Evaluation of Tumor-Suppressive Properties and Apoptotic Functions of Mad Honey and Vincristine Applications in a Rat Model of Breast Cancer

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ABSTRACT

In this study, the suppressive effects of vincristine and Turkish mad honey alone and in co-applications were biochemically, hematologically, and histopathologically investigated in a mammary tumor model induced with 7,12-dimethylbenz[a]anthracene (DMBA) in rats. A total of 72 rats, 43-49 days old, were divided into 6 groups of 12 rats each. The control group (CG) consisted of healthy rats. The vehicle group (VG) received only vehicle substance and the cancer control group (CCG) was given only DMBA. DMBA and the honey group (HG) given group. DMBA and the vincristine (VinG) given group, and DMBA, the vincristine-honey group (VHG) received both Turkish mad honey and vincristine. Turkish mad honey and/or vincristine was given in the last 4 weeks of the 13-week trial period. White blood cell and lymphocyte counts differed significantly in the CCG and VG groups. Alanine transaminase and total protein levels were higher in the CCG and VinG groups. Aspartate transaminase was higher in the CCG, HG and VG groups. Caspase-3 and Bax protein levels were in the HG and VHG groups significantly higher than CCG. In caspase-8 protein level VHG significantly higher than other groups. Caspase -9 protein level was in CG and VG groups significantly lower than other groups. Bcl-xL increased more in the CCG group. Anaplasia was reduced in the HG and VinG groups, although apoptosis and other cellular damages increased. It was concluded that mad honey and vincristine could be considered together as effective therapeutic agents in this model of DMBA-induced breast cancer.

Introduction

Breast cancer is one of the most common cancers in the world. As a progressive disease with high mortality rates, it is one of the leading causes of death in both Turkey and the world as a whole and it impairs patients' quality of life. Breast tumors are the most common type of tumor among all cancer types in women (39). In veterinary medicine, breast cancer is also frequently encountered in dogs and cats, although mammary tumors are particularly more common in unspayed female dogs. This adversely affects the quality of life of animals, similarly to humans, and it brings great economic costs. In recent years, veterinarians who have adopted the "single health care" principle have been undertaking important tasks in terms of developing effective treatment methods against neoplastic diseases with poor prognoses. For this reason, in vivo breast cancer models have become a popular subject of research for human physicians and veterinarians alike (3, 42).

Chemotherapy is a treatment method used alone or in combination treatment of breast tumors (43). However, the toxicity of chemotherapeutics used in the treatment of tumors and the resistance of tumor cells to

chemotherapeutic drugs are the main obstacles for these treatment applications. To avoid those disadvantages, studies are being carried out to develop protocols for the combination of various chemotherapeutics used in cancer treatment or for alternative therapeutic agents applied together with chemotherapeutic agents. It has been predicted that the side effects of chemotherapeutic drugs can be reduced and their anti-carcinogenic potential can be increased by using combinations that include alternative substances with antioxidant properties (8, 15).

Vincristine is an important chemotherapeutic agent used in various stages of breast cancer treatment (8). However, in addition to the effectiveness of vincristine against cancerous cells, it also has toxic effects against rapidly proliferating healthy cells. In recent years, researchers have considered the use of antioxidants such as quercetin, genistein, and geraniol separately or in combination to reduce the side effects of chemotherapeutic agents without reducing the antitumoral effectiveness of those agents (43).

The mad honeys popularly known as "mad honey" or "rhododendron honey" are obtained from regions of the Black Sea provinces where Rhododendron ponticum and R. luteum are found in the flora. These honeys are mainly produced in the provinces of Artvin, Kastamonu, Zonguldak, Rize, Ordu, Tokat, and Sinop. Said to be a source of healing when consumed in measured amounts, these honeys have traditionally been used for health purposes and are also called "medical honey." Grayanotoxin types I-III are most typically found in mad honey (17, 35) and these substances exert cytotoxic effects without harming healthy cells. The phenolic compounds in mad honey are the most significant group among the honey's total compounds. They prevent mitotic catastrophe and scavenge free radicals. In this way, oxygenation is increased within the mitochondria of the cells and the microenvironment, helping to preserve cellular morphology by balancing the oxidative capacity (1, 6, 26). Many in vivo and in vitro studies have been conducted to evaluate the antioxidant properties of mad honey and Rhododendron ponticum extracts.

The main reasons for the frequent occurrence of breast cancer are inflammation, angiogenesis, the rapid cell cycle of breast tissue, and hormonal and genetic factors. The main factors that cause a predisposition to breast cancer are genetic variants in breast tissue cells, hormonal changes and the responses of breast tissue receptors to those changes, and variations in cell signaling pathways that change according to environmental factors (37).

This study aimed to evaluate whether the administration of vincristine and mad honey separately or together was an effective treatment for rats in a model of breast cancer. Changes in complete blood counts, blood serum parameters, and oxidative stress parameters were clinically assessed and regressive changes in breast tissue masses were evaluated in histopathological examinations.

Materials and Methods

Animal and Experimental Design: This study included 4 experimental and 2 control groups created using 72 inbred female Wistar albino rats that weighed approximately 195-240 g and had reached sexual maturity. A minimum of 12 animals were included in each group so as not to distort the statistical analysis with small sample sizes. The animals were cared for, housed, and fed for the duration of the experiment in the Experimental Animal Unit of the Ankara University, Turkey. Considering animal welfare, appropriate numbers of animals were kept in each cage and food was provided ad libitum with appropriate temperature and ventilation conditions on the basis of a 12/12-hour cycle of light and darkness. Ethical permission was obtained from the relevant review board (Decision No: 2021-13-113).

A total of 72 rats were examined in six groups, 12 rats in each group. Administration of honey using gavage, and intraperitoneal administration of vincristine were performed during the last four weeks of the 13-week study period. (Figure 1).

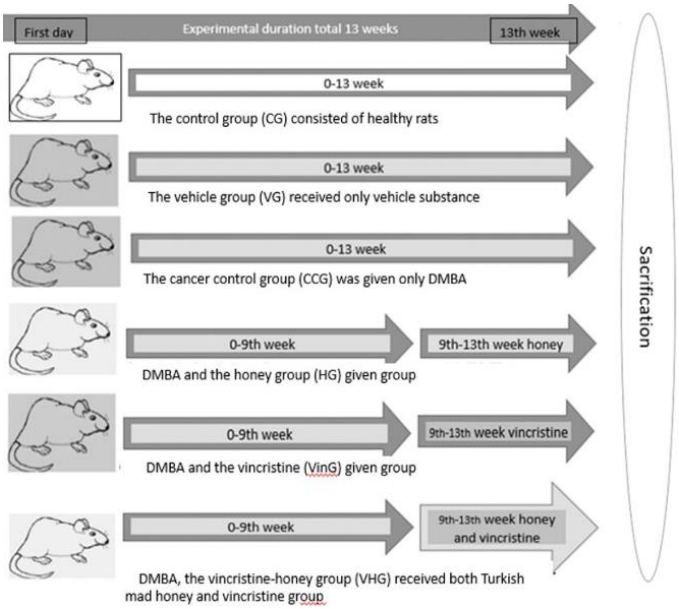

Figure 1. Experimental procedure.
1. Rat: The control group (CG) consisted of healthy rats.
2. Rat: The vehicle group (VG) received only vehicle substance
3. Rat: The cancer control group (CCG) was given only DMBA.
4. Rat: DMBA and the honey group (HG) given group.
5. Rat: DMBA and the vincristine (VinG) given group,
6. Rat: DMBA, the vincristine-honey group (VHG) received both Turkish mad honey and vincristine.

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Mammary Cancer Induction Modelling: After DMBA administration, 6 pairs of mammary glands were examined weekly in each group to monitor the experimental protocol. The presence of mass formations was monitored by inspection, palpation, and micrometer measurements. After DMBA administration, the tumors in breast tissues were considered based on a volume of 200 mm3, but the expected mass size did not develop and so the treatment protocol was started. Therefore, considering that masses developing in the mammary glands may be micromorphometric, the standard formula for calculating tumor volume of $V = 0.5 \times (W2 \times L)$ was not applied (V = tumor volume, W = length of the shorter side of the tumor; L = length of the longer side of the tumor).

Blood Sampling and Biochemical Analysis: A total of 7 mL of blood, with 5 mL collected into a tube without anticoagulant and 2 mL into a tube with EDTA, was obtained from each rat. Serum samples were separated within 3 hours of the collection of blood samples and stored at -80 °C until analysis. Hematological analyses were performed using an automated blood count device (Mindray BC-5000) within 3 hours of blood collection. Using serum samples, C-reactive protein (CRP), total protein, albumin, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine levels were measured with an automated biochemistry analyzer (Mindray BS-300).

Bcl xL level (Bioassay Technology Laboratory Cat No: E3340Ra, Zhenjiang, China) and Bax level (Bioassay Technology Laboratory Cat No: E1869Ra, Zhenjiang, China) and caspase-3 level (Bioassay Technology Laboratory, Cat No: E1648Ra Zhenjiang, China) caspase 8 level (Bioassay Technology Laboratory Cat No: E1370Ra, Zhenjiang, China) caspase 9 level (Bioassay Technology Laboratory Cat No: E1898Ra, Zhenjiang, China), and survivin level (Bioassay Technology Laboratory Cat No: E0191RA, Zhenjiang, China) ELISA test kits were measured spectrophotometrically (Sunrise RS-232, Tecan, Grödig, Austria).

Superoxide dismutase (SOD) and malondialdehyde (MDA) were also evaluated as parameters of oxidative stress. SOD activity was measured using a commercial test kit according to the method developed by Sun et al. (40). MDA level was measured using a commercial test kit according to the method developed by Ohkawa et al. (28).

End of Experiment: At the end of the experiment, the rats were anesthetized by intramuscular injections of xylazine (10 mg/kg IP) and ketamine (100 mg/kg BW), and then the rats were sacrificed by taking a large amount of blood from the heart with injectors. The suggested changes has been made. After the animals were euthanized, necropsies were performed in a species-specific manner.

Necropsy and Histopathological Examinations: Tumor development in the affected breast lobes was detected along the incised breast lines. Samples were taken from various points of the tissue to determine whether there was metastasis around the breast lobe or into the lymph nodes draining the area. Information about the size, color, shape, and appearance of the upper and sectional faces of tumoral masses in the affected area was recorded. Liver and kidney tissues were collected and fixed in 10% buffered formalin (pH 7.2) for 48 hours. Tissue samples were trimmed, passed through ethanol and xylene series and liquid paraffin series with an automatic tissue processer (Leica TP1020, Germany), and blocked in paraffin (EG1150, Thermo Shandon, Germany). Sections were taken from blocks with thicknesses of 5 µm (AS325, Shandon, Germany). Tissue sections were stained with hematoxylin and eosin (H&E) (22).

Sections were examined at magnification of ×200 to identify the presence of significant differences in the masses in breast lobes between the groups. Atypical malignancy criteria including pleomorphism, mitotic index, polychromasia, and islet formation were evaluated and scored in terms of vascularization and inflammatory cell infiltrations in anaplastic cells in a total of 10 different areas. Previous findings of relevant studies were taken into account in this process (13, 33). The findings were calculated according to equal percentiles obtained from mean calculation of all microscopic fields. The results were categorized with scores of 0 for no findings and 1, 2, 3, 4, 5, or 6 for cases of lesions according to the severity of the findings. According to this; numerics and clarification has been showed between parenthesis. (score 0): no finding 0%: (score 1) very light, 1-15%; (score 2) mild, 16-30%; (score 3) weak moderate, 31-45%, (score 4) moderate, 46-60%: (score 5) severe, 61-75%; (score 6) very severe, 76-100%.

Statistical Analysis: G*Power version 3.1.9.7 (44) was used to perform a priori power analysis to estimate the minimal sample size that is necessary to test the study hypothesis. To control Type I and Type II errors, $\alpha=0.05$ and $1-\beta=0.8$ levels were accepted and to indicate effectiveness of experimental conditions $d=0.5$ was determined. Thus, N=72 the obtained sample size was considered to be adequate to test the study hypothesis. The data were initially summarized with descriptive statistics and checked for whether assumptions were met. Results were evaluated by using Shapiro-Wilk test and Q-Q plot for normality and the Levene test for homogeneity of variances. One-Way Anova and Kruskal-Wallis test were used for comparisons between groups. In case of statistically significant difference between groups; Tukey, Games Howell or Dwass-Steel-Critchlow-Fligner pairwise comparisons analysis were used appropriately.

Results were analyzed and expressed as mean ± standard error (M±SE).

Histopathological data were converted into equally divided percentiles and scored, they were validated with one-way ANOVA and post hoc Tukey tests and statistically compared between groups.

P value of <0.05 was considered to be statistically significant result for all analyses. The data were analysed using IBM SPSS Statistics 26.0 (SPSS®, IL, USA) and Graph-pad (8.0.1, San Diego, California, USA).

Results

Macroscopic Findings: In the rats of all groups, no macroscopic findings were observed in terms of criteria for size, color, and shape in the areas of the breast lobes.

Histopathological Findings: The tissues of each of the animals in the experimental groups and the control group of healthy animals were scored in terms of islet formation, vascularization, inflammatory cell infiltration, and apoptotic-degenerative changes in the glands as well as malignancy criteria. In the control group (CG), the cells were not anaplastic, as they were in the VG group, and islets had formed. Cystic changes and other changes such as degeneration, necrosis, and apoptosis were not observed in this group. Vascular and inflammatory changes were also not observed.

Islet proliferation was evident in the cells of the groups with cancer, especially in the cancer control group (CCG). Rather than forming a glandular structure, the glands were separated from each other and malignant cells were scattered within glands. Malignant cell islets were observed to be intense with features of adenocarcinoma. However, there were no cystic changes. In these cells, destruction was extensive in the nuclei and cytoplasm in terms of parenchymal degeneration, necrosis, and apoptotic changes. Pleomorphism and polychromasia were evident in the cells and mitotic figures were observed. However, the mitotic index was not as high as expected. Vascular changes were also prevalent in capillaries parallel to the degree of anaplasia. Arterioles and venioles, as well as capillaries, were enlarged with erythrocytes and were hyperemic. In some cases in the CCG group, free erythrocytes were observed outside of blood vessels, signifying hemorrhage. numerous eritrocytes were observed at periphery of vessels. In some cases, edema was also encountered. No inflammatory changes were seen (Figure 2).

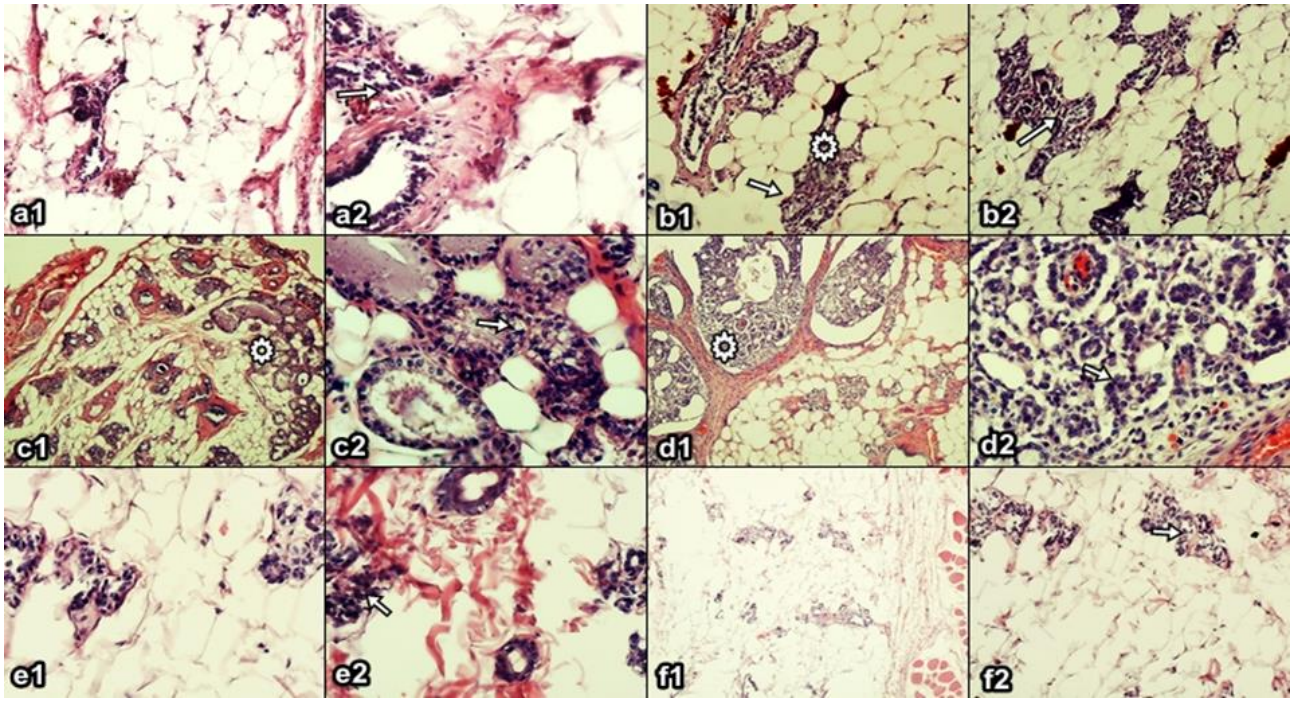

Figure 2. Histopathological changes in breast tissues of the experimental groups.
(a) CG: Mammary glands with normal appearance, ×100 (a1), ×200 (a2). (b) CCG: Anaplastic changes in the mammary glands with islets of proliferating cells (asterisk) and cells showing polychromasia and pleomorphism (arrow), ×100 (b1), ×200 (b2). (c) HG: Proliferating changes in the mammary glands (asterisk) and proliferating cells (arrow), some of which show degenerative findings, ×100 (c1), ×200 (c2). (d) VinG: Islet of prominently proliferating cells in the mammary glands (arrow), ×100 (d1), ×200 (d2). (e) VG: Normal appearance of mammary glands with degenerative changes in one or two areas (arrow), ×100 (e1), ×200 (e2). (f) VHG: Mild proliferative and marked degenerative changes in the mammary glands (arrow), ×100 (f1), ×200 (f2).

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Anaplastic changes with features of adenocarcinoma due to malignant cells, pleomorphism, and polychromasia were found at higher rates in the honey-administered group (HG) compared to the CCG group. Although the anaplastic activity in the glands was higher in this group, cystic changes were also more prevalent compared to the CCG group. The mitotic index was again higher than the value obtained for the CCG group. Inflammatory changes, apoptosis, and degenerative changes were absent (Figure 2).

Anaplasia in the islets formed by malignant mammary gland epithelial cells was not as intense in the vincristine group (VinG) in comparison to the CCG and HG groups. However, the cystic changes were intense. No apoptosis or degenerative changes were observed in the glands, as in the HG group. Pleomorphism, polychromasia, and vascular changes were common in many parts of the tissues, although less so than in the other group. The mitotic index was higher compared to the CCG group and lower compared to the HG group (Figure 2).

Cells in the vehicle control group (VG) were not anaplastic and cells showing mild polychromasia were found in some areas. Gland cells were smooth and formed islets. Cystic changes, degeneration, necrosis, and apoptosis were not observed in this group.

In the group of rats receiving both vincristine and honey (VHG), malignant mammary gland epithelial cells were seen in the form of islets in some cases. Pleomorphic changes were mild and polychromasia was generally mild to moderate. Gland structures were cystic in only one case. No vascular or inflammatory changes were found. However, degenerative changes in the gland epithelium were observed more intensely in both the nuclei and cytoplasm than in the other groups, except for the CCG group (Table 1, Table 2, Figure 2, Figure 3).

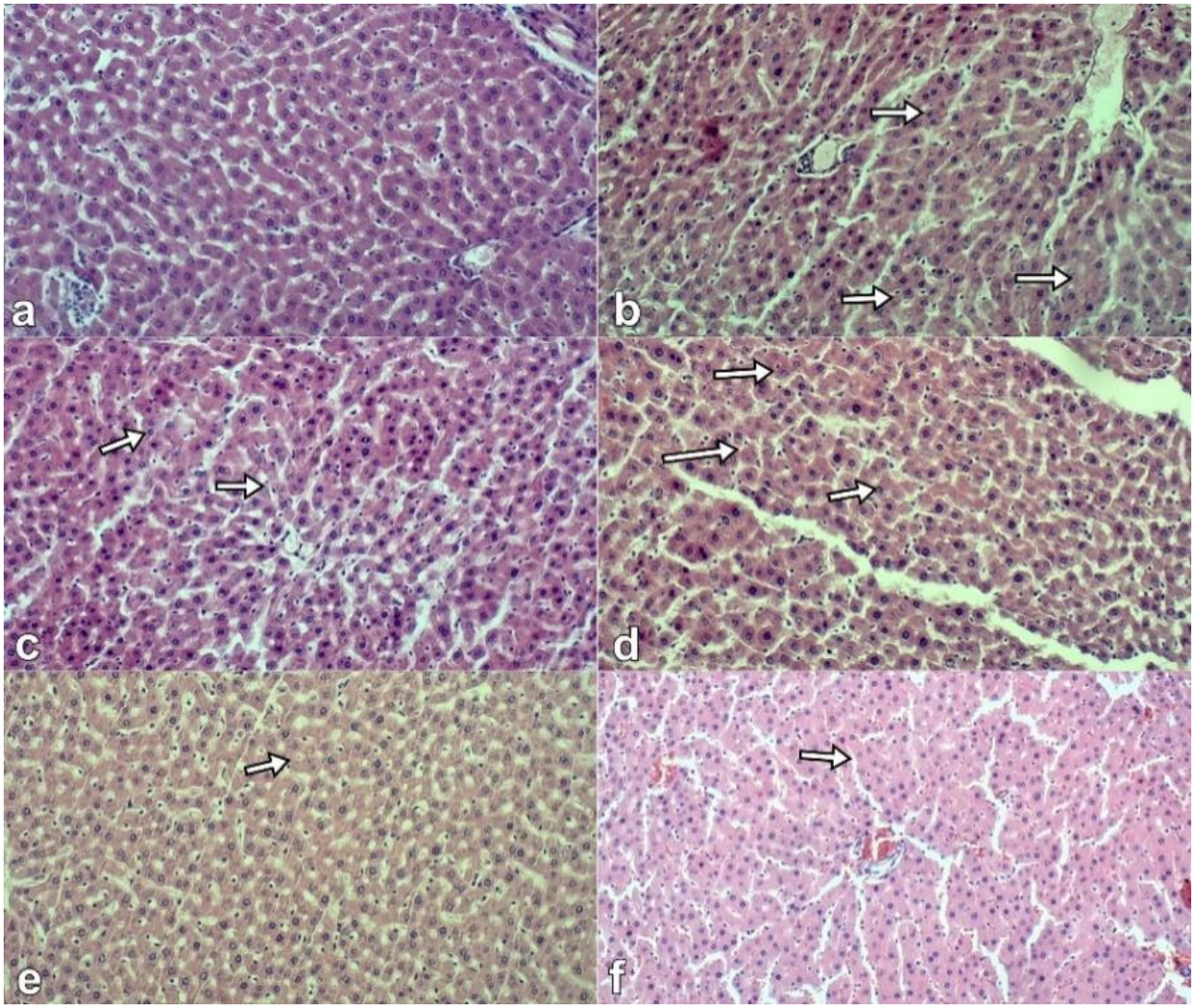

Figure 3. Histopathological findings in the liver in the experimental groups.
(a) Healthy appearance in hepatocytes in the control group (CG). (b) Intense degenerative and necrotic changes in hepatocytes in the cancer control group (CCG). (c) Moderate degenerative changes in hepatocytes in the group given honey (HG). (d) Intense degenerative and necrotic changes in hepatocytes in the group given vincristine (VinG). (e) Slight degenerative changes in hepatocytes in the group given the vehicle substance (VG). (f) Slight degenerative changes in hepatocytes in the group given vincristine and honey (VHG). ×100, H&E.

Table 1. Evaluations of histopathological lesions in breast tissue (mean ± standard error).

	Histopathological Parameters					
Groups	**CG (n=12)**	**VG (n=12)**	**CCG (n=12)**	**VinG (n=10)**	**HG (n=12)**	**VHG (n=7)**
Pleomorphism	1.51±1.51	0.00±0.00	56.23±13.33	69.41±4.95	42.39±5.20	4.5±7.24
Mitotic index	0.00±0.00	0.00±0.00	6.24±4.38	9.08±4.12	24.99±6.64	0.00±0.00
Polychromasia	18.17±5.24	11.10±4.73	64.80±11.92	36.33±5.86	76.36±5.21	28.33±5.83
Islet Anaplasia	55.55±14.26	61.10±13.34	66.63±9.20	33.31±5.49	67.42±4.16	23.33±6.18
Islet Cyst Formation	0.00±0.00	0.00±0.00	0.00±0.00	13.62±5.86	22.71±8.18	3.33±3.33
Vascular changes	0.00±0.00	0.00±0.00	22.90±11.32	15.14±8.54	27.76±4.73	3.33±2.20
Inflammation	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Apoptosis, degeneration, necrosis	20.82±6.52	0.00±0.00	75.9±6.28	0.00±0.00	0.00±0.00	33.33±6.50

Level of significance: * (mild), ** (moderate), *** (strong).

Table 2. Comparison of histopathological findings in breast tissues.

Tukey Multiple Correlation Test	Significance	Correlation	P-value
Pleomorphism vs. Mitotic index	-	-	0.3971
Pleomorphism vs. Polychromasia	-	-	0.3653
Pleomorphism vs. Islet anaplasia	-	-	0.6132
Pleomorphism vs. Islet cysts	-	-	0.4752
Pleomorphism vs. Vascular changes	-	-	0.4339
Pleomorphism vs. Inflammation	-	-	0.4260
Pleomorphism vs. Apoptosis-degeneration	-	-	0.9995
Mitotic index vs. Polychromasia	-	-	0.0700
Mitotic index vs. Islet anaplasia	Yes	*	0.0147
Mitotic index vs. Islet cysts	-	-	>0.9999
Mitotic index vs. Vascular changes	-	-	0.6051
Mitotic index vs. Inflammation	-	-	0.6982
Mitotic index vs. Apoptosis-degeneration	-	-	0.9371
Polychromasia vs. Islet anaplasia	-	-	0.9095
Polychromasia vs. Islet cyst	-		0.1140
Polychromasia vs. Vascular changes	Yes	*	0.0494
Polychromasia vs. Inflammation	-	-	0.1173
Polychromasia vs. Apoptosis-degeneration	-	-	0.8739
Islet anaplasia vs. Islet cysts	Yes	*	0.0291
Islet anaplasia vs. Vascular changes	Yes	*	0.0260
Islet anaplasia vs. Inflammation	Yes	*	0.0104
Islet anaplasia vs. Apoptosis-degeneration	-	-	0.4779
Islet cysts vs. Vascular changes	-	-	0.8551
Islet cysts vs. Inflammation	-	-	0.6896
Islet cysts vs. Apoptosis-degeneration	-	-	0.9492
Vascular changes vs. Inflammation	-	-	0.4197
Vascular changes vs. Apoptosis-degeneration	-	-	0.9824
Inflammationvs. Apoptosis-degeneration	-	-	0.6584

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Histopathological Findings in the Liver: In order to evaluate changes in catabolic metabolism triggered by DMBA, livers were evaluated histopathologically. degenerating hepatocytes, the nuclei were pushed to one side of the cell and were usually pycnotic or lytic. Their cytoplasm had either swollen or several vacuoles of varying sizes. Degeneration were present in the VinG and CCG groups at same appearence. The rate of degeneration was higher in the HG group compared to the VHG and VG groups; however, it was lower compared to the VinG and CCG groups. In addition, the VHG group had a lower rate of degeneration than all other groups excluding CG and VG. Necrotic changes were more prominent in the VinG and CCG groups compared to the HG group. No signs of degeneration were found in the VHG, VG, and CG groups. There were no remarkable findings in terms of vascular changes or inflammation between control and experimental group (Figure 3). First of all, the control group and then the experimental groups seem to be better.

Histopathological Findings in the Kidneys: To evaluate the impact of DMBA on catabolic metabolism, the kidneys were evaluated histopathologically and minor changes were observed. In the kidneys, the findings were concentrated in the cortex. In tissues that showed signs of degeneration in the epithelium lining of the cortical tubules, the cytoplasm was generally pale. The nuclei were lytic and some of them were pycnotic. In cases of acute cell swelling or in more advanced stages, vacuolations of varying widths were present in the cytoplasm. The numbers of cells in the kidneys showing such findings were higher in the VG group. However, these disorders were encountered at lower rates in the HG and CCG groups. The values obtained for those two groups were found to be close to each other. There was a significant decrease in the rate of such degenerative changes in the VHG and VG groups compared to the other experimental groups. Tubules were healthy in most cases. No findings were observed in the CG group. In terms of necrotic changes, higher values were observed in the VinG group and, to a lesser extent, in the CCG group, while these values were lower in the HG group. No findings were observed in the CCG, VG, or VHG groups. There were no remarkable pathological findings in terms of vascular changes or inflammation.

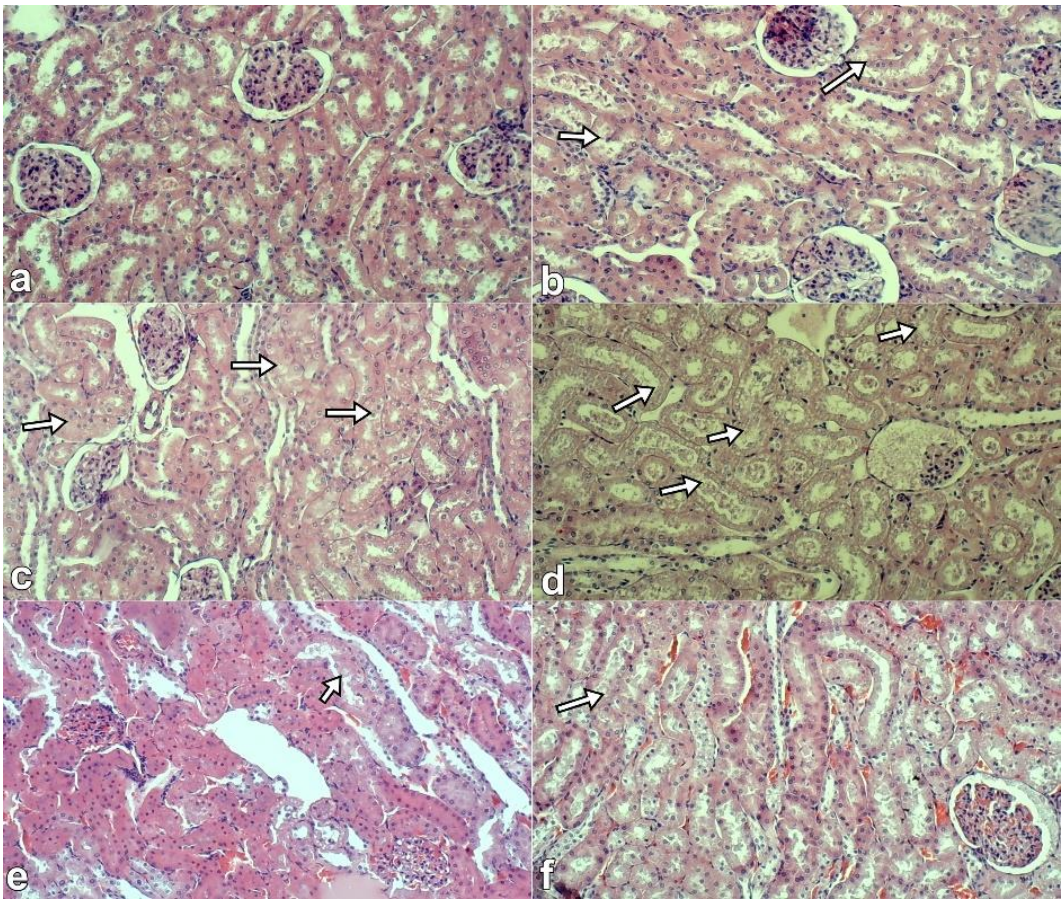

Figure 4. Histopathological findings in the kidneys in experimental groups.
(a) Healthy appearance in the kidney tubules in the control group (CG). (b) Degenerative and necrotic changes in the cortical tubules in the cancer control group (CCG). (c) Moderate degenerative changes in the cortical tubules in the honey-treated group (HG). (d) Intensive degenerative and necrotic changes in the cortical tubules in the vincristine group (VinG). (e) Degenerative changes in some cortical tubules in the group given the vehicle substance (VG). (f) Mild degenerative changes in the cortical tubules in the group given vincristine and honey (VHG) ×100, H&E.

Table 3. Classification of histopathological findings in the liver and kidneys in experimental groups.

Tukey multiple comparisons test (Liver)	Mean diff.	Significance	Adjusted P-value
Degeneration vs. Necrosis	0.2450	-	>0.9999
Degeneration vs. Vascular changes	25.32	-	0.1090
Degeneration vs. Inflammation	25.32	-	0.1090
Necrosis vs. Vascular changes	25.08	-	0.0979
Necrosis vs. Inflammation	25.08	-	0.0979
Vascular changes vs. Inflammation	0.000		
Tukey multiple comparisons test (Kidney)			
Degeneration vs. Necrosis	16.12	-	0.0968
Degeneration vs. Vascular changes	46.55	-	0.0850
Degeneration vs. Inflammation	46.55	-	0.0850
Necrosis vs. Vascular changes	30.43	-	0.2822
Necrosis vs. Inflammation	30.43	-	0.2822
Vascular changes vs. Inflammation	0.000	-	

Level of significance: * (mild), ** (moderate), *** (strong).

Table 4. Hematologic-Biochemical parameters according to experimental groups.

	Hematologic Parameters						
Groups	**CG (n=12)**	**VG (n=12)**	**CCG (n=12)**	**VinG (n=10)**	**HG (n=12)**	**VHG (n=7)**	**P**
WBC (10^9/L)	8.16±0.42^{a}	8.46±0.19^{a}	6.86±0.4^{b}	6.38±0.24^{b}	6.8±0.32^{b}	6.46±0.47^{b}	*P<0.001*
Lymphocyte (10^9/L)	5.4±0.18^{ab}	5.86±0.27^{a}	3.97±0.59^{bc}	4.43±0.2^{ac}	3.64±0.67^{cd}	2.04±0.4^{d}	*P<0.001*
Monocyte (10^9/L)	0.33±0.03	0.34±0.04	0.34±0.52	0.43±0.04	0.42±0.08	0.4±0.11	
Neutrophil(10^9/L)	2.16±0.25	2.12±0.3	2.27±0.26	1.61±0.18	1.74±0.14	1.88±0.23	
	Biochemical Parameters						
CRP (mg/L)	0.51±0.03	0.51±0.04	0.58±0.03	0.54±0.04	0.53±0.04	0.55±0.05	
TP (g/dL)	7.25±0.1^{b}	7.55±0.42^{ab}	8.17±0.19^{a}	7.68±0.14^{ab}	7.15±0.26^{ab}	6.92±0.27^{b}	*P=0.002*
Alb (g/dL)	3.15±0.13	3.29±0.19	3.55±0.09	3.5±0.06	3.09±0.11	3.58±0.1	
ALT(U/L)	44.08±4.11^{c}	47.85±2.54^{c}	83.88±1.77^{a}	71.19±5.62^{a}	60.39±3.73^{b}	53.68±2.61^{bc}	*P<0.001*
AST(U/L)	138.83±5.2^{c}	122.7±11.9^{c}	305.9±19.16^{a}	297.5±32.1^{a}	185.35±9.77^{b}	159.8±11.3^{bc}	*P<0.001*
Urea (mg/dL)	38.7±1.11	41.28±1.53	37.63±1.79	36.65±1.54	36.45±0.92	38.74±2.59	
Creatinine (mg/dL)	0,67±0.02^{ab}	0,75±0.03^{a}	0,72±0.02^{a}	0,64±0.05^{b}	0,65±0.01^{b}	0,71±0.02^{ab}	*P=0.009*
Survivin (ng/L)	38.04±0.91^{b}	38.75±1.39^{b}	46.03±1.43^{a}	35.67±1.91^{b}	39.8±1.85^{b}	36.61±1.02^{b}	*P<0.001*
Caspase-3 (ng/mL)	147.61±0.9^{a}	147.73±1.4^{a}	110.32±3.86^{c}	135.59±4.7^{ab}	129.6±2.18^{b}	126.17±1.1^{b}	*P<0.001*
Caspase-9 (ng/L)	6.72±0.06^{c}	6.22±0.08^{c}	8.87±0.27^{a}	7.92±0.2^{b}	8.03±0.09^{ab}	8.23±0.04^{a}	*P<0.001*
Caspase-8 (ng/mL)	120.95±1.1^{b}	121.09±1.6^{b}	117.38±1.57^{b}	117.65±0.8^{b}	132.59±3.18^{b}	136.15±2.5^{a}	*P<0.001*
Bax (ng/mL)	7.32±0.04^{a}	7.35±0.06^{a}	6.38±0.11^{c}	6.39±0.04^{c}	6.86±0.1^{b}	7.08±0.04^{b}	*P<0.001*
Bcl-xL (ng/mL)	7.27±0.06^{b}	6.2±0.03^{c}	8.31±0.23^{a}	7.11±0.23^{b}	7.24±0.11^{b}	7.48±0.05^{b}	*P<0.001*

Values in the table are given as arithmetic mean ± standard eror (M±SE).
*a,b,c,d indicate differences between groups in the same row.

WBC values determined in CG and VG group rats were found to be significantly higher than other groups. The lymphocyte values determined in the CG and VG group rats were found to be significantly higherer than the values determined in the VinG, HG and VHG group.

Total protein and AST values determined in CCG and VinG groups were significantly higher than the values determined in CG and VG groups. Although the total protein and AST values determined in the HG group were not as high as CCG and VinG, they were found to be significantly higher than the values determined in the other groups. ALT levels determined in CCG and VinG groups were found to be significantly higher than the values determined in other groups. The survivin value

determined in the CCG group was found to be significantly higher than the values determined in the other groups. The Caspase-3 value determined in the CCG group was found to be significantly lower than the values determined in the other groups. In addition, Caspase-3 values determined in the HG and VHG groups were found to be significantly lower than the values determined in the CG and VG groups. Caspase 9 levels determined in the CG and VG groups were found to be significantly lower than the values determined in the other groups. Caspase 8 levels determined in VHG group was found to be significantly higher than the values determined in other groups. Bax levels determined in the CG and VG groups were found to be significantly higher than the values determined in the other groups. Bax levels determined in CCG and VinG groups were found to be significantly lower than the values determined in other groups. The Bcl-xl level determined in the CCG group was found to be significantly higher than the values determined in the other groups.

Discussion and Conclusion

Breast cancer is the second most common type of cancer in humans after lung cancer and the most common type of cancer in women (24). *In vivo* breast cancer models are important research tools for understanding both the pathogenetic mechanism of the disease and the development of treatment options (16). It is extremely crucial to evaluate disease progression by looking at levels of relevant biomarkers in serum or tissue and deciding accordingly on main or alternative treatment applications for breast tumors (18). By determining the levels of prognostic biomarkers for patients with breast tumors, critical evaluations and operational or chemotherapeutical decisions can be properly made based on knowledge of whether the cells have acquired malignant atypia, the epithelial-mesenchymal transition state, vascularization in the microenvironment, changes indicating hypoxia, or dysplasia. In this way, decisions can be made considering the likelihood of the cancer accelerating with neoplastic development, regressing, or remaining stable. The important point here is whether mitotic cells in neoplastic tissues are experiencing death or remaining alive. The detection of cell proliferation and survival or the death of the cells via programmed cell death or apoptosis is very important in terms of making decisions about the severity of the patient's pathophysiological condition (31). While apoptosis may cause inhibition of the cancer to maintain homeostasis in tissues where cancer has developed, the inhibition of apoptosis is provided by survivin. However, there are still debates about whether the evaluation of survivin levels alone can allow an informed decision about cancer progression. Generally speaking, in the last 15 years it has been accepted that survivin is a good biomarker in terms of evaluating cell proliferation resulting from the disruption of homeostasis (27, 41).

Survivin is normally expressed in both the cytoplasm and nuclei of cells. Although survivin is expressed in the nuclei of different tumor types, it has been shown to be more intensely expressed in the cytoplasm of tissues in cases of breast cancer. Survivin has another function apart from maintaining neoplastic cells to facilitate their continuous division. In many *in vitro* studies and *in vivo* experiments, it has been shown that survivin may inhibit the promoter activity of caspase-9, an initiator of apoptosis, which is a critical step in apoptosis. It has been shown *in vivo* that apoptosis inhibits the activation process in mitochondrial pathways, and it is thought to do so by inhibiting secondary cancer initiation (21, 27, 29). Again, in studies on survivin, it was stated that the rate of survivin expression ranges between 60% and 90% according to differences in the methods used to evaluate breast cancer in humans (19, 20, 27, 34). However, survivin is accepted as a reliable marker in the early diagnosis of breast cancers and in determining treatment procedures according to expression levels. The cytoplasmic expression of survivin is stated to be highly effective in determining the prognosis of the disease (27). In the current study, the results showed that survivin levels were slightly increased in the cancer control group. The survivin levels determined in the groups given vincristine, vincristine and honey, and the vehicle substance were close to the levels obtained for the control group. However, when compared among themselves, the survivin levels in the vincristine group and the vincristine-honey group were slightly lower than the values determined in the control group or the vehicle group. These results showed that survivin levels were notably triggered in the presence of rapidly proliferating anaplastic cells. The decrease in survivin levels in the group given vincristine and the group given vincristine and honey showed that these substances suppressed the anti-neoplastic effects of the cancer by slowing down the transitions among the G0, G1, S, G2, and M phases of anaplastic cells during the division and proliferation stages. Therefore, the survivin levels began to decrease. This selected marker is also valuable for the determination of cancer prognosis and evaluations of co-treatment with grayanotoxin as well as vincristine chemotherapy.

Apoptosis, on the other hand, is an important mechanism for the determination of cell death or survival following different chemotherapy treatments. For this mechanism, there are mainly internal and external pathways. The internal pathways in apoptosis are extremely vital, particularly the one known as the mitochondrial pathway. These pathways generally act by increasing or decreasing the function of Bcl-2 protein family members (9, 11). Proteins that promote or inhibit

apoptosis interact with each other and play roles in the death or survival of cells. Bax is also a member of the Bcl-2 family and it is known as a proapoptotic protein that plays a key role in promoting apoptosis. Bcl-2 is an antiapoptotic protein that inhibits Bax function in the initiation of cell death. An increase in Bax expression increases the susceptibility of cells to apoptotic stimuli and reduces tumor growth (2). In this sense, it has been suggested that the Bcl-2/Bax ratio alone may be an effective prognostic factor in determining apoptosis. Components that stimulate or inhibit apoptosis also determine the death or survival of cells. Under the influence of apoptotic stimuli, the release of cytochrome c from the mitochondria of the cell is triggered. With the help of ATP, Apaf-1, and cytochrome c a complex is formed and caspases are activated. Stimuli are commonly reported to be trigged by initiator caspases (caspase-2, -8, -9, and -10) with propagation via effector caspases (caspase-3, -6, and -7). As a result, researchers (36, 38) predict decreases in caspase-9 levels and high Bcl-2/Bax ratios in resistant cells.

In vitro studies of breast cancer have shown that dysregulated caspase activity plays a role not only in the progression or proliferation of breast tumor cells but also in chemotherapeutic resistance. It was previously shown that the MCF-7 breast cancer cell line may be susceptible to apoptosis due to restoration of caspase-3 expression and to doxorubicin in the event of caspase-3 deficiency, and this suggests that caspase-3 deficiency may be a possible mechanism for chemoresistance (46). In addition, activation mediated by pro-caspase-9, cytochrome c, and caspase-8 was shown to be regulated in MCF-7 cells (4). However, no association of caspase-8 with breast cancer-specific survival was found. Blazquez et al. (5) and Pu et al. (32) observed that caspase-3-dependent overexpression and apoptotic activity were more common in breast carcinomas, but in terms of prognosis, no significant relationship could be demonstrated between these findings and baseline caspase-3 expression. In the present study, in the process of apoptosis, Bax and Bcl-xL protein levels were close to each other in all groups. While the initial values of caspase-9 were close to each other in the control group, the group given honey, and the group given the vehicle substance, these values were seen to decrease in the cancer control group, the group given vincristine, and the group given vincristine and honey. Although it was observed that these changes were triggered by caspase-3, values were relatively lower in the cancer control group, the group given honey, the group given vincristine, and the group given vincristine and honey, in contrast to expectations. Although the Bax and Bcl-xL levels remained stable in apoptosis, the decreases in caspase-9 and caspase-3 in the cancer control group and in the vincristine group were interpreted as signs of intense anaplastic changes. Therefore, it could be suggested that the apoptosis-triggering genes did not function sufficiently and did not initiate signaling as adequately as in the healthy groups. In line with the histopathological data, the cells were destroyed in the cancer control group and partially so in the group given vincristine and honey, and the changes were more apt to take the forms of degeneration and necrotic changes instead of apoptosis. Although anaplastic changes were common in the group given honey, cystic changes were observed and grayanotoxin functioned to trigger apoptosis even though degeneration was also observed. However, the induction of apoptosis in the group that received vincristine and honey was not observed at this critical level. In this groups, histopathological alterations were more prevalent than apoptosis. Furthermore, in this study, no changes related to anaplasia were found in the healthy control group; in those animals, the mammary gland cells had a regular order and gland structures were appropriately formed. It was thus thought that mild apoptotic changes may have been encountered in the cell nuclei in some areas as a result of physiological effects. In the cancer control group and the group given honey, intense anaplastic changes and islet formation due to the proliferation of cells lining the glands were detected. On the other hand, oxygenation and the feeding of the blood supply are important processes for cell survival during cancer development. Hence, we also observed capillarization and blood supply in medium-sized vessels in the mammary tissue. In addition, degenerative and necrotic changes were intense in the cancer control group. However, rather than degeneration and necrosis in the honey-administered group, cystic structures were noted, which were seen at high levels in all groups. In the vincristine group, anaplasia was reduced and islet formations were weaker, and vascular changes were also reduced compared to other groups. In addition, no degenerative or necrotic changes were found in the mammary tissue of this group. Gland structures in the vehicle group were normal, as in the control group. Apart from this, no remarkable findings were noted. In the group given vincristine and honey, anaplastic changes in mammary gland epithelial cells were considerably reduced, glandular cell proliferations were not sufficient for the formation of islets, cells were close to normal in appearance, and vascular changes were weak. On the other hand, degenerative and necrotic changes were observed, although not as much as in the cancer group. Inflammatory changes were not observed in any experimental groups.

As a result of chemical interactions, certain DMBA metabolites and excessive reactive oxygen species are produced in the cellular microenvironment. This situation creates carcinogenicity because it disturbs DNA and mitotic activities. After cumulative exposure, these

metabolites create a deficit of cytochrome P450 enzymes, particularly in hepatocytes and kidney cells (10, 25). The cycles of tricarboxylic acids are also disrupted by metabolites of DMBA and free radicals. The first changes occur in biochemical parameters because the toxic compounds have catastrophic effects via cellular organelles (25). All cellular organelles, and particularly mitochondria, continue to shift toward catabolism (7, 10). As a result of these interactions, some enzymes, proteins, and macromolecules were found to be elevated in serum in the present study. At the same time, nuclear and cytoplasmic changes developed in cells, seen as degeneration, apoptosis, or necrosis. In our study, total protein levels were found to be relatively higher in the CCG, VinG, and VG groups when compared to the others. Values of ALT and AST were nearly the same. Levels were also higher in the CCG and VinG groups compared to CG, VG, and VHG for total protein. However, differences in CRP, urea, and creatinine were not significant. These findings confirm that the liver and kidneys are affected similarly by the toxicity of DMBA and vincristine. Plasma protein levels were elevated dramatically as a result of major damage in the cells. On the other hand, the groups that received honey containing grayanotoxin did not experience increases as large as expected for biochemical parameters. Hence, we believe that mad honey may be useful and effective in fighting cancer cells.

We encountered histopathological alterations as expected. Normally, the histological results obtained from the liver and kidneys provide evidence of biochemical alterations (27). We observed parallel results in our study. In previous studies of DMBA-induced liver damage, it was stated that necrosis, vascular changes, and non-purulent inflammation were observed in rat livers (12, 23, 30). In our study, degeneration and necrosis were also observed in the livers, but there was no inflammation. Vascular changes were not remarkable. The histopathological results obtained from the livers of the VinG and CCG groups were similar. Liver cells were also affected in the HG group, while the opposite was seen for VHG. In the VG and CG groups, the rates of degeneration were lowest. In the kidneys, cortical tubules were more affected by degeneration and necrosis. The cells were affected in VinG and CCG as well as the HG group. Preservation of cellular morphology in the kidneys was similar in the VG and CG groups. There were no remarkable pathological changes in terms of vascular changes or inflammation.

In conclusion, it was understood that in the group given honey alone, degeneration and apoptosis were triggered. Thus, the development of breast tumors was reduced. Additionally, in the group given vincristine alone and in the group given vincristine and honey together, it was observed that these applications did not have significant regressive effects on breast tumors. It is clearly important to evaluate the relationship between the results obtained in the group given honey and the findings of apoptosis and degeneration in more depth, as the amount of grayanotoxin in particular honey samples may be a determining factor. It would also be useful to look at dose-response curves and Bax, Bcl-xL, caspase-9, and caspase-3 levels after selecting grayanotoxin applications in amounts close to the grayanotoxin levels in honey in mouse models and the frequently preferred human MCF-7 breast cancer line in *in vitro* trials. Evaluation of Bcl-2 antiapoptotic proteins with dose-response curves proportioned to Bax proteins, together with results obtained from literature reviews, will also be valuable. Although grayanotoxin targets the cell mitochondrial pathway with intrinsic apoptosis, it may also be effective on extrinsic pathways. Another result obtained from this study is that when cells are under the influence of cancer-inducing DMBA, vincristine, and vincristine-grayanotoxin, cellular toxicity develops and cell homeostasis is disrupted, and this affects the viability of the cells, resulting in strong effects of degeneration and necrosis instead of apoptosis. We believe that the most effective grayanotoxin types, namely I and III, which are found at high concentrations in Turkish mad honey, can be successfully applied as co-therapeutics with vincristine chemotherapy, which is classically preferred for mammary cancer therapy, after the proper therapeutic dosages are determined.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

EK, MEA, AMA, BB, NK and EG conceived and planned the experiments. EK, MEA, AMA, BB, NK and EG carried out the experiments. EK, MEA, AMA, BB, NK and EG planned and carried out the simulations. EK, MEA, AMA, BB, NK and EG contributed to sample preparation. EK, MEA, AMA, BB, NK and EG contributed to the interpretation of the results. EK, MEA, AMA, BB, NK and EG took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Permission to conduct the study was granted by the ethics committee decision numbered 2021-13-113 of Ankara University Animal Experiments Local Ethics Committee, Türkiye.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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