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ORIGINAL PAPER

Cell cultures of *Maytenus ilicifolia* Mart. are richer sources of quinone-methide triterpenoids than plant roots *in natura*

Juliana S. Coppede · Edieidia S. Pina · Tiago A. Paz · Ana L. Fachin · Mozart A. Marins · Bianca W. Bertoni · Suzelei C. França · Ana Maria S. Pereira

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Abstract The quinone-methide triterpenoids (QMTs) are chemotaxonomic markers of the family Celastraceae and many compounds of this class possess important anticancer and anti-inflammatory activities. However, the levels of QMTs in the roots of Celastraceous species are typically very low (ca. 0.0003 %) and commercial production by extraction from such tissues would not be commercially viable. With the aim of determining if cells cultured in vitro might provide alternative sources of these bioactive triterpenoids, we have quantified and compared the concentrations of QMTs accumulated by the roots of plants of *Maytenus ilicifolia* Mart. ex Reissek (Celastraceae) aged 0.5–10 years, and in callus and suspension cultures derived from a cell line that had been subcultured over a period of 10 years. Comparing plants cultivated *in natura*, the highest levels of QMTs were detected in the root bark of 5-year old specimens. However, cell suspensions derived from the long-term cell line retained their capacity to synthesize and accumulate QMTs, and presented levels of maytenin, 22β-hydroxymaytenin, celastrol and pristimerin that were, respectively, 1.96-, 2.48-, 8.85- and 3.29-times higher than those in the roots of 5-year old plants. The results presented herein open up new possibilities for the large-scale production of QMTs and for the development of novel pharmaceuticals.

Keywords *Maytenus ilicifolia* · Cell suspensions · Maytenin · Celastrol · Pristimerin

Abbreviations

QMT	Quinone-methide triterpenoid
MS	Murashige and Skoog
HPLC	High performance liquid chromatography

Introduction

The family Celastraceae encompasses 98 genera comprising approximately 1,210 species that are widely distributed throughout Asia, Africa and the Americas (Simmons et al. 2008; Coughenour et al. 2011). Many members of the Celastraceae exhibit important therapeutic properties and a number are used in the traditional medicines of the indigenous populations (Burkill 1985; Marchese et al. 2009). The significant anti-inflammatory activities associated with these species have been attributed to the presence of triterpenes and quinone-methide triterpenoids (QMTs) (Ohashi et al. 1994; Alvarenga and Ferro 2006).

In Brazil, extracts of *Maytenus ilicifolia* Mart. ex Reissek, one of the 132 Celastraceous species native to the country, are used widely as phytotherapeutic drugs and are even made available through the publicly funded Brazilian health care system. Teas prepared with leaves of *M. ilicifolia* are used to treat stomachaches and gastric ulcers (Camparoto et al. 2002; Jorge et al. 2004), while the roots, which produce the QMTs maytenin, 22β-hydroxymaytenin, celastrol and

J. S. Coppede · E. S. Pina · A. L. Fachin · M. A. Marins · B. W. Bertoni · S. C. França · A. M. S. Pereira (✉)
Departamento de Biotecnologia, Universidade de Ribeirão Preto, Av. Costabile Romano 2201, Bairro Ribeirania, Ribeirão Preto, SP 14096-380, Brazil
e-mail: apereira@unaerp.br

T. A. Paz
Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais, Departamento de Química Orgânica, Instituto de Química, Universidade Estadual Paulista, Araraquara, SP 14801-970, Brazil

pristimerin, exhibit antifeedant (Avilla et al. 2000), anti-inflammatory (Kim et al. 2009), antimicrobial (Luo et al. 2005), antitumor (Costa et al. 2008), antiviral (Murayama et al. 2007), antioxidant (dos Santos et al. 2010), cytotoxic (Almeida et al. 2010) and trypanocidal (dos Santos et al. 2013) activities.

Currently, QMTs are considered one of the most promising chemical classes for the development of new anti cancer drugs (Shanmugam et al. 2012; Wang et al. 2012). However, agricultural production of *M. ilicifolia* for the extraction of QMTs is impractical since the plant is a slow-growing shrubby evergreen tree and the amount of QMTs produced by the roots is very low at around 0.0003 % (Buffa-Filho et al. 2004). Cultivation in a controlled environment, especially in the form of cell or tissue cultures, offers an attractive alternative to conventional agriculture. The advantages of such an approach include circumvention of the phenotypic and seasonal variations that influence the production and accumulation of secondary metabolites, and the possibility of selecting for highly productive clones. Additionally, cell cultures are particularly recommended for the production of candidate compounds that may serve as precursors for new drugs (Smetanska 2008; Hussain et al. 2012).

In this context, we hypothesize that in vitro cultures of *M. ilicifolia* offer stable production of QMTs with higher productivities than plant roots *in natura*. In order to test this hypothesis, the aims of the present study were: (1) to quantify QMTs in roots of plants of *M. ilicifolia* aged 0.5–10 years; (2) to quantify QMTs in cells that had been subcultured in vitro over a period of 10 years; and (3) to compare the productivity of roots *in natura* with those presented by in vitro systems.

Materials and methods

Plant materials

Specimens of *M. ilicifolia* were collected in Jardinópolis, São Paulo, Brazil, and authenticated by Dr. Rita Maria de Carvalho Okano (Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Minas Gerais, Brazil). A voucher specimen (reference number 00755) was deposited at the Herbarium of Medicinal Plants of the Universidade de Ribeirão Preto (Ribeirão Preto, São Paulo, Brazil).

Roots from plants ($n = 3$) aged 0.5, 1, 2, 5 and 10 years (Fig. 1a–e) were separated and washed thoroughly under running water, taking care to avoid mechanical damage. Roots from 10-year old plants ($n = 3$) were separated according to diameter (0.1, 0.3, 0.6 and 1.0 cm; Fig. 1a1–a4) and representative samples were dissected into bark, heartwood (root without bark and pith) and pith (Fig. 2). All plant material was subsequently dried at 50 °C for 48 h in a forced air oven (Marconi, Piracicaba, São Paulo, Brazil).

In vitro cultures

Callus cultures were initiated from leaves of plants that had been micropropagated in vitro over a period of 5 years following the methodology described by Pereira et al. (1994). The callus and cell suspensions employed in the assays were obtained from callus material that had been subcultured every 45 days over a 10-year period. Calli were maintained in glass flasks (8 cm high × 5 cm diameter) on semi-solid MS (Murashige and Skoog 1962) medium (pH 6.0 ± 0.05) containing 0.25 % Phytagel® (Sigma-Aldrich, St. Louis, USA) and supplemented with 3 % (w/v) sucrose, 4.51 μM 2,4-dichlorophenoxy acetic acid and 2.32 μM kinetin. Cell suspensions were cultured in 250 mL Erlenmeyer flasks containing 60 mL of liquid MS medium, supplemented as described above, and maintained on an orbital shaker (model IS 971R, Lab Companion, Seoul, Korea) with agitation at 90 rpm. Cultures were incubated in the growth room at 25 ± 2 °C under a 16 h photoperiod with a photon flux density of 20–70 mmol m^{-2} s^{-1}.

Growth curves and culture sampling

In order to determine growth profiles, an inoculum of callus material equivalent to 1.0 ± 0.10 or 2.0 ± 0.20 g (for calli and suspension growth curves, respectively) was transferred to appropriate MS medium containing supplements and incubated under the conditions described above. Sampling was performed by removing all of the callus material or cells from the growth medium, weighing and drying in a forced air oven at 50 °C for 48 h. The totally randomized experiment was conducted in triplicate with three repetitions ($n = 9$) at each sampling point commencing at day 0 and continuing at 4-day intervals until day 48.

Preparation of extracts

Dried plant material (roots, root parts, calli or cells) was ground to a fine powder (40 mesh), suspended in dichloromethane (1:100, w/v) and submitted to sonication (3×) in an ultrasonic bath (Unique, Indaiatuba, São Paulo, Brazil) for 20 min at room temperature. The suspension was subsequently filtered through filter paper and the filtrate evaporated to dryness according to an adaptation of the method described by Paz et al. (2013).

Quantitative determination of QMTs by high performance liquid chromatography (HPLC)

The concentration of QMTs present in each extract was determined using the method based on Corsino et al.

Fig. 1 Roots excised from *Maytenus ilicifolia* plants of different ages: **a** 10-year old plants: diameter of the roots—0.1 cm (**a1**), 0.3 cm (**a2**), 0.6 cm (**a3**) and 1.0 cm (**a4**); **b** 5-year old plants; **c** 2-year old plants; **d** 1-year old plants; **e** 0.5-year old plants

(1998). Prior to HPLC analysis, the dried extract was dissolved in methanol/water (95:5; v/v) to a final concentration of 2.5 mg mL^{-1}, and an aliquot (1 mL) of the solution was submitted to solid-phase extraction using a Supelco® SPE 6 mL cartridge (Sigma Aldrich, St. Louis, USA) containing 500 mg of C18 silica gel (40 µm particle size) that had been preconditioned with methanol and methanol/water (95:5; v/v). Analytes of interest were eluted from the cartridge with 4 mL of methanol/water (95:5; v/v) while interfering compounds were retained on the stationary phase. The eluate was transferred to a 5 mL volumetric flask, the volume adjusted to the mark with methanol/water (95:5; v/v), and the resulting solution filtered through a 0.22 µm nylon membrane.

The HPLC system consisted of a Shimadzu (Kyoto, Japan) model LC-10-AVP instrument equipped with a SIL-10AF autoinjector, a SPD-M20A photodiode array detector and a Phenomenex (Torrance, CA, USA) Luna C18 column (250 × 4.6 mm i.d.; 5 µm particle size). The mobile phase was methanol/water/formic acid (85:15:0.1; v/v/v) and isocratic elution was performed for 30 min at a flow rate of 1 mL min^{-1}. The injection volume was 20 µL and the detection wavelength was 420 nm. Internal standards were maytenin and 22β-hydroxymaytenin (HPLC purity >98 %) prepared by one of the authors (TAP), and celastrol and pristimerin purchased from Sigma-Aldrich.

Statistical analysis

Multiple comparisons of mean values of analyte concentrations in the extracts were performed using the Scott Knott test at the 5 % confidence level with the aid of Sisvar software (Ferreira 2005).

Fig. 2 Root parts dissected from 10-year old *Maytenus ilicifolia* plants: **a** whole root; **b** root pith, **c** root heartwood; **d** root bark

Table 1 Accumulation of QMTs in whole roots and root bark of *Maytenus ilicifolia* plants of different ages

QMTs (μg g^{-1} dry weight)	Whole roots from plants aged					Root bark from plants aged				
	10 years	5 years	2 years	1 year	0.5 year	10 years	5 years	2 years	1 year	0.5 year
Maytenin	653.7^{Ba}	$2{,}182.5^{Aa}$	182.4^{Ca}	105.1^{Da}	132.5^{Da}	$18{,}957.3^{Ba}$	$30{,}556.1^{Aa}$	$2{,}554.8^{Ca}$	$1{,}157.1^{Da}$	$1{,}060.5^{Da}$
22β-Hydroxymaytenin	86.9^{Cb}	263.2^{Ab}	93.2^{Cb}	60.5^{Db}	119.4^{Ba}	$2{,}703.3^{Bb}$	$3{,}685.4^{Ac}$	$1{,}217.5^{Cb}$	666.1^{Eb}	955.66^{Da}
Celastrol	12.8^{Cc}	122.4^{Ad}	104.8^{Ab}	63.9^{Bb}	$38{,}1^{Cb}$	$2{,}689.6^{Bb}$	$6{,}031.7^{Ab}$	$2{,}462.7^{Ba}$	220.2^{Cc}	129.3^{Cc}
Pristimerin	92.7^{Cb}	430.8^{Ac}	175.9^{Ba}	20.0^{Dc}	16.1^{DB}	371.9^{Cc}	$1{,}713.8^{Ad}$	$1{,}467.6^{Ab}$	703.1^{Bb}	305.2^{Cb}
Total	824.7^{B}	$2{,}634.3^{A}$	530.5^{B}	234.7^{C}	301.8^{C}	$24{,}722.1^{B}$	$41{,}987.0^{A}$	$7{,}702.6^{C}$	$2{,}746.5^{D}$	$2{,}450.7^{D}$

Mean values followed by dissimilar superscript uppercase (rows) and lowercase (columns) letters are significantly different according to the Scott-Knott test ($P < 0.05$)

Results and discussion

Accumulation of QMTs in whole roots and root bark of *M. ilicifolia* plants of different ages

The concentrations of QMTs accumulated in whole roots and in root bark of *M. ilicifolia* were influenced by the age of the plant (Table 1). Thus, the total concentration of QMTs in roots of 5-year old plants was significantly higher than the levels detected in roots of plants aged 0.5, 1, 2 or 10 years. The onset of flowering in *M. ilicifolia* occurs some 5 years after cultivation, at which stage the plants have reached maturity. It would appear, therefore, that full physiological development of the plant is one of the main factors contributing to the increased levels of QMTs in the root tissue. In general, the age of a plant is associated qualitatively and quantitatively with the production of secondary metabolites (Doan et al. 2004; Gobbo-Neto and Lopes 2007). Such correlations have been observed in a number of plant species, including *Artemisia annua* (Asteraceae) in which production of the antimalarial artemisinin and of various bioactive flavonoids is enhanced by flowering (Baraldi et al. 2008). Furthermore, in *Panax ginseng* (Araliaceae), the levels of ginsenosides are relatively low during the first 3 years after cultivation, but increase from the fourth year onwards such that root biomass and concentration of the dammarane-type glycosides are positively correlated (Soldati and Tanaka 1984).

Accumulation of QMTs in whole roots and root bark, heartwood and pith of 10-year old *M. ilicifolia* plants

The concentrations of QMTs that accumulated in the root tissues of *M. ilicifolia* varied significantly according to root

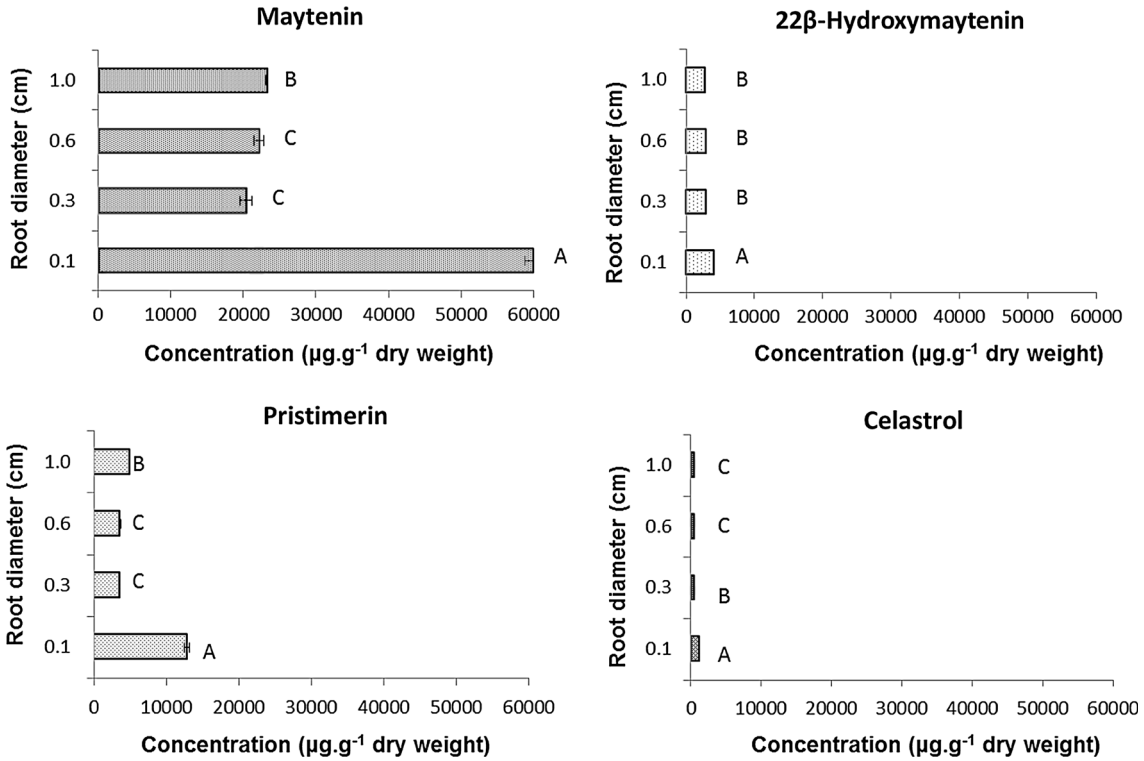

Fig. 3 Accumulation of QMTs in the root bark of 10-year old *Maytenus ilicifolia* plants. Analyses were performed in roots with different diameters. Dissimilar *uppercase letters above bars* indicate that the mean values are significantly different according to the Scott-Knott test ($P < 0.05$)

layer. In 10-year old plants, the average accumulations in whole roots, root bark and root heartwood were 824.70, 61,805.75 and 0.04 µg g^{-1} dry weight, respectively, while no QMTs were detectable in the root pith. These results demonstrate that the sites of accumulation of QMTs are in the external layers of the root, and that accumulation in the root bark is 6×10^4-fold greater than in the root heartwood.

Accumulation of QMTs in the root bark of 10-year old *M. ilicifolia* plants as a function of root diameter

The bark of young roots was thicker than that of older roots, a feature associated with the defense mechanism of the plant. Moreover, in 10-year old plants, the proportion of bark decreased with increasing diameter of the root as follows: 0.1 cm (13.8 %), 0.3 cm (8.6 %), 0.6 cm (6.9 %) and 1.0 cm (3.0 %). These differences were statistically significant (except between roots of 0.3 and 0.6 cm diameter) according to the Scott-Knott test ($P < 0.05$). The accumulation of QMTs in the root bark of 10-year old *M. ilicifolia* plants was significantly greater in bark derived from roots of the smallest diameter (Fig. 3). However, irrespective of root diameter, the concentration of maytenin present was always significantly higher than the levels of 22β-hydroxymaytenin, celastrol and pristimerin.

It is generally accepted that young leaves and roots produce higher amounts of secondary metabolites in comparison with their older counterparts for the purpose of protection (Meldau et al. 2012). Since QMTs are known to be highly toxic to microorganisms (Lima et al. 1969), it is assumed that the production of these compounds by the tender roots of *M. ilicifolia* serves as a form of defense against soil pathogens.

It has been reported that the leaves of *M. ilicifolia*, unlike those of *M. aquifolium*, do not synthesize or accumulate QMTs (Nossack et al. 2004). The results obtained in the present study show that QMTs are always present in the roots of *M. ilicifolia*, even though the quantities may vary according to the morphotype (Buffa Filho et al. 2002a). This finding suggests that QMTs form part of the constitutive defense strategy of the root system of *M. ilicifolia*. The underground environment contains not only a multiplicity of microorganisms but also a large array of root-feeding herbivores, all of which may elicit chemical defense responses from the plant. According to Rasmann and Agrawal (2008), this type of defense reaction is mediated by constitutive metabolites produced/accumulated by the vulnerable tissues. Thus, the most susceptible parts of the plants normally contain high levels of constitutive defense chemicals while, in the less vulnerable parts, the defense reaction is provided by chemicals elicited by

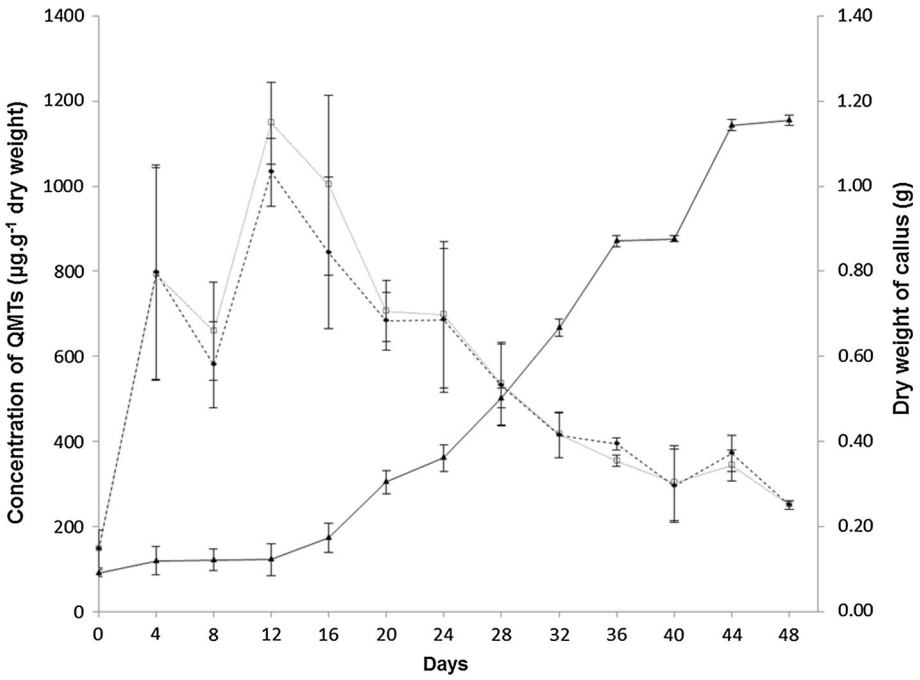

Fig. 4 Growth curve of *Maytenus ilicifolia* callus cultured in MS medium (Murashige and Skoog 1962) supplemented with 3 % (w/v) sucrose, 4.51 µM 2,4-dichlorophenoxy acetic acid, 2.32 µM kinetin, 0.25 % Phytagel (pH 6.0), 16 h photoperiod, and concomitant accumulation of QMTs. *Symbols* Dry biomass (*triangle symbol*); Maytenin (*square symbol*); 22β-Hydroxymaytenin (*circle symbol*)

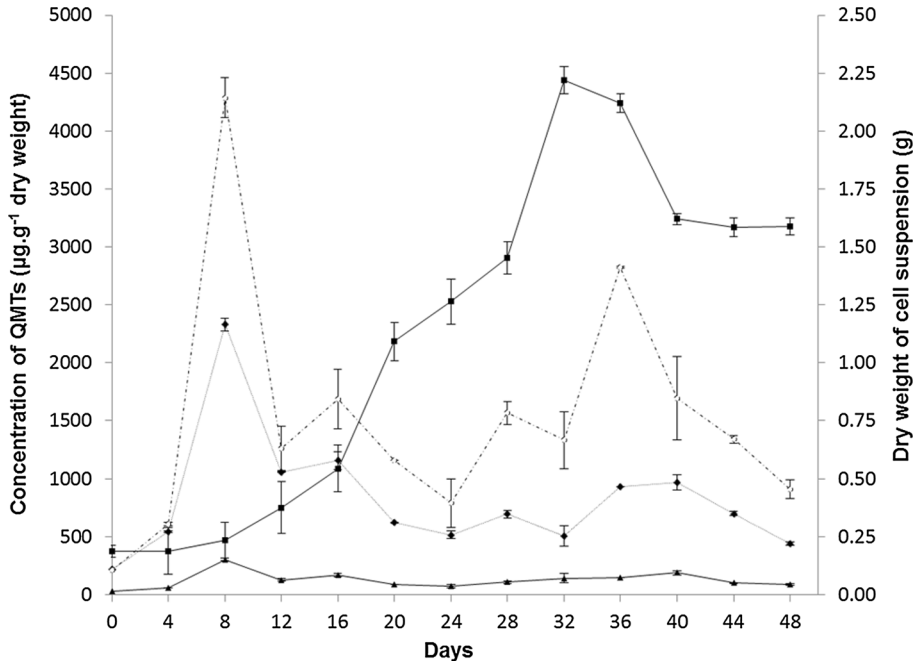

Fig. 5 Growth curve of *Maytenus ilicifolia* cell suspensions cultured in MS medium (Murashige and Skoog 1962) supplemented with 3 % (w/v) sucrose, 4.51 µM 2,4-dichlorophenoxy acetic acid and 2.32 µM kinetin (pH 6.0), 16 h photoperiod, and concomitant accumulation of QMTs. *Symbols* Dry biomass (*square symbol*); Maytenin (*circle symbol*); 22β-Hydroxymaytenin (*diamond symbol*); Celastrol (*triangle symbol*)

injury. This may explain why QMTs are concentrated mainly in the root bark rather than in the inner layers of the root.

Accumulation of QMTs by in vitro cultures

In growth experiments, the quantities of dry biomass produced were maximal at 32 and 48 days after inoculation for suspension and callus cells, respectively, and represented tenfold increases in relation to the masses of inoculum employed (Figs. 4, 5).

The production of maytenin and 22β-hydroxymaytenin by *M. ilicifolia* callus cells was relatively stable during the entire experimental period, and the values recorded at all samplings (except for that at day 36) did not differ significantly one from another (Fig. 4). In absolute terms, the highest accumulations of maytenin (1,147.90 ± 95.09 µg g^{-1} dry weight) and 22β-hydroxymaytenin

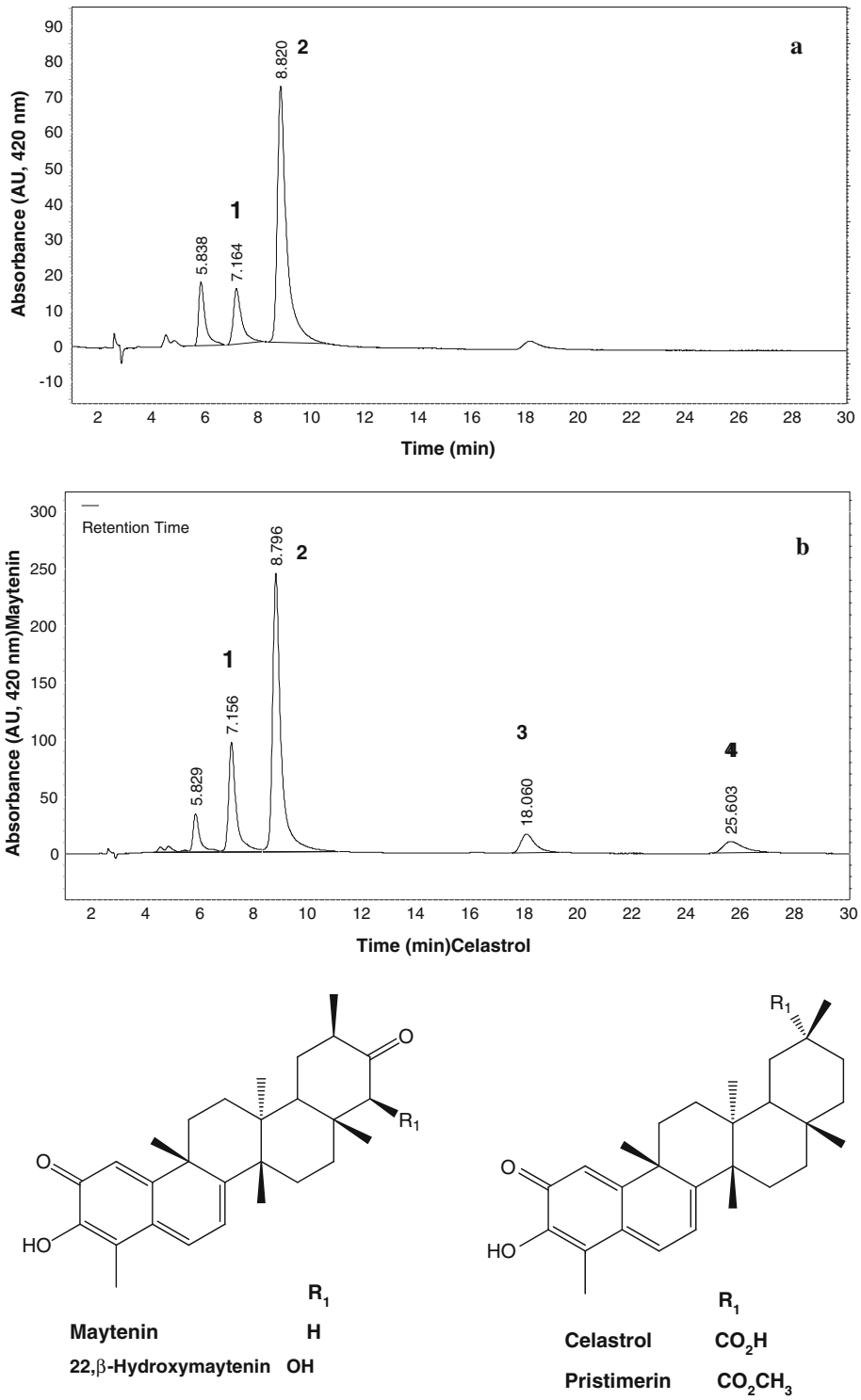

Fig. 6 Chromatographic profile of the dichloromethane extracts from *Maytenus ilicifolia* cultures at day 8 of the growth cycle: **a** callus, **b** cell suspensions. QMTs represented in the spectra are 22β-hidroxymaytenin (**1**), maytenin (**2**), celastrol (**3**) and pristimerin (**4**)

(1,032.89 ± 80.50 μg g^{-1} dry weight) in callus cultures were observed 12 days after inoculation. Such high accumulation of QMTs in the first 2 weeks of culture appears to be a consistent pattern in members of the Celastraceae since it was also verified in root cultures of *Peritassa campestris* (Paz et al. 2013). HPLC analyses of extracts of callus cells sampled on day 8 of the growth cycle (Fig. 6) confirmed the presence of maytenin, 22β-hydroxymaytenin and celastrol, but no pristimerin could be detected.

Biomass production and accumulation of QMTs tended to be inversely proportional in *M. ilicifolia* calli, such that maximum accumulation was observed at the beginning of

Table 2 Accumulation of QMTs in cell suspensions of *Maytenus ilicifolia* obtained from 10-year old callus cultures

QMTs (μg g^{-1} dry weight)	Maytenin	22β-Hydroxymaytenin	Celastrol	Pristimerin
Day 8	$4{,}288.1^{Aa}$	$2{,}329.9^{Ba}$	303.7^{Ca}	130.7^{Ca}
Day 36	$2{,}777.8^{Ab}$	965.3^{Bb}	147.1^{Cb}	20.3^{Db}

Mean values followed by dissimilar superscript uppercase (rows) and lowercase (columns) letters are significantly different according to the Scott-Knott test ($P < 0.05$)

the exponential phase and decreased with increasing biomass. Similar results have been reported for other classes of secondary metabolites produced in vitro or *in natura*, including anthraquinones (Abdullah et al. 1998), flavonoids (Hofmann and Jahufer 2010), sesquiterpene lactones (Yann et al. 2012), lignins (Novaes et al. 2010) and saponins (Fulcheri et al. 1998). These results may support the evolutionary resource availability hypothesis that the costs and benefits of investing in defense (in this case, the production of secondary metabolites) depend on the inherent growth rate of the organism (Endara and Coley 2010; Grime 2001).

Suspension cells of *M. ilicifolia* accumulated predominantly maytenin, followed by 22β-hydroxymaytenin and celastrol, and peaks of production were observed at days 8 and 36 of the culture cycle (Fig. 5; Table 2). Regarding pristimerin, the levels attained were much lower than those of the other QMTs, and peak production was observed at days 8, 12 and 16 of the culture cycle (130.70 ± 6.56, 109.82 ± 10.36 and 98.08 ± 1.69 μg g^{-1} dry weight, respectively). The presence of all four QMTs in extracts of suspension cells sampled at day 8 of the growth cycle was confirmed by HPLC analysis (Fig. 6). Considering the biomass and total QMT productivity profiles in cell suspension cultures, it was concluded that the best time to terminate the culture cycle was at day 36.

The major obstacle to the large-scale production of valuable secondary metabolites by in vitro culture relates to instability regarding the production/accumulation of these compounds and the increase in biomass of the cultures. Such unreliability has been attributed to the repetitive subculture of the cells. Whilst this is true for cell cultures of *Taxus* sp. (Taxaceae) and *Panax ginseng* (Kim et al. 2004; Reshetnyak et al. 2008), in vitro cultures of other species have shown greater stability. For example, *Beta vulgaris* (Amaranthaceae) cells that had been subcultured more than 45 times presented a 1.8-fold increase in the production of betaxanthins (Trejo-Tapia et al. 2008), while cells of *Tabernaemontana divaricata* (Apocynaceae) retained a stable production of alkaloids over the long-term when the levels of growth regulators were appropriately adjusted (Sierra et al. 1992).

The factors associated with the instability of in vitro cultures have yet to be clarified, but among those commonly cited are callus friability (Bolta et al. 2000), inoculum size, exogenous sugar levels, polyploidy (Qu et al. 2011; Patil et al. 2013) and diversity of biosynthesized compounds (Callebaut et al. 1997). Certainly, these issues, along with many others, complicate the standardization of response of long-term cultures with respect to stability.

It is known that callus cultures submitted to repetitive culture cycles undergo somaclonal variation leading to the reduction of biomass and productivity. However, Bourgaud et al. (2001) stated that, following an interval that may vary from months to years, callus cultures reach genetic stability and can be considered as homogenous cell aggregates. Nevertheless, the long-term subculture of callus cultures may favor the stable production of some compounds and hinder the formation of others. For instance, the repetitive subculture of callus from *Erythrina americana* (Fabaceae) exerted a negative influence on the production of alkaloids in general, although one compound of this class, namely erysovine, was only detectable at the ultimate subculture (García-Mateos et al. 2005).

The in vitro production of QMTs was first described by Kutney et al. (1981) in cell suspension cultures of *M. buchananii* that accumulated 0.01 % dry weight of maytenin and 22β-hydroxymaytenin. Later, it was reported that a cell line of *M. ilicifolia* that had been subcultured for a 3-year period, had retained stable growth and productivity, and accumulated 0.004 % dry weight of maytenin and 0.04 % dry weight of 22β-hydroxymaytenin (Buffa-Filho et al. 2002b, 2004). In the present study, we have been able to demonstrate that cell suspensions obtained from the 10-year old *M. ilicifolia* cell line accumulated levels of QMTs that were 27-fold higher than those detected by Buffa-Filho et al. (2004) in the 3-year old callus.

Comparison of *in natura* and in vitro systems for the production of QMTs

Suspensions of *M. ilicifolia* cells produced 1.96-, 2.48- and 8.85-times more maytenin, 22β-hydroxymaytenin and celastrol, respectively, than the roots of 5-year old plants cultivated in the field. Moreover, the production of pristimerin in cell suspensions was 3.29- and 0.71-fold higher, respectively, in comparison with roots of 5- and 10-year old plants (Tables 1, 2). It would appear that *M. ilicifolia* cells cultured in vitro can accumulate more QMTs than roots *in natura* as long as the growth conditions are favorable. Similar findings have been reported for callus cultures of *Centella asiatica* (Mackinlayaceae), which produced 2.3-times more of the triterpenoid saponin madecassoside than 4-month old plants (Kiong et al. 2005). In addition, the concentrations of tyrosol and its glucoside

salidroside produced by suspension cultures of *Rhodiola crenulata* (Crassulaceae) were significantly higher (by 2.95- and 2.88-fold, respectively) than those in the roots of the native plant (Shi et al. 2013).

Conclusion

Callus and cell suspensions of *M. ilicifolia* were qualitatively and quantitatively different with respect to the accumulation of QMTs. Suspensions cultures derived from callus subcultured for more than a decade retained the capacity to synthesize and accumulate QMTs, the levels of which were 2.7-fold higher than those found in whole roots of 5-year old plants. It is concluded that suspensions of *M. ilicifolia* cells represent very promising systems for the production of QMTs since they display the capacity to produce all four QMTs present in plant roots *in natura*. The possibility of in vitro production of QMTs on a large-scale would open new possibilities for the development of novel pharmaceuticals with anti-cancer and anti-inflammatory activities.

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