Supporting Information for

Brominated extracts as source of bioactive compounds

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Content:

Materials

Chemical reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Solvents were analytical grade or were purified by standard procedures prior to use. Infrared spectra (IR) were recorded on a Shimadzu Prestige 21 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker avance at 300 MHz in $CDCl_3$, in the presence of TMS (0.00 ppm) as the internal standard. ^{13}C NMR spectra were recorded on the same apparatus at 75 MHz with $CDCl_3$ as solvent and reference (76.9 ppm), ^{13}C NMR assignments were made on the basis of chemical shifts and proton multiplicities (from DEPT spectra). The concentration used for NMR of the extracts was 40 mg of sample per mL. Mass spectra were performed at LEM (Laboratorio de Espectrometría de Masas(, CCT-CONICET.HPLC was performed using a Hewlett-Packard 1050 instrument, coupled to a HP G1306AX DAD. Acetonitrile was acquired from J. T. Baker and formic acid from Merck. The water used was Milli Q. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} pre-coated aluminum sheets (Merck). Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh).

β-Glucosidase from almonds, Xanthine Oxidase from bovine milk and Acetylcholinesterase from *Electrophorus electricus* (electric eel**)** were purchased from Sigma (St. Louis, MO, USA; product numbers: 49290, X-1875 and C3389 respectively). Agar was purchased from Britania (Buenos Aires, Argentina). Sodium acetate, ferric chloride hexahydrate and glacial acetic acid were purchased from Cicarelli (San Lorenzo, Argentina). Esculin hydrate, ethylenediaminetetraacetic acid (EDTA), nitrobluetetrazolium (NBT), tris(hydroxymethyl)aminomethane, Fast Blue, 1-naphthyl acetate were purchased from Aldrich (Milwaukee, WI, USA). Aluminium-backedsilica gel 60 F254 TLC layers were purchased from Merck (Darmstadt, Germany). Acetylthiocholine iodide (ATCI), 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB) and reference compounds galanthamine hydrobromide and physostigmine hemisulfate were purchased from Sigma-Aldrich Chemical (St. Louis, MA, USA)

Virtual extracts

Searching strategy for double bonds

The number of structures containing non-aromatic ring double bonds was calculated as follows: "**α NOT β = γ**; **γ OR δ= ε**". Where **α** represents the number of molecules in the DNP that contain at least one double bond (aromatic or non-aromatic) in their structure

β represents the number of molecules in the DNP that contain at least one aromatic ring in their structure, and **δ** represents the number of molecules in the DNP that contain at least one aromatic double bond and one non-aromatic double bond in their structure. **β** was subtracted from **α** to obtain **γ** that represents the number of molecules in the DNP that contain only non-aromatic double bonds in their structure. This operation eliminated all those structures that contained at least one aromatic double bond but also, all those structures that contained at least one non-aromatic double bond and an aromatic ring double bond. For this reason, **γ** was combined with **δ** to give **ε**, which represents the total number of molecules in the DNP that contain at least one non-aromatic double bond in their structure. **α**=124,233 structures, **β**=59,467 structures, **γ** 64,766 estructuras, **δ**=31,646 structures, **ε**=96,412 structures.

The average frequency of molecules containing aromatic rings and non-aromatic double bonds were standardized to the total number of molecules contained in the DNP (147,852 structures).

Generation of Virtual Extracts

The frequencies were normalized respect to the number of structures belonging to each virtual mixture.

The plant species and genus selected were those with higher number of compounds described in the database.

Aromatic ring distribution

Set 1. Selected plant species (frequency of aromatic ring containing molecules) are as follows: *Withania somnifera* (L.) Dunal (0.04), *Tripterygium wilfordii* Hook. f. (0.61), *Taxus mairei* (Lemée & H. Lév.) S. Y. Hu ex T. S. Liu (0.54), *Taxus baccata L.* (0.69), *Buxus sempervirens* L. (0.31), *Foeniculum vulgare* Mill. (0.29), *Glycyrrhiza uralensis* Fisch. ex DC. (0.74), *Halimium viscosum* (Willk.) P. Silva (0.07), *Glycyrrhiza glabra* L. (0.76), *Cannabis sativa* L. (0.88), *Azadirachta indica* A. Juss. (0.31), *Helianthus annuus* L. (0.20), *Panax ginseng* C. A. Mey. (0.03), *Clausena excavata Burm. f.* (0.99), *Lupinus albus L.* (0.69), *Melia azedarach* L. (0.31) and *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (0.39).

Set 2. Selected genus (frequency of aromatic ring containing molecules) are as follows: Thalictrum (0.84), Taxus (0.62), Strychnos (0.96), Solanum (0.15), Senecio (0.15), Piper (0.85), Pinus (0.44), Sophora (0.68), Juniperus (0.37), Euphorbia (0.40),

Glycyrrhiza (0.72), Citrus (0.71), Cassia (0.83), Baccharis (0.21), Artemisia (0.22), Annona (0.23) and Aconitum (0.47).

Set 3. Selected types of metabolites (frequency of aromatic ring containing molecules) are as follows: aliphatics (0.15), alkaloids (0.64), aa and peptides (0.46), benzofuranoids (0.80), benzopyranoids (0.91), carbohydrates (0.27), flavonoids (1.00), lignans (0.93), oxygen heterocycles (0.22), polycyclic aromatics (0.96), polyketides (0.31) polypyrroles (0.07), simple aromatics (0.95), steroids (0.06), tannins (1.00), terpenoids (0.14) and monoterpenoids (0.28).

Set 4. Two initial numbers molecular weight (frequency of aromatic ring containing molecules) are as follows: 10*(0.25), 15*(0.31), 20*(0.41), 25*(0.44), 30*(0.47), 35*(0.57), 40*(0.46), 45*(0.33), 50*(0.38), 55*(0.53), 60*(0.56), 65*(0.57), 70*(0.43), 75*(0.55), 80*(0.47), 85*(0.31) and 90*(0.36).

Non-aromatic double bond distribution

Selected plant species (frequency of non-aromatic ring containing molecules) are as follows: *Withania somnifera* (L.) Dunal (0.95), *Tripterygium wilfordii* Hook. f. (0.57), *Taxus mairei* (Lemée & H. Lév.) S. Y. Hu ex T. S. Liu (0.84), *Taxus baccata L.* (0.86), *Buxus sempervirens* L. (0.66), *Foeniculum vulgare* Mill. (0.19), *Glycyrrhiza uralensis* Fisch. ex DC. (0.91), *Halimium viscosum* (Willk.) P. Silva (0.98), *Glycyrrhiza glabra* L. (0.92), *Cannabis sativa* L. (0.65), *Azadirachta indica* A. Juss. (0.77), *Helianthus annuus* L. (0.35), *Panax ginseng* C. A. Mey. (0.79), *Clausena excavata Burm. f.* (0.65), *Lupinus albus L.* (0.78), *Melia azedarach* L. (0.96) and *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (0.68).

Selected genus (frequency of non-aromatic ring containing molecules) are as follows: Thalictrum (0.16), Taxus (0.90), Strychnos (0.58), Solanum (0.88), Senecio (0.58), Piper (0.38), Pinus (0.77), Sophora (0.81), Juniperus (0.77), Euphorbia (0.79), Glycyrrhiza (0.97), Citrus (0.71), Cassia (0.92), Baccharis (0.78), Artemisia (0.68), Annona (0.80) and Aconitum (0.90).

Selected types of metabolites (frequency of non-aromatic ring containing molecules) are as follows: aliphatics (0.64), alkaloids (0,61), aa and peptides (0.46), benzofuranoids (0.64), benzopyranoids (0.90), carbohydrates (0.39), flavonoids (0.74), lignans (0.45), oxygen heterocycles (0.84), polycyclic aromatics (0.46), polyketides (0.79) polypyrroles (0.97), simple aromatics (0.49), steroids (0.78), tannins (0.19), terpenoids (0.87) and monoterpenoids (0.84).

Two initial numbers molecular weight (frequency of non-aromatic ring containing molecules) are as follows: 10*(0.62), 15*(0.49), 20*(0.55), 25*(0.63), 30*(0.68), 35*(0.69), 40*(0.73), 45*(0.67), 50*(0.67), 55*(0.74), 60*(0.70), 65*(0.63), 70*(0.71), 75*(0.64), 80*(0.76), 85*(0.74) and 90*(0.58).

Bromine distribution

Selected plant species (frequency of bromine containing molecules) are as follows: *Withania somnifera* (L.) Dunal (0.00), *Tripterygium wilfordii* Hook. f. (0.00), *Taxus mairei* (Lemée & H. Lév.) S. Y. Hu ex T. S. Liu (0), *Taxus baccata L.* (0), *Buxus sempervirens* L. (0), *Foeniculum vulgare* Mill. (0), *Glycyrrhiza uralensis* Fisch. ex DC. (0), *Halimium viscosum* (Willk.) P. Silva (0), *Glycyrrhiza glabra* L. (0), *Cannabis sativa* L. (0), *Azadirachta indica* A. Juss. (0), *Helianthus annuus* L. (0), *Panax ginseng* C. A. Mey. (0), *Clausena excavata Burm. f.* (0), *Lupinus albus L.* (0), *Melia azedarach* L. (0) and *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (0).

Selected genus (frequency of non-aromatic ring containing molecules) are as follows: Thalictrum (0), Taxus (0), Strychnos (0), Solanum (0), Senecio (0), Piper (0), Pinus (0), Sophora (0), Juniperus (0), Euphorbia (0), Glycyrrhiza (0), Citrus (0), Cassia (0), Baccharis (0), Artemisia (0), Annona (0) and Aconitum (0).

Selected types of metabolites (frequency of bromine containing molecules) are as follows: aliphatics (0.028), alkaloids (0.033), aa and peptides (0.015), benzofuranoids (0.010), benzopyranoids (0.002), carbohydrates (0.001), flavonoids (0), lignans (0.001), oxygen heterocycles (0.020), polycyclic aromatics (0.003), polyketides (0.044) polypyrroles (0), simple aromatics (0.025), steroids (0.001), tannins (0), terpenoids (0.013) and monoterpenoids (0.045).

Two initial numbers molecular weight (frequency of bromine containing molecules) are as follows: 10*(0.020), 15*(0.005), 20*(0.004), 25*(0.008), 30*(0.018), 35*(0.021), 40*(0.024), 45*(0.012), 50*(0.014), 55*(0.007), 60*(0.021), 65*(0.023), 70*(0.013), 75*(0.014), 80*(0.010), 85*(0.005) and 90*(0).

^{1}H NMR and PCA analysis of NEs and BEs.

All spectra were recorded in $CDCl_3$ solutions of 40 mg of sample per mL (Bruker 300 MHz). The ^{1}H NMR spectra were automatically pre-processed using a modified version of PROMETAB.[1] Each spectrum was segmented into 0.005-ppm chemical shift 'bins' between δ=0.2 and δ =8.9 ppm, and the spectral area within each bin was integrated.

Bins between δ =7.25 ppm to δ =7.27 ppm corresponding to chloroform peak were removed. The total spectral area of the remaining bins was normalized to the TSA (Total Spectral Area). Principal component analyses (PCA) of the pre-processed data was conducted using a MATLAB written code, TOMCAT (TOMCAT: A MATLAB toolbox for multivariate calibration techniques).[2] The routines have been developed under MATLAB 6.5 (Release 13).

The scatter plot showed discrimination between two groups by principal components (PCs) 1 and 2 (Figure S1). Natural extracts (NEs) showed a positive PC2 value, whereas the brominated extracts (BEs) showed a positive PC2 value. The loading plot of PC2 shows that the signals between 5.40–5.25 ppm corresponding to double bonds, one of the target groups, have a positive effect in PC2 (Figure S1). On the contrary some signals located between 4.70–3.20 ppm, which could correspond to H *gem* to Br, have a negative effect in PC2 (Figure S2).

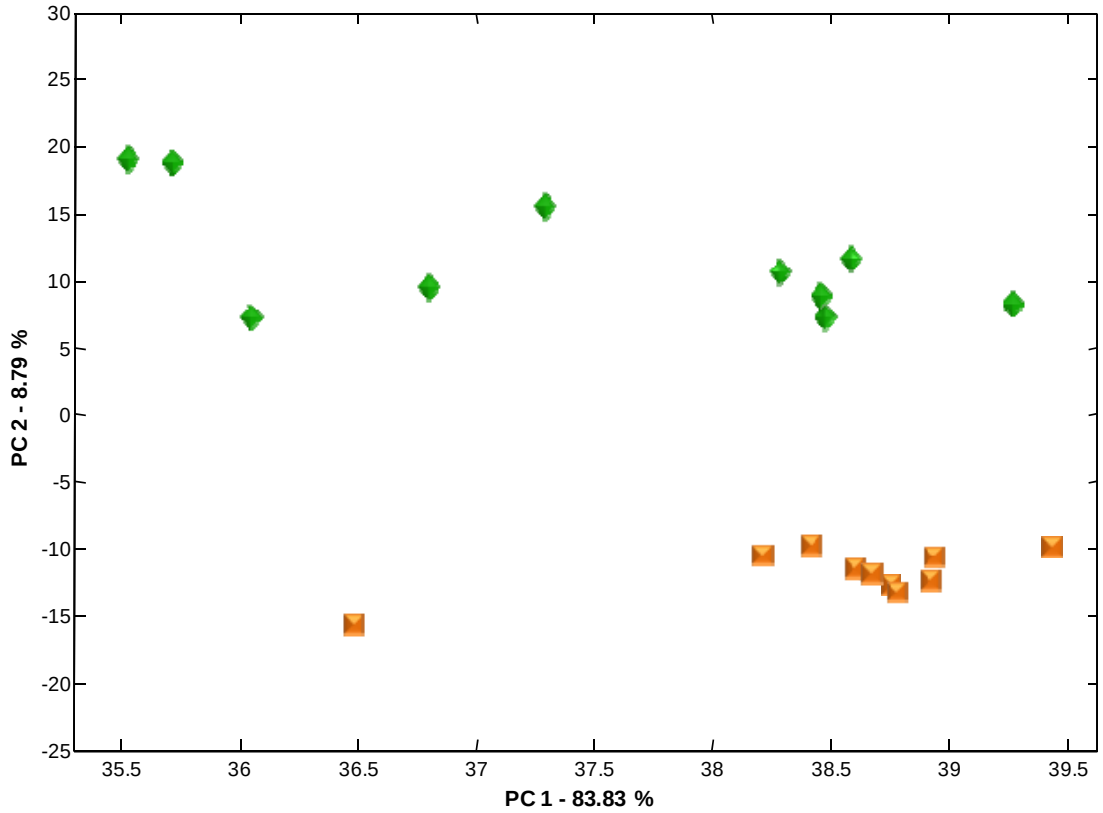

Figure S1. Score plot of PCA of NMR data: natural extracts (♦) and brominated extracts (■).

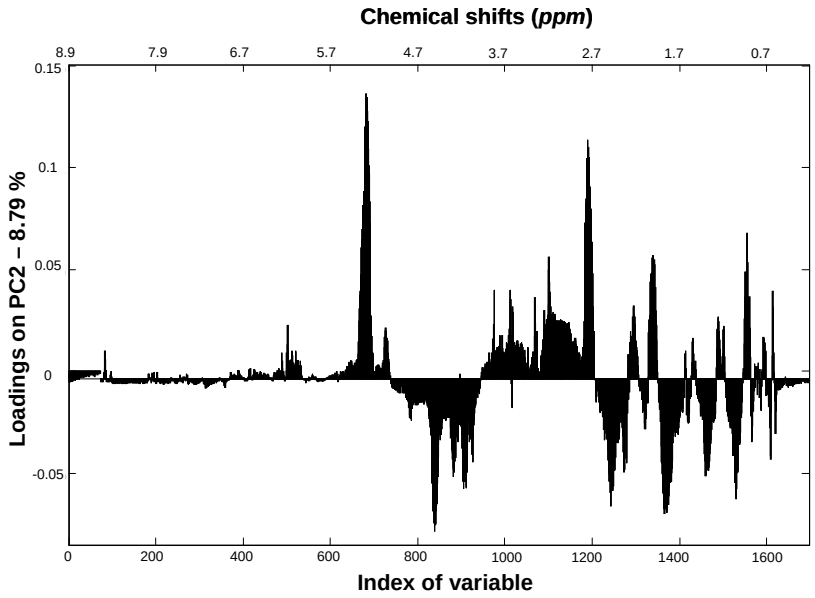

Figure S2. Loading plot on PC2 of NE and BE.

SI-MS analysis of compound 1.

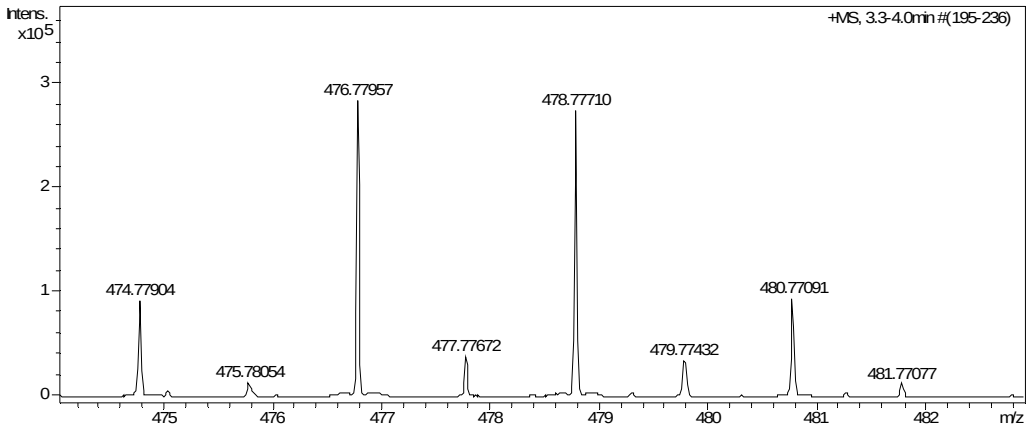

Figure S3. Isotope pattern observed for the parent ion of tribrominated compound **1** ($C_{12}H_7Br_3NaO_4^+$).

Expected m/z (%)	Observed m/z (%)
474.7787 (34)	474.7790 (32)
475.7821 (5)	475.7805 (4)
476.7767 (100)	476.7795 (100)
477.7800 (13)	477.7767 (13)
478.7747 (98)	478.7771(97)
479.7780 (13)	479.7743(12)
480.7729 (33)	480.7709 (33)
481.7761 (4)	481.7707 (4)

Table S1. m/z and intensities calculated and observed for the parent ion of tribrominated compound **1.**

^{1}H and ^{13}C NMR of compound 1

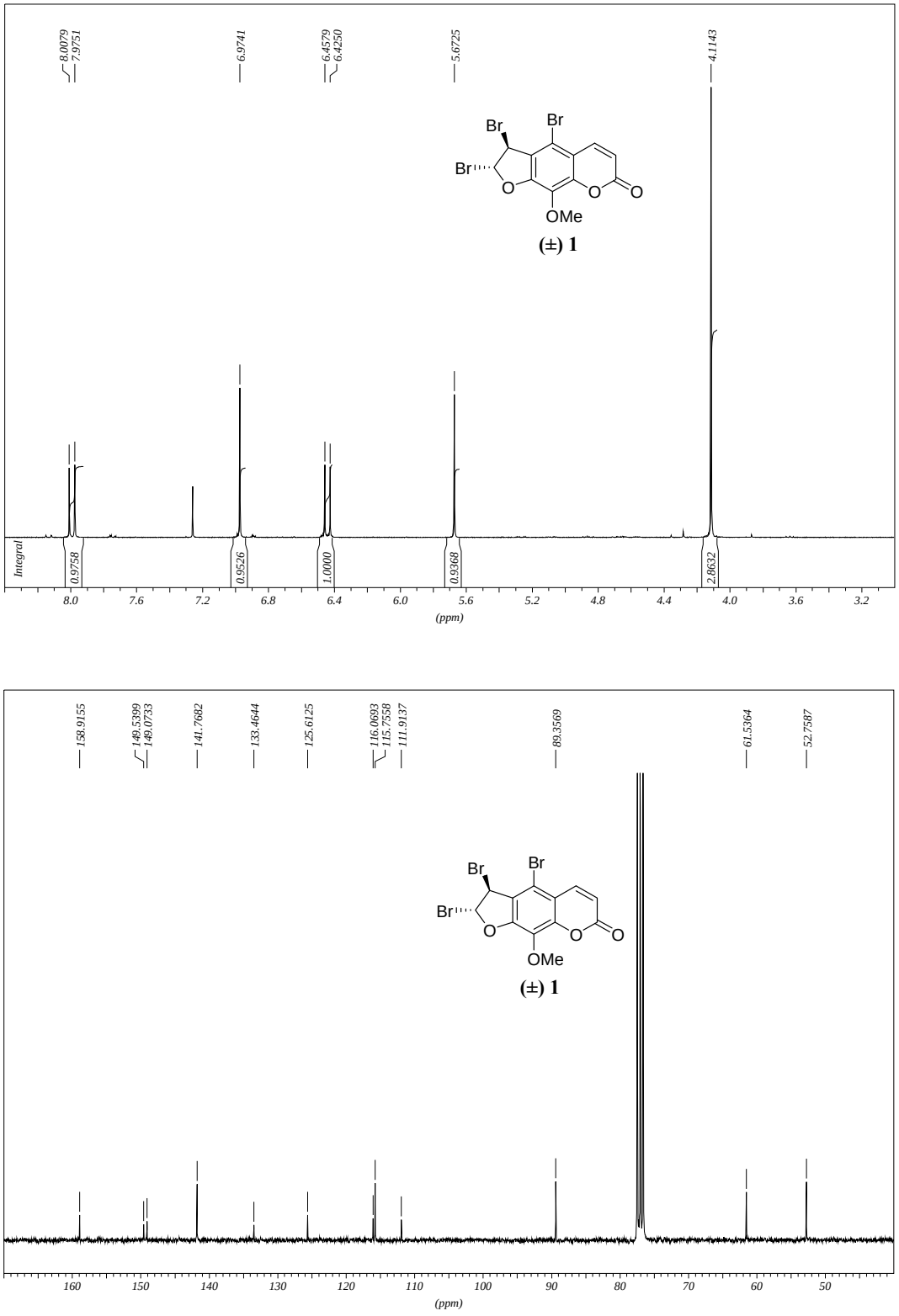

Figure S4. ^{1}H NMR and ^{13}C NMR spectra of compound **1**.

HPLC analysis

HPLC was performed using a Hewlett-Packard 1050 instrument, coupled to a HP 1050 DAD.

HPLC Parameters

Flow rate: 1.000 mL/min

Column: Waters Symmetry C18, 150 × 4.6 mm, 5 µm.

Mobile phase: acetonitrile with 0.05% formic acid (solvent A) acetonitrile:milliQ water (70:30) with 0.05% formic acid (solvent B).

Time (min.)	Solvent A	Solvent B
0	0	100
45	100	0
50	100	0
53	0	100
55	0	100

Table S2. Gradient elution used for HPLC analysis.

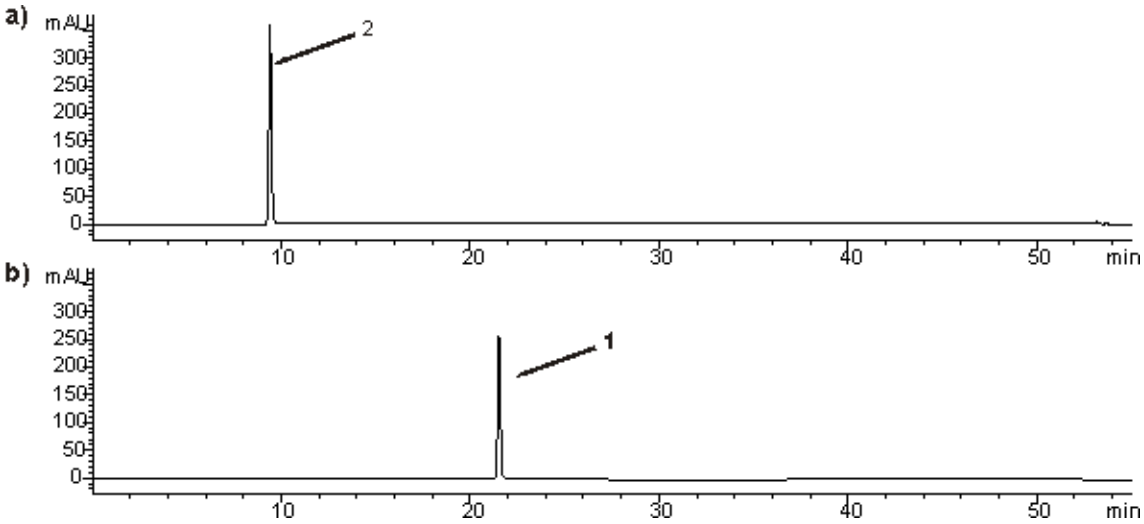

Figure S5. a) HPLC-UV-300nm of commercial 8-methoxypsoralen. b) HPLC-UV-300nm of synthetic tribrominated compound **1**.

Comparison of ^{1}H NMR spectra of 1, 2, BE and NE.

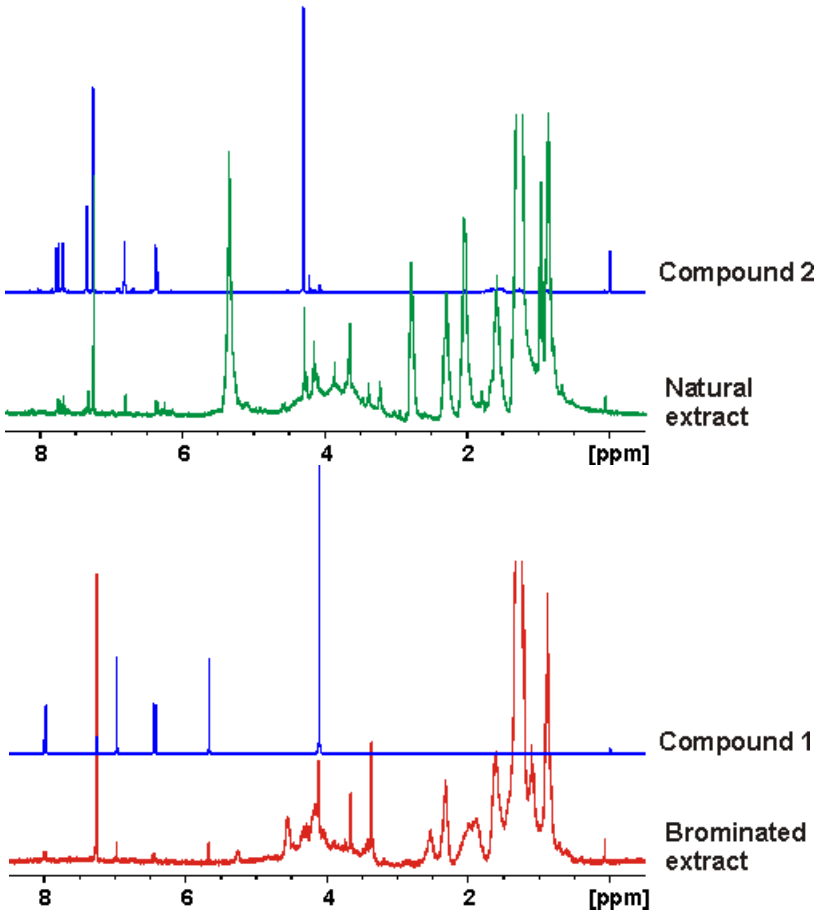

Figure S6. a) Comparison of the ^{1}H NMR spectra of compound **2** and *C. maculatum* NE, b) Comparison of the ^{1}H NMR spectra of compound **1** and *C. maculatum* BE.

β-glucosidase bioautography

Typical procedure for the detection of β–glucosidase activity. The amount of sample loaded was 500 µg of extract/spot. We used the methodology previously reported by Salazar *et al*.[3]

Xantine oxidase bioautography

Typical procedure for the detection of xantine oxidase activity. The amount of sample loaded was 500 µg of extract/spot. We used the methodology previously reported by Ramallo *et al*.[4]

Acetylcholinesterase bioautography

Staining solution for acetylcholinesterase. Agar (170 mg) was dissolved at 80ºC in Tris-hydrochloric acid buffer (50 mM, pH = 8, 16.3 mL). The solution was allowed to cool to 50ºC and acetylcholinesterase solution in 50 mM Tris-hydrochloric acid buffer (60 U/mL, 437 μL) was added and the obtained solution mixed by inversion.

Typical procedure for detection of acetylcholinesterase activity. Samples were separated on 10 x 10 cm silica gel layers. The amount of sample loaded was 500 μg of extract / spot. After migration of the sample with hexane:EtOAc (70:30), the TLC was allowed to dry and then sprayed with a mixture of Fast Blue B salt (30 mg) in distilled water (12 mL) and naphtyl acetate (7.5 mg) in ethanol (3 mL). The TLC plate was dried and acetylcholinesterase staining solution was distributed over the TLC layer. After solidification of the staining solution, the TLC plate was incubated at 37ºC during 20 min. White inhibition zones were observed against a purple background.

Acetylcholinesterase quantitative bioassays

TLC assay: Compounds **1** and **2** were dissolved in CH_2Cl_2 and were spotted in decreasing quantities in the TLC plate. We employed the acetylcholinesterase inhibitor physostigmine as a positive control. This methodology has been published previously by Marston *et al*.[5]

Microplate assay: AchE activity was measured using a 96-well microplate assay based on Ellman's method reported in Di Giovanni *et al*.[6] The test samples were dissolved in 3μL of DMSO (1.15% final concentration DMSO). The controls contained the corresponding volume of DMSO instead of compound solutions. Percentage of inhibition was calculated by comparing the rates for each sample to the control and the IC_{50} was estimated by using Prism V5.01, GraphPad Software Inc.

References

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