Biochemical and histopathological effect of propolis and nanopropolis supplementation on alleviating dietary *Microcystis aeruginosa* toxicity on Nile tilapia, *Oreochromis niloticus*

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A B S T R A C T

The objective of this research was to clarify the potential impacts of propolis and its nanoparticles in protecting an edible and economically significant tilapian fish, *Oreochromis niloticus* in Egypt after feeding *M. aeruginosa* cells mixed with their food on biochemical variables and histopathological changes of liver under laboratory conditions. The results showed that the diet containing *M. aeruginosa* cells caused aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), creatinine and urea to increase significantly compared to the control groups. Various histopathological modifications were noted in the liver tissues which were described by marked degenerative changes within the hepatic and pancreatic portion. Whereas propolis and nanopropolis co-administration causes these parameters to significantly decrease with best result for nanopropolis. The study concluded that the uptake of *M. aeruginosa* orally had deleterious impacts on some clinical and biochemical parameters of the blood of Nile tilapia that ultimately impacted the health of fish. The current findings evoked that the administration of propolis and its nanoparticles increases fish health and soothes *M. aeruginosa* induced toxicity. Nano form of propolis is more efficient in competing toxicity of *m. aeruginosa* and as hepatoprotective agent.

Keywords: *Oreochromis niloticus; Microcystis aeruginosa; Propolis; Nanopropolis; Histopathological; Biochemical.*

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1. INTRODUCTION

Toxin-producing cyanobacterial blooms, which are primarily driven by anthropogenic pollution from sources such as urban development, industrial emissions, transportation and the synergistic impacts of global climate change , are growing worldwide (Lu *et al.*, 2018). The blooms have an effect on human health and ecosystems by generating powerful toxins or hypoxic areas, that influence water and habitat quality, drinking water safety and food webs (Song *et al.*, 2017; Qian *et al.*, 2019).

Microcystis aeruginosa (M. aeruginosa) is the main species in blooming Cyanobacteria and a ubiquitous toxin-producing cyanobacterium widely distributed in freshwater lakes and

reservoirs worldwide (Liu *et al.*, 2018). Various Cyanobacteria genera known to produce Microcystins (MCs) which considered to be the primary toxic metabolites damaging to aquatic biota, specially fish (Xu *et al.*, 2016). Microcystin ingestion can cause gastrointestinal distress, liver failure, neurological problems, and death in humans and other mammals, and skin contact can cause contact dermatitis (US EPA 2014). MCs are regarded to be the most hazardous group, primarily because they accumulate powerful hepatotoxins (Ikehara *et al.*, 2015) in a wide range of aquatic biota such as fish (Bieczynski *et al.*, 2013). The toxicological impacts of the toxic cyanobacteria and their toxins in fish have been extensively evaluated and discussed before. Whereas there are various indications of hepatotoxicity and renal toxicity histopathological signs were shown in *Cyprinus carpio* (Fischer and Dietrich 2000), *Oreochromis mossambicus* (Kanchana *et al.* 2012), and in *Oreochromis niloticus* (Sanad *et al.* 2015; Abdel-Latif and Abou Khashaba 2017).

Propolis is a brownish resinous material gathered from the leaf buds of trees by worker bees. Because of its antioxidant and preservative impacts, propolis may both extend some aquatic organisms physiological functions and add to the health advantages of consumers of aquatic animals (Gulhan *et al.* 2012). The flavonoids contained in propolis react to antibacterial activities (Barud *et al.*, 2013). (Schmidt *et al.* 2014) and (Kothai and Jayanthi 2014) indicated that propolis is effective against the inhibitory effects of free radicals and can behave as an antibacterial. In fish, propolis was widely used as immunostimulant (Talas and Gulhan, 2009) and hepatoprotective agent (Deng *et al.*, 2011).

Nanotechnologies have broad application in fishery industry (Huang *et al.*2015). The body absorbs nanopropolis more readily because it has a lower size. Thus in terms of antibacterial and antifungal activity nano-propolis may be more effective than propolis. It has been very efficient in treating of rat mammary gland tumors, breast cancers (Hasan *et al.*2016) and against negligible illness such as leishmaniosis (Nascimento *et al.* 2016).Yet to our knowledge there seems to be no literature about using propolis or its nanoparticles to control *M. aeruginosa* toxicity.

In this context, the current research was conducted to assess the potential protective impacts of propolis and propolis nanoparticles on dietary *M. aeruginosa* cells toxicity on biochemical variables as well as to assess the histopathological changes on hepatic tissue of Nile tilapia to provide data about the public health issue arising from human consumption.

2. Materials and methods

Experimental fish:

180 tilapia fish (*Oreocromis niloticus*) of a body weight 30 g±5 has been obtained from National Institute of Oceanography and Fishries (NIOF) Serw farm and transferred to the Wet lab in NIOF. All fish were acclimated for two weeks in stock aquaria and then randomly divided into 6 groups ten fish each in triplicate (3 aquaria/ treatment). During the acclimation period, fish were fed daily with commercial fish food (Hidrax- 40% protein). The eighteen aquaria were supplied with air pumps, 60 L. dechlorinated tap water and thermostatic heaters .

M. aeruginosa was kindly obtained from Reference lab. of the holding company of water and waste water in Cairo while growth employment was achieved at Algal Biotechnology unit, National Research Centre, Cairo, Egypt as following :

The blue green alga Microcystis aeruginosa was cultured through autotrophically growig in 5L polyethylene bottles containing the

original growth medium BG-11(Stainer et al. 1971). Nitrate nitrogen (1.5 g.L-1) was substituted by (0.53g.L-1) of urea nitrogen at the same content m M/L. Aeration was performed by free oil compressed air. Illumination was provided from one side light bank of white fluorescent lamps to give a light intensity of 120 M.e. When cultured of microcystis alga reached the maximum (1.0 g.L-1); harvesting was performed by laboratory centrifuge (HERAEUS-MEGAFUGE, 40 Centrifuge) at 3000 rpm/5min. The obtained biomass drying was done using freeze-dryer (Christ, Alpha 1-4 LSC plus, Germany). The freeze-dried biomass was fine grinded by Retsch- RM 200 electric mortar.

Propolis:

Dark – colored powder was purchased from Imtenan Pharma Cairo, Egypt.

Nanopropolis preparation propolis was made into Nano-sized particles in crushed by using ball milling technique (Hamdi et al. 2019) for 24 hours till reach to size 58, 6± 1nm at Nanotechnology Center, Cairo University, Sheikh Zaid branch .

Diet preparation and feeding:

Commercial basal diet was split into six portions. The first one was left as control, while the second to six portions were carefully blended with propolis (Prop.) 2.5 (g/Kg food pellets) and 1.25 (g/Kg food pellets) for nanopropolis, lyophilized M. aeruginosa 1.9 (g cells/kg food pellets) (Sanad et al. 2015) individually and in combination with Prop. and nanopropolis, respectively. To generate rigid dough and repelled, adequate amounts of water have been added The moist pellets were left to dry for 24 h at room temperature, then packed and stored at 4 °C until used (Abbass et al.2012). All fish received diet twice daily at rate of 3% of the body weight, seven days weekly for 4 weeks (28 days) according to the type of the treatment as follow :

Group 1: (control): fish fed basal diet.

Group 2: fish fed propolis supplemented diet

Group 3: fish fed nanopropolis supplemented diet

Group 4: fish fed M. aeruginosa

Group 5: fish fed M. aeruginosa plus propolis incorporated diet

Group 6: fish fed M. aeruginosa plus nanoproplis incorporated diet

The excreta and uneaten food particles were siphoned daily and their water was changed partially daily (siphoning) and totally three times weekly (every other day) .

Analysis of blood biochemistry:

Fish blood samples were used to biochemical analysis for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity according to Huang et al.(2006(, alkaline phosphatase (ALP) was assayed by the method described by EL-Aaser and EL-Merzabani, (1975), serum creatinine and urea were determined according to the method described by Young and Woodside 2001 and Vassault, (1986), respectively.

Statistical analysis:

All obtained data were expressed as means ±S.E. and statistically using SPSS, 18.0 software, 2011. Mean value are significant at which $p < 0.05$

Histopathological studies:

Tissue samples (livers) were gathered from fish of the experimental and control groups after the exposure duration ended (28 days) and then quickly resolved inadequate amount 10 percent neutral buffered formalin for several hours, dehydrated, paraffin-embedded, and archived. Prepared Paraffin blocks were used, and of 3-5 mm were mounted and stained with hematoxylin and eosin stains (Reddy and Rawat, 2013).

3. RESULTS

The results in table (1) cleared: That, serum AST, ALT, ALP enzymes activities showed significant increase in fish fed with *M. aeruginosa* cells compared with control

groups. However supplementation of diet containing *M. aeruginosa* cells with nanopropolis and propolis showed reduced activities of serum ALT, AST, and ALP compared with fish fed with *M. aeruginosa* cells and higher than those of fish fed the control or nanopropolis, propolis -enriched diets only with no significant changes observed between the later mentioned groups.

The results for creatinine and urea metabolites levels increased significantly in serum of fish fed on diet mixed with *M. aeruginosa* cells compared to other groups. Fish groups fed with diet mixed with *M. aeruginosa* cells concomitant with propolis, and propolis nanoparticles showed marked decrease in creatinine and urea levels and had relative values to the control groups compared with fish fed with *M. aeruginosa* cells. The lowest activities were observed in specimens of fish fed with nanoproplis.

Mortality and macroscopic observations:

No fish died during the entire exposure time in the experiments. Exposed fish have acted like the control one, except in some fish, whereas evident changes have occurred in swimming, lethargy, accumulation to one side of the aquaria, and rest on the aquaria ground.

Macroscopic lesions were noted during examination of the liver. Liver discoloration and brittleness were recorded macroscopically. No pathological modifications were noted in the control specimens.

Histopathology under light microscopy:

Histopathological changes were noted in the liver of the fish specimens of experimental group compared to the control ones as follow: Liver of Nile tilapia fed *M. aeruginosa* supplemented diet showed marked degenerative changes within the hepatic and the pancreatic portion as shown in Fig.4. While liver of Nile tilapia fed *M. aeruginosa* plus propolis (G5) showed marked decrease of degenerative changes within the hepatic and pancreatic fig.4. Liver of Nile tilapia fed *M. aeruginosa* plus nanopropolis (G6) showed that both hepatic and pancreatic portions were within normal limits Fig.6. However, no differences were observed between control groups**.**

Table1: Effect of propolis and propolis nanoparticles on serum ALT, AST, ALP enzymes activities and Urea, Creatinine metabolites levels of fish fed on diet containing M. aeruginosa.

parameters	ALT		AST		ALP		Urea		Creatinine	
groups	means	S.E.	means	S.E.	means	S.E.	means	S.E.	means	S.E.
B.D. control (G1)	16.4^{e}	0.88	36.15^{d}	1.67	90.37^{e}	2.37	0.55^{e}	0.04	0.39^{e}	0.03
B.D.+Prop. (G2)	15.55^{e}	0.84	34.71^{d}	1.69	89.73^{e}	2.53	0.58^{e}	0.03	0.38^{e}	0.06
B.D.+Nanoprop. (G3)	14.61^{e}	0.92	32.39^{d}	1.34	87.80^{e}	2.30	0.5^{e}	0.03	0.32^{e}	0.06
*B.D+M. aeruginosa (*G4)	41.4^{a}	1.67	76.8^{a}	2.28	154.09^{a}	3.75	2.63^{a}	0.16	2.35^{a}	0.10
B.D.+M. aeruginosa+ prop. (G5)	29.13^{b}	0.54	53.26^{b}	1.32	124.46^{b}	1.30	1.4^{b}	0.09	1.23^{b}	0.06
B.D.+M. aeruginosa+ nano prop. (G6)	23.69^{c}	0.60	45.15^{c}	1.11	112.00^{c}	1.25	1.01^{c}	0.07	0.84^{c}	0.05

Data are presented as (Mean ± SE). SE = Standard error of mean.

Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$).

Histopathology results

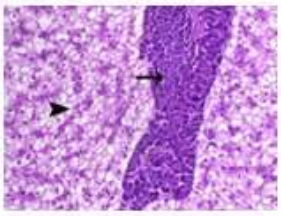

Fig. 1. Liver of G1(control) fish fed on basal diet fish showed normal hepatic portion (arrowhead) and pancreatic portion (arrow), H&E, X200.

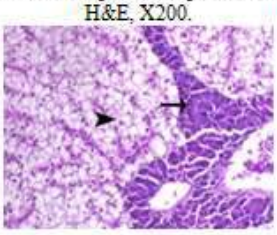

Fig.2. Liver of Nile tilapia fed propolis supplemented diet (G2) showed normal hepaticnormal hepatic portion (arrowhead) and pancreatic portion (arrow), H&E, X200.

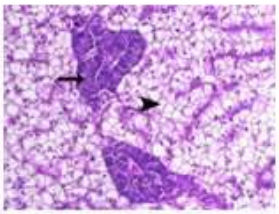

Fig. 5. : Liver of Nile tilapia fed M. aeruginosa plus propolis (G5) showed marked decrease of degenerative changes within the hepatic and pancreatic (arrowhead and arrow respectively), H&E, X200.

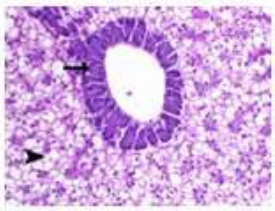

Fig. 3. Liver of Nile tilapia fed nanopropolis supplemented diet (G3) showed normal hepatic portion (arrowhead) and pancreatic portion (arrow), H&E, X200.

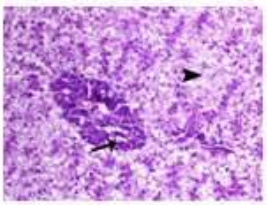

Fig. 4. Liver of Nile tilapia fed M. aeruginosa (G4) showed marked degenerative changes within the hepatic portion (arrowhead) and the pancreatic portion (arrow), H&E, X200.

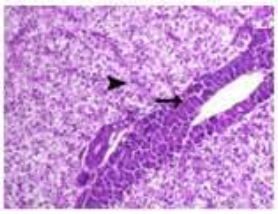

Fig. 6. : Liver of Nile tilapia fed M. aeruginosa plus nanopropolis (G6) showed that both hepatic and pancreatic portions were within normal limits (arrowhead and arrow respectively), H&E, X200.

4. DISCUSSION

This research was particularly focused on the hazardous effects of the cyanobacterial algae, *M. aeruginosa* not only on the histopathological modifications in liver as well as serum biochemical parameters of Nile tilapia. Fish liver is an outstanding organ for studying the environmental quality biomarkers, as in the mammalian cycle system and also plays a significant role in organism's metabolism (Qu *et al.*, 2018) which include the proteins production, the oxidation, methylation, conjugation, inactivation or detoxification of substances, or rather the excretion of pollutants (Ahmed *et al.* 2017).

In the current study, the serum ALT and AST enzymes activities of fishes fed on diet mixed with *M. aeruginosa* cells significantly increased compared to other groups. When cells in the liver are harmed or hyper permeable stimulated by microcystin produced by *M. aeruginosa* cells, the evident indication is the leakage of hepatic enzymes into plasma, leading to increased activities of ALT and AST (Ming *et al.*, 2018). Thus, alterations in aminotransferase activities may indicate interference with the cellular energy supply for fish fed with diet contained *M. aeruginosa* cells. Our results are agree with (Xiaoyu *et al.*,2019) whose results showed that cyanotoxin exposure promoted the

activities of fish serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicating the hepatotoxicity of cyanobacteria on the silver carp. On contrast Pradhan and Das (2015) reported that dietary *M. aeruginosa* did not induced the liver enzymes e.g. AST and ALT which could attributed either lacking or less availability of microcystin.

Furthermore the results of the present study proved that the diet mixed with *M. aeruginosa* cells increased ALP activity in the liver of *O. niloticus* compared with the control groups. These increment indicate that the membrane characteristics are disturbed by interaction with toxic *M. aeruginosa* cells because alkaline phosphatases are plasma membrane enzymes intrinsically involved in membrane transport activities and in bone formation (Mazorra *et al.* 2002). These results are in harmony with Pradhan and Das (2015) who reported elevated ALP in *Labeo rohita* fish fed with diet containing *M. aeruginosa* and ascribed increased phosphatase activity to greater breakdown of energy reserved which are used for growth and survival of fishes. Also agreed with (Lin *et al.* 2017) who observed important rises in the activity of serum ALP in Zebrafish subjected to elaveted conc. (10-30µg/l) to microcystin. In contrast Marzouk *et al.* (2013) recorded that ALP concentrations did not demonstrate any major distinction between *O. niloticus* fed toxic *M. aeruginosa* and control. Meanwhile fish groups fed with diet mixed with *M. aeruginosa* cells concomitant with propolis, and propolis nanoparticles showed reduced activities of serum ALT, AST, and ALP, than the group fed on diet mixed with *M. aeruginosa* and had relative values to the control groups. The lowest activities showed in specimens of fish fed with nanoproplis. The results of this study are in harmony with the results of Selamoglu *et al.* (2015) who indicated that ALT, AST, values increased when exposed *Cyprinus carpio* to arsenic but reduced by combination of arsenic and propolis. As well as Orun *et al.* (2014) showed that application of propolis neutralized cypermethrin (CYP) negative effect on the biochemical variables of the fish such as: the enzymes: ALT, AST, ALP.

Concerning to creatinine and urea which are classical monitoring indices for kidney function and renal structural integrity their values increased significantly in group of fish fed on diet mixed with *M. aeruginosa* cells compared to other groups. Marzouk *et al.* (2013) reported higher concentrations in *O. niloticus* fed with toxic *M. aeruginosa* than control. The increment of creatinine level in the present experiment may indicate kidney damage or malfunction which confirmed more by elevation of urea upon feeding fish with *M. aeruginosa.* This last result agreed with Lone *et al.* (2017) who speculated that microcystin-LR treatment resulted in a significant elevation of urea level in serum, suggesting damage to kidney by MC-LR. Contrary to the outcomes of Kopp *et al.* (2010) which indicated that the creatinine levels of the common carp *Cyprinus carpio* subjected to toxic *Microcystis* were reduced, whilst Carbis *et al.* (1996) found no changes in Creatinine levels in that exposed carp. The difference in fish response in some blood parameters between the present study and other studies may be due to different susceptibility of fish species, algae strain, toxins produced and their congeners, dose and period of exposure. On the other hand fish groups fed with diet mixed with *M. aeruginosa* cells concomitant with propolis, and propolis nanoparticles showed marked decrease of creatinine and urea level than the group fed on diet mixed with *M. aeruginosa* and had relative values to the control groups. The lowest activities showed in specimens of fish fed with nanoproplis. The results of this

study are in harmony with Selamoglu *et al.* (2015) who indicated that urea value increased when exposed (Cyprinus carpio, *Linnaeus 1758*) to arsenic but reduced by combination of arsenic and propolis. As well as Orun *et al.* (2014) showed that application of propolis neutralized cypermethrin (CYP) negative effect on the biochemical variables of the *Oncorhynchus mykiss* such as: creatinine, urea levels.

Indeed, fish histopathology is commonly used as a biomarker to assess water quality and prospective hazard. Concerning the liver histopathology, in the current study the fish liver fed on toxic cells of *M. aeruginosa* mixed with their basal food fish exhibited marked degenerative changes within the hepatic and the pancreatic portion. This indicated that cyanobacteria produce potent hepatotoxins toxins (Cyanotoxins), which, not only be accumulated in the tissues especially the liver of Nile tilapia but also can alter the architecture of the hepatocytes and impair its functions (Abdel-Latif and Abou Khashaba 2017). Our result cope with (Sanad *et al.* 2015) who observed various histopathological changes in the liver tissue of tilapia, *O. niloticus* represented by cyroplasm degeneration and vacuolation, nuclei pyknosis, fibrotic connective tissue patches, central vein dilation and thickened walls blood vessels were congested with blood after feeding diet incorporated with *M. aeruginosa* cells for 30 days with fish food plus toxic cells of *M. aeruginosa* at dose (1.869 g *M. aeruginosa* cells/kg food pellets). Also supported by Li *et al.* (2007) who reported that cyanotoxicity could be resulted in two types of structural modifications, one is the immediate toxic effect of the pollutant, which results in degeneration and necrosis of tissue, and the other is the production of compensatory processes, such as cellular hyperplasia, to overcome the stressor. Hepatic tumors and serious hepatic hemorrhages, as well as the hepatic cytoskeleton distribance and the subsequent, progressive necrosis of the liver and apoptosis, have been commonly reported in fish as a result of microcystin toxicity (Fischer *et al.* 2000). The degree of the toxin-induced impacts caused by toxin relies on the path of exposure. However, most of the research conducted on microcystin were operated using an IP injection or uptake orally as the path of exposure. On the other hand, when fish fed with diet mixed with *M. aeruginosa* cells plus propolis there was marked decrease of degenerative changes within the hepatic and pancreatic. Whereas liver of fish fed on dietary propolis with *M. aeruginosea* showed that both hepatic and pancreatic portions were within normal limits. These results showed that although the natural form of propolis give acceptable results in minimizing damage of liver and improved hepatic functions, the nanoform was more effective in the same respect as hepatoprotective agent. This may be attributed to diverse pharmacological activities of propolis (Olczyk *et al. 2013*) and more effectiveness of nanoparticles due to smaller size and good absorbance.

5. Conclusion

To sum up, *M. aeruginosa* has toxic effects on tilapia and the mechanism underlying this toxicity might be liver damage even though this toxicity may be attenuated by diet supplemented with propolis or its nanoparticles with more effectiveness for the latter one. Serum biochemical enzymes activities and histopathological alterations of supplemented fish with dietary toxic *M. aeruginosa* tended to decline with dietary propolis and propolis nanoparticles suggesting that these dietary additives have the ability to overcome the toxic effects of dietary *M. aeruginosa* on *O. neloticus* fish and could maintain the structural integrity of the *O. niloticus* liver. Hence our suggestions

include the avoidance and tracking of organic and inorganic pollution that favors the harmful algal blooming through the using of early preventive interventions to avoid toxic impacts of cyanobacteria on fish and therefore on humans.

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