Secondary Metabolite of Horse Chestnut in Vitro Culture

Dušica Ćalić-Dragosavac, Svetlana Stevović, Snežana Zdravković-Korać, Jelena Milojević, Aleksandar Cingel and Branka Vinterhalter

1Department of Plant Physiology, Institute for biological research “Siniša Stanković”, University of Belgrade, Serbia
2Department of Environmental Protection, Faculty of Ecology and Environmental Protection, University Union, 11000, University of Belgrade, Serbia

Dušica Ćalić-Dragosavac, Svetlana Stevović, Snežana Zdravković-Korać, Jelena Milojević, Aleksandar Cingel and Branka Vinterhalter

ABSTRACT

Plants are source of many compounds useful in medicine, pharmaceutical and cosmetic industry. Horse chestnut (Aesculus hippocastanum L., Hippocastanaceae) seed extract is widely used for the treatment of peripheral vascular disorders, and in cosmetics as a remedy against cellulites. It has a general vasoprotective role by protecting collagen and elastin. By protecting these key vessel proteins, veins and capillaries stay strong and maintain their structural integrity when exposed to stress. Aescin was found to be effective as anti-inflammatory, anti-tumor and anti-viral agent. Horse chestnut extracts are more powerful anti-oxidants than vitamin E, and also exhibit potent cell-protective effects, which are linked to the well-known anti-ageing properties of anti-oxidants. The present study is aimed to determine the content of aescin in horse chestnut androgenic embryos growing on media supplemented with various hormones. Aescin content was determined in cotyledonary embryos grown with media supplemented with various hormones. Aescin content was determined in cotyledonary embryos grown on media supplemented with various hormones. Aescin content was found to be dependent on the stage of androgenic embryo development and the type of the phytoregulator supplemented to the nutritive medium. In the absence of the phytoregulators, androgenic embryos at the globular stage of development contained approximately four times less aescin than those at the cotyledonary stage. Inclusion of various phytoregulators in the nutritive media stimulated aescin production. In conclusion, horse chestnut androgenic embryos produce high amount of aescin, which can be manipulated by the addition of phytoregulators. We find this approach promising for resolving the problems associated with commercial production of aescin.

Key words: Aesculus hippocastanum, androgenic embryo, triterpenoid saponins.

Introduction

Plants serve as a valuable source of many compounds used in medicine, pharmaceutical and cosmetic industry. Horse chestnut (Aesculus hippocastanum L., Hippocastanaceae) seed extract is widely used for the treatment of peripheral vascular disorders [1], and in cosmetics as a remedy against cellulitis [2]. It exhibits potent anti-inflammatory [2], antitumor, antiviral [3] and antioxidative [4, 5] effects as well.

An active component of the horse chestnut seed extract is a group of chemically related triterpenic saponoids, known as aescin. The main source of...
these compounds are the seed cotyledons, but they are also present in trace amounts in the seed integuments, the barks, buds, leaves and the immature fruit pericarps of *A. hippocastanum* [1] and other species within the genus *Aesculus* [6,7]. A small amount of aescin was detected in native roots of *Aesculus turbinata* Blume [8] and seedling roots of *Aesculus parviflora* Walt. [9].

As the zygotic embryos are the main site of aescin accumulation, horse chestnut seeds are the sole industrial source of aescin. This raw material is available only for a short period during the year and the pharmacologically active tissue (zygotic embryo) presents only a small part of the seed (up to 500 mg per seed fresh weight). Furthermore, Caldas and Machado [10] found high levels of lead in leaves and seed extracts of horse chestnut. Taking all this into consideration, plant cell and organ culture may be an alternative solution for aescin production.

Profumo et al. [11] and Gastaldo et al. [12] demonstrated the presence of substantial amount of aescin in embryogenic calli and somatic embryos of *A. hippocastanum*, which remained high after 2 years of culture [11], and could be increased further by applying certain plant growth regulators [13].

Our previous studies showed significant growth and multiplication, as well as simple nutritive demands of androgenic embryos [14] and horse chestnut hairy roots [15]. Therefore, we assume that these cultures can be used as an alternative source for large-scale aescin production. The present study was aimed at determining the content of aescin in horse chestnut androgenic embryos grown on media supplemented with various phytoregulators.

**Materials and Methods**

**Plant material:**

Anthers were obtained from the 100-year-old horse chestnut tree (N, P1/15) growing in the Botanical Garden “Jevremovac”, University of Belgrade, according to previously described protocol [14]. In brief, anthers with uninucleated microspores were macerated, suspended in 100 ml of liquid MS [16] medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (Kin), 1.0 mg/l each, and sieved through a 50 μm mesh. The cultures were shaken on a platform shaker (85 rpm) in darkness for 4 weeks. Cell suspensions produced after 4 weeks were mixed with equal volumes of cooled (30°C) MS medium supplemented with 0.01 mg/l 2,4-D, 1 mg/l Kin and 0.7 % agar and dispensed in Petri dishes. Solid medium of the same composition was used to cultivate regenerated embryos and to maintain the cultures. Development of androgenic embryos is asynchronous, so that embryos at different developmental stages (globular, heart-like, torpedo-like and cotyledonary) were observed in the same culture (figure 1). Embryos at early heart-like and torpedo stages were transferred to phytoregulator-free MS medium and cultivated for a month. After one month, those among them reaching the cotyledonary stage (5-10 mm, figure 1) were transferred to MS medium supplemented with 0.1, 0.2 and 0.5 mg/l each of various phytoregulators: 2,4-dichlorophenoxyacetic (2,4-D), 6-furfurylaminopurine (Kin), or abscisic acid (ABA). Basal medium contained MS mineral salts, 2 % sucrose, and was supplemented with the following (mg/l): pantothentic acid 10, nicotinic acid 5, vitamin B1 2, adenine sulphate 2, myo-inositol 100 and casein-hydrolysate 200. All media were sterilized by autoclaving at 0.9 x 10⁵ KPa and 114°C for 25 min. The cultures were maintained at 25 ± 1°C under 16-h photoperiod, at irradiance of 33 - 45 μmol m⁻² s⁻¹ provided by cool white fluorescent tubes.

**Analysis of aescin:**

Amount of aescin was determined in cotyledonary androgenic embryos grown on media with various phytoregulators. Leaves and seeds collected from the nature were used as a control. Leaves were harvested in June and seeds were collected in October. The seeds were stored in a dry and cooled storage till January, and then the zygotic embryos were isolated and subjected to aescin analysis.

Aescin analysis was conducted according to the German Pharmacopoeia (Deutsches Arzneibuch 10) [17]. Plant material was lyophilised and 1g of powdered tissue was extracted with 100 ml of 65% methanol (v/v) 30 minutes on a water bath under reflux. Then, 30 ml of extract was evaporated till dryness and 20 ml 0.1 N HCl was added. Reextraction was done with mixture of 20 ml n-propanole and 50 ml chlorophorm and extract was evaporated till dryness and then washed with ether. Dried residue was dissolved in anhydrous acetic acid into 50 ml volumetric flask. Five ml of solution was transferred in 25 ml volumetric flask and ferry chloride reagents (75 mg of ferry chloride with 50 ml anhydrous acetic acid and 50 ml concentrated sulfuric acid, freshly prepared) was added till 25 ml. Solution was heated at 60°C on a water bath for 25 minutes and then cooled to room temperature. Control/blank was prepared with 5 ml of anhydrous acetic acid. Absorbance was measured on 540 nm. The amount of aescin was calculated from the expression:

\[
\% \text{ of aescin} = \frac{A_{540} \times 13.89}{m}
\]

where \(A_{540}\) is the absorbance and \(m\) is the amount of sample in grams.
Three replicates, taken from different Petri dishes, were used for each treatment, and the experiment was repeated three times.

**Results and Discussion**

Androgenic embryos at the cotyledonary stage (figure 1) were used for studies. Since aescin content in the zygotic embryo cotyledons of stored horse chestnut seeds was found to increase gradually, reaching the maximal value in January [18], we used the zygotic embryos at this stage as a control for comparison with our samples. Cotyledonary embryos cultured on phytoregulator-free medium produced almost three times lower amount of aescin than zygotic embryos and similar amount as the leaves, presented in table 1. The inclusion of various phytoregulators (2,4-D, Kin and ABA) in the nutritive media increased aescin production in cotyledonary androgenic embryos about 2 times (table 1). The different concentration (0.1, 0.2 and 0.5 mg/l) of phytoregulators were tested. The best results for aescin production showed concentration of 0.5 mg/l for all type of phytoregulators. Similarly, Profumo et al. [13] found that embryogenic calli produced 2-3 times more aescin when grown on phytoregulator-supplemented media compared to phytoregulator-free medium. 2,4-D (0.5 mg/l) showed the most pronounced effect, with aescin production almost reaching that of the zygotic embryos, table 1. However, 2,4-D is a synthetic auxin and strong herbicide and is considered as a stressor that provokes numerous physiological responses to greater extent than naturally occurring auxins [19]. This study also confirmed that aescin formation is an intrinsic embryo feature, independent of their origin and the ploidy level. Somatic embryos induced from leaves, cotyledons and stem segments [11,20] produced similar amounts of aescin as haploid androgenic embryos induced from uniculated microspores (this study) and the zygotic embryos from the nature.

Commercial *in vitro* production of any particular compound demands stable cell lines exhibiting high yields and growth potential. Although plant cell suspension cultures have been recognized as a method of choice in some cases [21,22], insufficient yield and metabolic instability of the suspension cultures often limit this approach. However, differentiated tissues display both genetic and biochemical stability [23].

![Fig. 1: Horse chestnut androgenic embryos at the globular (g) and cotyledonary (c) stage of development.](image)

### Table 1: Aescin content in horse chestnut androgenic embryos.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Hormone free</th>
<th>2,4-D</th>
<th>Kin</th>
<th>ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves (control)</td>
<td>5.05 ± 0.02</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Zygotic embryos (control)</td>
<td>6.96 ± 0.10</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Cotyledonary androgenic embryos</td>
<td>2.43 ± 0.10</td>
<td>4.55 ± 0.11</td>
<td>5.16 ± 0.22</td>
<td>6.77 ± 0.45</td>
</tr>
</tbody>
</table>

The aescin content is expressed as percentages on the basis of dry weight. Data indicate means ± SD of three independent experiments.

**Acknowledgments**

This work was supported by the Ministry of Science and Technological Development, Serbia, grants No. 143026 and No. EE18031.

**References**