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In vivo assessment of the hepatotoxicity of a new *Nostoc* isolate from the Nile River: *Nostoc* sp. strain NRI

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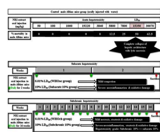

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1 **_In vivo_ assessment of the hepatotoxicity of a new _Nostoc_ isolate from the Nile River: _Nostoc_**
2 **sp. Strain NRI**

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36 **Abstract**

37 *Nostoc* sp. is one of the most widely distributed cyanobacterial genera that produce
38 potentially protein phosphatase (PP) inhibitor; microcystins (MCs). MCs have posed a worldwide
39 concern due to predominant hepatotoxicity to human health. We have previously isolated a
40 *Nostoc* strain (NR1) from the Nile River (the main water supply in Egypt) and this strain exerted
41 production of rare and highly toxic MC; demethylated microcystin-LR. There is no data
42 concerning risk factors of liver diseases for human and animal exposure to NR1-contaminated
43 drinking water yet. It is thus important to evaluate acute (LD_{50} dose), subacute (0.01% and 10%
44 of LD_{50} dose) and subchronic (0.01% and 10% of LD_{50} dose) hepatotoxicity's NR1 extract using
45 experimental mice. Mice groups, who orally received 0.01% LD_{50}, represented a permissible
46 concentration of the World Health Organization (WHO) for MC in drinking water. Several
47 parameters were detected, including hepatotoxicity (i.e. PP activity, liver function, oxidative
48 stress markers and DNA fragmentation), pro-inflammatory cytokine (TNF-α) and liver
49 histopathology. Our results demonstrated LD_{50} of NR1 extract was at 15350 mg/kg body weight
50 and caused hepatotoxicity that attributed to PP inhibition and a significant increase of hepatic
51 damage biomarkers with lipid accumulation. Moreover, NR1 extract induced hepatic oxidative
52 damage that may have led to DNA fragmentation and production of TNF-α. As demonstrated
53 from the histopathological study, NR1 extract caused a severe collapse of cytoskeleton with
54 subsequent focal degeneration of hepatocytes, necroinflammation and steatosis. The grade of
55 hepatotoxicity in subacute (10% of LD_{50}) group was higher than that in the subchronic (10% of
56 LD_{50} and 0.01% of LD_{50}, WHOch, respectively) groups. No significant hepatotoxicity was
57 detectable for subacute (0.01% of LD_{50}, WHOac) group. NR1 is therefore considered as one of
58 the harmful and life-threatening cyanobacteria for Egyptian people being exposed to dose above
59 WHO guideline. Thus, biological indicators and thresholds for water treatment are extremely
60 needed.

61 **Key words:** *Nostoc* sp. NR1; Microcystins; Hepatotoxicity; Protein phosphatase; Oxidative
62 damage; Necroinflammation; Steatosis.

63 **1. Introduction**

64 The massive growth of certain cyanobacteria in freshwater and marine ecosystems has
65 become a worldwide environmental problem. These bloom formations, followed by the
66 production of toxic secondary compounds, called cyanotoxins, causing harm to aquatic
67 ecosystems, animals and human health (Rastogi, 2014; Carmichael and Boyer, 2016). The
68 cyanotoxins can be categorized, based on biological effects, into hepatotoxins (microcystins),
69 neurotoxins, dermatoxins, irritant toxins (lipopolysaccharides) and cytotoxins (Zegura et al.,
70 2011). The potential harmful accumulative impacts on aquatic species have also been
71 investigated previously by using species sensitivity distributions methods (Chen et al., 2014). In
72 humans and animals, exposure to cyanotoxins can occur through direct contact or by means of
73 intake of contaminated drinking water or foods that is the main route for cyanotoxin intoxication
74 (Lee et al., 2017; Zhang et al., 2009). Previous studies have been reported that the Nile River, the
75 longest river in the world and the main source of drinking water for the Egyptian population, is
76 contaminated with one of the most widely distributed cyanobacteria species, *Nostoc* sp (Amer et
77 al., 2013; Mohamed et al., 2006).

78 *Nostoc* sp. is a cosmopolitan cyanobacterial genus occurring in both terrestrial and aquatic
79 ecosystems (Dodds et al., 1995). Globally, it was observed as increasing evidence of the
80 abundance of *Nostoc* sp. as a hepatotoxin (microcystins)-producing organism (Kurmayer, 2011;
81 Genuario et al., 2010; Oudra et al., 2009). However, *Nostoc* is used as a source of healthy food
82 for humans in some countries and bio-fertilizer for rice fields in Egypt (Abed et al., 2009; Yanni
83 and Carmichael, 1998). We isolated and identified microcystins (MCs)-producing *Nostoc* sp.
84 strain (*Nostoc* sp. NR1) from the Nile River (Amer et al., 2013) that is considered the main water
85 source for more than 90 million inhabitants. A previous study also reported on the production of
86 MCs from isolated *Nostoc spongioforme* and *Nostoc muscorum* from the Nile River (Mohamed et
87 al., 2006). Thus, *Nostoc* is considered a potentially toxic species (Carmichael et al., 2001).
88 *Nostoc* sp. NR1 was proved to produce demethylated MC-leucine arginine (MC-LR) which is the
89 most toxic and commonly encountered MC variants (Amer et al., 2013). Due to critical risks by
90 the existence of MCs in drinking water, the World Health Organization (WHO) proposed a
91 guideline value for MC-LR that is equivalent to one microgram per Liter (WHO, 1998).

92 Among all cyanotoxins, hepatotoxins (particularly, microcystins) are the most prevalent
93 cyanotoxins in surface and drinking water and thoroughly-studied toxin. The hepatotropism of

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94 MCs (cyclic heptapeptides) is due to selective uptake by liver cells which possess a high
95 concentration of MCs transporters in their membrane (Zanchett and Oliveira-Filho, 2013,
96 Falconer, 1999). MCs have been proved to be inhibitors of serine/threonine protein phosphatase 1
97 and 2A (PP1&2A) within the liver cells, which are vital components, controlling the cell
98 structure and function (Alverca et al., 2009; Li et al., 2005). The degree of inhibition can,
99 therefore, be used as a tool to detect MCs concentration (MacKintosh, 1990). Inactivation of
100 protein phosphatase by hepatotoxins disturbs the normal balance of cell processes, resulting in
101 cancer production, or cell death (Herfindal and Selheim, 2006; Mankiewicz et al., 2001). In
102 addition, several cellular mechanisms referred to the mitochondria pathway and oxidative stress
103 that have been proposed for MCs-induced cell death (Alverca et al., 2009). MCs have been
104 shown to induce overproduction of free radicals that might cause serious cellular damage (Ding
105 and Ong, 2003). Oxidative damage is recognized as an important cause of hepatic injury in a
106 variety of liver toxicoses (Li et al., 2010).

107 Moreover, MCs are very stable; hence it resists high temperature and extreme pH. WHO
108 (1999) reported the persistence of MCs at a temperature above 100 °C, a sign that water treatment
109 by boiling does not guarantee an absence of the persistent biotoxin, hence the toxin persists for
110 long periods of time, causing ecological and health devastations. Apart from well-known
111 hepatotoxicity of MCs, previous studies regarded cyanotoxins-mediated liver injuries to
112 lipopolysaccharides (Choi and Kim, 1998) or another hepatotoxin; nodularin (Ohta et al., 1994).

113 Accordingly, the Egyptian cyanobacteria isolate *Nostoc* sp. NR1 may present a real risk
114 factor for liver diseases in the population but there is no information about the hepatotoxicity of
115 the demethylated MC-LR produced by *Nostoc* sp. NR1 yet. All of these stimulated the research
116 team to conduct this study which constitutes the first report of *in vivo* hepatotoxicity of *Nostoc*
117 sp. NR1. In this study, we investigated hepatotoxicity that may result from animal exposure to
118 contaminated drinking water with crude extract of *Nostoc* sp. NR1 at above equivalent MCs dose
119 of the permissible limits assigned by the WHO guidelines at different time intervals for acute,
120 subacute and subchronic toxicity studies.

121 2. Materials and Methods

122 2.1. Preparation of *Nostoc* sp. NR1 extract

123 2.1.1. Sampling and isolation of *Nostoc* sp. NR1

124 Water samples were collected previously by Amer et al. (2013) from the Delta of the Nile
125 River at Kafr El Zayat city. It is one of the heaviest agricultural and industrial cities in Egypt.

126 **2.1.2. Extraction of cyanotoxins from *Nostoc* strain NR1**

127 *Nostoc* sp. strain NR1 was cultured on BG11 in continuous low light intensity at 25°C
128 (Amer et al., 2013). *Nostoc* cells were collected by centrifugation at 4000 rpm for 20 min then
129 pellets were frozen and lyophilized. Freeze-dried pellets were suspended in Milli-Q water,
130 sonicated to extract the toxins and filtered to remove cell debris (Heresztyn and Nicholson,
131 2001). The produced crude extract was then ready to be used in the ensuing experiments
132 according to the required doses.

133 **2.1.3. Determination of MCs concentration in *Nostoc* strain NR1 extract**

134 The MCs concentration in *Nostoc* sp. strain NR1 extract was calculated from the standard
135 curve of MC-LR for PP-inhibition according to Heresztyn and Nicholson (2001). Twenty
136 microliters of the lysed NR1 cells and serial concentrations of MC-LR standard (Sigma, USA)
137 were incubated with 20 µl of PP2A enzyme solution at 37°C for 5 min. Two hundred microliters
138 of 60 mM p-nitrophenol phosphate was added to the reaction and incubated at 37°C for 90 min.
139 The color was measured at 405 nm using a microtitre plate reader (BMG LabTech, Germany).

140 In addition, the identification and concentration of MC-LR in the crude extract of *Nostoc*
141 sp. NR1 was detected using C18 analytical column HPLC (Agilent, USA) by injection of 10
142 µg/mL standard MC-LR (Sigma, USA) and MALDI-TOF/MS (Amer et al., 2013).

143 **2.2. Experimental animals**

144 Male Albino mice (weight of 20-25g) were obtained from the animal house of MISR
145 University for Science and Technology (animal welfare assurance no. A5865-01), Egypt. The
146 mice were maintained at approximately 25°C with a 12-h light/dark cycle and received basal diet
147 and tap water ad-libitum for 2 weeks (acclimation period) before the experiments.

148 **2.2.1. Acute toxicity of cyanotoxins extract of *Nostoc* strain NR1**

149 Mice were orally administered a single dose of lysed *Nostoc* cells in Milli-Q water. The
150 single dose of serial dilutions of lysed NR1 extract (50-30700 mg/kg body weight) was
151 equivalent to the estimated concentrations of MCs (120-80000 µg/kg, respectively) and was
152 injected by gavage, i.e. dosing directly into the stomach through the mouth. One millimeter of
153 Milli-Q water was injected orally into mice (control). The time to death was observed within 7

154 days after the administration of the extract to detect lethal dose which enabled the extracts to be
155 ranked as toxic if an animal death was caused by 50%. LD_{50} was calculated according to
156 arithmetical method of Karber (Turner 1965). Where, LD_{50} equals to the apparent least lethal
157 dose minus (the sum of probit divides by the number of animals in each group "n=8"); probit is
158 the dose difference multiplies by the mean of mortality.

159 The livers of dead mice (at LD_{50} dose) were fixed in 10% formalin in phosphate buffer
160 saline for histopathological investigation.

161 **2.2.2. Subacute and subchronic toxicity study of cyanotoxins extract of *Nostoc* strain NR1**
162 **on animal liver tissues**

163 Sixty male Albino mice were divided randomly into five groups (twelve mice each).
164 Control group (Control) was orally administered Milli-Q and two subacute groups (subacute
165 0.01% (WHOac) and subacute 10%) in which mice were injected orally with 0.09 and 90 mg
166 NR1 extract (0.216 and 216 µg MCs are 0.01% and 10% of LD_{50}, respectively) per kg body
167 weight daily for two weeks. In addition to two subchronic groups (subchronic 0.01% (WHOch)
168 and subchronic 10%), the mice were orally administered with a daily dose (0.011 and 11.25 mg
169 NR1 extract/kg body weight) that is equivalent to 0.027 µg MCs (0.01% LD_{50}) and 27 µg MCs
170 (10% of LD_{50}), respectively for 16 weeks. The 0.01% of LD_{50} is equivalent to the permissible
171 WHO concentrations of MCs.

172 At the end of the experimental period, all mice were sacrificed by decapitation under
173 diethyl ether anesthesia. Plasma samples and liver tissues were collected. The liver tissues were
174 washed with chilled phosphate buffer, pH 7.4 and then divided into three portions: one was used
175 for biochemical assays, second part was lysed for DNA extract and fragmentation assay and the
176 third was fixed in 10% formalin in phosphate buffer saline for histopathological study.

177 **2.2.2.1. Hepatotoxicity markers**

178 **2.2.2.1.1. Determination of hepatic PP activity**

179 The PP was detected according to the method described by McAvoy and Nairn (2010).
180 Fifty microliters of 10 mM p-nitrophenol phosphate was added to 50 µl of liver homogenates
181 (diluted in 20 mM Tris, pH 7.5 containing 5 mM $MgCl_2$, 1 mM EGTA, 0.02% β-mercaptoethanol
182 and 0.1 mg/ml BSA) and incubated at 37°C for 1 h. The color was measured at 405 nm using a
183 microtitre plate reader (BMG LabTech, Germany). The PP activity was calculated using standard

184 curve of p-nitrophenol and expressed as U/mg protein. Protein content (mg) of liver samples was
185 detected using a colorimetric kit obtained from Biosystem, Egypt.

186 **2.2.2.1.2. Transaminases, lactate dehydrogenase (LDH), alkaline phosphatase (ALK)**

187 These enzyme activities (U/L) were measured in plasma samples using spectrophotometric kits
188 (Biosystem, Egypt).

189 **2.2.2.1.3. Alteration in metabolic function (protein synthesis and in lipid metabolism) of**
190 **liver**

191 Albumin is a major protein in plasma and synthesized exclusively by liver. Albumin was
192 measured in plasma using commercial kits (Biosystem, Egypt). The change in lipid metabolism
193 can be detected by determination of hepatic levels of total cholesterol (TC) and triglyceride (TG)
194 using spectrophotometric kits (Biosystem, Egypt).

195 **2.2.2.1.4. Determination of oxidative stress markers in liver tissues**

196 **2.2.2.1.4.1. Determination of hepatic NO level and products of lipid and protein oxidation**

197 Nitric oxide was detected in liver homogenate supernatants according to Ding et al. (1988)
198 using Griess reagent. The product of lipid peroxidation or malondialdehyde (MDA) of liver
199 homogenates was determined according to the method by Devasagayam et al. (2003) with a
200 modification of the method by Sinnbhuber et al. (1958), using thiobarbituric acid reagent. The
201 concentration of oxidized protein product (OPP) in liver homogenate supernatants was quantified
202 using the method described by Witko et al. (1996).

203 **2.2.2.1.4.2. Determination of hepatic non-enzymatic and enzymatic antioxidants**

204 The reduced form of glutathione (GSH) was determined by the method of Ellman (1959).
205 Hepatic GPx activity was measured in supernatants of liver homogenates according to the method
206 described by Rotruck et al. (1973) using GSH, cumene hydroperoxide and Ellman´s reagent. The
207 SOD activity in liver homogenate supernatant was detected according to using pyrogallol
208 autooxidation method described by Marklund (1974). The enzyme activities were estimated as
209 U/mg protein that was quantified using a spectrophotometric kit obtained from Biosystem, Egypt.

210 **2.2.2.1.5. Detection of DNA fragmentation**

211 Detection of DNA fragmentation was carried out as described by Wyllie (1980) with
212 some modifications as follow; liver cells were lysed in 250 µl DNA lysis buffer (TTE) (1 M Tris-
213 HCI pH 8, 0.5 M EDTA, and 0.2% Triton X-100) and centrifuged at 15000 rpm for 10 min at
214 4°C. Then the supernatants were transferred in a new tube and 0.5 ml of TTE solution was added

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215 to the pellets. Ice-cold 5M NaCl was mixed vigorously and chilled isopropanol was incubated
216 with supernatants and pellets overnight at -20ºC. DNA was recovered by centrifugation for 10
217 min at 15000 rpm at 4°C and rinsed by chilled 70% ethanol and centrifuged at 15000 rpm for 10
218 min at 4°C. The sediment DNA was air dried and dissolved in 30 µl deionized water-RNase
219 solution. DNA fragmentation was quantified in supernatants and pellets by diphenylamine assay
220 according to Burton (1956).

221 Also, DNA fragmentation was detected, after extraction, by running on 1.5% ethidium
222 bromide stained agarose gel according to the method of Miller et al. (1988) and visualized under
223 the UV trans-illuminator gel documentation system.

224 **2.2.2.2. Detection of proinflammatory cytokine**

225 Liver tissues were homogenized in phosphate buffer saline containing 0.05 % sodium azide, 0.5
226 % Triton X-100 and protease inhibitor cocktail, pH 7.2 and centrifuged at 12,000 xg for 10 min.
227 Tumor necrosis factor-α (TNF-α) concentrations were measured in the supernatants using a rat
228 TNF-α sandwich ELISA kit (RayBio, USA).

229 **2.2.2.3. Histopathological study**

230 Formalin-fixed liver tissue was dehydrated in ascending grade of alcohol, cleaned in
231 xylene and embedded in paraffin to form blocks. Five micrometers thick sections were cut using
232 microtome and sections were stained with conventional hematoxylin and eosin stain. Sections
233 were investigated and changes were recorded (Griffith and Farris, 1942).

234 **2.3. Statistical analysis**

235 Data were expressed as the mean ± SEM (standard error of mean) and estimated by the
236 multiple comparisons post-hoc Bonferroni analysis of variance (ANOVA) using the SPSS16
237 program. The differences were considered statistically significant at $*P < 0.05$, $**P < 0.01$ and
238 $***P < 0.001$.

239 **3. Results and discussion**

240 **3.1. Acute toxicity of NR1 extract**

241 Figure 1, PP inhibition assay and our previous study (Amer et al., 2013) demonstrated
242 that one milligram of lyophilized *Nostoc* sp. NR1 lysate contained MCs (2.4 µg), with MC-LR
243 which is the major variant of the lysate. The concentration of MC-LR was equivalent to 80 µg in
244 the crude extract obtained from 1L culture. It is thus important from a general public health

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245 perspective to determine the toxicity of this strain *in vivo* and to assess the risk that it may pose to
246 humans and animals by exposure to uncontrolled doses above the WHO guideline. In this study,
247 the mortality percentage was increased in a NR1 dose-dependent manner and reached 50 % by
248 the third day in mice groups exposed to 15350 mg lysed NR1 extract/kg body weight. This
249 amount of lysed NR1 contained about 40 mg of MCs (Fig. 2). The estimated oral LD_{50} value of
250 crude extract of NR1, using the Karber equation, was 30.23 mg MCs/kg body weight.
251 Accordingly, the crude extract of NR1 can be ranked as lethal (Bernard et al., 2003; Blaha and
252 Marsalek, 2000). This acute toxicity of NR1 extract may be mediated via irreversible inhibition
253 of PP by 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4, 6-decadienoic acid of MC (Campos
254 and Vasconcelos, 2010; Omoregie, 2017). Oudra et al. (2008) reported that the estimated
255 intraperitoneal LD_{50} of *Nostoc muscorum* isolated from Morocco's Oukaimeden River ranged
256 from 15 to 125 mg MCs/kg body weight. Mohamed et al. (2006) also isolated the same species
257 (*Nostoc muscorum*) from the Nile River which recorded LD_{50}=50±3.4 mg MCs/kg body weight
258 for intraperitoneal administration. Our isolate NR1 recorded LD_{50} (30.23 mg MCs/kg body
259 weight) which is lower than the results obtained by Mohamed et al (2006). This study indicates
260 higher toxicity of *Nostoc* sp. NR1 than *Nostoc muscorum* despite the oral administration of NR1
261 lysate.

262 **3.2. Subacute and subchronic hepatotoxicity of NR1 extract**

263 **3.2.1. Suppression of hepatic PP activity**

264 Microcystins are well known as PP inhibitors. One of the most studied PP inhibition
265 mechanisms of MCs is interacting with the catalytic subunits of PP at three sites; the hydrophobic
266 groove, C-terminal groove and the catalytic site (Maynes et al., 2006; Campos and Vasconcelos,
267 2010), where the toxin first binds to the enzyme inactivating it and subsequently forms covalent
268 adducts during prolonged reaction-time (Craig et al., 1996; MacKintosh et al., 1990). As shown
269 in Fig. 3, the administration of crude MCs extract of NR1 into subacute 10 % and subchronic 10
270 % groups caused a significant decrease ($P<0.001$) in PP activity by 47.9 % and 37.9 %,
271 respectively, when compared to control group (70.52±1.12 U/mg). Data revealed that lower doses
272 of NR1 extract in WHO groups showed an insignificant effect for 2 weeks by 2.3 % ($P=0.852$)
273 while 16 weeks showed a significant decrease by 8.7 % ($P<0.01$) in PP activity. This refers to a
274 significant inhibition in PP activity in all animal groups except in WHOac group compared to
275 control group (Fig. 3). These current results are in agreement with our previous *in vitro* study on

276 NR1 (Amer et al., 2013) and *in vivo* study of Runnegar et al. (1993) that illustrated the MC-
277 mediated PP inhibition was dose-dependent.

278 **3.2.2. Biochemical alterations in liver functions**

279 It was previously demonstrated that the inhibition of PP enzymes by hepatotoxic MCs
280 resulted in an excessive phosphorylation of cytosolic and cytoskeletal proteins, alterations in
281 cytoskeleton and loss of cell shape with subsequent destruction of liver cells causing leakage of
282 liver enzymes, ALT, AST, LDH and ALK, from the liver into the bloodstream (van Apeldoorn et
283 al., 2007; Solter et al., 1998). As shown in Fig. 3 and Table 1, PP inhibition was dose-dependent
284 and proportional to the severity degree of the liver damage. Other studies reported a relation
285 between MCs exposure and an elevation in blood parameters of liver enzymes that associated
286 with hepatic cellular injury (Hilborn et al., 2013; Giannuzzi et al., 2011; Li et al., 2011; Chen et
287 al., 2009). In accordance, Table 1 shows significant elevation of these soluble enzymes which are
288 indicators of the hepatic dysfunction and damage particularly ($P<0.001$) in subacute 10 % group
289 than subchronic 10 % group and at lower extent ($P<0.01$) in WHOch group. There was no
290 significant difference observed between the above-mentioned enzymes of WHOac group and
291 control group. Moreover, Fig. 4 shows an observable diminishing of albumin in subchronic 10%
292 group (12.9±0.15 g/L) in comparison with other groups (20 g/L). Albumin represents a major
293 synthetic protein and a marker for the degree of chronic liver damage (Yasmin et al., 1993). On
294 the other hand, the albumin level did not change significantly in WHOch group in comparison
295 with control untreated group. In accordance with our results, a previous study has reported
296 alteration in several serum biochemical tests, including increased in AST, sorbitol
297 dehydrogenase, gamma-glutamyl transferase and ALK, as well as a decrease in albumin that
298 occurred in a dose-dependent fashion after intraperitoneal exposure of rats to sublethal
299 concentrations of 0, 3, 6, or 9 μg of MC-LR for 28 days (Solter et al., 1998).

300 Furthermore, our statistical analysis showed that accumulative levels of TC and TG in
301 liver tissues of subchronic 10 % group (81.9 ± 1.5 mg/dL and 347.2 ± 3 mg/dL, respectively)
302 were significantly higher ($P<0.001$) than those of other groups, i.e. < 59 mg/dL and < 176 mg/dL
303 respectively (Fig. 4). Previous studies also demonstrated that chronic exposure to MC induced
304 elevation in serum concentrations of TC and TG either in human populations (Hilborn et al.,
305 2013; Chen et al., 2009) or animals (Zhang et al., 2016) and the presence of fatty vacuoles in

306 murine livers (steatosis) (Zhang et al., 2016; Guzman and Solter 1999). A recent study showed
307 that oral MC-LR exposure can induce hepatic lipid metabolism disorder by induction of
308 endoplasmic reticulum stress and peroxisome proliferator activated receptor-stimulated
309 unsaturated fatty acids and steroid biosynthesis (Zhang et al., 2016).

310 **3.2.3. Hepatic oxidative damage and the subsequent DNA damage**

311 Our results indicate that *Nostoc* sp. NR1 extract induced hepatotoxicity through oxidative
312 stress by increasing the formation of free radicals and modifying intracellular antioxidant factors,
313 resulting in the elevation of lipid peroxidation (MDA) and protein oxidation products as shown in
314 Table 2. Wei et al. (2008) found that MC-LR directly interacts with mitochondria and induces
315 production of free radicals leading to liver damage (Wei et al., 2008). Table 2 illustrates a
316 significant elevation of radical species (NO), MDA and OPP as well as marked suppression of
317 GSH level, GPx and SOD activities in liver tissues of subacute 10 % group that was significantly
318 ($p<0.001$) followed by that of subchronic 10 % group ($p<0.01$) and WHOch mice group ($p<0.05$).
319 The sharp increase ($p<0.001$) of oxidative products (MDA and OPP) were recorded for subacute
320 10 % group in comparison with other groups. In WHOac dose-exposed animal group, depletion
321 of antioxidant parameters and elevation of NO, MDA and OPP levels indicated a non-significant
322 change ($P > 0.05$) in comparison with the control group (Table 2).

323 These findings show a strong correlation with those of a recent study by Shi et al. (2015)
324 and Zhang et al. (2013) in which MDA and free radical concentrations increased and GSH
325 content decreased in livers of frogs and carp (*Cyprinus carpio* L.) with sublethal exposure to
326 MCs (Zhang et al., 2013, Shi et al., 2015). Also, a study by Ji et al. (2011) demonstrated that
327 MC-LR stimulated NO elevation via activation of induced nitric oxide synthase (iNOS) (Ji et al.,
328 2011) and Jiang et al. (2014) found that MCs inhibit aldehyde dehydrogenase which may result in
329 the elevation of MDA (Jiang et al., 2014). The depletion of hepatic GSH content is mainly due to
330 the conjugation reaction with MCs and the subsequent excretion of this conjugate which lead to
331 alter the intracellular redox status and favor the abnormal production of free radicals (Jiang et al.,
332 2011). This depletion led to decrease the activity of GPx that requires GSH in removing radical
333 species and subsequently, the role of GSH/GPx system in detoxifying nitrosative stress and lipid
334 peroxidation was arrested (Chen et al., 2015; Jablonska et al., 2015). The accumulated hydrogen
335 peroxide, as result of defective GPx, mediates an irreversible inactivation of SOD (Gottfredsen et
336 al., 2013). The decrease antioxidant defense against free radicals may be responsible for the

337 abnormal production of lipid and protein oxidation (Khare et al., 2014). Therefore, MDA and
338 OPP increase abnormally in the subacute group (10%) rather than other animal groups being
339 exposed to a lesser dose of NR1 extract (Table 2).

340 In addition, cellular GSH is an important factor for the regulation of cytoskeletal
341 organization, by acting as a buffer to maintain the reduced form of cytoskeleton protein
342 sulfhydryls that are essential for their proper polymerization state (Leung and Chou, 1989).
343 Perturbing the cellular redox status by MCs-depleting intracellular GSH provoked disruption of
344 cytoskeletal structures (Pflugmacher et al., 1999). Therefore, it is represented as another
345 mechanism, besides protein phosphorylation, which contributes to MCs disrupted cytoskeleton
346 elements.

347 Moreover, MCs-induced oxidative stress plays a critical role in DNA damage. Zegura et
348 al. (2004) have revealed that MC-LR induced the oxidation of nitrogenous bases, the pyrimidines
349 and purines, using human hepatoma (HepG-2) cell line. These results indicated that oxidative
350 stress is an important mediator of MC-LR-induced genotoxicity and the oxidized purines were
351 not repaired. This refereed that MC-LR-induced formation of oxidized purines was faster than
352 their removal by cellular DNA repair mechanisms, leading to accumulation of these lesions. Free
353 radicals attack DNA generating different types of DNA damage; modified DNA bases and DNA
354 strand breaks (Zegura et al., 2011; Zegura et al., 2004). In accordance with studies of Zegura et al
355 (2004), NR1 extract induced oxidative DNA damage as shown in Fig. 5A,B. Fig. 5A
356 demonstrates significant elevation ($p<0.001$) of DNA fragmentation percentage in subacute 10 %
357 group and subchronic 10 % group (34.29 % and 28.26 %, respectively) was higher ($p<0.01$) than
358 that of WHOac and WHOch groups (5.86 % and 9.17 %, respectively) in comparison with
359 control mice group (0.49 %). Fig. 5B demonstrates highly dense smears of extensive fragmented
360 DNA of subacute 10 % and subchronic 10 % groups compared to intact DNA band of the control
361 group and slightly smear of WHOac and WHOch groups. The excessive accumulation of free
362 radicals and lipid hydroperoxides under intracellular GSH depletion (as shown in Table 2) may
363 promote giant DNA fragmentation and induce cell death through necrosis (Higuchi 2004).

364 **3.2.4. Elevation of proinflammatory cytokine**

365 Actually, MC-LR has been linked to hepatotoxicity and inflammatory response in
366 hepatocytes through activation of NF-κB which in turn induces the expression of inflammatory
367 cytokines such as TNF-α (Christen et al., 2013; Zhang et al., 2013; Zegura et al., 2011) that

368 represents the master proinflammatory cytokine responsible for liver toxicity (Geier et al., 2003).
369 However, in mouse leukocytes, MC-LR dysregulated TNF-α mRNA levels, which elicited an
370 immunosuppressive effect in immunocytes (Chen et al., 2004). In this study via ELISA assay,
371 crude MCs of *Nostoc* sp. NR1 was found to induce production of TNF-α significantly ($p<0.001$)
372 in liver tissues of subacute 10% (33.67 ± 0.85 pg/mg, $p<0.001$), subchronic 10% (23.79 ± 1.96
373 pg/mg, $p<0.001$) and WHOch (12.1±0.3 pg/mg, $p<0.01$) mice groups in comparison to control
374 (2.35±0.19 pg/mg) mice group (Fig. 6). The primary event in different types of liver injuries is
375 the production of TNF-α, which further enhances the production of other cytokines, that together
376 recruit inflammatory cells and induce hepatocytes death via necrosis (Schwabe and Brenner,
377 2006; Ni et al., 2016).

378 **3.2.5. Histopathological changes**

379 All the above tested biochemical parameters indicate that crude extract of *Nostoc* sp. NR1
380 caused hepatic damage. This was confirmed by the observed histopathological changes that are
381 useful tools to assess the degree of hepatotoxicity. Figure 7A shows normal hepatocytes of
382 control mice compared to mice injected with LD_{50} dose of crude MCs of NR1 (acute group),
383 showing severe grade of architectural distortion and disorganization with complete collapse of
384 the reticulin framework with lytic necrosis of the hepatocytes (Fig. 7B). This cytoskeletal
385 collapse may be secondary to the potent inhibitory effects of LD_{50} dose of MCs on hepatic PP
386 activity that have been correlated with rapid loss of the sinusoidal architecture and subsequently
387 mice death from hemorrhagic shock (van Apeldoorn et al., 2007, Beasley et al., 2000). There
388 were no pathological changes rather than mild congestion of central vein in WHOac group in
389 comparison with control group (Fig. 7C). In the subacute 10% group, the severe changes were
390 observed in the form of a marked distortion of hepatic architecture with confluent areas of
391 necrosis, hyperplastic Kupffer cells and cell swelling as it shown in Fig. 7D and E. On the other
392 hand, liver tissues of WHOch mice group demonstrated the lowest grade of necrosis and steatosis
393 (Fig. 7F). In the subchronic 10% group, a mild grade of injury was seen in the form of focal
394 macrovesicular steatosis and necroinflammation with congestion veins as it was observed in Fig.
395 6G and H. These changes may be attributed to alteration in PP activity, cellular redox status and
396 hepatic lipid metabolism. Our findings are similar to those of previous studies (Berillis et al.,
397 2014; Kujbida et al., 2008; Fischer et al., 2000; Guzman and Solter, 1999).

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398 **4. Conclusions**

399 This first *in vivo* study showed the extent acute, subacute and subchronic hepatotoxicity
400 of the Nile River *Nostoc* sp. NR1 extract on mice. The acute oral dose of crude extract resulted in
401 lethal hepatic degeneration. The subacute 10 % dose of this NR1 extract caused hepatic
402 necroinflammation and oxidative damage with severe alteration in cytoskeleton that were higher
403 than subchronic (10 % of LD_{50}) group and WHOch (0.01 % of LD_{50}) group, respectively. The
404 highest grade of lipid accumulation (steatosis) was recorded in liver of subchronic 10 % group.
405 No significant change was observed between WHOac (0.01 % of LD_{50}) group and control group.
406 This indicates that people (especially the Egyptian population) who are exposed to NR1-
407 contaminated drinking water above the WHO guideline of MCs dose, by 1000 times even for
408 short duration (2 weeks), may be at risk of major health problems. This *in vivo* assessment of
409 hepatotoxicity of crude cyanotoxins of the Nile River *Nostoc* sp. NR1 is deemed critical, as it
410 provides crucial insights in determining future suitable biological indicators and thresholds for
411 water treatment in the region and highlights the potential human and animal health risks if proper
412 measurements are not taken.

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416 **Conflict of interest**

417 The authors declare no conflict of interest and state that all applicable international, national,
418 and/or institutional guidelines for the care and use of animals were followed.

419 **References**

420 Abed, R., Dobretsov, S., Sudesh, K., 2009. Applications of cyanobacteria in biotechnology. J.
421 Appl. Microbiol. 106(1), 1-12.

422 Alverca, E., Andrade, M., Dias, E., Sam Bento, F., Batoreu, M.C., Jordan P, Silva, M.J., Pereira,
423 P., 2009. Morphological and ultrastructural effects of microcystin-LR from *Microcystis*
424 *aeruginosa* extract on a kidney cell line. Toxicon 54, 283-294.

425 Amer, R., Shehawy, R., Saad El-Dien, S., Abu Serie, M., Shaker, K., 2013. Isolation and characterization of cyanobacterial community including a microcystin-producing *Nostoc* sp. strain in the Nile River, Egypt. Adv. Microbiol. 3, 38-46.

428 Beasley, V.R., Lovell, R.A., Holmes, K.R., Walcott, H.E., Schaeffer, D.J., Hoffmann, W.E., Carmichael, W.W., 2000. Microcystin-LR decreases hepatic and renal perfusion, and causes circulatory shock, severe hypoglycemia, and terminal hyperkalemia in intravascularly dosed swine. J. Toxicol. Environ. Health 61, 281-303.

432 Berillis, P., Papadimitriou, T., Petridou, E., Konstantinos, K., Ifigenia, K., 2014. Brain and liver histopathological examination of *Carassi*us *gibelio* from a newly reconstructed lake with toxic cyanobacteria. Turk. J. Fish. Aquat. Sci. 14, 213-219.

435 Bernard, C., Harvey, M., Briand, J.F., Bire, R., Krys, S., Fontaine, J., 2003. Toxicological comparison of diverse *Cylindrospermopsis raciborskii* strains: evidence of liver damage caused by a French *C raciborskii* strain. J. Environ. Toxicol. 18(3), 176-186.

438 Blaha, L., Babica, P., Marsalek, B., 2009. Toxins produced in cyanobacterial water blooms-toxicity and risks. Interdisc. Toxicol. 2(2), 36-41.

440 Blaha, L., Marsalek, B., 2000. Methods for detection and quantification of cyanobacterial toxins - a review. Algol. Stud. 99, 1-22.

442 Burton, K., 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62(2), 315-323.

444 Campos, A., Vasconcelos, V., 2010. Molecular mechanisms of microcystin toxicity in animal cells. Int. J. Mol. Sci. 11, 268-287.

446 Carmichael, W.W., Azevedo, S.M.F.O., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environ. Health Persp. 109, 663-668.

449 Carmichael, W.W., Boyer, G.L., 2016. Health impacts from cyanobacteria harmful algae blooms: Implications for the North American Great Lakes. Harmful Algae 54, 194-212.

451 Chen, J., Bhandar, B., Kavdia, M., 2015. Interaction of ROS and RNS with GSH and GSH/GPX Systems. FASEB J. 29(1), 636-637.

453 Chen, J., Liu, Y.M., Zhang, J.Y., 2014. Aquatic ecological risk assessment of microcystins and nitrogen pollution based on species sensitivity distribution. Chin. J. Appl. Ecol. 25 (4), 1171-1180.

456 Chen, J., Xie, P., Li, L., Xu, J., 2009. First identification of the hepatotoxic microcystins in the
457 serum of a chronically exposed human population together with indication of hepatocellular
458 damage. Toxicol. Sci. 108(1), 81-89.
459 Chen, T., Zhao, X., Liu, Y., Shi, Q., Hua, Z., Shen, P., 2004. Analysis of immunomodulating
460 nitric oxide, iNOS and cytokines mRNA in mouse macrophages induced by microcystin-
461 LR. Toxicol. 197, 67-77.
462 Choi, S.H., Kim, S.G., 1998. Lipopolysaccharide inhibition of rat hepatic microsomal
463 epoxidehydrolase and glutathioneS-transferase gene expression irrespective of nuclear
464 factor-Kb activation. Biochem. Pharmacol. 56, 1427-1436.
465 Christen, V., Meili, N., Fent, K., 2013. Microcystin-LR induces endoplasmatic reticulum stress
466 and leads to induction of NFB interferon-alpha, and tumor necrosis factor-alpha. Environ.
467 Sci. Technol. 47 (2013), 3378-3385.
468 Craig, M., Luu, H.A., McCready, T.L., Williams, D., Andersen, R.J., Holmes, C.F., 1996.
469 Molecular mechanisms underlying he interaction of motuporin and microcystins with type-
470 1 and type-2A protein phosphatases. Biochem. Cell Biol. 74, 569-578.
471 Devasagayam, T., Boloor, K., Ramasarma, T., 2003. Methods for estimating lipid peroxidation:
472 An analysis of merits and demerits. Indian J. Biochem. Biophys. 40(5), 300-308.
473 Ding, A., Nathan, C., Stuehr, D., 1988. Release of reactive nitrogen intermediates from mouse
474 peritoneal macrophages: Comparison of activating cytokines and evidence for independent
475 production. J. Immunol. 141, 2407-2412.
476 Ding, W.X., Ong, C.N., 2003. Role of oxidative stress and mitochondrial changes in
477 cyanobacteria–induced apoptosis and hepatotoxicity-MiniReview. FEMS Microbiol. Lett.
478 220, 1-7.
479 Dodds, W.K., Gudder, D.A., Mollenhauer, D., 1995. The ecology of *Nostoc*. J. Phycol. 31, 2-18.
480 Ellman, G.L. 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70-77.
481 Falconer, I.R. 1999. An overview of problems caused by toxic Blue-Green Algae
482 (Cyanobacteria) in drinking and recreational water. Environ. Toxicol. Wat. Qual. 14, 5-12.
483 Falconer, I.R., Smith, J.V., Jackson, A.R., Jones, A., Runnegar, M.T., 1988. Oral toxicity of a
484 bloom of the cyanobacterium *Microcystis aeruginosa* administered to mice over periods up
485 to 1 year. J. Toxicol. Environ. Health 24, 291-305.

486 Fischer, W.J., Hitzfeld, B.C., Tencalla, F., Eriksson, J.E., Mikhailov, A., Dietrich, D.R., 2000.
487 Microcystin-LR toxicodynamics, induced pathology, and immunohistochemical
488 localization in livers of blue-green algae exposed rainbow Trout (*Oncorhynchus mykiss*).
489 Toxicol. Sci. 54, 365-373.
490 Geier, A., Dietrich, C.G., Voigt, S., Kim, S.K., Gerloff, T., Kullak-Ublick, G.A., Lorenzen,
491 J, Matern S, Gartung C., 2003. Effects of proinflammatory cytokines on rat organic anion
492 transporters during toxic liver injury and cholestasis. Hepatol. 38(2), 345-54.
493 Genuario, D.B., Silva-Stenico, M.E., Welker, M., Beraldo Moraes, L.A., Fiore, M.F., 2010.
494 Characterization of a microcystin and detection of microcystin synthetase genes from a
495 Brazilian isolate of *Nostoc*. Toxicon 55(4), 846-54.
496 Giannuzzi, L., Sedan, D., Echenique, R., Andrinolo, D., 2011. An acute case of intoxication with
497 cyanobacteria and cyanotoxins in recreational water in Salto Grande Dam, Argentina. Mar
498 Drugs 9, 2164-2175.
499 Gilroy, D.J., Kauffman, K.W., Hall, R.A., Huang, X., Chu, F.S., 2000. Assessing potential health
500 risks from microcystin toxins in blue-green algae dietary supplements. Environ. Health
501 Perspect. 108, 435-439.
502 Gottfredsen, R.H., Larsen, U.G., Enghild, J.J., Petersen, S.V., 2013. Hydrogen peroxide
503 induce modifications of human extracellular superoxide dismutase that results in enzyme
504 inhibition. Redox. Biol. 1(1), 24–31.
505 Griffith, J., Farris, E., 1942. The osseous system. In: Griffith, J., Farris, E (Eds), The rat in
506 laboratory investigation. Lippincott Co., USA, pp. 418-419.
507 Guzman, R.E., Solter, P.H.F., 1999. Hepatic oxidative stress following prolonged sublethal
508 microcystin LR exposure. Toxicol. Pathol. 27(5), 582-588.
509 Heresztyn, T., Nicholson, B.C., 2001. Determination of cyanobacterial directly in water using a
510 protein phosphatase protein inhibition assay. Wat. Res. 35(13): 3049-3056.
511 Herfindal, L., Selheim, F., 2006. Microcystin produces disparate effects on liver cells in a dose
512 dependent manner. Mini Rev. Med. Chem. 6(3), 279-285.
513 Higuchi, Y., 2004. Glutathione depletion-induced chromosomal DNA fragmentation associated
514 with apoptosis and necrosis. J. Cell. Mol. Med. 8 (4), 455-464.

515 Hilborn, E.D., Soares, R.M., Servaites, J.C., Delgado, A.G., Magalhaes, V.F., Carmichael, W.W.,
516 Azevedo, S., 2013. Sublethal microcystin exposure and biochemical outcomes among
517 hemodialysis patients. PLoS ONE 8(7), 1-9.
518 Jablonska, E., Gromadzinska, J., Peplonska, B., Fendler, W., Reszka, E., Krol, M.B., Wieczorek,
519 E., Bukowska, A., Gresner, P., Galicki, M., Quispe, O.Z., Morawiec, Z., Wasowicz, W.,
520 2015. Lipid peroxidation and glutathione peroxidase activity relationship in breast cancer
521 depends on functional polymorphism of GPX1. BMC Cancer 15, 657-686.
522 Ji, Y., Lu, G., Chen, G., Huang, B., Zhang, X., Shen, K., Wu, S., 2011. Microcystin-LR induces
523 apoptosis via NF-κB /iNOS pathway in INS-1 cells. Int. J. Mol. Sci. 12, 4722-4734.
524 Jiang, J.L., Gu, X.Y., Song, R., Zhang, Q., Geng, J.J., Wang, X.R., Yang, L.Y., 2011. Time-
525 dependent oxidative stress and histopathological alterations in *Cyprinus carpio* L. exposed
526 to microcystin-LR. Ecotoxicol. 20, 1000-1009.
527 Khare, M., Mohanty, C., Das, B.K., Jyoti, A., Mukhopadhyay, B., Mishra, S.P., 2014. Free
528 radicals and antioxidant status in protein energy malnutrition. Int. J. Pediatr. 2014, 1-6.
529 Kujbida, P., Hatanaka, E., Campa, A., Curi, R., Poliselli-Farsky, S.H., Pinto, E., 2008. Analysis
530 of chemokines and reactive oxygen species formation by rat and human neutrophils
531 induced by microcystin-LA, -YR and -LR. Toxicon 51, 1274-1280.
532 Kurmayer, R., 2011. The toxic cyanobacterium *Nostoc* sp. strain 152 produces highest amounts
533 of microcystin and nostophycin under stress conditions. J. Phycol. 47(1), 200-207.
534 Lee, J., Lee, S., Jiang, X., 2017. Cyanobacterial Toxins in Freshwater and Food: Important
535 Sources of Exposure to Humans. Annu. Rev. Food Sci. Technol. 8, 281-304.
536 Leung, M.F., Chou, I.N., 1989. Relationship between 1-chloro-2,4-dinitrobenzene-induced
537 cytoskeletal perturbation and cellular glutathione. Cell. Biol. Toxicol. 5, 51-66.
538 Li, L., Xie, P., Chen, J., 2005. *In vivo* studies on toxin accumulation in liver and ultrastructural
539 changes of hepatocytes of the phytoplanktivorous bighead carp i.p.-injected with extracted
540 microcystins. Toxicon 46, 533-545.
541 Li, L., Xie, P., Guo, L.G., 2010. Antioxidant response in liver of the phytoplanktivorous bighead
542 carp (*Aristichthys nobilis*) intraperitoneally-injected with extracted microcystins. Fish
543 Physiol. Biochem. 36, 165-172.

544 Li, Y., Chen, J.A., Zhao, Q., Pu, C., Qiu, Z., Zhang, R., Shu, W., 2011. A cross-sectional
545 investigation of chronic exposure to microcystin in relationship to childhood liver damage
546 in the Three Gorges Reservoir Region, China. Environ. Health Perspect. 119, 1483-1488.
547 MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial
548 microcystin-LR is a potent inhibitor of protein phosphatases 1 and 2A from both mammals
549 and higher plants. FEBS Lett. 264, 187-192.
550 Mankiewicz, J., Walter, Z., Tarczynska, M., Fladmark, K.E., Doskeland, S.O., Zalewski, M.,
551 2001. Apoptotic effect of cyanobacterial extract on rat hepatocytes and human
552 lymphocytes. Environ. Toxicol. Wat. Qual. 3, 225-233.
553 Mariappan, N., Soorappan, R.N., Haque, M., Sriramula, S., Francis, J., 2007. TNF-alpha-induced
554 mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase
555 mimetic Tempol. Am. J. Physiol. Heart Circ. Physiol. 293(5), H2726-H2737.
556 Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the
557 autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J.
558 Biochem. 47, 469-474.
559 Maynes, J.T., Luu, H.A., Cherney, M.M., Andersen, R.J., Williams, D., Holmes, C.F., James,
560 M.N., 2006. Crystal structures of protein phosphatase-1 bound to motuporin and
561 dihydromicrocystin-LA: Elucidation of the mechanism of enzyme inhibition by
562 cyanobacterial toxins. J. Mol. Biol. 356, 111–120.
563 McAvoy, T., Nairn, A.C., 2010. Serine/Threonine Protein Phosphatase Assays. Curr. Protoc.
564 Mol. Biol. 18, 1-13.
565 Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting
566 DNA from human nucleated cells. Nucleic Acids Res. 16, 1215-1215.
567 Mohamed, Z.A., El-Sharouny, H.M., Ali, W.S.M., 2006. Microcystin production in benthic mats
568 of Cyanobacteria in the Nile River and irrigation canals, Egypt. Toxicon 47, 584-590.
569 Ni, H.M., McGill, M.R., Chao, X., Woolbright, B.L., Jaeschke, H., Ding, W.X., 2016. Caspase
570 inhibition prevents tumor necrosis factor-α–induced apoptosis and promotes necrotic cell
571 death in mouse hepatocytes *in vivo* and *in vitro*. Am. J. Pathol. 186 (10), 2623–2636.

572 Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., Tatematsu, M., Kim,
573 S.J., Carmichael, W.W., Fujiki, H., 1994. Nodularin, apotent inhibitor of protein
574 phosphatase 1 and 2A, is a new environmental carcinogen in male F344 rat liver. Cancer
575 Res. 54, 6402-6406.
576 Omoregie, I.P., 2017. Oxidative stress biomarkers in Clarias gariepinus (Burchel, 1822) exposed
577 to Microcystin-LR. Beni-Suef University J. Basic Appl. Sci. 6, 69-75.
578 Oudra, B., Dadi-El Andaloussi, M., Vasconcelos, V.M., 2009. Identification and quantification of
579 microcystins from a *Nostoc muscorum* bloom occurring in Oukaimeden River (High-
580 Atlas mountains of Marrakech, Morocco). Environ. Monit. Assess. 149(1-4), 437-44.
581 Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A., Steinberg,
582 C.E.W., 1999. Identification of an enzymatically formed glutathione conjugate of the
583 cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. Biochem.
584 Biophys. Acta. 1425, 527-533.
585 Pillotto, L.S., Burch, M.D., Douglas, R.M., Cameron, S., Roach, G.J., Cowie, C.T., Beers, M.,
586 Robinson, P., Kirk, M., Hardiman, S., Moore, C., Attewell, R.G., 1997. Health effect of
587 recreational exposure to cyanobacteria (blue-green algae) during recreational water
588 activities. Aust. N Z J Public Health 21, 562-566.
589 Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: current
590 overview. Rev. Environ. Sci. Bio. Technol. 13, 215-249.
591 Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Haffman, D.G., Hoekstra, W.G., 1973
592 Selenium: Biochemical role as a component of glutathione peroxidase. Sci. 179, 588-590.
593 Runnegar, M.T, Kong, S., Berndt, N., 1993. Protein phosphatase inhibition and *in vivo*
594 hepatotoxicity of microcystins. Am. J. Physiol. 265, G224-G230.
595 Schwabe, R.F., Brenner, D.A., 2006. Mechanisms of Liver Injury. I. TNF-alpha-induced liver
596 injury: role of IKK, JNK, and ROS pathways. Am. J. Physiol. Gastrointest. Liver
597 Physiol. 290(4), G583- G589.
598 Shi, Y., Jiang, J., Shan, Z., Bu, Y., Deng, Z., Cheng, Y., 2015. Oxidative stress and
599 histopathological alterations in liver of Cyprinus carpio L. induced by intraperitoneal
600 injection of microcystin-LR. Ecotoxicol. 24(3), 511-519.

601 Sinnhuber, R.O., Yu, T.C., Yu, T.C., 1958. Characterization of the red pigment formed in the 2-
602 thiobarbituric acid determination of oxidative rancidity[a,b]. J. Food Sci. 23(6), 626-634.
603 Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic
604 cyanobacteria in water: A guide to their public health consequences, monitoring and
605 management. Spon, London, pp. 41-111.
606 Solter, P.F., Wollenberg, G.K., Huang, X., Chu, F.S., Runnegar, M.T., 1998. Prolonged sublethal
607 exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-
608 dependent hepatotoxic effects. Toxicol. Sci. 44, 87-96.
609 Turner, R., 1965. Acute toxicity: The determination of LD50. In: Screening methods in
610 pharmacology. Academic press, New York, pp. 61-63.
611 van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J., Bakker, G.J., 2007. Toxins of
612 cyanobacteria. Mol. Nutr. Food Res. 51, 7-60.
613 Wei, Y., Weng, D., Li, F., Zou, X., Young, D.O., Ji, J., Shen, P., 2008. Involvement of JNK
614 regulation in oxidative stress-mediated murine liver injury by microcystin-LR. Apoptosis
615 13, 1031-1042.
616 Weng, D., Lu, Y., Wei, Y., Liu, Y., Shen, P., 2007. The role of ROS in microcystin-LR-induced
617 hepatocytes apoptosis and liver injury in mice. Toxicol. 232, 15-23.
618 World Health Organization (WHO), 1998. Guidelines for drinking-water Quality, 2nd edn,
619 Health Criteria and Other Supporting Information. World Health Organization, Geneva.
620 World Health Organization (WHO), 1999. Toxic cyanobacteria in water: a guide to their public
621 health consequences, monitoring and management. Routledge, London and New York.
622 Witko-Sarsat, V., Friedlander, M., Capelliere-Blandin, C., Nguyen-Khoa, T., Nguyen, A.T.,
623 Zingraff, J., Jungers, P., Descamps-Latscha, B., 1996. Advanced oxidation protein
624 products as a novel marker of oxidative stress in uremia. Kidney Int. 49, 1304-1313.
625 Wyllie, A.H. 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251-306.
626 Yanni, Y.G., Carmichael, W.W., 1998. Screening of cyanobacteria isolated from soil, rice fields
627 and water resources of Nile River Delta for production of cyanotoxins. In: Reguera, B.,
628 Blonco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Vigo and Paris: Xunta de
629 Galicia and Intergovernmental Oceanographic Commission (IOC) of UNESCO, pp. 943-
630 949.

631 Yasmin, M.Y., Aziz, B., Nazim, M., Madhavan, R.K., 1993. Prealbumin rather than albumin is a
632 more sensitive indicator of acute liver disease. Malays. J. Pathol. 15(2), 147-50.
633 Zanchett, G., Oliveira-Filho, E.C., 2013. Cyanobacteria and cyanotoxins: From impacts on
634 aquatic ecosystems and human health to anticarcinogenic effects. Toxins 5, 1896-1917.
635 Zegura, B., Lah, T.T., Filipic, M., 2004. The role of reactive oxygen species in microcystin-LR-
636 induced DNA damage. Toxicol. 200(1), 59-68.
637 Zegura, B., Straser, A., Filipic, M., 2011. Genotoxicity and potential carcinogenicity of
638 cyanobacterial toxins- a review. Mut. Res. 727(1-2), 16-41.
639 Zhang, D., Xie, P., Liu, Y., Qiu, T., 2009. Transfer, distribution and bioaccumulation of
640 microcystins in the aquatic food web in Lake Taihu, China with potential risks to human
641 health Sci. Total Environ. 407, 2191-2199.
642 Zhang, H., Cai, C., Fang, W., Wang, J., Zhang, Y., Liu, J., Jia, X., 2013. Oxidative damage and
643 apoptosis induced by microcystin-LR in the liver of *Rana nigromaculata in vivo*. Aq.
644 Toxicol. 140-141:11– 18.
645 Zhang, J., Chen, J., Xia, Z., 2013. Microcystin-LR exhibits immunomodulatory role in mouse
646 primary hepatocytes through activation of the NF-κB and MAPK signaling pathways.
647 Toxicol. Sci. 136, 86-96.

648 **Figure legends**

649 Fig. 1. HPLC chromatogram of MCs of *Nostoc* sp. NR1 culture.

650 Fig. 2. Acute toxicity study (n=8) of *Nostoc* sp. strain NR1 extract

651 Fig. 3. Alteration in hepatic PP activity (U/mg protein) in different animal (n=12) groups
652 (Significance $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group).

653 Fig. 4. Alteration in liver metabolic functions (albumin, TC and TG, mg/dL) in different animal
654 (n=12) groups (Significance $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group).

655 Fig. 5. (A) Percentage of DNA fragmentation in different animal (n=12) groups (Significance $^*P$
656 < 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group). (B) Analysis of DNA
657 fragmentation pattern using agarose gel electrophoresis in different animal groups (M refers to
658 100 bp DNA ladder).

659 Fig. 6. Level of hepatic TNF-α (pg/mg tissue) in various animal (n=12) groups (Significance $^*P <$
660 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group).

661 Fig. 7. Hematoxylin-eosin sections of liver tissues of various animal groups showing (A) normal
662 architecture in control group (n=12), (B) complete collapse of the reticulin framework in acute
663 mice group (n=8), (C) limited area of congested central vein (CV) in WHOac (0.01% of LD_{50})
664 group (n=12), (D, E) areas of acute inflammatory cell infiltration and confluent necrosis (N) with
665 brownish granular pigment (Bp) of hyperplastic Kupffer cells in the subacute (10% of LD_{50})
666 group (n=12), (F) limited areas of necrosis (N) with macrovesicular steatosis (S) in WHOch
667 (0.01% of LD_{50}) mice group (n=12), and (G,H) areas of macrovesicular steatosis (S),
668 inflammation (I) and necrosis (N) with CV in subchronic (10% of LD_{50}) group (n=12).
669 (Magnifications x 200).

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Table 1 Alteration in activities (U/L) ALT, AST, LDH and ALK in NR1 extract-exposed animal groups compared to control group

Animal group / Parameter	Control	Subacute		Subchronic	
		0.01% (WHOac)	10%	0.01% (WHOch)	10%
ALT	75±2.4	79.92±1.27	209.4±9.1***	103.9±3.4**	135.5±2.67***
AST	182.19±1.81	184.4±2.2	354.44±4.9***	201±2.6**	252.22±2.8***
LDH	571.9±4.7	587.2±8.5	944.3±8.1***	616.3±7.1**	833.6±12.4***
ALK	78.75±1.2	81.54±2.12	145.83±2.2***	98.33±2.2**	113.33±2.2***

All values (n=12) are expressed as mean±SEM. Significance *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group.

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Table 2 Hepatic levels (mg/g tissue) of NO, MDA, OPP (nmol/g tissue) and GSH as well as activities (U/mg protein) of GPx and SOD of various mice groups

Animal group / Parameter	Control	Subacute		Subchronic	
		0.01% (WHOac)	10%	0.01% (WHOch)	10%
NO	1.122±0.028	1.13±0.26	1.88±0.027***	1.24±0.018*	1.31±0.034**
MDA	117.41±1.5	122.29±3.11	211.22±0.72***	132.3±3.4*	137.7±4.7**
OPP	51.47±0.53	51.69±0.61	109.39±1.3***	57.9±1.52*	60.75±2.4**
GSH	0.466±0.11	0.44±0.003	0.227±0.003***	0.414±0.007*	0.393±0.014**
GPx	4.146±0.03	4.06±0.08	2.266±0.03***	3.89±0.05*	3.71±0.06**
SOD	3.022±0.03	3.0197±0.03	2.492±0.05***	2.83±0.04*	2.78±0.038**

All values (n=12) are expressed as mean±SEM. Significance *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group.

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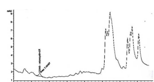

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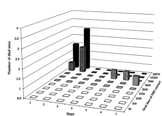

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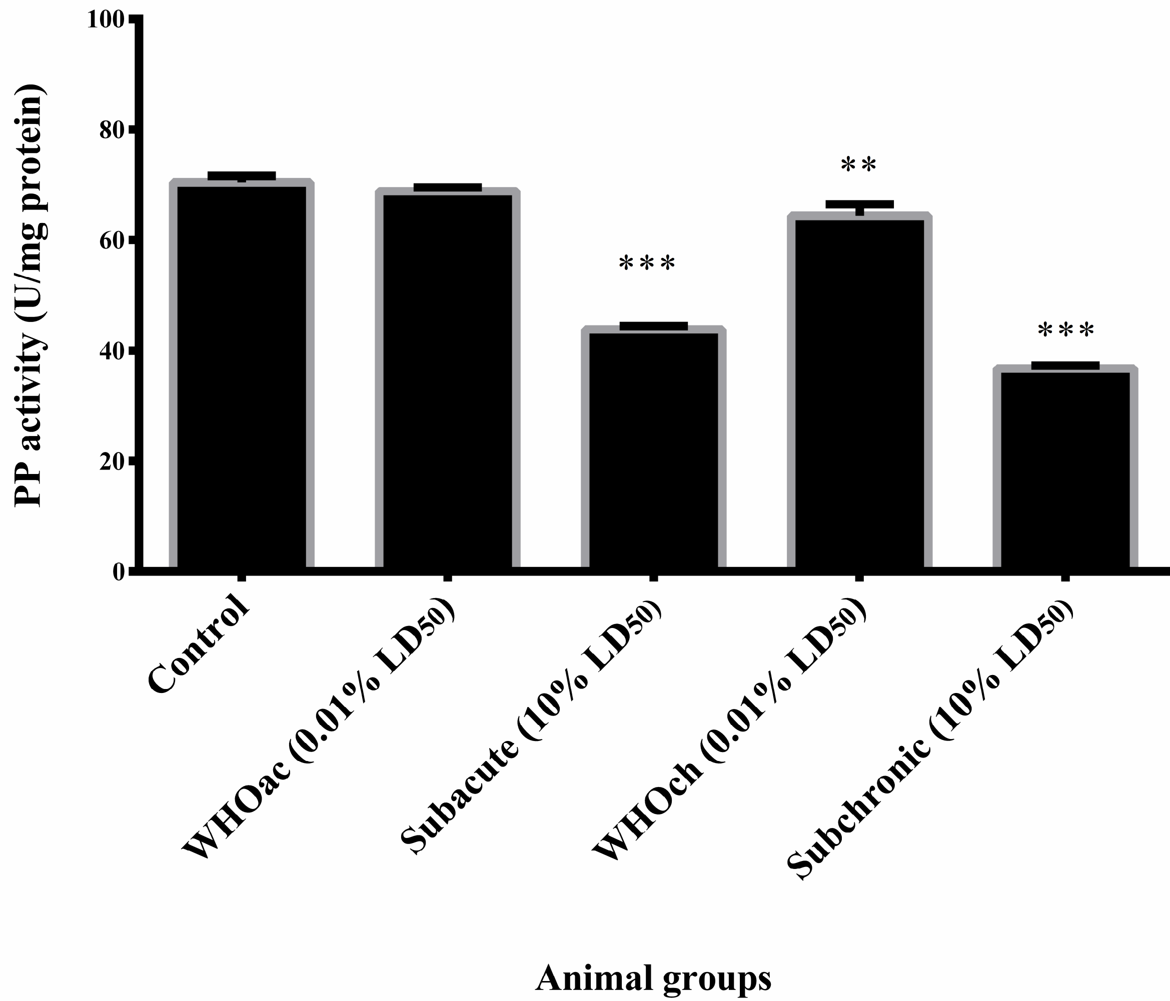

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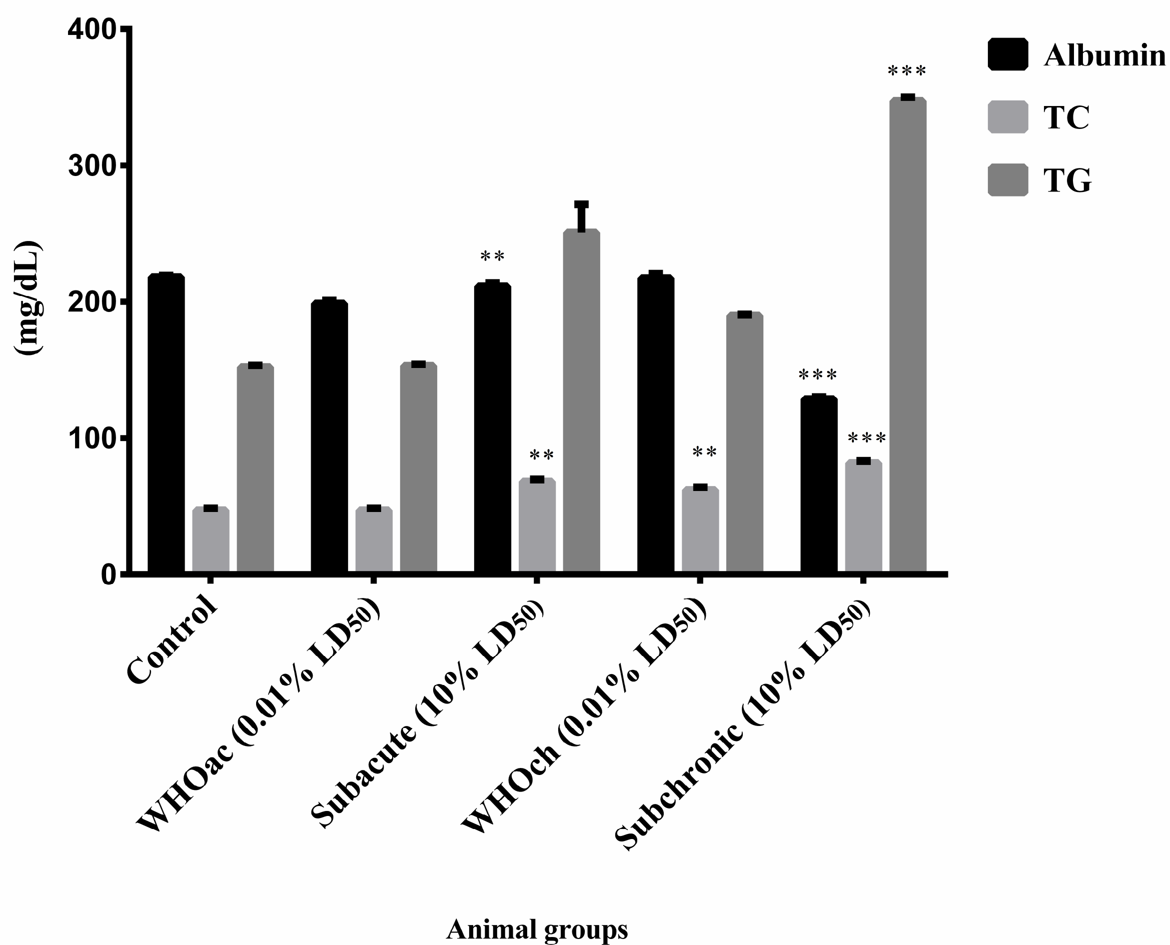

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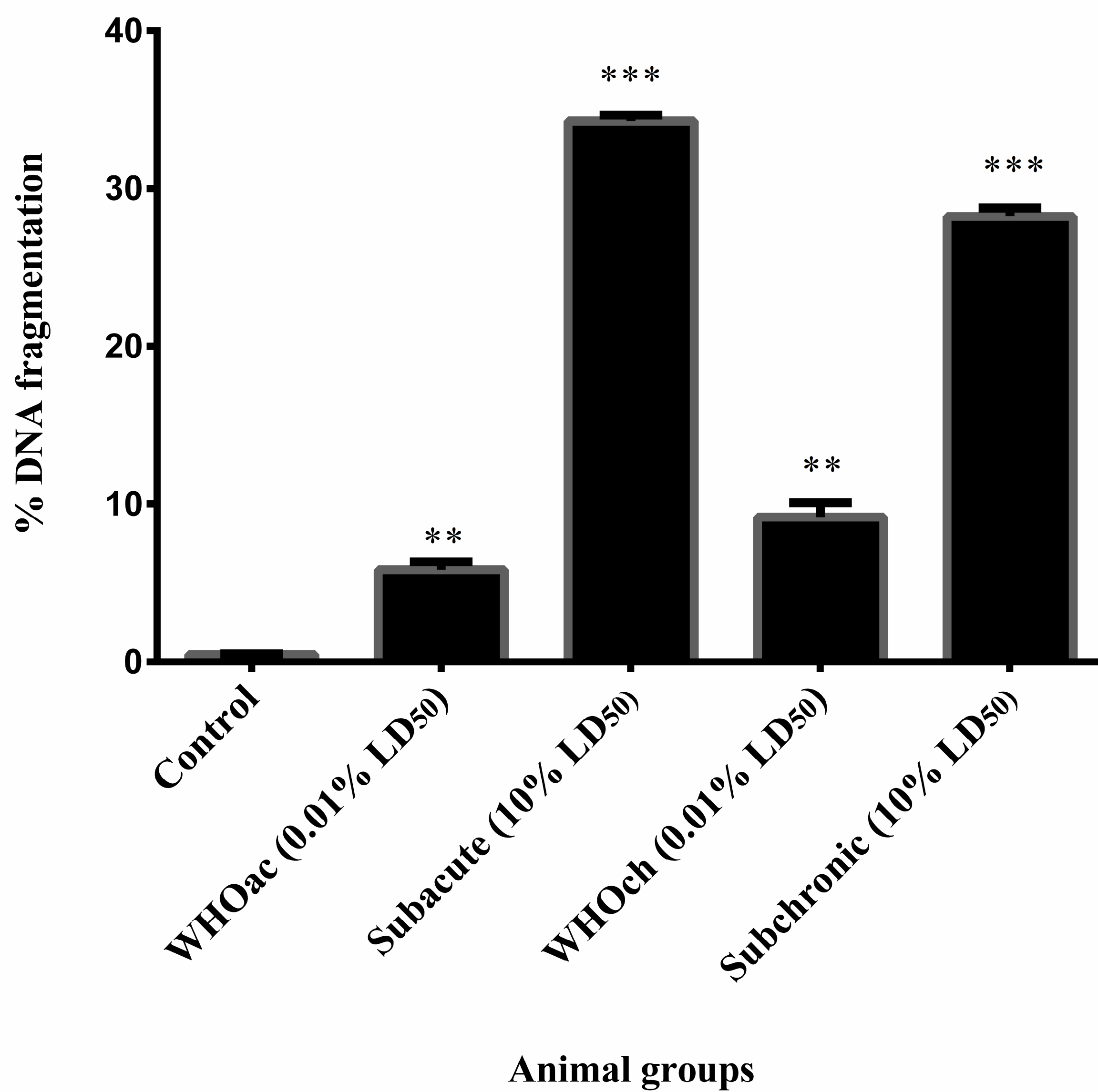

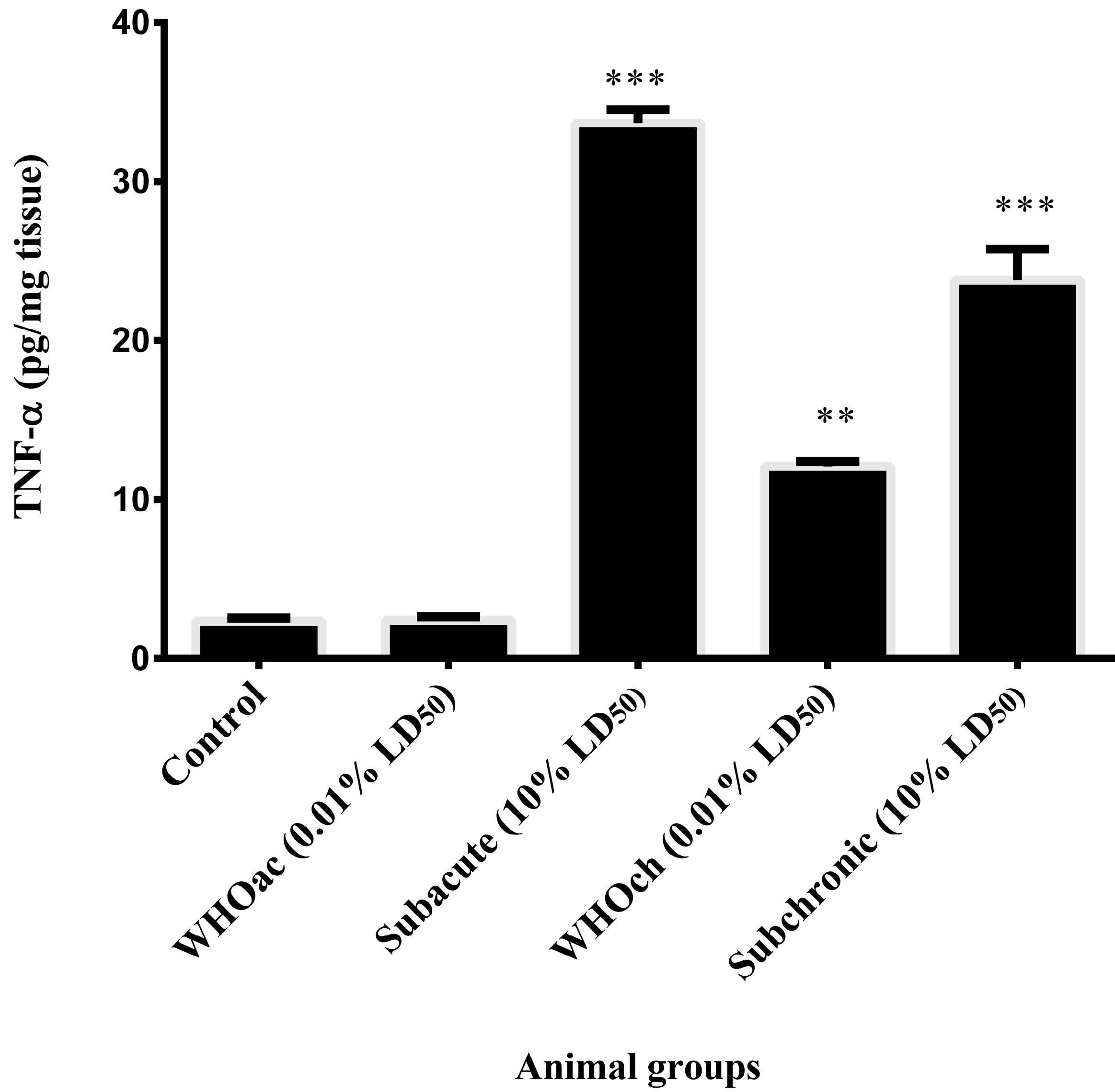

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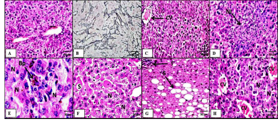

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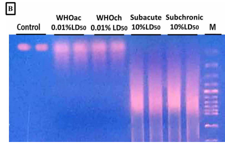

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- *In vivo* investigation of hepatotoxicity of our identified Nile River Nostoc sp. strain (*Nostoc* sp. NR1) extract.
- Hepatotoxicity signs of *Nostoc* sp. NR1 extract-injected mice included cytoskeleton alteration, oxidative damage and necroinflammation.
- Hepatotoxicity of *Nostoc* sp. NR1 extract is highly related to its microcystins.
- Severe hepatotoxicity grade was recorded in 10% LD_{50} Nostoc sp. NR1 extract-administered mice of subacute group than subchronic group.
- Human and animals who being exposed to excessive *Nostoc* sp. NR1-contaminated drinking water, may be at risk of major health problems.

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