UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Farmácia (Fisiopatologia e Toxicologia)
Área Toxicologia

Extraction, stability and bioavailability methods of guanitoxin in *Sphaeropermopsis torques-reginae* cultures analyzed by mass spectrometry

Métodos de extração, estabilidade e biodisponibilidade da guanitoxina em cultivos de *Sphaeropermopsis torques-reginae* analisadas por espectrometria de massas

Kelly Afonsina Fernandes

Tese para obtenção de título de DOUTOR
Orientador: Prof. Dr. Ernani Pinto

São Paulo
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Comissão Julgadora Tese para obtenção de título de DOUTOR

Prof. Dr. Ernani Pinto Junior, orientador/presidente

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1o. examinador

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4o. examinador

São Paulo, _____ de _____ 2020

Dedico esta pesquisa ao meus pais e familiares que sempre me incentivaram e me apoiaram durante esta jornada.

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“Eu quase que nada não sei. Mas desconfio de muita coisa... ”

O senhor já sabe: viver é etcétera…

"O correr da vida embrulha tudo, a vida é assim: esquenta e esfria, aperta e daí afrouxa, sossega e depois desinquieta. O que ela quer da gente é coragem. O que Deus quer é ver a gente aprendendo a ser capaz de ficar alegre a mais, no meio da alegria, e ainda mais alegre ainda no meio da tristeza! A vida inventa! A gente principia as coisas, no não saber por que, e desde aí perde o poder de continuação porque a vida é mutirão de todos, por todos remexida e temperada. O mais importante e bonito, do mundo, é isto: que as pessoas não estão sempre iguais, ainda não foram terminadas, mas que elas vão sempre mudando. Afinam ou desafinam. Verdade maior. Viver é muito perigoso; e não é não. Nem sei explicar estas coisas. Um sentir é o do sentente, mas outro é do sentidor..."

A gente quer passar um rio a nado, e passa: mas vai dar na outra banda é um ponto muito mais embaixo, bem diverso do em que primeiro se pensou. Viver nem não é muito perigoso? Dói sempre na gente, alguma vez, todo amor achável, que algum dia se desprezou...”

“Qualquer amor já é um pouquinho de saúde, um descanso na loucura..."

“Quem desconfia fica sábio...”

Trechos de Grande sertão: veredas, de João Guimarães Rosa. Rio de Janeiro: Nova Fronteira, 2001.

RESUMO

FERNANDES, K. A. **Métodos de extração, estabilidade e biodisponibilidade da guanitoxina em cultivos de *Sphaeropermopsis torques-reginae* analisadas por espectrometria de massas**. 2020, 112f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

A guanitoxina (GNT) é uma neurotoxina produzida por algumas cepas de cianobactérias dos gêneros *Dolichospermum* e *Sphaerospermopsis*. A GNT é o único organofosforado natural, capaz de causar a morte de animais selvagens e domésticos devido à inibição irreversível da acetilcolinesterase. Apesar de sua alta toxicidade, o diagnóstico da GNT em amostras biológicas ainda é um grande desafio. A dificuldade para sua detecção está diretamente ligada à sua instabilidade em altas temperaturas e pH alcalino, tornando difícil seu monitoramento em corpos d'água. Por isso, esta pesquisa objetivou estudar a estabilidade e biodisponibilidade da GNT em amostras aquosas, com intuito de obter mais informações sobre a natureza química e biológica dessa potente neurotoxina. Para realizar este estudo, a cepa ITEP-24 (*S. torques-reginae*) produtora de GNT foi cultivada em laboratório sob condições controladas, para obter biomassa para os experimentos de extração, semi-isolamento, estabilidade, ensaio *in vitro* e identificação por LC-MS/MS. Primeiramente foram realizados testes de extração da GNT partir de células liofilizadas da cepa ITEP-24 utilizando água, metanol e etanol em pH ácido. Depois utilizou-se dois métodos de extração em fase sólida (SPE) com cartuchos preenchidos com fases estacionarias C18 em fase reversa e sílica gel em fase normal, com objetivo de avaliar qual método de SPE seria melhor para extrair e concentrar a GNT. Nós também testamos métodos para lisar as células com sondas de ultrassom, misturador e centrifugação. Além dos métodos de extração, nós avaliamos a estabilidade da toxina em diferentes temperaturas, para isso a biomassa seca contendo a GNT ficou condicionada a 4 °C, 23 °C, -20 °C, -80 °C durantes seis meses, e análises de identificação foram realizadas dentro período de 150 dias em uma sequência de 30 dias. A estabilidade da toxina foi analisada também a partir de extrações em soluções com diferentes valores de pH (1,5; 3,0; 5,0; 7,0; 8,5; 10,5) e temperatura (23 ºC e 37 ºC). Depois, analisou-se a biodisponibilidade da GNT em células frescas da linhagem ITEP-24 através de teste de dissolução *in vitro*. O objetivo deste teste foi avaliar a liberação da toxina intracelular em meio simulado do conteúdo gástrica e intestinal com e sem enzimas digestivas para compreender e estimar a disponibilidade da GNT *in vivo*. Os resultados de todos experimentos descritos neste estudo, foram obtidos a partir de análises por cromatografia

líquida de interação hidrofílica (HILIC) acoplado ao espectrômetro de massas do tipo triplo quadrupolo LC-QqQ-MS/MS utilizando as transições 253>58, 253>159 e 159>58 $[M+H]^+$ utilizando coluna com fase estacionária zwitteriônica (ZIC). A identificação da GNT foi realizada também por cromatografia líquida acoplada ao espectrômetro de massas de alta resolução (LC-HR-QTOF-MS) com coluna Luna C18, Hydro-RP C18 e ZIC-HILIC. Dos protocolos de extração testados, a combinação de metanol/água (70:30 v/v) com ácido acético (0.3%) extraiu maior quantidade relativa da GNT a partir de células frescas e liofilizadas da cepa ITEP-24 e a concentração da toxina foi maior em amostras de células frescas. Em relação aos métodos de lise celular, as extrações realizadas em sonda de ultrassom com banho-maria e centrifugação por 1h foram estatisticamente significantes para liberar a toxina intracelular. Não houve diferença significativa entre os testes de SPE, no entanto, a semipurificação da toxina foi melhor com cartucho preenchido com sílica gel em fase normal e adaptação desse método em coluna aberta permitiu obter uma fração enriquecida com GNT. A GNT mostrou ser mais estável em pH ácido, sendo o pH 3,0 o melhor para manter e extrair a toxina em amostras aquosas e a toxina intracelular presente em células secas podem degradar em temperatura de 23 °C por um período de 150 dias mesmo em solução com pH 3,0. Durante os testes de extração e purificação foi observado também a degradação da toxina em processos de secagem e ressuspensão. As análises realizadas no LC-HR-QTOF-MS com diferentes métodos cromatográficos possibilitou a identificação da GNT, porém o método realizado com coluna ZIC-HILIC mostrou melhor resolução cromatográfica dos picos relativos *m/z* e tempo de retenção de toxina. Os resultados obtidos nos testes de dissolução *in vitro* mostraram que a GNT fica mais disponível no simulado gástrico com e sem a enzima pepsina, mas também pode ser absorvida no intestino. Portanto, o teste de dissolução *in vitro* pode ser uma ferramenta útil para a avaliação de risco de cianotoxinas *in vivo*, devido ao seu potencial de monitorar qualitativa e quantitativamente substâncias dissolvidas em fluidos gastrointestinais. Os resultados apresentados neste estudo fornecem informações valiosas para uma melhor compreensão da estabilidade e biodisponibilidade do GNT. Além disso, os métodos apresentados neste estudo podem ser úteis para diversas aplicações projetadas para identificar a toxina em amostras ambientais, bem como orientações para procedimentos de purificação da GNT.

Palavras chaves: neurotoxina, organofosforado natural, acetilcolinesterase, cianotoxinas

ABSTRACT

FERNANDES, K. A**. Methods of extraction, stability, and bioavailability of guanitoxin in *Sphaeropermopsis torques-reginae* cultures analyzed by mass spectrometry,** 2020, 112f. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

Guanitoxin (GNT) is a neurotoxin produced by some strains of cyanobacteria of the genus *Dolichospermum* and *Sphaerospermopsis*. GNT is the only natural organophosphate, capable of causing the death of animals from wild and domestic animals due to irreversible inhibition of acetylcholinesterase. Despite its high toxicity, the diagnosis of GNT in biological samples is still a significant challenge. The difficulty in its detection is directly linked to its instability at high temperatures and alkaline pH, making it difficult to monitor in bodies of water. Therefore, this research aimed to study the stability and bioavailability of GNT in aqueous samples to provide more information about the chemical and biological nature of this molecule. The strain ITEP-24 (*S. torques-reginae*) producing GNT was grown in the laboratory under controlled conditions to obtain biomass for the extraction, semi-isolation, stability, *in vitro* tests, and toxin identification by LC-MS/MS. Firstly, tests were carried out to extract GNT from lyophilized cells strain ITEP-24 using water, methanol, and ethanol at acidic pH and, two SPE methods in cartridges with stationary phases of C18 reverse phase and normal phase gel silica, to evaluate which would be better to extract and concentrate the GNT. We also tested different methods of cell lysis, such as ultrasound probes, mixers, and centrifugation. In addition to the extraction methods, the stability of the toxin was evaluated at different temperatures, for this, the dry biomass containing the toxin was conditioned at 4 °C, 23 °C, -20 °C, -80 °C for 150 days and analysis of the identification of the GNT was carried out within that period in a sequence of 30 days. The toxin stability was also analyzed from extractions in solutions with different pH values (1.5; 3.0; 5.0; 7.0; 8.5; 10.5) and temperature (23 ºC and 37 ºC). In addition, we performed dissolution tests with fresh cells of the ITEP-24 strain to evaluate the bioavailability of GNT in simulated gastric and intestinal fluids with and without digestive enzymes to understand and estimate the availability of GNT *in vivo*. The results of all experiments described in this study were obtained from analyzes by hydrophilic interaction liquid chromatography (HILIC) coupled to the LC-QqQ-MS/MS triple quadrupole mass spectrometer using the transitions *m/z* 253> 58, *m/z* 253> 159 and *m/z* 159> 58 $[M + H]^+$ using a column with the zwitterionic stationary phase (ZIC). Liquid

chromatography coupled to the high-resolution mass spectrometer (LC-HR-QTOF-MS) with Luna column C18, Hydro-RP C18, and ZIC-HILIC carried out the identification of the GNT. From the extraction protocols tested, the combination of methanol/water (70:30 v/v) with acetic acid (0.3%) extracted a greater relative amount of GNT from fresh and lyophilized ITEP-24 cells, and the concentration of the toxin is higher previously fresh. Concerning cellular methods, the ultrasound probe with a water bath and centrifugation for 1h ware statistically significant to release the intracellular toxin. There was no significant difference between the SPE tests. However, the semi-purification of the toxin was better with a cartridge filled with gel silica in the normal phase and adaptation of this method in an open column allowed to obtain a fraction enriched with GNT. GNT was more stable at acid pH, with pH 3.0 being the best to maintain and the intracellular toxin present in dry cells can degrade at a temperature at 23 °C for 150 days even in pH 3.0 solution. The toxin can also hydrolyze in the drying and resuspension processes. The analyzes carried out in LC-HR-QTOF-MS with different chromatographic methods made it possible to identify the GNT itself, however, the ZIC-HILIC column method showed excellent chromatographic resolution of the relative *m/z* peaks and toxin retention time. The results obtained in the *in vitro* dissolution tests showed that GNT is more available in the gastric simulation with and without the enzyme pepsin, but it can also be absorbed in the intestine. Thus, *in vitro* dissolution tests can be used as a useful tool for the risk assessment of cyanotoxins *in vivo* due to their potential to qualitatively and quantitatively monitor substances dissolved in gastrointestinal fluids. The results presented in this study provide valuable information for a better understanding of the stability and bioavailability of GNT. Besides, the methods presented in this study can be useful for various applications designed to identify the toxin in environmental samples, as well as guidance on procedures for purifying GNT.

Keywords: neurotoxin, natural organophosphate, acetylcholinesterase, cyanotoxins

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CONTENT

THESIS PRESENTATION

The data presented in this doctoral thesis consist of research on methods of extraction, identification, and bioavailability of GNT in aqueous samples analyzed by liquid chromatography coupled with mass spectrometry. The thesis was divided into chapters so that the reader can better understand the data available during the text.

Since 1978, studies on GNT have been carried out, the data presented in the first chapter show that the number of studies has gradually increased. Although the number of published studies is lower than that of other cyanotoxins, there has been a rise in published data on the occurrence of toxin-producing cyanobacteria, in cases of accidental intoxication of birds and mammals and *in vivo* and *in vitro* tests. However, we observed a lack of data on methods of extraction and isolation of the toxin. Therefore, this study dedicated to testing some methodologies as described in the following chapters in order to provide more information for the identification of the toxin in water bodies and, thus, to minimize the impacts on aquatic and terrestrial biota.

The second chapter refers to methods of extracting GNT from lyophilized cells of the ITEP-24 strain. In this chapter, we test some methods of extraction and identification of GNT described in the literature and, thus, we evaluate which is more efficient to extract the intracellular toxin. In addition, in this chapter, based on our experiences and data available in the literature, we recommend some methodologies that can be useful for the extraction, purification, and isolation of GNT.

In the third chapter, we evaluated the availability of GNT from dissolution tests with cells of the ITEP-24 strain in simulated solutions of gastric and intestinal fluid with and without digestive enzymes. According to the data available in the literature, GNT was directly associated with several cases of intoxication in animals that consumed water containing cells of toxin-producing species. Therefore, dissolution tests were used in this study as a model for risk assessment of cyanobacteria containing GNT that can be accidentally ingested by animals and humans.

In the fourth chapter, it comprises the results of the study of the stability of the molecule at different temperatures and pH, carried out for six months. The stability study was developed as a way to suggest protocols for adequate storage of aqueous samples or dry biomass with GNT. Other details of this research are described in this document.

CHAPTER 1. Guanitoxin: occurrence, physicochemical and toxicological properties

1.1 INTRODUCTION

Cyanobacteria are prokaryotic and photosynthetic microorganisms with fossil records from 3.5 billion years ago (Altermann et al., 2006). The evolutionary success of these organisms is due to their high adaptive and metabolic plasticity, making them capable of inhabiting aquatic and terrestrial environments under extreme environmental conditions (Altermann et al., 2006; Billi et al., 2017). These microorganisms may also have played essential roles in the biological and geochemical cycles that contributed to the evolution of life on Earth (Mur et al., 1999).

However, human interference with natural ecosystems has resulted in significant impacts on biogeochemical cycles, contributing to increased concentrations of greenhouse gases, nitrogen (N) and phosphorus (P) in the atmosphere and aquatic systems (Smil, 2000). These interferences have had a negative impact on the effects of global warming, soil acidification and artificial eutrophication of water bodies (Smil, 2000). Consequently, these anthropic actions have also contributed to the predominance of cyanobacteria in different ecosystems (Garcia-Pichel and Pringault, 2001; Singh and Elster, 2007).

The abundance of N and P in the environment can be incorporated by cyanobacteria to perform metabolic processes essential for their development (O'Neil et al., 2012). Other environmental variables such as high temperatures, low circulation of atmospheric winds and neutral-alkaline pH also favor the proliferation of cyanobacteria (Paerl and Ustach, 1982; Robarts and Zohary, 1987; Zhou et al., 2016).

Cyanobacteria produce a variety of active compounds with toxicological, pharmacological, industrial and cosmetic properties (Singh et al., 2005). However, the production of potentially toxic metabolites has been a target of worldwide concern, due to the cases of accidental poisoning involving domestic animals, wild animals and humans (Carmichael and Gorham, 1978; Onodera et al., 1997; Pouria et al., 1998; Jochin). In addition, the exacerbated growth of cyanobacteria can significantly alter the balance of native communities of different trophic levels (Sukenik et al., 2015).

Particularly in the 70s and 90s there was a huge explosion of published case studies on intoxication and animal deaths, as well as diseases caused by cyanobacterial toxins (Carmichael and Gorham, 1978; Francis, 1878; Odriozola et al., 1984; Turner et al., 1990). However, the most severe case of cyanotoxin poisoning occurred in 1996, when patients from

a hemodialysis clinic in Caruaru/PE (Brazil) died after contact with water containing the microcystin-LR toxin (MC-LR) (Azevedo et al., 2002). From this case, cyanobacteria came to represent a problem directly linked to public health.

Before this case in Caruaru/PE (Brazil), several studies had already been published on the action of cyanotoxins in several aquatic and terrestrial organisms (Carmichael and Falconer, 1993; Falconer, 1991; Humpage et al., 1994). However, after this case of human intoxication, the studies were intensified mainly for chemical description and structural classification of the toxins. The mechanism of action of cyanotoxins has been described from bioassays with rats and mice, and it is possible to determine the Lethal Dose (LD_{50}) and describe different diagnoses based on the intoxication symptoms observed in the animals (Mahmood and Carmichael 1986; Cook et al., 1990; Hyde and Carmichael, 1991).

Thus, cyanotoxins were classified into four main groups. Hepatotoxins, comprising the group of microcystins and nodularins, which have the liver as the target organ (Pearson et al., 2010). Neurotoxins, represented by saxitoxins, BMAA, and anatoxins (anatoxin-a, guanitoxin, and homoanatoxin-a), which are responsible for interferences in the nervous system and the transmission of signals between neurons (Brand et al., 2010; Pearson et al., 2010; Sivonen and Jones, 1999). Cytotoxin (cylindrospermopsin), an inhibitor of protein synthesis, which can affect all cells, but especially the kidneys and liver of mammals (Pearson et al., 2010). Dermatoxins (lyngbyatoxin, lipopolysaccharides, and aplysiatoxin), responsible for skin irritations (Sivonen and Jones, 1999)**.**

The frequent events of cyanobacterial blooms and reports of animal and human intoxication, among other relevant environmental impacts, resulted in the elaboration of a guide for the monitoring of cyanotoxins dissolved in water for human supply by the World Health Organization (WHO) (Sivonen and Jones, 1999). The WHO has established detection limits of up to 1µg/L for MC-LR, the most frequently occurring cyanotoxin in water bodies (Sivonen and Jones, 1999). However, there is still no regulation established by the WHO for other variants of MCs or other cyanotoxins, due to the deficiency of toxicological data and consistent information (Ibelings et al., 2014). However, the values established for MC-LR serve as guidance for several countries for monitoring MCs and other cyanotoxins in their bodies of water (Ibelings et al., 2014).

The presence of other cyanotoxins in water bodies has been increasingly common, particularly cylindrospermopsin, saxitoxin, and anatoxins (Akcaalan et al., 2014; Faassen et al., 2012; Smith et al., 2011). Available data show an alarming decline in water quality, suggesting a reassessment by the regulatory bodies for monitoring other cyanotoxin classes,

especially neurotoxins due to their rapid mode of action (Ibelings et al., 2014; Sivonen and Gary Jones, 1999). This chapter will focus on guanitoxin (GNT) belonging to the neurotoxin class, due to the high toxicity that this toxin represents, but above all due to the lack of information that makes it difficult to monitor it by environmental inspection bodies.

GNT was widely reported in the 1980s, after which various cases were attributed to this toxin. On the other hand, the presence of this toxin may have been neglected for a long time. Probably due to the lack of analytical standard necessary for its quantification in bodies of water. Recently, data have been published showing evidence of the presence of this cyanotoxin in aquatic and terrestrial environments (Chatziefthimiou et al., 2014; Metcalf et al., 2018, 2012). Therefore, the objective of this study is to show the information available in the literature about GNT, such as producing species, occurrence in the world, cases of intoxication, methods for identification and quantification, toxicological tests, and the challenges for scientists and environmental inspection agencies for identification of this toxin in lakes, rivers, and reservoirs.

1.2 MATERIALS AND METHODS

The data available here comes from extensive research on GNT on search engines such as Google Scholar, Pubmed.Gov and Web Of Science. The figures were developed in the Landscape and ChemDraw Ultra 12.0 software. Graphs and statistical analyzes were performed using Graphical Pad Prism version 7.

1.3 RESULTS

The data presented here correspond to an intense search in the literature that includes the period of discovery of the GNT until the present day (Figure 1). In this research, forty articles were used that approached different studies on GNT. From these data, it the data presented here correspond to an intense search in the literature that includes the period of discovery of the GNT until the present day (Figure 1). In this research, forty articles were used that approached different studies on GNT. From these data, it was possible to observe that the number of publications on GNT is still low compared to information available other cyanotoxins.

The most significant percentage of studies concentrated mainly on *in vivo* and *in vitro* tests (33%), followed by cases of occurrence of producing species by GNT (24%), which,

consequently, are directly associated with events of accidental poisoning of mammals and birds in the environment (12 %) (Figure 2). Articles on GNT identification methods, such as AChE, biosensors, HPLC, and LC/MS inhibition tests accounted for 9%, as well as review articles on GNT, also representing 9%. Among the published studies, the lowest percentage was represented by studies of synthesis/biosynthesis (7%) and purification and isolation of GNT (3%) (Figure 2).

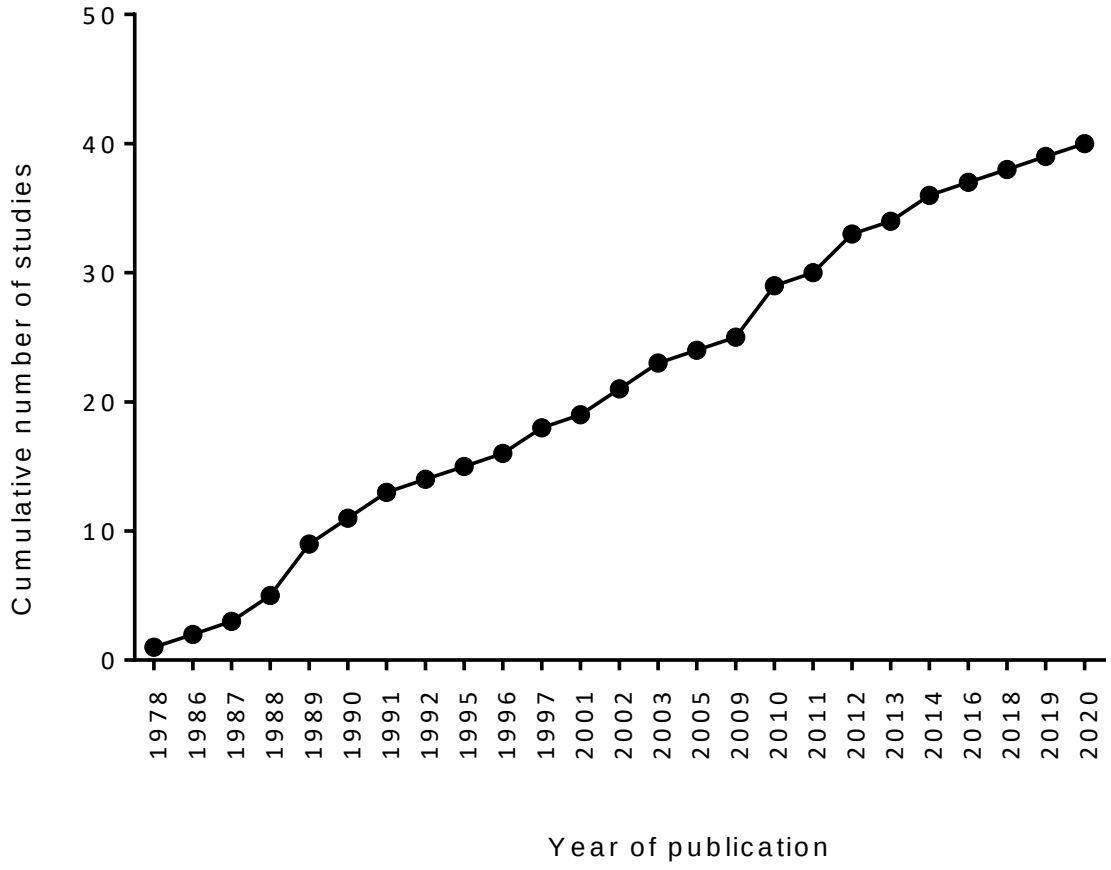

Figure 1: Cumulative data from published studies on guanitoxin from 1978 to 2020.

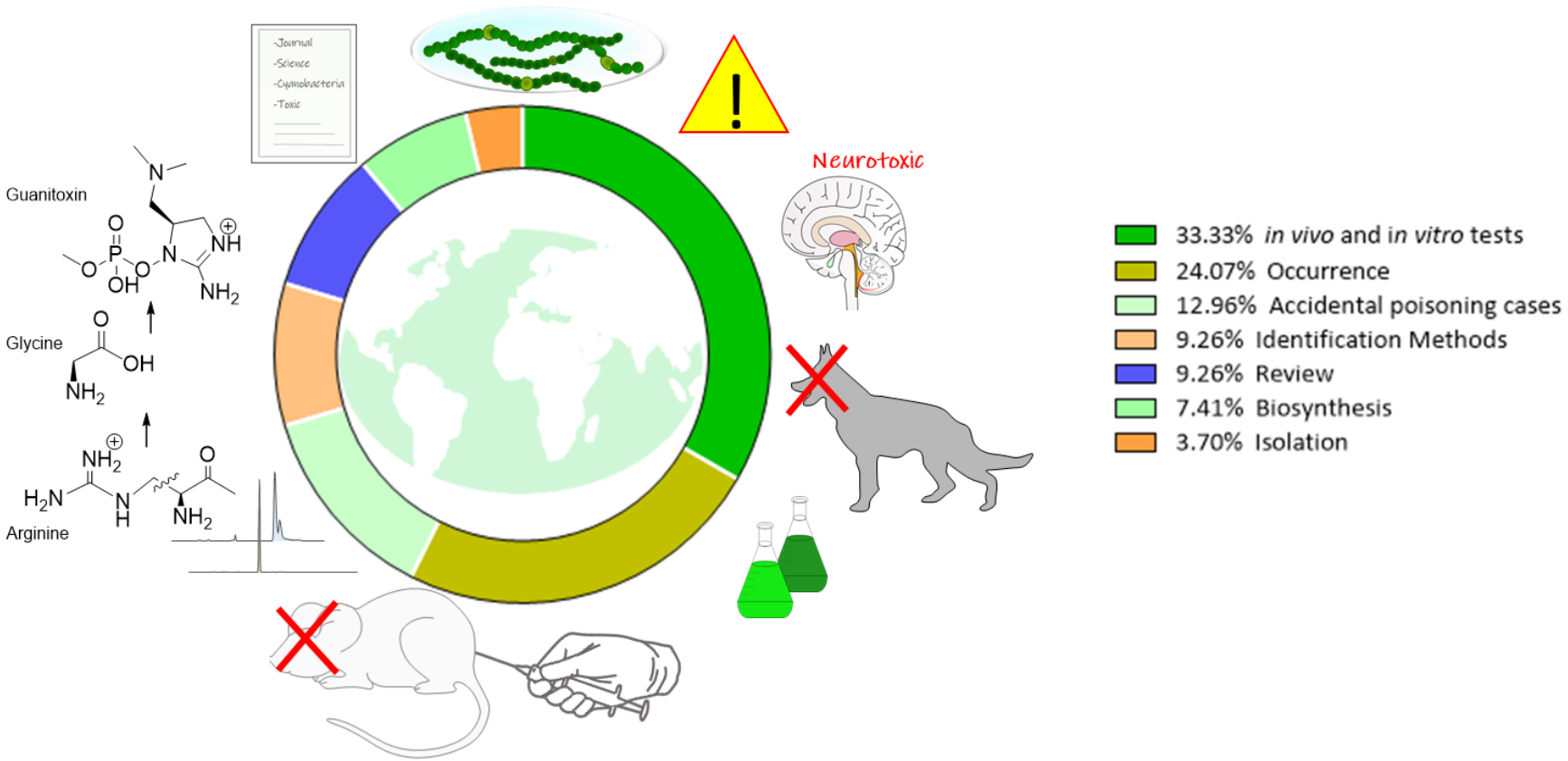

Figure 2: The figure shows the percentage of the main topics covered in the literature on guanitoxin. Toxicity tests *in vivo* and *in vitro* (33.33 %), cases of occurrence of species producing guanitoxin (24.07 %) followed by cases of accidental poisoning of wild and domestic animals (12.96 %), methods of identification and review (9.26 %), synthesis and biosynthesis of GNT (7.41 %) and published articles on guanitoxin isolation (3.70 %).

1.4 DISCUSSION

1.4.1 Guanitoxin nomenclature

GNT was discovered in 1978 through *in vivo* tests with samples of cyanobacteria (*Dolichospermum flos-aquae*-strain NRC-525-26-4) collected in 1965 at Buffalo Pound Lake (Canada) (Carmichael and Gorham, 1978). The animals treated with lyophilized cells of NRC-525-26-4 showed clinical signs similar to those of anatoxin-a, but with excessive salivation and lacrimation, thus creating the hypothesis that the NRC-525-26-4 strain produced a new toxin. Thus, the authors named it anatoxin-a plus the letter “S” due to excessive salivation observed in animals (Carmichael and Gorham, 1978).

After *in vivo* and *in vitro* tests, it was found that the sites of action of GNT and anatoxin-a were different. Anatoxin-a had an agonistic action on nicotinic acetylcholine receptors (ACh), functioning as congeners of ACh, but not being recognized by acetylcholinesterase (AChE) (Carmichael, 1994), while GNT was characterized as an AChE blocker, preventing ACh hydrolysis (Carmichael, 1994). Also, other significant differences between cyanotoxins (anatoxin-a and GNT) have been reported, such as molecular structure and biosynthesis (Devlin et al., 1977; Hyde and Carmichael, 1991; Koskinen and Rapoport, 1985; Macallan et al., 1988; Matsunaga et al., 1989).

Although the researchers who described GNT recommended the need for further studies to adjust the toxin to an appropriate nomenclature (Carmichael and Gorham, 1978), the name anatoxin-a (S) remained for a long time, even after diversified studies pointed out differences between anatoxin-a and GNT. However, the name change from anatoxin-a (S) to Guanitoxin occurred only in January 2020 (Fiore et al., 2020). The change in the name of the toxin was mainly justified by the similarity between the terms "anatoxin-a and anatoxin-a (S)," which caused much confusion and compromised the correct identification of GNT (Fiore et al., 2020).

1.4.2 Guanitoxin-producing species and Occurrence places

Most records of occurrence of GNT have been reported in aquatic environments covered by filamentous cyanobacterial scums with neurotoxic action, which results in the death of domestic and wild animals. GNT was first identified in samples from Lake Buffalo

Pound (Canada) and then in North America, Europe, South America, and, more recently, in Asia (Table 1).

Currently, the main GNT-producing strains belong to the genera *Dolichospermum* spp. and *Sphaerospermopsis* spp. (Table 1). Before, the species identified as GNT producers were classified within the genus *Anabaena*. However, due to some taxonomic divergences, s planktonic species from the *Anabaena* group have undergone phylogenetic reviews and are reclassified to the genera *Dolichospermum* and *Sphaerospermopsis*. The reclassification of these species consisted of molecular analyses based on a sequence of 16S rRNA genes and morphological characterization (Komárek, 2013; Komárek and Mareš, 2012; Wacklin et al., 2009; Werner et al., 2012; Zapomělová et al., 2012, 2009).

The morphology of species that occur in natural populations is generally characterized by spiral trichomes (Figure 3). However, some strains grown in the laboratory can alter the original morphology according to the culture conditions and can silence the expression of some morphological structures, such as heterocysts, akinetes or gas vesicles (Komárek and Zapomělová, 2007). In general, the species classified in the *Dolichospermum* and *Sphaerospermopsis* group share common morphological characteristics, filamentous forms composed of trichrome with a mucilage sheath and specialized cells (akinetes and heterocysts) (Li et al., 2016; Werner et al., 2012).

The species *D. flos-aquae* (NRC525-17) was widely used in *in vivo* and *in vitro* tests until the '90s. Then other *Dolichospermum* spp. and S*phaeropermopis* spp. GNT producers have been described, and other studies have been published from the species *D.lemmermannii* isolated from the lake of KnudSø in Denmark, *S. torques-reginae*, and *S. oumiana* identified in the North and South of Brazil, respectively (Werner and Laughinghouse IV, 2009). Currently, the only strain of cyanobacteria that produces GNT present in banks of cyanobacteria cultures is the strain ITEP-24 (*S. torques-reginae*) (Figure 3).

In most cases, the cyanobacterial species characterized as producing the GNT toxin were subjected to AChE inhibition tests (Table 1) (Mahmood and Carmichael, 1987; Villatte et al., 2002). Currently, this method is still used (Metcalf et al., 2012), even though it is not the most recommended, because it is a method of low selectivity and specificity for the GNT molecule. Other analysis were performed by HPLC-DAD, where the wavelength of 220-230 nm was used to identify the GNT (Cook et al., 1989a). The detection of GNT in environmental samples can also be performed by the molecular mass of GNT, through liquid

chromatography coupled to LC/MS mass spectrometer (Dörr et al., 2010). However, this method was developed after the majority of reported cases of GNT occurrence.

Table 1: Main species of cyanobacteria producing guanitoxin.

Cyanobacteria specie	Collect location	Date	Geographical Coordinates	Strains	Identification methods	Author
Dolichospermum flos-aquae	Canada - Buffalo Pound Lake, Saskatchewan	June, 1965	50°39′00″N 105°30′50″W	NRC-525-26-C-4	*In vitro* in AChE assays HPLC and NMR	(Carmichael and Gorham, 1978)
Dolichospermum flos-aquae	United States - Richmond Lake, Brown County, South Dakota	August and September, 1985	45°32'12.6"N 98°37'09.7"W	NRC 525-17	*In vitro* in AChE assays and HPLC	(Mahmood et al., 1988)
Dolichospermum lemmermannii	Denmark - Lake KnudSø, Ry, Skanderborg, Jutland	July, 1993	56°06'4"N 9°45'57"E	PH 160 B, PH-160 D and PH 177	*In vitro* in AChE assays HPLC and NMR	(Henriksen et al., 1997; Onodera et al., 1997)
Dolichospermum flos-aquae	Scotland - Loch Muidhe, Rogart	1998	58°00'57.4"N 4°15'35.2"W	–	*In vitro* in AChE assays	(Codd et al., 1995)
Sphaerospermopsis torques-reginae	Brazil- Lake inside the Fundation University Campus Federal University of Rio Grande, Rio Grande, Rio grande do sul	October, 1995	32°04'16.0"S 52°09'50.9"W	–	*In vitro* in AChE assays	(Monserrat et al., 2001; Yunes et al., 2003)
Sphaerospermopsis torques-reginae	Brazil - Tapacurá Reservoir, Recife, Pernambuco	March, 2002	8°02'11.3"S 35°10'04.6"W	ITEP-24, ITEP-25 and ITEP-26	*In vitro* in AChE assays and LC/MS	(Dörr et al., 2010; Renato J R Molica et al., 2005)
Dolichospermum crassum	Brazil - Faxinal Reservoir, Caxias do Sul, Rio Grande do Sul	January, 2004	29°05′00″S; 51°03′30″W	–	*In vitro* in AChE assays	(Becker et al., 2010)
Sample of cyanobacteria collected in cryptogamic crust	Qatar (Arabian Gulf) - North of Doha; Al Wukair and Al Dawodyu.	July 2007 and February 2008	25°12'38.6"N 51°09'28.0"E	–	*In vitro* in AChE assays	(Metcalf et al., 2012)
Dolichospermum flos-aquae	United States - St. Lucie Canal, Lake Okeechobee, Florida	June, 2016	27°12’12’’N,80°15’29’’ W and 27°10’25’’N, 80°15’40’’W	–	*In vitro* in AChE assays	(Metcalf et al., 2018)

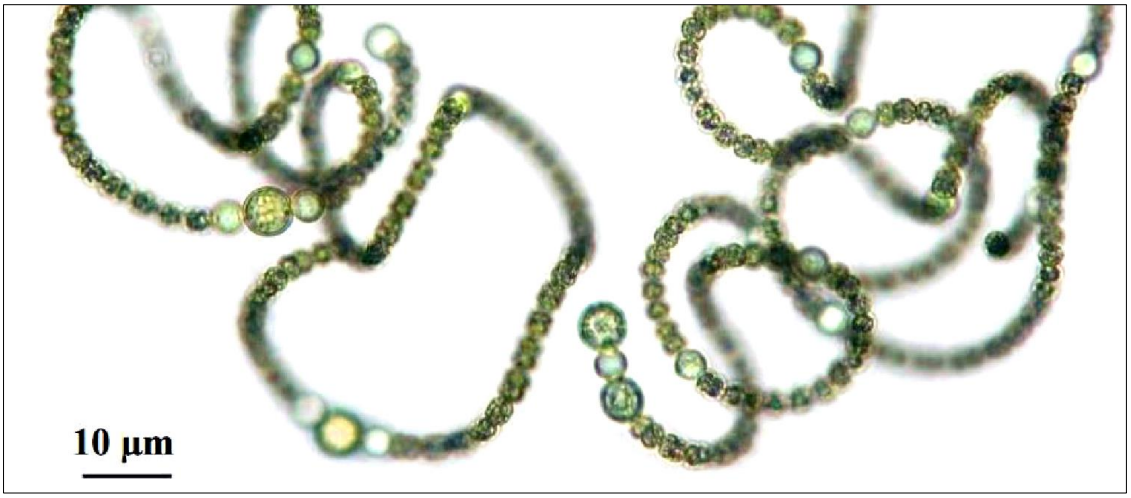

Figure 3: Morphology of the species *Sphaerospermopsis torques-reginae*, characterized by trichomes with spherical akinetes adjacent to the heterocysts (Werner et al., 2012; Werner and Laughinghouse IV, 2009).

1.4.3 Guanitoxin's mechanism of action

Guanitoxin has in its molecular structure a methyl phosphate group equal to some organophosphates (Figure 4) and is therefore considered the only natural organophosphate already described (Prugh et al., 2017). Despite having a chemical structure different from synthetic and organic organophosphates, its mechanism of action is the same and toxicity may even be greater than most of them, blocking AChE activity (Mahmood and Carmichael, 1986).

The mechanism of action of guanitoxin occurs at the neuromuscular junction during the transmission of nerve impulses from the motor neuron to the muscle fibers (Carmichael, 1994). When neurons release the neurotransmitter ACh, it quickly spreads to muscle cells, binding to receptors containing a local binding neurotransmitter and an ion channel that covers the cell membrane (Tuček, 1985). When neurotransmission to muscle fiber ceases, Ach is removed from the synaptic cleft, where it moves away from the motor plate and is broken down by the enzyme AChE in acetic acid and choline (Tuček, 1985).

The choline is transported to the axonal terminal for the synthesis of new ACh, and the process is repeated. However, when the GNT molecule becomes available in the neuromuscular system, it can interact with the enzyme AChE, blocking its activity and consequently preventing its hydrolysis from Ach (Carmichael, 1994). AChE inhibition allows ACh to remain available in muscle stimulation, leading to muscle over excitation (Figure 5). Therefore, the transmission of nerve impulses to muscle cells becomes continuous in muscle cells.

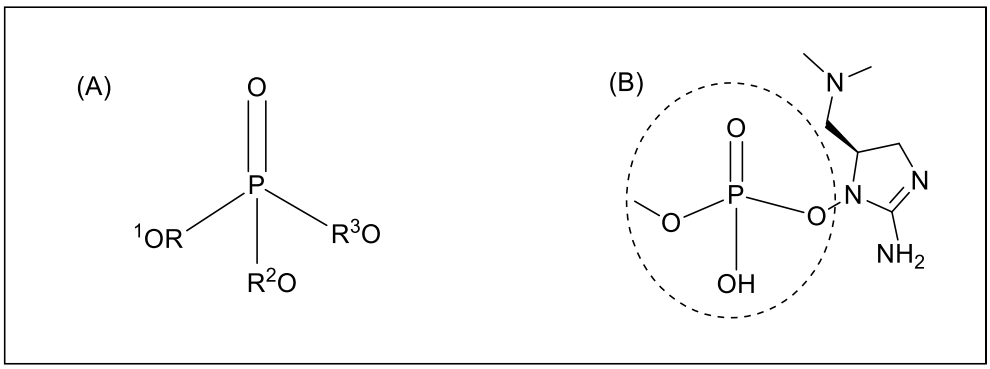

Figure 4: (A) Molecular Structure Common to Organophosphate with Phosphate Functional Group, (B) Guanitoxin molecular structure with phosphate functional group.

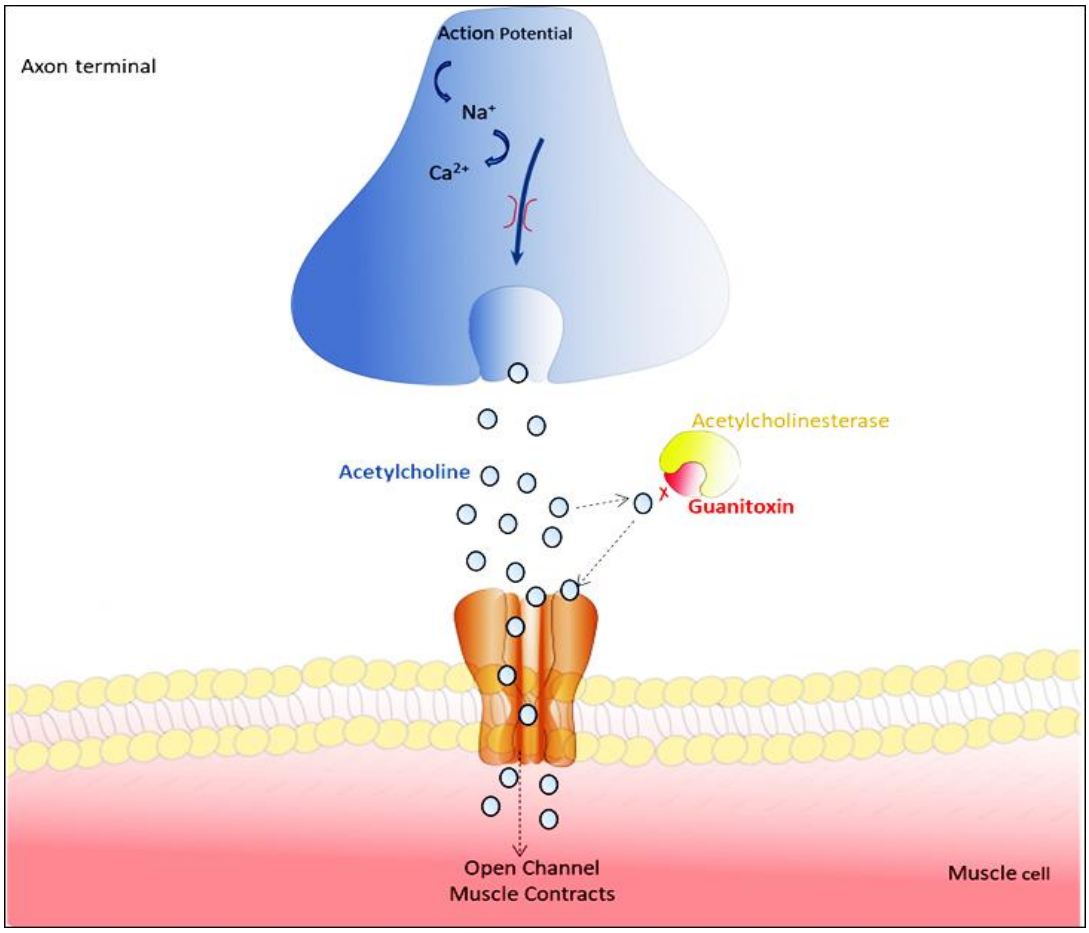

Figure 5: Scheme of a neuromuscular synapse showing the processes involved in the inhibition of acetylcholinesterase by guanitoxin, adapted from Carmichael (1994).

1.4.4 Potential use of guanitoxin in industry

Organophosphates are widely used in agriculture and are classified in the class of pesticides. Organophosphates are esters, amides, or thiol derivatives of phosphoric acids,

containing various combinations of carbon, hydrogen, oxygen, phosphorus, sulfur, and nitrogen (Chapalamadugu and Chaudhry, 1992). Organophosphates were developed to replace chlorinated recalcitrant pesticides considered extremely dangerous for aquatic and terrestrial biota due to the power of organic matter chronic accumulation (Chapalamadugu and Chaudhry, 1992).

However, its chemical residues and secondary metabolites can be as toxic as the first compound, and can accumulate in cell membranes and lipid-rich tissues, and can overcome placental and blood-brain barriers (Chapalamadugu and Chaudhry, 1992). Organophosphates can also be found in food, causing transfer problems via the food chain, leading to severe environmental damage and human health problems (Favari et al., 2002). To date, there are no reports of synthetic products that effectively combat undesirable organisms without causing environmental damage, and although there is a ban on the use of organophosphates in some countries, they are still widely used (Ballantyne and Marrs, 2017). As an alternative, one could invest in studies with natural compounds whose effect for combating undesirable organisms is already known, to minimize toxic effects on ecosystems.

GNT is a highly polar compound, being soluble in water, with a relatively short half-life in the environment (Cook et al., 1988). Also, studies have shown that it is more potent than some synthetic organophosphates (Cook et al., 1988). Thus, the possibility of using GNT as a raw material for new pesticides could bring more significant benefits to the environment, as it is a natural and biodegradable compound. On the other hand, it may also be less able to accumulate in animal tissue or insect exoskeletons.

Some studies have already been carried out to evaluate the effect of GNT on cockroaches, showing effects on the locomotor, cardiac and behavioral activity of organisms (Santos et al., 2019; Rodríguez et al., 2012). The use of other cyanobacteria compounds is already known in the pharmaceutical, food, and cosmetic industry (Kasting and Siefert, 2002), and cyanobacteria biomass is also used for the production of sustainable energy. In this perspective, the use of GNT could be used as a tool for biological pest control. However, to support this projection, new studies need to be carried out to affirm the action of the molecule as a substitute for synthetic organophosphates.

1.4.5 Effects of guanitoxin *in vivo* and *in vitro*

The presence of GNT in the environment poses severe risks to the life of wild and domestic animals. The detection of GNT in most cases was associated with animal deaths.

For this reason, there was a great effort by researchers who committed to studying the adverse effects that GNT could cause in animals, especially in mammals, due to a significant number of incidents involving different animals of this class.

Between events of animal intoxication and analysis of environmental samples of cyanobacterial blooms, species of the genus *Dolichospermum* were always dominant. The analyzes performed to detect GNT consisted mainly of anti-AChE colorimetric tests, observational analyzes of clinical signs in animals, physiological and histological analyses. These analyses were commonly used to diagnose GNT in biological samples.

GNT LD_{50} was determined in mice with a range of 20 µg/Kg to 50 µg/Kg, with a survival time of 10 to 30 minutes, being considered ten times more toxic than anatoxin-a (Cook et al., 1991; Mahmood et al., 1988; Mahmood and Carmichael, 1986). Due to its high toxicity and fast action, the potential of this toxin as a weapon in chemical warfare has already been discussed (Pita et al., 2003b)

Other effects were observed in *in vitro* tests, in blood and phrenic nerve-hemidiaphragm of rats, muscle of the abdominal rectus of the frog *Rana pipiens*, and nerve-muscle of the biventer cervicis (Mahmood and Carmichael, 1986). The results showed that GNT was able to inactivate serum cholinesterase activity in muscle tissue and blood samples (Mahmood and Carmichael, 1986). Thus, the authors hypothesized that GNT would be the first cyanobacterial toxin capable of inhibiting the enzyme acetylcholinesterase, suggesting complementary studies.

Then the same authors carried out a specific study of acute toxicity in rats treated with semi-purified GNT (Mahmood and Carmichael, 1987). The colorimetric method (Ellman et al., 1961) was used to evaluate the inhibition of the enzymatic activity of acetylcholinesterase by GNT. It was concluded then that GNT was a small alkaloid, with high polarity, unstable in alkaline conditions, and with anti-AChE activity (Mahmood and Carmichael, 1987). Later on, the alkaloid characteristic was excluded from the chemical characteristics of the GNT molecule.

In 1985, cyanobacterial blooms with *D. flos-aquae* species were associated with the death of nine dogs in Lake Richmond/USA. To confirm whether the GNT had caused the animals to die, *in vivo* tests were realized out with a fraction of the semi-purified toxin from the strain NCR525-15 and with a crude extract from the samples of the lake's cyanobacteria blooms (Mahmood et al., 1988). Rodents treated with samples from Lake Richmond showed characteristic symptoms of intoxication equal to samples of semi-purified GNT (Mahmood et al., 1988).

Until then, most tests were performed from intraperitoneal injection rats and mice with purified or semi-purified GNT extract or sample. Then, *in vitro* toxicological tests were performed with mice brains and blood using the crude and purified extract of the NRC525-17 strain to compare the effects with synthetic organophosphates (Cook et al., 1988; Cook et al., 1989a).

The results showed that GNT was unable to cross the blood-brain barrier. This fact could be explained by the high polarity of the molecule (GNT), which may have resulted in slower absorption or degradation, thus reflected in the delay for direct bioactivation on the cholinesterase in the central nervous system (Cook et al., 1988).

Then, Cook and collaborators (1989) carried out several experiments to evaluate the effects of GNT on different animals. Acute toxicity tests were performed on mice, ducks, pigs, and different doses of crude extract and purified with GNT to study and simulate the intoxication of domestic and wild animals that occurred in the field. The samples used in this study were acquired from cyanobacterial blooms in the Tolono and Griggsville lagoons in Illinois/USA and collected in July and September 1986. The species were predominant *D. flos-aquae* in the samples, and GNT was confirmed by anti-AChE enzyme tests and HPLC tests (Cook et al., 1989a)

Cook and collaborates (1989a) observed that animals treated with samples (Tolono and Griggsville) containing GNT had some symptoms of toxicosis similar to other reported cases, such as salivation, muscle tremors, dyspnea, and death. Other effects were specific to each group of animals evaluated (Cook et al., 1989a). Ducks and pigs treated with samples (Tolono and Griggsville) by endotracheal intubation showed lesions in the digestive system, profuse nasal and mucoid salivation, algal regurgitation, polydipsia diarrhea, muscle tremors, rectal sphincter dilation, bruxism, depression, stress, dyspnea, and cyanosis followed by death (Cook et al., 1989a). The steer that received doses of the extract (Griggsville sample) via intra-ruminal and parenteral was susceptible to toxicosis similar to that observed in other species (Cook et al., 1989a). *In vitro* assays with tissues of animals treated with the purified toxin and extract had an apparent inhibition of AChE (Cook et al., 1989a).

Pathophysiological effects were evaluated in rats, a group of animals received atropine sulfate intraperitoneally before exposure to a toxin (GNT). The rats pretreated with atropine had a longer survival time and response to the parasympathetic system compared with rats treated with toxin (Cook et al., 1990). Neuromuscular blockade of the respiratory muscles was also observed, being pointed out as the leading cause of death in rats (Cook et al., 1990; Cook et al., 1989b). Histopathological changes were not observed in animals

treated with GNT (Cook et al., 1991), probably due to the process of acute intoxication and rapid death, causing tissue deformity.

Cyanobacterial blooms with *D. lemmermannii* species caused bird deaths in lakes in Denmark. Tests were performed with rats, showing that the samples were positive for AChE inhibition (Henriksen et al., 1997). Subsequently, the GNT was isolated from samples in Lake KnudSø, using SPE, HPLC techniques, and analyzed by NMR, giving more support to previously published data on the presence of GNT in Lake KnudSø (Onodera et al., 1997).

The toxicity of GNT was also assessed in the mouse, fish brain (*Odontesthes argentinensis*), and crab thoracic ganglia (*Callinectes sapidus*), resulting in toxic effects on the neuromuscular system and 50% inhibition of AChE activity (Monserrat et al., 2001). These tests were performed with extract of the species *S. torques-regiane* isolated from eutrophic environments in Brazil between 1995 and 2002 (Monserrat et al., 2001; Yunes et al., 2003). The *in vitro* and *in vivo* results obtained with *S. torques-regiane* extract were similar to the organophosphate pesticides and corroborated with the effects described for *D. flos-aquae* and *D. lemmermannii* GNT-producing species (Henriksen et al., 1997; Mahmood and Carmichael, 1986).

After several studies with rodents, showing that GNT has a similar mode of action to synthetic organophosphates, Rodríguez et al. (2012) and Santos et al. (2019) evaluated the toxicity of crude extract of strain ITEP-24 (*S. torques-regiane*) on cockroaches. Effects on heart rate decreased locomotor activity, behavioral changes, and significant neuromuscular failures were observed in cockroaches (Santos et al., 2019; Rodríguez et al., 2012).These studies suggested the use of cockroaches as an alternative biological model for the biomonitoring of GNT in aquatic systems (Santos et al., 2019; Rodríguez et al., 2012).

In addition to mammals, fish, and insects, the effect of GNT was also analyzed in Cladocerans. The toxicity of GNT was analyzed in *Daphniidae* from exposure in aqueous medium with intact cells and ruptured cells of the strain ITEP-24 (Abreu and Ferrão-Filho, 2013; Freitas et al., 2014; Freitas et al., 2016). The effects observed in *Daphnia similis* exposed to intact cells were more pronounced than in the assay with disrupted cells of the ITEP-24 strain. However, in both assays, the growth and survival of the organisms were affected (Abreu and Ferrão-Filho, 2013; Freitas et al., 2014). Other effects have been observed in other species of *Daphina* ssp., such as AChE inhibition, changes in individual and population parameters of organisms exposed to the extract (ITEP-24) containing GNT (Freitas et al., 2016).

In November 2013 and March 2014 in Doha (Qatar), four dogs were referred for emergency care with functional changes typical of GNT poisoning (Chatziefthimiou et al., 2014). Two of these dogs died and the others managed to recover after treatment. The contamination of the animals occurred after the consumption of rainwater accumulated in the depressions of the desert. The local samples were analyzed one week after animal poisoning, showing inhibition of AChE and esterases. The fact that the samples were analyzed after the accident with animals may have underestimated the initial concentration of toxins (Chatziefthimiou et al., 2014)

Previously, in the case of dog intoxication in Qatar, cyanotoxins have been reported in cryptogamic samples collected from dry and ephemeral riverbeds and on the coast between tides (Metcalf et al., 2012). The samples were analyzed for AChE inhibition (70% to 98%), but the site is close to agricultural areas, and other anti-AChE compounds may have influenced the analysis. On the other hand, the samples were composed mainly of cyanobacteria so that the presence of GNT in this environment could not be discarded (Metcalf et al., 2012)

In the past, the identification of GNT was restricted to enzymatic tests of AChE inhibition. The diagnosis of intoxicated animals was very complicated due to the rapid action of the toxin followed by death. The deterioration of the samples in the tissues of the animals added with, and the absence of specific macroscopic or histological lesions made a diagnosis and proper treatment difficult (Cook et al., 1989a). The lack of specific methodologies for the collection, storage, and even extraction of samples collected in the field may have negatively influenced the non-detection of GNT in biological samples.

The deficiency of analytical methods for chemical detection of GNT in the period from 1980 to 2000 may have made it challenging to diagnose GNT in cases of intoxication *in vivo*, *in vitro*, and environmental samples. Most cases of GNT poisoning consisted of clinical signs that were characterized by excessive salivation (Mahmood and Carmichael, 1986). The diagnosis was generally confirmed by analysis of the stomach contents of animals composed of filamentous species of the genus *Dolichospermum* spp. and *in vitro* tests to assess AChE inhibition (Mahmood et al., 1988).

The excessive salivation observed in animals intoxicated by GNT in the field, and the laboratory was of the viscous and aqueous type, characteristic of cholinergic stimulation over salivary glands and digestive tract. Excessive salivation can initiate an acute dehydration process, leading the intoxicated individual to develop other symptoms, for example, polydipsia, observed in ducks treated with GNT (Cook et al., 1989a). The other symptoms,

such as tremors, convulsions, respiratory and cardiovascular failure, were caused by acetylcholine dysfunction, resulting in the inactivation of nicotinic receptors (in the autonomic ganglia and skeletal muscle) and muscarinic receptors (in the myocardium and smooth muscle) (Cook et al., 1989b).

The data showed that acute toxicity of GNT is the same for both animals intoxicated in the environment and the laboratory. However, the dose-response of GNT was different between the groups evaluated in the laboratory. The divergence among other published studies LD_{50} or LC_{50} is due to the non-standardization of the test substance (some studies used the pure toxin, others tested fractions with the semi-purified toxin, and others the crude extract of GNT-producing species). The performance of other biomolecules present in the crude extract and semi-purified sample must be considered (Cook et al., 1988; Yunes et al., 2003). Although the results presented have a significant contribution to the knowledge of the adverse effects of GNT, there is still a need to carry out new tests with the purified toxin in different organisms.

1.4.6 Treatment for animals intoxicated by Guanitoxin

The drugs atropine or atropine sulfate and pralidoxime (2-PAM) are used as antidotes for the treatment of anticholinesterase poisoning. Atropine acts as a muscarinic blocker in the central and peripheral system, acting as a minimizer of seizures caused by AChE blocking substances, while pralidoxime (2-PAM) acts in the reactivation of acetylcholinesterase (Jokanović, 2015).

Atropine sulfate therapy was used and evaluated in rodents exposed to GNT, showing increased survival and stabilization of parasympathomimetic effects in animals, but did not prevent death from the respiratory arrest of the animals (Mahmood and Carmichael, 1986). On the other hand, the pretreatment and prolonged dose with intravenous atropine in rats promoted survival by avoiding the death of animals exposed to GNT (Cook et al., 1990).

In vivo tests with AChE reactivators have shown that GNT can be resistant to reactivation of the oxime, due to the structure of its enzyme adduct (Hyde and Carmichael, 1991) (Figure 6). GNT binds to two regions of the AChE active site, causing changes in the conformation of the enzyme (Hyde and Carmichael, 1991). On the other hand, other studies have shown that the use of high concentrations of pyridine-2-aldoxima (2-PAM) was effective in the treatment of GNT DL_{50} (Cook et al., 1990; Hyde and Carmichael, 1991).

In the case of accidental *in vivo* intoxication by GNT ingestion, activated charcoal is recommended to assist in the absorption of the toxin and use of medications to control convulsive conditions (Khan, 2014). In a case study, a dog showed signs of neurotoxic after drinking water from a lake in the United States with filamentous cyanobacteria. The animal was panting and salivating excessively, and the body temperature was 39.7 °C. The animal also vomited cyanobacterial cells during medical care (Khan, 2014). The dog was submitted to treatment with different dosages of the drugs, atropine sulfate, diazepam, propofol, and phenobarbital after the animal treatment showed significant improvements.

The number of cases of cyanotoxin poisoning in dogs is quite (Chatziefthimiou et al., 2014; Faassen et al., 2012; Mahmood et al., 1988). On the other hand, the LD_{50} for dogs is not yet known (Khan, 2014). Generally, the diagnosis is realized according to the previous symptoms and analyzes of the animal's stomach contents. There is still no approved therapy to treat animals and humans affected by GNT. However, the use of anticonvulsant drugs and with effects on parasympathetic systems has proved to be an alternative to control and reverse the effects caused by GNT.

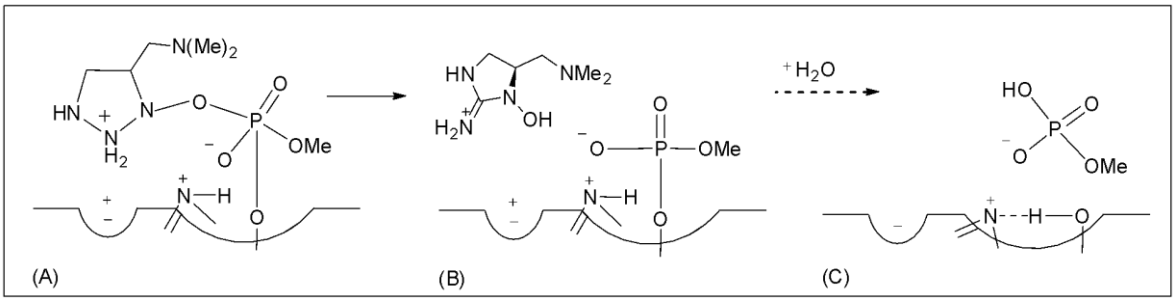

Figure 6: Proposed hydrolysis of Guanitoxin adapted from Hyde and Charmachael (1991).A: Intermediate enzyme-inhibitor, B: Phosphorylated enzyme and C: The adduct formed by GNT with hydroxyl serine resembling an aged organophosphate.

1.4.7 Guanitoxin identification through the inhibition of acetylcholinesterase

After GNT was characterized as an AChE inhibitor, the amperometric method proposed by Ellman et al. (1961) became an alternative to identify and quantify GNT in biological samples. The method consists on the stoichiometric reaction between GNT and cholinesterase enzymes AChE and butyrylcholinesterase (BuChE) (Mahmood and Carmichael, 1986; Mahmood and Carmichael, 1987; Villatte et al., 2002). When AChE is hydrolyzed, thiocoline is released, which is measured by reaction with 5,5'-dithio bis (2-nitrobenzoic acid) (DTNB), producing a yellow color with absorbance at 412 nm (Ellman et al., 1961).

However, colorimetric biosensors are considered methods of low sensitivity and low specificity and can generate false positive results (Devic et al., 2002). The fact that GNT is a natural organophosphate would probably be confused with synthetic organophosphates present in the environment. In the study by Devic et al. (2002), they optimized a method using a set of AChE mutants obtained in *Drosophila* ssp. to improve the specificity and sensitivity of GNT. Devic et al. (2002), used an electrochemical biosensor used to monitor organophosphates in environmental samples (Bachmann et al., 2000), obtaining AChE more sensitive to GNT.

Devic et al. (2002) used a GNT-producing strain as a positive control and compared it with other strains *Dolichospermum* spp. collected from several freshwater lakes in Europe that had cyanobacterial blooms, with AChE inhibition being observed in all analyzed samples. GNT is among the most toxic organophosphate compounds, since AChE inhibition rates were higher than some pesticides (Devic et al. 2002).

The use of biochemical biomarkers for environmental risk assessment is advantageous only to indicate the presence or absence of specific groups of contaminants in aquatic environments (Adams, 2001). In the case of GNT, its concentration in biological samples is estimated by methods that assess AChE inhibition. This method can be applied as a tool for initial tests aimed at determining the presence of GNT in samples of cyanobacteria.

1.4.8 Guanitoxin extraction, purification and identification methods

Since the discovery of GNT in 1978, several studies have been carried out to describe the chemical nature and physiological processes involved by GNT (Cook et al., 1988; Hyde and Carmichael, 1991; Mahmood and Carmichael, 1986; Matsunaga et al., 1989). The first method of GNT extraction, separation and isolation was carried out using lyophilized cells from the NCR 525-17 strain grown with BG-11 medium (Mahmood and Carmichael, 1986)

GNT extraction was performed first by mixing ethanol:water (80:20 v/v) with 1.0 M acetic acid, followed by filtration on gel resin (Sephadex G-15) with 0.1 M acetic acid (Mahmood and Carmichael, 1986). Then, the samples were subjected to solid phase extraction (SPE-Sep-Pak C18) followed by another SPE with weak cation exchange (CM Sephadex C-25) (Mahmood and Carmichael, 1986). The SPE process elutions were performed with water and different concentrations of acetic acid (Mahmood and Carmichael, 1986). The purification of the GNT was completed on a semi-preparative HPLC in reverse

phase with a cyanopropyl (CN) column (9.8 x 250 mm), with the peak of the GNT being collected at 220-230nm UV (Mahmood and Carmichael, 1986).

The method developed by Mahmood and Carmichael (1986) has been used in several studies to confirm GNT in samples of cyanobacterial blooms. However, small adaptations were made using the method proposed by Mahmood and Carmichael (1986), such as column size, flow and mobile phases aiming at a better analysis performance (Mahmood et al., 1988; Mahmood and Carmichael, 1987; Matsunaga et al., 1989; Onodera et al., 1997)

The structural elucidation of the GNT molecule occurred 8 years after the date of description of GNT as a new cyanotoxin. Previously, several pharmacological studies, analysis of taxonomic description of producer species and purification tests by SPE and HPLC were carried out. The molecular structure, as well as its chemical characteristics, were published in 1989 by researchers who were closely linked in the study of this toxin (Matsunaga et al., 1989).

To isolate the GNT, the culture of the NRC 525-17 strain was added 50% of ^{13}C and 90% of ^{15}N. The toxin extraction followed the method initially proposed by Mahmood and Carmichael (1986) with new adaptations; the lyophilized cells of the NRC525-17 strain were extracted with 0.05N AcOH/EtOH (Matsunaga et al., 1989). Subsequently, a liquid-liquid extraction (LLE) was performed with the filtered extract supernatant, adding water and dichloromethane, where the aqueous phase was washed with n-BuOH and evaporated *in vacuo*, and resuspended in 0.05 N AcOH/MeOH and 0.05 N AcOH/EtOH to concentrate the toxin (Matsunaga et al., 1989).

Then, solid-phase extraction (SPE) was performed on TOYOPEARL HW-40S resin (Supelco), and the purification process was carried out on semi-preparative HPLC with reverse-phase column CN and octadecylsilyl (ODS) (Henriksen et al., 1997; Matsunaga et al., 1989; Onodera et al., 1997). After the isolation of the toxin, tests were carried out to evaluate the anti-AChE activity promoted by GNT (Matsunaga et al., 1989).

The structural elucidation of the GNT molecule occurred through analyzes by Fast Atom Bombardment Mass Spectrometry (FABMS) and Nuclear Magnetic Resonance (NMR) in positive mode *m/z* 253 $[M + H]^+$ and negative *m/z* 251 $[M-H]^-$ resulting in the molecular formula of $C_7H_{17}N_4O_4P$, with maximum absorption at λmax= 220-230nm (Mahmood and Carmichael, 1986; Matsunaga et al., 1989).

GNT is the only methyl phosphate ester of an *N*-hydroxyguanidine produced by cyanobacteria with cholinesterase inactivation induced by the phosphate group (Matsunaga et al., 1989; Moura and Pinto, 2010). It is a polar molecule capable of decomposing slowly

at -20 °C and rapidly in alkaline solutions at temperatures >40 °C (Barros et al., 2004; Cook et al., 1988; Mahmood and Carmichael, 1986; Matsunaga et al., 1989). The GNT degradation compound was proposed by Matsunaga et al. (1989) being synthesized in four stages from D and L-asparagine, resulting in Da 158 (4S-2-imino-4 (dimethyl aminomethyl) -imidazolidine) (Figure 7).

After a decade, Dorr et al. (2010) developed a method of identifying GNT LC-MS with collision-induced dissociation (CID) using hydrophilic interaction liquid chromatography (HILIC) using a Zwitterionic (ZIC) column. The method was developed using strains producing GNT PH-160B (*D. lemmermanii*) and ITEP-025, ITEP-026, and ITEP-024 (*S. torques reginae*) proposing the fragmentation profile of GNT through transitions *m/z* 253> 58, 253> 159, 235> 98 and 235> 96 $[M+H]^+$ (Dörr et al., 2010).

After the isolation of GNT by Matsunaga et al. (1989), only one study was published on the purification of toxins, with adaptations in the original methodology (Onodera et al., 1997). The separation of the toxin by semi-preparative HPLC with an absorbance of the CN column at maximum= 220-230 nm, precedes a series of other procedures that are difficult to reproduce. In addition, the GNT chromophore group does not appear to be strong enough to be detected on HPLC-DAD systems. Some solvents used in the mobile HPLC phases, thus increasing the signal noise in the analyses (Devic et al., 2002; Dörr et al., 2010), can absorb the 220-230 nm wavelength used to detect GNT.

Another fact that also justifies the difficulty of purifying and isolating GNT is a large amount of biomass needed to perform all procedures. In tests with the ITEP-24 strain, low production of the toxin was observed when compared to other secondary metabolites (unpublished data). GNT can degrade during the purification processes, making the yield quite low (Hyde and Carmichael, 1991). The sum of these factors may be strongly linked to the lack of an analytical standard for GNT.

Currently, the LC/MS method with ZIC-HILIC is the most used to identify the GNT in aqueous samples, and the selectivity and sensitivity of the method provide to identify the GNT with precision (Dörr et al., 2010; dos Santos et al., 2019). Luna C18 reverse phase columns that promote hydrophobic interactions may work well in LC/MS analysis to detect GNT (Dörr et al., 2010). The identification of GNT *m/z* by LC/MS has been an excellent tool for monitoring GNT in water bodies. However, it is necessary to develop studies that aim to isolate the GNT to produce the analytical standard.

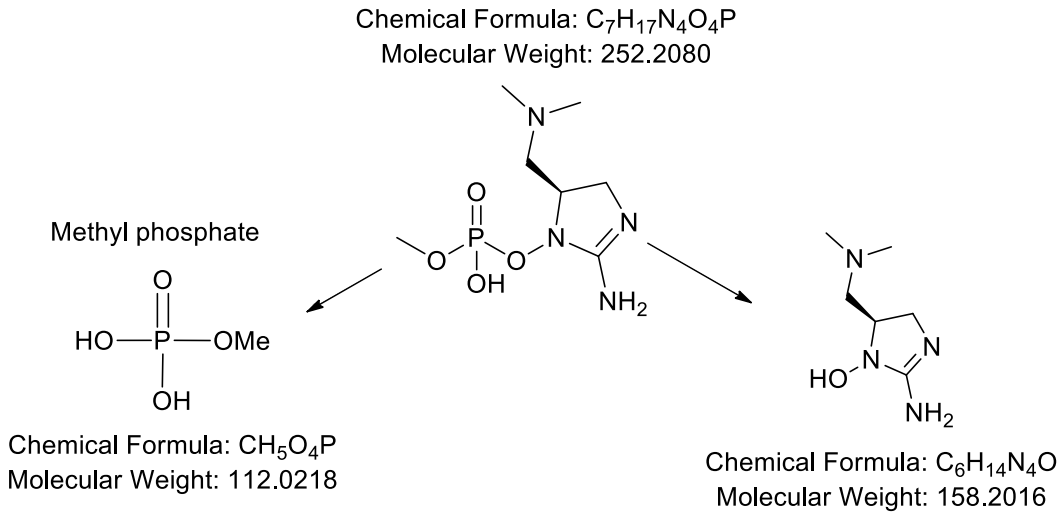

Figure 7: Scheme referring to the degradation of guanitoxin, in which there is loss of the methyl phosphate group and formation of the compound Da 158.

1.4.9 Guanitoxin biosynthesis

GNT biosynthesis was first proposed by Moore et al. (1992) through NMR analysis and addition of radiolabeled precursors ([metil-^{14}C]-L-methionine, [2-^{14}C]-glycine, [U-^{14}C]-serine, [guanido-^{14}C]-L-arginine, [U-^{14}C]-L-arginine, [U-^{14}C]-L-ornithine, [4,5-3H]-L-arginine, [1,2-^{14}C]- sodium acetate and [U-^{13}C]-L-arginine) to NRC 525-17 strain cultures producing GNT in an attempt to explain the origin of the carbon present in the GNT molecule. They then proposed that the C-2, C-4, C-5 and C-6 carbons of GNT are derived from guanido carbon, C-5, C-4 e C-3 de [guanido-14C]-L-arginine, and the three O, N-methyl groups originate from the tetrahydrofolate pool C1 and suggesting that erythro-4-hydroxy-1-arginine could be a precursor of biosynthesis (Moore et al., 1992).

Still trying to understand better the pathway of GNT biosynthesis Hemscheidt et al. (1995) proposed that 4-hydroxyarginine would be an intermediate compound in the cyclization of L-arginine and suggested that ring closure present in the molecule could be via bimolecular nucleophilic substitution (SN2). After, Shimizu (1996) proposes a discussion on the biosynthetic mechanisms of toxic metabolites produced by cyanobacterial species and, in this case, a new biosynthesis pathway for GNT from unusual arginine catabolism by a retroClaisen condensation mechanism has been suggested, where arginine loses the part where glycine is present (Figure 8).

Subsequent, Moura and Pinto (2010) proposed a biosynthesis for GNT through cyclic guanidine in racemic and chiral form and obtained some analog compounds from this

procedure. The synthesis of cyclic *N*-hydroxyguanidine showed two different forms of the route starting from the amino acid asparagine and another racemic form using benzyl alcohol and further suggested that *N*-hydroxyguanidine synthesis could be used as an internal standard to quantify the toxin since there is no commercial standard for quantifying GNT (Moura and Pinto, 2010) (Figure 9).

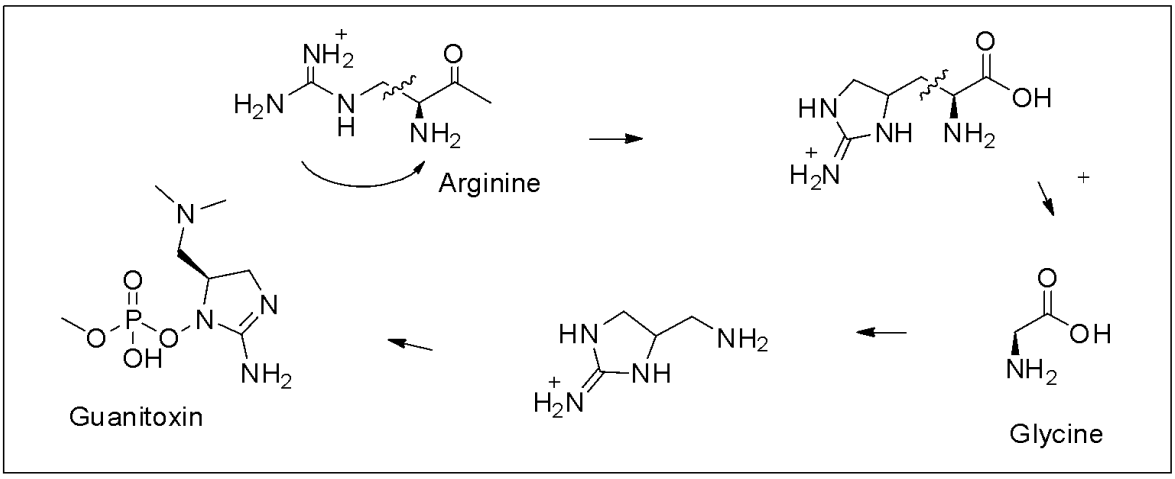

Figure 8: Guanitoxin biosynthesis pathway adapted from SHIMIZU (1996).

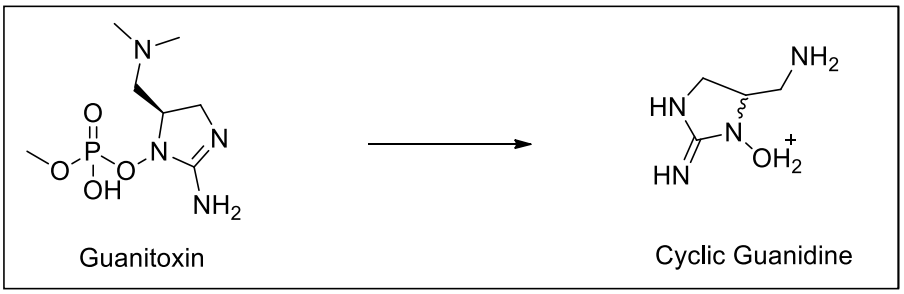

Figure 9: Cyclic guanidine for the total synthesis of guanitoxin adapted from Moura and Pinto (2010).

1.5 FINAL CONSIDERATIONS

The data available in the literature showed a significant number of studies on the diverse effects of GNT *in vivo* and *in vitro* and cases of animal poisoning by GNT in the natural environment. GNT is among the most toxic cyanotoxins and can occur naturally in eutrophic environments. In this way, monitoring of GNT by environmental control agencies can reduce impacts on water quality and prevent the accidental death of aquatic and terrestrial animals. In addition, the standardization of methodologies and the development of a GNT

analytical standard would guarantee the identification of GNT in the environment and biological samples.

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Chapter 2: Evaluation of guanitoxin extraction protocols in *Sphaerospermopsis torques-reginae* samples (ITEP-24)

ABSTRACT

In this study, we evaluated different guanitoxin (GNT) extraction tests in aqueous samples of the ITEP-24 strain. The identification of GNT was performed by LC-QqQ-MS/MS using hydrophilic interaction liquid chromatography (HILIC) in positive mode and multiple reaction monitoring (MRM). The initial extractions performed with 70% methanol and 0.3% acetic acid, followed by cell lysis in an ultrasound probe with a water bath, provided better results for the extraction of GNT (p-value <0.05). After optimizing the initial extraction of the GNT, we tested two methods of extraction in the solid phase (SPE): cartridge C18 in the reverse phase and a cartridge with gel silica in the normal phase. Statistical tests showed no differences between the two SPE methods (p-value > 0.05). In the SPE processes, there were losses of GNT compared to the initial sample (p-value <0.001); however, it possible to remove non-polar compounds and pigments present in the crude extract of the ITEP-24 strain. We adapted the SPE method (silica gel in a normal phase) for column chromatography (CC) to optimize the extraction time and minimize the use of organic solvents and the generation of solid waste, resulting in 1.2 mg of GNT semi-purified. We evaluated the stability of GNT after drying procedures at room temperature (23 °C) over nitrogen gas and resuspension in mobile phase B (80% ACN). The results showed that GNT could degrade rapidly compared to samples resuspended in H_2O with 0.3% acetic acid (-20 °C) and dried in a lyophilizer. Besides, we observe the gradual hydrolysis of the toxin contained in lyophilized cells stored at -20 °C for one year compared to freshly dried samples. Here, we tested three chromatographic methods with different LC-HR-QTOF-MS analytical columns as an option to detect GNT in water samples. However, the use of a ZIC-HILIC column we recommended because it provides better peak resolutions for polar compounds like GNT. The methods presented in this study can be useful for various applications aimed at identifying the toxin in environmental samples, as well as guidance on procedures for purifying GNT.

Keywords: sample preparation, multiple extraction, mass spectrometry, cyanotoxin.

2.1 INTRODUCTION

Guanitoxin (GNT) is a potent neurotoxin produced by planktonic cyanobacteria that occur in freshwater environments. The presence of GNT-producing strains in aquatic ecosystems can affect the quality of water for human consumption and the risks to the life of different aquatic and terrestrial species due to their high toxicity (Cook et al., 1989; Mahmood and Carmichael, 1986). However, monitoring this toxin in the environment remains a challenge that can be directly related to the methodologies applied for its detection. Therefore, studies of methodological bases are essential, as they guarantee the identification of substances that present risks to the environment and provide information that can be useful to guarantee environmental quality.

The advancement of analytical techniques with the coupling of Liquid Chromatography (LC) and Gas Chromatography (GC) to the Mass Spectrometer (MS) allows the rapid processing of samples and the accurate identification of toxic substances available in water, atmosphere, and soil (Andreu and Picó, 2004; Aráoz et al., 2005; Spoof et al., 2003). Therefore, LC/MS has become an essential tool for studies of monitoring cyanotoxins in water bodies (Zervou et al., 2017). However, to be successful in LC/MS analysis, it is necessary to develop appropriate methodologies for applications, from the collection of samples *in situ* to the laboratory analysis processes (Kaushik and Balasubramanian, 2013; Rippka, 1988; Zastepa et al., 2015).

The identification of toxins, as well as other secondary compounds of the cyanobacteria metabolites, is preceded by different extraction processes (Haque et al., 2017). The release of specific metabolites is generally impeded by the intrinsic cell wall rigidity of most species of cyanobacteria (Haque et al., 2017). Therefore, it is necessary to subject the cells to some stress, by either mechanical or chemical to promote the early release of intracellular metabolites(Cerasino et al., 2016; Wu et al., 2011). There are several extraction methods for natural products. However, generally, for initial extraction studies, organic solvents with properties compatible with the compound of interest are used, so that the extraction is carried out with the entire volume/concentration of the solvent to extract the compound of interest at once (Filho, 2018). Nevertheless, in particular studies, it is necessary to perform multiple extractions that involve two or more simple extractions (Filho, 2018).

Depending on the analyte properties and the type of samples, it is usually necessary to apply more than one type of extraction. In this sense, solid-phase extraction (SPE) is very versatile and can be applied in different scenarios, minimizing the cost of solvents and

reducing the extraction time. SPE has become a widely used laboratory technique for the concentration of toxins available in aqueous samples and complex extraction processes, aiming at the purification or isolation of bioactive compounds (Haque et al., 2017; Simpson, 2000). The SPE technique consists of the use of disposable absorbent cartridges filled with silica or polymers, in which the separation of the analyte occurs in eluents for which the compound has an affinity, allowing the fractionation of complex samples. This technique is very efficient for processing samples of dense blooms of cyanobacteria, for example, acting in the removal of interferents. Also, the SPE technique reduces the likelihood of peaks co-eluting with the analyte during LC/MS analyzes (Simpson, 2000).

Therefore, the identification of cyanotoxins in environmental samples or the isolation of active compounds requires planning and in-depth studies on the chemical and biological nature of the analyte in question. The lack of this knowledge can cause problems in the analysis and, consequently, interfere in the identification of compounds of environmental risk, as is the case of some cyanotoxins, mainly GNT, which present peculiarities of stability over in the temperature and pH variations. Therefore, our study proposed to test the extraction methods described in the literature to direct the most appropriate methodologies for the extraction and identification of GNT in biological samples.

2.2 MATERIAL AND METHODS

2.2.1 Reagents

All reagents used were of high purity. The salts used to prepare the ASM-1 medium were of analytical grade (Vetec, Rio de Janeiro, Brazil). The water used was of the ultra-pure Miliq-Q (Merck, Darmstadt, Germany). Ammonium formate, ammonium acetate, formic acid (Sigma-Aldrich, Darmstadt, Germany), acetic acid JT Baker (Phillipsburg, New York, USA). The solvents used in the LC/MS analyses were analytical grade obtained from JT Baker (Phillipsburg, New York, USA) and Merck (Sigma-Aldrich, Darmstadt, Germany).

2.2.2 Cultivation of the guanitoxin-producing strain *Sphaerospermopsis torques-reginae* (ITEP-24)

The experiments were conducted from the cyanobacterial strain *Sphaerospermopsis torques-reginae* (Komárek) Werner et al., (2012) strain ITEP-24, obtained from the

collection of cultures of the Toxins and Natural Laboratory Algae and Cyanobacteria Products (LTPNA) at the Faculty of Pharmaceutical Sciences from University of São Paulo (FCF-USP).The cultures were maintained at a pH of 7.5; a temperature of ± 24 °C; 12:12 photoperiod with a light intensity of 40µmol.fotons.m-^{2}.s^{-1} and continuous aeration. After 20 days, cultures were stopped and centrifuged (10.000 RPM, four ºC, for 10 minutes - condition used in all analyzes). The biomass was stored at -20 °C and then lyophilized.

2.2.3 Simple extraction of guanitoxin with water, ethanol, and methanol

According to the literature, GNT is soluble in water, ethanol, and methanol, so we started the extraction tests with these solvents to evaluate their efficiency in the initial extraction of GNT. We used 25mg lyophilized cells of the ITEP-24 strain in triplicate and extracted with ultrapure water, 20% ethanol, and 20% methanol, all solutions acidified with 0.3% acetic acid.

Then, the samples were sonicated in an Omni Sonic Ruptor 400 Homogenizer ultrasound probe (OMNI International, USA) with a 30% intensity for 5 minutes. Then, the samples were centrifuged and filtered through a 0.45-µm PVDF filter (Nova Analítica, São Paulo, BR).

2.2.4 Multiple extraction of guanitoxin with methanol

Still, in order to optimize the initial extraction of GNT in lyophilized cells, we use only methanol in different concentrations (20%, 50%, 70% and 100%) with 0.3% acetic acid. Extractions were performed with 10 mg of lyophilized cells in triplicate. In this step, we evaluate the intracellular release of GNT in three processes, using the same sample, that is, we reuse the pellet three times, adding the extraction solution and subjecting the samples to sonication and centrifugation according to item 2.2.2.

2.2.5 Methods of cell lysis

In this study, experiments were carried out to evaluate what would be the best method to release GNT from the dry cell of the ITEP-24 strain without compromising the concentration of the toxin in the intracellular medium. Five methods of cell lysis were chosen, which are usually in the routine of laboratories for cell lysis of several organisms. We

prepared the extracts with 10 mg of lyophilized cells and 70% of methanol acidified with 0.3% of acetic acid and submitted the samples to the following conditions/equipment:

- Cell lysis method in an ultrasonic homogenizer (CLMU), with an intensity of 30% in 5 minutes for three consecutive times with a total duration of 1 hour, (Omni Sonic Ruptor 400 OMNI International, USA).
- Cell lysis method in an ultrasonic homogenizer with a water bath (CLMUW), with a temperature of ± 15 °C duration of 1 hour, Elma E60 Emasonic (Nova Analitica, São Paulo, SP).
- Cell lysis method in the mixer (CLMM), temperature ± 10 °C, and stirring at 1400 RPM for 1h, Thermomixer comfort (Eppendorf, Hamburg, Germany).
- Cell lysis method on centrifugation (CLMC), at ±1 °C, 3.500 RPM, the samples were mixed at 15-minute intervals (4X) for 1 h, Centrifuge 5810R (Eppendorf, Hamburg, Germany).

To evaluate the efficiency of cell lysis methods, we used a sample of 70% methanol as a negative control. The extract was homogenized manually for about 1 minute and left for 1 hour. All samples from the respective cell lysis protocols were subjected to centrifugation and were filtered for analysis in an LC-QqQ-MS/MS system.

2.2.6 Comparison between guanitoxin extraction from fresh cells and lyophilized cells

After experiments to evaluate the most appropriate solvent to extract the GNT from the ITEP-24 strain, we evaluated the viability of intracellular extraction of GNT from conditioned lyophilized cells (-20 ° C for 1 year) and cells recently dried by lyophilization. For experiments on newly lyophilized cells, we use 20 ml of cultures, centrifuges, and lyophilizes. Then we weigh, resulting in a dry weight of 6.83 mg.

The same weight was used for lyophilized cells stored for 1 year. The experiments were carried out with six samples for both experiments, and then the samples were extracted with 1 ml of methanol/water and 0.3% acetic acid (70:30 v/v). The samples were submitted to an ultrasound probe with a water bath for 1 hour, and then they were centrifuged, filtered, and analyzed by LC-QqQ-MS/MS.

2.2.7 The pre-concentration of guanitoxin developed using the solid phase extraction (SPE) technique

Samples

We used 10 mg of lyophilized cells of the strain ITEP-24 (triplicate) extracted with 1 mL of 70% Methanol and 0.3% acetic acid in both experiments of SPE. The samples homogenized by ultrasound and centrifugation according to the conditions mentioned in item 2.2.2. The samples were dried over nitrogen at room temperature. However, for the cartridge experiment with silica in the normal phase, the samples were resuspended in 1 ml of 100% acidified methanol, while the samples prepared for C18 extraction in the reverse-phase were resuspended in 1 ml of MeOH/H_2O (10:90 v/v) acidified.

Normal phase SPE

We used syringes for SPE (Supleco Analytical, Bellefonte, USA) with a capacity of 3 ml. The SPE cartridge in the normal phase was prepared manually, using 500 mg of silica gel, 60Å pore with 230-400 mm particle (Sigma-Aldrich, Darmstadt, Germany), and inserts of polyethylene frits in the lower and upper part of the syringes (Supleco Analytical, Bellefonte, USA). The activation and conditioning of the SPE were carried out with 100% methanol (6 mL). Then, the cartridge was loaded with 1 ml of sample, and then washes were carried out with 100%, 70%, 50%, 20% methanol (3 ml), and H_20 with 0.3% acetic acid and 10mM acetate ammonium (6 mL).

Reverse phase SPE

Reverse phase SPE extractions were performed with a 3 mL cartridge, with C18 500 mg stationary phase, mean particle size 40 µm, mean pore diameter 60 Å (Waters, Milford, USA). After activating the SPE with 100% methanol (3mL) and conditioning with ultrapure H_2O (3 mL), 1 mL of sample was loaded. Elutions were followed with H_2O with 0.3% acetic acid and 10mM ammonium acetate (6mL), 20%, 50%, 70% and 100% methanol (3 mL).

Eluate Post-treatment - Normal and Reverse-Phase SPE

The fractions resulting from washing with H_2O, 0.3% and acetic acid and 10mM of ammonium acetate obtained from both SPE experiments were conditioned (-20 °C) and then lyophilized. The samples were resuspended in 1mL of acetonitrile/water (80:20 v/v) acidified with 0.01% formic acid and 5mM ammonium formate (Mobile Phase B). Then the samples were filtered through a 0.22 μm polyvinylidene fluoride (PVDF) filter (Millipore) and analyzed by LC-QqQ-MS/MS.

2.2.8 Column chromatography

After the experiment with SPE cartridges, we adapted the SPE method in the normal phase for column chromatography (CC), using a glass column of 18.75 x 2.00 cm. The column was packing with 10 g of silica gel using cotton at the bottom and top of the column.

The extracts were prepared with 0.300 mg of lyophilized cells, and 70% acidified methanol (30 mL). The samples were subjected to cell lysis for 1 hour in an ultrasound with a water bath and then centrifuged.

After activating and conditioning the CC with methanol 100%, the sample was applied in the column, and then washes were performed with 30 mL of 100%, 70%, 50%, 20% and 60mL of H_2O, with 0.3 % acetic acid and 10mM ammonium acetate. The fractions with water were conditioned to (-20 °C) and then lyophilized to concentrate the toxin.

2.2.9 Drying test of pre-concentrated samples with GNT

The samples obtained from the SPE extractions were frozen and then lyophilized to concentrate the toxin and dry compound storage. Then, the dry material was resuspended in 1 ml of mobile phase B for LC/MS analysis. After that, the samples were dried again, but on nitrogen gas at room temperature (23 °C). Samples resuspended in mobile phase B and analyzed. We repeated this drying procedure on nitrogen twice in a row, in order to assess the losses and degradation of the toxin.

2.2.10 LC-QqQ-MS/MS Analyses

The samples referring to all extraction protocols were analyzed by high-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (HPLC-QqQ) Agilent 6460 (Agilent Technologies, Santa Clara, USA) with ionization by electrospray (ESI), in positive mode 3500V. Nitrogen was used as the gas nebulizer (45 psi) and drying gas (5 mL/min at 300 °C).

The separation of compounds was performed on hydrophilic chromatographic column ZIC-HILIC, 150 x 2.0mm, 5µm (Merck, Darmstadt, Germany). The mobile phases consisted of A) water containing 10 mM ammonium formate and 0.04% formic acid and B) acetonitrile/water (80:20 v/v), containing 5 mM ammonium formate 0.01% formic acid (Dörr et al., 2010). The injections were of 5 µL, and the chromatographic separation was carried out in a linear gradient with a flow of 0.150 mL/min.

The gradient started with 90% to (B) in 10 min., and then maintained at 40% (B) 10-12 min, increasing to 90% (B) in 12-12.5 min. maintaining 90% (B) up to 20 min. The results were obtained by multiple reaction monitoring (MRM) and the identification of the GNT was performed by the retention time, *m/z* 253 (PM of [GNT + H^+]) and the quantifier and qualifier ions $[M + H]^+$ *m/z* 253> 58 and *m/z* 253> 159. Data analysis was performed using Software Qualitative Analysis B06.00 (Agilent Technologies, Santa Clara, USA).

2.2.11 Statistical Analysis

The statistical and graphical tests were performed using the GraphPad Prism software version 7.0. The differences between the data, the variance test (ANOVA) was first applied, followed by the Tukey test for multiple comparisons of the means. The confidence level adopted in the analyzes was 95%, results with a *p*-value $\leq$ than 0.05 were considered significant.

2.2.12 LC-HR-QTOF-MS Analyses

The following experiments were carried out using different chromatographic methods with different analytical columns in order to evaluate the efficiency of these methods on the

correct identification of GNT. The identification of GNT was performed from dry cells (10 mg) of the strain ITEP-24 extracted 1 ml of 70 % acidified methanol pH=3.0.

Equipment

The identification of GNT was performed in an ultra-high performance liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to a high resolution mass spectrometer and hybrid quadrupole flight time (LC-HR-QTOF-MS) (MicroTOF- QII, Bruker Daltonics Corporation, Germany) equipped with an electrospray ionization (ESI) in positive mode.

C18 Column

The data were obtained under the following conditions: ionization source with 4000 V capillary, potential, nitrogen was used as nebulizer gas (40 psi), and drying gas (6 ml/min at 220 °C). Mass spectra were obtained using electrospray ionization in the range of *m/z* 50 to 1500 with a collision energy of 20 to 50 eV to induce maximum ion fragmentation.

The separation of the compounds was performed using a Luna C18 column, 5 μm 100 Å, LC column 150 x 4.6 mm (Phenomenex, Torrance, CA, USA), under a linear gradient. Where the mobile phases are composed of A) water with 0.1% formic acid and B) acetonitrile/water (90:10 v/v) containing 0.1% formic acid, under a flow of 0.300 ml/min. The gradient started in 0-2 min. with 10% (B), then 2-10 min for 95% (B) and 10-13 min with 95% (B). The sample extracts were injected in 4 μL to obtain the data.

C18 Column Hydro-RP

The following chromatographic method was carried out according to the protocol of Geraldes et al. (2020), following the parameters: ionization source with 4500 V capillary potential, drying temperature nitrogen gas (9mL/min. and 200 °C), a nebulizer pressure of 60 psi. Mass spectra were acquired using ESI in the positive in a range of *m/z* 50 to 500 with a collision energy of 35 to 52.5 eV.

The separation of the compounds in the crude extracts of the ITEP-24 strain was carried out with a Synergi Hydro-RP column, 4μm, 80Å, 150 × 2.0 mm (Phenomenex, Torrance, USA) using mobile phase A) H_2O with 0.1% formic acid and 2 mM ammonium format and mobile phase B) acetonitrile/water with 0.1% formic acid (80:20 v/v) (Geraldes

et al., 2020). The analytes were eluted at a flow rate of 0.4 mL/min. The gradient used to start with 100% (A) in 0-2 min. increasing to 95% (B) in 2-6min. where gradient 95 (B) was maintained for 4 min. until the end of the analysis. Sample extracts were injected 10 μL to obtain the data.

ZIC-HILIC Column

The following chromatographic method was carried out according to the protocol of Dorr et al. (2010) with adaptations, following the parameters: ionization source with 4000 V capillary potential, drying temperature nitrogen gas (6mL/min. at 200 °C), a nebulizer pressure of 35 psi. Mass spectra were acquired using ESI in the positive in a range of *m/z* 50 to 500 with a collision energy of 35 to 52.5 eV.

The separation of compounds was performed on hydrophilic chromatographic column ZIC-HILIC, 150 x 2.0mm, 5μm (Merck, Darmstadt, Germany) at a flow rate of 0.200 mL/min. The gradient started with 95% (B) up to 12 minutes, then was maintained at 40% (B) in 12-13.5 min., increasing to 95% (B) up to 20 min. Data were collected from *m/z* 50 to 800 with an acquisition rate of 1 Hz, and the variable number of ions was selected by auto MS/MS scan fragmentation with a cycle time of 2.5 seconds.

MS/MS data acquisition

The MS/MS data were acquired using the Bruker Compass Data Analysis 4.3 software (Bruker Daltonics Corporation, Bremen, Germany), allowing the acquisition of the molecular formula calculated from the experimental mass in [M + H]+ with error in ppm <5 and millisigma value (mSigma) <20. The chemical structure of the GNT and its respective characteristic ions were illustrated in version 12.0.2.10.7627 of the ChemDraw software.

2.3 RESULTS

2.3.1 Evaluation of guantoxin extraction methods by Analyzes were performed by liquid chromatography – triple quadrupole-tandem spectrometry (LC–QqQ-MS/MS)

The data represented in figures 10 to 15 were obtained through analyses in the LC/MS-QqQ, using the MRM method. The chromatographic separation was obtained by

Hydrophilic Interaction Chromatography (HILIC) of the reverse phase, with a column filled with a zwitterionic stationary phase (ZIC). The identification of the GNT was performed through the area and the peak intensity referring to the transitions $[M + H]^+$ *m/z* 253> 58 and *m/z* 253>159 $[M + H]^+$ detected between 7.0-8.0 minutes. The degradation product of GNT was monitored by the transition $[M + H]^+$ *m/z* 159>58 with a retention time between 14 and 15 minutes.

2.3.2 Extractions

Initially, simple extraction tests were performed with three solutions, 100% H_2O, 20% Methanol, 20% ethanol, all with 0.3% acetic acid. The results showed that a solution with 20% Methanol extracts the intracellular GNT better compared to the solution of 100% H_2O (*p*-value <0.05), but there was no significant difference between the solutions of 20% ethanol and 20 % methanol (*p*-value = 0.1) (Figure 10). The treatment with 20% ethanol and 100% H_20 were statistically equal in the extraction of GNT; there was no difference between these two treatments. In general, the graph shows better results for extraction with 20% methanol even if there is no statistical difference compared to treatment with 20% ethanol, but we consider the methanol solvent to perform GNT extractions from ITEP-24 strain cells.

After the results obtained from the initial extractions (Figure 10), multiple extractions were performed with different concentrations of methanol. The sum of the extractions performed 50%, 70%, 100% were statistically equal and showed significant differences compared to the extractions performed with H_2O and 20% methanol (*p*-value <0.05) (Figure 11). But, the sum of the extractions of the treatment with Methanol 70% made it possible to extract a higher relative concentration of intracellular GNT compared to the other treatments (Figure 11) and therefore, we carried out the next experiments using 70% methanol with 0.3% acidic acid pH = 3.0.

In addition to evaluating the efficiency of different solvents in the extraction of GNT, we tested the feasibility of extractions from fresh, freeze-dried cells and dry cells that were stored in a freezer -20 °C for 1 year (Figure 12). These experiments aimed to evaluate the degradation of intracellular GNT in samples stored at -20 °C for long periods. That is why we used lyophilized cells from an old sample and a "fresh" sample from cultures of the ITEP-24 strain that were kept under the same conditions. The results that the relative concentration of GNT obtained from recently lyophilized (fresh) cells was higher compared to the extractions performed from dry samples (*p*-value <0.0001).

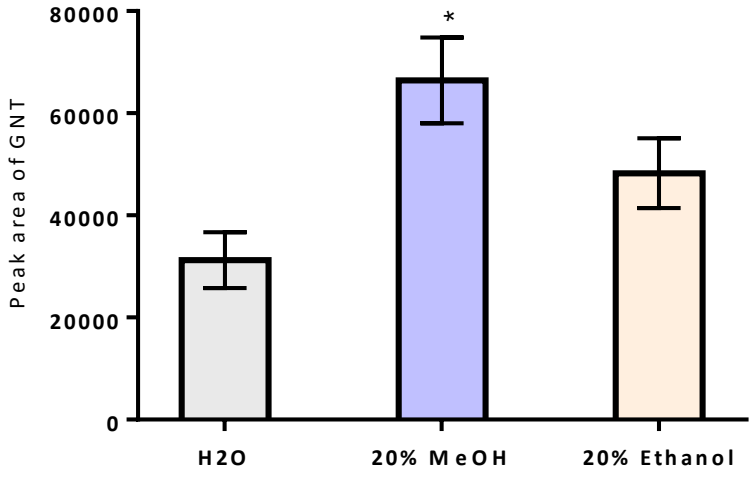

Figure 10: Simple extractions performed to extract the GNT from lyophilized cells of the ITEP-24 strain with H_2O, 20% methanol and 20% ethanol acidified with 0.3% acetic acid. The results show that extractions performed with 20% methanol extracted higher concentrations of intracellular GNT compared to extractions performed with H_20 (*p*-value <0.05).

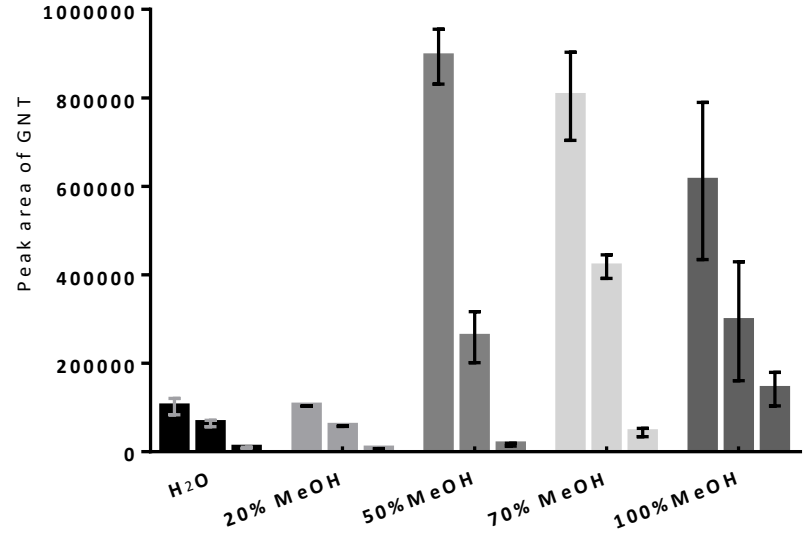

Figure 11: Multiple extractions performed from lyophilized cells of the strain ITEP-24 extracted with different concentrations of Methanol with 0.3% acetic acid. The sum of the extractions performed with 50%, 70% and 100% were statically the same and different compared with the solutions of 20% Methanol and H_2O (*p*-value <0.05).

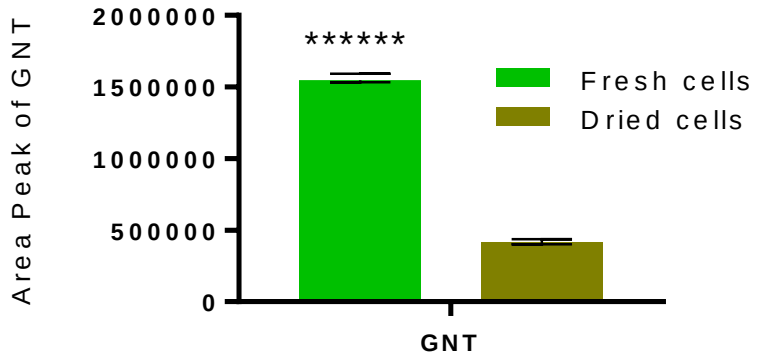

Figure 12: GNT extraction performed with lyophilized cells stored for 1 year (-20 °C) and fresh cells that were lyophilized and immediately analyzed. The results show that extractions performed directly from fresh cells are statistically significant at (*p*-value <0.0001).

2.3.3 Cell lysis

Figure 13 shows the results of cell lysis methods, the protocols of CLMC and CLMUB were better in the extraction of GNT compared with control (*p*-value <0.05). The control (70% Methanol) sample was used as a reference to evaluate the efficiency of the equipment used to promote the intracellular release of GNT. Nevertheless, all methods help to break the cell and can be entirely used in laboratory routines; however, the results shown here are to optimize the extraction of GNT in the best conditions.

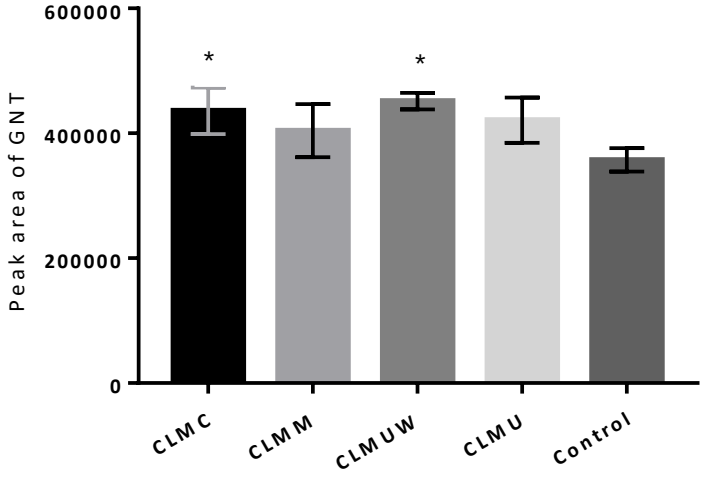

Figure 13: Cell lysis methods used to extract GNT. Centrifugation (CLMC), mixer (CLMM), water bath with an ultrasound probe (CLMUW), ultrasound probe (CLMU), and negative control extract in Methanol 70% with 0.3% acetic acid (without lysis). The results showed that CLMC and CLMUW are statistically different from the control sample (*p*-value<0.05)

2.3.4 SPE extractions

After completing the initial extraction tests, we proceed to SPE extractions, to eliminate pigments and hydrophobic compounds and thus obtain a fraction concentrated in GNT. We tested the extraction with cartridge C18 and cartridge with silica gel. The results show that there is no statistical difference between the two methods (Figure 14).

However, extraction in the normal phase with silica gel extracts a higher concentration of the toxin and therefore we use this metadata to concentrate the GNT. Significant losses of GNT were observed concerning the initial sample (Control); however, the losses were higher in the C18 test (Figure 14). Regarding the experiments using CC, 1.375 g of dry biomass was used, resulting in 1.2 mg of the semipurified compound.

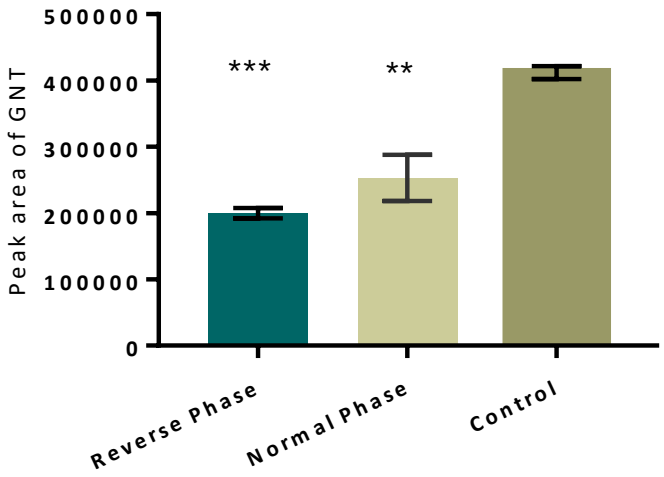

Figure 14: Results show that there are losses of GNT in both SPE tests compared to the control sample. However, extraction with silica gel in a normal phase obtained fewer losses of GNT during the extraction process compared to SPE in the reverse phase.

2.3.5 Drying test

After the SPE tests, the samples eluted in H_2O were frozen and then lyophilized for the GNT concentration. However, during laboratory experiments for drying and resuspending samples with GNT, we observed that GNT hydrolyzed after nitrogen gas drying procedures. Therefore, we decided to evaluate the GNT losses during the nitrogen gas drying procedures, in order to inform the possible problems during these procedures and alternatives to minimize the GNT losses during the molecular purification procedures.

Figure 15 shows the total ion chromatogram (TIC) in MRM mode using the transitions *m/z* 253> 58 $[M+H]^+$ and *m/z* 253> 159 $[M+H]^+$ to identify the GNT with a retention time of 7.5 min. Degradation product was monitored using the transition $[M+H]^+$ *m/z* 159> 58 with retention time in 13 min. The data presented as GNT I refers to the initial sample that had not yet been dried over nitrogen. The GNT II and GNT III samples refer to drying the initial sample 1x and 2x, respectively. The results show that drying over nitrogen influences the GNT hydrolysis. The chromatogram shows that as the concentration of GNT decreases, the peak for the product of its degreasing increases (Figure 15).

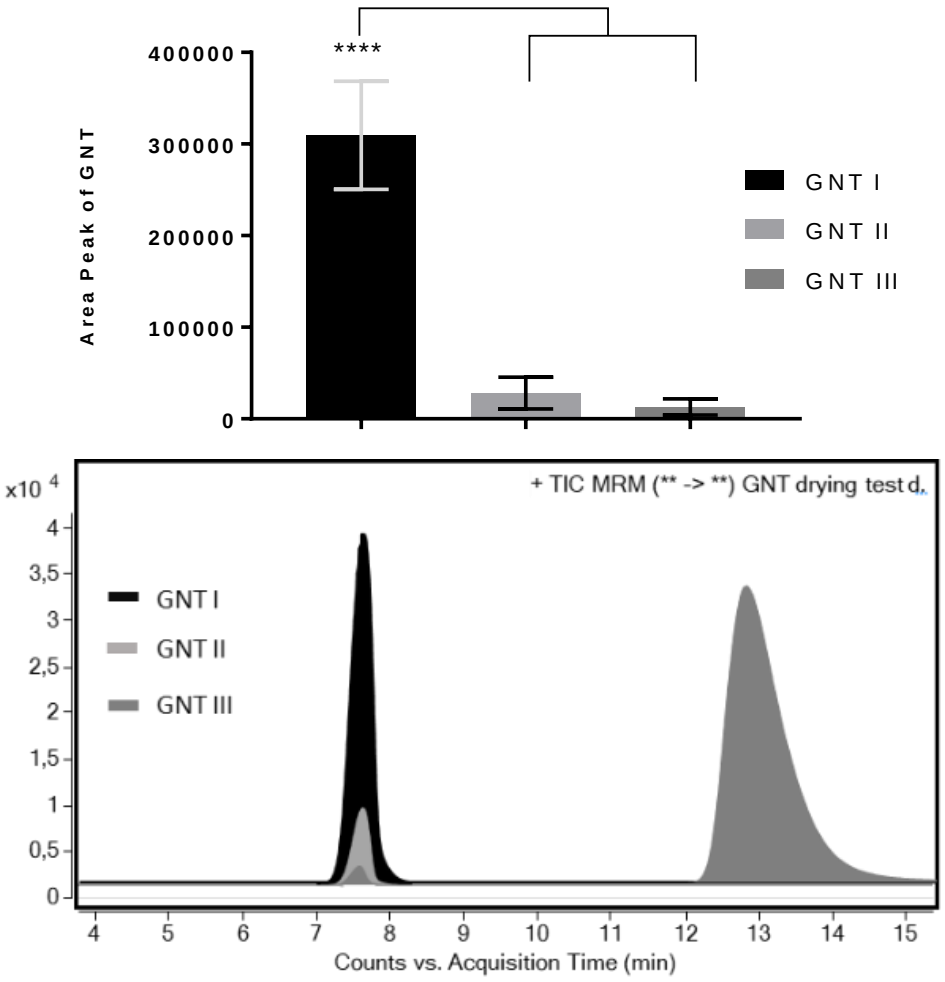

Figure 15: Sample-drying test with semi-purified GNT. The graph and chromatogram shows significant losses of the toxin after continuous drying processes under nitrogen gas at room temperature 23 °C. In the third drying step (GNT III), the product of degradation monitored by the transition *m/z* 159> 58 $[M + H]^+$ increases significantly concerning the initial sample (GNT I).

2.3.6 Guanitoxin identification using ultra-high performance liquid chromatography coupled to high resolution mass spectrometer (LC-HR-QTOF-MS)

We tested three different chromatographic methods to identify the GNT in aqueous samples. Samples of crude extract of the strain ITEP-24 extracted in 70% methanol with acetic acid at 0.3% pH 3.0. In all samples, it was possible to identify the toxin. However, we show the chromatograms that showed better resolution. In the method of column C18, GNT was identified at the retention time of 0.6 min. (Figure 16), in the chromatographic method of the Hydro-RP C18 column, the GNT retained in 1.1 min. (Figure 17) and the ZIC-HILIC method was detected in 7.7 minutes (Figure 18).

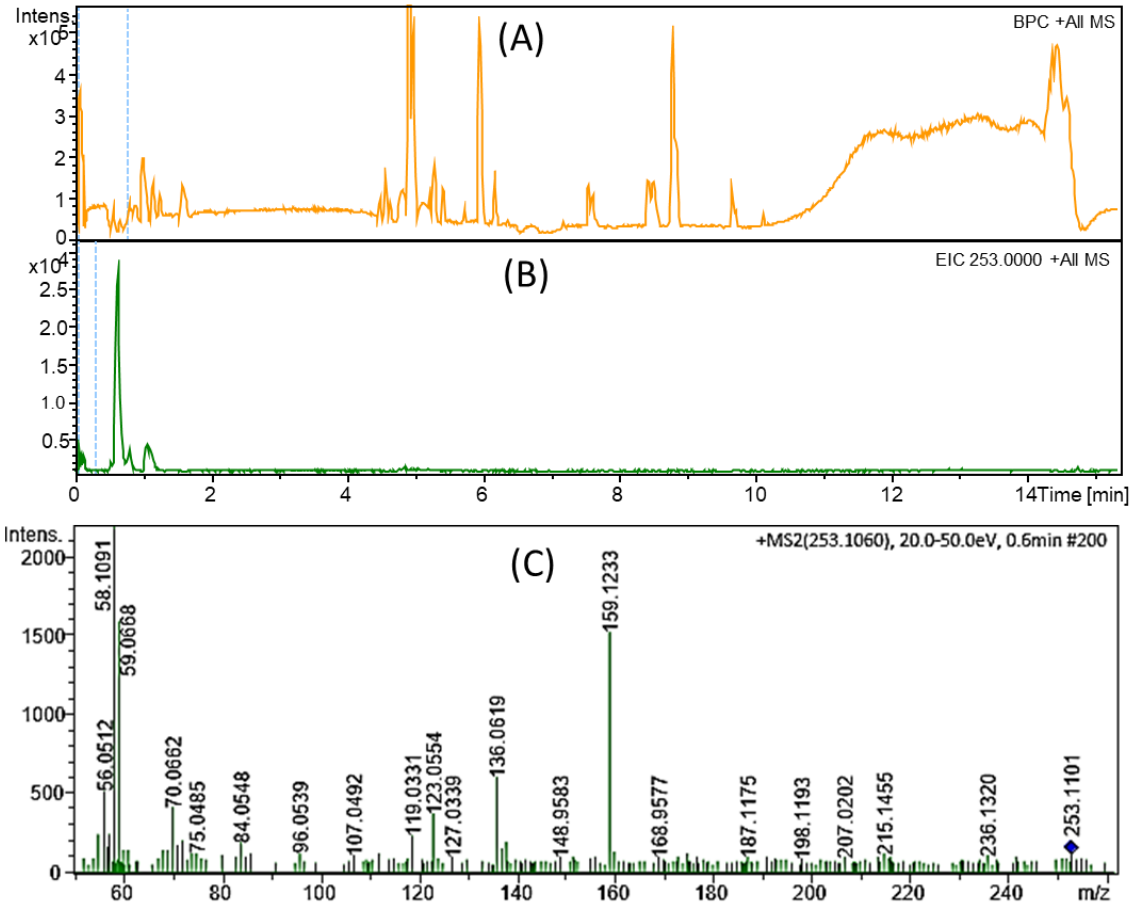

Figure 16: Chromatographic profile acquired by the chromatographic method with a C18 column crude extract from the ITEP-24 strain. Base Peak Chromatogram (A). The chromatogram of ions extracted from the molecular weight of GNT in 0.6 min (B). Mass spectrum of compound (MS2) *m/z* 253.1060 $[M+H]^+$ (C).

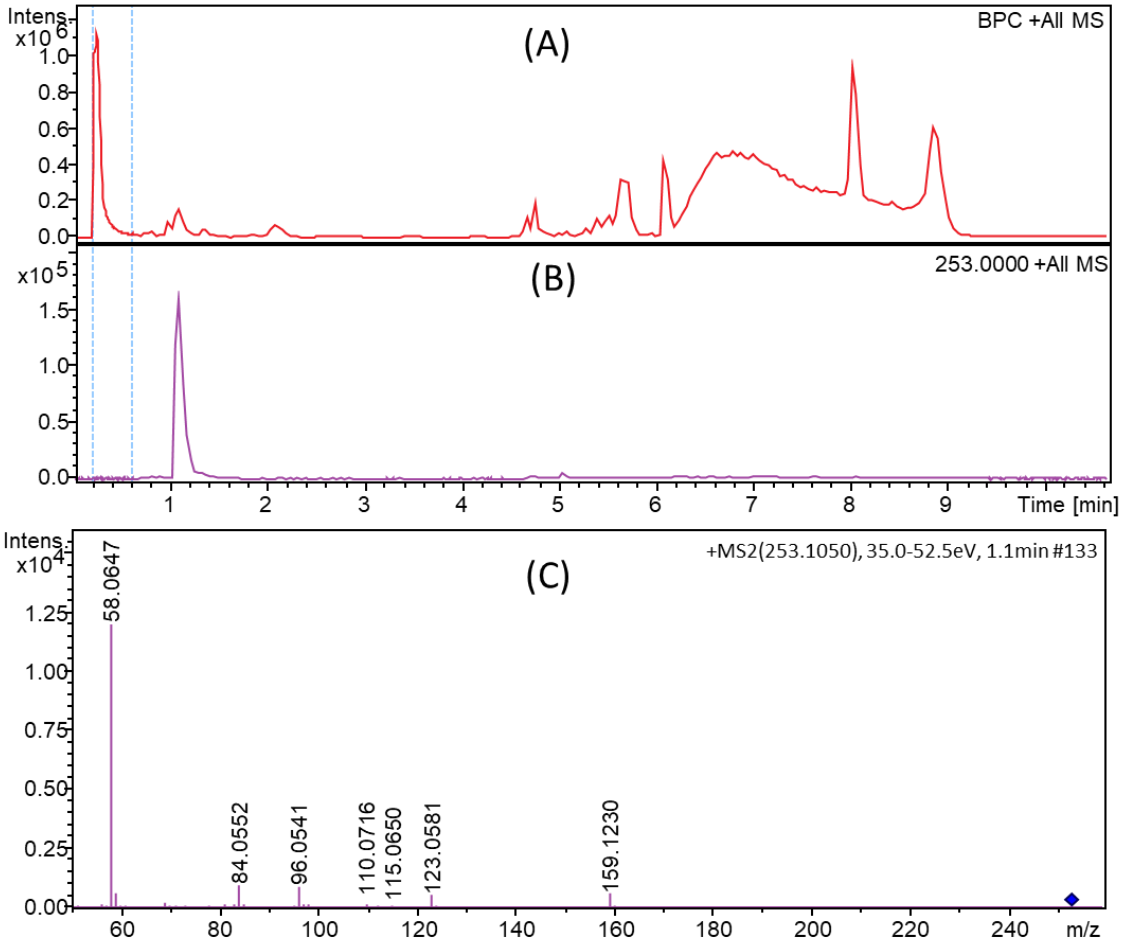

Figure 17: Chromatographic profile acquired by the chromatographic method with a Hydro-RP column crude extract from the ITEP-24 strain. Base Peak Chromatogram (A). The chromatogram of ions extracted from the molecular weight of GNT in 1.1 min (B). Mass spectrum of compound (MS2) *m/z* 253.1050 $[M + H]^+$ (C).

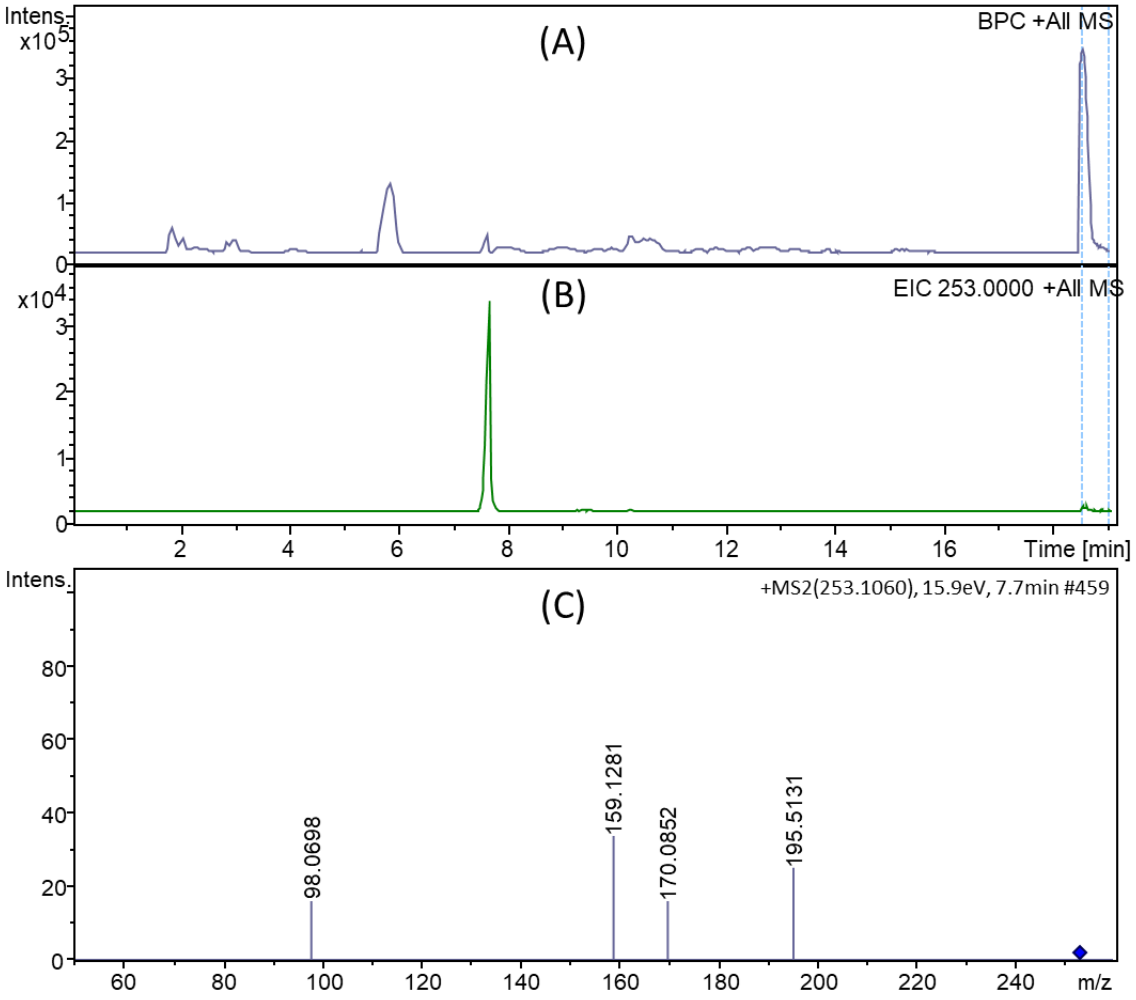

Figure 18: Chromatographic profile acquired by the chromatographic method with a ZIC-HILIC column crude extract from the ITEP-24 strain. Base Peak Chromatogram (A). The chromatogram of ions extracted from the molecular weight of GNT in 7.7 min (B). Mass spectrum of compound (MS2) *m/z* 253.1060 $[M + H]^+$ (C).

The mass and formula of the GNT molecule calculated in *m/z* 253.1060 $[M+H]^+$ ($C_7H_{18}N_4O_4P$) in all chromatographic methods (Figure 19). In the mass spectra (MS2), the ion *m/z* 58.0651 $[M+H]^+$ (C_3H_8N) and *m/z* 159.1240 $[M+H]^+$ ($C_6H_{15}N_4O$) were the most intense ions present in the MS2 chromatograms (Figure 19). It was possible to calculate the molecular formula of other ions that were more abundant in the MS2 spectra of the C18 and Hydro RP C18 column methods, such as *m/z* 70.0651 $[M+H]^+$ (C_4H_8N), *m/z* 72.0808 $[M+H]^+$ ($C_4H_{10}N$), *m/z* 84.0808 $[M+H]^+$ ($C_5H_{10}N$), *m/z* 96.0556 $[M+H]^+$ ($C_4H_6N_3$) and *m/z* 123.0553 $[M+H]^+$ ($C_6H_7N_2O$). However, ion *m/z* 110.0410 $[M+H]^+$ ($C_5H_8N_3$) appeared only in the MS2 spectrum of the Hydro RP C18 column chromatographic method, and ions *m/z* 98.0713 $[M+H]^+$ ($C_4H_8N_3$), *m/z* 170.0542 $[M+H]^+$ ($C_7H_{13}N_3P$) and *m/z* 195.5141 $[M+H]^+$ (formula

not calculated) appear exclusively in the fragmentation spectrum MS2 of the ZIC-HILIC column.

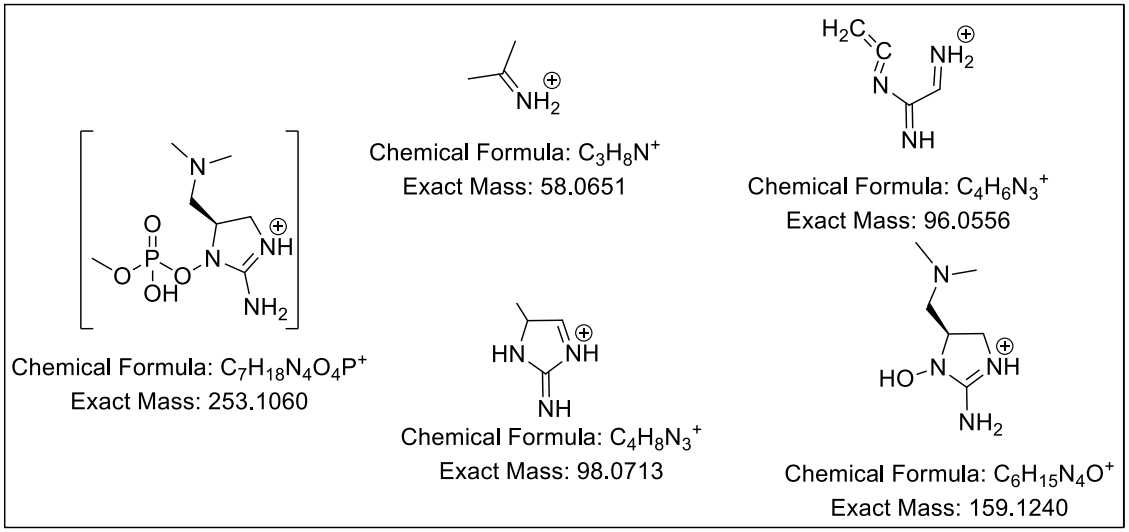

Figure 19: Molecular structure of the main diagnostic ions of GNT.

2.4 DISCUSSION

Most studies that reported intracellular GNT extraction used water and ethanol (20% to 80%) plus acetic acid (0.1 M to 0.5 N), followed by an ultrasound probe to promote cell lysis and, consequently, the release of toxins (Dörr et al., 2010; Mahmood and Carmichael, 1986; Shigeki Matsunaga Walter P. Niemczura, Wayne W. Carmichael et al., 1989). However, there are no comparative studies of GNT extractions with other solvents.

GNT is also soluble in methanol (Cook et al., 1988) and, according to the results of our study, the initial extraction with 20% methanol extracts the toxin more than 20% ethanol and water (Figure 10). We tested other concentrations of methanol (multiple extractions), in which the 70% acidified methanol solution extracts almost all-intracellular GNT (Figure 11). In addition, all extractions were performed in an acid medium to ensure the stability of the GNT molecule. Besides, the results of this study showed that GNT could gradually degrade in lyophilized cell samples of the ITEP-24 strain even when conditioned at low temperatures (Figure 12). Thus, for studies directed at isolating the toxin, it is recommended to use fresh cells or freshly lyophilized cells.

In addition to the use of solvent, we tested five techniques to promote the release of intracellular GNT. The treatments that used ultrasound probe in a water bath (CLMUW) and centrifugation (CLMC) showed better results when compared to control samples that

contained only 70% acidified methanol (Figure 13). Ultrasonic applications generate vibrations and mechanical stresses in the cell wall, promoting cell disruption (Safi et al., 2014). In the centrifugation method, the cells were rotated and subjected to a slight thermal shock, between the temperature of the centrifuge (1 °C) and the environment at 23 °C during the process. This procedure may have led to cellular lysis due to the formation of crystals in the cytoplasmic matrix (Safi et al., 2014) .

The SPE techniques adopted in this study, made it possible to exclude less polar pigments and compounds. However, during this procedure there were large losses of the toxin compared to the initial sample (Zervou et al., 2017). The first studies carried out for the semipurification of GNT consisted of SPE with C18 stationary phases, cationic exchange polymer based resin gel filtration and hydroxylated methacrylic polymer resin (Henriksen et al., 1997; Mahmood et al., 1988; Mahmood and Carmichael, 1986; Shigeki Matsunaga Walter P. Niemczura, Wayne W. Carmichael et al., 1989). However, most of these studies do not provide complete information on the yield and losses of the analyte during the process.

Here our study tested only two methods of SPE that are normally used for the concentration of cyanotoxin and other cyanobacterial metabolites. The SPE method consisting of stationary silica gel phase and normal phase showed better results for pre-purification of GNT (Figure 14). GNT is among the low-production compounds by the ITEP-24 strain, requiring much biomass to obtain sufficient concentrations for the isolation of the molecule. Therefore, it is recommended to test other SPE techniques with different stationary phases, to standardize the method of extraction of GNT by SPE and, thus, to minimize the losses of the toxin during the extraction and purification processes.

Analyte stability is another crucial parameter that must be studied before extractions. In the case of GNT, it is unstable in alkaline pH, so the extraction solution must be in acidic pH (Mahmood and Carmichael, 1987). Besides, other procedures need to be carefully studied before proceeding with the steps of extraction, purification, and isolation of molecules. Many variables can be strictly linked to the instability of a compound or its metabolites so that every process becomes essential to be successful in the analysis of samples by LC-MS/MS (Li et al., 2011). As it is a high-cost technique, procedures that precede the LC/MS analyzes such as in situ sample collection, processing, storage, extraction must be strictly considered to avoid enabling incorrect diagnosis of the compound (Li et al., 2011).

Our study showed that the concentration of GNT present in lyophilized cells of the strain ITEP-24 decays after long periods conditioned to -20 °C. GNT is unstable at high

temperatures and the conditioning (-20 °C) of the sample containing the toxin it did not prevent its degradation. This sample remained in the freezer for one year (-20 °C), and, during this period, it was used in other experiments, being exposed quickly to 23 °C (room temperature). This procedure of removing the sample from the freezer at room temperature has not been calculated (Mahmood and Carmichael, 1986). This process has been repeated many times, and this may have resulted in the gradual decomposition of the molecule. Temperature variations, even for short periods, may have generated extracellular exposure of the toxin caused by cellular stress. While the toxin is still within the cell *in vivo*, it is protected by cellular mechanisms that guarantee the stability of the molecule, as shown in our study, where high levels of the extracted toxin were obtained directly in the cell.

We also observed the decomposition of the semipurified toxin after drying and resuspending procedures, but we do not know exactly the causes of decomposition of the toxin (Figure 15). The drying and resuspension procedure itself may have caused losses of the toxin, but the temperature and humidity of the environment may also be related to the hydrolysis of the GNT. In this case, the development of analytical methods with internal standards (Li et al., 2011) could help to quantify the GNT and minimize uncertainties introduced in the analysis of their identification by LC/MS.

In this study, we also tested different chromatographic methods to identify GNT. The C18 column method was developed during this study to identify the GNT and other secondary metabolites produced by the ITEP-24 strain (Figure 16). In the method of column C18, GNT is identified at the beginning of the analysis (0.6 min.) similar results were also reported by Dörr et al., (2010). The ITEP-24 strain produces a variety of secondary metabolisms, but only a few have been described (Geraldes et al., n.d.; Lima et al., 2017; Sanz et al., 2017, 2015).

In the chromatographic method with the Hydro-RP column, the detection of GNT was successful. However, its detection was also at the beginning of the analysis (1.1 min.); but the peak showed an adequate resolution to detect GNT (Figure 17). The fact of limiting the molecular mass of the compounds to *m/z* 500 and injections with 10uL of the samples may have influenced the excellent resolution of the data, as in other methods, we used 5 µl of sample for the analyzes.

Chromatographic methods using C18 and Hydro-RP columns have full application in the screening of cyanotoxins and cyanopeptides, mainly for microcystin variants, being, therefore, an alternative to identify GNT in environmental samples. Among the tested chromatographic methods, the use of the ZIC-HILIC column is recommended, as it has an

excellent chromatographic resolution of the *m/z* relative peaks and toxin retention time (Figure 18) (Dörr et al., 2010). In the mass spectra (MS2) of the three chromatographic methods, it was possible to identify the characteristic ions of the GNT fragmentation (*m/z* 58, 98, 159 $[M+H]^+$) and other abundant ions that can be useful for the correct identification of GNT by LC/MS (Figure 19) (Dörr et al., 2010) .

The MRM method used with the ZIC-HILIC column in LC/MS-QqQ is an excellent tool for the rapid identification of GNT (Dörr et al., 2010). It is usually used as a quantify method, which consists of detecting specific ions of a molecule present in samples with other constituents, minimizing the matrix effect and cross-contamination (Yang and Herold, 2017). In this study we select the transitions *m/z* 253> 58 $[M+H]^+$, *m/z* 253> 159 $[M+H]^+$ to identify the GNT and *m/z* 159> 58 $[M+H]^+$ for the product of its degradation proposed in this study. The MRM method made it possible to identify GNT in the extraction, semi-purification, and hydrolysis samples of the toxin in a qualitative way, based on the area and intensity of the peaks. Therefore, the methods pointed out in this study can be strong allies in the identification of GNT.

2.5 CONCLUSIONS

The results of this study showed that multiple extractions with 70% acidified methanol extract higher relative concentrations of GNT in lyophilized cells with the aid of ultrasound in a water bath. Extraction techniques from the normal phase silica gel cartridge and the reverse phase C18 cartridge can be effective in concentrating the toxin and removing the initial sample of interference. In addition, we recommend performing extractions directly from fresh cells and successive drying and resuspension procedures in nitrogen gas at room temperature (23 °C), which causes the hydrolysis of the GNT molecule.

2.6 REFERENCES

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Chapter 3: Availability of Guanitoxin in water samples containing *Sphaerospermopsis torques-reginae* (ITEP-24) cells submitted to dissolution tests

ABSTRACT

Guanitoxin is a potent neurotoxin produced by freshwater cyanobacteria with a high incidence in the deaths of wild and domestic animals. Through reports of animal intoxication by cells of cyanobacteria that produce GNT, this study aimed to investigate the bioavailability of GNT in simulated solutions of the gastrointestinal content, to understand the process of toxicosis promoted by GNT *in vivo*. The dissolution tests were conducted with 30% culture of the ITEP-24 strain and 70% of gastrointestinal solutions with and without proteolytic enzymes, at a temperature of 37 °C and 100 RPM for 2 hours. The identification of GNT was performed by LC/MS-QqQ through the transitions *m/z* 253> 58 $[M + H]^+$ and *m/z* 253> 159 $[M + H]^+$ showing high concentrations of GNT in solutions of gastric content (*p*-value <0.001) in comparison with simulated solutions of intestinal content. The gastric solution with pepsin promoted more excellent stability of the GNT (*p*-value <0.05) compared to the simulated solution of gastric fluid at the same pH. Although the concentration of GNT has been much lower in trials with intestinal solution, the results show that GNT can also be absorbed in the intestine, and the presence of pancreatin can influence higher absorption.

Key words: toxicosis, neurotoxin, environmental monitoring, bioavailability.

3.1 INTRODUCTION

Guanitoxin (GNT) is a potent natural neurotoxin produced by freshwater cyanobacteria. Its mode of action is the same as synthetic organophosphates, in which the phosphate ester functional group binds to the active serine site of acetylcholinesterase (AChE), ultimately causing AChE block (Carmichael 1994). The result of the irreversible inactivation of AChE is the accumulation of acetylcholine in the synaptic clefts, thus causing cholinergic hyperstimulation that, in most cases, is lethal for organisms (Mahmood et al. 1988).

In the past, GNT has been associated with the death of domestic and wild animals that accidentally consumed water containing cyanobacterial cells (Mahmood et al. 1988; Henriksen et al. 1997). The clinical signs observed in the animals consisted mainly of excessive salivation, muscle tremors, convulsions, fasciculation convulsions, and respiratory failure. LD_{50} from GNT was determined in mice comprising a range of 20 µg/kg to 50 µg/kg with a survival time of 10 to 30 minutes, being considered ten times more toxic than other cyanotoxins of the same class (Mahmood and Carmichael 1987; Carmichael et al. 1990). Other LD_{50} and IC_{50} values were observed in fish, Cladocera, and insects, showing symptoms of intoxication common to those observed in mammals (Cook et al. 1989; Monserrat et al. 2001; Rodríguez et al. 2012; Abreu and Ferrao-Filho 2013; Freitas et al. 2016; dos Santos et al. 2019).

There are no known variants of GNT; it is known that species of the genus *Dolichospermum* spp. and *Sphaerospermopsis* spp is the producer of this cyanotoxin (Fiore et al. 2020). AChE has been used as a biomarker to assess the presence of the toxin in aqueous samples (Devic et al. 2002; Villatte et al. 2002). However, enzymatic methods can generate false-positive results and can be influenced by the presence of synthetic organophosphates available in the environment. For this reason, analytical methodologies by LC/MS are more indicated due to the high specificity and sensitivity that they provide for the correct identification of GNT (Dörr et al. 2010).

Although the presence of this toxin is less common than other cyanotoxins like microcystins, extremely high levels of GNT have already been detected in water samples (Henriksen et al. 1997; Sivonen 1999). Besides, there are recent reports of the occurrence of this toxin through cases of accidental poisoning in dogs that, after drinking water with cyanobacterial cells, showed clinical signs of acute intoxication characteristic of GNT (Metcalf et al. 2012). However, monitoring of GNT in bodies of water for human use is not

yet mandatory, and there are no limits for the detection of GNT established by the World Health Organization (WHO). The lack of consistent toxicological data and an analytical standard for quantifying GNT are the main factors that limit mandatory monitoring of GNT in water bodies (Villatte et al. 2002; Ibelings et al. 2014; Bartram et al. 2015).

Environmental factors also imply a lack of data on the occurrence of GNT in water bodies, such as the instability of the toxin at variations in high temperatures and slightly alkaline pH (Barros et al. 2004). However, there are contradictory results regarding the time of degradation of the molecule, and it is not known whether GNT has resistance to other chemical substances. On the other hand, the occurrence of GNT was reported in eutrophic environments with slightly alkaline pH and with of predominance of species producing GNT. There is no precise information on the toxin's half-life in the environment. However, even if the environment prevails for a short time, it can be sufficiently lethal depending on the available concentrations; it can cause severe impacts on aquatic and terrestrial biota.

Therefore, our work aimed to investigate the availability of GNT in simulated solutions of gastric and intestinal contents with digestive enzymes and without enzymes through *in vitro* tests, following guidelines established by the United States Pharmacopeia (USP) (Rockville 1995; Anand et al. 2011). From the dissolution tests, we hope to provide information's on the bioavailability of GNT in the gastrointestinal system *in vivo*, especially for wild and domestic animals that are generally the most affected by toxic cyanobacteria available in eutrophic environment.

3.2 MATERIAL AND METHODS

3.2.1 Cultivation of the *Sphaerospermopsis torques-reginae* (ITEP-24) strain producing Guanatoxin

The species *Sphaerospermopsis torques-reginae* from the strain ITEP-24 that produced GNT was cultivated in the ASM-1 medium for ~20 days (Gorham et al. 1964). The cultures were maintained at a pH between 7.5; a temperature of ± 24 °C; photo 12:12 period with the light intensity of 40 μmol.fotons.m^{-2}.s^{-1}. After reaching exponential growth, cultures were stopped and centrifuged at 10.000 RPM, 4 ºC, for 10 minutes. The resulting biomass was stored at -20 °C and then lyophilized.

Before the dissolution experiments, 5 mL of the ITEP-24 strain culture was removed and fixed with Lugol. Then, the sample counting fixed in Lugol on the Neubauer Chamber

(hemocytometer) was performed with the aid of an optical microscope Zeiss Axiovert 135M (Carl Zeiss, Göttingen, Germany). Cell counting was performed based on the number of cells per filament, and cell density was performed according to Blakefield and Harris (1994).

3.2.2 Dissolution tests

Reagents

The enzymes used in the test solutions were pepsin, from pig stomach and pancreatin, from the porcine pancreas 4X P-1500 (Sigma-Aldrich, Darmstadt, Germany). Sodium hydroxide, monobasic potassium phosphate, sodium chloride, hydrochloric acid, and phosphoric acid (Vetec, Rio de Janeiro, BR). Ultrapure water Milli-Q water system, (Millipore Corporation, Bedford, MA) was used to prepare all solutions.

Preparation of simulated gastric and intestinal fluid solutions

The dissolution tests were designed to understand how the GNT would react after being released from the S. torques-reginae cells (ITEP-24) from the extracellular medium in different simulated media of gastric and enteric fluid, with and without the presence of enzymes that help in metabolism processes of the stomach and intestine. The solutions used in the dissolution tests were prepared according to the Pharmacopoeia standards (Rockville 1995). The simulated gastric fluid was prepared with 2.0g of sodium chloride dissolved in ultrapure water, and then 6.0 ml of hydrochloric acid was added, and the pH was adjusted to 1.2 ml with ultrapure water for 1000 ml. For the medium with the enzyme pepsin, the same solution was prepared (simulated gastric fluid), and 3.2g of pepsin was added.

The intestinal solution was prepared with 6.81g of monobasic potassium phosphate and 1.70 g of sodium hydroxide dissolved with ultrapure water to the volume of 1000 ml. The pH was adjusted with 1M sodium hydroxide and 1M phosphoric acid to reach a pH of 7.5. Then the same medium (simulated intestinal fluid) was used to prepare the solution with the pancreatin enzyme, using 2.5g of pancreatin for 1000ml of the simulated solution of the intestinal content.

Assays with cells of the ITEP-24 strain

The tests were carried out in a 708-DS dissolution apparatus (Argilent) equipped with 6 glass cylinders with a capacity of 1000 mL, using the rowing method at 100 RPM with a temperature of 37 ± 1 ° C (Moreno et al., 2004). The experiments were conducted with 30% culture of the ITEP-24 strain and 70% of gastric fluid medium and simulated intestinal fluid with and without enzymes with a final volume of 1000mL.

The dissolution tests lasted 2 hours and the samples were obtained through manual collections with 5 ml syringes connected to cannulas. The collections were performed in 5, 10, 15, 20, 30, 45, 60, 90, 120 min, total of 3 ml of solution for each time (triplicate). After the collections, the samples were filtered with a 0.45µm nylon filter and stored on dry ice until the time of analysis in the LC/MS-QqQ. The low figure is a schematic representation of the dissolution tests on the adopted conditions (Figure 20).

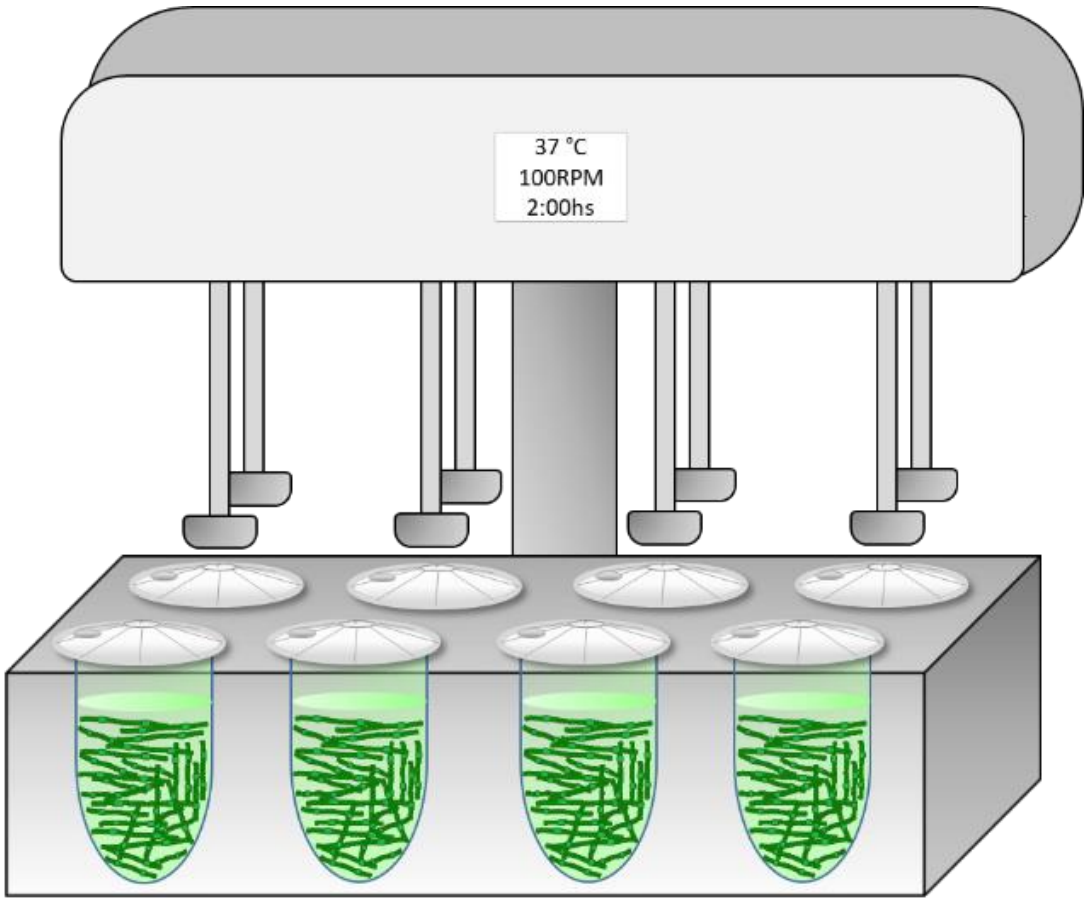

Figure 20: Experimental design of the dissolution tests, in which a 30% culture of the ITEP-24 strain was established, 70% of simulated gastrointestinal fluid with and without proteolytic enzymes at 100 RPM, 37 °C, lasting 2 hours.

3.2.3 LC-QqQ-MS/MS Analyses

Reagents

All reagents used were of high purity. Ammonium formate, ammonium formate, formic acid (Sigma-Aldrich, Darmstadt, Germany). The solvents used in the HPLC and LC/MS analyzes were chromatographic grade obtained from JT Baker (Phillipsburg, New York, USA) and Merck (Darmstadt, Germany).

Chromatographic equipment and conditions

The samples referring to all extraction protocols were analyzed by high-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (HPLC-QqQ) Agilent 6460 (Agilent Technologies, Santa Clara, USA) with ionization by electrospray (ESI), in positive mode 3500V. Nitrogen was used as the gas nebulizer (45 psi) and drying gas (5 mL/min. at 300 °C).

The separation of compounds was performed on hydrophilic chromatographic column ZIC-HILIC, 150 x 2.0mm, 5µm (Merck, Darmstadt, Germany). The mobile phases consisted of A) water containing 10 mM ammonium formate and 0.04% formic acid and B) acetonitrile/water (80:20 v/v), containing 5 mM ammonium formate 0.01% formic acid (Dörr et al., 2010). The injections were of 5 µL, and the chromatographic separation was carried out in a linear gradient with a flow of 0.150 mL/min.

The gradient started with 90% to (B) in 10 min., and then maintained at 40% (B) 10-12 min, increasing to 90% (B) in 12-12.5 min. maintaining 90% (B) up to 20 min. The results were obtained by multiple reaction monitoring (MRM) and the identification of the GNT was performed by the retention time, *m/z* 253 (PM of [GNT + H]) and the quantifier and qualifier ions *m/z* 253> 58 $[M + H]^+$ and *m/z* 253> 159 $[M + H]^+$. Data analysis was performed using Software Quatitative Analysis B06.00 (Agilent Technologies, Santa Clara, USA).

3.2.4 Statistical analysis

All values in this study were presented as mean ± standard deviation (SD). The significant difference was assessed by the two-way Anova test and the Tukey test for multiple comparisons (p-value<0.05) and (p-value <0.001). Statistical tests were performed using R

Statistical software version 3.1.2 (R CORE TEAM, 2012) and graphs using Prism 7 (GraphPad Software, CA, USA).

3.3 RESULTS

The data on the bioavailability of GNT in simulated solutions of the gastrointestinal content were acquired from analyzes by the LC/MS-QqQ system in MRM mode. The results shown in figure 21 refer to the peak intensity and area using the transitions *m/z* 253> 58 $[M + H]^+$ and *m/z* 253> 159 $[M + H]^+$ (Figure 22) that make it possible to obtain the qualitative concentration of GNT in the test samples dissolution.

The graphs represented in Figures 23 and 24, show the results obtained through an *in vitro* dissolution test according to the standards established by USP, where the cells of the strain ITEP-24 (3.29×10^6) were subjected to treatments with simulated means of gastric content with enzyme pepsin and without the enzyme (Figure 23) and simulated medium of the intestinal content with pancreatin enzyme and without the enzyme (Figure 24).

Treatment with the enzyme pepsin (pH 1.2) shows that the concentration of GNT increased significantly up to 0-10 min (*p*-value <0.05) and continued to increase slowly, with a tendency to stabilize the concentration of the toxin. The test by Anova Two-way and Tukey showed that the concentration of GNT in 10 min was statistically different from all times, except 5 and 15 min. In the times that comprised 0 to 15 minutes, there was the period of greatest activity of the enzyme pepsin on cell breakdown of the strain ITEP-24 and consequently availability of GNT in the extracellular environment (Figure 23).

The results obtained from the treatment with the enzyme pepsin (pH 1.2) was also compared with treatment of the gastric solution (pH 1.2), showing that the concentration of GNT in the times 20 and 30 min., were statistically equal in both treatments (Figure 23). However, while the concentration of GNT starts to increase in times of 0-15 min. the toxin concentration falls in the same period in the treatment with gastric solution (*p*-value <0.05). The results of treatments with simulated stomach contents (with and without enzyme) GNT concentrations were statistically (*p*-value <0.001) higher than treatments of simulated intestinal contents with pancreatin enzyme and without enzyme (pH 7.5) (Figure 24).

The graph (Figure 24) shows the results obtained in the treatments with simulated solution of the intestinal content with and without the pancreatin enzyme. The concentration of GNT increased significantly in the period of 10 and 15 min. (*p*-value <0.05), and then the

concentration decreases in 20 minutes, increasing slightly from 30 to 60 minutes tending to stabilize until the end of the experiment.

The treatments of the intestinal solution with the pancreatin enzyme (pH 7.5) were compared with the results obtained in the test with simulated solution of the intestinal content without the enzyme, the statistical test of Tukey showed difference in the concentration of GNT represented in the 10 and 15 min., and then 90 and 120 min. (p-value <0.05), the concentrations expressed at the other times were statistically equal at both times in both tests (Figure 24).

At the end of the experiments, pH measurements were made for all test solutions, there were no significant changes in the acidic (1.20 to 1.33) and alkaline (7.50 to 7.56) solutions. Microscopic analyzes of the samples resulting from the dissolution tests were carried out to evaluate the cell lysis of the strain ITEP-24. Microscopic analysis revealed a small number of isolated cells still intact.

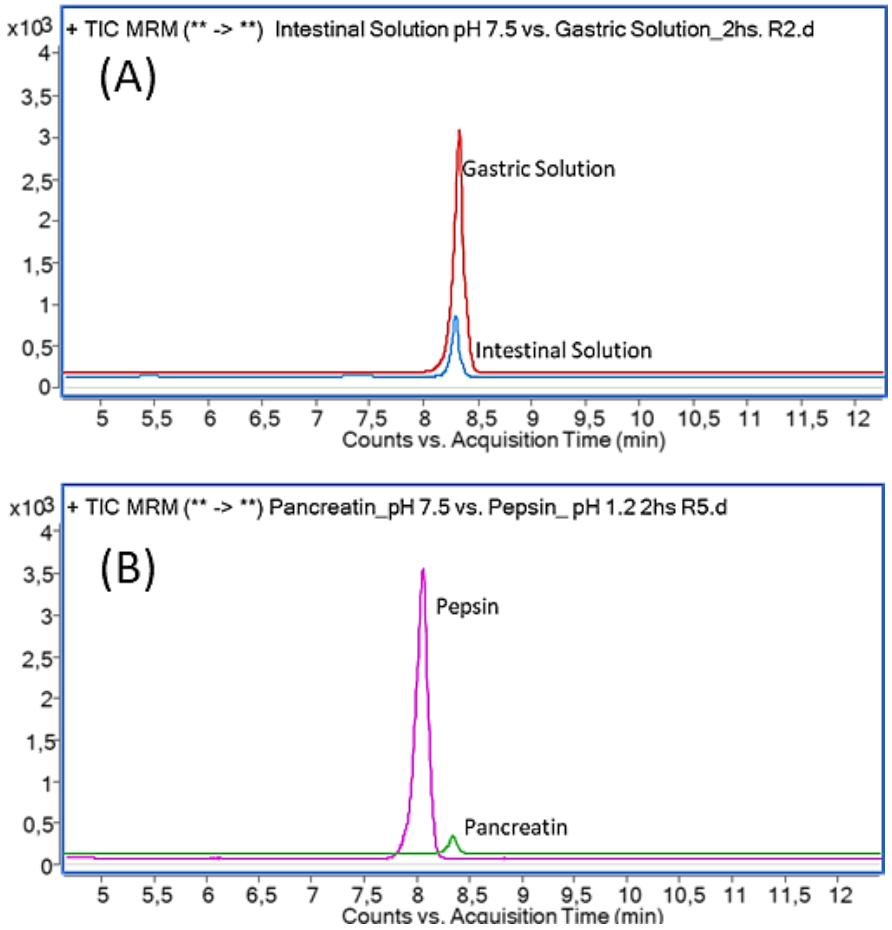

Figure 21: Total ion chromatogram (TIC) and Monitoring of multiple reactions (MRM) using the transitions *m/z* 253> 58 $[M+H]^+$ and *m/z* 253> 159 $[M + H]^+$. Chromatograms show the GNT concentrations were higher in tests with simulated stomach content solution (A) compared to simulated solutions of intestinal content (B).

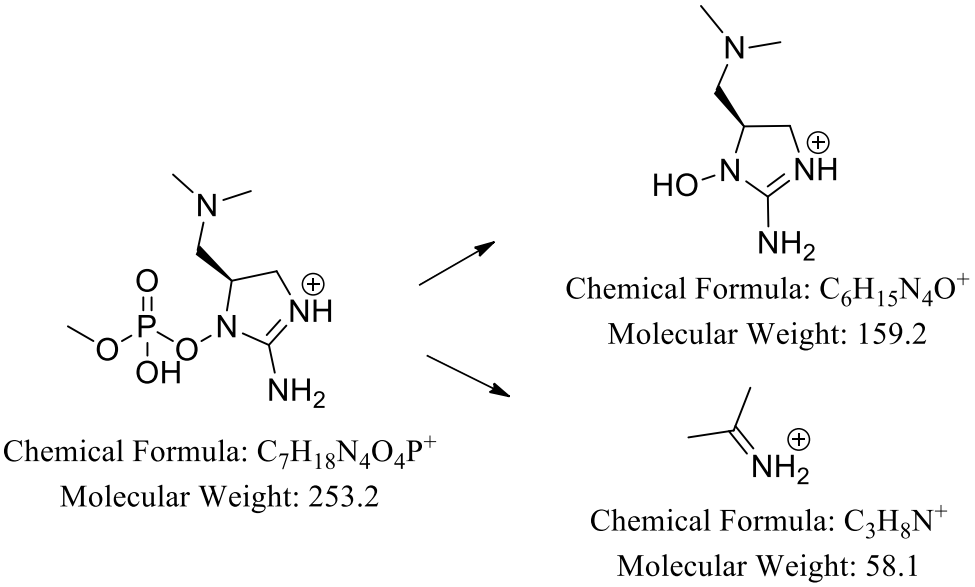

Figure 22: Molecular structure of guanitoxin *m/z* 253 (PM of [M + H]) and ions products *m/z* 159 and *m/z* 58 (PM of [M + H]) used in QqQ-MS/MS analyzes with ionization electrospray in positive mode through the MRM method to identify guanitoxin in dissolution test samples.

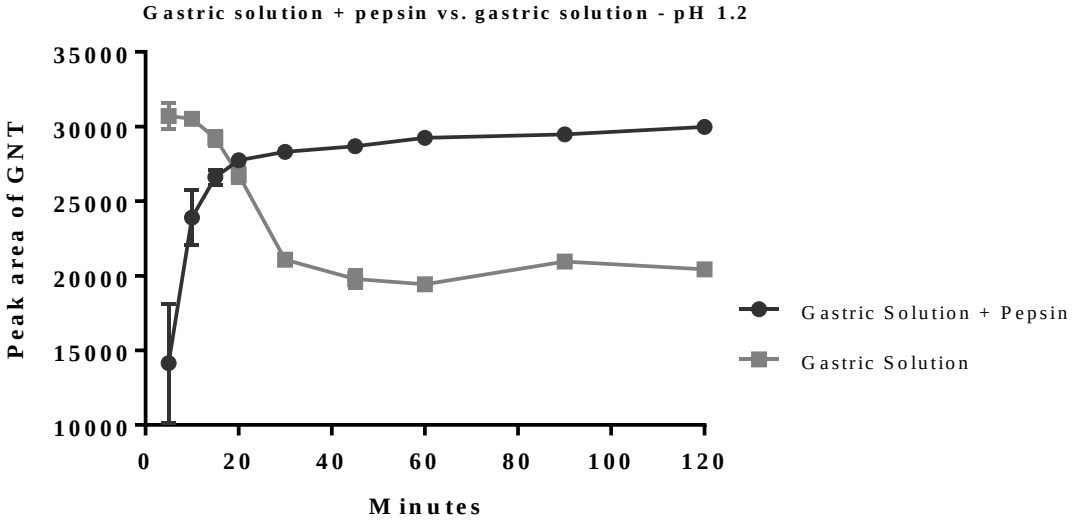

Figure 23: The graph shows the relative value of the guanitoxin concentration released by the cells of the ITEP-24 strain during the dissolution tests. Values are represented as the mean ± SD (n = 6). The GNT concentrations were higher after 20 min in the tests with the simulated solution of the stomach contents with the enzyme pepsin compared to the test with the simulated solution of the gastric contents without the enzyme (*p*-value <0.05).

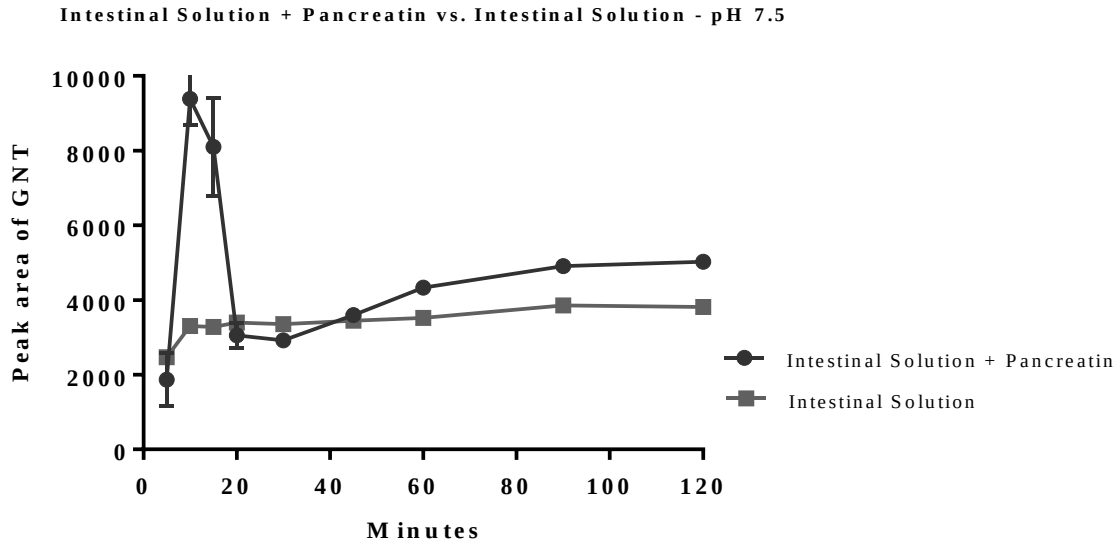

Figure 24: Comparative test to evaluate the release of GNT through fresh cells of the strain ITEP-24 submitted to a dissolution test in the simulated medium of the intestine, with pancreatin enzyme at pH 7.5. Values are represented as the mean ± SD (n = 6). The results showed that the release of GNT was greater in the test with a summarized medium from the intestine with the pancreatin enzyme in the periods of 10 and 15 minutes and after 60 minutes, tending to stabilize until the end of the experiment (*p*-value <0 05).

3.4 DISCUSSION

The dissolution test was developed in order to study the time needed for drug solubility in the human and animal organism (Anand et al. 2011). The dissolution method is also used to evaluate and guarantee the quality of medicines made available for use, in which it is established that at least 80% of solid formulations are dissolved in a short period of time (Qureshi and Shabnam 2001; Tsong et al. 2004; Anand et al. 2011). In general, this method aims to predict whether solid formulations will be dissolved in simulated aqueous media of the stomach and intestine, leaving the active ingredient available to be absorbed into the bloodstream and thus have an effect on the individual (Kasim et al. 2004). In this way, dissolution tests are used as primitives for other *in vivo* tests (Klein 2010).

Based on the principles of the dissolution method, the solubility of GNT in acidic (pH 1.2) and basic (pH 7.5) media was evaluated in order to understand the bioavailability of GNT in the gastrointestinal tract *in vivo*. These unprecedented results show the release of GNT by cells of the ITEP-24 strain in a simulated solution of the gastrointestinal content with and without the action of enzymes. Dissolution tests with proteolytic enzymes are normally used for formulations that are coated with some gelatinous membrane that does not

dissolve in aqueous media, due to the cross-linking that can interfere with the dissolution of the drug (Marques 2014).

In the case of our study, GNT was protected by cyanobacterial cells. Cyanobacteria have a cell wall made up of peptidoglycan, proteins and lipopolysaccharides, which have protective and compliance functions (Stanier and Cohen-Bazire 1977; Palinska and Krumbein 2000). In addition, the cell wall forms a boundary between cell constituents, so some stimulation is needed to break the cell wall to release intracellular components of interest.

According to the conditions of the dissolution method, the use of the enzymes pepsin and pancreatin, together with the constant rotation promoted by the rotating blades at 100 RPM, could act in the cell lysis of cyanobacteria. In studies of extraction of secondary cyanobacteria metabolites, organic solvents and suitable equipment are normally used, such as ultrasound probes or thermal shock cells, freezing and thawing; these methods assist in cell breakdown, promoting the release of intracellular metabolites (Safi et al. 2014).

The results of this study showed that the concentration of GNT in the dissolution test with the enzyme pepsin expressed an exponential growth character (5 to 20 min.) Keeping it high and constant after 20 min. (Figure 23). While in the test, only simulated solution of the stomach contents was used, the concentration was high and then decreased in the first 20 min of collection and after 30 min established that the concentration remained practically the same until the end of the experiment (Figure 23).

The enzyme pepsin probably acted in the breakdown of proteins present in the cyanobacterium cell wall, thus enabling the continuous release of GNT in the extracellular medium. Pepsin acts on the metabolism of proteins by transforming them into simpler peptides; it catalyzes the hydrolysis of peptide bonds adjacent to amino acids with side chains, aromatic amino acids (phenylalanine, tryptophan and tyrosine) and branched chain and methionine (Murray et al. 2003). Pepsin may also have had a greater role in degrading other metabolites present in the cell of the ITEP-24 strain, thus making GNT more available. However, these are hypotheses that require more specific studies to answer questions about the interaction of GNT with other molecules, especially digestive ones.

On the other hand, in the dissolution test with simulated solution of gastric content (pH 1.2) the concentration of GNT started high and then decreased between 10 to 30 min. and kept the concentration constant until the end of the experiment. GNT is stable in acidic solutions, but the optimum pH for GNT is pH 3.0. Studies carried out to assess the stability of the GNT molecule at different pH and temperature > 23 °C, have shown that the toxin can

be slightly degraded even though it is at acidic pH (pH 1.5 and 5.0), in addition the temperature of 37 °C can have contributed to obtaining this profile (article submitted).

In dissolution tests with simulated solutions of the stomach contents it usually lasts for 60 min., we extend the time to 120 min in order to make a comparison with the tests carried out with simulated solutions of the intestinal content. The data show that the higher concentrations of GNT remain more available in the stomach compared to the tests with alkaline solutions (Figure 21). However, GNT was not totally degraded in the solutions of the intestinal content (Figure 24), so that the toxin can be reabsorbed in the intestine.

In the pancreatin enzyme assay, there was an increase in the concentration of GNT in the range of 5 to 20 min. without statistically significant (p-value <0.05) with the displayed results of the intestinal solution (Figure 24). The pancreatin used in the dissolution assay is composed of a mixture of several enzymes, including trypsin, amylase and lipase, ribonuclease and protease. Pancreatic enzymes such as trypsin act primarily in the hydrolysis of lysine and arginine esters (Murray et al. 2009). So that it may also have acted in the cell break making it possible to release GNT, however GNT is not stable in a basic environment, so that GNT was released and hydrolyzed simultaneously.

The remains of cells still intact after 2 hours indicated that the solutions of the gastrointestinal content with and without enzymes managed to release only a part of the intracellular toxin. Depending on the morphology and metabolism of the digestive system *in vivo*, the presence of intact cells after 2 hours could result in the continuous release of GNT in the body and cause prolonged toxic effects, even if in low concentration.

Cyanobacteria produce a variety of secondary metabolites that are toxic to many organisms including man. Most of the GNT-producing cyanobacteria have been identified in freshwater environments accompanied by animal poisoning. Birds and domestic mammals were the most affected groups; the consumption of water containing cyanobacterial cells containing GNT caused the deaths of several animals (Mahmood et al. 1988; Cook et al. 1989; Henriksen et al. 1997).

Toxicological tests carried out on mice and rats through intraperitoneal injections with GNT, showed that the toxic effects are more pronounced between 7 to 30 min it can cause rapid death up to 60 min (Mahmood and Carmichael 1986, 1987; Mahmood et al. 1988). The results presented here, showed that in the tests with the enzymes pepsin and pancreatin the highest concentrations of GNT were also registered at the beginning of the tests specifically between 10 and 20 min. The highest concentrations were observed in simulated solutions of the stomach contents (Figures 21 and 24).

Tests carried out with other animals treated with crude extracts of cyanobacteria producing GNT showed that the toxic effect of GNT can vary according to the method of exposure and the dose administered (Mahmood and Carmichael 1987; Cook et al. 1989). Nevertheless, both tests with the pure toxin and tests that used cells containing the toxin showed similar clinical effects.

3.5 CONCLUSION

Dissolution tests showed that GNT is more available in solutions of stomach contents and the presence of the enzyme can influence the stability of the toxin in acidic solution. Although GNT is more stable in acidic solutions, this study showed that it can also be available in solutions of the intestinal content (pH 7.5), but in a concentration 30 times lower.

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Chapter 4: Stability studies of guanitoxin isolated from *Sphaerospermopsis torques-reginae* by mass spectrometry

ABSTRACT

This study investigated the stability of Guanitoxin (GNT) at different temperature and pH. Analyses were performed by ESI-Q-TOF-MS and LC-QqQ-MS/MS in MRM mode. In addition to the fragments ions described in the literature, the ion *m/z* 123 $[M + H]^+$ ion described here can also be used for the correct diagnosis of the toxin. The data showed that GNT is stable at acid pH; pH 3.0 is best for GNT stability and extraction. GNT degrades at pH > 9.0 and temperature > 23 °C in an interval ≥ 96 hours, generating the product *m/z* 159 $[M + H]^+$ detected by LC/MS from transition *m/z* 159> 58 $[M + H]^+$. From these data, it is recommended to store GNT samples below 4 °C. Toxin extracted directly from fresh cells has greater stability compared to dry cells. As it does not exist in the GNT commercial standard, the information presented in this study can contribute to the identification and isolation of GNT in the environment.

Keywords: hydrolysis, sample preparation, neurotoxin, cyanobacteria.

4.1 INTRODUCTION

Guanitoxin (GNT) is a neurotoxin produced by cyanobacterial species belonging to the group of anatoxins. This toxin was isolated from *Dolichospermum flos-aquae* strain NRC 525-17 and its molecular structure consists of a methyl phosphate ester of a cyclic N-hydroxyguanidine, whose molecular formula is $C_7H_{17}N_4O_4P$ and molecular mass is 252 Da.(Matsunaga et al. 1989). From laboratory experiments with rats, it was possible to evaluate the lethal dose (DL) of GNT as well as other effects adding to lethality (Carmichael et al. 1977; Mahmood and Carmichael 1986; Cook et al. 1988). The action mechanism consists of the irreversible inhibition of the enzyme acetylcholinesterase, a mechanism similar to that of organophosphate insecticides (Hyde and Carmichael 1991). Cholinesterase inhibition prevents acetylcholine hydrolysis, inducing excessive stimulation at the cholinergic synapse to cause muscle exhaustion and acute effects are characteristic of a cholinergic syndrome due to excess acetylcholine at the neuromuscular junction (Cook et al. 1988).

Some cases of intoxication involving GNT have been described from characteristic clinical signs and deaths of mammals and birds following accidental consumption of contaminated water containing GNT (Henriksen et al. 1997; Onodera et al. 1997; Briand et al. 2003). GNT has been reported in the North America, South America, Europe and the most recent reports recent studies have been documented in Asia and again in North America (Florida/USA) from samples of cyanobacterial blooms in freshwater and terrestrial environments (Carmichael and Gorham 1978; Mahmood et al. 1988; Monserrat et al. 2001; Molica et al. 2005; Becker et al. 2010; Metcalf et al. 2012, 2018; Chatziefthimiou et al. 2014). The cumulative worldwide data on this toxin is still very low compared to other known cyanotoxins (Dörr et al. 2010). GNT can hydrolyse at alkaline pH and high temperatures and probably its instability on these environmental variables makes it difficult to identify it in the environment.

Hydrolysis methods measure at a base line loss rate of any chemical in an aqueous environment in the function of time and temperature (Speight 2018). Many chemicals can be altered by a direct reaction of the chemical with water (hydrolysis), in which a chemical bond is cleaved and two new bonds are formed, each having the hydrogen component (H) or the hydroxyl component (OH-) of the water molecule (Speight 2018). Hydrolytic reactions can be influenced by the presence of acids (hydrogen ions) and bases (hydroxyl ions)

denominated as catalysts (Speight 2018) which may result in the degradation or neutralization of the compound under study.

The degradation of substances by the environment is extremely important, especially for substances harmful to the environment. In this process, the environment can degrade a specific chemical structure to a simpler form, making it less lethal or not harmful to natural resources (Mabey and Mill 1978).Although GNT is more sensitive to some environmental variables and can be easily degraded, it is a fast-acting toxin with LD_{50} measured in mice from 20 μg kg^{-1} to 50 μg kg^{-1}.(Mahmood and Carmichael 1987a; Mahmood et al. 1988; Cook et al. 2001). According to data available in the literature, the presence of cyanobacteria producing GNT in the environment can cause serious impacts on the potability of water for human consumption, in addition to non-measurable damage to the life of organisms of different trophic levels.

Therefore, although there is information in the literature on GNT instability at alkaline pH and high-temperature, here we investigate the half-life of GNT based on methodologies proposed by the present study in order to estimate environmental conditions and establish ideal laboratory conditions to identify extract and even isolate GNT. Therefore, the results of this study will provide valuable information for water body GNT monitoring and reliable sample preparation methodologies, as well as the identification of GNT by LC/MS.

4.2 MATERIAL AND METHODS

4.2.1 Cultivation of ITEP-24 Strains

The strain ITEP-24, classified as producer of cyanotoxin Guanitoxin and of mycosporins, spimigines and namalides, was isolated in Tapacurá Reservoir/PE/Brazil; this strain is part of the collection of cyanobacteria in the laboratory of Toxins and Natural Products (LTPNA) at the School of Pharmaceutical Sciences, University of Sao Paulo/Brazil. To perform the experiments with fresh culture and dried cells, ITEP-024 strain was cultured ASM-1, pH 7.5–8.0, under 40 μmol of photons m^{-2} s^{-1} light intensity, 12 h photoperiod, continuous aeration and 22.0 ± 1.0 °C (Gorham et al. 1964) (Nova technical, São Paulo, BR). ITEP-024 strain cultures were centrifuged Eppendorf 5804R (Eppendorf, Sao Paulo, BR) at

10.000 RPM for 10 min at 4 °C stored at -20 °C and then lyophilized for to get of dried cells for experiments.

4.2.2 Stability of Guanitoxin

According to the literature, GNT is a very sensitive molecule to pH and temperature variations, and therefore, its detection in water bodies with cyanobacteria blooms is very rare compared to other cyanotoxins (Matsunaga et al. 1989; Hyde and Carmichael 1991; Barros et al. 2004). Based on this information, the study presented here investigated the stability of GNT over a wide pH range (acid-neutral-base) as a function of temperature and exposure time. Here, our goal was only to evaluate toxin semi-integration after it was released from the cell.

4.2.3 Stability of intracellular Guanitoxin in dry cells (ITEP-24) exposed to different temperatures

The methodology used was to evaluate the viability of dry cell storage (ITEP-24) for long periods for subsequent extraction of GNT at different temperatures. Therefore, the experiments were performed from a single dry cell pool of the ITEP-24 strain and several 10 mg samples (triplicate) were aliquoted from this stock sample and conditioned at -80, -20, 4 and 23 °C for 6 months.

Each month, three 10 mg samples were collected from each environment and extracted with ultra-pure Miliq-Q H_2O (Merck, Darmstadt, Germany) and 0.3% Acetic Acid (JBaker Phillipsburg, New Jersey, USA). Extraction was performed by freezing and thawing. Then, the samples were centrifuged Eppendorf 5804R (Eppendorf, Sao Paulo, BR) at 10.000 RPM, 4 °C for 10 min. Supernatants were filtered on 0.45 μm PVDF membranes (Nova Analítica, Sao Paulo, BR) and analysed by LC-QqQ-MS/MS**.** This centrifugation and samples filtration procedure was followed in all of the other experiments described here.

4.2.4 Stability of Guanitoxin extracted from dry cells pH 1.5 to 9.5

We used 10 mg of dry cells (triplicate) and prepared solutions for toxin extraction at pH 1.5, 3.0, 5.0, 7.0 and 9.5. Acid pH solutions were prepared with 1 M hydrochloric acid

and the pH adjusted as needed with 0.1 M sodium hydroxide. For the neutral solution, we used only ultra-pure water (pH 6.8–7.0). The pH 9.5 solution was prepared with 1 M monobasic potassium phosphate and 1 M sodium hydroxide and the pH was adjusted with 0.1 M sodium hydroxide. The extracts were frozen at -20 °C and thawed at 23 ºC.

Freeze-thaw cycles (-20 °C and 25 °C) were performed for six consecutive years at consecutive intervals of up to one hour. After cell lysis, the samples were centrifuged and filtered as described in the previous experiment. LC-QqQ-MS/MS analysed samples, making injections at 0, 24, 48, 72 and 96 hours. The samples remained at room temperature (±23 °C) during all analyses.

4.2.5 Stability of Guanitoxin extracted from fresh cells pH 1.5 to 10.5

5 ml of the ITEP-24 strain (triplicate) culture was used, the samples were centrifuged (10.000 RPM, 4 °C, 10 min) the supernatant was discarded and only the pellet was used in the experiments. Solutions pH were 1.5, 3.0, 5.0, 7.0, 8.5 and 10.5. The acidic solutions were prepared with 0.1 M hydrochloric acid with 1 M sodium hydroxide adjustment. The neutral solution pH 7.0 was prepared using only ultra-pure water (pH 6.8–7.0); pH 8.5 and 10.5 solutions were prepared with 1 M monobasic potassium phosphate and 1M sodium hydroxide and adjusted using 0.1 M sodium hydroxide. In an ultrasonic homogenizer Elma E60 Emasonic (Nova Analitica, São Paulo, BR) with a water bath at 37 °C extracted samples for 1 hour. After extraction, the samples were centrifuged, filtered and LC-QqQ-MS/MS analysed samples for 96 hours at ±23 °C, injecting at 0, 24, 48, 72 and 96 hours.

4.2.6 Degradation of Guanitoxin

To evaluate the degradation of GNT at alkaline pH as a function of time and temperature, we separated 10 mg (triplicate) samples of dried cells from the ITEP-24 strain. Then, we extracted the toxin with 1 mL ultrapure H_2O with 0.3% acetic acid, pH 3.0. The samples were submitted ultrasonic Homogenizer Omni Sonic Ruptor 400 (OMNI International Company, Georgia, USA) at 30% pulse for 3 min per three cycles, and then centrifuged and filtered.

After extracting the GNT, we add sodium hydroxide (pH= 10.5). Then, the samples were analysed by LC-QqQ-MS/MS, performing injections at 0, 24, 48, 72, 96, 120 and 144

hours. During the experiment, the samples were at ± 23 °C. The data for this experiment were plotted from the peak area of the GNT degradation product transition *m/z* 159> 58 $[M+H]^+$ in order to monitor the time that the toxin takes to be hydrolysed completely.

4.2.7 ESI-Q-TOF-MS Analyses

Samples were analysed using an ultra-high performance liquid chromatograph (Shimadzu, Kyoto, Japan) coupled with a high-resolution hybrid quadrupole flight time mass spectrometer, micrOTOF-Q II, equipped with a positive mode electrospray ionization source (ESI-Q-TOF-MS) (Bruker Daltonics Corporation, Bremen, Germany). The ESI-Q-TOF mass spectrometer was operated in the positive and negative ionization modes, set at 4500 and 3000 V, respectively; with an endplate offset potential of −500 V. The dry gas parameters were set to 9 L min^{-1} at 200 °C with a nebulization gas pressure of 5 bar.

The separation of the ions was carried out by Hydrophilic Interaction Chromatography (HILIC) using a SeQuant ZIC-HILIC 150 x 2.1 mm column, 5 µm particles, 200 Å (Merck, Darmstadt, Germany). The mobile phase was delivered at a flow rate of 0.15 mL min^{-1} and consisted of a mixture of solvents **A** (H_2O with 0.04% formic acid; 10 mM ammonium formate v/v) and **B** (acetonitrile: H_2O [80:20] with 0.01% formic acid and 5 mM ammonium formate, v/v) (Sigma-Aldrich, Darmstadt, Germany). The identification of GNT was performed on a linear gradient with 90% (B) of 0-10 min, 90-40% (B) in 10-14 min, 40-90% (B) 14-20 min at a temperature of 40 °C.

Data were collected from *m/z* 60 to 1800 at an acquisition rate of 2 Hz, and the variable number of ions was selected by automatic full scan and autoMS/MS scan fragmentation with a cycle time of 2.5 seconds. MS/MS data were analysed using Bruker Compass Data Analysis 4.3 software (Bruker Daltonics Corporation, Bremen, Germany), and it was possible to calculate the experimental mass of the compound $[M + H]^+$ and the molecular formula with errors <5 ppm and millisigma value (mSigma) <10. The exact mass and fragment ions structures of GNT were designed by ChemDraw software version 12.0.2.10.7627

4.2.8 Liquid Chromatography-Tandem Mass Spectrometry LC-QqQ-MS/MS Analyses

The samples were analysed and quantified in an Agilent 1260 Infinity chromatographic system coupled to a triple quadrupole mass spectrometer 6460 Triple Quadrupole LC-MS (Agilent Technologies, Santa Clara, USA), with ionization by electrospray (ESI), in positive mode at 3500 V. Nitrogen was used as the gas nebulizer (45 psi) and drying gas (5 mL min^{-1} at 300 °C). Chromatographic separation was performed using a SeQuant ZIC-HILIC column—150 x 2.1 mm, 5µm, 200 Å (Merck, Darmstadt, Germany)—at a flow rate of 0.15 mL min^{-1}. The mobile phases and the gradient used to identify the GNT followed the same conditions described in the method of ESI-Q-TOF-MS analyses.

The injection volume used for the analyses was 5µL. Identification of GNT and qualitative analysis were performed by retention time and peak area, determined by MS/MS tandem analysis in multiple reaction monitoring (MRM) mode, monitoring the ionic products of *m/z* 253 > 58 $[M + H]^+$, *m/z* 253> 159 $[M + H]^+$ and degradation product of GNT$[M + H]^+$ *m/z* 159> 58 $[M + H]^+$. Data were acquired using MassHunter Qualitative B.07.00 software (Agilent Technologies, Santa Clara, USA).

4.2.9 Statistical analysis

Statistical and graphical tests were performed using the GraphPad Prism software version 7.0. All data are represented as mean and standard deviation. Comparisons between samples were performed using ANOVA tests followed by Tukey's multiple comparison tests for comparisons of various groups with a 95% confidence interval.

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis by high-performance liquid chromatography-tandem mass spectrometry LC-QqQ-MS/MS and ESI-Q-TOF-MS/MS

GNT identification analyses were performed using liquid chromatography-tandem mass spectrometry analytical methods LC-QqQ-MS/MS Analyses and ESI-Q-TOF-MS/MS. The chromatographic conditions and analytical columns were the same for methods. However, the analyses were directed to the different equipment according to the specific

objectives of each experiment. A quadrupole mass analyser (QqQ) is a widely used apparatus for quantification and identification of molecules in biological matrices due to its high sensitivity (Xu et al. 2008). A quadrupole-flight time mass spectrometer (Q-TOF) has high-resolution mass analysers and mass/charge accuracy, providing reliability in molecular ion fragmentation results and can result in high-quality information for known or even unknown compounds (Chernushevich et al. 2001; Milman and Zhurkovich 2014).

The analyses performed on LC-QqQ-MS/MS were in full scan mode and MRM (multiple reaction monitoring) using precursor ion *m/z* 253 $[M + H]^+$ and its characteristic ions *m/z* 253> 58 and *m/z* 253>159 $[M + H]^+$. We also monitored the GNT *m/z* 159> 58 $[M + H]^+$ degradation product (Supplementary information - Figure S1). Due to its high sensitivity, the quadrupole triple mass analyser allowed us to identify GNT even when the molecule was in low concentration in the samples (see Figures 25–28). Although it is a low-resolution mass analyser, it is an excellent tool for scanning water samples and assessing the presence of cyanotoxins such as Therefore.

We use ESI-Q-TOF-MS/MS to detect and identify the molecular formula based on the calculated exact mass of GNT. The identification of the GNT molecule was performed based on the mass exact $[M + H]^+$, isotopic pattern and MS2 fragmentation spectrum (Supplementary information - Figure S2A), where the measured *m/z* was m/z 253.1057, calculated *m/z* was 253.1060, the ion formula was $C_7H_{18}N_4O_4P$, error [ppm] = 1.3 and mSigma= 1.3. In the mass spectra (MS), it shows the ions *m/z* 58.0651 (C_3H_8N) $[M + H]^+$, *m/z* 96.0556 ($C_4H_6N_3$) $[M + H]^+$ and *m/z* 159.1240 ($C_6H_{15}N_4O$) $[M + H]^+$ that are characteristic fragmentation of the GNT (Supplementary information - Figure S2B) (Dörr et al. 2010). The spectrum MS2 shows other ions that can also be used for the diagnosis of GNT, however, it was possible to calculate only the molecule of the ions *m/z* 98.0713 ($C_4H_8N_3$) $[M + H]^+$ and *m/z* 123.0553 ($C_6H_7N_2O$) $[M + H]^+$ (Supplementary information - Figure S2C).

The analyses were performed in LC/MS with a stationary phase analytical column based on zwitterionic polymers (ZIC) due to its success in retaining polar compounds, including acids, alkaline, neutral, environmental, pesticides and biologicals, such as urine and blood plasma (Dörr et al. 2010; Xiong and Liu 2016). Normally, in HILIC methods, the compound separation is realized by normal phase eluents, but the ZIC-HILIC column was adapted to use reverse-phase eluents. This type of adaptation allows numerous advantages by uniting aqueous mobile phases and separation with normal phase principles.(Lindberg et al. 2015). The union of these parameters allows more excellent retention of small polar

molecules such as GNT. Although there is no analytical standard to quantify the toxin in environmental samples, the chromatographic method and analysis in high-resolution mass spectrometer becomes especially important for the correct diagnosis of the molecule.

4.3.2 Guanitoxin stability

The data obtained in this study aimed to evaluate toxin stability and compare with existing data in the literature. Data were evaluated considering the pH and temperature parameters and their influence on the relative peak area of GNT. The information available in the literature reports that the toxin is unstable in alkaline solutions (half-life of approximately one hour at pH > 8.5).[20] Our results show that the toxin decomposes quickly in alkaline solutions, but the half-life differs from the data in the literature; in our experiments, the toxin hydrolysed in an interval ≥ 96 hours. Here, we evaluate the degradation at different time intervals from those reported in the literature; however, if we compare the initial time with the later time, the peak area decays in all pH solutions and is significant in neutral to alkaline pH solutions.

In Figure 25, there are data from stored, dried cells at four temperatures. Samples that were conditioned at 23 °C had significant GNT degradation, *p*-value < 0.05, after one month. At the end of the 150 days, the relative concentration of GNT showed a decreasing profile in samples conditioned to 23 °C. There was no statistical difference between -80, -20 and 4 °C. However, peak area values were higher at -80 and -20 °C. Some studies report the loss of biological activity of GNT after six and four months of storage at -20 ºC, and two weeks without preservation (Onodera et al. 1997; Barros et al. 2004). Nevertheless, our study only evaluated the feasibility of storing dry biomass containing the toxin at different temperatures, and this must be considered.

Figure 26 shows that GNT is more stable at acid pH and pH 3.0 proved to be the best for toxin stability (*p*-value <0.05). The multiple comparison tests (Tukey) were used to compare the peak GNT area and the respective treatments, showing that there was no statistical difference between pH 1.5 and 5.0, *p*-value = 0.86, treatments and pH 7.0 and 9.5, *p*-value = 0.81. Then, a comparison was made between temperature and peak area of the GNT, showing that the 0-h time is statistically different from the others, *p*-value <0.05.

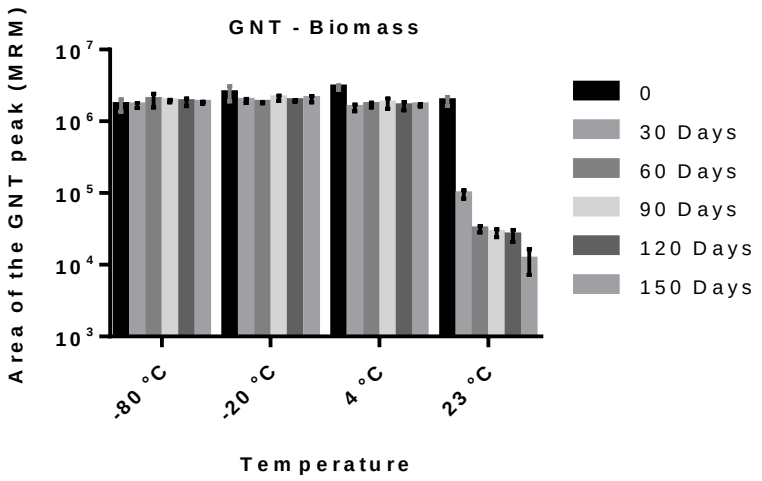

Figure 25: Stability of intracellular GNT in lyophilized cells of the ITEP-24 strain stored for up to 150 days between temperatures of -80, -20, 4 and 23 °C. The graph shows that the peak area of samples stored at 23 °C decreases significantly (p-value <0.05) in the GNT concentration compared to samples stored at temperatures - 80, -20 and 4 °C.

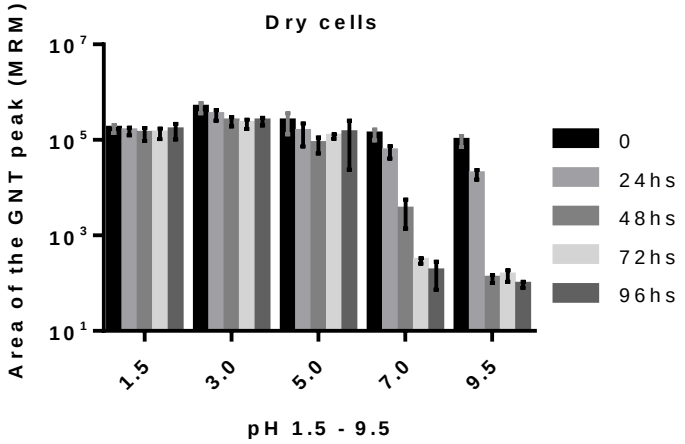

Figure 26: Stability of GNT extracted from lyophilized cells of the ITEP-24 strain at different pH with intervals of 24 hours for 96 hours. The graph shows that GNT is more stable at acidic pH, with pH 3.0 being better for its stability (*p*-value <0.05). There was no statistical difference in the concentration of GNT extracted in solutions with pH 1.5 and 5.0 and between pH 7.0 and 9.5.

In Figure 27, it shows that from 0 to 96 h, the solutions at pH 1.5 and pH 3.0 were statistically different from each other, *p*-value <0.05, while pH 5.0, 7.0, 8.5, and 10.5 showed no statistical difference between them. The results show that the stability of the GNT extracted in dry and fresh cells was similar. However, the concentration of the toxin is higher in samples extracted directly from fresh cells. In summary, the results shown in Figures 25 to 27 show that extracellular GNT can gradually degrade at alkaline pH and a temperature higher than 23 °C. However, the molecule did not degrade totally under the conditions used

in the study. According to Matsunaga *et al* [1] and Barros *et al* (Barros et al. 2004) GNT is a high-temperature sensitive molecule > 40 °C. In our study, we analysed freeze-dried and fresh samples at different temperatures in order to study the best condition to store samples in the laboratory and evaluate the half-life of the extracellular toxin at temperatures observed in cyanobacterial blooms (Rapala and Sivonen 1998).

Figure 28 shows the degradation profile of the GNT for the ITEP-24 strain referring to samples that were extracted in acidic pH 3.0 and then alkalized pH 10.5. The peak area of the GNT gradually decreased, while the peak of the GNT degradation product increased significantly after 96 to 144 hours (Figure 28 and Supplementary information - Figure S3). Ions *m/z* 159> 58 $[M+H]^+$ were also incorporated into the MRM method because ion *m/z* 159 $[M+H]^+$ is the GNT degradation product after the neutral loss of a phosphate residue (Figure 29) (Matsunaga et al. 1989; Hyde and Carmichael 1991; Dörr et al. 2010). Throughout the analysis, we observed that the GNT degradation product was more common in dry cell samples. In fresh cell samples, this ion (*m/z* 159) was rarely observed; basically, it was present in the samples after extractions in solution with pH 7.0 to 10.5 and the samples of dry cells kept at room temperature.

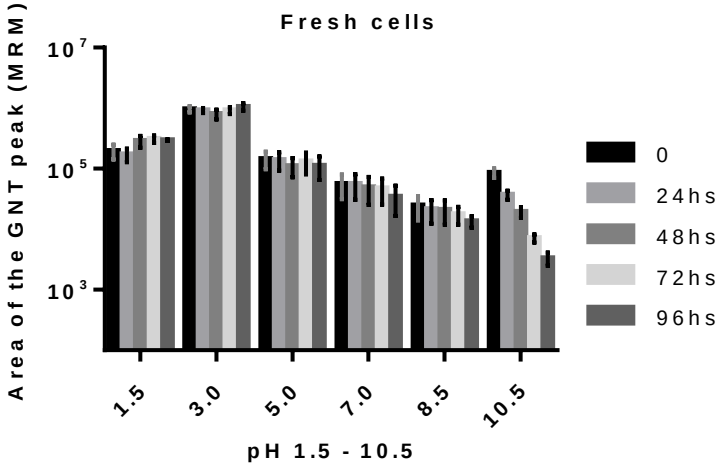

Figure 27: GNT extracted from fresh cells of the ITEP-24 strain at 37 °C in pH of 1.5 to 10.5. The pH 3.0 differed statistically (*p*-value<0.05) from other pH solutions at all sampling times. In neutral solutions and alkaline pH, the extracted toxin was degraded as a function of exposure time. Statistical analysis showed that 0–96hs at pH 1.5 and 3.0 were statistically different from others, while pH 5.0, 7.0, 8.5 and 10.5 have no statistical difference per se.

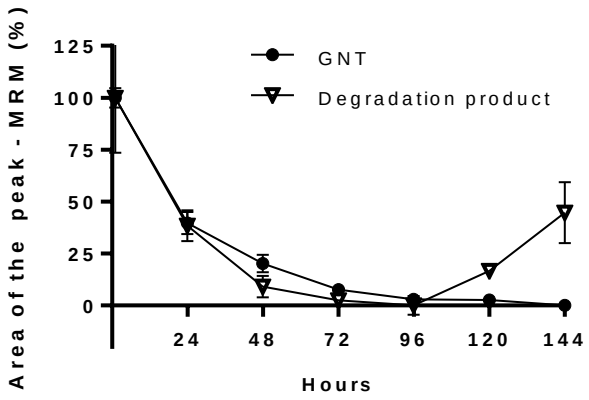

Figure 28: Degradation profile of GNT present in lyophilized cells of strain ITEP-24 analysed by LC-QqQ-MS/MS in MRM mode. The graph shows that the peak area of GNT for transitions *m/z* 253> 159 $[M + H]^+$ and *m/z* 253> 59 $[M + H]^+$ decreases after treatment with sodium hydroxide pH 9.0, while the degradation of the product *m/z* 159> 58 $[M + H]^+$ increases significantly (*p*-value <0.05) after 96 hours.

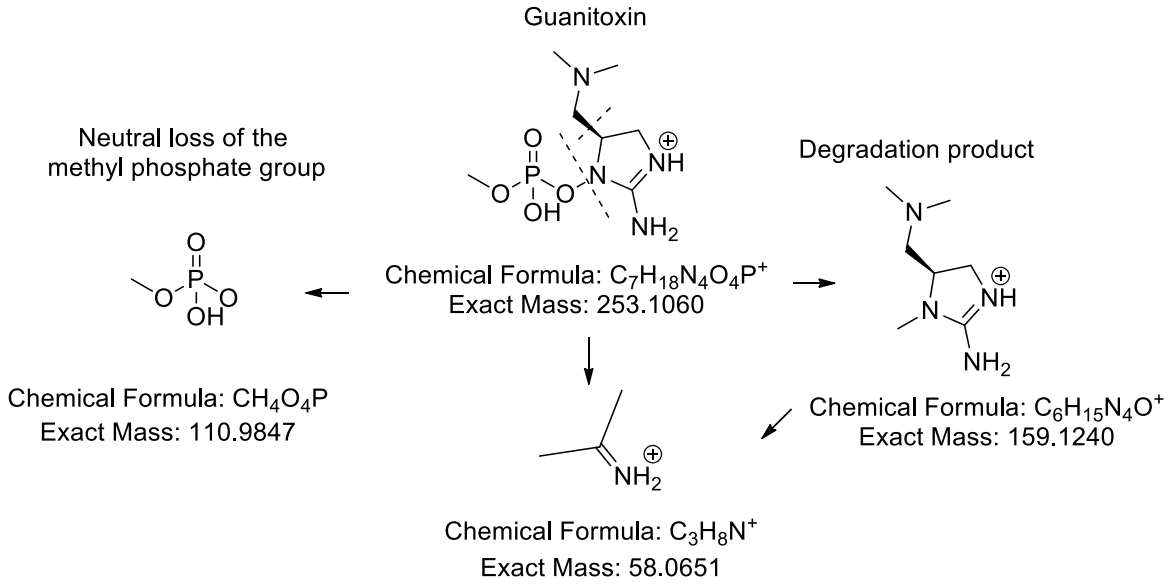

Figure 29: Scheme the of hydrolysis of Guanitoxin adapted from Hyde and Carmichael 1991, showing the formation of ion *m/z* 159 [M+H]+ after the neutral loss of a methyl phosphate residue. Ions *m/z* 253> 159 $[M+H]^+$, *m/z* 253> 58 $[M+H]^+$ were used to identify guanitoxin (Dörr et al., 2010) and *m/z* 159> 58 $[M+H]^+$ was used to detect the guanitoxin degradation product proposed in this study.

In the natural environment, the cyanobacterial blooms are directly related to the climatic conditions of a given region and the availability of nutrients in the water (nitrogen and phosphorus) (Walker 1983; Cong et al. 2006; Yang et al. 2016). Most cyanobacterial blooms reports occur in summer and early autumn with temperatures > 15 °C.(Organisation et al. 1999). GNT has been identified in different regions of America (Carmichael and Gorham 1978; Mahmood and Carmichael 1987b; Molica et al. 2005), Europe (Henriksen et

al. 1997), and recently in Asia (Metcalf et al. 2012; Chatziefthimiou et al. 2014) in eutrophic environments of fresh and terrestrial water.

The predominance of these organisms in the water body directly interferes with some important physicochemical variables for the balance of aquatic ecosystems (Miller et al. 2012). Eutrophic environments are generally characterized by oxygen depletion, and pH increase observed in; the pH present can vary from neutral to alkaline (Ji et al. 2017). This fact also justifies the success and dominance of specific cyanobacterial species and, consequently, the relationship of toxins identified in bloom events (Karjalainen et al. 2007). The occurrence of cyanobacteria is an environmental problem of worldwide concern due to the predominance of toxin-producing species that can cause lethality for several organisms and mainly compromise the potability of water for human consumption.

Although GNT has a short half-life, its presence in the environment was responsible for the mortality of birds and mammals after consumption of water containing toxin-producing cyanobacterial cells. The results of our study showed that extracellular GNT could remain at neutral and alkaline pH for more than 24 hours. Although there is no specific legislation for monitoring this toxin in aquatic systems, nor an analytical standard for its quantification, the methodologies shown in this study are sufficient for the correct diagnosis of the molecule in biological samples.

4.4 CONCLUSIONS

The data presented here showed that cyanotoxin GNT is more stable at acidic pH ranging from 1.5 to 5.0, with pH 3.0 being the best for maintaining and extracting toxin in aqueous solutions. We recommend keeping dry cell samples at temperatures below 4 °C for up to six months, our study showed that GNT degrades rapidly in samples stored at 23 °C. In addition, extracting the toxin directly from fresh cells is more advantageous since there is no formation of the degradation product *m/z* 159 $[M+H]^+$. The LC/MS identification methods used here were essential for the development of the study, as it allows a detection of the GNT even at low concentrations in alkaline solutions.

4.5 SUPPLEMENTARY INFORMATION

Complementary information, total ion chromatograms (TIC) in various reaction monitoring modes (MRM) and extracted ion chromatogram (EIC) of the qualifying, quantifying ions, and the guanitoxin degradation product.

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Supplementary Information

Chapter 4: Stability studies of Guanitoxin isolated from *Sphaerospermopsis torques-reginae* by mass spectrometry

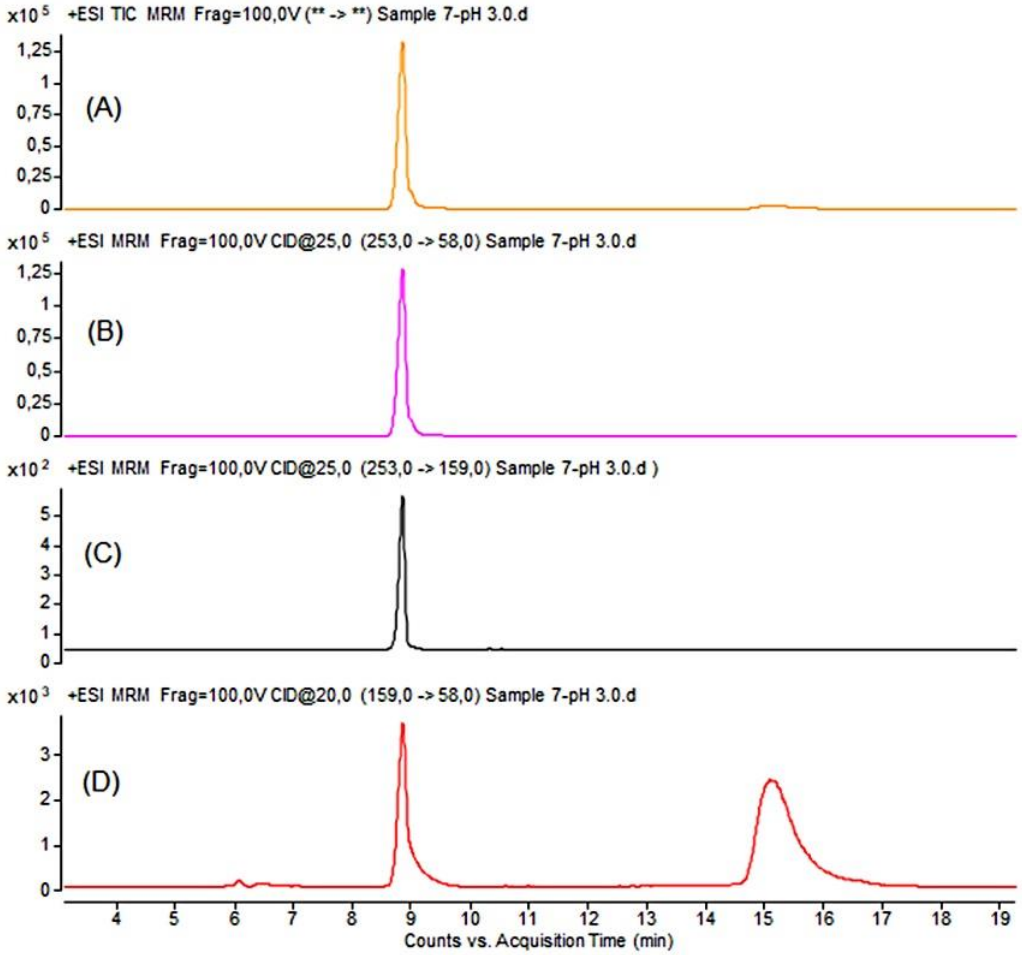


Figure S1: Chromatographic profile referring to the analytical method in LC-QqQ-MS/MS performed in various reaction monitoring (MRM) modes to identify guanitoxin, and its degradation product in the experiments conducted out in this study. The data represented here is from a sample of lyophilized cells extracted at pH 3.0. Total Ion Chromatogram (TIC) (A), Extracted Ion Chromatogram (EIC) of the transitions used to identify guanitoxin $[M+H]^+$ *m/z* 253> 58 (B), $[M+H]^+$ *m/z* 253> 159 (C) with retention time in 8.0 min and in $[M+H]^+$ *m/z* 159> 58 (D) degradation product of its degradation retention time between 13.0 to 14 minutes.

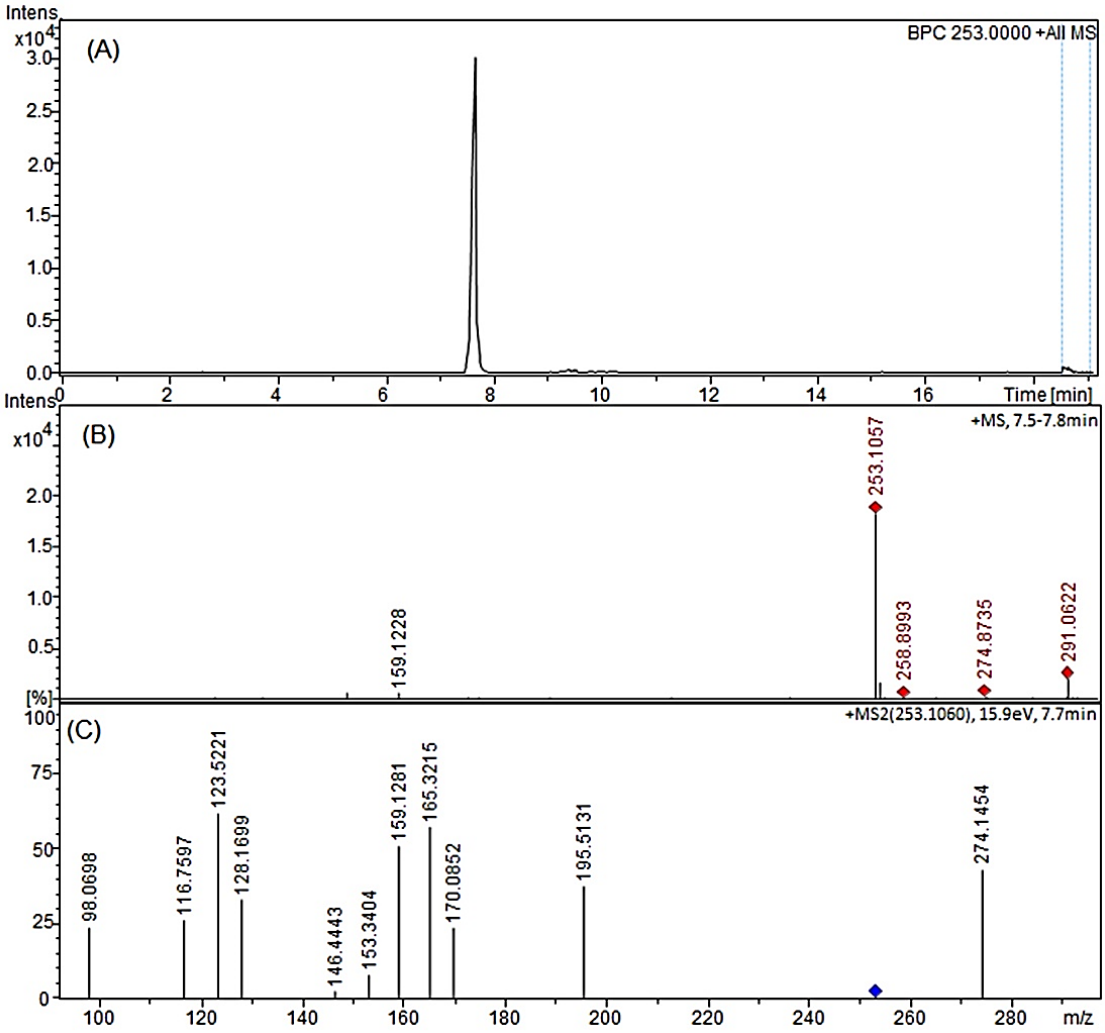
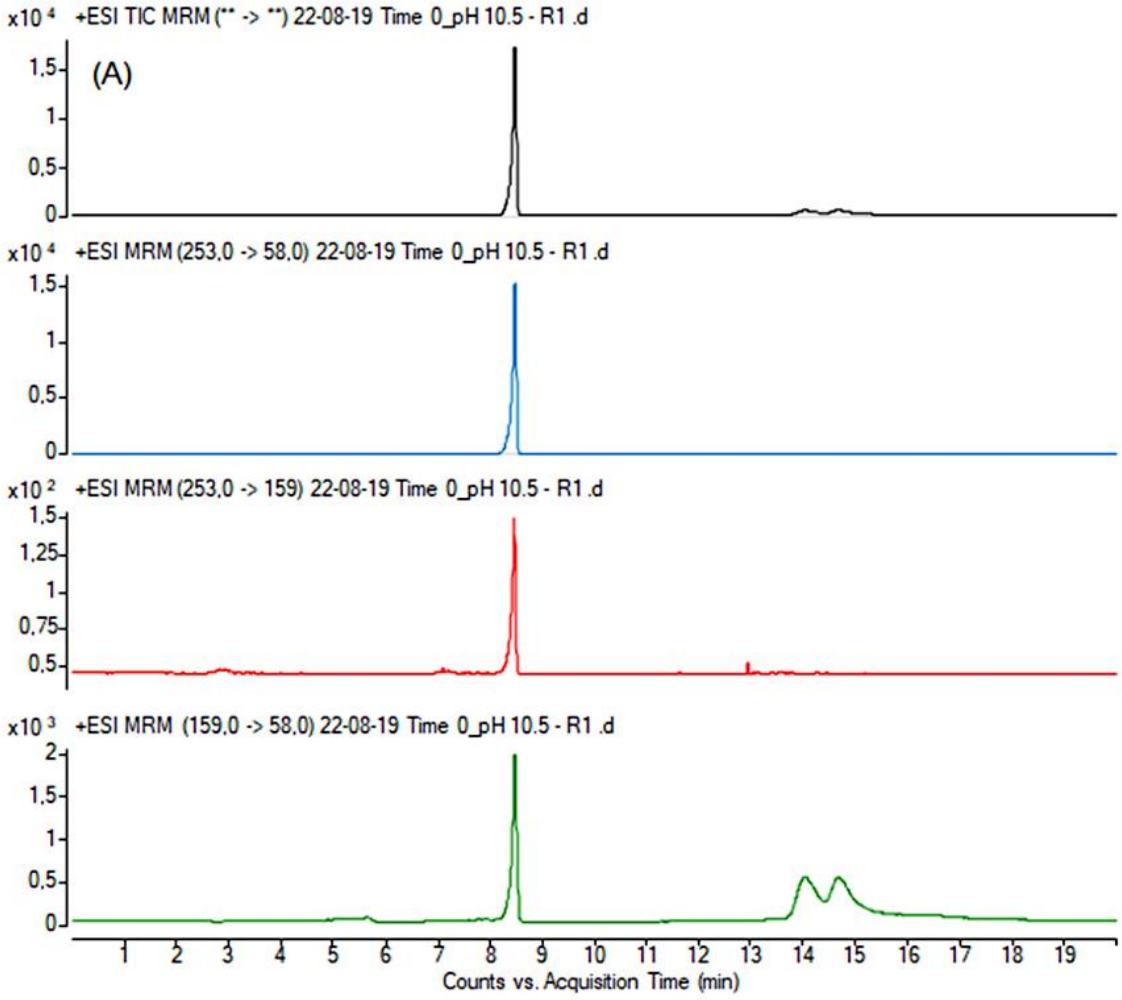


Figure S2: Identification of GNT in ITEP-24 strain samples by ESI-Q-TOF-MS: (A) Extracted Ion Chromatogram (EIC) $[M+H]^+$ *m/z* 253.1060 and the molecular formula calculated from the DataAnalysis Bruker software, (B) Spectrum MS showing the ion $[M+H]^+$ *m/z* 253.1057 and (C) Spectrum MS2 product ions $[M+H]^+$ *m/z* 253.1060.


x10 4 +ESI TIC MRM (** -> **) 22-08-19 Time 0_pH 10.5 - R1 .d
(A)
1.5
1
0.5
0
x10 4 +ESI MRM (253,0 -> 58,0) 22-08-19 Time 0_pH 10.5 - R1 .d
1.5
1
0.5
0
x10 2 +ESI MRM (253,0 -> 159) 22-08-19 Time 0_pH 10.5 - R1 .d
1.5
1.25
1
0.75
0.5
x10 3 +ESI MRM (159,0 -> 58,0) 22-08-19 Time 0_pH 10.5 - R1 .d
2
1.5
1
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0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Counts vs. Acquisition Time (min)

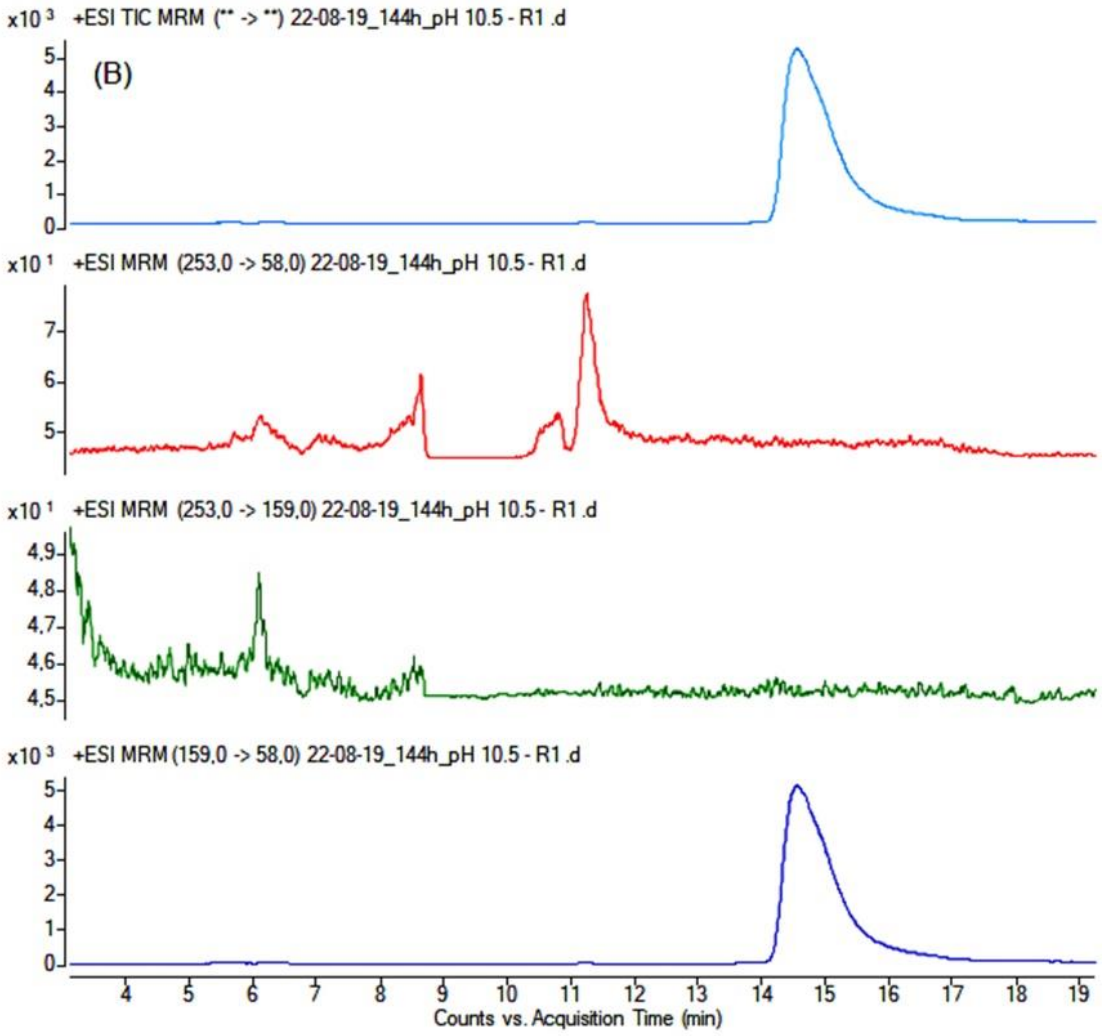

Figure S3: Chromatographic profile of samples of the strain ITEP-24 submitted to the treatment in alkaline pH it is analyzed in multiple reaction monitoring (MRM) modes by LC-QqQ-MS/MS. The chromatograms show the initial time (A) and at 144 hours (B), where the peak of the degradation product ($[M+H]^+$ *m/z* 159> 58) is higher than the GNT peak ($[M+H]^+$ *m/z* 253> 58 and $[M+H]^+$ *m/z* 253> 159).