Preliminary Investigation on Arbutin Production in Mulberry (*Morus nigra*) Callus Culture

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ABSTRACT

Arbutin, a phenolic glucoside, can be extracted from plants to use as bioactive compounds. This work investigated the production of arbutin in callus of mulberry, *Morus nigra.* Tissue culture of mulberry was carried out from the lateral buds via sterilization using different combinations of chemicals. Soaking the explants in carbendazim for 10 min, followed by 70% ethanol (v/v) for 5 min, sodium hypochlorite (NaOCl) solution twice for 10 min each and finally 0.1% (w/v) of mercuric chloride ($HgCl_2$) for 5 min offered the highest survival percentage of 72.50±17.08. The sterilized buds were subsequently placed in Murashige and Skoog media (MS) supplemented with 2,4-dichlorophenoxy (2,4-D) at 1.0 mg/l for 4 weeks to establish the culture. The leaves from the sterile explants were directly transferred to MS supplemented with 3 different growth regulators including 2,4-dichlorophenoxy acetic (2,4-D), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) at concentrations of 1.0, 2.0 and 3.0 mg/l to investigate arbutin production. Arbutin in callus culture was analyzed in comparison with native leaves via high performance liquid chromatography (HPLC) on C18 Hypersil ODS column using water: methanal: hydrochloric acid (89:10:1, v/v) as mobile phase. The average arbutin content in callus cultured in MS supplemented with 3.0 mg/l IAA was the highest at 35.67 ± 0.27 µg/mg dry weight when compared with the controls at 13.43 ± 4.29 µg/mg dry weight and native leaves at 31.14 ± 13.86 µg/mg dry weight. Due to the fewer pigments present in the callus than in the native counterpart, the IAA elicited mulberry callus is, thus, an alternative source for arbutin production with the advantage of the separation convenience.

Keywords: Arbutin, Callus, Mulberry, *Morus nigra*

INTRODUCTION

Arbutin, a phenolic glucoside, can be found in nature as either α-arbutin or β-arbutin (Figure 1). Arbutin is a secondary metabolite present in many plants such as bearberry (*Arctostaphylos uva-ursi*), cranberry (*Vaccinium macrocapron*) and blueberry (*Vaccinium corymbosum*) (Kittipongpatana N., 2007). Arbutin has been reported to have potential to stimulate alcohol metabolism, decrease blood alcohol levels and hangover symptoms. Arbutin contained in the leaves of the Wild Himalayan pear, *Pyrus biossieriana Buhse,* was concluded to contribute to antioxidant, antihyperglycaemic and antihyperlipidemic activities in hyperglycaemia and was an effective cryoprotective agent for osteochondral allografts. In cosmetics, arbutin is a compatible and effective natural whitening substance without skin stimulation and allergy. The skin whitening property of arbutin is due to its tyrosinase inhibitory effect. It has been shown that α-arbutin was much more effective than β-arbutin as whitening agent (Chun-Qiao L. et al. 2013).

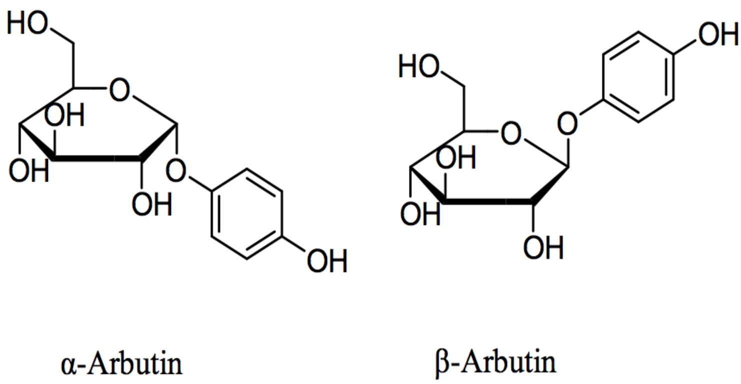

Figure 1. Chemical structures of α-arbutin and β-arbutin.

Mulberries belong to the Moraceae family and are native to temperate Asia and North America. Several species are cultivated for their fruits and as ornamentals (Burlando *et al*., 2017). Mulberry plants are also important as food for silkworms. Almost all of the parts of mulberry trees are used for pharmacological purposes all over the world. The leaves have been shown to possess diuretic, hypoglycemic and hypotensive activities, whereas the root bark has long been used as anti-inflammatory, antitussive and antipyretic agent. Mulberry fruits can be used as a warming agent, as a remedy for dysentery, and as a tonic, sedative, laxative, odontalgic, anthelmintic, expectorant and emetic agent (Pawlowska *et al*., 2008). Extract of mulberry has also been shown to have tyrosinase inhibitory effect which can be applied for cosmetic uses (Burlando *et al*., 2017). The quantitative analysis of arbutin is commonly carried out via high performance liquid chromatography (HPLC). Arbutin contents in callus cultures of *Capsicum annuum* L., *Solanum aculeatissimum*, *Datura fastuosa*, *basilicum* L. and *Allamanda cathartica* L. have been analyzed and reported by HPLC based on arbutin standard (Kittipongpatana N., 2007).

Plants can produce secondary metabolites such as phenolic compounds that possess diverse biological activities and are often used as medicinal substances. Some of the phenolic compounds can also be synthesized in plant cells and tissue culture *in vitro*. In this relation, the culture conditions need to be optimized to assist their accumulation. Such components of nutrient media, hormones and hormone-like compounds have combined effect on the synthesis of secondary metabolites in cell cultures of the plants. In this work, various MS media with growth regulators were prepared to investigate arbutin production in callus of mulberry. The contents of arbutin in the samples will be quantified via HPLC. Both tissue culture technique and analytical method developed here will be useful as alternative means for arbutin production and quantification, respectively.

MATERIAL AND METHODS

Plant materials and chemicals

Mulberry plants, *Morus nigra*, were purchased from a local supplier in Chiang Mai Province, Thailand for tissue culture preparation. Plant tissue growth regulators including 2,4- dichlorophenoxy (2,4- D) and 1-naphthaleneacetic acid (NAA) were obtained from Fulka, USA, while indole-3- acetic acid (IAA) was from Sigma- Aldrich, Germany. Other reagents for plant tissue culture were of analytical grade. Arbutin standard was purchased from Sigma Chemical Co. (St. Louis, USA). All chemicals used for chromatographic purposes were of HPLC grade.

Explant surface sterilization methods

Mulberry lateral buds from fresh branches were cut and rinsed thoroughly by tap water and used as explants in three different sterilization methods as shown in Table 1 with steps in the order from left to right. The bud segments were cultured on sterile MS agar medium, pH 5.8 containing 3% sucrose and 0.2% (w/v) gelatin in the presence of 2,4-D at 1.0 mg/l. The cultures were incubated under a photoperiod of 16 h light and 8 h dark at room temperature (25 ± 2 °C). The percentages of explants contaminated or necrotic or survived were calculated as follows:

$$\frac{\text{Number of explants contaminated or necrotic or survived}}{\text{Total number of explants cultured}} \times 100$$

Table 1. Bud surface sterilization methods

Treatment	**Disinfectants**				
	carbendazim (%v/v)	**Ethanol (%v/v)**	**NaOCl (%v/v) with a few drops of Tween 20**		**$HgCl_2$ (%v/v)**
1	1 (10 min)	70 (1 min)	20 (10 min)	10 (10 min)	-
2	-	70 (1 min)	20 (10 min)	10 (10 min)	0.1 (5 min)
3	1 (10 min)	70 (1 min)	20 (10 min)	10 (10 min)	0.1 (5 min)

Callus induction from leaf segments

Leaves (5 cm long) were removed from 4 weeks old *in vitro* explants. Without any further sterilization the leaves were cut around the edge and placed onto MS agar media supplemented with different growth regulators including 2,4-D, NAA and IAA at concentrations of 1.0, 2.0 and 3.0 mg/l for callus induction and to investigate arbutin production. The cultures were incubated under a photoperiod of 16 h light and 8 h dark at room temperature (25 ± 2 °C) and harvested at 4 weeks old.

Determination of arbutin contents in the callus culture by high performance liquid chromatography (HPLC)

Instrumentation and chromatographic conditions

Arbutin quantification by HPLC was performed on Agilent LC-1100 and run on reversed-phase C18 Hypersil ODS column (125 x 4 mm ID, 5 µm) at room temperature (25 ± 2 °C) and monitored by UV detector at 240 nm. The mobile phase was methanol: water: hydrochloric acid (89:10:1, v/v) with a flow rate of 1.0 ml/min. The mobile phase was filtered through a nylon membrane filter (0.45 µm) and degassed prior to use. The injection volume of all samples was 10 µl. Quantitative analysis was performed using the peak area based standard curve.

Standard arbutin solution preparation

Standard stock solutions of arbutin were prepared at the concentration of 5, 10 and 15 mg/ml by weighing and dissolving arbutin standard in 1.0 ml of absolute methanol accordingly. The injection volume of all standard samples was 10 µl. The standard curve was constructed using peak area reported by the HPLC analysis versus known amounts of arbutin.

Extraction of abutin from callus culture

Callus at 4 weeks old was harvested and dried to constant weight in an oven at 50 ºC. The dried callus was ground in a mortar and stored as powder. Five hundred milligrams of callus powder were soaked in 1.0 ml of absolute methanol at room temperature for 24 hrs for arbutin extraction. The mixture was then centrifuged at 6,700 x g for 15 min and filtered through a 0.45 µm nylon membrane filter. The filtrate was analyzed by high performance liquid chromatography.

Statistical Analysis

Statistical analysis for this work was performed using One Way Analysis of Variance (ANOVA) from SPSS software version 17.0. The multiple range significantly different LSD Test was performed at $p < 0.05$. All experiments were repeated and the results were given as the mean of three independent experiments ± standard error.

RESULTS

Mulberry lateral bud surface sterilization

Effectiveness of surface sterilization was evaluated after 2 weeks of culturing by observing and recording the number of explants with contamination of microbes. Some pieces did not have contamination, but they did not survive and were labelled as necrotic. The ones without contamination and demonstrated growth of leaves were counted as explants that survived. The number of explants for each category was used to calculate the percentage based on the number of explants originally cultured. Surface sterilization in treatment 1 performed by carbendazim pretreatment, followed by immersing the explants in ethanol, sodium hypochlorite (NaOCl) solution with a few drops of Tween 20 without employing mercuric chloride ($HgCl_2$) solution in this study resulted in contamination of 30.00 ± 11.55 % and offered 47.50 ± 9.57 % survival. Without carbendazim pretreatment, immersing explants in ethanol, followed by $HgCl_2$ solution provided slightly higher percentage of survival of 55.00 ± 17.31 (Table 2, treatment 2). Out of the three surface sterilization treatments carried out, pretreatment of mulberry buds with carbendazim for 10 min, followed by immersing in 70% ethanol (v/v) for 5 min, NaOCl solution twice for 10 min and finally by 0.1% (w/v) $HgCl_2$ for 5 min was the most effective procedure resulting in survival of 72.50 ± 17.08 % (Table 2, treatment 3) with significant difference. The percentage of contamination and necrotic explants resulted from treatment 3 was also low at 15.00 ± 12.91 and 12.50 ± 5.00, respectively. Representative results of mulberry bud and leaf development after treatment 3 are shown in Figure 2.

Table 2. Effect of different treatments in mulberry lateral bud surface sterilization after culturing for 2 weeks

Treatment	Contamination (%)	Survival (%)	Necrotic (%)
1	30.00 ± 11.55^{a}	47.50 ± 9.57^{b}	22.50 ± 5.00^{a}
2	32.50 ± 17.08^{a}	55.00 ± 17.31^{ab}	12.50 ± 5.00^{b}
3	15.00 ± 12.91^{b}	72.50 ± 17.08^{a}	12.50 ± 5.00^{b}

- Values are expressed as means ± SD of triplicate experiments.
- $^{a-b}$ Means in the column followed by different letters are multiple range significantly different reported by LSD test *($p < 0.05$)*

Figure 2. Lateral bud from mulberry, *Morus nigra* (a), bud development after culturing for 2 weeks (b) and leaf formation from buds after culturing for 4 weeks (c)

Arbutin production in mulberry callus culture

Callus Induction

Callus formation was obtained from mulberry leaf segments placed in MS supplemented with different plant growth regulator, 2,4-D, IAA and NAA at concentrations of 1.0, 2.0 and 3.0 mg/l. Young leaves cut around the edge were used to produce callus as shown in Figure 3. All callus culture in different media had similar yellow-ochre color and was friable (Figure 4).

Figure 3. A young leaf cut around the edge used for callus induction (a), and the formation of callus on MS supplemented with 0.3 mg/l IAA after 4 weeks (b)

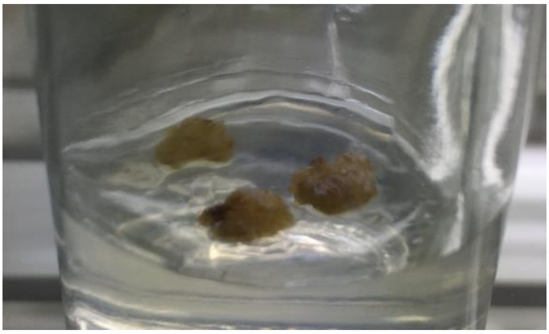

Figure 4. Appearance of mulberry callus on MS medium with 0.3 mg/l IAA after 4 weeks

Determination of arbutin in the callus culture by high performance liquid chromatography

Arbutin standard at different amounts were used to construct standard curve for arbutin content determination in the samples. The HPLC of the standard arbutin showed signal peak at retention time of 1.325 min (Figure 5), while the callus that was cultured in MS supplemented with 3.0 mg/l IAA revealed signal peak at 1.313 min (Figure 6) within the range of that obtained for arbutin standard.

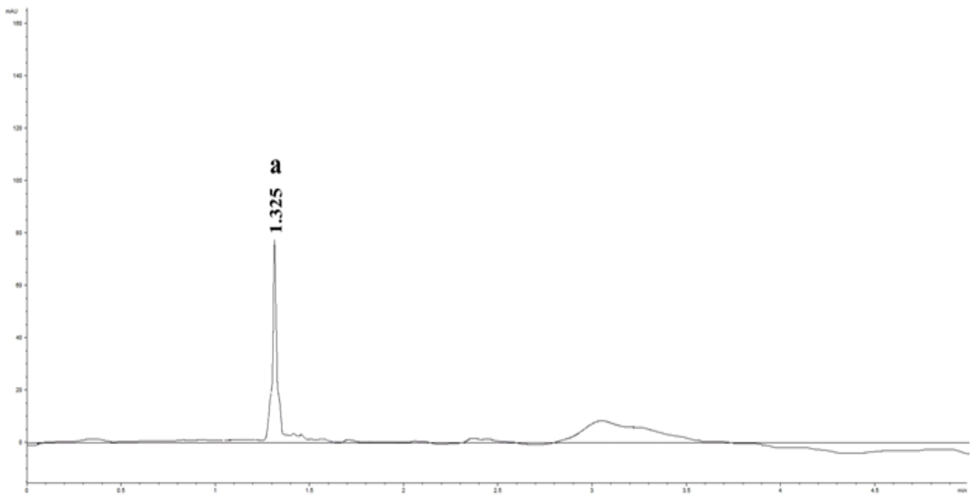

Figure 5. Representative HPLC chromatogram of standard arbutin analysis performed on C18 Hypersil ODS column (125 x 4 mm ID, 5 µm), UV 240 nm, mobile phase - methanol: water: hydrochloric acid (89:10:1, v/v), flow rate of 1.0 ml/min.

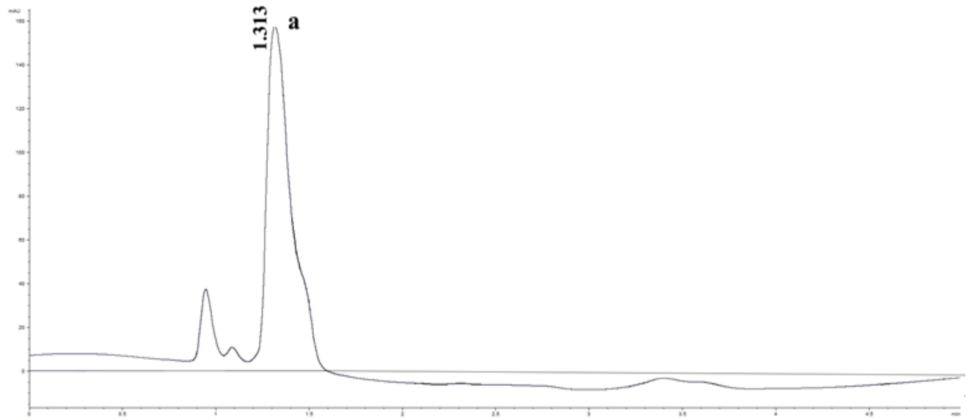

Figure 6. Representative HPLC chromatogram of arbutin content analysis of callus cultured on MS medium supplement with 3.0 m/l IAA performed on C18 Hypersil ODS column (125 x 4 mm ID, 5 µm), UV 240 nm, mobile phase - methanol: water: hydrochloric acid (89:10:1, v/v), flow rate of 1.0 ml/min.

The content of arbutin for each sample was analyzed based on the standard curve plotted using the known amounts of arbutin versus the peak area.

Table 2. Arbutin contents in callus cultured in MS medium with 2,4-D, NAA and IAA at various concentrations after 4 weeks.

Culture Media	Arbutin content (µg/mg dry weight)
MS only (controls)	13.43 ± 4.29[c]
MS with 2,4-D at 1.0 mg/l	29.67 ± 12.39[abc]
MS with 2,4-D at 2.0 mg/l	23.10 ± 5.77[abc]
MS with 2,4-D at 3.0 mg/l	34.56 ± 0.10[ab]
MS with IAA at 1.0 mg/l	17.19 ± 1.61[bc]
MS with IAA at 2.0 mg/l	28.75 ± 9.01[abc]
MS with IAA at 3.0 mg/l	35.67 ± 0.27[a]
MS with NAA at 1.0 mg/l	32.75 ± 16.24[ab]
MS with NAA at 2.0 mg/l	26.44 ± 1.87[abc]
MS with NAA at 3.0 mg/l	34.31 ± 0.11[ab]
Native leaves	31.14 ± 13.86[abc]

- Values are expressed as means ± SD.
- [a-c] Means in the column followed by different letters are multiple range significantly different reported by LSD test *($p < 0.05$)*.

At concentration of 3 mg/l the amounts of arbutin were detected at 34.56 ± 0.10 µg/mg dry weight, 35.67 ± 0.27 µg/mg dry weight and 34.31 ± 0.11 µg/mg dry weight in callus grown in MS supplements with plant growth regulator 2,4-D, IAA and NAA, respectively. The average arbutin content in callus that was cultured in MS supplemented with 3.0 mg/l IAA was the highest at 35.67 ± 0.27 µg/mg dry weight when compared with the controls at 13.43 ± 4.29 µg/mg dry weight (approximately 2.6 fold higher) and native leaves at 31.14 ± 13.86 µg/mg dry weight. Although arbutin content in the IAA elicited mulberry callus was not much higher than that present in the native leaf sample, the clear extract of the callus was much easier to handle than the extract full of pigments from native leaves if arbutin was to be separated from other substances.

DISCUSSION

In this study, the explants were prepared from lateral buds of *Morus nigra*. The mulberry buds were pretreated with carbendazim which was effective against fungi. We further achieved surface sterilization using ethanol and mercuric chloride solution. These sterilizing agents have been reported as effective for *Morus nigra* (Anis M., 2003). Pretreatment of buds with carbendazim for 10 min, followed by 70% ethanol (v/v) for 5 min, sodium hypochlorite solution twice for 10 min and by 0.1% (w/v) $HgCl_2$ 5 min (treatment 3) provided sterile and healthy explants of *Morus nigra.*

All growth regulators including 2,4-dichlorophenoxy acetic (2,4-D), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) at concentrations of 1.0, 2.0 and 3.0 mg/l stimulated callus growth on the leaf segments. This likely resulted as the growth regulators selected were auxins which could stimulate cell elongation and division. Thus, they likely had similar effects and were able to generate callus. However, detailed studies can be achieved from monitoring arbutin production generated from different growth regulators in time intervals or over longer periods of time. Callus induction of mulberry by 3.0 mg/l IAA gave the highest arbutin content at 35.67 ± 0.27 μg/mg dry weight. Tissues culture of mulberry which is native to Thailand can be used as a source of arbutin and other useful phenolic compound production and with the friable nature of the mulberry callus the suspension cell culture can be developed for larger scale production of arbutin.

CONCLUSION

Mulberry bud surface sterilization employing carbendazim for 10 min, followed by 70% ethanol (v/v) for 5 min, sodium hypochlorite solution twice for 10 min and by 0.1% (w/v) mercuric chloride 5 min offered explants with survival of 72.50 ± 17.08%. Callus induction of *Morus nigra* by 0.3 mg/l IAA under photoperiod of 16 h light and 8 h dark gave the highest arbutin content of at 35.67 ± 0.27 µg/mg dry weight. With the ability of elicited mulberry callus to produce arbutin and the relatively lower contents of pigments and other substances found in native leaves, the production of arbutin via callus culture is an alternative application that can be optimized and used as tool to obtain this biologically active compounds.

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