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A rapid and effective method for obtaining ardicrenin from Ardisia crenata Sims var. bicolor plants

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Abstract

Ardisia crenata Sims var. *bicolor* has high ornamental and medical value, especially because it contains the compound ardicrenin, which is a possible alternative drug for Taxol/camptothecin. To protect wild germplasm resources and obtain more ardicrenin, a plant regeneration system was established via calli, and ardicrenin content was quantified by reversed-phase high-performance liquid chromatography (RP-HPLC). The results showed that a Murashige and Skoog (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg L^{-1}) and kinetin (KT, 0.01 mg L^{-1}) was suitable for callus induction, with an induction rate of 95.31 ± 1.62%; a MS medium with 2,4-D (0.5 mg L^{-1}) and 6-benzylaminopurine (6-BA, 0.2 mg L^{-1}) was suitable for callus multiplication, with a multiplication coefficient of 359.26; a MS medium with 6-BA (1.0 mg L^{-1}) and 1-naphthaleneacetic acid (NAA, 0.5 mg L^{-1}) was suitable for callus differentiation, with a differentiation rate of 89.36 ± 1.39%; and 1/2 MS with indole-3-butyric acid (IBA, 0.2 mg L^{-1}) was suitable for rooting, with a rooting rate of 93.74 ± 1.38%. Additionally, garden soil: vermiculite: perlite (v: v: v = 1:1:1) was appropriate for acclimatization and transplanting, with a survival rate of 87.38%. RP-HPLC analysis revealed that the ardicrenin content in one-month-old plantlets was higher than that in one-year-old plants and was nearly the same as the content observed in three-year-old plants and two-month-old transplanted plantlets, especially in the roots (17.69 mg g^{-1} DW, 1.77% DW). In summary, a rapid propagation system for *A. crenata* Sims var. *bicolor* was successfully established for the first time, and the roots of one-month-old plantlets were used to effectively obtain ardicrenin.

Introduction

The rapid current rate of urbanization has led to severe health problems (Pedersen and Mortensen 2001; Poortinga 2006; Velarde et al. 2007; Peen et al. 2010). At the same time, with advances in sustainable development, garden plants must serve more than ornamental functions to meet people's needs for ecological safety and a better life (Al-hader et al. 1994; Papiez et al. 2009; Zhu et al. 2019). Obtaining the germplasm resources of garden plants with ornamental, medicinal and health care value is an emerging direction in garden plant cultivation and application research (Qiao and Zhang 2012; Lin 2020; Xu et al. 2022). Studies on the exploration and application of the resources of garden plants with medicinal and health care functions are focused not only on selecting flower seedlings with long flowering periods and high ornamental value but also on identifying garden plants with high levels of medicinal or health care functions (Jia et al. 2009; Tan and Gao 2017).

Currently, cancer and acquired immunodeficiency syndrome (AIDS) are two leading causes of human death (Douce et al. 2014). At this time, plant medicine studies are focused on drugs that target tumours, such as Taxol from *Taxus chinensis* (Oberlies and Kroll, 2004). However, this compound is expensive and in short supply. Therefore, there is an urgent need to develop alternative drugs to supplement Taxol. Previous studies have reported that triterpenoid saponins, especially the pentacyclic triterpenoid saponins of the genus *Ardisia*, significantly inhibit cancer cell proliferation and have direct anticancer and cytotoxic effects (Chen et al. 2007; Deng et al. 2006, 2011, 2012; Hu et al. 2016, 2020a; Huang et al. 2017). Among these compounds, ardicrenin, which has the molecular formula of $C_{53}H_{86}O_{22}$, a relative molecular mass of 1074, and a melting point of 239–241°C (Fig. 1, Zhen et al. 2007, 2009; Ma et al. 2009b; Zhen 2009), is one of the triterpenoid saponins in *Ardisia crenata* Sims var. *bicolor* (Zou et al. 2009; Ye et al. 2022).

Ardisia crenata Sims var. *bicolor* of the family Myrsinaceae and genus *Ardisia* is a small evergreen perennial shrub that is distributed in the understorey of subtropical evergreen broad-leaved forests (Chen et al. 2001; Zhao et al. 2006). The upper surface and lower surface of its leaves are green and purplish red, respectively, and the red foliage colour lasts a long time; it is a rare bicoloured tree species (Peng 2006; Peng et al. 2008; Ai et al. 2022). *A. crenata* Sims var. *bicolor* produces one of the best-selling Chinese New Year flowers in China, and it can also be used in landscape construction for understorey planting in forests, in courtyard clump planting, in bridge beautification, and in potted plantings (Cui 2007, 2008). In China, *A. crenata* Sims var. *bicolor* is usually used as an indigenous and traditional medicinal plant (Tian et al. 1996; Yin and Zhao 2004; Zhang et al. 2007a, b; Wei et al. 2016, 2017, 2018). Wang et al. (2011) found that the triterpenoid saponins (ardisiacrispins B) of *A. crenata* var. *bicolor* have strong tumour metastasis activity. Hu et al. (2020b) reported that extracts of *Ardisia crenata* var. *bicolor* inhibited the proliferation, migration and invasion of MDA-MB-453 breast cancer cells and promoted apoptosis. However, a rapid and effective method for obtaining ardicrenin is needed.

Currently, extraction and purification techniques for ardicrenin have been determined, but the commercial development and utilization of this compound is still complicated by an important practical question (You and Yu 2006; Zhen et al. 2007, 2009; Zhen et al. 2009; Wang et al. 2012; Wu and Chen 2014; Zhu et al. 2015): how can higher rates of ardicrenin be obtained without damaging the wild plant resources and without threatening the survival of this species? Tissue culturing is advantageous because it shortens the propagation process, saves space, achieves year-round production, can maintain the favourable properties of the parent plant and facilitates large-scale production to meet market demands (Ruben 2017; Long et al. 2022). In addition, secondary metabolites can be obtained by tissue culturing plantlets and calli (Sanyal et al. 2022; Sudheer et al. 2022). Unfortunately, plant regeneration and ardicrenin production by *A. crenata* Sims var. *bicolor* have not been reported. Therefore, to address this issue, we successfully established a plant regeneration system for *Ardisia crenata* Sims var. *bicolor* plants for the first time using young stem explants and obtained numerous plantlets. Additionally, we determined ardicrenin content by reversed-phase high-performance liquid chromatography (RP-HPLC) to better measure the content of the active medicinal ingredient. This study not only overcomes the limitations of germplasm resources but also provides experimental methods for further genetic transformation and genetic engineering. Furthermore, it provides theoretical support and suitable guidance for the improvement of sprout propagation and germplasm resource protection of *Ardisia crenata* Sims var. *bicolor*. In summary, this study provides new insights into ardicrenin and protects its natural sources by using a rapid and effective method for obtaining ardicrenin from *Ardisia crenata* Sims var. *bicolor*.

Materials And Methods

Plant materials

A. crenata Sims var. *bicolor* natural plants were collected from the evergreen broad-leaved forests of the Qishanling Mountains (110°20'E, 22°42'N; Luchuan, Yulin, Guangxi, China) and Tunyanjiaoling Mountains (109°64'E, 25°83'N; Sanjiang, Liuzhou, Guangxi, China) in December 2019. Plant morphology was identified by Dr. Xiaokai Yan from the Plant Research Center of Chengdu Botanical Garden, Sichuan Province, China. All plants were transplanted and cultured in a chamber and were maintained in a greenhouse at the College of Biology & Pharmacy, Eastern campus, Yulin Normal University, Guangxi, China. A preliminary experiment showed that the dedifferentiation capacity of *A. crenata* Sims var. *bicolor* was strong and calli were induced in the root, stem and

leaf, but the stem segments had the best induction effect. Therefore, a strong and healthy potted parent plant (Fig. 2-a) was used in this study.

Establishment of a plant regeneration system for obtaining plantlets

Young stem explants (basal shoots) that were obtained from a strong and healthy parent plant (Fig. 2-a, the part indicated by the red arrow) were surface-sterilized based on a previously reported protocol (Hu et al. 2019, 2020a). The details are shown in Supplementary Method S1. Then, the explants were inoculated into a Murashige and Skoog (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1, 0.5, 1.0 mg L^{-1}) and kinetin (KT, 0.01, 0.05, 0.1 mg L^{-1}) for callus induction (one explant/bottle and a total of 30 bottles; the same protocol was used below). After 30 days of dark culturing, calli that did not show browning were inoculated into a MS medium supplemented with 2,4-D (0.5, 1.0, 2.0 mg L^{-1}) and 6-benzylaminopurine (6-BA, 0.1, 0.2, 0.5 mg L^{-1}) for callus multiplication. After another 30 days of dark culturing, the calli were inoculated into a MS medium supplemented with 6-BA (0.5, 1.0, 2.0 mg L^{-1}) and naphthaleneacetic acid (NAA, 0.1, 0.5, 1.0 mg L^{-1}) for plantlet induction. Thirty days later, single heathy, strong and rootless seedlings with a stem length of more than 1.5 cm were inoculated in 1/2 MS supplemented with 3-indolebutyric acid (IBA, 0.1, 0.2, 0.5, 1.0, 1.5 mg/L) for plantlet rooting. After another 30 days, rooted plantlets with root lengths of 1.5–2.0 cm, plantlet heights of 3.0–4.0 cm, and healthy and strong root systems were selected for acclimatization and transplanting as described in Supplementary Method S2. In this protocol, unless otherwise specified, all media were solidified with 6.5 g L^{-1} agar supplemented with 30 g L^{-1} sugar, and pH was adjusted to 5.8–6.0. All experiments were repeated three times and performed at 25 ± 2°C with a 12 h/day photoperiod and an illumination intensity of 1500–2000 lx.

Ardicrenin quantification by RP-HPLC

This analysis was completed at the Natural Products Laboratory, Dujiangyan Campus of Sichuan Agricultural University (SICAU). The ardicrenin standard (purity ≥ 99.8%) was donated by professor Mingdong Ma of SICAU and stored at 4 °C. The equipment and chromatographic conditions were based on a previously reported protocol (Hu et al. 2020a). The details are shown in Supplementary Method S3 of the standard stock solution and sample solution preparation was based on a previously reported protocol (Ma et al. 2009; Hu et al. 2020a), which is described in Supplementary Method S4. The tested sample materials for *A. crenata* Sims var. *bicolor* were provided by the College of Biology & Pharmacy, Yulin Normal University, which included compact- and friable-type calli (30 days old), roots, stems, leaves, whole five-year-old, three-year-old and one-year-old plants, two-month-old transplanted plantlets, and one-month-old plantlets.

Statistics and Analysis

Excel 2016 and IBM SPSS Statistics 24 software were used for data processing, and the means were compared using Duncan's multiple-range test at the 5% level.

Results

Analysis of rapid propagation and plantlets

In this study, the stem segments of *A. crenata* Sims var. *bicolor* were laid flat on the medium and began to expand at one end or both ends after 7 days, and calli began to appear approximately 10 days later (Fig. 2-b). The stem segments had the highest callus induction rates (95.31 ± 1.62%) and the lowest callus contamination rates (7.88 ± 0.95%), browning rates (2.01 ± 0.57%) and browning grade (1) on MS supplemented with 2,4-D 1.0 mg L^{-1} and KT 0.01 mg L^{-1} (Table 1). The calli included compact types (nubbly, Fig. 2-c) and friable types (loose, Fig. 2-d), and the compact calli (++ or +++) grew better than the friable calli (+) (Table 1). The highest callus multiplication coefficient (359.26) was observed in MS with 2,4-D 0.5 mg L^{-1} and 6-BA 0.2 mg L^{-1} (Fig. 3). The calli had highest differentiation rates (89.36 ± 1.39%), highest average number of differentiation buds (6.60 ± 0.36), and the best growth status (+++, Fig. 2-e) in MS with 6-BA 1.0 mg L^{-1} and NAA 0.5 mg L^{-1}, respectively (Table 2). The plantlets had the highest rooting rates (93.74 ± 1.38%), the highest average number of roots (4.24 ± 0.46), the longest average length of roots (4.51 ± 0.07 cm), and the best growth status of roots (long, thick and strong with many fibrous) in 1/2 MS with 0.2 mg L^{-1} IBA (Fig. 2-f, Table 3). Transplanted plantlets had the highest survival rates (87.38%) in the transplanted garden soil: vermiculite: perlite (v: v: v = 1: 1: 1) (Fig. 4, Fig. 2-g). Therefore, the most suitable hormone combinations for callus induction, multiplication and differentiation, and plantlet rooting were MS with 2,4-D 1.0 mg L^{-1} and KT 0.01 mg L^{-1}, MS with 2,4-D 0.5 mg L^{-1} and 6-BA 0.2 mg L^{-1}, MS with 6-BA 1.0 mg L^{-1} and NAA 0.5 mg L^{-1}, and 1/2 MS with IBA 0.2 mg L^{-1}; the most suitable transplanted substrate for plantlet transplanting was garden soil: vermiculite: perlite (v: v: v = 1: 1: 1).

Table 1

Effect of different hormone concentrations on callus induction. Induction rate (%) = the total number of induced callus explants/number of inoculation explants × 100%. Contamination rate (%) = the total number of contaminated callus explants/number of induced callus explants × 100%. Browning rate (%) = the total number of browned callus explants/number of induced callus explants × 100%. Browning grade (A) = callus browning area/callus area, and the classification criteria are as follows: grade **0** (A = 0, fresh appearance, faint yellow or chartreuse, no browning), grade **1** (A ≦ 1/4, slight browning, basically faint yellow or chartreuse), grade **2** (1/4 A ≦ 1/2, browning, yellowish brown), grade **3** (1/2 A ≦ 3/4, more severe browning, brown), and grade **4** (A > 3/4, severe browning, dark brown) (He and Lai 2010; Deng et al. 2012). **+** Calli generally grew, **++** calli grew well, and **+++** calli grew very well. Data represent standard deviations (SDs) of the mean (n = 3). The different lowercase letters indicate that the mean values are significantly different among the strains for the same parameter at $P < 0.05$ according to Duncan's test. The same convention is followed below.

Treatments	2,4-D (mg L^{-1})	KT (mg L^{-1})	Induction rate (%)	Contamination rate (%)	Browning rate (%)	Browning grade (A)	Cell morphology	Growth status
1	0.1	0.01	33.79 ± 0.53 f	15.52 ± 1.29 a	14.10 ± 1.08 ab	3	Friable type, loose	+
2	0.1	0.05	44.32 ± 1.31 e	13.62 ± 1.42 b	14.03 ± 0.57 ab	3	Friable type, loose	+
3	0.1	0.1	79.18 ± 4.53 d	12.28 ± 0.40 bc	13.35 ± 0.89 ab	3	Friable type, loose	+
4	0.5	0.01	82.63 ± 1.91 bcd	11.10 ± 0.39 c	5.93 ± 0.68 c	1	Compact type, nubbly	++
5	0.5	0.05	85.92 ± 3.45 b	11.70 ± 0.51 c	14.89 ± 0.29 a	2	Friable type, loose	+
6	0.5	0.1	84.29 ± 2.82 bc	10.90 ± 0.58 c	13.82 ± 0.46 ab	3	Friable type, loose	+
7	1.0	0.01	95.31 ± 1.62 a	7.88 ± 0.95 d	2.01 ± 0.57 d	1	Compact type, nubbly	+++
8	1.0	0.05	82.30 ± 3.06 bcd	12.40 ± 0.37 bc	5.33 ± 1.85 c	1	Compact type, nubbly	++
9	1.0	0.1	80.49 ± 2.19 cd	11.96 ± 0.37 c	12.26 ± 1.40 b	2	Friable type, loose	+

Table 2

Effect of different hormone concentrations on plantlet induction. Callus differentiation rate (%) = the total number of differentiated calli/number of inoculated calli × 100%. **+** plantlets generally grew, **++** plantlets grew well, and **+++** plantlets grew very well. Criteria: differentiation buds had to be clearly visible, and the length had to be more than 1.5 cm.

Treatments	6-BA (mg L^{-1})	NAA (mg L^{-1})	Differentiation rate (%)	Average number of differentiated buds	Growth status
1	0.5	0.1	41.57 ± 1.18 f	2.45 ± 0.19 f	+
2	0.5	0.5	33.06 ± 1.38 g	2.77 ± 0.31 ef	+
3	0.5	1.0	22.09 ± 1.24 h	1.45 ± 0.16 g	+
4	1.0	0.1	61.51 ± 1.32 c	4.46 ± 0.38 c	++
5	1.0	0.5	89.36 ± 1.39 a	6.60 ± 0.36 a	+++
6	1.0	1.0	74.49 ± 1.03 b	5.51 ± 0.53 b	++
7	2.0	0.1	53.37 ± 1.02 d	3.78 ± 0.17 d	+
8	2.0	0.5	48.60 ± 0.85 e	3.42 ± 0.45 de	+
9	2.0	1.0	34.26 ± 1.24 g	2.89 ± 0.50 ef	+

Table 3

Effect of different IBA concentrations on plantlet rooting. Rooting rate (%) = the total number of rooting plantlets/number of inoculated plantlets × 100%. Criteria: the roots had to be clearly visible, and the length had to be more than 1 cm.

Treatments	IBA (mg L^{-1})	Rooting rate (%)	Average number of roots	Average length of roots (cm)	Growth status of roots
1	0.1	85.25 ± 1.53 b	2.36 ± 0.07 b	4.19 ± 0.02 b	Short and thick with many fibrous roots
2	0.2	93.74 ± 1.38 a	4.24 ± 0.46 a	4.51 ± 0.07 a	Long, thick and strong with many fibrous roots
3	0.5	80.70 ± 0.44 c	2.41 ± 0.27 b	3.84 ± 0.09 c	Short and slender
4	1	79.49 ± 1.71 c	2.43 ± 0.07 b	3.02 ± 0.06 e	Short and slender
5	1.5	72.85 ± 2.36 d	2.06 ± 0.07 b	3.17 ± 0.05 d	Short, slim and fragile

Analysis of ardicrenin content in A. crenata **Sims var.** bicolor

The regression equation y = 4048.1 x − 6.0 (R^2 = 0.9997) was obtained by using the injected concentrations (x, mg mL^{-1}) and peak areas of the chromatographs (y, mAUs) as the abscissa and the ordinate, respectively. A maximum peak value reached at a retention time of approximately 8.8 or 8.9 min for each sample (Fig. 5). The

data (Table 4) showed that the ardicrenin content of the friable calli (3.07 mg g^{-1} DW, 0.31% DW) was more than 4 times that of the compact calli (0.71 mg g^{-1} DW, 0.07% DW) at one month of age. Irrespective of whether the tissue was from the seedlings (one-, three- or five-year-old) or plantlets (one-month-old, two-month-old transplanted), there was a tendency in the ardicrenin content to decrease in the following order: the roots had the highest content, followed by the whole plants, stems, and leaves. Interestingly, the ardicrenin content in the root, stem and leaves of one-month-old plantlets (17.69 mg g^{-1} DW, 1.77% DW; 1.85 mg g^{-1} DW, 0.19% DW; 0.36 mg g^{-1} DW, 0.04% DW) was higher (nearly 2-fold) than that of one-year-old seedlings (9.05 mg g^{-1} DW, 0.91% DW; 0.97 mg g^{-1} DW, 0.10% DW; 0.19 mg g^{-1} DW, 0.02% DW) and was close to the values seen in three-year-old seedlings (20.23 mg g^{-1} DW, 2.02% DW; 1.97 mg g^{-1} DW, 0.20% DW; 0.35 mg g^{-1} DW, 0.03% DW) and two-month-old transplanted plantlets (20.06 mg g^{-1} DW, 2.01% DW; 1.94 mg g^{-1} DW, 0.20% DW; 0.32 mg g^{-1} DW, 0.03% DW) but was lower (nearly 1/3rd) that in five-year-old seedlings (65.12 mg g^{-1} DW, 6.51% DW; 6.94 mg g^{-1} DW, 0.69% DW; 1.09 mg g^{-1} DW, 0.11% DW). However, the ardicrenin content in the whole plants or plantlets was nearly equal. Overall, it was evident that the ardicrenin content was higher in one-month-old plantlets and two-month-old transplanted plantlets than in one-year-old plants, and the plantlets rather than three-year-old plants could be used to obtain ardicrenin. Additionally, and ardicrenin content was highest in the roots.

Table 4
Ardicrenin content of different parts of *A. crenata* Sims var. *bicolor* at various ages and from different origins.

Origins	Ages	Parts/types	Content (mg g^{-1} DW)	Percentage content (% DW)
Calluses	30-day-old	Compact	0.71	0.07%
		Friable	3.07	0.31%
Plants	Five-year-old	Root	65.12	6.51%
		Stem	6.94	0.69%
		Leaf	1.09	0.11%
		Whole-plant	3.45	0.34%
	Three-year-old	Root	20.23	2.02%
		Stem	1.97	0.20%
		Leaf	0.35	0.03%
		Whole-plant	1.54	0.15%
	One-year-old	Root	9.05	0.91%
		Stem	0.97	0.10%
		Leaf	0.19	0.02%
		Whole-plant	1.52	0.15%
Plantlets	Transplanted two-month-old	Root	20.06	2.01%
		Stem	1.94	0.20%
		Leaf	0.32	0.03%
		Whole-plant	1.59	0.16%
	One-month-old	Root	17.69	1.77%
		Stem	1.85	0.19%
		Leaf	0.3	0.04%
		Whole-plant	1.57	0.16%

Discussion

A protocol for organogenesis has been reported for *A. crenata* Sims (Ma et al. 2009a), but in *A. crenata* Sims var. *bicolor*, it is reported here for the first time, specifically via calli. Organogenesis is a process in which different plant organs directly induce adventitious buds and roots that differentiate into complete plants, and it includes

direct and indirect ways (Xiong et al. 2022; Shashi and Vishnu 2022; Chauhan and Taylor 2018). The former directly induces adventitious buds and roots from explants, while the latter produces calli first and then adventitious buds and roots (Shin et al. 2020; Srinivasan et al. 2021; Li et al. 2022). Here, young stems (basal shoots, Fig. 2-a) of *A. crenata* Sims var. *bicolor* were selected as explants because they show a certain degree of lignification, and the contamination rate was controlled by two successive disinfection steps with $HgCl_2$ based on previous studies (Hu et al. 2019, 2020a).

In addition, the different hormones that were selected improved the propagation rates (Fig. 3, Tables 1–3). Calli are groups of nonpolar and vigorously dividing parenchyma cells formed by explants *in vitro* (Zhou et al. 2022). Almost all organs and tissues in higher plants can induce calli, but there are substantial differences in the degree of difficulty of induction, in the time taken for callus formation, and in the growth of calli (Dong et al. 2021). A study showed that hormones have a very significant effect on callus induction and that a high concentration of auxin combined with a low concentration of cytokinin could yield a high callus induction rate (Liu 2007). Additionally, appropriate hormone types and concentration ratios are required for plant regeneration from calli (Wang et al. 2017). This study showed similar results, and two types (friable type and compact type) of calli were induced (Fig. 2-c, d). The former was yellow green (turquoise), dry and compact (Fig. 2-c), with strong differentiation ability, and could be cultured for organ regeneration (Liu 2007). The latter was light yellow, moist, loose and easy to disperse (Fig. 2-d), with fast proliferation, and could be used for cell culturing to extract secondary metabolites (Liu 2007; Zhen et al. 2007). Regenerated plantlets were obtained from compact calli via bud induction and rooting in this study (Fig. 2-c, e, f).

The growth conditions of plantlets in the culture bottles and outdoors were very different (Liu et al. 2019). The plantlets had to be acclimated and transplanted to improve their adaptability to the external environment so they could grow better in nurseries (Ye et al. 2019). For a long time, the low survival rate during acclimatization and transplanting has been a bottleneck for the large-scale production of plantlets (Zhang et al. 2019). During these processes, the type and proportion of the matrix is one of the main factors affecting the survival rate of plantlet acclimatization and transplanting (Yao et al. 2020; Li et al. 2018; Thaniarasu et al. 2016). We obtained the most suitable matrix type and proportion for plantlet acclimatization and transplanting, and the survival rate was greater than 90% (Fig. 4). Therefore, through a stable hormones-assisted propagation system involving the organogenesis of calli, callus multiplication, bud induction, bud rooting, acclimatization and transplanting, numerous plantlets of *A. crenata* Sims var. *bicolor* were obtained for the further study of ardicrenin production and quantification in this study (Fig. 2-b-g).

Ardicrenin has attracted increasing amounts of interest in recent years due to its pharmaceutical properties (Song et al. 2021; Hu et al. 2016, 2020a; Liu et al. 2016; Shen 2003; Koike et al. 1999; Jia et al. 1994). RP-HPLC methods for the determination of this compound have been reported (Zhen et al. 2007; Zhen 2009; Hu et al. 2020a). We used the method based on Hu et al. (2020a) to analyse ardicrenin. RP-HPLC analysis of the standard and sample solutions revealed that ardicrenin is present in *A. crenata* Sims var. *bicolor* (Fig. 5). The results demonstrated that ardicrenin is a major compound in *A. crenata* Sims var. *bicolor* (Zou et al. 2009; Ye et al. 2022), and it can be found in the aerial and underground parts of the plantlets, plants and calli (Table 4). The results also indicated that the content was greatest in the roots, as has been reported for other *Ardisia* species (Liu et al. 2007; Zhen et al. 2009; Hu et al. 2016, 2022a). Moreover, the ardicrenin content of one-month-old plantlets greatly exceeded (two times) that of one-year-old plants and was nearly equal to that of three-year-old plants and two-month-old transplanted plantlets, but it was lower (nearly 1/3rd) than that of five-year-old plants (Table 4). A possible reason

for this result is that the employed hormones promoted accumulation of ardicrenin in the plantlets (Deshpande et al. 2015; Hu et al. 2019). However, such an explanation needs verification through further studies.

Although the extraction of secondary metabolites from calli have been reported (Jan et al. 2018; Verardo et al. 2019; Pan et al. 2020), to the best of our knowledge, this is the first report of an ardicrenin study on callus cultures of *A. crenata* Sims var. *bicolor*. The results showed that the ardicrenin content in friable calli was higher than that in compact calli, which is similar to the results of Rafiq and Dahot (2012) and Zare et al. (2011). Although the calli induced in this study grew rapidly, the ardicrenin content obtained from the calli was much lower than that from the roots of plants or plantlets (Table 4). We speculated that ardicrenin synthesis-related enzymes or genes were not activated due to the short culture cycle of the calli, leading to a very low yield of ardicrenin (Fang et al. 2014; Xiao et al. 2020; Hazrati et al. 2022). In the future, calli cultures of *A. crenata* Sims var. *bicolor* can be carried out for the selection of high-quality cell lines, optimization of callus culture conditions and culture methods, key gene regulation, gene engineering and molecular biology (Verpoorte et al. 2000; Wang et al. 2004; Huang et al. 2017; Ghasempour et al. 2019).

As is well established, rapid propagation is not only convenient for factory production but can also significantly improve the development and utilization of plants, particularly for obtaining secondary metabolites and preserving plant resources (Li et al. 2012; Shasmita et al. 2018; Lin et al. 2020; Wang et al. 2021). The reported methods are easy to operate and do not decrease the ardicrenin content. In fact, the ardicrenin content was enhanced, as has been reported in *A. crenata* (Liu et al. 2007; Zhen et al. 2007, 2009; Hu et al. 2016, 2020a). This approach could not only preserve existing *A. crenata* Sims var. *bicolor* resources and other *Ardisia* genus plants in their habitats but could also provide alternative sources of natural secondary metabolites to meet the increasing demand for ardicrenin in the pharmaceutical industry worldwide. Accordingly, this rapid and effective propagation approach is a potentially suitable strategy for rapidly enhancing ardicrenin production by greatly shortening the culture time and production cycles. In summary, the use of an *in vitro* system for the production of ardicrenin can guarantee its continuous supply and contribute to the preservation of the natural plant resources of *A. crenata* Sims var. *bicolor* and other plants used in the production of ardicrenin.

Declarations

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Author contributions JH and FL designed the research. CYM, HCL, LJK, SCX, HYZ, and SRP conducted the experiments. ZL, XLY, and LL analysed the data. ZL wrote the manuscript, JH and FL provided helpful suggestions and helped revise the manuscript carefully during the course of major revisions and minor edits. All authors read and approved the manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they do not have any commercial or associated interests that could present a conflict of interest related to the work submitted.

Ethical approval The experiments in this study were performed according to the current laws in China.

Data Availability Statements The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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Figures

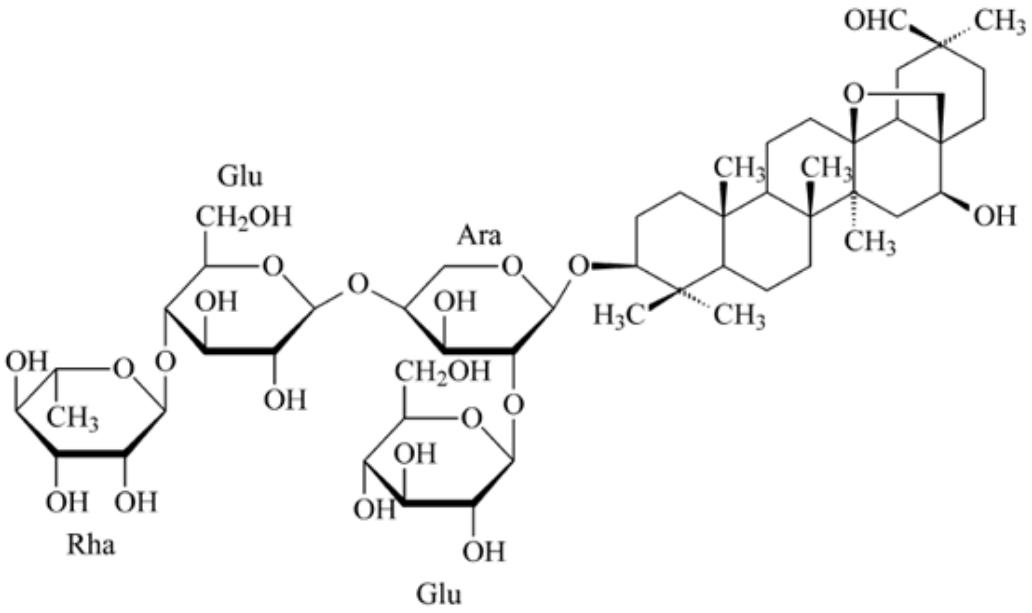


Figure 1

Chemical structure of ardicrenin.

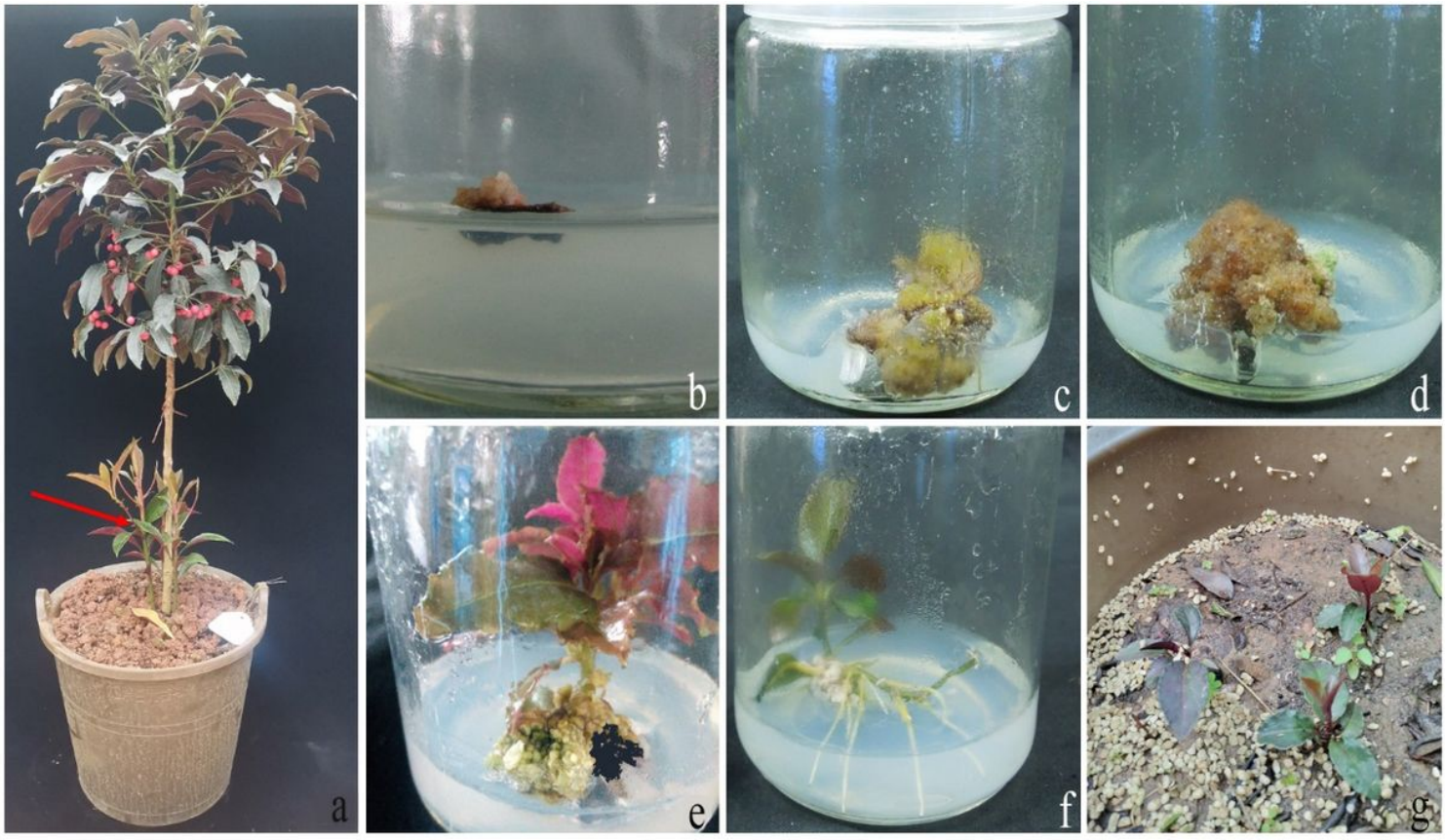


Figure 2

Establishment of a rapid propagation system for *A. crenata* Sims var. *bicolor* by an organogenesis pathway. **a** parent plant (3-year-old), **b** callus induction, **c** callus multiplication (compact type), **d** callus multiplication (friable

type), **e** plantlet induction, **f** plantlet rooting and **g** acclimatization and transplanting. The redarrow represents the explants (basal shoots) used in this experiment.

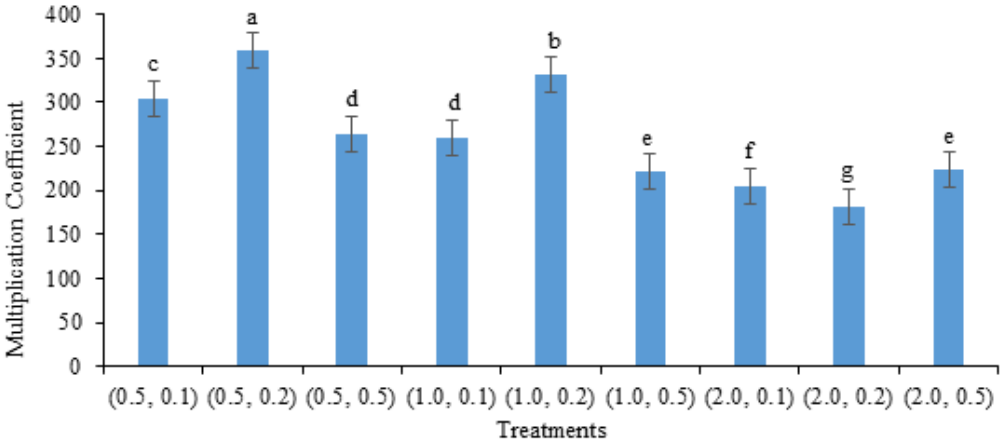


Figure 3

Analysis of different hormone combinations for callus multiplication. The first value in the brackets of the abscissa represents the concentrations of 2,4-D (mg L^{-1}), while the other value represents the 6-BA concentrations (mg L^{-1}). The same lowercase letters show insignificant differences, while the different lowercase letters show significant differences ($P < 0.05$). The same convention is followed in the figures below. Multiplication coefficient = total number of visible calli/number of inoculated calli.

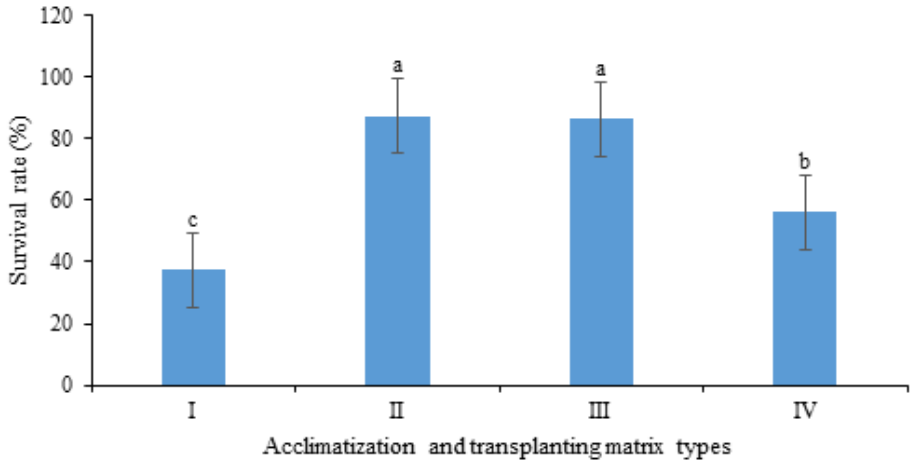


Figure 4

Analysis of different matrices on the survival rates of plantlets acclimatized and transplanted. I indicates that garden soil and river sand were prepared at a ratio of 1:1. ☒ shows that garden soil, vermiculite and perlite were prepared at a ratio of 1:1:1. ☐ denotes that garden soil, river sand, vermiculite and perlite were prepared at a ratio of 1:1:1:1. ☐ specifies that garden soil and humus soil were prepared at a ratio of 1:1. Survival rate (%) = total number of survivingplantlets/numbers of transplanted plantlets ×100%.

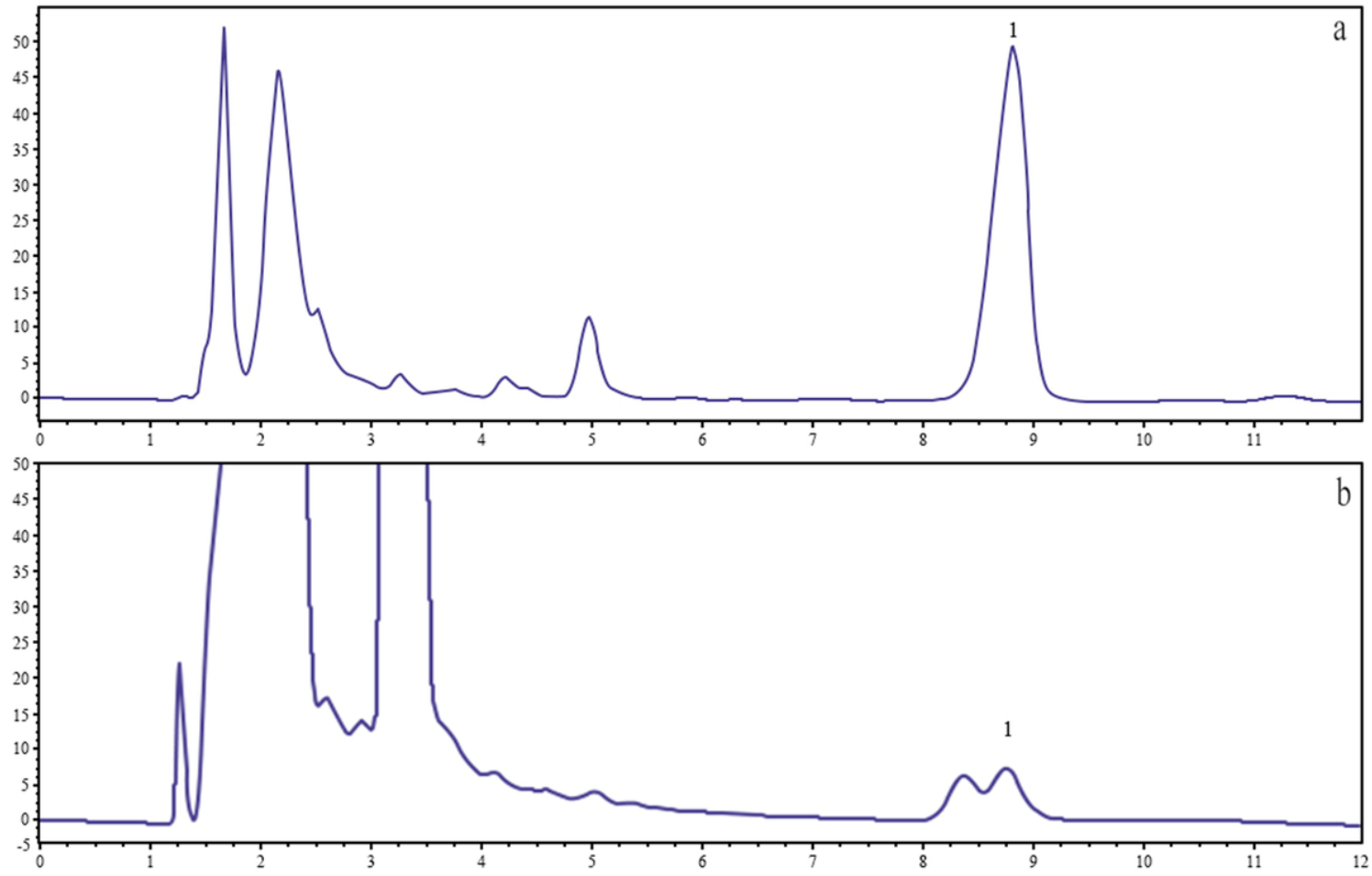


Figure 5

RP-HPLC analysis for the quantification of ardicrenin in *A. crenata* Sims var. *bicolor*. **a** Standard ardicrenin solution and **b** sample solution.

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