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Comparison of antibacterial and cytotoxic activities of *Achillea filipendulina* plant extracts obtained using deep eutectic solvent and ethanol

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Abstract

Achillea filipendulina is a flowering perennial herb belonging to Asteraceae family that grows in meadows and roadsides. It is used in traditional medicine and is reported to have antimicrobial and antioxidant activities. Deep eutectic solvent (DES) is considered as alternative-systems due to its non-toxic structure. This study aimed to compare the antibacterial and cytotoxic activities of DES and ethanol extracts of flowers of *A. filipendulina*. In antibacterial assays, MIC values of DES and ethanol extracts were found to be 12.5 and 6.25 mg/mL for *E. coli*, 25 and 12.5 mg/mL for *S. aureus*, respectively. In the disk diffusion method, ethanol extracts are more effective than DES extracts. The IC_{50} value of ethanol extract was 239.1±2.6 µg/mL while that of DES extract was 1272.6±101.3 µg/mL in T24 bladder cancer cell line after 48 h. In the healthy BJ dermal fibroblast cell line, the IC_{50} values of ethanol and DES extracts were 426.7±9.8 and 1304.3±102.8 µg/mL, respectively after 48 h. The cytotoxic effect of both extracts on T24 cells is greater than BJ cells. Although ethanol extracts have higher cytotoxic and antibacterial activities, there is potential for different results to be obtained after extractions using different DES components due to their properties.

Introduction

The importance of traditional medicinal herbs, which have been used in the treatment of diseases for many years, has been increasing today. The fact that drugs derived from plants are safe, cheaper, effective, and rarely have side effects has led researchers to investigate the potential use of these plants in modern medicine. Plants especially rich in phenolic compounds are interesting for these studies (Khan *et al.*, 2019). Among these plants, *Achillea* L., which belongs to the Asteraceae family, has an important role. Even though it is native to Southwest Asia and Southeast Europe, it has a wide distribution from Eurasia to North America. These wild-growing perennial plants usually bloom in summer. There are approximately 140 species of this plant worldwide and 48 species are known in Türkiye, 24 of which are endemic. Various parts of different species belonging to the *Achillea* genus are used in traditional medicine for gastrointestinal disorders, fever, ulcers, and colds and are known to exhibit antimicrobial, anti-inflammatory, antiallergic, and antioxidant activities (Salehi, 2020; Şirin, 2023; Vojoudi *et al.*, 2024).

Achillea filipendulina, a member of the genus *Achillea* L., is also commonly known as milfoil, yellow yarrow, and nosebleed. It is a perennial plant that likes to grow in loamy and sandy soils and can be grown as drought tolerant (Asnaashari *et al.*, 2023; Vojoudi *et al.*, 2024). It has a woody, hairy, straight, and strong trunk and can reach 120 cm in length. It has green colored dense flat leaves on its few branches. The leaves are thin, long, and hairy on both sides. It usually blooms in summer and has yellow, small, densely aggregated flowers. *A. filipendulina* can grow at altitudes between

1000 and 4000 m, especially in meadows, mountain slopes, and roadsides (Liu *et al*., 2020). The aromatic leaves and flowers of *A. filipendulina* are an important resource for pharmacological and biological studies (Ebadollahi, 2017). It is known that *A. filipendulina* exhibits good anti-inflammatory, antiseptic, antiviral, antitumor, antihepatitis, anticoagulant, and antiallergic properties as well as antimalarial, antimicrobial, antifungal, and antioxidant activities (Hamzeloo *et al*., 2019; Hasimi *et al*., 2015; Kaur *et al*., 2017). In traditional medicine, it is used in the treatment of cardiovascular diseases, urinary tract disorders, gastrointestinal disorders, arthritis, gout, and malaria. It is known that this plant was used in the past as an emmenagogue, expectorant, and cough suppressant (Asghari *et al*., 2020; Asnaashari *et al*., 2023; Khan *et al*., 2019).

Plants contain specific secondary metabolites, such as phenolics and terpenoids, which can exhibit a wide range of biological activity from cancer and cardiovascular diseases to various pathogens and insect pests. The therapeutic benefit of secondary metabolites on humans makes these plants to be considered as medicinal plants (Afshari & Rahimmalek 2021). *Achillea* plant has been found to contain over a hundred compounds. The most important of these compounds are phenolics, flavonoids, tartaric esters, and low molecular weight monoterpenes and sesquiterpenes (Dokhani *et al*., 2012). It is thought that *A. filipendulina* contains secondary metabolites at different rates at various stages of the development period. In a recent study, it was found that the plant contained maximum phenolic and flavonoid content in the middle of the flowering period and these contents decreased towards the end of this period. Total phenolic content reached 93.7 mg tannic acid equivalent/g dry weight and total flavonoid content reached 14.2 mg quercetin equivalent/g dry weight in the middle of the flowering period (Afshari & Rahimmalek, 2021). The extracts obtained from flowers and leaves were found to contain 11 different essential phenolics and flavonoids, mainly chlorogenic acid, cinnamic acid, and apigenin. In addition to flavonoids, the extracts contain significant amounts of tannins, saponins, alkaloids, glycosides, terpenoids, and steroids (Asghari *et al*., 2020; Khan *et al*., 2019). In the study on the essential oil content of flowers, leaves, stems, and above-ground parts of *A. filipendulina*, it was determined that 0.67%, 0.77%, 0.11%, and 0.67% yields were obtained, respectively. This may change the ratio of essential oil components in different parts (Vojoudi *et al*., 2024). However, the main components are known to be santolina alcohol, borneol, α-pinene, 1,8-cineole, and chrysanthenyl acetate (Asghari *et al*., 2020; Şirin, 2023). It can also contain a significant amount of 2,7-Dimethyl-4(E),6-octadiene-2-ol, terpinene-4-ol, geraniol, bornyl acetate, especially in the middle of the flowering period (Afshari & Rahimmalek, 2021).

Investigations on the therapeutic efficacy of *A. filipendulina* have been limited. Studies on the bioactive properties of its extracts are quite scarce and mainly focused on the bioactivity of the essential oil content (Khan *et al*., 2019). Basically, essential oils from *A. filipendulina* are reported to be an easily accessible source of natural antioxidants (Hasimi *et al*., 2015). Among the studies, methanol extract from the plant leaf was found to be highly effective in terms of both DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity and hydrogen peroxidase radical scavenging activity (Khan *et al*., 2019). In addition to its antioxidant potential, the high content of monoterpenes in the essential oil components indicates strong antimalarial activity (Asnaashari *et al*., 2023). Studies have shown that *A. filipendulina* oil extracts exhibited antimalarial activity against chloroquine-sensitive D6 strain and chloroquine-resistant W2 strain of *Plasmodium falciparum* without being cytotoxic to healthy mammalian cells. IC_{50} values against these strains were calculated as 0.68 mg/mL and 0.9 mg/mL, respectively. It also showed antifungal activity against *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides* pathogens (Şirin, 2023). Essential oils of *A. filipendulina* displayed high antibacterial activity against different gram-positive and gram-negative bacteria (Salehi, 2020). In addition to all these bioactivity properties, *A. filipendulina* has been found to have acaricidal and insecticidal effects. Essential oils isolated from the above-ground part of the plant demonstrated strong toxicity against important plant pests such as *Oryzaephilus surinamensis* and *Tetranychus urticae* (Ebadollahi, 2017).

Organic solvents used in extraction systems are obtained from non-renewable sources and have toxic properties for both the environment and human health. As alternative extraction solvents, deep eutectic solvent (DES) systems are accepted as green solvents (Alam *et al*., 2021). DESs show some advantages as solvents, especially considering that they are easy and cheap to prepare, non-flammable, non-toxic, natural, biodegradable, and the precursors used are renewable. A DES consists of a mixture of a hydrogen acceptor (usually choline chloride) and a hydrogen bond donor (usually natural plant-based organic ions such as amino acids, carboxylic acids, sugars, etc.) combined by hydrogen bonding in the solid state (García *et al*., 2016).

Green solvents, including deep eutectic solvents, bio-based solvents, and supercritical fluids, offer various advantages, such as reduced environmental impact, low toxicity, and biodegradability, compared to the traditional solvents (Almohasin *et al*., 2023). Previous studies on *A. filipendulina* have focused on traditional solvents. Information on the effects of ethanol and DES extracts of this plant on cancer cells (T24) and healthy cells (BJ) and their antibacterial properties is quite limited. The aim of this study was to compare the antibacterial and cytotoxic activities of flowers of *A.*

filipendulina extracts obtained using DES (choline chloride:urea (1:2)) and ethanol. Herein, the total phenolic, total flavonoids, and total sugar contents, and antioxidant activities of the extracts were also determined.

Materials and Methods

Plant preparation and extraction

The floral parts of the dried *A. filipendulina* were ground into powder and ultrasound-assisted extraction was carried out using DES (choline chloride:urea (1:2)) and ethanol. In order to reduce the viscosity of the DES solution, 30% distilled water by volume was added. The ethanol solution was diluted to 70% by using distilled water. 1 g of plant powder was mixed with 10 ml of solvent, vortexed, and extracted in an ultrasonic water bath for 40 min at room temperature by applying 100 W power. Solid-liquid separation of the obtained solution was carried out by centrifugation (10 min at 3500 rpm). The supernatant was taken, and further analysis was carried out.

Biometabolite analysis of the extracts

Total sugar amounts, total phenolic content, total flavonoid content, and antioxidant activities of the extracts were determined and compared.

Total sugar amounts

Dubois method was used to measure the total sugar content in the extracts. The standard solution was glucose. Firstly, 0.5 mL of the extract was taken and transferred to a glass tube. DES and ethanol solutions were used for the blank. 0.5 mL of 5% phenol solution was added to the tubes. Then 2.5 mL of concentrated sulphuric acid was added and vortexing was performed. After 15 min in a water bath, which is at room temperature, the absorbance value was recorded in a spectrophotometer at 490 nm wavelength. The results were calculated according to the glucose standard (Dubois *et al.*, 1956).

Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method. 200 µL of the 0.2 N Folin-Ciocalteu reagent was added to 100 µL of the extracts. Then 2 mL of distilled water and 1 mL of 6% sodium carbonate solution (Na_2CO_3) were added and vortexed respectively. The mixture was kept in dark conditions and at room temperature for 2 h. The absorbance of the resulting solution was read at 765 nm in a spectrophotometer. Gallic acid was used as the standard phenolic compound. The total phenolic contents of the samples were calculated as the gallic acid equivalent (GAE) (Miceli *et al.*, 2009).

Total flavonoid content

The total flavonoid content of the extracts was evaluated by aluminium chloride colorimetric assay. 25 µL sample extract was mixed with 100 µL of distilled water and 7 µL of 5% $NaNO_2$ in 96-well plates. The plate was kept at room temperature for 5 min. Then 7 µL of10% $AlCl_3$ was added, and incubated for 5 min. Afterwards, 50 µL of 1M NaOH and 60 µL of distilled water were added to the mixture. The absorbance of the mixture was measured at 490 nm. Total flavonoid content in the sample extracts was expressed as catechin equivalent (CE) (Molan *et al.*, 2014).

Antioxidant activity analysis

The antioxidant activity test was performed according to DPPH method. 50 µL of the extracts were taken into test tubes and the final volume was made up to 200 µL with methanol solution. Then 3.8 mL of 0.1 mM DPPH solution was added. The tubes were vortexed and kept in the dark for 30 min. Absorbance values were then read at 515 nm in a spectrophotometer. The same procedure was carried out with the DES and ethanol, respectively as a positive control. The results obtained in this method were expressed as inhibition percentages. For comparison, the same procedure was applied for BHA (Butylated hydroxyanisole) (Seyrekoğlu & Temiz, 2020).

$$\text{Inhibition\%} = \frac{(Absorbance_{\text{positive control}} - Absorbance_{\text{sample}})}{Absorbance_{\text{positive control}}} \times 100$$

Antibacterial activity analysis

The antibacterial activities of the extracts were determined by minimum inhibitory concentration (MIC) and disk diffusion methods using gram-negative bacteria *Escherichia coli* (ATCC 25922) and gram-positive bacteria *Staphylococcus aureus* (ATCC 25923). Firstly, bacteria were cultured in Nutrient Broth medium at 37 °C for 24 h. Then, to determine the MIC in 96-well plates, samples prepared at different concentrations were incubated with a bacterial culture containing 5×10^5 CFU/mL cells in each well for 24 h at 37 °C. After incubation, the MIC value was determined by measuring absorbance in a spectrophotometer at 600 nm wavelength. In the disk diffusion method, the bacteria's turbidity was diluted so that the absorbance at 600 nm wavelength corresponded to 0.6, and then it was inoculated onto Mueller Hinton Agar. After inoculation, the samples were placed on agar by impregnating sterile antibacterial susceptibility (Oxoid) disks with a diameter of 6 mm in a volume of 25 µL. Petri dishes were then incubated at 37 °C for 24 h, and antibacterial inhibition zones were determined by measuring the zone diameters around the disks. The experiment was repeated three times. Gentamicin antibiotic was used as a positive control and the solvent of each sample was used as a negative control (Abdel-Mohsen *et al.*, 2016; Maltaş *et al.*, 2010; Saravanan *et al.*, 2018; Singh *et al.*, 2023).

Cytotoxicity analysis

The cytotoxic activities of the extracts on T24 human bladder cancer cell line (ATCC HTB-4, passage 25) and BJ dermal fibroblast cell line (ATCC CRL-2522, passage 24) were determined by MTT method. The cells were grown as monolayer cultures in 75 T-flasks at 37 °C, in an atmosphere of 5% CO_2 in air. The culture medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine (200 mM), and 1% penicillin/streptomycin (10,000 units/mL penicillin, 10,000 µg/mL streptomycin). For MTT assay, 6×10^3 cells/well were plated into 96-well plates and incubated for 24 h before addition of extracts. After incubation time, the concentration of 10, 100, 500, 1000, 2500, and 5000 µg/mL of ethanol and DES extracts of *A. filipendulina* were treated against each cell line. The plates were incubated at 37 °C for 48 h and 72 h. At the end of the incubation period, 10 µL of MTT reagent (5 mg/mL) in phosphate-buffered saline (PBS) was added to each well. The plates were incubated at 37 °C for 3.5 h. After this period, the medium was removed and 100 µL of DMSO was added to each well. The formazan salts were quantified via reading the absorbance at 570 nm using a microplate reader. The cytotoxicity value was presented as IC_{50} of the extracts compared to control.

Statistical analysis

Statistical analysis of the total sugar, total phenolic, and total flavonoid content was performed by student t-Test after comparison of the data distribution by Shapiro-Wilk normality test ($P < 0.05$). One-way ANOVA using the Kruskal-Wallis test was applied for statistical analysis of the antioxidant activity data depending on the type of extraction solvent ($P < 0.05$) (Afshari & Rahimmalek, 2021). In the antibacterial test, MIC values were evaluated statistically based on bacteria type for each extract, while disk diffusion method was analyzed according to extraction method considering solvent control groups. In cytotoxic activity test, statistical analysis of the IC_{50} data was performed via two-way ANOVA to determine based on the incubation period and type of solvent ($P < 0.05$) (Khanavi *et al.*, 2012).

Results and Discussion

A. filipendulina is among the plants frequently used in traditional medicine and can exhibit important biological activities, especially due to its phenolic content. In a few studies conducted with this plant, researchers focused on antibacterial and antioxidant activities (Afshari *et al.*, 2018; Aminkhani *et al.*, 2020). Especially the biological activities of essential oils obtained from this plant have been evaluated (Aminkhani *et al.*, 2020; Afshari & Rahimmalek, 2021). In this study, total phenolic, total flavonoid, and total sugar contents of DES and ethanol extracts obtained from *A. filipendulina* were determined and their antioxidant, antibacterial, and cytotoxic activities were compared.

Biometabolite analysis of the extracts

Total sugar, total phenolic, and total flavonoid contents of the extracts were determined according to the relevant methods (Table 1).

Table 1. Total sugar, total phenolic and total flavonoid contents of ethanol and DES extracts of *A. filipendulina*

	DES extract	Ethanol extract
Total sugar (mg/mL)	0.431 ± 0.09[a]	0.472 ± 0.03[a]
Total phenolic (mg GAE/mL)	0.569 ± 0.06[a]	0.532 ± 0.03[a]
Total flavonoid (mg CE/mL)	0.518 ± 0.05[a]	0.337 ± 0.02[b]

Sugars or their derivatives are used as raw materials in the synthesis of many bioactive compounds in plants such as phenolic compounds and flavonoids. The total amount of sugar content in the plant can increase the synthesis and bioavailability of bioactive compounds.

Also, sugars can indirectly support antioxidant activity because they are involved in the production of many antioxidant compounds (Xie *et al.*, 2016). Therefore, the total sugar content of the extracts was investigated. The total sugar content of the extracts was 0.472 ± 0.03 mg/mL for the ethanol extract and 0.431 ± 0.09 mg/mL for the DES extract. Total phenolic content was close in both extracts (0.569 ± 0.06 mg/mL for DES extract; 0.532 ± 0.03 mg/mL for ethanol extract), while flavonoid content was much richer in DES extract (0.518 ± 0.05 mg/mL). At this point, it is quite remarkable that DES extract obtained higher flavonoid content than ethanol extract (0.337 ± 0.02 mg/mL).

It is reported in the literature that the total phenolic and flavonoid content of *A. filipendulina* may present varied results at different developmental stages. Samples taken in the middle of the flowering period have the highest phenolic and flavonoid content, while variables such as location of the collection and extraction method can also affect the results (Afshari & Rahimmalek, 2021; Khan *et al.*, 2019).

Antioxidant activity analysis

The antioxidant activities of DES and ethanol extracts of *A. filipendulina* were evaluated by the DPPH free radical scavenging method (Figure 1). The results showed that DES and ethanol extracts inhibited free radicals by 88.74% and 92.23%, respectively. On the other hand, the inhibition % value of BHA standard was 97.1%. It has been shown that both extracts possess a high level of antioxidant activity and can effectively scavenge free radicals. Especially the ethanol extract exhibited a higher antioxidant capacity. DES extract also displayed a significant level of activity. The BHA control exhibited statistically significant results than the DES extract ($P < 0.05$). However, no statistical difference was observed between ethanol and DES extracts.

Antioxidants are critical in the treatment of cancer, coronary heart disease, diabetes, and various degenerative diseases. In addition to many biological-activities, the antioxidant potential of *Achillea* spp. is also known.

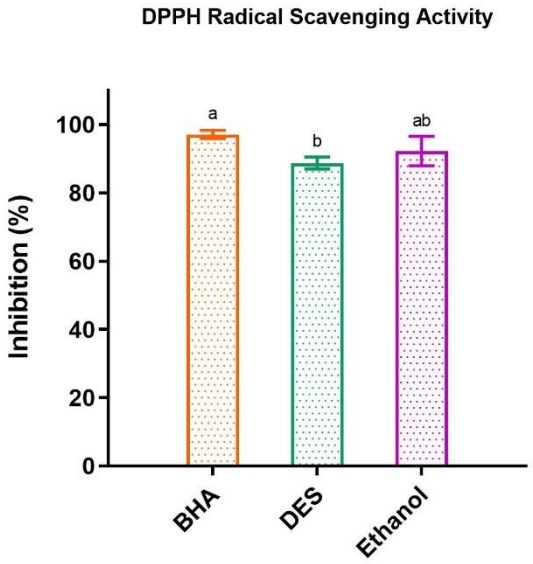

Figure 1. Antioxidant activity values of BHA and ethanol-DES extracts of *A. filipendulina*.

To date, a few studies have been performed on the antioxidant activity of *A. filipendulina* (Afshari & Rahimmalek, 2021; Asghari *et al*., 2020). Asnaashari and colleagues (2023) found that *A. filipendulina* methanol extracts and fractions of these extracts had significant antioxidant activity compared to the control group. In another study, the antioxidant effects of *A. filipendulina* methanol extracts and essential oils were analyzed by both DPPH and ferric thiocyanate (FTC) methods. By analyzing samples taken at different stages of the plant, antioxidant activity values were found at the 50% flowering stage with IC_{50} = 466.1 µg/mL, followed by the five-leaf appearance stage with IC_{50} = 727.9 µg/mL, which were the closest values to the butylated hydroxytoluene (BHT) control (Afshari & Rahimmalek, 2021). In addition to this result, Gharibi and colleagues (2015) calculated the IC_{50} value of essential oils against DPPH to be 340.62 µg/mL in their antioxidant activity test. In a different study, the antioxidant potentials of *A. filipendulina* essential oils and ethanol extracts were determined by DPPH and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) methods. As a result of the study, ethanol extracts obtained from the flower and leaf parts were found to be highly effective between 35.03-53.9 mg Trolox equivalent/g. Furthermore, essential oils also showed a strong radical scavenging activity between 15.40-25.87 mg Trolox equivalent/g (Asghari *et al*., 2020). A study evaluating the antioxidant activity of *A. filipendulina* grown under stress conditions over a wide range used 3 different model systems: DPPH test, FTC model, and linoleic model. While *A. filipendulina* showed 53.21% inhibition in DPPH test, this effect remained at low levels in FTC model and linoleic model (Gharibi *et al*., 2016). Hasimi and colleagues (2015) also found that the essential oils of *A. filipendulina* showed 55.3% inhibition in DPPH test antioxidant activity. In a study conducted by Tunç and colleagues (2024), among n-hexane, ethanol, water, and ethyl acetate extracts of *A. filipendulina,* ethanol extract demonstrated the highest FRAP value of 432.32±3.43 mg/g, and the lowest antioxidant activity with DPPH value of 18.13±1.53 µg/mL.

Antibacterial activity analysis

The antibacterial activities of the extracts were determined by both disk diffusion and MIC assays. To represent a broad spectrum of bacteria, the activity of the extracts against *E. coli* as gram-negative bacteria and *S. aureus* as gram-positive bacteria were compared. In the disk diffusion test, the results were given as zone diameter compared to the solvent control (Table 2). The lowest concentrations of the samples inhibiting the bacteria were determined by calculations (Figure 2).

Table 2. Zone diameter (mm) of DES and ethanol extracts of *A. filipendulina* against *S. aureus* and *E. coli* bacteria in disk diffusion test

	Inhibition zone (mm)	
	S. aureus	***E. coli***
***A. filipendulina* DES extract**	10 ± 0.4[a]	13 ± 0.5[b]
DES solvent control	8 ± 0.2[c]	11 ± 0.4[d]
***A. filipendulina* Ethanol extract**	9 ± 0.3[a]	15 ± 0.5[b]
Ethanol solvent control	6.5 ± 0.2[c]	7 ± 0.1[d]

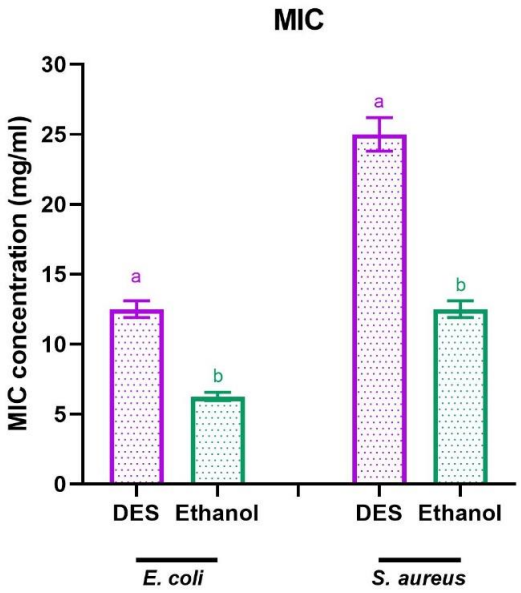

Figure 2. MIC values of DES and ethanol extracts of *A. filipendulina* against *S. aureus* and *E. coli* bacteria.

Disk diffusion test results showed that the extracts exhibited significant antibacterial activity. All tests were performed in triplicate. The highest zone diameter was detected in ethanol extract against *E. coli* (zone diameter = 15 ± 0.5 mm). When the ethanol extract was compared to the control, the difference indicated that the effect resulted from the extract. *A. filipendulina* DES extract also had a remarkable effect against the same bacteria with a zone diameter of 13 ± 0.5 mm. Against *S. aureus*, DES extract of *A. filipendulina* (zone diameter = 10 ± 0.4 mm) gave a larger zone diameter with the effect of DES solvent than ethanol extract (zone diameter = 9 ± 0.3 mm).

According to the MIC test results, DES and ethanol extracts were more effective against *E. coli*. The ethanol extract of *A. filipendulina* gave the most significant inhibition result with a MIC value of 6.25 mg/mL against this bacterium. This extract was followed by DES extract against *E. coli* and ethanol extract against *S. aureus* with a concentration value of 12.5 mg/mL. DES extract against *S. aureus* had a 25 mg/mL MIC concentration. The results of the MIC test were generally in parallel with the disk diffusion test.

A. filipendulina is an aromatic plant with antibacterial and anti-inflammatory effects, especially owing to its essential oils. Borneol, isoborneol, and their acetate derivatives that can be found in this plant are commercially used in anti-inflammatory and antibacterial creams. In addition, studies show that secondary metabolites such as santolina alcohol, carvacrol, and 1,8-cineole also contain in this plant have antimicrobial effects (Aminkhani *et al.*, 2020; Vojoudi *et al.*, 2024). In a study, the effectiveness of essential oils obtained from the stem, leaf, and flower parts of *A. filipendulina* against six gram-positive and gram-negative bacteria was evaluated by disk diffusion, MIC, and Minimum Bactericidal Concentration (MBC) tests. The essential oils extracted from different parts of the plant were found to be rich in 1,8-cineole, bormeol, and santolina alcohol, and also exhibited activity against certain bacteria. The essential oil of the stem showed antibacterial activity against *Bacillus anthracis, S. aureus*, and *E. coli*, while the essential oil of the leaf was effective against *S. aureus*, *B. anthracis*, *E. coli*, *Enterococcus faecalis,* and *Salmonella paratyphi B.* In the disk diffusion test, the extracts obtained from the stem and flower parts formed a zone diameter of 10 mm against *E. coli*, while the leaf part sample formed a zone of 15 mm. In *S. aureus*, the zone diameters were found to be 10 mm against the stem part sample and 15 mm against the leaf and flower part samples (Aminkhani *et al.*, 2020). Within the scope of this current study, the zone diameters obtained against *E. coli* and *S. aureus* are confirmed by the study. In a study using the MIC method, the antibacterial activity of *A. filipendulina* methanol extract was determined against five gram-positive and gram-negative bacteria including *S. aureus*, *Bacillus subtilis*, *Streptococcus epidermidis*, *E. coli,* and *Salmonella typhimurium*. The most effective results were obtained against *E. coli* and *S. aureus* with a MIC concentration of 32.5 µg/mL, while a MIC concentration of 50-82.5 µg/mL was observed against other bacteria (Afshari *et al.*, 2018). In the study conducted by Aminkhani and colleagues, it was observed that samples of different parts of the plant showed an effect between 12.5-25 µg/mL against *E. coli* and *S. aureus* (Aminkhani *et al.*, 2020). Tunç and colleagues (2024) explored the antibacterial activity of n-hexane, ethanol, water, and ethyl acetate extracts of *A. filipendulina* against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. aureus, E. faecalis, Streptococcus mutans, Bacillus cereus,* and *Candida albicans.* Ethanol and hexane extracts, 50 µg/mL, were effective on *S. mutans.* Also, the ethanol extract was effective against *C. albicans* and *E. faecalis.* It is predicted that by increasing the extraction yield of DES, the MIC concentration will be optimised, and the antibacterial activation potential will increase.

Cytotoxicity analysis

Bladder cancer is one of the most common malignancies in humans, especially in men, and can exhibit a high mortality rate. It has been reported that this cancer is showing an increasing tendency (Ye *et al.*, 2020). On the other hand, BJ cells, which are fibroblasts isolated from the foreskin, are good skin cell models (Radomir *et al.*, 2023). In several studies, a healthy BJ cell line was used as a control (Łukawski *et al.*, 2020; Pawlicka et al., 2022), and to evaluate the possible biological properties and cosmetic effects of extracts on the human skin (Nawrot, *et al.*, 2021). These cells were selected to determine the cytotoxicity against bladder cancer cells to investigate the potential of *A. filipendulina* extract for use in pharmaceuticals and to investigate its non-cytotoxicity against skin cells for use in medical materials targeting the skin.

The cytotoxic activities of the extracts obtained from *A. filipendulina* were evaluated on T24 human bladder cancer cell line and BJ healthy dermal fibroblast cell line following MTT assay. Percent viability results of the samples were determined both after 48 h and 72 h (Figure 3) and IC_{50} values were calculated (Table 3).

The ethanol extract of *A. filipendulina* displayed the lowest IC_{50} value in T24 cell line. In the bladder cancer cell line T24, the IC_{50} value was 239.1 ± 2.6 µg/mL at 48 h and 236.4 ± 1.1 µg/mL at 72 h. On the other hand, the IC_{50} of ethanol extract was calculated as 426.7 ± 9.8 µg/mL at 48 h and 400.6 ± 12.1 µg/mL at 72 h in the healthy cell line BJ. In DES extract of *A. filipendulina*, IC_{50} values were observed as 1272.6 ± 101.3 µg/mL at 48 h and 712.5 ± 35.8 µg/mL at 72 h in T24 cells. By contrast, in BJ cells, IC_{50} = 1304.3 ± 102.8 µg/mL at 48 h and IC_{50} = 1262.1 ± 77.1 µg/mL at 72 h. Cytotoxicity of all samples increased at 72 h. This increase in *A. filipendulina* DES extract in T24 cells is an important result which is statistically significant ($P < 0.05$). In addition, ethanol extracts have significant results compared to DES extract

Table 3. IC_{50} values of DES and ethanol extracts of *A. filipendulina* against T24 and BJ cell lines after 48 h and 72 h

	T24 cell line		BJ cell line	
IC_{50} (µg/mL)	**48 h**	**72 h**	**48 h**	**72 h**
***A. filipendulina* Ethanol extract**	239.1 ± 2.6[a]	236.4 ± 1.1[a]	426.7 ± 9.8[a]	400.6 ± 12.1[a]
***A. filipendulina* DES extract**	1272.6 ± 101.3[b]	712.5 ± 35.8[c]	1304.3 ± 102.8[b]	1262.1 ± 77.1[c]

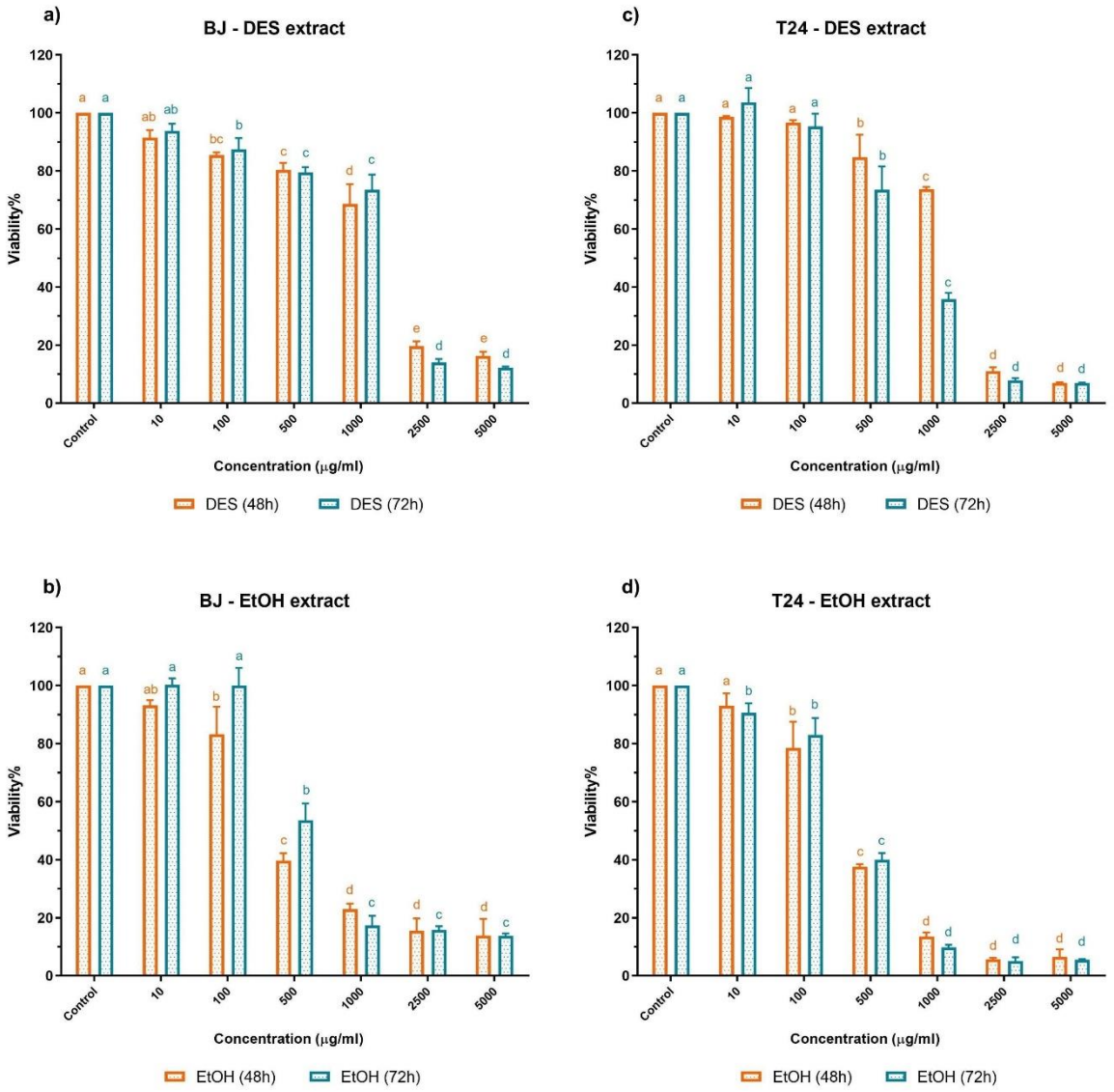

Figure 3. Percent viability of different concentrations of DES and ethanol extracts of *A. filipendulina* against T24 and BJ cell lines after 48 h and 72 h, **a)** DES extract on BJ cell line, **b)** ethanol extract on BJ cell line, **c)** DES extract on T24 cell line, **d)** ethanol extract on T24 cell line.

in both cell lines at both 48 and 72 h. Both extracts were more cytotoxic in the T24 cancer cell line than healthy BJ cell line. These results have also promoted the investigation of the use of these extracts in bladder cancer.

Species of the Asteraceae family have been reported to exhibit cytotoxic activity on various cell lines. Although cytotoxicity studies on *A. filipendulina* are quite limited, the experiments have focused on its potential. In a study, the cytotoxicity of the methanol extract obtained from this plant was determined by the MTT test in MCF-7 and MDA-MB-468 breast cancer cell lines. IC_{50} values were calculated as 386 and 248 µg/mL IC_{50} in MCF-7 and MDA-MB-468 cells, respectively. It was also shown that *A. filipendulina* was able to induce cell death through apoptosis in these two cell lines (Hamzeloo *et al.*, 2019). In another study, the antiproliferative effect of ethanol extracts and essential oils obtained from flowers and leaves of *A. filipendulina* was evaluated in Hep-G2 and MCF-7 cancer cell lines. The ethanol extracts of flowers inhibited cell proliferation by 38.16% and 26.48% against Hep-G2 and MCF-7 cell lines, respectively. The inhibition values of ethanol extracts obtained from leaves were 24.14% in Hep-G2 cells and 12.17% in MCF-7 cells, which were lower compared to flower ethanol extracts. On the other hand, the antiproliferative effect of the essential oils obtained could not be determined (Asghari *et al.*, 2020). Tunç and colleagues (2024) assessed the apoptotic effects of n-hexane, ethanol, water, and ethyl acetate extracts of *A. filipendulina.* The ethanol extract induced 14.7% apoptosis in HCT116 colon carcinoma cells, and 42.9% apoptosis in HT29 colon adenocarcinoma cells. In addition, necrosis was found to be 8.70% in HCT116 cells, and 4.25% in HT29 cells.

In this study, *A. filipendulina* plant, which has very rich activities in terms of biometabolites, was extracted for the first time with choline chloride:urea (1:2), which

is in the green solvent class and has many advantages and compared the activities with ethanol. The results obtained are promising for future studies. Although ethanol solvent is more successful in some bioactivities in the extraction, activities can be increased by changing the parameters or components in DES systems, which have recently attracted interest due to their advantages such as non-flammability, volatility, biodegradability, and biocompatibility.

Conclusion

The discovery of DESs has been an important breakthrough in the field of green chemistry. Herein, ethanol as a traditional solvent and DES as a green solvent were used to evaluate the biological activities of flowers of *A. filipendulina.* While ethanol extracts showed stronger antibacterial and cytotoxic activities, DES extracts had higher total phenolic and total flavonoid contents. Additionally, the extracts demonstrated more cytotoxic activity in the bladder cancer cell line than in the dermal fibroblast cell line. In conclusion, these properties highlight *A. filipendulina*'s potential for use in developing novel treatments for bacterial infections and cancer. However, further studies, including *in vivo* experiments as well as various cell lines and bacteria, are needed to understand their mechanisms and to isolate and characterize the specific compounds responsible for these activities.

Author Contributions

Conceptualization: TÖÖ, Aİ, Data Curation: TÖÖ, Aİ, FOÇ, Formal Analysis: TÖÖ, Aİ, FOÇ, Funding Acquisition: TÖÖ, Investigation: TÖÖ, Aİ, Methodology: TÖÖ, Aİ, Project Administration: TÖÖ, Resources: TÖÖ, Aİ, Supervision: TÖÖ, Visualization: TÖÖ, Aİ, FOÇ, Writing -original draft: TÖÖ, Aİ, FOÇ, Writing -review and editing: TÖÖ, Aİ, FOÇ

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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