Effects of Propolis on microbiologic and biochemical parameters of Rainbow trout (*Oncorhynchus mykiss*) after exposure to the pesticide

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Abstract

Cypermethrin is a potential toxic pollutant that directly threatens the aquatic ecosystems and environment. Propolis, a natural product collected from plants by honey bees has many functions, such as antimicrobial, antioxidant, and anti-inflammatory. This study attempted to detrmine some biochemical and microbiological parameters of rainbow trout fillets after exposure to propolis and cypermethrin. The results of the current study revealed that levels of malondialdehyde (MDA), lactic acid, total volatile base-nitrogen (TVB-N), total counts of psychrophilic, and mesophilic bacteria increased in cypermethrin groups ($P<0.05$), compared to control group. Furthermore, there were no significant differences in pH levels between groups. Additionally, levels of MDA, lactic acid, TVB-N, count of psychrophilic, and mesophilic bacteria in cypermethrin+propolis treated groups were significantly reduce in comparison to exposed groups with cypermethrin ($P<0.05$). The findings also showed that the fillet quality some biochemical and microbiologic functions of fishes could be changed in groups that exposed to cypermethrin by propolis.

Keywords: Biochemical parameter, Cypermethrin, Microbiological parameter, Propolis, *Oncorhynchus mykiss*

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Introduction

Physio-chemical parameters of water resources can be changed by pesticides, which carried by rains and floods. Due to the intensive use of synthetic pyrethroids, water resources are being constantly polluted, directly threatening the life of many aquatic animals ,and indirectly the human life (Das et al., 2003; Borges et al., 2007). Pyrethroids are synthetic analogues of natural pyrethrins, which originated by the ornamental plant *Chrysanthemum cinerariafolium*.They were developed to protect grain crops and other agricultural products against pests in 1970s and later on, they were also used to control animal ectoparasites (Polat et al., 2002; Dobsikova et al., 2006). Cypermethrin is a synthetic pyrethroid used as an insecticide and it plays a vital role in agricultural and in healthly pest control (Velisek et al., 2006). After the application of cypermethrin, it is released directly into the environment, and then it will be entered the water body by runing off, affecting many aquatic organisms (Muthuviveganandavel et al., 2008).

Several researchers have probed the effects of natural therapeutics on cypermethrin damage in fish. (Rao et al., 1995; Koru et al., 2007; Kanbur et al., 2009). Cases in point Kolankaya(2002) and Sforcin (2007) believed that propolis is one of these natural agents. If toxic agents are overrun against the natural protective systems, then exogenous antioxidant and protective compounds must be taken into consideration (Rao et al., 1995; Kanbur et al., 2009). Furthermore, the investigation of a new antioxidant as a potential therapeutic agent is an active field of biochemistry. Recently, varieties of organic forms of antioxidant molecules have been studied as natural therapeutic and preventive agents in general, and propolis, which has an important place among these natural agents, in particular, has been excited (Rao et al., 1995; Kanbur et al., 2009).

Propolis (bee glue) is a solid substance which has a natural dark colour produced by honey bees after mixing their own waxes with resinous collected from plants. Afterward, it is utilized for sterilizing hives of honey bees (Cuesta et al., 2005). Traditionally, propolis has been used as a medicine, due to its biological properties such as antimicrobial, antifungal, antiprotozoal, and antiviral (Cuesta et al., 2005). The antimicrobial effect of propolis resulted from the synergistic effects of its components (Koru et al., 2007). Propolis influences the cytoplasmic membrane and inhibits enzyme activity as well as bacterial motility (Mirzoeva et al., 1997). Moreover, its phenolic components extend the capability of cells both to prevent apoptosis contributing and neutralize oxidative stress, due to its anti-inflammatory and antioxidative effects (Geckil et al., 2005).

Fish is one of the most important aquatic organisms which can produce important sources of protein for human nutrition (Duran et al., 2008; Akbary et al., 2011). With regard to its developmental process, the microbial contamination of fish should be considred as highly importance. Fresh fish is a highly perishable product and there are a large

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number of bacteria in the mucus, gills, and gut (Duan et al., 2011). To determine the freshness of fish fillet ,various physical and chemical methods are being used in microbial diagnostics (Scherer et al., 2006). These measurements are pH (Gonzalez-Rodriguez et al., 2001), total volatile basic nitrogen (TVBN), malondialdehyde (MDA*)* (Ruff et al., 2002), and mesophilic and psycrophilic bacteria counts (Scherer et al., 2006).

Distortion process is made initially by muscle enzymes ,and then by microbial enzymes. The most widely used assay for lipid peroxidation is the malondialdehyde (MDA) formation which represents the secondary lipid peroxidation product with the thiobarbituric acid reactive substances test (Draper et al., 1993). Malondialdehyde (MDA) is the final product of lipid peroxidation. The concentration of MDA is the direct evidence of toxic processes, caused by free radicals (Janero, 1990).

Inspired by the afore-mentioned literature, the current study aimed at investigating propolis effects on biochemical parameters (pH, lactic acid, TVB-N, MDA) and microbiological parameters (psychrophilic and mesophilic bacteria) in fillets of rainbow trout which exposed to cypermethrin.

Materials and methods

Experimental phase

Fish were purchasd from Camardı, Ecemis fish farm (Nigde, Turkey) and hold for 15 days in a 8 x 5 x 1.5 m stock pond to be acclimatized. The mean fish weight and length were 248.42± 5.18 g and 29.25 ± 3.94 cm, respectively. Afterward, 8 fish were transferred to 200 L tank filled with distilled water. Physical and chemical parameters of water are illustrated in Table 1.

Table 1: Amount of physical and chemical parameters of water in the current study

Parameter	Before treatment	After treatment
Dissolved oxygen, DO (ppm)	7.8 ± 0.20	7.6 ± 0.10
Chemical oxygen demand, COD	15.1 ± 0.10	16.2 ± 0.20
(ppm)	36.8 ± 1.20	40.1 ± 1.70
Suspended solids (ppm)	126.0 ± 1.50	114.1 ± 1.10
Calcium (ppm)	22.4 ± 0.80	19.7 ± 0.70
Sodium (ppm)	16.0±1.50	18.0±1.40
Chloride (ppm)	5.8 ± 0.20	6.8 ± 0.30
Total nitrogen (ppm)	174.3 ± 3.10	168.2 ± 2.80
Hardness ($CaCO_3$) (ppm)	11.5 ± 10	12 ± 0.70
Temperature (ºC)	7.7 ± 0.10	7.7 ± 0.10
pH		

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Preparation of propolis extractive solution

Propolis was collected from a farm at village Kocaavsar in Balikesir, Turkey. Then, it was dissolved to 30 % in ethanol (Mani et al., 2006). Obtained solution was protected from light and moderately shaken for 1 day at room temperature. Also, the extracts were filtered twice, dried and stored in sealed bottles at 4°C until using (Mani et al., 2006).

Experimental design

Throughout the study, 8 fish were indiviually distributed among 10, 20, 30 ppm propolis treated groups (Talas and Gulhan, 2009). Each rainbow trout was weighted just before the beginning of the study; 10 ppm propolis was treated to the animals in group I for 96 h and not fed for 12 h before; 20 ppm propolis was treated to the animals in group II for 96 h and not fed for 12 h before; 30 ppm propolis was treated to the animals in group III for 96 h and not fed for 12 h before. Totally, 56 specimens in groups exposed to cypermethrin (CYP) and in cypermethrin+propoplis treated groups and in control group (the average weights and lengths of rainbow trouts were 240-250 g and 29.25 ± 3.94 cm, respectively). Forty eight rainbow trouts were then exposed with 0.0041 (CYP I), 0.0082 (CYP II), 0.0123 ppm cypermethrin (Atamanalp et al., 2002a; Atamanalp et al., 2002b) (CYP III) and also administrated groups, 0.0041 ppm cypermethrin + 10 ppm propolis (CYP I + propolis), 0.0082 ppm cypermethrin + 10 ppm propolis (CYP II + propolis), 0.0123 ppm cypermethrin + 10 ppm propolis (CYP III + propolis). Eight fish in last group were used as a control and were killed 96 h later.

Mesurment of pH

In order to determine the pH values of fish fillets, the researchers adopted Ockerman ' method for the puprposes of this study (Ockerman, 1985). Measurements were performed to fillet homogenate, using a pH 211 instrument with a stick probe (Hanna Instruments, Kehl, Germany). Probe insertion was always performed in the same position.

Analytical methods

Measurement of Lactic acid

Lactic acid was measured by the use of a method described by Keller (Keller, 1974). The sample homogenized by a blender (Waring Laboratory, Torrington, CT, USA) was titrated by an Isolab Digitrate 50 ml (Isolab Laborgerate, Wertheim, Germany) and pH was then adjusted to 8.3 with 0.1 N NaOH.

Measurement of TVB-N

The TVB-N content was determined in the fillets by the Kjeldahl method as described by Schormüller (Schormüller, 1968). The homogenized samples were made alkaline by adding MgO (2 g) during distillation,and then distillates were collected in flasks containing 0.1 N H2SO4 and finally titrated with NaOH. The results were presented as mg TVB-N/100 g fillet.

Measurement of Malondialdehyde (MDA)

Lipid oxidation was measured by means of a distillation-colorimetric technique, which was the 2-thiobarbituric acid method (Schormüller, 1969). Absorbance was read

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at 530 nm using a spectrophotometer (6100, Jenway Ltd, Dunmow, UK). Thiobarbituric acid reactive substance levels were expressed as malondialdehyde (MDA, mg/kg) equivalents.

Microbiological analyses

Microorganisms in samples composed of mesophilic and psychrophilic plate counts were enumerated according to a certain method (Anonymous, 1990). For all analytical analyses, flesh samples (10 g of fillet with skin but without scales) were aseptically obtained by cutting slices from the dorsal, ventral, and tail areas. The samples were mixed with 90 ml of serum physiological water solution (0.85% NaCl) ,and then homogenized for 3 min, followed by blending in 0.1% (w/v) sterile peptoned water for 2 min. Further decimal solutions were made up to 10-6 and then 0.1 ml of each dilution was pipetted onto the surface of plate count agar plates in triplicate (Labm-L1010, LabM Ltd, Bury, UK) based on the pour plate method (Anonymous, 1990). Mesophilic and psychrophilic plate counts were determined by counting the colony forming units (CFU) after plates had been incubated at 35°C for 48 h and 10°C for 10 days, respectively (Anonymous, 1990). All counts were expressed as $\log_{10}$ cfu.g^{-1}.

Statistical analyses

Biochemical and microbiological data were analyzed by SPSS 9.0. One-way ANOVA was employed for analysis of data. Means were separated by Duncan's New Multiple Range Test, and were set significantly at $P<0.05$.

Results

pH *Development and Lactic acid content*

The pH value was affected by microbial and enzymatic activities in fillets. These effects on the fish fillets, that were observed through biochemical and microbiological parameters, are depicted in Table 2. The level lactic acid in fish exposed to 10 ppm did not change ($P>0.05$) in comparison to the control group (Table 2), however, there were increases ($P<0.05$) in 20 ppm and 30 ppm propolis concentrations. The findings also showed that there were no statistically significant changes ($P>0.05$) in levels of pH in 10, 20 , and 30 ppm concentrations groups (Table 2). The lactic acid level was increased exposing to cypermethrin ($P<0.05$) but the pH value was not changed ($P>0.05$) as compared to control and propolis groups (Table 3). With regards to the groups together administered cypermethrin and propolis (CYP I + Propolis, CYP II +Propolis, CYP III +Propolis); there were significant decreases ($P<0.05$) in the lactic acid levels as compared to only treated cypermethrin groups (CYP I, CYP II, CYP III). However, no statistically significant changes ($P>0.05$) in pH level have been reported.

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Table 2: Changes on the biochemical and microbiological parameters of rainbow trout fillets in different concentrations of propolis

Groups	Parameters					
	pH	Lactic acid (%)	TVB-N (mg/100g)	MDA (µg/g)	Psychrophilic bacteria Cfu/g	Mesophilic bacteria Cfu/g
Control	7.46±0.11	0.44±0.02^{c}	0.11±0.03^{c}	1.88±0.11^{c}	30.86±0.29^{c}	26.82±1.33^{c}
Propolis (10 ppm)	7.44±0.05	0.46±0.01^{c}	0.13±0.02^{c}	2.02±0.14^{c}	29.14±0.22^{a}	27.10±0.96^{c}
Propolis (20 ppm)	7.42±0.10	0.49±0.02^{b}	0.19±0.02^{b}	2.50±0.10^{b}	27.90±0.18^{b}	33.20±0.65^{b}
Propolis (30 ppm)	7.44±0.08	0.51±0.02^{a}	0.24±0.01^{a}	2.86±0.13^{a}	26.10±0.22^{c}	35.47±0.44^{a}

**Values are presented as mean±SD. Value in the same column having different superscripts are significantly different (P<0.05)

TVB-N content

The TVB-N level in fish exposed to 10 ppm propolis did not change (P>0.05) in comparison to the control group (Table 2), but there were increases (P<0.05) in 20 and 30 ppm propolis groups. Also,there were increases (P<0.05) in the TVB-N levels in cypermethrin groups as compared to both the control and propolis groups (Table 3). there were significant decreases (P<0.05) in the TVB-N level as compared to cypermethrin groups (CYP I, CYP II, CYP III) in the groups together administered cypermethrin and propolis (CYP I + Propolis, CYP II +Propolis, CYP III +Propolis);.

Malondialdehyde (MDA) content

Fish exposed to 10 ppm did not change (P>0.05) the level of MDA as compared to the control group (Table 2), but there were statistically increases (P<0.05) in 20 and 30 ppm propolis concentrations. Cypermethrin groups caused statistically significant increases (P<0.05) in the MDA level in comparison to the control group and propolis groups (Table 3). In the groups, together administered cypermethrin and propolis (CYP I + Propolis, CYP II +Propolis, CYP III +Propolis); there were statistically significant decreases (P<0.05) in the MDA level as compared to only exposed to cypermethrin concentrations (CYP I, CYP II, CYP III).

Microbiological analyses

The counts of mesophilic and psychrophilic bacteria in fish exposed to 10 ppm did not change (P>0.05) as compared to the control group (Table 2), but there were significant increases (P<0.05) in 20 and 30 ppm propolis groups. Therefore, the most convenient concentration is chosen as 10 ppm. Exposing to cypermethrin caused statistically significant increases (P<0.05) in the counts of mesophilic and psychrophilic bacteria as compared to control and propolis groups (Table 3). In the groups together administered cypermethrin and propolis (CYP I + Propolis, CYP II +Propolis, CYP III +Propolis); there were statistically significant decreases (P<0.05) in the counts of mesophilic and psychrophilic

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bacteria as compared only to administered cypermethrin (CYP I, CYP II, CYP III).

Table 3: Changes of biochemical and microbiological parameters in rainbow trout fillets with propolis and different concentrations of cypermethrin

Groups	parameters					
	pH	Lactic acid (%)	TVB-N (mg/100g)	MDA (µg/g)	Psychrophilic bacteria Cfu/g	Mesophilic bacteria Cfu/g
Control	7.46±0.11	0.44±0.02^{c}	0.11±0.03^{c}	1.88±0.11^{c}	30.86±0.29^{c}	26.82±1.33^{c}
Propolis	7.44±0.05	0.46±0.01^{c}	0.13±0.02^{c}	0.02±0.14^{c}	29.14±0.22^{a}	27.10±0.96^{c}
CYP I	7.55±0.02	0.56±0.03^{a}	0.26±0.04^{a}	2.96±0.19^{a}	35.80±0.30^{a}	30.12±0.20^{a}
CYP II	7.37±0.05	0.69±0.01^{a}	0.29±0.02^{a}	3.50±0.12^{a}	36.10±0.24^{a}	33.90±0.34^{a}
CYP III	7.51±0.04	0.71±0.01^{a}	0.32±0.03^{a}	3.91±0.16^{a}	36.02±0.17^{a}	37.20±0.60^{a}
CYP I + Propolis	7.45±0.04	0.47±0.02^{b}	0.19±0.02^{b}	2.26±0.13^{b}	32.50±0.40^{b}	28.40±0.32^{b}
CYP II +Propolis	7.46±0.03	0.53±0.02^{b}	0.22±0.03^{b}	3.02±0.14^{b}	32.20±0.56^{b}	29.24±0.36^{b}
CYP III +Propolis	7.44±0.03	0.58±0.02^{b}	0.24±0.02^{b}	3.36±0.18^{b}	33.40±0.30^{b}	34.12±0.20^{b}

**Values are presented as mean±SD. Value in the same column having different superscripts are significantly different (P<0.05).

Discussion

Toxic matters can have a major role in the development and progression of many diseases and damage processes in fish tissues. Pesticide damage is an important factor in many pathological and toxicological processes that can be taken into account. Accumulation of pesticides in organisms living in contaminated water is an important aspect for environment because it may affect all the members of food chain, including fish (Tarras-Wahlberg et al., 2001). Industrial and agricultural activities are among the potential sources of pollutant accumulation in aquatic environment (Tarras-Wahlberg et al., 2001). Thus, fish is an important source of pollutant which is transferred to humans via food chain (Tüzen, 2003). The concentration of contaminants in commercial fish play as important role in human heath issues (Cid et al., 2001). To predict the impact of environmental pollution on living systems, there has been an increasing trend toward using pollutants in controlled medium to monitor the biological changes occurring in organisms (Lemly, 2002; Lal Shah, 2010). Also, Certain concentrations and periods are being utilized, and target tissues are examined for required parameters in such studies. Aquatic organisms were chosen as test species because of their filtration capacity, ease of caging, and sensitivity to oxidative damage from concerning chronic exposure or sublethal concentrations. Aquatic organisms can provide model

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systems for the investigation of how reactive oxygen species damage cellular compounds, how cells respond, how repair mechanisms treatment this damage, and how oxidative stress can lead to disease (Rostamzad et al., 2010). Oxidative stress has become an important item for aquatic toxicology (Di Giulio et al., 1989; Winston and Di Giulio, 1991; Livingstone 2001). Most recent studies have confirmed that natural protective compounds have gained popularity day by day as some of the widely used synthetic pharmaceuticals ,and therapeutics might have some unexpective effects (Sforcin et al., 2000; Mani et al., 2006). One can think that certain natural food ingredients would be better and safer than synthetic ones. Many of these compounds, such as plant phenolics, often shown up antioxidant and antimicrobial characteristics; therefore, the addition of these compounds into food products may be helpful to health of the consumers and also to the stabilization of food products (Sforcin et al., 2000; Mani et al., 2006). The use of antibiotics in disease prevention can bring about the emergence of drug-resistant microorganisms and leave antibiotic residues in the fish and in the environment (Esiobu et al., 2002). This study noticeably showed the antimicrobial effect of ethanolic propolis extract in rainbow trout fillets. Previous studies indicated that the antimicrobial and/or antioxidant activities of the components of ethanolic propolis extract could result in better intestinal health and improved digestion and absorption, and thereby improved the growth performance of chick (Seven, 2008; Abd-El-Rhman et al., 2009). The findings of the current study showed that propolis exerted had a significanly effect on some fillet indices. In the same vein, the increased innate immune responses in carp (Chu, 2006) and Nile tilapia (Abd-El-Rhman, 2009) have been also reported after *in vivo* or *in vitro* treatment with propolis. Moreover, results indicated a potential use as antioxidant, antimicrobial agents and immunostimulant of propolis for rainbow trout. Fish are popularly recognized as an excellent source of lipids that are composed of a wide range of important fatty acids. MDA is the most widely occured formation for lipid peroxidation product, with the thiobarbituric acid reactive substances test (Janero, 1990; Draper et al., 1993). The concentration of MDA is the direct evidence of toxic processes caused by free radicals (Sieja and Talerczyk, 2004). MDA is considered to be one of the quality criteria for evaluating the fish freshness (Caggiano, 2000; Haghparast et al., 2010). The rancidity level as a consequence of auto-oxidation formed during the storage of fatty foods like fish is measured by MDA analysis (Kilinc and Cakli, 2004). MDA levels of fish arised at the accordance with increase in concentration of cypermethrin. Consequently, concentration of MDA is the direct evidence of toxic processes, caused by free radicals. Mesophilic and psychrophilic bacteria, and TVB-N increased in cypermethrin concentration groups. Increase in TVB-N levels may be due to bacterial metabolism (Pacquit et al., 2007). Besides, TVB-N content has been shown to be a common indicator of freshness and quality in a variety of fish such as Atlantic

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cod (Botta et al., 1994), sardine (Ababouch et al., 1996; Ozogul et al., 2004) and European eel (Ozogul et al., 2005). The major factor in determining the freshness of seafood is the pH value in fillet,providing that this value above or below of optimal conditions allows the growth of some bacteria. Long-term stress and excessive muscle activity lead to insufficient amount of oxygen. Eventually, lactic acid accumulates in the cells. In order to determine the effect of propolis extract, the researchers used three concentrations (10, 20 and 30 ppm). The relationship between lactic acid accumulation and pH in the tissues is well established (Bodwell et al., 1965). Therefore, lactic acid formation and resultant pH assessment can be regarded as an important indicator for freshness of fish fillet. In this study, there were increases in the lactic acid levels of cypermethrin groups (P<0.05) as compared to control group. But, there was no significant changes (P>0.05) in pH level.

In this study, the researchers considered the determination of fish flesh quality which was influenced by cypermethrin and propolis. The results showed that cypermethrin attributes to the elevation of TVB-N, MDA, lactic acid, mesophilic and psychrophilic bacteria counts. Propolis in trout fillet showed better results in terms of mesophilic and psychrophilic bacteria counts, TVB-N and MDA. Mesophilic and psychrophilic bacteria counts, MDA, TVB-N and lactic acid levels in the fillets fish that administrated propolis were lower as compared to other groups.

Exposure to pesticide will depend on the particular dietary and ecological life styles of the aquatic organisms. Accumulation of pesticides on organisms in contaminated water is an important aspect of environment, because it may affect all members through food chain, including fish. There are natural therapeutic and preventive agents against to cypermethrin damage. Propolis is one of this preventive agents. These natural antioxidants are essential for homeostasis in many biological systems both in fish and in human. Due to antioxidant and preservative properties of propolis, it may both prolong the physiological functions of some aquatic living organisms and contribute to the health benefit of consumers who consume aquatic animals. Totally, we can say that propolis may also significantly influence the certain biochemical and microbiologic functions in fish fillet exposed to toxic matter, especially the fish flesh quality.

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