TRITERPENES AND PHENOLICS IN CALLUS OF *Maytenus aquifolium* MART.

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Keywords: tannins, friedelin, friedelin-3-ol, anti-ulcer, espinheira santa, *Maytenus aquifolium*

Abstract

The antiulcerogenic effects of *Maytenus aquifolium* Mart. extracts have been confirmed by biological assays and are apparently related to total phenols, more specifically tannins and triterpenes. Leaves of *M. aquifolium* have been widely consumed in Brazil as a tea against ulcer. The present work reports the production of *M. aquifolium* callus on semi-solid media and cell suspension cultures. Both were evaluated for their ability to accumulate triterpenes and phenolics. Dried cells were extracted and phenolics and triterpenes were analyzed by spectrometry and gas chromatography. Friedelan-3-ol was detected in cell suspension and friedelin in callus. Cells cultured in semisolid medium accumulated higher yields of phenolics than cell suspensions.

1. Introduction

*Maytenus aquifolium* Mart., belongs to the Celastraceae family and is popularly known in Brazil as 'Espinheira Santa'. The antiulcerogenic effects of this species have been confirmed by Souza-Formigoni et al. (1991), and are apparently related to total phenols, more specifically the tannins, and the triterpenes (Pereira et al., 1993).

Aqueous extracts of *M. aquifolium* leaves have been widely consumed in Brazil as a tea against ulcer. Extracts of plants cultivated in Ribeirão Preto - SP produce an average of 5% of total phenols and 0.8% of triterpenes, being 0.2% friedelin and 0.6% friedelin-3-ol (Pereira et al., 1995). *M. aquifolium* is a large shrub which shows considerable genetic variability and slow growth admitting the first harvest only 5 years after planting. A micropropagation protocol was established by Pereira, et al. (1994), to obtain *M. aquifolium* clones and to accelerate this species growth.

Studies involving cell cultures of plants from the genus *Maytenus* have been developed in the search for maytenine an ansamacrolide antileukemic compound, however it has not been found in cultures (Kutney et al., 1981).

The objective of the present work was the production of *M. aquifolium* callus on semisolid media and investigation of the presence of triterpenes and total phenols in established cultures.

2. Materials and methods

Axenic leaf segments (1 cm²) from micropropagated plantlets produced following methodology described by Pereira et al. (1994), were inoculated on MS medium (Murashige & Skoog, 1962) supplemented with KH₂PO₄ (final concentration 510 mg/L) solution, 3.0% (w/v) sucrose, 0.5–5.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1–1.0 mg/L Kinetin (Kin) in 16 different concentration levels.
Callus induced on MS medium containing 5.0 mg/L 2,4-D and 0.5 mg/L Kin, were monthly subcultured in the same medium for one year. After this period callus biomass was collected every 3 days during 1 month for establishment of the growth curve and also triterpene and total phenol quantitation. The experiments were conducted with 13 replicates.

Fourteen-month-old callus cultured in MS medium containing 5.0 mg/L 2,4-D and 0.5 mg/L Kin were transferred to media containing the following growth regulator combinations: a) 0.5 mg/L 2,4-D and 0.1 mg/L Kin, b) 0.5 mg/L 2,4-D and 0.5 mg/L Kin, c) 1.0 mg/L 2,4-D and 0.1 mg/L Kin, d) 1.0 mg/L 2,4-D and 0.5 mg/L Kin. Thirty replicates were used for each different medium.

All in vitro cultures were carried out in 250 mL-glass flasks (12 cm x 6.5 cm i.d.) containing medium gelled with 0.2% (w/v) Phytagel (Sigma), pH adjusted to 5.7 autoclaved at 121°C and 98 KPa for 15 min. Flasks were covered with aluminum foil sealed with plastic film. Cultures were kept at 25 ± 2°C under a 16-h day photoperiod (40 μmol m⁻² s⁻¹ 85 W cool-white GE fluorescent lamps) light regime.

After 30 days cells were harvested and the growth index: \( (f.w.)_f - (f.w.)_i / (f.w.)_i \) was determined.

Dried cells were submitted to extraction and phenolics and triterpenes were analyzed by spectrometry and gas chromatography respectively as already described by Pereira et al. (1995).

*M. aquifolium* leaves from ten-year old intact plants harvested in June of 1996, were dried in oven under circulating air at 50°C, ground (0.21-0.35 mm in diameter) and analyzed for triterpene and total phenol determination.

Yields of target molecules in intact plants were compared to those of *M. aquifolium* callus.

### 2.1. Cell suspension cultures

Callus subcultured during 14 months in MS semi solid medium supplemented with 5.0 mg/L 2,4 D and 0.5 mg/L Kin were transferred to MS liquid medium added with 0.5 mg/L 2,4 D and 0.1 mg/L naphthalene-acetic acid (NAA). The initial inoculum consisted of 3 g of fresh biomass/50 mL of culture medium. Liquid cultures were filtered under vacuum and kept under agitation (120 rpm) for 30 days. After this period cells were dried, extracted and analyzed for triterpene contents.

### 2.2. Triterpene determination

Samples of dried leaves and callus (1 g) were extracted 3 times with hexane (10 ml) at the boiling point. Hexane extracts were pooled and submitted to a dechlorofiltration with active carbon (100 mg) followed by a second filtration. Crude extract was concentrated to dryness. The solvent was eliminated from the extract in a rotary evaporator under vacuum. The residue was dissolved in chloroform and the volume was adjusted to 1 ml in a volumetric flask.

The final solution was directly analyzed by high resolution gas chromatography (HRGC) using a Varian 3400 ex gas chromatograph with a flame ionization detector (FID). The column was a DB-1, 30 m long, 0.256 mm i.d. and 0.1 μm film thickness with a cross-linked methylpolysiloxane stationary phase. Samples were injected using the split mode (split ration 1:30) with the injector temperature at 300°C and detector at 315°C. Column temperature was held isothermal at 315°C. The resulting data were processed on a varian 4400 integrator. Quantitative levels were determined by comparison to an external reference standard. The response factors of friedelin (purity
92.08%) and friedelin-3-ol were correlated to standards in the same range. Every analysis was performed at least 3 times.

2.3. Total phenol determination

The official method of the AOAC (1984) adapted to plant material was used for phenol determination. The pulverized plant material (200 mg) was extracted 5 times with 10 ml of boiling MeOH/H2O (1:1 v/v). The extract was filtered and reconstituted to 100 ml. Samples (0.2ml) of the reconstituted extract were utilized to determine total phenols by comparison with a tannic acid standard of known concentrations (AOAC, 1984). The absorbance of the extract at 730 nm was determined using a U-110 in Hitachi spectrometer and quartz cells of 1 cm of path length.

3. Results and Discussion

Explants cultured in medium supplemented with growth regulators, produced homogeneous callus mass in only one of the tested combinations, 5.0 mg/L 2,4-D and 0.5 mg/L Kin, however callus were compact, no proliferative, and it took 12 months of subculture to get friable callus.

The growth curve of callus cultured under the above conditions, revealed that the logarithmic phase of M. aquifolium callus development occurred during the third week of cultivation. Quantitation of triterpenes performed simultaneously evidenced that higher accumulation of total phenols (2.68%) happened around 27 days of culture and no triterpene could be detected in callus cultured on medium containing 5.0 mg/L 2,4-D and 0.5 mg/L Kin (Figure 1).

Accumulation of phenolics is not growth related since increased level of those compounds is reached after 20 days of culture, i.e., during the stationary phase of the growth cycle.

Addition of Kinetin to the medium influenced positively the biomass accumulation. The increase in Kin level from 0.1 to 0.5 mg/L induced a negative effect on friedelin production, reducing yield in 70%. According to Fernandes-Ferreira et al. (1992), the production of triterpenes in callus of Euphorbia characias was also strongly influenced by the type and concentration of growth regulators.

Callus cultured in 1.0 mg/L of 2,4D and 0.5 mg/L of kinetin, produced the highest growth ratio (2.252) and phenolics (3.9%) but the lowest production of triterpene (0.0067%). Callus exposed to lower concentrations of 2,4D produced friedelin and the combination of 0.5 mg/L of 2,4D and 0.1 mg/L of kinetin induced enhanced yield of friedelin (0.0197%) (Table 1).

Friedelan-3-ol is usually the predominant type of triterpene found in M. aquifolium leaves (0.64%); however it was undetectable in callus. Yield of friedelin in cultured callus was 8% of the amount present in adult leaves (0.18%).

Concentration limit of Kin enhanced to 0.5mg/mL also increases the production of phenolics (Table 1). This result is in accordance with that previously related by Shah et al. (1976) for Cassia tissue cultures.

Accumulation of phenolics and triterpenes was strongly affected by the ratio of 2,4D and kinetin supplementation tested (Figure 2). There is an inverse correlation (r = -0.9733) at level 5% between accumulation of phenolics and triterpenes. The linear regression equation is y = 0.0397-0.0081x.

Callus produced in all tested media presented dark coloration (dark blue, half black). The cells maintained that aspect until the 20th day of culture when aggregates of white cells appeared and overlaid the dark cells.
Callus heterogeneity concerning to coloration seems to be a characteristic related to genus *Maytenus*, since Kutney et al. (1981) also observed this characteristic when working with *M. buchananii* callus.

Cells produced in suspension cultures also showed the dark blue coloration and diversely of callus only produced friedelan-3-ol (0.02%). Besides, growth and yields of total phenols were relatively lower.

The production and accumulation of friedelin and friedelan-3-ol in *Maytenus* cells cultured *in vitro* is important because those substances are related to the healing of gastric ulcers, considered potent antiinflammatory (Shimizu and Tomoo, 1994) and present confirmed citotoxic action (Zheng, 1994).

4. References


Table 1 - Yields of Triterpenes and Phenolics in 27-day old callus cultures of *M. aquifolium*

<table>
<thead>
<tr>
<th>Growth Regulators</th>
<th>Growth index</th>
<th>Friedelan-3-ol %</th>
<th>Friedelin %</th>
<th>Phenolics %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 2.4D + 0.1 Kinetin</td>
<td>0.98 bc</td>
<td>0.02a</td>
<td>2.44 b</td>
<td></td>
</tr>
<tr>
<td>0.5 2.4D + 0.5 Kinetin</td>
<td>1.85 ab</td>
<td>0.01b</td>
<td>3.73 a</td>
<td></td>
</tr>
<tr>
<td>1.0 2.4D + 0.1 Kinetin</td>
<td>0.22 d</td>
<td>0.02 a</td>
<td>2.56 b</td>
<td></td>
</tr>
<tr>
<td>2.4D + 0.5 Kinetin</td>
<td>2.25 a</td>
<td>0.01 b</td>
<td>3.91 a</td>
<td></td>
</tr>
</tbody>
</table>

Cell Suspension Culture

| 0.5 2.4D + 0.1 NAA | 0.31 | 0.02 | 0.70 |

In columns means followed by the same letter do not differ statistically at \( p \leq 0.05 \) according the Tukey test.

![Graph](image)

**Figure 1 - Yield of phenolic compounds in *M. aquifolium* callus**
Figure 2 - Yield of phenolics and triterpenes in callus cultures of *M. aquifolium*