(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 August 2017 (31.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/144065 A1

(51) International Patent Classification:
A01H 4/00 (2006.01) *C12P 5/00* (2006.01)
C12N 5/00 (2006.01) *C12P 17/04* (2006.01)

(21) International Application Number:
PCT/DK2017/050049

(22) International Filing Date:
24 February 2017 (24.02.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2016 70113 26 February 2016 (26.02.2016) DK

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) Title: SUSTAINABLE AND INDUSTRIAL PRODUCTION OF GUAIANOLIDES BASED ON ORGAN TISSUE CULTURE

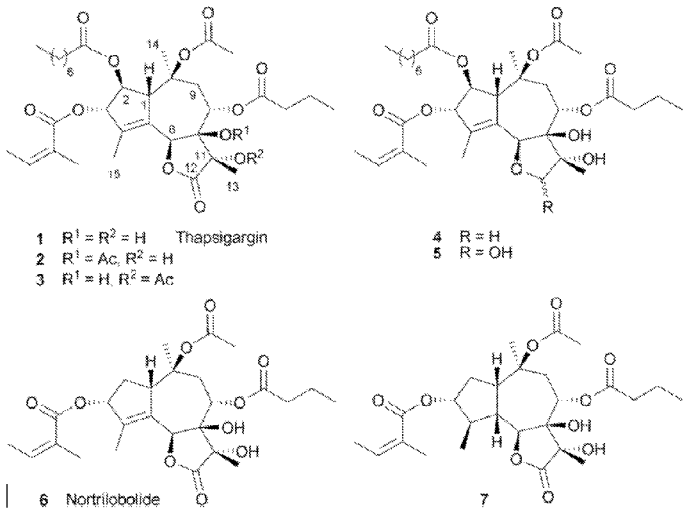

Fig. 1

(57) Abstract: The present invention reports a method of producing complex guaianolides from the plant genus *Thapsia*. The invention comprises a temporary immersion bioreactor (TIB) and cultures conditions for mass micropropagation of *Thapsia spp*. The innovation is advantageous in that it can be used to produce large quantities of biomass of *Thapsia spp* while providing suitable conditions for said plant to produce significant amount of guaianolides, including but not restricted to thapsigargins. This process is a new production platform to supply these molecules of commercial interests at industrial level. At the same time, we also provide a micropropagation protocol of *Thapsia* via direct and indirect organogenesis as a conservation tool of this important medicinal plant.

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

Sustainable and industrial production of guaianolides based on organ tissue culture

FIELD OF THE INVENTION

The present invention relates to a method of producing complex guaianolides, and in particular guaianolides having a thapsigargin backbone which are characterized with a guaiene backbone and a lactone ring (see Formula I below), herein collectively termed "thapsigargin backbone guaianolides". Thapsigargin backbone guaianolides and Nortrilobolid backbone guaianolides are a family of complex guaianolides, which can be isolated from plants of the sub-family *Apioideae*, for example from plants of the genus *Thapsia or Laser.* Thapsigargin backbone guaianolides are characterized with a guaiene backbone and a lactone ring (see Formula I below). We present a method for the culture and mass propagation of *Apioideae* in temporary immersion bioreactors (TIB) as an approach for a sustainable production of these sesquiterpenes lactones at industrial level.

BACKGROUND OF THE INVENTION

Sesquiterpenoids (C_{15}) are a large group of specialized metabolites widespread within the genus *Thapsia.* But it is thaspsigargin and fifteen closely related hexa- and penta- oxigenated guaianolides (herein collectively termed "thapsigargin backbone guaianolides" which are of most interest.

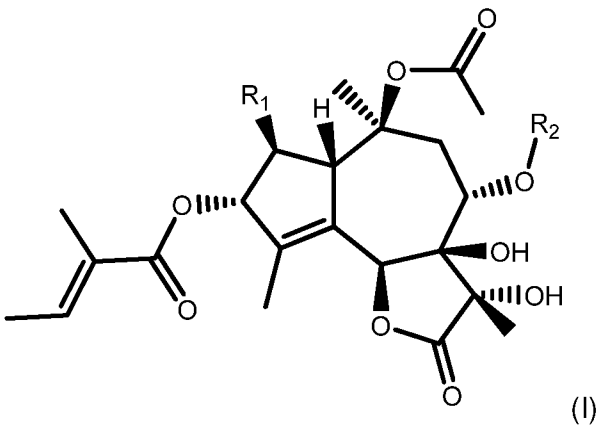
Among this large family of compounds, hexaoxygenated guaianolides such as thapsigargin and pentaoxygenated guaianolides such as nortrilobolide are potent inhibitors of the SERCA (sarco-endoplasmic reticulum calcium transport ATPase) pump. The irreversible inhibition of SERCA leads to elevated cytoplasmic Ca^{2+} levels that induce apoptosis in mammalian cells. As such, they have become powerful tools in the study of Ca^{2+} signaling pathways.

Both types of guaianolides have the same binding site in the SERCA pump and are equipotent [1] (Table 2). However, the subnanomolar affinity for SERCA and the lacking of the large octanate group at C-2 in nortrilobolide has made the thapsigargin the most prominent and intensely studied member of the family.

In addition, the effect on SERCA has also been utilized in the treatment of solid tumors. A recently discovered prodrug comprising thapsigargin linked to an antigen specific for prostate cancer target the molecule only into blood vessels of cancer cells; the death of these blood vessels then leads to tumor necrosis. The first clinical trials of this drug were initiated in 2008,

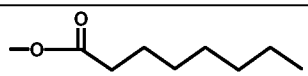
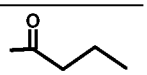
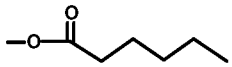
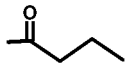
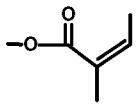
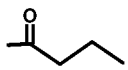
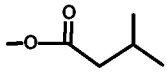
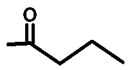
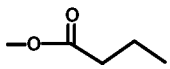
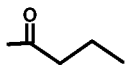
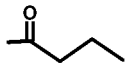
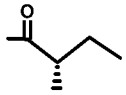
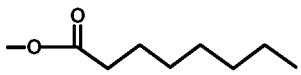
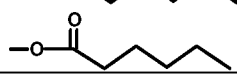
and the potent drug is expected to enter the market in the near future under the generic name Mipsagargin (G-202)[2] (Figure 3). The notion of thapsigargin as a real and viable prostate cancer treatment has therefore greatly elevated the need for its chemical synthesis.

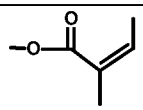
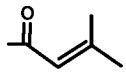
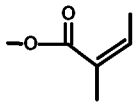
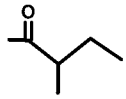
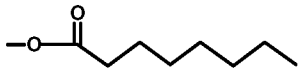
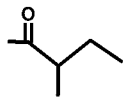
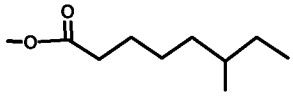
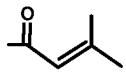
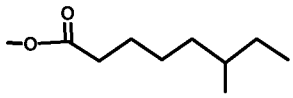
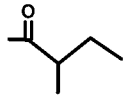
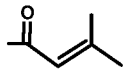
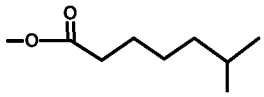
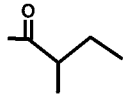
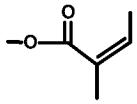
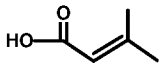
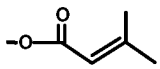
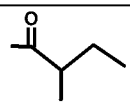
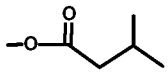
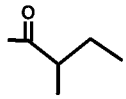
The core structure of thapsigargin is provided as formula I below:

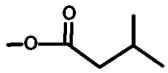
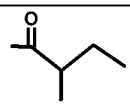


(I)

Table 1: Thapsigargin and similar guaianolides reported within *Thapsia*. The table is amended from Drew et al [4].

Species	Compound	R1	R2
T. garganica L.	Thapsigargin		
	Thapsigargicin		
	Thapsivillosin C-E	See below	
	Thapsivillosin I		
	Thapsivillosin J		
	Thapsivillosin L		
	Nortrilobolid	H	
	Trilobolid	H	
	not named		H
	not named		H
Species	Compound	R1	R2
T. gymnesica Rosselló & A.	Thapsigargin	See above	
	Thapsigargicin	See above	

Pujadas	Nortrilobolid	See above	
T. villosa L.	Thapsivillosin A		
	Thapsivillosin B		
	Thapsivillosin C		
	Thapsivillosin D		
	Thapsivillosin E		
	Thapsivillosin F	H	
	Thapsivillosin G		
	Thapsivillosin H	 or  Exact positions undefined	
	Thapsivillosin K		
	Thapsitranstagin		
	Trilobolide	See above	

Species	Compound	R1	R2
T. transtagana Brot.	Thapsitranstagin		
	Thapsivillosin B	See above	
	Trilobolid	See above	
	Thapsivillosin K	See above	
T. smittii Simonsen, Rønsted, Weitzel and Spalik	Thapsivillosin A, B, H	See above	

Thapsigargin was first isolated from *Thapsia garganica* L. (Figure 2) in 1978. This bioactive compound is the major constituents of the roots and fruits of this Mediterranean species and currently, all of the commercially available thapsigargin is obtained from fruits and roots of wild populations of *T.garganica*. Due to *T. garganica* difficulty to germinate from seeds and to maintain under greenhouse conditions, ThapsIbiza, a Spanish company, is the only company in the world, which has started a small production of *T. garganica* plants. The reliable and sustainable supply of Thapsia biomass is therefore extremely limited.

On the other hand, the total chemical synthesis of Thapsigargin was reported in 2007 [5] obtaining Thapsigargin in 42 steps from (s)-carvone with an overall yield of 0.6 %. Recently, a method with 10-11 steps has been described [15]. Despite the successful synthesis of Thapsigargin, approaches utilizing semi-synthesis or total synthesis are currently far from being economically feasible, therefore, the world-wide chemotherapeutic use is not assured; so there are strong reasons for exploring and developing alternative means of thapsigargin production.

As an alternative to solve said problems, the *in vitro* propagation method for Thapsigargins production has been proposed, however establishment of such methods has proved to be very difficult.

The difficulties in achieving Thapsigargins production from in vitro plants of *T. garganica* have been reflected in the only five reports about this subject. Jäger et al. initiated calli, cell and embryogenic suspension cultures and regenerated roots and shoots of *Thapsia garganica* [6], but were unable to detect any thapsigargin in this in vitro plant material; they only found trilobolide and nortrilobolid in regenerated roots and shoots and in embryos which were developed a little further into the cotyledonary stage. In addition to that, these authors used elicitors such as chitosan or a fungal homogenate without any successful in the synthesis of thapsigargins.

Later, Makunga *et al.* describes the formation of shoots directly from petiole and leaflet explants and tested different medias for micropropagation of this specie, the most successful media containing 1,5 mg l^{-1} benzyl-6-adenine with 0,5 mg l^{-1} α-naphthaleneacetic acid [7] . They also

reported rooting conditions and a fungal treatment for the acclimation of *ex-vitro* plants. These workers describes in 2005 an improved system for the *in vitro* regeneration of *T. garganica* via direct organogenesis [8] and improved rooting and hyperhydricity in regenerating tissues of *T. garganica* in 2006 [9]. However, in none of this publications was described the presence of Thapsigargin in any *in vitro* plant material.

Recently, international patent application WO/2015/082978 described the production of Thapsigargin using undifferentiated cell cultures of *Thapsia* spp. The inventors disclosed that the unique feature allowing them to produce Thapsigargin was to use non-embryogenic undifferentiated cell cultures of *Thapsia*. However, this patent application, while detecting for the first time Thapsigargin in an *in vitro* propagation method of *Thapsia* spp, does not quantify the amount of this specific compound in undifferentiated cell cultures of *Thapsia*. Based on their results, and the detection methods used, a person skilled in the art would believe that the yield is too low to be commercially and industrially relevant. Thus, as described in Example 1 non-embryogenic undifferentiated cells of *Thapsia garganica* produce less than 50ng/g d.w.

Therefore, a strong need in a sustainable production method with high yields of Thapsigargin is needed in order to supply significant amount of this compound to pharmaceutical companies.

SUMMARY

The present invention provides a method for the production of thapsigargins from *in vitro* plant material of genius *Thapsia* with high yields. The advantages of the *in vitro* propagation methods for Thapsigargin production are many: (i) an *in vitro* propagation method contributes to the plant's conservation; (ii) an *in vitro* propagation method ensures a limitless, continuous and uniform supply of product, and is not subject to pests, disasters and seasonal fluctuations; (iii) an *in vitro* plant material can be cultivated in large bioreactors, and can be induced to overproduce thapsigargins by manipulating environmental conditions; (iv) a tissue culture stage is a prerequisite for current transformation protocols for the ultimate recovery of transgenic plants.

The present invention provides a method for the production of thapsigargins from *in vitro* plant material of genius *Thapsia*. The invention comprises a temporary immersion bioreactor (TIB) for biomass micropropagation, including means for incorporating chemical inducing agents (salt, metals, organic compounds, hormones, elicitors, etc.) and adequate conditions.

An object of the present invention is, therefore, to supply the commercial and pharmacological need of thapsigargins from *in vitro* plant of *Thapsia* at industrial levels.

Another object of the invention is to provide a protocol of micropropagation of the genus *Thapsia* via direct and indirect organogenesis which offer a viable tool for mass multiplication and germplasm conservation of these rare and threatened medicinal plants.

Thus, the invention provides methods of producing guaianolides, said methods comprising the steps of

a. providing plant tissue derived from a plant of the sub-family *Apioideae,* wherein said plant tissue is embryogenic callus and/or shoots
b. culturing said embryogenic callus, and/or shoots in a manner involving temporary immersion in basal medium B containing at least one auxin and at least one cytokinin which together are capable of inducing plant shoot micropropagation, thereby inducing plant shoot micropropagation;
d. optionally isolating produced guaianolides.

The invention also provides methods for producing a pharmaceutical composition, said method comprising the steps of

i) preparing guaianolides by the method according to the invention
ii) formulating said thapsigargin into a pharmaceutical composition.

The invention also provides methods for producing a prodrug, said method comprising the steps of

i) preparing a guaianolide by the methods of the invention;
ii) attaching a peptide, which is cleavable by a PSA, an hK2 or PSMA protease to said guaianolide optionally via a linker

thereby producing a prodrug.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Relative potencies of thapsigargin and related analogues [1]. Concentration of inhibitor required to elicit 50% of SERCA. The table shows the equipotency between thapsigargin and nortrilobolide.

Figure 2: Structure of G-202 [2]. Prodrug, that comprises thapsigargin linked to an antigen specific for prostate cancer.

Figure 3: Retrosynthesis of 2-acetoxytrilobolid (4) from Nortrilobolide (3).

Figure 4: a and b, leaflet explants cultured in the basal medium A for 3 months in light conditions; c, in vitro plant cultured in the basal medium B. Subcultures of *in vitro T.garganica* plants.

Figure 5: *Thapsia* garganica *in vitro* plant after 3 weeks cultured in tubes with the basal medium C.

Figure 6: Biomass production stages of *in vitro Thapsia garganica* plants cultured in TIBs with the basal medium B.

Figure7: FW increased of *Thapsia garganica in vitro* shoots after 3 weeks in TIBs cultured with the basal medium B.

Figure 8: HPLC profile of the thapsigargin and nortrilobolide extracted from the *in vitro* plants.

Figure 9: Thapsigargin calibration curve (12, 60, 600, 120 and 1200 mg/L).

Figure 10: Nortrilobolide calibration curve (11, 54, 168, 504 and 1075 mg/L).

Figure 11: UV-UPLC chromatrogram of *T. garganica in vitro* plant (LC-MS) 230 nm. A: nortrilobolide peak D: thapsigargin peak.

Figure 12: Mass spectra of peak A (negative mode).

Figure 13: Mass spectra of peak D (negative mode).

Figure 14: Mass spectra of peak B (negative mode).

Figure 15: Mass spectra of peak E (negative mode).

DETAILED DESCRIPTION

Plants have long provided important sources of pharmaceuticals. The resin from *Thapsia* spp. has been used in traditional medicine in the Mediterranean region for thousands of years. The effects of *Thapsia* spp. are due to the presence of specialized metabolites, such as sesquiterpenoids, which are found in all members of the genus [10].

Although, the most interesting molecule is thaspsigargin, recently *Christensen and col.* have discovered a possible pathway for accessing the hexaoxygenated guaianolides using as a starting material nortrilobolide [11]. More concretely, they synthesized 2-acetoxytrilobolide from nortrilobolide after a few subsequent chemical modifications (Figure 4). These outcomes could provide an expedient access to a wide library and related thapsigargins as potential anticancer agents. Therefore, leads us to highlight the importance not only of thapsigargin but also the importance of other guaianolides, in particular pentaoxygenated guaianolides, such as nortrilobolide.

Both thapsigargin and nortrilobolide are present in the genus *Thapsia* and nortrilobolide is also presents in the genus *Laser* [10].

The present invention is based in an efficient micropropagation system, by using temporary immersion bioreactors (TIB), that implies the rapid multiplication of plant material to produce large number of progeny plants and different cultures medias or treatments that enhance significantly the amounts of guaianolides.

Definitions

The term “approximately” as used herein in relation to numbers, mean that it may be the given number of any minor derivations from said number e.g. +/- 10%, preferably +/- 5%, more preferably +/- 1%.

As used herein the term “basal medium” refers to medium capable of supporting propagation and/or growth of plant tissue, such as plant cells, roots and/or shoots. The basal medium may be an aqueous solution comprising one or more, for example all of the mineral salts, vitamins, organic nitrogen sources and/or carbon sources described herein in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. In addition the basal medium may comprise other components as described herein e.g. plant hormones, and/or elicitors.

As used herein the term “elicitors” refers to molecules capable of enhancing the production of secondary metabolites with phytoalexinic properties as well as to obtain more insight into the regulation of their biosynthetic pathways.

As used herein the term “embryogenic callus” refers to plant cells, either cultivated on a solid media, or in liquid media, in which case they may be referred to as embryogenic cell suspensions. Both kinds must be capable of producing somatic embryos when placed into contact with specific plant hormones.

As used herein, the term "explant" is the plant material used to produce plant clones from a "mother plant" and can be parts of leaves, of roots, seeds, flowers, stems, bark and buds.

As used herein, the term "explant" is the plant material used to produce plant clones from a "mother plant" and can be parts of leaves, of roots, seeds, flowers, stems, bark and buds.

As used herein in relation to plants, the term “multiplication” is used to refer to plants that are multiplied by cloning the plants themselves. In such manner, a large number of specimens can be obtained, which are identical to each other and to the specimen introduced *in vitro* at the beginning of the process.

As used herein the term “organogenic” refers to plant material that have the capacity for organogenesis. Organogenesis is the creation of new form and organisation, where previously it was lacking.

As used herein, the term “rooting” refers to the process to obtain complete plants, such as shoots with developed roots.

As used herein the term “shoots” refers to plant leaves and stems capable of photosynthesis and usually denotes the presence of at least one shoot meristem.

A method of producing guaianolides

The present invention provides methods for producing guaianolides. Said methods may comprise *in vitro* propagation of plant tissue of a plant of the subfamily *Apioideae,* e.g. a plant of the genus *Thapsia* or the genus *Laser*. In particular the invention provides methods of producing guaianolides, wherein the methods comprise the steps of

a) providing plant tissue, which is in the form of embryogenic callus and/or shoots, said tissue being derived from a plant of the subfamily *Apioideae,* which for example may be any of the plant tissues described herein below in the section “Plant tissue derived from a plant of the subfamily *Apioideae*”

b) culturing said embryogenic callus and/or shoots in a basal medium B which for example may be performed in any of the manners described herein below in the section “Cultivation in basal medium B”, thereby inducing plant shoot micropropagation;

d) optionally isolating produced guaianolides, which for example may be done in any of the manners described herein below in the section “Purification of guaianolides”.

In addition to the steps outlined above, the method may also comprise additional steps. In particular, the methods may comprise an additional step performed after step b., but prior to step d. This step may be referred to as step c), which may comprise or consist of steps c1) and/or c2). Said step c1) may comprise culturing the plant shoots generated in step b) on or in a basal medium C, which for example may be performed in any of the manners described herein below in the section "Cultivation in basal medium C", thereby inducing root formation. Said step c2) may comprise culturing the plant shoots generated in step b) on or in a basal medium D, E and/or F, which for example may be performed in any of the manners described herein below in the section "Cultivation in basal medium D", "Cultivation in basal medium E" or "Cultivation in basal medium F", thereby increasing the production of secondary metabolites such as guaianolides. Said step c) may comprise steps c1) and c2), i.e. culturing the plant shoots generated in step b) on or in a basal medium C and/on or in a basal medium D, E and/or F, as described in any of the aforementioned sections.

The embryogenic callus and/or shoots provided in step a) may be obtained by culturing a plant tissue of a plant of the sub-family *Apioideae* on a basal medium A containing one or more plant growth regulators (PGRs) in any of the manners described herein below in the section "Cultivation on basal medium A".

Alternatively, the embryogenic callus and/or shoots provided in step a) may be shoots obtained after cultivation in basal medium B. Thus, after microprogation in basal medium B, all or a fraction of the shoots obtained may be sub-cultured in fresh basal medium B to obtain even further plant shoots.

Originally, the embryonic callus and/or shoot are typically obtained by cultivation of plant tissue (e.g. an explant) of a plant of the sub-family *Apioideae* on basal medium A, however once obtained, embryogenic callus and/or shoots may be micropropagated in basal medium B for many generations.

When cultivated as described herein in basal medium B, the plant shoot obtained by micropropagation produces high levels of guaianolides, including thapsigargin backbone guaianolides, such as thapsigargin. Thus, preferably the plant shoots obtained by the micropropagation of step b) comprises at least 0.1 mg, preferably at least 0.5 mg, more preferably at least 1 mg, for example at least 5 mg, such as at least 10 mg, for example in the

range of 10-15 mg guaianolides per g dry weight plant shoot. In particular, the plant shoots obtained by the micropropagation of step b) may comprises at least 0.1 mg, more preferably at least 1 mg, for example at least 5 mg, for example in the range of 5-15 mg thapsigargin per g dry weight plant shoot. The high yield is obtained by cultivating differentiated material (i.e. embryogenic callus and/or shoots) in a temporary immersion bioreactor (TIB). In contrast to pure non-embryogenic callus (as e.g. described in international patent application WO/2015/082978), then embryogenic callus and/or shoots comprises high levels of guaianolides, and the present invention provide means for cultivation of said embryogenic callus and/or shoots in an efficient and industrially relevant manner in a TIB.

Thus, guaianolides (e.g. thapsigargin or other thapsigargin backbone guaianolides) may be purified from the plant shoots obtained by the micropropagation of step b).

When cultivated as described herein in basal medium B and basal medium C, the plants obtained produces high levels of guaianolides, including thapsigargin backbone guaianolides. Thus, preferably the plants obtained after the induction of root formation of step c) comprise at least 1 mg, preferably at least 2 mg, more preferably at least 3 mg more preferably at least 5 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg guaianolides per g dry weight plant. In particular, the plants obtained after step c) may comprise at least 1 mg, more preferably at least 3 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg thapsigargin per g dry weight plant.

Thus, guaianolides (e.g. thapsigargin backbone guaianolides, thapsigargin or nortrilobolid) may be purified from the plant shoots obtained after induction of root formation in step c).

When cultivated as described herein in basal medium B and basal medium D, E and/or F, the plants obtained produces high levels of guaianolides, including thapsigargin backbone guaianolides. Thus, preferably the plants obtained after enhancing secondary metabolite production of step c) comprise at least 1 mg, preferably at least 2 mg, more preferably at least 3 mg more preferably at least 5 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg guaianolides per g dry weight plant. In particular, the plants obtained after step c) may comprise at least 1 mg, more preferably at least 3 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg thapsigargin per g dry weight plant.

Thus, guaianolides (e.g. thapsigargin backbone guaianolides, thapsigargin or nortrilobolid) may be purified from the plant shoots obtained after enhancing secondary metabolite production in step c).

When cultivated as described herein in basal medium B and basal medium C and basal medium D, E and/or F, the plants obtained produces high levels of guaianolides, including thapsigargin backbone guaianolides. Thus, preferably the plants obtained after the induction of root formation and enhancing secondary metabolite production of step c) comprise at least 1 mg, preferably at least 2 mg, more preferably at least 3 mg more preferably at least 5 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg guaianolides per g dry weight plant. In particular, the plants obtained after step c) may comprise at least 1 mg, more preferably at least 3 mg, yet more preferably at least 10 mg, for example in the range of 10-15 mg thapsigargin per g dry weight plant.

Thus, guaianolides (e.g. thapsigargin backbone guaianolides, thapsigargin or nortrilobolid) may be purified from the plant shoots obtained after induction of root formation and enhancing secondary metabolite production in step c).

In some embodiments, the method comprises step b) as described above, and step c) as described above. In some embodiments, step c) comprises or consists of step c1). In some embodiments, step c) comprises or consists of step c2). In some embodiments, step c) comprises or consists of step c1) and step c2).

For all of these embodiments, the method may additionally comprise a step prior to step a), comprising culturing plant tissue in basal medium A as described in the section “Cultivation on basal medium A. Step a) is typically useful for establishing or starting a new culture, and can usually be dispensed with once the culture has been initiated for a given plant.

Plant tissue derived from a plant of the subfamily *Apioideae*

The methods of the invention comprises the step of providing plant tissue derived from a plant of the sub-family *Apioideae.* Said plant may for example be a plant of the genus *Thapsia* or said plant may for example be a plant of the genus *Laser.*

The methods comprises cultivating plant tissue, which is embryogenic callus and/or shoot in basal medium B. Said embryogenic callus and/or shoot may originally be obtained from plant tissue taken directly from a plant of the sub-family *Apioideae.* Said plant tissue may be from any useful part of a plant of the sub-family *Apioideae*, e.g. it may be parts of leaves, roots, seeds, flowers, stems or buds. The plant tissue taken directly from a plant of the sub-family *Apioideae* to be used as starting material for generating embryogenic callus and/or shoots may also be referred to as "explant".

In one embodiment of the invention, said plant tissue is part of leaves of a plant of the sub-family *Apioideae,* e.g. of the genus *Thapsia* or the genus *Laser*.

The plant from which the plant tissue is obtained may have been propagated by any useful method, e.g. it may have been propagated in the field, in nature or by *in vitro* propagation, e.g. by cultivation in basal medium B and/or basal medium C and/or basal medium D, E and/or F described below.

The plant of the sub-family *Apioideae* may for example be a plant of the genus *Thapsia* or the genus *Laser*. The plant of the genus *Thapsia* may be any plant of said genus. For example said plant of the genus *Thapsia* may be selected from the group consisting of: *T. leucotricha, T. tenuifolia, T.garganica, T. gymnesica, T. transtagana, T. thapsioides, T. gummifera, T. smittii, T. asclepium, T. scabra, T. maxima, T. villosa, T. minor and T. laciniata.*

The plant of the genus *Laser* may be any plant of said genus. For example said plant of the genus *Laser* may be selected from the group consisting of: *L. trilobum, L.siler, L. aquilegifolium, Laser divaricatum, Laser rechingeri* and *L. cordifolium.*

The plant from which the plant tissue is obtained may be a wild-type plant or a genetically modified plant. While wild-type plants can be used in the present methods to produce guaianolides (e.g. thapsigargin backbone guaianolides, thapsigargin or nortrilobolid), genetically modified plants may be designed as is known in the art to further increase the yields, e.g by increasing biomass or increasing guaianolide levels. Methods of genetically modifying plants are known in the art and include random mutagenesis and subsequent selection of plants with the desired characteristics, nuclear transformation, chloroplast transformation. The plants can also be modified as is known in the art so that production of guaianolides (e.g. thapsigargin backbone guaianolides, thapsigargin or nortrilobolid) is transient.

Cultivation on basal medium A

The invention relates to methods involving culturing embryogenic callus and/or shoots in basal medium B (see herein below). Said embryogenic callus and/or shoots may be obtained by culturing plant tissue taken directly from a plant of the sub-family *Apioideae,* e.g. by culturing an explant of a plant of the sub-family *Apioideae* in basal medium A.

The basal medium A is a medium capable of supporting growth of a plant. The basal medium A comprises one or more plant growth regulators (PGRs). In some embodiments the PGRs present together are capable of inducing embryogenic callus and/or shoot formation. Thus, cultivation on the basal medium A preferably results in formation of embryogenic callus and/or shoot formation.

In one embodiment the basal medium A comprises at least one plant growth regulator which is an auxin. Preferably, the auxin is capable of inducing differentiation.

It is preferred that the basal medium A comprises the plant growth regulator 2,4-Dichlorophenoxyacetic acid (2,4-D). Typically, the concentration of 2,4-D in the first basal medium may be in the range of 0.01-3.0 mg l^{-1}, for example in the range of 0.05 – 1 mg l^{-1}, such as approximately 0.1 mg l^{-1}.

In one embodiment the basal medium A comprises a plant growth regulator, which is a cytokinin. Said cytokinin may for example be selected from the group consisting of phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ) and 6-benzylaminopurine (BAP).

It is also comprised in the invention that the basal medium A may comprise the plant growth regulator phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ) or 6-benzylaminopurine (BAP). Typically, the concentration of TDZ or BAP in the basal medium A may be in the range of 0.1-5.0 mg l^{-1}, for example in the range of 0.1 to 2 mg l^{-1}, such as in the range of 0.1-1 mg l^{-1}.

In addition to the PGRs, the basal medium A in general also comprises additional components promoting plant growth and/or viability. Thus, the basal medium A may contain typical ingredients of plant basal media. Frequently, the basal medium A may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium A may

also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium A may also further comprise one or more organic nitrogen sources, including any of the organic nitrogen sources described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium A may also further comprise one or more carbon sources, including any of the carbon sources described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". Media for cultivation of plant material are known in the art. Thus, the basal medium A may be a conventional plant growth medium, such as MS further comprising sugar and the PGRs described above.

The basal medium A may be in solid of semisolid phase, i.e. it may be a gel. In embodiments of the invention where the basal medium A is a gel, then the basal medium A may also comprise a gelling agent, e.g. agar. For example, the basal medium A may comprise in the range of 1 to 50 g l^{-1}, preferably in the range of 1 to 25 g l^{-1}, such as in the range of 1 to 15 g l^{-1}, for example in the range of 3 to 13 g l^{-1}, such as in the range of 5 to 9 g l^{-1}, for example approximately 7 g l^{-1}, such as 7 g l^{-1} gelling agent, e.g. agar.

The pH of the basal medium A may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation on the basal medium A is preferably performed for a time period sufficient to allow embryogenic callus and/or shoot formation. Typically, this may require several days, thus the cultivation may be for at least 1 week, preferably for at least 1 month, more preferably at least two months, for example in the range of 2 to 5 months, such as for approximately 3 months.

Cultivation on the basal medium A may be at any useful temperature allowing growth and/or differentiation of the plant tissue. Typically, the temperature may be room temperature, e.g. a temperature in the range of 15 to 30°C, such as in the range of 20 to 26°C, for example in the range of 22 to 24°C.

The cultivation on the basal medium A is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the

range of 4 to 16 hours dark periods. Thus, cultivation on basal medium A may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day. Exposure to light may aid in differentiation into embryogenic callus and/or shoots.

Cultivation in basal medium B

The methods of the invention may comprise cultivation of embryogenic callus, and/or shoots in a basal medium B. Said embryogenic callus and/or shoots may be obtained by cultivation of a plant tissue obtained from a plant of the genus *Thapsia* on a basal medium A as described above. Thus, the new embryogenic callus and/or shoots obtained from cultivation on the basal medium A, may be cultured in a basal medium B in an amount sufficient to ensure shoots micropropagation. The embryogenic callus and/or shoots are in general organogenic plant material.

Alternatively, the embryogenic callus and/or shoots may be derived from a culture of embryogenic callus and/or shoots in basal medium B, in which case the step of cultivation in basal medium B also could be regarded as a subcultivation.

Basal medium B is capable of supporting plant shoot micropropagation. The basal medium B comprises at least one auxin and at least one cytokinin. The at least one auxin and at least one cytokinin are preferably together capable of promoting direct or indirect shoot formation. Thus, cultivation on or in the basal medium B preferably results in plant shoot micropropagation.

Cytokinins are a group of chemicals that primarily influence cell division and shoot formation but also have roles in delaying cell senescence, are responsible for mediating auxin transport throughout the plant, and affect internodal length and leaf growth.

Cytokinins comprise a class of growth regulators, they particulary stimulate protein synthesis and participate in cell cycle control. It is perhaps for this reason that they can promote the maturation of chloroplasts and delay the senescence of detached leaves. The effect of cytokinins is most noticeable in tissue cultures where they are used, often together with auxins, to stimulate cell division and control morphogenesis. Added to shoot culture media, these compounds overcome apical dominance and release lateral bud from dormancy. The cytokinin may be any natural or artificial cytokinin, for example a cytokinin belonging to the

adenine-type or the phenylurea-type. Preferably, the cytokinin is selected from the group consisting of kinetin, zeatin, 6-benzylaminopurine (BAP), diphenylurea (Ph_2Urea), thidiazuron (TDZ), and isopentiladenina (2-Ip). The cytokin may also be a derivative of any of the aforementioned, which has cytokinin activity

It is comprised within the invention that the basal medium B comprises the cytokinin 6-benzylaminopurine (BAP). Typically, the concentration of BAP in the basal medium B is in the range of 0.1-5.0 mg l^{-1}, for example in the range of 1 to 2 mg l^{-1}, such as approximately 1.5 mg/L.

Auxins are compounds that positively influence cell enlargement, bud formation and root initiation. They also promote the production of other hormones and in conjunction with cytokinins, they control the growth of stems, roots, fruits and convert stems into flowers.

Said auxin may be any useful auxin, such as the naturally occurring auxins. For example, the auxin may be selected from the group consisting of 4-chloro-indoleacetic acid (4-CPA), phenylacetic acid (PAA), indole-3-butyric acid (IBA), chloroindole-3-acetic acid (Cl-IAA) and indole-3-acetic acid (IAA). The auxin may also be a synthetic auxin, e.g. an auxin selected from the group consisting of 1-naphthaleneacetic acid (NAA), 3,6-dichloroanisic acid (dicamba), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D).

It is also comprised in the invention that the basal medium B may comprise the auxin 1-naphthaleneacetic acid (NAA). Typically, the concentration of NAA in the basal medium B is in the range of 0.01-3.0 mg l^{-1}, for example in the range of 0.1-1 mg l^{-1}.

In addition to the cytokinin(s) and auxin(s), the basal medium B in general also comprises additional components promoting plant shoot formation and micropropagation. Thus, the basal medium B may contain typical ingredients of plant basal media. Frequently, the basal medium B may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium B may also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium B may also further comprise one or more organic nitrogen sources, including any of the organic nitrogen sources described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium B

may also further comprise one or more carbon sources, including any of the carbon sources described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". Media for cultivation of plant material are known in the art. Thus, the basal medium B may be a conventional plant growth medium, such as MS further comprising sugar and the cytokinin(s) and auxin(s) described above.

The pH of the basal medium B may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation in basal medium B is generally performed using a method involving temporary immersion in basal medium B. Thus, the cultivation in basal medium B may be performed using an immersion bioreactor, e.g. a temporary immersion bioreactor. Temporary immersion bioreactors are also known as temporary liquid immersion culture systems.

The use of temporary liquid immersion culture systems (e.g. temporary immersion bioreactors or TIBs) is known, for example from Etienne & Berthouly (2002) Plant Cell, Tissue and Organ Culture 69, 215-231 , Hanhineva & Karenlampi (2007) BMC Biotechnology 7, 11-23, and also from Ducos et al (2007) In Vitro Cellular & Developmental Biology - Plant 43: 652-659 and any of these systems may be used with the invention except that basal medium B should be used.

The temporary immersion bioreactor may comprise at least two vessels, one containing the embryogenic callus and/or the plant shoots and one for the liquid basal medium B, wherein the vessels are coupled together in a manner allowing the flow of the liquid media from one vessel to the other. This flux may for example be driven by a pump or by gravity through a see-saw movement. The temporary immersion bioreactor will ensure alternation between immersion of the embryogenic callus and/or shoots in the basal medium B and leaving the embryogenic callus and/or shoots without medium. Typically, the embryogenic callus and/or shoots are immersed in sufficient amounts of basal medium B to completely cover the embryogenic callus and/or shoots. The immersion may take place every 2 to 10 hours, e.g. every 4 to 8 hours, e.g. approximately every 6 hour, and the immersion may last for in the range of 1 to 15 min., such as in the range of 2 to 10 mins, for example for in the range of 2 to 5 min, such as for approximately 3 min.

When subcultivating plant material a fraction of plant shoots are transferred to fresh basal medium B. Said plant shoots may have been manipulated to remove dead tissue or to separate individual shoots. This can be done manually or automatically.

Cultivation in basal medium B is preferably performed for a time period sufficient to allow sufficient plant shoot micropropagation. For example the incubation may be for a time sufficient to obtain at least 2x, preferably at least 3x, more preferably at least 4x, such as at least 5x, for example approximately 5x increase in biomass. Typically, this may require several days, thus the cultivation may be for at least 1 week, preferably for at least 2 weeks, for example at least 3 weeks, such as for approximately 3 weeks.

Cultivation in the basal medium B may be at any useful temperature allowing micropropagation of plant shoots. Typically, the temperature may be in the range of 15 to 30°C, such as in the range of 20 to 28°C, for example in the range of 22 to 26°C, such as at approximately 24°C.

The cultivation in the basal medium B is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the range of 4 to 16 hours dark periods. Thus, cultivation on basal medium B may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day. Exposure to light may aid shoot micropropagation.

Cultivation on basal medium C

The methods of the invention may comprise cultivation of plant shoots on or in a basal medium C., e.g. cultivation of the shoots obtained after micropropagation in basal medium B.

Thus, subsequent to step b) of micropropagation of plant shoots in basal medium B, the methods of the invention may comprise a step c), which may comprise or consist of step c1) of cultivating the shoots obtained in step b) in basal medium C, Preferably cultivation in basal medium C results in root formation.

Cultivation in basal medium C may involve temporary immersion of the plant shoots in basal medium C, Alternatively, the cultivation may be performed on basal medium C in gel or liquid

form. In this case, individual single shoots may be transferred to basal medium C and incubated to allow root formation.

The basal medium C is capable of supporting growth of plants and comprises at least one auxin. Preferably, the auxin is capable of inducing formation of roots. For example, the basal medium C may comprise one auxin, which is capable if inducing formation of roots. Thus, cultivation on the basal medium C preferably results in formation of roots.

It is preferred that the basal medium C comprises one or more auxins selected from the group consisting of 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA). In particular, basal medium C may comprise an auxin selected from the group consisting of NAA, IAA and IBA.

Thus, the basal medium C may comprise IBA. Typically, the concentration of IBA in the basal medium C may be in the range of 0.1-8.0 mg l^{-1}, for example in the range of 1 to 5 mg l^{-1}.

The basal medium C may comprise NAA. Typically, the concentration of NAA in the basal medium C may be in the range of 0.1-8.0 mg l^{-1}, for example in the range of 1 to 5 mg l^{-1}.

The basal medium C may comprise IAA. Typically, the concentration of IAA in the basal medium C may be in the range of 0.1-8.0 mg l^{-1}, for example in the range of 1 to 5 mg l^{-1}.

In addition to the auxins, the basal medium C in general also comprises additional components promoting plant growth and/or viability. Thus, the basal medium C may contain typical ingredients of plant basal media. Frequently, the basal medium C may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium C may also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium C may also further comprise one or more amino acid(s), including any of the amino acid(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium C may also further comprise one or more carbon sources, including any of the carbon sources described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. Frequently, the basal medium C comprises a lower level of mineral salts than basal medium A and basal medium B. The low

level of mineral salts may aid in induction of root formation. Media for cultivation of plant material are known in the art. Thus, the basal medium C may be a conventional plant growth medium further comprising an auxin as described above. Since the basal medium C frequently contains low levels of mineral salts, the basal medium C may also be a diluted conventional plant growth medium, such as diluted MS, e.g. 40-60% MS in water, further comprising sugar and an auxin as described above.

The basal medium C may be in solid or semisolid phase, i.e. it may be a gel. In embodiments of the invention where the basal medium C is a gel, then the basal medium C may also comprise a gelling agent, e.g. agar. For example, the basal medium C may comprise in the range of 1 to 50 g l^{-1}, preferably in the range of 1 to 25 g l^{-1}, such as in the range of 1 to 15 g l^{-1}, for example in the range of 3 to 13 g l^{-1}, such as in the range of 5 to 9 g l^{-1}, for example approximately 7 g l^{-1}, such as 7 g l^{-1} gelling agent, e.g. agar.

Cultivation in basal medium C may also be performed using a method involving temporary immersion in basal medium C. Thus, the cultivation in basal medium C may be performed using an immersion bioreactor, e.g. a temporary immersion bioreactor. Temporary immersion bioreactors are also known as temporary liquid immersion culture systems. Any of the methods for temporary immersion described herein above in the section “Cultivation in basal medium B” may also be employed for cultivation involving temporary immersion in basal medium C.

Thus, typically, the embryogenic callus and/or shoots are immersed in sufficient amounts of basal medium C to completely cover the embryogenic callus and/or shoots. The immersion may take place every 2 to 10 hours, e.g. every 4 to 8 hours, e.g. approximately every 6 hour, and the immersion may last for in the range of 1 to 15 min., such as in the range of 2 to 10 mins, for example for in the range of 2 to 5 min, such as for approximately 3 min.

The pH of the basal medium C may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation on the basal medium C is preferably performed for a time period sufficient to allow root formation. Typically, this may require several days, thus the cultivation may be for at least 1 week, preferably for at least 2 weeks, more preferably at least 1 month, such as for approximately 1 month.

Cultivation in the basal medium C may be at any useful temperature allowing micropropagation of plant shoots. Typically, the temperature may be in the range of 15 to 30°C, such as in the range of 20 to 28°C, for example in the range of 22 to 26°C, such as at approximately 24°C.

The cultivation in the basal medium C is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the range of 4 to 16 hours dark periods. Thus, cultivation on basal medium C may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day.

Cultivation on basal medium D

The methods of the invention may comprise cultivation of plant shoots on or in a basal medium D., e.g. cultivation of the shoots obtained after micropropagation in basal medium B.

Thus, subsequent to step b) of micropropagation of plant shoots in basal medium B, the methods of the invention may comprise a step c), which may comprise or consist of step c2) of cultivating the shoots obtained in step b) in basal medium D. Preferably cultivation in basal medium D results in increased production of thapsigargins.

Cultivation in basal medium D may involve temporary immersion of the plant shoots in basal medium D. The basal medium D composition is based on “basal medium B” further comprising an elicitor.

Elicitors are compounds that stimulate any type of plant defense, thereby promoting secondary metabolism. These secondary metabolites include terpenes, steroids, phenolics and alkaloids.

Elicitors, abiotic or biotic include: $AgNO_3$, $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, KCl, $MgSO_4$, $NiSO_4$, $VOSO_4$, Zn, microbial enzymes, bacterial lysates and polysaccharides from microorganism cell walls, polysaccharides arising from pathogen degradation of the plant cell wall, intracellular proteins, and small molecules synthesized by the plant including plant hormones such as jasmonates or salicylic acid.

The basal medium D is capable of promoting production of secondary metabolites such as thapsigargins. In addition to at least one auxin and at least one cytokinin (described in the section "Cultivation on basal medium B"), the basal medium D also comprises an elicitor belonging to the abiotic or biotic types. As described in aforementioned section, the at least one auxin and at least one cytokinin are preferably together capable of promoting direct or indirect shoot formation

Preferably, the elicitor is selected from the biotic group: microbial enzymes, bacterial lysates, polysaccharides from microorganism cell walls, polysaccharides arising from pathogen degradation of the plant cell wall, intracellular proteins, and small molecules synthesized by the plant including plant hormones such as ujasmonates or salicylic acid.

Jasmonate and its derivatives, commonly referred to as jasmonates, are lipid-based hormones that regulate a wide range of processes in plants, ranging from growth and photosynthesis to reproductive development. Jasmonates are oxylipins, i.e. derivatives of oxygenated fatty acids. Jasmonate itself can be further metabolized into active or inactive derivatives. Methyl jasmonate (MeJA or MeJ) is a volatile compound. Jasmonate conjugated with isoleucine results in JA-Ile. Decarboxylation of jasmonate yields cis-jasmone.

In some embodiments, the elicitor is a jasmonate. In particular, the jasmonate may be selected from the group of: jasmonate, methyl jasmonate (MeJ), JA-Ile and cis-jasmone. In one embodiment, the elicitor is methyl jasmonate.

In some embodiments, the concentration of MeJ in the basal medium D is in the range of 1-1000 µM, for example in the range of 10 to 600 µM, such as approximately 400 µM.

The basal medium D may contain typical ingredients of plant basal media. Frequently, the basal medium D may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium D may also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium D may also further comprise one or more amino acid(s), including any of the amino acid(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium D may also further

comprise one or more carbon sources, including any of the carbon sources described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”.

The basal medium D may be in solid or semi-solid phase, i.e. it may be in the form of a gel. In embodiments of the invention where the basal medium D is a gel, the basal medium D may also comprise a gelling agent, e.g. agar. For example, the basal medium D may comprise in the range of 1 to 50 g/L, preferably in the range of 1 to 25 g/L, such as in the range of 1 to 15 g/L, for example in the range of 3 to 13 g/L, such as in the range of 5 to 9 g/L, for example approximately 7 g/L gelling agent, e.g. agar.

Cultivation in basal medium D may also be performed using a method involving temporary immersion in basal medium D. Thus, the cultivation in basal medium D may be performed using an immersion bioreactor, e.g. a temporary immersion bioreactor (TIB). Temporary immersion bioreactors are also known as temporary liquid immersion culture systems. Any of the methods for temporary immersion described herein above in the section “Cultivation in basal medium B” may also be employed for cultivation involving temporary immersion in basal medium D.

In some embodiments, the shoots are immersed in sufficient amounts of basal medium D to completely cover the shoots. The immersion may take place every 2 to 10 hours, e.g. every 4 to 8 hours, e.g. approximately every 6 hour, and the immersion may last for in the range of 1 to 15 min, such as in the range of 5 to 10 min, for example for in the range of 2 to 5 min, such as for approximately 3 min.

The pH of the basal medium D may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation on the basal medium D is preferably performed for a time period sufficient to increase thapsigargin production. In some embodiments, this may require several days, thus the cultivation may be for at least 5 days, preferably for at least 10 days, more preferably at least 15 days, even more preferably at least 18 days, such as for approximately 18 days.

Cultivation in the basal medium D may be at any useful temperature allowing micropropagation of plant shoots and increase of thapsigarginproduction. Typically, the temperature may be in the

range of 15 to 30°C, such as in the range of 20 to 28°C, for example in the range of 22 to 26°C, such as at approximately 24°C.

The cultivation in the basal medium D is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the range of 4 to 16 hours dark periods. Thus, cultivation on basal medium D may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day.

Cultivation on basal medium E

The methods of the invention may comprise cultivation of plant shoots on or in a basal medium E, e.g. cultivation of the shoots obtained after micropropagation in basal medium B.

Thus, subsequent to step b) of micropropagation of plant shoots in basal medium B, the methods of the invention may comprise a step c) comprising or consisting of step c2) of cultivating the shoots obtained in step b) in basal medium E, Preferably cultivation in basal medium E results in increased production of thapsigargins.

Cultivation in basal medium E may involve temporary immersion of the plant shoots in basal medium E. Alternatively, the cultivation may be performed on basal medium E in gel or liquid form. In this case, individual single shoots may be transferred to basal medium E and incubated to allow increased production of thapsigargins.

The basal medium E is preferably devoid of elicitors such as hormones and is thus capable of increasing thapsigargin production without any elicitor or hormone in the medium.

The basal medium E in general comprises additional components promoting plant viability and thapsigargin production. Thus, the basal medium E may contain typical ingredients of plant basal media. Frequently, the basal medium E may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium E may also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium E may

also further comprise one or more amino acid(s), including any of the amino acid(s) described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”. The basal medium E may also further comprise one or more carbon sources, including any of the carbon sources described herein below in the section “Mineral salt, vitamin, organic nitrogen source and carbon source”.

The basal medium E, similar to basal medium C, comprises a lower level of mineral salts than basal medium A, B and basal medium D. Without being bound by theory, it is hypothesized that the low level of mineral salts may aid in increasing thapsigargin production.

Since the basal medium E frequently contains low levels of mineral salts, the basal medium E may also be a diluted conventional plant growth medium, such as diluted MS, e.g. 40-60% MS in water.

The basal medium E may be in solid or semi-solid phase, i.e. it may be in the form of a gel. In embodiments of the invention where the basal medium E is a gel, then the basal medium E may also comprise a gelling agent, e.g. agar. For example, the basal medium E may comprise in the range of 1 to 50 g/L, preferably in the range of 1 to 25 g/L, such as in the range of 1 to 15 g/L, for example in the range of 3 to 13 g/L, such as in the range of 5 to 9 g/L, for example approximately 7 g/L, such as 7 g/L gelling agent, e.g. agar.

Cultivation in basal medium E may also be performed using a method involving temporary immersion in basal medium E. Thus, the cultivation in basal medium E may be performed using an immersion bioreactor, e.g. a temporary immersion bioreactor. Temporary immersion bioreactors are also known as temporary liquid immersion culture systems. Any of the methods for temporary immersion described herein above in the section “Cultivation in basal medium B” may also be employed for cultivation involving temporary immersion in basal medium E.

In some embodiments, the shoots are immersed in sufficient amounts of basal medium E to completely cover the shoots. The immersion may take place every 2 to 10 hours, e.g. every 4 to 8 hours, e.g. approximately every 6 hour, and the immersion may last for in the range of 1 to 15 min., such as in the range of 5 to 10 min, for example for in the range of 2 to 5 min, such as for approximately 3 min.

The pH of the basal medium E may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation on the basal medium E is preferably performed for a time period sufficient to allow high amounts of thapsigargins. In some embodiments, this may require several days, thus the cultivation may be for at least 5 days, preferably for at least 10 days, more preferably at least 15 days, even more preferably at least 18 days, such as for approximately 18 days.

Cultivation in the basal medium E may be at any useful temperature allowing viability of plant shoots and high thapsigargin production. Typically, the temperature may be in the range of 15 to 30°C, such as in the range of 20 to 28°C, for example in the range of 22 to 26°C, such as at approximately 24°C.

The cultivation in the basal medium E is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the range of 4 to 16 hours dark periods. Thus, cultivation on basal medium E may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day.

Cultivation on basal medium F

The methods of the invention may comprise cultivation of plant shoots on or in a basal medium F, e.g. cultivation of the shoots obtained after micropropagation in basal medium B.

Thus, subsequent to step b) of micropropagation of plant shoots in basal medium B, the methods of the invention may comprise a step c) comprising or consisting of step c2) of cultivating the shoots obtained in step b) in basal medium F. Preferably cultivation in basal medium F results in increased production of thapsigargins.

Cultivation in basal medium F may involve temporary immersion of the plant shoots in basal medium F, Alternatively, the cultivation may be performed on basal medium F in gel or liquid form. In this case, individual single shoots may be transferred to basal medium F and incubated to allow increased production of thapsigargins.

The basal medium F is capable of increasing thapsigargin production and comprises at least one elicitor. Preferably, the elicitor is capable of promoting secondary metabolism, especially thapsigargins. As shown in the examples, in the hands of the inventors cultivation on basal medium F resulted in the highest production of thapsigargins.

Basal medium F comprises an elicitor. Elicitors are compounds that stimulate any type of plant defense, thereby promoting secondary metabolism. These secondary metabolites include terpenes, steroids, phenolics and alkaloids.

Elicitors, abiotic or biotic include: $AgNO_3$, $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, KCl, $MgSO_4$, $NiSO_4$, $VOSO_4$, Zn, microbial enzymes, bacterial lysates and polysaccharides from microorganism cell walls, polysaccharides arising from pathogen degradation of the plant cell wall, intracellular proteins, and small molecules synthesized by the plant including plant hormones such as jasmonates or salicylic acid.

The basal medium F is capable of promoting production of secondary metabolites such as thapsigargins. The basal medium F also comprises an elicitor belonging to the abiotic or biotic types.

Preferably, the elicitor is selected from the biotic group: microbial enzymes, bacterial lysates, polysaccharides from microorganism cell walls, polysaccharides arising from pathogen degradation of the plant cell wall, intracellular proteins, and small molecules synthesized by the plant including plant hormones such as ujasmonates or salicylic acid.

Jasmonate and its derivatives, commonly referred to as jasmonates, are lipid-based hormones that regulate a wide range of processes in plants, ranging from growth and photosynthesis to reproductive development. Jasmonates are oxylipins, i.e. derivatives of oxygenated fatty acids. Jasmonate itself can be further metabolized into active or inactive derivatives. Methyl jasmonate (MeJA or MeJ) is a volatile compound. Jasmonate conjugated with isoleucine results in JA-Ile. Decarboxylation of jasmonate yields cis-jasmone.

In some embodiments, the elicitor is a jasmonate. In particular, the jasmonate may be selected from the group of: jasmonate, methyl jasmonate (MeJ), JA-Ile and cis-jasmone. In one embodiment, the elicitor is methyl jasmonate.

In some embodiments, the concentration of MeJ in the basal medium F is in the range of 1-1000 µM, for example in the range of 10 to 600 µM, such as approximately 400 µM.

The basal medium F in general comprises additional components promoting plant viability and thapsigargin production. Thus, the basal medium F may contain typical ingredients of plant basal media. Frequently, the basal medium F may comprise one or more mineral salt(s), including any of the mineral salt(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium F may also further comprise one or more vitamin(s), including any of the vitamin(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium F may also further comprise one or more amino acid(s), including any of the amino acid(s) described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source". The basal medium F may also further comprise one or more carbon sources, including any of the carbon sources described herein below in the section "Mineral salt, vitamin, organic nitrogen source and carbon source".

The basal medium F, similar to basal medium C and E, comprises a lower level of mineral salts than basal medium A, B and basal medium D. Without being bound by theory, it is hypothesized that the low level of mineral salts may aid in increasing thapsigargin production.

Since the basal medium F frequently contains low levels of mineral salts, the basal medium F may also be a diluted conventional plant growth medium, such as diluted MS, e.g. 40-60% MS in water.

The basal medium F may be in solid or semi-solid phase, i.e. it may be in the form of a gel. In embodiments of the invention where the basal medium F is a gel, then the basal medium E may also comprise a gelling agent, e.g. agar. For example, the basal medium F may comprise in the range of 1 to 50 g/L, preferably in the range of 1 to 25 g/L, such as in the range of 1 to 15 g/L, for example in the range of 3 to 13 g/L, such as in the range of 5 to 9 g/L, for example approximately 7 g/L, such as 7 g/L gelling agent, e.g. agar.

Cultivation in basal medium F may also be performed using a method involving temporary immersion in basal medium F. Thus, the cultivation in basal medium F may be performed using an immersion bioreactor, e.g. a temporary immersion bioreactor. Temporary immersion

bioreactors are also known as temporary liquid immersion culture systems. Any of the methods for temporary immersion described herein above in the section “Cultivation in basal medium B” may also be employed for cultivation involving temporary immersion in basal medium F.

In some embodiments, the shoots are immersed in sufficient amounts of basal medium F to completely cover the shoots. The immersion may take place every 2 to 10 hours, e.g. every 4 to 8 hours, e.g. approximately every 6 hour, and the immersion may last for in the range of 1 to 15 min, such as in the range of 5 to 10 min, for example for in the range of 2 to 5 min, such as for approximately 3 min.

The pH of the basal medium F may be adjusted before cultivation, for example it may be adjusted to a pH in the range of 5 to 7, such as to a pH in the range of 5.5 to 6.5, for example to a pH of approximately 5.8.

Cultivation on the basal medium F is preferably performed for a time period sufficient to allow high amounts of thapsigargins. In some embodiments, this may require several days, thus the cultivation may be for at least 5 days, preferably for at least 10 days, more preferably at least 15 days, even more preferably at least 18 days, such as for approximately 18 days.

Cultivation in the basal medium F may be at any useful temperature allowing viability of plant shoots and high thapsigargin production. Typically, the temperature may be in the range of 15 to 30°C, such as in the range of 20 to 28°C, for example in the range of 22 to 26°C, such as at approximately 24°C.

The cultivation in the basal medium F is preferably performed at least partly under light, e.g. it may be performed under lamps providing a regulated photoperiod of light/darkness. Typically, the cultivation is performed under a light/darkness circle with alternating light periods and darkness periods, for example in the range of 8 to 20 hours light periods interrupted by in the range of 4 to 16 hours dark periods. Thus, cultivation on basal medium F may involve exposure to light for at least 8 hours, such as in the range of 8 to 20 hours, for example in the range of 12 to 18 hours per day.

Mineral salt, vitamin, organic nitrogen source and carbon source

As described above the basal medium A, B, C, D, E and F may comprise mineral salt(s), vitamin(s), organic nitrogen sources and/or carbon sources. Furthermore, the basal medium A, B, C, D, E and F may also comprise other compounds, such as organic compounds, hormones and elicitors.

The organic nitrogen source may be any organic compound comprising at least one N-atom. For example the nitrogen source may be one or more amino acids, e.g. glycine. Thus, the basal medium A, B, C, D, E and/or F may comprise in the range of 1 to 10 mg/L, such as in the range of 1 to 5 mg/L, for example in the range of 1 to 3 mg/L, such as approximately 2 mg/L glycine.

The carbon source may be any organic compound, however typically the carbon source is a sugar. Preferably the carbon source is sucrose. Thus, the basal medium A, B, C, D, E and/or F may comprise in the range of 2 to 5%, such as in the range of 2 to 4%, for example approximately 3% (w/w) sucrose.

The basal medium A, B, C, D, E and/or F may also comprise mineral salt(s) and/or vitamin(s). In general, the basal medium A, B and D may comprise more mineral salts than basal medium C, E or F for example basal medium C, E or F may comprise in the range of 40 to 60%, such as approximately 50% of the mineral salts of basal medium B.

Thus, the basal medium A and/or B and/or D may comprise $CoCl_2.6H_2O$, for example in the range of 0.01 to 0.1 mg/L, such as in the range of 0.01 to 0.05 mg/L, for example in the range of 0.02 to 0.03 mg/L, such as approximately 0.025 mg/L $CoCl_2.6H_2O$. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The basal medium A and/or B and/or D may comprise $CuSO_4.5\ H_2O$, for example in the range of 0.01 to 0.1 mg/L, such as in the range of 0.01 to 0.05 mg/L, for example in the range of 0.02 to 0.03 mg/L, such as approximately 0.025 mg/L $CuSO_4.5\ H_2O$. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise FeNaEDTA, for example in the range of 5 to 200 mg/L, such as in the range of 10 to 100 mg/L, for example in the range of 20 to 60 mg/L, such as approximately 37 mg/L FeNaEDTA. The

basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise H_3BO_3, for example in the range of 1 to 20 mg/L, such as in the range of 1 to 10 mg/L, for example in the range of 4 to 8 mg/L, such as approximately 6 mg/L H_3BO_3. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise KI, for example in the range of 0.1 to 10 mg/L, such as in the range of 0.1 to 5 mg/L, for example in the range of 0.5 to 2 mg/L, such as approximately 0.8 mg/L KI. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise $MnSO_4$ H_2O, for example in the range of 1 to 200 mg/L, such as in the range of 5 to 100 mg/L, for example in the range of 10 to 30 mg/L, such as approximately 17 mg/L $MnSO_4$ H_2O. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise $Na_2MoO_4.2$ H_2O, for example in the range of 0.1 to 1 mg/L, such as in the range of 0.1 to 0.5 mg/L, for example in the range of 0.2 to 0.3 mg/L, such as approximately 0.25 mg/L $Na_2MoO_4.2$ H_2O. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise $ZnSO_4.7$ H_2O, for example in the range of 1 to 20 mg/L, such as in the range of 3 to 15 mg/L, for example in the range of 5 to 10 mg/L, such as approximately 8.6 mg/L $ZnSO_4.7$ H_2O. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D and/or F may comprise $CaCl_2$, for example in the range of 50 to 2000 mg/L, such as in the range of 100 to 1000 mg/L,

for example in the range of 200 to 600 mg/L, such as approximately 332 mg/L $CaCl_2$. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D and/or F may comprise KH_2PO_4, for example in the range of 10 to 2000 mg/L, such as in the range of 50 to 1000 mg/L, for example in the range of 100 to 300 mg/L, such as approximately 170 mg/L KH_2PO_4. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise KNO_3, for example in the range of 100 to 20000 mg/L, such as in the range of 500 to 10000 mg/L, for example in the range of 1000 to 3000 mg/L, such as approximately 1900 mg/L KNO_3. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D and/or F may comprise $MgSO_4$, for example in the range of 10 to 2000 mg/L, such as in the range of 50 to 1000 mg/L, for example in the range of 100 to 300 mg/L, such as approximately 180 mg/L $MgSO_4$. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A and/or B and/or D may comprise NH_4NO_3, for example in the range of 100 to 20000 mg/L, such as in the range of 500 to 10000 mg/L, for example in the range of 1000 to 3000 mg/L, such as approximately 1650 mg/L NH_4NO_3. The basal medium C and/or E and/or F may comprise 40 to 60%, such as approximately 50% of aforementioned concentrations.

The first, second and/or third and/or basal medium A, B, C, D, E and/or F may comprise myo-Inositol, for example in the range of 10 to 500 mg/L, such as in the range of 30 to 300 mg/L, for example in the range of 50 to 200 mg/L, such as approximately 100 mg/L myo-Inositol.

The first, second and/or third and/or basal medium A, B, C, D, E and/or F may comprise Nicotinic acid, for example in the range of 0.05 to 5 mg/L, such as in the range of 0.1 to 2 mg/L, for example in the range of 0.2 to 0.8 mg/L, such as approximately 0.5 mg/L Nicotinic acid.

The first, second and/or third and/or basal medium A, B, C, D, E and/or F may comprise pyridoxine HCl, for example in the range of 0.05 to 5 mg/L, such as in the range of 0.1 to 2 mg/L, for example in the range of 0.2 to 0.8 mg/L, such as approximately 0.5 mg/L pyridoxine HCl.

The first, second and/or third and/or basal medium A, B, C, D, E and/or F may comprise Thiamine, for example in the range of 0.1 to 5 mg/L, such as in the range of 0.3 to 3 mg/L, for example in the range of 0.5 to 2 mg/L, such as approximately 1 mg/L Thiamine.

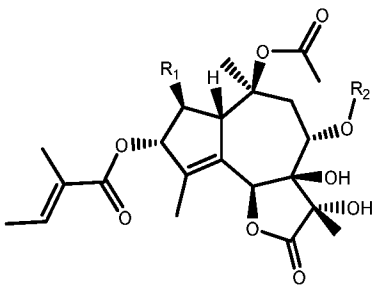
In specific embodiments the basal medium A, B, C, D, E and/or F do not comprise additional components apart from the components defined herein and water.

Guaianolides

The invention relates to methods for production of guaianolides. Said guaianolide may for example be a hexaoxygenated guaianolide or a pentaoxygenated guaianolide.

In particular, the guaianolide may be a guaianolide comprising a thapsigargin backbone. Such guaianolides are also referred to as thapsigargin backbone guaianolides herein.

In one embodiment, the guaianolide is a guaianolide comprising a core structure of formula I:



R_1 of formula I may for example be –H, –O-(C=O)-C_{2-10}-alkyl or –O-(C=O)-C_{2-10}-alkenyl. For example R_1 may be selected from the group consisting of –O-(C=O)-C_{2-10}-alkyl or –O-(C=O)-C_{2-10}-alkenyl. For example R_1 may be any of the R_1 groups listed in table 1 herein above.

R_2 of formula I may for example be –H, –(C=O)-C_{2-12}-alkyl or –(C=O)-C_{2-12}-alkenyl. For example R_2 may be –H, –(C=O)-C_{2-5}-alkyl or –(C=O)-C_{2-5}-alkenyl. For example R_2 may be any of the R_2 groups listed in table 1 herein above.

In particular, the guaianolide may be a thapsigargin backbone guaianolide. Thapsigargin backbone guaianolides comprises a core structure of formula I. In particular, the thapsigargin backbone guaianolide may be a compound of formula I, wherein

R_1 is –O-(C=O)-C_{2-10}-alkyl or –O-(C=O)-C_{2-10}-alkenyl, for example R_1 may be –O-(C=O)-C_{5-8}-alkyl; and

R_2 may for example be –H, –(C=O)-C_{2-12}-alkyl or –(C=O)-C_{2-12}-alkenyl, such as any of the R_2 groups listed in table 1 herein above

In one embodiment, the guaianolide may be a nortrilobolide backbone guaianolide. Nortrilobolide backbone guaianolides are compounds of formula I, wherein R_1 is –H, and

R_2 for example may be –H, –(C=O)-C_{2-12}-alkyl or –(C=O)-C_{2-12}-alkenyl, such as any of the R_2 groups listed in table 1 herein above

In one embodiment the guaianolide may be selected from the group of guaianolides mentioned in Table 1 herein above. For example, the guaianolide may be selected from the group consisting of thapsigargin, nortrilobolide, thapsivillosin I and thapsivillosin C.

In another embodiment the guaianolide may be selected from the group of guaianolides shown in figure 1.

In one embodiment the guaianolide is nortrilobolide, the structure of which is provided in figure 1.

In one preferred embodiment the guaianolide is thapsigargin. The structure of thapsigargin is provided in figure 1.

Purification of guaianolides

The guaianolides may be purified according to any useful method. For example, the purification may involve freeze drying the biomass, e.g. the plant shoots obtained in step b) and/or the plants obtained in step c), grinding the freeze dried material to a powder, followed by extraction with a solvent such as ethanol. The mixture may be centrifuged and the guaianolides purified from the supernatant by chromatography.

The methods of purification may also involve freezing involve lyophilising the biomass, e.g. the plant shoots obtained in step b) and/or the plants obtained in step c), homogenising the lyophilised biomass with a solvent in a bead mill, followed by centrifugation to obtain a crude extract and purification of the components of the crude extract for example as described in Ollivier A et al. 2013 Ollivier, A. 2013; J Chromatogr B Analyt Technol Biomed Life Sci.; 926: 6-20].

A method of producing a pharmaceutical composition or a prodrug

In one embodiment the invention relates to a method for producing a prodrug, said method comprising the steps of

i) preparing a guaianolide by any one of the methods described herein;
ii) attaching a peptide, which is cleavable by a PSA, an hK2 or PSMA protease to said guaianolide optionally via a linker
iii) thereby providing a prodrug.

The prodrug may thus comprise or consist of a guaianolide, a linker and a peptide. Said guaianolide may be any of the guaianolides described herein above in the section "Guaianolides", and in particular in may be thapsigargin.

The linker may for example be an alkyl, such as C_{1-12}-alkyl, for example C_{1-12} linear alkyl, such as linear octyl. The linker is preferably attached to the R_2 group of formula I. Thus, the prodrug may be a guaianolide of formula I, wherein the R_2 group is attached to a linker, and the linker is attached to said peptide. The linker may be attached to the guaianolide by substitution of R_2 with a substituent of the formula -–(C=O)-C_{8-12}-alkyl. Useful methods for preparing the prodrug starting from a guaianolide, e.g. thapsigargin are also described in US8772226.

The peptide of the present invention may be any peptide cleavable by a PSMA protein or

derivative thereof. In particular embodiments, the peptide may comprise the sequence Asp-Glu-Glu-Glu-Glu. In other embodiments, the peptide may comprise the sequence Asp-Glu.

Alternatively, the peptide of the present invention may be any peptide cleavable by a PSA protein or a derivative thereof. In particular embodiments the peptide may be any of the peptides having one of the following SEQ ID numbers in US 8772226: SEQ ID NO:42, SEQ ID NO:43,SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO: 55, or SEQ ID NO:56.

Alternatively, the peptide of the present invention may be any peptide cleavable by a hK2 protein or a derivative thereof. In particular embodiments the peptide may be any of the peptides having one of the following SEQ ID numbers in US 8772226: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 or SEQ ID NO:54.

In some embodiments, the peptide further comprises a capping group attached to the N-terminus of the peptide, wherein the capping group inhibits endopeptidase activity. In particular embodiments, the capping group is selected from the group consisting of acetyl, morpholinocarbonyl, benzyloxycarbonyl, glutaryl, and succinyl substituents.

The prodrug may be any of the prodrugs described in US8772226, such as any of the compounds described therein, In a preferred embodiment the prodrug is G-202, the structure of which is shown in figure 2.

EXAMPLES

The invention is further illustrated by the following examples, which however should not be construed as being limiting for the invention.

Example 1

Explant preparation and culture initiation

In this example, we used leaves explant that were harvested from a *Thapsia garganica* stock plant growing under greenhouse conditions (University of Copenhagen, Denmark). The leaves were washed to remove loose dirt and placed in 0,5% NaOCl for 15 min to thoroughly remove the surface decontamination agent, the plant material was washed three times with sterile water in a laminar flow. Leaflet explants were aseptically transferred to petri dishes (90 mm) containing 25 mL MS nutrient medium supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar. Leaflet explants were placed with de abaxial surface directly on the medium. A combination of auxins (0.01-3.0 mg l^{-1} 2,4-D, e.g. 0.1 mg l^{-1} 2,4-D) and cytokinins (0.1-5.0 mg l^{-1} TDZ or BAP, e.g. 0.1, 0.5 or 1 mg l^{-1} TDZ or BAP) were used to induce cellular differentiation. The PH of all media was adjusted to 5.8 before autoclaving at 121 ℃ and 103 kPa for 20 min. The explants were placed in a growth room (22-24 ℃) fitted with cool white fluorescent lamps automated to provide a photoperiod of 16h light (15 µmol $m^{-2}s^{-1}$) and 8 h darkness or 24 h darkness. These treatments had resulted in callus, embryos and shoots elicitation (Figure 5a and 5b).

Shoot multiplication

After 3 months in culture, *T.garganica* shoots, buds and callus that had regenerated from the leaflet explants (Figure 5a and 5b) were transferred to a micropropagation fresh medium containing MS nutrient medium supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar and a combination of auxins (0.5 mg/L NAA) and cytokinins (1,5 mg/L BAP) to induce shoots multiplication. All regenerated shoots (Figure 5c) derived directly or indirectly from explant were left to multiply and grow in culture vessels with solid media or in temporary immersion bioreactors (TIB) with liquid media to use them as a highly regenerative continuous system.

Plant shoots cultivated in TIBs can be harvested or subcultured after being cultivated in temporary immersion bioreactors (TIB) for 3 weeks with a 5-fold increase of biomass (FW) of about 500% (Figure 7 and 8). In order to subcultivate them, the plants clusters of shoots are separated into individual shoots Between 2-4 g of individual shoot bases are inoculated on TIBs with fresh liquid subculture medium containing 200 mL MS nutrient supplemented with 30 g l^{-1} sucrose and a combination of auxins (0.5 mg/L 1-Naphthaleneacetic acid (NAA)) and cytokinins (1.5 mg/L 6-Benzylaminopurine (BAP)) to induce multiplication (basal medium B) .

Rooting of shoots

Healthy single shoots (4 cm or more) were excised from multiple shoot clusters growing on MS medium supplemented with combination of 0,5 mg/L NAA and 1.5 mg/L BAP were transferred to half strength MS media containing 30 g l^{-1} , 7 g l^{-1} agar (PH= 5.8) and supplemented with 1 mg/L NAA, IBA or IAA (basal medium C) for a month. After 6 weeks roots were present in 65% of the shoots (Figure 6). After 4 weeks in the third basal media, the plants were transferred to a half strength MS media containing 30 g l^{-1} , 7 g l^{-1} agar (PH=5.8) (basal medium E) without hormones to remove any traces of auxine.

Table 3: Thapsigargin content in rooted plants

Basal medium C (IAA)	mg Thapsigargin/g DW	mg Nortrilobolid/g DW
Aerial part (shoots)	3,56±0,33	13,54±0,64
roots	0,77±0,22	8,27±0,69

Enhancement of thapsigargin production

Basal medium D

Healthy single shoots (4 cm or more) were excised from multiple shoot clusters growing on MS medium supplemented with combination of 0,5 mg/L NAA and 1.5 mg/L BAP (basal medium B) were transferred to MS media containing 30 g l-1 , 7 g l-1 agar (PH= 5.8) and supplemented with 0,5 mg/L NAA, 1.5 mg/L BAP and 400 µM MeJ (basal medium D) for 18 days. After 18 days thapsigargin production raised to 2.15 mg thapsigargin/g plant DW and 17.42 mg nortrilobolid/g plant DW (Table 4).

Basal medium E

Healthy single shoots (4 cm or more) were excised from multiple shoot clusters growing on MS medium supplemented with combination of 0,5 mg/L NAA and 1.5 mg/L BAP (basal medium B) were transferred to half strength MS media containing 30 g l-1 , 7 g l-1 agar (PH= 5.8) (basal medium E) for 18 days. After 18 days thapsigargin production raised to 2.45 mg thapsigargin/g plant DW and 13.35 mg nortrilobolid/g plant DW (Table 4).

Basal medium F

Healthy single shoots (4 cm or more) were excised from multiple shoot clusters growing on MS medium supplemented with combination of 0,5 mg/L NAA and 1.5 mg/L BAP (basal medium B) were transferred to half strength MS media containing 30 g l-1 , 7 g l-1 agar (PH= 5.8) (basal

medium F) and supplemented with 400 µM MeJ for 18 days. After 18 days thapsigargin production raised to 3.37 mg thapsigargin/g plant DW and 21.50 mg nortrilobolid/g plant DW (Table 4).

Table 4

	mg Thapsigargin/g plant DW	mg Nortrilobolid/g plant DW
Basal medium B	1,31±0,16	9,98±0,71
Basal medium D	2,15±0,16	17,42± 1,60
Basal medium E	2,45±0,16	13,35±1,10
Basal medium F	3,37±0,23	21,50±1,87

Analytical methods

Thapsigargins, produced from the culture of *T. garganica in vitro* plants after micropropagation in basal medium B and D, E and/or F as described above, were quantitatively assayed by employing high performance liquid chromatography (HPLC). The identity of these molecules has also been confirmed using a LC-MS method.

Plant material and thapsigargins extraction

T.garganica in vitro plants were collected from the growth chamber in order to be analyzed. The samples were weighted (fresh weight) and frozen in a -80 °C refrigerator. The frozen tissues were dried in a freeze drier (LABCONCO FreeZone 2.5 plus) for 48 hours. The completely dried tissues were weighted to determine the dry weights and ground into fine powders. About 0.05 g of the powders of each sample were placed into brown Eppendorfs and extracted with 1.5 mL EtOH 70% under agitation overnight (25 °C). The mixtures were then centrifuged (13000 rpm) for 10 min. 1 mL of the supernatant underwent evaporation by speed vacuum. The extracts were resuspended in 250 µL MeOH 80% (concentration x4).The extracts were then filtered through a 0.45 µm filter, and placed into 1.5 mL vials and kept in a -20 °C refrigerator until HPLC analysis.

Standard solution

Standard solutions of thapsigargin and nortrilobolide were prepared in the range 12-1200 mg/L and 11-1007 mg/L respectively, corresponding to the concentration range of the plant extracts.

High Performance Liquid Chromatography analysis of Thapsigargins

Analytical high performance liquid chromatography (HPLC) system consisted of a quaternary pump (JASCO-2089 Plus pump), a thermoregulated autosampler set at 4°C (Intelligent autosampler JASCO AS-2059) and a PDA detector (UV/VIS detector JASCO MD-2018 Plus). Column temperature was regulated at 30° C. Separation of compounds was achieved on a Luna C18 column (5µ, 4.6 mm X 25 cm) (Phenomenex, USA). The flow rate was set at 0.5 mL/min and the mobile phases consisted of A: water+0.01% o-phosphoric acid, B: acetonitrile+0.01% o-phosphoric acid. The binary gradient elution scheme as follows:

Scheme 1: HPLC binary gradient elution used.

Time	% Eluant A	% Eluant B	Flow
0	80	20	0.5 mL/min
15	100	0	*
25	100	0	*
27.5	80	20	*
Eluant **A**= 0.01% o-phosphoric acid + H_2O			
Eluant **B**= 0.01% o-phosphoric acid + Acetonitrile (ACN)			

The HPLC profile of the thapsigargin and nortrilobolide extracted from the *in vitro* plant material cultured in TIBs and standards are shown in figure 8. Thapsigargin elutes between 17 and 18 min and nortrilobolide between 9 and 10 min. Both molecules were detected at 230 nm wavelength.

The *in vitro* plant tissue was derived from the leaflet explants from a *T.garganica ex-vitro* plant. The plant tissue was developed after being cultivated in the first described basal medium for about 3 months and being later cultivated in TIBs with the second described basal medium.

Calculation

Calibration curves were generated by plotting the peak area versus the concentration of standard thapsigargins, and the linear regression equations were found as follows: Tg) Y = 19127 X + 302617 (Figure 9) and Nt) Y = 11034 X – 250342 (Figure 10) (X is mg/L and Y is peak area of the peak). In both instances the calibration graphs were linear with a correlation

coefficient of 0.99, this value indicated appropriate correlation between the investigated thapsigargin and nortrilobolide concentration and the peak area values of unknown sample concentrations. The results were converted to amount per percent dry weight.

Table 2 shows Thapsigargin and Nortrilobolid quantification in plant shoots after micropropagation in basal medium B. The data provided are an average of 18 *Thapsia* samples grown in basal medium B in TIBs. In addition, Table 2 shows Thapsigargin and Nortrilobolid quantification in undifferentiated cell cultures and in embryogenic cell cultures prepared essentially as described in international patent application WO/2015/082978. It is clear that the undifferentiated cell cultures and embryogenic cell cultures produces very low amounts of both Thapsigargin and Nortrilobolid, whereas plant shoots produces both compounds in significant amounts.

Table 2

	TIB biomass	*Undifferentiated cell cultures*	*Embryogenic cell cultures*
mg Tg/g DW plant	**1,22±0,50**	**< 0,00005***	**< 0,00005***
mg Nt /g DW plant	**6,71±1,68**	**< 0,00005***	**< 0,00005***

- * equal to less than 50ng/g d.w, or 0,00005mg/g d.w., which was the Limit Of Quantification.
- Tg= thapsigargin. Nt = nortriloboloid

Wild plants of T. garganica have a concentration of thapsigargin of 0.2%–1.2% of the dry weight of the roots and 0.7%–1.5% of the dry weight of the ripe fruits, whilst the dried stems and leaves contain a total concentration of 0.1%–0.5% and 0.1% respectively [12]. As shown in table 2, the *in vitro* plant tissue (leaves and stems) contained about 1,22 mg/g DW of thapsigargin and about 6,71 mg/g DW of nortrilobolide representing around the 0.12% of thapsigargin and 0.67% of nortrilobolide of the dry weight of the *in vitro* leaves and stems (average 18 plants). The results demonstrated that the *in vitro* plant tissue cultured in TIBs contained as much as in

naturally occurred dried leaves of *T. garganica* and therefore could be used as a valuable source of naturally produced thapsigargin.

HPLC-MS confirmation of thapsigargins

Qualitative analysis of thapsigargins from the *in vitro* plants was carried out using a Waters Acquity UPLC-TQD-MS (triple quadrupole mass spectrometer) system associated with a PDA detector. The system was directed by the software MassLynx 4.1. 7.5 µL of extracts were separated on a C18 Luna (2)-HST (100 x 2.0 mm, 2.5 µm) column (Phenomenex, USA). The flow rate was set at 0.6 mL/min and the mobile phases consisted of water modified with formic acid (0.1%) for A and acetonitrile (ACN) modified with formic acid (0.1%) for B. The binary gradient elution scheme was:

Scheme 2: UPLC-TQD_MS binary gradients elution used for fraction mass analysis.

Time	% Eluant A	% Eluant B	Flow
0	90	10	0.6 mL/min
0.5	90	10	*
7	0	100	*
10	0	100	*
10.2	95	5	*
13	95	5	*

Eluant **A**= 0.1% formic acid + H_2O
Eluant **B**= 0.1% formic acid + Acetonitrile (ACN)

The PDA swept wavelength from 200 to 400 nm with a 2.4 resolution. Mass spectra were obtained in positive and negative modes, using an electrospray ionization (ESI) source on a triple quadripole instrument (Waters Acquity) in full scan (50 to 2000 m/z). The mass conditions were as follows, capillary voltage: 3000V, cone voltages: 30 and 60V, desolvatation temperature: 450°C, source Temp: 150°C, gaz flow cone: 50 l/min, desolvatation gas: 800 l/min.

Five major peaks of the chromatogram were fragmented and injected trough the UPLC-TQD-MS (figure 9). Peak A corresponds to nortrilobolide (figure 12) (MW= 508.5) (14), peak B correspond to thapsivillosin I (figure 14) (MW=605.4) [13], peak C correspond to an indeterminate molecule with a MW=506, peak D correspond to thapsigargin (figure 13) (MW=650.7) [13] and peak E correspond to thapsivillosin C (figure 15) (MW=663.5) [13]. In figures 10 to 11 it can be shown the mass spectra of the different peaks.

Concerning nortrilobolide the spectrum at 30 V exhibits a major $[M-H]^-$ fragment at *m/z* 507.5 while at 60 V, the major fragment is *m/z* 1015.8 which matches with the nortrilobolide source dimer which is formed because of the high concentration of this molecule. Regarding thapsigargin, at 30 and 60 V, the major $[M-H]^-$ fragment at *m/z* 649.6. At 60 V is also present in the spectrum a major $[M-H]^-$ fragment at *m/z* 1299.9 corresponds to source dimer. Thapsivillosin I at 30 V exhibits a major $[M-H]^-$ fragment at *m/z* 605.5 while at 60 V the major $[M-H]^-$ fragment is *m/z* 651.5 which matches with a formic acid adduct *m/z* 649 + 46 $[M-H]^-$In the case of thapsivillosin C, at 30 V the major $[M-H]^-$ fragment is at *m/z* 663.5 and in 60 V the major $[M-H]^-$ fragment is 709.5 also due to a formic acid adduct 664 + 46 $[M-H]^-$. These results demonstrate the presence of these molecules in our *thapsia in vitro* plants.

The demand of thapsigargins will increase in the next decade given its potential medical application as a chemotherapeutic prodrug. The present invention provides an alternative source for the production of thapsigargins. The future need of these molecules can be met by our plant tissue culture that, thanks of the HPLC analyses, has been proved.

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ITEMS

The invention may for example be as defined in the following items.

1. A method of producing guaianolides, said method comprising the steps of
   a. providing plant tissue derived from a plant of the sub-family Apioideae, wherein said plant tissue is embryogenic callus and/or shoots
   b. culturing said embryogenic callus, and/or shoots in a manner involving temporary immersion in basal medium B comprising at least one auxin and at least one cytokinin which together are capable of inducing plant shoot micropropagation, thereby inducing plant shoot micropropagation;
   d. optionally isolating produced guaianolides.

2. The method according to item 1, wherein the cytokinin is natural or artificial cytokinin belonging to the adenine-type or the phenylurea-type.

3. The method according to item 1, wherein the basal medium B comprises the cytokinin 6-benzylaminopurine (BAP).

4. The method according to any one of the preceding items, wherein the basal medium B comprises an auxin selected from the group consisting of 4-chloro-indoleacetic acid, phenylacetic acid (PAA), indole-3-butyric acid and indole-3-acetic acid and 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid.

5. The method according to any one of the preceding items, wherein the basal medium B comprises the auxin 1- naphthaleneacetic acid (NAA).

6. The method according to any one of the preceding items, wherein the embryogenic callus and/or shoots provided in step a) are obtained by culturing a plant tissue of a plant of the sub-family Apioideae on a basal medium A comprising one or more plant growth regulators (PGRs) capable of inducing embryogenic callus and/or shoot formation, thereby inducing embryogenic callus, and/or shoot formation.

7. The method according to one of the preceding items, wherein the basal medium A comprise a plant growth regulator, which is an auxin capable of inducing differentiation.

8. The method according to any one of the preceding items, wherein the basal medium A comprises the plant growth regulator 2,4-Dichlorophenoxyacetic acid (2,4-D).

9. The method according to any one of the preceding items, wherein the basal medium A comprises a plant growth regulator, which is a cytokinin selected from the group consisting of phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ) and BAP.

10. The method according to any one of the preceding items, wherein the method further comprises the step c) performed after step b) and prior to step d), said step c) comprising or consisting of step c1) and/or step c2), wherein

- step c1) is a step of culturing the plant shoots generated in step b) on or in a basal medium C comprising at least one auxin capable of inducing formation of roots, thereby inducing root formation, and/or
- step c2) is a step of culturing the plant shoots generated in step b) on or in
    I. a basal medium D comprising at least one auxin and at least one cytokinin and at least one elicitor capable of promoting production of secondary metabolites such as thapsigargins; and/or
    II. a basal medium E devoid of elicitor and comprising mineral salt(s), wherein the level of mineral salts is 40 to 60% of the level of mineral salts in basal medium B; and/or
    III. a basal medium F comprising at least one elicitor capable of promoting production of secondary metabolites such as thapsigargins.

11. The method according to any one of the preceding items, wherein the basal medium C and/or D comprises one or more auxins selected from the group consisting of indole-3-butyric acid (IBA), 1- naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA).

12. The method according to any one of the preceding items, wherein the basal medium C and/or F comprises mineral salt(s), wherein the level of mineral salts in basal medium C and/or F is 40 to 60% of the level of mineral salts in basal medium B.

13. The method according to any one of the preceding items, wherein the elicitor is an abiotic or biotic elicitor such as $AgNO_3$, $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, KCl, $MgSO_4$, $NiSO_4$, $VOSO_4$, Zn, microbial enzymes, bacterial lysates, cell wall polysaccharides derived from microorganisms, polysaccharides derived from pathogen degradation of the plant cell wall, intracellular proteins, or small molecules synthesized by the plant, for example plant hormones such as jasmonates or salicylic acid.

14. The method according to item 13, wherein the elicitor is selected from the group consisting of jasmonates and salicylic acid.

15. The method according to item 14, wherein the elicitor is selected from jasmonate, methyl jasmonate (MeJ), JA-Ile and cis-jasmone.

16. The method according to any one of the preceding items, wherein said culturing of step c1) and/or c2) involves temporary immersion in basal medium C, and/or temporary immersion in basal medium D, and/or temporary immersion in basal medium E, and/or temporary immersion in basal medium F.

17. The method according to any one of the preceding items, wherein said culturing of steps b) and/or c) is performed in a temporary immersion bioreactor (TIB).

18. The method according to any one of the preceding items, wherein the plant of the sub-family *Apioideae* is a plant of the genus *Laser* or the genus *Thapsia*.

19. The method according to any one of the preceding items, wherein said plant of the sub-family *Apioideae* is a plant of the genus *Thapsia* selected from the group consisting of: *T. leucotricha, T. tenuifolia, T.garganica, T. gymnesica, T. transtagana, T. thapsioides, T.*

gummifera, T. smittii, T. asclepium, T. scabra, T. maxima, T. villosa, T. minor and T. laciniata.

20. The method according to any one of the preceding items, wherein said plant of the sub-family *Apioideae* is a plant of the genus *Laser* selected from the group consisting of: *L. trilobum, L.siler, L. aquilegifolium, Laser divaricatum, Laser rechingeri* and *L. cordifolium*

21. The method according to any one of the preceding items, wherein the concentration of TDZ in the basal medium A is in the range of 0.1-5.0 mg l^{-1}.

22. The method according to any one of the preceding items, wherein the concentration of 2,4-D in the basal medium A is in the range of 0.01-3.0 mg l^{-1}.

23. The method according to any one of the preceding items, wherein the concentration of BAP in the basal medium B or in the basal medium D is in the range of 0.1-5.0 mg l^{-1}.

24. The method according to any one of the preceding items, wherein the concentration of NAA in the basal medium B or in the basal medium D is in the range of 0.01-3.0 mg l^{-1} .

25. The method according to any one of the preceding items, wherein the concentration of IBA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}.

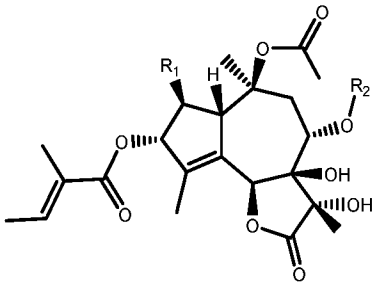
26. The method according to any one of the preceding items, wherein the concentration of NAA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}.

27. The method according to any one of the preceding items, wherein the concentration of IAA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}.

28. The method according to any one of the preceding items, wherein the plant shoots obtained in step b) or the plants obtained in step c) comprise at least 1 mg, preferably at least 3 mg, more preferably at least 5 mg thapsigargin per g dry weight plant.

29. The method according to any one of the preceding items, wherein the method comprises purifying one or more guaianolides from the plant shoots obtained in step b) or from the plants obtained in step c).

30. The method according to any one of the preceding items, wherein the guaianolide comprises a core structure of formula I:



31. The method according to item 25, wherein R_1 is –H, –O-(C=O)-C_{2-10}-alkyl or is –O-(C=O)-C_{2-10}-alkenyl.

32. The method according to any one of items 25 and 26, wherein the R_2 is –H, –(C=O)-C_{2-12}-alkyl or –(C=O)-C_{2-12}-alkenyl.

33. The method according to any one of the preceding items, wherein the guaianolide is any of the guaianolides mentioned in Table 1.

34. The method according to any one of the preceding items, wherein the guaianolide is thapsigargin or nortrilobolide.

35. The method according to any one of the preceding items, wherein the method comprises the step of purifying thapsigargin from the plant shoots obtained in step b) or from the plants obtained in step c), c1) and/or c2).

36. A method for producing a pharmaceutical composition, said method comprising the steps of

i) preparing guaianolides by the method according to any one of the preceding items
ii) formulating said thapsigargin into a pharmaceutical composition.

37. A method for producing a prodrug, said method comprising the steps of

i) preparing a guaianolide by the method according to any one of items 1 to 35;
ii) attaching a peptide, which is cleavable by a PSA, an hK2 or PSMA protease to said guaianolide optionally via a linker

thereby producing a prodrug.

38. The method according to item 37, wherein the guaianolide is a guaianolide as defined in any one of items 33 to 34.

39. The method according to any one of items 37 to 38, wherein the peptide comprises or consists of the sequence Asp-Glu-Glu-Glu-Glu.

40. The method according to any one of items 37 to 39, wherein the prodrug is G-202.

41. A method for cultivating plants of the sub-family Apioideae or cells or tissues thereof, the method comprising the steps of

a. providing plant tissue derived from a plant of the sub-family Apioideae, wherein said plant tissue is embryogenic callus and/or shoots
b. culturing said embryogenic callus, and/or shoots in a manner involving temporary immersion in basal medium B comprising at least one auxin and at least one cytokinin which together are capable of inducing plant shoot micropropagation, thereby inducing plant shoot micropropagation.

42. The method according to item 41, wherein the method is as defined in or comprises the steps as defined in any one of items 2 to 35.

CLAIMS

1. A method of producing guaianolides, said method comprising the steps of
    a. providing plant tissue derived from a plant of the sub-family Apioideae, wherein said plant tissue is embryogenic callus and/or shoots
    b. culturing said embryogenic callus, and/or shoots in a manner involving temporary immersion in basal medium B comprising at least one auxin and at least one cytokinin which together are capable of inducing plant shoot micropropagation, thereby inducing plant shoot micropropagation;
    d. optionally isolating produced guaianolides.

2. The method according to claim 1, wherein the cytokinin is natural or artificial cytokinin belonging to the adenine-type or the phenylurea-type or wherein the basal medium B comprises the cytokinin 6-benzylaminopurine (BAP), and/or wherein the basal medium B comprises an auxin selected from the group consisting of 4-chloro-indoleacetic acid, phenylacetic acid (PAA), indole-3-butyric acid and indole-3-acetic acid and 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid, preferably the auxin is NAA.

3. The method according to any one of the preceding claims, wherein the embryogenic callus and/or shoots provided in step a) are obtained by culturing a plant tissue of a plant of the sub-family Apioideae on a basal medium A comprising one or more plant growth regulators (PGRs) capable of inducing embryogenic callus and/or shoot formation, thereby inducing embryogenic callus, and/or shoot formation, such as an auxin capable of inducing differentiation, or 2,4-Dichlorophenoxyacetic acid (2,4-D), or a cytokinin selected from the group consisting of phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ) and BAP.

4. The method according to any one of the preceding claims, wherein the method further comprises the step c) performed after step b) and prior to step d), said step c) comprising or consisting of step c1) and/or step c2), wherein

- step c1) is a step of culturing the plant shoots generated in step b) on or in a basal medium C comprising at least one auxin capable of inducing formation of roots, thereby inducing root formation, and/or
- step c2) is a step of culturing the plant shoots generated in step b) on or in

i) a basal medium D comprising at least one auxin and at least one cytokinin and at least one elicitor capable of promoting production of secondary metabolites such as thapsigargins; and/or

ii) a basal medium E devoid of elicitor and comprising mineral salt(s), wherein the level of mineral salts is 40 to 60% of the level of mineral salts in basal medium B; and/or

iii) a basal medium F comprising at least one elicitor capable of promoting production of secondary metabolites such as thapsigargins,

wherein optionally

the basal medium C and/or D comprises one or more auxins selected from the group consisting of indole-3-butyric acid (IBA), 1- naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA), and

the basal medium C and/or F comprises mineral salt(s), wherein the level of mineral salts in basal medium C and/or F is 40 to 60% of the level of mineral salts in basal medium B.

5. The method according to any one of claims 10 to 12, wherein the elicitor is an abiotic or biotic elicitor such as $AgNO_3$, $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, KCl, $MgSO_4$, $NiSO_4$, $VOSO_4$, Zn, microbial enzymes, bacterial lysates, cell wall polysaccharides derived from microorganisms, polysaccharides derived from pathogen degradation of the plant cell wall, intracellular proteins, or small molecules synthesized by the plant, for example plant hormones such as jasmonates or salicylic acid, preferably the elicitor is selected from the group consisting of salicylic acid and jasmonates such as jasmonate, methyl jasmonate (MeJ), JA-Ile or cis-jasmone.

6. The method according to any one of claims 10 to 12, wherein said culturing of step c1) and/or c2) involves temporary immersion in basal medium C, and/or temporary immersion in basal medium D, and/or temporary immersion in basal medium E, and/or temporary immersion in basal medium F and/or wherein said culturing of steps b) and/or c) is performed in a temporary immersion bioreactor (TIB).

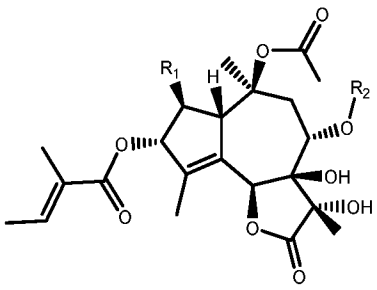
7. The method according to any one of the preceding claims, wherein the plant of the sub-family *Apioideae* is a plant of the genus *Laser,* such as a plant of the genus *Thapsia* selected from the group consisting of: *T. leucotricha, T. tenuifolia, T.garganica, T. gymnesica, T. transtagana, T. thapsioides, T. gummifera, T. smittii, T. asclepium, T.*

scabra, T. maxima, T. villosa, T. minor and T. laciniata, or of the genus *Thapsia* such as *L. trilobum, L.siler, L. aquilegifolium, Laser divaricatum, Laser rechingeri* and *L. cordifolium*.

8. The method according to any one of claims 9 to 14, wherein:
   - the concentration of TDZ in the basal medium A is in the range of 0.1-5.0 mg l^{-1}, and/or
   - the concentration of 2,4-D in the basal medium A is in the range of 0.01-3.0 mg l^{-1}, and/or
   - the concentration of BAP in the basal medium B or in the basal medium D is in the range of 0.1-5.0 mg l^{-1}, and/or
   - the concentration of NAA in the basal medium B or in the basal medium D is in the range of 0.01-3.0 mg l^{-1} , and/or
   - the concentration of IBA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}, and/or
   - the concentration of NAA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}, and/or
   - the concentration of IAA in the basal medium C is in the range of 0.1-8.0 mg l^{-1}.

9. The method according to any one of the preceding claims, wherein the plant shoots obtained in step b) or the plants obtained in step c) comprise at least 1 mg, preferably at least 3 mg, more preferably at least 5 mg thapsigargin per g dry weight plant, and/or wherein the method comprises purifying one or more guaianolides from the plant shoots obtained in step b) or from the plants obtained in step c).

10. The method according to any one of the preceding claims, wherein the guaianolide comprises a core structure of formula I:



wherein optionally:
- R_1 is –H, –O-(C=O)-C_{2-10}-alkyl or is –O-(C=O)-C_{2-10}-alkenyl, and/or
- R_2 is –H, –(C=O)-C_{2-12}-alkyl or –(C=O)-C_{2-12}-alkenyl.

11. The method according to any one of the preceding claims, wherein the guaianolide is any of the guaianolides mentioned in Table 1, such as thapsigargin or nortrilobolide.

12. The method according to any one of the preceding claims, wherein the method comprises the step of purifying thapsigargin from the plant shoots obtained in step b) or from the plants obtained in step c), c1) and/or c2).

13. A method for producing a pharmaceutical composition, said method comprising the steps of

i) preparing guaianolides by the method according to any one of the preceding claims

ii) formulating said thapsigargin into a pharmaceutical composition.

14. A method for producing a prodrug, said method comprising the steps of

i) preparing a guaianolide by the method according to any one of claims 1 to 12;

ii) attaching a peptide, which is cleavable by a PSA, an hK2 or PSMA protease to said guaianolide optionally via a linker

thereby producing a prodrug,

wherein optionally

the guaianolide is a guaianolide as defined in any one of claims 1 to 12 and/or

the peptide comprises or consists of the sequence Asp-Glu-Glu-Glu-Glu.

15. The method according to claim 14, wherein the prodrug is G-202.

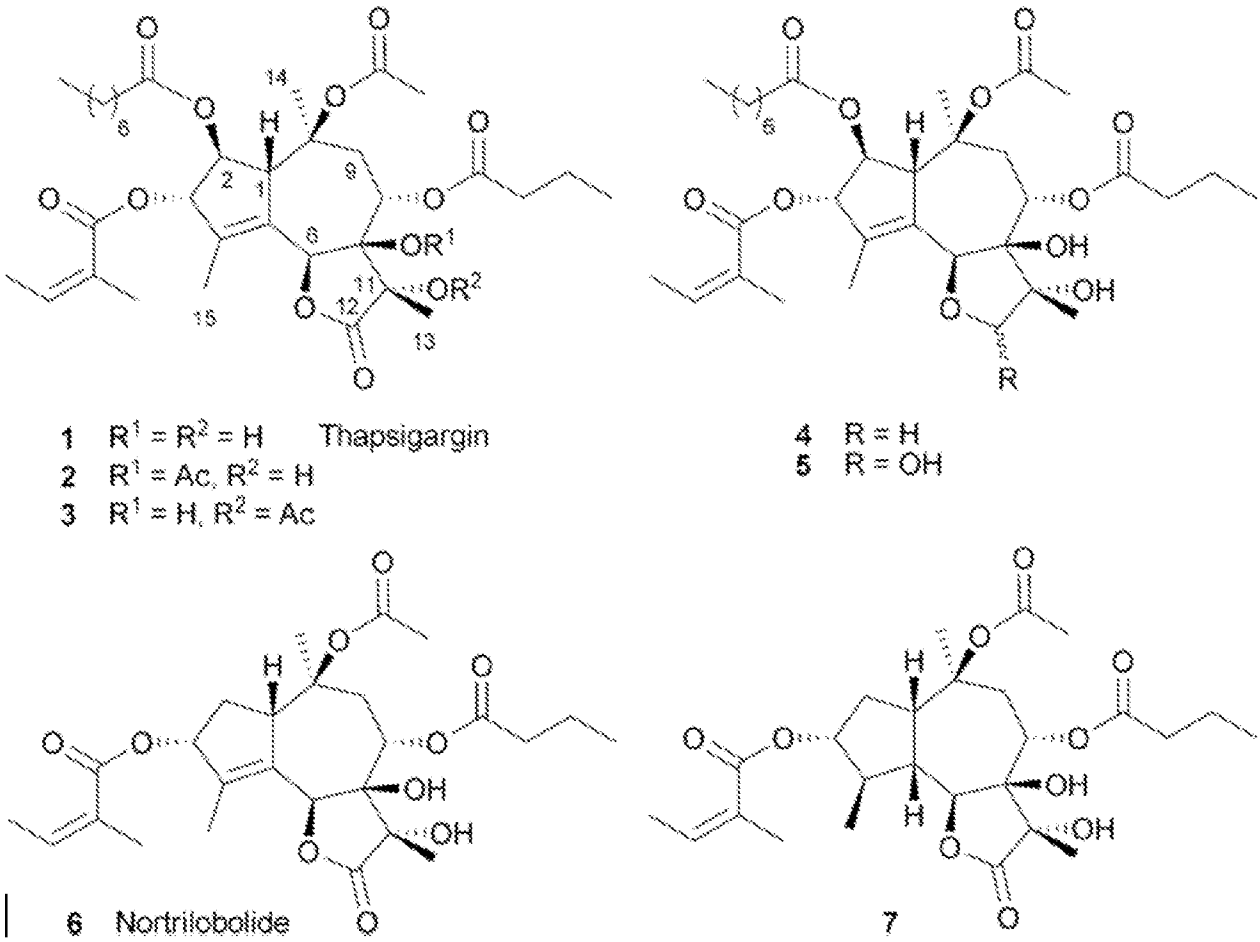

1 $R^1 = R^2 = H$ Thapsigargin
2 $R^1 = Ac$, $R^2 = H$
3 $R^1 = H$, $R^2 = Ac$
4 R = H
5 R = OH
6 Nortrilobolide
7

Fig. 1

Compound	IC_{50}(analogue)/IC_{50}(**1**) [a]
1	1.0
2	2.8
3	2.6
4	1.1
5	1.6
6	1
7	0.1
C-8-*epi*-**1**	3124

[a] IC_{50} = concentration of inhibitor required to elicit 50% inhibition of SERCA.

Fig. 1 (Cont.)

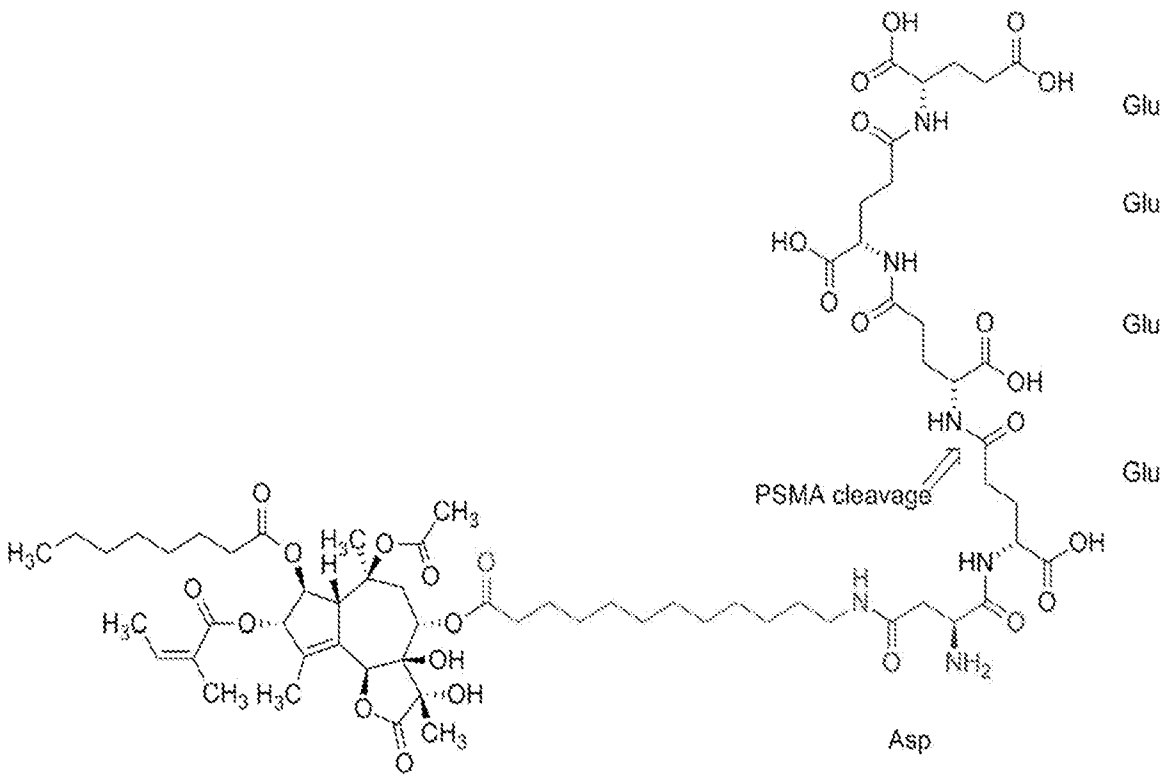


Fig. 2

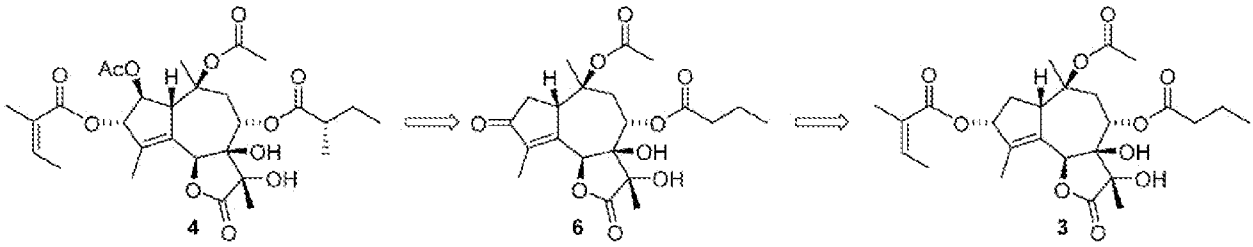


Fig. 3

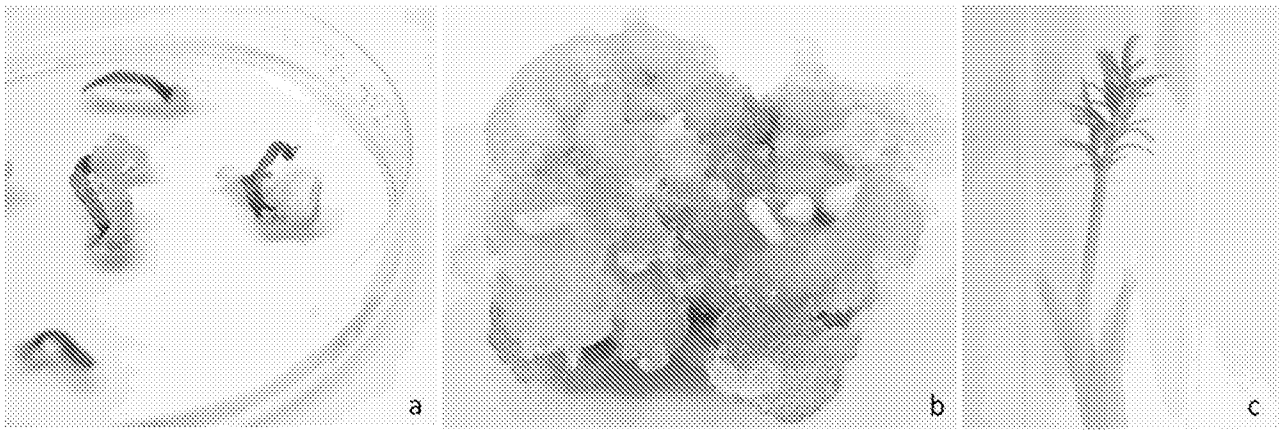


Fig. 4

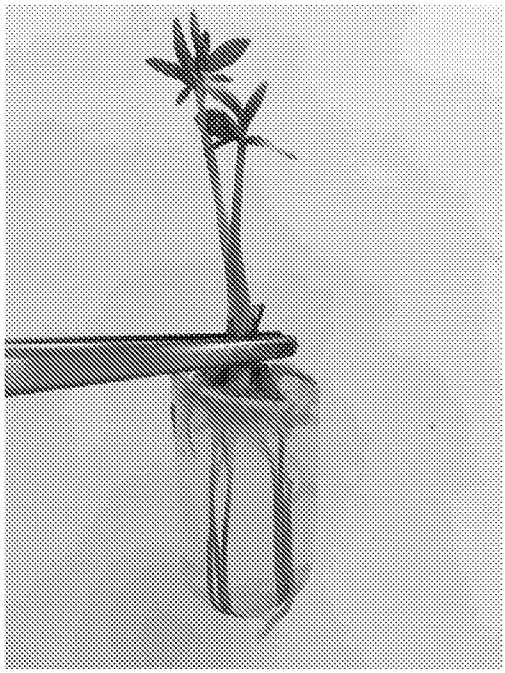


Fig. 5

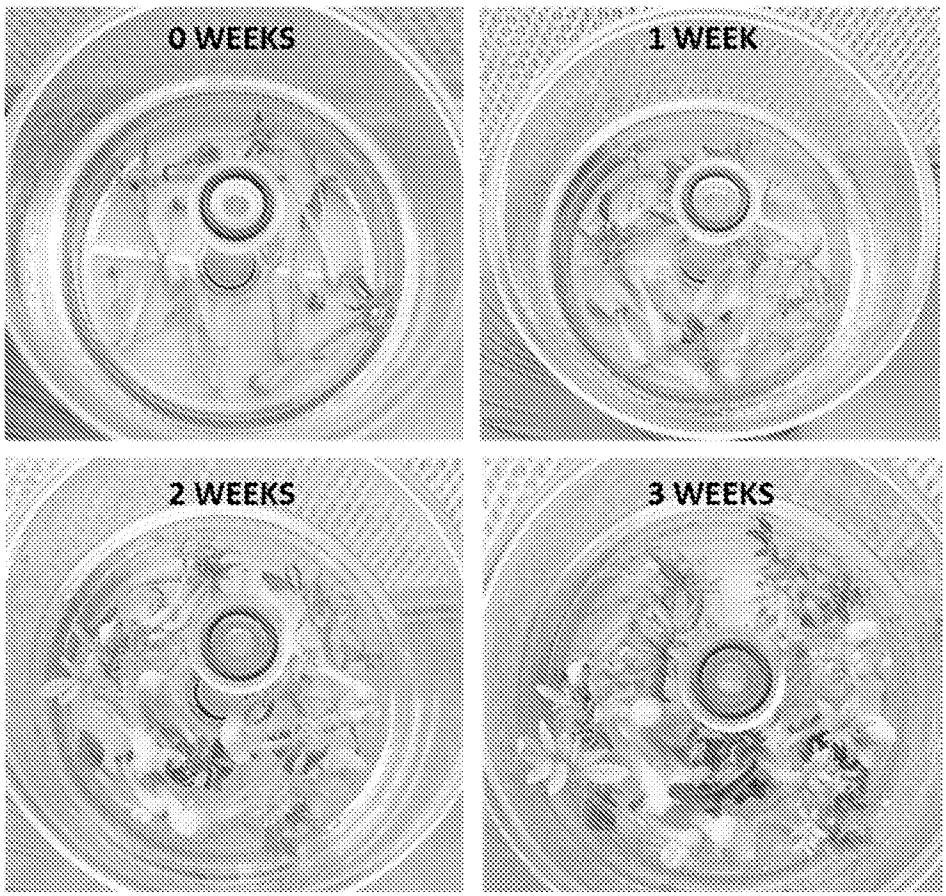


Fig. 6

	Initial FW (g)	FW at 3 weeks (g)	Increase (%)
TIB 1	2,13	9,37	439,91
TIB 2	2,60	11,77	452,69
TIB 3	2,48	10,00	403,23
TIB 4	2,18	15,20	697,25
TIB 5	2,60	13,76	529,23
Average	**2,40**	**12,02**	**501,25**

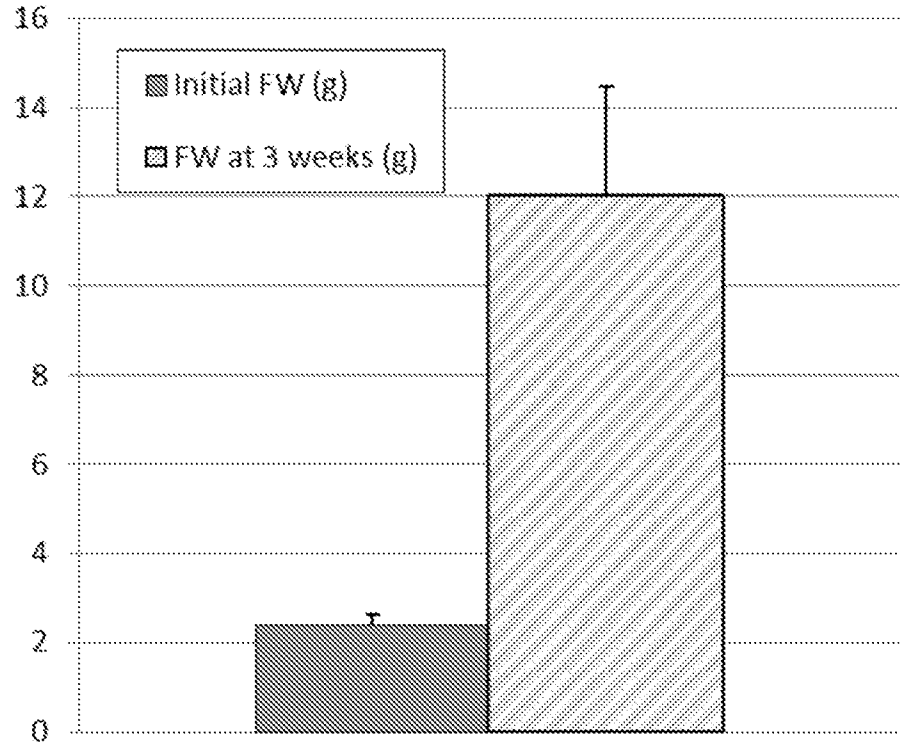


Fig. 7

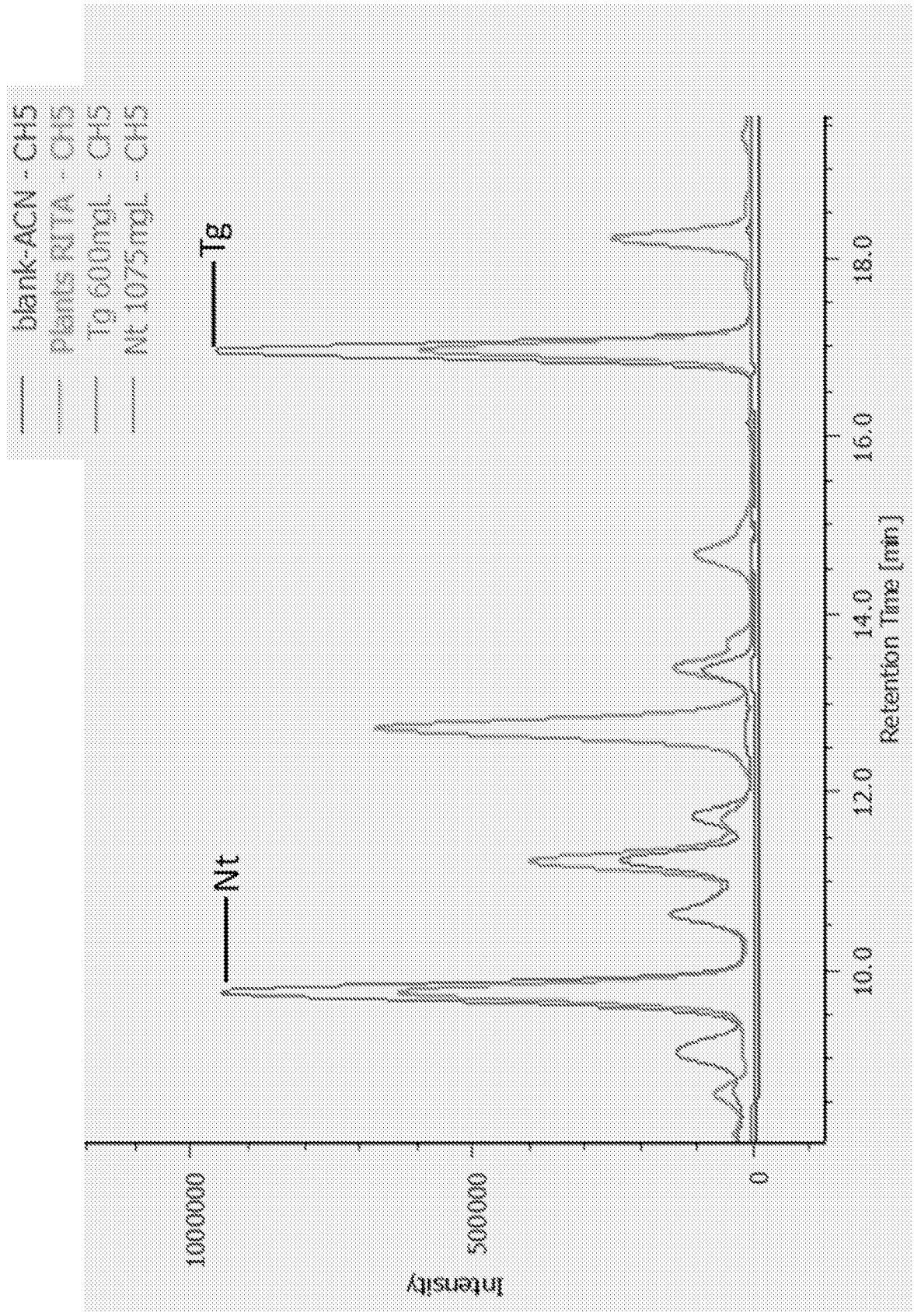

blank-ACN - CH5
Plants NTA - CH5
Tg 600mgL - CH5
Nt 1075mgL - CH5
Tg
Nt
Intensity
1000000
500000
0
10.0
12.0
14.0
16.0
18.0
Retention Time [mm]

Fig. 8

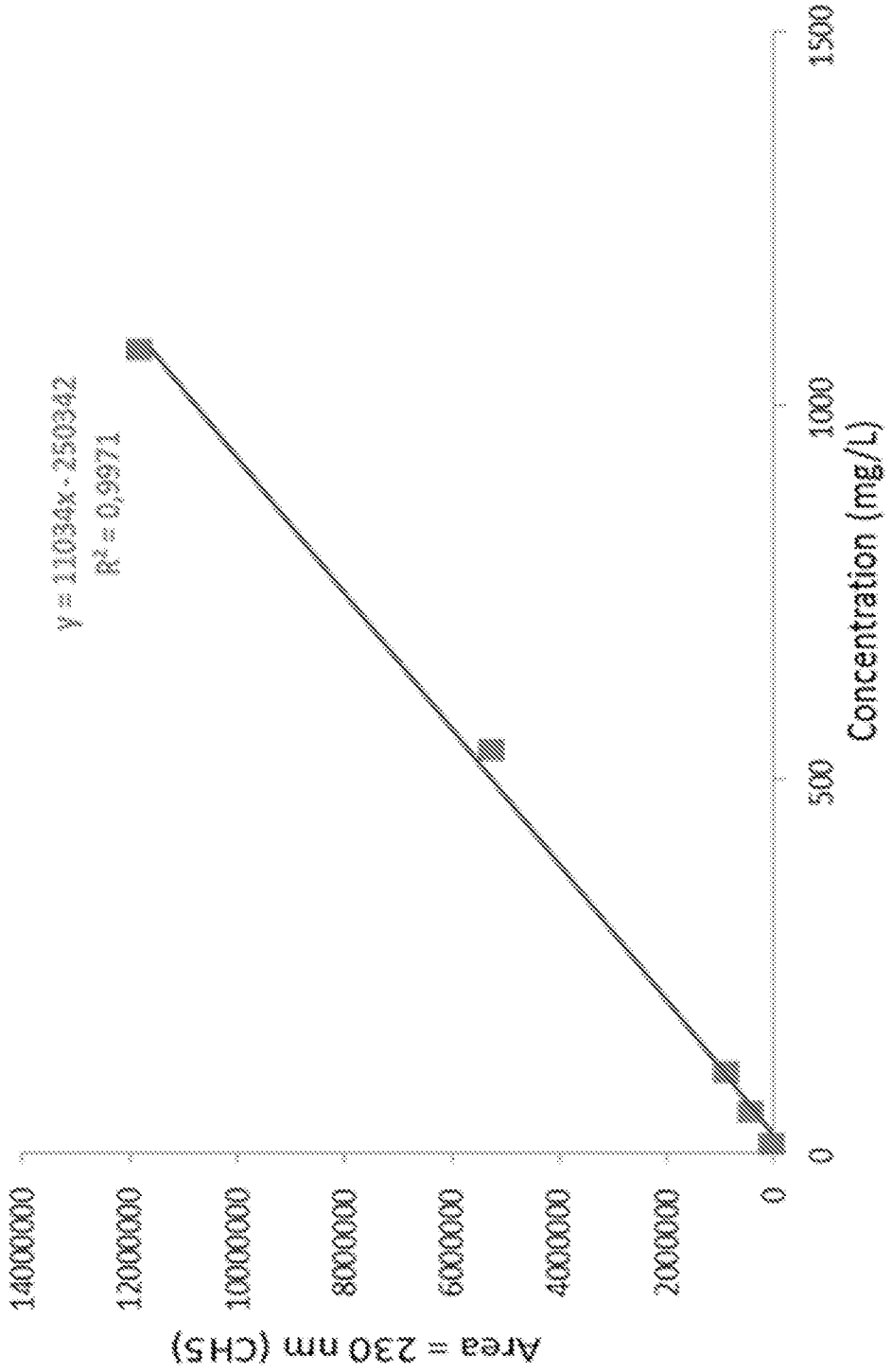

y = 11034x - 250342
R² = 0,9971
Area = 230 nm (CHS)
Concentration (mg/L)
0
500
1000
1500
0
200000
400000
600000
800000
1000000
1200000
1400000

Fig. 9

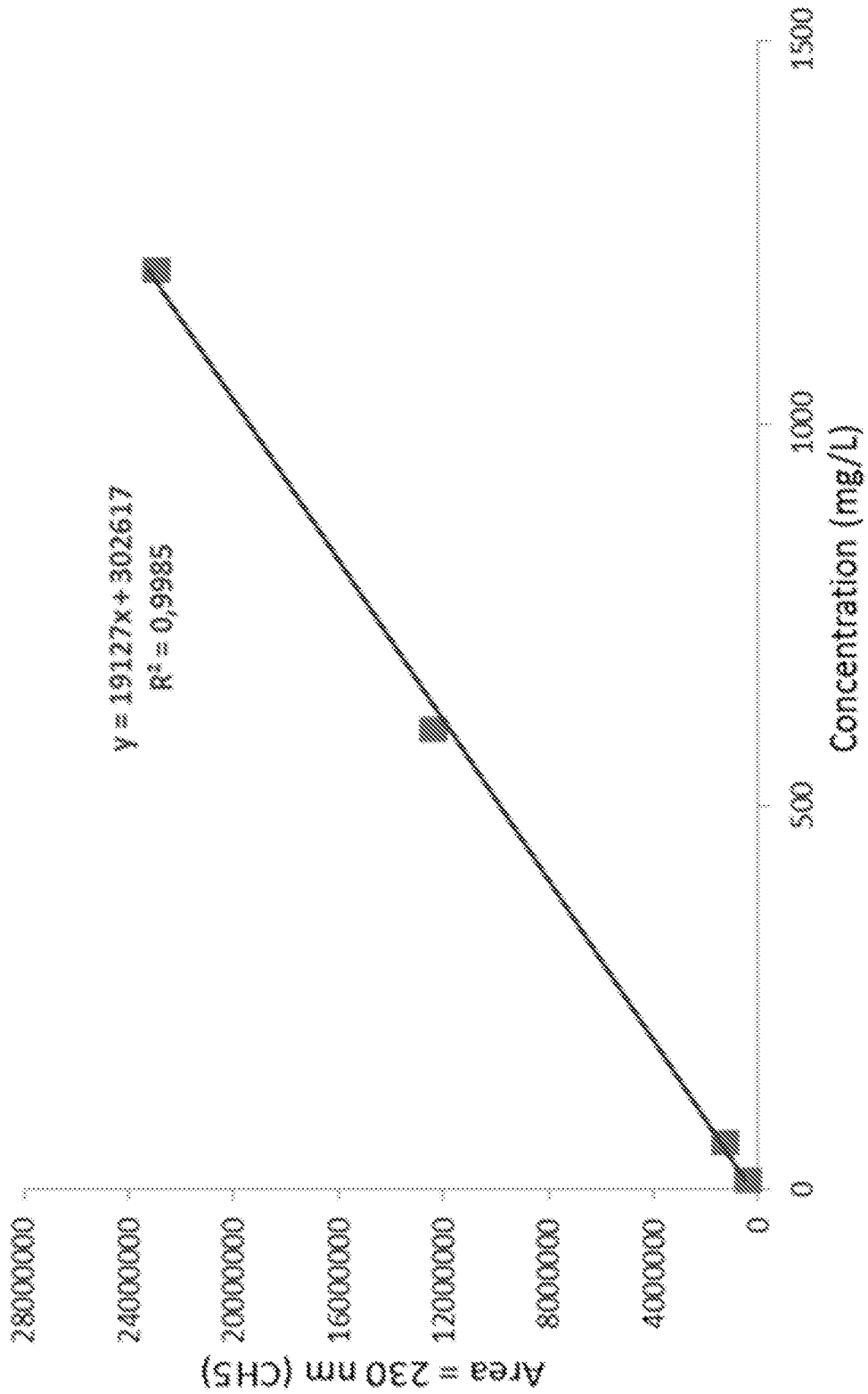

Area = 230 nm (CHS)
2800000
2400000
2000000
1600000
1200000
800000
400000
0
0
500
1000
1500
Concentration (mg/L)
y = 19127x + 302617
R² = 0,9985

Fig. 10

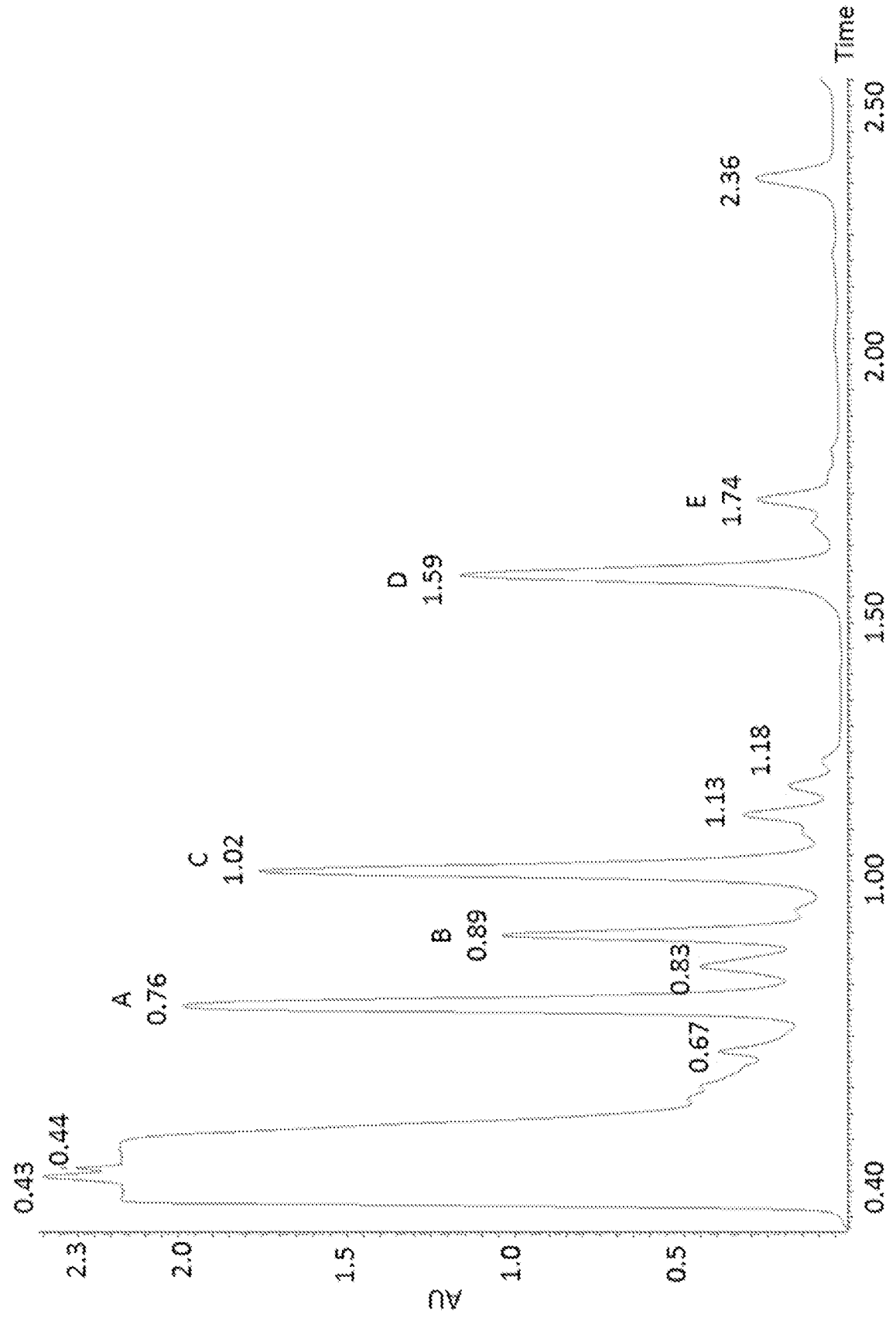

Time
2.50
2.00
1.50
1.00
0.40
AU
2.3
2.0
1.5
1.0
0.5
0.43
0.44
A
0.76
0.67
B
0.89
0.83
C
1.02
1.13
1.18
D
1.59
E
1.74
2.36

Fig. 11

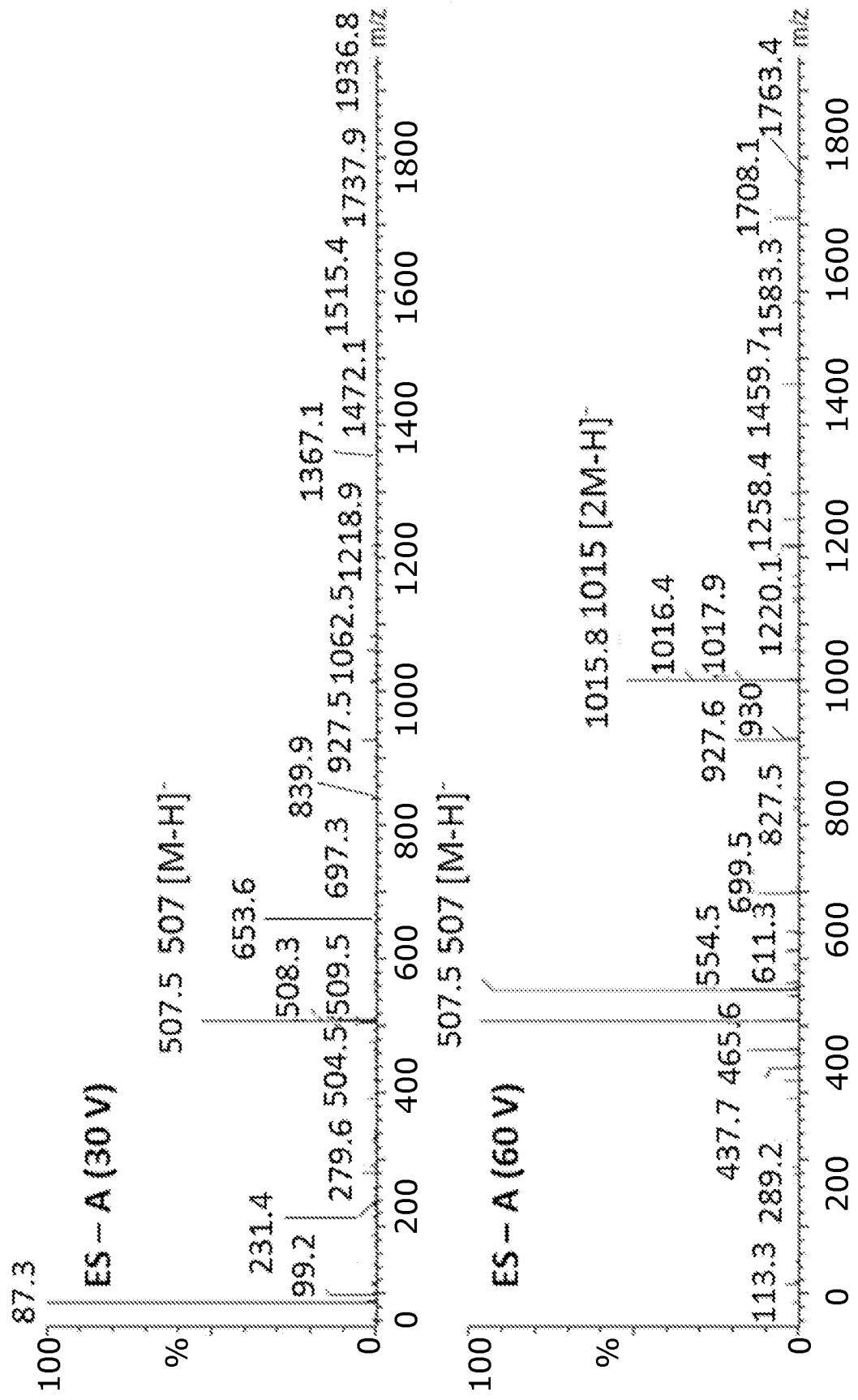

Peak A = Nortriloboloide
ES – A (30 V)
87.3
231.4
99.2
279.6
504.5
507.5
507 [M-H]-
653.6
508.3
509.5
697.3
839.9
927.5
1062.5
1218.9
1367.1
1472.1
1515.4
1737.9
1936.8
m/z
ES – A (60 V)
113.3
289.2
437.7
465.6
507.5
507 [M-H]-
554.5
611.3
699.5
827.5
927.6
930
1015.8
1015 [2M-H]-
1016.4
1017.9
1220.1
1258.4
1459.7
1583.3
1708.1
1763.4
m/z
%
100
0
0
200
400
600
800
1000
1200
1400
1600
1800

Fig. 12

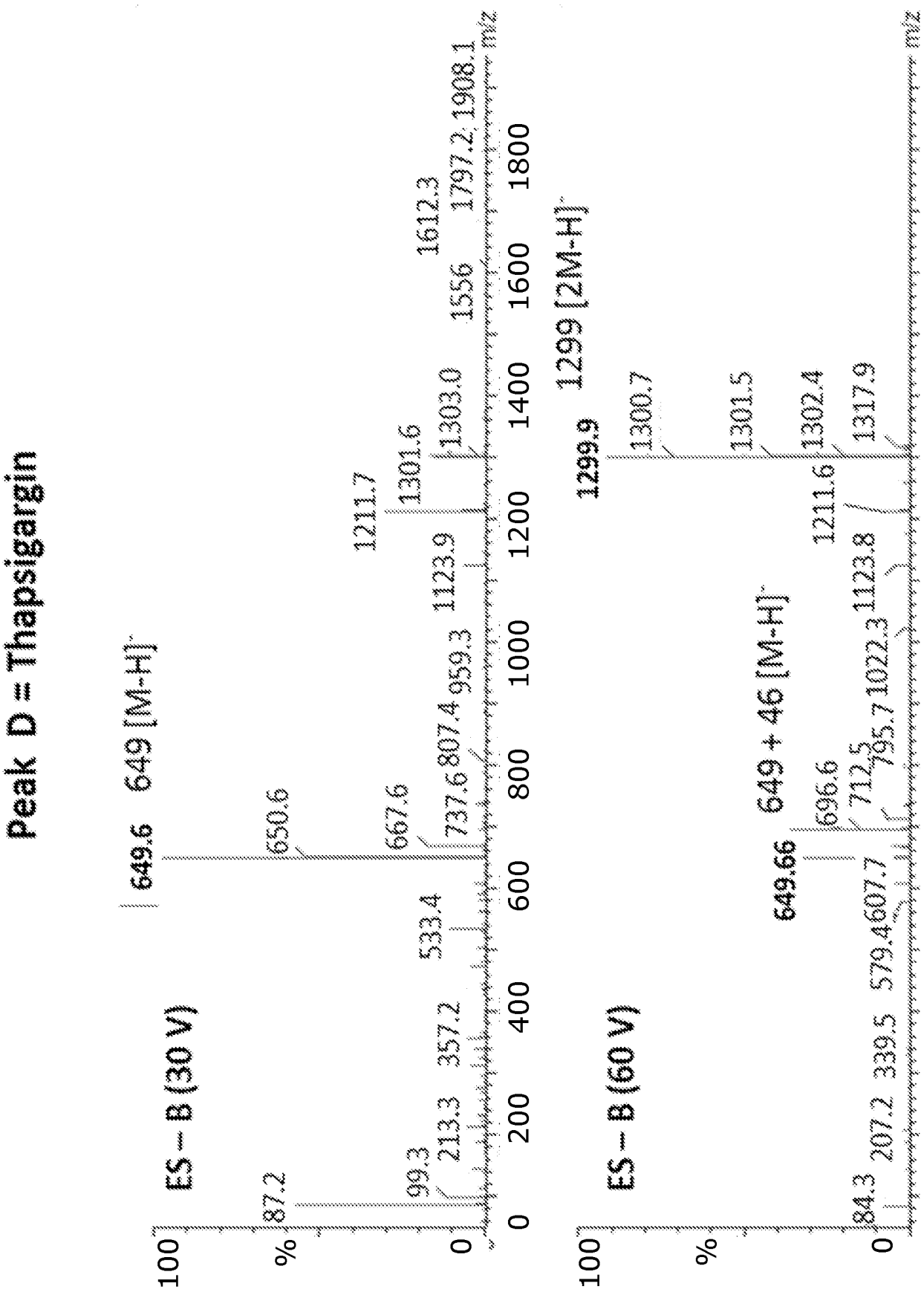

Peak D = Thapsigargin
ES – B (30 V)
649.6
649 [M-H]-
650.6
667.6
737.6
807.4
959.3
1123.9
1211.7
1301.6
1303.0
1556
1612.3
1797.2
1908.1
533.4
357.2
213.3
99.3
87.2
ES – B (60 V)
649.66
649 + 46 [M-H]-
696.6
712.5
795.7
1022.3
1123.8
1211.6
1299.9
1299 [2M-H]-
1300.7
1301.5
1302.4
1317.9
579.4
607.7
339.5
207.2
84.3
100
%
0
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

Fig. 13

Peak B = Thapsivillosin I

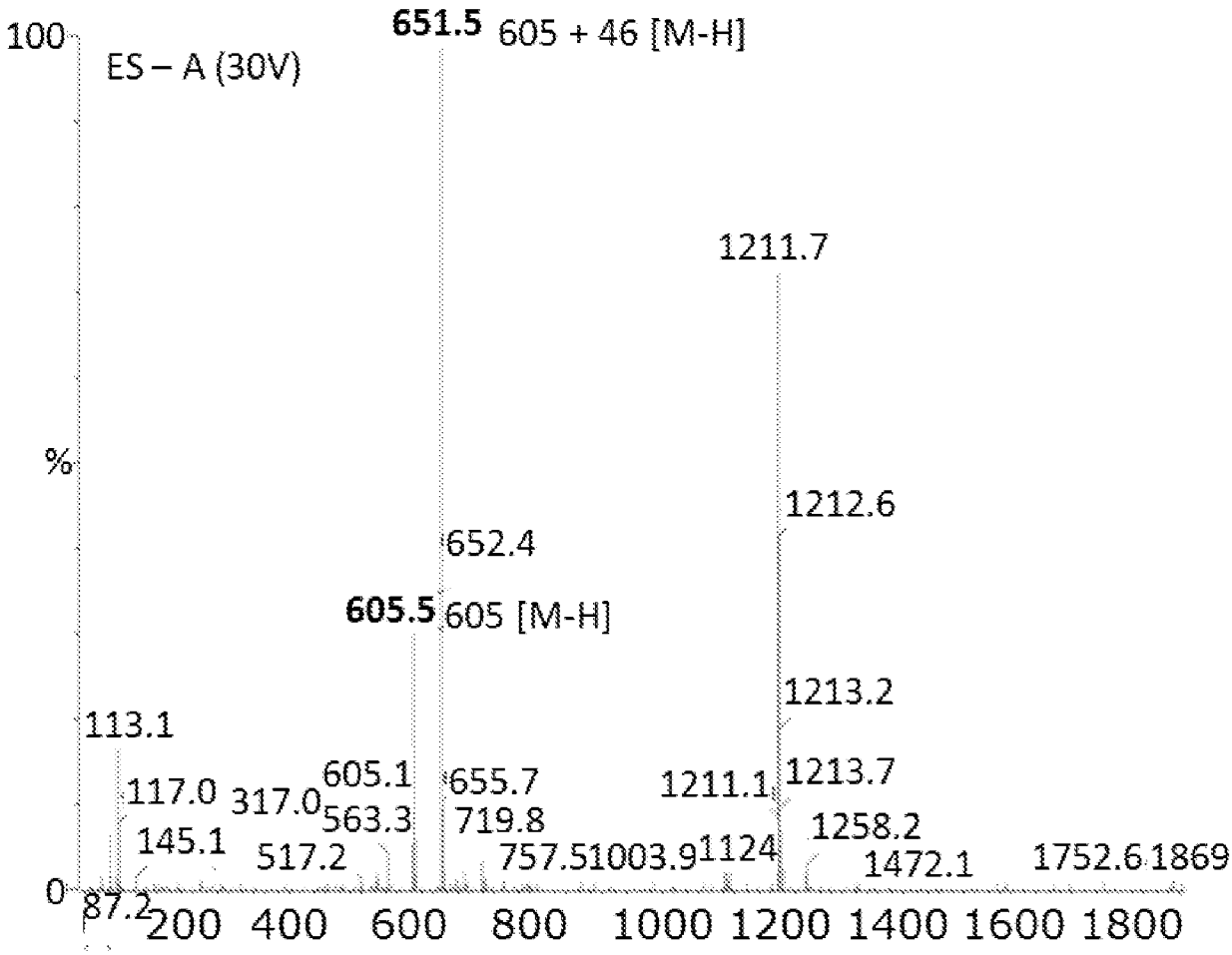


Fig. 14

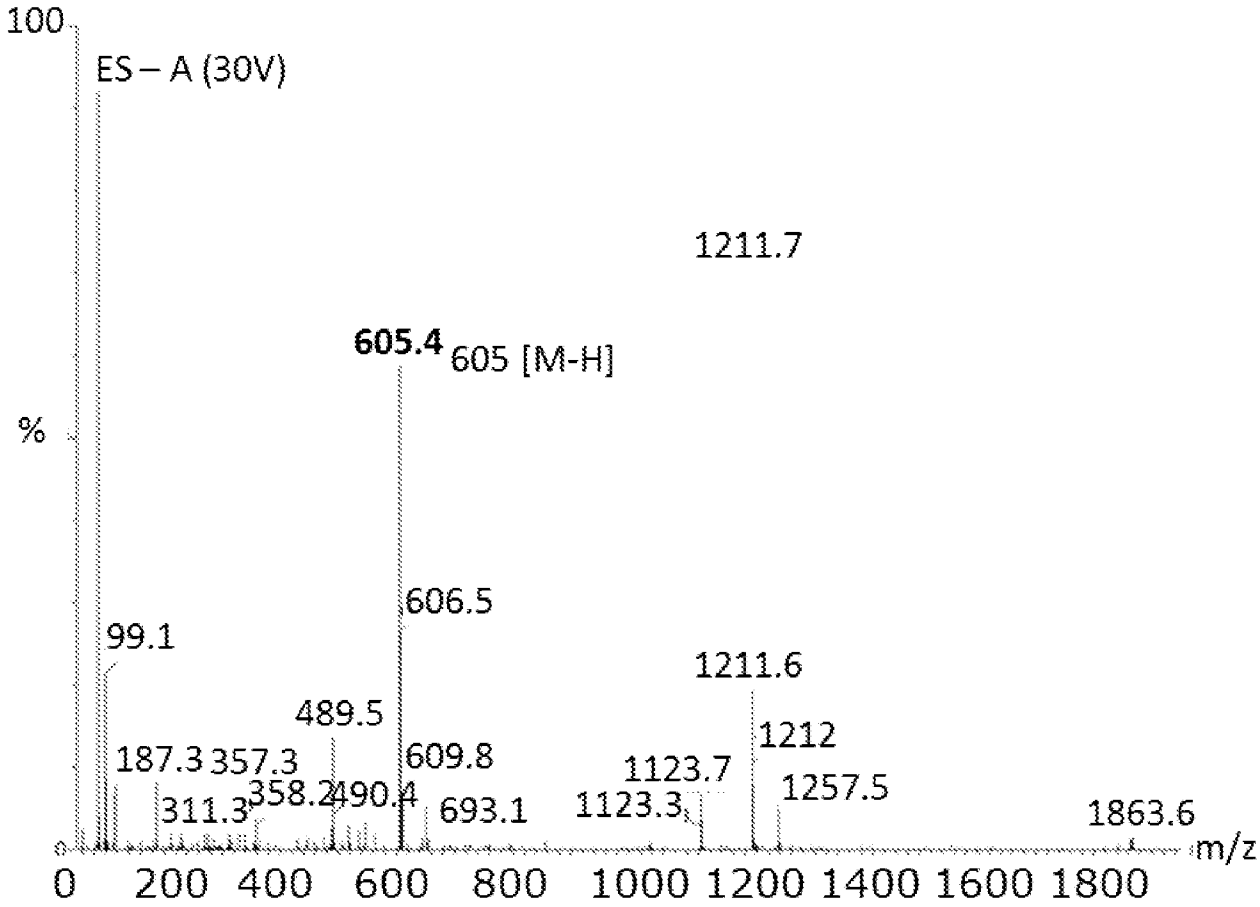

100
ES – A (30V)
1211.7
605.4
605 [M-H]
%
606.5
99.1
1211.6
489.5
1212
187.3
357.3
609.8
1123.7
1257.5
358.2
490.4
311.3
693.1
1123.3
1863.6
m/z
0
200
400
600
800
1000
1200
1400
1600
1800

Fig. 14 (Cont.)

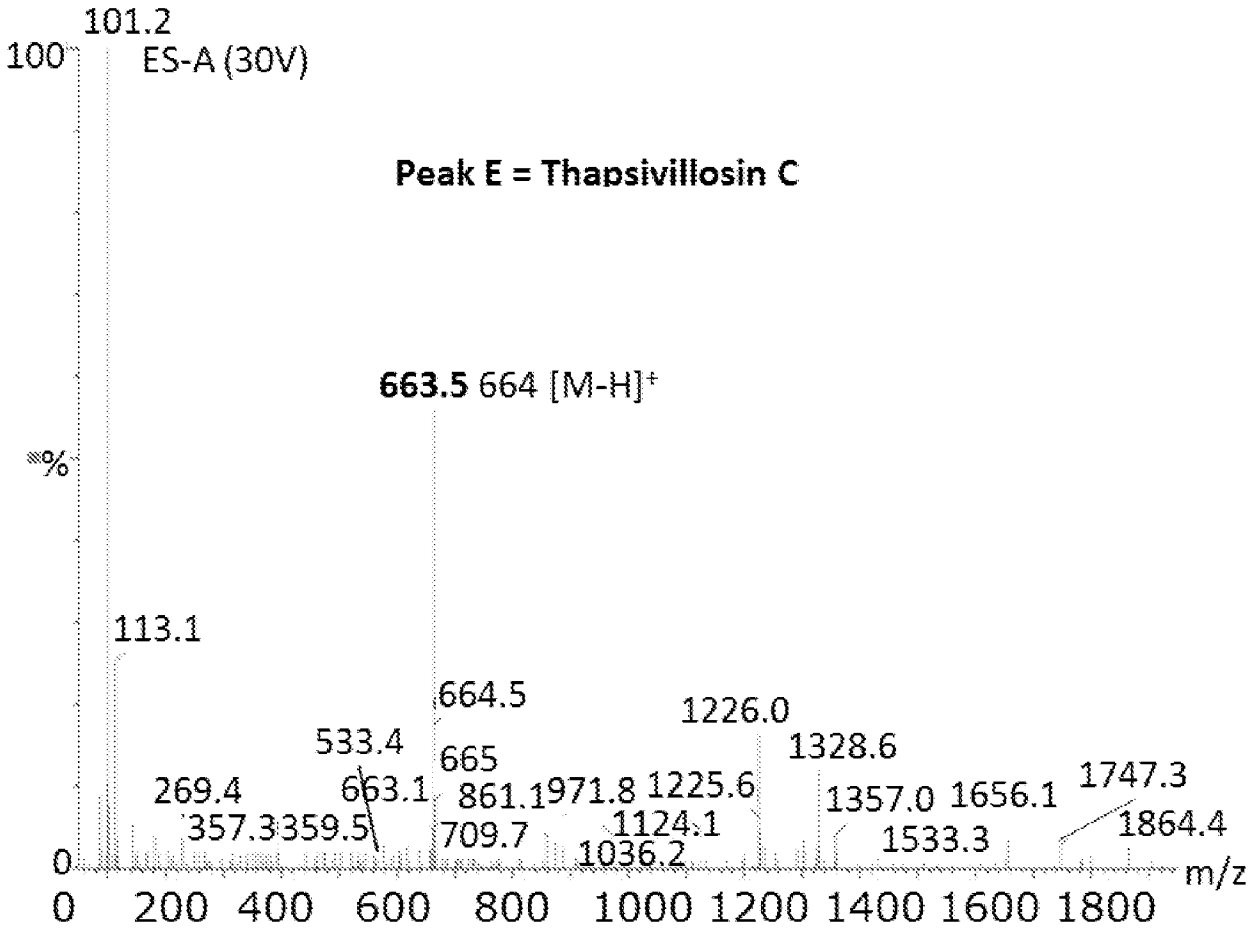

101.2
100
ES-A (30V)
Peak E = Thapsivillosin C
663.5 664 [M-H]+
%
113.1
664.5
533.4
665
1226.0
1328.6
269.4
663.1
861.1
971.8
1225.6
1747.3
1357.0
1656.1
357.3
359.5
709.7
1124.1
1864.4
1533.3
1036.2
0
m/z
0 200 400 600 800 1000 1200 1400 1600 1800

Fig. 15

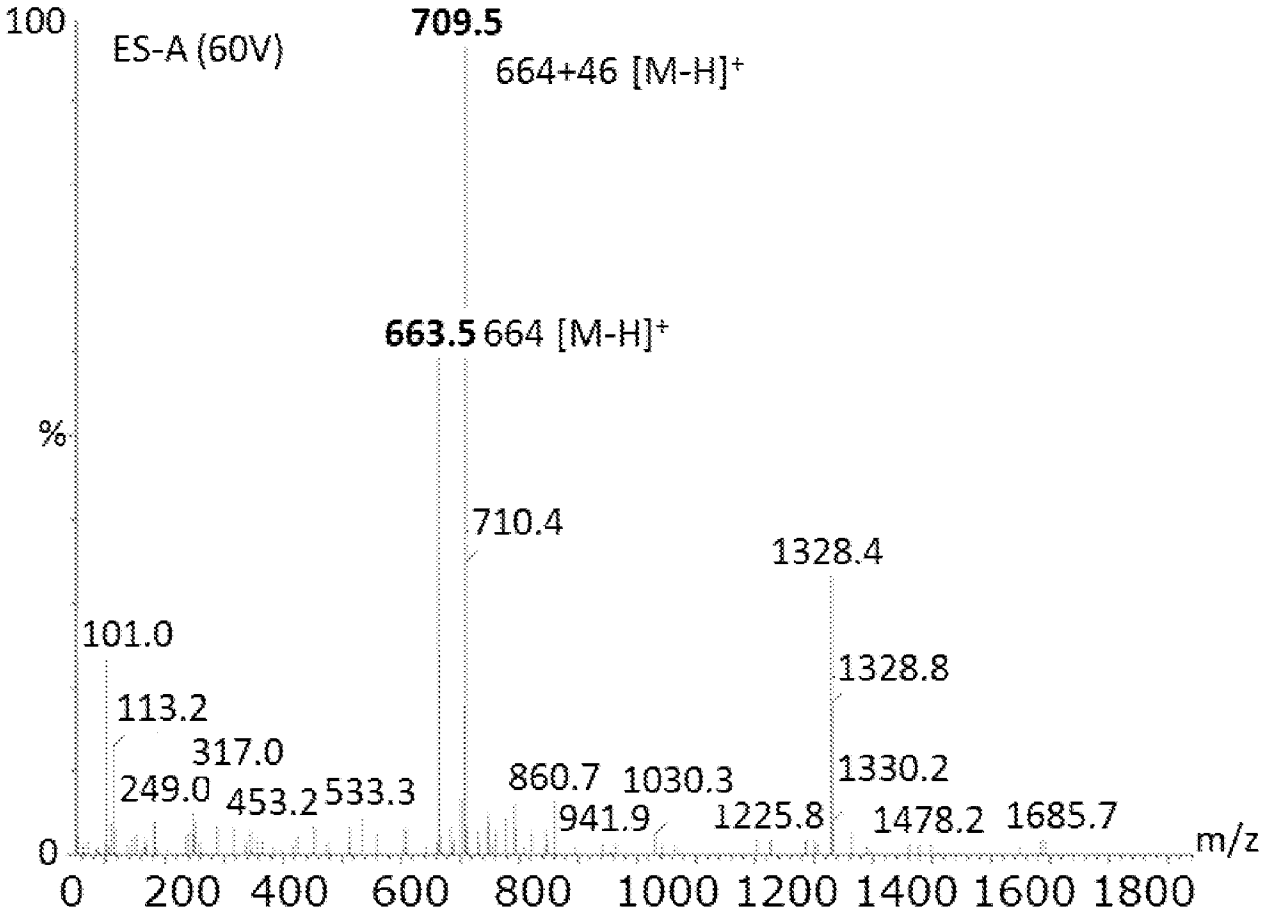

100
ES-A (60V)
709.5
664+46 [M-H]+
663.5 664 [M-H]+
%
710.4
1328.4
101.0
1328.8
113.2
317.0
249.0
453.2
533.3
860.7
1030.3
1330.2
941.9
1225.8
1478.2
1685.7
m/z
0
0 200 400 600 800 1000 1200 1400 1600 1800

Fig. 15 (Cont.)

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2017/050049

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01H4/00 C12N5/00 C12P5/00 C12P17/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01H C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/082978 A1 (PHYTON HOLDINGS LLC [US]) 11 June 2015 (2015-06-11) cited in the application	1-4,7-12
Y	page 9, paragraph 0039 - paragraph 0042; claims 1-11; examples 3-7	5,6, 13-15
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2017	27/06/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Marchesini, Patrizia

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2017/050049

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nokwanda P Makunga: "GENETIC TRANSFORMATION AND MICROPROPAGATION OF THAPSIA GARGANICA L.- A MEDICINAL PLANT", , 1 November 2003 (2003-11-01), XP055380910, Retrieved from the Internet: URL:http://researchspace.ukzn.ac.za/xmlui/bitstream/ handle/10413/10057/Makunga_Nokwanda_P_2003.pdf? sequence=1 [retrieved on 2017-06-13]	1-4,7-12
Y	page 6, paragraph 2.3 - page 20, paragraph 2.7 page 48, paragraph 3.1 - page 63; table 3.2 page 64, paragraph 4.1 - page 87; table 4.1	5,6, 13-15

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2017/050049

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International application No
PCT/DK2017/050049

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