Effect of wood betony (*Stachys lavandulifolia* Vahl) extract on some serum biochemical changes and acute stress response in juvenile common carp (*Cyprinus carpio*)

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Abstract

The effects of four different levels of wood betony (WB) (*Stachys lavandulifolia* Vahl) extract (0, 2, 4 and 8% W/W in the diet) were investigated on some serum enzymatic activities and acute stress response of juvenile common carp (*Cyprinus carpio*). After 10 weeks post feeding, two groups of fish (0 and 8% WB) were subjected to handling and crowding stress and the stress responses including serum cortisol, glucose and some electrolytes (Na^+, K^+ and Ca^{+2}) levels were assayed. The results revealed that the fish fed on 8% WB had significantly lower levels of lactate dehydrogenase (LDH) and alanine transaminase (ALT) compared to the control group ($P<0.05$). Although cortisol and glucose levels were increased significantly shortly after stress ($P<0.05$), the elevation level was significantly lower for fish fed on 8% WB ($P < 0.05$). In control group, sodium level decreased ($P < 0.05$) at 3 h post stress but did not show any change in 8% WB ($P > 0.05$). The potassium and calcium levels showed insignificant fluctuations post stress ($P>0.05$). Dietary inclusion of WB seems to improve liver function and response to acute stress in juvenile common carp.

Keywords: stress, enzyme activity, ion regulation, wood betony, common carp.

Introduction

Fish cultivation under intensive conditions has been increased all around the world. High rearing densities, poor water quality, weighing, transportation and grading are common stressors which can both affect fish welfare and increase the risk of disease under intensive fish cultivation. Different levels of stress reactions has been well-defined in fish including primary (hormonal response), secondary (metabolic and haematological changes) and tertiary as a last stage which leads to exhaustion, growth decline or death (Barton 2002). Urbinati & Carnerio (2001) defined handling as a practice including chasing, capture and exposing the fish to air which can cause explicit physiological and endocrinological responses in fish. Dobšikova, Svobodova, Blahova, Modra & Velišek (2006) observed that 12 h transportation could cause significant changes in some biochemical and haematological indices of three years old common carp. However, McCormick, Shrimpton, Carey, O'Dea, Sloan, Moriyama & Björnsson (1988) found out that handling stress decreased growth rate of Atlantic salmon (*Salmo salar*). Different practices such as water quality optimization, optimal fish density and administration of natural or synthetic compounds could be used to improve fish resistance to stressor. Synthetic feed additives have some adverse effects such as bioaccumulation and environmental pollution. Due to these problems, much more attention has been focused on the use of medicinal plants in aquaculture (Citarasu 2010; Chakraborty & Hancz 2011). Most of the studies about the effect of using herbal biomedicine in aquaculture has been focused on their application such as growth promoter, immune stimulator or antipathogenic agent in different cultivated fish species like African catfish (*Clarias gariepinus*) (Dada & Ikuerowo 2009; Soosean, Marimuthu & Sudhakaran 2010), Tilapia

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(*Orechromis mossambicus*) (Immanuel, Uma & Iyapparaj 2009), common carp (*Cyprinus carpio*) (Alishahi, Ranjbar & Ghorbanpour 2010; Pakravan, Hajimoradloo & Ghorbani 2012) and olive flounder (*Paralichthys olivaceus*) (Cho & Lee 2012). However, little information is available on the effects of herbs on stress responses in fish (Xie, Liu, Zhou, Su, He, Pan, Ge & Xu 2008; Shahsavani, Baghshani & Alishahi 2010). Wood betony (*Stachys lavandulifolia* Vahl) which has been belonged to family Lamiaceae, is grown in many parts of Iran, Turkey, Iraq, Syria, Armenia as well as Georgia (Javidnia, Mojab & Mojahedic 2004). Fresh and dried areal parts such as leaves, flowers and roots have been used as traditional drugs for treatment of wounds and bruises, mouth ulcers, gum inflammations (Ody 1997) as well as treating arthritis and respiratory inflammatory disorders (Rezazadeh, Zaringhalam, Manaheji & Kebryaeezadeh 2009). Alkanoids (including stachydrine and trigonelline), tannins, saponines, nicotinic acid, polyphenols, organosulfids and steroids are the main components of wood betony (Vundac, Brantner & Plazibat 2007). Some of the Stachys components' have shown a variety of biological activities (Chakraborty & Hancz 2011; Ghasemi Pirbalouti, Jahanbazi, Enteshari, Malekpoor & Hamedi 2010). The biological activity of WB has not yet been studied in fish. Moreover, the ability of herbal biomedicine on osmoregulation and stress response has not been studied seriously. Hence, this study was aimed to evaluate the effects of dietary inclusion of WB extract on some serum biochemical characteristics and stress responses in juvenile common carp, which has been exposed to handling and crowding stress.

Materials and Methods

Fish

Two hundred and fifty juvenile common carp (35 ± 5.2 g) were obtained from Isfahan Fish Propagation and Breeding Center in summer 2012. The fish were kept under environmental condition, placed in 10 m^3 rectangular concrete tanks for 2 weeks for acclimatisation. Then, they fed on a commercial carp feed (Isfahan Mokkamel, Iran) with proximate composition (wet basis %) as the following conditions: humidity (9.2%), protein (32%), lipid (10.2%) and ash (11.1%). These amounts were obtained based on our analyses (data not shown).

Plant extract

In spring 2012, the Wood betony aerial parts including flowers and leaves were collected from natural habitat, Isfahan province. The plants were delivered to the Central Herbarium of Isfahan University of Technology, Department of Natural Resources for final identification and analyses. Hydro-alcoholic plant extraction was done based on Ghasemi Pirbalouti *et al*. (2010) with some modification. Briefly, aerial parts of the plants were washed thoroughly with distilled water and dried at room temperature under shading. Then, the plants were grounded into powder. A 100 g of powdered plant material was soaked in 500 mL of ethanol (75%) for 48 h, shacked vigorously to allow for proper extraction. After filtering of the extract through Whatman paper no. 1, filtrate was concentrated using a rotary evaporator at 50 ˚C. Finally, 20 mL of concentrated liquid extract was obtained from l00 g of the plant powder; each mL of the concentrated extract was almost equal to 5 g of the plant powder.

Feed preparation and feeding trails

In order to prepare the diets, the commercial pellet diet was crushed and mixed with the appropriate WB liquid extract concentration (the extract volumes were adjusted by adding distilled water to final volume of 100 mL for each kg of diet), remade into the pellets, were allowed to be dried for 72 h at room temperature and then stored at refrigerator until it was used. After 2 weeks of acclimatization, 180 individual fish (44±0.62 g) were randomly divided into four groups, with three replicate each. The first treatment was fed on normal diet without S. lavandulifolia and kept as control group. The second, third, and fourth ones were fed on normal diet containing 2, 4 and 8 % of *S. lavandulifolia*, respectively (defined as 2 WB, 4 WB and 8 WB). Each replicate contained 15 individuals in a fibereglass tank (110 L water volume, 50% renewed each day).

Water quality was monitored throughout the experimental period at daily intervals; temperature 25 ± 1°C, pH 7.21 ± 0.5 and dissolved oxygen concentration at 7.5 ± 0.06 mg L^{-1}. Fish were fed frequently on a diet of 32% crude protein (CP) at a rate of 2% body weight, three times a day for 70 days.

Stress challenge

The stress challenge was performed according to Tahmasebi-Kohyani, Keyvanshokooh, Nematollahi, Mahmoudi & Pasha-Zanoosi (2011) with some modifications. Briefly, at the end of 10 weeks, 30 fish from control and 8 WB were subjected to stress challenge. For this purpose, the fish were remained on experimental diets and acclimatized for 1 week as defined before. After the acclimation period, three fish per tank were removed for sampling (time 0) prior to subjecting the remaining fish in the tank to an acute stress. The stress consisted of netting the remaining fish from the tank, holding them out of water for 5 min, and then crowded at an approximate density of 150 g L^{-1} in a plastic mesh bucket in their original tank for 3 h without any aeration or water exchange. The experimental fish were sampled after 1 h of crowding, as they were released from the crowding stress (3 h) and at 8, 24, and 48 h.

Blood sampling and biochemical analysis

At the end of the 10-week experiment, for biochemical analyses, at least 3 fish from each replicate were anaesthetized with clove powder (100 ppm) and blood sampling (1.5-3 mL) was performed individually from caudal puncture. The blood was centrifuged at 3000 rpm for 10 min and the supernatant serum was collected. The serum was kept frozen at -80 °C until analyses for enzymatic activity of lactate dehydrogenase (LDH), aspartate transaminase (AST), alkaline phosphatise (ALP), and alanine transaminase (ALT) activities (Peyghan & Azary Takamy 2002).

To evaluate the stress response of the fish, they were captured with minimal disturbance at the time described above (stress challenge). Cortisol and glucose levels were determined using radioimmunoassay method (Immunotech, France) and colorimetric glucose oxidize procedure (Benfey & Biron 2000). The ions levels were assayed based on Braun, Lima, Baldisserotto, Dafre & Nuner (2010).

Statistical analysis

Statistical analysis was performed by one way ANOVA at 5% significant level. A multiple comparison test (Duncan multiple range test, DMRT) was conducted to compare the statistically significant differences among the groups using SPSS Version19. Values were presented as mean ± standard errors. To evaluate the differences between stress related analysed parameters at the same time between two experimented groups, paried T-test was utilized.

Results

At the end of the experiment (10 weeks), levels of serum ALP and AST did not change significantly among groups ($P>0.05$). LDH showed significant decrease in group of fish fed on 8% WB (256.25 ±40.30 UL^{-1}) compared to control group (605.5± 78.98 UL^{-1}) plus other groups treatments (Table 1; $P<0.05$).

The levels of ALT were in the range of 3.5-8.25 UL $^{-1}$ (Table 1). Inclusion of WB in the diet, higher than 2% could decline ALT level significantly (Table 1; $P<0.05$). Regarding the stress challenges, the fish treated by control diet had an initial cortisol level of 9.23 ng mL^{-1} which rose significantly to 27 ng mL^{-1} at 1 h and reduced to 16 ng mL^{-1} at 3 h and remained in a plateau state without any significant changes until 48 h (Fig. 1).

The fish fed on 8% WB had an initial cortisol level at 6.53 ng mL^{-1} which increased significantly to 18.66 ng mL^{-1} after 1h and then reduced to 12.2 ng mL^{-1} which were similar to basal level after 8 h (Fig. 1). Cortisol level showed very limited changes during 8-48 h post stress in 8WB group (Fig. 1). In general, the hormone levels were higher in fish fed control diet than that of 8 % WB (Fig. 1). However, only significant differences were observed 1 h and 8 h post-stress (Fig. 1; $P<0.05$). Glucose concentrations in fish fed control diet were 62.66 mg dL^{-1} which significantly rose up to 157 and 264 mg dL^{-1}at 1 h and 3 h, respectively (Fig. 2; $P<0.05$).

Table 1 Serum enzymes (UL) in common carp fed different levels of dietary wood betony extract for 10 weeks

Variable (U/L^{-1})*	Control (0)	2%	4%	8%
ALP	87.75 ± 6.68^{a}	81.75 ± 11.23^{a}	80.50 ± 6.68^{a}	78.50 ± 1.19^{a}
AST	178.00 ± 51.96^{a}	115.00 ± 35.12^{a}	114.50 ± 17.93^{a}	70.75 ± 11.98^{a}
LDH	605.50 ± 78.98^{a}	580.25 ± 106.08^{a}	557.00 ± 102.10^{a}	256.25 ± 40.30^{b}
ALT	8.25 ± 1.60^{a}	6.75 ± 0.75^{ab}	4.75 ± 0.85^{bc}	3.50 ± 0.28^{c}

Alkaline phosphatase, ALP; asparate transaminase, ASP; lactate dehydrogenase, LDH and alanine transaminase, ALT. Values are mean ± SEM. Mean values with different superscripts are significantly different from each other (P<0.05).

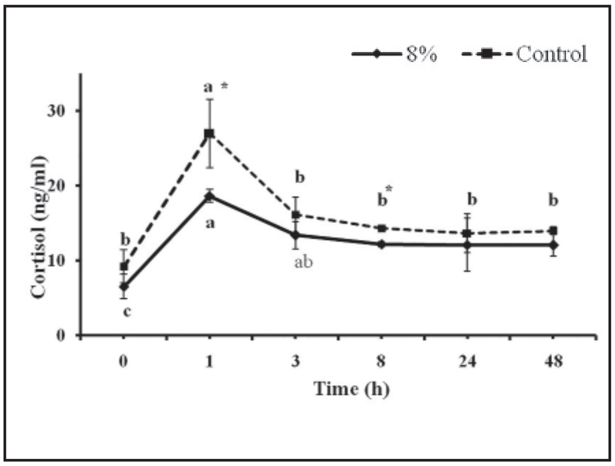

Figure 1 Serum cortisol (ng mL^{-1}) levels of common carp subjected to acute handling followed by 3 h of crowding stress in fish fed the control and 8% diets. Data are presented as mean ± standard error as error bars; Significant differences between different time of blood sampling in the same group (P<0.05; Duncan test) or between two groups (0 and 8% of WB) at the same time (P<0.05; t-test) are indicated by unlike letters, and *,respectively.

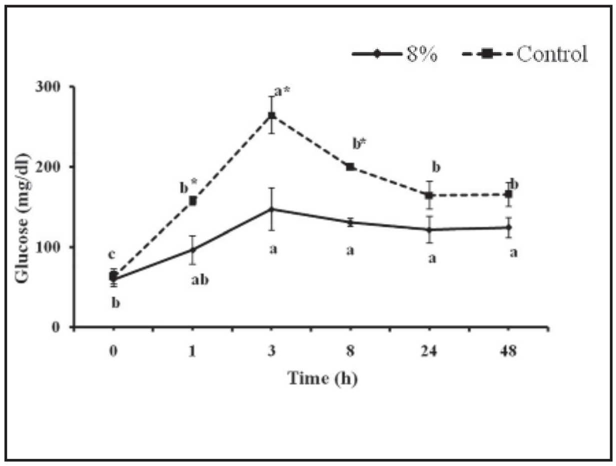

Figure 2 Serum glucose (mg dL^{-1}) levels of Common carp subjected to acute handling followed by 3 h of crowding stress in fish fed the control and 8% diets. Data are presented as mean ± standard error as error bars; Significant differences between different time of blood sampling in the same group (P < 0.05; Duncan test) or between two groups (0 and 8% of WB) at the same time (P < 0.05; t-test) are indicated by unlike letters and *, respectively.

As Figure 1 illustrates, the glucose level did not return to the basal level until 48 h post-stress (P<0.05). In fish treated with 8% WB, the initial glucose level was reported as 59.33 mg dL^{-1}, that is very similar to those which have been measured in control group (Fig. 1; P > 0.05). The glucose concentration was elevated in a very gradual route and reached to 96 and 146.66 mg dL^{-1} at 1 and 3 h post-stress, respectively (Fig. 1). Similar to those reported for control group, the glucose level for 8WB fish did not changed significantly during 3h to 48h (Fig. 1; P > 0.05). At all intervals, the glucose levels were higher in control group compared to 8 WB, but significant differences were only observed at 1, 3 and 8 h post-stress (Fig. 2; P < 0.05). At both treatments prior to stress induction, serum sodium concentrations were 156 mmol L^{-1} in fish. In control group, the sodium level dropped to the lowest level at 3 h, then elevated to initial level throughout the experiment (Fig. 3A).

In fish fed on 8% WB, the sodium level did not change significantly during the experiment (time 0 to 48h; Fig. 3A; P > 0.05). In all sampling times, the sodium levels in fish fed on 8% WB were higher than those of measured in control group (Fig. 3A), but these differences were insignificant (Fig. 3A; P>0.05). For control group, the potassium and calcium levels were in the range of 0.53-0.73 and 2.55-3 mmol/L, respectively and did not show any significant differences during the sampling time (Fig. 3B, C; P > 0.05). Similar to the control group, the concentration of potassium and calcium levels were too low, that is, in the range of 0.6-1 and 2.81-2.94 mmol/L, respectively. The concentrations of these ions did not change throughout the experiment (Fig. 3B, C; P > 0.05). At the most sampling time, the potassium and calcium levels for fish fed on 8WB were higher than those measured for control fish (Fig. 3B, C) without any significant differences

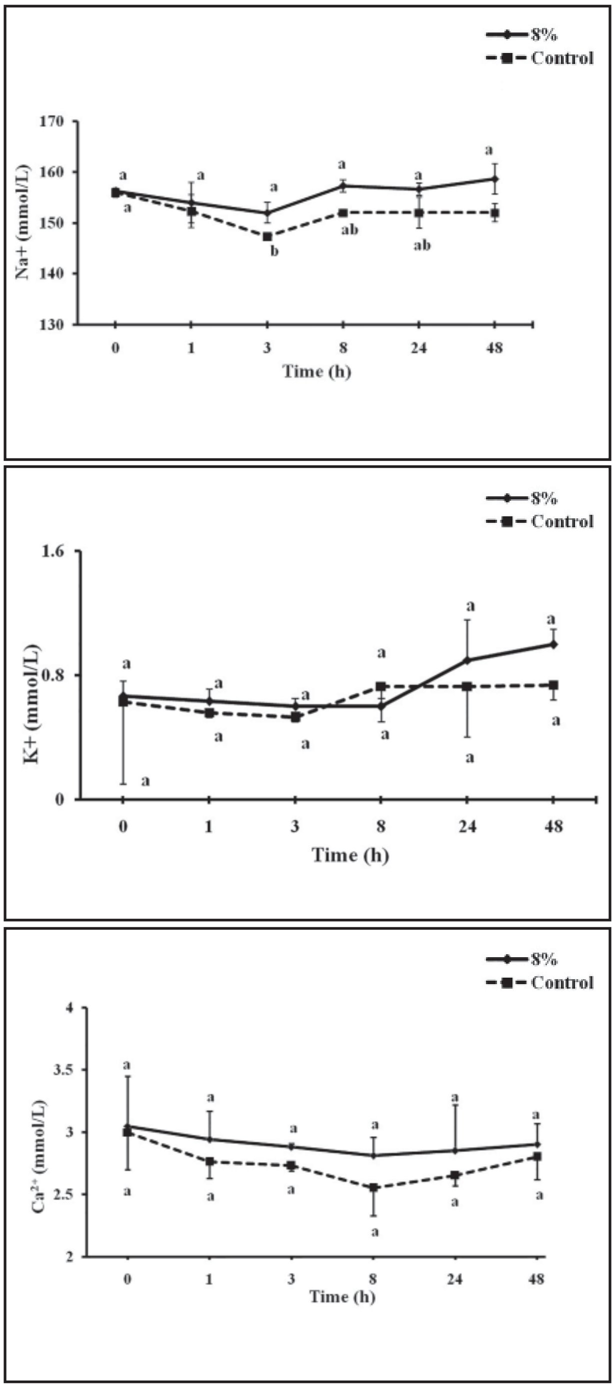

Figure 3 The effects of dietary wood betony extract on some ion concentrations Na+ (A), K+ (B) and Ca++ (C) in common carp exposed to acute handling followed by 3 h of crowding stress. Data are presented as mean ± standard error as error bars; Significant differences between different time of blood sampling in the same group ($P<0.05$; Duncan test) is indicated by unlike letters.

(Fig. 3B, C; $P > 0.05$).

Discussion

Many research works have been conducted on the use of different herbal medicines in aquaculture both in finfish (Chakraborty & Hancz 2011) and shellfish species (Citarasu 2010). Despite many studies have been investigated the effects of dietary phytochemical on growth or immunity parameters, less information is available about the use of these environmental friendly compounds such as anti-stress agent in aquaculture industry. Different stressors can influence hypothalamus- pituitary- internal axis of the aquatics, mainly fish like other vertebrates and impact on dynamic balance of organisms. In this study, at least the level of two serum enzymes including LDH and ALT were significantly reduced by inclusion of 8 % WB in the diet of carp. Also fish fed with 2% and 4% WB had a tendency of decrease in these enzymes. None significant decreasing pattern was also reported for ALP and AST by elevating the WB in the diet. Although mentioned enzymes have been obtained in different tissues such as heart, skeletal muscle and kidneys in vertebrates, liver usually considered as a main source of these enzymes. Also, the elevation of these enzymes in fish serum, in response to exogenous compounds such as food additive and heavy metals, may be considered as an indication for liver dysfunction (Talas & Gulhan 2009). The decline of some serum enzyme at least in groups received the highest dose of WB could explain potential benefit of wood betony for fish welfare by improving liver function. Recently, Yılmaz, Ergün & Çelik (2013) revealed the positive effects of dietary thyme (Thymus vulgaris), rosemary (*Rosmarinus officinalis*) and fenugreek (*Trigonella foenum graecum*) on some physiological characteristics of sea bass (*Dicentrarchus labrax*). The fish fed on these medicinal herbal extracts had better liver function, which has been evaluated as lower total liver fat, visceral fat index and hepatosomatic index (HSI). Some researchers (e.g., Cho, Lee, Park, Ji, Lee, Bae & Oh 2007; Zheng, Tan, Liu, Zhou, Xiang & Wang 2009) conducted experiments with channel catfish, *Ictalurus punctatus*, fed a basal diet containing Greek oregano, *Origanum heracleoticum* essential oil and olive flounder, *Paralichthys olivaceus*, fed diets supplemented with green tea, *Camellia sinensis*, respectively and showed significant improvement in the fish liver function. Furthermore, Srinivasan (2005) reported different active compounds of herbs stimulated digestion, accompanied by enhanced bile acid concentration, stimulated the pancreas, and increased the secretion of digestive enzyme activities in chicken broilers. It is possible that different antioxidant compounds available in the wood betony extract can influence liver function in fish. Frankič, Volǰc, Salobir & Rezar (2009) reported that antioxidants enhance the synthesis of bile acids in the liver; However, due to the limited scientific data about the effect of herbal biomedicine on liver enzyme activity (Xie *et al*. 2008), more research is needed to expand our knowledge concerning the physiological mechanisms of fish phenomenon.

It is well-documented that fish like other vertebrate try to cope stress by changing some biochemical compositions such as blood cortisol, glucose as well as different ions (Fevolden & Roed 1993; Barton 2002). Cortisol elevation usually considered as a first smart signal for fish under stress (Hsieh, Chen & Kuo 2003). The blood cortisol level of common carp was as low as 10-20 ng mL^{-1} before stress challenge, abruptly increased just 1 h after stress exposure, and the sharply decreased to basal level at 3 or 8 h. Regardless, the pattern of the hormone changes, the blood cortisol levels of fish fed on 8% WB apparently reported lower than the control group in the most time intervals. This indicates that WB can mitigate cortisol charge, which is motivated by handing stress. These findings are in conformity with the report of Xie *et al*. (2008) on common carp. Blood glucose elevations imply to stress usually considered as atypical secondary stress response (Barton & Iwama 1991). Usually, the major reason for blood glucose rise is dissimilation and dissolution of liver glycogen for providing energy during stress process, caused by elevation of different hormone level, especially in cortisol (Hsieh *et al*. 2003). The current study indicated that blood glucose levels increased significantly after stress and reached the highest concentration at 3 h; 2 h after highest level of blood cortisol level. Nonetheless, the augmentation of blood sugar in fish fed on 8% WB was much lower than control group indicating that WB could also assuage blood glucose load probably because of the hormone diminish. Significant reduction in serum glucose levels were also reported for Tilapia (*Oreochromis mossambicus*) fed on diet supplemented from four different medicinal plant (Immanuel *et al*. 2009) and common carp received diet containing rhubarb (*Rheum officinale*) extract (Xie *et al*. 2008). Ion concentrations could be used deliberately as a useful tool to evaluate secondary stress response in fish. It is under control of several neuroendocrine pathways in fish which some of them are similar to those affect stress responses. It is well understood that releasing cathecholamines and cortisol can modify ion regulations (McCormick 2001). In this study, the ion concentration level leaved unaffected in both control and 8WB all tested interval. However, the only exception was lower sodium concentration of the control fish at 3 h in comparison to time zero which may show some positive effects of dietary WB on sodium regulation in common carp. Nevertheless, the ion disturbance in fish, after stress exposure, has been influenced by several factors such as type of stress, stress intensity, duration, and fish species (Barton 2002; Tejpal, Pal, Sahu, Kumar, Muthappa, Vidya & Rajan 2009). Based on our knowledge, the effects of dietary phytochemical on ion regulatory capacity of fish are poorly understood (Immanuel *et al*. 2009) and further research is needed in order to find out the exact mechanisms.

In conclusion, the findings of this study indicated that a dietary addition of wood betony could improve some of the physiological conditions of common carp. Thus, this study provides a new perspective for the use of medicinal herbs, which can be added to fish feed literature. Wood betony containing a number of different active components, which possibly play an important role in improving organ function and stress responses. However, the mechanism by which this occurs needs further and more detailed research.

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اثر عصاره چای کوهی (*Stachys lavandulifolia*) بر برخی پارامترهای بیوشیمیایی سرم و پاسخ به استرس حاد در کپور معمولی (*Cyprinus carpio*)جوان

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چکیده

تاثیر چهار سطح مختلف عصاره چای کوهی (*Stachys lavandulifolia*) شامل ۰، ۲، ۴ و ۸ درصد در جیره بر سطح برخی آنزیم‌های سرمی‌و پاسـخ به اسـترس حاد در کپور معمولی جوان (*Cyprinus carpio*) مورد بررسی قرار گرفت. در پایان دوره غذادهی به مدت ۱۰ هفته، ۲ گروه (۰ و ۸ درصد عصاره چای کوهی) در معرض اسـترس دسـتکاری و تراکم قرار گرفتند و پاسخ‌های استرسی سرم شامل مقادیر کورتیزول، گلوکز و سطوح برخی الکترولیت‌ها (K^+، Na^+ و Ca^{2+}) ارزیابی شد. ماهیانی که با ۸ درصد عصاره چای کوهی تغذیه شده بودند به طور معنی‌داری مقادیر کمتری از لاکتات دهیدروژناز (LDH) و آلانین ترانسمیناز (ALT) را در مقایسه با گروه شاهد نشان دادند ($P<0/05$). در هر دو گروه آزمایشی، میزان کورتیزول و گلوکز سرم مدت اندکی پس از استرس افزایش یافت ($P<0/05$)، با این وجود ماهیان تغذیه شده با جیره حاوی ۸ درصد عصاره چای کوهی، مقادیر کمتری از این دو ترکیب را در مقایسه با گروه شاهد نشان دادند . ($P<0/05$) در گروه شاهد میزان سدیم ۳ ساعت پس از استرس کاهش یافت ($P<0/05$) در حالی که تغییر معنی‌داری در میزان سدیم سرم ماهیان تغذیه شده با عصاره چای کوهی مشاهده نشد ($P>0/05$). در هیچ یک از گروهای آزمایشی، پتاسیم و کلسیم سرم تحت تاثیر استرس تغییر نکرد ($P>0/05$). با توجه به نتایج به نظر می‌رسد که عصاره چای کوهی می‌تواند تا حدودی منجر به بهبود عملکرد کبد و پاسخ به استرس در ماهیان جوان کپور معمولی شود.

واژه‌های کلیدی: استرس، فعالیت آنزیمی، تنظیم یونی، چای کوهی، کپور معمولی.

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