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Effects of different concentrations of pollen extract on brain tissues of *Oncorhynchus mykiss*

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PEER REVIEW

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Comments

This is an interesting study. The results suggest that pollen may possess antioxidant properties that could influence serum oxidant and antioxidant balance in treated fish.
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ABSTRACT

Objective: To determine the antioxidant capacities of pollen extract applied at different concentrations on biochemical parameters in brain tissues of rainbow trouts.

Methods: The effective concentration of pollen was determined with some biochemical parameters in brain tissues of fish treated at various concentrations of the pollen extract (0.5, 2.5, 5, 10, 20 and 30 mg/L) for 96 h. The malondialdehyde levels, total antioxidant status, total oxidant status, oxidative stress index and amounts of total free sulfhydryl groups were analyzed in fish brain.

Results: The malondialdehyde levels decreased in groups of 0.5, 2.5, 5, 10, 20 and 30 mg/L pollen-treated compared to control group ($P<0.05$). The highest level of total antioxidant status ($P<0.05$) and the lowest value ($P<0.05$) of the total oxidant status was 10 mg/L concentration of pollen. Oxidative stress index and level of sulfhydryl groups showed lowest values ($P<0.05$) in 10 mg/L pollen treated group compared with control group.

Conclusions: To apply the pollen to fish reduces the detrimental effects and modulates oxidative status via activating antioxidant defense systems at brain tissue. As a result, pollen can be added up to 10 mg/L to the medium of rainbow trout to improve health of fish.

KEYWORDS
Antioxidant, Brain, Pollen, Rainbow trout

1. Introduction

Fish are commonly used to estimate the influences of environmental compounds due to the sensitivity of their biochemical parameters under conditions of environmental stress[1]. As a sign of stress, the use of biochemical methods provides valuable knowledge about physiological reactions occuring against changing environmental conditions, especially understanding the physiological and biochemical changes occuring at various concentrations of compounds, to predict the possible level of threat to life[2]. Fish are an important aquatic organism. Fish products are an important source of protein for human consumption[3]. Long chain polyunsaturated fatty acids (PUFA) are conditionally essential nutrients for adequate growth, development and function in humans[4]. Among them, omega-3 PUFA (ω3 PUFA) have gained popularity due to their various health promoting and diseases preventing attributes. For example, ω3 PUFA are reported to be highly effective against cardiovascular diseases, cancer and other metabolic diseases[5]. Aquatic

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organisms can provide model systems for investigation of how reactive oxygen species damage cellular compounds, how cells respond, how repair mechanisms reverse this damage, and how oxidative stress can lead to diseases. Oxidative stress has become an important item for aquatic toxicology. Factors of stress in the aquatic organisms can be tolerable with antioxidant molecules. Recently, many investigations have been concerned over the different nutritional products due to their antioxidant potential to prevent or treat the diseases of aquatic animals[6,7]. In recent years, several organic forms of antioxidant molecules have been studied as possible natural therapeutic and preventive agents[8-10]. Antioxidant ability has usually been attributed to the activity of antioxidant enzymes as well as to the content of low molecular antioxidants such as carotenoids, tocopherols and phenolic substances[11]. Honeybee products, particularly rich in flavonoids, have been the subject of researches[12]. Especially, one of the natural agents attracted attention of researchers was pollen. Bee pollens are the male generative cells gathered by honeybees from flower stamens[13]. It provides nutrition through its remarkable quantity of proteins, sterols, fatty acids, vitamins, carbon hydrates, lipids, vitamins, ashes, minerals, phenolic compounds and flavonoids which are regarded as protective agent[14]. 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[6]. Malondialdehyde (MDA), which is the last product of lipid peroxidation is used as an indicator of local tissue damage and brain injury[15]. Lipid peroxides can change properties of biological membranes, resulting in free radical damage[16]. The change of protein conformation correlates with exposure of functional groups such as sulfhydryl groups and hydrophobic groups. However, according to the changes of total sulfhydryl groups contents in proteins, we can understand more about the conformation changing and formation of disulfide bonds[17]. Phenolic compounds such as phenolic acid, flavonoids and tannins are thought to be an important subscribe to the antioxidant capacity of foods[6]. After some antioxidant treatments, the assays of total antioxidant status (TAS) and total oxidant status (TOS) can reflect the biochemical changes in tissues[18]. Our study aimed to examine the effective concentrations of pollen extract on biochemical parameters (MDA, TAS, TOS and total free sulfhydryl groups) in brain tissue of rainbow trout [*Oncorhynchus mykiss* (*O. mykiss*)].

2. Materials and methods

2.1. Animals

Forty nine rainbow trouts (*O. mykiss*) with average weight of (248.54±5.12) g were obtained from Camardi, Ecemis fish farms in Nigde, Turkey in 2011. They were then transferred to research station in Nigde University under optimum conditions, distributed to seven stock ponds (7 fish each) with the dimension of 8.0×5.0×1.5 m and acclimated for 15 d. They were fed with commercial food once daily.

2.2. Preparation of pollen extractive solution

Pollen was obtained from a farm at village Kocaavsar in Balikesir, Turkey and diluted to 30% in ethanol. It was kept in dark at room temperature and moderately shaken for one day. Afterward, the extracts were filtered twice, dried and stored in sealed bottles at 4 °C until use[6].

2.3. Experimental design

The fish treated for 96 h with 0.5 mg/L pollen extract in group I, with 2.5 mg/L in group II, with 5 mg/L in group III, with 10 mg/L in group IV, with 20 mg/L in group V, with 30 mg/L in group VI and untreated fish in control group were used. Fish used in this study had an average weight of (248.54 ±5.12) g and length of (29.75±3.81) cm. Physical and chemical properties of water are depicted in Table 1. Then, they were sacrificed in accordance with the guidelines approved by the Committee of Animal Experiments at Cumhuriyet University, Sivas, Turkey.

Table 1
Some parameters of the water used in the experiment.

Parameter	Before treatment	After treatment
Dissolved oxygen (mg/L)	7.8±0.2	7.6±0.1
Chemical oxygen demand (mg/L)	15.1±0.1	16.2±0.2
Suspended solids (mg/L)	36.8±1.2	40.1±1.7
Calcium (mg/L)	126.0±1.5	114.1±1.1
Sodium (mg/L)	22.4±0.8	19.7±0.7
Chloride (mg/L)	16.0±1.5	18.0±1.4
Total nitrogen (mg/L)	5.8±0.2	6.8±0.3
Hardness ($CaCO_3$)	174.3±3.1	168.2±2.8
Temperature (°C)	11.5±1.0	12±0.7
pH	7.7±0.1	7.6±0.8

2.4. Biochemical assay

After these treatments, fish were anaesthetised with clove oil 40 mg/L[19]. Brain tissues of fish were removed and frozen in liquid nitrogen. Tissues were stored at −80 °C until used. The tissues were separated into two parts for determination of MDA, TAS, TOS and levels of total free sulfhydryl groups. Tissues were weighed and then homogenized in 100 mL of 2 mmol/L phosphate buffer, pH 7.4 using homogenizer. Homogenized samples were then sonicated for 1.5 min (30 seconds sonications interrupted with 30 seconds pause on ice). Samples were then centrifuged at 3 500 r/min for 5 min at 4 °C and supernatants, if not used for enzyme assays immediately, were kept in the deep freeze at −80 °C.

Supernatants were used for determination of TAS, TOS and total free sulfhydryl groups. The second part of tissues was used for lipid peroxidation analysis. Tissues were washed three times with ice-cold 0.9% NaCl solution and homogenized in 1.15% KCl. The homogenates were assayed for MDA, the last product of lipid peroxidation.

2.5. Malondyaldehyde level

Lipid peroxidation in brain tissue of rainbow trout was measured according to the concentration of thiobarbituric acid (TBA) reactive substances. The amount of produced MDA was used as an index of lipid peroxidation[20]. In the thiobarbituric acid test reaction, MDA or MDA-like substances and thiobarbituric acid react with the production of pink pigment with a maximum absorbtion at 532 nm. The reaction was performed at pH 2-3 at 90 °C for 15 min. The results were expressed as nmol/g wet tissue.

2.6. Total antioxidant status

TAS in serum were determined based on the method of Erel[18]. This method used the hydroxyl radical, which is the most forceful radical for biological molecules. The test has highly sensitive values of <3%. Data were expressed as millimoles of trolox equivalent per liter (mmol trolox equiv./L).

2.7. Total oxidant status

TOS in serum were determined using a novel automated measurement method, developed by Erel[21]. Oxidants presented in the samples oxidize the ferrous ion-o-dianisidine complex to ferric ion. 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).

2.8. Oxidative stress index (OSI)

The ratio of TOS to TAS was accepted as the OSI. For calculation, the resulting unit of TAS was changed to mmol/L, and the OSI value was calculated according to the following formula: OSI (arbitrary unit)=TOS (μmol H_2O_2 equiv./L)/TAS (mmol trolox equiv./L)[22].

2.9. Total free sulfhydryl groups

Free sulfhydryl groups of tissue samples were assayed according to the method of Ellman[23] followed by Hu *et al*[24]. Absorbances of samples were read at 412 nm on a spectrophotometer (Cecil 3000). The concentration of sulfhydryl groups was calculated using reduced glutathione as free sulfhydryl group standard and the results were expressed as mmol/mg protein[25].

2.10. Statistical analysis

Data were analyzed with SPSS 16.0 for Windows using nonparametric Kruskal-Wallis test. Differences between ranks were determined using Mann-Whitney test in which the significance level is defined as $P<0.05$.

3. Results

In order to determine the effects of various concentrations of pollen extract, we prepared pollen extracts as six concentrations. The effects on the fish brain, observed through biochemical parameters, are summarized in Table 2. MDA levels in brain tissues of fish treated with 0.5, 2.5, 5, 10, 20 and 30 mg/L pollen extract significantly decreased ($P<0.05$) compared with control group (Table 2). The lowest MDA levels were detected in brain of fish applied to 10, 20 and 30 mg/L pollen extract (Table 2). There were no significant changes in TAS levels of fish treated with 0.5 and 2.5 mg/L pollen extract compared with control group ($P<0.05$). Changes in TAS levels in brain tissues of rainbow trouts in groups of 5, 10, 20 and 30 mg/L pollen extract have been showed in Table 2. There were statistically significant increases ($P<0.05$) in TAS values of these groups compared to control group (Table 2). The highest TAS level was detected in the brain of fish treated with 10 mg/L pollen extract (Table 2). There were statistically significant decreases in TOS and OSI data in brain tissues of fish administered to 0.5, 2.5, 5, 10, 20 and 30 mg/L pollen extract compared to control group

Table 2

Changes on the biochemical parameters in brain tissues of rainbow trout treated with various concentrations of pollen extract (n=7, mean±SD).

Groups and Concentrations	MDA (nmol/g wet tissue)	TAS (mmol trolox equiv./g protein)	TOS (μmol H_2O_2 equiv./L)	OSI (arbitrary units)	Total free sulfhydryl group (mmol/mg protein)
Control	17.49±1.62^a	2.20±0.05^c	2.05±0.27^a	0.93±0.01^a	0.61±0.19^b
0.5 mg/L (Group I)	13.84±0.41^b	2.18±0.03^c	1.80±0.19^b	0.82±0.14^b	0.63±0.10^b
2.5 mg/L (Group II)	12.99±1.42^b	2.22±0.02^c	1.74±0.16^b	0.78±0.13^b	0.94±0.10^a
5 mg/L (Group III)	11.02±0.45^b	2.39±0.04^b	1.62±0.16^b	0.67±0.07^b	0.90±0.11^a
10 mg/L (Group IV)	4.41±0.45^c	2.74±0.07^a	1.35±0.09^c	0.49±0.05^c	0.31±0.07^c
20 mg/L (Group V)	6.27±0.60^c	2.47±0.16^b	1.71±0.33^b	0.69±0.06^b	0.85±0.14^a
30 mg/L (Group VI)	7.15±0.65^c	2.43±0.32^b	1.84±0.11^b	0.75±0.01^b	0.89±0.13^a

Data with different superscript letters (a, b, c) means statistically significant ($P<0.05$).

(P<0.05) (Table 2). The lowest levels of TOS and OSI were detected at 10 mg/L concentration of pollen extract (Table 2). The level of total free sulfhydryl group in fish treated to 0.5 mg/L pollen extract did not change (P>0.05) compared with control group (Table 2). Total free sulfhydryl groups in fish applied to 2.5, 5, 20 and 30 mg/L pollen extract significantly increased (P<0.05) compared to control group (Table 2). The lowest level of total free sulfhydryl group detected at 10 mg/L concentration of pollen etraxct (Table 2).

4. Discussion

The aquatic environment plays a vital role for function of ecosystem and is intimately related to human health. Fish are a valuable source of protein in the human diet[5]. Due to rapid growth and easy accommodation to environmental conditions, rainbow trout is an important fish consumed economically and commonly. In recent years, there have been a great deal of studies carried out on therapeutic and antioxidant effects of natural products. To investigation the effects and useful concentration of these products such as pollen on biochemical parameters of aquatic animals is the first time in this study. We studied for the first time the effective concentration of pollen extract using the *O. mykiss*, one of the most popular cultural fish in Turkey. This study opens a new perspective on the test of the biological effects of pollen, mainly with respect to biochemical parameters in brain tissues of rainbow trout. Thus, this study aimed to discuss the effective concentration of this bee product on biochemical parameters (MDA, TAS, TOS, OSI and total free sulfhydryl group) in brain tissues of rainbow trout.

Biochemical data showed that pollen at various concentrations exerted certain influences on some parameters of the studied brain tissues. Pollen may enter to the aquatic environments from agricultural and rural fields by rain water, wind, insects and have long-term effects in fish. These effects appear as physiological and biochemical reactions in population or ecosystems. Pollen is thought to have a wide range of health benefits. Pollen has nonspecific esterase and cholinesterases which might be useful to neutralize the stopped acetylcholinesterase activity by oxidant molecules[12]. Membrane phospholipids of aerobic organisms are permanently subjected to oxidant challenges from endogenous and exogenous sources, while peroxidized membranes and lipid peroxidation products represent constant threat to aerobic cells. The most widely used assay for lipid peroxidation is MDA formation as a secondary lipid peroxidation product, with the thiobarbituric acid reactive substances test. The concentration of MDA is the direct evidence of toxic processes caused by free radicals[26,27]. The high levels of MDA may cause renal, liver and brain dysfunctions, and the changes of lipid metabolism[28]. The reduction in MDA levels of fish applied to 0.5, 2.5, 5, 10, 20 and 30 mg/L pollen extracts may be considered as powerful antioxidant effect of pollen.

In a study that done by Hamre *et al*., it has demonstrated the effects of different natural antioxidants (rosemary extract, crystalline ascorbic acid, tocopherol mix, spermine *etc*.) in an experimental fish feed, made directly from marine raw materials. The researchers have referred them that their powerful antioxidant properties are also effective in removing free radicals and have protective effects against lipid peroxidation in cell membranes[29]. The other researchers have found that there is a positive correlation between proportional reduction of MDA production with the amount of flavonoids[30,31].

Sahin *et al.* suggests that supplementation of different doses of lycopene to high density-stressed fish causes a dose-dependent decreased level of MDA as well as increased activities of antioxidant enzymes (glutathione peroxidase, superoxide dismutase and catalase) in the liver. Their results indicate the important role of lycopene in the reduction of oxidative stress. However, the mechanism by which lycopene in a dose-response manner could affect free radical production, as well as catalase, superoxide dismutase and glutathione peroxidase activities, is due to its highly efficient antioxidant with a singlet-oxygen and free radical scavenging capacity[30]. 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 TAS and TOS using various concentrations of pollen. Because of the number of different antioxidants in plasma, serum or other biological samples are difficult to measure separately each antioxidant. Therefore, several methods have been developed to determine the antioxidative capacity of various biological samples[8]. The measurement of TAS can only reflect the antioxidative status of tissues[18]. According to TAS measurements made in serum, 10 mg/L treatment group has the highest TAS level among six concentrations (0.5, 2.5, 5, 10, 20 and 30 mg/L) of pollen extract. In 10 mg/L pollen group, pollen can behave as the best antioxidant for this concentration.

Talas and Gulhan investigated the biochemical and hematological parameters in blood of rainbow trout treated with various concentrations of propolis (0.01 g/L, 0.02 g/L and 0.03 g/L) for 96 h. The study showed the protective effect of 0.01 g/L propolis against to oxidative modification on blood parameters[2]. Protein denaturation is associated with the formation of disulfide bonds and conformational changes in proteins (*i.e.* changes in secondary and tertiary structure), resulting in an increase in aggregated β-sheet structure and a decrease in α-helical structure of

proteins[32]. This includes changes in reactive groups, such as loss of hydrophilic surface, exposure of hydrophobic areas and sulfhydryl groups that are buried or blocked in native proteins[33]. Since the sulfhydryl groups of proteins are exposed, more disulfide bonds are formed due to the increase in interaction of the interior and exterior amino acids[34]. In this study, total free sulfhydryl values of group treated with 10 mg/L pollen extract were determined which is statistically significantly higher than other experimental groups in brain tissue. Levels of total free sulfhydryl groups were increased with the application of 2.5, 5, 20 and 30 mg/L pollen extracts and therefore pollen can play a role as antioxidant agent. Results of our study show conformity with other researchers's data[19,35].

Most recent studies have shown that natural preventive compounds have gained popularity day by day as some of the widely used synthetic pharmaceuticals and therapeutics might have some undesirable effects. We can think that certain natural food ingredients would be better and safer than synthetic ones. Many of these compounds, such as plant phenolics, often exhibit antioxidant activities; therefore the addition of these compounds into food products may be helpful to the health of consumers and also to the stabilization of food products. Due to antioxidant and preservative properties of pollen, it may not only prolong the physiological functions of some aquatic living organisms, but also contribute to the health benefit of consumers who consume aquatic animals.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Honeybee-collected pollen is an apicultural product containing considerable amounts of polyphenolic compounds which may act as potent antioxidants. Due to antioxidant and preservative properties of pollen, it may not only prolong the physiological functions of some aquatic living organisms, but also contribute to the health benefit of consumers who consume aquatic animals.

Research frontiers

For the first time, the present work investigates the effects and useful concentration of pollen on biochemical parameters of aquatic animals.

Related reports

There are many evidences of effectiveness of different natural antioxidants (rosemary extract, crystalline ascorbic, acid tocopherol mix, spermine *etc*.) in removing free radicals and their protective effects and antioxidant properties.

Innovations and breakthroughs

This study opens a new perspective on the test of pollen biological properties, mainly with respect to biochemical parameters in brain of rainbow trout.

Applications

Due to antioxidant and preservative properties of pollen, this study suggests that pollen may not only prolong the physiological functions of some aquatic living organisms, but also contribute to the health benefit of consumers who consume aquatic animals.

Peer review

This is an interesting study. The results suggest that pollen may possess antioxidant properties that could influence serum oxidant and antioxidant balance in treated fish.

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