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# The Amount of Flavonoids in *Glycyrrhiza glabra* L. Plants Artificially Infected with Fusarium velticilliodes in *Invitro* Conditions

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**ABSTRACT**

In this research, the dynamics of flavonoids were studied by infecting the *in vitro* plant Glycyrrhiza glabra L with the fungus Fusarium velticilliodes. The amounts of apigenin, gallic acids, kaempferol, quercetin, and rutin in the leaves, stems, and roots of these plants were determined using high-performance liquid chromatography (HPLC). When infected with the fungus, apigenin exhibited antifungal properties in the stems of Glycyrrhiza glabra L, and its amount decreased compared to the control. Gallic acids and rutin, which are considered strong antioxidants, were found to be higher in the leaves than in other parts, and increased to 472.502 mg/L and 933.703 mg/L, respectively, when infected with the fungus. The decrease in the amount of apigenin in the leaves to 14.218 mg/L, in the stems to 107.636 mg/L, and in the roots to 14.133 mg/L after fungal infection indicates the manifestation of the antifungal properties of this flavonoid. The increase in the amount of kaempferol and quercetin in the leaves to 84.088 mg/L and 69.398 mg/L after infection with Glycyrrhiza glabra L, respectively, scientifically confirms that flavonoids are an important part of the antioxidant defense system in plant tissues, especially in response to stress factors, the synthesis of phenolic compounds is activated.

## Introduction

Fungi are the most common plant pathogens that negatively affect agricultural crop production. A wide range of fungal diseases in plants significantly reduce crop yields. However, plants have evolved to develop mechanisms to combat biotic stress (1).

Phenolic compounds are actively studied for their involvement in plant defense mechanisms against pathogens, including bacteria, fungi, and viruses, as well as against major abiotic stresses such as drought, salinity, and UV radiation. Phenolic compounds have antimicrobial and antioxidant properties, which help the plant to protect itself from infections and also protect the underlying tissues from the toxic effects of active oxygen forms. Rapid activation of phenylpropanoid biosynthesis pathway genes and accumulation of phenols can be observed in response to environmental stress (2).

Researchers have reported an increase in flavonoids in plants during fungal diseases (3). Some flavonoids are effective against certain diseases and pathogens (4). The most important function of flavonoids is to protect plants from environmental stressors such as temperature, ultraviolet radiation, herbivores, heavy metals, pathogenic bacteria, and fungi (5). Some flavonoids protect plants from parasitic infestations (6).

There is also literature data on flavonoids that decrease in quantity under stress, according to which (7) apigenin caused membrane disruption in the fungus Candida albicans, resulting in cell mass loss due to the leakage of ions and sugar from the cell to the outside. Thus, apigenin demonstrated antifungal properties.

The dynamics of flavonoids under salinity stress in Glycyrrhiza glabra L (8,9) and other (10) plants grown in saline soil conditions in the Syrdarya region have been determined, but the dynamics of flavonoids under the influence of fungi has not been sufficiently studied. The aim of this research was to determine the amount of apigenin, gallic acids, kaempferol, quercetin, and rutin in plant organs based on infecting of Glycyrrhiza glabra L plants grown *in vitro* for 8 weeks with F. velticilliodes.

## Materials and Methods

### Plant Material

The object of the study was a cultured plant (11) Glycyrrhiza glabra L. This plant was grown in *in vitro* for 8 weeks at +22±1 0 C, in a 16/8 (light/dark) regime, and artificially infected with the fungus *F. velticilliodes* according to the method (12).

### Sample Preparation

1 g of leaves, stems and roots of a 5-day-old plant infected with *F. velticilliodes* fungus were extracted with 20 ml of 96% ethanol at 30°C for 75 minutes on a magnetic stirrer. After filtration, the extracts were stored at 4°C.

### HPLC Analysis

Flavonoids were quantified using an Agilent HPLC system with a Zorbax guard cartridge (4.6 mm ID × 12.5 mm) and a Perkin Elmer C18 column (250 × 4.6 mm, 5 µm). The mobile phase (0.5% acetic acid in water (A) and acetonitrile (B)) followed a gradient: 60% A/40% B (1 min), 70% A/30% B (3 min), 55% A/45% B (6 min), 80% A/20% B (10 min), stop (12 min), at 0.8 mL/min and 40°C. Detection occurred at 254 nm, with standards at 0.025 and 0.05 mg/mL.

### Statistical Analysis

Data were analyzed using ANOVA and Tukey's test ($p < 0.05$), reported as means ± SD.

## Results and Discussion

### Flavonoid Content

Phenolic compounds are involved in the defense mechanisms of plants against bacteria, fungi and viruses, as well as against major stresses such as drought, salinity and UV radiation.

Phenolic compounds also have antimicrobial and antioxidant properties, which help the plant to protect itself from infections and also protect the underlying tissues from the toxic effects of reactive oxygen species. Rapid activation of phenylpropanoid biosynthesis pathway genes and accumulation of phenols can be observed in response to environmental stress (13).

Taking this into account, during the research, G. glabra plants grown in vitro were artificially infected with F. velticilliodes. After a week, the amount of flavonoids in the plants were analyzed (Table 1 and chromatograms). The results of HPLC-PDA (254 nm) analysis of *G. glabra* leaves grown in *in vitro* under fungal infection showed the activation of flavonoid metabolism (Fig. 1).

According to the results, when the fungus was artificially infected, gallic acids (RT≈3.34 min) were detected in the highest concentration in G. glabra leaves, which was 472.502 mg/L. This indicator indicates the rapid synthesis of phenolic acids, especially gallic acids, in *G. glabra* leaves in response to fungal infection. Since gallic acids have strong antioxidant and antimicrobial properties, their high content is considered to be related to the plant's defense strategy against pathogens. Rutin (RT≈4.62 min) was recorded as the second largest peak, and its concentration was determined at around 933.7 mg/L. Rutin, as a flavonol glycoside, is important in maintaining membrane stability, neutralizing reactive oxygen species, and strengthening the cell wall. The increase in rutin content under fungal attack indicates an increase in the antioxidant defense system in *G. glabra* leaves.

Quercetin (RT ≈ 9.97 min, 69.398 mg/L), apigenin (RT ≈ 13.62 min, 14.218 mg/L) and kaempferol (RT ≈ 14.77 min, 84.088 mg/L) were also detected in *G. glabra* leaves. Although these flavonoids are present in relatively low amounts, their presence indicates a broadened flavonoid spectrum and a complex activation of biosynthetic pathways. The detection of quercetin and kaempferol may play an important role in chelating metal ions, indirectly supporting the activities of peroxidases and other antioxidant enzymes (9).

HPLC-PDA (254 nm) chromatogram and tabular data for flavonoids in *G. glabra* leaves grown in vitro (control) showed that the main flavonoid components in the plant extract were accurately identified qualitatively and quantitatively. The chromatographic separation was effective, with all major compounds clearly separated from the baseline, and the peaks were symmetrical and highly reproducible, confirming the methodological reliability (Figure 2).

The earliest major peak in the chromatogram is gallic acid, with a retention time of RT≈3.22 min. The area of this peak (Area ≈ 1 692 953) and the calculated concentration in the leaf (control) are 427.368 mg/L, indicating that gallic acid is the dominant phenolic component in the extract.

The second major peak detected in the leaf is rutin, detected around RT≈ 4.60 min. Its concentration (in the control) is 308.94 mg/L, indicating that rutin is the second most abundant flavonoid in the extract.

Quercetin in the leaf of *G. glabra* (in the control) is observed at RT≈ 9.90 min and is equal to 66.695 mg/L, which is lower than gallic acid and rutin, but is considered to be very biologically active.

In the next part of the chromatogram, peaks of apigenin and kaempferol were observed. In the leaf (control), apigenin was detected at RT ≈ 14.02 min, with a concentration of 14.23 mg/L, and kaempferol at RT ≈ 15.43 min, with a concentration of 11.64 mg/L. Despite the relatively low content of these two flavonoids, their regulatory significance is high: apigenin, as a signaling molecule, is involved in chemical interactions with rhizobia, and kaempferol is involved in stabilizing the photosynthetic apparatus and antioxidant system.

The results of chromatographic analysis of the control root of G. glabra grown in in vitro clearly showed that flavonoids are naturally synthesized in the root tissues. The main peaks in PDA Ch1 (254 nm) detection corresponded to gallic acid, rutin, quercetin, apigenin and kaempferol, and their retention times were determined in agreement with the standard substances (Fig. 3).

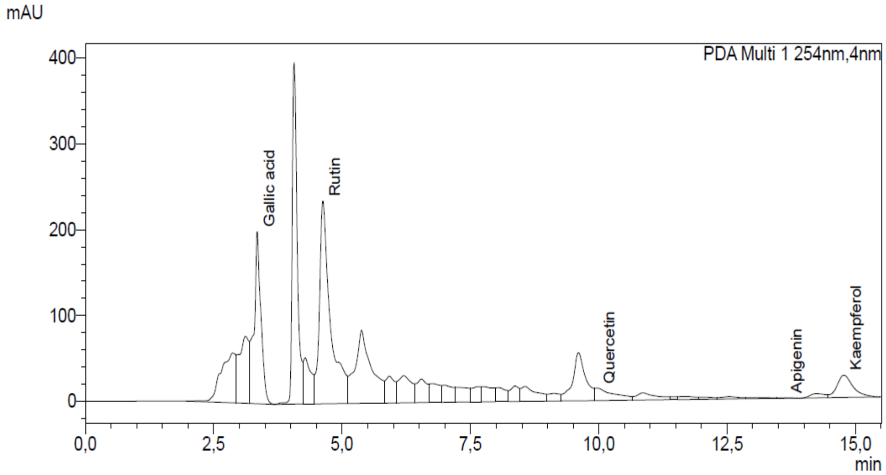
According to the results, gallic acid was detected in the highest amount in the root tissue (in the control) (≈194.8 mg/L), indicating that phenolic acids play a key role in the antioxidant defense system. Also, the relatively high content of rutin (≈73.8 mg/L) and quercetin (≈61.1 mg/L) indicates that the flavonol group is actively synthesized in the roots. Apigenin (≈21.9 mg/L) and kaempferol (≈15.9 mg/L) were constantly present in the root tissue, although in smaller amounts.

Analysis of flavonoid content in *G. glabra* roots infected with the fungus using HPLC-PDA (254 nm) revealed an activation of flavonoid metabolism in response to stress. The chromatogram showed distinct peaks corresponding to gallic acid, rutin, quercetin, apigenin, and kaempferol, indicating the synthesis of a wide range of polyphenolic protective compounds in root tissues (Fig. 4).

According to quantitative data, gallic acid was detected in the root tissue in the highest concentration (≈126.97 mg/L), which is the main antioxidant component among phenolic acids.

The content of rutin was also high (≈79.53 mg/L), which indicates the active accumulation of flavonoid glycosides under stress conditions. The concentration of quercetin was ≈105.58 mg/L, which indicates its important role in neutralizing reactive oxygen species in the root. Although apigenin (≈14.13 mg/L) and kaempferol (≈13.55 mg/L) were detected in relatively low amounts, their presence provides a functional diversity of the flavonoid profile.

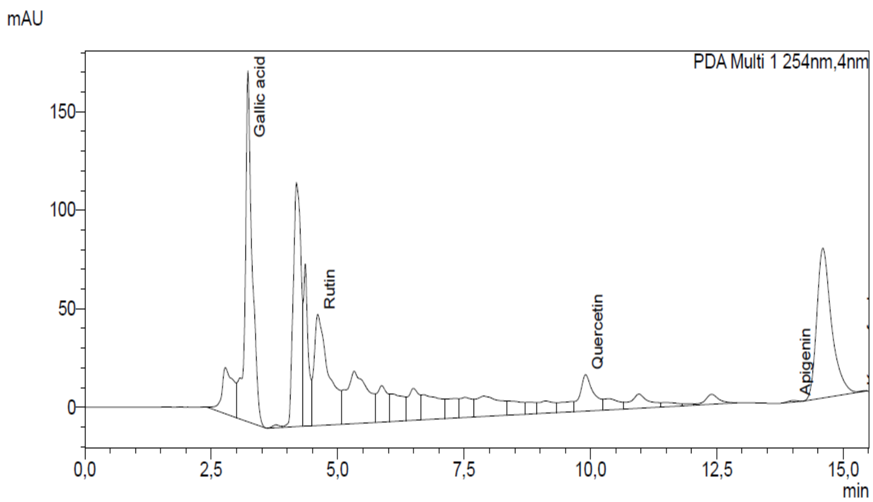
**Figure.1** Results of chromatographic analysis of flavonoids in G. glabra leaves (infected with fungus) grown in vitro. Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 3 | 3,345 | 1874180 | 200417 | 472,502 | mg/L | V | Gallic acid |
| 6 | 4,624 | 3526096 | 235922 | 933,703 | mg/L | V | Rutin |
| 21 | 9,971 | 375633 | 14435 | 69,398 | mg/L | V | Quercetin |
| 28 | 13,618 | 10088 | 584 | 14,218 | mg/L | V | Apigenin |
| 30 | 14,773 | 571291 | 25974 | 84,088 | mg/L | V | Kaempferol |
| Total | | 6357289 | 477332 | | | | |

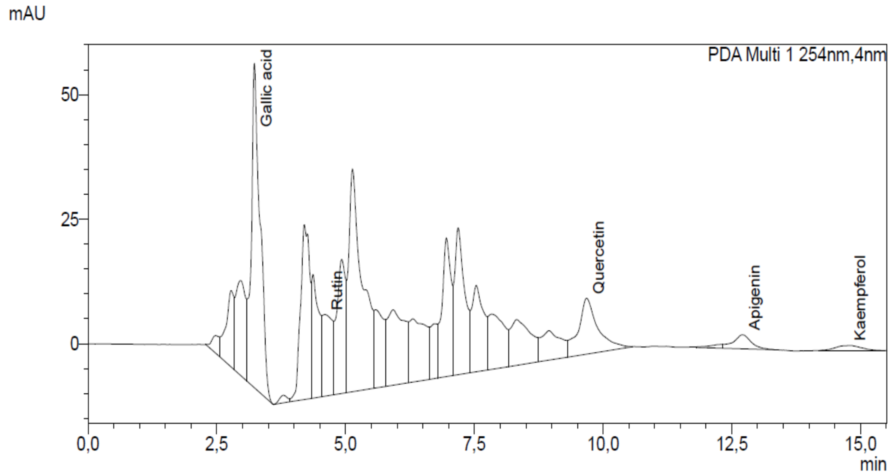
**Figure.2** Results of chromatographic analysis of flavonoids in *G. glabra* leaves (control) grown in vitro. Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 2 | 3,224 | 1692953 | 177953 | 427,368 | mg/L | V | Gallic acid |
| 6 | 4,604 | 1126064 | 56371 | 308,940 | mg/L | V | Rutin |
| 19 | 9,903 | 351267 | 18461 | 66,695 | mg/L | V | Quercetin |
| 25 | 14,015 | 10159 | 807 | 14,227 | mg/L | | Apigenin |
| 27 | 15,431 | 1489 | 187 | 11,640 | mg/L | T | Kaempferol |
| Total | | 3181933 | 253779 | | | | |

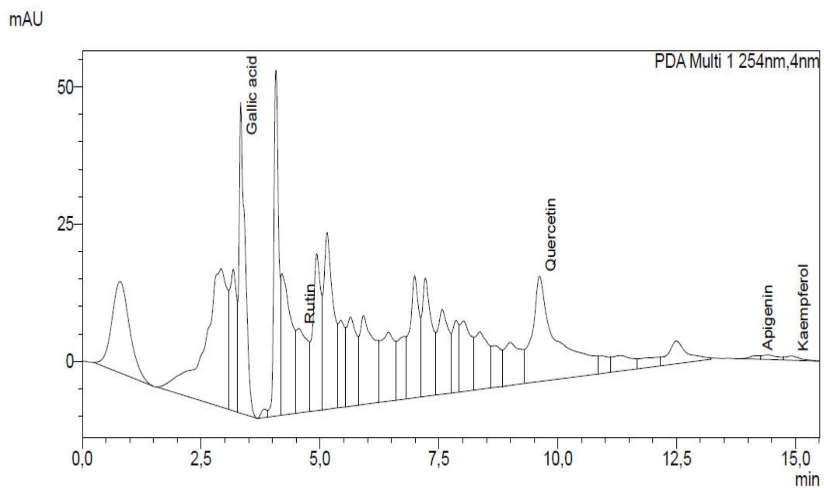
**Figure.3** Results of chromatographic analysis of flavonoids in the roots of *G. glabra* grown in vitro (control). Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 4 | 3,228 | 759243 | 65129 | 194,829 | mg/L | V | Gallic acid |
| 8 | 4,602 | 222843 | 16400 | 73,818 | mg/L | V | Rutin |
| 21 | 9,679 | 300602 | 11186 | 61,074 | mg/L | V | Quercetin |
| 23 | 12,706 | 68344 | 2823 | 21,936 | mg/L | V | Apigenin |
| 24 | 14,760 | 35041 | 1030 | 15,906 | mg/L | | Kaempferol |
| Total | | 1386073 | 96568 | | | | |

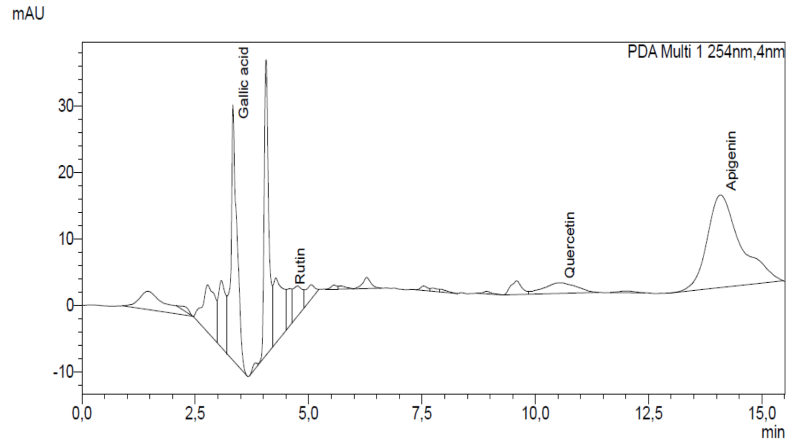
**Figure.4** Results of chromatographic analysis of flavonoids in the roots of *G. glabra* (infected with fungus) grown in vitro. Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 4 | 3,331 | 486797 | 56704 | 126,977 | mg/L | V | Gallic acid |
| 8 | 4,551 | 244792 | 15369 | 79,532 | mg/L | V | Rutin |
| 24 | 9,616 | 701798 | 19210 | 105,583 | mg/L | V | Quercetin |
| 29 | 14,176 | 9450 | 626 | 14,133 | mg/L | | Apigenin |
| 31 | 14,924 | 16477 | 774 | 13,545 | mg/L | V | Kaempferol |
| Total | | 1459314 | 92681 | | | | |

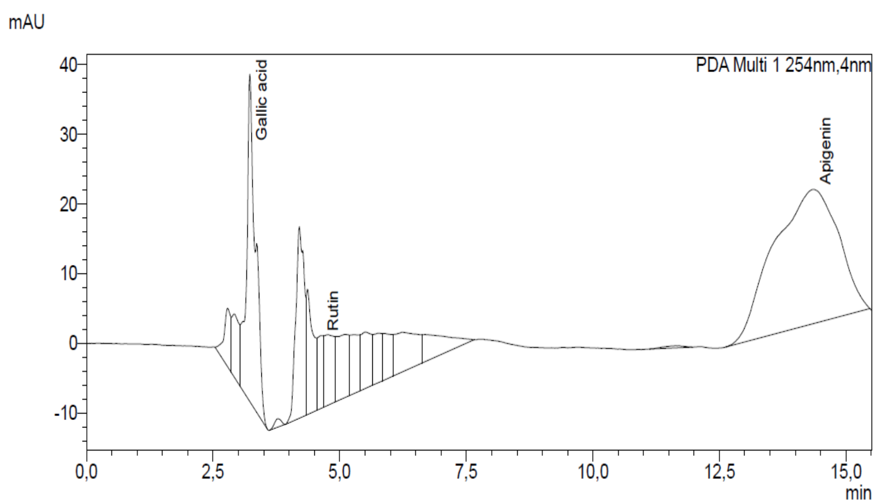
**Figure.5** Results of chromatographic analysis of flavonoids in *G. glabra* plant stems (infected with fungus) grown in vitro. Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 5 | 3,331 | 345867 | 38430 | 91,878 | mg/L | V | Gallic acid |
| 9 | 4,578 | 43561 | 5616 | 27,148 | mg/L | V | Rutin |
| 20 | 10,528 | 81505 | 1611 | 36,767 | mg/L | V | Quercetin |
| 22 | 14,085 | 715190 | 13924 | 107,636 | mg/L | | Apigenin |
| Total | | 1186123 | 59581 | | | | |

**Figure.6** Results of chromatographic analysis of flavonoids in the stem of G. glabra plant (control) grown in vitro. Mobile phase flow rate 1 ml/min. Detection wavelength 254 nm.




PDA Ch1 254nm

| Peak# | Ret. Time | Area | Height | Conc. | Unit | Mark | Name |
|---|---|---|---|---|---|---|---|
| 3 | 3,224 | 566178 | 46981 | 146,746 | mg/L | V | Gallic acid |
| 7 | 4,629 | 86531 | 10446 | 38,334 | mg/L | V | Rutin |
| 17 | 14,359 | 1724314 | 19242 | 241,333 | mg/L | | Apigenin |
| Total | | 2377023 | 76670 | | | | |

**Table.1** Content of flavonoids in Glycyrrhiza glabra L. plants artificially infected with F. velticilliodes under in vitro conditions

| In plant organs | Option | Amount of flavanoids mg/L | | | | |
|---|---|---|---|---|---|---|
| | | Apigenin | Gallic acids | Kayempferol | Quercetin | Rutin |
| Leaves | Control | 14.227±0.550 | 427.368±7.507 | 11.640±0.542 | 66.695±1.903 | 308.940±4.622 |
| | + F.v | 14.218±0.564 | 472.502±8.336 | 84.088±1.125 | 69.398±1.502 | 933.703±5.16 |
| Stems | Control | 241.333±4.968 | 146.746±5.014 | ND | ND | 38.334±0.844 |
| | + F.v | 107.636±2.432 | 91.878±2.5 | ND | 36.767±0.844 | 27.148±0.612 |
| Roots | Control | 21.936±0.934 | 194.829±3.6 | 15.906±0.704 | 61.074±0.732 | 73.818±1.784 |
| | + F.v | 14.133±0.612 | 126.977±2.8 | 13.545±0.632 | 105.583±2.606 | 79.532±2.016 |

*Explanation: F.v- F. velticilliodes.*
*ND- not determined*

Compared to the control variant, an increase in the content of gallic acid and quercetin was observed in the roots infected with the fungus, indicating that the plant has strengthened its antioxidant and defense systems in response to biotic stress. Overall, the results obtained confirm that under conditions of fungal infection, the accumulation of flavonoids and phenolic acids in licorice roots increases, which play an important role in protecting cells from oxidative damage and increasing resistance to other general stresses.

According to the results of the study of the flavonoid content in the stem of a fungus-infected *G. glabra* plant using the HPLC–PDA (254 nm) method, the main peaks in the chromatogram corresponded to gallic acid (RT ≈ 3.33 min), rutin (RT ≈ 4.58 min), quercetin (RT ≈ 10.53 min), and apigenin (RT ≈ 14.09 min), and their quantitative content differed significantly in the stem tissue (Figure 5).

According to quantitative analysis, apigenin was detected in the highest concentration (107.636 mg/L), which indicates an increase in the biosynthesis of flavones in response to stress in the stem tissue.

The high accumulation of apigenin is associated with its strong antioxidant and antifungal properties, which play an important role in maintaining cell wall stability and neutralizing ROS under conditions of fungal infection. Quercetin was also found in relatively high amounts (36.767 mg/L), which indicates the activation of a mechanism to limit oxidative stress through flavonols in the stem tissue.

The presence of rutin (27.148 mg/L) and gallic acid (91.878 mg/L) confirms the formation of a complex defense system in the stem tissue with the participation of phenolic acids and glycosidic flavonoids. In particular, a significant amount of gallic acid is explained by its antioxidant and antimicrobial effects, which is an important component of the response to fungal infection.

These results indicate that the flavonoid spectrum in the stem of the fungus-infected sweetgum, although relatively simpler than in the leaf, is redistributed in a protective direction due to active compounds such as apigenin and quercetin. This scientifically substantiates the fact that flavonoids in the stem tissue mainly act as a mechanical and biochemical barrier, that is, serve to limit the spread of infection.

The results of HPLC-PDA (254 nm) chromatographic analysis of *G. glabra* (control variant) indicate that flavonoids are synthesized at a physiological level in stem tissues (Fig. 6).

The main detected compounds in the chromatogram were gallic acid, rutin and apigenin peaks. Of these, the highest concentration was attributed to apigenin, its amount was 241.33 mg/L, which indicates that apigenin is one of the main flavonoid components in stem tissues.

The amount of gallic acid was 146.75 mg/L, which indicates that phenolic acids serve an antioxidant and protective function. Rutin was detected in a relatively low amount (38.33 mg/L), indicating the presence of flavonol glycosides in the stem at a basic level.

The absence or very low levels of quercetin and kaempferol peaks in the control variant indicate that these compounds are not actively synthesized in stem tissues under normal conditions. This is consistent with general laws confirming that flavonoid biosynthesis is enhanced under the influence of stress factors (e.g., salt or biotic stress).

As a result of the conducted studies, in the in vitro conditions of the plant *Glycyrrhiza glabra* L. infected with a fungus, the amount of flavonoids in the organ section was found to change differently. In particular, the concentration of flavonoids (gallic acids, rutin, quercetin, kaempferol) in leaf tissues was significantly increased compared to the control variant. This phenomenon indicates the activation of the biosynthesis of secondary metabolites, including flavonoids, as a protective-adaptive response of the plant under the influence of a fungus infection. The increase in the amount of flavonoids studied under the influence of a fungus indicates the strengthening of the antioxidant defense system in the plant under the influence of a pathogen.

The quantitative increase in the amount of flavonoids studied in the leaves, quercetin and rutin in the roots compared to the control is of great importance due to their activity in maintaining membrane stability and strengthening the cell wall. The decrease in the amount of flavonoids (apigenin, gallic acids, kaempferol) in the stem and root of *Glycyrrhiza glabra* L. when artificially infected with Fusarium showed dynamic shifts.

In particular, the decrease in the amount of apigenin in all organs when infected with fungi showed antifungal properties, in accordance with the literature data. This phenomenon can be explained by the redistribution of metabolites between organs, selective changes in biosynthetic processes under stress conditions.

The results obtained indicate the formation of an organ-specific biochemical response to fungal infection in the licorice plant and confirm the important role of flavonoids in the defense mechanism against phytopathogens.

## Author Contributions

Ismoilova Кaromatkhon Makhmudjonovna: Investigation, formal analysis, writing—original draft. Safarov Karimdjon: Validation, methodology, writing—reviewing. Inoyatov Islombek Ilhom-ugli:—Formal analysis, writing—review and editing. Dilorom Turabekova Bakhtiyarovna: Investigation, writing—reviewing. Djurayev Tulkin Arzikulovich: Resources, investigation writing—reviewing.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethical Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent to Publish** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

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