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Rhamnetin improves antioxidant status in the liver of Ehrlich solid tumor bearing mice

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Abstract

Objective: Rhamnetin, a flavanol, is in the subclasses of the flavonoids existing in plants. The antioxidant properties of several plants containing flavonoids have been extensively studied in several diseases including cancer. This study investigated the effects of rhamnetin on tumor masses, oxidant and antioxidant status in the livers of mice bearing Ehrlich solid tumor.

Material and Methods: Fifty male Balb/C mice weighing 25-30 g were used in the study. Ten mice were kept for Ehrlich ascites tumor (EAT) cells production and the remaining mice were randomly assigned to four groups containing 10 mice in each as healthy control and treatments receiving 1x106 EAT cells and EAT cells plus 100 µg/kg/day or 200 µg/kg/day rhamnetin via subcutaneous route. The tumor inhibition rates of rhamnetin treatments were calculated. The livers were analyzed for malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels.

Results: Compare to tumor control, both levels of rhamnetin suppressed tumor masses throughout the experiment. The MDA levels were increased whereas SOD and CAT activities were reduced by EAT cells injection in the liver of mice. The 100 µg/kg/day rhamnetin treatment decreased MDA level but 200 µg/kg/day rhamnetin had no significant effect on increased MDA level. The reduced liver SOD ($p<0.001$) and CAT ($p<0.01$) activities were elevated by both levels of rhamnetin injection.

Conclusions: The results of this study have revealed that rhamnetin suppresses tumor progression and improves antioxidant status in the livers of solid tumor-bearing mice.

Key words: Ehrlich solid tumor, lipid peroxidation, antioxidant enzymes, rhamnetin

Introduction

Cancer, which DNA damage based malformation, is a major public health problem worldwide. Cancer which is accepted among chronic diseases is frequent and is the second leading cause of mortality after cardiovascular diseases (1). Surgical removal of the tumor masses, chemotherapy and radiotherapy or their combinations are common applications for the treatment of cancer cases. Unfortunately, chemotherapeutic agents may have many side effects and chemotherapy treatments takes very long time. Therefore, in recent years, there is a growing intention to use the plant products along with the chemotherapeutic agents or radiotherapy or as a possible alternative in cancer therapy (2-4). The most commonly used alternative methods in cancer cases are herbal therapies.

Nowadays, phytotherapy is defined as a complementary and alternative treatment method (3,4). Several natural products have been investigated for their anticancer activities. Flavonoids have attracted considerable interest in recent years due to their various pharmacological properties, including their protective effects against cytotoxicity and cancer. Most of the flavonoids are considered to be safe and have limited side effects or toxicities (5,6). Flavonols are the subclasses of the flavonoids (7). Flavonols, plant-derived polyphenolic compounds, are commonly consumed in the diet . Rhamnetin [2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxychromen-4-one], O-methylated flavonol, can be extracted particularly from cloves and many other plants species such as fruit, vegetables, tea and coffee (7-9).

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Although antioxidant (6,10-12), reactive oxygen species (ROS) scavenging (12-14) and anti-inflammatory (15-17) properties of rhamnetin have been shown in previous studies, most of these studies have been conducted at in vitro conditions.

Park et al.(6) have investigated the protective effect of rhamnetin on cell viability, apoptosis and ROS production and found that rhamnetin protected the H9c2 cardiomyoblast cells against H_2O_2 induced cell death. These authors also determined that rhamnetin increased CAT and Mn-SOD expression and inhibits intracellular ROS production. Oak et al. (11) demonstrated in vitro anticancer activity, antioxidant and anti-proliferative ability of rhamnetin by determining increases in the expression of caspase-3 and caspase-9, which induce the apoptosis as well as reduce the intracellular ROS levels in prostate cancer cells.

Although, flavonoids, found in many food items, play a beneficial role in disease prevention, further studies are needed to investigate whether pure forms have similar beneficial effects at in vivo conditions (5,18).

Therefore, in the present study, the effects of different doses of the rhamnetin on MDA levels, which is the indicator of lipid peroxidation, and antioxidant enzymes, SOD and CAT, were determined in the liver of Balb/C mice bearing Ehrlich solid tumor.

Materials and Methods

Animals, management and experimental design

Fifty, 8 week old, male Balb/C mice weighing 25-30 g were used in the study. The mice were provided by Erciyes University Experimental and Clinical Research Center (DEKAM).

Animals (five mice per cage) were maintained in polycarbonate cages sized 42x26x15 cmand 21±2^{o}C room temperature, 50±5 % humidity, environmental ventilation system with air flow rotation of 12 per hour and 12 hours of light/dark cycle were provided for the highest welfare conditions throughout the study. Water and commercially available pellet diet that met or over the daily nutritional requirement of the mice were provided ad libitum during the study.

Ten mice were kept as cancer stock to obtain Ehrlich ascites tumor (EAT) cells. The remaining 40 animals were evenly distributed into four experimental groups as healthy control, tumor control and rhamnetin treatments.

On the first day of the experiment, mice in all groups except the mice kept as healthy control were inoculated with 0.1 ml of ascites fluid containing 1x10^{6} EAT cells via subcutaneus (s.c.) route through nape skinfor solid tumor developmentandthe mice in healthy control group received 0.1 ml of sterile physiologic saline solution via s.c. route.

A 24 hour later, a daily dose of either 100 µg/kg or 200 µg/kg rhamnetin (in 0.1 ml) was injected to each mouse in treatment groups via intra peritoneal (i.p.) route for 15 days. A 0.1 ml of sterile physiologic saline solution was administered via i.p. route to each mouse in healthy and tumor control groups every day throughout the experiment.

Preparation of Ehrlich Ascites Tumor Cell and Stock Mice

The EAT cells, previously used in the studies conducted in our laboratory and preserved in cryovials at -80^{o}C, were thawed at room temperature and 0.1 ml of EAT cell suspension was inoculated into the peritoneal cavity of a mouse.

Following EAT inoculation, the mouse was controlled every day for abdominal ascitic fluid volume for 11 days. On day 11, the ascitic fluid was collected by an injector and 0.1 ml of this ascitic fluid was inoculated into the peritoneal cavity of another mouse to provide more aggressive EAT cells.

This animal was also observed for 11 days and on day 11, approximately 3 ml of ascitic fluid was collected and preserved in cryovials. The EAT cell count was performed. For this purpose ascitic fluid was diluted with PBS (1/1 v/v) and inoculated onto cell culture then 100 µl of cell culture fluid was stained with the trypan blue staining technique for determination of cell viability. The cells were counted undera light microscope (Olympus CX31, Tokyo, Japan) and the inoculum size was determined.

Preparation of rhamnetin solution

Rhamnetin was purchased from Sigma Aldrich (Cat no: 17799, Sigma-Aldrich). A five mg of rhamnetin was dissolved in 0.5 ml of 1% methanol and then filled up to 10 ml with distilled water. The rhamnetin solution was sterilized by filtering through a 0.45 micrometer filter.

Measurements of body weights, tumor volumes and tumor inhibition rate

Mice were weighed and body weightswere recorded daily. Animals were palpated every day for the solid tumors development. The tumor sizes were measured by a digital caliper with 0.01 mm sensitivity (A Brand Digital Caliper 300 mm, China). Tumor sizes were recorded every day (Figure 1).

Tumor volumes were determined with the following formula: Tumor volume (mm^3)=width²xlengthx0.52 (19). For determination of the efficacy of rhamnetin levels, the tumor inhibition rates (TIR) were calculated with the following formula: Tumor inhibition rate=(mean tumor volume value of control group-mean tumor volume value of treatment group)/(mean tumor volume value of control group)x100 (20).

Sample collection and preparations

At the end of the experiment (on day 16), animals were sacrificed with 50 mg/kg ketamine/15 mg/kg xylazine mixture under general anesthesia. The tumor masses were removed and their sizes were measured. The livers of animals were collected into sterile plastic bags and they were transferred immediately to the laboratory under cold chain and stored at -80^{o}C until biochemical analyses.

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Figure 1. Measurements of tumor sizes and appearance of Ehrlich solid tumor

Homogenization of the livers

The 500 mg of liver samples were homogenized in a glass-glass homogenizer with physiological saline solution (p H=7.4) (1/10, w/v). The homogenates were centrifuged at 12 000 rpm for 20 minutes at 4 °C and used for MDA, SOD and CAT analyses.

Biochemical analysis

Determination of malondialdehyde concentration

Malondialdehyde levels of the livers were determined with the method described by Ohkawa et al. (21). Freshly prepared 10, 20, 40, 60, 80 and 100 nMol/ml of 1,1,3,3-tetramethoxypropane (density: 0.99 g/mL) solutions were used as standards. The method was briefly as follow: A 100 µl of liver homogenate was mixed with 8.1% of sodium dodecyl sulfate (SDS), 20% of acetic acid (pH 3.5) and 0.8% of thiobarbituric acid (TBA) (pH 3.5) and incubated at 95°C for 30 minutes. Then cooled and n-butanol-pyridine solution and distilled water were added and strongly vortex mixed. The supernatant was separated following the centrifugation at 4000 rpm for 10 minutes. The absorbance of the complex developed after heat treatment at 95°C was measured at 532 nm by a UV-Visible spectrophotometer (Shimadzu, UV1601, USA). The result was expressed as nMol/mg protein.

Determination of superoxide dismutase activity

The liver was homogenized with 1/10 of distilled water. The sample was mixed with the chloroform/ethanol mixture 1/1 (v/v) and centrifuged at 12000 rpm for 2 hours at +4 ºC. Supernatant was separated to determine SOD activity. The activity of SOD was measured spectrophotometrically according to the method described by Sun et al. (22). This method was briefly as follows: A 50 µl of tissue supernatant and 50 µl XO in 2 M ammonium sulfate solution (1/100, v/v) were added to 2.9 ml of the reagent mixture consisting of xanthine solution+ NBT+ Na2CO3+ BSA.

After incubation at 25 °C for 20 minutes, 1 ml of 0.8 mM CuCl2 was added to the tube and the optical density of the sample was read at 560 nm. The SOD activity was expressed as Unit/mg protein (1 unit=50% inhibition of NBT reduction) and % inhibition was calculated with the following formula: % inhibition = [(blank abs-tissue abs)/blank abs] x100.

Determination of catalase activity

The CAT activity was determined with the method previously described by Aebi (23). The CAT assay was briefly as follows: Liver homogenate was mixed with H2O2 solution (30 mM) and freshly prepared phosphate buffer (50 mM, pH=7.0) then the absorbance was measured spectrophotometrically at 240 nm.

The extinction coefficient was 0.004 (0.0039) mM-1mm-1. The CAT activity was expressed as U/mg protein/min for tissue.

Statistical analysis of the data

Statistical analyses of the data were performed with IBM SPSS Statistics 22.0 (IBM Corp., Armonk, New York, USA) program. The normality of the data was evaluated byhistogram, q-q graphs and Shapiro-Wilk test. The variance homogeneity was tested by the Levene test. One way ANOVA and Kruskal Wallis test were used in the group comparisons where appropriate.

When the F values were significant, Tukey and Dunn-Bonferroni tests were applied for multiple comparisons. The data were evaluated using the R 3.2.3 program. Data were presented as means ± standard deviation of the means and median (25%-75% percentiles) where appropriate. Significance level was accepted as $p < 0.05$.

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Results

Body weight changes, solid tumor development, tumor volumes and tumor inhibition rates

The body weights of all animals increased during the study. The body weight changes of the mice in rhamnetin treated groups were close to the ones in healthy controlgroup (Table 1). Tumor development started only in tumor control group on the 5th day of the experiment, which could not be measured until day 7. On day 7, measurable solid tumor was developed in 5 mice in tumor control group whereas in rhamnetin treated groups 3 mice exhibited tumor masses. Statistically significant differences were determined between tumor control and rhamnetin treated groups after day 9. Tumor volumes were significantly lower in rhamnetin injected mice than the tumor control mice from day 8 to the end of the study.

There was no significant difference between 100 and 200 µg/kg rhamnetin treated groups (Table 2, Figure 2). However, the tumor inhibition rate of 200 µg/kg rhamnetin was higher than 100 µg/kg rhamnetin treatment (Table 3).

Liver MDA levels, SOD and CAT activities

Compare to healthy control mice, a significant increase was determined in MDA level ($p<0.001$) of the tumor control mice. The SOD ($p<0.001$) and CAT ($p<0.01$) activities were lower in tumor control mice than healthy controls.

The injection of 100 µg/kg rhamnetin decreased the elevated MDA level but 200 µg/kg rhamnetin had no significant effect. Both levels of rhamnetin increased the reduced SOD and CAT activities (Table 4, Figure 3).

Table 1. Body weight (g) changes of controls and rhamnetin treatedmice bearing Ehrlich solid tumor

Days	Healthy control	Tumor control	Rhamnetin		p
	n:10	n:10	100 µg/kg n:10	200 µg/kg n:10	
1- 15	8.31±1.50^{ab}	3.53±2.56^{c}	8.43±2.78^{a}	5.61±1.98^{cb}	0.000

$^{a-c}$ The values within the same row with different superscript differ significantly.

Table 2. Solid tumor volumes (mm^3) of tumor control and rhamnetin treated mice bearing Ehrlich solid tumor

Days	Tumor Control		Rhamnetin 100 µg/kg /day		Rhamnetin 200 µg/kg/day		p
	n		n		n		
7	5	76.09±35.96	3	92.89±22.11	3	93.06±19.03	0.651
8	8	221.14±168.25	6	121.80±48.84	6	108.25±71.70	0.170
9	10	546.92±470.47^{a}	9	143.40±91.28^{b}	7	135.20±88.86^{b}	0.011
10	10	1283.78±933.85^{a}	10	224.47±164.14^{b}	7	216.05±46.64^{b}	0.001
11	10	1668.38±1198.44^{a}	10	357.70±356.45^{b}	8	219.72±148.78^{b}	0.000
12	10	1996.09±1510.30^{a}	10	494.62±422.62^{b}	9	328.83±213.58^{b}	0.001
13	10	3404.43±2899.53^{a}	10	690.71±611.12^{b}	10	495.89±442.46^{b}	0.001
14	10	5052.48±4541.40^{a}	8	887.61±655.41^{b}	10	687.79±592.44^{b}	0.003
15	10	6278.52±5015.10^{a}	7	909.55±275.24^{b}	10	913.67±731.32^{b}	0.001

^{a,b} The values within the same row with different superscript differ significantly.

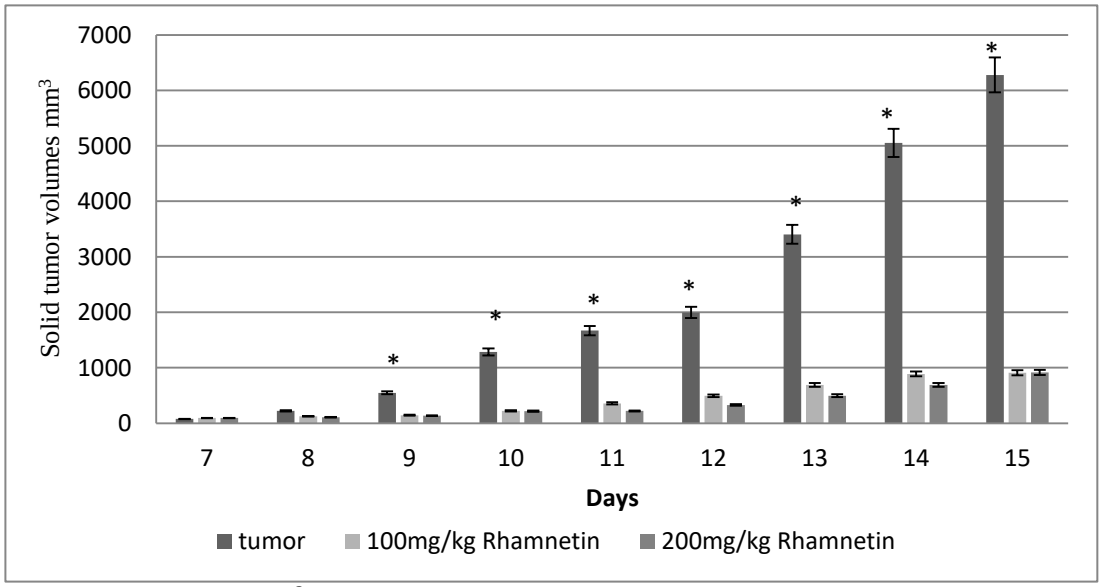

Figure 2. Solid tumor volumes (mm^3) of all groups

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Liver MDA levels, SOD and CAT activities

Compare to healthy control mice, a significant increase was determined in MDA level ($p<0.001$) of the tumor control mice. The SOD ($p<0.001$) and CAT ($p<0.01$) activities were lower in tumor control mice than healthy controls.

The injection of 100 µg/kg rhamnetin decreased the elevated MDA level but 200 µg/kg rhamnetin had no significant effect. Both levels of rhamnetin increased the reduced SOD and CAT activities (Table 4, Figure 3).

Table 3. Tumor inhibition rates of 100 µg/kg and 200 µg/kg rhamnetin levels in the mice bearing Ehrlich solid tumor

Days	Rhamnetin	
	100 µg/kg /day	200 µg/kg/day
7	-22.08	-22.30
8	44.92	51.05
9	73.78	75.28
10	82.51	83.17
11	78.56	86.83
12	75.22	83.53
13	79.71	85.43
14	82.43	86.38
15	85.51	85.44

Table 4. Liver MDA levels, SOD and CAT activities in rhamnetin treated mice bearing Ehrlich solid tumor

Parameters	Healthy Control	Tumor Control	Rhamnetin 100 µg/kg/day	Rhamnetin 200 µg/kg/day	p
MDA	12.06±0.53^{a}	15.81±0.94^{c}	14.30±0.90^{b}	16.42±1.00^{c}	0.000
SOD	6.60±0.26^{c}	4.44±0.16^{a}	5.57±0.22^{b}	6.94±1.00^{c}	0.000
CAT	47.96±3.10^{b}	41.76±5.96^{a}	47.27±3.07^{ab}	50.16±2.73^{b}	0.003

$^{a-c}$The values within the same row with different superscript differ significantly.

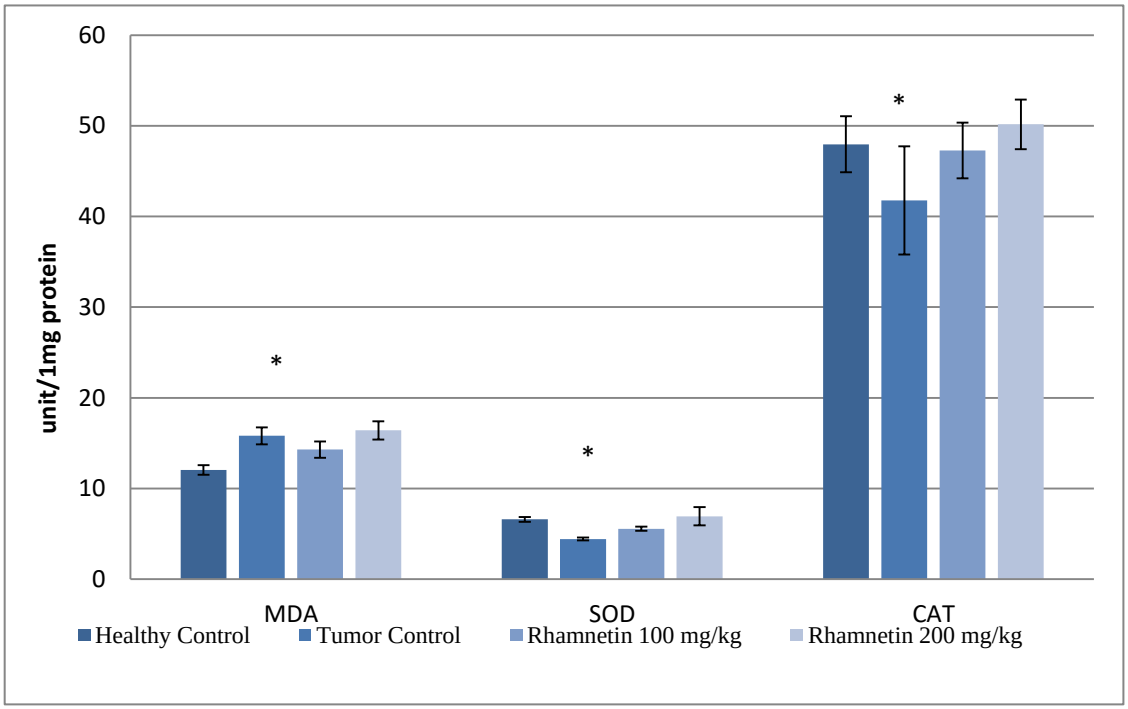

Figure 3. Liver MDA levels, SOD and CAT activities all of the groups

Discussion

Cancer cells increase production of ROS compare to normal cells and it is speculated that tumorigenic signaling also increases expression of antioxidant proteins to balance the high ROS production to maintain redox homeostasis (24,25). Reuter et al. (26) have reported that oxidative stress, chronic inflammation and cancer are closely related. Ehrlich ascites carcinoma, a spontaneous murine mammary adenocarcinoma, is adapted to ascites form by serial intraperitoneal passages (27). Because Ehrlich ascites tumor (EAT) cells do not contain H-2 histocompatibility antigens, they rapidly proliferate in almost all mouse species (28). Ehrlich tumor cells cause morphological and metabolic changes including alterations in oxidant and antioxidant status in the animals (29). Therefore, Ehrlich solid tumor model was chosen to investigate the effect of rhamnetin on lipid peroxidation and antioxidant status in the liver tissue of tumor bearing mice in the present study.

The efficacy of anti-carcinogenic agents can be determined via body weight, tumor volumes or tumor inhibition rate (20). The body weights of mice in all groups increased during the study but the changes in body weights in healthy and rhamnetin treated mice, particularly in 100 µg/kg rhamnetin injected group, were very close to each other. The least weight changes were observed in mice in tumor control group ($p<0.001$) (Table 1). In the present study, the tumor masses showed rapid growth rate and reached the palpable size on day 5 following the SC injection of 0.1 ml of ascitic fluid containing $1x10^6$ EAT cells. The volume of tumor masses became measurable on day 7 in mice kept as tumor control and rhamnetin injected groups. The solid tumor was developed in all animals in tumor control after day 9 whereas tumor masses reached to measurable size in all animals on day 10 in 100 µg/kg rhamnetin treated group. In 200 µg/kg rhamnetin injected group, all animals exhibited solid tumor after day 13.Rapid development of solid tumor in all EAT injected mice is due to aggressive behavior andthe rapid proliferation of EAT cells because of the lack of H-2 histocompatibility antigens (28). During the experimental period, the tumor volumes increased in all groups but both levels of rhamnetin treatments reduced the elevated tumor volumes significantly ($p<0.05$- $p<0.001$) (Table 2). The decrease in tumor volume was more pronounced with 200µg/kg rhamnetin. The decreases in tumor volumesdue to rhamnetin treatments may result from the decreased rRNA gene expression capacity which indicates the suppression of tumor formation (30) and induced apoptosis (11). In the present study, tumor inhibition rates of both levels of rhamnetin increased with the increasing length of their use. However, 200 µg/kg rhamnetin treatment was found to be more efficient throughout the experiment (Table 3).

Many previous studies have emphasized the antioxidant capacities of flavonoids which are found in various fruits, vegetables, seeds, tea and red wine (6,11). The antioxidant property is due to the hydroxylation status of the aromatic ring of the flavonoids. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) exert beneficial and deleterious effects. ROS act as secondary messenger in intracellular signaling cascade (31) thus ROS are involved in the initiation, progression and malignancy of tumors (11,32).

Malondialdehyde, a secondary product of lipid peroxidation, is a uniqueindicator of lipid peroxidation. In the present study, liver MDA concentration was increased by tumor development as previously indicated by Kabel et al. (29) who also subcutaneously implanted EAT cells into mice and found increases in MDA levels in tumor tissue. In human prostate cancer cells, 5-80 µM rhamnetin reduced the ROS production in dose dependent manner in the study of Oak et al (11). In a rat study of Igarashi and Ohmuma (33), TBARS content of the liver of rats receiving cholesterol free diet was reduced by feeding with 0.01% and 0.2% rhamnetin without any significant difference between the rhamnetin levels and rhamnetin treatment had no effect on liver SOD and CAT activities. However, in the present study, the IP injection of 100 µg/kg rhamnetin decreased the elevated MDA level but interestingly 200 µg/kg rhamnetin had no significant effect. In the present study, injection of EAT cells resulted in significant decreases in liver antioxidant enzymes. Kabel (29) has reported that subcutaneous implantation of Ehrlich tumor cells into mice decreases tumor tissue CAT activity. Similarly, in the present study, tumor formation significantly reduced liver SOD ($p<0.001$) and CAT ($p<0.01$) activities and the reduced liver SOD activity was increased by both levels of rhamnetin whereas a significant increase was achieved in CAT activity with 200 µg/kg rhamnetin treatment (Table 4). Antioxidants alleviate the oxidative damage directly by reacting with free radicals or indirectly by suppressing radical generating enzyme or enhancing the activity and/or synthesis of antioxidant enzymes (32). Rhamnetin enhanced the expression of catalase and Mn-SOD, thus inhibits production of intracellular ROS in the study of Park et al. (6). Majewska et al. (12) have also shown radical scavenging property and antioxidant activity of rhamnetin at in vitro test conditions. On the other hand, in the present study, rhamnetin elevated antioxidant enzymes without affecting lipid peroxidation which indicates that the protective effect of rhamnetin against oxidative stress is not mediated by direct radical scavenging (5).

Conclusions

This study has shown that rhamnetin suppresses tumor progression and improves antioxidant status in the livers of solid tumor-bearing mice.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in this study were in accordance with the ethical standards of the Erciyes University Experimental Animals Local Ethics Committee (approval no: 14/30, date: 12.02.2014).

Author Contributions: MS, OB, TE, DC, Nİ, ÖA, HG, EU; study design and concept, **MS;** Biochemical analysis, statistic, writing, revisions

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