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Research Article

Separation Of Endophytic Microorganisms from Licorice Plants (Glycyrrhiza Glabra L), Analysis and Characteristics of Their Biologically Active Substances

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Abstract

Endophytic microorganisms play an important role in the growth, stability and metabolism of plants, including medicinal species. The aim of this study was to isolate and identify strains of endophytic bacteria and fungi associated with licorice plants (Glycyrrhiza glabra and Glycyrrhiza uralensis) collected from different natural conditions. A total of 142 isolates were isolated, including 92 bacterial and 50 fungal. Their morphological and molecular identification was carried out, as well as an assessment of antagonistic activity against phytopathogens (Fusarium oxysporum, Alternaria alternata). The results show a high biodiversity of licorice endophytic microflora and confirm its potential use in biotechnology and agriculture.

Key Words: endophytes; licorice; Glycyrrhiza; microbiota; biocontrol; antagonism; biotechnology

1.Introduction

"The process of isolating endophytic microorganisms from licorice roots (Glycyrrhiza glabra L.) and examining their biologically active compounds involves multiple steps. These include the aseptic collection of roots, surface sterilization, tissue homogenization, cultivation on nutrient media, purification of microbial cultures, their identification, and the evaluation of both the microorganisms and their metabolites for biological activity [1-5].

Licorice (Glycyrrhiza spp.) is one of the most important medicinal plants traditionally used in medicine and the pharmaceutical industry. The main pharmacologically active substances (e.g. glycyrrhizin) are produced largely in the root system, where intensive interactions between the plant and the microbiota occur.

The genus consists of nearly 20 species, with some examples being:

- Glycyrrhiza acanthocarpa (Lindl.) J.M. Black
- Glycyrrhiza anthocarpa (Lindl.) J.M. Black
- Glycyrrhiza aspera Pall., commonly known as Rough Licorice
- Glycyrrhiza astragalina Hook. & Arn.
- Glycyrrhiza bucharica Regel, or Bukhara Licorice
- Glycyrrhiza echinata L., also called Licorice Bristlecone
- Glycyrrhiza eglandulosa X.Y. Li
- Glycyrrhiza eurycarpa P.C. Li
- Glycyrrhiza foetida Desf.
- Glycyrrhiza foetidissima Tausch
- Glycyrrhiza glabra L., widely known as Licorice or Naked Licorice
- Glycyrrhiza gontscharovii Maslenn., or Goncharov's Licorice
- Glycyrrhiza iconica Hub.-Mor.
- Glycyrrhiza inflata Batalin
- Glycyrrhiza korshinskyi Grig., also known as Korzhinsky's Licorice
- Glycyrrhiza lepidota Pursh
- Glycyrrhiza macedonica Boiss. & Orphanides, or Macedonian Licorice

- Glycyrrhiza pallidiflora Maxim.
- Glycyrrhiza squamulosa Franch.
- Glycyrrhiza triphylla Fisch. & C.A. Mey., commonly referred to as Trifoliate Licorice
- Glycyrrhiza uralensis Fisch. ex DC., known as Ural Licorice

Over the past five years, a series of reforms have laid the political, legal, socio-economic, and scientific-educational foundations essential for building the New Uzbekistan. As part of the state program for implementing the Development Strategy of New Uzbekistan—designated as the "Year of Human Dignity and Active Mahalla" for the period 2022–2026—the following objective has been outlined: the creation of a program to regulate the production of 17 types of compounds and biologically active substances derived from the processing of medicinal plants. In particular, to establish the production of biologically active substances based on the processing of 83.8 tons of medicinal plant extracts (pomegranate peel, licorice root, chamomile fruits, grape seeds and skins) per year" [1]. Also, the tasks have been set for growing licorice root, modernizing production facilities, organizing the production of import-substituting and export-oriented products, rational use of natural resources, attracting investments from local and foreign companies for growing and increasing industrial processing of licorice root, organizing the production of export products with high added value, creating industrial plantations of up to 3970 hectares in the Republic of Karakalpakstan, 700 hectares in the Khorezm region by 2022. As well as harvesting licorice root up to 30,966 tons in the Republic of Karakalpakstan, and 5600 tons in the Khorezm region by 2026 [2]. Accordingly, it is important to use efficient and energy-saving technology to obtain highly concentrated biologically active substances from licorice root based on compressed CO2 gas at high critical pressures that meet international requirements. This dissertation research contributes, to some extent, to the implementation of the objectives outlined in various decrees, resolutions, and regulatory documents adopted in this field—such as the Presidential Resolution of the Republic of Uzbekistan No. PP-2492 dated April 12, 2016, "On measures to further improve the management structure of the food industry," and the Cabinet of Ministers Resolution No. 63 dated January 27, 2018, "On measures to further develop the production and industrial processing of licorice and other medicinal plants in the Republic of Uzbekistan."

In Russia there are 7 species, mainly in the steppe, semi-desert and desert zones. The most common are Naked Licorice (Glycyrrhiza glabra) - in the south of the European part, the Caucasus and Ural Licorice (Glycyrrhiza uralensis) - in the south of the Urals and Western Siberia.

Endophytes are microorganisms that colonize internal plant tissues without causing disease symptoms. They can stimulate plant growth, increase resistance to abiotic stresses and pathogens, and influence the metabolism of secondary compounds. In recent years, there has been increasing interest in the study of endophytic bacteria and fungi associated with medicinal plants, including the possibility of using them as biocontrol agents or biostimulants. However, despite the widespread use of licorice, its endophytic microbiota remains poorly understood. Therefore, the aims of this study are:

(1) isolation and identification of endophytic microorganisms from various licorice organs;

(2) evaluation of their antagonistic activity against phytopathogens.

2. Materials and methods

2.1. Collection and preparation of samples

Glycyrrhiza glabra and Glycyrrhiza uralensis plant samples were collected in the natural conditions of the southern part of Khorezm and Karakalpakstan in June–July 2024. At least 5 individual plants were selected from each point. The selected samples were washed with running water, the roots, stems and leaves were separated.

2.2. Sterilization and isolation of endophytes

Surface sterilization was carried out according to the standard method:

– 70% ethanol – 1 minute,

– 2% sodium hypochlorite – 3 minutes,

– three-fold rinsing with sterile distilled water.

Sterility control was carried out by applying the last wash water to the nutrient media. Then, the sterile fragments were crushed in a mortar with the addition of sterile saline.

The suspension was sown on:

nutrient agar (NA),

P2A medium – for bacteria;

potato dextrose agar (PDA) – for fungi.

Incubation was carried out at a temperature of 28 °C for 7-14 days.

A study of the component composition of aqueous-alcoholic extracts of naked licorice and Ural licorice herb was carried out using the HPLC method with a detection wavelength of 290 nm. During the qualitative analysis using HPLC, the extracts were examined with the help of standard reference compounds: liquiritigenin (1), genistein (2), pinocembrin (3), and isoglabranin (4). A 2 µl sample volume was used for both the extracts of naked licorice and Ural licorice herb, as well as for the standard solutions of the mentioned flavonoids [3–8]. According to the results of the chromatographic analysis conducted in gradient mode (Table 10), it was determined that diagnostically significant flavonoids such as pinocembrin, liquiritigenin, genistein, and isoglabranin could be identified in the licorice herb extract (Figure 1).

Time, min	Area of fraction A, %	Area of fraction B, %
0-5	30	70
5-15	50	50
15-20	60	40
20-25	80	20

Table 1: Gradient elution profile for chromatographic analysis

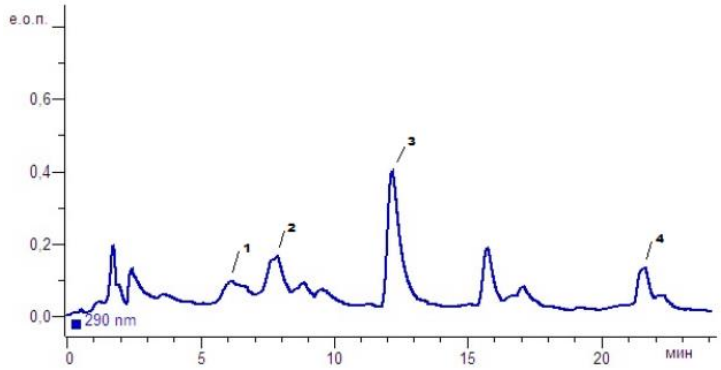

Figure 1: HPLC chromatogram of licorice herb extract: 1 – liquiritigenin; 2 – genistein; 3 – pinocembrin; 4 – isoglabranin.

Flavonoid	Retention time on chromatogram, min	
	Standard sample	**Separation**
Liquiritigenin (peak 1)	6,57	6,52
Genistein (peak 2)	7,43	7,64
Pinocembrin (peak 3)	12,22	12,34
Isoglabranin (peak 4)	21,60	21,54

Table 2: Comparison of retention times between extract peaks and standard reference compounds

The addition of a standard pinocembrin solution to the licorice herb extract resulted in an increased intensity of the pinocembrin peak, confirming the presence of this compound in the sample (Figure 2).

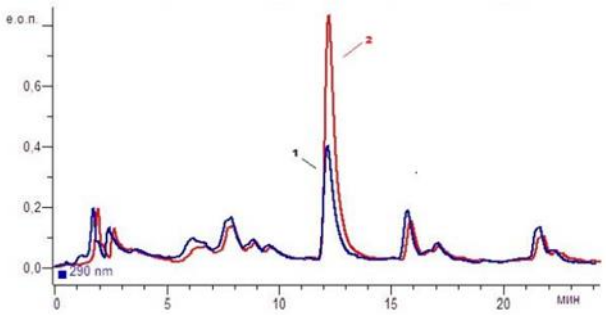

Figure 2: HPLC chromatogram of naked licorice herb extract before and after the addition of pinocembrin: 1 – original naked licorice herb extract; 2 – naked licorice herb extract with added pinocembrin.

A comparison between the chromatograms of naked licorice and Ural licorice herb extracts [5–12] reveals differences in their component composition (Figure 3).

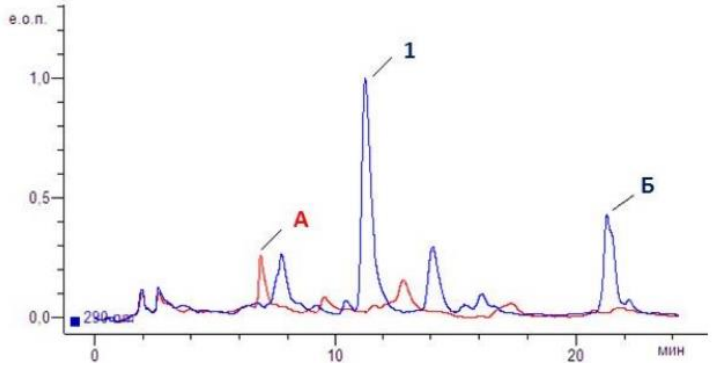

Figure 3: Comparative HPLC chromatogram of the extract of naked licorice and Ural licorice herbs: A – Ural licorice; B – naked licorice; 1 – pinocembrin SS.

The comparative HPLC analysis revealed distinct differences in the component profiles of naked licorice and Ural licorice herbs. Notably, pinocembrin was identified as a key diagnostic flavonoid specific to naked licorice herbs.

2.3. Identification of isolates

Primary identification was carried out by morphological features (shape, color, colony structure). Then, DNA extraction and PCR amplification were carried out:

• for bacteria – the 16S rRNA gene;

• for fungi – the ITS region.

Sequencing and comparison with the NCBI BLAST database allowed us to determine the genus and species affiliation of the isolates [13].

2.4. Evaluation of antagonistic activity

Antagonistic activity was determined in a double culture with phytopathogens Fusarium oxysporum and Alternaria alternata. The diameter of the pathogen growth inhibition zone was measured after 5–7 days of incubation.

3. Results

3.1. Total number of isolates

A total of 142 endophytic isolates were isolated from 60 plant samples:

• bacteria – 92 strains,

• fungi – 50 strains.

The frequency of endophyte isolation was higher in the roots than in the above-ground parts.

It was observed that adding aluminum chloride to the test solution, as well as to pinocembrin and pinostrobin solutions, caused a bathochromic shift in the long-wavelength absorption spectrum. Additionally, differential spectrophotometry showed that the absorption maxima of pinostrobin and pinocembrin solutions overlap at 310 nm in the short-wavelength region (Figures 7 and 8), while their maxima differ in the long-wavelength region. This finding supports the recommendation of 310 nm as the analytical wavelength. When using pinostrobin CO, the total flavonoid content is recalculated for pinocembrin by applying a conversion coefficient in the formula [14–15]. If pinostrobin CO is unavailable, the experimentally determined theoretical specific absorption value of pinostrobin is used instead.

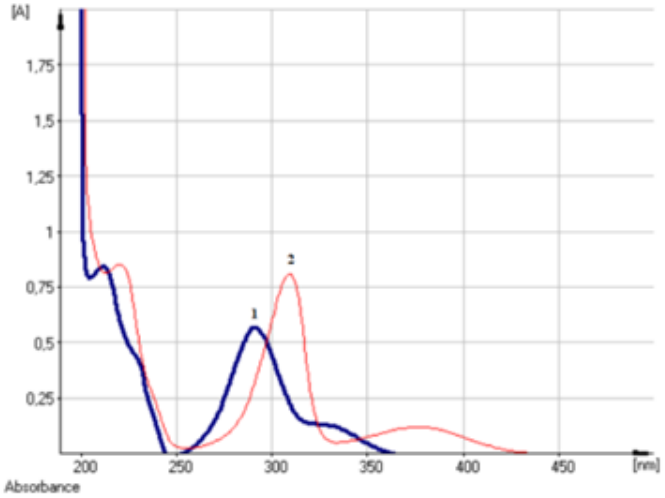

Figure 4 : Electronic spectra of pinocembrin solution Designations: 1 – pinocembrin solution (direct spectrophotometry); 2 – pinocembrin solution with the addition of aluminum chloride

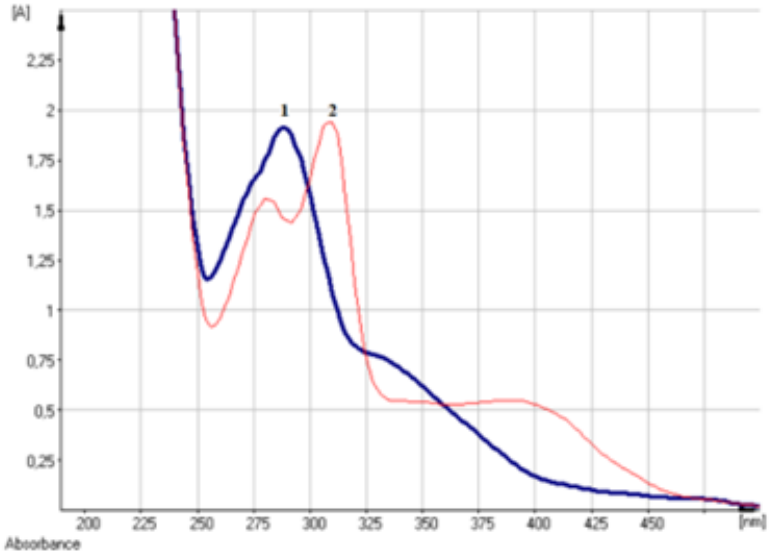

Figure 5: Electronic spectra of solutions of water-alcohol extract from licorice grass Designations: 1 – extract solution (direct spectrophotometry); 2 – extract solution with the addition of aluminum chloride

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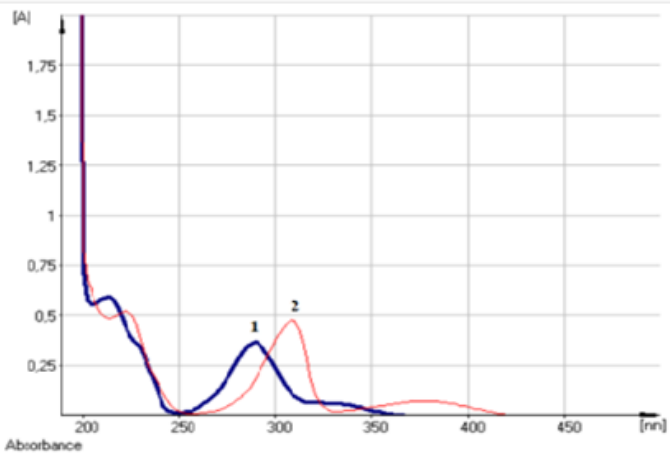

Figure 6: Electronic spectra of pinostrobin solution Designations: 1 – pinostrobin solution (direct spectrophotometry); 2 – pinostrobin solution with the addition of aluminum chloride

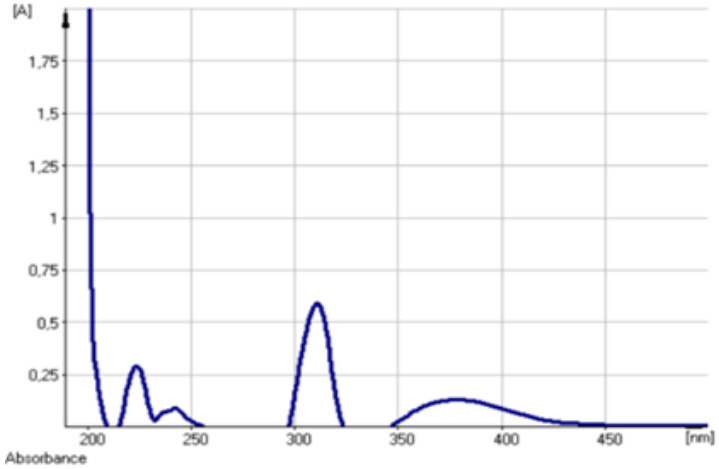

Figure 7: Electronic spectrum of pinocembrin solution (differential spectrum)

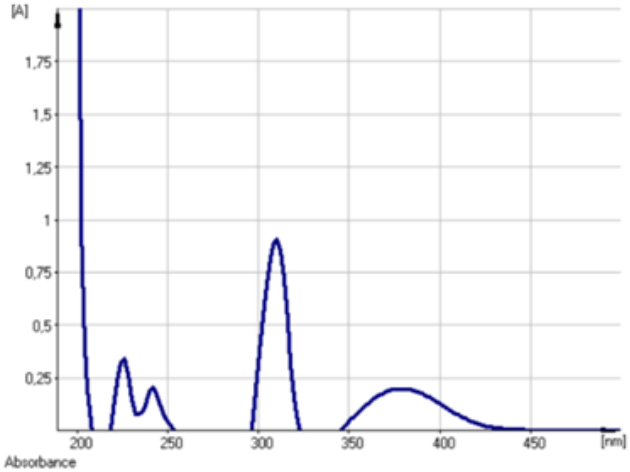

Figure 8: Electronic spectrum of pinostrobin solution (differential spectrum).

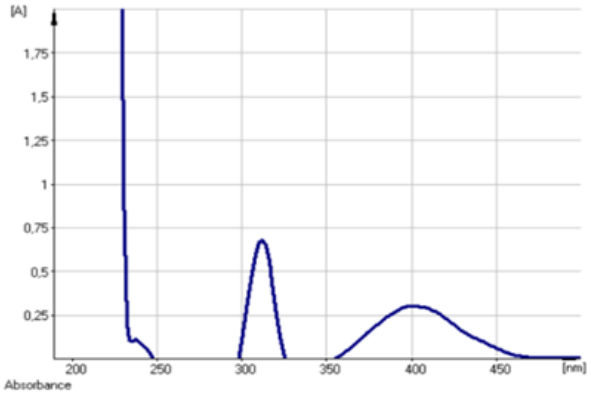

Figure 9: Electronic spectrum of a solution of water-alcohol extract from licorice grass (differential spectrum)

Experimental results showed that the most efficient extraction of flavonoids from naked licorice herb is achieved using 90% ethyl alcohol. The subsequent experiment identified the optimal raw material-to-extractant ratio as 1:50. Extraction time was then optimized, with maximum flavonoid extraction occurring within 60 minutes [23]. Finally, the ideal particle size for thorough flavonoid extraction was determined to be 2 mm. The relationship between optical density and pinostrobin concentration was represented by a linear regression curve over the concentration range of 0.016 to 0.16 mg/ml (0.016; 0.032; 0.08; 0.16) using aluminum chloride at a wavelength of 310 nm (Figure 1).

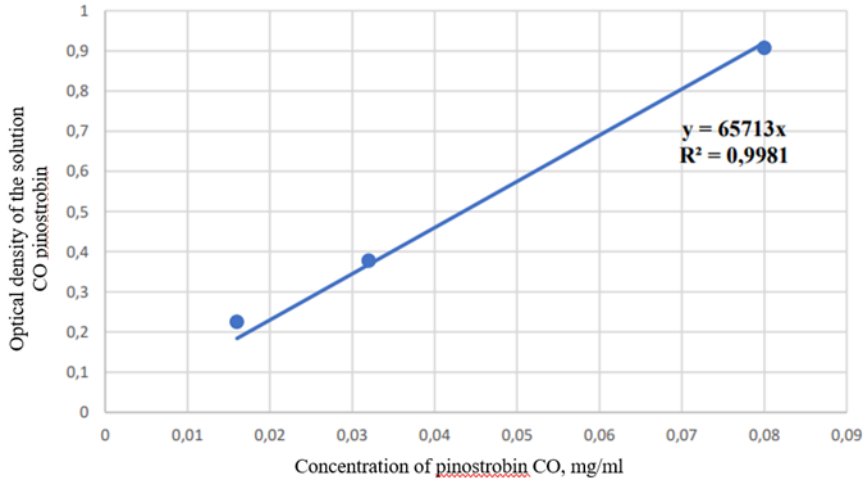

Figure 10: Dependence of the optical density values of the pinostrobin solution with aluminum chloride on the concentration of pinostrobin (differential variant)

3.2. Molecular identification

The most common bacterial genera were:

• Bacillus subtilis,

• Bacillus amyloliquefaciens,

• Pseudomonas fluorescens,

• Streptomyces spp.

The most common fungi were:

• Trichoderma harzianum,

• Penicillium spp.,

• Aspergillus niger.

3.3. Antagonistic activity

Seven isolates showed high antagonistic activity (>15 mm inhibition zone):

• B. amyloliquefaciens Gg-27 suppressed the growth of both test pathogens,

• T. harzianum Gu-11 effectively inhibited F. oxysporum.

4. Discussion

The obtained data demonstrate a significant biodiversity of the licorice endophytic microbiota, especially in the root system. The prevalence of Bacillus and Trichoderma strains correlates with their known ability to produce antibiotic substances and stimulate plant growth [16-22]. The results are consistent with the literature on the importance of these genera in biocontrol.

The identified endophytes can promote the synthesis of secondary metabolites, including flavonoids and saponins, which is especially important for the pharmacological quality of raw materials. Further studies are aimed at identifying metabolic pathways and interactions of microorganisms with the host plant at the molecular level.

5. Conclusion

In the course of the work, endophytic microorganisms from Glycyrrhiza Glabra L. collected in natural conditions were isolated and characterized for the first time. A number of strains demonstrated significant antagonistic

activity against phytopathogens and have high potential for use in plant biotechnology and environmentally friendly means of protection.

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