University of Tripoli
Faculty of Sciences
Department of Zoology

Evaluation of aerial parts of *Echium angustifolium* extracts as an antidote for Ciguatoxins toxicity using molecular modelling and Albino mice models.

BY

Abdelraouf O. Algheryane

Supervised by:

Dr. Massaud S. Maamar
Department of Zoology,
Faculty of Science,
University of Tripoli.

&

Prof. Abdulfatah M.Gbaj
Department of Medicinal Chemistry,
Faculty of Pharmacy,
University of Tripoli.

A research thesis submitted in partial fulfillment of the requirements of the degree of Master of Science in Zoology.

29/1/2022

Declaration

I am Abdelraouf Othmane Algheryane the undersigned hereby confirm that the work contained in this thesis, unless otherwise referenced, is the researcher's work, and has not been previously submitted to meet requirements of an award at this University or any other higher education or research institution, I furthermore, cede copyright of this thesis in favour of the University of Tripoli.

Student name: Abdelraouf Othmane Algheryane.

Signature:……..

Date...../...../.....

Evaluation of aerial parts of *Echium angustifolium* Mill extracts as an antidote for Ciguatoxins toxicity using molecular modeling and Albino mice models.

Abdelraouf Othman Algheryane

University of Tripoli (2020-2021)

Dr Massaud S. Maamar (Supervisor)

Prof Abdulfatah M.Gbaj (Co-supervisor)

Abstract.

Background: Using molecular modeling simulations and Albino mice model ,accumulating evidence indicates a role for Ciguatoxins (CTXs) in protecting from many neurological problems mainly pathognomonic features of postingestion paraesthesiae, dysaesthesiae, heightened nociperception pruritis, arthralgia, myalgia, cerebellar dysfunction, neuropathy and polymyositis. Evidence shows that *Echium angustifolium* Mill constituents can be used to prevent CTX toxicity. Further chemical synthesis of analogues and *in vivo* studies, were necessary to substantiate the obtained results. The aim of the study is to explore the effect of *Echium angustifolium* Mill constituents on ciguatoxins toxicity produced by microalgae (*dinoflagellate, Gambierdiscus* spp) using molecular modelling strategy and *in vivo* mice model.

Methods: A total of 30 adult male Swiss albino mice (2 to 2.6 months old, N=6) were randomly divided into five groups and treated orally as follows. The first five group received only 200 µl (0. 2 g/ml) of the CTXs extract (LD_{50} 0.45µg/ kg, in mice). Groups 2 -5 were given an equivalent amount of extract with 100 µl, 200µl, 300µl and 400µl of aqueous *Echium angustifolium* Mill extract orally (0.043 g/ml), respectively. The number of deaths was recorded within twenty-four hours. Similar experiments were conducted with liver, brain and viscera extracts using groups 6 to 20. Molecular modeling as a tool was used to aid the understanding of the fundamental concepts of structure-activity relationships, and to elucidate the mechanism of action of experimental *Echium angustifolium* Mill constituents with the ciguatoxins toxicity. The physico-chemical properties as well as three-dimensional visualization of electronic and steric molecular properties elucidation of the interaction between *Echium angustifolium* Mill constituents and macromolecules target sodium (Na^+) and potassium (K^+) voltage-gated channels can be calculated and/or suggested by molecular modeling programs. Molecular

dockings of *Echium angustifolium* Mill components with (sodium (Na^+) and potassium (K^+) voltage-gated channels) were accomplished by Auto Dock 4.2 software.

Results: The *Sarpa salpa* toxin introduced to the experimental mice, caused different histopathological effects on the four different tissues and different LD_{50} values. The mice affected by the toxin exhibited typical signs of neurotoxin disorders including hypothermia, considerably reduced locomotors activity during the first three hours and eventually breathing failure showing a significant difference in toxicity between the four extracts. Clearly, the concentrations of toxins in organs were different and can be ranked in ascending order; flesh, brain, liver, and lastly the viscera extract. The *Echium angustifolium* Mill aqueous extract significantly increases mean survival time up to 5±1 days and protects animals from death if compared with the control. *Echium angustifolium* Mill aqueous extract if used at a higher dose was found to be more effective against *Sarpa salpa* toxin. The binding energies of *Echium angustifolium* Mill constituents to voltage-gated Na^+ (6AGF) and K^+ (1BL8) channels obtained by the molecular docking strategy are significant. The modeling studies show that there are *van der Waals*, hydrogen bonding and electrostatic interactions between *Echium angustifolium* Mill constituents with voltage-gated channels.

Conclusion: Ciguatera fish poisoning is seafood intoxication due to the consumption of tropical coral reef fishes that have built up CTXs in their tissues. *Echium angustifolium* Mill aqueous extracts exhibit a positive activity in treating CTX toxicity. The results indicated that *Echium angustifolium* Mill constituents form hydrogen bonds with active sites of Na^+ and K^+ channels and protect the mice from CTX toxicity. The positive activity in mice suggests a promising detoxifying action caused by ciguaintoxication.

Keywords: *Echium angustifolium* Mill aqueous extracts inhibitor, CTXs toxicity.

تأثير المستخلص المائي لنبات *حنة العقرب* (*Echium angustifolium* Mill) كترياق لسمية سيجواتوكسين (Ciguatoxins) CTXs بأستخدام النمذجة الجزيئية ونمادج الفئران المُهق.

عبدالروؤف عثمان الغرياني

جامعة طرابلس (2020-2021)

د مسعود سالم معمر (مشرف أول)

ا.د.عبدالفتاح مصباح قاباج (مشرف ثان)

المستخلص

تمهيد: بأستخدام محاكاة النمذجة الجزيئية في الجانب النظري والتجارب علي الفئران المُهق في الجانب العملي، تشير النتائج بأن سيجواتوكسين Ciguatoxins (CTXs) له دورفي العديد من الأضطرابات العصبية بشكل رئيسي، وصفة أعراضه مثمتلة في الوخز والتنميل، وخلل الحس، والتهاب الحكة الشديدة، وألم المفاصل، والألم العضلي، والخلل الوظيفي المخيخي، والتهاب الأعصاب. تشير الدلائل إلى أنه يمكن استخدام مستخلص نبات *حنة العقرب* *Echium angustifolium* Mill لمنع سمية CTX. لإثبات النتائج التي تم الحصول عليها. الهدف من الدراسة هو استكشاف تأثير مستخلاصات نبات *حنة العقرب* *Echium angustifolium* Mill على سمية مادة سيجواتوكسين ciguatoxins التي تنتجها الطحالب الدقيقة (dinoflagellate، *Gambierdiscus* spp) باستخدام برامج النمذجة الجزيئية واختبار الفئران .

المواد وطُرق العمل: تم تقسيم 30 من الفئران الذكور البالغين من العمر (من 2 إلى 2.6 شهرًا ، N = 6) إلى خمس مجموعات وتم أعطاها الجرعات عن طريق الفم على النحو التالي. أعطيت كل المجموعات الخمس الأولى 200 ميكرولتر (0. 2 جم/مل) من مستخلص مادة CTXs (0.45 LD_{50} ميكروغرام/كجم في الفئران). أعطيت المجموعات 2 -5 كمية مكافئة من مستخلص نبات *حنة العقرب* *Echium angustifolium* Mill المائي عن طريق الفم بجرعات 100 ميكرولتر ، 200 ميكرولتر ، 300 ميكرولتر و 400 ميكرولتر (0.043 جم/مل)، على التوالي.

تم تسجيل عدد الوفيات خلال أربع وعشرين ساعة، وتم إجراء تجارب مماثلة على مستخلصات الكبد والدماغ والأحشاء باستخدام المجموعات من 6 إلى 20. وأستخدِمت النمذجة الجزيئية كأداة مساعدة في فهم العلاقات الاساسية بين البنية والنشاط ، وتوضيح آلية عمل مكونات نبات *حنة العقرب* *Echium angustifolium* Millمع سمية سيجواتوكسين ciguatoxins. يمكن أن تكون الخصائص الفيزيائية والكيميائية بالإضافة إلى التصور الثلاثي الأبعاد والخصائص الجزيئية الإلكترونية والفراغية تقوم بتوضيح تأثير ودرجة التفاعل بين

مكونات نبات *حنة العقرب* *Echium angustifolium* Mill والجزيئات الكبيرة المستهدفة لقنوات بوابات الجهد للصوديوم (Na^+) والبوتاسيوم (K^+). وأستطعنا حساب و/أو المقترح بواسطة برامج النمذجة الجزيئية. تم عمل الإرساء الجزيئي لمكونات نبات *حنة العقرب* *Echium angustifolium* Mill مع قنوات الصوديوم (Na^+) و البوتاسيوم (K^+) ذات بوابات الجهد بواسطة برنامج Auto Dock 4.2.

النتائج: تم أختبارالسم المستخلص من أنسجة سمكة *ساربا سالبا* *Sarpa salpa* على الفئران المعملية مما أدى إلى نتائج LD_{50} مختلفة. أظهرت الفئران المصابة التى أعطيت هدا السم أعراض مرضية للسموم العصبية بما في ذلك انخفاض درجة حرارة الجسم ، وتضاؤل كبير في النشاط الحركي خلال الساعات الثلاث الأولى وفشل التنفس في النهاية مما أظهر اختلافًا كبيرًا في السمية بين الانسجة الأربعة المستخلصة من السمكة. من الواضح أن تركيزات السموم في الأعضاء كانت مختلفة ويمكن ترتيبها بترتيب تصاعدي؛ اللحم والدماغ والكبد وأخيرًا خلاصة الأحشاء وهي الأقوى في التركيز. يزيد المستخلص المائي لنبات *حنة العقرب* *Echium angustifolium* Mill بشكل كبير من متوسط وقت البقاء على قيد الحياة حتى 5 ± 1 يومًا ويحمي الحيوانات من الموت إذا ما قورنت بمجموعة التحكم. المستخلص نبات *حنة العقرب* *Echium angustifolium* Mill المائي إذا تم استخدامه بجرعة أعلى سوف يكون أكثر فعالية ضد سم *Sarpa salpa*. تعد طاقات الربط لمكونات نبات *حنة العقرب* *Echium angustifolium* Mill لقنوات (Na^+,6AGF) و(K^+,1BL8) ذات بوابات الجهد التي تم الحصول عليها قوية الارتباط بواسطة استراتيجية الالتحام الجزيئي. تظهر دراسات النمذجة أن هناك روابط *van der Waals* فان دير وال الهيدروجينية وتفاعلات كهروستاتيكية بين مكونات نبات *حنة العقرب* *Echium angustifolium* Mill مع قنوات الجهد كهربائي.

الخلاصة: سمية سيجواتوكسين ciguatoxins هو تسمم المأكولات البحرية بسبب استهلاك أسماك الشعاب المرجانية الاستوائية التي تراكمت CTXs في أنسجتها. تُظهر المستخلصات المائية نبات *حنة العقرب* *Echium angustifolium* Mill نشاطًا إيجابيًا في علاج سمية CTXs. أشارت النتائج إلى أن مكونات نبات *حنة العقرب* *Echium angustifolium* Mill تشكل روابط هيدروجينية مع مواقع نشطة لقنوات Na^+ و K^+ وتحمي الفئران من سمية CTX. يشير النشاط الإيجابي في الفئران إلى إجراء واعد لإزالة السموم بسبب تسمم سيجواتوكسين ciguatoxins.

الكلمات الدالة: المستخلص المائي، نبات *حنة العقرب*، *Echium angustifolium* Mill ، مثبط لسمية سيجواتوكسين CTXs.

Dedication

I dedicate this work to

my parents and all my family members.

Acknowledgments

First and foremost, my gratitude to almighty Allah for giving me the health, ability, patience and opportunity to complete this thesis.

I wish to express my deep appreciation to my supervisors Dr Massaud Salem Maamar Associate Professor at the Zoology Department, Faculty of Sciences, University of Tripoli, and Prof. Abdulfatah M. Gbaj, Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, Libya. For their advice, ideas, support, patience and continuous encouragement throughout this study.

I am very thankful to Prof. Mohammed Abuhadra, Botany Department, University Of Tripoli Libya, and Prof. Taher Ahmed Shaibi, Zoology Department, Faculty of Sciences, University of Tripoli, and Dr Inass A. Sadawe, and Dr Nisreen H. Meiqal. Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, Libya. For all advice and support in my experimental work.

Finally, I wish to express my sincere thanks to everybody whom I forgot to mention by name and who has helped me by whatsoever means that gave me assistance to accomplish this work.

Contents

List of Figures

List of Tables.

Abbreviations

CFP	Ciguatera Fish Poisoning.
CTXs	Ciguatoxins.
AP	Action potentials.
MTX	Maitotoxin.
GI	Gastrointestinal.
TSRI	The Scripps Research Institute.
RMS	Root Mean Square.
TTX	Tetrodotoxin.
Na^+ / K^+	The sodium-potassium ion.
Na^+ / K^+ pump	The sodium-potassium pump.
LD_{50} ‘Lethal dose50	Fifty percentage lethal dose

Chapter 1. Introduction

The toxin is a poisonous substance produced within living cells or organisms. It causes harm to organisms when sufficient quantities are absorbed, inhaled or ingested. Some poisons make an organism sick, others may cause it to die and yet others may lead to subtle changes in health that may not be noticed for years.

Toxicology is the study of harmful effects of chemicals on living organisms. For example, dioxins, some pesticides and nerve gases are poisonous manufactured chemicals, whereas, belladonna, Botulinum and Tetrodotoxin and Ciguatoxins.

The poisonous are naturally produced chemicals. There are also poisonous substances that occur naturally in the ground, such as asbestos and lead. The venom contains toxins that are injected by a bite (for example, from a spider) or snakes (for example, from a wasp) to cause their effect.

Venom is defined as a substance produced in glands of snakes, spiders, scorpions, centipedes, wasps, bees, etc., and endowed with the means of injecting toxic substances into the aggressor or prey through stings or teeth, which can be solid, grooved or canaliculated. Conversely, poison, in general, is used solely for defense and refers to substances produced by animals like amphibians (Mailho-Fontana *et al.*, 2020).

The poisonous toad *Rhinella icterica* has honeycomb- like internal architecture of the parotoid, where each alveolus accommodates a large poison gland. Taking into account the existence of other types of toxin production and delivery mechanisms that do not perfectly fit the definitions of venom or poison, (Nelsen *et al.*, 2014). Toxungen was coined to describe poisonous biological fluids given to the victim's body surface without an accompanying wound. "Whereas poison distribution is largely passive and relies mostly on the victim's efforts to introduce the toxins, Toxungen delivery is dependent on actions made by the toxic organism," the scientists add". This group includes the fire salamander (*Salamandra salamandra*), spitting snakes (*Naja* spp. and *Hemachatus haemachatus*), the Texas horned lizard (*Phrynosoma cornutum*), and invertebrates like bombardier beetles (family Carabidae) (Nelsen *et al.*, 2014).

Poisons and venoms are both poisonous compounds that, while they enter the victim's body in different ways, share the same mechanism of action. Venom is defined as a chemical generated in the glands of snakes, spiders, scorpions, wasps, bees, and other insects and capable of being injected by stings or teeth that can be solid, grooved, or hollow. By contrast, poison, in general, acts only for defense and refers to substances produced by animals such as amphibians (Mailho-Fontana *et al.,* 2020). They do not have injection mechanisms, depending exclusively on the work of the aggressor. Poisons in animals also consist of glands (or stored in reservoirs), which run through the mucous membrane, especially the oral mucosa, or directly through healthy skin but what happens in CTX is the toxins are passed on to the food chain and after they reach an adequate concentration they incite human poisoning known as ichthyosarcotoxism (Nelsen *et al.,* 2014; Zhang 2015). Toxins may be classified as exotoxins (those excreted by an organism, for example, bufotoxin) or endotoxins (toxins that are structurally part of bacteria, for example, botulinum).

An example of the most toxic compound is the toxic substance botulinum, which is produced by bacteria. *Clostridium botulinum*, is a million times more deadly than the most deadly manufactured compound dioxin. Botulinum is the compound that is used in Botox® a popular method of reducing wrinkles from the skin. The toxin blocks nerve impulses and temporarily paralyses the muscles that cause wrinkles, giving the skin a smoother appearance. Botulinum is also used for medical procedures such as the treatment of muscle spasms. Toxins cause harm to organisms when the toxic compound comes in contact with or is absorbed by body tissues. These compounds interact with parts of the body. Toxins vary greatly in the severity of their effect, ranging from minor but acute (bee stings) to almost immediately deadly (botulinum) (Nelsen *et al.,* 2014).

Some food can contain harmful natural occurring toxins which may lead to adverse reactions in humans and have a carcinogenic effect. The human being is exposed to several types of toxins in the surrounding environment, including food toxins that cause him many diseases to the point of death, and these types of marine toxins that reach him through the food chain such as Ciguatera Fish Poisoning and Ciguatera food poisoning; Ciguatera is an unusual form of food poisoning most

typically caused by fish that live in warm ocean waters. The poisoning is caused by eating fish containing the ciguatera toxin. The contaminated fish have eaten smaller fish that eats the algae that shelter a tiny organism responsible for producing a toxin that causes ciguatera (Nelsen *et al.,* 2014).

Ciguatera Fish Poisoning is the most often reported seafood poisoning globally, causing significant physical and functional effects in people. Ciguatera toxins CTXs are lipid-soluble, heat-stable cyclic polyethers generated by Gambierdiscus toxicus, a dinoflagellate found in marine benthic algal plankton (Achaibar *et al.,* 2007).

Ciguatera Fish Poisoning is a problem originally occurring tropical and subtropical Pacific and Indian Ocean regions, and in the tropical Caribbean (Lehane & Lewis 2000) CFP is a problem originally occurring in tropical and subtropical Pacific and Indian Ocean regions, and in the tropical Caribbean (Lehane 2000; Lewis 2001).

Fishes that cause CFP are those that inhabit warm seas around the world, particularly in the vicinity of coral reefs (Stewart *et al.,* 2010). Herbivorous fish and the macrophytic algae on which it lives devour the microalgae. Toxins are transferred via the food chain, and when they reach a high enough concentration, they cause ichthyosarcotoxism, a type of human poisoning. Contaminated seafood is difficult to distinguish by odor, sight, or taste, making it difficult to avoid.

All fish species associated with coral reefs may be hazardous, especially those at the top of the food chain (sea perch, groupers, barracudas, sharks, moray eels, and so on (Grant 1997). With no treatment for this poisoning, research and work had to be done to find an antidote to this poison. It was noticed at the local level that there is a folk remedy where a local seasonal plant works to stop the effect of scorpion venom called *Echium angustifolium* Mill scorpion henna, which made us study the effect of the water extract of this plant on marine toxins. To emphasize these common popular concepts *Echium angustifolium* Mill (family: Boraginaceae) is a Mediterranean wildflower found in Libya, Algeria, Tunisia, Greece, and other Mediterranean countries.

The plant includes Allantoin and pyrrolizidine alkaloids (including Heliosupin), phenolic acid derivatives, flavonoids, and other substances with a variety of biological

actions, yet it is only mildly poisonous to small warm-blooded animals. It is not harmful to humans, because sheep's stomachs neutralize the active elements. This medicinal herb is used as a diuretic, anti-inflammatory, astringent, and antirheumatic in small dosages. However, long-term consumption may cause liver damage or be carcinogenic (Pan *et al.,* 2018) The results of molecular docking can be used to guide and develop a wide range of investigations *(Inserra et al.,* 2017). Hesperidin on Na^+ voltage-gated channels and Ellagic acid on K^+ voltage-gated channels were explored among all the compounds tested utilizing molecular modeling against CTX.

The findings demonstrated that the elements of *Echium angustifolium* Mill establish hydrogen bonds with the active sites of Na^+ and K^+. The ability of an aqueous extract of *Echium angustifolium* Mill to lessen or revoke the effect of CTXs in mice was tested in this study. The interaction of *Echium angustifolium* Mill aqueous extract with CTXs may result in CTXs' actions being neutralized or inhibited

Hesperidin on Na^+ voltage-gated channels and Ellagic acid on K^+ voltage-gated channels were explored among all the compounds tested utilizing molecular modeling against CTX. The findings demonstrated that the elements of *Echium angustifolium* Mill establish hydrogen bonds with the active sites of Na^+ and K^+. The ability of an aqueous extract of *Echium angustifolium* Mill to lessen or revoke the effect of CTXs in mice was tested in this study. The interaction of *Echium angustifolium* Mill aqueous extract with CTXs may result in CTXs' actions being neutralized or inhibited.

1.1. Aims and objectives.

The aim of this study is to explore the effect of *Echium angustifolium* Mill constituents on ciguatoxins toxicity produced by microalgae (dinoflagellate, *Gambierdiscus* spp.) using molecular modelling strategy and in vivo mice model. One of the therapeutic strategies for neutralizing the ciguatoxins toxic components (agonists at receptors on voltage-gated channels) comes from the knowledge of the mechanism of action of the different types of ions channel agonists and this strategy can aid in developing drugs with greater specificity and effectiveness. In this context, natural products such as *Echium angustifolium* Mill present itself as formidable ion channel

protector. They are a promising source for adjuvant molecules in combination therapies and may minimize ciguatoxins damage prior to the patient reaching the hospital.

Through testing on the theoretical side using Swiss ADME Programs, it was found that there is no toxicity in the components of *Echium angustifolium* Mill extract. The simulation process was carried out using Auto Dock 4.2 software. It was found that the strength of the association between the sodium and potassium gate and plant components is stronger than CTX good results.

Chapter 2 Literature Review

2.1 Ciguatoxins.

2.1.1 History.

Ciguatoxins poisoning has been documented since the Tang Dynasty (AD 618 to 907), (Achaibar *et al.,* 2007; Goodman *et al.,* 2013; Gingold *et al.,* 2014). Many conventional methods for identifying toxicity in fish, such as discoloration of silver and copper metals or flies and ants aversion, have been debunked(Lange *et al.,* 1992).

2.1.2 Ciguatoxins chemistry.

Multiple CTXs have been discovered and are often used. CTXs is a word used to describe a group of chemicals (Yasumoto 2001). The chemistry and biological function of natural marine toxins Maitotoxin MTX, lysophosphatidylcholine, scaritoxin, and CTX-associated adenosine triphosphatase inhibitor are some of the other chemicals that may be involved in ciguatera toxicity. CTX is a crystalline, colorless, lipophilic, heat-stable substance that can't be frozen or cooked away (Yasumoto 2001; Achaibar *et al.,* 2007; Barrett *et al.,* 2017) (Figure1).

2.1.3 Dosing.

Dosing recommendations for ciguatera toxin are unavailable due to a lack of proof of any clinical application. CTXs concentrations in fish ingested as meals have not been shown to be safe (Friedman *et al.,* 2008; Dickey & Plakas 2010).

2.1.4 Uses and Pharmacology of Ciguatoxins.

Ciguatoxins major pharmacologic activity is to cause persistent depolarization and current leakage by increasing cell permeability to sodium *via* voltage-gated sodium channels in cell membranes (Dickey & Plakas 2010; Friedman *et al.,* 2017). CTX's major pharmacologic activity is to cause persistent depolarization and current leakage by increasing cell permeability to sodium *via* voltage-gated sodium channels in cell

membranes, (Friedman *et al.,* 2017). The action of Anti-cholinesterase activity and cholinomimetic action are said to be involved in the mechanism of action in humans. The Ciguatoxins CTX3C has been chemically synthesized, allowing for more research into its mechanism of action (Hirama 2005).

2.1.5 Pregnancy/Lactation.

Several published case reports of ciguatera poisoning during pregnancy claim that baby symptoms began at the same time as the mother. Tumultuous fetal movements and an intermittent, strange fetal shivering were among the symptoms. There appeared to be no long-term repercussions from CTX exposure in any of the live born infants (one fetus was aborted during the acute phase of poisoning); however, long-term detrimental effects could not be ruled out in one infant exposed just before birth. Ciguatera toxin is secreted in breast milk, and infants who were breastfed during their illness experienced (GI) and pruritic symptoms. To avoid symptoms in breast-feeding newborns, breastfeeding should be stopped (Pearn *et al.,* 1982).

2.1.6 Toxicology.

Ciguatoxins are among the most toxic marine toxins known to science, with a median intraperitoneal fatal dosage (LD_{50}) of 0.45 g/kg in mice (corresponding to 2 to 5 g of original fish meat) (Friedman *et al.,* 2017). CTXs have been linked to negative effects at doses as low as 0.1g/kg. in mice. (Friedman *et al.,* 2017). CTXs fish have a normal appearance, smell, and taste. Freezing or frying does not neutralize the poison. In human samples, there are no good biomarkers for determining toxin exposure.(Friedman *et al.,* 2008; Boada *et al.,* 2010). Methods for identifying CTXs in fish have, however, been established (Dickey & Plakas 2010). The most extensively used assay for detecting CTXs in fish is the mouse bioassay (Friedman *et al.,* 2008). CFP has a wide range of symptoms; diagnosis is based mostly on clinical signs and symptoms, as well as a history of fish consumption (Achaibar *et al.,* 2007; Boada *et al.,* 2010b; Goodman *et al.,* 2013).

In mice, there have been changes in electroencephalogram activity (Kumar *et al.,* 2017). GI effects (nausea, vomiting, diarrhea), neurologic effects (numbness, tingling,

joint pain, headache, disorientation, temperature inversion [cold objects seem hot]), and cardiovascular effects (dysrhythmia, elevated heart rate, hypotension) are some of the symptoms (Goodman *et al.,* 2013). Shock, muscle paralysis, and, in rare situations, death may result in severe cases (Pottier *et al.,* 2001).

Symptoms normally develop 1 to 6 hours after intake; however, the time it takes for symptoms to appear varies widely, with studies finding start times ranging from less than 1 hour to 48 hours (Achaibar *et al.,* 2007; Friedman *et al.,* 2008; Goodman *et al.,* 2013). Neurological symptoms can last for weeks or months, and a tiny percentage of people who are afflicted acquire chronic ciguatera, a multisystem chronic condition (Pottier *et al.,* 2001). Patients should avoid seafood and alcohol for 3 to 6 months during their recovery period (Achaibar *et al.,* 2007; Friedman *et al.,* 2008; Goodman *et al.,* 2013). CTXs toxicity has no antidote; treatment consists on adequate rehydration as well as symptomatic and supportive therapies.

The well-studied treatment for CFP toxicity is mannitol. Mannitol's action is assumed to be achieved by the osmotic decrease of neuronal edema; it may also serve as a scavenger of free radicals created by CTXs, lowering CTX's effect on sodium and/or potassium channels. Mannitol should only be administered as an osmotic diuretic in sufficiently hydrated individuals (Friedman *et al.,* 2008).

2.1.7 Herbal treatment of Ciguatoxins.

The cytotoxicity of mouse neuroblastoma cells induced by ouabain, veratridine, and/or brevetoxin-3 or Pacific ciguatoxin-1 was examined using 31 plant extracts, the majority of which are historically used to treat ciguatera fish poisoning in the Pacific area. A quantitative colorimetric approach was used to measure cell viability. Seven of the 31 plant extracts analyzed showed significant cytotoxicity. Despite this, active compound(s) against brevetoxin (2 extracts), brevetoxin, ouabain, and/or veratridine (3 extracts), or just against ouabain and/or veratridine cytotoxicity was suspected in these plant extracts (2 extracts). 22 of the 24 plant extracts that showed no cytotoxicity on their own were efficacious against the effect of brevetoxin or combined veratridine and brevetoxin (Loeffler *et al.,* 2021).

When the seven most active plant extracts were retested with CTXs instead of brevetoxin, similar findings were observed. In conclusion, the present work reports the first activity assessment of some plant extracts, achieved in vitro on a quite large scale. The fact that 27 plant extracts were found to exert, in vitro, a protective effect against the action of CTXs and/or brevetoxin, paves the way for finding new active compounds to treat ciguatera fish poisoning, provided these compounds also reverse the effects of sodium channel activators Finally, the current study presents the first in vitro activity assessment of various plant extracts on a wide scale. The discovery of 27 plant extracts that protect against the action of CTX and/or brevetoxin *in vitro* opens the door to the development of novel active compounds to treat CFP, provided that these compounds also reverse the effects of sodium channel activators (Wong *et al.,* 2005; Goodman *et al.,* 2013).

2.1.8 Ciguatoxins mechanisms of action.

Activation of neuronal voltage-sensitive Na^+ channels and inhibition of K^+ channels, leading to neuronal excitability, and increased neurotransmitter release.

Elevation of intracellular calcium ion concentration and in addition, causing edema of axons and Schwann cells leading to repetitive action potentials.

2.1.9 Ciguatera Fish Poisoning

The Ciguatera Fish Poisoning (CFP) most commonly observed seafood intoxication in humans has a significant physical and functional impact. CFP toxins are lipid-soluble, heat-stable cyclic polyether's generated by *Gambierdiscus* spp' toxicus, a kind of dinoflagellate found in marine benthic algal plankton. Herbivorous fish, as well as the macrophytic algae on which it lives, devour the microalgae. Toxins are transferred via the food chain, and when they reach a high enough concentration, they cause ichthyosarcotoxism, a kind of human poisoning. Contaminated seafood is difficult to distinguish by odor, sight, or taste, making it difficult to avoid. All fish species associated with coral reefs may be hazardous, especially those at the top of the food chain (seaperch, groupers, barracudas, sharks, moray eels, *etc*) (Chagas *et al.,* 2018).

Several ciguateric toxins have been identified to be involved in the etiology of ciguatera fish poisoning. CTXs and Maitotoxin MTX are the two main poisons (Friedman *et al.,* 2008; Chagas *et al.,* 2018). CTX, a potent marine toxin that is unaffected by freezing or heating, with an LD_{50} of 0.45g/kg in mice Intraperitoneally (i.p.) (Boada *et al.,* 2010a; Kryuchkov *et al.,* 2020). MTX is much more poisonous LD_{50} = 0.13g/kg, in mice (i.p.) and can be biosynthesized in *Gambierdiscus toxicus* cultures.

Gam-bierdiscus toxicus, a tiny marine creature that grows on and near coral reefs in subtropical and tropical waters, produces CTXs and MTX. Herbivorous fish consume *Gambierdiscus toxicus*, which eats bigger carnivorous fish, and both poisons get more concentrated as they go up the food chain. CTXs and MTX are produced by *Gam-bierdiscus toxicus*, a small marine critter that lives on and around coral reefs in subtropical and tropical seas. *Gambierdiscus toxicus*, which consumes larger carnivorous fish, is consumed by herbivorous fish, and both toxins become more concentrated as they go up the food chain (Parsons *et al.,* 2011). CFP is a polymorphous emergency characterized by a wide spectrum of cardiovascular, gastrointestinal (GI), and neurological symptoms, including vomiting, nausea, diarrhea, ataxia, joint pain, reversal of temperature perceptions, coma, and death (47-480 hours) (Gillespie *et al.,* 1986).

Symptoms of gastrointestinal distress usually appear within twenty-four hours after ingesting ciguatera-toxin-contaminated fish.

Even though mortality is uncommon (about 2%), entire recovery takes anything from a few days to a week in light intoxications to many weeks to months, if not years, in severe episodes (Barrett *et al.,* 2017). Aside from the availability of a particular immunological method for assessing toxins in seafood products, clinical diagnosis aids in the identification of persons who have consumed poisonous fish (Gillespie *et al.,* 1986; Blythe *et al.,* 1992).

Potentials CTXs cause neuronal excitability, increased neurotransmitter release, impaired synaptic vesicle recycling, increased intracellular calcium ion concentration,

and edema of axons and Schwann cells, all of which lead to spontaneous and repetitive action potentials (Lewis *et al.*, 1999; Lehane 2000).

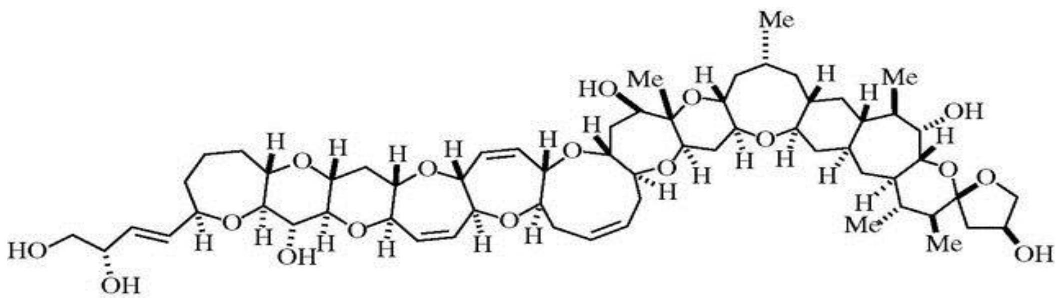


Figure 1 Pacific ciguatoxin-1 (P-CTX-1) chemical structure (Hamilton *et al.*, 2002).

Ciguatera Fish Poisoning is the most frequent natural marine toxin-related sickness in the world; Symptoms include gastrointestinal, neurologic, and cardiac issues (Parsons *et al.*, 2011).

Poisonous fish that contain natural toxins called CTXs cause damage to humans' CTXs. CFP happens when the poison spreads among fish and no storage, preparation, or cooking procedure can stop it. CTXs are odorless, tasteless, colorless, heat-stable, and acid-stable for at least six months at commercial freezing temperatures of -2, -3 degrees Fahrenheit. As a result, preventing and managing CFP necessitates a comprehensive approach. Microscopic algae are known as CTXs and their precursors generate CTXs and their predecessors (dinoflagellate, *Gambierdiscus* spp) Dinoflagellates are bottom-dwelling dinoflagellates (Grant 1997).

They are commonly found in shallow tropical and subtropical seas, clinging to seaweeds, live and dead corals, and other substrates (*i.e.,* surfaces). CTXs production occurs in shallow coastal settings due to *Gambierdiscus* spp. for light and substrate (*e.g.*, reefs, atolls) Oceanic fish that get their nutrition from pelagic (*i.e.*, food chains that occur in open sea waters far from the shore) rather than shallow/coastal food webs are less sensitive to the buildup of contaminants CTXs.

Ciguatoxins are transmitted and processed across the food chain since *Gambierdiscus* cells are eaten by herbivorous fish, which are subsequently eaten by piscivorous fish, who are both eaten by humans. It is thought that CTXs are bioaccumulated and concentrated, with the greatest CTX concentrations seen in fish higher in the food chain (Silver *et al.*, 2014; El Rokh *et al.*, 2018) (Figure 1). Recent research from French Polynesia and Hawaii, on the other hand, show that for many species and families of fish, there is no correlation between fish size and ciguatera toxicity, suggesting that fish size alone is not a sufficient predictor of toxicity (Tiwari *et al.*, 2009; Dong *et al.*, 2014). Membrane Sodium channels are closed when the membrane potential of axons is at rest.

Ciguatoxins molecules cause these channels to open at resting membrane potential, allowing sodium to flood in and depolarize the axonal membrane, resulting in spontaneous and recurrent action potentials (AP). Normally, a regulated inflow of sodium is followed by an outflow of potassium, which keeps the axon electroneutral and facilitates water passage across the membrane (Mattei *et al.*, 2010).

Swelling at the nodes of Ranvier occurs when the dynamic mechanism of influx/efflux is disrupted by CTX (the unmyelinated sections of axons exposed to extracellular fluid in myelinated axons) (Benoit *et al.*, 1996). The enlargement of the Ranvier nodes decreases sensorimotor conduction velocities and inhibits salutatory conduction along the axon (Lewis *et al.*, 1999). Body CTXs are rapidly absorbed from the gastrointestinal system and transported throughout the body, according to an animal laboratory study (Lewis 2001). Furthermore, CTX-sensitive voltage-gated sodium channels have been discovered in all of the CFP-affected systems (*i.e.*, brain, skeletal muscle, heart, peripheral nervous system, and sensory neurons) In addition; CTX-sensitive voltage-gated sodium channels have been found in all (CFP)-affected systems (brain, skeletal muscle, heart, peripheral nervous system, sensory neurons).

These pathways may have a role in the symptomatology of CFP (Benoit *et al.*, 2002; Yamaoka *et al.*, 2004). Cold allodynia, for example, might be caused by a CTX-induced change in the voltage-gated sodium channels in A-delta and C fibers (Cameron & Capra 1993). which convey thermal and pain signals via the spinal cord to the brain

(Cameron & Capra 1993). Nerve conduction investigations in individuals with acute CFP show that both sensory and motor conduction are disrupted (Lewis *et al.*, 1999). In terms of the central nervous system, one animal investigation found that CTX delivered orally and intraperitoneally was detectable in the liver, muscle, and brain four days after treatment.

Ciguatoxins has been shown to pass the blood-brain barrier. This backs up the fact that the central nervous system is involved in human clinical situations of (CFP) (Friedman *et al.*, 2017).

2.1.10. Ciguatera Fish poisoning in the world.

In the United States, 94 outbreaks (418 cases) have been reported to the Center for Disease Control and Prevention (CDC) through the national food-borne surveillance system from 1970 to 1980. This is believed to be a much under-reported disease and therefore it is probable that the number of cases should be much higher (Morris, 1980). From 1983 to 1992 there were 129 outbreaks that involved 508 persons that were reported to the (CDC). Several of these reports came from Hawaii (111) and Florida (10), but there were also sporadic reports from California, Vermont, New York and Illinois. No deaths have been reported (Html 5). 19 In Australia 50-100 cases are reported each year and this is estimated to be about 20% of the actual cases. There are usually not any reports of deaths but it is likely to be one every ten years in Australia (Lehane 2000). Data have been collected from the years 1965 to 1988 and showed 617 cases from 225 outbreaks. The risk of being poisoned by eating coral trout (member of the Serranidae family), a fish often associated with ciguatera, is less than 1/5000 (Lehane 2000).

2.1.11. Maitotoxin.

Maitotoxin (MTX) is a toxin that is often discussed concerning CFP. It is a very potent toxin and it is present in some of the ciguatoxic fish and can be produced by the dinoflagellate *G. toxicus*. The structure is shown in figure 2. In the surgeon fish (*Ctenonhaetus striatus*), which is often associated with CFP, MTX is the dominating toxin in the alimentary tract. Both MTX and CTXs are present in the liver but only

CTXs are present in the meat. It is unlikely that MTX accumulates in the meat of these fish or that this causes human poisonings because of this poor accumulation and it's rather a low potency when administered orally (Lehane & Lewis 2000).

Figure 2 shows the structure of MTX, is a toxin that can be involved in CFP (Pisapia *et al.*, 2017). In culture, *G. toxicus* may produce MTX but little or no CTX (Lehane 2000). Different cultured strains of *G. toxicus* produce different types of MTX, with one strain only producing one single type. MTXs have up to 32 ether rings and even though they are analogous in structure they have no partial structure corresponding to CTX. After intraperitoneal injection, MTX is much more toxic than CTX, but MTX is about 100 times less toxic than CTX, when administered orally (Lewis 2001).

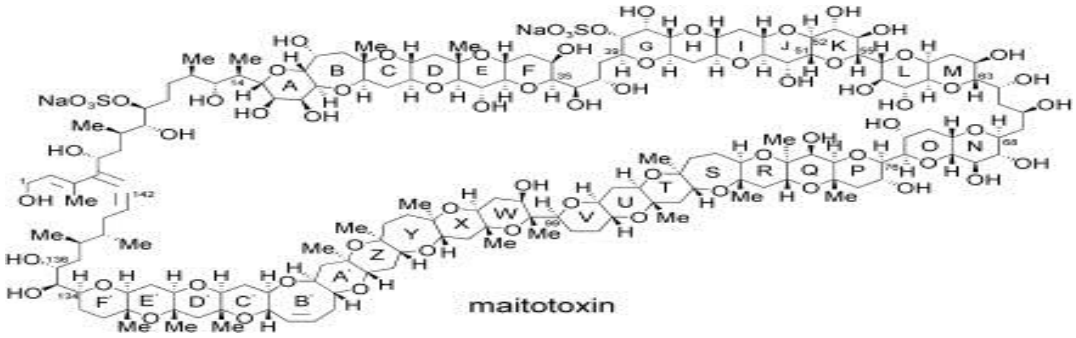


Figure 2 The structure of MTX, a toxin that can be involved in CFP(Nicolaou & Aversa 2011).

2.2 *Echium angustifolium* Mill.

2.2. 1 Taxonomic classification

Kingdom : Plantae

Division : Spermatophyta

Calss : Dicotyledonae

Order : Tubiflorae

Family : Boraginaceae

Genus : *Echium*

Species : *E. angustifolium* Mill.

The family Boranginaceae of 100 genera and 2000 species, distributed chiefly in temperate and tropical regions of the world: represented by 23 genera and 53 species in Libya.

The genus *Echium* L. (Boraginaceae) consists of 67 recognised species, represented in Libya by 12 species, which are native to North Africa, mainland Europe, and the Macaronesia region (the Azores, Madeira, Canary Islands, and Cape Verde). (Zohary, 1978).

2.2.2. Description and morphology:

Perennial herb, 30 – 80 cm, grey, usually branched from base, all plant organs covered with dense hairs. Leaves linear or linear – lanceolate. Inflorescences rather short, terminal or axillary along branches; Corolla narrowly trumpet – shaped. 15 – 25 mm, reddish purple – purplish violet, hairy all over the outside. Fruit nutlet, grayish, glossy, tuberculate, keeled on one side (Fig.3 a,b) (Qaiser, 1979)

Figure 3.a. Habit of *Echium angustifolium* Mill

Figure 3.b. Branch of *Echium angustifolium* Mill

2.2.3. Synonym (s)

E. sericeum vahl. , 2, 35. 1791 : Durand & Barratte, l. c. 171; Pamp. l. c. 196; ll. cc. 383 : Keith, l. c. 447. 1965.: *E. sericeum* var. diffusum (Sibth & Sm.) Boiss. Fl. Or. 4 : 207. 1879 ; *E. diffusum* Sibth. & Smith, Fl. Grace. 2: 69 .i. 182. 1816: *E. elegans* Lehm. , Asperif. 459. 1818; ? *E. distachyum* Viv. , Fl. Libyc. Spec. 8 t. 5.

2.2.4 Local name

Hennat al agrab, idma, Twade Omi.

2.2.5 life-form

Perennial erect herb.

2.2.6 Reproduction

Flowering March to August , seeds.

2.2.7. Economic interest:

From the side of medical use, *Echium* species can be used as folk medicine in Mediterranean region, utilised predominately for their sedative, anti-inflammatory, antioxidant, and anxiolytic properties, treating ailments including fissures of the hands, general abrasions, and even snakebites but unfortunately, the details for treatment of snake bites and cough is poorly documented, with no detail of preparations available (Sharma and Esler, 2008), (Konazewski. *et al.,* 2012; Qaiser, 1979).

2.2.8.Distribution:

This species *E. angustifolium* is very common throughout Libya it can be found in Jebel Nefusa range, Gharyan to Nalut, Sirte, Jebel al-Akhdar , native to North Africa, mainland Europe, and the Canary Islands. In general, they are hardy plants that adapt well to harsh environments. Due to their ability to grow and thrive in austere environments, they have particularly thrived in Australia, South Africa, and America. (Qaiser, 1979).

2.3. Treatment of Ciguatera Fish poisoning.

The treatment involves a 1g/kg intravenous infusion of 10 % or 20% mannitol solution over 30 minutes, given as soon as possible after CFP has been diagnosed. Some patients report a dramatic improvement in their neurological symptoms but some do not respond at all to the treatment. The reasons for these differences are unknown and require further studying but can be related to how long after the meal the mannitol was administered or to the size of the given dose. In non-responding patients, a second infusion is recommended (Friedman *et al.,* 2007). Mannitol does not remove the CTX in the molecule from its site of binding, but it reduces swelling of the Schwann cells, which is induced by the CTXs.(Lewis, 2001). Australian studies show that the diuresis that normally follows an infusion of mannitol, does not appear when it is used to treat CFP. The mannitol infusion should be administered as soon as possible but not until the patient is adequately hydrated (Stewart *et al.,* 2010).

2.4 Prevention.

When eating tropical fish from the affected regions, there is no certain way to avoid CFP since there is no simple way to detect the toxin. The toxic fish is normal in taste and appearance and is therefore impossible to detect (Lehane 2000). Still, there are a few precautions that can be taken to reduce the risk. It is usually safer to eat many small servings (< 50 g) from several different fishes than to eat one large portion (>200g) from a single fish. The concentration of CTXs in the liver, viscera and roe is usually high and consumption of these parts should therefore be avoided (Lewis *et al.*, 1999).

Chapter 3 Materials and Methods.

Laboratory experiments were conducted in the research laboratories of the Department of Medicinal Chemistry, Faculty of Pharmacy-University of Tripoli.

3.1 Materials.

Table 1 show the equipment and consumables using the study.

Table1 Tools used in the study.

Used equipment	The manufacturing company—Enterprise
Kate's anatomy.	Delta Med Surgical. (Pakistan).
Gloves.	Apex Glove Sdn.Bhd. (Malaysia).
Syringes.	Elver- (Turkey).
Eppendorf tubes.	Sigma-Aldrich (Germany).
Whatman. (Filter paper)	Sigma-Aldrich (Germany).
Micropipette 1-1000	Exispin (Korea).

3.1.1 Toxicity screening of the *Echium angustifolium* Mill components using Swiss ADME Programmers.

Accessing http://www.swissadme.ch in a web browser displays directly the submission page of Swiss ADME, where molecules are to be estimated for ADME, physicochemistry, drug-likeness, pharmacokinetics, and medicinal chemistry friendliness properties can be input. As shown in (Figure 4), a black toolbar at the top of the Web page allows the user to navigate within the different Swiss Drug Design tools. A second bar gives admittance to dissimilar information regarding Swiss ADME, amongst which are the FAQ and Help pages as well as legal disclaimer and contacts. The ADME study was carried out using the SWISS ADME predictor. This is a free web tool to evaluate the pharmacokinetics, drug likeness and medicinal chemistry friendliness of small molecules.

The target attention was accessing the molecules which fit into the rule of drug-likeness. The properties like the molecular weight of less than 500 g/mol, less than 5 numbers of hydrogen bond donors, less than 10 numbers of hydrogen bond acceptors and less than 10 rotatable bonds were chosen as criteria, while the selection of molecules to be synthesized (Dong *et al.*, 2014). The search engine further gave a compiled result on lipophilicity and hydrophilicity of these molecules by integrating results obtained from various Log P and S prediction programs called ILOGP, XLOGP3, WLOGP, ESOL, and SILICOS-IT. Log P, a measure of lipophilicity of the molecule is the logarithm of the ratio of the concentration of drug substance between two solvents in a unionized form.

The Lipinski rule prescribes an upper limit of 5 for druggable compounds. The lower the log P values the stronger the lipophilicity of the chemical substance. The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. On the other side, low water solubility goes along with bad absorption, and therefore, the general aim was to avoid poorly soluble compounds. Log S is a unit of expressing solubility in itself, which is the 10-based logarithm of the solubility measured in mol/L. Distribution of Log S in traded drugs reveals a value somewhere between -1 to -4, will be optimized for better absorption and distribution of drugs in the body. (In silico Toxicity Assessment of *Echium angustifolium* Mill components).

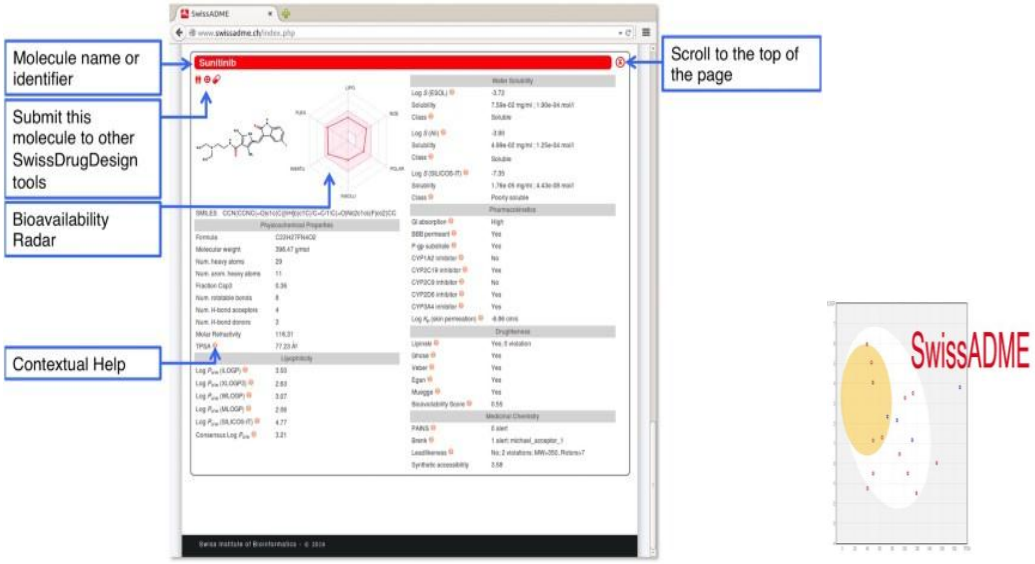


Figure 4 Swiss ADME Programmes (Dong *et al.*, 2014; Banerjee *et al.*, 2018).

3.1.2 Molecular modelling using Computer software.

The starting geometry of the *Echium angustifolium* Mill components were constructed using *chem3D Ultra* (version 8.0, Cambridge soft Com., USA).

The Crystal structures of voltage-gated Na^+ (6AGF, Nav1.4) and K^+ (1BL8, KcsA) channels were downloaded from the Protein Data Bank (Velankar *et al.*, 2021).

The molecular dockings of *Echium angustifolium* Mill components with voltage-gated Na^+ and K^+ were accomplished by *Auto Dock 4.2* software from the Scripps Research Institute (TSRI) (Velankar *et al.*, 2021).

The starting geometry of the *Echium angustifolium* Mill components was constructed using chem3D Ultra (version 8.0, Cambridge soft Com., USA). The optimized geometry with the lowest energy was used for molecular docking. Crystal structures of voltage-gated Na^+ (6AGF, Nav1.4) and K^+ (1BL8, KcsA) channels in a complex with transition-state analogues were downloaded from the Protein Data Bank Https://www.rcsb.org/structure/6AGF and https://www.rcsb.org/structure /1BL8. For sodium and potassium channels, respectively (Figure 5).

Molecular dockings of *Echium angustifolium* Mill components with 6AGF and 1BL8 was accomplished by Auto Dock 4.2 software from the Scripps Research Institute (TSRI) (http://autodock.scripps.edu/). Firstly, polar hydrogen atoms were added to protein molecules. Then, partial atomic charges of 6AGF and 1BL8 and *Echium angustifolium* Mill components were calculated using Kollman methods (Tiwari *et al.*, 2009). In the process of molecular docking, the grid maps of dimensions (62Å X 62Å X 62Å) with a grid-point spacing of 0.376Å and the grid boxes centered were used.

The number of genetic algorithm runs and the number of evaluations was set to 100. All other parameters were default settings. Cluster analysis was performed on the results of docking by using a root mean square (RMS) tolerance of 2.0Å, which was dependent on the binding free energy. Lastly, the dominating configuration of the binding complex of *Echium angustifolium* Mill components and 6AGF and 1BL8 channels fragments with a minimum energy of binding were determined which relied strongly on the information of 3D structures of 6AGF and 1BL8 ion channels binding sites and ultimately generated a series binding complexes, respectively (Figure 5).

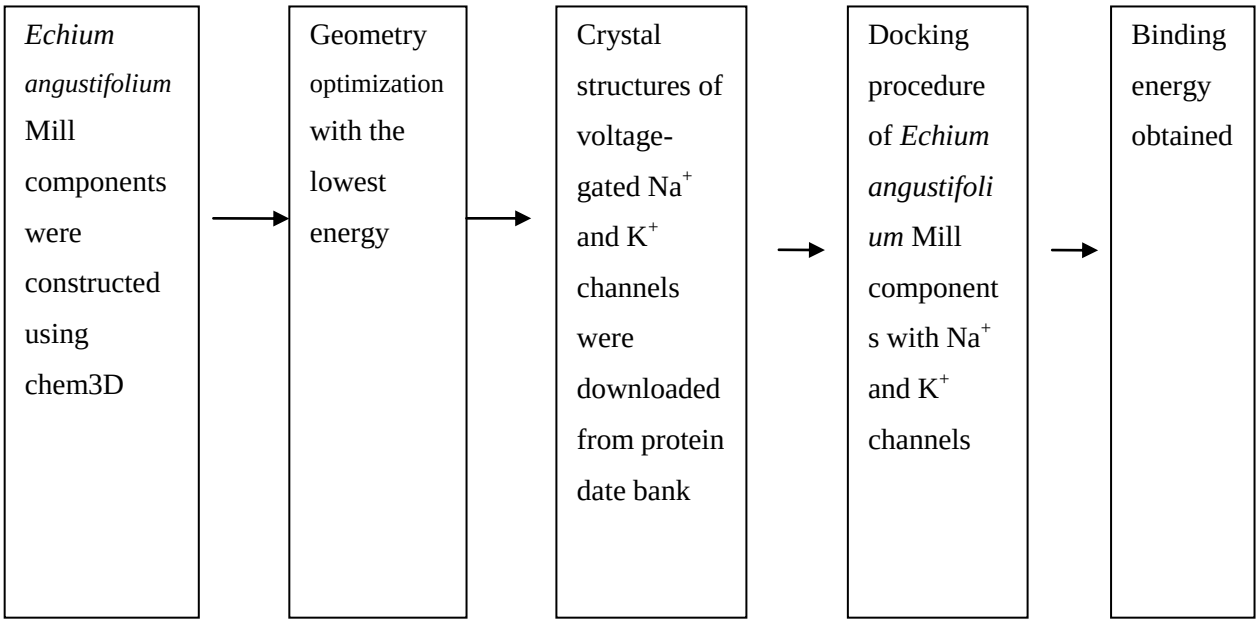


Figure 5 Schematic sketch for molecular docking process.

3.1.3 Animals.

Laboratory bred adult male Swiss albino mice were used for this study. The experimental protocols were approved by the Ethical committee of the Biotechnology Research

3.1.4 Experimental design.

The mice were housed in cages with free access to food and water and allowed to acclimate to animal room conditions, relative humidity of 50-60%, and 12 hours of light and dark cycle for one week before experimental work. After adaptation, the mice at age of 6-8 weeks old with a weight range of 24-30 g were randomly divided into 5 groups (at least 6 mice/group) (Table 2). Animals were bred and experimentation was carried out in the animal house of the Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli

Table 2 Doses of mice in the study**.**

Groups	Condition	Numbers mice	Treatments
Group 0µL	200.µL (0. 2 g/ml) of the extract (CTX) (LD_{50} 0.45µg/ kg in mice)	6	No treatment
Group 100µL	200.µL (0. 2 g/ml) of the extract (CTX) (LD_{50} 0.45µg/ kg in mice) + 100µl (0.043 g/ml) of *aqueous Echium angustifolium* Mill extract orally.	6	100µL (0.043 g/ml) of aqueous *Echium angustifolium* Mill extract orally
Group 200µL	200 µL (0. 2 g/ml) of the extract (CTX) (LD_{50} 0.45µg/ kg in mice) + 200µl (0.043 g/ml) of *aqueous Echium angustiflium* extract orally.	6	200µL (0.043 g/ml) of aqueous *Echium angustifolium* Mill extract orally
Group 300µL	200.µL (0. 2 g/ml) of the extract (CTX) (LD_{50} 0.45µg/ kg in mice) + 300µl (0.043 g/ml) of *aqueous Echium angustiflium* extract orally.	6	300µL (0.043 g/ml) of aqueous *Echium angustiflium* extract orally
Group 400µL	200 µL (0. 2 g/ml) of the extract (CTX) (LD_{50} 0.45µg/ kg in mice) + 400µl (0.043 g/ml) of *aqueous Echium angustiflium* extract orally.	6	400µL (0.043 g/ml) of aqueous *Echium angustiflium* extract orally

3.2 Methods.

3.2.1 Collection of plant material and preparation of aqueous extract.

Plants were collected from the (Garabolle Zone, Tripoli, Libya) in March 2020 and *Echium angustifolium* Mill were identified and authenticated by Prof. Dr. M Abuhadra, in the herbarium of department of Botany, Faculty of Science, University of Tripoli, Libya. Voucher specimens of the identified plant were deposited in herbarium of Botany department, Faculty of Science, UoT (Voucher number: D6868123).

The sample was initially rinsed with distilled water at room temperature. The leaves with the stems were cut into smaller pieces and 1.29 g of the sample was taken. The cut leaves and stems were then grinded in a homogenizer (HO4A Edmund Buhler GmbH, UK) along with 30 ml of distilled water Figure 6. The resulting aqueous solutions were filtered under vacuum using a Millipore filter (0.45 µm, GHD Acrodisc GF, UK) and the filtrate was stored at 4°C.

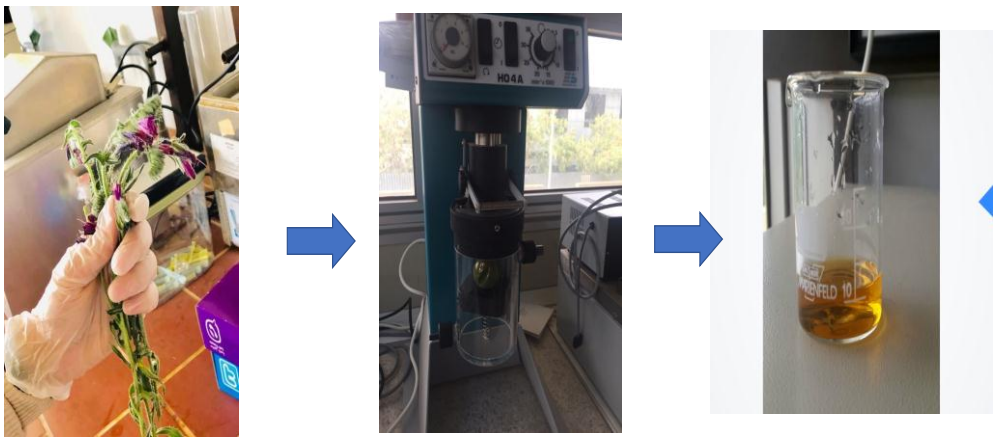


Figure 6 Plant materials and preparation of aqueous extract.

3.2.2 Sampling and toxin extraction.

Specimens of fish (n=6) *Sarpa salpa*, known commonly as the dream fish, *salema salema*, cow bream, porgy or gold line were used for experiments. The fishes were caught at different locations on the Tripoli-Libya coast during January 2020. Immediately following the collection, the fishes were eviscerated and frozen at -20°C until use.

3.2.2.1 Extraction procedure.

One gram from each organ [(flesh (including muscles and skin), liver, brain and viscera)] was homogenized with 5ml of 0.1% acetic acid Figure 7. The samples were boiled in a water bath for 10min at 50°C and then cooled to room temperature. The samples were centrifuged at 3000 RPM for 10 minutes at 10°C. The obtained supernatant from specimens was collected. Each aliquot was conserved at -20°C until further use Figure 8.

Figure 7 *Sarpa salpa* tissue sampling.

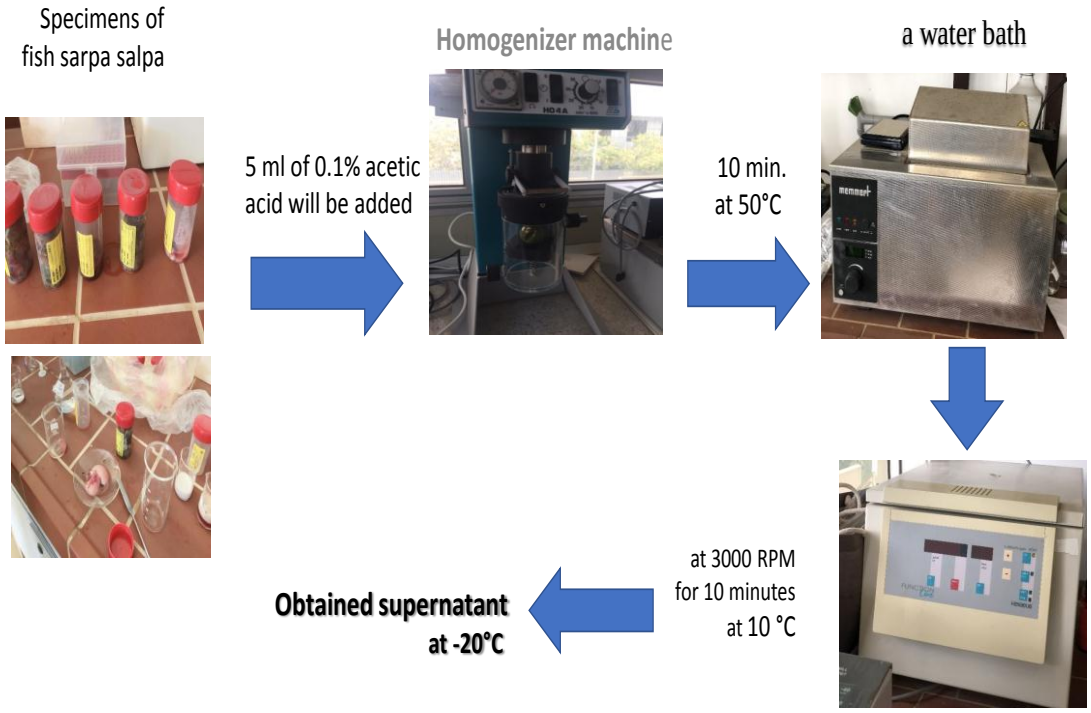


Figure 8 Toxin extractions.

3.2.3 Molecular docking.

The starting geometry of the *Echium angustifolium* Mill components was constructed using chem3D Ultra (version 8.0, Cambridge soft Com., USA). The optimized geometry with the lowest energy was used for molecular docking.

Crystal structures of voltage-gated Na^+ (6AGF, Nav1.4) and K^+ (1BL8, KcsA) channels in a complex with transition-state analogues were downloaded from the Protein Data Bank (Velankar *et al.*, 2021) (Figure 10) for sodium and potassium channels, respectively (Figure 5). Molecular dockings of *Echium angustifolium* Mill components with 6AGF and 1BL8 was accomplished by Auto Dock 4.2 software from the Scripps Research Institute (TSRI) (http:// autodock.scripps.edu/). Firstly, polar

hydrogen atoms were added to protein molecules. Then, partial atomic charges of 6AGF and 1BL8 and *Echium angustifolium* Mill components were calculated using Kollman methods.22 In the process of molecular docking, the grid maps of dimensions (62Å X 62Å X 62Å) with a grid-point spacing of 0.376Å and the grid boxes centered were used.

The number of genetic algorithm runs and the number of evaluations was set to 100. All other parameters were default settings. Cluster analysis was performed on the results of docking by using a root mean square (RMS) tolerance of 2.0Å, which was dependent on the binding free energy. Lastly, the dominating configuration of the binding complex of *Echium angustifolium* Mill components and 6AGF and 1BL8 channels fragments with a minimum energy of binding were determined which relied strongly on the information of 3D structures of 6AGF and 1BL8 ion channels binding sites and ultimately generated a series binding complexes, respectively (Figure 9, 10).

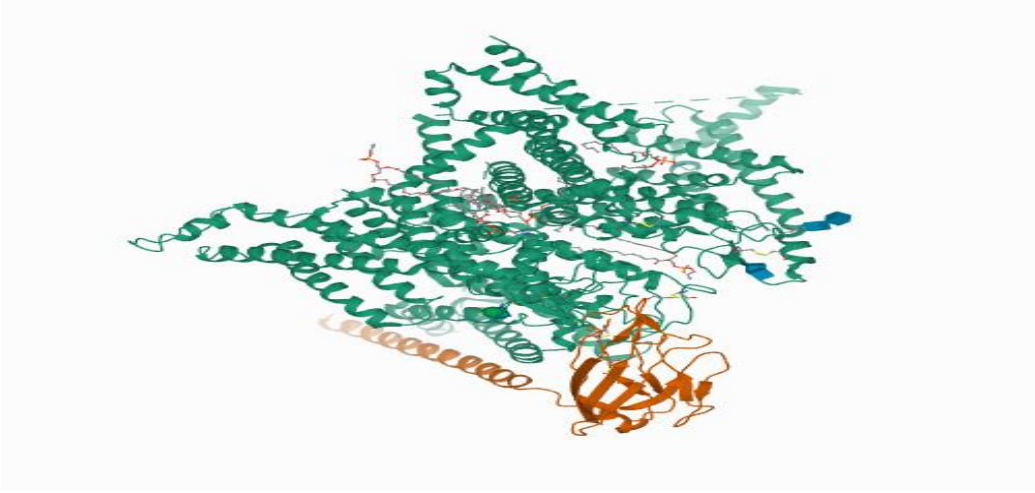


Figure 9 Structure of the human voltage-gated sodium channel (6AGF)

(Pan *et al.*, 2018).

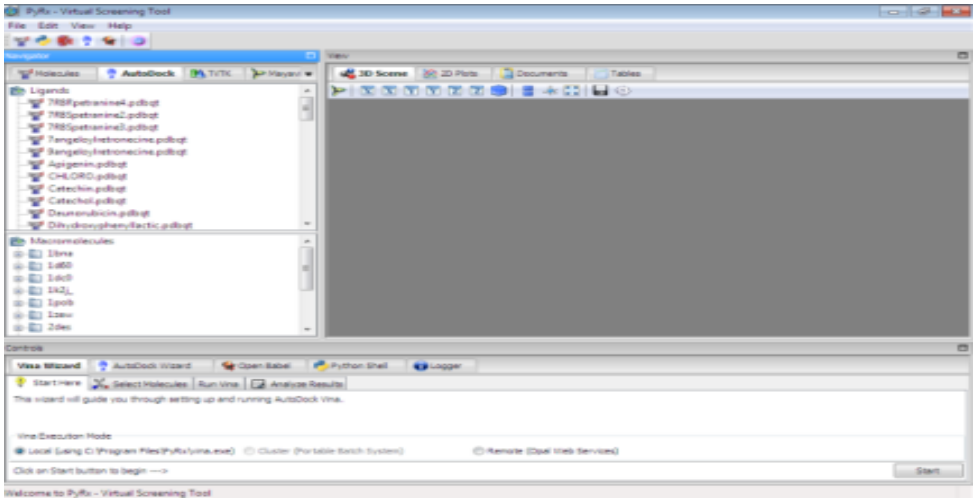


Figure 10 Protein Data Bank (Berman *et al.*, 2000)

3.2.4 Acute toxicity study.

Experimental design.

Acute toxicity studies were performed to determine the LD_{50} value in the experimental animals. The intention of performing acute toxicity studies were to establish the therapeutic index of *Echium angustifolium* Mill and to guarantee *in vivo* safety. The male mice were randomly allocated into four groups.

The first group served as control and was given 0.9 % normal saline orally at 0.2 ml/kg body weight. The remaining groups were given a single oral dose of either 50, 100 or 400 or 800m mg/kg body weight *Echium angustifolium* Mill extract respectively, (Marrelli *et al.*, 2020) (Figure11) Similar acute toxicity studies were performed for the flesh (keletal muscles and fat), liver, brain and viscera extracts. Acute toxicity experiments were also performed with twenty male mice randomly allocated into five groups (n=5). The first group served as control and was given 0.9% normal saline orally at 0.2ml/kg body weight. The remaining four groups were given a single oral dose of either 50, 100, 300 or 400µl of the flesh extract (1g/5ml 0.1 acetic acid). A similar protocol was used for liver, brain and viscera extracts.

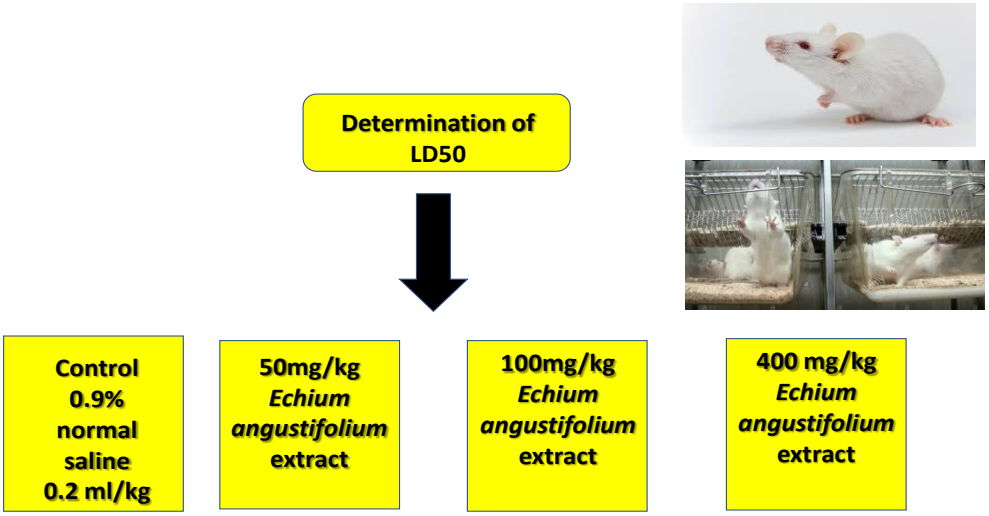


Figure 11 Acute toxicity study.

3.2.5 Detoxication of CTXs by *Echium angustifolium* Mill extract.

Experimental design.

The animals (albino mice) utilized in this investigation were separated into five groups, each with six mice, after a week of acclimation to laboratory settings (male). The first five group wase given just 200 µl (0. 2 g/ml) of the *Sarpa salpa* extracts (1g/5ml, 0.1% acetic acid with LD_{50}.45g/kg in mice) and the dose was confirmed and compared with previous study(Sanders, Jr. 1987; Friedman *et al.*, 2008). Groups(2-3-4-5) were given a similar quantity of extract orally (0.043 g/ml) with 100µl, 200µl, 300µl, or 400µl of aqueous *Echium angustifolium* Mill extract, respectively.

Within twenty-four hours, the number of deaths had been documented. Using groups 6 to 20, similar tests were repeated using liver, brain, and viscera extracts. The remaining four group wase received a single oral dose of either 100µl, 200µl, 300µl or 400µl of the flesh extract (1g/5ml 0.1 acetic acid). A similar protocol was used for liver, brain and viscera extracts (Figure 12).

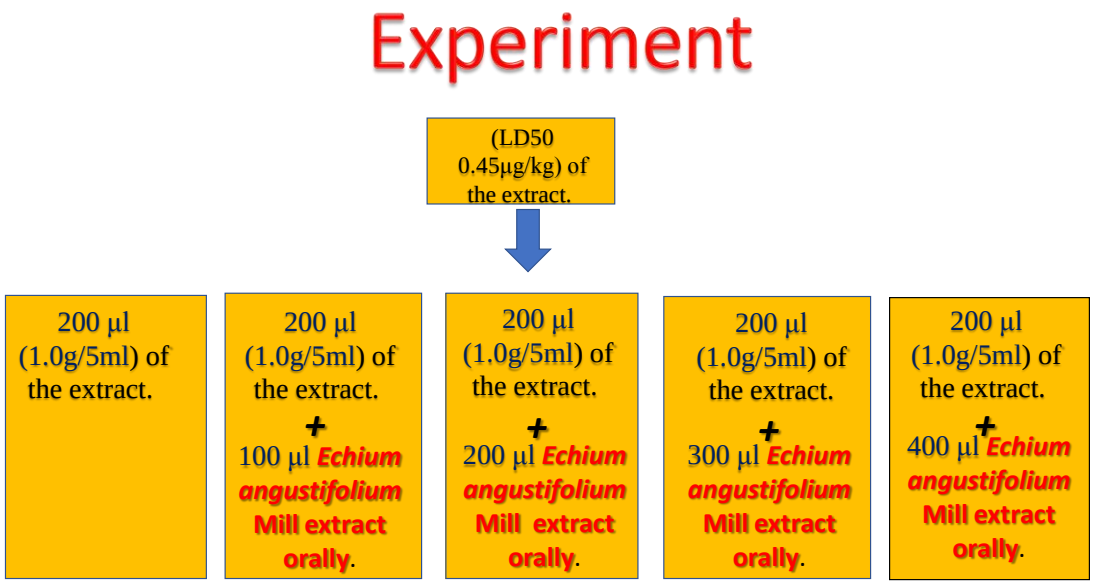


Figure 12 Detoxication of CTXs by *Echium angustifolium* Mill extract.

3.2.6 In silico toxicity Assessment of *Echium angustifolium* Mill components.

The online program ProTox-II: a webserver for the prediction of chemical toxicity was used to examine the *in silico* toxicity of all *Echium angustifolium* Mill chemical constituents (http://tox. charite.de/protox II (Banerjee *et al.*, 2018).

3.2.7 Statistical analysis.

The difference among various treatment groups and control groups were analyzed using followed using unpaired Student's t-test as the one-way analysis of variance (ANOVA) which is used to determine whether there are any statistically significant differences between the means of independent (unrelated) groups. The results were expressed as the mean ± SEM of the number of experiments done, with $P < 0.05$ indicating a significant difference between groups.

Chapter 4 Results

4.1 Acute toxicity study of *Echium angustifolium* Mill aqueous extract (1.29g/30ml) given to albino mice.

Within two weeks, an *Echium angustifolium* Mill aqueous extract was determined to be safe up to 140 mg/kg orally (Table 3), no mortality among mice.

Table 3 The effect of *Echium angustifolium* Mill extract (1.29g in 30 ml distilled water (single dose)) on mice

Evaluation of doses difference group		No. of animals	No. of survivors
Group 1	0.9% normal saline at 0.2 ml/kg	5	5
Group 2	50 mg/kg	5	5
Group 3	100 mg/kg	5	5
Group 4	400 mg/kg	5	5
Group 5	800 mg/kg	5	5

4.1.1 LD_{50} and lethality testing.

Lethal Dose 50 tests (tests LD_{50}) is defined as "the animal research procedure in which any material or substance is administered to animals for the purpose of determining the concentration or dose of the material or substance which will achieve any predetermined death rate. The value of LD_{50} for the four extracts is the dose required to kill half the mice after a specified test duration. LD_{50} figures are frequently used as a general indicator of a substance's acute toxicity. A lower LD_{50} is indicative of increased toxicity. Tables 4-7 represent the LD_{50} of flesh, brain, liver and viscera extracts respectively.

Tables 4-7 Summarizes the LD_{50} of the brain, liver and viscera organ extracts. This study, it is established a-log (dose) regression models and solved them by the maximum likelihood method using Microsoft Excel. The z- and $\chi 2$-tests were used. The LD_{50} of the brain, liver and viscera organs was shown in Table 8.

Table 4 Determination of LD_{50} using acute toxicity study of the flesh extract (2g/5 ml 0.1 acetic acid, single dose concentration was adjusted to 100 µl) to 8 mice. Note: Data presented as Mean: n = 8, One-way ANOVA, *significantly different from the Distilled Water (DW) control at $p<0.05$. .

Concentration of the flesh extract, g/ 200 µl	Number of death (out of 8 mice)	% death
Distilled water	0	0
0.1	1	12.5
0.5	2	25
0.75	2	25
1	3	37.5
1.25	4	50
1.5	4	50
1.75	6	75
2	8	100

Table 5 Determination of LD_{50} using acute toxicity study of the brain extract (2g/5 ml 0.1 acetic acid, single dose concentration was adjusted to 100 µl) to 8 mice.

Note: Data presented as Mean: n = 8, One-way ANOVA, *significantly different from the Distilled Water (DW) control at $p<0.05$.

Concentration of the brain extract , g/ 200 µl	Number of death (out of 8 mice)	% death
Distilled water.	0	0
0.1	1	12.5
0.5	2	25
0.75	3	37.5
1	4	50
1.25	5	62.5
1.5	6	75
1.75	7	87.5
2	8	100

Table 6 Determination of LD_{50} using acute toxicity study of the liver extract (2g/5 ml 0.1 acetic acid, single dose concentration was adjusted to 100 µl) to 8 mice. Note: Data presented as Mean: n = 8, One-way ANOVA, *significantly different from the Distilled Water (DW) control at $p<0.05$.

Concentration of the liver extract g/ 200 µl	Number of death (out of 8 mice)	% death
Distilled water	0	0
0.1	2	25
0.5	4	50
0.75	5	62.5
1	6	75
1.25	7	87.5
1.5	8	100
1.75	8	100
2	8	100

Table 7 Determination of LD_{50} using acute toxicity study of the viscera extract 2g/5 ml 0.1 acetic acid, single dose concentration was adjusted to 100 µl) to 8 mice. Note: Data presented as Mean: n = 8, One-way ANOVA, *significantly different from the Distilled Water (DW) control at $p<0.05$.

Concentration of the viscera extract, g/ 200 µl	Number of death (out of 8 mice)	Death %
Distilled water	0	0
0.1	4	50
0.5	5	62.5
0.75	5	62.5
1	6	75
1.25	7	87.5
1.5	8	100
1.75	8	100
2	8	100

Tables 8 Summarizes the LD_{50} of flesh, brain, liver and viscera organs extracts

Crude ciguatoxin (neurotoxins) Extracts 1g/5ml 0.1% acetic acid	LD_{50} fitting equation dosage-mortality relationship	LD_{50} g/kg (orally)
Flesh extract	y = 1.872ln(x) + 4.078	51±2.6
Brain extract	y = 2.228ln(x) + 4.890	33±3.4
Liver extract	y = 2.176ln(x) + 6.381	16±2.6
Viscera extract	y = 1.456ln(x) + 6.630	8.2±1.1

4.2 Acute toxicity of *Sarpa salpa* extracts and its neutralization by *Echium angustifolium* Mill aqueous extract.

The toxin-affected mice displayed classic neurotoxic symptoms such as hypothermia, significantly decreased locomotor activity within the first three hours, and subsequently respiratory failure. The toxicity of the four extracts differs significantly, as seen in Table 9-12. Toxic amounts in organs were distinct and could be rated in ascending order, flesh, brain, liver, and viscera extract come last. The outcomes are comparable to those achieved by Elfeki (Bellassoued *et al.*, 2013; Bellassoued *et al.*, 2015).

Table 9 Detoxication of 200 µl (1.0g) of the flesh extract of *Sarpa salpa* by *Echium angustifolium* Mill extract (1.29g in 30 ml distilled water). Note: Significantly different from the Distilled Water (DW) control at $p<0.05$. DW = distilled.

Doses of *Echium angustifolium* Mill 1.29g in 30 ml distilled water	Number of survivors (out of 6 mice)	Survivors %
Group 1 (Dose of 0 µl (DW)) control	0	0
Dose of 100 µl *Echium angustifolium* Mil.	2	3.33
Dose of 200 µl *Echium angustifolium* Mil	6	100
Dose of 300 µl *Echium angustifolium* Mil	6	100
Dose of 400 µl *Echium angustifolium* Mil	6	100

Table 10 Detoxication of 200 µl (1.0g) of the brain extract of *Sarpa salpa* by *Echium angustifolium* Mill extract. Note: Significantly different from the Distilled Water (DW) control at $p<0.05$. DW = distilled.

Doses of *Echium angustifolium* Mill 1.29g in 30 ml distilled water	Number of survivors (out of 6 mice)	Survivors %
Group 1 (Dose of 0 µl (DW)) control	0	0
Dose of 100 µl *Echium angustifolium* Mil.	0	0
Dose of 200 µl *Echium angustifolium* Mil	4	66.67
Dose of 300 µl *Echium angustifolium* Mil	6	100
Dose of 400 µl *Echium angustifolium* Mil	6	100

Table 11 Detoxication of 200 µl (1.0g) of the liver extract of *Sarpa salpa* by *Echium angustifolium* Mill extract. Note: Significantly different from the Distilled Water (DW) control at $p<0.05$. DW = distilled.

Doses of *Echium angustifolium* Mill 1.29g in 30 ml distilled water	Number of survivors (out of 6 mice)	Survivors %
Group 1 (Dose of 0 µl (DW) **)** control	0	0
Dose of 100 µl *Echium angustifolium* Mil.	0	0
Dose of 200 µl *Echium angustifolium* Mil	0	83.33
Dose of 300 µl *Echium angustifolium* Mil	5	100
Dose of 400 µl *Echium angustifolium* Mil	6	100

Table 12 Detoxication of 200 µl (1.0g) of the Viscera extract extract of *Sarpa salpa* by *Echium angustifolium* Mill extract. Note: Significantly different from the Distilled Water (DW) control at $p<0.05$. DW = distilled

Doses of *Echium angustifolium* Mill 1.29g in 30 ml distilled water	Number of survivors (out of 6 mice)	Survivors %
Group 1 (Dose of 0 µl **(**DW) **)** control	0	0
Dose of 100 µl *Echium angustifolium* Mil.	0	0
Dose of 200 µl *Echium angustifolium* Mil	1	16.67
Dose of 300 µl *Echium angustifolium* Mil	1	16.67
Dose of 400 µl *Echium angustifolium* Mil	6	100

Tables 13 Summaries the protective dosage of *Echium angustifolium* Mill aqueous extract needed to reverse the toxic effect of flesh, brain, liver, and viscera extracts. The *Echium angustifolium* Mill aqueous extract significantly increases mean survival time up to 5 ±1 days and protects animals from death if compared to the mice who had *Sarpa salpa* toxin only. *Echium angustifolium* Mill aqueous extract if used at a higher doses was found to be more effective against *Sarpa salpa* toxin (Figure 13).

Tables 13 summarizes the toxicity studies of *Echium angustifolium* Mill at different doses, determination of LD_{50} using acute toxicity study of different organs extract and detoxication of different organs extracts by *Echium angustifolium* Mill extract.

Crude ciguatoxin (neurotoxins) Extracts 1g/5ml 0.1% acetic acid	LD_{50} g/kg (orally)	Protective dosage of *Echium angustifolium* Mill aqueous extract (1.29g/30 ml) in µl given to Albino mice
Flesh extract	51±2.6	125±2.3
Brain extract	33±3.4	220±6.3
Liver extract	16±2.6	302±7.4
Viscera extract	8.2±1.1	411±11

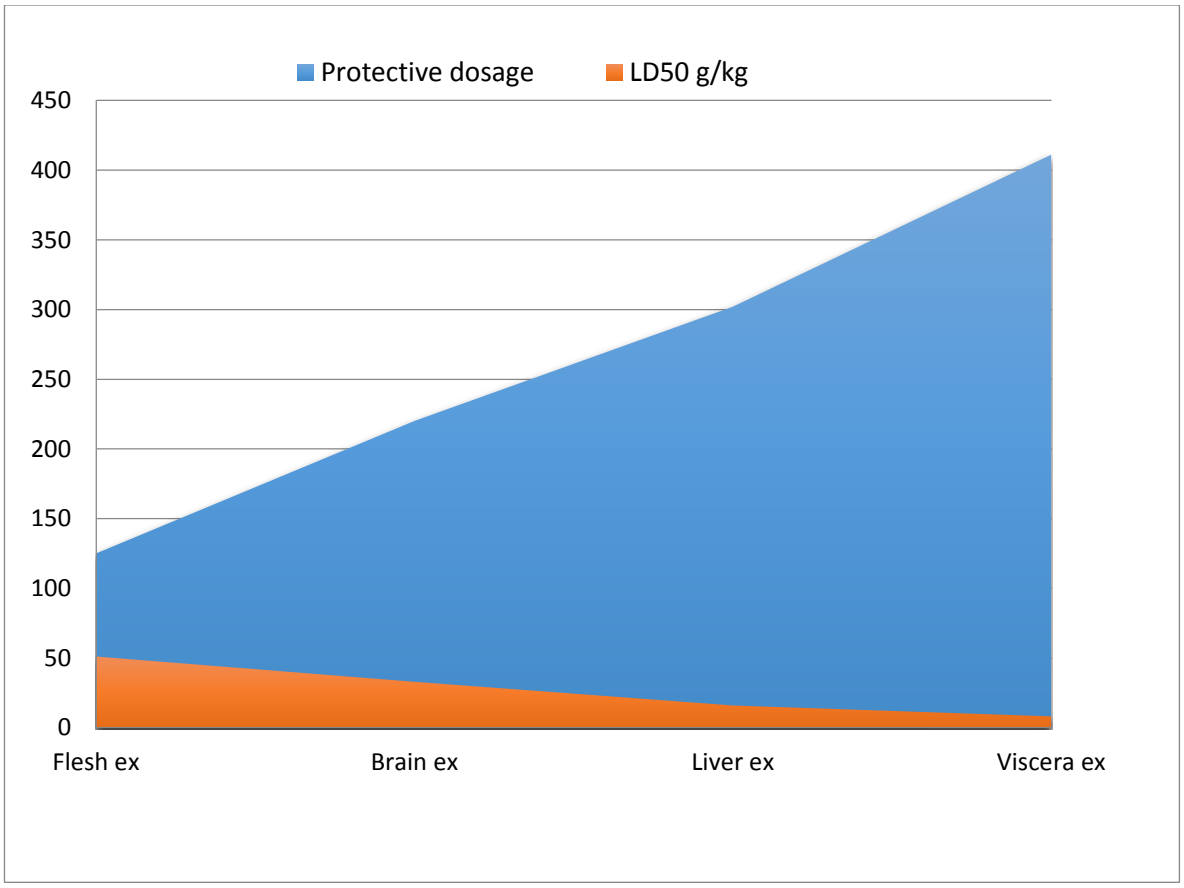
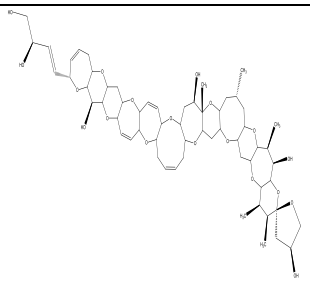
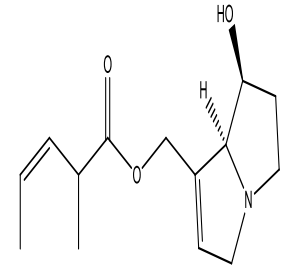
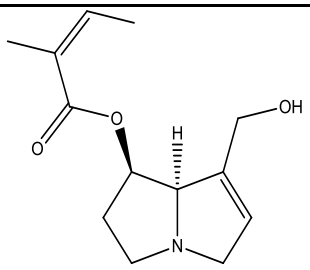


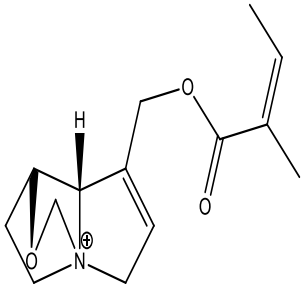
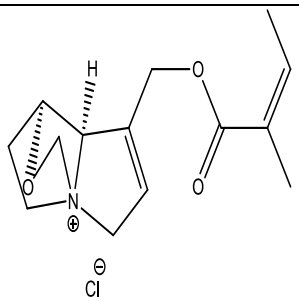
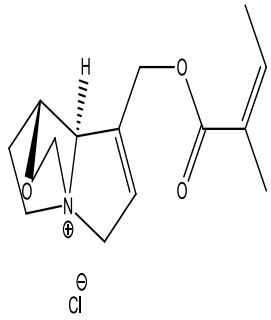
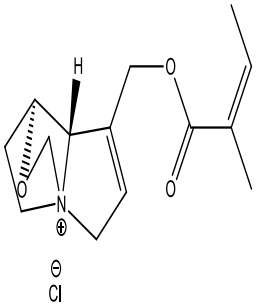
Figure 13.Summarizes the LD $_{50}$ of flesh, brain, liver and viscera organs extracts

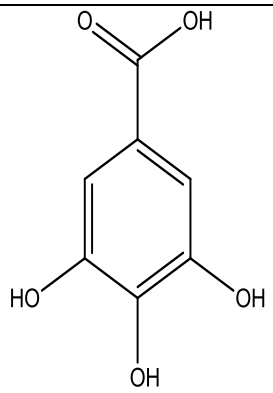
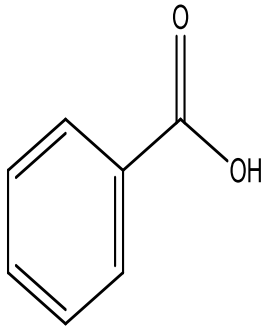
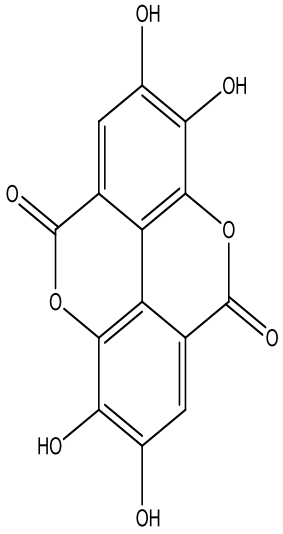
4.3 Molecular docking results.

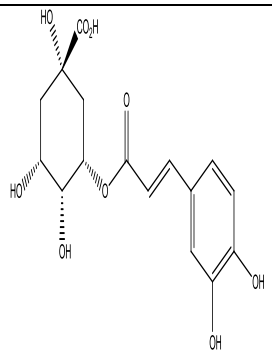
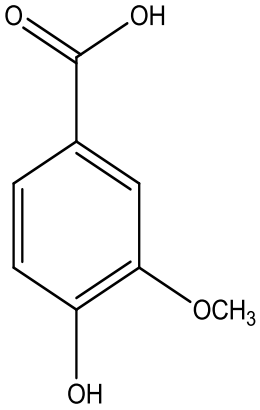
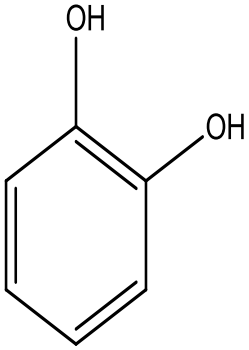
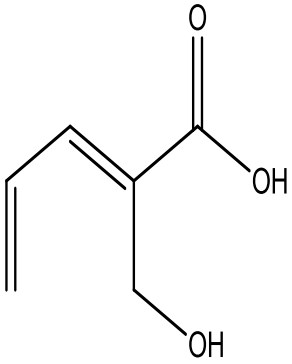
Table 14 shows the findings of the molecular docking. According to the modeling research, *Echium angustifolium* Mill components with voltage-gated channels have *van der Waals*, hydrogen bonding, and electrostatic interactions. Because the total of *van der Waals* energy, hydrogen bonding energy, and desolvation free energy is more than the electrostatic energy, which is consistent with the literature, the contribution of van der Waals and hydrogen bonding interaction is significantly bigger than that of electrostatic interaction (Gilad & Senderowitz 2014; Chagas *et al.,* 2018). Table 14 Depicts the interactions between *Echium angustifolium* Mill compounds such as Ellagic acid and voltage-gated K^+ (1BL8) channels. As shown in Table 5, the binding energy of Ellagic acid (-9.0kcal/mol) was substantially greater than that of ciguatoxin-1 (-8.60kcal/mol) in *Echium angustifolium* Mill constituents. In Figure 14, Ellagic acid and K^+ voltage-gated sodium form six hydrogen bonds, but ciguatoxin-1 forms just one.

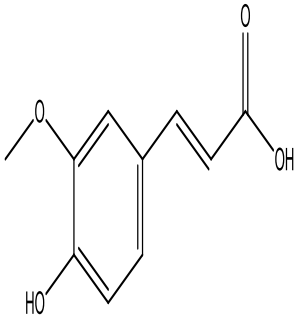
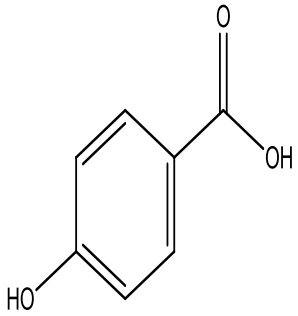
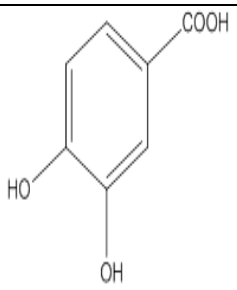
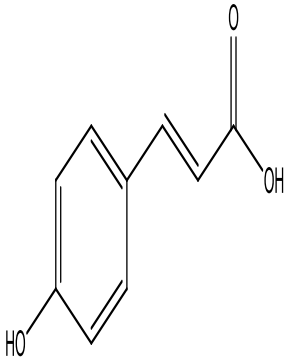
Table 14 Various energies in the binding process of Echium angustifolium constituents and voltage -gated Na^+ (6AGF) and K^+ (1BL8) channels obtained from molecular docking. The unit of all energies (ΔG) is kcal/mol

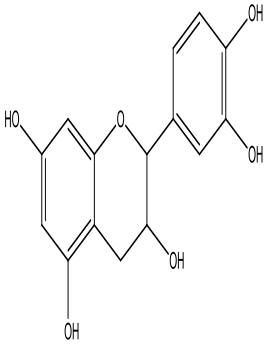
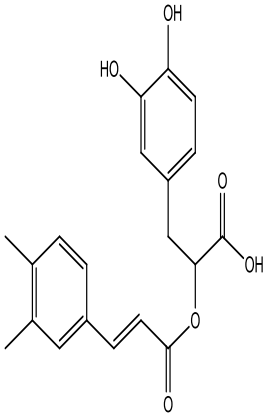
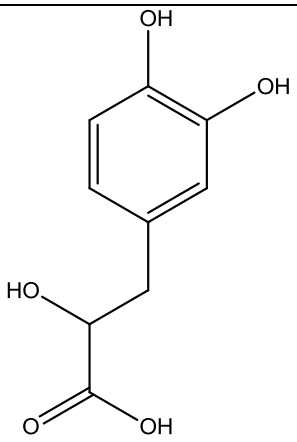
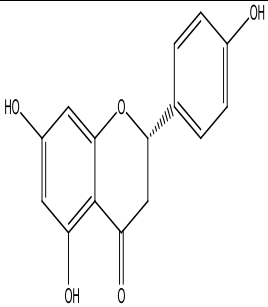
-Chemical structures	-No. - Chemical nature.	Name of ligand	Potassium channel 1bl8 Binding energy	Sodium channel (6AGF) Binding energy Nav channel
	NO.1 ciguatoxin-	P-CTX 1B	-8.6	-12.3
	NO.2 pyrrolizidie alkaloids	9-angeloylretronecine	No binding	-5.5
	NO.3 pyrrolizidie alkaloids	7-angeloylretronecine	-5.0	-5.6

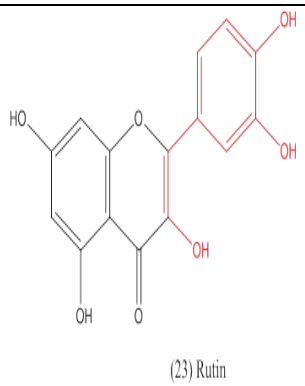
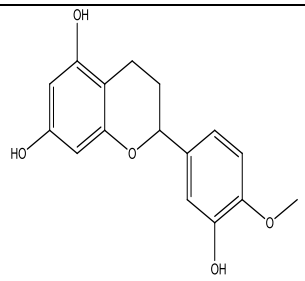
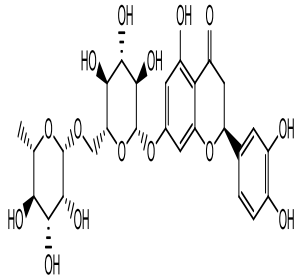
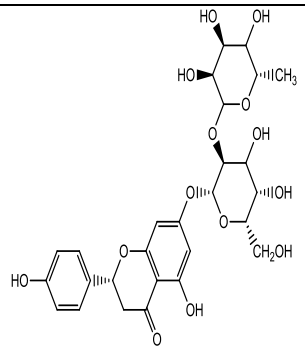
	NO.4 pyrrolizidie alkaloids	(7R, 8S)-petranine 3	-5.0	-6.1
	NO.5 pyrrolizidie alkaloids	(7S, 8R)-petranine 1	-5.0	-6.8
	NO.6 pyrrolizidie alkaloids	(7R, 8R)-petranine 4	-5.0	-6.1
	NO.7 pyrrolizidie alkaloids	(7R, 8S)-petranine 2	-4.9	-6.4

	NO.8 Phenolic acid derivatives	Gallic acid	-6.1	-6.0
	NO.9 Phenolic acid derivatives	Benzoic acid	-4.3	-5.8
	NO.10 Phenolic acid derivatives	Ellagic acid	-9.0	-7.8

	NO.11 Phenolic acid derivatives	Chlorogenic acid	-7.6	-7.0
	NO.12 Phenolic acid derivatives	Vanillic acid	-5.7	-5.0
	NO.13 Phenolic acid derivatives	Catechol	-5.3	-4.9
	NO.14 Phenolic acid derivatives	Salicylic acid	-5.6	-6.0

	NO.15 Phenolic acid derivatives	Ferulic acid	-6.1	-6.4
 p-hydroxy benzoic acid	NO.16 Phenolic acid derivatives	P- hydroxy-benzoic	-5.2	-5.5
 (12) protocatechuic acid	NO.17 Phenolic acid derivatives	Protocatechuic acid	-5.9	-5.7
	NO.18 Phenolic acid derivatives	P-Coumaric acid	-5.1	-6.0

	NO.19 Phenolic acid derivatives	Catechin	-6.0	-7.7
	NO.20 Phenolic acid derivatives	Rosmarinic acid	-5.6	-7.6
	NO.21 Phenolic acid derivatives	Dihydroxyphenyl lactic acid	-5.6	-8.9 No H-bond
	No.22 Flavonoid	Naringin	-6.8	-8.2

 (23) Rutin	No.23 Flavonoid	Rutin	-7	-8.4
	No.24 Flavonoid	Hesperetin	-6.2	-8.5
	NO.25 Flavonoid	Hesperidin	-6.5	-7.3
	NO.26 Flavonoid	Naringenin	-5.8	-7.3

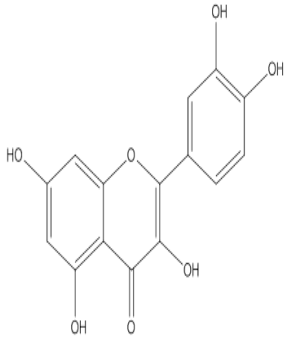
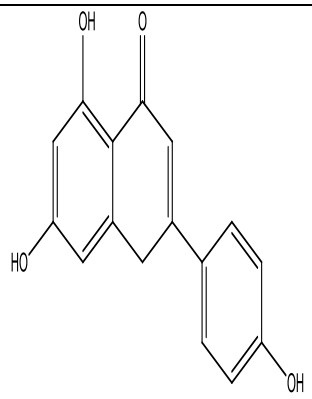
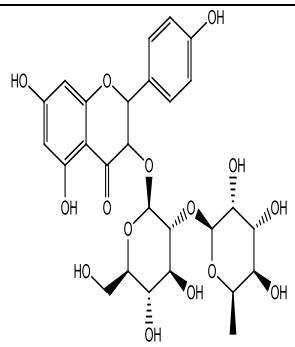
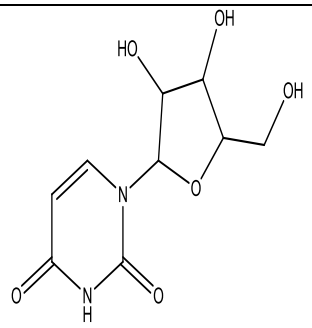
 (9) Quercetin	NO.27 Flavonoid	Quercetin	-5.7	-7.6
	NO.28 Flavonoid	Apigenin	-5.7	-8.3
	NO.29 Flavonoid	Kaempferol-3-neohesperidoside	-8.4	-7.9
	NO.30 pyrimidine-analog	Uridine	-7.5	-6.2

Figure 14 (A) Shows the interaction model between Ellagic acid and CTXs with K^+ channel (1bl8, KcsA (K^+ channel of streptomyces)). **(B)** Shows the interaction model between Ellagic acid with the K^+ channel active site. **(C)** Shows the interaction model between CTXs with the K^+ channel. The hydrogen bonds are represented using green broken lines. The figure was obtained with the help of a molecular visualization tool (discovery studio software 2.4).

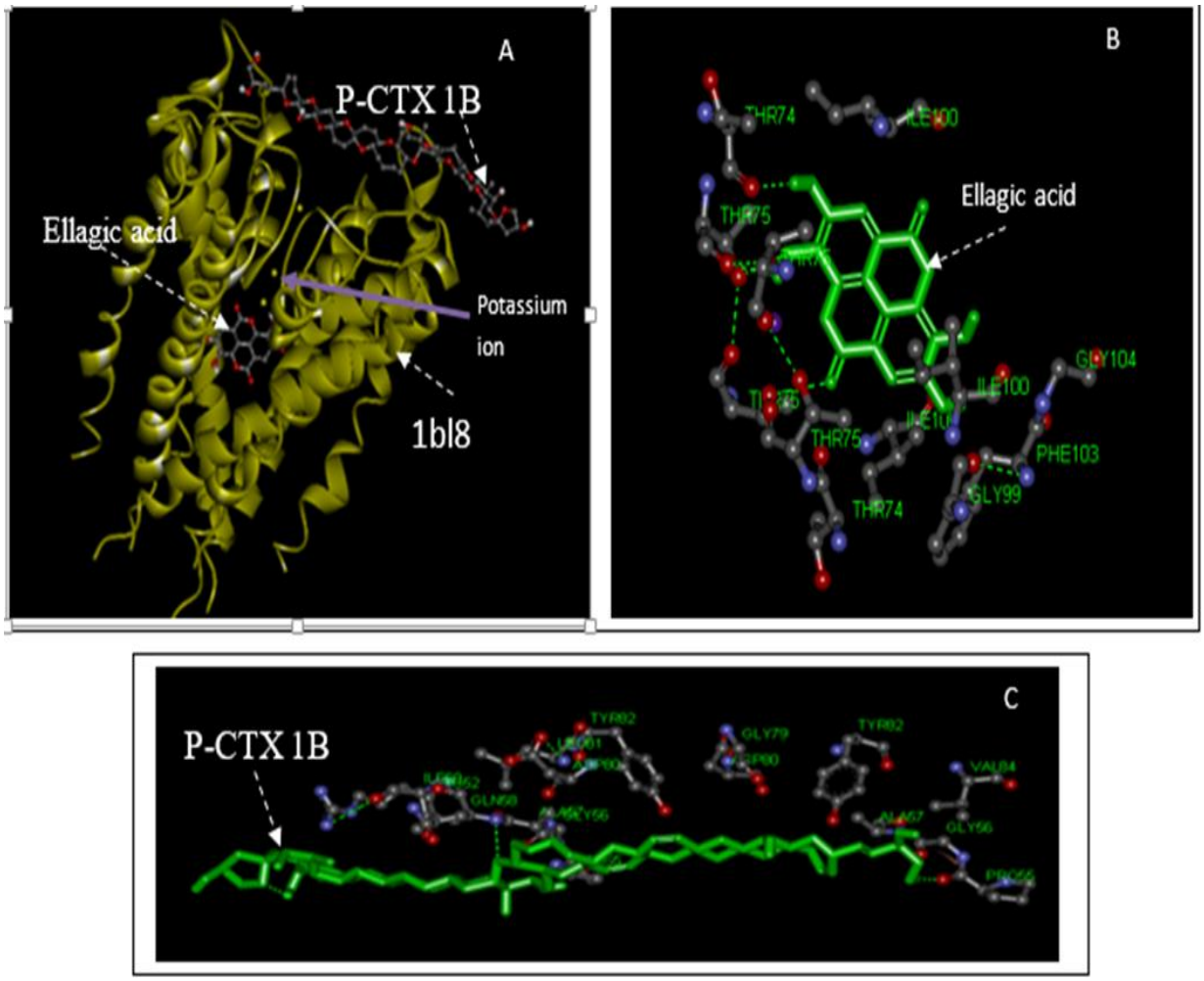


Figure 14 Ellagic acid and voltage-gated K^+ (1BL8) channel interactions are shown.

Chapter 5 Discussion.

The current study is compared to prior Boraginaceae plant family investigations in which a single intravenous dosage of 561 mg/kg of rosmaric acid did not cause acute toxicity in mice (Rossi *et al.*, 2012).

The results of this study revealed that *Echium angustifolium* Mill constituents form hydrogen bonds with active sites of Na^+ and K^+ channels and protect the mice from ciguatoxin toxicity with a statistically significant difference in the extract compared to controls with a p-value less than 0.01. Hence it is proposed that *Echium angustifolium* Mill constituents can be used to prevent ciguatoxin toxicity. When compared to mice that only got *Sarpa salpa* toxin, the *Echium angustifolium* Mill aqueous extract considerably enhances mean survival time up to 51 days and protects animals from death. Higher dosages of *Echium angustifolium* Mill aqueous extract were shown to be more efficient against *Sarpa salpa* toxin.

A similar investigation was carried out on mouse neuroblastoma cells employing H. Foertherianum aqueous extract containing rosmarinic acid as the main component, which was found to be capable of reversing P-CTX-1B-induced cytotoxicity (Rossi *et al.*, 2012). P-cytotoxicity CTX-1B's was decreased by H. Foertherianum at doses up to 2734g/ml and rosmarinic acid at concentrations up to 607g/ml, amounts at which they began to become cytotoxic (Bellassoued *et al.*, 2013). The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide MTT assay, a colorimetric assay for assessing cell metabolic activity and it reflects the number of viable cells present), LDH assays (lactate dehydrogenase assay for cell viability testing using cell lines and primary cultured astrocytes), and neutral red assays (lactate dehydrogenase) a cytotoxicity assay used to detect cell viability or drug cytotoxicity based on the detection of viable cells via the uptake of the dye neutral red) (Rossi *et al.*, 2012). Demonstrating its ability to treat CFP and was consistent with our results. On the other hand; pharmacological tests were essential to decide whether or not *Echium angustifolium* Mill indeed have direct 'detoxifying' action on the ciguatoxin itself in human and that is our program's goal

to assess the therapeutic potential of the *Echium angustifolium* Mill aqueous extract in human.(Chagas *et al.,* 2018)

5.1 Molecular docking analysis.

The voltage-gated Na^+ channel (denominated Nav1.4) was employed for molecular docking. This channel is responsible for action potential generation and is implicated in a variety of human illnesses. The pore do-main, the voltage-sensing domains of the -subunit, and the 1 subunits were all modelled, giving the molecular foundation for Na^+ ion penetration and kinetics(Pan *et al.,* 2018) The K^+ channel employed was Streptomyces lividans' KcsA, which is a fundamental membrane protein having sequence similarities to several known K^+ channels.

The primary chain carbonyl oxygen atoms are held open and offer an ion filter by structural constraints to organize passing K^+ but not smaller Na^+ ions in K^+ channels, which are made up of four distinct units connected together to create the ion pore. This arrangement promotes ion conduction by leveraging electrostatic repulsive forces to overcome attraction forces between K^+ ions and carbonyl oxygen atoms in the main chain, as well as assisting in molecular docking of *Echium angustifolium* Mill components.(Doyle *et al.,* 1998)

The predisposition is created by the pore's structure (Pan *et al.,* 2018) Discharge In the majority of excitable cells, voltage-gated Na^+ channels play a crucial role in the creation and propagation of electrical impulses. They are single membrane-spanning proteins with an ion pore that causes a quick and brief increase in membrane Na^+ ion conductance, which is responsible for the depolarizing portion of action potentials. Persistent channel activation eventually leads to depolarization block and action potential discharge suppression (Inserra *et al.,* 2017; Pan *et al.,* 2018).

Stimulation CTXs causes spontaneous action potentials that can be inhibited at low nanomolar doses. This action was mediated through voltage-gated sodium channels, according to TTX (Tetrodotoxin: is a particular Na^+ channel blocker).

Action potential suppression hinders nerve cells from transmitting messages, preventing muscles from contracting in response to neural stimulation (Inserra *et al.,* 2017; Pan *et al.,* 2018). The components of *Echium angustifolium* Mill can counteract this effect. Calcium-activated potassium channels, inwardly rectifying potassium channels, tandem pore domain potassium

channels, and voltage-gated potassium channels are the four primary types of potassium channels. KcsA, which was employed in this study, is more closely linked to voltage-gated potassium channels (Gomez-Sanchez & Oki 2014; Pan *et al.,* 2018).

Ciguatoxins has been shown to affect neuronal excitability by blocking K^+ channels, which contributes to membrane hyperpolarization. The activation of voltage-gated Na^+ channels causes membrane depolarization when voltage-gated K^+ channels are blocked. Furthermore, the lack of the hyperpolarizing force induced by the K^+ conductance adds to a lower action potential threshold when K^+ channels are blocked (Chen *et al.,* 2008). *Echium angustifolium* Mill components can counteract both CTX's effects on voltage-activated Na^+ and K^+ channels. Table No 8.

The molecular docking technique revealed the binding energies of *Echium angustifolium* Mill components to voltage-gated Na^+ (6AGF) and K^+ (1BL8) channels. In this study, Auto Dock 4.2 was used to perform molecular dockings of *Echium angustifolium* Mill constituents to voltage-gated channels to investigate the binding mode of *Echium angustifolium* Mill constituents and obtain information about interaction forces between *Echium angustifolium* Mill constituents and voltage-gated channels. Voltage-gated channels and *Echium angustifolium* Mill constituents were retained as flexible molecules and docked into seven types of stiff ion channels to get their preferred binding site to *Echium angustifolium* Mill constituents.

With voltage-gated K^+ channels, there are five hydrogen bonds. Furthermore, Ellagic acid demonstrated a strong docking contact with voltage-gated K^+ channels. Figure 19 shows the binding location (threonine 75 and threonine 74). CTX-1, on the other hand, had a good docking contact with the K^+ channel. The interaction of Ellagic acid with the K^+ channel binding site (proline55 and glutamine58) is required for the successful reversal of CTX-1 blocking activity. Based on sequence function and similarity, the voltage-gated potassium (Kv) channels family may be separated into many subfamilies. Kv1 (Shaker), Kv2 (Shab), Kv3 (Shaw), and Kv4 (S) are four of these subfamilies (Robertson & Stevens 2017; Luckert *et al.,* 2018).

Reported that there is a subtype-specific difference in the role of Kv1 channels and only Kv4 channels are involved in repolarizing the narrow action potential of mouse somatosensory

cortex cells and this could explain the protective results obtained with KcsA. Therefore, some *Echium angustifolium* Mill constituents may be considered as the effective agents of reversing the blocking action of CTX-1.Pyrrolizidine alkaloid are a class of naturally occurring alkaloids that are based on the structure of pyrrolizidine and are generated by plants as a defense mechanism against insect herbivores. Some of them are hepatotoxic when taken persistently. Pyr-rolizidine alkaloids are necine bases that have been esterified with necic acid. Pyrolizidine, a bicyclic aliphatic hydrocarbon composed of two fused five-membered rings with nitrogen at the bridgehead, is a distinctive component of the necine base. Pyrrolizidine alkaloids are mostly found as N-oxides (Table 14), which are water-soluble and act as hydrogen bonding groups with the amino acids of Na^+ and K^+ voltage-gated channels (Robertson & Stevens 2017; Luckert *et al.*, 2018).

In this work, we assessed the capacity of *Echium angustifolium* Mill aqueous extract to reverse the P-CTX-1B-induced toxicity in mice. The toxicity produced by CTXs was inhibited by *Echium angustifolium* Mill at dosages up to 411µl (1.29g/30ml).

A comparison of the structures and activities of pyrrolizidine alkaloids, phenolic acid derivatives, flavonoids andpyrimidine-analog indicates that the carboxyl moiety of phenolic acid derivatives constitutes a significant substituent required for activity against CTXs toxicity, these derivatives lacking this carboxyl functional group were not very potent in the case of potassium voltage-gated channels.

In addition, flavonoids that contain phenols were a considerable substituent required for activity against CTXs toxicity in the case of sodium voltage-gated channels. Additionally, other phenolic acid derivatives and flavonoids had positive binding energies. The phenolic compounds are still the most potent on both channels suggesting that the phenolic moieties were required for a significant positive activity against CTXs toxicity. Furthermore, the similar activity of phenolic acid derivatives and flavonoids derivatives confirmed that the positive activity results from both the phenolic and carboxyl moiety.

Lastly, the number of hydroxyl substitutions on thephenolic moieties was important. Indeed, the difference of activity between gallic acid, benzoic acid, ellagic acid, chlorogenic acid, vanillic acid, catechol, salicylic acid, ferulic acid, p-hydroxy-benzoic acid, protocatechuic

acid, p-coumaric acid, catechin, rosmarinic acid, dihydroxyphenyl lactic acid, naringin, rutin, hesperetin and hesperidin showed that they needed at least two hydroxyl substitutions. Thus, a diphenol was required to obtain inhibition of the CTXs toxicity. The comparison with all these derivatives indicates that the structure of phenol was significant for its biological activity. This specificity of action provides a basis for the improved acceptance of the wide utilization of *Echium angustifolium* Mill aqueous extract, which contains pyrrolizidine alkaloids, phenolic acid derivatives, flavonoids and pyrimidine analog, for future treatment of CTXs toxicity.(Doyle *et al.,* 1998)

5.2 In silico toxicity assessment of *Echium angustifolium* Mill components.

Echium angustifolium Mill components were evaluated for drug likeness and toxicity. Examination of *Echium angustifolium* Mill components for drug likeness was performed by computational prediction of ADME-Tox properties (adsorption, distribution, metabolism, excretion, and toxicity). All *Echium angustifolium* Mill components were found to be non-carcinogenic and acceptable as drugs. Refractivity Furthermore, all *Echium angustifolium* Mill components were found to follow the Lipinski Rule of five for drug likeness, with molecular masses less than 500 Daltons, no more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, Log P scores less than 5, and molar refractivity less than 5 (Daina *et al.,* 2017; Chagas *et al.,* 2018). The limitation of this study is the cytotoxicity assay; which still needs to be an essential part of evaluating the safety of *Echium angustifolium* constituents because it affords direct information at the cellular level which may be significant in assessing the true toxicity of *Echium angustifolium* Mill constituents.

Chapter 6 Conclusion.

Ciguatera Fish Poisoning is seafood intoxication due to consumption of tropical coral reef fishes that have built up ciguatoxins in their tissues. *Echium angustifolium* Mill aqueous extracts exhibit a positive activity in treating ciguatoxin toxicity. The results indicated that *Echium angustifolium* Mill constituents form hydrogen bonds with active sites of Na^+ and K^+ channels and protect the mice from ciguatoxin toxicity. The positive activity in mice suggests a promising detoxifying action caused by cigua-intoxication. Furthermore, the obtained results confirm the potential of *Echium angustifolium* Mill in the treatment of Ciguatera Fish Poisoning. Detailed clinical studies in this direction are needed to potentiate this claim in human beings.

References

Achaibar, K.C., Moore, S. & Bain, P.G. 2007. Ciguatera poisoning. *Pract. Neurol.* 7, 316-322.

Banerjee, P., Eckert, A.O., Schrey, A.K. & Preissner, R. 2018. ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res.* 46, W257-W263.

Barrett, K.A., Nakao, J.H., Taylor, E.V., Eggers, C. & Gould, L.H. 2017. Fish-Associated Foodborne Disease Outbreaks: United States, 1998-2015. *Foodborne. Pathog. Dis.* 14, 537-543.

Bellassoued, K., Hamza, A., Van Pelt, J. & Elfeki, A. 2013b. Seasonal variation of Sarpa salpa fish toxicity, as related to phytoplankton consumption, accumulation of heavy metals, lipids peroxidation level in fish tissues and toxicity upon mice. *Environ. Monit. Assess.* 185, 1137-1150.

Bellassoued, K., Van Pelt, J. & Elfeki, A. 2015. Neurotoxicity in rats induced by the poisonous dreamfish (Sarpa salpa). *Pharm. Biol.* 53, 286-295.

Benoit, E., Juzans, P., Legrand, A.M. & Molgo, J. 1996. Nodal swelling produced by ciguatoxin-induced selective activation of sodium channels in myelinated nerve fibers. *Neuroscience* 71, 1121-1131.

Benoit, E., Mattei, C., Ouanounou, G., Meunier, F.A., Suput, D., Le Gall, F., Marquais, M., Dechraoui, M.Y. & Molgo, J. 2002. Ionic mechanisms involved in the nodal swelling of myelinated axons caused by marine toxins. *Cell Mol. Biol. Lett.* 7, 317-321.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N. & Bourne, P.E. 2000. The Protein Data Bank. *Nucleic Acids Res.* 28, 235-242.

Blythe, D.G., De Sylva, D.P., Fleming, L.E., Ayyar, R.A., Baden, D.G. & Shrank, K. 1992. Clinical experience with i.v. Mannitol in the treatment of ciguatera. *Bull. Soc. Pathol. Exot.* 85, 425-426.

Boada, L.D., Zumbado, M., Luzardo, O.P., Almeida-GonzÃ¡lez, M., Plakas, S.M., Granade, H.R., Abraham, A., Jester, E.L. & Dickey, R.W. 2010c. Ciguatera fish poisoning on the West Africa Coast: An emerging risk in the Canary Islands (Spain). *Toxicon* 56, 1516-1519.

Bury, D., Langlois, N. & Byard, R.W. 2012. Animal-related fatalities--part II: characteristic autopsy findings and variable causes of death associated with envenomation, poisoning, anaphylaxis, asphyxiation, and sepsis. *J. Forensic Sci.* 57, 375-380.

Cameron, J. & Capra, M.F. 1993. The basis of the paradoxical disturbance of temperature perception in ciguatera poisoning. *J. Toxicol. Clin. Toxicol.* 31, 571-579.

Chagas, C.M., Moss, S. & Alisaraie, L. 2018. Drug metabolites and their effects on the development of adverse reactions: Revisiting Lipinski's Rule of Five. *Int. J. Pharm.* 549, 133-149.

Chen, L., Liu, C. & Liu, L. 2008. The modulation of voltage-gated potassium channels by anisotonicity in trigeminal ganglion neurons. *Neuroscience* 154, 482-495.

Daina, A., Michielin, O. & Zoete, V. 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci. Rep.* 7, 42717.

Dickey, R.W. & Plakas, S.M. 2010. Ciguatera: a public health perspective. *Toxicon* 56, 123-136.

Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K. & Zhorov, B.S. 2014. Molecular biology of insect sodium channels and pyrethroid resistance. *Insect Biochem. Mol. Biol.* 50, 1-17.

Doyle, D.A., Morais, C.J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. & MacKinnon, R. 1998. The structure of the potassium channel: molecular basis of K+ conduction and selectivity. *Science* 280, 69-77.

El Rokh, A.R., Negm, A., El Shamy, M., El Gindy, M. & Abdel-Mogib, M. 2018. Sucrose diester of aryldihydronaphthalene-type lignans from Echium angustifolium Mill. and their antitumor activity. *Phytochemistry* 149, 155-160.

Friedman, M.A., Arena, P., Levin, B., Fleming, L., Fernandez, M., Weisman, R., Bernstein, J., Schrank, K., Blythe, D., Backer, L. & Reich, A. 2007. Neuropsychological study of ciguatera fish poisoning: a longitudinal case-control study. *Arch. Clin. Neuropsychol.* 22, 545-553.

Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T. & Fleming, L.E. 2017. An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. *Mar. Drugs* 15.

Friedman, M.A., Fleming, L.E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M.Y., Backer, L., Ayyar, R., Weisman, R., Watkins, S., Granade, R. & Reich, A. 2008. Ciguatera fish poisoning: treatment, prevention and management. *Mar. Drugs* 6, 456-479.

Gilad, Y. & Senderowitz, H. 2014. Docking studies on DNA intercalators. *J. Chem. Inf. Model.* 54, 96-107.

Gillespie, N.C., Lewis, R.J., Pearn, J.H., Bourke, A.T., Holmes, M.J., Bourke, J.B. & Shields, W.J. 1986. Ciguatera in Australia. Occurrence, clinical features, pathophysiology and management. *Med. J. Aust.* 145, 584-590.

Gingold, D.B., Strickland, M.J. & Hess, J.J. 2014. Ciguatera fish poisoning and climate change: analysis of National Poison Center Data in the United States, 2001-2011. *Environ. Health Perspect.* 122, 580-586.

Gomez-Sanchez, C.E. & Oki, K. 2014. Minireview: potassium channels and aldosterone dysregulation: is primary aldosteronism a potassium channelopathy? *Endocrinology* 155, 47-55.

Goodman, D.M., Rogers, J. & Livingston, E.H. 2013. JAMA patient page. Ciguatera fish poisoning. *JAMA* 309, 2608.

Grant, I.C. 1997. Ichthyosarcotoxism: poisoning by edible fish. *J. Accid. Emerg. Med.* 14, 246-251.

Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A. & Lewis, R.J. 2002. Isolation and characterisation of Indian Ocean ciguatoxin. *Toxicon* 40, 685-693.

Hirama, M. 2005. Total synthesis of ciguatoxin CTX3C: a venture into the problems of ciguatera seafood poisoning. *Chem. Rec.* 5, 240-250.

Inserra, M.C., Israel, M.R., Caldwell, A., Castro, J., Deuis, J.R., Harrington, A.M., Keramidas, A., Garcia-Caraballo, S., Maddern, J., Erickson, A., Grundy, L., Rychkov, G.Y., Zimmermann, K., Lewis, R.J., Brierley, S.M. & Vetter, I. 2017b. Multiple sodium channel isoforms mediate the pathological effects of Pacific ciguatoxin-1. *Sci. Rep.* 7, 42810.

Konarzewski, T.K., Murray, B.R., Godfree, R.C. (2012). Rapid development of adaptive, climate-driven clinal variation in seed mass in the invasive annual Forb *Echium plantagineum* L. *PLoS ONE.* 2012;7:e49000

Kryuchkov, F., Robertson, A., Miles, C.O., Mudge, E.M. & Uhlig, S. 2020. LC-HRMS and Chemical Derivatization Strategies for the Structure Elucidation of Caribbean Ciguatoxins: Identification of C-CTX-3 and -4. *Mar. Drugs* 18.

Kumar, G., Au, N.P.B., Lei, E.N.Y., Mak, Y.L., Chan, L.L.H., Lam, M.H.W., Chan, L.L., Lam, P.K.S. & Ma, C.H.E. 2017. Acute Exposure to Pacific Ciguatoxin Reduces Electroencephalogram Activity and Disrupts Neurotransmitter Metabolic Pathways in Motor Cortex. *Mol. Neurobiol.* 54, 5590-5603.

Lange, W.R., Snyder, F.R. & Fudala, P.J. 1992. Travel and ciguatera fish poisoning. *Arch. Intern. Med.* 152, 2049-2053.

Lehane, L. 2000. Ciguatera update. *Med. J. Aust.* 172, 176-179.

Lehane, L. & Lewis, R.J. 2000. Ciguatera: recent advances but the risk remains. *Int. J. Food Microbiol.* 61, 91-125.

Lewis, R.J. 2001. The changing face of ciguatera. *Toxicon* 39, 97-106.

Lewis, R.J., Jones, A. & Vernoux, J.P. 1999. HPLC/tandem electrospray mass spectrometry for the determination of Sub-ppb levels of Pacific and Caribbean ciguatoxins in crude extracts of fish. *Anal. Chem.* 71, 247-250.

Loeffler, C.R., Bodi, D., Tartaglione, L., Dell'Aversano, C. & Preiss-Weigert, A. 2021. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). *Harmful. Algae.* 103, 101994.

Luckert, C., Braeuning, A., Lampen, A. & Hessel-Pras, S. 2018. PXR: Structure-specific activation by hepatotoxic pyrrolizidine alkaloids. *Chem. Biol. Interact.* 288, 38-48.

Mailho-Fontana, P.L., Antoniazzi, M.M., Alexandre, C., Pimenta, D.C., Sciani, J.M., Brodie, E.D., Jr. & Jared, C. 2020. Morphological Evidence for an Oral Venom System in Caecilian Amphibians. *iScience.* 23, 101234.

Marrelli, M., Statti, G. & Conforti, F. 2020. A Review of Biologically Active Natural Products from Mediterranean Wild Edible Plants: Benefits in the Treatment of Obesity and Its Related Disorders. *Molecules.* 25.

Mattei, C., Marquais, M., Schlumberger, S., MolgÃ³, J., Vernoux, J.P., Lewis, R.J. & Benoit, E. 2010. Analysis of Caribbean ciguatoxin-1 effects on frog myelinated axons and the neuromuscular junction. *Toxicon* 56, 759-767.

Nelsen, D.R., Nisani, Z., Cooper, A.M., Fox, G.A., Gren, E.C., Corbit, A.G. & Hayes, W.K. 2014. Poisons, toxungens, and venoms: redefining and classifying toxic biological secretions and the organisms that employ them. *Biol. Rev. Camb. Philos. Soc.* 89, 450-465.

Nicolaou, K.C. & Aversa, R.J. 2011. Maitotoxin: An Inspiration for Synthesis. *Isr. J. Chem.* 51, 359-377.

Pan, X., Li, Z., Zhou, Q., Shen, H., Wu, K., Huang, X., Chen, J., Zhang, J., Zhu, X., Lei, J., Xiong, W., Gong, H., Xiao, B. & Yan, N. 2018. Structure of the human voltage-gated sodium channel Na(v)1.4 in complex with Î²1. *Science* 362.

Parsons, M.L., Settlemier, C.J. & Ballauer, J.M. 2011. An examination of the epiphytic nature of Gambierdiscus toxicus, a dinoflagellate involved in ciguatera fish poisoning. *Harmful. Algae.* 10, 598-605.

Pearn, J., Harvey, P., De Ambrosis, W., Lewis, R. & McKay, R. 1982. Ciguatera and pregnancy. *Med. J. Aust.* 1, 57-58.

Pisapia, F., Sibat, M., Herrenknecht, C., Lhaute, K., Gaiani, G., Ferron, P.J., Fessard, V., Fraga, S., Nascimento, S.M., Litaker, R.W., Holland, W.C., Roullier, C. & Hess, P. 2017. Maitotoxin-4, a Novel MTX Analog Produced by Gambierdiscus excentricus. *Mar. Drugs* 15.

Pottier, I., Vernoux, J.P. & Lewis, R.J. 2001. Ciguatera fish poisoning in the Caribbean islands and Western Atlantic. *Rev. Environ. Contam Toxicol.* 168, 99-141.

Qaiser M., (1979). Boraginaceae vol.(68), Flora of Libya, Jafri S.M.H. & El. Gadi A., Al Faateh University, Faculty of Science. Department of Botany, Tripoli.

Robertson, J. & Stevens, K. 2017. Pyrrolizidine alkaloids: occurrence, biology, and chemical synthesis. *Nat. Prod. Rep.* 34, 62-89.

Rossi, F., Jullian, V., Pawlowiez, R., Kumar-RoinÃ©, S., Haddad, M., Darius, H.T., Gaertner-Mazouni, N., Chinain, M. & Laurent, D. 2012. Protective effect of Heliotropium foertherianum (Boraginaceae) folk remedy and its active compound, rosmarinic acid, against a Pacific ciguatoxin. *J. Ethnopharmacol.* 143, 33-40.

Sanders, W.E., Jr. 1987. Intoxications from the seas: ciguatera, scombroid, and paralytic shellfish poisoning. *Infect. Dis. Clin. North Am.* 1, 665-676.

Silver, K.S., Du, Y., Nomura, Y., Oliveira, E.E., Salgado, V.L., Zhorov, B.S. & Dong, K. 2014. Voltage-Gated Sodium Channels as Insecticide Targets. *Adv. In Insect Phys.* 46, 389-433.

Sharma G., Esler K. (2008). Phenotypic plasticity among *Echium plantagineum* populations in different habitats of Western Cape, South Africa. *S. Afr. J. Bot.* 2008;74:746–749.

Stewart, I., Lewis, R.J., Eaglesham, G.K., Graham, G.C., Poole, S. & Craig, S.B. 2010. Emerging tropical diseases in Australia. Part 2. Ciguatera fish poisoning. *Ann. Trop. Med. Parasitol.* 104, 557-571.

Tiwari, R., Mahasenan, K., Pavlovicz, R., Li, C. & Tjarks, W. 2009. Carborane clusters in computational drug design: a comparative docking evaluation using AutoDock, FlexX, Glide, and Surflex. *J. Chem. Inf. Model.* 49, 1581-1589.

Velankar, S., Burley, S.K., Kurisu, G., Hoch, J.C. & Markley, J.L. 2021. The Protein Data Bank Archive. *Methods Mol. Biol.* 2305, 3-21.

Wong, C.K., Hung, P., Lee, K.L. & Kam, K.M. 2005. Study of an outbreak of ciguatera fish poisoning in Hong Kong. *Toxicon* 46, 563-571.

Yamaoka, K., Inoue, M., Miyahara, H., Miyazaki, K. & Hirama, M. 2004. A quantitative and comparative study of the effects of a synthetic ciguatoxin CTX3C on the kinetic properties of voltage-dependent sodium channels. *Br. J. Pharmacol.* 142, 879-889.

Yasumoto, T. 2001. The chemistry and biological function of natural marine toxins. *Chem. Rec.* 1, 228-242.

Zhang, Y. 2015. Why do we study animal toxins? *Dongwuxue. Yanjiu* 36, 183-222.

Zhu, X., Skoneczny, D., Weidenhamer, J.D., Mwendwa, J.M., Weston, P.A., Gurr, G.M., Callaway, R.M., Weston, L.A. (2016) Identification and localization of bioactive naphthoquinones in the roots and rhizosphere of Paterson's curse (*Echium plantagineum*), a noxious invader. *J. Exp. Bot.* 2016;67:3777–3788

Zohary, M., (1978). Flora Palaestinea , Part three text, Ericaceae to Compositae

Jerusalem, The Israel Academy of Sciences and Humanities.

جامعة طرابلس

كلية العلوم

قسم علم الحيوان

تأثير المستخلص المائي لنبات *حنة العقرب* (*Echium angustifolium* Mill) كترياق لسمية سيجواتوكسين (CTXs (Ciguatoxins بأستخدام النمذجة الجزيئية ونمادج الفئران المُهق.

الطالب عبدالرووف عثمان الغرياني

د مسعود سالم معمر (مشرف أول)

قسم علم الحيوان كلية العلوم

كلية العلوم جامعة طرابلس

ا.د.عبدالفتاح مصباح قاباج (مشرف ثان)

قسم الكيمياء الطبية

كلية الصيدلة جامعة طرابلس

بحث مقدم استيفاء لمتطلبات نيل درجة الاجازة العالية (الماجستير) في العلوم من قسم علم الحيوان

29/11/2022