

Histopathological and Immunohistochemical Changes in the Liver Caused by Oregano Essential Oil Added to the Rations of Lambs at Different Doses

Recep Gumus^{1*}, Kubra Asena Terim Kapakin², Selim Comakli², Yavuz Selim Saglam², Halit Imik³

¹Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Cumhuriyet University, TR-58140, Sivas – Turkey

²Department of Pathology, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum – Turkey

³Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum – Turkey

Original Research Article

*Corresponding author

Recep Gumus

Article History

Received: 13.02.2018

Accepted: 21.02.2018

Published: 28.02.2018

DOI:

10.36347/sjavs.2018.v05i02.008



Abstract: Many plants have been used for years to treat various diseases. One of the plants used for this purpose is oregano. In this study, by adding different doses of oregano essential oil to lamb rations, histopathological and immunohistochemical changes in the liver were investigated. In the study, 3 groups (8 lambs in each group) were formed using 24 male lambs at the age of 3 months; Control group given basal ration, Oregano-1 given basal ration+200 mg/kg oregano essential oil and Oregano-2 given basal ration + 400 mg/kg oregano essential oil. In conclusion, it was determined that there were slight degenerative changes in hepatocytes in Oregano-2, in addition, heat shock protein 27 (HSP-27) release and 8-hydroxy-2-deoxyguanosine (8-OHdG) immunosuppressivity were higher in hepatocytes, vascular endothelial cells, and gland epithelial cells than in other groups.

Keywords: Histopathological, HSP-27, Lamb, Liver, Oregano essential oil.

INTRODUCTION

The liver is one of the most important organs of the body in terms of biochemical activity, and any functional disorder that will occur in this organ affects all systems. Chemical substances, drugs, and various diseases are the most important causes of possible liver damage [1]. The amount of toxic substances in the bloodstream increases depending on the damage to the liver, impairing the liver as well as other parts of the body [2]. In addition, oxidants caused by increased stress due to various causes during daily life negatively affect the liver [3].

Medical treatment is needed to rapidly heal liver damage caused by various effects. However, the higher side effects of drugs used in liver treatment has increased the interest in herbal products.

Herbal treatments are safe because they provide a harmless alternative to natural and traditional medicine. Many plants in nature are used to treat various diseases. Some of these plants are oregano and thyme species belong to *Lamiaceae* family. One of the oregano species is *Origanum vulgare subsp. hirtum*, which has been reported to have a wide biological activity due to its numerous phytochemical compounds [4]. The antioxidant [5], anti-inflammatory [6], and antibacterial [7] effects of essential oils obtained from the oregano have been reported to be due to numerous polyphenol compounds, including carvacrol, thymol, p-simen and γ -terpinen [8]. In studies on mice, it was

reported that *Origanum vulgare* [9] and *Origanum majorana* had protective effects on the liver [10].

Some studies on effects of oregano or its products (extract, essential oil and powder) on liver damage in experimental animals can be found in the literature, but no studies on its effect on the ruminant liver in normal cases were encountered. In this study, we aimed to investigate the effect of oregano essential oil on the liver in different doses in lamb's ration, by histopathological and immunohistochemical methods.

MATERIALS AND METHODS

Animals, Experimental design, and Feed

The study was conducted at the Animal Husbandry Research and Application Unit of Ataturk University, Faculty of Veterinary Medicine. 24 male Akkaraman lambs (which were weaned when they were 3 months old on average) were divided to 3 groups as Control and two treatment (Oregano-1 and Oregano-2)

groups, each consisting of four replicates of eight lambs. The lambs two animals were allocated to each compartment measuring 280×200×120 cm. This study was approved by the ethic committee of Faculty of Veterinary Medicine in Ataturk University [Decision No: 2010/700 (Decision No: 2006/5g)].

In this study the Control group was fed a basal lamb diet, the Oregano-1 group was fed a basal lamb ration + 200 mg/kg ration oregano essential oil and the Oregano-2 group was fed a basal lamb ration + 400 mg/kg ration oregano essential oil (Table-1). The animals were provided with pre-weighed feed, twice a day at 08:00 am and 05:00 pm. The study lasted for 70 days (14 days were used as adaptation followed by 56 days for data collection). The daily amount of roughage

provided to the lambs was 125 g of wheat straw per animal (the chemical composition of wheat straw on the basis of dry matter content was as follows: crude protein: 3.1, crude ash: 6.69, Neutral Detergent Fiber (NDF): 77.45, Acid Detergent Fiber (ADF): 50.32) Concentrate feed and water was supplied ad libitum during the trial. The rations fed to the animals were formulated in accordance with the recommendations of the NRC [11] (Table 1). The raw feed materials used in the study were crude protein and crude ash analysis according to the Weende Analysis System of AOAC [12] and if the crude fibre according to Crampton and Maynard [13]. NDF and ADF analysis were according to Van Soest and Robertson [14] with Goering and Van Soest [15].

Table-1: Composition of lamb diets used in the study %

| Ingredient, % | Groups | | |
|------------------------------------|---------|-----------|-----------|
| | Control | Oregano-1 | Oregano-2 |
| Barley | 30 | 30 | 30 |
| Corn | 20 | 20 | 20 |
| Sunflower seed meal | 13.33 | 13.33 | 13.33 |
| Cotton seed meal | 13.2 | 13.2 | 13.2 |
| Bran | 9.7 | 9.68 | 9.66 |
| Corn gluten | 5 | 5 | 5 |
| DDGS ¹ | 5 | 5 | 5 |
| Marble powder | 2.05 | 2.05 | 2.05 |
| Molasses | 1.12 | 1.12 | 1.12 |
| Salt | 0.5 | 0.5 | 0.5 |
| Vitamin mineral premi | 0.1 | 0.1 | 0.1 |
| Oregano essential oil ³ | - | 0.02 | 0.04 |
| Rates of nutrient, % | | | |
| Crude protein | 18.53 | 18.52 | 18.51 |
| Crude fibre | 12.74 | 12.74 | 12.73 |
| Crude ash | 7.2 | 7.2 | 7.2 |
| Acid detergent fiber | 13.67 | 13.67 | 13.67 |
| Neutral detergent fiber | 27.3 | 27.3 | 27.3 |

¹ DDGS: Dried Distillers Grains with Solubles.

² The vitamin & mineral premix provided the following (per kg): 4,000,000 IU vitamin A, 800,000 IU vitamin D3, 5,000 IU vitamin E, 400 mg vitamin B2, 2 mg vitamin B12, 5,000 mg vitamin PP, 1,000 mg D-pantothenic acid, 20,000 mg choline, 50 mg Co, 5,400 mg Fe, 185 mg I, 6,900 mg Mn, 800 mg Cu, 6,400 mg Zn, 14 mg Se.

³ The oregano essential oil were added in place of marble powder (Oregano essential oil was obtained from Ecopharm Hellas S.A. (Kilkis, Greece). Oregano oil was in the form of a powder called Oregano-Stim (Meriden Animal Health Ltd. that contains 5% essential oil of *Origanum vulgare subsp. Hirtum* plants and 95% natural feed grade inert carrier).

Histopathological Examination

At the end of the study, 6 animals were slaughtered from each group. Liver samples of animals were taken after the necropsy for histopathological and immunohistochemical examinations.

The liver tissue samples were fixed in 10% buffered formalin and routinely processed for histological examination by embedding in paraffin wax. Tissue sections were cut 4 µm in thickness and stained by the Haematoxylin-Eosin for observation under a light microscope.

Image Analysis

Tissue sections were evaluated by high-power light microscopic examination using an Olympus Bx51 with a DP72 camera system. Each specimen was examined in 10 randomly selected areas of approximately an X40 objective. The scores were derived semi-quantitatively using light microscopy on the preparations from each rat and were reported as follows: Grade 0 = - (negative); Grade 1 = +1 (mild); Grade 2 = +2 (moderate); Grade 3 = +3 (severe); Grade 4 = +4 (most severe) (40).

Immunohistochemical Examinations

Four μm sections from all of the tissue samples were cut and processed for immunohistochemical examination by a standard avidin-biotin-peroxidase method that the producer described. Rabbit polyclonal antibodies that react with rat 8-OHdG (sc-66036) the dilution of 1:200 and HSP-27 (clone: ab78806) the dilution of 1: 100 were used for for 60 minutes. A secondary antibody was used according to the manufacturer's protocol (expose mouse and rabbit-specific HRP/DAB detection IHC Kit, Abcam Cat. No. ab80436). After three washes with 0.1% Tween 20 in PBS, the sections were incubated with 3,3-diaminobenzidine (Dako Cytomation) and counterstained with Mayer's hematoxylin (Dako Cytomation).

Image Analysis

Tissue sections were evaluated by high-power light microscopic examination using an Olympus Bx51 with a DP72 camera system. Each specimen was examined in 10 randomly selected areas of approximately an X40 objective. The scores were derived semi-quantitatively using light microscopy on the preparations from each rat and were reported as follows: Grade 0 = - (negative); Grade 1 = +1 (mild); Grade 2 = +2 (moderate); Grade 3 = +3 (severe); Grade 4 = +4 (most severe) (40).

Statistical Analysis

The data obtained were assessed using SPSS 20 package software [16]. Differences between the groups were determined the nonparametric Kruskal-Wallis Test with the $p < 0.05$ value for significance.

RESULTS

There were no histopathological findings in Control and Oregano-1 groups (Figures 1 a-b), whereas mild degenerative changes in hepatocytes were observed in Oregano-2 group ($p < 0.05$) (Figure 1c).

In all groups, HSP-27 release was observed in hepatocytes, vascular endothelial cells, and epithelial cells. It was found that HSP-27 was the highest in Oregano-2 group ($p < 0.05$). When compared the Oregano-1 and Control groups with each other, HSP-27 release were found higher in the Control group ($p < 0.05$) (Figures 2 a-c).

The 8-OHdG immunopositivity was observed in hepatocytes, vascular endothelial cells and glandular epithelial cells in all groups, which was found to be higher in the Control group when compared to the Oregano-1 group ($p < 0.05$), while to be highest in the Oregano-2 group ($p < 0.05$) (Figures 3 a-c).

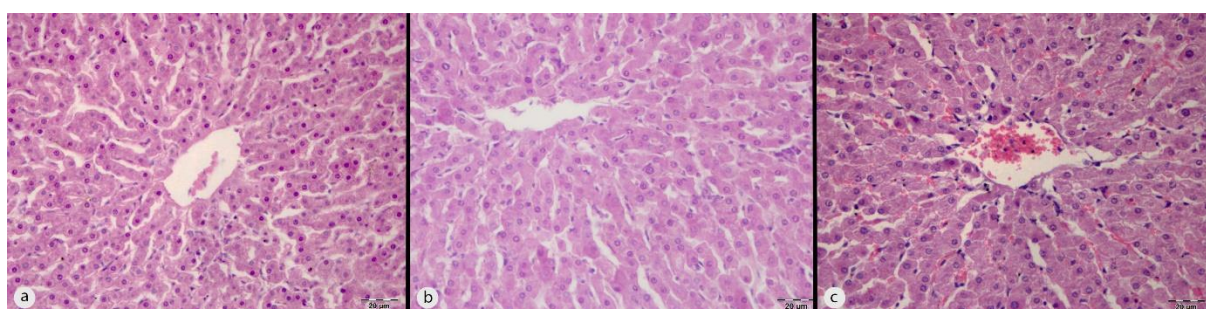


Fig-1: Normal appearance in the hepatocytes, a. Control group, b. Oregano-1 group, c. Mild degenerative changes in the hepatocytes (Oregano-2 group), HXE, Bar: 20 μm

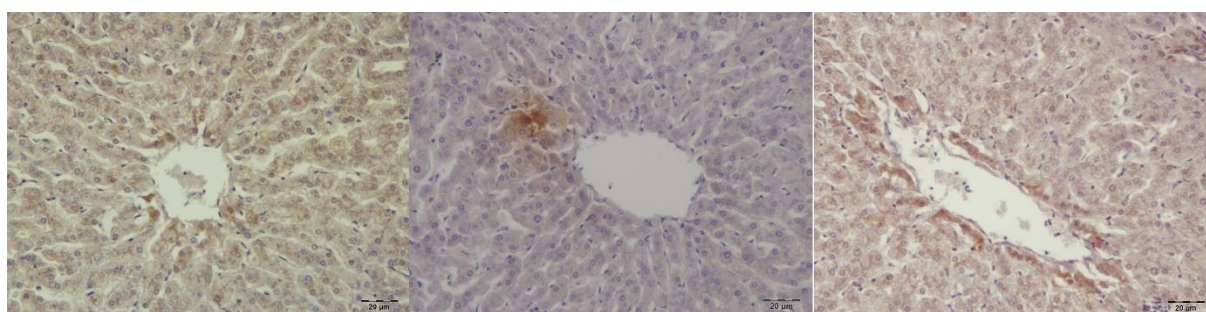


Fig-2: Immunohistochemistry stain; HSP-27 expression in the hepatocytes a. Mild, (Control group), b. Mild, (Oregano-1 group), c. Moderate, (Oregano-2 group), Bar: 20 μm .

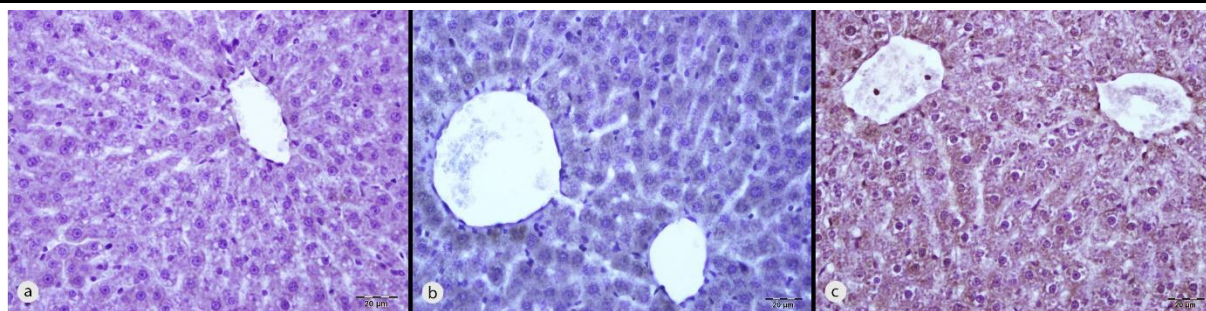


Fig-3: Immunohistochemistry stain; immunopositivity for 8-OHdG in the hepatocytes a. Mild, (Control group), b. Mild, (Oregano-1 group), c. Moderate, (Oregano-2 group), Bar: 20 µm.

DISCUSSIONS

As in humans, there is a complex antioxidant system in many animals to alleviate oxidative stress. However, excessive reactive species derived from oxygen and nitrogen can cause oxidative damage to tissues and organs. Oxidative stress is a compound pathological mechanism that causes liver damage to begin and progress. Many risk factors, such as drugs for treatment, environmental pollutants, radiation, and temperature, can cause liver oxidative stress and cause serious liver diseases [17]. The application of antioxidants means an appropriate treatment strategy to prevent and cure oxidative stress-induced liver diseases.

Phenolic compounds such as thymol and carvacrol contained in thyme essential oil have antioxidant properties and have a protective effect on liver [18, 19]. In previous studies on mice, *Origanum vulgare* [9] and *Origanum majorana* have been reported to have protective effects on liver [10]. However, it has been shown that thyme essential oil can have toxic effects due to the excessive dose, in addition to its protective effects. In a study conducted by Luaibi and Mousa [20], rats were given thyme extract at doses of 500 and 750 mg/kg bw by injection and investigated for histopathological changes in the liver tissue during the days 10, 20 and 30. In that study, it was reported that 500 mg/kg dose did not show side effects on the day 10, but slight degenerative changes in the veins as well as hyperemia in 20 days. However, in 30 days hepatocytes near the portal area showed degenerative changes as necrosis and decrease of glycoproteins in the cytoplasm of hepatocytes. The administered 750 mg/kg dose resulted in congestion and mild inflammatory changes in the blood vessels at day 10; degenerative and necrotic changes were observed in the portal region in day 20 as well as glycoproteins in the hepatocyte cytoplasm decreased. At the same time, it has also been observed that an inflammation with mononuclear cell infiltrations occurred in animals in this group. All of these lesions observed in day 30 were found to get more severe [20]. Ruiz-Cabello *et al.*, [21], in another study, reported that oregano essential oil at the dose of 50, 100 and 200 mg/kg bw in rats liver didn't cause a significant change histopathologically. In the present study, there was no significant histopathological change in the liver of the animals in the Control group and Oregano-1

group given 200 mg/kg oregano essential oil, but a congestion and slight degenerative changes in the veins of hepatocytes were observed in the Oregano-2 group given 400 mg/kg oregano essential oil.

Heat shock proteins (HSP) are highly preserved protein groups presented in humans, animals, and plant cells with various functions, and their common property is that they increase when encountered by sudden temperature changes or other stress factors [22, 23]. Heat shock proteins are divided into classes according to the molecular weights, structures, and functions. These are HSP-100, HSP-90, HSP-70, HSP-60 and small heat shock proteins (HSP-27, HSP-25, etc.) [22]. Heat shock proteins provide the protection of functional structures of proteins under stress factors such as high temperature, pH changes, and lack of oxygen [24, 25]. In a study, 25 mg/kg oregano essential oil was added to the pigs' diet, and HSP-27 release in the intestinal tissue (jejunum) of pigs exposed to transport stress decreased significantly [26]. In the literature review, we could not find any histopathological study investigating the effect of thyme applied to animals on the release of HSP proteins in tissues, except for a study of Zou *et al.* [26]. In our study, HSP-27 release was observed in hepatocytes, vascular endothelial cells and gland epithelial cells in all three groups. It was found that HSP-27 was the highest in Oregano-2 group. When compared the Oregano-1 and Control groups with each other, HSP-27 release were found higher in the Control group. Considering these results, we can suggest that the dose of 200 mg/kg had a more positive effect on the liver.

Reactive oxygen species produced in excess as a result of exposure to various stresses lead to the formation of more than 20 oxidative base damage products in DNA [27]. The 8-hydroxy-2-deoxyguanosine (8-OHdG) is a highly sensitive and most frequently encountered oxidative DNA damage marker among these base products [28]. For this reason, 8-OHdG measurement is considered a direct indicator of oxidative damage in DNA and is the most commonly used method for detecting oxidative DNA damage [29]. Studies have reported that carvacrol reduces the level of 8-OHdG in the pancreas of rats [30], similarly oregano essential oil (OEO) in fish sperm [31]. In the present

study, the 8-OHdG immunopositivity was observed in hepatocytes, vascular endothelial cells and glandular epithelial cells in all groups, which was found to be higher in the Control group when compared to Oregano-1 group, while to be highest in the Oregano-2 group.

CONCLUSIONS

Based on this results, we can say that oregano essential oil added to the ration of lambs in 400 mg/kg dose increased the amount of 8-OHdG in liver immunohistochemically, reducing the amount of HSP-27 that is one of the proteins responsible for protecting the cells against damage, leading to degenerative changes in hepatocytes, although the dose of 200 mg/kg have more positive effects on the liver. This study showed that, by histopathological and immunohistochemical methods, oregano may have a toxic effect due to overdosage, despite its known positive effects at the appropriate doses.

REFERENCES

1. Okatani, Y, Wakatsuki A, Enzan H, Miyahara Y. Edaravone protects against ischemia/reperfusion-induced oxidative damage to mitochondria in rat liver. *European Journal of Pharmacology*. 2003; 465(1): 163-170.
2. Öztürk C, Avlan D, Cinel I, Cinel L, Ünlü A, Camdeviren H, Oral U. Selenium pretreatment prevents bacterial translocation in rat intestinal ischemia/reperfusion model. *Pharmacological Research*, 2002; 46(2): 171-177.
3. Serteser M, Koken T, Kahraman A, Yilmaz K, Akbulut G, Dilek ON. Changes in hepatic TNF- α levels, antioxidant status, and oxidation products after renal ischemia/reperfusion injury in mice. *Journal of Surgical Research*. 2002; 107(2): 234-240.
4. Vladimir-Knežević S, Blažeković B, Kindl M, Vladić J, Lower-Nedza AD, Brantner AH. Acetylcholinesterase inhibitory, antioxidant and phytochemical properties of selected medicinal plants of the *Lamiaceae* family. *Molecules*. 2014; 19(1): 767-782.
5. Asensio CM, Grosso NR, Juliani HR. Quality preservation of organic cottage cheese using oregano essential oils. *LWT-Food Science and Technology*. 2015; 60(2): 664-671.
6. Bukovská A, Cikoš Š, Juhás Š, Il'ková G, Reháč P, Koppel J. Effects of a combination of thyme and oregano essential oils on TNBS-induced colitis in mice. *Mediators of Inflammation*. 2007; 2007: 23296.
7. Govaris A, Botsoglou E, Sergelidis D, Chatzopoulou PS. Antibacterial activity of oregano and thyme essential oils against *Listeria monocytogenes* and *Escherichia coli* O157: H7 in feta cheese packaged under modified atmosphere. *LWT-Food Science and Technology*. 2011; 44(4): 1240-1244.
8. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*. 2004; 94(3): 223-253.
9. Habibi E, Shokrzadeh M, Chabra A, Naghshvar F, Keshavarz-Maleki R, Ahmadi A. Protective effects of *Origanum vulgare* ethanol extract against cyclophosphamide-induced liver toxicity in mice. *Pharmaceutical Biology*. 2015; 53(1): 10-15.
10. El-Ashmawy IM, El-Nahas AF, Salama OM. Protective effect of volatile oil, alcoholic and aqueous extracts of *Origanum majorana* on lead acetate toxicity in mice. *Basic & Clinical Pharmacology & Toxicology*. 2005; 97(4): 238-243.
11. National Research Council (NRC). Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids. (sixth edition), National Academy Press, Washington, 2007.
12. AOAC. Official Methods of Analysis of AOAC International. 18th ed. Rockville, MD, USA: Association of Official Analytical Chemists; 2005.
13. Crampton EW, Maynard LA. The relation of cellulose and lignin content to the nutritive value of animal feeds. *The Journal of Nutrition*. 1938; 15(4): 383-395.
14. Van Soest PJ, Robertson JB. Analysis of forages and fibrous foods (A laboratory manual). Cornell University. Ithaca, New York, 1985.
15. Goering H, Van Soest PJ. Forage fiber analyses (apparatus, reagents, procedures, and some applications), US Agricultural Research Service, Washington, USA, 1970.
16. SPSS. Statistical packages for the social sciences. 20th ed. IBM, Chicago, 2011.
17. Correa M, Roig-Navarro AF, Aragon CM. Motor behavior and brain enzymatic changes after acute lead intoxication on different strains of mice. *Life Sciences*. 2004; 74(16): 2009-2021.
18. Baranauskienė R, Venskutonis PR, Viškelis P, Dambrauskienė E. Influence of nitrogen fertilizers on the yield and composition of thyme (*Thymus vulgaris*). *Journal of Agricultural and Food Chemistry*. 2003; 51(26): 7751-7758.
19. Grosso C, Figueiredo AC, Burillo J, Mainar AM, Urieta JS, Barroso JG, Palavra AM. Composition and antioxidant activity of *Thymus vulgaris* volatiles: comparison between supercritical fluid extraction and hydrodistillation. *Journal of Separation Science*. 2010; 33(14): 2211-2218.
20. Luaibi NM, Mousa AH. A study effect of thyme on biochemical and histological changes in liver of male rats. *Advances in Environmental Biology*. 2016; 10(10): 217-227.
21. Llana-Ruiz-Cabello M, Maisanaba S, Puerto M, Pichardo S, Jos A, Moyano R, Cameán AM. A subchronic 90-day oral toxicity study of *Origanum vulgare* essential oil in rats. *Food and Chemical Toxicology*. 2017; 101: 36-47.

22. Henle KJ, Jethmalani SM, Nagle WA. Stress proteins and glycoproteins. *International Journal of Molecular Medicine*. 1998; 1(1): 25-57.
23. Moseley P. Stress proteins and the immune response. *Immunopharmacology*. 2000; 48(3): 299-302.
24. Locke M, Noble EG. Stress proteins: the exercise response. *Canadian Journal of Applied Physiology*. 1995; 20(2): 155-167.
25. Terim Kapakin KA, Imik H, Gumus R, Kapakin S, Saglam YS. Effect of vit E on secretion of HSP-70 in testes of broilers exposed to heat stress. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*. 2013; 19(2): 305-310.
26. Zou Y, Hu XM, Zhang T, Wei HK, Zhou YF, Zhou ZX, Peng J. Effects of dietary oregano essential oil and vitamin E supplementation on meat quality, stress response and intestinal morphology in pigs following transport stress. *Journal of Veterinary Medical Science*. 2017; 79(2): 328-335.
27. Dizdaroglu M. Facts about the artifacts in the measurement of oxidative DNA base damage by gas chromatography-mass spectrometry. *Free Radical Research*. 1998; 29(6): 551-563.
28. De Martinis BS, Bianchi MDLP. Methodology for urinary 8-hydroxy-2'-deoxyguanosine analysis by HPLC with electrochemical detection. *Pharmacological Research*. 2002; 46(2): 129-131.
29. Helbock HJ, Beckman KB, Ames BN. 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. In *Methods in Enzymology*. 1999; 300: 156-166.
30. Kılıç Y, Geyikoglu F, Çolak S, Turkez H, Bakır M, Hsseinigouzdagani M. Carvacrol modulates oxidative stress and decreases cell injury in pancreas of rats with acute pancreatitis. *Cytotechnology*. 2016; 68(4): 1243-1256.
31. Liu Q, Duan RJ, Zhou YF, Wei HK, Peng J, Li JL. Supplementing oregano essential oil to boar diet with strengthened fish oil: Effects on semen antioxidant status and semen quality parameters. *Andrologia*. 2017; 49(10): 12764.