SUPPLEMANTARY INFORMATION

Analytical methods

Determination of Cd and Cr

Total metal concentration was determined in 0.3 g of lyophilised whole plant sample which was previously frozen in liquid nitrogen and homogenate. The sample aliquot was treated with 5 ml of HNO_3 (Suprapur reagent grade, Merck, USA) and 5 ml of H_2O_2 (Suprapur reagent grade, Merck, USA) in polytetrafluoroethylene (PTFE) bombs and submitted to acid digestion in a microwave oven (Milestone MLS-1200 Mega microwave laboratory unit). Four heating steps of 5 min each (200, 350, 550, 250 W power respectively), followed by a ventilation step of 25 min, were applied. A clear solution was obtained at the end of the treatment. The solutions was diluted to 50 ml with ultrapure water and analysed without any further treatment by Inductively Coupled Plasma-Quadrupole Mass Spectrometry (ICP-QMS, Agilent 7500) equipped with a V-groove nebulizer and a cooled spray chamber (2°C), RF power 1400 W, sample gas 1.20 L min^{-1}, sample flow rate 500 mL min^{-1}, dwell time 20 ms, and 3 points per peak. Blanks were simultaneously run and showed a chromium concentration below the detection limit, and a cadmium concentration of 0.57 µg L^{-1}. Quantification was carried out by rhodium internal standard and mach calibration curve. A seven points calibration curve was achieved by standard additions to a sample in the range 5 - 1000 µg L^{-1}. The coefficient of determination (R^2) was always better than 0.998. The recovery test was carried out by the CRM tomato lives (NIST 1573a), and was 89% for Cr at 1.99 mg/kg level, and 83% for Cd at 1.52 mg/kg, with a coefficient of variation of 8 and 11%, respectively. Mean concentrations of the samples were the average of five replicate measurements, with a typical of10-15% CV% for exposed plants, and 25-40 % for the control ones. Chromium determination showed higher CVs due to the interference of ^{12}C ^{40}Ar.

Determination of phytohormones

The procedure has been described elsewhere [1s]. In brief, a known amount of D_5-IAA and D_6-S-ABA standard solutions was added to a 0.15 g aliquot of lyophilised whole plant sample which was

previously frozen in liquid nitrogen and homogenate. The sample aliquot was then subjected twice to cold extraction (-28°C) with methanol/water/formic acid mixture (75:20:5 v/v). IAA, S-ABA, D_5-IAA and D_6-S-ABA were supplied by OlChemIm Ltd., Czech Republic. Methanol Pesticides Grade, acetonitrile RS Plus Pesticides grade, 99% formic acid and acetonitrile RS Plus Pesticides grade were supplied by Carlo Erba Reagents (Italy) and hexadecane was from Sigma Aldrich (USA). Primary standard solutions were prepared in methanol at a concentration of 1 mg/ml and stored at –28°C.

Analyses were performed by a Perkin Elmer 200 liquid chromatograph coupled with an Analytical Biosystem Sciex mod API 4000 mass spectrometer, with triple quadrupole and turbo spray ion source. A Symmetry Waters C18 column (2.1 mm x 50 mm, 5 µm particles diameter) was used, and gradient chromatographic separation was performed at a flow rate of 0.2 ml/min as follows: 95% eluent A (H_2O + 0.1%HCOOH), 3% eluent B (CH_3OH + 0.1% of HCOOH) and 2% eluent C (CH_3CN + 0.1% of HCOOH) isocratic for 1 min, then 30% eluent B and 70% eluent C in 5 min, isocratic for 1 min and finally 95% eluent A, 3% eluent B and 2% eluent C in 0.5 min, isocratic for 5 min. The injected volume was 10 µl.

The most important instrumental parameters were as follows: spray voltage 4 kV, sheath and auxiliary gas N_2; sheath gas flow-rate 55 mL/min; auxiliary gas flow-rate 20 mL/min; capillary voltage 35 V; capillary temperature 280 °C. The values of the most important instrumental parameters and m/z ratio for precursor and product ions are reported in Table 1s.

Table 1s. Precursor and product ion m/z values, and instrumental parameters values selected for the determination of phytohormones by HPLC-MS/MS.

Phytohormone	**Precursor ion m/z**	**Product ion m/z**	**Declustering potential (V)**	**Collision energy (eV)**	**Collision cell exit potential (V)**
IAA	176.10	130.10	41	17.0	10.0
[D_5]-IAA	181.20	134.20	41	17.0	10.0
S-ABA	265.20	247.30	51	9.0	22.0
[D_6]-S-ABA	271.20	253.30	51	9.0	22.0

The investigated linear concentration range and coefficient of determination (R^2) for each analyte were the following: IAA (5 – 100 pg/µl, 0.997), S-ABA (10 – 100 pg/µl, 0.999). Primary standard solutions were prepared in methanol at a concentration of 1 mg/ml and stored at –28°C. The recovery was evaluated on five aliquots of a sample, which were added with a known amount of D5-IAA and D6-S-ABA, and resulted always in the range 87-96%, with a typical relative standard deviation of 10%. Finally, the instrumental detection limit (S/N=5) was evaluated on chromatograms of real samples and resulted equal to 3 ng/g f.w. of the original sample for both IAA and S-ABA.

Determination of shikimic and salycilic acids.

Shikimic acid (SCI) and salycilic Acid (SAL) were extracted from a 0.1 g aliquot of lyophilised whole plant sample which was previously frozen in liquid nitrogen and homogenate. The sample aliquot was added with the internal standard (deuterated salycilic acid-d_4) and 1.6 ml of the extracting solution (HCl 99.9%, methanol 0.1%). Shikimic acid and salycilic acid (purity≥ 99,0 %), acetic acid ultrapure for HPLC/MS and hydrochloric acid (37%) analytical grade were from Sigma-Aldrich (Steinheim, Germany); methanol UPS HPLC/MS Ultra Pure Solvent) was from Romil Ltd. (Cambridge, U.K.); salycilic acid D_4 (isotopic enrichment 98,5%, purity 98%) was from Isotopes Inc. (Pointe Claire, Quebec, Canada). The extraction was repeated 3 time and the extracts were joined. The solution was concentrated under a gentle flux of nitrogen to 0.5 ml and diluted with ultrapure water to a final volume of 1 ml, which was filtered by a PTFE membrane filter cartridge (filter 15 mm diameter, 0.45 µm pore size), to determine shikimic and salycilic acids aliquots of the solution were then further diluted 100 time with ultrapure water. The content of SCI and SAL was determined by HPLC-ESI-MS/MS. A liquid chromatograph (series 4100, Agilent) was coupled with a mass spectrometer with triple quadrupole and turbo spray ion source (Analytical Biosystem Sciex mod. API 4000). Analyses were carried out in negative ion mode, using the enhanced resolution and the MS/MS experiments. The Q1 and Q3 quadrupoles operate in unit mass resolution by keeping a full width at half maximum of 0.7±0.1 amu and a dwell time of 200 msec for each monitored transition. All the HPLC and mass spectrometer functions, including the acquisition and processing of chromatograms, were controlled by the Analyst software version 1.5 (AB Sciex).

Shikimic, salycilic acids and the deuterated salycilic acids ionize as $[SCI-H]^-$, $[SAL-H]^-$, $[D_4\text{-}SAL-H]^-$ and $[D_3\text{-}SAL-H]^-$, respectively. Table 2s shows the mass spectrometric experimental conditions and the values of the most important instrumental parameters.

Table 2s. Precursor and product ion m/z values and instrument parameter values selected for the determination of the Shikimic and Salycilic acids by MS/MS.

	Q1 m/z	**Q3 m/z**	**Declustering potential (V)**	**Collision energy (eV)**	**Collision cell exit potential (V)**
$[SCI-H]^-$	172.9	154.90	-88	-12	-13.5
		136.80	-88	-16.0	-10.5
		110.90	-88	-14.5	-8.5
		93.00	-88	-17.0	-6.4
		73.00	-88	-19.0	-4.8
$[SAL-H]^-$	136.80	93.00	-90	-22.0	-15.0
		64.90	-90	-40.0	-11.0
$[D_4SAL-H]^-$	140.90	97.00	-53	-22.0	-7.0
		69.00	-53	-40.0	-4.8
$[D_3SAL-H]^-$	139.80	96.00	-53	-22.0	-7.0
		68.10	-53	-40.0	-4.7

A Synergy-Hydro (Phenomenex, USA) column (2.1 mm x 50 mm, 4 µm particles diameter) was used, a gradient chromatographic separation using H_2O + CH_3COOH 0,1% (solvent A) and 100% CH_3OH (solvent B) was performed at a flow rate of 0.5 ml/min as follows: 100% eluent A isocratic for 3 min, then a gradient until 40% eluent A and 60% eluent B % in 2 min, isocratic for 4 min, then a second gradient until 1% A and 99% B in 5 min and finally the same eluent was maintained in isocratic for 4 min.. The injected volume was 20 µl.

A seven points calibration curve was achieved by standard additions to a sample in the range 1 - 100 ng/ml. The coefficient of determination (R^2) was always better than 0.989. The recovery was verified by addition of 2.0 µg of SCI and 0.50 µg of SAL to 0.1 g aliquots of lyophilised material, and resulted 71% and 89% for SCI and SAL, respectively. The CV% (n=5) was always better than 10%.

Determination of polyphenols and antiradical activity

Polyphenols and antiradical activity were determined on an aliquot of lyophilised whole plant sample which was previously frozen in liquid nitrogen, homogenate and then stored at -80°C until analysis was performed. Chlorogenic acid, (+)-catechin, caffeic acid, gallic acid, p-coumaric acid, scopoletin, rutin and quercetin standards were supplied by Sigma-Aldrich (MO, USA); HPLC grade methanol and HPLC grade water were purchased from J.T. Baker (Deventer, Holland). Sodium fluoride, sodium carbonate, formic acid, Folin-Ciocalteu (F-C) reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were obtained from Merck (Germany). Polytetrafluoroethylene (PTFE) membranes (porosity 0.2 μm) for the filtration of the extracts before HPLC analysis were obtained from Sartorius (Germany).

About 50 mg of the freeze dried material was homogenised in an ice bath under magnetic stirring with 15 ml of a methanol/water solution 80/20 (v/v) containing 10 mM NaF to inactivate polyphenol oxidase; the mixture was centrifuged at 1500 × g for 10 min and the supernatant recovered. In order to evaluate the recovery of the extraction procedure, five sequential extractions on five aliquots of the same thesis were carried out and the F-C total polyphenols were determined in each extract. The results showed that the fifth extraction accounted for 4.6±0.9% of the whole recovery and therefore did not contribute significantly to the recovery of total polyphenols; according to these findings four sequential extractions were chosen for the recovery of analytes. The resulting extract was used for the following analysis: total polyphenols, selected monomeric polyphenols and antiradical activity. Five aliquots of each thesis were analyzed.

Total polyphenols

Total polyphenols were spectrophotometrically determined with the F-C method using chlorogenic acid as reference standard, according to a procedure described elsewhere [2s]. In brief, the extract (0.8 ml of the methanol/water extract) was mixed with 200 μl of F-C reagent; after 3 min. 400 μl of a supersaturated sodium carbonate solution were added and the obtained mixture was made up to 10 ml with milliQ water. After dark incubation for 1 h the spectrophotometric determination was carried out at λ=740 nm.

Selected monomeric polyphenols

1.5 ml of the methanol/water extract was loaded on the top of a Supelclean LC-18 SPE Tubes (Supelco, USA) and eluted with 4.5 ml of methanol. The cartridge was previously preconditioned with 10 ml of methanol and 10 ml of ultra pure water acidified at pH = 4 with acetic acid. A Prominence HPLC system (Shimadzu, Japan) consisting of two solvent delivery pumps LC-20AD Ultra Fast Liquid Chromatography (UFLC), an autoinjector SIL-20A HT, a column thermostat CTO/20A and a system controller CBM-20A UFLC coupled with a 3200QTrap™ mass detector (AB Sciex, CA, USA) by a Turbo V™ interface equipped with a heated nebuliser and a turbo ion spray (TIS) probes, was used for the determination of the selected monomeric polyphenols in the eluate.

Chromatographic separation was performed on an Acquity UPLC BEH C18 (100 mm x 2.1 mm i.d.; particle size 1.7 μm) (Waters, MA, USA) column. The following solvents were used: solvent A (99.5/0.5 v/v water/acetic acid); solvent B (methanol/acetic acid 99.5/0.5). Gradient elution was carried out at 40°C as follows: 0-0.1 min, isocratic 5% B; 0.1-30 min, linear gradient 5-30% B; 30-32 min, linear gradient 30-100% B; 32-38 min, isocratic 100% B; 38-40 min, linear gradient 100-5% B. The flow rate was 0.3 ml/min and the injection volume 10 μl. Table 3s shows the optimized values of instrument parameters and the m/z ratios of precursor and product ions.

Table 3s. Precursor and product ion m/z values and instrument parameter values selected for the determination of the selected polyphenols by HPLC–MS/MS.

Polyphenol	Precursor ion m/z	Product ion m/z	Declustering potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Gallic acid	168.9	125.1	-40	-20.0	0.0
Catechin	289.1	245.1	-55	-18.0	-2.0
Chlorogenic acid	353.0	191.1	-30	-28.0	-2.0
Caffeic acid	178.9	135.2	-30	-22.0	0.0
p-Coumaric acid	163.0	119.0	-30	-22.0	0.0
Scopoletin	190.9	176.0	-30	-22.0	0.0
Rutin	609.1	299.9	-75	-52.0	-4.0
Quercetin	301.0	151.0	-55	-32.0	0.0

MS-MS analyses were carried out in negative ion mode, keeping a full width at half maximum of about 0.7 amu and a dwell time of 100 msec for each monitored transition. All the HPLC and mass

spectrometer functions, including the acquisition and processing of chromatograms, were controlled by the Analyst software version 1.5 (AB Sciex). The instrumental detection limit (S/N=5) were: 10 pg injected for chlorogenic acid, p-coumaric acid, scopoletin and rutin; 20 pg injected for gallic acid and caffeic acid; and 70 pg injected for catechin. The linearity of the calibration curves was investigated within the following concentration range: 0.01 – 5.0 ng/µl (caffeic acid, p-coumaric acid, scopoletin, rutin, quercetin); 0.01 – 10.0 ng/µl (gallic acid, catechin), 0.1 – 50.0 (chlorogenic acid), achieving in all cases correlation coefficients higher than 0.995.

The recovery was evaluated by spiking 1.5 ml-aliquots of five extracts of the untreated wild type *Nicotiana langsdorffii* with 10 µl of a standard solution containing chlorogenic acid at a concentration of 5 µg μl^{-1} and the other target polyphenols at a concentration of 0.5 µg μl^{-1}. According to this procedure, recovery percentage of each spiked polyphenol was in the range 73 – 106 % (Table 4s).

Table 4s. Recovery percentage of the selected monomeric polyphenols in wild type *Nicotiana langsdorffii* extract and of fortified extract. Standard deviation (n=5) is reported in brackets.

Polyphenols	**Concentration in the extract (mg l^{-1})**	**Total concentration in spiked extract (mg l^{-1})**		**Mean recovery (%)**
		Expected	**Measured**	
Gallic acid	n.d.	3.3	2.4 (0.2)	73
Catechin	n.d.	3.3	2.8 (0.2)	85
Chlorogenic acid	175 (8)	208	210 (1)	101
Caffeic acid	0.4 (0.1)	3.7	3.2 (0.3)	86
p-Coumaric acid	n.d.	3.3	3.5 (0.3)	106
Scopoletin	0.8 (0.2)	4.1	3.5 (0.3)	85
Rutin	n.d.	3.3	3.2 (0.2)	97
Quercetin	n.d.	3.3	3.3 (0.1)	100

n.d. = not detected

Antiradical activity

Antiradical activity was spectrophotometrically determined using the free radical DPPH [3s], suitably modified [2s]. This radical is largely adopted for the evaluation of the radical scavenging activity of

different vegetal matrixes since it strongly reacts with reducing species and has a characteristic absorbance at λ = 517 nm [4s-6s]. According to this method IC_{50} is defined as the amount of the plant sample (mg d.w.) in 1 ml of reaction mixture, necessary to decrease the initial DPPH concentration (which was kept constant in all the experiments) by 50%. Therefore, higher IC_{50} values correspond to lower radical scavenging activities. Radical scavenging activity of plant samples (RSA_{sample}) can be derived from IC_{50} values (mg d.w./ml DPPH solution) determined on *Nicotiana* extracts (IC_{50},sample) by using the equation 1, where the factor 100 refers to the polyphenolic measured in plants- By this way RSA increases with the increase of the antiradical activity.

$$RSA_{sample} = \frac{100}{IC_{50,sample}} \qquad \text{(eq. 1)}$$

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