The potent therapeutic effect of novel cyanobacterial isolates against oxidative stress damage in redox rats

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Abstract
Aims: Cyanobacteria are immense sources of several pharmacological active compounds such as flavonoids and carotenoids with anti-inflammatory and antioxidant activity. The potential therapeutic effect of two novel cyanobacterial isolates, Cronbergia siamensis (KY296358.1) and Sphaerospermopsis aphanizomenoides (KU212886.1), against hydrogen peroxide (H₂O₂)-induced oxidative stress damage in the rat model was determined in this study.

Methods and Results: In vitro antioxidant activity of the two studied isolates was evaluated by radical scavenging assay and ferric reducing power. The possible prophylactic activity of S. aphanizomenoides (KU212886.1) against H₂O₂-induced oxidative stress in the rat model was assessed in vivo. Serum alanine transaminase and aspartate transaminase were measured for the liver functions in redox rats. Liver malondialdehyde (MDA), glutathione, oxidized glutathione, nitric oxide, superoxide dismutase (SOD) and catalase (CAT) were assessed as oxidative stress markers. The effect of S. aphanizomenoides on the transcripts level of superoxide dismutase (Mn-SOD) and catalase (CAT) genes in the rat’s liver tissues was measured using qRT-PCR. Oral administration of S. aphanizomenoides extract in low and high doses (100, 200 mg kg⁻¹ b.w) resulted in significant improvement in biochemical parameters of liver functions and oxidative stress markers. Also, the endogenous antioxidant defence enzymes and the expression of their related genes (Mn/SOD, CAT) were upregulated. Immunohistochemistry of Caspase-3, an apoptotic marker, showed potent amelioration in the liver tissues.

Conclusions: The novel isolate S. aphanizomenoides proved in vitro and in vivo antioxidant activity against redox rat model.

Significance and Impact of the Study: This isolate provides a new source of pharmacological compounds with great importance in pharmacological and medical fields.

Keywords
antioxidant activity, Caspase-3 qRT-PCR, cyanobacteria, liver functions, oxidative stress, redox rats.

Introduction
Oxidative stress is a common indicator of toxicity that could promote tissue damage of all vital molecules threatened organism health. It occurs when the production of free radicals and reactive oxygen species (ROS) exceed the cellular antioxidant defence system (Mecocci et al. 2018). The released free radicals were considered the main endogenous damage in the biological systems (Thajuddin and Subramanian 2005). This damage affects all the essential bio-compounds like proteins, DNA and membrane lipids. The produced ROS correlated directly with over than 100 disease states either as source or outcome like cancer, diabetes, obesity, cardiovascular disease, neurodegenerative diseases, immune function decline and ageing (Wijeratne et al. 2005; Gagné 2014; Furukawa et al. 2017; Mecocci et al. 2018).

Antioxidants work as free radical scavengers that could hunt all ROS and prevent oxidative damage in living organisms. They can interact safely with free radicals to

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terminate the chain reaction, enhance the immune system, remove the contaminants and pollutants, and reduce inflammation and hypertension (Safafar et al. 2015). In the cell, the antioxidants could be enzymatic factors like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutaredoxins, glutathione reductase and thioredoxins besides nonenzymatic antioxidant factors, such as beta-carotene, carotene, selenium, zinc, taurine, hypotaurine, glutathione and vitamins like vitamin C and E (Agarwal et al. 2008; Mulgund et al. 2015; Mecocci et al. 2018). Despite all organisms have their own antioxidant defence and repair systems, at the time that body ages, antioxidant levels decrease dramatically offering a disturbance in the balance between oxidants and oxidant factors. As a result of that rising the oxidative stress damage and the shortage of natural repair system that will fail to overcome the entire damage (Chandrika and Kumara 2015).

The crude extracts of vegetables, fruits and plants are the main sources of natural antioxidants (Hossain et al. 2014). However, the medical use of these sources is limited as their attainment to optimum growth takes a long time (Nakao et al. 2009; Aydaş et al. 2013). Hence, these sources must be replaced by nonexploited natural products that can be converted into medical products under high technical condition.

Recently, cyanobacteria have attracted the attention of the scientific community as they represent themselves strongly as new natural sources for numerous pharmacologically active compounds (Hossain et al. 2016; Singh et al., 2017). Cyanobacteria produce a variety of secondary metabolites, flavonoids, carotenoids, pigments, phenols, tannins, terpenes and vitamins with antioxidant, antidiabetic, anti-inflammatory, antifungal, anticancer, antibiotic and antiviral activity (Guihéneuf et al. 2016; Singh et al., 2017). Interestingly, it is estimated that more than 15 000 innovative compounds have been chemically determined in cyanobacterial biomass (Munir et al. 2013). Nowadays cyanobacteria are regarded as good candidates for drug discovery, where they are noncompetetive with economic crops, can multiply in volume nightly and cropped on daily basis. Hence, they are efficient, unconventional and unexplored sources for different antioxidants with multiple applications in the pharmaceuticals and cosmetic sectors (Hossain et al. 2016).

The identification and characterization of cyanobacteria have been widely carried out in many previous studies (Gugger et al. 2002; Rajaniemi et al. 2005; Hayes et al. 2007; Nayak et al. 2007; Li et al. 2008; Zapomelová et al. 2010; Galhano et al. 2011; Ponnuswamy et al. 2013). However, few investigations have been differentiated and quantified the antioxidants in cyanobacteria (Chacón-Lee and González-Mariño 2010; Cirulis et al. 2013; Safafar et al. 2015). The biological and therapeutic efficiency of cyanobacteria have been established in some recent studies (Li et al. 2015; Carfagna et al. 2015; Bashandy et al., 2016; Hossain et al. 2016; Singh et al., 2017). Although, the molecular and therapeutic responses to administration of cyanobacteria received limited attention.

This study aimed to determine in vitro and in vivo biological activity of two novel cyanobacterial isolates (Cronbergia siamensis, KY296358.1, and Sphaerospermopsis aphanizomenoides, KU212886.1) against hydrogen peroxide (H$_2$O$_2$)-induced oxidative stress in a rat model. The antioxidant effect of S. aphanizomenoides (KU212886.1) on liver functions and oxidative stress markers was evaluated and its ability to inhibit Caspase3 interaction was determined. In addition, the mRNA transcripts level of Mn/SOD and CAT genes were quantified as an antioxidant biological marker to confirm the therapeutic effect of the potent isolate on redox rats via qRT-PCR.

Materials and methods

Two cyanobacterial isolates, C. siamensis (KY296358.1) and S. aphanizomenoides (KU212886.1), were used in this study. These isolates were obtained from Badr et al. (2018) study as probable new species that were isolated from irrigated agriculture canals at Kafr Elsheikh governorate, stressed aquatic system. Samples were purified using serial dilutions method according to Lee et al. (2014). Where, a nutrient selective media (BG-11) was optimized for the growth of cyanobacterial species (Rippka et al., 1979).

Cyanobacterial extract preparation

For each cyanobacterial isolate, the fresh biomass was harvested by centrifugation at 2318 g for 10 min. The obtained biomass was freeze-dried by Lypohilizer (NAN-BEI; Model Number: LGJ-10-3; Mainland of China, Zhengzhou, China). The freeze-dried biomass was extracted by homogenization at 11 5216 g for 10 min in 70% ethanol. The obtained crude extract was centrifuged at 12 3622 g for 10 min, the aqueous supernatant was lyophilized. The dried sample was kept in 5 ml brown sealed bottles, at –20°C, until use. This method was modified from Carvalho et al. (2013) methodology.

Chromatographic analysis of phenolic acids, flavonoids, carotenoids and vitamins in cyanobacterial isolates

The phenolic compound was measured by Agilent HPLC 1260 Infinity II, system; the system consisted of autosampler, quaternary pumps, UV detector, an interface, reversed-phase column alternative for LiChrospher 100 RP-c18, 250 mm × 4.0 mm I.D., 5 µm particle size. The
system was controlled by Chemstation HPLC Manager. After lyophilization, the cyanobacterial samples were ground in methanol-dimethyl sulphoxide (DMSO) (v/v) using homogenizer at 14 5216 g then centrifugation was done at 14 1304 g for 10 min. The prepared samples were stored at 4°C till proceeding of analysis.

HPLC conditions

The detector monitored the eluent at 285 nm, two-solvent gradient system was used. The gradient program presented as follow: A (0.01 mol l⁻¹ phosphoric acid) and B (methanol), 1st 0–16.5 min, 70–55% (v/v); 2nd 16.5–28.5 min, 55–0% A in B; 3rd 28.5 : 30 min, isocratic, 100% B. The resulting chromatographic data on the absorbing peaks was integrated up to 30 min. The flow-rate was 2 ml min⁻¹ and the column was operated at 40°C. The sample injection volume was 20 μl.

Flavonoids were determined according to Nogata et al. (1994). The mobile phase contains gradient elution with 0.01 mol l⁻¹ phosphoric acid–methanol. The flow rate was adjusted to 0.6 ml min⁻¹, the column was thermostatically controlled at 40°C and the injection volume was kept at 20 μl. A gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elution was changed from 10% to 40% B in a linear fashion for duration of 28 min, from 40 to 60% B in 39 min, from 60 to 90% B in 50 min. The mobile phase composition back to initial condition (solvent B: solvent A: 10: 90) in 55 min and allowed to run for another 10 min, before the injection of another sample. HPLC chromatograms were detected using a UV detector 285 nm wavelength. Each compound was identified by its retention time and by spiking with standards under the same conditions.

Detection of β-carotene and Zeaxanthin was done by HPLC according to Ahamad et al. (2007). The mobile phase was acetonitrile, dichloromethane and methanol by the ratio of 70 : 20 : 10 respectively. The elution was performed at a flow-rate of 2 ml min⁻¹. The column was reversed-phase C18 (150 mm × 4.6 mm, 5 μm). The injection volume was 20 μl. The analytical column was kept at 25°C and the detection was performed at 452 nm.

Determination of vitamins A and E was done by HPLC according to Gimeno et al. (2000). The mobile phase contains methanol–water (96 : 4, v/v) and the elution was performed at a flow-rate of 2 ml min⁻¹, the column was RP-column (Nucleosil® C18; 10 μm). The injection volume was 20 μl. The analytical column was kept at 45°C. Detection was performed at 325 for vitamin A and 292 nm for vitamin E (Switch the wavelength after 7 min.). While, water-soluble vitamins were detected using Ekinici and Kadakal (2005) methodology. The mobile phase was 0.1 mol l⁻¹ KH₂PO₄ (pH 7)-methanol, 90 : 10. The elution was performed at a flow-rate of 1 ml min⁻¹ the column was reversed-phase Discovery C18 (150 mm × 4.6 mm, 5 μm) from Supelco (Bellefonte, PA). The injection volume was 20 μl. The analytical column was kept at 45°C. Detection was performed using UV detector at 254 nm wavelength.

Antioxidant activity evaluation

Total phenolic content

The total phenolic content of the cyanobacterial extract was determined by the Folin–Ciocalteu method (Kaur and Kapoor 2002). Briefly, 200 μl of the cyanobacterial extract suspension (1 mg ml⁻¹) was adjusted to 3 ml with distilled water, mixed thoroughly with 0.5 ml of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 ml of 20% (w/v) sodium carbonate. The mixture set for 60 min in the dark and the absorbance was measured at 650 nm (UV-2450, UV-VIS Spectrophotometer; Shimadzu Tokyo, Tokyo, Japan). Total phenolic content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction and it was calculated from the calibration curve.

DPPH radical scavenging assay

The ability of different cyanobacterial extract to act as hydrogen donors was measured by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay according to Brand-Williams et al. (1995). Different concentrations from cyanobacterial extracts were prepared (50, 100 and 200 μg ml⁻¹). 50 μl of each sample was mixed with 450 μl of Tris-HCl (pH 7) and 1 ml of DPPH then incubated at 37°C for 30 min. Spectrophotometric absorbance was 517 nm and gallic acid was used as standard. Percentage of Inhibition was calculated according to formula:

\[
\text{Inhibition} \% = \frac{\text{absorbance of DPPH} - \text{absorbance of sample}}{\text{absorbance of DPPH}} \times 100
\]

Ferric reducing power assay

Ferric Reducing Antioxidant Power (FRAP) of the tested isolates was determined following Oyaizu (1986) methodology. The FRAP reagent was freshly prepared and the absorbance was measured at 700 nm after 10 min of incubation at 25°C using rutin hydrate as standard.

Experimental design and In vivo study

The in vivo study was established depending on in vitro antioxidant activity results of the obtained cyanobacterial isolates. Where, the most potent isolate S. aphanizomenoides
was used for oral administration in this part of the study. A
total of 48 rats (Sprague Dawley) weighed 200 ± 20 g,
9–12 weeks of age were used in this study. Experiments
were done following the instructions of the National Regu-
lations on Animal Welfare and Institutional Animal Ethical
Committee (IAEC). All animals get care in agreement with
the rules of the animal care. The animals were protected in
a quiet place and allowed free access to water and food dur-
ing the study period. Rats were randomly separated into
eight groups, six each. The 1st group received tap water as a
control. The 2nd group received vitamin C as an antioxi-
dant reference drug (250 mg kg\(^{-1}\) body weight) according
to Paget and Barnes (1964). The 3rd group received a low
dose of \(S.\ aphanizomenoides\) extract (100 mg kg\(^{-1}\) b.w.).
The 4th group received a high dose of \(S.\ aphanizomenoides\)
extract (200 mg kg\(^{-1}\) b.w.). The 5th group received \(H_2O_2\)
0.5% as a positive control in drinking water. The 6th group
received \(H_2O_2\) (0.5%) in drinking water simultaneously
with VC. The 7th group received \(H_2O_2\) (0.5%) as well as
low-dose extract of \(S.\ aphanizomenoides\). The 8th group
received \(H_2O_2\) (0.5%) simultaneously with high dose of \(S.\ aphanizomenoides\) extract. The experiment lasted for 2
weeks and all animals received cyanobacterial extract and
other treatments by oral administration. After 2 weeks,
blood samples were gathered through 1 h from 8:00 to 9:00
am under light ether anaesthesia by a retro-orbital punct-
ure. Serum was separated for liver function analysis. After
general anaesthesia, all animals were immolated by cervical
dislocation. The liver was anatomized in ice quickly, cleaned
and stored at −80°C till analysis.

The effect of \(S.\ aphanizomenoides\) on rats challenged by
\(H_2O_2\)
Liver tissues of all groups were homogenized in phos-
phate buffer saline pH 7.4 and the homogenates were used
for the determination of oxidative stress markers malondialdehyde (MDA), glutathione (GSH), oxidized
 glutathione (GSSG) and nitric oxide (NO) by HPLC
according to Karatas et al. (2002), Jayatilleke and Shaw
(1993) and Papadoyannis et al. (1999) respectively.
While, endogenous antioxidant enzymes (SOD and CAT)
were analysed by spectrophotometer according to Mark-
lund and Marklund (1974) and Aebi (1984) respectively.
Hepatic dysfunction was measured by calculating the ele-
vation in serum levels of serum alanine transaminase (ALT), aspartate transaminase (AST) using commercially
available kits and the results were expressed in interna-
tional units/litre (IU l\(^{-1}\)) (Reitman and Frankel 1957).
For immunohistochemical staining of caspase-3, slide
preparation and antigen antibody reaction were per-
formed as described by Abd-Elrazez and Ahmed-Farid
(2018). The Adequate amount of haematoxylin stain was
added to the slide to cover the entire tissue surface for
counterstaining (Bancroft and Cook 1994).

RNA extraction and differential expression analysis of
Mn/SOD and CAT genes
Total RNA was isolated from liver tissues of all groups
using SV Total RNA Isolation System (Promega Corpora-
tion, Madison, WI). Complementary DNA (cDNA) was
synthesized from RNA using High Capacity cDNA reverse
transcriptase kit (Thermo Fisher Scientific, Waltham,
MA). Conventional PCR was performed to test the primer
specificity of target genes using cDNA as a template (data
not shown).

Triplicate qRT-PCR was performed for each sample,
nontemplate control (NTC) and negative cDNA template.
PCR reaction contained of 500 ng per reaction of cDNA
(except for NTC and cDNA control), 12.5 μl Maxima
SYBR Green qPCR Master Mix (Maxima SYBR Green
qPCR; Thermo Fisher Scientific), 0.3 μmol l\(^{-1}\) of each for-
ward and reverse primer, 10 nmol l\(^{-1}\)/100 Nm ROX Solu-
tion, nucleases-free water to a final volume of 25 μl.
Reactions were analysed on an AriaMx Real-Time PCR
System (Agilent Technologies, Santa Clara, CA), Two-step
cycling protocol, under the following conditions: 95°C
for 10 min and 40 cycles of 95°C for 15 s followed by 60°C for
60 s. The expression levels of the antioxidant enzyme genes
including \(Mn/SOD\) and CAT were normalized to the
housekeeping gene glyceraldehyde-3-phosphate dehydro-
genase (\(GAPDH\)), the names and oligonucleotide
sequences of the primers are listed in Table S1. All changes
in the expression of the studied genes were obtained as n-
fold changes relative to the corresponding controls. Rela-
tive gene expression ratios (RQ) between treated and con-
tral groups were calculated using the formula:
\[ RQ = 2^{-\Delta\Delta\text{Ct}} \] (Livak and Schmittgen 2001).

Statistical analysis
All values were stated as a mean ± SE for the rats in each
group. Differences between rat groups were evaluated by
one-way analysis of variance using SAS software (SAS,
2004). The obtained data was analysed using the general lin-
ear model. Duncan multiple ranges test (Duncan 1955) was
used to evaluate the significant differences among means.

Results
Nutritional profile and antioxidant activity of the
isolates
Nutritional profile results of the cyanobacterial isolates
are presented in Table 1. The isolate \(S.\ aphanizomenoides\)
Table 1 Nutritional profile of cyanobacterial isolates powder

<table>
<thead>
<tr>
<th>Contents concentration (µg g⁻¹ powder)</th>
<th>Cronbergia siamensis</th>
<th>Sphaerospermopsis aphanizomenoides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine HCL</td>
<td>0.03 ± 0.002</td>
<td>0.05 ± 0.003</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.02 ± 0.001</td>
<td>0.14 ± 0.009</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.18 ± 0.011</td>
<td>0.04 ± 0.002</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.15 ± 0.009</td>
<td>0.16 ± 0.010</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>0.03 ± 0.002</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>211.00 ± 12.83</td>
<td>309.00 ± 19.29</td>
</tr>
<tr>
<td>IU g⁻¹ powder</td>
<td>0.15 ± 0.009</td>
<td>0.05 ± 0.003</td>
</tr>
<tr>
<td>β-carotene</td>
<td>0.06 ± 0.004</td>
<td>0.20 ± 0.012</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.89 ± 0.053</td>
<td>1.34 ± 0.079</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>ND</td>
<td>0.83 ± 0.050</td>
</tr>
<tr>
<td>Coumaric acid</td>
<td>0.23 ± 0.014</td>
<td>0.44 ± 0.027</td>
</tr>
<tr>
<td>Ferulic</td>
<td>0.67 ± 0.043</td>
<td>1.21 ± 0.072</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.11 ± 0.127</td>
<td>4.12 ± 0.257</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>ND</td>
<td>1.39 ± 0.082</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD for five replicates for nutritional profile of different cyanobacterial isolates.

Table 2 Total phenolics content in the extracts of obtained cyanobacterial isolates

<table>
<thead>
<tr>
<th>Cyanobacterial extracts µg ml⁻¹</th>
<th>Total phenolics content (µg gallic per g dried algae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cronbergia siamensis</td>
<td>2.46 ± 0.05</td>
</tr>
<tr>
<td>Sphaerospermopsis aphanizomenoides</td>
<td>14.96 ± 0.30</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD for five replicates for total phenolics content of different cyanobacterial isolates.

Table 3 % DPPH radical scavenging activity of cyanobacterial isolates

<table>
<thead>
<tr>
<th>Cyanobacterial concentration µg ml⁻¹</th>
<th>DPPH scavenging activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cronbergia siamensis</td>
<td></td>
</tr>
<tr>
<td>Sphaerospermopsis aphanizomenoides</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5.32 ± 0.15</td>
</tr>
<tr>
<td>100</td>
<td>8.95 ± 0.32</td>
</tr>
<tr>
<td>200</td>
<td>13.74 ± 0.49</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD for five replicates for scavenging activity of different cyanobacterial isolates.

The effect of novel cyanobacterial isolate on reducing oxidative stress in rats

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Table contained higher levels of caffeic, ferulic, coumaric, gallic, ginnamic, salicylic acids as well as vitamin A, riboflavin, pyridoxine and zeaxanthin, than C. siamensis isolate. Consequently, it appeared the highest total phenolic content (Table 2). On another hand, S. aphanizomenoides showed the highest scavenging DPPH activity (58.74%) at concentration of 200 µg ml⁻¹ compared with C. siamensis (Table 3). The reducing powers of the isolates demonstrated that the scavenging ability was positively correlated with cyanobacterial concentrations (Fig. 1). Depending on these findings, the S. aphanizomenoides isolate seemed to be the best as an antioxidant compound in the cell, for its higher antioxidant activity than C. siamensis. Therefore, its therapeutic effect against H₂O₂-induced oxidative stress in rat model was evaluated in vivo.

Effect of S. aphanizomenoides against redox status in rat liver

Oral administration of S. aphanizomenoides low dose (100 mg kg⁻¹ b.w) and vitamin C revealed no significant differences in all biochemical parameters in comparison with the control group (Table 4). Whereas, the H₂O₂ treatment (P) caused a high toxicity alteration in all biochemical parameters compared with the control group.

On another hand, the groups that received S. aphanizomenoides treatments simultaneously with H₂O₂ (P+T1 and P+T2) exhibited a marked amelioration in liver function (AST, ALT), a decrease in oxidative stress markers (MDA, GSSG, NO), increase in endogenous antioxidant defence enzymes (SOD, CAT) compared with positive control group, P < 0.05 (Table 4). Interestingly, the high dose of S. aphanizomenoides (200 mg kg⁻¹) showed marked amelioration for all biochemical parameters compared to H₂O₂ group. Similarly, the low dose of S. aphanizomenoides (100 mg kg⁻¹) showed marked enhancement for all biochemical parameters in comparison with the H₂O₂ group.

Immunohistochemistry of Caspase-3 in the liver tissue

Histopathological examination of liver tissues of normal rats in control groups showed normal hepatic tissue without any injury and no interaction of Caspase 3 (Fig. 2a,b). Liver tissues treated with H₂O₂ showed strong positive interaction of Caspase 3 (Fig. 2c). Whereas, oral administration of high and low doses (200, 100 mg kg⁻¹) of S. aphanizomenoides extract strongly reduced the interaction of Caspase 3 in redox rat livers (Fig. 2d–f). Remarkably, the reduced patterns of Caspase 3 caused by the high dose of S. aphanizomenoides (200 mg kg⁻¹) was nearly reached to that created by the vitamin C, standard drug, (Fig. 2e,f).

Effect of S. aphanizomenoides on the expression of Mn/SOD and CAT

The effects of H₂O₂ and S. aphanizomenoides extract on the mRNA levels of two antioxidant enzyme encoding genes, manganese superoxide dismutase (Mn/SOD) and
catalase (CAT), were determined by qRT-PCR (Fig. 3a, b). There were no significant differences in gene expression levels of Mn/SOD and CAT among the groups that received vitamin C (VC), low and high doses of S. aphanizomenoides (T1 and T2). In contrast, the groups that received hydrogen peroxidase (P) reduced Mn/SOD and CAT transcripts significantly by 1.89- and 1.45-fold, respectively, compared to untreated groups. Where, Treatments of low and high doses of S. aphanizomenoides upregulated mRNA levels of Mn/SOD by 2.42- and 2.89-fold, respectively, compared with the positive control group (P). Similarly, the transcripts amount of CAT gene increased by 2.62- and 2.92-fold in response to treatments of low and high doses S. aphanizomenoides respectively.

Discussion

Cyanobacteria are enormously diverse group of prokaryotic organisms that live in a widespread environmental systems (Hu et al. 2008). They are rich sources of secondary metabolites with potential biotechnological applications in the field of pharmacology (Vijayakumar and Menakha 2015; Singh et al., 2017). It is clearly known that environmental pollution and diverse stress factors elicit cyanobacteria to produce high levels of secondary metabolites (Jimenez-Garcia et al. 2013). The cyanobacterial isolates in this study obtained from irrigated wastewater canals that characterized with the presence of various types of contaminants. The continuous environmental stresses in this site may be promoting the development of its cyanobacterial community through upregulating of secondary metabolites production. Consequently, these isolates were expected to have upregulated secondary metabolites with probable antioxidant activity.

Carotenoids and phenolic compounds are major contributors of antioxidant capacities for microalgae (Jimenez-Escrig et al. 2001; Li et al. 2007; Goh et al. 2010; Hajimahmoodi et al. 2010). Additionally, flavonoids are capable of inhibiting and reducing free radicals to terminate the radical chain reactions (Rahul et al. 2016). Nutritional profile results of the cyanobacterial isolates revealed that both isolates contained different antioxidant constituents like phenolic compounds, flavonoids, carotenoids and vitamins. The highest levels of vitamin A, gallic acid, salicylic acid, ferulic acid (FA), cinnamic acid, coumaric acid, riboflavin, pyridoxine and zeaxanthin appeared in the crude extract of S. aphanizomenoides. Interestingly, it recorded high levels of caffeic acid in disagreement with Goiris et al. (2014) who investigated flavonoids in different cyanobacterial species and reported that the flavanone eriodyctiol and its precursor caffeic acid were not detected in the algal biomass samples. However, the recent study of Singh et al. (2017) evidenced that cyanobacteria have a wide range of carotenoids, flavonoids and phenolic compounds.

The DPPH radical scavenging assay is used usually for estimating natural antioxidants due to its constancy, simplicity and reproducibility (Kuda et al. 2007). The DPPH scavenging ability of a compound depend on its ability to pair with the unpaired electron of a radical (Park et al. 2004). Hence, The DPPH scavenging ability is a reliable and effective indicator of possible antioxidant activity (Hossain et al. 2016). Jerez-Martel et al. (2017) investigated the antioxidant activity of Nostoc sp., L. protosira, N. spumigena and Phormidiochaete sp., where DPPH radical ranged from 7.65% (L. protosira) to 27.89% (Nostoc sp.). While, other investigations reported that S. marginatum and T. conoides scavenged DPPH by 11 and 17-2%, respectively (Chandini et al. 2008) as well as F. vesiculosus and A. nodosum scavenged DPPH by 31-2 and 25-6% respectively (O’Sullivan et al. 2011). In comparison with all previous results, S. aphanizomenoides in this study appeared the highest scavenging DPPH activity (58.74%) at concentration of 200 µg ml⁻¹. These findings approved that, S. aphanizomenoides could be a promising natural source of antioxidants with high pharmacological and medical importance. Depending on
these results, *S. aphanizomenoides* was selected to study its antioxidative effect against H$_2$O$_2$-induced oxidative stress in the rat model.

Cyanobacteria antioxidative defense system consists of both enzymatic (SOD, CAT, glutathione reductase and ascorbate peroxidase) and nonenzymatic compounds (ascorbic acid, reduced glutathione, tocopherols, carotenoids and phycocyanin) (Ohki et al. 2008). In this study, the oral administration of *S. aphanizomenoides* significantly reduced free radical damage induced by H$_2$O$_2$ which is an extremely reactive free radical, can damage almost every molecule in living cells (Hochstein and Atallah 1988). There was a marked amelioration on liver function (AST, ALT), a decrease in oxidative stress markers (MDA, GSSG, NO), increase in endogenous antioxidant defence enzymes (SOD, CAT). These results supported by the study of El-Tantawy (2016) who revealed that *Spirulina* oral administration revealed a significant decrease in serum ALT, AST activities as compared to lead treated animals. The effect of *Spirulina* administration was strong as the effect of vitamin C and these results are similar to the obtained results in this study. Moreover, the study of El-Baz et al. (2018) reported that the antioxidant activity of *Dunalieila salina* and *Haematococcus pluvialis* against colon, breast, lung and liver cancer cell lines could be due to the carotenoid fraction in the crude extracts of these isolates. While, the study of Chen et al. (2016) clearly confirmed that glutathione has the ability of scavenging free radicals and protecting the liver from oxidative stress.

The antioxidant activity of *S. aphanizomenoides* could be due to high levels of FA that appeared in the crude extracts of the isolate. Ferulic acid is a phenolic compound biosynthesized from caffeic acid by the action of the enzyme caffeate O-methyltransferase. It exhibits a strong antioxidant activity via donating one hydrogen atom from its phenolic hydroxyl group (Kikuzaki et al. 2002; Ou and Kwok 2004; Rosa et al. 2013a, 2013b). Its antioxidant activity probably due to the specific structure of the compound, the hydroxyl group, the location—a couple, a methoxy group in the meta-position of the phenyl ring, (Mielczarek et al. 2010). Some investigations revealed that the regular absorption of FA to provide protection associated with a range of oxidative stress related diseases. Jung et al. (2009) reported that ferulate effectively suppresses redox-sensitive, proinflammatory NF-κappaB activation via NF-kappa-B-inducing kinase/eNOS-kappa-B kinase and MAPKs by reducing oxidative stress in aged rats. Moreover, Khanduja et al. (2006) indicated that FA helped in reduction in lipid peroxidation in peripheral blood mononuclear cells induced by H$_2$O$_2$. Hence, the presence of FA and other phenolic compounds in the crude extract of *S.
aphanizomenoides may be the main cause of decreasing the levels of ALT, AST, MDA, GSSG, NO and increasing GSH, SOD, CAT in redox rats.

In this study, immunohistochemical examination for caspase-3 (proapoptotic marker) of liver revealed pathological alteration in the H2O2 group and ameliorative

Figure 2 Immunohistochemical staining of Caspase 3 in liver of rats from the studied groups, (a) control group (tap), (b) vitamin C group (VC), (c) H2O2 group (P), (d) the group of H2O2 with VC (P + VC). (e) The group of H2O2 with low dose of algae (P + T1). (f) The group of H2O2 with high dose of algae (P + T2), immunopositivity indicated by brown colour, (X 400).

Figure 3 (a) Expression profiling of CAT gene in response to cyanobacterial treatments. (b) Expression profiling of Mn-SOD gene in response to cyanobacterial treatments. Changes in gene expression were denoted as relative change (ratio of target gene/reference gene). GAPDH was used as reference gene.
effect of S. aphanizomenoides at low and high doses. 
H2O2-induced apoptosis was associated with activation of caspase-3 as assessed by cleavage of specific fluorogenic substrate peptide, processing of pro-caspase-3 and poly (ADP-ribose) polymerase. However, the treatment of S. aphanizomenoides caused a decrease in pro-apoptotic markers and this may be due to the presence of FA in the isolate crude extract which could increase the stability of cytochrome c, and hence inhibits the apoptosis. These results agreed with the results of Wang et al. (2016) who studied the effect of FA on the expression of Bcl-2, an anti-apoptotic Bcl-2 family protein, and Bax, a pro-apoptotic Bcl-2 family protein, in osteosarcoma cells using immunostaining. The Bax/Bcl-2 ratio affects caspase 3 and controls the cell apoptosis. He reported that FA upregulated Bax, downregulated Bcl-2, and subsequently enhanced caspase-3 activity.

These results were confirmed by the expression analysis of antioxidant enzyme genes, Mn/SOD and CAT. The mRNA transcript levels of Mn/SOD and CAT raised significantly in the H2O2 induced groups that received S. aphanizomenoides extract. Thus, the induction of mRNA patterns was in accordance with the changes in levels of antioxidant enzymes that observed in the liver. Storz (2011) results stated that Mn/SOD and CAT enzymes are directly induced by FOXO3a which is an iso-type of FOXO transcription factors. FOXO3a binding sites have been identified in the promoters of Mn/SOD and CAT genes (Kops et al. 2002; Li et al. 2008; Tan et al. 2008). Thus, the induction of Mn/SOD and CAT transcripts may be due to the activation of PI3K/Akt/FOXO3a pathway via administration of S. aphanizomenoides that contain high levels of FA.

In conclusion, the newly identified isolate S. aphanizomenoides can be of pharmacological and medical importance. The observed antioxidant activity of S. aphanizomenoides may be due to a presence of different antioxidant constituents. Especially, the presence of high contents of FA in its crude extract could be the main factor responsible for the antioxidant activity through induction of PI3K/Akt/FOXO3a pathway. Consequently, increasing the levels of Mn/SOD and CAT transcripts and recovering caspase-3 in redox rats.

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Availability of data and materials
The datasets used and/or analysed during this study are available on request from the corresponding author.

Author contributions
Omnia A. Badr, Mahmoud M. Moustafa and Omar Farid performed the in vitro and in vivo tests to measure the biochemical and physiological parameters and analysed the related data. Omnia A. Badr, I.I.S. El-Shawaf, Hoda A. El-Garhy and Mahmoud M. Moustafa quantitatively examined the expression of SOD and CAT genes and performed the immunohistochemical examination. All authors wrote and drafted the corresponding sections of the manuscript and revised the complete manuscript.

Ethics approval and Consent to participate
The protocol for the conducted animal experiments was approved by the Research Ethics Committee of the Faculty of Pharmacy, Ain Shams University which followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication no. 85–23, revised 1996).

Consent for publication
Not applicable.

Competing Interests
The authors declare that they have no competing interests.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for qPCR amplification.