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In Vitro Vitamin C Equivalent Antioxidant Capacity, Cytotoxicity and Anti-Cancer Activity of *Methanolic Urtica dioica* L. Leaf Extract as a Food Supplement

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HIGHLIGHTS

- The average Vitamin C Equivalent Antioxidant Capacity was calculated as 42.3 µg/mg methanolic Urtica dioica L. leaf extract.
- Cytotoxic Concentration 50% was calculated as 15.71 mg/ml and 5.14 mg/ml for mammalian kidney cells.
- Inhibition Concentration 50% was calculated as 2.46 mg/ml for human hepatocellular carcinoma.
- This dose-response study presented the effects of methanolic Urtica dioica L. leaf extract on kidney cytotoxicity and the proliferation of liver cancer cells.

Abstract

Antioxidant capacity, cytotoxicity on two vital cell lines and the anti-cancer activity of methanolic *Urtica dioica* L. leaf extract (UDE) collected from Duzkoy, Giresun, Turkey were studied by determining safe concentration. The antioxidant capacity of the extract was expressed as vitamin C equivalency by spectrophotometric MTT assay. The cytotoxic concentration 50% was measured by the linearity between UDE concentrations (CC50) and the cell viability of non-cancer kidney cell lines (BHK-21, MDBK). The anti-cancer activity was conducted on human hepatocellular carcinoma cells (HepG2) by determining inhibition concentration (IC50) on cell proliferation. The vitamin C equivalence of UDE increased linearly by increasing the concentration. The cytotoxic and non-toxic concentrations of UDE were determined on BHK-21 and MDBK with 15.71 mg/ml and 5.14 mg/ml of CC50 respectively. The extract inhibited the proliferation of human hepatocellular carcinoma cells with a 2.46 mg/ml of IC50. In conclusion, the present study tried to explain in detail the dose-dependent activity of *Urtica dioica* L. leaf extract. The dose-response results showed that *Urtica dioica* L. leaf extract could have low cytotoxicity, but potential anti-cancer activity at safe concentrations.

Keywords: Anti-cancer; Cytotoxicity; Food additive; Nettle; *Urtica dioica* L.

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1. 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 (Upton, 2013; Dhouibi et al., 2020; European Commission, 2022). 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 and Aksu, 2010). Phytochemical studies have mainly focused on its bioactive compounds and activities related to antioxidant contents (Körpe et al., 2013; Dhouibi et al., 2020; Veiga et al., 2020). Therefore, 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 antioxidant activities at their safe concentrations (Alp and Aksu, 2010; Körpe et al., 2013; Dhouibi et al., 2020).

In addition to antioxidant capacity, several in vitro and in vivo studies of plants aimed to determine the non-toxic doses for preventing adverse effects before using them including Urtica dioica L. as a potential therapeutic agent in modern and traditional medicine (Özkol et al., 2012; Sayhan et al., 2012; Dhouibi et al., 2020; Veiga et al., 2020). In recent, the aerial and subsoil parts of Urtica dioica L. were experimentally used to treat many cancer types by inhibiting cell proliferation (Konrad et al., 2000; Gözüm et al., 2003). Also, the cancer treatment has a risk of toxicity in different tissues including kidney and liver by inducing oxidative stress and free radical generation (Özkol et al., 2012; Dhouibi et al., 2020). So, the present study aimed to assess dose-dependently the antioxidant capacity, cytotoxicity on non-cancer kidney cell culture lines and anti-cancer activity on liver cancer cells of methanolic U. dioica L. leaf extract (UDE).

2. Materials and Methods

The Materials and Methods should be described with sufficient details to allow others to replicate and build on the published results. Please note that the publication of your manuscript implies that you must make all materials, data, computer code, and protocols associated with the publication available to readers. Please disclose any restrictions on the availability of materials or information at the submission stage. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.

2.1. Extraction of U. dioica L.

The sampling and extraction methods used in this study were adapted from the thesis of Aydın (2022) (Aydin, 2022). Briefly, U. dioica L. leaf samples 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 in Marmara University, and an herbarium record was created with the code "MARE 23334". The samples were dried in cool room conditions avoiding direct sunlight.

Solid/liquid extraction and evaporation methods were used for the extraction of dried samples. Dried samples were ground by a water-cooled miller. Fifty grams of ground leaves were 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/m2). 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 stored at +4 °C for further analysis.

2.2. Handling of Cell Lines

Baby hamster kidney fibroblast (BHK-21, CCL-10), bovine kidney epithelial (MDBK. CCL-22) and human hepatocellular carcinoma (HepG2. HB-8065) cell lines were from ATCC, USA. All cell lines were cultured with Eagle's Minimum Essential Medium (EMEM) in the incubator with the standard condition (SC) of 37 °C and 5% CO2. The medium contained foetal 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 the anti-cancer and cytotoxicity

assay, the stock viable cell suspension (3x105 cell/ml) (100 µl) was transferred in each well (3x104 cell/well) and kept in SC for 24 h to achieve the confluence of at least 90% in 96-well microplates.

2.3. Vitamin C equivalent antioxidant capacity

Vitamin C (l-ascorbic acid. HPLC grade) was two-fold diluted as 600. 300. 150. 75 and 37.5 µmol with ultra-distilled water. UDE was two-fold diluted as 0.62. 1.25. 2.5. 5. 10 and 20 mg/ml with ultra-distilled water. MTT (2.5-diphenyl-2H-tetrazolium bromide, 1 mg/ml) (380 µl) and each Vitamin C or extract dilution (20 µl) were mixed in an Eppendorf tube and incubated at 37 °C for 4 h. After incubation, DMSO (400 µl) was added to all tubes and mixed well to solve the blue formazan salt formed during the incubation. 100 µl of each mixture was added quadruplicated to the U-bottom 96-well microplate. The microplate was read at 570 nm.

2.4. Anti-cancer and cytotoxicity assays 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, each dilution (100 µl) was added to the microplates with monolayer cell culture at six-replicated wells. The cell control wells contained only fresh medium and cells. The blank wells contained the medium without cells. 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).

2.5. 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".

The 50% cytotoxic concentration (CC50) was calculated from concentration-based-curves after non-linear regression analysis (y = mebx). The vitamin C standard curve was generated with optic density (OD. nm) values of five vitamin C dilutions by linear regression analysis (vitamin C equivalence = m x OD570 + b. R^2). The equivalence to vitamin C of extract dilutions was calculated concerning the standard curve (Figure 1). Statistical analyses were conducted by using SPSS version 15 software (IBM. USA). The graphs were generated by using Office Excel 2016 (Microsoft. USA.

3. Results

3.1. Vitamin C equivalent antioxidant capacity of UDE

The natural antioxidants in herbal additives such as carotenoids, tocopherol (vitamin E), some phenolic compounds, and ascorbic acid inhibit oxidative damage by free-radical scavenging (Dini, 2019; El-Saber Batiha et al., 2021). Previous research suggested that fresh *U. dioica* L. leaves contained various amounts of total ascorbic acid content from 16 to 112.8 mg/100 g fresh weight at different harvest times (Skalozubova and Reshetova, 2013; Shonte et al., 2020). The highest vitamin C was measured in August while the lowest was in September (Paulauskienė et al., 2021). As the vegetation period and drying methods influenced the vitamin C content and antioxidant capacity of *U. dioica* L. leaves, it was not varied by meteorological conditions. The highest vitamin C content was determined with low-temperature drying compared to owen drying (Shonte et al., 2020; Garcìa et al., 2021; Paulauskienė et al., 2021). In this study, *U. dioica* L. leaves were harvested in May, dried under cool-dry room conditions and extracted by methanol. The vitamin C equivalence of UDE was calculated with regards to the standard curve by linear regression analysis (vitamin C equivalence. µmol = 1191.6 x OD of UDE - 1.4723. R^2 = 0.998). The vitamin C equivalence of UDE increased by increasing the concentrations of UDE with a linearity of R^2=0.690 (vitamin C equivalence, µmol = 29.578 x UDE mg/ml + 501.51) (Figure 1). In harmony with previous studies, the average vitamin C content was calculated as approximately 42.3 µg/mg in methanolic extract of *U. dioica* L. leaves collected from Giresun region in Turkiye.

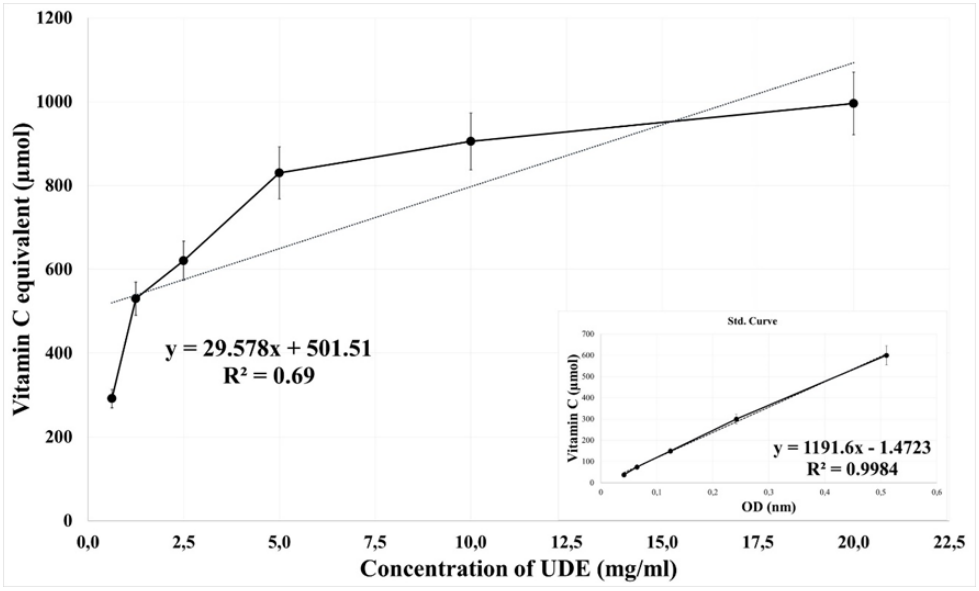

Figure 1. The vitamin C equivalency antioxidant capacity of UDE based on concentration.

3.2. Cytotoxicity of UDE

The cytotoxicity and safety levels of plant extracts vary related to the extraction method and are generally tested with cell lines of mammalian kidney and liver. A previous study suggested that methanolic extract (CC50:0.702-0.803 mg/ml) was safer than aqueous extract (CC50:0.37-0.49 mg/ml) of *U. dioica* L. on BHK-21 (Flores-Ocelotl et al., 2018). Also. the ethanolic extracts of *U. dioica* L. showed higher cytotoxicity than the aqueous extracts (Mannila et al., 2022). In this study, the cytotoxic effects of methanolic *U. dioica* L. leaf extract experimented on the viabilities of non-cancer (BHK-21 and MDBK) cell lines with concentration-response curves (Table 1). The relation between the cell viability and the concentration was the cell viability %=99.474e-0.044 x UDE (mg/ml) (R^2 = 0.936) for BHK-21 and the cell proliferation %=163.76$e^{-0.23}$ x UDE (mg/ml) (R^2 = 0.913) for MDBK (Figure 2). The CC50, was calculated as 15.71 mg/ml and 5.14 mg/ml for BHK-21 and MDBK respectively (Table 1). The Higher CC50 than the previous study indicated that the methanolic extract of *U. dioica* L. leaves might be a safer and potential food supplement and additive.

Table 1. The dose-response effects of UDE on cell viability %.

UDE Conc. (mg/ml)	Non-Cancer Cells		Cancer Cell
	BHK-21	MDBK	HepG2
	Mean±SD (%)	Mean±SD (%)	Mean±SD (%)
20	44.66±5.00c	1.13±4.44e	2.37±3.38d
10	56.36±6.50bc	43.21±4.30d	22.65±2.30c
5	73.59±9.15b	61.73±8.35c	34.49±5.25bc
2.5	93.45±4.25ab	79.42±4.01b	44.05±2.30b
1.25	99.82±8.35ab	101.54±2.02a	60.05±6.61a
0.62	99.95±5.51a	102.82±1.51a	60.13±5.45a
P value	<0.01	<0.01	<0.01
CC_{50} (mg/ml)	15.71	5.14	2.46

3.3. Anti-cancer activity of UDE

Various studies have recently demonstrated the cytotoxic and anti-cancer properties of *U. dioica* L. in particular against colon, gastric, lung, prostate and breast cancers (Esposito et al., 2019). Aqueous extract of *U. dioica* L. leaf cultured in Iran inhibited the proliferation of human breast cancer (MCF-7) at 2 mg/ml after 72h treatment (Fattahi et al., 2013, 2018). Its aqueous extract (from 5 to 30 µg/mL) harvested in Turkey has shown a dose-dependent inhibition effect on three breast cancer cell lines (MCF-7, MDA-MB-468, and MDA-MB-231) with IC50s of 14-18 µg/ml (Karakol et al., 2022). For Its dichloromethane extract against both mouse and human breast cancer cells, IC50 was determined between 31.37 and 38.14 mg/ml. Similarly, its anti-cancer

activity was measured by inhibiting the metastasis of breast cancer with 20 mg/kg daily injection treatment in vivo mouse models (Mansoori et al., 2017; Mohammadi et al., 2017) (Mohammadi 2017 Mansoori 2017). For its anti-cancer activities on human prostate cancer, the dichloromethane and aqueous extracts inhibited the proliferation of PC3 and LNCaP cells with 15.54 µg/ml and 42-50 µg/ml of IC50, respectively (Durak et al., 2004; Levy et al., 2013; Mohammadi et al., 2016). Meanwhile, no cytotoxicity was determined in non-cancer human prostate stromal cells (Konrad et al., 2000). *U. dioica* L. hydroalcoholic (50:50 v/v) extract inhibited 25.4% of human hepatocellular carcinoma (HepG2) cell viability at the concentration of 0.35 mg/ml (Carvalho et al., 2017). In this study. The anti-cancer effect of UDE on the proliferation of HepG2 cells was determined by spectrophotometric MTT assay with the concentration-response curve (cell proliferation % = $74.941e^{-0.163}$ x UDE (mg/ml), R^2 = 0.965) (Figure 2). The IC50 was calculated as 2.46 mg/ml for human hepatocellular carcinoma (Table 1). These IC50 values present the potential anti-cancer activity through hepatoprotective and reducing tumorigenesis activities of methanolic extract of *Urtica dioica* L. leaf used at safe concentrations.

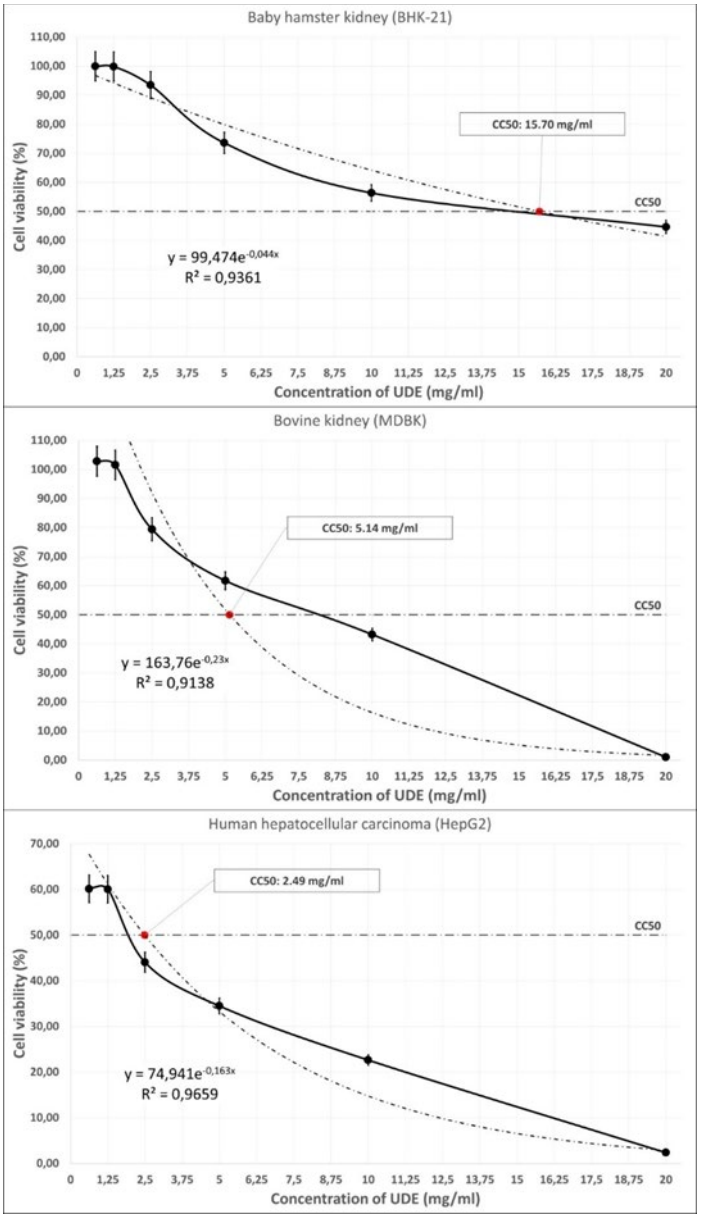

Figure 2. The cell viability and inhibition with CC50 (mg/ml) of UDE

4. Conclusions

In conclusion, the present study gives information about the bioactivity of U. dioica L. leaf harvested during the growing season in Giresun city of Turkey. The extract had dose-dependently antioxidant capacity expressed as vitamin C equivalency. Non-toxic and safe concentrations of the extract were determined in detail on vital cell lines originating from kidney tissue which are generally used in food and drug research for consumer safety. The dose-response results indicated that the extract was less toxic to kidney cells while inhibiting the proliferation of liver cancer cells at a lower concentration.

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