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Large scale purification of the SERCA inhibitor Thapsigargin from *Thapsia garganica* L. roots using centrifugal partition chromatography

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ABSTRACT

Thapsigargin (Tg) is a selective and irreversible inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)-dependent pump at subnanomolecular concentrations. As such, it has become a powerful tool in the study of Ca^{2+} signaling pathway. Purification of Tg from *Thapsia* species requires repeated chromatographic steps with normal-phase alumina or silica and reverse phase chromatography. We thus developed an innovative procedure coupling high pressure automatized extraction with centrifugal partition chromatography allowing a fast and safe large-scale isolation of highly pure Tg, in two steps from *Thapsia garganica* L. roots. Comparison of influence of extraction procedures, storage conditions and harvesting areas on Tg content in different Algerian specimens of *Thapsia garganica* L. roots has been precised by mean of HPLC quantification procedure. Highest Tg content were found in the fresh material of the sample from Setif area.

1. Introduction

Thapsigargin (Tg) is a sesquiterpenic lactone isolated from *Thapsia* species (Apiaceae). Tg has been a subject of intensive research because of its ability to inhibit the sarco-endoplasmatic reticulum calcium ATP-ase (SERCA) at subnanomolar range (Ki ca 2 *p*M). Subsequent depletion of the endothelial-reticulum calcium induces cell apoptosis [1]. Thapsigargin is thus a very useful tool to study intracellular calcium-dependent signaling pathways involved in calcium homeostasis, apoptosis, oxidative stress [2,3]. Moreover, its ability to restore apoptosis in human prostatic cancer cell lines led to the development of promising prodrugs targeting androgen-independent prostate cancer [4–6].

Commercial Tg is mainly obtained by extraction from vegetal sources, although one total synthesis of Tg has been described by Ley's group [7,8]. Tg occurs exclusively in *Thapsia* genus, in association with about twenty penta- and hexaoxygenated guaianolides (thapsigargins), thapsanes, phenylpropanoids, and coumarins [9–12]. Among the seven described *Thapsia* species [13], chemotaxonomic studies showed that Tg has been identified both in fruits and roots of *T. garganica*, *T. gymnesica and T. villosa* [14]. However, first multi-grams isolation of Tg described by Appendino et al. reported the highest yields of Tg in *Thapsia garganica* L. roots. [15,16]. Procedures applied in this large-scale isolation involved classical extraction methods followed by repeated solid–liquid separations using normal or reverse phase silica gel column chromatography, or alumina gel column chromatography. Due to its high toxicity and histamine-releasing properties, Tg manipulation is highly hazardous and development of efficient and safe purification procedure is prioritary. In this context, we have thus optimized the process and developed an innovative two step large-scale isolation of Tg from the vegetal material, associating automatized high-pressure extraction and liquid–liquid centrifugal partition chromatography. For this purpose, we also evaluated effect of extraction procedures, storage conditions and harvesting areas on Tg content in our starting vegetal material, using HPLC quantification.

2. Experimental

2.1. Chemicals and reagents

Acetone for extraction (ACS RPE grade), acetonitrile (HPLC grade), formic acid (ACS reagent grade) and thapsigargin (ACS

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Table 1
Thapsigargin (Tg) content in *Thapsia garganica* L. roots.

Sample	Localization	Harvesting period	Storage/Tp °C	Water[a] (%)	Extraction yields[b] g (%)		Tg content[c]	
					Classical	SpeedEx	Classical	SpeedEx
a	Oued Zenati (36.31°N, 7.16°E)	March 2010	Dried/RT	11.8	0.677 (3.40)	–	0.49	–
b	Souk-Ahras (36.28°N, 7.9°E)	March 2010	Dried/RT	9.8	0.731 (3.65)	–	0.29	–
c	Jijel (36.82°N, 5.76°E)	March 2010	Dried/RT	10.7	0.418 (2.09)	–	0.30	–
d	Bou-Saada (35.21°N, 4.18°E)	September 2010	Dried/RT	10.5	0.490 (2.45)	0.567 (2.84)	0.50	1.00
e	Setif (36.18°N, 5.41°E)	April 2011	Frozen/-18 °C	59.6	0.492 (2.46)	0.527 (2.64)	1.65	1.92
f	Setif	April 2011	Dried/RT	13.2	–	0.655 (3.28)	–	0.98
g	Setif	December 2011	Frozen/-18 °C	66	0.648 (3.24)	0.477 (2.39)	1.65	1.77
h	Setif	December 2011	Dried/RT	12.5	–	0.504 (2.52)	–	0.60

[a] % loss on drying calculated as follows: ((mb−ma)/mb) × 100.
[b] Expressed over 20 g of the starting vegetal material.
[c] % expressed on dried material.

reagent grade) were purchased from Acros Organic, Fisher Scientific France (Illkirch). Cyclohexane, methanol (MeOH) and ethyl acetate (EtOAc) were purchased from Carlo Erba – SDS. Cyclohexane and EtOAc were distilled before use. Water was distilled from deionized water.

2.2. Apparatus

2.2.1. HPLC analysis

HPLC analysis was performed on LaChrom Elite apparatus provided by VWR (Fontenay-sous-Bois, France) with a D-7000 interface, a L-7200 autosampler, a L-7100 pump, a L-7400 UV detector, and running on EZChrom Elite 3.3 software. ODS reverse phase column (phenomenex, 150 mm × 4.6 mm, 5 μm) was used for analysis.

2.2.2. Spectroscopic analysis

^{1}H and ^{13}C NMR experiments were performed in methanol-*d4*. Spectra were recorded on a Bruker AC 300 MHz spectrometer or a Bruker Avance 400 MHz spectrometer (Wissembourg, France). MS experiments were performed on a mass spectrometer ZQ 2000 Waters (Saint-Quentin, France). The ESI source conditions were capillary cone voltage 50 V in positive mode, spray voltage 3.4 kV.

2.2.3. Extraction apparatus

High pressure extractions were performed on a Speed Extractor E-914 apparatus provided by Büchi (Flawil, Switzerland) equipped with four extraction cells (120 mL) and a collector with four flat bottom vials (220 mL). Maximum pressure and temperature can be adjusted from 50 to 150 bars and from 30 to 200 °C, respectively.

2.2.4. CPC apparatus

CPC was performed on a SCPC-250+1000-B apparatus provided by Armen Instrument (Saint-Avé, France) fitted with two rotors (total volume: 250 mL or 1 L), containing respectively 1953 or 2016 twin-cells. Rotation speed can be adjusted from 0 to 3000 rpm for the 250 mL rotor and from 0 to 1500 rpm for the 1 L rotor. A valve incorporated in the CPC apparatus allowed operation in ascending or descending mode. The system is equipped with a gradient pump, and a 10 mL or a 50 mL loop injection 6-way valve. Fractions were collected by a Büchi 684 fractions collector (Flawil, Switzerland). The experiments were conducted at room temperature.

2.3. Plant material

Roots of *T. garganica* L. were collected in different regions of the east coast of Algeria, in five different areas (Oued Zenati **a**, Souk-Ahras **b**, Jijel **c**, Bou-Saada **d** and Setif **e**-**h**). The Setif specimens were harvested at 2 different periods (April and December). A total of eight samples was obtained (Table 1). Voucher specimens were deposit in the herbarium of the department of Pharmacognosy, University Paris-Descartes, France (voucher specimen no. MKOSB2012a to MKOSB2012h).

2.4. Samples preparation

Fresh harvested roots were either dried or frozen. Samples **a**-**d**, **f** and **h** of *T. garganica* L. roots (Table 1) were directly dried away from light and stored at room temperature before being crushed using a universal cutting mill Pulverisette 19 provided by Fritsch (Idar-Oberstein, Germany). Fresh Setif samples **e** and **g** (Table 1) were maintained between 10 and 15 °C during transport, frozen at −18 °C and cryo-crushed after brief contact with liquid nitrogen using the same apparatus. Then, they were stored at −18 °C until extraction. A small aliquot of each sample was dried at 100 °C for 24 h, in order to determine water content. Loss on drying value was expressed as a percentage and calculated as follows: $((mb - ma)/mb) \times 100$, where *ma* and *mb* represent respectively sample weight after and before desiccation. Tg content in each sample was expressed on dried material extrapolated from this value (Table 1).

2.5. Extraction procedures

2.5.1. Extraction of T. garganica L. roots for HPLC quantification

Classical maceration: samples of *T. garganica* L. roots (20 g) were extracted with acetone (2 × 200 mL, room temperature, 12 h, under magnetic stirring). After filtration, the acetonic extracts were concentrated under reduced pressure to obtain crude extracts.

Speed Extractor: samples of *T. garganica* L. roots (20 g) were mixed with sand (40 g). Extraction cells were filled with the mixture and charged in the apparatus. Extraction program consisted in two distinct cycles of 20 min. Each cycle comprised a pre-heat period of 5 min, pumping of acetone through the extraction cell until final pressure gets to 100 bar, a hold-on time fixed at 5 min and a final flush of the acetonic extract into the corresponding vial. Extraction parameters are shown in Table 2. Extraction yields are presented in Table 1.

Table 2
Extraction method on the Speed-Extractor E-914.

Parameter	Value
Temperature	30 °C
Pressure	100 bar
Cells volume	120 mL
Vials volume	240 mL
Cycles	2
Heat-up	1 min
Hold	5 min
Discharge	2 min
Flush with solvent	1 min
Flush with gas	2 min