Original Article

Examining the Antitumoral Effect of Cornelian Cherry (*Cornus mas*) in Ehrlich Ascites Tumor-induced Mice

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Abstract

Introduction: Different doses of *C. Mas* concentrated syrup on ascitic tumors was investigated in the Ehrlich Ascites Tumor model (EAT). **Material and Methods:** A total of 46 Balb/C mice were used in our study, 6 of which were stock animals and the other were in ascitic tumor groups. EAT cells (1x106 EAT cells) were injected intraperitoneally into all of the mice. Mice in the treatment groups with ascitic tumors received 100 mg/kg and 200 mg/kg *Cornus Mas* extract intraperitoneally for 9 days. **Results:** Counts after the 3 and 24-hour incubations in the EAT cell line that the average number of the dead cells was less in the group to which 100µg/ml *C. Mas* was administered when compared with the control group, and that this difference was significant at a statistical level ($P<0.05$). The purpose was also to determine the in vitro cytotoxic effects of *Cornus Mas* on EAT cells, to define the alive and dead cell rates, and to compare the 3-hour and 24-hour incubation in groups to which *Cornus Mas* (syrup) extract were given at different concentrations (50, 100, 250µg/ml). **Discussion and Conclusion:** EAT model is one of the animal tumors induced empirically, it has been the subject matter of many other studies. In the group in which EAT was applied together with high-dose C. mas fruit syrup, it was observed that the EAT cells were not as intense as they were in the tumor control group. Our study showed the anti-tumor effect of *C. Mas* in assisted tumor development with EAT cells.

Keywords: *Antitumoral, Cornus mas, tumor-induced mice*

Introduction

Cancer cases are increasing in the world and cancer treatment modalities are changing day by day.[1,2] Herbal agents are among the most used products in complementary medicine. Medicinal plants that are used today have been known by people of ancient cultures around the world and have largely been considered due to their medicinal properties. *Cornus mas*, which belongs to Cornaceae family and is known as Cornelian cherry, is one of these medicinal plants with high level of antioxidant activity. Cornelian cherry is a plant that has high nutritional value and also has therapeutic properties. It grows in Asia and Europe. Especially in Turkey, it is grown in gardens for its fruit as it grows wild in Northern Anatolian forests.[3]

C. mas ranges from a shrub to a small tree of about 3–5 m in height. The fruits are 12–15 mm long and the color is red in maturity.[3] There are 1,585,000 Cornelian cherry trees in Turkey with a yield of approximately 14,000 tons per year. Cornelian cherry fruits have high levels of natural antioxidants such as ascorbic acid, anthocyanin, and phenolic contents.[4] Organic acids (malic acid and citric acid) and also mucilage are present in the fruit.[1] The Cornelian cherry fruits, which have sour and sweet-tasting juice, contain a high amount of Vitamin C. Furthermore, the fruits are not only consumed fresh but also used to produce jam, stewed fruit, marmalade, dried fruit roll-ups (a locally dried fruit pulp product), syrup, and several types of soft drinks. For medicinal purposes in Turkey, fresh or dried fruit boiled in water is used in diarrhea treatment. The cortex of the body and the shells of the *C. mas* shrub are used in diarrhea, fever, and intestinal parasite treatment as an infusion. Dried and powdered *C. mas* leaves are used as dryers and wound healing agents on wounds. Furthermore, infusion prepared from flowers is used for asthma treatment.[5] In Iran, conventionally, the fruits have been used as a remedy for diarrhea, inflammatory bowel disease, fever, malaria, kidney stones, urinary tract infections, cancer, and sunstroke.[6]

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Cornelian cherry fruit has also been used in Chinese herbal medicine for its tonic, analgesic, and diuretic properties. Extracts are also used for cosmetic determination in Europe and are claimed to have a positive effect on human skin.[7] In folk medicines, the fruits and other parts of the plant have been used for the prevention and treatment of a wide range of diseases. Most of the biological effects of *C. mas* are attributed to its chemical constituents.[6]

Scientific studies have focused on researching the anticancer potential of naturally derived compounds. Antitumoral effects of plant extracts have been tested on several cancer models, one of which is the Ehrlich ascites tumor (EAT) model.[8,9] The EAT model first appeared as a spontaneous breast adenocarcinoma in a female mouse. Tumor pieces were transplanted subcutaneously into mice and transformed into experimental tumors. Then, another tumor form was obtained growing in liquid form in the peritoneum of the mice. This tumor is called the EAT because it produces ascitic fluid like cells in the peritoneum. This model has been included in many studies.[2,10]

In the literature, the cytotoxic effect of *C. mas* on several cancer cell lines mainly focused on its flower and leave extracts. Due to frequent consumption of Cornelian cherry fruits among the population and use for anticancer purposes in folk medicine, the aim of this study is to investigate the antitumor effect of *C. mas* fruit, which has not been tested in this model on the experimental EAT model in Balb/C mice.

Material and Methods

The study on experimental animals was made in accordance with the decision of the Local Ethics Committee of Animal Experiments, Erciyes University, dated October 12, 2016, with the number 16/119. Balb/C type male mice with 8–10 weeks old and with average weights of 25–30 g were used in the study. Four groups which included 10 mice each were identified. During the study, mice were kept in specially prepared, automatically air-conditioned chambers which had 12-h light/dark periods and a constant temperature of 21°. EAT cells from stock mice were used for *in vitro* cell culture with liquid tumor formation *in vivo*.

Formation of the stock mice

The stock cells were thawed at room temperature and were administered to stock animal as 0.1 ml in intraperitoneal way the stomach area. It was expected that ascites tumor would occur in the stock animal within 7–8 days [Table 1]. The 1×10^6 EAT cells in the ascitic fluid drawn with the help of an injector from the stock animal were administered to the mice intraperitoneally in 0.1 ml.

Preparation of *Cornus mas* syrup

Fresh *C. mas* fruits were boiled in water approximately 15 min. After then, the fruits were grinded and filtered with filter aid. The filtrate was further boiled for a while to prepare the concentrated syrup. The concentrated syrup was frozen at −80° degrees after it became cold and then lyophilized (Labconco FreeZone 4.5). The powder of the concentrated syrup was stored at −18°C until analyses.

The powder of the concentrated syrup was weighed each day for each animal experiment after being adjusted to 0.2 ml of phosphate-buffered saline (PBS) and filtered to 100 and 200 mg/kg/day *C. mas* extract for experimental groups and then injected intraperitoneally.

Total phenolic and flavonoid contents

Total phenols were estimated as gallic acid equivalents, expressed as mg of gallic acid/$g_{extract.}$ 100.0 µL of the sample was transferred in a 10.0 mL volumetric flask, to which 500.0 µL undiluted Folin–Ciocalteu reagent was added subsequently. After 1 min, 1.5 mL 20% (w/v) Na_2CO^3 was added and the volume was made up to 10.0 mL with H_2O. After 2 h incubation at 25°C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses.

Total flavonoid analysis of the extracts was made by modifying the method used by Zhishen *et al*.[11,12] Accordingly, 1 mL extract with 0.3 mL of 5% NaNO2 solution mixed at t = 0 min, after addition of 0.3 mL of a 10% $AlCl_3.6H_2O$ solution at t = 5 min., 2 mL of 1 M NaOH solution and 2.4 mL of water was added at t = 6 min. At 510 nm, the absorbance against the blind was measured. The total flavonoids contained in the extracts were calculated as

Table 1: Creation of experiment groups

Experiment groups	Day	Type of injection	PBS	EAT cell	*C. mas* 100 mg/kg	*C. mas* 200 mg/kg
Group 1 (negative control group)	9	i.p	0.1 ml/day	_	_	_
Group 2 (positive control group)	9	i.p	0.1 ml/day	1×10^6 (single dose the first day)	_	_
Group 3 (treatment group with 100 mg *C. mas*)	9	i.p	0.1 ml/day	1×10^6 (single dose the first day)	100 mg/kg/day (throughout the experiment)	_
Group 4 (treatment group with 200 mg *C. mas*)	9	i.p	0.1 ml/day	1×10^6 (single dose the first day)	_	200 mg/kg/day (throughout the experiment)

C. mas=Cornus mas, PBS=Phosphate-buffered saline, EAT=Ehrlich ascites tumor

catechin equivalents in mg CA/$g_{extract}$. Calibration curve of catechin was prepared in the same manner using ethanol. *C. mas* total phenolic and flavonoid contents are shown in Table 2. The anticarcinogenic effect of Cornelian cherry was evaluated by cell counting in this liquor. All groups were taken under general anesthesia using ketamine (50 mg/kg) and xylazine (15 mg/kg). In addition, tissues from the abdominal organs of the liver, spleen, kidney, stomach, small intestine, and large intestine were examined histopathologically in order to evaluate the effect of EAT cells on the organs. For this, the tissues were evaluated by routine histological procedures and by staining with hematoxylin and eosin.

In vitro experimental group

The effects of *C. mas* fruit syrup doses of 50, 100, and 250 μg/ml on EAT cells for cell culture were investigated in the study. *C. mas* fruit syrup was dissolved in PBS. Culture medium was prepared with 20% fetal bovine serum, 80% Dulbecco's middle eagle medium (DMEM) and 1 ml penicillin/streptomycin: 10,000 units/ml penicillin and 10 mg/ml streptomycin. 96-well plates were divided into four groups as tumor control, 50 μg/ml, 100 μg/ml, and 250 μg/ml Cornelian cherry extract treatment groups as 24 wells in each group. 104,000 EAT cells were seeded in each well, and the effect of different doses of *C. mas* fruit syrup was assessed by alive and dead cell counts 3 and 24 h later.

Cell count procedure

The EAT cells that were in the suspension form in the medium in each well were laced in the Eppendorf tube. 100 μl trypan blue solution was added to the EAT cells and pipetted. The Thoma slide was placed on a smooth surface, and the lamellae were covered on the counting area frame to which the lamellae would be glued. 50 μl cell solution was pipetted from the middle line to the counting area by placing the edge of the pipet to the point where the slide and the lamellae join in the counting area which is in the middle of the channels placed on both sides of the Thoma slide; the objective of the microscope was adjusted to ×40. The cells were counted one by one.

Table 2: Total phenolic and flavonoid contents of *Cornus mas* fruit syrup

Extract	Total phenol (mgGAE/gextract)	Total flavonoid (mgCA/gextract)
C. mas fruit syrup	44.14±2.62	10.68±0.37

C. mas=*Cornus mas*

Statistical analysis

The IBM SPSS Statistics version 22.0 (IBM Inc., ILL, USA) program was used for statistical analysis. The Kruskal–Wallis tests were applied in weight-related analyzes between groups and in dose comparison in *in vitro* experimental groups. Statistical analysis was significant when $P < 0.05$. In variables with normal distribution, two-way analysis of variance was performed on repeated measures for groups of time-based comparisons.

Results

Body weight changes of ascites tumor groups during the experiment

When the daily body weights of the animals belonging to the groups were examined during the experiment (9 days), it was observed that there was an increase in weight in the tumor control group and *C. mas* fruit syrup treatment groups. It was observed that the tumor control group had a greater increase than the treatment groups [Table 3]. During the experiment, the circumference of the abdomen of the animals in the groups was measured. The maximum abdominal circumferential extension length was measured as 12 cm in the tumor control group [Table 4].

At the end of the experiment, the cells in the ascitic fluid from the animals that constituted the positive control and treatment groups were stained with trypan blue and counted. The average number of viable cells in 1 ml ascitic fluid was 66.50 × 10^6 in the positive control group, 56.09 × 10^6 in the group administered 100 mg/kg *C. mas* fruit syrup, and 48.38 × 10^6 in the group administered 200 mg/kg *C. mas* fruit syrup.

Histopathological findings

After the experiment, EAT cells in the kidney, liver, and spleen tissues belonging to the healthy, tumor control, and

Table 3: Average weight changes

Day	Negative group	Positive group	100 mg/kg *C. mas* fruit syrup	200 mg/kg *C. mas* fruit syrup	*P*
1	28.0 (27.6-29.7)	29.1 (28.0-30.5)	28.5 (28.0-30.7)	30.0 (28.2-30.7)	0.400
2	28.3 (28.0-29.4)[a]	29.5 (28.4-31.8)[a,b]	29.0 (28.8-29.0)[a,b]	31.0 (29.4-32.0)[b]	0.041
3	28.8 (28.1-29.6)[a]	30.5 (30.0-32.4)[a,b]	30.5 (28.5-34.2)[a,b]	31.7 (31.0-32.7)[b]	0.038
4	28.8 (28.5-30.2)[a]	32.5 (30.1-33.8)[a,b]	32.0 (29.7-34.7)[a,b]	33.0 (31.0-34.7)[b]	0.010
5	30.2 (29.1-30.1)[a]	33.0 (31.2-33.9)[a,b]	32.6 (31.9-36.2)[b]	34.1 (32.7-35.3)[b]	0.003
6	29.7 (29.3-31.7)[a]	34.0 (33.1-35.5)[b]	34.2 (33.0-36.5)[b]	34.3 (33.2-36.3)[b]	0.001
7	30.5 (29.6-32.1)[a]	36.0 (34.8-37.1)[b]	34.5 (32.5-37.9)[b]	35.1 (34.2-36.5)[b]	0.001
8	31.5 (29.9-32.5)[a]	37.7 (36.3-38.0)[b]	36.4 (33.0-38.7)[b]	36.2 (35.1-36.8)[b]	0.001
9	33.5 (30.9-34.9)[a]	40.0 (39.1-41.0)[b]	38.0 (35.7-40.5)[b]	37.1 (36.8-37.4)[a,b]	0.001

The data are median. Expressed as 1. Quarter and 3. Quarter. The parameters with significant differences ($P<0.05$). [a,b]Different superscripts indicate significant differences in each column ($P<0.05$). *C. mas*=*Cornus mas*

treatment groups were seen as invasive in the connective tissue capsule. However, EAT cells were invasive in the tunica serosa layer of the stomach and thin and large intestine tissues, and the EAT cells found here had large hyperchromatic nucleus and eosinophilic cytoplasm. While the EAT cells showed a dense aggregation in the tissues from the animals belonging to the tumor control group, they were observed as small and scattered around the connective tissue capsule in the treatment groups [Figures 1-5].

In vitro effect of Cornus mas fruit syrup applied on Ehrlich ascites tumor cells

The effects of different doses of *C. mas* fruit syrup on EAT cells on live and dead cell counts after 3- and 24-h incubation were evaluated. After 3- and 24-h incubation times, counts showed that the largest number of dead cells was in the group given 100 μg/ml *C. mas* fruit syrup. Obtained numerical values as average cell number and standard deviation are given in Table 5.

Discussion

Induced cancer models and cell lines obtained from tumors are used frequently in studies conducted on cancer.[13] One of these models is the EAT model. Although EAT model is one of the animal tumors induced empirically, it has been the subject matter of many other studies. The EAT model, which was initially developed in a female mouse as spontaneous breast adenocarcinoma, was made into a form that could be used in empirical tumor models after being transplanted subcutaneously from mice to mice by Ehrlich and Apolant (1905). Some of the studies conducted on EAT are conducted on benefiting from the plants that may be used for treatment purposes to obtain possible effects on treatment.[14,15,16]

In the present study, 100 and 200 mg/kg *C. mas* fruit syrup was administered i. p. as the treatment dose to the groups in which i. p EAT cells were induced. The highest increase in animal weights was detected in the tumor control group, and the weight increase was determined to be less in treatment groups. EAT cell communities with eosinophilic cytoplasm in different shapes with large hyperchromatic nucleus around the tissue in the tumor control group after the histopathologic examination in the small and large intestine tissues, kidney, liver, spleen, and stomach tissues taken from the tumor-positive and treatment groups. In the group in which EAT was applied together with high-dose *C. mas* fruit syrup, it was observed that the EAT cells were not

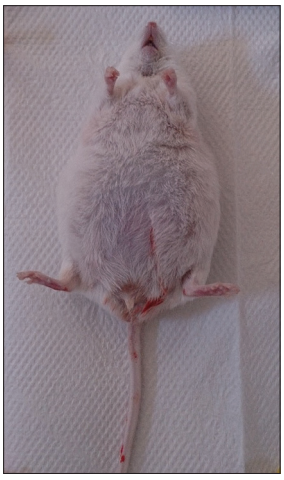

Figure 1: Expansion in the abdominal region due to the accumulation of ascites in the Ehrlich ascites tumor cell given animal

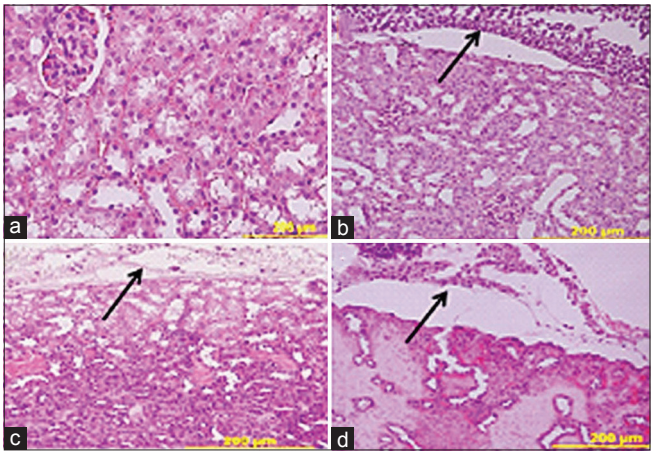

Figure 2: Histopathological findings of the kidney tissue (a) Negative control group. (b) Tumor control group. (c) Tumor and 100 mg/kg *Cornus mas* fruit syrup applied group. (d) Tumor and 200 mg/kg *Cornus mas* fruit syrup applied group (H and E, ×20)

Table 4: Abdominal circumference measurement results

Day	Negative group	Positive group	100 mg/kg *C. mas* fruit syrup	200 mg/kg *C. mas* fruit syrup	*P*
1	8.0 (8.0-8.4)	8.0 (7.6-8.5)	8.0 (7.6-8.4)	8.0 (7.1-8.9)	0.979
2	8.0 (8.0-8.5)	9.0 (8.1-9.4)	8.2 (8.0-9.0)	8.0 (8.0-8.9)	0.117
3	8.7 (8.5-9.0)	9.2 (8.5-9.5)	8.5 (8.5-9.0)	8.5 (8.0-8.9)	0.070
4	9.0 (8.6-9.4)	9.2 (9.0-9.5)	9.0 (8.5-9.4)	8.7 (8.5-9.0)	0.313
5	9.0 (9.0-9.4)^{a}	9.5 (9.5-10.0)^{b}	9.0 (9.0-9.5)^{a,b}	9.0 (9.0-9.5)^{a,b}	0.027
6	9.2 (9.0-9.5)^{a}	10.5 (10.0-10.9)^{b}	9.5 (9.0-9.9)^{a,b}	9.5 (9.0-9.5)^{a}	0.003
7	9.5 (9.5-10.0)^{a}	10.5 (10.5-11.0)^{b}	9.7 (9.5-10.0)^{a,b}	10.0 (9.5-10.5)^{a,b}	0.009
8	9.7 (9.1-10.0)^{a}	11.0 (11.0-11.5)^{b}	10.5 (10.1-11.0)^{a,b}	10.5 (10.0-11.0)^{a,b}	0.001
9	9.7 (9.5-10.5)^{a}	12.0 (11.1-12.3)^{b}	11.0 (10.5-11.3)^{a,b}	10.7 (10.5-11.5)^{a,b}	0.001

The data are median. Expressed as 1. Quarter and 3. Quarter. The parameters with significant differences (P<0.05). ^{a,b}Different superscripts indicate significant differences in each column (P<0.05). *C. mas*: *Cornus mas*

Table 5: Values of average living and dead cell numbers calculated in *in vitro* experimental groups

	Average cell count±SD			
	Tumor control	Treatment 50 µg/ml *C. mas* fruit syrup	Treatment 100 µg/ml *C. mas* fruit syrup	Treatment 250 µg/ml *C. mas* fruit syrup
Live cell (at 3 h)	6.61±0.20	6.67±0.15	6.63±0.16	6.72±0.16
Dead cell (at 3 h)	6.53±0.19	6.63±0.19	6.79*±0.20	6.63±0.20
Live cell (at 24 h)	6.69±0.19	6.62±0.15	6.50±0.15	6.60±0.16
Dead cell (at 24 h)	4.98±2.43	4.84±2.06	5.61*±1.80	4.93±2.30

*P<0.05 compared with tumor control. SD=Standard deviation, *C. mas*=*Cornus mas*

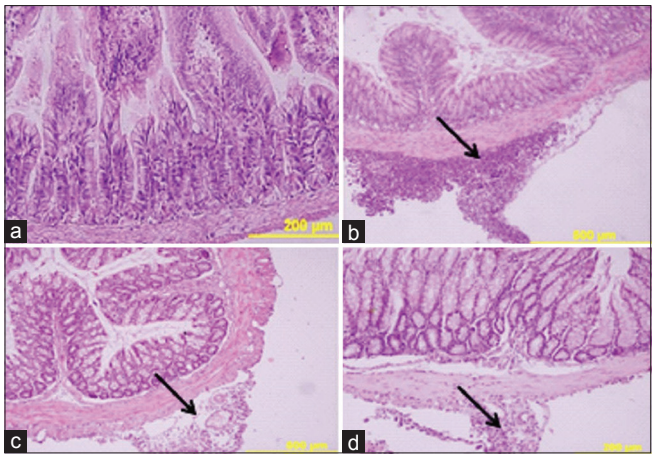

Figure 3: Histopathological findings of the tissues taken from the middle tract of the large intestine in the healthy control and treatment groups. (a) Negative control group. (b) Tumor control group. (c) Tumor and 100 mg/kg *Cornus mas* fruit syrup applied group. (d) Tumor and 200 mg/kg *Cornus mas* fruit syrup applied group (H and E, x10, x20)

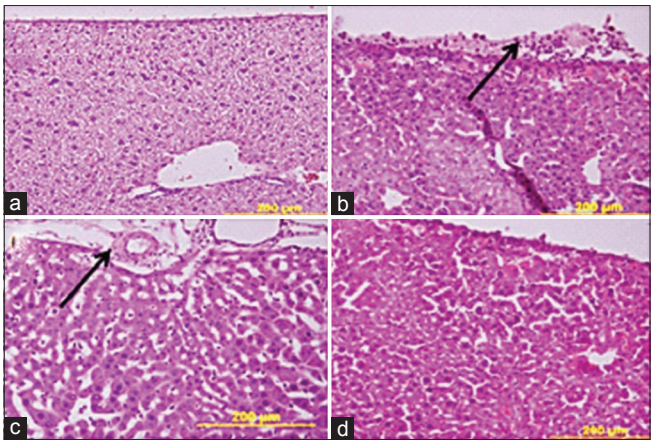

Figure 4: Histopathological findings of the liver tissue. (a) Negative control group. (b) Tumor control group. (c) Tumor and 100 mg/kg *Cornus mas* fruit syrup applied group. (d) Tumor and 200 mg/kg *Cornus mas* fruit syrup applied group (H and E, ×20)

as intense as they were in the tumor control group. In this group, the structures of the tissues showed normal histologic properties. In the group to which 100 mg/kg *C. mas* fruit syrup was administered, it was observed that there were more EAT cell connective tissue capsules when compared with the other treatment group. In the literature, when 1×10^6 EAT cell suspension was injected subcutaneously to the host mice, it was reported that ascites tumor was formed at a measurable level after 1-week duration. In our study, 1×10^6 EAT cells were injected i. p. When the EAT cells were counted in the fluids taken from the animals after the trial, it was observed that the number of the EAT cells was reduced in the group to which 200 mg/kg *C. mas* fruit syrup was applied. In our study, it was determined in the counts after the 3- and 24-h incubations in the EAT cell line that the average number of the dead cells was less in the group to which 100 µg/ml *C. mas* was administered when compared with the control group and that this difference was significant at a statistical level ($P < 0.05$).

Alavian *et al*.[16] conducted a study and examined the serum biomarkers of *C. mas* in male mice in which hepatotoxicity was induced and administered it orally for 14 days as 200 and 500 mg *C. mas*. They reported that the liver functions were preserved and membrane integrity was ensured in the group to which *C. mas* was administered as 200 mg. Francik *et al*.[17] conducted a study and investigated the neuroprotective effect of *C. mas* on the brain tissue. They added *C. mas* dust at a rate of 10% to the daily rations of the study groups and reported that the catalase activity decreased. Zarei *et al*.[18] investigated the effect of *C. mas* extract on the fertilization potential in male mice that were treated with methotrexate. They gave 250, 500, and 1000 mg/kg oral *C. mas* daily to the study groups and reported that sperm damage was reduced in the group to which they gave 1000 mg/kg when compared with the control group. In the study conducted by Forman *et al*.,[19] they investigated the antiproliferative activities of water infusion from *C. mas* L. leaves and measured the antiproliferative effects that depended on time (24, 48, and 72 h) of 50–750 µg/mL *C. mas*. Savikin *et al*.[20] examined the cytotoxicity and antioxidant properties of methanol extracts of the leaves of *C. mas* in human breast adenocarcinoma cell lines. They reported the total phenol amount in the *C. mas* flower as 181.7 ± 6.9 and as 56.9 ± 3.2 in the leaves. They also reported the apoptotic effects of 200 µg/ml *C. mas*. In their study, Bahman *et al*. applied 20 µg/ml *C. mas* L. after 3- and 34-h incubation in the human breast cell line and reported 81.85% suppression in the growth of the cell line. Gayatri *et al*.[21] investigated *Sphaeranthus amaranthoides* on EAT cells in *in vitro* conditions. They reported that the cells were suppressed at a rate of around 82% in the EAT cell line in the group to which they applied 15 µM treatment and that the treatment caused a reduction in cell line. Ceylan

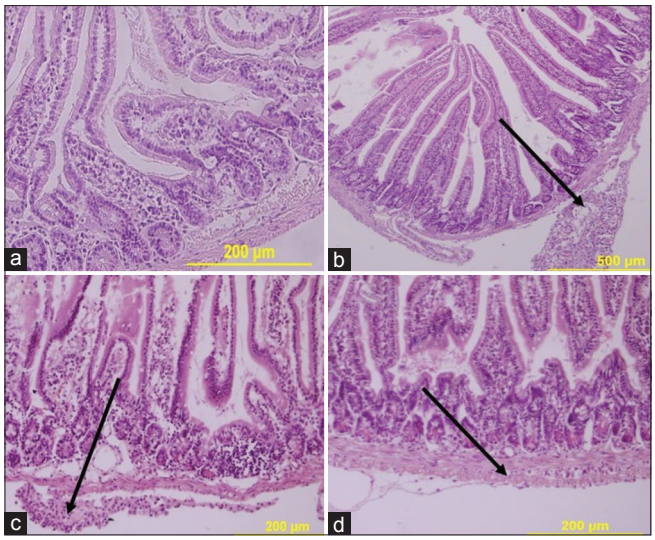

Figure 5: Histopathological findings of the tissues taken from the middle tract of the small intestine in the healthy control and treatment groups. (a) Negative control group. (b) Tumor control group. (c) Tumor and 100 mg/kg *Cornus mas* fruit syrup applied group. (d) Tumor and 200 mg/kg *Cornus mas* fruit syrup applied group (H and E, ×20)

et al.[22] conducted a study and investigated the effects of highbush cranberry (*Viburnum opulus*) on empirical cancer induced in mice with EAT cells. They reported that the EAT cells caused metastasis in the liver and kidney tissues in the tumor control group, and there was metastasis in the treatment groups; however, EAT cell communities were not as intense as in the control group around the tissue capsule. In the study conducted by Facchini *et al*.,[23] they investigated the effects of polysaccharide fractions of *Pleurotus ostreatus* (a fungus species) on the mice to which 5 × 106 EAT cells were administered intraperitoneally. They reported that there was tumor inhibition at a high level. Ozaslan *et al*.[24] investigated the antitumoral effects of *Plantago major* plants in Balb/C mice in which EAT was induced in *in vitro* conditions. They applied 1 × 106 EAT cells intraperitoneally to the study groups.

Conclusion

In our study, it was determined that *C. mas* induced apoptosis in the rate by which EAT model was formed. We believe that our study will be a reference to future studies that will be conducted on *C. mas*.

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Conflicts of interest

There are no conflicts of interest.

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