TURKISH REPUBLIC
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

REGENERATION OF *Cichorium intybus* L. IN TISSUE CULTURE AND ANALYSIS OF ITS SECONDARY METABOLITES

M.Sc. THESIS

PREPARED BY: Yousif Abdullah ABAS
SUPERVISOR: Prof. Dr. Musa TÜRKER
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ACCEPTANCE and APPROVAL PAGE

This thesis entitled "**REGENERATION OF *CICHORIUM INTYBUS* L. IN TISSUE CULTURE AND ANALYSIS OF ITS SECONDARY METABOLITES**" presented by Yousif Abdulah ABASS under supervision of Prof. Dr. Musa TÜRKER and co-supervision of Dr, Ayten Eroglu in the department of Molecular Biology and Genetics has been accepted as a M.Sc. thesis according to Legislations of Graduate Higher Education on ……/……./………. with unanimity / majority of votes members of jury.

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THESIS STATEMENT

All information presented in the thesis were obtained according to the ethical behaviors and academic rules frame. And also, all kinds of statement and source of information that does not belong to me in this work prepared in accordance with the rules of theses, were cited to the source of information absolutely.

Signature

Yousif Abdullah ABAS

ABSTRACT

REGENERATION OF *Cichorium intybus* L. IN TISSUE CULTURE AND ANALYSIS OF ITS SECONDARY METABOLITES

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Cichorium intybus L. was collected from field and regenerated *in vivo* in soil and *in vitro* in three different media; Murashige and Skoog (MS), Gamborg B5 and White medium supplemented with different concentration and combinations of auxins and cytokinins. Callus production was also achieved in the basic media supplemented with plant growth regulators (PGRs). Plant was regenerated by direct organogenesis from leaf explant in MS and B5 medium supplemented with Indole-3-butyric acid (IBA), and Naphthalene acetic acid (NAA). Root and shoot regeneration occurred. Plantlets were maturated in growth chamber in sterile condition and acclimatized in laboratory in ambient conditions and transferred to soil. Callus was produced from leaf explant in MS, B5 and White medium supplemented with auxin, cytokinin and gibberellic acid. The best result for callus production was provided from MS medium supplemented with Indole-3-acetic acid IAA and Benzyl amino purine (BAP). Secondary metabolites provided from *in vivo* and *in vitro* samples were analysed by High Performance Liquid Chromatography (HPLC) and Spectrophotometry. Phenolic compounds (esculine, caftaric acide, chlorogenic acid and cichoric acid) were extracted in ethanol/water (80:20 v/v) and quantified by HPLC. Inulin was quantified by spectrophotometry.

The highest caftaric acid concentration was found in flowers from *in vivo* samples. However, the highest concentration of esculine was found in leaf extract of *in vitro* regenerated plants. Chlorogenic acid concentration was higher *in vivo* samples then that of *in vitro* whereas chichoric acid concentration was found to be higher in *in vitro* samples than that of *in vivo*. Inulin was found to be higher in *in vivo* samples.

Keywords: HPLC, Inulin, *in vitro,* Phenolic compounds.

ÖZET

Cichorium intybus L. (HİNDİBA) BİTKİSİNİN DOKU KÜLTÜRÜNDE REJENERASYONU VE SEKONDER ÜRÜNLERİNİN ARAŞTIRILMASI

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Hindiba (*Cichorium inythibus* L.) bitkisi araziden toplanarak laboratuvar şartlarında yetiştirilmiştir. Oksin, sitokinin ve giberellik asit Bitki Büyüme Düzenleyicileri (BBD) ile desteklenmiş, Murashige ve Skoog (MS), Gamborg B5 ve White ortamlarında *in vitro* olarak rejenerasyon ve kallus üretimi çalışmaları yapılmıştır. *İn vivo* ve *in vitro* örneklerden eskulin, kaftarik asit, klojenik asit, kikorik asit ve inulin ekstre edilerek sıvı kromatografi ve spektrofotometrede kantitatif analizleri yapılmıştır. Bitki yaprak eksplantından İndol-3-bütirik asit (IBA) ve Naftalen asetik asit (NAA) ile desteklenmiş MS ve B5 ortamlarında direk organogenez yöntemi tüm bitki elde edilmiştir. Doku kültüründe kök ve sürgün geliştikten sonra fideler laboratuvar ortamında geliştirilmiş, ortama alıştırılmış ve saksılara ekilerek canlı kalmaları ve saksıda büyümeleri sağlanmıştır. Yaprak eksplantından farklı konsantrasyon ve kombinasyonlarda oksin, sitokinin ve giberellik asit kullanılarak MS, B5 ve White ortamlarda kallus geliştirme çalışmaları yapılmıştır. En verimli kallus üretimi IAA+BAP ile desteklenmiş MS ortamından elde edilmiştir. Eskulin, kaftarik asit, klojenik asit ve kikorik asit etanol/su (80:20 v/v) içerisinde *in vivo* ve *in vitro* bitki örneklerinden ekstre edilerek HPLC'de, inulin ise spektrofotometrede kantitatif olarak analiz edilmiş ve karşılaştırılmıştır. En yüksek kaftarik asit konsantrasyonu *in vivo* ortamdan toplanmış bitkilerin çiçeklerinde bulunmuştur. Eskulin konsantrasyonunun, *in vitro* ortamda yetişen bitki yapraklarında *in vivo* ortamda yetişen bitki örneklerindeki miktardan daha fazla olarak tespit edilmiştir. Klorojenik asit ve inulin miktarlarının *in vivo* örneklerde daha yüksek olduğu tespit edilirken, kikorik asit miktarı *in vitro* örneklerde daha yüksek olarak tespit edilmiştir.

Anahtar kelimeler: HPLC, Fenolik bileşikler, inulin, *in vitro*.

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2018

Yousif Abdullah ABAS

TABLE OF CONTENTS

Pages

ÖZET ... iii
ABSTRACT ... i
ACKNOWLEDGMENT ... v
TABLE OF CONTENTS ... vii
LIST OF TABLES ... xi
LIST OF FIGURES ... xiii
SYMBOLS AND ABBREVIATIONS ... xv
1. INTRODUCTION ... 1
1.1. *Cichorium intybus* L. ... 1
1.2. Significance of the study ... 2
2. LITERATURE REVIEW ... 3
2.1. Botany of *Cichorium intybus* L. ... 3
2.2. Taxonomic Classification ... 4
2.3. Vernacular Names ... 4
2.4. Geographical distribution ... 5
2.5. Utilization of *Cichorium intybus* L ... 5
2.6. Phytochemical of *Cichorium intybus* L. ... 7
2.7. Inulin of Cichorium intybus L ... 7
2.8. Phenolic Compounds in *Cichorium intybus* L. ... 8
2.8.1. Esculin in *Cichorium intybus* L. ... 8
2.8.2. Chicoric acid ... 9
2.8.3. Caftaric acid ... 9
2.8.4. Chlorogenic acid (CGA) ... 9
2.9. Plant Tissue Culture Technique ... 9
2.9.1. *In vivo* culture conditions of Cichorium intybus L. ... 10
2.9.2. *Cichorium intybus* L. in tissue culture ... 11
2.10. Secondary Metabolites in *Cichorium intybus* L. ... 12
2.10.1. HPLC (High Pressure Liquid Chromatography) ... 12

Pages

3. MATERIALS AND METHODS 15

3.1. Sample of Study 15

3.1.1. In vivo Sample (from field) 15

3.1.2. *In vitro* Sample (grown in tissue culture) 15

3.2. Plant Culture Media 15

3.2.1. MS medium (Murashige and Skoog Basal Medium) 17

3.2.2. White medium composition 18

3.2.3. B5 medium composition 19

3.3. Sterilization 21

3.3.1. Sterilization of Equipment and Glassware. 21

3.3.2. Explant Sterilization 21

3.3.3. Working environment sterilization 21

3.3.4. Media Sterilization 22

3.4. *In vitro* Culture 22

3.5. Plant Growth Regulators 23

3.6. Establishment of Culture Regeneration Protocols in Tissue Culture: 23

3.6.1. Plant regeneration 25

3.6.2. Plant Regeneration Protocol 25

3.7. Extraction and Analysis of Phenolic Composition and Polysaccharide (inulin) of *Cichorium intybus* L. 25

3.7.1. Identification of phenolic compounds by liquid chromatography-diode array Mass spectrophotometer (LC-DAD-MS/MS) 26

3.7.2. Quantification of phenolic compounds by high performance liquid chromatography-diode array detector (HPLC-DAD) 27

3.8. Statistical Analysis 28

4. RESULTS 29

4.1. Callus Production 29

4.2. Root and Shoot Regeneration in Tissue Culture From Leaf Explant Via Direct Organogenesis 34

4.3. Quantitative Analysis of Phenolic Composition 37

Pages

4.3.1. Caftaric acid 38
4.3.2. Esculin 38
4.3.3. Chlorogenic acid 38
4.3.4. Chicoric acid 39
4.4. Polysaccharide (Inulin) Analysis of *Cichorium Intybus* L. in Spectrophotometer.. 40
5. DISCUSSION AND CONCLUSION 41
5.1. *In vitro* Regeneration of Cichorium Intybus 41
5.2. Production of Leaves and Root *in Vitro* (direct organogenesis)...................... 42
5. 3. Secondary Metabolite Production.. 42
5.3.1. Extraction and analysis of phenolic composition.. 43
5.3.2 Polysaccharide (inulin) of cichorium intybus by using spectrophotometer... 45
REFERENCES 47
APPENDIX 551
EXTENDED TURKISH ABSTRACT (GENİŞLETİLMİŞ TÜRKÇE ÖZET) 551
CURRICULUM VITAE 555

LIST OF TABLES

Tables **Pages**

Table 2.1. Vernacular names of *Cichorium intybus* L. 4

Table 2.2. Utilization of *Cichorium intybus* L. 7

Table 3.1. MS medium (Murashige and Skoog Basal Medium) 16

Table 3.2. White medium composition 17

Table 3.3. B5 medium composition 18

Table 4.1. *In vitro* callus production from *Cichorium intybus* L. 28

Table 4.2. Dry weight and yield percentage of calli for extraction. 32

Table 4.3. Result for direct organogenesis of *Cichorium intybus* L. leaf explant 33

Table 4.4. Phenolic compositions of *Cichorium intybus* L. 35

Table 4.5. Concentration of Inulin of *Cichorium intybus* L. using spectrophotometer 38

LIST OF FIGURES

Figure **Pages**

Figure 2.1. *Cichorium intybus* L... 3

Figure 2.2. Distribution area of *Cichorium* species... 5

Figure 2.3. HPLC Devices.. 12

Figure 3.1. *In Vivo* Sample of Study (dried weight)... 15

Figure 3. 2. Plant Culture Media Preparation.. 19

Figure 3.3. Culturing *Cichorium intybus* L.... 22

Figure 3.4. Ethanol-based hydrophilic extracts of *Cichorium intybus* L. samples......... 24

Figure 4.1 Calluses production from leaf explant after 7 days of incubation…………29

Figure 4.2. Callus production from leaf explant.. 30

Figure 4.3. Shoot and root regeneration from leaf explant (direct organogenesis) 31

Figure 4.4. Shoot and root regeneration from leaf explant (direct organogenesis) 33

Figure. 4.5.The chromatogram of phenolic composition of *Cichorium intybus* L. from leaf explant.. 35

Figure 4.6. Caftaric acid concentration in the sample of study using HPLC 36

Figure 4.7. Esculin concentration in the sample of study using HPLC......................... 37

Figure 4.8. Chlorogenic acid concentration in the sample of study using HPLC 37

Figure 4.9. Chicoric acid concentration in the sample of study using HPLC 38

SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
HPLC	high performance liquid chromatography
BA	Benzyl adenine
BAP	Benzyl amino purine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
NAA	Naphthalene acetic acid
ST	Shoot tip
PGR	Plant Growth Regulator
WT	White medium
MS	Murashige and Skoog Medium
Hr	Hour
N	Not used
HCL	Hydrogen Chloride
NaOH	Sodium Hydroxide
pH	Hydrogen Potential
NaOCl	Sodium hypochlorite
UV	Ultraviolet
Lab.	Laboratory

Symbols	Description
Ml	millilitre
w/v	Weight/volume
˚C	Centigrade degree
mg/l	Milligram/ liter
g	gram

1. INTRODUCTION

1.1. *Cichorium intybus* L

Cichorium intybus L. is the earliest herbs cited in recorded literature. Thousands of years, the study of herbs had been handed down from generation to generation as long as the history of humankind (Nandagopal and Kumari, 2006). Nowadays, farmers plant chicory in wild widely. On the other hand, breeders are attempting to produce chicory for medicinal purposes due to its ability for clonal propagation *in vitro* culture (Doliński and Olek, 2013). In recent years, the demand for secondary metabolites has increased dramatically, because the source of plants is limited due to climate changes, disease, economic development and shrinking habitat. Hence, plant tissue culture offers an alternative source for controlled production of these products (Mulabagal and Tsay, 2004).

Plant tissue culture technique, is a significant method in the study of basic areas of plant biology, biochemistry, agricultural biotechnology as well as commercial application. Development of *in vitro* technology led scientist to produce more expansion in tissue culture application for different plants species (Thorpe, 2007). It was confirm that, root culture technique is a significant method to obtain rapid growth rate, easy preparation, maintenance and increasing plant production. Tissue culture is widely used to increase clonal propagation of superior plants, to produce virus-free plant and also to use in field of genetics and plant breeding (Illg, 1991).

Scientist have provided a large number of different materials called secondary metabolites to make life easier for humankind. Secondary plant metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism. They play an important role in pollination, defence against pathogens and abiotic stress. However, they have great importance for human in medicine, agriculture and industry. Plant derived compounds have become a topic of great interest, owing to their versatile application (Agarwal and Sarin, 2014). To obtain secondary metabolites, plant material resources, extraction techniques have been developed, In addition to extraction processes, some new techniques for the separation

of compounds from plant material have been developed depend on the time of extraction, temperature, nature, polarity and concentration of solvent (Pandey, 2013), such as solvent extraction, steam extraction, supercritical extraction, pervaporation and microwave heating (Starmans and Nijhuis, 1998).

1.2. Significance of the study

From the theoretical point of view, *Cichorium* is widely used in medicine and culinary aspects. Inulin, esculin, caftaric acid, chicoric acid and chlorogenic acids are the main component of Cichorium. The present study was aimed to investigate different *in vitro* regeneration strategies of *Cichorium* and to increase the amount of secondary compunds (inulin, esculin, caftaric acid, chicoric acid and chlorogenic) *in vitro* regenerated samples of plant. The study will fill the gap in this field. From the practical point of view, the results of the study will provide wide perspective on the optimal methods to cultivate plant and to produce target concentration of inulin, esculin, caftaric acid, chicoric acid and acids of Cichory plant.

2. LITERATURE REVIEW

2.1. Botany of *Cichorium intybus* L.

Chicory (*Cichorium intybus* L.) is a small aromatic biennial or perennial herb belongs to the family of Asteraceae. *Chicory* contains a number of medicinally important compounds such as inulin, esculin, volatile compounds (monoterpenes and sesquiterpenes), coumarins, flavonoids and vitamins (Nandagopal and Kumari, 2007). Flowers of chicory are blue, lavender, sometimes white colour (Wang and Cui, 2011). Chicory is a diploid (2n = 18) perennial herb plant (Liang, et al., 2014)

Figure 2.1: *Cichorium intybus* L.

2.2. Taxonomic Classification

Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Asterales
Family: Asteraceae, Compositae
Genus: Cichorium
Species: *C.intybus* L.

2.3. Vernacular Names

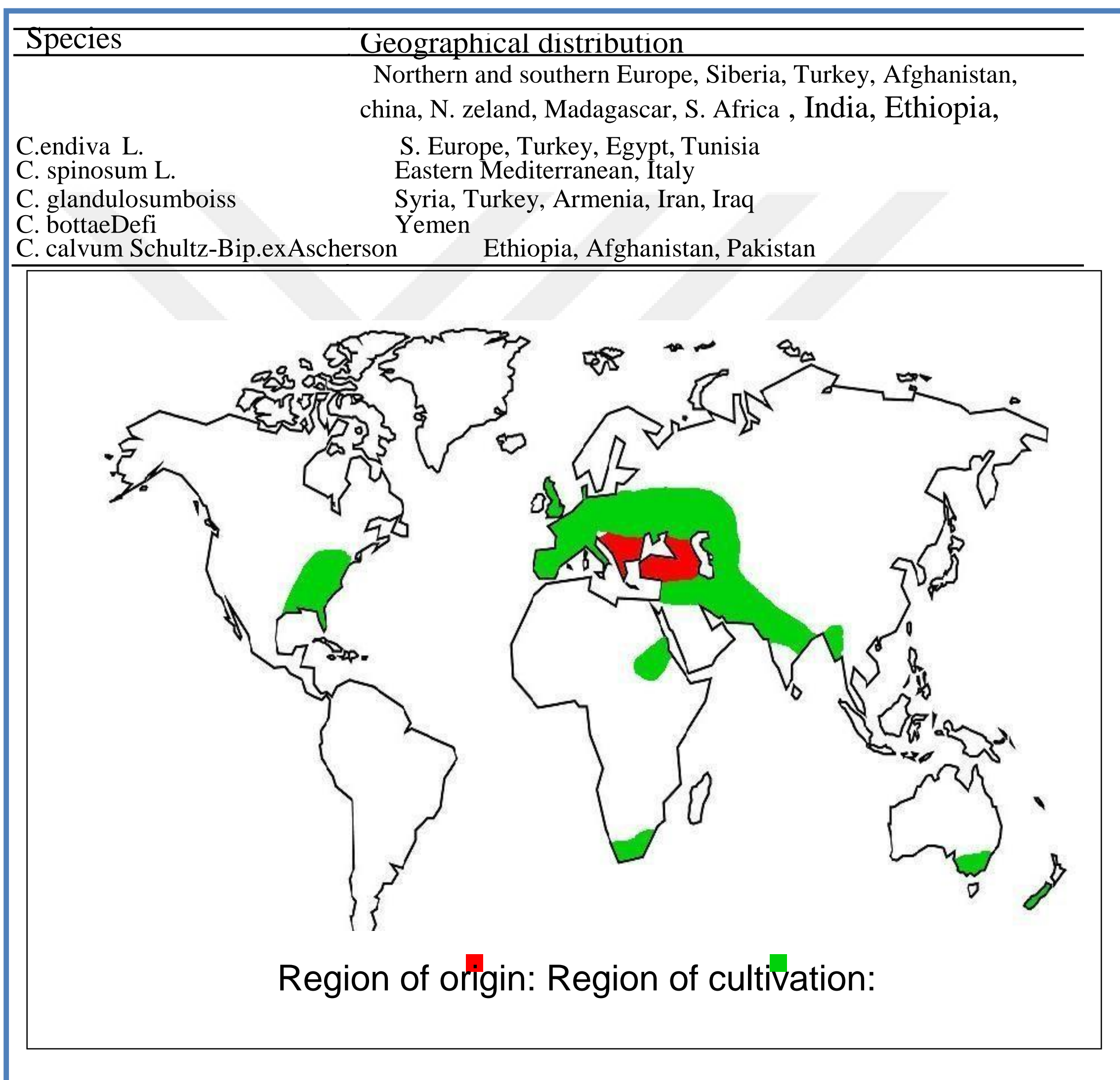
The name of *Cichorium intybus* L. is derived from Greek and Latin (Al-Snafi, 2016). Table 2.1 shows common names of *Cichorium intybus* L.

Table 2.1. Vernacular names of *Cichorium intybus* L.

	Language	Name
1	Arabic	Shikoryah, Hidaba, Hindaba Bariah;
2	Chinese	JuJu
3	English	Belgium endive, Chicory, Coffee Chicory, French Endive, Succor, Witloof
4	French	Chicon, Chicorée, Chicorée À Café, Chicorée De, Bruxelles,Chicorée Sauvage, Endive, Endive Witloof, Witloof
5	German	Chicorée, Fleischkraut, Kaffeezichorie, Salatzichorie, Wegwarte, Wurzelzichorie
6	Italian	Cicoria, Radicchio
7	Japanese	KikuNigana;
8	Spanish	Achicoria de Bruselas, Achicoria de café, Achicoria de raz
9	Turkish	Hindiba
10	Swedish	Cikoria

2.4. Geographical distribution

Historically, chicory was grown by the ancient Egyptians as a medicinal plant, coffee substitute, vegetable crop, and occasionally for animal forage (Wang and Cui, 2011). The plant is distributed in temperate and semi-arid regions of the world such as Mediterranean region, Mid-Asia and Northern Africa (Liang, et al., 2014).



Species	Geographical distribution
	Northern and southern Europe, Siberia, Turkey, Afghanistan, china, N. zeland, Madagascar, S. Africa , India, Ethiopia,
C.endiva L.	S. Europe, Turkey, Egypt, Tunisia
C. spinosum L.	Eastern Mediterranean, Italy
C. glandulosumboiss	Syria, Turkey, Armenia, Iran, Iraq
C. bottaeDefi	Yemen
C. calvum Schultz-Bip.exAscherson	Ethiopia, Afghanistan, Pakistan

Figure 2.2. Distribution area of *Cichorium* specie**.**

2.5. Utilization of *Cichorium intybus* L

Historically, the ancient Egyptians grew the plant for medicinal purpose, coffee substitute, vegetable crop, and occasionally for animal forage. Nowadays, *Cichorium*

has been used in many application such as medicinal, pharmaceutical and industrial uses as shown in Table 2.2 (Wang and Cui, 2011). *Cichorium* roots are used as coffee substitutes and additives, while young leaves can be added to salads and vegetable dishes, as well as chicory extracts are used for the production of invigorating beverages. On the other hand *Cichorium* was used for the treatment of diseases such as diarrhoea, cough, cancer, hangover, for purification of biliary tract, liver disease, as spasmolytic, to relief of symptoms related to mild digestive disorders (such as feeling of abdominal fullness, flatulence, and slow digestion) and temporary loss of appetite. On the other hand chicory was used in sore throat, haemorrhoids, tuberculosis, abdominal cramps, melancholy, deafness, and rashes and as laxative for children (Al-Snafi, 2016).

Cichorium intybus, as herbaceous perennial plant, known as a coffee substitute, it has a wide range of healing characteristics because of inulin (one of its components) (Bais and Ravishankar,2001), and grown as a crop for livestock forage (WenYing and JinGu, 2012). The best known using aspects of the plant is perhaps the roasted roots used as the traditional coffee substitute with no caffeine (Barbara et al., 2007).

Recently, *Cichorium intybus* L. is cultivated in Europe and North America with many commercial uses, e.g.; the US imports more than 1.9 million kilograms roasted chicory roots for coffee. It is prospective forages and cash crops with high production, multiple function and good quality. It can be used as health care food and medicine (Wang and Cui, 2011).

Table 2.2. Utilization of *Cichorium intybus* L (Wang & Cui, 2011).

Part of chicory	Utilization	Reference
Leaves and shoots	Salads, vegetable dishes Forage Boiled and eaten Dried and roasted and used as a coffee substitute and additive: Sources of inulin For ethanol production by direct fermentation	Hermann, 1978; Hocking and Withey, 1987 Hur and Park 1995; Wang and Cui, 2009a,b Zanner, 1988 Bais and Ravishankar, 2001; Van Waes et al., 1998 Herck and Baert, 1999 Kyazze et al., 2008

2.6. Phytochemical of *Cichorium intybus* L.

All parts of *Cichorium intybus* plant possess great medicinally important compounds such as alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterols, flavonoids, saponins and tannins (Khalil et al 2015). Dried chicory contains approximately 98 % inulin and 2 % other compounds. But fresh chicory typically contains 68 % inulin, 14 % sucrose, 5 % cellulose, 6 % protein, 4 % ash, and 3 % other compounds Abbas et al.,2015).

Wenying and Jingu (2012), confirm that two varieties of seeds of *Cichorium intybus* L. contained substantial amounts of crude proteins (19 %), crude fat (22 %) and carbohydrate (31 %). The essential fatty acid, linoleic acid was the predominant fatty acid accounted for over 76 % of the total fatty acids in the two chicory seeds, with lower saturated/unsaturated ratios (0.11) making them potentially a superior source of nutritional oil, compared with alfalfa. A Mineral analysis showed that chicory seeds possess high values of K, Ca, P, Mg, Cu, Zn and Mn. All parts of chicory contain considerable amounts of phytochemicals and are good source of antioxidants (Khalil et al 2015).

2.7. Inulin of *Cichorium intybus* L

Inulin is a natural food element, found in different percentages in dietary foods. It is available in a number of vegetables and plants such as wheat, onion, bananas, garlic and chicory. Recently most of the commercially available inulin is either synthesizes

from sucrose or extracted from chicory roots. The root of the *Cichorium intybus* L. plant contains 15 to 20 % inulin (Niness, 1999). Inulin is fructan and mostly found in the Asteraceae family as chicory. The root of chicory is considered as one of the most important crops for the commercial extraction of inulin on industrial scale (Maroufi1, et al., 2012).

Inulin chains consists of up to 100 D-fructofuranose units linked via β-(2→1) glycosidic bonds (Milala, et al., 2009). Inulin is a reserve carbohydrate, and used to replace fat or sugar and reduce the calories of food. It is suitable for consumption by diabetics and is also used in inulin clearance test to measure glomerular filtration rate-GFR. Recent pharmacological investigation of the root extract of this plant revealed immunomodulatory, antitumor and anticancer properties (Coussement,1999).

Nandagopal and Kumari,(2007).Inulin is a natural polysaccharide, a polyfructosane containing 27-35 fructose residues in furanose form and glucose residue. When inulin is in the alimentary tract it passes to the stomach and the small intestine unchanged. In large intestine inulin is fermented by bifid bacteria and then converted into a great number of short-chain fatty acids. Due to the process it stimulates the growth of bifid bacteria population and reduces pathogenic microorganisms. The increase of healthy intestinal pool flora normalizes the passage of feces mass through the intestine, improves immunologic status, regulates carbohydrate and lipid metabolism (Ev, 2013). From chemical point of view, inulin is a multi molecular element with a polydisperse β (2—1) fructan, linear fructose polymer types, with glucose molecule resides at the end of each fructose chain and is linked by an (1—2) bond, at 2 to 60 units length, with an average 10 DP (Niness, 1999).

2.8. Phenolic Compounds in *Cichorium intybus* L.

2.8.1. Esculin in *Cichorium intybus* L

Esculin is a phenolic compounds group. It has multiple medicinal, pharmaceutical and industrial uses. It is a well-known natural UV-B protective agent. Esculin possesses various biological activities such as inhibiting oxidative DNA damage and formation of aberrant crypt foci and tumours (Saini, et al., 2014).

2.8.2. Chicoric acid

The importance of chicoric acid in the medical health benefit, lead researcher to give attention to this compound in 1958. Recently chicoric acid is found out at least 63 genera and species. According to the studies of Scarpati and Oriente (1958), Lee et al., (2013), leaves of *Cichorium intybus* L. poses a phenolic compound that is a tartaric acid ester of two caffeic acids. Chicoric acid have protection role in plants, mainly to protect plant from insects and infection from viruses, bacteria, fungi, and nematode.

2.8.3. Caftaric acid

Caftaric acid is non-flavonoid phenolic and bioactive compound. It is well known as cinnamates (hydroxycinnamic acids). It was recorded that, the caftaric acid is responsible for yellowish-gold color seen in some white's drinks, and it has a good bioavailability when fed in rats (Cecotti et al., 2007).

2.8.4. Chlorogenic acid (CGA)

Chlorogenic acid (CGA) is known as svetol in market, and biologically active polyphenol that can be soluble in ethanol and acetone. CGA has also been found as a phenolic component of bamboo, green coffee bean extract and tobacco. It is present in various type of fruits such as peach and prunes and vegetables such as potato. CGA poses range of pharmacological activities such as anticancer, antioxidant, anti-inflammatory, cardiovascular, hepatoprotective, renoprotective, anti-diabetic and anti-lipidemic (Bukhari et al., 2016).

2.9. Plant Tissue Culture Technique

Plant tissue culture technique is a technique which utilizes either single plant cells, group of unorganized cells (callus) or organized tissues or organs put in culture under controlled sterile condition (Illg, 1991). Plant tissue culture also referred to

growing plant cells, tissues, organs, seeds, or other plant parts in a sterile environment on a nutrient medium (Touchell et al., 2008).

Plant tissue culture, is a significant method in the study of basic areas of plant biology, biochemistry, agricultural biotechnology as well as commercial application. It is originated from the idea of the German scientist, Haberlandt at the beginning of the 20th century. Developments of *in vitro* technology in 20th led scientist to produce more expansion in the tissue culture application to different species of plants. It is divided to five broad area as follows; cell behavior, plant modification and improvement, pathogen-free plants and germ plasm storage, clonal propagation, and product (mainly secondary metabolite) formation (Thorpe, 2007).

Amer et al., (2016) confirm that, the root culture technique is a significant method to obtain rapid growth, easy preparation and maintenance and to increase plant production. Tissue culture is widely used to increase clonal propagation of superior plant material, and to produce virus free plant. It is also used in field of genetics and plant breeding (Illg, 1991), as well as obtaining healthy plants, free from bacterial and fungal diseases, increase propagation of plants those are difficult to propagate, somatic hybridization, and genetics improvement of commercial plants. Generally there are three main methods used in tissue culture; micro propagation through the enhanced multiplication of axillary bud, organogenesis, somatic embryogenesis.

2.9.1. *In vivo* culture conditions of *Cichorium intybus* L.

Chicory is a hardy biennial plant that grows in almost any soil. The seeds are sown in May or June in drills about 1 inch deep, about 12 inches apart in the rows (Torres, 1989). According to Barry (1998)*Cichorium intybus* L. is grown on well-drained soils with high fertility at pH ranging from 4.8 to 6.5. The growth rate of *Cichorium intybus* L. in warm seasons is higher than in winter. Nowadays, farmers plant chicory in wild widely. On the other hands, breeders are attempting to produce chicory for medicinal purposes due to its ability for clonal propagation *in vitro* culture (Doliński and Olek, 2013).

2.9.2. *Cichorium intybus* L. in tissue culture

Special compounds are used in plant tissue culture. The components of the culture should be easy to clean and reduce contamination as much as possible. Supplies of both tap and distilled water and gas should be provided. Special devices are required to get high performance such as a refrigerator, freezer, hot plate, stirrer, pH meter, electric balances with different weighing ranges, heater, bunsen burner in addition to glassware and chemicals. It is very important to take care about accuracy in media preparation process. Therefore it is necessary to clean glassware, to use high quality water, pure chemicals and needs careful measurement of media components (Saad and Elshahed, 2012).

The traditional method of plant propagation produce limited and uncertain quantity and quality of plant, Therefore the researchers give more attention to find out optimum plant growth media. Moreover optimal growth of tissues is different from plant to plant based on their living nutrient requirements. In the meantime, tissues from different parts of same plant is also have different requirements (Murashige and Skoog, 1962). Media composition is a key element for the successful application of tissue culture. Changes in the composition of plant media can induce stem, root, callus, or somatic embryo from explant or inhibit growth for long-term storage (Touchell et al., 2008).

Generally plant tissue culture media should contain; macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents (Saad and Elshahed, 2012). Firstly tissue culture media were developed from nutrient solutions for culturing whole plants from root in White medium and callus culture in Gautheret medium, while the frequently used media are Murashige and Skoog (MS), Linsmaier and Skoog (LS), Gamborg (B5) and Nitsch (NN) (Saad and Elshahed, 2012). On the other hand, Nandagopal and Ranjitha (2012) confirm that, micropropagation, cell/ root, and hairy root culture were considered as biotechnological methods for optimal ways to produce mature plant and increase production of medical drugs in laboratory from *Cichorium intybus* L. without depending on field cultivation.

2.10. Secondary Metabolites in *Cichorium intybus* L.

Scientists have provided large number of different materials to make life easier for humankind. However, scientists still cannot make some compounds as efficiently as Mother Nature does. Plant cells synthesize a vast supply of natural compounds that are not strictly necessary for growth or reproduction, but their presence can be demonstrated genetically, physiologically or biochemically importance. Plant derived compounds have become a topic of great interest, owing to their versatile application in medicine, agriculture and industry (Agarwal, 2014).

The raw materials for special chemicals such as pharmaceuticals may be made synthetically or extracted from plants. The plant are crushed and shaken for long periods with a suitable solvent. Many different compounds may dissolve. The mixture is filtered to separate the insoluble parts of the plant from the solution (Handa, 2008). The phytochemicals extracted from plant depend on the nature of the plant material, its origin, degree of processing, moisture content, and particle size (Pandey, 2013).

2.10.1. HPLC (High Pressure Liquid Chromatography)

High pressure/performance liquid chromatography (HPLC) is a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. It makes the process much faster. All chromatographic separations, including HPLC operate under the same basic principles consist of separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. Hence it is a method used to separate mixtures to their individual component. There are many types of HPLC, depending on the phase system (stationary) in the process: Normal Phase HPLC, Reverse Phase HPLC, Size-exclusion HPLC, and Ion-Exchange HPLC (Nuutila,, 2002). HPLC device consist of a pump, injector, column (heart of system), detector and integrator or acquisition and display system, as shown in Figure 2.3.

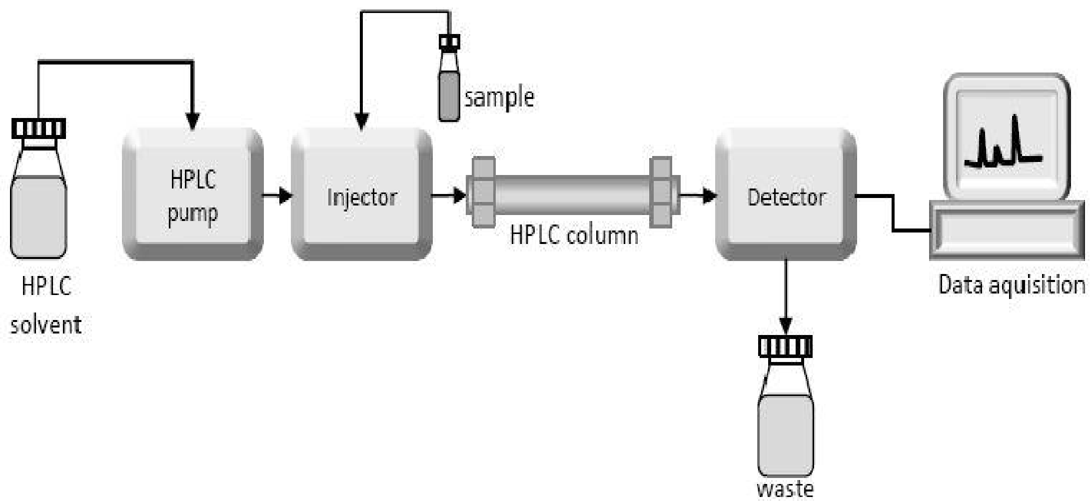


Figure 2.3. HPLC Device (Giri, 2015).

HPLC is a separation technique consists of the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. HPLC particularly RP-HPLC, is the method of choice in the chromatographic analysis of phenolic acids. The solvent systems used in the analytical HPLC usually include binary gradient elution using solvents of aqueous acetic, formic, or phosphoric acids with methanol or acetonitrile as an organic modified solvents (Kulevanova et al., 2003).

3. MATERIALS AND METHODS

3.1. Sample of Study

Two type of sample have been selected from plant *Cichorium intybus* L. for the purpose of secondary metabolites as follows:

3.1.1. *In vivo* Sample (from field)

Cichorium intybus L. was collected from the field of the campus of Van Yuzuncu Yil University, Van- Turkey on October, 20, 2016, when the plant was in flowering stages. The samples then dried in the dark room to protected sun light in laboratory of Faculty of Science in Yuzuncu Yil University. The collected sample of the plant separated to: root, stems, leaves and flowers as shown Figure (3.1). The dried samples were ground into powder and kept in refrigerator -20 ^{o}C until analysis

3.1.2. *In vitro* Sample (grown in tissue culture)

Different type of medium were used to obtain different plant growth environment under controlled condition in tissue culture such as MS Medium, B5 Medium, and White Medium with different concentration of plant growth regulator. Callus, shoot and root were obtained from leaf explant *in vitro*.

3.2. Plant Culture Media

In the current study three type of media (Murashige and Skoog (MS), White and B5 media) supplemented with 30 g L^{-1} sucrose, 3-6 g L^{-1} agarose were used. pH f was adjusted between 5.7 – 5.8, using 1M HCL and1M of NaOH. Pure double distilled water was used for media and stock solutions to protect media composition. The selected media contain all nutrients requirement such as, mineral salts, amino acids and vitamins.

Stems: 80.36 g

Roots:184.71 g

Cichorium intybus L.

Leaves: 33.87g

Flowers: 8.76 g

Figure 3.1. *In vivo* samples of the plant (dry weight).

3.2.1 MS medium (Murashige and Skoog Basal Medium)

MS originally formulated by Murashige and Skoog in 1962 to optimize of bioassay system of tobacco callus for facilitating the study of cytokinins. It is widely used for micropropagation, organ culture, callus culture and suspension culture.

Table 3.1. Basic Murashige and Skoog (1962) (MS) medium composition.

Medium Compound	Concentration (mg / l)
$CaCl_2 . 2H_2O$	440
KNO_3	1900
$MGSO_4\ 7H_2O$	370
KH_2PO_4	170
NH_4NO_3	1650
$ZNSO_4 . 7H_2O$	8.6
H_3BO_3	6.2
$MNSO_4 . 4H_2O$	22.3
KI	0.83
$Na_2MoO_4 . 2H_2O$	0.25
$CuSO_4 .5H_2O$	0.025
$COCL_2 . 6H_2O$	0.025
na2edta. 2 H_2O	37.3
$FeSO_4 .\ 7\ H_2O$	27.8
Glycine	2.0
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Myo- inositol	100
Thiamine-HCl	0.1
Total GM/l	4.736

3.2.2. White medium composition

White medium is a defined medium, which consists of inorganic salts, vitamins and carbohydrate. Potassium nitrate and calcium nitrate serves as the nitrate sources. Sucrose serves as the carbohydrate source.

Table 3.2. White medium composition.

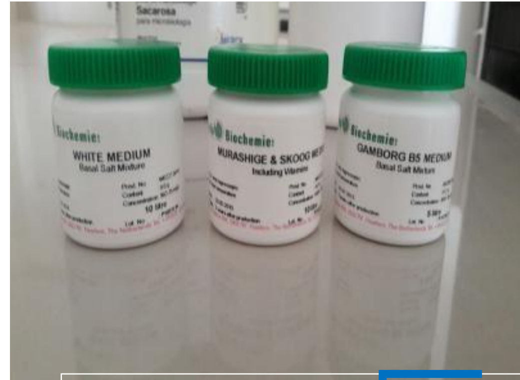
Ingredients	Concentration (mg / l)
Potassium nitrate	80
Calcium nitrate	221.96
Magnesium sulphate	360
Sodium phosphate monobasic	18.98
Potassium chloride	65
Sodium sulphate	200
Manganese sulphate.H2O	5.04
Boric acid	1.5
Potassium iodide	0.75
Molybdenum trioxide	0.001
Zinc sulphate.7H2O	2.67
Copper sulphate.5H2O	0.01
Ferrous sulphate.7H2O	2.5
Myo- Inositol	100
Thiamine hydrochloride	0.1
Pyridoxine hydrochloride	0.1
Nicotinic acid (Free acid)	0.5
Glycine (Free base)	3
Sucrose	30000
Total (gram/liter)	31.06

3.2.3. B5 medium composition

Developed by Gamborg, B5 medium originally designed for cell suspension and callus cultures. At present with certain modifications, this medium used for protoplast culture (Jha, 2016).

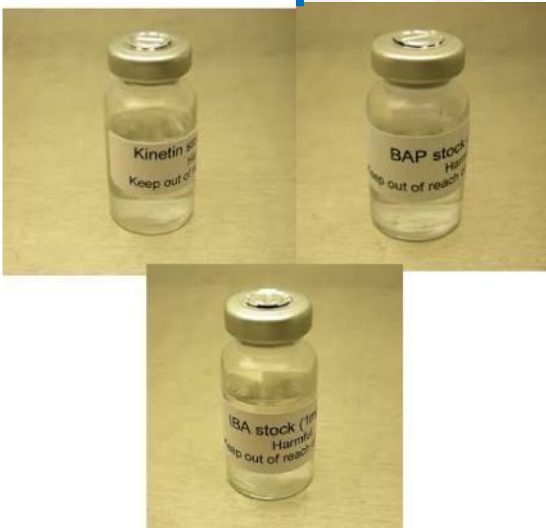
Table 3.3. B5 medium composition.

Ingredients milligrams/ litter	milligrams/ litter
MACROELEMENTS	
Ammonium sulphate	113.23
Calcium chloride	122.09
Magnesium sulphate	2500
Potassium nitrate	130.42
Sodium phosphate monobasic	
MICROELEMENTS	3
Boric acid	0.025
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	37.3
EDTA disodium salt dihydrate	27.8
Ferrous sulphate heptahydrate	10
Manganese sulphate monohydrate	0.213
Molybdic acid (sodium salt).	0.75
Potassium Iodide	2
Zinc sulphate heptahydrate	
VITAMINS	100
myo-Inositol	1
Nicotinic acid (free acid)	1
Pyridoxine HCl	10
Thiamine hydrochloride	
CARBOHYDRATE	20000
Sucrose	
Total (gm/liter)	23.2



powder of MS,white and B5

Supplement (sucrose and agarose)



Plant growth Regulators

Figure 3. 2. Plant Culture Media Preparation.

3.3. Sterilization

Sterilization is very important step in tissue culture to protect the laboratory and explants culture from microorganisms as fungi and bacteria that cause to contaminate of explants culture and loss of labour. In this study, the nutrient media, equipment, glassware, explants and plant material were sterilized for the safe germination and incubation of the explants.

3.3.1. Sterilization of Equipment and Glassware.

All material were washed with water and detergent then submerged in distilled water to dispose of salts or minerals in water to prevent change the concentration of medium. The metal and glassware covered in aluminium foil. Then all the equipment were incubate in autoclave at 121 ° C and 1.5 atmosphere. Sterilization was carried out in 25 minutes for media and one hour for metal and glassware.

3.3.2. Explant Sterilization

All part of the plants (seed, root, stems and leafs) surface may carry fungi and bacteria and reproductive cells. Therefore the surfaces need to be sterilized. In this study explants were sterilized to produce healthy plant which is free from viruses, bacterial and fungi. The explants were soaked in tape water about 30 min. After that, their surface were sterilized with 70 % ethanol for 30 second, washed by distilled water 3- 4 times, then the explants put in sodium hypochlorite 5 % commercial bleach (NaOCl- ACE-Turkey) for 3 min as confirmed by Yıldız (2000). Finally the explants were washed by distilled water 4-5 times.

3.3.3. Working environment sterilization

In the current study, the UV radiation device is used to sterilize working area. It is necessary to be mentioned here that before UV application in the LFC (Laminar Flow Cabinet), must be empty from human because the UV radiation is very dangerous on the

human health. Then air flow and fluorescent light were turned on in LFC. After that, LFC were washed with 10 % of commercial sodium hypochlorite solution and 70 % of ethanol at least 10 to 15 minutes.

3.3.4. Media Sterilization

In this study, the selected plant growth media (MS, B5 and White) were sterilized under 1.5 atmospheres pressure and 121 ° C for 25 minutes after the nutrient and plant growth regulators added.

3.4. *In vitro* Culture

After glassware, petri dishes, instruments, environment workplace, plant growth regulators, media nutrient sterilized, the explants of *Cichorium intybus* L. were cultured *in vitro*.

In order to stimulate plant growth *in vitro*, the plant growth regulator (PGRs) were added to media. The media were autoclaved at 121 ° C and 1.5 atm pressure for 25 min. The leaf explants were divided to small part and sterilized. The sterilized explants were cultured in the three selected media. The explants were cultured in petri dishes. Petri dishes were covered by Para film to avoid of contamination as shown in figure (3.3). The prepared petri dishes were put in the growth chamber. The growth chamber was characterized as manufacturer mentioned; Phytotron, Sanyo, Gellenkamp PLC, UK, at 25 ^{o}C ± 2 ^{o}C under 16 hours light/8 hours dark, provided by cool white fluorescent lamps.

3.5. Plant Growth Regulators

The plant growth regulators (PGRs) added to the culture media to increase *in vitro* sample growth rate as much as possible, PGRs used in this study are IAA, NAA, 2, 4-D and IBA, BAP, KIN and GA3.

3.6. Establishment of Culture Regeneration Protocols in Tissue Culture

The whole plants were collected from field and planted in the pots in the laboratory. Plants were acclimated survived in the laboratory conditions and subcultured during the experimental period. Plant regeneration studies were applied on different explants and leaf was found to be the most suitable explant for whole plant regeneration and callus production. Therefore experiments are based on leaf explant.

Figure 3.3. Culturing *Cichorium intybus* L.

3.6.1. Plant regeneration

The young leaf taken from field collected plants were used to produce whole plant via indirect organogenesis *in vitro* culture by using different media and plant growth regulators in different concentrations and combinations. Root and shoot development was observed in petri dishes in two weeks time. The plantlets are transferred to jars for maturation in PGRs free MS medium. Plant regeneration was developed under controlled *in vitro* culture conditions. Plant maturation was occurred in 4-5 weeks in jars depending on PGRs used. Plants were transferred to soil and acclimated in ambient environment. During acclimation/hardening stage the plant in soil was supported by 0.0073 mg/l NAA and BAP for shoot and root regeneration with tap water. All regenerated plants were survived.

Plants regenerated in B5 medium supplemented with 0.124 g/l IBA+0.1 g/l NAA was prolific and selected for secondary metabolite analysis.

3.6.2. Plant regeneration protocol

Leaf explants were incubated in MS, B5 and White medium supplemented with PGRs in different concentrations and combinations. Callus production was observed in 3 weeks time. At the end of the experiments solely callus without shoot and root was produced in MS medium supplemented with 0.16 g/l IAA+0.14 g/l BAP and was selected for secondary metabolite analysis. Callus was dried and ground into powder and used for analysis.

3.7. Extraction and Analysis of Phenolic Composition and Polysaccharide (inulin) of *Cichorium intybus* L.

In the current study, the extraction process was carried out in different parts of the plant both *in vivo* and *in vitro* as shown below:

1- Roots *in vivo*
2- Leaf *in vivo*
3- Stems *in vivo*
4- Flowers *in vivo*

5- Leaf *in vitro*

6- Callus *in vitro*

100 mg of pulverized plant material were weighed into Eppendorf tubes and extracted with 1 ml of aqueous ethanol (80% ethanol diluted in water). The extracts were sonicated for 15 minutes, centrifuged 10 minutes, 10000 rpm, (FA-45-24- 11 rotor, Eppendorf, Germany), and the pellets were re-extracted two times. Supernatant was stored -20°C until analysis (Figure 3.4).

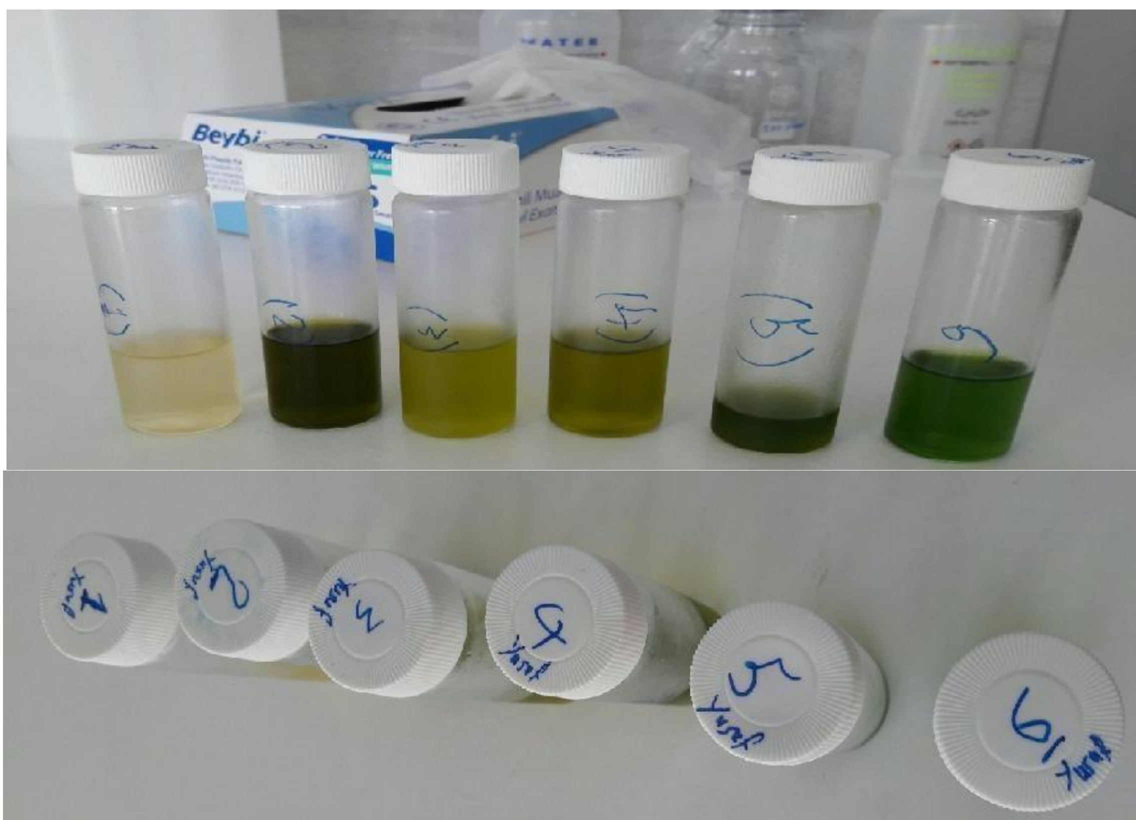


Figure 3.4. Ethanol-based hydrophilic extracts of *Cichorium intybus* L. samples.

3.7.1 Identification of phenolic compounds by liquid chromatography-diode array Mass spectrophotometer (LC-DAD-MS/MS)

LC-DAD-MS/MS analysis was conducted as described previously with minor modifications (Dalar and Konczak, 2013), on a Quantum triple stage quadrupole (TSQ) mass spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an auto sampler. An aliquot (3 µL) of each ethanolic extract was injected in chromatographic column (150x2.1 mm i.d., 5 µm Luna Synergy Hydro, Phenomenex)

which was heated to 30°C. Samples were separated using 0.5 % formic acid in purified water (A) and 0.5 % formic acid in acetonitrile (B) under a flow rate of 200 µl/min. The gradient employed was 0. % B for 2 min, then 40 % B for for 6 min, following 60 % of B for 8 min and 100% B for 4 min. Then the gradient was held for 4 min at the rate of 100 % of B. The photodiode array detector was used to acquire data from 190-520 nm. Ions for mass spectrophotometer were generated using an electrospray source in either the positive or negative mode (depending on analytic) under conditions set following optimization using Chlorogenic acid (negative) or quercetin-3-glucoside (positive). MS experiments in the full scan (parent and product- specific) and the selected reaction monitoring (SRM) mode were conducted. The composition of phenolic compounds was characterized based on their UV spectrum, retention time, co-chromatography with commercial standards, when available and MS fragmentation patterns.

3.7.2 Quantification of phenolic compounds by high performance liquid chromatography-diode array detector (HPLC-DAD)

Quantification of phenolic compounds by the HPLC was conducted as described previously with minor modifications (Dalar and Konczak, 2013) on HPLC system, which consisted of two LC-10ADVP pumps, SPD-M10ADVP diode array detector, CTO-1- ADVP column oven, DGU-12A degasser, SIL-10ADVP auto injector, and SCL-10A system controller (Shimadzu Corporation, Kyoto, Japan) equipped with an Atlantis column (dC18, 4.6 mm i.d x 100 mm length, 5 µm particle size, Waters Associates, Chippendale, NSW, Australia). Analytical HPLC was run at 30 °C and monitored at 280 nm, 326 nm and 370. Injection volume was 10 µl. The following solvents in purified water with a flow rate of 1.0 ml/min were used: A, 0.5% trifluoroacetic acid (TFA) and B, 0.5% TFA + 95% acetonitrile. The elution profile was a linear gradient elution for B of 0 % to 20 % for 3 min, to 40 % for 5 min, to 60 % for 5 min, to 80 % for 5 min and to 100 % for 5 min. The gradient elution was held at the rate of 100 % B for 5 min. The identity of compounds was confirmed by co-chromatography and comparison of their spectral characteristics with those of authentic standards of esculin, chlorogenic acid and chicory acid.

The method of Gibson *et al*., (1994) was used with some modifications for the extraction and analysis of inulin from chicory (root, stems, leaf, and flowers *in vivo* and

leaf, callus *in vitro*). 100 mg of dried material was extracted separately in 10 ml of warm water to 1 ml of sample, equal volume of concentration of HCl and 0.1 ml resorcinol were added and made up to 10 ml with distilled water. The mixture was warmed on a water bath for 10 min and the absorbance was read at 490 nm in a spectrophotometer. The results were expressed as mg of inulin content (inulin equivalent) per gram of dry weight of the lyophilized powder (mg inulin equivalent/g DW), based on inulin standard curve and against a blank control. The analyses were conducted in triplicate.

3.8. Statistical Analysis

The mean values were calculated based on at least three determinations (n = 3). One way ANOVA followed by the Bonferroni *post-hoc* test were performed to assess differences between the samples at the level of $p<0.05$. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA).

4. RESULTS

4.1. Callus production

A. The leaf explants were cultured in B5 medium, supplemented with 0.14 mg/l BAP+0.16 mg/l IAA. The fresh and dry weight of callus was measure 0.61g and 0.13g respectively.

B. The leaf explants were cultured in MS medium, supplemented with 0.17 mg/l IBA+0.17 mg/l NAA. The fresh and dry weight of callus was measure 8.4 g and 0.6 g respectively.

C. The leaf explants were cultured in MS medium, supplemented with 0.16 mg/l IAA+0.14 mg/l BAP. The fresh and dry weight of callus was measure 0.61 g and 0.13 g respectively.

D. The leaf explants were cultured in MS medium, supplemented with 0.16 mg/l IBA+0.02 mg/l NAA. The fresh and dry weight of callus was measure 1.4 g and 0.13 g respectively.

E. The leaf explants were cultured in MS medium, supplemented with 0.16 mg/l IBA+0.024 mg/l BAP. The fresh and dry weight of callus was measure 1.3 g and 0.007 g respectively.

F. The leaf explants were cultured in B5 medium, supplemented with 0.055 mg/l IBA+0.17 mg/l IAA. The fresh and dry weight of callus was measure 10 g and 0.78 g respectively (Figures 4.1, and 4.2).

Table 4.1. *In vitro* callus production from *Cichorium intybus* L.

Type of Media	Type of PGRs concentration	Type of explant	Type of regeneration
B5	BAP 0,14 mg L^{-1} IAA 0,16 mg L^{-1}	Leaf	Callus +shoot
MS	NAA 0,17mg L^{-1} IBA 0,17 mg L^{-1}	Leaf	Callus +shoot
MS	IAA 0,16 mg L^{-1} BAP 0,14 mg L^{-1}	Leaf	Callus
MS	IBA 0,16 mg L^{-1} NAA 0,02mg L^{-1} GA_3 0,06 mg L^{-1}	Leaf	Callus +shoot
MS	IBA 0,16 mg L^{-1} BAP0,024mg L^{-1}	Leaf	Callus +root
B5	IBA 0,055mgL^{-1} IAA10,17mg L^{-1}	Leaf	Callus +shoot

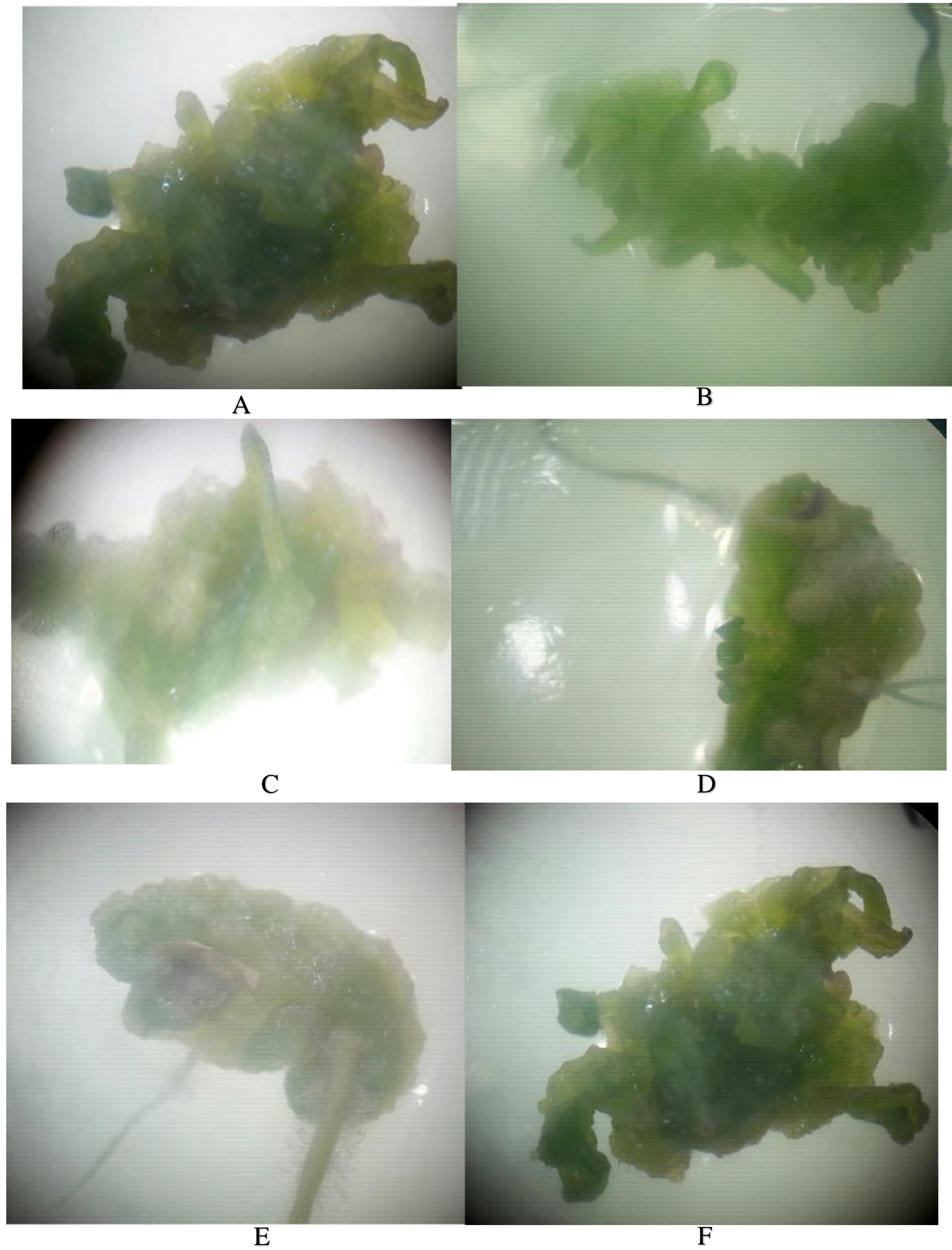


Figure 4.1. Calluses production from leaf explant after 7 days of incubation.

A

B

D

C

E

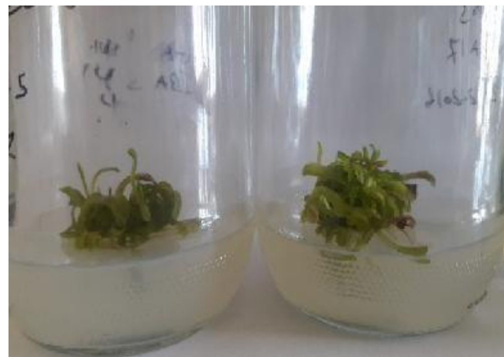


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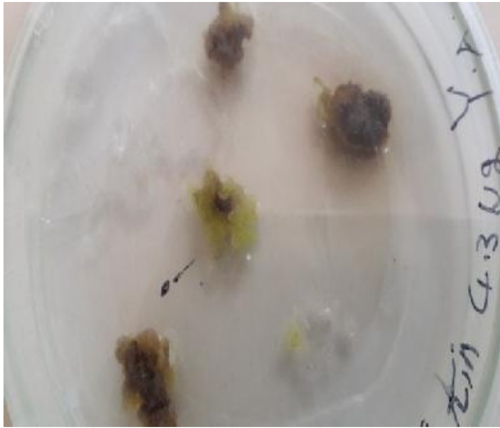
Figure 4.2. Callus production from leaf explant (Continued).



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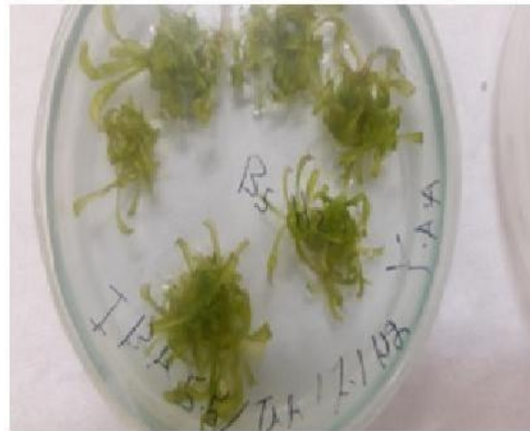

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Figure 4.2. Callus production from leaf explant.

Table 4.2. Dry weight and yield percentage of calli for extraction.

Type of media	Type of PGRs and concentration	Fresh weight by grams	Dry weight by grams	Yield % for extraction
B5	BAP 0,14 mg L^{-1} IAA 0,16 mg L^{-1}	0.61	0.13	21.31
MS	NAA 0,17 mg L^{-1} IBA 0,17 mg L^{-1}	8.4	0.6	7.14
MS	IAA 0,16 mg L^{-1} BAP 0,14 mg L^{-1}	0.61	0.13	21.31
MS	IBA 0,16 mg L^{-1} NAA 0,02 mg L^{-1} GA_3 0,06 mg L^{-1}	1.4	0.13	9.29
MS	IBA 0,16 mg L^{-1} BAP 0,024 mg L^{-1}	1.3	0.007	0.54
B5	IBA 0,055 mg L^{-1} IAA 10,17 mg L^{-1}	10	0.78	8.7

4.2. Root and Shoot Regeneration in Tissue Culture From Leaf Explant Via Direct Organogenesis

The young leaves taken from field samples of the plant were surface sterilized and incubated in MS and B5 medium supplemented with IBA and NAA. In two weeks time root and shoot regeneration occurred (Table 4.3, Figure 4.4). Plantlets were transferred to PGRs free MS and B5 medium in the bottles for maturation. Mature plants were transferred to soil and acclimated to the natural condition in the laboratory. B5 medium supplemented with 0.125 g/l IBA+0.1 g/l NAA was found to be more prolific for plant regeneration.

Table. 4. 3. Results for direct organogenesis of *Cichorium intybus* L. leaf explants.

Type of Medium	Type of PGRs and concentration	Number of roots	Length of roots (cm)	Number of leafs	Time (day)
MS	IBA 0,16 mg L^{-1} NAA 0,1 mg L^{-1}	2	5,5	7	54
B5	IBA 0,125 mg L^{-1} NAA 0,1 mg L^{-1}	2	10	27	77

Figure 4.3. Shoot and root regeneration from leaf explant.

Figure 4.4. Shoot and root regeneration from leaf explant (direct organogenesis).

4. 3. Quantitative analysis of Phenolic Composition

The main phenolic compositions; caftaric, chlorogenic, chicoric acids and esculin of *Cichorium intybus* L. were extracted from the sample of *in vivo* and *in vitro* regenerated plants and analysed in HPLC (Table 4.4) The chromatograms of standards are given in Figure 4.5.

Table 4.4. Phenolic Compositions of *Cichorium intybus* L.

Sample ID	Concentration of phenolic compounds (mg/g)			
	Caftaric acid	**Esculin**	**Chlorogenic acid**	**Chicoric acid**
Root in vivo	0.2±0.01f	0.2±0.001c	12.1±0.1c	6.3±0.1d
Stems in vivo	5.4±0.15b	0.4±0.005b	4.6±0.1d	3.9±0.08f
Leaf in vivo	2.0±0.01c	0.5±0.005b	24.2±0.1b	10.4±0.2c
Flower in vivo	17.3±1.2a	0.2±0.002c	65.2±0.1a	12.2±0.1b
Leaf in vitro	1.1±0.005d	6.1±0.4a	2.3±0.002e	15.4±0.1a
Callus in vitro	0.3±0.003e	0.4±0.004b	0.7±0.01f	5.6±0.1e

All data represent the mean ± standard deviation of at least three independent experiments.
Means with different letters in the same column were significantly different at the level (p<0, 05); n=3.

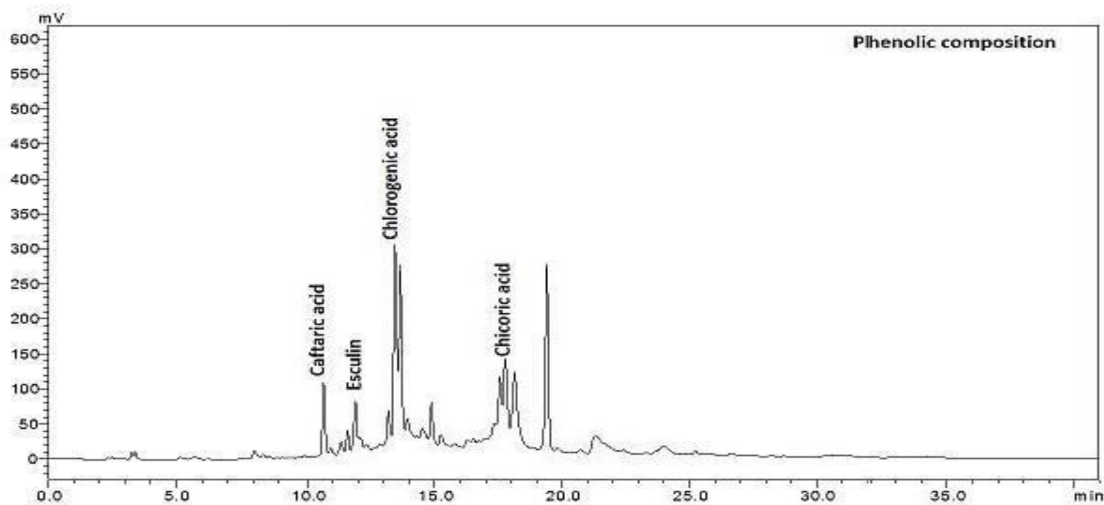

Figure. 4.5. The chromatogram of phenolic composition of *Cichoruim intybus* L. from leaf explant.

4.3.1. Caftaric acid

The caftaric acid concentrations were analysed in different samples (Figure 4.6). The highest value was found in flowers *in vivo* (17.3±1.2a mg/g-dw). The lowest value was found in root of *in vivo* samples (0.2±0.01f mg/g-dw).

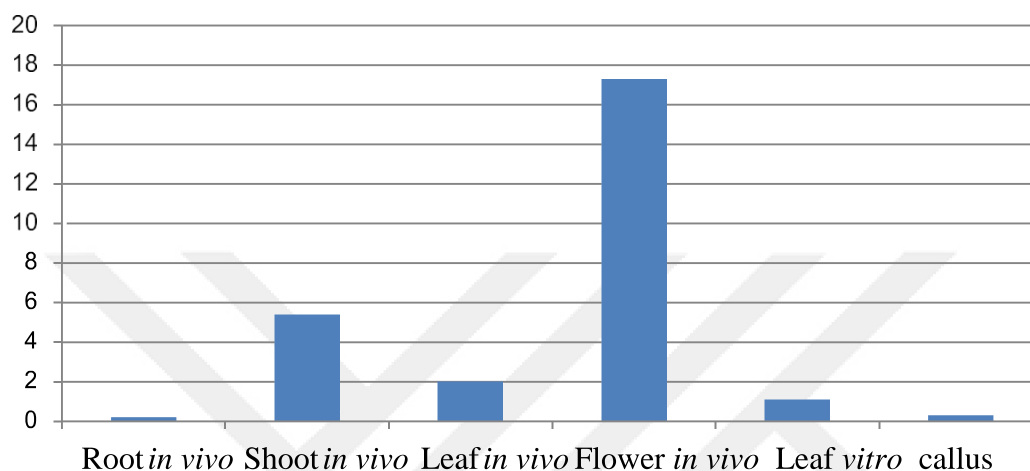

Figure 4.6. Caftaric acid concentration in (mg/g) the sample of study using HPLC.

4.3.2. Esculin

The highest esculin concentration was foundin leaf of in *vitro* regenerated plant (6.1±0.4a mg/g-dw). (Figure 4.7). Esculin concentration in roots and flowers of *in vivo* samples was in the same concentration (0.2±0.01c mg/g-dw) and less than all other parts.Its concentration in stems *in vivo* and callus *in vitro* samples was in the same concentration (0.4±0.004b mg/g-dw). The lowest esculin production was detected *in vitro* proliferated callus sample.

4.3.3. Chlorogenic acid

The lowest chlorogenic acid concentration was detected in callus was (0.7±0.01f mg/g-dw) and it is less than that of all other parts. On the other hand the highest concentration of chlorogenic acid (65.2±0.1a mg/g-dw) was found in flower (Figure 4.9).

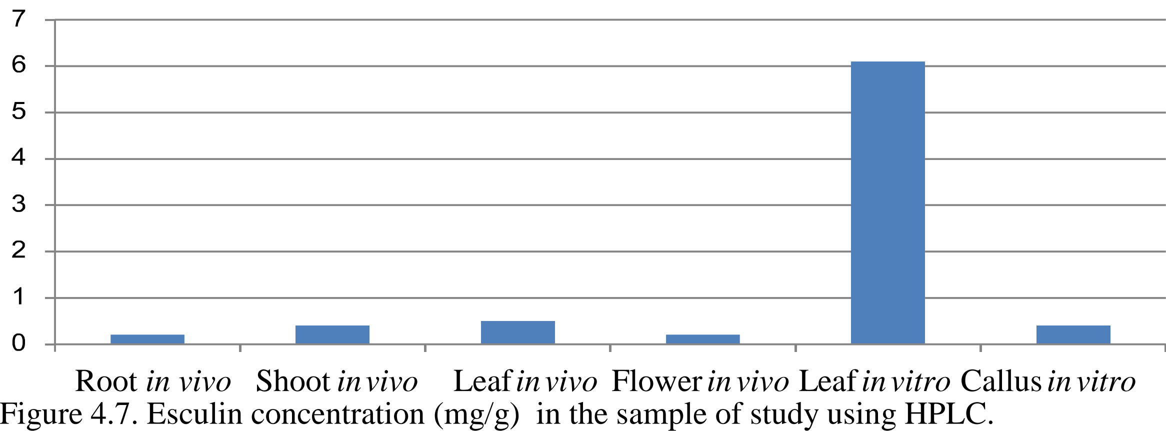

Figure 4.7. Esculin concentration (mg/g) in the sample of study using HPLC.

4.3.4. Chicoric acid

The lowest chicoric acid value was detected in stems *in vivo* (3.9±0.08f mg/g-dw). The high chicoric acid concentration was detected in leaves *in vitro* (15.4±0.1a mg/g-dw) (Figure 4.10).

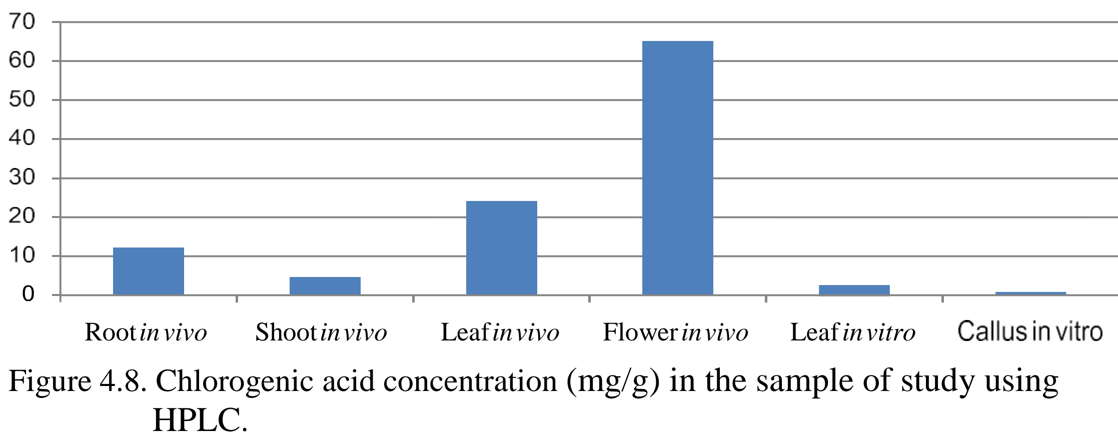

Figure 4.8. Chlorogenic acid concentration (mg/g) in the sample of study using HPLC.

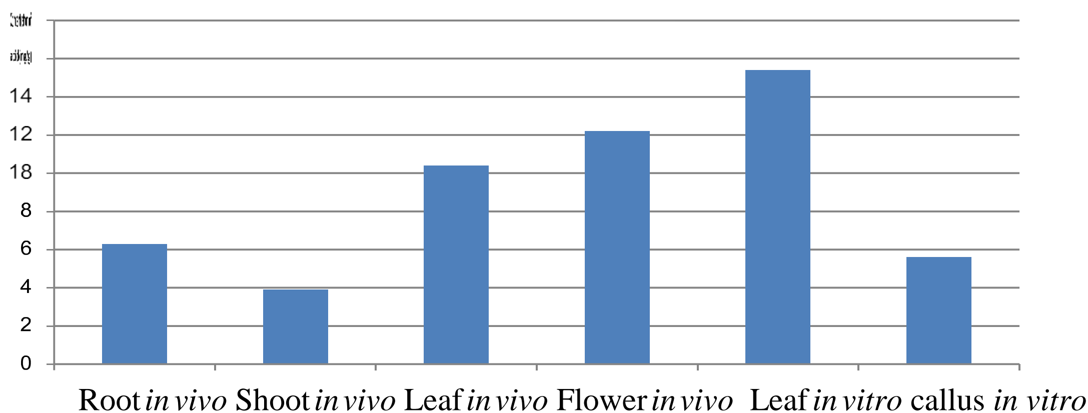

Figure 4.9. Chicoric acid concentration(mg/g) in the sample of study using HPLC.

4.4. Polysaccharide (Inulin) Analysis of *Cichorium intybus* L.in Spectrophotometer

Inulin as a main polysaccharide component in *Cichorium intybus* L was extracted and analysed in spectrophotometer. The high inulin concentration was found in root of *in vivo* sample (37.9±0.5a mg/g-dw). The lowest inulin concentration was found in callus (14.3±0.5e mg/g-dw) (Table 4.5).

Table 4.5. Concentration of Inulin of *Cichorium intybus* L. using spectrophotometer.

Sample ID	Concentration of *Inulin* (mg/g dry weight)
Root *in vivo*	37.9±0.5a
Stems *in vivo*	16.1±1.2d
Leaf *in vivo*	33.4±0.5b
Flower *in vivo*	17.9±1.6d
Leaf *in vitro*	28.8±0.5c
Callus *in vitro*	14.3±0.5e

All data represent the mean ± standard deviation of at least three independent experiments.
Means with different letters in the same column were significantly different at the level ($p < 0.05$); n=3.

5. DISCUSSION AND CONCLUSION

5.1. *In vitro* Regeneration of *Cichorium intybus*

Chicory is the medicinally important plant, due to its chemical compounds such as: inulin, esculin, volatile compounds (monoterpenes and sesquiterpenes), coumarins, flavonoids and vitamins (Nandagopal and Kumari, 2007). It is used against inflammation, as digestive, diuretic, vomiting, for the treatment of diarrhoea, cough, cancer, hangover, for purification of biliary tract, liver complaints, as spasmolytic, to relief of symptoms related to mild digestive disorders such as feeling of abdominal fullness, flatulence, and slow digestion, temporary loss of appetite, sore throat, haemorrhoids, tuberculosis, abdominal cramps, melancholy, deafness, and laxative for children (Al-Snafi, 2016). Due to the versatility, the regenerating of the plant in tissue culture is important. Collecting plants from field causes extinction of the distinguished specie and to be faced environmental challenges.

Callus production from leaf, shoot and root of *Cichorium intybus* L. were studied and increased based on the type of media and PGRs. MS and LS basal media, containing different concentration of 2, 4-D caused high amount of callus proliferation (Salehian, 2011). In the present study the leaf was used as explant and incubated in three different media supplemented with different concentration and combination of PGRs. The prolific result was provided from MS medium supplemented with IAA and BAP. PGRs type, concentration and combination play important role on the quality and quantity of callus production. Here one auxin and one cytokinin in combination with low concentration caused the best callus formation. It is usually expected that auxin produce more amount of callus due to its physiological role in cell proliferation. The result was supported by (Nandagopal and Kumari, 2006) indicating that high amount of callus was produced from leaf explants in MS medium adding different concentrations of IAA, IBA, NAA and 2, 4-D in combination with BAP.

5.2. Production of leaves and root *in vitro* (direct organogenesis)

Leaf was incubated in three different media supplemented with IBA and NAA shoot and root regeneration was observed in two weeks time. Plantlets were isolated from petri dishes and incubated in PGRs free MS medium in jars. Plantlets were maturated in 3-4 weeks time and transferred to soil, acclimated in uncontrolled environmental conditions. The result was supported by the study reported that leaf explant incubated in MS medium supplemented with auxin and cytokinin initiates shoot and root development. Auxins are reported to initiate root and cytokinins initiate shoot. The combination of the two PGRs initiate both root and shoot simultaneously (Nandagopal and Kumari, 2007). IAA is an auxin and has important property contributing to plant growth. It can be used for large scale plant propagation (Chavda et al., 2015). Plant growth regulators such as IBA play an important role in the rooting stage of tissue culture (Gateable´ and Pastor, 2006). In the present study, combining of cytokinin and auxin provided a useful effect on direct organogenesis. The combination of NAA with IBA resulted to direct shoot and root regeneration. Parallel results were reported by Annapurna and Rathore, (2010).

Amer et al., (2016) confirmed that in the case of culturing explants in MS medium with different PGRs at various concentration of IAA, IBA and NAA, high amount of roots were produced by direct organogenesis. In the current study MS and B5 media and same PGRs in different concentration are used and high amount of shoot and root were produced by direct organogenesis.

5. 3. Secondary Metabolite Production

In the present study, secondary metabolites extracted from *in vivo* and *in vitro* regenerated plant samples were quantitatively analysed. The least secondary metabolites production were provided from callus with some minor exceptions from *in vivo* specimens. Although callus tissue has advantages for the first step for suspension culture and bioreactor, long-term culture continuity with subcultures cause insufficient production of secondary metabolites. This can be explained by the fact that genes responsible for the synthesis of secondary metabolites in some non-differentiating

tissues, are not expressed, the metabolite is disintegrated immediately, the substrate is shifted to another pathway, and the produced metabolite is not at the storage site (Charlwood et al., 1990). There are many studies on the production of secondary metabolites in differentiated callus tissues to produce shoots or roots (Berlin et al., 1983). At the same time, callus is not a stable structure. Somaclonal variation may occur throughout subcultures. However, it can also cause undesirable properties to appear loss of yield. Also infertility is a problem that can be caused by somaclonal variations. For these reasons, studies on the production of adventitious shoots and roots from *Cichorium intybus* L. plant were carried out in tissue culture to obtain more secondary metabolites from shoots and roots than that of callus.

5.3.1 Extraction and Analysis of Phenolic Composition

In the present study the highest total phenolic compounds were analysed in flowers, roots, leaves and stems. The results are also parallel to the report of Dalar and Konczak, (2014) concluded that, the high concentration of total phenolic were found in leaves and flowers. Gallori et al., (2005) concluded that, the phenolic compounds in green chicory, red chicory and witloof chicory (Belgian endive) were the same.

Nandagoopal and Kumari (2007) concluded that the concentration of esculin found in callus was more than that of *in vivo* roots, and the maximum concentration of esculin was determined *in vitro* leaves and roots, while the minimum concentration of esculin was found in callus and *in vivo* leaf. In the present study the maximum esculin concentration was determined *in vitro* leaves while the minimum concentration of esculin found *in vivo* leaf and root. This is an important result for the present study by means of increasing secondary metabolite production *in vitro* cultures. Producing secondary metabolites in controlled the tissue culture was achieved. All of the samples regenerated in tissue culture produced secondary metabolites even in some case more than that of *in vivo* regenerated specimens. In tissue culture substances can be produced with a certain standard. Mass production of valuable chemicals in economic and medicinal terms can be achieved. So destruction of wild plants in nature can be reduced to minimum and less land use can be achieved.

In the current study, the highest concentration of caftaric acids was found in flowers *in vivo*, and the lowest concentration was found in root *in vivo*. Although the best results were provided from *in vivo* specimens, producing the compound *in vitro* applications is a promising result. The amount of the compound can be increased in tissue culture with some applications and modifications. Caftaric acid is well known as cinnamates (hydroxycinnamic acids) and responsible for yellowish-gold colour seen in some white's drinks (Cecotti and Passamonti, 2007).

Chlorogenic acid is an important phenylpropanoid with antioxidant properties. In the study, the concentration of chlorogenic acid *in vivo* samples was found more than that of *in vitro*. The highest chlorogenic acid concentration was determined in flowers *in vivo*, and the lowest one was in callus. The concentration of chlorogenic acid *in vivo* leaves was found more than that of *in vitro* leaves. Phenylpropanoids have very important functions in plants, as building elements, protectants against biotic and abiotic stresses, pigments and signalling molecules (Weisshaar and Jenkins, 1998). Factors such as pathogen invasion, UV, light density, injury, nutrient deficiency, temperature have been reported to increase the production of phenylpropanoids (Dixon and Paive, 1995). The amount of chlorogenic acid was increased by UV in *Hypericum perforatum* L. (Eray, 2016). In this study the amount of chlorogenic acid was very low *in vitro* samples. It can be attributed to the lack of abiotic and biotic stress factors. *Cichorium intybus* L. plant growing in field conditions produced more chlorogenic acid because of exposing hard environmental conditions as a protective agent against stress factors. Among the samples analysed here, the highest chlorogenic acid was detected in parts exposed to sunlight such as leaves and flowers.

The highest chicoric acid concentration was found in leaves *in vitro* of Cichory. *In vitro* samples here produced more chicoric acid than that of *in vivo* samples. The data is another remarkable result in the study. Chicoric acid production was accomplished in the samples regenerated through tissue culture and the amount is more than in the specimens provided from the plant grown in the field. Media compositions and PGRs in different combination and concentration may trigger secondary metabolite production. Callus also produced the compound. Chicoric acid protects plants from bacteria, virus, fungi, nematodes and insects that cause to wound in plants. Also these factors cause to produce more chicoric acid (Lee et al, 2013).

5.3.2 Polysaccharide (inulin) of *Cichorium intybus* by using spectrophotometer

In current study the highest inulin concentration was found *in vivo* root and leaves while the lowest in callus and flowers *in vivo*. The reason may be attributed to storage of the compound in roots and leaves. The finding is also in some extend parallel to Nandagoopal and Kumari, (2007) reported the maximum concentration of the compound in root of *in vivo* and *in vitro* regenerated plants. Inulin is fructan and mostly found in the Asteraceae family as chicory. The root of chicory is considered as one of the most important crops for the commercial extraction of inulin on industrial scale (Maroufi1 et al., 2012). Inulin is a reserve carbohydrate, and used to replace fat or sugar and reduce the calories of food. It is suitable for consumption by diabetics and is also used in inulin clearance test to measure glomerular filtration rate. Recent pharmacological investigation on the root extract of the plant revealed immunomodulatory, antitumor and anticancer properties (Nandagopal and Kumari, 2007). In this study, the concentration of inulin was found to be different in different part of the plant. It was found that the concentration of inulin was maximum in root *in vivo*. The amount of inulin in the leaves of plants grown *in vitro* is found to be more than that of flower and stem *in vivo*. The result is remarkable for commercially mass production of an important medicinal chemical such as inulin.

As a conclusion, the production of secondary metabolites in tissue culture even in callus has remarkable and promising result and also has the potential to be increased with some external application in controlled environment in tissue culture. For future perspective abiotic stress factors are suggested to apply on *in vitro* sample to increase the amount of medicinally important compounds.

REFERENCES

Abbas, Z. K., Saggu, S., Sakeran, M. I., Zidan, N., Rehman, H., and Ansari, A. A., 2015. Phytochemical, antioxidant and mineral composition of hydro alcoholic extract of chicory (*Cichorium intybus L.*) leaves. ***Journal of Biological Sciences***, **22**: 322–326.

Agarwal, Sarin, M., 2014. Phytochemical screening of sterols extracted from. ***World Journal of Pharmacy and Pharmaceutical Sciences***, **3**(1): 20-24.

Ali, Z., 2012. ***Transformation and Agrotechnology Strategies for Improved Levels of Secondary Metabolite in Cichorium intybus L***. PhD Thesis, Hamdard Unıversıty, New delhı-110062, Indıa.

Al-Snafi, A. E., 2016. Medical importance of *Cichorium intybus L.* ***IOSR Journal of Pharmacy***, **6** (3): 41-56.

Amer, A., Ibrahim, M., Askr, M., 2016. Establishment of *in vitro* root culture of *Cichorium endivia* subsp, pumelum L. a multipurpose medicinal plant. ***International Journal of Chem Tech Research***, **9** (12): 141-149.

Annapurna, D., Rathore, T. S., 2010. Direct adventitious shoot induction and plant regeneration of Embelia ribes Burm F. ***Plant Cell, Tissue and Organ Culture* (PCTOC)**, **101**(3): 269-277

Barbara, M. S., Nebojsa, I., Alexander, P., Ilya, R., 2007. Toxicological evaluation of chicory root extract. ***Food and Chemical Toxicology***, **45**: 113-139.

Barry, T. N., 1998. The feeding value of chicory (*Cichorium intybus*) for ruminant livestock. ***The Journal of Agricultural Science***, **131**(3): 251-257.

Bais, H. P., & Ravishankar, G. A., 2001. *Cichorium intybus* L–cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. ***Journal of the Science of Food and Agriculture***, **81**(5): 467-484.

Bukhari, M., Shah, Z., Syed, A., Kausar, A., 2016. Chlorogenic Acid: A pharmacologically potent molecule. ***Journal of Agricultural and Food Chemistry***, **73**(4): 851--854.

Berlin, J., Forche, E., Wray, V., Hammer, J., Hosel, W., 1983. Formation of benzophenanthiridine alkaloids by *suspension cultures of Eschscholtzia californica*. ***Z Naturforsch***, **38**: 346–52.

Charlwood, B. V., Rhodes, M. J., 1990. ***Secondary Products From Plant Tissue Culture***. *Oxford University Press.London United kingdom.*

Chavda, J., Dwsai, B., Hha, S., Tandel, M., Patel, D., 2015. Effect of PGR on clonal propagation of tion of madhunashini (Gymnema Sylvestre R. BR) through rooted cutting. ***An International Quarterly Journal of Life Sciences***, **10** (4): 1645-1648

Cecotti, AM, Passamonti, V., 2007. The fate of trans-caftaric acid administered into the rat stomach**.** ***Journal of Agricultural and Food Chemistry***, **8**(2): 15-20.

Coussement, P., 1999. Nutritional and health benefits of inulin and oligofructose. inulin and oligofructose: Safe Intakes and Legal Status. ***The Journal of Nutrition***,**10**: 5-7.

Dalar, A., Konczak, I., 2013. Phenolic contents, antioxidant capacities and inhibitory activities against key metabolic syndrome relevant enzymes of herbal teas from Eastern Anatolia. ***Industrial Crops and Products***, **44**: 383-90.

Dalara A, Konczaka, A., 2014. *Cichorium intybus* from eastern anatolia: Phenolic composition, antioxidant and enzyme inhibitory activities. ***Industrial Crops and Products*.1**: 79- 85.

Dixon, R.A., Paiva, N., 1995. Stressed induced phenyl propanoid metabolism**. *Plant Cell culture*, 7**: 1085-97.

Doliński, R., Olek, A., 2013. Micro propagation of wild chicory (*Cichorium Intybus* L. var. silvestre bisch. from leaf explants. ***Acta Sci. Pol., Hortorum Cultus***, **12**(6): 33-44.

Eray, N., 2016. ***In Vitro Şartlarda Yetiştirilen Hypericum Perforatum L. (Sari Kantaron) Bitkisinde Abiyotik Stres Faktörlerinin Fenolik Bileşik Miktarlarina Etkileri*** (yüksek lisans tezi, basılmamış). YYÜ, Fen Bilimleri Enstitüsü, Van.

Ev, L., 2013. Food value of *Cichorium intbus* L. ***Voprosy pitaniia***, **82**(2): 20-24.

Franklin, G., Dias, A. C. P., 2006. Organogenesis and embryogenesis in several *Hypericum perforatum* genotypes. ***In vitro Cellular and Developmental Biology-Plant*, 42**(4): 324-330.

Gâteblé, G., and Pastor, M., 2006. Ontogenic stage, auxin type and concentration influence rooting of oxera sulfurea stem cuttings. In international symposium on the labiatae***:*** *Advances in Production,* ***Biotechnology and Utilisation 723***: 269-272.

Gallori, I., Ieri, G., Milina, V., 2005. Evaluation of the phenol content in the aerial parts of cichorium using HPLC. ***Agricuture. Food Chemistry***., **53**: 6497-6502.

Gibson, G., Willis, C. L., & Van Loo, J., 1994. Non digestible oligosaccharides and bifidobacteria: implications for health. ***International Sugar Journal***, **96:** 381-387.

Illg, R. D., 1991. Plant tissue cultur techniques. ***Mem Inst. Oswaldo Gruz de Janeiro***, **86**: 21-24.

Jha, N., 2016. Plant tissue culture media: types, constituents, preparation and selection. Biology discussion. ***National Academy of Sciences 86***(2): 407-413

Khalil, M. M., Abouraya, M. A., Ibrahim, F. Y., AbdELrasool, E., AbdElall, E. E., 2015. Chemical and technological studies on some medicinal plants. ***Journal Food and Dairy Science*, 6**(2): 119 – 136.

Kulevanova, S., Svetl, M., 2003. HPLC Analysis of flavonoids. ***Macedonian pharmaceutical bulletin,* 58**: 39 – 44.

Lee, J. and Scagel, C. F., 2013. Chicoric acid: chemistry, distribution, and production. ***Frontiers in chemistry*, 1**, 40:1-17

Liang, X. Y., Zhang, X. Q., Bai, S. Q., Huang, L. K., Luo, X. M., Ji, Y., Jiang, L. F., 2014. Genetic diversity and relationship of chicory (*Cichorium intybus* L.) using sequence-related amplified polymorphism markers. ***Genetics and Molecular Research* 13**(3): 7736-7746.

Maroufi1, A., Bockstaele, E. V., Loose, M. D., 2012. Differential expression of fructan 1-exohydrolase genes involved in inulin biodegradation in chicory (*Cichorium intybus*) cultivars. ***Australian Journal of Crop Science*** (**AJCS**), **6** (9): 1362-1368.

Milala, G., Juśkiewicz, Z., Joanna, K., Jerzy, Z., 2009. Composition and properties of chicory extracts rich in fructans and polyphenols. ***Journal of Food and Nutrition Sciences***, **59**(1): 35-43.

Mulabagal, V., Tsay, H. S., 2004. Plant cell cultures-an alternative and efficient source for the production of biologically important secondary metabolites. ***International Journal Application Science Engineering***, **2**(1): 29-48.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures**.** ***Journal of Physiol. Plant***., **15**: 473-479.

Nandagopal, S., Ranjitha Kumari, B., 2007. Phytochemical and antibacterial studies of chicory (*Cichorium intybus* L.) - A multipurpose medicinal plant. ***Advances in Biological Research*** **1 (1-2):** 17-21.

Nandagopal, S. Ranjitha Kumari, D. B., 2006. Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cichorium intybus* L. cv. Focus - a potent medicinal plant. ***Acta agricultural Slovenica***.**87** (2): 415-425.

Nuutila, A. M., Kammiovirta, K., Oksman-Caldentey, K. M., 2002. Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. ***Food chemistry,*** **76**(4): 519-525.

Ranjitha Kumari, B. D., Velayutham, P. Anitha, S., 2007. A comparative study on inulin and esculin content of *in vitro* and *in vivo* plants of Chicory (*Cichorium intybus* L. cv. Lucknow Local). ***Advances in Biological Research***, **1**(1): 2.

Nandagopal, S., Ranjitha Kumari, D. B., 2007. Effectiveness of auxin induced *in vitro* root culture in chicory. ***Journal of Central European Agriculture***, **8**(1): 73-79.

Niness, K. R., 1999. Inulin and oligofructose: what are they? ***The Journal of Nutrition***, **129**(7): 1402-1406.

Pandey, A., 2013. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. ***Journal of Pharmacognosy and Phytochemistry***, **2**(5): 7-15.

Pullaiah, T., 2002. Medicinal Plants in India, an efficient *in vitro* plant regeneration system for *Cichorium.* ***Journal of Agricultural Technology,*** **5**(1): 287- 298.

Saad, A. I., Elshahed, A. M., 2012. ***Plant Tissue Culture Media***. Faculty of Science, Sebha University, Libya: INTECH.

Saini, M., ALi Khan, A., Bala, M., Abdin, M., Farooqi, H., 2014. Development of a validated hptlc method for quantification of esculin in different fractions of *cichorium intybus* leaf extract. ***International Journal of Pharmacy and Pharmaceutical Sciences,*** **6**(1): 478-482.

Salehian, H., 2011. Investigation of *in vitro* potential of callus induction in *Cichorium intybus L.*of medicinal plant. ***National Biotechnology***. **2**:190-201.

Spina, C., S., Acciarri, E., Fiore, M. A., 2008. Comparative evaluation of flavonoid content in assessing quality of wild and cultivated vegetables for human consumption. ***Journal of the Science of Food and Agriculture***, **88**: 294–304.

Starmans, D. A., Nijhuis, H. H., 1998. Extraction of secondary metabolites from plant materia. ***Trends in Food Science and Technology,*** **7**(6): 191-197.

Thorpe, T. A., 2007. History of plant tissue culture. ***Molecular biotechnology*****,** **37**(2): 169-180.

Torres, K. C., 1989. Tissue culture media composition and preparation**.** ***Tissue Culture Techniques for Horticultural Crops*** **25**: 26-51.

Touchell, D., Smith, J. Ranne, T., 2008. Novel applications of plant tissue cultur. ***Combined Proceedings International Plant Propagators***, **58**: 22-25.

Wang, Q., Cui, J., 2011. Perspectives and utilization technologies of chicory (*Cichorium intybus* L.): ***African Journal of Biotechnology,*** **10**(11): 1966-1977.

Weisshaar, B., Jenkins, G. I., 1998. Phenylpropanoid biosynthesis and its regulation. ***Current Opinion in Plant Biology*, 1**(3): 251-257.

WenYing, G., JinGu, L., 2012. Chicory seeds: a potential source of nutrition for food and feed. **Journal of Animal and Plant Sciences .13**(2): 1736-1746.

Yıldız, M., 2000. ***Keten (Linum Usitatissimum L) Bitkisinde Adventif Sürgün Rejenerasyonu Ve Agrobacterium Tumefaciens Aracılığıyla Gen Aktarımı.*** Doktora tezi. Doktora Tezi, Ankara Üniversitesi Fen Bilimleri Enstitüsü, Ankara.

Yucesan, B., Turker, A. U. Gurel, E., 2007. TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). ***Plant Cell, Tissue and Organ Culture*, 91**(3): 243-250.

APPENDIX

EXTENDED TURKISH ABSTRACT
(GENİŞLETİLMİŞ TÜRKÇE ÖZET)

Tezin Adı: Hindiba (*Cichorium Intybus* L.) Bitkisinin Doku Kültüründe Rejenerasyonu ve Sekonder Ürünlerinin İncelenmesi
Yüksek Lisans Öğrencisi: Yousif Abdullah ABAS
Danışman: Prof. Dr. Musa TÜRKER
İkinci Danışman: Dr. Öğr. Ü. Ayten EROĞLU

Bitkinin laboratuvar ortaminda doku kültürü ile üretilmesi

Bitkiler araziden toplanarak laboratuvar ortamına taşınmış ve saksılara ekilerek hayatta kalmaları ve canlılıklarının devam etmesi sağlanmıştır. Saksılara ekilen örnekler daha sonra bitkininin *in vitro* rejenerasyon çalışmalarında kullanılmıştır.

Bitkinin yaprakları eksplant olarak kullanılarak MS, B5 ve White ortamlarında oksin ve sitokinin BBD ile desteklenerek inkübe edilmiştir. *İn vitro* ortamda direkt organogenez NAA ve IBA varlığında MS ve B5 ortamlarında gerçekleştirilmiştir. 0,125 mg L^{-1} IBA+0,1 mg L^{-1} NAA içeren B5 ortamında gelişen bitkinin daha verimli olduğu tespit edilmiştir ve bunlardan alınan örnekler sekonder metabolit analizlerinde kullanılmıştır. Kallus gelişimi ise B5, MS ve white ortamlarında farklı BBD'ler kullanılarak gerçekleştirilmiştir. Sekonder ürün analizlerinde 0.16 mg/l IAA+0.14 mg/l BAP ile desteklenmiş MS ortamında gelişen kallus örnekleri kullanılmıştır. Elde edilen kalluslar, *in vivo* ve *in vitro* örneklerden etanol ekstraksiyonu yapılmış, liyofilize edilmiş ve sekonder metabolit analizi yapılıncaya kadar -20°C'de saklanmıştır.

Tablo. 4.1. *Cichorium intybus* L. yaprak eksplantlarından direkt organogenesis

Ortam Çeşiti	BBD ve konsantrasyonları	Kök Sayısı	Kök Uzunluğu (cm)	Yaprak Sayısı	Zaman (gün)
MS	IBA 0,16 mg L^{-1} NAA 0,1 mg L^{-1}	2	5,5	7	54
B5	IBA 0,125 mg L^{-1} NAA 0,1 mg L^{-1}	2	10	27	77

Tablo 4.2. *Chicorium intybus* L. bitkisinden kallus üretimi

Ortam Çeşiti	BBD ve konsantrasyonları	Eksplant Tipi	Rejenerasyon Tipi
B5	BAP 0,14 mg L^{-1} IAA 0,16 mg L^{-1}	Yaprak	Kallus+ Sürgün
MS	NAA 0,17 mg L^{-1} IBA 0,17 mg L^{-1}	Yaprak	Kallus+ Sürgün
MS	IAA 0,16 mg L^{-1} BAP 0,14 mg L^{-1}	Yaprak	Kallus
MS	IBA 0,16 mg L^{-1} NAA 0,02 mg L^{-1} GA_3 0,06 mg L^{-1}	Yaprak	Kallus+ Sürgün
MS	IBA 0,16 mg L^{-1} BAP 0,024 mg L^{-1}	Yaprak	kök +Kallus
B5	IBA 0,055 mg L^{-1} IAA 10,17 mg L^{-1}	Yaprak	Kallus+ Sürgün

Tablo 4.3. Ektraksiyon işlemi yapılacak kallusların liyofilizasyon sonrası verimleri

Ortam Çeşiti	BBD ve konsantrasyonları	Taze Ağırlık (gr)	Kuru ağırlık (gr)	Verim Yüzdesi (%)
B5	BAP 0,14 mg L^{-1} IAA 0,16 mg L^{-1}	0.61	0.13	21,31
MS	NAA 0,17 mg L^{-1} IBA 0,17 mg L^{-1}	8.4	0.6	7,,14
MS	IAA 0,16 mg L^{-1} BAP 0,14 mg L^{-1}	0.61	0.13	21,31
MS	IBA 0,16 mg L^{-1} NAA 0,02 mg L^{-1} GA_3 0,06 mg L^{-1}	1.4	0.13	9,29
MS	IBA 0,16 mg L^{-1} BAP 0,024 mg L^{-1}	1.3	0.007	0,54
B5	IBA 0,055 mg L^{-1} IAA 10,17 mg L^{-1}	10	0.78	7,8

Yaprak eksplantlarından doğrudan kök ve sürgün gelişimi 2 haftada gerçekleşmiştir. Fideler kavanozlara aktarılarak hormonsuz MS ortamında olgunlaştırılmış ve saksılarda dış ortama uyumları sağlanmıştır. *In vivo* ve *in vitro* ortamda yetişen bitkiler hasat edilmiş, organları kurutma kağıtları arasında direkt güneş ışığına maruz kalmadan kurutulup toz haline getirilmiştir. Sekonder metabolit analizi bu toz örneklerden yapılmıştır.

Sekonder ürünlerin analizleri

Araziden toplanan bitkilerin tamamına yakını organlarına göre parçalanarak sınıflandırılmış ve gölgede kurutulmuştur. Daha sonra *in vitro* ve *in vivo* örnekler toz haline getirilerek analizler için fenolik bileşikler ve inulin ekstraksiyonu etanol çözeltisinde gerçekleştirilmiştir. Sekonder ürünlerin kantitatif tayinleri HPLC cihazında gerçekleştirilmiştir. İnulin tayini ise spektrofotometirk olarak belirlenmiştir.

Tablo 4.4. *Cichoruim intybus* L. nin fenolik madde içeriği

Analiz Edilen Örnek	Fenolik bileşik konsantrasyonu (mg/g)			
	Kaftarik Asit	Eskulin	Klorojenik Asit	Kikorik Asit
Kök *in vivo*	0.2±0.01f	0.2±0.001c	12.1±0.1c	6.3±0.1d
Gövde *in vivo*	5.4±0.15b	0.4±0.005b	4.6±0.1d	3.9±0.08f
Yaprak *in vivo*	2.0±0.01c	0.5±0.005b	24.2±0.1b	10.4±0.2c
Çiçek *in vivo*	17.3±1.2a	0.2±0.002c	65.2±0.1a	12.2±0.1b
Yaprak *in vitro*	1.1±0.005d	6.1±0.4a	2.3±0.002e	15.4±0.1a
Kallus *in vitro*	0.3±0.003e	0.4±0.004b	0.7±0.01f	5.6±0.1e

Tüm veriler en az üç bağımsız deneyin ortalama ± standart sapmasını temsil etmektedir. Aynı kolondaki farklı harfler istatistiksel olarak anlamlıdır ($p < 0.05$); n = 3

En yüksek düzeyde kaftarik asit *in vivo* çiçek örneklerinden elde edilmiştir. En düşük düzeyde ise yine *in vivo* örneklerde kökten elde edilmiştir. Yani doku kültüründen elde edilen örneklerde belirlenen kaftarik asit miktarları *in vivo* kök örneklerinden daha fazla bulunmuştur. En yüksek eskulin miktarı *in vitro* ortamda yetişen yapraklardan elde edilmiştir. En düşük oranda ise *in vivo* ortamda araziden toplanan bitkilerin kök ve çiçeklerinden elde edilmiştir. *İn vitro* ortamda en yüksek düzeyde eskulin eldesi oldukça ümit verici bir sonuçtur. Doku kültüründe sekonder ürün artışı konusunda başarı sağlanmıştır.

En yüksek klorogenik asit miktarı *in vivo* örneklerde çiçeklerden elde edilmiştir. En düşük kloreogenik asit miktarı *in vitro* kalluslardan elde edilmiştir. Tüm kallus örneklerinin fenolik bileşikleri ve inulini üretiyor olması yine çalışmanın amacı bakımından oldukça ümit verici ve başarılı bir sonuçtur.

En yüksek düzeyde kikorik asit *in vitro* ortamda yetişen bitkinin yapraklarından elde edilmiştir. En düşük düzeyde ise *in vivo* gövde örneklerinden elde edilmiştir. Muhtemelen gövdede sentezlenen kikorik asit taşınmış ve gövdede minimum miktarda

kalmıştır. Yine *in vitro* örneklerde kikorik asitin en yüksek düzeyde elde edilmesi sonuçlar açısından dikkate değer ve ayrıntılı çalışmalar için ümit vericidir.

Bu çalışmada inulin HPLC ölçümlerinde sağlıklı olarak elde edilemediği için spektrofotometri yöntemi ile belirlenmiştir. En yüksek inulin miktarı *in vivo* köklerden elde edilmiştir. İnulin bir polisakarit olduğu için kökte depolanmış olma ihtimali yüksektir. En düşük inulin miktarı *in vitro* kallus örneklerinden elde edilmiştir.

Çalışmadan elde edilen önemli sonuçlar:

Bitki *in vitro* ortamda rejenere edilebilmiş ve kitlesel sekonder metabolit üretimine imkan sağlayan kallus kültürleri elde edilmiştir. Fenolik bileşikler ve inulin etanolde ekstre edilerek kantitatif tayinleri sonucunda, analizi yapılan tüm bileşiklerin doku kültürü örneklerinde varlığı tespit edilmiştir. En önemlisi bazı bileşiklerin doku kültürü ortamında daha fazla üretildiği tespit edilmiştir.

Tablo 4.5. *Cichoruim intybus* L. bitkisi *in vivo* ve *in vitro* örneklerinde spektorofotometrik olarak belirlenen inulin konsantrasyonları

Analiz Edilen Örnek	İnulin Konsantrasyonu (mg/g kuru ağırlık)
Kök *in vivo*	37.9±0.5a
Gövde *in vivo*	16.1±1.2d
Yaprak *in vivo*	33.4±0.5b
Çiçek *in vivo*	17.9±1.6d
Yaprak *in vitro*	28.8±0.5c
Kallus *in vitro*	14.3±0.5e

Tüm veriler en az üç bağımsız deneyin ortalama ± standart sapmasını temsil etmektedir.
Aynı kolondaki farklı harfler istatistiksel olarak anlamlıdır ($p < 0.05$); n = 3.

CURRICULUM VITAE

Yousif Abdullah ABAS was born in Naınaw North Iraq in 1980. He completed primary and secondary school in Akre Naınaw City. He completed undergraduate study in Horticulture Department, Faculty of Agriculture, and University of Duhok in 2009. He was registered to Master of Science program in Molecular Biology and Genetics Department, Yüzüncü Yıl University, Van-Turkey in 2015.

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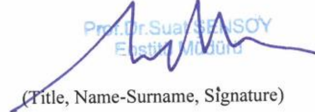

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