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Research Article

Preliminary phytochemical analysis and DNA protective activities of *Ephedra major* and *Ephedra equisetina* species

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Abstract: Worldwide, the *Ephedra* plant is one of the oldest medicinal herbs used in traditional alternative medicine. The medicinal varieties are utilized as stimulants and antiasthmatic agents for the treatment of colds, bronchial asthma, cough, fever, influenza, headache, edema, and allergies. In our study, it was aimed to determine the preliminary phytochemical composition of *Ephedra major* and *Ephedra equisetina* species belonging to the *Ephedra* genus and to determine their antioxidant and DNA protective activity. *Ephedra major* is known to contain components such as flavonoids and tannins with a moderate ephedrine content and has been traditionally used in the treatment of respiratory diseases. *Ephedra equisetina*, on the other hand, contains higher concentrations of ephedrine and other bioactive compounds and is widely used in traditional medicine for the treatment of asthma, bronchitis, and allergic reactions. When we look at the study findings, the total phenolic and flavonoid substance amounts in both plant species were determined at close levels in both tests, and it was seen that they contained phenolic and flavonoid substances and had antioxidant activity and DNA protection effect. In addition, it was observed that there was a DNA protection effect at a concentration of 0.5 mg/mL in the presence of Fenton in the medium.

1. INTRODUCTION

Plant species constitute valuable sources of bioactive compounds. Nowadays, an increasing trend towards the use of natural products can be observed in high demand from food, cosmetic, and pharmaceutical manufacturers, as the phytochemicals found in plant extracts generally show low toxicity and are effective in micromolar concentrations (González-Juárez *et al*., 2020). In addition, many of the plants are widely used in traditional medicine around the world to treat various diseases. Of these plants, the *Ephedra* genus (in the Ephedraceae family) is one of the oldest medicinal plants known to mankind (Hollander *et al*., 2010). The family Ephedraceae has a single genus, *Ephedra* spp.., with about 50 species worldwide (Mellado *et al*., 2019; Dousari *et al*., 2022). The Ephedraceae family is native to temperate and subtropical

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regions of Asia, Europe, Central and North America (Elhadef *et al*., 2020). At least 247 different chemical compounds, including a wide variety of alkaloids and non-alkaloids, have been found in different species of *Ephedra* genus. (Osmic *et al*., 2024). The plant has a mixture of alkaloids, including ephedrine, pseudoephedrine, norephedrine, and methylephedrine (Ibragic & Sofić, 2015; Hung *et al*., 2021). *Ephedra* is a shrub plant that has been used in China for medicinal purposes for several thousand years (Dewick *et al*., 2002). It comprises the whole plant or the aerial parts of numerous *Ephedra* species. The medicinal and therapeutic effects of these plants are related to alkaloids such as ephedrine, pseudoephedrine, norephedrine and methylephedrine, and also contain tannins and flavonoids (Dousari *et al*., 2022). The pharmacological and toxicological effects depend on the individual ephedrine alkaloid type, enantiomeric form, and receptor binding properties. Ephedrine stimulates heart rate, increases blood pressure, promotes bronchodilation, and exerts pronounced effects on the central nervous system (CNS) by binding to adrenergic receptors. It acts similarly to pseudoephedrine, but with less CNS effects (Ibragic & Sofić, 2015). Many plants with stimulant effects on the CNS synthesize substances containing phenylethylamine or xanthine structures that can enhance Catecholaminergic effects and/or act on adrenoreceptors (Carlini *et al*., 2003). In the past, ephedrine alkaloids have been used in the treatment and/or prophylaxis of various conditions, including asthma, nasal congestion, hypotension induced by spinal anesthesia, and urinary incontinence (Ibragic & Sofić, 2015). Additionally, various secondary metabolites such as alkaloids (amphetamine type, imidazole, quinoline, pyrrolidine, and others), flavonoids (flavonols, dihydroflavonol, flavanone, flavonols, flavones, anthocyanin), tannins (dimmer, trimmer and tetramer of proanthocyanidins), lignans, naphthalenes, esters, terpenoids, phenolic acids, and quinones have been reported in plants of the genus *Ephedra*. However, some *Ephedra* species have anti-inflammatory, antiviral, hepatoprotective, antibacterial, and antifungal activities as well as anticancer activities (Zhang *et al*., 2018; Mellado *et al*., 2019; Zhu *et al*., 2023).

When looking at the studies, the medicinal and therapeutic properties of various *Ephedra* species were evaluated through pharmacological research. The aim of this study was to determine the compound content in the *Ephedra major* Host species growing in Türkiye and the *Ephedra equisetina* Bunge species growing endemic in Kazakhstan by various phytochemical analyses and to investigate their DNA protective activities.

2. METHOD

2.1. Plant Materials and Extraction Process

Ephedra equisetina: Kazakhstan, Almaty, Kungei Alatau (Kolsai) N 42°59'43,1" E 78°19'36,7". *Ephedra major*: Türkiye, Van, Gürpınar, Taşdöndüren village roadsides N 38°16'59" E 43°48'52,90". Dr. Murat Ünal identified both species. Plant samples were dried and crushed for extraction. 350 mL of methanol was combined with approximately 7 g of material to create the extract, which was maintained at room temperature and in darkness while being magnetically stirred for 10 days. The solution was subsequently filtered using filter paper. Methanol was evaporated using an evaporator apparatus. Resultant samples were preserved at -40°C until utilized.

2.2. Reagents

Folin-Ciocalteu Reagent, Sodium carbonate (Na2CO3), Gallic acid, Quercetin, Trolox, Sodium nitrite ($NaNO_2$), Aluminum Chloride (AlCl3), Sodium hydroxide (NaOH), $CuCl_2$, ethanolic neocuprine, Ammonium acetate, Sodium acetate, $FeCl_3$, DPPH, Ethanol, Methanol, pBR322 plasmid DNA, ascorbic acid, $FeCl_3$, Hydrogen peroxide. All chemicals used were supplied by Sigma Aldrich.

2.3. Preparation of Extracts and Biochemical Analyses

Dry plant samples were pulverized. 4 grams of sample were mixed using 40 mL of methanol on a rotary shaker at 350 rpm for 3 days. After centrifuging at 5000 rpm for 10 min and passing

through a 0.45 µm membrane filter, it was filtered into tared flasks. Then, the solvent was evaporated with the help of an evaporator at 40°C. Extracts were dissolved at a concentration of 1 mg/mL with 1% DMSO and used for further analysis.

2.3.1. *Total phenolic substance amount*

The determination of total phenolic substance amount is carried out by measuring the absorbance of the blue color formed by the reduction of Folin-Ciocalteu reagent (Slinkard & Singleton, 1977). The color intensity formed is directly proportional to the phenolic substance concentration. Thus, the total amount of phenolic substance in the analyzed sample is calculated. In this method, 0.5 N Folin-Ciocalteu reagent and 10% concentration Na_2CO_3 are prepared. Dissolve 1 g of plant extracts in 46 ml of distilled water, then add 1 ml of Folin-Ciocalteu reagent. Thoroughly combine after three minutes. Introduce 3 ml of 10% sodium carbonate (Na_2CO_3) and allow the mixture to stand for 1 hour with periodic agitation, then measure the absorbance at a wavelength of 760 nm (Singleton & Rossi, 1965). The phenolic compound gallic acid is used in the preparation of the standard chart. Different concentrations of gallic acid within methanol (10.0-8.0-6.0-4.0-2.0-1.0-0.5-0.25-0.1 mg/mL) were prepared and their absorbances were read. A graph of absorbance versus concentration was plotted. According to the graph, the total phenolic substance amount of the samples was determined as Gallic acid equivalent.

2.3.2. *Total flavonoid content*

In the determination of total flavonoid amount, pink color formation is observed in direct proportion to the concentration of flavonoid amount. Thus, the total amount of flavonoids in the sample analyzed is calculated. In this method, 1 g of the extracts was dissolved in 1.5 mL of ethyl alcohol, followed by the addition of an equivalent volume of $AlCl_3$ (10% in 100 mL of methanol). The absorbance of the samples was read at 510 nm after 15 minutes of incubation.

Quercetin is used in the preparation of the standard graph. Different concentrations of quercetin standard within methanol (1-2.5-5.0-10.0-20.0-40.0 mg/mL) were prepared, and their absorbances were read. An absorbance graph against concentration was drawn. According to the graph drawn, the total flavonoid amount of the samples was determined as quercetin equivalent (Park *et al*., 2008).

2.3.3. *Determination of antioxidant activity by DPPH method*

The method suggested by Benvenuti *et al*. (2004) was used to determine antioxidant activity using the DPPH method. The method is based on measuring the decrease in color resulting from the inhibition of the DPPH radical at a wavelength of 517 nm. 140 µL of ethanol was added to 10 µL of the sample, and 50 µL of 1 mM DPPH radical was added and incubated for 15 minutes at room temperature in the dark. Absorbance values of the samples were recorded on a UV-Vis Spectrophotometer at a wavelength of 517 nm.

Different concentrations of Trolox with ethanol (0.2-0.5-1.0-2.0-4.0 mg/mL) were prepared as a standard, and absorbance values were recorded, and absorbance versus concentration graphs were drawn. According to the graph, the antioxidant activity of the samples was determined in terms of Trolox equivalent.

2.3.4. *Determination of antioxidant activity by CUPRAC method*

The determination of antioxidant activity with the CUPRAC method is based on the principle of reducing Cu+2 ions. Concentration amounts were modified based on the method of Apak *et al*. (2007). In the method, 0.015 M ethanolic neocuproine was dissolved in ethanol. 0.02 M CuCl2 and 2 M Ammonium acetate buffer were dissolved in pure water, and the buffer pH was adjusted to 6.5. Different concentrations of Trolox with ethanol (9.0-6.0-3.0-1.8-1.4-1.0-0.6-0.3 mg/mL) were prepared as a standard, and after 1/2 h absorbance values were recorded (A450), and absorbance versus concentration graphs were drawn. According to the graph, the antioxidant activity of the samples was determined in terms of Trolox equivalent.

2.3.5. *DNA preservation activity of samples*

It was investigated whether plant extracts had a protective effect on pBR322 (Thermo Sci) plasmid DNA. The DNA protection and DNA breakage effects of the samples were determined according to the method of Akkemik *et al*. (2022). Briefly, plant extracts were prepared in a solution consisting of a mixture of methanol (10%) and water (90%). Two different extract concentrations (0.5 mg/mL and 1 mg/mL) were applied for each plant and for experimental design, negative (plasmid DNA only) and positive control (Fenton + plasmid DNA) groups were created. The incubation reaction mixtures consisted of 3 µL of pBR322 plasmid DNA, 5 µL of Fenton's solution (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$), and 5 µL of plant extracts at varying concentrations (0.5 mg/mL and 1 mg/mL), with the total volume adjusted to 20 µL using distilled water. The mixture was thereafter incubated at 37°C for 30 minutes. Subsequent to incubation, 5 µL of 6x loading dye was incorporated into the reaction mixture. 10 µL of the mixture were extracted and applied on a 0.8% agarose gel stained with ethidium bromide. The agarose gel was electrophoresed at 100 volts for 60 minutes. The gel image was captured using the gel imaging apparatus.

3. RESULTS and DISCUSSION

3.1. Total Phenolic and Total Flavonoid Content of *Ephedra equisetina* and *Ephedra major*

Phytochemicals such as flavonoids and phenolics obtained from plants have been reported to have positive effects on many diseases (Kumar & Goel, 2019; Sankaranarayanan *et al*., 2019). The importance and interest in plants rich in phytochemicals is increasing day by day, not only in the medical and pharmaceutical industries but also in the cosmetics, beverage, paint, and food sectors. Although the *Ephedra* genus is widely used in traditional medicine, it carries potential side effects and addiction risks due to its ephedrine content (Osmic *et al*., 2024). Therefore, it should be used in a controlled and careful manner in modern medicine. It is important to reveal the relationship between the potential for pharmacological activity and the phytochemical composition of plant extracts.

Literature indicates that studies measure the total polyphenol, total flavonoid, and total anthocyanin content of *Ephedra* sp. utilized gallic acid and cyanidin-3-lucoside (chrysanthemin, 12) as standard compounds, expressed as quercetin or catechin equivalents. Approximately 0.29% of the principal components of the *Ephedra* species comprised flavonoids, flavanols, dihydroflavonols, dihydroflavonoids, flavonols, and anthocyanins. (Elhadef et al., 2020; Shuang-Man *et al*., 2020; Dousari *et al*., 2022). In another study conducted with *Ephedra* species, the highest alkaloid content was found in E. equisetina (Chen *et al*., 2019).

The total phenolic amount of the samples was determined in terms of Gallic acid as shown in Table 1. The standard graph created using gallic acid is shown in Figure 1. The total flavonoid amount of the samples was determined in terms of Quercetin as shown in Table 1. The standard graph created using Quercetin is shown in Figure 2. When looking at the amount of flavonoids according to Table 1, it was determined that the *E. major* species was higher than the *E. equisetina* species. Again, when looking at Table 1, it was seen that the *E. equisetina* plant had a higher phenolic substance amount than *E. major*.

Table 1. The total phenolic and flavonoid substance amount of the samples.

	TPC mg GAE/mL	TFC mg QE/mL
Ephedra equisetina	0.262 ± 0.009	0.184 ± 0.007
Ephedra major	0.248 ± 0.010	0.185 ± 0.014

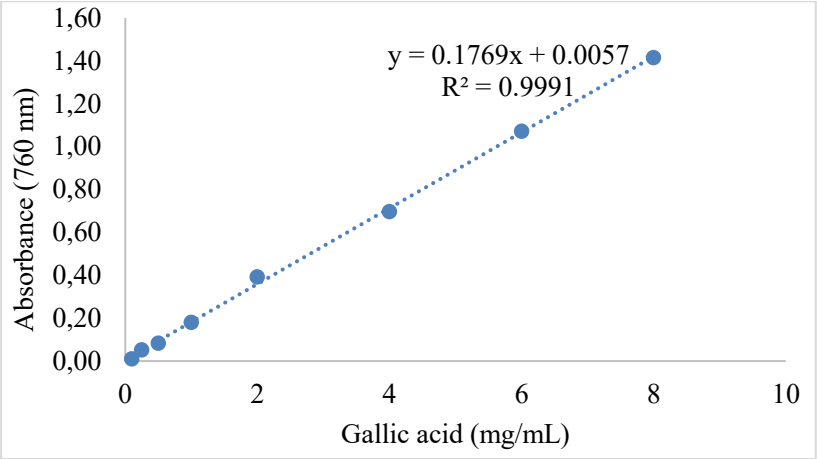

Figure 1. Gallic acid standard chart.

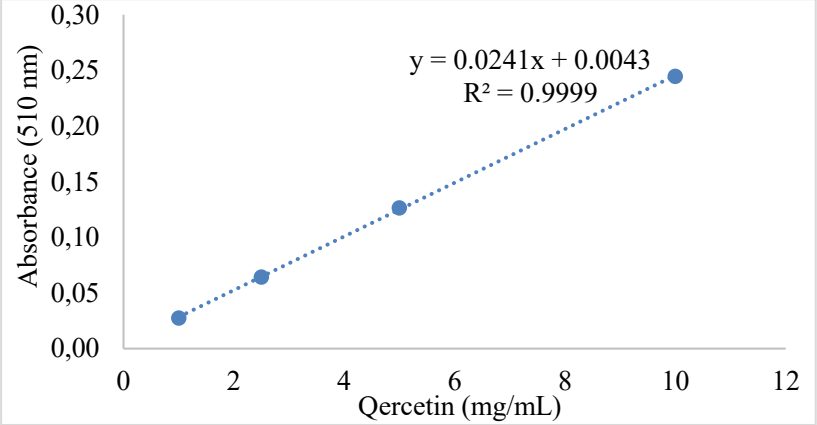

Figure 2. Quercetin standard chart.

3.2. Antioxidant Activity of *Ephedra equisetina* and *Ephedra major* Samples

Plants are the main source of natural antioxidants. Plants have strong antioxidant potential with the phenolic acids, flavonoids, terpenes, vitamins and nitrogenous compounds they contain. Various methods have been developed to evaluate the effects of these antioxidant components and their DNA protection activities have begun to be revealed (Zheng *et al*., 2023).

DPPH and CUPRAC antioxidant activities of the samples were determined in terms of Trolox as shown in Table 2. The standard graph created using Trolox is shown in Figure 3. Table 2 shows that DPPH and CUPRAC antioxidant activities were higher in *E. equisetina* than in *E. major*.

Table 2. Antioxidant activity of samples.

	DPPH mg TE/mL	CUPRAC mg TE/mL
Ephedra equisetina	0.498 ± 0.031	2.718 ± 0.128
Ephedra major	0.457 ± 0.008	2.657 ± 0.269

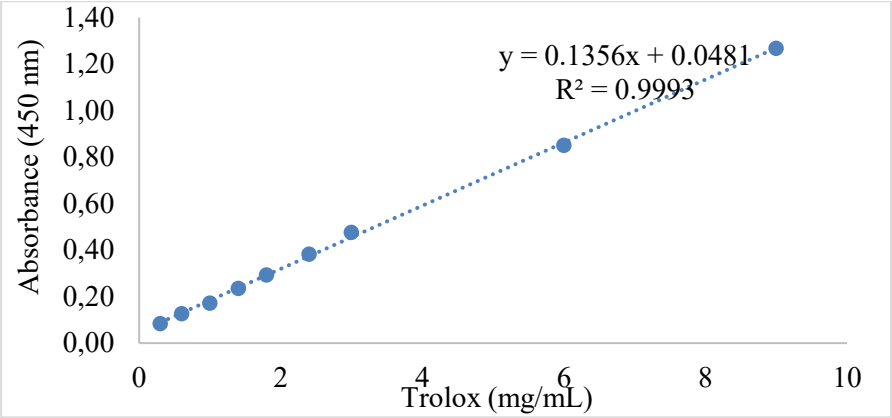

Figure 3. Trolox standard chart.

3.3. DNA Protection Activity of *Ephedra equisetina* and *Ephedra major* Samples

When DNA is exposed to hydrogen peroxide (H_2O_2), it causes open-ended DNA breakage and DNA breaks (chromatid and chromosome breaks). Genetic disorders can occur as a result of breaks in the DNA chain. Irreversible DNA damage can lead to cancer, aging, and other degenerative diseases (Tao *et al*., 2003). Therefore, it is extremely important to investigate the effects of pharmacologically active substances of plants on DNA protection. Since there is no study in the literature on how *E. equisetina* and *E. major* affect the genetic material, DNA protective activity was examined.

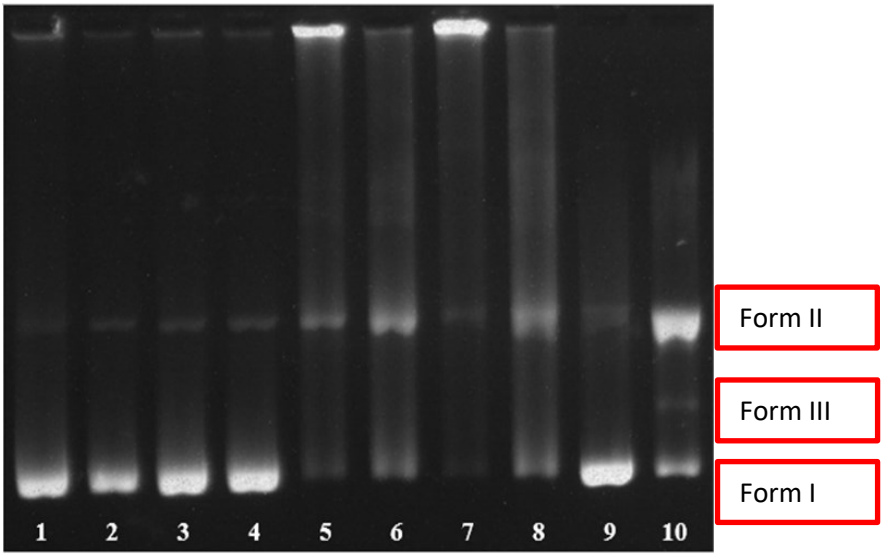

Figure 4. DNA protection gel image.

Note: 1-2) *Ephedra equisetina* (1-0.5 mg/mL), 3-4) *Ephedra major* (1-0.5 mg/mL), 5-6) *Ephedra equisetina* (1-0.5 mg/mL) + Fenton, 7-8) *Ephedra major* (1-0.5 mg/mL) + Fenton, 9) Negative control (without Fenton), 10) Positive control (with Fenton).

The protective/cleavage effect of *E. equisetina* and *E. major* extracts on plasmid pBR322 DNA against the Fenton reaction was examined using agarose gel electrophoresis (see Figure 4). pBR322 plasmid DNA runs as Form I and Form II on agarose gel. However, when plasmid DNA is damaged by the hydroxyl radical (OH-) formed by the Fenton reaction with H_2O_2 and Fe(II) or Fe(III), the resulting forms (Form I superhelical; Form II relaxed, Form III linear) have different walking speeds in the agarose gel. Form III represents the double-stranded breaks that occur after damage, hence the linear form, and appears between Form I and Form II on the agarose gel (Zhao *et al*., 2005). DNA damage is characterized by a decrease in supercoiled DNA forms or an increase in relaxed or linear DNA forms after oxidative attack.

When the DNA Gel image is examined in wells 1-4, it is seen that *E. equisetina* and *E. major* extracts do not cause DNA damage and are similar to the negative control well no. 9. When we look at the wells containing Fenton, Form III, which was seen in the positive well number 10, was not detected in wells number 5-8. This shows that *E. equisetina* and *E. major* extracts prevent the formation of the linear DNA form formed by the Fenton reaction, thus preventing the formation of DNA damage. In wells 5 and 7, it is shown that 1 mg/ml extract in the presence of Fenton does not allow plasmid DNA to leave the well, it is easier to walk in the gel at lower concentrations, and the protective effect continues at lower concentrations.

4. CONCLUSION

To ensure the conscious use of *Ephedra* spp. in terms of efficacy and safety in both traditional and modern medical practices, it is of great importance to understand the phytochemical content of these plants. In this context, our study aimed to determine the preliminary phytochemical composition of *Ephedra equisetina* and *Ephedra major* and to determine their DNA protective activity. As a result of the analyzes made in this direction, it was seen that both species of the *Ephedra* genus contain phenolic and flavonoid substances and have antioxidant activity and DNA protection effect. The phytochemical profile and DNA protective effect of *Ephedra* species have shown that these plants can be evaluated in traditional medicine practices. However, further research is needed to determine their safe use as medicine.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Farkhad Yeskendirov: Investigation, Resources. **Nashtay Mukhitdinov**: Supervision. **Murat Ünal**: Methodology, Supervision, and Validation. **Nasip Demirkuş**: Supervision. **Bedia Bati**: Writing – original draft, review. **Ayşe Yenilmez**: Conceptualization, investigation, methodology, software, writing – original draft, review and editing.

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