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Research Article

Detailed Investigations on the Solid Cell Culture and Antimicrobial Activities of the Iranian *Arnebia euchroma*

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In pursuit of strong shikalkin-producing cell lines, seeds of the Iranian *Arnebia euchroma* were collected from Dena altitudes in the central Zagross. Chemical analysis showed that the dried root of the plant contained about 8.5% (w/w) shikalkin pigment. The root explants of the young plantlets, obtained from the germinated seeds, were used for establishing callus. Then, parameters effective on proliferation and pigment production of the resulting calli were studied in detail. Accordingly, two modified media called mLS and mM9 were optimized for propagation and pigment production, respectively. Using these media, the biomass of the *A.euchroma* calli was increased to 600%, and the pigment production reached to a maximum of 16.3 mg per gram of the wet biomass in a period of a subculture (21 days). Parallel to these experiments, the antimicrobial activity of shikalkin pigment was examined on some fungi and Gram-positive and Gram-negative bacteria. Results indicated that the pigment was almost ineffective on fungi and Gram-negative bacteria, but it was meaningfully effective against *Micrococcus luteus*.

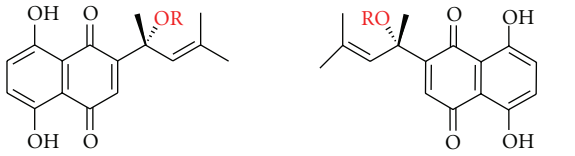
1. Introduction

The colorful mixture of shikonin and alkannin, Figure 1, is called shikalkin [1]. These compounds are 1,4-naphthoquinone derivatives usually found alongside with some of their corresponding esters, in different ratios, in the roots of some plants of *Boraginacea* family such as *Lithospermum erythrorhizon*, *Arnebia euchroma,* and *Alkanna tinctoria* [2]. Shikalkin is famous because of its natural red-purple color and medicinal properties. It was being used for dying foods and fabrics as well as curing burnt skin and ulcers in traditional medicine of East Asia [2–4]. Modern medicinal studies also support its old-known uses and promise additional applications in combats against cancers, HIV and risky microorganisms [5].

While there is an increasing demand for shikalkin in pharmaceutical, food, and cosmetic industries, a synthetic procedure for mass production of any of the enantiomers has remained impracticable [6]. In contrast, plant biotechnology methods based on cell and tissue culture of *L. erythrorhizon* root earned success in early 1980s [7]. Expectedly, the costs of the production and geographical limitation of *L. erythrorhizon* encouraged scientists to search for other accessible and more capable shikalkin producing cell lines among the other members of *Boraginacea* family [2]. Scientific reports indicate that *A. euchroma*, a perennial herbaceous plant, could be a valuable competitor for *L. erythrorhizon* [8, 9]. In addition to shikalkin derivatives, pigment extracted from the *A. euchroma* root contains some novel substances which seem important from both biodiversity and biological points of views [10, 11]. This paper presents results of a five-year study on the solid culture of the callus obtained from the Iranian *A. euchroma* root and antimicrobial properties of its pigment.

2. Materials and Methods

2.1. Plant Samples and Chemicals. A. euchroma specimens were collected and determined as described earlier [12]. Thiamine, inositol, 2,4-Dichlorophenoxyacetic acid (2,4-D), 3-indoleacetic acid (IAA), and N-(2-furanylmethyl)-1H-Purin-6-amine (kinetin) were purchased from Sigma-Aldrich Company. Other chemicals used in this work were



S-enantiomer
R = −H = alkanin

R-enantiomer
R = −H = shikonin

R = −H = shikalkin =
R and S- 5, 8-dihydroxy-2-(1′- hydroxy-4′-methylpent-3′-enyl)
-1, 4-naphthaquinone

Figure 1: Chemical structures of shikonin and alkanin.

taken from the authentic samples. Murashige-Skoog (MS), Linsmaier-Skoog (LS), White, SH and M9 media were prepared according to the literature [13]. Shikalkin pigment was extracted by THF from the powder of the dried *A. euchroma* roots and calli. The extracts were dried in the dark under a moderate vacuum. The dried pigment was weighed and identified according to the reported procedure [12].

2.2. Seed Germination and Callus Formation. *A. euchroma* seeds were sterilized by the conventional method using NaOCl solution [13]. The sterilized seeds were sprouted on hormone-free MS medium from one to eight weeks. Callus induction was examined on two groups of explants at two different ages (10 and 20 days old) excised from the young plantlets (Figure 2(b)). The explants were put on LS medium containing 3% sucrose. Then, callus induction was examined in the absence and presence of light and phytohormones. Each group had explants, of different parts of the plantlets, in two different sizes (2–4 and 4–6 mm). Based on the literature [7, 8, 14–16], the culture medium of those samples which had to be studied in the presence of phytohormones was supplemented with 2,4-D (10^{-6} M) and kinetin (10^{-5} M). The resulting callus cells were primarily transferred on solid LS medium containing sucrose 3%, 2,4-D 10^{-6} M, and kinetin 10^{-5} M and solidified with 0.8% agar. The fast growing cell-lines were subcultured every three weeks.

2.3. Induction of Shikalkin Production. The induction was successfully carried out via transferring the *A. euchroma* calli onto White medium supplemented by sucrose (25 g/L), IAA (10^{-6} M), and kinetin (10^{-5} M) and maintained under darkness at 25°C. Modifications to the propagation and the pigment production media were made according to the results presented in this paper.

2.4. Antimicrobial Tests. All the species showed in Table 2 were obtained from the Bacteriology Department of Shahid Beheshti Pharmaceutical Faculty. Two methods of antibiogram disks and growth inhibition in suspension culture were used in these experiments. The antibiogram tests were carried out by impregnating the paper disks in THF solutions containing various amounts, in a range of 50–500 μg/mL, of the pigment. The THF solvent was considered as the blank and clotrimazole and ciprofloxacin at different concentrations of 0.1–0.5 mg/mL were used as the standards.

The suspension cultures were carried out in 50 mL flasks containing various amounts of the THF extract in a range of 50–300 μg/mL. The growth rate of the desired bacteria was measured by reading the optical density of the diluted (1/20) cultures at 600 nm. The growth of the desired species in the liquid culture in the absence of THF and THF extract was considered as 100% in inhibition calculations. Solid and liquid Mueller-Hinton media were used for the antibiogram and suspension cultures, respectively. All of the tests were assayed after 24 h incubation under darkness at 25°C.

2.5. Statistical Considerations. Results are average of, at least, three replicates for each treatment. Data were analyzed by Microsoft Excell 2003, and P values less than .05 were regarded significant.

3. Results and Discussion

3.1. Description of the Plant and Results of Chemical Analysis. Two species of *A. euchroma*, Grandis and Ordinary, have been reported from Iran [17]. They are found at Dena altitudes in the central Zagross and Shah mountains in Kerman district. The dark red root of the Ordinary species, locally named Ho'e-Cho'e, grows up to 2 cm in diameter and exceeds 40 cm in length. Its acicular leaves hardly grow longer than 10 cm. It usually blooms in mid-June and its petals are dark-purple with yellow limb apex, Figure 2(a). In contrast, the Grandis species has thinner and shorter root in bright red color. Its leaves are softer and exceed 15 cm in length. This one blooms in early June and gives yellow flowers. However, the flowers structures of both species are similar.

There are two more discernible points about Ho'e-Cho'e species. It is only found at altitudes higher than 3300 m and grows beside stones. It seems that its root has penetrated into the stones, but Grandis *A. euchroma* is found at lower altitudes on sandy soil. Yet, none of them is seen in shadow and locals use the former for flourishing their foods and healing wounds. Therefore, this species was selected for this research. Chemical analysis showed that the dried root of the Iranian *A. euchroma* contained about 8.5% (w/w), in average, shikalkin pigment (Table 1).

3.2. Seedling and Establishing Callus Formation. Germination of 30 sterilized *A. euchroma* seeds on hormone-free MS medium was studied in a four-month plan. The first and the last seeds germinated on the 7th and 54th day of the experiment, respectively, and about 30% of the seeds never germinated. The observations indicated that despite the rough coat of *A. euchroma* seed, the conventional sterilizing method was efficient enough, because only one of the sprouted seeds showed fungal contamination. The observations also proved that there is no need to apply sophisticated methods for germination of *A. euchroma* seeds. Yet, it is possible to raise the germination possibility close to 100% by exposing seeds to current water (data is not showed here); however, keeping the sprouted seeds clean under this condition is cumbersome.

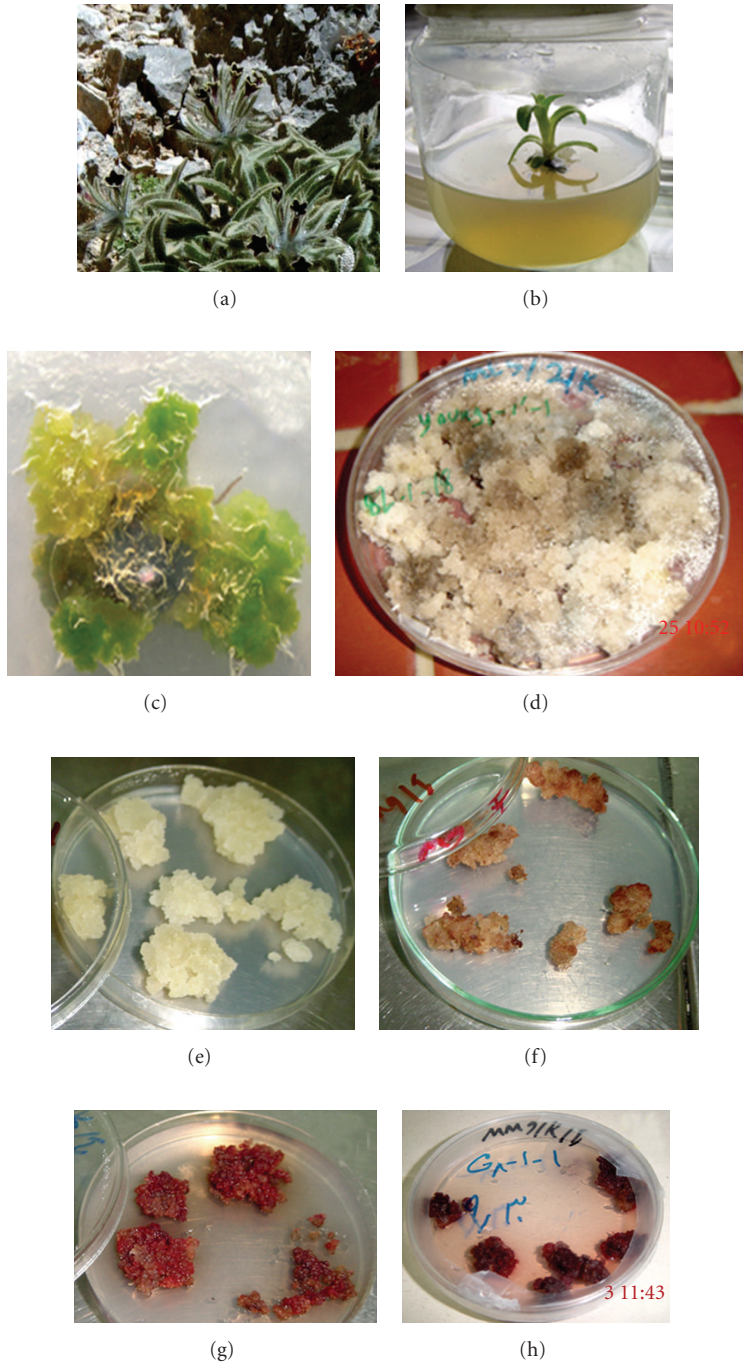


Figure 2: (a) *A. euchroma* found in central Zagross altitudes locally called Ho'e-Cho'e, (b) A young *A. euchroma* plantlet on hormone-free MS medium, (c) *A. euchroma* callus subcultured on LS medium containing 2,4-D in the light, (d) *A. euchroma* callus subcultured in the dark on a modified LS medium ($[NH_4NO_3] = 16.5$, $[KNO_3] = 1900$ mg/L) containing 2,4-D, (e) *A. euchroma* callus subcultured in the dark on mLS medium ($[NH_4NO_3] = 0$, $[KNO_3] = 2850$) containing 2,4-D, (f) *A. euchroma* callus subcultured in the dark on LS medium containing IAA, (g) *A. euchroma* callus subcultured in the dark on White medium containing IAA, (h) *A. euchroma* callus subcultured in the dark on mM9 medium containing IAA. The auxin concentration in experiments 2C-2H was adjusted to 10^{-6} M and the corresponding media contained kinetin (10^{-5} M).

Callus induction can be a bottle neck in plant cell and tissue culture [18]. Nonetheless, running the program introduced in the Materials and Methods section proved it was not bothersome in this case. Results of examining different explants employed in this experiment were strongly in favor of the younger and shorter (10 days old, 2–4 mm in length) collar and root explants which had been maintained in the dark. In fact, some of the explants, which had been

Table 1: The average amount of shikalkin pigment extracted from 5 samples of the natural Iranian *A. euchroma* roots and 5 pieces (taken from 5 different petri dishes) of the *A. euchroma* calli subcultured on mM9 [supplemented by IAA (10^{-6} M), kinetin (10^{-5} M), and sucrose 25 g/L) maintained in the dark at 25°C for 3 weeks.

Dried root (g)	Extraced pigment (g)	Pigment/dried root (%)	Wet callus (g)	Dried callus (g)	Produced pigment (g)	Pigment/dried callus (%)
9.55	0.8116 ± 0.031	8.5	0.2074	0.0156 ± 0.0076	0.0219 ± 0.0103	140.4

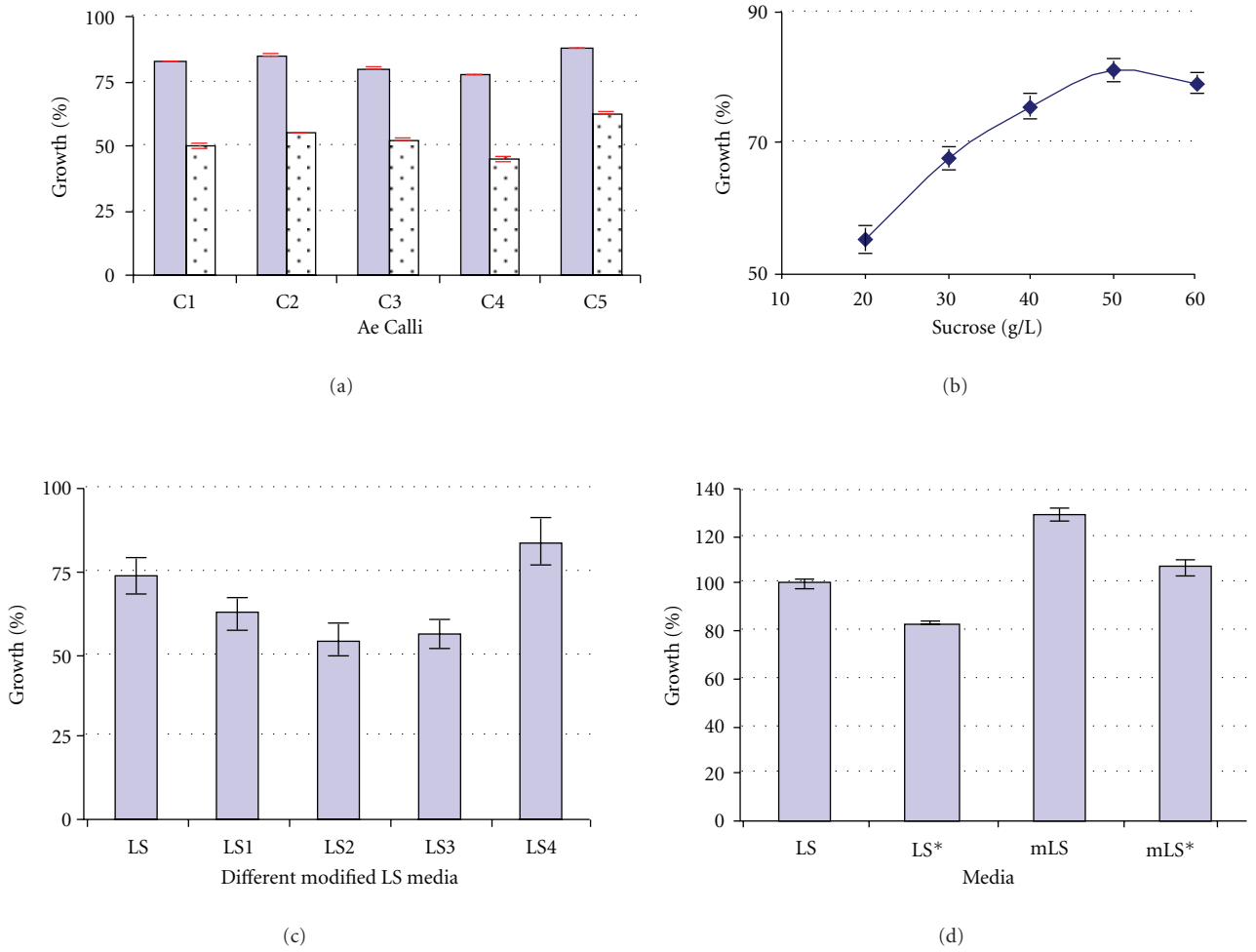


Figure 3: (a) The growth of 5 *A. euchroma* calli obtained from the root explants of 5 different seeds and subculturd on LS medium containing 2,4-D (10^{-6} M), kinetin (10^{-5} M), and sucrose (30 g/L) in the dark (filled bars) and the light (spotted bars). The growth of the *A. euchroma* callus subculturd on: (b) LS medium containing 2,4-D (10^{-6} M), kinetin (10^{-5} M), and different amounts of sucrose in the dark, (c) modified LS media [LS (normal LS), LS1 ($[NH_4NO_3] = 0$, $[KNO_3] = 1900$ mg/L), LS2 ($[NH_4NO_3] = 0$, $[KNO_3] = 950$ mg/L), LS3 ($[NH_4NO_3] = 16.5$, $[KNO_3] = 1900$ mg/L), LS4 ($[NH_4NO_3] = 0$, $[KNO_3] = 2850$ mg/L)] containing 2,4-D (10^{-6} M), kinetin (10^{-5} M) in the dark, (d) LS, mLS and vitamins-free media (LS* and mLS*). Except for the experiments of 3b, all the media applied in the other experiments contained 30 g/L of sucrose.

under the effect of 2,4-D (10^{-6} M) and kinetin (10^{-5} M), started to show callus formation in the dark after 10 days. But all the explants which had been maintained in the light or on phytohormones-free media failed to form callus and gradually started to darken. These results are in parallel to those reported for *L. erythrorhizon* and Chinese *A. euchroma* [8, 14].

3.3. Callus Propagation. Callus propagation rate of *A. euchroma* was optimized through examining the effects of light, carbon source concentration, nitrogen source, vitamins, growth regulators, and the type of the medium culture. Results of these studies are illustrated in Figure 3. It is understood from Figure 3(a) that *A. euchroma* cells which had been propagated in the dark produced about

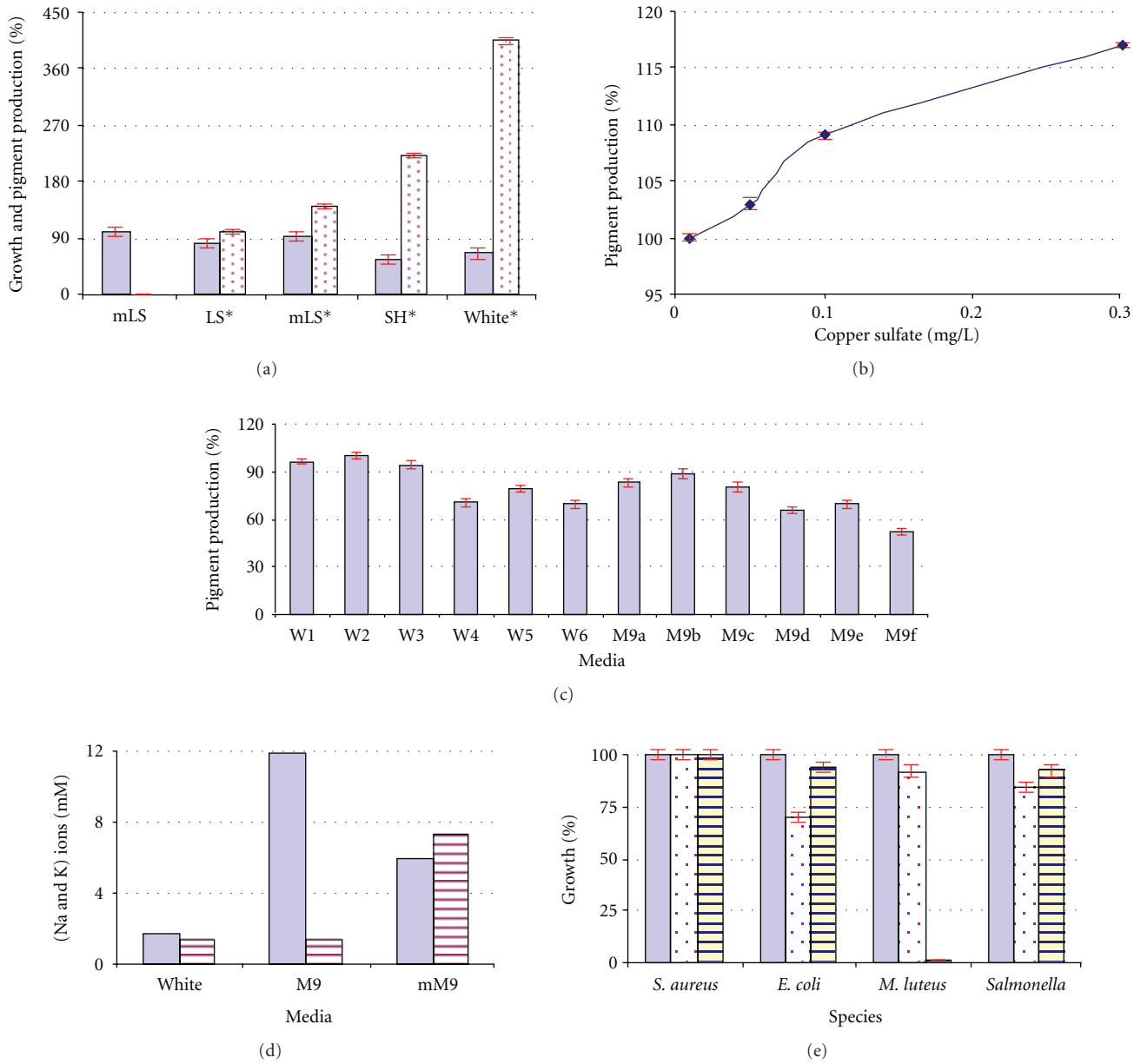


FIGURE 4: (a) The growth (filled bars) and the pigment production (spotted bars) of the *A. euchroma* callus on mLS medium containing 2,4-D (10^{-6} M), kinetin (10^{-5} M), and stared media [LS, mLS, SH and White media containing IAA (10^{-6} M), kinetin (10^{-5} M)] in the dark. The pigment production of the *A. euchroma* callus subcultured (b) on White medium containing IAA (10^{-6} M), kinetin (10^{-5} M), and different amounts of copper sulfate in the dark, (c) in dark on White and M9 media containing [W1&M9a (IAA 10^{-7} M, kinetin 10^{-5} M), W2&M9b (IAA 10^{-6} M, kinetin 10^{-5} M), W3&M9c (IAA 10^{-5} M, kinetin 10^{-5} M), W4&M9d (IAA 10^{-7} M, kinetin 10^{-6} M), W5&M9e (IAA 10^{-6} M, kinetin 10^{-6} M), W6&M9f (IAA 10^{-5} M, kinetin 10^{-6} M)], (d) The amounts of the K^+ and Na^+ ions in White, M9, and mM9 media, (e) The growth of the selected bacteria in the liquid Mueller-Hinton (filled bars) containing THF (spotted bars), 200 μg/mL THF extract (dashed bars). All the media applied in the experiments of 4a-d contained 30 g/L of sucrose.

30%, in average, higher biomass in comparison with those propagated in the light. It seems that light not only inhibits shikalkin production [15, 19], but it also bothers faster growth of *A. euchroma* cells. One reason for this growth decline could be initiation of chlorophyll formation in these cells in the presence of light (Figure 2(c)).

Figure 3(b) shows the positive impact of increasing the sucrose concentration on *A. euchroma* callus propagation. Apparently, sucrose concentrations higher than 50 g/L perturb the right balance of carbon to nitrogen ratio [20] and cause a decline in the growth of *A. euchroma* cell.

Literature indicates that similar to light, ammonium inhibits shikalkin production [8, 21]. In order to examine the effect of this cation on *A. euchroma* callus propagation, the growth rate of these cells was measured on normal LS and some modified LS media. As it is seen in Figure 3(c), the

Table 2: Results of the antimicrobial tests on the THF extract (300 μg/mL) of Iranian *A. euchroma* root. Control efficiency (CE) was calculated according to (%CE = {[(measured diameter of the sample/measured diameter of the blank)] -1} $\times$ 100.

Species	%CE	*P*-value
Candida albicans	8.32	.38
Aspergillus niger	6.25	.353
Helicobacter pylori	0	0
Salmonella tvpiminurim	2.97	.68
Pseudomonas aeruginosa	2.2	.82
Escherichia. coli	12.9	.14
Staphylococcus aureus	8.7	.298
Micrococcus luteus	18.25	.031
Listeria mnocytognesis	14.3	.364
Streptococcus salivarius	5.5	.15
Bacillus cereus	6.1	.131

propagation rate drops to the lowest level when the nitrogen source of LS medium is at the lowest concentration (NH_4^+ is omitted and NO_3^- is halved). Interestingly, keeping the NO_3^- concentration at its normal amount in LS is more efficient when the NH^{4+} is totally omitted in comparison with those containing decreased NH_4^+ concentration (down to 0.01 of its normal level). This result suggests that the imbalanced ratio of NO_3^- to NH_4^+ could play a negative role in *A. euchroma* callus propagation. This idea is more supported by the observations revealing that *A. euchroma* calli grown on such a medium (LS medium with imbalanced ratio of NO_3^- to NH_4^+) gradually browned (Figure 2(d)). However, those cells propagated on the ammonium-free LS medium containing higher (1.5 fold) nitrate remained friable and colorless (Figure 2(e)) and even showed growth rate about 14% higher than those propagated on normal LS. So, this modified medium was nominated mLS and used in next studies.

In the next step, the growth rate of *A. euchroma* calli on normal LS and mLS was studied in the presence and absence of the vitamins (thiamine and inositol) during 4 subcultures in three months. Results in Figure 3(d) indicate that omitting the vitamins has caused about 7% decrease in the growth rates of *A. euchroma* calli subcultured on both LS and mLS media. Therefore, solid mLS medium containing vitamins, sucrose (50 g/L), 2,4-D (10^{-6} M) and kinetin (10^{-5} M) was selected for the propagation of *A. euchroma* callus under darkness at 25°C. It is noteworthy to mention that the grown calli under the mentioned conditions were cream in color, friable, and showed about 6 folds increase in biomass during a subculture period (21 days). Applying treatments containing other combinations of the mentioned phytomormones' concentrations caused vitrification and morphological changes which is not presented and discussed here.

3.4. Pigment Production versus Biomass Growth. Based on cell culture technique, the existing literature suggests a two-step plan for shikalkin production. The aim of the first step is increasing the biomass of the desired cells in known media such as LS and SH in the presence of 2,4-D and kinetin. In the second phase, instead of biomass growth, shikalkin production is pursued. Hence, the cells are transferred into media like White or M9 to start pigment production [22–24]. It is known that similar to light and ammonium cation, 2,4-D inhibits shikalkin production. This is why 2,4-D is substituted by IAA in the second phase of the shikalkin producing cell cultures [7, 8, 21–24]. To evaluate the efficacy of mLS medium (developed in this research), the biomass growth and the pigment production of *A euchroma* callus were investigated on mLS, SH, and White media.

Results in Figure 4(a) supports the idea that 2,4-D inhibits shikalkin production, while IAA induces it. As a matter of fact, substituting 2,4-D by IAA triggered the pigment production even on LS medium which contained $NH4^+$. However, browning was the prominent phenomenon under that conditions (Figure 2(f)). It has been showed that $NH4^+$ does not inhibit directly the shikonin biosynthetic pathway, but it causes a hindrance through accumulation of glutamine in the cells [19].

Results in Figure 4(a) also suggest that the callus propagation rate declines in the presence of IAA regardless of the type of the medium and White medium can be considered as a suitable medium for pigment production. So, further studies on the shikalkin production by *A. euchroma* calli were carried out on White medium.

3.5. Enhancing the Pigment Production. In addition to a starvation shock through decreasing the sucrose concentration to 25 g/L [20, 24], the literature introduces some elicitors for enhancing shikalkin production. The most famous chemical elicitor is copper ion [21–25]. Figure 4(b) confirms that increasing the copper ion concentration has caused enhancement in the shikalkin formation. Comparing the composition of M9 with White reveals the obvious difference in the copper ion concentration of these media (0.3 mg/L in M9 and 0.01 mg/L in White), but experiments in this lab indicated *A. euchroma* callus was able to produce higher amounts of shikalkin on White medium in comparison with M9. Figure 4(c) illustrates the results of shikalkin production by *A. euchroma* callus on White and M9 media under different conditions of the phytohormones. It is understood from these results that the combination of IAA (10^{-6} M) and kinetin (10^{-5} M) which had been selected for *L. erythrorhizon* and Chinese *A. euchroma* is similarly effective on the Iranian *A. euchroma* cell culture, but M9 which had been engineered for the pigment production by *L. erythrorhizon* [22] does not work as efficient as White medium does for these cells (Figure 2(g)).

In order to see what kind of factor(s) influences the efficacy of M9 and White media for the Iranian *A. euchroma* shikalkin production, the compositions of these media were reviewed. In addition to the difference in copper concentrations, it seems that White medium enjoys a kind of balance between the concentrations of potassium and sodium cations, while the sodium concentration is about 9 times higher than the potassium in M9 medium, Figure 4(d). So, the pigment production of *A. euchroma* callus was studied on a modified M9 (mM9) in which the concentrations of sodium sulfate and potassium sulfate were adjusted at 740

and 907 mg/L, respectively. Results of these experiments in comparison with the results of the pigment analysis of the natural root of Iranian *A. euchroma* have been summarized in Table 1. It is evident that *A. euchroma* callus on the solid mM9 has produced shikalkin 16 times higher than the natural root in 21 days (Figure 2(h)). This result is slightly higher than the results reported by other researchers [8, 22, 24, 26].

3.6. Antimicrobial Activities of Shikalkin Extracted from Iranian A. euchroma. Shikalkin is famous because of its healing effect on burnt and damaged skin tissue [27]. Some proposed that shikalkin helps recovery of damaged tissues through its antimicrobial activity. So, there are several reports now on antimicrobial activity of shikonin, alkanin, shikalkin, their different derivatives, and even various extracts of the roots of different plants of the *Boraginacea* family [2, 5, 28]. Although some of these results have been commercialized [29] and some are promising, the review of the literature indicates that there are still serious works to be done to explain the healing effect or selective antimicrobial ability of the shikalkin derivatives.

In addition to the shikalkin derivatives, the *A. euchroma* root contains some novel compounds [10]. This makes the *A. euchroma* root extract more precious to be studied. In this research, the THF-extract of the *A. euchroma* root was applied in the antimicrobial experiments on 2 fungi, 4 Gram-negative and 5 Gram-positive bacteria species showed in Table 2. The highest control efficiency of the extract was obtained on *M. luteus*. More importantly, the results on this Gram-positive bacterium produced the lowest *P* value (.031), while the data on *S. aureus* resulted in a *P* value of .298. These results are interesting when compared with the existing reports on the *A. euchroma*. Sasaki et al. had announced that acetylshikonin obtained from *A. euchroma* could inhibit *Candida albicans* [30]. Two years later, Shen et al. reported that the *A. euchroma* extract had little effect on fungi and Gram-negative bacteria, but it was effective on some Gram-positive bacteria specially *S. aureus* [31]. Comparing this conclusion with the results showed in Table 2, it seems that the THF-extract of Iranian *A. euchroma* has not showed meaningful effect on the fungi and Gram-negative bacteria but has affected a Gram-positive bacterium, *M. luteus*, and not *S. aureus*.

To validate the results of Table 2, the *A. euchroma* extract was applied in the liquid culture of *S. aureus*, *M. luteus*, *E. coli*, and *Salmonella*. It is evident from the results, illustrated in Figure 4(e), that the extract of Iranian *A. euchroma* has almost completely inhibited the *M. luteus* growth, while the *S. aureus* growth has remained unchanged. Considering these results and the previous scientific reports, it can be concluded that the *A. euchroma* root extract has a greater inhibiting potential toward Gram-positive bacteria. However, this potential has to be formulated according to the biodiversity of the plant source and the extraction method.

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