Research on the Content of Plumbago auriculata (L.) Hairy Roots Mediated by Agrobacterium Rhizogenes and Its Plumbagin

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Abstract

Agrobacterium rhizogene-mediated genetic transformation of hairy roots is an effective method to obtain secondary metabolites. In accordance with different genotypes, it is very specific and difficult to set up a stable genetic transformation system. The plumbagin is found in the roots of *Plumbago auriculata* L., a secondary metabolite with significant medicinal value, but the common root grows slowly, its accumulation period is lengthy (2–6 years). In this paper, we first explored the most effective *A. rhizogene*-mediated (A4, ATCC 15834, and LBA 9402) genetic transformation to induce hairy root of *P. auriculata*, and evaluated the plumbagin concentration in different root. The results showed that the leaves were soaked with bacterial solution for 25–30 min and then transferred to 1/2 MS + AS 100 μmol·L^{-1} solid medium without preculture for 2-5d. After co culture, the leaves were transferred to 1/2 MS + Cef and sterilized with cefotaxime sodium. Under this scheme, all strains can induce hairy roots, with ATCC 15834 having the highest hairy root induction rate (86.78 ± 0.74%) and the earliest root emergence time (8.33 ± 0.58 d). 1-month-grown hairy root showed an increase in plumbagin content compared with the root of the same age group and 1-year-old live seedlings, with PAHR 15834 having the highest content of 38.95 mg·g^{-1} DW, which was 72.13 times higher than the same age group and 3.95 times higher than that of 1-year-old live seedlings. This is an important experimental basis for further investigation of the biosynthesis mechanism of plumbagin and the feasibility of subsequent commercial production.

1. Introduction

It is well known that plants and their extracts are considered as rich sources of medicinal substances (Gerszberg and Smagur 2022). For example, Plumbagin, a secondary metabolite from Plumbaginaceae, is a naturally occurring bioactive compound (5-hydroxy-2-methyl-1,4-naphthoquinone, abbreviated as PL). It has a variety of pharmacological properties, including anti-inflammatory, anti radiation, anti-rheumatoid arthritis, tumor treatment, nerve protection, and blood lipid lowering (Onodera et al 2015; Checker et al2019; Khichariya et al 2022; Sun et al 2022; Jangra et al 2021; Pai 2019). It has been reported that the PL of undergraduate plants mainly comes from plant roots, which has been proved in *Plumbago zeylanica*(*P. zeylanica*), *Plumbago indica P. indica* , *P. auriculata* (Singh et al 2020; Gangopadhyay et al 2011;Katoch K et al 2022). With the increasing demand for natural medicinal products, especially the secondary metabolites of medicinal plants (Chen et al 2016), the supply of natural sources of PL is insufficient, which limits its therapeutic use (Nayak et al 2015). For example, the *P.auriculata* is collected as the drug source of PL. The traditional method is root digging (Mallavadhani et al 2002). This method of obtaining roots in the way of destroying the whole plant not only leads to destructive destruction of natural populations and germplasm resources, but also limits the harvest of useful ingredients due to low root yield and long growth cycle. Since a small amount of PL is produced after 2–6 years of plant growth (Beigohamadi et al 2021), the in vitro biological production of PL has attracted more attention. For example, the method of producing PL (Komaraiah et al., 2001; Sharma and Agrawal 2018) from *Plumbago indica* and *Plumbago zeylanica* by callus or cell suspension culture was reported earlier. However, the stability of cell lines, and the inconsistent production and storage of metabolites in cells or vacuoles because of the low yield, this method has high time and economic costs and is difficult to apply to efficient and stable commercial progresses.

A. rhizogenes mediated “hairy root” culture has been considered as a common tool to advance plant secondary metabolites (Veena and Taylor 2007). It has the characteristics of high proliferation rate, high lateral branch rate and strong genetic stability in the medium without plant hormones (Yang et al 2020). Through this transformation, individuals can transfer interested genes to make plant cells produce the required proteins (Gerszberg and Hnatuszko-Konka 2022), metabolites (Bahmani et al 2021) and other target substances. For example, in the Plumbagaceae plants, *A. rhizogenes* can be used to induce hairy roots to extract Plumbagin. This technology has been reported in *P. zeylanica* (Wei et al 2006)d *indica* (Gangopadhyay et al 2010), but so far there is no research report on this technology in *P. auriculata*.

Although it has been reported that the hairy roots of plants of the same family and genus were successfully induced to extract Plumbagaceae, the technology of setting up genetic transformation system has strong pertinence and specificity. Even if the same family and genus have different genotypes, the reference value of the technology is very small (Ninković et al 2010; Md-Setamam et al 2014). In this way, it is necessary to make specific exploration and research to successfully establish genetic transformation system for different plants or varieties. Generally speaking, the main factors affecting the establishment of hairy root genetic transformation lines can be composed of strain type, explant selection, pre culture time, infection time and co culture method (Boobalan and Kamalanathan, 2020; Miao et al 2021; Sahai and Sinha 2022; Mahendran et al 2021; Piątczak et al 2019). In order to distinguish common roots from hairy roots, predecessors mainly adopted morphological identification to preliminarily screen hairy roots (Boobalan and Kamalanathan 2020). Then the gene expression was analyzed by PCR and q-PCR (Piątczak et al 2019) to determine whether the transformation was made. It can be seen that for a new species or variety, due to the technical difficulties in each link, the exploration and optimization of technical parameters and environmental conditions are worthy of in-depth research in the process of successfully establishing a complete set of hairy root genetic transformation lines. In this way, this research was the first to explore the *P. auriculata* hairy root genetic transformation system that was mediated by *A. rhizogenes*, so as to explore the technical parameters and environmental conditions successfully mediated. The hairy roots were identified by morphological and molecular means. This paper concentrated on the detection of the content of plumbagin and compared the content of plumbagin with the roots obtained of tissue culture and sowing, which aims to further verify the effectiveness of hairy roots and the advantages of producing plumbagin. The research results can lay an important experimental foundation for further understanding the biosynthesis mechanism of plumbagin and the potential commercial development and application feasibility.

2 Materials And Methods

2.1 Plant materials and bacteria

Leaf explants from 20-d to 30-d aseptic seedlings (Fig. 1) of *P. auriculata* obtained via seeds aseptic germination, which were used for hairy roots induction at the Biotechnology Laboratory.

Three *A. rhizogenes* strains A4, ATCC 15834 and LBA 9402, which were used to transfect purchased from the ATCC (American Type Culture Collection, Rockville, Maryland, USA), and stored at − 80°C. The bacteria was activated on yeast mannitol broth (YMB, yeast extract 1 $g{\cdot}L^{-1}$, mannitol 10.0 $g{\cdot}L^{-1}$, K_2HPO_4 0.5 $g{\cdot}L^{-1}$, $MgSO_4{\cdot}7H_2O$ 0.2 gL^{-1}, NaCl 0.1 $g{\cdot}L^{-1}$, agar 12 $g{\cdot}L^{-1}$, pH 7.2) media at 28 ℃ for three times as it was described previously protocol. In the last time of liquid culture of bacteria, the liquid YMB media was supplemented with acetosyringone (AS, 100μmol· L^{-1}) to increase the frequency of hairy roots induction.

2.2 Different species of *A. rhizogenes*-mediated genetic transformation of hairy roots

In order to facilitate the description of hairy root systems induced by three different *A. rhizogenes* A4, ATCC 15834 and LBA 9402, they were named PAHR 4, PAHR 15834 and PAHR 9402 respectively in this paper, while the roots of the control group were named PAR TK.

The hairy root induction formula can be seen as follows: (1) immerse about $1cm^2$ of sterile leaves in active bacteria (OD600 about 0.5 to 0.8) for 25 to 30 minutes, and shake gently during this period; (2) Take out the explants and absorb the water with sterile filter paper. Then it was transferred to the medium added with 1/2 MS + AS 100 μ $mol{\cdot}L^{-1}$, and incubated at 28 ℃ in complete darkness for 2–5 days; (3) After obvious plaques appeared around the explants, they were transferred to 1/2 MS + Cef medium containing cefotaxime in complete darkness at 25 ± 1 ℃ until they were completely sterilized. Cef concentration changes were adjusted to 500, 400, 300, 200, 100 and 0 $mg{\cdot}L^{-1}$/week. The control group was replaced with sterile water, and the biological experiment was repeated three times.

2.3 Identification of hairy roots

Morphological identification.

Morphological characteristics of hairy roots and untransformed roots (namely sterile tissue culture roots, as the control) were observed, including initial state, color, thickness, presence or absence of branches, villi, and geotropism.

Organization structural identification.

Hairy roots (30 d) and tissue culture roots (30 d, as control) took for paraffin section analysis. The steps were as follows: (i) Picking and fixing: Samples that were collected and placed in FAA fixative (70% ethanol: glacial acetic acid: formalin was 18:1:1), and fixed at 4℃ for more than 24h. (ii) Dehydration and transparency Samples dehydrated with 75% alcohol for 4 h, 85% alcohol for 2 h, 90% alcohol for 2 h, 95% alcohol for 1 h, absolute ethanol I for 30 min, then absolute ethanol II for another 30 min. Then material was then transparented for 5 ~ 10 min for alcohol-benzene, 5 ~ 10 min for xylene I and 5 ~ 10 min for xylene II. (iii) Wax dipping and embedding Samples was placed in a beaker with melted wax powder and dry 1 h at 60°C, and this step needs to repeat three times. Then samples conduct of embedding. (iv)Sectioning and gluing Samples slice to a thickness of 3 μm and stick to a glass slide with one drop of 10 $g{\cdot}L^{-1}$ gelatin solution. (v) Dewaxing and staining The slices were placed in xylene for 20 min and another 20 min, absolute ethanol for 5 min and another 5 min, 75% alcohol for 5 min. Then staining with 5 $g{\cdot}L^{-1}$ sarranine solutions for 1–2 h .Then slices were placed in 50%, 70%, and 80% gradient alcohol for 3–8 s each.Then put it into 5 $g{\cdot}L^{-1}$ fast green staining solution for 30–60 s, and dehydrate with absolute ethanol. (vi) Cover slides and microscopy: The slices need to be sealed with neutral gum and dried for 12 h at 40°C. Finally, we carry out microscopic examination and analysis.

PCR molecular identification.

Appropriate hairy roots and un-transformed roots were collected randomly, Using the modified CTBA method (štorchova et al., 2000; Nathar & Gadge, 2013) extracted genomic DNA from each root system, SanPrep column plasmid DNA small volume extraction kit was used to extract plasmid genomic DNA from *A. rhizogenes*. The *rol*A, *rol*B, *rol*C genes on the T-DNA of *A. rhizogenes* and the virD gene outside the T-DNA fragment were detected, and the forward and reverse primers used for amplification are shown in Table 3. The known nucleotide sequence was designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Table 1

Statistics for dynamic parameters and morphological characteristics of hairy roots and tissue culture roots of *P. auriculata*. PAR C was the logogram of *P. au* PAHR was the logogram of *P. auriculata* hairy roots. Induction rate (%) = number of rooting explants / number of inoculation explants × 100%. Browning rate explants / number of inoculation explants × 100%. Callus rate (%) = number of callus explants / number of inoculation explants × 100%.Pollution rate (%) = nu number of inoculation explants × 100%.Mortality rate (%) = number of mortality explants / number of inoculation explants × 100%. Rooting time (d) represent from the first day of co-culture. Slender (or thick) represented that diameter of roots was between 0.5 to 1.0 cm. −, there was no root grew; +, roots grew well; ++ detected. Control group were conducted in sterile water. Data represented standard deviations (SDs) of the mean (n = 3). Different lowercase letters indicated different among the treatments at $P < 0.05$ according to Duncan's test. For description conveniently, hairy roots induced by A4, ATCC 15834 and LBA 9402 we 15834 and PAHR 9402, and the tissue culture roots (the control) was named as PAR TC. The ND is an abbreviation for no detected, indicating that it is not dete means average growth; "++" means good growth; "+++" means good growth; The values in the table are mean ± standard error, and different lower case letters significant difference between treatments ($P < 0.05$).

Strains	Naming	Co-culture (d)	Induction rate (%)	Browning rate (%)	Callus rate (%)	Pollution rate (%)	Mortality rate (%)	Rooting time (d)	Colour	Thick/Slender	Branches	Tomentum
Control	PAR TC	3 4	0.00 ± 0.00 a	23.17 ± 1.48 d	4.95 ± 0.21 d	0.12 ± 0.03 a	68.01 ± 0.43 d	ND	ND	ND	ND	ND
A4	PAHR 4	3 4	46.18 ± 1.62 b	12.08 ± 2.11 c	3.99 ± 0.12 c	0.1 ± 0.01 a	15.8 ± 0.43 c	14.33 ± 0.58 c	White	Minuteness	D	D
15834	PAHR 15834	3 4	86.78 ± 0.74 d	2.07 ± 0.32 a	2.04 ± 0.08 a	0.11 ± 0.02 a	5.43 ± 0.69 a	8.33 ± 0.58 a	White	Minuteness	D	D
9402	PAHR 9402	2 3	54.23 ± 2.34 c	8.71 ± 0.63 b	3.57 ± 0.40 b	0.12 ± 0.01 a	14.06 ± 0.58 b	11.67 ± 0.58 b	White	Minuteness	D	D

Table 2

Plumbagin content in different roots of *P. auriculata*. DW meant dry.

Roots	Actual injection volume (μL)	Retention time (min)	Peak area (mAUs)	Content (mg/g)	Percentage content (%)
PAR TC	20	21.55	26.15	0.54	0.05
PAR NC	20	21.55	422.59	9.84	0.98
PAHR 4	20	21.54	1043.28	24.72	2.47
PAHR 15834	20	21.55	1436.61	38.95	3.90
PAHR 9402	20	21.55	1135.46	24.90	2.49

Table 3

Amplication primer of gene *rolA*, *rolB*, *rolC* and *virD*

Num.	Gene names	Forward/Rreverse	Primer sequences	Length (bp)	References
A	*rol*A	F	5'-CGT TGT CGG AAT GGC CCA GAC C-3'	258	Hu J *et al*., 2016.
		R	5'-CGT AGG TCT GAA TAT TCC GGT CC-3'		
B	*rol*B	F	5'-ATG GAT CCG AAA TTG CTA TTC CTT CCA CGA-3'	780	Gangopadhyay et al., 2008.
		R	3'-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-5'		
C	*rol*C	F	5'-ATG GCT GAA GAC GAC CTG TGT-3'	550	Thiruvengadam et al., 2014.
		R	5'-TTA GCC GAT TGC AAA CTT GCA-3'		
D	*vir*D	F	5'-ATG TCG CAA GGC AGT AAG CCC-3'	438	Mohammad *et al*., 2016.
		R	5'- GGA GTC TTT CAG CAG GAG CAA – 3'		

The reaction system (25 μL) included Buffer (2.5 μL), dNTPs (2.0 μL), forward primer (1.0 μL), reverse primer (1.0 μL), Taq enzyme (0.35 μL), ddH2O (16.2 μL), and template DNA (2.0 μL). After vortex mixing and centrifugation, PCR reaction: predenaturation at 95 °C for 5 min, (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s) repeated for 34 cycles, extension at 72 °C for 5 min; 4°C terminal extension ∞. DL 2000 DNA Marker, plasmid DNA and un-transformed roots DNA amplification products used as reference, positive and negative control, respectively, and electrophoresed on a 1% agarose gel electrophoresis. Then gel stained with ethidium bromide (EB) for 10 min and scanned by UV light scanning on a gel imaging system (UV 260 nm).

2.4 Plumbagin content quantification of hairy roots

This experiment was performed by high-pressure liquid chromatography (HPLC) .

Chromatographic condition. The mobile phase was 0.1% H_3PO_4 : methanol (v : v, vacuum filtered through a 0.45 μm filter before use), and the elution gradient was 0 min (0 : 40), 21 min (25 : 75), 23 min (10 : 90), 30 min (10 : 90), the flow rate was 1 mL·min^{-1}, the detection wavelength was 254 nm, the column temperature was 40 ℃, the detection temperature was room temperature, the sensitivity was 0.16 AUFS, the injection volume was 20 μL, and the retention time was about 21 min. The operating temperature was kept at room temperature.

Standards and Sample Preparation. The following are the specific steps for preparing the standard: (1) In the first step, we need to weigh 5.1mg of the standard plumbagin and place it in a 10mL volumetric flask. Then, add an appropriate amount of methanol into the bottle to dissolve and fix the volume. Finally, shake it well to obtain standard stock solution A (0.510 mg·mL^{-1}). (2) First, we need to take 2mL of standard stock solution A and put it into a 10mL volumetric flask. Then, add an appropriate amount of methanol to the bottle to constant volume. Finally, shake it well to obtain standard stock solution B (0.102 mg·mL^{-1}). (3) First, we need to take 1mL of standard stock solution B and put it into a 25mL volumetric flask. Then, add an appropriate amount of methanol to the bottle to constant volume. Finally, shake it well to obtain standard stock solution C (0.004 mg·mL^{-1}). The standard stock solution of each concentration shall be stored at 4 ℃ for standby. The following are the specific steps of sample preparation: (1) In the first step, we need to randomly select an appropriate amount of roots from different sources PAHR 4, PAHR 15834, PAHR 9402, tissue culture seedling root system (PAR TC) and seedling root system (PAR NG) in triplicate. It is dried in an oven at 65 ℃ for 24 hours. Then it is dried to constant weight at 45 ℃, and then it is fully ground and crushed into coarse powder, which is then sieved through a 40 mesh sieve. (2) In the first step, 0.10 (± 0.02) g of dried powder of each root system needs to be weighed, dissolved in methanol solution and fixed to 25 mL. After the fresh-keeping film is sealed and placed in the ultrasonic cleaner for ultrasonic extraction at room temperature for 30 min, the sample solution is replenished to 25mL with methanol solution again. (3) It is necessary to mix three solutions from the same source with 0.22 μM water soluble filter membrane is filtered and stored in 10mL EP tube.

For the determination of the sample plumbagin, it is necessary to draw an appropriate amount of solution from each EP tube to an Agilent bottle (1.5mL), and then make use of the machine for HPLC analysis with the injection volume of 20 μL.

2.5 Statistics and Analysis

All data were analyzed using SPSS Statistics 22 (SPSS Inc., Chicago, USA) software statistical analysis, one-way ANOVA and least significant difference test (LSD), Excel 2016 and Origin 9.0 (OriginLab Inc., Massachusetts, USA) Software tabulation. The significance level was set to α = 0.05.

3 Results

3.1 Effects of different strains on hairy root induction and growth

Growth parameters of hairy roots. According to the statistical analysis (as shown in Table 1), although the three tested *A. rhizogenes* can induce the hairy root of Plumbago paniculata (Fig. 3), the transformation efficiency (average days of hairy root induction) and transformation frequency of different strains have significant differences. It was found that ATCC 15834 had the highest hairy root induction rate (86.78 ± 0.74%), followed by LBA 9402 (54.23 ± 2.34%) and A4 (46.18 ± 0.74%). In addition, compared with A4 and LBA 9402, ATCC 15834 induced the lowest browning rate (2.07 ± 0.32%), callus rate (2.04 ± 0.08%), pollution rate (0.11 ± 0.02%) and mortality rate (5.43 ± 0.69%). Moreover, its rooting time was the earliest (8.33 ± 0.58 d) (Fig. 2B). At 30 days, a large number of hairy roots were produced (Fig. 2D).

Table 4
Statistics for results of plumbagin standard substance A of peak area

Standard substance stock solution	Injection volume/μL	Concentration/mg·mL^{-1}	Peak aera/mAU*s
A	1	0.051	179.897
A	5	0.205	948.407
A	10	0.510	2074.254
A	15	0.715	3003.085
A	20	1.020	4139.224
A	40	2.040	8207.259
B	1	0.010	34.800
B	5	0.051	183.289
B	10	0.102	359.141
C	1	0.004	14.7983
C	5	0.020	72.401
C	15	0.061	216.409

Induction dynamic process of hairy roots. After the explants were co cultured and cultured on the sterile medium for 3 to 4 days, the hairy roots of blue flower lead developed/appeared from the wound infection site (Table 1, Fig. 2B). The browning, callus, pollution and mortality of all hairy roots are low, but the induction rate is relatively high (Table 1). In accordance with morphological characteristics, hairy roots with branches and villi but no geotropism are white and slender. Through elongation (Fig. 2C) and hormone autotrophy, it can reproduce in large numbers (Fig. 2D) and grow well. During the whole induction process, the morphological characteristics and dynamic records of hairy roots are shown in Fig. 2.

3.2 Identification of hairy roots

Morphological analysis. Seen from Fig. 3, the three lines of hairy roots induced by the different strains respectively were all white or milk while. However, hairy roots were significantly different tissue culture roots on morphology, namely, hairy roots which have mass root hair and diameter of roots was smaller, but tissue culture roots were almost root-hairless or with little of short tomentum. Thus, we could preliminary infer that the roots of *P. auriculata* were typical hairy roots.

Organization structural analysis. After stained with safranine and fixed green, the root tissue structure was clear and complete under fluorescence microscope. (Fig. 4). Seen from the image, the hairy roots were anatomically similar to the tissue culture root generally, while the hairy roots had root hair but without phloem and cortex. Thus, this could structurally distinguish both clearly. Meanwhile, we determined that the average diameter of hairy roots (361.0767 μm) was 3/4 of tissue roots (499.7935 μm).

PCR analysis. It demonstrated that the T-DNA of *A. rhizogenes* had been presented in the hairy roots and the hairy roots genomic DNA amplified the same size-specific DNA fragments with the Ri plasmid genomic DNA by PCR analysis (Fig. 5).

In this way, the morphology, tissue structure and PCR molecular marker detection showed that hairy roots of *P. auriculata.* were the results of transformation of *A. rhizogenes* Ri plasmid. It presents typical morphological characteristics. Although its shape and structure are different from ordinary roots, it has the function of ordinary roots and is a real root.

3.3 Plumbagin content analysis of hairy roots

According to the relationship between the peak area value of each sample and the injection volume, we can further calculate and analyze the content of plumbagin according to the linear equation y = y = 4044.7 x + 4.9262 (r^2 = 0.9995). The results showed that the content of Plumbagin in hairy roots cultured for 30 days was higher than that in control roots. We found that PAHR 15834 had the highest content (38.95mg·g^{-1} DW or 3.90% DW), followed by PAHR 9402 (24.90 mg·g^{-1} DW or 2.49% DW), and PAHR 4 had the lowest content (24.72 mg·g^{-1} DW or 2.47% DW). They were 72.13, 45.78, 18.22 times of PAR TC (0.54 mg·g^{-1} DW or 0.05% DW) cultured for 30 days and 3.95, 2.53, 2.51 times of PAR NG (9.84 mg·g^{-1} DW or 0.98% DW) grown for 1 year, respectively. The results showed that the hairy roots induced by ATCC15834 were beneficial to the accumulation of plumbagin.

4. Discussion

4.1Effects of different strains on induction and transformation efficiency

Due to different sensitivity to the host, different strains have different infectivity (Brijwal and Tamta 2015; Khazaei et al 2019). The rhizobia taken in this research (A4, ATCC 15834 and LBA 9402) are all agroalkaline. They are highly toxic and are the first choice for hairy root system (Alagarsamy et al 2018; Henzelyov and Čellárová 2018; Akhgari et al 2015). Therefore, they can successfully induce hairy roots, but their inducing abilities are different. The results showed that compared with other strains, ATCC 15834 strain had a significant effect on the accumulation of hairy roots and its *P. auriculata*. The results of this research are slightly different from those of the same family and genus. Nayak (2015) believed that LBA9402 strain had better induction effect on hairy roots of Plumbago paniculata.

In previous studies, different strains of *A. rhizogenes* have specificity in plants (Bahmani et al 2021). At the same time, the selection of *A. rhizogenes* strains will also affect the growth of hairy roots of different species and the accumulation of metabolites (Ooi et al 2013; Gupta et al 2016; Tavassoli and Safipour Afshar, 2018). For example, both are “chilies”. *Capsicum frutescens* and *Capsicum annuum* have specific recognition for different strains. In pepper, ATCC 15834 and ATCC 13333 strains can better induce hairy root growth, while in pepper, ATCC 43056 and ATCC 43057 strains have the best induction efficiency (Md Setamam et al 2014). Therefore, when selecting strains to establish genetic transformation lines, the reference value in different genotypes is still limited, even if it is near species.

4.2 Effect of *rol* gene in *A. rhizogenes* on the production of plant secondary metabolites

According to this research, we detected three *rol*A, *rol*B and *rol*C genes in three transformed roots by PCR. On the basis of previous research reports, these genes are directly related to the production of plant hair roots and secondary metabolites. For example, Shkryl et al(2008) showed that the transfer of *rol*A gene into *Rubia cordifolia* callus can stably express, thus producing more secondary metabolite anthrone and positively regulating the vigorous growth of callus. At the same time, by transferring *rol*B and *rol*C genes, it can enable transgenic plants of *Artemisia annua* to metabolize more artemisinin (Dilshad et al 2015). These genes are usually stably expressed in the long-term culture of plants, so they are often used to stimulate their secondary metabolism. However, further exploration of these genes cannot separate from the research of biochemical and genetic functions (Zhou et al 2011).

Because *rol*A, *rol*B, and *rol*C genes stimulate the production of secondary metabolites (Matveeva et al, 2015), hairy roots tend to produce more secondary metabolites than common roots (Chandra S, 2012). In this experiment, it was found that at 30 days, the content of plumbagin in the three transformed roots was dozens of times higher than that in the ordinary group, and even more plumbagin was harvested than that in the roots of annual seedlings. This may be caused by the transfer of *P. auriculata* into the *rol* gene through *A. rhizogenes*. This shows that the transfer of rol gene has a very important impact on the secondary metabolism of most plants. *A. rhizogenes* mediated genetic transformation lines can be used for commercial production of medicinal plants, which can bring higher economic benefits.

4.3 HPLC method is suitable for quantitative analysis of plumbagin

Nayak *et al*. in this research, HPLC was also used to quantify plumbagin in hairy roots. The pre experiment results showed that the extraction efficiency of plumbagin was higher and the purification effect was better than the traditional ultrasonic treatment. The separation of wave peaks is good and there is no influence of stray peaks during HPLC detection (Fig. 8). Therefore, 30 min ultrasonic treatment can replace the long time extraction process of ethyl alcohol. At the same time, the analysis of this research verifies that the precision of the instrument is good. As the plumbagin concentration is within the range of 4.08×10^{-3} 2.04 mg·mL^{-1}, the linear relationship is favorable with wider relative linear range (Fig. 9 & Table. 4). In addition, we also investigated the stability and repeatability of this method. The experimental results showed that the relative standard deviation (RSD) was 4.3, and the stability of plumbagin standard stock solution was good within 12 hours (Table 5). The repeatability of the test was favorable, and the relative standard deviation (RSD) was 0.79% (Table 6). The sample adding recovery shows that the method is reliable and the RSD is small (Table 7), which shows that the method has good sensitivity. Therefore, the hairy root system, tissue culture seedling root system and natural growth root system of *P. auriculata* can be accurately determined by this method.

Table 5 Statistics for results of plumbagin standard substance of stability

Determination time (h)	0	2	4	6	8	10	12
Peak area (mAU*s)	4498.3	4035.2	4497.9	4601.3	4579.1	4513.5	4553.7
RSD (%)				4.3			

Table 6 Statistics for results of samples of repeatability

NO. of sample	Weight/g	Injection volume/μL	Peak aera/mAU*s	Percentage content/%	Average content/%	RSD
1	0.500	20	6294.017	0.619		
2	0.500	20	6182.138	0.610		
3	0.500	20	6211.794	0.613	0.612	0.790
4	0.500	20	6201.993	0.612		
5	0.500	20	6119.954	0.604		
6	0.500	20	6199.812	0.612		

Table 7 Statistics for results of sample of recover rate

Treatment NO.	Sample content/mg	Standard content/mg	Total content/mg	Recovery rate/%	Average recovery rate/%	RSD%
1	26.70		42.27	94.56		
2	28.15	18.0	43.475	94.20	94.14	0.48
3	29.15		44.415	93.67		
4	27.40		54.79	100.72		
5	26.35	27.0	52.28	97.08	98.71	1.97
6	27.60		53.695	98.34		
7	28.10		55.98	101.6		
8	29.05	36.0	55.105	98.31	99.38	1.93
9	27.25		53.295	98.24		

4.4 Hairy roots formed by genetic transformation become feasible to obtain plumbagin

Researches have confirmed that hairy roots are considered as one of the potential sources of various secondary metabolites, which usually synthesize high levels of secondary metabolites (Sevón and Oksmancaldentey 2002). Previous studies showed that syringin in hairy roots of *Saussurea involucrata* increased 40 times (Fu et al 2005); *Gentiana macrophylla* hairy roots have a two-fold increase in gentiopicroside production compared with normal roots (Tiwari et al 2007). This research shows that compared with untransformed roots (including tissue culture and seedling roots), three different hairy roots of *P. auriculata* have higher content of plumbagin, which is 72.13, 45.78, 18.22 times higher than that of the ordinary group cultured for 30 days, and 3.95, 2.53, 2.51 times higher than that of the one-year seedlings of *P. auriculata*. Compared with the plants of the same family and genus, the content of plumbagin extracted from the hairy root system of plumbagin, which was cultured for 50 days by predecessors, was only 2.50 times and 3.12 times higher than that of the untransformed roots (Verma et al 2002; Gangopadhyay et al 2010). In contrast, the content of plumbagin extracted from hairy roots of *P. auriculata* at 30 days was 72.13 times higher than that of untransformed roots, which indicates that the efficiency was significantly higher than that of the same genus.

5. Conclusion

Through genetic transformation, this research obtained a considerable amount of plumbagin from hairy roots, which can be harvested in a short time. The results showed that the induction of hairy roots of *P. auriculata* could be an effective and reliable source of plumbagin. Its active ingredient content is high, the

time cost is low, and the future commercial development prospect can be expected. At the same time, it shows that the genetic transformation hairy root method for obtaining plumbagin can replace the traditional root digging method for obtaining plumbagin.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ju Hu, Ting Lei and Yunzhu Zhou. The first draft of the manuscript was written by Zian Zhao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest The authors declare no confict of interest.

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Figures

Figure 1

20- to 30-d old aseptic seedlings of *P. auriculata* via seed aseptic germination.

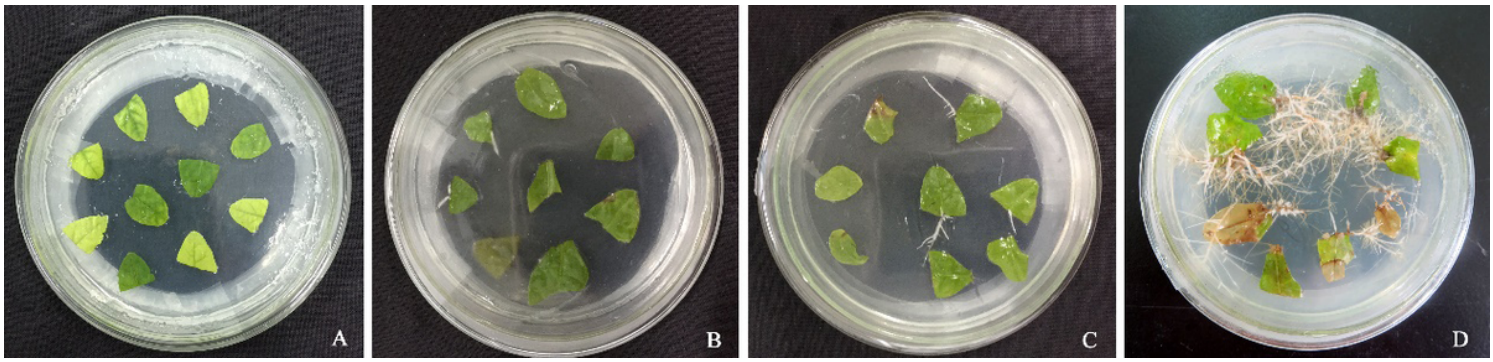

Figure 2

Dynamic process for PAHR 15834 induction and growth of *P. auriculata*. A, co-culture; B, hairy-root appearance; C, hairy-root elongation; D, hairy-root multiplication.

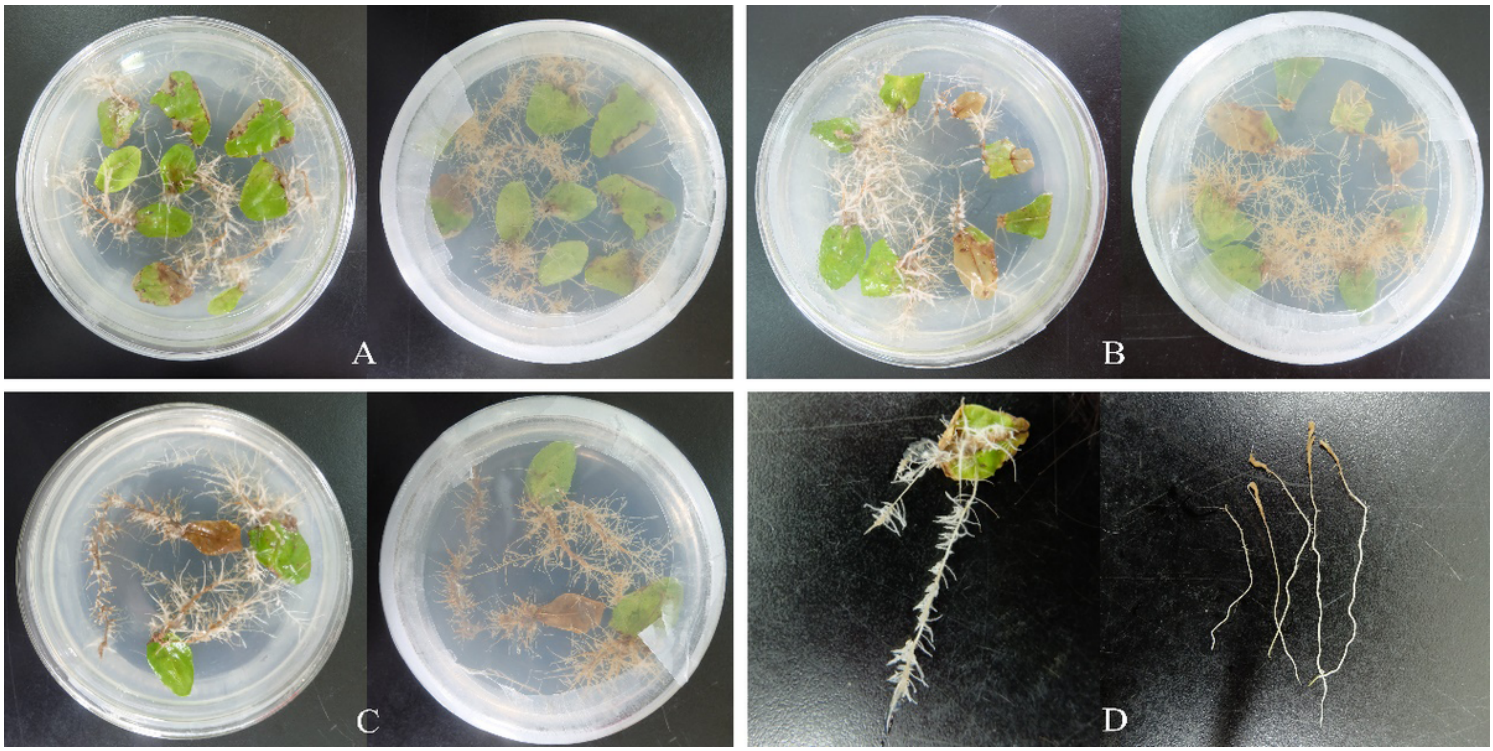

Figure 3

Different lines of hairy roots and tissue culture roots of *P. auriculata*. A, PAHR 4 induced by A4; B, PAHR 15834 induced by ATCC 15834; C, PAHR 9402 induced by LBA 9402; D, PAHR (left) and PAR TC (right). PAHR meant hairy roots of *P. auriculata*, and PAR TC meant tissue culture roots of *P. auriculata*.

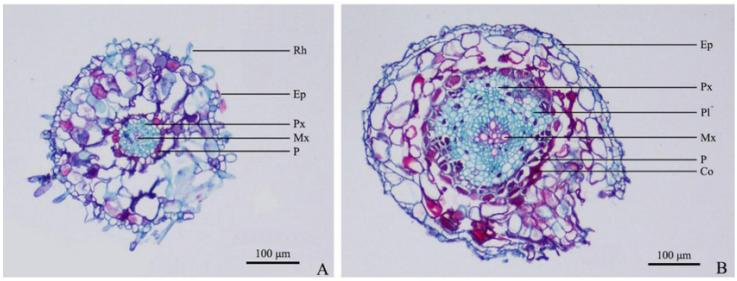

Figure 4

Paraffin section of roots tissue (A, hairy root; B, tissue culture root). Rh, roothair; Ep, epidermis; Px, protoxylem; Mx, metaxylem; P, pericycle; Pl, phloem; Co, cortex. Bar = 100 μm.

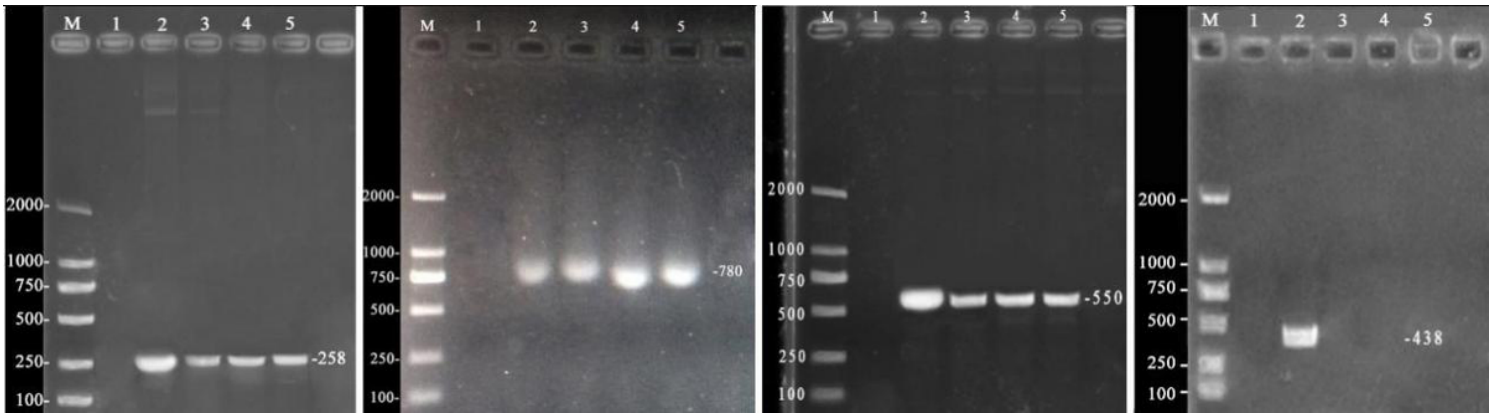

Figure 5

Gel electrophoresis analysis of PCR amplification products of *P. auriculata* hairy roots. M, DL 2000 DNA Marker (100-2000 bp); 1, DNA amplification products of *P. auriculata* sterile seedlings tissue culture roots (negative control); 2, DNA amplification products of ATCC 15834 (positive control); 3, DNA amplification products of hairy roots PAHR A4; 4, DNA amplification products of hairy roots PAHR 15834; 5, DNA amplification products of hairy roots PAHR 9402. About 258 bp, 780 bp and about 550 bp specific *rolA*, *rolB* and *rolC* DNA fragments were amplified from the total DNA of the three hairy roots, which were consistent with the specific T-DNA fragments amplified from the total DNA of the positive control strain Ri plasmid. The *virD* gene is located outside the T-DNA segment, so it is only detected in the positive control.

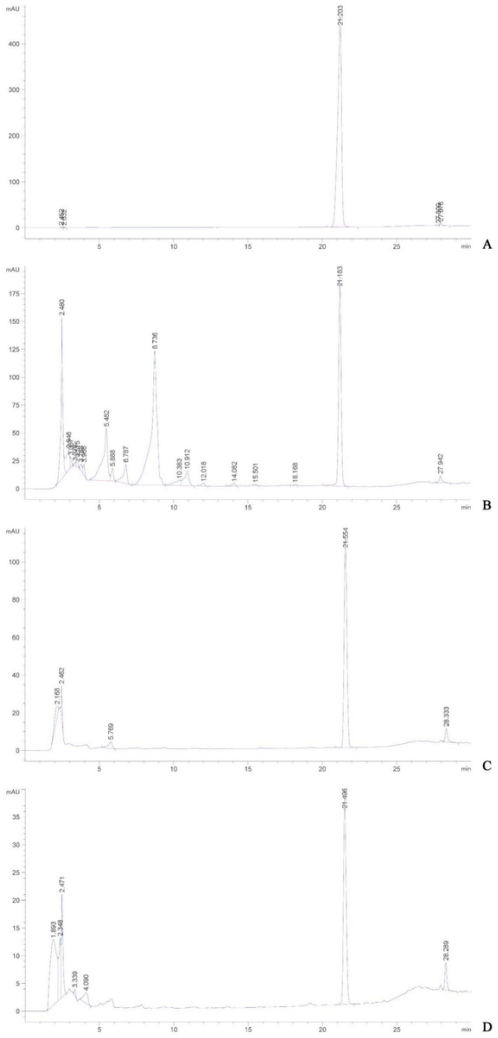

Figure 6

The HPLC chromatogram of plumbagin standard compound and different roots sample of *P. auriculata*. A, chromatogram of plumbagin standard compound; B, chromatogram of hairy roots; C, chromatogram of tissue roots.

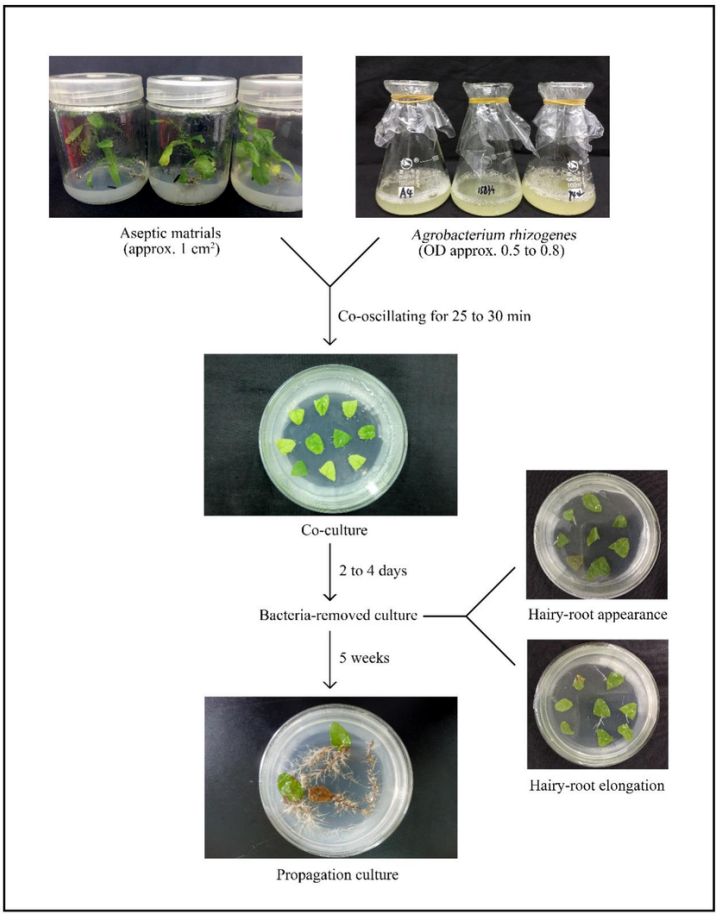

Figure 7

A summary of the technique for hairy roots inducing and propagating of *P. auriculata*.

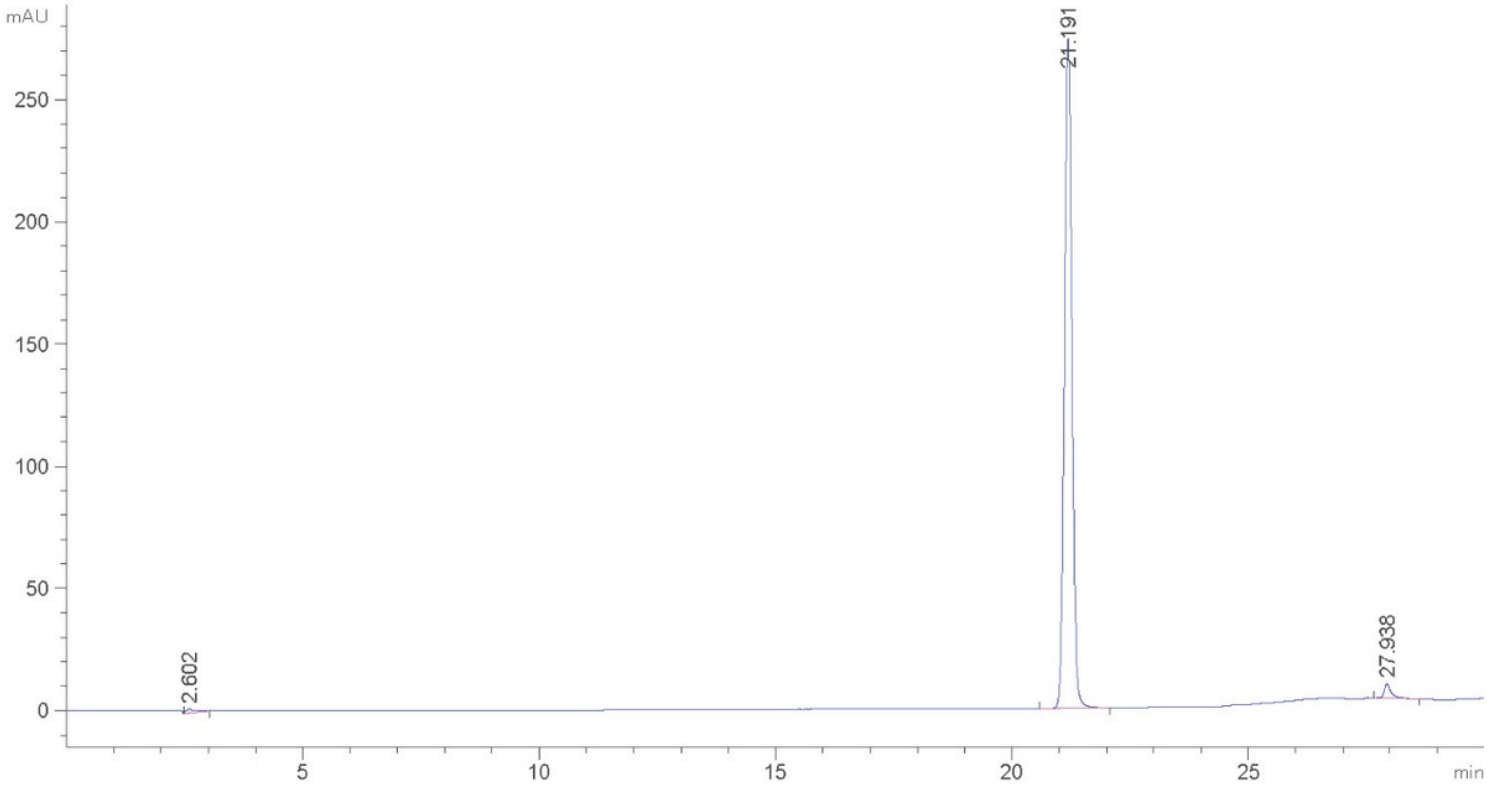

Figure 8

The HPLC chromatogram of standard substance of plumbagin the plumbagin of standard stock solution A, press 1 μL,5 μL,15 μL,20 μL,40 μL Inject samples separately.

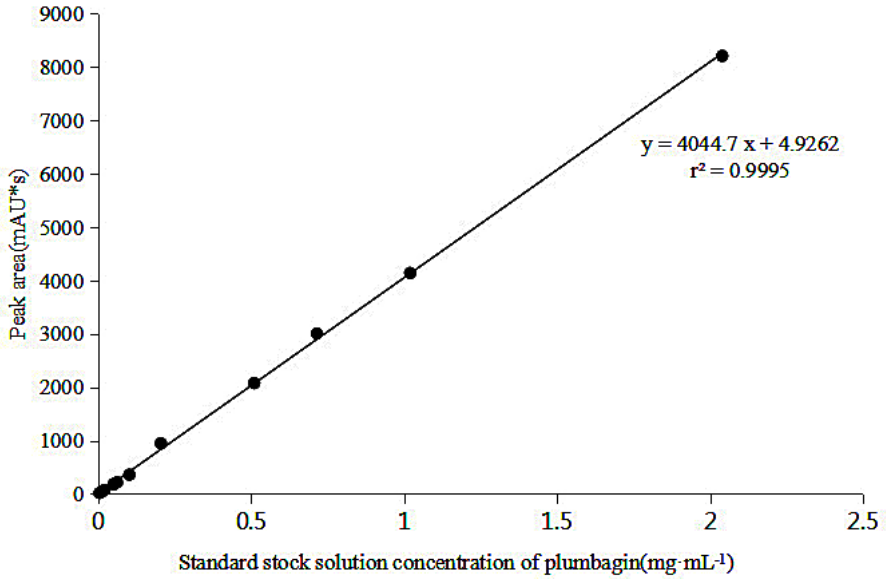

Figure 9

The A, B and C standard curves of plumbagin standard stock solution. Drawing the standard curve with the standard concentration ($mg \cdot mL^{-1}$) as the abscissa and the measured peak area (mAU*s) as the ordinate. The linear regression equation was obtained by investigating the linear relationship between the sample concentration and the peak area with the method of linear regression.