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Phytochemical profile of *Eranthis longistipitata* Regel from three study sites in the Kazakhstan part of the Western Tien Shan

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Abstract. *Aimenova ZE, Matchanov AD, Esanov RS, Sumbembayev AA, Duissebayev SE, Dzhumanov SD, Smailov BM. 2023. Phytochemical profile of* Eranthis longistipitata *Regel from three study sites in the Kazakhstan part of the Western Tien Shan. Biodiversitas 24: 6031-6038.* The aim of this study was to determine the phytochemical profile, the antifungal and antiradical potential of *Eranthis longistipitata* Regel from three study sites in the Kazakhstan part of the western Tien Shan region (in the territory of Aksu Jabagly State Nature Reserve). Sample collection was carried out in Taldybulak Gorge, Zhetymsai Gorge, and the valley of the Irsu River. The largest population of *E. longistipitata* was found in the valley of the Irsu River due to the high humidity and the higher availability of sunlight. The flavonoid compounds of the leaves and tubers of *E. longistipitata* collected in the Irsu River valley were analyzed by HPLC. Antifungal activity against *Trichoderma lignorum*, *Fusarium oxysporum* and *Aspergillus niger* was determined. The free radical activity was determined using DPPH. The results showed that the leaves and tubers of *E. longistipitata* growing in the Kazakh part of the western Tien Shan region contained rutin, apigenin, and gallic acid. The leaves of *E. longistipitata* had the highest rutin content (15.971 mg/g). The highest free radical scavenging activity was observed in the ethanol extract (3) of *E. longistipitata* leaves (57.16 % at 1.0 mg/ml) compared to chloroform (26.95% at 1.0 mg/ml) and ethyl acetate extracts (28.69% at 1.0 mg/ml). The free radical scavenging activity of the extracts was lower than that of gallic acid. The *E. longistipitata* leaves extract had good antifungal activity against *F. oxysporum* and *A. niger* (18.8 mm and 18.6 mm zones of inhibition, respectively).

Keywords: Antifungal activity, DPPH, *Eranthis longistipitata*, HPLC, Western Tien Shan

INTRODUCTION

Eranthis longistipitata Regel is an endemic plant of Central Asia (Erst and Erst 2019). *Eranthis* Salisb (Ranunculaceae tribe Helleboreae) comprises nine or ten species in Europe and East Asia (Mabberley 2017). *Eranthis* flowers early in the spring and is commonly known as winter aconite. It was previously identified as *Helleborus* (Linnaeus 1753) but later recognized as *Eranthis* (Salisbury 1807). The genus *Eranthis* Salisb. is characterized by thickened tuberous underground stems, separate palm-like basal leaves, a leafless stem with leaf-like sheeting on the upper part, and actinomorphic single flowers with an unusual petal structure. The genus *Eranthis* is a flowering herbaceous plant with thickened tuberous roots.

Several previous studies have indicated that *Eranthis* exhibits pharmacological activity. *Eranthis* sp. has been used to treat urolithiasis and diuresis (Hao 2018). The tubers of *Eranthis* plants have significant pharmacological activity. *E. cilicica* tubers contain triterpene glycosides of cycloartan and oleanane groups and saponins. These substances are toxic to human promyelocytic leukemia cells (HL-60) (Watanabe et al. 2019). Isolated chromones from the tubers of *E. cilicica* possess antioxidant properties (Kuroda et al. 2009). Chromones and lectins in *E. hyemalis* tubers have anti-inflammatory (Malik et al. 2017), antitumor, insecticidal, antifungal, and antiviral effects (McConnell 2015; Djafari et al. 2018). A study by Kumar et al. (1993) showed that the genus *Eranthis* possesses antibacterial and antiviral activity (Kumar et al. 1993).

However, there are still limited studies on the profile and levels of polyphenols in *Eranthis* plants. Plant extracts, herbs, and spices rich in polyphenolic compounds have been used for thousands of years in traditional oriental medicines. The beneficial effects of polyphenols are their potential antioxidant activity, that is, their ability to scavenge oxygen radicals and other reactive species (Abbas et al. 2017).

In a study by Kostikova et al. (2021), flavonoids in the leaves of *E. longistipitata* from natural populations of Kyrgyzstan and Uzbekistan were investigated by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) and showed that there are 18 flavonoid compounds in the leaves of *E. longistipitata*, including flavonols, flavones, flavans, flavanones, and chalcones.

Currently, there is increasing multiresistance of infectious agents to antimicrobial drugs. Therefore, the search for new antibiotic and antiviral compounds is

urgently needed. Medicinal plants serve as a promising source of antimicrobial agents of natural origin. Synthetic drugs and antibiotics have many adverse effects, such as a narrow spectrum of action, rapid adaptation, and individual intolerance. Substances of different chemical natures synthesized by plants are the wealthiest source of therapeutic and prophylactic operators with bactericidal and fungicidal properties. Medicinal plant extracts contain a certain proportion of biologically active substances that contribute to optimal effects on the human body with the possibility of using them for a long time.

In this regard, searching for new antimicrobial, antifungal, and free radical scavenging agents of natural origin is very important. The aim of this was to investigate the potential of *E. longistipitata* populations from the Kazakhstan site in the western Tien Shan region as an antifungal and free radical scavenger and to identify flavonoid compounds.

MATERIALS AND METHODS

Study area

Eranthis longistipitata populations grow on the slopes of the western Tien Shan Mountains, i.e. Aksu Jabagly State Nature Reserve, Turkistan State, Kazakhstan (Figure 1). The western Tien Shan region has unique ecological regions characterized by the richness and uniqueness of flora and vegetation and the concentration of endemic and rare plant species (Ryabushkina et al. 2008).

Field expeditions in the western Tien Shan region (Aksu Jabagly State Nature Reserve) were carried out from the end of February to the beginning of March 2023, with the total project area of the Reserve being approximately 75,000 hectares. The climate is moderately continental. This project area is located in the northern part of the western Tien Shan region, characterized by an uneven distribution of precipitation, i.e., a maximum in winter and spring and a minimum in summer. The average temperature of the coldest month of January is -5.4°C; the warmest month is August, with an average temperature of 21°C. The lowest temperature is -34°C, the highest is +37°C. The precipitation in winter, spring, summer, and autumn was 30%, 40%, 10%, and 20%.

To characterize the ecological requirements of species, descriptions of ecological factors were used as recommendations (Brown et al. 2014). The state of *E. longistipitata* populations in the Kazakhstan part of the western Tien Shan range was assessed according to recommendations (Sumbembayev et al. 2022).

Procedures

Plant material and extract preparation

Leaves and tubers of *E. longistipitata* were collected during the flowering-fruiting period in March 2023. Undamaged plants that were in a generative state (blooming as much as possible) were selected for collection. The plants were sorted by discarding yellowed, pest-damaged, and dirty parts of plants and then dried no later than 1 hour after collecting the material to avoid accelerated destruction of biologically active substances. Drying of the material was carried out by an air-shadow method. The aboveground parts of the plant were hung in well-ventilated, dry rooms, tied in bundles, or laid out on dry sheets of paper. The plant materials were protected from direct sunlight because it could lead to a decrease in the content of flavonoids and other biologically active substances. Drying was carried out for 2-3 weeks, and the dried plants were packed in paper bags and stored in a clean, dry room. After separation from the stem, the tubers were cleaned and washed under distilled water and dried for 2 weeks using the air-shade method. The brown tuber skin was peeled using a knife, and the white pulp inside the tubers was crushed into a powder. This white powder was used for extraction.

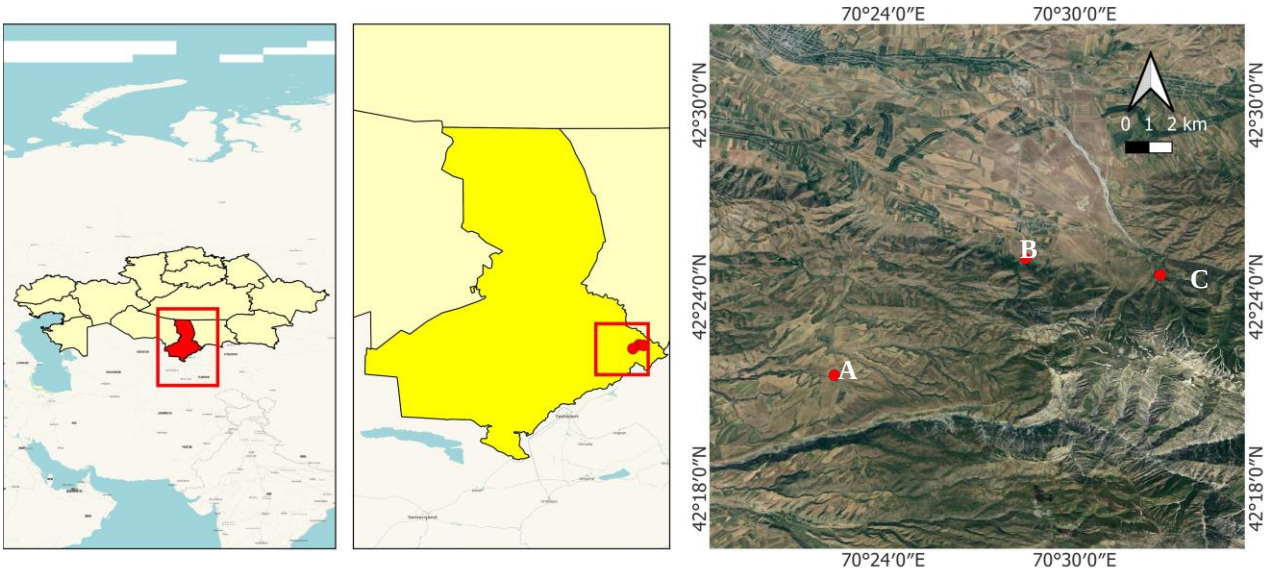

Figure 1. Map of study site in western Tien Shan Mountains, i.e. Aksu Jabagly State Nature Reserve, Turkistan State, Kazakhstan. Notes: Red dots represent *Eranthis longistipitata*. A. Valley of the Irsu River; B. Taldybulak Gorge; C. Zhetimsay Gorge

The 70% aqueous-ethanol extracts of the leaves and tubers were used to characterize the ecological requirements of the species and then concentrated using a water bath at 72°C. The concentrated extract was then analyzed for flavonoids: 0.2 g of the crushed air-dried leaves and tubers was extracted twice (30 mL for 30 min and 20 mL for 20 min). The filtrate was composited, and the flask and filter residue were washed with 5 mL of 70% ethyl alcohol. The mixed extract was concentrated to 5 mL. The combined extract was concentrated in porcelain dishes to 5 ml. The concentrated extract was filtered to remove undissolved particles and stored in a refrigerator at 4°C.

Flavonoid analysis by high-performance liquid chromatography

An Agilent 1260 HPLC system (detector-diode matrix, ChemStation system, USA) was used to determine phenolic compounds using an Eclipse XDB column (5 microns, 4.6 × 250 mm), and t =25°C was selected. The mobile phase of an aqueous solution of H_3PO_4 (0.1%) was 50-52%, 56 min (Van Beek 2002). The eluent flow rate was 1 mL/min. The detection wavelengths were 254, 320, and 381 nm, and the groups of phenolic substances were identified by their spectral characteristics (Erst et al. 2020). For identification of the phenolic components in plant extracts, standard samples of salicylic and chlorogenic acids, quercetin, kaempferol, orientin (Sigma-Aldrich Chemie GmbH, Munich, Germany), gentisic and caffeic acids (Serva Heidelberg, Germany), hyperoside and vitexin (FlukaChemie AG, Buchs, Switzerland) were used to identify the phenolic compounds in the extracts. The samples were analyzed twice.

Free radical scavenging activity

The free radical scavenging activity of *E. longistipitata* leaf extract was determined by DPPH assay (Takatsuka et al. 2022). DPPH (2,2-diphenyl-1-picrylhydrazyl radical) was inhibited by 0.1 ml of an ethanol solution of *E. longistipitata* leaf extracts in the concentration range of 0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml by adding 3 ml of $6*10^{-5}$ M radical solution. The test tubes were in a rack wrapped in black polyethylene. After vigorous stirring, the solutions were incubated in the dark for 30 minutes; then, optical densities were measured at 520 nm using a Cary-50 UV Visible Area spectrophotometer (Agilent Technologies). The values of the free radical scavenging activity (ARA) were determined using the following formula:

$$ARA\ (\%) = A_0 - A_t / A_0 * 100 \quad \text{(i)}$$

Where:
A_0 = the optical density of the control sample; and
A_t -= the optical density of the test sample

Antifungal activity

The antifungal activity of *E. longistipitata* leaf extract against the pathogens *T. lignorum*, *F. oxysporum*, and *A. niger* was determined. The working culture was prepared in nutrient broth by transferring a loopful of stock cultures into a test tube and kept in an incubator at 37°C for 24 hours. The antifungal assay of *E. longistipitata* leaf extract was performed by agar disc diffusion using Chapek medium. Sterilized media in Petri dishes were inoculated with fungus culture using a sterilized cotton bud. Discs of different extract concentrations (15, 20, 25, 30 µL) were placed on media using sterilized pincers. Next, the Petri dishes were incubated at 37°C for 3 days. After incubation, the diameter of the inhibitory zone was measured.

Chemicals and reagents

Chemical references for external standards, i.e., rutin, apigenin, gallic acid, robinin, hyperoside, luteolin, and quercetin, were purchased from Extrasynthese (France).

RESULTS AND DISCUSSION

Habitat of *E. longistipitata* populations

The collected samples of E. longistipitata populations were obtained from the Aksu Jabagly State Nature Reserve territory (Figure 2). Populations of *E. longistipitata* (Figure 3) were collected from three habitats (study sites) in the Kazakhstan site of the western Tien Shan range in the Aksu Jabagly State Nature Reserve. Environmental conditions ranged from deciduous forest to mixed forest edges (1,198-1,454 m asl.). *E. longistipitata* grows in semilight or fully isolated areas with moist soils.

The first habitat of *E. longistipitata* populations is in Taldybulak Gorge (Figure 4), on the left bank of the river with geographical coordinates 42º 25’12N 70°28’28E (1198 m a.s.l.). Community: Deciduous-shrubby. Trees and shrubs-*Crataegus turkestanica* Pojark, *Lonicera tianschanica* Pojark., *Spiraea hypericifolia* L., *Rosa kokanica* Regel, *Rosa fedtschenkoana* Regel, *Ephedra equisetina* Bunge. Herbaceous plants - *Ziziphora bungeana* Juz, *Hypericumper foratum* L., *Eremurus regelii* Vved, *Achillea millefolium* L., *Crocus alatavicus* Regel & Semen., *Gagealutea* (L.) Ker Gawl, *Arum korolkowii* Regel, *Hypericum perforatum* L.

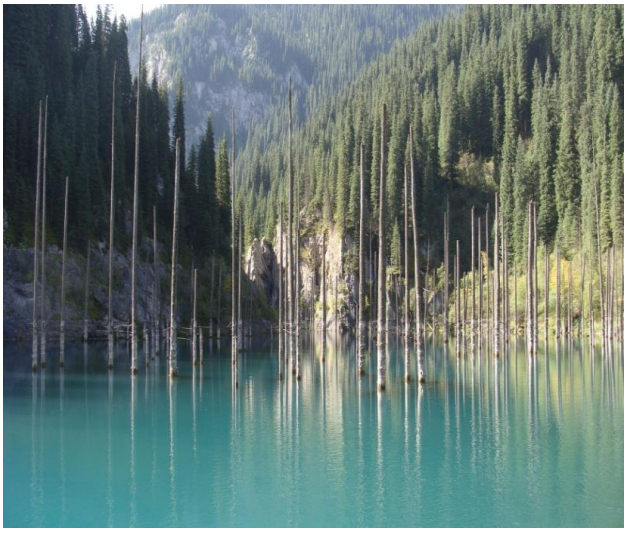

Figure 2. Aksu Jabagly State Nature Reserve, Kazakhstan

The second habitat of *E. longistipitata* populations is the Zhetimsay Gorge (Figure 5), on the left bank of the river with geographical coordinates 42º 24'41N 70°32'41E (1452 masl). Community: Deciduous-shrubby. Trees and shrubs-*Crataegus turkestanica* Pojark, *Lonicera tianschanica* Pojark., *Lonicera nummulariifolia* Jaub. & Spach, *Spiraea hypericifolia* L., *Rosa kokanica* Regel ex Juz., *Rosa fedtschenkoana* Regel, *Salix babylonica* L., *Malus sieversii* (Ledeb.) M.Roem. Herbaceous plants - *Ziziphora bungeana* Juz., *Hypericum perforatum* L., *Eremurus regelii* Vved, *Achillea millefolium* L., *Crocus alatavicus* Regel & Semen., Gagealutea (L.) Ker Gawl., *Hypericum perforatum* L., *Leontice albertii* Regel, *Corydalis ledebouriana* Kar. & Kir., *Verbascum songaricum* Schrenk., *Hordeum bulbosum* L., *Tulipa kaufmanniana* Regel.

The third habitat of *E. longistipitata* populations is in the valley of the Irsu River (Figure 6) with geographical coordinates 42º 21'33 N70°22'28E (1454 m asl). Community: Savannoid. Trees and shrubs-*Spiraea hypericifolia* L., *Rosa kokanica* Regel ex Juz.Herbaceous plants - *Ziziphora bungeana* Juz., *Hypericum perforatum* L., *Eremurus regelii* Vved, *Achillea millefolium* L., *Crocus alatavicus* Regel & Semen., Gagealutea (L.) Ker Gawl., *Leontice albertii* Regel, *Corydalis ledebouriana* Kar. &Kir., *Verbascum songaricum* Schrenk., *Hordeum bulbosum* L, *Tulipa kaufmanniana* Regel, *Tulipa greigii* Regel, *Rhinopetalum karelinii* Fisch. ex D. Don, Sedum alberti Regel.

In all subpopulations, the number of *E. longistipitata* plants was 150±8 individuals. The average density of the subpopulations ranged from 14-22 individuals/2m^2 in the 3 habitats. The largest and tallest *E. longistipitata* individuals were found in the valley of the Irsu River. This might be due to the high humidity levels in this area, including a large amount of precipitation and a higher position above sea level. All 3 collection sites of *E. longistipitata* are characterized by hilly terrain, high humidity, and sunlight exposure.

Flavonoid analysis by HPLC

Among the wide variety of natural compounds, one of the most common and numerous classes with high biological activity is phenol compounds, which include flavonoids. Phenols show strong protection against the development and progression of many chronic pathological conditions, including cancer, diabetes, cardiovascular problems, and aging. The low toxicity and high pharmacological activity of phenols make them promising for preventing and treating several serious diseases. These natural substances are essential for the body, so a constant intake of food or supplements is needed (Chand et al. 2023).

Eranthis longistipitata populations grow in early spring (March). Due to weather conditions, frosts and insolation may still be observed at this time of the year, harming plant growth. Flavonoids found in ephemeral plants of *E. longistipitata* protect plant tissues from damage caused by external factors and participate in redox processes in the plant (Mazibuko-Mbejeet al. 2019).

Populations growing in the Irsu River valley were selected for flavonoid analysis using HPLC since these populations have the largest stem height, number of leaves, and size of tubers. Table 1 presents the flavonoid content in the leaves and tubers of *E. longistipitata* from the Kazakhstan part of the western Tien Shan region. This table shows the presence of rutin, apigenin, and gallic acid in the leaves and tubers of *E. longistipitata*, whereas robinin, hyperoside, luteolin, and quercetin were absent in leaves and plants. The rutin concentration in the leaves of *E. longistipitata* was the highest among all identified flavonoids and was almost 15 times higher than that in the tubers.

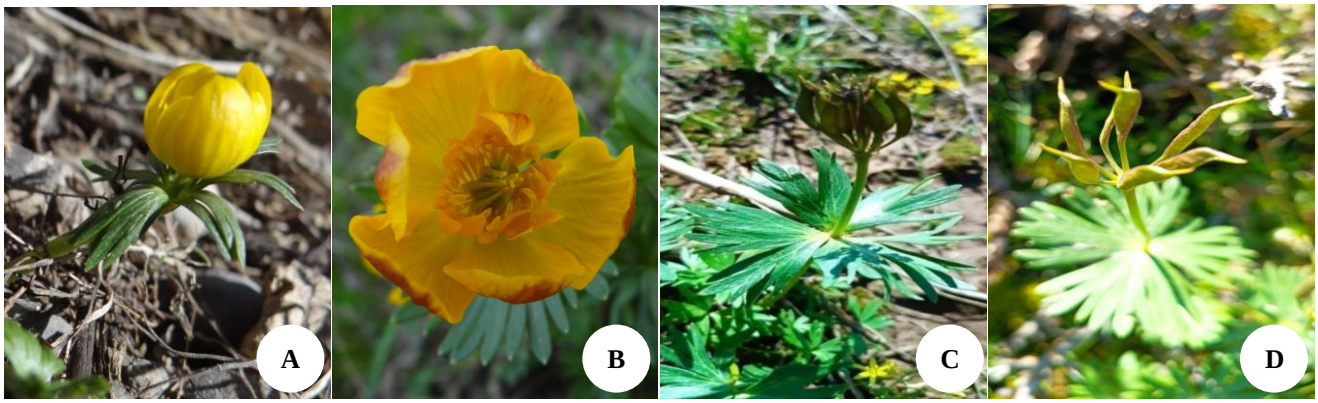

Figure 3. *Eranthis longistipitata* in the natural habitat in the Kazakhstan part of the western Tien Shan range (Aksu Jabagly State Nature Reserve): A. The beginning of flowering (end of February); B. Flowering period (March); C. The end of flowering (beginning of April); D. Complete coincidence of petals, the beginning of the dormant phase and the ripening of seeds (mid-April)

Table 1. The amount of some identified flavonoids in *E. longistipitata* leaves and tubers (mg/100g of air-dried matter)

Sample	Rutin	Apigenin	Gallic acid	Robinin	Hyperoside	Luteolin	Quercetin
E. longistipitata leaves	15.971 ± 0.04	7.518 ± 0.05	0.596 ± 0.06	-	-	-	-
E. longistipitata tubers	0.202 ± 0.03	22.592 ± 0.04	8.935 ± 0.04	-	-	-	-

Note: The data are presented as means and standard error (n=3)

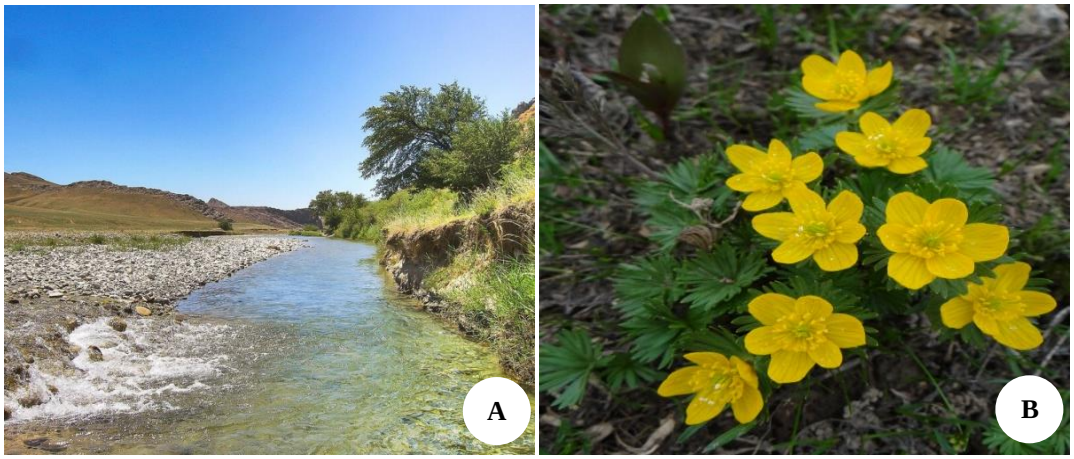

Figure 4. Habitat of *E. longistipitata* populations in the Taldybulak Gorge (left bank of the river): A. Taldybulak River; B. Flowering period of *E. longistipitata*

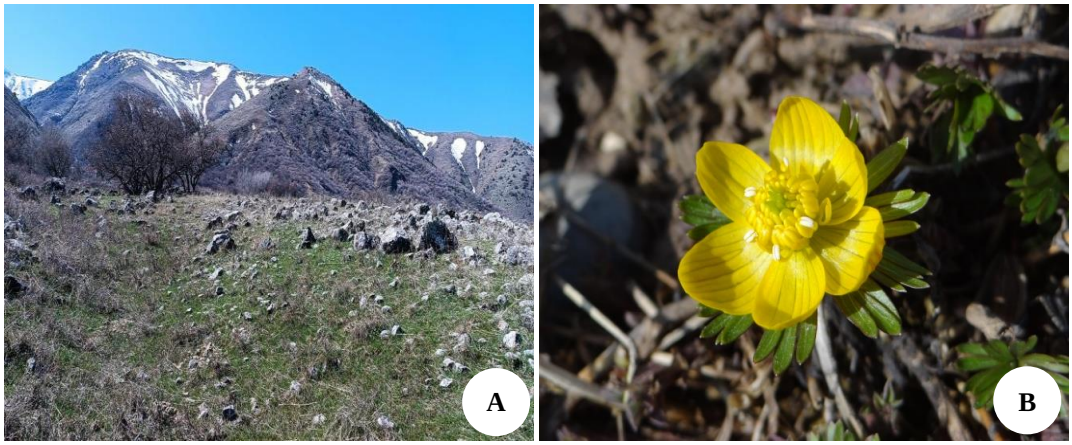

Figure 5. Habitat of *E. longistipitata* populations in the Zhetimsay Gorge (left bank of the stream): A. Zhetimsay Gorge; B. Flowering period of *E. longistipitata*

Figure 6. Habitat of *E. longistipitata* populations in the Irsu River Valley: A. Irsu River Valley; B. Flowering period of *E. longistipitata*

A previous study by Kostikova et al. (2021) showed that rutin was present in *E. longistipitata* leaves from the Kyrgyz and Uzbek parts of the western Tien Shan region in the highest amount. The rutin content in *E. longistipitata* from Kyrgyzstan was 2.46-3.20 mg/g, while that from Uzbekistan was 1.50-3.01 mg/g. The rutin content in the leaves of *E. longistipitata* from the Kazakh part of the western Tien Shan was 15.971 mg/g. The biological

activities of rutin are antioxidant, cytoprotective, vasoprotective, neuroprotective, anticarcinogenic, and cardioprotective effects (Ganeshpurkar and Saluja 2017). Rutin participates in redox processes, improving connective tissue and oxygen saturation. Rutin optimizes metabolism, is responsible for vascular strength, prevents premature aging, and protects against allergic reactions by inhibiting histamine synthesis.

Antiradical activity

Free radicals and reactive oxygen species are involved in cell pathogenesis (Halliwell 2020). Lipid peroxidation products harm the human body (Mao et al. 2022). An increase in the lipid peroxidation products in the cell leads to a change in intra- and intercellular relationships, the breakdown of ATP and amino acids, depolarization of DNA, changes in the activity of cytoplasmic enzymes, changes in osmotic pressure, disruption of membrane structure and cell function and can lead to cell death (Usukhbayar et al. 2023). Pharmacology currently employs the repair of the membrane peroxidation process, which involves comprehensive research on the biological properties of flavonoids in cells (Onaolapo et al. 2023). Therefore, the next step of research is to study the free radical scavenging activity of *E. longistipitata* leaf extract because there is a proven link between the presence of flavonoids in the plant and the anti-free radical properties it exhibits (Albayrak and Aksoy 2023).

The free radical scavenging activity of the extract was compared to that of gallic Acid (GA). The optical density of test solutions at different concentrations is shown in Table 2. The values of the extracts' free radical scavenging activity are presented in Table 3 and Figure 7. Table 3 and Figure 7 show that the ethanol extract (3) of *E. longistipitata* leaves at concentrations of 0.1, 0.25, and 0.5 mg/ml has below-average antiradical activity; at a concentration of 0.75 mg/ml, the average antiradical activity level; and at a concentration of 1 mg/ml, the above-average amount of antiradical activity.

Chloroform (1) and ethyl acetate extract (2) of *E. longistipitata* leaves at all concentrations had low antiradical activity compared to the antiradical activity of gallic acid.

Antifungal activity

The antifungal activity of *E. longistipitata* leaves against several pathogenic fungi at different concentrations was determined. The leaf ethanol extract was tested against several fungi by the disc diffusion method. The diameter of zone inhibitions of leaf extract against *F. oxysporum* and *A. niger* were 18.8 mm and 18.6 mm, respectively. The inhibition zone of leaf extract against other fungi is presented in Figure 8.

Discussion

Among the three study sites, the largest population of *E. longistipitata* was in the valley of the Irsu River. This result might be associated with increased humidity in this highland, including high precipitation. The results of a comprehensive study of the coenopopulations of *E. longistipitata* in the Kazakhstan part of the western Tien Shan region provide information about the morphobiological, ecological, and cenotic features and structure of the coenopopulations of this species. The flavonoid profile of *E. longistipitata* includes rutin, apigenin and gallic acid. The highest concentration of rutin reached 15.971 mg/g in the leaves of *E. longistipitata*. Rutin has been shown to slow cellular aging processes and enhance the effectiveness of chemotherapy against cancer (Liu et al. 2023). Apigenin has broad therapeutic potential in treating diabetes, amnesia, cancer, etc. (Salehi et al. 2019). In animal models, gallic acid suppresses the growth of cancer cells (Verma et al. 2013).

Table 2. The optical density of test solutions at different concentrations

Test substances	Optical density values by concentration (mg/ml)				
	0.1	**0.25**	**0.5**	**0.75**	**1.0**
Gallic acid (GA)	0.1670	0.1624	0.1624	0.1492	0.1358
Chloroform extract (1)	0.7298	0.7234	0.6884	0.6377	0.5962
Ethyl acetate extract (2)	0.7419	0.7225	0.6724	0.6246	0.5842
Ethanol extract (3)	0.7377	0.6721	0.6314	0.4762	0.3514

Table 3. Free radical scavenging activity (%) of *E. longistipitata* leaf extract at various concentrations

Test substances	Extract concentration (mg/ml)				
	0.1	**0.25**	**0.5**	**0.75**	**1.0**
Gallic acid (GA)	80.59	81.13	81.13	82.66	84.21
Chloroform extract (1)	10.59	11.36	15.66	21.87	26.95
Ethyl acetate extract (2)	9.45	11.81	17.92	23.76	28.69
Ethanol extract (3)	10.07	18.07	23.02	41.94	57.16

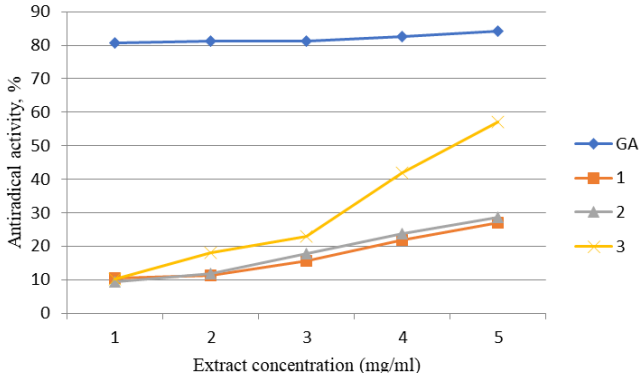

Figure 7. The free radical inhibition percentage of *E. longistipitata* leaf extract at various concentrations (blue line - Gallic acid, red line - Chloroform extract, green line - Ethyl acetate extract, violet line - Ethanol extract)

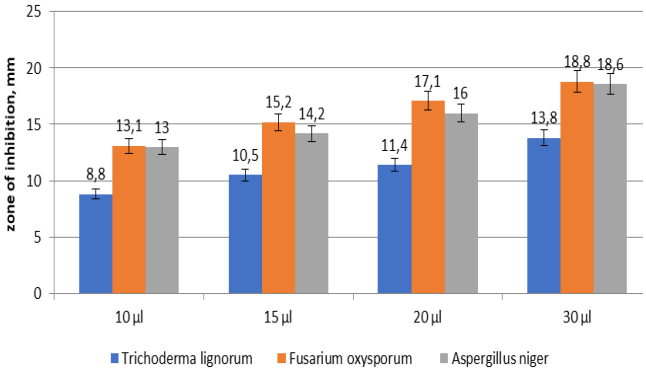

Figure 8. Antifungal activity of *E. longistipitata* leaf extract against pathogenic fungal strains

The highest free radical scavenging activity was obtained from the ethanol extract (3) of *E. longistipitata* leaf extract (57.16% at 1.0 mg/ml) in comparison with the chloroform (26.95% at 1.0 mg/ml) and ethyl acetate extracts (28.69% at 1.0 mg/ml), which had lower-than-average activity compared to gallic acid. A possible explanation for the high antiradical properties of *E. longistipitata* leaves in ethanol extract is that DPPH radicals may be neutralized by nonphenolic antioxidants (Foti and Amorati 2009), which might be better extracted by ethanol solvent. In this way, ethanol extract from *E. longistipitata* leaf can be recommended as a preventive dietary supplement that helps reduce the level of free radical processes in the organism.

Antifungal studies of *E. longistipitata* leaves extract against the pathogens *T. lignorum*, *F. oxysporum*, and *A. niger* have revealed that at a concentration of 30 µL, the zone of inhibition of *F. oxysporum* was 18.8 mm, and the zone of inhibition of *A. niger* was 18.6 mm. Wu et al. (2008) and Michielse et al. (2012) reported that phenolic acids inhibit conidia production in *F. oxysporum*. There are different mechanisms by which phytochemicals exert antifungal activities. Phenolic acids and flavonoids found in E. longistipitata can form complexes with pathogen cell walls and extracellular and soluble proteins, causing valuble antifungal effects (Scalbett 1991).

By this line of reasoning, we can recommend the leaves and tubers of *E. longistipitata* as prospective antioxidant, antiradical, and antifungal agents.

ACKNOWLEDGEMENTS

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