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GC-MS Analysis and Apoptotic Effect of *Paliurus spina-christi* Mill. Leaf and Flower Extracts against Breast Cancer Cells

Ferdi OGUZ[1], Çisil ÇAMLI PULAT[1], Süleyman İLHAN*[1], Harika ATMACA[1]

Abstract

In recent years, herbal medicines have become a significant novel source of treatment for various types of cancer, including breast cancer. Various investigations have declared that *Paliurus spina-christi* Mill. (PSC) shows antioxidant, antifungal, antimicrobial, and antibacterial properties, but its effect on cancer cells is unknown. This study purposed to evaluate the possible anti-cancer effects of the ethanolic extract of the PSC in human MCF-7 and MDA-MB-231 breast cancer cells. The leaf and flower extracts of PSC were prepared in ethanol and volatile compounds were determined by GC-MS analysis. The possible cytotoxic effects of extracts were evaluated via MTT assay. Apoptotic effect was examined using the PI Annexin V Apoptosis Detection Kit. Significant cytotoxic effects were detected after 72 h treatment of ethanolic leaf and flower extracts in MCF-7 cells but not in MDA-MB-231 cells. Both leaf and flower extracts of PSC induced apoptotic cell death in MCF-7 cells. On phytochemical screening, it was shown that the leaf extract of PSC contains pyrrolidine, 2-decenal, 2-undecanal, phytol, oleic acid, oleamide, squalane, vitamin E, and gamma-sitosterol and the flower extract contains pyrrolidine, 2-decenal, 2-undecenal, oleic acid, lupeol, and gamma-sitosterol. These data report that PSC leaf and flower extracts have cytotoxic and apoptotic effects in MCF-7 breast cancer cells. Moreover, this study can be considered an *in vitro* background for further *in vivo* cancer experiments.

Keywords: *Paliurus spina-christi*, breast cancer, anti-cancer, GC-MS, MCF-7.

1. INTRODUCTION

Breast cancer is both the most frequently diagnosed cancer in females and the second cause of cancer-related death in women [1]. Therefore, considering the limitations of present chemotherapeutic drugs such as various undesirable side effects and multi-drug resistance, novel treatment methods and agents are needed. In recent years, natural compounds have become a promising potential resource in cancer therapy [1-3]. Plants containing chemical compounds with therapeutic potential have a long history of use" in the treatment of various diseases. Most current anticancer drugs, such as taxol, vincristine, and vinblastine, originate from plants or their synthetic derivatives [4, 5]. *Paliurus spina-christi* Mill. (PSC), also defined as Jerusalem thorn or Christ's thorn, is generally

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distributed in dry and rocky areas in the Mediterranean region and Asia [7]. The plant belongs to the Rhamnaceae family, which has five known species, and only PSC grows in the flora of Turkey. PSC is traditionally used as a diuretic, anti-rheumatic, tonic, hypocholesterolemic, anti-diarrheal. PSC fruit extract is also known to have an anti-diabetic effect [8]. The plant extract contains biologically active chemical compounds such as alkaloids, flavonoids, tannins, and sterols. These components in the PSC aqueous extract show antimicrobial, antibacterial, antifungal, hypoglycemic, and antioxidant properties. In addition, the fruits of the PSC generally are used as an anti-inflammatory in kidney stones, chest and face infections, and the leaves are usually used as a therapeutic against boil inflammation [6-8]. Based on all these significant biological activities of PSC, here we conducted the first study in the literature investigating the possible cytotoxic and apoptotic effects of the ethanolic extract of *Paliurus spina-christi* in human breast cancer cells. First, we characterized the extracts of leaves and flowers by gas chromatography–mass spectrometry (GC-MS) analysis. The possible cytotoxic effects were investigated via MTT assay and apoptotic effects of extracts were examined via flow cytometry.

2. MATERIALS AND METHODS

2.1. Preparation of PSC Extracts

Dried and washed plant material (*Paliurus spina-christi* Mill.) was purchased from a local company (Manisa, Turkey) and held at room temperature until used. The dried PSC flowers and leaves were powdered to homogeneous size with a homogenizer. Dried powder (2 gr) of the PSC leaf and flower were extracted separately in 50 ml ethanol (EtOH) at room temperature and ultrasonically extracted for 1 h and then filtration was performed with Whatman filter paper and stored at 4 °C until the experiments were carried out.

2.2. Cell Culture

MCF-7 and MDA-MB-231 cells were provided from Interlab Cell Line Collection (Genova, Italy). Human embryonic kidney cells (HEK-293) were provided from the American Type Cell Culture Collection (ATCC, USA). MCF-7 and MDA-MB-231 cells were grown in RPMI 1640 medium and human embryonic kidney cells were cultured in Eagle's Minimum Essential medium. Each medium included 10% heat-inactivated fetal bovine serum supplemented with 1% L-glutamine, and 1% penicillin-streptomycin. All cell lines were kept at 37 °C in a humidified atmosphere with 5% CO_2. The development and morphology of the cells were monitored daily with an inverted microscope.

2.3. Cytotoxicity Analysis

The cytotoxic effects of PSC leaf and flower extracts in MCF-7 and MDA-MB-231 cells were analyzed with MTT assay. Briefly, the breast cancer cells were placed at 10^4 cells per well in 100 µL media and increasing concentrations of PSC leaf and flower extracts (100, 250, 500, 750, 1000 µg/mL) were added to the cells in well-plate for 24, 48, and 72 h. After incubation periods, cells exposed to PSC leaf and flower extracts were subjected to 10 µL MTT and held at 37 °C for 4 h. Then, the solutions were withdrawn from wells and DMSO was implemented to the cells to dissolve formazan crystals. Lastly, the optical density of each well was evaluated at 570 nm wavelength with a spectrophotometer (TECAN Infinite 200 Pro).

2.4. Flow Cytometric Apoptosis Assay

PI Annexin V Apoptosis Detection Kit (BD, Biosciences) was utilized following the manufacturer's directions to verify the apoptotic effects of the PSC flower and leaf extracts. To determine early apoptotic cells, staining with Annexin V FITC is generally utilized in combination with a vital dye such as Propidium Iodide (PI). Viable cells externalize PI since they have undamaged membranes, while the membranes of dead cells are pervious to PI. Briefly, the cells were placed at 10^6 cells per well in a 6-well plate and were subjected to the most effective concentrations of PSC flower and leaf extracts for 72 h. Following the washing cells with cold PBS and resuspending in 1 mL of 1X

Binding Buffer, 5 µL of Annexin V FITC and 5 µL of PI were transferred to the solution. Then, the solution was shaken with vortex and maintained for 15 minutes at RT (25 °C) in the dark. Finally, 400 µl of 1X Binding Buffer was applied to each tube and the detection of apoptosis was evaluated utilizing flow cytometry. (BD Accuri C6 Flow Cytometer).

2.5. Detection of Volatile Components by GC-MS

Volatile compounds in the PSC flower and leaf extracts were qualitatively examined in electron ionization (EI) mode using an Agilent Technology 7890 Gas Chromatography (GC) Mass spectrometer (MS). The ethanol (EtOH) PSC extracts were centrifuged at 15.000 rpm for 10 min, and the supernatants were transported to the autosampler vial for GC-MS analysis. MS was registered at 70 eV ionization energy in full scan mode 35-550 amu range. The ionization source and transfer route temperatures were adjusted to 230 and 290 °C, respectively. The chromatographic pillar was an Agilent HP-5 MS capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). The kiln temperature was launched at 40 °C with 2 min hold, then warmed to 300 °C at the rate of 5 °C min-1 and kept for 5 min. Helium gas was utilized as a carrier gas and the constant flow ratio was 1 mL min-1 and the injector temperature was adjusted as 220 °C. The PSC extracts were inserted in splitless mode (200:1) Evaluation of the mass spectrum of GC-MS was carried out utilizing the database of National Institute Standard and Technology (NIST).

2.6. Statistical Analysis

Statistical analysis was done via one-way analysis of variance (ANOVA) followed by a Dunnett's t-test using Graph Pad Prism 5. Values with a $p<0.05$ were considered statistically significant.

3. RESULTS

3.1. Volatile Components of PSC Extracts by GC-MS

The volatile component composition of PSC leaf and flower ethanolic extracts was identified by GC-MS analysis and the relative percentage amount of each component was measured by comparing the average peak area with the total areas. The analysis of PSC leaf extract volatile components exhibited a total of 27 defined components (Table 1) and the flower extract exhibited a total of 19 compounds (Table 2). On phytochemical screening, it was demonstrated that the leaf extract of PSC contains pyrrolidine, 2-Decanal, 2-Undecenal, phytol, oleic acid, oleamide, squalane, vitamin E, and gamma-sitosterol and the flower extract contains pyrrolidine, 2-Decanal, 2-Undecenal, oleic acid, lupeol, and gamma-sitosterol. Tables 1 and 2 display molecular formulas, similarity, and retention times of these components. Based on the present literature concerning components in GC-MS some compounds such as phytol, lupeol, gamma-sitosterol, oleamide, and squalane have been declared as anticancer agents.

3.2. Cytotoxic Effects of PSC Extracts on Breast Cancer Cells

To examine the efficacy of PSC leaf and flower extracts on the viability of human breast cancer cells, we exposed cells to increasing concentrations (100-1000 µg/mL) of PSC leaf and flower extracts for 24, 48, and 72 h and then MTT assay was carried out. As seen in Figure 1, PSC leaf extract did not have a significant cytotoxic effect on human breast cancer cells at 24 and 72 h ($p>0.05$), however, increasing PSC leaf extract concentrations inhibited the viability of MCF-7 and MDA-MB-231 cells at 48 h ($p<0.05$). As demonstrated in Figure 2, PSC flower extract suppressed the viability of MCF-7 breast cancer cells in a concentration- and time-dependent manner and the highest cytotoxic effect was obtained at 72 h. As shown in Figure 2, PSC flower extract showed no significant cytotoxic effect on MDA-MB-231 cells. Based on the MTT

viability assay, significant cytotoxic effects were observed after 72 h treatment of ethanolic leaf and flower extracts in MCF-7 cells but not in MDA-MB-231 cells.

Table 1 GC/MS analysis of ethanolic PSC leaf extract. The table lists the most common compounds in order of retention time (tR). The NIST database was used to interpret the mass spectrum of the GC-MS, which was expressed as % similarity

No	Source	Compound	tR (min)	Molecular formula	NIST match (similarity, %)
1	Leaf	Isopropoxycarbamic acid	5.431	C6H13NO3	71.31
2	Leaf	3-Amino-2-oxazolidinone	6.202	C3H6N2O2	80,62
3	Leaf	Pyrrolidine-Alpha	6.460	C4H5D4N	84.25
4	Leaf	Heptanal	8.377	C7H14O	92.86
5	Leaf	1,2-Cyclopentanedione	8.94	C5H6O2	89.72
6	Leaf	Octanal	10.997	C8H16O	95.17
7	Leaf	Formic acid	13.102	C9H18O2	94.48
8	Leaf	Nonanal	13.818	C9H18O	93.69
9	Leaf				
10	Leaf	2-Decenal	18.613	C10H18O	94.83
11	Leaf	Nonanoic acid	19.238	C9H18O2	97.09
12	Leaf	Cyclohexanone	20.072	C10H14O	78.54
13	Leaf	2-Undecenal	22.316	C13H18O2	93.38
14	Leaf	Trans-2-Dodecenoic acid	26.52	C12H22O2	80.41
15	Leaf	(-)-Loliolide	30.919	C11H16O3	81.67
16	Leaf	Hexadecanoic acid	34.72	C16H32O2	93.95
17	Leaf	Phytol	37.576	C20H40O	85.24
18	Leaf	Oleic Acid	37.999	C18H34O2	87.47
19	Leaf	Ethyl Oleate	38.507	C20H38O2	96.11
20	Leaf	9,12-Octadecadienoic acid	40.529	C18H32O2	75.72
21	Leaf	Oleamide	41.807	C18H35NO	84.53
22	Leaf	2,3-Dihydroxypropyl elaidate	46.866	C21H40O4	83.84
23	Leaf	13-Docosenamide	48.117	C22H43NO	95.27
24	Leaf	Squalene	48.784	C30H50	90.94
25	Leaf	Quercetin	49.715	C15H10O7	74.51
26	Leaf	Vitamin e	53.467	C29H50O2	90.27
27	Leaf	Gamma.-Sitosterol	57.456	C29H50O	73.85

Table 2 GC/MS analysis of ethanolic PSC flower extract. The table lists the most common compounds in order of retention time (tR). The NIST database was used to interpret the mass spectrum of the GC-MS, which was expressed as % similarity

No	Source	Compound	tR (min)	Molecular formula	NIST match (similarity, %)
1	Flower	Silane	5.410	C4H12O2Si	75.26
2	Flower	3-Amino-2-oxazolidinone	6.195	C3H6N2O2	80.46
3	Flower	Pyrrolidine-alpha	6.453	C4H5D4N	84
4	Flower	N Heptenal	8.377	C7H14O	92.53
5	Flower	Formic acid, octyl ester	13.13	C9H18O2	93.34
6	Flower	Nonanal	13.832	C9H18O	98
7	Flower	2-Decenal, (E)-	18.585	C10H18O	94.25
8	Flower	2-Undecenal	21.337	C11H20O	96.81
9	Flower	2H-Pyran-2-one	26.562	C12H22O2	80.23
10	Flower	1-Octadecanol	31.308	C18H38O	85.42
11	Flower	Hexadecanoic acid	34.740	C16H32O2	72.21
12	Flower	2,4(1H,3H)-Pyrimidinedione	35.387	C4H3N3O4	77.42
13	Flower	Oleic Acid	37.965	C18H34O2	72.04
14	Flower	Octadecanoic acid, ethyl ester	38.972	C20H40O2	83.17
15	Flower	Lupeol	43.551	C30H50O	79.26
16	Flower	2-Myristynoyl pantetheine	44.149	C25H44N2O5S	73.16
17	Flower	13-Docosenamide, (Z)-	48.151	C22H43NO	92.32
18	Flower	Butanoic acid	49.138	C24H34O6	78.15
19	Flower	Gamma.-Sitosterol	57.518	C29H50O	66.42

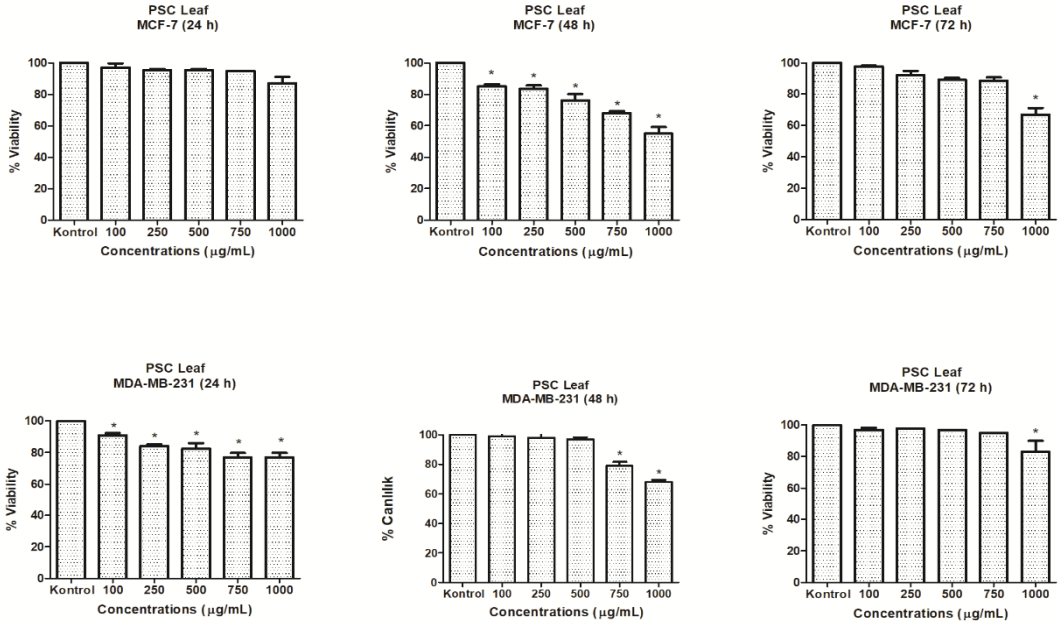

Figure 1 Effect of PSC leaf extract on the viability of MCF-7 and MDA-MB-231 breast cancer cells at 24, 48 and 72 h ($p<0.05$)

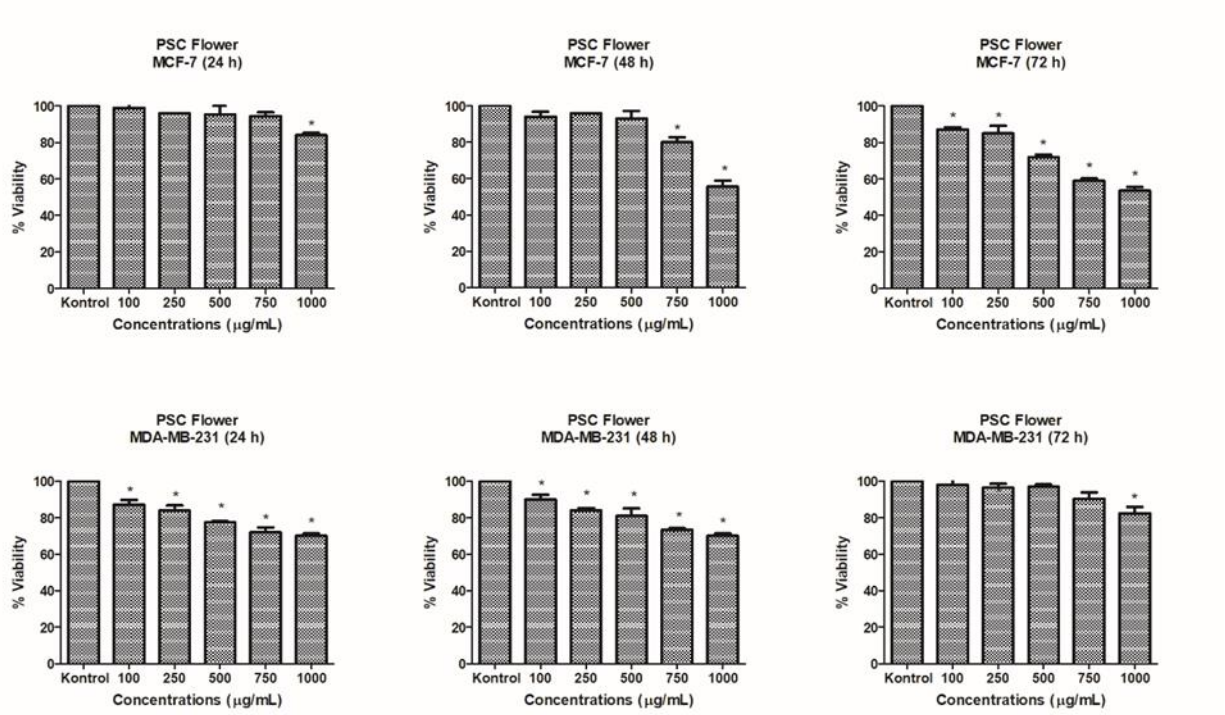

Figure 2 Effect of PSC flower extract on the viability of MCF-7 and MDA-MB-231 breast cancer cells at 24, 48 and 72 h ($p<0.05$)

3.3. Induction of Apoptosis by PSC Extracts in MCF-7 Breast Cancer Cells

To identify whether ethanolic PSC leaf and flower ethanolic extracts can trigger apoptosis in MCF-7 breast cancer cells, cells were exposed to 750 and 1000 µg/mL PSC extracts for 48 h. Then, we evaluated the potential apoptotic effect of PSC extracts on MCF-7 cells by flow cytometry using the PI Annexin V Apoptosis Detection Kit. Annexin V FITC has a high affinity to phosphatidylserine so Annexin V-FITC+/PI- cells specify early apoptotic cells and Annexin V-FITC+/PI+ cells specify late apoptotic cells. Based on a comparison of MCF-7 breast cancer cells exposed and not exposed to PSC flower and leaf extracts, dot plots of flow cytometric apoptosis analysis displayed the rate of early and late apoptosis. As shown in Figure 3, the percentage of early and late apoptotic cells in MCF-7 were 15.8% and 74.9% by 750 µg/mL PSC flower extract, respectively. The percentage of early apoptotic cells in MCF-7 breast cancer cells exposed to the PSC leaf extract was 0.2%, while the percentage of late apoptotic cells was 91.9% by 1000 µg/mL PSC flower extract, respectively. However, the rate of early apoptotic cells was 79% and the rate of late apoptotic cells was 0.8% in 750 µg/mL PSC flower extract treated MCF-7 breast cancer cells. The percentage of early and late apoptotic cells in MCF-7 breast cancer cells subjected to the 1000 µg/mL PSC leaf extract was 93.1% and 2.2%, respectively. These results revealed that both leaf and flower PSC extracts significantly induce apoptosis in MCF-7 breast cancer cells.

4. DISCUSSION

Multidisciplinary approaches such as surgery, adjuvant therapy, and radiotherapy are used in the treatment of breast cancer. In the effective treatment of breast cancer, maximum therapeutic effects and minimum undesirable effects such as recurrence, resistance, and toxic effects are aimed [12]. Plants have been widely used for therapeutic purposes in cancer treatment in recent years, due to their unique biological structures and the source of bioactive components [4].

Paliurus spina-christi Mill. (PSC) is a plant that grows in the Mediterranean region and Asia and has antimicrobial, antibacterial, antifungal, and

antioxidant properties [6-8]. PSC has attracted the attention of scientists in recent years due to these features. Takım et al. examined the inhibitory effect of PSC fruit against diabetes-stimulated inflammation caused by oxidative stress in vivo on diabetic rats. Researchers determined that PSC fruit extract has rich phenolic content and PSC fruit extracts showed a more effective antioxidant activity than standard antioxidants [9]. In another study, Takım et al. investigated the effects of PSC fruits on blood glucose, insulin, and glycated hemoglobin levels of streptozotocin-induced diabetic rats. They found that the groups that received insulin and PSC fruit extract had statistically lower blood sugar and glycosylated hemoglobin levels compared to the diabetic control group. Moreover, the outcomes of the investigation reported that the PSC fruit extract is rich in phenolic and mineral content [11]. Zor et al. analyzed the antigenotoxicity of compounds obtained from extracts of PSC fruits. The researchers examined the cytotoxicity and antigenotoxic efficiency of the components in Chinese hamster lung fibroblast (V79) cell lines. The IC_{50} values of catechin, gallocatechin, and rutin, which are components isolated from the PSC fruit extract, were calculated as 734 µg/mL, 220 µg/mL, and 1004 µg/mL, respectively. They found that the methanolic extract of PSC alone did not stimulate DNA single-stranded breaks, while catechins, gallocatechin, and rutin considerably decreased DNA damage caused by H_2O_2. Therefore, it is predicted that PSC fruits may be beneficial in the prevention of DNA damage-related disturbances [10]. Mosaddegh et al. investigated the effect of PSC fruit extract on lowering serum lipid content. The researchers administered streptozocin to male rats and then fed them a high cholesterol diet, and the rats were simultaneously exposed to various doses of PSC extract. Researchers analyzed serum levels of cholesterol, triglyceride, and HDL after 8 days and found that PSC extract decreased total

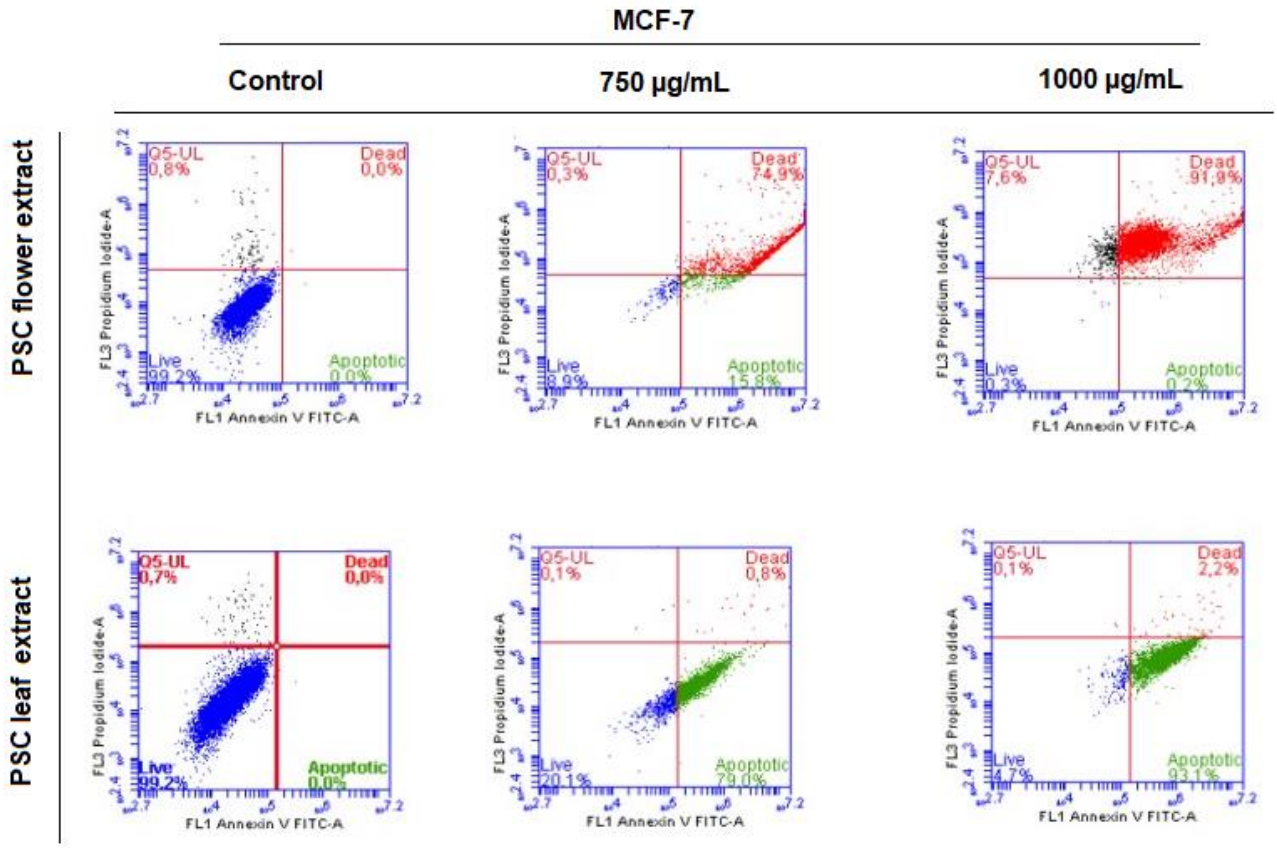

Figure 3 Apoptotic effects of PSC leaf and flower extract on MCF-7 cells at 48 h

cholesterol and triglyceride amount, but no significant increase was observed in HDL amount [13].

In the literature, antioxidant, antifungal, antimicrobial properties of PSC are known however its effects on cancer cells are not known [7]. Therefore, in this investigation, we purposed to investigate the possible anti-cancer effects of ethanolic extract of the PSC in human breast cancer cells. According to MTT analysis results, significant cytotoxic effects were observed in MCF-7 cells after 72 h of treatment of ethanolic leaf and flower extract, but not in MDA-MB-231 cells. This may be due to the different molecular phenotypes of the MCF-7 and MDA-MB-231 cell lines. MDA-MB-231 is an aggressive, invasive triple-negative breast cancer cell line as it lacks estrogen receptor, progesterone receptor expression, and HER2. MCF-7 cells are estrogen receptor-positive, progesterone receptor-positive, and HER2 negative [14]. Consistent with the MTT analysis results, flow cytometry data showed that both PSC leaf and flower extracts induced apoptosis in MCF-7 cells. We also performed GC-MS analysis to determine the volatile compound composition, and the results of the analysis detected the presence of active substances that may have anticancer potential in both leaf and flower extracts. These preliminary data showed that PSC is a potential herb to be used in the treatment against estrogen receptor-positive MCF-7 breast cancer cells. In the next stages, the active ingredients in the PSC extract can be purified and their effects on different cancer cells can be investigated individually or in combination.

5. CONCLUSION

Paliurus spina-christi Mill. has been used in folk medicine for its antimicrobial, antibacterial, antifungal, and antioxidant effects. Various in vitro studies have confirmed these effects of PSC extract, but to the best of our knowledge, the possible cytotoxic effect of ethanolic PSC extract on cancer cells had not been investigated before. This in vitro study reported that ethanolic PSC leaf and flower extracts have a significant cytotoxic effect on MCF-7 cells, but not in MDA-MB-231 cells. According to the results of flow cytometric analysis, PSC leaf and flower extracts also induced apoptosis in MCF-7 cells. GC-MS analysis results indicated the presence of active substances that may have anticancer effects in PSC leaf and flower extracts. These components in combination or separately may have behaved as an anticancer agent by inhibiting breast cancer cells. Therefore, there is a need for investigations in which the active ingredients in the PSC extract are purified and used on cancer cells individually or in combination.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

Under this heading, the authors contributed equally to the study

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission. The Declaration of Research and Publication Ethics The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic

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