Ameliorative effect of propolis and nanopropolis supplementation against oxidative stress induced by Microcystis aeruginosa in Oreochromis niloticus

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ABSTRACT
The present study was planned to evaluate whether dietary supplementation with propolis or its nanoparticles was able to reduce the hepatic oxidative damage of the Nile tilapia, Oreochromis niloticus, feeding M. aeruginosa cells mixed with their food. Fish used in the current study were collected from the National Institute of Oceanography and Fisheries (NIOF) Serw farm and transferred to the Wet Lab of NIOF. Six groups of fish; each of three replicate, received diet twice daily at a rate of 3% of the body weight, for 4 weeks (28 days) according to the type of the treatment as follow: First Group was left as a control; the second one fed on fish meal (basal diet) plus propolis. In the third group, fish were fed on fish meal plus nanopropolis, while in the fourth group, fish were fed on fish meal plus toxic cells of M. aeruginosa, and in the fifth group, fish fed on fish meal plus toxic cells of M. aeruginosa plus propolis. The sixth group fed on fish meal plus toxic cells of M. aeruginosa, plus nanopropolis. Samples from liver were taken from all fish groups for assessing hepatic antioxidant enzymes activities and gene expressions of the Nile tilapia. The results showed that levels of malondialdehyde (MDA) and reduced glutathione (GSH) conc. as well as (SOD) and catalase (CAT) activities decreased significantly due to dietary M. aeruginosa cells compared to the control group. Also significant reductions in the mRNA relative expressions of Superoxide dismutase (SOD), (CAT) and glutathione peroxidase (Gpx) in the liver of O. niloticus. It was noticed that propolis and nanopropolis co-administration reduced all alterations elicited by diet mixed with M. aeruginosa cells and the analyzed variables and they were nearly relative to control groups. Nano form of propolis is more effective on competing for toxicity of M. aeruginosa and as an antioxidant agent. In summary, this supplementation can be an intriguing approach in preventing M. aeruginosa induced liver damage.
INRODUCTION

The global expansion of cyanobacterial harmful algal blooms (CyanoHABs) is a serious threat to the ecological integrity, ecosystem services, safe use, and sustainability of inland and coastal waters (Paerl and Otten, 2013) which has become more frequent in many countries of the world and they are predicted to be a rapidly expanding global problem (Shao et al., 2014). In recent years, microalgal blooms and their ecotoxicological potential have drawn substantial attention, particularly because of the threat they pose to human health and the environment (Corbel et al., 2015).

*Microcystis aeruginosa* (*M. aeruginosa*) is a ubiquitous toxin-producing cyanobacterium caused cyanobacterial blooms in freshwater lakes and reservoirs worldwide (Xu et al., 2016). Microcystins is a family of toxins produced by cyanobacteria primarily of the genus *M. aeruginosa*, but also of the genera *Anabaena*, *Nostoc*, and *Oscillatoria*. 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 considered to be the most dangerous group, mainly because they are potent hepatotoxins (Ikehara et al., 2015).

Many studies have revealed that oxidative stress is also a toxicological consequence of the exposure to MCs in different aquatic organisms (Guzmán-Guillén et al., 2014) which results in lipid peroxidation (LPO), protein oxidation and DNA damage (Qian et al., 2014). In natural environments, MCs can accumulate in a wide range of aquatic biota such as fish (Bieczynski et al., 2013).

Elimination of cyanobacteria and their toxins from freshwater sources during the water treatment process is essential in order to meet water supply standards for cyanotoxins (Westrick and Szlag, 2010). Chlorination has been the main strategy for disinfecting drinking water but it has minor effect on the removal of cyanotoxins of the microcystin contingent (Szlag et al., 2015). However, this process targets only soluble toxins but not the toxic cells of the cyanobacteria.

Propolis is a brownish resinous material collected by worker bees from the leaf buds of trees. Due to its antioxidant and preservative effects, propolis may both prolong the physiological functions of some aquatic organisms and contribute to the health benefits of consumers of aquatic animals (Gulhan et al., 2012). (Schmidt et al., 2014) and (Kothai and Jayanthi 2014) showed that propolis has efficacy against the inhibitory effects of free radicals and as an antibacterial. In fish, propolis has been extensively used as hepatoprotective agent (Deng et al., 2011).

Nanotechnologies have broad application in fishery industry (Huang et al., 2015). Nano-propolis is more easily absorbed by the body because it has a size smaller. Thus Nano-propolis may be more effective than propolis in terms of antibacterial and antifungal activity. It was very effective for treatment of rat mammary gland tumors, breast cancers (Hasan et al., 2016). Some studies stated the role of nanoparticles as antibacterial agent (Hasan et al., 2014) effective against negligible diseases such as leishmaniosis (Nascimento et al., 2016), but yet to our knowledge there is no literature about using propolis or its nanoparticles to control *M. aeruginosa* toxicity.

Therefore, the present study was carried out to evaluate the possible protective effect of propolis and propolis nanoparticles against dietary *M. aeruginosa* cells toxicity induced oxidative stress in Nile tilapia.
**MATERIALS AND METHODS**

**Experimental fish:** 180 apparently healthy tilapia fish (*Oreocromis niloticus*) of a body weight 30 g±5 three months old has been collected from National Institute of Oceanography and Fisheries (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 pellets. The eighteen aquaria were supplied with air pumps, 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:

**Culturing:** The blue green alga *Microcystis aeruginosa* was autotrophic ally grown 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 Me.

**Harvesting** 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.

**Drying** of the obtained biomass was done using freeze-dryer (Christ, Alpha 1-4 LSC plus, Germany). The freeze dried biomass was fined grinded by Retsch- RM 200 electric mortar.

**Propolis**

Propolis brown powdered color was purchased from Imtenan Company Cairo, Egypt.

**Nanopropolis preparation** propolis was made into Nano-sized particles in crushed by using ball milling technique for 24 hours till reach to size: 58.6 ± 1.1 at Nanotechnology Center, Cairo University, Sheikh Zaid branch (Hamdi et al., 2019).

**Nanopropolis size analysis**

NanoSight NS500 Instrument (Malven, UK) was used to analyze the particle size and zeta potentials for the obtained nanoparticles. The obtained data for propolis nanoparticles are as following: **Particle size: 58.6 ± 1.1**

**Diet preparation and feeding regimen**

Crushed commercial basal diet were thoroughly mixed with propolis (2.5 g/Kg food pellets), nanopropolis (1.25g/Kg food pellets) and lyophilized *M. aeruginosa* (1.9 g cells/kg food pellets). Adequate amount of water was added to the ingredients of each diet to produce stiff dough and re-pelleted. 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 a daily feeding rate of 3% of the actual body weight, seven days weekly for 4 weeks (28 days) according to the type of the treatment as follow:

**Group 1:** (control) fish fed on basal diet (B.D.).

**Group 2:** fish fed on B.D. plus Prop.
**Group 3:** fish fed on B.D. plus nanopropolis.
**Group 4:** fish fed on B.D. plus toxic cells of *M. aeruginosa*
**Group 5:** fish fed on B.D. plus toxic cells of *M. aeruginosa* plus Prop.
**Group 6:** fish fed on B.D. plus toxic cells of *M. aeruginosa* plus nanopropolis.

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).

**Tissue samples** of liver were quickly removed and weighted, then perfused with cold saline to exclude the blood cells and then blotted on filter paper.

**Antioxidants assay and molecular investigation:** was carried out at the biotechnology lab. at the faculty of vet. Medicine, Kafr El Sheikh Univ.

**i- For biochemical analysis:**
The Preparation of liver tissue samples for determination of malondialdehyde (MDA), Reduced glutathione (GSH), Catalase (CAT) and Superoxide dismutase (SOD), has been done as follow: tissues were cut, weighed and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH 7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4°C. The resultant supernatant was directly used for the determination of the following parameters: malondialdehyde conc. (MDA), according to method of (Esterbauer and Cheeseman 1990), according to the method of (Beutler et al., 1963), Catalase (CAT) activity by the method of Aebi, (1984), and Super oxide dismutase (SOD) activity according to the method (Lawrence and and Burk 1976).

**ii- For Molecular analysis:**
About .5g of liver tissues were collected from all fish groups put in eppendorf tubes and immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for determination of the following gene expression: Catalase (CAT), Superoxide dismutase (SOD) and glutathione peroxidase (GPX). Pure RNA was extracted using total RNA Purification Kit following the manufacturer protocol (Thermo Scientific, Fermentas, #K0731). This technique was done using Revert Aid H minus Reverse Transcriptase which is a genetically modified M-MuLV RT, to convert RNA into complementary DNA (cDNA). To quantify the concentration of RNA and cDNA to be sure that the concentrations are pure enough to conduct real time PCR (El-Magd et al., 2013). For very pure samples, the absorption of Ultra-Violet (UV) light by the ring structure of purines and pyrimidines can be used to measure the amount of nucleic acids. The Q5000 (Uv-Vis spectrophotometer Q5000/USA) automatically performs all necessary measurements and calculations. Real-time PCR with SYBR Green was used to measure expression of mRNAs of target genes in the liver tissues, with β- actin as an internal reference (Daliri et al., 1999). The isolated cDNA were amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific, USA, #K0221) and gene specific primers. Forward and reverse primers sequence for real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (‘5------’3)</th>
<th>Reverse primer (‘5------’3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>AAGGGAGACGTGACAACACA</td>
<td>AGGAGCCAAAGTCCCGTTTG</td>
</tr>
<tr>
<td>CAT</td>
<td>GAACTTGCCCGGTTTCTAA</td>
<td>CGTGCAAAGTTGCATCTCTC</td>
</tr>
<tr>
<td>GPx</td>
<td>GTGCCCTGCAAATCAGTTTGG</td>
<td>CGAGGAGCTGGAACCTTGGT</td>
</tr>
</tbody>
</table>
The final reaction mixture was placed in a StepOnePlus real time thermal cycler (Applied Biosystems, Life technology, USA) and the PCR program was carried out with the PCR conditions as detailed in Table 2.

Table 1: The thermal cycler condition used during real time PCR.

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C/ 10 min</td>
<td>95°C/ 15 sec</td>
<td>60°C/ 30 sec</td>
<td>72°C/ 30 sec</td>
<td>40</td>
</tr>
</tbody>
</table>

At the end of the last cycle temperature was increased from 60 to 95 °C to produce a melt curve. The house keeping gene (β-actin) represented as normalize that used to calculate the relative gene expression or fold change in target gene.

Statistical analysis for real time PCR

All the data were expressed as means ±S.E. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, 18.0 software, 2011 and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when p<0.05.

RESULTS AND DISCUSSION

The results obtained showed that nanopropolis and propolis supplementation to Nile tilapia significantly minimized oxidative stress and damage of liver via antioxidant pathways. These results may be because propolis has diverse pharmacological activities amongst them antioxidant (Ozan et al., 2007).

The results of this study indicated that dietary cyanobacteria (M. aeruginosa cells) induce oxidative stress in live tilapia nelotica and altered activity of antioxidant enzymes. In the present study, the increase of LPO, as assessed by the formation of MDA in the liver of O. neloticus fed with M. aeruginosa suggests oxidative stress during M. aeruginosa intoxication. MDA has a strong bio toxicity and can damage cell structure and function (Ming et al., 2018). As shown in table (3), (MDA) in group 4 (dietary M. aeruginosa cells treated) was significantly increased compared with groups fed with basal diet lonely (control) or enriched with propolis and nanopropolis (1, 2, and 3). This was in agreement with the results of (Martins et al., 2017) who reported increases in lipid peroxidation (LPO) indicating oxidative damage in the liver of Brycon amazonicus after microcystin exposure.

However, the antioxidant enzymes activities (CAT, SOD) and (GSH) conc. in liver tissue of fish supplemented with diet containing M. aeruginosa cells (group 4) decreased significantly compared with groups fed with basal diet lonely (control) or enriched with propolis and nanopropolis (1, 2, 3 ). These finding agreed with (Liu et al., 2014) who stated that activities of SOD, CAT drastically decreased in microcystin treated groups compared with the control. The inhibition of SOD-CAT system in the liver may have been the major mechanism responsible for the establishment of oxidative damage in our study. Under normal conditions, the antioxidant defense system is capable of eliminating ROS produced by basal metabolism, thus protecting tissues against oxidative damage (Oliveira et al., 2004). Some studies reported elevation of antioxidant enzymes with the MC-LR toxicity in Nile tilapia (Jos et al., 2006; Prieto et al., 2007; Al-Kahtani and Fathi
2008) and in early life stages of the zebrafish (Wiegand et al., 1999). The previous reviews were based mainly on time-dependent manner.

On the other hand, Tilapia fed the diet containing propolis nanoparticles and propolis combined with M. aeruginosa cells supplementation showed significant increment in the studied antioxidant activity levels and decrement in MDA level, respectively compared to (group 4) fish fed on diet mixed with M. aeruginosa cells only. These results are in cope with, (Yonar et al., 2014) who reported that simultaneous treatment with propolis decreased the severity of the oxidative stress induced by chromium in different tissues of common carp.

**Table(2): Effect of propolis and nanopropolis on liver tissue oxidative stress biomarkers of fish fed Microcystis aeruginosa cells mixed diet.**

<table>
<thead>
<tr>
<th>groups</th>
<th>parameters</th>
<th>MDA mean S.E.</th>
<th>CAT mean S.E.</th>
<th>SOD mean S.E.</th>
<th>GSH mean S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.D. control (G1)</td>
<td></td>
<td>43.16 e1.43</td>
<td>11.85 a0.52</td>
<td>205.17 a34.6</td>
<td>34.6 a1.04</td>
</tr>
<tr>
<td>B.D.+Prop. (G2)</td>
<td></td>
<td>41.8 e1.66</td>
<td>10.73 a0.84</td>
<td>204.8 a4.33</td>
<td>34.28 b1.11</td>
</tr>
<tr>
<td>B.D.+Nanoprop. (G3)</td>
<td></td>
<td>40.52 e1.40</td>
<td>11.39 a0.59</td>
<td>209.25 a4.13</td>
<td>36.05 a1.32</td>
</tr>
<tr>
<td>B.D.+M. aeruginosa (G4)</td>
<td></td>
<td>82.19 e2.80</td>
<td>6.3 d0.28</td>
<td>140.71 c2.38</td>
<td>17.22 ca0.52</td>
</tr>
<tr>
<td>B.D.+M. aeruginosa + nano prop. (G5)</td>
<td>66.6 b1.03</td>
<td>8.75 b0.33</td>
<td>179.22 c2.07</td>
<td>26 c0.54</td>
<td></td>
</tr>
<tr>
<td>B.D.+M. aeruginosa + nano prop. (G6)</td>
<td>57.82 c1.12</td>
<td>9.84 b0.26</td>
<td>187.84 b1.94</td>
<td>30.18 b0.68</td>
<td></td>
</tr>
</tbody>
</table>

(Data are presented as (Mean ± S.E). S.E=Standard error. Mean values with different superscript letters in the same row are significantly different at (P ≤ 0.05). It is known that MCs can cause DNA damage and produce genotoxicity (Zegura et al., 2011). The results of this study showed that the transcriptional changes of hepatic (CAT, SOD, and GPx) genes expression of tilapia are shown in table (4) markedly downregulated in liver of fish fed on (M. aeruginosa cells) under the same conditions as compared with group control and groups fed with basal diet lonely or enriched with propolis and nanopropolis (P < 0.05). However, comparing with group (4) fish fed on (M. aeruginosa cells) transcriptional level of CAT, SOD, and GPx genes were significantly upregulated in groups (1, 2, 3, 5, 6) with no significant differences between the control group (1) and fish fed with propolis (2) or propolis nanoparticles (3) which may be due to the balanced environmental conditions at which fish were kept during the experiment. It is noticed that the co-administration of diet containing M. aeruginosa cells with nanopropolis and propolis, respectively upregulated the tested antioxidant genes compared to the fish fed on diet containing M. aeruginosa cells. The lower GPx transcription, which may have led to lower GPx activity, could be due to a decline in GSH concentration.

**Table(3): Effect of propolis and nanopropolis on the relative expression of SOD,CAT, GPx genes in liver tissue of fish fed Microcystis aeruginosa cells mixed diet.**

<table>
<thead>
<tr>
<th>groups</th>
<th>parameters</th>
<th>SOD Gene Means S.E.</th>
<th>CAT Gene Means S.E.</th>
<th>GPx Gene Means S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.D. control (G1)</td>
<td></td>
<td>1.00 e0.06</td>
<td>1.00 a0.06</td>
<td>1.00 a0.07</td>
</tr>
<tr>
<td>B.D.+Prop. (G2)</td>
<td></td>
<td>1.01 e0.07</td>
<td>1.09 a0.08</td>
<td>1.04 a0.09</td>
</tr>
<tr>
<td>B.D.+Nanoprop. (G3)</td>
<td></td>
<td>1.08 e0.09</td>
<td>0.19 a0.09</td>
<td>1.14 a0.08</td>
</tr>
<tr>
<td>B.D.+M. aeruginosa (G4)</td>
<td></td>
<td>0.14 a0.01</td>
<td>0.11 e0.01</td>
<td>0.28 e0.53</td>
</tr>
</tbody>
</table>
Ameliorative effect of propolis and nanopropolis supplementation in *O. niloticus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catalase (c)</th>
<th>Glutathione (d)</th>
<th>Peroxidase (e)</th>
<th>Aspartate (f)</th>
<th>Glutathione peroxidase (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.D. + <em>M. aeruginosa</em> + prop (G5)</td>
<td>0.21</td>
<td>0.02</td>
<td>0.33</td>
<td>0.02</td>
<td>0.59</td>
</tr>
<tr>
<td>B.D. + <em>M. aeruginosa</em> + nano prop (G6)</td>
<td>0.62</td>
<td>0.04</td>
<td>0.73</td>
<td>0.04</td>
<td>0.81</td>
</tr>
</tbody>
</table>

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

This result showed that antioxidant enzyme activities were closely related to their mRNA expression levels, similar to the previous reports in grass carp (Xu et al., 2016). It also indicated that although *O. niloticus* supplemented with diet containing *M. aeruginosa* cells suffered from oxidative stress, resulting in the decrease of these enzymes activities and mRNA expression levels, the diet supplemented with a certain amount of propolis and their nanoparticles could improve its antioxidant capacity and reduce oxidative damage. This result is in agreement with the previous reports such as the protection induced by green tea polyphenols in grass carp (Ming et al., 2018).

In conclusion nanopropolis have higher significant effect on competing toxicity of *M. aeruginosa* although the natural form of propolis give acceptable results in the same respect as antioxidant, and hepatoprotective agent.

**CONCLUSION**

Based on the results from this study it's concluded that *M. aeruginosa* has toxic effects on tilapia and the mechanism underlying this toxicity might be oxidative stress and the basal diet supplemented with propolis and nanopropolis scavenges ROS by enhancing the activity of the endogenous antioxidant defense system activity for conferring liver protective effects. The studied materials attenuate liver toxicity via antioxidant pathway, however the positive effect of propolis and nanopropolis showed more ability to overcome the toxic effects of dietary *M. aeruginosa* on *O. niloticus* fish liver.

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