Original article

Investigation of Chemopreventif Properties of Urtica Dioica L., in MCF-7 and MDA 231 Breast Cancer Cell Lines

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ÖZET

MCF-7 ve MDA 231 hücre hatlarında Urtica Dioica'nın kemopreventif özelliklerinin araştırılması

Giriş: Gelişmekte olan dünyanın en büyük sağlık problemi tedavide yetersiz kalınan kanser hastalığıdır. Çalışmanın amacı Türkiye'de yetişen ısırgan otunun var olabilecek kanser oluşumunu engelleyici özelliklerinin ve etki mekanizmalarının araştırılmasıdır.

Materyal ve Metot: Isırgan sulu ve asit bitki özütlerinde, antioksidan aktivite tayin metotlarından toplam fenol, flavon ve serbest radikal süpürücü aktivitesi çalışılmıştır. U. dioica özütünün, 48 saat süreyle, artan konsantrasyonlarda MCF7 ve MDA-231 hücre kültürleri üzerindeki sitotoksik etkisi XTT yöntemi ile tespit edildi.

Sonuç: Çalışmada kullanım gören hücre kültürü, fenol flavon tayini ve HPLC sonuçlarına göre araştırma bulguları: ısırgan bitkisinin etki mekanizmalarıyla kanser oluşumunu engelleyici özelliğini göstermektedir.

Anahtar Kelimeler: Kemoprevansiyon; Urtica diocia; antioksidan; DPPH; total fenol; XTT; MCF-7; HPLC; antikanser etki

ABSTRACT

Introduction: Cancer is a major health problem in developing world with mostly unsufficient treatment. The interest of present research is to check traditional Turkish herb "Urtica diocia L" which seems to provide promising agents with the potential protection against cancer and search for their mechanism of action.

Material and Method: Total phenol and flavon contents and radical scavenging activity (RSA) were also determined to survey antioxidant potential of U. diocia aqueous and acid extracts. Cells were incubated with the extract in the variying concentration range for 48 h and the cytotoxicity was determined using the XTT assay.

Conclusion: According to the results of determination of free radical scavenging activity and cytotoxicity assay, HPLC-DAD profiles of "U.dioica" which seems to provide promising agents with the potential protection against cancer and findings from its mechanism of action.

Key Words: Chemoprevention; Urtica diocia; antioxidant; DPPH; total phenolics; XTT; MCF-7; HPLC; anticancer effect

INTRODUCTION

DNA accumulates changes that activate proto-oncogenes and inactivate tumour-suppressor genes. The genetic instability driving tumorigenesis is fuelled by DNA damage and by errors made by the DNA machinery[1].

The researchers identified adenosine deaminase inhibition by U. dioica including phytochemicals as the key effect in the prevention of prostate cancer cell growth[2]. In study has been proposed that the additive and synergistic effects of phytochemicals in U.dioica are responsible for these potent anticancer and antioxidant activities. Flavanoid and Lignan phytoestrogens have antioxidant activity[3].

In present study, total phenol and flavon contents, and radical scavenging activity (RSA) were also determined to survey antioxidant potential of U. diocia aqueous and acid extracts.

The U. diocia extract was fractionated with, chloroform, ethyl acetate and n-butanol by partitioning into solvents of increasing polarity.

HPLC analyses of phenolic, compounds in n-butanol and ethyl acetate fractions of Urtica diocia L were carried out in Varian ProStar HPLC. U.dioica water extract was investigated for cytotoxic activity against MCF-7; MDA-231 breast cancer cell lines. Cells were incubated with the extract in the varrying concentration range for 48 h and the cytotoxicity was determined using the XTT assay.

Antioxidant capacity of U.dioica needs further extensive investigation for it's gastrointestinal tract absorption and metabolism. Dietary active parent compounds in U.dioica are metabolized extensively in the body into partly conjugated compounds and their contribution to the antioxidant defense in vivo is uncertain. We also properly evaluated the stomach acid (pH 2) and colonic microflora (pH 7.4) how convert most of these dietary phenols or flavon in U. dioica are a group of polyphenolic compounds into metabolites that then reach the circulation.

MATERIAL AND METHOD

Extraction and Fractionation of U. dioica

Plant material U. dioica were harvested according to pharmacognostic collection guidelines from Erzurum, Şenkaya, Ardahan, Ankara-Eymir at their flowering time and taken their GPS records[4].

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Aerial part of U. dioica were press-dried according to herbarium techniques and identified by "Flora of Turkey"[5].

Dried plant to distilled water ratio was 1:10 (w/v) (g/mL) in a beaker by maceration method. The acidic extraction of soluble active constituents of U.dioica was performed by maceration of leaves in acidified water at pH 2.0.

The air dried aerial parts of the plant (30g) were extracted with methanol for 4 h at 40^0 C in Cole-parmer 8851, sonicator. After evaporation of the combined methanol extracts of U.dioica under vacuum, the extract was fractionated with chloroform, ethyl acetate and n-butanol by partitioning into solvents of increasing polarity. N-butanol extracts were kept to -80°C at SANYO freezer for overnight and dried in lyophilizator (HETO).

Free Radical Scavenging Activity by DPPH Method

The free radical scavenging activities were determined by DPPH Method[6]. An ethonolic solution (0.05 mg/mL) of the radical DPPH was prepared daily and protected from light. Different concentrations (0.5 mg/mL up to 8 mg/mL) of extract mg powder/mL are prepared in water. The absorbance at 517 nm was recorded after 30 min of incubation at room temperature.

Determination of Total Flavonoid Content

Aluminum chloride colorimetric method was used for flavonoid determination[7]. Lyophilized U. dioica extracts (0.5 mL) were separately mixed with 1.5 mL of water, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water and vortexed vigorously. Mixture remained at room temperature for 30 min; the absorbance of mixture was monitored at 415 nm with a single beam Schimadzu 1240 UV/ Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 µg/mL, 50 µg/mL up to 100 µg/mL in ethanol.

Determination of Total Phenol Content

The Folin-Ciocalteu (FC) method based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products was used to determine the phenolic compounds[8]. Aqueous solution (1 mg/mL and 2 mg/mL) of lyophilized extract (100 µl) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (1 mL, 1:9 diluted with distilled water) and aqueous Na_2CO_3 (800 µl, 1 M). The absorbance values were measured at 765 nm at room temperature. Total phenol values are calculated using the standard curve and expressed in terms of gallic acid equivalent (mg per g of dry mass of U. dioica water extract).

HPLC -DAD Analyses

HPLC-DAD analyses were performed on an Varian Prostar HPLC equipped with a ProStar 330 Photo diode Array Detector managed between UV-VIS-NIR regions (190-700 nm).

Chromatographic conditions
Injected volume 10µl
Column Pursuit C18 (150X 4,6 mm i.d, 5 µm)
Mobile phase A (formic acid in water, 2,5% v/v): B (methanol)
0-7 min: 0:100 (elution step)
7-42 min: 20:80 (elution step)
2-67 min: 60:40 (elution step)
68-73 min: 0:100 (flushing step)
Flow rate 1mL/min
Temperature 35 °C
Detection conditions
Detection wavelenght Gallic acid 280nm, Quercetin 360 nm Caffeic+Chlorogenic acid 320nm.

Cell Proliferation by XTT Assay

The monolayer cells were maintained as an attached type monolayer culture in complete RPMI 1640 Cell Culture Medium with phenol red containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biochrom AG). Cell culture media were supplemented with 0.2% (v/v) gentamycin (Biological Industries). Antiproliferative effects U. dioica on MCF-7 and MDA 231 cells were evaluated by means of the Cell Proliferation Kit (Biological Industries) according to manufacturer's instructions. In brief, XTT reagent was added to each well and soluble product was measured at 415 nm with an Spectromax 340 96-well plate reader (Molecular Devices, USA). MCF-7 and MDA 231 cells (10^4 cells/well) were cultivated in RPMI 1640 for 24h, and then varying concentrations (0-73 µg/mL) of extracts were added and continued incubation for 48h. XTT reagent were added and incubated for 4 hours. The intensity of orange colour formed was measured with an ELISA plate reader using filter at 415 nm.

UV-VIS Absorbance Spectra of U. dioica

The absorbance spectrum of 1 mg/mL aqueous solution of U.dioica were recorded between 250-800 nm wavelengths.

RESULTS and DISCUSSION

The results of the present study established that Radical Scavenging Activity (RSA) varied from 15% to 65% in the different extracts which performed for 6, 12, 24 and 36 hours, at 50°C, 37°C and 25°C for optimization of maceration condition. The highest RSA was found in 12 hour at 25°C extract with %65 RSA value and the (IC50) inhibitory concentration of 300 mg/L. In literature[9], IC_{50} value of methanolic extract of U. dioica was found as 335 mg/L[9] This study confirms that IC_{50} value of methanolic extract of U. dioica was quite similar to IC_{50} value of aqueous extract of U. dioica and highlights the

positive correlation between these two studies[8]. The slight difference (35 mg/L) among IC_{50} values of aqueous and methanol extracts of U. dioica was probably related to solvent polarity. Similar RAS was also observed in macerated acidic and aqueous extracts of U. dioica at the optimum maceration condition. While the RSA value of 12 hour at room temperature aqueous macerated U. dioica was 62%, that of acid macerated U. dioica was 66%, which is not a significant difference.

The flavonoid contents of extracts in terms of quercetin equivalent were between 14.5±1.33 and 28.9±0.93 mg quercetin per g of extract. Figure 1 also show 12H at room temperature macerated U. dioica sample has the richest flavonoid content (28.9±0.93mg quercetin/g extract). An asignificant change in flavonoid content, a maximum of 2.4 fold change was observed ($p<0.05$) by using one way ANOVA (Figure 1).

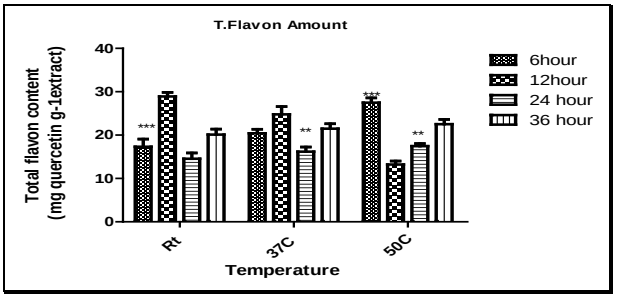


Figure 1. The total flavonoid content of U. dioica extract prepared under different conditions. In this graph,*,** and *** represent statistically significant difference at ($p<0.05$), ($p<0.001$) and ($p < 0.01$), respectively

Phenol content of extracts in terms of GaE equivalent varied from 28.8± 0.57 to 74.2±2.24 mg g^{-1} calculated from standard curve equation: $y = 0.0054x +$, $r^2=0.9986$ (Figure 2). Total Phenol Amount of U. dioica extract which macerated at 12H room temperature has (74.2 mg g^{-1}**±**2.24mg GaE) and this was approximately 2,6 folds more than 24 h at room temperature macerated U. dioica extract (28.8± 0.57 mg GaE/ g extract) in Figure 2.

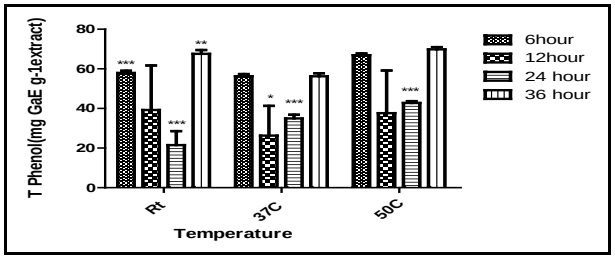


Figure 2. The total phenol content of U. dioica extracts prepared under different conditions

Results were listed in the Table 1. as the acid macerated U. dioica extracts, having higher phenolic content, with an 77.3±1.91 mg GaE/g extract.

Table 1. Comparision of %DPPH Radical Scavenging Activitiy, Total Phenolic Content of *U. Dioica, A*cid and Aqueous Extract

Maceration Condition; 25°C, 12 hour	Total Phenol (mg GAE/ g extract)	Total Flavon (mg/quercetin g extract)	DPPH scavenging IC_{50} (mg/mL)	XTT MCF7& MDA231 (µg/mL)
Aqueous macerated UD	70.7±1.46	22.7±0.32	0,30	34
Acid macerated UD	77.3±1.91	20.9±0.48	0,37	ND (not determined)

A relatively higher flavonoid content (22.7±0.32 mg quercetin/g extract) was observed in aqueous U. dioica extract obtained from maceration at room temperature for 12 hour. This is slightly higher compared to the acid U.dioica extract obtained from maceration at room temperature for 12 hour (20.9±0.48 mg quercetin/g extract) in Table 1. Acid extract of U.dioica has higher Total Phenol Amount than its water extract. In contrast, DPPH-RSA, acid extract are lower than water extract were provided in Table 1. The breakdown of flavonoids and phenolic acids into smaller molecules through ring cleavage and β-oxidation in the colon and liver unfortunately lowers their antioxidant activity[10].

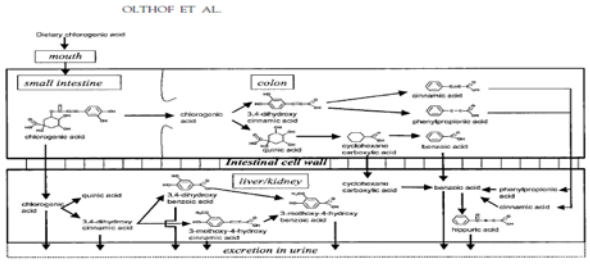


Figure 3. Proposed metabolic pathway of chlorogenic acid in human[12]

According to our HPLC results Chlorogenic acids found in U.dioica including two ferrulic acid, are also major derivatives of hydroxycinnamic acids present in plants. Chlorogenic acids are the ester of caffeic acids and are a substrate for enzymatic oxidation and metabolized in liver/kidney and colon of humans as given in Figure 3[12].

Subsequently, phenols and their metabolites are conjugated with glucuronic acid, sulfates or glycine, which also lowers their antioxidant activity[11]. Phenolics compounds in U. dioica are usually identified by standard HPLC. HPLC analyses of 12 h at 25°C U. dioica extract (14.4mg/mL) revealed that the extract contained 7.10 $^{-3}$ mg/mL caffeic acid and 5.10^{-3} mg/mL quercetin. $3x10^{-2}$ mg/mL gallic acid was found in n-butanol fraction of U. dioica. Caffeic and chlorogenic acid were found at $1.9X\ 10^{-2}$ mg/mL in acid macerated extract of U. dioica. Neither gallic acid nor the caffeic acid and quercetin were found at detectable levels in chloroform extract of U.dioica.

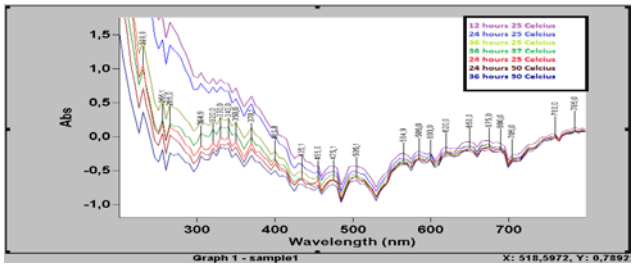


Figure 4. Spectra of aqueous extracts of all macerated U. dioica samples.

The spectra results showed that 320 nm band due to the caffeic acid belonging to hydroxycinnamic acid with bands centered at 300 and 340 nm, as chlorogenic acid in same family (Figure 4). The spectrum of exracts of all dwater macerated U. dioica samples exhibited bands centered at 226 and 240 nm are presented for secoisolariciresinol from lignan main classes of phyto-oestrogens. The UV data showed that 280nm band due to the gallic acid shows the hydroxybenzoic acid which are part of a phenolic acid in the samples as seen in Figure 4. The presence of quercetin from flavonols group derived from flavonoids in the sample was confirmed by peak at 360 nm. The band at 350nm was shown the presence of 1-Scopoletin. From spectra data, it clearly appears that the peak at 450 nm confirms the carotenoids in U. dioica. Anthocyanidins from flavonoids group was due to the peaks between 510 and 530 nm.

U.dioica water extract was investigated for cytotoxic activity against MCF-7, MDA-231 breast cancer cell lines. Inhibitory concentration (IC_{50} doses) value is the U. dioica concentration at which 50% of cells are viable were calculated from the XTT cell cytotoxicity assay. IC_{50} was as calculated IC_{50}: 34µg/mL for MCF-7 cells in 48 th hour as seen in Figure 5. Increasing the concentration of U. dioica aqueous extract up to 29,2 µg/mL was observed to decrease MDA-231 cell viability to 43%. However, further increase of extract concentration (up to 49 and 73 µg/mL) did not cause a marked decrease in percent cell viability. Since percent viable MDA-231 cell was not reduced below 35% with respect to untreated cells, in order to prevent misinterpretations, IC_{50} value of U. dioica extract was not cosidered as a proper indicator of cytotoxic dose of the extracts in this cell line**.** It was reported by Harputs that U. dioica water extract was investigated for cytotoxic activity against KB, B16, HeLa and HLA tumor cell lines[13]. Cells were incubated with the extract in the concentration range 800–1 µg/mL for 48 h and the cytotoxicity was determined using the MTT assay. Since the extract did not affect cell viability in any tested concentrations, immuno-modulatory activity was proposed to be responsible for the anticancer usage of the plant[13].

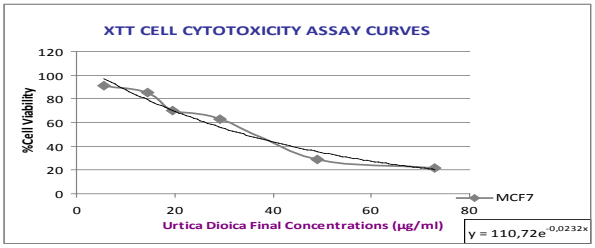


Figure 5. Effects of varying U. dioica concentration on viability of MCF-7 cells which were treated with U. dioica for 48 hours

CONCLUSION

Recent data suggests that the U. dioica phytochemical extracts exhibit strong antioxidant and antiproliferative cancer activities and that the major part of total antioxidant activity is from the combination of phytochemicals. Furthermore, significant correlations existed between phenols and flavonoids and free radical scavenging activity, cytotoxicity test and high performance liquid chromatography (HPLC) findings. The major findings from our research; Additive and synergistic effects of phytochemicals in U. dioica were responsible for their potent antioxidant and anticancer activities.

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