DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ELUCIDATION OF PLANT GROWTH PROMOTERS IN THE EXTRACT OF *CAULERPA RACEMOSA*

by
Ezgi Melis EKİCİ

December, 2012
İZMİR

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"ELUCIDATION OF PLANT GROWTH PROMOTERS IN THE EXTRACT OF *CAULERPA RACEMOSA*"** completed by **EZGİ MELİS EKİCİ** under supervision of **Assoc. 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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Ezgi Melis EKİCİ

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ABSTRACT

Caulerpa racemosa var. *cylindracea* (Sonder) Verlaque, Huisman, et. Boudouresque (Verlaque, Durand, Huisman, Boudouresque & Parco, 2003), is an algae species that has been reported as an invasive species. It has been invading many coastlines and destroying the habitat of hundreds of different species since 1991. The aim of the thesis is to investigate possible plant growth regulators in the extracts of *C. racemosa.* This species was collected from the coastlines of Dikili-Turkey. The samples were extracted with distilled water and ethyl acetate. The seeds of *Allium cepa* L., *Helianthus annuus* L., and *Portulaca olerace* L. were chosen for biological testing. The seeds of these species were treated with the extract of *C. racemosa*. The plant growth regulating effect of algal extracts was investigated by measuring root length, shoot length and root number, shoot number and germination percentage of the seeds. Growth experiments were carried out in 2 different ways; first by water soaking-extract treating and the second by extract soaking-water treating. After growth experiments, crude extract were separated to fractions with silica column chromatography. UPLS-ESI/MS spectrometric detections were carried out to indicate the presence of caulerpin in the extract. Afterwards structural elucidation was carried out with H^1-NMR spectrometry. In conclusion, since *C. racemosa* extract was found to stimulate the growth of the seeds, biomass of *C. racemosa* can be evaluated as natural growth promoter in organic agriculture.

Keywords: *Caulerpa racemosa* var *cylindracea*, plant growth stimulation

CAULERPA RACEMOSA EKSTRAKTINDAKİ BİTKİ BÜYÜME DÜZENLEYİCİLERİNİN AYDINLATILMASI

ÖZ

Caulerpa racemosa var. *cylindracea* (Sonder) Verlaque, Huisman, et. Boudouresque (Verlaque et al., 2003), yayılımcı karakterde olduğu rapor edilmiş bir makro alg türüdür. 1991 yılından beri yüzlerce türün yaşam alanlarını yok etmiş ve birçok kıyı kesiminde yayılımcı özellik göstermiştir. Bu tezin amacı *C. racemosa* ekstraktlarındaki olası bitki büyüme düzenleyicilerinin araştırılmasıdır. *C. racemosa* örnekleri Dikili-Türkiye sahillerinden toplanmıştır. Toplanan alg destile su ve etil asetat ile özütlenmiştir. Biyolojik testler için *Allium cepa* L., *Helianthus annuus* L., *Portulaca olerace* L. türleri kullanılmıştır. Türlerin tohumları *C. racemosa* özütü ile muamele edilmiştir. Özütlerin bitki büyümesine etkileri kök uzunluğu, gövde uzunluğu, kök sayısı, gövde sayısı ve tohumların çimlenme yüzdesinin ölçülmesi ile araştırılmıştır. Büyüme deneyleri 2 farklı yöntemle gerçekleştirilmiştir; birincisi suda bekletip özüt ile muamele, ikincisi ise özütte bekletip su ile muameledir. Büyüme deneylerinin ardından ham özüt silika kolon kromatografisi ile fraksiyonlara ayrılmıştır. Ardından özütteki caulerpin varlığının belirlenmesi için UPLS-ESI/MS ile spektrometrik tayinler gerçekleştirilmiştir. Sonrasında H^1-NMR spektrometrik yöntemiyle yapısal tayin gerçekleştirilmiştir. Sonuç olarak, *C. racemosa* özütleri çalışılan bitki tohumlarındaki büyümeyi uyardığından dolayı, *C. racemosa* biyokütlesi organik tarımda doğal büyüme tetikleyicisi olarak değerlendirilebilir.

Anahtar sözcükler: *Caulerpa racemosa* var *cylindracea*, bitki büyümesinin uyarılması

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CHAPTER ONE

INTRODUCTION

1.1 *Caulerpa racemosa* var. *cylindracea*

Caulerpa racemosa var. *cylindracea* (Sonder) Verlaque, Huisman, et. Boudouresque (Verlaque et al., 2003), is a species of green alga which is widely distributed either tropical or warm-temperate sea zones (Verlaque et al., 2003). Inside the borders of Mediterranean Sea, the species *C. racemosa* was first observed in Sousse Harbor, Tunisia (Hamel, 1926). Additionally, it was also reported that the species has not had any invasive potentiality in the Eastern Mediterranean Sea (Hamel, 1931; Mayhoub, 1976; Verlaque et al., 2003).

Figure1.1 *Caulerpa racemosa* (Image is retrieved from; Cengiz, Çavaş & Yurdakoc, 2008)

It was considered as a Lessepsian species, which means a migrant species from Red Sea to Mediterranean Sea, until 1990 (Por, 1978; Verlaque, 1994). Afterwards, in the year of 1991, Nizamuddin was observed a new unknown species of *C. racemosa* in Libya.

Then this unknown species has been reported in 13 Mediterranean countries. These mentioned countries were Albania, Algeria, Croatia, Cyprus, France, Greece, Italy, Libya, Malta, Monaco, Spain, Tunisia, and Turkey. Additionally, it was also observed in large islands which were Balearic Islands, Corsica, Crete, Cyprus, and Sicily, and it was also reported in the Canary Islands of Atlantic in 2003 (Verlaque et al., 2003; Verlaque et al., 2004).

After the genetic and morphological identifications, invasive form of *C. racemosa* was named as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman, et Boudouresque (Verlaque et al., 2003). In addition to this information, it was reported that *C. racemosa* var. *cylindracea* has more invasion capacity than *Caulerpa taxifolia* (Vahl) C. Agardh which is a very well published species in the Mediterranean Sea as "killer algae" (Meinesz et al., 2001; Verlaque et al., 2003).

C. racemosa var. *cylindracea* (here after *C. racemosa*) can cover the sheltered and unprotected areas and also can invade between the depth of 0-70m in any kinds of marine habitats (Argyrou, Demetropoulos, & Hadjichristophorou, 1999; Klein & Verlaque, 2008; Piazzi & Cinelli, 1999; Zuljevic, Antolic, & Onofri, 2003). Coral-reefs which are the most important oxygen supply of the seas are also under the threat of *C. racemosa* invasion (Piazzi, Balata & Cinelli, 2007; Klein & Verlaque, 2008). *C. racemosa* can cover almost 100% of other macroalgal species, and it easily invades many kinds of areas (Piazzi, Ceccherelli & Cinelli, 2001a; Balata, Piazzi & Cinelli, 2004). The species can change the structure and composition of the sea floor with its dominancy and also cause to reduction of species diversity between habitats (Piazzi & Balata, 2008).

There are several methods to eradicate the invasive *Caulerpa* species. Manual removing, physico-chemical methods, and biological control by sea slugs can be some examples for these eradication trials. The invasion of the species still continues in the Mediterranean Sea because of the strong competition abilities of the invasive species (Verlaque & Fritayre, 1994; Piazzi, Ceccherelli & Cinelli, 2001b). In addition, the invasion can not be possible to control by grazing because the *C.*

racemosa has some toxic secondary metabolites (Boudouresque, Lemée, Mari & Meinesz, 1996; Dumay, Pergent, Pergent-Martini & Amad, 2002). Also, it is a difficult task to control this invasion in limited areas such as in bays and harbors (Anderson, 2005; Bax et al., 2001; Kuris & Culver, 1999).

When the scientific literature is examined, many industrial evaluation methods have been proposed by many researchers such as; boron sorption from polluted areas, removal of malachite green, removal of methylene blue, antiprolifetative and apoptotic activity, in vitro anti-herpetic activity, antitumor activity etc. (Ant Bursali, Çavaş, Seki, Seyhan Bozkurt, & Yurdakoc, 2009; Bekci, Seki, & Çavaş, 2009; Çavaş, Baskin, Yurdakoc & Olgun, 2006; Cengiz & Çavaş, 2008; Ghosh et al., 2004; Ji, Shao, Zhang, Hong, & Xiong, 2008;). Since *C. racemosa* is a considerably important species depending on its secondary metabolites, it seems obviously a brilliant option to investigate its important metabolites instead of attempting to eradicate the species. The real aim of present MSc thesis was to utilize the huge algal biomass of *C. racemosa* and its metabolites in a beneficial way and all the experiments were carried out to reach this goals.

1.2 Caulerpin

C. racemosa has important chemical metabolites. One of the most important of these metabolites is caulerpin (CPN) which we were focused on.

Meissner was the first scientist who was suggested the term of “Alkaloid” in 1819. (Pelletier, 1970; Trier, 1931). According to Bentley, (1957) the description of an alkaloid is “One chemical structure which has N atoms in one ring-cycle” (Kasim, Aline & Ekrem, 2010; Boopathy & Kathiresan, 2010).

Caulerpin (Dimethyl 5,12-dihydroindolo[2',3':5,6]cycloocta[1,2-B]indole-6,13-dicarboxylate) is an alkaloid comes from a family of bisindole natural products. It is an algal pigment included in the indole group alkaloids which refers to alkaloids containing a benzopyrrole (derived from tryptophan) (Figure 1.2). Between the two

indole rings which are incorporated with the carbonyl group, it has an extra eight-member ring (da Matta et al., 2011). It can be isolated from various *Caulerpa* species, especially from *C. racemosa* (Anjaneyulu, Prakash & Mallavadhani, 1991).

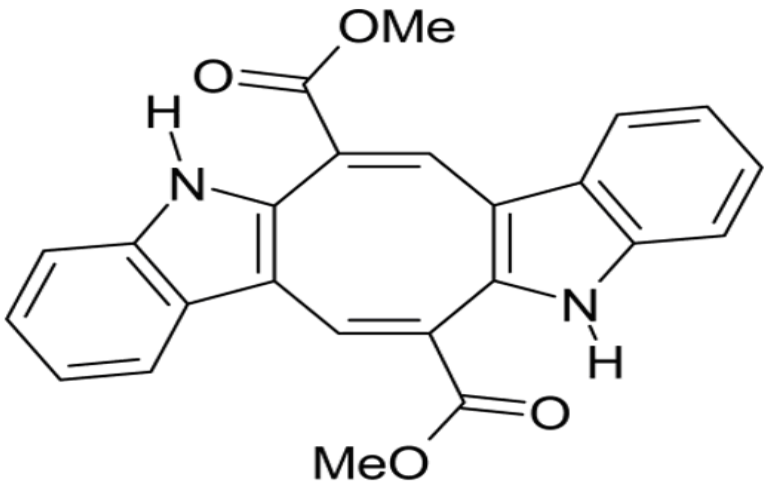


Figure 1.2 Chemical structure of caulerpin (Image is retrieved from de Souza et al., 2009).

In the literature there are several publications about important biological activities of this alkaloid such as antitumor activity (Ayyad & Badria, 1994), growth regulatory effects (Xu & Su, 1996), the plant root growth stimulant properties (Raub, Cardellina & Schwede, 1987), *in vivo* antinociceptive and anti-inflamatory activities (da Matta et al., 2011).

As it can easily be seen in the Figure 1.2, CPN has a quite similar chemical structure with the plant growth hormones auxins. The structure of CPN is almost like double indole-3-acetic acid form which seems widely remarkable similarity to investigate.

1.3 Auxin

Auxins (AUX) are the first discovered and most popular plant hormones which have been investigating for hundreds of years. Plant growth and development properties of AUXs have been shown in many researches for years (Hobbie, 1998).

AUXs belong to chemically diverse compounds and most of them have an aromatic system such as indole, phenyl or naphthalene ring with a side chain containing a carboxyl group attached (Andrzej & Alicja, 2007).

AUXs can effect the plant growth on a whole-plant level like; tropisms, apical dominance and root initiation. AUXs have also effect on cellular level of the plant, such as cell enlargement, division, and differentiation (Hagen & Guilfoyle, 1985). In addition to these properties, AUXs are included in many physiological and growth-related processes. Formation of patterns, elongation of cells, branching of roots and shoots are some examples for these processes (Benjamins & Scheres, 2008; Fukaki & Tasaka, 2009; Kepinski and Leyser, 2003; Kieffer et al., 2010; Kuhlemeier & Reinhardt, 2001; Tromas & Perrot-Rechenmann, 2010; Benjamins & Scheres, 2008; Fukaki & Tasaka, 2009; Kepinski & Leyser, 2003; Kieffer, Neve & Kepinski, 2010; Kuhlemeier & Reinhardt, 2001; Tromas & Perrot-Rechenmann, 2010; Vanneste & Friml, 2009).

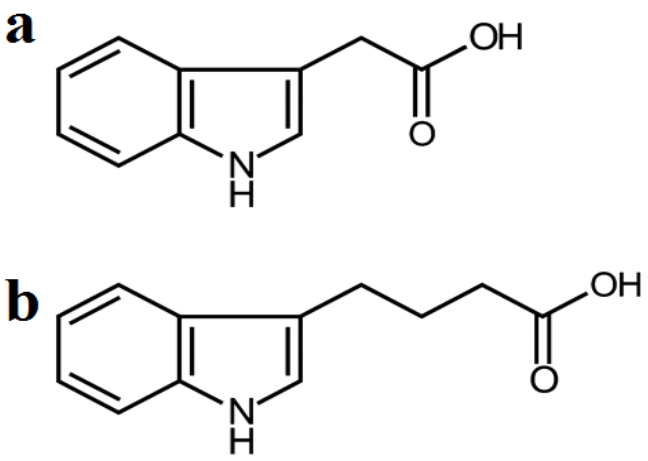


Figure 1.3 a. Structure of IAA b. Structure of IBA (Image is retrieved from; Strader & Bartel, 2011)

The natural AUX is called as indole-3-acetic acid (IAA). IAA occurs in all vascular and lower plants mostly (Cooke, Poli, Sztein & Cohen, 2002). In addition to the indolic AUXs, phenylacetic acid has also been reported as an active AUX in plants (Ludwig-Muller & Cohen, 2002). Some IAA precursors, like indole-3-

acetonitrile and indole-3-pyruvic acid, has also plant growth and development efficiency because they can easily be converted in the tissues to IAA (Cohen, Slovin & Hendrickson, 2003). Except for two methylene groups of it, indole-3-butyric acid (IBA) is also a plant hormone which is identical to IAA in their side chains which is efficient in the bio-assays (Bartel, LeClere, Magidin & Zolman, 2001; Dziczkowski & Soucek, 2010).

Because of its similarity with algal metabolite CPN and importance on the plant growth, AUX was one of the most important research parts of the experiment. It is obviously important to see these growth effects of either AUX or CPN in very early growth phase of the seed. Germination is clearly the best option to start to search for these effects on growth.

1.4 Seed Germination

Germination is the transformation of an embryonic plant inside the seed to a seedling. Seeds mostly go through the dormancy period. In this period, seeds have not any growing activity. During this period, seeds can safely move to a new location or survive in extreme climate conditions. Dormant seeds are the ripe seeds which do not germinate. These seeds wait until the right time for cell growth during the adverse external environmental conditions and prevent the initiation of metabolic processes of growth. When the seeds reach optimum conditions for cell growth, seeds begin to germinate. Then, the embryonic tissues resume their growth. Seeds start to develop towards a seedling. The most important period of the life cycle of a plant is the germination of the seeds. Germination has the central position for the life time of higher plants because seed formation determines the properties of next generations. Seeds have approximately 5–15% of water content depended on the humidity of environment. However seeds need water uptake for initiation of germination. In the presence of water, metabolic reactions can occur to accommodate for germination (Wang, Moller & Song, 2012).

Water uptake of a seed includes three phases. The rapid initial period is called “Phase I”. Afterwards, a plateau phase follows this phase. In in the Phase II; a small water uptake occurs. In this 2nd phase; seed becomes a living organism. Production of vital molecules like enzymes, hormones, proteins etc. initiates. Then, an over-flow of radicals start and the water content of the seed increases. In the last phase, the seedling starts to grow which is called as Phase “III” (Bewley, 1997; Perino & Come, 1991; Nonogaki, Chen & Bradford, 2007; Nonogaki, Bassel & Bewley, 2010).

One of the most important periods of the life time of a plant is germination (Thompson & Ooi, 2010). Each species has different and specific environmental requirements for the initiation of germination (Simons & Johnston, 2006). Temperature and light are important ecological factors that regulate seed germination of many plant species (Baskin & Baskin, 1998; Botha, Grobbelaar & Small, 1982; Jarvis & Moore, 2008). Also, in some species, low and high temperatures were reported to inhibit seed germination (Amri, 2010; Teketay, 1994).

Some seeds germinate equally well in light and darkness (Baskin & Baskin, 1998), while others germinate more readily either only under light (Baskin & Baskin, 1990) or darkness (Baskin & Baskin, 1990; Thanos, Georghiou & Skarou, 1989). In addition, light requirements for seed germination may vary with changes in temperature (Baskin & Baskin, 1998). Although many studies have been conducted on the effects of temperature and light on seed germination, temperature and light germination requirements are relatively unexplored in many plant species (Cony & Trione, 1996).

Germination is probably one of the most important phase of the plant growth. Therefore, our experiments were designed to observe the effects of the metabolites coming from *C. racemosa* on germination and experiments were carried out until the late stages of growth.

1.5 The Aims of the Thesis

C. racemosa is an invasive marine algae which still can not be eradicated or controlled with any kinds of eradication methods. *C. racemosa* threats many other marine species by covering and destroying their habitats. In this thesis it is aimed to evaluate this huge algal biomass of *C. racemosa* in a beneficial way such as using it the agriculture as an organic bio-fertilizer. To reach this goal, we investigated the plant growth stimulating effects of *C. racemosa* extracts and attempted to find out the metabolites which are responsible of this stimulation.

CHAPTER TWO

MATERIALS AND METHODS

Table 2.1 Abbreviations used in the text.

A. cepa	*Allium cepa* L.
AUX	Auxin
CPN	Caulerpin
C. racemosa	*Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman, et. Boudouresque (Verlaque et al., 2003)
CREE	*C. racemosa* ethyl acetate extract
CRWE	*C. racemosa* water extract
DEE	Diethyl ether
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
PE	Petroleum ether
P. oleracea	*Portulaca oleracea* L.
RL	Root length
RN	Root number
SL	Shoot length
SN	Shoot number

2.1 Statistical Tests

All the experiments were statistically tested with the Minitab 16.0 program. Different letters on the error bars of the graphics show statistical differences at $p<0.05$

2.2 Sterilization of the Seeds

All the seeds were sterilized previously with 10% of sodium hypochlorite for 15 minutes. Afterwards the seeds were washed with deionized water.

2.3 Growth Experiments

In order to observe the effect of *C. racemosa* on plant growth, growth experiments were carried out. Two different extract solutions were prepared from *C. racemosa* by using water and ethyl acetate.

2.3.1 Collection of Alga

To prepare the stock solutions for the further procedures, *C. racemosa* biomass was collected from Dikili, İzmir – Turkey from the depth between 30 and 60 cm. As soon as collecting the alga, the wet biomass was transported to laboratory immediately within the sea water. Then, the biomass was washed with tap water and afterwards distilled water to remove the salt and ephyphites. Then, seaweeds were laid on a filter paper to remove the excess water and then, separated into polyethylene bags and stored at -14 ºC until experiments.

2.3.2 Preparation of Water Extract (CRWE)

CRWE was prepared by using the procedure in the research of Caparkaya, Cavas & Kesercioglu, (2009) with 1 kg of alga. Initially, the biomass of *C. racemosa* was weighed and homogenized with mortar and pestle in 25 ºC until all the cellular material comes out. 1 L of deionized water was added to 1 kg of homogenized alga and mixed with magnetic stirrer about 100 rpm at 90 ºC for 1 h. Emulsion was cooled and filtrated with cheese cloth. Filtrate was stored at +4 ºC as stock solution for further experiments.

2.3.3 Growth Experiments of Allium cepa with CRWE

The growth experiments were started with *A. cepa*, because of its fast growth rate and relatively good adaptation to different growth conditions. Changes on root length, shoot length, root number and shoot numbers of plants were observed to explain the effects on the growth of *A. cepa.*

Starting from the stock CRWE; 5%, 15% and 20% solutions were prepared with distilled water. 15 mL tubes filled with the each concentrations of extract. One tube was prepared as control and filled with only distilled water. The experiments were carried out with 3 independent replicates. An *A. cepa* with the approximately height of 3.5 cm and the diameter of 2 cm was put on the top of each tube. Each *A. cepa* was fixed to the top of the tube from 2 cm down the sprig by using parafilm. As soon as the level of extract decreases, tubes were filled with the extract on their own concentration until 15 mL. The difference in root and shoot length of each *A. cepa* was determined at every 24 h at 25 ºC. All the samples were observed for 7 days.

2.3.3.1 Root and Shoot Length

All the *A. cepa* samples from each test tube were removed and roots were separated from the *A. cepa* bulbs. Then, root lengths were measured precisely. All the data obtained from root lengths were statistically evaluated and differentiations of the root lengths were shown in graphics by using the data from the root samples.

2.3.3.2 Root and Shoot Number

All the test tubes were prepared with the same method for length experiment. On the 7th day, *A. cepa* samples were removed. All the separated roots and shoots were counted for each *A. cepa*.

2.3.4 Growth Experiments of Portulaca oleracea with CRWE

P. oleracea is one of the very well known the Mediterranean species. It grows perfectly in the Mediterranean climate and has numerous benefits to human health such as analgesic and anti-inflammatory activities, anti-hypoxic action, bronchodilatory effect, type-2 diabetes mellitus treatment, antioxidant properties, etc. (Chan et al., 2000; Chen et al., 2009; El-Sayed, 2011; Lim & Quah, 2007; Malek, Boskabady, Borushaki, & Tohidi, 2004). On the other hand, it has a quite good tolerance to salinity in the growth conditions in contrast to *A. cepa* (Kilic, Kukul &

Anac, 2008). Due to its advantages, *P. oleracea* can be used as a perfect species which could directly be treated with the extract of *Caulerpa* biomass. By considering that, *P. oleracea* was chosen as a species which will be treated with the *C. racemosa* extract for the further experiments.

According to method of Sivasankari, Venkatesalu, Anantharaj & Chandrasekaran, (2006), healthy and uniform *P. oleracea* seeds with the diameter of approximately 850 µm and dark brown color were chosen. Seeds were divided into eight groups as it is shown in Figure 2.1. First four groups of the selected seeds were soaked in the different concentrations of CRWE and treated with distilled water. Additionally the second 4 groups were soaked in distilled water and treated with the extract.

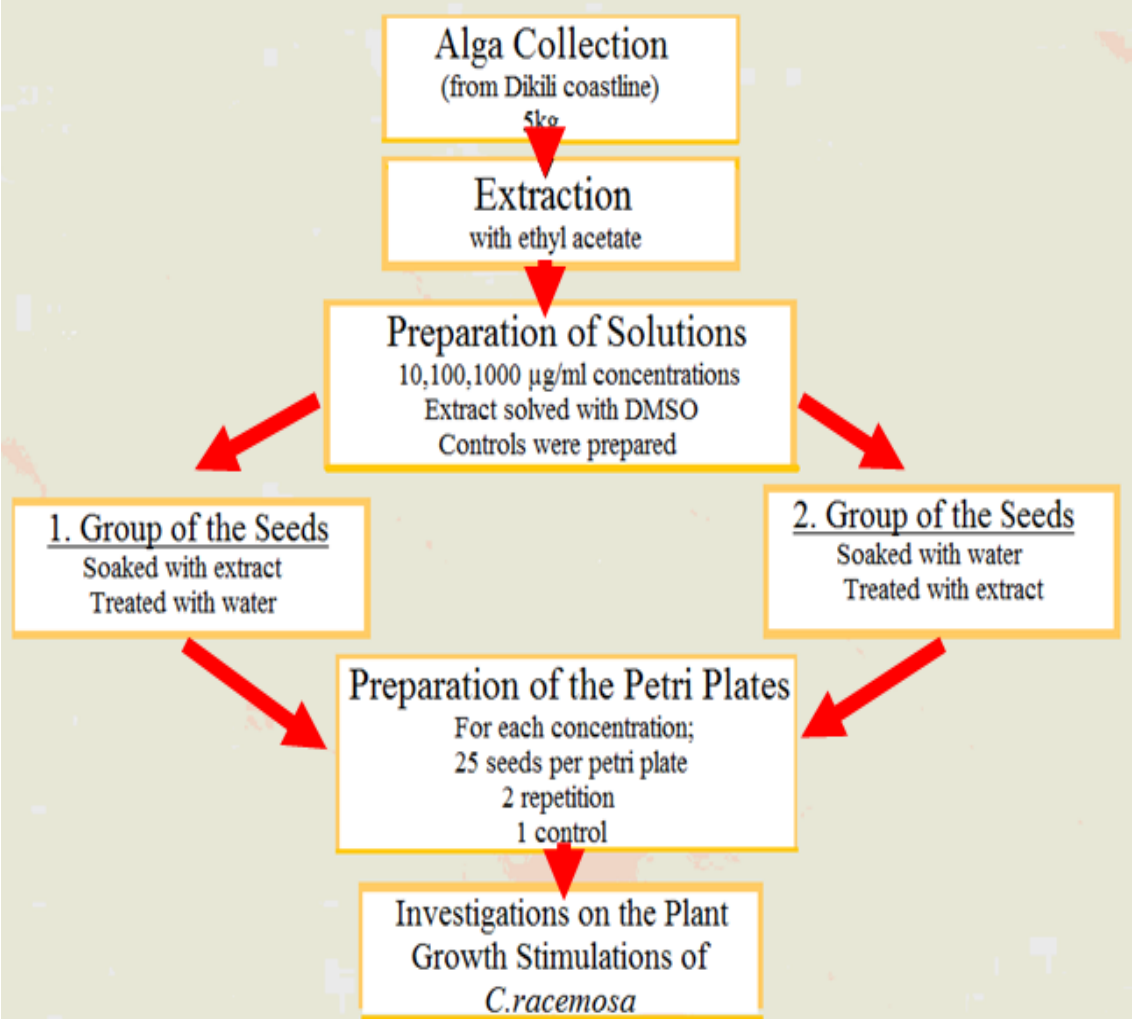


Figure 2.1 Experimental chart of *C. racemosa* extract bio-assays.

2.3.4.1 Extract Soaked P. oleracea Plants

First group of the seeds were sterilized with 10% of sodium hypochlorite and then, soaked with 2.5%, 5%, 10% and 20% of CRWE for 24 h at +4 ºC. Dilutions were carried out with distilled water. Afterwards, for each petri plate, 20 uniform seeds were chosen by their sizes, and then, aligned to form lines in each petri plate which has already 2 layers of moistened filter paper (Figure 2.2).

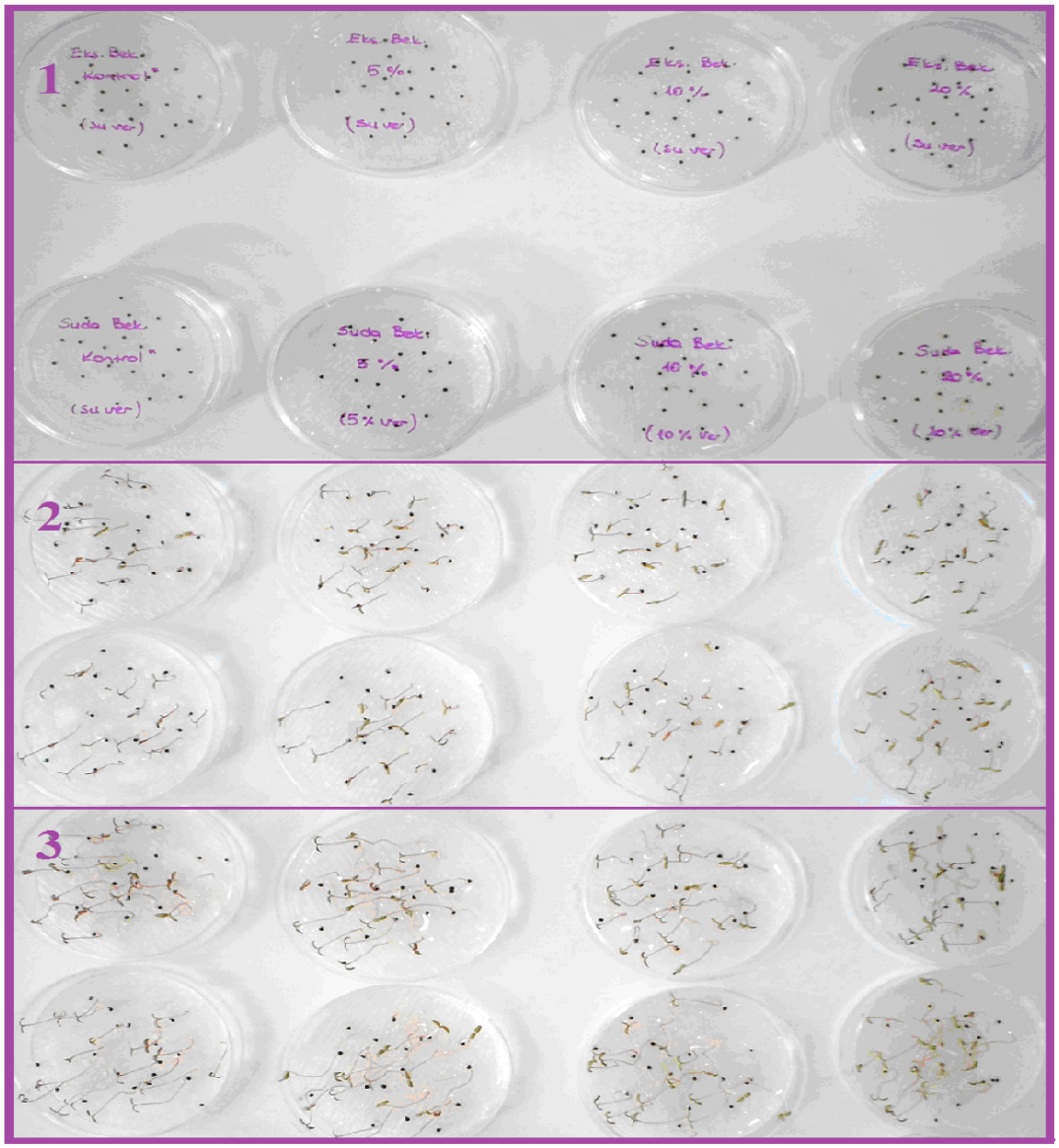


Figure 2.2 Images refer to 1st, 4th, 8th days of *C. racemosa* extract treatment of *P. oleracea* from up to down. first row demonstrates the experiment group. 2nd raw refers to control group. The concentration of the extract increases from left to right.

All petri plates in the experiment were treated with 1 mL of tap water at every 24th hour regularly. For water control, one of the petri plates was soaked with distilled water and treated with 1 mL of distilled water at each 24th hour as well. Plants were let to grow in stable humidity in 28 ºC. 5 days later from the beginning of the experiment, grown plants in every petri plate were harvested. Root and shoot lengths of plants were measured.

2.3.4.2 Water Soaked P. oleracea Plants

P. oleracea seeds with the diameter of approximately 850 µm and dark brown color were chosen carefully. Selected seeds were soaked in water for 24 hours in +4 ºC. Bottom of each petri plate was filled with 2 layers of watered filter paper and 20 uniform *P. oleracea* seeds were laid in lines. One plate was chosen as control and watered with 1 mL of tap water regularly. Other 3 plates were treated with 1 mL of 2.5%, 5%, 10% and 20% of *C. racemosa* extract. Seeds were let to grow for 5 days in +28 ºC.

Growth in each petri plate was checked at every 24th hour. After 5th day of the experiment, plants were harvested and shoot and root lengths of plants were measured. Results were evaluated statistically and shown in the graphics.

2.3.5 Growth Experiments with Indole-3-butyric acid (IBA)

As a positive control for extract; same procedure of Sivasankari et al. (2006) was repeated with IBA. Uniform *P. oleracea* seeds were chosen in same sizes and weights by using binocular.

5, 50 and 100 µg/ml of IBA were prepared. IBA was solved before with dimethyl sulfoxide (DMSO). Final concentrations of DMSO were 1:4000, 1:400, and 1:40 (v:v) respectively. Solutions were filled with distilled water. Soaking experiments with *C. racemosa* extract were carried out again in the same growth conditions and

as positive control, procedure repeated with IBA. Differences between the growths of plants were determined.

2.3.5.1 IBA Soaked P. oleracea Plants

Petri plates were prepared with the same procedure with growth experiments of *C. racemosa* extract. Seeds were chosen identically and soaked in 5, 50 and 100µg/ml of IBA for 24 hour in +4 ºC. Afterwards, soaked seeds were transferred to petri plates which include 2 layers of filter paper. The seeds were treated with 1 mL of distilled water every day and let to grow for 5 days.

At the end of 5th day, root and shoot lengths of plants measured and compared with the values from the plants which were grown in same procedure with *C. racemosa* extract.

2.3.5.2 IBA Treated P. oleracea Plants

In order to observe the effect of IBA on the growth of the water soaked *P. oleracea* seeds, petri plates were prepared with the same procedure of Sivasankari et al. (2006) like the previous growth experiments. 5, 50 and 100 µg/ml of IBA was prepared. Identically selected seeds were soaked with distilled water for 24 hour at +4 ºC. Then, prepared IBA solutions were applied on the water soaked seeds and let them grow in 25 ºC for 5 days.

At the end of 5th days, the root and shoot lengths of plants were measured and compared with the values from the plants which were grown with CREE by the same procedure.

2.4 Germination Experiments

In order to determine the growth stimulating effect of *C. racemosa* ethyl acetate extract (CREE) on earlier phases of plant, germination experiments were planned for

P. oleracea seeds. *H. annus* is a typical Mediterranean and very well produced species like *P.oleracea.* As the control species, all the experiments were carried out with additional *H. annuus*. *H. annuus* seeds were selected with approximately height of 0.95 cm and the diameter of 0.3 cm. *P. oleracea* seeds were choosen with diameter of approximately 850 µm.

The efficiency of the CRWE was observed at low levels in the growth experiments. Therefore, *C. racemosa* biomass was extracted by using ethyl acetate as solvent. The aim of the chosen procedure was to obtain more hydrophobic compounds from *C. racemosa* biomass. Effects of CREE on germination of *P. oleracea* and *H. annuus* seeds were determined.

2.4.1 Preparation of Ethyl Acetate Extract (CREE)

The fresh *C. racemosa* biomass was transferred to laboratory immediately in sea water. Then alga was washed with first tap water in laboratory and rinsed with distilled water to remove the salt and ephyphites. Afterwards, 3 kg of the alga was dried with paper tissues and shock-freezed with liquid nitrogen.

The alga was grinded in the liquid nitrogen with blender. Freezed alga fragments were poured in the precooled (-25 °C) ethyl acetate and this suspension was stirred until it reached to 25 °C. For faster filtration, silica gel was added to one -third of the weight of the grinded algae and filtered with a Büchner funnel and the filter cake was extracted two times with ethyl acetate. Lastly, it was dried with $MgSO_4$ and the solvent was removed. Approximately, 10 ml of CREE was obtained.

2.4.2 Germination Experiments of H. annuus

2.4.2.1 Preparation of Solutions

First of all the solvent inside of the crude CREE was completely dried with rotary evaporation at 40 °C. Starting with the stock CREE; 10, 100 and 1000 µg/ml of

extract were prepared like the Figure 2.3. Crude extracts were solved first with 1:40 (v:v) of DMSO. Concentration of DMSO was optimized in order to the tolerance of the plants. Fresh solutions were prepared for each experiment with the same method in each day.

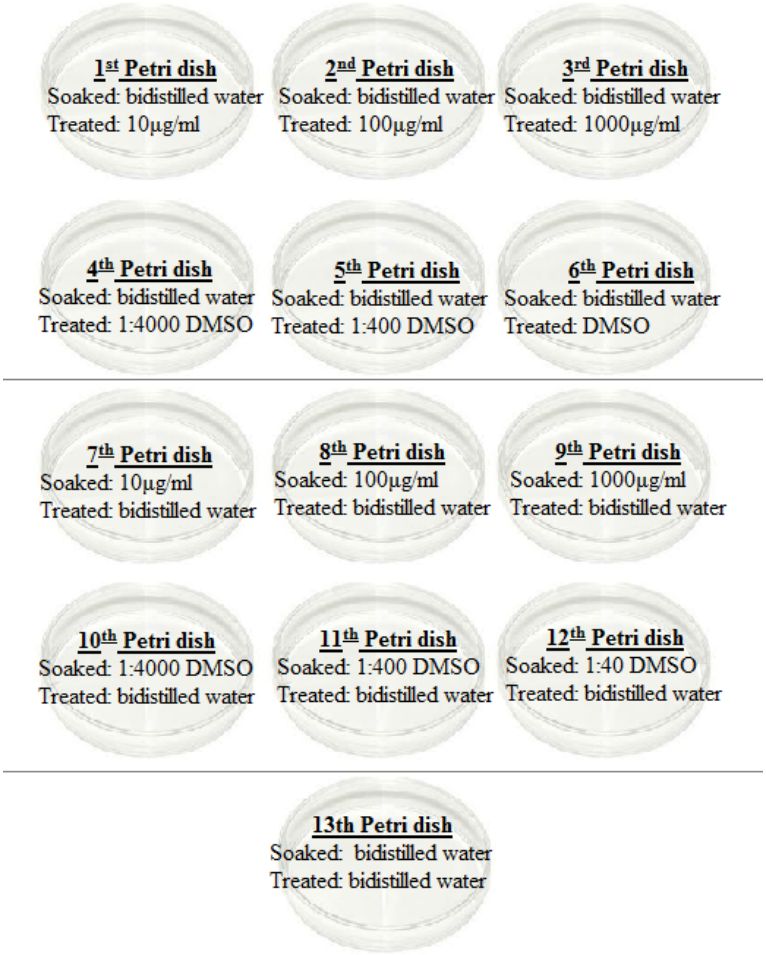


Figure 2.3 The petri plates for both species of *P. oleracea* and *H. annuus.* For the each group, 1st rows demonstrate the experiment groups and the second rows represent the DMSO control groups. 13th petri plate is the water control.

2.4.2.2 Germination of Extract Soaked H. annuus Seeds

20 uniform seeds of *H. annuus* with the approximately height of 0.95 cm and the diameter of 0.3 cm were selected by using binocular. Experiment was proceeded in 2 different procedures. Germination stages of *H. annuus* was shown in the Figure 2.4.

Figure 2.4 Germination of *H. annuus*. Pictures were taken respectively at the 30h, 35h and 40h after soaking of the seed.

As the first procedure; sterilized seeds were soaked with 3 different concentrations (1000, 100, and 10 µg/ml) of CREE for 24 hours at +4 ºC. Afterwards, seeds were laid on the bottom of petri dishes containing 2 sheets of Watman no. 1 filter paper moistened initially with distilled water. Seeds were germinated in stable humidity conditions in 28 ºC for 36 hours. Experiment groups were monitored for 36 hours and germination percentage (GP) was determined at every hour (Yeh, Hung & Huang, 2003).

2.4.2.3 Germination of Water Soaked H. annuus Seeds

Seeds were prepared with the same procedure with the previous germination experiment. Sterilized seeds were soaked with water and treated with 1000, 100, and 10 µg/ml of CREE for 24 hours at +4 ºC. Then, soaked seeds were laid on the filter paper and germination percentages was determined at 28 ºC for 36 hours.

2.4.3 Germination Experiments of P. oleracea

2.4.3.1 Preparation of Solutions

Solutions for the germination experiments of *P. oleracea* were prepared with the same method for *H. annuus*. 10, 100, and 1000 µg/ml of CREE were prepared with respectively 1:4000, 1:400 and 1:40 (v:v) of DMSO. Fresh solutions were prepared for each experiment with the same method in each day.

2.4.3.2 Germination of Extract Soaked P. oleracea Seeds

For each petri plate 20 unique *P. oleracea* seeds were selected. The seeds were chosen in similar weight and size by using binocular. Germination stages of *H. annuus* was shown in the Figure 2.5.

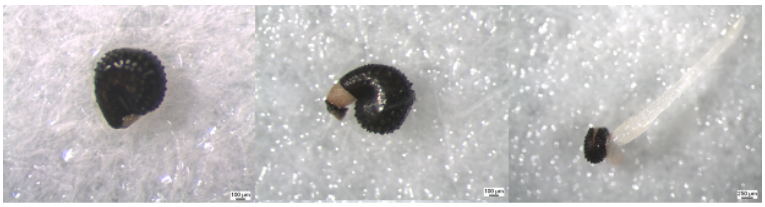


Figure 2.5 Germination of *P. oleracea*

Sterilized seeds were soaked in 10, 100 and 1000 µg/mL of CREE for 3 hours at +4 ºC. Then transferred to the petri plates and germination percentages were observed each hour.

2.4.3.3 Germination of Water Soaked P. oleracea Seeds

Seeds were prepared using the same procedure with the previous germination experiments. Seeds were soaked with distilled water for 3 hours. The seeds placed to the petri plates. Each petri plate was treated with 1 mL of the different concentrations (1000, 100, and 10 µg/ml) of CREE. GP of each petri plate was determined once in an hour and shown in the graphics.

2.5 Growth Experiments of the Fractions of CREE

Crude extract of *C. racemosa* includes quite large amounts of different chemical compounds from the biological material of the alga or residues from growth conditions. In order to eliminate the reducing effect of these components on the plant growth, the crude extract was separated to the fractions. Afterwards, the following chromatography methods were used to obtain the active fractions.

2.5.1 Thin Layer Chromatography (TLC)

In order to determine the fractions of *C. racemosa* crude extract first of all a TLC experiment were carried out. Silica TLC plates were used as stationary phase. Starting with 100% petroleum ether (PE) and 10% diluted with diethyl ether (DEE) mobile phase contain up to 100% DEE were prepared. All the visible bands on the plates were marked after separation. Then, TLC plates were put under UV light to see the separated UV visible compounds. In order to observe separated oxidizable compounds, TLC plates were put into Seebuch solution. Then, plates were heated by using drier until the new bands on TLC were appeared.

2.5.2 Column Chromatography

In order to separate the extract of *C. racemosa*, a column chromatography experiment was carried out.

2.5.2.1 Preparation of the Column

For stabilization of flow rate, a small piece of glass wool was placed at the bottom of the column. Then, the column filled with silica gel-100% petroleum ether mixtures. During the preparation, column was knocked continuously for uniform silica gel distribution. Top of the column was filled with approximately 10 cm^3 sea sand. Sea sand was previously washed with distilled water to remove the residues. *C. racemosa* crude extracts were applied to the column and the valve was opened to start the flow.

2.5.2.2 Separation

As it is shown in Figure 2.6, extraction was started with 100% PE. All the extraction solvents were prepared with 10% percent DEE dilutions. 1 L of each extraction solvents was passed through the column as a gradient elution with flow rate approximately 1 mL/min.

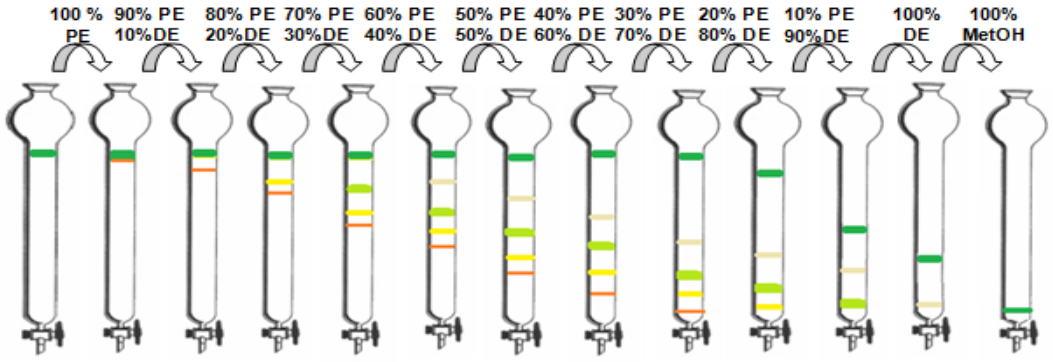


Figure 2.6 Fractionation of *C. racemosa* crude extract with silica column separation. DEE: Diethyl ether, PE: Petroleum ether, MetOH: Methanol. From left to right petroleum ether concentration decreases and diethyl ether concentration increases. Different color in the each color symbolizes a different compound.

Fractions which were determined with thin layer chromatography were collected into 15 mL test tubes. Afterwards, the content of all the tubes were determined with TLC plates by the same method before and tubes which has similar chemical content were pooled. Pooled fractions were evaporated by using rotary evaporator. At the end of the separation, 10 different fractions of the CREE were obtained.

2.5.3 LC-MS Measurements

2.5.3.1 UPLC- ESI/MS

All fractions of crude CREE were qualitatively determined for presence of CPN by using UPLC-ESI/MS. For all fractions, 0.33 mg/mL of fractions were prepared.

Waters Acquity Ultra Performance LC was used for the separation. The system includes the column of 50 mm Acquity UPLC BEH C_{18}. The C_{18} column has the size of 2.1mm, 1.7µm. The temperature of the column was stabilized at 30 °C. The liquid chromatography device an AcquityTM Ultra Performance LC (Waters, Milford, MA, USA) was used. The system has a C_{18} column with the brand of Acquity UPLC BEH. The system was connected to a Q-ToF Micro mass spectrometer (Waters Micromass, Manchester, England). It was run with an ESI (+) source. The measurements were

carried out with a scan rate of 1 scan s^{-1}, Interscan delay of 0.1 s, and a scan range from 100 to 1000 m/z.

1–5µl loop injector was used for sample injection. The temperature of the auto sampler was stabilized at 4ºC and the temperature of column was stabilized at 27ºC. All the mass evaluations were carried out in ESI(+) mode. It was recorded at the mass range between 100-1000 m/z. Scan rate was 0.6s and the inter-scan delay was 0.1s. And the following MS parameters were applied; capillary voltage 3000 V, sample cone 10.0V, source temperature 120 ºC, desolvation gas temperature 300 ºC, collision energy 5.0 V, collision gas argon, and ion energy 1.8 V (Spielmeyer & Pohnert, 2010, http://www.lcresources.com/training/training.html). The existence of CPN was observed in the *C. racemosa* fractions which had been obtained by using more polar solvent mixtures, at the m/z value of 399.

2.5.4 Coleoptile Cuttings Experiments with Isolated CPN

In order to investigate the plant growth stimulating effect of CPN, an bioassay experiment was developed with *P. oleracea* by using the coleoptile cuttings method of Nitsch & Nitsch (1954).

2.5.4.1 CPN isolation

The publication of Aguilar-Santos, (1970), was used for CPN isolation. The collected *C. racemosa* was dried on a paper tissue at 25 ºC. Algae were cut and powdered in a mill. 1.4 kg of the dried species was extracted with soxhlet by using 4 L of petroleum ether. The extract was concentrated to 300 mL and set aside to cool in vacuum evaporator. Red prisms of CPN molecules were separated.

2.5.4.2 The Effects of CPN on Plant Growth

The coleoptile sections were cut in 4 mm by following the method of Nitsch & Nitsch (1954), and put into the different concentrated solutions of the fractions.

Seeds were grown in a climate chamber 28ºC in the dark for 5 days. When the coleoptile reached about 2.5 cm in length, the coleoptiles which have equal lengths were selected. Then, they were cut in 4 mm sections, from 3 mm below of the tip. Primary leaf was left inside the sections.

These sections were floated for 3 hours in glass distilled water containing 1 mg/L of $MnSO_4.H_2O$. 10 sections were put in 0.5 mL of the isolated CPN solutions with the concentration of 0.01, 0.1, 1, and 10 µg/ml. CPN was solved with 1:40 (v:v) DMSO:Water (v:v) solution. All the CPN solutions were prepared containing pH 5.0 buffer (K_2HPO_4 1.794 g/L, and citric acid monohydrate 1.019 g/L) and 2% sucrose.

The sections were incubated about 20 hours in the dark at 25ºC in horizontal shaker to have a more uniform growth. Differences of the lengths were observed by taking pictures with binocular and measured with ImageJ 1.46 software.

2.5.5 Coleoptile Cuttings Experiments with the Fractionated CREE

After the detection of the CPN existence in CREE, all the fractions of crude extract were applied on *P. oleracea*. The coleoptile sections were prepared the same method with CPN assay.

10 sections were put in 0.5 ml of the fractionated CREEs (250µg/ml) which contain pH 5.0 buffer (K_2HPO_4 1.794 g/L, and citric acid monohydrate 1.019 g/L) and 2 % sucrose. The sections were incubated about 20 hours in the dark at 25ºC in horizontal shaker.

Differences of the lengths were shown by taking pictures with binocular. Taken pictures of the sections were shown in the Figure 2.7. After 20 hours in shaker, lengths of all cuttings were measured by using ImageJ 1.46 software.

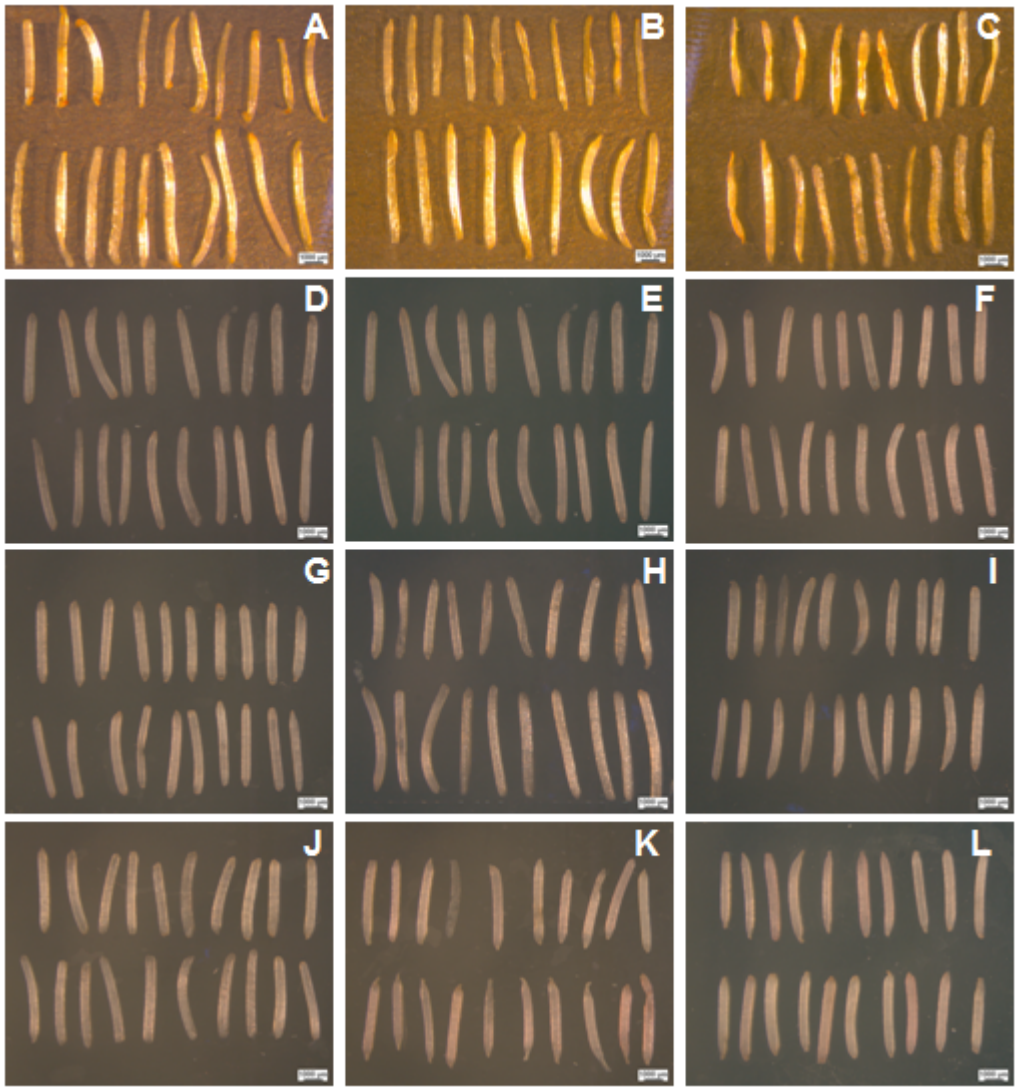


Figure 2.7 Effect of CREE's fractions on the *P. oleracea* coleoptile cuttings. (Pictures were taken by using Binocular with the scale of 0.63). From A to K; fragment separation concentrations of petroleum ether increases from 0% to 100 %. L refers to the control group. For each image 1st row represents the control and the 2nd row is for the coleoptile cuttings which were treated with the fraction of extract.

2.5.6 Coleoptile Cuttings Experiments with the Separated Fractions of 60% PE *Fraction*

Like the previous coleoptile cutting experiments, it has been followed the procedure of Nitsch & Nitsch (1954). Active fraction of *C. racemosa* (60% PE) was separated by column chromatography. Silica was used as stationary phase. 250 µg/ml of each fraction prepared with the method of Nitsch & Nitsch (1954).

The sections were incubated about 20 hours in the dark at 25ºC in horizontal shaker to have a more uniform growth. Differences of the lengths were determined by taking pictures with binocular and measured with ImageJ 1.46.

2.5.7 NMR Measurements

The active fraction of *C. racemosa* extract which is 60% PE fraction was fractionated again with column chromatography starting with 100% diethyl ether. The chromatography was carried out with 10% petroleum ether dilutions until reaching to 100% petroleum ether. All the separated fractions of the active fraction were solved in deuterated chloroform ($CDCl_3$) for the NMR measurements as NMR solvent. All the solutions were prepared as 3µg/ml with $CDCl_3$. Measurements was achieved with Bruker AC 400 and Bruker AC 600 Spectrometer. Chemical shifts of ^{1}H- and ^{13}C NMR are given in ppm.

CHAPTER THREE

RESULTS

3.1 The Effects of CRWE on Growth of *A. cepa*

Salts such as sodium chloride or sodium sulphate in the growth media of plants prevent absorption of nutrients, particularly potassium, and cause nutrient deficiencies (Tchiadje, 2007). The CRWE was directly obtained from the seaweed. Since the salinity of the extract was considerably high, the growth rates of plants were reduced instead if increasing, due to the salt intolerance of *A. cepa* (Mansour & Salama, 2004; Teerarak, Bhinija, Thitavasanta & Laosinwattana, 2009). According to our experiments the images of *A. cepa* growth were shown in Figure 3.1-3.

Figure 3.1 1st day of *C. racemosa* extract treatment to *A. cepa.* From left to right tubes refer to the different concentrations (Control, 5%, 15%, 20%) of the crude CRWE, with 3 replicates.

Figure 3.2 7th day of *C. racemosa* extract treatment to *A. cepa.* From left to right tubes refer to the different concentrations (Control, 5%, 15%, 20%) of the crude CRWE, with 3 replicates.

Figure 3.3 11th day of *C. racemosa* extract treatment to *A. cepa.* From left to right tubes refer to the different concentrations (Control, 5%, 15%, 20%) of the crude CRWE, with 3 replicates.

As it is seen in the Figure 3.2 and 3.3, there is an increase on the growth of *A. cepa* bulbs which were treated by up to 15% of CRWE. However, as much the

concentration of extract increased, the growth rate of the plants decreased after the concentration of 15%.

3.1.1 Root Length

Although the root number of *A. cepa* increased with the 5% of CRWE, higher concentrations caused a decrease on the growth. This decrease demonstrated the negative effects of the high salt concentration in the CRWE which is shown in Figure 3.4.

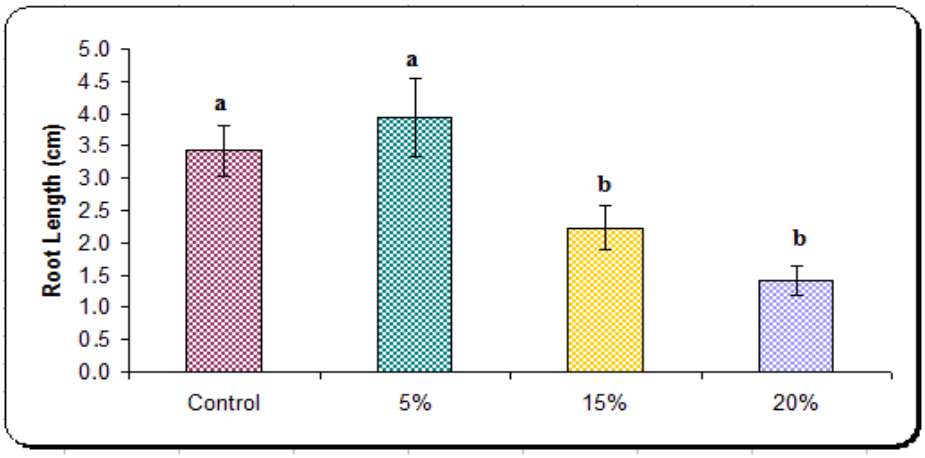


Figure 3.4 Effects of *C. racemosa* extracts on the root length of *A. cepa* Error bars are calculated with the standard deviation values of the length of the roots for 3 replicates. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.1.2 Shoot Length

Contrary to the root length until the 15% of CRWE, there is a presence of growth rate increased. Roots are probably the most important organs of a plant for the growth by taking important minerals from earth. The longer roots mean better growth. Nevertheless the negative effects of the salts on the growth showed itself with the concentration of 20% (Figure 3.5). It demonstrated the importance of the extract with different solvents to see the growth effect without existence of salts.

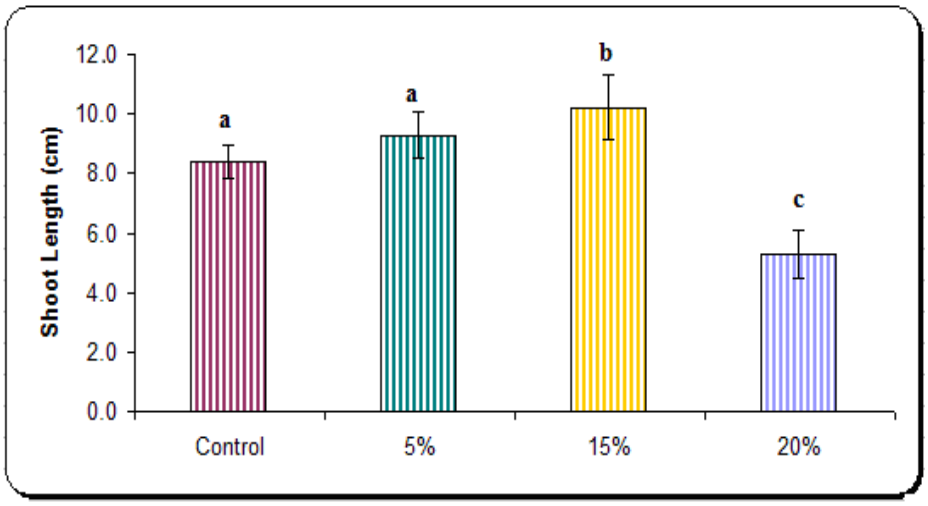


Figure 3.5 Effects of *C. racemosa* extracts on the shoot length of *A. cepa*. Error bars are the means of 3 different experiments. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.1.3 Root and Shoot Number

As it is shown in the Figure 3.6 and 3.7, there was no significant effect of the CRWE on the root and shoot numbers of *A. cepa.* The error bars were relatively high and the decrease on the numbers can be easily seen with the increase of concentration.

To sum up all these information, it is obvious that there was a positive effect on the plant growth but contaminations were blocked this effect. Therefore, it is obligatory to change the type of the experiments to see the effect of *C. racemosa* on the plant growth.

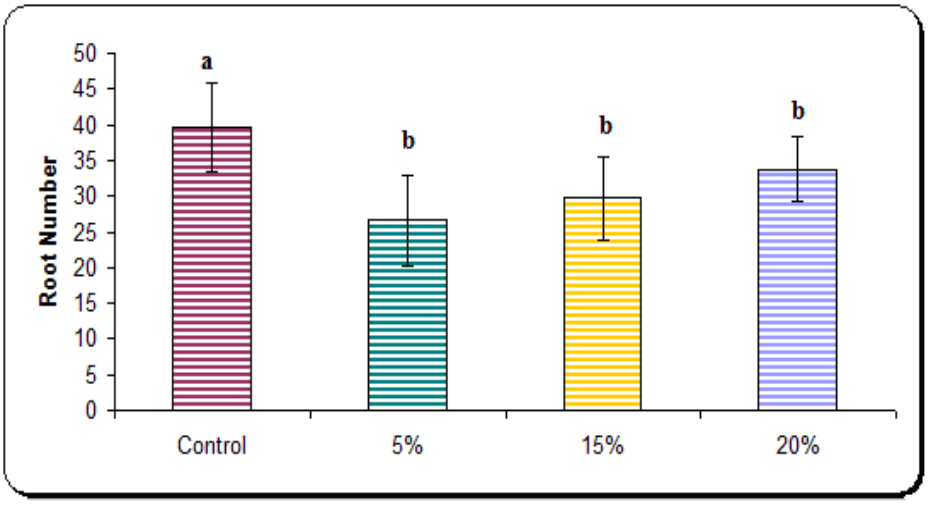


Figure 3.6 Effects of *C. racemosa* extracts on the root number of *A. cepa*. Error bars are the means of 3 different experiments. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

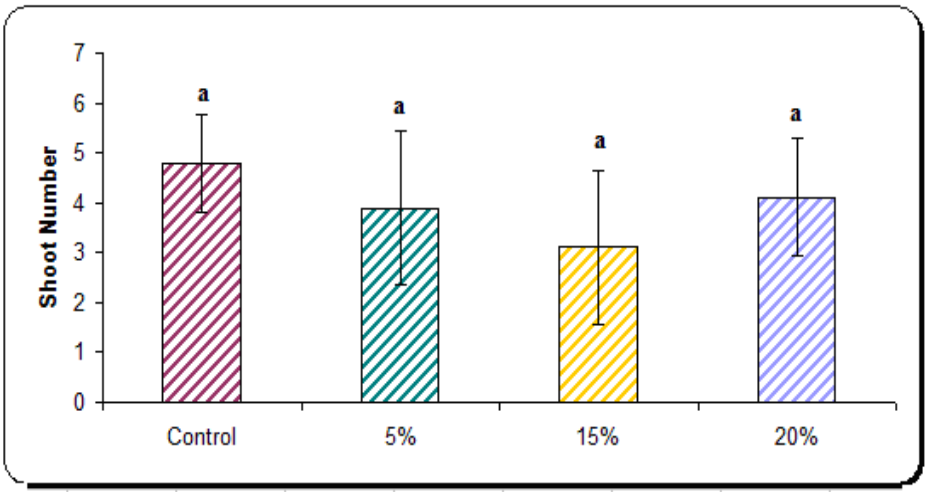


Figure 3.7 Effects of *C. racemosa* extracts on the shoot number of *A. cepa*. Error bars are the means of 3 different experiments. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.2 The Effects of *C. racemosa* on Growth of *P. oleracea*

In order to determine the effects of *C. racemosa* extract on the growth of *P. oleracea* the root length and shoot length experiments were carried out.

3.2.1 The Growth of Extract Soaked P. oleracea Plants

Root and shoot length experiments were carried out previously with the *C. racemosa* expract soaked seeds.

3.2.1.1 Root Length

Figure 3.8 and 3.9 demonstrated the effect of *CRWE* on the shoot and root lengths of *P. oleracea.*.Regardinging the effect on root length there is a considerably significant increase on the length with the increase of the extract concentration. In addition to this, there was also been found a significant increase on the shoot length of *P. oleracea.* This effect, which seems quite important to investigate particularly, was planned to examine with the further experiments.

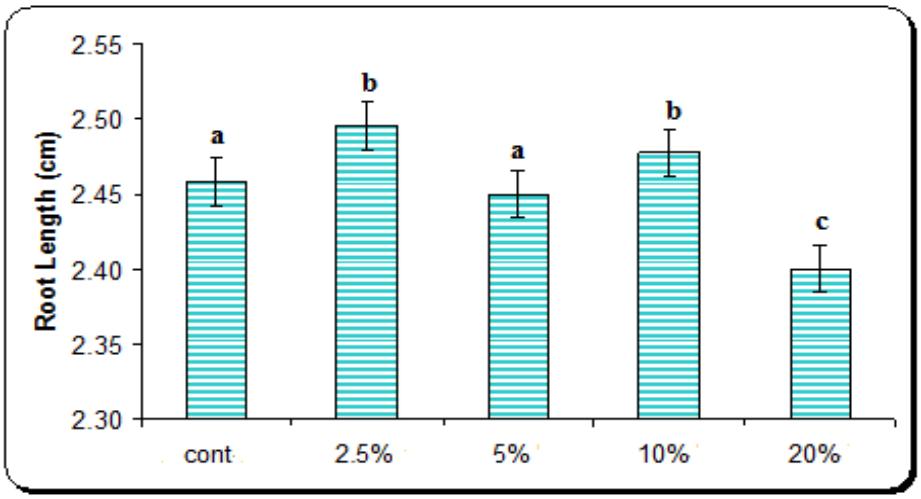


Figure 3.8 Effects of *CRWE* on the root length of extract soaked *P. oleracea* seeds. Soaking concentrations of *C. racemosa* extract increases from left to right. Error bars refers to the standard deviation of the root length values of each group. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.2.1.2 Shoot Length

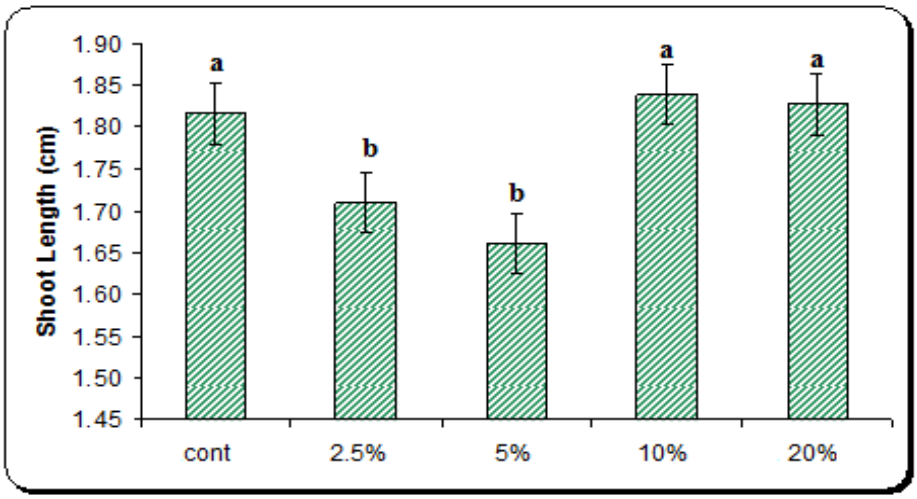


Figure 3.9 Effects of *CRWE* on the shoot length of extract soaked *P. oleracea*. Soaking concentrations of *C. racemosa* extract increase from left to right. Error bars refers to the standard deviation of the shoot length values of each group. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.2.2 The Growth of Water Soaked P. oleracea Seeds

Root and shoot length experiments were carried out secondly with the water soaked seeds. Graphics of the experiments were shown below.

3.2.2.1 Root Length

As it can be seen in the Figures 3.10 and 3.11, growth stimulating effect of the *C. racemosa* extract on *P. oleracea* was observed more significantly in comparison with the experiments with *A. cepa*. Proportionally with the increase in extract concentration; it was observed a rise on shoot length of the *P. oleracea* plants. However, the increase of root lengths of the seeds, especially which were soaked with extracts did not demonstrate any proportion with neither the increase of concentration nor soaking conditions.

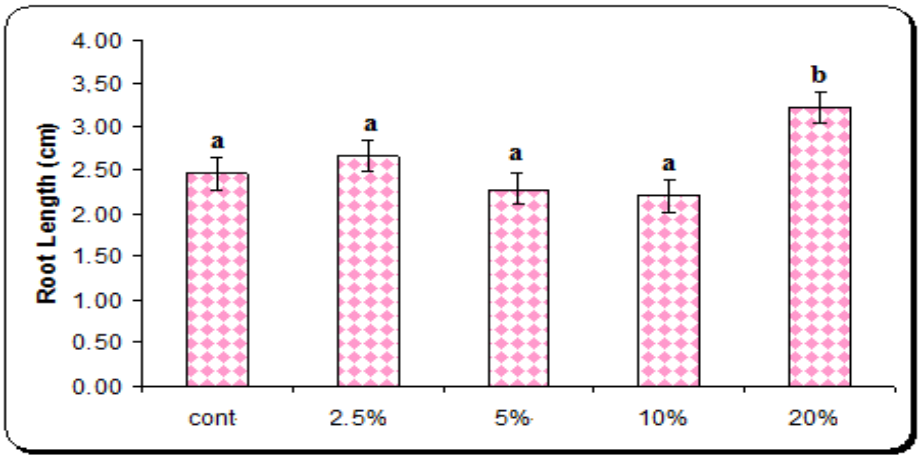


Figure 3.10 Effects of *C. racemosa* extracts on the root length of water soaked *P. oleracea*. Watering concentrations of *C. racemosa* extract increase from left to right. Error bars demonstrate the standard deviation of root lengths. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.2.2.2 Shoot Length

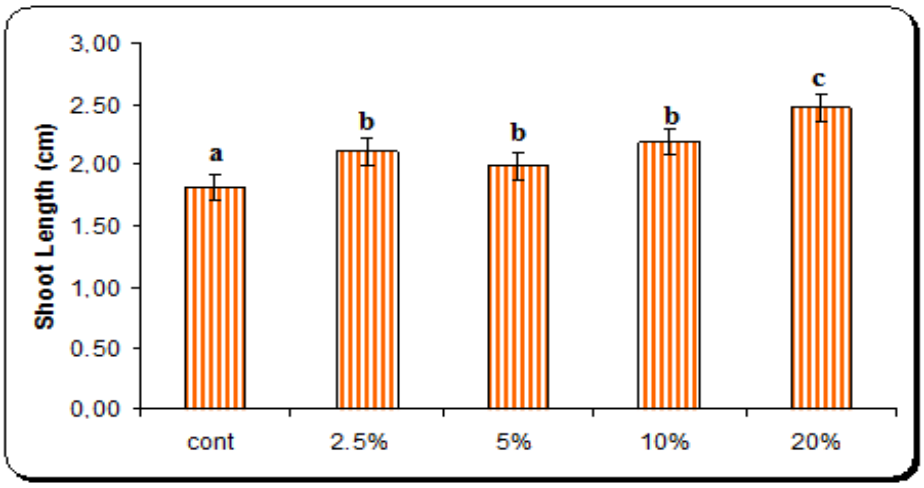


Figure 3.11 Effects of *C. racemosa* extracts on the shoot length of water soaked *P. oleracea*. Watering concentrations of *C. racemosa* extract increase from left to right. Error bars demonstrate the standard deviation of shoot lengths. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.3 The Effects of Indole-3-Butyric acid (IBA) on Growth of *P. oleracea*

In order to determine the effects of indole-3-butyric acid on the growth of *P. oleracea,* root length and shoot length experiments were carried out.

3.3.1 The Growth of IBA Soaked P. oleracea Plants

For the root length experiments on *P. oleracea*, length differentiation was shown at Figure 3.12 and 3.13. The experiments were carried out with CRWE and IBA at the same time.

3.3.1.1 Root Length

IBA was chosen as positive control. The both experiments were achieved with different soaking concentrations and afterwards water treatment. As it can be easily seen in the Figure 3.12 and 3.13; in the lower concentrations of extract soaking, there was an effect of similar growth stimulation for extract treated and IBA treated *P. oleracea* plants.

When the concentration of soaking extract increased, the stimulation effect in root growth decreased in the case of both solutions. This similarity demonstrates us that there is a metabolic similarity between IBA and CREE.

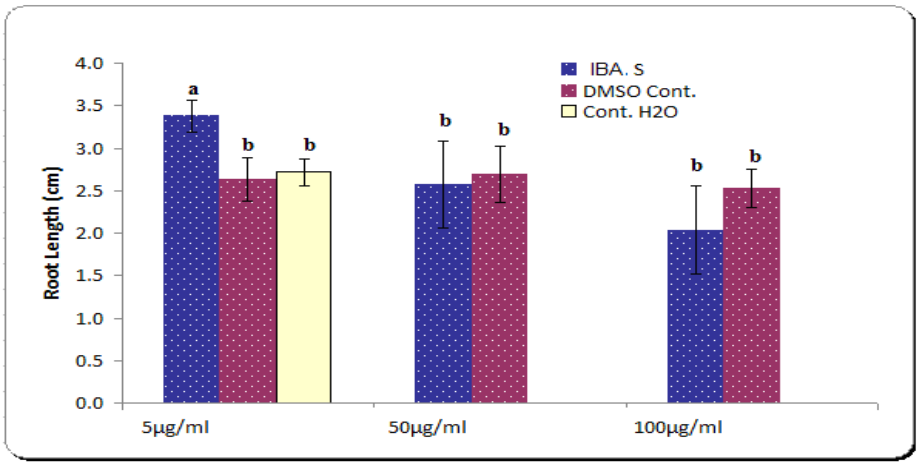


Figure 3.12 Root Length of *P. oleracea* - Soaked with IBA, Treated with Water. IBA-S refers to "IBA soaking concentration" and the graphic bars refer to root length of each group. Error bars demonstrate the standard deviation of root lengths. Blue bars show IBA soaked seeds, pink ones are DMSO controls and the white one refers to water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

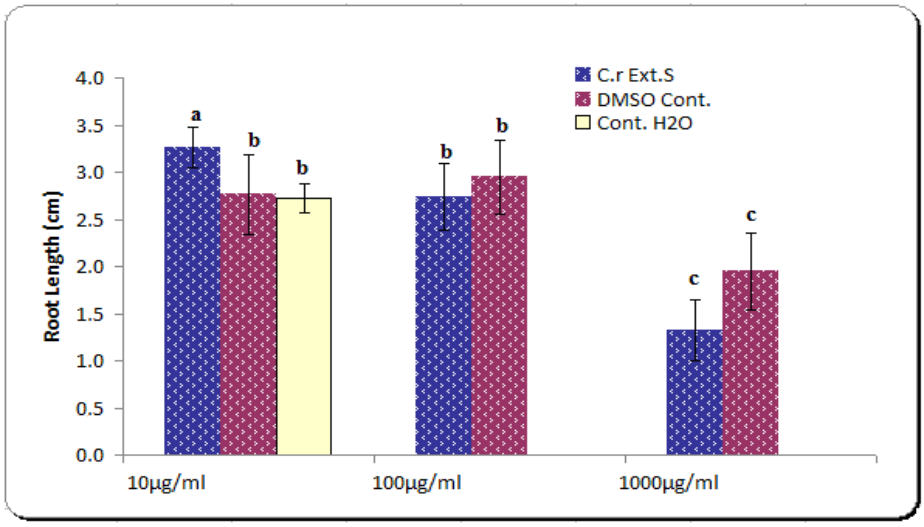


Figure 3.13 Root Length of *P. oleracea* - Soaked with *C. racemosa* Extract, Treated with Water. ExtS refers to "extract soaking concentration", C.r refers to *"C. racemosa*" and the graphic bars refer to root length of each group. Error bars demonstrate the standard deviation of root lengths. Blue bars show Extract soaked seeds, pink ones are DMSO controls and the white one refers to water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.3.1.2 Shoot Length

The obtained data for IBA and extract soaked plants demonstrated in Figure 3.14 and 3.15. The growth graphics were similar for roots and shoot lengths. Lower concentrations of both soaking solutions had an ability to increase the growth rate but when the concentration increased to higher levels, this stimulation effect disappeared. In the same way, the results clearly showed us that there growth similarity effect between IBA and *C. racemosa* extract.

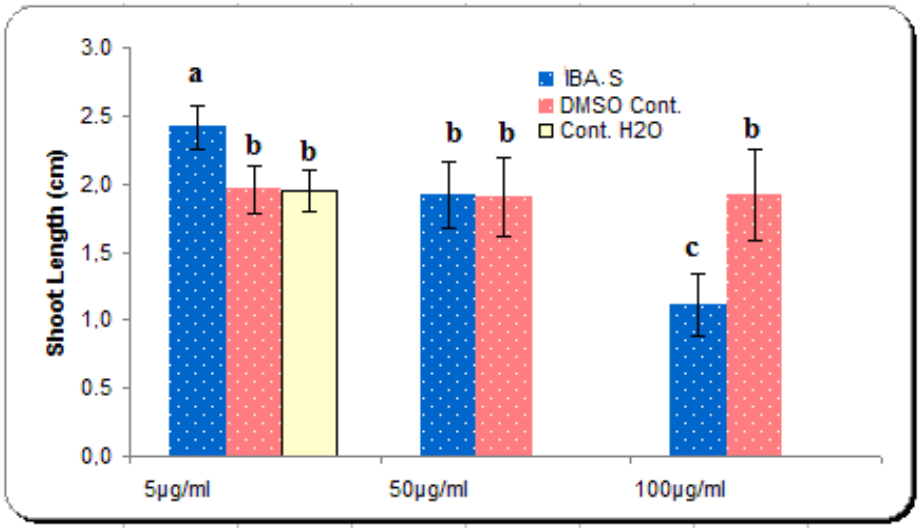


Figure 3.14 Shoot Length of *P. oleracea* - Soaked with IBA, Treated with Water. IBA-S refers to "IBA soaking concentration" and the graphic bars refer to shoot length of each group. Error bars demonstrate the standard deviation of shoot lengths. Blue bars show IBA soaked seeds, pink ones DMSO controls and the white one refers to water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

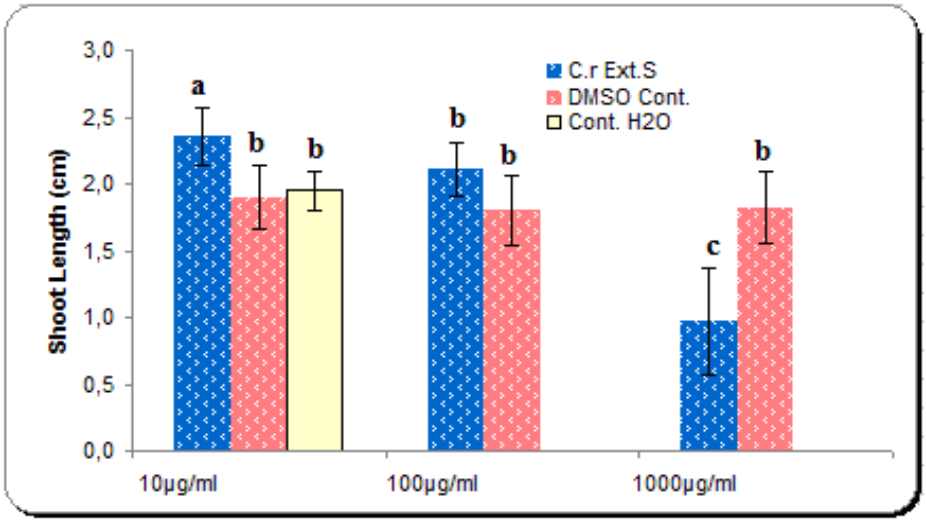


Figure 3.15 Shoot Length of *P. oleracea* - Soaked with *C. racemosa* extract, Treated with Water. ExtS refers to "extract soaking concentration", C.r. refers to *"C. racemosa"* and the graphic bars refer to shoot length of each group. Error bars demonstrate the standard deviation of shoot lengths. Blue bars show Extract soaked seeds, pink ones DMSO controls and the white one refers water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.3.2 The Growth of IBA Treated P. oleracea Plants

In order to determine the effects of indole-3-butyric acid on the growth of *P. oleracea,* root length and shoot length experiments were carried out for the previously IBA treated seeds.

3.3.2.1 Root Length

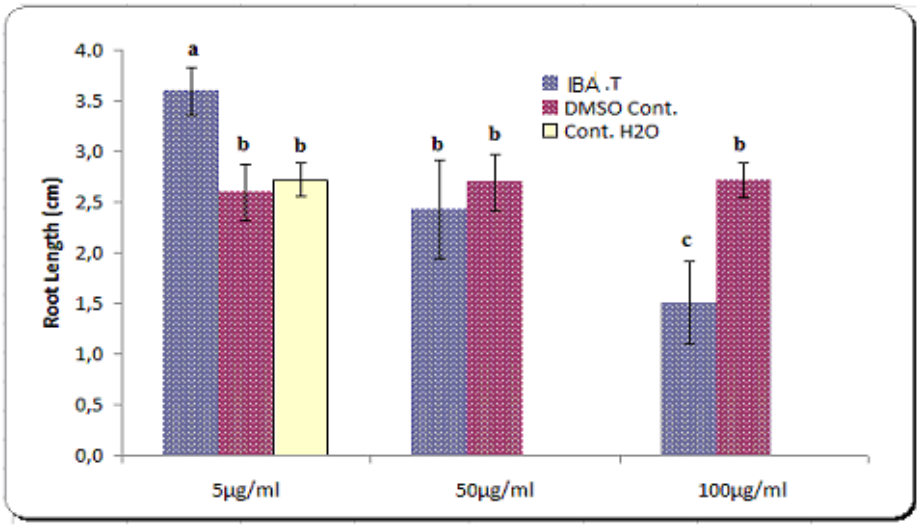


Figure 3.16 Root Length of *P. oleracea* – Soaked with Water, Treated with IBA. IBA-T refers to "IBA treatment concentration" and the graphic bars refer to root length of each group. Error bars demonstrate the standard deviation of root lengths. Blue bars show IBA treated seeds, pink ones DMSO controls and the white one refers water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

For the second part of the experiment soaking conditions were changed and soaking was carried out with water. Afterwards, all the petri plates were treated with different concentrations of *Caulerpa* extract or IBA. The results were quite similar with the first experiments (Figure 3.16).

This experiment were also proved us from Figure 3.17 the similarity between *Caulerpa* extract and IBA. However, it could easily be easily seen that there was no significant effect of soaking conditions on the root or shoot length of *P. oleracea* plants.

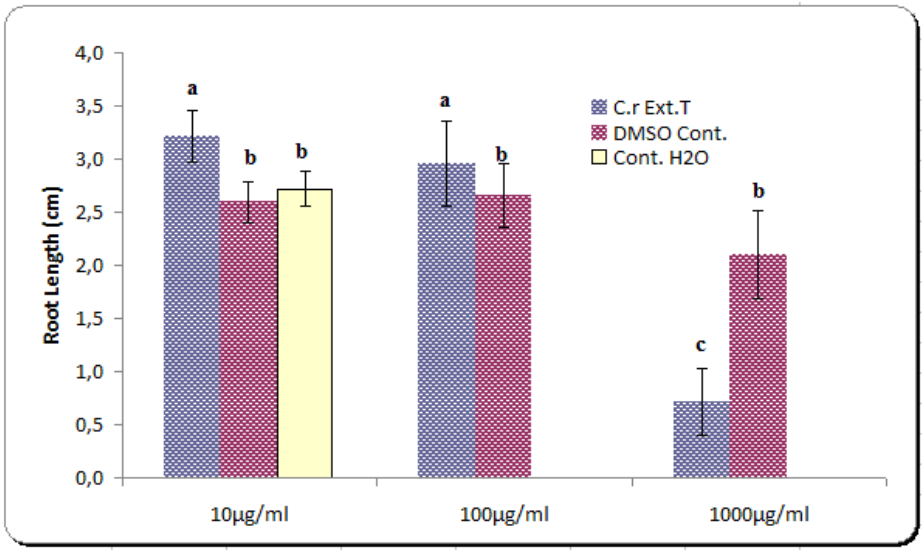


Figure3.17 Root Length of *P. oleracea* – Soaked with Water, Treated with *C. racemosa* Extract. Ext.T refers to "extract treatment concentration", C.r refers to "*C. racemosa"* and the graphic bars refer to root length of each group. Error bars demonstrate the standard deviation of root lengths. Blue bars show Extract treated seeds, pink ones DMSO controls and the white one refers water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.3.2.2 Shoot Length

Regarding the Figure 3.18 and Figure 3.19, the significant effect of soaking was not seen but it is obvious that there was a stimulation effect of CREE in lower concentrations for the growth of *P. oleracea*. Also, it was thought that this increase of root and shoot length could be results of the contaminations coming from CRWE like salts. Furthermore, following experiments were carried out with CREE.

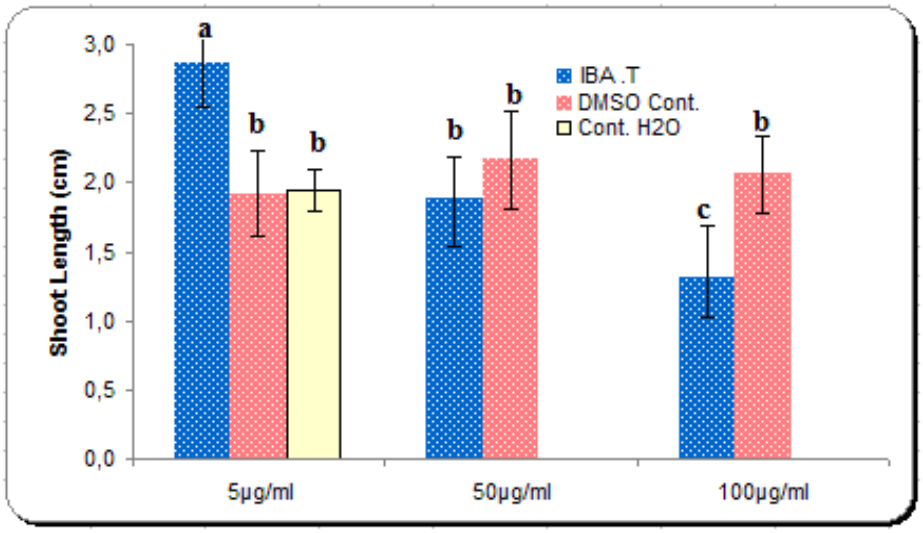


Figure 3.18 Shoot Length of *P. oleracea*- Soaked with Water, Treated with IBA. IBA-T refers to "IBA treatment concentration" and the graphic bars refer to shoot length of each group. Error bars demonstrate the standard deviation of shoot lengths. Blue bars show IBA treated seeds, pink ones DMSO controls and the white one refers water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

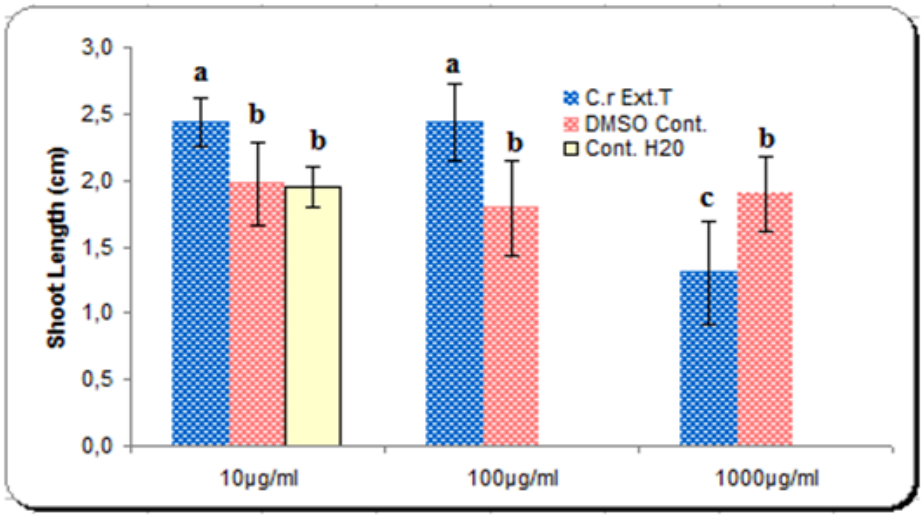


Figure 3.19 Shoot Length of *P. oleracea* – Soaked with Water, Treated with *C. racemosa* Extract. ExtT refers to "extract treatment concentration", C.r refers to *"C. racemosa* "and the graphic bars refer to shoot length of each group. Error bars demonstrate the standard deviation of shoot lengths. Blue bars show Extract treated seeds, pink ones DMSO controls and the white one refers water control. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.4 The Effects of CREE on Germination

After the experiments on the plant growth, germination experiments were carried out with two different species *H. annuus* and *P. oleracea*. In order to see the effects of the CREE on the germination were aimed.

3.4.1 The Effects of C. racemosa on Germination of H. annuus

The germination experiments were first carried out with the species of *H. annus*. The seeds were treated with CREE and stimulations were observed.

3.4.1.1 The Germination of CREE Soaked H. annuus Seeds

The germination experiments were carried out with two different ways which were described before. The data were shown in Figure 3.20-22. An increase was seen in the 100µg/ml soaked seeds, but the increase was not statistically significant.

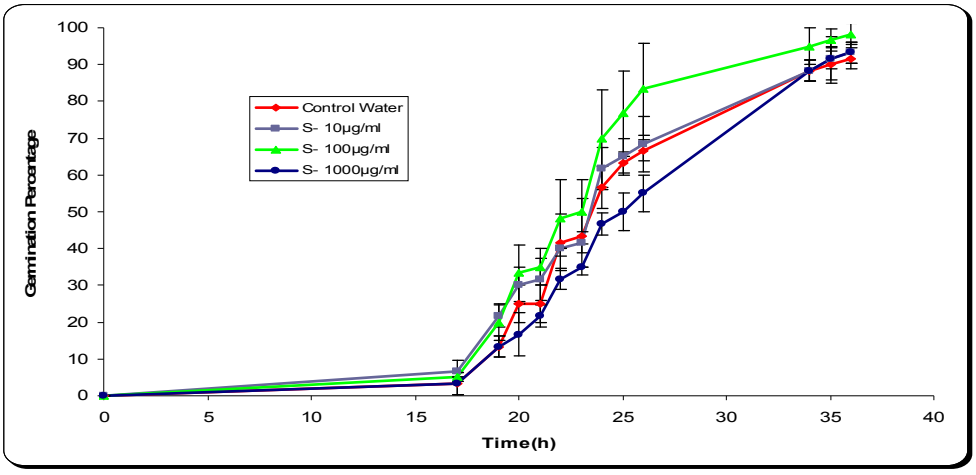


Figure 3.20 Germination graphic of *H. anuus* – seeds were soaked with CREE - treated with water. Red line in the graphic refers to germination of water control, grey one refers to 10µg/ml extract soaked seeds' germination, green line refers to germination of 100µg/ml extract soaked seeds and the dark blue line refers to the germination of 1000µg/ml soaked seeds. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

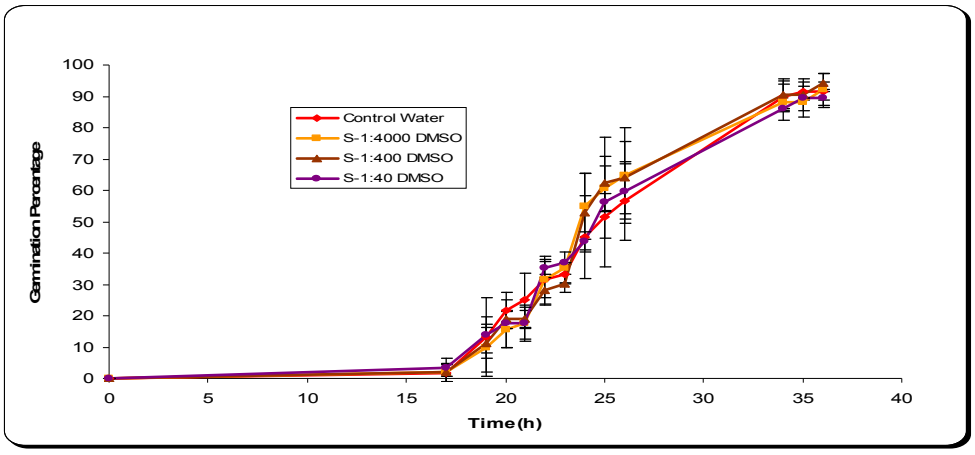


Figure 3.21 Germination graphic of *H. anuus* – seeds were soaked with DMSO – treated with water. Red line in the graphic refers to germination of water control, orange one refers to 1:4000 (v:v) DMSO soaked seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO soaked seeds and the purple line refers to the germination of 1:40 (v:v) DMSO soaked seeds. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

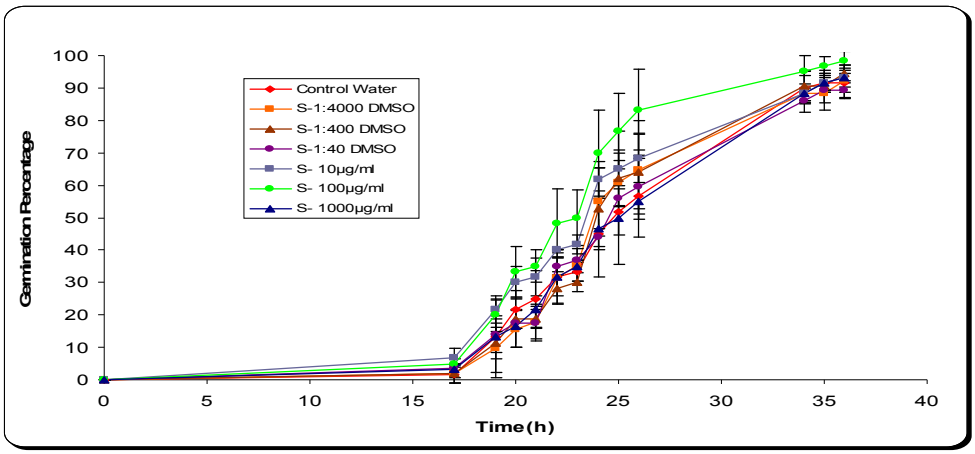


Figure 3.22 Germination graphic of *H. anuus* – seeds were soaked with CREE – treated with water (including DMSO controls). Red line in the graphic refers to germination of water control, orange one refers to 1:4000 (v:v) DMSO soaked seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO soaked seeds and the purple line refers to the germination of 1:40 (v:v) DMSO soaked seeds, grey one refers to 10µg/ml extract soaked seeds' germination, green line refers to germination of 100µg/ml extract soaked seeds and the dark blue line refers to the germination of 1000µg/ml soaked seeds. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.4.1.2The Germination of Water Soaked H. annuus Seeds

The data were shown in Figure 3.23-25. An increase was seen in the 100µg/ml treated seeds, but the increase was not statistically significant.

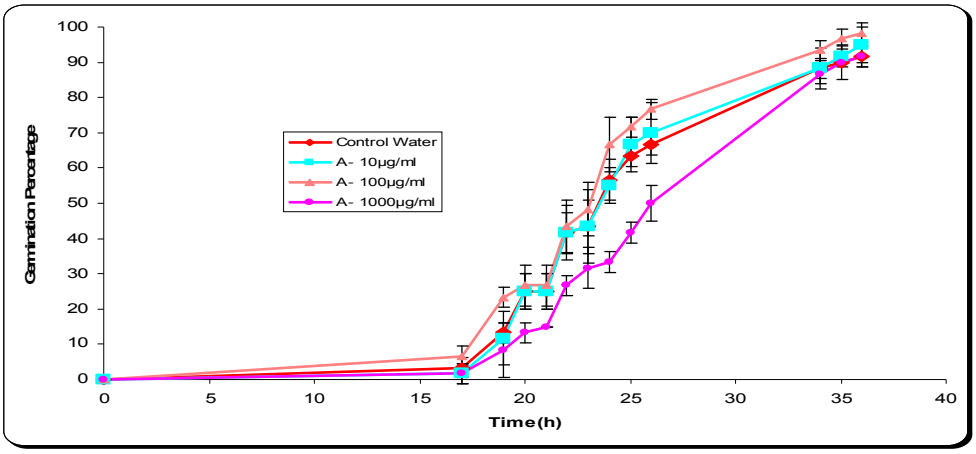


Figure 3.23 Germination graphic of *H. anuus* – seeds were soaked with water - treated with CREE. Red line in the graphic refers to germination of water control, blue one refers to 10µg/ml extract soaked seeds' germination, light pink line refers to germination of 100µg/ml extract soaked seeds and the dark pink line refers to the germination of 1000µg/ml soaked seeds.

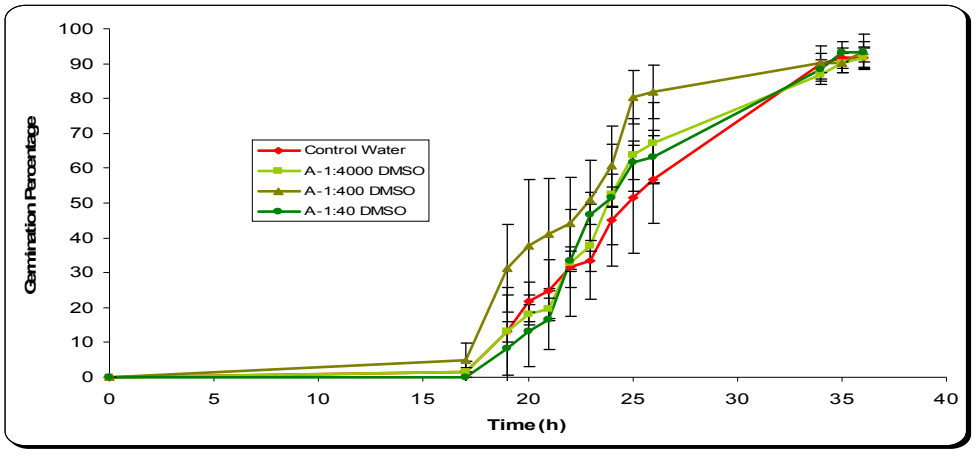


Figure 3.24 Germination graphic of *H. anuus* – seeds were soaked with water – treated with DMSO. Red line in the graphic refers to germination of water control, light green one 1:4000 (v:v) DMSO soaked seeds' germination, green line 1:400 (v:v) DMSO soaked seeds and thedark green line refers to the germination of 1:40 (v:v) DMSO soaked seeds.

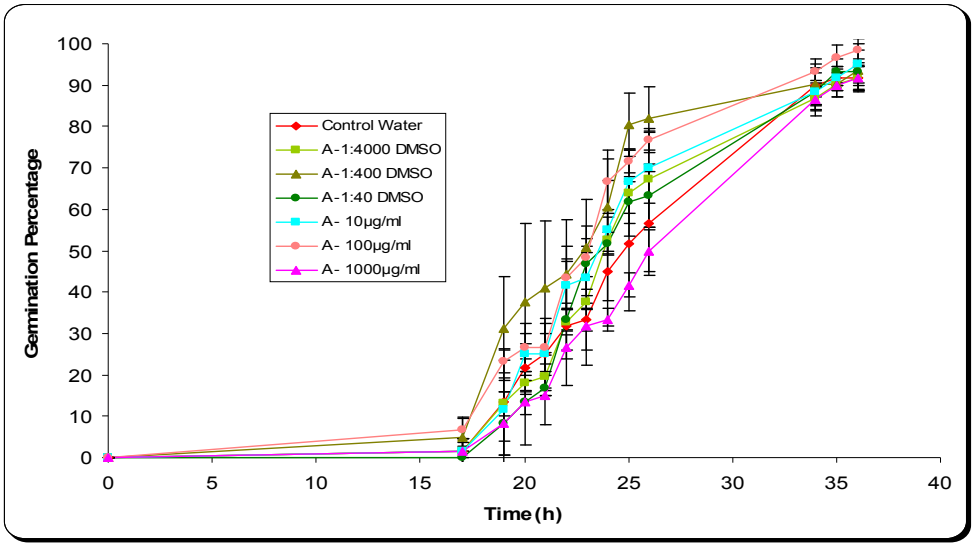


Figure 3.25 Germination graphic of *H. anuus* – seeds were soaked with water– treated with CREE (including DMSO controls). Red line in the graphic refers to germination of water control, light green one refers to 1:4000 (v:v) DMSO soaked seeds' germination, green line refers to germination of 1:400 (v:v) DMSO soaked seeds and the dark green line refers to the germination of 1:40 (v:v) DMSO soaked seeds, blue one refers to 10µg/ml extract soaked seeds' germination, light pink line refers to germination of 100µg/ml extract soaked seeds and the dark pink line refers to the germination of 1000µg/ml soaked seeds.

3.4.2 The Effects of C. racemosa on Germination P. oleracea

The germination experiments were secondly carried out with the species of *P. olercea*. Seeds were treated with CREE and stimulations were observed.

3.4.2.1 The Germination of CREE Soaked P. oleracea Seeds

The results were similar with the germination results of *H. annuus*.The obtained data were shown in Figure 3.26-28 below. An increase was seen in the 100µg/ml treated and soaked seeds, but the increase was not statistically significant.

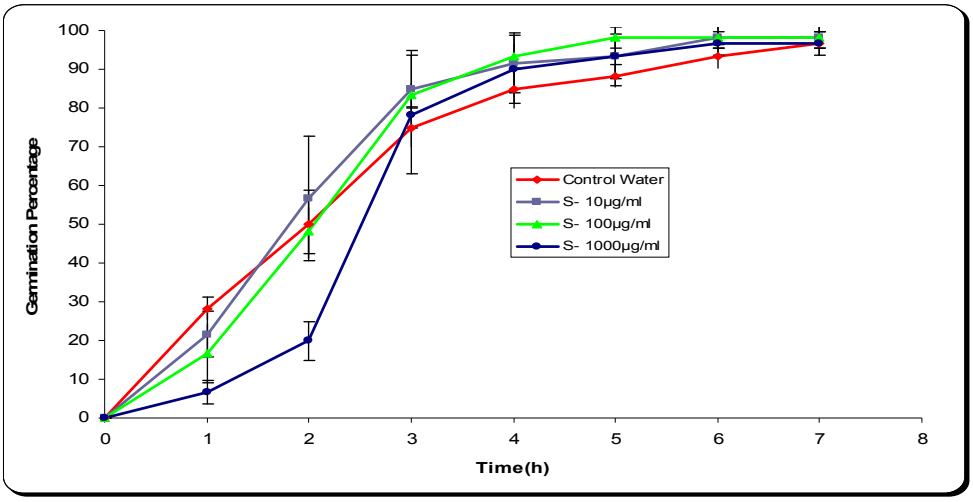


Figure 3.26 Germination graphic of *P. oleracea*; seeds were soaked with CREE – treated with water. Red line refers to water control , grey one refers to 10µg/ml extract soaked seeds' germination, green line refers to germination of 100µg/ml extract soaked seeds and the dark blue line refers to the germination of 1000µg/ml soaked seeds.

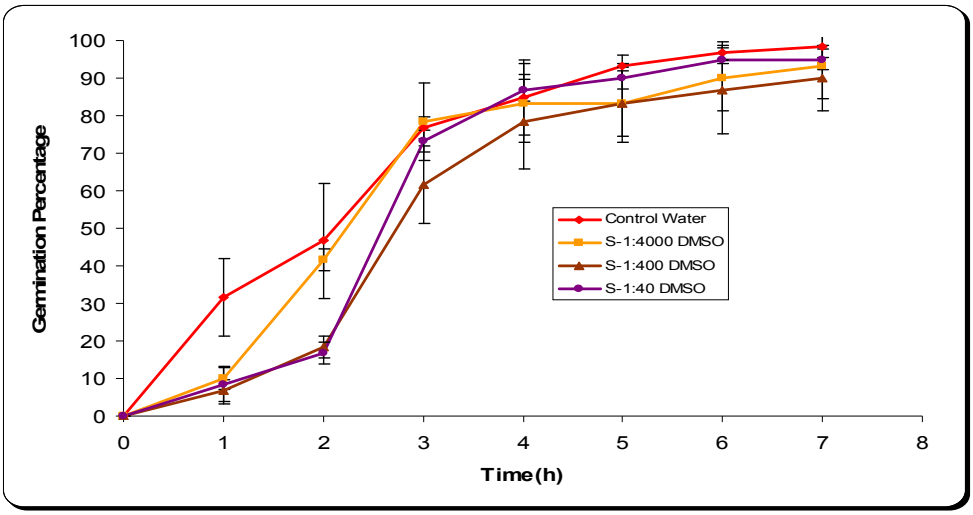


Figure 3.27 Germination graphic of *P. oleracea*; seeds were soaked with DMSO – treated with water. Red line in the graphic refers to germination of water control, orange one refers to 1:4000 (v:v) DMSO soaked seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO soaked seeds and the purple line refers to the germination of 1:40 (v:v) DMSO soaked seeds.

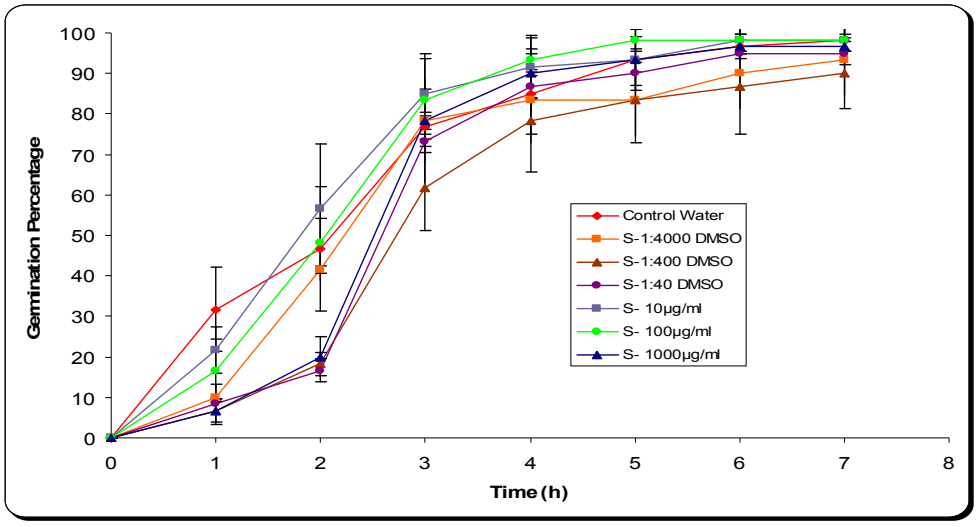


Figure 3.28 Germination graphic of *P. oleracea*; seeds were soaked with CREE– treated with water (including DMSO controls). Red line in the graphic refers to germination of water control, orange one refers to 1:4000 (v:v) DMSO soaked seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO soaked seeds and the purple line refers to the germination of 1:40 (v:v) DMSO soaked seeds, grey one refers to 10µg/ml extract soaked seeds' germination, green line refers to germination of 100µg/ml extract soaked seeds and the dark blue line refers to the germination of 1000µg/ml soaked seeds.

3.4.2.2 The Germination of Water Soaked P. oleracea Seeds

As it could be seen in Figure 3.29-30, it is possible to realize that there was no any significant effect on the germination of soaking conditions. For all the cases of *P. oleracea* in the germination experiments, 100 µg/ml caused a relatively significant stimulation on germination. However, for the control species *H. annuus*, there was no significant stimulation effect. It might be caused by the thick shelf of the species which could not permit the penetration of the chemicals.

Germination which is one of the most important growth phases of a plant was decided to be investigate regarding to the existence of a growth stimulating effect of *C. racemosa.* Regarding the germination of the plant 2 different species were treated with CREE. Species; *H. annuus* and *P. oleracea,* they were chosen because of their importance in Turkish agriculture and relatively fast growth rate. 100µg/ml concentration of extract affected the GP slightly in positive direction.

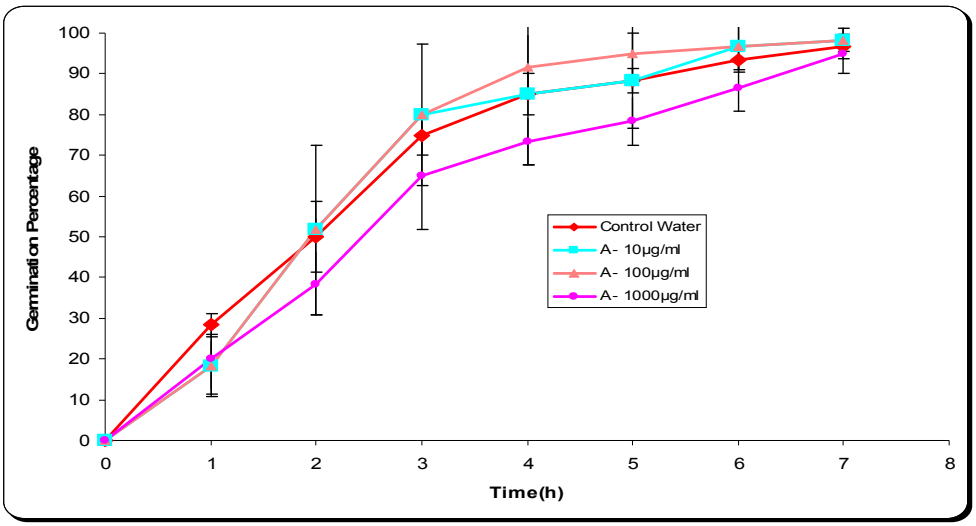


Figure 3.29 Germination graphic of *P. oleracea*; seeds were soaked with water – treated with CREE. Red line in the graphic refers to germination of water control blue one refers to 10µg/ml extract applied seeds' germination, light pink line refers to germination of 100µg/ml extract applied seeds and the dark pink line refers to the germination of 1000µg/ml applied seeds.

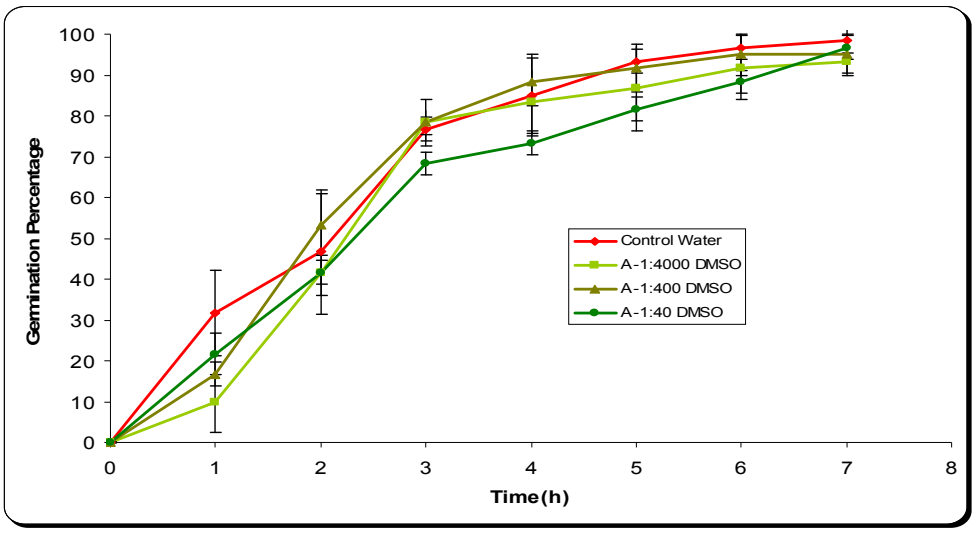


Figure 3.30 Germination graphic of *P. oleracea*; seeds were soaked with water – treated with DMSO. Red line in the graphic refers to germination of water control, light green with square one refers to 1:4000 (v:v) DMSO applied seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO applied seeds and the dark green line refers to the germination of 1:40 (v:v) DMSO applied seeds.

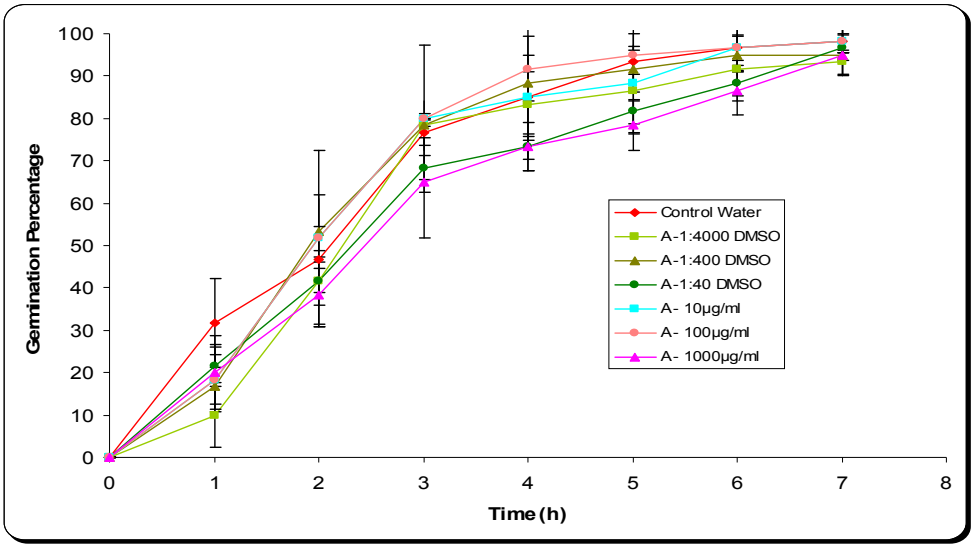


Figure 3.31 Germination graphic of *P. oleracea*; seeds were soaked with water – treated with CREE (including DMSO controls). Red line in the graphic refers to germination of water control blue one refers to 10µg/ml extract applied seeds' germination, light pink line refers to germination of 100µg/ml extract applied seeds and the dark pink line refers to the germination of 1000µg/ml applied seeds, , light green with square one refers to 1:4000 (v:v) DMSO applied seeds' germination, brown line refers to germination of 1:400 (v:v) DMSO applied seeds and the dark green line refers to the germination of 1:40 (v:v) DMSO applied seeds.

3.5 The Effects of the Fractions of Extract on Plant Growth

CPN is an algal pigment exists in *Caulerpa*cea family which has already investigated for its plant growth regulatory effect in the literature (Caparkaya, Cavas & Kesercioglu, 2009). Therefore, in order to find the responsible molecule for the stimulation in *C. racemosa* extract, the extract was fractionated with column chromatography and UPLC-MS experiments were carried out.

3.5.1 UPLC-MS Measurements

As a result of the UPLC-MS measurements, CPN existence was observed. Following by using the procedure of Nitsch & Nitsch (1954) coleoptile cutting assay, plant growth stimulating effect of CPN was observed and highly positive effect was detected.

3.5.1.1 UPLC- ESI/MS

In order to determine the existence of CPN in the separated extracts of *C. racemosa,* UPLC- ESI/MS experiments were carried out.

3.5.1.1.1 UPLC Chromatograms. At the retention time of 1.83 the caulerpin existence was observed in the fractions. The UPLC chromotograms were shown below in the Figure 3.32-44.

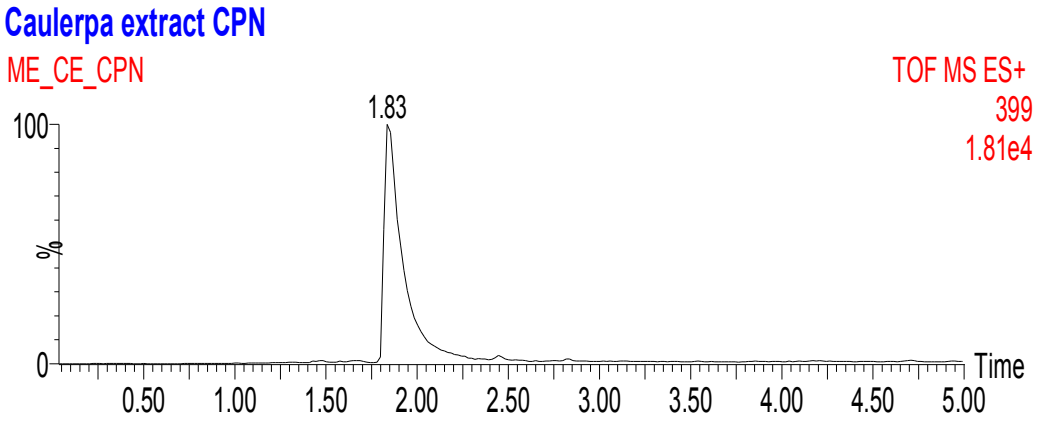


Figure 3.32 UPLC Chromatogram of isolated CPN from *C. racemosa*. Experiments were carried out at ES+.

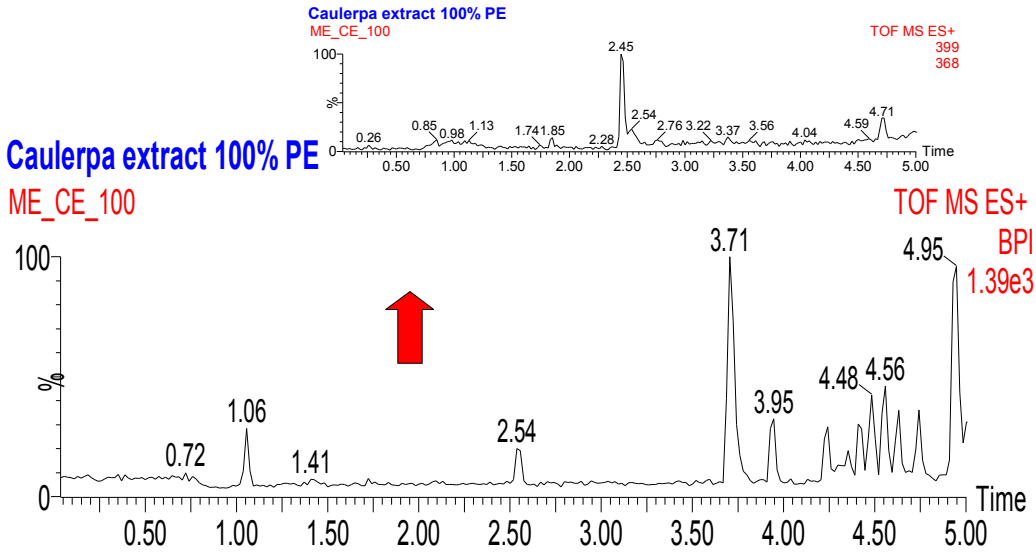


Figure 3.33 UPLC Chromatogram of 100% PE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

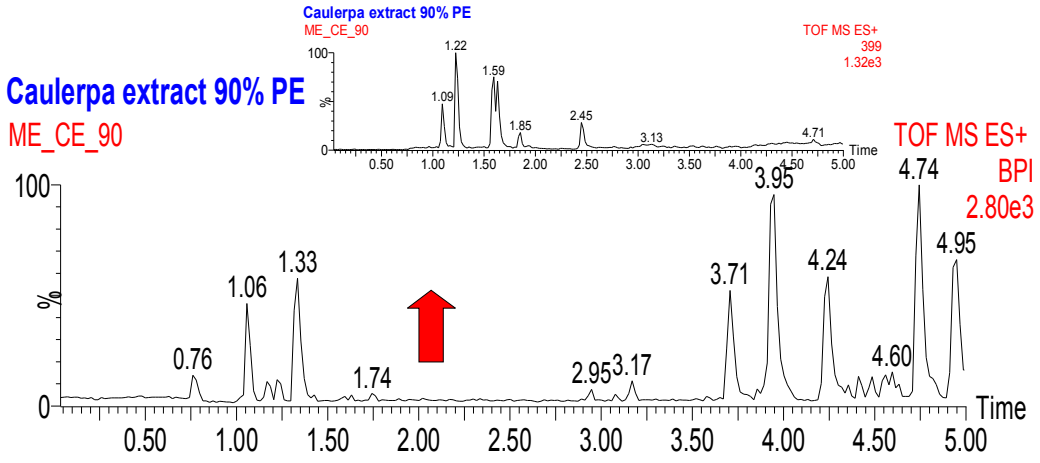


Figure 3.34 UPLC Chromatogram of 90% PE - 10% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

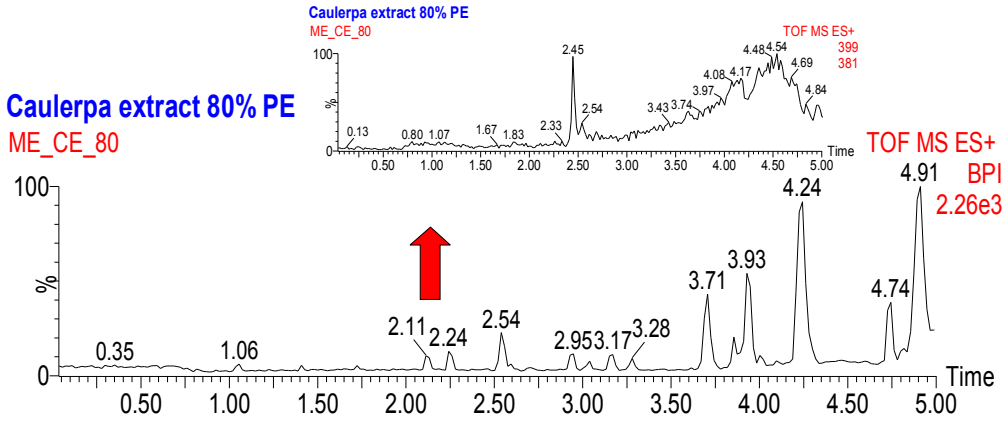


Figure 3.35 UPLC Chromatogram of 80% PE - 20% DEE Fraction of CREE Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

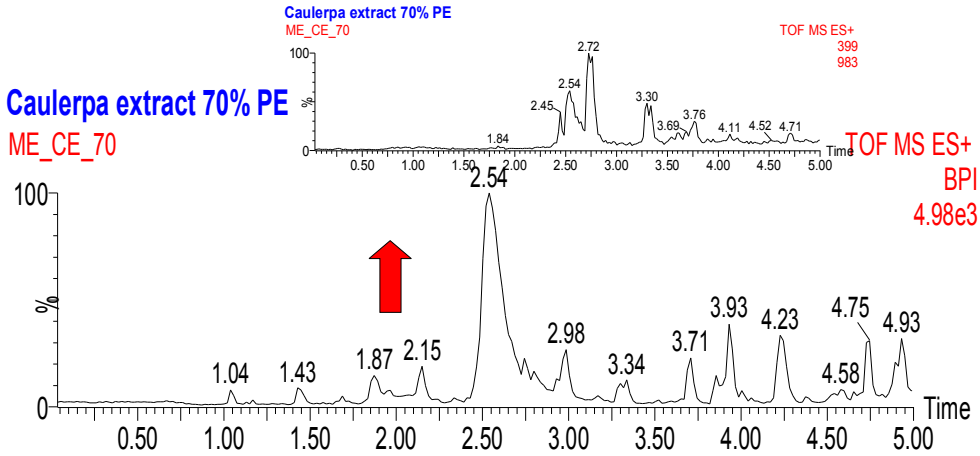


Figure 3.36 UPLC Chromatogram of 70% PE - 30% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

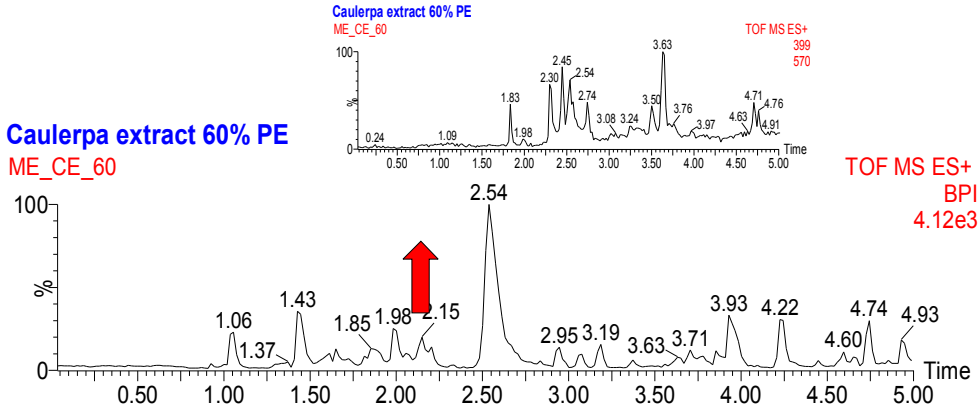


Figure 3.37 UPLC Chromatogram of 60% PE - 40% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

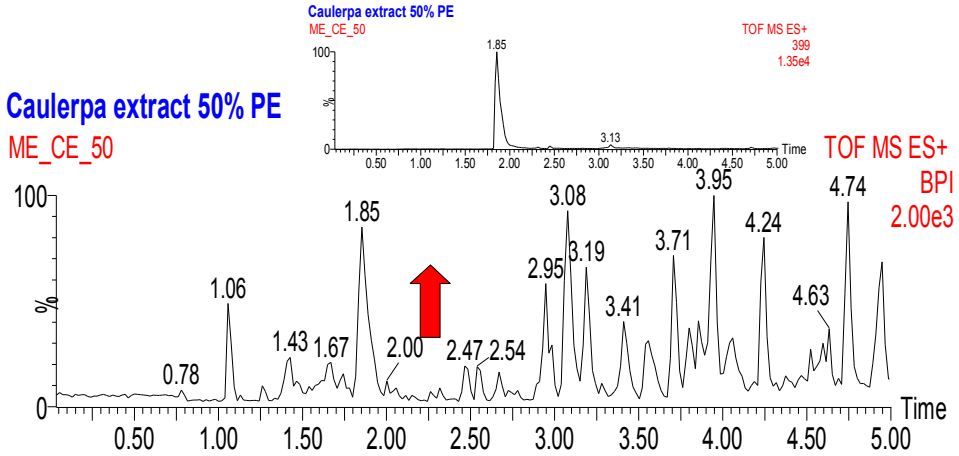


Figure 3.38 UPLC Chromatogram of 50% PE - 50% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

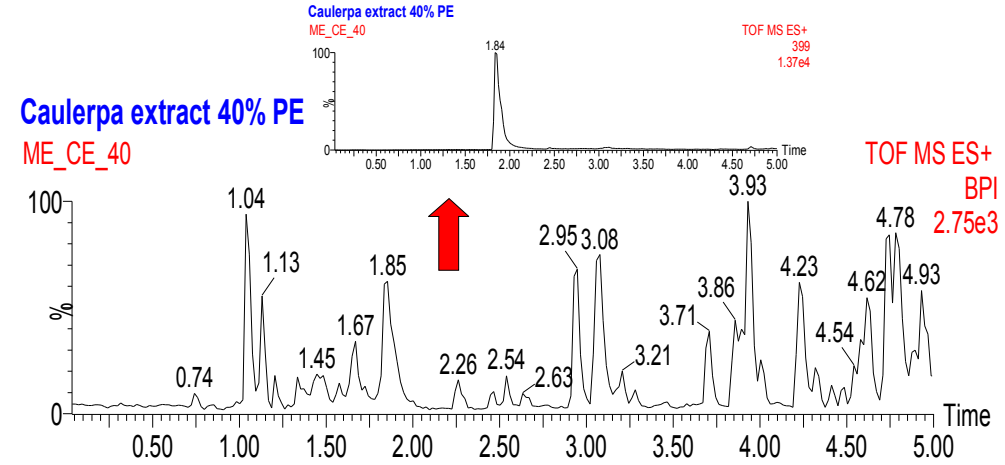


Figure 3.39 UPLC Chromatogram 40% PE - 60% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

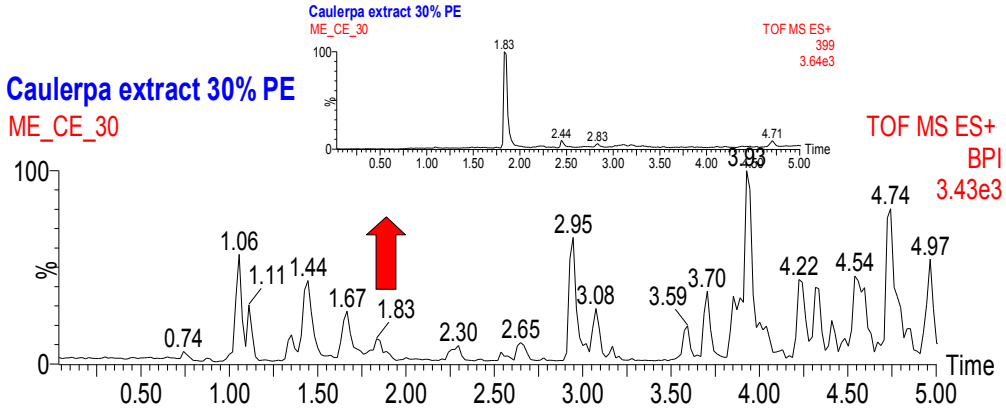


Figure 3.40 UPLC Chromatogram of 30% PE - 70% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

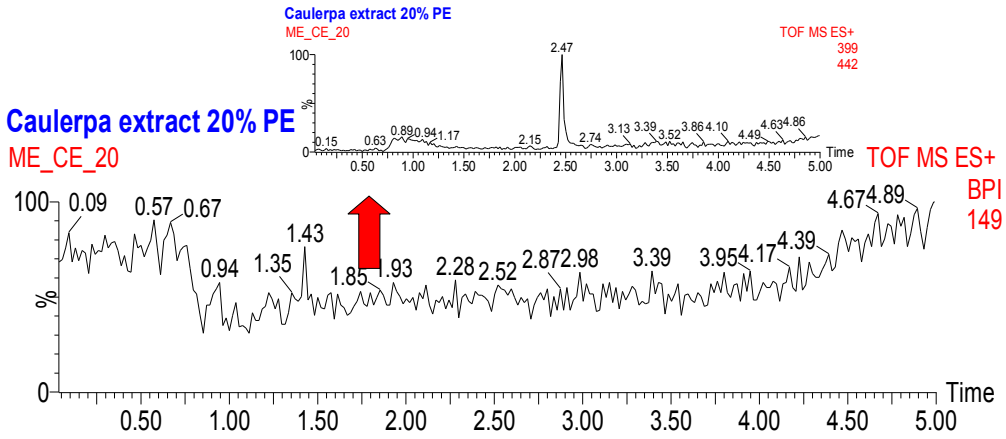


Figure 3.41 UPLC Chromatogram of 20% PE - 80% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

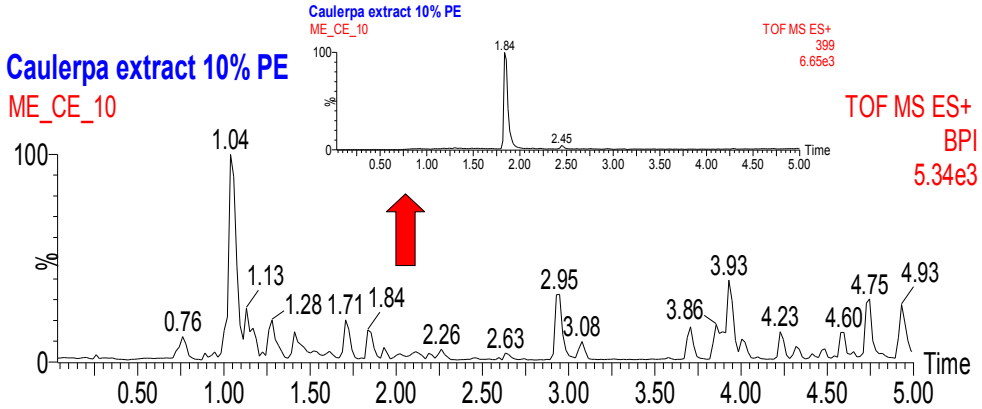


Figure 3.42 UPLC Chromatogram of 10% PE - 90% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

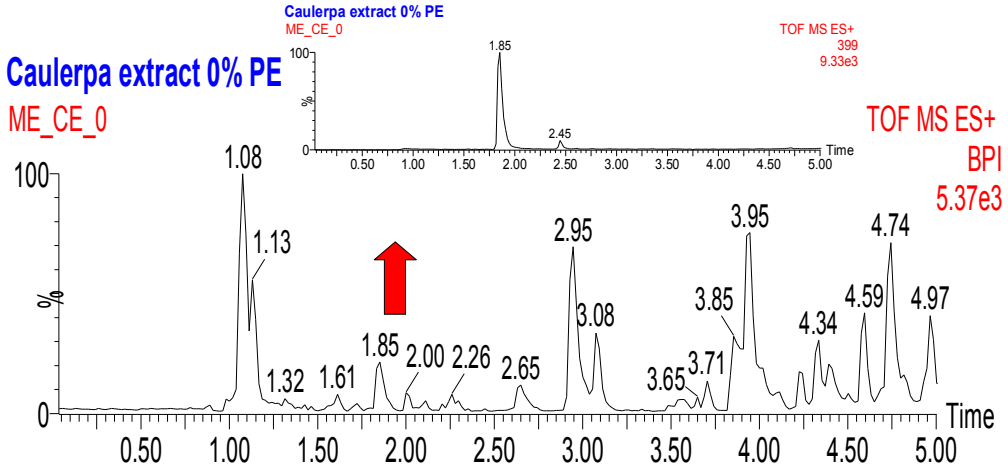


Figure 3.43 UPLC Chromatogram of 100% DEE Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

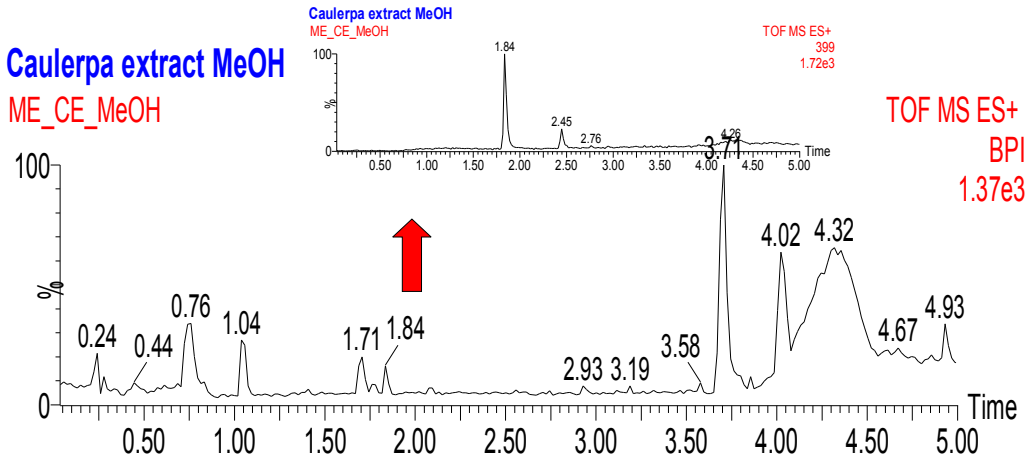


Figure 3.44 UPLC Chromatogram of 100% MetOH Fraction of CREE. Experiments were carried out at ES+. Red arrow demonstrates the abbreviated spectrum for CPN.

3.5.1.1.2 ESI/MS Spectrums. At the m/z value of 399 the caulerpin existence was observed in the fractions. The MS spectrums were shown below in the Figure 3.45-57.

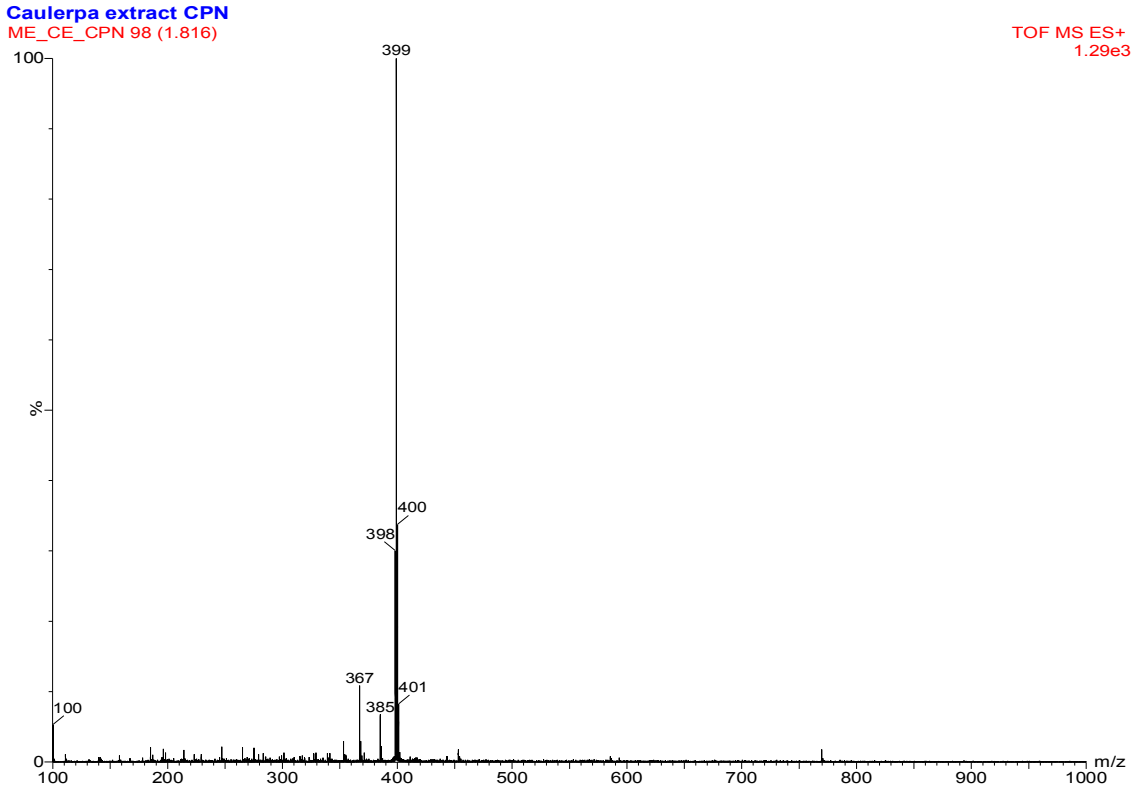


Figure 3.45 ESI/MS Spectrum of isolated CPN (CPN) from *C. racemosa*. Experiments were carried out at ES+.

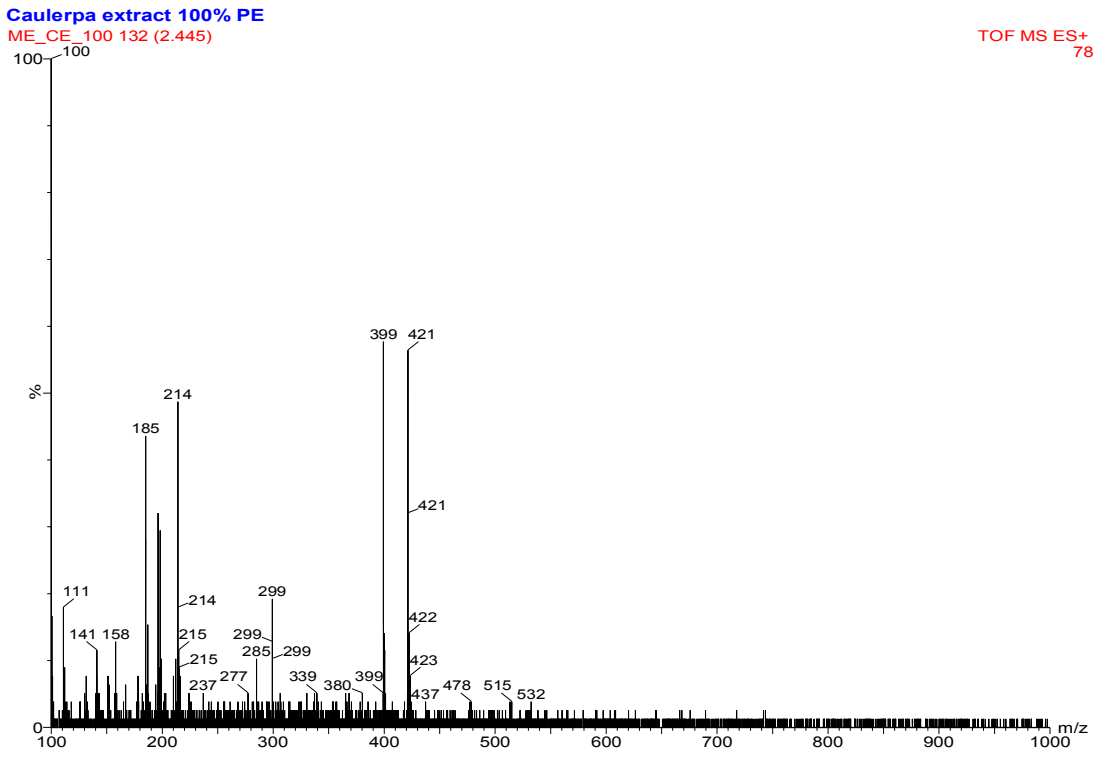


Figure 3.46 Spectrums of 100% PE Fraction of CREE. Experiments were carried out at ES+.

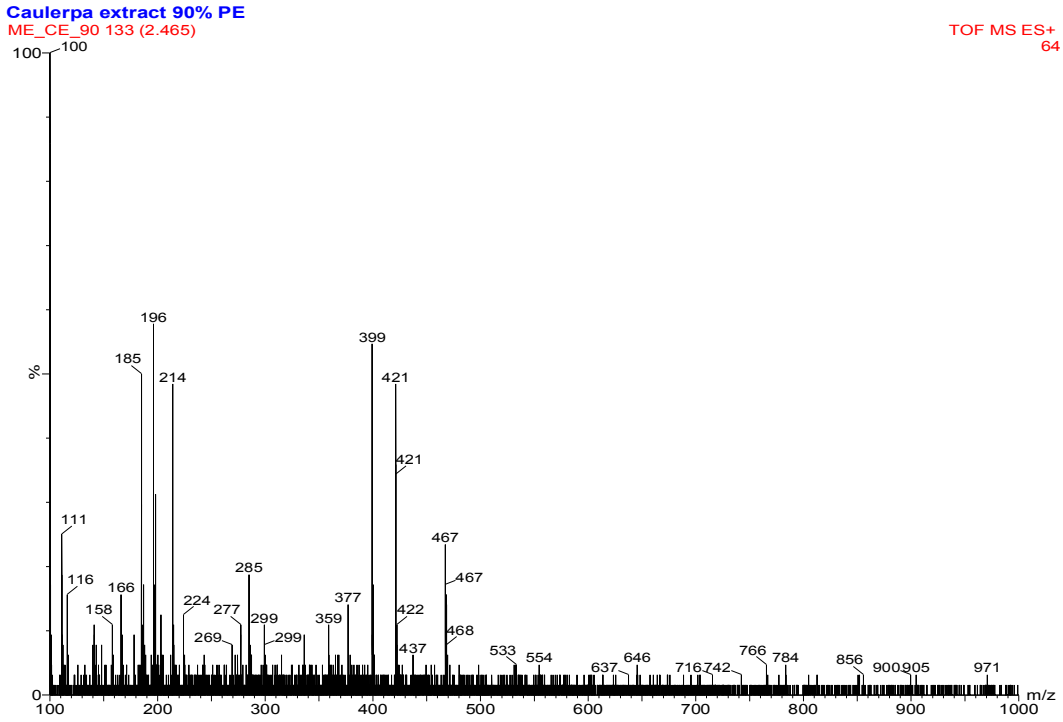


Figure 3.47 Spectrums of 90% PE - 10% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

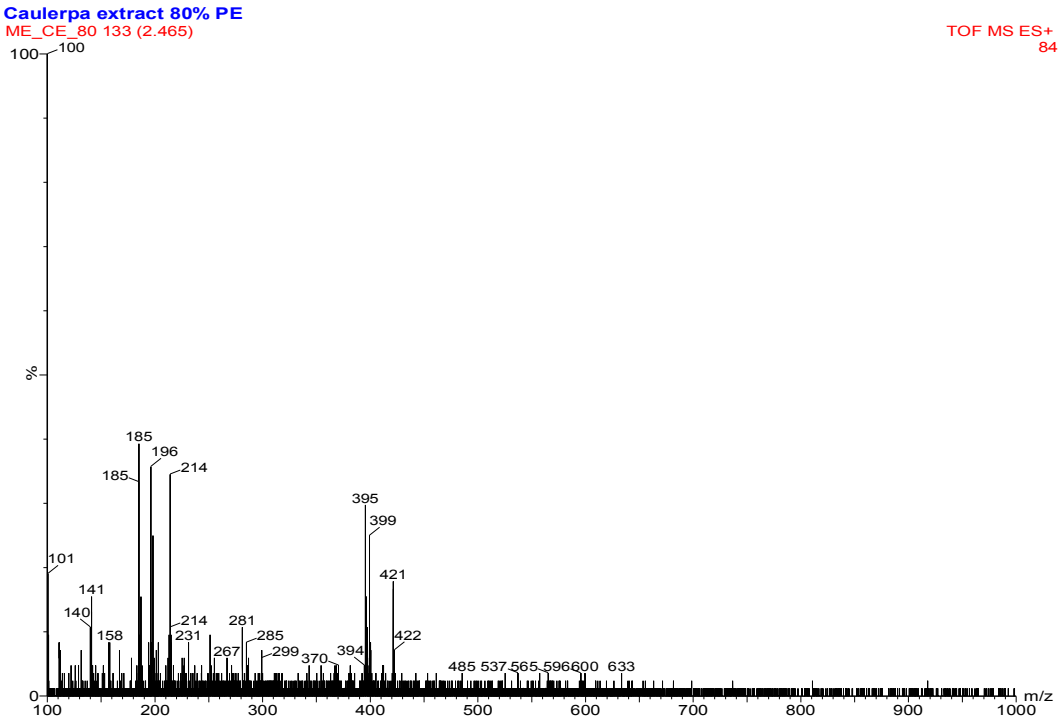


Figure 3.48 Spectrums of 80% PE - 20% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

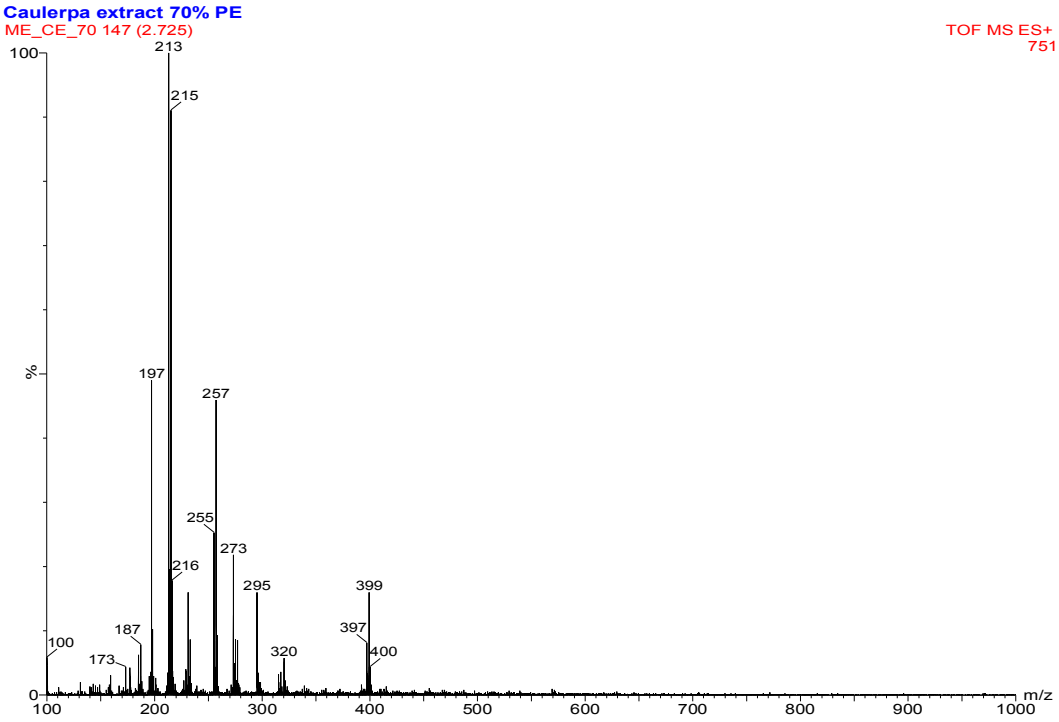


Figure 3.49 Spectrums of 70% PE - 30% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

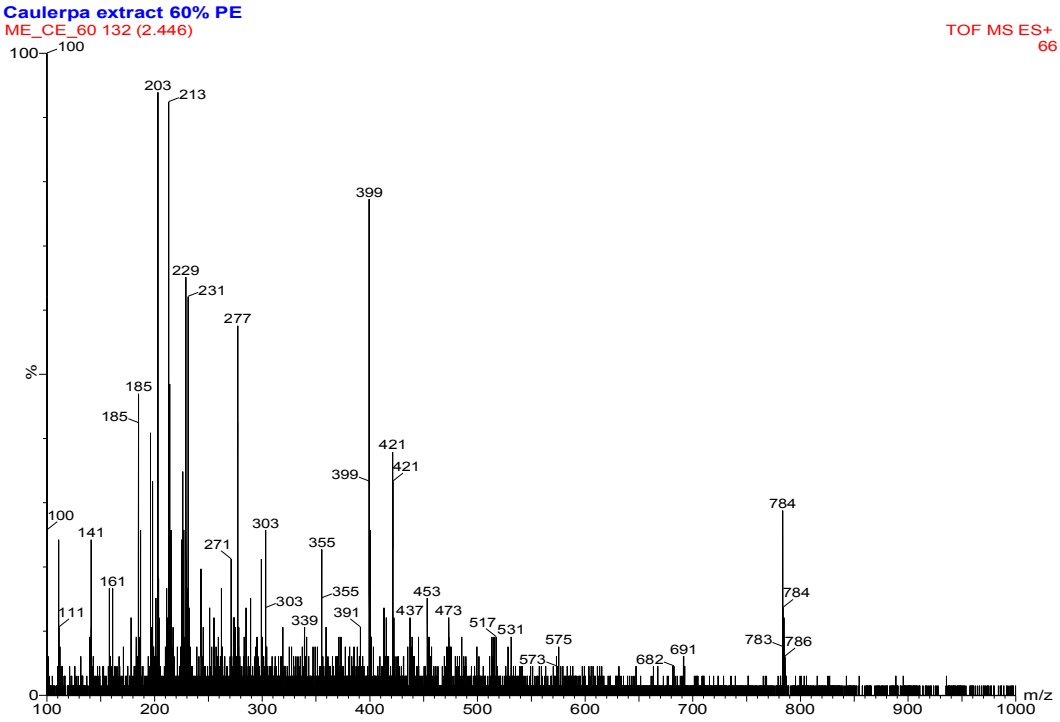


Figure 3.50 Spectrums of 60% PE - 40% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

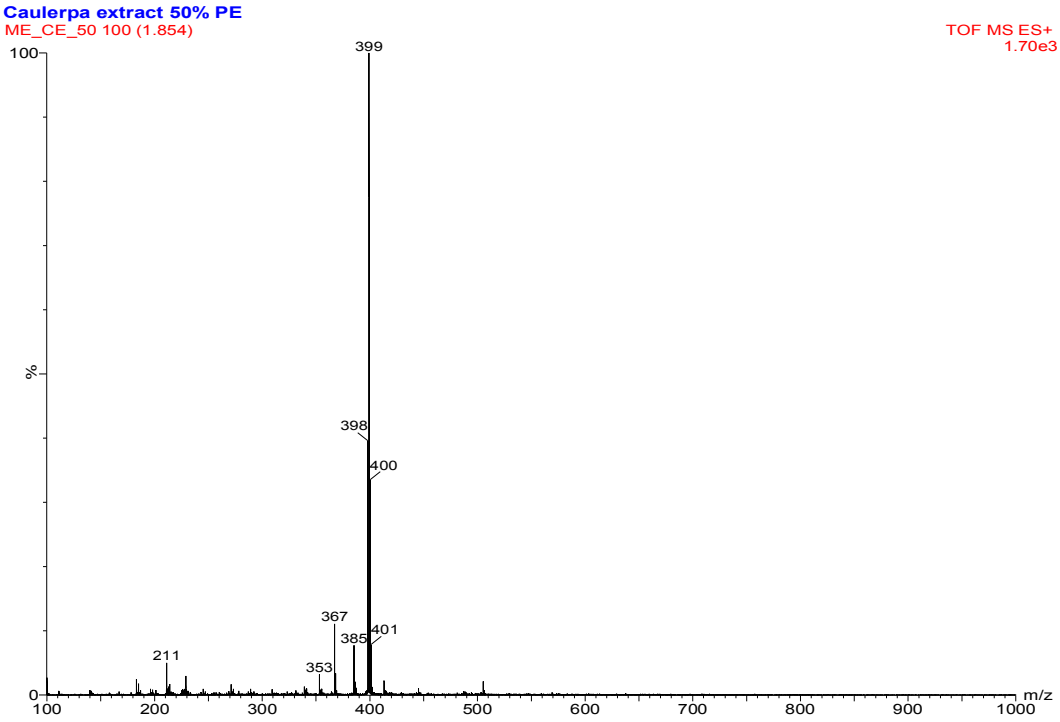


Figure 3.51 Spectrums of 50% PE - 50% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

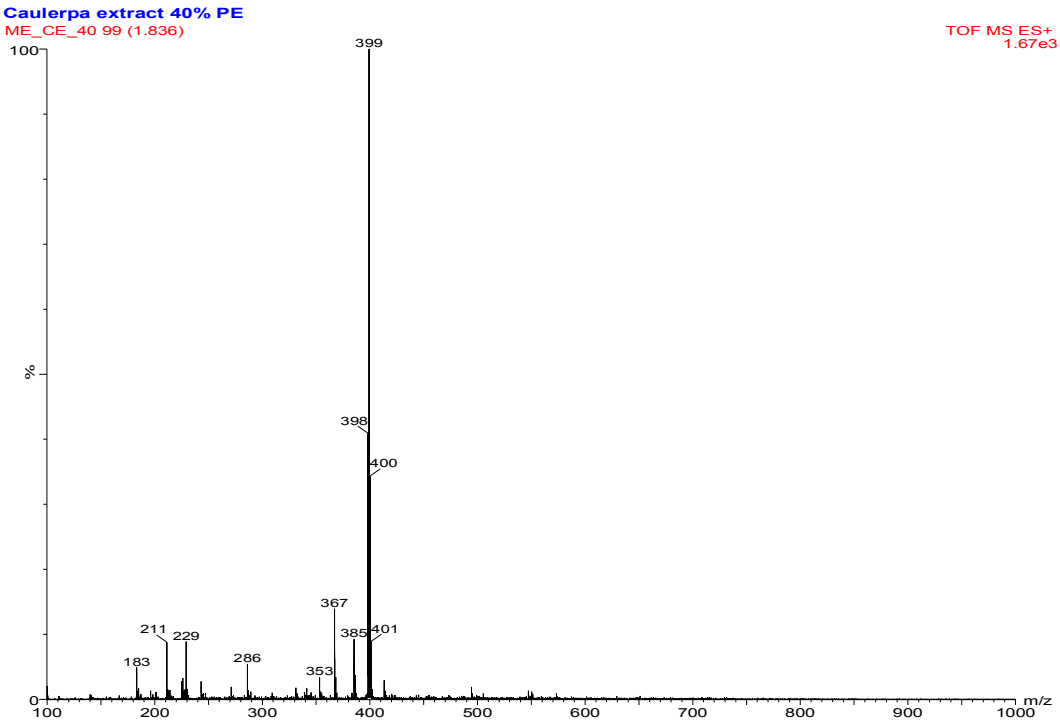


Figure 3.52 Spectrums of 40% PE - 60% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

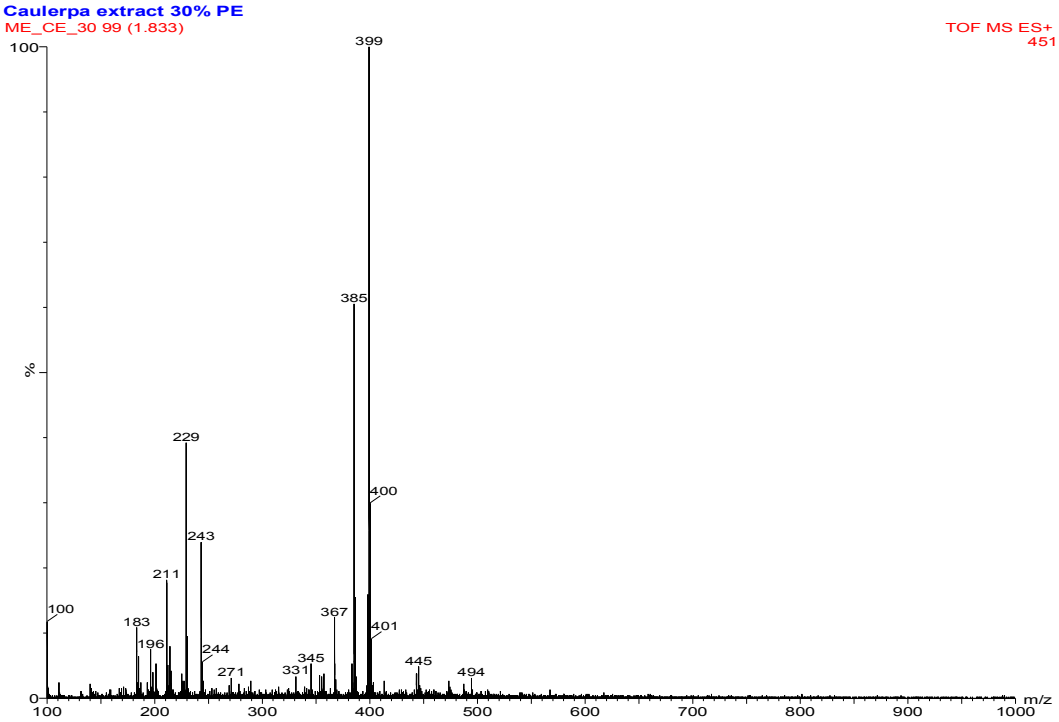


Figure 3.53 Spectrums of 30% PE - 70% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

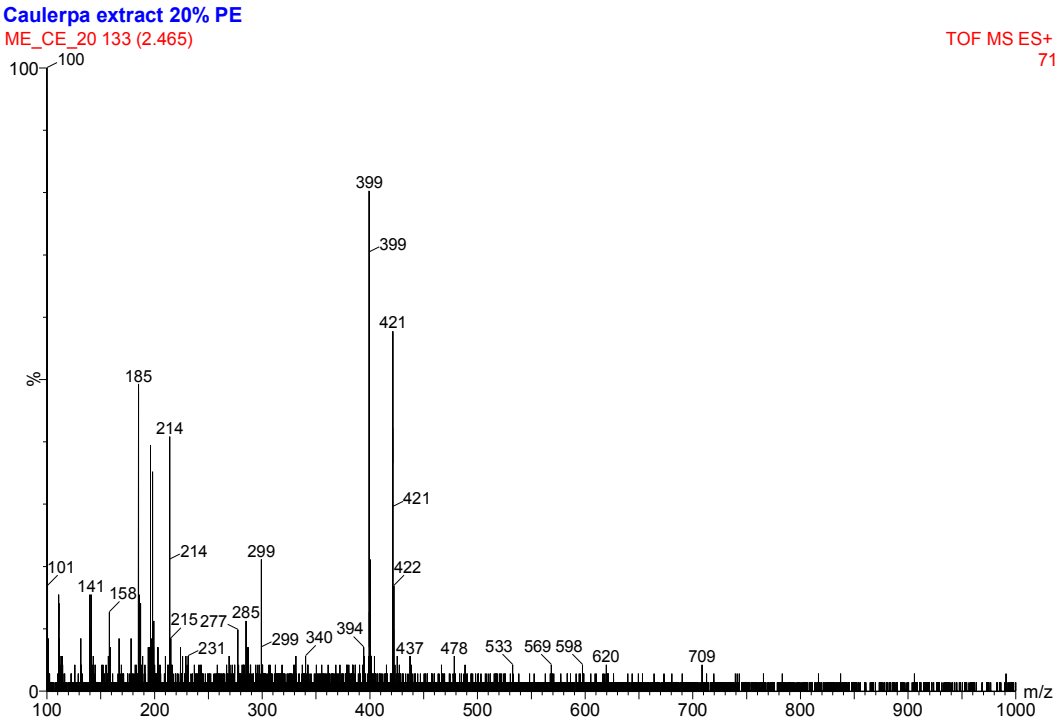


Figure 3.54 Spectrums of 20% PE - 80% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

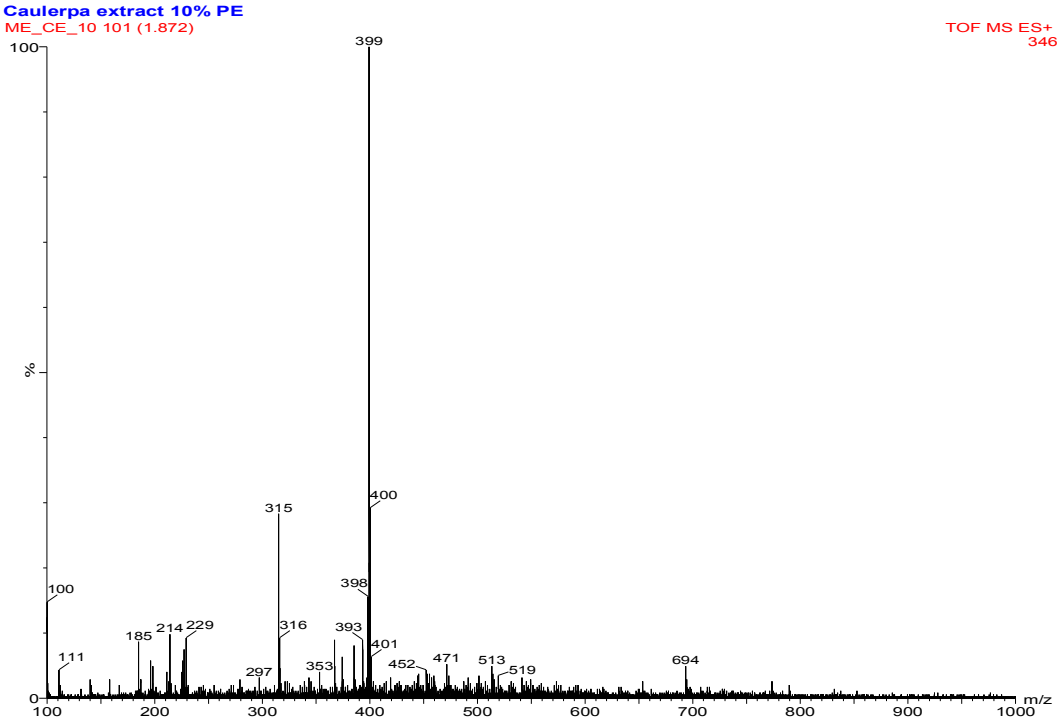


Figure 3.55 Spectrums of 10% PE - 90% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

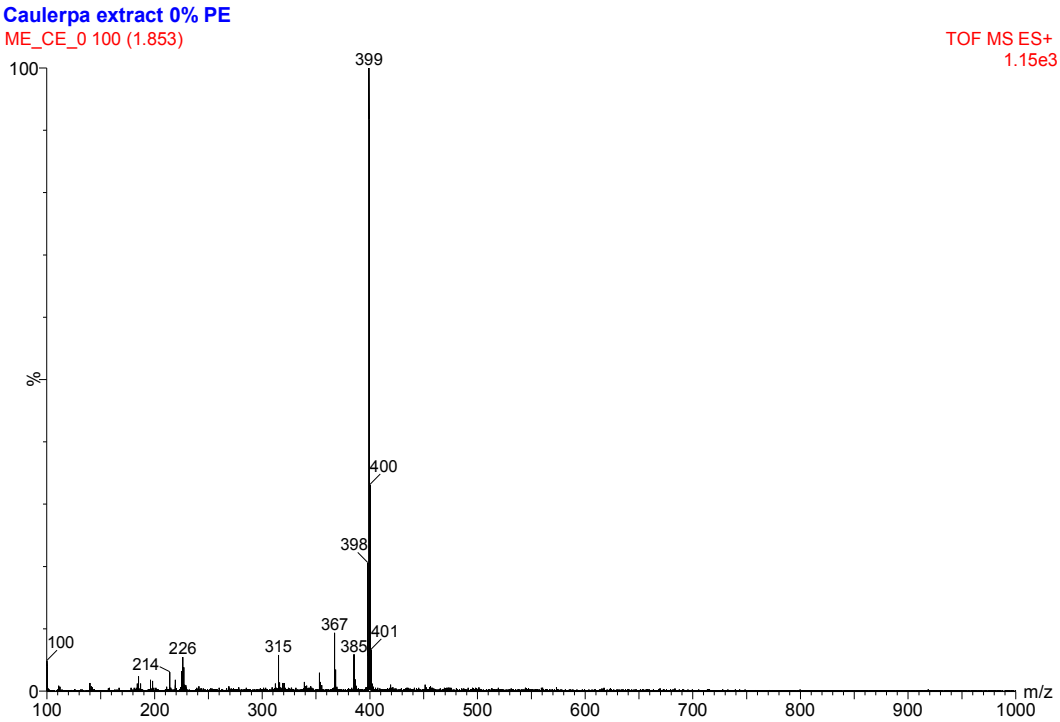


Figure 3.56 Spectrums of 100% DEE Petroleum Ether Fraction of CREE. Experiments were carried out at ES+.

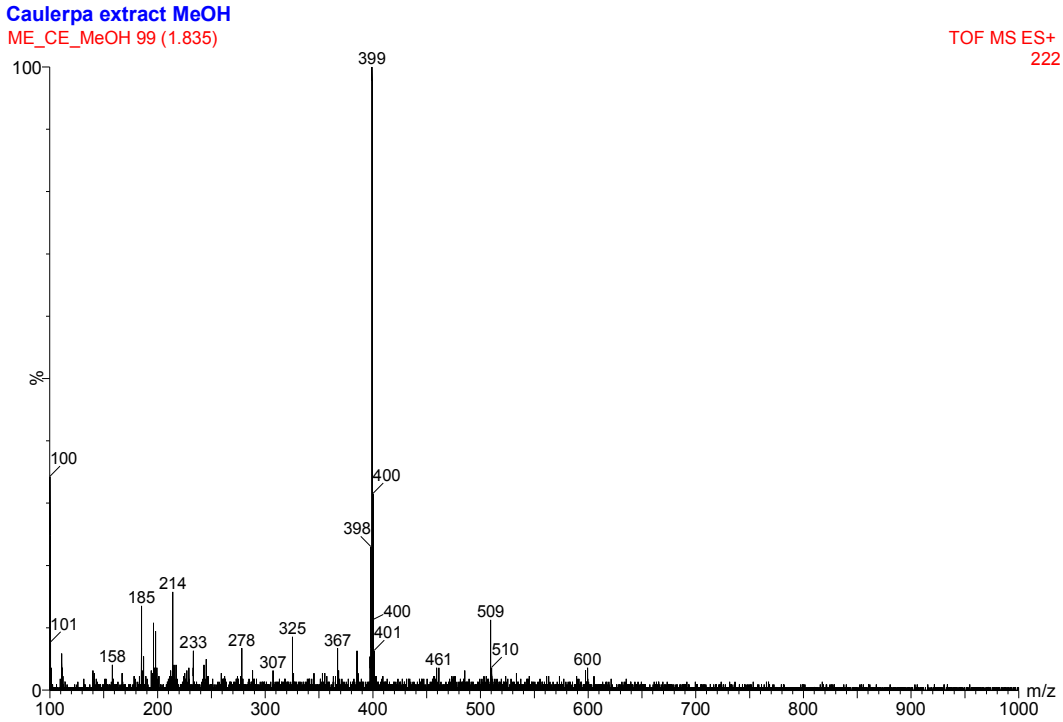


Figure 3.57 Spectrums of 100% Methanol Fraction of CREE. Experiments were carried out at ES+.

According to UPLC-ESI/MS measurements, CPN content of each fraction was demonstrated in Figures 3.32-57. UPLC-MS raw data were analyzed by MassLynx V4.1 (Waters). This application integrates peaks using ApexTrack peak detection the date were detected and noises were reduced in both of LC and MS graphics (http://prosolia.com).

As it has been demonstrated in each LC and MS graphics for all fractions, from non-polar fraction to polar fraction; CPN content was increased. Between 50%- 0% PE fractions, the existence of high amount of CPN content can be easily realized in 399 m/z.

In order to see the plant growth stimulating effect of each fraction, the growth experiments were carried out in following experiments. The fractions which include higher CPN concentrations significantly affected plant growth and existence of different active fractions was examined.

3.6 The Effects of Isolated CPN on the Growth of *P. oleracea* Coleoptile Cuttings

After the results of LC-MS, all the fractions of CREE were treated to the cuttings from *P. oleracea* and to see the effect of the fractions instead of whole extract. Surprisingly, a growth stimulation effect was seen in a fraction (60% PE) which has already been detected with low CPN content regarding to the other fractions.

In order to investigate the reason of this effect, new active fraction was separated with column chromatography. Then, to monitor the growth stimulating effect, another coleoptile cutting assay was carried out with the fractions of 60%. The results of this assay showed us that there was a stimulation effect on coleoptile elongation by an unknown chemical.

3.6.1 The Effects of CPN on Plant Growth

A positive growth stimulating effect of CPN was observed with the experiment group 0.1 and 1 µg/ml of CPN. By increasing the concentration of the CPN, a decrease was observed for the coleoptile cuttings. This information leads quite important information about the metabolites of *C. racemosa* (figure 3.58).

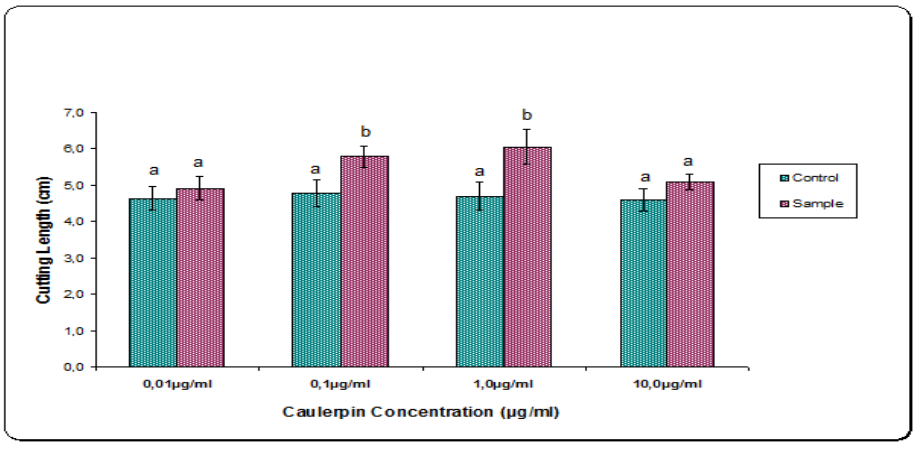


Figure 3.58 Effect of CPN on the growth of *P. oleracea* coleoptile cuttings. Blue bars demonstrate the controls and the red ones demonstrate length differentiations with concentrations of CPN. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

3.6.2 The Effects of Fractionated CREE*s on the Growth of P. oleracea Coleoptile Cuttings*

The growth stimulating effect on the fractions was observed between 0%-40% petroleum ether fractions which are previously detected with higher CPN content.

It was expected an increase of growth for this experimental group because of the plant growth stimulating effect of CPN which was demonstrated in prior growth experiments. However, there was a really interesting result of growth stimulation which shows itself in the experiment for 60% fraction of extract. 60% fraction has a definitely low CPN content but there was a significant growth stimulation effect existed on coleoptile cuttings (Figure 3.59).

This result was absolutely important to investigate structural elucidation methods. For the further experiments, it was attempted to find out the chemicals which caused this stimulation effect which was observed in 60% fraction.

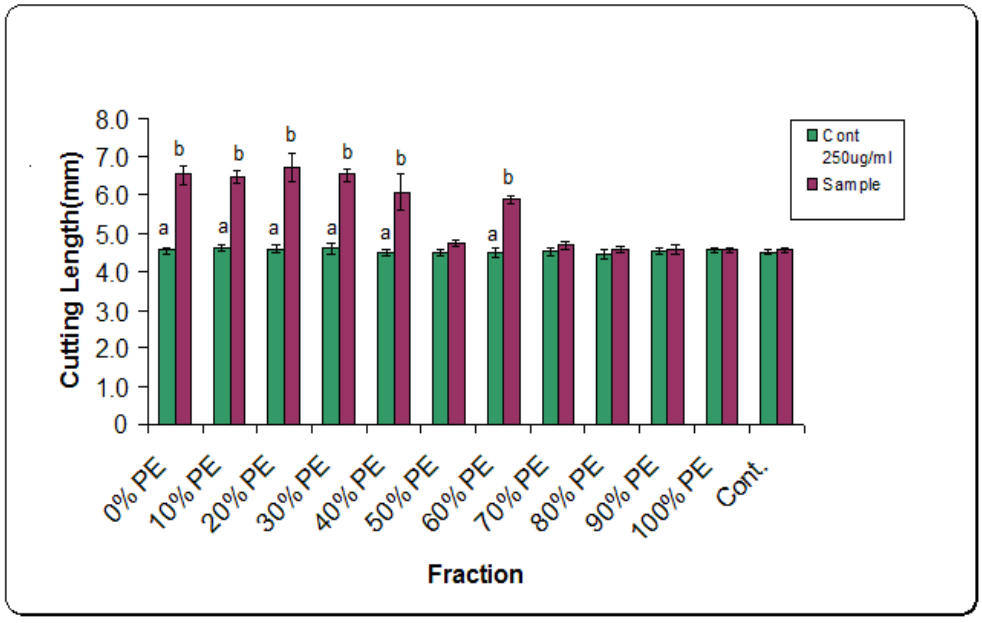


Figure 3.59 Effect of fractionated *C. racemosa* extracts on the *P. oleracea* cuttings (with the concentration of 250µg/ml). Green bars demonstrate the controls and the purple ones demonstrate length differentiations with the fractions. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

In order to determine the molecules which were responsible of this stimulation, CREE was separated one more time with the same procedure. New fractions were investigated by the coleptile cutting assay and the unknown molecules inside of active 60% fraction was attempted to identify by using NMR spectroscopy. Obtained data was shown in following parts.

3.7 The Effects of Separated Fractions of 60% *C. racemosa* Extract on the Growth of *P. oleracea* Coleoptile Cuttings

Figure 3.60 belonged to the fraction of 60% CREE. Figure showed that especially 40%, 50 %, 70%, 80% and 90% fragments caused a highly important stimulation effect. For a complex tissue extract, these results were obviously important and at the same time quite difficult task to do. There was no CPN content in 60% fraction; therefore it was important to investigate the fraction. In order to prove this aim NMR spectrums were determined with the following experiments.

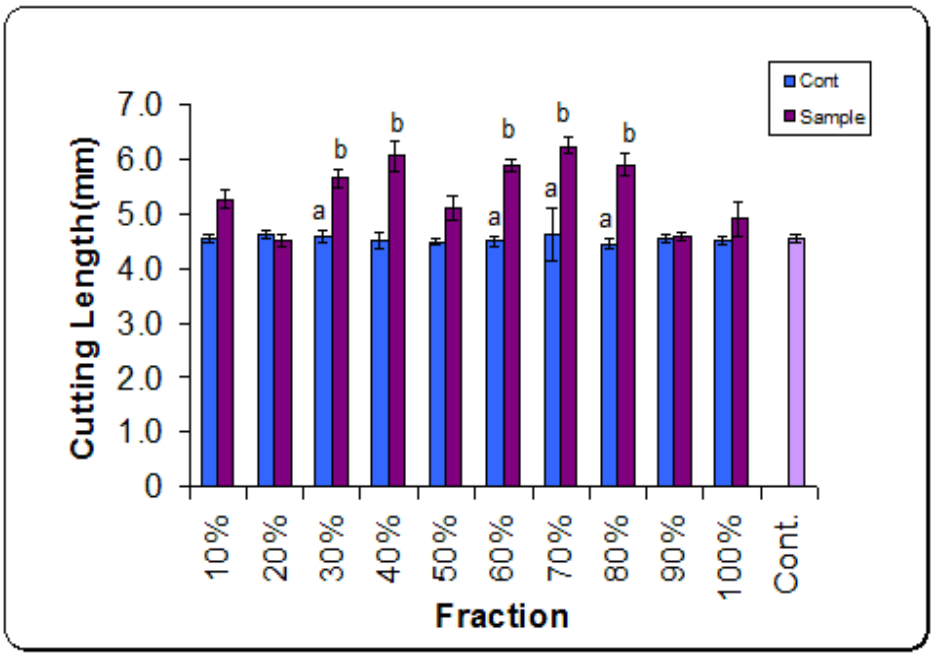


Figure 3.60 The effect of separated fractions of 60% *C. racemosa* extract on shoot cuttings of the plant *P. oleracea*. Blue bars demonstrate the controls and the purple ones demonstrate cutting length differentiations with the fractions. Different letters on the error bars of the graphics show statistical differences at $p<0.05$.

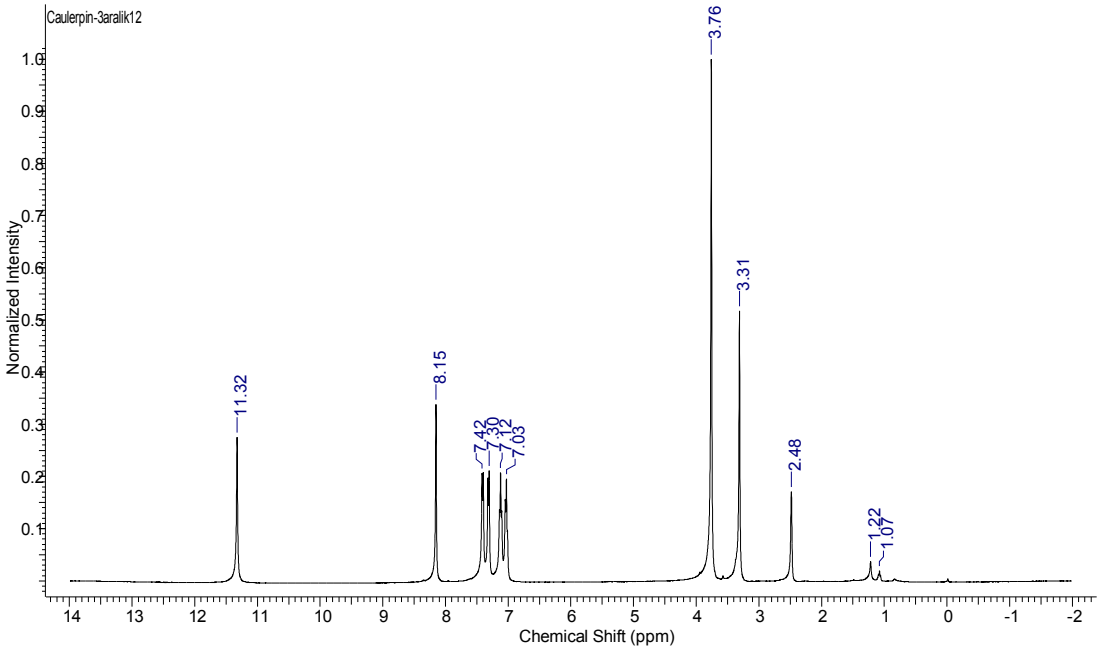
3.8 NMR Measurements

3.8.1 Caulerpin

When we compare the obtained ^{1}H NMR data that shown in figure 3.81, it was possible to get some information about the structure basically. The peak at the point of 11.32 ppm demonstrates us the proton bounds to the nitrogen atom in the cycle which was shown in Figure 3.82 with the number of 5.

The peak on the 8.15 ppm was the signal from the proton of olefinic CH group with the number of 7 in the Figure 3.82. The peaks on the ppm value of 7.03, 7.12, 7.30 and 7.42 are the protons from the benzene group of the indole ring. These protons are shown in the Figure 3.82 with the numbers of 1, 2, 3 and 4. Splitting on the peaks demonstrates the interactions between these protons, as a result of confirmation of the chemical structure.

The peak on the 3.76 ppm demonstrates the proton of the methoxy group with the number of 6. The peak of 3.31 shows us the protons coming from the water inside of DMSO. Deuterated dimethyl sulfoxide was used as NMR solvent. Because of this, the peak which was shown in 2.48 ppm was related to the signal of the solvent for the measurements. The signals on the 1.22 and 1.07 were the results of the residues from the membrane bounding aliphatic chains of the CPN molecule.



No.	(ppm)	(Hz)	Height
1	1.07	429.9	0.0190
2	1.22	486.8	0.0376
3	2.48	992.7	0.1709

No.	(ppm)	(Hz)	Height
4	3.31	1323.4	0.5176
5	3.76	1502.8	1.0000
6	7.03	2810.6	0.1952

No.	(ppm)	(Hz)	Height
7	7.12	2847.6	0.2075
8	7.30	2920.6	0.2113
9	7.42	2965.8	0.2059

No.	(ppm)	(Hz)	Height
10	8.15	3259.9	0.3384
11	11.32	4528.6	0.2756

Figure 3.81 ^{1}H NMR spectrum of isolated Caulerpin.

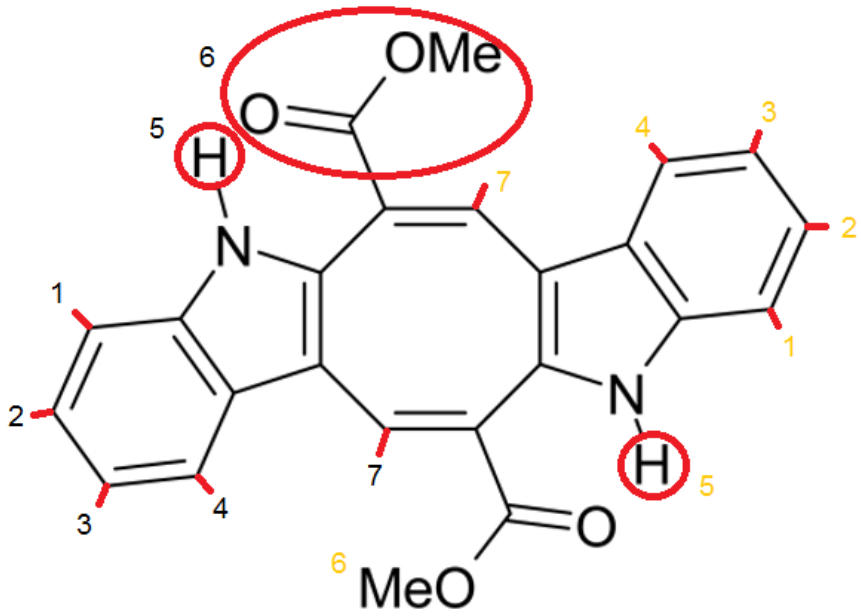
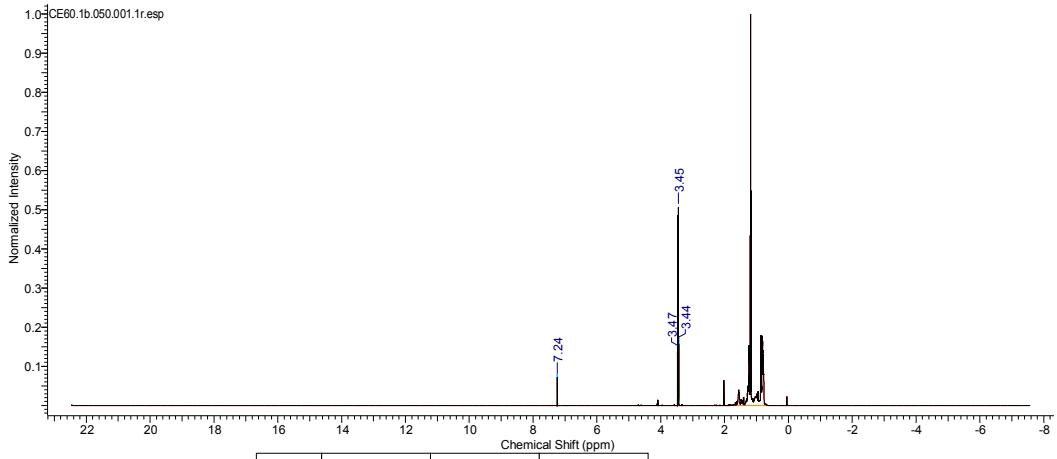


Figure 3.82 Structure of Caulerpin.

3.8.2 The Fractions of 60% PE Active Fraction

As it was discussed and interpreted ^{1}H NMR results of CPN in the last part, the existence of CPN was demonstrated and the structure was elucidated by using the NMR and UPLC-MS data. Afterwards in order to investigate the bio-stimulation effect of the active fractions on the plant growth, new active fractions were separated with column chromatography and examined with NMR spectroscopy. In addition to this, it is possible to see from the results, there were some active compounds exist in the *C. racemosa*. Considering the complication of the alga tissue, it was quite challenging task to elucidate all the active compounds which were effective on the plant growth. According to the results of these NMR experiments, it was not possible to demonstrate the positive effect of the fractions of 60% PE *C. racemosa* extract. The visible results of all experiments indicate that, there was an existence of important metabolites related to plant growth. As a future plan, it will more deeply be investigated by our team.

In order to determine the unknown molecules, new fractions of 60% were examined with NMR spectroscopy. The peak on the 7.24 ppm demonstrates the signal of chloroform as the NMR solvent. However, the rest of the peaks were not identified because of the low sample concentration. The COSY graphics was also shown above with the ^{1}H NMR results. However, more detailed structure elucidation was needed. The COSY figures were added to to this thesis so that the readers of this thesis can benefit from the data. These studies can be carried out by scientists who were interested in this field. For the further researches of our experimental group, these topics will investigate more deeply.



No.	(ppm)	(Hz)	Height
1	3.44	2062.3	0.1565
2	3.45	2069.4	0.4981
3	3.47	2083.7	0.1393
4	4.09	2455.1	0.0139
5	7.24	4345.0	0.0716

Figure 3.62 ^{1}H spectrum of 10% fraction of 60% PE *C. racemosa* extract.

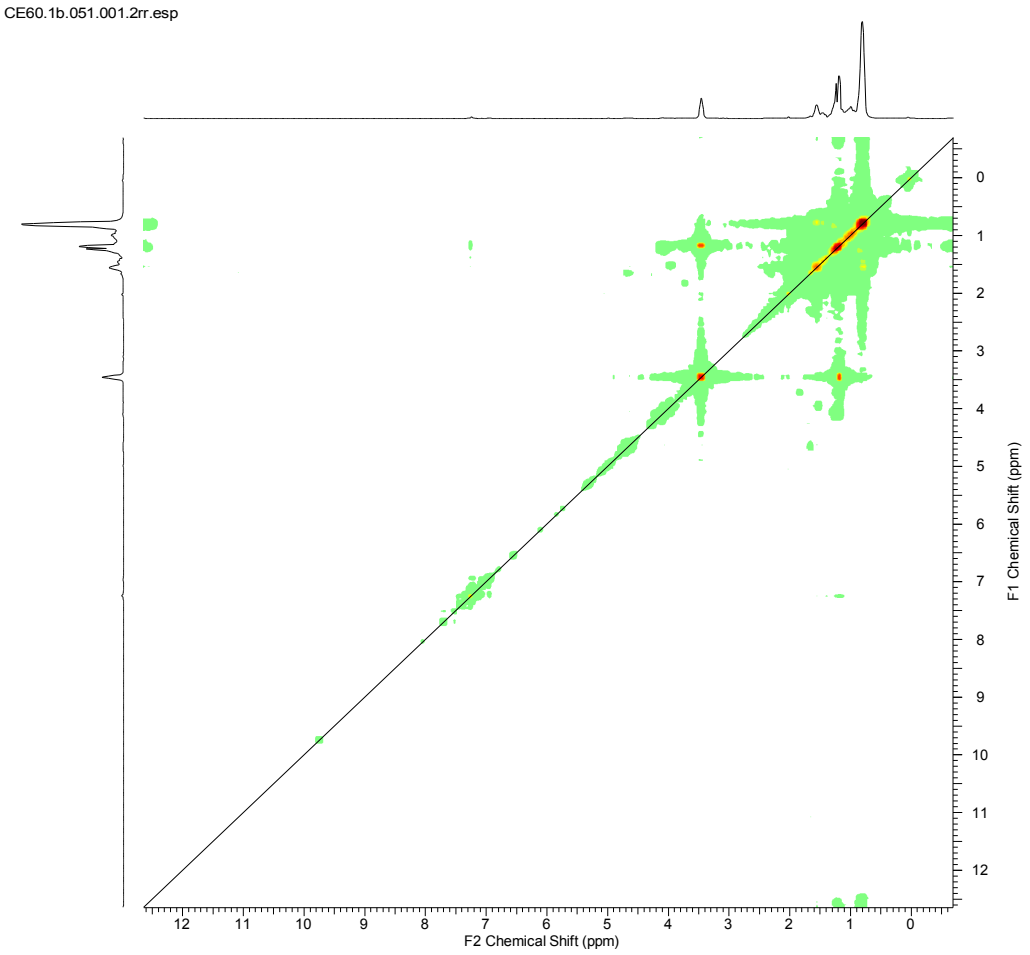
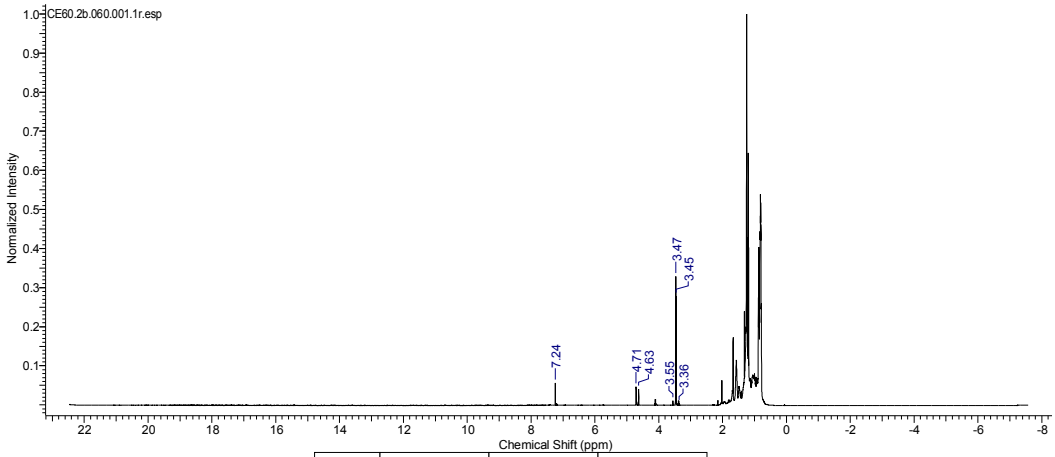


Figure 3.63 COSY spectrum of 10% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.36	2017.6	0.0042
2	3.45	2073.2	0.2784
3	3.47	2080.3	0.3283
4	3.55	2133.1	0.0105
5	4.63	2779.1	0.0411
6	4.71	2825.8	0.0466
7	7.24	4344.4	0.0558

Figure 3.64 ^{1}H spectrum of 20% fraction of 60% PE *C. racemosa* extract.

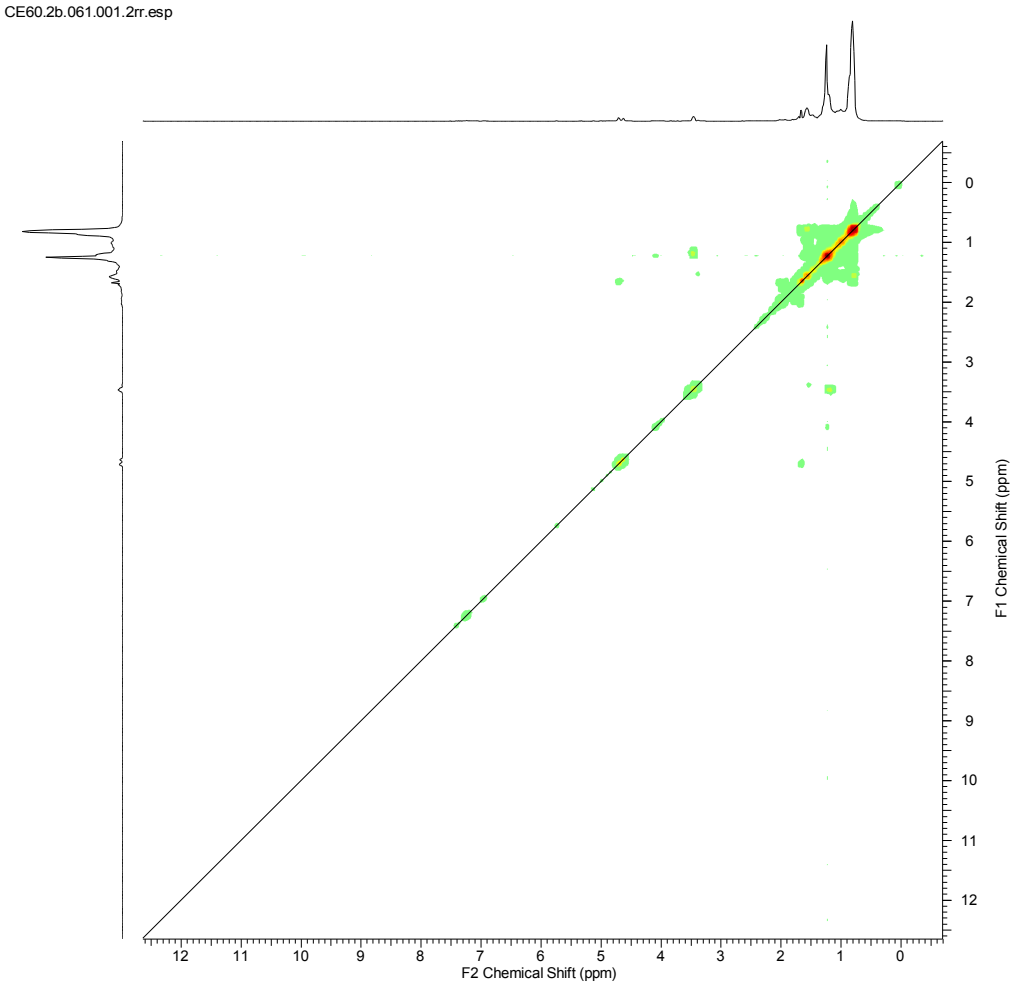
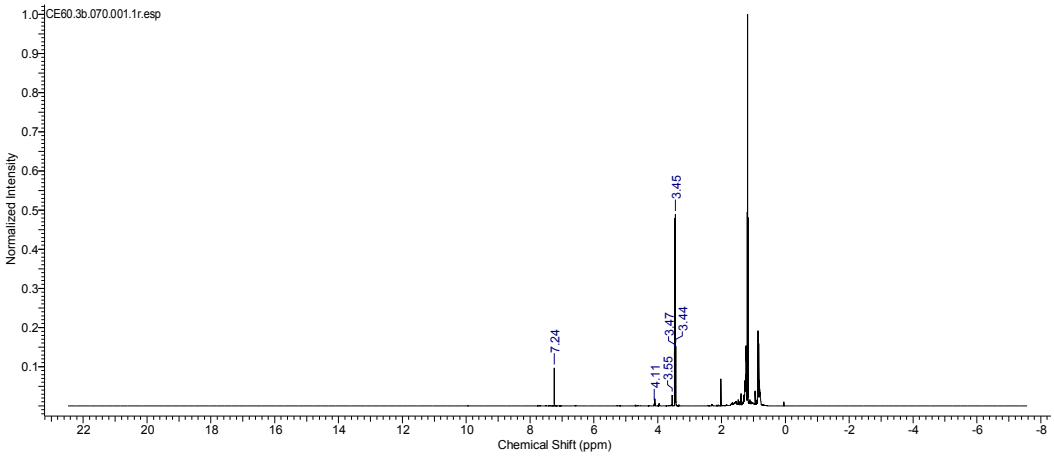


Figure 3.65 COSY spectrum of 20% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.44	2062.1	0.1530
2	3.45	2069.2	0.4896
3	3.47	2083.5	0.1399
4	3.55	2128.1	0.0278
5	4.11	2469.2	0.0058
6	7.24	4344.8	0.0966

Figure 3.66 ^{1}H spectrum of 30% fraction of 60% PE *C. racemosa* extract.

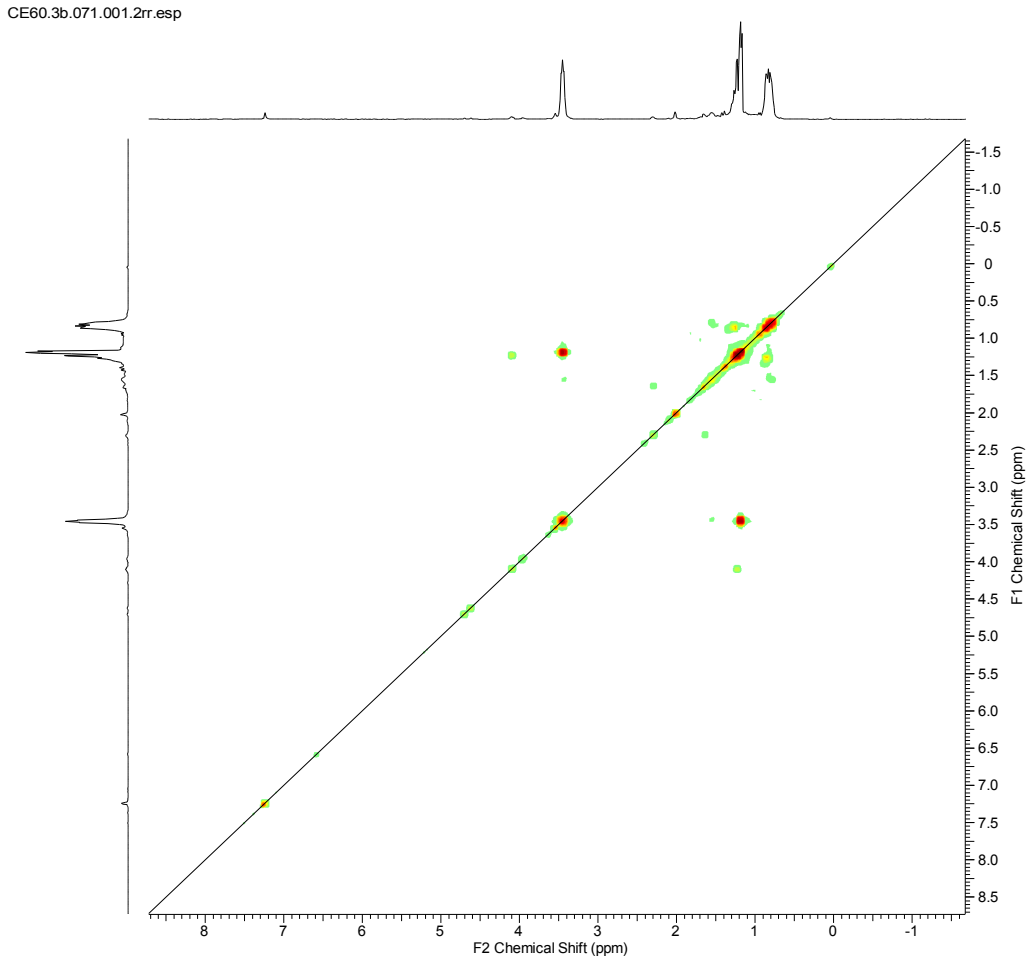
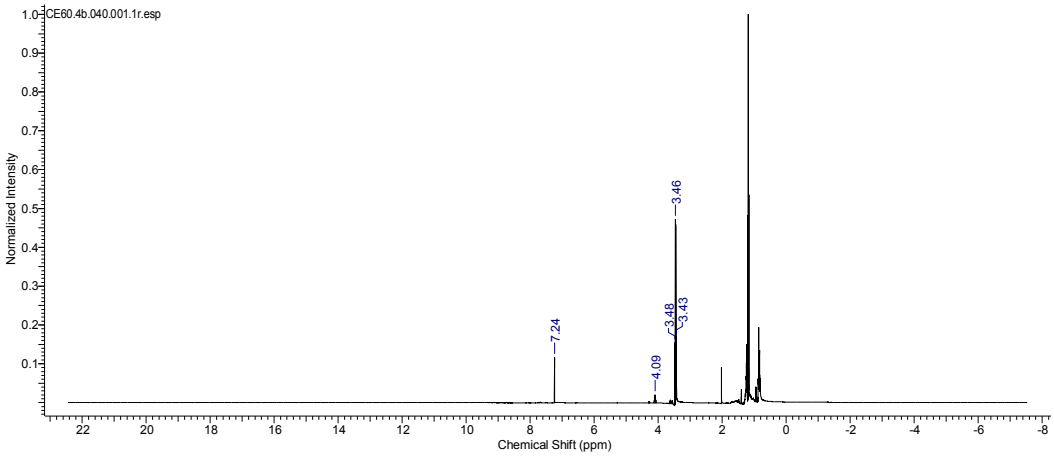


Figure 3.67 COSY spectrum of 30% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.43	1371.7	0.1697
2	3.46	1385.9	0.4729
3	3.48	1392.9	0.1543
4	4.09	1635.9	0.0209
5	7.24	2897.2	0.1165

Figure 3.68 ^{1}H spectrum of 40% fraction of 60% PE *C. racemosa* extract.

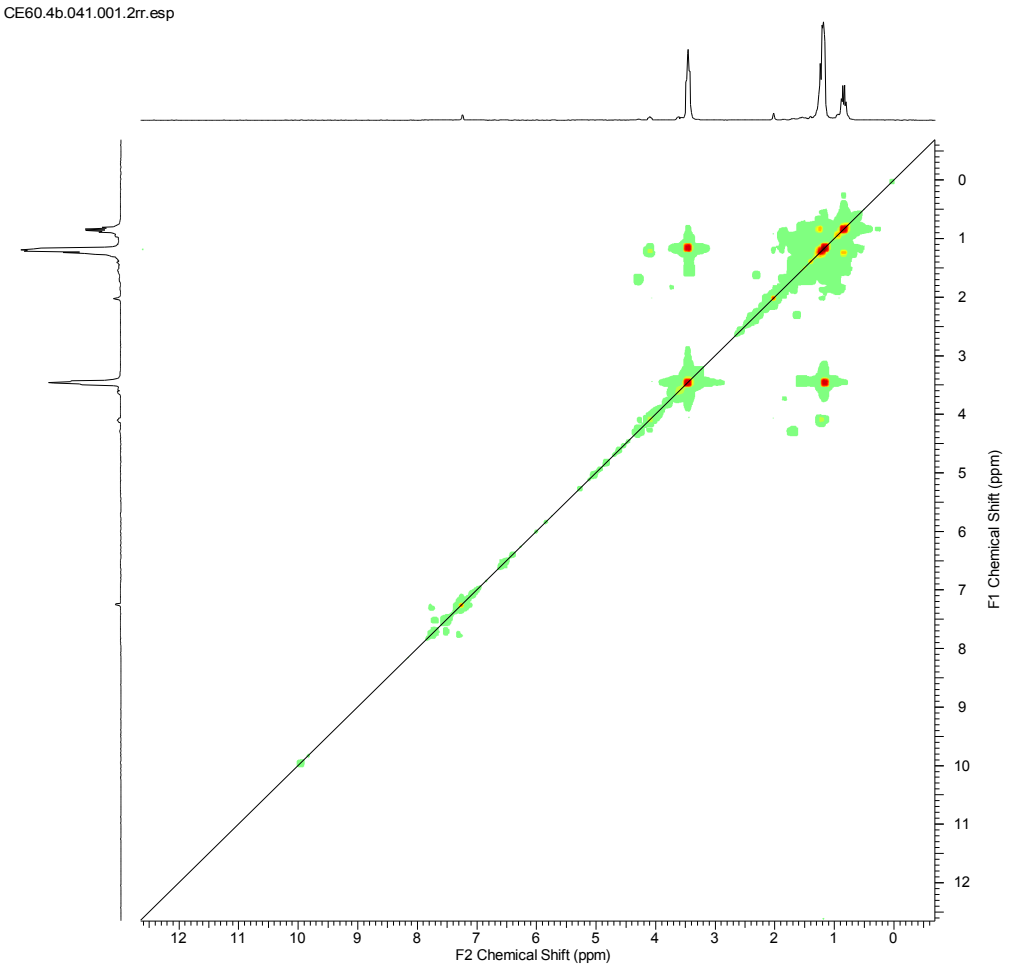
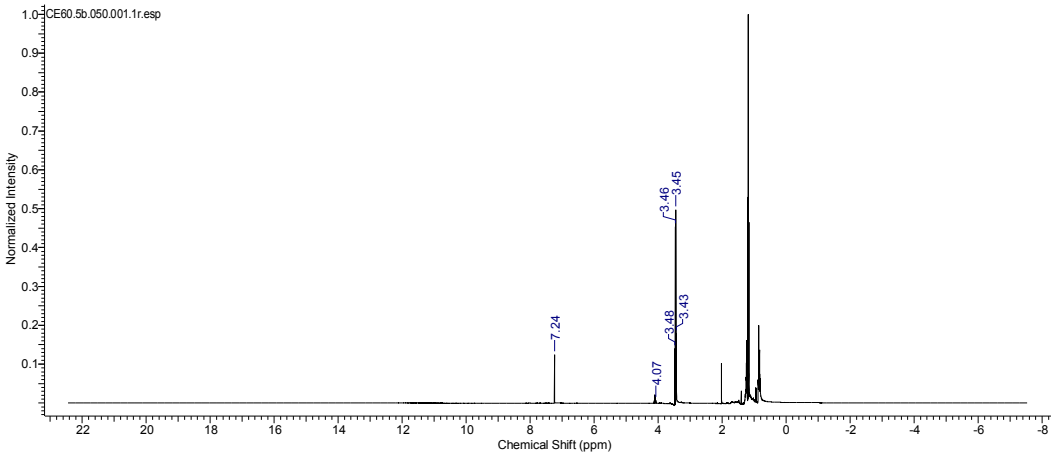


Figure 3.69 COSY spectrum of 40% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.43	1372.0	0.1787
2	3.45	1378.9	0.4975
3	3.46	1385.9	0.4537
4	3.48	1393.2	0.1403
5	4.07	1628.9	0.0073
6	7.24	2897.1	0.1242

Figure 3.70 ^{1}H spectrum of 50% fraction of 60% PE *C. racemosa* extract.

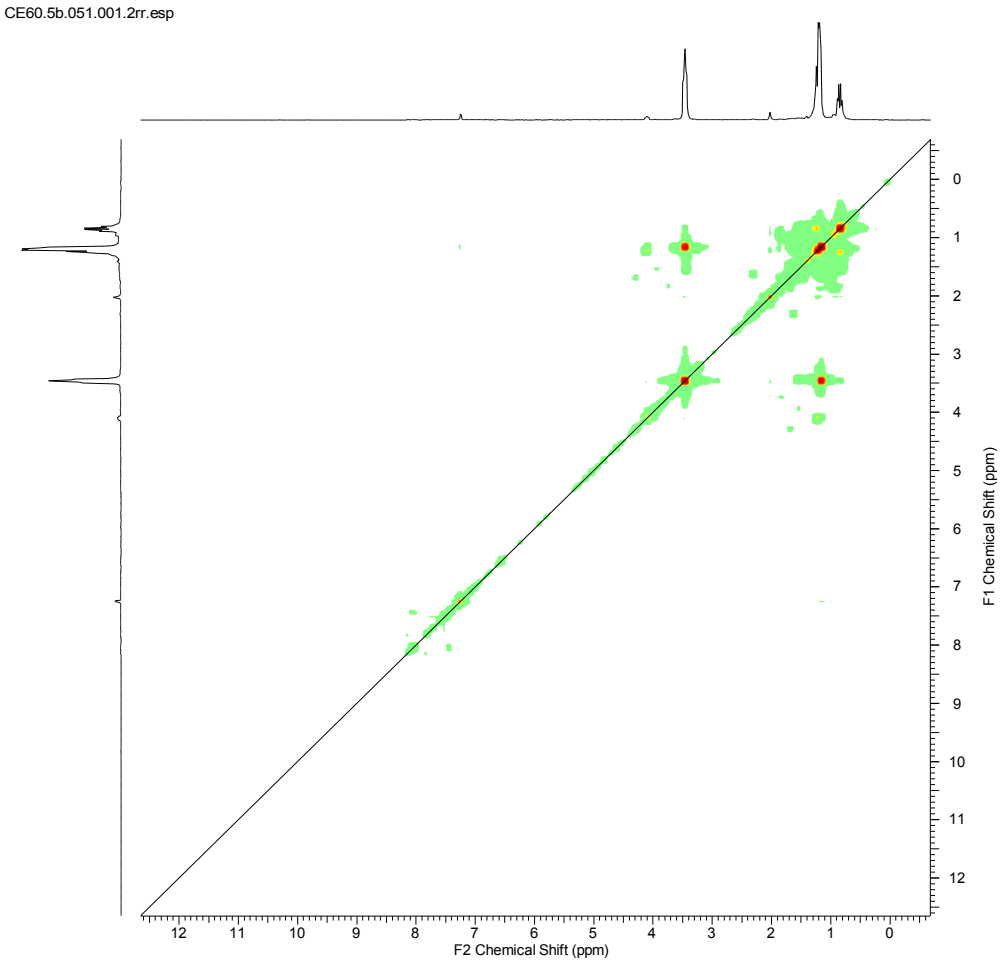
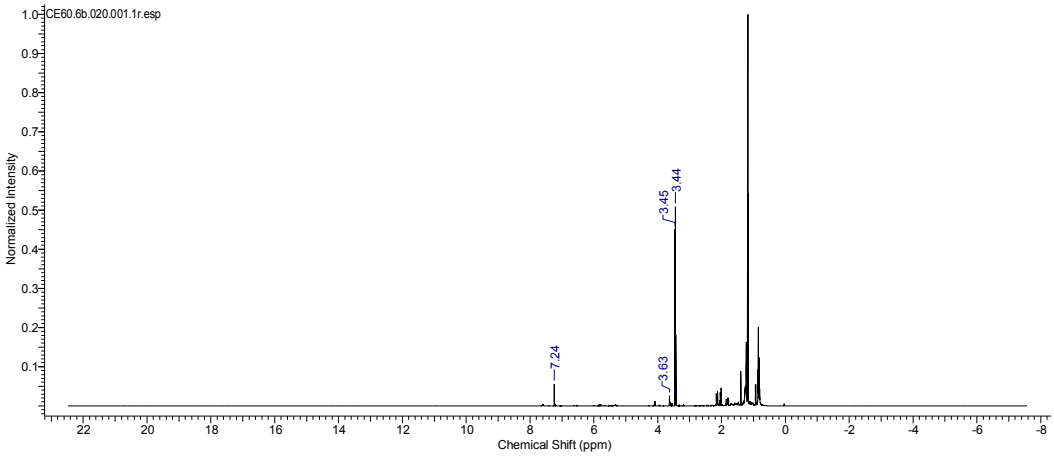


Figure3.71 COSY spectrum of 50% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.44	2065.4	0.5090
2	3.45	2072.0	0.4506
3	3.47	2080.2	0.0222
4	3.63	2179.8	0.0255
5	7.24	4344.3	0.0554

Figure 3.72 ^{1}H spectrum of 60% PE fraction of 60% PE *C. racemosa* extract.

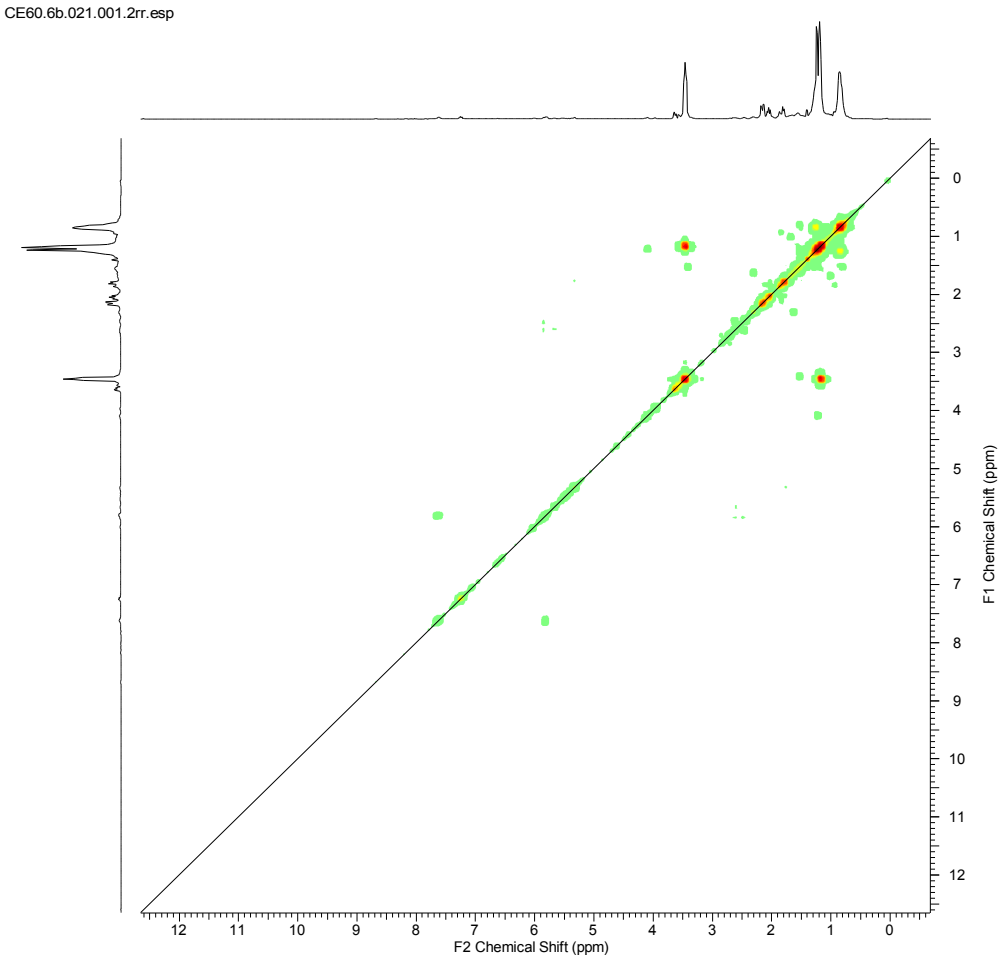
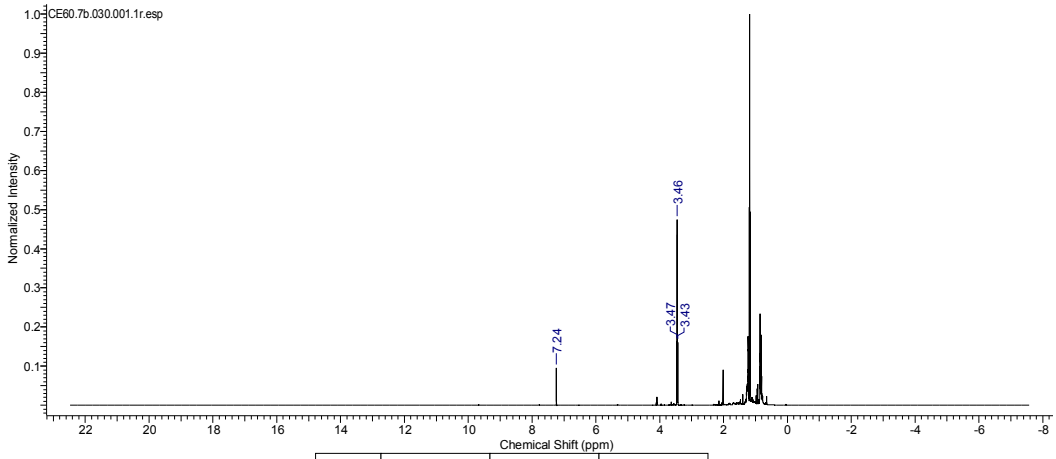


Figure 3.73 COSY spectrum of 60% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.43	2060.9	0.1611
2	3.46	2074.7	0.4745
3	3.47	2081.8	0.1639
4	7.24	4343.7	0.0958

Figure 3.74 ^{1}H spectrum of 70% fraction of 60% PE *C. racemosa* extract.

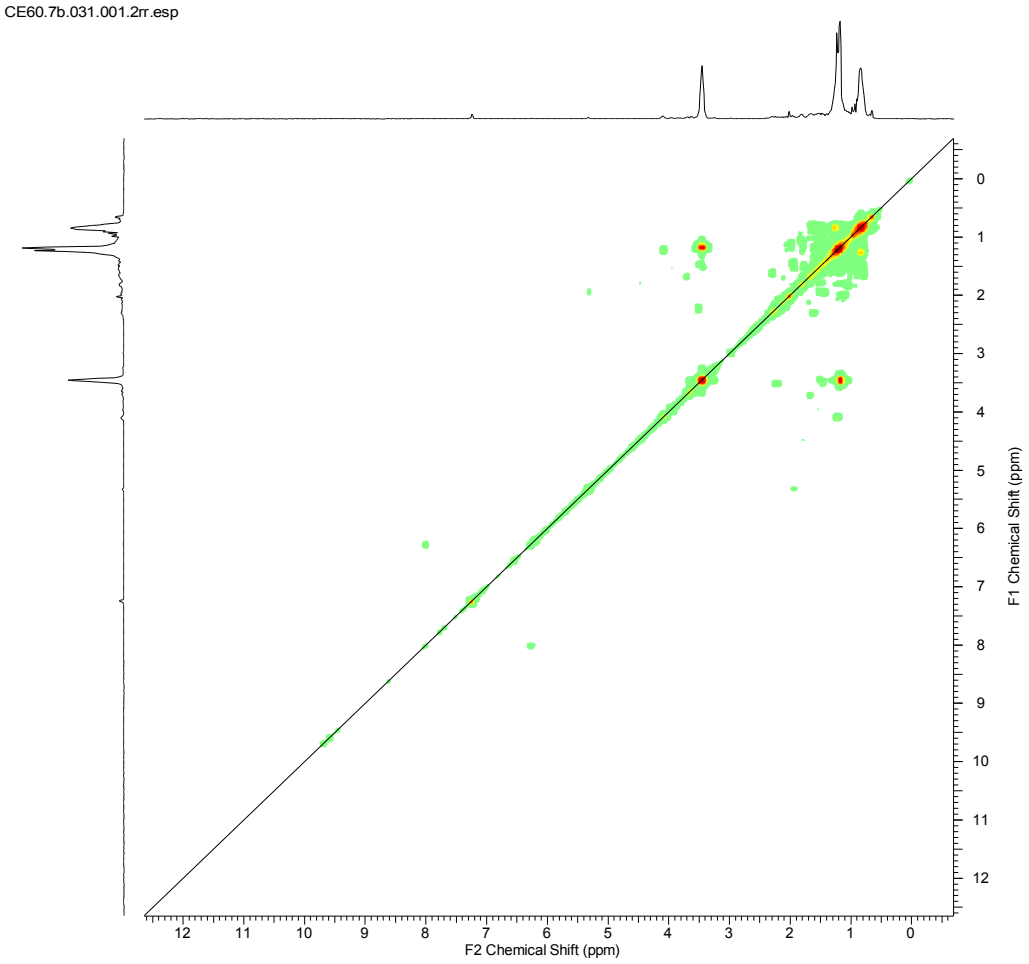
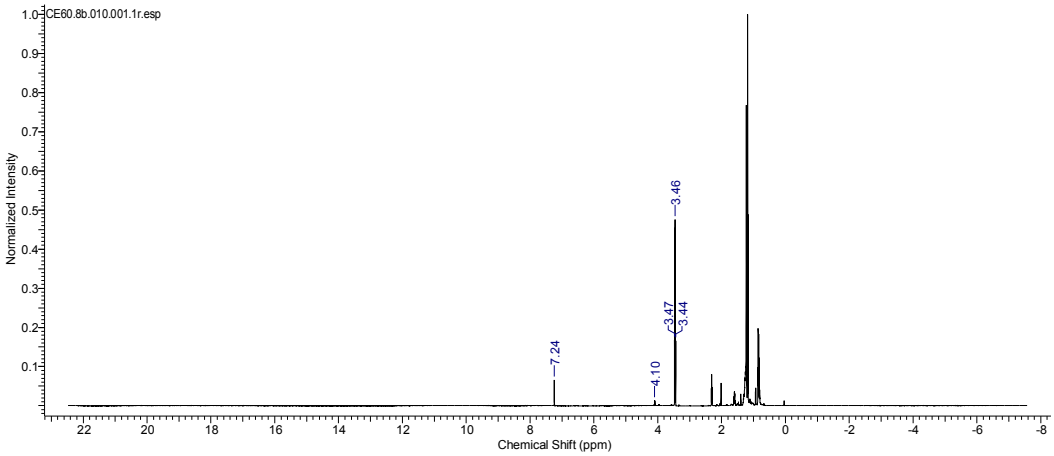


Figure 3.75 COSY spectrum of 70% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.44	2062.8	0.1658
2	3.46	2076.5	0.4759
3	3.47	2083.7	0.1676
4	4.10	2460.0	0.0126
5	7.24	4344.4	0.0658

Figure 3.76 ^{1}H spectrum of 80% fraction of 60% PE *C. racemosa* extract.

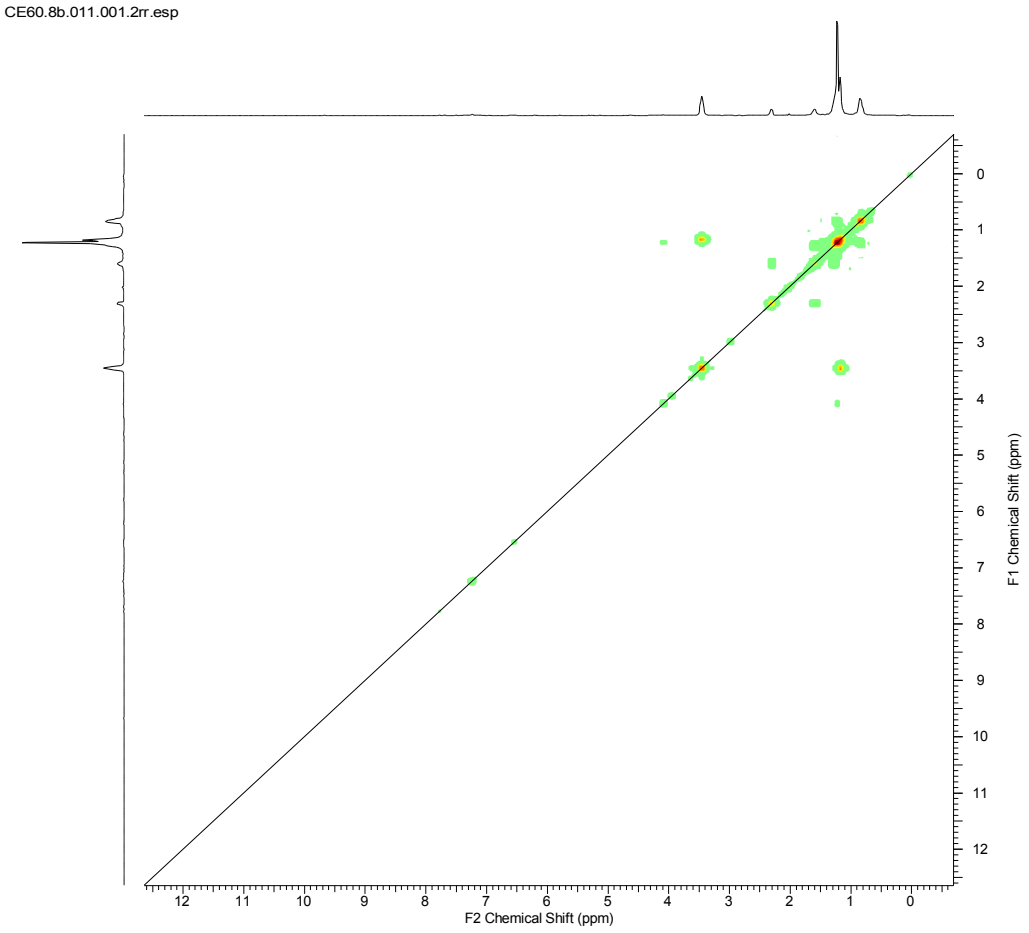
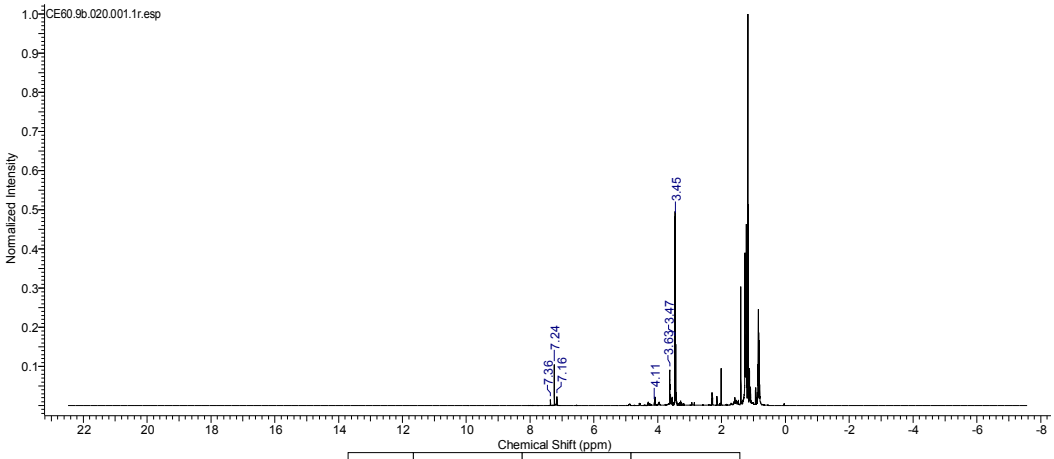


Figure 3.77 COSY spectrum of 80% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.45	2067.8	0.4841
2	3.47	2082.1	0.1689
3	3.63	2175.6	0.0912
4	4.11	2466.1	0.0077
5	7.16	4296.6	0.0234
6	7.24	4345.0	0.1049
7	7.36	4419.3	0.0158

Figure 3.78 ^{1}H spectrum of 90% fraction of 60% PE *C. racemosa* extract.

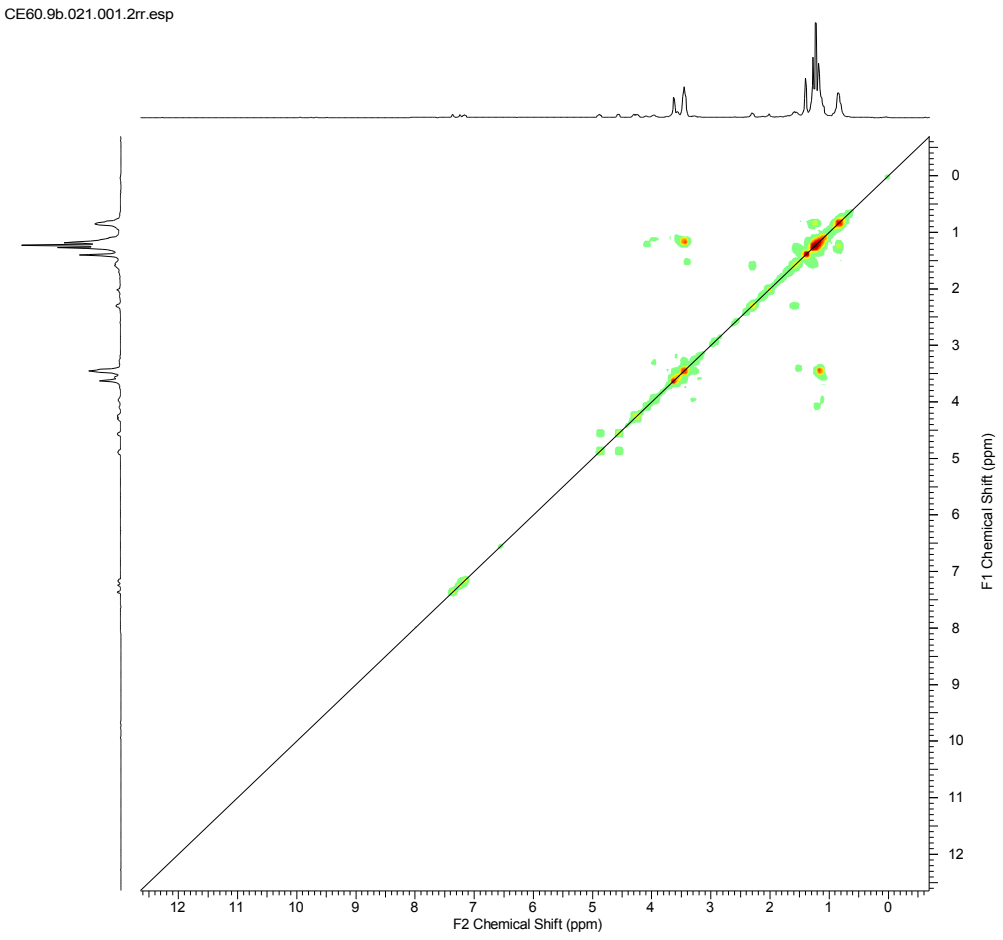
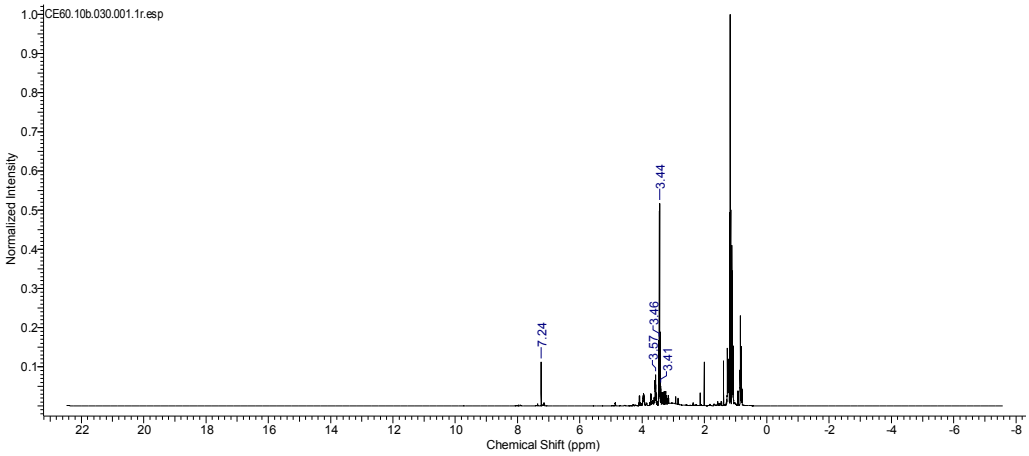


Figure 3.79 COSY spectrum of 90% fraction of 60% PE *C. racemosa* extract.



No.	(ppm)	(Hz)	Height
1	3.41	2046.2	0.0512
2	3.44	2063.8	0.5172
3	3.46	2077.5	0.1680
4	3.57	2143.0	0.0796
5	7.24	4344.9	0.1120

Figure 3.80 ^{1}H spectrum of 100% fraction of 60% PE *C. racemosa* extract.

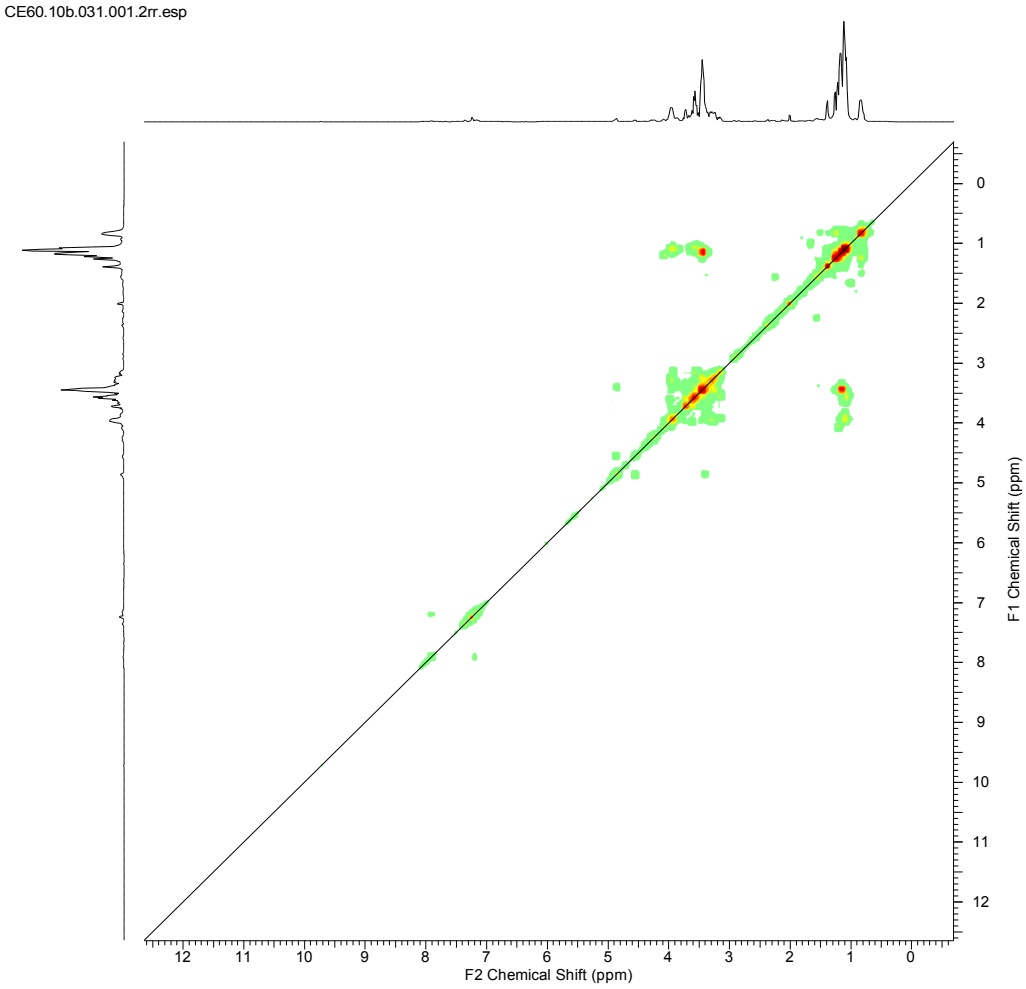


Figure 3.81 COSY spectrum of 100% fraction of 60% PE *C. racemosa* extract.

CHAPTER FOUR

DISCUSSION

New methods for the better agriculture have been developing for many decades to produce healthier and productive crops. Bio-fertilizers are one of the most valuable plant additives in order to get better products. However bio fertilizers can not get the attention they deserve. Whereas plant additives are required by plants because of their hormone, aminoacid etc. contents (Sivasankari et al, 2006; Zahid, 1999). It's likely to find many reports in the literature about the importance of the seaweed extracts as plant growth stimulating fertilizers. As an example Crouch and Staden investigated the effects of *Ecklonia maxima* on the growth of tomato seedlings (Crouch & Straden, 1992). As a result of their study *E. maxima* caused an increase on the fruit production and growth rate of the extract treated tomato seedlings(Crouch & Straden, 1992).

The plant growth stimulation effects of *wightii* and *Caulerpa chemnitzia* seaweeds were investigated by Sivasankari et al. (2006). According to their results *S. wightii* has more biostimulation effect than *C. chemnitzia.* This was reported in the article as the plants might have absorbed the biochemically important molecules from the seaweed extract.

Caparkaya, Cavas & Kesercioglu, (2009) were investigated the effects of *C. racemosa* extract on *Vigna sinensis* and *Phaseolus vulgaris* (Fabaceae). According to their results on root and shoot lengths, they observed a biostimulation effect on *Vigna sinensis.* They reported that *C. racemosa* extract could be used as an antioxidant supply. By that way they might have an oxidative stress which causes to produce higher quality crops (Caparkaya, Cavas & Kesercioglu, 2009).

Statistically significant differences were observed for shoot length and root length of water soaked *P. oleracea* seeds. A positive response was observed at 20% applied water soaked seedlings. When we compare our result with Caparkaya, Cavas &

Kesercioglu (2009), there are parallel results with root and shoot lengths of *P. oleracea* in the lower *C. racemosa* concentrations. However, our findings were more parallel with the results of Sivasankari et al. (2006). When the extract concentration increases (20%) an inhibition occurs like the report of Sivasankari et al (2006). According to Sivasankari et al. (2006), the increase in the germination percentage in lower concentration might have been caused by several plant growth promoters like IAA, IBA etc. (Challen & Hemingway, 1965; Sivasankari et.al., 2006; Taylor & Wilkinson, 1977).

In the literature, similar results were reported for several different species such as; *Cajanus cajan, Vigna catajung, Dolichos biflorus, Trigonella foenum-graecum* L. (Mohan et al., 1994; Anantharaj & Venkatesalu, 2001; Anantharaj & Venkatesalu, 2002; Pise & Sabale, 2010). It was also reported by Dhargalkar and Untawale (1983) on the extracts of *Hypnea musciformis*, *Spatoglossum asperum, Stoechospermum marginatum* and *Sargassum* on the growth of green chillies, turnips and pineapple.

We have found a high concentration of CPN in the fractions between %0-40 PE of extract. Relatively higher concentrations of plant growth regulators in certain seaweeds have been reported by Kingmann & Moore (1982). This might be the most important reasons of popularity of the seaweed fertilizers in the entire world.

Prasad et al. (2010) were quantified several plant growth regulators in fresh state and after 1 year of storage of *Kappaphycus alvarezii* by ESI-MS without chromatographic separation. Quantification was validated against HPLC data. They investigated the existence of several plant hormones such as; IAA, kinetin, zeatin, giberellin in the extract of *K. alvarezii* by using ESI-MS.By the same way we identified the existence of CPN in *C. racemosa* extract. This attempt was the first scientific contribution on the plant growth regulators from *C. racemosa* along Turkish coastlines.

CHAPTER FIVE

CONCLUSION

C. racemosa, as it was mentioned before, is an invasive green marine alga in Mediterranean Sea. This species is able to grow either in warm or colder sea water and can be easily invade between 0-70 meters depths. It grows over sea bottom and destroys the habitat of numerous other species. Therefore *C. racemosa* is a widely important danger for the future of sea ecosystem (Klein & Verlaque, 2008).

Recently several eradication methods were applied to control the invasion of this species. However, none of these methods were able to become an exact solution for that serious problem. Since it was not possible to save the ecosystem with any physical, chemical or biological methods, we thought that this huge biomass can be evaluated in the organic agriculture.

Turkey is such a country which is surrounded by sea from three sides as a peninsula. Agriculture is one of the most important economical sources of Turkey. Synthetic and chemical growth stimulators have been highly used in Turkey instead of natural biomaterials. Unfortunately, these synthetic chemicals, such as ammonium sulphate, ammonium phosphate, ammonium nitrate, urea, and ammonium chloride, are indirectly enter the food chain.

C. racemosa was investigated by terms of its plant growth stimulating effect. It was thought that *C. racemosa* as a bio-fertilizer can be easily used directly in the agricultural fields. It was aimed to transform a natural problem, invasion of *C. racemosa*, to a solution of a better agricultural growth without chemicals like hormones which are quite harmful to human health.

C. racemosa var. *cylindracea* has been invading the subliteral ecosystem of the Mediterranean Sea since 1991. Inasmuch as there has been no validated eradication method on this species in the scientific literature, the biomass of this species could be evaluated in different areas such as organic agriculture, adsorption science and pharmacy. The present MSc thesis confirms the importance of the extract from this species in the field of organic agriculture.

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