

Isolation and Utilization of Toxins from Marine Invasive Species towards the management of their population

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Abstract

The pufferfish *Lagocephalus sceleratus* and lionfish *Pterois miles*, are two of the most important marine alien species of the Mediterranean basin. *L. sceleratus* is one of the most toxic species on the planet, as its tissues contain Tetrodotoxin (TTX), a neuroparalytic toxin that can cause death if consumed, while lionfish is also venomous and causes a wide range of symptoms when its venom is injected through the hard thorns of its fins. Both species have rapidly developed large populations in the Eastern Mediterranean basin, while showing increasing signs of spread in the Central and Western part, causing a wide range of socioeconomic and environmental impacts. Finding innovative methods for their economic exploitation is a basic condition in order to create incentives for their targeted fishing and to achieve the limitation of their populations. A study was conducted on the isolation and identification techniques of toxins from both species. In the context of the utilization of the tetrodotoxin (TTX) contained in *L. sceleratus*, an attempt was made to integrate it into products of high added value, such as cosmetics, after the study of its encapsulation in a polymer matrix through the electro-hydrodynamic process of electrospraying.

Keywords: marine alien species, population management, cosmetics

1. Introduction

The destruction of the natural environment by anthropogenic factors is increasingly leading to the dispersal of species in ecosystems that are foreign. The impact of this dispersion clearly affects the native species and the balance of both terrestrial and marine ecosystems, leading to evolutionary pathways that are not yet fully understood [1]. These invasive species can lead to a reduction in the number of native species, to the disruption of certain important processes, to financial losses as well as to the development of diseases and pathogens. In fact, in some extreme cases, the native species may become extinct due to competition or prey by invasive ones [2]. Invasive species are often discovered after being in the new habitat for a long time, making it difficult to determine how and when the invasion began. Of course, there are cases where a species fails to establish a viable population in a particular habitat. In recent years the problem of climate change has further complicated the issue of alien species invasion due to its uncertain effect on species distribution, abundance and behavior [3], [4]. In particular, cases have already been recorded in which species have migrated due to environmental change which is related to human climate change. Given that the invasion of alien species in the Mediterranean will continue to take place in the coming years and since changes in

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ecosystems and coastal fisheries have already been reported, the whole issue is a serious ecological and economic issue that needs to be addressed immediately. [5].

Early detection and rapid response of invasive species is much more effective than trying to control a widespread infestation. In the case of *Lagocephalus sceleratus* and *Pterois miles* (commonly known as silver-cheeked toadfish and lionfish, respectively) that have already established in parts of the Mediterranean Sea and are spreading unaided towards its western and northern basins, there is a need for new customised measures in order to control their population. Within that notion, the establishment of a targeted fishing towards those species can be considered as one of the major alternatives. In order for that to be a sustainable approach we suggest the establishment of a “turn waste to profit” action, where the catch that otherwise would be destroyed, will be a source of profit. The exploitation of those species by using their bioactive compounds towards the pharmaceutical and cosmeceutical industry which answer to a well-established consumers’ audience will provide the need for these species’ long term targeted fishing and therefore contribute to their population control.

The present study focused on the isolation of toxins for the alien species *L.sceleratus* and *P.miles*. In the context of the utilization of *L.sceleratus*’s toxin, an attempt was made to integrate it into high value-added products (such as cosmetics). Before that it had been investigated the possibility of encapsulating the fish’s toxin in a polymeric matrix via electrohydrodynamic process (electrospraying). The species *Lagocephalus sceleratus* (Gmelin, 1789) and *Pterois miles* (Bennett, 1828) are two of the most important invasive alien species of the Greek sea [6,7]. The invasive Lessepsian species, i.e those that have entered the Mediterranean and Greek waters through the Suez Canal, have rapidly developed large populations in the Eastern Mediterranean basin, while they are showing increasing signs of spreadability in the central and western part. *L.sceleratus* is one of the most toxic species on the planet, as its tissue contain Tetrodotoxin (TTX), a neuroparalytic toxin that can cause death in case of consumption. For this reason, its marketing and distribution throughout the European Union has been banned. In many areas of the Eastern Mediterranean and South Aegean, accidental catches of the species often constitute a large percentage of the weight of the fish, especially in coastal fishing. As for the lionfish *P.miles*, despite the fact that does not consist an immediate danger to human life, this specie is also venomous and causes a wide range of symptoms including severe pain especially when its venom is injected through the hard thorns of its fins. The rise in temperature of the Mediterranean Sea due to the climate change favors the spread of the Lessepsian invasive species. With zero commercial value and exploitation opportunities, invasive species such as the silver-cheeked toadfish and then the lionfish are aggravating factors for Greek fishery. On the one hand finding innovative methods for their economic exploitation is a basic condition in order to create incentives for targeting fishing and on the other hand in order to achieve the limitation of their populations in the Greek sea. Regarding the lionfish *P.miles*, an attempt was made in order to isolate and identify the venom which is contained in its thorns. The protocol has to do with the uptake of venom from the thorns and tissue of the outer surface and base of the lionfish via ultrasonic extraction (Sample A, Sample B).

2. Methods and Materials

2.1 Poison extraction from spines and tissue of lionfish *P.miles* through ultrasound assisted extraction

Dorsal, lateral and abdominal spines were cut from their base and kept at -18°C until the day of extraction. The outer casing and base tissues of the spines were earlier removed. Spines were then cut into small pieces, mashed using a porcelain mortar and weighed (1.15 g). The tissues from the outer surface and base of the spines were collected in a vial to be extracted separately (0.36 g).

1.15 g of the spines were mixed with 2.3mL of phosphate buffer 10 mM (pH 7.0) containing 1 mM *CaCl*₂, followed by extraction using ultrasound (Hielscher UP 100H, 100W, 30kHz) at intensity 80% for 2 min. Centrifugation of the mixture was done (3500 rpm) for 20 min from which the supernatant was collected, containing the crude venom (Sample A).

The same extraction procedure was performed for the external tissues of the surface and base of the spines (Sample B).

2.2 Determination of total protein concentration in the spines of lionfish

To find the concentration of total protein in samples A and B, the specialized kit “Pierce™ Microplate BCA Protein Assay Kit Reducing Agent Compatible” of Thermo Scientific was used. The Pierce BCA test is based on the known reduction of Cu^{2+} to Cu^{+} of proteins to alkaline medium and its subsequent sensitive and selective colorimetric detection of the copper cation using dicinochonic acid (BCA).

2.3 Tetrodotoxin extraction from muscle tissue and internal organs of the pufferfish *L. sceleratus*

For the isolation of TTX of *L.sceleratus* from muscle tissue and internal organs, the aqueous acetic acid solution 0.1% v/v was used. 30 g of muscle tissue were weighed and chopped in a blender. They were transferred to a 250 mL conical flask and mixed with 100 mL of 0.1% aqueous acetic acid solution v/v and then homogenized using a BagMixer (Interscience) for 10 min followed by ultrasonication (Witeg A22H Ultrasonic Bath) for 10 min. The mixture was then frozen at a temperature of -18°C for 10 min and centrifuged for 30 min at 3500 rpm in order to separate the toxin precipitate.

2.4 Encapsulation of *L.sceleratus* tetrodotoxin in a zein matrix via electrospraying

After the isolation of TTX, next step was to encapsulate the toxin for the purpose of utilizing it in products of high added value such as cosmetic products. The encapsulation study was performed using the electrohydrodynamic process of electrostatic spraying (electrospraying). The polymeric matrix used was zein.

Zein flakes were dissolved in a methanol / water solution (70% MeOH, 30% H_2O) with a magnetic stirrer for 3 hours at a concentration of 10% w/w. The extracted tetrodotoxin was added to this solution with a ratio of 1: 12. Stirring was continued for another hour in order to ensure complete dissolution of the materials before the process of electrospraying (FluidNatek, BioInicia S.L., Spain).

After an operating conditions' optimization, the best results were obtained when its surface collector was placed at 13 to 15 cm from the capillary tip, the volumetric supply ranged between 200 and 300 $\mu\text{L}/h$ and the trend ranged from 25 to 27 kV. The volumetric flow of acetic acid (position 2) was constant at 100 $\mu\text{L}/h$. Optimal conditions were displayed at surface collector position of 15 cm, volumetric flow of 200 $\mu\text{L}/h$ and applied voltage at 26 kV.

2.5 Development of cosmetic creams - Physicochemical characterization

The development and analysis of cosmetic products was done in sight of investigating the effect of adding the enclosed TTX as an additive ingredient in a standard cosmetic cream formula. For this purpose, an attempt was made to integrate the particles (with TTX encapsulated by the electrospraying process) in a cosmetic base cream without additives. Essentially the final product will be a cosmetic cream that will mimic the action of Botox, which acts directly on the skin muscles preventing their contractions.

The desired amount of active ingredients (TTX) to be contained in the cream is equivalent to 1% of its weight. Therefore, for the needs of the experiment two types of cream will be analyzed: the cream base and cream base with encapsulated zein particles containing TTX. Acidity (pH) and color measurements were made as well as rheological oscillation tests (frequency scan tests) were performed using the method of Dynamic Mechanical Analysis (DMA) in order to evaluate changes in the storage rate (G'), loss factor (G'') and composite viscosity (η^*). The procedure followed to determine the acidity of creams includes the preparation of Ringer's solution by dissolving one tablet in 500mL deionized water. 1g of each cream was sampled and placed in 9 mL of Ringer's solution followed by pH measurement for each sample using an electronic pH meter (MARTINI Instruments 180 Bench Meter). The control of the change of color of the creams took place using a colorimetric spectrophotometer (MiniScan XE Hunter, Associates Laboratory Inc, Reston, Virginia).

3. Results and Discussion

3.1 Total protein concentration of *Pterois miles*' thorns

Table 1: Protein Concentration ($\mu\text{g}/\text{mL}$) of samples A and B obtained by the calibration curve

Protein Sample	Dilution	Protein Concentration ($\mu\text{g}/\text{mL}$)
A	-	580
A	1:4	668
A	1:10	692
B	-	3334
B	1:4	3447
B	1:10	4132

As shown in Table 1, the highest protein concentration is found in sample B (base thorn tissue). This was to be expected, as the fish's tissues and skin debris are considered to be rich in protein.

From the concentrations found through the calibration curve for the diluted samples, the initial protein concentration (before dilution) of the samples was calculated. This was then compared to the concentration found in non-diluted samples to test for the possibility of interference with the analysis of proteins by the specific methodology. For sample A the initial concentrations calculated were similar to the concentration of the undiluted sample, with relative standard deviation (RSD) 9%. The same happened for sample B, where the measurements in the initial solution and its dilutions showed RSD 12%. Since the concentration of each sample and the volume and mass of the extracted tissues are known, the total amount of protein per gram of tissue was calculated.

Table 2: Protein concentration of samples A and B in lionfish biomass (mg /gr)

Sample	mg Protein /gr Biomass
A	760
B	6062

3.2 Characterization of final encapsulation TTX products

3.2.1 Morphology of electrostatic spray products with SEM

The final is presented morphology of the particles after their treatment by Microscopy Electronic Scanning (SEM).

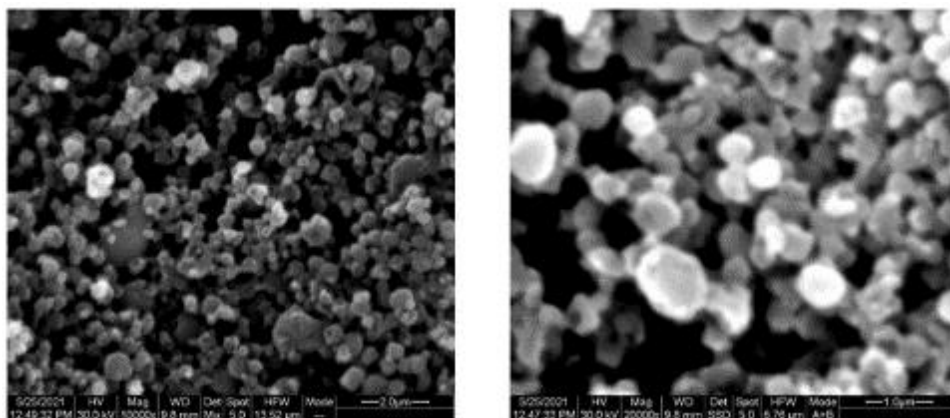


Figure 1: Imaging of the particles that emerged through the electro spraying process (operating conditions: ASA 15 cm, volumetric flow 300 μ L / b, voltage 26 kV) using Electron Scanning Microscopy.

After processing the images with ImageJ Software, it was calculated that the average particle diameter is 540 ± 53 nm. The size and the shape of the encapsulation product are the two main factors for its interaction with different skin blocks. Regarding the shape, it is considered that spherical nanoparticles have better capacity penetration into the skin compared to other forms (e.g., ellipsoids) due to their symmetry in all dimensions and their smaller surface [8]. In terms of the size, they can be absorbed by the skin either through the skin appendages or through the keratin layer [9]. Particles of 500-1000 nm are capable of penetrating skin surface layers, while smaller ones can go even deeper releasing the desired compound [10]. On the other hand, the skin exposure in nanoparticles of very

small size (<100 nm) and especially in those with size less than 30 nm can be extremely dangerous as it can lead to prolonged erythema (redness of the skin), swelling and even partial necrosis of the skin (eschar) [11]. Therefore, the particles in terms of size are suitable and can be used in cosmetics.

3.2.2 PH Measurements of the produced cosmetic product

The optimal pH for human skin is around 5.5 [12]. Aim of most care product companies are creating formulas where their pH is friendly to the skin so that they do not disturb the skin's defense mechanism (secretion of oil, sweat and lymph fluids). So, in the case where this mechanism is disrupted (basically due to poor quality cosmetics) the skin loses its balance and at the same time the ability to absorb nutrients.

The pH of cosmetics is an important quality factor. The pH value of human skin ranges from 5.4-5.9 (neutral skin pH), but shows changes depending on age, gender, race and metabolic rate (depending on the time of day and the season). The cosmetics preparations should have a neutral pH, always in relation to that of the skin [13].

Table 3 shows the acidity measurements performed for the two types of cream.

Table 3: Acidity measurements (pH) of the base cream (C) and the TTX added-cream product (E)

Cream sample	pH
C	6.78
E	6.23

3.2.3 Color Measurement of the produced cosmetic product

Color measurements included the measurement of brightness (L), the measurement of the red-green color change (a) and the measurement of its blue-yellow color change (b). Table 4 shows all the measurements (L, a, b) performed for both types of cream.

Table 4: Color measurements brightness (L), red-green color change (a), blue-yellow color change (b) of the base cream (C) and the TTX added-cream product (E)

Sample	L	a	b
C	75.13	-0.54	-0.57
E	73.76	-2.55	9.68

Regarding the brightness (L) similar values are observed between the two samples. Regarding the change of the green-red color (a), it is observed that the base cream (sample C) is close to the zero zone while the cream with the encapsulated TTX (sample E) gets negative values (below -1.5), a fact that represents shades of green. In the case of factor b which indicates the change of blue-yellow color, the dominance of the yellow color in the cream with the encapsulated substance (positive values with values above 5) is evident. This fact is completely normal as the matrix that was used for the realization of the encapsulation is yellow.

3.2.4 Dynamic Mechanical Analysis (DMA)

Rheology is a valuable tool in the effort to evaluate the performance of a cream

when is spread to the skin. According to this, measurements regarding rheology provide significant data about the cream structure and stability [14]. For the study of factors related to rheology and viscoelasticity, the measurements of the storage coefficient G' , the loss factor G'' and the composite viscosity η^* were taken. The stability of cosmetics depends on the intramolecular forces of interaction that form a three-dimensional network. Concerning the dynamic parameters of these products, the G' storage coefficient must be greater than the loss rate throughout the frequency range. Figure 18 and Figure 19 show the change of dynamic parameters (G' , G'' , and η^*) as a function of the angular frequency for samples C and E at time zero.

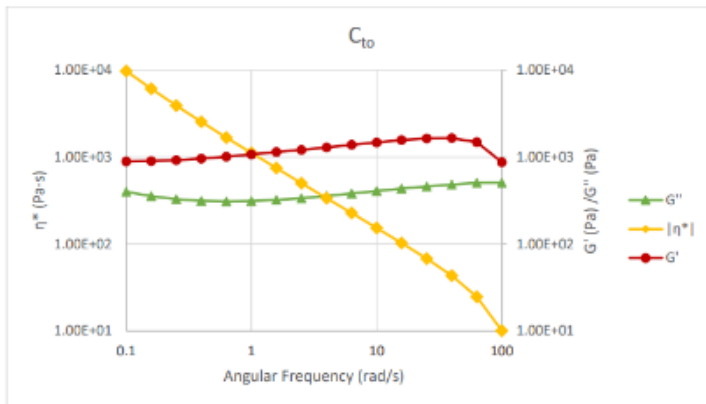


Figure 2: Presentation of G' , G'' and η^* as a function of angular frequency at a constant frequency $\gamma = 0,1\%$ for sample C

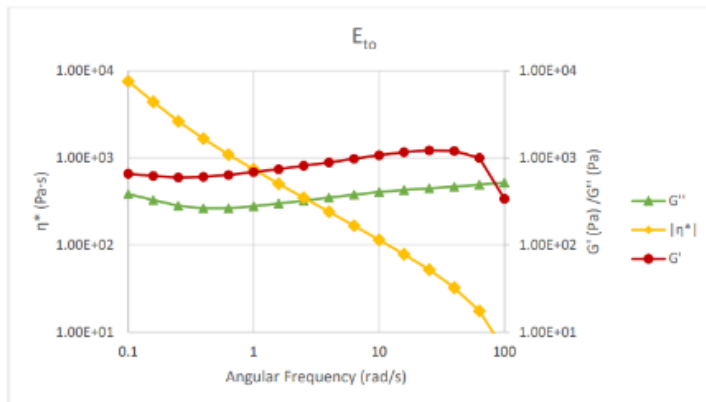


Figure 3: Presentation of G' , G'' and η^* as a function of angular frequency at a constant frequency $\gamma = 0,1\%$ for sample E

In both cases the curves G' , G'' are almost parallel throughout frequency range with G' consistently greater than G'' . The value G' in different angular frequencies provide valuable information. In particular, the value G' at low angular frequency ($\omega \leq 0.1 \text{ rad / s}$) is related to the performance of the sample at rest.

According to an empirical rule applied in the formation of cosmetics formulations, when $G' \geq 10$ Pa then the dispersion is considered as stable, whereas when $G' \leq 1$ Pa it is unstable [15].

In this case the G' value is greater than 10 Pa in both cases (891 and 655 Pa respectively), wherein the formulations show satisfactory stability.

Low deformation values in combination with low G' and G'' values are a sign of a soft sense that characterize the "soft" creams. On the other hand, "hard" creams require equally low values of deformation in order to be applied on the skin, but the higher G' and G'' values show a sense of hardness. Therefore, there is a need to know the viscoelastic parameters of cosmetics since they can act as prediction factors regarding the performance of different products.

Table 5: Values G'_0 and G''_0 for the samples C and E, for angular frequency 0.1 rad/ s

Cream Sample	Storage Temperature (°C)	Storage Time (days)	G'_0 (Pa)	G''_0 (Pa)
C	25	0	891	397
E	25	0	655	384

Regarding the values of Table 5 it is observed that sample C shows higher values G' and G'' . According to this fact, the base cream C can be described as "hard" cream while E as "soft". Nevertheless, the important thing is that both creams meet all needed specifications for commercial cosmetic products. Viscosity measurements also verify this fact. The viscosity can be used as a crucial factor for cosmetics to determine the changes that materials may undergo. In some cases, a slight increase in the value of viscosity is observed due to the growth and further balancing the emulsified structures, while at other times such as long storage conditions, the viscosity can be significantly reduced due to product degradation [16]. The following diagrams show the viscosity values as a function of the angular frequency for both types of cream.

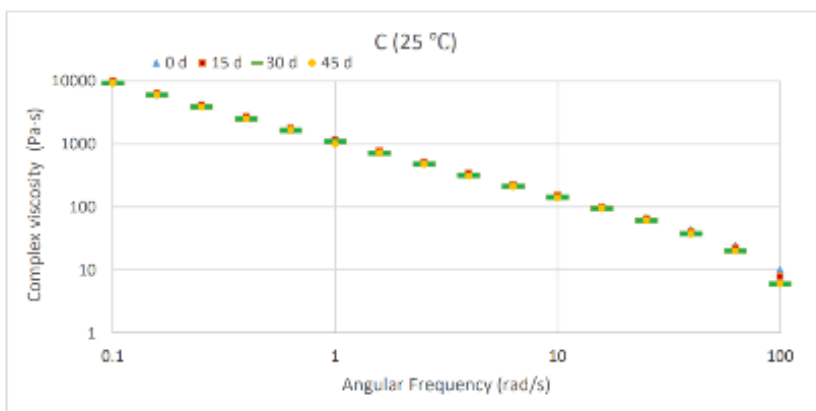


Figure 4: Change of the composite viscosity over the whole frequency range (ω) under constant $\gamma = 0,1\%$ for the base cream

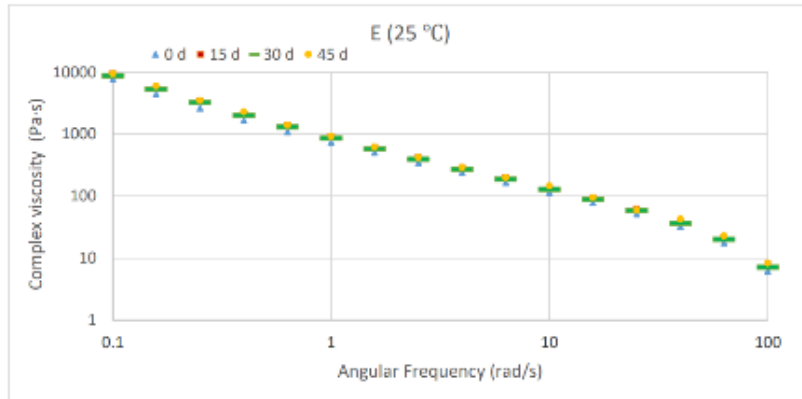


Figure 5: Change of the composite viscosity over the whole frequency range (ω) under constant $\gamma = 0,1\%$ for the cream E with added TTX

Charts concerning the base cream show that the composite viscosity is not significantly affected. A similar performance is observed for the cream with the enclosed product.

4. Conclusions

The major achievements of the presented study were:

- i. The selection and implementation of the optimum protocols through literature [17] for the isolation of venom from the lionfish's thorns
- ii. The selection and application of the optimum protocols through literature [18] for the isolation of tetrodotoxin from the muscle tissue and organs of the *L. sceleratus*
- iii. The optimization of electrospraying encapsulation technique and the development of stable formulation with potential use as ingredients in cosmeceutical applications
- iv. Incorporation of the developed formulation in cosmetic creams for facial use with acceptable organoleptic and physicochemical characteristics

The encapsulation of tetrodotoxin and the formulation of nanoparticles using electrospraying technique as well as the incorporation of these encapsulated formulations in cosmeceutical products is a scientific breakthrough since no published research can be found up to now. Based on these successful results, this work could contribute by turning the current problem of invasive alien species into a "win-win" solution for fisheries and companies operating in the field of nutrition and cosmetology. In addition to the innovation that would be brought to the pharmaceutical and food sectors by opening up a new market for innovative formulations based on unique products, their exploitation is expected to create a new type of fishery that will bring economic benefits to professional fishermen themselves. The next research steps will be focused on safety issues regarding the developed cosmeceutical products, the implementation of clinical trials that will prove the safety and functionality of the new products following the EU and national regulations in matter of Cosmetic products.

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