Research Article

Vigna radiata as a New Source for Biotransformation of Hydroquinone to Arbutin

Zahra Tofighi[1], Mohsen Amini[2], Mahzad Shirzadi[1], Hamideh Mirhabibi[1], Negar Ghazi Saeedi[1], Narguess Yassa[1*]

[1]*Department of Pharmacognosy, Faculty of Pharmacy and Medicinal Plant Research Center, Tehran University of Medical Sciences, Tehran, Iran*

[2]*Department of Medicinal Chemistry, Faculty of Pharmacy and Medicinal Plant Research Center, Tehran University of Medical Sciences, Tehran, Iran*

A B S T R A C T

Background: The suspension culture of *Vigna radiata* was selected for biotransformation of hydroquinone to its β-D-glucoside form (arbutin) as an important therapeutic and cosmetic compound.

Methods: The biotransformation efficiency of a *Vigna radiata* cell culture in addition to different concentrations of hydroquinone (6-20 mg/100 ml) was investigated after 24 hours in comparison to an *Echinacea purpurea* cell culture and attempts were made to increase the efficacy of the process by adding elicitors.

Results: Arbutin was accumulated in cells and found in the media only in insignificant amounts. The arbutin content of the biomass extracts of *V. radiata* and *E. purpurea* was different, ranging from 0.78 to 1.89% and 2.00 to 3.55% of dry weight, respectively. *V. radiata* demonstrated a bioconversion efficiency of 55.82% after adding 8 mg/100 ml precursor, which was comparable with result of 69.53% for *E. purpurea* cells after adding 10 mg/100 ml hydroquinone (P>0.05). In both cultures, adding hydroquinone in two portions with a 24-hour interval increased the biotransformation efficiency. Different concentrations of methyl jasmonate (25, 50, and 100 μM) and chitosan (50 and 100 μg/ml) as elicitors increased the bio-efficiency percentage of the *V. radiata* culture in comparison with the flask containing only hydroquinone.

Conclusion: This is the first report of the biotransformation possibility of *V. radiata* cultures. It was observed the bioconversion capacity increased by adding hydroquinone in two portions, which was comparable to adding an elicitor.

Introduction

Arbutin, hydroquinone β-D-glucoside, is a natural valuable phenolic compound existing in the leaves of some species of *Ericaceae, Rosaceae, Saxiferaceae, Rubiaceae* and *Fabaceae* families, as well as other taxa.[1]

Arbutin is used for its important therapeutic and cosmetic properties. Arbutin containing plant extracts are widely used for their urogenital disinfectant activity and strong antitussive effects in traditional and modern medicine.[2] Arbutin is also an efficient tyrosinase inhibitor and can block melanin biosynthesis in the human skin. Cosmetic and pharmaceutical industries are focused on the use of arbutin because of more or equal lightening properties and less toxicity and side effects in comparison with hydroquinone.[3]

Several attempts have been made for production of arbutin in a biotechnological way instead of chemical synthesis. The capability of microorganisms to carry out β-D-glucosylation is weaker than α-D-glucosylation.[4] Biotransformation of arbutin by plant cell and tissue culture is one of the most promising procedures that lead to detoxification of exogenous hydroquinone through glucosylation. Several plant cell and tissue culture have demonstrated the capability of forming glucosyl conjugates of secondary metabolites such as phenolics, steroids, cardenolides, flavonoids, and anthraquinones.[5,6]

Various plants have been selected for biotransformation of hydroquinone to arbutin. The yields of biotransformation in earlier experiments on plant cell culture were relatively low,[7,8] but the yields increased in recent years due to technology improvement, which suggests economical production of many compounds.[9] The highest production of arbutin has been reported with *Catharanthus roseus* cells, up to 9 g pure arbutin/l cell suspension.[10] A similar process has been developed with *Rauwolfia* cell suspension, enhancing and the amount of the formed arbutin considerably by up to 18 g/l of nutrition medium.[11]

Vigna radiata (L.) Wilczek (Mung bean) is one of the important pulse crops widely grown in Asian countries.

Its seeds contain 51% carbohydrate, 24–26% protein, 4% mineral, and 3% vitamins.[12] The mung bean has many pharmacological properties like antitumor activities, angiotensin I-converting enzyme inhibitory effects, and antioxidant and antifungal properties.[13]

In vitro cultures of *Vigna radiata* were reported earlier.[14-17] The present research investigated the biotransformation possibility of hydroquinone to arbutin for the first time. On the other hand, it evaluated its bioconversion capacity by adding elicitors like chitosan and methyl jasmonate to *V. radiata* cell culture.

Materials and Methods
Chemicals
Murashige and Skoog (MS) medium and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were obtained from Duchefa Biochemie (Netherland). Arbutin and plant agar were prepared from Merck Company (Germany). Hydroquinone and elicitors, methyl jasmonate and chitosan, were purchased from Sigma–Aldrich, USA.

Establishment of plant in-vitro cultures
The seeds of *Vigna radiata* (L.) R. Wilczek and *Echinacea purpurea* (L.) Moench were obtained from the market and herboratum of Faculty of Pharmacy, Tehran University of Medical Sciences (PMP-748 and 84159, respectively).

The seeds were surface-sterilized with 1.25% (w/v) calcium hypochlorite solution for 15 to 20 minutes. After rinsing with sterile distilled water, the seeds were germinated on Murashige and Skoog (MS) medium containing 1 ppm 2, 4-dichlorophenoxy acetic acid (2, 4-D), 3% (w/v) sucrose, and 1% (v/v) vitamins solution (Vitamin B_1 0.1 g, B_2 0.05 g, B_6 0.2 g, Folic acid 0.05 g, Biotin 0.1 g, Nicotinamide 0.2 g and Calcium pantothenate 0.1 g) (PH 6.0). The obtained seedlings were transferred to another jar for callus production. The young and healthy calluses were subcultured every 4 weeks. Suspension cultures were formed by inoculating friable calluses from generation 4 of *V. radiata* and generation 7 of *E. purpurea* into 500 ml erlenmeyer flasks containing 100 ml liquid MS medium supplemented with 0.5 ppm 2, 4-D and other components in the same concentration of agar medium. All cell suspensions were subcultured every 3 weeks. The ratio between the volume of inocula and the volume of fresh medium was 1:2 or 1:3. The suspension cultures were placed on a rotary shaker gyrating at 100 rpm and maintained in the 12 h light/dark cycle at 25± 2 °C.

Biotransformation experiments
For biotransformation experiments, the day 20 of the culture stage of *V. radiata* and *E. purpurea* cell suspension cultures were used. Hydroquinone was dissolved in bi-distilled water (10 mg/ml) and administered aseptically through a 0.2 μm membrane

filter to obtain final concentrations (6, 8, 10, 15, and 20 mg/100 ml). Each experiment was carried out in three replicates. The cultures were incubated in the dark under the aforementioned condition. Twenty-four hours after adding of last dose of hydroquinone, the biomass was separated from the medium by vacuum filtration. The biomass was rinsed with distilled water, dried at room temperature, and weighed to achieve its dry weight. The supernatants of all different tests (media) were collected separately and each one was freeze dried, weighed, and kept for further analysis.

Elicitation experiments
The elicitation process was carried out with chitosan (β-1, 4-poly-D-glucosamine) and methyl jasmonate on day 20 of *V. radiata* culture.

The chitosan was dissolved in 1% aqueous acetic acid by stirring and the pH of the solution was adjusted to 5.5 by adding 1 M sodium hydroxide. Then, the stock solution was autoclaved at 120 °C for 15 minutes.[18] Different concentrations of the chitosan solution (25, 50 and 100 μg/ml) were added individually to *V. radiata* cell cultures in the presence of 8 mg/100 ml hydroquinone. After 72 hours, the biomasses were separated and their dry weights were determined separately.

Methyl jasmonate was first dissolved in ethanol: H_2O (13:12) to obtain a final concentration of 10,000 μM.[19] Then, various concentrations of methyl jasmonate (25, 50 and 100 μM) were made up, filter sterilized, and fed along with 80 mg/l hydroquinone to the *V. radiata* cultures. After 24 hours of treatment, the cells were harvested, dried at room temperature, and weighed. All dried masses were kept until extract preparation and all tests were carried out in triplicate.

Extraction
The dried biomass of each culture was milled and extracted three times with boiling methanol. The methanol extracts were concentrated and the remainder was dissolved in 20 ml methanol (CME: cell methanol extract). The lyophilized media were dissolved in 20 ml methanol, too (ME: media extract).

Analysis of arbutin
Qualitative analysis of arbutin and hydroquinone was performed using the method proposed by Skrzypczak-Pietraszek et al. with some modification.[1] Methanol extracts were spotted on TLC plates (Merck, Silicagel 60 F_{254}) and chromatographed with ethyl acetate: methanol: water (100:13.5:10) solvent. After development, the plates were sprayed with anisaldehyde sulfuric acid reagent for the detection of spots. On the chromatograms, arbutin was represented by a brown spot with $R_f = 0.48$ and hydroquinone was represented by an orange spot with $R_f = 0.91$.

For quantitative assay of arbutin and hydroquinone, all methanol extracts were analyzed by HPLC using the method proposed by Piekoszewska et al. with slight

modifications.[20] For this reason, 100 µl of CME and ME was dissolved in 5 ml water and 20 µl of the mixture was injected to the RP-18 column (250 × 4 mm; 5 µm); then, the column was eluted with methanol: water (15:85). The flow rate was 1 ml/min. Arbutin and hydroquinone were both detected at 285 nm (retention times: 6.2 and 10.2 min, respectively). The amount of arbutin was calculated from the calibration curve.

The capacity of bioconversion of hydroquinone to arbutin was calculated using the following formula:

Efficiency (%) = 100 × (mg of produced arbutin/ maximum mg of arbutin can be obtained) per flask. Eq.(1)

Statistical analysis

The results of arbutin content and efficiency of biotransformation are presented as mean ± standard deviation. Student's *t*-test was used to evaluate the statistical significance between the groups, and *P* values less than 0.05 were considered significant.

Results

To establish experimental cultures, calli were transferred to a liquid medium under aseptic conditions. The callus culture of *V. radiata* formed a brown friable mass, but the calli of *E. purpurea* formed a light cream mass soft in texture. The cells in suspension cultures of *V. radiata* were very small in size, fine and friable with a brown color. The cells of *E. purpurea* were grown with isolated cells and small aggregates which showed different colors in various generations (cream to light gray).

Arbutin as the biotransformation product was found in biomass extracts of both plant cultures. The amount of arbutin in lyophilized media (supernatants of filtered cultures) was insignificant. The arbutin content of different samples was measured based on the calculation curve (y=11.61x-16.91; r^2=0.999).
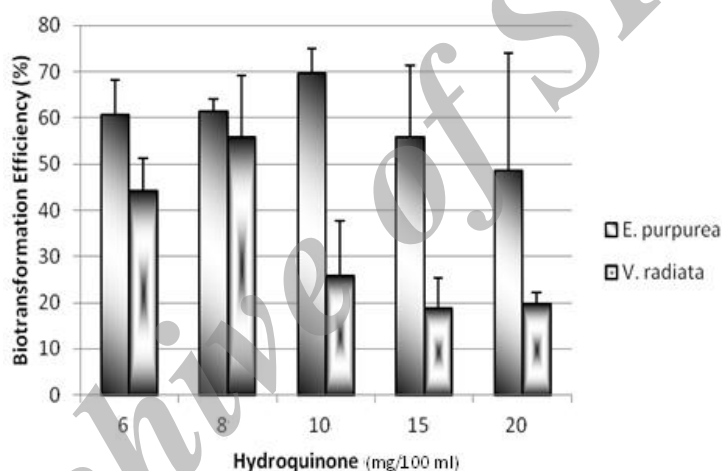

Figure 1. Bioconversion of different concentrations of hydroquinone to arbutin by *E. purpurea* and *V. radiata*.

The biotransformation efficiency of *V. radiata* and *E. purpurea* cells after adding different concentrations of hydroquinone (6-20 mg/100 ml) after 24 hours has been shown in Figure 1. *V. radiata* and *E. purpurea* cultures demonstrated the best efficiency of the process after adding 8 and 10 mg/100 ml precursor.

The arbutin content and the biotransformation efficiency of *V. radiata* and *E. purpurea* cultures in the presence of different concentrations of hydroquinone, in one or two portions, are demonstrated in Table 1. *V. radiata* and *E. purpurea* cultures yielded maximum amounts of arbutin by adding 8 and 15 mg/100 ml hydroquinone in two portions, respectively.

In *V. radiata* culture, biotransformation induction with chitosan and methyl jasmonate was performed in the presence of 8 mg/100 ml hydroquinone. Table 2 shows the efficiency of biotransformation and the arbutin content in presence of various concentrations of chitosan (25, 50 and 100 µg/ml) and methyl jasmonate (25, 50 and 100 µM). It was observed that 100 µM

methyl jasmonate enhanced the biotransformation rate more than other (*P*<0.05).

Discussion

Glycosylation is often the last modification reaction in the biosynthesis of plant secondary metabolites.[21] The glycosyl transferases are enzymes which transfer activated sugars to low molecular weight substrates. Sugar acceptors include all major classes of natural products, including phenolics, terpenoids, cyanohydrins, thiohydroximates and alkaloids.[22] There are reports of the existence of glycosyl transferase in *Vigna radiata* and *E. purpurea*.[1,23-24] Since *V. radiata* is an edible and easily accessible plant so it was selected for conversion of hydroquinone to arbutin in our experiment.

The results showed that the bioconversion capacity quickly diminished by increasing the hydroquinone dose.

Table 1. Arbutin content and biotransformation efficiency of hydroquinone to arbutin in of *V. radiata* culture compared to *E. purpurea* culture.

Hydroquinone concentration (mg/100 ml)	Method of hydroquinone supply	Arbutin content (g/100 g dry weight)		Arbutin content (mg/100 ml)		Efficiency of Biotransformation (%)	
		V. radiata	*E. purpurea*	*V. radiata*	*E. purpurea*	*V. radiata*	*E. purpurea*
6	single dose	0.78±0.18	2.19±0.77	6.53±3.16	8.72±3.38	44.11±7.11	60.58±7.63
6	2 portions	18.78±0.98	-	12.36±1.26	-	83.05±2.82	-
8	single dose	1.89±0.23	2.00±0.67	11.04±7.88	12.14±1.64	55.82±13.28	61.35±2.78
8	**2 portions**	**32.54±3.23**	-	**18.92±1.04**	-	**95.66±1.75**	-
10	single dose	1.24±0.77	3.55±0.23	6.35±8.85	17.16±4.08	25.71±11.96	69.53±5.51
10	2 portions	14.62±0.65	2.27±0.50	10.01±0.85	18.73±6.64	40.42±1.14	75.89±8.97
15	single dose	1.46±0.25	3.15±0.70	6.80±7.08	20.28±16.9	18.73±6.50	55.86±15.5
15	2 portions	9.53±1.33	3.24±0.61	7.02±2.49	22.83±12.5	18.91±2.24	67.08±12.4
20	single dose	1.71±0.37	2.97±1.24	9.66±3.50	23.75±37.4	19.71±2.37	48.47±25.5
30	single dose	-	1.37±0.46	-	7.96±14.0	-	14.45±1.27
40	single dose	-	1.74±1.23	-	10.87±32.2	-	4.74±9.20

The data were represented as Mean±SD from 3 replicates.

Table 2. Arbutin content and bioconversion percentage of *V. radiata* culture in presence of 8 mg/100 ml hydroquinone.

Elicitor	Elicitor concentration	Arbutin content (g/100 g dry weight)	Arbutin content (mg/100 ml)	Efficiency of Biotransformation (%)
[a]Chitosan	25 µg/ml	10.50±1.56	9.20±1.41	46.49±2.38
	50 µg/ml	14.04±0.51	13.26±1.74	67.03±2.94
	100 µg/ml	16.65±0.41	17.03±1.98	86.10±3.34
[b]Methyl Jasmonate	25 µM	12.21±0.54	12.21±1.61	61.70±2.72
	50 µM	16.20±0.73	15.10±1.41	76.35±2.36
	100 µM	19.97±0.78	18.83±1.33	95.20±2.25

The data were represented as Mean±SD from 3 replicates. The biotransformation ability was investigated after 72 h ([a]) and 24 h ([b]).

E. purpurea demonstrated the highest efficiency of the process (69.53%) by adding 10 mg/100 ml of the precursor. This finding is comparable with the result of 55.82% for *V. radiata* cells by adding 8 mg/100 ml hydroquinone (*P*>0.05). The maximum reported yield of bioconversion for *E. purpurea* has been 58.10% after 36 hours.[1]

The arbutin contents of the extracts of *V. radiata* and *E. purpurea* biomass were different, ranging from 0.78 to 1.71% and 1.37 to 3.55% of dry weight, respectively. Skrzypczak-Pietraszek et al (2005) demonstrated that the maximal scale of hydroquinone biotransformation to arbutin in the suspension culture of *E. purpurea* was 4.01 g/100 g cell dry weight after 24 hours.[1]

Because of the cytotoxic effect of hydroquinone on plant cells, Terasaka et al. (2005) showed that utilization of high concentrations of the precursor at a single portion in the culture medium could reduce the production of arbutin.[25] Addition of hydroquinone to the media in divided quantities causes less cell death and allows the viable cells to produce more arbutin.[7] Therefore, *V. radiata* and *E. purpurea* cultures with best bio-efficiency percentages, were selected for the evaluation the effect of adding hydroquinone in divided doses on biotransformation. In *V. radiata* and *E. purpurea* cultures, adding 8 and 10 mg/100 ml hydroquinone in two portions with a 24 hour interval

increased the biotransformation efficiency by up to 71.4% and 9.1%, respectively. Also, the production of arbutin per flask was increased by about 41.6% and 8.4%, respectively. However, in previous studies, optimization of biotransformation conditions such as hydroquinone dose partitioning into three or four portions st 24-hour intervals led to obtaining the maximum amount of arbutin.[26,27]

Induction of biotransformation with chitosan and methyl jasmonate has been reported in previous studies.[28,29] Addition of 25 µg/ml chitosan to *V. radiata* culture did not increase the bioconversion percentage after 72 hours, but addition of 50 and 100 µg/ml chitosan to *V. radiata* culture enhanced the biotransformation efficacy by up to 20.1% and 54.9%, respectively. Moreover, the content of arbutin per flask was elevated by about 20.1% and 54.2%, respectively. The results demonstrated that chitosan enhanced both the bioconversion efficacy and arbutin content in comparison to 8 mg/100 ml hydroquinone (*P*<0.05). Methyl jasmonate was used as another elicitor in the *V. radiata* culture. Different concentrations of methyl jasmonate (25, 50 and 100 µM) increased the bio-efficiency percentage and arbutin content of the flask by up to 10.5%, 36.8%, and 70.5% respectively in comparison to hydroquinone alone (*P*<0.05). This result strongly suggests that the presence of methyl

jasmonate promotes the arbutin production in *V. radiata* culture. A previous investigation demonstrated endogenous jasmonic acid mediated to induce glucosyl transferase activation.[26] It could be the reason for the best obtained biotransformation efficacy by methyl jasmonate in our study.

Conclusion

The arbutin producing biotransformation systems were developed based on the cell suspension culture of *V. radiata* and *E. purpurea* using hydroquinone as a precursor.

V. radiata culture showed a lower sensitivity for the biotransformation of hydroquinone, but the bioconversion capacity was increased by dividing the hydroquinone intake or application of elicitors.

Acknowledgments

Conflict of interests

The authors claim that there is no conflict of interest.

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