EXPERIMENTAL STUDY

The investigation of the antitumoral effect of *Cornus mas* *L* in mice with ehrlich solid tumor

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ABSTRACT

AIM: *Cornus mas* *L* is commonly used due to its anti-inflammatory, anti-carcinogenic and anti-oxidant properties. In the study, the effects of *C. mas* *L* extract on a solid tumor were examined in the Ehrlich solid tumor model developed in Balb/C type mice.

METHODS: Ehrlich acid tumor (EAT) cells (1x10⁶ EAT cell) from the stock animal were injected subcutaneously (s.c.) through the nape of the mice. Treatment groups of solid tumor-induced animals received 100 mg/kg and 200 mg/kg of *C. mas* *L* extract intraperitoneally (i.p.) for 14 days.

RESULTS: Tumor volumes and animal weights were found to be statistically significant compared to the control group (p < 0.05). AgNOR staining was performed in tumor tissues. Statistically significant differences were observed between the groups in terms of TAA/NA ratio (p < 0.05). Immunohistochemical and biochemical parameters were also evaluated. An estimation of tumor proliferation of the lung, liver, brain, kidney, testis and tumor antioxidant parameters viz. lipid peroxidation, reduced glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) was made.

CONCLUSIONS: Our study showed that the anti-tumor effect of *C. mas* *L* in assisted tumor development with EAT cells, was mediated by the enhancement of oxidative stress with multiple mechanisms (Tab. 6, Fig. 12, Ref. 38). Text in PDF www.elis.sk.

KEY WORDS: Ehrlich solid tumor, *Cornus mas* *L*, anti-tumor, AgNOR.

Introduction

Recent breakthroughs in genomic and proteomic technologies enabled us to develop a better understanding of the underlying causes of various cancers (1). Nowadays, the cause of cancer disease is shown as multifactorial. Causes such as: smoking, microorganisms, some chemical substances, and hormones can start the formation of cancer (2, 3). Some cancers produce angiogenic factors. A large number of veins growing in cancer tissue provide the nutrients needed for the growth of cancer (4). Because of rapidly developing resistance against various therapeutic op-

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without tumor specific transplantation antigens (TSTA) (3, 12). In studies, it was observed that the number of cells on average increased exponentially until the 9th day after the i.p injection of the 1x10^6 EAT cells and that the plateau phase was entered on the 9th and 10th days (13).

The cytotoxic effect of C. mas L on various cancer cell lines is found in particular, in its flower and plant extracts. Oxidative stress is defined as an increase in oxidants and/or a decrease in antioxidants functionally. Therefore, the total oxidant and antioxidant levels should be measured at the same time to assess the overall or clear oxidative state in the body. Oxidative stress plays a role in the pathogenesis of cancer and has been reported to affect tissue damage in cancer patients (14, 15). High lipid peroxidation and impaired antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) are generally determined in cancer patients. In order to determine the overall antioxidant status, total antioxidant status (TAS) is frequently measured. Similarly, the total oxidant state (TOS) is used to measure the total oxidation state. The ratio of TOS to TAS, which is called the oxidative stress index (OSI), is generally used to measure the net oxidative stress in the body (14).

Nucleolus organizer regions (NOR) are regions of the ribosomal gene in chromosomes. These regions are composed of ribosomal DNA (rDNA) and some proteins with argyrophilic properties. These regions are copied to the ribosomal RNA, which is transformed into the immature ribosomes in the nucleolus and mature ribosomes in the cytoplasm, respectively (16). These regions can be stained with silver, when active. Depending on the silver, these proteins are called argyrophilic NOR (AgNOR) related proteins, and the silver staining method is the most suitable to indicate nucleoli in the interphase nuclei (16, 17). There are several studies on the importance of interphase AgNOR quantification in tumor pathology for prognostic and diagnostic definitions of different types of cancer (18).

To the best of our knowledge, in literature, there has been no research on apoptosis, angiogenesis, AgNOR protein amount of different doses of C. mas L treatment on the EAT model. On the other hand, we performed this study since there has been no experimental report about the antitumor activity on lungs, brain, kidneys, testis and tumor issues.

**Method**

This study was conducted adhering to the decision of the Local Ethics Committee of Animal Experiments, Erciyes University dated 13/09/2017 with the number 17/086. Eight to ten weeks old Balb/C type male mice with the average weight of 25–30 gr were used in the study. The mice were grouped into four groups of 10 mice each. The mice were kept in specially-prepared and automatically air-conditioned chambers with a constant temperature of 21 °C and had 12 hours light/dark periods.

**Formation of the stock mice**

EAT cells stored at –80 degrees were used in the production of stock animals. The stock cells were thawed at room temperature to give the stock animal 0.1 cc as i.p through the joining place of the left hind leg and the abdominal region. After 7 days, 1x10^6 EAT cell in ascites fluid, drawn with the help of an injector from the stock animal, was administered to the nape area in 0.1 ml phosphate buffer saline (PBS) and the tumor was formed.

**Preparation of C. mas L syrup**

Fresh C. mas L fruits were boiled in water for approximately 15 min and were then grinded and filtered with filter aid. The filtrate was further boiled to prepare the concentrate syrup, which was then frozen at –80 °C after it became cold, and then lyophilized (Labconco Freezone 4.5). The powder of the concentrated syrup was stored at –18 °C until study analysis.

**Formation of experimental groups**

The weights of the animals were measured from the first day of the experiment until the day they were sacrificed. At the same time, the solid tumor region was palpated every day by hand and the tumor diameter was measured by electronic caliper after the tumor was formed. Tumor volume (mm³) = Width² x Length x 0.52 formula was used to measure tumor volumes (19).

Group 1: Negative control group (n = 10): Cancer was not formed. The animals were fed with a normal diet for 14 days. 0.1 ml of saline (SF) was administered as i.p for 14 days.

Group 2: Positive control group (n = 10): On day 0, 0.1 ml of ascites fluid with 1x10^6 EAT cells were applied to the nape area as s.c. From day 0, the mice were injected with 0.5 ml of saline as i.p for 14 days.

Group 3: Treatment group (100 mg/kg C. mas L) (n = 10): On day 0, 0.1 ml of ascites fluid with 1x10^6 EAT cells were applied to the nape area as s.c. From day 0, 100 mg/kg/day C. mas L was injected to the mice as i.p for 14 days.

Group 4: Treatment group (200mg/kg C. mas L) (n = 10): On day 0, 0.1 ml of ascites fluid with 1x10^6 EAT cell was applied subcutaneously. From day 0, the mice were injected with 200 mg/kg/day of C. mas L as i.p for 14 days.

**Immunohistochemistry**

**Factor VIII**

In solid tumor tissues, the immunoreactivity of the Von Willebrand factor (1/50, ab9378; Abcam, UK) molecule was determined using the avidin-biotin-peroxidase method. The Large Volume Detection System (Thermo Scientific, TP-125-HL) immunohistochemistry staining kit was used for the following steps: Images were examined on an Olympus BX51 microscope. 10 different areas taken from the random section of the solid tumor tissues were included in the measurement in the x40 magnification. Von Willebrand factor immunoreactivity capillaries were counted using Image J software.

**Apoptosis**

Apoptotic cell death in situ was evaluated using the TdT-mediated X-dUTP nicked labeling (TUNEL) reaction and the ApopTag Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany), which was used to determine the apoptotic cell. The staining was performed with a Nuclei 4’, 6-diamidino-
2-phenylindole kit (DAPI). The tissues sealed with the glycerol sealing solution were visualized by the Olympus BX51 immunofluorescence microscope. All incubation steps were carried out in the humidity chamber.

For the TUNEL-positive apoptotic cell count, apoptotic cells in ten different areas were counted at 40X objective from each section.

**AgNOR staining procedure**

5 μm sections were placed in slides from the 10 % neutral formalin-fixed and paraffin embedded tissues. Sections were deparaffinized, rehydrated and stained. AgNORs were stained according to the method recommended by the International Committee on AgNOR Quantitation, revised by Trerè (20). Silver stained samples were evaluated under light microscope (Eclipse E-600, Nikon, Japan) using an image analysis system (NIS Elements Nikon, Japan). 50 nuclear AgNOR protein images per sample were assessed. The mean AgNOR number and the total AgNOR area/total nuclear area (TAA/TNA) ratio for each nucleus were calculated.

**Tissue preparation**

Homogenization was conducted on all brain lung, kidney, liver, tumor and testis tissues with a RIPA buffer, which contained 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l EDTA, pH 7.4, 1 % of aprotinin, 1 % of Nonidet P-40, 1 % of sodium deoxycholate, 0.1 % of SDS, 50 mmol/l of NaF and 0.1 mmol/l of Na3VO4; together with a proteinase inhibitor cocktail (Merck KGaA, Darmstadt, Germany). After centrifugation (Beckman Coulter, Krefeld, Germany) for 40 min at 4 °C at 14,000 rpm, the supernatant produced was used for assays. The tissue samples were measured with Xylenol orange. The assay was calibrated with a standard hydrogen peroxide solution of 39.16 μmol/l. The subsequent results were expressed in μmol H2O2 equivalents/l (μmol H2O2 equiv/l). The TOS level of the tissues were measured by the Erel 2005 TOS method (23) that relied on the oxidation of ferrous ions into ferric ions together with various oxidative species inside an acidic medium. Ferric ion concentrations were measured with Xylenol orange. The assay was calibrated with a standard hydrogen peroxide solution of 39.16 μmol/l. The subsequent results were expressed in μmol H2O2 equivalents/l (μmol H2O2 equiv/l). The TOS/TAS ratio was defined as an oxidative stress index (OSI). The superoxide dismutase (SOD) levels of the tissues were obtained by the nitroblue tetrazolium reduction technique based on Beauchamp (24). The quantity of protein in each sample were reported for the SOD measurements.

**Measurement of CAT, GPx**

The methods that were modified by Ozturk et al (25) were used to measure the activities of CAT and Se-GSH-Px and the levels of GSH and GSSG in tissues.

**Statistical analysis**

SPSS for Windows® 23.0 (SPSS, Chicago, IL, USA) was used to analyze data. The results are presented as the mean ± standard deviation (Mean ± SD) of replicates. Data in all the experiments were analyzed for statistical significance using analyses of variance (One-Way ANOVA). Post hoc analyses were conducted to compare Tukey test parameters in multicomponent comparisons. Either the Tukey or Dunn–Bonferroni tests were used for multiple comparisons. For intergroup comparisons, the Kruskal–Wallis analysis was used. For factor VIII, and Apoptosis between the groups, ANOVA or the Kruskal–Wallis was used. p < 0.05 was considered statistically significant. Another program used was GraphPad Prism for Windows (version 7.00 La jolla California USA).

**Results**

**Body weight changes of experimental groups**

When the data regarding the daily body weights of the animals belonging to the groups were observed during the experiment (14 days), it was determined that there was an increase in weights in the tumor control group and C. mas L treatment groups and this increase was higher in the tumor control group compared to the treatment groups (p < 0.05) (Fig. 1).

<table>
<thead>
<tr>
<th>Day</th>
<th>Tumor control group Median (25–75%)</th>
<th>Treatment group 100mg/kg C. mas L Median (25–75%)</th>
<th>Treatment group 200mg/kg C. mas L Median (25–75%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>76.09 (38.19–131.29)</td>
<td>–</td>
<td>–</td>
<td>0.683</td>
</tr>
<tr>
<td>8</td>
<td>221.14 (10.67–472.29)</td>
<td>165.28 (21.28–448.68)</td>
<td>148.77 (19.03–354.76)</td>
<td>0.075</td>
</tr>
<tr>
<td>9</td>
<td>546.92 (73.40–1683.29)</td>
<td>331.61 (113.09–751.75)</td>
<td>178.18 (28.68–478–14)</td>
<td>0.001</td>
</tr>
<tr>
<td>10</td>
<td>1283.78 (126.36–2971.10)</td>
<td>601.71 (123.99–1387.33)*</td>
<td>339.03 (94.16–471.54)*</td>
<td>0.055</td>
</tr>
<tr>
<td>11</td>
<td>1668.37 (305.79–4036.82)</td>
<td>1419.14 (127.99–2642.32)*</td>
<td>692.19 (164.13–1722.57)*</td>
<td>0.004</td>
</tr>
<tr>
<td>12</td>
<td>1996.08 (354.12–4594.50)</td>
<td>1492.62 (320.40–3642.28)*</td>
<td>678.32 (268.89–1722.57)*</td>
<td>0.051</td>
</tr>
<tr>
<td>13</td>
<td>3404.42 (1069.62–11000.21)</td>
<td>1718.43 (373.79–3049.79)*</td>
<td>870.67 (274.45–1621.89)*</td>
<td>0.055</td>
</tr>
<tr>
<td>14</td>
<td>5052.47 (1263.38–17175.30)</td>
<td>2043.57 (545.97–3715.41)*</td>
<td>1127.23 (325.69–2609.11)*</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Values measured in mm3. Compared with tumor control group * p < 0.05
Tumor volume changes of experimental groups

Tumor measurement started from the 7th day in the tumor control group and these measurements could be performed from the 8th day in the treatment groups. On the last day of the experiment, tumor volumes were 5052.47 mm$^3$ in the tumor control group, 2043.57 mm$^3$ in the 100 mg/kg C. mas L group, and 1127.23 mm$^3$ in the 200 mg/kg C. mas L group. All of the tumor sizes in all animals were compared among each other in a volumetric manner. When the treatment groups were compared with the tumor control group, the increase in tumor volume was found to be statistically significant (p < 0.05) and the findings of this comparison are given in Table 1.

Angiogenesis findings

The mean vascular density determined by Factor VIII in the sections obtained from the tissues belonging to the groups is shown in the Table 2. Positive staining was observed in vessel endothelial cells in sections of the control and treatment groups by immunohistochemical staining for factor VIII (Fig. 2). Necrotic regions of tumor tissues were not evaluated. When the vessel density between the groups was considered, the mean vascular density in the control group was calculated as 9.28, while the mean vascular density in the group treated with 100 mg/kg C. mas L was 7.54, and in the 200 mg/kg C. mas L treated group it was calculated as 7.36. When the treatment groups were compared with the control group in terms of the expression intensity of Factor VIII, a statistically significant difference was determined (p < 0.05).

Apoptotic findings

In solid tumor tissue sections, apoptotic cells were observed as distributed throughout the tumor tissue (Fig. 3). Apoptotic cell numbers are shown in Table 3. According to the results obtained, a significant increase in apoptotic cell number was observed in both treatment groups. In the group, where we applied a high dose C. mas L, it was observed that there were more apoptotic cells than in the control group and the other treatment groups. When the treatment groups were compared with the control group, the difference among them was found statistically significant (p < 0.05).

Ag-NOR results

An example of AgNOR staining cells is shown in Figure 4. The TAA/NA ratio and the average number of AgNOR were de-
determined in C. mas L (100 and 200 mg/kg) groups and positive control (Tab. 4). For the TAA/NA ratio ($x^2 = 86.333, p = 0.000$) and the mean number of AgNOR ($x^2 = 211.740, p = 0.000$), statistically significant differences were determined among three groups (Tab. 5, Fig. 5). In the double comparison of the groups, no significant difference was found in TAA/NA ratio among the differences of the C. mas L (100 mg/kg) groups with positive control ($Z = -2.801, p = 0.005$), and C. mas L (200 mg/kg) groups with positive control whereas it was significant for TAA/NA ratio among ($p = 0.000$) and C. mas L (100 mg/kg) and C. mas L (200 mg/kg) groups ($Z = -7.325, p = 0.000$) (Tab. 6).

**Biochemical results**

**Measurement of CAT**

CAT activity was measured for all lung, brain, kidney, liver, testis and tumor tissues. In the tumor control group, CAT was found to have the highest value and the negative control group was found to have the lowest value ($p < 0.05$) (Fig. 6).

**Oxidative stress markers result**

While the lowest GSH, SOD and TAS values were observed in the tumor control group in all tissues, the highest value was found among all tissues in the control group (Figs 7, 8, 9, respectively). Other oxidative stress markers of GSSG, TOS and OSI showed negative values. Thus, the lowest value for GSSG, TOS and OSI was found in the control group and the highest value was observed in the tumor control group (Figs 10, 11, 12, respectively). The dose-dependent administration of C. mas L showed a significant decrease in OSI, GSSG and TOS levels ($p < 0.05$) in all tissues compared to the tumor group.

**Discussion**

In addition to the treatments applied in the treatment of cancer in recent years, complementary medicine is also used. Some plants were shown to be useful in the treatment of cancer. Plants and vegetables used in various countries by the public have shed light on the progression of chemotherapy of cancer (26). With the previous studies considered, C. mas L is a promising plant in terms of possible anti-carcinogenic effects. Cornelian cherry Cornus mas L belongs to the family of Cornaceae. In different cancer models, the effects of C. mas L extract at different doses were performed in studies. Although it is known that it is not toxic in rats even at a dose of 3000 mg/kg body weight, its bioavailability and stability in the cell is very low (27). Numerous scientific reports showed that C. mas L has anti-tumoral activity and targets different oncogenic pathways (18). They reported that C. mas L had a cytotoxic effect on tumor cells, but did not fully approach the antitumor activity in terms of apoptosis and angiogenesis (28).

Limtrakul et al, in their studies gave curcumin, which has an antioxidant characteristic, to the mice orally. For this reason, cur-

![Fig. 4. A demonstrative examples of the AgNOR staining cells (A) positive control; B) C. mas L (100 mg/kg) group and C) C. mas L (200 mg/kg group).](image_url)
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cumin with a dose of 115 mg/kg/day, was added to the diets of the mice and the dose of curcumin was given to the mice daily for 3 months. In their studies, it was stated that after a 3 month period, the dose of curcumin did not reach toxic values and it induced apoptosis in cancerous cells in mice (29).

Ceylan investigated the effects of (Viburnum opulus) highbush cranberry juice in his study of the experimental cancer created with...
and angiogenetic effects of curcumin in mice given 1x10^6 EAT cell and stated that the extract, which has antioxidant characteristic, induces apoptosis and suppresses angiogenesis (3). Facchini et al, investigated the effects of polysaccharide fractions belonging to Pleurotus ostreatus (a fungal species) in mice given 5x10^6 EAT cell intraperitoneal and stated in their studies that there is a high degree of tumor inhibition (31). Alavian, Seyed et al. examined the serum biomarkers of C. mas in their study of male rats formed hepatotoxicity and 200 mg and 500 mg C. mas was given orally for 14 days. They stated that liver function was maintained and membrane integrity was achieved in the 200 mg given group (32). In their study, Forman et al, evaluated the anti-proliferative activity of water infusions from the leaves of five Cornus L. types and their in vitro anti-proliferative activities proceeded on water extracts (lyophilisates) of the selected Cornus types, in human breast carcinoma MCF-7 cells. The current results indicated that all the extracts tested showed dose and time dependent anti-proliferative effects and the strongest of the selected types were obtained from CO, CM and CA types (33). In recent studies it was reported that C. mas L extract in all the cancer cells reduced cell viability below 26 % even at the lowest doses (34, 35).

NORs act as functional subunits of nucleoli and are related to a large number of regulatory proteins in the interphase. Reliable information provides knowledge on the proliferative and metabolic activity of cells by calculating the NOR field and core area values. It is important to identify new biomarkers to differentiate benign and malignant lesions. In addition, the selection of the most reliable therapeutic strategy for cancer treatment is very important for the management of the treatment strategy and to improve success (16, 18, 36). We aimed to determine the average number of AgNOR in our study. We used the TAA/NA ratio as the new approach in routine cytopathology to determine the proliferation activity of benign and malignant lesions. We aimed to show whether C. mas L extract has an antitumor effect and whether AgNOR proteins could be used for the selection of the most reliable dose and for the detection of new metabolites for a potential for cancer treatment.

As far as we know, this study is the first to determine the amount of AgNOR in EAT at different doses of C. mas L concentrations.

EAT cells in mice and it was reported that the EAT cells metastasize in liver and kidney tissue in the tumor control group and that metastasis was observed in treatment groups, however, the EAT cell population around the tissue capsule was not as dense as in the tumor control group (30). Yilmaz et al, studied the apoptotic...
In addition to biochemical research, animal experiments showed that dried fruit powder and fruit extracts can increase the level of physiological antioxidants such as: superoxide dismutase or catalase, as well as protecting cells and tissues against free radicals here (37). In the studies, it was observed that the catalase activity was increased in the brain tissue within the experimental groups given the dried C. mas L fruits, which were rich in terms of polyphenol. Similar studies showed that the increased activity of paraoxonase-1 (PON1) increased the protective effect of PON1 on tissues of tumor induced Balb/C mice was made. Furthermore, the possible role of the C. mas L fruit extract was investigated with regards to the prevention of cancer-related antioxidant systems, such as: the increase in TAS, SOD and GSH in the antioxidant defense system. The study findings indicate a significant reduction in catalase activity and an enhancement in all tissue values in the tumor control group, but which were reversed approximately in the control levels. These results indicate that the C. mas L extract is protective of oxidative stress induced by EAT modeling.

Conclusion

Our study showed that the synthesis capacity of AgNOR proteins decreased due to C. mas L concentration and caused a decrease in TOS, OSI and GSSG levels in suppressing synthesis causing tumor formation in the presence of CAT. In our study, we stated that the C. mas L fruit extract showed beneficial effect on testis, brain, liver kidney, lung and tumor tissues. In addition, our study is the first study, which demonstrated that C. mas L induced apoptosis and suppressed angiogenesis.

References


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