Research Article

Comparative Study of Antioxidant Status in Androgenic Embryos of Aesculus hippocastanum and Aesculus flava

Dubravka Štajner,1 Boris M. Popović,1 Dušica Čalić,2 and Marijana Štajner3

1 Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21 000 Novi Sad, Serbia
2 Department of Plant Physiology, Institute for Biological Research “Siniša Stanković,” University of Belgrade, Despota Stefana Boulevard 142, 11000 Belgrade, Serbia
3 Emergency Centre, Clinical Centre of Vojvodina, Hajduk Veljkova 1, 21000 Novi Sad, Serbia

Correspondence should be addressed to Dušica Čalić; calic@ibiss.bg.ac.rs

Received 20 August 2013; Accepted 7 November 2013; Published 3 February 2014

In vivo (leaves and seed embryos) and in vitro (androgenic embryos) antioxidant scavenging activity of Aesculus hippocastanum and Aesculus flava medical plants was examined. Here we report antioxidant enzyme activities of superoxide dismutase, catalase, guaiacol peroxidase and glutathione peroxidase, reduced glutathione quantity, flavonoids, soluble protein contents, quantities of malondialdehyde, and $\cdot$OH radical presence in the investigated plant samples. Total antioxidant capacity of all the samples of A. hippocastanum and A.flava was determined using FRAP, DPPH, and NO$^\cdot$ radical scavenger capacity. The leaves of A. flava collected from the botanical garden exhibited stronger antioxidant activity (higher activities of SOD, and higher quantities of GSH, TSH, TPC, and scavenging abilities of DPPH and NO$^\cdot$, and higher FRAP values and lowest quantities of $\cdot$OH and MDA) than in vitro obtained cultures. However, the leaves of A. flava showed higher antioxidant activity than the leaves of A. hippocastanum, and therefore they have a stronger tolerate of oxidative stress. Androgenic embryos of both species had low amount of antioxidants due to controlled in vitro environmental conditions (T, photoperiod, humidity, nutritive factors, and pathogen-free). Our results confirmed that we found optimal in vitro conditions for producing androgenic embryos of both Aesculus species. Also, we assume that horse chestnut androgenic embryos can be used as an alternative source for large-scale aescin production.

1. Introduction

Horse chestnut (Aesculus hippocastanum L.) grows under varying ecological conditions in many European cities in the northern temperate zone [1, 2]. Yellow buckeye (A. flava Marshall) is a species of buckeye native to Florida, USA. A. flava as well as many American Aesculus species is resistant to the C. ohridella leaf miner. A. hippocastanum and A. flava have a slow and difficult reproduction cycle under natural conditions, which can be overcome via in vitro androgenesis.

Aesculus species have different medicinal or cosmetic uses, and the bark of the horse chestnut contains low amounts of gallic and tannic acids which are used in industrial applications. The bark and leaves of A. hippocastanum have been employed as an astringent to treat diarrhea and hemorrhoids, venous insufficiency, and postoperative edema in order to pass kidney stones and to ease stomach aches, while a fraction of the seed was swallowed to alleviate hemorrhoidal symptoms [3]. A. hippocastanum increases the antioxidative defense system of the body and prevents HFD-induced lipid peroxidation in male mice [4]. In mainland China, the seeds of A. chinensis have been used as stomachic and analgesic in the treatment of distention and pain in the chest and abdomen and in the treatment of malaria and dysentery and heart diseases [5].

Saponins from A. hippocastanum have been reported to show anti-inflammatory activity [6]. It was proven that Japanese horse chestnut (Aesculus turbinata Blume) suppresses the blood glucose levels using the oral starch tolerance test and long-term antiobesity effects in obese mice fed a high-fat diet. Recently, it was reported that seed shells of A. turbinata contain higher levels of polyphenolic antioxidants.
than typical foods such as cranberry, blueberry, almonds, hazelnut, and chestnut [7–9]. The antioxidant compounds can be recycled in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances [10, 11].

Plant in vitro cultures are able to produce and accumulate many medicinally valuable secondary metabolites [12–17]. Many different in vitro approaches have been used for increased biosynthesis and for the accumulation of antioxidant compounds in plant cells. In vitro technology offers some or all of the following benefits: simpler extraction and purification from interfering matrices, novel products not found in nature, independence of climatic factors and seasons, more control over biosynthetic routes for obtaining the most desired variants compounds, shorter and more flexible production cycles, and easier fulfillment of the high-profile pharmaceutical production [18]. Biotechnological methods based on in vitro tissues and plants are considered as raw material for producing standardized material, independent of environmental factors [19–22]. The presence of substantial amounts of aescin in androgenic embryos of A. hippocastanum, which remained high after a few years of culture and could be increased further by applying certain plant growth regulators, was detected [23].

In the present paper, we evaluated the antioxidant capacities of extracts obtained from leaves and zygotic embryos in vivo and androgenic embryos in vitro of A. hippocastanum and A. flava. Antioxidant activities of the extracts from in vitro cultures were compared with those of extracts of A. hippocastanum and A. flava grown in nature. The aim of this research was to study the antioxidant scavenging activity in globular and cotyledonary androgenic embryos of A. hippocastanum and A. flava with the goal of improving the experimental in vitro culture growth conditions.

2. Material and Methods

2.1. Plant Material. Leaves, seed embryos (as control), and anthers were collected from elite A. hippocastanum and A. flava trees. A. hippocastanum and A. flava were harvested during April. Inflorescences with closed flower buds were transported and stored in the dark at 4°C.

2.2. Extraction Procedures. Plant material (1 g) was extracted with 25 mL 70% aqueous ethanol (0.1 M HCl) and sonicated for 30 min in an ultrasonic bath at ambient temperature. The extracts were rapidly vacuum-filtered through a sintered glass funnel and kept refrigerated. This extract was used for total phenolic content, DPPH and NO° radical scavenger capacity (RSC), and total antioxidant power determinations.

For lipid peroxidation, antioxidant enzymes, hydroxyl radical quantity and soluble protein content, and phosphate buffer (pH 7) extracts were used. One gram of plant material was extracted with 50 mL 0.1 M K₂HPO₄ at pH 7.0 after

| Aesculus flava samples | TPC (mg catechin/100 g) | DPPH RSC (%) | NO RSC (%) | FRAP (FRAP units) | °OH (nmol/mg protein) | LP (nmol MDA/mg protein) |
|------------------------|-------------------------|-------------|-----------|-------------------|----------------------|------------------------|
| In vitro androgenic embryos in cotyledonary stage | 61.23ᵃ | 16.38ᵇ | 28.11ᵇ | 237.0ᵃ | 38.44ᵃ | 18.95ᵃ |
| In vitro androgenic embryos in globular stage | 47.70ᵇ | 12.71ᵃ | 17.11ᵇ | 185.4ᵇ | 62.39ᵇ | 33.83ᵇ |
| Leaves in vivo (control) | 173.8ᵃ | 28.95ᵇ | 51.38ᵇ | 744.3ᵇ | 15.36ᵃ | 11.68ᵇ |

Values in rows marked with different letters (a, b, c, and d) were significantly different according to Duncan t-test P < 0.05. For each parameter, experiments and measurements were also recorded in triplicate; TPC: total phenol content; RSC: radical scavenging capacity; FRAP: Ferric reducing antioxidant power; LP: lipid peroxidation.
30 min of sonication in an ultrasonic bath at ambient temperature. After 10-minute centrifugation at 4 °C and 15,000 g, the aliquots of the supernatant were used for the above-mentioned determinations.

2.3. Total Phenol Content. Total phenol content (TPC) was determined spectrophotometrically using the Folin and Ciocalteu assay described by [25]. Aliquots of plant extracts (250 μL) were mixed with 4.0 mL distilled water and 250 μL of previously diluted Folin and Ciocalteu reagent. Aliquots of saturated Na₂CO₃ solution (500 μL) were added to this mixture to produce basic conditions. The mixture was diluted to 10 mL with distilled water. The absorbance versus a prepared blank was read at 760 nm until it reached steady state. The same procedure was applied for six standardsolutions of catechin (50–300 mg/100 mL). Final results were expressed as mg catechin equivalent per 100 g dry sample.

2.4. Total Antioxidant Capacity

2.4.1. FRAP. Total antioxidant capacity was estimated according to the FRAP (Ferric reducing antioxidant power) assay [26]. The FRAP reagent was prepared by mixing: acetate buffer (300 mM pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) reagent (10 mM in 40 mM HCl), and FeCl₃·6H₂O (20 mM) in ratio 3:1:1. Sample (100 μL) was mixed with 3 mL of working FRAP reagent and absorbance (593 nm) was measured at 4 minutes after vortexing. FRAP value was calculated using the formula:

\[
\text{FRAP value} = \frac{A_{\text{sample}}(0-4 \text{ min}) - A_{\text{standard}}(0-4 \text{ min})}{A_{\text{standard}}(0-4 \text{ min})},
\]

FRAP unit is equal to 100 μM Fe²⁺/dm³ Fe²⁺/100 μM Fe²⁺.

2.4.2. DPPH radical Scavenging Capacity. DPPH· RSC assay was based on measurement of the loss of DPPH (2,2'-diphenyl-1-picrylhydrazyl) color after reaction with test compounds [27]. The DPPH· radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH·. The ability can be evaluated by measuring the decrease of its absorbance. The widely used decoloration assay was first reported by [28]. Each extract (5, 10, 20, 30, and 40 μL) was mixed with 90 μM DPPH· in methanol making up a final volume of 3.0 mL. The mixtures were shaken vigorously and were stored in dark for 30 min at room temperature. The decrease of absorbance of the reaction mixtures for the control was monitored spectrophotometrically at 515 nm.

RSC was calculated by following:

\[
\text{RSC} = \frac{(A_0 - A_1)}{A_0} \times 100.
\]

2.4.3. NO Radical Scavenging Capacity. NO RSC was evaluated by measuring the accumulation of nitrite (formed by the reaction of NO with oxygen), according to the Griess reaction [29]. NO· was generated by sodium nitroprusside in buffered aqueous solution. Each prepared extract (10, 25, 50, 75, and 100 μL) was mixed with freshly prepared solution of sodium nitroprusside (0.5 mL, 0.01 M in NaH₂PO₄·Na₂HPO₄ buffer, 0.067 M, pH 7.4) and NaH₂PO₄·Na₂HPO₄ buffer (0.067 M, pH 7.4) making a final volume of 1.0 mL. These mixtures were prepared at 25 °C for 10 min and illuminated at 3000 lx. After illumination, each reaction mixture (1 mL) was mixed with Griess reagent (1 mL, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDA) in distilled water and 1% sulfanilamide in 5% H₃PO₄). Reduction of nitrite by the extracts was determined spectrophotometrically at 546 nm, by measuring the decrease of absorbance of the reaction mixtures for the control (containing the same chemicals except for the sample).

RSC was calculated by following:

\[
\text{RSC} = \frac{(A_0 - A_1)}{A_0} \times 100.
\]

2.5. Lipid Peroxidation. Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient at 532 nm of 153,000 mol⁻¹ cm⁻¹ for the chromophore was used to calculate the MDA-like TBA produced. The colour intensity of the MDA-TBA complex in the supernatant was measured by its absorbance at 532 nm [30].


Table 2: Soluble protein content, antioxidant enzyme activities (SOD, GPx and CAT), and glutathione, and total thiol content in A. flava.

| Aesculus flava samples | Proteins (mg/g) | SOD (U/mg protein) | GPx (nmol/mg protein) | CAT (nmol/mg protein) | GSH (µmol/mg protein) | TSH (µmol/mg protein) |
|------------------------|-----------------|--------------------|----------------------|-----------------------|-----------------------|-----------------------|
| In vitro androgenic embryos in cotyledonary stage | 1.0<sup>a</sup> | 1024<sup>a</sup> | 1515<sup>a</sup> | 31.6<sup>a</sup> | 2.7<sup>a</sup> | 2.7<sup>a</sup> |
| In vitro androgenic embryos in globular stage | 2.1<sup>b</sup> | 333.8<sup>b</sup> | 1493<sup>a</sup> | 14.3<sup>b</sup> | 1.0<sup>b</sup> | 1.0<sup>b</sup> |
| Leaves in vivo (control) | 0.9<sup>a</sup> | 1586.0<sup>c</sup> | 36.9<sup>b</sup> | 15.3<sup>b</sup> | 4.1<sup>c</sup> | 4.1<sup>c</sup> |

Values in rows marked with different letters (a, b, c, and d) were significantly different according to Duncan’s test for independent observations. For each parameter, experiments and measurements were also recorded in triplicate; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; GSH: reduced glutathione; TSH: total thiols.

2.6. Antioxidant Enzymes. Enzyme specific activity is expressed as µmol of the substrate transformed in minute/mg protein. Superoxide dismutase (SOD, EC 1.15.1.1.) activity was determined by the method based on the inhibition of adrenaline transformation to adrenochrome at pH 10.2. SOD units can be regarded as the amount of enzyme which causes a 50% inhibition in the extinction change in 1 min as compared to the control [31]. Measurements were made at 480 nm.

Guaiacol peroxidase (GPx, EC 1.11.1.7.) activity was determined using guaiacol as substrate at 436 nm [32]. Glutathione peroxidase (GSH-Px, EC 1.11.1.9.) activity was determined using cumene hydroperoxide and reduced glutathione (GSH) as substrates at 412 nm [33]. Catalase (CAT, EC 1.11.1.6.) activity was determined at 240 nm. The decomposition of H₂O₂ was followed by decrease in absorbance [34].

The amount of reduced GSH and total thiols (TSH) was determined with Ellman’s reagent at 412 nm [35]. Soluble protein content was determined [36]. Hydroxyl radical (·OH) was determined by the inhibition of deoxyribose degradation [37].

2.7. Statistical Analysis. For each parameter, experiments and extraction procedures were performed in triplicate. All measurements for each extract were also recorded in triplicate. Statistical comparisons between samples performed with Duncan’s test for independent observations were done using STATISTICA 9.1. Differences were considered significant at P < 0.05.

3. Results and Discussion

3.1. TPC and Total Antioxidant Status In Vitro and In Vivo Tissues of A. flava. This is the first report about antioxidant scavenging activity in androgenic embryos of A. hippocastanum and A. flava. We chose two Aesculus species because they are related. A. flava is often grafted on A. hippocastanum for improving cold and insect resistance [2].

The results obtained from the study are presented in four comparative tables containing data concerning in vivo control samples (leaves and seed embryos), in vitro globular, and in vitro cotyledonary embryos of A. hippocastanum (Figure 1(a)) and A. flava (Figure 1(b)).

Significant differences in MDA, ·OH, FRAP, NO· RSC, DPPH· RSC, and TPC were observed (Table 1) in all investigated samples of A. flava. TPC was the highest in leaves of control plant 173.8 (mg gallic acid/100g) and the lowest in globular in vitro embryos 477.7 (mg gallic acid/100g). Apart from the TPC leaves of the control plant exhibited the highest values of DPPH· RSC (28.9%), NO· RSC (51.4%), and FRAP values (744.3 FRAP units). The lowest scavenging activities of DPPH· (12.7%) and NO· (171%) were observed in globular in vitro embryos. The highest MDA (33.8 nmol MDA/mg protein) and ·OH (62.4 nmol/mg protein) quantities were observed in globular in vitro embryos which indicate greater disintegration of membrane lipids [10]. On the other hand, accumulation of the ·OH radical was the highest in globular androgenic embryos which agrees with statements of other authors who observed that the O₂· − generation rate and H₂O₂ level, (H₂O₂ could be decomposed and generate ·OH radicals) increased in tissue culture, respectively, and were higher than in the normal tissue [38]. The lowest MDA (11.7 nmol MDA/mg protein) and ·OH (15.4 nmol/mg protein) quantities were observed in leaves of a A. flava control plant which is the consequence of high scavenging activities and TPC content. Similar results were obtained by other authors on Centaurea L. species [39, 40].

The results presented showed that the investigated samples of A. flava were exposed to the negative influence of oxidative stress but also showed that they possess effective antioxidant capacity indicating a possible benefit which may be explored in future.

Comparative data concerning antioxidative enzymes activities reduced glutathione and total thiol content in A. flava in vivo and in vitro samples are presented in Table 2. SOD (1586.1 U/mg protein) activity was the highest in leaves of A. flava control plant, as well as quantities of GSH (3.1 µmol/mg protein) and TSH (4.1 µmol/mg protein). SOD present in leaves removes O₂· − in the compartments where O₂· − radicals are formed including chloroplast and mitochondria, controlling oxidative stress in plants [41]. CAT activity was the highest in androgenic embryos in cotyledonary stage (31.6 nmol/mg protein), as well as GPx activity (1515.2 nmol/mg protein). Content of soluble proteins was the highest in globular androgenic embryos 2.1 (mg/g).

The results presented showed that all investigated samples of A. flava suffered from the negative consequences of oxidative stress but also showed that they possess effective antioxidant capacity indicating a possible benefit which should
be further explored. On the basis of almost all parameters of antioxidant status, we could conclude that leaves of *A. flava* exhibited stronger antioxidant activity (higher activities of SOD, higher quantities of GSH, TSH, TPC, scavenging abilities of DPPH and NO, higher FRAP values, and lower quantities of 'OH and MDA) than *in vitro* obtained cultures. Our previous results showed that plant leaves possess the highest antioxidant activity comparing to other plants organs [42–44].

### 3.2. TPC and Total Antioxidant Status *In Vitro* and *In Vivo* Tissues of *A*. hippocastanum

Results concerning *A. hippocastanum* are presented in Tables 3 and 4. Results from Table 3 clearly indicated that seed embryos control exhibited the highest antioxidant ability due to the highest TPC content (194.4 mg gallic acid/100 g), scavenging abilities of DPPH and NO, higher FRAP values, and lower quantities of 'OH and MDA) than *in vitro* obtained cultures. Our previous results showed that plant leaves possess the highest antioxidant activity comparing to other plants organs [42–44].

### 3.3. Total Phenolic Content *In Vitro* and *In Vivo* Tissues of *A*. hippocastanum

Results presented in Table 4 indicate that SOD activity was the highest (3197 U/mg protein) as well as quantities of GSH (3.4 μmol/mg protein) and TSH (4.1 μmol/mg protein) which together with the lowest 'OH and MDA quantities indicate that their high antioxidative capacity (Tables 3 and 4) in leaves of *A. hippocastanum* was similar as in leaves of *A. flava*. This is in agreement with the finding of previous studies that GSH and TSH are necessary to maintain the normal reduced state of cells and that they are potential scavengers of the most dangerous 'OH radical [41].

On the basis of our results for antioxidant power *in vitro* *A. hippocastanum* samples, we could conclude that both control samples seed embryos and leaves exhibited high antioxidative power because they employ antioxidant defense systems to protect themselves against ROS. If we compared leaves of *A. flava* and *A. hippocastanum*, we observed that leaves of *A. flava* exhibited higher antioxidant ability and therefore a stronger tolerance of oxidative stress.

Researching the resources of plants may bring new and safe natural products into pharmaceutical, cosmetic, and food industries [48]. Research showing that combinations of different natural antioxidants present in medicinal plants work better than separate antioxidants alone [49] has increased interest among scientists towards exploring natural antioxidants from botanical sources and those produced in tissue cultures. Our results indicated that extracts of *A. flava* and *A. hippocastanum* control samples and tissue culture materials exhibited antioxidant and scavenging abilities. Our investigations could be the starting point for further phytochemical investigations of *A. flava* and *A. hippocastanum* *in vitro* plants. Androgenic embryos of *A. hippocastanum* and *A. flava* had low amount of antioxidants due to the controlled environmental conditions we employed (T, photoperiod, humidity, nutritive factors, and pathogen-free). It can be concluded that tissue culture methods produce optimal condition for the growth of *Aesculus* androgenic embryos.

### Table 3: Total phenolic content, DPPH and NO RSC, FRAP, 'OH quantity, and lipid peroxidation in *A. hippocastanum* samples.

| *Aesculus hippocastanum* samples | TPC (mg gallic acid/100 g) | DPPH RSC (%) | NO RSC (%) | FRAP (FRAP units) | 'OH (nmol/mg protein) | LP (nmol MDA/mg protein) |
|---------------------------------|---------------------------|--------------|------------|-------------------|----------------------|------------------------|
| *In vitro* androgenic embryos in cotyledonary stage | 72.0b | 14.0b | 35.7b | 152.3b | 11.6b | 10.4a |
| Leaves *in vivo* (control) | 35.6c | 16.3b | 25.4c | 134.6b | 59.2c | 29.7b |
| Seeds *in vivo* (control) | 194.4c | 36.5c | 39.2b | 338.6c | 4.5d | 2.8c |

Values in rows marked with different letters (a, b, c, and d) were significantly different according to Duncan t-test *P* < 0.05. For each parameter, experiments and measurements were also recorded in triplicate; TPC: total phenol content; RSC: radical scavenging capacity; FRAP: ferric reducing antioxidant power; LP: lipid peroxidation.

### Table 4: Soluble protein content, antioxidant enzyme activities (SOD, GPx and CAT), and glutathione, and total thiol content in *A. hippocastanum*.

| *Aesculus hippocastanum* organs | Proteins (mg/g) | SOD (U/mg protein) | GPx (nmol/mg protein) | CAT (μmol/mg protein) | GSH (μmol/mg protein) | TSH (μmol/mg protein) |
|---------------------------------|----------------|-------------------|---------------------|----------------------|----------------------|----------------------|
| *In vitro* androgenic embryos in cotyledonary stage | 2.2b | 500.5b | 1290b | 11.9b | 0.9b | 0.9b |
| Leaves *in vivo* (control) | 0.6c | 3197c | 55.9c | 30.9a | 3.4c | 4.1c |
| Seeds *in vivo* (control) | 8.4d | 123.8d | 327.7d | 478e | 0.79b | 0.5b |

Values in rows marked with different letters (a, b, c, and d) were significantly different according to Duncan t-test *P* < 0.05. For each parameter, experiments and measurements were also recorded in triplicate; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; GSH: reduced glutathione; TSH: total thiols.
4. Conclusions

In vivo control samples (leaves) of both species showed higher antioxidant activity than in vitro obtained androgenic embryos. However, A. flava leaves had better antioxidant activity than the leaves of A. hippocastanum, and therefore they have a stronger tolerance of oxidative stress.

The optimization of in vitro conditions for mass production of androgenic embryos could improve cultivation techniques and achieve diversity protection, conservation of these species, and protection from leaf miner Cameraria ohridella.

These results could be also beneficial for growing Aesculus plants with a high tolerance to oxidative stress and also for producing a physiology stable standardized material independent of environmental factors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This research was supported by the Ministry of Education, Science, and Technology Development of Serbia, Grants no. III43002 and no. 173015.

References

[1] K. J. Steadman and H. W. Pritchard, "Germination of Aesculus hippocastanum seeds following cold-induced dormancy loss can be described in relation to a temperature-dependent reduction in base temperature (Tb) and thermal time," New Phytologist, vol. 161, no. 2, pp. 415–425, 2004.

[2] G. Grabenweger and R. Grill, "On the place of origin of Cameraria ohridella Deschka & Dimic (Lepidoptera: Gracillariidae)," Beiträge zur Entomofaunistik, vol. 1, pp. 9–17, 2000.

[3] C. R. Sirtori, "Aescin: pharmacology, pharmacokinetics and therapeutic profile," Pharmacological Research, vol. 44, no. 3, pp. 183–193, 2001.

[4] I. Küçükkurta, S. Inceb, H. Keleş et al., "Beneficial effects of Aesculus hippocastanum L. seed extract on the body’s own antioxidant defense system on subacute administration," Journal of Ethnopharmacology, vol. 129, no. 1, pp. 18–22, 2010.

[5] Z. Zhang, S. Li, S. Zhang, and D. Gorenstein, "Triterpenoid saponins from the fruits of Aesculus pavia," Phytochemistry, vol. 67, no. 8, pp. 784–794, 2006.

[6] S. Apers, T. Naessens, L. Pieters, and A. Vlietinck, "Densitometric thin-layer chromatographic determination of aescin in a herbal medicinal product containing Aesculus and Vitis dry extracts," Journal of Chromatography A, vol. 1112, no. 1-2, pp. 163–170, 2006.

[7] T. Delgado, R. Malheiro, J. A. Pereira, and E. Ramalhosa, "Hazel nut (Corylus avellana L.) kernels as a source of antioxidants and their potential in relation to other nuts," Industrial Crops and Products, vol. 32, no. 3, pp. 621–626, 2010.

[8] H. Kimura, S. Ogawa, A. Sugiyama, M. Jisaka, T. Takeuchi, and K. Yokota, "Anti-obesity effects of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut (Aesculus turbinata Blume)," Food Research International, vol. 44, no. 1, pp. 121–126, 2011.

[9] G. Vázquez, A. Fernández-Agulló, C. Gómez-Castro, M. S. Freire, G. Antorrena, and J. González-Álvarez, "Response surface optimization of antioxidants extraction from chestnut (Castanea sativa) bur," Industrial Crops and Products, vol. 35, no. 1, pp. 126–134, 2012.

[10] B. Halliwell and J. M. C. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, Oxford, UK, 4th edition, 2007.

[11] H. K. Obied, M. S. Allen, D. R. Bedgood, P. D. Prenzler, K. Robards, and R. Stockmann, "Bioactivity and analysis of bio-phenols recovered from olive mill waste," Journal of Agricultural and Food Chemistry, vol. 53, no. 4, pp. 823–837, 2005.

[12] M. Singh and R. Chaturvedi, "Improved clonal propagation of Spilanthes acmella Murr. for production of scopoletin," Plant Cell, Tissue and Organ Culture, vol. 103, no. 2, pp. 245–253, 2010.

[13] D. Štajner, B. M. Popović, D. Čalić-Dragosavac, D. Malenčić, and Š. Zdravković-Korać, "Comparative study on Allium schoenoprasum cultivated plant and Allium schoenoprasum tissue culture organs antioxidant status," Phytotherapy Research, vol. 25, no. 11, pp. 1618–1622, 2011.

[14] W. Al Khateeb, E. Hussein, L. Qouta, M. Aluđatt, B. Al-Shara, and A. Abu-zaiton, "In vitro propagation and characterization of phenolic content along with antioxidant and antimicrobial activities of Cichorium pumilum Jacq.," Plant Cell, Tissue and Organ Culture, vol. 110, no. 1, pp. 103–110, 2012.

[15] S. O. Amoo, A. O. Aremu, and J. Van Staden, "In vitro plant regeneration, secondary metabolite production and antioxidant activity of micropropagated Aloe arborescens Mill," Plant Cell, Tissue and Organ Culture, vol. 111, no. 3, pp. 345–358, 2012.

[16] E. García-Pérez, J. A. Gutiérrez-Uribe, and S. García-Lara, "Luteolin content and antioxidant activity in micropropagated plants of Poliomintha glabrescens (Gray)," Plant Cell, Tissue and Organ Culture, vol. 108, no. 3, pp. 521–527, 2012.

[17] L. E. B. Savio, L. V. Astarita, and E. R. Santarém, "Secondary metabolism in micropropagated Hypericum perforatum L. grown in non-aerated liquid medium," Plant Cell, Tissue and Organ Culture, vol. 108, no. 3, pp. 465–472, 2012.

[18] A. Matkowski, "Plant in vitro culture for the production of antioxidants: a review," Biotechnology Advances, vol. 26, no. 6, pp. 548–560, 2008.

[19] B. M. Panda and S. Hazra, "In vitro regeneration of Senecarpus anacardium L. from axenic seedling-derived nodal explants," Trees Structure and Function, vol. 24, no. 4, pp. 733–742, 2010.

[20] I. Grzegorczyk, A. Matkowski, and H. Wysokińska, "Antioxidant activity of extracts from in vitro cultures of Salvia officinalis L.," Food Chemistry, vol. 104, no. 2, pp. 536–541, 2007.

[21] P. Sansberro, H. Rey, L. Mroginski, and C. Luna, "In vitro plantlet regeneration of Schinopsis balansae (Anacardiaceae)," Trees Structure and Function, vol. 17, no. 6, pp. 542–546, 2003.

[22] S. Shukla, S. K. Shukla, and S. K. Mishra, "In vitro plant regeneration from seedling explants of Stereospermum personatum D.C.: a medicinal tree," Trees Structure and Function, vol. 23, no. 2, pp. 409–413, 2009.

[23] D. Čalić-Dragosavac, S. Zdravković-Korać, K. Šavkink-Fodulović, L. Radiojević, and B. Vinterhalter, "Determination of escin content in androgenic embryos and hairy root culture of Aesculus hippocastanum," Pharmaceutical Biology, vol. 48, no. 5, pp. 563–567, 2010.
