DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MIGRATION OF CAULERPIN FROM *CAULERPA* SPP. TO *SARPA SALPA*

by

Songül TURHAN

July, 2018

İZMİR

MIGRATION OF CAULERPIN FROM *CAULERPA* SPP. TO *SARPA SALPA*

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in Partial Fulfilment of the Requirements for the Degree of Master of Science
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İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **MIGRATION OF CAULERPIN FROM *CAULERPA* SPP TO *SARPA SALPA*** completed by **SONGÜL TURHAN** under supervision of **PROF. DR. LEVENT ÇAVAŞ** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Songül TURHAN

MIGRATION OF CAULERPIN FROM *CAULERPA* SPP. TO *SARPA SALPA*

ABSTRACT

Sarpa salpa is a fish species which is consumed in the Mediterranean Sea. It has been reported that *S. salpa* includes marine algae-based toxic molecules and this is why it shows neurotoxic and hallucinogenic effects on human. Invasive *Caulerpa cylindracea* and non-invasive *Caulerpa prolifera* are the most important members of *Caulerpa* genus in the Mediterranean Sea. Caulerpin is one of the secondary metabolites of this species which has important biological activities such as anti-tuberculosis, anti-proliferative and anti-nociceptive activity. On the other hand, this molecule has a detrimental effect on native fish species in the Mediterranean Sea. *S. salpa* is one of the fish species which consumes *Caulerpa* spp. In this study, caulerpin levels were investigated in *Caulerpa* spp and *S. salpa* tissues collected from İzmir coastlines in four seasons. Firstly, caulerpin was isolated from *C. cylindracea* and characterized by 1H-NMR, UV, MALDI-TOF/MS and melting point. The levels of caulerpin in the *Caulerpa* species and *S. salpa* tissues were analysed by RP-HPLC. Caulerpin was not detected in *C. prolifera*. While caulerpin levels in *C. cylindracea* and *S. salpa* tissues reached the highest value from the end of summer to the beginning of autumn, it decreased from the winter to spring. Finally, caulerpin was determined in the liver, brain and muscle tissues of *S. salpa*. It has been reported that *S. salpa* has negative effects on human health depending its *Caulerpa*-based diet. In this first study for Turkish coastlines, determination of caulerpin in *S. salpa* tissues is a proof for consuming of *Caulerpa* species by S. *salpa*. It is recommended not to consume *S. salpa* to avoid a possible health problem during the month August in which caulerpin concentration reached maximum level.

Keywords: Caulerpin, *Caulerpa cylindracea, Caulerpa prolifera,* RP-HPLC, *Sarpa salpa*

CAULERPA TÜRLERİNDEN CAULERPİNİN *SARPA SALPA'* YA GEÇİŞİ

ÖZ

Sarpa salpa balığı Türkiye'de tüketilen balık türlerinden birisidir. Bu balığa yönelik literatürde deniz algleri kaynaklı toksik metabolitleri barındırdığı ve dolayısıyla halusinojenik ve nörotoksik etkiler gösterdiği rapor edilmiştir. Akdeniz'de bulunan yayılımcı *Caulerpa cylindracea* ve yayılımcı olmayan *Caulerpa prolifera*, *Caulerpa* genusunun iki önemli türüdür. Caulerpin anti-tüberküloz, anti-proliferatif ve ağrıya karşı duyarlılığı azaltıcı gibi oldukça önemli biyolojik aktivitelere sahip bir moleküldür. Öte yandan bu molekülün Akdeniz'deki yerel balık türleri üzerinde ölümcül etkileri olduğu yapılan çalışmalarca gösterilmiştir. *S. salpa, Caulerpa* türlerini tüketen balıklardan biridir. Bu çalışmada, 4 mevsimde İzmir kıyılarından toplanan *Caulerpa* örneklerinde ve *S. salpa* dokularında caulerpin düzeyleri incelenmiştir. Ticari amaçla satılan caulerpin standardı olmadığından bu metabolit öncelikle *C. cylindracea*'dan izole edilmiş ve 1H-NMR, UV, MALDI-TOF/MS ve erime noktası tayini analizleri ile karakterize edilmiştir. *Caulerpa* türlerindeki ve balık dokularındaki caulerpin düzeyleri RP-HPLC analizleri ile belirlenmiştir. Yapılan deneysel çalışmalar sonrasında, maksimum caulerpin düzeyleri *C. prolifera*' da caulerpin saptanmamıştır. *C. cylindracea* ve *S. salpa* dokularındaki caulerpin düzeyleri yaz sonu ve sonbahar başında en yüksek seviyelere ulaşırken kıştan bahara doğru bir azalış göstermektedir. Sonuç olarak, ülkemiz kıyılarında yoğun olarak yayılış gösteren ve ekonomik değeri olan *S. salpa* balığının kas, beyin ve karaciğer dokularında caulerpin tespit edilmiştir. Literatürde *S. salpa* balığının *Caulerpa* türleri tüketimine bağlı olarak insan sağlığı üzerinde olumsuz etkiler oluşturduğu rapor edilmiştir. Caulerpin konsantrasyonun en yoğun olarak gözlendiği Ağustos ayında *S. salpa* balıklarının tüketilmemesi olası bir sağlık probleminin engellenmesi amacıyla tavsiye edilebilir.

Anahtar kelimeler: Caulerpin, *Caulerpa cylindracea*, *Caulerpa prolifera*, RP-HPLC, *Sarpa salpa*

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CHAPTER ONE

INTRODUCTION

1.1 *Caulerpa* J.V. Lamouroux

In the Mediterranean Sea, there are about 700 invasive species (Galil et al., 2014) which are constituted higher than 5% of flora and fauna. In the light of this information, it can be said that the Mediterranean Sea is one of the most affected marine ecosystem from invasive species. Introduction of non-indigenous species to new ecosystem has negative effects on population and protection of native species and structural and functional properties of the ecosystem. Also, it affects the economy and human health negatively. Therefore, invasive species became a global problem in the worldwide. Several native species have been negatively affected by non-indigenous species. For example, American coastline origin *Mnemiopsis leidyii* has caused a significant decrease in the population of a native species *Engraulis encrasicolus* in the Black Sea. It is known that *M. leidyii* was introduced to the Black Sea via ballast waters of ships (Knowler, 2007) and *M. leidyii* has rapidly consumed the zooplankton which is the main food source of *E. encrasicolus*. There are many reasons for the introduction of non-indigenous species to new ecosystems. These reasons are ballast waters of ships, artificial sea channels, human-based activities and global climate changing. The most studied non-indigenous marine algae in the Mediterranean Sea are *Caulerpa* taxa. A green algae species, *Caulerpa* genus has some invasive species which prefer hot and tropical seas and show rapid colonization when they grow outside their local regions (Varela- Álvarez et al., 2012). Three taxa belong to this genus are spreading in the Mediterranean Sea. These taxa are *Caulerpa taxifolia* (Figure 1.1a), *Caulerpa cylindracea* (Figure 1.1b) and *Caulerpa taxifolia* var. *distichophylla* (Figure 1.1c). *C. taxifolia* has accidentally introduced the Mediterranean Sea through the Oceanographic Museum of Monaco, in 1984. Soon after its first introduction to the Mediterranean Sea, this species was observed on the coastlines of 6 Mediterranean countries. These algae, also known as 'Killer algae' had great attention all around the world (Meinesz & Hesse, 1991) and international workshops have been organized just for this species (Boudouresque, Meinesz & Gravez, 1994). After *C. taxifolia* has become famous in the Mediterranean Sea,

another invasive species *C. cylindracea* previously known as *C. racemosa* var. *cylindracea* has emerged (Klein & Verlaque, 2008). A different invasive strain of *C. taxifolia* has been found in 2007 by Cevik et al.(2007). The invasive potential of new strain of *C. taxifolia* from the İskenderun Bay has not spread efficiently like the *C. taxifolia* has done. As a result of morphological and genetic researches, this strain was determined as a new subspecies and it was called as *C. taxifolia* var. *distichophylla* (Jongma et al., 2013). There is also another species of *Caulerpa* genus called *Caulerpa prolifera* in the Mediterranean Sea, but this taxon is not invasive and it is native for the Mediterranean Sea.

Members of *Caulerpa* genus produce several secondary metabolites for various purposes such as protection against the herbivores and interspecific competition. These secondary metabolites are great importance for the medicinal and cosmetic industries. Because of these properties, macro algae are characterized as "pharmaceutical warehouse". In this context, *Caulerpa* genus has great biotechnological importance. Sesquiterpene-based caulerpenyne (CYN) (Figure 2b), one of the major secondary metabolites of the *Caulerpa* genus, acts as a chemical deterrent against herbivores and plays an allelochemical role in the interspecific race (Gorbi et al., 2014). In case of possible injury, CYN is secreted as a wound healer avoids to releasing of genetic material (Adolph, Jung, Rattke, & Pohnert, 2005). CYN is transformed to oxytocin after several steps reactions by esterase enzyme activity. The final product acts as a cross-linking agent to closure the wound in the membrane of *C. cylindracea* (Jung & Pohnert, 2001; Cavas & Pohnert, 2010). CYN, which has various biological activities such as inhibiting the xanthine oxidase enzyme, which plays an important role in the treatment of gout disease (Cengiz, Cavas, Yurdakoc & Aksu, 2012).

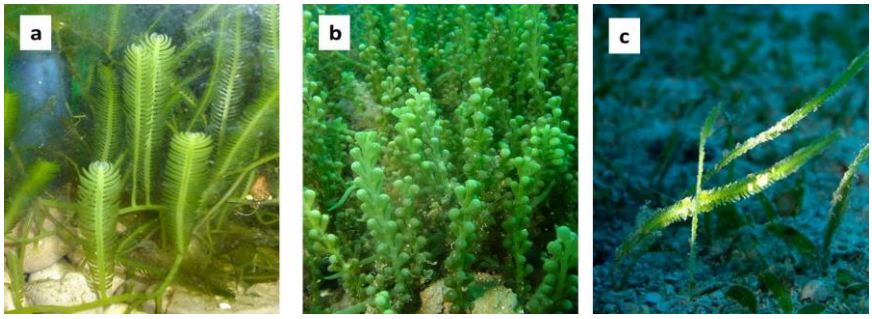

Figure 1.1 a) *C. taxifolia*, b) *C. cylindracea* (Levent ÇAVAŞ, Personal archive, 2010) and c) *C. taxifolia* var. *distichophylla* (Özgür DARA, Personal archive, 2010)

1.2 *Caulerpa prolifera (Forsskål) J. V. Lamouroux*

C. prolifera, a member of the *Caulerpa* genus, is a native species in the Mediterranean Sea and is predominantly distributed in shallow regions. *C. prolifera* is recognized as a simple leaf-like frond that develops horizontally, without any indentation in the thallus (Figure 1.2a). The thallus is a single, multi-nuclear cell with many chloroplasts and no wall (coenocyte). These leaf-like fronds are usually oval or long linear. New leaves develop on the back of old ones, usually from the point closest the upper edge of the thallus. This development is characteristic for *C. prolifera* and it's called as ‘ramification'.

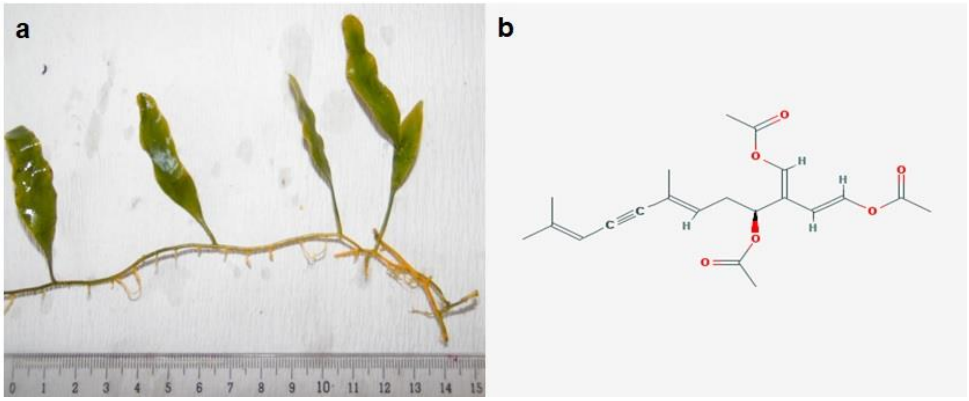

Figure 1.2 a) *C. prolifera* (Personal archive, 2017) and b) Molecular structure of caulerpenyne

C. prolifera loses the green colour through the end of reproduction season and its surface is covered by nipple like the green of yellow colour circles. These circles secreted mucus-like material include zoospores onto the slits. Female reproductive

cells are taller when compare with male cells and both of them include zoospores. Following the assimilation, period zygote loses its zoospores and produces the cell wall. During the germination zygote groves, numbers of chloroplasts increase and it keeps until the production of the coenocytic thallus (Einav, 2007).

1.3 *Caulerpa cylindracea Sonder*

C. cylindracea is widely spread in the Mediterranean Sea. This species was previously known as *C. racemosa* var. *cylindracea* (Klein & Verlaque, 2008), the name of this taxon was changed as *Caulerpa cylindracea* after the morphologic and genetic studies made by Belton et al. (2014). The most important side effect of the invasive *Caulerpa* taxa is to affect the indigenous species in the same region. According to scientific reports, the most vulnerable species affected by invasive taxa is *Posidonia oceanica*, which is endemic to the Mediterranean Sea (de Villele & Verlaque, 1995; Ceccherelli & Cinelli, 1999; Pergent, Boudouresque, Dumay, Pergent-Martini &Wyllie-Echeverria, 2008). *P. oceanica* has a great importance for the Mediterranean ecosystem. It is certain that a possible collapse in the *P. oceanica* population causes the decreasing bio-diversity in this ecosystem. This is why observation of this species regularly is very important. Based on the scientific reports, *P. oceanica* is under the threat of invasive species. The invasion of *C. cylindracea* in the Mediterranean Sea was started in 1992 (Nizamuddin, 1991). However, it is observed that its invasion has reached the balance nowadays. Our field of study in Dikili-İzmir (Turkey) proves that this species does not expand its spreading areas. Furthermore, during the winter season this species disappear and then it emerges again with the beginning of the summer season (unpublished data).

Figure 1.3 *C. cylindracea* fronds which are spreading into *C. reniformis* pores in Turkish coastline ((a) Levent ÇAVAŞ, Personal archive, 2010 and (b) Müjdat TURAN, Personal archive, 2017)

Chondrosia reniformis is a marine sponge species which is affected by *C. cylindracea* invasion. *C. cylindracea* groves in a way to cover *C. reniformis* (Figure 1.3a). The pores on *C. reniformis* which allow the organism to receive the necessary nutrients from the sea environment are closing and *C. reniformis* loses its vitality in time. The intensive spread of *C. cylindracea* onto *C. reniformis* in Turkish coastline has been seen in the videos of fisherman Müjdat Turan (Figure 1.3b). On the other hand, the study of Schröder et al. (1998) has shown that *C. cylindracea* and *C. taxifolia* have a detrimental effect on another marine sponge species *Geodia cydonium*. A water pollutant tributyltin which has an apoptotic effect on *G. cydonium* at 3 µM and higher concentration has shown its apoptotic effect at lower concentration via the contribution of *Caulerpa* extract.

There are many omnivore species that consume *C. cylindracea* in the Mediterranean Sea. *Sarpa salpa* located on the Turkish coastline is one of the important species consuming *C. cylindracea*. It was also observed with the nacked eye that *S. salpa* consumed *C. cylindracea* (Figure 1.4) (Ruitton, Verlaque, Aubin, & Boudouresque, 2006). During the scientific diving, it was observed that *S. salpa* selectively consumes *C. cylindracea*. There are several studies show that caulerpin is accumulated in fish tissues (Felline et al., 2017; Gorbi et al., 2014). Also, it has been reported that there are many species that consume *C. cylindracea* such as *Boops boops* (Klein & Verlaque, 2008), *Pagellus acarne* (Klein & Verlaque, 2008), *S. salpa* (Ruitton, Verlaque, Aubin, & Boudouresque, 2006) and *Diplodus sargus* (Gorbi et

al., 2014). It is known that sea urchin species *Paracentrotus lividus* and *Sphaerechinus granularis* also consume *C. cylindracea* (Ruitton, Verlaque, Aubin, & Boudouresque, 2006). Observations indicate that the consumption on the *C. cylindracea* meadows begins through the end of summer and increases at the beginning of autumn which *C. cylindracea* invasion reaches the maximum level. Several studies have performed to eradicate the *C. cylindracea*. A nudibranch species *Lobiger serradifalci* (Figure 1.5a) was used in these studies. However, experimental studies have demonstrated contrary effect to expectations. Since *L. serradifalci* fragmented *C. cylindracea* to small pieces that enough to regenerate itself, it raised an effect that would greatly enhance this spread, rather than eradicate *C. cylindracea* (Zuljevic, Thibaut, Elloukal, & Meinesz, 2001). It has been observed in our laboratory that another nudibranch species *Oxynoe olivacea* (Figure 1.5b) has opened a hole on the *Caulerpa* species and it has consumed the *Caulerpa* spp. via sucking cell materials but, it could not consume fast enough. On the other hand, it has been revealed that nudibranch species protect themselves against to fish by transferring the toxin metabolite (caulerpenyne) to more toxic form via consumption these marine algae, after the investigation of why nudibranch species consume marine algae include toxic metabolite rather than avoiding it. The more toxic derivatives of caulerpenyne were detected in mucus and para podiums of nudibranch (Thibaut et al., 2001).

Figure 1.4 *S. salpa* species on the *C. prolifera* meadows (Levent ÇAVAŞ, Personal archive, 2010; Dikili, Bademli Village, Depth: -5m)

Figure 1.5 a) *Lobiger serradifalci* and b) *Oxynoe olivacea* (Levent ÇAVAŞ, Personal archive, 2006)

The studies show that consumption of *Caulerpa* spp. has negative effects on fish health. Terlizzi et al. (2011) and Gorbi et al. (2014) have investigated the molecular effects of *C. cylindracea* on *D. sargus* by measuring of antioxidant activities. At the same time, it has been shown by Fellini et al (2014) that *Caulerpa*-based diet reduces the production of polyunsaturated fatty acids which are of great importance for human health.

1.4 *Caulerpin*

Caulerpin is one of the important metabolites of *Caulerpa* genus. The basic role of this metabolite is not fully explained, but it is reported as a secondary metabolite in the literature (Nagappan & Vairappan, 2013). However, we consider that this metabolite acts as secondary pigment during the photosynthesis and it might be an antioxidant molecule. Caulerpin is an alkaloid that contains the bis-indole ring in its structure. The molecular structure of caulerpin is given by Figure 1.6.

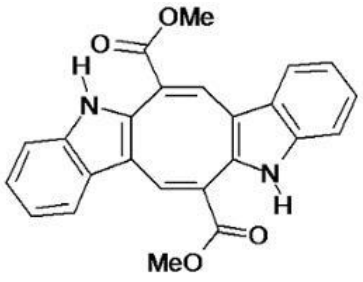

Figure 1.6 Molecular structure of caulerpin

There has not been sufficient number of scientific report on *Caulerpa* spp. along Turkish coastline. Moreover, no publication has been appeared in scientific literature

related to caulerpin levels and its relationships with local species. Caulerpin has industrial important thanks to its biological activities. Plant growth stimulation and regulation effects of caulerpin were patented by Schwede (1986). Also, Kamal & Sethuraman (2012) showed that caulerpin in 1M HCl is a green inhibitor that prevents corrosion of steel. Biological activities of caulerpin are given below. In a study on pig ileum, caulerpin showed an effect to decrease the spasm on ileum (Cavalcante-silva et al., 2013). Also, there are so many studies on anti-nociceptive (de Souza et al., 2009; Cavalcante-silva et al., 2014) and anti-inflammatory (de Souza et al., 2009; Bitencourt et al., 2015) effects of caulerpin. Due to these properties, caulerpin could be considered as a painkiller/ antiplatelet medicine. In the study of Canché Chay, Cansino, Pinzon, Torres-Ochoa & Martinez (2014), caulerpin showed a strong activity against to *Mycobacterium tuberculosis*-based tuberculosis. The activity of caulerpin against many cancer mechanisms has also been investigated. Yu et al. (2016) revealed that caulerpin causes mitochondrial disorders via inhibition of oxidative phosphorylation and mitochondrial complex-1 and destroying mitochondrial membrane potential. In the same study, caulerpin wiped out the colorectal cancer cell through the damaging of glucose metabolism because of the long-term activation of AMP-activated protein kinase enzyme by caulerpin. Thus, it can be said that caulerpin has therapeutic potential against colorectal cancer. In a similar study by Ferramosca et al., (2016), it has been observed that caulerpin acts against to ovarian cancer by causing mitochondrial disorders and proliferation. It is known that caulerpin has inhibitory activity on monoamine oxidase B which has an important role in Alzheimer disease (Lorenzo, Barbasa Filho, Scotti, L. & Scotti, M. T., 2015). Also, caulerpin has anti-viral activity against the herpes simplex virus type-1 (Macedo et al., 2012) and Bovine viral diarrhea virus which caused a serious economic problem in the worldwide (Pinto et al., 2012).

According to the 2014 data, there are 366 million diabetes patients in the world and this number is gradually increasing (Sharma, Kim & Rhyu, 2017). Numerous studies are being conducted to treat this disease which is of worldwide significance. Among to these studies, there is *Caulerpa lentillifera* extract. In the studies conducted by Sharma & Rhyu (2014) and Sharma, Kim & Rhyu (2017), it has been

observed that *C. lentillifera* inhibits the dipeptidyl peptidase-IV and α-glycosidase enzymes which have key importance in the treatment of diabetes and regulates the insulin secretion by protecting pancreatic β-cells from possible damage. While traditional medicines which use in the treatment of diabetes inhibit dipeptidyl peptidase-IV or α-glycosidase, *C. lentillifera* extract exhibits an inhibitory effect on both enzymes. In addition to that, *C. lentillifera* extract reduces glucose through increasing glucose uptake to adipose tissues. In these studies, the direct effect of caulerpin on diabetes has not been investigated. However, it can be recommended that further experiments could be done only for caulerpin because it exists in the *C. lentillifera*. In Far East, *C. lentillifera* is known as green caviar. It has been using as a salad and known with its potential against diabetes (Nagappan & Virappan, 2014).

On the other hand, there are several studies on impairment of fish health as a result of *Caulerpa*-based diet. The accumulation of caulerpin is fish tissues is an indicator of trophic exposure of *Caulerpa* spp. Depending on increasing accumulation of caulerpin in fish tissues, changes in the glutathione reductase enzyme and glutathione levels in the basic antioxidant defense mechanism, increased activity in cytochrome P-450, glutathione-S-transferase and acyl-CoA oxidase enzymes, and increased expression of peroxisomal proliferator-activated receptor alpha (P4501A) and vitellogenin 1 genes have been observed (Terlizzi et al., 2011; Gorbi et al., 2014). Based on these results, it can be said that caulerpin could be toxic to fish. In the study conducted by Fellini et al. (2014), fatty acids productions were investigated in the case of caulerpin accumulation in fish tissues. In caulerpin accumulated fish, the production of polyunsaturated fatty acids was decreased while the production of saturated fatty acids was increased. Polyunsaturated fatty acids synthesized by fish are very important for human health functions such as intellectual development, prevention of cardiovascular diseases. However, the decreasing of polyunsaturated fatty acid production causes decreasing the nutritional and economic value of fish.

Additionally, accumulation of secondary metabolites belong to *Caulerpa* spp. in fish tissue could be dangerous for human health. According to Haro & Pommier

(2006), it has been reported that serious health problem such as hallucinogenic effects, tachycardia, and insomnia was observed after consumption of *S. salpa*. In this study, accumulation of caulerpin in *S. salpa* tissues was investigated for the first time in Turkish coastline. The caulerpin level in liver, brain and muscle tissues of *S. salpa* and *C. cylindracea* and *C. prolifera* have been determined.

1.5 *Sarpa salpa* (Linnaeus)

S. salpa is an herbivorous fish species which abundant in our coastline. This fish species colloquially known as *sarpa* (Figure 1.7a) has very high nutritional value and it is consumed in our country. According to 2016 statistical data, 263,724 tones fish have been caught in Turkey and 127 tons of them are *S. salpa*. In our country, fish consumption per person is 5 kg on average. There is no research on dietary habits and the caulerpin level in the tissues of *S. salpa* known to consume *Caulerpa* species in our country (de Haro et al., 1993; Ruitton, Verlaque, Aubin & Boudouresque, 2006). The determination of caulerpin in *S. salpa* tissues is very important for human health because it has been reported that hallucinogenic effects were observed after the consumption of *S. salpa* (Haro & Pommier 2006). In the relevant report, in addition to hallucinogenic effects, serious health problems have been indicated such as insomnia and tachycardia depend on mental disorders. These symptoms started about 2 hours after the fish consumption and continued until 3 days. *S. salpa* is also known as hallucinogenic fish because of these effects. In literature, the food poisoning based on fish consumption is known as ichthyosarcotoxism. Ichthyosarcotoxism is characterized by central nervous system disorders. Ciguatera food poisoning is more extensive and widely studied form of ichthyosarcotoxism and presence of toxin compounds synthesized by benthic algae in fish tissue is the sources of this problem. *S. salpa* preferably consumes *P. oceanica.* It is considered that co-ingestion of ciguateric species which live on *P. oceanica* as epiphyte is the crux of the ciguatera food poisoning based on *S. salpa* consumption (Bellassoued, Van Pelt & Elfeki, 2015). It is known that *P. oceanica* which is very important for Mediterranean Sea ecosystem is the most affected species by *Caulerpa* spp. (Villele & Verlaque, 1995; Ceccherelli & Cinelli, 1999; Pergent, Boudouresque, Dumay,

Pergent-Martini &Wyllie-Echeverria, 2008). *C. cylindracea* is generally presence in *P. oceanica* meadows (Figure 1.7b) in İzmir coastlines and also a collapse in the population of *P. oceanica* has been observed. This is why *S. salpa* consumes *Caulerpa* spp. during the alimentation and as a result of that metabolites particular to the *Caulerpa* spp. were accumulated in *S. salpa* tissues. It is considered that the reason for the health problems is based on *Caulerpa* spp. In the study conducted by Felline et al. (2017), stomach content of *S. salpa* was investigated and *C. cylindracea* was observed in all sample's stomach. Also, caulerpin has been determined in muscle and liver tissues of *S. salpa*. In the study conducted by Bellassoued, Van Pelt & Elfeki (2015), liver, muscle, brain and viscera extracts of *S. salpa* were given to the laboratory mouse by gastric gavage to understand neurotoxic effects based on *S. salpa* consumption. Fish samples have been collected in the period that health problems were observed frequently. There is no death after the 7 days long exposure to fish extracts. However, a serious increase in oxidative stress and significant decrease in the activity of superoxide dismutase, catalase and glutathione peroxidase enzymes which are located in cerebral cortex were observed especially in the mouses exposure to viscera and liver extracts. Based on these results, *S. salpa* has toxic effects on especially the brain cerebral cortex which is the most important part of the human brain and responsible for memory, movement, speech, intelligence and decision mechanism. Therefore, evaluation of caulerpin contents of *S. salpa* collected in Turkish coastline and served to human consumption is of great importance in health.

Figure 1. 7 a) *S. salpa* species and b) *C. cylindracea* in *P. oceanica* meadows (Levent ÇAVAŞ, Personal archive, 2008)

1.6 *The Internet News on S. salpa*

The internet news about *S. salpa* are given in Figure 1.8a and Figure 1.8b. The common point of this news is emphasizing on hallucinogenic effects of *S. salpa*. It has been indicated that hallucination and LSD-like effects have emerged after *S. salpa* consumption and they continue for 36 hours to a couple days in the cases happened in the Mediterranean Sea, especially in French coastline. This feature of the fish is very old; it is said that it stood until the time of the Roman Empire. In the Roman Empire, *S. salpa* was used as a narcotic drug in party nights and it was known as psychedelic/dream fish.

Figure 1.8 The internet news about *S. salpa* ((a) David DOOCHIN, Personal archive, 2016, (b) Phillip SMITH, Personal archive, 2016 and (c) Angelo PETRONE, Personal archive, 2017)

Fish that triggers LSD-like hallucinations is caught off Cornwall

By DAILY MAIL REPORTER
UPDATED: 16:15 BST, 14 May 2009

A fish that can trigger LSD-like hallucinations when eaten has been discovered in UK waters.

The sarpa salpa species of bream is normally found in the Mediterranean and around South Africa.

But fisherman Andy Giles has told how he hauled a sarpa salpa, instantly recognisable by its gold stripes, near Polperro, Cornwall.

Netted in the UK: The sarpa salpa species of bream that triggers hallucinations was found in waters off Cornwall

Sarpa salpa, il pesce 'allucinogeno' con gli effetti dell'LSD

Il pesce Sarpa salpa ha effetti simili alle droghe, ma solo se ne viene consumata la testa.

AMBIENTE

Angelo Petrone 0 17:10 3 marzo 2017

Gli effetti "stupefacenti" della **Sarpa salpa** sono conosciuti fin dall'antichità. Si tratta di un **pesce** che vive in una vastissima zona del Mediterraneo, nell'Oceano Atlantico orientale, fino in Sudafrica, ma soprattutto nelle cose di Tenerife, Malta e Cipro. Mangiare la **Sarpa salpa** significa andare incontro ad effetti allucinogeni molto marcati, per certi aspetti all'**LSD** con incubi notturni, voci e molto altro. E proprio come le **droghe** più potenti, gli effetti possono durare per alcuni giorni come accaduto alcuni anni a due uomini che la consumarono un un ristorante nel sud della Francia.

Figure 1.8 continues (Patrick BENNET, Personal archive, 2013)

1.7 *Artificial Neural Network (ANN)*

Artificial neural network (ANN) is a machine-learning technique that is used in modeling and estimating to non-linear system behaviors. ANN is computer programs that act like human brain while data processing. Instead of fitting input and output parameters to a programmed model, it learns via experiences like the human did. ANN collects data by determining the patterns and relationships within the data set and creates a suitable model for these parameters. In ANN, data processing is similar to neuron's (Figure 1.9).

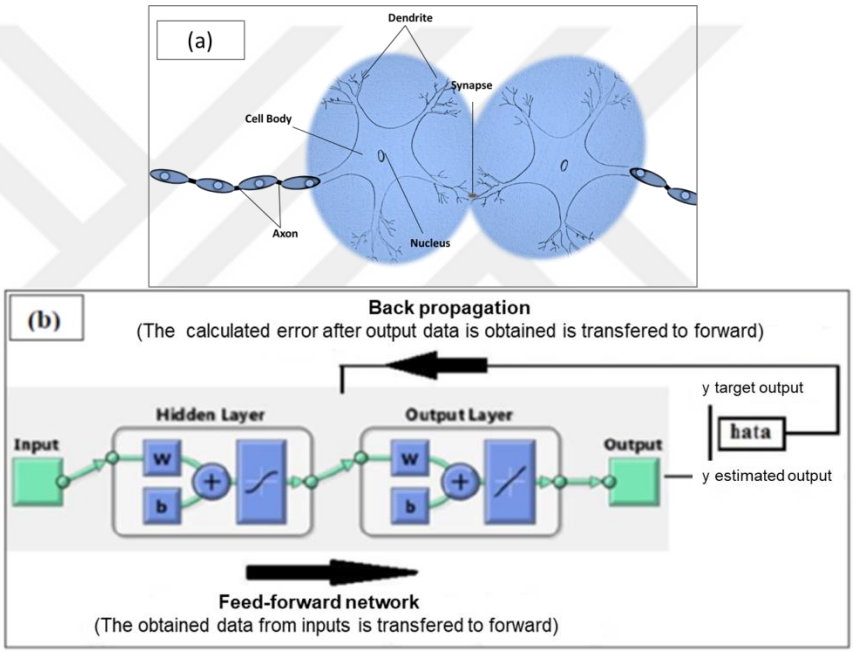

Figure 1.9 Relationship between (a) biologic neuron cell and (b) artificial neuron cell

In a biologic neuron, the signals are transmitted to the nucleus via dendrites and gathered here then transmitted to the axon. These collected signals are processed by axons and transmitted to synapses. After that, synapses transmit newly produced signals to other neurons. ANN is a network system that consists of artificial neurons connected to coefficients (weight –w). After inputs are introduced to the system, they are summed by multiplied by the weight of the links they arrive. This is called as the activation function. Thereafter, an output is obtained for relevant neuron by passing a transfer function. In MATLAB, data processing occurs within two layer feed forward

network (Figure 1.9). The output from the sigmoid transfer function in the hidden layer is the input quality for the output layer. Here again, after the activation function, it is passed through the linear transfer function and the estimated output is obtained. Because, artificial neurons are related to weights, weights are the model's memory system (Agatonovic-Kustrin & Beresford, 2000). In ANN, there is no limitation for the number of inputs and when the number of the data increases, the system's learning ability increases. In this study, ANN modeling occurs within 3 stages.

1.7.1 Optimization of Hidden Neuron Number

During the learning stage, inputs transform their weight. Data sets in the input layer are transferred to artificial neurons in the hidden layer through the link-weight. This is why the number of links and so hidden neuron number affect the network's performance. So, very few number hidden neurons can block the learning process. On the other hand, a very large number of the hidden neuron can cause over learning and to closely following of learning pattern by the model (Agatonovic-Kustrin & Beresford, 2000). This situation causes over fitting in the model and so moving away from the general model and decreasing of estimation ability. Therefore, optimization of hidden neuron number is of great importance.

1.7.2 Optimization of The Percentage of Training, Validation, and Testing

In ANN model, experimental data is divided 3 parts in percentage randomly determined by the program. The dataset in validation and testing stages is not seen by the system in training stage. The dataset in training stage is used for regulation of link-weight and finally producing a pattern or model. This prevenient model in training stage is verified by using validation data and model’s estimation ability is proven by testing data. These stages proceed in parallel and minimum square error (MSE) between the target and estimated output is calculated. In training stage, rearranging of weights and model is provided by sending back the error to the network (back propagation) to obtain minimum MSE value (Figure 1.9). Also, the

model should not deviate from the generalized model. In other words, a very good fitting model to training data could give very wrong results in estimation the data that system has never seen because of the over fitting (The Matworks, 2016). In this stage, obtained data from validation data set is used to understand if there is any deviation from the generalized model. Epoch on iteration in training stage is stopped when obtained MSE from validation dataset increased. Testing dataset is used for independently evaluate the model performance during and after training stage. Therefore, optimization of the percentage of training, validation, and testing is important for ANN.

1.7.3 Back Propagation Algorithms in MATLAB

Back propagation algorithms are training algorithms in ANN. ANN is a system that learns with errors. The calculated error during one training epoch is fed back through the network. The error is transferred from output layer to hidden layer then input layer within several algorithms. Accordingly, repetition of error is prevented and model is improved by rearrangement of link-weights (Agatonovic-Kustrin & Beresford, 2000). This is why optimization of back propagation algorithms is important.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals

RP-HPLC grade methanol and ethanol, silica gel for column chromatography and TLC Silica gel 60 F_{254} plates were bought from Merck. Ultrapure water was bought from VWR. Diethyl ether was bought from Macron. High-purity ethyl acetate, acetone, and hexane were bought from Tekkim. Intersil ODS_3 RP-HPLC column (150 mm x 4,6mm x 5 μm) from GL Science was used for RP-HPLC analyses.

2.2 Sampling

A necessary official permission was taken from Ministry of Food, Agriculture and Livestock, General Directorate of Agricultural Research (TAGEM) and General Directorate of Fisheries and Aquaculture. Because the collection of any *Caulerpa* spp in any means along Turkish coastlines have been banned for a long time to prevent invasion of this species (Figure 2.1). *Caulerpa* samples (*Caulerpa cylindracea* and *Caulerpa prolifera*) were collected from Dikili and Çeşme region via free-diving on 0-5 meter depth. After removing of impurities, samples were dried under the sunlight. The sampling periods were determined as of February'2017, May'2017, August'2017 and November'2017.

T.C.
GIDA TARIM VE HAYVANCILIK BAKANLIĞI
Balıkçılık ve Su Ürünleri Genel Müdürlüğü

Sayı :67852565-140.03.03-E.560523 08.03.2017
Konu :Araştırma İzni (Prof.Dr.Levent ÇAVAŞ)

DOKUZ EYLÜL ÜNİVERSİTESİ REKTÖRLÜĞÜNE
(Fen Fakültesi Dekanlığı)
Buca/İZMİR

İlgi : a) 07.03.2016 tarihli ve bila sayılı Dilekçe.
b) 11.03.2016 tarih ve 67852565-140.03.03-706-988 sayılı yazı.
c) 02.02.2017 tarih ve 90554282/900/359 sayılı yazı.
ç) 02.03.2017 tarihli ve 57514100-425 sayılı yazınız.

Dokuz Eylül Üniversitesi, Fen Fakültesi Öğretim üyesi Prof.Dr.Levent ÇAVAŞ tarafından yürütülecek olan "Caulerpa Türlerinden Caulerpin'in Sarpa salpa Dokusuna Geçişi" isimli projenin TÜBİTAK başvurusu için ilgi (a) yazı ve ekli form ile müracaat edilmiş, ilgi (b) yazımız ile ön izin verilen projenin, TÜBİTAK tarafından kabul edildiğinin bildirildiği ilgi (c) yazı ve ekleri incelenmiş, güncel Su Ürünleri Araştırma İzinleri Başvuru Bilgileri Kontrol Listesi'nin olmadığı, ilgi (a) dilekçe ekindeki formunda güncel olmaması sebebiyle proje yürütücüsünden güncel başvuru formu istenmiştir.

Üniversiteniz tarafından gönderilen, bahse konu projenin arazi çalışmaları için izin talep eden ilgi (ç) yazı ve ekindeki güncellenmiş başvuru formu incelenmiştir.

Söz konusu çalışma kapsamında; Prof.Dr.Levent ÇAVAŞ, ile Yüksek Lisans öğrencisi Songül TURHAN'ın; Mart 2017 ile Mart 2018 tarihleri arasında mevsimlik periyotlarda İzmir İl'i Dikili, Çeşme, Seferihisar ilçelerindeki 0-5 metre derinlik konturlarındaki denizel alanlardan el ile *Caulerpa türlerinden* 10 kg. örnek toplanmasına;

Çalışma yapılacak güne ait; çalışma takvimi programının çalışmaların yapılacağı ilde bulunan Gıda Tarım ve Hayvancılık İl Müdürlüğüne önceden bildirilmesi, İl Müdürlüklerinde görevli bir personelin imkanlar dahilinde çalışma bölgelerindeki proje çalışmalarına katılması, " Ticari ve Amatör Su Ürünleri Avcılığını Düzenleyen Tebliğ"lerde belirtilen diğer hükümlere riayet edilmesi, çalışmalara yabancı araştırmacının katılmaması, proje çalışma sonuçlarının Bakanlığımıza gönderilmesi, elde edilen su ürünlerinin hiçbir suretle satılamayacağı ve örneklerin izin alınmadan yurt dışına çıkartılamayacağı şartlarına uyulması kaydı ile uygun görülmüştür.

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Not: 5070 sayılı Elektronik İmza Kanunu gereği bu belge elektronik imza ile imzalanmıştır.
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Eskişehir Yolu 9. Km. Lodumlu Mevkii 06800 Çankaya/ Ankara
Tel: (0312) 258 30 19 Faks: 0 (312) 258 30 75
http://www.tarim.gov.tr/BSGM
Bilgi için:Tolga AYDOSLU
Mühendis
Telefon No:(312) 258 30 86

15.3

1 / 2

Figure 2.1 The legal permission was taken from Ministry of Food, Agriculture, and Livestock, General Directorate of Agricultural Research (TAGEM)

S. salpa samples were provided in same sampling periods. 10 individual fish samples were taken for each sampling periods and mean weight of *S. salpa* samples were measured as 391.46 ±49.76, 491.59 ± 71.25, 257.85 ± 60.25 and 331.22 ± 37.40 g for winter, spring, autumn, and summer respectively. Length measurements of *S. salpa* were taken and recorded as shown in Figure 2.2. After the length measurements and the total weight were recorded, the fish were cut according to steps shown in Figure 2.3 and the muscle, liver and brain tissues were separated. These tissues were stared at -14 ^{o}C until extraction step. Muscle, liver and brain weights were also recorded after the tissues were separated. The stomach contents of fish were maintained in ethanol and then investigated via microscope to identify ingredients to the lowest possible taxonomic level.

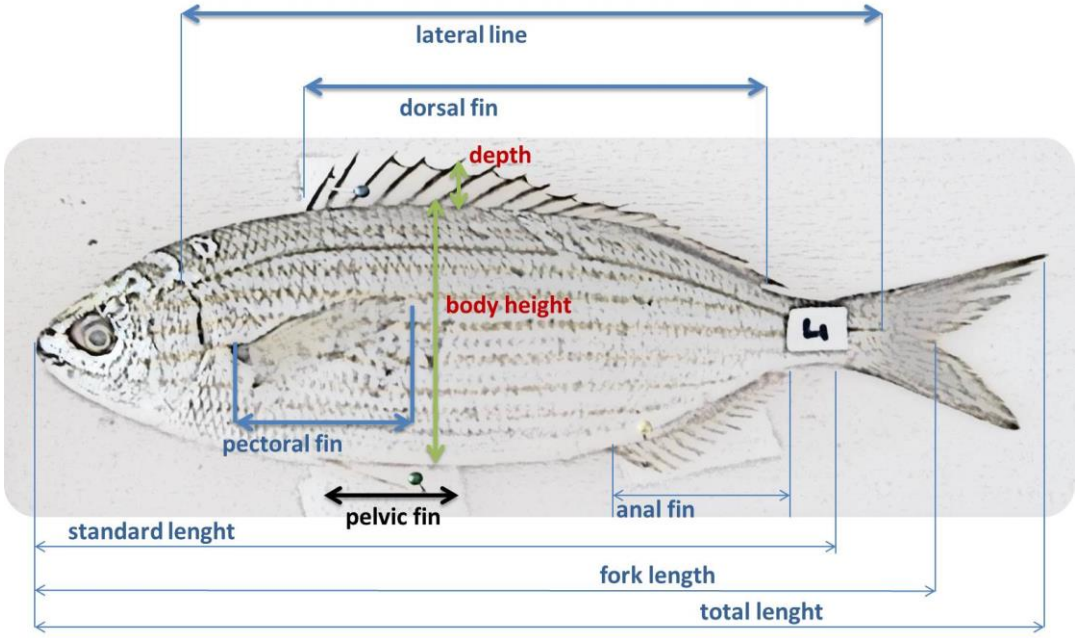


Figure 2.2 The measurement of fish size

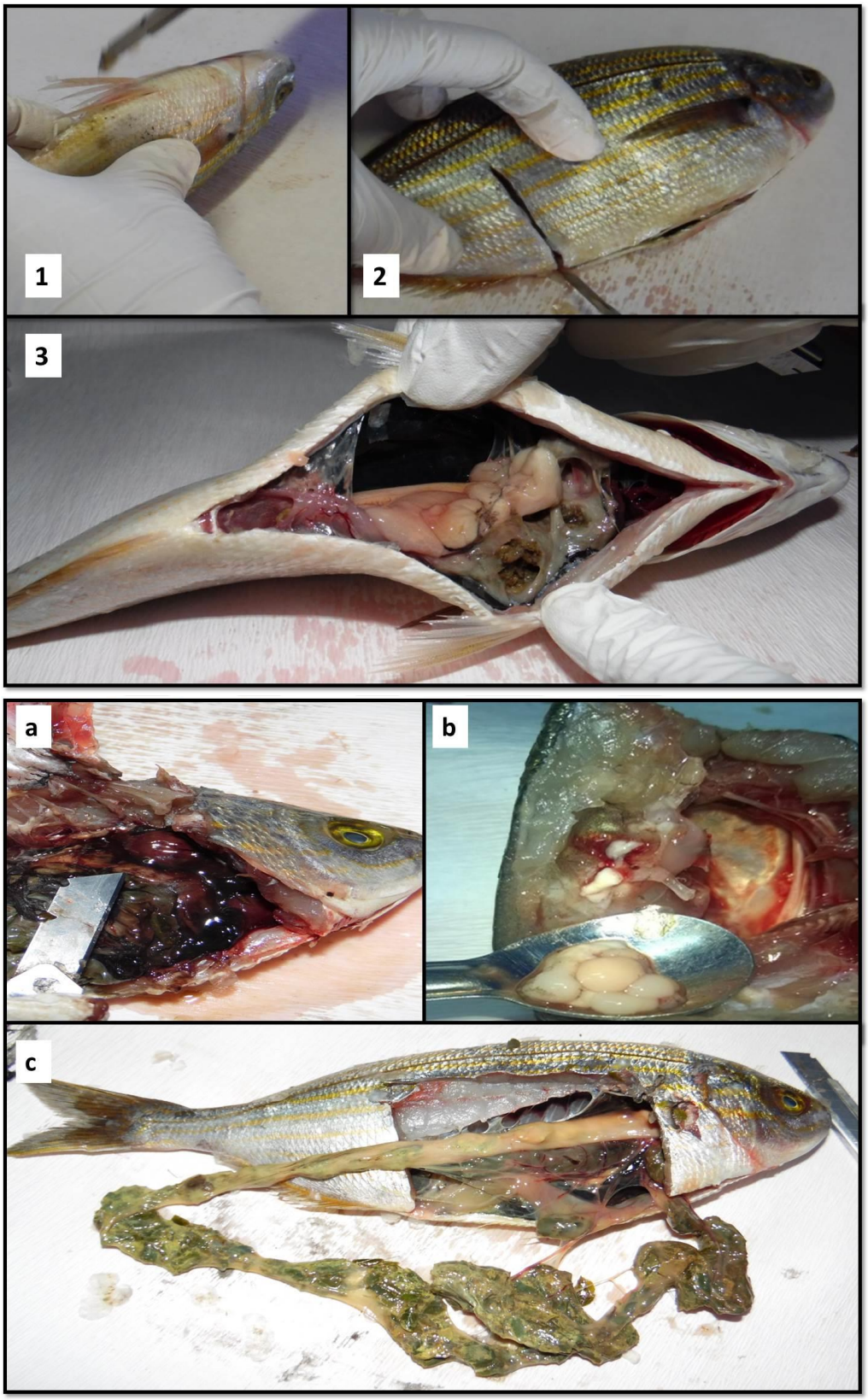


Figure 2.3 The dissection of fish tissues (1, 2, and 3) and liver (a), brain (b) and digestive tract of *S. salpa* (Personal archive, 2017)

2.3 Extraction and Isolation of Caulerpin

2.3.1 Caulerpa prolifera and Caulerpa cylindracea

Dried and ground *Caulerpa* samples were extracted with ethyl acetate via magnetic stirring at 100 rpm. The ethyl acetate extract was evaporated via rotary evaporator until dryness and the dark-green color residue was observed. Methanol was added and the red crystalline pigment was obtained. The red pigment was characterized as caulerpin by ^{1}H-NMR, MALDI-TOF/MS, UV-Vis spectrophotometer and melting point analysis.

2.3.2 Sarpa salpa

S. salpa samples were extracted according to Fellini et al (2012). Muscle tissues were separately extracted for each 10 individual. However, liver and brain tissues were combined in groups of five, because of the difficulty to determine caulerpin in low amount tissue. The fish tissues were mixed with acetone in a ratio of 1:5 and this mix was homogenized by the high-speech mixer and ultrasonic bath. It was centrifuged and the supernatant was removed. The acetone extract was evaporated via rotary evaporator until dryness and the yellowish color residue was obtained. After removing the organic solvent, the residue was further extracted 3 times with ethyl acetate. The ethyl acetate extract was concentrated under nitrogen gas until dryness. The obtained residue was dissolved in methanol and analyzed by RP-HPLC.

2.4 Chromatographic Applications

2.4.1 Column Chromatography

The *C. cylindracea* extract was performed to column chromatography with silica gel. Initially, the ethyl acetate: hexane (1:1 v/v) mix was used as mobile phase. After removing of chlorophyll, 100 % ethyl acetate was added to the column. 15 fractions

were obtained and then these fractions were performed on thin layer chromatography.

2.4.2 Thin Layer Chromatography (TLC)

All fractions were performed to silica gel TLC plate. Ethyl acetate was used as mobile phase. These fractions were performed to TLC plate with caulerpin standard at the same time and Rf values were compared.

2.4.3 Analysis of Fatty Acid Composition

In this study, the fatty acid composition was investigated, because of the negative effects of caulerpin on fatty acid composition. 2 *S. salpa* samples were selected from the samples collected in November'2017 according to caulerpin level in their tissues. The lipids were extracted from 10 g muscle tissue for each sample according to Bligh and Dyer (1959). The lipid extract was weighed and 0.1 g of the lipid was mixed with 10 mL of hexane. After strongly stirring, 2 N KOH was added and mixed. After sufficient time for the phase separation to occur, a sample was taken from the upper phase to 2 mL amber vial and analyzed by Shimadzu GC-2010/FID Dedector (GC conditions: detector: FID, column: TR-CN 100 capillary column (length: 60.0 m, inner diameter: 0.25 mm ID, film thickness: 0.20 µm, column temperature: 185.0 ^{o}C, injection volume: 1.0 µL, carrier gas: N_2 and time: 25 minutes).

2.5 RP-HPLC Analysis

2.5.1 Method Validation

Since there is no authenticate standard, firstly we isolated caulerpin from C. *cylindracea* and then it was characterized. Isolated caulerpin pigment was used as a standard for RP-HPLC analysis. These analyses were performed according to Movahheddin et al (2014) methods include methanol-water gradient elution system. Elution system: 0-5 minute, 10 % methanol-water; 5-35 minute, 10-100 % methanol-

water; 35-45 minute, 100 % methanol; flow rate: 1.0 mL/min; detection: 235 and 280 nm; injection volume: 50 µL and retention time for caulerpin: 30.90 minute.

2.5.1.1 Calibration interval and linearity

The standard caulerpin solutions were freshly prepared in methanol:water (80:20) mix at the concentration levels of 0.25, 0.50, 1.00, 2.50, 5.00 and 7.50 µg/mL. These solutions were analyzed by RP-HPLC with UV detector at 235 and 280 nm and then the calibration curves were created with 6 plots and 3 replicates.

2.5.1.2 LOD and LOQ

After caulerpin standards were analyzed, calibration was prepared on Shimadzu LC Solution software. Limit of detection (LOD) and limit of quantification (LOQ) values of methods were determined by blank determination technique (Guideline, I.H.T., 2005).

2.5.1.3 Precision and Accuracy

For accuracy, caulerpin standard solutions at the concentration levels of 1.25, 2.50 and 5.00 µg/mL were used. Each solution was analyzed with three replicate. The accuracy of the method was expressed as Recovery % (R %). R % formulation is given in 2.1.

$$\text{R \%} = \frac{\text{Recoveried concentration}}{\text{Injected concentration}} x100 \qquad (2.1)$$

The precision of the method was expressed as Relative Standard Deviation % (R.S.D. %). For precision, 2.50 µg/mL caulerpin standard solution was analyzed by RP-HPLC with 6 replicate. R.S.D. % formulation is given 2.2.

$$\text{R. S. D \%} = \frac{\text{Standard deviation of analyses}}{\text{Mean of analyses}} x100 \qquad (2.2)$$

2.5.1.4 Specificity

Firstly, the retention time of caulerpin at related wavelength was determined to determine the specificity of the method. After then, UV spectrum scanning was recorded of the peak that appearing at this retention time. The obtained results were compared with the wavelengths which caulerpin exhibits maximum absorbance levels. UV scanning was recorded at the same retention time during the analyses of *Caulerpa* and S. *salpa* tissues and then the correlation between the scanning of caulerpin standard and *Caulerpa* and S. *salpa* tissues was investigated.

2.5.2 Determination of Caulerpin Levels in Caulerpa spp. and S. salpa tissues

The levels of caulerpin in *Caulerpa* spp. and the liver, brain and muscles tissues of *S. salpa* have been determined with RP-HPLC analysis. RP-HPLC analyses have been conducted with SHIMADZU-Prominence LC-20A RP-HPLC equipped with DGU-20A5 solvent degasser, SPD-20A UV/VIS detector and CTO-10ASUP column oven according to Movahheddin et al. (2014) methods includes metanol and water in its gradient elution system. The elution program: 0-5 minute, 10% methanol-water; 5-35 minute, 10-100% methanol-water; 35-45 minute, 100% methanol; flow rate: 1.0 mL/minute; detection: 235 and 280 nm; injection volume: 50 µL and retention time of caulerpin: 30.90 minute.

2.6 Generation of Artificial Neural Network

RP-HPLC optimization of caulerpin was modeled using artificial neural network (ANN) (2016b version) tool of MATLAB. In this study, ANN structure consists of two-layer feed-forward networks with sigmoid and linear functions (Figure 2.4).

The algorithms of linear (purelin) and sigmoidal (tansig) functions mentioned in the tool-box in 2.3 and 2.4 respectivelyy.

$$a = \text{purelin}(n) = n \quad (2.3)$$

$$a = \text{tan sig}(n) = 2/((1+\exp(-2*n))-1) \quad (2.4)$$

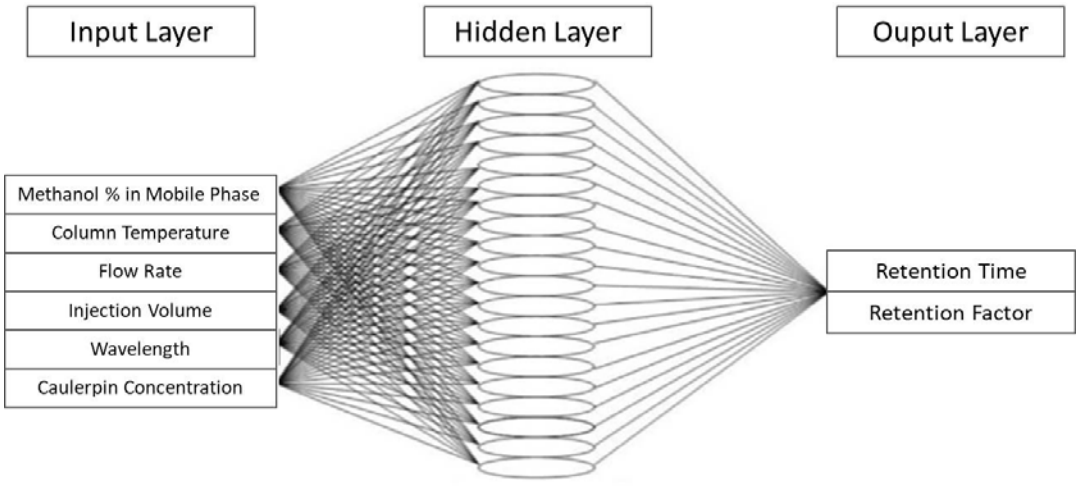


Figure 2.4 Optimal ANN structure of caulerpin analysis in RP-HPLC

2.6.1 RP-HPLC Data for ANN

Firstly, 2.5 µg/mL caulerpin solutions were prepared in metanol:water (80:20). These solutions were analyzed by RP-HPLC according to optimized method in this study. The input parameters were selected as methanol % in the mobile phase, column temperature, flow rate, injection volume, and wavelength and caulerpin concentration. Retention time and retention factor were considered as output data. Statistical data of variables was given Table 2.1. The retention time and the retention factor that obtained from totally 111 RP-HPLC chromatogram were recorded in excel program. These data sets were divided to for group as input, sample input, output and sample output for each output parameters. 15 data of these 111 data were selected as sample parameters to use after ANN optimization as estimating data. Other 96 data were evaluated in MATLAB separately for retention time and retention factor. Thereafter, percentage of training, validation and testing, hidden neuron number were optimized. Following that sample output was evaluated with different algorithms. The flow chart of ANN optimization is given in Figure 2.5.

Table 2.1 Data Statistics of Model Variables

Variables	Statistical Data	
	Range	**Mean ± S. D.**
Input Layer Methanol % in Mobile Phase	10.00-70.00	40.00 ± 21.60
Column Temperature (°C)	25.00-40.00	32.29 ± 5.41
Flow Rate (mL/minute)	0.50-1.50	1.00 ± 0.39
Injection Volume (µL)	50.00-100.00	57.14 ± 28.85
Wavelength (nm)	193.00-326.00	266.88 ± 46.77
Caulerpin Concentration (mg/L)	1.00-15.00	6.83 ± 5.16
Output Layer Retention Time	31.08- 6.63	11.27 ± 7.10
Retention Factor	0.96-0.88	0.88 ± 0.04

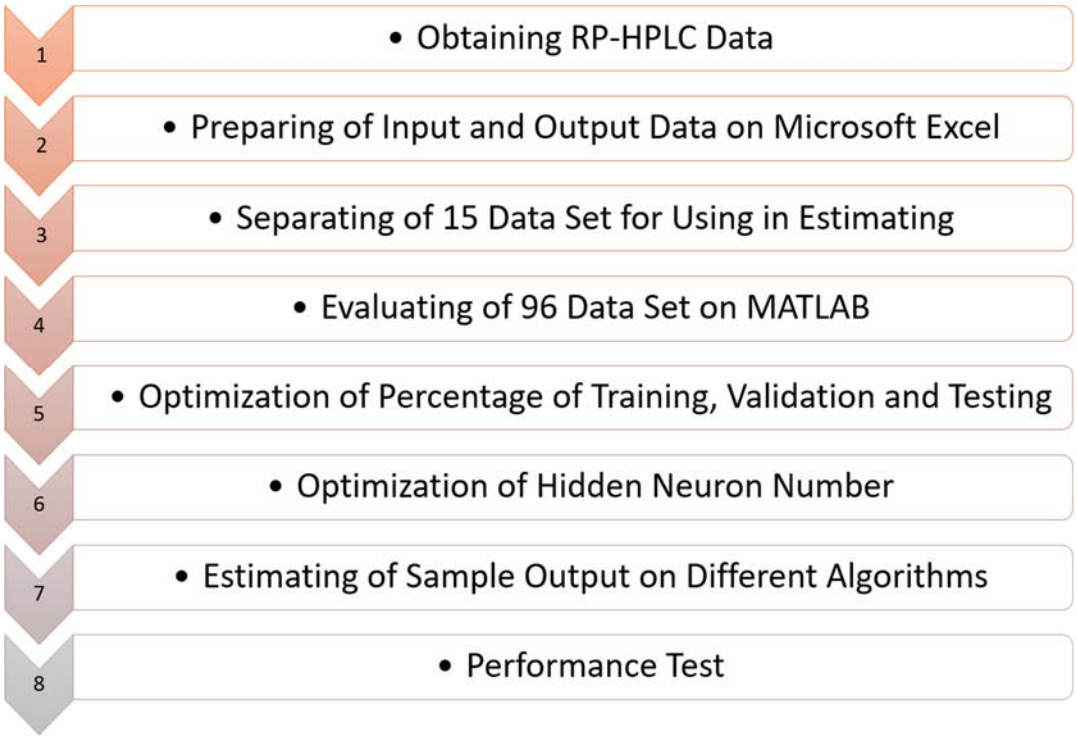


Figure 2.5 Flow chart of ANN optimization

2.6.2 Guideline for ANN tools of MATLAB

2.6.2.1 Uploading of Data to System

The obtained RP-HPLC data were uploaded to MATLAB using 'Import Data' icon then input, sample input and output data set were opened one by one. 'Numeric Matrix' option was chosen just before import data (Figure 2.6).

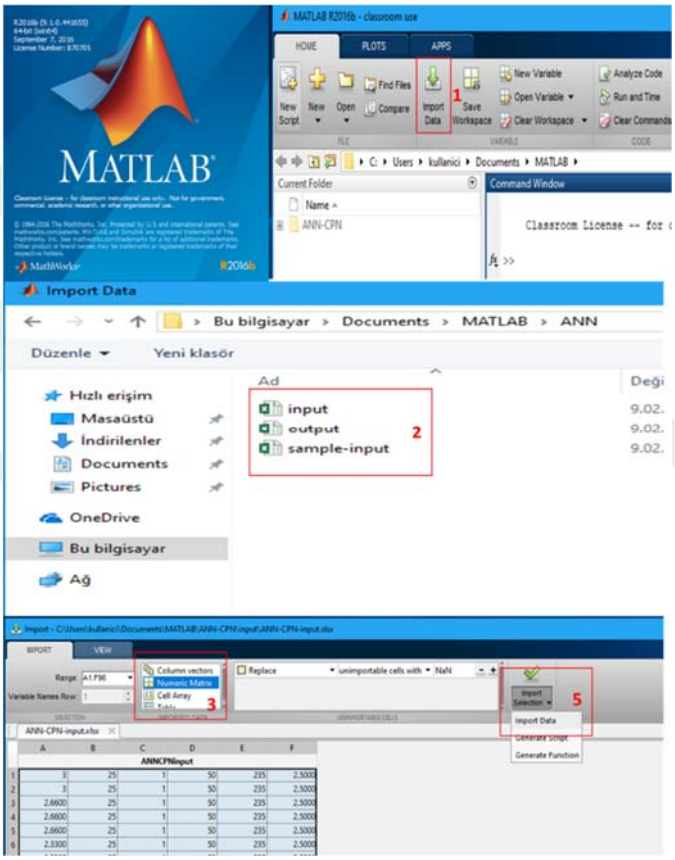


Figure 2.6 Uploading of the data to system

After data was imported, they are seen in the right site of the screen with 96x6, 96x1 and 15x6 for input, output and sample input respectively. The data sets were selected and transpose to the 6x96, 1x96 and 6x15 for input, output and sample input respectively by using transpose icon (Figure 2.7).

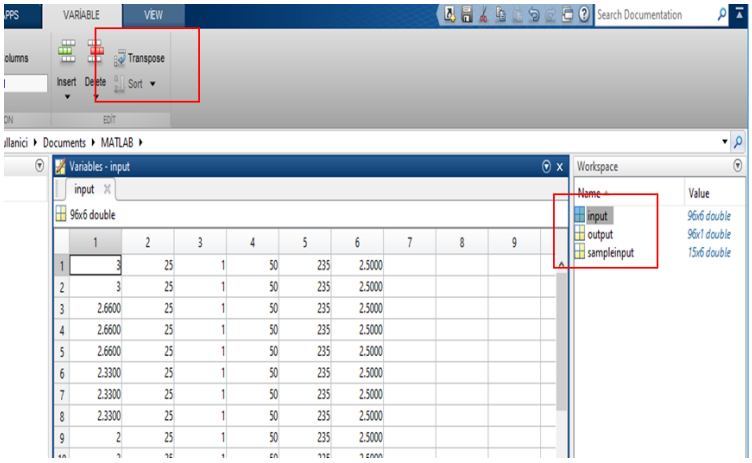


Figure 2.7 Transposition of the data sets

2.6.2.2 nftool Function

After entering 'nftool (neural network fitting tool)' to program, the window will open to enter input and output data for optimization (Figure 2.8).

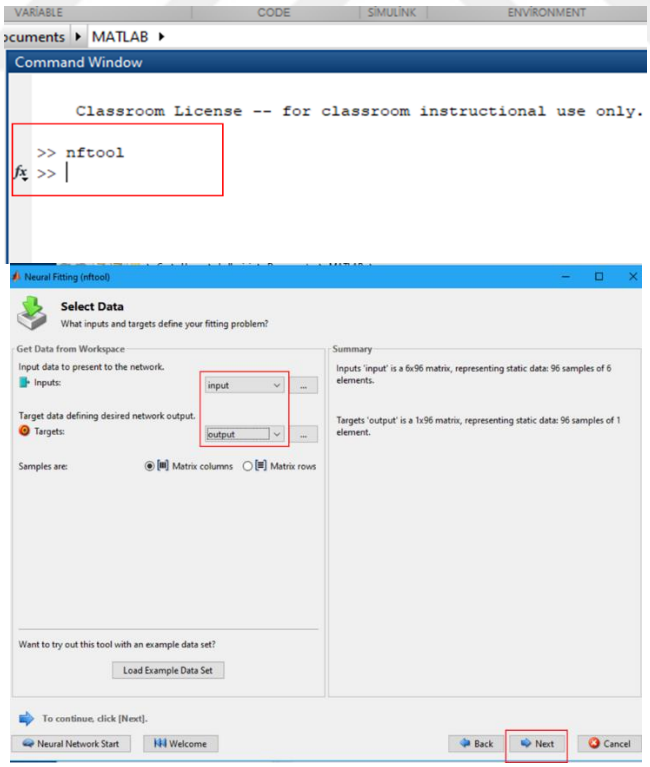


Figure 2.8 Entering of nftool function and Data set

2.6.2.3 Switching of Percentage of Training, Validation and Testing and Hidden Neuron Number

Following the introduction of data set to the system, an interface that allows for changing the percentage of validation, testing, and training data as well as hidden neuron numbers will show up (Figure 2.9a and Figure 2.9b). The ANN model and the graphs and data related to the model were obtained. The obtained graphs were interpreted. R^2 and MSE values were recorded (Figure 2.10).

The epoch values indicate how many cycles the model are created. Model performance and regression graphs can be viewed from the PlotPerform and PlotRegression icons (Figure 2.11).

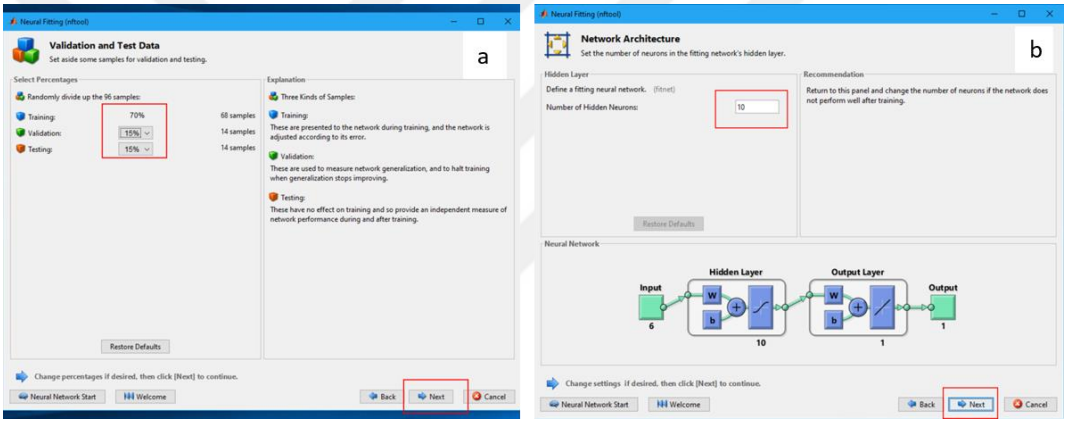


Figure 2.9 Optimization of a) the percentage of training, validation and testing b) the hidden neuron number

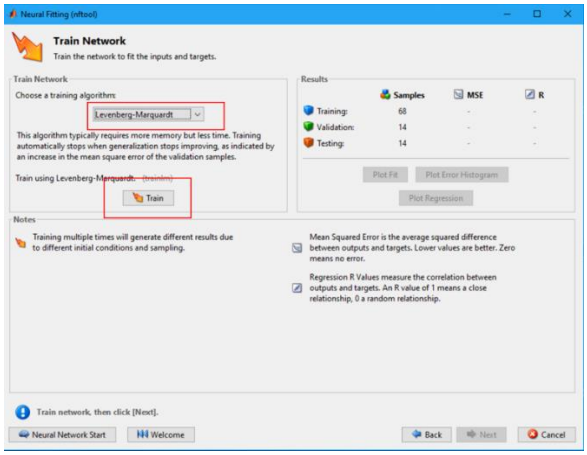


Figure 2.10 The creation of ANN model and obtaining of the R^2 and MSE values related to training, validation and testing data

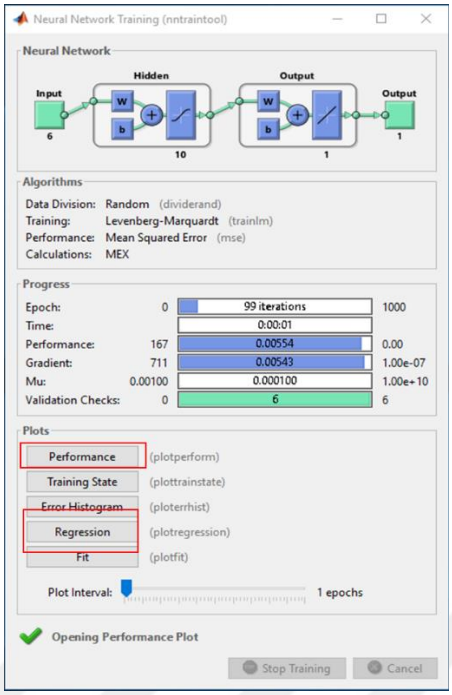


Figure 2.11 Screen shoot of nntraintool

2.6.2.4 Performance Test

The R^2 values of training, validation and testing are obtained with regression graphs (Figure 2.12a). The MSE values of training, validation and testing are obtained with performance graphs (Figure 2.12b). These graphs also provide information on the best validation performance.

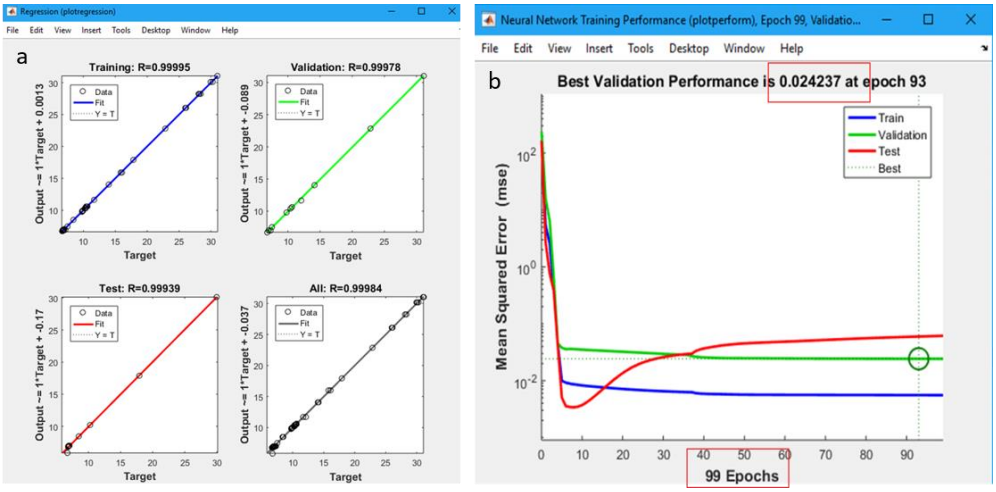


Figure 2.12 A sample view from a) regression graph and b) a performance graph

2.6.2.5 nntool Function

After entering 'nntool (neural network toolbox)' to program, the window opens for matching input data as input and output data as target and creating a model with different algorithms (Figure 2.13a). When the all data are introduced, 'new' icon is clicked.

When the input and output data are assigned, the optimum neuron number is entered and number of layers is selected as 2 (Figure 2.13b). The obtained models with different training functions can be seen in networks section.

In the window that opens with double click on newly created model, input and output data are selected with train icon (Figure 2.14a). Thereafter, sample input data set is used in simulate section and estimated output data is obtained. The estimated output data can be seen in output data section and with double click on newly obtained estimated output data; data set will be opened and saved (Figure 2.14b). There are 14 different algorithms that are used in the estimation of sample output. The list of these algorithms is given in Table 1.2.

Table 1.2 The algorithms used in estimation of sample output

	Abbreviation	**Name of Algorithms**
1	trainbfg	BFGS Quasi-Newton Back propagation
2	trainbr	Bayesian Regulation Back propagation
3	traincgb	Conjugate Gradient with Powell/Beale Restarts Back propagation
4	traincgf	Fletcher-Powell Conjugate Gradient Back propagation
5	traincgp	Polak-Ribiére Conjugate Gradient Back propagation
6	traingd	Gradient Descent Back propagation
7	traingdm	Gradient descent with momentum Back propagation
8	traingda	Gradient Descent with Adaptive Learning Rate Back propagation
9	traingdx	Gradient Descent with Momentum and Adaptive Learning Rate Back propagation
10	trainlm	Levenberg-Marquardt Back propagation
11	trainoss	One Step Secant Back propagation
12	trainr	Random Order Incremental Training with Learning Functions
13	trainrp	Resilient Back propagation
14	trainscg	Scaled Conjugate Gradient Back propagation

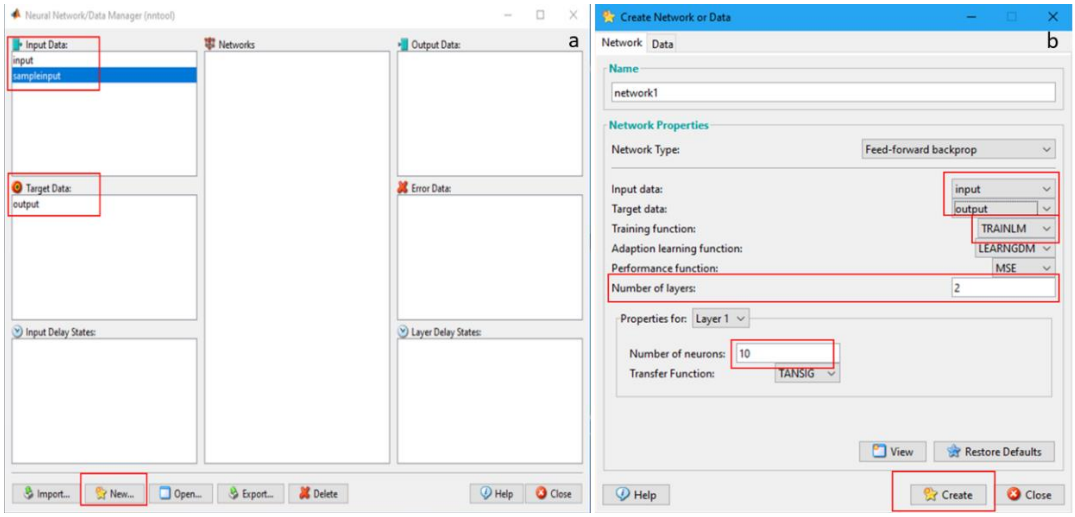


Figure 2.13 View of a) nntool function for entering input and output data and b) creation of a network in nntool

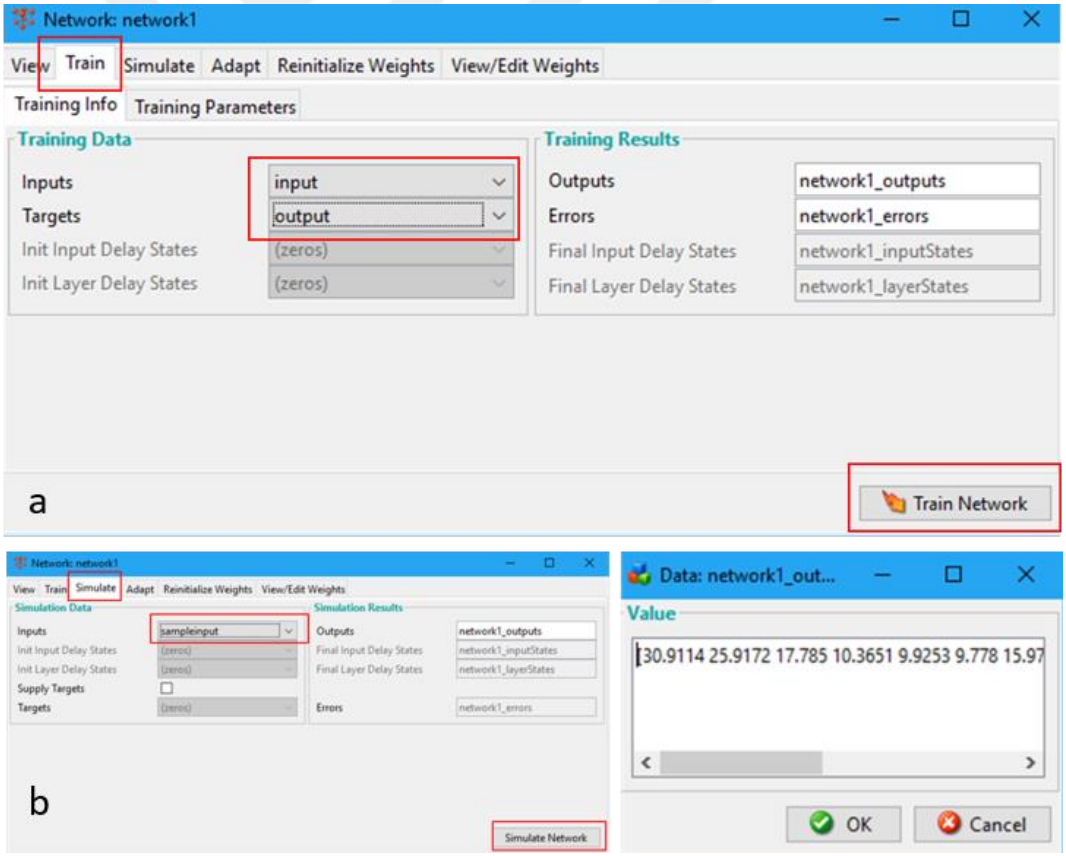


Figure 2.14 Screen shoots of a) nntool function for training and b) obtaining of the estimated data in nntool

CHAPTER THREE

RESULTS

3.1 Sampling

3.1.1 S. salpa

S. salpa samples were collected in February'2017, May'2017, August'2017 and November'2017. 10 individuals were provided each sampling periods. After fish samples were brought to the laboratory, total, standard and fork length and the length of lateral line and all fins were recorded. Thereafter, brain, liver and muscle tissues of *S. salpa* were dissected and their weights were also recorded. The length and weight measures of *S. salpa* samples were given in between Table 3.1 and Table 3.8.

Table 3.1 The length of *S. salpa* collected on 21 February 2017

FISH NUMBER	LENGTH (cm)		FIN LENGTH (cm)		CADUAL FIN LENGTH (cm)	BODY DEPTH (cm)
1	Total	30	Pectoral	4.9	5.5	9.5
	Lateral Line	22	Anal	5		
	Fork Length	27	Dorsal	2.7 **(depth)**		
				13.2		
	Standard	26	Pelvic	3.7		
2	Total	29	Pectoral	4.2	5	9
	Lateral Line	20	Anal	5		
	Fork Length	27	Dorsal	2.2 **(depth)**		
				13.2		
	Standard	25	Pelvic	3.7		
3	Total	29	Pectoral	4.5	5.2	9.3
	Lateral Line	19	Anal	5		
	Fork Length	26	Dorsal	2.4 **(depth)**		
				12.5		
	Standard	25	Pelvic	3.2		
4	Total	29	Pectoral	4.5	5.5	9.5
	Lateral Line	19	Anal	4.8		
	Fork Length	25	Dorsal	2.3 **(depth)**		
				13		
	Standard	24	Pelvic	3.5		
	Total	28	Pectoral	4.5		
	Lateral Line	19	Anal	4.8	5.3	8.8

Table 3.1 continues

	Fork Length	26	Dorsal	2.4 **(depth)**		
				12		
	Standard	24	Pelvic	3.6		
6	Total	34	Pectoral	5.2		
	Lateral Line	21	Anal	5.6		
	Fork Length	29	Dorsal	3.0 **(depth)**		
				14	6	10
	Standard	27	Pelvic	4		
7	Total	30	Pectoral	4.5		
	Lateral Line	20	Anal	5.1		
	Fork Length	27	Dorsal	2.4 **(depth)**	5	9.2
				13.5		
	Standard	25	Pelvic	3.7		
8	Total	30	Pectoral	5		
	Lateral Line	21	Anal	4.8		
	Fork Length	26	Dorsal	2.7 **(depth)**		
				14.4	5.2	8.5
	Standard	28	Pelvic	3.7		
9	Total	29	Pectoral	5		
	Lateral Line	19	Anal	5		
	Fork Length	25	Dorsal	2.4 **(depth)**	5.5	8.7
				13		
	Standard	27	Pelvic	3.9		
10	Total	28	Pectoral	4.7		
	Lateral Line	19	Anal	4.9	4.9	8.5
	Fork Length	25	Dorsal	2.2 **(depth)**		
				12		
	Standard	24	Pelvic	3.7		

Table 3.2 The weight of *S. salpa* collected on 21 February 2017

FISH NUMBER	TOTAL WEIGHT (g)	MUSCLE WEIGHT (g)	LIVER WEIGHT (g)	BRAIN WEIGHT (g)
1	395.34	187.34	1.01	0.92
2	371.96	181.6	0.76	0.75
3	359.12	157.08	1.95	0.59
4	350.81	168.56	1.75	0.62
5	337.79	156.6	1.15	0.62
6	509.11	275.23	2.88	0.72
7	406.83	200.17	1.38	0.79
8	428.61	192.88	1.33	0.87
9	392.74	169.91	1.91	0.81
10	362.24	142.4	1.61	0.77

The length and weight measures of *S. salpa* samples that collected in February 21, 2017 were given in Table 3.1 and Table 3.2 respectively. The mean length and weight of these individuals were recorded as 29.50 ± 1.56 cm and 391.46 ± 49.76 g, respectively.

Table 3.3 The length of *S. salpa* collected on 25 May 2017

FISH NUMBER	LENGTH cm)		FIN LENGTH (cm)		CADUAL FIN LENGTH (cm)	BODY DEPTH (cm)
1	Total	37	Pectoral	5.7	6	10
	Lateral Line	24	Anal	6		
	Fork Length	31	Dorsal	2.5 **(depth)**		
				16		
	Standard	29	Pelvic	4		
2	Total	33	Pectoral	5.2	6	9.5
	Lateral Line	21	Anal	4.5		
	Fork Length	29	Dorsal	2.5 **(depth)**		
				13		
	Standard	26	Pelvic	4		
3	Total	35	Pectoral	5.5	6.7	9.5
	Lateral Line	22	Anal	5.5		
	Fork Length	29	Dorsal	2.5 **(depth)**		
				14.5		
	Standard	26	Pelvic	4.2		
4	Total	35	Pectoral	5.5	5.5	9.5
	Lateral Line	23	Anal	6		
	Fork Length	29	Dorsal	2.4 **(depth)**		
				15		
	Standard	27	Pelvic	4.3		
5	Total	31	Pectoral	5.2	5.5	8.5
	Lateral Line	20	Anal	4.8		
	Fork Length	27	Dorsal	2.7 **(depth)**		
				13		
	Standard	25	Pelvic	3.7		
6	Total	30	Pectoral	5	5	10.5
	Lateral Line	19	Anal	5		
	Fork Length	26	Dorsal	2.2 **(depth)**		
				12.5		
	Standard	23	Pelvic	3.7		
7	Total	31	Pectoral	4.7	5.5	9.5
	Lateral Line	20	Anal	5		
	Fork Length	27	Dorsal	2.5 **(depth)**		
				13		
	Standard	24	Pelvic	3.5		
	Total	36	Pectoral	5.5	6	10.5
	Lateral Line	24	Anal	5		
8	Fork Length	31	Dorsal	2.5 **(depth)**		

Table 3.3 continues

				15.4		
	Standard	28	Pelvic	4.3		
9	Total	32	Pectoral	5	5.3	9.2
	Lateral Line	22	Anal	5.5		
	Fork Length	28	Dorsal	2.0 **(depth)**		
				14		
	Standard	24	Pelvic	4.2		
10	Total	33	Pectoral	5	5.7	10.3
	Lateral Line	22	Anal	5.5		
	Fork Length	29	Dorsal	2.5 **(depth)**		
				14		
	Standard	25	Pelvic	3.8		

Table 3.4 The weight of *S. salpa* collected on 25 May 2017

FISH NUMBER	TOTAL WEIGHT (g)	MUSCLE WEIGHT (g)	LIVER WEIGHT (g)	BRAIN WEIGHT (g)
1	587.95	124.51	0.72	1.1
2	443.34	103.62	0.65	0.75
3	473.34	110.08	0.66	1.15
4	493.68	106.83	0.95	0.97
5	359.42	88.23	0.44	1.01
6	422.92	120.07	0.76	0.61
7	413.78	106.15	0.67	0.7
8	569.12	147.29	1.43	1.05
9	418.28	110.51	1.41	0.97
10	437.76	113.02	0.75	1.05

The second sampling was on May 25, 2017. The lengths and weights of *S. salpa* samples were given in Table 3.3 and Table 3.4, respectively. The mean length and weight of these individuals were recorded as 33.04 ± 2.24 cm and 461.96 ± 71.25 g, respectively.

Table 3.5 The length of *S. salpa* collected on 03 August 2017

FISH NUMBER	LENGTH (cm)		FIN LENGTH (cm)		CADUAL FIN LENGTH (cm)	BODY DEPTH (cm)
1	Total	25.5	Pectoral	4	4	8.2
	Lateral Line	16.5	Anal	4.2		
	Fork Length	23	Dorsal	2.4 **(depth)**		
				9		
	Standard	21.5	Pelvic	3		
2	Total	28.7	Pectoral	4.7	4.5	9
	Lateral Line	18.2	Anal	4.7		
	Fork Length	25.5	Dorsal	2.0 **(depth)**		
				13		
	Standard	24.3	Pelvic	3.5		
3	Total	26.2	Pectoral	4.5	4.7	8
	Lateral Line	17.1	Anal	4.2		
	Fork Length	23	Dorsal	2.2 **(depth)**		
				11.5		
	Standard	22	Pelvic	3.3		
4	Total	25	Pectoral	4.2	4.2	8.2
	Lateral Line	16	Anal	4		
	Fork Length	22.5	Dorsal	1.8 **(depth)**		
				12		
	Standard	21	Pelvic	3.3		
5	Total	25.5	Pectoral	4.2	4.2	8.5
	Lateral Line	17	Anal	4		
	Fork Length	22.2	Dorsal	1.8 **(depth)**		
				11.5		
	Standard	21	Pelvic	3.5		
6	Total	27	Pectoral	4.5	4.5	8.5
	Lateral Line	17.2	Anal	5.5		
	Fork Length	24.2	Dorsal	1.6 **(depth)**		
				11.5		
	Standard	23	Pelvic	3.5		
7	Total	29.5	Pectoral	4.5	4.5	10
	Lateral Line	19.5	Anal	5		
	Fork Length	26.5	Dorsal	1.5 **(depth)**		
				12.7		
	Standard	25	Pelvic	3.5		
8	Total	25	Pectoral	4	4.5	7.7
	Lateral Line	16	Anal	4.2		
	Fork Length	22	Dorsal	1.3 **(depth)**		
				11		
	Standard	20.5	Pelvic	3		
9	Total	25.2	Pectoral	4	4	7.5
	Lateral Line	16	Anal	4		

Table 3.5 continues

	Fork Length	22.2	Dorsal	1.3 **(depth)**		
				10.5		
	Standard	21	Pelvic	3		
10	Total	24	Pectoral	4	4.5	7.7
	Lateral Line	15.5	Anal	4		
	Fork Length	21	Dorsal	1.2 **(depth)**		
				10.5		
	Standard	20	Pelvic	3.5		

Table 3.6 The weight of *S. salpa* collected on 03 August 2017

FISH NUMBER	TOTAL WEIGHT (g)	MUSCLE WEIGHT (g)	LIVER WEIGHT (g)	BRAIN WEIGHT (g)
1	240.47	46.61	0.69	0.61
2	321.33	67.39	1.23	0.73
3	245.59	60.02	0.52	0.65
4	228.51	64.37	1.06	0.69
5	255.69	57.28	0.73	0.62
6	280.72	45.91	0.7	0.61
7	392.64	104.83	1.28	0.75
8	203.38	54.06	0.44	0.54
9	198.02	72.59	1.19	0.64
10	212.15	49.94	0.52	0.62

The next sampling was on August 03, 2017 and the length and weight of *S. salpa* were given in Table 3.5 and Table 3.6, respectively. The mean length and weight were recorded as 26.16 ± 1.75 cm and 257.85 ± 60.25 g, respectively.

Table 3.7 The length of *S. salpa* collected on 09 November 2017

FISH NUMBER	**LENGHT (cm)**		**FIN LENGTH (cm)**		**CADUAL FIN LENGTH (cm)**	**BODY DEPTH (cm)**
1	Total	28.7	Pectoral	5	4.5	8.5
	Lateral Line	19	Anal	5		
	Fork Length	26.4	Dorsal	2.5 **(depth)**		
				13		
	Standard	24.7	Pelvic	3		
	Total	29.5	Pectoral	4.5		
	Lateral Line	19	Anal	4.5		
2	Fork Length	26	Dorsal	2.4 **(depth)**	4.5	9.5

Table 3.7 continues

				13.2		
	Standard	24.6	Pelvic	3.5		
3	Total	29.4	Pectoral	4.5	5	8.3
	Lateral Line	19.5	Anal	5		
	Fork Length	26.3	Dorsal	2.3 **(depth)**		
				13.5		
	Standard	24.5	Pelvic	3.5		
4	Total	28.9	Pectoral	4.5	5	9
	Lateral Line	18.8	Anal	5.2		
	Fork Length	26	Dorsal	2.6 **(depth)**		
				13		
	Standard	24	Pelvic	3.7		
5	Total	28.7	Pectoral	4.7	5.2	8.7
	Lateral Line	17.8	Anal	5		
	Fork Length	25	Dorsal	2.3 **(depth)**		
				12		
	Standard	23.5	Pelvic	3.5		
6	Total	27.2	Pectoral	4.5	4.5	8
	Lateral Line	17.5	Anal	4.8		
	Fork Length	24.1	Dorsal	2.4 **(depth)**		
				11.6		
	Standard	23	Pelvic	3.4		
7	Total	30	Pectoral	5	5	9
	Lateral Line	19.6	Anal	5		
	Fork Length	26.5	Dorsal	2.5 **(depth)**		
				13.3		
	Standard	23	Pelvic	3.7		
8	Total	30.5	Pectoral	4.7	5.6	9
	Lateral Line	20	Anal	5		
	Fork Length	27	Dorsal	2.5 **(depth)**		
				13.2		
	Standard	25.7	Pelvic	3.9		
9	Total	32	Pectoral	5.5	5.5	9
	Lateral Line	21.5	Anal	5.5		
	Fork Length	28	Dorsal	2.6 **(depth)**		
				14		
	Standard	26.3	Pelvic	4.3		
10	Total	29	Pectoral	4.5	4.8	9.6
	Lateral Line	19	Anal	4.5		
	Fork Length	25.7	Dorsal	2.4 **(depth)**		
				13		
	Standard	24	Pelvic	3.6		

Table 3.8 The weight of *S. salpa* collected on 09 November 2017

FISH NUMBER	TOTAL WEIGHT (g)	MUSCLE WEIGHT (g)	LIVER WEIGHT (g)	BRAIN WEIGHT (g)
1	304.5	79.19	1.12	0.68
2	374.14	110.25	0.98	0.59
3	325.98	97.5	0.61	0.44
4	330.68	112.5	0.62	0.62
5	287.17	80.43	0.79	0.56
6	270.42	77.65	0.61	0.74
7	327.43	110.83	0.57	0.73
8	344.91	99.84	1.23	0.39
9	390.66	104.3	0.93	0.47
10	356.32	11.99	0.84	0.7

The last sampling was on November 09, 2017. The length and weight of *S. salpa* samples were given in Table 3.7 and Table 3.8, respectively. The mean length and weight of the samples were recorded as 29.39 ± 1.27 cm and 331.22 ± 37.40 g, respectively.

3.1.2 Caulerpa spp.

Caulerpa spp. samples were seasonally collected via free-diving from İzmir, Çeşme and Dikili regions which were determined in the previous studies. However, *Caulerpa* samples could not be observed in certain periods because of the sea water temperature and changing in nutrient enrichment. In first sampling period, February'2017, no *C. prolifera* neither *C. cylindracea* were observed. On the other hand, it was observed that another invasive species *Halophila stipulacea* invaded the *Caulerpa* meadows and widely spread despite the very low sea water temperature in February'2017 (Figure 3.1). In the next sampling period, May'2017, *Caulerpa* spp. were in the regrowth phase and still *H. stipulacea* samples were observed in the same area (Figure 3.2 and Figure 3.3). In August'2017, it was observed that population of *C. cylindracea* and *C. prolifera* reached to the saturation; number of roots, branched and length of fronds were increased. In final sampling period, November'2017,

population of *Caulerpa* spp. was decreased. Also, number of branched and frond lengths were decreased.

Figure 3.1 *C. prolifera*, *C. cylindracea* and *H. stipulacea* samples collected from İzmir in February'2017, May'2017, August'2017 and November'2017 (Personal archive, 2017)

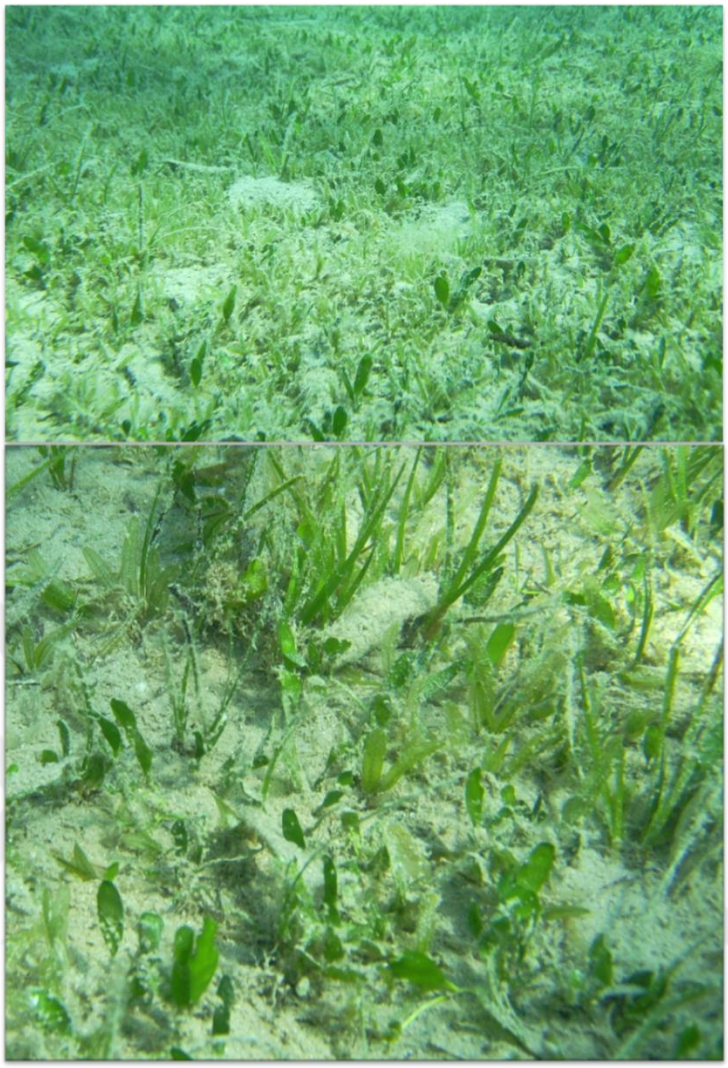


Figure 3.2 *H. stipulacea* invasion on *Caulerpa* spp. meadows (Levent CAVAS, Personal archive, 2017)

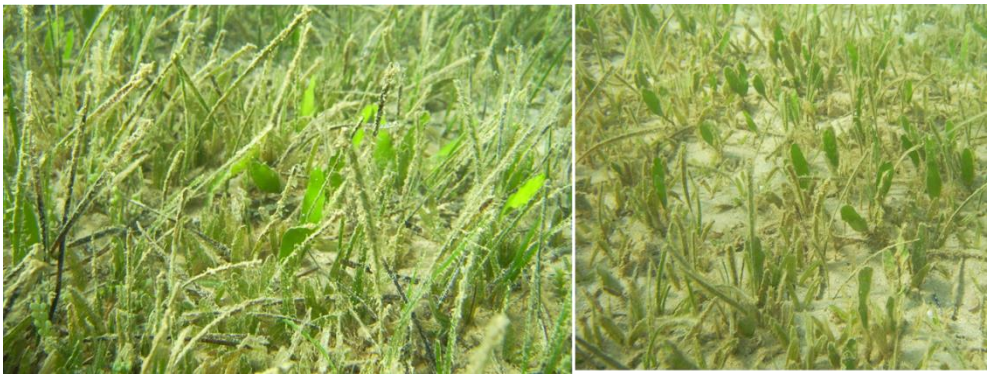


Figure 3.3 *H. stipulacea* invasion on *Caulerpa* spp. meadows (Levent CAVAS, Personal archive, 2017)

Caulerpa samples were carried to the laboratory in sea water. After removing of the impurities, samples were weighed and dried under the sunlight. Also dried samples were weighed and percentage of water in wet weight was determined as over than 80 %. *Caulerpa* samples were ground until they become dust formation before the extraction step (Figure 3.4).

Figure 3.4 Wet (1), dried (2) and grinded (3) *Caulerpa* spp. samples (Personal archive, 2017)

3.2 Extraction and Isolation of Caulerpinin

3.2.1 C. cylindracea and C. prolifera

As described in material and method section, caulerpin was isolated from *C. cylindracea* using chromatographic techniques and then characterized by MALDI-TOF/MS, ^{1}H-NMR, UV-Vis spectrometry.

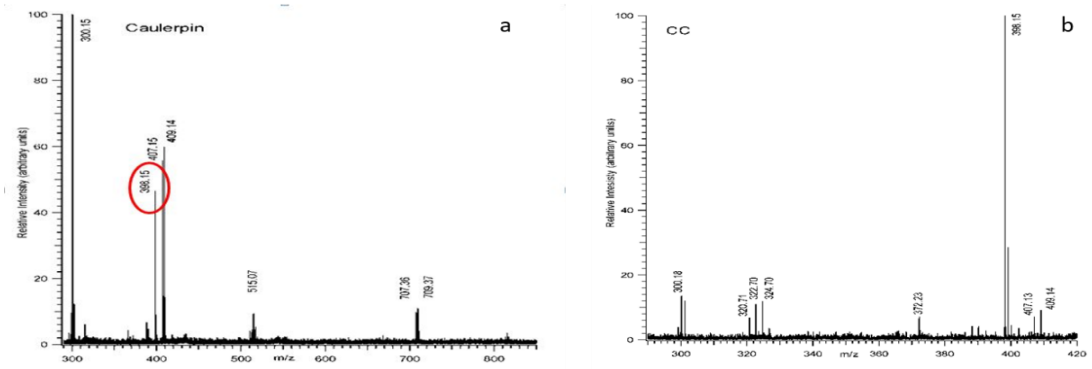


Figure 3.5 MALDI-TOF/MS spectrum of a) caulerpin and b) the diethyl ether extract of *C. cylindracea*

The MALDI-TOF/MS spectrum of caulerpin and the diethyl ether extract of *C. cylindracea* are given in Figure 3.5a and Figure 3.5b respectively. The molecular weight of caulerpin is 397 g/mol and the peak at M^+: 398 (in positive mode) belongs to caulerpin. The peak at M^+: 398 was observed in both spectrums which meant caulerpin presence in *C. cylindracea.*

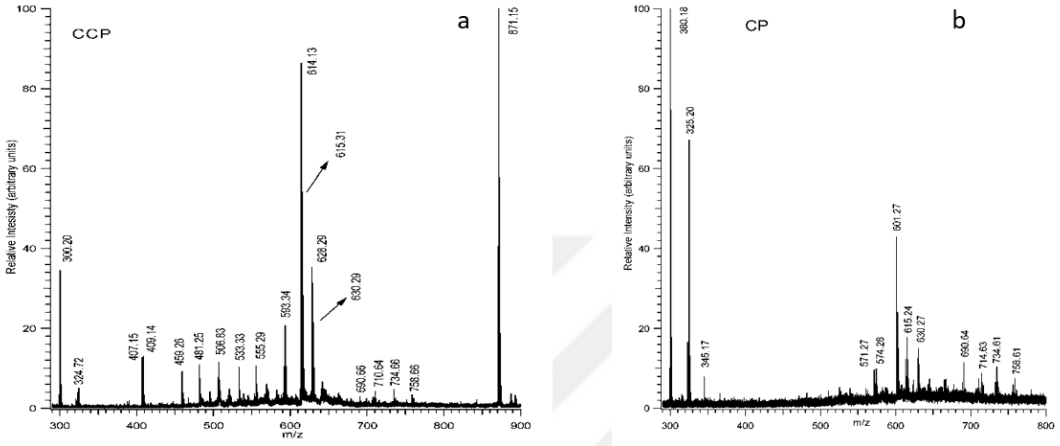


Figure 3.6 MALDI-TOF/MS spectrum of the diethyl ether extract of *C. prolifera*

MALDI-TOF/MS spectrum of the diethyl ether extract of *C. cylindracea* was given in Figure 3.6a and Figure 3.6b. There was no peak that indicates caulerpin presence in *C. prolifera.*

The ^{1}H NMR spectrum of isolated caulerpin from *C. cylindracea* is given in Figure 3.7b. According to ^{1}H NMR results, the peak at 10.45 ppm indicated to the proton bounds to the nitrogen atom in indole-ring that was shown in Figure 3.7a with the number 5. The peak at 8.22 ppm indicates the proton of olefinic carbon atom with the number 7 in Figure 3.7a. The peaks on 7.06, 7.14, 7.38 and 7.45 ppm belong to the protons bound to benzene ring that were shown in Figure 3.7a with the number 1, 2, 3 and 4. The splitting on these peaks indicated the interactions between these protons by confirmation of the chemical structure. The peak at 3.81 ppm indicated the proton bounds to methoxy group that is shown in Figure 3.9 with the number 6. The peaks at 2.80 and 2.77 ppm demonstrated the protons coming from the water

inside of the Acetone-d6 which was used as NMR solvent. The peaks at 2.05 and 1.97 were noise signals.

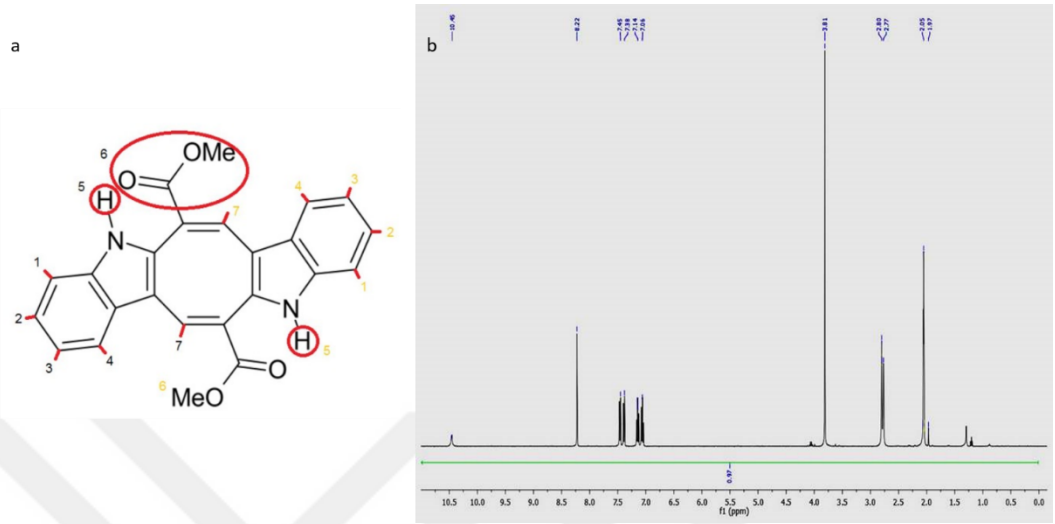


Figure 3.7 a) Molecular structure of caulerpin and b) ^{1}H NMR spectrum of isolated caulerpin from *C. cylindracea*

The melting point of isolated caulerpin was determined as 317 ^{o}C.

The UV-Vis spectrum of caulerpin that was isolated from different extracts of *C. cylindracea* was given in Figure 3.11. The maximum absorbance wavelengths that obtained from UV-Vis spectrum was comparable with the literature (Movahheddin et al., 2014).

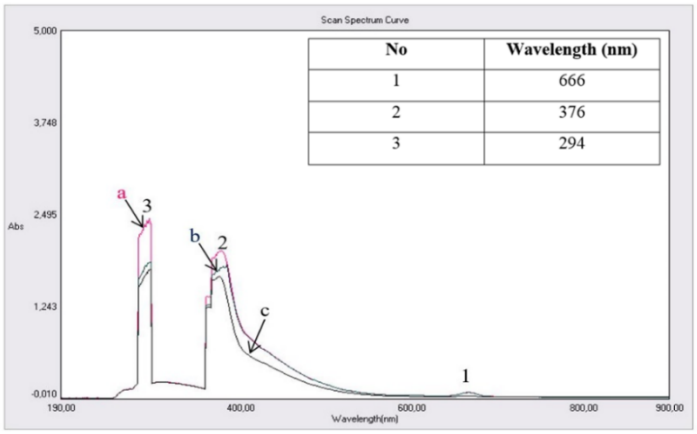


Figure 3.8 UV-Vis spectrum of caulerpin that was isolated from a) and b) ethyl acetate and c) diethyl ether extracts of *C. cylindracea*

3.2.2 Determination of caulerpin in Sarpa salpa tissues

The presence of caulerpin in *S. salpa* tissues is showed by MALDI-TOF analyses. The MALDI-TOF spectrum of the liver extract of *S. salpa* is given in Figure 3.9. The peak at M^+: 398 which demonstrates us the presence of caulerpin is observed in MALDI-TOF spectrum.

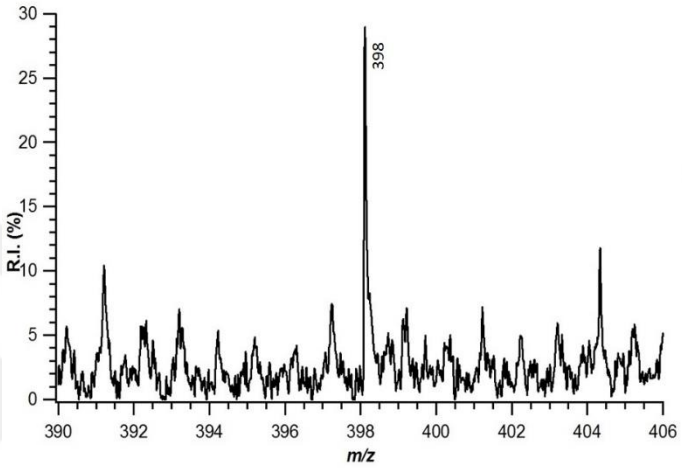


Figure 3.9 The MALDI-TOF spectrum of the liver extract of *S. salpa*

3.3 Chromatographic Techniques

3.3.1 Column and Thin Layer Chromatography (TLC)

The *C. cylindracea* extract was performed to column chromatography filled silica gel to isolate caulerpin. The ethyl acetate and hexane mix (1:1 v/v) was used as mobile phase at the beginning but, after removing of chlorophyll 100% ethyl acetate was used. 15 fractions were obtained from column chromatography. The obtained fractions were applied to thin layer chromatography (TLC) (Figure 3.10). The orange colour bands that specific to caulerpin were observed on the TLC plate with the number 7^{th}, 8^{th}, 9^{th}, and 10^{th} fractions (Figure 3.10). The Rf values of these bands were calculated and compared with the Rf value of isolated caulerpin in previous studies.

Figure 3.10 The column chromatography and TLC application of *C. cylindracea* extract (Personal archive, 2017)

TLC applications were always performed after each extraction step. The TLC results are given in Figure 3.11. The green colour bands observed in Figure 3.11a and Figure 3.11b indicated the chlorophyll and the orange colour bands were caused by caulerpin and carotenoids. The *C. cylindracea* extracts and caulerpin standard were applied to TLC at the same time to observe if caulerpin is presence in *C. cylindracea.*

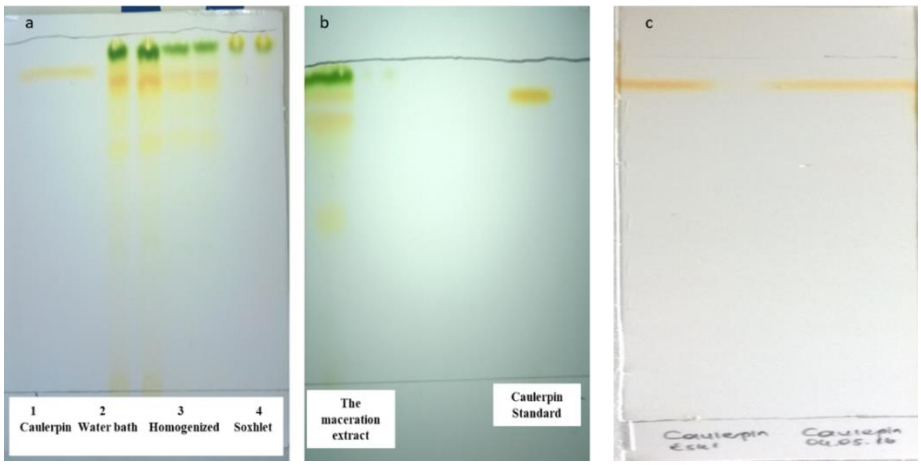
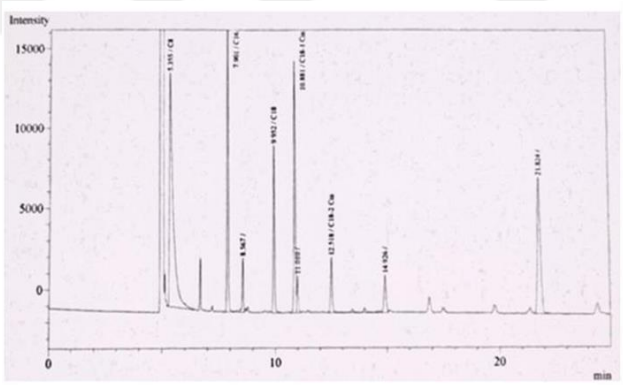


Figure 3.11 a, b) The TLC applications of *C. cylindracea* extracts and c) caulerpin standard (Songül TURHAN, Personal archive, 2017)

In our laboratory, we had previously isolated caulerpin. We also wanted to compare newly isolated caulerpin to check its purity. TLC was applied to new and previously isolated caulerpin. We found that they had same Rf values. Therefore, we concluded that caulerpin was pure.

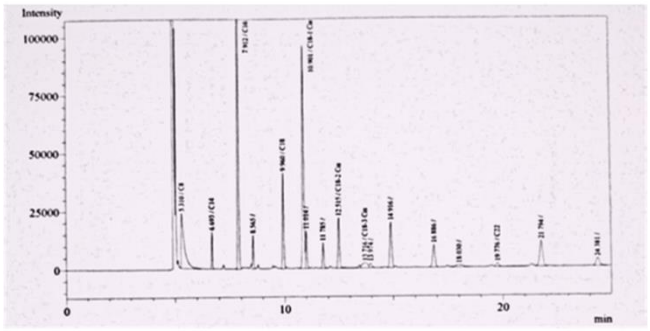
3.3.2 *Determination of Fatty Acid Composition of S. salpa Muscle*

Two *S. salpa* samples were selected from November'2017 group based on the caulerpin level in their muscle tissue. One of the samples was labeled as CPN (-) which contains no caulerpin in its tissues. Second sample was labeled as CPN (+) which contain caulerpin in its tissues. The fatty acids were extracted from muscle tissues according to Bligh and Dyer (1959) method and then analyzed by Gas Chromatography (GC). The GC chromatograms of number 1 and 2 samples are given in Figure 3.12 and Figure 3.13. The fatty acid composition of these samples is given in Table 3.9.



Compound Name	Retention Time	Area	Height	Concentration (%)
C8	5.355	161838	14450	31.636
C16	7.901	101824	32078	19.904
C18	9.952	41928	10203	8.196
C18:1 Cis	10.881	68861	15478	13.461
C18:2 Cis	12.518	19820	3402	3.784

Figure 3.12 GC chromatograms of CPN (+) *S. salpa* sample-01 November 2017



Compound Name	Retention Time	Area	Height	Concentration (%)
C8	5.310	239232	23694	10.497
C14	6.693	43464	15865	1.907
C16	7.912	654468	200864	27.716
C18	9.960	168127	40660	7.377
C18:1 Cis	10.901	449348	95387	19.716
C18:2 Cis	12.515	116162	21622	5.097
C18:3 Cis	13.716	38771	2330	1.701
C22	19.776	16043	1586	0.704

Figure 3.13 GC chromatograms of CPN (-) *S. salpa* samples-01 November 2017

Table 3.9 Fatty acid composition of CPN (-) and CPN (+) *S. salpa* samples -01 November 2017

Compound Name/ Concentration (%)	CPN (+)	CPN (-)
C8	31.636	10.497
C14	X	1.907
C16	19.904	27.716
C18	8.196	7.377
C18:1 Cis	13.461	19.716
C18:2 Cis	3.784	5.097
C18:3 Cis	X	1.701
C22	X	0.704

3.4 RP-HPLC Analyses

3.4.1 Method Validation

3.4.1.1 Calibration interval and Linearity

Calibration interval and linearity were studied for method validation. The calibration graphs of caulerpin standard at 235 and 280 nm were given in Figure 3.14 a and 3.14 b. The calibration curves were created with 6 plots and 3 replicates. The R^2 values of calibration curves were 0.9977 and 0.9976 for 235 and 280 nm, respectively. The results are calculated based on the calibration curve equation at 235 nm since 280 nm was problematic because of absorbance of proteins at this wavelength.

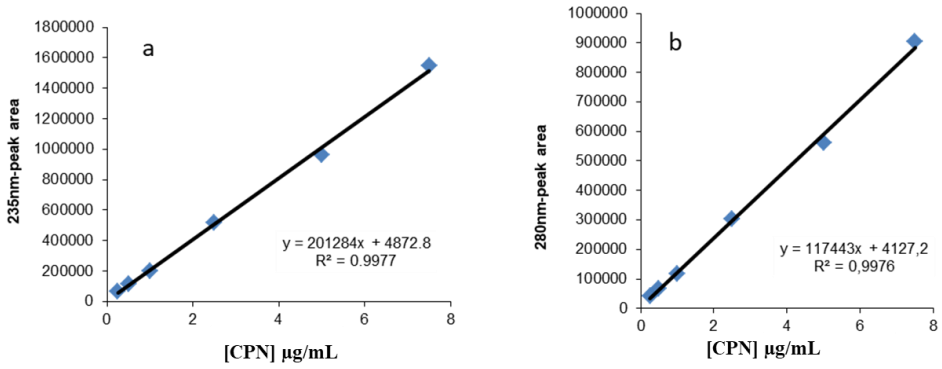


Figure 3.14 The calibration curve of caulerpin at a) 235 nm and b) 280 nm

The RP-HPLC chromatogram of caulerpin solutions that prepared for calibration curve was given in Figure 3.15. It was observed that peak areas increased dependent on the caulerpin concentration. The condition of RP-HPLC analyses: Intersil C18 column (150x4.6mm, 5µm), mobile phase: MeOH:H_2O (80:20 v/v), wavelength: 235 nm, flow rate: 1.0 mL/minute, column temperature: 25 ^oC, injection volume: 50 µL, loop volume: 100 µL.

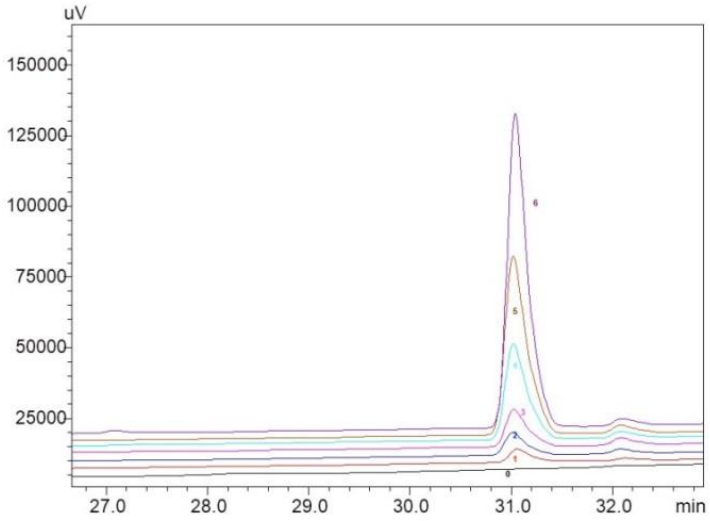


Figure 3.15 RP-HPLC chromatogram of (0) blank (MeOH:H2O (80:20)) and caulerpin solutions at (1) 0.25, (2) 0.5, (3) 1.0, (4) 2.5, (5) 5.0 and (6) 7.5 µg/mL

3.4.1.2 LOD and LOQ

The blank determination technique was used to determine the limit of dedection (LOD) and limit of quantification values. The blank sample (MeOH:H_2O (80:20)) was injected to the HPLC for 10 times and then the standard deviation of the peak areas were calculated. LOD and LOQ values were 0.00179 and 0.00543 µg/L respectively at 235 nm. LOD and LOQ values were 0.00425 and 0.01288 µg/L respectively at 280 nm (Table 3.10).

Table 3.10 Validation results obtained with caulerpin standard

Validation Parameter	**235 nm**	**280 nm**
Calibration Equation	y = 201284x + 4872.8	y = 117443x + 4127.2
Linearity (R^2)	R^2 = 0.9977	R^2 = 0.9976
Slope	201284	117443
Shear	4872.8	4127.2
Measuring Range (µg/L)	0.25-7.5	0.25-7.5
LOD (µg/L)	0.00179	0.00425
LOQ (µg/L)	0.00543	0.01288
Recovery (%)	108.8	108.2
RSD (%) (*n=3, k=3*)	7.8	7.9

3.4.1.3 Accuracy

The recovery (R %) has been calculated to determine the accuracy of the method. The R % values are calculated as 108.8% and 108.2% for 235 and 280 nm respectively.

3.4.1.4 Specifity

Firstly, the retention time of caulerpin in RP-HPLC was determined. Thereafter, the HPLC pump was stopped at the point of this peak that observed at this retention time and the UV-Vis spectrum of this peak was recorded. The maximum absorbance wavelengths of caulerpin were observed in Figure 3.16. The obtained results from UV-Vis spectrum were compatible with the literature (Aguilar & Santos, 1984). The UV-Vis scanning of the peaks that observed in the relevant retention time during the analyses of *Caulerpa* spp. and the liver, brain and muscle tissues of *S. salpa* were also recorded. Thereafter, the correlation between the UV-Vis spectrum of caulerpin standard and extracts has been investigated. If the correlation coefficient is around 1 then it was accepted that caulerpin is presented in these extracts.

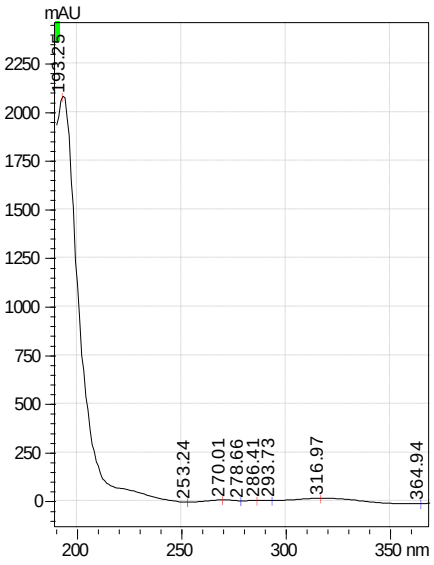


Figure 3.16 UV-Vis scanning of caulerpin (Retention time: 30.90)

3.4.2 Determination of Caulerpin Levels in Caulerpa spp. and S. salpa Tissues

The caulerpin levels in *Caulerpa* spp. and liver, brain and muscle tissues of *S. salpa* were determined by RP-HPLC analyses. RP-HPLC analyses were conducted with SHIMADZU-Prominence LC-20A RP-HPLC equipped with DGU-20A5 solvent degasser, SPD-20A UV/VIS detector and CTO-10ASUP column oven according to Movahheddin et al. (2014)'s method includes metanol and water in its gradient elution system. The elution program: 0-5 minute, 10% methanol-water; 5-35 minute, 10-100% methanol-water; 35-45 minute, 100% methanol; flow rate: 1.0 mL/minute; detection: 235 and 280 nm; injection volume: 50 µL and retention time of caulerpin: 30.90 minute. During this study, 10 individual *S. salpa* samples were collected in February'2017, May'2017, August'2017 and November'2017. The muscle tissues of *S. salpa* were extracted separately. The caulerpin levels in the muscle tissues of *S. salpa* were given in Table 3.11. Based on the results obtained from the preliminary tests, the liver and brain tissues of *S. salpa* were extracted in two groups of contain 5 individuals each, because of the caulerpin levels in the single individual's liver and brain tissues were below the LOD values. The caulerpin levels in the liver and brain tissues of *S. salpa* were given in Table 3.12 and Table 3.13.

Table 3.11 The caulerpin levels in the muscle tissues of *S. salpa* that were collected in February'2017, May'2017, August'2017 and November'2017. The caulerpin levels were calculated as µg caulerpin in per gram wet tissues

Fish Number/ Sampling Periods	Caulerpin (µg/g) FEBRUARY	MAY	AUGUST	NOVEMBER
1	0.04	0.011	0.285	0.317
2	0.10	0.011	0.380	nd
3	0.02	nd	0.075	0.031
4	nd	0.012	0.357	0.053
5	0.12	0.015	0.190	0.049
6	0.34	0.007	0.056	0.003
7	0.09	0.010	0.151	nd
8	0.04	0.003	0.147	0.043
9	0.07	0.007	0.257	nd
10	0.03	nd	0.119	0.054
Mean	0.085	0.007	0.202	0.055
Std. Dev.	0.097	0.005	0.113	0.095

(nd: not detectable)

Table 3.12: The caulerpin levels in the liver tissues of *S. salpa* that were collected in February'2017, May'2017, August'2017 and November'2017. The caulerpin levels were calculated as µg caulerpin in per gram wet tissues

Fish Number/ Sampling Periods	Caulerpin (µg/g) FEBRUARY	MAY	AUGUST	NOVEMBER
1st Group	0.420	3.092	0.619	5.379
2nd Group	11.28	1.838	0.881	0.149
Mean	5.850	2.465	0.750	2.764
Std. Dev.	7.679	0.887	0.185	3.698

Table 3.13: The caulerpin levels in the brain tissues of *S. salpa* that were collected in February'2017, May'2017, August'2017 and November'2017. The caulerpin levels were calculated as µg caulerpin in per gram wet tissues

Fish Number/ Sampling Periods	Caulerpin(µg/g) FEBRUARY	MAY	AUGUST	NOVEMBER
1st Group	0.12	0.066	0.291	0.500
2nd Group	0.21	0.196	0.228	0.471
Mean	0.165	0.131	0.26	0.485
Std. Dev.	0.064	0.092	0.045	0.021

A collapse on the *Caulerpa* population in February'2017 was occurred dependent on a decrease on seawater temperature and *Caulerpa* samples could not be observed. The caulerpin levels in *Caulerpa* spp. were given in Table 3.14. While caulerpin was determined in the level of mg caulerpin in per g dried tissue in *C. cylindracea*, it could not be determined in *C. prolifera.* This result is also compatible with MALDI-TOF /MS spectrums.

Table 3.14: The caulerpin levels in the *Caulerpa* spp. that were collected in February'2017, May'2017, August'2017 and November'2017. The caulerpin levels were calculated as mg caulerpin in per gram dried tissues

Sampling Periods	Caulerpin (mg/g) MAY	AUGUST	NOVEMBER
C. cylindracea	1.09 ± 0.01	11.82 ± 0.21	11.10 ± 0.15
C. prolifera	**nd**	**nd**	**nd**

(nd: not detectable)

The highest caulerpin levels were observed in August'2017.When the caulerpin levels were high in the end of summer and autumn, it decreased from the winter to spring. The RP-HPLC chromatograms of *S. salpa* and *C. cylindracea* were given in Figure 3.17, 3.18, 3.19 and 3.20. The chromatogram that was shown in Figure 3.17 with the number 1 belonged to 15 µg/mL caulerpin standard and the other chromatogram that is shown in Figure 3.17 with the number 2 belongs to 2nd group of *S. salpa* liver collected in February'2017. The caulerpin level in the relevant tissue is 11.28 µg/g wet tissues.

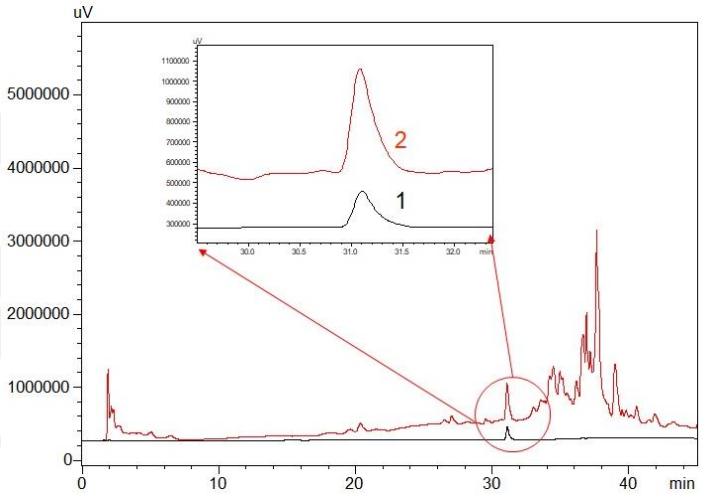


Figure 3.17 The RP-HPLC chromatogram of (1) the 15 µg/mL caulerpin standard and (2) the 2nd group liver extract of *S. salpa* collected inFebruary'2017, at 235 nm

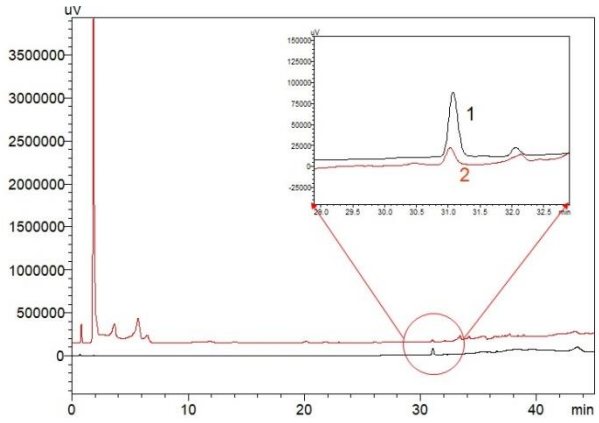


Figure 3.18 The RP-HPLC chromatogram of (1) the 5 µg/mL caulerpin standard and (2) the muscle extract of *S. salpa* collected inFebruary'2017 with number 6, at 235 nm

The RP-HPLC chromatogram of 5 µg/mL caulerpin standard was represented by number 1 and the RP-HPLC chromatogram of muscle extract of *S. salpa* collected in February'2017 was represented by number 2 in Figure 3.18. The caulerpin level in the relevant tissue is 0.34 µg/g wet tissues.

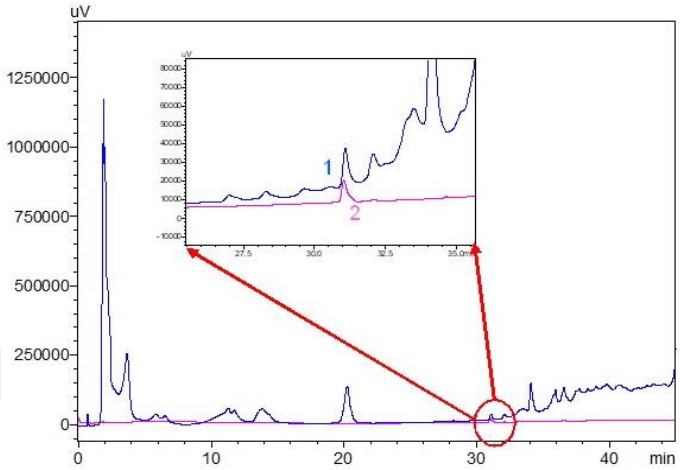


Figure 3.19 The RP-HPLC chromatogram of (1) the 2nd group brain extract of *S. salpa* that collected in February'2017 and (2) the 1 µg/mL caulerpin standard, at 235 nm

The chromatogram that was shown in Figure 3.19 with the number 1 was represented to the 2nd group brain extract of *S. salpa* collected in February'2017 and the other chromatogram that is shown in Figure 3.19 with the number 2 was represented to the 1 µg/mL caulerpin standard. The caulerpin level in the relevant tissue was 0.21 µg/g wet tissues.

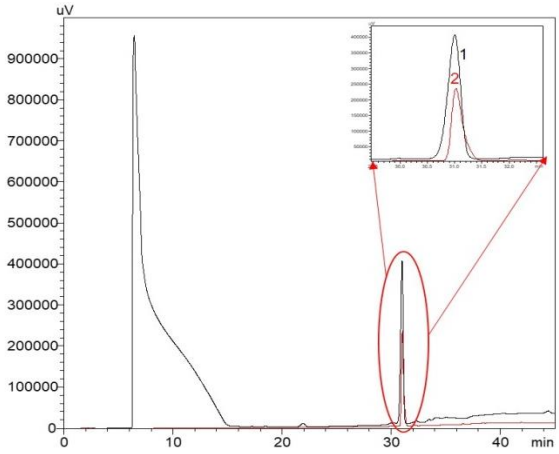


Figure 3.20 The RP-HPLC chromatogram of (1) *C. cylindracea* extract that collected in August'2017 and (2) the 15 µg/mL caulerpin standard, at 235 nm

The chromatogram that was shown in Figure 3.20 with the number 1 was indicated to the *C. cylindracea* extract collected in August'2017 and the other chromatogram that was shown in Figure 3.20 with the number 2 was indicated to the 15 µg/mL caulerpin standard. The caulerpin level in the relevant tissue is 11.82 µg/g dried tissues.

The areas of the peaks which were observed at the retention time of caulerpin were measured and these area values were used in calibration equation to calculate caulerpin level in relevant tissues. The UV-Vis spectrum technique which was explained in the material method section was applied to understand if the peak belonged to caulerpin in *S. salpa* and *Caulerpa* spp. tissues extracts.

3.5 Optimization of ANN

3.5.1 RP-HPLC Data

RP-HPLC optimization of caulerpin was modelled using neural network toolbox of MATLAB. The input parameters were selected as methanol % in the mobile phase, column temperature, flow rate, injection volume, and wavelength and caulerpin concentration. Each parameter has been studied in 7 levels. Rt and Rf values were recorded for each level. 111 data were obtained after experimental studies for both output parameters. 15 data were selected from this data set as sample input and output. The most effective input parameters on output parameter were methanol range in mobile phase and flow rate. The effects of the changes in input parameter on Rt values are given in the following figures.

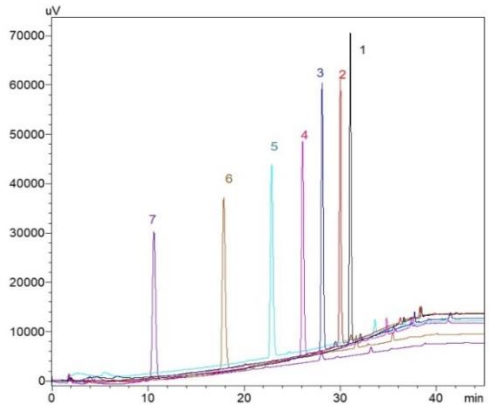


Figure 3.21 RP-HPLC chromatograms of 2.5 µg/mL caulerpin that obtained with the different initial methanol range in mobile phase: (1): 10% MeOH; (2): 20% MeOH; (3): 30% MeOH; (4): 40% MeOH; (5): 50% MeOH; (6): 60% MeOH; (17): 70% MeOH. The other conditions: T: 25 ºC, flow rate: 1.0 mL/minute, injection volume: 50 µL, λ: 235 nm

It was observed in Figure 3.21 that Rt values decreased depend on the increasing of initial methanol range in mobile phase. However, a decrease in the peak heights was observed.

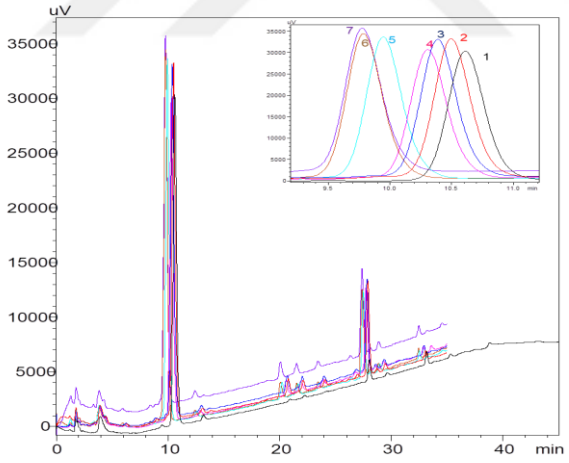


Figure 3.22 RP-HPLC chromatograms of 2.5 µg/mL caulerpin that obtained with different column temperature: (1): 25 ºC; (2): 27 ºC; (3): 30 ºC; (4): 32 ºC; (5): 35 ºC; (6): 37 ºC (7): 40 ºC. The other conditions: Methanol % in mobile phase: 70% MeOH, flow rate: 1.0 ml/minute, injection volume: 50 µL, λ: 235 nm

The RP-HPLC chromatogram about the effects of column temperature on Rt was given in Figure 3.22. Even though the Rt values decreased dependent on the increasing of column temperature. This was not a significant effect.

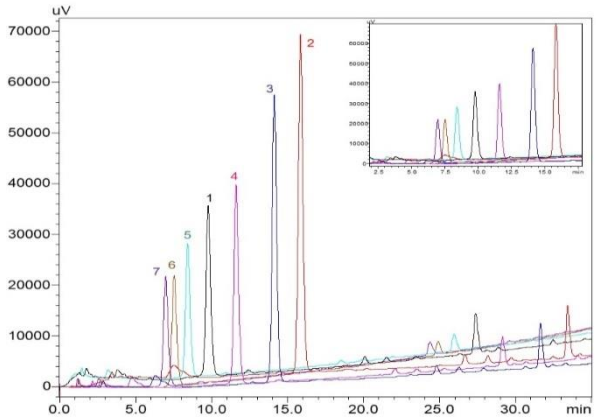


Figure 3.23 RP-HPLC chromatograms of 2.5 µg/mL caulerpin that obtained with different flow rate: (1): 1.0 mL/minute; (2): 0.5 mL/ minute; (3): 0.6 mL/ minute; (4): 0.8 mL/ minute; (5): 1.2 mL/ minute; (6): 1.4 mL/ minute (7): 1.5 mL/ minute. The other conditions: Methanol % in mobile phase: 70% MeOH, T: 40 ºC, injection volume: 50 µL, λ: 235 nm

It was observed in Figure 3.23 that Rt values decreased dependent on the increasing of flow rate. A decrease in the peak heights also was observed. The peak height significantly increased at 0.5 mL/minute flow rate.

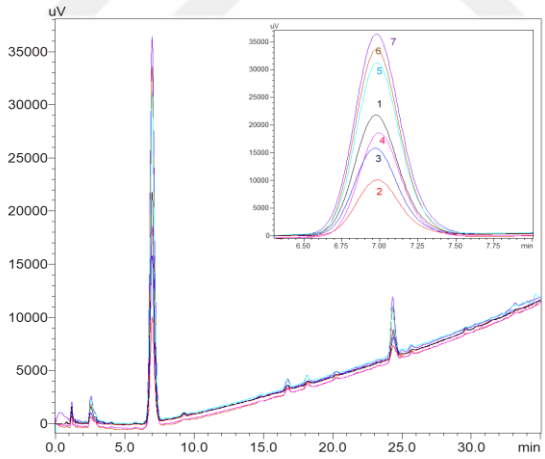


Figure 3.24 RP-HPLC chromatograms of 2.5 µg/mL caulerpin that obtained with different injection volume: (1): 50 µL; (2): 20 µL; (3): 35 µL; (4): 40 µL; (5): 70 µL; (6): 85 µL, (7): 100 µL. The other conditions: Methanol % in mobile phase: 70% MeOH, T: 40 ºC, flow rate: 1.5 mL/minute, λ: 235 nm

The RP-HPLC chromatogram that obtained with different injection volume was given in Figure 3.24. There was no significant effect on the Rt values.

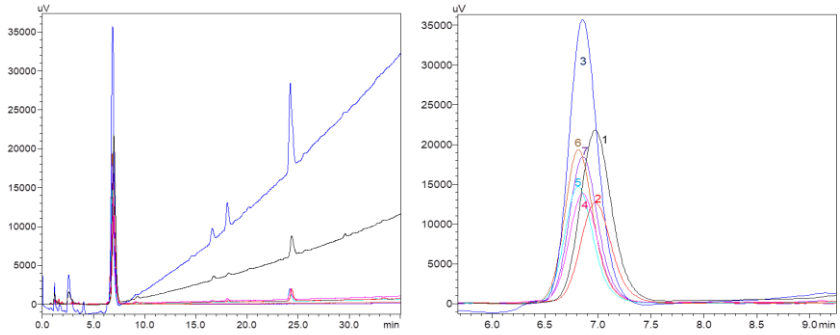


Figure 3.25 RP-HPLC chromatograms of 2.5 µg/mL caulerpin that obtained at different wavelengths: (1): 235 nm; (2): 280 nm; (3): 222 nm; (4): 270 nm; (5): 292 nm; (6): 317 nm, (7): 326 nm. The other conditions: Methanol % in mobile phase: 70% MeOH, T: 40 ºC, flow rate: 1.5 mL/minute, injection volume: 50 µL

The different wavelengths were also investigated as input parameters. It can be seen in Figure 3.25 that wavelengths had no effect on Rt values. It just affects the peak heights. Also, several interferences were observed during the analyses of fish tissues at 280 nm because of the proteins. That is why, all calculations were conducted based on the results at 235 nm.

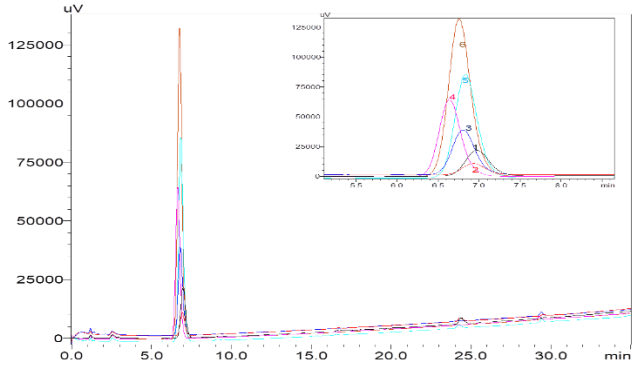


Figure 3.26 RP-HPLC chromatograms of caulerpin standard at different concentration levels: (1): 2.5 µg/mL; (2):1.0 µg/mL; (3): 5.0 µg/mL; (4): 7.5 µg/mL; (5): 10 µg/mL 292 nm; (6): 15.0 µg/mL. The other conditions: Methanol % in mobile phase: 70% MeOH, T: 40 ºC, flow rate: 1.5 mL/minute, injection volume: 50 µL, λ: 235 nm

The caulerpin standards were prepared at different concentration levels and analysed in RP-HPLC. The concentration levels had no effect on Rt values as it is

expected. In the optimization of ANN, some parameters known that have no effects on Rt should also be used to obtain well performed ANN model.

3.5.2 Optimization of Hidden Neuron Number

Firstly, the optimum neuron number was determined by changing the hidden neuron number between 1 and 20. The MSE and R^2 values were recorded. In this study, percentages of training, validation and testing were selected as 70-15-15 and Levenberg-Marquardt algorithm was used. The maximum R^2 and minimum MSE values were obtained when the hidden neuron number was 20.

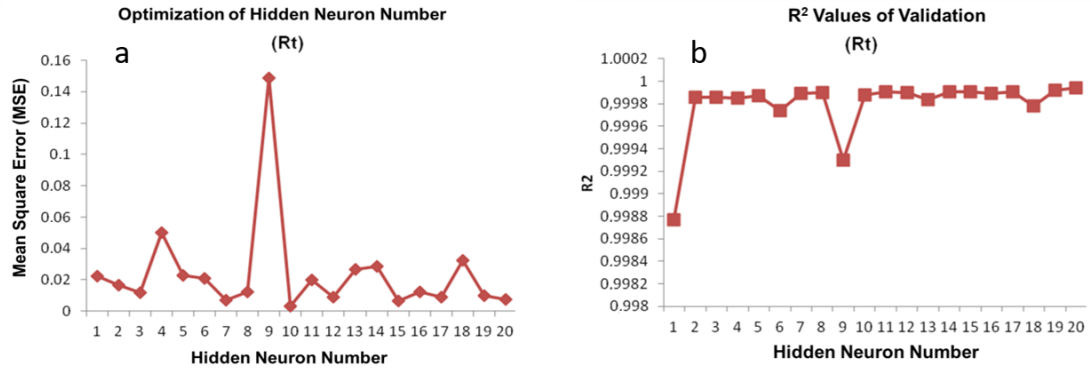


Figure 3.27 The graphics of the a) MSE and b) R^2 values obtained during the optimization of the hidden neuron number for Rt parameter

The graphics of the MSE and R^2 values obtained during the optimization of the hidden neuron number for Rt parameter were given in Figure 3.27a and Figure 3.27b. The optimum hidden neuron number is 20 which the case the minimum MSE and the maximum R^2 values were found as 0.0075933 and 0.99994 respectively. The all results about the obtained MSE and R^2 values during the optimization of the hidden neuron number were given in Table 3.15.

Table 3.15 The MSE and R^2 values obtained from different hidden neuron number

% 70-15-15	R^2				Mean Square Error (MSE)			Best Validation Performance		
Hidden Neuron Number	Training	Validation	Testing	All	Training	Validation	Testing	MSE	Iteration	Epoch
1	0.99869	0.99896	0.99896	0.99877	0.139912	0.022552	0.144121	0.022552	11	5
2	0.99986	0.99981	0.99994	0.99986	0.0134463	0.00165898	0.010519	0.01659	37	31
3	0.99989	0.99986	0.99942	0.99986	0.0116774	0.0165391	0.022904	0.0116539	23	17
4	0.99989	0.99949	0.99996	0.99985	0.00967749	0.0500359	0.00968097	0.050036	27	21
5	0.9999	0.99994	0.99951	0.99987	0.00839893	0.022845	0.0337876	0.022845	16	10
6	0.99968	0.99985	0.99988	0.99974	0.0293674	0.020763	0.0210115	0.020736	16	10
7	0.99991	0.99996	0.99968	0.99989	0.0102835	0.00698581	0.0289557	0.0069858	17	11
8	0.99993	0.99894	0.99985	0.9999	0.00838483	0.0121997	0.0118839	0.0122	19	13
9	0.99993	0.99611	0.99657	0.9993	0.00872565	0.148942	0.291272	0.14894	14	8
10	0.99991	0.99998	0.99967	0.99988	0.0107794	0.00318199	0.0299611	0.003182	11	5
11	0.99997	0.99981	0.99967	0.99991	0.00345548	0.0201393	0.026377	0.020139	11	5
12	0.99993	0.99989	0.99972	0.9999	0.00689271	0.00888446	0.0281458	0.008845	17	11
13	0.99992	0.99938	0.99947	0.99984	0.00970358	0.0264894	0.0405089	0.026489	14	8
14	0.99997	0.99919	0.99989	0.99991	0.0037221	0.0286064	0.0133963	0.028606	42	36
15	0.99994	0.99995	0.99982	0.99991	0.00610068	0.00669449	0.0327686	0.0066945	11	5
16	0.99994	0.9999	0.9994	0.99989	0.00723114	0.0124899	0.0342783	0.01249	12	6
17	0.99995	0.9998	0.99976	0.99991	0.00625659	0.0091592	0.020594	0.0091592	15	9
18	0.99981	0.99953	0.99815	0.99978	0.0310839	0.0323453	0.0151704	0.032345	11	5
19	0.99994	0.99994	0.99973	0.99992	0.00535445	0.0097741	0.0224179	0.0097741	13	7
20	0.99997	0.99992	0.99977	0.99994	0.00341269	0.00759329	0.0198297	0.0075933	28	22

3.5.3 Optimization of the Percentage of Training, Validation and Testing

Several range for the percentage of training, validation and testing data have been studied and the obtained the MSE and R^2 values have been recorded. The optimum hidden neuron and Levenberg-Marquardt algorithm have been used.

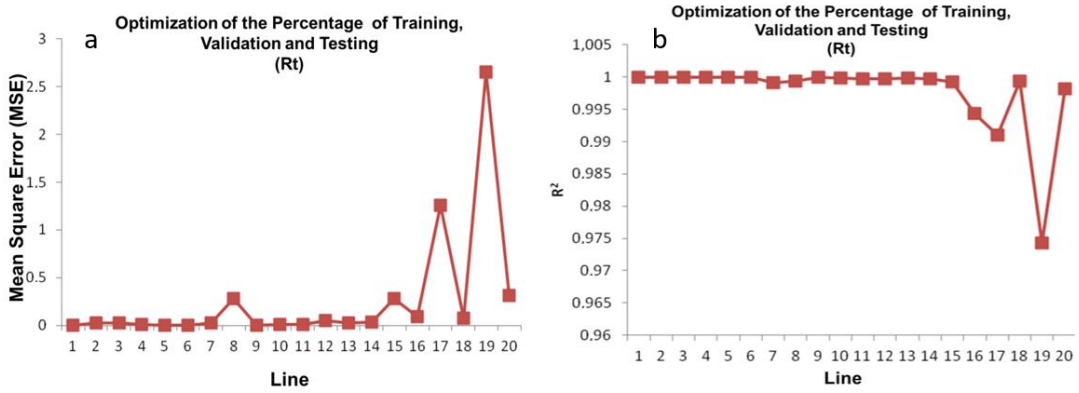


Figure 3.28 The graphics of the a) MSE and b) R^2 values obtained during the optimization of the percentage of training, validation and testing data for Rt parameter

The graphics of the MSE and R^2 values obtained during the optimization of the percentage of training, validation and testing data for Rt parameter were given in Figure 3.28a and Figure 28b. The optimum percentage of training, validation and testing data set 75-5-20, respectively in which the minimum MSE and the maximum R^2 values are found as 0.00071764 and 0.99994, respectively. The all results about the obtained MSE and R^2 values by changing the percentage of training, validation and testing data set were given in Table 3.16.

The sample input data set was investigated with using different algorithms and the estimated output data was recorded. The correlation also was investigated between estimated and experimental output data set. The highest correlation coefficients were obtained with Levenberg – Marquardt (trainlm) and Polak – Ribiére Conjugate Gradient Back propagation (traincgp) algorithms. The correlation coefficients were found as 0.9994 and 0.9949 respectively (Figure 3.29a and Figure 3.29b).

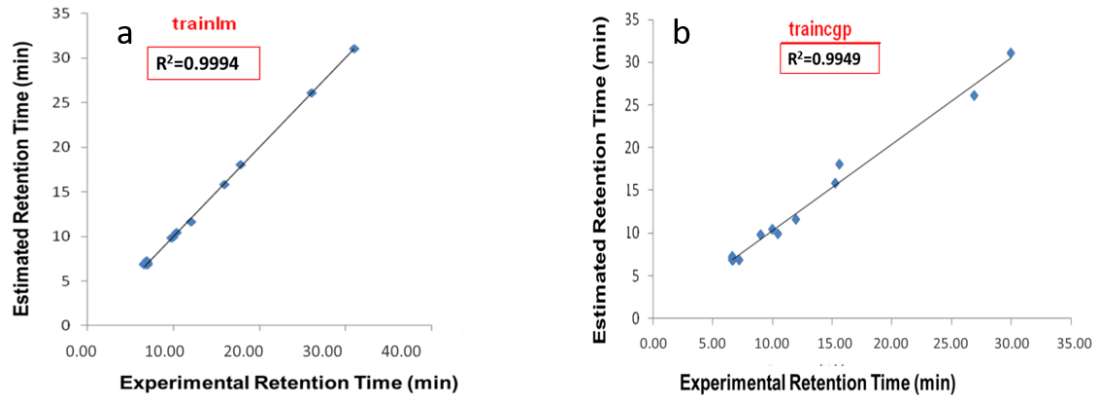


Figure 3.29 The graph of the correlation between experimental and estimated data by using a) trainlm (Levenberg – Marquardt Back propagation) and b) traincgp (Polak – Ribiére Conjugate Gradient Back propagation) algorithms

Table 3.16 The obtained MSE and R^2 values from optimization of the percentage of training, validation and testing data set

Hidden Neuron Number	Training %	Validation %	Testing %	R^2				MSE			Best Validation Performance		
				Training	Validation	Testing	All	Training	Validation	Testing	MSE	Iteration	Epoch
17	90	5	5	0.99993	0.99999	0.99995	0.99994	0.0066334	0.0017878	0.00426915	0.0017878	11	5
17	80	10	10	0.99995	0.99979	0.99994	0.99993	0.00439736	0.0265657	0.0133312	0.026566	32	26
17	80	5	15	0.99997	0.99494	0.99958	0.99993	0.00308194	0.0231863	0.0256645	0.023186	59	50
17	80	15	5	0.99994	0.99991	0.99958	0.99993	0.00623074	0.0114375	0.0096434	0.011438	12	6
17	75	10	15	0.99996	0.99999	0.99902	0.99994	0.00429093	0.00418189	0.0162504	0.0041819	62	56
17	75	5	20	0.99995	1	0.99991	0.99994	0.00441384	0.000717641	0.0156333	0.00071764	56	50
17	70	10	20	0.9999	0.99989	0.99653	0.99911	0.00905226	0.0262329	0.405924	0.026233	10	4
17	65	15	20	0.99981	0.99812	0.99735	0.99932	0.0354227	0.277132	0.121832	0.27713	10	4
17	60	20	20	0.99995	0.99998	0.99983	0.99992	0.00399829	0.00316511	0.0237239	0.0031651	22	16
17	60	15	25	0.99996	0.99986	0.99944	0.99989	0.00564075	0.0110319	0.0230907	0.011032	14	8
17	55	15	30	0.99995	0.99967	0.99939	0.9997	0.00418902	0.0129185	0.0870976	0.012919	31	25
17	50	25	25	0.99987	0.9996	0.99972	0.99974	0.0115265	0.0521231	0.0360527	0.052123	13	7
17	50	20	30	0.9996	0.99977	0.99988	0.99989	0.00436421	0.0249572	0.0123949	0.024957	12	6
17	50	15	35	0.99995	0.99971	0.99948	0.99971	0.00510127	0.034489	0.060548	0.034489	11	5
17	45	25	30	0.99996	0.9985	0.9998	0.99929	0.00384841	0.282613	0.029089	0.28261	7	7
17	40	30	30	0.9999	0.99942	0.98614	0.99441	0.00813382	0.094528	1.9319	0.094528	9	3
17	40	25	35	0.99998	0.99276	0.97441	0.99106	0.00205439	1.26033	1.95674	1.2603	8	5
17	35	35	30	0.99999	0.99945	0.99769	0.99942	0.00121228	0.0721034	0.110145	0.072103	16	10
17	30	35	35	0.9998	0.98071	0.90146	0.9743	0.228452	2.65482	4.30265	2.6548	9	3
17	35	30	35	1	0.99813	0.99712	0.99818	0.00084848	0.315665	0.343708	0.31567	6	5

3.5.4 Performance Test

The relevant figure for the performance test of the optimum hidden neuron number and the percentage of training, validation and testing data set were given in Figure 3.30 and Figure 3.31.

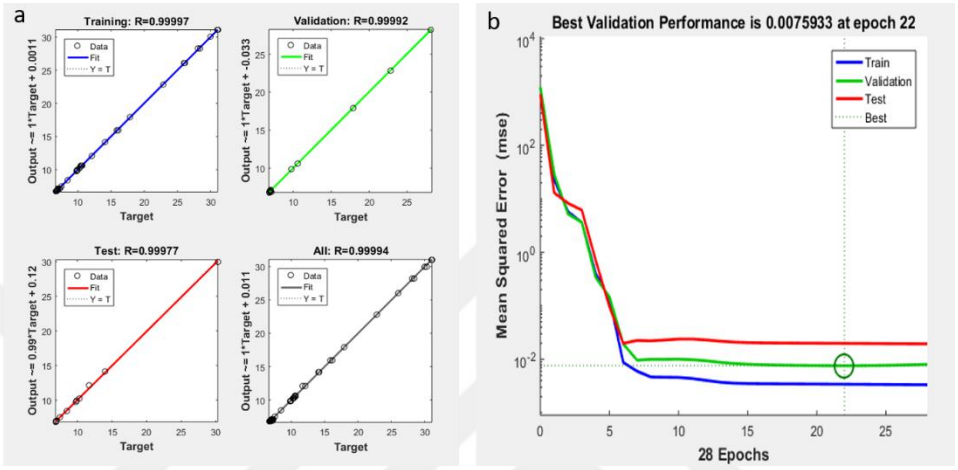


Figure 3.30 a) The regression and b) the performance graphs of the optimum hidden neuron number

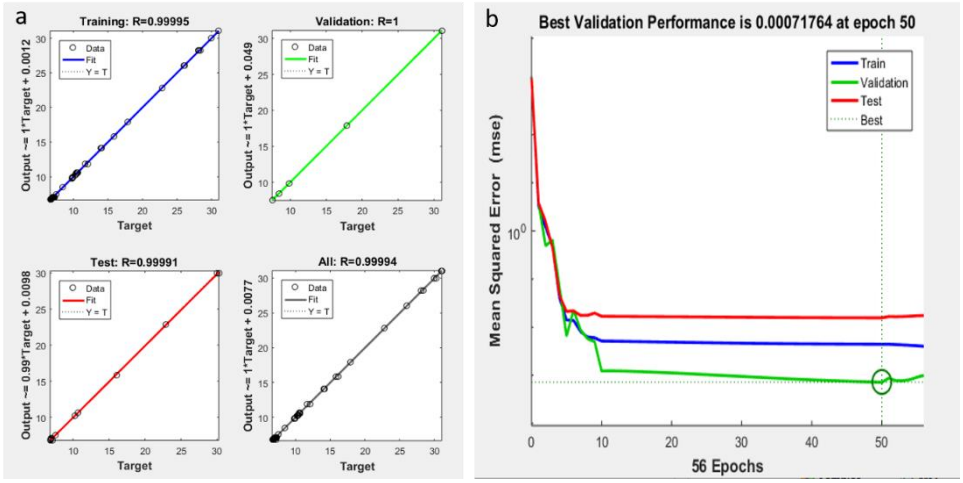


Figure 3.31 a) The regression and b) the performance graphs of the optimum percentage of training, validation and testing data

The all steps of the optimization of the RP-HPLC data of caulerpin by using ANN tool of MATLAB were also applied for the Rf output parameter. The optimum hidden neuron number and the percentage of training, validation and testing data set

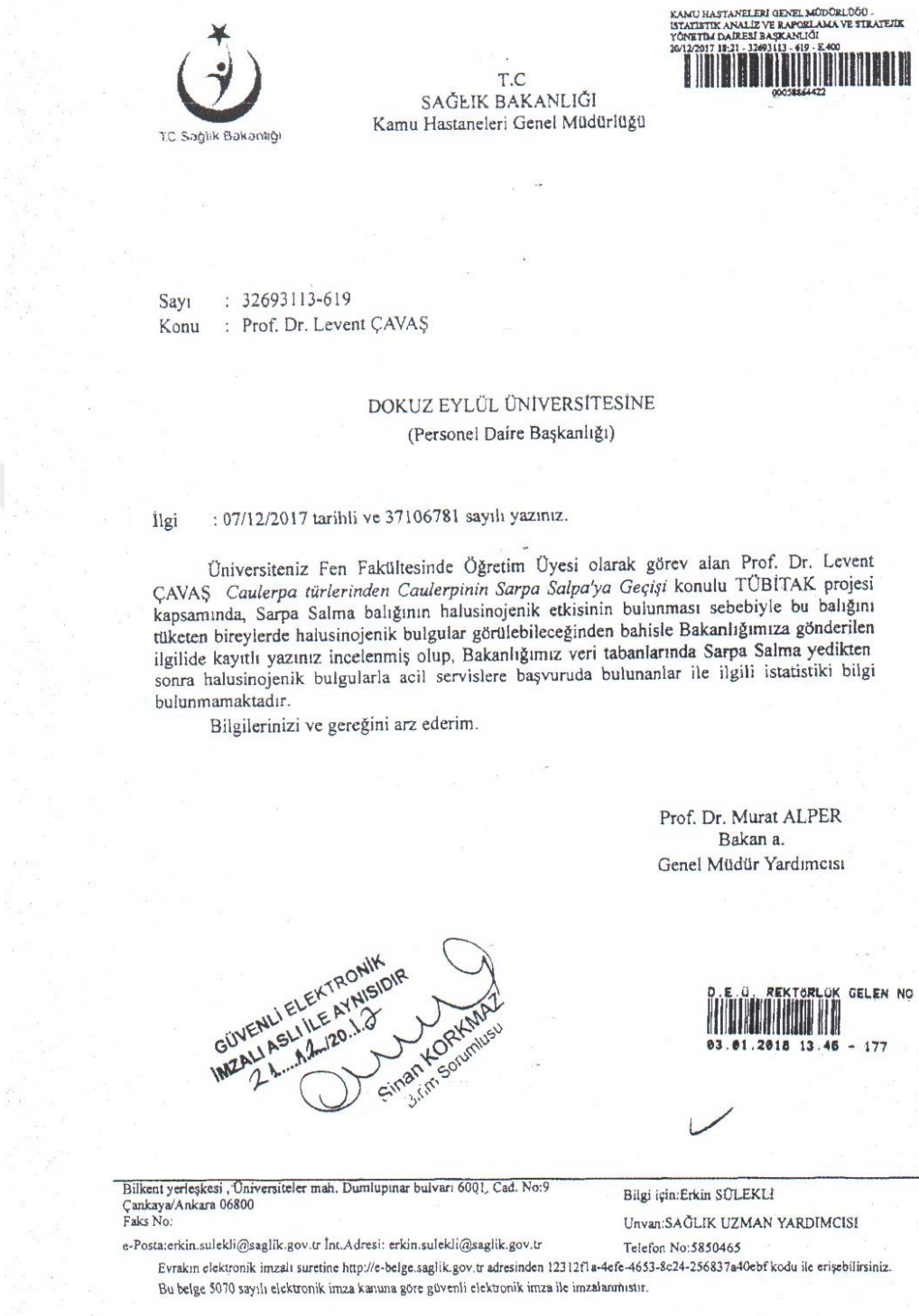
were found as 17 and 80-15-5, respectively. However, the estimated output data obtained with using different algorithms were not relevant with the experimental data set. The highest correlation coefficients were as 0.79 and 0.78. In the light of this information, it can be said that while Rt output data could be modelled well by ANN tool of MATLAB, but Rf output data could not.

CHAPTER FOUR

CONCLUSION

In this study, *C. cylindracea, C. prolifera,* and *S. salpa* samples were collected from Çeşme and Dikili regions in February'2017, May'2017, August'2017 and November'2017. The levels of caulerpin in *C. cylindracea, C. prolifera* and muscle, brain and liver tissues of *S. salpa* were investigated. Since there is no available authenticate standard for caulerpin, it was firstly isolated from *C. cylindracea* then characterized by ^{1}H-NMR, UV-VIS scanning, MALDI-TOF/MS and melting point analysis. The results of ^{1}H-NMR, UV-VIS scanning are compatible with the study conducted by Kamal and Sethuraman (2012). The peak at 397 (in the positive mod: 398) M^{+} that indicates caulerpin molecular weight was observed in MALDI spectrum. Also, the melting point of caulerpin was determined at 317 °C. Vidal (1984) found the melting point of caulerpin as 317 °C.

S. salpa is known as hallucinogenic fish because of its hallucinogenic and other negative effects on human health. The aim of this study is the determination of caulerpin levels in invasive *C. cylindracea* and non-invasive *C. prolifera* tissues and muscle, brain and liver tissues of *S. salpa* that consumes *Caulerpa* spp. *S. salpa* samples were collected from above the *Caulerpa* stations. The determination of caulerpin in *S. salpa* tissues is an indicator that trophic exposure to *Caulerpa* spp. In this study, it is aimed at establishing a relationship between consumption of *Caulerpa* spp. by *S. salpa*, accumulation of caulerpin in *S. salpa* tissues. In addition to that, we also investigated the relationship between caulerpin accumulation in *S. salpa* tissues and negative effects of *S. salpa* on human health. In this respect, it has been investigated that if there has been any application to emergency services because of hallucinogenic symptoms as a result of *S. salpa* consumption in Ministry of Health databases by contacting with the Ministry of Health, Public Hospitals, General Directorate. However, it was shared that there has been no statistical information about this subject in the report which of Public Hospitals, General Directorate (Figure 4.1).



T.C Sağlık Bakanlığı

T.C
SAĞLIK BAKANLIĞI
Kamu Hastaneleri Genel Müdürlüğü

KAMU HASTANELERİ GENEL MÜDÜRLÜĞÜ - İSTATİSTİK ANALİZ VE RAPORLAMA VE STRATEJİK YÖNETİM DAİRESİ BAŞKANLIĞI
20/12/2017 18:21 - 32693113 - 619 - E.400

Sayı : 32693113-619
Konu : Prof. Dr. Levent ÇAVAŞ

DOKUZ EYLÜL ÜNİVERSİTESİNE
(Personel Daire Başkanlığı)

İlgi : 07/12/2017 tarihli ve 37106781 sayılı yazınız.

Üniversiteniz Fen Fakültesinde Öğretim Üyesi olarak görev alan Prof. Dr. Levent ÇAVAŞ *Caulerpa türlerinden Caulerpinin Sarpa Salpa'ya Geçişi* konulu TÜBİTAK projesi kapsamında, Sarpa Salma balığının halusinojenik etkisinin bulunması sebebiyle bu balığını tüketen bireylerde halusinojenik bulgular görülebileceğinden bahisle Bakanlığımıza gönderilen ilgilide kayıtlı yazınız incelenmiş olup, Bakanlığımız veri tabanlarında Sarpa Salma yedikten sonra halusinojenik bulgularla acil servislere başvuruda bulunanlar ile ilgili istatistiki bilgi bulunmamaktadır.

Bilgilerinizi ve gereğini arz ederim.

Prof. Dr. Murat ALPER
Bakan a.
Genel Müdür Yardımcısı

GÜVENLİ ELEKTRONİK İMZALI ASLI İLE AYNISIDIR 21.12.2017
Şinan KORKMAZ
Birim Sorumlusu

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03.01.2018 13.46 - 177

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Figure 4.1 The report of Public Hospitals, General Directorate with the number 32693113-619 and dated 21 December 2017

The levels of caulerpin in *Caulerpa* samples and *S. salpa* tissues were determined by RP-HPLC analyses. Caulerpin was not detected in *C. prolifera*. It was observed that caulerpin levels in *C. cylindracea* and *S. salpa* tissues changed seasonally and caulerpin levels were higher in August'2017 compared to other seasons. The nutritional enrichment in coastal water affects spread and regrowth of *Caulerpa* spp. In the study conducted by Uya et al (2017), *C. cylindracea* exhibited two different phase, one fast-growing summer phase, and latent winter phase. It was indicated that nutritional content of sea water affects the growth of invasive *C. cylindracea* while indigenous species is not affected by this situation. The nutritional richness increases the regrowth while this does not affect biomass, the number, and length of fronds. The collapse of *C. cylindracea* and *C. prolifera* population were occurred during the winter because of decreasing of nutritional content. Caulerpin is a secondary metabolite specific to *Caulerpa* spp. It is an alkaloid that includes the bis-indole ring in its structure. Based on the literature, secondary metabolite production also could be changed seasonally, the highest levels were observed at the end of the summer through the beginning of autumn while the lowest levels were observed in winter season concurrently the vegetative activity (Piazzi & Balata 2008; Klein & Verlaque 2008). In this study, caulerpin was analysed in invasive *C. cylindracea* and indigenous *C. prolifera*. The presence of caulerpin was quantitatively and qualitatively detected in invasive *C. cylindracea*, while it was not determined in indigenous *C. prolifera*. In the light of these experimental results, it can be said that caulerpin contributes the invasion of *C. cylindracea*. It should be considered that caulerpin could be act like secondary pigment in photosynthesis mechanism. Moreover, it can play a role for scavenging of superoxide radicals that produce in consequence of a long time exposure to UV light caused by sun. Therefore, it could be considered that caulerpin contributes invasion of *Caulerpa* spp. to the new ecosystem by increasing the UV resistance. It is known that levels of caulerpenyne another metabolite of *Caulerpa* species is also changed depending on seawater temperature, depth, and herbivore activity (Cavas et al., 2012). The caulerpin levels in the *C. cylindracea* and *S. salpa* tissues differed seasonally due to changing in population and secondary metabolite production of *Caulerpa* spp. In this study, *C. cylindracea* and *C. prolifera* were not observed in February'2017. However, newly

growing *C. cylindracea* and *C. prolifera* samples were observed in May'2017. The population of *Caulerpa* spp. were reached to the saturation in August'2017. In November'2017, a collapse in *Caulerpa* population was observed. The length of fronds and branches was decreased. In parallel with these developments, the levels of caulerpin were high at the end of summer and the beginning of autumn, while it decreased from winter to spring.

The studies conducted by Haro and Pommier (2006) and Bellassoued, Van Pelt & Elfeki (2015) showed that *S. salpa* exhibited its toxic effects in the certain periods of the year. The aim of the study conducted by Bellassoued, Van Pelt & Elfeki (2015) was to investigate the cytotoxic effects of the compounds that accumulate in *S. salpa* tissues and not identified yet. Several studies showed that *S. salpa* consumes *Caulerpa* spp. In the report published by Bauchot (1987), it was indicated that toxic effects of *S. salpa* emerged after consumption of *Caulerpa* spp. by *S. salpa*. In other studies like this, there is much information about toxic effects of *S. salpa* on human based on its *Caulerpa*-based diet (Meinesz, 1991; Ruitton et al., 2006; Haro & Pommier, 2006; Dinesh et al., 2017; Felline et al., 2012). On the other hand, even if it is accepted that these toxic effects are caused from *Caulerpa* spp, it has not been fully understood. It is only known that caulerpin is accumulated in *S. salpa* tissues as a result of *Caulerpa* consumption.

The secondary metabolite production is changed seasonally in *Caulerpa* spp. in parallel with vegetative activity. Thereby, the levels of caulerpin in *S. salpa* tissue is changed based on season and the amount of *Caulerpa* that consumed. Additionally, health problems based on *S. salpa* consumption are peaked in certain periods (Bellassoued, Van Pelt & Elfeki, 2015). So, the accumulated caulerpin is *S. salpa* tissues can be transferred to human body by nutrition. In the light of this information, it could be said that the reason of the negative effects of *S. salpa* on human is the caulerpin accumulation in its tissues. The highest caulerpin level was observed in liver tissue of *S. salpa*. The liver is detoxification centre of organisms. Several important antioxidant enzymes are located in liver such as cytochrome P-450, glutathione-S-transferase, and acyl-CoA oxidase. The toxic compounds that are

produced during the chemical transfer of bioactive compounds such as hormones and toxin are transformed to the less toxic formation. Thus, these compounds can be eliminated from the body. This is why, while there is no or very small amount of toxic compounds are exist in another part of the body, this is different for the liver. It can be explained in this way that the level of caulerpin in liver tissue of *S. salpa* is higher than other tissues.

In the light of this information, it can be said that consumption of *S. salpa* can affect human health. However, further researches are necessary to understand the main reason for this problem. To understand if toxic effect caused by caulerpin that accumulated in *S. salpa*, a similar study can be applied to rats like the study conducted by Bellassoued, Van Pelt & Elfeki (2015). In that study, liver, muscle, brain and viscera extracts of *S. salpa* were injected to lab rat and then neurologic and vital changes were observed. A similar study can only be performed using caulerpin solutions instead of *S. salpa* extracts. In this way, clear information can be obtained about neurotoxic effects of caulerpin. In addition to measuring caulerpin levels in *S. salpa* tissues, the activities of cytochrome P-450, glutathione-S-transferase, acyl-CoA oxidase and 7-ethoxysorufine-O-deethylase enzymes can be determined and these results can be correlated with caulerpin levels. Thereby, further information can be obtained if caulerpin is a stress factor in *S. salpa* mechanism and so can affect human health. As a result of this study, it is advisable not to be consumed *S. salpa* by the human in August and November, when caulerpin levels in *S. salpa* tissues are high. For this purpose, an information note was sent to TUBİTAK-ÇAYDAG and the Ministry of Health and the Ministry of Food, Agriculture, and Livestock (Appendix-1).

Several eradication studies have been applied because of the negative effects of invasive *Caulerpa* spp. on the marine ecosystem. Despite the 18 mounts regularly hand picking on *Caulerpa* meadows, *Caulerpa* could not be eradicated. There is no permanent and for large areas eradication technique for this species (Ceccherelli and Piazzi, 2005). In spite of there has been no eradication technique for *Caulerpa* spp., this biomass that presents in Turkish coastline is of great importance for

biotechnology. Caulerpin secondary metabolite specific to *Caulerpa* spp. has several important biological activities. Caulerpin is great white hope as a new generation and herbal medicine thanks so its biologic activities such as anti-cancer, anti-tuberculosis and anti-viral activities. Also, it's stimulating and regulating effects on plants growth was patented (Schwede, 1986; Cavas et al., 2010). Chemical fertilizers cause serious pollution in soil and these pollutants are reached to the all aquatic ecosystems via rainwater. In this respect, several studies are being conducted to generate biocompatible fertilizer. Caulerpin can be used as fertilizer and or additive in fertilizer with benefit from its stimulating and regulating effects on plant growth. Thus, invasion of *Caulerpa* spp. which causes serious problems in the aquatic ecosystem can be transformed into an economically valuable product.

S. salpa is known as unsavoury fish in the last years. The obtained results from GC analyses were indicated changes in *S. salpa*'s taste can be originated from consumption of *Caulerpa* spp. It was observed that percentage of palmitic (C16) and oleic (C18:1 and C18:2) acids are higher in sample 2 without caulerpin in its tissue. While the long chain docosanoic acid (C22) was not observed in sample 1, it was observed in 0.704 % ratio in sample 2. In the study conducted by Felline et al. (2014), a decreasing in polyunsaturated fatty acid production and an increasing saturated fatty acid production were observed depend on increasing caulerpin concentration in fish tissue. In this study, the obtained data as a result of the oil analyses correspond to this information in the literature. Fatty acid content sample 2 which does not contain caulerpin in its tissues is richer than the fatty acid content sample 1 containing high amounts of caulerpin in its tissues. In the light of this data, trophic exposure to *Caulerpa* species of fish could affect human health indirectly and negatively, because fishes are a very important food source for human health. In spite of healthy nourishment, especially the individuals in the age of development may not get polyunsaturated fatty acids which are very important for the development of intelligence sufficiently. This is why further researches should be applied to understand the negative effects of invasive species on ecosystem and precautions should be taken to prevent the spread of these species. The simplest precaution to prevent indirect effects of *C. cylindracea* on human health is not recommended that

the consumption of *S. salpa* in August and November which caulerpin accumulation is the highest level in *S. salpa* tissue.

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APPENDIX-1: Information note provided to TÜBİTAK-ÇAYDAG and Ministry of Health and Ministry of Food, Agriculture and Livestock

Project Number: 116Y359
Supporting Institution: The Scientific and Technological Research Council of Turkey (TÜBİTAK)
Project Name: Migration of caulerpin from *Caulerpa* spp to *Sarpa salpa*
Project Manager: Prof. Dr. Levent ÇAVAŞ, Dokuz Eylül University
Intern: Songül TURHAN, Dokuz Eylül University

(Songül TURHAN is a master student at Dokuz Eylül University, Institute of Science and Technology, Master of Biochemistry. Prof. Dr. Levent ÇAVAŞ is an academic member of Dokuz Eylül University, Institute of Science and Technology, Department of Chemistry.)

Invasive *Caulerpa* species (killer and terrorist algae) and their effects

A green algae genus *Caulerpa* contains a significant number of invasive species. Invasive *Caulerpa* species is a great threat for the Mediterranean ecosystem. *Caulerpa cylindracea* (Cavas et al. 2006; Cavas & Pohnert 2010), *Caulerpa taxifolia* (Turan et al. 2011) and *Caulerpa taxifolia* var. *distichophylla'dir* (Cevik et al. 2007) are the invasive *Caulerpa* taxons that spreaded in Turkey coastlines. *Sarpa salpa* is a fish species abundantly consumed in Turkey. It has been reported that *S. salpa* consumes *Caulerpa* spp. Also, it has been mentioned in several literatures that secondary metabolites particular to *Caulerpa* spp. were accumulated in *S. salpa* tissues (Terlizzi et al. 2011; Felline et al. 2012; Gorbi et al. 2014; Felline et al. 2017). As a result of *Caulerpa*-based diet, the nutritional value of *S. salpa* is decreased (Felline et al. 2014). Moreover, it has been reported that *S. salpa* has hallucinogenic effect on human (Haro & Pommier 2006). According to 2016 statistical data, 127 tones *S. salpa* have been caught in Turkey.

So what is the situation in our country?

Prof. Dr. Levent ÇAVAŞ from Dokuz Eylül University, Faculty of Science, Department of Chemistry and his team are focusing on the invasive *Caulerpa* species. They proposed a project titled “Migration of caulerpin from *Caulerpa* spp to *Sarpa salpa*" on 27.03.2016 in order to reveal the situation in our country in response to the striking results in the foreign articles on this subject. The project was accepted on 15.04.2017 by TÜBİTAK.

In this project, non-indigenous *C. cylindracea* and indigenous *C. prolifera* were observed in Çeşme during the scientific diving. Also, caulerpin was detected in the tissues of *S. salpa* collected from the same region by RP-HPLC analysis. These results reveal that *S. salpa* consumes *Caulerpa* species.

The hallucinogenic effects of *S. salpa* on humans have not yet been fully elucidated, but ın the related literature, it has been mentioned that *S. salpa* consumes *C. prolifera* (Chevaldonne 1990). During the scientific diving, it was that *C. prolifera* is abundant in Çeşme and an intense *S. salpa* population was observed on *C. prolifera* meadows (Figure 1).

Figure 1 *Caulerpa prolifera* spread and *S. salpa*, Çeşme Harbor

Results

In this project, it aims that investigation of caulerpin levels in *Caulerpa* spp. and *S. salpa* tissues on February, May, August and November. 10 individuals *S. salpa* was collected from Çeşme region and liver, brain and muscle tissues were separated. Caulerpin was extracted from *S. salpa* tissues according to the Felline et al. (2012) and then the caulerpin levels were determined by RP-HPLC. Muscle tissues were separately extracted for each 10 individual, but liver and brain tissues were combined in groups of five, because of the low amount tissue. The RP-HPLC chromatograms of the liver, brain and muscle tissues of *S. salpa* collected on February are given in Figure 2, Figure 3 and Figure 4, respectively.

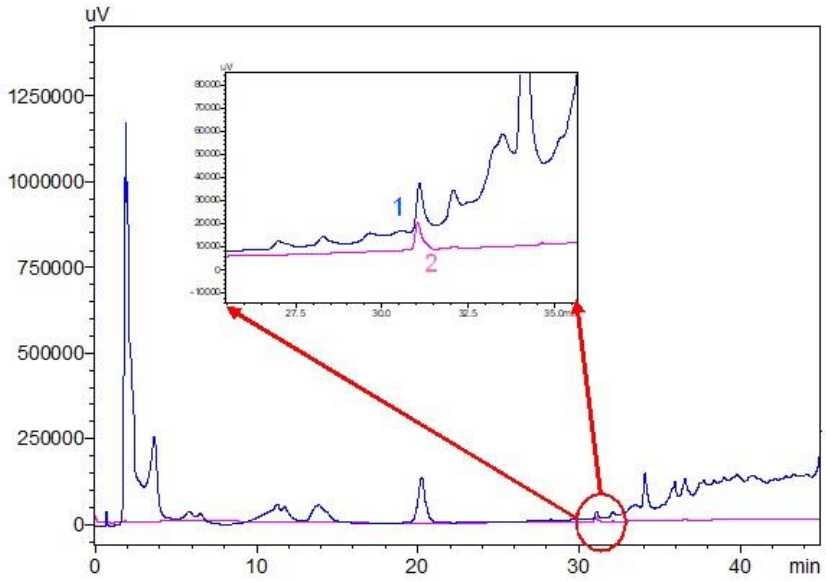

Figure 2 The RP-HPLC chromatogram of (1) the brain extract of *S. salpa* and (2) 1 µg/mL caulerpin, at 235nm

In Figure 2, the RP-HPLC chromatogram of the brain extract of *S. salpa* is shown with number 1 and the RP-HPLC chromatogram of the 1 µg/mL caulerpin is shown with number 2. The caulerpin level in the liver tissues is 0.21 µg/g wet tissues.

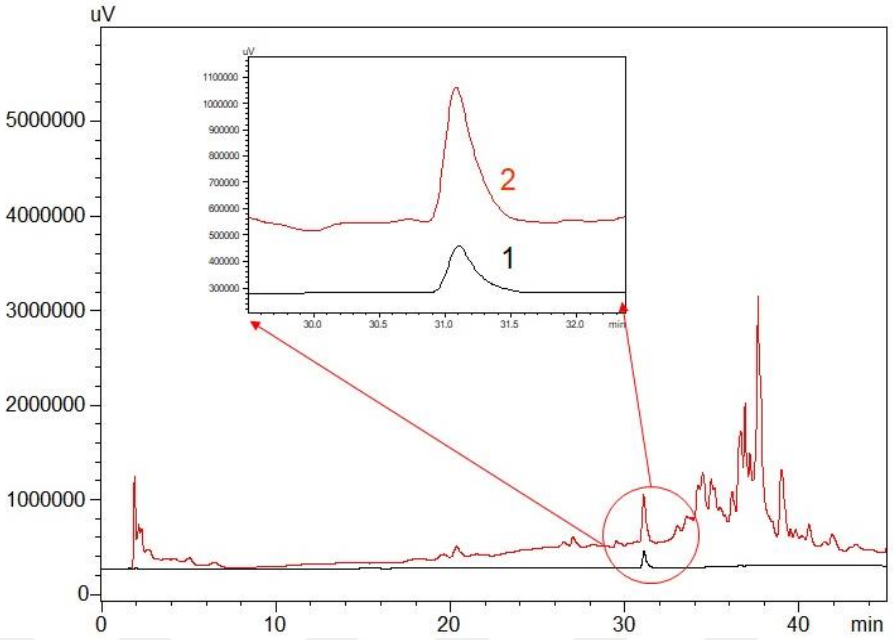

Figure 3 The RP-HPLC chromatogram of (1) 15 µg/mL caulerpin and (2) the liver extract of *S. salpa*, at 235nm

The RP-HPLC chromatogram of the liver extract of *S. salpa* is given in Figure 3 and the caulerpin level in this tissue is 11.28 µg/g wet tissues.

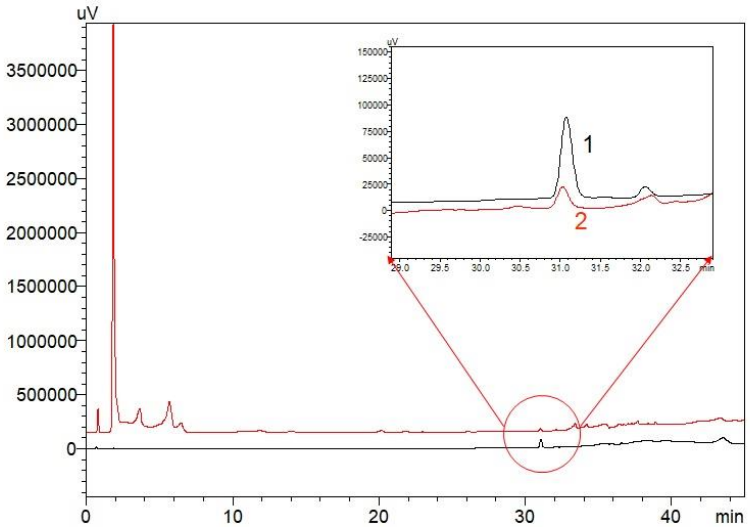

Figure 4 The RP-HPLC chromatogram of (1) 5 µg/mL caulerpin and (2) the muscle extract of *S. salpa*, at 235nm

The RP-HPLC chromatogram of the muscle extract of *S. salpa* is shown in Figure 4 with the number 2. The caulerpin level in the muscle tissue is 0.34 µg/g wet tissues.

The RP-HPLC chromatograms of the liver, brain and muscle tissues of *S. salpa* collected on August are given in Figure 5, Figure 6 and Figure 7 respectively.

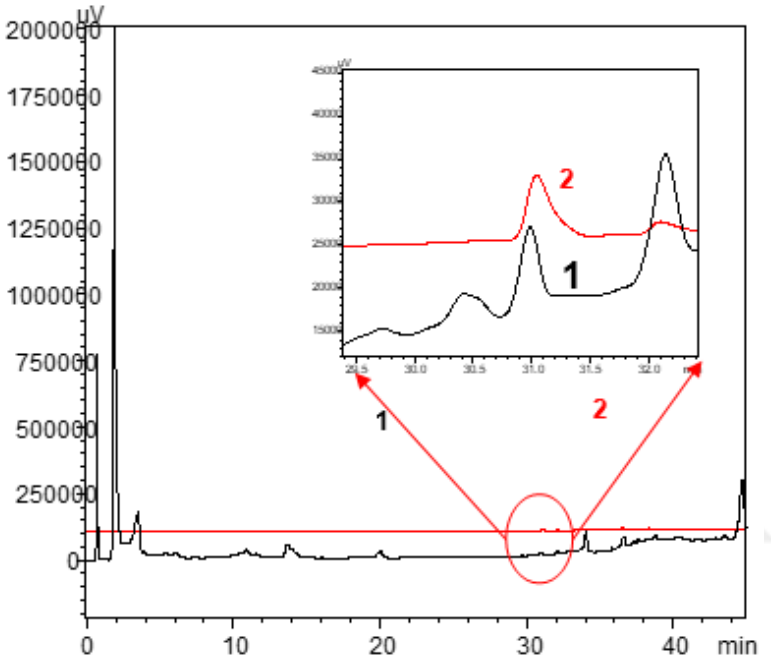

Figure 5 The RP-HPLC chromatogram of (1) the brain extract of *S. salpa* and (2) 0.5 µg/mL caulerpin, at 235nm

The RP-HPLC chromatogram of the brain extract of *S. salpa* is given in Figure 5 with the number 1 and the caulerpin level in this tissue is 0.29 µg/g wet tissues.

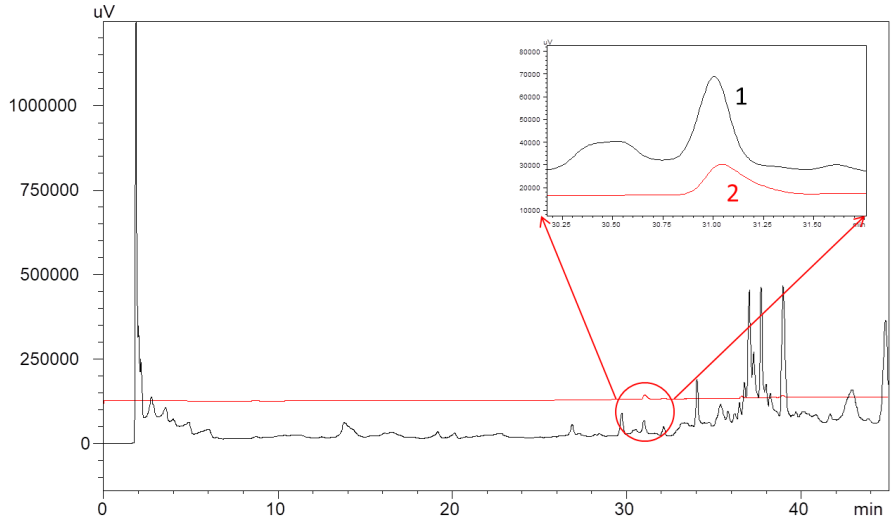

Figure 6 The RP-HPLC chromatogram of (1) the liver extract of *S. salpa* and (2) 1.0 µg/mL caulerpin, at 235nm

The RP-HPLC chromatogram of the liver extract of *S. salpa* is given in Figure 6 with the number 1. The caulerpin level in the liver tissues is 0.88 µg/g wet tissues.

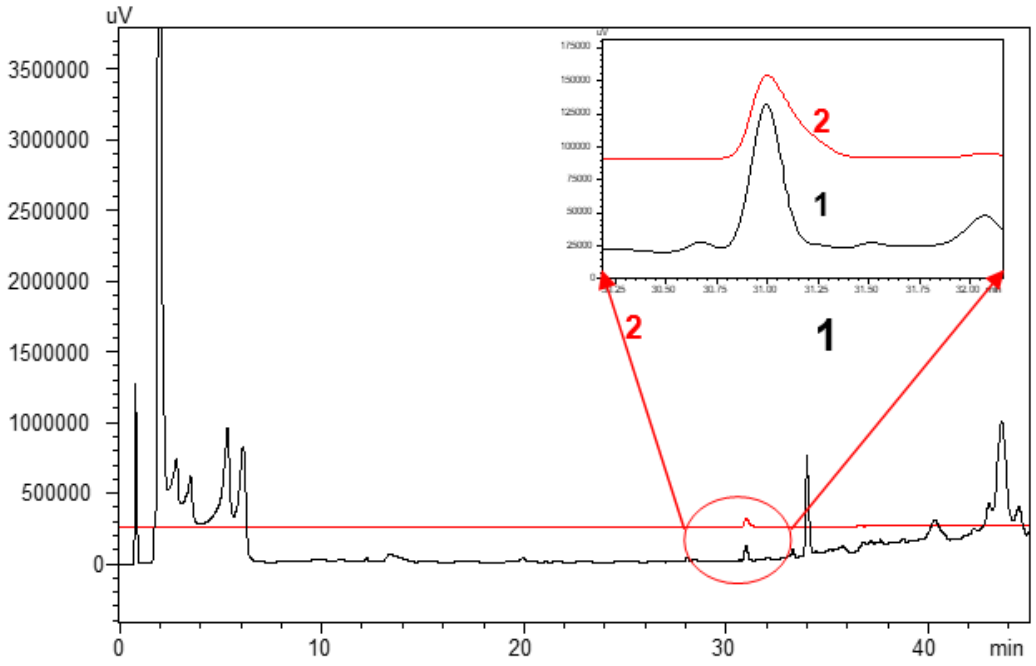

Figure 7 The RP-HPLC chromatogram of (1) the muscle extract of *S. salpa* and (2) 5 µg/mL caulerpin, at 235nm

The RP-HPLC chromatogram of the muscle extract of *S. salpa* is given in Figure 7 with the number 1. The caulerpin level in the muscle tissue is 0.38 μg/g wet tissues.

It has been reported that *S. salpa* has negative effects on human health such as hallucination, tachycardia, insomnia (Haro & Pommier 2006). In another study, neurotoxicity, shrinkage in important organs such as brain, liver and kidney and oxidative stress were observed on lab rats that exposure to the brain, liver, muscle and viscera extracts of *S. salpa.* In the light of the literature, we determined the caulerpin in the liver, brain and muscle tissues of *S. salpa* collected on February, May and August, 2017 (The samples collected on November are still being investigated). The accumulation of caulerpin in *S. salpa* tissues is an evident of the trophic exposure to *C. cylindracea.* It has been shown that accumulation of caulerpin in fish tissues decreases the polyunsaturated fatty acids production (Felline et al., 2014).

In view of the fact that these results, it can be said that consumption of *S. salpa* causes negative health effects. At the very least, consumption of *S. salpa* may not be temporarily proposed to remove the public health hazard until the invasive *C. cylindracea* is completely eradicated. We thought that the findings were important and urgently wanted to communicate ÇAYDAG. We need the views on the strategies of sharing our findings with our public.

Sincerely,
Prof. Dr. Levent ÇAVAŞ
13.09.2017, İzmir