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Triterpenes and β-agarofurane sesquiterpenes from tissue culture of *Maytenus boaria*

[Triterpenos y β-agarofuranos sesquiterpenos desde cultivo de tejidos de *Maytenus boaria*]

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Abstract: Leaf explants of *Maytenus boaria* were induced towards callus tissue culture with different mixture of cytokinins and auxins. MeOH extract of callus was partitioned with AcOEt and water, and through repeated chromatography procedures were isolated and identified, four triterpenes and three β-agarofuran sesquiterpenes.

Keywords: *Maytenus boaria*, tissue culture, Celastraceae, terpenoids production

Resumen: Explantes de hojas de *Maytenus boaria* fueron inducidos a formar callos mediante diferentes mezclas de citoquininas y auxinas. Un extracto metanólico de los callos fue fraccionado con AcOEt y agua, y mediante repetidas cromatografías fueron aislados e identificados siete compuestos, cuatro triterpenos y 3 sesquiterpenos del tipo agarofurano.

Palabras clave: *Maytenus boaria*, cultivo de tejidos, Celastraceae, producción de terpenoides

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INTRODUCTION

Plants produce a wide variety of so called secondary metabolites (Berenbaum, 2002). The compounds are frequently accumulated by plants in smaller quantities than are primary metabolites (Croteau *et al*., 2000; Dewick, 2009). Plant secondary metabolites are synthetized in specific pathway and sites of production can vary between kinds of compounds as well as between plant species. Moreover, some molecules can be synthetized by all tissues, whereas other are produced in a specific tissue or even cell-specific fashion (Yazdani *et al*., 2011). Production of natural product by plants is not always satisfactory. Some plants are difficult to cultivate, or may grow very slowly. Another way is by the synthesis chemistry, but many compounds present complex structures. For these reasons, different biotechnological tools can be used to produce plant secondary metabolites of commercial interest (Verpoorte *et al*., 2002). Considerable advances have been made in the production of secondary metabolites in plant cell suspension cultures. In general, these advances were developed through the manipulation of media ingredients, especially plant growth hormones, through the development of an efficient cloning procedure and the use of sensitive analytical methods. The use of medium manipulation is generally a random approach and is, therefore, time-consuming; however it has showed good results in plant cell culture system (Heinstein, 1985; Ramachandra & Ravishankar, 2002; Matkowski, 2008; Sree *et al*., 2010)

The family Celastraceae encompasses 98 genera comprising approximately 1210 species that are widely distributed throughout Asia, Africa and the Americas, these species are trees and shrubs and sometime climbing or vining. In Chile the Celastraceae family comprises one genus, *Maytenus*, with four species. Particularly from plants of Celastraceae family a large number of highly oxygenated dihydro-β-agarofuran sesquiterpenoids and triterpenoids have been isolated (Brüning & Wagner, 1978). Many are considered to be important chemotaxonomic indicators. Members of this group of natural products are of particular interest because have antifeedant, insecticidal, cytotoxic, immunosuppressive, anti-HIV, and antitumor activities, as well as their ability to reverse the P-glycoprotein-dependent multidrug resistance (MDR) phenotype of several human cancer cells (Spivey *et al*., 2002).

Many pharmaceuticals are produced from plant such as L-DOPA, morphine, codeine, reserpine and the anticancer drugs vincristine, vinblastine and taxol. Some of these secondary metabolites are quite expensive because of their low abundance in the plant, often less than 1% of the total carbon, or storage usually occurring in dedicated cell or organs. The plant cell, tissue and organ cultures are considered as an alternative way to produce the corresponding secondary metabolites. This progress has notably concerned knowledge of enzyme activities and regulation of biosynthetic pathways (Kirakosyan & Kaufman, 2009).

In Chile, the genus *Maytenus* is represented by fours species, *M. boaria*, *M. chubutensis*, *M. disticha*, and *M. magellanica*. In a previous paper, have been reported on the isolation of dihydro-agarofuran sesquiterpenes from seeds of *M. boaria*, and from aerial part of *M. disticha*, *M. chubutensis* and *M. magellanica* (Gonzalez *et al*., 1989; Alarcon *et al*., 1993; Muñoz *et al*., 1993; Gonzalez *et al*., 1994; Alarcon *et al*., 1995; Alarcon *et al*., 1998). For another hand, the inhibition of the acetylcholinesterase has been reported by agarofurans isolated from Chilean *Maytenus* (Cespedes *et al*., 2001; Alarcon *et al*., 2008).

In a continuation of our studies on these plants, the objectives of the present work was induced callus in first time and then evaluate the capacity by to produce characteristic compounds of these plants under an exploration program forward production of bioactives metabolites with promissory pharmacological applications.

MATERIAL AND METHODS

General experimental procedures

The NMR spectra (400MHz for ^{1}H and 100 MHz for ^{13}C) were measure in $CDCl_3$ (which also provided the lock signal) with Bruker spectrometers. The chemical shift was assigned with distortion-free enhancement of polarization transfer (DEPT) using a flip angle of 135°. IR spectra were recorder on Shimadzu FTIR-8400 instrument.Analytical plates (silica gel, Merck 60G) were rendered visible by spraying with H_2SO_4-Ac_2O followed by heating to 120° C.

Plant material

Young leaves from plant of *M. boaria* were thoroughly washed with tap water, surface sterilized in 70% EtOH for 1 min, rinsed twice with sterile distilled water, immersed in 1.5% sodium hypochloride for 10 min and rinsed four times with sterile distilled water. Callus tissue was induced on MS medium (Murashige & Skoog, 1962) and Hiroshi-Harada supplemented with different 2,4-dichlorophenoxy acetic acid (0, 2.5 and 5.0 mg/L), indol acetic acid (0.25 to 1.0 mg/L), indol butyric acid (0.25 to 1.0 mg/L), kinetin (0.5 mg/L), 2-isopentenyladenine (0.5 mg/L), 6-bencilaminopurine (0.25 to 0.75 mg/L), giberellic acid (0,5 to 1.0 mg/L) concentrations. The pH of the medium was adjusted to 5.7 with 1N NaOH and agar added at 7 g/L before autoclaving for 20 min at 121º C. The cultures were maintained at 25 ± 1º C, under a 16 h photoperiod provided by cool white fluorescent lamps (45 $mmol.m^{-2}.s^{-1}$). Calluses were routinely transferred at 3 week intervals.

Isolation of products

Fresh cells (200 g) were extracted with MeOH at room temperature overnight. After filtration the residue was further extracted with hot MeOH for 3 h. The resulting methanol extracted was concentrated at reduced pressure in rotatory evaporator at 40° C and 250 mb to yield a syrupy methanol extract (3.5 g). The methanol extract was dissolved in distilled water, diluted with methanol to a ratio 60:40 methanol:water, placed in separator funnel, and washed with ethyl acetate (EtOAc). The EtOAc phases combined and concentrated under reduced pressure (1.5 g). The concentrated EtOAc was repeatedly chromatographed on a silica gel column (column diameter 2.5 cm, height 55 cm, 200-425 mesh) using n-hexane, CH_2Cl_2:MeOH, and MeOH afford **1** (0.020 g, 0.57%), **2** (0.025 g, 0.71%), **3** (0.030 g, 0.86%), **4** (0.032 g, 0.91%), **5** (0.006 g, 0.17%), **6** (0.003 g, 0.086%), and **7** (0.008 g, 0.23%). The known compounds were identified by NMR experiment and comparison with published spectral data.

Lupeol

1: was obtained as amorphous white solid. mp 215°C.IR(KBr) ν_{max} cm^{-1} 3311, 2946, 2870, 1638, 1464, 1189, 1035, 996. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm (∫, m, *J*(Hz); assignment): 3.13 (1H,), 4.50(1H, d), 4.65 (1H, d). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 38.4 (C-1), 27.9(C-2), 78.60(C-3), 38.8(C-4), 55.10(C-5), 17.8 (C-6), 35.3(C-7), 40.9(C-8), 50.3 (C-9), 37.4(C-10), 22.80(C-11), 25.10(C-12), 36.40 (C-13), 42.28(C-14), 26.4(C-15), 35.6(C-16), 48.6(C-18), 41.28(C-21), , 33.40, 31.10, 27.90 , 19.10, 14.80 150.8(C-22), 110.10 (C-30). MS: *m/z* (%) $[M]^+$ 426(14), 411(8), 383(1), 218(30), 207(8), 189(21), 149(15), 125(10), 97(56), 69(87), 43(100).

Betulin

2: was obtained as amorphous white solid. Mp. 261°C. IR(KBr) ν_{max} cm^{-1} 3440, 2868, 1638, 1432, 1388. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm (∫, m, *J*(Hz); assignment): 4.67 (1H,s, H-29), 4.57(1H, s, H-29), 3.8 (1H, d, *J*= 12 Hz, H-28), 3.31 (1H, d, *J*=12 Hz, H-28), 3.17 (1H, m, H-3), 1.67 (3H, s, H-30), 1.01 (3H,s), 0.97(3H,s), 0.96(3H, s), 0.81 (3H, s), 0.73(3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.4 (C-1), 27.4(C-2), 78.5 (C-3), 38.8 (C-4), 55.1 (C-5), 17.3 (C-6), 35.2(C-7), 40.9(C-8), 50.3(C-9), 37.4(C-10), 21.7 (C-11), 25.3(C-12), 38.2(C-13), 42.9(C-14), 26.7(C-15), 29.5(C-16), 47.8(C-17), 48.7 (C-18), 48.3(C-19), 30.1(C-20), 34.8(C-21), 150.3(C-22), 23.4(C-23), 23.4(C-24), 16.1(C-25), 18.5(C-26), 15.0(C-27), 60.5(C-28), 21.7(C-29), 110.6(C-30). MS: *m/z* (%) $[M]^+$442(2), 411(65), 222(8), 220(28), 203(70), 207(80), 189 (100).

Oleanolic acid

3: was obtained as amorphous white solid. mp 293-296. IR(KBr) ν_{max} cm-^{1} 3383, 2937,1686, 1460. ^{1}H NMR(400 MHz, CDCl3), δ ppm (∫, m, *J*(Hz); assignment): 0.75(3H,s), 0.78 (3H,s), 0.92 (3H,s), 0.94 (3H,s), 0.98 (3H,s), 1.08 (3H,s), 1.13 (3H,s), 4.45 (1H, dd, *J*=4.56 Hz, H-3), 5.25 (1H, t, *J*=3.76 Hz, H-12). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 38.9(C-1), 28.4(C-2), 79.2 (C-3), 39.5(C-4), 55.4(C-5), 18.5 (C-6), 33.3(C-7), 39.8(C-8), 47.8(C-9), 37.4(C-10), 23.8(C-11), 122.7(C-12), 144.8(C-13), 41.8 (C-14), 28.4(C-15), 23.7(C-16), 46.8(C-17), 41.2(C-18),46.6(C-19), 31.6(C-20), 34.0(C-21), 32.8(C-22), 28.8 (C-24), 16.5(C-25), 17.4(C-26), 26.2(C-27), 183.5(C-28), 33.4(C-29), 23.1(C-30). MS: m/z (%) $[M]^+$ 456(5), 412(3), 248(100), 203(50), 167(25), 44(51).

β-amyrin

4: was obtained as amorphous white solid.mp 186°C. IR ν_{max} 3450, 2945, 2850, 1460, 1385. ^{1}H NMR (400 MHz, CDCl3) (∫, m, *J*(Hz); assignment): δ 5.18 (1H, t, *J* = 3.2 Hz, H-12), 3,22 (1H, dd, *J* = 4.4,10.8 Hz, H-3),1.93(1H, dd, *J*=4.0, 13.7 Hz, H-19β), 1.89(1H, td, *J*=4.0, 14.0 Hz, H-15β), 1.80 (1H, m, H-22), 1.70(1H, td, *J* =4.0, 14 Hz, H-16β) 1.13 (3H, s), 0.99(3H,s), 0.96(3H,s), 0.92 (3H, s), 0.86 (6H,s), 0.83(3H, s), 0.78(3H, s),0.68(1H, d, *J*=11 Hz, H-5). ^{13}C NMR(100 MHz, $CDCl_3$) 38.27(C-1), 26.49, 78.54(C-3), 38.14(C-4), 54.75(c-5), 17.96(C-6), 30.78(C-7), 39.34(C-8), 47.28(C-9), 36.46(C-10), 23.04(C-11), 121.26(C-12), 144.68(C-13), 41.62(C-14), 27.64(C-15), 26.16(C-16), 31.99(C-17), 47.18(C-18), 46.37(C-19), 30.57(C-20), 33.26(C-21), 36.68((C-22), 27.89(C-23), 15.12(C-24), 15.12(C-25), 16.34(C-26), 25.26(C-27), 27.64(C-28), 32.83(C-29), 22.78(C-30). MS: *m/z* (%) $[M]^+$426(6), 218(100), 207(29), 203(47), 189(25).

2β,6β-diacetoxy-1α,9β-dibenzoyl-3β-hydroxy-dihydro-β-agarofuran

5: was obtained as white amorphous powder. M.p. 176-178°C. IR (CHCl3) ν_{max} cm^{-1} 3520, 3480, 3010, 2950, 2910, 1750, 1740, 1720, 1600, 1400, 1270, 1112, 1049. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm (∫, m, J(Hz); assignment): 1.19 (3H, d, *J*=7.6 Hz, H-14), 1.46 (3H, s, H-12), 1.48 (3H, s, H-13), 1.57(3H, s, H-15), 1.86(3H, s), 2.15(3H, s), 2.24 (1H, m, H-8), 2.42 (1H, m, H-4), 4.0(1H, t, H-3), 5.01(1H, d, *J*=6.4 Hz, H-9), 5.36(1H, dd, J=2.6,11 Hz, H-2), 5.44 (1H, s, H-6), 6.36 (d, J=11.0 Hz, H-1). ^{13}C NMR (100 MHz,$CDCl_3$) δ 69.01 (C-1), 71.40(C-2), 73.70 (C-3), 41.61(C-4), 91.45(C-5), 79.85(C-6), 48.11(C-7), 32.10(C-8), 73.30(C-9), 53.38(C-10), 85.59(C-11), 30.65(C-12), 26.34(C-13), 20.22(C-14), 16.22(C-15). MS: *m/z* (%) 579.2[M-Me](6), 552(19), 472(2), 457(3), 416(10), 397(2), 352(2), 248(5), 105(100).

1α,2α,6β,8α-tetraacetoxy-9β-benzoyl-15-hydroxy-dihydro-β-agarofuran

6: was obtained as oil. IR($CHCl_3$) ν_{max} cm^{-1} 3565, 3020, 2920, 2840, 1735,1365, 1270, 1230, 1090, 710. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm (∫, m, J(Hz); assignment): 5.75 (1H, d, *J*=3.30, H-1), 5.50 (1H, dd, *J*=4.0, 6.8 Hz, H-2β), 1.83 (1H, ddd, *J*=1.2, 2.5, 15.0 Hz, H-3α), 2.49(1H, ddd, J=3.9, 6.5, 15.0 Hz, H-3β), 2.39(1H, ddq, *J*=1.2, 6.4, 7.5 Hz, H-4), 5.58(1H, d, *J*=1.0 Hz, H-6), 2.38 (1H, d, *J*=3.0 Hz, H-7), 5.32 (1H, d, *J*=4.0 Hz, H-8), 5.90 (1H, d, *J*=1.0 Hz, H-9), 1.44 (3H, s, H-12), 1.40 (3H, s, H-13), 1.23 (3H, s, H-14), 4.32(1H, d, J=12.5 Hz, H-15), 4.24 (1H, d, J=12.5 Hz, H-15). ^{13}C NMR(100 MHz, $CDCl_3$) δ 69.02 (C-1), 71.42 (C-2), 73.70 (C-3), 41.61 (C-4), 91.45 (C-5), 79.87 (C-6), 48.11(C-7), 32.10 (C-8), 73.33 (C-9), 53.38(C-10), 85.59 (C-11), 30.65 (C-12), 26.34 (C-13), 20.22 (C-14), 16.22 (C-15). MS : *m/z* (%) $[M]^+$ 590(2), 548(2), 530(4), 488(2), 485(1), 468(1), 428(2), 408(2), 348(3), 306(6), 261(2), 202(3), 105(100).

1α,2α,6β,8α,15-pentaacetoxy-9β-benzoyl-dihydro-β-agarofuran

7: was obtained by crystallization from *n*-hexane.Mp 250-251°C. IR(nujol) ν_{max} cm^{-1} 2950, 1725,1715,1460, 1380, 720. ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm (∫, m, *J*(Hz); assignment): 5.71(1H, d, *J*=3.5 Hz, H-1), 5.59(1H, dd, *J*=2.35, 3.5 Hz, H-2), 1.77 (1H, m, H-3α), 2.49 (1H, m, H-3β), 2.39 (1H, ddq, *J*=1.2, 6.5, 7.5 Hz, H-4), 6.38 (1H, d, *J*=1.0, H-6), 2.38 (1H, brd, *J*=3.0 Hz, H-7), 5.27 (1H, d, *J*=3.0 Hz, H-8), 5.52(1H, s, H-9), 1.56(3H, s, H-12), 1.43(3H, s, H-13), 1.16 (3H, 6, *J*=7.5 Hz, H-14), 5.10 (1H, d, *J*=12.5 Hz, H-15), 4.53 (1H, d, *J*=12.5 Hz, H-15).^{13}C NMR (100 MHz, $CDCl_3$) δ 71.6(C-1), 69.2(C-2), 31.9(C-3), 32.7(C-4), 89.8(C-5), 74.9(C-6), 53.1(C-7), 77.8(C-8), 72.9(C-9), 52.6(C-10, 77.5(C-11), 25.8(C-12), 30.3(C-13), 10.9(C-14), 65.3(C-15).MS: *m/z* (%) $[M]^+$632, 617[M-Me](2), 105(100), 77(2).

RESULTS AND DISCUSSION

Many plant species that provide medicinal herbs have been scientifically evaluated for their possible medical applications. It has been mentioned that natural habitats for medicinal plants are disappearing fast and together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into cell cultures as alternative supply for the production of plant pharmaceuticals. The tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions.

For many years, synthetic chemist have been afforded the challenge of developing synthesis of

such components but often due structural complexity the resulting multi-step synthesis rarely find application in large scale production as required. On this way, our goal is to know the condition for the production of promissory secondary metabolites from Celastraceae plants, since these have shown potential pharmacological properties for pharmacological applications such as antitumoral, insecticidal and other (Alarcón *et al*., 1998). It is very important consider that the synthesis conventional of agarofurans it has only allowed a few compounds (Spivey *et al*., 2002; Ishiyama *et al.*, 2013). Therefore is necessary development new alternatives for have molecules by biological studies.

Table 1
The callus inducing media (CIM) with Gamborg's B5 salts, vitamins and plant growth regulators.

	CIM	Growth regulators (mg/L)	Callus formation
	CIM -1	IAA(0.25); IBA (0.25);2-iP (0.5)	-
	CIM- 2	IAA(0.50); IBA (0.50);2-iP (0.5)	+
	CIM-3	IAA(0.75); IBA (0.75);2-iP (0.5)	+
	CIM-4	IAA(1.00); IBA (1.00);2-iP (0.5)	+
	CIM 5	IAA(0.50):BAP(0.5):GA3(0.75)	-
	CIM-6	IAA (0.75); BAP(0.5); GA3 (0,75)	+
	CIM-7	IAA (1.0); BAP(0.5); GA3 (0,75)	+
	CIM-8	IAA (1.5); BAP(0.5); GA3 (0,75)	+
	CIM-9	IAA (1.0);IBA (1.0); GA3 (0.50)	+
	CIM-10	IAA (1.5);IBA (1.5); GA3 (0.50)	+
	CIM-11	IAA (1.0);BAP (1.0); GA3 (0.75)	+
	CIM-11	IAA (1.0);IBA (1.0); GA3 (1.0)	+

IAA = indol acetic acid; IBA = indol butyric acid; 2-iP = 2-isopentenyladenine; BAP= bencyl aminopurine; GA3 = giberellic acid

Plant cell suspension cultures are the most popular strategy among the in vitro plant culture techniques- it is simple, provides homogenous and fast growing material, and is easy to scale up. However, the biosynthetic machinery necessary to generate the various target metabolites may not be available or may afford only small amounts of the desired compounds. We verified initially formation from the leaf explants from *M. boaria* plants maintained under sterile condition was used to establish callus cultures. They treated with IBA, IAA, 2-iP, and GA3 in different proportion resulted into good callus formation and maximum callus biomass with friable features after 8 weeks of culture (Figure 2 and Figure 3). The concentration used in the induction of callus formation (Table 1) did not induced organogenesis. The callus material was subcultures monthly and after 8 months appeared

green, yellow less and white. The combination used in callus inducing medium (CMI), CIM 6, 7, 8 and 9 have the best biomass formation. The other CIM present formation of callus but the velocity of upgrowth biomass formation is slowly. The crude methanol extract of the callus was partitioned between ethyl acetate and water. Repeated chromatography of ethyl acetate fraction yield four triterpene identified as lupeol (**1**), betuline (**2**), oleanolic acid (**3**), and β-amyrine (**4**), and three sesquiterpene agarofuran (**5-7**) (Figure 1 and Figure 4). The compounds **5** was previously isolated aerial part of *Maytenus magellanica* with 0.032% yield (Gonzalez *et al*., 1992), and the compound **6** and **7** were isolated from *M. chubutensis* with 0.13% and 0.12% respectively. Our results indicate that the yield find with our procedures show a production five time higher than occur in plant, thus leaf explant cultures of *M. boaria* are a promising source of terpenoids because the yield are greater in the cell cultures than in the plant, and a base for scaling-up adaptation in view of its future production in bioreactors.

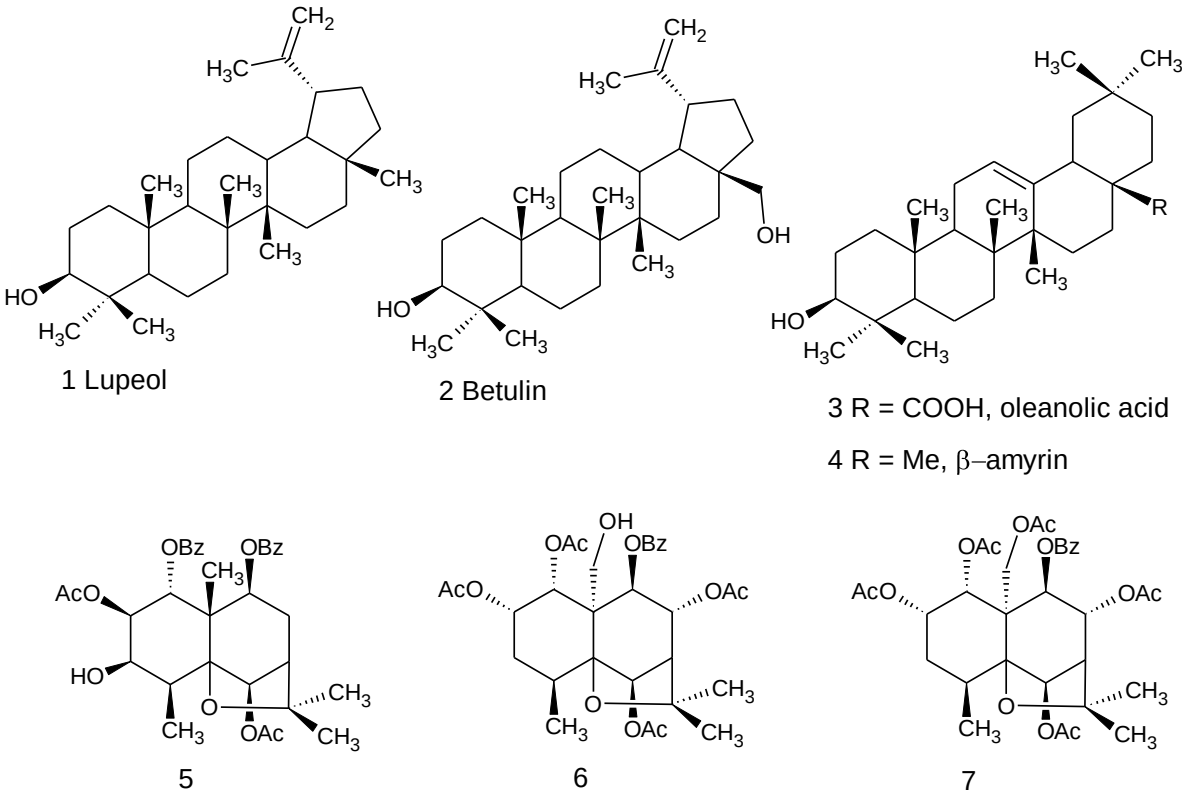

Figure 1
Compounds isolated from callus of *Maytenus boaria*

Previously, best callogenic response have been observed on NAA in combination with BAP, using explant of *Maytenus ilicifolia*, *Peritassa campestris*, or *Tripterygium wilfordii* (Nakano *et al*., 1997; Buffa *et al*., 2004; Antunez *et al*., 2013) which, in accordance with our results, show the combination of cytokinins and auxins is a good mixture by inducing friable callus in leaf explant of *M. boaria*. Tissue cultures of some species of the family Celastraceae seem to be excellent sources of quinonemethide triterpenoids (QMTs) (Kutney *et al*., 1981; Corsino *et al*., 1998; Antunez *et al*., 2013). These studies have been oriented to the production of QMTs therefore developing tissue cultures have from roots, as these compounds accumulate in the roots of plants belonging to this family. The results shows that it is possible to achieve concentrations of metabolites to 8 times larger than it is possible to obtain from the roots (Coppede *et al*., 2014). Other works has been oriented to the resource conservation (Chen, 2009; Sanchez-Chuquicusma *et al*., 2015).

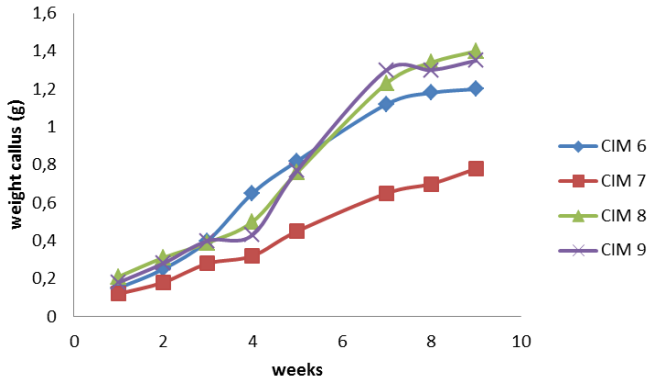

Figure 2
Growth kinetics of callus culture of *Maytenus boaria* on MS medium supplemented with IBA, IAA, 2-iP, and GA3.

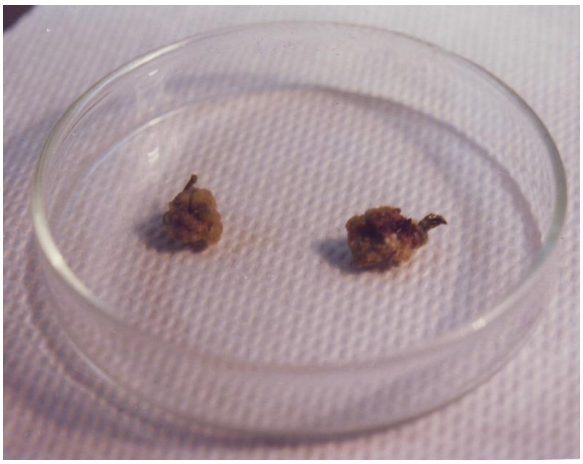

Figure 3
Callus from explants of leaf *M. boaria*

The major advantage of the cell cultures include synthesis of bioactive secondary metabolites, running in controlled environment, independently of climate and soil conditions. The use of in vitro plant cell culture for the production of chemicals and pharmaceutical has made great strides building on advances in plant science.

From this study a best response was obtained with mixture of BAP and NAA *in vitro* production of secondary metabolites under a large-scale would open new possibilities for development of novel pharmaceuticals with several biological activities.

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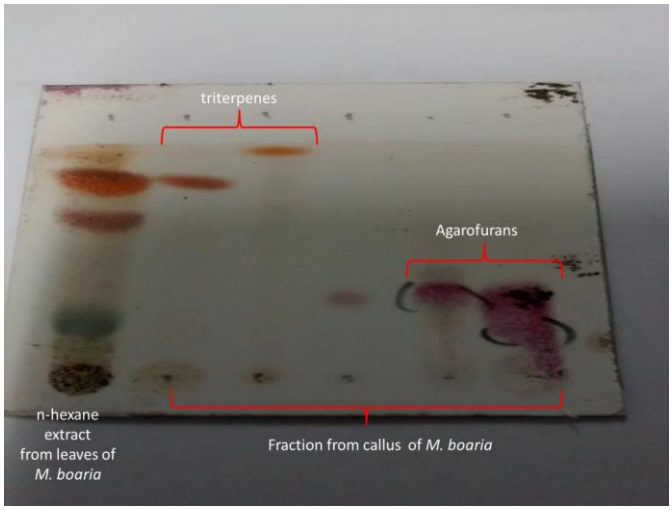

Figure 4
Comparative TLC between apolar extract of leaves of *M. boaria* and fraction from extract of callus of *M. boaria*.

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