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Chemical Composition and In Vitro Cytotoxic, Genotoxic Effects of Essential Oil from *Urtica dioica* L.

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Abstract The aim of this study was to determine the chemical composition of *Urtica dioica* essential oil, and to evaluate its cytotoxic and genotoxic effects, using cytogenetic tests such as the cytokinesis-block micronucleus assay and chromosomal aberration analysis in human lymphocyte cultures in vitro. GC–MS analysis of *U. dioica* essential oil identified 43 compounds, representing 95.8% of the oil. GC and GC–MS analysis of the essential oil of *U. dioica* revealed that carvacrol (38.2%), carvone (9.0%), naphthalene (8.9%), (E)-anethol (4.7%), hexahydrofarnesyl acetone (3.0%), (E)-geranyl acetone (2.9%), (E)-β-ionone (2.8%) and phytol (2.7%) are the main components, comprising 72.2% of the oil. A significant correlation was found between the concentration of essential oil and the following: chromosomal aberrations, micronuclei frequency, apoptotic cells, necrotic cells, and binucleated cells.

Keywords *Urtica dioica* · Essential oil · Chemical composition · Cytotoxic · Genotoxic

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Plants have always been used as a common source of medicine, both for traditional remedies and in industrialised products. *Urtica* L. Stinging nettle (Urticaceae), consists of annual and perennial herbs, distinguished by stinging hairs. *Urtica dioica* has been long consumed as a medicinal plant in many parts of the world, and is one of the most commonly used plants by the local people of Turkey (Gozum et al. 2003; Cakilcioglu and Turkoglu 2010). It is traditionally used as an expectorant, purgative, diuretic, haemostatic, and vermifuge, as well as for the treatment of eczema, rheumatism, haemorrhoids, hyperthyroidism, bronchitis and cancer (Davis 1982; Sezik et al. 2001). Although several studies, including genotoxicity (Graf et al. 1994), have been conducted on these species, their antioxidant and antimicrobial characteristics are particularly striking (Aksu and Kaya 2004; Gulcin et al. 2004; Karabacak and Bozkurt 2008). Infusions of *U. dioica* are commonly used as an herbal medicine by cancer patients (Gozum et al. 2003). In fact, stinging nettle root extract of *U. dioica* has been shown to have antiproliferative effects on human prostate cancer cells (Konrad and Muller 2000).

The present study was conducted: (1) to determine the chemical composition of *U. dioica* essential oil; and (2) to evaluate the cytotoxic and genotoxic effects of *U. dioica* essential oil, using cytogenetic tests such as the cytokinesis-block micronucleus assay and chromosomal aberration analysis in human lymphocytes culture in vitro.

Materials and Methods

Urtica dioica plants were collected from Tokat: Erbaa, Tortepe, Turkey (September 2009). The voucher specimen was identified by a senior plant taxonomist, Dr. H. Askin Akpulat at the Department of Biology, Cumhuriyet

University, Sivas-Turkey, and has been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher No: AA 3901).

Air dried aerial parts of the plant were hydro-distilled for 3 h using a Clevenger-type apparatus, to produce a small amount of essential oil which was trapped in *n*-hexane.

GC–MS analysis was performed using an Agilent 5975 GC–MSD system. An Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used, with helium as a carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. The Mass range was from m/z 35 to 450.

GC analysis was conducted using an Agilent 6890 N GC system. The FID detector temperature was 300°C. To obtain the same elution order as for GC-MS, simultaneous auto-injection was performed on a duplicate of the same column, applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. Results are given in Table 1.

Identification of components of the essential oil was carried out by comparison of their relative retention times with those of authentic samples, or by comparison of their relative retention index (RRI) to a series of n-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty and Stauffer 1989) and in-house "Başer Library of Essential Oil Constituents" (composed of genuine compounds and components of known oils), as well as MS literature data (Joulain and Koenig 1998), was used for the identification.

Human peripheral blood cells were used as a test system. Peripheral venous blood was collected from healthy, non-smoking adults aged 18–22 years. Donors provided written, informed consent at the time of donation for use of their blood samples in this study. Whole blood (0.4 mL) was added to 5 mL chromosome medium (Biochrome). Essential oil was dissolved in acetone (1 part essential oil in 9 parts acetone). Because longer exposure times caused considerable inhibition of cell division, the essential oil of *U. dioica* was added after 48 h of culture initiation. Human lymphocytes were exposed to different treatments (0.10, 0.15, 0.20, 0.25 μL/mL) of essential oil. A solvent control (Acetone 2.3 μL/mL) and a positive control (mitomycin-C(MMC), 0.3 μg/mL) were included in every experiment (Lazutka et al. 2001; Roncada et al. 2004). Colchicine (0.06 μg/mL) was added to each culture at 70 h. Cells were

Table 1 The volatile composition of *Urtica dioica*

RRI	Compound	%
1,093	Hexanal	0.3
1,192	2-Heptanone	0.2
1,194	Heptanal	0.2
1,244	2-Pentyl furan	0.4
1,280	*p*-Cymene	0.1
1,400	Nonanal	0.8
1,497	α-Copaene	0.5
1,535	β-Bourbonene	0.4
1,553	Linalool	1.9
1,562	Octanol	0.1
1,565	Linalyl acetate	0.5
1,612	β-Caryophyllene	2.2
1,638	β-Cyclocitral	0.4
1,687	Methyl chavicol	1.3
1,706	α-Terpineol	0.3
1,709	α-Terpinyl acetate	0.6
1,719	Borneol	0.4
1,741	β-Bisabolene	1.7
1,751	Carvone	9.0
1,763	Naphthalene	8.9
1,773	δ-Cadinene	0.4
1,776	γ-Cadinene	0.3
1,783	β-Sesquiphellandrene	0.3
1,786	*ar*-Curcumene	0.2
1,802	Cumin aldehyde	0.9
1,845	(*E*)-Anethol	4.7
1,849	Calamenene	0.5
1,868	(*E*)-Geranyl acetone	2.9
1,884	1-Methyl naphthalene	0.3
1,958	(*E*)-β-Ionone	2.8
2,008	Caryophyllene oxide	1.5
2,030	Methyl eugenol	0.2
2,131	Hexahydrofarnesyl acetone	3.0
2,179	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	1.4
2,186	Eugenol	1.0
2,198	Thymol	1.2
2,200	3,4-dimetil-5-pentyl-5H-furan-2-one	1.4
2,218	4-Vinyl guaiacol	0.4
2,239	Carvacrol	38.2
2,384	Farnesyl acetone	0.9
2,500	Pentacosane	0.1
2,622	Phytol	2.7
2,900	Nonacosane	0.3
	Total	95.8

RRI Relative retention indices calculated against n-alkanes

% calculated from FID data

collected by centrifugation (377×*g*, 10 min), and resuspended in a hypotonic KCl solution (0.4%) for 30 min at 37°C. Following this process, cells were centrifuged again and fixed in a cold methanol:acetic acid (3:1) mixture for 35 min at +4°C. At the end of this procedure, cells were treated two times with fixative. Then, slides were made by dropping concentrated cell suspensions followed by air drying. Air-dried slides were stained for 15–20 min with 5% Giemsa stain (pH 6.8) prepared in Sorensen buffer. One hundred metaphases per culture were analysed for the presence of chromosomal aberrations (CA). The number of CAs was obtained by calculating the percentage of metaphases at each concentration and treatment period that significantly different from the negative control howed structural and numerical chromosome aberrations. Chromatid and chromosome breaks, chromosome exchange and chromatid union, and polyploid cells were screened at all the treatments (Fenech et al. 1999).

For the cytokinesis-block micronucleus assay (CBMN), blood samples were added to 5 mL of chromosome medium (Biochrome). Cultures were incubated at 37°C for 72 h. Twenty-eight hours before harvest, cytochalasin-B (6 μg/mL) was added to each culture. Human lymphocytes were exposed to different treatments (0.10, 0.15, 0.20, 0.25 μL/mL) of essential oil for the CBMN test. Cells were exposed to the chemical after 48 h of culture initiation. Cells were harvested by centrifugation (167×*g*, 10 min), and pellets were resuspended in a hypotonic solution of 0.075 M KCl for 5 min at +4°C. The cells were again centrifuged and fixed in a cold methanol:acetic acid (3:1) mixture for 15 min. The fixation procedure was applied three times. Formaldehyde (1%) was added to the last fixative to preserve the cytoplasm. Slides were made by dropping concentrated cell suspension and air drying. For CBMN analysis, staining was done using 5% Giemsa (pH = 6.8), prepared in Sorensen buffer solution, for 20–25 min. Slides were then washed in distilled water, and dried at room temperature. Mitomycin-C(MMC), 0.50 μg/mL, was used as a positive control. In accordance with standard criteria (Fenech et al. 1999), CBMN analysis was performed on coded slides by scoring 2,000 binucleate lymphocytes for each subject. The nuclear division index (NDI) was calculated using the following formula: $NDI = (M_1 + 2(M_2) + 3(M_3) + 4(M_4))/N$, where M1 − M4 indicates the number of cells with 1–4 nuclei and *N* indicates the total number of cells scored. The NDI of each cytochalasin B-treated culture was determined by screening 2,000 interphase cells for the number of nuclei included (Lazutka et al. 2001).

Apoptotic and necrotic cells were identified by light microscopy, using morphological characteristics of the nucleus. In order to distinguish between apoptotic and necrotic cells, we checked the properties of necrotic cells, which exhibit a pale cytoplasm or a loss of cytoplasm, numerous vacuoles, and a damaged/irregular nuclear membrane with a partially intact nuclear structure. 2,000 cells were counted for each sample. The nuclear division cytotoxicity index (NDCI) = $(Ap + Nec + M_1 + 2(M_2) + 3(M_3) + 4(M_4))/N$ was calculated according to Fenech et al. (1999), where Ap = the number of apoptotic cells, Nec = the number of necrotic cells, M1 − M4 = the number of viable cells with 1–4 nuclei and N = the total number of cells scored.

All statistical analyses were performed using GraphPad InStat version 3.05 for Windows 95. The frequency of chromosomal aberrations in the cell cultures was analysed using *Fisher's* exact test. CBMN data were statistically analysed using the *F* test for analysis of variance (ANOVA), and the significance of differences between the negative control and a series of treated groups was determined with Dunnett's *t* test (GraphPad Software, San Diego California USA).

Results and Discussion

GC–MS analysis of *Urtica dioica* essential oil resulted in identification of 43 compounds, representing 95.8% of the essential oil. Based on GC and GC–MS analysis data for *U. dioica* essential oil: carvacrol (38.2%), carvone (9.0%), naphthalene (8.9%), (*E*)-anethol (4.7%), hexahydrofarnesyl acetone (3.0%), (*E*)-geranyl acetone (2.9%), (*E*)-*β*-ionone (2.8%) and phytol (2.7%) were found to be the main components, comprising 72.2% of the sample. Other components were present in amounts <2% in the oil (Table 1).

Two parameters (CA and CBMN) were used to evaluate the genotoxic effects of *U. dioica* essential oil on human lymphocyte cells. A significant increase in the frequency of chromosomal aberration was observed for all treatments with *U. dioica* essential oil (0.10, 0.15, 0.20, 0.25 μL/mL) at 24 h compared with treatment with a solvent control (Acetone) and mitomycin C (MMC, 0.3 μg/mL), which was used as a positive control (Table 2). Examination of lymphocyte chromosomes revealed chromosome and chromatid union, as well as chromatid and chromosome breaks in both the positive control and at all treatments except for 0.10 μL/mL. Polyploidy was observed at some oil treatments (0.20 and 0.25 μL/mL), and upon treatment with the positive control.

For all the treatment groups and in a dose dependent manner, statistical analysis demonstrated significant increases in CA frequency (after 24 h) after addition of essential oil, compared with the negative control ($r = 0.98$). Mitomycin treatment also caused a significant increase ($p < 0.001$) in the frequency of aberrant cells. Treatment with 0.10 μL/mL oil did not increase the CA frequency in human lymphocyte cells. All essential oil

Table 2 Chromosome aberrations produced by *U. dioica* essential oil in human lymphocytes in vitro

Test substance	Treatment		Structural aberrations				Numerical aberrations	Frequency of aberrant cell ± SEM(%)	Mitotic index ± SEM(%)
	Period (h)	Doses(μL/mL)	ctb	csb	cse	cu	p		
NC	24	1	1	–	–	–	–	0.2 ± 0.2	4.3 ± 0.3
SC (Acetone)	24	2.3	1	–	–	1	–	0.4 ± 0.2	4.1 ± 0.4
MMC	24	0.3	17	2	3	8	1	6.2 ± 2.9*	1.7 ± 1.2*
U. dioica essential oil	24	0.10	1	–	1	4	–	1.2 ± 0.7	3.6 ± 0.5*
		0.15	6	1	3	4	–	2.8 ± 1.0*	3.2 ± 0.4*
		0.20	8	1	4	6	1	3.8 ± 1.4*	2.2 ± 0.6*
		0.25	10	2	4	8	1	5.0 ± 1.7*	1.9 ± 0.8*

ctb chromatid break, *csb* chromosome break, *cu* chromatid union, *cse* chromosome exchange, *nc* negative control (%1 distilled water), *MMC* (0.3 μg/mL mitomycine-C (24 h), *p* polyploidy

* $p < 0.05$ as compared to solvent control. *Fisher's* exact test

treatments clearly inhibited the mitotic index in human lymphocytes (Table 2).

Urtica dioica essential oil significantly increased the frequency of micronuclei in a dose dependent manner ($r = 0.97$). Table 3 demonstrates that the nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI) were significantly influenced by the oil. NDI and NDCI values were lower than in controls. The lowest NDI values were observed in 0.15 μL/mL essential oil-treated cultures. We demonstrated that the presence of a micronucleus in binucleated cells correlates with induction of apoptosis ($r = 0.60$) and necrosis ($r = 0.75$).

Our results show that there is a significant correlation between the concentration of essential oil applied, and chromosomal aberrations, micronuclei frequency, apoptotic cells, necrotic cells and binucleated cells (Table 3).

Essential oils have been used in many fields, such as medicine and the pharmaceutical, perfume, cosmetic, agricultural and food industries. In addition, it is important to determine the chemical composition of the plants containing essential oils that are used in ethnopharmacology. Our study demonstrates that the major compounds of *U. dioica* are carvacrol (38.2%), carvone (9.0%) and naphthalene (8.9%).

Carvacrol, or cymophenol, $C_6H_3CH_3(OH)(C_3H_7)$, is a monoterpenoid phenol. Interestingly, carvacrol has been shown to possess remarkable antibacterial, antiviral, antifungal and antiparasital effects. In addition, it does not appear to have any long-term genotoxic risks. Furthermore, Ipek et al. (2003) showed that carvacrol exhibits significant anti-genotoxic activity in mammalian cells, indicating its potential use as an anti-genotoxic agent.

Carvone is the major constituent of the essential oils of the plants commonly used as condiments. Such plants are cultivated in several countries for the commercial production of spearmint oil (Kokkini et al. 1995). Carvone exerted weak mutagenic effects in the Ames test. DNA-repair tests showed that carvone displays the most marked differential toxicity against the DNA repair-deficient strain, CM871. This effect, however, became apparent only at doses 10 times higher than that used for other plant volatiles tested (Stammati et al. 1999).

In vivo and in vitro studies have demonstrated that naphthalene possesses cytotoxic and genotoxic effects when applied to human and animal cells (Chichester et al. 1994; IARC 2002; Saeed et al. 2009). In particular, exposure to Naphthalene caused sister chromatid exchanges and chromosomal aberrations in hamster ovary cells, as well as wing mutations in *Drosophila melanogaster* (Delgado-Rodriguez et al.1995).

The naphthalene content of soil generally ranges from 0 to 3.1 μg/kg (Wild et al.1990), although Canadian marine sediments have been reported to have naphthalene treatments ranging from 0.1 to 115 mg/kg dry sediment (Simpson et al.1995). Fish samples can contain naphthalene ranging from 5 to 176 μg/kg (Bender and Huggett 1989).

Many studies on the chemical composition of the plants have been reported (Davis 1982; Sezik et al. 2001; Gozum et al. 2003; Aksu and Kaya 2004; Gulcin et al. 2004; Karabacak and Bozkurt 2008; Cakilcioglu and Turkoglu 2010), which show that the leaves, branchlets, flower buds and flowers have a very wide distribution of mono- and sesquiterpenoids and other compounds. However, in all of these studies, naphthalene was not detected. In fact, the highest naphthalene content reported in the plants has been found in *Magnolia* species (Azuma et al. 1996).

Although many mutagenic and cytotoxic investigations on the chemical contents of essential oils have been reported (Lazutka et al. 2001; Bakkali et al. 2008; Peres et al. 2009; Patharakorn et al. 2010), naphthalene was never mentioned. Interestingly, in our study, naphthalene

Table 3 The micronucleus frequency, nuclear division and nuclear cytotoxic division index in human lymphocytes treated with *U. dioica* essential oil

Test substance	Treatment		Binucleated cells scored	Distribution of cells according to number of nuclei				Distribution of BN cells according to the no. of MN				MN/cell (%) ± SEM	NDI	Apoptotic cells	Necrotic cells	NCDI
	Period (h)	Dose µL/mL		1	2	3	4	1	2	3	4					
Negative Control	–	–	2,000	744	978	93	185	1	0	0	0	0.05 ± 0.01	1.86 ± 0.34	0	0	1.86 ± 0.34
Solvent Control (Acetone)	24	2.3	2,000	708	1,021	103	168	0	1	0	0	0.05 ± 0.01	1.87 ± 0.34	1	0	1.87 ± 0.35
PC (MMC)	24	0.5	2,000	1,186	690	64	60	132	19	2	2	7.75 ± 2.4	1.49 ± 0.90	22	25	1.51 ± 1.15
Essential oil	24	0.10	2,000	942	810	151	97	8	2	1	0	0.60 ± 0.42*	1.70 ± 0.34	8	7	1.70 ± 0.40
	24	0.15	2,000	1,043	730	140	87	22	3	0	0	1.25 ± 0.51*	1.63 ± 0.48	8	6	1.63 ± 0.35
	24	0.20	2,000	1,105	743	105	47	26	2	0	0	1.40 ± 0.62*	1.54 ± 0.68	11	10	1.55 ± 0.80
	24	0.25	2,000	1,124	715	90	71	32	5	0	0	1.85 ± 0.97*	1.51 ± 0.55	9	9	1.52 ± 0.39

NDI nuclear division index, *NCDI* nuclear cytotoxic division index, *SEM* standard error of the mean

* Significantly different from the negative control $p < 0.05$ (Dunnett's *t* test)

was one of the main components identified, comprising 8.9% of the essential oil. According to data from the literature, the mutagenicity and cytotoxicity found in this study for the essential oil of *U. dioica* may be due to the presence of naphthalene.

Finally, the results of our study can be summarised as follows:

1. GC and GC–MS analysis of *U. dioica* essential oil demonstrates that carvacrol (38.2%), carvone (9.0%), naphthalene (8.9%) are the main constituents.
2. *U. dioica* essential oil increases chromosomal aberration rates, MN formation and cytotoxicity in human lymphocyte cells.

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