The Combined Effects of 5-Fluorouracil and Turkish Propolis Extract on EPGF and KRAS Expressions and Apoptotic Cascade Changing in Rat Colon Cancer Model

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ABSTRACT

This study aimed to investigate the effectiveness of 5-fluorouracil (5-FU) and Turkish propolis extract on the colon cancer model induced by azoxymethane (AOM) in Wistar rats. Study groups were designed as cancer group (AOM), control group, AOM+5-FU group, AOM+propolis group, AOM+Propolis+5-FU group. The findings showed that there was a significant decrease in WBC, and lymphocyte levels in the treatment groups AOM+5FU and AOM+Propolis+5FU compared to the AOM group (P<0.0001, P<0.005, respectively). The increase in TAS level and TOS level were found to be significant in the AOM+Propolis+5FU group compared to the AOM group (P<0.0001). Compared to the the AOM group, caspase-3, caspase-8 and bax levels were significantly increased and Bcl-2 evel was significantly lower in the 5 AOM+Propolis+5FU group (P<0.0001, P<0.05, P<0.0001, P<0.0001, respectively). In the 5-FU and propolis used-therapy groups, decreased anaplasia in colonic cells. In conclusion, we strongly believe that as a chemotherapeutic, 5-FU, and propolis might have a stopper effect on cellular anaplasia over extrinsic apoptotical pathway. We also believe that this antiproliferative effect on cellular anaplasia can appear if the combined therapy is preferred.

Introduction

Colorectal cancers (CRCs) have been reported to be amongst the health problems in developed and developing countries most associated with a positive socioeconomical status (40). In the pathogenesis of CRCs, there are complex interactions between genes, signaling cascade systems, specific molecules and receptors. One of these factors is genetic mutation. Some specific genes play an pivotal role during critical events in carcinogenesis. In other words, activations / inhibitions of genes and molecules assist in triggering cancer cell proliferation (15, 45).

Epidermal growth factor (EPGF) is a transmembrane protein that include a receptor (EPGR) amongst members of the epidermal growth factor family (EGF family). In epithelial origined cancers, it is released plenty of this transmembran protein after activated of EPG receptor. Kirsten rat sarcoma (KRAS) oncogene produce KRAS protein and is a member of RAS/MAPK signaling cascade. It is a fact that KRAS is an oncogene stimulated after activation by several cancer cascade molecules. EPGR-KRAS receptor are indeed homolog signaling receptor which playing role are among the key factors in the pathogenesis (9, 30, 45, 54). This leads, firstly, to an

upstream at receptor tyrosine kinase (RTK) and secondly at mitogen-activated protein kinases (MAPKs) / extracellular signal regulated kinases (ERKs) (MAPKs/ERK) signal transduction pathway. After intracellular tyrosine kinase receptor undergoes stimulation, it is then activated by extracellular ligand to the transmembrane receptor of EPGF. In such, cellular growth and proliferation are provided under control of chief oncogenes in colon cells. Also The KRAS gene is a member of the rat sarcoma viral oncogene family (RAS). In CRC cells eventually die and then go under the regulation of apoptosis through activation RAS protein as well as Protein kinase B (PKB/AKT)-phosphoinositide 3-kinases (PI3K) pathway. Firstly, RAS activates PI3K, which results in AKT activation. After AKT-PI3K pathway triggered, proapoptotic proteins and anti-apoptotic proteins are indirectly activated (30, 35).

One of the mechanisms of apoptosis, caspase -3,-8 and 9, is inhibited by AKT upregulation (3). On this point, RAS can also interact with anti-apoptotic bcl-2 protein (37). Cell death cannot happen alone when Bcl-2 protein is knock downed and bax and bak proteins influence growth factors (28).

5-FU, which has been a widely preferred chemotherapeutic agent against colorectal cancer malignancies to date, continues to be used effectively and frequently against the developmental stages of colorectal cancers that have acquired malignant properties through genetic mutations such as the KRAS oncogene (7, 18, 36).

On the other side, there have been many natural supplements and complementary therapeutics for cancer therapies nowadays. Herein, propolis is thought providing crucially role for clinical applications of anti-cancer treatments and lessen side effects of adjuvant therapy to main cancer chemotherapeutics. Its chemical composition is made up of polyphenols-flavonoids and phenolic acids (27, 33, 49). Also it has potential to be both chemotherapeutic and chemopreventive for pre-cancerous changes and cancer progressions (2, 17, 34). Its anti-cancer effectivities have been shown both in-vivo and in-vitro (12, 14, 25).

During cancer progression in the body, continuously exposure of oxidative stress on cells can prone to proliferation by disturbing cellular and microenvironmental homeostasis. For this purpose, important data regarding oxidative stress and oxidant/antioxidant balance in the body are obtained by revealing the total oxidant status (TOS) and total antioxidant status (TAS) of the body (8, 20).

The hypothesis of this study was based on investigating whether the separate and combined use of 5-FU, a chemotherapeutic agent, and propolis would cause mutations and apoptosis-related changes in cancer tissue and antiproliferative effects on cellular anaplasia in an experimentally created colon cancer model in rats.

In order to decide on the propositions in this hypothesis, it was aimed to investigate the changes in hemogram and biochemical parameters (various protein and enzyme concentrations) and histopathological results with the presence of chemotherapeutic 5-FU and propolis extract in AOM-induced CRC modeling in rats. It was also planned to evaluate how the expressions of KRAS and EGFR in colon cancer cells and their levels in apoptotic state (caspases, pro-apoptotic Bax, and anti-apoptotic Bcl-2 proteins) could be affected.

Materials and Methods

The study was conducted upon permission given from the ethics committee decision numbered 2022/05/23 of Kırıkkale University Animal Experiments Local Ethics Committee, Türkiye. In the study, 6 weeks old (average weight of 150–180 g), 30 male Wistar albino rats were used. They were given standard feed and water ad libitum throughout the study and maintained an air-conditioned animal facility under constant temperature and humidity with a 12 h day-night cycle.

The 30 rats used in the study were randomly divided into 5 groups of 6 each. The first group was the group in which cancer was induced by giving AOM. The second group was the healthy control group to which no application was made. The third group was the group in which AOM and 5-FU were given. The fourth group was the group in which AOM and propolis were given. The fifth group was the group in which AOM and 5-FU and propolis were given. The applications made in these experimental groups are explained below. In first group (AOM group) (n=6), Azoxymethane (AOM) in 0.5 ml physiological saline solution (PSS) were dissolved and administered at 15 mg/kg (Body Weight-BW) intraperitoneally once a week for three weeks. Additionally, this group was administered orally distilled water three times a week for four weeks. In the second group of the study (Control group) (n=6), a PSS was administered intraperitoneally once a week for three weeks. Additionally, this group was administered distilled water orally three times a week for four weeks. In the third group of the study (AOM+5-FU treatment) (n=6), AOM was administrated at the same dosage and duration to be in AOM group. Additionally, 12.5 mg/kg/BW 5-fluorouracil (5-FU group) given intraperitoneally three times a week for four weeks. In the fourth group (AOM+propolis administration group) (n=6), AOM and PSS were administered as they were in the previous groups. In addition, 0.3 mg/kg/BW propolis dissolved in distilled water was administered orally by gavage to this group, occurring three times a week for four weeks (12). In the last group (AOM +propolis+5-FU group) (n=6), AOM, 5-FU and propolis were administered the same dosage

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Table 1. Experimental design in groups.

Experimental Groups	Physiological saline solution (once /every week during 3 weeks)	Azoxymethane (cancer induction) (once /every week during 3 weeks)	5-Flourouracil Chemotherapy (three times / every week during 4 weeks)	Propolis Additive therapy (three times /every week during 4 weeks)
AOM		✓		
Control	✓			
AOM+5-FU		✓	✓	
AOM+propolis		✓		✓
AOM+5FU+propolis		✓	✓	✓

and duration as in the previous treatment groups. Study design described in Table 1.

At the end of the experiment, the rats were anesthetized by intraperitoneally injections of xylazine (10 mg/kg) and ketamine (90 mg/kg). The blood (tube without anticoagulant and tube with EDTA) was taken from the V. Cava Caudalis of anesthetized animals. After blood was taken from the animals, the animals were sacrificed, and tissues were taken. Serum samples were removed from blood samples and stored at -80 °C until analysis.

Propolis samples were collected after honey harvest in beekeeping areas in the Black Sea region of Türkiye. Obtained from herbalist and beekeeping commercial products from sales points, the propolis was stored at -4°C until the samples were studied. Before the study, the samples were removed from the cold and allowed to reach room temperature. 5 g of propolis the solution obtained by adding 100 mL of 70% ethanol and shaken regularly placed in ultrasonic device (Bandelin electronic device, Sonorex D12207, Berlin Germany) at 220 W and 40 kHz for 30 min. The extract was filtered to a volume of 100 ml to obtain the extract for further analysis. After ultrasound extraction, the mixtures were centrifuged for 10 min at 1,600 g. Supernatants of different extracts were placed in an orbital shaker (Stuard, orbital incubator S1500, Bibby Scientific, Staffordshire, UK) 24 h at 40°C. The extract was filtered to a volume of 100 ml to obtain the extract for further analysis.

The hematological analyses were performed using an automatic blood count device (Mindray BC 5000, China) within three hours of blood collection.

TAS and TOS, which are oxidative stress parameters, were determined in the colon samples collected from the all the study groups. Total antioxidant activity was calculated based on the free radical scavenging effects of *1,1-diphenyl-2-picrylhydrazyl* (DPPH) in propolis using the indirect method of the prepared extracts. Using ascorbic acid as a reference, the measurement was carried out in a spectrophotometer at 520 nm. (29, 41, 50).

Antioxidant activity was expressed as the percentage of inhibition of free radical DPPH and calculated using the formula specified above.

B-cell lymphoma 2 (Bcl-2) protein, b-cell lymphoma-extra-large (Bcl-xl) protein, caspase 3, 8, 9 (Bioassay Technology Laboratory, Bcl-2 Cat No: AP00861, Bcl-xL Cat No: E3340Ra, caspase-3 Cat No: E1648Ra, caspase-8 Cat No: E1370Ra, caspase-9 Cat No: E1898Ra, Zhenjiang, China) levels were measured spectrophotometrically (Sunrise RS-232, Tecan, Grödig, Austria).

In the macroscopic and histopathological examinations the rats were humanely euthanasied according to general protocol for rats, necropsies were performed. Possible cancer developing areas in colon and intestines, and pathological changes in the livers and kidneys as well as other tissues and organs were evaluated according to general macroscopic definition criteria. Samples were fixed in a 10%-neutral buffered formalin (NBF, Merck, Germany) solution for 48 hours. Then, for histopathological examination, the tissues were followed-up at alcohol series and xylene in an automatic vacuum tissue processor (TP1020, Leica, Germany) and embedded in paraffin in a paraffin dispenser (Leica, EG1150H, Germany). Next, 5-µm-thick sections were taken from the paraffin blocks (Shandon AS320, Germany). All tissue sections were stained according to the standard hematoxylin–eosin (H&E) staining method. Sections were covered with the cover slip using the mounting medium Entellan® (Merck). Tissue sections were evaluated semiquantitatively by counting 10 high power fields (HPFs) under a digital optical light microscope (Olympus BX51, Germany) at 400× magnification. Differences in the numbers of the groups were evaluated statistically. Eligible areas chosen under microscopy were photographed (Olympus DP5 camera attachment, Germany). Additionally, colon epithelial and glandular cells were scored in terms of anaplastic criteria (including chromatin density, nuclei/cytoplasm proportion and mitotic activity) and other preneoplastic changes (such as dysplasia, metaplasia, hyperplasia etc.) according to Quickscore (QS) method modified by Detre et al. 1995 (11).

In EPGF and KRAS expressions in colon tissue avidin-biotin complex peroxidase (ABC-P) method were preferred according to the manual instruction of the immunoperoxidase detection kit (Novocastra- RE7110-K, Leica, Germany). EPGF and KRAS primary antibodies were utilized for detection of precancerous and cancerous proliferation in colon. For this purpose, tissue sections were taken 5-micron thick on positive charged slides. The slides were deparaffinized in xylenes and dehydrated by passing through ethanol series. Then, they were placed in a citrate buffer (pH=6.0, 10x, Bioptica, Italy) solution and kept in a microwave oven at 800-Watt power for 25 minutes to reveal the antigen in the tissue. To eliminate endogenous peroxidase activity, the slides were kept in 3% hydrogen peroxide-methanol mixture at room temperature for five minutes, then taken into a humid chamber, where one drop of normal blocking serum was added. They were kept in the oven at 37°C for 25 minutes. In the following stage, primary serums containing commercially available antibodies were dropped onto the sections and incubated in an oven at 37°C for 60 minutes. Then, appropriate secondary antibodies labeled with biotinylated horse radish peroxidase (HRP) were used and incubated in the oven at the specified temperature and time. For the reaction to become visible, *3,3'-Diaminobenzidine* (DAB) chromogen was dropped onto the sections and given five minutes to set in. Finally, Gill's hematoxylin was used for ground staining. The slides were passed through degraded ethanol series and xylene and covered with a coverslip using Entellan (Merck, Germany). The findings were evaluated under a digital light microscope (Olympus BX51, Germany) as in other histopathological examinations, and the results were scored according to Quickscore (QS) method modified by Detre et al. 1995 (11).

Statistical Analysis: In the study, the normality test was used to check whether the groups were distributed parametrically or not. The results were expressed as mean ± standard error (M±SE). A P value of <0.05 was criteria was considered statistically significant for all analyses. Data showing parametric distribution (TAS, TOS, Caspase3, caspase9, bax, Bcl-2, caspase8, neutrophil, eosinophil, monocyte, PCT and lower values) One-Way ANOVA, groups Duncan test (post hoc) was performed to check the significance of the difference between the biochemical data were analyzed using the SPSS statistical software program (PASW Statistics for Windows, ver. 18.0. Chicago, USA). Caspase-8, creatine, and lymphocyte values showed nonparametric distribution; Kruskal-Wallis test followed by Mann-Whitney *U* (post-hoc, Bonferroni correction; P<0.005) test was performed to check the significance of the difference between groups. One-Way ANOVA was applied for histopathological changes, with Tukey (post-hoc) to check significance between groups.

Results

The hematological parameters table for the study groups is given in Table 2. In the hemogram profile, WBC levels in the AOM group were significantly higher than AOM+5-FU and AOM+Propolis+5-FU groups (P<0.0001). Lymphocyte levels decreased significantly in the AOM+5FU and the AOM+5FU+propolis groups compared to the AOM group (P<0.005).

In the oxidative stress parameters, the increase in TAS level and TOS level were found to be significant in the AOM+Propolis+5-FU group compared to the AOM group (P<0.001). The oxidative stress parameters (TAS and TOS) of colon tissue in the study groups are given in Table 2.

Table 2. Hemogram profile, TAS and TOS levels, Apoptosis status (M±SE) in all experimental groups.

Groups	AOM	Control	AOM+5FU	AOM+Propolis	AOM+Propolis+5-FU	P value
Hemogram profile						
WBC	8.32±0.30^{ab}	7.42±0.67^{bc}	5.66±0.42^{d}	8.97±0.54^{a}	6.56±0.15^{cd}	**P<0.0001**
Neutrophil	1.80±0.12^{ab}	1.37±0.18^{b}	2.56±0.38^{a}	2.31±0.42^{a}	1.39±0.06^{b}	**P<0.05**
Lymphocyte	6.00±0.32^{a}	5.52±0.47^{ad}	2.42±0.14^{c}	5.99±0.11^{a}	4.75±0.11^{bd}	**P<0.005**
Monocyte	0.37±0.03	0.34±0.05	0.36±0.02	0.35±0.07	0.27±0.04	P>0.05
Eosinophil	0.11±0.01	0.14±0.04	0.13±0.02	0.16±0.02	0.13±0.02	P>0.05
PCT	2.58±0.13	2.17±0.24	2.00±0.36	2.62±0.15	2.51±0.25	P>0.05
TAS and TOS levels						
TAS	1.60±0.03^{b}	1.52±0.05^{b}	1.59±0.02^{b}	1.55±0.03^{b}	1.86±0.06^{a}	**P<0.0001**
TOS	15,15±0.23^{b}	14.78±0.44^{b}	15.31±0.19^{b}	15.46±0.28^{b}	17.81±0.55^{a}	**P<0.0001**
Apoptosis status						
Caspase3	101.11±0.58^{d}	84.66±0.58^{c}	121.72±1.12^{b}	96.94±0.66^{d}	136.90±3.00^{a}	**P<0.0001**
BCL2 protein	6.35±0.09^{a}	7.83±0.08^{c}	5.99±0.09^{d}	6.64±0.10^{e}	5.32±0.11^{b}	**P<0.0001**
Caspase9	5.35±0.25	5.30±0.07	5.03±0.07	5.44±0.19	5.54±0.03	P>0.05
Bax protein	6.72±0.03^{d}	6.45±0.05^{c}	7.36±0.02^{a}	7.37±0.11^{a}	7.67±0.02^{b}	**P<0.0001**
Caspase8	138.45±0.79^{bd}	129.68±0.64^{bc}	133.97±2.12^{b}	134.33±0.52^{bcd}	157.22±2.58^{a}	**P<0.005**

$^{(a,b,c,d)}$ **P<0.05, P<0.005, P<0.0001** were statistically significant between different captions.

In this study, compared to the AOM group, caspase 3, and bax levels were significantly increased, and bcl-2 levels were significantly lower in the AOM+5FU and AOM+propolis+5-FU groups (P<0.0001). The caspase-8 levels increased significantly in the AOM+propolis+5-FU group compared to the AOM group (P<0.05). Caspase-3, -8, -9 and bcl-2, bax protein levels of colon tissue in the study groups are given in Table 2.

In the AOM group, there was anaplastic proliferation having high mitosis in the epithelial cells lining the colon surface and in the glandular epithelial cells in all cases. Some hyperplastic and mild to moderate degenerative-necrotic changes were detected in the cells. There were moderate inflammatory changes in the propria mucosa. In control group, tissue morphologies were intact and normal epithelial cell proliferation were just seen in almost all cases. In some areas, degeneration and necrosis were also seen. In AOM+5-FU, although anaplasic changes and mitosis ranged from mild to severe, mild to moderate hyperplasia as well as higher inflammatory cell infiltration in the propria mucosa, were observed as seen in the AOM group. Degeneration and necrosis, unlike the previous control group, there were moderate to severe alterations in only two cases and moderate in the remaining cases. In AOM+propolis, the general view was similar to AOM. However, anaplasia was generally moderate to severe and did not have a mitotic index as low as AOM+5-FU. Although the general view of AOM+ propolis+ 5-FU group was similar to AOM+5-FU, anaplasia was mild or mild-moderate and degenerative-necrotic activities were again more mild-moderate. The results of the histopathological examinations of the study groups are presented in Table 3, while the histopathological changes observed in the colon tissues are illustrated in Figure 1.

Liver, kidney, and heart were evaluated in terms of degeneration, necrosis, vascular changes, inflammation, and fibrosis as related to acute and chronic histomorphological changes. In the liver, degeneration, and necrosis were found higher in the AOM group when compared to mainly control and the others. AOM+5-FU and AOM+propolis findings were relatively higher than AOM+ propolis+5-FU and similar to AOM group. Vascular changes were elevated in all groups excepting negative control cases. Other findings were not observed. In the kidney, the general view was similar to liver histopathology obtained from all groups. However, in the heart, degeneration, and necrosis in AOM+5-FU group were elevated when compared to control and AOM+proopolis+5FU groups. These findings of control and AOM+propolis groups were again decreased relatively according to other groups. In heart, vascular changes including hyperemia were found relatively at low level when compared to vascular changes in liver and kidney. The illustrations showing findings were given in Figure 2.

EPGF expressions were more elevated in AOM group when compared to healthy Control group. In treatment groups including AOM+propolis and AOM+ propolis+ 5-FU, expressions were decreased when compared to AOM group. KRAS expressions were more elevated in AOM group. The expressions in other groups were found similar. However, AOM+5-FU and AOM+propolis group expressions were slightly increased when compared to AOM and AOM+ propolis+ 5-FU. No statistical difference was found in the EPGF and KRAS expression results between the study groups. The KRAS and EPGF expression results are given in Graphic 1. The EPGF and KRAS expressions in the colon surface and gland epithelium of the study groups are given in Figure 3 and Figure 4.

Table 3. Histopathological scoring of groups.

Group	AOM	Control	AOM+5FU	AOM+Propolis	AOM+Propolis+5FU	P value
Inflammation	4.17 ± 0.60^{b}	1.50 ± 0.22^{a}	4.50 ± 0.50^{b}	2.83 ± 0.17^{ab}	3.17 ± 0.40^{ab}	P<0.001
Degeneration	2.83 ± 0.31^{a}	0.33 ± 0.33^{c}	4.33 ± 0.21^{b}	2.67 ± 0.58^{a}	2.50 ± 0.22^{a}	P<0.001
Necrosis	1.00 ± 0.00^{a}	0.33 ± 0.33^{c}	3.00 ± 0.26^{b}	2.17 ± 0.48^{ab}	2.17 ± 0.60^{ab}	P<0.001
Hyperplasia	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	2.83 ± 0.17^{a}	3.00 ± 0.00^{a}	2.83 ± 0.17^{a}	P<0.001
Anaplasia	4.17 ± 0.31^{b}	0.00 ± 0.00^{c}	3.33 ± 0.33^{ab}	3.33 ± 0.49^{ab}	2.50 ± 0.22^{a}	P<0.001
Mitosis	3.17 ± 0.31^{b}	0.00 ± 0.00^{c}	1.00 ± 0.00^{a}	1.00 ± 0.37^{a}	1.00 ± 0.00^{a}	P<0.001

$^{(a,b,c)}$P<0.0001 were statistically significant between different captions.

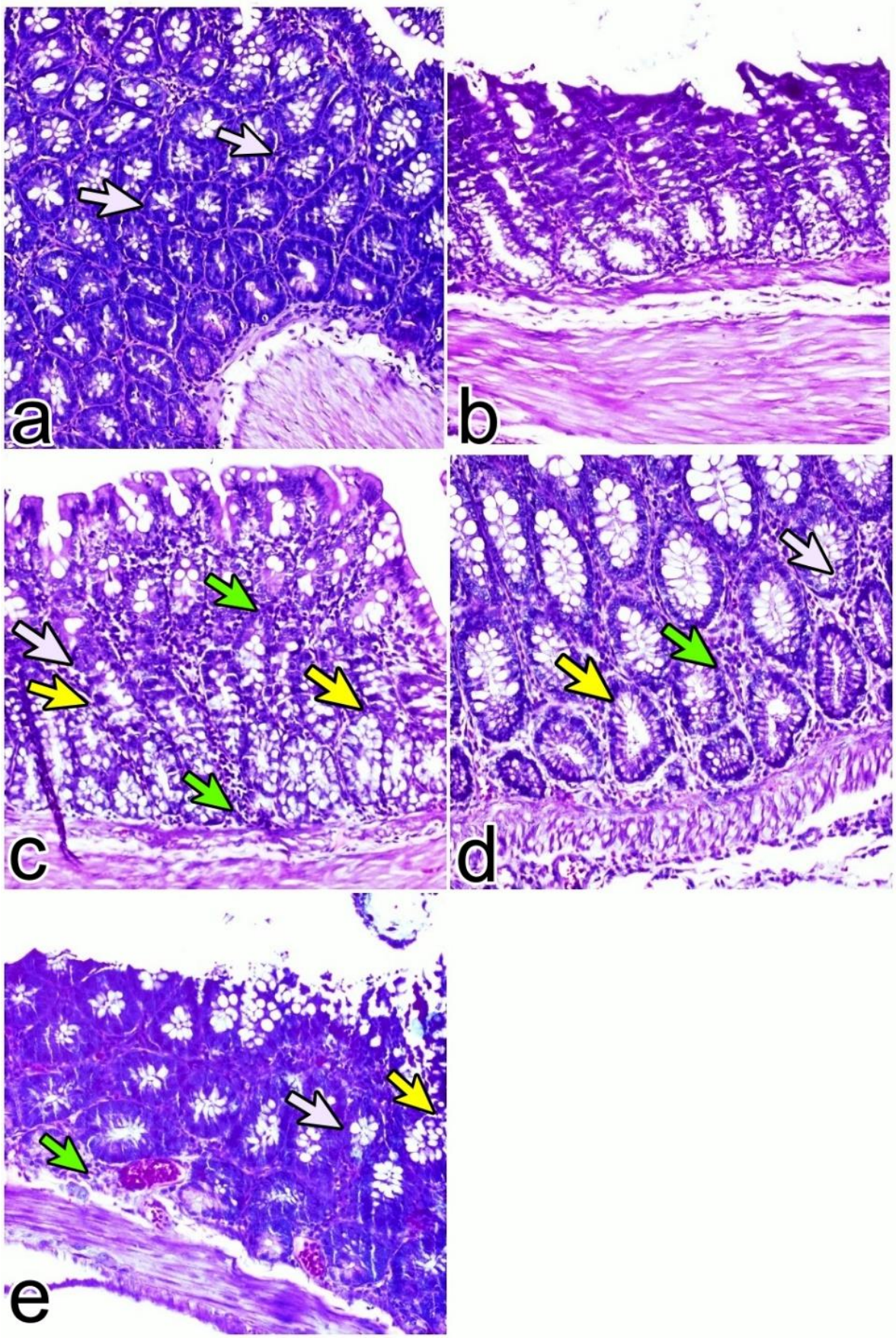

Figure 1. Histopathological findings in the experimental groups.

Anaplasic colon epitheliums (white arrows), degenerated cells (yellow arrows), inflammation (green arrows), group numbers consequtively corresponding to captions: AOM(a), Control(b), AOM+5-FU (c), AOM+propolis (d), AOM+ propolis +5-FU (e), x200, H&E staining).

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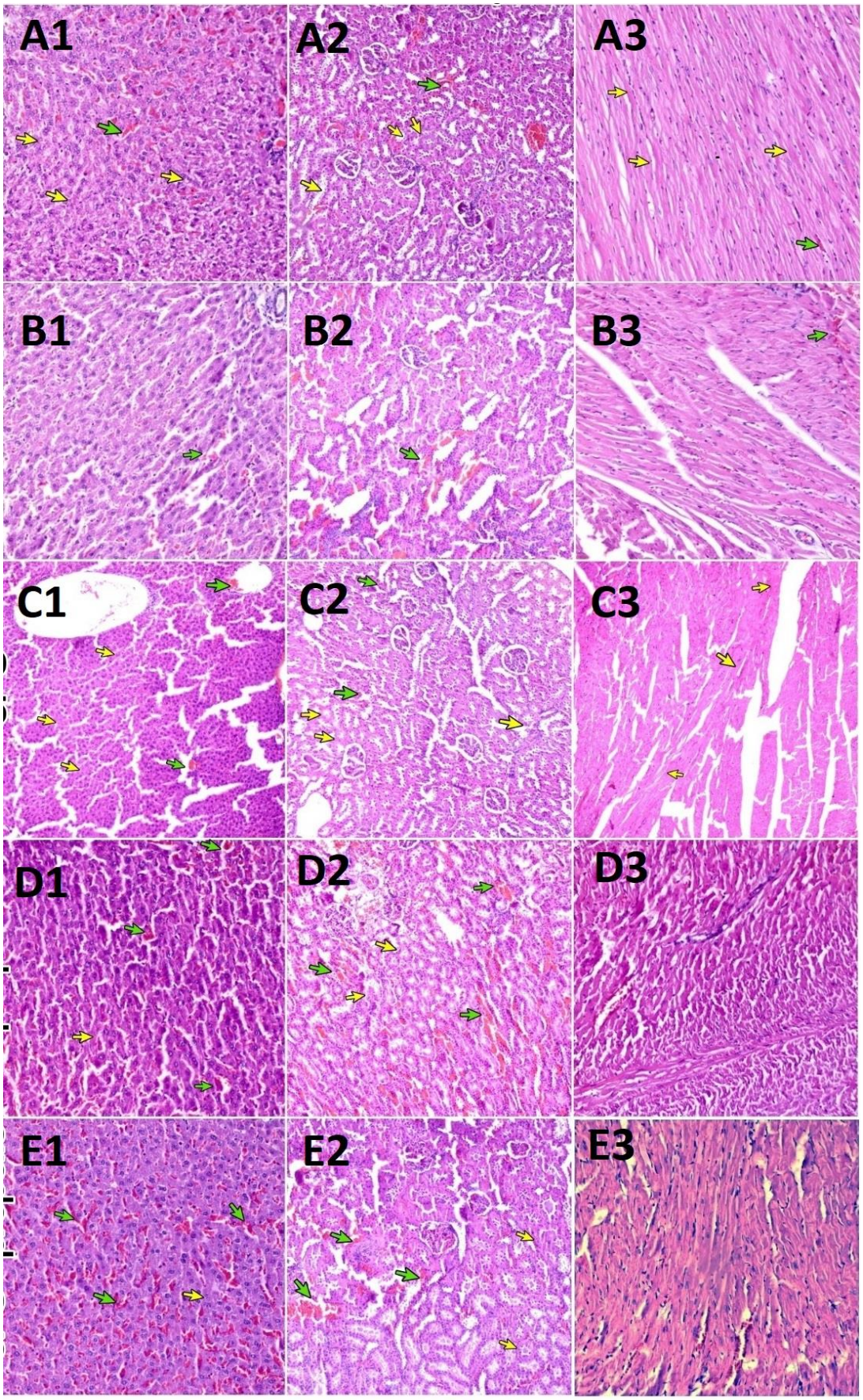

Figure 2. Histopathological findings in other organs including liver, kidney and heart.

Cell swelling and vacuolar degeneration (yellow arrows) and vascular changes including hyperemia (green arrows) in the experimental groups (numbered in vertical axis), x200, H&E staining.

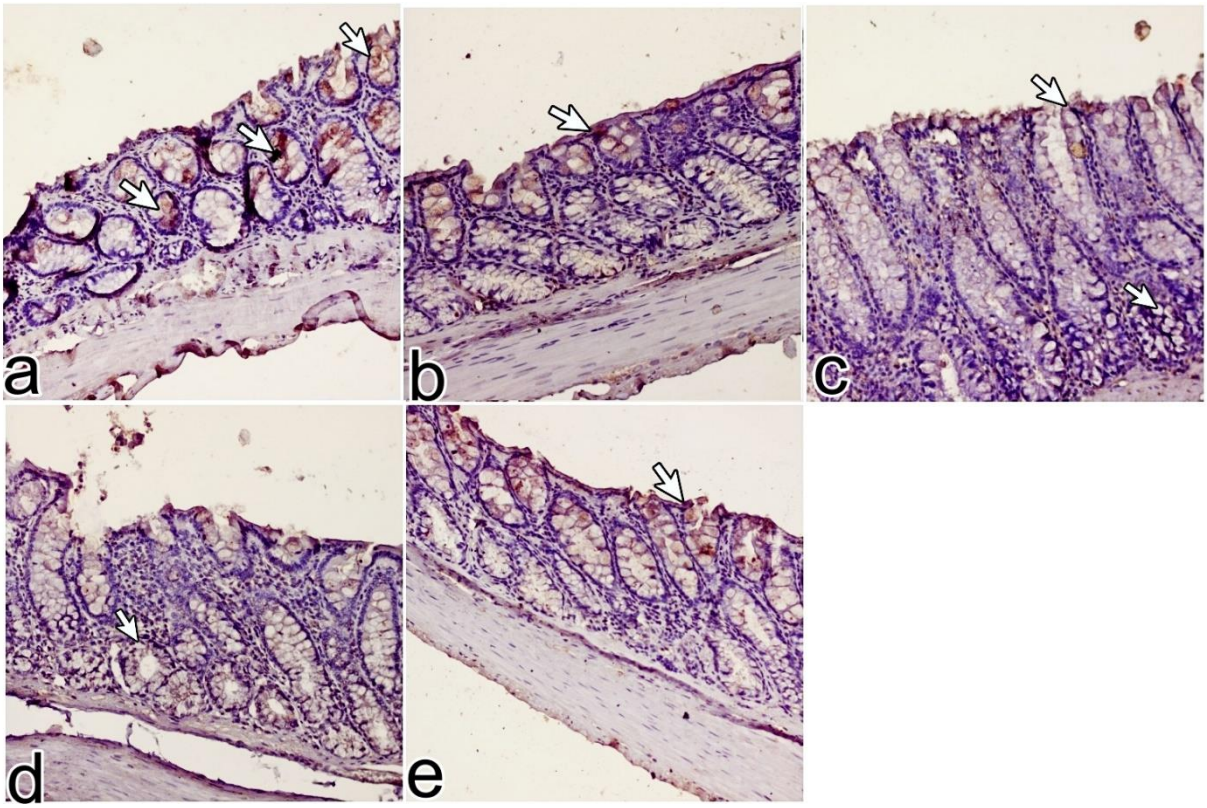

Figure 3. EPGFexpressions in colon surface and gland epitheliums (arrows).

AOM(a), control (b), AOM+5-FU(c), AOM+propolis(d) and AOM+ propolis+ 5-FU groups, ABC-Peroxidase staining, Gill's hematoxyline counterstain and DAB chromogene, x200.

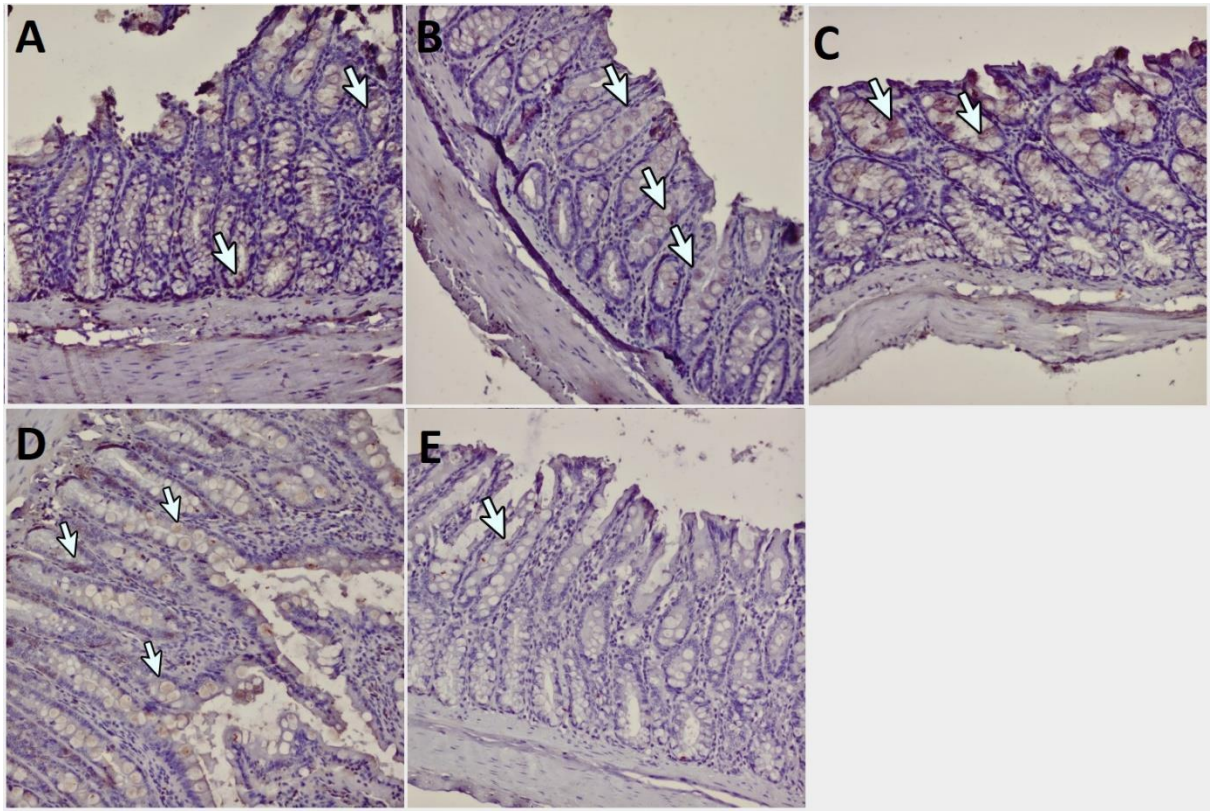

Figure 4. KRAS expressions in colon surface and gland epitheliums (arrows).

AOM(A), control (B), AOM+5-FU(C), AOM+propolis(D) and AOM+ propolis+ 5-FU groups (E), ABC-Peroxidase staining, Gill's hematoxyline counterstain and DAB chromogene, x200.

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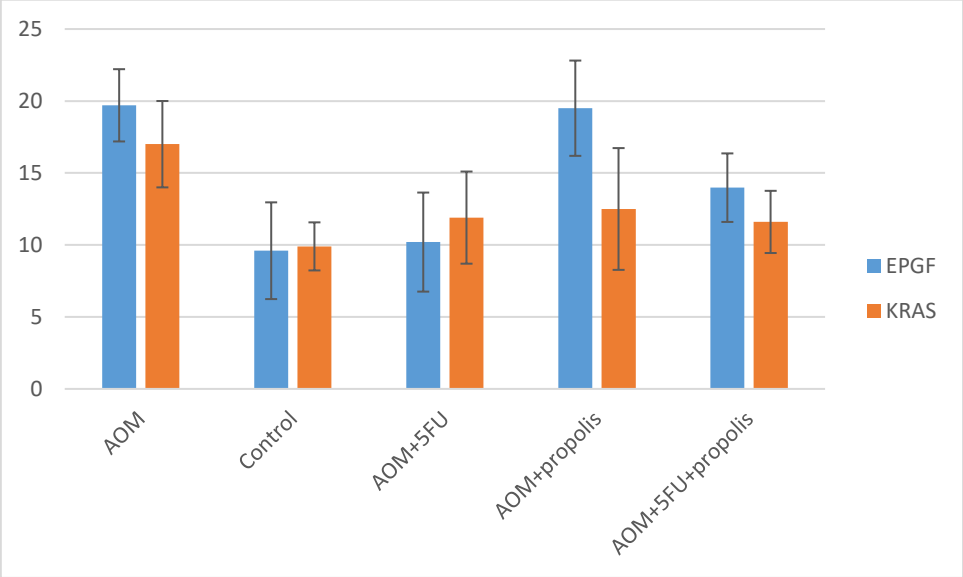
Graph 1. KRAS and EPGFexpressions.



Discussion and Conclusion

The study found that (1) AOM triggers anaplastic changes and mitotic activity in colon surface and gland epitheliums. This is why AOM is considered a useful model chemical for CRC in rats; (2) treatments with both 5-FU and propolis might be effective individually and co-effective in preventing cancer cell proliferation in the colon (3) 5-FU, used both alone and in the combined treatment, led to much more suppression in expressions due to the chemicals increasing TOS levels in the cellular microenvironment. Therefore, cancer cells with low TOS levels and high mutation and receptor activity were exposed to much more apoptosis in the cancer group compared to the treatment groups (5-FU, propolis, propolis+5-FU); (4) The chemicals using treatment of CRC direct the cells to programmed death over apoptosis pathway. 5-FU and propolis can be more successful fighting cancer cell progression in colon.

Colon cancer is one of most common types of malignancies. It is responsible for a high mortality rate around the world. In the pathogenesis of colon cancer, the formation of abnormal crypt foci, mucosal and gland cell clusters constituting adenocarcinoma are evident (24, 26, 42).

Colon and other gastrointestinal cancer types may progress rapidly because of speedy mitosis and epithelial cell cycle. Some genetic and cell signaling pathways as well as growing factors take control the cycle during the development of CRC (19, 23, 51). Due to its higher malignancy and rapid cell cycle, prognosis is unfortunately negative. Metasis can be easily developed in the gastrointestinal system and other organs. For this reason, remedies continue to seek out the best effective chemical or combined therapy.

It is worth noting that 5-fluorouracil (5-FU) has been used frequently in the treatment of colon cancer for the last 50 years. 5-fluorouracil is considered an important therapeutic agent in various stages of colon cancer (6, 53). However, there is evidence of toxic effects on all rapidly proliferating cells as well as normal tissue. Thus, its combined treatment with antioxidants can be potent in fighting cancer cells and relieving side effects. Quercetin, genistein, and geraniol have been frequently studied in recent years as agents with the potential to eliminate the side effects of cancer drugs without reducing their effectiveness (13, 47). Propolis obtained from honey bees (nom. Apis mellifera) is one of these anti-oxidants. It has a phytochemical composition, which includes polyphenols-flavonoids and phenolic acids (33). By powerful antioxidant effects, propolis provides bioactivation and prevention in cancer cells. The presence of Bcl-2 and surviving antiapoptotic protein properties revealed their excessive production in cancer cells (21, 31). In the evaluation of apoptosis, caspase-3 has regulatory properties in the proliferation and survival of tumor cells. It has also been stated that caspase-3 has an important role in recurrence and the effectiveness of radiotherapy and chemotherapy (38). In cancer, apoptotic expressions such as bax, bax-xl proteins and caspase-8 and -9 levels can be changed. Consequently, cancer cells drift preferring mitochondria-dependent apoptosis go to death through the upregulation of bax and caspase-3 and caspase 8 (39). In our study, we thought that propolis and 5-FU would be prone to the cells to death. Accordingly, the levels of mimic and effector caspases (caspase-8 and -3) changed much more than caspase-9 and bax protein. The changing levels for caspase-8 and -3 among cancer and treatment groups vary according to the levels of pro-

apoptotic bax and anti-apoptotic Bcl-2 proteins between the groups. Therefore, the results showed us that imitator to effector caspase signaling cascade gave a more sensitive reaction. The cells undergoing apoptosis used the extrinsic pathway. This did not change between treatment groups including 5-FU and propolis alone.

Some studies have reported direct relationships between oxidative stress and changes in cell structures after AOM application and the severity of carcinogenesis (22, 48). In the colon cancer model created with AOM, a significant increase was determined in the TAS levels in the treatment group compared to the TAS level in the AOM applied group (22). In their colon cancer model using AOM, Thirupurasundri et al. (43) emphasize that treatment applications significantly eliminate the effects of oxidative stress. In addition, they suggest that the treatment protocol they applied prevents the development of malignant morphology of AOM-induced cancer and the emergence of ultrastructural changes by producing apoptosis-like changes. Thus, the applied treatment method induces the antioxidant defense system, as well as inhibiting neoplastic transformation by inducing apoptotic-like changes. As a result, the anti-cancer role of this treatment method becomes apparent. Pallem et al. (32) determined a correlation between the decrease in hydroxyl radicals in the reactive oxygen radicals formed and the frequency of KRAS mutation in their evaluation of neoplastic lesions caused by AOM in rats. Akcakavak and Ozdemir (1), in the colon cancer model they created with AOM in rats, determined that the KRAS mutation detected in the treatment group was lower than the KRAS mutation determined in the AOM applied group. Fichera et al. (16) and Akcakavak and Ozdemir (1) reported that the increase in EGFR signals in rats treated with AOM triggered KRAS. We attest that the decrease in KRAS levels in the group to which we administered propolis and 5-FU in our study is due to the positive effect of propolis and 5-FU on the formation of EGFR signals. Additionally, we found that the TAS and TOS levels in the group to which we applied propolis and 5-FU were significantly higher than the values determined in the AOM applied group. As a result, it was suggested that the application of 5-FU together with propolis may have positive effects on the development of carcinogenesis in the treatment of the colon cancer model we created with AOM in rats.

In some cancer reports over the last decade, propolis extracts have been claimed to be an advantageous therapy option to complement classic chemotherapeutics. Its increasing apoptotic activity in cancer cells was documented as Egyptian propolis as additive therapeutic (13, 38) in several cancer cells, Algerian propolis (48) as additive in lung cancers, Philippine propolis (10) in gastric cancer, Brazilian propolis (12) in CRC, Portuguese propolis in several cancer cells (5), Iranian propolis (3) in gastric cancer, and Omani propolis (46) in CRC. Turan et al. (44) reported that Turkish propolis has potent cytotoxic effects on human cancer cell lines, liver, colon, breast, prostate, and cervical cancers thanks to its higher polyphenolic and flavonoid contents, antioxidant properties, and cytotoxicity. We also found effective it to be in co-treatment against CRC even though propolis obtained from the Black Sea region in Turkiye is not very effective on its own by scoring method. We inferred from results that chemical content in association with polyphenols in propolis can be effective on cancer cells and more effective on cancer cells of different origins. The antioxidants and some biological substance ratios contained in propolis samples produced in different parts of the world and in our country show partial differences according to the regions where they are collected. Medically, these substances contained in propolis are supportive substances that positively affect body health.

Various studies have been conducted in the world and in our country on the local and systemic effects of propolis against experimental or natural tumoral disorders that occur in different parts of the body and their results have been evaluated (44, 52). These studies did not aim to investigate whether propolis has a curative therapeutic effect like an antitumoral chemotherapeutic drug. However, it was aimed to investigate whether propolis can reduce the severity of the pathological lesion in the patient and cause the lesion to regress by increasing body resistance. For this reason, researchers did not find it meaningful to categorize the use of propolis samples produced in different parts of the world for supportive treatment purposes in tumoral disorders in terms of superiority in effectiveness.

In this study, a propolis sample produced in the Black Sea region of our country, which has similar basic content characteristics to propolis samples produced in different countries or regions, was collected. It was investigated whether this substance has an indirect slowing and/or regressive effect on pathological lesions in experimentally induced colorectal cancer, and it was concluded that propolis, a product of the Black Sea region, is a substance that can suppress the severity of local lesions in colorectal cancer cases and support general health status.

In conclusion, anaplastic changes and higher mitotic index in CRC cells are progressed by high receptor activation in epithelial growth factor and genetic mutation. Amongst several treatment choice, 5-FU and propolis might be co-effective more when compared to administration alone against cancer cell fighting. By implementing both 5-FU and propolis treatment, cancer cells can undergo cell death and preserve morphology in cells by higher TAS capacity. Without any chemical induction, TOS capacity could not stop proliferation of CRC cells. In contrast to what has been suggested, we think that apoptotic reactions are more connected to the

extrinsic pathway (caspase-8 and -3) in AOM+propolis+5-FU group. Cellular toxicity by 5-FU can be more effective in extrinsic pathway drifting because high toxic content is accumulated in cell membrane. According to the resultswe believe that propolis can be investigated under co-administration with 5-FU, trying different doses.

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Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 2022/05/23).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

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

Data Availability Statement

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

Animal Welfare

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

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