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# Aescin formation in calli and embryoids from cotyledon and stem explants of *Aesculus hippocastanum* L.

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**Abstract**—Aescin in calli and embryoids obtained from both cotyledon and stem explants of *Aesculus hippocastanum* were investigated by HPLC. Determinations were carried out on tissues cultured in agarized medium supplemented with growth substances (2,4-dichlorophenoxyacetic acid; kinetin; 1-naphthaleneacetic acid). The results indicate that aescin was produced in all the analysed samples. The amount of active principle present in some samples was higher than that found in horse-chestnut seeds.

Somatic embryogenesis had been obtained starting from quite different types of *Aesculus hippocastanum* L. explants; primary leaves (Dameri et al 1986), anthers (Radojević 1978), cotyledons (Profumo et al 1990), staminal filaments (Jörgensen 1989) and stem (Gastaldo et al 1994) were used for such a goal. These studies have a pharmacobotanic interest because of the great industrial importance of aescin, the active principle of the horse-chestnut, which has a large application in pharmaceutical and cosmetic preparations. In previous studies, we estimated aescin concentration in calli and embryos that were obtained from primary leaves (Profumo et al 1991). The results encouraged us to plan the experiments reported in the present work, the principal aim of which was to investigate aescin in proliferations obtained from explants of very different origin: cotyledons, that are the main natural accumulation site of the active principle and its sole industrial source, and stem, part of the plant that in-vivo contains very small quantities of active principle (Kartning et al 1965, 1966; Kartning & Hiermann 1968).

## Materials and methods

*In-vitro culture.* The explants used in the experiments reported here were obtained from an *Aesculus hippocastanum* L. plant growing in the Genoa University Botanic Garden.

Cotyledonary fragments were excised from ripe seeds. Stem explants were obtained by cutting fragments of about 2 cm excised from young branches (about 0·5 cm in diameter). The stem portions were placed in their natural position in the culture medium.

For the formation of calli, both types of explants were cultured in agarized medium (0·9% Difco Bacto-Agar) supplemented with growth regulators (Murashige & Skoog (1962)); kinetin, 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid were used at a concentration of 2 mg $L^{-1}$ each.

For each group of explants, 60 test-tubes were prepared; the experiments were repeated twice in two different years (1992, 1993). The cultures were kept in a growth room at 25 ± 1°C under diffuse fluorescent light (3000 lx) in 12-h photoperiods. As described previously (Gastaldo et al 1994), within a month the explants produced a white compact callus in great quantity ($E_1$ callus). Later, a friable green-yellow callus ($E_2$ callus) formed on the top of the compact white callus or directly on the explant; after about one month, this callus produced low frequency embryoids. The embryogenetic callus was transferred into flasks on the same nutritional medium without growth substances; within two weeks it grew abundantly and produced high frequency embryoids.

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*Aescin extraction and determination.* Aescin extraction and determination were performed according to Wagner et al (1985), modified as described previously (Profumo et al 1991). Briefly, each finely powdered tissue sample (1 g) was extracted overnight with 60% methanol (25 mL) at room temperature (21°C). The extract was filtered and adjusted to 30 mL with 60% methanol in a calibrated flask; 6 mL of this solution was diluted to 12 mL with water and adsorbed on Sep-Pak C-18 cartridges (Waters, Milford, MA, USA). Aescin was eluted from the resin with methanol (4 mL) and subjected to HPLC analysis.

Perkin-Elmer (Norwalk, CT, USA) HPLC equipment with a UV detector (Model Series 10/LC-15) and a Shimadzu (Kyoto, Japan) data processor (Model C-R6A Chromatopac) was used. The column was a Hibar LiChrospher 100 CH-18/2, 10 $\mu$m (Merck, Darmstadt, Germany). The mobile phase was 35% $CH_3CN$ with 10 mL 0·1 M $H_3PO_4$ per litre and flow rate of 1·2 mL $min^{-1}$. The detection wavelength was 254 nm. Recovery, tested with authentic aescin, was 98%. Samples of each type of tissue (1 g) were dried in an oven at 80°C and weighed to obtain percentage dry weight.

For each type of tissue examined, five pools of samples were collected from different flasks. All the samples were processed in duplicate together with external standards of authentic aescin (Inverni della Beffa, Milano, Italy). Aescin identification and quantitative determination were carried out as described previously (Profumo et al 1991). Aescin content was expressed in percent of dry weight.

## Results

Fig. 1 shows HPLC analysis of a purified extract of embryoids from stem explants as an example of the results obtained with horse-chestnut proliferations. Peaks A–E are due to $\beta$-aescin which is a mixture of saponins that, in the analytical conditions

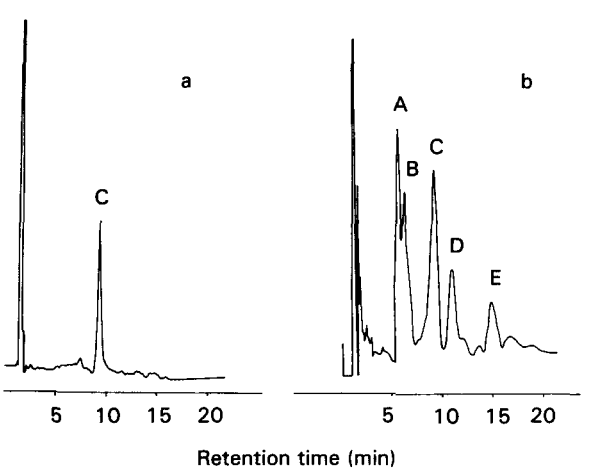


FIG. 1. High-pressure liquid chromatograms of aescin. a. Standard solution (25 mg $L^{-1}$); b. somatic embryos obtained from stem explants cultured in agarized MS medium supplemented with kinetine, 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid (2·0 mg $L^{-1}$ of each). Quantitative determination of aescin was made by the one-point calibration curve method grouping peaks A–E.

Table 1. Aescin content in calli and somatic embryos from cotyledon and stem explants of *Aesculus hippocastanum* L. cultured in-vitro in agarized MS medium supplemented with phytoregulators (kinetine, 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid, 2·0 mg $L^{-1}$ of each); ripe cotyledons in-vivo were used as control.

| Material | Sample | Dry wt (%) | Aescin content/ dry wt (%/dry wt) |
|---|---|---|---|
| Explants from stem | $E_1$ callus | 7·0 | 37·3 ± 0·8 |
| | $E_2$ callus | 4·0 | 47·1 ± 7·7 |
| | Embryos | 7·8 | 9·8 ± 0·1 |
| Explants from cotyledons | $E_1$ callus | 5·8 | 31·4 ± 7·2 |
| | $E_2$ callus | 5·9 | 38·2 ± 8·7 |
| | Embryos | 5·2 | 7·5 ± 1·4 |
| Seed | Ripe cotyledons | 56·7 | 10·7 ± 0·5 |

Data are mean ± s.e.m.; the number of observations is six for each type of sample.

used, have different retention times (Wagner et al 1985). Table 1 shows aescin concentration (as percentage of dry wt) in the initial $E_1$ calli, in $E_2$ calli derived from them and in embryoids obtained from both cotyledons and stem explants cultured in-vitro; aescin content in ripe cotyledons in-vivo was used as a control value.

The data reveal that $E_1$ calli, derived either from cotyledons or from stem, have an aescin content higher than the control; the amount of active principle is slightly increased in the corresponding $E_2$ calli and significantly decreased in embryoids, where it reaches values similar to those found in ripe cotyledons.

Standard error is relatively high for $E_1$ and $E_2$ calli because it is sometimes very difficult to separate the samples clearly; furthermore, $E_2$ samples can contain newly formed embryos that cannot be easily separated from the surrounding tissues.

## Discussion

The results confirm that in-vitro cultured tissues are an excellent aescin source for possible future industrial applications because they are rich in aescin, the material used as explant source is easy to find, and extraction procedures are less expensive than those necessary when horse-chestnut seeds are used. In all the neoformations obtained in-vitro, aescin is present in concentrations similar to or even higher than those found in horse-chestnut seeds in nature. Aescin concentration values in precursor callus ($E_1$) derived from stem and cotyledons are similar; the same is true for embryogenic callus ($E_2$). We emphasize this point as, in nature, the embryonal tissues are rich in aescin while the stem contains very small quantities of aescin. Aescin concentration seems to be directly correlated to the intensity of the cellular multiplication; during the growth stages of the proliferation (which in-vitro, evolve from $E_1$ to $E_2$ and, then, to somatic embryos) the active principle concentration increases when $E_1$ precursor callus evolves to $E_2$ embryogenetic callus and decreases in well-formed embryos derived from $E_2$ callus. Thus, during callogenesis and somatic embryogenesis which require very high activities of both metabolic and morphological differentiation, aescin concentration reaches its highest values, confirming a direct relationship between tissue differentiation and active principle production.

Our data are in agreement with the results obtained from leaf-derived neoformations with the exception of those related to somatic embryos; the embryos obtained from stem and cotyledon explants have an active principle content lower than that found in the embryos obtained from leaf expalnts (Profumo et al 1991). This difference is probably because those collected for the chemical assay were completely developed. It is relatively simple to modify the culture conditions (light, nutrients, phytoregulators) in a controlled manner to influence the secondary metabolism of the neoformations to study the importance of factors and to increase active principle production. Studies of this sort are more difficult to carry out on the plant in-vivo.

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