Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2020; 27 (2): 120-133

Ethanolic Extract of Propolis from Kerman Area Triggers Apoptosis and Arrests Cell Cycle in Three Human Breast Cancer Cell Lines MDA-MB-231, SKBR and MCF-7

Vagiheh Soltaninejad, DVM [1], Nasrin Kazemipour, Ph.D.[2], Mohammad Mehdi Yaghoobi, Ph.D.[3], Abbas Pardakhty, Ph.D.[4]

1- Ph.D. student, Department of Pathobiology School of Veterinary Medicine, Shiraz University, Shiraz, Iran

2- Associate Professor, Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

3- Associate Professor, Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran (Corresponding author; E-mail: m.yaghoobi@kgut.ac.ir)

4- Professor, Department of pharmaceutics, Faculty of pharmacy and pharmaceutical sciences, Kerman University of Medical Sciences, Kerman, Iran

Received: 5 December, 2019 Accepted: 3 February, 2020

ARTICLE INFO

Article type:
Original Article

Keywords:
Propolis
Breast cancer
Cytotoxicity
Apoptosis
Cell cycle

Abstract

Background: Cancer is one of the major health problems worldwide and natural resources are being explored to develop anticancer drugs with fewer side effects. Iranian propolis contains components including flavonoids and polyphenols and has various medicinal properties. The aim of this study was to investigate the effect of Ethanolic Extract of Sirch Propolis (EESP) on three breast cancer cell lines.

Methods: The MDA-MB-231, SKBR-3 and MCF-7 cells were treated for 24 and 48 h at the presence of 1% and 10% fetal bovine serum (FBS) concentration. MTT, BrdU and flow cytometry assays were used for measuring cytotoxicity, cell proliferation and apoptosis.

Results: The highest cytotoxicity was seen on MDA-MB-231 cell at the presence of 1% and 10% FBS respectively following 48 h treatment. BrdU assay showed that treatment with 200 µg/ mL of EESP at the presence of 1% FBS for 48 h, reduced proliferation of MDA-MB-231 cell to 75% and that of MCF-7 and SKBR-3 cells to 70% and 60% respectively. Cell cycle analysis by flow cytometry showed that EESP at 200 µg/mL for 48h, induced G_0/G_1 phase arrest in MCF-7 and SKBR-3 cells and G_2/M, S phase arrest in MDA-MB-231 cell. The cytotoxic effects of EESP were primarily found to be due to the induction of early stage apoptosis on SKBR-3 cell and early and late stage apoptosis on MCF-7 and MDA-MB-231 cells.

Conclusion: The results demonstrated that EESP is a natural anticancer mixture capable of reducing breast cancer cells proliferation and inducing cell cycle arrest and apoptosis in them.

Introduction

Breast cancer is the primary cause of cancer-related death in females younger than 45-years-old and the second most common cancer diagnosed in women worldwide (1-3). According to the global cancer statistics, 2.1 million new breast cancer cases emerged and 627,000 people died in 2018 (4). Chemotherapy, radiotherapy, hormonal therapy and surgery are the main treatment methods for breast cancer. Due to severe side effects and multidrug resistance, these treatment approaches are being ineffective (5). Therefore, it is important to develop effective management strategies against chemotherapy and radiotherapy-induced side effects. A large number of researches are now focusing on the natural and dietary compounds as new and more effective drug sources for breast cancer patients. Natural products including crude extracts, bioactive components-enriched fractions, and pure

compounds derived from herbs as well as herbal formulas have been widely shown to prevent and treat cancers (6) Natural compounds have almost fewer side effects, with many therapeutic benefits (7). Natural compounds fight against aggressiveness of breast cancer, inhibit cancer cell proliferation, and modulate cancer related pathways (5). Till now, more than 50% of approved drugs have been extracted or designed from natural compounds. Among them, 75% of anticancer drugs have been designed and developed from plant or other natural resource ingredients (8).

Propolis is a mixture of resin produced by honey bees through collecting exudate from plants, vegetation and pollen and mixing with their enzyme's secretions. It is also mixed with bees wax to create a sealing material (9, 10). Propolis contains polyphenols, flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes and ketones. The composition of propolis varies depending on the vegetation, climate, season and environmental conditions of the area from where it is collected (11). Overall, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other various substances, including organic debris (12). Undoubtedly, propolis can be considered as a potential source for drug discovery. Anticancer activities of propolis, against various human breast cancer cell lines such as MDA-MB-231, MCF-7 and SKBR-3 have been widely reported (13-16). The ethanolic extract of propolis was found to be more effective in inhibiting mammary carcinogenesis (17). In a study, the phenolic and flavonoid contents of Turkish propolis showed statistically significant cytotoxic effects on both non-aggressive and aggressive breast cancer cell lines (18).

There are few studies published about the composition and anticancer properties of Iranian propolis. Here we investigated the anticancer effects of ethanolic extract of propolis (EESP) collected from Sirch/ Kerman/ Iran on three breast cancer cell lines (MCF-7, MDA-MB-231 and SKBR-3) *in vitro*.

Material and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher, USA). Dimethyl sulfoxide (DMSO) and trypsin and Propidium Iodide (PI) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] powder was purchased from (Melford, UK). BrdU cell proliferation ELISA Kit was purchased from Abcam (Cambridge, Sudbury, UK). Apoptosis Detection Kit FITC eBioscience™ Annexin V Kit was purchased from Thermo Fisher (USA).

Preparation of propolis extracts

Propolis sample was collected during May 2018 from (Sirch area at 50 Km East of Kerman city/ Iran). EESP was prepared according to the method described by Muli et al (19). In brief, the collected propolis sample was powdered, and 2g powdered sample was admixed with 70% ethanol (10 mL) and macerated for a week. Then, ethanolic extract was stored in sterile, airtight glasses at 4°C in the dark and used in the next experiments.

Cells and Culture Conditions

Three human breast cancer cells (MCF-7, SKBR-3 and MDA-MB-231) were purchased from National Cell Bank of Iran (NCBI) at Pasteur Institute of Iran. The cells were grown in DMEM supplemented with 10% or 1% heat FBS, 100 U/mL

penicillin G, and 100 µg/mL streptomycin and incubated at 37°C in humidified air with 5% CO_2.

Cytotoxicity of EESP

Cytotoxicity of EESP against the three cell lines was determined by MTT assay. The cells were plated in 96-well tissue culture plate at a density of 6×10^3 cells/well in triplicate rows and fed with 100µL/ well medium. On the next day, the cells were treated with different concentrations (10, 50, 100, and 200 µg/mL) of EESP for 24 and 48 h at the presence of 1% and 10% FBS. Then, 10 µL MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C in a cell culture incubator. Further, the supernatant was removed, and the insoluble Formosan crystals were dissolved in 150 µL DMSO. The absorbance was measured at 570 nm using micro plate reader (Bio-Tek USA). The mean of the three experiments (3 wells/ plate) was considered for analysis. Untreated cells were considered as the control plate. The percentage of viability was calculated using the following formula:

(%) Cell viability = OD_{570} of treated cells / OD_{570} of control cells × 100

Incorporation of BrdU

To measure cell proliferation under treatment with EESP, the cells were seeded at 2×10^5 cells/mL density in 96-well plates and fed with 100 µL/well appropriate cell culture media for 24 h. The proliferation rate was measured using Cell Proliferation ELISA BrdU kit (Abcam UK) and according to the manufacturer's instructions. Briefly, after treatment with three concentrations (50, 100 and 200 µg/mL) of EESP for 48 h, the cells were labeled with BrdU at a final concentration of 10 µL/well, 24 h before the end of treatment. The cells were then denatured with Fix Denaturing solution. The wells were aspirated and washed with Wash buffer three times and incubated for 60 min with 100 µL (1:50 diluted) mouse anti-BrdU antibody at room temperature. Following the removal of the antibody and washing it with a washing solution (PBS 1X) for three times, the wells were incubated with peroxidase conjugated goat anti-mouse IgG conjugate for 30 min at room temperature. The wells were washed with Wash buffer three times. The substrate solution (TMB peroxidase) was added for 30 min at room temperature in the dark. After this period, the reaction was stopped by adding stop solution. Absorbance was measured at 450 nm using an ELISA plate reader (Bio-Tek USA). The blank corresponded to 100 µL of culture medium without BrdU, and the control was produced in the presence of cells in culture media without any treatment. Assay was performed in triplicates. Results were presented as plot area and compared with the control. A well containing cell/medium without adding BrdU reagent was considered as background.

Cell death assay

Occurrence of apoptotic or necrotic cell death was assayed using eBioscience™ Annexin V Apoptosis Detection FITC Kit (Thermo Fisher Scientific, USA) and according to the manufacturer's instructions. Briefly, the cells were seeded in 6-well plates at 6×10^4 cells/mL density in 6-well plates and fed with 2 mL/well appropriate cell culture media and 10% FBS for 24 h. After reaching 80% confluency, they were treated with EESP at 100_µg/mL for 48 h. The cells were harvested and centrifuged at 200 × *g* for 5 min. The cells were washed with PBS and then binding buffer (1X) and re-suspended in 1 mL binding buffer (1X). Five µL FITC-conjugated Annexin V was added to 100 µL of the cells and incubated for 15 min at room temperature. The cells were washed and re-suspended in 200

μL binding buffer. Five μL PI was added and they were incubated for 30 minutes on ice in the dark. The percentage of apoptotic/necrotic cells was analyzed by running the samples on flow cytometry machine (CyFlow Space, Partec, Germany). FSC and SSC were recorded linearly while FL1 and FL2 histograms were recorded on a log scale. Untreated unstained cells were used to adjust gain and to define the boundaries of quadrants. Annexin V only control ($Annexin^+ PI^-$) and PI only control ($Annexin^- PI^+$) were used to setup the required parameters. Double labeling data of 10000 cells per treatment was recorded and analyzed by FloMax 2.70 software (Partec, Germany). Data were expressed as mean percentage of apoptotic/necrotic cells ±SEM.

Flow Cytometry Analysis of Cell Cycle

Cancer cells were seeded in 6-well plates at 6×10^4 cells/mL density in 6-well plates and fed with 2 mL/well appropriate cell culture media and 10% FBS for 24 h at 37 °C. Following a 48-h treatment with EESP at100μg/mL, 1×10^6 cells were gently harvested by trypsin treatment and centrifuged for 5 min at 200 × *g*. The pellets were re-suspended in PBS and fixed in chilled 70% ethanol for 2 h. After ethanol removal, the cells were suspended in 0.5 mL PBS. The cell pellets were stained in 1 mL PBS containing PI, Triton X-100 and RNase A and incubated for 30 minutes at room temperature in the dark or for 15 min at 37°C. FSC, SSC and FL2 histograms were recorded linearly by running the samples on CyFlow Space (Partec, Germany). Untreated unstained cells were used to adjust gain. The percentage of cells in G_1, S and G_2/M phases were determined by FloMax 2.70 software (Partec, Germany).

Statistical Analysis

Data obtained from MTT and BrdU assays were analyzed by Graph Pad Prism 8 (GraphPad Prism Software, San Diego, CA) and using ANOVA followed by Dunnett's multiple comparison tests to identify statistically significant differences. Data obtained from cell cycle were analyzed by ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered as significant level.

Results

MTT assay

In the first part of the study, we studied the cytotoxic effects of a range of EESP on three breast cancer cell lines (MDA-MB-231, MCF-7and SKBR-3) by MTT assay. The cells were treated for 24 h and 48 h, at the presence of 1% and 10% FBS. We observed that EESP suppressed growth of cancer cells in a dose and time-dependent manner (Fig. 1). After 24 h and 48 h, 50% inhibitory concentration (IC_{50}) was observed in the 22.49 to 198 μg/mL range (table1). The results showed that EESP treatment for 24 h at the presence of 10% FBS did not alter the MDA-MB-231 and MCF-7 cells' viability markedly. But, we observed a decrease in the viability of SKBR-3 cells at 200 μg/mL with a growth inhibition of 12% ($P < 0.01$). Nevertheless, treatment with 50–200 μg/mL EESP for 24 h at the presence of 1% FBS inhibited the growth of MDA-MB-231 cells in a dose-dependent manner (IC_{50} = 50.58 μg/mL) (Figure 1 and table 1). Furthermore, the cytotoxic effects on MCF-7 occurred at 200 μg/mL (IC_{50} = 198 μg/mL) and the growth inhibition was 77%. Moreover, following a 48-h treatment with EESP at the presence of 10% FBS the following results were obtained: at the range of 10–200 μg/mL, cytotoxic effects were induced on MDA-MB-231 cells in a dose-dependent manner (IC_{50} = 95.07

μg/mL) and the growth inhibition range was 30-63%. Likewise, the cytotoxic effects on SKBR-3 cells took place at the range of 100–200 μg/mL (IC_{50} = 93.59 μg/mL) and growth inhibition was 54-56%. Finally, a 48-h treatment at the presence of 1% FBS, led to cytotoxic effects on MDA-MB-231 cells at the range of 50–200 μg/mL (IC_{50} = 22.49 μg/mL), and growth inhibition was 46-58%. In addition, the effects on SKBR-3 cells occurred at 50 and 200 μg/mL (IC_{50} = 42.62 μg/mL) and growth inhibition was 54-58%. Also, the effects on MCF-7 cells took place at 200 μg/mL with (IC_{50} = 160 μg/mL) and growth inhibition was 84%. Moreover, following treatment with 200 μg/mL EESP (and 1% FBS) for 24 h, viability of MCF-7, MDA-MB-231 and SKBR-3 cells was reduced to about 77%, 44% and 21%, respectively. While, this reduction in viability following the same treatment for 48 h for MCF-7, MDA-MB-231, and SKBR-3 cells was 84%, 64% and 53%, respectively. Thus, EESP at 200 μg/ mL was more effective on MCF-7 cell than MDA-MB-231 and SKBR-3 cells. Collectively, IC_{50} values demonstrated that EESP had the highest growth inhibitory effects on MDA-MB-231 cells. Although some cytotoxic effect was already observed at 50-100 μg/mL EESP, maximal effect was obtained at 200 μg/mL and 1% FBS in all the three cancer cells. Accordingly, treatment with 200 μg/mL EESP and 1% FBS were used for further study.

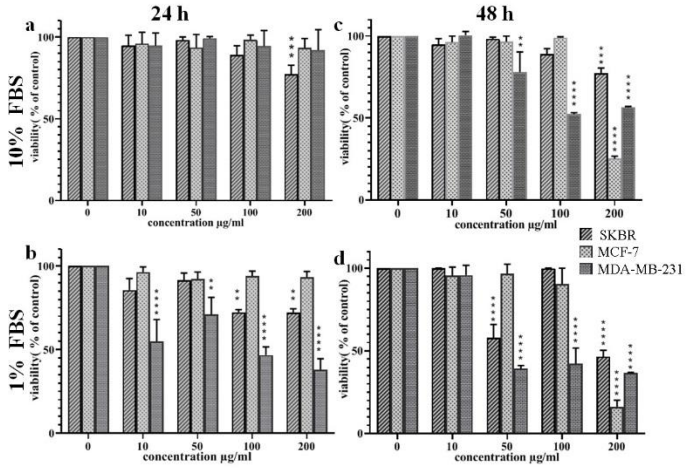

Figure1. Cytotoxic effect of EESP on viability of three cancer cell lines in a dose dependent manner using MTT assay; a) 24 h ,10% FBS, b) 24 h ,1% FBS, c) 48 h ,10% FBS, d) 48 h 1% FBS. Values have been presented as mean ± SEM of three replicates.

****: $P < 0.0001$, ***: $P < 0.001$, **: $P < 0.01$, and *: $P < 0.05$

With IC_{50} values of EESP ranging from 22.49 to 198 μM, the three breast cancer cell lines exhibited differential EESP sensitivity (Table 1). MCF-7 Cell line showed a relative resistance compared to the sensitive cell lines MDA-MB-231and SKBR-3.

Table 1. IC_{50} values calculated by GraphPad Prism (µg/mL) of EESP against MDA-NB231, SKBR-3 and MCF-7 cells for 24 h and 48 h at the presence of 1% and 10% FBS

Cell line / Treatment	MDA-MB231	SKBR-3	MCF-7
24 h,1% FBS	50.58	60.98	198
24 h,10% FBS	97.78	95.9	112
48 h,1% FBS	22.49	42.62	116.4
48 h,10% FBS	60	83.59	110

BrdU assay

Proliferation inhibition of EESP at 200µg/mL and 1% FBS for 48 h, was observed using BrdU assay. Results indicated that following treatment with EESP, DNA proliferation of SKBR-3, MDA-MB-231 and MCF-7 cells decreased to 40%, 25% and 29%, respectively. The highest cancer cell proliferation was seen on MDA-MB-231 cell line.

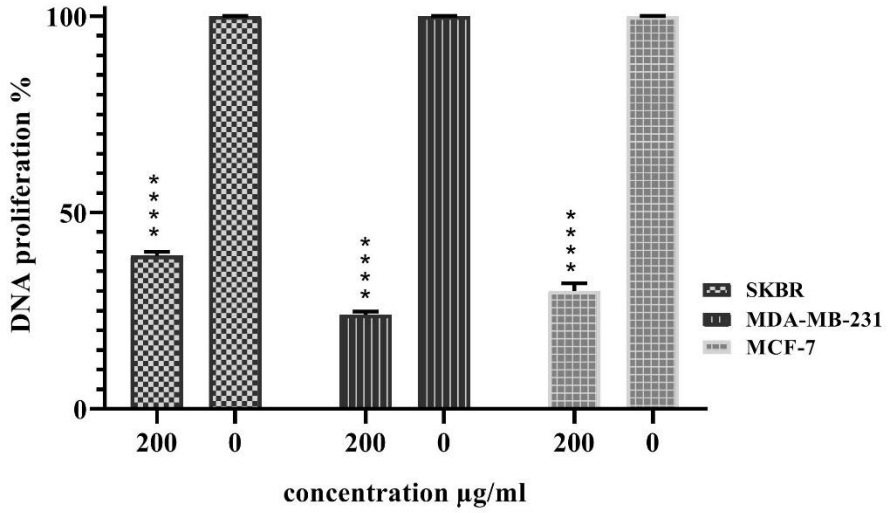

Figure 2. The effect of a 48- h treatment with 200 µg/mL EESP and 1% FBS on MCF-7, SKBR- and MDA-MB-231 cell proliferation assessed by BrdU incorporation assay. Data were presented as mean ± SEM. ****: p<0.0001 according to Dunnett's Test

Apoptosis

To investigate the apoptotic effect of EESP, three breast cancer cells were treated with 200 µg/mL EESP for 48 h at the presence of 1% FBS, and apoptosis/necrosis cell death was assessed by Annexin V and PI staining and flow cytometry. The results showed that the rate of apoptosis was significantly increased in all three cancer cells compared with non-treated control group. The early apoptotic cell populations of MCF-7, MDA-MB-231and SKBR-3 in the lower right quadrant were 9.96% ± 0.52,

11.5% ± 0.45 and 21.86%, respectively. In addition, the percentage of late apoptotic cells for MCF-7, MDA-MB-231 and SKBR-3 in the upper right quadrant were 13.72% ± 0.54, 14.66% ± 0.20 and 1.30%, respectively. Collectively, Total apoptosis rates observed in MCF-7, MDA-MB-231 and SKBR-3 cells were 22 ± 0.32%, 26 ± 0.16%, 23 ± 0.52% respectively. Whereas, the same values were 5.5 ± 0.12%, 0.70 ± 0.2%, 1.8 ± 0.04% in non-treated control group respectively. Even though, early and late stage apoptosis was observed in MCF-7 and MDA-MB-231 cells, early stage apoptosis was more prominent in SKBR-3 cells. Collectively, these data suggest that EESP suppresses cell viability in all of the three breast cancer cell lines via induction of apoptotic pathway.

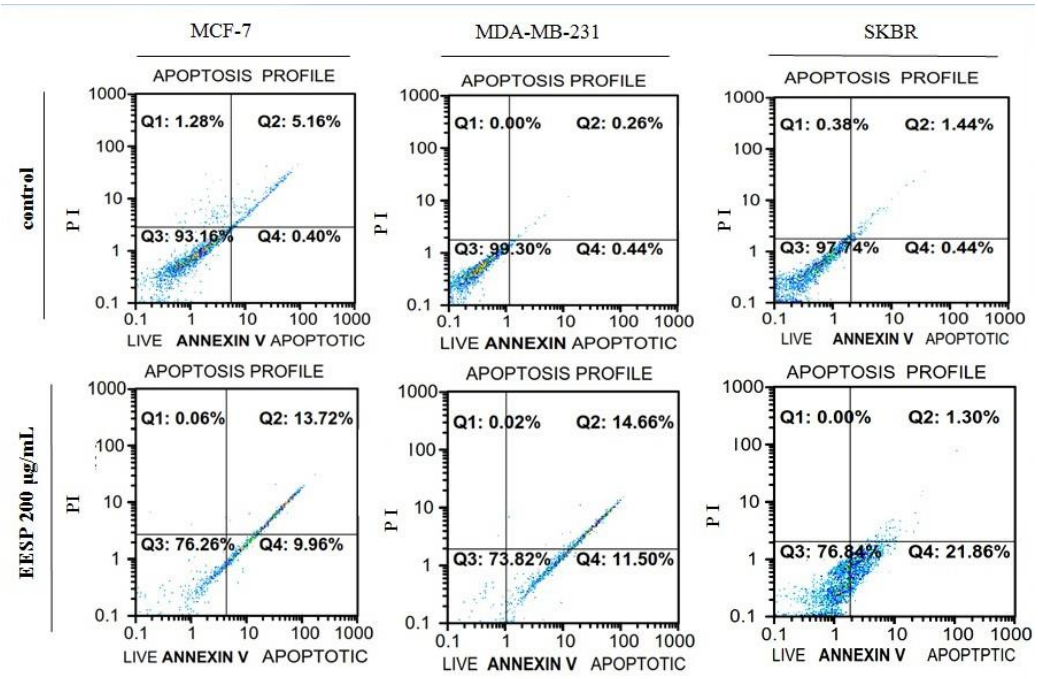

Figure 3. Apoptotic effect of EESP on three cancer cell lines (MDA-MB-231, SKBR-3 and MCF-7) following a 48-h treatment, measured by eBioscience Annexin V and Dead Cell assay. EESP induced apoptosis in all breast cancer cells. Early stage apoptotic cells are shown in the lower right quadrant and late stage apoptotic cells are illustrated in the upper right quadrant of the plot. Data were presented as mean ± SEM.

Cell cycle analysis

The effect of EESP on the cell cycle phases was determined using flow cytometry analysis. In this step, cell cycle progression of three cancer cells was examined after 48 h treatment with 200 µg/mL EESP with 1% FBS. Treatment of MCF-7, SKBR-3 and MDA-MB-231 cells resulted in significantly higher percentages of 90.48% and 63.960% and 79.085% respectively, of cells in the G_1 phase. While, in the control group (74.05%, 63.40% and 59.530%), Reduction in the percentage of cells in the S phase (18.9% and 18.19%) was occurred in MCF-7 and MDA-MB-231 cells respectively compared to the control group (21.9% and 23.26 %). In addition, G_2/M phase arrest was observed in MDA-MB-231 and SKBR-3 cells (7.3 %, 3.5 %) compared to the control group (12.18%, 11.5% respectively). Also, S phase in SKBR-3 cell was increased to 24.070 % compared to the control (18.640 %) (Fig.

4). These data suggest that inhibition of cell proliferation or induction of cell death in the three breast cancer cell lines by EESP is associated mainly with the induction of G_1 or S, arrest. The different proliferation rates of breast cancer cells were seen when exposed to 200 µg/mL of EESP in comparison with the untreated control cells which is partially due to the differences in cell cycle regulation.

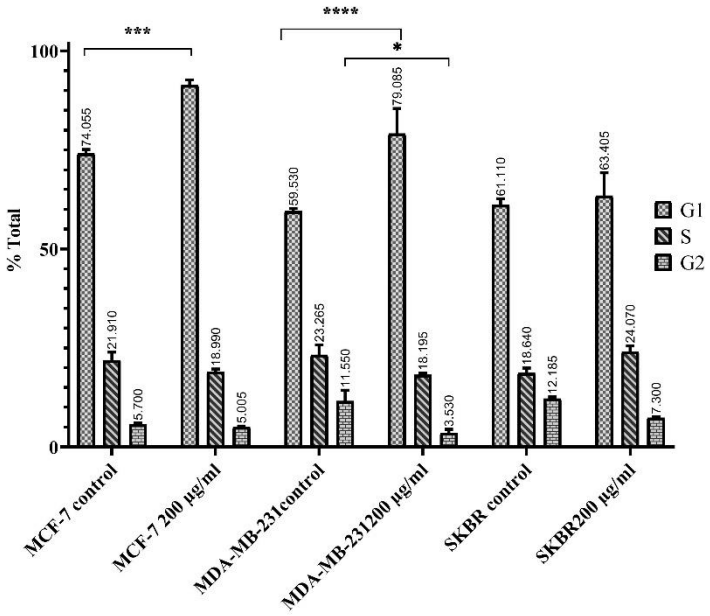

Figure 4. EESP effect on the cell cycle phases in MDA-MB-231, MCF-7 and MDA-MB-231 cells

The cells were treated with 200 µg/mL of EESP and 1% FBS for 48 h and were stained with PI. Untreated was indicated as (Control). Vertical bars represent the standard error of means (mean ± SEM), (n=3 experiments), ****: $P < 0.0001$, ***: $P < 0.001$, and *: $P < 0.05$

Discussion

There have been several efforts to treat cancer using various natural materials. Natural anticancer agents in the human diet are safe, and have long-lasting beneficial effects on human health (8, 20). Results from several studies indicate that propolis, as a natural product, and its components have anticancer properties (21-24). In the present study, we evaluated for the first time the anticancer effects of EESP on three breast cancer cell lines. Our results showed dose and time-dependent cytotoxic effect of EESP (i.e. 50, 100, 200 µg/mL) at 24 and 48 h in the presence of 1% and 10% FBS. MTT assay results demonstrated that following 48 h at 200 µg/mL with 1% FBS, EESP resulted in significant cytotoxicity in all of the three breast cancer cell lines ($p < 0.0001$). The IC_{50} of the EESP at 48 h with 1% FBS was 22.49 µg/mL for MDA-MB-231, 116.04 µg/mL for MCF-7 and 42.46 µg/mL for SKBR-3 cell lines. These results indicate that EESP has the highest significant cytotoxicity against MDA-MB-231 and the least cytotoxicity against MCF-7 cell ($p < 0.0001$). Previous studies indicated that propolis and its active compounds from various origins significantly inhibit cell growth and reduce proliferation of breast cancer cells (25). In addition, different cell lines are diversely affected by the ethanolic extracts of propolis with various origins. Thus, it can be conceived that the main

determinants of the properties of propolis against cancer cells are the diversity of its components and concentrations. Similarly, ethanolic extracts of Brazilian red propolis significantly reduced the viability of MCF-7 cell (26). Likewise, the antiproliferative activities of ethanolic extract of brown Cuban propolis exhibited a significant antiproliferative activity on MCF-7 rather than MDA-MB 231 cells, in a dose (1–25 µg/mL) and time-dependent (24 –48 h) manner. (13). Additionally, Nemorosone, which is found in Brown Cuban propolis, inhibited the viability of MCF-7, but not of MDA-MB-231 cell (27). In contrast, we observed that cytotoxic effect of EESP on MDA-MB-231 was more than that on MCF-7 cell. Our results are similar to some published studies. Hongzhuan Xuan *et al.* Investigated the sensitivity of MCF-7 and MDA-MB-231 cells to ethanolic extracts of Chinese propolis in 25-200 µg/mL concentrations using MTT assay at 24 and 48 h. This extract significantly inhibited MCF-7 and MDAMB-231 cells proliferation in a dose- and time-dependent manner. Notably, the inhibitory effect of Chinese propolis on MDAMB-231 cells was significantly higher than that on MCF-7 cells (14). In addition, Zhou et al. showed that MDA-MB-231 was more sensitive to cafeic acid phenyl ester as a propolis component, than MCF-7 cells (28). Furthermore, Vatansever et al. showed that all propolis extracts from Turkey inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. Their results revealed that some Propolis extracts at 0.125 mg/mL concentration were more effective in inhibiting MCF-7 cell growth (29).

Besides, Seyhan et al. showed that Turkey, Argentina and Chinese propolis extracts induced cytotoxicity on MCF-7, MDA-MB-231 and for the first time on SKBR-3 cells in a dose and time dependent manner. Interestingly they found that the sensitivity of different types of breast cancer cells to propolis varies from region to region (18).

In addition, Omene et al. showed decreased MCF-7, MDA-MB-231, and SKBR-3 cells viabilities in a concentration and time-dependent manner by propolis (IC_{50} of about 10 µg/mL for all the cell lines) at 72 h (16). In the present study, the EESP was cytotoxic on SKBR-3 cell at 200 µg/mL and 1% FBS following 24 h, which is similar to Seyhan et al. findings (18). After 48 h EESP treatment, cytotoxicity was observed at 10-200 µg/mL with 10% FBS and 50-200 µg/mL with 1% FBS respectively. In addition, On MDA-MB-231 toxicity was seen at 24 and 48 h at 50-200 µg/mL with 1% FBS. However, following 48 h, MDA-MB-231 showed cytotoxicity at lower dose (10-200 µg/mL) with 10% FBS. Furthermore, on MCF-7 cell, 200 µg/mL of EESP showed potent cytotoxicity after 24 and 48 h in the presence of 1% FBS. Accordingly, IC_{50} values obtained in our study are different from those reported in above studies. Collectively, EESP has high cytotoxicity against MCF-7 at all times and moderate toxicity against MDA-MB-231 and SKBR-3 cells. Thus, along with above studies, these observations indicate that the inhibitory effects of propolis on breast cancer cells depend on propolis type and dose, as well as incubation time.

Also, we evaluated the effect of FBS amount on EESP cytotoxic effect against breast cancer cells. Some reports have indicated that FBS has a potent role on cytotoxicity assay results such as MTT. Zhang *et al.* showed that albumin alone reduced XTT and MTT to the corresponding formazan and observed a 20% higher formazan signal when cells were assayed in 10% FBS rather than in 5% FBS (30). MTT assay is typically affected by the FBS because FBS has albumin protein that binds to formazan and leads to an increase in the signal (31). Besides,

enzymes that may be found in FBS, such as glutathione S-transferase, as well as energy sources required for Na^+ pumping may also affect MTT reduction and cause increase in absorption (32). On the other hand, as albumin or other proteins with free SH groups lead to the artificial increase in the assay signal, Funk *et al.* advised to be careful with data from MTT assays when they are present in the medium. This could lead to an underestimation of the cytotoxicity of the compounds to be tested (33).

Collectively, Our MTT assay results agree with Zhang et al (30) and clearly showed that cytotoxic effect of EESP against all three cancer cells at both 24 and 48 h was more in the presence of 1% FBS than 10% FBS .

In the next step, BrdU assay results showed about 60-75% proliferation inhibition of EESP on the three breast cancer cells following 48 h ($p< 0.0001$). The proliferation of SKBR-3 cell was less inhibited by EESP than those of MDA-MB-231 and MCF-7 cell lines (Fig. 2). Our results on the SKBR-3 cell is similar to the results published earlier (18).

Apoptosis is one of the defense mechanisms used by organisms against cancer, which eliminates potentially deleterious, mutated cells. Many dietary cancer preventive compounds, including propolis and its active derivatives, induce apoptosis in cancer cells. The mechanism of occurring apoptosis by propolis may depend on the concentration of the applied extract. Our results strongly suggest that 200 µg/mL EESP with 1% FBS triggered about 22%, 23% and 26% apoptosis in MCF-7, SKBR-3 and MDA-MB-231 cells respectively following 48 h. The results supported that MDA-MB-231 cell was more sensitive than SKBR-3 and MCF-7 cells (Fig. 5). In contrary, EESP induced the highest early apoptosis on SKBR-3 cell. Collectively, our finding indicates that cytotoxicity of EESP on three breast cancer cells seems to be related to apoptotic effects. There are some reports that propolis could induce apoptosis in breast cancer cells (34, 35) Syhan et al. showed that Turkey type 3 propolis (at 100 and 250 µg/mL) inhibited MCF-7, SKBR-3, and MDA-MB-231 cells growth via its apoptotic effects (18). Ethyl acetate extract of propolis at the concentration of 47.45 µg /mL also induced 13.21% apoptosis in MCF-7 cells, during the 24 h incubation (36). Likewise, Brazilian red propolis significantly reduced MCF-7 cell viability through induction of apoptosis (26). Ethanolic-extract of Chinese propolis again inhibited MDA-MB-231 breast cancer cell proliferation through activating apoptosis (37). Our finding along with the above reports demonstrated that apoptosis triggering rate of different propolis on breast cancer cells is related to the propolis type-, concentration- and incubation time.

Additionally, Cell cycle arrest is another major cause that can inhibit breast cancer cells survival. Here, we found that EESP induced G_0/G_1 phase arrest in MCF-7 and MDA–MB-231 cells and S phase arrest in SKBR-3 cell. Many other propolis extracts have the same effects as EESP on MDA-MB-231 and MCF-7 cells. Popolo et al. showed that Brown Cuban propolis exhibited anti-proliferative activity and significant G_1 phase arrest on MCF-7 cells in a dose and time-dependent manner (13). Also, Noureddine et al. showed cell cycle arrest in the Sub G_0 fraction in MDA-MB-231 cells after 24 h incubation with Lebanese propolis (38). Furthermore, Brazilian red propolis significantly increased sub G_1-G_0 phase of MCF-7 (26). Besides, flavonoid of propolis induced apoptosis on MCF-7 and MDA-MB-231 cancer cell lines and induced G_1 phase arrest in MCF-7 cells. Despite our finding, G_2/M phase arrest also occurred on MDA-MB-231 cell (15).

Along with the above reports, our results demonstrated that EESP induced cell cycle arrest on breast cancer cells including SKBR-3 cell which was not evaluated previously. Also, ethanolic extract of propolis from various regions induced differential cell cycle arrest on breast cancer cells. Small differences may have arisen due to different methods of propolis extraction, geographic region, harvest season and races of honeybee involved. The induction of cell cycle arrest at a specific checkpoint, and thereby inducing apoptosis, is a common mechanism for the cytotoxic effects of anticancer drugs (39-41).

Collectively, the results demonstrated that EESP has anticancer, anti-proliferation and pro-apoptotic effects and causes cell cycle arrest in the three breast cancer cells. Our finding suggests that this extract contains compounds with anticancer activities. Other studies suggested that propolis compounds such as flavonoids in propolis have anticancer effect (15) (42) It is recommended to identify and isolate the active compounds of this extract which have anticancer properties. The molecular anticancer mechanisms of Iranian propolis have not been fully elucidated and need to be deeply clarified.

Acknowledgment

This work has been supported by the University of Shiraz. The authors declare no conflict of interest.

References

1. Coughlin SS, Ekwueme DU. Breast cancer as a global health concern. Cancer Epidemiol 2009; 33(5):315-8.
2. Ferlay J, Hery C, Autier P, Sankaranarayanan R. Global burden of breast cancer. In: Li C. Breast Cancer Epidemiology. New York: Springer; 2010. p.1-19.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66(2):115-32.
4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68(6):394-424.
5. Mitra S, Dash R. Natural products for the management and prevention of breast cancer. Evid Based Complement Alternat Med 2018; 2018:8324696.
6. Sanders K, Moran Z, Shi Z, Paul R, Greenlee H. Natural products for cancer prevention: clinical update 2016. Semin Oncol Nurs 2016; 32(3):215-40.
7. Zhang QY, Wang FX, Jia KK, Kong LD. Natural product interventions for chemotherapy and radiotherapy-induced side effects. Front Pharmacol 2018; 9:1253.
8. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016; 79(3):629-61.
9. Pietta PG, Gardana C, Pietta AM. Analytical methods for quality control of propolis. Fitoterapia 2002; 73(Suppl 1):S7-20.
10. Bankova V, Christov R, Kujumgiev A, Marcucci MC, Popov S. Chemical composition and

antibacterial activity of Brazilian propolis. Z Naturforsch C J Biosci 1995; 50(3-4):167-72.

11. Orsolic N, Basic I. Water-soluble derivative of propolis and its polyphenolic compounds enhance tumoricidal activity of macrophages. J Ethnopharmacol 2005; 102(1):37-45.

12. Burdock GA. Review of the biological properties and toxicity of bee propolis (propolis). Food Chem Toxicol 1998; 36(4):347-63.

13. Popolo A, Piccinelli LA, Morello S, Cuesta-Rubio O, Sorrentino R, Rastrelli L, et al. Antiproliferative activity of brown Cuban propolis extract on human breast cancer cells. Nat Prod Commun 2009; 4(12):1711-6.

14. Xuan H, Li Z, Yan H, Sang Q, Wang K, He Q, et al. Antitumor activity of chinese propolis in human breast cancer MCF-7 and MDA-MB-231 cells. Evidence-Based Complementary and Alternative Medicine 2014; 2014:280120.

15. Kabala-Dzik A, Rzepecka-Stojko A, Kubina R, Iriti M, Wojtyczka RD, Buszman E, et al. Flavonoids, bioactive components of propolis, exhibit cytotoxic activity and induce cell cycle arrest and apoptosis in human breast cancer cells MDA-MB-231 and MCF-7 – a comparative study. Cell Mol Biol (Noisy-le-grand) 2018; 64(8):1-10.

16. Omene C, Kalac M, Wu J, Marchi E, Frenkel K, O'Connor OA. Propolis and its Active Component, Caffeic Acid Phenethyl Ester (CAPE), modulate breast cancer therapeutic targets via an epigenetically mediated mechanism of action. J Cancer Sci Ther 2013; 5(10):334-42.

17. Padmavathi R, Senthilnathan P, Chodon D, Sakthisekaran D. Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7,12 dimethyl benz(a)anthracene-induced breast cancer in female Sprague Dawley rats. Life Sci 2006; 78(24):2820-5.

18. Seyhan MF, Yilmaz E, Timirci-Kahraman O, Saygili N, Kisakesen HI, Gazioglu S, et al. Different propolis samples, phenolic content, and breast cancer cell lines: Variable cytotoxicity ranging from ineffective to potent. IUBMB Life 2018; 71(5):619-31.

19. Muli EM, Maingi JM, Macharia J. Antimicrobial properties of propolis and honey from the Kenyan stingless bee, Dactylurina schimidti. Apiacta 2008; 43:49-61.

20. Yemis O, Bakkalbaşi E, Artik N. Antioxidative activities of grape (Vitis vinifera) seed extracts obtained from different varieties grown in Turkey. Int J Food Sci Technol 2009; 43(1):154-9.

21. Barlak Y, Deger O, Colak M, Karatayli SC, Bozdayi AM, Yucesan F. Effect of Turkish propolis extracts on proteome of prostate cancer cell line. Proteome Sci 2011; 9:74.

22. Chan GC, Cheung KW, Sze DM. The immunomodulatory and anticancer properties of propolis. Clin Rev Allergy Immunol 2013; 44(3):262-73.

23. Frion-Herrera Y, Diaz-Garcia A, Ruiz-Fuentes J, Rodriguez-Sanchez H, Mauricio Sforcin J. Mechanisms underlying the cytotoxic effect of propolis on human laryngeal epidermoid carcinoma cells. Nat Prod Res 2018; 32(17):2085-91.

24. Aguero MB, Svetaz L, Sanchez M, Luna L, Lima B, Lopez ML, et al. Argentinean Andean propolis associated with the medicinal plant Larrea nitida Cav. (Zygophyllaceae). HPLC-MS and GC-MS characterization and antifungal activity. Food Chem Toxicol 2011; 49(9):1970-8.

25. Frion-Herrera Y, Diaz-Garcia A, Ruiz-Fuentes J, Rodriguez-Sanchez H, Sforcin JM. The cytotoxic effects of propolis on breast cancer cells involve PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential, and reactive oxygen species generation. Inflammopharmacology 2019; 27(5):1081-9.

26. Kamiya T, Nishihara H, Hara H, Adachi T. Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. J Agric Food Chem 2012; 60(44):11065-70.

27. Popolo A, Piccinelli AL, Morello S, Sorrentino R, Osmany CR, Rastrelli L, et al. Cytotoxic activity of nemorosone in human MCF-7 breast cancer cells. Can J Physiol Pharmacol 2011; 89(1):50-7.

28. Zhou K, Li X, Du Q, Li D, Hu M, Yang X, et al. A CAPE analogue as novel antiplatelet agent efficiently inhibits collagen-induced platelet aggregation. Pharmazie 2014; 69(8):615-20.

29. Seda Vatansever H, Sorkun K, Ismet Deliloglu Gurhan S, Ozdal-Kurt F, Turkoz E, Gencay O, et al. Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. Acta Histochem 2010; 112(6):546-56.

30. ZD Z, Cox G. MTT assay overestimates human airway smooth muscle cell number in culture. Biochem Mol Biol Int 1996; 38(3):431-6.

31. Peters T. All About Albumin: Biochemistry, Genetics and Medical Applications. USA: Academic Press; 1995.

32. Newman JM, DiMaria CA, Rattigan S, Steen JT, Miller KA, Eldershaw TP, et al. Relationship of MTT reduction to stimulants of muscle metabolism. Chem Biol Interact 2000; 128(2):127-40.

33. Funk D, Schrenk HH, Frei E. Serum albumin leads to false-positive results in the XTT and the MTT assay. Biotechniques 2007; 43(2):178.

34. Rzepecka-Stojko A, Kabala-Dzik A, Mozdzierz A, Kubina R, Wojtyczka RD, Stojko R, et al. Caffeic Acid phenethyl ester and ethanol extract of propolis induce the complementary cytotoxic effect on triple-negative breast cancer cell lines. Molecules 2015; 20(5):9242-62.

35. Frozza CO, Ribeiro Tda S, Gambato G, Menti C, Moura S, Pinto PM, et al. Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells. Food Chem Toxicol 2014; 63:195-204.

36. Ratna D, Simanjuntak P, Abdillah S, Heffen WL. Apoptosis of human breast cancer cells induced by ethylacetate extracts of Propolis. American Journal of Biochemistry and Biotechnology 2010; 6(2):84-8.

37. Chang H, Wang Y, Yin X, Liu X, Xuan H. Ethanol extract of propolis and its constituent caffeic acid phenethyl ester inhibit breast cancer cells proliferation in inflammatory microenvironment by inhibiting TLR4 signal pathway and inducing apoptosis and autophagy. BMC Complement Altern Med 2017; 17(1):471.

38. Noureddine H, Hage-Sleiman R, Wehbi B, Fayyad-Kazan H, Hayar S, Traboulssi M, et al. Chemical characterization and cytotoxic activity evaluation of Lebanese propolis. Biomed Pharmacother 2017; 95:298-307.

39. Sawicka D, Car H, Borawska MH, Niklinski J. The anticancer activity of propolis. Folia Histochem Cytobiol 2012; 50(1):25-37.

40. Ahn MR, Kumazawa S, Hamasaka T, Bang KS, Nakayama T. Antioxidant activity and constituents

of propolis collected in various areas of Korea. J Agric Food Chem 2004; 52(24):7286-92.

41. Li LK, Rola AS, Kaid FA, Ali AM, Alabsi AM. Goniothalamin induces cell cycle arrest and apoptosis in H400 human oral squamous cell carcinoma: a caspase-dependent mitochondrial-mediated pathway with downregulation of NF- kb. Arch Oral Biol 2016; 64:28-38

42. Lirdprapamongkol K, Sakurai H, Abdelhamed S, Yokoyama S, Maruyama T, Athikomkulchai S, et al. A flavonoid chrysin suppresses hypoxic survival and metastatic growth of mouse breast cancer cells. Oncol Rep 2013; 30(5):2357-64.