PROSPECTS
IN PHARMACEUTICAL SCIENCES

Prospects in Pharmaceutical Sciences, 22(1), 1-10
https://prospects.wum.edu.pl/

Original Article

NATURAL- AND *IN VITRO*-GROWN *FILIPENDULA ULMARIA* (L.) MAXIM: EVALUATION OF PHARMACEUTICAL POTENTIAL (ANTIBACTERIAL, ANTIOXIDANT AND TOXICITY) AND PHENOLIC PROFILES

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Received: 21.11.2023 / Accepted: 06.01.2024 / Published: date

ABSTRACT

Meadowsweet (*Filipendula ulmaria* (L.) Maxim) is Rosaceae family perennial herb. Traditional uses of *F. ulmaria* include inflammatory problems like rheumatism, arthrosis, and arthritis), gastrointestinal disorders, liver malfunction, and gout. The pharmaceutical industry has been very interested in this plant because of its health benefits. The presence of phenolic secondary metabolites is thought to be the primary cause of meadowsweet's biological activity and therapeutic efficacy. This study was intended to compare and evaluate the biological activities (antibacterial, antioxidant, antitumor and toxicity) and phenolic profiles (total phenol-flavonoid content and individual phenolic constituents) of *F. ulmaria* aerial parts obtained from two different sources (naturally- and *in vitro*-grown). Antibacterial activity was evaluated using 17 different bacteria (10 human and 7 fish pathogens) with disc diffusion method. Methanolic extracts of *in vitro*-grown parts showed higher antibacterial effect than naturally-grown parts with all tested bacterial pathogens (human and fish). On the other hand, other extracts (aqueous, ethanol, hexane, and ethyl acetate) demonstrated higher antibacterial potential with field-grown parts. The highest sensitivity was observed with *Staphylococcus epidermidis*, *S. aureus* and *Enterobacter cloacae* to aqueous extract of field-grown plants. The potent antibacterial activity of *F. ulmaria* extracts rendered the *Agrobacterium tumefaciens*-induced potato disc tumor assay inapplicable. Toxicity assay (brine shrimp) showed that aqueous extract of both sources had the lowest toxicity. Methanolic extract of both parts had a strong antioxidant potential (DPPH radical scavenging activty) having IC_{50} values as 205.65 µg/mL and 206.74 µg/mL, respectively, and similar level of overall total phenol-flavonoid contents. Individual phenolic analysis with HPLC-DAD showed that the most prevalent phenol was rutin in both sources. While the quantities of chlorogenic acid in both aerial parts were similar, the parts that were grown naturally had higher levels of salicylic acid and rutin. These findings demonstrated the effective antibacterial and antioxidant properties, high phenolic content, and low toxicity of both *F. ulmaria* sources, indicating that they can be utilized in nutraceutical industry due to their high health promoting potential.

KEYWORDS: Antibacterial, antioxidant, brine shrimp, *Filipendula ulmaria*, meadowsweet

1. Introduction

Filipendula ulmaria (L.) Maxim, known as 'Çayır kraliçesi' in Turkish and meadowsweet in English, is a perennial herb belonging to Rosaceae family. It can be found in forests, wet meadows, riverbanks and also wetlands and limestones and is seen all over Europe, North America, and Siberia [1,2]. Aerial parts of *F. ulmaria* have antiseptic, alterative, aromatic, analgesic, diuretic, diaphoretic, febrifuge, stomachic, and tonic properties [3-6]. Traditional uses of *F. ulmaria* include fever, inflammatory diseases (arthritis, arthrosis, and rheumatism), pain, gastric disorders, liver malfunction, and gout [7]. It is an important plant in herbal medicine due to its salicylic acid and salicylate content [5]. The medicinal parts of *F. ulmaria* (flowers, leaves, the whole herb, and the rhizomes) contain phenolic compounds such as phenolic acids (gallic acid, salicylic acid, ellagic acid, caffeic acid derivatives), flavonoids (catechin, kaempferol, quercetin, astragalin hyperoside, quercitrin, rutin, spiraeoside), hydrolysable and condensed tannins, and salicylate aglycons and glycosides [8]. The phenolic content of *F. ulmaria* is related to its therapeutic effect. *F. ulmaria* has been demonstrated to have anti-arthritic, anti-

inflammatory, anti-coagulant, anti-oxidant, anti-cancer, anti-microbial, analgesic, immunomodulatory, hepato-protective, and gastroprotective effects in pharmacological studies [7,8].

Reactive oxygen and nitrogen species induce some oxidative damage to cellular biomolecules, including proteins, lipids and nucleic acids, which can initiate the variety of diseases such as cancer, cardiovascular disease, atherosclerosis, hypertension, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, and aging. Antioxidants as defensive compounds have a significant role to prevent the oxidation of molecules. It has also been demonstrated that the addition of antioxidants to foods prevents free radical-induced lipid oxidation and extend their shelf-life [9]. The antioxidant properties of *F. ulmaria* components are associated with the presence of a number of phenolic compounds including flavonoids, phenolic acids, tannins, salicylate aglycons and glycosides [8,10,11]. On the other hand, the utilization of plant extracts as antimicrobial agents for food preservation has been considered [12]. It was reported that plant extracts and isolated pure natural compounds from plants were potentially effective in use as additives for food preservation due to antimicrobial properties [8,13]. The clear antimicrobial activity of *F. ulmaria* extracts against some selected bacteria has been reported by several researchers [14,15]. Lately, a few studies reported on the cytotoxicity of *F. ulmaria* extracts [16,17].

F. ulmaria is particularly well-known for its therapeutic and health-promoting properties. *In vitro* culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of many plant species, including many medicinal plants. The hypothesis was tested that *in vitro* propagated *F. ulmaria* aerial parts exhibit similar biological activity and phenolic profiles to naturally grown ones. This study evaluated and compared the naturally-grown and *in vitro*-grown *F. ulmaria* aerial parts in terms of phenolic profiles, biological activities including antibacterial, antioxidant, antitumor, using some selected bioassays, and toxicity (brine shrimp assay).

2. Matherials and Methods

2.1 Plant Material and Extraction

Field-grown *F. ulmaria* aerial parts (stem and leaves) were collected from Abant Lake, Bolu, Turkey (Figure 1). Identification of the plant was done using "Flora of Turkey and the East Aegean Islands" [8] and voucher specimens were deposited at Bolu Abant Izzet Baysal University (AIBU) Herbarium, Bolu, Turkey. *In vitro*-grown aerial parts of *F. ulmaria* were collected from microropagated plants according to the previously developed *in vitro* culture protocol in our laboratory [19] (Figure 1). *In vitro* culture studies were carried out with the seeds of *F. ulmaria* growing in its natural environment, from which we collected the aboveground parts. This way, genetic variability was to be kept to a minimum level.

The extraction was performed with oven-dried (40ºC) aerial parts of field-grown and *in vitro*-grown *F. ulmaria* in the vegetative growth stage. Plant material was extracted with different solvents (water, ethanol, methanol, ethyl acetate, and hexane) at 40ºC in water bath for 18h and then filtered. After extraction, organic solvents were removed using rotary evaporator at 40°C to obtain the crude extracts. Yield (%) for each extraction was presented in Table 1. For water extraction, frozen filtrate was lyophilized by using freeze-drier at -65ºC. When extracts were used for biological activity studies and HPLC analyses, each extract was dissolved in extraction solvent to get a known final concentration.

Figure 1. Field-grown (A) and *in vitro*-grown (B) *F. ulmaria* plants.

2.2 Antibacterial Assay

Antibacterial activity was determined using a disc diffusion assay as indicated by Turker et al. [20]. As human pathogens, 3 gram positive [*Streptococcus pyogenes* (ATCC© 19615), *Staphylococcus aureus* (ATCC© 25923) and *Staphylococcus epidermidis* (ATCC© 12228)] and 7 gram negative [(*Escherichia coli* (ATCC© 25922), *Pseudomonas aeruginosa* (ATCC© 27853), *Salmonella typhimurium* (ATCC© 14028), *Serratia marcescens* (ATCC© 8100), *Proteus vulgaris* (ATCC© 13315), *Enterobacter cloacae* (ATCC© 23355) and *Klebsiella pneumoniae* (ATCC© 13883)] bacteria were investigated, and as fish pathogens, 3 gram positive (*Enterococcus faecalis*, *Lactococcus garvieae* and *Streptococcus agalactiae*) and 4 gram negative (*Aeromonas hydrophila*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Yersinia ruckeri*) bacteria were tested utilizing disc diffusion assay [21]. The McFarland Densitometer (Biosan®) was used to adjust the turbidity of each broth culture of bacteria to 0.5 before using cotton swabs to inoculate Mueller Hinton agar plates. Sterile paper discs (Glass microfibre filters, Whatman®; 6 mm diameter) were impregnated with 13 µL filter-sterilized extracts (100 mg/mL), placed on the surface of each inoculated plate, and then incubated overnight at 37ºC. Two plates were used, and each plate consisted of five replicates for each extract that was tested against each bacterium. Antibiotic discs (Bioanalyse®) were used as a positive control (for human pathogens - erythromycin, ampicillin, carbenicillin, tetracycline and chloramphenicol; for fish pathogens - furazolidone, sulphamethoxazole, erythromycin and tetracycline). Solvents were used as a negative

control. After the incubation period of 24 hours at 37°C, diameter of inhibition zones was determined for each disc. The experiments were repeated three times.

2.3 Toxicity (Brine Shrimp Bioassay)

Brine shrimps have been used to determine toxicity of *F. ulmaria* extracts through the estimation of the medium lethal concentration (LC_{50} value) according to Meyer et al. [22]. In the present study, artificial seawater was obtained by dissolving 36 g seasalt (Sigma®) in 1 liter of distilled water and MS-222 (tricaine methane sulfonate), common fish anesthetic, was prepared at different concentrations (1, 10, 100 and 1000 mg/L) in seawater as a positive control. Seawater alone was also used as a negative control. Stock solutions of the extracts were prepared in seawater to obtain 100000 mg/L solution. Then, serial dilutions were carried out with seawater to obtain the concentrations of 10000, 1000, 100 and 10 mg/L of prepared extracts. Different concentrations of the extracts or controls (2.5 mL) were added into each well of twenty-four well culture plates. In the meantime, brine shrimp eggs were incubated in seawater to hatch and mature as nauplii for 48-72 h. After the incubation, 10 hatched nauplii were placed into each well containing test solutions and incubated for 24 h at room temperature. Each concentration was tested in triplicate. After incubation, lethality to larvae for each concentration was determined by counting the dead nauplii. Using the Reed-Muench method, the lethal concentration for 50% mortality after a 24-hour exposure (LC_{50}) and 95% confidence intervals were calculated [23].

2.4 Antitumor Assay

Antitumor activity was assessed performing *A. tumefaciens*-induced potato disc tumor assay regarding McLaughlin's study [24,25] as described by Coker et al. [26]. In this method, suspensions of *Agrobacterium tumefaciens* (ATCC 23341) in phosphate-buffered saline (PBS) were standardized to $1x10^9$ Colony Forming Units (CFU). All extracts were dissolved in sterile water (100 mg/mL) and control solutions were filter sterilized with 0.22 µm filter. Camptothecin was used as a positive control for tumor suppressant and water was used as a negative control. The test solutions included 600 µl of extracts or controls, 150 µl of water, and 600 µl of adjusted bacterium suspension. Briefly, potato discs of 10-mm diameter were placed on 24 well-plates containing water-agar. Each disc was overlaid with 50 µl of prepared extract or control solutions and inoculated plates were incubated at 28 ºC in the dark for 2 weeks.

In order to demonstrate that the extract under test should not have antibacterial action toward *A. tumefaciens*, a bacterial viability test was also carried out for the *A. tumefaciens*-induced potato disc tumor assay. *A. tumefaciens* ($1×10^9$ CFU in PBS) was serially diluted until $1×10^3$ CFU and then 0.1 mL of inoculum (bacteria + extract) was inoculated on YEM media using spread plate technique after 30 min incubation. After inoculating plates for 24 hours at 28°C, colony counts were performed.

2.5 Antioxidant assay

Antioxidant potential of *F. ulmaria* methanol extracts were determined by DPPH (2,2-diphenyl-1-picrylhydrazil) photometric assay using a modified version of the Blois [27] method as described by Turker et al. [20]. To test the antioxidant capacity of the extracts, different concentrations of the extracts and quercetin were prepared in MeOH and combined with a DPPH solution (0.13 mM). Quercetin was used as an antioxidant standard. There were 3 replicates for each concentration of them. After 30 minutes in the dark, the decrease in absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Hitachi U-1900®). DPPH· scavenging effect (% inhibition) was calculated as $[(A_0-A_1/A_0) \times 100]$ where A_0 is the absorbance of the control reaction and A_1 is the absorbance after the reaction with *F. ulmaria* extracts. Each experiment was repeated 3 times.

2.6 Determination of total phenolic and flavonoid content

Folin-Ciocalteu and aluminum chloride colorimetric methods described in Turker et al. [20] were used to evaluate the total phenolic and flavonoid content of *F. ulmaria* extracts, respectively.

In the Folin-Ciocalteu method, gallic acid was used as a reference phenol for the calibration curve. The extracts were prepared as a 2000 µg/mL solution. In brief, 20 µl of the extract, gallic acid at various concentrations (0, 12.5, 25, 50, 100, 200, and 400 µg/mL) or MeOH (as a blank), 1.58 mL of distilled water, and 100 µL of Folin-Ciocalteu solution (1:1 ratio) were mixed, and after 2 minutes, 300 µL of 20% sodium carbonate (w/v) was added and mixed again, and the solution was kept at room temperature for 2 hours. At the end of 2 hours, absorbance values were measured at 765 nm, and total phenolic amounts were given as gallic acid equivalents (mg GAE/g extract).

In the aluminum chloride colorimetric method, quercetin was used as a reference flavonoid for the calibration curve. The plant extracts solutions were prepared at a concentration of 2000 µg/L. For the measurement, 500 µL of extract, quercetin solution at various concentrations (0, 6.25, 12.5, 25, 25, 50, 100, and 200 µg/mL) or MeOH were thoroughly mixed with 2 mL of MeOH and 150 µL of 5% sodium nitrite (w/v). After waiting for 5 minutes, 150 µl of 10% aluminum chloride was added to the mixture. At the 6th minute, 1 mL NaOH was added, and the solution was made up to 5 mL with 1.2 mL MeOH and measured at 410 nm in a spectrophotometer. The results are given as the equivalent amount of quercetin (mg QE/g extract).

2.7 HPLC analysis of methanol extracts

Eight different phenolic substances [1,3-dicaffeoylquinic acid (Carbosynth®), gallic acid monohydrate, chlorogenic acid, vanillic acid, rutin hydrate, rosmarinic acid, salicylic acid and apigenin (Sigma®)] were quantitatively analyzed in MeOH extracts using high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) (VWR-Hitachi LaChrom Elite®). HPLC method was performed as described by Turker et al. [20]. Separation was performed in the gradient mode, using acetonitrile (solvent A) and water containing 0.1% orthophosphoric acid (solvent B). Elution was performed with 10% of A and 90% of B at 0 min and adjusted to 20%, 40%, 60%, 80% and 10% A at 5, 10, 15, 20 and 20.1 min, respectively, at a flow rate of 1 mL/min and 25 ºC oven temperature. All chromatographic data were recorded at 270, 324, 327, 260, 255, 330, 280, and 338 nm for gallic acid, 1,3-dicaffequinic acid, chlorogenic acid, vanillic

acid, rutin hydrate, rosmarinic acid, salicylic acid and apigenin standards, respectively. HPLC analysis was carried out in triplicate.

2.8 Statistical analysis

All experiments were set up in a completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range tests using SPSS vers. 26 (SPSS Inc, Chicago, IL, USA) were used for data analysis. All data in the tables were presented as a mean number ± standard error (SE). The assessment of correlations was done using the analysis of Pearson correlation.

3. Results and discussions

3.1 Antibacterial Assay

Water, ethanol, methanol, ethyl acetate and hexane extracts of field-grown and *in vitro*-grown aerial parts were used to screen for antibacterial potential of *F. ulmaria* against 10 human pathogenic bacteria. Generally, results revealed that field-grown plant extracts, except methanolic ones, showed better antibacterial activities than that of *in vitro*-grown plants. Conspicuously, methanolic extracts of *in vitro*-grown materials showed better activity than these of field-grown materials against 8 tested bacteria, and same activity was observed against *S. pyogenes* for both extracts. Generally, *F. ulmaria* extracts exhibited broad-spectrum inhibitory activity against tested bacteria and gram-positive bacteria seemed to be more susceptible to the inhibitory effects of the extracts than gram-negative bacteria. Of all the extracts that were evaluated, none of the ethyl acetate extracts exhibited antibacterial inhibition against any bacterium (Table 1).

Although field-grown plant extracts in water, ethanol, and hexane showed efficacy against *S. marcescens*, methanolic extract was ineffective against this bacterium. The highest susceptibility was observed with *S. epidermidis* to aqueous extract (25.3 ± 0.2 mm), ethanolic extract (20.4 ± 0.6 mm) and hexane extract (18.8 ± 0.4 mm) of field-grown plants. Generally, aqueous extract of field-grown plant exhibited the best inhibitory effect against used bacteria, except *S. pyogenes* and *E. coli* (Table 1).

Among the *in vitro*-grown plant extracts, ethanol and methanol extracts were more effective against the tested bacteria than aqueous, hexane and ethylacetate extracts. Ethanol extracts of *in vitro* plant showed the best antibacterial activity against *S. epidermidis* (17.2 ± 0.4 mm) followed by *E. cloacae* (14.4 ± 0.4 mm), while methanol extract of *in vitro* plant showed the best antibacterial activity against *P. vulgaris* (15.8 ± 0.2 mm) followed by all tested gram-positive bacteria (ranging from 14 to 14.67 mm) (Table 1).

The use of antimicrobial drugs in aquaculture has led to the emergence of more resistant bacterial strains, and

Table 1. Antibacterial potential of various extracts of *F. ulmaria* from two different sources (naturally- and *in vitro*-grown) against ten human pathogens. Means with the same letter within columns are not significantly different at $P>0.05$. FW, FE, FM, FH and FEA: aqueous, ethanol, methanol, hexane and ethylacetate extract of field-grown plants; IW, IE, IM, IH and IEA: aqueous, ethanol, methanol, hexane and ethylacetate extract of *in vitro*-grown plants.

		Mean Diameter of Inhibitory Zones (mm ± SE)									
Treatments	**Yield***	***S. auerus***	***S. epidermidis***	***S. pyogenes***	***S. marcescens***	***S. typhimurium***	***P. aeruginosa***	***P. vulgaris***	***K. pneumonia***	***E. cloacae***	***E. coli***
FW	18.9	18.00 ± 0.63 ^{c}	25.30 ± 0.21 ^{c}	-	10.90 ± 0.28 ^{d}	8.00 ± 0.15 ^{e}	10.10 ± 0.23 ^{c}	15.60 ± 0.16 ^{e}	12.40 ± 0.27 ^{c}	17.90 ± 0.23 ^{e}	-
FE	15.0	14.00 ± 0.26 ^{de}	20.40 ± 0.56 ^{d}	14.70 ± 0.21 ^{e}	8.80 ± 0.13 ^{e}	-	9.50 ± 0.17 ^{d}	13.00 ± 0.21 ^{g}	10.60 ± 0.48 ^{d}	15.80 ± 0.51 ^{f}	-
FM	14.53	12.00 ± 0.37 ^{g}	11.83 ± 0.17 ^{h}	14.33 ± 0.21 ^{e}	-	-	-	11.67 ± 0.21 ^{h}	-	-	-
FH	6.0	13.40 ± 0.34 ^{ef}	18.80 ± 0.42 ^{e}	8.60 ± 0.16 ^{g}	8.10 ± 0.18 ^{e}	7.10 ± 0.10 ^{f}	-	11.10 ± 0.48 ^{h}	8.90 ± 0.31 ^{fg}	15.40 ± 0.31 ^{fg}	8.40 ± 0.16 ^{g}
FEA	5.34	-	-	-	-	-	-	-	-	-	-
IW	17.6	-	10.90 ± 0.31 ^{i}	-	-	-	-	-	8.20 ± 0.25 ^{g}	9.60 ± 0.40 ^{i}	-
IE	15.5	12.60 ± 0.22 ^{fg}	17.20 ± 0.39 ^{f}	9.50 ± 0.17 ^{f}	-	-	-	12.10 ± 0.10 ^{h}	8.20 ± 0.39 ^{g}	14.40 ± 0.37 ^{g}	-
IM	15.1	14.50 ± 0.22 ^{d}	14.00 ± 0.00 ^{g}	14.67 ± 0.21 ^{e}	-	8.67 ± 0.21 ^{e}	8.33 ± 0.21 ^{e}	15.83 ± 0.17 ^{e}	9.00 ± 0.37 ^{ef}	10.67 ± 0.21 ^{h}	10.17 ± 0.17 ^{f}
IH	4.5	-	9.20 ± 0.42 ^{j}	-	-	-	-	-	-	10.20 ± 0.4 ^{hi}	-
IEA	3.8	-	-	-	-	-	-	-	-	-	-
Chloramp		31.00±0.37 ^{b}	34.83±0.17 ^{a}	42.33±0.76 ^{d}	29.50±0.22 ^{a}	30.00±0.00 ^{a}	-	26.50 ±0.22 ^{c}	30.17±0.31 ^{a}	31.00±0.00 ^{a}	29.00± 0.00 ^{a}
Carbenicillin		34.50±0.22 ^{a}	27.00±0.37 ^{b}	59.17±0.31 ^{a}	20.50±0.22 ^{b}	26.00±0.00 ^{b}	16.33±0.21 ^{a}	35.00 ±0.37 ^{a}	-	30.00±0.00 ^{b}	24.33± 0.21 ^{c}
Erythromycin		30.50±0.22 ^{b}	35.00±0.37 ^{a}	47.67±0.21 ^{b}	11.50±0.22 ^{c}	12.00±0.00 ^{d}	-	14.00 ±0.37 ^{f}	12.00±0.45 ^{c}	-	13.33± 0.21 ^{e}
Tetracycline		31.00±0.00 ^{b}	8.67±0.21 ^{j}	44.67±0.42 ^{c}	20.50±0.22 ^{b}	26.00±0.00 ^{b}	13.00±0.00 ^{b}	31.00 ±0.37 ^{b}	26.00±0.63 ^{b}	27.00±0.00 ^{c}	27.00± 0.45 ^{b}
Ampicillin		34.50±0.22 ^{a}	24.67±0.21 ^{c}	59.00±0.37 ^{a}	0.00±0.00 ^{d}	24.50±0.22 ^{c}	-	25.67 ±0.42 ^{d}	9.00±0.45 ^{ef}	22.50±0.22 ^{d}	17.50± 0.22 ^{d}

*Yield (%) = Weight of extract (g) / 20 g of powdered plant sample * 100
'-' indicates no antibacterial activity

Table 2. Antibacterial potential of methanolic extracts of *F. ulmaria* from two different sources (naturally- and *in vitro*-grown) against seven fish pathogens. Means with the same letter within columns are not significantly different at *P*>0.05. FM: methanol extract of field-grown plants; IM: methanol extract of *in vitro*-grown plants.

	Mean Diameter of Inhibitory Zones (mm ± SE)						
Treatments	***S. agalactiae***	***L. garviaeae***	***E. faecalis***	***A. hydrophila***	***V. anguillarum***	***Y. ruckeri***	***A. salmonicida***
FM	9.00±0.00 ^{d}	8.00±0.00 ^{e}	8.33±0.21 ^{f}	10.67±0.21 ^{f}	8.00±0.00 ^{f}	-	10.67±0.21 ^{d}
IM	12.00±0.00 ^{c}	10.00±0.00 ^{d}	10.67±0.21 ^{e}	15.33±0.21 ^{e}	10.00±0.00 ^{e}	15.50±0.22 ^{d}	12.33±0.21 ^{c}
Furazolidone	-	13.33±0.21 ^{c}	18.50±0.22 ^{c}	28.67±0.21 ^{d}	17.67±0.21 ^{c}	17.00±0.63 ^{c}	33.00±0.63 ^{a}
Sulphamethoxazole	-	-	25.17±0.31 ^{a}	33.17±0.31 ^{c}	25.50±0.22 ^{a}	35.50±0.22 ^{a}	24.00±0.45 ^{b}
Erythromycin	35.33±0.21 ^{a}	29.50±0.22 ^{a}	21.33±0.21 ^{b}	36.67±0.56 ^{a}	22.67±0.21 ^{b}	14.00±0.00 ^{e}	13.50±0.22 ^{c}
Tetracycline	33.33±0.21 ^{b}	28.50±0.22 ^{b}	13.50±0.22 ^{d}	35.67±0.21 ^{b}	14.00±0.00 ^{d}	33.00±0.63 ^{b}	24.50±0.67 ^{b}

'-' indicates no antibacterial activity

the continued use of synthetic antibiotics poses a hazard to consumer health, non-target organisms, and the environment. As consequently, treating bacterial fish diseases with natural chemicals may be risk-free for all concerned organisms [28]. All tested fish pathogens were sensitive to methanolic extract of *in vitro*-grown *F. ulmaria* parts. Naturally-grown aerial parts showed lesser activity than *in vitro*-grown parts against fish pathogens. The highest antibacterial activity was observed against *Y. ruckeri* (15.50 ± 0.22 mm) and *A. hydrophila* (15.33 ± 0.21 mm) with *in vitro*-grown plants. One previous study showed lower antibacterial activity of methanol extract of naturally-grown leaf and flower parts as 11.13 ± 0.30 mm against *A. hydrophila* and 10.25 ±0.31 mm against *Y. ruckeri* as well as no activity against *S. agalactiae*, *E. faecalis* and *L. garviae* [28]. Similarly, methanol extract of naturally grown aerial parts showed antibacterial activity against *A. hydrophila* (10.67 ± 0.21 mm) but no activity against *Y. ruckeri* in the present study. Also, weak antibacterial effect of this extract was observed against *S. agalactiae*, *E. faecalis* and *L. garviae* (Table 2).

Positive controls (reference antibiotics) generally showed antibacterial activity to our test microorganisms (Table 1 and 2). Because final concentrations of all extracts were adjusted with extraction solvent (water, ethanol, methanol, ethyl acetate and hexane), they were used as a negative control and there was no inhibition with these solvents.

Similar to our findings, antimicrobial activity of *F. ulmaria* aerial part was screened against some microbial species using the Cylinder diffusion method and results of this study showed that aqueous methanolic extract had a bacteriocidal activity against *S. aureus* and *E. coli* [14]. Katanic et al. [8] showed strong antibacterial activity of methanolic extract of *F. ulmaria* aerial part against *E. coli*, *E. faecalis*, *P. aeruginosa* and *K. pneumonia*. In another study, Denev at al. [29] investigated aerial parts of *F. ulmaria* against some foodborne pathogens and observed that acetone extract (in 2% formic acid) has shown strong activity against *S. aureus*, *P. vulgaris* and *K. pneumonia*. Sokolov et al. [30] revealed significant antibacterial activity of aqueous extract of aerial part of *F. ulmaria* against gram-positive bacteria of *S. aureus* and *B. cereus*. In parallel to this result, aqueous extract of field-grown aerial parts in our study showed strong antibacterial potency againts *S. aureus* (Table 1). Savina et al. [31] evaluated aqueous ethanol extract of various parts of *F. ulmaria* and observed susceptibility of *P. aeruginosa* to upper leaves, fruits, and flowers. Similarly, *P. aeruginosa* showed moderate susceptibility to aqueous and ethanol extract of field-grown *F. ulmaria* aerial parts (Table 1). On the other hand, Woods-Panzaru et al. [32] studied aqueous extract of *F. ulmaria* leaves against some microorganisms and reported that no antimicrobial activity was observed on the growth of *S. aureus*, *P. aeruginosa*, *E. coli* and any microorganisms.

Meadowsweet is used in folk medicine to treat a variety of conditions, including arthritis, rheumatism, joint pains, urinary tract infections, fever, chronic lung infections and bronchitis [3-7]. Antibacterial effect of *F. ulmaria* extracts against *S. epidermidis*, *S. aureus*, S. pyogenes, *E. cloacae*, *P. vulgaris*, *K. pneumonia*, *P. aeruginosa* and *S. marcescens* may help to explain the traditional uses of this plant. Furthermore, as dried leaves and flowers of *F. ulmaria* are used for boils treatment in folk medicine [33], the remarkable sensitivity of *S. aures* to various extracts of *F. ulmaria* (Table 1) may justify the folkloric usage of this plant as a remedy for boils that are caused by *S. aureus*.

Aqueous extract of the plants may contain active components such as antocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectines [34]. The effectiveness of the aqueous extract of naturally-grown plant materials of *F. ulmaria* may be explained by the inhibitory effect of these compounds on bacteria (Table 1). The salicylic acid concentration of meadowsweet may be responsible for *F. ulmaria*'s considerable antibacterial effect. However, a salicylic acid-rich extract of goat willow (*Salix caprea*) displayed very limited efficiency against *S. aureus* and *E. coli* and no activity against *S. epidermidis* [14].

3.2 Toxicity (Brine Shrimp Bioassay)

Toxicity of *F. ulmaria* extracts was tested with brine shrimp bioassay. Results of this bioassay showed that extracts of *F. ulmaria* were toxic at higher doses (around 2000-70000 mg/L) by comparing with MS-222 (positive

control). Among all extracts, aqueous extracts of field grown (FW) and *in vitro* grown (IW) plant with higher LC_{50} values (70103 mg/L and 62243 mg/L, respectively) were less toxic than other extracts. Moreover, hexane extracts (FH and IH) with lower LC_{50} values (2151 mg/L and 2275 mg/L, respectively) were more toxic than other extracts.

Table 3. LC_{50} (lethal concentration for 50% mortality after 24 hr exposure) values for meadowsweet extracts and MS-222 (a positive control). FW, FE, FH and FEA: aqueous, ethanol, hexane and ethylacetate extract of field-grown plants; IW, IE, IH and IEA: aqueous, ethanol, hexane and ethylacetate extract of in vitro-grown plants.

Treatments	LC_{50} (mg/L)	Confidence Intervals		
FW	70103	44360	-	110786
FE	3877	2454	-	6128
FH	2151	1361	-	3399
FEA	3898	2466	-	6159
IW	62243	39386	-	98364
IE	3536	2238	-	5589
IH	2275	1440	-	3595
IEA	3494	2211	-	5522
MS-222	47	30	-	74

The traditional use of this plant is by preparing it with water (infusion or decoction) and toxicity of aqueous extract is very low comparing with other solvents (Table 3). This result proved the most trustworthy method of utilizing this herb in traditional medicine. The German Commission E Monographs does not mention any restrictions on the administration of *F. ulmaria*, which is consistent with our findings that the extracts from the plant were toxic at high concentrations [7].

3.3 Anti-tumor Assay

According to the results of potato disc method, strong antitumor activity was observed for all extracts of meadowsweet (data not shown). When compared with negative control (water), the percentage inhibition of all extracts was more than 40% in three separate experiments. However, two mechanisms can inhibit the formation of crown galls on potato discs: either the reduction of the viability of *A. tumefaciens* or the inhibition of tumorogenesis. All extracts underwent viability testing in order to differentiate between these possibilities. It was observed that *F. ulmaria* extracts affected the viability of the bacterium. Thus, the observed suppression of tumor formation for these extracts was due to bacterial viability rather than tumor formation.

It was inferred that *A. tumefaciens*-induced potato disc tumor assay was not applicable to *F. ulmaria* extracts because of the strong antibacterial activity against *A. tumefaciens*.

3.4 Antioxidant assay

An antioxidant and phenolic profile analysis was carried out using the methanolic extract of *F. ulmaria* because phenols are the primary antioxidant components and methanol extracts have a higher concentration of phenolic compounds than other extracts [35].

The antioxidant activity of *F. ulmaria* methanolic extracts (FM and IM) was evaluated by the ability to scavenge 50% of the DPPH (IC_{50} value) free radicals comparing with quercetin. The methanolic extract of field-grown and *in vitro* grown plant presented similar level of antioxidant potential with IC_{50} of 205.65 µg/mL and 206.74 µg/mL, respectively (Table 4). Pukalskiene et al. [36] reported the DPPH scavenging activity of aerial parts of *F. ulmaria* extracts obtained with acetone, methanol and water. They found that methanol extract was the strongest DPPH scavenger with the lowest IC_{50} (0.25 mg/mL) that is similar to our result (Table 4). Katanic et al. [8] reported that methanolic extract of *F. ulmaria* aerial part possessed very strong DPPH scavenging activity (IC_{50} 16.41 µg/mL) that is higher than the present study. Sukhikh et al. [37] investigated the antioxidant activity of *F. ulmaria* leaves extracted with some organic solvents like ethanol, methanol and ethyl acetate. They found that methanol extract showed strong DPPH radical scavenging activity as 451.08 ± 24.45 µmol TE (Trolox Equivalents)/g). Savina et al. [31] showed antioxidant potential of aqueous ethanol extract of upper, middle, and lower leaves of *F. ulmaria* as 172.3, 118.8 and 127.8 mg of ascorbic acid equivalents per gram of dry weight of the plant.

3.5 Determination of total phenolic and flavonoid content

Total phenolic and flavonoid contents of methanol extracts were expressed as mg of gallic acid (GAE) and quercetin equivalents (QE), respectively, by using a standard curve. According to the findings, *F. ulmaria* aerial parts grown in the field had a higher phenolic content (196.80 mg GAE/g dry extract) than those grown *in vitro* (170.31 mg GAE/g dry extract), and the total flavonoid content of the *in vitro F. ulmaria* aerial parts was found to be higher (92.93 mg QE/g dry extract) than that of the field-grown *F. ulmaria* parts (75.43 mg QE/g dry extract).

Antioxidant compounds contained in plant materials have an essential role in free radical scavenging and inhibition. The radical scavenging activity of both *F. ulmaria* aerial parts was very strongly correlated with overall amount of total phenolics and flavonoids ($r = -1.0$, $P < 0.05$). The findings of the study indicated that overall amount of total phenolic and flavonoid content of both parts were about the same level. This fact may explain the same antioxidant activity observed for both parts in the current study (Table 4).

In parallel to our results, Pukalskiene et al. [36] assessed aqueous, acetone, and methanol extract of *F. ulmaria* aerial part, and found the values of total phenolic content as 22.50 mg GAE/g, 40.84 mg GAE/g and 106.81 mg GAE/g extract, respectively. Neagu et al. [38] found a total phenolic content of ethanol extract of dried *F. ulmaria* obtained from a national producer as 103.0 µg GAE/mL. In another study, Harbourne et al. [39] investigated the effect of drying temperatures on the amount of total phenolic contents in aqueous extract of *F. ulmaria*. They found that drying condition had no significant effect on the total phenolic content and reported total phenolic content of around 110-120 mg GAE/g in the extract of *F. ulmaria*. The values of phenolic content in the present study were found to be slightly higher compared to the literature. Katanic et al. [8]

Table 4. Free radical scavenging activity and total phenolic-flavonoid content of methanolic extracts of *F. ulmaria* from two different sources (naturally- and in vitro-grown). Means with the same letter within columns are not significantly different at $P>0.05$. FM: methanol extract of field-grown plants; IM: methanol extract of *in vitro*-grown plants.

Treatments	IC_{50} (µg/mL)	Total Phenol (mg GAE/g dry extract)	Total Flavonoid (mg QE/g dry extract)	Overall amount
FM	205.65±3.84 [b]	196.80±2.98 [a]	75.43±1.33 [b]	272.23
IM	206.74±4.36 [b]	170.31±4.75 [b]	92.93±1.36 [a]	263.24
Quercetin	40.98±0.67 [a]			

determined total phenolic and flavonoid content in methanolic extract of *F. ulmaria* aerial part as 249.53 mg GAE/g and 45.47 mg R(Rutin)E, respectively, that is higher phenolic but lower flavonoid content comparing with our study. On the other hand, Savina et al. [31] evaluated total phenolic (62.87 mg GAE/g, 59.62 mg GAE/g and 61.29 mg GAE/g, respectively) and flavonoid content (117.42 mg RE/g, 74.55 mg RE/g and 66.14 mg RE/g, respectively) of aqueous ethanol extract of upper, middle, and lower leaves of *F. ulmaria*. Their findings were lower in terms of total phenolic content than in our study (Table 4).

While our results on antioxidant activity and total phenolic-flavonoid content of *F. ulmaria* were generally in agreement with other studies' findings, variations may have occurred due to different ecotypes, separate plant parts, the location of the plants, the season in which the plants were collected, the postharvest conditions, and the type of extraction technique employed.

3.6 HPLC analysis of methanol extracts

The presence of some phenolic compounds (gallic acid, 1,3-dicaffeoylquinic acid, chlorogenic acid, vanilic acid, rutin hydrate, rosmarinic acid, salicylic acid and apigenin) in methanol extracts of field- and *in vitro*-grown *F. ulmaria* aerial parts were investigated by HPLC-DAD system (Table 5). Chromatogram of phenol standards is presented in Figure 2. It was figured out from the results that neither of the two *F. ulmaria* sources contained gallic acid, 1,3-dicaffeoylquinic acid, vanilic acid, rosmarinic acid, or apigenin. When the rest of the phenols in the extracts were compared, rutin hydrate was found to be the most abundant phenolic compound and its content of field-grown plant extract (49.72 mg/g dry extract) was found to be almost 1.5 times higher than its content in *in vitro*-grown plant extracts (34.45 mg/g dry extract). The amount of salicylic acid in field-grown plant extract (1.80 mg/g dry extract) was found to be almost five times higher than its amount in *in vitro*-grown plant (0.38 mg/g dry extract). However, detected chlorogenic acid content was higher in *in vitro*-grown plant extract (3.74 mg/g dry extract) than field-grown plant extract (3.44 mg/g dry extract) (Table 5).

Similar to our results, Papastavropoulou et al. [40] reported that gallic acid and apigenin was not detected in aqueous methanol extract of *F. ulmaria* flower. Besides, Proestos et al. [41] reported that hydrolyzed aqueous methanol extract of *F. ulmaria* leaves contained gallic acid and vanillic acid which were not present in our study. Additionally, findings of their study demonstrated that apigenin and rutin was not detected in the same extract while rutin hydrate was the most dominant phenol in our study (Table 5).

Katanic et al. [8] found that methanol extract from meadowsweet aerial part contained low concentrations of gallic acid while the amount of gallic acid increased in the hydrolyzed aerial part extract. In another study, gallic acid and chlorogenic acid were detected in the methanol extract of *F. ulmaria* leaves [37]. In contrast to our study (Table 5), in the aqueous ethanol extract of the upper, middle, and lower leaves of *F. ulmaria*, Savina et al. [31] detected a small amount of gallic acid but no salicylic acid.

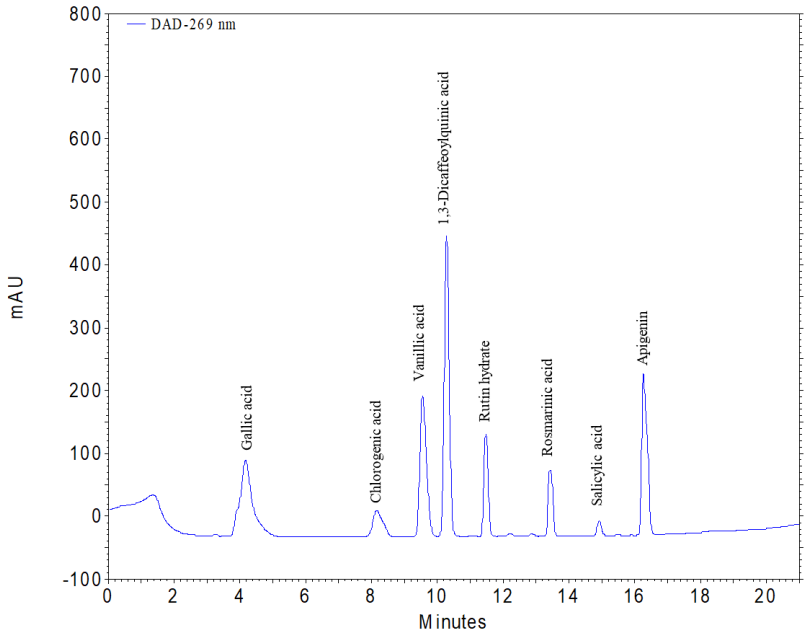

Figure 2. HPLC chromatogram of eight phenolic standards and their retention times.

Table 5. Quantitative analysis of individual phenolic constituents in methanolic extracts of *F. ulmaria* from two different sources (naturally- and *in vitro*-grown) by HPLC-DAD. Means with the same letter within columns are not significantly different at $P>0.05$. FM: methanol extract of field-grown plants; IM: methanol extract of *in vitro*-grown plants.

Extracts	Phenolic Compounds (mg/g dry extract)							
	Gallic acid	Chlorogenic acid	Vanillic acid	1,3-Dicaffeoylquinic acid	Rutin hydrate	Rosmarinic acid	Salicylic acid	Apigenin
FM	-	3.44±0.00 [b]	-	-	49.72±0.07 [a]	-	1.80±0.01 [a]	-
IM	-	3.74±0.00 [a]	-	-	34.45±0.02 [b]	-	0.38±0.01 [b]	-

'-' indicates undetected phenolic

4. Conclusions

All tested *F. ulmaria* extracts obtained from two different sources (field- and *in vitro*- grown plants) except ethyl acetate extract exhibited good antibacterial potential against all tested bacteria and a broad spectrum of antibacterial activity was observed for these extracts. The antibacterial impact of methanol extract of *in vitro*-grown aerial parts was higher than that of naturally-grown parts; nevertheless, aqueous, ethanol, and hexane extracts of natural parts outperformed these of *in vitro*-grown leaves. Strong antibacterial effects of *F. ulmaria* extracts of both aerial parts against *S. epidermidis*, *S. aureus*, *S. pyogenes*, *E. cloacae*, *P. vulgaris*, *A. hyrophila* and *Y. ruckeri* were very remarkable. The medium effect against *S. marcescens*, *P. aeruginosa*, *K. pneumonia*, *E. coli*, *S. agalactiae*, *E. faecalis*, *V. anguillarum* and *A. salmonicida* was noticeable as well. Extracts from *F. ulmaria* exhibited high antibacterial action against *A. tumefaciens*, making the potato disc tumor assay caused by this pathogen inapplicable. Low toxicity was observed while performing brine shrimp assay with *F. ulmaria* extracts especially with aqueous ones of both sources. *F. ulmaria* has strong antibacterial properties, and the fact that it is not toxic, or harmful at high concentrations, to brine shrimp larvae suggests that it may be a safe and useful substitute for antibiotics. The aerial parts obtained from both sources had a high antioxidant capacity due to their high phenolic content. The findings of this study highlight the potential of *F. ulmaria* raised in both ways as valuable natural source of bioactive compounds, with prospective applications in the pharmaceutical and nutraceutical industries. Further research is suggested to explore the different therapeutic potential of this species with their mechanisms of action.

Author Contributions: Conceptualization, A.U.T.; methodology, A.U.T., A.B.Y., A.C., Y.B.; validation, A.U.T., A.B.Y.; investigation, A.U.T., A.B.Y., A.C., Y.B.; resources, A.U.T.; data curation, A.U.T., A.B.Y., A.C., Y.B.; writing—original draft preparation, A.U.T., A.B.Y.; writing—review and editing, A.U.T.; visualization, A.U.T., A.B.Y.; supervision, A.U.T.; project administration, A.U.T.; funding acquisition, A.U.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Bolu Abant Izzet Baysal University Research Foundation, BAP 2005.03.01.219.

Conflicts of Interest: The authors declare no conflict of interest.

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