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Can bioactive peptides of *Lagocephalus sceleratus* be evaluated in the functional food industry?

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Abstract

Observations of Lessepsian migrant *Lagocephalus sceleratus* has been increasing along the Turkish coastline. Because of its toxins, it is known as a poisonous fish and not recommended to consume. To overcome this problem, an *in silico*-based biotechnological approach is proposed to evaluate the bioactive peptides from this species. The bioactive peptide contents of cytochrome oxidase subunit 1 in *L. sceleratus* with BIOPEP parameters were investigated in this study. The results show that there are many bioactive peptides such as the peptides with DPP-IV, ACE, alpha-glucosidase inhibition activities, antioxidant and antiamnestic and their levels are comparable in well-consumed species such as *Gallus gallus domesticus* and *Bos taurus*. In conclusion, after removal of the toxin, the biomass of the *L. sceleratus* can be used to produce bioactive peptides for the production of functional foods which will be very important for food industry to provide multi-functional properties to foods. The paper can be used as a model methodology to exploit new bioactive peptides when new proteins are explored from *L. sceleratus*.

Introduction

Introduction of invasive species into the new ecosystem creates important problems on the indigenous species. The Mediterranean Sea is under threat of introduction of invasive species due to the Suez Channel and also heavy maritime traffic (Galil, 2009). Although *Lagocephalus sceleratus* (Gmelin, 1789) is a very common species in the tropical waters such as Indian and Pacific Ocean, it is being one of the well invaded species in the Mediterranean Sea via Suez Channel (Leonardo et al, 2019). This alien fish has been commonly observed in almost all the Mediterranean Sea region (Akyol et al., 2005; Kalogirou, 2013; Kasapidis et al., 2007). Since this species has poisonous compounds in its tissues, it is not under stress of human consumption and fisheries (Leonardo et al, 2019). Therefore, this species has increased its habitat along the Mediterranean Sea. The scientific reports and also observations by fishermen revealed that this species consumes the eggs of local fish species and destroys the fisheries in the Mediterranean Sea (Kalogirou, 2013; Yaglioglu et al., 2011). According to Ulman et al. (2015), while 5% of fish caught in 2003 were *L. sceleratus*, this increased up to 50% after 5 years. *L. sceleratus* is a successful invasive species as it has a high reproduction rate, can hunt natural predators, benefit from food resources and adapt well to environmental conditions (Yaglioglu et al., 2011). They can reach 15-60 cm in length and mostly live at depths of 18-100 m (Yaglıoglu et al., 2011). *L. sceleratus* is very difficult to be hunted by its predators since it can inflate itself with the water and air in the environments and is highly poisonous (Golani et al., 2006). *L. sceleratus* includes tetrodotoxin (TTX) and this toxin is considered as one of the most effective chemicals among the marine toxins. Since no developed antidote or antitoxin related to TTX is existed, it is very dangerous to touch or consume *L. sceleretus*.

According to scientific publications, *L. sceleratus* in the Mediterranean Sea also includes the toxin at lethal doses (Bentur et al., 2008). As the fish grows in size, its toxicity increases (Katikou et al., 2009). Katikou et al. (2009) reported that while liver, gastrointestinal system and gonads had the highest level of toxicity in all of their samples, the toxicity in muscles and tissues was lower. The toxicity distribution is species-specific, but within the same species, local, seasonal and individual variations and also variations in toxin composition are observed (Katikou et al., 2009; Noguch & Arakawa, 2008; Yu & Yu; 2002). Kosker et al. (2019) investigated TTX levels in different *L. sceleratus* and found the TTX level in tissues in the range of 0.69-35.6 µg/g and most in gonad and liver tissues.

TTX is used as a channel blocker in physiological and neurological studies in many laboratories (Narahashi, 2001; Saoudi et al., 2010). TTX has an analgesic effect in advanced patients, shows an antitumor effect and is used for the treatment of drug addiction (Bragadeeswaran et al., 2010; Haque et al., 2008; Saoudi et al., 2010; Schwartz et al., 1998; Yu, 2008). Also, TTX is used for rheumatism treatments in Japan (Noguchi & Arakawa, 2008). Due to the possible inhibitory effect of TTX on neural conduction, it is examined for developing anesthetic drugs (Schwartz et al., 1998).

Food derived bioactive peptides are the hot research topics in functional food industry. Bioactive peptides are important sources for essential amino acids, and they have biological activities such as antioxidant, antimicrobial, antihypertensive, anticancer and antihyperglycemic (Karami et al., 2019; Tonolo et al., 2020; Valencia-Mejía et al., 2019; Zhou et al., 2020). Bioactive peptides can be obtained through the enzymatic hydrolysis, fermentation or food processing (Liu et al., 2020). Most of the bioactive peptides consist of 2-20 amino acids and they display different activities based on their amino acid compositions and secondary structures (Bechaux et al., 2019; Ryan et al., 2011). Bioactive peptides have been classified via *in silico* or *in vitro* studies from dairy products, mushroom, fish, meat, and seaweed etc. (Barati et al, 2020; Cipolari et al., 2020; Lafarga et al., 2020; Sayd et al., 2018; Zhou et al., 2020). The number of bioactive peptides according to BIOPEP-UWM is 4056 and this number is increasing with the new studies (Minkiewicz et al., 2019). Since they are easily absorbed in the intestine and can go into the circulatory system, they can exhibit physiological effects (Martínez-Sánchez et al., 2020). Moreover, bioactive peptides have been the subject of research in the development of functional foods and medicines (Jauset & Beaulieu, 2019; Tadesse & Emire, 2020). Fish based bioactive peptides are reviewed by Cipolari et al. (2020). They underlined that fish venoms and poisons are still undiscovered resources for bioactive peptides.

In this study, we aimed to investigate bioactive content of *L. sceleratus* by selecting a model protein (cytochrome c oxidase subunit 1) and then the results of *L. sceleratus* were compared with the traditional protein sources. This model biotechnological method proposes a novel *in silico* approach to evaluate the biomass of *L. sceleratus.*

Methods

The proteins were searched by using the keyword "*Lagocephalus sceleratus*" in Uniprot.org (The UniProt Consortium, 2019). 45 entries were found in uniprot.org related to *L. sceleratus*. From the entries, it is understood that they are unreviewed that means "*records that await full manual annotation*" according to Uniprot.org. The min and max length of amino acids in the entries were found in major capsid protein and NADH-ubiquinone oxidoreductase chain 5 (EC 7.1.1.2) to be 51 and 612, respectively. Cytochrome c oxidase subunit 1 was selected as a model protein for our research due to *i)* it contains relatively high number of amino acids, *ii)* it is also found in well-consumed animal sources such as bovine (*Bos taurus*) and chicken (*Gallus gallus domesticus*), iii) it is an important protein in electron transport chain. Bovine and chicken sequences were selected for comparison purposes. Moreover, this protein is one of the vital components of the mitochondria. The catalytic activity is based on the electron transfer and it catalyzes the reduction of oxygen to water via reduced cytochrome c. The aim of this report is to exhibit a model study for *L. sceleratus*. When new proteins from this fish species are discovered, bioactive peptide contents can be identified by using this methodology. The flowchart of the study was given in Figure 1.

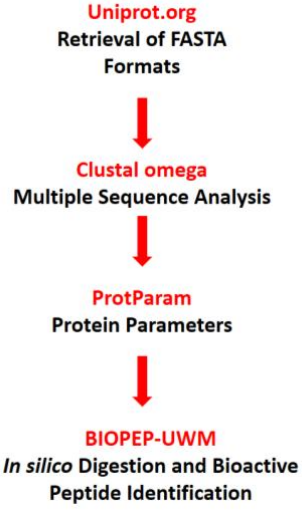

Figure 1. Flowchart of the methodology.

Amino acid sequence in FASTA format of cytochrome c oxidase subunit 1 protein found in *Lagocephalus sceleratus* (F2EN11)*, Bos taurus* (P00396) *and Gallus gallus* (P18943) were retrieved from Uniprot.org (The UniProt Consortium, 2019; Morgat et al., 2019).

Multiple sequence analysis of cytochrome c oxidase proteins was performed using Clustal omega

(Sievers et al., 2011). The similarity between sequences and preserved regions were shown by using the outputs of Clustal omega.

ProtParam tool was used for defining the physical and chemical characteristics of studied proteins (Gasteiger et al., 2005). Molecular weight, percentage and number of amino acids, instability index, net charge and theoretical pI value were determined via this tool.

Bioactive peptides found in *L. sceleratus* were investigated using BIOPEP-UWM database (Minkiewicz et al., 2019). Chymotrypsin, trypsin and pepsin (pH=1.3) enzymes were used for *in silico* digestion of the proteins studied in the report. These enzymes were chosen in the study because they are involved in the gastrointestinal track of humans. Cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* were also *in silico* digested by using the same methodology in the present study.

Results

Clustal omega database was used for determining the similarity between *Lagocephalus sceleratus, Bos taurus* and *Gallus gallus domesticus*. The asterisk symbol (*) shows conserved residues while colons (:) indicates groups with similar characteristics and period (.) shows groups with little similar characteristics. The comparisons between cytochrome c oxidase subunit 1 proteins of *L. sceleratus, B. taurus* and *G. gallus domesticus* can be seen in Figure 2a-b. The multiple sequence analysis results of the cytochrome c oxidase subunit 1 proteins found in *L. sceleratus, B. taurus* and *G. gallus domesticus* were shown in Figure 2a-b. From Figure 2a and b, it could be said that there is very high similarity among these species.

Physical and chemical characteristics of cytochrome c oxidase subunit 1 proteins found in *L. sceleratus, B. taurus* and *G. gallus domesticus* were determined by using ProtParam tool (Gasteiger et al., 2005). According to Table 1, most abundant amino acids found in cytochrome c oxidase subunit 1 protein of *L. sceleratus* are leucine, alanine and glycine to be 12.10, 9.20, and 9.10%, respectively. There is a similar order for the number of amino acids found in cytochrome c oxidase subunit 1 proteins of *B. taurus* and *G. gallus domesticus*. The theoretical pI values of cytochrome c oxidase proteins found in *L. sceleratus* and *G. gallus domesticus* were found nearly the same as 6.21 and 6.23 (Table 2).

Table 1. Number and percentage of amino acids found in cytochrome c oxidase subunit 1 proteins of *Lagocephalus sceleratus*, *Bos taurus* and *Gallus gallus domesticus*

	Lagocephalus sceleratus		***Gallus gallus domesticus***		***Bos taurus***	
	#	**%**	**#**	**%**	**#**	**%**
Ala (A)	48	9.20	46	8.90	40	7.80
Arg (R)	9	1.70	9	1.70	8	1.60
Asn (N)	14	2.70	15	2.90	19	3.70
Asp (D)	14	2.70	15	2.90	16	3.10
Cys (C)	1	0.20	1	0.20	1	0.20
Gln (Q)	7	1.30	9	1.70	6	1.20
Glu (E)	12	2.30	10	1.90	9	1.80
Gly (G)	47	9.10	46	8.90	47	9.10
His (H)	21	4.00	19	3.70	17	3.30
Ile (I)	37	7.10	41	8.00	37	7.20
Leu (L)	63	12.10	62	12.00	59	11.50
Lys (K)	9	1.70	9	1.70	9	1.80
Met (M)	27	5.20	26	5.00	34	6.60
Phe (F)	42	8.10	43	8.30	42	8.20
Pro (P)	28	5.40	31	6.00	28	5.40
Ser (S)	28	5.40	26	5.00	30	5.80
Thr (T)	37	7.10	40	7.80	38	7.40
Trp (W)	17	3.30	17	3.30	17	3.30
Tyr (Y)	18	3.50	18	3.50	19	3.70
Val (V)	40	7.70	32	6.20	38	7.40

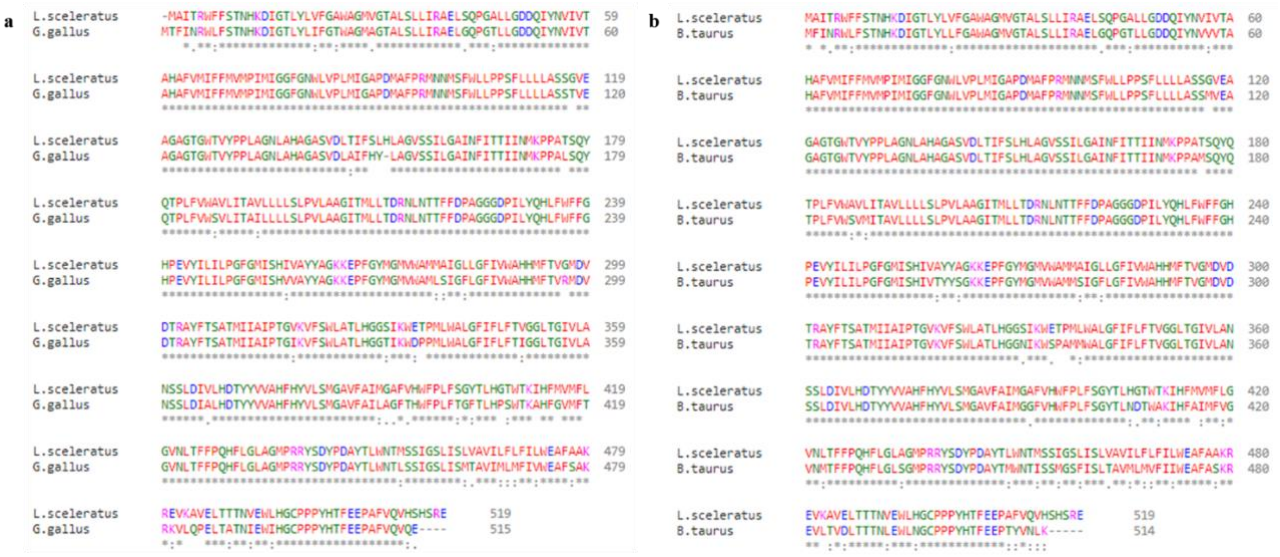

Figure 2. Multiple sequence analysis of **(a)** *Lagocephalus sceleratus* and *Gallus gallus domesticus* **(b)** *Lagocephalus sceleratus* and *Bos taurus.*

Table 2. Protein parameters of cytochrome c oxidase subunit 1 proteins found in *Lagocephalus sceleratus*, *Bos taurus* and *Gallus gallus domesticus* (aa: amino acids)

Species	# aa	Mw (Kda)	Theoretical pI	Negatively Charged Residues (Asp+Glu)	Positively Charged Residues (Arg+Lys)	Net Charge	Instability Index
Lagocephalus sceleratus	519	57314.59	6.21	26	18	-8	26.54
Gallus gallus domesticus	515	57051.23	6.23	25	18	-7	29.60
Bos taurus	514	57032.31	6.06	25	17	-8	25.81

The net charge of cytochrome c oxidase subunit 1 protein of *L. sceleratus* was found to be -8 while cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* were determined as -7 and -8, respectively. Instability index is a value that corresponds to the stability of the protein found in the test tube. Protein is stable when the stability index is below 40 (Guruprasad et al., 1990). According to Table 2, the instability index of cytochrome c oxidase proteins found in *L. sceleratus* is 26.54 and other studied proteins have similar instability index values.

Table 3. *In silico* hydrolysis of cytochrome c oxidase subunit 1 protein of *Lagocephalus sceleratus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 35.1393					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Antiamnestic	0.0015	0.3261	3.28850E-5	1
2	Inhibitor	0.0015	1.0000	0	
3	ACE inhibitor	0.0495	0.1266	0.00404	0.14986
4	Stimulating	0.0077	0.1158	0	
5	Antioxidative	0.0108	0.1247	0	0
6	Renin inhibitor	0.0062	0.2672	0.00017	0.98743
7	CaMPDE inhibitor	0.0015	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0634	0.1100	0.00013	0.21510
9	Alpha-glucosidase inhibitor	0.0031	0.1179	0.00014	0.65774
10	Dipeptidyl peptidase III inhibitor	0.0124	0.1542	0	

In silico hydrolysis of studied proteins were performed using BIOPEP-UWM tool and chymotrypsin, trypsin and pepsin (pH=1.3) enzymes were used. DH_t value is a theoretical degree of hydrolysis and in this study DH_t value of cytochrome c oxidase subunit 1 proteins found in *L. sceleratus* is 35.1393 (Table 3). A_E value corresponds to the frequency of release of fragments by selected enzymes (Minkiewicz et al., 2019). According to Table 3, A_E value was found for dipeptidyl peptidase IV inhibitor as 0.0634, ACE (Angiotensin-converting enzyme) inhibitor as 0.0495 and dipeptidyl peptidase III inhibitor as 0.0124. Similar activities and A_E values were obtained with the hydrolysis of cytochrome c oxidase proteins of *B. taurus* and *G. gallus domesticus* (Table 4-5).

Table 4. *In silico* hydrolysis of cytochrome c oxidase protein of *Gallus gallus domesticus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 35.1823					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Immunostimulating	0.0016	0.5000	0	
2	Antiamnestic	0.0016	0.3404	3.36655E-5	1
3	ACE inhibitor	0.0522	0.1320	0.00382	0.13687
4	Stimulating	0.0079	0.1280	0	
5	Antioxidative	0.0127	0.1639	0	
6	renin inhibitor	0.0079	0.2500	0.00017	0.98746
7	CaMPDE inhibitor	0.0016	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0633	0.1102	0.00018	0.29338
9	Alpha-glucosidase inhibitor	0.0032	0.1190	0.00014	0.49511
10	Dipeptidyl peptidase III inhibitor	0.0142	0.1547	0	

Table 5. *In silico* hydrolysis of cytochrome c oxidase protein of *Bos taurus* using BIOPEP-UWM database (CaMPDE: Calmodulin-dependent phosphodiesterase 1; ACE: Angiotensin Converting Enzyme)

DH_t [%] 36.2041					
No	**Activity**	**A_E**	**W**	**B_E**	**V**
1	Antiamnestic	0.0016	0.3333	3.38799E-5	1
2	Inhibitor	0.0016	1.0000	0	
3	ACE inhibitor	0.0478	0.1261	0.00391	0.14212
4	Stimulating	0.0064	0.1087	0	
5	Antioxidative	0.0096	0.1257	0	0
6	Renin inhibitor	0.0064	0.3092	0.00017	0.98743
7	CaMPDE inhibitor	0.0016	1.0000	0	
8	Dipeptidyl peptidase IV inhibitor	0.0621	0.1068	0.00013	0.20641
9	Alpha-glucosidase inhibitor	0.0032	0.1339	0.00014	0.65819
10	Dipeptidyl peptidase III inhibitor	0.0127	0.1564	0	

Discussion

The importance of bioactive peptides is being increased since the several health effects of bioactive peptides are shown in numerous papers. Bioactive peptides occur via enzymatic catalysis, digestion in

gastrointestinal track or fermentation via several microorganisms (Chalamaiah et al, 2018; Gorguç et al., 2020). Bioactive peptides are generally characterized via wet lab studies. On the other hand, *in silico* tools developed under the umbrella of bioinformatics have been providing significant contributions to wet-lab studies and also, they have been accelerating the discoveries of novel bioactive peptides. One of the most important and well known in silico tools is found in BIOPEP-UWM Database. (Minkiewich et al., 2019). By using BIOPEP-UWM Database, several bioactive peptides such as ACE inhibitor, activating ubiquitin-mediated proteolysis, alpha-glucosidase inhibitor, antiviral, dipeptidyl peptidase IV inhibitor, heparin binding, HMG-CoA reductase inhibitor, Protein Kinase C inhibitor and vasoconstrictor can be obtained under *in silico* conditions. More activities are listed by Minkiewich et al. (2019). The tool provides scientists to compare the protein sources efficiently before wet-lab conditions. From this perspective, the bioactive peptide contents of cytochrome c oxidase subunit 1 from invasive *L. sceleratus* are compared with cytochrome c oxidase subunit 1 from *Bos taurus* and *Gallus gallus domesticus*.

Red meat consumption is becoming very problematic due to many factors such as the contribution to increased emission of greenhouse gases, environmental pollution problems and also health problems caused by processed meat products (Gonzales et al., 2020). Instead of increasing red meat consumption from terrestrial livestocks, the use of *Lagocephalus sceleratus* may provide an alternative source after removal of its toxin.

In this study, protein parameters of cytochrome c oxidase subunit 1 protein from *L. sceleratus* were compared with *G. gallus domesticus* and *B. taurus*. From the results, it could be said that essential amino acids are almost same levels in these species. Therefore, *L. sceleratus* could be used to supply essential amino acid resources for functional food industry. However, it is important to note that the toxin should be removed before use of the bioactive peptides or the essential amino acids from *L. sceleratus*. The concentration of TTX varies based on many different factors such as season, length and also organs (Kosker et al., 2016; Kosker et al., 2019). Therefore, optimized conditions should be developed before utilization of the bioactive peptides from this alien species in the Mediterranean Sea.

The model protein, cytochrome oxidase subunit 1, from *L. sceleratus* was hydrolyzed in the study by using digestion enzymes such as chymotrypsin, trypsin and pepsin. Many bioactive peptides such as antiamnestic, inhibitor, ACE inhibitor, stimulating, antioxidative, renin inhibitor, Calmodulin-dependent phosphodiesterase 1 (CaMPDE) inhibitor, dipeptidyl peptidase IV inhibitor, alpha-glucosidase inhibitor, dipeptidyl peptidase III inhibitor were obtained after *in silico* hydrolysis. The results were also compared with *B. taurus* and also *G. gallus domesticus* (Table 3-5).

Although we observe slightly increased DHt (%) in *B. taurus*, the values of *L. sceleratus* and *G. gallus domesticus* were very close. It is very interesting to note that A_E value related to ACE inhibition in *G. gallus domesticus* was significantly higher than those of other studied animals in the study. Stimulating bioactive peptides are defined as the peptides that stimulate various biological processes. Since the values of *G. gallus domesticus* and *L. sceleratus* were very close, *L. sceleratus* can be used as an alternative source for *G. gallus domesticus*. Antioxidant property of *L. sceleratus* showed similar trend, AE value was very close to *G. gallus domesticus*, on the other hand, it was higher than that of *B. taurus*. Since there are many well defined antioxidant molecules in scientific literature, antioxidant property of an industrial food component can be supplied from different plant based sources. Renin-angiotensin system (RAS) has important physiological role for blood pressure and related homeostasis (Crowley et al., 2012). Increased activity of renin is associated with elevated blood pressure (Fu et al., 2017). Renin inhibitor property of *L. sceleratus* was found to be close to *B. taurus,* on the other hand, it was lower than that of *G. gallus domesticus*.

The bioactive peptides with CaMPDE inhibitor property may be of importance in the diseases associated with excessive inflammatory signaling since CaMPDE plays critical roles in cyclic nucleotide metabolism (O'Brien et al., 2020). Obtaining similar values compared to *G. gallus* and *B. taurus* might show the importance of *L. sceleratus*.

For example, DPP-IV is one of the target enzymes in the therapy of Type II Diabetes. Its inhibition increases the insulin stimulation (Craddy et al., 2014). Similar inhibition parameters were found within degraded protein of *L. sceleratus* confirms that the isolated bioactive peptides (TTX-free) from this alien can be proposed for consumption in diabetes therapy. Another medicinally important enzyme is ACE. This enzyme is associated with hypertension and its inhibition may provide a contribution to the therapy of hypertension. We also observed that ACE inhibitor property is also existed in the results (Table 3).

Alpha-glucosidase inhibition is an important topic in therapeutic approaches for diabetes mellitus due to involvement of alpha-glucosidase in the digestion of dietary starch into glucose (Papoutsis et al., 2020). The A*E* values related to alpha-glucosidase inhibition were found quite similar. Therefore, it could be said that there is no difference among the species studied in this study for alpha-glucosidase inhibition.

At first glance, it could be thought that use of this alien species for the Mediterranean Sea could be dangerous due to TTX. On the other hand, several studies reveal that the tissues of the fish even with TTX may be used for the treatment of various diseases.

In an interesting study carried by Hong et al (2018), it is shown that oral TTX pellets inhibited resiniferatoxin-induced postherpetic neuralgia in a rat model. TTX was

also reported as an analgesic for various pains and also cancer (Nieto et al., 2012). TTX combined with lidocaine is also proposed for severe arrhythmias by Hong et al. (2019). From these publications, it could be inferred that bioactive pellets including bioactive peptides mentioned in this study can be exploited in these diseases.

As it is mentioned in the materials section, limited numbers of protein sequences exist in the protein databases related to *L. sceleratus*. Liver and gonads of this species may include novel proteins and also novel bioactive peptides. After wet lab-based techniques such as 2D-electrophoresis, the novel proteins in this fish can be explored. After sequence analysis, enzymatically digested proteins can be studied *in silico* and *in vitro* to understand their functional properties. However, it must be noted that TTX should be removed from *L. sceleratus* based products for possible human consumption because of its toxic effects on human metabolism. Alternatively, TTX containing formulas like in Hong et al. 2018 and Hong et al. 2019 may be prepared to evaluate the biomass of the species (for therapeutic purposes).

Conclusion

Observation of *L. sceleratus* in the Mediterranean coastline of Turkey has been in increasing trend. Since no human consumption is proposed due to its highly effective toxin, an alternative utilization method should be proposed to create a stress on the *L. sceleratus* population. The present paper reveals that there are many bioactive peptides and their BIOPEP parameters are in comparable levels with well-consumed *B. taurus* and *G. gallus domesticus*. Moreover, TTX have been recently associated with promising therapeutic effects (Hong et al., 2018; Hong et al., 2019; Nieto et al., 2012). Therefore, more wet lab-based studies are strongly recommended to exploit the idea on the utilization of widely distributed *L. sceleratus* in the Mediterranean Sea.

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