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Phytochemical Profiling and Biological Activities of Two *Helianthemum* Species Growing in Greece

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Abstract: *Helianthemum nummularium* (HN) and *Helianthemum oelanticum* subsp. *incanum* (HO) are plant species, among Cistaceae, that are highly distributed in the Mediterranean region. In the current study, extracts of the aerial parts from both species have been analyzed phytochemically. The non-polar extract analysis resulted in the identification of 15 compounds in each species, mainly terpene and fatty acid derivatives, through GC–MS. The methanolic extract analysis, conducted through UHPLC–MS/MS, led to the identification of 39 metabolites in HN and 29 in HO, respectively, the majority of which were phenolics. Among the identified compounds, several have also been isolated and structurally determined (from HN: rutin, linoleic acid, gallic acid, and isoquercetin, and from HO: quercetin-3-*O*-(2″-*O*-galloyl)-galactopyranoside, methyl gallate, catechin-3-*O*-glucopyranoside, and astragalin, while hyperoside, and *cis*- and *trans*-tiliroside have been determined in both species). Furthermore, the methanolic extracts of HN and HO displayed a high total phenolic content (177.2 mg GA/g extract and 150.6 mg GA/g extract, respectively) and considerable free-radical scavenging activity against the DPPH radical (94.6% and 94.0% DPPH inhibition, respectively). Antimicrobial testing showed stronger inhibition of HN against Gram (+) bacterial strains (MIC values 0.07–0.15 mg/mL), while both extracts exhibited low tyrosinase-inhibitory activity. Considering the lack of studies conducted on the chemistry and biological activities of the genus *Helianthemum*, the chemical characterization of extracts could contribute to new sources of bioactive metabolites to be explored and exploited for further potential applications such as food and/ or the cosmetic industry.

Keywords: *Helianthemum nummularium*; *Helianthemum oelanticum*; UHPLC–MS/MS; antioxidant activity; antimicrobial activity; tyrosinase inhibition

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1. Introduction

The genus *Helianthemum* (Cistaceae) is a monophyletic lineage with about 136 heliophytic species and subspecies of evergreen or semi-evergreen shrubs, subshrubs, and annual herbs [1,2]. It is the largest and most diverse genus in the Cistaceae family, thriving from sea level up to approximately 3000 m in various substrates, including limestone, dolomite, marl, gypsum, saline, and sandy soils [2]. This genus ranges from the Macaronesia region (North Atlantic archipelagos) to Central Asia, spanning the Mediterranean floristic region and Europe, with its highest diversity of species concentrated in the western Mediterranean area [2].

Helianthemum species have been traditionally used as natural remedies in folk medicine in Spain, Turkey, and America [3,4]. The aerial parts of different *Helianthemum* species have been used as anti-inflammatory, antiulcerogenic, antidiabetic, wound-healing, antiprotozoal, antimicrobial, analgesic, and psychiatric remedies [3,5,6]. In general, the plants

of this genus are used in preparations of decoctions and teas for ingestion for gastrointestinal problems [3], while extracts are embodied into poultices and ointments for direct application on infected wounds or burns [7]. The conducted pharmacological studies have confirmed the benefits of the species regarding their traditional use and have enriched their biological activities known to date by also adding strong antioxidant, cytotoxic, and spasmolytic effects [3,8,9].

Regarding the phytochemistry of *Helianthemum* species, the majority of the literature data primarily focus on polar extract analysis, while limited studies have been conducted on essential oils [10,11]. The most characteristic chemical class of the genus is the phenolic derivatives including a variety of phenolic acids (derivatives of benzoic acid or hydroxycinnamic acid) [12–14], flavonoids [9,12,14,15], and polyphenols (hydrolyzed tannins and proanthocyanidins) [6,16], while to a lesser extent, lignans and coumarin derivatives have been reported [12,13]. In addition to these compounds, other chemical categories like aliphatic derivatives (aliphatic and fatty acids), sterols, and alkaloids are present in some species [12,13,16].

H. nummularium (L.) Mill (HN) is a species that consists of evergreen, dwarf shrubs that produce multiple racemes of yellow or pink flowers in early summer [2]. It has been traditionally used as part of the flower remedies, a popular form of complementary medicine often employed to address psychological issues and stress [12,17]. *H. oelanticum* subsp. *incanum* (synonym of *H. canum*) (HO) is a perennial dwarf shrub [2], with traditional use as an anti-inflammatory and wound-healing medicine, especially in the eastern Mediterranean region [18].

Both plants are widely distributed in Greece, where the *Helianthemum* genus is represented by 11 species [19]. Both studied species are native to Greece and are also encountered in the Prespa Lakes' region, a protected National Park northwest of Greece with an exceptional richness and diversity of habitats, fauna, and flora unique to Greece and Europe [20].

According to our knowledge, no *Helianthemum* species growing in Greece have been previously studied. Therefore, the objective of this study was to conduct a comprehensive phytochemical analysis of the aerial parts of *H. nummularium* and *H. oelanticum* subsp. *incanum*, encompassing both non-polar and polar extract analysis, as well as the isolation and identification of their most abundant metabolites. Additionally, evaluating antioxidant, antimicrobial, and tyrosinase-inhibitory activities can provide insights into new sources of bioactive extracts.

2. Materials and Methods

2.1. Chemicals and Reagents

For the extraction, all solvents were purchased from Merck (Darmstadt, Germany) (c-hexane, dichloromethane, methanol). For the column chromatography (CC) stationary phases, silica gel (Kieselgel 60 H Merck) and microcrystalline cellulose (cellulose microcrystalline, Merck) were used, with gradient elution with the solvent mixtures indicated in each case. The solvents used were HPLC grade and were purchased from Fisher Chemical (Fisher Scientific, Loughborough, Leics, UK). For all column chromatographic procedures, fractionation was monitored via TLC: Merck silica gel 60 F254 (0.2 mm layer thickness), Merck RP-18 F254S, and Merck cellulose. For preparative thin-layer chromatography (prepTLC), 60 F254 (Merck) silica gel was used. Detection on TLC plates was enabled using UV light (254 and 366 nm), H_2SO_4–vanillin spray reagent on silica gel, followed by heating, and Naturstoff spray reagent on cellulose. For the biological assays, the chemicals and reagents were purchased from Merck (Darmstadt, Germany) (Folin–Ciocalteu reagent, gallic acid, sodium carbonate (Na_2CO_3)), Carlo Erba Reagents (Val-de-Reuil, France) (dimethyl sulfoxide (DMSO)), and Glentham Life Sciences (Corsham, UK) (2,2-diphenyl-1-picrylhydrazyl (DPPH•).

2.2. Plant Material

The crude plant materials (aerial parts of *H. nummularium* (HN) and *H. oelanticum* subsp. *incanum* (HO)) were collected from the Prespa Lake Park during the flowering period in 2021 (Table 1) and were botanically identified by F.N. Sakellarakis (Society for the Protection of Prespa, Agios Germanos, Greece). The samples were kept in a shaded and dry environment until fully dried, then ground using a laboratory mill and stored in darkness at room temperature.

Table 1. Collection data of the two studied species *H. nummularium* and *H. oelanticum* subsp. *incanum*.

Species	Abbreviation	Date	Location	Elevation (m)
Helianthemum nummularium (L.) Mill.	HN	05/2021	Mt. Devas, woodland *Quercus macedonica*, *Juniperus excelsa* and *Carpinus* sp., Prespa National Park, NW Greece	1067
Helianthemum oelandicum (L.) DC. in Lam. and DC. subsp. *incanum* (L.) Bonnier	HO			1065

2.3. Preparation of Extracts

The air-dried aerial parts of HN (52 g) and HO (66 g) were successively extracted with solvents of increasing polarity: cyclohexane (C-hex), dichloromethane (DCM), and methanol (MeOH) (3 times in each solvent with 500 mL) for 24 h at room temperature, receiving three extracts from each species (HN-C/HO-C cyclohexane extracts, HN-D/HO-D, dichloromethane extracts, HN-M/HO-M methanolic extracts). The extracts were evaporated under reduced pressure to dryness.

2.4. GC–MS Analysis

The chemical compositions of the cyclohexane and dichloromethane extracts were analyzed using GC–MS. Analysis was conducted using an Agilent Technologies Gas Chromatograph 7820A connected to an Agilent Technologies 5977B mass spectrometer system (Agilent, Santa Clara, CA, USA) with electron impact (EI) ionization at 70 eV. The gas chromatograph featured a split/splitless injector and an HP5MS capillary column (30 m, 0.25 mm internal diameter, and 0.25 μm film thickness). For cyclohexane extracts, the temperature program started at 60 °C for 5 min, increased at 3 °C/min to 280 °C, and held for 15 min, totaling 93 min. For dichloromethane extracts, the program started at 60 °C, increased at 3 °C/min to 300 °C, and held for 10 min, totaling 90 min. Helium was used as the carrier gas at a flow rate of 0.7 mL/min, with an injection volume of 2 μL, a split ratio of 1:10, and an injector temperature of 280 °C. Compounds were identified by comparing mass spectra with the Wiley Registry of Mass Spectral Data.

2.5. UHPLC–HRMS Analysis

Analyses of methanolic extracts were performed on a UHPLC–HRMS/MS Orbitrap Q-Exactive platform (Thermo Scientific, San Jose, CA, USA). A full scan in centroid mode with a mass range of 100–1200 Da was utilized. HRMS data at a resolution of 70,000 were collected in both negative and positive ionization modes under the following conditions: capillary temperature at 320 °C, spray voltage at 2.7 kV, S-lens RF level at 50 V, sheath gas flow at 40 arb units, auxiliary gas flow at 5 arb units, and auxiliary gas heater temperature at 50 °C. Separations were performed using a Hypersil Gold UPLC C18 reverse-phase column (2.1 × 100 mm, 1.9 μm; Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of solvent A: ultra-pure H2O with 0.1% (*v*/*v*) FA, and solvent B: MeOH with 0.1% (*v*/*v*) FA. A gradient method with a total run time of 18 min was employed: 0–1 min, 5% B (isocratic); 1–8 min, 50–50% B (linear gradient); 8–13 min, 100% B (isocratic for column cleaning); 14–16 min, 95–5% B (linear gradient); and 16–18 min, 5% B (isocratic for column

equilibration). The column temperature was maintained at 40 °C and the sample tray temperature at 10 °C, with a flow rate of 0.3 mL/min and an injection volume of 5 μL.

2.6. Fractionation and Purification Procedures of Methanolic Extracts

2.6.1. *H. nummularium* (HN-M)

HN-M (2.00 g) was subjected to column chromatography with microcrystalline cellulose and eluted with CH_3COOH 3% up to 30% (gradient analysis); 219 fractions (HNA1–HNA219) were collected. Fractions HNA8–HNA14 (6.3 mg) were identified as quercetin-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranose (rutin) and fractions HNA15-HN27 (3.8 mg) identified as gallic acid, all of which were eluted with CH_3COOH 3%. Fractions HNA214–HNA215 were eluted with CH_3COOH 30% and were further extracted with ethyl acetate. From the ethyl acetate residue, linoleic acid (3.5 mg) was obtained. Furthermore, fractions HNA36–HNA148 (215 mg) were subjected to silica-60 gel column chromatography eluted with DCM: MeOH (98:2–80:20) (gradient method) to afford 176 fractions (HNB1–HNB176). Fractions HNB5-HNB45 (22.6 mg) were eluted with DCM: MeOH (95:5) and identified as the racemic mixture of *cis*- and *trans*-kaempferol-3-*O*-*β*-D-(6″-p-coumaroyl-glucopyranoside (*cis*/*trans*-tiliroside). Fractions HNB152–HNB159 (7.8 mg) were eluted with DCM: MeOH (90:10) and identified as the mixture of metabolites quercetin-3-*O*-*β*-D-glucopyranoside (isoquercetin)/quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside).

2.6.2. *H. oelanticum* subsp. *incanum* (HO-M)

HO-M (1.15 g) was subjected to column chromatography with microcrystalline cellulose and eluted with CH_3COOH 5% up to 30% (gradient analysis); 300 fractions (HOA1–HOA300) were collected. Fraction HOA39 (2.9 mg) was eluted with CH_3COOH 5% and was identified as quercetin-3-*O*-(2″-*O*-galloyl)-*β*-D-galactopyranoside. Fractions HOA25-HOA38 (25.9 mg) were subjected to a preparative TLC silica plate (EtOAc (100):AcOH (11):FA (11):H_2O (26)) and three bands were received. The first band was the racemic mixture of *cis*- and *trans*-kaempferol-3-*O*-*β*-D-(6″-p-coumaroyl-glucopyranoside (*cis*/*trans*-tiliroside) (7.6 mg), the second band was the metabolite kaempferol-3-*O*-*β*-D-glucopyranoside (astragalin) (4.2 mg) and the third band was identified as quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside) (4.5 mg). Furthermore, fractions HOA8–HOA14 (225 mg) were subjected to silica-60 gel column chromatography eluted with $CHCl_3$: MeOH (90:10-80:20) (gradient analysis) to afford 162 fractions (HOB1–HOB162). Fractions HOB4-HOMB5 (6.1 mg) were identified as methyl gallate and fractions HOB50–HOB61 (6.9 mg) were identified as catechin-3-*O*-*β*-D-glucopyranoside, all of which were eluted with $CHCl_3$: MeOH (90:10).

2.7. Nuclear Magnetic Resonance (NMR)

One-dimensional (^{1}H-NMR, ^{13}C-NMR) and two-dimensional NMR spectra (COSY, HSQC, HMBC) were recorded on a Bruker Avance III 400 MHz (Bruker BioSpin, Rheinstetten, Germany) spectrometer using methanol-d4 (D024ES, Methanol-d4, Eurisotop, Chembiotin S.A., Voula, Greece) as the solvent. Chemical shifts are reported in ppm relative to the solvent in which the spectra were recorded [^{1}H: δ (CD_3OD) = 3.31 ppm; ^{13}C:δ (CD_3OD) = 49.15 ppm].

2.8. Total Phenolic Content (TPC)

The total phenolic content of the samples was determined by the Folin–Ciocalteu method [21]. A 96-well plate was used to dilute 25 μL of various concentrations (4, 2, 1 mg/mL) of extract or standard solutions of gallic acid (2.5, 5, 10, 12.5, 20, 25, 40, 50, 80, 100 μg/mL) in dimethylsulfoxide (DMSO). Both the extract and standard solutions were mixed with 125 μL of 10% Folin–Ciocalteu solution, followed by the addition of 100 μL of 7.5% sodium carbonate. The plate was incubated in darkness at room temperature for 30 min. Absorbance was measured at 765 nm using a TECAN Infinite m200 PRO multimode reader (Tecan Group, Männedorf, Switzerland). All measurements were performed in

triplicate, and the mean values were plotted on a gallic acid calibration curve. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per gram of dry extract.

2.9. DPPH (2,2-DiPhenyl-1-PicrylHydrazyl) Assay

The antioxidant activity of the samples was evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, according to the literature [21]. In a 96-well plate, 10 µL of various concentrations (4, 2, 1, 0.5, 0.25, 0.125, 0.0625 mg/mL) of extracts dissolved in DMSO were added to 190 µL of DPPH solution (12.4 mg/100 mL in ethanol). The plate was then incubated in darkness at room temperature for 30 min. Absorbance was measured at 517 nm using an Infinite M200 Pro TECAN photometer (Tecan Group, Männedorf, Switzerland). All evaluations were performed in triplicate, with gallic acid serving as the positive control (IC50 = 2.6 µg/mL). The percentage inhibition of the DPPH radical for each dilution was calculated using the following formula: % inhibition = [(A-B) − (C-D)]/(A-B) × 100, where A is the control (without sample), B is the blank (without sample, without DPPH), C is the sample, and D is the blank sample (without DPPH). Samples were evaluated in triplicate at final concentrations of 200, 100, 50, 25, 12.5, and 6.25 µg/mL, with results expressed as means ± standard deviation (n = 3).

2.10. In Vitro Tyrosinase Assay

The methanolic extracts were investigated for their ability to inhibit the oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine) to dopaquinone and subsequently to dopachrome by the enzyme tyrosinase according to the literature [21]. The fractions were dissolved in DMSO (10 mg/mL) and then diluted in 1/15 M phosphate buffer (NaH2PO4/Na2HPO4), pH 6.8, ensuring that the final DMSO concentration in each well did not exceed 2%. In 96-well plates, 40 µL of sample in the phosphate buffer (1/15 M, pH 6.8) was mixed with 80 µL of the same buffer and 40 µL of mushroom tyrosinase (92 Units/mL) in the buffer. The mixture was incubated for 10 min at 25 °C, followed by the addition of 40 µL of 2.5 mM L-DOPA in the same buffer. The plates were then incubated for 5 min at 25 °C, and the absorbance of each well was measured at 475 nm. Extracts were tested in triplicate at a concentration of 300 µg/mL, with blank samples for each fraction also measured. Kojic acid was used as a positive control at a final concentration of 2 µg/mL, resulting in 52.84 ± 2.39% inhibition.

2.11. Antimicrobial Activity

A total of eleven microorganisms were assayed, including four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus mutans* (ATCC 31989), and *Streptococcus viridans* (ATCC 19952); four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883), and *Pseudomonas aeruginosa* (ATCC 227853); and three pathogenic fungi: *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13801), and *Candida glabrata* (ATCC 28838). The antimicrobial activity was tested using the micro-dilution broth method to determine the minimal inhibitory concentration (MIC), following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Sterile 96-well microtiter plates were prepared by dispensing 100 µL per well of appropriately diluted extracts in broth medium, with two-fold dilutions to achieve final concentrations ranging from 0.50 to 10 mg/mL. Microbial inoculums, prepared in sterile 0.85% NaCl to match the turbidity of the 0.5 McFarland standard, were added to the wells to achieve final densities of 1.5×10^6 CFU/mL for bacteria and 5×10^4 CFU/mL for yeasts. The plates were then incubated at 37 °C for 24 h. MICs were determined visually as the lowest concentration of the extracts that completely inhibited the growth of the reference microbial strains. Each microplate included a DMSO control (at a final concentration of 10%), a positive control (inoculum without tested samples), and a negative control (tested samples without inoculum). Standard antibiotics, including netilmicin (at concentrations of 4–88 µg/mL) for bacteria and sanguinarine for oral bacteria, were used as controls. For fungi, 5-flucytosine

(at concentrations of 0.5–25 μg/mL) and amphotericin B (at concentrations of 30, 15, and 10 μg/mL) were used as controls (Sanofi Paris, France, Diagnostics Pasteur). Pure solvent was also used as a blind control. All experiments were repeated three times, and results were expressed as mean values [22].

2.12. Statistical Analysis

All the experiments were conducted in triplicate and average values along with their standard deviations (SDs) were calculated using the Excel software package (Microsoft Office 2021).

3. Results

3.1. Identification of Metabolites from the Non-Polar Extracts (HN-C, HO-C, HN-D, and HO-D)

GC–MS analysis of HN-C, HO-C, HN-D, and HO-D is given in Tables 2 and 3. Twelve and nine compounds were tentatively identified from the cyclohexane extracts as well as four and six compounds from the dichloromethane extracts.

Table 2. Volatile metabolites of the extracts HN-C and HO-C using GC–MS.

Compound	Area (%)	
	HN-C	HO-C
α-Thujene	0.63	-
α-Pinene	35.77	40.77
Sabinene	12.05	14.74
β-Pinene	1.03	-
Myrcene	2.70	2.97
δ-3-Carene	6.47	7.18
p-Cymene	1.45	1.04
Limonene	28.95	28.93
γ-Terpinene	1.25	1.11
Terpinolene	1.43	1.25
Thujone isomer	<0.1	<0.1
Palmitic acid methyl ester	0.76	-

Table 3. Volatile metabolites of the extracts HN-D and HO-D using GC–MS.

Compound	Area (%)	
	HN-D	HO-D
1-Nonadecene	-	7.74
Octadecane	-	5.63
Eicosane	-	19.46
Hexadecanoic acid (Palmitic acid)	1.35	1.42
9,12-Octadecenoic acid (Linoleic acid)	1.14	-
γ-Sitosterol	1.11	1.19
β-Sitosterol	0.83	0.90

3.2. Identification of Metabolites from Methanolic Extracts

Based on the chromatographic analysis of the methanolic extracts HN-M and HO-M, ten metabolites were isolated from the studied species through several chromatographic techniques and identified through NMR spectral analysis in comparison with

the literature data: quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose (rutin), gallic acid, linoleic acid, *cis*/*trans*-kaempferol-3-*O*-β-D-(6″-p-coumaroyl-glucopyranoside (*cis*/*trans*-tiliroside), quercetin-3-*O*-β-D-glucopyranoside (isoquercetin), quercetin-3-*O*-β-D-galactopyranoside (hyperoside), quercetin-3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside, kaempferol-3-*O*-β-D-glucopyranoside (astragalin), methyl gallate, and catechin-3-*O*-β-D-glucopyranoside. Furthermore, mass spectral analysis was performed to identify the bioactive compounds in both studied extracts (Figures S1–S4). Thirty-nine and twenty-nine compounds (Table 4) were tentatively identified from HN-M and HO-M extracts, respectively, using UHPLC–HRMS and comparing the mass spectral data with the literature.

Table 4. Metabolites of the methanolic extracts of HN-M and HO-M using UHPLC–HRMS in negative and positive modes.

Rt (min)		Adduct Ions	Observed *m/z*	MS/MS	Mass	Molecular Formula	Identified Compounds	Determined by
HN-M	**HO-M**							
3.54	3.51	$[M-H]^-$	169	169, 125	170	$C_7H_6O_5$	Gallic acid	[12], NMR
4.02		$[M+H]^+$	365	203, 185	364	$C_{17}H_{16}O_9$	Xanthotoxol glucopyranoside	[12,23]
4.05	4.03	$[(M-H)+HCO_2H]^-$	387	341, 179, 119, 101, 89	342	$C_{15}H_{18}O_9$	Caffeic acid hexoside	[24]
4.29		$[M-H]^-$	312	312, 184, 183	313		Methylgallate derivative	[25]
14.16		$[M-H]^-$	315	179, 153, 152, 108	316	$C_{13}H_{16}O_9$	Dihydroxybenzoic acid hexoside	[26]
	14.44	$[M-H]^-$	343	191, 169	344	$C_{14}H_{16}O_{10}$	*O*-galloylquinic acid	[27]
	14.60	$[M-H]^-$	153	153, 109	154	$C_7H_6O_4$	Dihydroxybenzoic acid	[28]
14.88	14.88	$[M-H]^-$	183		184	$C_8H_8O_5$	Methyl gallate	[12], NMR
14.93		$[M-H]^-$	455	409, 325, 307, 265, 205, 163,	456		p-Coumaric acid derivative	[26]
15.38		$[M-H]^-$	305	179,174, 139, 137, 125	306	$C_{15}H_{14}O_7$	(Epi)gallocatechin	[27]
15.47		$[M+H]^+$	355		354	$C_{16}H_{18}O_9$	Chlorogenic acid	[12]
15.90		$[M-H]^-$	153	153, 135, 109, 65	154	$C_7H_6O_4$	Dihydroxybenzoic acid	[28]
	15.95	$[M-H]^-$	325	169, 125	326	$C_{14}H_{14}O_9$	Galloylshikimic acid isomer	[29]
16.12		$[M-H]^-$	783	783, 765, 301, 275, 247, 169	784	$C_{34}H_{24}O_{22}$	Pedunculagin isomer (Ellagitannin)	[30,31]
	16.28	$[M-H]^-$	451	451, 329, 313, 289, 271	452	$C_{21}H_{24}O_{11}$	Catechin-3-*O*-glucopyranoside	[32], NMR
16.66	16.64	$[M-H]^-$	483		484	$C_{20}H_{20}O_{14}$	Digalloyl-hexoside	[29]
17.05	17.05	$[M-H]^-$	431	385, 223, 205, 161, 153,	432	$C_{17}H_{22}O_{10}$	Sinapic acid hexoside	[26]
17.16		$[M-H]^-$	367	367, 193, 134	368	$C_{17}H_{20}O_9$	5-O-Feruloyl-quinic acid	[33]
17.35		$[M-H]^-$	337	191, 163, 119	338	$C_{16}H_{18}O_8$	Coumaroyl-quinic acid	[34]

Table 4. *Cont.*

Rt (min)		Adduct Ions	Observed *m/z*	MS/MS	Mass	Molecular Formula	Identified Compounds	Determined by
HN-M	**HO-M**							
	17.57	$[M-H]^-$	633	633, 463, 301, 275, 257, 245	634	$C_{27}H_{22}O_{18}$	Galloyl-HHDP-hexoside	[30,31]
17.64	17.64	$[(M-H)+HCO_2H]^-$	461	415, 269. 161, 101	416	$C_{21}H_{20}O_9$	Dicaffeoyl glycerol	[35]
	18.47	$[(M-H)+HCO_2H]^-$	567	567, 521, 359, 341, 329, 179,	522	$C_{21}H_{30}O_{15}$	Syringyl dihexoside	[36]
18.52	18.52	$[M-H]^-$	631	631, 479, 317, 179	632	$C_{28}H_{24}O_{17}$	Myricetin-O-galloyl-hexoside	[37]
18.67		$[M-H]^-$	463	463, 301, 300, 271, 255	464	$C_{21}H_{20}O_{12}$	Isoquercetin	[38], NMR
18.68		$[M-H]^-$	609	301, 300, 271	610	$C_{27}H_{30}O_{16}$	Rutin	[38], NMR
18.82		$[M-H]^-$	761	609, 305	762	$C_{37}H_{30}O_{18}$	(Epi)gallocatechin-*O*-galloyl(epi)gallocatechin	[33]
19.15		$[M-H]^-$	436	436, 316, 273, 145, 119	437	$C_{25}H_{31}N_3O_4$	N, N″-di-p-coumaroylspermidine	[39–41]
19.23		$[M-H]^-$	593	593, 571, 447, 384, 327, 285,	594	$C_{27}H_{30}O_{15}$	Kaempferol rutinoside	[12,42]
19.28	19.31	$[M-H]^-$	463	463, 301, 300, 271, 255	464	$C_{21}H_{20}O_{12}$	Hyperoside	[38], NMR
19.31		$[M-H]^-$	615	463, 301, 300, 271, 255, 169	616	$C_{28}H_{24}O_{16}$	Quercetin galloylhexoside	[43]
	19.33	$[M-H]^-$	615	463, 301, 300, 271, 255, 243	616	$C_{28}H_{24}O_{16}$	Quercetin-3-*O*-(2″-*O*-galloyl)-galactopyranoside	[43], NMR
	19.76	$[M-H]^-$	447	285, 284, 255, 227	448		Kaempferol-3-O-glucopyranoside	[38], NMR
	19.80	$[M-H]^-$	599	517, 447, 429, 415, 301, 285	600	$C_{28}H_{24}O_{15}$	Kaempferol galloyl hexoside	[44]
19.85	19.85	$[M-H]^-$	137	137, 93	138	$C_7H_6O_3$	4-Hydroxybenzoic acid	[28]
19.94	19.90	$[M-H]^-$	447	315, 299, 284, 255, 227	448	$C_{21}H_{20}O_{11}$	Isorhamnetin-pentoside	[45]
	20.25	$[M-H]^-$	625	463, 301	626	$C_{27}H_{30}O_{17}$	Quercetin dihexoside	[46]
	20.40	$[M-H]^-$	477	301	478	$C_{21}H_{18}O_{13}$	Quercetin glucuronide	[46]
21.22		$[(M-H)+HCO_2H]^-$	493	447, 399, 315, 161	448	$C_{21}H_{20}O_{11}$	Isorhamnetin pentoside isomer	[47]
21.43		$[M-H]^-$	419	153, 152, 108, 109	420		Procatechuic acid derivative	[24]
21.97	21.95	$[M-H]^-$	593	593, 447, 307, 285, 255, 145,	594	$C_{30}H_{26}O_{13}$	*cis*/*trans*-Tiliroside	[12,47], NMR
22.24	22.24	$[M-H]^-$	593		594	$C_{30}H_{26}O_{13}$	*cis*/*trans*-Tiliroside	
23.39	23.41	$[M-H]^-$	327	327, 291, 239, 229, 211	328	$C_{18}H_{32}O_5$	Oxo-dihydroxy-octadecenoic acid	[48]
24.12	24.11	$[M-H]^-$	329	329, 229, 211	330		Linoleic acid derivative	[49]

Table 4. *Cont.*

Rt (min)		Adduct Ions	Observed *m/z*	MS/MS	Mass	Molecular Formula	Identified Compounds	Determined by
HN-M	**HO-M**							
25.36	25.40	$[M-H]^-$	739	739, 593, 453, 285, 255, 227,	740	$C_{36}H_{36}O_{17}$	Kaempferol coumaroyl rutinoside	[47]
	25.63	$[M-H]^-$	739	593, 285, 284, 255, 227, 145	740	$C_{36}H_{36}O_{17}$	Kaempferol coumaroyl rutinoside isomer	[47]
26.60		$[M-H]^-$	593	593, 315, 277, 241, 152	594	$C_{27}H_{30}O_{15}$	Isorhamnetin deoxyhexosyl-pentoside	[50]
27.71	27.70	$[(M-H)+HCO_2H]^-$	721	675, 415, 397, 277, 235, 179	676	$C_{33}H_{56}O_{14}$	DGMG (18:3) galactolipid	[51,52]
27.79		$[M-H]^-$	577	577, 441, 299, 225, 94, 80	578	$C_{30}H_{26}O_{12}$	Procyanidin dimer	[53]
28.19	28.16	$[M-H]^-$	595	415, 315, 279, 214, 152	596	$C_{27}H_{49}O_{12}P^-$	Lysophospatidylinositol 18:2	[52,54]
29.45		$[(M-H)+HCO_2H]^-$	647	601, 571,341, 323, 277, 265			Fatty acid derivative	[55]
32.37		$[M-H]^-$	279		280	$C_{18}H_{32}O_2$	Linoleic acid	[56], NMR

3.3. Determination of Total Phenolic Content (TPC), DPPH Free Radical, and Tyrosinase-Inhibitory Activity

The total phenolic content was measured in the methanolic extracts HN-M and HO-M using the DPPH assay. Tyrosinase-inhibitory effects of methanolic extracts were tested according to the L-DOPA oxidation method. The results of the determination of the TPC as well as the DPPH free radical and tyrosinase-inhibition rates are presented in Table 5.

Table 5. Determination of TPC, DPPH free radical, and tyrosinase-inhibitory activity of the methanolic extracts of HN-M and HO-M and standards gallic acid (for DPPH assay), and kojic acid (for tyrosinase-inhibition assay).

Samples	TPC (mg GAE/g)	% Inhibition of DPPH•						Tyrosinase % Inhibition
		200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	300 µg/mL
HN-M	177.21 ± 2.35	94.58 ± 0.11	86.93 ± 2.45	59.40 ± 0.67	48.81 ± 1.58	19.66 ± 2.25	16.05 ± 0.97	42.72 ± 0.61
HO-M	150.63 ± 4.72	93.96 ± 1.00	85.66 ± 4.38	57.11 ± 2.84	47.83 ± 2.25	15.84 ± 2.54	13.95 ± 1.23	8.46 ± 1.12
Gallic acid (2.6 µg/mL)		53.56 ± 2.47						
Kojic acid (2.0 µg/mL)								52.84 ± 2.39

3.4. Antimicrobial Activity

The methanolic extracts were evaluated for their antimicrobial activity via the dilution method. The results, expressed as the minimum growth inhibitory concentrations (MICs) of the extracts and the reference antimicrobial agents, are shown in Table 6.

Table 6. Antimicrobial activity of the extracts HN-M and HO-M measured in terms of the MIC (mg/mL).

Sample/ Standards	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*K. pneumoniae*	*E. cloacae*	*E. coli*	*S. mutans*	*S. viridans*	*C. albicans*	*C. tropicalis*	*C. glabrata*
HN-M	0.10	0.08	0.75	0.82	0.93	0.78	0.12	0.07	0.67	0.70	0.79
HO-M	0.12	0.10	0.72	0.97	1.00	0.80	0.15	0.12	0.70	0.77	0.90
Netilmicin	3.5×10^{-3}	4×10^{-3}	8.5×10^{-3}	7.7×10^{-3}	7.5×10^{-3}	9×10^{-3}					
Sanguinarine							0.017	0.013			
5-flucytocine									0.12×10^{-3}	1.2×10^{-3}	10×10^{-3}
Amphotericin B									1.5×10^{-3}	0.7×10^{-3}	0.25×10^{-3}

4. Discussion

Despite being the largest genus in the Cistaceae family, with an exceptionally diverse distribution and a history in folk medicine, only limited data concerning the phytochemical profile and biological activities of the *Helianthemum* species have been identified in the literature. We proceeded with an integrated phytochemical analysis of the species HN and HO, growing wild in the Prespa Lakes region, northwest of Greece, including non-polar and polar extracts of their aerial parts.

Cyclohexane (HN-C, HO-C) and dichloromethane extracts (HN-D, HO-D) were analyzed through GC–MS and the cyclohexane extracts showed the presence of monoterpenes, probably attributed to their volatile content received through the extraction procedure. In both species, the most notable monoterpenes were α-pinene, sabinene, myrcene, δ-3-carene, and limonene, with slight quantitative differences (Table 2). Specifically, in HN-C, α-pinene (35.77%), sabinene (12.05%), myrcene (2.70%), δ-3-carene (6.47%), and limonene (28.95%) were detected, while in HO-C, α-pinene (40.77%), sabinene (14.74%), myrcene (2.97%), δ-3-carene (7.18%), and limonene (28.93%) were detected. On the other hand, the volatile fraction of the dichloromethane extracts differed chemically (Table 3). In HN-D, the fatty acids palmitic and linoleic acid were detected, while in HO-D, hydrocarbons and palmitic acid were detected. In both species, the sterol derivatives β- and γ-sitosterol were identified.

Only a few studies have been conducted on the volatile compounds of *Helianthemum* species, and these concern essential oils from the aerial parts of *H. kahiricum* Del. from Iran [10] and *H. canum* (L.) Baumg. from Turkey [11]. The essential oil from *H. kahiricum* consisted mainly of the fatty acids like palmitic acid, myristic acid, lauric acid, and linoleic acid, as well as oxygenated monoterpenes and oxygenated sesquiterpenes [10]. The essential oil from *H. canum* appeared to contain myristicin (29.4%) as the dominant component [11], which was not detected in the studied species in the current study. Other reported minor compounds were saturated fatty acids (palmitic acid, myristic acid, lauric acid), hydrocarbons, sesquiterpenes, oxygenated sesquiterpenes, and monoterpenes similar to those found in the studied cyclohexane extracts (α-pinene, myrcene, limonene, 1,8-cineole, (*E*)-β-ocimene, camphor, bornyl acetate) [11].

The presence of sterols has been previously reported in the species *H. sessiliflorum*, presenting the isolation of β-sitosterol and daucosterol [13], and in *H. ruficomum,* referring to β-sitosterol and stigmasterol isolations [15].

Overall, our analysis of the chemical composition of the non-polar extracts HN-C, HO-C, HN-D, and HO-D have revealed the presence of monoterpenes, fatty acids, and hydrocarbons similar to those found in essential oils from the genus [10,11], as well as sterols that have been reported in other *Helianthemum* species [13,15].

According to the UHPLC–HRMS study of the methanolic extracts (HN-M, HO-M) (Table 4), the majority of the determined metabolites are new for the species to the best of our knowledge. There were significant similarities in both studied samples in terms of

qualitative analysis. Thirty-nine and twenty-nine compounds were identified from HN-M and HO-M extracts, respectively, based to the comparison of mass spectral data with the literature. In both species, phenolic derivatives including phenolic acids, flavonoids, and polyphenols were abundant, while other chemical categories (such as fatty acids) were also detected.

Regarding phenolic acids, benzoic and hydroxycinnamic acid derivatives were present in both plants (4-hydroxybenzoic acid, dihydroxybenzoic acid isomers, gallic acid, methyl gallate, chlorogenic acid, 5-*O*-feruloylquinic acid, syringoyl dihexoside). Previous studies in various *Helianthemum* species including *H. canum* [4], *H. nummularium* [9], *H. lippii* [12,14], *H. sessilifolium* [13], *H. ruficomum* [15], and *H. getulum* [57], have also confirmed the existence of phenolic acids in their aerial parts. Dihydroxybenzoic acid hexoside and sinapic acid hexoside, most likely glycosylated derivatives, were detected in HN-M, marking the first report of their presence in the genus. Similarly, metabolites such as the galloylshikimic acid isomer and *O*-galloylquinic acid, identified in HO-M alongside caffeic acid derivatives, caffeic acid hexoside, and dicaffeoylglyceride, were detected in both studied plants and have also been reported for the first time in the genus. Among the detected phenolic acid derivatives, gallic acid and methyl gallate were isolated from HN-M and HO-M, respectively. Common phenolic acid derivatives from HN-M, HO-M, and previously studied *Helianthemum* spp. are presented in Table S1.

Flavonoids, especially flavonol derivatives, were detected in both species such as quercetin and kaempferol derivatives. Rutin (quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranose) was detected and isolated from HN-M, while it has been previously reported in the same species [17] as well as in *H. lippii* [12] and *H. ruficomum* [15]. Furthermore, isoquercetin (quercetin-3-*β*-D-glucopyranoside) was isolated from HN-M, previously reported in this species [9], as well as in *H. sessilifolium* [13] and *H. lippii* [14]. Hyperoside (quercetin-3-*β*-D-galactopyranoside) was isolated from both studied species, and it has been previously detected in *H. nummularium* [17], but it was isolated from HO for the first time.

Among the kaempferol derivatives, the racemic mixture of *cis-/trans*-tiliroside was isolated from both studied species. This metabolite is one of the most widespread in the genus, having been reported previously in many species such as *H. nummularium* [9,17], *H. lippii* [12], *H. getulum* [57], *H. ruficomum* [15], *H. sessilifolium* [13], and *H. kahiricum* [58]. Kaempferol coumaroyl rutinoside, identified in both studied species, and kaempferol rutinoside, detected in HN-M, appeared for the first time in the species.

Interestingly, galloyl-flavonol derivatives were detected in both plants for the first time in the genus. Specifically, quercetin galloylhexoside, and myricetin-*O*-galloylhexoside were identified in both species, while from HO-M, the quercetin galloylhexoside was isolated and identified as quercetin-3-*O*-(2″-*O*-galloyl)-galactopyranoside. In HO-M, kaempferol galloyl hexoside was also detected. Galloylflavonols have been previously reported in only two species in the whole Cistaceae family: *Fumana montana* Pomel. [59] and *Cistus salvifolius* [60].

The flavan derivative catechin-3-*O*-*β*-D-glucopyranoside was isolated from HO-M, not previously reported in the species, while in the *Helianthemum* genus, only catechin rutinoside has been detected so far in *H. lippii* [12]. In HN-M, epigallocatechin was detected with previous reference in *H. lippii* [12], *H. getulum* [57], and *H. sessilifolium* [13]. Common flavonoid derivatives from HN-M, HO-M, and previously studied *Helianthemum* spp. are presented in Table S2.

Hydrolyzed and condensed tannins were detected in both studied species. Galloyl-HHDP-hexoside, was found in HO-M, digalloyl-hexoside was detected in both species, and the pedunculagin isomer was found in HN-M. These polyphenols are reported for the first time in the genus. The flavan-3-ol dimers (epi)gallocatechin-*O*-galloyl(epi)gallocatechin and procyanidin dimer were also detected in HN-M. In the genus *Helianthemum*, the presence of polyphenols has been confirmed in *H. helianthemoides* [6], *H. lippii* [12], and *H. ordosicum*, from which gallocatechin-(4a → 8)-epigallocatechin was identified [16]. Ellagitannins

and gallotannins have been reported in the genus *Cistus*, with galloyl-HHDP-hexoside and digalloyl-hexoside found in *C. creticus* L., and the compound pedunculagin in *C. incanus* L. [60].

Apart from the abovementioned phenolic derivatives, several fatty acid derivatives were detected in the studied species, including oxo-dihydroxy-octadecenoic acid, which is an oxidated oleic acid derivative; DGMG (digalactosylmonoacylglycerol) (18:3) galactolipid, which is a digalactosyl alpha-linolenic acid derivative; and lysophosphatidylinositol (LPI) 18:2, which belongs to the class of lysophospholipids. These compounds are most probably cellular components with either a structural role as components of cell membranes or organelles such as chloroplasts, or they may have a functional role in participating in the regulation of biological functions by transmitting or transferring signals through transcript G-protein-coupled receptors (GPCRs) [52,54,61]. To the best of our knowledge, there are no previous reports in the Cistaceae family regarding these compounds, while their presence has been reported in the fruits of *Corylus avellana* (Betulaceae) [54] and the leaves and fruits of *Castanea sativa* (Fagaceae) [52]. From this class of derivatives, linoleic acid was isolated from HN-M in the present study.

The last chemical class detected was hydroxycinnamate amides, with the detection of the metabolite N, N″-di-p-coumaroylspermidine in HN-M. Nitrogen-containing secondary metabolites are not prevalent in the genus *Helianthemum*, nor the wider family of Cistaceae. However, the presence of alkaloids has been reported in *H. ordosicum*, from which the alkaloid 7-phenyl-pyrazino[1,2-a] azepine was isolated [16]. In a recent study on the aerial parts of *H. lippii*, the metabolite spermatheridine was detected through GC–MS analysis [7], while the presence of alkaloids in *H. lippii* was additionally confirmed by detection testing in the Laib et al. study [14]. N,N″-di-p-coumaroylspermidine was detected in both the negative and positive ionization modes with parent ions at *m*/*z* 436 and 438, respectively, at a retention time of 19.15 min. (Figure S5). In the figure below (Figure 1), the chemical structure for this compound identified in HN-M, reported for the first time in Cistaceae family, is presented.

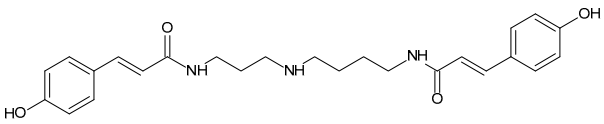

Figure 1. N,N″-di-p-coumaroylspermidine identified by UHPLC–MS/MS.

The antioxidant capacity of the methanolic extracts of the studied species has been determined. The extracts displayed high TPC values, with 177.2 mg GA/g extract for HN-M and 150.6 mg GA/g extract for HO-M. The DPPH assay revealed similarly high inhibition for both species, with maximum inhibition occurring at 0.2 mg/mL (94.6% for HN-M and 94.0% for HO-M). The IC_{50} was observed at 0.25 mg/mL for both species. In the Baldemir et al. study, the methanolic extract of *H. canum* (HO) displayed a higher total phenolic content with a TPC value of 284.13 mg GA/g extract, and the IC_{50} for the DPPH assay was 0.19 mg/mL [4]. Pirvu et al. estimated the scavenging effect of the 70% ethanolic extract of *H. nummularium* with the chemiluminescence assay and indicated IC_{50} was 1.27 μg/mL, using gallic acid (IC_{50} 0.85 μg/mL) as a positive control [17].

Since *Helianthemum* species have been used traditionally for dermal applications, with no supportive literature referring to in vitro assays on skin disorders, we demonstrated a screening on the extracts' inhibitory activity against tyrosinase due to the lack of such data and/or experiments on the genus *Helianthemum*.

The assayed extracts exhibited low tyrosinase-inhibitory activity with inhibition percentages of 42.7% for HN-M and 8.5% for HO-M at the concentration of 300 μg/mL. Comparing both results, the higher inhibition for HN-M could be attributed to the presence of rutin with known anti-tyrosinase activity, which was not identified in the HO-M extract [62].

The antimicrobial activity against free-living microbial strains is expressed in terms of MIC in mg/mL and is shown in Table 6. The methanolic extracts of both species noted stronger inhibition against the Gram-positive bacterial strains *S. aureus*, *S. epidermidis*, *S. mutans*, and *S. viridans*, while between the two species, HN-M showed lower MIC values (0.07 and 0.08 mg/mL against *S. epidermidis* and *S. viridans*, respectively). Moderate-to-weak activities were exhibited against the examined *Candida* spp. and Gram-negative bacterial strains *P. aeruginosa, K. pneumoniae, E. cloacae,* and *E. coli* (MIC range of 0.72–1.00 mg/mL). However, inhibition in fungal strains was slightly higher compared to that of Gram-negative bacteria.

According to the literature, quercetin glycosides (hyperoside, isoquercetin, rutin) and kaempferol derivatives (tiliroside, kaempferol rutinoside) combined with gallic and/or caffeic phenylcarboxylic acid derivates may contribute to the exerted microbial inhibition [17]. Both studied species have been previously evaluated in terms of their antimicrobial activities. Baldemir et al. reported, that the methanolic extract of *H. canum* (HO) showed strong inhibition against *Bacillus* sp. strains with an MIC value of 0.62 mg/mL. In contrast, the MIC values for *E. coli, S. aureus*, and *P. aeruginosa* were found to be comparatively higher (1.25 and <2.5 mg/mL) than the MICs for the same strains in our study [4]. In the study of Pirvu et al., the 70% ethanolic extract of HN was evaluated using the dilution method, exerting strong activity against *S. aureus* and *E. coli*, with inhibition zones of 15.5 and 21.5 mm, respectively [17].

The antimicrobial activity and the ability of phenolic compounds to scavenge free radicals is a property that makes these compounds extremely attractive. Phenolic extracts, including flavonol compounds such as glycosides of quercetin and kaempferol, and phenolic acids such as gallic acid and its derivatives have been shown to constitute interesting tools as natural preservatives, increasing the shelf life and stability of fresh food products [63,64]. Moreover, several reports indicate an antimicrobial effect of flavonols identified in the studied extracts including kaempferol-3-*O*-rutinoside (HO-M), rutin (HN-M), isoquercitrin (HN-M), and hyperoside (HN-M, HO-M) [17,63]. Dietary polyphenols have demonstrated the ability to modulate gut microbiota composition and function, as well as interfere with bacterial quorum sensing and exhibiting prebiotic-like activity [65]. Taken together, these data indicate extracts rich in these compounds can be a valuable source for functional food products, food supplements, and natural food preservatives.

The antioxidant and antimicrobial activities of phenolic compounds are also of cosmeceutical significance. The use of antioxidants in cosmetics helps to mitigate oxidative damage, offering an effective approach for preventing and treating premature aging, while the antimicrobial activity aims to minimize the degradation of a product caused by microorganisms [66]. The inhibition of the microbial proliferation of *S. aureus*, *S. epidermis*, and *S. mutans*, especially from HN-M, alongside its high antioxidant and moderate tyrosinase-inhibitory activity suggest some potential application in cosmetics.

5. Conclusions

The current study conducted an integrated phytochemical analysis of the aerial parts of the *Helianthemum* species HN and HO. The analysis included the identification of metabolites from the non-polar extracts (GC–MS) and the chemical profile from methanolic extracts through UHPLC–HRMS, while the latter were evaluated for their total phenolic content, and antioxidant, tyrosinase-inhibition, and antimicrobial activities. Both species have limited references towards their phytochemistry and biopotential, while they consist of a considerable source of natural remedies and are used traditionally against various health disorders. The current study is the first comprehensive analysis for *Helianthemum* species growing wild in Greece.

Both studied species seem to bear essential oil in their aerial parts, which is in accordance with a previous study on volatile compounds from HO. Specifically, HN-C appeared to have a characteristic odor, with no previous reports on its volatile compounds, making it an interesting candidate for such an analysis. The chemical profile from the methanolic

extracts showed similarities with other species of the genus previously studied, with the most prominent chemical class being the phenolic derivatives, something that was also confirmed by the TPC determination. However, new derivatives from the species and the genus were identified, such as galloyl flavonol glycosides, flavonoid and catechin glucosides, caffeic acid derivatives, and polyphenols, while several compounds are reported for the first time in the family like the hydroxycinnamate amide, N,N″-di-p-coumaroylspermidine, and the fatty acid derivatives DGMG (18:3) galactolipid and lysophosphatidylinositol (LPI) 18:2. Although the compounds were tentatively identified by comparing the mass spectral data with the literature, the isolation of some of them (quercetin-3-*O*-(2″-*O*-galloyl)-galactopyranoside, catechin-3-*O*-*β*-D-glucopyranoside) further confirms their existence.

In our effort to broaden, the already tested biological properties, we proceeded to the evaluation of the tyrosinase-inhibitory activity. This is in addition to the antioxidant and antimicrobial activities, both of which showed promising results with high inhibition percentages for the free radical and low MIC values against Gram-positive microbial strains. Although the inhibition of tyrosinase was moderate for HN and low for HO, the noticeable difference between them indicates qualitative and/or quantitative differences that have a crucial impact on the ability to interact with the enzyme, representing a hypothesis that could be further studied.

Overall, the rich phenolic content of both species alongside their high antioxidant and antimicrobial activities indicates that species of this widespread genus can consist of new sources of bioactive compounds to be used as natural food preservatives or as part of functional food products, food supplements, and cosmeceutical products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm92030042/s1, Figure S1: LC chromatogram (-cESI) of *H. nummularium* by UHPLC–HRMS analysis; Figure S2: LC chromatogram (+cESI) of *H. nummularium* by UHPLC–HRMS analysis; Figure S3: LC chromatogram (-cESI) of *H. oelanticum* subsp. *incanum* by UHPLC–HRMS analysis; Figure S4: LC chromatogram (+cESI) of *H. oelanticum* subsp. *incanum* by UHPLC–HRMS analysis. Figure S5. (a) ESI (-) and (b) ESI (+) mass spectrum of N, N″-di-p-coumaroylspermidine; Table S1. Phenolic acids of the genus *Helianthemum* according to the literature; Table S2. Flavonoids of genus *Helianthemum* according to the literature.

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Abbreviations

AcOH	Acetic acid
C-hex	Cyclohexane
CFU	Colony-forming unit
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	Ethyl acetate
FA	Formic acid
GA	Gallic acid

GAE	Gallic acid equivalent
GC–MS	Gas chromatography–mass spectrometry
HN	*H. nummularium*
HN-C	*H. nummularium* cyclohexane extract
HN-D	*H. nummularium* dichloromethane extract
HN-M	*H. nummularium* methanolic extract
HO	*H. oelanticum* subsp. *incanum*
HO-C	*H. oelanticum* subsp. *incanum* cyclohexane extract
HO-D	*H. oelanticum* subsp. *incanum* dichloromethane extract
HO-M	*H. oelanticum* subsp. *incanum* methanolic extract
MeOH	Methanol
MIC	Minimum inhibitory concentration
UHPLC–MS/MS	Ultra-high-performance liquid chromatography–MS/MS

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