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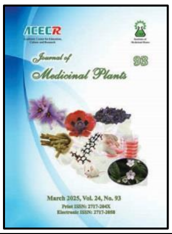

Research Article

The optimization of *in vitro* culture of *Lithospermum erythrorhizon* and shikonin production

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ABSTRACT

Background: *Lithospermum erythrorhizon* is a medicinally valuable plant with diverse biological activities. **Objective**: This research focused on optimizing the in-vitro cultivation of *L. erythrorhizon* and establishing an efficient protocol for extracting shikonin from callus tissue. **Methods**: leaf explants were subjected to various concentrations of Kinetin (Kin), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA), and 6-Benzylaminopurine (BAP) in the LS culture medium to study callus induction. Different concentrations of LS, M9, and MS media, along with varying amounts of BAP, NAA, Kin, and IAA, were employed to investigate callus regeneration. The rooting was examined in concentrations of Indole-3-butyric acid (IBA). For shikonin production, callus was cultivated in LS, MS, and M9 media containing 0.6 $mg.l^{-1}$ Kin and 2 $mg.l^{-1}$ NAA. **Results**: The findings shown that the highest callus induction rate occurred in LS medium with 0.6 $mg.l^{-1}$ Kin and 2 $mg.l^{-1}$ NAA (93.3%). full-strength LS medium containing 2 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA led to the highest shoot regeneration. Using 1 $mg.l^{-1}$ IBA in the LS medium let to improve rooting percentage (> 70%). The suitable substrate for the acclimatization was vermiculite. The shikonin content analysis in callus indicated that the M9 medium was more effective than MS and LS media in producing shikonin-containing calli. The results demonstrated that the concentration of shikonin in calli grown in M9 medium was increased with the number of day's post-explant cultivation. **Conclusion**: This research can serve as a model for future investigations into optimizing the cultivation conditions of *L. erythrorhizon*.

1. Introduction

In natural therapies, medicinal plants have stood the test of time, providing comfort and healing for countless diseases. Their enduring legacy continues to grow in the modern era, where the increasing demand for these botanical

Abbreviations: Kin, Kinetin; NAA, 1-Naphthaleneacetic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; IAA, Indole-3-acetic acid; BAP, 6-Benzylaminopurine; IBA, Indole-3-butyric acid.

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treasures, especially for industrial applications, has placed them at the center of attention, reflecting a revival in herbal medicines [1]. Among these esteemed plants, *Lithospermum erythrorhizon* from the Boraginaceae family is recognized as a plant of significant biological and economic value. It is revered in Asian countries for its dual use as a raw medicinal material and a source of natural dye [2]. This plant has gained fame in plant biotechnology, particularly since the 1980s when its cell suspension cultures were first used for the industrial production of the secondary metabolite shikonin, a testament to the plant's adaptability and the importance of this compound [3]. Shikonin, exclusively synthesized in *L. erythrorhizon* root bark, is a hydrophobic red naphthoquinone pigment [4]. This substance is the primary bioactive compound extracted from the roots, although it is also found in other members of the Boraginaceae family. Shikonin possesses a range of medicinal properties, including antibacterial, anti-inflammatory, tumor-inhibitory, anti-topoisomerase [5], antiviral, and wound healing capabilities [6]. Recently, shikonin and its derivatives have recognized as useful natural dyes and new pharmaceutical scaffolds [6]. Moreover, shikonin acts as an active inhibitor of the TMEM16A chloride channel, identified as a potential agent to reduce the acute respiratory effects of COVID-19 by inhibiting SARS-CoV-2's CLpro, and is widely used in traditional Chinese medicine as a supportive therapy for the virus [2, 7, 8]. Despite its notable properties, cultivating *L. erythrorhizon* presents significant challenges, including slow seed germination, susceptibility to viral infections, and sensitivity to disinfectants. Optimal cultivation requires moderate sunlight, minimal fertilization, and careful watering to prevent soil contact with lower leaves, making cultivation difficult. Moreover, it takes three years of growth to harvest roots with sufficient shikonin content [5]. However, cell suspension cultures can produce shikonin in quantities up to 10 percent of the dry cell weight or ten times more than what accumulates in undisturbed roots [3]. Common culture media used in laboratory conditions include Murashige and Skoog (MS), Gamborg (B5), Linsmaier and Skoog (LS), and Nitsch and Nitsch (NN). To establish a new protocol with specific objectives, it is best to first select the most suitable medium based on preliminary tests. Plant growth regulators (PGRs), especially cytokinins and auxins, are among the most important variables in culture media. While full-strength salt concentrations are typically used, sometimes half or quarter strength yields better results [9]. Numerous studies have focused on optimizing laboratory plant cultures and determining the appropriate growth medium [10, 11, 12]. This study is an effort to refine the laboratory cultivation of *L. erythrorhizon* and optimize a protocol that maximizes shikonin production, paving the way to harness the full potential of this plant and introduce its benefits to the current era of medical science.

2. Materials and Methods

2.1. Preparation of sample

Leaves were detached from seed-grown plantlets of *L. erythrorhizon* Obtained from Zhong Wei Horticultural Products Company with plant code P665240. After washing leaves with running water, they were sterilized with sodium hypochlorite solution (1.5% - 5 minutes). Then sterile distilled water, mercuric chloride solution (0.1 % - 5 minutes) and carbendazim solution (500 mg.l^{-1} – 6 minutes) were used to complete sterilization.

2.2. Culture medium preparation

Three base culture media, MS, LS, and M9, were utilized in this research. All media contained 3% sucrose (w/v) and 0.75% agar (w/v), pH=5.8. All media were autoclaved at 121◦C for 20 minutes.

2.3. Callus induction

0.5×0.5 cm pieces of leaves were used for callus induction and cultured on LS medium supplemented with various combinations of plant growth regulators (PGRs). The composition of the culture medium was reviewed and selected according to various articles [5, 13]. Callus induction percentage and callus volume (with submerging the callus in a known volume of liquid and measuring the volume of liquid displaced) were evaluated 30 days after culturing. This experiment was conducted according to a completely randomized design with three replicates. each replicate containing 10 samples. The treatments has been shown in Table 1.

2.4. Callus regeneration

To assess the regeneration percentage of calli, and considering finding of previous experiment, the suitable compounds were selected for regeneration. Therefore, three base culture media, including MS, LS, and M9, with different plant growth regulators were used (Table 2). This experiment was designed as a completely randomized design with three replicates, each replicate containing 10 explants. After 30 days of culturing, shoots number per explant, regeneration percentage, and plant length were recorded.

2.5. Rooting

A half-strength LS medium with IBA (0, 0.5, 1, and 1.5 $mg.l^{-1}$) is utilized for rooting. Each treatment had 10 replicates; each replication has 3 explants. After one month, rooted plantlets number, root length, and root number were evaluated.

Table 1. PGRs treatments in callus induction media

Treatments	
0.2 $mg.l^{-1}$ Kin + 0.8 $mg.l^{-1}$ 2,4-D	1 $mg.l^{-1}$ Kin + 1 $mg.l^{-1}$ IAA
0.2 $mg.l^{-1}$ Kin + 6 $mg.l^{-1}$ NAA	2 $mg.l^{-1}$ Kin + 0.2 $mg.l^{-1}$ IAA
0.6 $mg.l^{-1}$ Kin + 2 $mg.l^{-1}$ NAA	0.2 $mg.l^{-1}$ BAP + 0.8 $mg.l^{-1}$ 2,4-D
0.6 $mg.l^{-1}$ Kin + 6 $mg.l^{-1}$ NAA	0.6 $mg.l^{-1}$ BAP + 6 $mg.l^{-1}$ NAA
0.6 $mg.l^{-1}$ Kin+ 0.8 $mg.l^{-1}$ 2,4-D	1 $mg.l^{-1}$ BAP + 0.2 $mg.l^{-1}$ NAA

Table 2. The base culture media and PGRs combinations for callus regeneration

Treatments
LS (full concentration) (without PGR) (Control)
LS (full concentration) + 1 $mg.l^{-1}$ NAA + 2 $mg.l^{-1}$ BAP
LS (full concentration) + 4 $mg.l^{-1}$ NAA + 1.2 $mg.l^{-1}$ Kin
LS (half concentration) + 1 $mg.l^{-1}$ NAA + 2 $mg.l^{-1}$ BAP
M9 (double concentration) + 1 $mg.l^{-1}$ IAA + 1 $mg.l^{-1}$ Kin
M9 (full concentration) + 0.2 $mg.l^{-1}$ NAA + 1 $mg.l^{-1}$ BAP
M9 (full concentration) + 0.2 $mg.l^{-1}$ IAA + 2 $mg.l^{-1}$ Kin
M9 (double concentration) + 2 $mg.l^{-1}$ NAA + 0.6 $mg.l^{-1}$ Kin
MS (half concentration) + 0.2 $mg.l^{-1}$ NAA + 2 $mg.l^{-1}$ BAP

2.6. Seedling compatibility

Rooted plantlets were transferred to perlite, vermiculite, cocopeat, cocopeat-perlite (1:1), and vermiculite-perlite (1:1) substrates to evaluate the most beneficial material for seedling compatibility. Each treatment in this experiment had 20 replicates, each replication had 3 explants, and the survival percentage was calculated 20 days after acclimation. To ensure plant survival, the plants were maintained on the same substrate for three months.

2.7. Shikonin concentration analysis in callus medium

In order to assay the effect of salt compounds of media on shikonin induction, induced calli in callus induction experiment were utilized as explants. The selected culture medium included liquid MS, LS, and M9 media containing Kin (0.6 $mg.l^{-1}$) + NAA (2 $mg.l^{-1}$). Different studies have mentioned various culture media for this purpose [5, 14]. To enhance shikonin production, culture flasks were placed in the dark, as shikonin is produced in dark conditions [15] and on a shaker at 120 rpm. Qualitative assessment of shikonin was done based on the the color of the culture medium to turn red. Shikonin extraction and assay was performed according to Tatsumi [16]. Initially, 3 ml of the culture medium was added to 3 ml liquid paraffin overnight. Then, 5 ml of hexane was added, and the upper solution was transferred to a new tube. Afterward, 2.5% KOH solution was added to the supernatant. At this stage, shikonin dissolves as a blue pigment. The color change of the solution was considered indicative of shikonin presence. Finally, to determine the variation in shikonin concentration in the culture medium on different days, 300 µl of the solution was sampled on days 0, 7, 14, and 21 after culturing and measured at a wavelength of 650 nm. A culture medium without explants (callus) served as the control.

2.8. Statistic analyze

All the experiments were performed in completely randomized designs. Each treatment consisted of three replications and ten explants per replication. One-way analysis of variance (ANOVA) is used for analyzing, and LSD test ($P < 0.05$) is used for mean comparison. Data analyzing is done by JMP-8.0 software. Data normalization was carried out prior to analysis according to the Compton method [17].

3. Results

3.1. Callus induction

The study of callus induction in leaf explants of *L. erythrorhizon* showed that the use of NAA in combination with Kin had a positive effect on inducing callus. The application of 0.6 $mg.l^{1}$ Kin + 2 $mg.l^{-1}$ NAA in LS culture medium led to the highest percentage of callus induction and the greatest callus volume. In addition, the callus induction increased to over 90% with applying 0.2 $mg.l^{-1}$ Kin + 6 $mg.l^{-1}$ NAA (Fig. 1). Qualitatively, the calli grown in these two treatments were green, compact, and well-structured. The use of 2,4-D and IAA in the culture medium was not capable of inducing callus in leaf explants (Table 3). Also, in these plant growth regulator combinations, a high percentage of explant loss was observed, and there was a significant presence of vitrified and blackened calli.

3.2. Callus regeneration

Callus regeneration was influenced by various treatments. The results showed that using LS culture medium at full concentration containing 2 $mg.l^{-1}$ BAP + 1 $mg.l^{-1}$ NAA led to the highest regeneration in calli. This treatment increased the regeneration percentage by 98.5% compared to the control treatment. Regarding regenerated shoots number and shoot length, the data indicated that the control treatment performed better than the other treatments (Table 4). However, between treatments, the treatment with LS culture medium at full concentration containing 2 mg.l^{-1} BAP + 1 mg.l^{-1} NAA had the highest number of regenerated shoots (13.00) (Fig. 1). Moreover, the longest shoot length after the control treatment (68.00 mm) was observed in this treatment (18.66 mm). The results also revealed that using M9 and MS culture media did not yield successful regeneration indices compared to LS culture medium. Furthermore, using LS culture medium at half concentration also did not show suitable efficiency in this regard (Table 4).

Table 3. The impact of various PGRs on induction of callus and callus volume

PGR	Callus induction (%)	Callus volume (ml)
0.2 mg.l^{-1} Kin + 6 mg.l^{-1} NAA	91.66 ± 3.05^{a}	3.81 ± 0.03^{b}
0.6 mg.l^{-1} Kin + 2 mg.l^{-1} NAA	83.33 ± 3.05^{a}	3.93 ± 0.06^{a}
0.6 mg.l^{-1} Kin + 6 mg.l^{-1} NAA	32.00±3.60^{e}	2.57 ± 0.03^{d}
0.6 mg.l^{-1} Kin+ 0.8 mg.l^{-1} 2,4-D	6.00 ± 1.00^{g}	0.88 ± 0.06^{h}
0.2 mg.l^{-1} Kin + 0.8 mg.l^{-1} 2,4-D	4.00 ± 1.00^{g}	1.25 ± 0.03^{g}
1 mg.l^{-1} Kin + 1 mg.l^{-1} IAA	74.00 ± 2.00^{c}	3.07 ± 0.09^{c}
2 mg.l^{-1} Kin + 0.2 mg.l^{-1} IAA	5.33 ± 1.52^{g}	1.26 ± 0.05^{g}
0.2 mg.l^{-1} BAP + 0.8 mg.l^{-1} 2,4-D	8.00 ± 1.00^{g}	1.88 ± 0.03^{f}
0.6 mg.l^{-1} BAP + 6 mg.l^{-1} NAA	24.66 ± 2.08^{f}	2.25 ± 0.10^{e}
1 mg.l^{-1} BAP + 0.2 mg.l^{-1} NAA	57.00 ± 3.60^{d}	2.98 ± 0.10^{c}

In each column similar letters shows that there is no significant difference at the 5% probability level based on the LSD test. Three replicates in each treatment, and each replicate consisted of 10 samples

Table 4. The impact of different treatments on regeneration, regenerated shoot number, and shoot length

Treatments	Regeneration (%)	Number of regenerated shoots	Shoot length (mm)
LS (full concentration) (without PGR) (Control)	46.00 ± 1.00^{c}	18.00 ± 1.00^{a}	68.00 ± 2.00^{a}
LS (full concentration) + 1 mg.l^{-1} NAA + 2 mg.l^{-1} BAP	91.33 ± 1.53^{a}	13.00 ± 1.00^{b}	18.66 ± 1.15^{b}
LS (full concentration) + 4 mg.l^{-1} NAA + 1.2 mg.l^{-1} Kin	31.66 ± 1.53^{d}	8.33 ± 0.58^{d}	8.00 ± 2.00^{d}
LS (half concentration) + 1 mg.l^{-1}NAA + 2 mg.l^{-1} BAP	53.00 ± 1.00^{b}	10.66 ± 1.15^{c}	13.66 ± 1.53^{c}
M9 (double concentration) + 1 mg.l^{-1} IAA + 1mg.l^{-1} Kin	19.66 ± 1.53^{f}	1.33 ± 0.58^{e}	5.66 ± 0.58^{de}
M9 (full concentration) + 0.2 mg.l^{-1} NAA + 1 mg.l^{-1} BAP	12.00 ± 2.00^{g}	1.06 ± 0.90^{e}	4.00 ± 1.00^{ef}
M9 (full concentration) + 0.2 mg.l^{-1}IAA + 2 mg.l^{-1} Kin	3.66 ± 1.53^{h}	0.73 ± 0.46^{e}	2.00 ± 1.00^{f}
M9 (double concentration) + 2 mg.l^{-1} NAA + 0.6 mg.l^{-1} Kin	10.00 ± 1.00^{g}	0.73 ± 0.46^{e}	2.66 ± 1.53^{f}
MS (half concentration) + 0.2 mg.l^{-1} NAA + 2 mg.l^{-1} BAP	22.66 ± 1.53^{e}	8.33 ± 0.53^{d}	17.33 ± 2.52^{b}

In each column similar letters shows that there is no significant difference at the 5% probability level based on the LSD test. Three replicates in each treatment, and each replicate consisted of 10 samples.

3.3. Rooting

The study of various IBA concentrations in the rooting study of regenerated plantlets showed that the application of 1 mg.l^{-1} IBA in LS culture medium increased the rooting percentage to over 70% (Fig 1). Moreover, the number of roots produced at this IBA concentration was three times higher compared to the control treatment (Table 5). Increasing the IBA concentration in the culture medium to 1.5 mg.l^{-1} decreased the rooting percentage and root number per plantlet. However, the root length at this concentration was the highest, which is likely related to the reduced root number per plantlet (Table 5).

3.4. Seedling compatibility

The study of different substrates showed that using vermiculite as a substrate for seedling compatibility was the best choice (Fig. 1), as the highest seedling compatibility was observed in this substrate (96%). In addition, the seedling compatibility percentage in the vermiculite + perlite substrate was also over 80%. Cocopeat and perlite substrates, either alone or in combination, were not suitable choices for seedling compatibility (Table 6).

3.5. Analysis of shikonin content in calli

The study of different culture media showed that MS medium was not useful for shikonin extraction as no color change was observed in the medium. In LS medium, the color change to red was very faint, while in M9 medium, the culture medium turned a dark red (Fig. 2). In M9 medium, the extracted shikonin was observed to be a dark blue color (Fig. 2). The results showed that with an increase in the number of days from the culturing of the explants and the production of calli, the concentration of shikonin in the calli also increased (Fig. 3).

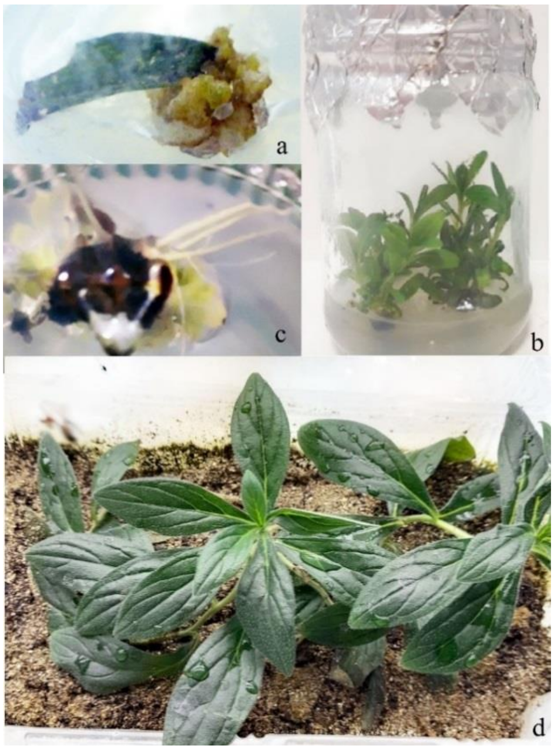

Fig 1. Indirect regeneration of *Lithospermum erythrorhizon* leaf explants. a) Callus induction, b) Regeneration and aerial organ growth, c) Rooting, and d) Seedling compatibility

Table 5. The impact of various IBA concentrations on rooting, root length, and number of roots per plantlet

IBA (mg.l^{-1})	Rooting percentage (%)	Root Length (mm)	Number of Roots per Plantlet
0	31.00 ± 1.00^{d}	6.66 ± 0.58^{c}	3.00 ± 1.00^{b}
0.5	35.00 ± 1.00^{c}	12.66 ± 2.08^{b}	2.66 ± 1.15^{b}
1	73.00 ± 2.00^{a}	8.66 ± 2.52^{c}	9.66 ± 0.58^{a}
1.5	42.33 ± 1.53^{b}	27.33 ± 1.53^{a}	4.00 ± 1.00^{b}

In each column, similar letters shows that there is no significant difference at the 5% probability level based on the LSD test. There were 10 replicates in each treatment.

Table 6. The seedling compatibility in different substrates

Substrate	Seedling compatibility (%)
Vermiculite	96.00 ± 1.00[a]
Vermiculite + Perlite	84.66 ± 2.08[b]
Perlite	55.00 ± 1.00[c]
Cocopeat	40.00 ± 1.00[d]
Cocopeat + Perlite	34.66 ± 1.53[e]

Similar letters in each column indicate no significant difference at the 5% probability level according to the LSD test. Each treatment had 20 replicates.

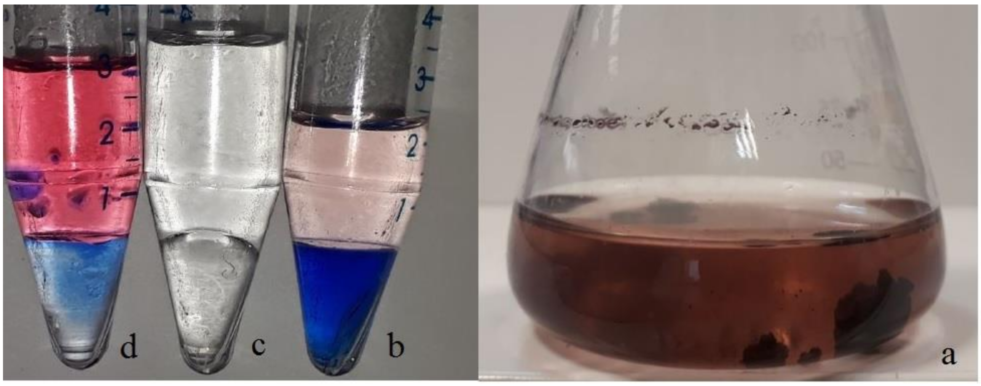

Fig. 2. a) Culturing of calli in M9 medium and the reddening of the culture medium and extraction of shikonin in the medium b) Shikonin extracted from M9 medium c) Culture medium without explants d) Adding KOH to the culture medium and beginning of extraction.

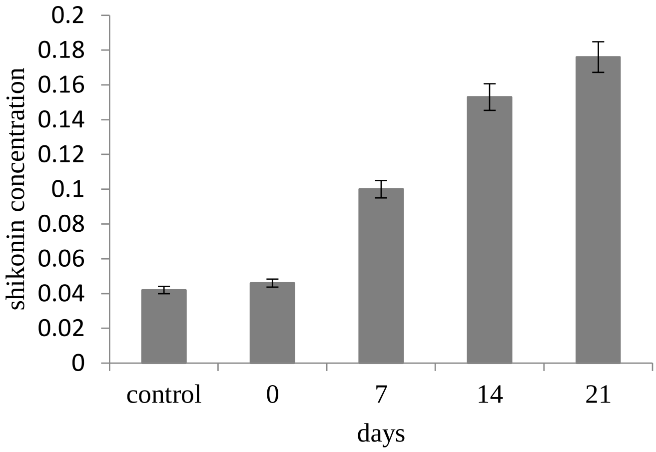

Fig. 3. Variation in shikonin concentration in M9 culture medium over different days after culturing the explants, measured at 650 nm wavelength. Medium without explants was used as a control.

4. Discussion

Plant propagation *in vitro* is significantly influenced by plant growth regulators (PGRs) [18]. This research demonstrated that explants placed in LS culture medium containing 0.2 mg.l^{-1} Kin + 6 mg.l^{-1} NAA and 0.6 mg.l^{-1} Kin + 2 mg.l^{-1} NAA produced the most callus. The positive role of PGRs in callus induction has been confirmed in other studies [19, 20, 21]. Generally, auxins such as NAA, IAA, and 2,4-D has impact on callus induction [22, 23, 24, 25]. In Lisianthus plants, the use of NAA effectively led to callus induction [26, 27]. The use of 2,4-D and BA was the best combination to induce callus from the leafbase explant in *Iris ferdowsii* [19]. In *Thalictrum minus*, culturing leaf explants in LS medium containing BA and NAA induced callus formation [28]. The positive effect of BAP on callus regeneration has also been confirmed in other studies [29, 30, 31]. Additionally, the positive impact of NAA on inducing shoot formation is noteworthy [32]. In this research, the use of 2 mg.l^{-1} BAP + 1 mg.l^{-1} NAA in full-strength LS medium resulted in the highest regeneration percentage. Consistent with these findings, application of 0.25 mg.l^{-1} NAA + 1 mg.l^{-1} BAP significantly increased regenerated plantlets in Caladium [33]. The combination and concentration of PGRs affect stem length because of their impact on cell division and development [34]. It has been stated that 1 or 2 mg.l^{-1} BAP had a decisive impact on the growth and development of lateral buds and shoot formation [35]. Cytokinins are commonly used to induce shoot formation *in vitro* [36, 37, 38]. The use of cytokinins activates buds and promotes the growth of lateral shoots. Moreover, application of cytokinins can stimulate bud growth, either in combination with auxins or alone [39, 40, 41]. The positive effect of cytokinins is likely due to the stimulation of cell division and induction of lateral shoot growth *in vitro* [42]. This process occurs following the induction of protein and RNA synthesis by BAP, which subsequently stimulates enzymatic activity associated with cell division and development [43]. In the process of plant propagation *in vitro*, root production in regenerated plantlets is crucial for proper establishment in subsequent growth stages. In many plant species, inducing rooting and plant growth can influence by auxins [44]. Most of the time, IBA is chosen because of more success in the rooting process [40]. The influential role of IBA in inducing rooting has been mentioned by other researchers in various plant species [45, 46, 47]. In line with the results of this research, Ren et al. [48] stated that the use of 0.02 mg.l^{-1} IBA and 0.4 mg.l^{-1} IAA was effective in inducing roots in *Casuarina equisetifolia*. The research results showed that the vermiculite culture substrate led to the highest acclimatization percentage compared to other tested treatments. Due to the unique structural, chemical, and mineralogical properties of vermiculite, this clay mineral is used for various purposes in agriculture, environmental applications, and industry. The improvement in plantlet acclimatization percentage in the vermiculite culture substrate is likely related to its lower bulk density, excellent aeration, suitable water permeability, and buffering capacity under different environmental conditions. Furthermore, the high absorption capacity of the culture substrate allows for the slow release of nutrients, facilitating plant nutrient uptake [49]. The mentioned factors contribute to improved root growth and, subsequently, an increase in plantlet acclimatization percentage. It has been stated that adding vermiculite and humic acid to Green waste compost improved the physicochemical properties of the culture substrate, such as water-holding capacity, bulk density, electrical conductivity, porosity, nutrient concentration, and pH, and significantly enhanced the growth indices of the cornflower plant [50]. Callus is a source of secondary metabolite production, suspension culture, and plant propagation through organogenesis and embryogenesis [51]. Various factors, including explant type, genotype, and ratio of auxins and cytokinins, play a role in callus induction [52]. The combination of PGRs in the culture medium significantly affects the size and morphology of callus cells. These compounds exhibit different functions during the mitotic cell division stage due to their varying effects on the cytoskeleton and microtubules when determining the division direction in callus cells [53]. The finding of this study are similar to study by Touno et al. [15], which observed that shikonin is not produced in LS or MS culture media and reported that this phenomenon might be due to the presence of nitrogen in the form of nitrate in these media, inhibiting shikonin production. Additionally, the red pigment extracted from M9 medium turns blue when dissolved in a 2.5% KOH solution. This color change confirm presence of shikonin [54]. The fluorescent spectrum measurement of shikonin with a nanodrop showed the highest concentration at 650 nm [16, 55]. This wavelength is consistent with the known absorption peak of shikonin, confirming the presence and concentration of shikonin in the calli. The use of nanodrop technology for measuring the fluorescent spectrum provides a precise and efficient method for quantifying shikonin, which is crucial for optimizing its production. Our result is confirmed by previous research [16, 55]. They have utilized similar techniques to measure shikonin concentration, further validating the effectiveness of the M9 medium for shikonin extraction.

5. Conclusion

The findings indicate that the use of LS culture medium with appropriate hormonal combinations can most effectively induce callus and shikonin production. Additionally, the M9 medium was identified as the best option for shikonin extraction. Given the existing challenges in the direct cultivation of *L. erythrorhizon*, this study has presented effective methods for improving shikonin production under laboratory conditions. Utilizing calli as a source for secondary metabolite production and plant propagation through organogenesis and embryogenesis offers a novel approach to accessing valuable medicinal compounds and reducing reliance on traditional cultivation. Ultimately, this research can serve as a model for future investigations into optimizing the cultivation conditions of *L. erythrorhizon* and other similarly important medicinal plants. With the development of effective cultivation protocols, there is hope that shikonin and other similar compounds will be utilized as accessible and sustainable resources for the pharmaceutical and dye industries in the future. This could make a crucial effect on advancing the field of pharmacology and related technologies.

Author contribution

A. SH and Z. S. designed the study. Z. S and N. S. performed the experiment and wrote the article. M. KH and A. KH. contributed to the research design and implementation.

Conflicts of interest

The authors are not affiliated with any organization with a direct or indirect financial interest in the topics discussed in the manuscript.

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