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Propolis: Intrinsic Pathway-Induced Apoptosis, G1 Cell Cycle Arrest, Reduced Chemotherapeutic Resistance in Adenocarcinoma, and Healthy Cell Preservation

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ABSTRACT

Objective: This study investigated the potential synergistic effects of propolis, an antitumor and antioxidant natural product, and carboplatin, a frequently used chemotherapeutic agent for endometrial adenocarcinoma, one of the most common gynaecological cancers treatment.

Materials and Methods: Ishikawa endometrial adenocarcinoma and healthy fibroblast (3T3) cell lines were treated with 0.5 µL of carboplatin and 5 µL of propolis. Cell count, viability, migration, ultrastructure, apoptosis, and cell cycle changes were assessed using cytological and immunocytochemical methods, flow cytometry, and transmission electron microscopy (TEM).

Results: Propolis and carboplatin exhibited cytotoxic effects on Ishikawa cells. The combination of the two agents further reduced cell viability and migration. Propolis induced apoptosis through the intrinsic pathway and arrested the cell cycle in the G1 phase. TEM analysis revealed apoptosis in Ishikawa cells treated with propolis or carboplatin, while the carboplatin+propolis combination resulted in severe cell budding, apoptosis, and vacuolization. Migrasome-like structures were only observed in the Ishikawa carboplatin group. Minimal effects were observed on 3T3 cells.

Conclusion: Propolis demonstrated cytotoxic, anti-proliferative, and proapoptotic effects on tumor cells without harming healthy cells. Its ability to prevent migrasome formation suggests it may reduce chemotherapeutic resistance. Therefore, propolis shows promise as a potential enhancer of anticancer treatments.

Keywords: 3T3, apoptosis, electron microscopy, endometrium, Ishikawa, migrasome

INTRODUCTION

Endometrial cancer is the most common cancer affecting women in both developed and developing countries worldwide, with the potential to cause infertility and even death in affected individuals (1-4). The Ishikawa endometrial epithelium continuous cell line, which expresses both estrogen and progesterone receptors, is a widely used model to study endometrial adenocarcinoma, while the 3T3 fibroblast cell line was often employed as a control group in various *in vitro* studies (4, 5). The platinum group chemotherapeutic agent, carboplatin, is the preferred treatment option for endometrial cancer, with the aim of controlling the disease and increasing patient survival rates (6, 7).

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There is promising evidence to suggest that combining carboplatin with herbal agents can improve cytotoxicity and increase survival rates in patients with endometrial cancer (8, 9). One such herbal supplement is propolis, a resinous substance collected by honeybees from plants, which has been shown to have anticarcinogenic, antioxidant, antifungal, and antibacterial effects, and have cytotoxic effect on various cancer cell lines (10-14). However, there is gap of research investigating the effects of propolis on endometrial cancer at a structural and ultrastructural level, and its mechanism of action remains poorly understood.

In this study, we aimed to investigate the potential of propolis extract to enhance the cytotoxic, apoptotic, and antitumor effects of carboplatin on the Ishikawa endometrial adenocarcinoma cell line, and to assess its effect on healthy 3T3 fibroblast cells. By elucidating the mechanism of action of propolis and carboplatin on both cancerous and healthy cells, we hope to improve the efficacy of cancer chemotherapy and contribute to the development of new treatment options for endometrial adenocarcinoma.

MATERIALS AND METHODS

Cell Culture

Ishikawa endometrial adenocarcinoma cell line derived from human endometrium (99040201, European Collection of Authenticated Cell Culture ECACC, Merck, Darmstadt, Germany), and 3T3 cell lines from mouse embryo fibroblast cells (86052701, European Collection of Authenticated Cell Culture ECACC, Merck, Darmstadt, Germany). Cell lines were cultured in 10% fetal bovine serum (FBS-11A, Capricorn, Germany), 100 µg/mL streptomycin, 100 IU/mL penicillin and 0.2 µM glutamine were filtered into sterile DMEM/F-12 (12500062, Thermo, Massachusetts, USA) medium. Cells were allowed to proliferate for 72 hours in a humidified incubator at 37°C containing 95% air and 5% CO_2.

After conducting preliminary studies to determine the optimal propolis and carboplatin dosages at various time intervals according to the literature, a total of 8 experimental groups were established (15, 16). In these groups, propolis (5 µL) (Eğriçayır, Istanbul, Turkiye) and carboplatin (0.5 µL) (150 mg/15 mL) (Koçak Farma, Tekirdag, Turkiye) were administered individually and in combination (Table 1). All experiments were conducted in triplicate on Ishikawa cells and 3T3 cells, and each experiment was repeated three times.

Table 1. Experimental groups.

Group 1	Ishikawa Control
Group 2	Ishikawa+Propolis
Group 3	Ishikawa+Carboplatin
Group 4	Ishikawa+Propolis+Carboplatin
Group 5	3T3 Control
Group 6	3T3+Propolis
Group 7	3T3+Carboplatin
Group 8	3T3+Propolis+Carboplatin

Cell Viability Assay

$1x10^5$ numbers of Ishikawa and 3T3 cells were cultured in 12-well cell culture plates. The trypan blue test was performed to determine viability at 24 hours. Cell counts were conducted by using a Neubauer slide under the microscope (Olympus CKX41, Tokyo, Japan).

The viability ratio was calculated as follows: Viable cell ratio (%) = Count of unstained cells/Total number of cells x 100

Measurement of Apoptosis in Cells by Flow Cytometry

The Annexin V-FITC Apoptosis Staining/Detection kit (Abcam-14085, Cambridge, Massachusetts, USA) was used to $1x10^5$ Ishikawa and 3T3 cells following the manufacturer's recommended protocol. 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI) were added to 500 µL of Annexin V binding buffer, followed by a 5-minute incubation in the dark at room temperature. At the 24th hour, the apoptotic effect in the experimental groups were assessed using a flow cytometry device (NovoCyte, ACEA, BD Biosciences, New Jersey, USA).

Cell Migration Assay

The migration of cells was monitored using the wound healing assay. A suspension of $2x10^5$ cells/mL was prepared. Ishikawa and 3T3 cells were seeded in 6-well cell culture plates and allowed for 48 hours to adhere to the ground until they reached confluence. Subsequently, scratches were created with a pipette tip in the wells where the cells were seeded. The wells were then washed with medium to remove cell

Table 2. Effects of drugs on viability at 24th hour in Ishikawa and 3T3 cell lines.

Viability %	Control	Propolis	Carboplatin	Propolis+Carboplatin	p-value
Ishikawa	96.23 ± 1.6	82.24 ± 6.5*	83 ± 3.9*	81.53 ± 3.8*	p<0.01
3T3	95.15 ± 0.7	95.30 ± 2.1	96.30 ± 1.6	95.79 ± 2.0	p>0.05

Cell lines are compared with the control group; values were given as mean ± standard deviation; *p<0.05.

debris, and fresh medium was added and dosed accordingly. The distance of the cells at specified points determined at 0 hours (initial), 24 hours and 48 hours were followed under the inverted microscope at 10x magnification and photographed. Results were measured with the Image J program.

Rate of cell migration was measured as follows:

% of change = - (Initial wound width – Wound width at 24 hours) / Initial wound width x 100

% of change = - (Initial wound width – Wound width at 48 hours) / Initial wound width x 100

Immunocytochemistry

Cytochrome-c was examined to determine the effects of propolis and carboplatin on the intrinsic pathway of apoptosis. P27 showed the effects of propolis and carboplatin on the G1 phase of the cell cycle.

$3x10^4$ Ishikawa and 3T3 cells were seeded and kept in the incubator for 1 hour to adhere to the ground. 5 µL propolis and 0.5 µL carboplatin doses were applied to the cells and incubated for cytochrome-c staining for 24 hours and for p27 staining for 48 hours at 37 °C with 5% CO_2. Cells were fixed with 4% paraformaldehyde. They were then incubated in hydrogen peroxide (H_2O_2) and block serum (TA-125-UB, Thermo Scientific, Massachusetts, USA).

For immunocytochemistry procedures, primary antibodies cytochrome-c (1:100, mouse anti-human, SC-13560, Santa Cruz, California, USA) and p27 (1:100, rabbit anti-human, SC-528, Santa Cruz, California, USA) were applied and incubated at +4°C overnight. A secondary antibody (Biotinylated Goat Anti-Polyvalent, Thermo Scientific, Massachusetts, USA) was applied for 1 hour and washed with PBS. After Streptavidin (TS-125-HR, Thermo Scientific, Massachusetts, USA) application, the cells were stained with AEC chromogen (TA-007-HAC, Thermo Scientific, Massachusetts, USA). Mayer's hematoxylin was used in cytochrome-c-stained preparations for counterstaining. Nuclear counterstaining was not done for p27 due to the staining localization of the primary antibody. At the end of the procedures, cells were sealed with an aqueous mounting medium (TA-125-AM, Thermo Scientific, Massachusetts, USA) and examined under a light microscope (Leica, DMLB, Wetzlar, Germany) and photographed. Two histologists unaware of the experimental groups counted ~400 cells, double-blind, and the mean of stained and unstained cell counts were noted in each preparation.

Staining was calculated as follows: Ratio of Stained Cells (%) = Stained cell count / Total counted cells x 100.

Table 3. Apoptosis rates of Ishikawa and 3T3 cell lines at 24th hour.

Apoptotic Cell %	Control	Propolis	Carboplatin	Propolis+Carboplatin	p-value
Ishikawa	5.68 ± 0.2	12.26 ± 0.4***	6.47 ± 0.2*	12.04 ± 0.3***	p<0.001
3T3	4.15 ± 0.4	3.61 ± 0.02	4.16 ± 0.05	3.69 ± 0.1	p>0.05

Cell lines are compared with the control group; values were given as mean ± standard deviation; *p<0.05, ***p<0.001.

Table 4. Migration rates of Ishikawa and 3T3 cell line at 24th and 48th hour.

Cell Migration %	Control n=30	Propolis n=30	Carboplatin n=30	Propolis+Carboplatin n=30	p-value
Ishikawa 24th hour	75.27 (86.99, 33.34)	54.95** (77.04, 33.42)	44.96** (74.63, 10.16)	38.06** (56.76, 13.20)	p<0.01
Ishikawa 48th hour	98.47 (99.68, 94.95)	77.18** (90.38, 50.80)	80.51** (95.52, 59.06)	76.35** (88.40, 60.65)	p<0.01
3T3 24th hour	62.67 (82.39, 22.37)	76.70* (94.62, 33.03)	79.26* (92.90, 53.52)	86.01*** (95.52, 35.39)	p<0.001
3T3 48th hour	86.11 (96.50, 64.11)	95.48** (98.40, 65.25)	91.99 (96.19, 75.26)	96.63** (98.77, 83.83)	p<0.01

Cell lines are compared with the control group; values were given as median (max, min); *p<0.05 **p<0.01 ***p<0.001.

Table 5. Cytochrome-c ratios of Ishikawa and 3T3 cell lines at 24th hour.

Cytochrome-c %	Control	Propolis	Carboplatin	Propolis+Carboplatin	p-value
Ishikawa	5.67 ± 0.7	11.63 ± 0.8***	7.01 ± 0.3	13.34 ± 0.9***	p<0.001
3T3	4.73 ± 0.1	3.91 ± 1.3	4.74 ± 1.2	4.00 ± 0.3	p>0.05

Cell lines are compared with the control group; values were given as mean ± standard deviation; ***p<0.001.

Table 6. p27 ratios of Ishikawa and 3T3 cell lines at 48th hour.

p27 %	Control	Propolis	Carboplatin	Propolis+Carboplatin	p-value
Ishikawa	9.96 ± 3.5	16.30 ± 2.7	15.33 ± 3.7	22.15 ± 3.4**	p<0.05
3T3	15.68 ± 4.0	15.15 ± 4.2	16.35 ± 1.6	16.80 ± 0.7	p>0.05

Cell lines are compared with the control group; values were given as mean ± standard deviation; **p<0.01.

Investigation of Ultrastructural Changes in Cells by Transmission Electron Microscopy (TEM)

$5x10^5$ live cells from each group were used for the TEM examination. The samples collected at the 24th hour from the experimental groups were prepared for investigation. Cells were fixed in 2.5% glutaraldehyde (104239, Merck, Darmstadt, Germany) solution for primary fixation and 1% osmic acid (124505.0100, Merck, Darmstadt, Germany) in PBS for secondary fixation. Cells were washed with PBS in between the steps. A graded series of alcohol were used for dehydration. After the propylene oxide/epon mixtures, cells were embedded in epon (45359-1EA-F, Epon 812, Sigma, Missouri, USA) and polymerized for 18 hours at 60°C. Thin sections of 60 nm were taken with an ultramicrotome (EM UC7, Leica, Wetzlar, Germany) to pyeloform-coated copper grids. Contrast was performed with uranyl acetate and lead citrate. Three histologists evaluated the specimens using TEM (JEM 1011, JEOL, Tokyo, Japan).

Statistical Analyses

The statistical analyses of the study were conducted using GraphPad Prism 9.2.0 (GraphPad Software, San Diego, California, USA) program and MS-Excel 2013. Normal distribution was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests for all data. For normally distributed groups, one-way ANOVA test and post-hoc Tukey tests were employed, while Kruskal-Wallis and post-hoc Dunn's tests were used for groups that did not exhibit normal distribution. Evaluations between Ishikawa and 3T3 groups were performed using Student's t-test and Mann-Whitney U test. A significance level of p<0.05 was considered statistically significant. The results presented in the tables included mean ± standard deviation for normally distributed groups and median, R (max, min) for non-normally distributed groups. The average of three replicates was taken for the study. Changes in wound width during migration experiments were calculated using the percentage change

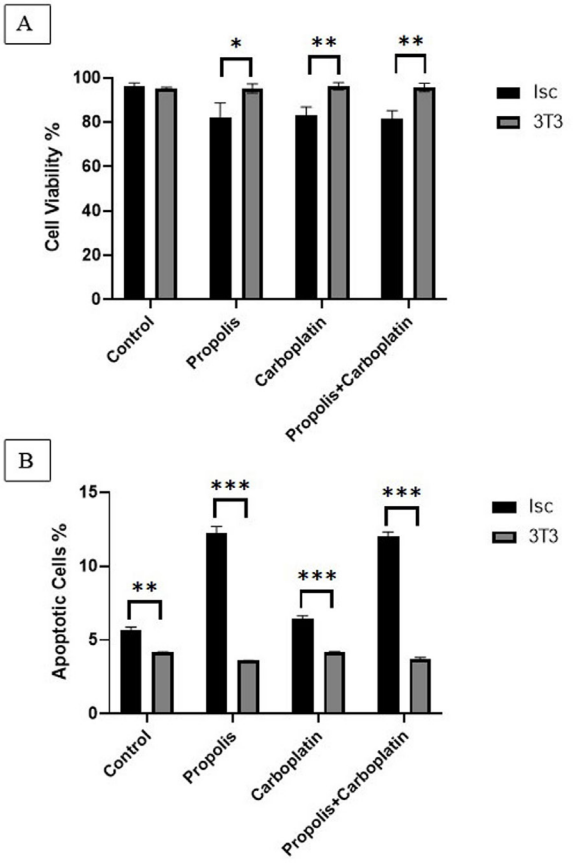

Figure 1. Comparison of Ishikawa (Isc) and 3T3 cell line A) viability and B) apoptosis at 24 hours between groups. Data was expressed as mean and standard deviation, ***p<0.001, **p<0.01, *p<0.05.

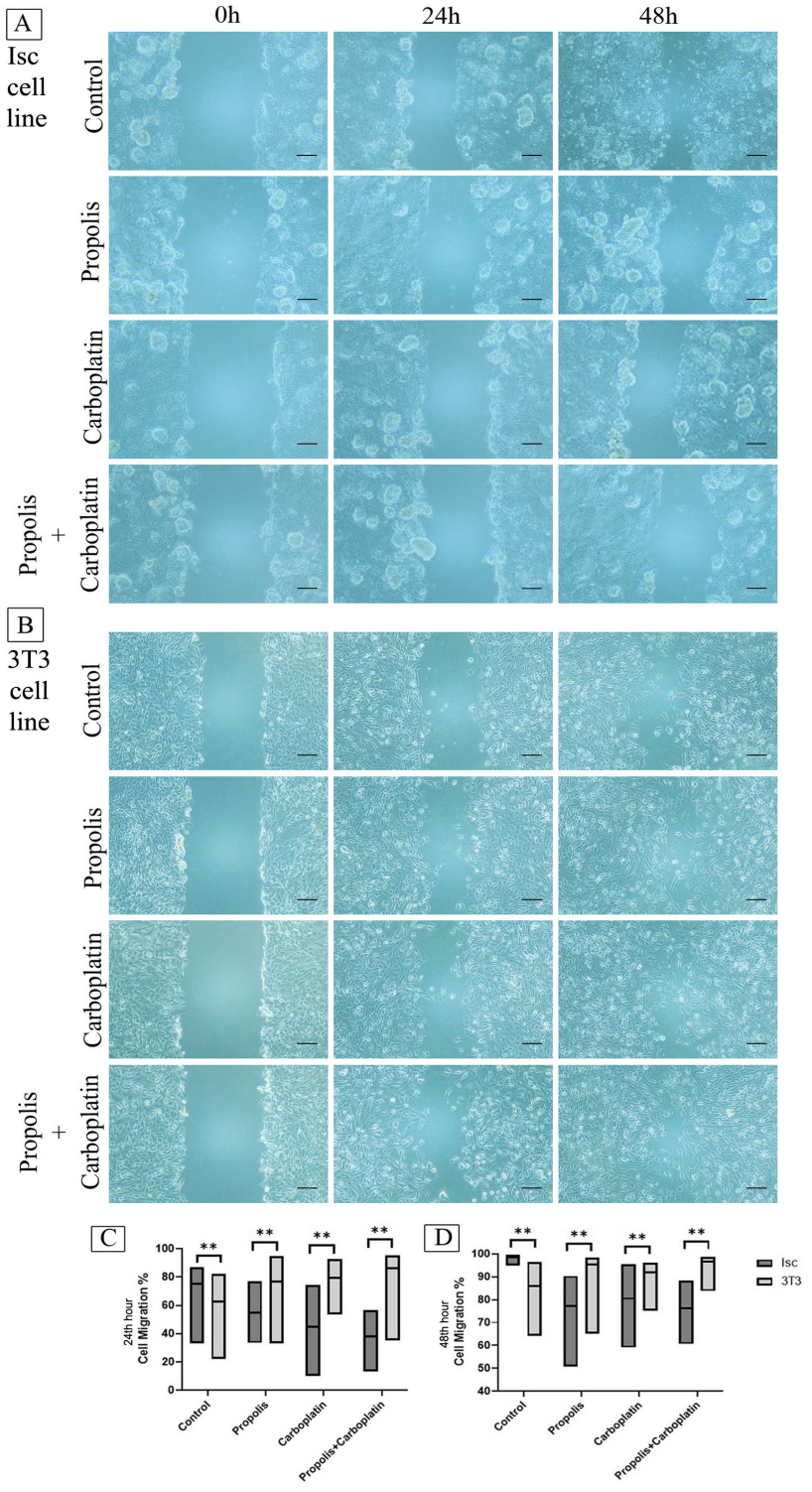

Figure 2. Phase contrast micrographs of cell migration assays at 0, 24 and 48 hours A) Ishikawa (Isc) cell line. Single treatments of propolis or carboplatin reduce migration and most significant migration deteriorating effect was observed in the propolis+carboplatin group. B) 3T3 cell line. There were no difference in cell migration in the 3T3 cell line experimental groups compared to the control group. C) 24 and D) 48-hour migration rates. 10x magnification. Bar: 100 µm; **p<0.01

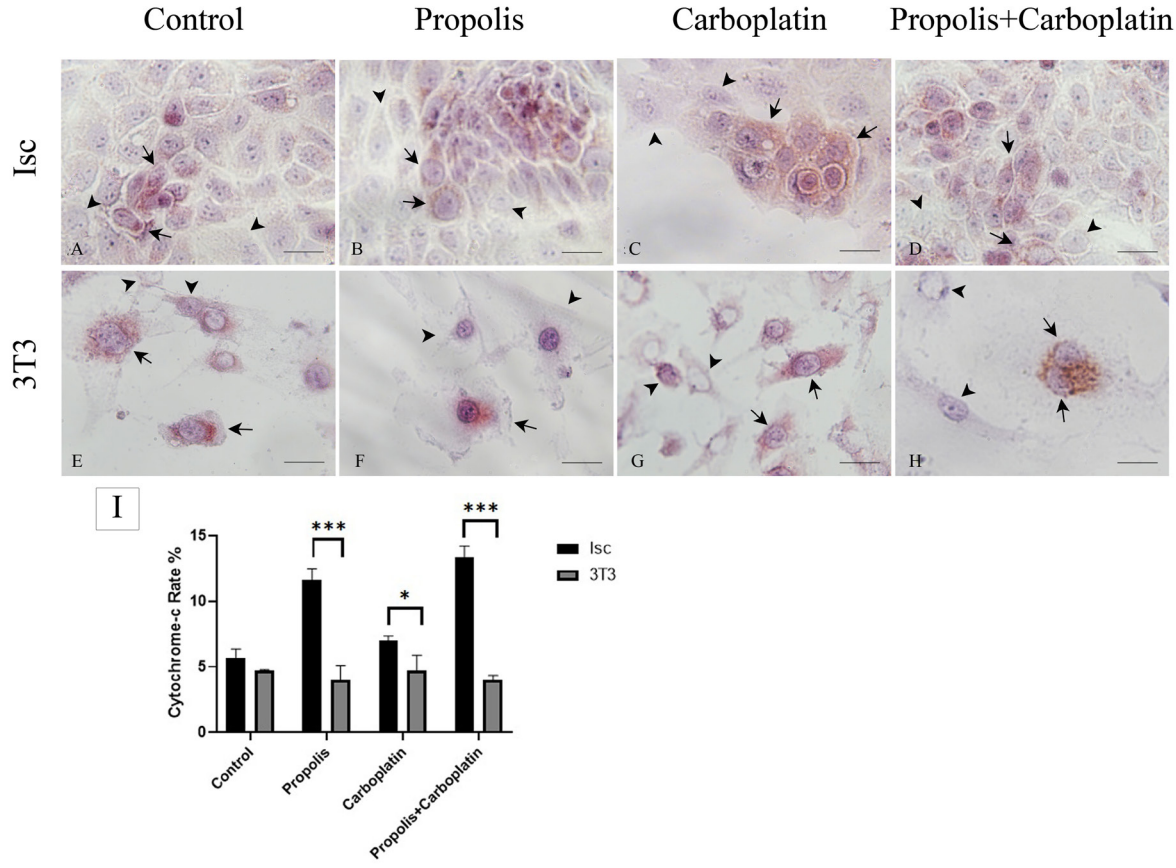

Figure 3. Cytochrome-c immunohistochemistry of A, B, C, D Ishikawa cells and E, F, G, H 3T3 cells at 24 hours. Cytoplasmic cytochrome-c positive cells with red cytoplasm (arrow) and cytochrome-c negative (arrowhead) cells are seen in all groups in different counts. Nuclei was counterstained with hematoxylin (blue) in all cells. I. Cytochrome-c ratios show the comparison of stained cell counts. While cytochrome-c staining was observed in all groups of Ishikawa cells, it was noted that the stained cell count was more in the propolis+carboplatin group. There was no difference in 3T3 cells cytochrome-c staining in experimental groups. The difference between Ishikawa and 3T3 is most profound in propolis+carboplatin groups. 40x magnification. Bar: 100 µm; ***p<0.001, *p<0.05.

formula. Apoptosis data were analyzed using NovoExpress software.

RESULTS

The Exposure of Propolis for 24h Decreased Cell Viability of Ishikawa Cells

The viability of the Ishikawa experimental groups were statistically different from each other (p=0.0086). A pairwise comparison of experimental groups with the control group showed a significant decrease in propolis (p=0.0169), carboplatin (p=0.0226) and propolis+carboplatin (p=0.0129). There was no difference in viability between 3T3 cell groups (Table 2).

The 24th hour viability test results for Ishikawa and 3T3 line experimental groups were significantly different in propolis groups (p=0.0292), carboplatin groups (p=0.0054), and propolis+carboplatin groups (p=0.0044). Carboplatin and/or propolis decreased the viability of Ishikawa cells (Figure 1A).

The Exposure of Propolis for 24h Increased Apoptosis of Ishikawa Cells

Propolis (p=0.0002), carboplatin (p=0.0385), and propolis+carboplatin groups (p=0.0003) had increased apoptosis in Ishikawa cells compared to the control group. There was no difference in the apoptosis rates in experimental groups of 3T3 cells (Table 3).

When apoptosis values of the two cell lines were compared with each other, the difference was significant between control groups (p=0.0044), propolis groups (p=0.0002), carboplatin groups (p=0.0005), and propolis+carboplatin groups (p=0.0003), and apoptosis was increased in the Ishikawa cell line (Figure 1B).

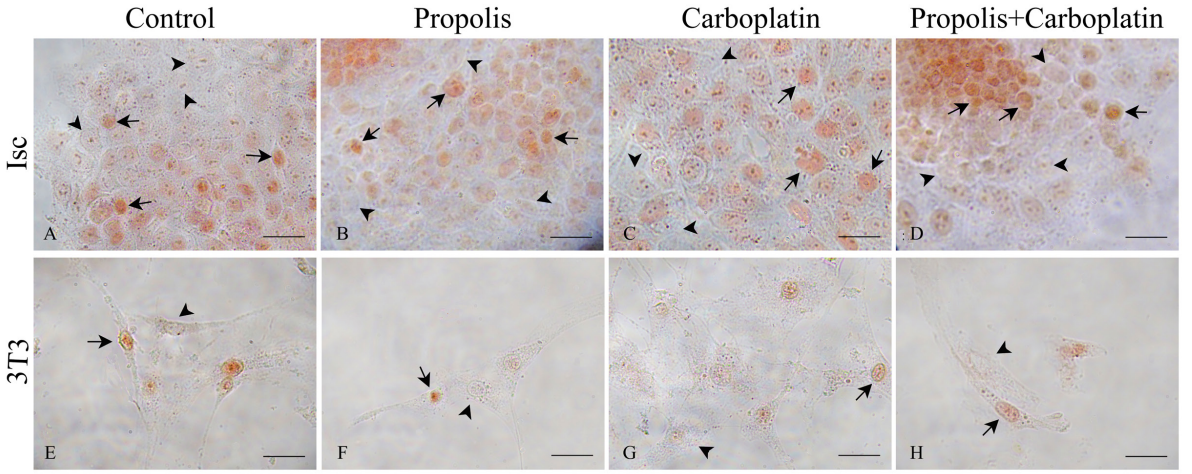

Figure 4. P27 Immunohistochemisry of A, B, C, D Ishikawa cells and E, F, G, H 3T3 cells at 48 hours. The p27 staining (red) intensity of Ishikawa cells was higher in treatment groups than in the control group with the most intense staining in the propolis+carboplatin group. P27 staining of 3T3 cells does not differ between groups. Nuclei of p27 positive (arrow) and p27 negative (arrowhead) cells are seen. 40x magnification. Scale bar: 100 µm.

Propolis Increased Migration in 3T3 Cells and Decreased Migration in Ishikawa Cells

Cell migration assay showed a statistically significant difference between groups of Ishikawa cells at 24 hours ($p<0.01$) and 48 hours ($p<0.01$). Carboplatin and/or propolis decreased the rate of migration of Ishikawa cells. The migration of Ishikawa cells at the 24th hour in propolis ($p=0.0035$), carboplatin ($p=0.0023$), and propolis+carboplatin ($p=0.0015$) experimental groups were lower when compared with the control group. The migration of Ishikawa cells at the 48th hour in propolis ($p=0.0029$), carboplatin ($p=0.0019$) and, propolis+carboplatin ($p=0.0011$) experimental groups were also lower than the control group.

The differences in migration between groups of 3T3 cells were significant at 24 hours ($p<0.001$) and 48 hours ($p<0.01$). However, propolis increased the rate of migration in 3T3 cells. The migration of 3T3 cells at 24th hours was increased in propolis ($p=0.0243$), carboplatin ($p=0.0188$) and propolis+carboplatin ($p=0.0002$) experimental groups than in the control group. The migration rate of 3T3 cells was increased in the propolis ($p=0.0031$), and propolis+carboplatin ($p=0.0018$) experimental groups at 48 hours (Table 4).

The difference between the same groups of Ishikawa and 3T3 cell lines was significant at 24 hours, and the migration in the Ishikawa cell line was lower in the propolis groups ($p=0.0062$), carboplatin groups ($p=0.0032$) and propolis+carboplatin groups ($p=0.0015$). The migration of the control groups at 24 hours was different, and it was lower in the 3T3 cell line ($p=0.0073$). At the 48th hour, migration was less in the Ishikawa cell line and the difference was significant between propolis groups ($p=0.0023$), carboplatin groups ($p=0.0015$) and propolis+carboplatin groups ($p=0.0011$). The difference was significant between the control groups at 48 hours, and the migration was lower in the 3T3 cell line ($p=0.0031$, Figure 2).

Propolis Increased Cytochrome C in Ishikawa Cells

The cytochrome c ratio of Ishikawa cells was statistically different ($p<0.001$). The propolis ($p=0.0006$) and propolis+carboplatin ($p=0.0002$) experimental groups exhibited a higher cytochrome c ratio compared to the control group. However, there was no significant difference in the cytochrome c ratio among the experimental groups of 3T3 cells (Table 5, Figures 3A-H).

Significant differences were observed between the Ishikawa and 3T3 cell lines in the same experimental groups. The cytochrome c levels in the propolis groups ($p=0.0006$), carboplatin groups ($p=0.0323$), and propolis+carboplatin ($p=0.0002$) were higher in the Ishikawa cell line. No significant difference was found between the control groups (Figure 3I).

Propolis+carboplatin Exposure Increased p27 in Ishikawa Cells

Comparing all groups, the p27 ratio of Ishikawa cells showed statistical differences ($p=0.0146$). Pairwise comparison indicated a significant difference only between the control and propolis+carboplatin experimental groups ($p=0.0093$). However, there was no significant difference in the p27 ratio of 3T3 cells (Table 6, Figures 4A-H).

The p27 ratio between the groups of Ishikawa and 3T3 cell lines groups displayed relative, but not statistically significant differences in the propolis groups ($p=0.7079$) and propolis+carboplatin ($p=0.0563$).

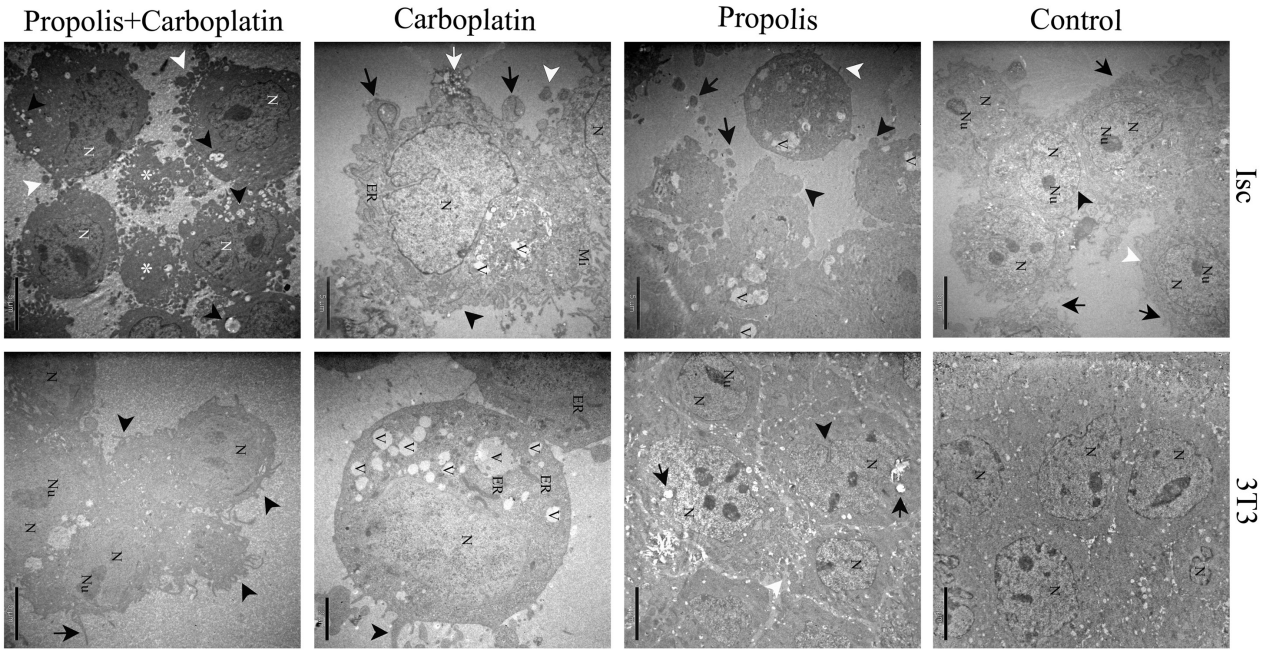

Figure 5. Electron micrographs of Ishikawa and 3T3 cells. In the left panel, electron micrographs of Ishikawa cells are shown. Control group electron micrograph: Nucleus (N) and nucleolus (Nu) are seen in normal structure, nuclear membrane (black arrowhead) and cell membrane (white arrowhead) and smooth microvilli (black arrow) are seen in intact structure. 5000x magnification. Scale bar: 5 µm. Propolis group electron micrograph: Increased number of vacuoles (V), cell budding (black arrowhead), apoptotic bodies (black arrow) and loss of microvilli (white arrowhead) are seen. 5000x magnification. Scale bar: 5 µm. Carboplatin group electron micrograph: Invaginated nucleus (N), mitochondria in normal morphology (Mi), degenerated endoplasmic reticulum (ER), vacuoles (V), cell budding (black arrow), loss of microvilli (black arrowhead), apoptotic body (white arrowhead), and migrasome-like structure (white arrow) is shown. 7500x magnification. Scale bar: 5 µm. Propolis+carboplatin group electron micrograph: Invaginated nucleus (N), vacuole (black arrowhead), apoptotic bodies (white arrowhead), and apoptotic cell remnant (asterisk) that have lost their organelles and have nuclear fragmentation are seen. 5000x magnification. Scale bar: 5 µm. In the right panel, electron micrographs of 3T3 cells are shown. Control group electron micrograph: Many cells with generally uniform morphology are seen with their nuclei (N). 5000x magnification. Scale bar: 5 µm. Propolis group electron micrograph: Although there are regular nuclei (N), nucleolus (Nu), little nuclear intussusception (black arrowhead), occasional vacuoles (black arrow), and rarely apoptotic bodies (white arrowhead), cells with smooth morphology, in general, attract attention. 5000x magnification. Scale bar: 5 µm. Carboplatin group electron micrograph: Nucleus (N), damaged endoplasmic reticulum cisterns (ER), vacuoles (V) and few cell budding (arrowhead) are seen. 10000x magnification. Scale bar: 2 µm. Propolis+carboplatin group electron micrograph: Nucleus (N), nucleolus (Nu), microvilli (arrowhead) and filopod (arrow) are seen in normal morphology. 5000x magnification. Scale bar: 5 µm.

Propolis and Carboplatin Enhanced Ishikawa Cell Apoptosis Ultrastructurally

The control group of Ishikawa cells exhibited typical cell morphology. The propolis group showed vacuoles and typical morphological features of apoptosis, including cell budding. Cell surfaces presented many apoptotic bodies and loss of microvilli. Carboplatin group displayed degeneration of endoplasmic reticulum and nucleus. The cisternae of the endoplasmic reticulum were fragmented. Migrasome-like structures were detected. Cell budding and apoptotic bodies were more prevalent. In the propolis and carboplatin combined treatment group, the nuclei were partially condensed, their size was reduced and invaginations were observed. The cells shrank, decreased in size, moved apart, and the spaces between the cells widened. Apoptotic bodies, degenerative cells, and remnants of late-phase apoptotic cells, which had already lost their organelles and had fragmented nuclei, were frequent. The apoptotic effect was severe in the propolis+carboplatin group when compared with other experimental groups (Figure 5).

The 3T3 cell line had normal morphology in the control group. In the propolis group, TEM generally showed cells with normal morphology, except for some small and few vacuoles. In the 3T3 cells carboplatin group, endoplasmic reticulum with swelling in their cisternas was found. A few cell budding and vacuoles were noted. In the propolis and carboplatin combination group, the microvilli and filopods were in regular morphology and number, and nuclei were uniform.

DISCUSSION

In our study, we investigated the effect of propolis on endometrial adenocarcinoma using cell culture techniques in combination with carboplatin, a chemotherapeutic agent commonly used in standard treatment.

Carboplatin exerts a cytotoxic effect by inhibiting cell proliferation of endometrial cancer cells (17). It increases cytochrome c release in the cytoplasm of human ovarian cancer cells and induces apoptosis in cervical carcinoma cells (18, 19). Additionally, it has an antimetastatic effect on the laryngeal carcinoma cell line (20). In our study, carboplatin increased the rate of apoptosis, reduced cell migration, and relatively elevated the rate of cytochrome c in Ishikawa cells. Since cytochrome c is associated with the intrinsic pathway of apoptosis, our study suggests that carboplatin is effective in triggering this intrinsic pathway. Although carboplatin is theoretically assumed to act on the S phase, it is not specific to the cell cycle. As an alkylating antineoplastic agent, it forms reactive platinum complexes within DNA molecule chains in the cell, leading to changes in DNA structure and inhibition of DNA synthesis. This can impact the cell cycle in every phase (21-23). Our results regarding the effect of carboplatin were consistent with the existing literature. TEM showed cell budding, increased vacuolization, the presence of apoptotic bodies, loss of microvilli, condensation of nuclei, the presence of migrasome-like structures, and organelle degeneration. These effects were minimal in the 3T3 cell line, which consists of healthy cells.

Migrasomes are classified as extracellular vesicles that mediate intercellular communication by removing unwanted molecules from cells and transferring cargo molecules to other recipient cells. Recently, migrasomes have been used to prevent resistance to cancer drugs (24-26). Our study is a first in the literature reporting that carboplatin-caused migrasome-like structure formation of Ishikawa cells is not produced in propolis treatment. The reason why migrasome structures were found only in the carboplatin experimental group of Ishikawa cells may be that tumor cells are resistant to the chemotherapeutic agent and are expelled from the cell, philopod structures are more uniform and more in number than other experimental groups, and the migration rate is high. In addition, no migrasome-like structures were found in the Ishikawa cells propolis+carboplatin treatment group, which suggested that propolis might play a role in a possible mechanism preventing the excretion of the chemotherapeutic agent carboplatin by tumor cells. However, the migrasomes observed and confirmed by three histologists with TEM were not verified by another method.

Propolis and its phenolic compounds can serve as a strong adjunct to radiotherapy and chemotherapy (27, 28). It has no known severe, moderate, or mild interactions with other drugs. Propolis decreases viability in MCF-7 and MDA-MB-231 breast cancer cell lines in a dose- and time-dependent manner but has no such effect on HUVEC cells (29). In our study, we found that propolis had a cytotoxic effect by reducing the viability of Ishikawa cells. It also had no such effect on 3T3 cells. Propolis inhibits the cell cycle in G0/G1 in human gastric cancer cells and in the G1 phase in human breast cancer cells (30, 31). As a result of our study, there was a relative increase in the p27 ratio in Ishikawa cells, which was not statistically significant. This suggested that different doses of propolis could be tested to arrest the cell cycle in the G1 phase, in line with the literature.

Propolis provides the mechanism of apoptosis both by stimulating the caspase cascade (intrinsic pathway) through the release of cytochrome-c from the mitochondria to the cytosol and by stimulating the TRAIL signalling pathway (extrinsic pathway) (32, 33). It has an apoptotic effect on the human leukemia cancer cell line U937 (34). The effect of propolis on human leukemia HL-60 cells involves the release of cytochrome-c from the mitochondria to the cytoplasm, stimulating the mitochondrial pathway, reducing cancer cell proliferation, and inducing apoptosis (35). We demonstrated that propolis induces apoptosis in the Ishikawa cell line through flow cytometry and confirmed that this occurs via the intrinsic pathway using our cytochrome-c immunocytochemical staining results. In Ishikawa cells treated with propolis; we observed increased cell budding, apoptotic bodies and vacuoles in TEM examinations, indicating the apoptotic effect of propolis. However, we did not observe apoptotic effects of propolis in 3T3 cell line experimental groups. Therefore, we concluded that propolis, which affects cancer cells, does not induce apoptosis in fibroblast cells.

Propolis inhibits cell migration in a dose-dependent manner at 48 hours on breast cancer cells. Supplements to anticancer treatments were studied in order to prevent metastasis. In this study, we investigated the metastatic activity of cancer cells using the migration test in accordance with the literature (29). Propolis slowed down cell migration of Ishikawa cells significantly when compared with the control group. Propolis was effective against metastasis.

The strengths of this study include the use of *in vitro* methods to investigate the effects of propolis and carboplatin on cancerous and healthy cells, with dosages selected based on relevant literature and preliminary studies. Another advantage is the observation of ultrastructural effects on cells without interference from *in vivo* conditions. However, a limitation is the need for further *in vivo* studies to determine the therapeutic dose for humans. Additionally, it should be noted that the composition of endemic propolis may vary geographically, which could impact its efficacy and safety in different regions. For future studies on chemotherapy resistance, a resistant cell line can be utilized, and a combination index analysis can be conducted to assess the efficacy of propolis and carboplatin in combination (36, 37).

Propolis combined with carboplatin has stronger cytotoxic and apoptotic effects on Ishikawa cells. Additionally, we observed the possibility of inhibiting treatment resistance.

This combination treatment resulted in severe apoptotic consequences for Ishikawa cells, including an increase in vacuole size and number, cell budding, apoptotic bodies, condensed relocated nuclei, a decrease in the number of microvilli, reduced cell sizes with degenerations in cytoplasmic organelles, and an increased number of advanced apoptotic degenerative cells. Our results showed that propolis and carboplatin have synergistic effects. They increased apoptosis via the intrinsic pathway and blocked the cell cycle in the G1 phase. In our examinations, we did not observe any finding suggesting any cytotoxic effect on 3T3 cells when propolis and carboplatin were administered together. In the literature, propolis did not cause cytotoxic effects in human dermal fibroblast cells; however, it increases viability, cell migration and migration rate. Hence, the increase in the migration rate of the 3T3 propolis and combination group in our study was suggested as the positive reported effect of propolis on wound healing (38). Other mechanisms of propolis on cancer cells are still under study (39).

In conclusion, propolis has demonstrated its synergistic effects with carboplatin on the endometrial cancer cell line, with no moderate or severe effects on fibroblast cells. These findings regarding the potential benefits of propolis as a dietary supplement suggest that it could be incorporated into the treatment regimens to enhance the prognosis and success of cancer chemotherapy for endometrial adenocarcinoma. Furthermore, it may warrant further investigation as a complementary approach in other chemotherapeutic studies.

Ethics Committee Approval: This research includes commercial cell lines Ishikawa and 3T3. An ethical approval is not needed because human or animal subjects or primary cell culture from human or animal subjects are not involved.

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