

Supplementary Materials

Chemical Profile and Antioxidant and Tyrosinase Inhibitory Activity of *Chamaemelum nobile* L. Green Extracts

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List of Supplementary Materials

1. UPLC-HRMSMS analysis
2. Multivariate Data Analysis of LC-MS data
3. Total Phenolic Content, TEAC and DPPH assays
4. Tyrosinase inhibition assay
5. LC-ESI/QTrap/MS/MS Analysis

Figure S1. UPLC-HRMS base peak profiles of *C. nobile* EtOH 100% and EtOH/H₂O (50%, 60%, 75%) extracts by maceration.

Figure S2. UPLC-HRMS base peak profiles of *C. nobile* EtOH 100% and EtOH/H₂O (50%, 60%, 75%) extracts obtained by long maceration.

Figure S3. UPLC-HRMS base peak profiles of *C. nobile* EtOH 100% and EtOH/H₂O (50%, 60%, 75%) extracts obtained by SLDE-Naviglio.

Figure S4. UPLC-HRMS base peak profiles of *C. nobile* EtOH 100% and EtOH/H₂O (50%, 60%, 75%) extracts obtained by UAE.

Figure S5. Compounds identified in *C. nobile*.

Table S1. Phenolic content and antioxidant activity of “green extracts” of *C. nobile* and apigenin.

Table S2. Correlation between TPC evaluated by Folin–Ciocalteu and antioxidant activity evaluated by the ABTS and DPPH methods. The correlation coefficients among means were determined using Pearson’s method.

Table S3. Tyrosinase inhibition activity of “green extracts” of *C. nobile* and apigenin.

1. UPLC-HRMSMS analysis

All obtained extracts were analyzed using liquid chromatography coupled with electrospray ionization and a high-resolution mass spectrometer (Q Exactive: hybrid quadrupole-Orbitrap mass spectrometer, Thermo Fisher, Waltham, MA, USA), operating in positive ion mode. LC-MS analysis was carried out on a Kinetex 2.6 μm C18 100 Å (100 \times 2.1 mm) column (Phenomenex, Aschaffenburg, Germany), using a flow rate of 0.2 mL/min. A binary solvent system was used (eluent A: water with 0.1% formic acid (99.9:0.1, v/v) and eluent B: acetonitrile with 0.1% formic acid (99.9:0.1, v/v)). The HPLC gradient started at 5% B, and after 30 min, % B was at 95%; this percentage was maintained for another 5 min before returning to the starting percentage. The autosampler was set to inject 5 μL of each extract (0.5 mg/mL). The ESI source parameters were the following: capillary voltage + 35 V; tube lens voltage + 50 V; ion source temperature 280 °C; sheath and auxiliary gas flow (N_2), 12.50 and 5; and sweep gas 0. The full range m/z adapted to the acquisition of MS spectra was 150–1500. For the fragmentation study, a data-dependent scan was set up, through which the precursor ions corresponding to the most intensive peaks were fragmented in the MS analysis with a collision energy of 30%. Xcalibur software version 2.2 was used for instrument control, data acquisition, and data analysis.

2. Multivariate Data Analysis of LC-MS data

LC-ESI/QExactive/MS/MS chromatograms of all obtained extracts were subjected to the chemometric study of a multivariate statistical approach such as principal component analysis (PCA). Raw data were first filtered using MZ mine 2.8 software and then processed using SIMCA-P+ software (version 12.0, Umetrics, Umeå, Sweden). MZMine software was used to filter the noise and detect and align the peaks observed in the LC-MS profiles (noise level 3.0×10^4 ; all data points below this intensity level were ignored) after exporting the processed data in tabular format (.csv file). Each extract was analyzed in triplicate. For the targeted analysis of specialized metabolites, the peak area obtained from the LC/MS analysis was considered. Logarithmic transformation and UV scaling were applied before multivariate data analysis; in detail, the matrix was composed of 32 variables, corresponding to the peak area of m/z values of specific specialized metabolites of *C. nobile*, and 16 observations, represented by different extracts.

3. Total Phenolic Content, TEAC and DPPH assays

The total phenolic content of the extracts was determined by the Folin-Ciocalteu assay. Gallic acid was used as a reference compound (calibration equation: $y = 0.0016x + 0.0146$, $R^2 = 0.992$). For the calibration curve, 30, 40, 50, 100, 200, 400, 600, and 800 $\mu\text{g/mL}$ solutions of gallic acid were prepared and submitted to the assay following the same procedure used for the extracts. All the experiments were performed in triplicate, and the results were expressed as the means of gallic acid equivalents (GAE mg/g dried extract).

The radical scavenging activity of the green extracts of *C. nobile* was determined by two different spectrophotometric assays, DPPH and TEAC. In the TEAC assay, the antioxidant activities of the analyzed extracts (range= 0.25–1.00 mg/mL) were expressed as TEAC values in comparison with the TEAC activity of quercetin; TEAC values were expressed as the concentration (mM) of a standard Trolox solution exerting the same antioxidant activity of a 1

mg/mL solution of the tested extract (calibration equation for Trolox: $y = 30.942x + 50.893$, $R^2 = 0.99$).

Apigenin was diluted with MeOH to produce solutions of 0.3, 0.5, 1, and 1.5 mM. The TEAC value is defined as the concentration of a standard Trolox solution with the same antioxidant capacity as a 1 mM concentration of the investigated compound.

All the experiments were performed in triplicate. For each well, 10 μ L of the extracts at the concentration of 750, 500, and 200 μ g/mL were added to 150 μ g/mL of the work solution (prepared with PBS, ABTS, and $K_2S_2O_8$) and the absorbance at 734 nm was measured immediately on a UV-visible spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, Milan, Italy).

For the DPPH assay, the percentage of DPPH• radical scavenging activity (%) was plotted against the extract concentration (μ g/mL) to determine the IC_{50} . In brief, stock solutions (1 mg/mL) of all the obtained extracts were used in the range of 50–200 μ g/mL, and an aliquot (5.0 μ L) of the methanol solution containing different amounts of each extract was added to 195 μ L of daily prepared DPPH• solution. Absorbance at 517 nm was measured immediately on a UV-visible spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, Milan, Italy). All the experiments were performed in triplicate. Range = 50–200 μ g/mL was tested for each extract, concentrations of apigenin 5–100 μ g/mL. Vitamin C (concentration 1–25 μ g/mL) was used as a positive control and analyzed by linear regression.

4. Tyrosinase inhibition assay

The tyrosinase inhibitory activity was evaluated as described. Moreover, 30 microliters of the sample (final concentrations of 50 and 25 μ g/mL) and 50 μ L of 100 U/mL mushroom tyrosinase were treated in 96-well plates and incubated at 37 °C for 15 min. Subsequently, 50 μ L of 1 mM L-tyrosine was added and then reacted at 37 °C for 15 min. The amount of dopachrome formed was measured at 495 nm using a Thermo Scientific™ Multiskan SkyHigh microplate spectrophotometer. Each sample has been tested in triplicate and the tyrosinase inhibitory activity was calculated using the following equation:

$$\text{tyrosinase inhibition (\%)} = [1 - (S - S_0) / (C - C_0)] \times 100$$

where S is the absorbance of the sample, tyrosinase, and L-tyrosine; S_0 is the absorbance of the sample and L-tyrosine; C is the absorbance of tyrosinase and L-tyrosine; and C_0 is the absorbance of L-tyrosine. Kojic acid, a known tyrosinase inhibitor, was used as a positive control.

5. LC-ESI/QTrap/MS/MS Analysis

Quantitative analysis was performed on an LC-ESI/QTrap/MS/MS system, using the MRM method, on a C18 reversed-phase (RP) column (50 mm \times 2.1 mm; Luna Omega C18 1.6 μ m; Phenomenex, Aschaffenburg, Germany) kept at 30 °C, using water as phase A and acetonitrile as phase B, both with 0.1% of formic acid, at a flow rate of 0.3 mL/min. The autosampler was set to inject 5 μ L of each sample (1.0 mg/mL). Each extract was analyzed in positive ion mode, and a linear gradient was used, starting from 5% B and increasing to 40% B in 2.9 min, holding at 40% for 1 min, then successively rising to 45% B in 1.0 min, holding at this percentage for 1 min, and finally reaching 95% B at 8.00 min, holding at this percentage for 1 min and returning to 5% B in 1 min. Linearity was evaluated by the correlation values of calibration curves. The limit of quantification (LOQ; equivalent to sensitivity) was evaluated by injecting a series of increasingly diluted standard solutions until the signal-to-noise ratio was reduced to 10. The limit of detection (LOD) was estimated by

injecting a series of increasingly diluted standard solutions until the signal-to-noise ratio was reduced to 3 [30]. The LOD was 0.0004 ng/mL, and the LOQ was 0.0012 ng/mL. A stock solution (1 mg/mL) of apigenin was used as an external standard (ES). This solution was diluted with methanol to obtain eight solutions of different ES concentrations (0.05, 0.1, 1.0, 2.5, 5.0, 10.0, 20.0, and 50.0 µg/mL). A declustering potential (DP) of +25.70 eV, an entrance potential (EP) of +6.76 eV, a collision energy (CE) of 35%, and a collision cell exit potential (CXP) of +20.10 eV were selected for apigenin. In this way, a calibration curve, analyzed by linear regression ($y = 0.00675x + 0.904$, $R^2: 0.993$), by Analyst 1.6.2 software provided by the manufacturer (AB Sciex), was obtained for apigenin. For the calibration curves, 5 µL of each standard solution at each concentration level in triplicate was used. The ratio of the peak area of the ES to those of the IS was calculated and plotted against the corresponding concentrations of the standard compounds using weighted linear regression to generate standard curves.

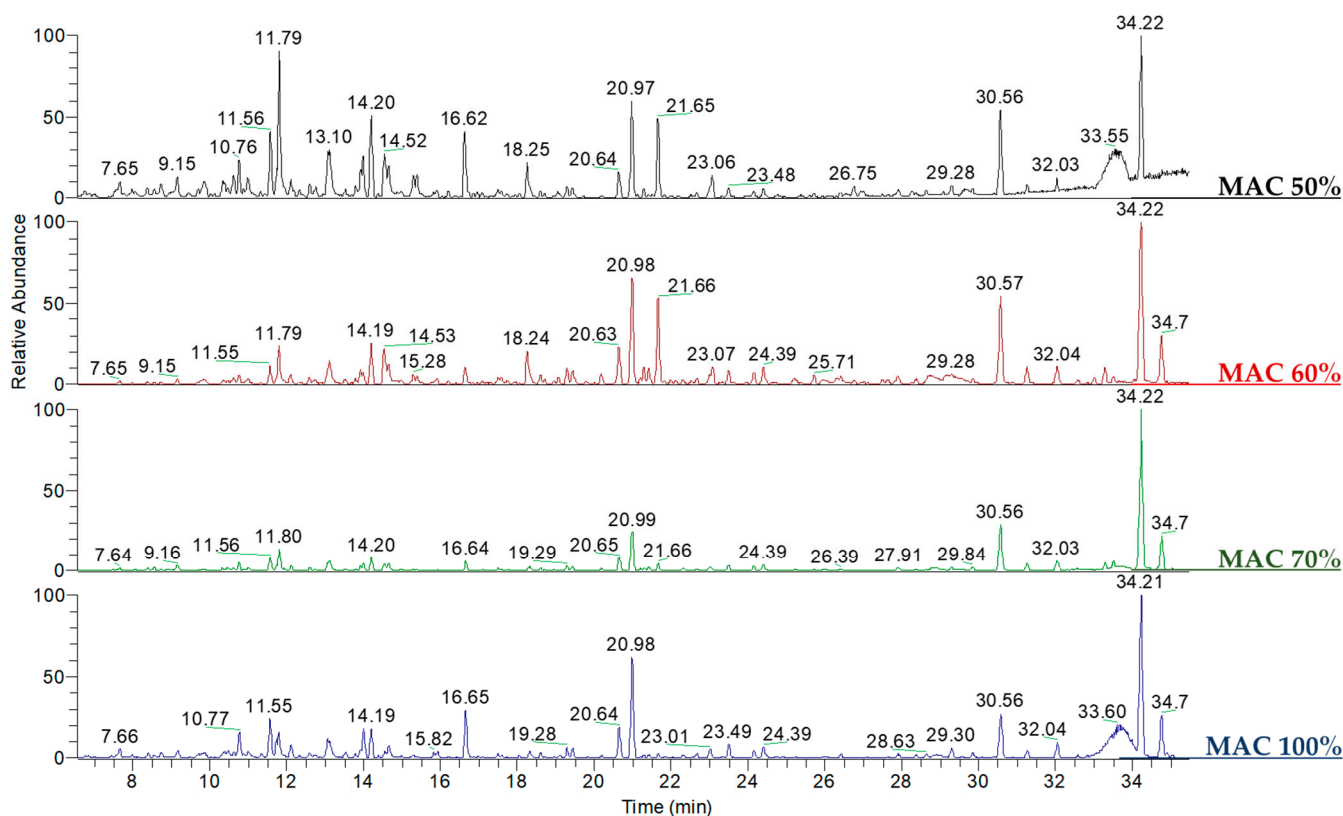


Figure S1. UPLC-HRMS base peak profiles of *C. nobile* macerations in positive ion mode.

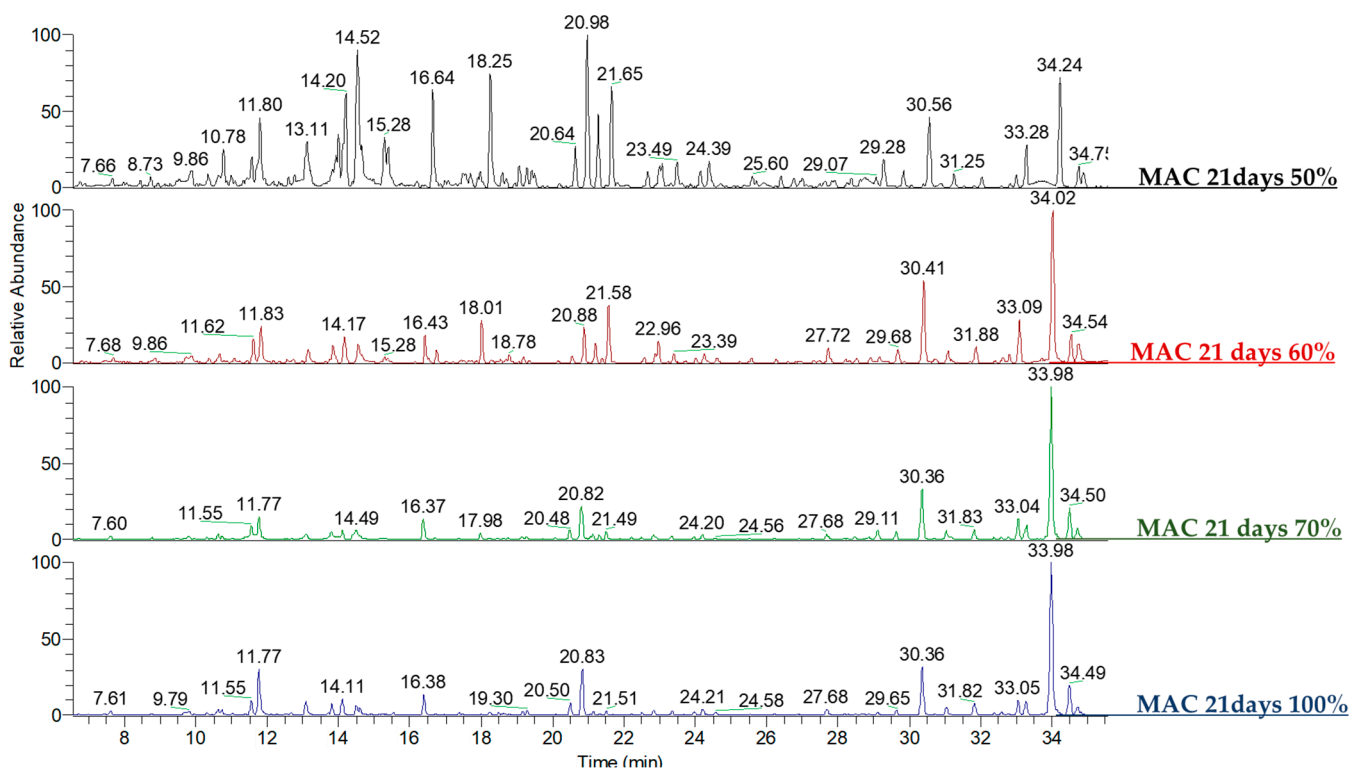


Figure S2. UPLC-HRMS base peak profiles of *C. nobile* long macerations in ion mode.

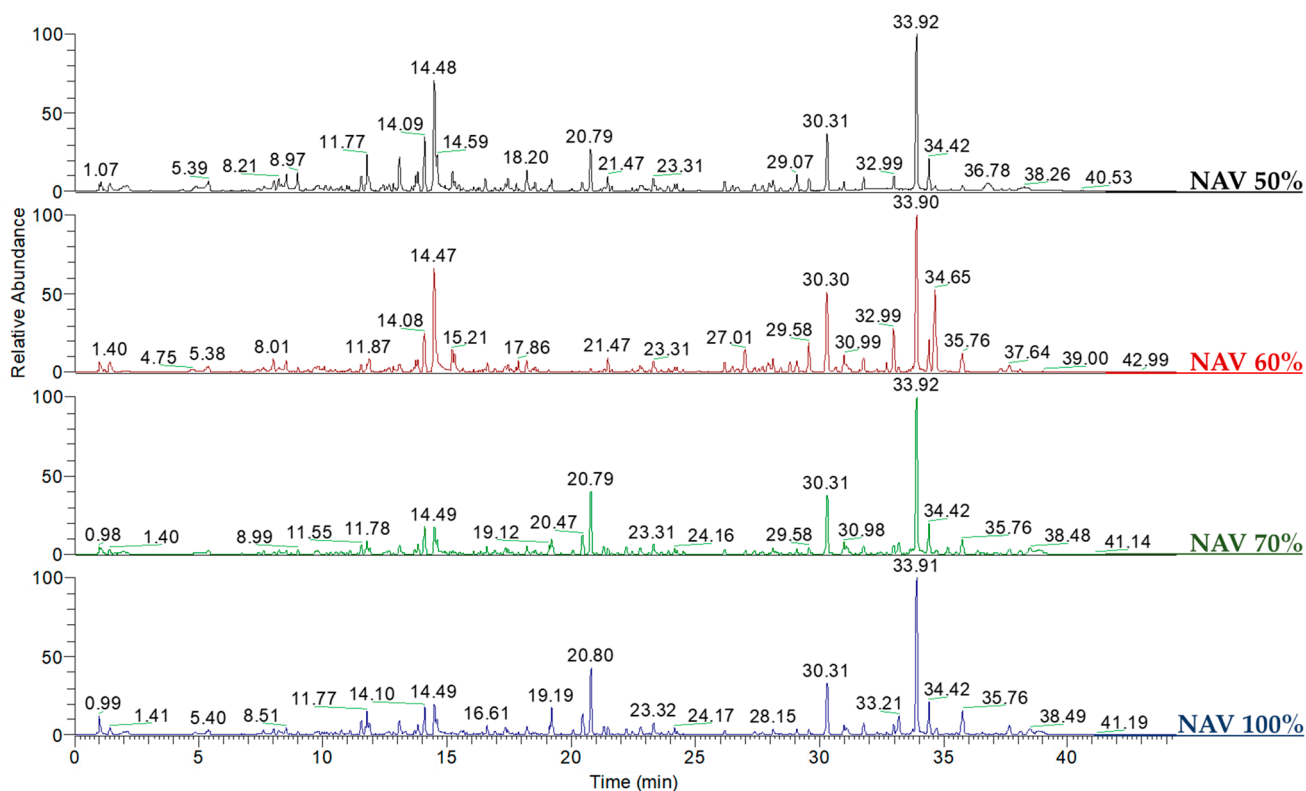


Figure S3. UPLC-HRMS base peak profiles of *C. nobile* SLDEs-Naviglio in positive ion mode.

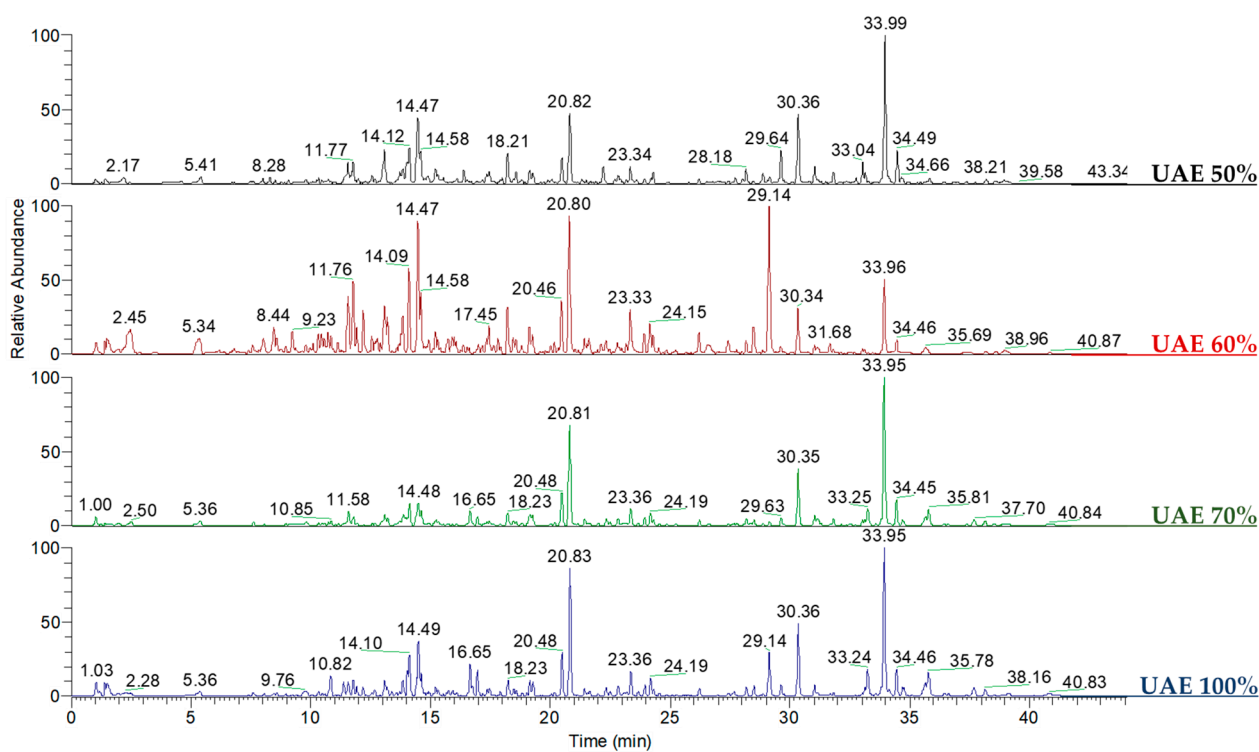


Figure S4. UPLC-HRMS Base Peak profiles of *C. nobile* UAEs in positive ion mode.

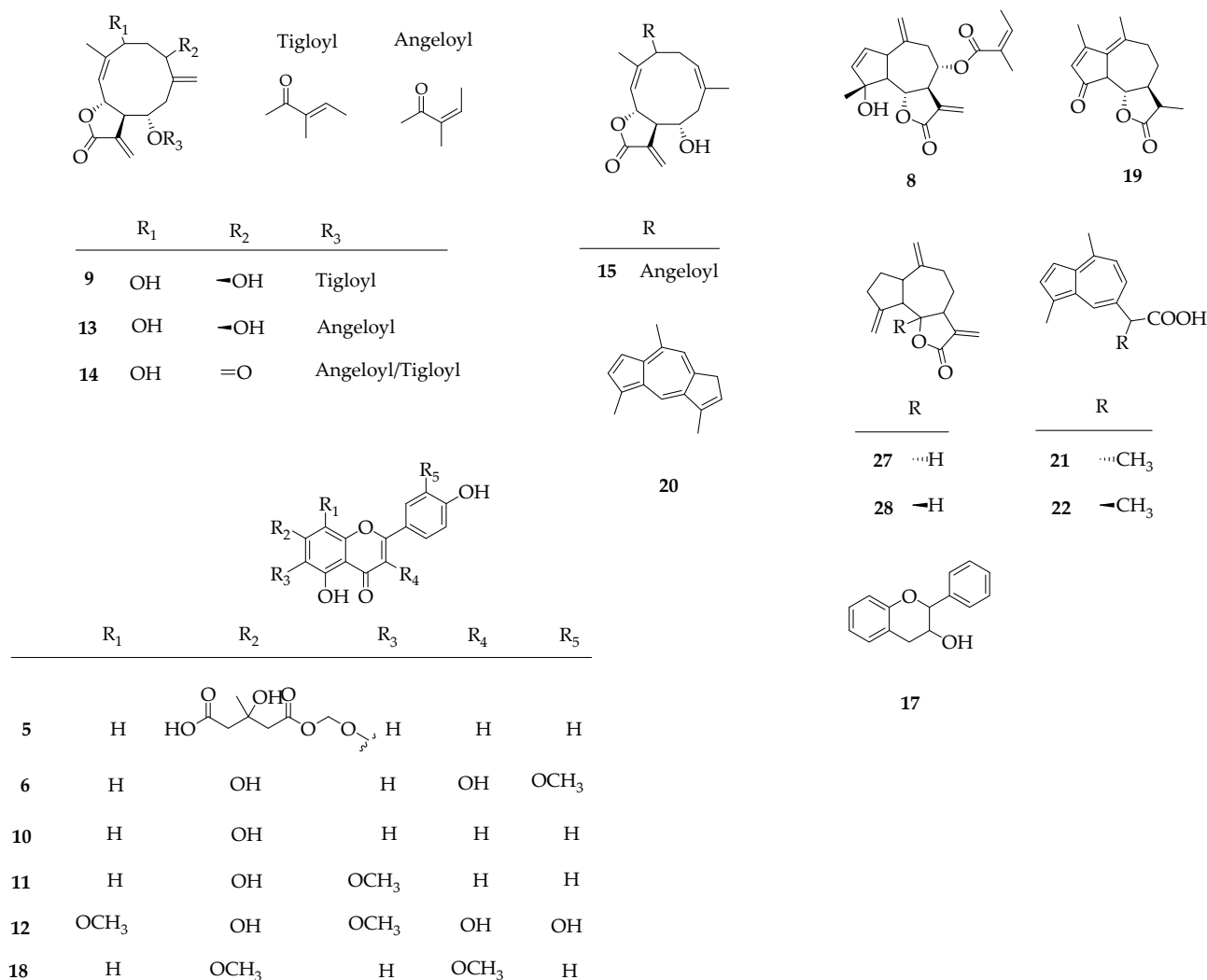


Figure S5. Compounds identified in *C. nobile*.

Table S1. Phenolic content and antioxidant activity of “green” extracts of *C. nobile* and apigenin.

<i>C. nobile</i> “green ” extracts	Total phenolic content	DPPH•	ABTS•+
	GAE ^a ±SD ^b	IC ₅₀ ±SD ^b	TEAC ^c ±SD ^b
UAE 100%	116.40 ± 1.53***	507.79 ± 5.53***	1.55 ± 0.05**
UAE 75%	105.73 ± 1.66***	483.88 ± 6.08***	1.57 ± 0.01***
UAE 60%	121.73 ± 1.58***	416.05 ± 2.14**	1.66 ± 0.06***
UAE 50%	157.73 ± 1.97***	311.22 ± 6.15***	1.67 ± 0.02***
NAV 100%	133.07 ± 1.64***	432.73 ± 2.28***	1.60 ± 0.05***
NAV 75%	135.07 ± 2.85***	353.70 ± 3.16***	1.65 ± 0.04***
NAV 60%	235.07 ± 2.07***	295.54 ± 1.32***	1.73 ± 0.06***
NAV 50%	223.07 ± 1.76***	310.12 ± 5.33**	1.69 ± 0.03***
MAC 100% 21days	117.07 ± 1.88***	591.28 ± 5.12***	1.62 ± 0.01***
MAC 75% 21days	159.73 ± 1.47***	461.32 ± 4.92***	1.62 ± 0.04***
MAC 60% 21days	162.40 ± 2.56***	346.54 ± 1.02**	1.62 ± 0.02***
MAC 50% 21days	188.40 ± 2.38***	338.75 ± 3.68***	1.66 ± 0.06***
MAC 100%	185.73 ± 1.97***	489.98 ± 5.63***	1.57 ± 0.08***
MAC 75%	190.41 ± 2.22***	428.29 ± 3.77***	1.61 ± 0.04***
MAC 60%	205.73 ± 1.38***	370.45 ± 3.21***	1.63 ± 0.01***

MAC 50	232.42 ± 2.44 ^{***}	363.77 ± 5.43 ^{***}	1.68 ± 0.05 ^{***}
Vitamin C ^d		13.62 ± 0.28	
Quercetin ^e			3.70 ± 0.06 ^{***}
Apigenin		26.34 ± 0.88 ^{***}	1.15 ± 0.05 ^{***}

^a Values are expressed as milligrams of gallic acid equivalents (GAEs) per gram of dried extract (mg GAE/g dried extract); ^b SD: results are expressed as mean of three experiments; SD, standard deviation.; ^c values are expressed as concentration (mM) of a standard Trolox solution exerting the same antioxidant activity of a 1 mg/mL solution of the tested extract, concentration of extracts 0.25-1.0 mg/ml. ^d Standard compound for DPPH assay; ^e standard compound for TEAC assay. ^{**} $p < 0.002$, ^{***} $p < 0.001$ vs. control, one-way ANOVA followed by Dunnett's multiple comparison test.

Table S2. Correlation between TPC evaluated by Folin–Ciocalteu and antioxidant activity evaluated by the ABTS and DPPH methods. The correlation coefficients among means were determined using Pearson's method.

Assays	<i>C. nobile</i> "green " extracts R ²
TEAC	0.62
DPPH	- 0.66

Table S3. Tyrosinase inhibition activity of "green extracts" of *C. nobile* and apigenin.

<i>C. nobile</i> "green " extracts	IC ₅₀ ^a ±SD ^b
UAE 100%	65.91 ± 1.80 ^{***}
UAE 75%	65.61 ± 0.84 ^{***}
UAE 60%	61.10 ± 4.04 ^{***}
UAE 50%	69.31 ± 1.62 ^{***}
NAV 100%	60.62 ± 1.61 ^{***}
NAV 75%	62.02 ± 5.38 ^{***}
NAV 60%	61.08 ± 4.02 ^{***}
NAV 50%	56.15 ± 4.74 ^{**}
MAC 100% 21days	70.36 ± 3.88 ^{***}
MAC 75% 21days	63.66 ± 3.86 ^{***}
MAC 60% 21days	63.75 ± 2.04 ^{***}
MAC 50% 21days	69.31 ± 2.03 ^{***}
MAC 100%	56.28 ± 4.95 ^{**}
MAC 75%	63.99 ± 1.99 ^{***}
MAC 60%	63.82 ± 2.04 ^{***}
MAC 50%	64.88 ± 3.45 ^{***}
Kojic acid ^c	39.44 ± 3.80
Apigenin ^d	57.76 ± 0.85 ^{***}

^aValues are expressed as micrograms per milliliter (µg/mL), concentrations of extracts 25-300µg/ml.^b SD: results are expressed as mean of three experiments; SD, standard deviation. ^c positive control, concentrations of control 10-100µg/ml; concentrations of apigenin 10-50 µg/ml ^{***} $p < 0.001$ vs. control, one-way ANOVA followed by Dunnett's multiple comparison test.