak no. 4, luteolin-glucoside, peak no. 8, luteolin-glucuronide, and peak no. 10, luteolin. Luteolin-glucoside and luteolin-glucuronide had absorption maxima at 336 and 344 nm, with corresponding [M-H]- ions at *m/z* 447 and 461, respectively, along with a fragment at *m/z* 285, corresponding to luteolin aglycone. All of these three luteolin derivates were previously reported in the *Apiaceae* family [7, 32, 34]. Further, three naringenin derivatives were detected, *viz.* peak no. 1 naringenin-hexoside, peak no. 11, naringenin and peak no. 11, naringenin methyl ether. Naringenin-hexoside showed an absorption maximum at 290 nm, with the corresponding $[M-H]^-$ ion at *m/z* 443 and a fragment at *m/z* 271; naringenin methyl ether had an absorption maximum at 290 nm, with the corresponding $[M-H]^-$ ion at *m/z* 443 and a fragment at *m/z* 271; finally, naringenin showed a characteristic absorption maximum at 345 nm with the corresponding $[M-H]^-$ ion at *m/z* 271. None of the naringenin derivates identified had ever been reported as constituents of the *Apiaceae* family.

TABLE 2 Quantitative performance of the polyphenolic standards investigated in this study

Reference material	Chemical class	LOD (μg/ml)	LOQ (μg/ml)	Precision (RSD%)	Accuracy (A%)
Caffeic acid	Cinnamic acid-like	0.01	0.03	0.19	4.8
p-Coumaric acid	Hydroxycinnamic acid-like	0.15	0.45	0.28	6.5
Naringin	Flavanone-like	0.05	0.15	0.25	−3.5
Luteolin	Flavone-like	0.02	0.06	0.15	−7.7
Apigenin	Flavone-like	0.02	0.06	0.18	−7.2
Isorhamnetin-3-*O*-glucoside	Flavonol-like	0.18	0.54	0.19	5.8
Quercetin	Flavonol-like	0.02	0.05	0.11	5.7

TABLE 3 Migration distance and percentage of inhibition of glycation in spectrofluorometric assay and native polyacrylamide gel electrophoresis (PAGE-NATIVE) electrophoresis

	Migration distance on PAGE-NATIVE gel (mm)			% of inhibition Fluorescence intensity			Migration distance		
Concentration (mg/ml)	1.5	3.5	10	1.5	3.5	10	1.5	3.5	10
Extract	42.8 ± 0.42	49.1 ± 0.23	64.1 ± 0.31	61.86 ± 0.56	51.86 ± 0.33	41.71 ± 0.29	39.72 ± 0.34	30.85 ± 0.25	9.72 ± 0.44
Control test (BSA+MG+MET)				84.01			67.94		

The developed HPLC method in combination with photodiode array and ESI-MS detection was then validated to assess its quantitative reliability. The reference standards employed along with LODs, LOQs, RSDs of peak areas, and accuracy values for each analyte for each available standard are reported in Table 2. LOQ and LOD were calculated considering an S/N ratio of 10 and 3, respectively. The LOQ/LOD values for the polyphenolic reference materials were extrapolated from the S/N value, observed at the lowest calibration level (10 ppm). As can be observed in Table 2, sensitivity was in general satisfactory, much higher than that necessary, and in agreement with literature data for this kind of application [1]. For instance, LOD and LOQ values were lower than 18 and 54 ppb, respectively. The RSD values were lower than 0.28% highlighting highly repeatability. Accuracy (A%) values, which were determined as relative error deviation between the values observed in the spiked sample and the expected values, were below 8.5%. In terms of quantification, luteolin-glucoside turned out to be the most abundant one (281.32 ± 0.34 mg/kg ± SD), followed by apigenin-hexoside (235.06 ± 0.29 mg/kg ± SD) and luteolin with a concentration of 202.41 ± 0.40 mg/kg.

3.3 | Antioxidant activity

In terms of antioxidant activity, the seeds hydroalcoholic extract of *A. leucotrichus* yielded a half-maximal inhibitory concentration value of 0.39 ± 0.003 mg AAE/ml, w/v (half-maximal inhibitory concentration ascorbic acid = 0.53 ± 0.003 mg AAE/ml, w/v). Such value reveals a very high antioxidant activity in comparison with previous works carried out on *A. leucotrichus* by other authors, although carried out of either fruits or aerial parts, for example, Halla et al., 5.70 ± 0.37 mg AAE/ml (w/v) [26], Hellal et al., 26.26 AAE/ml (w/v) [27], Rached et al., 45.79 ± 1.07 mg AAE/ml (w/v) [36] and Louail et al., 97.89 ± 2.91 mg AAE/ml (w/v) [28].

3.4 | Antiglycation activity

The fluorimetric test showed the best inhibition activity with the lowest extract concentration (1.5 mg/ml), in comparison to elevated concentrations (3.5 and 10 mg/ml) where the glycation of BSA was decreased. Likewise, similar results were obtained in the PAGE-NATIVE electrophoresis assay, which aimed to separate the glycated proteins on the basis of their charge. Table 3 represents the different percentages of glycation inhibition represented by this species, as well as the migration profile on PAGE-NATIVE gel (Table 3). The electrophoretic migration toward the anode can be positively increased due to the following factors; the increase of sugar and/or protein concentration and the period or temperature of incubation [37]. In fact, protein glycation decreases its positive charge which is translated into further migration toward the anode [38]. The results obtained elucidates the effect of glycation on apparent protein charge in bands treated with increasing concentrations of ethanolic extract. Indeed, compared with the control, the more the extract concen-

tration is increased, the further the band migrates. The inhibition percentages from the migration distance of 1.5, 3.5, and 10 mg/ml were decreased by 39.72, 30.85, and 9.72%, respectively. The antiglycation effect of the extract of *A. leucotrichus* was lower than that of control, as demonstrated by a loss of effectiveness with the increase of the extract concentration to 10 mg/ml. Since there are only a few studies that have investigated this species, a comparison of the present results was performed with other species belonging to the same family. Studies on *C. sativum* showed that this plant has a hypoglycemic effect, as well [39], whereas other studies on *C. sativum* revealed an antiglycation activity in its methanolic and ethanolic extracts of 50% at 0.42 mg/ml and 38.8% at 10 mg/ml, respectively [38, 40, 41].

The obtained results can be explained by a high content of RS compared to other species previously studied [42].

4 | CONCLUDING REMARKS

This study aimed to highlight the relationship between the phytochemical profile and biological activities of the seeds hydroalcoholic extract of *A. leucotrichus* using HPLC-DAD/ESI-MS. Quantitative and qualitative results of the phytochemical screening showed that *A. leucotrichus* contains a remarkable number of novel phenols and flavonoids, with an interesting antioxidant activity. In regards to antiglycation activity, which was less studied in this species, activation of protein glycation when the extract concentration exceeds 1.5 mg/ml in both fluorescent measurement and electrophoresis assay, was attained. Hence, it is worthwhile to further investigate the biological activity by studying other parameters to be able to identify the mechanism leading to the switch on the protein glycation leading to AGEs formation.

ACKNOWLEDGMENT

The authors thank Merck Life Science and Shimadzu Corporations for their continuous support.

Open Access Funding provided by Universita degli Studi di Messina within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Paola Dugo https://orcid.org/0000-0002-9432-1782
Luigi Mondello https://orcid.org/0000-0002-8890-675X
Francesco Cacciola https://orcid.org/0000-0003-1296-7633

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How to cite this article: Selama G, Cadi HE, Ramdan B, Majdoub YOE, Dugo P, Mondello L, et al. Determination of the polyphenolic content of *Ammodaucus leucotrichus* Cosson and Durieu by liquid chromatography coupled with mass spectrometry and evaluation of the antioxidant and antiglycation properties. J Sep Sci. 2022;45:3301–3309.
https://doi.org/10.1002/jssc.202200235