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Bioconversion of hydroquinone to arbutin in F3K *Digitalis purpurea* cell line

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Abstract. The capacity of the F3K *Digitalis purpurea* cell line to biotransform hydroquinone into β-arbutin was tested. The experimental protocol involved the sequential addition of hydroquinone to the culture medium, and testing different precursor (4-, 5-, and 6 mM) and sucrose concentrations (3 % and 6 %) under different lighting conditions (16-hour photoperiod and darkness). The biotransformation process was positively influenced by the photoperiod regime and increased sucrose concentration. The highest arbutin production (1 g/L) has been achieved in cell suspensions fed with 6% sucrose and 5 mM hydroquinone, under 16 hours daily light exposure.

***Keywords*:** hydroquinone; arbutin production; *Digitalis purpurea* (L.); cell suspensions.

1. Introduction

Chemically, β-arbutin (or simply arbutin) is the O-β-D-glucopyranoside of hydroquinone. It was first discovered in 1852 by Kawalier in the leaves of *Arctostaphylos uva-ursi* and later, in 1903, by Kanger in the leaves of *Vaccinium vitis-idaea* [1]. Arbutin possesses several therapeutically relevant properties, particularly as a urinary disinfectant and antibacterial agent. It is commonly used in the treatment of urinary tract infections, cystitis, and kidney stones, as well as serving as a diuretic [2, 3]. In traditional medicine, aqueous extracts from plants containing arbutin are commonly used, most often in the form of infusions [4]. However, some authors recommend preparing cold macerations to obtain aqueous solutions with a lower tannin content [4]. In recent decades, modern medicine has begun to use arbutin in its purified form [5]. Subsequently, arbutin was found to exhibit another therapeutic action: melanin synthesis inhibition in human skin, making it useful in the cosmetic industry as an ingredient in skin-whitening creams [5]. It is used for the depigmentation of hyperpigmented scars and freckles in topical applications. Moreover, it has excellent safety with no adverse effects for health such as toxicity [5]. The property of inhibiting melanin synthesis appears to be related to its inhibitory effect on the activity of melanocyte tyrosinase, with another hypothesis suggesting that it intervenes by suppressing the expression and synthesis of the enzyme tyrosinase [6]. In addition to its whitening properties, it is also notable for its sun protection and antioxidant effects. Arbutin can help prevent severe sunburns caused by the accumulation of melanin in the subcutaneous tissues [6]. Plants represent an almost unlimited source of chemical compounds, such as primary and secondary metabolites that provide the therapeutic value of medicinal plants [7]. Although plants are renewable resources, there are situations where certain species are difficult to cultivate in sufficient quantities to meet the increasing demand [8]. The destruction of natural habitats, along with the challenges in cultivating certain plants, has led to a significant reduction of these resources [9, 10]. Arbutin occurs naturally in plants and it has been suggested that it may contribute to their stress hardiness [11]. High quantities of arbutin were found in several plant species, like *Myrothamnus flabellifolia* Welw. (Myrothamnaceae), native to southern Africa, and *Leucodendron* spp., which can endure drought. Species like *Vaccinium* spp., *Arctostaphylos* spp., and *Bergenia crassifolia* can withstand low temperatures [12, 13]. The physiological role of arbutin in plants is not fully understood, but it is believed to contribute to the protection of membrane components in dry states through its antioxidant action and by inhibiting phospholipase A2 activity in dehydrated systems [13]. This reinforces the idea that this compound is a key factor in the protective mechanisms of plants [13]. In Romania, the plant species with the highest arbutin content is *Arctostaphylos uva-ursi* (bearberry), but it is

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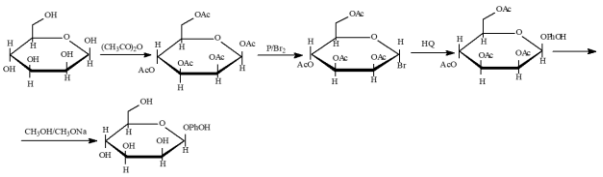
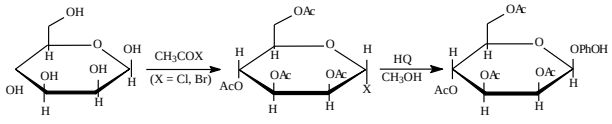
legally protected, which prohibits its harvesting [14]. According to the 11th edition of the European Pharmacopoeia [15], the official source of arbutin is the medicinal product *Uvae-ursi folium* which should contain not less than 7 % arbutin. As a substitute, lingonberry leaves *Vitis-idaeae folium*, are listed in both the European [15] and Romanian Pharmacopoeias [16], although it has a lower arbutin content of 3 %. Therefore, arbutin extraction from various plant sources is hindered by its low content, the complicated extraction process, and low purity of the extracted product [17]. Producing this therapeutically important compound in large quantities requires the development of alternative methods [18-20].

Over time, the possibility of obtaining β-arbutin through chemical synthesis has been tested, but from the very first attempts, two major disadvantages became evident: the long reaction time and the low yield. Until 2004, the shortest method for the chemical synthesis of arbutin consisted of four successive steps, according to the reaction scheme below [18]:



In the reaction scheme presented, the use of 2,3,4,6-tetra-O-acetyl-α-D-glucosyl bromide can be observed, an electrophilic substance that has proven to be an important and widely used intermediate in the synthesis of many pharmaceutical compounds, contributing to the formation of glycosidic bonds in saccharides chemistry. The reaction of this compound with hydroquinone, followed by the deacetylation of the –OH groups, resulted in β-arbutin with a reaction yield of only 27 %. The halogenating agents used were quite varied: $AlCl_3$, PCl_5, BCl_3, 4-toluenesulfonyl chloride (TsCl) etc.

Later, Huang et al. [18] proposed a two-step synthesis method consisting of only two steps, according to the scheme below:



The first step consisted of preparing 2,3,4,6-tetra-O-acetyl-α-D-glucosyl chloride or bromide through the reaction between glucose and acetyl chloride or bromide. In the second step, the 2,3,4,6-tetra-O-acetyl-α-D-glucosyl halide (chloride or bromide) reacted with hydroquinone, with methanol as the reaction solvent and an optimal pH of 9.5-10. These reaction conditions somewhat increased the reaction yield to 38 % and shortened the reaction time to 2 hours. Therefore, regardless of the synthesis route, producing β-arbutin through chemical synthesis is challenging because it requires blocking the hydroxyl groups of glucose via acetylation, etherifying the protected glucose with hydroquinone, and finally, deacetylation. The etherification reaction with hydroquinone necessitates high temperatures, has a long reaction time, and the overall yield is low.

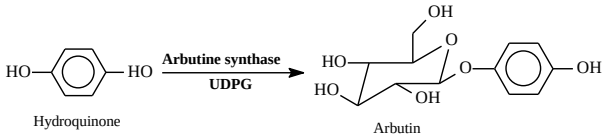
Xue et al. [19] developed a rapid method for the synthesis of arbutin through the chaining of two chemical reactions. The first step involves the glycosylation reaction of hydroquinone with penta-O-acetyl-β-D-glucopyranoside in the presence of $BF_3 \cdot Et_2O$ as a catalyst, and the resulting intermediate is subsequently subjected to a hydroxyl group deprotection reaction by hydrolysis in a basic medium. The glycosylation reaction was carried out by microwave irradiation, which significantly shortened the reaction time and achieved a maximum yield of 62.1 %. Additionally, the beta anomer was selectively obtained [19].

Another approach focused on obtaining arbutin esters (arbutin ester with undecylenic acid) through transesterification reactions, most of which were catalyzed by enzymes (such as proteases), and testing their activity on melanin suppression [20].

Phenolic compound-derived glucosides represent a significant percentage of the total glucosides commonly found in plants. Plants therefore have a large capability to glucosylate a wide range of different chemical structures [21]. The isolation and purification of such glucosides from plants are difficult and time-consuming, with very low yields. In organic chemistry, glucosylation is very important, especially concerning the production of natural β-D-glucosides. Most existing synthetic strategies are characterized by the formation of by-products, and the production of a mixture of α- and β-glucosides, instead of the pure β-glucoside. For this reason, an enzymatically catalyzed glucose transfer could be a viable alternative to chemical synthesis for obtaining this category of substances. However, this requires the presence of an appropriate glucosyltransferase [21, 22].

Plant tissue and cell cultures offer a promising alternative for obtaining large quantities of biologically valuable secondary metabolites [23]. The biotechnological exploitation of plant cell and tissue cultures offers numerous advantages, such as the ability of plant cells to grow indefinitely in an undifferentiated state, the production of the compound of interest regardless of season or climatic conditions, and the capability to maintain the biogenetic potential of the cell line [23]. This allows the use of cell suspensions to produce secondary metabolites. The accumulation of large quantities of metabolites results from a complex interplay of biosynthesis, transport, storage, and degradation processes, among others. The biosynthesis mechanism of β-arbutin in plants involves a glycosylation process, which consists of attaching glucose to various substrates [24]. This reaction is most often the final step in the biosynthesis of plant secondary metabolites, resulting in an extremely large number of glucosides. The conjugation of various compounds with glucose significantly increases their solubility, allowing them to be stored in different cell compartments, such as vacuoles [24]. Arbutin synthase belongs to the glycosyltransferase class, being the enzyme that attaches glucose to hydroquinone (HQ). Its presence in the cells of certain plants is surprising, as those plants does not

naturally contain arbutin. Further research revealed that it has low substrate specificity and uses UDP-glucose (UDPG) as the glucose donor. The glycosylation reaction of HQ under the action of arbutin synthase can be schematized as follows [24]:



The identification of this glycosyltransferase in plants that do not produce β-arbutin through their metabolic pathways led to the initiation of experiments to obtain this compound in *in vitro* cell cultures derived from various species. Notable results were achieved in cell suspensions of *Catharanthus roseus* (9.2 g/L of β-arbutin) and in high-density cell suspensions of *Rauwolfia serpentina* (18 g/L) [25-27]. Hydroquinone biotransformation into arbutin has also been reported in plant cell suspensions of *Aronia melanocarpa* [28] and *Capsicum annuum* [29], as well as in hairy roots cultures of *Brugmansia candida* [30].

Based on the preliminary results obtained by our team from *Digitalis lanata* cell suspensions [31, 32], which demonstrated the ability of this species to biotransform the exogenously added HQ into arbutin, we initiated cell suspension cultures of *Digitalis purpurea*, assuming that this plant also possesses the enzymatic equipment necessary for HQ biotransformation [33]. Following a laborious experimental plan, previously described [34], a *Digitalis purpurea* cell line (designated DpHQ), with high resistance to elevated hydroquinone concentrations, was successfully selected. The biotransformation experiments involving this cell line resulted in the production of maximum 3.05 g/L arbutin at 5 mM HQ concentration, 6 % sucrose supplementation and 16 - hour photoperiod regime.

The aim of this work was to test the ability of another *Digitalis purpurea* cell line, grown under different experimental conditions, to biotransform HQ into arbutin.

2. Experimental

2.1. Materials and reagents

Digitalis purpurea cell cultures. The steps for obtaining *Digitalis purpurea in vitro* cell cultures were described in detail in a previous study [33, 34]. As a starting point, *Digitalis purpurea* seeds collected from the "Alexandru Borza" Botanical Garden in Cluj-Napoca were used. A voucher specimen (126.14.1.1) is available in the herbarium of the Pharmaceutical Botany Department of the Faculty of Pharmacy in Cluj-Napoca. A 5 mL aliquot of the F3 parent cell culture [33, 34] was transferred in Erlenmeyer flasks containing 50 mL of Murashige and Skoog [34] culture medium supplemented with 0.2 g/L glutamine, 0.2 g/L hydrolyzed casein, 3% sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 2 mg/L kinetin (K). The resulting cell line, with 10% (v/v) inoculum size, was designated F3K and underwent six subcultures at two-weeks intervals, maintaining the same subculture ratio, before the biotranformation experiments. Some samples (3 Erlenmeyer flasks/treatment) were kept under a 16-hour photoperiod lighting regime, while others were maintained in complete darkness. All cultures were kept for continuous agitation at 100 rpm on the orbital shaker and incubated at 25 ± 1 °C, while those exposed to a 16-hour photoperiod were illuminated by cool white, fluorescent light (30 μmol/m^2/sec).

Hydroquinone feeding experiments. Hydroquinone (Merck, Germany) was introduced fractionally into the culture medium on days 10, 11 and 12, in the form of a sterile aqueous solution, so that the final substrate concentration reached 4-, 5-, and 6 mM. HQ addition was divided in 3 doses as follows: 1-1-2, 1-2-2 and 2-2-2 ml to reach 4-, 5- and 6 mM concentration respectively. The influence of hydroquinone concentration on the growth of cell suspensions and the biotransformation process was tested, as well as the impact of using a 3 % sucrose supplement. Biomass and medium samples were collected on day 14. The biomass was weighed after drying, while the liquid medium was lyophilized. Both the biomass and medium samples were subjected to extraction for the quantitative determination of arbutin.

2.2. Methods

HPLC-MS Analysis of Arbutin: Arbutin was extracted and quantified from dried, powdered samples following the method described by Pop et al. [34, 36]. The extraction involved sonicating 50 mg of sample in 20 mL of a methanol/water mixture (5:95) at 25 °C for 30 minutes. High-performance liquid chromatography–tandem mass spectrometry (HPLC-MS) was employed for arbutin quantification. The analysis was carried out using an Agilent 1100 Series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA), which included a G1322A degasser, G1311A quaternary gradient pump, and a G1313A autosampler, coupled with an Agilent Ion Trap 1100 SL mass spectrometer.

Chromatographic separation was achieved on a Zorbax SB-C18 reversed-phase column (100 mm × 3.0 mm i.d., 3.5 μm particle size) at 45 °C. The mobile phase consisted of water with 50 μM sodium acetate, delivered at a flow rate of 1 mL/min, with an injection volume of 5 μL. Arbutin was detected using single ion monitoring (SIM) mode with positive ion electrospray ionization (ESI), and its retention time under these conditions was 1.6 minutes.

Mass spectrometric parameters included a nebulizing gas pressure (nitrogen) of 60 psi, a drying gas flow rate of 12 L/min, a drying gas temperature of 300°C, and a capillary voltage of + 4000 V. Chromatographic data were processed using ChemStation (vB01.03) and Data Analysis (v5.3) software from Agilent Technologies, Inc. Arbutin content was measured in both dry biomass and lyophilized culture media, providing a comprehensive evaluation of its distribution and concentration.

Experimental design and statistical analysis. A full factorial experimental design was implemented to evaluate the effect of independent variables (Table 1) on the dry biomass and total arbutin production. Model analysis considered the goodness of fit (R^2), predictive

capacity (Q^2) and reproducibility. Surface contour plots were represented for the interpretation of effects. Data analysis was performed using Modde 13 Pro (Sartorius Stedim Biotech, Germany).

Table 1. The independent variables of the experimental design

Variables		**Levels**
Independent variables (factors)	Unit	
Sucrose concentration (X_1)	%	3 6
Hidroquinone concentration (X_2)	mM	4 5 6
Illumination (X_3)		photoperiod darkness
Dependent variables (responses)		
Dry biomass (dry weight) (Y_1) (g/L)		
Arbutin content (Y_2) (mg/L)		Dry biomass
		Liquid medium
		Total amount

3. Results and discussion

The experimental results are presented in Table 2.

Table 2. The worksheet of the experimental design

Experiment name	**Sucrose concentration**	**Hydroquinone concentration**	**Illumination**	**Dry biomass (g/L)**	**Arbutin content (mg/L)**
N1	3	4	Photoperiod	9.57	662.67
N2	6	4	Photoperiod	8.97	821.37
N3	3	5	Photoperiod	9.06	750.433
N4	**6**	**5**	**Photoperiod**	**10.26**	**1009**
N5	3	6	Photoperiod	9.25	754.5
N6	6	6	Photoperiod	10.3	820
N7	3	4	Darkness	5.26	152.67
N8	6	4	Darkness	7.3	642.37
N9	3	5	Darkness	8.6	626.9
N10	6	5	Darkness	7.69	841.933
N11	3	6	Darkness	8.97	725.57
N12	6	6	Darkness	9.35	786.23
N13	3	5	Photoperiod	9.06	749
N14	3	5	Photoperiod	8.9	751.3
N15	3	5	Photoperiod	9.22	751

3.1. Growth profile

The purpose of establishing the growth curve for the F3K line was to test the influence of the phytohormone combination used (1 mg/L 2.4 D and 2 mg/L K) on cell multiplication and to identify the optimal time for hydroquinone (HQ) introduction. As previously mentioned, the suspensions were subcultured six times to ensure that the cells adapted to the new environmental conditions. Biomass samples were collected every two days, up to and including day 16 of culture, dried, and weighed. The results obtained for 50 mL of culture medium were extrapolated to 1 L, thus calculating the amount of dry biomass, expressed in g/L (Figure 1).

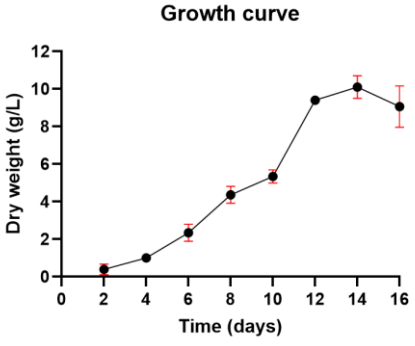


Figure 1. *Digitalis purpurea* F3K cell line growth profile from 2 to 16—harvesting days. Data are presented as mean value ± SD (in red)

Biomass yield was seven times higher by the 8th day of culture compared to the beginning of the experiment (exponential phase). The growth rate was substantially slower between days 8-10 of culture, as for the previously tested DpHQ cell line [34]. The stationary phase was reached between days 12 and 14, while from day 16 onward, the decline phase became evident. The highest biomass amount was registered on day 14 of culture (10.1 g/L). In our previous experiments [34], the DpHQ cell line exhibited higher biomass yield (13 g/L) on the 14th day of culture, at the same inoculum size (10 % v/v). This might be explained by the specific effects of different phytohormone classes and combinations (1 mg/L of 2,4-D and 2 mg/L of 6-benzylaminopurine - BA). In this regard, it is known that 2,4-D has the most potent auxinic action, its introduction into culture media resulting in a strong stimulation of cell division, and particularly an increase in cell volume [37].

3.2. Feeding protocol outcome

Hydroquinone itself, as well as its oxidation products, exerts a toxic effect on cells [27]. It was interesting to observe in our experiments how exposure to increased hydroquinone concentrations impacts cell proliferation. Therefore, to prevent cell damage, we opted for the sequential addition of HQ, after the strategy described by Yokoyama and Inomata [25, 26]. By applying this strategy, we registered positive outcomes in biomass production and, consequently, in arbutin yield in

previous experiments [34]. Additionally, since the stability of hydroquinone is affected by lighting conditions [4], we varied this parameter by maintaining the suspensions under a 16-hour photoperiod as well as in complete darkness. The toxic effect of hydroquinone was revealed also by the impact on dry biomass accumulation (Figure 2). However, overall, the F3K cell line was not particularly susceptible to its harmful effects. There were no significant differences in biomass production between control suspensions and variants treated with different hydroquinone doses, indifferent of lighting conditions. However, it can be observed that light exposure had an impact on biomass accumulation, regardless of the sucrose concentration used. Light-grown suspensions exhibited greater biomass yields, showing similar behavior to DpHQ cell line [34].

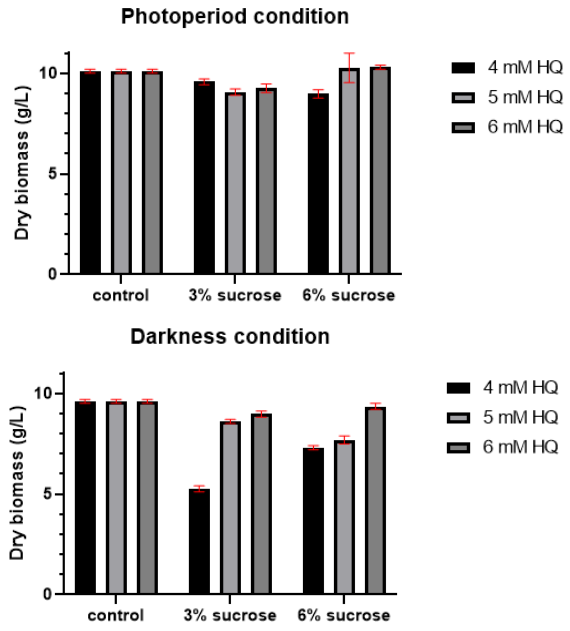


Figure 2. Dry biomass yields from hydroquinone sequential feeding protocol – studies using 3 % and 6 % sucrose, suspensions maintained under photoperiod conditions or in darkness.

The result registered for the suspension with 3 % sucrose and 4 mM hydroquinone is interesting, as the dry biomass accumulation was the lowest. However, we cannot correlate this decrease with the toxicity of hydroquinone, since at higher hydroquinone concentrations, the growth of the suspensions was only slightly influenced compared to the control. Thus, in the case of suspensions with 3 % sucrose, for the control suspension, the amount of dry substance reached a value of 9.6 ± 0.1 g/L, while for the hydroquinone treated suspensions, the biomass amounts were slightly lower: 8.6 ± 0.1 g/L for 5 mM and 8.9 ± 0.15 g/L for 6 mM of hydroquinone.

Several research teams have tested the effect of high concentrations of carbon sources, typically sucrose or glucose, on cell multiplication. For example, in the case of *Hancornia speciosa*, *Salvia leriifolia*, and *Melastoma malabathricum* cell suspensions, higher biomass yields were obtained, with similar results achieved in callus cultures [38-40]. In the case of the F3K line of *Digitalis purpurea* an uneven behavior is observed, akin to that characteristic of the DpHQ cell line, considering that the 3 % sucrose supplementation did not lead to increased biomass accumulation in all variants. We can conclude that, increased sucrose concentrations may not necessarily contribute to the increase in cellular biomass but, as indicated by arbutin yields, it does stimulate the biotransformation process of hydroquinone into arbutin probably by enhancing cellular viability in all treatments.

3.3. Biotransformation experiments results

The impact of sucrose on the glucosylation of exogenous substrates was first documented by Mizukami et al. [41], who utilized salicylic alcohol as a substrate in their study. This initial finding prompted subsequent investigations into how sucrose enhances the biotransformation process of hydroquinone into arbutin [41]. Early hypotheses suggested that sucrose might serve as a nutrient via the Embden-Meyerhof pathway or as a precursor for UDP-glucose formation through the glucose-phosphate pathway. However, Yokoyama et al. [42] later refuted these hypotheses. Several studies argument that saccharides, broadly speaking, act as scavengers for hydroxyl radicals [42]. Upon entering the cell, hydroquinone undergoes oxidation to form a transient and highly unstable semiquinone radical, which quickly converts to *p*-quinone [42]. In animal cells, *p*-quinone can be enzymatically reduced back to semiquinone by flavin-containing enzymes like NADPH-cytochrome P450 reductase, constituting a pivotal step in the oxidation-reduction cycle. The oxidation of semiquinone to *p*-quinone also yields superoxide radicals due to semiquinone's avidity for oxygen [42]. These superoxide radicals can subsequently react with hydrogen peroxide, either spontaneously or catalytically generated from superoxide radicals, releasing potent reactive oxygen species such as hydroxyl radicals that can inflict substantial cellular damage [42]. Given the adverse effects of hydroquinone, similar processes likely occur in plant cells [42]. While saccharides shield cells from hydroxyl radicals, they do not interact with superoxide radicals. In light of these findings, the beneficial influence of saccharides on arbutin production may stem, in part, from their capacity to scavenge hydroxyl radicals, thereby safeguarding cellular integrity and viability [43, 44].

Table 3 illustrates the amounts of arbutin obtained through the biotransformation experiments.

Table 3. Arbutin yield in F3K cell line cultured under varying concentrations of sucrose and sequential hydroquinone feeding, photoperiod and darkness.

	Arbutin amount (mg/L)					
	4 mM HQ *		**5 mM HQ**		**6 mM HQ**	
Photoperiod	**3% sucrose**	**6% sucrose**	**3% sucrose**	**6% sucrose**	**3% sucrose**	**6% sucrose**
Dry biomass	662.67 ± 6.11	821.37 ± 3.49	750.43 ± 1.25	1009.01 ± 1.01	754.07 ± 3.17	820.01 ± 4.02

	Arbutin amount (mg/L)					
	4 mM HQ *		**5 mM HQ**		**6 mM HQ**	
Liquid medium	-	-	-	-	0.432 ± 0.01	-
Total	662.67 ± 6.11	821.37 ± 3.49	750.43 ± 1.25	1009.01 ± 1.01	754.50 ± 3.17	820.01 ± 4.02
Conversion rate	60.9 %	75.5 %	55.17 %	74.19 %	46.2 %	50.2 %
	Arbutin amount (mg/L)					
	4 mM HQ		**5 mM HQ**		**6 mM HQ**	
Darkness	**3% sucrose**	**6% sucrose**	**3% sucrose**	**6% sucrose**	**3% sucrose**	**6% sucrose**
Dry biomass	152.67 ± 1.53	642.37 ± 1.76	626.90 ± 1.15	841.93 ± 1.10	720.02 ± 1.01	786.23 ± 2.36
Liquid medium	-	-	-	-	5.57 ± 0.28	-
Total	152.67 ± 1.53	642.37 ± 1.76	626.90 ± 1.15	841.93 ± 1.10	725.57 ± 0.96	786.23 ± 2.36
Conversion rate	14.03 %	59.04 %	46.09 %	61.9 %	44.46 %	48.17 %

Our results revealed that there are no significant differences between the amounts of arbutin produced in cell suspensions with 3 % sucrose, grown under a 16-hour photoperiod, indifferent of HQ concentration tested. Low overall arbutin yields were registered at low hydroquinone concentrations (4 mM), while the highest amount was achieved at 6 mM HQ supplementation. However, for 5- and 6 mM HQ concentrations, the differences between arbutin production yields is unsignificant. In the medium samples, the presence of arbutin was not detected for the concentrations of 4- and 5 mM hydroquinone. In liquid medium, arbutin was detected in small amounts (0.432 ± 0.01 mg/L) when testing the concentration of 6 mM hydroquinone. In the suspensions with 6 % sucrose, the amounts of arbutin obtained were higher by 1.2 times for 4 mM, 1.33 times for 5 mM, and 1.1 times for 6 mM hydroquinone for all tested concentrations. The optimal concentration of hydroquinone was found to be 5 mM, with the amount of arbutin reaching 1009.01 ± 1.01 mg/L.

Under dark conditions, for the suspensions with 3 % sucrose at a concentration of 4 mM, the amount of arbutin was 4 times lower than that obtained under light. The differences were not as pronounced at the concentrations of 5- and 6 mM. Thus, when adding 5 mM hydroquinone, the amount of arbutin was 1 % higher under light conditions, while at 6 mM hydroquinone, the levels of arbutin were practically equal. Therefore, we find that the highest amount of arbutin in the dark was obtained with the addition of 6 mM hydroquinone, identical to that in light conditions. Regarding the medium samples from the dark conditions, arbutin was only detected at the concentration of 6 mM HQ, in a higher amount than in the light samples (5.57 ± 0.28 mg/L). The small amount of arbutin corresponding to the concentration of 4 mM is directly correlated with the low biomass obtained. In the suspensions with 6 % sucrose, a significant difference was observed between the control suspensions and samples at a concentration of 4 mM HQ. As previously mentioned, the small amount of arbutin in the control suspensions at 4 mM HQ cannot be correlated with the substrate's toxicity, considering the results corresponding to the concentrations of 5- and 6 mM. Therefore, the fact that the addition of sucrose stimulated the biotransformation process by 4.2 times is not relevant for the concentration of 4 mM. However, for the concentrations of 5- and 6 mM, the results are significant, as the sucrose supplement led to an increase in the amount of arbutin by 1.3 times and 1.1 times, respectively. The analysis of the medium samples highlighted an important aspect: both in the samples maintained in light and those placed in darkness, arbutin was not identified. This distinguishes the F3K line fundamentally from the other cell line DpHQ studied [34]. The scientific literature contains numerous data indicating that phytohormones can play a major role in the biosynthesis of secondary metabolites. However, the way in which they intervene in these biosynthetic pathways is extremely poorly understood. Moreover, there are many other unknowns, as the same hormone can stimulate or inhibit the production of certain secondary compounds depending on the species that has been cultured *in vitro*. It has been found that 2,4-D stimulated the glucosylation of scopoletin into scopoline in *Nicotiana tabacum* cell cultures by enhancing the activity of the enzyme responsible for this process [45]. There is a possibility that the F3K cell line may behave somewhat differently compared to the other two tested, precisely due to the modification of the phytohormone combination. Although the amounts of arbutin obtained under light were higher than those in darkness, the results were not very different, allowing us to state that light does not significantly influence the biotransformation process in the case of this cell line. However, a 3 % sucrose supplement contributes to enhancing the biotransformation of hydroquinone into arbutin.

Many published biotransformation studies emphasize the importance of the carbon source and its concentration in obtaining secondary metabolites with high yields. Regarding arbutin production, various research teams have tested the influence of glucose and sucrose, as well as the concentration of HQ, in different plant species over time. The amount of arbutin produced in the cell suspensions of the DpHQ line of *Digitalis purpurea* was higher when hydroquinone was added gradually [34]. Similar results were obtained in cell suspensions of *Vigna radiata*, where low HQ concentrations (0.5-1.8 mM) were added in two portions. Consequently, the bioconversion rate of hydroquinone increased from 55.82% with a single-dose administration to 95.66% with a two-dose administration [46]. In *Datura innoxia* cell suspension cultures, the sequential addition of HQ in small doses did not inhibit cellular growth and ensured the production of higher quantities of arbutin [47]. Yokoyama et al. [25] documented successful arbutin production in cell cultures of *Catharanthus roseus*. Their research spanned nearly a decade, with subsequent milestones in 1991 involving successful arbutin production in 5 L fermenters [25, 26]. Yokoyama

emphasized the potential of biotransformation to substitute chemical synthesis due to its high hydroquinone bioconversion rate (98%) and absence of concurrent reactions leading to unwanted by-products. Coste et al. [31] successfully achieved arbutin production in cell cultures of *Digitalis lanata* and *Catharanthus roseus*. Their study investigated the effects of hydroquinone on cell growth, viability, enzyme activity, and various cell densities. Pop et al. [32] expanded their research by conducting quantitative analyses on samples derived from *Digitalis lanata* suspensions. They reported obtaining arbutin percentages ranging from 0.17% to 3.1% in dry biomass. Importantly, they highlighted that arbutin accumulates solely within cells, with no presence detected in the culture medium. Pop et al. [33] indicated the feasibility of obtaining arbutin through the biotransformation of hydroquinone in cell suspensions of *Digitalis purpurea*. Similar to the related species *Digitalis lanata*, arbutin accumulates exclusively within cells and cannot be detected in the culture medium when using a low concentration of hydroquinone (just 1 mM HQ/L). However, in the case of the hydroquinone-resistant DpHQ cell line of *Digitalis purpurea*, depending on the concentration of hydroquinone, arbutin was found both in the biomass and in the culture medium [34]. Although we failed in performing a scaling up of the process by using the F3K cell line, there are some elements of novelty as the influence of different lighting conditions and different HQ concentrations, which might contribute to the optimization of the biotransformation process at largest scales.

3.4. Results of fitting the data with the model

Model performance parameters suggested that more than 80% of response variation was linked to the independent variables (R^2 dry biomass = 0.835; R^2 arbutin content = 0.819). The predictive capacity was satisfactory ($Q^2 > 0.5$), and the reproducibility was high. ANOVA tests identified significant models, having *p* values below the threshold for statistical significance (< 0.05).

3.5. The influence of experimental conditions on arbutin content

The influence of the experimental setup on arbutin production is depicted below as diagrams with scaled and centered coefficients (Figures 3 and 4).

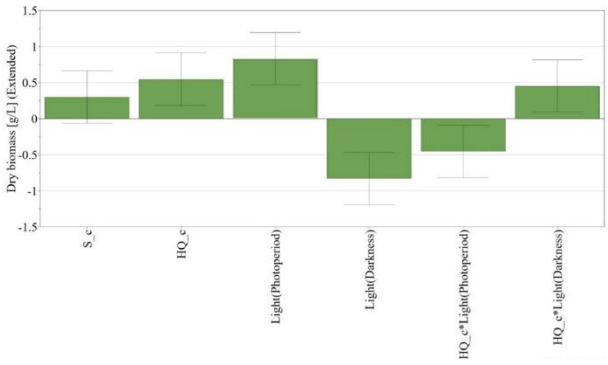


Figure 3. Coefficient column plot for dry biomass production. S_c represents the sucrose concentration; HQ_c indicates the hydroquinone concentration; HQ_c*Light presents the interaction between these two factors.

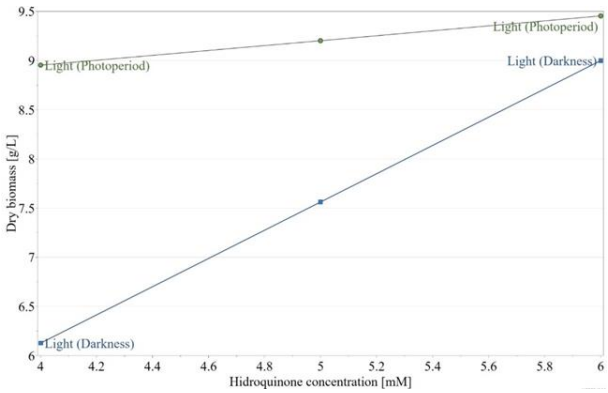


Figure 4. The effect of hydroquinone concentration and light exposure interaction on dry biomass production

The outcomes illustrated in Figures 3 and 4 revealed that sucrose concentration had no significant effect on dry biomass (g/L). On the other hand, the coefficient plots indicated a positive correlation between biomass production and both HQ concentration and light exposure (photoperiod). Furthermore, it was proved that increasing HQ levels exhibited an even more marked influence on biomass production, even in the absence of light, than HQ concentration and light exposure together. This result is supported by the last two columns which depict the interaction between these two independent factors. Darkness conditions led to lower dry biomass production, this variable presenting a negative effect on the experimental outcome.

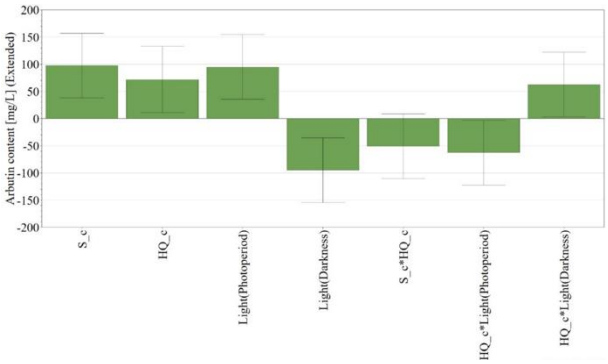


Figure 5. Coefficient column plot for dry arbutin content. S_c indicates the sucrose concentration; HQ_c represents the hydroquinone concentration; HQ_c*Light depicts the interaction between these two factors.

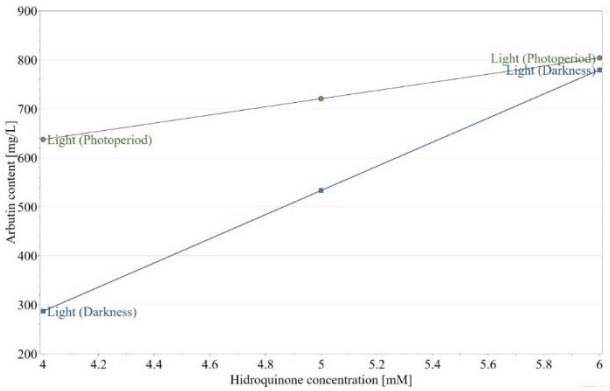


Figure 6. The effect of hydroquinone concentration and light exposure interaction on arbutin content

Sucrose concentration, HQ levels and light exposure (16 h photoperiod), all showed a positive influence on

arbutin content. Higher sucrose (S) concentrations have been shown to promote arbutin production. Increased HQ dosage (5 mM) significantly enhanced arbutin yield, which was to be expected since it serves as a precursor in arbutin biosynthesis, and its increased availability may directly support this process by providing more substrates for the enzymatic reactions involved [40]. Increased HQ doses had an even more pronounced effect on arbutin production under total darkness, which was demonstrated by the coefficient value for the interaction between HQ concentration and light exposure. This suggests that, while each factor independently enhances arbutin synthesis, the effect of increasing HQ concentration on arbutin production yield at the cellular level is amplified by the absence of light. These results are in agreement with those obtained when evaluating the influence of factors on dry biomass, which is to be expected considering that the level of dry biomass is strongly correlated with arbutin production yield.

Surface contour plots were generated to visualize the effects of multiple combinations of independent variables on responses (Figure 7). These diagrams provide a graphical representation of interaction effects and help to understand the optimal conditions for desired outcomes. By analyzing these diagrams, the specific conditions under which the cell culture system exhibits the highest yield for arbutin synthesis can be identified, thus providing a comprehensive tool for exploring the multidimensional space of experimental factors.

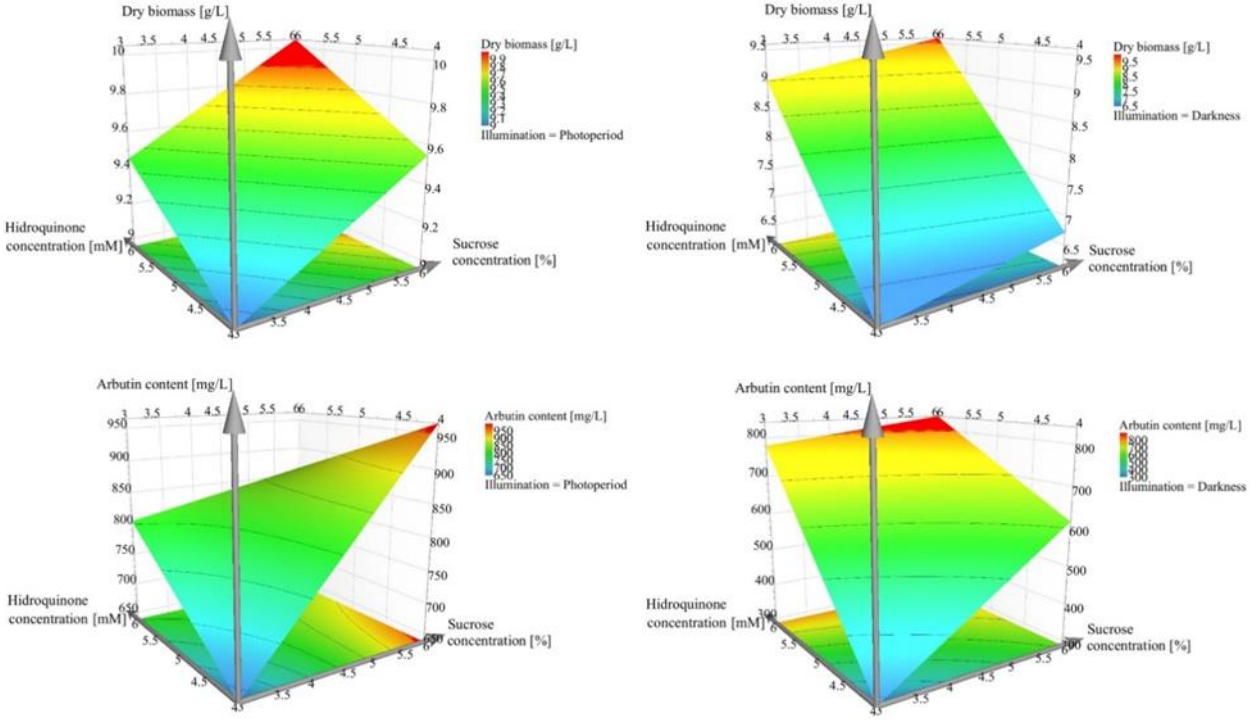


Figure 7. Surface contour plots for Dry biomass and Arbutin content under photoperiod (left) and darkness (right) conditions

The analysis of surface contour plots revealed distinctive patterns in arbutin production influenced by the sucrose and HQ concentrations and illumination conditions. Specifically, higher sucrose levels consistently resulted in higher total arbutin production across both light exposure settings. In addition, a notable interaction between HQ levels and illumination conditions was observed. Under photoperiod conditions, the concentration of HQ, a precursor in arbutin synthesis, influenced arbutin output to a limited extent, which may suggest a saturation point or a limited rate of conversion under these conditions. The highest yield of arbutin production (1009.01 ± 1.01 mg/L) was obtained under light exposure, with 6 % sucrose as carbon source and 5 mM HQ. Conversely, the level of the carbon source showed a positive correlation with arbutin production, highlighting its role as a critical factor in supporting or enhancing the biosynthetic pathway under light-exposed conditions.

Under darkness conditions, the effect of sucrose and HQ levels was similar, exhibiting a positive impact on dry biomass and arbutin production, respectively. Nonetheless, the total amount of arbutin is lower when cell culture is deprived of light, which could indicate an inhibition or different biosynthesis pathway utilization in the absence of light. In this case and consistent with the results for photoperiod, the highest arbutin content (841.93 ± 1.10) was found when 6% sucrose and 5 mM HQ were used. In addition, and similar to photoperiod conditions, an increased level of carbon source continued to positively affect arbutin production, supporting the fundamental role of carbon availability in arbutin biosynthesis regardless of light conditions.

These observed effects on arbutin production were also identified in dry biomass measurements, suggesting a consistent response of cell cultures to these experimental variables, both in terms of biochemical production and growth parameters. This consistency in results supports the reliability of the observed trends as well as the biological relevance of the experimental conditions and evaluated variables.

4. Conclusions

The experimental plan applied to test the biotransformation capacity of HQ into arbutin in the F3K cell line of *Digitalis purpurea* yielded good results, both for biomass multiplication and for obtaining corresponding biotransformation yields. The multifactorial analysis of the tested parameters (carbon source concentration, hydroquinone concentration, and lighting conditions) revealed the importance of a high carbon source quantity. Biomass quantity was not significantly influenced, but the hydroquinone-to-arbutin conversion rate was higher in all cases compared to suspensions without sucrose supplementation. Light proved to be a factor that stimulated both cell growth and the biotransformation process, and the optimal hydroquinone concentration was 5 mM. Arbutin was found almost exclusively in the biomass.

Conflict of interest

The authors declare no conflicts of interest.

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References

[1]. A. Kawalier, On the leaves of *Arctostaphylos uva-ursi*, American Journal of Pharmacy 25 (1835) 68.

[2]. W.H. Xu, Q. Liang, Y.J. Zhang, P. Zhao, Naturally occurring arbutin derivatives and their bioactivities, Chemistry & Biodiversity 12 (2015) 54–81. Doi: 10.1002/cbdv.201300269

[3]. K. Jurica, I. Gobin, D. Kremer, D.V. Čepo, R.J. Grubešić, I.B. Karačonji, I. Kosalec, Arbutin and its metabolite hydroquinone as the main factors in the antimicrobial effect of strawberry tree (*Arbutus unedo* L.) leaves, Journal of Herbal Medicine 8 (2017) 17–23. Doi:10.1016/j.hermed.2017.03.006.

[4]. J. Bruneton, Pharmacognosie, phytochimie, plantes médicinales (4e éd.), Lavoisier, 2009.

[5]. M.J. Kang, H.W. Ha, H.G. Kim, D.H. Lee, M.J. Kong, Y.T. Ahn, D.H. Kim, B.S. Shin, H.G. Kang W. Jeong, T.C. Jeong, Role of metabolism by intestinal bacteria in arbutin-induced toxicity in vitro, Archives of Pharmacal Research 34 (2011) 687–693. Doi: 10.1007/s12272-011-0420-9.

[6]. Y.C. Boo, Arbutin as a skin depigmenting agent with antimelanogenic and antioxidant properties, Antioxidants 10 (2021) 1129. Doi: 10.3390/antiox10071129.

[7]. J.O. Erhabor, R. Komakech, Y. Kang, M. Tang, M.G. Matsabisa, Ethnopharmacological importance and medical applications of *Myrothamnus flabellifolius* Welw. (Myrothamnaceae) - A review, Journal of Ethnopharmacology 252 (2020) 112576. Doi: 10.1016/j.jep.2020.112576

[8]. N. Chaachouay, L. Zidane, Plant-derived natural products: a source for drug discovery and development, Drugs and Drug Candidates 3 (2024) 184-207. Doi: 10.3390/ddc3010011

[9]. S. Marcelino, S. Hamdane, P.D. Gaspar, A. Paço, Sustainable agricultural practices for the production of medicinal and aromatic plants: evidence and recommendations, Sustainability 15 (2023) 14095. Doi: 10.3390/su151914095

[10]. A. Pathak, A.P. Gupta, P. Pandey, in: S.C. Izah, M.C. Ogwu, M. Akram (Eds), Herbal medicine phytochemistry. Reference series in phytochemistry, Springer Cham 2024, pp. 1457-1482. Doi: 10.1007/978-3-031-43199-9_48

[11]. Y. Chen, Z. Dan, S. Li, Growth regulating factor 7-mediated arbutin metabolism enhances rice salt tolerance, The Plant Cell 36 (2024) 2834-2850. Doi: 10.1093/plcell/koae140

[12]. R. Suau, A. Cuevas, V. Valpuesta, M.S. Reid, Arbutin and sucrose in the leaves of the resurrection plant *Myrothamnus flabellifolia*, Phytochemistry 30 (1991) 2555-2556. Doi: 10.1016/0031-9422(91)85098-K

[13]. U. Rockinger, M. Funk, G. Winter, Current approaches of preservation of cells during (freeze-) drying, Journal of Pharmaceutical Sciences 110(8) (2021) 2873-2893. Doi: 10.1016/j.xphs.2021.04.018

[14]. I. Sârbu, N. Ştefan, A. Oprea, Plante vasculare din Romania: determinator ilustrat de teren, Ed. Victor B. Victor, Bucureşti, 2013 (in Romanian).

[15]. European Directorate for the Quality of Medicines European Pharmacopoeia; 11th ed., Council of Europe, Strasbourg, France, 2022.

[16]. Farmacopeea Română, ed. a 10-a.; Editura Medicală, București, 2010 (in Romanian).

[17]. K.X. Xu, M.G. Xue, Z. Li, B.C. Ye, B. Zhang, Recent progress on feasible strategies for arbutin production, Frontiers in Bioengineering and Biotechnology 10 (2022) 914280. Doi: 10.3389/fbioe.2022.914280

[18]. S.L. Huang, Y.L. Zhu, Y.J. Pan, S.H. Wu, Synthesis of arbutin by two-step reaction from glucose, Journal of Zhejiang University-Science A 5 (2004) 1509-1511. Doi: 10.1631/jzus.2004.1509.

[19]. J.L. Xue, J.W. Yang, Q. Deng, X.P. He, G.R. Chen, A shortcut to the preparation of naturally occurring arbutin, Bulletin of the Korean Chemical Society 31 (2010) 1825-1826. Doi: 10.5012/bkcs.2010.31.7.1825

[20]. Y. Tokiwa, M. Kitagawa, T. Raku, S. Yanagitani, K. Yoshino, Enzymatic synthesis of arbutin undecylenic acid ester and its inhibitory effect on melanin synthesis, Bioorganic & Medicinal Chemistry Letters 17 (2007) 3105-3108. Doi: 10.1016/j.bmcl.2007.03.039

[21]. T. Hefner, J. Arend, H. Warzecha, K. Siems, J. Stöckigt, Arbutin synthase, a novel member of the NRD1β glycosyltransferase family, is an unique multifunctional enzyme converting various natural products and xenobiotics, Bioorganic & Medicinal Chemistry 10 (2002) 1731-1741. Doi: 10.1016/S0968-0896(02)00029-9

[22]. P.R. Jones, B.L. Møller, P.B. Høj, The UDP-glucose: p-hydroxymandelonitrile-O-glucosyl-transferase that catalyzes the las step in synthesis of the cyanogenetic glucoside dhurrin in *Sorghum bicolor*. Isolation, cloning, heterologous expression and substrate specificity, The Journal of Biological Chemistry 274 (1999) 35483-35491.

[23]. T. Wu, S.M. Kerbler, A.R. Fernie, Y. Zhang, Plant cell cultures as heterologous bio-factories for secondary metabolite production, Plant Communications 2 (2021) 100235. Doi: 10.1016/j.xplc.2021.100235

[24]. J. Arend, H. Warzecha, J. Stöckigt, Hydroquinone: O-glucosyltransferase from cultivated *Rauvolfia* cells: enrichment and partial amino acid sequences, Phytochemistry 53 (2000) 187-193. Doi: 10.1016/S0031-9422(99)00539-7

[25]. M. Yokoyama, S. Inomata, in Y.P.S Bajaj (Ed.), Medicinal and Aromatic Plants X Biotechnology in Agriculture and Forestry, Springer 1998, pp. 67–80.

[26]. M. Yokoyama, S. Inomata, M. Yanagi, Y. Wachi, Change of maximal cellular productivity of arbutin by biotransformation depending on the culture stage of *Catharanthus roseus* cells, Plant Tissue Culture Letters 13 (1996) 285–290. Doi: 10.5511/plantbiotechnology1984.13.285

[27]. R. Lutterbach, J. Stöckigt, High-yield formation of arbutin from hydroquinone by cell-suspension cultures of *Rauwolfia serpentina*, Helvetica Chimica Acta 75 (1992) 2009–2011, Doi: 10.1002/hlca.19920750625.

[28]. I. Kwiecień, A. Szopa, K. Madej, H. Ekiert, Arbutin production via biotransformation of hydroquinone in *in vitro* cultures of *Aronia melanocarpa* (Michx.) Elliott, Acta Biochimica Polonica 60 (2013) 865–870. Doi: 10.18388/abp.2013_2074

[29]. N. Kittipongpatana, P. Maneerat, P. Pattanakitkosol, O. Kittipongpatana, Effect of some factors on the growth of *Capsicum annuum* L. cell suspension culture and biotransformation of hydroquinone to arbutin, Chiang Mai University Journal of Natural Sciences 6 (2007) 207–218.

[30]. D.A. Casas, S.I. Pitta-Alvarez, A.M. Giulietti, Biotransformation of hydroquinone by hairy roots of *Brugmansia candida* and effect of sugars and free-radical scavengers, Applied Biochemistry and Biotechnology 69 (1998) 127–136. Doi: 10.1007/BF02919394.

[31]. A. Coste, C.E. Pop, A. Butiuc-Keul, C. Munteanu-Deliu, C. Deliu, A. Halmagyi, Aspects concerning hydroquinone effect on cell cultures of *Arctostaphyllos uva-ursi*, *Catharantus roseus* and *Digitalis lanata*, Contribuţii Botanice 38 (2003) 93–104.

[32]. C.E. Pop, C. Deliu, M. Tămaș, Biotransformarea hidrochinonei în arbutozidă în culturi celulare in vitro de *Digitalis lanata*, Clujul Medical 78 (2005) 182–187.

[33]. C.E. Pop, C. Deliu, M. Tămaș, A. Coste, Use of *Digitalis purpurea* cell cultures for the biotransformation of hydroquinone into arbutin, Proceedings of the 4th Conference on Medicinal and Aromatic Plants of South-East European Countries (2006) 220–225.

[34]. C.E. Pop, A. Coste, A.-M. Vlase, C. Deliu, M. Tămaș, T. Casian, L. Vlase, Selection of a *Digitalis purpurea* cell line with improved bioconversion capacity of hydroquinone into arbutin, Life 14 (2024) 84. Doi:10.3390/life14010084.

[35]. T. Murashige, F.A. Skoog, Revised medium for rapid growth and bio assays with tobacco tissue cultures, Plant Physiology 15 (1962) 473–497. Doi: 10.1111/j.1399-3054.1962.tb08052.x.

[36]. C.E. Pop, L. Vlase, M. Tămaș, Natural resources containing arbutin. Determination of arbutin in the leaves of *Bergenia crassifolia* L. Fritsch. acclimated in Romania, Notulae Botanicae Horti Agrobotanici 37 (2009) 129–132. Doi: 10.15835/nbha3713108

[37]. Q. Ma, P. Grones, S. Robert, Auxin signaling: a big question to be addressed by small molecules, Journal of Experimental Botany 69 (2018) 313-328. Doi: 10.1093/jxb/erx375

[38]. L.A. Dantas, P.S.A. Faria, B.M.M. Dário, A.L.M. Arantes, F.G. Silva, R.G. Avila, P.S. Pereira, A.R. Neto, The impact of carbon source on cell growth and the production of bioactive compounds in cell suspensions of *Hancornia speciosa* gomes, Scientific Reports 11 (2021) 24315. Doi: 10.1038/s41598-021-03845-0.

[39]. M. Modarres, S. Esmaeilzadeh Bahabadi, M.E. Taghavizadeh Yazdi, Enhanced production of phenolic acids in cell suspension culture of *Salvia leriifolia* Benth. using growth regulators and sucrose, Cytotechnology 70 (2018) 741–750. Doi: 10.1007/s10616-017-0178-0.

[40]. K. See, A. Bhatt, C. Keng, Effect of sucrose and methyl jasmonate on biomass and anthocyanin production in cell suspension culture of *Melastoma malabathricum* (Melastomaceae), Revista de Biología Tropical 59 (2011) 597–606.

[41]. H. Mizukami, T. Terao, A. Amano, H. Ohashi, Glucosylation of salicyl alcohol by *Gardenia jasminoides* cell cultures, Plant and Cell Physiology 27 (1986) 645-650. Doi: 10.1093/oxfordjournals.pcp.a077144

[42]. M. Yokoyama, S. Inomata, S. Seto, M. Yanagi, Effects of sugars on the glucosylation of exogenous hydroquinone by *Catharanthus roseus* cells in suspension culture, Plant and Cell Physiology 31 (1990) 551–555. Doi: 10.1093/oxfordjournals.pcp.a077945.

[43]. M. Singh, R. Chaturvedi, Evaluation of nutrient uptake and physical parameters on cell biomass growth and production of spilanthol in suspension cultures of *Spilanthes acmella* Murr, Bioprocess and Biosystems Engineering 35 (2012) 943–951. Doi: 10.1007/s00449-012-0679-3.

[44]. C.O. Akalezi, S. Liu, Q.S. Li, J.T. Yu, J.J. Zhong, Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*, Process Biochemistry 34 **(**1999) 639-642. Doi:10.1016/S0032-9592(98)00132-0.

[45]. F. Hino, M. Okazaki, Y. Miura, Effect of 2,4-dichlorophenoxyacetic acid on glucosyilation of scopoletin to scopolin in tobaco tissue culture, Plant Physiology 62 (1982) 810-813. Doi: 10.1104/pp.69.4.810

[46]. Z. Tofighi, M. Amini, M. Shirzadi, H. Mirhabibi, N. Ghazi Saeedi, N. Yassa, *Vigna radiata* as a new source for biotransformation of hydroquinone to arbutin, Pharmaceutical Sciences 22 (2016) 126–131. Doi: 10.15171/PS.2016.20

[47]. T. Suzuki, T. Yoshioka, M. Tabata, Y. Fujita, Potential of *Datura innoxia* cell suspension cultures for glucosylating hydroquinone, Plant Cell Reports 6 (1987) 275–278. Doi: 10.1007/BF00271997.

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