Effects of propolis on biochemical and microbiological parameters in carp (*Cyprinus carpio*) fillets exposed to arsenic

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Received: April 2013 Accepted: June 2014

Abstract

The purpose of this study was to investigate the therapeutic effects of propolis on biochemical and microbiological parameters in muscle tissues of carp (*Cyprinus carpio*, Linnaeus 1758) exposed to arsenic. A sublethal concentration of arsenic (0.01 ppm) and/or 0.01 ppm propolis were administered to fish for seven days. Biochemical parameters [pH, lactic acid, total volatile basic nitrogen (TVB-N), and malondialdehyde (MDA) values] and microbiological changes (mesophilic and psychrophilic bacteria count) were determined in fillet of carp in control, arsenic only, propolis only and arsenic+propolis treatment groups. Results showed that the levels of MDA, lactic acid and TVB-N increased (p<0.05) while there were decreases total counts of psychrophilic, mesophilic bacteria and level of pH in arsenic group compared to the control group. Additionally, levels of pH, lactic acid, TVB-N, counts of psychrophilic and mesophilic bacteria in arsenic (0.01 ppm)+propolis (10 ppm) group significantly reduced compared to arsenic group (p<0.05). In conclusion, propolis can affects some biochemical and microbiologic functions and quality in the fillet of carp exposed to arsenic.

Keywords: Arsenic, Biochemical parameter, Carp, Fillet, Microbiological analysis, Propolis

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Introduction

Arsenic (As) is the most widespread environmental contaminant which arising through natural phenomena such as the weathering of geochemical sources and from anthropogenic activities such as mining, metal working and coal burning (Zhang *et al*., 2012). Perhaps because of the widespread nature of this toxic substance, biological systems have evolved a mechanism which permit them to survive the presence of reasonable levels of the element by its methylating (Talas *et al*., 2014). Besides the direct exposure of people to As by drinking contaminated water, the As might also be biologically exist to aquatic organisms, such as fish which are used as human food thereby supply an supplement source of As (Datta *et al*., 2009). Therefore, studies concerned to As content in aquatic organisms and sea fish in especially, have interested significant attentions. In the aquatic environment, As exists either as, arsenite and arsenate forms which are inter converted through redox and methylation reactions. These types of As can accumulate in many aquatic organisms which may catalyse the oxidation of arsenite to arsenate and promote the formation of methylarsines through biomethylation reaction (Kavitha *et al*., 2010). Finally, oxidative stress may occur partially with arsenic toxicity (Banerjee *et al*., 2009). Although As is not biomagnified through the food chain, bioconcentration has been observed in various aquatic organisms such as a fish. Freshwater fish uptake As not only through diet by benthic-feeding but also with waterborne across the gill (Talas *et al*., 2012).

To eleminate toxic effects and oxidative damages caused by As, cells have improved defense systems including antioxidant molecules. When toxic agents against the natural protective systems are exceed, exogenous antioxidative and protective compounds must be taken. Thus, the searching of new antioxidant molecules as potential therapeutic agents is an active and interesting field of biochemistry. In recent years, several organic forms of antioxidant molecules have been studied as preventive agents and natural therapeutics (Nabavi *et al*., 2012, 2013; Talas and Gulhan, 2013) particularly, researchers attracted in propolis. It has high antioxidant capacity and is an important agent among the natural antioxidants (Talas and Gulhan, 2009; Kashkooli *et al*., 2011; Gulhan *et al*., 2012; Talas *et al*., 2012). Propolis has noticeable biological activities, particularly antimicrobial effects with rich chemical composition including flavonoids, volatile organic compounds phenolic acids, aglycones and their esters, alcohols, phenolic aldehydes and ketones, quinones, coumarins, steroids, aminoacids from various geographical and botanical origins of the world (Kumazawa *et al*., 2004; Mascheroni *et al*., 2010; Ishida *et al*., 2011). The most studies have been accomplished to investigate the relationship with polyphenolic composition of propolis

due to its antioxidant properties (Talas and Gulhan, 2009; Gulhan *et al*., 2012; Talas *et al*., 2012). There are some physical and chemical methods to analyze freshness of fish fillet (Scherer *et al*., 2006). These measurements include assays of some parameters such as pH (Gonzalez-Rodriguez *et al*., 2001), total volatile basic nitrogen (TVB-N), malondialdehyde (MDA) (Ruff *et al*., 2002) and mesophilic and psycrophilic bacteria count (Scherer *et al*., 2006). Distortion process is made by muscle enzymes and followed by microbial enzymes. MDA is ultimate product of lipid peroxidation. The level of MDA is the direct evidence of toxic process induced by free radicals (Gulhan *et al*., 2012). The aim of present study was to investigate the effects of propolis on biochemical parameters (pH, lactic acid, TVB-N, MDA) and microbiological criteria (psychrophilic and mesophilic bacteria) in the muscle of carp exposed to arsenic.

Materials and methods

Samples

The carp were obtained from Azatli Dam Lake (Nigde, Turkey) and fed for 15 days in a 8 × 5 × 1.5 m stock pond to be acclimated. They were transferred to 200 L tank filled with water. Airflow in tank was continuously provided and fish were given artificial dry food once daily. Average weight of fish was determined as 500-600 g. Water quality parameters were given in Table 1.

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

Parameters (ppm)	Before treatment	After treatment
Dissolved oxygen (mg/L)	7.7 ± 0.3	7.5 ± 0.2
Chemical oxygen demand (mg/L)	14.8 ± 0.2	16.6 ± 0.1
Suspended solids (mg/L)	36.7 ± 1.0	39.8 ± 1.5
Calcium (mg/L)	125.7 ± 1.3	113.9 ± 1.0
Sodium (mg/L)	21.8 ± 0.5	20.1 ± 0.6
Chloride (mg/L)	15.9±1.2	18.3±1.6
Total nitrogen (mg/L)	6.0 ± 0.1	6.9 ± 0.2
Hardness ($CaCO_3$) (mg/L)	173.9 ± 3.0	167.8 ± 2.6
Temperature (°C)	18.5 ± 0.5	20 ± 1.0
pH	7.6 ± 0.4	7.6 ± 0.2

Preparation of propolis extraction solution

Propolis extraction methods may influence on its activity, since various solvents solubilize and extract different compounds. In this work, propolis was collected from a farm at the village, Kocaavsar in Balikesir, Turkey. Propolis was dissolved to 30% in ethanol (30 g of propolis, completing the volume to 100 mL with 70% ethanol), protected from light and moderately shaken for 1 day at room temperature. Afterward, the extracts were filtered by filter paper (Whatman no 4 filter paper) twice, evaporated in a rotary evaporator (Heildolph Heizbad

HB Digit) and stored in sealed bottles at 4°C until use (Talas and Gulhan, 2009).

Experimental design

After acclimatization, fish were randomly assigned to one of 4 experimental treatment groups, each consisting of 28 fish (4 replicate tanks per treatment, each tank containing 7 fish). The average weight of the fish was between 500 and 600 g. Fish in the first group were used as controls and were given no treatment. Propolis at an antioxidant concentration of 10 ppm (Talas and Gulhan, 2009), was administered to the tank water for the second group for seven days, and the fish were not fed for 12 h before the application. Arsenic (As_2O_3) (98% pure; Aldrich) at 0.01 ppm (Schlenk *et al*., 1997) was added to the third group of tanks for seven days, and they were not fed for 12 h before. Both 10 ppm propolis and 0.01 ppm arsenic (As_2O_3) were added to the last group of tanks for seven days; these fish were also not fed for 12 h before (Talas *et al*., 2012). At the end of the treatment period, we randomly sampled 2 fish from each tank for assessment of biochemical parameters; thus 8 fish (4 replicates) were sampled per treatment. The experiments were carried out in accordance with the guidelines for fish research from National Institude of Health and approved by the Ethical Committee of Science Institute at Nigde University, Nigde, Turkey. After these treatments, fish were anaesthetised with clove oil (Mylonas *et al*., 2005). The fillets of carp were removed and used for some biochemical and microbiological analyses.

Biochemical analyses

Mesurement of pH

The method reported by Ockerman (1985) was used to determine the pH values of carp fillets. Measurements were performed to fillet homogenates of carp, using a pH meter with a stick probe (Hanna Instruments, Kehl, Germany).

Measurement of lactic acid and TVB-N

Lactic acid was measured using the method described by Keller (Keller, 1974) and the TVB-N content was determined in the fillets of carp by the Kjeldahl method as described by Schormüller (1968).

Measurement of MDA level

The lipid oxidation in fillets of fish was measured by means of a distillation-colorimetric technique, the 2-thiobarbituric acid method (Schormüller, 1969). Absorbance was read at 530 nm using a spectrophotometer (6100, Jenway Ltd, Dunmow, UK). Levels of thiobarbituric acid reactive substance were expressed as malondialdehyde (MDA, mg/kg) equivalents.

Microbiological analyses

Microorganisms in fillet samples composed of mesophilic and psychrophilic plate counts were enumerated according to a certain method (Gulhan *et al*., 2012). For all analytical analyses, flesh fillet 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 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 triplicates (Labm-L1010, LabM Ltd, Bury, UK) by the pour plate method (Gulhan *et al.*, 2012). 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 (Gulhan *et al.*, 2012). All counts were expressed as log10CFU/g^{-1}.

Statistical analyses

Biochemical and microbiological data were analyzed using SPSS 16.0 for Windows using one-way analysis 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

pH value and lactic acid content

The pH value and level of lactic acid are affected from microbial and enzymatic activities in fish fillets. The effects on the fillets of carp applied to propolis, arsenic and arsenic+propolis observed by biochemical and microbiological assays are shown in Table 2. The pH levels decreased in all of experimental groups compared to control group ($p<0.05$) (Table 2). The lactic acid levels significantly increased in group exposed to arsenic compared to control, propolis and arsenic+propolis groups ($p<0.05$) (Table 2). There was significant decrease in lactic acid in arsenic+propolis group compared to arsenic group ($p<0.05$) (Table 2).

TVB-N content

The microbiological and biochemical activities affected the TVB-N levels in carp fillets. The TVB-N levels increased in fish exposed to arsenic compared with control, propolis and arsenic+propolis groups ($p<0.05$) (Table 2). There was decrease in the TVB-N amount in arsenic+propolis group according to arsenic group ($p<0.05$) (Table 2).

MDA level

MDA level of arsenic group significantly increased according to control, propolis and arsenic+propolis groups ($p<0.05$) (Table 2). MDA level in fillets of fish exposed to arsenic was significantly decreased by treated to propolis ($p<0.05$) (Table 2).

Table 2: Changes of the biochemical parameters in fillets of carp exposed to arsenic by propolis.

Parameters / Groups	pH	Lactic acid (%)	TVB-N (mg/100g)	MDA (mg/kg)
Control	7.21±0.02^{a}	0.242±0,003^{d}	0.046±0.001^{c}	3.04±0.02^{b}
Propolis (10 ppm)	6.59±0.02^{cb}	0.474±0.006^{c}	0.050±0.001^{cb}	3.03±0.19^{b}
Arsenic (0.01ppm)	6.61±0.01^{b}	0.563±0.002^{a}	0.067±0.001^{a}	4.31±0.35^{a}
Arsenic + Propolis (0.01ppm+10 ppm)	6.54±0.01^{c}	0.528±0.001^{b}	0.053±0.001^{b}	3.26±0.26^{b}

All data show the average of n=8 with ±SD. ^{abc}statistically significant (p<0.05)

Microbiological analyses

The quality of fish fillets was affected from the changes in mesophilic and psychrophilic bacteria counts. Mesophilic and pshycrophilic bacteria counts significantly decreased in fillets of carp in all of experimental groups compared with control group (p<0.05) (Table 3). The highest levels of decreaeses in mesophilic and pshycrophilic bacteria counts were observed in arsenic+propolis group (Table 3). Mesophilic and pshycrophilic bacteria counts in arsenic+propolis group significantly decreased according to arsenic group (p<0.05) (Table 3).

Table 3: Changes of microbiological parameters in carp fillets exposed to arsenic by propolis.

Parameters / Groups	Psychrophilic bacteria (Cfu/g)	Mesophilic bacteria (Cfu/g)
Control	227.63±2.49^{a}	538.33±40.86^{a}
Propolis (10 ppm)	20.66±2.75^{c}	332.95±13.68c
Arsenic (0.01ppm)	118.59±3.08^{b}	429.54±3.93^{b}
Arsenic + Propolis (0.01ppm+10 ppm)	13.41±1.07^{c}	168.18±6.42^{d}

All data show the average of n=8 with ±SD. ^{abc}statistically significant (p<0.05)

Discussion

Aquatic areas are the last set for most chemical matters and water can be the vehicle for exposure to many toxic agents. The existing of contaminants in aquatic habitats affects the health and survival of fish and the immune system (Datta *et al*., 2009). However, it is extremely sensitive to both chemical and microbiological degredations, for its high water activity, neutral pH, comparatively great quantities of free amino acids, and existence of autolytic enzymes (Duan *et al*., 2010). Carp (*C. carpio*) is one of the main culturing species of fishes. Stressful environment renders the fish highly sensitive to some diseases (Sheikhzadeh *et al*., 2011). Besides, it has been very rich in omega-3 fatty acids (Olsen *et al*., 2013). This study showed the antimicrobial effect of ethanolic extract of propolis in carp

fillets. Earlier studies indicated that the antimicrobial and antioxidant capacities of propolis extract including various components could be very good for intestinal health and improved absorption and digestion, and therefore developed the evolution process of fish (Abd-El-Rhman, 2009; Yonar *et al*., 2012). The findings of the present work indicated that propolis as natural agent has significant effects on fish fillet. The increased innate immune responses in carp (Chu, 2006) and Nile tilapia (Abd-El-Rhman, 2009) after *in vivo* or *in vitro* treatment with propolis have been also reported. Results of our investigation show a potential use in carp muscle as antioxidant and antimicrobial agents of propolis. 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 occurred by lipid peroxidation and assayed with the thiobarbituric acid reactive substances (Gulhan *et al*., 2012). The concentration and formation of MDA are the direct evidence of toxic process caused by free radicals (Sieja and Talerczyk, 2004). MDA level increased in muscle tissue of fish exposed to arsenic. Consequently, It has been observed that concentration of MDA as an evidence of result of toxic effects caused by free radicals increased. In the secondary oxidation stage, volatile compounds (e.g. alcohols and aldehydes) are formed by the decomposition of lipid hydroperoxides. In particular, volatile aldehydes have great importance as an indicator of oxidation due to their considerable contribution to the aroma and flavor deterioration of the final products. Lactic acid, MDA and TVB-N levels increased in arsenic group. TVB-N content has been shown to be a common indicator of freshness and quality of fillets in a variety of fish such as Atlantic cod (Botta *et al*., 1994), sardine (Ababouch *et al*., 1996), hybrid catfish (Chomnawang *et al*., 2007) and rainbow torut (Gulhan *et al*., 2012), *Anguilla anguilla* (Ozogul *et al*., 2005). The pH value in fish fillet is an important factor to determine the freshness of seafood if 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. Finally, lactic acid accumulates in the cells. The relationship between lactic acid accumulation and pH in the tissues is well established (Bodwell *et al*., 1965). Therefore, lactic acid formation and pH assessment can be used as an important indicator for freshness of fish fillet. In the present study, there was an increase in the lactic acid level of arsenic group ($p<0.05$) compared to control group. On the contrary, pH level decreased in arsenic group ($p<0.05$) compared to control group. In this work, it has been paid attention to determine the flesh quality of fish influenced by arsenic and propolis. The results indicated that arsenic attributes the elevation of TVB-N, MDA, lactic acid levels. Propolis has the positive effects on carp fillet in respect of biochemical and microbiological analyses. Mesophilic and psychrophilic

bacteria counts, MDA, TVB-N and lactic acid levels in the fillets of fish treated with propolis were lower compared to other experimental groups. Results of this study are in well accordance with the data of previously reported (Duran and Talas, 2009; Gulhan *et al*., 2012; Talas and Duran, 2012; Talas *et al*., 2012). The exposure to arsenic is depend on the particular dietary and ecological lifestyles of the aquatic organisms. The accumulation of arsenic in organisms in contaminated water is an important aspect of environment, because it may affect all members through food chain, including fish. A large number of natural agents have been used in traditional medicine for the treatment and control of several diseases (Sheikhzadeh *et al*., 2011; Talas and Gulhan, 2013). There are natural therapeutic and preventive agents against arsenic damage too. Propolis is one of these natural preventive agents. These natural antioxidants are essential for homeostasis in many biological systems such as fish and 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.

In conclusion we can say that propolis may significantly influence the certain biochemical and microbiologic functions in the fillets of fish exposed to toxic matter, especially the flesh quality of fish. Arsenic may cause oxidative stress and alterations in some biochemical and microbiological properties of the muscle tissue of carp. Our data indicated that propolis can repair the deterioration caused by arsenic in carp which was showed by analyses of biochemical and microbiological levels. The biological effects of propolis are mostly attributed to the phenolic components such as flavonoids. It has been emphased that flavonoids have biological activities, including antibacterial, antiviral, anti-inflammatory and antioxidant effects (Talas and Gulhan, 2009; Kashkooli *et al*., 2011; Gulhan *et al*., 2012). The treatment of propolis showed a protective effect against arsenic damage in carp fillet. Propolis influences the cytoplasmic membrane and inhibits enzyme activities as well as bacterial motility (Mirzoeva *et al*., 1997). The antimicrobial properties of propolis depend on the synergistic effects of its components (Koru *et al*., 2007). The phenolic components of propolis have the capability of neutralize the oxidative stress due to its antiinflammatory and antioxidative activities (Gulhan *et al*., 2012; Talas *et al*., 2012). The antioxidant compounds can be used to eleminate the damages of toxic matters such as arsenic. The propolis exists in natural environmental conditions and has biologically effective functions on living organisms that live in these natural areas. In conclusion, we suggest that propolis may use as a protector agent against toxic materials such as arsenic and their oxidative damage on fillets of carp.

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