ORIGINAL RESEARCH

DOI: 10.17944/interdiscip.1466355
Interdiscip Med J 2024;15(52):80-85

Investigation of apoptotic efficacy of propolis in MCF-7 cell line

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Abstract

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Objective: Propolis, also known as bee glue, is a resinous compound collected by honey bees from various plants and processed by their saliva enzymes. Propolis and its components have been studied for their cytotoxic effects on cell lines in vitro, and recent studies have shown that they also have an antitumor effect in vivo. This study aimed to investigate the in-vitro apoptotic effects of propolis on the human breast cancer cell line (MCF-7).

Method: The MTT test was used to determine the effect of propolis on cell viability and the doses to be administered. The GraphPad Prism Version 6.01 program was used to analyze the MTT results, while the qRT-PCR method was used to determine the expression levels of Caspase-8, Caspase-9, and Bcl-2 genes. The RT2 profiler PCR Assay Data Analysis version 3.5 was used to analyze gene expression data.

Results: This study it was found that doses of 3.9 and 7.8 µg/ml of propolis showed no cytotoxic effect, while doses of 15.625 µg/ml and above had a cytotoxic effect. There was no change in the expression levels of genes at concentrations of 3.9 µg/ml and 7.8 µg/ml of propolis. However, at 15.625 µg/ml of propolis, Caspase-9 gene expression increased 11.89-fold ($p=0.033$). Although there was no significant difference in Caspase-8 gene expression in the extrinsic pathway of apoptosis ($p=0.437$), a 0.04-fold decrease in anti-apoptotic Bcl-2 gene expression was observed ($p=0.000098$).

Conclusion: In conclusion, propolis showed a dose-dependent cytotoxic effect on the MCF-7 cell line, induced apoptosis, and did so via the intrinsic pathway of apoptosis. The study suggests that propolis has high potential as an anticancer agent since its apoptotic effects have been demonstrated in the MCF-7 cell line.

Keywords: Propolis, MCF-7, Apoptosis, Caspase-9, Bcl-2

INTRODUCTION

Propolis is a resinous bee product manufactured by honey bees *Apis mellifera* L.(1). This natural product, also known as bee gum, is used by bees to repair the hive to keep the temperature and humidity constant by covering the inner surface of the hive and to protect the hive from external factors. Additionally, bees use propolis for hive sterilization (2).There are many active compounds in the structure of propolis, and the composition of the active compounds it contains varies depending on the region and season where propolis is collected (3). Studies have reported that propolis has antibacterial (4-6), antioxidant (7), anti-inflammatory (7), antifungal (8-9), antiviral (10-12), and anticarcinogenic (13-16) effects. Studies conducted in recent years have demonstrated the cytotoxic effects of propolis on cancer cell lines and its anticarcinogenic effects in in vivo studies (17-18). In molecular studies, it has been published that propolis components stop the cell cycle and exhibit anticarcinogenic effects by directing cancer cells to apoptosis (19-20). This effect of propolis has been reported to be due to its anti-proliferative properties, as it arrests the cell cycle in many cancerous cells and induces both death receptor-mediated and mitochondrial apoptosis. (20-21).

Cancer is characterized simply by genetic changes accumulating in the cell and is still one of the most dangerous diseases today (22-23). According to 2020 records, 10 million cancer-related deaths occurred worldwide, and 19.3 million new cancer cases were reported. Among cancer types, breast cancer surpassed the number of lung cancer cases with 2.3 million new cases (11.7%) and was accounted as the most common cancer type (24). Cancer can also be defined as an imbalance between cell gain and cell loss, in which the rate

Cite this article: Duran GG. Investigation of apoptotic efficacy of propolis in MCF-7 cell line. Interdiscip Med J. 2024;15(52):80-85. https://doi.org/10.17944/interdiscip.1466355

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Received: Apr 8, 2024
Accepted: Jun 30, 2024

of increasing mutant tumor cells exceeds the rate of dying cells. Apoptosis, the most well-known form of programmed cell death, is a significant physiological mechanism that limits the expansion of the cell population to maintain tissue homeostasis or to eliminate potentially harmful cells that have persistent DNA damage (25). From the onset of cancer, the apoptosis mechanism is the first process that prevents the proliferation of tumor cells. Apoptosis relies on the activation of different signaling pathways that are frequently released in cancer. Therefore, investigating the apoptotic compounds underlying their expression in carcinogenesis is meaningful and helpful in monitoring the progression of the disease (26).

In this study, it was aimed to investigate whether propolis triggers apoptosis in MCF-7 (breast cancer cell line) cells in vitro, if so, through which apoptotic pathway it occurs and whether it can have an anticarcinogenic effect by causing MCF-7 cancer cells to undergo apoptosis.

METHOD

Cell Culture:

MCF-7 cells, a breast cancer cell line, were used in the experiments. The cell line was obtained from the cell culture stock of Hatay Mustafa Kemal University, Faculty of Medicine, Department of Medical Biology. All experiments were carried out in Hatay Mustafa Kemal University, Faculty of Medicine, Medical Biology laboratories. The cell line was cultivated in DMEM (Dulbecco's Modified Eagle Medium, DMEM, Gibco, UK) containing 10% FBS and 1% penicillin/streptomycin. Incubation of the cells was carried out at 37 °C in an atmosphere of 5% carbon dioxide and 95% humidity. The medium was changed when necessary depending on the change in pH, and it was used in activity studies when the cells grew to cover 70-80% of the culture vessel surface.

Preparation of Propolis:

Propolis samples were supplied from Hatay Mustafa Kemal University, Faculty of Agriculture, Department of Animal Sciences. The propolis extract was obtained by dissolving 300 grams of propolis, which was collected from Hatay province and then powdered, with a 70% ethanol/water solution. The homogenization processes of propolis samples were carried out according to the method described by Mendonca et al (27). Alcohol in the samples was evaporated by shaking in the evaporator for 36 hours at 45 rpm at 50-55 °C. Stock solutions of the propolis were prepared by dissolving solid propolis samples in 1% DMSO.

Cell Viability Analysis:

We performed two separate MTT assays to evaluate the effect of DMSO and propolis on cell viability. Firstly, to determine the non-cytotoxic concentrations of DMSO, the cells were exposed to medium containing different concentrations of DMSO (%1-5) for 24 hours. The cell density used was 1x105 cells/ml, and the experiments were conducted in 24-well plates under specific cultivation conditions, including 95% humidity, 5% CO2, and 37°C temperature. Once the plates reached 70-80% coverage of the culture vessel surface, DMSO dilutions were added to the culture medium and incubated for 24 hours. Following the incubation period, the medium was removed, and 1 mg/ml MTT (Sigma) was added to each well and then incubated for four hours. Next, 0.5 ml DMSO solution was added to each well and incubated at room temperature for five minutes. Then, to detect the colorimetric change, the plates were measured by reading the plates at 590 nm and 670 nm with a spectrophotometer (MultiScan Go, ThermoFisher, Finland). To evaluate the effect of propolis on cell viability, dilutions of propolis in serum-free DMEM medium were prepared at concentrations of 500 - 250 - 125 - 62.5 - 31.25 -15.625 - 7.8 - 3.9 and 0 µg/mL and the above analysis steps were applied.

Table 1. Primer sequences of apoptosis-related genes

Genes	Forward Primers	Reverse Primers
Caspase-8	5'-AGGGCTCAATTTCTGCCTAC-3'	5'-GGCACTGGCTGTTTGCTT-3'
Caspase-9	5'-GTCACAAGACCTTGACACCCG-3'	5'-ACCAGGTGGTCTAGGGGTTT-3'
β-actin	5'-TCAACACCCCAGCCATGTA-3'	5'-AGTACGGCCAGAGGTGTACG-3'
Bcl-2	QuantiTect Primer Assay Cat No: QT00025011, Lot No: 286236904	

Gene Expression Analysis

Propolis dilutions ranging from 15.625 to 3.9 were appended to MCF-7 cells that had grown to cover 70-80% of the culture plates and were treated for 24 hours. At the end of the incubation, total RNA was isolated from the cells using a total RNA isolation kit (Thermo Scientific GeneJET) as per the manufacturer's instructions. The quality and concentration of the obtained RNAs were determined, and the RNA concentration was adjusted to 0.2 µg/µl. Subsequently, cDNA was obtained using the cDNA synthesis kit (Applied Biosystems). The expression levels of Caspase 8, Caspase 9, Bcl-2, and the housekeeping gene β–actin were calculated using the qRT-PCR method (QIAGEN Rotor-Gene Q ,Germany). Gene expressions in MCF-7 cells without propolis added were used as a control group. The primers used for qRT-PCR are given in Table 1. The first denaturation was carried out for 10 minutes at 95°C, followed by qRT-PCR for 40 cycles (15 seconds at 95°C, 60 seconds at 60°C). The expression levels were calculated using the 2-ΔΔCt method, and the results are presented as fold changes.

Statistical analysis

GraphPad Prism Version 6.01 software was used to analyze cell viability assay. Shapiro-Willk test was used to examine whether there is any normal distribution within groups. The Kruskal-Wallis test was performed to compare the differences

among groups. The statistical difference between two groups was determined with Dunn test. Gene expression data was analyzed with RT2 profiler PCR Assay Data Analysis version 3.5 (Qiagen,online service). β–actin house-keeping gene was used for the normalization of the data. The gene expression results were expressed as "fold change" compared to the control group. $p < 0.05$ was considered as significant for statistical comparisons.

RESULTS

Cell Viability

It was determined that 1% DMSO solution did not have a cytotoxic effect on MCF-7 cells (p>0.05) (Figure 1). Considering the effects of propolis samples on cell viability, the concentrations determined by the MTT method were 3.9 and 7.8 µg/ml. There is no statistically significant difference in cell viability between these two concentrations and the control group (p>0.05). Propolis concentrations >15,625 µg/ml were toxic (Figure 2). Cell viabilities were determined as 95.6%, 95.4%, and 79.7% at propolis concentrations of 3.9, 7.8, and 15.625 µg/ml, respectively. When the propolis concentration was increased further (31.25, 62.5, 125, 250, and 500 µg/ml), deeper decreases in cell viability were detected (36.8, 8, 5.4, 6.1, and 2.7%, respectively). For this reason, in gene expression experiments, two concentrations (3.9, 7.8 µg/ml), which were statistically indifferent to the control group cells in terms of cell viability, and propolis concentrations of 15.625 µg/ml, which were above the IC_{50} value, were selected.

Gene Expression

At 3.9 and 7.8 µg/ml concentrations of propolis, Caspase 8 expression, which is involved in the extrinsic apoptosis pathway, was found to be 0.57 and 0.09 fold, respectively. There was no statistically significant difference compared with the control group (p>0.05). At the same concentrations, 0.51- and 0.63-fold increases were detected in the expression of Caspase 9, which is involved in the intrinsic apoptosis

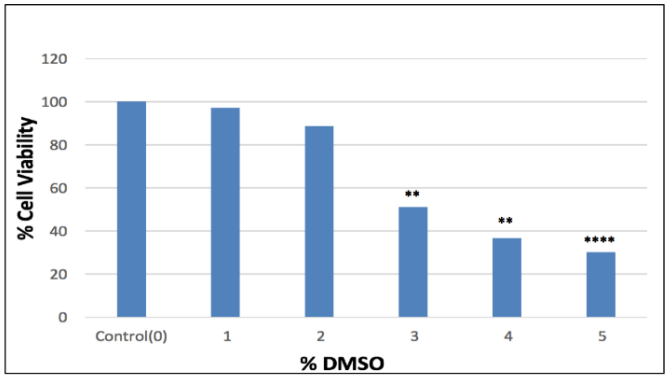

Figure 1. Effect of DMSO on cell viability in MCF-7 cell line. (Statistical significance level: *p<0.05, **p<0.01 and ***p<0.001). *p<0.05, **p<0.01 and ***p<0.001)

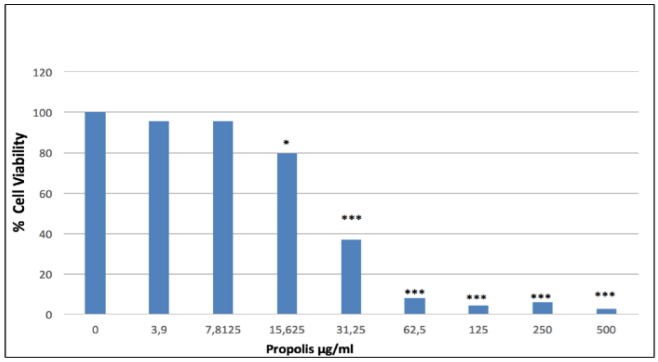

Figure 2. Effect of propolis on cell viability in MCF-7 cell line. (Statistical significance level: *p<0.05, **p<0.01 and ***p<0.001). *p<0.05, **p<0.01 & ***p<0.001)

pathway, respectively. Similarly, there are no significant differences between these groups and the control group (p>0.05). A 1.45-fold and 0.15-fold increase in Bcl-2 expression levels was detected in cells treated with 3.9 and 7.8 µg/ml concentrations of propolis, respectively, and there was no significant difference compared to the control group. Although a decrease was detected in the expression level of cells treated with a propolis concentration of 7.8 µg/ml, it was not statistically significant (p>0.05). When propolis concentration was increased (15.625 µg/ml), the level of Caspase 8 expression was found to increase 0.7-fold, similarly, there is no significant difference with the control group (p>0.05). At the same concentration, an 11.89-fold increase in Caspase 9 expression level was detected. This concentration of propolis significantly increased Caspase 9 gene expression compared to the control group ($p = 0.033$). This propolis concentration (15.625 µg/ml) affected Bcl-2 expression by

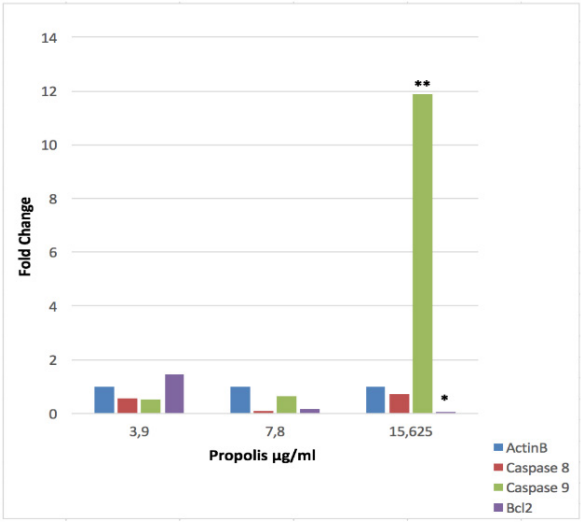

Figure 3. Effect of propolis on the expression levels of apoptotic genes in MCF-7 cell line. (Statistical significance level: *p<0.05, **p<0.01 & ***p<0.001).

0.04-fold, leading to a significant decrease compared to the control group (p = 0.000098) (Figure 3).

DISCUSSION

Breast cancer is one of the leading causes of death among women worldwide. These malignant tumors have very diverse characteristics, including their clinical development, prognosis, and molecular mechanisms (28). According to the latest published cancer statistics of the Ministry of Health of the Republic of Turkey, breast cancer is the most common type of cancer in women (29). In addition to surgical methods, chemotherapy, radiotherapy, and immunotherapy can also be applied in the treatment of breast cancer. However, the majority of patients experience relapses after treatment (30). Moreover, the currently available anticancer drugs have significant problems such as drug resistance and severe side effects, which limit their use (31). Therefore, researchers worldwide are conducting studies on new therapeutic agents of natural origin that are selectively effective against cancer cells. Propolis, a plant-derived honey bee product in the form of resin, is one of many natural products that exhibit anticancer potential. The unique feature of propolis that has attracted the attention of many researchers is that it selectively targets cancer cells, which makes it an alternative to traditional chemotherapeutic drugs (28).

The results of propolis applied in the literature were contradictory. This is because the content of propolis, a natural product, varies depending on the region where it is collected (32). For this reason, the MTT test was taken as the basis for determining the propolis doses to be used. In a study conducted by Amalia et al., it was reported that propolis had a cytotoxic effect on MCF-7 cells (33). In this study, firstly the cytotoxic effects of propolis were examined. Propolis was detected to have a cytotoxic effect on MCF-7 cells at concentrations above 15,625 µg/ml. An inhibition of 20.3% was detected at a concentration of 15.625 µg/ml. It was determined that as propolis concentration increased, the decrease in cell viability increased in direct proportion. Unlike the study conducted by Amalia et al., serious cytotoxic effects were detected even at low propolis concentrations. We think that this difference may be due to factors such as the geographical region where propolis is collected, vegetation, and climatic conditions affecting the composition of propolis. In a similar study by Xuan et al., it was reported that propolis may have a cytotoxic effect on the MCF-7 cell line, depending on dose and time (34).The results of this study are parallel to the study of Xuan et al., and dose-dependent cytotoxicity was detected. The results of another study conducted by Seyhan et al., with propolis samples collected from different countries were also compatible with our findings. In the studies of Seyhan et al., strong cytotoxicity was reported in two different propolis samples at a concentration of 2.5 µg/ml at 24 hours. It has also been reported that cellular apoptosis was triggered in a time-related manner in a sample (35).

Many studies are reporting that propolis has an anti-carcinogenic effect by inducing apoptosis in cancer cells. In a study conducted by Vatansever et al., the MCF7 cell line was treated with various propolis extracts (36). The presence of apoptotic cells was detected using the TUNEL method, and caspase activation was demonstrated through immunohistochemical methods. Mısır et al. reported that propolis induces apoptosis in MCF-7 cells by arresting the cell cycle in the G1 phase, increasing the expression of pro-apoptotic genes, and decreasing the mitochondrial membrane potential (37). Xuan and colleagues reported that propolis induced apoptosis in the MCF-7 cell line. (34). Niyomtham and his colleagues showed in their study that propolis induces apoptosis in the HNSCC (head and neck squamous carcinoma) cell line. (38). Similarly, in the study conducted by Amalia et al., it was reported that propolis initiated apoptosis in the MCF-7 cell line (33). Jiang et al also found that propolis significantly inhibited the growth of human gastric cancer SGC-7901 cells and they reported that it causes cell apoptosis and cell cycle arrest in the S phase, with increased production of reactive oxygen species (ROS) and decreased mitochondrial membrane potential. (20). This study findings, consistent with all these studies in the literature, showed that propolis induces apoptosis in a dose-dependent manner.

Although there are many studies in the literature showing that propolis induces apoptosis in the MCF-7 cell line, no study measuring the expression levels of apoptotic genes has been found. This study is unique by measuring the expression levels of two different caspase genes, which allows distinguishing between intrinsic and extrinsic pathways. It was determined that propolis increased the Caspase-9 expression level by 11.89 times at a concentration of 15.625 µg/ml, while it decreased the expression level of the anti-apoptotic Bcl2 gene by 0.04 times. These results are compatible with all other studies showing that propolis induces apoptosis, as well as showing that it induces apoptosis through the (intrinsic) mitochondrial pathway.

CONCLUSION

The search for natural therapeutic agents with minimal or no side effects in cancer treatment has been ongoing for several years. Propolis is a strong candidate for such a therapeutic agent. In the MCF-7 cell line, it has demonstrated a dose-dependent cytotoxic effect and induced apoptosis through the intrinsic pathway. Its apoptotic effects have been demonstrated in the MCF-7 cell line, which suggests that propolis has high potential as an anticarcinogenic agent. In order to reveal the this potent potential of propolis, further molecular pathway studies in cancer cells need to be carried out.

Limitations of the study

The limitation of this study is that apoptotic cells cannot be demonstrated with more advanced techniques.

ACKNOWLEDGEMENT

Peer-Review

Both externally and internally peer reviewed.

Conflict of Interest

The authors declare that they have no conflict of interests regarding content of this article.

Financial Support

The Authors report no financial support regarding content of this article.

Previously Presented

The authors declare that some part of this study was presented as oral presentation at "the XVI. Medical Biology and Genetics Congress" held in Bodrum/Muğla city, entitled as "Investigation of Anti-Carcinogenic Efficacy of Propolis in MCF-7 Cell Line".

Ethical Declaration

Since this study was conducted with commercially obtained cell culture, an ethics committee certificate is not required.

Authorship Contributions

Concept: GGD, Design: GGD, Supervising: GGD, Financing and equipment: GGD, Data collection and entry: GGD, Analysis and interpretation: GGD, Literature search: GGD, Writing: GGD, Critical review: GGD

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