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Effects of *Rheum palmatum* L. Root Extract on the Blood Parameters in Responses to Two High Heat Stress and Lipid Oxidation of *Rutilus frisii kutum*

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Abstract: It is well known that certain blood parameters and cortisol serve as reliable indicators of fish health. So, in the present investigation we were focused on hematological parameters and cortisol as indicator to evalute the effect of extract from *Rheum palmatum* on health and resistance of Kutum (average initial body weight of 6.74) to high temperature stress. Also, we used thiobarbituric acid (TBA) analysis to the measurement of *Rheum palmatum* extract effect's on lipid oxidation. Kutum fingelings were divided randomly into four groups: a control group on basal diet, three treatment groups fed a basal diet supplemented with 0.5%, 1% and 2% *R. palmatum* extract, respectively. After 60 days feeding, blood samples were obtained in two stages (before and after stress) for Complete blood count test and evaluate cortisol. To create stress, water temperature was set up at to levels: 31°C and 26°C. Also, after feeding period, samples of each treatment were kept in-20°C for 1 month and TBA values were evaluated. Eventually, we were concluded that 0.5-2% extract form *Rheum palmatum* increase immune capability and health of kutums.

Key words: Blood Parameters · *Rheum palmatum* L. Extracts · Temperature Stress · Lipid Oxidation

INTRODUCTION

Kutum (*Rutilus frisii kutum*) is one of the most important and economical fish in the south shores of the Caspian Sea. Unfortunately, during previous years, its resources have been declined because of various reasons such as over exploitation of brood stocks, changes of rivers, decrease of water flow of rivers, pollution increase, gravel and sand removals in rivers which are all caused the decrease of natural breeding of fishes. Also, Kutums during culture and transportation face with kinds of stressors that can affect its growth and its resistance to the types of deceases.

The stress response in teleosts has been receiving a great deal of attention because of the perceived impact of stress on the welfare and productivity in aquaculture systems. The response to stress in fish is characterized by the stimulation of the hypothalamus, which results in the activation of the neuroendocrine system and subsequently metabolic and physiological changes [1]. Under conditions of stress, the body of fish emits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNC) and the release of the stress hormones, cortisol and catecholamines into the bloodstream by the endocrine system. According to Martinez-Porchas *et al*. [2], Secondary responses occur as a consequence of released stress that causing changes in blood and tissue chemistry. Cortisol is the principal corticosteriod in teleost fishes and its concentrations in blood rise dramatically during stress [3]. On the other hand, The hematological parameters of fish can be used as indicators of physiological conditions and monitoring diseases and the stress caused by handling [4, 5], pollutants, metals, hypoxia, etc [6-8]. The study of blood parameters in fishes has been widely used for detection on physiopathological alterations in different conditions of stress [9]. Also, cortisol is the principal corticosteriod in teleost fishes and its concentrations in blood rise dramatically during stress [3].

Rhubarb is commonly used worldwide herb and it is officially stipulated in Chinese pharmacopoeia. In Chinese pharmacopoeia, the official rhubarb is prescribed as the dried rhizome and root of *Rheum palmatum* L., *Rheum tanguticum* Maxim. Ex Balf. and *Rheum officinale* Bail. from the family of polygonaceae [10]. The anthraquinone extracts of *R. palmatum* have many

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pharmacological activities such as purgative [11], anti inflammatory [12], anticancer [13, 14], nephric protection [15, 16], liver protection [17], antimicrobial and hemostasis [10]. The roots and stems of Rhubarb are rich in anthraquinones, such as emodin and rhein. These substances are cathartic and laxative, which explains the sporadic abuse of Rhubarb as a slimming agent. Its astringent qualities making Rhubarb an excellent agent for improving the tone and health of the digestive tract; on the other hand, its laxative effects make it a valuable aid in the treatment of chronic constipation, hemorrhoids and gastroenteritis [13]. In this study, according to the two recently works that show the effect of another species of rhubarb, *R. officeinale*, on promote the capability of common carp *Cyprinus carpio* and freshwater prawn to resist stress and reduce the pathogenic infection [18, 19] and to anthraquinone components of *R. palmatum*, that is be exist in *R. officeinale* too and also to pharmacological activities of anthraquinone extract from *R. palmatume* that described, so we selected *R. palmatume* and hypothesized that it can have positive effects on some blood parameters of Kutums in responses to the two high heat stress and lipid oxidation of this fish. As, no studies have investigated on effects of this specie on fish.

MATERIALS AND METHODS

Experimental Fish and Diets: Kutums (*Rutilus frisii kutum*) were selected from Voshmgir Dam (one of breeding centers) in Iran. They were allocated to 12 aquaria (70 × 30 × 20 cm) and acclimatized for 30 days. Then kutums, an average initial body weight of 6.74, randomly were divided into four groups i.e., a control group on a basal diet and three treated groups fed a basal diet supplemented with 0.5%, 1.0% and 2.0% of *Rheum palmatum* extracts, respectively.

Preparation of Herbal Extract: The dry roots of *R. palmatum* were obtained from local market in Iran and were identified by Doctor Mazandarani, a botanist at Azad Universitiy of Gorgan and Doctor Amin, a botanist at Tehran University and then they were ground to powder. Then, Rhubarb powder was extracted in Soxhlet extractor. According to Soxhlet extraction method, 25 g of the dried ground roots in 300 ml methanol for 8 h followed by removal of the solvent on rotary evaporator (Type: HB 4B-German) and oven (below 40°C) gave a yellow solid material [13].

Rearing Management: Kutums were acclimatized in aquaria (15 fish in each aquarium) for 30 days and they were fed by trial diet with the feeding amount about 2-4.5% body weight (BW). Feeding was conducted twice a day, 0ne at 8:00-9:00 in the morning, the other at 16:00-17:00 in the afternoon. The water in each aquarium was exchanged twice a week; a tow-third volume was exchanged each time as the water quality. The water quality in the experiment was as follows: average water temperature 20±2°C, DO□5 mg/l, pH 6.5-7.5. The amount of feeding was adjusted according to BW measurements every 15 days. After 60 days rearing experiment, feeding was suspended 48 h prior to testing and fish were not fed during the experiments. Blood samples were collected before and after stress.

Stress Experiment: Water temperature was set up at to level: 26°C and 31°C. 12 aquaria were selected for each temperature and during one week temperatures were set up. After feeding period and 48 h suspended feeding, 8 fish of each replication randomly were exposed to the temperature stress. After 24 hour exposure to temperature stress three blood samples were taken from each replication.

Blood Sample Collection and Analysis: Fish were subjected to the anesthesia material (clove flower), but as the results of anesthetics effects on fish were contrast and there was a few studies on effect of clove flower on *Rutilus frisii kutum* so to avoided of probably affecting this material on blood parameters we planned a medium status and we didn't allow to fish that completely were anesthetized by clove flower and used of the results of the publication by Imanpour *et al.* [20]. Three blood samples from each replication (9 samples from each treatment) were obtained using heparinized microhematocrit tubes from caudal artery. Then, blood samples immediately (less than 1 h) were transferred to the Landa lab and total leukocytes (WBCs), erythrocytes (RBCs), hematocrit (Hct), hemoglobin (Hb), mean cell hemoglobin concentration (MCHC) and mean cell volume (MCV) were counted in this lab.

For cortisol determination, plasma was separated by centrifugation (1000 rpm for 10 min), removed, a liquated into 1.5ml Eppendorf tubes and frozen at-20°C until analysis of cortisol [21]. Then plasma was thawed (allowed to reach ambient temperature) and cortisol was measurement with ELISA (modle Plate Screen-Italy) according to description of Monobind Cortisol EAI kit (Product code: 3625-300).

Sample Preparation and Lipid Peroxidation Assay: After feeding period and bleeding, fish were beheaded, eviscerated [22] and filleted by using common household methods in medium weight of 16.91±0.24 (g). Then, samples of each treatment were kept in-20°C for 1 month. After that, lipid hydrolysis was determined in the lipid extract by the Egan *et al.* [23] method. The basic principle of the method is the reaction of one molecule of malonaldehyde and two molecules of TBA to form a red malonaldehyde-TBA complex, which can be quantitated spectrophotometrically (538 nm).

Data Statistics and Analysis: We used SPSS (version 16.0) software Duncan's multiple range tests to determine the differences between groups. All the results were expressed as means ± standard error (SE.).

RESULTS

Before stress, the red blood cell was higher in groups supplemented with 0.5% and 1% extracts compared with control group (Fig. 1A). After 26°C high temperature stress, tendency to increase in the number of red blood

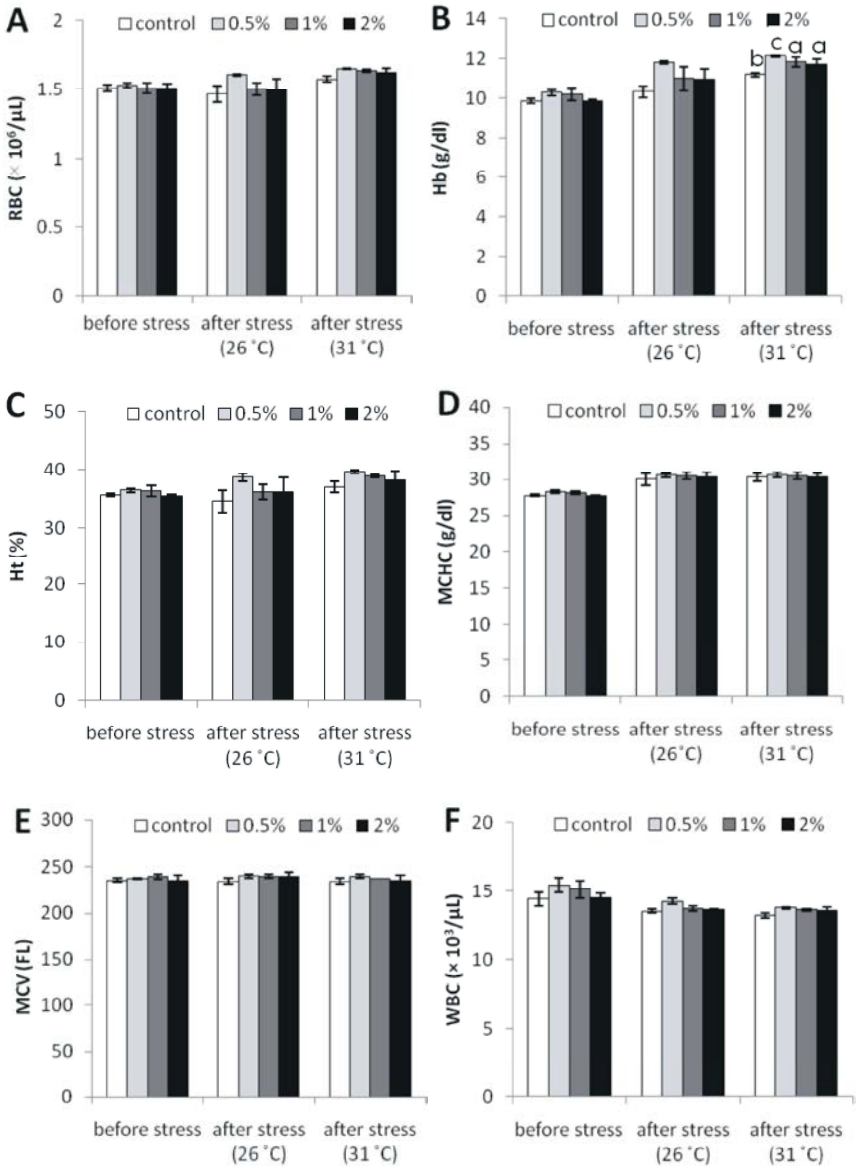

Fig. 1: Red blood cells count (RBCs), hematocrit (Ht), hemoglobin concentration (Hb), Mean Cell Volume (MCV) and Mean Cell Hemoglobin Concentration (MCHC) of *Rutilus frisii kutum* fed with experimental diets for 60 days. Bars indicate mean±SE. Means with different letters indicate significant differences (p□ 0.05)

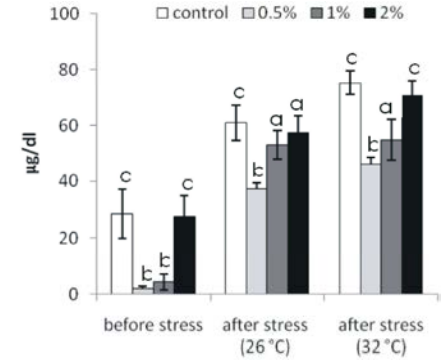

Fig. 2: Blood cortisol level of *Rutilus frisii kutum* fed with experimental diets for 60 days. Legends are the same as in Fig. 1

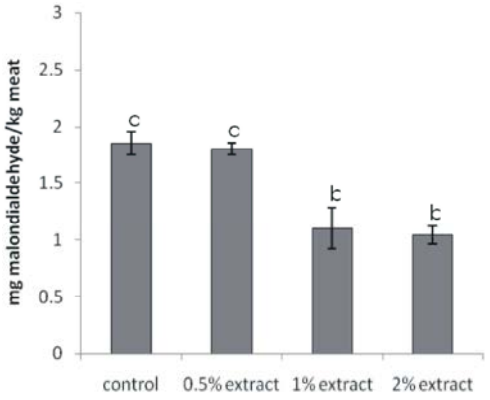

Fig. 3: Malondialdehyde contents of *Rutilus frisii kutum* kept in-20°C for 1 month after feeding experimental diets for 60 days. Bars indicate mean±SE. Means with different letters indicate significant differences (p□0.05)

cells was higher in treated groups though this increasing was not significant (Fig. 1A). After 31°C high temperature stress, the red blood cells had similar changes, but it had higher values (Fig. 1A).

Before stress, the hemoglobin level of blood was higher in groups supplemented with 0.5% and 1% extracts compared with control (Fig. 1B). After 26°C high temperature stress, the hemoglobin level was higher in treated groups compared with control group, but it was not significant (Fig. 1B). After 31°C high temperature stress, the hemoglobin level increased in all groups. Although, the highest level was found in treated fish with 0.5% *R. palmatum* extract compared with control but there was no significant differences among the groups (Fig. 1B).

The hematocrit of the tested groups supplemented with 0.5% and 1% extract had an increasing trend compared with control, but this increasing was not significant (Fig. 1C). After both 26°C and 31°C high temperature stress, the hematocrit value of the test groups supplemented with extract was higher compared with control but it was not significant (Fig. 1C).

The MCHC value did not show significant difference in all groups before and after stress although it had the highest values in group supplemented with 0.5% and 1% extract from *R. palmatum* (Fig. 1D).

Before and after stress no differences were found in MCV of all groups (Fig. 1E).

Before stress, no significant differences were found between groups in WBCs of Kutums, although The WBCs levels were higher in supplemented groups compared with control (Fig. 1F). After stress 26°C high temperature stress, the level of WBCs reduced in all groups although this reducing was lower in treatment. The changes in WBCs after 31°C high temperature stress were similar to its changes after 26°C high temperature stress (Fig. 1F).

Before stress, the blood cortisol levels had significant increase in control and group that supplemented with 2% extract, but its levels in groups supplemented with 0.5% and 1% extract from *R. palmatum* were low (P□0.05) (Fig. 2). After 26°C high temperature stress, the blood cortisol level had tendency to increase in all groups. Although this increase was lower in treated groups, especially groups supplemented with 0.5% and 1% extract, but no significant difference was found among the groups (Fig. 2). After 31°C high temperature stress, the blood cortisol levels significantly increased in control group and group supplemented with 2 % *R. palmatum* extract but its levels in group fed with 0.5% and 1% *R.palmatum* were low (P□0.05) (Fig. 2).

TBA values showed significant decrease in groups fed with 1% and 2% *R. palmatum* extract after one month frozen storage at-20°C (P□0.05) (Fig. 3).

DISCUSSION

Fish are frequently exposed to stressors under culture conditions, which cause a series of physiological responses, known as stress, which are divided in primary, secondary and tertiary responses [24, 25]. Some secondary effects of hormones, such as hyperglycemia, increase of total protein, hematological and plasma ions changes are important parameters to assess the fish health conditions [26]. The hematocrit percentage, hemoglobin rate and erythrocyte count are good indicators for oxygen transportation capacity of fish thus making it possible to establish relationships with the oxygen concentration available and the health status of

these fish [27]. On the other hand, the white blood cells afford protection against infectious agent caused by microbial and chemical factors [28]. In this study, we could see effects of *R. palmatum* extracts on hematological parameters. Although, we didn't found significant differences between groups in some parameters for instance in red blood cells but we could see a tendency to increase in some treated groups that could be indices to fish health and it ability to contrast with stressor factors (Fig. 1A). The hematocrit, hemeglobin and MCHC values showed affected with the red blood cells values. In this research we could see that after temperature stress (26°C and 31°C) compared with before stress, the red blood cells, hematocrit, hemoglobin and MCHC increased in all groups. On the other hand, after stress creation, we found that this increasing trend in groups supplemented with *R. palmatum* extract is higher compared with control group. The differences between groups in hemoglobin level were clearer than other parameters as Fig. 1B confirmed this statement. Although the differences between groups in these parameters were not significant, but these little variations probably can be an index for fish health and it ability to contrast with stressor factors as in this study after cortisol evaluation, we could find out those groups had higher values of the red blood cells, hematocrit, hemoglobin and MCHC, groups supplemented with 0.5% and 1% extract, had lower values of cortisol.

After high temperature stress, the level of WBC reduced in all groups that were relevant to a few previous works had done on fish [29, 30], although this reducing was lower in treated groups. There are no previous studies on effects of *R. palmatum* on leukocytes in fish but this study and a few study with the other species of polygonaceae family [31, 32] may be a reason for the effect of *R. palmatum* extracts on leukocytes in *Rutilus frisii kutum* that can induce a protection against diseases and improve the health mechanisms in stress conditions. However in this specific high temperature stress, leukocytes were not significant but increasing trend in the number of leukocyte show that more researches are necessary on effects of *R. palmatum* on leukocytes.

The primary targets of cortisol action are the gills, intestine and liver, which reflect the two main adaptive functions of cortisol identified to date: osmoregulation and the maintenance of a balanced energy metabolism [33]. Although, cortisol appears to play several roles in the stress response including energy mobilization, stimulation of ionoregulatory processes and facilitate ion of oxygen up take under hypoxic conditions but prolonged cortisol elevation can also have severe, debilitating, consequences for disease resistance, growth and reproduction. For example, the rising cortisol level can cause reducing in the number of lymphocyte and white blood cells [34] and damaging immune organs such as spleen, thymus [35]. Different feeding regimens may alter the cortisol response to stressors [36, 37]. Fig. 2 indicated that *rheum palmatum* extract could affect blood cortisol level of *Rutilus frisii kutum.* Results showed that 0.5% and 1% extract of *R. palmatum* had significant effect on cortisol level and reduced its level before stress. Also, after 31°C high temperature stress, 0.5% extract had significant effect on reducing blood cortisol level.

Oxidative Stress Is Defined: An imbalance between oxidants, reactive oxygen species (ROS) and antioxidants in favour of the oxidants, potentially leading to damage [38]. ROS are produced naturally during metabolism and experimental evidence shows that organisms often use these radicals for advantageous biological effects [39], though they can also cause molecular damage. Oxidation of polyunsaturated lipid materials caused to produce the 3-carbon compound malonaldehyde (MDA) that is a major carbonyl decomposition product [40]. As Tokur *et al.* [41] expressed Spectrophotometric detection of the malonaldehyde-thiobarbutiric acid (TBA) complex has been widely used for measuring lipid oxidation in food and biological tissues. In our research Fig. 3 shows that 1% and 2% of *R. palmatum* extract supplemented to basal diet can have significant effect on TBA values and reduce lipid oxidation.

So, according to the results of cortisol and TBA content, we found that *R. palmatum* extract can have positive effects on immune system of kutums. Although, this is first research on effects of *R. palmatum* on blood parameters and TBA values kutums and data's about mechanism of *R. palmatum* act on immune system is low but it is justifiable. Effects of *R. palmatum* extract on cortisol level could occur because of its anthraquinone derivatives. As, late research on *common carp* by Xie *et al.* [18] showed that anthraquinone extract from *R.officinale* Bail reduced cortisol level of this fish after crowding stress. On the other hand, the effect of extracts from *R. palmatum* on immune system can carry out by the effect on lysozyme activity. This probable effect of *R. palmatum* can occur from its anthraquines compounds according to Xie *et al*. [18] and Liu *et al.* [19] findings that

showed the effects of anthraquinone extract on increasing of lysozyme activity. Lysozyme is one of several anti-microbial proteins associated with front line, innate immunity in all vertebrates [42]. Lysozyme available in the lysosomes of neutrophils and macrophages and secreted into the blood by these cells [43-46]. The decrease of lysozyme activity due to handling and transport persisted for 24 h, but the activity returned to normal within 2 weeks following confrontation with the stressor [46]. Moreover, the review article by Halliwell *et al*. [47] expressed many work with plant established that a compounds derived from plants, especially phenols such as quercetin, carnosol, thymol, carnosic acid, hydroxytyrosol, gallic acid derivatives, tannins, catechins, rutin, morin, ellagic acid, eugenol and rosemarinic acids, are of considerable interest from the view point of dietary antioxidant supplementation and food preservation. As, this study showed the effect of *R. palmatum* on TBA value and could confirm with Xie *et al.* [18] publication that reported that anthraquinone extracts from *R. officinale* could promote antioxidant enzymes system, superoxidsse dismutase and hepatic catalase and reduce oxidation, similar to the role of Garlic on antioxidant system in Tilapia nilotica (*Oreochromis niloticus*) [48].

CONCLUSION

According to cortisol level, we concluded that 0.5% *Rheum palmatum* extract could have a greatest effect in resistance to high temperature stress. On the other hand, according to TBA values, we found that 1% and 2% *Rheum palmatum* extract improve anti-oxidation capability of Kutums. So we could conclude that 0.5-2% extract form *Rheum palmatum* increased immune capability and health of kutums.

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REFERENCES

1. Lowe, C.J. and W. Davison, 2005. Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic nototheniid fishes to acute and chornic thermal change. J. Fish Biol., 67: 752-766.
2. Martinez-Porchas, M., L. Martinez-Cordova and R. Ramos-Enriquez, 2009. Cortisol and Glucose: Reliable indicators of fish stress? Pan-American J. Aquatic Sci., 4(2): 158-178.
3. Mommsen, T.P., M.M. Vijayan and T.W. Moon, 1999. Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. Reviews in Fish Biology and Fisheries 9: 211-268.
4. Sebastiao, F.A., D. Nomura, R. Sakabe and F. Pilarski, 2011. Hematology and productive performance of Nile Tilapia, *Oreochromis niloticus*, naturally infected with Flavobacterium columnare. Brazilian J. Microbiol., 42: 282-289.
5. Gbore, F.A., O. Oginni, A.M. Adewole and J.O. Aladetan, 2006. The effect of transportation and handling stress on haematology and plasma biochemistry in fingerlings of *Clarias gariepinus* and *Tilapia zillii*. World J. Agric. Sci., 2(2): 208-212.
6. Savari, A., A. Hedayati, A. Safahieh and A. Movahedinia, 2011. Characterization of blood cells and hematological parameters of yellowfin Sea Bream, *Acanthopagrus latus*, in some creeks of Persian Gulf. World J. Zool., 6(1): 26-32.
7. Blaxhall, P.C., 1972. The hematological assessment of the health of freshwater fish. J. Fish Biol., 4: 593-605.
8. Duthie, G.C. and L. Tort, 1985. Effects of dorsal aortic cannulation on the respiration and haematology of the Meediterranean dog-fish, *Scyliorhinus canicula* L. Comparative Biochemistry and Physiol., 81A: 879-883.
9. Nussey, G., J.H.J. Van Vuren and H.H. Preez, 1995. Effect of copper on the haematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus*. Comp, Biochem, Physiol., 111C: 369-380.
10. Wang, J., W. Kong, H. Wang, H. Zhao, H. Xiao, C. Dai, X. Xiao, Y. Zhao, C. Jin, L. Zhang, F. Fang and R. Li, 2010. Toxic effects caused by rhubarb, *Rheum palmatum* L., are reversed on immature and aged rats. J. Ethnopharmacol., 134: 216-220.
11. Huang, K.C., 1993. The pharmacology of Chinese Herbs. CRC Press, Boca Raton, FL, pp: 233-234.
12. Ding, Y., L. Zhao, H. Mei, S.L. Zhang, Z.H. Huang, Y.Y. Duan and P. Ye, 2008. Exploration of emodin to treat alpha-naphthylisothiocyanate-induced cholestatic hepatitis via anti inflammatory pathway. European J. Pharmacol., 590: 377-386.
13. Ashnagar, A., N. Gharib naseri and H. Haidari nasab, 2007. Isolation and Identification of Abthralin from the Roots of Rhubarb Plant, *Rheum palmatum*. J. Chemistry, 4: 546-549.

14. Cai, J., A. Razzak, J. Hering, A. Saed, T.A. Babcock, S. Helton and N.J. Espat, 2008. Feasibility evaluation of emodin (rhubarb extract) as an inhibitor of pancreatic cancer cell proliferation *in vitro*. J. Parenteral and Entereral Nutrition, 32: 190-196.
15. Yokozawa, T., N. Suzuki, I. Okuda, H. Oura and I. Nishioka, 1985. Changes in the urinary constituents in rats with chronic renal failure during oral administration of rhubarb extract. Chemical and Pharmaceutical Bulletin, 33: 4508-4514.
16. Wang, J., Y. Zhao, X. Xiao, H. Li, H. Zhao, P. Zhang and C. Jin, 2009. Assessment of the renal protection and hepatotoxicity of rhubarb extract in rats. J. Ethnopharmacol., 124: 18-25.
17. Zhao, Y.L., J.B. Wang, G.D. Zhou, L.M. Shan and X.H. Xiao, 2009. Investigations of free anthraquinones from rhubarb against naphthylisothiocyanate-induced cholestatic liver injury in rats. Basic and Clinical Pharmacology and Toxicol., 104: 463-469.
18. Xie, J., B. Liu, Q. Zhou, Y. Su, Y. He, L. Pan, X. Ge and P. Xu, 2008. Effects of anthraquinone extract from rhubarb, Rheum officinale, on the crowding stress response and growth of common carp, Cyprinus carpio var. Jian. Aquaculture, 281: 5-11.
19. Liu, B., J. Xie, X. Ge, P. Xu, A. Wang, Y. He, Q. Zhou, L. Pan and R. Chen, 2010. Effects of anthraquinone extract from rhubarb, *Rheum officinale* Bail, on growth performance and physiological responses of *Macrobrachium rosenbergii* under high temperature stress. Fish and Shellfish Immunol., 29: 49-57.
20. Imanpoor, M., T. Bagheri and S.A.A. Hedayati, 2010. The Anesthetic effects of clove essence in Persian Sturgeon, *Acipenser persicus*. World J. Fish and Marine Sci., 2: 29-36.
21. Imanpoor, M., T. Bagheri and A. Azimi, 2010. Serum Biochemical Change Induced by Soybean Meal in Diet on Persian Sturgeon, *Acipenser persicus*. Global Veterinaria, 5: 61-64.
22. Gamal, F.M., E.M. Hegazy and M. Abdellatef, 2011. Physicochemical properties and Mycotoxins contents of Tilapia fish fillets after solar drying and storage. Global Veterinaria, 7(2): 138-148.
23. Egan, H., R. Kirk and R. Sawyer, 1997. Pearson's Chemical Analysis of Foods, 9th ed. pp: 609-634. Edinburgh, Scotland, UK: Churchill Livingstone.
24. Wedemeyer, G.A., 1996. Physiology of fish in intensive culture systems. Chapman and Hall, New York, pp: 232.
25. Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol., 42: 517-525.
26. Gusmao Affonso, E., E. Da Costa Silva, M. Tavares-Dias, G. Cruz de Menezes, C. Suely Melo de Crvalho, E. Da Silva Santiago Nunes, D. Rebelo Ituassu, R. Roubach, E. Akifumi Ono, J. Daniel Indrusiak Fim and J. Luiz Marcon, 2007. Effect of high levels of dietary vitamin C on the blood responses of matrinxa, *Brycon amazonicus*. Comparative Biochemistry and Physiol., 147A: 383-388.
27. Lamas, J., Y. Santos, D.W. Bruno, A.E. Toranzo and R. Anadon, 1994. Non-specific cellular responses of rainbow trout to *Vibrio anguillarium* and its extracellular products (ECPs). J. Fish. Biol., 45(5): 839-854.
28. Harikrishnan, R., M. Nisha Rani and C. Balasundaram, 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture, 221: 41-50.
29. Langston, A.L., R. Hoare, M. Stefansson, R. Fitzgerald, H. Wergeland and M. Mulcahy, 2002. The effect of temperature on non-specific defense parameters of three strains of juvenile Atlantic halibut, *Hippoglossus hippoglossus* L., Fish and Shellfish Immunol., 12: 61-76.
30. Verma, A.K., A.K. Pal, S.M. Manush, T. Das, R.S. Dalvi, P.P. Chandrachoodan, P.M. Ravi and S.K. Apte, 2007. Persistent sub-lethal chlorine exposure augments temperature induced immunesuppression in *Cyprinus carpio* advanced fingerlings. Fish and Shellfish Immunol., 22: 547-555.
31. Chansue, N., A. Ponpornpisit, M. Endo, M. Sakai and Y. Satoshi, 2000. Improved immunity of tilapia, *Oreochromis niloticus*, by C-UP III, an herb medicine. J. Fish Pathol., 35: 89-90.
32. Chen, X., Z. Wu, J. Yin and L. Li, 2003. Effects of four species of herbs on immune function of *Carassius auratus* gibelio. J. Fish Sci., 10: 36-40.
33. Wendelaar-Bonga, S.E., 1997. The stress response in fish. Physiological Review, 77: 591-625.
34. Pickering, A.D. and T.G. Pottinger, 1987. Crowding causes prolonged leucopenia in salmon fish despite interregnal acclimation. J. Fish Biol., 32: 701-712.
35. Demers, N.E. and C.J. Bayne, 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Developmental and Comparative Immunol., 21: 363-373.

36. Van Anholt, R.D., F.A.T. Spanings, W.M. Koven, O. Nixon and S.E. Wendelaar-Bonga, 2004. Arachidonic acid reduces the stress response of gilthead sea bream. J. Experimental Biology, 207: 3419-3430.
37. Belo, M.A.A., S.H.C. Schalch, F.R. Moraes, V.E. Soares, A.M.M.B. Otoboni and J.E.R. Moraes, 2005. Effect of dietary supplementation with vitamin E and stocking density on macrophage recruitment and giant cell formation in the teleosts fish, *Piaractus mesopotamicus*. J. Comparative Pathol., 133: 146-154.
38. Sies, H., 1997. Oxidative stress: oxidants and antioxidants. Experimental Physiol., 82: 291-295.
39. Droge, W., 2002. Free radicals in the physiological control of cell function. Physiological Reviews, 82: 47-95.
40. Crawford, D.L., T.C. Yu and R.O. Sinnhuber, 1966. Reaction mechanism and reaction of malonaldehyde with glycine. J. Agriculture and Food Chemistry, 14: 184.
41. Tokur, B., K. Korkmaz and D. Ayas, 2006. Comparison of Two Thiobarbituric Acid (TBA) method for monitoring lipid oxidation in fish. J. Fisheries and Aquatic Sci., 23: 331-334.
42. Marsh, M.B. and C.D. Rice, 2010. Development, characterization and technical applications of a fish lysozyme-specific monoclonal antibody (mAb M24-2). Comparative Immunology, Microbiology and Infectious Diseases, 33: e15-e23.
43. Goldstein, I.M., S.T. Hoffstein and G. Weissmann, 1975. Mechanisms of lysosomal enzyme release from human polymorphonuclear leucocytes. J. Cell Biol., 66: 647-652.
44. Murray, C.K. and T.C. Fletcher, 1976. The immunohistochemical localization of lysozyme in plaice, *Pleuronectes platessa* Tissue. J. Fish Biol., 9: 329-334.
45. Gallin, J.I., 1982. Role of neutrophil lysosomal granules in the evolution of the inflammatory response. In phagocytosis past and future (M.L. Karnovsky and L. Bolis, eds). pp: 519-541.
46. Mock, A. and G. Peters, 1990. Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling transport and water pollution. J. Fish Biol., 37: 873-885.
47. Halliwell, B., J. Aeschbacht, J. Loliger and O.I. Aruoma, 1995. The Characterization of Antioxidants. Food Chemistry and Toxicol., 33: 601-617.
48. Metwally, M.M.A., 2009. Effects of Garlic, *Allium sativum*, on some antioxidant activities in Tilapia nilotica, *Oreochromis niloticus*, World J. fish and Marine Sci., 1(1): 56-64.