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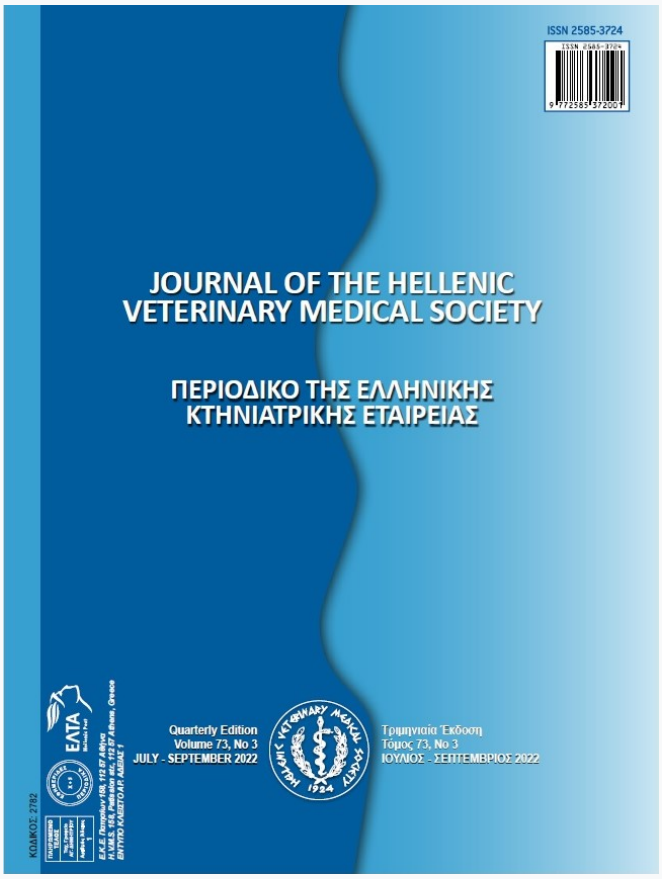

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Research article
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The influence of phytogenic additive on the antioxidant capacity, immunity and liver functions in stress-induced male rats

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ABSTRACT: Global animal feeding strategies have been modified due to several ecosystem changes that cause stress and decline in health, growth, and yields.According to the literature, natural additives have been fundamental to animal health from day to day. The present study measured changes in antioxidant status, immunity, liver functions and organ weights tested under stress conditions to determine whether dietary supplementation with phytogenic additive could provide beneficial effects. Forty-eight adult,male Sprague-Dawley rats were randomly separated into four groups;- *Control(C),Stress(S),Treatment(Tr),Treatment and Stress(TrS)*. Rats in groups Tr and TrS received phytogenic additive by adding into water (2mL/L) 5days a week for 28days. All rats were exposed to prolonged light phase conditions (18h light: 6h dark) for 14 days. Also, two chronic stresses, isolation and crowded environments, were applied to animals in the *Stress* and *TrS* groups. There was a significant decline in the oxidant status in untreated stress group, while phytogenic additive fed rats maintained a significantly higher total antioxidant status. This study also showed a significant increase in IL-4 and decrease in IFN-γ in the untreated *Stress* group compared to the *Control* group. There were increases in liver enzymes in the *Stress* group in comparison to the *Control* group. After the phytogenic treatment, there was an increase in the weight of the liver, intestine, brain and testes. In conclusion, this study showed that supplementation of phytogenic additive containing milk thistle and artichoke with choline,carnitine, vitamin E and methionine describes the protective effects on antioxidant status, immunological parameters and liver functions under mixed stress conditions.

Keywords: Antioxidant status; feed additives; immunity; phytogenic ingredients;stress; rats

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INTRODUCTION

Stress can be described as the activation of the hypothalamus-pituitary-adrenal axis (HPA) and the sympathetic nervous system. The activation of the HPA axis has a remarkable role in physiological, neuroendocrine and behavioral responses against stress. Stress is also related to the release of the adrenal glands, which is an activity of the sympathetic nervous system. All of the responses of organisms are called the flight or fight mechanism which describes the adaptation of stress. When animals are subjected to stress, physiological mechanisms are activated to stabilize the internal environment through neuroendocrine and behavioral reactions. Activation of the neuroendocrine system affects the secretion of hormones and neurotransmitters that collectively act as a response mechanism to adapt to stress. Studies have shown that after acute and chronic stress, parallel induction of cortisol levels is consistent with the stress index (Lee et al., 2015). Environmental temperature, crowded environment or isolation are the most important stressors of animals which also may alter some conditions of animals such as growth, immunity and oxidation, and thereby animal welfare and health (Sejian et al., 2011; Coldizt and Hine, 2016; Li et al., 2019). It was reported that rats exposed to variable chronic stress, such as isolation and food/water deprivation for 10 days reduced the weight gain (Marin et al., 2007). Meddings and Swain (2000) noted an increase in gastrointestinal mobility and permeability in Wistar albino rats following environmental stress for 3 weeks. Studies about abnormal lightening regimens in adult rats to reproductive performances were evaluated, and it was found that short photoperiod resulted in reproductive inhibition (Kus et al., 2004). According to literatures, overcrowding stress is the most important stress factor in animal production, especially poultry. It was reported that crowding stress is focused on decreased body weight, feed intake, leg problems, and immune system and behavioral changes (Guardia et al., 2011;Saymore et al., 2011;Housmand et al., 2012; Gomes et al., 2014).

As the liver is the primary side of the metabolism, regulation of the immune system and stress, the effect of chronic stress on the integrity of the liver is important. It can also eliminate toxic substances of metabolism via the activities of liver cells. The mitochondrion, microsomes and peroxisomes in parenchymal cells can produce ROS and those are primary cells subjected to oxidative stress-induced injury in the liver (Sanchez-Valle et al., 2012). Moreover, Kupffer cells, hepatic stellate cells and endothelial cells are potentially more exposed or sensitive to oxidative stress-associated molecules (Cichoz-Lach and Michalak, 2014). When the ROS is excessive, oxidative stress triggers inflammation and immune responses, and can also cause damage to extra hepatic organs. In mammals, a sophisticated antioxidant system has been developed to maintain the redox homeostasis in the liver. Also, Kupffer cells in the liver have sensitivity to oxidative stress, which increase the inflammation and apoptosis. In order to combat the damaging effects of ROS, liver cells have exogenous and endogenous antioxidant enzymes. The liver enzymes ALT (alanine aminotransferase), AST (aspartate aminotransferase) and GGT (gamma-glutamyl transferase) are excreted to blood in oxidative stress (Zlatkovic et al., 2014; Samarghandian et al., 2014). It has been shown that administration of social isolation in male rats resulted in a harmful increase in the markers of oxidative stress, such as ALT, AST and MDA (Zlatkovic et al., 2014). The reviewed literature suggests that exposure to chronic social isolation for 21 days in male Wistar rats altered the protein expression/activity of the liver antioxidant enzymes.

Cytokines are signaling molecules which participate in the amplitude and duration of the immune and inflammatory responses. Excessive or insufficient production of cytokines is related to the pathophysiology of a range of diseases (Gulati et al., 2016). Stress is accompanied by altered production of neuropeptides and inflammatory cytokines. There are contradictory results indicating the relationship between stress and cytokines. Some researchers reported a decrease in IL-6 during stress (Mormède et al., 2002), while several investigators have found an increase (Glaser and Kiecollt-Glaser, 2005; Rohleder, 2012). On the other hand, it was reported that IL-4 production could be increased, decreased or unchanged by stress (Chuian et al., 2005; Yang et al., 2006; Murakami et al., 2007). In contrast to IL-4 and IL-6, it was reported that stress suppressed the production of IFN-γ in male mice (Curtin et al., 2009).

A balanced diet copes with stress and improves the organism's defense abilities. The usage of phytogenic ingredients, natural additives, antioxidants and vitamin-mineral complexes for animal nutrition can support the metabolic functions, growth and productivity. This means to ensure getting healthy nutrition in animal husbandry besides welfare (Quezada-Mendoza et al., 2011; Koseli et al., 2019; Seyidoglu and

Aydin, 2020; Seyidoglu et al., 2021; Koseli et al., 2021; Aydin et al., 2021).

The rapid development of the potential use of plant-based additives for animal feed is primarily due to the 2006 EU ban on antimicrobial agents as growth promoters. The use of phytogenic additives in extracts, most of which are essential oils, has been tested in many animal species. However, there remains a lack of scientific results on the effectiveness of their use or their effect on stress management. In this study, we examined the effects of a phytogenic additive containing milk thistle and artichoke with choline, carnitine, vitamin E and methionine on liver health, antioxidant status, immunity and organ weights in rats during mixed stress such as crowded environment and isolation.

MATERIALS AND METHODS

Experimental Animal

The experimental protocols were approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Animal Care and Use Committee of Bursa Uludag University (ApprovalNo: 2018-07/01).

In this study, forty-eight male, Sprague Dawley rats weighing 150-200 g and aged 7-8 weeks were used for experiment. Research with experimental animal models is a complex undertaking that includes many considerations, adaptations and precautions. Livervital contain B vitamins, methionine, vitamin E, L carnithine, milk thistle and artichoke. These ingredients support lipid metabolism and are central to chicken laying performance. But also a complementary feed for all animal species for the highest antioxidant capacities. Rats were chosen as a model due to ability to adjust a stress protocol and similarity to humans physiologically. Besides, rats have a rapid metabolic rate, and studies may result in a short time. The rats were housed under standard laboratory conditions (22 ± 1.0 C; 55 ± 10% humidity) in plastic and clear 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 commercial pelleted rodent diet (Korkuteli Yem Gida San. A.S. Turkey) and tap water. Basal diet contained 2000-2500 kcal/kg energy, 23.50% raw protein, 5.92% crude cellulose, 2.95% crude oil and 6.36% ash. The diet included lysine 1.35%, methionine 0.43 %, sodium 0.05 %, calcium 0.85% and phosphorus 0.98%. Prior to the experiment rats were given one week to acclimatize to laboratory conditions.

The rats in treatment groups (Tr and TrS) received a phytogenic additive (Livervital, Miavit, Germany) containing phytogenic ingredients from milk thistle and artichoke with choline, carnitine, vitamin E, methionine in drinking water at concentration of 2ml/l concentration, 5 days a week for 28 days.

Livervital is a commercial special combination which can support the liver protection and its regeneration for poults. Carnitin, methionine, choline and vitamins are used to improve the lipid metabolism of the liver. Especially high vitamin E offers oxidative protection to liver tissues. Furthermore, milkthistle and artichokeprovidelipidmetabolism and help to minimize the formation of liver fat.

Experimental Design

The rats were randomly divided into four experimental groups. The total experimental protocol was maintained for twenty-eight days. The rats were allocated randomly into four experimental groups of twelve rats each that are: *Control (C), Stress (S), Treatment (Tr) ,Stress + Treatment (TrS)* . Livervital was given at 2 mL/l/daily by adding to water five days a week (Monday, Tuesday, Thursday, Friday, Saturday) to Tr and TrS.

Experimental Set

The trial lasted twenty-eight days. The first week, it was the adaptation of the rats. Following the first and second week, Livervital was applied to water in the Tr and TrS groups. During the final two weeks of the trial, in addition to feeding with Livervital, all animals were exposed to light: dark cycle. This model (18h light: 6h dark) is based upon modified literatures (Ten Hoor et al., 1980; Gancarczyk et al., 2004; Park et al., 2015; Seyidoglu et al., 2019a; Seyidoğlu et al., 2021). In the last two weeks, two chronic stresses have been exposed to rats in Stress and TrS. No food or water was provided during this stressful application:

Isolation Stress: The rat was left alone for 30 minutes on Monday, Wednesday, Friday, and Sunday of the 3rd week on the trial. This stress was formed in a separate cage with four sides and ground covered with white paper.

Crowded Environment Stress: Social overcrowding stress was formed by leaving six rats in a cage

(50cm x 50cm), which was designed for three rats, for 30 minutes. This stress was implemented on Tuesday, Thursday, and Saturday of the 4th week of trial.

Measurements

Blood samples were obtained by puncturing the heart under short (2-3 minutes) isoflurane anesthesia at the end of the study. Blood samples were centrifuged on the same day to separate the serum, and after that samples were kept at -80^{0}C until the analysis. Shimadzu UV-VIS Spectrophotometer 2600 device was used to measure serum triglyceride (Archem Diagnostic), ALT (alanine aminotransferase, Archem Diagnostic), AST (aspartate aminotransferase, Archem Diagnostic) and GGT (gamma-glutamyltransferase, Archem Diagnostic).

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

Plasma cortisol (Elabsience, USA), Interleukine-4 (IL-4), IL-6 and interferon-gamma (IFN-γ) were measured using a commercially available rat ELISA kit3 (Thermo Fisher), and the assays were performed colorimetrically using a plate reader. The serum percentage changes of all these parameters were calculated and figured.

The brain, heart, intestines (duodenum, ileum, colon), kidney, liver, spleen, and stomach of the rats were weighted (Sartorius, BL210S) immediately after sacrifice.

Statistical Assessment

Statistical analyses were performed with SPSS (Version 20.0, IBM, SPSS Inc., Chicago, USA). Data were tested for normality distribution and variance homogeneity assumptions. All the values were grouped, and means and standard errors were calculated. One-way ANOVA was completed on all parameters to examine the differences between the groups. Differences were considered significant at $P < 0.05$. If the difference between the groups was significant ($P < 0.05$), differences were then evaluated by Tukey's test. On the other hand, in non-homogenous groups, differences between the means were analyzed by Kruskal Wallis and following Mann Whitney U test between the groups one by one.

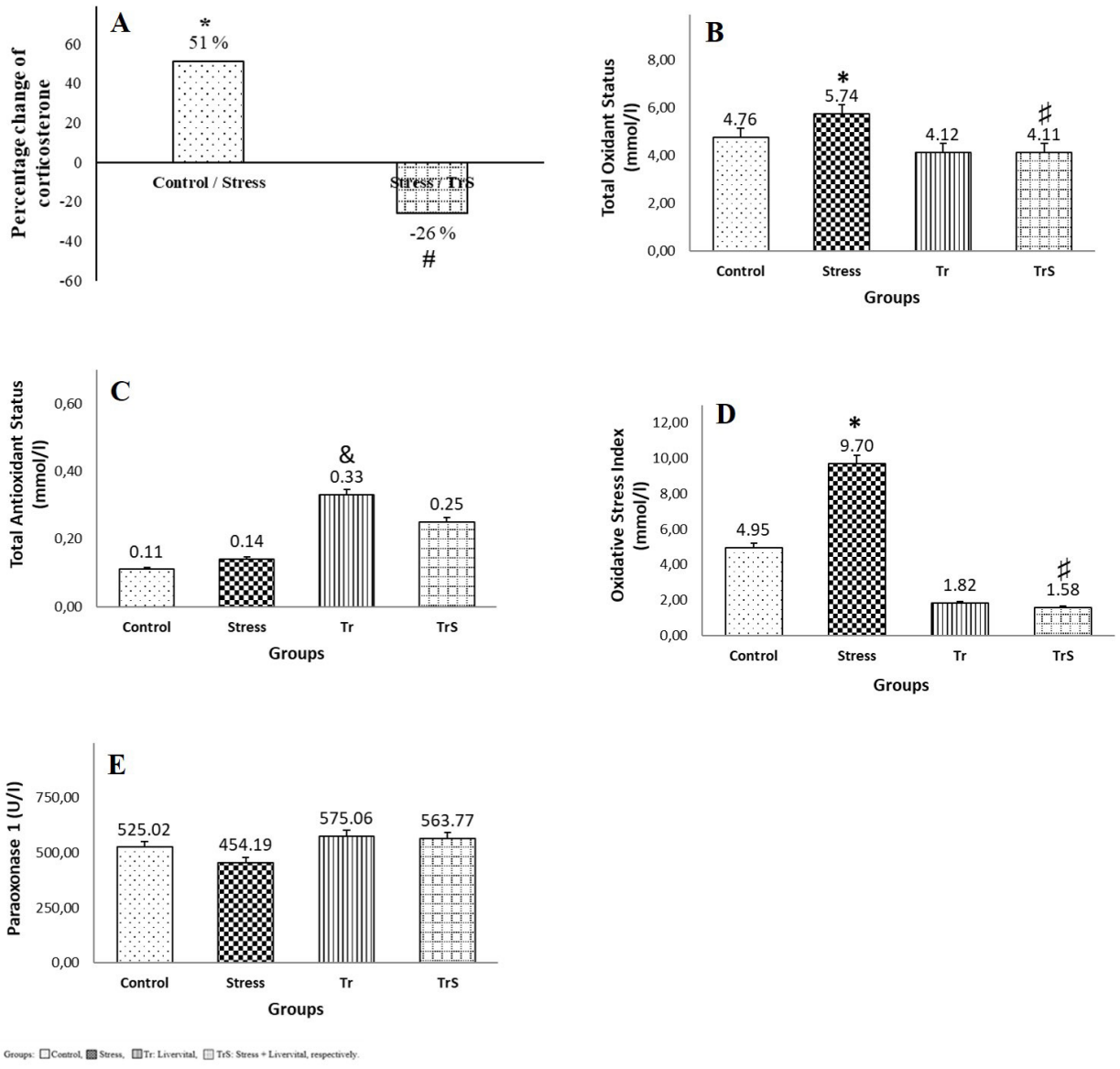
RESULTS

Our result in Figure-1A showed that the percentage changes of serum corticosterone were significantly increased in the stress induced rat group when compared to control group (the serum cortisol levels in control and stress group, 51.24±1.62 and 77.31±8.97, respectively). This means stress was occurred successfully. On the other hand, feeding with phytogenic additive reversed this change by reducing the cortisol level (the serum cortisol levels in Stress and TrS group, 77.31±8.97 and 57.47±2.45, respectively).

The oxidant-antioxidant status parameters, TOS, TAS, PON1 and OSI index in blood serum are shown in Figure-1B. In the present study, a highly significant increase was found in the mean TOS level in stress-induced rats compared those in the control group. Treatment rats (Tr) exhibited remarkable improvement in the TOS level compared to the stress-induced rats ($p<0.05$). There was a significant increase in the TAS level of Treatment rats compared to that of control rats ($p<0.05$, Figure 1C). Meanwhile, the TAS level of the TrS group decreased in comparison to the Tr group as shown in Figure 1C. Additionally, the oxidative stress index increased in stress-induced rats in comparison to Control group, shown in Figure 1D ($p<0.05$). However, rats with supplemented phytogenic additive has offered an enhancement of oxidative stress index values. On the other hand, there were no statistical differences found in PON value in all groups ($p<0.05$; Figure 1D).

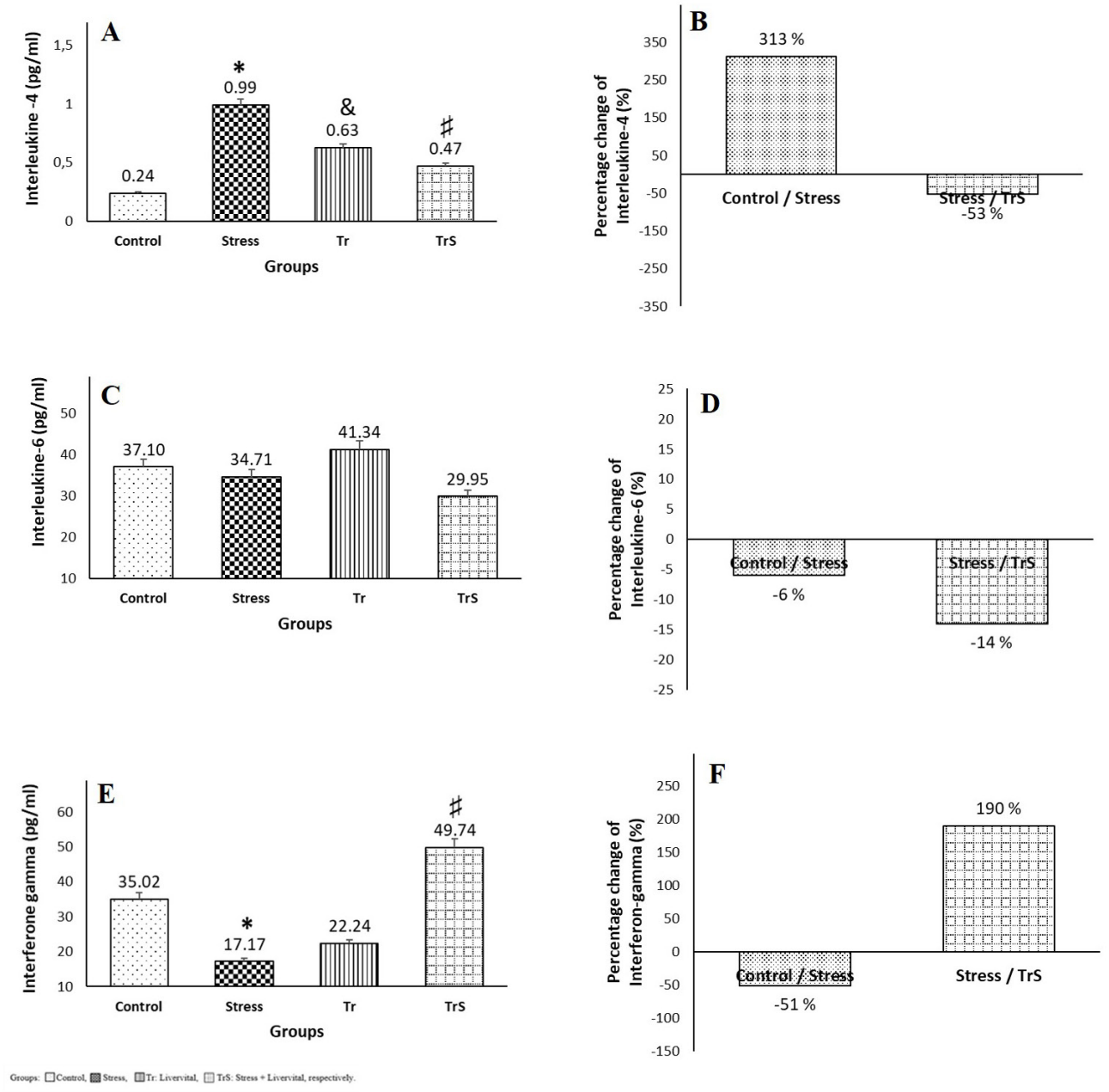
The IL-4, IL-6 and IFN-γ parameters of feeding with phytogenic additive in the stress induced rats are shown in Figure 2. There was a significant increase in IL-4 value in the stress group compared to control one ($p<0.05$; Fig 2A). Feeding with phytogenic additive reversed the IL-4 level of rats exposed to stress. There was a significant decrease in IFN-γ in the stress group than control rats ($p<0.05$; Fig 2E). Phytogenic ingredient supplemented rats ameliorated of IFN-γ as compared to stress group.

The triglyceride level of stress induced rats decreased, but feeding with phytogenic additive reversed this change by increasing the triglyceride level. The AST and ALT levels increased in Stress-induced group compared to the Control group. Feeding



* P < 0.05 ; Stress versus Control group
# P < 0.05 ; TrS versus Stress group
& P < 0.05 ; Treatment group versus Control group

Figure 1: Percentage change in mean serum cortisol level in groups of Control versus Stress and in groups of Stress + Treatment versus Stress (A); Effect of fed with *Livervital* for 28 days on serum total oxidant status (B), total antioxidant status (C), oxidative stress index (D) and paroxonase1 (E) in stressed rats. All data are presented as the mean ± SE (n=12).



* P < 0.05 ; Stress versus Control group
\# P < 0.05 ; TrS versus Stress group
& P < 0.05 ; Treatment group versus Control group

Figure 2: Effect of fed with *Livervital* for 28 days of the serum concentrations and percentage change in mean the serum of Interleukin-4, Interleukin-6 and Interferon gamma. 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. All data are presented as the mean ± SE (n=12).

with phytogenic additive compensated only the liver enzyme AST activity under stress conditions significantly ($p<0.05$; Table 1).

In the study, there were statistical differences in organ weights shown in Table 2. The weights of brain, intestine, liver and testis were found lower in stress-induced rats than control. Feeding with phytogenic additive reversed the organ weights of rats exposed to stress ($p<0.05$).

Table 1: Effect of fed with *Livervital* for 28 days on serum triglyceride and liver enzyme activities in stressed rats. All data are presented as the mean ± SE (n=12).

Parameters	Groups			
	Control	Stress	Treatment (Tr)	Stress + Treatment (TrS)
Triglyceride (mg/dl)	176.31±9.75	169.61±7.34	193.28±8.81	179.27±15.68
AST (U/L)	107.95±3.64	128.35±5.37*	120.82±6.05	113.26±2.23#
ALT (U/L)	55.00±2.25	66.22±2.46*	60.00±1.52	62.80±1.11
GGT (U/L)	0.65±0.03	0.67±0.07	0.58±0.04	0.66±0.04

AST= Aspartate Aminotransferase; ALT= Alanine aminotransferase; GGT= Gama glutamiltransferase. Different superscripts within a row indicate a significant difference.

* P < 0.05 ; Stress induced rats versus Control group
# P < 0.05 ; TrS versus Stress induced rats
& P < 0.05 ; Treatment group versus Control group

Table 2: Effect of fed with *Livervital* for 28 days on organ weights in stressed rats. All data are presented as the mean ± SE (n=12).

Organ weights (g)	Groups			
	Control	Stress	Treatment (Tr)	Stress + Treatment (TrS)
Brain	1.88±0.03	1.76±0.04*	1.91±0.03	1.92±0.02#
Heart	1.16±0.05	1.02±0.03	1.10±0.04	1.09±0.03
Intestines	28.25±0.82	24.76±1.01*	28.19±1.05	27.43±0.65#
Kidney	2.72±0.11	2.45±0.08	2.55±0.08	2.57±0.04
Liver	12.93±0.43	10.73±0.39*	11.27±0.4&	12.09±0.34#
Spleen	0.81±0.02	0.73±0.04	0.84±0.02	0.86±0.03
Stomach	5.96±0.51	5.79±0.59	8.88±0.60&	6.39±0.38
Testis	4.58±0.11	4.19±0.13*	5.08±0.08&	4.67±0.05#

Different superscripts within a row indicate a significant difference.

* P < 0.05 ; Stress versus Control group
# P < 0.05 ;TrS versus Stress group
& P < 0.05 ; Treatment group versus Control group

DISCUSSION

In this study, we assessed the association between feeding with phytogenic additive containing milk thistle and artichoke with choline, carnitine, vitamin E and methionine, and different stresses in a rat model. We observed a protective effect on serum cortisol, antioxidant status, cytokines, liver enzyme activities and organ weights under mixed stress conditions.

Stress occurs when animals suffer to make extreme and/or prolonged physiological and behavioral alterations in order to manage with their surroundings. Stress responses can be linked to corticosterone, oxidant-antioxidant status and cytokine levels. In the present study, serum corticosterone levels were significantly higher after the induced mixed stress. Similar results were observed byGong et al.(2015)that cortisol increased when rats were subjected to repeated restraint and unpredictable stresses. In another study, the feeding dietary 150, 200 and 400 mg/gr milk thistle with food restriction stress resulted in a significant decrease of cortisol levels in rats (Mahjoor and Denghan, 2008).

Oxidative stress is a result of increased generation of free radicals and/or reduced physiological activity of antioxidant defense mechanism. Studies have demonstrated that many antioxidants have protective effects in preventing chronic diseases that are mediated by oxidative stress and inflammation. The Total Oxidant Status (TOS) is usually used to estimate the overall oxidation state of the organism. Regarding the influence of feeding with phytogenic additive on oxidative stress, we observed that the serum TOS value decreased. This is probably associated with a positive correlation between dietary supplementation of phytogenic feed additive and the protection of antioxidant potential which may lead to regulation of the oxidant-antioxidant balance. Results from experimental studies confirm that the serum TAS is an emerging biomarker of overall antioxidant status. The protective effect of phytogenic ingredients against oxidative

damage is reported by Ahmed et al. (2019). In that study, in Wistar albino rats received supplementation of 750 and 1500 mg/kg of artichoke extract for 3 weeks inhibited the oxidative damage while improving the antioxidant balance. In the present study, we tested the effect of isolation and crowded environment stress on serum TAS values in male rats, and the statistical analysis showed that TAS serum levels showed an increase compared to non-stressed rats as shown in figure 1C. Stress reveals the efficiency of antioxidant status by altering the patterns of oxidative stress. The phyto additives (milk thistle and artichoke) used in the present formulation in drinking water with choline, carnitine, vitamin E, and methionine are recognized for their antioxidant properties (Qawami et al., 2013; Gostin and Waisundara, 2019; Koseli et al., 2019; Seyidoglu et al., 2021) in the present study. Paraoxonase-1 (PON1) is an antioxidant enzyme which is produced by the liver and regulates the metabolism in oxidative stress. PON1 have also shown to have activities of anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic, anti-microbial and organophosphate-hydrolyzing properties. In a meta-analysis which aims to analyze the clinical data quantitatively, it was reported that serum PON levels are lower than the controls in the depressed patients (Tao et al., 2015). In the present study, supplementation of 2ml/day phytogenic additive for 2 weeks in stressed male rats showed an increase in the PON-1 level compared to the stressed groups (Figure 1D). Ahmad et al. (2020) investigated the influence of dietary supplementation of milk thistle on heat stressed broilers. They reported that milk thistle supplementation can alleviate the harmful effect of heat stress and the improvement lowered PON-1 activity levels as compared to control group.

It is well documented that infections lead to inflammation, thereby releasing pro-inflammatory cytokines, chemokines, and adhesion molecules. Cytokines not only activate immune cells but also have an intense and degrading influence on animal health by reducing food intake and favoring catabolism of muscle tissues (Johnson and Escobar 2005). Some interleukins, which are produced following exposure to immunological and psychological challenges, play an important role in the neuroendocrine and behavioral stress responses in animals (Ménard et al., 2017; Takahashi et al., 2018; Picard and McEwen, 2018). Researchers reported that milk thistle acts as an immunostimulant which enhances lymphocyte proliferation related to increased IL-4 and IFN-γ cytokines (Wilasrusmee et al., 2002). In the present study, we tested the effect of isolation stress and crowded environment stress on IL-4, IL-6 and IFN-γ in male rats, and statistical analysis showed that IFN-γ serum levels decreased and IL-4 levels increased compared non-stressed rats. But the decrease of IL-6 was not statistically significant in stressed rats. The feeding dietary phytogenic additive resulted in an increase in serum IL-4 and IL-6 compared to the control. Phytogenic additive containing milk thistle and artichoke exerts a variety of immunomodulatory and anti-inflammatory activities by altering the pattern of IL-4 and IFN-γ.

Chronic stress and hepatic injuries can be associated with numerous enzymes that are produced in the liver. During stress conditions, Kupffer cells in liver are over activated, and hepatic blood flow decreases. Even more, elevation of serum liver enzymes is an important sensitive biomarker, and these specific enzyme activities can be considered as a diagnostic feature (Zlatkovic et al., 2014; Contreras-Zentella and Hernández-Muñoz, 2016). In the present study, serum ALT and AST levels increased in the liver, which was associated with oxidative stress. Phytogenic additive feeding developed an increase in the activities of liver enzymes: AST, ALT and GGT, which indicate the amelioration of liver damage. In another experiment, which was performed to demonstrate the protection of milk thistle against toxic effects of aflatoxin by using male broiler chicks, the serum enzyme activities of ALT and AST were found lower in the milk thistle administered groups (Dumari et al., 2014).

On the other hand, stress situations such as crowded environment or small cage size are also important for physical activity, feeding, and growth. It was reported that crowded environment stress suppressed the organ development and thereby growth (Mering et al., 2001; Seyidoglu et al., 2019b). This depression is also explained by basal metabolism and hormonal profile (Yildiz et al., 2007). Dietary supplementation of phytogenic feed additive causes morphologic changes in gastrointestinal tissues by increasing the height of villi in poultry's small intestine (Hong et al., 2012). This is expected to increase the absorptive surface area and effective digestion and nutrient absorption. A greater height of the villi can also increase the enzymes secreted from the tip of the villi, contributing to improved digestibility. (Baurhoo et al., 2007). In the present study, stress significantly decreased the weight of the liver, stomach, intestine, brain, and tes-

tes as compared to the unstressed control group. Results showed that during the four-week experiment, the weights of spleen, stomach, brain, and testes increased while kidney, liver, heart, and intestine decreased in phytogenic additive feeding rats compared to the control group. In our experiment, feeding with phytogenic additive in stress-induced animals showed an increase in the organ weights.

CONCLUSION

To improve the animal husbandry, nutrition is important as well as management of stress. The animal's growth, immunity and animal yield capacities are affected negatively when they are exposed to stressors. It was accepted that changes in animals' normal conditions decrease the growth performance, immunity and well-being. The phytogenic additive used in this study has rich contents especially phytogenic ingredients from milk thistle and artichoke which have a positive role on the organism. It can be said that phytogenic additive containing milk thistle and artichoke with choline, carnitine, vitamin E and methionine may be a useful supplement against stress condition.

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CONFLICT OF INTEREST

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

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