# Genotoxic, Cytotoxic, and Apoptotic Effects of *Hypogymnia physodes* (L.) Nyl. on Breast Cancer Cells

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**ABSTRACT:** The aim of this study is to determine the chemical composition, and evaluate the genotoxic, and anti-growth potency of the methanol extracts of lichen species *Hypogymnia physodes* (L.) Nyl. (HPE). Anti-growth effect was tested in two different human breast cancer cell lines (MCF-7 and MDA-MB-231) by the MTT and ATP viability assays and apoptosis was assayed by the caspase-cleaved cytokeratin 18 (M30-antigen). Genotoxic activity of HPE was studied using chromosome aberration and micronuclei tests in human lymphocytes culture *in vitro*. The chemical composition of *H. physodes* was analyzed by using direct thermal desorption method coupled with comprehensive gas chromatography–time of flight mass spectrometry (GCXGC-TOF/MS). Our results indicate that HPE has an anti-growth effect at relatively lower concentrations, while relatively higher concentrations are required for genotoxic activity. HPE, therefore, seems to represent a therapeutic potential and poses new challenges for medicinal chemistry.
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**Keywords:** *Hypogymnia physodes*; cytotoxicity; genotoxicity; apoptosis; chemical composition

## INTRODUCTION

Lichens are symbiotic organisms consisting of, at least, one fungus and one photosynthetic partner, alga or cynanobacterium, or possibly of all three (Oksanen, 2006). These associations contain a great number of organic compounds, the so-called lichen substances which are more typical of these groups (Huneck and Yoshimura, 1996). A great number of lichen substances have been identified and isolated with new experimental and analytical techniques to date (Molnar and Farkas, 2010). Due to their therapeutic properties, many cultures have utilized these compounds in traditional medicine for centuries (Muller, 2001). In addition to their role in lichen chemotaxonomy and systematic, lichen secondary metabolites have possible biological roles, including photoprotection against intense radiation, as well as allelochemical, antibiotic, antiviral, antitumor, antibacterial, antiherbivore, antioxidant, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects (Muller, 2001; Oksanen, 2006; Molnar and Farkas, 2010).

Several studies were carried out related with cytotoxic activity of different lichen extracts (Perry et al., 1999; Bezivin et al., 2003; Manojlovic et al., 2010). Zeytinoglu et al. (2008) reported the genotoxic, antigenotoxic, and cytotoxic potential of the acetone extract from *Cetraria aculeata in vitro*. Koparal et al. (2006) investigated

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cytotoxic and genotoxic activities of secondary metabolites isolated from the *Ramalina farinacea* and *Cladonia foliacea* on human lymphocytes and determined high cytotoxic activity against cancer cells. In addition, there are some studies related with antiproliferative effects of lichen metabolites and extracts on several cancer cells such as murine myeloma, colon cancer cells (Kumar and Müller, 1999; Bucar et al., 2004; Ren et al., 2009; Triggiani et al., 2009).

*Hypogymnia physodes* is a foliose form and belongs to the family of *Parmeliaceae*. *H. physodes* contain atranorin, chloratranorin, physodic acid, physodalic acid, 3-hydroxiphysodic acid, and protocetraric acid (Asahina and Shibata, 1971; Culberson, 1979; Huneck and Yoshimura, 1996). A significant antioxidant activity of *H. physodes* was reported by Stojanović et al. (2010) using two different free radical scavenger assays. In another study it was reported that these isolated metabolites from *H. physodes* had antimicrobial activity on human and animal pathogens (Ranković et al., 2007).

In this study, genotoxic and cytotoxic activities of the methanol extracts from *H. physodes* were investigated in human lymphocytes and breast cancer cell lines. To the best of our knowledge, this is the first report which shows genotoxic, cytotoxic, and apoptotic effects of *H. physodes* on breast cancer cell lines. We have demonstrated that HPE inhibited the growth of breast cancer cells by inducing apoptosis at relatively lower doses, while it is also genotoxic at relatively higher doses.

## MATERIALS AND METHODS

### Collections and Identification of Lichen Materials

*Hypogymnia physodes* was collected from Uludag Mountain (Bursa, Turkey) in May 2010 and identified aid of flora books (Purvis et al., 1994; Wirth, 1995). A voucher specimen was deposited in the Herbarium of Uludag University, (BULU), Bursa, Turkey.

### Extraction of Lichen Sample

The sample was air-dried at room temperature and carefully cleansed of extraneous materials. Next, *H. physodes* sample was ground into powder and 15 g of the material was extracted by adding 150 mL of solvent methanol (Merck) in Soxhlet apparatus for 24 h. The crude extracts were concentrated in rotary evaporator at 40°C. The residues were lyophilized and stored at −20°C until used.

### Direct Thermal Desorption

A GCXGC-TOF/MS system was used together with a thermal desorption injector. Empty glass thermodesorption tubes were conditioned at 400°C for 2 h prior to each use. Approximately 20–30 mg of sample was placed into the thermodesorption. Each desorption tube was then sealed with a stainless steel o-ring assembly and handled throughout the remaining analysis stages by robotic autosampler. Initial desorption of the sample was carried out by heating the TDU from 40°C (initial time 0.2 min) to 150°C at a rate of 120°C $min^{-1}$ with a final hold time of 5 min under a helium flow of 1.5 mL $min^{-1}$. Volatile analytes released from this heating were cryo-focused at −40°C in the CIS cooled with liquid nitrogen prior to injection. The CIS was then heated at a rate of 10°C $s^{-1}$ to a final temperature of 150°C. Analytes were transferred splitless to the GC column during the CIS temperature ramp.

### Chromatographic Analysis

The GCxGC–TOF/MS system consisted of an Agilent 6890 (Agilent Technologies, Palo Alto, CA) gas chromatograph and a Pegasus III TOF-MS (LECO, St. Joseph, MI). The first column was a nonpolar Rxi 5 ms (50 m × 0.32 mm i.d. × 1.0 $\mu$m film thickness) and the second column a Rtx 50 (2 m × 0.18 mm i.d. × 0.20 $\mu$m film thickness). Both columns were from Thames Restek (Sounderton, UK). The columns were connected by means of a press-fit connector. The first dimensional separation was based on separation by volatility a nonpolar column. The second dimensional separation was based on separation by polarity using a polar column. The combination of separations produces the overall two dimensional chromatograms. The initial temperature of the first dimension column was 70°C for 1 min and the subsequent temperature programme was a heating rate of 5°C $min^{-1}$ until 270°C was reached and held isothermally for a further 1 min. The initial temperature of the second dimension column was 85°C for 1 min and a 5°C $min^{-1}$ heating rate was used until 285°C was reached and held isothermally for a further 1 min. Cryogenic modulation was performed using a jet-type modulator which was installed at the front of the second dimension column. This consisted of two cold and two hot jets, with the nozzles providing the cold jets mounted orthogonal to the hot jets. Nitrogen gas was cooled by heat exchange through copper tubing immersed in liquid nitrogen outside the GC system and delivered through vacuum-insulated tubing to the cold jets to provide two continuous jets of approximately 10 L $min^{-1}$ of cold nitrogen gas. The modulation time was 5 s. Peak identification was made using TOF/MS with electron ionization. Mass spectra were compared against the NIST 2005 mass spectral library and Kovats indexes from the literature.

### Determination of Cytotoxicity

#### Chemicals, Cell Culture

Stock and final concentrations of *H. physodes* extract (HPE) were prepared in culture medium. Lichen extract

was used at different concentrations ranging from 0.09 to 100 μg/mL. Breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured in RPMI 1640 supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine, and 10% fetal calf serum (Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere containing 5% $CO_2$.

### The MTT Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) viability assay was performed as previously described (Ulukaya et al., 2008). Briefly, the cells were seeded in 200 μL culture medium in triplicates at a density of $5 \times 10^3$ cells per well of a 96-well plate. Cells were incubated either alone (for control) or in the presence of the HPE for 72 h. At the end of the incubation, the absorbance was determined by an ELISA plate reader (FLASH Scan S12, Analytic Jena, Germany) at 570 nm. Cell viability of treated cells was calculated in reference to the untreated control cells using the formula:

$$(\%) = [100 \times (\text{sample Abs})/(\text{control Abs})].$$

### The ATP Viability Assay

The ATP assay is based on the highly sensitive ''firefly'' reaction to determine the level of cellular ATP as a surrogate marker for the number of live cells (Andreotti et al., 1995). It was performed to confirm the results of the MTT assay due to the possibility of the interference of the MTT assay itself with the HPE. The seeding and treatment conditions as well as the calculation of viability were performed as similar to that is for the MTT assay (see above). At the end of the treatment period, the ATP content was determined according to the manufacturer's recommendations (DCS Innovative Diagnostika-Systeme, Hamburg, Germany).All in all ATP was extracted from the cells and then luciferin-luciferase was added. Luminescence was determined in a luminometer (Bio-Tek, USA). The result was expressed in U/L.

### Measurement of Programmed Cell Death (Apoptosis)

Apoptosis was measured by the detection of caspase-cleaved cytokeratin 18 (M30-antigen), which is a well known marker of apoptosis, by ELISA. Apoptosis was measured by a commercially available immunoassay kit (M30-Apoptosense ELISA kit, Peviva AB, Sweden), according to the manufacturer's instructions. About $5 \times 10^3$ cells were seeded per well of a 96-well plate in 200 μL culture medium in triplicates. Cells were treated for 72 h with the 100 μg/mL of the HPE. At the end of the treatment, the cells were lysed as described in the assay's instructions. The content of identical wells were pooled and centrifuged at 2000 rpm for 10 s to remove the debris and the supernatant was subsequently kept at −20°C until to be used. All of the samples were placed into wells coated with a mouse monoclonal antibody as a catcher. After washing, a horseradish peroxidase conjugated antibody (M30) was used for detection. The absorbance was determined with an ELISA reader at 450 nm (FLASH Scan S12, Analytik Jena, Germany).

### Fluorescence Imaging for Determination of Cell Death Mode

The detection of cell death mode was based on both the nuclear morphology of cells and the cell membrane integrity using a fluorescent microscope. MCF-7 and MDA-MB-231 cells were seeded in a six-well plate at the density of $5 \times 10^5$ cells per well, and then exposed to the HPE at the concentration of 100 μg/mL for 72 h. After the treatment, the cells were washed with PBS and incubated with the propidium iodide (PI) (2 μg/mL) solution for 15 min in the dark at 37°C. Apoptosis was decided on the basis of the presence of the chromatin condensation and/or nuclear fragmentation (Van Cruchten and Van Den Broeck, 2002).

## Genotoxicity Assays

Heparinized peripheral blood obtained from four healthy and nonsmoking donors (two males and two females, ages 18–23) was used in all experiments. The cultures were set up by adding 0.3 mL of whole blood to RPMI 1640 medium (1X, Sigma) supplemented with 20% fetal calf serum (Biochrom AG), 100 IU/mL penicillin, 100 μg/mL streptomycin (Biological Industries), 0.5 mg/mL L-glutamine and 6 μg/mL phytohemagglutinin (PHA-L, Biochrom AG). Lymphocytes were incubated at 37°C for 72 h. As positive control, mitomycine-C (MMC) was dissolved in sterile distilled water and drug solution was prepared immediately before the use to avoid degradation of the drug. HPE was dissolved DMSO 50%. The cells were treated separately with mutagenic agent, DMSO 50% as solvent control and diluted sterile HPE (125, 250, and 500 μg/mL), which were chosen after a preliminary study (50, 100, 125, 250, 400, 500, and 750 μg/mL), for 24 h prior to harvest. Metaphases were obtained by adding colcemid (0.2 μg/mL final concentration, Sigma) 2 h prior to harvest. For micronuclei (MN) assay, cytochalasin B (6 μg/mL) was added at 44 h to block the cytokinesis process, and lymphocyte cultures were harvested after 72 h (Fenech, 2000).

Chromosomes were prepared using standard procedures (Benn and Perle, 1992). Staining of the MN was performed by immersing the air-dried slides in a 2% Giemsa solution.

## Microscopic Evaluation

### Chromosomal Aberrations

The analysis of chromosomal aberrations (CAs) was performed in 50 metaphases for each culture. Both gaps and

**TABLE I. Chemical composition of *Hypogymnia physodes* (L.) Nyl. sample**

| Compound[a] | RI[b] | % Area[c] |
|---|---|---|
| Acetic acid | 600 | 0.56 |
| 2-Propanone, 1-hydroxy- | 672 | 0.20 |
| Butanoic acid | 763 | 0.28 |
| 2,3-Butanediol | 785 | 0.11 |
| Hexanal | 801 | 0.26 |
| Furfural | 828 | 0.36 |
| 2-Furanmethanol | 866 | 0.18 |
| 2(5H)-Furanone | 871 | 0.42 |
| 2-Heptanone | 889 | 0.43 |
| Heptanal | 901 | 1.36 |
| Pentanoic acid | 911 | 1.24 |
| 2-Cyclohexen-1-one | 914 | 3.24 |
| 2-Furancarboxaldehyde, 5-methyl- | 942 | 0.04 |
| Benzaldehyde | 952 | 0.06 |
| Hexanoic acid | 967 | 3.72 |
| Phenol | 980 | 0.28 |
| 2,5-Octanedione | 983 | 0.05 |
| 2(5H)-Furanone, 3-methyl- | 983 | 0.16 |
| 2-Octanone | 988 | 0.68 |
| Octanal | 998 | 0.92 |
| Benzyl alcohol | 1026 | 0.11 |
| Hexanoic acid, 2-ethyl- | 1027 | 0.08 |
| 1-Hexanol, 2-ethyl- | 1032 | 0.07 |
| Benzeneacetaldehyde | 1036 | 0.13 |
| Butyl 3-methylbutanoate | 1047 | 0.45 |
| Phenol, 2-methyl- | 1053 | 0.09 |
| $\gamma$-Caprolactone | 1055 | 7.63 |
| Acetophenone | 1059 | 0.05 |
| Octanenitrile | 1081 | 0.08 |
| Heptanoic acid | 1083 | 2.93 |
| 2-Nonanone | 1087 | 0.30 |
| Nonanal | 1100 | 1.70 |
| Phenylethyl alcohol | 1106 | 0.05 |
| 2(3H)-Furanone, dihydro-5-propyl- | 1130 | 0.36 |
| 2(3H)-Furanone, ethyl-4-hydroxymethyl- | 1139 | 0.64 |
| 2-Nonenal | 1157 | 0.28 |
| Octanoic acid | 1167 | 4.62 |
| 1-Dodecene | 1187 | 0.15 |
| 2-Decanone | 1190 | 0.51 |
| Decanal | 1204 | 0.33 |
| 2-Phenoxyethanol | 1245 | 0.06 |
| Nonanoic acid | 1267 | 6.52 |
| Benzoic acid | 1276 | 0.05 |
| 2-Undecanone | 1293 | 0.08 |
| Butyrolactone | 1299 | 0.90 |
| *n*-Decanoic acid | 1364 | 1.54 |
| Tetradecane | 1400 | 0.06 |
| Dimethyl phthalate | 1416 | 0.05 |
| 2-Propen-1-ol | 1448 | 2.30 |
| B-Farnesene | 1458 | 0.26 |
| Calamenene | 1523 | 0.05 |
| Tetradecanamide | 1526 | 0.14 |
| (−)-Spathulenol | 1577 | 0.40 |
| Caryophyllene oxide | 1582 | 0.24 |
| Diethyl phthalate | 1590 | 0.14 |
| Hexadecane | 1600 | 0.53 |
| Benzophenone | 1626 | 0.08 |
| Cubenol | 1645 | 0.25 |
| $\beta$-Eudesmol | 1649 | 0.13 |
| Pantolactone | 1685 | 0.07 |
| $\beta$-Farnesol | 1696 | 0.89 |
| Heptadecane | 1700 | 0.30 |
| 2,6-Diisopropylnaphthalene | 1728 | 1.57 |
| Octadecane, 2-methyl- | 1867 | 3.10 |
| Hexadecanoic acid, methyl ester | 1912 | 0.15 |
| Dibutyl phthalate | 1922 | 0.59 |
| Nonadecane, 2-methyl- | 1966 | 3.70 |
| Eicosane | 2000 | 13.21 |
| 1-Docosene | 2195 | 3.01 |
| Heptacosane | 2700 | 16.50 |
| Unknown | | 8.00 |

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 5 ms.

[c] Percentage of each component is calculated as peak area of analyte 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 analyte were combined to find the total area for this particular analyte).

pulverizations were included and both gaps and pulverizations were excluded from total CAs. The CAs was classified according to the Environmental Health Criteria (EHC) 51 for short-term tests for mutagenic and carcinogenic chemicals (IPCS, 1985).

## Micronuclei

Two thousand binucleated cells for each experimental point were examined following the scoring criteria adopted by the human micronucleus project (Bonassi et al., 2001). We evaluated the binucleated micronucleated lymphocytes (BNMN) frequency as the number of binucleated lymphocytes containing one or more MN per 1000 binucleated cells.

## Mitotic Index and Nuclear Division Index

The mitotic index was calculated from the number of metaphases in 2000 cells analyzed per culture for each dose group and donor in CA assay. Moreover, in MN assay, 500 lymphocytes were scored to evaluate the percentage of binucleated cells and the nuclear division index (NDI) was calculated according to the following formula:

$$NDI = [MONO + 2BN + 3TRI + 4TETRA]/500$$

**TABLE II. The cytotoxic activity of *Hypogymnia physodes* extracts (HPE) against MCF-7 and MDA-MB-231 breast cancer cells**

| Cells | $IC_{50}$ ($\mu$g/mL) | $IC_{90}$ ($\mu$g/mL) |
|---|---|---|
| MCF-7 | 50 | 100 |
| MDA-MB-231 | 44 | 100 |

where MONO, BN, TRI, and TETRA are mononuclear, binucleated, trinucleated, and tetranucleated lymphocytes, respectively.

## Statistical Analyses

The frequencies of CAs and MN in treated cultures and % viability were compared by using one-way analysis of variance (ANOVA) and Tukey honest significant difference (HSD) tests with 95% confidence intervals. Dose-response relationships were determined by regression analyses. A value of $p < 0.05$ was considered statistically significant. Results were expressed as mean ± SD (standard deviation). All of the statistical analyses were conducted using the SPSS 16.0 statistical software for Windows.

## RESULTS

Direct thermal desorption (DTD) has important advantages over the other methods such as the ability to be directly coupled to GC-MS, the requirement of only a small amount of sample and it is a rapid method for qualitative and quantitative analysis of volatiles compounds, and quantification of volatiles by DTD is possible (Ozel et al., 2006). However, there is very limited literature on this. GCxGC provides increased resolution and peak capacity over single column methods. The use of a spectrometric detector, in particular a MS, is highly desirable for identification of the numerous separated compounds found during a GCxGC run and provides a third dimension of specificity to the analysis. DTD with GCxGC-TOF/MS can be used to determine content in plant materials very quickly using a low volume of the starting sample and no prepreparation.

The chemical composition of *H. physodes* was analyzed by GCXGC-TOF/MS system and the qualitative and quantitative compositions were presented in Table I. Seventy-one compounds were identified in *H. physodes* and the major components of it were heptacosane (16.50%), eicosane (13.21%), $\gamma$-caprolactone (7.63%), nonanoic acid (6.52%), octanoic acid (4.62%), hexanoic acid (3.72%), nonadecane, 2-methyl-(3.70%), 2-cyclohexen-1-one (3.24%), octadecane, 2-methyl-(3.10%), 1-docosene (3.01%), heptanoic acid (2.93%), 2-propen-1-ol (2.30%), nonanal (1.70%), 2,6-diisopropylnaphthalene (1.57%), and *n*-decanoic acid (1.54%). All other components were present in less than 1%.

Anti-growth activity of the HPE was first assessed by the MTT assay. MCF-7 cells were treated for 72 h with increasing doses of HPE. $IC_{50}$ and $IC_{90}$ values for HPE are shown in Table II. The $IC_{50}$ values of the HPE were quite similar between MDA-MB-231 and MCF-7 cells.

The anti-growth effect of HPE (0.20–100 $\mu$g/mL, for 72 h) was assessed by the MTT viability assay [Fig. 1(A)]. As it was shown in Fig. 1(A), HPE reduced cell viability in a dose-dependent manner ($p < 0.05$). Given the results of both the MTT and ATP assay, only the relatively higher doses showed anti-growth activity in both cell lines. The intracellular ATP level significantly decreased after HPE treatments at 25, 50, and 100 $\mu$g/mL doses in both cell types ($p < 0.05$) [ Fig. 2(B)].

The morphological evaluation by phase contrast microscopy showed that the anti-growth effect that was judged by the MTT and ATP assays, was resulted from cell death (cytotoxicity) (Fig. 2). Cells were treated with HPE (100 $\mu$g/mL) as shown in Figure 2, the HPE was more cytotoxic when compared with the control in both MCF-7 and MDA-MB-231 breast cancer cells.

The growth inhibiting effect of the HPE seemed to be the result of the cytotoxic activity against the cells as stated above. Therefore, the apoptotic activity of HPE was further investigated by examining caspase-cleaved cytokeratin 18 (M30-antigen) which is a well-known marker of apoptosis. In Figure 3(A), we found that M30-antigen level was not changed after the treatment with HPE (100 $\mu$g/mL, for 72 h) in MDA-MB-231 cells. In contrast, M30-antigen level increased after HPE treatments (100 $\mu$g/mL, for 72 h) in MCF-7 cells. This clearly shows that HPE causes apoptosis in MCF-7 cells but not in MDA-MB-231 cells.

As shown above, HPE induced apoptosis in MCF-7 cells. Accordingly, we confirmed apoptosis by fluorescence imaging on the basis of nuclear morphology. We found that HPE resulted in nuclear fragmentation, which is one of the hallmarks of apoptosis, in MCF-7 cells as shown in Figure 3(A). However, there was no such change in MDA-MB-231 cells, which suggests that the mode of cell death was necrosis in this cell line [Fig. 3(C)].

The results of genotoxicity tests were summarized in Table III. Table III shows the effect of HPE (125, 250, and 500 $\mu$g/mL), positive (mitomycine-C, 0.25 $\mu$g/mL), solvent (DMSO, 50%), and negative controls on the mitotic index, the number of CAs and the frequency of abnormal metaphases in human lymphocyte cell cultures.

As presented in Table III, 250 and 500 $\mu$g/mL HPE treatment significantly increased CAs when gap and pulverized metaphase were both included and excluded compared with the solvent control group ($p < 0.005$ and $p < 0.01$ respectively). The doses lower than 125 $\mu$g/mL was not genotoxic effect (data not shown).

Chromatid gaps and breaks, iso-chromatid gaps and breaks and other subchromatid aberrations were the most common chromosomal abnormalities. A significant and

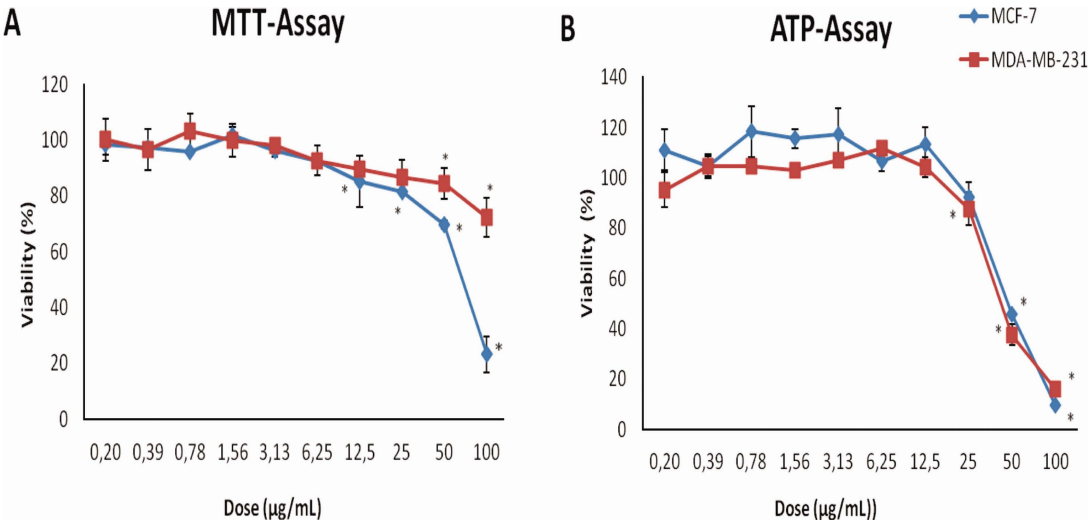



**Fig. 1.** Assessment of viability of MCF-7 and MDA-MB-231 cell lines after 72 h treatment with varying doses of *H. physodes* extract. *Denotes statically significant differences in comparison with control ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

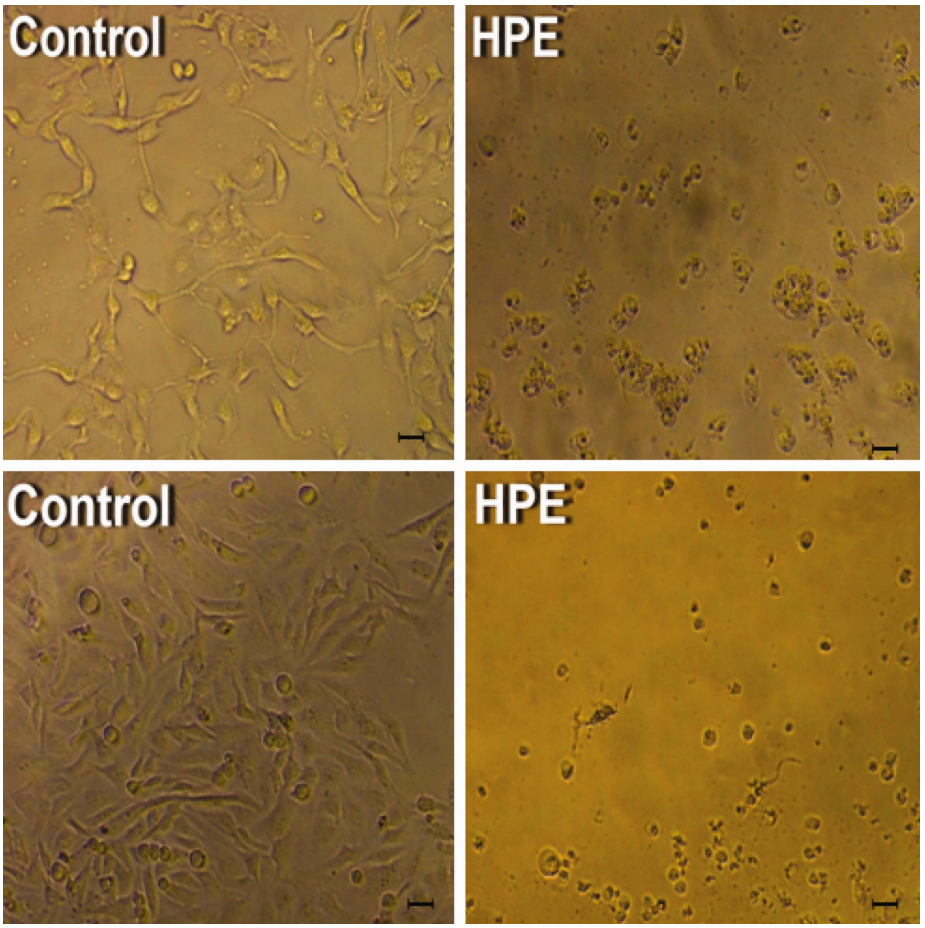



**Fig. 2.** Images of phase contrast microscopy. Cells were treated with 100 $\mu$g/mL *H. physodes* extract (HPE) for 72 h. Controls included untreated cells. Scale bars represents 10 $\mu$m. Upper panel indicates MCF-7 cells, while bottom panel indicates MDA-MB-231 cells for 72 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

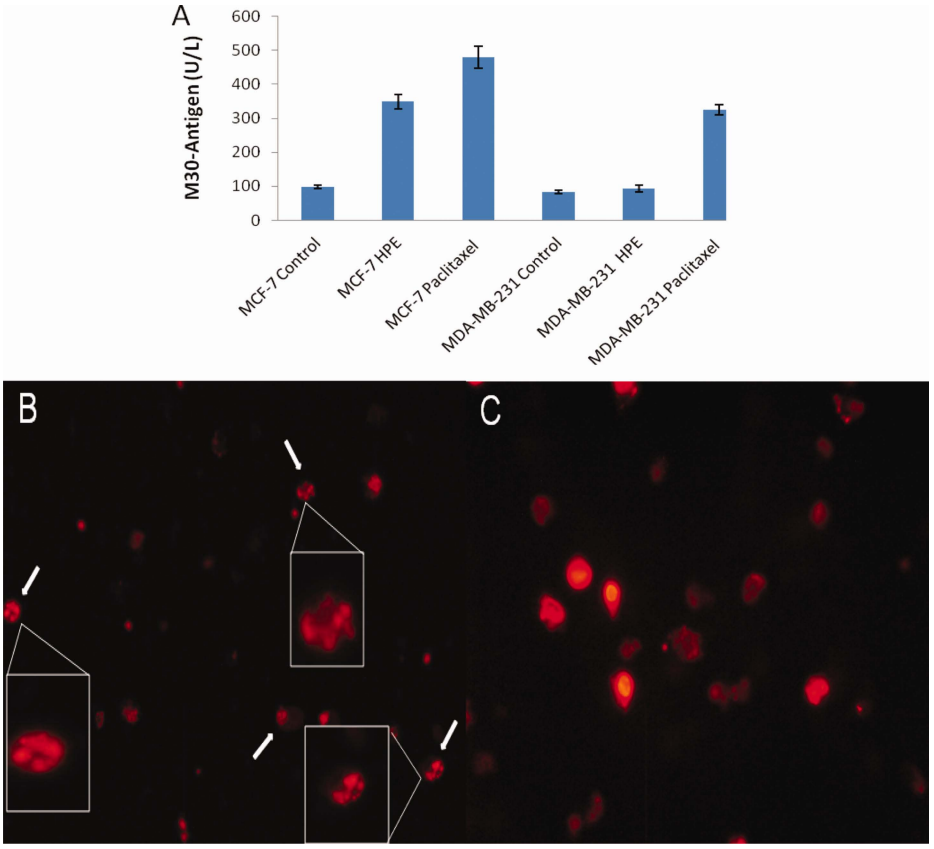



**Fig. 3.** M30 Assay results and fluorescence images stained with propidium iodide for determination of cell death mode (A) M30-antigen levels (U/L) 72 h after the *H. physodes* extract (HPE) and paclitaxel (3.12 $\mu$M, as positive control) treatments. (B) MCF-7 cells, (C) MDA-MB-231 cells. Arrows indicate the apoptotic cells with nuclear fragmentation. Inserts show higher magnification for better view of apoptotic nuclear fragmentations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dose-dependent decrease in the frequency of mitotic index was detected for the treatment with HPE ($r = -0.778$, $p < 0.0001$). A significant and dose-dependent increase in the frequency of the CAs when gap and pulverized metaphase were both included (TGAP, $r = 0.661$, $p < 0.0001$) and excluded (TGEP, $r = 0.584$, $p < 0.0001$).

The positive controls MMC at concentrations of 0.25 $\mu$g/mL was significantly increased in the number of abnormal metaphases and the total structural CAs, respectively when compared with the negative control ($p < 0.001$).

The results of the MN assay were also shown in Table III. All doses of HPE slightly increased the MN frequency and this increase was not statistically significant ($p > 0.05$). When we compared the NDI, we did not find any significant differences in all doses of HPE (Table III).

## DISCUSSION

To the best of our knowledge, surprisingly, there are no published data on genotoxicity, cytotoxic, and apoptotic activity of *H. physodes*. In this study, the cytotoxic and genotoxic effects of the methanol extract of *H. physodes* were investigated. It was demonstrated that HPE extract had significant genotoxic and clastogenic activities in a dose dependent manner (for TGA $r = 0.661$; $p < 0.0001$ and for TGEP $r = 0.584$; $p < 0.0001$, respectively) in human lymphocytes *in vitro* were evaluated using the genotoxicity tests. In addition, HPE had anti-growth effect on MCF-7 and MDA-MB-231 breast cancer cells at lower concentration (25–100 $\mu$g/mL) and induced apoptosis in MCF-7 cells at 100 $\mu$g/mL dose. This relatively lower dose-induced cytotoxicity without inducing genotoxicity may be a favorable property for an anticancer agent.

In some publications about lichen screening of extracts, some species of lichen were found to be highly active (Perry et al., 1999; Bezivin et al., 2003). In this study, different concentrations (ranging from 0.20 to 100 $\mu$g/mL) of HPE were tested in MCF-7 and MDA-MB-231 breast cancer cells. HPE showed a significant growth inhibiting effect at 25–100 $\mu$g/mL on breast cancer cells according to the MTT and ATP assays and these effects were confirmed by

**TABLE III. Frequencies of chromosome aberrations (CA), micronuclei (MN), nuclear division index (NDI) and mitotic index (MI%) in cultured human lymphocytes treated with *Hypogymnia physodes* (L.) Nyl. extract (HPE), positive and negative controls (mean ± SD)**

| Treatments | MI ± SD[a] | Number of Chromosome Aberration | | | | | | TA(G + P) ± SD[a] | TA(G − P) ± SD[a] | MN ± SD[a] | NDI ± SD[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CG | ICG | CB | ICB | EXC | SE | PLV | | | | |
| DW | 9.48 ± 0.61 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0.02 ± 0.02 | 0.00 ± 0.00 | 13.40 ± 1.22 | 5.09 ± 0.06 |
| DMSO | 8.05 ± 0.61 | 104 | 14 | 46 | 44 | 0 | 0 | 0 | 1.24 ± 0.23 | 0.65 ± 0.03 | 26.70 ± 0.08 | 5.17 ± 0.01 |
| 125 μg/mL HPE | 7.63 ± 0.02 | 128 | 21 | 64 | 84 | 0 | 11 | 0 | 1.65 ± 0.21 | 0.89 ± 0.12 | 27.20 ± 2.50 | 5.48 ± 0.14*** |
| 250 μg/mL HPE | 7.64 ± 0.22 | 140 | 43 | 91 | 76 | 0 | 16 | 0 | 1.94 ± 0.17** | 1.02 ± 0.07* | 31.60 ± 3.76 | 5.24 ± 0.09 |
| 500 μg/mL HPE | 6.55 ± 0.53** | 145 | 53 | 71 | 71 | 2 | 19 | 0 | 1.88 ± 0.39* | 0.90 ± 0.27 | 33.45 ± 6.08 | 5.11 ± 0.03 |
| MMC | 8.34 ± 1.92 | 32 | 14 | 26 | 18 | 31 | 2 | 7 | 0.61 ± 0.37 | 0.41 ± 0.19 | 27.5 ± 2.12 | 4.26 ± 0.34 |

CB, chromatid break; CG, chromatid gap; DW, distilled water as negative control; DMSO, 50% dimethyl sulphoxide as solvent control; MMC, mitomycine-C as positive control (0.25 μg/mL); EXC, exchange figure; HPE, *Hypogymnia physodes* methanolic extract; ICB, iso-chromatid break; ICG, iso-chromatid gap; MI, mitotic index; MN, micronuclei frequency(%); NDI, nuclear division index; PLV, pulverization; SD, standard deviation; SE, spiralization error; TA(G + P), total chromosome aberration including gaps and pulverizations; TA(G − P), total chromosome aberration excluding gaps and pulverizations.

[a]Significancy of HPE doses compared with DMSO solvent control.

*$p < 0.01$;

**$p < 0.005$;

***$p < 0.001$.

microscopic evaluation. In Figure 1, HPE decreased the viability of MDA-MB-231 cells by 70% in the MTT assay, while the level of decrease was about 20% in the ATP assay. The reason for this difference may be explained by the fact that the MTT assay might overestimate the viability (Ulukaya et al., 2008).

We also tried to elucidate the mode of cell death induced by HPE. Accordingly, we measured apoptosis via M30-antigen detection, which is a marker of apoptosis, after the treatment with HPE. We found that HPE induced apoptosis in MCF-7 cells at 100 μg/mL. Indeed, we also demonstrated apoptotic effects on MCF-7 cells by the propidium iodide staining. As the nuclear morphology showed characteristic feature of apoptosis such as nuclear fragmentation, we think that HPE clearly induced apoptosis in MCF-7 cells, while it induced totally different cell death mode in MDA-MB-231 cells which is necrosis. This suggests that HPE is able to induce different cell death modes depending on the cell type.

Previously, Bačkorová et al. (2011) showed that atranorin which major metabolites of *H. physodes* had cytotoxic on used all cancer cell lines but apoptotic effects on only HL-60 human promyelocytic leukemia cancer cell line. Similarly, in our study, the HPE induced apoptosis in only the MCF-7 cells but not in MDA-MB-231 cells. The possible reasons for this outcome may be related to the genetic and phenotypic differences between the cell lines. For example, caspase activity may not be active enough in MDA-MB-231 cells to cleave the intact cytokeratin, thereby no yield of caspase-cleaved cytokeratin 18 (M30 antigen) was observed. Estrogen receptor status may also indirectly play a role in this because MCF-7 cells have no estrogen receptor, while MDA-MB-231 cells have it. However, there is dearth of information of the link between receptor status and caspase activity. Given this gap in the literature, we aim to further explore this unexplained mechanism by which HPE induces different cell death modes in different cell lines. It is notable that the death mechanism of lichen extract on cancer cells is largely under researched. To the best of our knowledge, this is the first report showing the anti-growth activity and differentially-inducing cell death (apoptosis–necrosis) effect by HPE. The type of extract may also be important. Ren et al. (2009) reported that *Lethariella zahlbruckneri* acetone extract exerted HT-29 human colon cancer cell growth inhibition by the induction of apoptosis; however, methanol extract did not induce apoptosis.

The cytogenetic characterization and classification of different types of CAs in human peripheral blood lymphocytes have an important role in human genetics. The main chromosomal damage identification proved the importance of this biomarker in the field of carcinogenesis and mutagenesis (Rossner et al., 2005). To evaluate a possible DNA damage induced by HPE in the peripheral blood, we used the CA and MN assays. As seen from Table III, this study

showed a highly cytotoxic, genotoxic, and clastogenic potential of HPE at high concentration as demonstrated by decreases on mitotic index (MI) and increases chromosome abnormalities respectively.

MN are a complete chromosome or acentric fragments that fail to attach to the mitotic spindle during mitosis and were excluded from the main nuclei as separate entities. Different mechanisms may be involved in the formation of MN, either from damage to the mitotic spindle or chromosome breakage and increase in its frequency is widely accepted measure of genotoxicity (Majer et al., 2001). Our results of MN assay indicated that HPE was slightly increased the frequency of MN at doses of 250 and 500 $\mu$g/mL as dose dependent. Interestingly, in both of genotoxicity tests, we did not find any genotoxic activity at the doses lower than 125 $\mu$g/mL. This effect may be related to the different secondary metabolites in lichen extract and its actions with different thresholds of sensitivity.

In conclusion, in this study, HPE has shown cytotoxic and genotoxic activity at relatively higher concentrations. Our findings suggest that *H. physodes* can be an interesting source as an anticancer agent, thereby being potential biomedical applications that is, for cancer chemotherapy, which may be of vital importance.

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