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RESEARCH ARTICLE

Total Phenolic Content, Antioxidant and Cyto-/Genotoxic Activities of *Pelargonium quercetorum* Agnew in Human Breast Cancer Cells

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ABSTRACT

Objective: Because the current cancer treatment success rate is not sufficient, plants have been gaining importance as a possible source for anti-cancer compounds. This study aimed to investigate both the genotoxic and cytotoxic activities of methanol extracts of *Pelargonium quercetorum* Agnew (*P. quercetorum*), which is traditionally used for its antihelminthic activity, but has not yet been studied for its effect in breast cancer cells.
Methods: In this study, the cyto-genotoxic activities of methanol extracts of *Pelargonium quercetorum* were investigated in human breast cancer cells (MCF-7 and MDA-MB-231). The cytotoxic effect of the extract on these cells was evaluated by MTT and ATP viability assays. The mode of cell death (apoptosis/necrosis) was determined using fluorescence microscopy and biochemical methods. Genotoxic activity was studied with Comet assay. In addition, the total phenolic content and antioxidant capacity were determined by the Folin-Ciocalteu and ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] methods, respectively.
Results: Results of this study reveal that *Pelargonium quercetorum* has a large total phenolic content and high antioxidant capacity. *Pelargonium quercetorum* induced anti-growth effects in a dose-dependent manner in cancer cells. The extract killed the cells by apoptosis as evidenced by the presence of pyknotic nucleus and annexin V-FITC positivity. The extract also exerted genotoxic activity at relatively low doses.
Conclusion: These results suggest that *Pelargonium quercetorum* induces apoptosis-like cell death by causing DNA damage in breast cancer cells.
Key words: *Pelargonium quercetorum*, apoptosis, breast cancer cells, DNA damage.

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INTRODUCTION

Due to their therapeutic properties, medicinal plants have been used for many years as remedies for human diseases. Extracts of the Pelargonium (P.) species (Geraniaceae) are reported to have antimicrobial, antifungal, anticancer, antihelminthic, and antiplasmodial activities [1,2]. In addition, the essential oils of the P. species are used in the perfumery, cosmetics, and flavor industries [3]. Further, the root extracts of *P. sidoides* and *P. reniforme* have been used to treat respiratory infections such as sinusitis, tonsillitis, sore throat, bronchitis, and the common cold [4]. The root extract of *P. sidoides* has also been used to treat cold and flu, and is commonly known as "UMCA" (Abdi Ibrahim) in Turkey [5]. The root extract of *P. sidoides* is also known to activate innate immune defense in human monocytes [6]. Although a considerable amount of research has been performed on the biological activities of the Pelargonium species, knowledge on its anticancer activity is limited. Data on this subject suggest that fractions of *P. sidoides* have anti-growth effects on the Jurkat E6.1 human leukemic T cell lymphoblast cell line [7], while another study reported that *P. zonale* extract had minimal cytotoxicity against the HeLa cell line [2].

Pelargonium quercetorum Agnew (*P. quercetorum*) refers a group of plants in the subfamily Geraniaceae, and are known as "Tolk" in Gecitli, Hakkari, Turkey. Kaval et al. [8] reported that *P. quercetorum* ethnobotanical was used to treat intestinal worms in people from Gecitli. Although there are many traditional uses for *P. quercetorum*, there is no information concerning its cytotoxic and genotoxic activity in breast cancer cells.

In the present study, we aimed to investigate the cytotoxic (apoptosis-inducing) and genotoxic activity of *P. quercetorum* extracts collected from Turkey on MCF-7 and MDA-MB-231 human breast cancer cell lines. Our results indicate that the extract does have cytotoxic and genotoxic activity on both breast cancer cell lines.

METHODS

Collection and identification of *P. quercetorum*

P. quercetorum was collected from the Zap valley at Sumbul Mountain in the Hakkari province (located in the C10 square according to Turkey's grid square system) in May 2006, and was identified using the standard text, 'Flora of Turkey and The East Aegean lslands' [9]. A voucher specimen (number MF.10111) was deposited in the Herbarium.

Extraction of *P. quercetorum*

The sample was air-dried at room temperature, cleaned of extraneous materials, and ground into powder. Of the material (trunk and flower parts), 15 g was extracted by adding 150 ml of methanol (Merck) to the ground plant in a Soxhlet apparatus for 24 h. Then, the crude extract was concentrated in a rotary evaporator at 40°C. The residues were lyophilized and stored at -80 °C until further use. The content of the *P. quercetorum* extract was determined by GCXGC-TOF/MS at York University, England, and published by Aztopal et al. [10].

Determination of total phenolic content and antioxidant capacity

The Folin-Ciocalteu assay was used to quantify the total phenolic content of the *P. quercetorum* lyophilized extract [11]. The following reagents were prepared for the assay: Lowry A (2% aqueous Na_2CO_3 in 0.1 M NaOH), Lowry B (0.5% aqueous $CuSO_4$ in 1% $NaKC_4H_4O_6$ solution), and Lowry C (prepared fresh as a mixture of 50 mL Lowry A and 1 ml Lowry B). The Folin-Ciocalteu reagent was diluted with water at a volume ratio of 1:3 prior to use. The extract (0.1 ml), water (1.9 mL), and Lowry C solution (2.5 mL) were mixed, and was left to stand for 10 min at room temperature. Then, 0.25 ml of Folin reagent was added and left to sit at room temperature for 30 min (for stabilization of the blue color). The assay was done in duplicate. The absorbance was read at 750 nm with a spectrophotometer (Varian Cary 50, Australia). Results were expressed as milligrams of gallic acid equivalent (GAE) per gram of lyophilized extract.

The total antioxidant capacity of *P. quercetorum* lyophilized extract was determined with the ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] method, as described by Sahin et al. [12]. The working solution was prepared by mixing 20 mM ABTS and 2.45 mM potassium persulfate solution, which were allowed to react for 12–16 h at room temperature in the dark before use. Then, 0.10 ml extract, 3.90 ml of ethanol, and 1 ml of the ABTS+ radical cation solution, which was diluted with ethanol at a ratio of 1:10, were mixed, and the absorbance at 734 nm was recorded against a blank after 6 min. The results were expressed as milligrams Trolox equivalent (TE) per gram of lyophilized extract.

Determination of cytotoxicity

Cell culture and chemicals

The breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured in RPMI 1640 medium supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 5% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. *P. quercetorum* lyophilized extract was dissolved in DMSO at a concentration of 100 mg/ml as a stock solution, aliquoted, and stored at -80 °C. Further dilutions were made in culture medium.

The MTT Viability Assay: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cell viability assay was performed as previously described [13]. Cells were seeded at a density of 5x103 cells per well in a 96-well plate in 200 µl medium. The cells were treated with or without *P. quercetorum* (3.125-100 µg/ml) for 48 h; cells not treated with the extract received only medium and vehicle (0.1% DMSO final). Each experiment was carried out twice in duplicate. At the end of the 48 h treatment period, the absorbance (Abs) was read by an enzyme-linked immunosorbent assay (ELISA) reader (FLASHScan S12, Jena, Germany) at 570 nm. The viability of treated cells was calculated in reference to the untreated control cells using the following formula: (%) = [100 x (Sample Abs)/(Control Abs)].

The ATP viability assay

The ATP (Adenosine Tri Phosphate) assay, a highly sensitive luciferin: luciferase-based assay, was performed to determine the level of cellular ATP as an indirect marker for the number of living cells [14]. The cells were seeded, grown, and treated as in the MTT assay (see above). At the end of the treatment period (48 h), cell viability was determined by the ATP assay (ATP Bioluminescent Somatic Cell Assay Kit, Sigma, St. Louis, MO, USA) according to the manufacturer's standard protocol.

Determination of apoptosis

Fluorescence imaging for the determination of mode of cell death

To determine the mode of cell death, MCF-7 and MDA-MB-231 cells were seeded at a density of 1×10^4 cells. After 24 h, cells were treated with *P. quercetorum* (100 µg/ml) and incubated for 48 h. Following incubation, apoptosis was determined on the basis of both the nuclear morphology and the cell membrane integrity as visualized via fluorescence microscopy. The nuclear and membrane morphologies of living (not fixed) cells were examined after staining with Hoechst 33342 and Propidium Iodide (PI), respectively. Hoechst dye stains both the living and dead (primary necrotic or secondary necrotic) cells. However, PI can only pass through damaged cell membranes, and thus, stains only secondary necrotic cells. A cell was defined as having secondary necrosis if it is pyknotic or has fragmented nuclei with a damaged cell membrane.

Detection of caspase-cleaved cytokeratin 18 (M30): Apoptosis was determined by measuring the level of caspase-cleaved keratin 18 (ccK18, M30). For this assay, $1x10^4$ cells were seeded per well of a 96-well plate in 200 µl culture medium in duplicate. Cells were treated 48 h with *P. quercetorum* at 100 µg/ml, and Paclitaxel (3.21 µM) was used as a positive control for apoptosis. M30 levels were determined with an ELISA reader at 450 nm.

Annexin-V-FITC staining for the determination of apoptosis/necrosis: Annexin-V and its fluorescent conjugates (e.g., Annexin-V-FITC) can specifically bind to exposed phosphatidylserine, which is a feature of apoptosis. The seeding and treatment conditions for the Annexin-V-FITC staining were similar to those used for the fluorescence staining (see above). An Annexin-V-FLUOS staining kit was purchased from Roche (Mannheim, Germany), and was used to stain and visualize the cells following incubation (as above).

Determination of genotoxicity by comet assay

The alkaline version of the comet assay was used [15] with modifications. Briefly, breast cancer cells ($5x10^5$ cells/well) were seeded in 6-well plates. The cells were incubated with 12.5 µg/ml or 25 µg/ml *P. quercetorum* extract, or with 3.8 µg/ml cisplatin as a positive control for 24 h. Following incubation, the cells were trypsinized and suspended in 60 µl PBS (phosphate buffered saline). The cell suspension (containing $3x10^4$ cells) was mixed with 1% low melting point agarose and rapidly laid on slides previously coated with 1% normal melting point agarose. The cells were then lysed and neutralized, and then they were stained with ethidium bromide (2 µg/ml) and visualized under a fluorescence microscope. The images were analyzed with specialized software for COMET analysis (Kameram 21, Argenit, İstanbul, Turkey).

The following parameters were evaluated for each cell: comet square, comet length, comet density, tail length, tail DNA percentage, tail moment length, olive moment length, head DNA%, genetic damage index (GDI), and percent of damage per cell. GDI was calculated according to the following formula used in the COMET analysis program [16]:

$$GDI=(1x\Sigma Type1)+(2x\Sigma Type2)+(3x\Sigma Type3)+(4x\Sigma Type4)/(\Sigma Type0+\Sigma Type1+\Sigma Type2+\Sigma Type3+\Sigma Type4)$$

$$\%\ \text{of damage per cell} = \Sigma Type2+\Sigma Type3+\Sigma Type4$$

(ΣType 0: Almost no cells have damage; ΣType 1: Very few cells have damage; ΣType 2: Approximately half of the cells are damaged; ΣType 3: More than half of the cells are damaged; ΣType 4: Almost all of the cells are damaged).

Statistical analyses

All statistical analyses were performed using the SPSS 20.0 (SPSS Inc.; Chicago, IL, USA) statistical software package for Windows. Significance was calculated using a one-way analysis of variance (ANOVA). Values of $p<0.05$ were considered significant. Results are expressed as mean ±SD or SE (Standard Deviation or Standard Error).

RESULTS

Total phenolic content and antioxidant capacity of *P. quercetorum*

The total phenolic content of *P. quercetorum* was determined spectrophotometrically according to the Folin-Ciocalteu method, and was reported as gallic acid equivalents (GAE). For samples dissolved in methanol, the total phenolic content for the lyophilized extract of *P. quercetorum* was 576.6 ± 15.8 mg GAE/g, and was 460.6 ± 8.3 mg GAE/g when dissolved in water. Total phenolic content values were compared with those of tannic acid, which is used as a standard for total phenolic content. The potential antioxidant capacity of the extract was determined by the ABTS method and reported as milligrams TE/g lyophilized extract. Antioxidant capacity for the lyophilized extract of *P. quercetorum* was 668.4 ± 20.0 mg TE/g for the sample dissolved in methanol and 293.3 ± 4.1 for the sample dissolved in water. Interestingly, the methanol extracts had significantly higher total phenolic content and antioxidant capacity than the water extracts. Therefore, we used only the methanol extract of *P. quercetorum* for further analyses.

Anti-growth activity of *P. quercetorum*

The cytotoxic effect of *P. quercetorum* was assessed at six different concentrations ranging between 3.12 and 100 µg/ml by the MTT viability assay (Figure 1A). The *P. quercetorum* exhibited anti-growth effects in a dose-dependent manner, and was significant at the 50 and 100 µg/ml doses compared to the control (marked as in Figure 1A) ($p<0.05$). According to the dose response curves, *P. quercetorum* had similar growth-inhibitory effects on both types of breast cancer cells. Subsequently, the results were confirmed by a more sensitive ATP viability assay (Figure 1B). The ATP levels significantly decreased after *P. quercetorum* treatments at

25, 50, and 100 µg/ml doses in both types of breast cancer cells ($p<0.05$) (Figure 1B).

IC_{50} and IC_{90} values were calculated on the basis of the results of the ATP assay and are shown in Table 1. The IC_{50} values were 19.3 and 24.1 µg/ml for the MCF-7 and MDA-MB-231 cell lines, respectively.

Table 1. Anti-growth parameters for *Pelargonium quercetorum* determined by the ATP assay against MCF-7 and MDA-MB-231 cell lines.

Cells	*IC_{50} (µg/ml)	**IC_{90} (µg/ml)
MCF-7	19.3	35.3
MDA-MB-231	24.1	48.2

*IC_{50} is defined as the dose inhibiting 50% of viability
**IC_{90} is defined as the dose inhibiting 90% of viability

Apoptosis-inducing effects of *P. quercetorum*

The determination of cell death mode was first made on the basis of nuclear morphology and cell membrane integrity via fluorescence microscopy in MCF-7 and MDA-MB-231 cells treated with *P. quercetorum* (100 µg/ml, 48 h).

The *P. quercetorum* caused cell/nuclear shrinkage and chromatin condensation in both cell types, which are hallmarks of apoptosis (Fig. 2). Both cell lines were also secondary necrotic (late stage of apoptosis) as evidenced by pyknotic nuclei with PI staining positivity (Fig. 2).

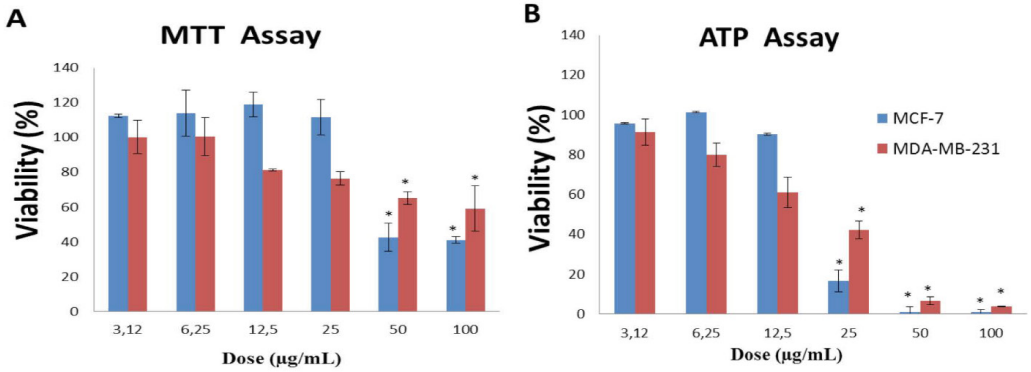

Figure 1. Assessment of viability of MCF-7 and MDA-MB-231 cell lines after 48 h treatment with varying doses of *Pelargonium quercetorum* extract by MTT (A) and ATP (B) viability assays. *Denotes statically significant differences in comparison with control ($p<0.05$).

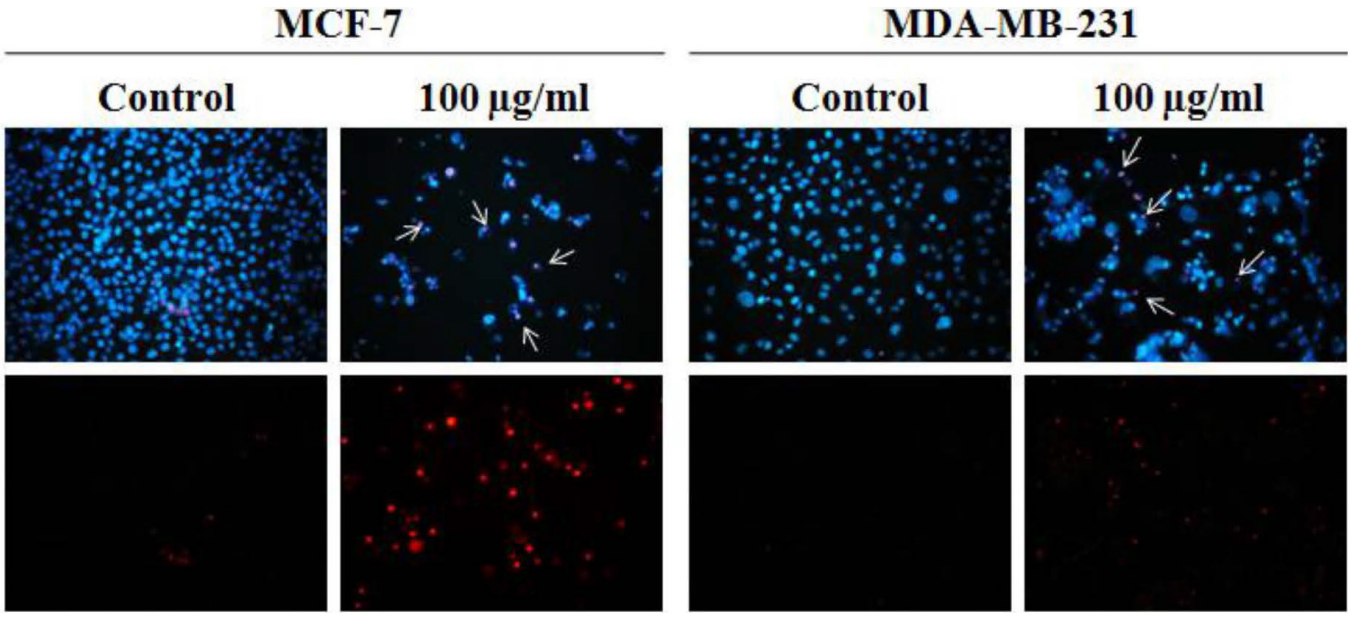

Figure 2. Fluorescence imaging for determination of cell death mode. Upper pictures show Hoechst dye 33342 staining; bottom pictures show propidium iodide staining of the corresponding areas. Arrows show pyknotic and late stage apoptotic nuclei. Magnification 100X.

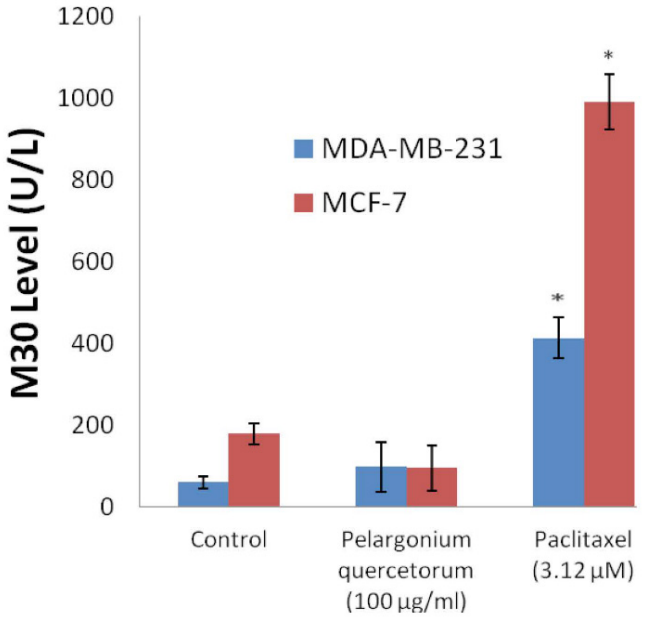

Figure 3. M30 (U/L) levels after treatment with *Pelargonium quercetorum* extract in MCF-7 and MDA-MB-231 breast cancer cell lines. Paclitaxel was used as a positive control for M30. * denotes statistically significant change from control ($p<0.05$).

It is clear that *P. quercetorum* caused pyknosis, which is a well-known feature of apoptosis. Apoptosis is a well-known cell death mode resulting from the action of chemotherapeutics. Therefore, we measured the levels of caspase-cleaved cytokeratin 18 (M30) as a marker of *P. quercetorum*-induced apoptosis. Paclitaxel (3.12 µM) was used as a positive control for apoptosis induction. Figure 3 shows that there was no increase in M30 levels due to *P. quercetorum* in both of the breast cancer cell types; however, the positive control drug (paclitaxel) increased the level of M30 significantly. Annexin-V-FITC staining was also used to confirm apoptosis. In MCF-7 cells, we observed Annexin-V-FITC staining positivity at both 12 and 24 h following *P. quercetorum* treatment (Figure 4).

Genotoxic effects of *P. quercetorum*

To our knowledge, first time in the literature, the current study evaluated the genotoxic effect of *P. quercetorum* using the comet assay in MCF-7 and MDA-MB-231 breast cancer cells. The effects of the *P. quercetorum* extract (12.5 and 25 µg/ml), positive controls (Cisplatin, 3.8 µg/ml and H_2O_2, 10 mM), and negative controls (distilled water) on comet frequency and genetic damage indexes in the breast cancer cells are presented in Table 2. The percentages of DNA in 'comet tails', which is indicative of the degree of DNA damage, were significantly higher at the 12.5 µg/ml dose of *P. quercetorum* extract in the MCF-7 cell line than in the negative control ($p<0.0001$, Table 2).

Table 2. Results of comet analysis in MCF-7 and MDA-MB-231 cell lines treated with Pelargonium quercetorum with positive and negative controls (Mean ± SE)

Cell Type	Treatment[a]	Comet Length[a]	Tail Length[a]	Olive Tail Moment[a]	% Tail DNA[a]	% Head DNA[a]	Genetic Damage Index[a]	% Damaged Cell[a]
MCF-7	**Negative Control**	39.51±2.22	10.07±2.53	3.25±1.27	9.94±3.06	90.06±3.06	0.29 ± 0.01	0.13 ± 0.01
	12.5 µg/ml *P. quercetorum*	47.96±2.77	19.87±3.14	7.86±1.52	25.65±4.07**	74.35±4.07**	1.08 ± 0.01***	0.46 ± 0.01***
	25 µg/ml *P. quercetorum*	49.42±4.32	19.96±4.56	8.09±2.36	18.56±4.57	81.44±4.57	0.97 ± 0.01***	0.24 ± 0.01***
	3.8 µg/ml Cisplatin	48.37±3.71	18.48±3.83	6.87±1.85	18.31±3.75	81.69±3.75	1.00 ± 0.01***	0.28 ± 0.01***
	10 mM H_2O_2	63.67±3.11***	40.33±3.20***	17.16±1.81***	53.10±3.13***	46.90±3.13***	2.50 ± 0.02***	0.98 ± 0.01***
MDA-MB-231	**Negative Control**	31.07±2.01	6.64±2.02	1.90±1.02	6.51±2.21	93.49±2.21	0.42 ± 0.01	0.02 ± 0.01
	12.5 µg/ml *P. quercetorum*	33.50±1.88	7.60±2.09	2.25±1.16	7.89±2.61	92.11±2.61	0.72 ± 0.01***	0.04 ± 0.02
	25 µg/ml *P. quercetorum*	34.77±1.60	6.89±1.48	1.46±0.54	6.35±1.48	93.65±1.48	0.6 ± 0.01***	0.06 ± 0.01*
	3.8 µg/ml Cisplatin	40.23±3.26	14.32±3.41	5.29±1.84	15.57±3.74	84.43±3.74	1.2 ± 0.02***	0.24 ± 0.03***
	10 mM H_2O_2	90.63±3.94***	63.86±4.07***	29.07±2.43***	64.90±3.65***	35.10±3.65***	3.02 ± 0.02***	1.00 ± 0.02***

Pelargonium quercetorum; SE, Standard Error; Cisplatin (3.8 µg/ml) and H_2O_2 (10 mM) as positive controls; Distilled water as negative control.
[a] significancy of *Pelargonium quercetorum* doses compared with negative control at ** $p < 0.005$; *** $p < 0.001$

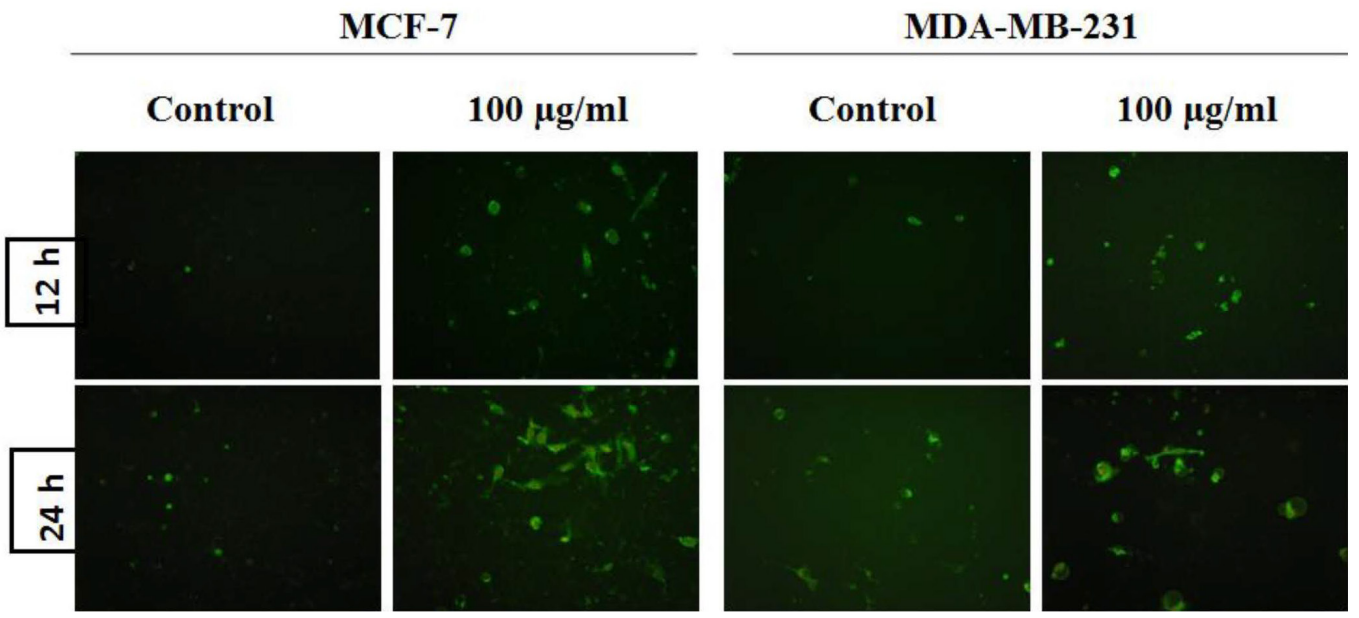

Figure 4. Annexin-V-FITC staining images with fluorescence microscope for MCF-7 and MDA-MB-231 cell lines. The cells stained with Annexin V-FITC. Magnification 100X.

ISCUSSION

Novel approaches/compounds are required for the better management of breast cancer. Therefore, we investigated *P. quercetorum* in terms of its possible cytotoxic and/or genotoxic activity against breast cancer cells. To our knowledge, although there are several studies regarding the anticancer activity of the Pelargonium genus, there are no published data on the genotoxic and/or cytotoxic activity of *P. quercetorum* in breast cancer cell lines. Thus, this study is the first to report a dose-dependent cytotoxic effect of *P. quercetorum* on two breast cancer cell lines (MCF-7 and MDA-MB-231). Results of this study indicate that the total phenolic content and antioxidant capacity of *P. quercetorum* were considerably higher in methanol extracts, rather than in water. Also, to our knowledge, there is no study investigating the phenolic content and antioxidant capacity of *P. quercetorum*. Therefore, we performed a detailed literature review of the Pelargonium genus. In the study of Čavar and Maksimović [17], the extracts and essential oils of *P. graveolens* showed radical scavenging activity and high antioxidant capacity. In another study, the total phenolic content of *P. geranium* leaf extract ranged from 1.65 to 8.23 mg GAE/g FW, and the ABTS scavenging capacity ranged from 23.15 (infusion) to 223.76 (heat reflux) µM TE/g FW [18]. In addition, it has been shown that the extracts of *P. graveolens* and *P. reniforme* have high antioxidant capacities [19,20].

In our current study, we observed that *P. quercetorum* exerts cytotoxic activity on MCF-7 and MDA-MB-231 cells in a dose-dependent manner (on the basis of MTT and ATP viability assays). The MTT assay revealed increased viabilities, especially at higher concentrations (25, 50 and 100 µg/ml), than those of the ATP assay. However, it has been reported that the MTT assay may suffer from false positive increases in viabilities in comparison with the ATP assay [13]. Cell viability significantly decreased after treatment with *P. quercetorum* at 25, 50, and 100 µg/ml doses in both cell types ($p<0.05$). Compared to the MDA-MB-231 cells, the MCF-7 cell line was slightly more sensitive to the *P. quercetorum*. The mechanism of action by which *P. quercetorum* causes cytotoxicity may be due to the Geranium essential oils that are derived from *P. quercetorum*. Geranium essential oils are known to exhibit anti-cancer activity [21,22]. Previous studies have shown that the Pelargonium species have cytotoxic activity on cancer cells [2,7, 23].

We also investigated the mode of cell death resulting from *P. quercetorum* treatment. Therefore, the level of caspase-cleaved cytokeratin 18 (M30) was measured for the detection of apoptosis. We found that *P. quercetorum* caused no increase in M30 levels in both cell types, although the positive control drug (paclitaxel) increased the level of M30. This implies that the mechanism of action of *P. quercetorum* is different from that of paclitaxel, which is also a plant-derived anti-cancer agent [24]. Since the results of our studies indicate that *P. quercetorum* causes apoptotic cell death, we hypothesize that the apoptosis may be caspase-independent. In fact, the occurrence of M30 fragments is believed to be dependent on caspase-activation. This may explain why there was no increase in M30 in our cells, even though they died by apoptosis. In one of our previous studies, we demonstrated that the extract of *P. quercetorum* has cytotoxic activity on lung cancer cells by inducing apoptosis [10].

The Comet assay can be used to determine cell death mechanisms and to detect any DNA damage (apoptotic or necrotic). In our

current study, damage frequency and genetic damage index were significantly increased by *P. quercetorum* extract compared with the control at all doses in both breast cancer cell lines ($p<0.0001$, Table 2). Therefore, we believe that *P. quercetorum* extract should be considered as highly genotoxic against breast cancer cells. Unfortunately, to our knowledge, there is no published data with which to compare our results.

In conclusion, our data demonstrate that the methanol extract of *P. quercetorum* inhibited the proliferation of human MCF-7 and MDA-MB-231 breast cancer cells. Additionally, *P. quercetorum* induced apoptosis in cells by causing DNA damage. These findings suggest that *P. quercetorum* might have strong antioxidant capacity, and may be able to be used as an effective therapeutic agent against breast cancer. Further *in vitro* and *in vivo* studies are needed to test this hypothesis.

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