academicJournals

Vol. 13(9), pp. 1086-1096, 26 February, 2013
DOI: 10.5897/AJB2013.13177
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Characterization of blue green algae isolated from Egyptian rice field with potential anti-hepatitis C active components

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Received 20 August 2013; Accepted 20 February, 2014

Several species of cyanobacteria has been recognized for its therapeutic value that can be used for treatment of malnutrition, cancer and viral infection. Many natural occurring cyanobacteria are known to produce toxins, for example, species of the genera *Microcystis, Nodularia, Nostoc, Anabaena, Aphanizomenon, Cylindrospermopsis, and Planktothrix* (*Oscillatoria*). Cyanotoxins are classified according to their mode of action in vertebrates as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritants. Microcystin is a hepatotoxin which commonly found in *Microcystis* and it was found to be produced by other genera, including *Anabaena, Nostoc, Nodularia*, and *Planktothrix*. In the present study cyanobacteria strain isolated from Egyptian soil was purified, characterized and identified as *Nostoc* sp. and named *Nostoc EGY*. PCR-based techniques targeting the toxin biosynthesis genes were used verifying absence of toxic genes in the newly purified cyanobacteria. Cell lysate was prepared from the purified strain; the efficacy of this lysate to prevent hepatitis C virus (HCV) replication in vitro was proved qualitatively and quantitatively. Lysate prepared from isolated cyanobacteria after 10 and 25 days of cultivation was able to prevent replication of *in vitro* cultivated HCV.

Key words: Hepatitis C, green algae, cyanobacteria, polymerase chain reaction.

INTRODUCTION

Cyanobacteria as one of the oldest fossils on earth, more than 3.5 billion years, they are aquatic and photosynthetic unicellular microorganisms (Botos and Wlodawer, 2003; Laura G. Barrientos, 2008). Cyanobacteria are growing in a wide range of habitats and were named for the blue-green pigment, phycocyanin. Usually, they grow in large colonies include unicellular, colonial, and filamentous forms and can be considered as one of the largest and most important groups of bacteria on earth. Cyanobacteria that lack toxins are widely used as food supplement as well as in complementary and alternative medicine (Gao, 1998; Liu Chenglong, 2009). Several

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Species of cyanobacteria has been recognized for its therapeutic value that can be used for treatment of malnutrition, cancer and viral infection due to presence of medicinally active component that varied according to cyanobacteria species (Helle et al., 2006). For example CV-N is an 11 kDa protein of known three-dimensional structure produced mainly by *Nostoc* strains of cyanobacteria, where CV-N blocks HIV infection by binding to the surface envelope glycoprotein, gp120. Further study of the mechanism of CV-N/gp120 interaction revealed that CV-N bound to high-mannose oligosaccharides on gp120, specifically, oligomannose-8 (Man-8) and oligomannose-9 (Man-9). Moreover CV-N was proved to be active against certain other viruses, such as the Ebola virus and HCV, whose envelope proteins possess similar oligosaccharide structures. Therefore in HCV infection, CV-N as mannose binding protein has the ability to bind to hepatitis C (HCV) viral particle where HCV envelop as mannose rich protein and prevent its entry to the host cells, consequently prevent viral replication. Also the same action by CV-N is applicable with other viruses with envelope similar to that of HCV viral particles (Buffa et al., 2009; Liu et al., 2009; O'Keefe et al., 2003; Tsai et al., 2004).

Many natural occurring cyanobacteria are known to produce toxins, for example, species of the genera *Microcystis, Nodularia, Nostoc, Anabaena, Aphanizomenon, Cylindrospermopsis, and Planktothrix* (*Oscillatoria*) (Haider et al., 2003; O.M. Skulberge et al., 1993; Rao et al., 2002) Cyanotoxins are classified according to their mode of action in vertebrates as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritants (Carmichael et al., 2001). Microcystin is a hepatotoxin which commonly found in *Microcystis* and it was found to be produced by other genera, including *Anabaena, Nostoc, Nodularia*, and *Planktothrix*. It is a cyclic heptapeptide produced non-ribosomally by a multifunctional enzyme complex consisting of peptide synthetase (PS)-polyketide synthase (PKS) modules and tailoring enzymes, they act by inhibiting protein phosphatase 1 and 2 (PP1 and PP2A) (Goldberg et al., 1995; MacKintosh et al., 1990).

PCR-based techniques were developed for targeting the toxin biosynthesis genes and they have become popular methods for detecting and quantifying toxic species due to their specificity, sensitivity, and speed (Dittmann and Wiegand, 2006; Furukawa et al., 2006; Ouellette et al., 2006). Characterization of the genes responsible for hepatotoxin biosynthesis has made it possible to design general PCR primers that target the aminotransferase (AMT) domains of the *mycE* and *ndaF* genes (Jungblut et al., 2006) allowing detection of microcystin and nodularin-producing cyanobacteria.

MATERIALS AND METHODS

Isolation and cultivation of blue green algae

Blue green algae had been isolated from soil of Rice field in river Nile Delta. The isolation of a single algal unit into medium for growth is required to establish a clonal, unialgal culture by streak plating on BG12 or BG13 media. This method serves to isolate an algal unit less than 10 µm diameter (Ferris and Hirsch, 1991). Plates were incubated at 30°C for 4 to 8 days under suitable growth conditions (light cycle). Using stereomicroscope the desired colonies that are free of other microorganism were selected for further isolation. The purity of the selected colonies was determined by examining samples from each selected colony using a fine wire needle and examined with a high power objective of the compound microscope. The pure colonies were transferred into liquid or agar media (BG12 or BG13) (Ferris and Hirsch, 1991).

The purified colonies of blue green algae were cultivated in a BG12 sterile modified medium (Elango, 2008); the algae were grown in a batch culture at 30°C illuminated by cool white lamp. The cells were allowed to grow for 10 days to density equal to 32 mg/ml and were harvested by cooling centrifugation; the pellets were stored at -20°C.

Light and scanning electron microscopy

The fresh unialgal culture from the isolated cyanobacteria was examined with an Olympus BX 40 Phase contrast light microscope. A part from the fresh culture was dried; the specimen was sputter-coated with gold and examined with a Joel scanning electron microscope (SEM, JSM-6360; Joel, Japan) at 10 kV. The morphological identification of cyanobacteria was done according to Komárek and Anagnostidis (Komárek, 1998; 2005).

Identification and amplification of 16S rDNA and ITS-23S rDNA genes

DNA was isolated from the samples as described by Tillett and Neilan (2000) and quantified using UV spectrophotometer (NanoDrop Technologies, Thermo Scientific, USA). All PCR was performed using 0.2 U of Dream Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, USA) in a 20 ml reaction. The PCR mixture contained 1x Taq polymerase buffer, 0.5 pmol of forward and reverse primers (Table 1), 0.2 mM dNTPs, and 100 ng template DNA. For the molecular identification of the cyanobacteria, 16S rDNA oligonucleotide primers, CYA106F-CYA781R (Nubel et al., 1997) and ITS-23S rDNA (Tillett et al., 2000) P322-P340, (Iteman et al., 2000) were used and synthesized by (Bioneer, Korea). PCR reactions were performed as previously described for each primer pair (Al-Tebrineh et al., 2011; Iteman et al., 2000; Jungblut et al., 2006; Nubel et al., 1997).

PCR amplification of HEP genes

The HEP-F and HEP-R oligonucleotide primers targeting the aminotransferase (AMT) domain of the hepatotoxin synthetase *mcyE* and *ndaF* genes (Bioneer, Korea) were used for determining whether that toxicity gene is present or absent in the isolated bacteria (Amer et al., 2009; Jungblut et al., 2006). The AMT primers HEP-F and HEP-R (Table 1) were used to amplify the 472 bp PCR product. DNA isolated from EGY isolate and positive control DNA isolated from toxin producing *Nostoc* sp were used as template for PCR amplification using this primers. The initial temperature of 94°C for 2 min, followed by 35 cycles at 92°C for 50 s, 52°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min.

Preparation of blue green algae (BGA) lysate

Cultivated cells of EGY strain were collected by centrifugation at

Table 1. List of primers used for PCR and sequencing in this study.

Primer ID	Primer sequence 5'-3'	Target	Reference
CYA106F	CGGACGGGTGAGTAACGCGTGA	16S rDNA	(Nubel et al., 1997)
CYA781R	GACTACTGGGGTATCTAATCCC(A/T)TT	16S rDNA	(Nubel et al., 1997)
P322F	TGTACACACCGCCCGTC	End of 16S rDNA	(Iteman et al., 2000)
P340R	CTCTGTGTGCCTAGGTATCC	23S rDNA	(Iteman et al., 2000)
HEPF	TTTGGGGTTAACTTTTTTGGGCATAGTC	mcyE/ndaF	(Jungblut et al., 2006)
HEPR	AATTCTTGAGGCTGTAAATCGGGTTT	mcyE/ndaF	(Jungblut et al., 2006)

5000 rpm for 15 min at 4°C followed by lypholization. Lyophilized cells were broken down into powder using liquid nitrogen (10 times volume of the dried cells) the resulted powder were dissolved in normal saline with EDTA free protease inhibitor cocktail (Roche, USA) at 20 mg/ml, sterilized by filtration to be ready for testing. In order to find out relation between the cell density/suitable cultivation time and anti-HCV activity, BGA lysate was also prepared from cultivated EGY cells and collected at time intervals from 5 up to 60 days. Cell density and dry cell weight were determined and anti HCV activity was determined.

Anti HCV activity

During the last few years, a number of cell culture systems showed to have the ability to harbor and support reliable and efficient progression of this virus. Among several human hepatocyte cell lines analyzed, the hepatocellular carcinoma HepG2 cell line was found to be most susceptible to the HCV infection (El-Awady et al., 2006). Also, human peripheral blood mononuclear cells (PBMC) showed the ability to harbor the replication of HCV *in vitro* and also HCV viral particles was reported to be harbored inside the PBMC of the HCV infected patients (el-Awady et al., 2005).

On the other hand, monitoring of the HCV viremia pre- and post-antiviral therapy through the detection of viral (+) and/or (-) RNA strands by the use of qualitative reverse transcription-polymerase chain reaction (RT-PCR) has become the most frequently-used, reliable and sensitive technique. Recently, it has been reported that the detection of the (-) strand HCV-RNA using the RT-PCR is a very important tool for understanding the life cycle of the HCV and provides a reliable marker for the diagnosis of HCV and monitoring the viral response to antiviral therapy (El-Awady et al., 1999).

Neutral red uptake assay to measure cytotoxicity on human cells

Peripheral blood lymphocytes (PBMC) were separated from whole blood of healthy volunteer using gradient separation by Ficoll-Paque™ Plus (MP Biomedicals, France), $10X10^4$ lymphocyte cells were seeded per well in 96 well plates and incubated in culture media (CM, RPMI1640 (Loza) media supplemented with 200 µM L-glutamine (Lonza), 25 µM HEPES buffer; N- [2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (lonza) and 10% Fetal bovine serum, FBS, Lonza) containing different concentrations of the BGA lysate for 24, 48, 72 and 96 h. The fraction of viable lymphocyte cells was measured by the Neutral Red assay (Guillermo Repetto, 2008). The neutral red assay is based on the initial protocol described by Borenfreund and Puerner (Borenfreund and Puerner, 1984) and determines the accumulation of the neutral red dye in the lysosomes of viable cells (Fotakis and Timbrell, 2006). Following exposure of isolated PBMC to different concentrations of BGA lysate, cells were incubated for 3 h with neutral red dye (40 µg/ml) dissolved in culture media RPMI. Cells were then washed with Phosphate Buffered Saline (PBS) and the addition of 1 ml of elution medium (50% ethanol /1% glacial acetic acid /49% water) followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to 96-well plates and absorbance at 490 nm was recorded using microtiter plate reader spectrophotometer (Biotek, USA). IC50 was estimated by plot x-y and fit the data with a straight line (linear regression) using IC50 was determined using Prism statistics software program.

Detection of the effect of the BGA lysate on HCV cultivated in human cancer cell line

Human cancer cell line, HepG2 cells, were washed twice in RPMI1640 (Loza) media supplemented with 200 µM L-glutamine (Lonza) and 25 µM HEPES buffer; N- [2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (lonza) and were suspended at 2×10^5cells ml^{-1}in RPMI CM. The cells were left to adhere on the polystyrene 6-well plates for 24 h in 37°C, 5% CO_2, 95% humidity incubator. After 24 h the cells were washed twice from debris and dead cells by using RPMI supplemented media. Cells were infected by incubation for 24 h with 4% HCV infected serum, followed by addition of culture media and depending on th value of the determined IC50, different concentrations of BGA (1, 10, 160, 1, 1.6 µg/ml) were added. Positive (HCV infected cells) and negative control (uninfected cells) cultures were included. Cultures were incubated for 96 h in 37°C, 5% CO_2, 95% humidity. The tested BGA concentration is considered to be active when it is capable of inhibiting the viral replication inside the HCV-infected HepG2 cells, as evidenced by the disappearance of the viral RNA-amplified products detected by the RT-PCR (compared with the positive control).

RNA extraction and RT-PCR of HCV RNA

Total RNA was extracted from HCV-infected HepG2 cells using the method described by El-Awady et al. (El-Awady et al., 1999). Briefly, culture cells were collected in 200 µl of Solution D (4 M guanidinium isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β- mercaptoethanol, 100 µl sodium acetate). The cell lysate was mixed with an equal volume of RNA phenol, chloroform and isoamyl alcohol mixture (pH 4, Fisher Scientific, USA), mixing vigorously by vortex, followed by centrifugation at 14 K rpm for 10 min at 4°C. The aqueous layer was collected and mixed with an equal volume of isopropanol then incubated overnight at -20°C. RNA was collected by centrifugation at 14 K rpm for 30 min at 4°C followed by washing twice with 70% ethanol then suspended in RNase free water. Concentration and purity (260/280 nm ratio) was determined using UV spectrophotometer. The complimentary DNA (cDNA) and the first PCR reaction of the

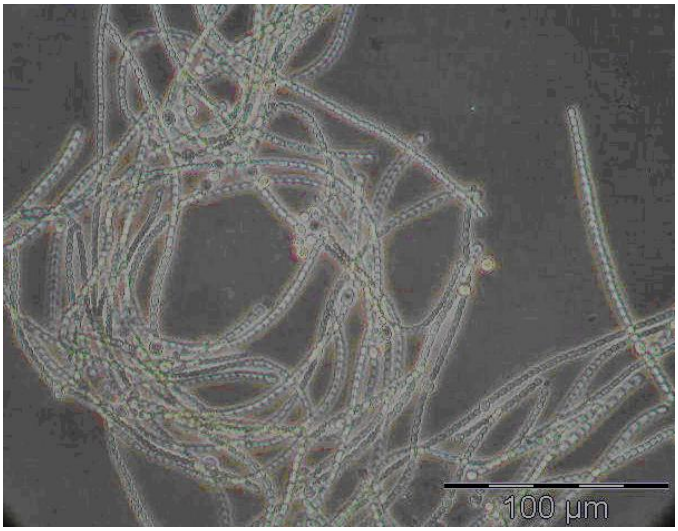

Figure 1. Ligth microscope filed showing the obtained pure culture EGY of isolate with magnification of 100X.

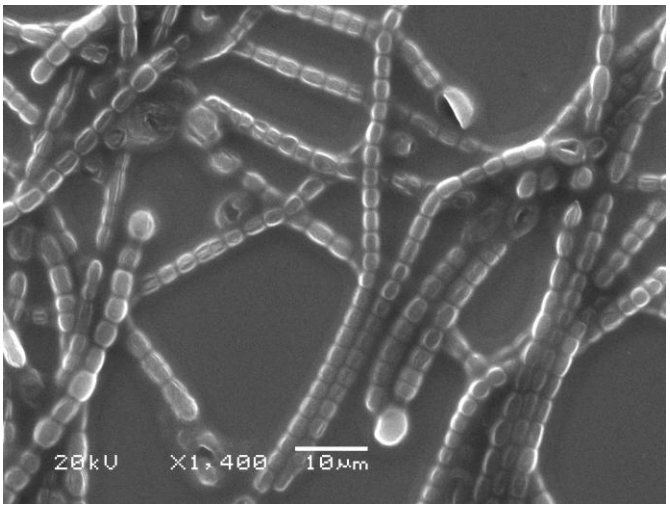

Figure 2. Scanning electron microscope for EGY by magnification 400 X.

nested PCR detection system for the HCV RNA was performed in a 50 µL volume single-step reaction using the Ready-To-Go RT-PCR beads (GE Healthcare, USA) followed the detailed protocol described by El Awady et al, 2002 (El Awady et al., 2002). The Final nested PCR product was detected using 2% agarose gel electrophoresis.

Quanititative *in-vitro* anti-HCV screening

Same BGA concentrations were examined for its anti HCV activity quantitatively using same culture system used for qualitative test and analytikjena HCV viral RNA extraction kit and Robogen HCV quantitative kit (Bometera, Germany)

RESULTS

Isolation and microscopic examination

The unialgal culture was obtained after primary and secondary screening on BG12 media plates and broth. The pure isolate EGY was examined for its morphological characterization under the light microscope (Figure 1) going to scanning electron microscope (SEM) (Figure 2). The figures showed that the isolated cyanobacterium was filamentous with the presence of hetercysts and akinetes that may belongs to group Nostocales.

Molecular identification of EGY by amplification of 16S rDNA gene

The DNA was extracted from EGY by the method described by Tillett and Neilan 2000. The cyanobacterial universal primers CYA106F-CYA781R were used for the identification of the pure isolate EGY. The obtained 16S rDNA PCR product was amplified and partially sequenced and analyzed by Bioneer Company, Korea. The resulting phylogenetic reconstructions were shown in Figure 3 on the basis of neighbor-joining analysis. The data showed in Figure 3 ensure the results of the microscopic examination that the EGY belongs to family Nostocales. It showed highest similarity to 91% to *Nostoc* sp. PCC 9426. The obtained sequence was added to the GenBank data base under the accession number JQ220408.1 and designated as *Nostoc* sp. EGY. As the similarity obtained from the partial sequence of the 16S rDNA is slightly low the 16S-ITS-23S rDNA which was amplified to confirm the 16S rDNA results and to confirm the genus of the isolated bacteria. As shown in Figure 4, the sequence of the PCR product of 16S-23S ITS showed 98% similarity with *Nostoc* sp. NR1 (2012) 16S-23S ribosomal RNA intergenic spacer from the GenBank. The obtained sequence was added to the data base under accession number JQ220407.1 and designated as *Nostoc* sp. EGY 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence.

Amplification of HEP genes

The identification of the hepatotoxin cyanobacteria is performed by amplification of AMT domain of hepatotoxin synthetase *mcyE* gene (Amer et al., 2009; Jungblut et al., 2006). The amplification of HEP genes of *Nostoc* sp. EGY versus the positive control (microcystine producing strain) was visualized on 2% agarose gel. The data showed the absence of 472 bp band for *Nostoc* sp. EGY and presence of the same band is the positive control. Therefore, *Nostoc* sp. EGY can be used for farther investigations.

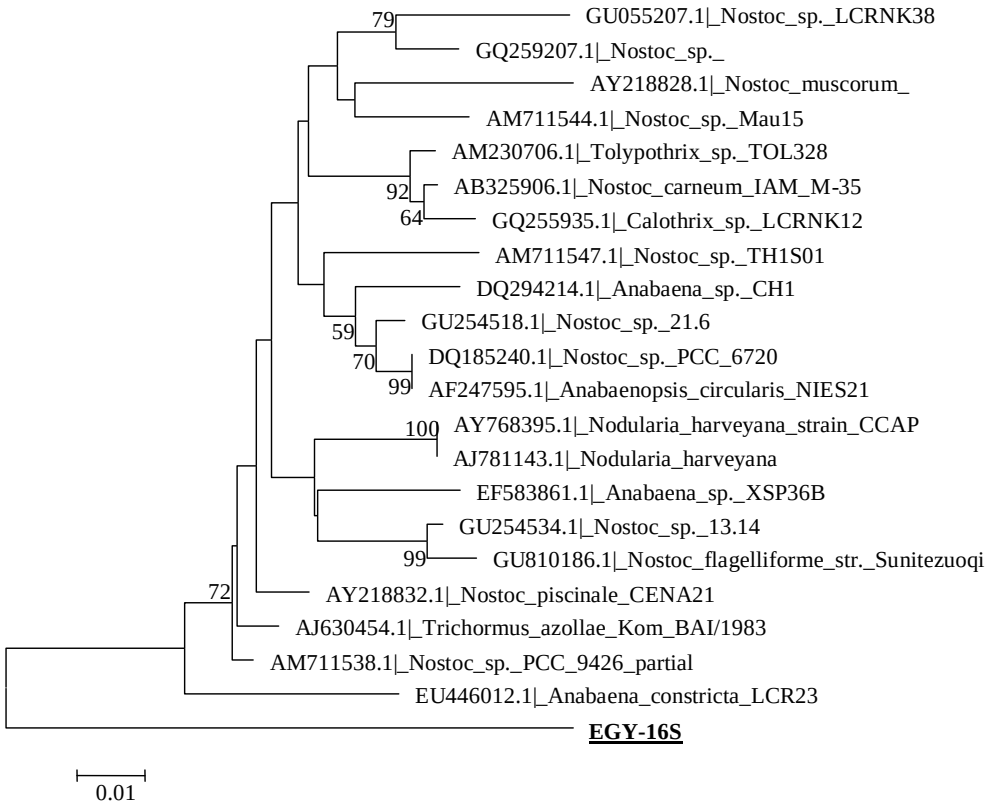

Figure 3. Neighbor-joining tree based on 16S rDNA sequences (CYA primers) of the EGY. Evolutionary distances were calculated using the Kimura 2 model using MEGA5 software. The numerals show the results of the bootstrap analysis values from 1000 replicates (only bootstrap values above 50% were shown).

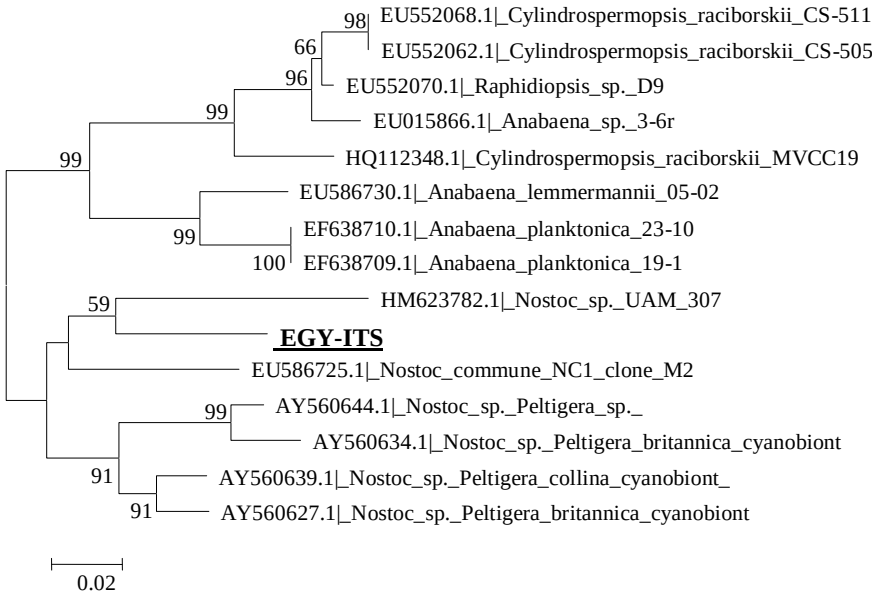

Figure 4. Neighbor-joining tree based on ITS sequences (P322-P340 primers) of Iso-1 and Iso-2 isolates isolated from Nile River. Evolutionary distances were calculated using the Kimura 2 model using MEGA5 software. The numerals show the results of the bootstrap analysis values from 1000 replicates. The sequence in bold was determined in this work.

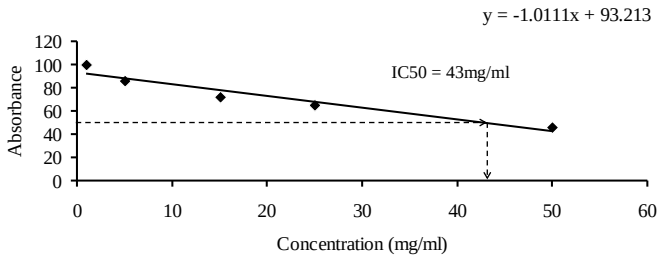

Figure 5. Cytotoxicity of BGA lysate and IC50 value.

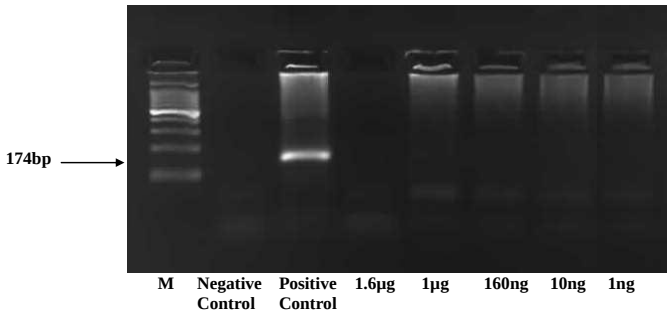

Figure 6. Anti HCV activity of different concentrations of BGA.

Table 2. Cyanobacteria growth curve and anti-HCV activity.

Cultivation interval (days)	Anti HCV activity
5	-ve
10	+ve
15	-ve
20	-ve
25	+ve
30	-ve
35	-ve
40	-ve
45	-ve
50	-ve
55	-ve
60	-ve

Anti HCV activity

As indicated in Figure 5 the IC50 of BGA lysate is 43 mg/ml. Safe concentration ≤ 1/100 the IC50 (0.4 mg/ml) was examined for the anti HCV activity. Safe concentration was able to prevent HCV replication (data not showed). Much Lower concentrations were tested for anti-HCV activity (1, 10, 160, 1, 1.6 µg/ml) all of selected concentrations were able to prevent HCV replication in the *in vitro* HCV culture system (Figure 6). Where, viral load was almost equal to zero at concentrations 10 160, 1, 1.6 µg/ml while 1 ng and 0.5 ng/ml showed very low viral load as compared to the positive control but the percent inhibition of viral replication was almost 100% at all used BGA concentrations (Table 3).

Table 3. Quantitative RT-PCR of anti HCV activity of the lowest BGA concentrations.

BGA concentration (ng/ml)	HCV viral load (IU/ml)	Percent inhibition
Positive control	$1.28X10^9$	
0.5	322	99.99999975
1	172	99.99999987

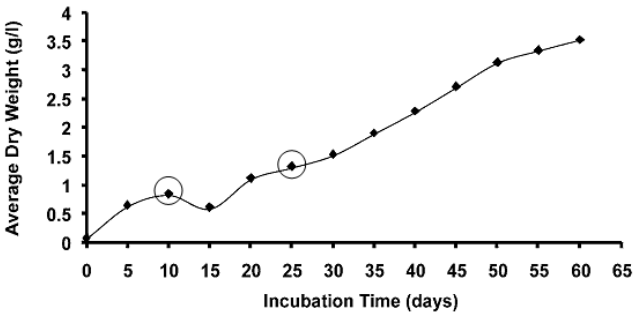

Figure 7. Cultivation of the organism in its selected suitable media for high cell density.

As presented in Table 2 and Figure 7 anti HCV activity was influenced by cultivation time where anti-HCV activity was detected with lysate of 10 and 25 days of culture while the lysate of the rest of cultivation time intervals have no anti HCV activity. This data indicated that cultivation time is very crucial for BGA activity against HCV while cell dynasty is not.

DISCUSSION

According to the taxonomic identification of Komárek and Anagnostidis (Komárek, 2005) the phenotype of the isolated cyanobacteria was characterized as a genus belongs to group of Nostocales as the filaments showed the presence of heterocysts and akinetes. The analysis of 16S rDNA and 23S-ITS rDNA together with the microscopic examination revealed that the isolated cyanobacterium was designated as *Nostoc* sp. EGY. The association of N2-fixing cyanobacteria is commonly known in the rice filed due to the lack of nitrogen. *Nostoc* as one of the N2-fixing cyanobacteria has been isolated and characterized by many authors from the roots of the rice plants (Nilsson et al., 2002). The absence of amplicon from the PCR amplification of aminotransferase (AMT) domain of the hepatotoxin synthetase *mcyE* and

ndaF genes by using HEP primers revealed that the isolated strain was not a hepatoxic strain. Whereas, these primers were used before for the characterization of the genes responsible for hepatotoxin biosynthesis (Amer et al., 2009; Jungblut et al., 2006).

Recently, Hashtroudi et al (Hashtroudi et al., 2013) was able to isolate four major carotenoids from *Nostoc* sp that can be used for nutrition or production of supportive medicine. Also, edible *Nostoc* sp was proven to have component that can act as anti herpes simplex virus (Kanekiyo et al., 2007). Other *Nostoc* sp was showed to have hypocholesterolemic efficacy (Rasmussen et al., 2009). Several reports have indicated the ability of small protein, cyanivirin N, of cyanobacteria *Nostoc* sp act as potent antivirus to HIV, Ebola, Influanza, and HCV (Barrientos et al., 2003; Boyd et al., 1997; Buffa et al., 2009; Dey et al., 2000; Helle et al., 2006; O'Keefe et al., 2003; Tsai et al., 2004). Also, cyanovirin-N has been recently evaluated for treatment of HIV infection (Buffa et al., 2009; Zappe et al., 2008). Accordingly, the new identified isolate presented in this study *Nostoc* sp. EGY may have the same anti viral component. However, more detailed study is required for the identification of detailed effect of the new isolated strain lysate on HCV replication. The absence of hepatotoxin synthetase *mcyE* gene indicate acceptable safety of the isolated *Nostoc* sp. EGY which increase the possibility of using this isolated strain for designing an effective anti HCV medication.

Conflict of interests

The author(s) have not declared any conflict of interests.

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