SUPPLEMENTARY MATERIAL

Anti-infective assessment of *Senecio smithioides* (Asteraceae) and isolation of 9-oxoeuryopsin, a furanoeremophilane-type sesquiterpene with antiplasmodial activity

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Abstract

The search for anti-infective activity in the antipyretic plant *Senecio smithioides* was conducted. Petroleum ether (PE), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and hydroethanolic (96% EtOH) extracts, and compounds 9-oxoeuryopsin (**1**), epoxydecompostin (**2**) and senecionine (**3**) were obtained from the aerial parts. All extracts and **1** were tested against chloroquine resistant strain of *Plasmodium falciparum* (ref. chloroquine), *Trypanosoma cruzi* (ref. nifurtimox), *Leishmania braziliensis*, *Leishmania amazonensis* and *Leishmania donovani* (ref. pentamidine), *Staphylococcus aureus* and *Escherichia coli* (ref. gentamicin), and, *Neurospora crassa* and *Candida albicans* (ref. ketoconazole). The PE extract exhibited the strongest *in vitro* activity against *Plasmodium falciparum* IC_{50} < 1.0 μg/mL. **1** was established as a potent antiplasmodial with an IC_{50} = 1.2 μg/mL, 5.2 μM. Other antiparasitic activities were recorded for all extracts and **1**. Antibacterial and antifungal activity was negligible.

Keywords: *Senecio smithioides*, *Plasmodium falciparum*, Furanoeremophilane-type sesquiterpene, Asteraceae, structural elucidation.

Experimental

General

^{1}H and ^{13}C NMR spectra were recorded in Bruker AC250, AV300 and AV500 spectrometers in $CDCl_3$ and CD_3OD with tetramethylsilane (TMS) as internal standard. The EIMS was measured in a VG Autospec spectrometer under 70 *e*V. The APCIMS spectrum was recorded with an ion trap mass spectrometer Thermo-Finnigan® LCQ Deca XP Max. The IR spectra were recorded in a Beckmann AcuLab 4 and Perkin-Elmer Spectrum BX FT-IR spectrometers. The UV spectra were recorded in a Hitachi-Perkin-Elmer 200 apparatus and an UV Helios α Spectrometric Instruments apparatus. Melting points were obtained in a Fisatom 430 D apparatus. Optical rotation $[\alpha]^{D}$ were measured with an Atago Polax-2L apparatus. TLC were developed on silica gel 60 F_{254} (Merck) precoated plates and detection was carried out by spraying with 50% H_2SO_4 plus heating, and Dragendorff's reagent.

Plant material

Senecio smithioides was collected in January 1999 and November 2013, in San Juan village, at the cross point of the San Juan River and the Potosí-Uyuni route, 65 km from Potosi city at 3,850 m.a.s.l., and in Cebadillas village, located on route No. 5 between the cities of Potosí and Uyuni, 20 km from Potosí city at 3,830 m.a.s.l. Department of Potosi, Bolivia. Samples were identified by Prof. Emilia García (Bolivian National Herbarium LPB. La Paz, Bolivia). Voucher specimens are deposited at LPB under the code NQ1.

Fractionation and isolation

Dried aerial parts of *S. smithioides* first sample (77.99 g) were pulverized and then macerated in Erlenmeyer flask (1000 ml) at rt in 400 ml of PE (40-60 °C) for 24 hours. The filtrate (PE extract) was evaporated under reduced pressure to give 1.33 g of dry matter (1.72%). PE extract was dissolved and evaporated under reduced pressure twice using PE (40-60 °C, 400 ml of each). Fine colourless crystalline needles (1), 605 (1st time) + 134 mg (second time), appeared as a precipitate in the extract of PE during evaporation; these were filtered before finishing the evaporation process. Compound **1** was re-crystallized in PE (20-40 ºC). The same plant sample was successively extracted with solvents of increasing polarity (24 hours of steeping, 500 ml of each). After evaporation under vacuum the corresponding extracts were obtained, namely: dichloromethane (2.94 g, 3.77%), ethyl acetate (0.53 g, 0.68%), and EtOH 95% (1.85 g, 2.37%). The crystals of **1** were obtained by filtration after redissolution (400 mL) and re-evaporation *in vacuo* to afford 141, 102

and 98 mg of **1** respectively. These crystals (**1**) were re-crystallized in petroleum ether (20-40 ºC). The total yield of **1** was 1.08 g (1.39%). These operations were monitored by TLC (SiO_2, 0.2 mm, $60F_{254}$, Merck, CH_2Cl_2/CH_3OH, 99.5:0.5, developer: H_2SO_4 50%). See Figure S1.

Dried aerial parts of the second sample of *S. smithioides* (276.2 g) were pulverized and extracted for 8 hours with PE (20-40 °C, 2000 mL) in a Soxhlet extraction system (1000 mL extractor and round bottom flask of 2000 mL, 30 °C mean temperature of boiling). During extraction, greenish crystals appeared in the round bottom flask of the Soxhlet system. Once completed extraction, the crystals were separated from the extract by filtration in a quantity of 2.1688 g. The crystals were washed with EtOH (95%) in a Buchner filtration system to provide pure needles designated as compound **2** (2.161 g). The extract was concentrated *in vacuo*, redissolved in EP (20-40 ° C, 200 mL) and concentrated again *in vacuo*. During the re-concentration of the solution, the presence of more crystals was noticed. These crystalline needles were filtered and washed with EtOH and separated as described above resulting in 0.9737 g of pure compound. The PE extract was re-dried *in vacuo*, redissolved in (20-40 ° C, 200 mL) and then concentrated *in vacuo*. During concentration of the extract PE, more crystals were formed (0.2074 g) which were washed and separated as already described for the previous crystals. All precipitates were combined resulting in a total of 3.3421 g (1.21%) for compound **2**. A sample of the crystals (39 mg) coming from the extract of PE of the second vegetal sample was submitted to NMR analyses for its ulterior structural identification. The PE extract after separation of compound **2** was concentrated to dryness under vacuum and weighed 8.887 g. The total weight of the extract (including **2**) was 12.2291 g (4.43%). See Figure S2.

Powdered plant material (second sample, 276.2 g), after degreasing with PE and after drying in an oven with air circulation at 25 °C for 24 hours to remove all traces of PE, weighed 264.0 g. The plant was basified by spraying with 850 ml 25% NH4OH (pH 8-9, pH paper by Merck) and then extracted for 22 hours (11+11) in a Soxhlet extraction system (extractor 500 ml and round bottom flask 1000 ml) using CH_2Cl_2 (2000 ml, 29° C). The CH_2Cl_2 extract after evaporation under vacuum weighed 5.8204 g (2.10% with respect to the source material, and about 2.20% of defatted material). This total dissolved in 175 ml of CH_2Cl_2 was extracted three times liquid to liquid against 150 ml of HCl (5%) each. The acidic aqueous layer was tested for alkaloid content after each manipulation using Dragendorff reagent (an aliquot of the H_2O layer in a test tube plus 2 drops of 5% HCl plus 2 or 3 drops of Dragendorff reagent to result in an orange precipitate, to the positive case). After the third extraction CH_2Cl_2-$HCl_{5\%}$ the Dragendorff test with the acidic aqueous layer showed a scarce precipitate; thus showing a CH_2Cl_2 layer free of alkaloids. Each of the three acid aqueous layers was basified with 25% NH_4OH (dropwise to pH 8-9 as measured by pH paper by Merck). The first basic aqueous layer was extracted liquid-liquid with 500 ml of CH_2Cl_2, and secondly and thirdly with 150 ml of CH_2Cl_2 each, until obtaining a negative Dragendorff test. This means an aliquot of the basic aqueous layer in a test tube, addition of a few drops of 5% HCl until pH 6 or 5 checked by pH paper, and 2 or 3 drops of Dragendorff reagent to yield a very small amount of orange precipitate. The second and third basic aqueous

layers were extracted in the same way as the first one. After each extraction with CH_2Cl_2 the presence or absence of alkaloids was tested with Dragendorff reagent as done with the first basic aqueous layer. All CH_2Cl_2 layers were combined and concentrated in a Büchi R-200 rotary evaporator and vacuum dried in a high vacuum pump Alcatel, for 24 hours to yield 0.350 g (0.13%) of total alkaloids (TCA, total content of alkaloids). A TLC analytical plate (SiO_2, 0.2 mm, $60F_{254}$, Merck) was run with the TCA in $CH_2Cl_2/CH_3OH/NH_3$, 85:14:1 (Sharma et al. 1965) and it was revealed under $UV_{254\ nm}$ radiation and sprayed with Dragendorff reagent. The chromatogram showed a single major alkaloid with Rf 0.56. A second and much smaller orange spot appeared at the beginning of the chromatogram (Rf 0). The TCA (0.350 g) was crystallized in $CH_2Cl_2/C_6H_5CH_3$, 80:20, by first dissolving the TCA in CH_2Cl_2 and then adding hot $C_6H_5CH_3$ and then heating the mixture for one minute. The mixture was placed in the refrigerator overnight. The precipitate formed is filtered on a Buchner system and dried in a high vacuum pump Alcatel, for 12 hours to yield 0.332 g of white plaques or compound **3**. No TLC was done with the product but 37 mg were dissolved in a NMR tube (5 mm internal diameter) for structural identification. See Figure S2.

Physicochemical data of compounds

Compound 1

1 (10)-en-9-oxo-furanoeremophilane [(+)-9-oxoeuryopsin] (**1**). Colourless needle-type crystals. MP: 123-124°C uncorrected. $[\alpha]_D$ +0.35 (*c* 1.3, $CHCl_3$, 589 nm) [8]. IR ($CHCl_3$)ν_{max} 1150 (C-O), 1420 (C-O), 1670 (C=O), 2970 (C-H), 3100 (C=C-H) cm^{-1}. For ^1H and ^{13}C NMR spectral data see Table S1. UV ($CHCl_3$) λ_{max} (log ε) nm: 301.6 (3.97) nm. EIMS 70 eV: *m/z* (rel. abund.): 230 $[M]^{+\cdot}$ (100), 215 (70), 201 (16), 197 (6), 187 (31), 173 (32), 161 (17), 145 (22), 141 (6), 128 (10), 123 (18), 115 (15), 107 (10).

Compound 2

1(10)α-epoxy-6β-acetoxy-9-oxo-furanoeremophilane [epoxydecompostin] (**2**)

Colourless needle-type crystals. MP: 169-170 °C uncorrected. $[\alpha]_D$ +0.05° (*c* 0.33, $CHCl_3$). FTIRν_{max} 1232 (C-O), 1411 (C-O), 1678 (C=O), 1747 (CH_3C=O). For ^1H and ^{13}C NMR spectral data see Table S1. UV ($CHCl_3$) λ_{max} (log ε) nm: 283.0 (2.08) nm. APCIMS: *m/z* (rel. abund.): 305 $[M+H]^+$ (100), $C_{17}H_{20}O_5$ positive APCI mode in neutral; sample dissolved in 10 mg/mL in CH_2Cl_2, ulterior dilution in CH_3OH in the ratio 1/10.

Compound 3

(1*R*,4*Z*,6*R*,7*R*,17*R*)-4-ethylidene-7-hydroxy-6,7-dimethyl-2,9-dioxa-14-azatricyclo[9.5.1.0^{14,17}]heptadec-11-ene-3,8-dione [senecionine] (**3**). Colourless plates. MP: 236 °C. $[\alpha]_D$ -55.0° (*c* 0.03, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$, CD_3OD): δ 6.10 (1H, *s*, H-2), 5.66 (1H, *q*, *J*=6.9 Hz, H-20), 5.40 (1H, *d*, *J*=11.8 Hz, H-9b), 4.96 (1H, *t*, *J*=3.5 Hz, H-7), 4.18 (1H, *dd*,

J=7.5, 2.4 Hz, H-8), 3.96 (1H, *d*, *J*=11.8 Hz, H-9a), 3.84 (1H, *dd*, *J*=15.7, 1.3 Hz, H-3b), 3.31 (1H, *dddd*, *J*= 11.3, 6.8, 5.8, 1.0 Hz, H-3a), 3.17 (1H, *t*, *J*=8.7 Hz, H-5b), 2.47 (1H, *ddd*, *J*=12.4, 9.6, 5.9 Hz, H-5a), 2.30 (1H, *dd*, *J*=14.0, 5.7 Hz, H-6b), 2.11 (1H, *brd*, *J*=12.2 Hz, H-14b), 2.04 (1H, *dddd*, *J*=16.2, 11.8, 7.9, 3.5 Hz, H-6a), 1.84 (3H, *d*, *J*=7.1 Hz, H-21), 1.76 (1H, *dd*, *J*=13.2, 10.6 Hz, H-14a), 1.57 (1H, *m*, H-13), 1.24 (3H, *s*, H-18), 0.83 (3H, *d*, *J*=6.7 Hz, H-19). ^{13}C NMR (125.8 MHz, $CDCl_3$, CD_3OD): δ 177.7 (C, C-11), 167.5 (C, C-16), 136.1 (CH, C-2), 134.6 (CH, C-20), 132.9 (C, C-15), 131.4 (C, C-1), 77.0 (CH, C-8), 76.9 (C, C-12), 74.9 (CH, C-7), 62.7 (CH_2, C-3), 60.3 (CH_2, C-9), 53.0 (CH_2, C-5), 38.4 (CH, C-13), 38.2 (CH_2, C-14), 34.7 (CH_2, C-6), 24.8 (CH_3, C-18), 15.0 (CH_3, C-21), 10.9 (CH_3, C-19).

In vitro Antiplasmodial assay

Cultures of *Plasmodium falciparum* (chloroquine-resistant strain INDO) were maintained in human erythrocytes. DMSO (50 μL) was added to samples of extracts or pure compound, which were then dissolved in RPMI 1640 medium with the aid of mild sonication in a sonicleaner bath (Branson Ltd.) and further diluted as required in medium between 1 and 0.05 μg/mL. The DMSO concentration for tested dilutions was no greater than 0.1%. The total culture medium (150 μL) was placed into the wells of 96-well microtiter plates with the diluted extract and the suspension of human red blood cells in medium (0+, 5% hematocrit) with 1% parasitemia. All tests were performed in triplicate. After 24 h of incubation at 37 °C using the candle-jar method, the medium was replaced fresh daily, and incubation continued for a further 48 h. On the third day of the test, a blood smear was taken from each well, and parasitemia counted. Each test included an untreated control, control with solvent, and chloroquine (99.98%; Sigma® USA) as an internal standard. The parasitemia was measured by the optical microtest method (Gakunju et al 1979), where the % of inhibition of parasitemia for each concentration of extracts with respect to the control. Linear regression analysis was used to determine the best fitting straight line from which IC_{50} values were determined (Deharo et al 1992).

In vitro antitrypanosomal assay

Trypanosoma cruzi strain Tulahuen for evaluation of trypanocidal activity. The strains were obtained from IBBA (La Paz, Bolivia), a WHO reference laboratory, and their identification was confirmed by isoenzyme analysis. In vitro procedure on the epimastigote form of *T. cruzi* were cultured in LIT (liver infusion tryptose) medium supplemented with 10% fetal calf serum at 28 °C with an inoculum of 106 cells/mL. Samples (4 mg) were aseptically dissolved in 50 μL of DMSO

and liquid medium to obtain final concentrations of 50, 20, 10, 1, and 0.1 μg/mL. All assays were carried out in triplicate. Final DMSO concentration was less than 0.5%. Parasites were counted after 48 h of contact with the samples in a haemocytometer, and the activity of the test substances was assessed by comparison with controls without extract and with nifurtimox-containing cells (Moretti et al. 1998).

In vitro antileishmanial assay

The following strains were used: *Leishmania amazonensis* IFLA/BR/67/PH8, *L.braziliensis* MHOM/BR/75/M 2903, and *L.donovani* MHOM/IN/83/HS-70, for determination of leishmanicidal activity. The strains were obtained from IBBA (La Paz, Bolivia), a WHO reference laboratory, and their identification was confirmed by isoenzyme analysis. In vitro test procedure on promastigote culture of *Leishmania* spp: samples were aseptically dissolved in liquid medium and DMSO (final concentration of DMSO less than 0.1%) to obtain final concentrations of 50, 25, 10, 1, and 0.1 μg/mL. The solution was filtered through a Millipore membrane (0.22 μm) and placed in Titertek 96 microcells (Flow Laboratories). All assays were done in triplicate. Each cell was cultured with 50 000 parasites/mL at 27 °C. The activity of the samples was evaluated after 72 h by optical observation on a drop of culture with an inverted phase microscope, by comparison with control cells without extracts and with pentamidine-containing cells. Reference compound was pentamidine (Moretti et al. 1998).

Antibacterial assay

Escherichia coli ATCC-8739 and *Staphylocouccus aureus* ATCC-25923/6538, were used. The strains were obtained from the Public Health Institute of Chile (Santiago, Chile). The culture of microorganisms was performed in tubes with 3 mL of tryptic soy broth medium (30 g/L) at 37 °C for 18 h. Samples were dissolved in a mixture of DMSO and water (1:1) to obtain final concentrations of 30 and 10 μg/mL. Petri dishes were prepared with 20 mL of tryptic soy broth medium (40 g/L) inoculated with 0.1 mL of test organisms (1 500 000 bacteria/mL). In all test plates, holes (Ø = 8 mm) were made and filled with 0.1 mL of solution of compound. Plates were incubated at 37 °C for 18 h. Diameters of inhibition were measured**.** Reference compounds was gentamicin (*E. coli* and *S. aureus*) (Bravo et al. 2001).

Antifungal assay

Plant extracts and pure compound (5 mg) were dissolved in the appropriate solvent (1 mL) and were placed in vials by separate aliquots of 200, 100 and 20 ml. The organic solvent was allowed to evaporate for 12 hours and the residues were re-dissolved in DMSO (20 mL) and deposited in borings (0.4 cm) made in *Sabouraud* agar (20 mL) in Petri dishes (10 cm diameter) containing each an inoculum of spores of *Neurospora crassa* and *Candida albicans* (20 mL of spores, 106 sperm / mL) and controls of ketoconazole, distilled water or DMSO. The measurement of the zones of inhibition (mm) was performed after incubation at 25 ° C for 18 hours.

Table S1. NMR data of 9-oxoeuryopsin (**1**), epoxydecompostin (**2**).

	Compound			
	1[a]	**2**[b]	**1**[a]	**2**[b]
Atom	δ^1H (*J*)	δ^1H (*J*)	δ^{13}C	δ^{13}C
1	6.96 *t* (3.8 Hz)	3.35 *d* (4.1 Hz)	136.5 *d*	62.5 *d*
2'ax	2.25*m*	2.05*m*	26.0 *t*	19.2 *t*
2eq	2.23 *m*	1.93 *m*		
3'ax	1.51 *m*	1.79 *m*	26.2 *t*	24.8 *t*
3eq	1.43 *m*	1.46 *m*		
4	1.79 *dqd* (10.9, 6.8, 4.0 Hz)	1.61 *dqd* (10.6, 7.2, 3.3 Hz)	39.9 *d*	32.1 *d*
5			40.3 *s*	45.1 *s*
6	2.45 *d* (16.5 Hz) 2.76 *d* (16.5 Hz)	6.62 *s*	34.1 *t*	69.3 *d*
7			121.3 *s*	121.5 *s*
8			146.9 *s*	146.4 *s*
9			175.8 *s*	181.0 *s*
10			142.4 *s*	65.4 *s*
11			137.3 *s*	136.8 *s*
12	7.38 *m*	7.46 *m*	145.1 *d*	146.7 *d*
13	2.00 *d* (1.1 Hz)	1.96 *s*	7.7 *q*	8.3 *q*
14	1.00 *s*	1.20 *s*	20.4 *q*	15.8 *q*
15	1.04 *d* (6.9 Hz)	1.03 *d* (7.2 Hz)	15.6 *q*	15.5 *q*
$\underline{CH_3}CO$		2.23 *s*		20.8 *q*
$CH_3\underline{CO}$				170.7 *s*

[a]250 and 62.9 MHz, $CDCl_3$; [b] 300 and 75 MHz, $CDCl_3$

Table S2. Antibacterial and antifungal evaluation of *Senecio smithioides*.

		Bacteria[§]		Fungi[§]	
Sample	Concentration*	*E.c.*[a]	*S.a.*[b]	*N.c.*[c]	*C.a.*[d]
PE	10	0	2	†	†
	8	†	†	14	0
CH_2Cl_2	10	0	4	†	†
	8	†	†	12	0
EtOAc	10	0	5	†	†
	8	†	†	14	0
95º-EtOH	10	0	5	†	†
	8	†	†	0	0
Comp. **1**	10	0	0	†	†
	8	†	†	0	0

[a]*Escherichia coli*, [b]*Staphylococcus aureus*, [c]*Neurospora crassa*, [d]*Candida albicans;* *mg/mL; [§]halo of inhibition in mm in Petri dish; †not evaluated

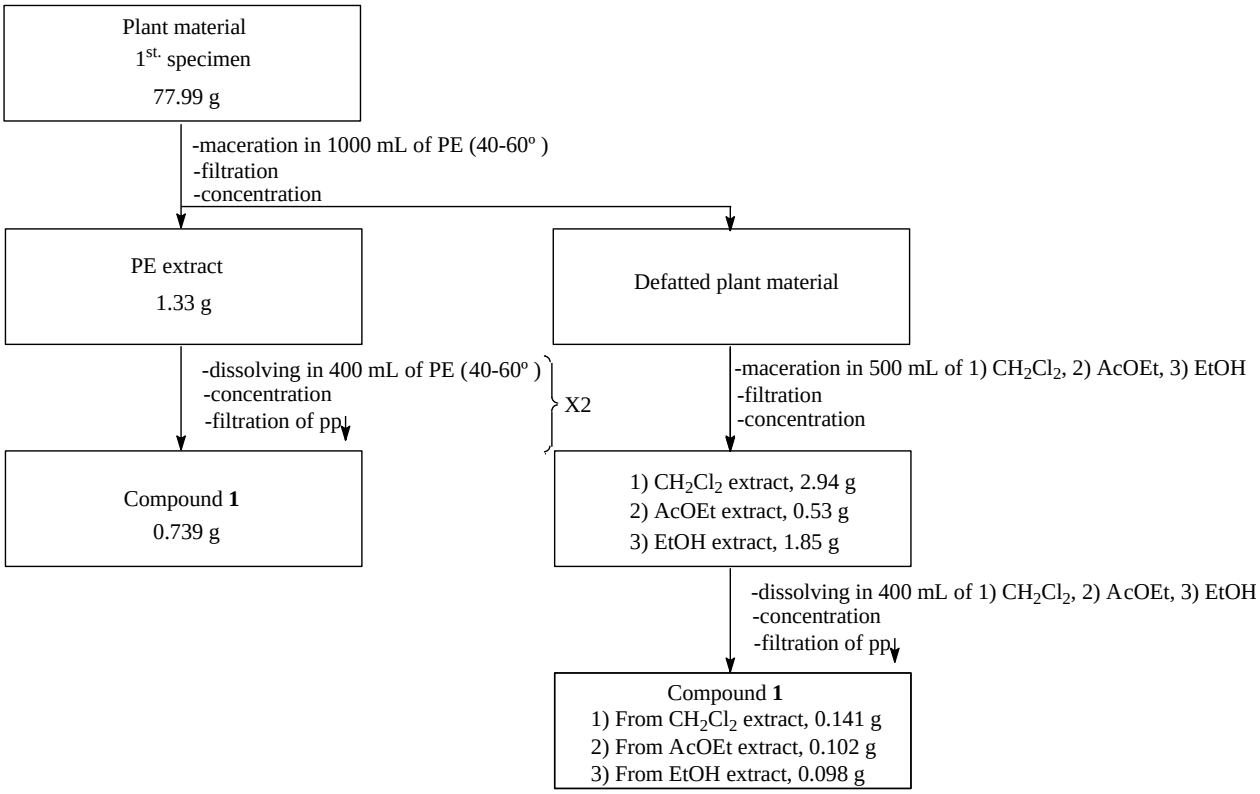

Figure S1. Extraction diagram, compound **1**, 9-oxoeuryopsin.

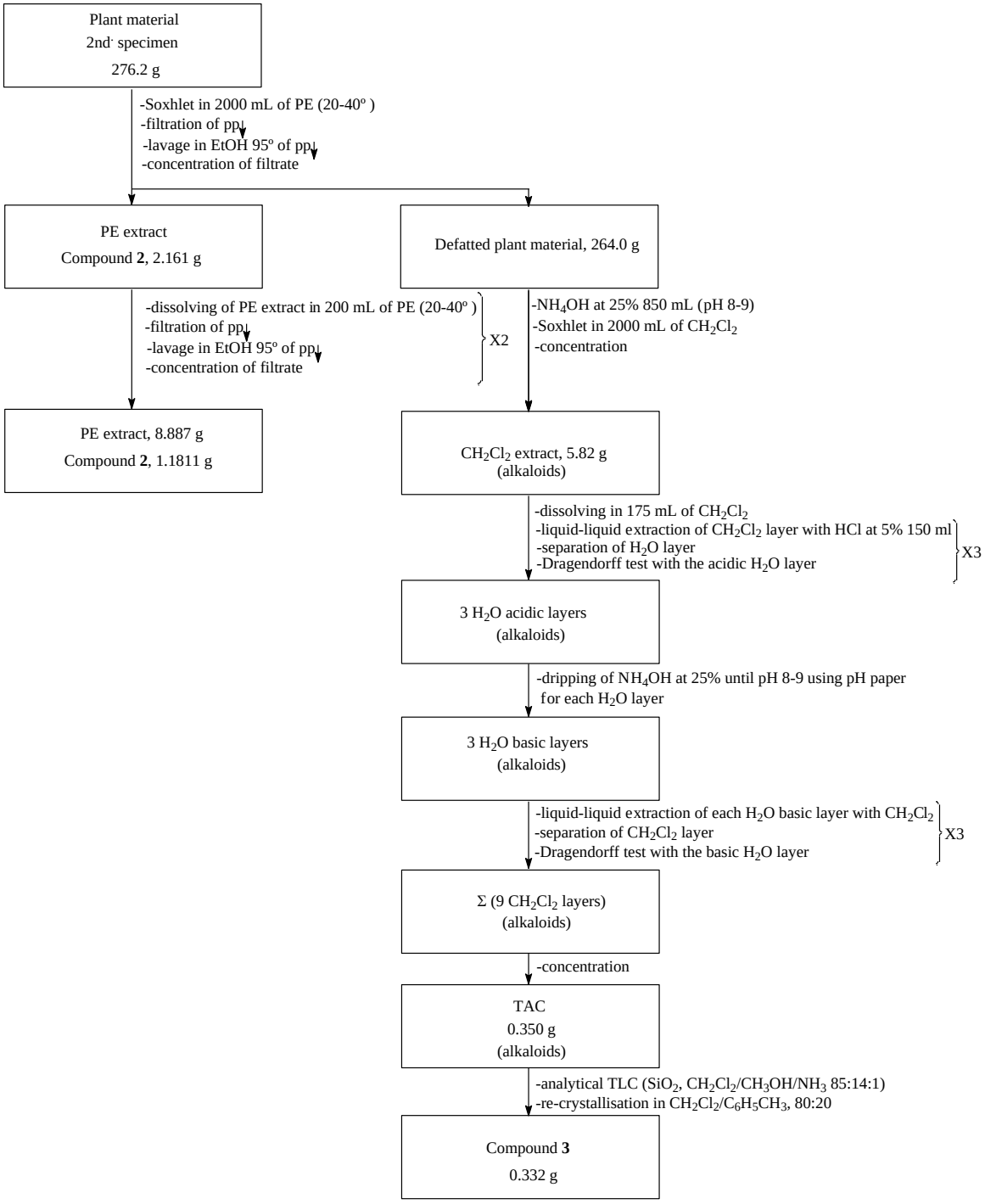

Figure S2. Extraction diagram, epoxydecompostin (**2**) and senecionine (**3**).

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