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Contributed Paper

Phenolic Compounds, Biological and Antioxidant Activities of *Onobrychis armena* Boiss. & Huet Flower and Root Extracts

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ABSTRACT

Onobrychis armena Boiss.&Huet (Fabaceae) is a plant species that is endemic to Turkey and grows in Aksaray. The aim of the present study is to examine antimicrobial and antioxidant activities of crude extracts of *O. armena* flower and root. The antimicrobial activities of the extracts were investigated by using disc diffusion and microdilution-broth methods against human and fish pathogen microorganisms. The results showed that all the extracts exhibited varying degrees of antimicrobial activity on the microorganisms tested. The antioxidant activity was determined with total antioxidant activity, free radical scavenging activity, ferric ion reducing power, and cupric ion reducing antioxidant capacity assays. In addition, the total phenolic compounds and flavonoids were measured in the extracts. The phenolic composition of the methanolic root extract was analyzed by HPLC. The HPLC analysis showed that the major component was rutin (428.80 μg/g). Cytotoxic effect of the methanolic root extract was also tested on human breast cell (MCF12A). The results showed that flower and root extracts of *O. armena* may be a potential source of natural antimicrobial and antioxidants and could be used as a natural source in food industry, drug discovery, clinical and food chemistry, and antimicrobial agents against human and fish pathogens.

Keywords: extraction, phenolic acids, HPLC, fish pathogens, clinical/food-borne pathogens, cytotoxic activity

1. INTRODUCTION

Turkey has an extraordinarily rich flora, and researchers have broad knowledge of their indigenous medicinal plants. Medicinal plants constitute an important component of flora and are widely distributed in different floristic regions of Turkey [1]. Throughout the recorded history, spices and herbs have been used for many purposes (beverages, flavoring foods and medicine) [2]. About 80% of the world population is dependent (wholly or partially) on herbal medicines

[3]. The preservative effect of many plant species and herbs suggests the presence of antioxidative and antimicrobial constituents [4]. Synthetic antioxidants and antimicrobials in use have been shown to have harmful side effects [5-7], therefore; there is a need for less or non-toxic antioxidants and antimicrobials obtained from natural sources.

Bacterial diseases can cause heavy mortality in cultured and wild fish. The use of antimicrobial agents in aquaculture has developed more resistant bacterial strains [8, 9]. In addition; continues use of synthetic antibiotics creates a danger for consumer health, non-target organisms, and the environment [10, 11]. The products from plants appear to be a possible alternative resource to prevent and treat of these bacterial infectious diseases.

The Fabaceae (Leguminosae) is a family of flowering plants including about 269 genera and 5100 species [12], and it is one of the largest plant families in Turkey and in the world. It has 68 genera and more than 900 species in the Flora of Turkey [13-15]. The family is important as food plants, especially leguminosae (beans, gram, peas), oil (soybean, ground nut), for tanbarks, timber, copal, gums, insecticides and cultivated or ornamentals, as well as medicinal plants [15, 16]. The genus *Onobrychis* is a member of the family Fabaceae represented throughout the world with 162 species and there are 52 species, 27 of which are endemic in Turkey [17]. Species of *Onobrychis* sp. (sainfoin) are used as animal feed in Turkey and in the world. There are 160 species (estimated) of sainfoin in the world [18].

The main objectives of this study on *O. armena* Boiss. & Huet (sainfoin), which is used as animal feed in the world and in Turkey, were to determine the antimicrobial and antioxidant properties and phenolic composition. In this study, flower and root extracts of *O. armena* were used in disc diffusion methods against 12 clinical and food-borne microorganisms and five fish pathogen strains to investigate the antimicrobial activity. Antioxidant activities were evaluated using four complementary assays methods, namely 2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging potential, ferric ion, cupric ion reducing antioxidant capacity, and total antioxidant capacity assays. Extracts were also analyzed for their total contents of flavonoid and phenolic components. The content of phenolic acid compounds of the methanolic root extract was observed with HPLC. The cytotoxic effect of the methanolic root extract from *O. armena* was determined on human breast cell.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

O. armena plants were collected during the flowering stage in June (east of Aksaray, 1219 m, Teksen 2543&Karaman) from Nizip-Aksaray in Turkey. The authenticated specimen of the plant was deposited at the herbarium of Department of Biology, Faculty of Arts and Sciences, Aksaray University, Turkey.

2.2 Preparation of Extracts

The harvested flowers and roots were rinsed with distilled water. The flowers and roots were air-dried (protected from sunlight), and dried plant samples were ground using a grinder (Waring) at room temperature. Then, the powdered plant materials (10 g) were extracted in a Soxhlet extractor with methanol (M), ethanol (E), water (W), dichloromethane (DCM) and n-hexane (H) for 24 h to obtain extracts. The samples were then filtered through Whatman No. 1 paper. Thereafter, the extracts were evaporated under vacuum with a rotary evaporator. After determining the yield, the extracts were dissolved in methanol and other solvents (ethanol,

water and dimethylsulfoxide (DMSO)) for this study.

2.3 Antimicrobial Evaluations Microbial Strains

The microorganisms used in this study were four Gram-positive bacteria, seven Gram-negative bacteria and one yeast: *Escherichia coli* (ATCC 11229), *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* (O157:H7), *Bacillus cereus* (RSKK 863), *Pseudomonas aeruginosa (*ATCC 27853), *Micrococcus luteus* (NRLL B-4375), *Shigella sonnei (*Mu:57), *Yersinia enterocolitica* (NCTC 11175) and *Candida albicans (*ATCC 10231).

The following fish pathogenic bacteria were used in the screening of antibacterial activity: one Gram-positive bacteria *Lactococcus garvieae* and three Gram-negative bacteria *Yersinia ruckeri*, *Vibrio anguillarum* (M1 and A4 strains, from two different companies) and *Vibrio alginolyticus*.

2.3.1 Microbiological assay The disc diffusion method was employed for the determination of the antimicrobial activity of the extracts [19]. The culture suspensions were adjusted with comparing with 0.5 McFarland. One hundred microlitres of suspension of the test microorganisms were spread on solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µL of the extracts (2.5 mg extract/ disc) and placed on the inoculated plates, which were stored at 4°C for 2 h and then incubated for 24 h. The diameters (mm) of the inhibition zones were measured, and each assay was repeated twice. Antibiotic discs of Ampicillin (Amp, 10 µg/disc), Gentamicin (CN, 10 µg/disc), and Amikacin (AK, 30 µg/disc) were also used as positive controls.

2.3.2 Minimal Bactericidal (MBC) or Fungicidal (MFC) Concentration MBC or MFC values of the extracts were determined by micro-dilution method using serially diluted (2 folds) plant extracts according to Chandrasekaran and Venkatesalu [20] with some modifications, and studied for the microorganisms, which are sensitive to the extracts in the disc diffusion assay. The serial dilutions of the extract were made in a concentration range from 1.41 to 180 mg/mL in the tubes. The inoculum of microorganisms was prepared by using 12 h cultures, and the suspensions were adjusted to 0.5 McFarland standard turbidity. The final volume in each tube was 100 µL. 2.5 µL of standardized suspension of each tested microorganism was transferred to each microtube. A positive control (containing 2.5 µL inoculum and 100 µL growth medium) and a negative control (containing 2.5 µL of extract, 100 µL growth medium without inoculum) were included on each microtube. The contents of the tubes were mixed by pipetting and were incubated 24 h. Then, 5 µL samples from tubes were placed on solid growth medium to confirm microbial growth because the plant extracts tested in this study were colored [21]. MBC or MFC is defined as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media [22]. So, the concentrations of the extracts that prevent the growth of a microorganism on the solid media were evaluated as MBC or MFC values in this study.

2.4 Assay for Total Phenolics

Total phenolic contents of the extracts were evaluated by using the Folin-Ciocalteu technique [23]. 0.2 mL of sample solution (1 mg/mL) was added to test tube containing 1 mL of Folin-Ciocalteu's reagent and 2 mL of Na_2CO_3 (7.5%). The final volume was raised to 7 mL with

deionized water. The mixture was incubated 2 h at room temperature. Then, the absorbance was measured at 765 nm with a spectrophotometer (HITACHI U-2000). The total phenolic content was expressed in terms of gallic acid equivalent (GAE) (mg GAE/g extract). Values are reported as means of separate experiments.

2.5 Total Flavonoid Analysis

The contents of flavonoids in the examined plant extracts were determined with using spectrophotometric method according to Arvouet-Grand et al. [24]. Briefly, 1 mL of 2% aluminium trichloride ($AlCl_3$) methanolic solution was mixed with the same volume of extract solution (1 mg/mL). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 mL extract solution with 1 mL methanol without $AlCl_3$. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line; then, the contents of flavonoids in extracts were expressed in terms of quercetin equivalent (QE)/g extract. Values are reported as means of separate experiments.

2.6 Antioxidant Activity

2.6.1 Determination of total antioxidant capacity by phosphomolybdenum assay The total antioxidant capacity of extracts was evaluated by phosphomolybdenum method by using Prieto et al. [25] method. The extracts were dissolved in methanol (2 mg/mL), and 0.3 mL of each extract was added to 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After 90 min at 95°C, the absorbance of reaction mixture at 695 nm was measured using a spectrophotometer. The antioxidant capacity of extracts was evaluated as equivalents ascorbic acid (mg AE/g extract). Values are reported as means of separate experiments.

2.6.2 Scavenging activity on DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical DPPH has long been used to quantify antioxidants in complex biological systems in recent years, and it's based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger [26]. The ability of the extracts to scavenge DPPH free radicals was estimated to the method of by the literature [27] with some modifications. 0.5 mL of test samples was mixed with 3 mL 6.10^{-5} M of a methanol solution of DPPH. After 30 min incubation in darkness and at ambient temperature, the absorbance was measured at 517 nm against a blank by a spectrophotometer. The inhibition of free radical, DPPH, in percent (I%) was calculated by using formula:

$$\text{DPPH Scavenging activity (\%)} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

where $Abs_{control}$ is the absorbance of the control reaction, and Abs_{sample} is the absorbance in the presence of the test compound. BHT (butylated hydroxytoluene) was used as positive controls. Values are reported as means of separate experiments.

2.6.3 Cupric ion reducing antioxidant capacity (CUPRAC) assay CUPRAC assay of the flower and root extracts obtained from *O. armena* was determined according to the method of Apak et al. [28]. One milliliter each of 10 mM $CuCl_2$, 7.5 mM neocuproine, and NH_4Ac buffer (1 M, pH 7.0) solutions were added into a test tube. Then, 0.5 mL of different concentrations of the extract was mixed, and the total volume was brought up to 4.1 mL with deionized water. Absorbance against a reagent blank was measured at 450 nm after 1 h. The same procedure was applied with BHT. Values are reported as means of separate experiments.

2.6.4 Ferric ion reducing power The ability to reduce ferric ions was measured using a modified version of the method described by Oyaizu [29]. Extract solution (30 μg/mL concentration of the each extract) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The reaction mixture was incubated in a water bath at 50°C for 20 min. After the incubation, 2.5 mL of 10% trichloroacetic acid was added. 2.5 mL of the reaction mixture was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%). The increase in absorbance at 700 nm was measured. The same procedure was applied with BHT. Values are reported as means of separate experiments.

2.7 High Performance Liquid Chromatography (HPLC) Analysis Conditions

HPLC analysis was performed by Süleyman Demirel University (Isparta, Turkey). The HPLC system consisted of a Shimadzu LC-10ADVP pump, a 20 μL sample loop, SCL-10A VP UV-DAD detector, and an Agilent Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm). The mobile phase was 3% acetic acid/methanol at a flow rate of 0.8 mL/min and UV detection was at 278 nm. The amount of each phenolic acid is expressed as micrograms per gram of dry extract.

2.8 Cytotoxicity Assay

Cytotoxicity assay was performed by Molecular Biology Research and Application Center, Gazi University (Ankara, Turkey). The methanolic root extract of *O. armena* was evaluated on human breast cell (MCF12A) in order to examine its cytotoxic effect on normal cells. The methanol root extract had the highest phenolic content among the other extracts. The cells were harvested (2×10^5 cells/well) and inoculated in 96 well micro titer plates. Cell proliferation was analyzed 48 h after MCF12A cells had been cultured with an extract of 250, 500, 1000 μg/mL in the final concentration using the trypan blue dye assay. After 48 h incubation, 0.4% trypan blue was added to each well and incubated 15 min. Dye was removed with phosphate buffered saline. Cells were lysis with 1% SDS (Sodium Dodecil Sulphate), and absorbance was read at 590 nm with micro-plate reader. The concentration that Gazi University utilized to determine the anticarcinogenic activity was chosen at the same values as the concentration that determines the cytotoxic activity in healthy human breast cells.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Activity

In this study, total extract yield of various flower and root extracts of *O. armena* collected from Aksaray in Turkey were determined. The extraction yields for the flower and root extracts varied from 0.54 to 95.80% (Table 1). Among all the flower extracts, water extract was found to have the maximum extractive yield (95.80%). In the root extracts, the methanol extract had the highest extractive yield (31.40 %).

The antimicrobial activities of the flower and root extracts were determined using a disc diffusion method [19] that is widely used for quick screening of natural products for antimicrobial activity. The results obtained in the current study relieved that the tested plant extracts possess potential antimicrobial activity against 11 bacteria and one yeast. The results of the antimicrobial screening of the flower and root extracts against all microorganisms are shown in Table 2. The inhibition zones formed by standard antibiotic discs, and those discs (Ampicillin, Gentamicin and Amikacin) were also listed in Table 2.

When tested by the disc diffusion method, the flower extracts showed

Table 1. The yield of *Onobrychis armena* extracts.

Parts	Extract yields (% w/w)				
	Methanol	Ethanol	Water	n-Hexane	DCM
Flower	22.70	13.36	95.80	1.69	2.94
Root	31.40	10.55	7.90	0.54	1.08

various antimicrobial activities (10.14 - 25.38 mm) against the tested microorganisms used in this study. The two highest inhibitory activities were determined against *Y. enterocolitica* (NCTC 11175) in the DCM and ethanol flower extracts. The two weakest inhibitory activities were determined against *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231) from the flower methanolic extract and the flower n-hexane extract, respectively. The flower water extract did not exhibit any activity against all the tested microorganisms whereas inhibitory activities of the water root

Table 2. Antimicrobial activity of flower and root extracts of *Onobrychis armena* against a set of microorganisms.

Parts		Inhibition zones of test microorganisms											
		863[b]	0157:H7	Mu:57[d]	4375[e]	11175[f]	11229[g]	27853[h]	25923[i]	35218[j]	13076[k]	10231[l]	7644[m]
FLOWER	M	-[n]	-	-	-	-	-	12.23± 0.02	10.14± 0.07	-	-	-	-
	E	21.02± 0.01	-	-	17.88± 0.45	23.86± 0.12	-	-	21.81± 0.65	-	-	-	-
	W	-	-	-	-	-	-	-	-	-	-	-	-
	H	12.02± 0.32	-	-	-	16.36± 0.45	13.84± 0.22	17.28± 0.56	-	10.98± 0.03	-	10.82± 0.33	-
	DCM	-	-	22.04± 0.70	23.82± 0.65	25.38± 0.79	-	22.60± 0.55	17.62± 0.63	-	-	21.18± 0.21	-
ROOT	M	17.12± 0.11	-	-	15.69± 0.54	10.28± 0.42	-	12.78± 0.87	15.72± 0.03	-	-	21.18± 0.65	-
	E	16.33± 0.08	12.38± 0.44	-	14.96± 0.23	-	8.68± 0.85	-	18.18± 0.23	10.85± 0.42	-	-	-
	W	-	-	-	-	-	-	-	-	-	-	13.79± 0.29	12.81± 0.54
	H	20.68± 0.11	26.80± 0.24	-	-	17.60± 0.11	13.16± 0.33	20.88± 0.21	20.48± 0.09	20.68± 0.65	-	-	-
	DCM	27.28± 0.07	8.88± 0.21	15.68± 0.65	14.44± 0.22	15.32± 0.05	8.08± 0.08	15.12± 0.12	37.08± 0.47	15.00± 0.55	12.6± 0.63	10.28± 0.55	-
Inhibition zone diameter (mm) Antibiotics	Ampicillin	37.68± 0.03	25.92± 0.15	38.43± 0.16	34.65± 0.12	11.58± 0.09	27.99± 0.14	-	34.82± 0.06	25.78± 0.19	29.49± 0.15	-	25.13± 0.06
	Gentamicin	18.02± 0.11	18.37± 0.17	19.49± 0.05	13.48± 0.22	16.17± 0.11	14.98± 0.12	15.89± 0.05	15.52± 0.14	12.17± 0.21	16.38± 0.17	-	20.63± 0.16
	Amikacin	18.7± 0.07	22.58± 0.09	27.07± 0.04	19.55± 0.14	21.19± 0.07	19.81± 0.13	19.71± 0.08	19.46± 0.16	20.03± 0.09	17.27± 0.11	-	20.52± 0.21

[a]: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of separate experiments.
[b]: *B.cereus* RSKK 863, [c]: *E. coli* O157:H7, [d]: *S. sonnei* Mu:57, [e]: *M. luteus* NRRL B-4375, [f]: *Y. enterocolitica* NCTC 11175, [g]: *E. coli* ATCC 11229, [h]: *P. aeruginosa* ATCC 27853, [i]: *S. aureus* ATCC 25923, [j]: *E.coli* ATCC 35218, [k]: *S. enteritidis* ATCC 13076, [l]: *C. albicans* ATCC 10231, [m]: *L. monocytogenes* ATCC 7644
[n]: indicate no antimicrobial activity

extract were determined against *C. albicans* (ATCC 10231, 13.79 mm) and *L. monocytogenes* (ATCC 7644, 12.81mm). The two highest inhibitory effect of the root DCM extract was 37.08 mm against *S. aureus* (ATCC 25923) and 27.28 mm against *B. cereus* (RSKK 863). The lowest inhibitory activities were determined in the root DCM extract (8.08 mm) and in the root ethanolic extract (8.68 mm) against *E. coli* (ATCC 11229). The DCM root extract has also shown better antibacterial activities against *Y. enterocolitica* (NCTC 11175) and *S. aureus* (ATCC 25923) when compared with the standard Ampicillin. (Table 2).

In their study, Babu et al. [30] used disc diffusion method and found that the methanolic flower extract (4 mg/disc) of *Clitoria ternatea* Linn. (Fabaceae) plant had antimicrobial effect on *E. coli* (20 mm), ETEC (Enterotoxigenic *E. coli*, 16 mm), EPEC (Enteropathogenic *E. coli*, 16 mm), and *P. auroginosa* (26 mm). In our study, the methanol flower extract didn't have any antimicrobial activity against *E. coli* strains (ATCC 11229, ATCC 35218, O157:H7), but it did have antimicrobial activity against *P. aeruginosa* (ATCC 27853, 12.23 mm). In the aforementioned study, the hexane flower extract didn't have any antimicrobial activity against these pathogens, but in our study, the antimicrobial activities of the n-hexane flower extract against *E. coli* strains (ATCC 11129, ATCC 35218) and *P. auroginosa* (ATCC 27853) were determined as 13.84 mm, 10.98 mm and 17.28 mm, respectively. Water flower extract of *C. ternatea* showed antimicrobial activity against the said pathogens (*E. coli,* ETEC, EPEC and *P. auroginosa*) but in our study, of the same extract of *O. armena* didn't have any activity against these microorganisms. Similarly, several researchers have generally reported that the aqueous extracts of plants do not have much antibacterial activity [31, 32]. The activity differences in these two studies may derive from the methods used in extraction of the plants, the difference of plants in species, and the different microorganism strains that were used.

The MBC or MFC values of the flower and root extracts against the set of 12 microorganism strains are shown in Table 3. MBC values for the microorganisms which were sensitive to flower and root extracts were in the range of 5.63-45.00 mg/mL and 2.82-45.00 mg/mL, respectively. The lowest MBC value of 5.63 mg/mL was found in the flower ethanol extract against *B. cereus* (RSKK 863). Among the flower extracts, the ethanol and n-hexane extracts showed the highest MBC value of 45.00 mg/mL against *M. luteus* (NRLL B-4375) and *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 35218). The lowest MBC values of 2.82 mg/mL were found in the root extracts against various microorganisms. In the root extracts, the ethanol extract showed the highest MBC value of 45.00 mg/mL against *E. coli* (ATCC 35218) and *E. coli* (O157:H7), and DCM extracts against *S. enteritidis* (ATCC 13076). The results of the MBC show that the root extracts seemed to be more effective than the flower extracts against the tested microorganisms used in this study.

In one of their studies, Buruk et al. [33] researched the antimicrobial activity of wi (water-insoluble) and ws (water-soluble) leaves extracts of *O. armena* against pathogen microorganisms (*E. coli, S. aureus, B. subtilis, C. albicans*) and found out that the wi extract showed no MIC value. In the extract with ws solvent, MIC values were recorded against the pathogens of *S. aureus* (0.625 mg/mL) and *B. subtilis* (>1000 mg/mL). In our study, no activity of the flower water extract was seen against the aforementioned microorganisms, but MFC and MBC values were determined for the root water extract against *C. albicans* (ATCC 10231, 22.50 mg/mL) and *L.*

monocytogenes (ATCC 7644, 5.62 mg/mL).

In our study, the extracts of *O. armena* which exhibited antibacterial properties against the five fish pathogen strains are shown in Table 4. These bacterial pathogens are the ones that commonly occur in aquaculture sector and cause serious infectious diseases and mortality in fish [34]. The extracts exhibited various antimicrobial activities against the fish pathogenic bacteria in the range of 7.59 mm (the ethanolic root extract, against *V. alginolyticus*) - 17.37 mm (the methanolic root extract, against *L. garviae*). The methanolic root extract has shown better antibacterial activities against *V. anguillarum* (A4) when compared with standard Ampicillin and Gentamicin (Table 4). The present results showed the root and flower extracts of *O. armena* may be used as antimicrobial agents for serious infectious fish diseases. The water and n-

Table 3. MBC or MFC values of *Onobrychis armena* flower and root extracts.

Parts		MBC or MFC (mg/mL) values[a] of test microorganisms											
		863[b]	O157:H7	Mu:57[d]	4375[e]	11175[f]	11229[g]	27853[h]	25923[i]	35218[j]	13076[k]	10231[l]	7644[m]
FLOWER	M	-	-	-	-	-	-	22.50	22.50	-	-	-	-
	E	5.63	-	-	45.00	11.25	-	-	11.25	-	-	-	-
	W	-	-	-	-	-	-	-	-	-	-	-	-
	H	11.25	-	-	-	11.25	22.50	45.00	-	45.00	-	22.50	-
	DCM	-	-	11.25	22.50	11.25	-	22.50	22.50	-	-	22.50	-
ROOT	M	2.82	-	-	11.25	22.50	-	22.50	2.82	-	-	22.50	-
	E	2.82	45.00	-	2.82	-	11.25	-	2.82	45.00	-	-	-
	W	-	-	-	-	-	-	-	-	-	-	22.50	5.63
	H	5.63	11.25	-	-	11.25	11.25	5.63	11.25	22.50	-	-	-
	DCM	11.25	11.25	11.25	22.50	22.50	11.25	11.25	2.82	22.50	45.00	22.50	-

[a]: Minimal Bactericidal Concentration (MBC), Minimal Fungicidal Concentration (MFC)
[b]:*B.cereus* RSKK 863, [c]: *E. coli* O157:H7, [d]: *S. sonnei* Mu:57, [e]: *M. luteus* NRRL B-4375, [f]: *Y. enterocolitica* NCTC 11175, [g]: *E. coli* ATCC 11229, [h]: *P. aeruginosa* ATCC 27853, [i]: *S. aureus* ATCC 25923, [j]: *E.coli* ATCC 35218, [k]: *S. enteritidis* ATCC 13076, [l]: *C. albicans* ATCC 10231, [m]: *L. monocytogenes* ATCC 7644

hexane root extracts were not studied against five fish pathogen strains in this study because of insufficient extracts.

In one of their studies, Turker *et al.* [35] studied the antimicrobial activity of water, MeOH and EtOH extracts of *Trifolium pannonicum* Jacq. subsp. *elongatum* (Willd.) Zoh. and *Astragalus gymnolobus* Fischer (family Fabaceae) against fish pathogens. While there was no activity of the water extract of *T. pannonicum* against *Y. ruckeri* and *L. garviae* pathogens, antimicrobial activity was recorded from the methanol (11.88 mm and 14.25 mm, respectively) and the ethanol (8.38 mm and 9.63 mm, respectively) extracts against these two fish pathogens. All three solvents of *A. gymnolobus* had no antimicrobial activity against two fish pathogens. In our study, the flower and root extracts had antimicrobial activity against these two fish pathogens, and the highest activity was of root ethanol extract with 17.37 mm against *L. garviae*.

The MBC values were in the range of 5.63-90.00 mg/mL for the flower extracts and in the range of 5.63-45.00 mg/mL for the root extracts (Table 4). The highest inhibitory activity for the DCM flower

Table 4. Antibacterial activity of flower and root extracts of *Onobrychis armena* against different bacterial fish pathogens.

	Diameter of zone of inhibition[a] (mm)								MBC[b] (mg/mL)								Inhibition zone diameter[a] (mm) Antibiotics		
	FLOWER					*ROOT*[c]			*FLOWER*					*ROOT*					
Test microorganisms	M	E	W	H	DCM	M	E	DCM	M	E	W	H	DCM	M	E	DCM	Amp[d]	CN[e]	AK[f]
V. anguillarum M1	10.77± 0.02	11.30± 0.09	8.24± 0.12	11.28± 0.32	8.72± 0.09	10.28± 0.11	12.28± 0.19	10.05± 0.22	45.00	11.25	11.25	90.00	11.25	22.50	22.50	5.63	9.02± 0.04	12.38± 0.09	9.46± 0.12
V. anguillarum A4	9.44± 0.13	13.79± 0.55	12.26± 0.71	9.27± 0.35	11.49± 0.68	13.32± 0.40	12.56± 0.51	10.88± 0.23	45.00	11.25	45.00	5.63	5.63	22.50	11.25	11.25	9.40± 0.11	15.13± 0.15	12.07± 0.13
V. alginolyticus	11.53± 0.80	-	8.54± 0.67	12.63± 0.45	10.99± 0.07	7.59± 0.32	11.81± 0.35	9.44± 0.07	22.50	-	45.00	11.25	5.63	5.63	11.25	11.25	13.57± 0.09	15.06± 0.07	15.03± 0.03
L. garviae	13.26± 0.31	14.30± 0.12	7.88± 0.10	11.87± 0.11	12.81± 0.33	12.21± 0.82	17.37± 0.13	13.18± 0.34	45.00	22.50	45.00	45.00	22.50	22.50	11.25	22.50	33.10± 0.12	15.19± 0.10	10.30± 0.08
Y. ruckeri	12.26± 0.56	13.12± 0.36	9.66± 0.47	11.37± 0.40	10.94± 0.04	9.74± 0.25	12.68± 0.11	11.36± 0.09	45.00	22.50	90.00	45.00	22.50	45.00	45.00	22.50	32.30± 0.15	18.85± 0.05	18.69± 0.12

[a]: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of separate experiments.
[b]: Minimal Bactericidal Concentration (MBC)
[c]: The root water and n-hexane extracts were not studied because of insufficient extracts.
[d]: Ampicillin, [e]:Gentamicin, [f]: Amikacin

extract was against *V. alginolyticus* and *V. anguillarum* (A4) which showed a lower MBC value (5.63 mg/mL). The lowest MBC values of 5.63 mg/mL were found in the root methanol and DCM extracts of *O. armena* against *V. alginolyticus, V. anguillarum* (M1), respectively. The results show that the analyzed plants may offer a potential as alternative therapy of microbial fish diseases. In conclusion, the present study shows that the extracts examined have variable antimicrobial activities.

3.2 Total Phenolics Content (TPC)

Phenolic compounds have been shown to be responsible for the antioxidant activity of plant extracts [36]. Thus, total phenol content (TPC) of the extracts was determined using the Folin-Ciocalteu technique and shown in Table 5. The content of total phenols was found as ranging from 11.18 to 128.23 mg GAE/g extract. As can be seen in Table 5, of all the flower extracts, the methanolic extract had the highest TPC (87.32 mg GAE/g extract) while the n-hexane extract had not TPC value. The methanol root extract of *O. armena* had the highest TPC while the water root extract showed the lowest value among the root extracts in the assay. In conclusion, the root methanol extract showed the highest TPC among the all extracts. Ince et al. [37] found out the phenol amount in the aerial parts of *Onobrychis viciifolia* as 36.78% in the methanol extract and 11.35% in water extract. In our study, the phenol content in flower and root methanol extracts were 87.32 mg GAE/g extract and 128.23 mg GAE/g extract, respectively. The TPC value was determined as 18.00 mg GAE/g extract for the root water extract, but the flower water extract had not TPC in this study. Phenolic composition of plants extracts is affected by different factors - variety, climate, storage, processing etc [38]. This can partly explain the wide range of variation in TPC values obtained from different studies which used the same evaluation methods.

3.3 Total Flavonoids Content (TFC)

The total flavonoid content in the flower and root methanol, ethanol, water, DCM and n-hexane extracts are summarized in Table 5 and expressed as mg quercetin equivalents per gram of extract (mg QE/g extract). The highest TFC in the flower extracts was found in methanol

extract with 23.92 mg QE/g extract and followed by DCM extract with 10.31 mg QE/g extract while the lowest TFC for flower extract can be found in ethanol extract with 5.82 mg QE/g extract. In the root extracts, the highest activity was found in methanol extract with 2.26 mg QE/g extract, and the lowest TFC for the root extract was found in ethanol extract with 1.54 mg QE/g extract.

3.4 Antioxidant Activity

3.4.1 Determination of total antioxidant activity (TAC)

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. [25]. This assay is based on the reduction of Mo (VI) to Mo (V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH, which is measured at 695 nm. TAC was reported as ascorbic acid equivalents and shown in Table 5. In the flower extracts, the highest TAC radical scavenging capacity value was identified for the methanolic extract (83.00 mg AAE/g extract), followed by DCM extract (48.38 mg AAE/g extract). In the root extracts, the two highest TAC radical scavenging capacity values were identified as methanol (208.77 mg AAE/g extract) and DCM (84.54 mg AAE/g extract) extracts. Ince et al. [37] determined the total antioxidant content of methanol and water extracts of *O. viciifolia* as 1739.50 and 521.85 mmol/g of extract, respectively.

3.4.2 Free radical scavenging activity (DPPH, 2, 2-diphenyl-1-picrylhydrazyl)

DPPH assays have been used by many researchers to determine free radical scavenging activity of antioxidant compounds [39, 40]. The antioxidant activity results were given in Figure 1a-b. The antioxidant potential varied widely and ranged from 7.96-94.21% and from 33.07-91.14% (at 2000 μg/mL concentration) for the flower and root extracts, respectively. The methanol and ethanol extracts of flower showed the highest DPPH scavenging activities 94.21% (IC_{50} 669.23 μg/mL) and 45.15% (IC_{50} > 1000 μg/mL), respectively. In the root extracts, methanol and ethanol extracts showed the highest DPPH scavenging activities 91.14% (IC_{50} 515.52 μg/mL) and 87.89% (IC_{50} 950.32 μg/mL), respectively. The free radical scavenging potentials of the flower and

Table 5. Total phenolics, flavonoid and antioxidant capacities of *Onobrychis armena* flower and root extracts.

Parts	Solvents	Total phenolic content[a] (mg GAE/g extract)	Total flavonoid content[a] (mg QE/g extract)	Total antioxidant capacity[a] (mg AAE/g extract)
Flower	M	87.32±10.29	23.92±0.22	83.00±7.07
	E	25.50±4.50	5.82±0.05	24.54±4.90
	W	-[b]	-	-
	DCM	11.18±4.18	10.31±0.31	48.38±9.25
	H	-	-	7.23±1.09
Root	M	128.23±1.93	2.26±0.48	208.77±10.88
	E	64.82±1.61	1.54±0.87	38.77±5.44
	W	18.00±6.11	-	2.62±4.35
	DCM	89.14±13.50	-	84.54±4.90
	H			

[a]:Values are reported as means ± S.D. of separate experiments.
[b]: Not determined

root extracts according to this assay were found in the order of M > E > W > DCM > H. As a result, the root extracts of *O. armena* generally showed better antioxidant activity compared with flower extracts according to DPPH method. Ince et al. [37] studied the DPPH activity in aerial parts of *O. viciifolia* plant and found out, as we did, that the methanol extract had a higher property of radical retention compared with water extract.

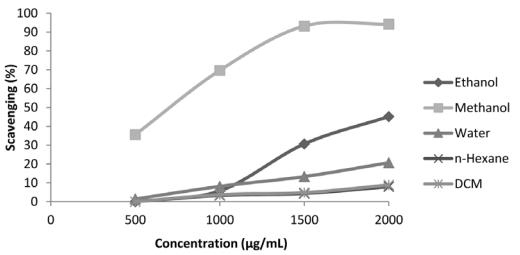

Figure 1a. DPPH scavenging activity of *Onobrychis armena* flower extracts.

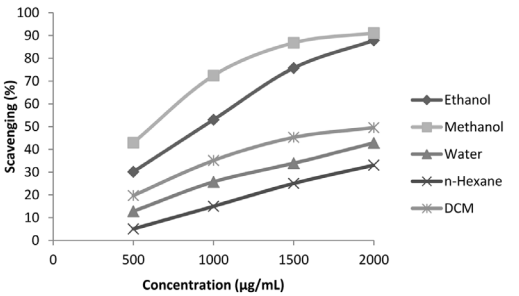

Figure 1b. DPPH scavenging activity of *Onobrychis armena* root extracts.

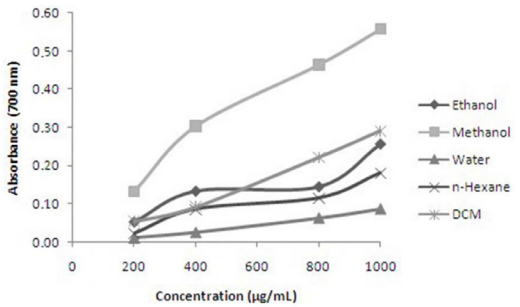

Figure 2a. Ferric ion reducing power of *Onobrychis armena* flower extracts.

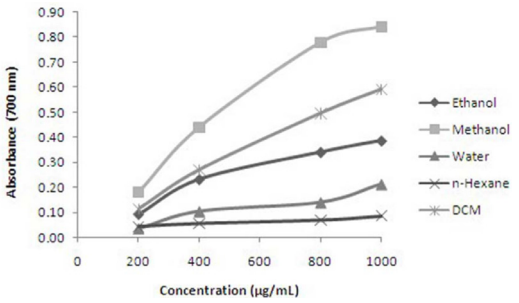

Figure 2b. Ferric ion reducing power of *Onobrychis armena* root extracts.

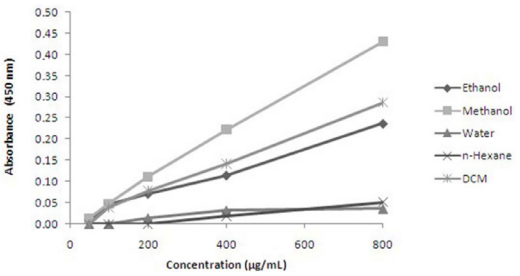

Figure 3a. Cupric ion reducing power of *Onobrychis armena* flower extracts.

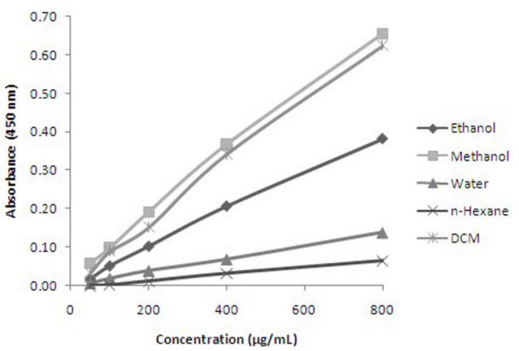

Figure 3b. Cupric ion reducing power of *Onobrychis armena* root extracts.

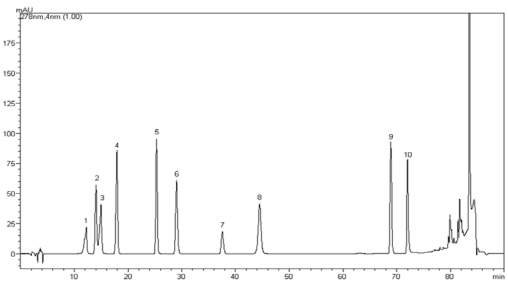

Figure 4a. Standard chromatography; 1-catechin, 2-p-hydroxy benzoic acid, 3-chlorogenic acid, 4-caffeic acid, 5-p-coumaric acid, 6-ferulic acid, 7-benzoic acid, 8-rutin, 9-cinnamic acid, 10-quercetin.

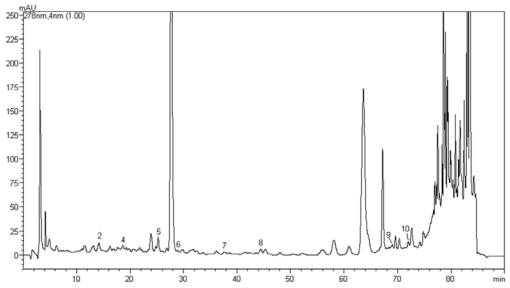

Figure 4b. HPLC analysis of methanolic root extract of *Onobrychis armena,* Peak Nos., 2-p-hydroxy benzoic acid, 4-caffeic acid, 5-p-coumaric acid, 6-ferulic acid, 7-benzoic acid, 8-rutin, 9-cinnamic acid, 10-quercetin.

Table 6. Amount of phenolic acid in the methanol root extract of *Onobrychis armena.*

	Phenolic acid (µg/g dry extract)									
	catechin	p-hydroxy benzoic acid	chlorogenic acid	caffeic acid	p-coumaric acid	ferulic acid	benzoic acid	rutin	cinnamic acid	quercetin
Methanolic root extract	-	76.70	-	66.70	84.10	30.50	418.70	428.80	19.20	141.30

-: Not determined

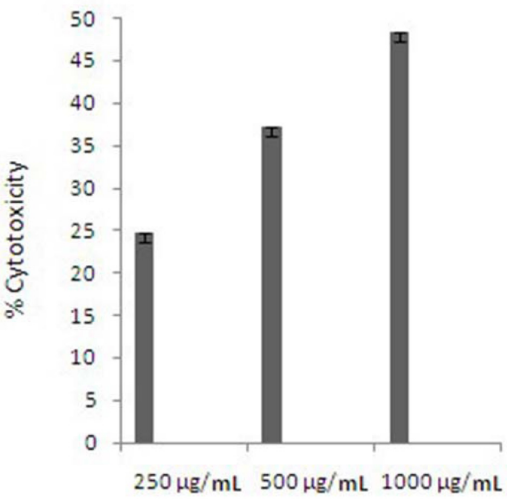

Figure 5. Cytotoxicity of the root methanolic extract from *Onobrychis armena* on human breast cell (MCF12A).

3.4.3 Ferric ion reducing power

All flower and root extracts interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity. Figure 2a-b show the dose response curves for the reducing power of the *O. armena* extracts. The strongest activity at a concentration of 1000 µg/mL was noticed in the methanol root extract (A_{700nm} 0.84) while the water flower extract (A_{700nm} 0.08) exhibited the lowest activity. The ferric ion reducing power of flower extracts of *O. armena* was found to decrease in the order of M > DCM > E > W > H. The ferric ion reducing power of the root extracts was found to decrease in the order of M > DCM > E > H > W. BHT, at 31.25 µg/mL the concentration, exhibited remarkably higher reducing power (A_{700nm} 0.61) than the extracts. As a result, the root extracts

presented a better activity of iron ion reduction capability than flower.

3.4.4 Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC assays have been used by many researchers to determine the reducing power of antioxidant compounds [28, 41, 42]. CUPRAC assay of the samples obtained from *O. armena* was determined according to the method of Apak et al. [28] in the present study; we use the cupric assay which is based on reduction of Cu^{2+} to Cu^{+} by antioxidants. Cupric ion (Cu^{2+}) reducing ability of the flower and root extracts is shown in Figure 3a-b. The highest Cu^{2+} reducing power, the methanolic root extract at the concentration of 800 μg/mL was found as A_{450nm} 0.65. According to the results of the present study, the reducing power in the flower extracts and standard compounds decreased in order of BHT > M > DCM > E > H > W, in the presence of 800 μg/mL test sample. On the other hand, the reducing power of the root extracts and standard compounds decreased in order of BHT > M > DCM > E > W > H at the same concentration.

3.5 HPLC Analysis

In this study, the methanolic root extract of *O. armena*, which had the highest total phenolic content, was investigated with regard to phenolic acids content (catechin, p-hydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, benzoic acid, rutin, cinnamic acid, and quercetin). The phenolic acids content is shown in Table 6. As a result, HPLC analysis of the methanol root extract among ten tested phenolic acids content showed eight different phenolic acids (p-hydroxy benzoic acid, caffeic acid, p-coumaric acid, ferulic acid, benzoic acid, rutin, cinnamic acid, and quercetin) while two acids (catechin, chlorogenic acid) were not present. HPLC chromatograms of the extract and standard are presented in Figure 4a-b.

The methanolic root extract showed the maximum amount of rutin (428.8 μg/g) followed by benzoic acid (418.7 μg/g) and quercetin (141.3 μg/g). The other phenolic acids were found as p-coumaric acid (84.1 μg/g), p-hydroxy benzoic acid (76.7 μg/g), caffeic acid (66.7 μg/g), ferulic acid (30.5 μg/g), and cinnamic acid (19.2 μg/g).

According to Bauer and Tittel [43] and Springfield et al. [44], HPLC fingerprinting as the best way for chemical characterization, and therefore, the current study also established HPLC fingerprinting for the active phenolic acids which can act as antioxidant, antimicrobial, and anti-inflammatory. Diverse pharmacological activities have been accredited to phenolic acids for instance, caffeic acid with anti inflammatory [45], antibacterial, antifungal [46], ferulic acid with anti-inflammatory [45], antifungal [47], cinnamic acid with antifungal [47], anthelmintic [48] and natural protection against infections by pathogenic microorganisms [49].

3.6 Cytotoxicity Assay

The cytotoxic activity was evaluated on human breast cell (MCF12A). The results of cytotoxic effects of the methanol root extract are shown in Figure 5. *O. armena* methanolic root extract at a concentration of 250-1000 mg/mL showed 24.71-48.35% cytotoxic effect on human breast cell (MCF12A).

In one of their studies, Khalighi-Sigaroodi et al. [50] examined the cytotoxic effect of methanol extracts of aerial parts of 23 plants from Fabaceae family against brine shrimp, and *Onobrychis altissima* Grossh among these plants had LC50 value of 51.38 (μg/mL). In their study, Oke and Aslim [32] studied the cytotoxic effect of water and methanol extracts (at concentrations of 10-100 μg/mL) of

Origanum minutiflorum, which is endemic plant to Isparta, against baby hamster kidney fibroblast (BHK 21) cells, and they observed no cytotoxic effect. In our study, only the methanolic root extract, which had the highest amount of total phenolic compound, was observed to effect healthy human breast cells. However, the cytotoxic effect of other extracts on healthy human and fish cells should be determined. It is aimed in the future studies to study the cytotoxic effect of other extracts and to determine the anticarcinogenic effect of the extracts which show no cytotoxic effect.

4. CONCLUSIONS

In this study, the antimicrobial effect of various extracts of *O. armena* (sainfoin), which is a significant animal feed in our country and in the world, against clinical and food-borne human and fish pathogens were determined. The antioxidant capacity of this plant was discovered as well. In general, the root extracts of *O. armena* showed better antimicrobial and antioxidant activities than the flower extracts and the methanolic root extract, had the highest total phenolic content, showed better antioxidant activity of all the other extracts. The activities of this plant in this study will be very much helpful to prevent various modern health disorders especially which are caused by or aggravated by free radicals and free iron as described above. The results of the study provide scientific basis for the use of flower and root of the plant extract in the future development as antioxidant and antibacterial, antifungal agent for human and fish.

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