INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 15, No. 1, p. 66-79, 2019

RESEARCH PAPER OPEN ACCESS

Toxicological investigation of a bloom of the blue - green alga *Lyngbya aestuarii* in Basra Governorate Southern of Iraq

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Key words: Cyanobacteria, Toxic bloom, Cyanotoxins, LC, MS/MS.

http://dx.doi.org/10.12692/ijb/15.1.66-79 Article published on July 06, 2019

Abstract

Many species of cyanobacteria can produce several toxic metabolites known as cyanotoxins that constitute a serious threat on aquatic organisms, wild life, domestic animals and human. The cyanobacterium *Lyngbya aestuarii* were collected from a unialgal bloom occurred in Al-Dawoodi River which is a branch of Garmat Ali River located in Basra governorate/southern Iraq during the desiccation period in Winter 2018. The current study is aimed to investigate the algal toxic compounds by using LC-MS/MS and ELISA technique and investigated its toxicity on laboratory mice. The water of sampling site for *L. aestuarii* was classified as saline water because its electrical conductivity and salinity was 18.67 and 14.93 ‰ which is in the range of 10 - 25 mmhos / cm and 7000 -15 000 mg /l for highly saline waters. The identified compounds with LC-MS/MS are Neosaxitoxin, Cryptophycin C and Dudawalamide B while the concentration of microcystins determined with ELISA kit was 0.156 µg/l. The median lethal dose reach to 560 mg/kg which indicated moderate to low toxicity of this alga. The symptom appeared on poisoning mice was typical of neurotoxicosis and no symptoms of hepatotoxicosis was appeared on them due to trace concentration of microcystins in the algal extract as emphased by ELISA kit. In conclusion, the current study investigated the dangerous impact of both desiccation and salinization of Basra Rivers which lead to a bloom of saline toxic blue-green alga *L. aestuarii* .

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Introduction

The frequent occurrence of cyanobacterial blooms in aquatic systems has been regarded as a serious global public health problem and a major environmental issue. This is because many species of cyanobacteria can produce several toxic metabolites known as cyanotoxins that constitute a serious threat on aquatic organisms, wild life, domestic animals, plants and human (Kopp *et al.,* 2010; Al-Sultan and Hatem, 2019). Many different aquatic organisms such as fish, bivalves, snails, and other macro-invertebrates may accumulate cyanotoxins via ingestion of cyanobacterial cells or dissolved toxins (Rücker *et al.*, 2007). Cyanotoxins could also be transmitted to plants from irrigation waters and can accumulate in edible parts (i.e. leaves, roots and fruits) (Al-Sultan and Hatem, 2019). According to WHO (2003) approximately 50- 75 % of the cyanobacterial blooms are toxic. Several Cyanobacterial species have been reported to be capable of producing potent toxins. *Microcystis* is the most commen toxic cyanobacterium in fresh water; other genera include *Anabaena*, *Anabaenopsis, Aphanizomenon, Cylindrospermopsis*, *hapalosiphon, Lyngbya*, *Nodularia*, *Nostoc*,*Oscillatoria*, *Plantothrix*, and *Phormidium* (Teneva *et al.*,2003; Homepage *et al.*, 2010; Lopes and Vasconcelos, 2011;AL-Sultan, 2017;AL-Sultan and Aubaed, 2017).

Cyanotoxins remain inside the cyanobacterial cells during growth phase, and released inside the environment with senescence of the bloom (maximal during late-logarithmic phase) and when the cells die or disintegrate (Masango, 2007). The occurrence of cyanotoxins in drinking water, depend on their level in raw surface water and the effectiveness of treatment methods for removing cyanobacteria and cyanotoxins. Cyanotoxins can cause severe poisoning and produce chronic diseases such as cancer, and even lead to death (Zanchett and Oliveira-Filho, 2013).

Lyngbya is a widespread genus of cyanobacteria, which is distributed worldwide throughout tropical and subtropical regions (Almaliti *et al.,* 2017). There are an increasing number of species of this genus that have been found to produce an admirable variety of structurally different compounds with various biological activities (Kumar *et al.*, 2014). Currently, the most important species of the genus *Lyngbya* in terms of production of secondary metabolites are: *L. majuscula*, *L. martensiana*, *L. aestuarii*, and *L. wollei*.There are a number of toxins investigated in *Lyngbya* species such as Antillatoxin (ATX), Kalkitoxin, Jamaicamide, Lyngbyatoxins, apratoxins,Barbamide andSaxitoxins (Rastogi *et al.,* 2015). So, the current study is aimed to investigate the toxicity of the bloomed alga *Lyngbya aestuarii*on laboratory mice and screen its toxic compounds by using LC-MS/MS technique.

Materials and methods

Algal and water sampling

An algal bloom has occurred in in Al-Dawoodi River durring the desiccation period in winter 2018 as showed below in Fig.1. Al-Dawoodi River is a branch of Garmat Ali River occurred in Al-Dawoodi village/Basrah governorate/Iraq. Water and algal samples were collected from this area during January /2018. The algal sample was identified with Leica microscope according to Desikachary, (1959); Prescott, (1975) and Stancheva *et al.*, (2014). It has been found the algal bloom was comprised of only one alga and the alga was identified as *Lyngbya aestuarii*.

Determination of chemical and physical properties of water

Water temperature, pH and electrical conductivity were measured in situ using Horiba multimeter in addition to collection of water sample and stored it in plastic bottle (500 ml) until arriving to the laboratory for determination of the following chemical analyses: total alkalinity, total hardness, inorganic nitrogen and phosphate.

Total Alkalinity was determined by titration with 0.025 N standard sulphuric acid andboth Phenolphthalein and Methyl orange indicators according to APHA (1998). Total hardness was

determined by titration with Na_2 EDTA as standard solution after the addition of Buffer solution (PH=10) and Erichrome black T indicator according to APHA (1998). While the inorganic nitrogen concentration (nitrate and nitrite) was done using the Hydrazine Reduction Method by spectrophotometer at 520 nm according to APHA (1998).Phosphate was determined as orthophosphate by spectrophotometer at 885nm according to APHA (1998).

Extraction of Lyngbya aestuarii

Twenty grams of lyophilized alga were weighed and extracted twice with 75 % methanol (1 liter) then it re-extracted twice with 1 % acetic acid (40 mililiter), then both extractions were mixed together before desiccation with Freeze dryer. The extraction procedure was repeated several times for extraction 100 grams of the lyophilized alga (Rangel*et al.*, 2013).

Screening for cyanotoxins with LC-MS/MS

The current procedure had been used for screening of toxic compounds in the algal extract. The crude extract of *Lyngbya aestuarii* is analyzed with LC-MS/MS at Center of Applied Research and Advanced Studies (CARAS) in Faculty of Pharmacy/Cairo University/Egypt according to Halme*et al.*,(2012).

LC-MS/MS was performed with positive mode electrospray ionization (ESI) source.

The chromatographic separation was carried out on BEH C18 150 × 2.1, 1.7 µm column. A mobile phase was 4 mM ammonium formate in H_2O-ACN 40:60 (v/v), and the pH of the eluent was adjusted to 3.5 with formic acid. The flow rate was 1 ml/min with an accurate post-column splitter (1:20) between LC and MS. Spray voltage of 5 kv was applied and nitrogen was used as sheath gas. Capillary temperature was set to 350 ˚C and the relative collision energy was 29%. Run time was 45 min.

Molecular weight is calculated according to Little *et al.*, (2011) by the following formula:

$$\text{Average molecular weight} = \frac{\Sigma(m/z \times intensity)}{\Sigma intensity} - 1.0074$$

Chemical structure and information of *Lyngbya* compounds were retrieved from PubChem database (http://www.ncbi.nlm.nih.gov/pccompound).PubChe mis an open chemistry database at the National Institutes of Health (NIH). It contains 96 546602 compounds.

Quantitative determination of microcystins

Extraction and purification of microcystins

Microcystins were extracted from 100 mg of freeze dried alga three times by 75 % methanol using magnetic stirrer for 1 h at room temperature. A 25 ml aliquot of the microcystin-rich concentrated sample was applied to a preconditioned silica gel column.

The preconditioning step included washing with 20 ml each of absolute methanol and by 20% aqueous methanol respectively. After that the microcystins were eluted using 5 ml of 80% methanol at 1ml/min. Then microcystins were concentration by rotary evaporation so as to expel methanol from the sample (Ramanan*et al.*, 2000).

Determination of microcystin concentration

Microcystin concentration was determined by enzyme-linked immunosorbent assay (ELISA) kit manufactured by Abraxis company according to Fischer *et al.*, (2001) and determined with Biotek ELISA reader at 450 nm. The concentrations of the samples are extrapolated from the standard curve of microcystin in µg/l.

Mouse bioassay

Calculation of median lethal dose test (LD_{50})

After weighting of pre-calculated amounts of the cyanobacterial crude extract. The weighted amounts were dissolved in a small volume of Tween-80 then diluted to the wanted volume with distilled water to prepare the following doses: (10 mg; 15 mg; and 20 mg for each mouse).

Adult male mice (weighting between 23 – 26 gram, and aging between 10-12 week) were chosen for determination of median lethal dose test (LD_{50}). Eighteen mice were randomly divided into three

groups, each group consists of six mice for intraperitoneal injection with the prepared solutions as follow:

Group I: six mice were injected with 0.1 ml containing 10 mg crude extract for each mouse.

Group II: six mice were injected with 0.1 ml containing 15 mg crude extract for each mouse.

Group III: six mice were injected with 0.1 ml containing 20 mg crude extract for each mouse.

The animals were observed for seventy two hours after injection. Then the dead ones were recorded and the LD_{50} was calculated according to Karber, (1937) equation as follow:

LD_{50} = Highest dose - (Σab/n).

Where:

LD_{50}: is the median lethal dose.

Highest dose: is the lethal dose to all mice in a group.

n: is the number of mice in each group.

a : is the value of difference between two successive doses.

b: is the mean mortality in mice for two successive doses.

Results

Algae

Lyngbya aestuarii is a blue-green alga found in lakes, ponds, rivers, and sea, also it found on soil, submerged substrates, stones and prefers saline waters. Its filaments (Fig.2) entangle to form a brown or dull blue-green thallus. Filaments are long, straight or curved.

Table 1. Environmental analyses for water sample.

Parameter	Result
Air temperature (°C)	20-22 °C
Water temperature (°C)	18 °C
pH	7.06
EC (mmhos/cm) at 25°C	18.672
Salinity (‰)*	14.93
Total alkalinity (as $CaCO_3$)	240
Total hardness (as $CaCO_3$)	2880
Nitrate + nitrite (mg/l)	High > 50
orthophosphate (mg/l)	7

* ‰ means part per thousand or gram per liter and it equivalents to 1000 milligram per liter.

Table 2. Results of LC-MS/MS chromatogram report for the *L. aestuarii* extract.

Peak number	Name of Chemical Compounds	Total Area %	Time (minutes)	Chemical Formula	Molecular Weight (g .mole $^{-1}$)
2	Neosaxitoxin	53.48	0.98	$C_{10}H_{17}N_7O_5$	315.12
4	Cryptophycin C	9.42	11.14	$C_{35}H_{43}ClN_2O_7$	639.18
5	Dudawalamide B	25.55	31.12	$C_{44}H_{59}N_5O_8$	785.98

The cells of trichomes are short. The length of cells were 2.1-4.4 µm ,1/3-1/11x as long as wide, and their width were 13.7-19.8 µm not constricted at cross-walls often with adjacent small granules sometimes have aerotopes. Apical cell wall either rounded, flat-rounded, or truncate. Filaments not or slightly attenuated at the ends. Younger filaments have thin, smooth sheaths initially, later with thick, wide, lamellated yellow-brown sheath, uneven on the outside; sometimes older filaments with outer sheaths colorless, inside only is yellow-brown.

Cellular division occurs by transversal centripetal growth of cross walls, where several rounds of division proceed simultaneously. Filament division occurs by self-immolation of one or groups of necridial cells at the breakage point leading to form short, sheath-less hormogonia.

Table 3. Median lethal dose (LD_{50}) values calculated according to Karber (1937).

Group	Dose	Number of dead animals	a	b	a*b
1	10 mg/mouse	0	0	0	0
2	15 mg/mouse	4	5	2	10
3	20 mg/mouse	6	5	5	25

Environmental analyses

The physico-chemical results of water sample analysis where *L. aestuarii* is collected are listed below in Table 1.

LC-MS/MS analysis

Total ion chromatograms (TIC) of *L.aestuarii* extract are shown in Fig.3 where it showed three peaks represent three chemical compounds only as illustrated below in Table 2, and the fragmentation spectra by tandem mass was illustrated in Fig.4 A, 4B and 4C respectively.

Fig. 1. Area of *L. aestuarii* collection.

Determination of microcystins

The standard curve of microcystins was done with four parameter logistic regression using https://www.aatbio.com/tools/four-parameter logistic-4pl-curve-regression-online-calculator as shown in Fig.5 below and the concentration of microcystins determined with ELISA kit was 0.156 µg/l.

Median lethal dose (LD $_{50}$)

The mouse bioassay is generally used to determine if a cyanobacterial bloom is toxic or non-toxic and also to identify the class of toxins present in it based on the observed clinical symptoms. In the current study, the mouse bioassay represents by Median lethal dose (LD $_{50}$) is illustrated below in Table 3.

Discussion

Algae

In the present study, the water of sampling site for *L. aestuarii* was classified as saline water because its electrical conductivity and salinity, as previously

shown in Table 1, was 18.67 and 14.93 ‰ which is in the range of 10 - 25 mmhos / cm and 7000 -15 000 mg /l for highly saline waters (Rhoades *et al.,* 1992). Salinization in Basrah Rivers is a real problem occurred usually as a result to low water discharge from Tigris and Euphrates. This problem yield a bloom of a toxic cyanobacterial bloom in Al-Dawoodi village at Hartha district. The bloom is caused by the cyanobacterium *Lyngbya aestuarii* only. *L. aestuarii* is a species with a large spectrum of occurrence in salty environments (Richter *et al.,* 2015).

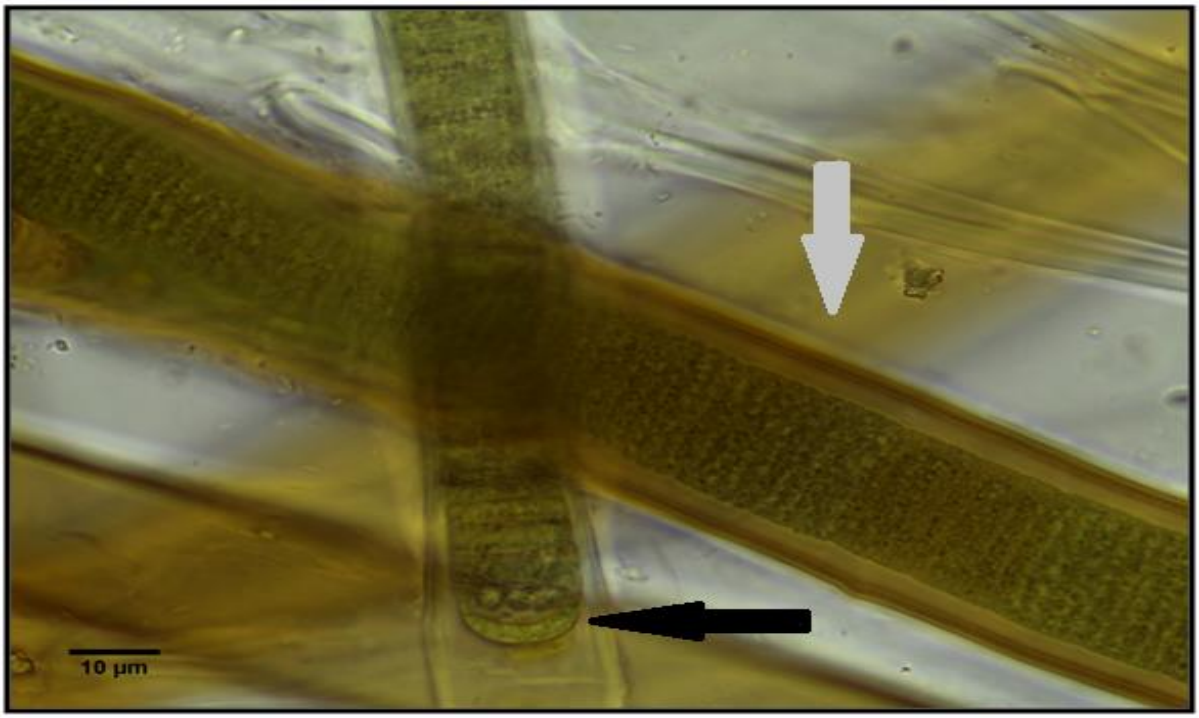

Fig. 2. A filament of *L. aestuarii* with yellow-brown multi - layered sheath as pointed with a gray arrow and another attenuated filament with rounded apical cell as pointed with a black arrow on 1000 X magnification.

In Nature, *L. aestuarii* forms extensive microbial mats in many marshes, marine intertidal muds and oceanic coasts. It presents in extreme environments with repeated cycles of desiccation and wetting, intense exposure to ultraviolet radiation, and changing regimes of salinity (as alga may be exposed to hypersaline marine waters to very dilute meteoric precipitation) (Baker, 1987; Kothari *et al.,* 2013; and Hassouani *et al.,* 2017).

This alga was listed by Palmer (1980) as toxic species in Palmyra Island located at Pacific Ocean of California. Large numbers of poisonous fishes are herbivorous. In the examination of majority of the poisonous fishes, the cyanobacteria were detected in their alimentary tract were *L. majuscule* and *L. aestuarii***.** The fish *Chelon vaigiensis* is an exception, this fish is omnivorous. The choice of algae was found to be very restricted, for only *L. aestuarii* (Dawson *et al.,* 1955).

Chemical compounds identified by LC-MS/MS

Mass spectrometry (MS) has become the method of choice for cyanotoxins detection and identification with the exception of lipopolysaccharides (Metcalf and Codd, 2014). In the current study, the identification of compounds with LC-MS/MS was based on calculation of their molecular weight by the equation of Little *et al.,* (2011) and the calculated molecular weight are a proximately 315, 639 and 785 for compounds 2, 4 and 5 respectively and they identified as Neosaxitoxin, Cryptophycin C and Dudawalamide B. The mass spectra of these compounds are shown in figure 4A, 4B and 4C respectively. The nearest ion to molecular ion of Neosaxitoxin is at mz 338.53 which represents

sodium adduct [M + Na]$^+$ of Neosaxitoxin (figure 4A). Both mz 205.68 and mz 221.65 represents product ions produced due to fragmentation of the molecular ion (M^+). According to fragmentation schemes of Sleno and Volmer (2004), the product ions at mz 220.08, mz 221.11 and mz 204.08 for Stx and Neosaxitoxin are nearest to mz 221.65 and mz 205.68 respectively produced in the current fragmentation pattern of compound 2. In figure 4B, the mz 640.9 represents $[M+H]^+$ for crypyophycin C while in figure 4C, the mz 831.84 represents $[M+2Na]^{2+}$for Dudawalamides B.

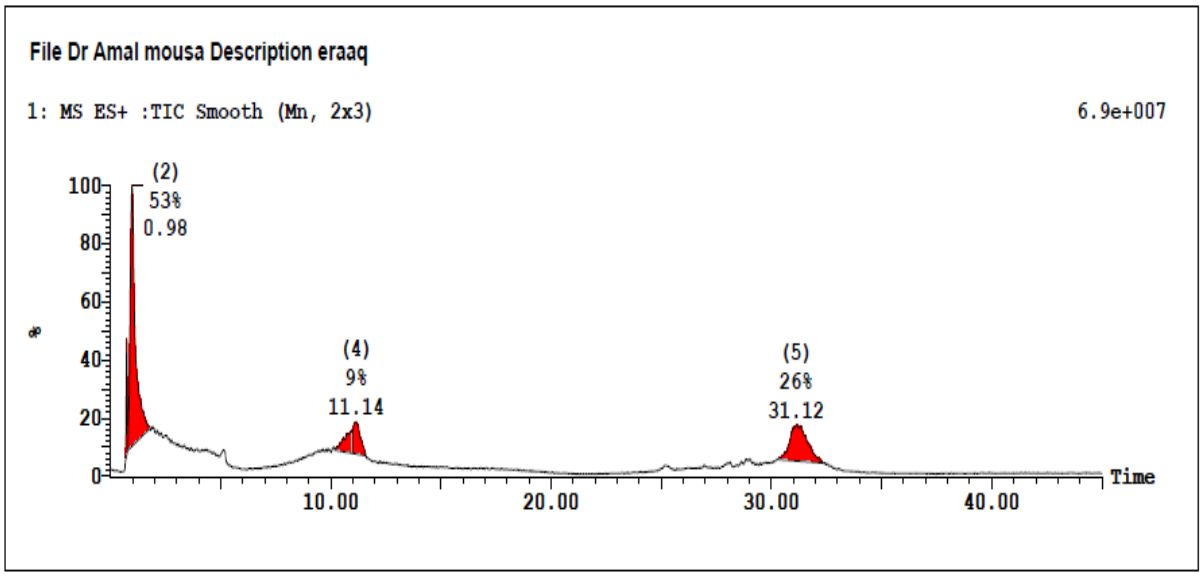

Fig. 3. LC-MS/MS chromatogram for the *L. aestuarii* extract.

Neosaxitoxin

Neosaxitoxin is an alkaloid neurotoxin belong to a family of compounds collectively referred to as Saxitoxins (STXs) or Paralytic Shellfish Toxins (PSTs). These compounds characteristic by tetrahydropurine structure and having two guanidinium groups which are responsible for their high polarity and toxicity (Wiese *et al.,* 2010; Durán-Riverol*et al.,* 2017).

Neosaxitoxin was discovered in marine dinoflagellates *Alexandrium andersoni*, *Alexandrium catenella*, *Alexandrium fundyense*, *Alexandrium tamarense*, *Gymnodinium catenatum*, *Pyrodinium bahamense* and in the cyanobacteria *Aphanizomenon flos-aquae*, *Aphanizomenon Gracile*, *Aphanizomenon Issatschenkoi*, *Cylindrospermopsis raciborskii* (Wiese *et al.*,2010;Foss *et al.,* 2012;Shearn-Bochsler *et al,*. 2014;Cirés *et al.,* 2014; Yilmaz *et al.,* 2018).In the present study, Neosaxitoxin is identified for the first time from *L. aestuarii*.

The ecotoxicological risk of STXs is high; they were found to induce toxicity in fish, mussels, copepods, mammals and birds (Shumway *et al.,* 2003; Perreault *et al.,*2011; Zagatto *et al.,*2012;Akmajian *et al.,* 2017). Intoxication with STXs may result in the severe and occasionally fatal illness known as paralytic shellfish poisoning (PSP) or sax toxin puffer fish poisoning (SPFP). This illness is caused when STXs reversibly bind voltage-gated Na^+ channels in axon of excitatory neurons.

This is mediated by the interaction between the positively charged guanidinium groups of STX with negatively charged carboxyl groups at site 1 of the Na^+ channel, thereby blocking its pore; therefore, STXs can cause inhibiting the propagation of depolarization and transmission of the nerve impulse, inducing neuromuscular paralysis and death by respiratory arrest. Manifested symptoms in paralytic shellfish poisonings include a paresthesia of the lips, numbness of extremities, gastrointestinal irritants, and troubles in breathing.

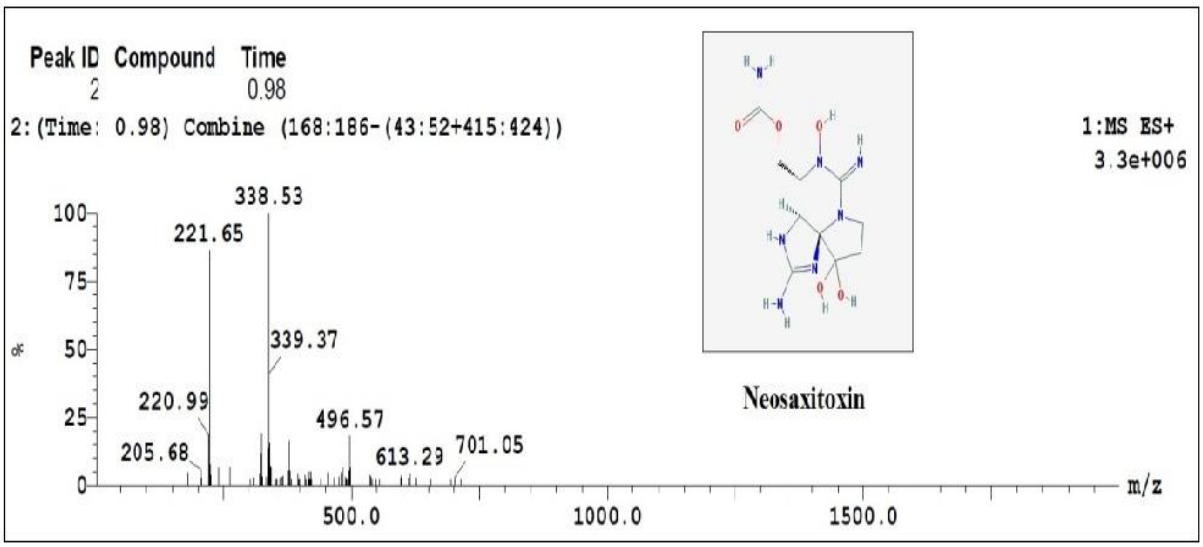

Fig. 4. A. Mass spectrum of Neosaxitoxin.

The beginning of PSP symptoms is fast, and death usually occurs within 12 hours. Respiratory paralysis due to paralysis of the thoracic diaphragm is usually the direct cause of death (Chang *et al.,* 1997; Botana, 2000; Callejas *et al.,* 2015; Knaack *et al.*, 2016).

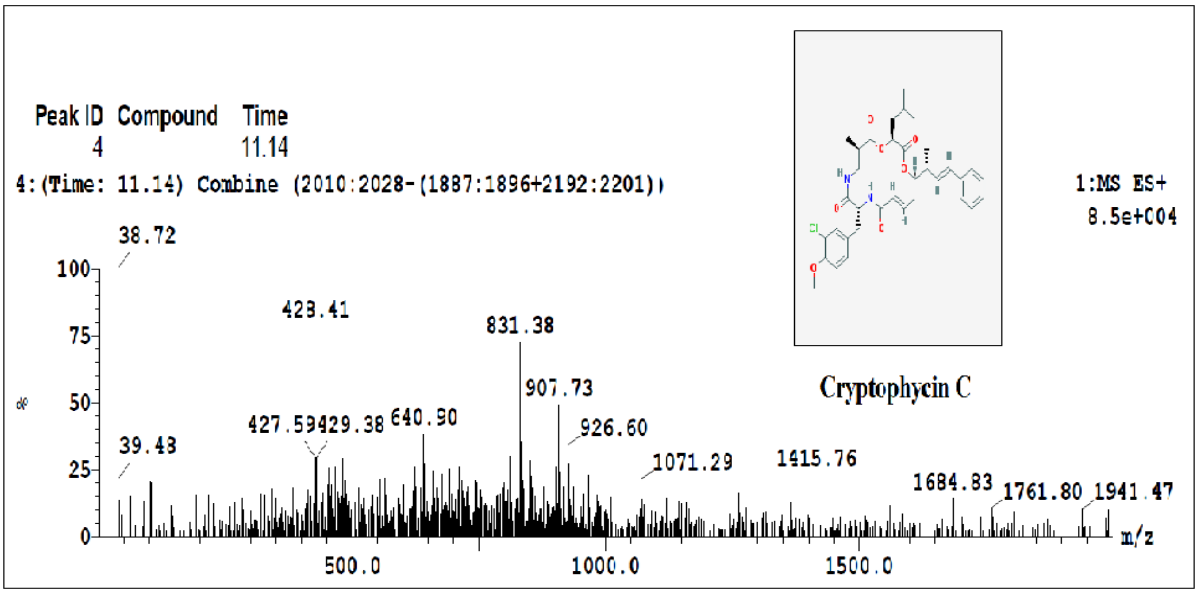

Fig. 4. B. Mass spectrum of Cryptophycin C.

Cryptophycin C

The cryptophycins are a unique family of sixteen-membered macrolide antimitotic agents. They were isolated from some species of *Nostoc sp*. and *Lyngbya sp*. (Eggen andGeorg, 2002; Liu and Rein, 2010).

The biological activity is based on their ability to interact with tubulin. Cryptophycins interfere with microtubule dynamics and prevent microtubules from forming correct mitotic spindles leading to cell-cycle arrest and apoptosis. Cryptophycins have been synthetically modified so as to increase their potency. The cryptophycin 1 potency is 100 to 1000 fold compared with that of anticancer drugs: paclitaxel and vinblastine (Weiss *et al.,* 2013). Hassouani *et al.,* (2017) investigated in vitro anticancer activity for crude extract of *L. aestuarii*, extracted by immersion on a 2:1 mixture of CH_2Cl_2/MeOH , on hepatocellular carcinoma human cell line HepG2, colon

adenocarcinoma human cell line HT-29, breast carcinoma human cell line T47D and osteosarcoma human cell line MG-63 using microculture tetrazolium test (MTT) assay. Moderate cytotoxicity was seen in HT29 and HepG2 cells only with reduced cell viability of 61.38 ± 3.26 and 62.78 ± 2.13%, respectively after 48h of incubation with *L. aestuarii* extract.

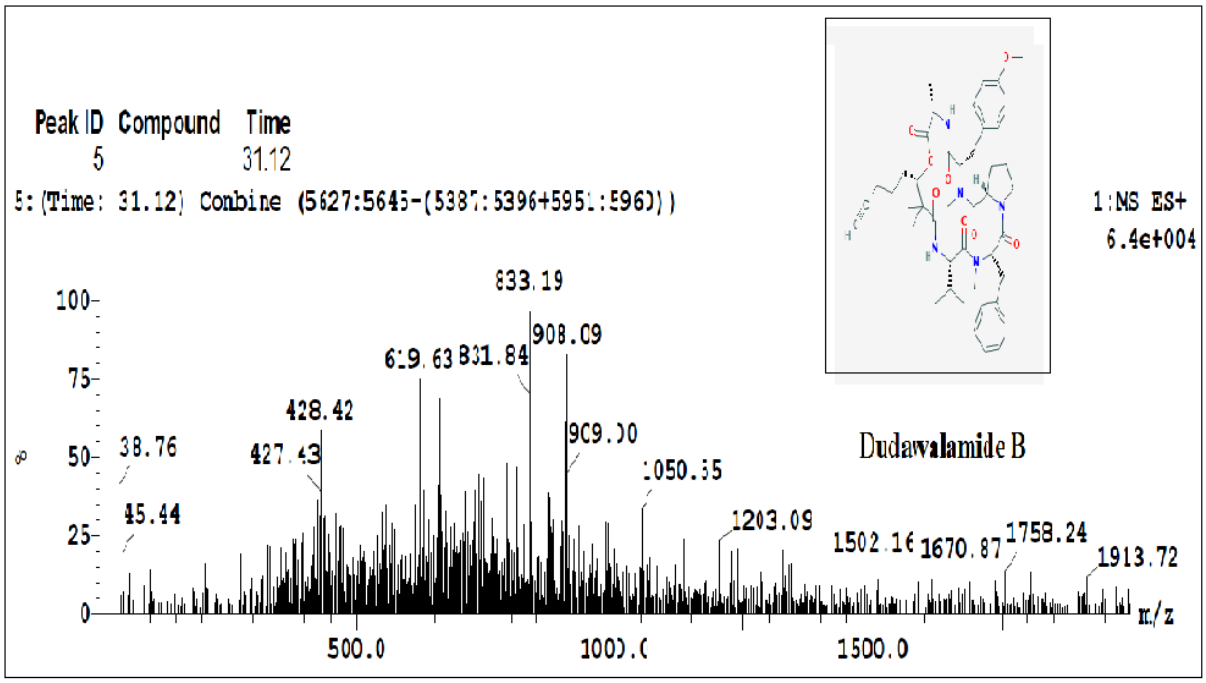

Fig. 4. C. Mass spectrum of Dudawalamide B.

Dudawalamide B

Dudawalamides A–D, Dhoya-containing cyclic depsipeptides belonging to the kulolide superfamily, were isolated from blue-green alga *L. majuscula*.These compounds have antiplasmodium and antiparasitic activities (Almaliti *et al.,* 2017).Veerabadhran*et al.,* (2014) showed that the crude extract of *L. aestuarii*, extracted using methanol: chloroform mixture (1:1 v/v), had antiplasmodial activity (IC_{50} = 18 µg/ml) against *Plasmodium falciparum*.

Determination of microcystins' concentration

The concentration of microcystins in the algal extract was rare and near the detection limits of Elisa kit. This result coincided with Teneva *et al.,* (2003) who found that *L. aerugineo-coerulea* contained trace amounts of microcystins.

Toxicity evaluation

Median lethal dose (LD_{50})

The crude extract provides status that closely mimics the environment (Kateregga *et al.,* 2014). The toxicity of the present crude extract is expressed as LD_{50} mg freeze dried cells weight per kg mouse body weight. The potency of cyanotoxins' extracts was classified into several classes depending on *f* factor where non toxic extracts have *f* values above 1000; low toxic extracts between 500 and 1000; the extracts with moderate toxicity have values ranging from 100 and 500, and finally the extracts with high toxicity have *f* factor value under 100 (Lawton *et al.,* 1994). However, some countries consider a cyanobacterium as non-toxic only when the *f* factor value is above 2000 (Harada *et al.,* 1999).

The LD_{50} of *L. aestuarii* extract is 560 mg/kg and according to the *f* factor described above, *L. aestuarii* extract is moderate to low toxicity. The symptoms appeared on mice were typical of neurotoxicosis and no symptoms of hepatotoxicosis were appeared on poisoning mice due to trace amount of microcystins in algal extract as investigated by ELISA kit. At the first non-lethal dose or LD_0 (10 mg/mouse), mice

sleep for a short period while at the second lethal dose, the mice became lethargic within minutes after dosing, with rapid abdominal breathing. They subsequently became immobile. Their respiration became irregular and the rate of respiration declined. Respiration rates continued to decrease until breathing ceased completely.

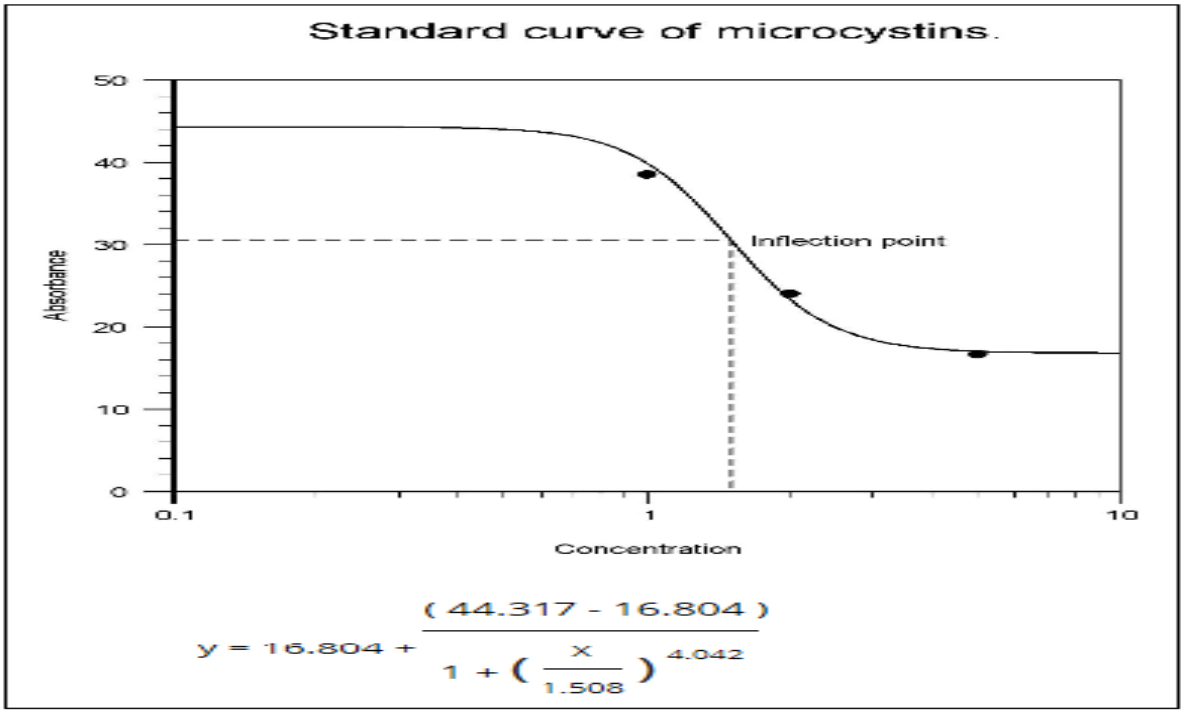

Fig. 5. Standard curve of microsystins as calculated by 4-parameterlogistic analysis.

These symptoms were coincided with those observed by Selwood *et al.* 2017. As mentioned earlier in results, there is a very small difference between the dose that does not kill any mouse (i.e. 10 mg/mouse) and the dose that kills four mice of a group consists of six mice (i.e. 15 mg/mouse). Both neurotoxicity symptoms and toxicity potency represented by LD_{50} bioassay are in agreement of both Munday *et al.,* (2013) and Delling *et al.,* (2017) where STX and its analogs had a very steep dose-response curve. Munday *et al.,* (2013) found that the LD_0 value for STXs is lower than LD_{50} value by 10 % only while Delling *et al.,* (2017) reported that the difference between the LD_{50} and LD_{100} was 6 µg / kg only. As emphasized by LC-MS/MS analysis, the potent toxicity of the crude extract was belonging to neosaxitoxin, an analogue of STXs.

On a global scale, around 2000 cases of STX poisoning are reported each year, with a mortality rate of approximately 15% (Selwood *et al.,* 2017).

Conclusion

The current study investigated a dangerous impact of both desiccation and Salinization of Basra Rivers which lead to a bloom of saline toxic blue-green alga *L. aestuarii*.

The alga is found to produce the neurotoxic neosaxitoxin which is more potent than the hepatotoxic microcystins.

Acknowledgements

We would like to acknowledge Department of Biology /College of Education for Pure Sciences for support us in our research.

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