Effects of various pollen concentrations on some biochemical and hematological parameters and paraoxanase activity in Rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Bee-collected pollen is reported as a health food with a vast range of nutritional and therapeutic effects. Biochemical (glucose, total protein, creatinine, urea, triglycerides, total cholesterol, ALT, AST, LDH, ALP, chloride, sodium, potassium and total antioxidant status) and hematological (total leucocyte count, erytrocyte count, hemoglobin, hematocrit, MCV MCH, MCHC) parameters in blood of rainbow trout (*Oncorhynchus mykiss*) treated to various pollen concentrations (0.5, 2.5, 5, 10, 20 and 30 ppm) for 96 h were determined. The results of this study demonstrated that the levels of total protein, creatinine, chloride and sodium in blood of fish which treated to different concentrations of pollen were not changed (P>0.05) compared with control group. In this study, the highest total antioxidant capacity of pollen was determined at 10 ppm concentration, but serum paraoxanase (PON) activities were not observed. It is concluded that concentration-dependent effects of pollen on blood of fish can be favorable in prevention of diseases.

Keywords: Pollen, *Oncorhynchus mykiss*, Paraoxanase, Total Antioxidant Status, Blood

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Introduction

In recent years, there has been a considerable rising in scientific researches about natural antioxidant agents and their potential protective effects (Nabavi et al., 2013). Pollen is one of these natural antioxidant agents. Bee pollens are the male generative cells gathered by honeybees from flower stamens. It provides nutrition through the remarkable quantities of proteins, sterols, fatty acids, vitamins, carbohydrates, lipids, vitamins, ashes, and minerals, phenolic compounds and flavonoids as protective agents (Le Blanc et al., 2009; Xu et al., 2009). Phenolic compounds such as phenolic acid, flavonoids and tannins are though to be an important subscribe to the antioxidant capacities of foods (Nabavi et al., 2012). Honeybee-collected pollen is an apicultural product which is composed of nutritionally valuable substances and contains considerable amounts of polyphenolic compounds, mainly flavonoids, which may act as potent antioxidants (Marghitas et al., 2009). Organisms have endogenous antoxidants as well as exogenous taken.

Paraoxanase (PON) is an endogenous antioxidant with protective effects. The PON multigene family as a basis has 3 members, which of PON1, PON2 and PON3 whose genes are located adjacent to each other on chromosome 7q21-22 in human and 6 in mice (Marsillach et al., 2008). Birds, fish and insects don't have gene of PON1. So, they can easily poisoned by insecticides (Folly et al., 2001). The physiological roles of these enzymes are still uncertain. Oxidatively modified lipoproteins, especially low-density lipoproteins (LDL) are consequences of the effects of reactive oxygen species (ROS). It is well known that high-density lipoprotein (HDL) particles exert as a part of their antiatherogenic effects by counteracting the oxidation of LDL particles (Deakin et al., 2011). Antioxidants, which containing components as rich of flavonoids may cause increases in PON activity by 20% in serum (Mackness et al., 1996). Because of the number of different antioxidants in plasma, serum or other biological samples to measure separately the each antioxidant is difficult. Therefore, since antioxidant components into plasma are additive, the measurement of total antioxidant status (TAS) can only reflect the antioxidative status of plasma (Erel, 2004). Fish are commonly used to estimate the influences of environmental factors due to the sensitivity of their biochemical and hematological parameters under certain conditions (Lopes et al., 2001). As a sign of stress, the using of biochemical and hematological methods provides valuable knowledge about physiological reactions occured against to changing environmental conditions.

In this study, the biochemical and hematological parameters in blood of rainbow trout treated to various concentrations of pollen for 96 h were determined.

Materials and methods

Animals and experimental design

The rainbow trouts (*Oncorhynchus mykiss*) were obtained from Camardı, Ecemis fish farm (Nigde, Turkey). Fish were hold for 15 days in a 8 x 5 x 1.5m stock tank to be acclimatized.

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After environmental adaptation period, forty nine rainbow trouts were distributed into seven groups, each consisting of seven animals. They were transfered to tank, which filled with 200 L well aerated water (physical and chemical parameters of water are shown in Table 1). Artificial dry food was provided for once daily. Fish, which are using in this study had an average weight of 248.54± 5.12 g and length of 28.65 ± 2.71 cm.

Table 1. Amount of physical and chemical parameters of water during the experiment period

Parameters	Before treatment	After treatment
Dissolved oxygen (ppm)	7.6 ± 0.6	7.4 ± 0.3
Chemical oxygen demand (ppm)	13.1 ± 0.4	15.5 ± 0.8
Suspended solids (ppm)	37.6 ± 1.5	41.1 ± 1.2
Calcium (ppm)	132.0 ± 1.8	109.1 ± 1.5
Sodium (ppm)	24.4 ± 0.4	17.7 ± 0.3
Chloride (ppm)	15.0±1.2	16.0±1.8
Total nitrogen (ppm)	5.3 ± 0.5	6.2 ± 0.7
Hardness (CaCO3) (ppm)	179.3 ± 3.6	163.2 ± 2.3
Temperature (^{o}C)	12.5 ± 1.6	11.0 ± 0.3
pH	7.6 ± 0.1	7.6 ± 0.1

Preparation of pollen extractive solution

The most common extracts used in biological assays are ethanol, methanol and water. In the present work, pollen was obtained from a farm at Kocaavsar village in Balikesir, Turkey. Pollen was prepared to 30% in ethanol (30 g of pollen, completing the volume to 100 mL with 70% ethanol) it protected from light and moderately shaken for 1 day at room temperature. Afterward, the extracts were filtered twice, dried and stored in sealed bottles at 4°C until use (Marghitas et al., 2009).

Experimental design

There were totally forty nine rainbow trouts, which treated to 0.5 ppm pollen (group I), 2.5 ppm pollen (group II), 5 ppm pollen (group III), 10 ppm pollen (group IV), 20 ppm pollen (group V), 30 ppm pollen (group VI) and control group. Forty nine rainbow trouts were divided into seven groups. Each group was consisting of seven fish. Fish were fed to Excel Pond trade mark pellet feed during experiments. Untreated fish in last group were used as control and were sacrificed 96 h later. Each rainbow trout was weighted just before the start of the study. Fish experiments were performed in accordance with the guidelines for approving by the Committee of Animal Experiments at Cumhuriyet University, Sivas, Turkey.

Biochemical assay

After application for 96 hours, fish were anaesthetised with 40 mg/L of clove oil (Mylonas et al., 2005) and 2 mL of blood was obtained from caudal vein. Blood samples were transferred to tubes, kept into cooled bath and immediately analysed. The blood was

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centrifuged at 3000 x g, at 4ºC for 5 minutes. All of the analyses were done with an Olympus Optical Corp. (Shizuoka-ken, Japan), using commercially available kits (Roche).

Measurement of total antioxidant status (TAS)

TAS levels in serum were determined using an automated measurement method, which developed by Erel (2004). The hydroxyl radical, which the most forceful radical for biological molecules was used in this method. In the experiment, a ferrous ion solution, was mixed with hydrogen peroxide which were existed in reagent 1 and 2, respectively. Another powerful radicals were produced, such as brown dianisidinyl radical cation, which was produced by the hydroxyl radicals. The method was aimed to measure antioxidant effect of the sample against to free radical reactions initiated by the hydroxyl radical. The test has highly sensitive values of < 3%. As a result, data were expressed as millimoles of Trolox equivalent per liter (mmol Trolox equiv./L).

Measurement of total oxidant status (TOS)

Levels of TOS in serum were determined using a novel automated measurement method, developed by Erel (2005). Oxidants presented in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is raised by glycerol molecules, which are plenty of present in the reaction medium. The ferric ion takes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be evaluated as spectrophotometric, is interrelated to the total quantities of oxidant molecules presented in the samples. The analysis is calibrated with hydrogen peroxide and the results are meaned in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv./L).

Measurement of paraoxanase (PON) activities

Measurements of PON activities were applied in the absence (basal activity) and presence of NaCl (salt-stimulated activity). The ratio of paraoxon hydrolysis (diethyl-p-nitrophenylphosphate) was estimated by monitoring the raise of absorbance at 412 nm at 25◦C. The quantity of generated p-nitrophenol was calculated from the molar absorptivity coefficient at pH 10.5, which was 18,290 M^{-1} cm^{-1} (Eckerson et al., 1983). PON activity was expressed as U/L serum. Paraoxonase phenotype distribution was established by a double substrate method, which calculates the ratio of salt-stimulated PON activity (Eckerson et al., 1983).

Hematological analyses

After treatments, the clove oil as anesthetic matter (40 mg/L) was applied to fish (Mylonas et al., 2005). Blood samples were collected from caudal vein of fish. The blood samples were transferred to tubes. Red blood cell counting was done after 1:200 dilution into Hayem solution. Counting leucocytes was done in blood samples after dilution into Turck solution (Blaxhall, 1981). Hemoglobin (Hb) concentration was determined according to the

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cyano-methemoglobin procedure (Kit 525- A; Sigma Chemical, St. Louis, MO, USA) (Blaxhall, 1973). Nonclotted blood (20 µL aliquots) was diluted with 1 mL of Drabkin solution and left to stand for 10 min at room temperature. The absorbance was read at 540 nm. The amount of Hb was calculated against to a Hb standard (Azizoglu and Cengizler, 1996). Hematocrit was determined according to Jewet et al. and Wilhelm Filho et al. (Jewet et al., 1991; Wilhelm Filho et al., 1992). Nonclotted blood was transferred to a microhematocrit capillary, centrifuged at 14,000g for 5 min and read against to a standart cart.

Statistical analysis

Hematological and biochemical data were analyzed with SPSS 16.0 for Windows using one-way analyses of variance (ANOVA). Differences between means were determined using Duncan's multiple range test in which the significance level was defined as $P<0.05$.

Results

The effects of pollen extracts with six different (0.5, 2.5, 5, 10, 20 and 30 ppm) concentrations were determined in blood of fish. The values of biochemical parameters are showed in table 2. The levels of total protein and creatinine in blood of fish treated 0.5, 2.5, 5, 10, 20 and 30 ppm compared control group did not change ($P>0.05$) (Table 2). There were significant increases ($P<0.05$) in the levels of triglycerides, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities and potassium levels in the all groups of pollen concentrations compared with control group. There were significant decreases in the levels of urea and glucose in 10 and 20 ppm concentrations compared with control group ($P<0.05$). There were no statistically significant changes in the levels of chloride and sodium in blood of rainbow trouts treated to six different concentrations of pollen compared with control group ($P>0.05$) (Table 2).

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Table 2. Changes on the biochemical parameters in rainbow trout blood treated to various pollen concentrations

Biochemical Parameters	Control	Group I (0.5 ppm)	Group II (2.5 ppm)	Group III (5 ppm)	Group IV (10 ppm)	Group V (20 ppm)	Group VI (30 ppm)
Metabolites							
Glucose (mg dL^{-1})	84.5±0.5^{a}	83.1±0.6^{a}	85.3±0.4^{a}	86.3±0.5^{a}	72.5±0.8^{b}	74.0±1.7^{b}	75.5±2.1^{b}
Total protein (g dL^{-1})	3.48±0.09	3.32±0.08	3.39±0.07	3.17±0.6	3.44±0.8	3.25±0.07	3.03±0.1
Creatinine (mg dL^{-1})	0.10±0.04	0.08±0.03	0.08±0.02	0.07±0.3	0.10±0.04	0.08±0.06	0.08±0.04
Urea (mg dL^{-1})	4.8±0.2^{a}	4.2±0.2^{c}	5.0±0.2^{a}	4.2±0.2^{b}	3.3±0.4^{c}	4.3±0.2^{b}	4.8±0.3^{a}
Triglycerides (mg dL^{-1})	168.8±16.2^{b}	158.1±13.1^{b}	217.7±2.3^{a}	198.2±2.1^{a}	220.7±14.6^{a}	192.7±14.2^{a}	197.7±10.6^{a}
Total cholesterol (mgdL^{-1})	188.3±11.5^{c}	213.4±12.2^{a}	202.1±7.9^{a}	205.1±8.1^{a}	225.7±10.4^{a}	232.7±6.9^{a}	206.1±9.8^{a}
Enzymes (IU L^{-1})							
ALT	28.5±4.46^{b}	23.3±6.3^{b}	30.6±5.4^{b}	33.3±3.4^{b}	34.8±4.2^{b}	33.6.1±2.4^{b}	62.3±1.8^{a}
AST	803.5±13.1^{c}	1025.6±16.5^{a}	1179.3±10.5b	1738.3±8.9^{a}	1800.1±7.6^{a}	1810.1±21.6^{a}	1805.1±23.2^{a}
LDH	1415.2±4.2^{b}	1485.1±9.1^{b}	1425.1±2.8^{b}	1565.7±7.5^{b}	1563.1±8.2^{b}	1792.2 ±8.6^{a}	1850.4±13.7^{a}
ALP	45.6±5.9^{b}	63.1±4.2^{a}	67.3±2.6^{a}	52.5±2.9^{a}	73.1±3.1^{a}	52.3±3.7^{a}	41.1±2.5^{b}
Electrolites (mmol L^{-1})							
Chloride	137.3±2.6	138.6±1.9	140.3±2.1	138.8±1.9	135.3±2.1	134.1±3.6	139.6±2.3
Sodium	152.8±2.5	155.5±1.7	158.0±3.2	154.8±3.4	152.6±4.1	153.1±3.7	156.5±2.8
Potassium	1.63±0.6^{b}	2.95±0.5^{a}	3.05±0.6^{a}	3.15±0.5^{a}	2.82±0.6^{a}	2.81±0.3 ^{a}	2.92±0.4^{a}
TAS	1.065±0.04^{c}	1.024±0.24^{c}	0,967±0.14^{c}	1.061±0.07^{c}	1.293±0.15^{a}	1.176±0.06^{b}	1.104±0.16^{c}

All data points are the average of n=7 with ± SD. ^{a,b,c}Statistically significant (P<0.05)

However, there were statistically significant increases (P<0.05) in potassium levels of fish in all of pollen concentrations compared with control group (Table 2). The highest TAS level occured in 10 ppm pollen group (Table 2). There were no PON activities and TOS values in blood serum of fish in all experimental groups. The effects of pollen on the hematological parameters are summarized in table 3. There were the significant increases (P<0.05) in MCV, MCH and MCHC values of fish treated to 30 ppm pollen compared to control and 0.5, 2.5, 5, 10, 20 ppm pollen groups (Table 3). There were significant increases (P<0.05) in total leucocyte counts and Hb levels of all of pollen groups compared to control group (Table 3). Significant changes in the erytrocyte counts and hematocrit values in blood of fish treated to 20 and 30 ppm pollen were observed compared to control group (P<0.05).

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Table 3. Changes on the hematological parameters in rainbow trout blood treated to various pollen concentrations

Hematological Parameters	Control	Group I (0.5 ppm)	Group II (2.5 ppm)	Group III (5 ppm)	Group IV (10 ppm)	Group V (20 ppm)	Group VI (30 ppm)
Total Leucocyte Count (10^3/mm^3)	7.75±0.11^c	10.83±0.15^b	12.4±0.17^a	11.5±0.17^a	13.6±0.24^a	9.9±0.13^b	9.79±0.17^b
Erytrocyte Count (10^6/mm^3)	0.87±0.06^b	0.85±0.05^b	0.77±0.05^b	0.87±0.02^b	0.88±0.02^b	1.11±0.03^a	0.67±0.08^c
Hemoglobin (g/dL)	8.5±0.24^b	9.6±0.12^a	9.02±0.13^a	8.9±0.25^a	10.0±0.11^a	11.1±0.42^a	9.0±0.53^a
Hematocrit (%)	12.5±1.54^b	14.3±1.23^b	12.7±2.66^b	13.2±1.30^b	31.2±0.91^a	31.9±2.12^a	11.8±2.25^b
Erytrocyte Indexes							
MCV (μ^3)	143.3±2.30^b	144.3±2.10^b	139.8±0.8^b	143.8±3.42^b	131.5±2.10^b	146.5±1.80^b	174.0±1.60^a
MCH (µg)	97.7±1.40^b	104.9±1.70^b	98.8±1.90^b	103.3±2.40^b	102.9±1.90^b	99.3±1.60^b	141.5±1.60^a
MCHC (%)	68.5±0.70^b	71.2±0.80^b	69.7±0.80^b	73.1±0.40^b	72.1±0.70^b	73.4±0.82^b	80.1±0.80^a

All data points are the average of n =7 with ± SD. ^{a,b,c}Statistically significant (P<0.05).

Discussion

This study investigated the effective and useful concentration of pollen, which has biologically benefits exists in natural environmental, on living organisms in natural area. In this respect, bee pollen is receiving a special attention in the field of functional foods and medicines (Xu et al., 2009). It is well known that polyphenols are responsible for the antioxidative and radical scavenging activities of plant food. Fish are considered as an avaluable source of protein and lipid in the human diet. There have not being studies on effective concentration of pollen on biochemical and hematological parameters of aquatic animals. Thus, this study was aimed to discuss the effective concentrations of this bee product on biochemical and hematological parameters in blood of rainbow trout. After analysed the biochemical and hematological parameters, data showed that pollen at various concentrations has a certain fluence on some blood indices studied. The levels of glucose, total protein, creatinine, urea, triglyceride, total cholesterol, ALT, AST and potassium in fish treated to pollen at different concentrations were evaluated. The elevation in their levels in blood of fish especially treated to 20 and 30 ppm pollen may be considered as the markers of renal and liver dysfunctions, and changes of lipid metabolism (Dobrowolski et al., 1991); 10 ppm pollen caused a significant (P<0.05) decreases in the most of these levels, therefore, it was appeared that have the certain potential to maintain of homeostasis in 10 ppm pollen-treated *Oncorhynchus mykiss.* Enzymes such as AST and ALT have been used to determine environmental changes and serve as good

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bioindicators (Lavanya et al., 2011). Our data showed the significant increases in the level of urea in the serum of fish with increases in concentration of pollen. The significant increases in urea levels of the blood serum noticed in this study suggest that the kidney was adversely affected by high concentration of pollen. Urea level can also be increased by many other factors such as dehydration, anti-diuretic drugs and diet. Beyraghdar Kashkooli et al. (2011) investigated the long-term effects of propolis administration on serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). To determine the possible toxicity and side effects of propolis, fish were fed on diets containing 0, 0.5, 1.5, 4.5 and 9 g propolis/kg diet for 8 weeks. The done experiments by Beyraghdar Kashkooli et al. and results of other studies show the parallelism for antioxidant effects of bee products on fish (Talas and Gulhan, 2009; Beyraghdar Kashkooli et al., 2011; Kelestemur, 2012; Talas et al., 2012).

Taken together, these data support the hypotheses that pollen may possess antioxidant properties that could influence serum oxidant and antioxidant balance. Thus, to test this hypothesis, we determined the values of TAS and TOS using various concentrations of pollen. The measurement of TAS can only reflect the antioxidative status of plasma (Erel, 2004). According to TAS measurements made in serum, 10 ppm pollen group has the highst TAS level among six concentrations (0.5, 2.5, 5, 10, 20 and 30 ppm) of pollen. 10 ppm pollen concentration has the best antioxidative effect in rainbow trout because of the highest TAS level in this concentration. There was no the paraoxanase activity in fish blood serum. In various trials, it was shown that PON prevents oxidative stress by inhibiting oxidation of cell membrane lipids induced by ROS, which develop in acute and chronic inflammation (Aviram and Rosenblat, 2004). PON activity of fish is indicated as very low even close to zero (Folly et al., 2001). PON activity was not observed in fish blood in our study. The study done by Folly et al. demonstrated the first experimental evidence of the association of PON activity with HDL in fish. It notifies that birds, fish and insects dont have gene of PON1 (Folly et al., 2001). It is known that exogen agents may change the hematological parameters such as erythrocyte number, Hb amount, hematocrit value and total leukocytes. Effective concentration of pollen may inhibit changes in hematological and biochemical parameters may exist at high concentration. Levels of MCV, MCH and MCHC in pollen groups with high concentration, especially 30 ppm, increased; erytrocyte count and hematocrit levels decreased. Decreases in erythrocyte, hematocrit levels at high concentrations (30 ppm) of pollen may be an indicator of anemia with inhibition of erythropoesis in the hemopoietic system (Kleinrok et al., 1978). The decrease in the Hb may be attributed to intravascular haemolysis, anemia or depression of the hemopoiesis. In addition, increases in MCV, MCH and MHCH values indicated the macrocytic type of anemia (Kleinrok et al., 1978). These results can be an implication of anemia and suppression of hemopoietic system. Some exogen agents may also lead to the development of anemia due to interference of hemoglobin biosynthesis and

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shortening of the life span of circulating erythrocytes. The usefulness of immunostimulants and antioxidants has been demonstrated in modern aquaculture and fish farmers use a wide range of immunostimulants and antioxidants that may need to be purified (vitamins, chitin, glucans, etc.) or not (microorganisms, animal and plant extracts, subproducts of other industries (Alberto et al., 2005). According to results of our study, 10 ppm pollen can act as immunostimulant and potent effective antioxidant. Data, which we get in this study show parallelism with results of studies on bee products (Talas and Gulhan, 2009; Beyraghdar Kashkooli et al., 2011; Gulhan et al., 2012; Kelestemur, 2012; Talas et al., 2012).

In conclusion, in the present study, the effective oncentrations of pollen were compared to control group in hematological and biochemical parameters. The data showed that pollen has important positive effects on biochemical and hematological parameters at 10 ppm level, whereas other concentrations of pollen appear to be unfavorable for blood tissue of rainbow trouts.

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