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Protective Effect of Propolis against Chromium Toxicity in Nile tilapia: Evaluation of Fillet Quality, Geno-oxidative and Pathological Alterations in Liver

Nermeen Borai El-Borai[1]*, Mostafa Abd El-Gaber Mohamed[2], Mohamed A. Khalaf[3], Mohamed Hamada[4], Hanim S.Heikal[5] and Hamada D.H. Mahboub[5]

[1]*Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt.*
[2]*Department of Pathology, Faculty of Veterinary Medicine, Menoufia University, Shebin Elkome, Egypt.*
[3]*Department of Aquatic Animals Medicine and Management, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt*
[4]*Department of Food Hygiene & Control, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt.*
[5]*Department of Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt.*
** Name and address of Corresponding Author:*
Nermeen Borai Ahmed El-Borai, PhD, Dep. of Forensic Medicine & Toxicology, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, 32897, Egypt.
Email: nermeenborai@yahoo.com

Abstract: *Using of natural products can improve fish health status and may protect fish against aquatic pollutants. The present study was designed to evaluate the impact of propolis- enriched diet on chromium affecting fillet quality and inducing oxidative, genetic and pathological alterations in the liver of Nile tilapia. Fish was exposed to propolis at 2% of diet 4 weeks before and 6 weeks concomitantly with potassium dichromate (PDC) at 9 mg.L^{-1}. Exposure of Nile tilapia to PDC induced deleterious changes in the keeping quality parameters of fish fillet with significant increase in its chromium content. Significant elevation of hepatic MDA level concurrently with significant reduction of GSH content; CAT and SOD activities were also recorded. Furthermore, PDC induced DNA damage of tilapia hepatocytes in association with hepatic vascular, inflammatory, degenerative and necrotic changes. Propolis- enriched diet improved fillet quality and ameliorated toxic effects induced by PDC. It was concluded that propolis can be used as a natural food additive for fish to improve fillet quality and could be of great value to introduce therapeutic strategies for treatment and prevention of fish toxicities.*

Keywords: *Chromium, Propolis, Nile tilapia, Fillet quality, DNA.*

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I. Introduction

The indiscriminate discharge of untreated waste water, originating from various industrial, agricultural and domestic sources, into the rivers is considered the ultimate cause of water pollution in Egypt[1]. Among pollutants, heavy metals are a core group of aquatic pollutants because of the incredible increase in the concentrations released into the aquatic environment that often exceed the permissible limits[2]. However, some of the metals including Cr, Ni, Pb and Hg are toxic, others such as Zn, Se, and Mn are biologically essential for growth and become toxic only at very high concentrations[3]. Anthropogenic activities increase discharge of both essential and nonessential metals into aquatic ecosystems[4].

Owing to their bio-accumulative and non-biodegradable properties[5], metals accumulate in aquatic organisms and persist in water and sediments[6]. Generally, fish are by far, the most relevant organisms for pollution monitoring in aquatic ecosystems[7] and are used to highlight the potential risk to the human beings exposed to polluted water or fish that because fish can accumulate large amounts of heavy metals from polluted water[8].

Chromium, a heavy metal, presents in the environment in several different forms. Among them, trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) chromium are the most stable forms and Cr^{6+} is the most toxic[9]. The effluents discharged from industrial activities mainly mining, printing, electroplating, polishing, dyeing, leather tanning, plastic, ceramics, fiberglass, photographic and pharmaceutical industries have chromium in different

valence state[10]. Its discharge into aquatic ecosystem exerts great threat to growth and survival of fish populations, and consequently, to humans.

Exposure of fish to Cr^{6+}induces biochemical and hematological[11], hepatic[12], genetic and mutagenic[13], behavioral[14] and reproductive[15] toxic effects. Pathological alterations in various tissues were recorded in chromium- exposed fish[12,16]. Toxic effects of chromium including its effect on fillet quality may be associated with reactive oxygen species (ROS) production and alterations of the endogenous antioxidants[17]. Natural antioxidants strengthen the endogenous antioxidants defenses and restore the optimal balance by neutralizing ROS[18]. So, a great attendance is directed toward the use of natural antioxidants for improving fish health status, and consequently, enhancing the resistance to aquatic pollutants.

Propolis, a natural antioxidant, is a dark-colored resinous hive product collected by honey bees from buds and plants secretions and contains a variety of chemical constituents, mainly, flavonoids, phenolics, terpenoids, amino acids and caffeic acid[19]. Propolis is a generic remedy in folk medicine due to its antimicrobial[20], anti-inflammatory[21], antioxidant[22] and anticancer[23] properties. Nowadays, supplementation of fish diet with propolis is promising benefits for fisheries due to its improving effect on the growth performance and immunity of fish[24]. Also, propolis is effective as a natural food additive to improve the quality of fish products; accordingly, they increase their shelf-life[25].

Nile tilapia (*Oreochromis niloticus)* is an important commercial fish in Egypt[26]. It is a vital component of human nutrition and widely consumed as a protein source so fish pose risk to human health via food chain. In this perspective, the present study was proceeded to determine the influence of propolis- enriched diet on chromium affecting fillet quality and inducing oxidative, genetic and pathological alterations in the liver of Nile tilapia.

II. Materials and Methods

The study was ethically approved by the International Animal Care and Use Committee IACUC, Faculty of Veterinary Medicine, University of Sadat City.

- **Experimental Fish and management:**

One hundred and twenty apparently healthy Nile tilapia (*Oreochromis niloticus*) fish with average weight 20±3g were obtained from a private fish farm in Kafr El Sheikh Governorate, Egypt. Fish were kept in 100 L glass aquarium supplied with running water and constant aeration. Fish were fed twice daily with commercial pellets. The water quality parameters were monitored and kept within the recommended ranges during the experimental period. Fish were acclimatized for 14 days prior to the beginning of the experiment.

- **Chemicals*:***

Chromium as potassium dichromate "PDC", K2Cr2O7, (MERCK, Mumbai, India; Purity 99%) was purchased from El-Nasr company, Egypt. Propolis in a powder form was obtained from apiary at Sadat City, Egypt. Chemicals used for assessment of hepatic total protein, MDA, GSH levels; CAT, SOD activities were obtained from Sigma Company, Egypt. All other chemicals and reagents were of analytical grade and commercially available.

- **Diet preparation:**

Crushed commercial basal diet was divided into two portions. The first one was kept as control, while the second portion was thoroughly mixed with propolis. Propolis was dissolved in distilled water and added to diet at 2%. Moist diet was left for 24 hours at room temperature for dryness then kept in glass jar. Fish fed twice daily at the rate of 3% of fish biomass and adjusted every two weeks according to fish weight.

- **Experimental Design:**

Following acclimatization, fish were divided randomly into four groups, with two replicates per treatment. **Control group** was fed basal diet for 10 weeks. **Propolis group** was fed propolis- supplemented diet (2%) for 10 weeks. **PDC group** was exposed to 9 $mg.L^{-1}$ PDC for the last 6 weeks. **Propolis+PDC group** was fed propolis- supplemented diet 4 weeks then 6 weeks concomitantly with PDC. The selected dose of PDC is equivalents to 1/10 LC50[27].

- **Collection of samples:**

At the end of the experimental period (10^{th} week), fish were euthanized with tricaine methanesulfonate and dissected for samples collection. Fillet of each fish was stored at −20°C for further estimation of chromium residues and keeping quality parameters. Liver from each fish was collected and divided into 3 parts, one part was kept at −20°C for assessment of oxidant/antioxidant biomarkers and the other part was placed in phosphate buffer saline (BPS) for comet assay. Additional part of liver was fixed for 3 days in 10% neutral buffered formalin for histopathological examination.

- **Biochemical analysis of tilapia fillet:**

Chromium residues:

Tilapia fillet samples were digested according to Staniskiene et al.[28]. Samples were analyzed for the presence of chromium residues by atomic absorption spectrophotometer (Pye Unicam SP9) according to Andreji et al.[29].

Keeping quality tests:

The pH, total volatile base nitrogen (TVB-N), thiobarbituric acid (TBA) and peroxide values were estimated according to the methods described by Pearson[30], FAO[31], Pikul et al.[32] and Asakawa and Matsushita[33], respectively.

- **Hepatic Oxidant/ antioxidant biomarkers:**

Liver specimens were homogenized[34] for estimation of total protein, malondialdehyde (MDA) and reduced glutathione (GSH) contents; catalase (CAT) and superoxide dismutase (SOD) activities according to the methods adopted by Lowry et al.[35], Mihara and Uchiyama[36], Beutler et al.[37], Aebi[38] and Giannopolitis and Ries[39], respectively.

- **Comet assay of hepatocytes:**

Comet assay was performed using the method of Ellahueñe et al.[40]. The prepared slides of hepatic cells were stained with Ethidium Bromide and out of 100 randomly selected cells in each slide were photographed and scanned. The obtained data were analyzed by DNA damage analysis software (Loats Associates Inc., USA). DNA damage was determined by evaluation of tail length and DNA% in tail and calculation of tail moment that expressed as, tail length × DNA% in tail /100.

- **Histopathological investigation:**

The formalin fixed hepatic tissues were routinely processed, embedded in paraffin, cut into 4 µm sections and stained with Hematoxylin and Eosin (H&E) [41].

- **Statistical analysis:**

The obtained data are presented as mean ± SE. Statistical analysis was determined by one-way ANOVA followed by Duncan's Multiple Range test for post hoc analysis. All statistical analyses were performed using SPSS, Version 16 released on 2007. Differences were considered statistically significant at $P< 0.05$.

III. Results

Chromium residues and keeping quality parameters of tilapia fillet:

Chromium residues were not detected (ND, under the detectible limit) in tilapia fillet of both control and propolis groups. Significant elevation in chromium content was recorded in PDC group when compared to control group at $P<0.05$. However, pre- and concomitant exposure to propolis with PDC significantly reduced chromium content in tilapia fillet, compared to PDC group (table 1).

Insignificant changes were recorded in the mean values of pH, TVBN, TBA and peroxide levels of tilapia fillet between control and propolis groups. Exposure to PDC induced significant ($P<0.05$) elevations in these values. Nonetheless, fish exposed to propolis+PDC showed reduction of the elevated values when compared with the corresponding values of PDC group and restored all values to be within normal control ranges (table 1).

Table 1. Chromium residues and keeping quality parameters levels in tilapia fillet of the different groups.

Group/Parameter	Chromium (mg/Kg)	pH	TVN-B (mg/100g)	TBA (mg MDA/Kg)	Peroxide (meqO2/kg fat)
Control	ND	5.83±0.031^{bc}	5.13±0.47^{b}	0.11±0.02^{b}	0.21±0.03^{b}
Propolis	ND	5.76±0.030^{c}	4.34±0.45^{b}	0.11±0.01^{b}	0.18±0.03^{b}
PDC	1.75±0.058^{a}	6.05±0.064^{a}	9.79±1.47^{a}	0.26±0.05^{a}	0.51±0.09^{a}
Propolis+PDC	1.06±0.064^{b}	5.93±0.039^{ab}	6.38±0.77^{b}	0.18±0.03^{ab}	0.34±0.06^{ab}

Values are expressed as Mean ± SE. Means in the same column followed by different superscripts are significantly different at P<0.05.

Oxidant/ antioxidant biomarkers in liver tissue of tilapia fish:

Regarding to control group, insignificant differences in the mean value of MDA level concomitantly with significant ($P<0.05$) elevation in the mean values of GSH content; CAT and SOD activities were recorded in hepatic tissue of propolis group. PDC- exposed fish showed the highest MDA level and the lowest GSH

content; CAT and SOD activities. However, these changes were ameliorated in propolis+PDC group and restored only the normal control value of MDA (table 2).

Table 2. Oxidant/ antioxidant biomarkers in liver tissue of tilapia fish of the different groups.

Group/Parameter	MDA (µmol/mg protein)	GSH (µmol/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)
Control	0.81±0.03^{b}	3.31±0.04^{b}	2.72±0.06^{b}	2.01±0.04^{b}
Propolis	0.77±0.02^{b}	3.43±0.06^{a}	2.91±0.04^{a}	2.57±0.05^{a}
PDC	1.45±0.04^{a}	1.81±0.02^{d}	1.22±0.01^{d}	0.88±0.02^{d}
Propolis+PDC	0.85±0.03^{b}	2.84±0.01^{c}	2.54±0.05^{c}	1.72±0.01^{c}

Values are expressed as Mean ± SE. Means in the same column followed by different superscripts are significantly different at P<0.05.

Comet assay findings of hepatocytes:

From the obtained results (Table 3 and Figure 1), the mean values of tail length, tail DNA% and calculated tail moment of tilapia hepatocytes did not show significant variations between control and propolis groups. Conversely, significant elevations were observed in the corresponding values of PDC group. Significant improvement in these values was recorded in propolis+PDC group, compared to PDC group. However, these values were still significantly higher (*P<0.05*) than control values.

Table 3: Comet assay parameters in hepatocytes of tilapia fish of the different groups.

Group/ Parameter	Tail Length (µm)	Tail DNA (%)	Tail Moment (Unit)
Control	7.7±0.232^{c}	0.81±0.048^{c}	0.06±0.004^{c}
Propolis	7.5±0.194^{c}	0.72±0.028^{c}	0.05±0.002^{c}
PDC	18.3±0.657^{a}	2.41±0.086^{a}	0.44±0.021^{a}
Propolis+PDC	10.4±0.410^{b}	1.59±0.014^{b}	0.17±0.006^{b}

Values are expressed as Mean ± SE. Means in the same column followed by different superscripts are significantly different at P<0.05.

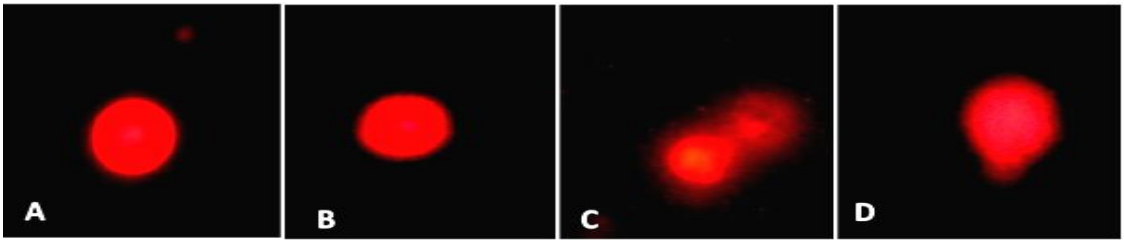

Fig.1. Comet assay images of tilapia hepatocytes, Ethidium bromide stain X400. **A** (control group), **B** (propolis group) showing intact DNA, **C** (PDC group) showing tailed DNA, and **D** (propolis+PDC group) showing slightly intact DNA.

Histopathological findings of liver:

Table (4) illustrated the semi-quantitative scoring of histopathological changes in liver of Nile tilapia of the different groups. Microscopical examination of liver of Nile tilapia of control (Fig. 2 A) and propolis (Fig. 2 B) groups showed no histopathological changes. Exposure of fish to PDC showed different hepatic pathological alterations (Fig. 2 C, D & E), including, severely congested blood vessels which were surrounded by mononuclear inflammatory cells (Fig. 2 C), vacuolar degeneration of hepatocytes with prominent nucleoli (Fig. 2 D), necrosis and lysis of some hepatocytes beside necrosis of hepato-pancreases (Fig. 2E). However, liver of propolis+PDC group revealed moderately congested blood vessels and hepatic sinusoids with more or less normal hepatic parenchyma (Fig. 2 F).

Table 4: Semi-quantitative scoring of histopathological changes in liver of Nile tilapia of the different groups.

Group/ Pathological changes	Vascular changes	Inflammatory changes	Degenerative & necrotic changes
Control	-	-	-
Propolis	-	-	-
PDC	+++	+++	+++
Propolis+PDC	++	-	-

The histopathological changes are graded as follows: (-) None, (+) mild< 20%, (++) moderate<50% and (+++) sever> 50% of the examined tissue.

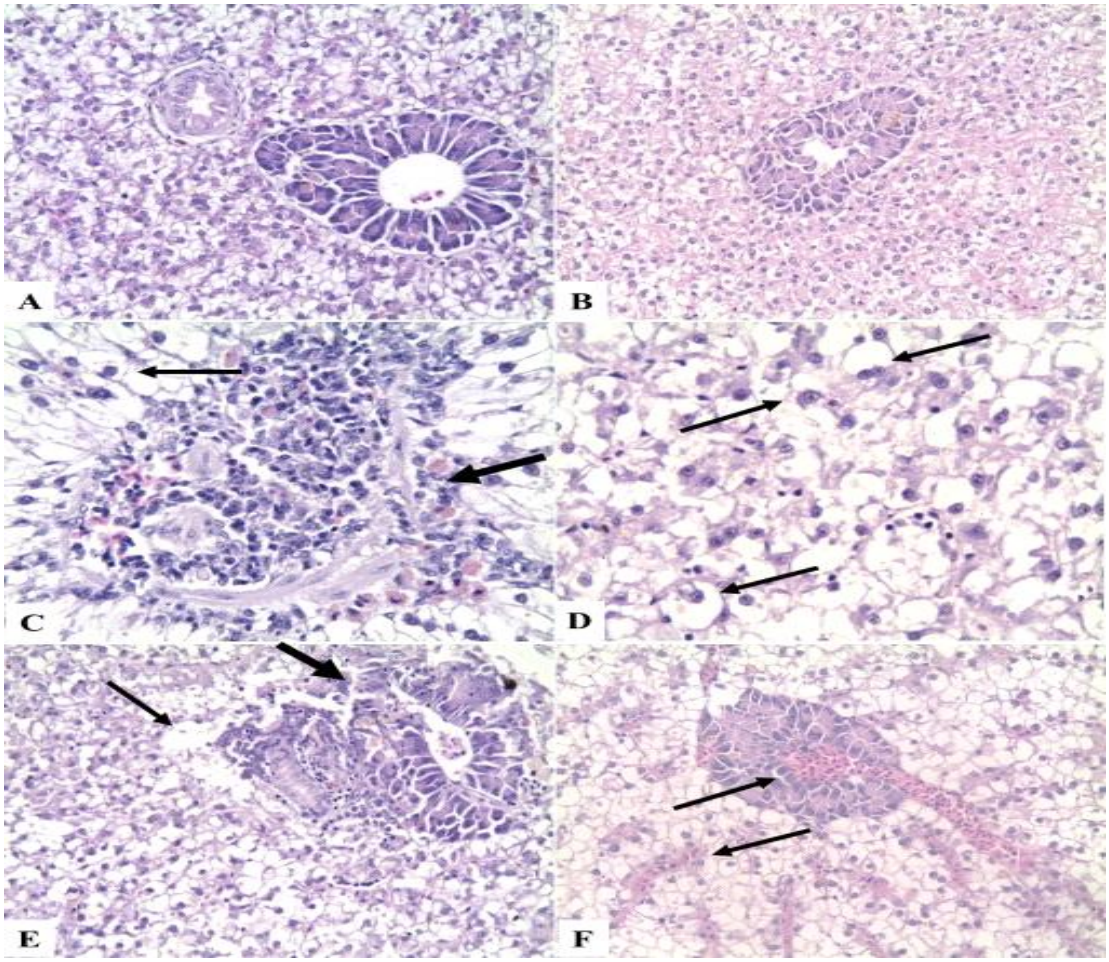

Fig. 2: Photomicrographs of liver tissue of Nile tilapia, **A**: Control group and **B**: Propolis group showing normal structure (H&E X20). **C, D & E**: PDC group, **C**: showing severely congested blood vessels surrounded by mononuclear inflammatory cells (thick arrow), vacuolar degeneration of hepatocytes with pyknotic nuclei (thin arrow) (H&E X40), **D**: showing vacuolar degeneration of hepatocytes and prominent nucleoli (black arrow) (H&E X40) and **E**: showing necrosis and lysis of hepatocytes (thin arrow) and necrosis of hepato-pancreases (thick arrow) (H&E X20). **F**: propolis+PDC group showing moderately congested blood vessels and hepatic sinusoids (thin arrow) (H&E X20).

IV. Discussion

Aquatic pollution by heavy metals from anthropogenic sources evoked major environmental and health concerns worldwide[10]. Such pollution exhibits deleterious effects on the fish and elicits public health problems[27]. Generally, heavy metals including, chromium exert their toxic effects in organisms by generating reactive oxygen species (ROS) resulting in oxidative stress[42].

Focusing on the effective role of natural antioxidants in controlling lipid peroxidation and restraining its harmful outcomes, this study was investigated to evaluate the protective effect of propolis- enriched diet on chromium affecting fillet quality and inducing oxidative, genetic and pathological alterations in the liver of Nile tilapia.

Fish are often at the top of the aquatic food chain but concentrate considerable amounts of certain metals from water[8]. Therefore**,** this may affect their fillet quality. In addition to the detection of metal content in fish fillet, estimation of various biochemical parameters, including, pH, TVB-N, TBA and peroxide levels may be valuable for assessment of fillet quality of fish.

The current study showed that PDC induced significant elevation of chromium, pH, TVB-N, TBA and peroxide values of fish fillet. El-Shafei[43] reported that chromium can accumulate in muscle of Nile tilapia. Fillet quality is affected by the rate of pH inclination and by the elevation in TVBN, TBA and peroxide values after scarifying, slaughtering or death. The decline of pH in fish fillet occurs due to anaerobic glycolysis, as the formed lactic acid reduces muscle pH and helps the muscle conversion to meat[44]. Exposure to PDC induced significant increase in pH value. Although, the recorded pH value was within the standard acceptable limit, it inclined to alkalinity and consequently, affects the quality and the shelf-life of tilapia fillet.

Total Volatile Bases Nitrogen (TVB-N) is a product of proteolytic changes in fish fillet that it can be considered spoiled if TVB-N level lies between 35–40mg/100g[45]. Thiobarbituric acid (TBA) and peroxide values are used as an index for lipid oxidation in fish and its products. Fish fillet is considered unfit for human consumption when TBA value is greater than 2.7 mg MDA/Kg[46] and peroxide value is greater than 10 meqO2/kg fat[47]. The recorded increase in TVB-N, TBA and peroxide values were within the standard acceptable limit, though it significantly inclined to unacceptability of fillet and may affect the shelf-life of fish fillet. In this study, the recorded alterations in the keeping quality parameters may be due to the ability of chromium to produce ROS that initiate lipid peroxidation[17].

Liver is the main site of xenobiotics detoxification or accumulation and, hence, more susceptible to damage. The studies of oxidative stress and DNA damage along with histopathological examination of liver may provide the complete risk assessment of the hepatotoxic potential of chromium to aquatic organisms, particularly on fish, and the prospective ameliorative effect of propolis as a natural antioxidant. Oxidant/ antioxidant imbalance is a biomarker of fish exposure to metals[48]. The present investigation demonstrated that exposure of fish to PDC caused oxidative stress in liver as evidenced by the significant increase of MDA level, suppression of GSH content; CAT and SOD activities. Previous literature recorded similar results in liver of goldfish[49], kidney of cat fish[50] exposed to chromium. Chromium- induced hepatotoxicity may be due to inhibition of cellular antioxidants, together with the augmented production of ROS that initiate lipid peroxidation and cause damage of cellular components[17].

Genotoxins as chromium are chemicals capable of causing DNA damage resulting in malignancies, reduction in fish growth, reproduction and survival. Eventually, genotoxins affect the economy of fish production and exert threats to human health via food chain[51]. Micronucleus test, chromosomal aberrations and comet assay have been regarded as helpful indicator for evaluating genotoxicity of chromium[13,52]. Particularly, comet assay is sensitive, reliable and a powerful test used for measuring the degree of DNA damage of aquatic organisms exposed to genotoxins[53]. The result of comet assay in the present study clearly demonstrated the genotoxic effect of chromium on liver of Nile tilapia fish. This was indicated by the significant increase in tail length, DNA % and tail moment in liver cells of the PDC- exposed fish. This also agrees with previous studies[13,16]. The DNA damage observed in the present investigation is the normal consequence of the recorded oxidative stress that may be due to the massive ROS production and the oxidant/ antioxidant imbalance during cellular reduction of Cr^{6+} [16].

Histopathological examination of fish organs, mainly liver, is considered a sensitive tool to reveal toxic effects of various xenobiotics[54]. Our study elucidated that exposure to PDC resulted in several hepatic pathological alterations, including inflammatory, vacuolar degenerative and necrotic changes. These recorded pathological alterations are in agreement with Velma and Tchounwou[16] and Muthukumaravel and Rajaraman[12] who observed pathological alterations in liver of chromium- exposed goldfish and *Labeo rohita*. Such observations were compatible with the recorded geno-oxidative hepatic damage that may be attributed to the higher accumulation of Cr associated with lipid peroxidation and consequently, pathological alterations of the liver.

The present investigation demonstrated that propolis reduced the chromium content in fish fillet. Moreover, propolis ameliorated chromium- induced changes of tilapia fillet quality as evidenced by reduction of the elevated values of pH, TVBN, TBA and peroxide. Similarly, Fuat Gulhan et al.[20] approved the ameliorative effect of propolis against cypermethrine- induced changes in fillet quality of Rainbow trout. Also, Hassanin and El-Daly[25] confirmed that propolis can be used as a natural food additive for tilapia fillet to improve their quality during freezing that may be due to its antimicrobial[20] and antioxidant[22] properties.

Furthermore, the obtained results verified the hepatoprotective effect of propolis against chromium toxicity as evidenced by reduction in MDA levels and elevations in GSH content; CAT, and SOD activities. Additionally, propolis alleviated the hepatic DNA damage and pathological alterations evoked by chromium exposure. In consistent with our result, Ferreira et al.[55] demonstrated that propolis reversed liver, brain and kidney oxidative damages induced by tebuconazole. Talas et al.[56], in turn, showed that propolis alleviated arsenic- induced oxidative damage and pathological alterations in liver of carp. Also, Kandiel et al.[57] recorded geno-protective effect of propolis against malathion toxicity in *Nile tilapia*.

The hepatoprotective effect of propolis could be attributed to its flavonoids and phenolic contents that elicit antioxidant and anti-genotoxic properties[57,58]. The antioxidant properties of flavonoids are attributed to their ability to reduce ROS production, scavenge free radicals and activate endogenous antioxidant enzymes[59].

V. Conclusion

Our current results afford important evidences for studying the effect of chromium toxicity on fish fillet quality and the mechanisms involved in chromium-induced liver damage. Moreover, it could be of great value in introducing propolis as one of the therapeutic strategies for improving fillet quality, protecting fish against toxicities and may provide health benefits for consumers.

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Conflict Of Interest

The authors declare that they have no conflict of interest.

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