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Role of essential oils in antioxidant capacity and immunity in a rat model of mixed stress

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Abstract

Animal wellbeing is a balance between environmental stress and nutrition that regulates homeostasis. Augmentation of animal feed with essential oils can promote homeostasis. The present study was designed to observe the biochemical, immunological, and biological effects of daily administration of a mixture of essential oils (EOM) in a stressed rat model. Forty-eight adult male Sprague Dawley rats were randomly allocated to four groups, namely a control group (C), a stressed group (S), a treated group (Tr), and a stressed group that received the treatment (TrS). The treatment was applied by adding EOM to the water (0.2 ml/l) three days per week for 28 days. Two chronic stressors (isolation and crowding) were applied to animals in groups S and TrS. Total oxidant status (TOS) increased in the S group compared with C, whereas it decreased when fed with EOM. Although TOS was the same in S and C, it increased in Tr compared with C. There was a significant increase in interleukin 4 (IL-4) in S compared with C, and EOM reversed the IL-4 level. Nevertheless, an increase was seen in the weights of the liver, intestine, brain, and testes in TrS compared with S. The increase in water intake was a result of stress, but feeding with EOM decreased water consumption gradually. This study showed that 0.2 ml/l EOM had protective effects on antioxidant status, immunity and liver function, and decreased water consumption under stress conditions.

Keywords: bodyweight, liver function index, water consumption
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Introduction

Stress responses affect the hypothalamus-pituitary-adrenal cortex (HPA) resulting in increased levels of corticosterone and adrenocorticotrophic hormones. Activation of the HPA axis also plays an important role in physiological, neuroendocrine, and behavioural responses, which are described as the fight or flight mechanism. Several stressors alter the living conditions of animals such as the upper and lower boundaries of the thermoneutral zone, a crowded environment, and isolation (Sejian *et al*., 2011; Lee *et al*., 2015). All these stress factors may cause physiological changes in animal welfare, that are detrimental to growth performance, immunological function, oxidation, and health (Colditz & Hine, 2016; Attia *et al*., 2017; Li *et al*., 2019; Saracila *et al.*, 2020). He *et al.* (2020) reported that rats exposed to chronic unpredictable mild stress for four weeks had reduced weight gain. Shoemaker and Heideman (2002) noted inhibition of the size of testes, food intake, and bodyweight in F344 rats that were exposed to short photoperiod stress for 52 weeks. Studies with various population densities in mice were evaluated in terms of corticosterone and blood lymphocyte subpopulations, and the corticosterone increased in the low and high crowding groups (Liu *et al.*, 2020). According to the literature, overcrowding is the most important stress factor in animal production, especially poultry. Crowding stress manifests in decreased bodyweight, feed intake, leg problems, and immune system and behavioural changes (Guardia *et al.*, 2011; Houshmand *et al*., 2012; Gomes *et al*., 2014).

All important functions in the liver are associated with free radicals from cellular metabolism, and an oxidative-antioxidative balance is developed (Muriel, 2009). Reactive oxygen species (ROS) stimulate the production of inflammatory cells during oxidative stress and cause alterations in mitochondrial function,

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hepatocellular injury, and liver fibrosis (Sanchez-Valle *et al.*, 2012). The main source of ROS is the mitochondria in hepatocytes, Kupffer cells and neutrophils. In oxidative stress, the parenchymal cells of the liver are injured, and lipid peroxidation occurs. Also, important liver enzymes such as ALT (alanine aminotransferase), AST (aspartate aminotransferase), and GGT (gamma-glutamyl transferase) are released into the blood. Markers of oxidative stress such as AST and ALT are increased when rats were subjected to crowd and noise stress (Shawer *et al.*, 2016). The literature suggested that the exposure of Wistar rats to chronic environmental stress for two weeks was closely associated with cell damage and showed an increase in the permeability of the cell membrane of the liver enzymes.

Cytokines are important signalling molecules of immunity. Consistent results were indicated for the relationship between stress and cytokines (Glaser & Kiecolt-Glaser, 2005; Ray *et al.*, 2016). Some researchers reported an increase (Glaser & Kiecolt-Glaser, 2005; Rohleder *et al*., 2012) in interleukin 6 (IL-6) during stress, whereas others found a decrease (Mormède *et al.*, 2002). On the other hand, IL-4 production could be decreased, increased or left unchanged by stress (Chuian *et al*., 2005; Yang *et al.*, 2006; Murakami *et al.*, 2007). In contrast to IL-4 and IL-6, stress suppressed the production of interferon gamma (IFN-γ) in male mice (Curtin *et al.*, 2009).

Homeostatic balance is maintained by water consumption. The reports indicated that water consumption and feed intake increased during stress conditions (Patki *et al.*, 2013). For example, acute and chronic stress caused an increase in the feed and water intake in Wistar albino rats. Feeding with essential oils and herbs increased water consumption and growth rate because of the rich phytochemical composition (Ramadan, 2007). Feeding with essential oils for 56 days increased the water intake and weight gain of Sprague Dawley rats (Tauseef Sultan *et al*., 2009). However, some pharmacological experiments reported that rats injected with pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α showed decreased motor activity and social withdrawal, and reduced food and water intake (Dantzer *et al.*, 2008). Generally, the induced level of IL-6 activated the HPA axis with increasing hyperthermia and stimulated inflammation. Intracerebroventricular administration of IL-6 to Wistar rats demonstrated a decrease in food and water intake (Schobitz *et al*., 1995; Lenczowski *et al*., 1999).

A balanced diet helps to cope with stress and improves the organism's defence abilities. The use of essential oils, natural additives, and vitamin-mineral complexes for animal nutrition can support metabolic functions, growth, and productivity. This is intended to ensure healthy nutrition in animal husbandry besides welfare (Quezada-Mendoza *et al.*, 2011; Attia & Al Harti, 2015; Seyidoglu & Aydin, 2020). The use of essential oils in the poultry industry in particular supports the digestive system and improves immunity (Nameghi *et al.*, 2019). Essential oils had a positive effect on egg quality, weight gain, feed, water intake and carcass yield (Attia & Al Harti, 2015; Attia *et al.*, 2017; Sevim *et al.*, 2020). Essential oils have antioxidant features for animals in environmental conditions, high temperature, stress, and over-nutrition (Nameghi *et al.*, 2019; El-Essawy *et al.*, 2019; Attia *et al.*, 2019; Seyidoglu *et al.*, 2019a). In this study, the effects of an EOM containing thyme, anise, peppermint, and eucalyptus with vitamins (B complex, AD_3E, E) and selenium on liver health, antioxidant status, immunity, and organ weight were examined in rats under stress from a crowded environment and from solation.

Materials and Methods

The experimental protocols were approved by the Turkish National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the National Animal Care and Use Committee of the University (Approval No 2018-07/01).

Forty-eight male Sprague Dawley rats aged 7 - 8 weeks were used. The initial weights were 217.00 g (C), 228.58 g (S), 223.75 g (Tr), 223.75 g (TrS) and the final weights were 311.09 g (C), 312.08 g (S), 313.50 g (Tr), 318.33 g (TrS). The rat model was chosen to reflect the types and responses of stress for animals. In addition, rats have a rapid metabolic rate, and studies may be concluded in a short time. The rats were housed under standard laboratory conditions (22 ± 1.0 °C; 55 ± 10% humidity) in clear plastic cages (42 cm x 21 cm 20 cm). Stainless steel food hoppers and wood shavings for bedding material were used.

The rats were given ad libitum access to tap water and a commercial pelleted diet (Korkuteli Yem Gida San, A.S. Turkey). The basal diet contained 2500 kcal/kg energy, 23.50% crude protein, 5.92% crude cellulose, 2.95% crude oil, and 6.36% ash. The diet included 1.35% lysine, 0.43% methionine, 0.05% sodium, 0.85% calcium, and 0.98% phosphorus. Prior to the experiment, the rats were acclimatized to laboratory conditions for one week. The rats were randomly allocated one of four experimental groups (n = 12), namely the control group (C), a stressed group (S), a treated group (Tr), and a stressed group that was also treated (TrS). The treatment groups (Tr and TrS) received a special essential oil mixture (EOM) (Miarom-L, Miavit, Germany) containing a mix of thyme, anise, peppermint, and eucalyptus oils with vitamins AD_3E(C), B complex and vitamin E, and selenium) in drinking water at 0.2 ml/l concentration three days a week for 28 days. The experimental protocol was implemented for 28 days. The first week constituted the

adaptation period to experimentation. The second and third weeks were the application period of the EOM. For the last two weeks, besides feeding EOM, all animals were held in a light to dark cycle with 18 hours light and 6 hours of darkness from 19h00 to 01h00 (Ten Hoor *et al.*, 1980; Gancarczyk *et al.*, 2004; Park *et al.*, 2015; Seyidoglu *et al*., 2019b). During this time, two chronic stresses were applied to the rats in S and TrS. Neither food nor water was given during these stress applications. Isolation stress was applied by placing a rat in a separate cage with four sides and a white ground and leaving it alone for 30 min on Monday, Wednesday, Friday, and Sunday of the third week of the experiment. The crowded environment stress was applied by placing six rats together in a cage (50 cm × 50 cm) that under normal conditions would have had a capacity of three rats for 30 min on Tuesday, Thursday, and Saturday of the fourth week of the experiment.

At the end of the study, blood samples were obtained by puncturing the heart under short (2–3 minutes) isoflurane anaesthesia. Blood samples were centrifuged on the same day to separate the serum, and then kept at -80 °C until analysis. A Shimadzu UV-VIS spectrophotometer 2600 was used to measure serum triglyceride, alanine aminotransferase (ALT) aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) (Archem Diagnostic) with the colorimetric method of Reitman and Frankel (1957).

The total antioxidant status (TAS) (Rel Assay), total oxidant capacity (TOS) (Rel Assay) and paraoxonase/arylesterase-1 (PON-1) parameters were measured according to commercial kits and colorimetrically with a spectrophotometer. Oxidative stress index (OSI) was calculated as the ratio (OSI =TOS/TAS) percentage of TOS to TAS (Erel *et al*., 2005).

Serum corticosterone (Elabsience, USA), interleukin-4 (IL-4), IL-6, and interferon-gamma (IFN-γ) were measured with the commercial rat ELISA kit 3 (Thermo Fisher), and the assays were performed colorimetrically with a plate reader according to the manufacturer's protocol (Dallak, 2018). The serum percentage changes of these parameters were calculated.

The brain, heart, intestines (duodenum, ileum, colon), kidney, liver, spleen, and stomach of the rats were weighed immediately after slaughter.

To examine the effects of EOM on bodyweight in stressed rats, all rats were weighed weekly, and daily water intake was recorded. The weight and water consumption percentages were evaluated as follows: weight percentages (%): at the beginning of the study (first weighing), before stress (second weighing), and before slaughter (third weighing); water consumption (%): at the beginning of the study (first consumption), before stress (second consumption), and before slaughter (third consumption).

Statistical analyses were performed with SPSS version 20.0 (IBM Corp., Armonk, New York, USA). For data on plasma variables and organ weights (n = 12/group), individually slaughtered rats were regarded as the experimental unit. Data were tested for normality distribution and variance of homogeneity assumptions. All values were grouped, and means and standard errors were calculated. One-way ANOVA was completed for all parameters to examine the differences between groups. Differences were considered significant at P <0.05. If the difference between groups was significant (P <0.05), the differences were evaluated by Tukey's test. On the other hand, in non-homogeneous groups, differences between means were analysed by Kruskal Wallis and Mann Whitney U tests between groups one by one. Nevertheless, the bodyweight (n = 12/group) and water consumption (n = 12; 6/animals; group) percentages before and after stress were compared within groups of rats using a paired sample T-test with a significance level of P <0.05.

Results and Discussion

A significant increase in the mean TOS level was observed in S compared with C (P <0.05) (Figure 1A). Also, TrS rats exhibited a remarkable improvement in the TOS level compared with S (P <0.05). There was a significant increase in the TAS level of Tr compared with C (P <0.05) (Figure 1B). Additionally, a significant increase in the TAS level of TrS rats was observed compared with S rats (P <0.05). Nevertheless, the OSI increased in S rats in comparison with C (P <0.05) (Figure 1C) group. However, Tr and TrS showed an enhancement in OSI values (P <0.05). There were no significant differences in PON-1 values in all groups (Figure 1D) (P >0.05). The oxidant-antioxidant status parameters TOS, TAS, PON1, and OSI index in blood serum are shown in Figure 1.

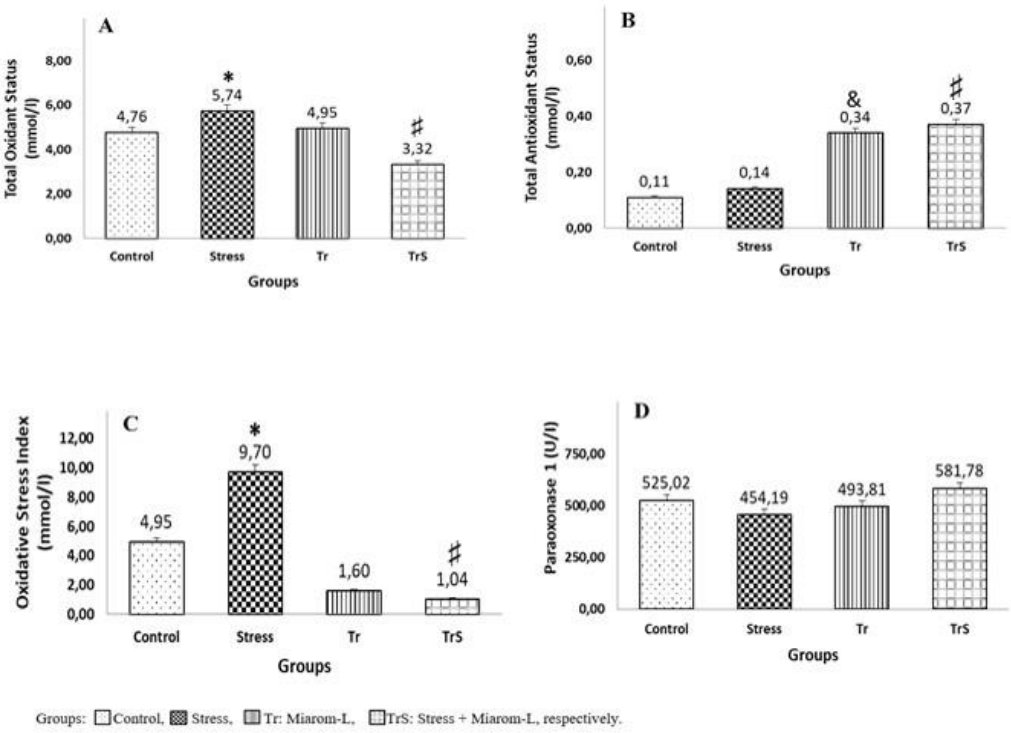

Figure 1 Effect of feeding a mixture of essential oils for 28 days on serum total oxidant status (A), total antioxidant status (B), oxidative stress index (C) and paraoxonase (D) in mixed stress rats

*Stress versus control: *P* <0.05; #Stress and treated (TrS) versus control: *P* <0.05; &Treated (Tr) versus control: *P* <0.05

The IL-4 level in S was significantly increased compared with C (*P* <0.05) (Figure 2A). Feeding with EOM reversed the IL-4 level of rats exposed to stress (*P* <0.05). Additionally, EOM supplementation increased the IL-4 level compared with the control (*P* <0.05). Although not significant, an increase in IL-6 was observed in the TrS group compared with stress-induced rats (Figure 2C). However, there was a significant decrease in IFN-γ in the stress group compared with the control (*P* <0.05) (Figure 2E). TrS had ameliorated IFN-γ levels compared with S (*P* >0.05). Also, feeding with EOM led to significant increase in IFN-γ compared with C (*P* <0.05). Effects of stress and supplementation with EOM on the immune response markers IL-4, IL-6, and IFN-γ in serum are shown in Figure 2.

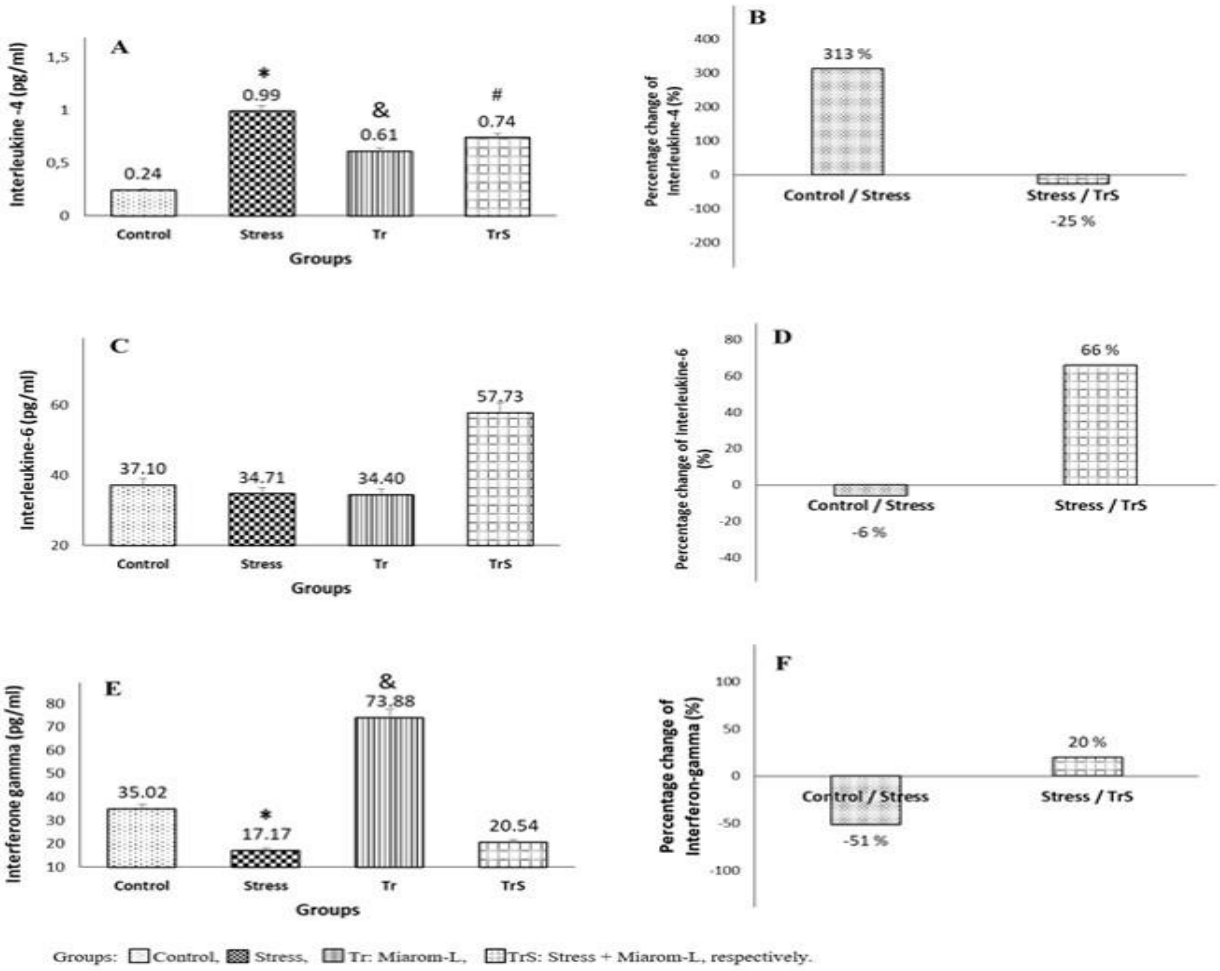

Figure 2 Effect of feeding a mixture of essential oils on A) interleukin-4 concentration; B) interleukin-4 percentage difference; C) interleukin-6 concentration; D) interleukin-6 percentage difference; E) interferon-gamma concentration; F) interferon-gamma percentage difference

*Stress versus control rats: *P* <0.05; #Stressed and treated (TrS) versus control rats: *P* <0.05; &Treated (Tr) versus control rats: *P* <0.05

There was a decrease in the serum triglyceride level of S rats (*P* >0.05) and feeding with the EOM reversed this effect (*P* <0.05). The liver enzyme AST and ALT levels were increased in S compared with C (*P* <0.05). However, feeding with EOM compensated only AST (*P* <0.05) under stress conditions. The ALT level was higher in the Tr group compared with C (*P* <0.05). However, no differences were found in GGT levels among groups. Serum concentrations of triglyceride, AST, ALT, and GGT are shown in Table 1.

There were significant differences among treatments in some organ weights (Table 2). The weights of the brain, intestine, liver, and testes were significantly lower in S rats in comparison with C (*P* <0.05). Feeding EOM reversed these effects on organ weights (*P* <0.05). However, the stomach weight was higher in TrS compared with S (*P* <0.05). Also, the stomach weight was high in the Tr group compared with C (*P* <0.05). No differences were found in the heart, spleen, and kidney among groups.

Table 1 Effect of feeding a mixture of essential oils for 28 days on serum triglyceride and the liver enzyme activities in the stressed rats

Constituents	Groups			
	Control (C)	Stress (S)	Treated (Tr)	Stressed + Treated (TrS)
Triglyceride (mg/dl)	176 ± 9.75	170 ± 7.34	200 ± 8.37	240$^{\#}$ ± 8.61
AST (U/l)	108 ± 3.64	128* ± 5.37	111 ± 4.44	100$^{\#}$ ± 5.89
ALT (U/l)	55.0 ± 2.25	66.2* ± 2.46	63.6$^{\&}$ ± 1.63	65.6 ± 1.21
GGT (U/l)	0.65 ± 0.03	0.67 ± 0.07	0.48 ± 0.05	0.59 ± 0.04

AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma glutamyl transferase
*Stress versus control rats: *P* <0.05; #stressed and treated (TrS) versus control rats: *P* <0.05; &treated (Tr) versus control rats: *P* <0.05

Table 2 Effects of feeding a mixture of essential oils for 28 days on organ weights in stressed rats (n=12)

Organ weights, g	Groups			
	Control (C)	Stress (S)	Treated (Tr)	Stressed + treated (TrS)
Brain	1.88 ± 0.03	1.76* ± 0.04	1.96$^{\&}$ ± 0.03	1.89$^{\#}$ ± 0.04
Heart	1.16 ± 0.05	1.02 ± 0.03	1.05 ± 0.02	1.07 ± 0.03
Intestines	28.3 ± 0.82	24.8* ± 1.01	28.1 ± 0.86	28.6$^{\#}$ ± 1.06
Kidney	2.72 ± 0.11	2.45 ± 0.08	2.61 ± 0.05	2.70 ± 0.07
Liver	12.93 ± 0.43	10.73* ± 0.39	12.84 ± 0.25	13.12$^{\#}$ ± 0.45
Spleen	0.81 ± 0.02	0.73 ± 0.04	0.81 ± 0.02	0.78 ± 0.05
Stomach	5.96 ± 0.51	5.79 ± 0.59	8.91$^{\&}$ ± 0.61	8.55$^{\#}$ ± 0.32
Testes	4.58 ± 0.11	4.19* ± 0.13	4.97$^{\&}$ ± 0.07	5.14$^{\#}$ ± 0.14

*Stress versus control rats: *P* <0.05; #stressed and treated (TrS) versus control rats: *P* <0.05; &treated (Tr) versus control rats: *P* <0.05

On average, across all groups, bodyweight gain was significantly greater between the second and third times that the rats were weighed compared with the earlier part of the study (Table 3). Likewise, water consumption increased significantly after the stress period (Table 3). The differences in relative water consumption before and after stress were significant for all groups except C.

Table 3 Effects of feeding a mixture of essential oils for 28 days on percentage changes in bodyweight and water consumption percentage

	Relative change, %			
	Between initiation of study and prior to imposition of stress		Between imposition of stress and end of study	
Groups	Bodyweight	Water intake	Bodyweight	Water intake
Control	26.0 ± 3.70	38.8 ± 6.83	36.8 ± 5.88	44.2 ± 5.42
Stressed	23.7 ± 3.30	31.5 ± 4.96	34.7 ± 7.29	83.3 ± 13.19
Treated	21.8 ± 1.49	26.2 ± 4.71	33.4 ± 3.18	71.2 ± 12.47
Treated + stressed	25.1 ± 2.34	26.3 ± 4.96	31.1 ± 4.26	38.1 ± 3.85
All groups	24.1 ± 1.37	28.0 ± 2.26	34.0 ± 2.61	54.7 ± 5.34

In this study, the effects of dietary supplementation with EOM were assessed with various stress models in male rats. The protective effect of the EOM was observed on serum corticosterone, antioxidant status, cytokines, liver enzyme activities, organ weight, and water consumption under mixed stress conditions.

Stress is an organism's natural way of responding to demands and events. Stress responses can be linked to corticosterone, oxidant-antioxidant status, and cytokine levels. The present results showed that the levels of serum corticosterone increased significantly in S compared with C. Feeding with EOM reversed the changes of parameters that were affected negatively because of stress, as shown by liver health, antioxidant status, immunity, and organ weights in the present study. Similar results were observed by Gong *et al.* (2015) that cortisol levels increased when rats were subjected to repeated restraint and unpredictable stresses. In another study, feeding with dietary 25, 50, and 75 mg/kg essential oil with restraint-induced immobilization stress in mice resulted in a significant decrease of corticosterone levels (Salehi & Setorki, 2017).

Oxidative stress is an important concept in redox biology and is associated with evoking biological stress responses. Application of chronic mixed stress leads to oxidative damage in organisms. In the present study, the TOS value was increased in S compared with C (P <0.05). Serum TOS decreased in TrS compared with S (Figure 1A) (P <0.05). Nevertheless, TAS serum levels increased in Tr compared with C, and increased in TrS compared with S (Figure 1B) (P <0.05). Supplementation with essential oils induced a non-specific stimulation of cellular immunity (Seirafy & Sobhanirad, 2017; Attia *et al.*, 2019). Similar to the present results, Faix *et al.* (2007) reported that supplementing male mice for 20 days with essential oils inhibited oxidative damage and the risk of inflammation and improved the antioxidant balance. In the present study, although not significant, EOM supplementation of stressed rats with 0.2 ml/day for two weeks showed an increase in the paraoxonase-1 level compared with untreated rats that were similarly subjected to stress. Paraoxonase-1 can inhibit lipoprotein oxidation, which is a marker of oxidative stress, because of the phenolic concentration of essential oils (Teissedre & Waterhouse, 2000; Cikman *et al.*, 2014). Vitamin C and folic acid, especially in combination, can alleviate the harmful effects of heat stress and improve lowered PON-1 activity levels (Gursu *et al.*, 2003). It could be concluded that EOM had a positive effect on antioxidant mechanisms in the current study because of its rich contents of vitamin C and vitamin E that functioned as non-enzymatic antioxidants, and essential oils. Vitamin E, which resides in cell membranes, has a lipophilic feature and helps to maintain membrane stability. Vitamin C is a hydrophilic antioxidant that protects the cells from peroxidative damage (Popovic *et al.*, 2015; Pehlivan, 2017; Saracila *et al.*, 2020). Hegab *et al.* (2019) also reported that selenium could counteract free radicals and preserve the structure and function of proteins.

In the present study, IL-4 levels increased in S compared with C. However, treatment with EOM reversed this effect (P <0.05) (Figure 2A). On the other hand, IFN-γ levels decreased in the S group compared with C, and supplementation with EOM resulted in an increase in IFN-γ compared with C (P <0.05) (Figure 1E). Although not significantly different, rats in the TrS group had numerically higher IFN-γ levels compared with S. No differences were found in IL-6 among groups. An EOM exerts a variety of immunomodulatory and anti-inflammatory activities by altering the patterns of IL-4 and IFN-γ. Similar to the results from the current study, researchers reported that thyme, peppermint, and eucalyptus essential oils had antioxidant and immunostimulant effects (Cook & Samman, 1996; Attia & Al-Harthi, 2015). These essential oils can extend the activity of vitamin C and act as antioxidants. Essential oils act as immunostimulants that enhance lymphocyte proliferation and have inflammatory activity related to increased interleukins and IFN-γ cytokines (Gopi *et al.*, 2014; De Lavor *et al.*, 2018). Vitamin E and selenium both regulate prostaglandin E synthesis and modulate immune functions.

Supplementation of EOM in water increased the serum triglyceride in the TrS group compared with S (P <0.05) (Table 1). Similarly, Bölükbasi *et al.* (2006) reported that thyme oil led to an increase in triglyceride in broilers. However, repeated stress led to a widespread metabolic response such as increased blood glucose and triglycerides levels, and might have other actions (Cockrem, 2007). Similar to triglycerides, the concentration of liver function enzymes also changed significantly in response to acute and chronic stress. In the present study, liver enzymes, ALT, AST (P <0.05), and GGT (P >0.05) increased in stressed rats, but feeding with EOM reversed this change, as was shown in TrS. The increasing levels of serum ALT and AST were associated with oxidative stress. Aspartate aminotransferase is present in mitochondria, whereas ALT is present in the cytoplasm of liver cells. Destruction of these cells leads to increased concentration of liver enzymes in the blood (Drotman & Lawhorn, 1978). Although the serum ALT level increased significantly in the EOM treatment group, serum AST level tended to increase, whereas GGT tended to decrease in the study. Similarly, Ayman *et al.* (2015) demonstrated the protective effects of several essential oils against hepatotoxicity in male rats and reported that the activities of ALT and AST were higher in the essential oil

group. The differences may be because of the amount of feed or the application. Nevertheless, Yanardag *et al.* (2007) indicated the protective effects of a combination of vitamin C, E, and selenium, which were associated with changes in lipid peroxidation and antioxidant enzyme activities in the liver.

In the present study, stress decreased the weight of the liver, intestine, brain, and testes significantly compared with the unstressed rats. The corticotrophin-releasing hormone (CRH) is released during stress and might be an aspect of suppression of food intake in stress-induced animals. Depression is explained by increased protein catabolism and restrained food consumption. A crowded environment compromises physical activity and growth and causes depression in body and organ weights, which is associated with mortality (Mering *et al.*, 2001; Yildiz *et al.*, 2007). In the present study, the weights of the stomach, brain, and testes increased, whereas the liver, spleen, heart, intestine and kidney weights decreased in Tr compared with C. In addition, stressed rats fed with EOM had increased weights of brain, intestines, liver, stomach, and testes (P <0.05).

In the present study, after the application of stress a significant increase was observed in the bodyweight percentage compared with before (P <0.05). Also, the mean water consumption after the stressed period was higher than before (P <0.05). In addition, a significant increase was observed in S rats compared with C. Feeding with EOM reversed this change in the period after stress (P <0.05). Plant extracts such as thyme apparently stimulate digestive secretions and enzymes (Williams & Losa, 2001; Nameghi *et al.*, 2019). However, other researchers observed that essential oils did not affect feed digestibility (Nameghi *et al.*, 2019) although some essential oils (clove, garlic, peppermint, and eucalyptus) reduced feed digestibility in animals (Patra & Yu, 2012). Increased feed and water consumption regulate homeostasis against stress (Nagaraja & Jeganathan, 2003). Thus, the homeostatic regulation of rats may have been reversed by increased IL-6 levels. In the present study, IL-6 levels increased in stressed rats supplemented with EOM (34.71 pg/ml and 57.73 pg/ml in S and TrS, respectively). Interleukin-6 plays a significant role in brain-mediated responses to stress such as decreased motor activity, reduced food intake, and decreased water consumption (Dantzer *et al.*, 2008). Mishra *et al.* (2019) reported that exogenous IL-6 reduced locomotor activity and food intake, but no effect was found on the water consumption of rats. Also, they indicated that the activity of IL-6 was demonstrated by increasing ACTH and corticosterone release.

Conclusions

Types and levels of stress affect growth performance, immunity, and wellbeing. The mechanisms and responses of animals to stress are difficult to identify and understand. There was no known stressed rat model for animal feeding that examined an EOM containing thyme, anise, peppermint, and eucalyptus with vitamin B complex, AD3E, vitamin E and selenium. This study showed that 0.2 ml/l EOM with vitamins played a positive role in animal wellbeing and this mixture may be useful in animal feeding.

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Authors' Contributions

NS and CA conceived and designed the study; NS, EK and RG collected and analysed the data and drafted the original manuscript, NS and CA revised the manuscript and approved the final version.

Conflict of Interest Declaration

The authors declare that they do not have any conflict, of interest.

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