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ANTIBACTERIAL AND ANTIVIRAL ACTIVITIES OF STINGING NETTLE (*URTICA DIOICA* L.) LEAF EXTRACT ON NOROVIRUS AND *CAMPYLOBACTER JEJUNI* AS FOODBORNE PATHOGENS

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ABSTRACT

Research background. The cytotoxicity on various vital cell lines and the activity on foodborne pathogens (murine norovirus 1 as a norovirus surrogate and *Campylobacter jejuni*) of methanolic *Urtica dioica* L. leaf extract (UDE) were studied.
Experimental approach. The cytotoxic concentration of 50% (CC_{50}) was measured by the linearity between UDE concentrations and cell viability. Antibacterial effects on *C. jejuni* were analyzed by the broth microdilution method with a spectrophotometer. The virucidal and antiviral activities of UDE were determined by the virus titration method on the host cell infectivity and expressed as the tissue cell infective dose of 50% using the method of Spearman–Karber.
Results and conclusions. The CC_{50} of UDE was determined on macrophage as the virus host cell. MIC and MBC of UDE were determined as 5 mg/ml and 20 mg/ml for both *C. jejuni* isolated from poultry meat and the standard strain. UDE inhibited MNV-1 on three pathways of host cell infectivity at approximately the same 50% inhibitory concentration (1.45-1.87 mg/ml). In conclusion, the present study tried to explain in detail the dose-dependent activity of *Urtica dioica* L. leaf extract on two important foodborne pathogens causing outbreaks worldwide. The results showed that it might be a safe and alternative food additive and supplement candidate at safe concentrations.

Keywords: cytotoxicity, campylobacter, norovirus, *urtica dioica* L., food safety

INTRODUCTION

Urtica dioica L. belongs to the Urticaceae family. It has been identified and widely distributed worldwide and is considered to be native to Europe, North Africa, Asia, and North America **(Dhouibi *et al.*, 2020; European Commission, 2022; Upton, 2013)**. It and its extracts are used in both pharmaceutical and food industries as a supplement and a food additive for extending shelf-life, ensuring microbial safety of foods, and higher consumer acceptability **(Alp & Aksu, 2010)**. Phytochemical studies have mainly focused on its bioactive compounds and activities related to antioxidant contents and effects against bacterial and viral pathogens including foodborne infections **(Dhouibi *et al.*, 2020; Flores-Ocelotl *et al.*, 2018; Körpe *et al.*, 2013; Veiga *et al.*, 2020)**.
Campylobacter and norovirus are the predominant cause of foodborne diseases resulting in acute gastroenteritis cases in all age groups worldwide. Both pathogens are the most commonly reported in foodborne outbreaks in Europe and the United States **(CDC, 2019, 2022; EFSA, 2021; WHO, 2021)**. In overall outbreaks, norovirus was mostly identified after salmonella, hepatitis A virus, and campylobacter worldwide **(CDC, 2022; EFSA, 2021; WHO, 2021)**. In norovirus outbreaks, the transmission routes are commonly person-to-person and foodborne. The main sources of infection are norovirus-contaminated water, vegetables, fruits, seafood and ready-to-eat raw foods **(van Beek *et al.*, 2018)**. Campylobacter outbreaks are mostly caused by the consumption of contaminated animal products, especially poultry and dairy **(Tang *et al.*, 2017, 2020)**. Campylobacter outbreaks ranked 2nd in foodborne outbreaks between 2015 and 2019 after salmonella **(CDC, 2021)**. Most campylobacter outbreaks reported resulted from contaminated chicken consumption **(Dogan *et al.*, 2019)**.
Because of low infection doses, no-licensed vaccines, antimicrobial resistance, high medical costs, and long-time infectivity on foods, water and fomites **(CDC, 2015; Hara-Kudo & Takatori, 2011)**, norovirus and campylobacter are still serious agents to cause millions of cases per year and treat public health. Currently, a wide range of plants and their extracts were applied as food additives and preservatives during food production and service to improve safety and quality by presenting antibacterial and antiviral activities **(Alp & Aksu, 2010; Dhouibi *et al.*, 2020; Körpe *et al.*, 2013)**. Therefore, the present study assessed the antiviral effects with three pathways on murine norovirus 1 (MNV-1) as a surrogate human norovirus and antibacterial effects on three *C. jejuni* strains of methanolic extract of *U. dioica* L. leaf (UDE).

MATERIAL AND METHODS

Sample Preparation

The nettle samples used in the study were collected from Duzkoy province (40°56'59.1"N, 38°36'06.5"E), the city of Giresun on the Black Sea coast of Turkey in May 2021. They were identified and authenticated as "*Urtica dioica* L." at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Marmara University, and a herbarium record was created with the code "MARE 23334". The samples were dried in cool room conditions avoiding direct sunlight.
The alcoholic extraction method of the dried sample was adapted from the solid/liquid extraction and evaporation methods of **Doukkali *et al.* (2015)** and **Simunovic *et al.* (2020)**. Dried samples were ground by a water-cooled miller. Fifty grams of ground nettle was macerated in 500 ml of methanol (reagent grade, ≥ 99.7%) on a magnetic stirrer for 24 hours. The alcoholic suspension was filtered twice through filter paper (FilterLab-50 g/m^2). The solvent in the remaining filtrate was evaporated in a rotary evaporator (Heidolph, Germany) at 40-45°C, 150 mbar, and 135 rpm. The extract was completely concentrated in a vacuum oven (Nüve EV 018, Turkey) at 45°C and -1 bar pressure. A UDE stock solution of 40 mg/ml was prepared in ultra-distilled water and filtered through a 33 mm diameter sterile syringe filter with a 0.22 µm pore size (Milipore, USA). The sterility of UDE stock solution was checked by inoculating in an agar plate containing MHA. pH was measured with a digital pH meter (Milwaukee MW 102, USA) before use in the antimicrobial assays. It was stored at +4°C for further analysis.

Bacteria, Virus and Cell Lines

The antibacterial activity of UDE was investigated with two isolates previously isolated from poultry meat in the field and biochemically identified as *C. jejuni* (Isolate 1 and Isolate 2) **(Ucar, 2020)** and also a standard strain (*Campylobacter jejuni* subsp. *jejuni*, ATCC 33560) of *C. jejuni*. The bacteria were sub-cultured on Mueller Hinton agar (MHA) at 42 °C in an anaerobic jar under micro-aerobic conditions supplied with CampyGen (OXOID) for 48 h. The cultured bacteria were added and vortexed in test tubes containing Mueller Hinton Broth (MHB) to attain a standard suspension of McFarland 0.5 (10^8 cfu/ml). The sterility of UDE stock solution was analysed by inoculating in an agar plate containing MHA and pH was measured with a digital pH meter (Milwaukee MW 102, USA) before use in the antimicrobial assays.

Murine norovirus 1 strain (MNV-1, VR-1937) and murine macrophage cell line (RAW 264.7, TIB-71) were from ATCC, USA. RAW 264.7 was cultured and maintained with Dulbecco's Modified Eagle Medium (DMEM) in the incubator with the standard condition (SC) of 37 °C and 5% CO_2. The medium contained fetal bovine serum (10%), L-alanyl-L-glutamine (200 mM) and 1% penicillin (10.000 unit/ml)-streptomycin (10 mg/ml)-amphotericin B (0.025 mg/ml). For virus titration in all experiments, the ten-fold serial dilutions method (10^{-1}-10^{-10}) was performed by adding microplate wells containing 100 µl host cell suspension ($3x10^5$ cell/ml) in six replicates. For cytotoxicity and antiviral assays, 100 µl of the stock viable cell suspension ($3x10^5$ cell/ml) was seeded in each well ($3x10^4$ cell/well) and kept in SC for 24 h to confluence at least 90% in 96-well microplates.

Antibacterial activity on *C. jejuni* isolates

The antibacterial activity of UDE was investigated with the two-fold dilutions of UDE on two isolates and one standard strain of *C. jejuni*. For the determination of the minimum inhibitory concentration (MIC) of UDE, the broth microdilution method was used in 96-well microplates. Briefly, the stock solution of UDE (40 mg/ml) was two-fold diluted with 100 µl of MHB/well in 96-well V-bottom microplates in six replicates. Then, 10 µl suspension of each *C. jejuni* (0.5 McFarland) was inoculated to each well of microplates except negative control wells. The positive control wells only consisted of 100 µl MHB and 10 µl bacteria suspension. The microplates were incubated in micro-aerobic conditions at 42 °C for 24 h. After incubation, the microplates were observed and read by a spectrophotometer (Byonoy Absorbance 96, Germany) at 600 nm for visible turbidity as microbial growth.
For the determination of the minimum bactericidal concentration (MBC) that caused completely the bacterial death, the inoculums from the microplate wells of MIC assay were inoculated in agar plates containing MHA in quadruple. The plates were incubated in micro-aerobic conditions at 42 °C for 48 h.

Cytotoxicity assay by MTT

The extract was two-fold serially diluted with the maintaining medium at the concentration of 0.62, 1.25, 2.5, 5, 10 and 20 mg/ml. Then, 100 µl of each dilution was added to the microplates with the monolayer host cell (RAW 264.7) at six-replicated wells. The fresh medium was only added to cell control wells (medium and cell) and blank wells (medium without cell). The microplates were incubated in SC for 24 h. 10 µl MTT in PBS (5 mg/ml) was added to each well. After a 4 h incubation in SC, the supernatant was discarded and DMSO (100 µl) was added to wells. The microplate was gently shaken to solubilize the formazan crystals and read at a wavelength of 570 nm (Absorbance 96, Byonoy, Germany).

Virucidal activity

The virucidal activity protocol was adapted from BS EN 14476:2013+A2:2019 standard method for the evaluation of virucidal activity in the medical area **(European Commission, 2019)**. Briefly, the stock virus suspension of 6.5 $TCID_{50}$/ml (1:10 v/v) was exposed to each extract dilution (7:10 v/v/) containing bovine albumin solution (BSA 0.3 g/l) as an interfering substance (2:10 v/v) at 20 °C for 30 s. After the contact time, 1 ml of each mixture (virus, extract, BSA) was transferred to a 9 ml ice-cold medium to stop the reaction. For the titration of the remaining virus, ten-fold serial dilutions were inoculated in 96-well microplate wells containing 100 µl the host cell (RAW 264.7) suspension in six replicates and incubated in SC for 72 h. Formaldehyde (0.7% in PBS) and PBS containing BSA were respectively used as positive and negative controls in parallel with the virucidal test.

Antiviral activity

Three types of antiviral strategies were performed to determine the effects of non-cytotoxic UDE dilutions on binding, penetration to host cell and prophylaxis of MNV-1 (**Figure 1**). All three experiment types were conducted with the host cell (RAW 264.7) in 24-well cell culture plates (12×10^4 cell/well) and kept in SC to confluence at least of 90% the day before. The negative (uninfected, untreated cell) control and positive (infected, untreated) control were conducted in parallel.

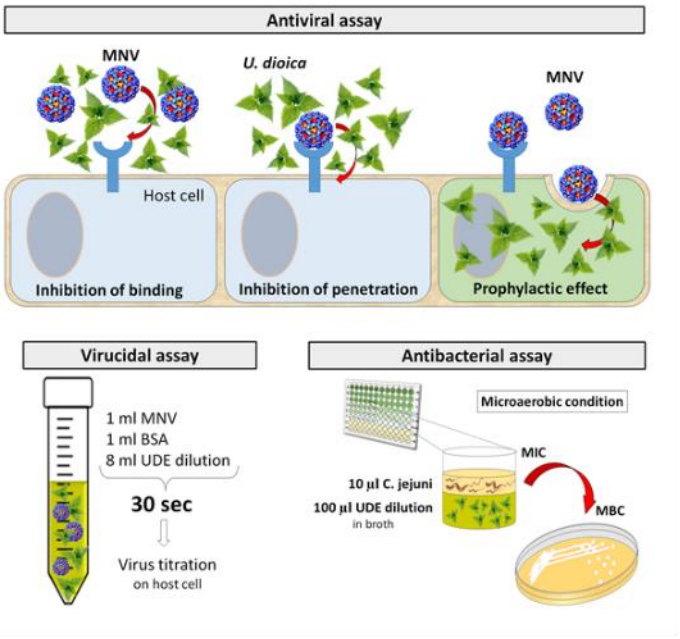

Figure 1 Summary of antiviral pathways and antibacterial protocols

Effect of UDE on virus binding to host cell

The host cell was treated with both MNV-1 and UDE at different concentrations during the 72-h incubation. Each non-cytotoxic dilution (0.625, 1.25 and 2.5 mg/ml medium) of the extract (1 ml) and the 200 µl virus suspension (MOI: 10 $TCID_{50}$/cell) were added to three-replicated wells except negative control wells.

Effect of UDE on virus penetration in the host cell

The host cell was treated with different concentrations of UDE after a one-hour adsorbing period of MNV-1. The virus suspension (MOI: 10 $TCID_{50}$/cell) of 200 µl was added to 24-well cell culture plates coated with the host cell except for negative control wells. The plate was incubated in SC for 1 h to adsorb the virus to host cells. Then, the unbound viruses were discarded by washing the wells with PBS. Then, non-cytotoxic dilutions (0.625, 1.25 and 2.5 mg/ml medium) of the extract were added to three-replicated wells.

Prophylactic effect of UDE against the virus

The host cell was infected with the virus titer of 6.5 $TCID_{50}$/ml after a 4-hour treatment period with different concentrations of UDE for prophylactic effect. Each non-cytotoxic dilution (0.625, 1.25 and 2.5 mg/ml medium) of the extract was added to 24-well cell culture plates coated with the host cell in three replicates. The plate was incubated in SC for 4 h to treat the host cell with UDE. Then, all mediums containing the extract were discarded by washing the wells with PBS. One millilitre of maintaining medium containing virus suspension (MOI: 10 $TCID_{50}$/cell) was added to all wells except negative control wells.
The plate was maintained in SC for 72 h with daily CPE observation. At the end of 72 h, the suspensions in 24-well plates were repeatedly frozen and thawed to perform the virus titration in the 96-well microplates with host cell suspension in SC for each extract dilution and the controls.

Data analysis

The percentage of cell inhibition was calculated using the equations as follows,

Cell viability (%) = (OD sample – OD blank) / (OD control – OD blank) x 100".

Virus titration was calculated as a tissue culture infective dose of 50% ($TCID_{50}$) using the method of Spearman–Karber on all experiments. The 50% cytotoxic concentration (CC_{50}), 50% virucidal concentration (VC_{50}) and 50% inhibitory concentration for antiviral activity (IC_{50}) were calculated from concentration-based-curves after linear regression analysis using Office Excel 2016 (Microsoft, USA). The selectivity indexes (SI) were also determined for antibacterial (CC_{50}/MIC), virucidal (CC_{50}/VC_{50}) and antiviral (CC_{50}/IC_{50}) capacity. Statistical analyses were conducted by using SPSS version 15 software (IBM, USA)

RESULTS AND DISCUSSION

MIC and MBC of UDE for *C. jejuni*

UDE was extracted from dried and ground *U. dioica* L. leaves with a yield of 16.2% by the methanolic extraction method in this study. The UDE stock solution was sterile and had a pH of 7.45±0.02.

C. jejuni is a foodborne pathogen that has the ability of quorum-sensing, motility, biofilm formation, adhesion to and invasion of surfaces and host cells. It was shown that ethanolic plant extracts including *U. dioica* L. could inhibit motility, adhesion to polystyrene surfaces, and invasion of host cells by *C. jejuni* through influencing quorum-sensing. And, MIC was determined as 1 mg/ml for *C. jejuni* NCTC 11168 **(Šimunović *et al.*, 2020)**. The pressurized liquid extracts (water, ethanol and acetone) of *U. dioica* L. leaf showed antioxidant activity and antimicrobial activity against *C. jejuni* NCTC 11168 strain with a MIC of 0.5 mg/ml and a MBC of 1 mg/ml **(Garofulić *et al.*, 2021)**. In this study, the methanolic extraction of *U. dioica* L. did not inhibit the growth of *C. jejuni* strains at the concentrations of 0.625, 1.25 and 2.5 mg/ml. But the concentrations of 5, 10 and 20 mg/ml inhibited all three strains resulting in an optically clarify as like negative control (Table 1). When comparing the results of the spectrophotometer assay, there were significant differences in optic density (OD, nm) of turbidity between the concentrations 5, 10 and 20 mg/ml and positive control for all *C .jejuni* strains ($p<0.01$). But, there was no growth on the agar plates only subcultured from the wells of 20 mg/ml UDE and negative control. So, MIC and MBC were respectively determined as 5 mg/ml and 20 mg/ml for all strains. Therefore, SI (CC_{50}/MIC) values for the three strains were the same and presented in **Table 1**.

Table 1 MIC and MBC of UDE against *C. jejuni* strains

UD extrract (mg/ml)	Field Isolate 1			Field Isolate 2			ATCC 33560		
	96-well Microplate	OD_{600} (nm)	Agar Plate	96-well Microplate	OD_{600} (nm)	Agar Plate	96-well Microplate	OD_{600} (nm)	Agar Plate
20	Clear	0.333±0.015c	-	Clear	0.378±0.038c	-	Clear	0.356±0.019c	-
10	Clear	0.640±0.060b	+	Clear	0.707±0.085b	+	Clear	0.674±0.062b	+
5	Clear	0.673±0.043b	+	Clear	0.755±0.132b	+	Clear	0.663±0.050b	+
2.5	Turbid	1.219±0.086a	+	Turbid	1.314±0.045a	+	Turbid	1.227±0.039a	+
1.25	Turbid	1.029 0.097a	+	Turbid	1.055±0.019a	+	Turbid	1.051±0.025a	+
0.625	Turbid	0.960±0.053a	+	Turbid	0.951±0.026a	+	Turbid	1.019±0.023a	+
NK	Clear	0.195±0.051c	-	Clear	0.179±0.035c	-	Clear	0.175±0.027c	-
PK	Turbid	0.934±0.038a	+	Turbid	0.925±0.017a	+	Turbid	1.000±0.035a	+
P value		<0.01			<0.01			<0.01	
MIC		5 mg/ml			5 mg/ml			5 mg/ml	
MBC		20 mg/ml			20 mg/ml			20 mg/ml	
SI (CC_{50}/MIC)*					2.53				

*for RAW 264.7 as host cell

Cytotoxicity of UDE

Before the antiviral assay, the effects of cell viability and non-cytotoxic concentrations of UDE were determined on the murine macrophage cell as the virus host cell. Previous studies generally used the cell lines of mammalian kidney and liver for the cytotoxicity of UDE. A study suggested that methanolic extract (CC_{50}:0.702-0.803 mg/ml) was safer than aqueous extract (CC_{50}:0.37-0.49 mg/ml) of *U. dioica* L. on BHK-21 **(Flores-Ocelotl *et al.*, 2018)**. For macrophage (RAW 264.7) and hepatocyte (HepG2) cell lines, the non-toxic concentration of the ethanolic extract was calculated as 0.20 and 0.35 mg/ml respectively **(Carvalho *et al.*, 2017)**. Also, the ethanolic extracts of *U. dioica* L. showed higher cytotoxicity than the aqueous extracts **(Mannila *et al.*, 2022)**. In this study, the cytotoxicity of UDE experimented on the viability of RAW 264.7 as the virus host cell. CC_{50} was calculated as 12.65 for RAW 264.7 (**Figure 2**). High CC_{50} values indicate the low cytotoxicity of *U. dioica* L. At the concentrations of 0.625, 1.25 and 2.5 mg/ml UDE, the cell viability rates of RAW 264.7 as MNV-1 host cell were 93.80%, 86.16% and 80.25% respectively (**Figure 2**). Therefore, 0.625, 1.25 and 2.5 mg/ml concentrations of UDE were selected to evaluate the antiviral activity assay.

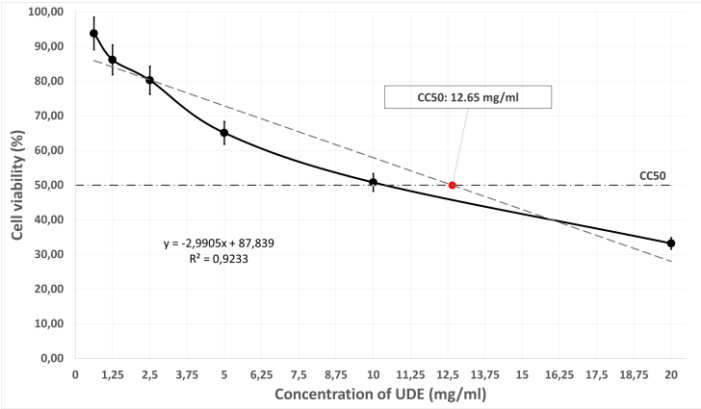

Figure 2 The effects of UDE on the viability of virus host cell

Virucidal activity of UDE

With Campylobacter, norovirus can stay infective for a long time on biological secretions, foods, food contact surfaces and fomites **(Cook *et al.*, 2016; Isakbaeva *et al.*, 2005; Trudel-Ferland *et al.*, 2021)**. The virucidal activity of UDE was investigated with the perspective of preventing norovirus contamination from contaminated biological fluids, food contact surfaces and fomites. Previously, the virucidal test of a mixture consisting of propylene glycol (79.0%), water (17.6%) and *U. dioica* L. extract (3.0%) was not effective against the African swine fever virus **(Juszkiewicz *et al.*, 2021)**.

This was the first study reporting the virucidal activity of *U. dioica* L. extract against norovirus tested by an in vitro suspension test. The test was valid with 0.7% formal aldehyde caused a logarithmic reduction of ≥4 $TCID_{50}$/ml in BSA soiling condition at least **(European Commission, 2019)**. In the study, 0.7% formal aldehyde caused a reduction of 5.5 $TCID_{50}$/ml (84.6%) as the positive control. Also, a reduction of 0.75 $TCID_{50}$/ml (11.5%) was measured in the negative control. The two-fold dilutions of UDE presented a titer reduction between 4.5 (69.2%) to 1.0 (15.4%) $TCID_{50}$/ml (**Figure 3**). The most effective concentrations of the tested was 20 mg/ml of UDE caused a reduction of ≥4. VC_{50} and IS for virucidal activity were calculated as 10.45 mg/ml and 1.21 with a linearity of $R^2=0.863$ ($y = -0.1606x + 4.9291$).

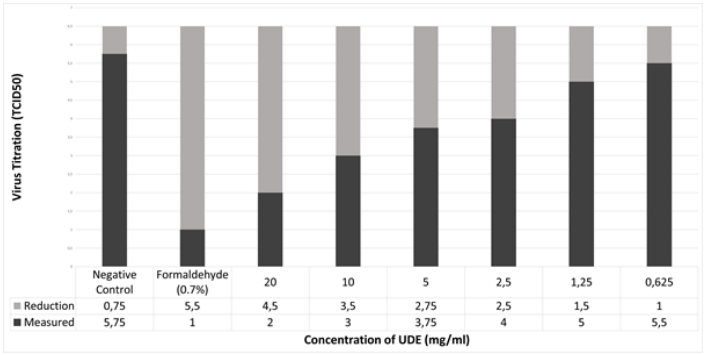

Figure 3 Virucidal activity of UDE

Antiviral activity of UDE

Plant extracts including *U. dioica* L. were studied as potent phytomedicines against a variety of foodborne RNA viruses (norovirus, coronaviruses, rotavirus) causing outbreaks and threatening food safety and public health **(Knipping *et al.*, 2012; Siddiqui *et al.*, 2020; Živković *et al.*, 2021)**. It was presented that the lectins from *U. dioica* L. inhibited the enveloped RNA viruses including coronaviruses with low cytotoxicity and high selectivity of antiviral activity **(van der Meer, 2014)**. At a lower inhibition concentration (IC_{50}<0.3 mg/ml) than many edible plants, the aqueous extract of *U. dioica* root presented a strong antiviral activity against rotavirus which is a non-enveloped RNA virus causing foodborne outbreaks **(Knipping *et al.*, 2012)**. The methanolic extracts of *U. dioica* L. leave inhibited the replication of RNA-genome dengue virus with IC_{50} values of 0.126 mg/ml and

SI of 6.01 **(Flores-Ocelotl *et al.*, 2018)**. Because of their non-enveloped structure with RNA genome in Caliciviridae, murine norovirus is usually used as a norovirus surrogate in foodborne infection models to investigate the adhesion, survival, infectivity, elimination, inactivation and control of norovirus **(Bozkurt *et al.*, 2021; Cook *et al.*, 2016; Mannila *et al.*, 2022; Trudel-Ferland *et al.*, 2021)**. Previously, the ethanolic and aqueous extracts of UD leaf showed a weak inhibition effect (reduction of log 0.63-0.67) on MNV-1 **(Mannila *et al.*, 2022)**. In this primary study on the antiviral effects of *U. dioica* L. leaf extract on norovirus, the three pathways of MNV-1 were investigated to set a control model for foodborne viral infection (**Figure 1**).

After 72-h incubation, the virus was tittered in the positive control (non-treated with UDE), but not in the negative control (non-treated with the virus). All concentrations of UDE significantly reduced the virus titer when compared to positive control for three pathways ($p<0.01$). The highest reduction rates were at the concentrations of 1.25 and 2.5 mg/ml for the virus penetration experiment and the concentration of 2.5 mg/ml for both virus binding and prophylaxis experiments ($p<0.01$) (**Table 2**). IC_{50} was 1.45, 1.87 and 1.60 mg/ml for the inhibition of virus binding and penetration effects and prophylaxis respectively. IS was calculated as 8.72, 6.76 and 7.91 for all three pathways of MNV-1 on host cells respectively. A higher SI value than 4 indicated that UDE had low cytotoxicity and high antiviral activity.

Table 2 Antiviral activity of UDE on the infectivity pathways of MNV

UDE (mg/ml)	Inhibition of virus penetration			Inhibition of virus binding			Prophylactic effect		
	Measured	Reduction	Inhibition %	Measured	Reduction	Inhibition %	Measured	Reduction	Inhibition %
2.50	3.00	3.50	53.85±7.69a	1.50	5.00	76.92±3.8a	1.50	5.00	76.92±3.8a
1.25	3.00	3.50	53.85±3.8a	2.75	3.75	57.69±3.8b	4.25	2.25	34.62±7.6b
0.625	4.75	1.75	26.92±3.8b	5.50	1.00	15.38±7.6c	4.75	1.75	26.92±7.6b
Pos. Control	6.75	-0.25	-3.8±7.6c	6.25	0.25	3.8±3.8d	6.5	0	0.00c
P value		<0.01			<0.01			<0.01	
IC_{50} (mg/ml)		1.45			1.87			1.6	
CC_{50} (mg/ml)*		12.65			12.65			12.65	
IS		8.72			6.74			7.90	

*for RAW 264.7 as host cell; Measured and Reduction were expressed as log TICD50/ml

CONCLUSION

The present study gives information about the antibacterial activity and antiviral activity of *U. dioica* L. harvested during the growing season in Turkey.

Dose-depend antibacterial and antiviral experiments were conducted on *C. jejuni* and MNV-1 as norovirus surrogates which are foodborne pathogens threatening food safety and public health. The extract showed an antibacterial activity on both standard strain and field isolates of *C. jejuni* in similar MIC and MBC manners. The results of IC_{50} and SI suggested that there was a slight difference between antiviral activities on virus infectivity pathways, however, the antiviral activity of UDE on the host cell could be stronger than the virucidal activity.

For inhibition of pathogens, maintaining hygiene and extending the shelf-life of foods, a wide range of plant-based food additives are used in the food industry. *Urtica dioica* L. might be considered an alternative natural supplement or additive in food. In conclusion, the present study contributed to further studies with novel data on the antimicrobial effects of *Urtica dioica* L. against foodborne pathogens.

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Authors' contribution: Conception and design, H.B.A., S.K. and B.I.O.K.; methodology, S.K. and B.I.O.K.; performing the analysis and data collection, H.B.A. S.K. and B.I.O.K..; data analysis, H.B.A. and S.K.; drafting the article, H.B.A. and S.K.; critical revision, H.B.A., S.K. and B.I.O.K.; supervision, B.I.O.K. All of the authors have read and agreed to the final approval of published version of the manuscript.

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