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Cytotoxic Evaluation of *Daphne pontica* L. Aerial Part Extracts on Three Cancerous Cell Lines by MTT Assay

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Abstract

Nowadays, cancer is the second prevalent cause of mortality after cardiovascular diseases in developed and the third one in developing countries. Adverse effects of chemotherapeutic agents bring the necessity of investigating about new medications with fewer side effects. *Daphne* L. genus is one of the natural sources with valuable reported anticancer effects. This study aimed to assess the cytotoxic effect of some extracts from the aerial parts of *Daphne pontica* collected from North of Iran on cancer cell lines.Extraction of the plant material was performed by maceration (3×72 h) of 200 g of sample with petroleum ether, chloroform, ethyl acetate, and methanol, respectively. The total extract was also obtained by maceration of the sample with 80% ethanol. Different concentrations of the dried extracts were prepared to assess their cytotoxic effect by 24 h incubation of cell lines with different extracts and then MTT (dimethyl thiazolyl diphenyl tetrazolium) assay on three cancerous cell lines (MDA-MB-231, MCF-7 and T47D), performed in triplicate. IC50 was then estimated from curves constructed by plotting cell survival (%) versus sample concentration (µg/ml). Results indicated that ethyl acetate fraction of *D. pontica* had the most potent cytotoxic effect in MTT assay with IC_{50} = 977.46 µg/ml; while other fractions were weaker in toxicity (IC_{50}>1000 µg/ml). By comparing to potent cytotoxic effects of other *Daphne* species, it seems that the cytotoxic properties of *D. pontica* is different from other species of this genus since according to this study, no significant antineoplastic properties against the three breast cancer cell lines were determined. Further studies on other pharmacological activities of this plant are recommended.

Keywords: Cytotoxic; MTT; *Daphne pontica*; Cancer cell line

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Introduction

Cancer is currently one of the most important challenges in medical sciences and despite all the advances made, no definitive treatment has been found. One of the approaches to find anticancer agents is the screening of plants to find natural compounds that have cytotoxic effects on human cancer cell lines, which has so far led to the discovery of remarkably effective therapeutic compounds in a variety of cancers including the alkaloids of *Vinca* rosea and *Taxus brevifolia*. Phytochemical studies of plants with a history of traditional or folk use for the treatment of cancer often results in the isolation of compounds with antitumor activity [1].

The genus *Daphne* belongs to the Thymelaeaceae family and has a history of therapeutic use in different countries. Various pharmacological effects have been reported from different species of this plant, such as analgesic and anti-inflammatory effects [2-4], wound healing properties [5], antimicrobial effects [6], and antioxidant effects [6,7].

Daphne pontica L., known with the local name of *Bargebooei*, is one of the *Daphne* species in Iran. The leaf infusion of *D. pontica* is used by native people in Mazandaran province to treat low back and abdominal pain, diarrhea, heartburn, and kidney infections [8]. In an in vivo study, anti-inflammatory and antinociceptive activity of ethyl acetate extract of the roots of *D. pontica* was reported [4]. Considering the cytotoxic effects of various *Daphne* species in different types of cancer cells, for example, *D. gnidium* and *D. genkwa* on lung cancer, and *D. mucronata* on breast and leukemia cell lines [9-18], the aim of this study was to investigate the cytotoxic effect of some extracts from the aerial part of *D. pontica* collected from North of Iran against T-47D، MDA-MB231، MCF-7 cancer cell lines.

Methods

Plant material

The aerial part (leave and stem) of the *Daphne pontica* was collected from the altitudes of Sari in Mazandaran province on June 2015 and a voucher specimen deposited in *Herbarium Ministrii Iranici Agriculturae* (Iran) (Code No. IRAN70100/1).

Extraction

The air-dried aerial parts of *D. pontica* (200 g) were powdered and extracted by maceration (3×72 h) with petroleum ether, chloroform, ethyl acetate and methanol, respectively. The total extract was also obtained by maceration of the plant with 80% ethanol. Removal of the solvents with a rotary evaporator resulted in the production of petroleum ether (PEE), chloroform (ChE), ethyl acetate (EAE) and methanol (MEE) extracts.

Cell lines and cell culture

Human breast cancer cell lines comprising MDA-MB231(ATCC^R HTB-26), MCF-7 (ATCC^R HTB-22) and T47D (ATCC^R HTB-133) cells were purchased from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran.

Determination of cell viability by MTT assay

The *in vitro* cytotoxic activity of all samples was assessed against three human breast cancer cell lines by dimethyl thiazolyl diphenyl tetrazolium (MTT; Merck, Germany) colorimetric test (19). Cancer cell lines were grown in RPMI-1640 medium supplemented with 10% FBS (fetal bovine serum protein) (Gibco BRL), 100 µg/ml streptomycin and 100 U/ml penicillin (Roche, Germany). All cell lines were cultured at 37° C in air/carbon dioxide (95:5) atmosphere.

Samples were dissolved in DMSO (dimethyl sulfoxide) and were further diluted with cell culture medium. The final DMSO concentration used was 1% of total volume of medium in all treatments, including the control group. For the MTT assay, 5×10^4 cells /wells were plated into 96-well plates (Nunc, Denmark) and incubated for 24 h before addition of the extracts. The plates were incubated overnight at 37°C with 5% CO_2. Then 5 µl of the media having various concentrations of the samples was added per well in triplicate. The plates were then incubated for 72 h. In each plate, there were three control wells (cells without test samples) and three blank wells (the medium with 0.1% DMSO) for cell viability. After treatment, the medium was removed and 200 µl phenol red-free medium containing MTT (1 mg/ml), was added to the wells, followed by 4 h incubation at 37°C. After incubation, the culture medium was then replaced with 100 µl of DMSO and the absorbance of each well was measured by using a microplate reader at 570 nm. The cytotoxicity value for each sample was presented as IC_{50} (the median growth inhibitory concentration) of the reagents compared with the control. IC_{50} values were calculated by Sigmaplot 9.0 software [20].

Results

The *in vitro* cytotoxic activity of total extract (TE) and other extracts including PEE, ChE, EAE, and MEE were assessed against three human breast cancer cell lines including MCF-7, T47D, and MDA-MB-231. The IC_{50} for the samples are represented in Table 1-3.

Table1. *In vitro* effect of *Daphne pontica* different extracts on viability (Mean ± SD) of MDA-MB 231 cell line.

Samples[a]	(Concentration (µg/ml)				IC_{50} (µg/ml)
	1000	100	50	10	
PEE	107.19±11.19	82.19±7.75	86.23±10.81	71.38±11.12	>1000
ChE	59.69±12.03	75.59±10.00	78.52±3.01	77.02±6.28	>1000
EAE	48.85±7.43	87.64±14.14	104.38±11.63	89.45±9.38	977.46
MEE	111.21±4.68	88.51±15.05	77.24±5.32	85.14±4.51	>1000
TE	104.70±12.36	102.89±4.80	90.14±10.27	92.50±4.70	>1000
CON+DMSO	94.18±5.71				
DMSO	3.83±0.16				

[a] Samples; PEE Petroleum ether extract, ChE: Chloroform extract, EAE: Ethyl acetate extract, MEE: Methanol extract, TE: Total extract, CON+DMSO: Control+DMSO

Table2. *In vitro* effect of *Daphne pontica* different extracts on viability (Mean±SD) of T47D cell line.

Samples[a]	(Concentration (µg/ml)				IC_{50} (µg/ml)
	1000	100	50	10	
PEE	54.06±4.35	79.85±6.87	90.00±8.54	68.13±7.18	>1000
ChE	63.07±1.86	115.60±4.97	96.92±13.96	92.63±3.88	>1000
EAE	59.19±2.21	120.54±12.27	97.50±14.27	93.62±11.18	>1000
MEE	94.26±8.59	107.03±12.74	73.73±9.16	73.84±10.56	>1000
TE	84.61±3.61	93.69±5.82	81.90±12.72	68.02±10.41	>1000
CON+DMSO	91.83±7.06				
DMSO	9.15±0.41				

[a] Samples; PEE Petroleum ether extract, ChE: Chloroform extract, EAE: Ethyl acetate extract, MEE: Methanol extract, TE: Total extract, CON+DMSO: Control+DMSO

Table3. In *vitro* effect of *Daphne pontica* different extracts on viability (Mean±SD) of MCF-7 cell line.

Samples[a]	(Concentration (µg/ml)				IC50 (µg/ml)
	1000	100	50	10	
PEE	70.41±6.17	113.88±4.49	107.19±6.74	94.37±6.06	>1000
ChE	81.85±1.53	129.67±23.22	112.28±3.04	95.94±12.10	>1000
EAE	77.58±0.12	171.23±18.60	153.72±22.07	85.39±15.01	>1000
MEE	120.32±18.47	135.69±7.12	101.57±4.63	96.91±3.33	>1000
TE	183.18±19.59	125.95±14.88	110.43±9.63	78.76±5.13	>1000
CON+DMSO	98.76±8.34				
DMSO	9.18±0.76				

[a] Samples; PEE Petroleum ether extract, ChE: Chloroform extract, EAE: Ethyl acetate extract, MEE: Methanol extract, TE: Total extract, CON+DMSO: Control+DMSO

Discussion

According to the results, among all samples, EAE showed the highest cytotoxic effect on MDA-MB231 cell line with IC_{50} value 977.46 µg/ml and the remaining samples did not show significant cytotoxicity (IC_{50} > 1000 µg /ml). A review of previous studies showed that the compounds in ethyl acetate, hydroalcoholic, and chloroform extracts of other *Daphne* species had significant cytotoxic effects. Ethyl acetate extract of *D. gnidium* inhibited the growth of lung cancer cell lines (A549), liver cancer cell line (SMMC-7721) and breast cancer cell line (MCF-7) by inducing apopto-

sis after 48 hours [18]. Also, daphnane diterpenoids from *D. genkwa* have been shown to inhibit proliferation of the A549 lung cancer cell line [9].

In other studies, diterpenoids with cytotoxic effects were isolated from *D. genkwa*, *D. mucronata, D. mezereum* and *D. linearifolia*, such as yuanhuahine, yuanhualine, yuanhuacine, yuanhuadine, yuanhuagine [9], gnidilatimonoein [12,13], mezerein [11], and bioflavonoids including 4-methylgenkwanol A, and 2-hydroxygenkwanol A [21].

Several compounds with diterpene ester and secolignans structure which had a moderate cytotoxic effect were isolated from the ethyl acetate fraction of *D. acutiloba* and *D. feddei* [10, 22]. The results of this study, which was performed on three breast cancer cell lines including T47D cell line which was evidently susceptible to progesterone [23], an estrogen-positive (MCF-7) and an estrogen-negative (MDA-MB-231) human breast cancer cell line [24], showed that the ethyl acetate extract had a poor effect on the MDA-MB-231.

Conclusion

Based on the results, the most potent effect of *D. pontica* ethyl acetate extract on MDA-MB231 cancer cell line was found, and the rest of the extracts did not show cytotoxicity on breast cancer cell lines. Future studies on pure compounds of the ethyl acetate extract as well as other cancer cell lines can be done to elucidate further biological effects of this plant.

Conflicts of Interest

The authors confirm that this article content has no conflicts of interest.

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