



Investigation of Total Phenolic Content of *Tilia dasystyla* and *Polygonatum orientale* Desf Extracts and Their Cytotoxic Effect on the Osteogenic Sarcoma (Saos-2) Cancer Cell Line

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Abstract

Background: Osteosarcoma; is one of the most common malignant tumors. Nowadays, because of the many side effects of cancer drugs, the usage of herbal medicine which can inhibit or eliminate cancer cells by their antioxidant compounds is increased.

Objectives: In the present study anticancer effect of *Tilia dasystyla* and *Polygonatum orientale* Desf different extracts on osteogenic sarcoma (Saos-2) cancer cell line was investigated and their polyphenolic compounds were identified by liquid chromatography-mass spectrometry (LC-MS) analysis. The cytotoxic effect of these extracts on Saos-2 cell line and identification of their phenolic compounds have not been reported so far.

Methods: Cancer cell lines were provided from Department of Biological Sciences, Bursa University, Turkey. Different concentrations of the methanol, ethanol, and diluted water extracts (0.5 - 5 mg/mL) were tested on Saos-2 cell line. After 24, 48, and 72 hours, the cell viability was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. For the investigation of total phenolic compounds of *T. dasystyla* and *P. orientale* Desf extracts LC-MS method was applied.

Results: According to the results diluted water extracts on the Saos-2 cancer cell line showed more cytotoxic effect than other solvents. The lowest IC₅₀ value was 0.58 ± 0.01 mg/mL within 72 hours belonged to *T. dasystyla* water extract.

Conclusions: *Tilia dasystyla* and *Polygonatum orientale* Desf extracts contain some polyphenolic compounds which showed cytotoxic effect on Saos-2 cancer cell line. The full potential of these herbal extracts is yet to be realized by further studies on animal models and subsequent trials.

Keywords: Anti-Cancer, LC-MS, *Polygonatum orientale* Desf, Saos-2, *Tilia dasystyla*

1. Background

Skeleton malignant tumor, defined as osteosarcoma, can metastasize to the other parts of the body, particularly lungs. Osteosarcoma, one of the most common malignant tumors, is more common in young age; this cancer is one of the most common types of cancer that occurs in mesenchymal bone marrow cells and affects the musculoskeletal system (1, 2). Prescription drugs suppress the immune system and have many side effects, for this reason, antioxidant compounds obtained from herbal extracts are preferred to prevent cancer or to support the treatment (3, 4).

Herbal medicine contains several antioxidant compounds, which neutralize free radicals and prevent lipid peroxidation. Plants are a rich source of antioxidant compounds that can protect cells from oxidative damages. Plants contain secondary metabolites, such as phenol and flavonoids, these compounds have antioxidant properties with strong potential for the clearance of free radicals in order to prevent diseases such as cancer (5, 6). *Tilia* is a genus in the Tiliaceae family with about 30 species of trees. These trees are found locally in Northern Hemisphere areas and known as a safe product as herbal medicine for symptoms of cold and anxiety. *Tilia* is used

as anti-inflammatory, anti-diarrhea, anti-spasmodic, anti-hair loss, anti-anxiety, and sedative medications since it contains the wide range of bioactive compounds. *Tilia* bioactive compounds include hydrocarbons, esters, terpenes, quercetin, kaempferol, tannins and scopoletin, and other polyphenolic compounds (7, 8).

P. orientale Desf belongs to Asparagaceae family which grows in North of Iran. The rhizome of *Polygonatum* is used as an herbal medicine in traditional medicine. The anti-depressant, anti-inflammatory, anticancer, anti-fever, antioxidant, and antimicrobial activities of the *Polygonatum* rhizome have been shown in many studies (9, 10). Variety of compounds such as polysaccharides, saponins, phytohormones, glycosides, flavonoids, and alkaloids have been isolated from *Polygonatum* rhizome. Polysaccharides of *Polygonatum* have the most variety among the other compounds in these plants that have shown anti-cancer properties. Pharmacological studies on several species including *P. odoratum*, *P. verticillatum*, *P. cyrtonema*, and *P. kingianum* have been reported (11, 12).

2. Objectives

The purpose of this study was to investigate the cytotoxic effect of *P. orientale* Desf and *T. dasystyla* methanol, ethanol, and diluted water extracts on osteogenic sarcoma (Saos-2) cancer cell lines and identification of their phenolic compound by liquid chromatography-mass spectrometry (LC-MS) analysis. The LC-MS method is used to accurately identify phenolic compounds.

The cytotoxic effect of *T. dasystyla* and *P. orientale* Desf extracts on the Saos-2 cell line and identification of their phenolic compounds by the LC-MS method has not been reported until now.

3. Methods

3.1. Material

Methanol (99.9%), ethanol (99.9%), acetonitrile, and other LC-MS grade solvents were from Merck.

Dulbecco's Modified Eagle's medium/nutrient mixture F-12 (DMEM F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and 1% Pen-Strep were from Gibco® and cell proliferation kit I were from Roche life science.

3.2. Preparation of Plant Extracts

P. orientale Desf and *T. dasystyla* species were purchased from Bagh Firuze, Tehran, Iran and were approved at Alzahra University Herbarium. In this study, the leaf of *T.*

dasystyla and rhizome of *P. orientale* Desf were used for investigation.

Amount of 1 g of the dried plant was added in 100 ml of 80% aqueous methanol, 80% aqueous ethanol, and diluted water solvents. The solvents were boiled in water bath for 60 min at temperature of 70°C. Extracts were centrifuged for 20 min at 2000 rpm, supernatant was used in lyophilization process. Methanol, ethanol, and water solvents were completely removed by lyophilization process and powder of plants was dissolved in PBS to determine the cytotoxic effect of the extracts on the cancer cell line. For sterilization of solution, 0.22 mm filters were used and then solution was diluted with DMEM F12 culture medium to concentrations of 0.5 - 5 mg/mL (13).

3.3. Cell Culture

Osteogenic sarcoma (Saos-2) cell line was provided by Bursa University of Turkey. Cells were grown in DMEM-high glucose containing 10% FBS and 1% Pen-Strep incubated in 5% CO₂ humidified atmosphere and 37°C incubator (13).

3.4. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

To determine the inhibition effect of extracts on the proliferation of cells, cell viability was detected by measuring MTT absorbance of viable cells. Eight-thousand cells for each well were seeded into 96-well plates for 24 h, after 24 h cells were treated with various concentrations of extracts, and were incubated for 24 h, 48 h, and 72 h in incubator. Subsequently, 10 mL of MTT (5 mg/mL) was added to each well and then cells were incubated for 4 hours. Then 100 mL of solubilization buffer was added to the wells and plates were kept in incubator for overnight, and then absorbance was measured at a wavelength of 570 nm using a microplate reader (Cytation™ Biotek, USA).

The percentage of cell viability was calculated using the formula:

$$(\text{Mean OD treated well}) \div (\text{mean OD control well}) \times 100 = \%$$

IC₅₀ was calculated by drawing curve using different concentrations of extract and cell viability percentage (14).

3.5. LC-ESI/MS Analysis

3.5.1. Extraction of Phenolic Compounds

The extracts were prepared with methanol solvent. Amount of 0.5 g of the dry sample added to 10 mL of 80% aqueous methanol solvent. The mixtures were heated for 60 min in water bath at 70°C. Then, the mixtures were centrifuged at 2000 rpm for 20 min at 25°C. The supernatants

were filtered through a 22 μ m filter then was used for the identification of total phenolic contents by LC-ESI/MS analysis.

Phenolic compounds in the 80% aqueous methanol extracts of *T. dasystyla* and *P. orientale* Desf were analyzed by the LCMS-8040 system (Shimadzu, Kyoto, Japan). The mobile phase consisted of 100% methanol (solvent A) and acetonitrile (solvent B) (1:1 v/v). The mobile phase flow rate was 0.3 mL/min. The column temperature was fixed at 40°C. Phenolic compounds were detected by a full scan mode ranged m/z 100 - 1000 amu. MS detection was applied using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. Data were acquired by Lab Solutions software (15).

3.6 Statistical Analysis

Statistical analysis was performed using the ANOVA test by SPSS version 24 program and the P Value < 0.05 was considered significant. Data were expressed as means $\pm$ standard deviation.

4. Results

Cytotoxic effect of the various concentrations of *T. dasystyla* and *P. orientale* Desf extracts on the Saos-2 cell line are given in Figures 1 and 2. Cytotoxic effect of three different solvent extracts (water, methanol, and ethanol) from 0.5 mg/mL to 5 mg/mL concentrations on Saos-2 cell line was tested by MTT assay. Cell viability was determined after treatment within different intervals of 24, 48, and 72 hours. Extracts reduced the cell proliferation in a dose and time-dependent manner. Among different solvents, water showed higher cytotoxicity to cancer cells. The most cytotoxic effect of water extract was at the concentration of 5 mg/mL at 72 h.

The maximum cell growth inhibition of the Saos-2 cell line was at 72 hours and at a concentration 5 mg/mL of *T. dasystyla* and *P. orientale* Desf water extracts with 14% and 29% of cell viability, respectively. According to Figure 3, the lowest values of IC₅₀ was 0.58 ± 0.01 mg/mL belonged to *T. dasystyla* water extract. The lowest IC₅₀ value of *P. orientale* Desf was 2.9 ± 0.01 mg/mL belonged to water extract. *T. dasystyla* showed more cytotoxic effect than *P. orientale* Desf and the water solvent was the most efficient in extracting antioxidants among the other solvents. The most effective solvents with anticancer properties were water, methanol, and ethanol extracts, respectively. Data statistical analysis (P value < 0.05) of Figures 1 and 2 indicated that

Table 1. Phenolic Compounds of *P. orientale* Desf and *T. dasystyla* identified by LC-ESI/MS Analysis

<i>T. dasystyla</i> Compound	<i>P. orientale</i> Desf Compound
5,6-dihydroxy-30	Quercetin-7-O-galloyl-glucoside
40,7-trimethoxyflavone	Corilagin derivative
Quercetin-7-O-galloyl-glucoside	Naringenin-40-methoxy-7-pyranoside
Corilagin derivative	Gallagic acid dilactone
Quercetin-7-b-O-diglucoside	Methyl-(S)-flavogallionate
Naringenin-40-methoxy-7-pyranoside	Oleanane type triterpenoid
Gallagic acid dilactone	Unknown Ellagitannin
Methyl-(S)-flavogallionate	Malic acid
trans-resveratrol-3-O-b-galloyl-glucoside	Chlorogenic acid
cis-resveratrol-3-O-b-galloyl-glucoside	Caffeic acid
Oleanane-type triterpenoid	Ferulic acid hexoside
Malic acid	Caffeoylquinic acid derivative
Aconitic acid	Caffeic acid derivative
Chlorogenic acid	Myricetin rhamnoside
Protocatechuic acid	Naringenin pentose
Hyperoside	Sinapic acid hexose
dicafeoylquinic acid	Prunetin
caffeoylquinic acid derivative	
Caffeic acid derivative	
Procyanidin dimer (type B1 or B2)	
(epi)catechin-(epi)gallocatechin	
Procyanidin trimer	
Catechin, Myricetin rhamnoside	
Sinapic acid hexose	

there was a significant difference between all solvents in *T. dasystyla* extracts and there was a significant difference between ethanol solvent with water and methanol solvents in *P. orientale* Desf extracts.

Table 1 shows the phenolic compounds identified by LC-ESI-MS. The most abundant phenolic compounds in *P. orientale* Desf were chlorogenic acid, naringenin, and prunetin, respectively and the most abundant phenolic compounds in *T. dasystyla* were dicafeoylquinic acid, procyanidin dimer (type B1 or B2), and chlorogenic acid. Simple and gradient elution-based ESI-LC/MS method was applied for the analysis of the phenolic compounds.

A solvent system consisting of methanol and acetonitrile was successful in the separation of phenolic compounds in the plant extracts with good resolution.

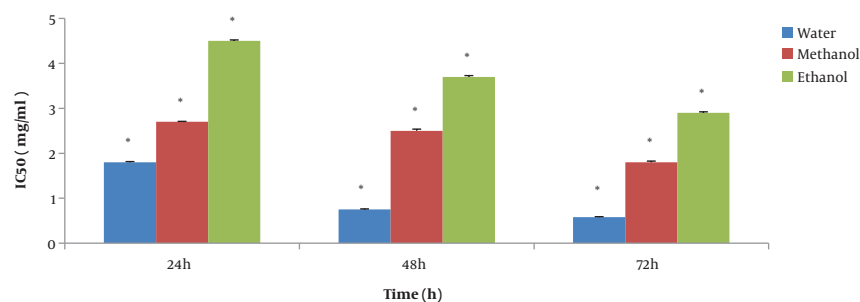


Figure 1. IC₅₀ values of different *T. dasystyla* extracts on the Saos-2 cancer cell for 24, 48, and 72 h. Experiments are performed in triplicate and express as the mean standard deviation. Values in each column marked with * show significant differences between solvent (water, methanol, and ethanol) in each time ($P < 0.05$).

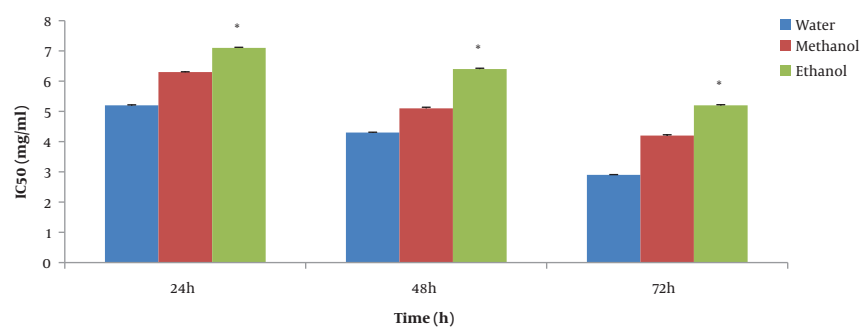


Figure 2. IC₅₀ values of the different *P. orientale* Desf extracts (water, methanol, and ethanol) on the Saos-2 cancer cell for 24, 48, and 72 h. Experiments are performed in triplicate and express as the mean standard deviation. Values in each column marked with * show significant differences between solvent (water, methanol, and ethanol) in each time ($P < 0.05$).

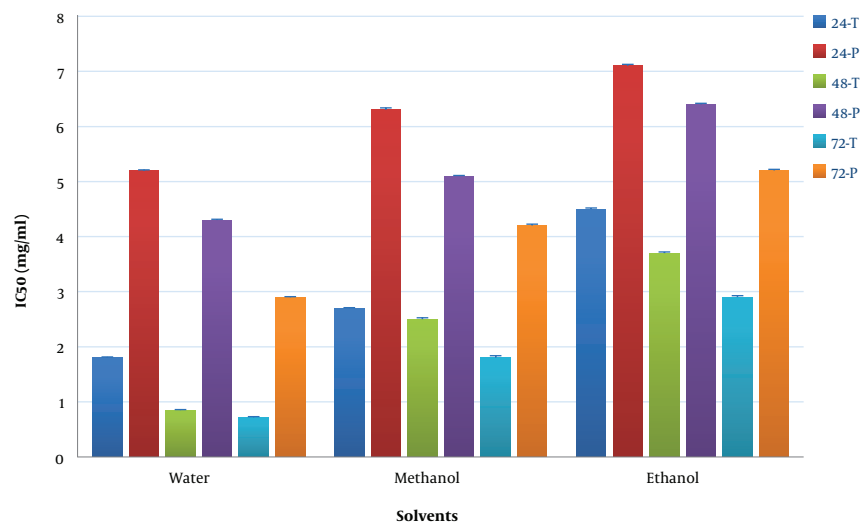


Figure 3. Comparison of the IC₅₀ values of the *T. dasystyla* (T) and *P. orientale* Desf (P) different extracts (water, methanol, and ethanol) on the Saos-2 cancer cell for 24, 48, and 72 h. Experiments are performed in triplicate and express as the mean standard deviation.

Results of identification and fragments are shown in Figures 4 and 5.

5. Discussion

Cytotoxic effect of various concentrations of *T. dasystyla* and *P. orientale* Desf extracts using three different solvents

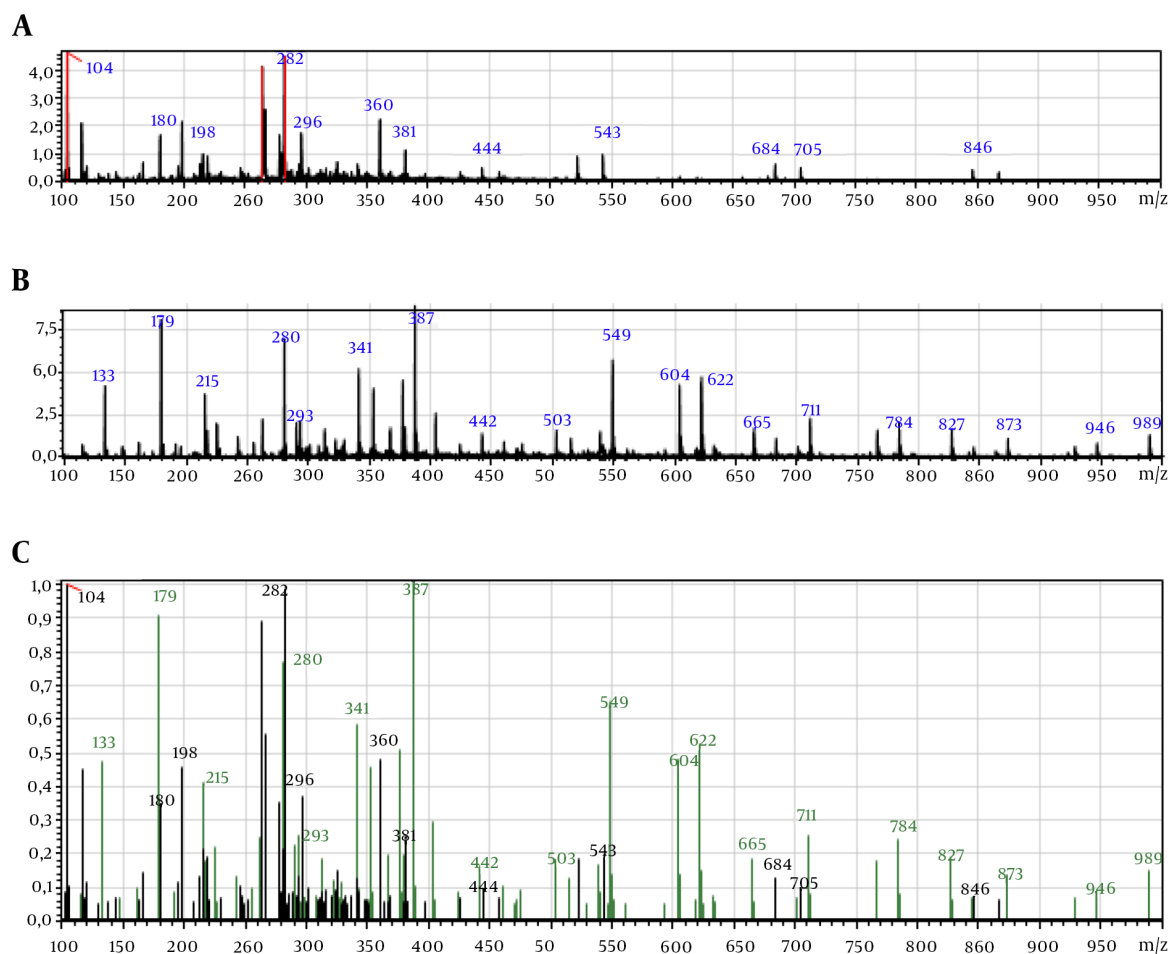


Figure 4. LC-ESI/MS analysis ion mass spectra of *P. orientale* Desf. A, positive ionization; B, negative ionization; C, total spectrum.

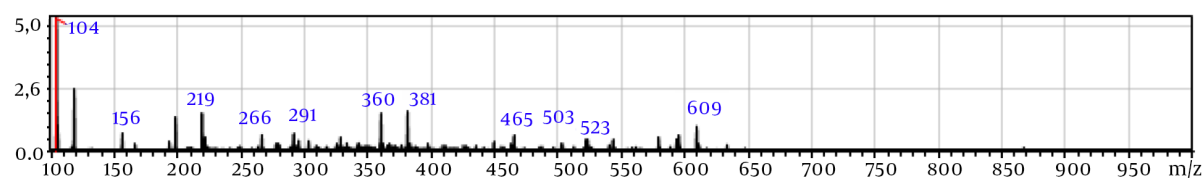
including water, methanol, and ethanol. According to the data statistical analysis (P value < 0.05) of data in Figures 1 and 2, water was the most efficient solvent in the extracting antioxidants, since water extract showed more cytotoxic effect on Saos-2 cancer cell line, after water, methanol was more efficient solvent among others. Also extracts showed time-dose depended manner in cytotoxic effect on Saos-2 cells. According to the data of Figure 3, the maximum inhibition of cancer cells growth was at 72 hours and at a concentration of 5 mg/mL of *T. dasystyla* water extract with the percentage of 86% cell growth inhibition.

Secondary metabolites in plants, such as phenols and flavonoids, are antioxidant compounds which have strong potential for cancer treatment and prevention (5). The yield of extraction depends on the solvents with varieties of polarity. The extraction yield increases with increasing

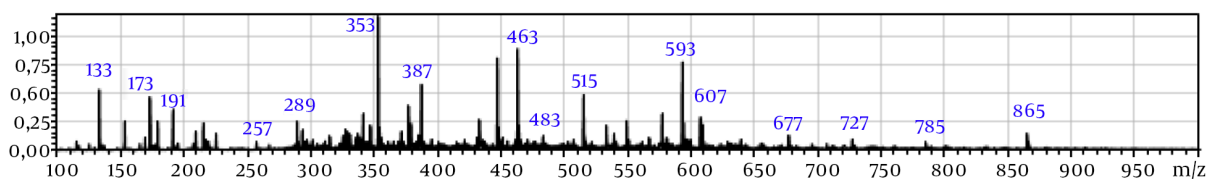
polarity of the solvent used in extraction. Therefore, the most effective solvents in the extraction of phenolic compounds were water, methanol and ethanol, respectively (16). The temperature of the extraction method is also important. The amount of phenolic compounds start to increase at 60°C. At this temperature, heavy polyphenols decompose to a variety of phenolic compounds with lower molecular weight. Also, the solubility of phenolic compounds increases in this temperature. So in this study, the water bath method was used due to the applied temperature within the process of extraction (17).

Phenolic compounds identification of *T. dasystyla* and *P. orientale* Desf were performed by LC-MS analysis for the first time in this study. But phenolic compounds of other species of *Tilia* and *Polygonatum* have been identified in other studies.

A



B



C

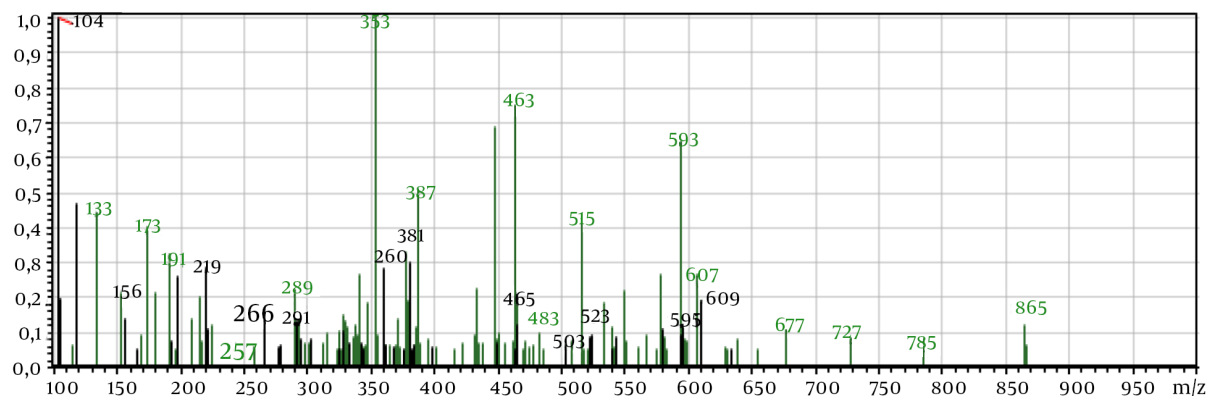


Figure 5. LC-ESI/MS analysis ion mass spectra of *T. dasystyla*. A, Positive ionization; B, negative ionization; C, total spectrum.

Catechin, naringenin, quercetin, protocatechuic, p-coumaric, chlorogenic, ferulic, and caffeic acids were determined in all *Tilia argentea* extracts and protocatechuic, ferulic, coumaric, gallic acids, naringenin and chlorogenic acid were identified in *Polygonum bistorta* extracts (15).

The cytotoxic effects of *T. dasystyla* and *P. orientale* Desf extracts on cancer cell lines have not been reported so far. However, some studies have reported the anticancer properties of other *Tilia* and *Polygonatum* species.

The structure of specific homoisoflavone has been identified from *Polygonatum odoratum* root which induces Bcl-2 phosphorylation and causes apoptosis in breast cancer cells (18). *Polygonatum cyrtoneura* lectin (PCL) also showed anti-proliferative and apoptosis-inducing activi-

ties. This component showed cytotoxic effect on murine fibrosarcoma L929 and melanoma A375 cancer cells by interrupting of a mitochondria-mediated ROS-p38-p53 pathway and this caused the growth inhibition of cancer cell lines (19, 20). *P. odoratum* extracts showed growth inhibition properties on human breast cancer MDA-MB-231 cells and induced apoptosis (9).

The extracts of *Tilia cordata* Mill flowers showed anti-proliferative activities on BW 5147 lymphoma tumor cells and *T. americana* var. *mexicana* had antitumor activities on K-562 leukemia cell line (21, 22). Extract from trunk of *Tilia amurensis* Rupr. (Tiliaceae) had cytotoxic effect on A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines and the *Tilia* × *viridis* extracts showed anti-proliferative properties on murine lymphocytes cancer cells (23, 24).

5.1. Conclusions

Based on the results, the extracts of *T. dasystyla* and *P. orientale* Desf contained polyphenolic compounds which never been isolated before from these species. Cytotoxic effect of extracts on Saos-2 cancer cell line may be due to the presence of antioxidant compounds such as phenol and flavonoids. In addition, the results showed water solvent is more efficient in extracting of antioxidants. *T. dasystyla* contained more polyphenolic compounds than *P. orientale* Desf and this could be one of the reasons for the anti-cancer properties of *T. dasystyla*. This study requires more detailed investigation in future studies. Isolation and purification of the polyphenolic compounds in these plants and their anti-cancer effect on different cell lines and finally how to use them in the pharmaceutical and health industries in future studies can be investigated.

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Not declared.

Footnotes

Authors' Contribution: Study concept and design: Ertuğrul Kaya, Parichehr Hanachi, and Roshanak Zarringhalami. Acquisition of data: Roshanak Zarringhalami, Aydan Fulden Ağan, Mert Donmez, and Kağan Ağan. Analysis and interpretation of data: Roshanak Zarringhalami, Aydan Fulden Ağan, Mert Donmez, and Kağan Ağan. Drafting of the manuscript: Roshanak Zarringhalami and Parichehr Hanachi. Critical revision of the manuscript for important intellectual content: Ertuğrul Kaya, Parichehr Hanachi, and Roshanak Zarringhalami. Statistical analysis: Roshanak Zarringhalami, Mert Donmez. Administrative, technical, and material support: Ertuğrul Kaya, Parichehr Hanachi, and Kağan Ağan. Study supervision: Ertuğrul Kaya and Parichehr Hanachi,

Conflict of Interests: The authors declare no conflict of interests.

Ethical Approval: The experiment was performed under the approval of Motamed Cancer Institute-Academic Center for Education, Culture and Research, Tehran, Iran (approval ID: IR.ACECR.REC.1398.006).

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