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Original research article

Toxicity assessment of *Hypericum olympicum* subsp. *olympicum* L. on human lymphocytes and breast cancer cell lines

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Abstract
There is a limited number of studies about the constituents of *Hypericum olympicum* subsp. *olympicum* and its genotoxic and cytotoxic potency. We examined the possible antigenotoxic/genotoxic properties of methanolic extract of *H. olympicum* subsp. *olympicum* (HOE) on human lymphocytes by employing sister chromatid exchange, micronucleus and comet assay and analyzed its chemical composition by GCxGC-TOF/MS. The anti-growth activity against MCF-7 and MDA-MB-231 cell lines was assessed by using the ATP viability assay. Cell death mode was investigated with fluorescence staining and ELISA assays. The major components of the flower and trunk were determined as eicosane, heptacosane, 2-propen-1-ol, hexahydrofarnesyl acetone and α-muurolene. HOE caused significant DNA damage at selected doses (250–750 μg/ml) while chromosomal damage was observed at higher concentrations (500 and 750 μg/ml). HOE demonstrated anti-growth activity in a dose-dependent manner between 3.13–100 μg/ml. Pyknotic nuclei were observed at 100 μg/ml concentration of HOE in both cell lines. In conclusion, HOE demonstrated cytotoxic effects in a cell type-dependent manner, however its genotoxic effects were observed at relatively higher doses.

Keywords: Breast cancer; Cytotoxicity; Folk medicine; Genotoxicity; Human lymphocyte; *Hypericum olympicum*

Highlights:
- Genotoxic and cytotoxic properties of *Hypericum olympicum* were investigated.
- Volatile compounds in methanol extract of *Hypericum olympicum* plant were revealed.
- Strong anti-growth activity was observed in a cell type dependent manner.
- Genotoxicity was observed at much higher concentrations than the doses required for cytotoxicity.

Introduction

Medicinal plants are considered as an ample source of ingredients which can be utilized in drug development and synthesis especially in cancer treatment. Eighty-three percent of the clinically approved small molecule anticancer drugs from the year 1981 to 2014 are classified as either a natural product or based thereon in one way or another (Newman and Cragg, 2016). A significant portion of these drugs are particularly associated with plants. Plant-derived traditional medicines along with plant extracts are sustaining the primary healthcare needs of a significant portion of the population in the developing countries (WHO, 1999). Appropriate usage of medicinal plant extracts requires the identification of the exact chemical composition, determination of effective and toxic concentrations (Briskin, 2000). In addition, genotoxicity studies may be invaluable where the medicinal plants bearing potential health risks have not been thoroughly elucidated (Bast et al., 2002).

Volatile substances found in plants are commonly investigated through an extraction step, a subsequent concentration, chromatographic analysis and detection steps. Direct thermal desorption (DTD) is a type of dynamic headspace technique coupled with GC-MS that enables rapid analysis and reduces required sample amount. DTD is therefore a more convenient method for prompt qualitative and quantitative analysis of compounds (Özel et al., 2006).

Hypericum genus is represented worldwide with over 400 species (Davis et al., 1988; Guner et al., 2000). Around eighty-four species of the genus are found throughout Turkey especially in Marmara region (Davis et al., 1988; Guner et al., 2000). Numerous species from *Hypericum* genus are being uti-

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lized as herbal remedies around the world and are investigated for medicinal applications. It has been shown that either whole extract or some biologically active constituents of different *Hypericum* species exhibit anti-inflammatory, anti-microbial, anti-depressant, anti-oxidant and anti-tumor activity (Bender et al., 2018; Ozkan et al., 2018; Tian et al., 2014; Zorzetto et al., 2015). Furthermore, cytotoxic, genotoxic and apoptosis-inducing effects against different cancer cells have also been highlighted in recent studies (Keskin et al., 2017; Mirmalek et al., 2015; Sarimahmut et al., 2016; Sousa et al., 2015). The most renowned *Hypericum* species, *Hypericum perforatum* (St. John's Wort), is extensively studied and utilized in traditional medicine due to its antidepressant activity and in burn-wound-healing with many pharmacological properties due to its active ingredients hypericin, hyperforin and other flavonoid compounds (Barnes et al., 2001; Baytop, 1984).

H. olympicum subsp. *olympicum*, a dwarf deciduous shrub, is native to Western Asia (Syria and Turkey) and Southeastern Europe (Albania, Bulgaria, Greece, Macedonia, Montenegro, Serbia) (Davis et al., 1988). Decoction from the aerial parts of *H. olympicum* subsp. *olympicum* is utilized in folk medicine for inflamed wounds, stomach ache, and cuts (Bingol et al., 2011; Tuzlacı and Aymaz, 2001). Available research on *H. olympicum* subsp. *olympicum* species shows that the crude ethanol extract of the plant exhibits a prominent antioxidant effect and inhibits acetylcholinesterase activity (Božin et al., 2013). Also, a growth inhibitory and programmed cell death inducing effects of *H. olympicum* subsp. *olympicum* extract in lung cancer cells was previously reported by our research group (Aztopal et al., 2016).

We examined the genotoxic and cytotoxic activities of *H. olympicum* subsp. *olympicum* extract (HOE) in human lymphocytes and breast cancer cell lines, respectively. The volatile components of the extract were detected by using the GCxGC-TOF/MS.

Materials and methods

Extraction and chromatographic analysis of H. olympicum *subsp.* olympicum

H. olympicum subsp. *olympicum* was collected from Mt. Uludag region in Bursa, Turkey. A methanolic extract from 30 g of the trunk and flowers of this plant was prepared by utilizing a Soxhlet apparatus as previously described (Aztopal et al., 2016). The sample was lyophilized and kept at –20 °C for further use.

Chromatographic analysis of HOE

The content of the trunk and flower parts of *H. olympicum* subsp. *olympicum* was determined by GCxGC-TOF/MS method. The GCxGC-TOF/MS system consisted of an Agilent 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph and a Pegasus III TOF-MS (LECO, St. Joseph, MI, USA). The chromatographic separation was made as described by Ari et al. (2015).

Lymphocyte cultures and cell harvesting

A total of four blood samples from non-smoker healthy donors between the ages of 18 and 25 were collected into EDTA tubes and used throughout the experiment. The lymphocytes were treated with 0.1% dimethyl sulfoxide (DMSO) as solvent control, 1.250 mg/ml ethyl methane sulfonate (EMS) as positive control, 250–750 μg/ml diluted sterile extract of *H. olympicum* subsp. *olympicum* and with lymphocyte culture medium as negative control. HOE concentrations were determined following the preliminary tests carried out with 10, 50, 100, 250, 500, 750, 1000, 1250, 1500 and 1750 μg/ml concentrations prepared from lyophilized powder (≥1000 μg/ml dose was highly toxic; data not shown). A dose range of 250–750 μg/ml was selected for further experiments. Stock HOE solution was diluted and sterile filtered with a 0.22 micron syringe filter before use in further experiments. Micronucleus (MN) assay was performed using the method described by Fenech with minor modifications as depicted in our previous work (Ari et al., 2015; Fenech, 2000). For sister chromatid exchange (SCE) analyses, lymphocyte cells were cultivated for 72 hours at dark in the presence of 10 μg/ml 5-bromodeoxyuridine (BrdUrd; Sigma) to detect cell proliferation via incorporating BrdUrd into the DNA after the first and second or more cell cycles. Addition of colcemid (Sigma; at 0.2 μg/ml final concentration) two hours before the completion of the treatment was followed by lymphocyte harvest. The slides with well spread chromosomes were stained according to the fluorescence plus Giemsa method (Perry and Wolff, 1974).

Comet assay was performed to assess DNA damage as detailed by Singh et al. (1988) with minor modifications. Electrophoresis conditions were set as previously described (Sarimahmut et al., 2016).

Microscopic evaluation

Microscopic evaluations for MN and comet assay were employed as previously described (Barnes et al., 2001). Sister chromatid exchange was scored according to the study of Carrano and Natarajan (1988). The frequency of SCE per cell was recorded in each group from a total of 50 well-spread, complete second division metaphases.

Proliferative index (PRI) was consecutively calculated by analyzing 200 cells in each group using the formula: PRI = $(M_1 + 2M_2 + 3M_3)/N$. Here, M_1, M_2 and M_3 depict the number of metaphases at the first, second and third cell division, respectively and N depicts the total number of scored metaphases (Lamberti et al., 1983).

Nuclear division index (NDI) was calculated among the 500 lymphocytes that were scored in MN assay according to the following equation: NDI = (MONO + 2BN + 3TRI + 4TETRA)/500. Here, MONO, BN, TRI and TETRA depicts the number of mononucleated, binucleated, trinucleated and tetranucleated lymphocytes, respectively.

Cell culture

MCF-7 and MDA-MB-231 breast cancer cell lines were incubated at 37 °C in RPMI 1640 medium. The medium was supplemented with 1% Pen/Strep solution (Invitrogen) and 5% fetal calf serum (Invitrogen). A stock solution at 100 mg/ml concentration was prepared from lyophilized HOE by dissolving it in DMSO. Obtained stock solution was further diluted in culture medium and sterilized by a 0.22 micron filter. The cells were treated with different concentrations of HOE ranging from 3.13 to 100 μg/ml.

Determination of anti-growth activity

The human MCF-7 or MDA-MB-231 breast cancer cell lines were seeded in 96-well plate containing 5×10^3 cells per well with 200 μl medium. Cells were incubated 72 h either with 0.1% DMSO for negative control or with different concentrations (3.13–100 μg/ml) of HOE. Two independent experiments run in triplicates were carried out for ATP viability assay as previously described (Ulukaya et al., 2008). ATP content was determined as per manufacturer's instructions (Sigma; product code FLASC). Shortly, the cells were lysed to extract

ATP, a luciferin-luciferase solution was added and the resulting luminescence was measured in a luminometer (Bio-Tek, USA). Untreated cells were used as a reference to calculate the viability of treated cells. The concentration that inhibits 50% of the cell viability (IC_{50}) was determined from the concentration versus % viability graph.

Determination of cell death mode

Fluorescence imaging for detection of cell death mode
MCF-7 and MDA-MB-231 cells were seeded at the density of 1×10^4 cells per well in 96-well plates and treated with HOE (100 μg/ml) for 72 h. The type of cell death was evaluated based upon the integrity of the cell membrane and the nuclear morphology. Hoechst 33342, propidium iodide (PI) and calcein-AM (Roche, Germany) were added into the wells subsequent to treatment and the cells were analyzed via fluorescent microscope (Olympus CKX41, Japan).

Assessing caspase-cleaved cytokeratin 18 (M30), active caspase-3 and cleaved PARP levels
The induction of apoptosis was investigated through measuring the levels of caspase-cleaved keratin 18 (M30), active caspase-3 and cleaved PARP. MCF-7 cells at the density of 1×10^4 cells per well were seeded in 96-well plate for M30 assay and 1×10^6 MCF-7 and MDA-MB-231 at the density of 1×10^6 cells were seeded in T25 flasks to determine the levels of active caspase-3 and cleaved PARP. The cells were incubated 72 h with 25–100 μg/ml of HOE in M30 assay and 100 μg/ml of HOE was selected in order to analyze active caspase-3 and PARP levels. Each experiment was performed twice. Paclitaxel (3.12 μM) was employed as positive control against the breast cancer cell lines in all experiments. M30 assay was not performed in MDA-MB-231 cells because of low cytokeratin 18 expression levels found in this cell line (Sommers et al., 1992). The ELISA assays were implemented as described previously (Kasımoğulları et al., 2014).

Statistical analyses
All statistical analyses were carried out using the SPSS 23.0 program (SPSS Inc., Chicago, IL, USA). To assess significance, one-way analysis of variance (ANOVA) and Tukey Honest Significant Difference (HSD) were conducted after performing a normality test. A p value less than 0.05 was considered statistically significant.

Results

Chemical analyses of HOE
DTD of volatiles obtained from flower and trunk of *H. olympicum* subsp. *olympicum* were listed in Table 1. Identification is based on a mass spectral library search using similarity and reverse factors above 750 and 800, respectively (Özel et al., 2004). The total detected number of volatiles for the flower and trunk were 106 and 100, respectively. The major components of the flower and trunk were determined as eicosane, heptacosane, 2-propen-1-ol, hexahydrofarnesyl acetone and α-muurolene.

Table 1. The results of chromatographic analysis of *Hypericum olympicum* subsp. *olympicum*

		% Area[c]	
Compound[a]	RI[b]	Flower	Trunk
Acetic acid	600	2.68	0.08
2-Propanone, 1-hydroxy-	672	[d]	1.15
Pyrrole	749	0.04	
Butanoic acid	763	0.22	0.33
Propanoic acid, 2-methyl-	763		0.96
2,3-Butanediol	785	0.20	
Hexanal	801		0.52
Methyl pyrazine	819		0.07
Furfural	828	0.55	2.01
Butanoic acid, 2-methyl-	832	0.51	0.27
2-Furanmethanol	866	0.50	1.22
2(5H)-Furanone	871	0.46	0.73
Heptanal	901		0.77
Pyrazine, ethyl-	907	0.10	
Pyrazine, 2,5-dimethyl-	908	0.46	0.11
Pentanoic acid	911	0.43	0.85
Pyrazine, 2,3-dimethyl-	915	0.10	
α-Pinene	939	0.17	
2-Furancarboxaldehyde, 5-methyl-	942	0.37	0.29
2(5H)-Furanone, 5,5-dimethyl-	951	0.86	0.11
Benzaldehyde	952	0.08	0.26
Hexanoic acid	967	2.74	0.91
Phenol	980	0.24	0.21
β-Pinene	981	0.09	
2(5H)-Furanone, 3-methyl-	983	0.08	0.22
5-Hepten-2-one, 6-methyl-	985	0.75	0.36
β-Myrcene	988	0.36	
2-Octanone	988	0.41	0.76
Furan, 2-pentyl-	992		0.72
Octanal	998		0.76
Pyrazine, 2-ethyl-3-methyl-	1001	0.19	
2,4-Heptadienal	1005	0.07	0.38
o-Cymene	1020		0.07
Benzyl Alcohol	1026	0.29	0.23
Hexanoic acid, 2-ethyl-	1027	0.15	0.21
1-Hexanol, 2-ethyl-	1032		0.14
2-Acetylpyridine	1035	0.50	0.12
Benzeneacetaldehyde	1036	0.58	0.61
Butyl 3-methylbutanoate	1047	0.38	0.20
Acetophenone	1059	0.12	0.12
2-Nonyne	1068		0.26
Pyrazine, 3-ethyl-2,5-dimethyl-	1082	0.08	0.06
Heptanoic acid	1083	1.05	1.89
2-Nonanone	1087	0.13	0.22
Nonanal	1100	0.41	1.28

Table 1 *(Continued)*

Compound[a]	RI[b]	% Area[c] Flower	% Area[c] Trunk
Undecane	1100	0.12	1.47
Phenylethyl Alcohol	1106	0.10	0.04
Maltol	1106	0.07	0.14
2(3H)-Furanone, dihydro-5-propyl-	1130	0.21	0.29
2(3H)-Furanone, ethyl-4-hydroxymethyl-	1139	0.20	0.27
Furaneol	1139	0.54	0.31
Camphor	1143	0.11	0.16
2,6-Nonadienal	1150		0.12
2-Nonenal	1157		0.23
Borneol	1165	0.08	
Octanoic Acid	1167	1.54	2.98
Melilotal	1181	0.11	0.11
2-Decanone	1190	0.07	0.27
1,2-Benzenediol	1197	0.11	0.14
Decanal	1204	0.21	0.57
2-Phenoxyethanol	1245	0.22	0.12
Nonanoic acid	1267	1.95	3.20
Thymol	1289	0.09	
Indole	1290	0.04	
2-Undecanone	1293	0.60	
Butyrolactone	1299	0.65	1.13
2-Methoxy-4-vinylphenol	1313	0.23	0.09
α-Cubebene	1345	1.01	0.16
n-Decanoic acid	1364	0.51	0.94
Biphenyl	1375		0.04
Tetradecane	1400	0.19	0.15
Diisobutyl phthalate	1442	1.91	1.15
2-Propen-1-ol	1448	2.81	3.77
α-Neoclovene	1454	0.16	
β-Farnesene	1458	0.80	1.75
Germacrene D	1484	2.02	1.21
β-Ionone	1485	0.51	0.47
Undecanoic acid	1490	0.61	0.63
2-Tridecanone	1495	0.04	
Pentadecane	1500	1.08	
Calamenene	1523	0.43	0.43
α-Muurolene	1524	3.85	2.72
(1-Butylhexyl)-benzene	1526	0.15	

RI; Kovats retention index
a As identified by GCxGC-TOF/MS software; names according to NIST mass spectral library, and by comparing their Kovats retention indices; **b** Kovats retention indices of each component was collected from the literature for column Rxi 5ms; **c** Percentage of each component is calculated as peak area of analyze divided by peak area of total ion chromatogram times 100 (In the case of multiple identification, the areas of the peaks that belong to one analyze were combined to find the total area for this particular analyze); **d** Not detected

Assessment of chromosomal damage, nuclear division index and proliferative index

The results of micronucleus assay and SCE frequencies in human lymphocyte cells treated with DMSO as solvent control, EMS as positive control and HOE doses at 250, 500 and 750 μg/ml are shown in Table 2. These three doses were selected after a preliminary study performed within the range of 50–1750 μg/ml to determine the concentrations that would induce genotoxic activity and would not decrease the mitotic index (data not shown). An elevation in the MN frequencies was observed in the cells treated with 250, 500 and 750 μg/ml concentrations of HOE compared to solvent control, however this increase was not significant at 250 and 500 μg/ml concentrations. A significant increase in the MN frequency was only observed at 750 μg/ml HOE. A significant increase in SCE ratio was detected when human lymphocytes were treated at a dose of 500 and 750 μg/ml ($p < 0.05$, $p < 0.005$ respectively). NDI and PRI remained the same at selected doses of HOE, which suggests that the extract does not act on the mechanisms pertaining to proliferation of the cells.

Comet assay

The outcomes of comet assay were expressed as the changes in the comet tail length, Olive tail moment, percentage of DNA in tail and head, DNA damage index (DDI) and percentage of damaged cells (Table 3). The results demonstrate that comet tail length, Olive tail moment, percentage of DNA in tail, DDI and percentage of damaged cells increased significantly at all concentrations compared to solvent control ($p < 0.005$ and $p < 0.0001$). EMS employed as positive control generated a more severe DNA damaging activity.

Anti-growth effects of HOE

The anti-growth effect of the HOE (3.13–100 μg/ml) against MCF-7 and MDA-MB-231 cell lines was analyzed by the ATP viability assay after a 72 h treatment (Fig. 1). HOE significantly inhibited the growth of the MCF-7 and MDA-MB-231 cells in a dose-dependent manner ($p < 0.05$). IC_{50} and 90% inhibitory concentration (IC_{90}) were calculated as 26.3 and 91.2 μg/ml for MCF-7 and 39.0 and 92.9 μg/ml for MDA-MB-231, respectively. The sensitivity of both cell lines to anti-growth effects of HOE was similar even though the IC_{50} value for MCF-7 cell line was slightly lower than that of MDA-MB-231.

Type of cell death induced by HOE

The mode of cell death triggered by HOE treatment was determined by fluorescence microscopy based on nuclear morphology and cell membrane integrity in breast cancer cells. We revealed that HOE treatment at 100 μg/ml concentration for 72 h resulted in nuclear shrinkage (pyknosis) in MCF-7 cells as illustrated in upper panel of Fig. 2. Likewise, nuclear shrinkage was noticed in some of the MDA-MB-231 cells as well. Pyknosis is a characteristic of apoptosis and pyknotic nuclei stained with Hoechst 33342 were indicated by white arrows in Fig. 2. However, these cells were also stained with PI signifying that membrane integrity was lost at this time point. Calcein-AM stains only viable cells and only a minority of the cells were stained with Calcein-AM in HOE treated MCF-7 and MDA-MB-231 cells.

Table 2. Results of Micronuclei (MN) and Sister Chromatid Exchange (SCE) assays in cultured human lymphocytes treated with HOE, positive and negative controls (Mean ± SE)

Treatment	Dose (µg/ml)	N[a]	‰ MN[b]	Nuclear division index (NDI)[b]	SCE/Cell[b]	Proliferative index (PRI)[b]
Solvent control	–	4	24.50 ± 3.12	1.16 ± 0.03	7.85 ± 1.21	1.92 ± 0.04
HOE	250	4	34.93 ± 6.67	1.17 ± 0.03	10.67 ± 1.10	1.96 ± 0.13
	500	4	37.20 ± 4.45	1.17 ± 0.03	12.47 ± 1.09*	1.76 ± 0.06
	750	4	42.35 ± 6.47*	1.19 ± 0.01	15.28 ± 0.82**	1.71 ± 0.05
Positive control[c]	1,250	4	45.00 ± 7.10	1.15 ± 0.01	25.37 ± 3.25	1.82 ± 0.09

HOE; *Hypericum olympicum* subsp. *olympicum* methanolic extract.
[a] Number of donors; [b] Significance compared with solvent control (0.1 % DMSO); [c] EMS was used as positive control.
* $p < 0.05$; ** $p < 0.005$.

Table 3. Results of comet analysis in cultured human lymphocytes treated with HOE, positive and negative controls in which 50 cells per donor were scored (Mean ± SE)

Treatment	Dose (µg/ml)	Tail length[a](µm)	Tail % DNA[a]	Olive tail moment[a]	Head % DNA[a]	DNA Damage index (DDI)[a]	Percentage of damaged cells[a]
Solvent control	–	4.23 ± 0.09	3.96 ± 0.15	0.53 ± 0.02	96.04 ± 0.15	0.21 ± 0.06	0.00 ± 0.00
HOE	250	15.74 ± 1.63***	11.88 ± 1.42***	4.09 ± 0.77**	88.12 ± 1.42***	0.65 ± 0.03***	0.17 ± 0.01***
	500	21.41 ± 2.12***	14.13 ± 1.67***	6.10 ± 0.96***	85.87 ± 1.67***	0.77 ± 0.07***	0.21 ± 0.01***
	750	24.00 ± 2.17***	18.15 ± 1.89***	7.66 ± 1.04***	81.85 ± 1.89***	1.05 ± 0.04***	0.29 ± 0.02***
Positive control[b]	1,250	110.71 ± 5.53	96.72 ± 4.84	56.94 ± 2.85	3.28 ± 0.16	3.92 ± 0.06	1.00 ± 0.01

HOE; *Hypericum olympicum* subsp. *olympicum* methanolic extract.
[a] Significance compared with solvent control at * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$.
[b] EMS was used as positive control.

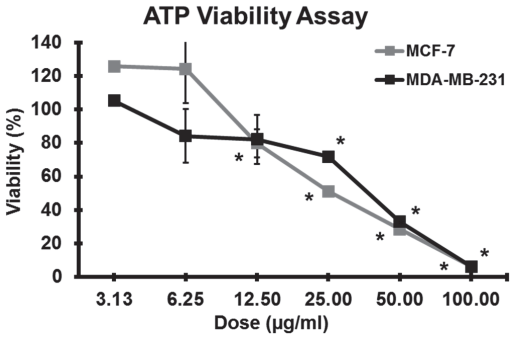

Fig. 1. Assessment of viability in MCF-7 and MDA-MB-231 cell lines after 72 h treatment with varying doses of HOE by the ATP viability assay.
* Denotes significant differences compared with control ($p < 0.05$).

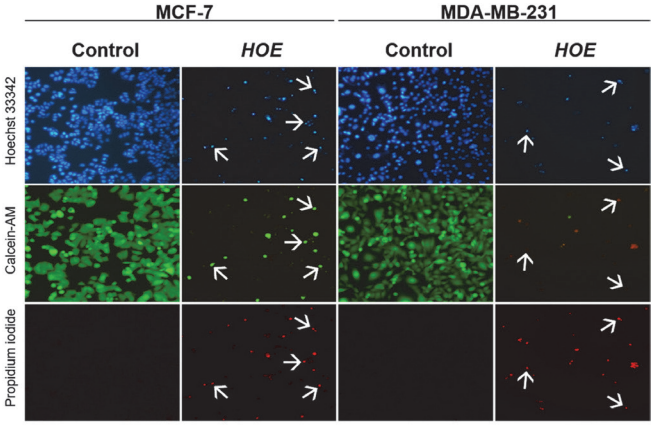

Fig. 2. Fluorescence imaging for determination of cell death mode in MCF-7 and MDA-MB-231 cells. Cells were treated with 100 µg/ml of the HOE for 72 h. Upper panel shows Hoechst 33342 staining while middle and lower panels show calcein-AM and propidium iodide (PI) staining, respectively. Arrows indicate the cells with pyknotic/fragmented nuclei.

To examine a possible apoptotic effect of HOE, we have measured the levels of M30, active caspase-3 and cleaved PARP. The level of M30 did not change following 25 and 50 µg/ml HOE treatment in MCF-7 cells, but its level was increased at 100 µg/ml concentration (Fig. 3). M30 assay was not performed in MDA-MB-231 cell line as these cells have low cytokeratin 18 expression (Sommers et al., 1992). To find out the involvement of caspase-3 activation and PARP cleavage in cell death, we further performed ELISA assays after 100 µg/ml HOE treatment. We have observed no caspase-3 activation or PARP cleavage in MDA-MB-231 cells (Fig. 4A and B). PARP cleavage also did not occur in MCF-7 cells (Fig. 4A). Caspase-3 activation was not assayed in MCF-7 cells as a result of a deletion in the gene that encodes procaspase 3 (Jänicke et al., 1998). A significant increase was observed in active caspase-3 and cleaved PARP levels after treating with paclitaxel (3.12 µM) which was selected as positive control.

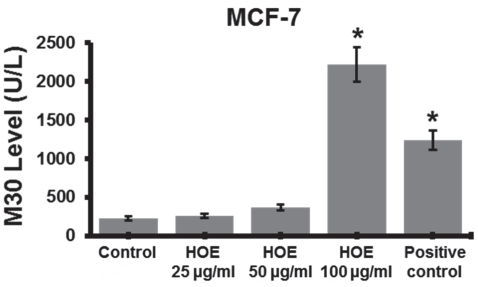

Fig. 3. The M30 levels (U/L) in MCF-7 breast cancer cells after treatment with HOE for 72 h. Paclitaxel (3.12 µM) was used as positive control for M30 increase. * Denotes significant differences compared with control ($p < 0.05$).

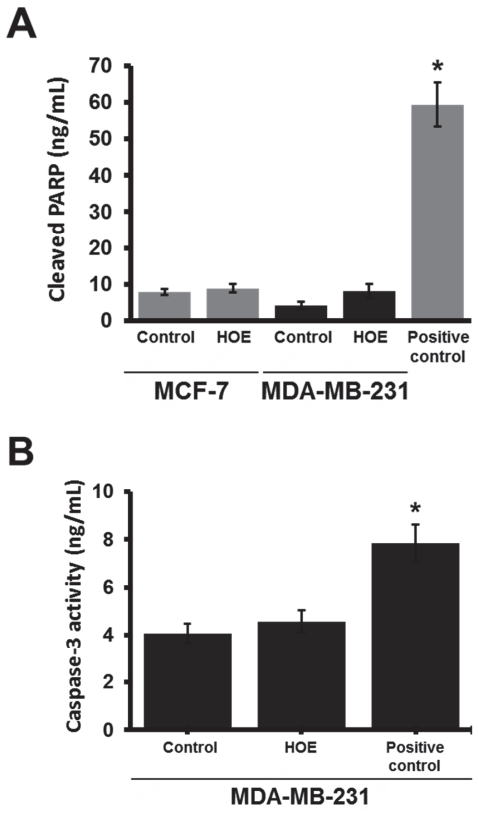

Fig. 4. Determination of cleaved PARP and active caspase-3 levels by ELISA. (**A**) Cleaved PARP levels in MCF-7 and MDA-MB-231 cell lines after 72 h treatment with 100 µg/ml concentration of HOE. Paclitaxel (3.12 µM) treated MCF-7 cells were used as positive control. (**B**) Active caspase-3 levels in MDA-MB-231 cells treated with 100 µg/ml HOE for 72 h. Paclitaxel (3.12 µM) treated MDA-MB-231 cells were used as positive control. * Denotes significant differences compared with control ($p < 0.05$).

Discussion

Medicinal plants are traditionally utilized worldwide against several diseases and eventually found their way into clinical practice due to their efficacy in cancer treatment (Cragg and Newman, 2005; Mann, 2002). Furthermore, genotoxicity analysis is also important to provide a risk assessment for human health (Bast et al., 2002). Hence, we evaluated both genotoxic and cytotoxic activities of HOE that still has use in traditional medicine regionally on human lymphocytes and breast cancer cell lines, respectively. We have shown that HOE had significant genotoxic activity at a higher dose range (250–750 µg/ml) based on SCE, MN and comet analyses in human lymphocytes *in vitro*.

No studies have been detected to focus on genotoxic activity of HOE, but there are several studies on other *Hypericum* species that still lacks agreement. For instance, Ramos et al. (2013) reported that *H. perforatum* presented a protective activity against oxidative damage in HT29 colon adenocarcinoma cell line; however, it was additionally revealed that the water extract of *H. perforatum* heightened the frequency of abnormal metaphases and chromosomal aberrations (Saadat, 2006). Water extract of a distinct species, *H. heterophyllum* was determined to elevate the MN levels in human lymphocytes. In general, these differences might arise from the interspecies differences, extraction methods or dose selection (Ocal and Eroğlu, 2012). Interestingly, some studies revealed that *H. perforatum* is not the most biologically active species in contrast to being the most widely known among the *Hypericum* genus (Stojanovic et al., 2013). Thus, it would be plausible to detect different levels of genotoxic/antigenotoxic activity in different species such as *H. olympicum* subsp. *olympicum*.

Our results demonstrated that HOE exhibited a similarly strong growth inhibitory effect on MCF-7 and MDA-MB-231 cell lines at relatively lower doses. Crude extracts of different species from *Hypericum* genus vary greatly in cytotoxic activity towards different cell lines. In our previous study, we have shown that *H. adenotrichum* methanol extract has superior cytotoxic activity against MCF-7 and MDA-MB-231 cell lines with IC_{50} values of 10.9 and 3.1 µg/ml, respectively (Sarimahmut et al., 2016). On the other hand, there are findings in which other *Hypericum* species have demonstrated weaker cytotoxic activity. For instance, *H. androsaemum* water extract exhibited anti-growth effects against HCT15 and CO115 colorectal carcinoma cells at relatively higher concentrations (IC_{50} values around 65 and 85 µg/ml) (Xavier et al., 2012). In addition, Sarrou et al. (2018) found out that methanol extract of *H. perforatum* displayed a cytotoxic behavior at 100 µg/ml concentration in Caco-2 intestinal cancer cell line. Also, according to the criteria established by the U.S. National Cancer Institute on cytotoxicity of crude extracts, the IC_{50} values of HOE lie close to the cut-off value (30 µg/ml) between an "active" and "moderately active" compound (Suffness and Pezzuto, 1990). In the light of these data, HOE might be considered as "active". Notably, we have demonstrated that it requires a much higher concentration of HOE to exert genotoxicity in human lymphocytes than the doses which result in cell death in breast cancer cells. This leads to an advantageous situation if this species is considered for medicinal uses.

Our research group previously has shown that HOE exerts cytotoxic and apoptotic effects against lung cancer cells (Aztopal et al., 2016). It was also clearly observed in the current study that presence of pyknotic nuclei suggests apoptotic cell death in MCF-7 and MDA-MB-231 cells after a treatment of 100 µg/ml concentration in accordance with the findings of the anti-growth assay. In addition, the cells with pyknotic nuclei being positive for PI indicates that the cells undergo late stage apoptotic cell death (Hammill et al., 1999). Further verification of apoptosis was made through the increase in M30 levels in MCF-7 cells. In addition, caspase 3 activation and degradation of PARP were examined in order to better understand apoptosis-inducing effect of HOE (Soldani and Scovassi, 2002). Analyses in MDA-MB-231 cells suggest that the resulting cell death might be independent from caspase-3 activation,

but additional analyses are needed. Absence of PARP cleavage which is commonly catalyzed by the effector caspases during apoptotic cell death implies caspase-3 independent cell death (Soldani and Scovassi, 2002).

HOE demonstrated its cytotoxic and genotoxic activities owing to a diverse chemical content. The methanol extract of the stamens from *H. olympicum* has hypericin and pseudohypericin components in approximately equal quantities (Kitanov, 2001). It was previously demonstrated that methanolic extract of *H. olympicum* subsp. *olympicum* to possess a rich flavonoid content such as rhamnetin, isorhamnetin and apigenin (Saddiqe et al., 2011). Recently, Llorent-Martinez et al. (2018) have identified 3-*O*-Caffeoylquinic acid as the most abundant phenolic acid and myricetin-O-deoxyhexoside as the dominant compound among flavonols in methanolic extract of *H. olympicum* in their study. There are a few reports in literature on the volatile compounds of HOE. Gudžić et al. (2001) identified 49 compounds and Pavlović et al. (2006) found 41 compounds including alkanes, monoterpenes, sesquiterpenes and oxygenated compounds. Moreover, Stojanovic et al. (2003) has determined 14 alkanes and 11 fatty acids in *H. olympicum*. It is apparent that DTD is superior in identifying a higher number of volatile compounds from *H. olympicum* subsp. *olympicum*. The major components of the flower and trunk were eicosane, heptacosane, 2-propen-1-ol, hexahydrofarnesyl acetone and α-muurolene. A number of furans, furanones and pyrazine compounds were also detected. These molecules were not original volatiles from *H. olympicum* subsp. *olympicum* which in fact were formed during thermal desorption unit heating process.

Some of the pharmacologically active constituents of *H. olympicum* L. spp. *auriculatum* were identified in a phytochemical profiling study and amentoflavone was detected as one of the active constituents while hypericin and hyperforin were not (Crockett et al., 2005). Another study that documents antioxidant potential and total phenolic and flavonoid content of *H. olympicum* and four other *Hypericum* species demonstrated that all plant extracts had a prominent antioxidant activity, but *H. olympicum* comprised the highest total flavonoid content compared to other four *Hypericum* species (Božin et al., 2013). Also, isolated acylphloroglucinols from *H. olympicum* L. cf. *uniflorum* were investigated by Shiu et al. (2011) for antibacterial activity and one of these compounds were found to have a strong antibacterial action.

Conclusions

In conclusion, a broad spectrum of polar and non-polar organic volatile compounds was analyzed from flower and trunk of *H. olympicum* subsp. *olympicum* using DTD coupled with GCxGC-TOF/MS. The methanolic extract of *H. olympicum* subsp. *olympicum* induced cell death in a dose-dependent manner against breast cancer cell lines and exhibited genotoxic activity at relatively higher doses in human lymphocytes. The cytotoxic activity of HOE determined in this study also points out the importance of dose selection if the plant extract is intended to be used as a remedy in traditional medicine.

Conflict of interests

The authors report no conflict of interests.

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