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Enhanced Eugenol Production in *Eryngium campestre* L. and *Eryngium caucasicum* Trautv. via *Agrobacterium rhizogenes*-Induced Hairy Roots

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ABSTRACT

Eryngium, an edible and medicinal plant from the Apiaceae family native to northern Iran, is traditionally used to treat inflammation, hypertension, and diabetes. This study investigated the effect of plant species, explant type and bacterial strain on hairy root induction of *Eryngium campestre* and *Eryngium caucasicum* via *Rhizobium rhizogenes* (formerly: *Agrobacterium rhizogenes*). Leaf, hypocotyl, and cotyledon explants were inoculated with *A. rhizogenes* (A4, ATCC-15834 and R318 strains). The transformation frequency and the number of roots generated per explant were recorded. The transformation of hairy roots was verified through PCR using gene-specific primers for *rolB* and *rolC*. The highest hairy root induction rate (65.8%) was obtained with the ATCC-15834 strain inoculated on the hypocotyls of *E. campestre*. The shortest time for root induction (7.5 days) was also observed in *E. campestre*. A high level of eugenol production in hairy roots induced by strain R318 in *E. caucasicum* shows that this strain has a significant potential to optimize secondary metabolite production in this species. HPLC analysis of the transgenic roots in both *Eryngium* species showed that the resulting hairy roots contained a significant amount of eugenol. By transferring desirable genes to these two plant species, the resulting hairy roots contain a significant amount of eugenol. Based on these results, this technique offers an effective, genetically stable, and sustainable alternative to conventional extraction or chemical synthesis methods for large-scale production of eugenol-rich secondary metabolites.

Keywords: Apiaceae, Medicinal plant, Secondary metabolite, Transgenic, *Rhizobium rhizogenes*

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INTRODUCTION

In recent decades, the utilization of medicinal plants for treating various health diseases has increased tremendously. These plants are rich in secondary metabolites with therapeutic properties that can improve human health [1-3]. *Eryngium* L. is one such medicinal plant, beneficial in treating many diseases. The genus *Eryngium* is the largest genus in the subfamily Saniculoideae and comprises approximately 250 species within the family Apiaceae. This genus is commonly found in North Africa, Central Asia, the Americas, Australia and Europe [4, 5]. Native tribes in the Americas have traditionally utilized different species of *Eryngium* for medicinal purposes, treating issues like digestive problems, toxicity, and body pain. Many of these plants also show significant antioxidant, anti-inflammatory, and blood sugar-lowering effects [6]. In the Mexican region, the genus *Eryngium* is commonly known as "Hierba del sapo" or "Frog grass" with the scientific name *E. carlinae* F.Delaroche. These species are typically distinguished by traditional herbalists (locally referred to as "hierberos") based on their location or state of growth [7]. *Eryngium* is commonly used for treating type 2 diabetes (T2D) and lipid disorders. It is traditionally ingested as an infusion (water extract), with an approximate intake of 20 g per individual (assuming an average body weight of 70 kg) [8].

Eryngium stands out as one of the most studied herbal remedies, with numerous studies demonstrating its medicinal properties, which correspond to the traditional uses documented among indigenous communities with extensive ethnobotanical knowledge [9]. For instance, *E. campestre* L., commonly known as “frog grass” has traditionally been employed in the treatment of heart disease and metabolic disorders such as diabetes. The aerial parts of *Eryngium* are typically used as infusions and have a long-standing history of use in treating diabetes, dyslipidemia, hypertension, and digestive disorders [10]. For example, the hydroethanolic extract of *Eryngium* spp. has been characterized to evaluate its anti-hyperlipidemic effects in rat models induced with diabetes [11]. While the hypolipidemic effects of various *Eryngium* species are established, many studies don’t specify which species were tested, complicating comparisons. However, a literature review highlights the potential of these plants as natural treatments for diabetic dyslipidemia, particularly in combating oxidative damage, insulin resistance, and lipid-related toxicity [12-14]. *Eryngium* species are rich in secondary metabolites, including compounds such as triterpenes, polyphenols, polyacetylenes, and volatile oils, which collectively contribute to their medicinal efficacy. Notably, certain *Eryngium* species contain eugenol as a key bioactive secondary metabolite [15]. Many investigations have demonstrated the significant effect of eugenol in cancer treatment. Research has shown that eugenol can inhibit tumor growth and induce apoptosis in cancer cells, such as those in lung cancer [16, 17]. *E. campestre* and *E. caucasicum* Trautv**.** are the predominant

species of *Eryngium* [18]. Both *E. campestre* and *E. caucasicum* hold considerable economic importance in Iran due to their medicinal uses in treating metabolic and cardiovascular disorders. These species are widely distributed across northern and western regions of Iran and are collected for use in traditional herbal formulations and local pharmaceutical industries [19, 20].

E. campestre is a notable species within the genus *Eryngium* and has been reported to possess a wide range of medicinal properties. However, despite previous studies, many of its biological and pharmacological characteristics remain insufficiently explored and warrant further investigation. This plant has a chromosome number of 2n = 2x = 14 in the diploid state and 2n = 4x = 28 in the tetraploid state. It is usually tetraploid and native to the northern part of Iran. The chromosome number of the *E. caucasicum* species is x = 8 [21].

Bioactive compounds in medicinal plants are derived from primary metabolic products and include substances such as phenolics, alkaloids, and flavonoids. The biosynthesis of these secondary metabolites is more complex than that of primary metabolites, resulting in their accumulation in limited amounts within specific plant tissues [22, 23]. One of the key techniques in plant biotechnology to improve the biosynthesis of bioactive compounds is the induction of hairy roots. This process involves introducing the DNA from Ri plasmids (root-inducing plasmids), containing rooting loci (*rol* genes) such as *rolA*, *rolB*, and *rolC*, into the plant genome. Transformed plants grow faster and also have a greater capacity to produce secondary metabolites. Additionally, this type of culture has demonstrated biochemical stability, as well as genetic and metabolic stability over extended periods. Research has shown that hairy root cultures exhibit increased growth and higher biosynthesis of secondary metabolites compared to non-transgenic plant tissues [24-26]. The natural production of secondary metabolites in *Eryngium* is relatively low; however, the hairy root induction method can significantly increase the biosynthesis of these medicinal compounds [27]. The aim of this research was to demonstrate that optimizing hairy root induction in *Eryngium* species can enable mass production of eugenol, an important compound for the development of natural and herbal medicines with antiviral and anti-inflammatory properties.

MATERIALS AND METHODS

Preparation of Sterile Seedlings

Seeds of two *Eryngium* species, *E. campestre* and *E. caucasicum*, both native to Iran, were obtained from the seed collection maintained by the Department of Genetics and Plant Breeding, Sari Agricultural Sciences and Natural Resources University (SANRU). Seeds were disinfected using 70% ethanol for 1 minute, followed by 0.2% sodium hypochlorite containing a few drops of Tween for 15 minutes. Murashige and Skoog (MS) ½ and MS media, supplemented with 30 g/L sucrose and 7 g/L agar, without hormones were used for seed cultivation [28]. The culture containers were preserved in a plant incubator at 20 °C with a light intensity of approximately 55.5 µmol/m²/s, a 16/8-hour light/dark cycle. On the fifth, seventh, tenth, and twelfth days after germination, hypocotyl, cotyledon, and leaf explants were excised from sterile seedlings using a sterile scalpel and transferred to fresh MS medium for inoculation with different *Agrobacterium* strains to induce hairy roots [29].

Preparation of Bacterial Suspension

Three strains of *Agrobacterium rhizogenes* (A4, ATCC15834, and R318) were obtained from the National Institute of Genetic Engineering and Biotechnology (NIGEB) of Iran. The bacteria were grown in liquid Luria-Bertani (LB) medium supplemented with 50 mg/L rifampicin at 28 °C on a rotary shaker at 120 rpm in the dark for 24 hours. The bacterial suspension was adjusted to an optical density (OD600) of 0.5–1.0 and used for explant inoculation [30]. Rifampicin was used to reduce the risk of contamination from non-target bacterial species present in the culture system [31].

Micropropagation and Hairy Root Induction

Seedlings of *E. campestre* and *E. caucasicum* were sampled on the fifth, seventh, tenth, and twelfth days post-germination. Hypocotyl, cotyledon, and leaf explants were excised from these seedlings for hairy root induction. Explants were first placed on MS medium for 2 hours. Wounds were created on the surfaces of the explants, which were then immersed in a bacterial suspension for 10 minutes. The samples were separated from the bacterial suspension and placed on sterile filter paper to dry. The sections were then placed on ½ strength MS medium for 48 to 72 hours in a growth room maintained at 25±2 °C in darkness. After co-cultivation, the sections were transferred to Half strength MS medium containing 400 mg/L cefotaxime to eliminate bacteria. The developed roots were transferred to liquid MS medium (pH 5.8, adjusted prior to autoclaving) lacking growth regulators and maintained on a rotary shaker at 120 rpm in darkness. Fresh root biomass was transferred to a new medium during subcultures conducted every two weeks. Hairy root biomass (fresh and dry weights) was measured after 30 days. Each hairy root line was subcultured in liquid medium without cefotaxime for one month to promote root growth.

Molecular Confirmation of Induced Hairy roots by *Agrobacterium rhizogenes*

Transformation was confirmed using PCR with specific primers for the *rolB* (forward primer: atggatcccaaattgctattccttccacga and reverse primer: ttaggcttctttcttcaggtttactgcagc, expected amplicon size: 790 bp) and *rolC* (forward primer atggctgaagacgacctgtgtt and reverse primer ttagccgattgcaaacttgcac, expected amplicon size: 550 bp) genes. DNA was isolated from both transgenic hairy root (Hairy roots are the same transgenic roots that result from inoculating the bacteria *Agrobacterium rhizogenes* with parts of sterile seedlings, such as leaves) and non-transgenic root or normal root samples (normal roots develop naturally at the end of the plant without genetic manipulation) using the CTAB extraction method [32]. DNA from normal roots of *Eryngium* (both *E. campestre* and *E. caucasicum*) served as negative controls, while DNA from bacteria served as positive control. Each PCR reaction consisted of sterile double-distilled water, magnesium chloride (2 mM), PCR buffer (1x), dNTP (0.2 mM), Taq polymerase enzyme (1 unit), forward primers *rolB* and *rolC* (50 ng), reverse primers *rolB* and *rolC* (50 ng), and 25 ng of the target DNA and the PCR conditions were as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles consisted of denaturation for 1 min at 94 °C, annealing for 1 min at 56 °C for *rolB* and 59 °C for *rolC*, extension for 1 min at 72 °C, and a further extension step for 10 min at 72 °C. Root induction experiments were conducted in a completely randomized design (CRD) with three replicates (petri dishes). The percentage of hairy root induction, number of hairy roots and number of days until hairy root induction were measured after four weeks. Ten explants (including parts of hypocotyls, cotyledons, and leaf segments from both *E. campestre* and *E. caucasicum*) were cultured in each petri dish. Statistical calculations and analysis of variance were performed using SAS 9.3 software. Mean comparisons were also performed using LSD and Duncan's test at

5% and 1% probability levels. Graphs were also drawn using Excel software. Prior to variance analysis, assumptions of normality and homogeneity of variances were verified. After confirming these assumptions, analysis of variance (ANOVA) was performed on the experimental data.

Measurement of Eugenol Content

A total of 10 mg of hairy root samples of *E. campestre* and *E. caucasicum* was accurately weighed and transferred to a 50 mL volumetric flask. The samples were then diluted to the mark with methanol to prepare the extract solutions for HPLC analysis. Eugenol standard (EUG-99, Arofine Chemical Co., India) solutions were prepared by weighing 10 mg of eugenol and dissolving it in methanol in a 100 mL volumetric flask. Serial dilutions were performed to prepare standard solutions with concentrations of 100, 50, 25, and 12.5 mg/L. The HPLC profiling was done using an Agilent 1260 system equipped with a DAD (Diode Array Detector). The chromatography-based separation was carried out using a C18 column (4.6 × 250 mm). The mobile phase was a mixture of acetonitrile and water (50:50, v/v) with a flow rate of 1 mL/min. The volume injected for both sample and standard solutions was 20 µL. Detection was performed at a wavelength of 280 nm. Chromatograms were compared to those of a standard eugenol solution and control plants [33]. The assessment of results, quantification, and calculation of the area under the curve were performed using KNAUER-Server/ ChromGate Client software (version 3.1.7). A calibration curve was prepared by weighing 10 mg of standard eugenol and dissolving it in methanol to a final volume of 100 mL. The absorbance at 280 nm was used to generate the calibration curve (Supplementary file).

RESULTS AND DISCUSSION

This study focused on the hairy roots of *Eryngium* species and evaluated the presence of medicinal compounds in these roots, highlighting their potential for mass production for medicinal purposes. In this study we investigated the induction and development of hairy roots in different explants, including hypocotyls, cotyledons and leaves, in two species of the medicinal plant *Eryngium*: *E. campestre* and *E. caucasicum*. Three strains of *A. rhizogenes* (ATCC-15834, A4, and R318), which contain genes such as *rolB* and *rolC* gene, were employed for transformation. The PCR method was used to confirm gene transformation efficiency, with amplification of the *rolB* and *rolC* gene fragments verifying the existence of T-DNA in the hairy roots. In contrast, PCR of normal root DNA did not yield any fragments (Fig. 1). The use of different *A. rhizogenes* strains is a key approach for enhancing the production of plant-derived compounds. In this study, all transgenic hairy roots contained *rol* genes, as confirmed by PCR amplification. The *rol* genes of the Ri plasmid in *A. rhizogenes* are important for the induction of hairy roots in different plant species [34-36].

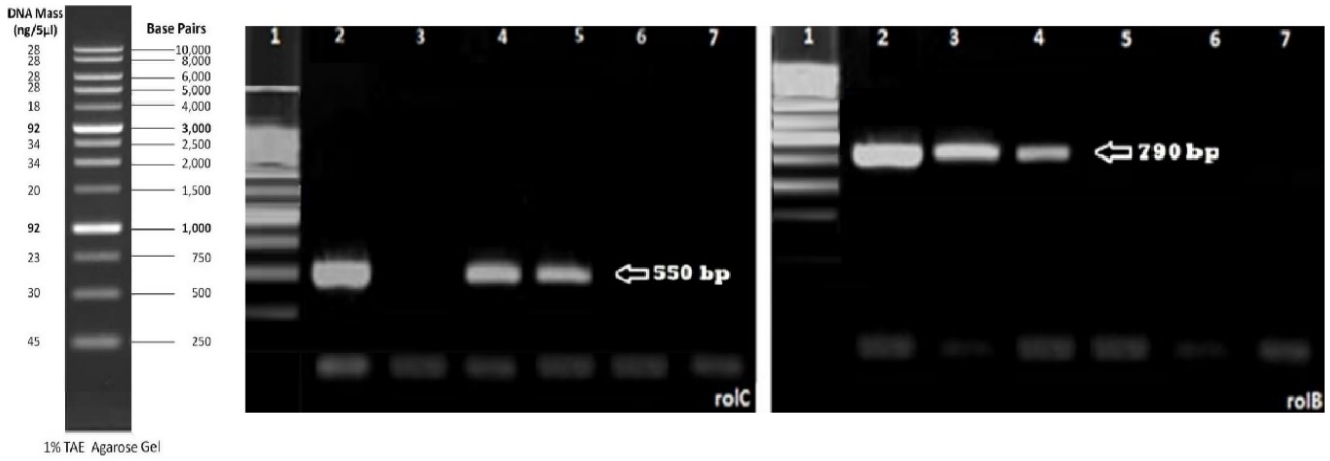

Fig. 1 Agarose gel electrophoresis image of PCR products. *rolC*: column 1, 1 kb marker, column 2, bacterial plasmid (positive control), column 3, distilled water, and columns 4 and 5, hairy roots of transgenics Plant with specific *rolC* primers, columns 6 and 7, non-transgenic *Eryngium* roots. *rolB*: column 1, 1 kb marker, column 2, bacterial plasmid (positive control), column 3 and 4, transgenic *Eryngium* roots with specific *rolB* primers, columns 5 and 6, non-transgenic *Eryngium,* and column 7, distilled water.

Non-transgenic roots emerged more rapidly than hairy roots, typically appearing around 5 days after the terminal part of the plant (The root tip is found at the very end of the root, where new growth occurs) was cultured. Hairy roots, however, developed approximately 15 to 21 days after inoculation at the wound sites intended for gene transfer (Fig. 2a). After transfer to a liquid MS culture medium, no bacterial contamination was observed in cultures without cefotaxime. To compare induction efficiency and growth performance, untransformed root cultures were used as controls. In this medium, the hairy roots exhibited a higher growth rate compared to normal roots and produced more branches with a brighter color and more elegant appearance (Fig. 2b).

To evaluate the impacts of plant genotype, explant type and bacterial strain interaction on hairy root induction, various characteristics of the hairy roots of medicinal plant (*E. campestre* and *E. caucasicum*) were assessed over time. The result of analysis of variance (Table 1) indicates that the effects of plant species and explant type on the number of days required for hairy root induction were significant at the 1% level. However, the effect of bacterial strain type was not significant. Additionally, all two-way and three-way interactions were significant at the 1% level.

Further analysis showed that both the two-way interaction between explant type and bacterial strain, and the three-way interaction, were significant at the 1% probability level. The comparison of means for the three-way interaction indicated that the highest number of hairy roots was obtained from inoculating bacterial strain R318 on the cotyledons of *E.caucasicum*, averaging 6.5 roots. In contrast, the lowest number, averaging two roots, was observed from inoculating bacterial strain R318 on the leaves of *E. campestre* (Fig. 4).

Table 1 Analysis of variance (ANOVA) for hairy root induction percentage, number of hairy roots, and days until hairy root induction in *Eryngium* species.

Sources of variation	Degrees of freedom	Days until hairy root induction	Number of hairy roots	Hairy root induction percentage
Plant species (A)	1	323.60 **	4.97 **	1765.82 **
Explant type (B)	2	22.07 **	1.92 *	576.71 **
A*B	2	35.31 **	0.19 ns	305.00 **
Bacterial strain (C)	2	2.84 ns	0.04 ns	230.11 **
A*C	2	67.55 **	0.21 ns	449.80 **
B*C	4	15.21 **	6.97 ns	120.91 **
A*B*C	4	33.44 **	3.45 ns	76.82 **
Test error	17	2.32	0.51	10.03 **
%CV	-	10.95	17.60	14.14

Analysis of the three-way interaction revealed that the longest duration for hairy root induction was observed with the ATCC bacterial strain inoculated on the leaves of *E. caucasicum*, averaging 25 days. Conversely, the shortest duration, averaging 7.5 days, was observed with the ATCC bacterial strain inoculated on the leaves of *E. campestre* (Fig. 3).

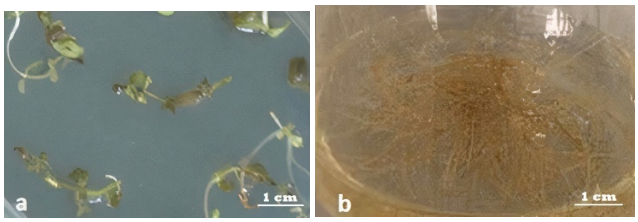

Fig. 2 a) Development of hairy roots from the wound site of leaf tissue. b) Development of hairy root branches in liquid culture medium.

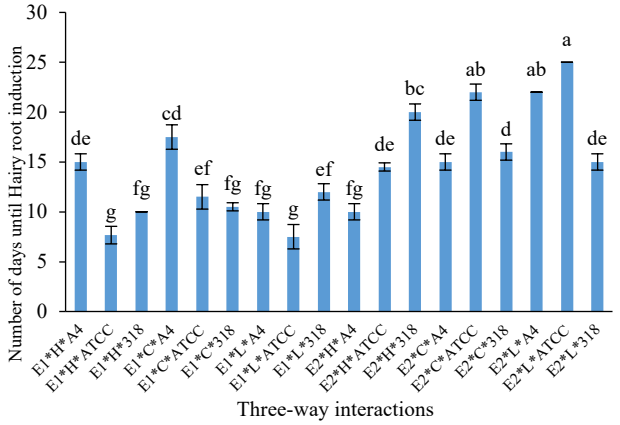

Fig. 3 Comparison of the three-way interaction effects among plant species (*E. campestre*: E1, *E. caucasicum*: E2), explant types (Hypocotyl: H, Leaf: L, Cotyledon: C), and *A. rhizogenes* strains (ATCC, A4, R318) on the number of days to hairy root induction. Error bars represent the standard error of the mean (SEM) calculated from three independent experiments. Similar lowercase letters above columns denote no significant differences between treatments (Duncan's test, $p < 0.05$).

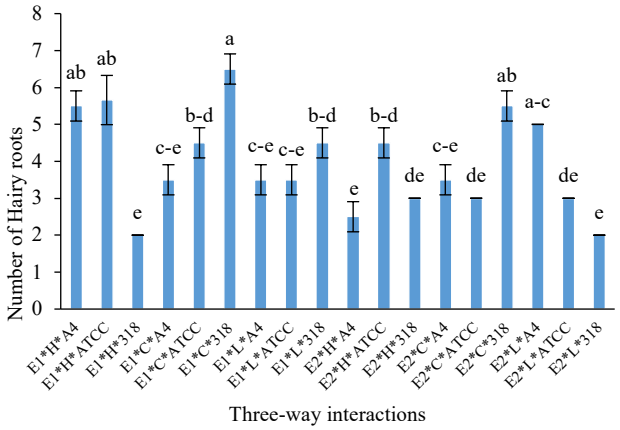

Fig. 4 Comparison of the three-way interaction effects among plant species (*E. campestre*: E1, *E. caucasicum*: E2), explant types (Hypocotyl: H, Leaf: L, Cotyledon: C), and *Agrobacterium rhizogenes* strains (ATCC, A4, R318) on the number of hairy roots in *Eryngium* species. Error bars represent the standard error of the mean (SEM) calculated from three independent experiments. Similar lowercase letters above columns denote no significant differences between treatments (Duncan's test, $p < 0.05$).

Also, ANOVA (Table 1) for percentage of root induction revealed that all main effects -plant species, explant type, and bacterial strain- were significant at the 1% level. Additionally, all two-way interactions (A*B, A*C, B*C) and the three-way interaction were also significant at the 1% level. These results suggest significant differences between plant species in hairy root induction percentage, potentially due to differential expression of *rol* genes in each species [37]. The mean comparison test for the three-way interaction showed that the maximum rate of hairy root induction, 65.8%, was obtained with the ATCC bacterial strain inoculated on the hypocotyls of *E. campestre*. The lowest percentage, 10%, was observed with the ATCC bacterial strain inoculated on the leaves of *E. caucasicum* (Fig. 5).

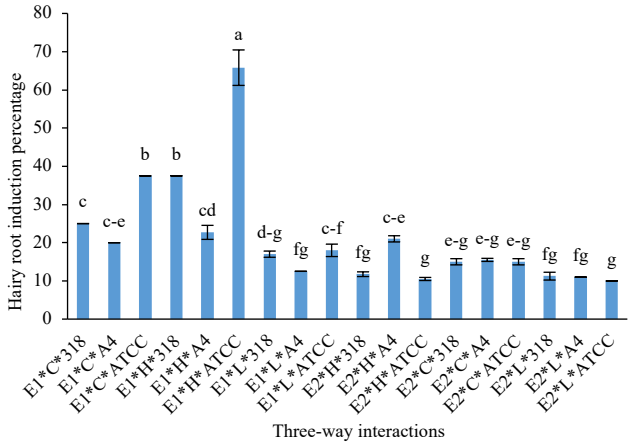

Fig. 5 Comparison of the three-way interaction effects among plant species (*E.campestre*: E1, *E. caucasicum*: E2), explant types (Hypocotyl: H, Leaf: L, Cotyledon: C), and *A. rhizogenes* strains (ATCC, A4, R318) on the percentage of hairy root induction. Error bars represent the standard error of the mean (SEM) calculated from three independent experiments (n=10 per group). Similar lowercase letters above columns denote no significant differences between treatments (Duncan's test, $p < 0.05$).

HPLC analysis was performed to determine the eugenol content in the extracts of normal and hairy roots of two *Eryngium* species. The results showed that the eugenol content was significantly higher in hairy roots compared to normal roots in both species (Table 2).

Table 2 Eugenol content of normal and hairy roots extracts of two *Eryngium* spp. using HPLC analysis

Samples	Conc. (mg/L)	Conc. (% w/w)
E. caucasicum -hairy roots	47.8724	23.9362
E. campestre -hairy roots	129.9312	64.9656
E. campestre -normal roots	2.236	1.118
E. caucasicum- normal roots	0.068	0.034

The highest eugenol concentration was observed in the hairy roots of *E. campestre* (Fig. 6), with 129.93 mg/L (equivalent to 64.97% w/w), while the hairy roots of *E. caucasicum* contained 47.87 mg/L (equivalent to 23.94% w/w). In contrast, the eugenol content in the normal roots of both species was considerably lower, with only 2.24 mg/L (equivalent to 1.12% w/w) in *E. campestre* and a negligible amount of 0.068 mg/L (equivalent to 0.034% w/w) in *E. caucasicum*. These findings indicate that hairy root induction significantly enhances eugenol production in both species and can be considered an efficient strategy for improving the production of secondary metabolites in medicinal plants. The higher eugenol content observed in hairy roots compared to normal roots may result from the expression of *rol* genes transferred by *A. rhizogenes*, which enhance phenylpropanoid metabolism and stimulate the accumulation of phenolic compounds such as eugenol [38, 39].

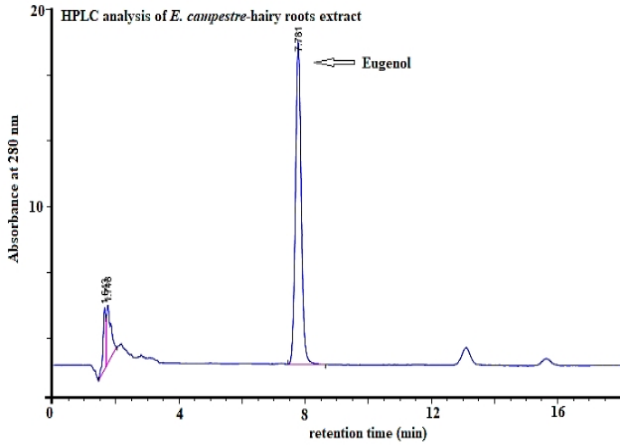

Fig. 6 HPLC profile, the presence of the active substance eugenol in Hairy root of *E. campestre*.

Variations in secondary metabolite yield in hairy roots induced by different strains of *A. rhizogenes* have been highlighted in numerous publications [29, 40-42], demonstrating that hairy roots induction is strain-dependent [34]. Differences in bacterial plasmids are considered a primary reason for variations in bacterial pathogenicity, unrelated to the taxonomic relationship between the plant host and bacterial strain [43]. However, in the past, literature has also described the A4 strain as producing hairy roots on other herbal plants such as *Aesculus hippocastanum* [44].

CONCLUSION

This study reports the first successful induction of hairy roots in *Eryngium* species using *A. rhizogenes*. Among the tested strains, ATCC-15834 achieved the highest hairy root induction rate (65.8%) in *E. campestre*, while the shortest induction time (7.5 days) was also observed in this species. Additionally, the R318 strain significantly enhanced eugenol accumulation in *E. caucasicum*, confirming its strong potential for secondary metabolite optimization. Overall, hairy root induction markedly increased eugenol production compared with natural roots, highlighting its promise as a biotechnological strategy for producing high-value medicinal compounds. Future research should focus on scaling up hairy root cultures for industrial eugenol production and exploring other pharmacologically active metabolites in *Eryngium* species.

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Authors' Contributions

Z.K. conducted the experiments, assessed and interpreted all data, and drafted the initial manuscript. S.A.S.N., and A.I.D. assisted with interpreting the results and provided discussions. S.A.S.N. also designed the study, interpreted the results, and revised the manuscript. M.S.H. assisted with rewriting, revising and submitting the manuscript and provided final review and approval for publishing.

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Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare no competing interests.

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