OPTIMIZATION OF HYPERICIN EXTRACTION FROM *HYPERICUM PERFORATUM* L.TISSUES AND EVALUATION OF ITS APPLICABILITY IN DYE-SENSITIZED SOLAR CELLS

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÖZGE AKTUNA

IN PARTIAL FULLFILMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEPTEMBER 2014

Approval of the thesis:

OPTIMIZATION OFHYPERICIN EXTRACTION FROM *HYPERICUM PERFORATUM* L. TISSUES AND EVALUATION OF ITS APPLICABILITY IN DYE-SENSITIZED SOLAR CELLS

submitted by **ÖZGE AKTUNA** in partial fulfillment of the requirements for the degree of **Masters of Science in Biotechnology Department**, **Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** ___________

Prof. Dr. Filiz Bengü Dilek
Head of Department **Biotechnology** ___________

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biology Department, METU** ___________

Prof. Dr. Füsun İnci Eyidoğan
Co-Supervisor, **Education Faculty, Başkent University** ___________

Examining Committee Members:

Prof. Dr. Meral Yücel
Biology Department, METU ___________

Prof. Dr. Hüseyin Avni Öktem
Biology Department, METU ___________

Prof. Dr. Füsun İnci Eyidoğan
Education Faculty, Başkent University ___________

Assoc. Prof. Dr. Remziye Yılmaz
MERLAB, Molecular Biology and Biotechnology R&D Dept., METU ___________

Assist. Prof. Dr. Kıvanç Bilecen
Genetics and Bioengineering Department, Okan University ___________

Date:05.09.2014

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Özge AKTUNA

Signature:

ABSTRACT

**OPTIMIZATION OF HYPERICIN EXTRACTION FROM *HYPERICUM PERFORATUM* L.
TISSUES AND EVALUATION OF ITS APPLICABILITY IN DYE-SENSITIZED SOLAR CELLS**

Aktuna, Özge

M.S., Department of Biotechnology

Supervisor: Prof. Dr. Hüseyin Avni Öktem

Co-supervisor: Prof. Dr. Füsun İnci Eyidoğan

September 2014, 75 pages

Medicinal plants which have been widely used in folk medicine are known to contain important biologically active compounds. *Hypericum perforatum* is one of the medicinal plants that grows in Europe, Western Asia and Northern Africa and is distinguished by its golden yellow flowers. The common name of the plant is St. John's wort. As a practical folk-remedy, it has been used widely to heal wounds, remedy kidney troubles, and alleviate nervous disorders. Nowadays, many application areas of hypericin are discovered such as antimicrobial, antiviral, antidepressive agent. Moreover, due to its photodynamic activity, the applications of hypericin in photodynamic therapy of cancer and in designing solar panels as dye-sensitized solar cell have been researched.

In order to extract hypericin from *H.perforatum,* ultrasonication and sonication methods were applied. Exposure times to ultrasonication and sonication, temperature, incubation time were the variables in the experiment. As a result, 20 minutes time to exposure to ultrasonication method gave the best results in analyses such as TLC, paper chromatography, spectrophotometry and LC-MS. Therefore, 20

minutes time to exposure to ultrasonication was determined as an optimum condition for extraction of hypericin. The extracted hypericin was successfully used as an organic dye in dye- sensitized solar cells.

Keywords: *Hypericum perforatum*, hypericin, TLC, LC-MS, dye-sensitized solar cells

ÖZ

*HYPERICUM PERFORATUM*L. DOKULARINDAN HİPERİSİN ELDESİNİN OPTİMİZASYONU VE HİPERİSİNİN BOYA DUYARLI GÜNEŞ HÜCRELERİNDE UYGULANABİLİRLİLİĞİNİN DEĞERLENDİRİLMESİ

Aktuna, Özge

Yüksek Lisans, Biyoteknoloji Bölümü

Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem

Ortak Tez Yöneticisi: Prof. Dr. Füsun İnci Eyidoğan

Eylül 2014, 75 Sayfa

Tıbbi bitkiler, günümüze kadar halk arasında ilaç olarak kullanılmış biyolojik aktiviteye sahip olan birçok madde içermektedirler. Günümüzde kullanılan birçok sentetik ilaç materyali bitkisel kaynaklı organik maddeler başlangıç maddesi alınarak sentezlenmektedir. *Hypericum perforatum* Avrupa, Batı Asya ve Kuzey Afrika'da yetişen bir tıbbi bitkidir ve altın sarısı çiçekleri ile tanınır. Bitkinin halk arasındaki ismi "sarı kantaron" dur. Bir halk ilacı olarak, hiperisin yaraların iyileşmesinde, yanıklarda, böbrek hastalıklarında ve sinir hastalıklarında kullanılmaktadır. Günümüzde ise *H.perforatum* ekstraktı olan hiperisinin bir çok alanda uygulamasına rastlanmaktadır. Hiperisin çeşitli alanlarda, antimikrobiyel, antiviral, antidepressan olarak kullanılmaktadır. Ayrıca fotodinamik özelliği kanıtlandığından beri kanser tedavisinde kullanımı ve güneş panellerinde boya duyarlı güneş hücreleri olarak kullanımı geliştirilmeye çalışılmıştır.

H.perforatum'dan hiperisin izole etmek için ultrasonikasyon ve sonikasyon yöntemleri denenmiştir Sıcaklık, inkübasyon süresi, ultrasonikasyon ve sonikasyona maruziyet süreleri deneyin değişkenlerini oluşturmaktadır. Sonuç olarak, TLC, kağıt

kromatografi, spektrofotometre, LC-MS gibi yöntemlerle analizler yapıldığında, ultrasonikasyona 20 dakika maruz bırakılarak yapılan deneme optimum koşul olarak belirlenmiştir. Ekstre edilen hiperisin boya duyarlı güneş hücrelerinin yapımında başarı ile kullanılmıştır.

Anahtar Kelimeler: *Hypericum perforatum*, hiperisin, TLC, LC-MS, boya duyarlı güneş hücreleri

To My Family Muratcan, Deniz & Cüneyt AKTUNA

&

Serkan KABAKCI,

For their support, encouragement & endless love…

ACKNOWLEDGEMENTS

I would like to thank to my supervisor Prof. Dr. Hüseyin Avni Öktem for his guidance and his valuable comments. He taught me how to be a member of a scientific project by giving responsibilities in every steps of the project and he also changed my vision about thinking not only in scientific area but also in industrial area.

I would like to thank to Prof. Dr. Ahmet Macit Özenbaş and his lab members in Metallurgical and Materials Engineering Department for their guidance. I was so lucky about studying with them.

I would like to thank to my co-supervisior Prof. Dr. Füsun Eyidoğan for her valuable suggestions and criticism during my thesis defense.

I would like to thank to Prof. Dr. Meral Yücel and Assoc. Prof. Dr. Remziye Yılmaz for their valuable comments in my thesis defense.

I would also thank to NANObiz Ltd. Co. members for providing me with all opportunities to conduct my study in their laboratory and for their support and guidance for this 3 years. Especially I would like to thank toDr. Ceren Caner Berkman, Yusuf Tolga Altuntaş, Sinan İncesu, Burhan Esen, Özge Kalman, I would never forget their supports. They always gave me moral and encouragement.

I wish thank to Assist. Prof. Dr. Kıvanç Bilecen, Batuhan Birol Keskin, Murat Kavruk for their valuable comments, guidance and moral supports.

As a member of Yücel-Öktem lab family, I would like to thank to Oya Ercan Akça, Ayten Eroğlu, Melih Onay, Işkın Köse, Dr. Tufan Öz for their moral and technical support. Especially, I would like to specially thank to Melih Onay who worked as hard as me in this study. I have learned a lot from him during this study.

I wish thank to my lovely friend Seren Atılgan. She was always with me when I need her support. Maybe her friendship is the most important gain for me in these 3 years.

During my master studies, the most beautiful, unbelievable memories were saved with her. I believe that this friendship will continue endlessly.

I owe my deepest gratitude to my best friend Yağmur Ünal. Her friendship is always invaluable for me. For 8 years, whenever I need her, she comes to me. Her laugh always gives me moral. I am sure that her shoulders are the most trustable place to cry. She always encourages and supports me not only for this study but also in every area of my life. I am very proud of being her best friend. Sometimes I see her more than my family. In these 8 years, all of the saved memories were beautiful and unbelievable because of her.

I wish thank to the most valuable things in my life, my mother Deniz Aktuna, my father Cüneyt Aktuna, my brother Murat Can Aktuna. If I can stand on my legs and achieve something in this life, it is totally the creation of my family. They always trust on me, encourage me and love me endlessly. In every step of my life, I know that they are always behind me. Whenever I achieve something, I see the honour in their eyes and this makes me encourage to achieve more. I am proud of being a member of this family. I would have been lost without them.

I would thank to the most special person in my life, Serkan Kabakcı. He always encourages me, believes me and loves me endlessly. He always makes me feel special. His love can always make me laugh. Distances never became a problem toreach his moral support to me. His trust and his love are the biggest power of my life.

It is my duty to express my gratitude to SANTEZ for the support that I have received as scholarships during my graduate education. This study was granted by SANTEZ (Project No: 00975.STZ.2011-2).

TABLE OF CONTENTS

ABSTRACT v

ÖZ..... vii

ACKNOWLEDGEMENTS x

TABLE OF CONTENTS xii

LIST OF FIGURES..... xiiiv

LIST OF TABLES xvii

LIST OF ABBREVATION..... xviii

CHAPTERS 1

1.INTRODUCTION..... 1

1.1 Medicinal Plants..... 1

1.2 *Hypericum Perforatum* L. 2

1.2.1Nomenclature of *Hypericum Perforatum* L. 4

1.2.2 The early uses of *Hypericum perforatum* L. as a medicinal plant 4

1.2.3Secondary metabolites of *Hypericum Perforatum* L. 4

1.3Hypericin and its Properties 6

1.3.1 Biosynthesis of Hypericin 8

1.3.2Application Areas of Hypericin 9

1.3.3. Production of Hypericin 14

1.4 Dye-sensitized Solar Cells 16

1.4.1Hypericin as dye for DSSCs..... 18

1.5Aim of the Study 18

2.MATERIALS AND METHODS 21

2.1 Materials 21

2.2 Methods 22

2.2.1 Reagents 22

2.2.2 Extraction of hypericin from *H.perforatum* 22

2.2.3 Separation of Carotenoid Pigments from *H.perforatum* 28

2.2.4 Extraction of *H.perforatum* 29

2.2.5 Incubation of *H.perforatum* on Stirrer 30

2.2.6 Evaporation of Methanol 30

2.2.7 Analyses of Hypericin 32

2.2.8 Extraction steps of hypericin from *H.perforatum* 35

3.RESULTS AND DISCUSSION 37

3.1 Pre-extraction of *H.perforatum* 37

3.1.1 Germination of plant material from seeds 37

3.1.2 Callus formation of *H.perforatum* from tissue culture 40

3.1.3 Plant material purchased from herbalist 45

3.2Separation of carotenoid pigments from *H.perforatum* 45

3.3Extraction of hypericin from *H.perforatum* 46

3.3.1 Sonication of *H.perforatum* & Incubation on Stirrer 46

3.3.2 Ultrasonication of *H.perforatum*& Incubation on Stirrer 48

3.3.3 Optimum Condition of Extraction of hypericin from *H.perforatum* 49

3.4Analyses of Hypericin 53

3.4.1Standard Graph of Hypericin 53

3.4.2 Analyses of Hypericin with Thin Layer Chromotography (TLC) 57

3.4.3 Analyses of Hypericin with Paper Chromotography 60

3.4.4 Analyses of Hypericin with LC-MS 61

3.5Designing of Solar Panels by Using Hypericin as Dye-Sensitized Solar Cell (DSSC) 64

4.CONCLUSION 67

REFERENCES 69

LIST OF FIGURES

FIGURES

Figure 1. Photo of *H.perforatum* 3
Figure 2. Chemical Structure of Hypericin (Molecular weight: 504.44 g/mole) 7
Figure 3.Chemical Structure of Pseudohypericin (Molecular weight: 520.44 g/mole) 7
Figure 4. Biosynthesis pathway of hypericin (Karppinen, 2010) 9
Figure 5. Designing of a Solar Panel 17
Figure 6. Dried *H.perforatum* from herbalist 23
Figure 7.Keeping *H.perforatum'*s seeds in cold 24
Figure 8.Planting *H.perforatum*'s cracked seeds 25
Figure 9. Covering the viols with stretch film 26
Figure 10. Germination of seeds 26
Figure 11. Removal of stretch film from viol 27
Figure 12.Preparing volumetric flask before evaporation of methanol 31
Figure 13. Transferring 2 mL extract to test tube 31
Figure 14 . Covering the viols with stretch film 38
Figure 15. Germination of seeds 39
Figure 16. Removing of stretch film from viol 39
Figure 17. Flowering of *H.perforatum* 40
Figure 18. Placing sterilized leaves of *H.perforatum* to liquid medium in shake flasks 41
Figure 19. Covering shake flasks with aliminium foil 42
Figure 20. Callus formation in Composition 3 43
Figure 21. Callus formation is Composition 2 44
Figure 22. Callus formation in Composition 1 44
Figure 23. OD vs Time Curve for Ultrasonication of *H.perforatum* 51
Figure 24. Dilutions of Hypericin Standard 54
Figure 25. Dilutions of Hypericin Standard 54
Figure 26.Standard Curve of Hypericin 56
Figure 27. TLC results of Optimum Conditions of *H.perforatum* 59

Figure 28. Paper Chromotography Results of Optimum Conditions of *H.perforatum* 61
Figure 29. LC-MS Results of Extraction of *H.perforatum* by the Optimum Condition for Ultrasonication (Black peak shows 0.125 ppm hypericin standard, red peak shows ¼ diluted sample) 62
Figure 30. Product ions of Hypericin Standard (Quantifier ion: 405.1m/z, Qualifier ion: 487.1m/z) 63
Figure 31. LC-MS Results of Extraction of *H.perforatum* by Incubation on Stirrer. 63
Figure 32. Product ions of hypericin standard (Quantifier ion: 405.1m/z, Qualifier ion: 487.1m/z) 64
Figure 33. Curves of hypericin based solar cells 66

LIST OF TABLES

TABLES

Table 1. Constituents of *Hypericum Perforatum* L. with main biological interest...... 5

Table 2. Flow chart of extraction method of hypericin.. 35

Table 3. Results of callus formation of *H.perforatum'* leaves.................................. 42

Table 4. Results of Extraction of Hypericin from *H.perforatum* by Sonication........ 47

Table 5. Results of Extraction of Hypericin by Ultrasonication from *H.perforatum.* 49

Table 6. Optimization of Ultrasonication Method for Hypericin Extraction from *H.perforatum*.. 50

Table 7. Results of Extraction of Other Starting Material Sources for *H.perforatum*52

Table 8. OD Values of Dilutions of Hypericin Standard at 590 nm.......................... 55

Table 9. LC-MS Results of Hypericin. 62

Table 10. Solar Cell Parameters by Using Hypericin based DSSC........................... 65

LIST OF ABBREVATION

MS medium	: Murashige and Skoog medium
TLC	: Thin Layer Chromatography
HPLC	: High Performance Liquid Chromatography
LC-MS	: Liquid chromatography–mass spectrometry
DSSC	: Dye-sensitized Solar Cell
PDT	: Photodynamic Therapy

CHAPTER 1

INTRODUCTION

1.1. Medicinal Plants

A medicinal plant is a plant which is used to relieve, prevent or cure a disease. A medicinal plant is also any plant used as a source of drugs or their precursors.

In recent years, side effects of conventional medicine, incorrect usage of synthetic drugs increase. Hence, there has been growing interest in medicinal plants (Vulto and Smet, 1988). In the world, approximately 25 percent of the drugs come from plant materials and 252 of them are shown as basic and essential by World Health Organization. Moreover, phytotherapy is considered in World Health Organization's health program (Vulto and Smet, 1988). Nowadays, large pharmaceutical companies, like Merk, CIBA, design drugs from plant materials (Reid et al., 1993).

Researching and developing therapeutic materials from plant origins is a very hard and expensive task. Designing of a new drug needs a large amount of investment and minimum ten years work (Boris, 1996). The most important thing is to select suitable plant by observing its traditional use as a medicine, its chemical content and toxicology (Williamson et. al., 1996).

Ethnopharmacology is known as observing the use of natural resources in folk medicine in different cultures. It is also the common strategy to select the most suitable medicinal plant. Preparation methods of plants used by ethnic groups may give an indication of appropriate extraction method of the plant. To extract the plant, firstly, the suitable plant should be chosen, collected and identified botanically. Thenthe next step should be the stabilization process. For stabilization process, the

plant material should be dried in a shady place in an optimum airflow and temperature. To do that, lyophilisation, freezing and alcohol vapor can be used (Williamson et. al., 1996).

After stabilization and drying of plant, plant material should be made as powder and a suitable extraction process should be applied. If the chemical composition of the plant is not known, the preparation and usage of the plant in folk medicine can be a guideline for the extraction. When extracting active components by chromatographic methods, components' structures are determined by using spectroscopic methods (Verpoorte, 1989).

1.2. *Hypericum Perforatum* L.

Hypericum perforatum L., is one of the most important traditional medicinal plant. *H.perforatum* is commonly known as St. John's wort and used in folk medicine for many different purposes for centuries (Nathan, 2001).

H. perforatum belongs to the *Hypericeae* family. *Hypericum* genus has approximately 450 species in it. This species includes herbaceous perennials, evergreen and deciduous shrubs as well as trees (Ayan & Çırak 2008, Kirakosyan *et al.* 2004). Although the herbaceous perennial *H. perforatum* belongs to Asia, Europe and North Africa, it is also seen in many temperate regions like North and South America, South Africa (Čellárová *et al.* 1995, Bruni & Sacchetti 2009). Generally, in these regions, *H.perforatum* is an aggressive weed (Mitich, 1994). Typically, *H.perforatum* is seen on sunny hillsides, fields, meadows and along roadsides (Bombardelli & Morazzoni 1995).

In Turkey, *H.perforatum* is known as ''sarı kantaron, binbirdelik otu, kuzukıran, kılıç otu, kan otu''. Turkey is the important center of *Hypericeae* family and *H.perforatum*is generally found almost everywhere, near the roads, forests and fields. In our country, 43 of 89 species are endemic (Çırak, 2006).The plant has opposite, sessile leaves andfive-petalled bright yellow flowers that are arranged in terminal

corymbs (Čellárová *et al.* 1995, Maleš *et al.* 2006). *H.perforatum* is shown in **Figure 1**;

Figure 1. Photo of *H.perforatum*

Among all medicinal plants, *H. perforatum* has always been a great interest for centuries. From the time of the ancient Anatolia down through the Middle Ages, the plant was considered to be imbued with magical powers and was used to ward off evil and protect against disease.*H.perforatum* is generallyused to heal wounds, cure kidney diseases and nervous disorders as a practical folk-remedy. At first, for nervous disorders *H.perforatum*'s antidepressant property is realized and the plant has been investigated increasingly to identify the antidepressant component of *H.perforatum* (Butterweck, 2003).

1.2.1. Nomenclature of *Hypericum Perforatum* L.

Since as a religious icon, the plant was considered as a symbol of protection, Linneus who described the genus thought that *Hypericum* came from yper (upper) and eikon (an image) (Jaeger, 1972). The golden flowers of the plant are believed in to symbolize the summer and the sun. The other name of *H.perforatum*, St.John's wort, comes from declaring the plant sacred to St John who was born in a summer day (Vickery, 1981). St John blessed the plant with healing power. The "wort" means that the plant is used as a medicinal plant.

1.2.2. The early uses of *Hypericum perforatum* L. as a medicinal plant

In the past, *H.perforatum* was considered as the most valuable herbal remedy and the best natural balsam to cure the deeper wounds by English herbalists (Gerard, 1633). In addition to healing property of deeper wounds, *Hypericum perforatum* has been used and is still used as an oil which was beneficial for the surgeons to clean foul wounds, for the remedy to burns, excoriations (Gerard, 1633). The oil of *H.perforatum* was prepared by macerating the flowering tops of the plant in oil and then placing them in the sun for two or three weeks. Nowadays, this preparation method became a guideline for extraction of active components of *H.perforatum*.

1.2.3. Secondary metabolites of *Hypericum Perforatum* L.

H.perforatum has many different types of active secondary metabolites belonging to different chemical groups. In the extracts of plant, Naphthodianthrones (hypericins), phloroglucinols (hyperforins), xanthones, essential oils, phenolic acids and a broad array of flavonoids, including flavonols, biflavones and procyanidins, have been identified (Greeson *et al.* 2001, Bilia *et al.* 2002). Naphthodianthrones, phloroglucinols and flavonoids are thought as the most important active compounds of *H.perforatum*. Especially for antidepressant activity, these secondary metabolites are the main contributors through different mechanisms (Nathan 2001, Medina, 2006). Secondary metabolites of *H.perforatum* are given in **Table 1** below;

Table 1. Constituents of *Hypericum Perforatum* L. with main biological interest.

Constituent groups	Names of constituents
Naphtodianhrones	Hypericin Psuedohypericin Protohypericin Protopsuedohypericin
Phloroglucinols	Hyperforin Adhyperforin
Phenolic carboxylic acids	Caffeic acid Chlorogenic acid Genistic acid Ferrulic acid
Flavonoids	Quercetin Hyperoside Quercitrin Isoquercitrin Rutin Campferol Myricetin Amentoflavone I3,II8-Biapigenin
Essential oils	α-pinene β-pinene Myrecene Limonene Alcohols
Procyanidins	Procyanidin Epictechin Epicatechin polymers
Tannins	Tannic acid
Aminoacids	GABA Cysteine Glutamine Luecine Lysine Ornithine Proline Threonine
Other water soluble Components	Peptides Polysaccharides

1.3. Hypericin and its Properties

Hypericin (Molecular weight: 504.44 g/mole) and pseudohypericin (Molecular weight: 520.44 g/mole) which are structurally found in chemical class of naphthodianthrones are typical secondary metabolite of *H.perforatum*. Naphthodianthrone content in *H.perforatum* ranges from 0.05% to 0.30%. This difference comes from the place where the plant grows because altitude, light conditions of the place and the period of year affect the naphthodianthrone amount of *H.perforatum* (Çırak,2006).

The plant also contains protohypericin, protopseudohypericin and cyclopseudohypericin. These secondary metabolites are located generally on dark granules stem, leaves, petals, stemans, ovules and flowering parts of the fresh plant known (Çırak, 2006). Pseudohypericin and hypericin are the most important metabolites of *H.perforatum*. Their biosynthetic precursors are protopseudohypericin and protohypericin, respectively. To convert precursors to active metabolites, pseudohypericin and hypericin, plant should be exposed to light. The optimal wavelength for the transformation of the protopigments in the extract and the flowertops of the plant was found to be 515 nm (Poutaraud, 2001). On the other hand, cyclopseudohypericin can be formed by transformation of pseudohypericin.

In *H.perforatum*, protopseudohypericin, cyclopseudohypericin and protohypericin are found in low concentrations. They are also formed the analytical term "total hypericins" or "total naphthodianthrones".

Generally, pseudohypericin is found more than hypericin in H.*perforatum*. 0.03-0.34% ratio belongs to pseudohypericin whereas hypericin has 0.03-0.09% ratio in the plant. This shows that the amount of pseudohypericin is two to four times more than the amount of hypericin (Kitanov, 2001). The chemical structures of hypericin and pseudohypericin are given below in **Figure 2** and in **Figure 3**;

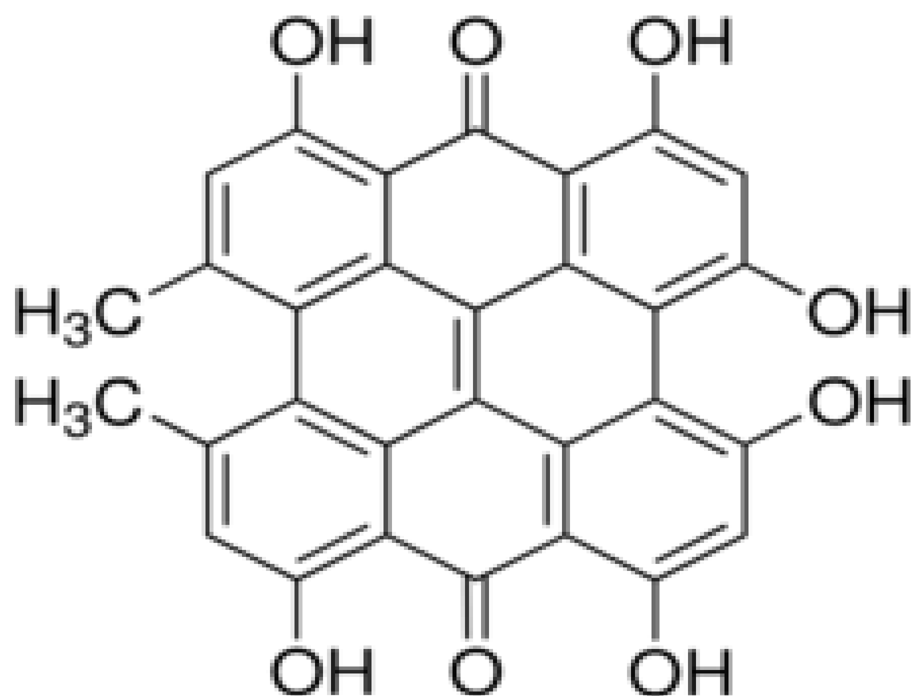


Figure 2. Chemical Structure of Hypericin (Molecular weight: 504.44 g/mole)

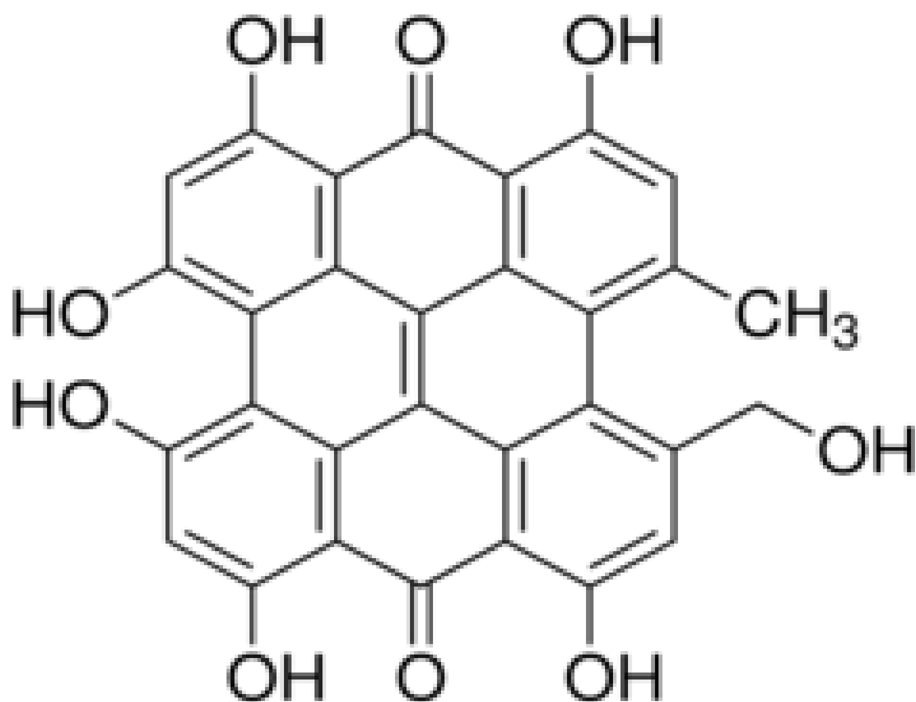


Figure 3. Chemical Structure of Pseudohypericin (Molecular weight: 520.44 g/mole)

1.3.1. Biosynthesis of Hypericin

The biosynthesis of hypericin and pseudohypericin, is presumed to follow the Polyketide Pathway. In this pathway, one molecule of acetyl CoA condensates with seven molecules of malonyl CoA in order to form an octaketide chain. This chain subsequently undergoes cyclizations and decarboxylation, leading to the formation of emodin anthrone (Huang et al, 2014). Emodin anthrone is considered to be the precursor of hypericin. When emodin anthrone is formed, it is oxidized to emodin, probably by the enzyme emodinanthrone-oxygenase and then through a condensation reaction yields a dianthrone. Successive oxidations lead to the formation of protohypericin, which upon irradiation with visible light yields hypericin. Oxidation of the methyl group of protohypericin is presumed to lead to protopseudohypercin, which is similarly transformed to pseudohypercin under visible light. This biosynthetic root is generally accepted today, even though further investigations that would prove it are necessary, and most importantly, the polyketide synthases that would play a crucial role have not yet been characterized (Karppinen, 2010). Pathway of biosynthesis of hypericin is given in **Figure 4**;

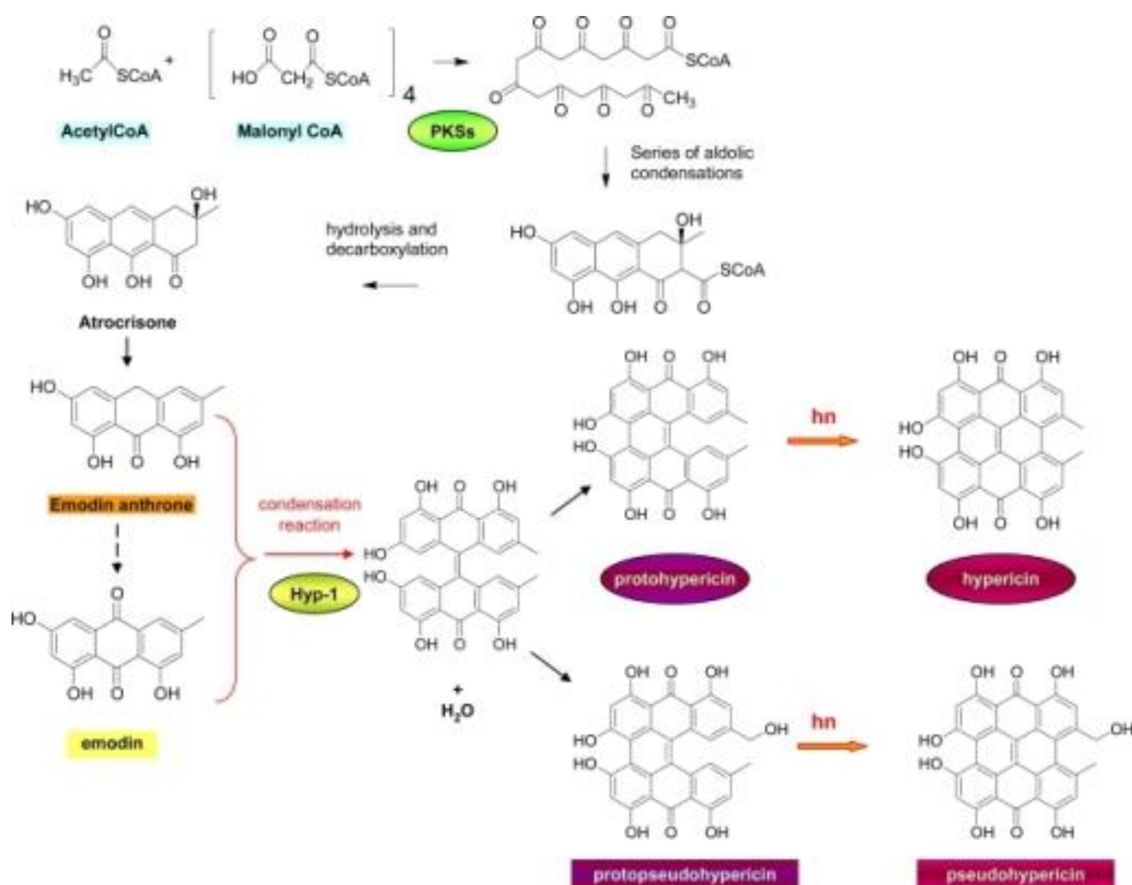


Figure 4. Biosynthesis pathway of hypericin (Karppinen, 2010)

1.3.2. Application Areas of Hypericin

Hypericin and *pseudohypericin* are biologically active compounds of *H.perforatum* which is shown in clinical demonstration. In 1998, the market value of St.John's wort exceeded US$ 210 million in USA alone and US$ 570 million worldwide. But, quality of active compound control, environmental conversation and safety are serious issue in medicinal plant (Mosaleeyanon et al, 2005). Today, plants that are grown in the field using for commercial St. John's wort products. However amount of *hypericin* that are handled from St. John's wort, is changed due to season, environmental conditions, abiotic & biotic contaminants and insects.

Hypericin and *pseudohypericin* contents in the leaves of St.John's wort may vary 50 fold in summer and winter grown plants (Mosaleeyanon et al, 2005). If plant material is used to pharmacological issue, plant growth should be done in controlled environments to ensure safety and efficacy. Also, in pharmacological studies of *hypericin,* growing the plant in controlled areas, prevent fluctuations.

Hypericin is used as pharmacological plant in many areas. Because of its dynamic properties, it is used in photo dynamic cancer therapy. *Hypericin* is also used as an antiviral and antibacterial component. In addition to these, *hypericin* is used as antidepressant component. Therefore, interest of *H. perforatum* is increasing in the world. In pharmacology, due to its remediation feature, the usage of *hypericin* is increasing. Nowadays, tablets, capsules, drops and teas of *H.perforatum*is sold in the market. It is oil also used in cure deeper wounds and burns.

1.3.2.1. Hypericin the photodynamic component of the plant

Hypericism which is a skin photosensitivity seen in cattle that ingested large amounts of hypericin containing plants and feed leaded to discovery of hypericin (Giese, 1971). The photosensitizing effects of skin by hypericin result in severe skin irritation, high body temperature and sometimes death of animal. Therefore, first research on hypericin's photodynamic property has been started.

There is no exact mechanism of the photosensitization of hypericin both *in vivo* and *in vitro* in literature. However, it has been confirmed that oxygen is needed for the hypericin photosensitization (Duran and Song, 2008).

Photodynamic action may be defined as the dye-sensitized photooxidation of cellular components. In photodynamic action oxygen, light and the sensitizing dye play an important role (Duran and Song, 2008).

Photodynamic property can lead to toxicity in living organisms. In the case of *Hypericum* toxicity, the compound hypericin is absorbed from the intestine and concentrates on the skin. An allergic reaction starts when the skin of the animal is exposed to sunlight. The photodynamic hemolysis which causes tissue damage

cannot be seen if there is no oxygen. Moreover, in the absence of sunlight, a reaction will not occur, and the compound does not show particular toxicity (Garrett, et. al., 1982). This first type of reaction is called “primary photosensitization” (Clare, 1952). Another more serious type is the“secondary photosensitization”, where the liver and other internal organs can be damaged (James and Johnson, 1976).

Since *H.perforatum* is being used a antidepressant in general, experts are warning people not to use excessive amount in order to prevent toxicity. On the other hand, photodynamic property of hypericin is thought to be used in Photodynamic Therapy of cancer.

1.3.2.2. Hypericin in Cancer Treatment

A photo sensitizer, molecule, that forms free superoxide which forms peroxide and hydroxyl radicals or non radical singlet oxygen molecules (Hunt et. al., 2001). In the presence of light, hypericin is a stong photosentisizer which can induce free radical production. This free radicals can lead to apoptosis of tumor cells (Piette, et. al., 2003). Thus, hypericin can be used in antitumor photodynamic therapy which sends visible light to tumor lesion to destroy it. With exporusing tumor cells to visible light, tumor vasculation is changed. In addition Photo Dynamic Threapy (PDT) create inflammatory response to releasing cytokines and antiflammatory mediators from treated cells.

Since PDT has minimum systemic toxicity and a highly selective photo destruction of tumor cells, PDT has more advantages than conventional therapeutic drugs or radiation cancer treatments. Especially, in skin cancer treatment PDT is the best way. Nowadays, usage of PDT in other types of cancer, such as esophageal, lung, bladder, has increased (Agostinis et. al., 2002).

While cancer treatment by using light and hypericin as PDT has been tried to improve, anti-tumoral activities of hypericin in dark conditions has been also studied

at the same time. These studies shows that hypericin can inhibit growth of tumor cells but there is no evidence that apoptosis is induced by hypericin (Blank, 2001).

Many photosensitizers used in PDT are lipophilic, this limits the administration of photosensitizers. On the other hand, systemic administration of photosensitizers can cause photosensitivity and the patient should avoid light exposure in short-term. As a result, in order to remove these restrictions of using photosensitizers in systemic administration, some strategies are studied. Conjugation of the photosensitizer to water soluble polymers, encapsulation the photosensitizer in nanoparticle carrier such as micelles can overcome the hydrophobicity and delivery limitation of hypericin as a photosensitizer (Lima et al, 2013).

1.3.2.3. Hypericin as an Anti-viral Agent

Hypericin and in lesser extent pseudohypericin have been the subject of intense research for their antiviral properties. In general, hypericin has shown antiviral activity *in vitro* against a great variety of viruses. It has shown virucidal activity by inhibiting viral infectivity in a hypericin-preincubation and light-dependent inactivation reaction and/or antiviral activity by inhibiting viral replication in cell cultures. This activity is influenced a great deal by the presence of light and oxygen. Both factors seem to be important for the antiviral/virucidal properties of the molecule (Karioti, 2010).

Some interaction studies of hypericin with the human immunodeficiency virus-1 reverse transcriptase and HIV-1 protease have been reported*in vitro*. However, administration of hypericin on HIV-1 and hepatitis C virus patients gave dissapointing results, since no antiviral effect was observed, and instead, patients showed phototoxicity with the dosage used. This contradiction between *in vitro* and *in vivo* studies could be explained in terms of light irradiation. The absence of light in many regions of the body limits the use of hypericin as a therapeutic compound for the treatment of viral infections *in vivo* (Hudson et. al., 1991).

1.3.2.4. Antimicrobial Activity of Hypericin

The traditional use of St John’s Wort includes the treatment for bacterial infections, respiratory conditions, skin wounds, peptic ulcers and inflammation. Several antimicrobial assays have been reported with extracts of different *Hypericum* species.

According to a study conducted in Hong Kong University, the photodynamic antimicrobial effect of hypericin was determined on clinically isolated *Staphylococcus aureus* and *Escherichia coli* cells. Bacterial cells (10^8 cells per mL) were incubated with hypericin (0-40 μM) for 30 min and followed by light irradiation of 600-800 nm at 5-30 J/cm^2. Cell survival was determined by colony counting, cellular hypericin uptake examined by flow cytometer, and cell membrane damage examined by scanning electron microscopy and leakage assay. Cellular structure and uptake of photosensitizer affected the effectiveness of hypericin-mediated photodynamic killing. The combination of hypericin and light irradiation could induce significant killing of Gram positive methicillin-sensitive and -resistant *S. aureus* cells (>6 log reduction), but was not effective on Gram negative *E. coli* cells (<0.2 log reduction). The difference was caused by different cell wall/membrane structures that directly affected cellular uptake of hypericin (Yow et al, 2012).

1.3.2.5. Antidepressive Effects of Hypericin

Usually known as St John's Wort, extracts of the plant *Hypericum perforatum*, have been used as an antidepressant for over 2000 years. In Germany St John's Wort is licensed for depression, anxiety and insomnia. There is increasing interest in the use of St John's Wort in the popular media (Bestic, 1999).

Currently, from all the scientific literature it is generally accepted that St. John’s wort preparations have a good profile of efficacy in mild to moderate depression and high tolerability (Maidment, 2000).

It is suggested that St John's Wort is more effective than placebo. However, St John's Wort is not as effective as therapeutic doses of standard antidepressants in severe depression. In clinical practice its use may be restricted to mild or moderate depression, where some data have shown that St John's Wort to have equivalent efficacy to low doses of conventional antidepressants. For such patients a possible advantage of St John's Wort is that it causes relatively few side-effects.The effective therapeutic dose and pharmacologically active components of St John's Wort require confirmation (Maidment, 2000).

1.3.3. Production of Hypericin

1.3.3.1. Tissue culture of *H.perforatum* L.

In vitro systems have been reported as an effective tool for obtaining genetically uniform plants, which can be a source of variable pharmaceutical preparations (Santarem and Astarita, 2003). Plant regeneration of *H.perforatum* L. has been achieved using as explants whole seedlings or their excised parts (Cellarova et al., 1994), hypocotyl sections (Murch et al., 2000a; Zobayed et al., 2004) and leaves using various types and concentrations of auxins and cytokinins.

Production of secondary metabolites via plant cell and tissue cultures yields various advantages, including standardization and quality. These criteria are also valid for the main economically important chemicals in St.John’s wort, namely hypericin, pseudohypericin and hyperforin (Zobayed et al., 2004).

Hypericumperforatum L. is a unique species that undergoes multiple forms of reproduction in the wild (Matzk et al., 2001). This reproductive flexibility has led to high chemical variability in field grown plants. Therefore, the application of in vitro techniques provides an approach for the production of standardized plant material (Murch et al., 2000).

Hypericin is generally extracted from flowers of wild or cultivated *Hypericum perforatum* L. plants. Both the limited area of occurrence of this plant and seasonal

harvesting necessitate a search for alternative methods for production of these compounds. In addition, plant tissue and cell cultures are important tools which allow extensive manipulation of the biosynthesis of the secondary compounds and yield a higher productivity compared to that of intact plants (Kirakosyan et al.,2004).

1.3.3.2. Extraction of hypericin from *H.perforatum* L.

Several extraction methods of naphthodianthrones from Hyperici herba have been described, including maceration, sonication, ultrasonication, Soxhlet extraction, supercritical fluid extraction and pressurized liquid extraction. Due to the very low content of naphthodianthrones in *H. perforatum* the extraction processes are of high cost and require multiple cycles and fast handling of the material, since hypericins are labile substances.

After the plant is dried to prevent the enzymatic hydrolysis of the compounds the plant material should be finely blended for effective extraction of the components.

During the extraction process the plant should be avoided form light for some compounds in the presence of light can be decomposed (Poole, 1991). The amount of solvent used, the time of the extractions and the number of subsequent extractions has significant effects on the amount of extract that can be obtained from the plant.

After filtration of the plant from the solvent the solvent is evaporized in a rotary evaporator or by lyophilization. It is important that the solvent is finely removed from the solid extract (Poole, 1991).

For the separation of the lipid soluble molecules from the plant material; the plant material may be extracted with chloroform, filtered and dried in an oven before the main extraction step (Çırak, 2006). The main extraction step can be made by using methanol (Anand et al, 2005).

1.3.3.3. Stability of *H.perforatum* L.

Tests on the stablity of hypericin and psuedohypericin showed that alcoholic solutions of the compounds were stable at -20°C for 140 days. Higher temperatures and light were observed to induce the degardation of psuedohypericin; where light was a more stronger stronger factor. Hypericin was more stable than psuedohypericin; that light was the only factor that led to the degradation of hypericin (Wirz et. al. ,2001).

Hypericin content of a juice of *Hypericum perforatum* and a powdered extract dropped by 14% during 1 year, and the dry extract remained stable, when stored at 20°C. When stored at 60°C, the hypericin content dropped 33%, 33%, and 47% from a powdered extract, tablets, and liquid juice, respectively (Adamski and Rekowska, 1971).

In one extensive study, up to 80% of the hypericin was shown to be destroyed by drying of the fresh plant in the presence of sunlight (Araya and Ford, 1981).

1.4. Dye-sensitized Solar Cells

Organic dyes such as 9-phenylxanthene dyes (e.g., rosebengal, fluorescein, and rhodamine B) were used asphotosensitizers for DSSCs in early studies. Recently, constructionof nanocrystalline DSSCs based on organic-dyephotosensitizers has been reported. Organic dyes haveseveral advantages as photosensitizers for DSSCs; they have larger absorption coefficients (attributed to an intramolecular *ð-ð** transition) than metal-complex photosensitizers (which are due to MLCT absorption), and these large coefficients lead to efficient light-harvesting properties; secondly, the variety in their structures provides possibilities for molecular design, e.g., the introduction of substituents, and thus allows for easy controlof their absorption spectra; there are no concerns about resource limitations, because organic dyes do not contain noble metals such as ruthenium. However, organic dyes have several disadvantages as photosensitizers. First, the emission lifetimes of their excited states are generally shorter than those of metal complexes (e.g., 0.4-5ns). However, if electron injection

from the organic dye to the conduction band of a semiconductor occurs much faster thanthe dye's emission lifetime, efficient charge separation can beachieved. A large electronic coupling between an organic dye and the conduction band of a semiconductor contributes toultra fast electron injection. Second, organic dyes have relatively sharp absorption bands in the visible region, which is disadvantage for the light harvesting of solar light. For example, DSSCs based on 9-phenylxanthene dyes such as eosin Y and mercurochrome adsorbed on TiO_2 and ZnO electrodes produced only 1.3-2.5% solar energy to-electricity conversion efficiencies,*è*, under AM 1.5, whereas they showed high incident photon-to-current conversion efficiencies (IPCEs) in the range from 400 to 500 nm. Using the molecular design of organic dyes to overcome their disadvantages is essential for the construction of highly efficient DSSCs based on the dyes. Organic-dye photosensitizers used in DSSCs, must have an anchoring group (e.g., -COOH,-SO3H) to be adsorbed onto the semiconductor surface with a large electronic coupling. In **Figure 5**, design of solar panel and the place where organic dyes are used are shown below;

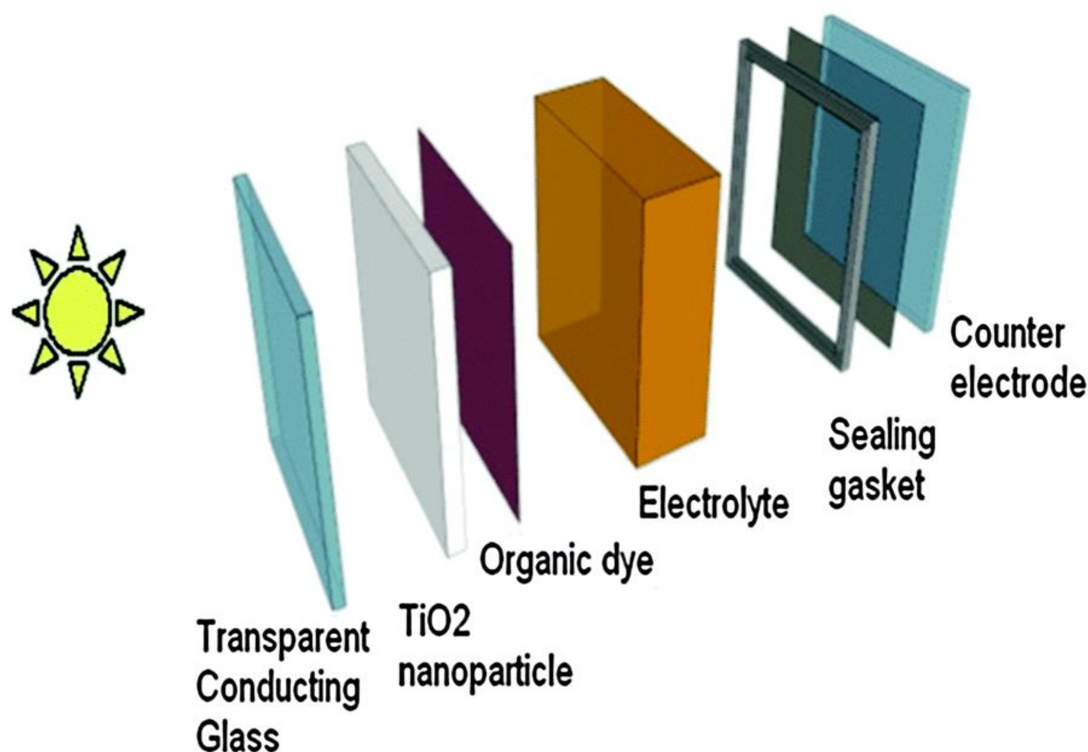


Figure 5. Designing of a Solar Panel

1.4.1. Hypericin as dye for DSSCs

Dye sensitized solar cell (DSSC) is a type technology for converting light energy into electrical energy based on the sensitization of wide band gap semiconductors. A DSSC is composed of a dye modified wide band semiconductor electrode such as TiO_2, ZnO, Nb_2O_5 and a counter electrode and the redox electrolyte. Similar to typical thin films photovoltaics, the performance of the cell mainly depends on the active layer; dye used as sensitizer (Suhaimi et al, 2013).

Alternatively, natural dyes can be used for the same purpose with an acceptable efficiency. A great attention has been paid to dye sensitized solar cells (DSSC) as cheap, effective and environmentally benign candidates for a new generation solar power devices. The sensitization of wide band gap semiconductor using natural pigments is usually ascribed to anthocyanins. The anthocyanins belong to the group of natural dyes responsible for several colors in red-blue range, found in fruits, flower and leaves of plants (Suhaimi et al, 2013).

Photosensitivity of hypericin makes hypericin as an alternative dye on conductive glasses of solar panels. Therefore, hypericin can be used as DSSC in solar panels (Zhou et al, 2011), in the place mentioned in **Figure 5** as organic dye. There are studies about application of plant extract as DSSC in solar panels in literature but there is not such an application in industry, yet.

1.5. Aim of the Study

In literature, extraction of hypericin from *H.perforatum* has been tried by different extraction methods since the biological active component of *H.perforatum,* hypericin, has many applications in literature. Hypericin play an important role in pharmacology as antimicrobial, antiviral, antidepressant agent. Moreover, hypericin has photodynamic activity. Therefore, it is used in cancer therapy.

This study aims to contribute to literature with a different application of hypericin, after extracting hypericin from *H.perforatum* by suitable extraction methods. Due to

photodynamic structure, hypericin can be used as dye-sensitized solar cell in designing of solar panels.

In both world and Turkey, fotovoltaic panels have being improved rapidly. The aim of this study is to investigate the application of extract of natural dyes on conductive glasses of these fotovoltaic panels. By this means, performance of dye-sensitized solar panels will increase and the usage of the dye originated from a plant in solar panels will decrease the cost of designing the solar panels in industry. Moreover, highlighting of a different application of hypericin may clear the way for using hypericin in different industrial areas.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Two types of *Hypericum perforatum* were used in this thesis. One type of them was grown from seeds. Seeds were purchasedfrom “Zengarden”. Their seeds are originated from England. These seeds were germinated and grown in soil with torfs. The other type of *H.perforatum* was purchased from herbalist as dried plant. For the extraction, ultrapure chloroform and 99.5% GC grade methanol are used. Chloroform was purchased from Applichem, methanol was purchased from Merck. For Thin Layer Chromotography (TLC) analysis, ethylacetate 99.5% GC grade, formic acid 99.5% GC grade, glacial acetic acid 99.5% GC grade and butanol 99% GC grade were purchased. Ethylacetate, formic acid and glacial acetic acid were obtained from Merck, whereas, butanol was from Riel-de Haen. TLC plates, TLC Silica Gel 60 F254 25 Aluminium Sheets 20 X 20 Cm, were from Merck. Chromotography papers, 100 sheets 46x57 cm, were obtained from Whatman Paper Ltd.Hypericin standard 10 mg 89.73% was purchased from HWI Analytik GMBH Pharma Solutions. For spectrophotometry, UV quartz cuvettes (micro rectangular, volume 0.6 mL, PTFE cover) were purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. Reagents

- 10% KOH: 10% KOH was prepared in methanol (99.5% GC grade)
- 10% PEG (Poly ethylene glycol): 10% PEG was prepared in dH_2O.
- 10% H_2SO_4: 10% H_2SO_4 was prepared in dH_2O.
- Ethylacetate: Formic Acid: Glacial Acetic Acid: Water
  (100: 11: 11: 26 v/v)
- Butanol: Acetic Acid: Water
  (28: 7: 35 v/v)

2.2.2 Extraction of hypericin from *H.perforatum*

2.2.2.1 Pre-extraction of *H.perforatum*

Three types of plant materials were used for extraction; namely, plant material bought from herbalist, plant material grown from seed and callus from tissue culture.

2.2.2.2 Dried *H.perforatum* purchased from herbalist

First of all, to make the optimization of extraction, the dried plants bought from herbalist were used. 1 g dried plant **(Figure 6)** was blended and made as powder. Therefore, the surface area was increased for enzymatic reactions.

Figure 6. Dried *H.perforatum* from herbalist

2.2.2.3 *H.perforatum* grown from seed

H.perforatum seeds were bought from "Zengarden". Their seeds were originated from England. In order to germinate the seeds different methods were used. Firstly, turf was put in a nylon bag and autoclaved at 121°C for 20 minutes so that turf became semi-sterile. Then, semi-sterile turfs were put into vases. On the other hand, seeds were washed in 3% sodium hypochloride solution for 20 minutes, next they were washed in dH_2O for 5 minutes. These washing steps were repeated three times so that seeds were surface-sterilized. The surface-sterilized seeds were planted in turfs. After planting, each one of vases was covered with one layered cheese-cloth and watered (Zobayed, 2004).

H.perforatum naturally germinates in spring. Therefore, seeds have dormancy in winter and it has to break their dormancies. To do that, planted vases were keeped in dark at 4°C for 2 days. Then they were placed in climate chamber and waited for germination **(Figure 7)**. Climate chamber was set at 20-25°C. This temperature is the optimum one for germination of *H.perforatum* (Çırak,2006).

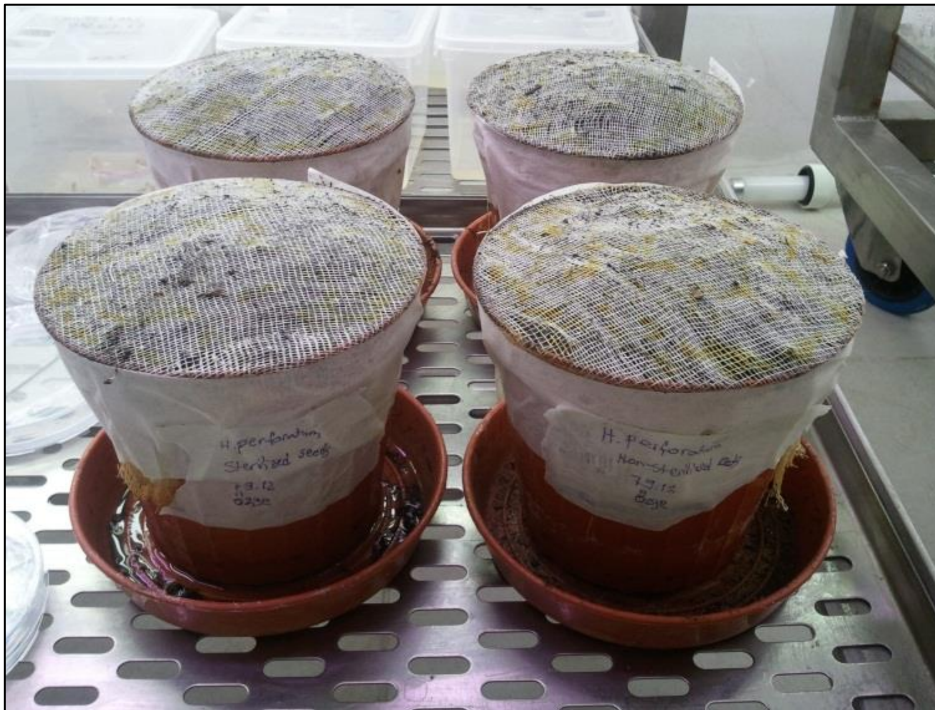


Figure 7. Keeping *H.perforatum'*s seeds in cold

As another method to break the dormancy, seeds were cracked by the aid of micropipette. This method was tried in Dicle University on *Hypericum triquetrifolium*and they broke the dormancy of plant by cracking (Karakas, 2005). Therefore, it was tried on *H.perforatum* **(Figure 8)**. This time, seeds were not surface-sterilized in order to mimic the natural conditions. Turfs were put in viols and seeds were planted on it. For control group, the seeds which were not cracked were used.

Figure 8. Planting *H.perforatum*'s cracked seeds

Last method was taken from Kaloorganik Tarim A.S. According to method, turfs were put into viols then seeds were placed on the top of turf. The key point was, there was not a slim layer of turf on the seeds. Seeds were just put onto the turf. Afterwards, the viols were covered by stretch-film **(Figure 9)** so as to provide humidity. When seeds started to germinate **(Figure 10)**, holes were made on stretch-film to get the air in it. Approximately 1 week later, stretch-film was removed **(Figure 11)**. After one week end, plants were watered every day.

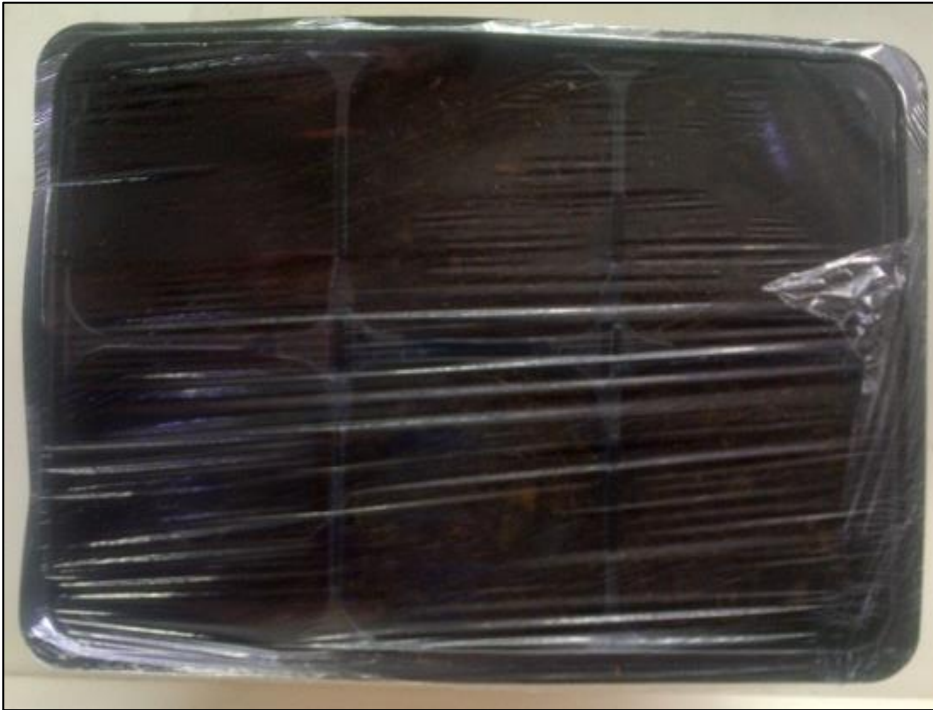


Figure 9. Covering the viols with stretch film

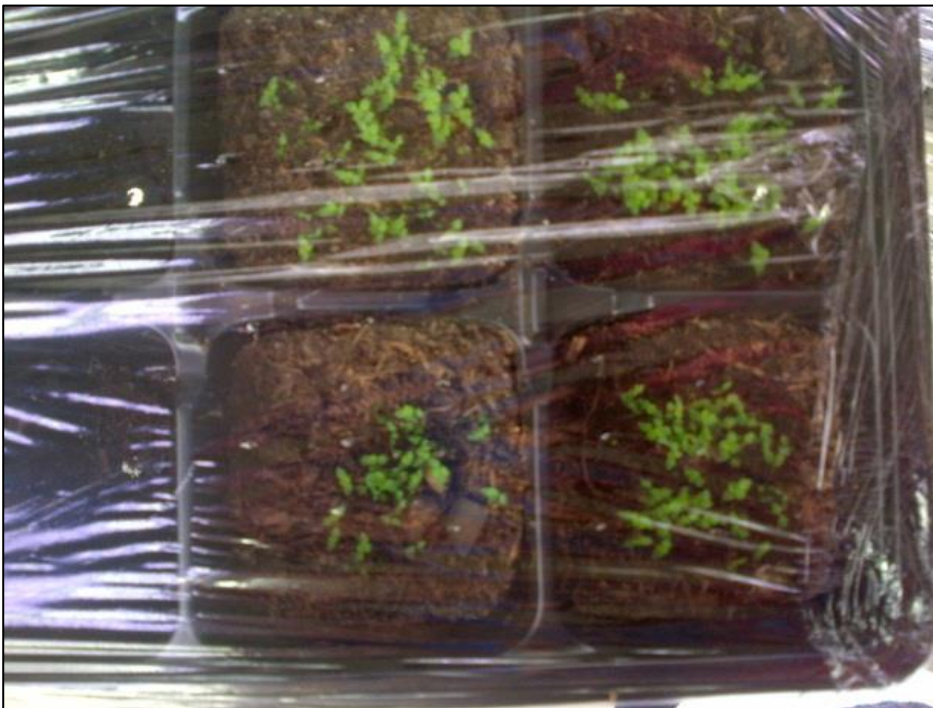


Figure 10. Germination of seeds

Figure 11. Removal of stretch film from viol

2.2.2.4 Callus from tissue culture of *H.perforatum*

Tissue culture was made from leaves from *H.perforatum* which was grown from seeds in turfs. Leaves were initially surface-sterilized. To do this, leaves were washed by 3% sodium hypochloride solution for 20 minutes. Then they were washed 3 times in dH_2O (Pretto and Santarem, 2000).

Callus formation in tissue culture were applied in both liquid medium by using shake flasks and solid medium by using sterile plates. Compositions of mediums were same for both except solidifying agent, Phytagel.

Murashige and Skoog (MS) medium with vitamins was prepared for bot liquid and solid mediums. Ingredients of MS medium are given below;

- MS with vitamins ⟶ 4,4 g/L
- Sucrose ⟶ 30 g/L
- Phytagel ⟶ 3 g/L (Only in solid medium)

This medium was prepared and then autoclaved at 121°C for 20 minutes. After sterilizing the medium, hormones were added and made filter sterilization. Three different hormone combinations were formed and MS medium with no hormones was set as control group. Hormone combinations are given below (Çırak,2006);

- 1. Composition: 1 mg/L 2-4-D + 0,1 mg/L Kinetin
- 2. Composition: 2 mg/L 2-4-D + 0,1 mg/L Kinetin
- 3. Composition: 3 mg/L 2-4-D + 0,1 mg/L Kinetin
- Control group: MS medium without hormones

After preparing mediums and sterilizing leaves, sterile leaves were cut and separated from each other. The important point was nodes should be transferred to medium with leaves, since callus formation began from nodes.

In shake flasks, medium were put into the flasks. Then sterile leaves were transferred to medium. Flasks were covered with aliminium foil due to photosensitivity of hypericin. Finally, flasks were placed into shaker in dark conditions and waited for 3 weeks to observe the callus formation.

2.2.3 Separation of Carotenoid Pigments from *H.perforatum*

Before extraction, it was needed to separate carotenoid pigments from plant. In order to separate carotenoid pigments, ultrapure chloroform was used (Çırak, 2006). 1 g *H.perforatum* powder was put into a flask and 50 mL ultrapure chloroform was added onto powder. And this flask was put on the stirrer (Brand: Wise Stir,Model: MSH-20A) for overnight.

The key point was keeping *H.perforatum* in the dark for whole experiment, because *H.perforatum* is photosensitive. After incubation, the powder of plant was filtered by filter paper and separated from chloroform. Therefore, carotenoid pigments were discarded with chloroform.

2.2.4. Extraction of *H.perforatum*

In order to optimize the extraction procedure, firstly dried plant materials bought from herbalist were used. After separating carotenoid pigments by chloroform, the remaining powder part of plant was collected from filter paper and put into 99.5% GC grademethanol (Çırak, 2006). Then, two different methods were tried; namely, sonication or ultrasonication. The aim was to give less sonic wave in short periods in a long time by using sonicator and give more sonic wave in long periods in a short time by using ultrasonicator. In this way, all parameters were fixed, just exposure time of ultrasonication and sonication were changed to optimize the procedure and to decide which procedure is the best for extraction (Zobayed et al, 2006). Besides extraction methods, incubation time and heat parameters were also optimized.

2.2.4.1. Sonication of *H.perforatum*

Sonicator was filled with ice-cold water to prevent heat formed during sonication which can damage enzymes. To find the optimum sonication exposure duration, 3 different durations for exposure were applied;

- 15 minutes sonication
- 30 minutes sonication
- 60 minutes sonication

2.2.4.2. Ultrasonication of *H.perforatum*

Like sonication, ultrasonication was also done on the ice in order not to damage enzymatic reactions. Suitable probe was submerged to the sample and ultrasonicator was set at suitable conditions. As suitable conditions, amplitude was set at 70, pulse was set at 9-10 sec. To find the optimum condition, different exposure times were tried. Different exposure times are given below;

- 5 minutes ultrasonication
- 15 minutes ultrasonication
- 20 minutes ultrasonication
- 30 minutes ultrasonication

2.2.5. Incubation of *H.perforatum* on Stirrer

After sonication, sample was put on the stirrer (Brand: Wise Stir,Model: MSH-20A). Different incubation times and different temperatures were tried to optimize the best conditions on stirrer. Different incubation times and temperatures are given below;

- 1 hour incubation at 50°C
- 2 hour incubation at 50°C
- 1 hour incubation at room temperature
- 2 hour incubation at room temperature
- Overnight incubation at room temperature

2.2.6. Evaporation of Methanol

After extraction, the target molecules were found in methanol. Therefore, methanol should be evaporated. To evaporate methanol, rotary evaporatorywas used from Middle East Technical University Central Laboratory. Firstly, the extract was put into volumetric flask and the flask was surrounded by aluminium foil in order to prevent light diffusion **(Figure 12)**. Then volumetric flask was placed to rotary evaporatory. Temperature was set at 40-45°C. Evaporation process was maintained until maximum 2 mL extract remained. The remaining 2 mL extract was transferred into test tube **(Figure 13)**.

Figure 12. Preparing volumetric flask before evaporation of methanol

Figure 13. Transferring 2 mL extract to test tube

The plug of the test tube left open for overnight so that all of the liquid part evaporated and just powder part of extract remained. 1 mg of powder was weighed and dissolved in methanol. With this procedure all of the extracts would be equalized.

2.2.7. Analyses of Hypericin

2.2.7.1. Hypericin Analyses with Spectrometry

OD of 1 mg of extract powder dissolved in methanol was measured by the aid of spectrophotometry. To measure UV quartz cuvette (micro rectangular, volume 0.6 mL, PTFE cover) was used. It was measured at 590 nm wavelength which is specific for hypericin (Çırak,2006). The blank was 99.5% GC grade methanol. At this wavelength, not only pure hypericin gives peak, but also other ring structures like derivatives of hypericin give peak. Hence, it was needed to separate the concentration of hypericin from this mixture. To do that, first of all "Standard Curve of Hypericin" was formed.

2.2.7.2. Hypericin Standard Curve

From HWI Analytik GMBH Pharma Solutions, 10 mg 89.73% hypericin standard was purchased. Graph of Standard was formed by using hypericin standard. The aim of using standard of hypericin was to compare the extracts with standard. Therefore, preparation of hypericin standard from powder should be same with the procedures of extracts. 1 mg of hypericin standard was weighed and dissolved in 1 mL 99.5% GC methanol so that 2 mM standard hypericin was prepared as stock solution. Since stock solution was too dense, it had to be diluted. As a result, 20 µL stock solution was completed to 2000 µL by 99.5% GC methanol. In this way, 100X dilution was made and the concentration decreased to 20 µM. This sample was measured at 590 nm by spectrophotometry. Then this sample was diluted several times and got

different molarities. When these different molarities were measured as OD, graph of standard could be drawn.

2.2.7.3. Hypericin Analyses with Thin Layer Chromatography (TLC)

For TLC analyses, two different solutions were tried which is given below (Vattikuti and Ciddi, 2005);

- Ethylacetate: Formic Acid: Glacial Acetic Acid: Water
  (100: 11: 11: 26 v/v)
- Butanol: Glacial Acetic Acid: Water
  (28: 7: 35 v/v)

Firstly, TLC plates were cut in suitable dimensions (length: 6cm, width: 4cm). In order to equalize all conditions for all samples, 1 mg was weighed from each *H.perforatum* extract powder and dissolved in 1 mL methanol. Maximum 5 µL of samples were loaded to TLC plates. 20 µM hypericin standard was also loaded to compare with samples. Then, TLC plate was observed under ultraviolet light and looked at if the extracts were at the same level with standard and brightness of extracts. But to be visible under UV, three different dyes were sprayed and tried to find the optimum solvent-dye system. After spraying the dye, TLC plate was heated for 5-6 minutes to dry the dye. Dyes were given below;

- %10 KOH
- %10 PEG (Poly ethylene glycol)
- %10 H_2SO_4

2.2.7.4 Hypericin Analyses with Paper Chromotography

For paper chromatography, same solvent and dye system with TLC were used. Firstly, chromatography papers were cut in suitable dimensions (length: 10 cm, width: 4 cm). As same as TLC, samples and standard were loaded side by side. After spraying dye and heating, samples were observed under UV and looked at if the

extracts were at the same level or not and the brightness of extracts (Vattikuti and Ciddi, 2005).

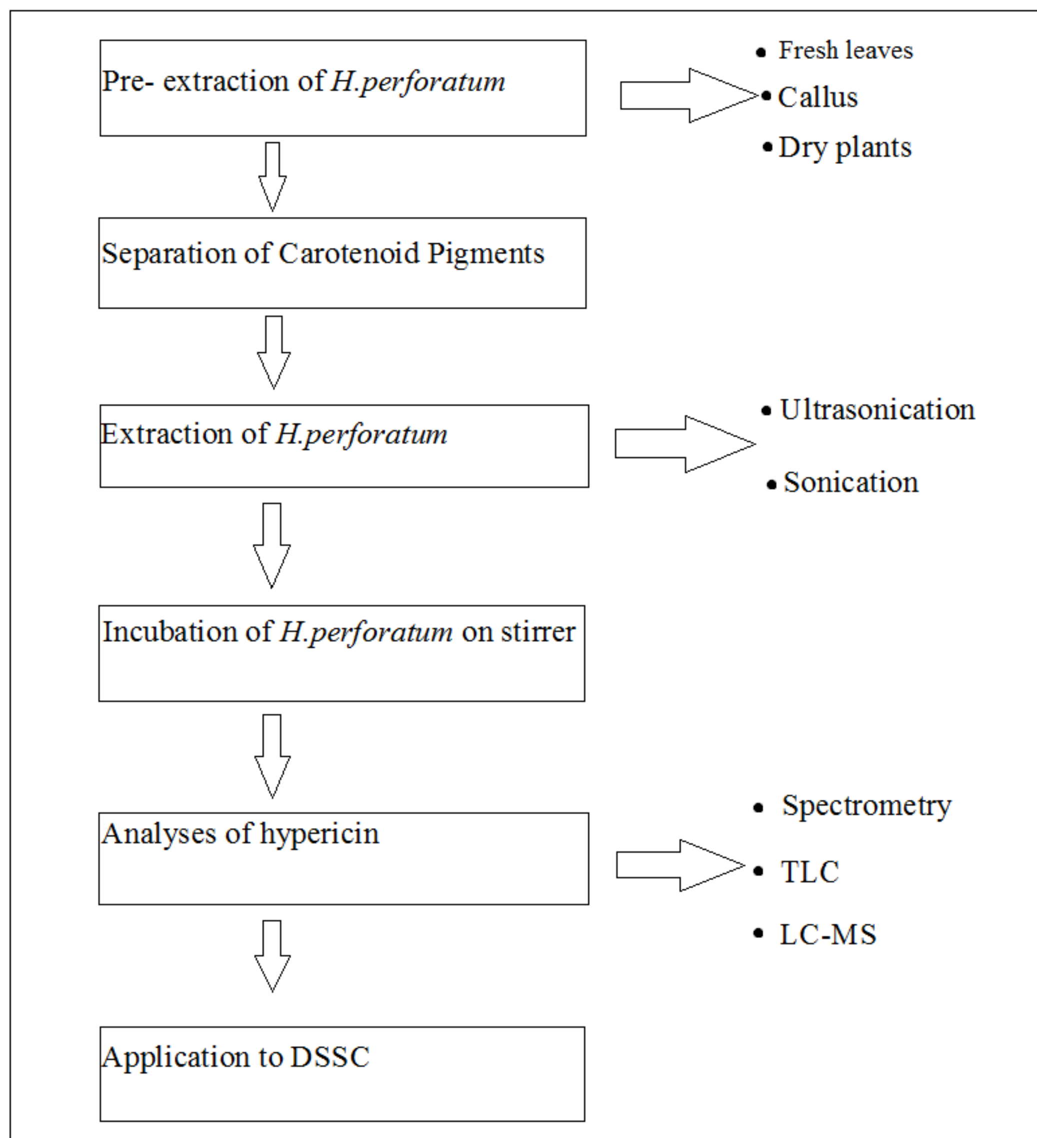
2.2.7.5 Hypericin Analyses with LC-MS

Liquid chromatography- mass spectrometry (LC-MS) is a type of HPLC (High performance liquid chromatography) system including a mass spec detector. Chemicals are separated by conventional chromatography on a column in HPLC (Brolis, 1998). The method is a reverse phase chromatography. In this chromatography, the metabolite binds to the column hydrophobically in the presence of a hydrophilic solvent. Then, the metabolite is eluted by a more hydrophobic solvent. After the metabolites are seen at the end of the column, they enter the mass detector. In the mass detector, the solvent is removed and the metabolites are ionized. Ionization step should be strictly conducted since the detector cannot work with neutral molecules. Ions can only go through a very good vacuum. Therefore, removal of solvent is the first step. Afterwards, mass detector scans the molecules and separates all ions that have different masses (Grebe, 2011).

After extraction of hypericin from *H.perforatum*, the purity of hypericin was analyzed by LC-MS. This experiment was conducted in Middle East Technical University Central Laboratory. For analyse, 2 mM hypericin standard, sample from optimum condition of ultrasonication (1mg extract powder dissolved in 1 mL 99.5% GC methanol) and sample from extraction by using strirrer (1mg extract powder dissolved in 1 mL 99.5% GC methanol) were given to Central Laboratory. Firstly, hypericin standard was ionized to its product ions. Molecular weight of hypericin standard is 504.44 g/mole. Hypericin standard's quantifier ion is 405.1 m/z, qualifier ion is 487.1 m/z. Hypericin standard's peak in LC-MS was constituted by using 0.125 ppm standard. In addition, during analyzing, samples were ¼ diluted. After constituting the standard's peak in LC-MS, samples peaks were compared with standard and if the samples' peaks overlap with the peak of standard, it can be interpreted that extracts include hypericin in their contents.

2.2.8. Extraction steps of hypericin from *H.perforatum*

Table 2. Flow chart of extraction method of hypericin



CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to isolate the major photoactive constituent, hypericin, from *Hypericum perforatum*. Hypericin extract was aimed to be used as organic dye in designing of dye-sensitized solar panels. As plant material three different sources were used; the germinated plant material from seed, callus from tissue culture, dry plants bought from herbalist.

3.1. Pre-extraction of *H.perforatum*

3.1.1. Germination of plant material from seeds

To germinate seeds different methods were tried. Firstly, semi-sterile turf and surface-sterilized seeds were applied. Sterilization of turf was made by autoclave at 121°C for 20 min, on the other hand, in addition to this, sterilization of seeds were made by 3% hypochloride solution and dH_2O. Seeds' washing step was repeated three times. When sterilization was over, semi-sterile turf was placed into vase and sterilized seed were planted on it. A thin layer of turf covered the top of the seeds. Then, after planting each one of vases was covered with one layered cheese-cloth and watered. Normally, seeds have dormancy and to germinate it had to break the dormancy. To do that, planted vases were placed in dark and cold room to mimic the winter conditions at 4°C for 2 days. After dark conditions, plants were put into climate chamber which had 20-25°C temperature. Approximately 20 days were waited for germination but germination was not observed under these conditions, which is most probably, due to unbroken dormancy of seeds.

Therefore another method was tried. To break dormancy of seeds, cracking method was applied. Seeds were cracked by the aid of micropipette before planting. This method was adopted from a previous study coming from Dicle University (Karakas, 2005). Since these two methods mentioned above could not germinate the seeds of *H.perforatum*, another method was investigated. Kaloorganik Tarim A.S. was contacted. According to their suggestion, after putting turfs in viols, seeds were just put on the top of the turf so that there was no a slim layer of turf on the seeds. Then, viols were covered by stretch film until germination began **(Figure 14)**. When this method was applied, germination was observed within a week **(Figure 15)** and holes were made on stretch film in order to start adaptation. In second week of germination stretch film was removed completely **(Figure 16)**. Watering was made once in every two days. Germinated plants were waited to reach approximately 15-20 cm to be used as fresh plant and tissue culture and when they reached to enough length flowering was seen **(Figure 17)**.

Figure 14 . Covering the viols with stretch film

Figure 15. Germination of seeds

Figure 16. Removing of stretch film from viol

Figure 17. Flowering of *H.perforatum*

3.1.2. Callus formation of *H.perforatum* from tissue culture

After plants reached enough length, they were utilized for tissue culture. Leaves of *H.perforatum* were used for tissue culture. Firstly, leaves were washed by 3% sodium hypochloride solution for 20 minutes, followed by 3 times wash in dH_2O. Therefore, leaves' surfaces were sterilized. As culture medium, three different types of MS medium containing different levels of hormones were tried for both liquid and solid mediums;

- 1. Composition: MS with vitamins + 1 mg/L 2-4-D + 0,1 mg/L Kinetin
- 2. Composition: MS with vitamins + 2 mg/L 2-4-D + 0,1 mg/L Kinetin
- 3. Composition: MS with vitamins + 3 mg/L 2-4-D + 0,1 mg/L Kinetin
- Control group: MS medium without hormones

3.1.2.1. Callus formation of *H.perforatum* in shake flasks

Sterilized leaves were put into liquid mediums in shake flasks **(Figure 18)**. Flasks were covered by aliminium foil due to photosensitivity of hypericin **(Figure 19)**. Then flasks were put into shaker and waited for 3 weeks to observe the callus formation. At the end of the 3 weeks, there was no callus formation in shake flasks. This was maybe due to structures of leaves. Probably, *H.perforatum*' sleaves need a solid surface to stick and therefore liquid medium is not suitable for callus formation of *H.perforatum*. Results of shake flasks were given below;

Figure 18. Placing sterilized leaves of *H.perforatum* to liquid medium in shake flasks

Figure 19. Covering shake flasks with aliminium foil

3.1.2.2 Callus formation of *H.perforatum* in solid medium

Leaves with nodal sections were cultured into the mediums. Callus formation started in two weeks. For each situation five leaves were planted to medium per plate and for each situation five repetitions were performed. Therefore, 25 leaves were used for each media composition. After two weeks, number of callus for each situation was counted so that the optimum medium conditions were determined. Number of callus was given below in **Table 3**;

Table 3. Results of callus formation of *H.perforatum'* leaves

(For each composition, repetition is made 5 times, each plate had 5 sterilized leaves)

	Number of leaves	Number of callus
1. Composition	25	6
2. Composition	25	7
3. Composition	25	13
Control group	25	1

According to the results, third composition exhibited the optimum conditions for *H.perforatum*'s tissue culture. The only difference between different compositions was the concentration of 2-4-D hormone. Representative callus formation patterns were shown below in **Figure 20-21-22**;

Figure 20. Callus formation in Composition 3

(MS with vitamins + 3 mg/L 2-4-D + 0,1 mg/L Kinetin)

Figure 21. Callus formation is Composition 2

(MS with vitamins + 2 mg/L 2-4-D + 0,1 mg/L Kinetin)

Figure 22. Callus formation in Composition 1

(MS with vitamins + 1 mg/L 2-4-D + 0,1 mg/L Kinetin)

When calluses were formed, they were taken out from medium and dried in the oven at 60°C. Then they were squeezed to make powder. Hence they were ready for extraction.

3.1.3. Plant material purchased from herbalist

Dried plant materials were purchased from herbalist. In order to optimize the extraction parameters, dried plant materials were used. Since using dried plants from herbalist was more practical than growing up plants from seed or tissue culture. The key point was that all extraction conditions should be conducted from same batch of dried plant material because hypericin concentrations of different batches collected from different regions may vary. Before starting extraction 1 g of dried plant material was blended and made as powder in order to increase surface area for diffusion of solutions easier.

3.2. Separation of carotenoid pigments from *H.perforatum*

Before extraction of hypericin from *H.perforatum*, carotenoid pigments should be removed. Since carotenoid pigments resemble the fluorescence of hypericin under UV light. Both of them give red fluorescence (Costes et al. 2004). Therefore, carotenoid pigments should be removed firstly. Carotenoid pigments are lipid soluble molecules and can be extracted from the plant bynon-polar solvents (Robinson, 1963). As nonpolar solvent chloroform was used (Çırak, 2006). Separation process was conducted in dark conditions due to photosensitivity of hypericin. Moreover, during separation, incubation was conducted at room temperature since during heating, chloroform vapor might be harmful.

After separating carotenoid pigments by using chloroform, all of the carotenoid pigments were removed from plant material. If there was any remaining carotenoid pigment on plant material, it could not be mixed with the hypericin extraction, because, hypericin extraction was made by using methanol which is a polar solvent.

Therefore, carotenoid pigments could not be dissolved in methanol and hypericin extract did not include any carotenoid pigments.

3.3. Extraction of hypericin from *H.perforatum*

Extraction method was optimized by plant material bought from herbalist. Since using plant material from herbalist was more practical than the other plant material choices. The powder of plant remaining on the filter paper was put into flask again and 99.5% GC methanol was added which is needed for extraction of hypericin as alcoholic solvent.

As extraction method, ultrasonication and sonication methods were evaluated. Ultrasonication gave more sonic waves in short time on the other hand sonicator gave less sonic waves in long periods (Zobayed et al, 2006). To make the optimization of extraction method, all parameters were fixed except exposure time of ultrasonication & sonication, incubation time and heat parameters. After extraction, methanol in the samples was evaporated by using rotary evaporatory until 2 mL methanol was remained. 2 mL methanol was transferred to test tube and test tube's plug left open to evaporate the remaining methanol and to collect the powder of plant material. Afterwards, 1 mg of powder was weighed and dissolved in 1 mL methanol. Thus all samples were equalized. Then, samples were measured at 590 nm in spectrophotometer.

3.3.1 Sonication of *H.perforatum* & Incubation on Stirrer

To sonicate the plant material, powder of plant in flask with methanol was put into the sonicator. Sonicator was filled with water and some ice particules were put into sonication bath in order to prevent the damage of extracts due to heating by sonic waves. To find the optimum condition, different exposure times were evaluated. Different exposure times are given below;

- 15 minutes sonication
- 30 minutes sonication
- 60 minutes sonication

Besides exposure times, incubation time on stirrer and heating temperature were evaluated in different conditions. As incubation time and heating temperature, the evaluated conditions were given below;

- 1 hour incubation at 50°C
- 2 hour incubation at 50°C
- 1 hour incubation at room temperature
- 2 hour incubation at room temperature
- Overnight incubation at room temperature

Therefore, for sonication of *H.perforatum*, different combinations were formed and each of them was tried separately. The results of are given below in **Table 4.** OD values which are measured in 590 nm are specific for hypericin and other ring structures of *H.perforatum*;

Table 4. Results of Extraction of Hypericin from*H.perforatum* by Sonication.

Measurements were made by spectroscopy. OD values were measured in 590 nm.

		INCUBATION				
		1 hr		2 hr		Overnight
SONICATION		50°C	Room Temperature	50°C	Room Temperature	Room Temperature
	15 min	0,98	0,67	0,95	0,37	0,88
	30 min	0,78	0,64	0,44	0,32	0,21
	60 min	0,66	0,62	0,34	0,30	0,19

When the results of sonication are observed, it is seen that 15 minutes sonication is the best for extraction of hypericin from *H.perforatum*. Because, when the temperature and incubation time are fixed and just looked at the exposure time of sonication, it was noticeable that 15 minute exposure is the best for sonication method. On the other hand, for all conditions 50°C temperature is better than room temperature and 1 hour incubation on stirrer is also better than 2 hours. Therefore, in order to extract hypericin from*H.perforatum*, optimum condition by using sonication is given below;

- **15 minutes exposure to sonicator at 50°C for 1 hour**

3.3.2. Ultrasonication of *H.perforatum* & Incubation on Stirrer

Ultrasonicator gives stronger sonic waves than sonicator in shorter time. Like sonicator, ultrasonication was also conducted on ice in case enzymes were not damaged due to heat from sonic waves. After setting amplitude at 70 and pulse at 9-10 sec, suitable probe was submerged to the plant material including methanol. Same as sonicator, different exposure times were tried for ultrasonicator. In addition, different incubation times and temperatures were evaluated. Different exposure times are given below;

- 5 minutes ultrasonication
- 15 minutes ultrasonication

Different incubation times and temperatures on stirrer are given below;

- 1 hour incubation at 50°C
- 2 hour incubation at 50°C
- 1 hour incubation at room temperature
- 2 hour incubation at room temperature
- Overnight incubation at room temperature

The results of different conditions are given below in **Table 5.**;

Table 5. Results of Extraction of Hypericin by Ultrasonication from *H.perforatum.* (Measurements were made by spectroscopy. OD values were measured in 590 nm)

		INCUBATION				
		1 hr		2 hr		Overnight
ULTRASONICATION		50°C	Room Temperature	50°C	Room Temperature	Room Temperature
	5 min	1,02	0,63	0,87	0,69	0,72
	15 min	1,22	0,71	0,85	0,78	0,60

According to the results of ultrasonication, it was observed that 15 minutes exposure to ultrasonication is the best condition for extraction of *H.perforatum*. As incubation time and temperature, the best condition is 1 hour incubation at 50°C. Therefore, best conditions for ultrasonication are given below;

- **15 minutes exposure to ultrasonicator at 50°C for 1 hour**

3.3.3. Optimum Condition of Extraction of hypericin from *H.perforatum*

Extraction of hypericin from *H.perforatum* was tried by using two different methods; namely, sonication and ultrasonication. Optimum condition of each of them was mentioned above but it was needed to make comparison between two methods. When comparing sonication and ultrasonication's optimum conditions, it is clear that OD values of ultrasonication of *H.perforatum* are better than OD values of sonication of *H.perforatum*. Since optimum condition of sonication gave 0.98 OD value whereas ultrasonication' optimum condition gave 1.22 OD value. Therefore, it is decided that ultrasonication is more efficient than sonication but as ultrasonication

method, only two different exposure times were tried and 15 minutes exposure time was better than 5 minutes. On the other hand, temperature was set at 50°C and 1 hour incubation on stirrer was preferred in further experiments. Different exposure times of ultrasonication given below were tried. As control group, samples were not ultrasonicated, they were just incubated on stirrer at 50°C for 1 hour.

- No ultrasonication
- 5 minutes ultrasonication
- 15 minutes ultrasonication
- 20 minutes ultrasonication
- 30 minutes ultrasonication

The results of different exposure times are given below in **Table 6**. and **Figure 23**;

Table 6. Optimization of Ultrasonication Method for Hypericin Extraction from *H.perforatum*

Measurements were made by spectroscopy. OD values were measured in 590 nm. Each parameters were tried 2 times.

Exposure Time	OD (590 nm)
No ultrasonication	0,49
5 min	0,56
15 min	0,65
20 min	0,78
30 min	0,68

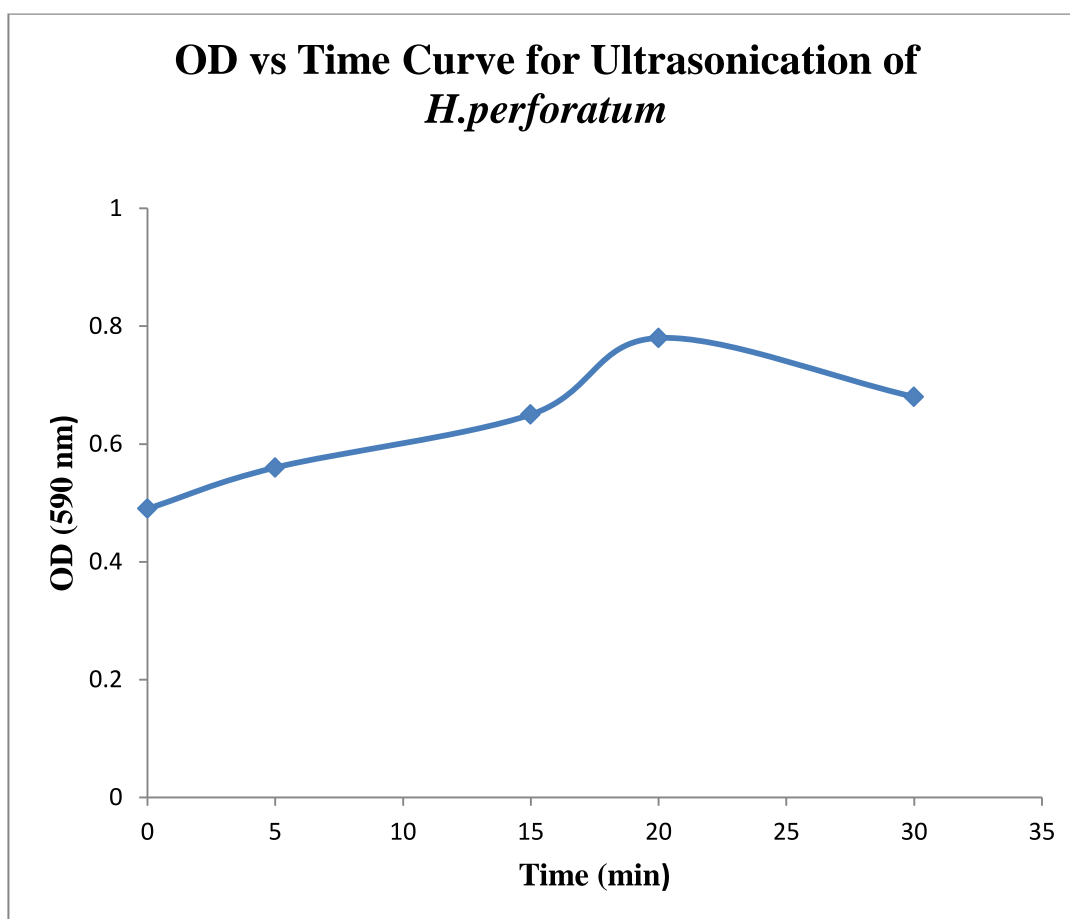


Figure 23. OD vs Time Curve for Ultrasonication of *H.perforatum*

(Experiments were repeated two times)

As **Table 6** and **Figure 23** are observed it is clear that 20 minutes exposure time to ultrasonication has the maximum result hence it is the optimum condition for extraction of *H.perforatum*. When ultrasonication is not applied, only incubation on stirrer extracts *H.perforatum* but ultrasonication enhances extraction efficiency. Until 20 minutes exposure efficiency increases but in 30 minutes exposure efficiency starts to decrease. Therefore, the optimum condition is;

- Extraction by ultrasonication for 20 minutes
- Incubation on stirrer at 50°C for 1 hour, then filtration by the aid of filter paper
- For the second time, extraction by ultrasonication for 20 minutes
- For the second time, incubation on stirrer at 50°C for 1 hour, then filtration by the aid of filter paper

After optimizing the best condition for extraction of *H.perforatum* by using dried plant material from herbalist; other plant materials, callus from tissue culture and plant material grown from seed, were extracted by using the optimum extraction condition. The results are given below in **Table 7**;

Table 7. Results of Extraction of Other Starting Material Sources for *H.perforatum* (Plant sources were provided from plants grown from seeds of *H.perforatum*. Repeats were made twice. Measurements were conducted with spectroscopy at 590 nm)

Type of Plant Material	**OD (590 nm)**
Callus from tissue culture	0,21
Fresh plant material grown from seed	0,33

For callus extraction, calluses were collected from tissue cultures and they were waited to be dried. In order to dry the calluses, calluses were heated at 60°C in the oven. Then they were squeezed by using mortar so to make them as powder. 1 mg of powder was weighed and used as starting plant material. Afterwards, optimum extraction method was applied to callus.

For plant material grown from seed, when seeds were germinated and plants' lengths reached at suitable length, they were cut and dried. Dried plant material was blended

and made as powder. Thus it was also ready to be extracted as starting plant material by using optimum extraction condition.

When callus and plant material grown from seeds' OD values are compared with dried plant material from herbalist, approximately 50% difference is seen. Dried plant from herbalist is 50% more efficient than the others. This may be because of hypericin and other ring structures' content, since different growing conditions (temperature, climate, place…) affect hypericin and other ring structures' concentrations (Vattikuti and Ciddi, 2005).

3.4. Analyses of Hypericin

3.4.1. Standard Graph of Hypericin

10 mg 89.73% hypericin standard was purchased from HWI Analytik GMBH Pharma Solutions. By using hypericin standard, standard graph was drawn to calculate the hypericin concentration of extracts. Since after extraction, measured OD values show not only the pure hypericin concentration but also other ring structures.

Firstly, 1 mg of hypericin standard was weighed and dissolved in 1 mL 99.5% GC methanol to prepare 2 mM stock solution. 100X dilution was made on stock solution and got 20 µM solution. Then this 20 µM solution was diluted several times. All dilutions including 20 µM solution were measured on spectrophotometry at 590 nm. During measuring all dilutions on spectrophotometry, measurement was made on duplications of samples to increase reliability of graph. Therefore, by using different molarities versus different OD values formed the Standard Graph of Hypericin. Dilutions and their OD values are given in **Figure 24-25**;

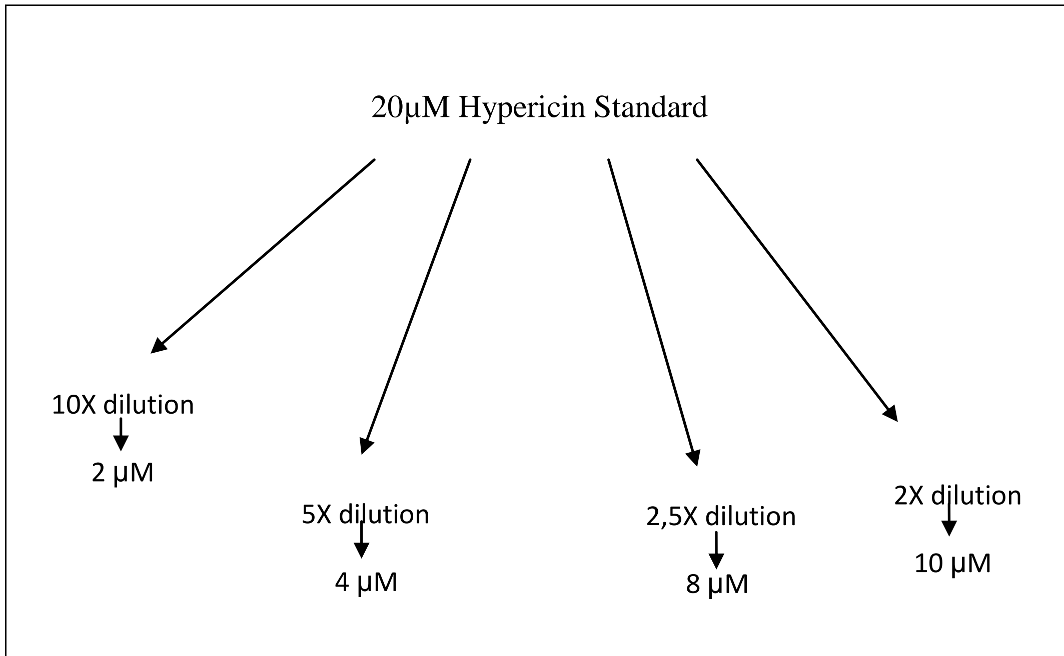


Figure 24. Dilutions of Hypericin Standard

Figure 25. Dilutions of Hypericin Standard

Table 8. OD Values of Dilutionsof Hypericin Standard at 590 nm

(Measurements were made by spectroscopy. Molecular weight of hypericin standard is 504,44 g/mole)

Concentrations (μM)	1.Measurement (OD)	2.Measurement (OD)	Average (OD)
0	0	0	0
2	0,13	0,15	1,14
4	0,33	0,31	0,32
8	0,66	0,65	0,65
10	0,86	0,77	0,81
20	1,52	1,50	1,51

Each concentration was measured as duplication and the average of each duplication was calculated. Then average values were used to draw Standard Curve of Hypericin. Standard Curve is given below in **Figure 26**;

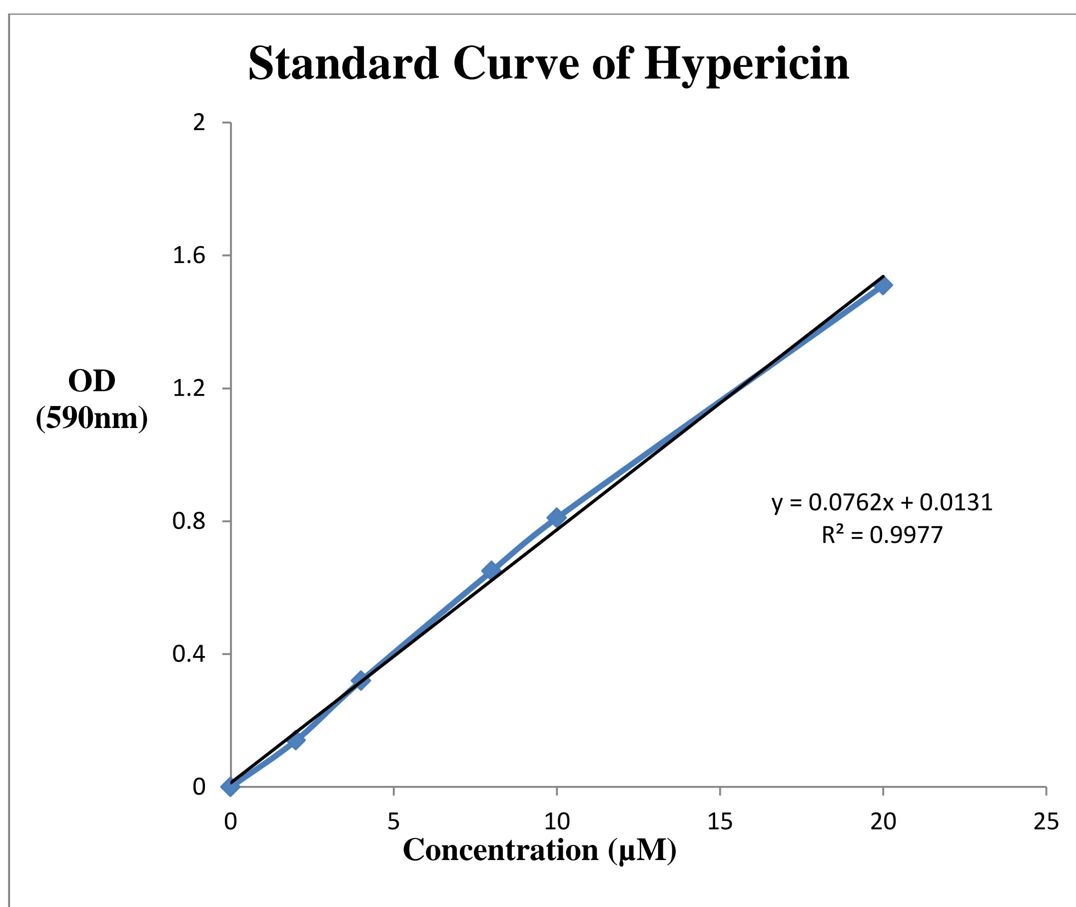


Figure 26. Standard Curve of Hypericin

Since extracts include not only hypericin but also other ring structures, OD values of extracts should be placed on curve to calculate pure hypericin. In order to measure OD values, 1 mg of powder extract was weighed and dissolved in 1 mL methanol. Therefore, when OD value places on Standard Curve, obtained concentration shows hypericin concentration in 1 mg dried extract.

To calculate the concentration of hypericin in 1 mg extract, equation of graph is used. "y-axis" represents OD values, whereas "x-axis" represents concentrations. Optimum condition of extraction given in Section 1.3.3 is used as an example of concentration calculation from graph. Calculation is given below;

Equation of Graph:

$y = 0{,}0762X + 0{,}0131$

OD value (590 nm) of Optimum Condition:

0,78

Calculation of Hypericin Concentration:

$0{,}78 = 0{,}0762X + 0{,}0131$

X= 10,06 μM Hypericin

Calculations show that optimum condition of extraction which has 0.78 OD value, includes approximately 10.06 μM hypericin.

3.4.2. Analyses of Hypericin with Thin Layer Chromotography (TLC)

As chromatographic analyses, thin layer chromatography and paper chromatography were applied. Although both methods are similar to each other, TLC uses a stationary phase of a thin layer of adsorbent, silica gel (SiO_2), instead of cellulose membrane which belongs to paper chromatography. In this study, TLC is more reliable than paper chromatography because silica gel is more durable to heavy metals like chloroform, methanol than paper chromatography. Cellulose membrane can be rent due to heavy metals.

For Thin Layer Chromotography (TLC), two different solutions (Vattikuti and Ciddi, 2005) given below were tried;

- Ethylacetate: Formic Acid: Glacial Acetic Acid: Water
  (100: 11: 11: 26 v/v)
- Butanol: Glacial Acetic Acid: Water
  (28: 7: 35 v/v)

TLC plates were cut in suitable dimensions (length: 6cm, width: 4cm). Dried powder *H.perforatum* extracts were prepared as mentioned before. 1 mg dried extract was weighed and dissolved in 1 mL methanol. Hypericin standard was prepared as 20 µM. Samples were loaded to TLC plate as maximum 5 µl. Hypericin standard was loaded to first line to compare with other samples. For approximately half an hour, samples were conducted in solvents. Afterwards, plates were dyed with different dyes to find the most effective dye for visualization (Vattikuti et al, 2005). Different dyes which were tried are given below;

- 10% KOH
- 10% PEG (Poly ethylene glycol)
- 10% H_2SO_4

After dying, samples were heated for 5-6 minutes to dry the dye. Then, plates were observed under ultraviolet light. The aim was to get the samples in the same line with the standard and the brightness of samples should be close to standard, either.

When two different solvent systems mentioned above were tried with the dyes mentioned above, 10% H_2SO_4 dye did not form any spot with both solvent systems. However, 10% KOH and 10%PEG dyes formed spots with both solvent systems. Therefore, to decide the optimum solvent-dye system, the important point was the brightness of the system. Accordingly, the brightest solvent-dye system mentioned below was chosen as the optimum one.

Optimum Solvent-Dye System:

Ethylacetate: Formic Acid: Glacial Acetic Acid: Water

+

10% KOH

By using optimum solvent-dye system; samples of hypericin standard, optimum extraction condition of ultrasonication, optimum extraction condition of sonication

and extraction with incubation on stirrer were observed under UV. TLC results are given in **Figure 27**;

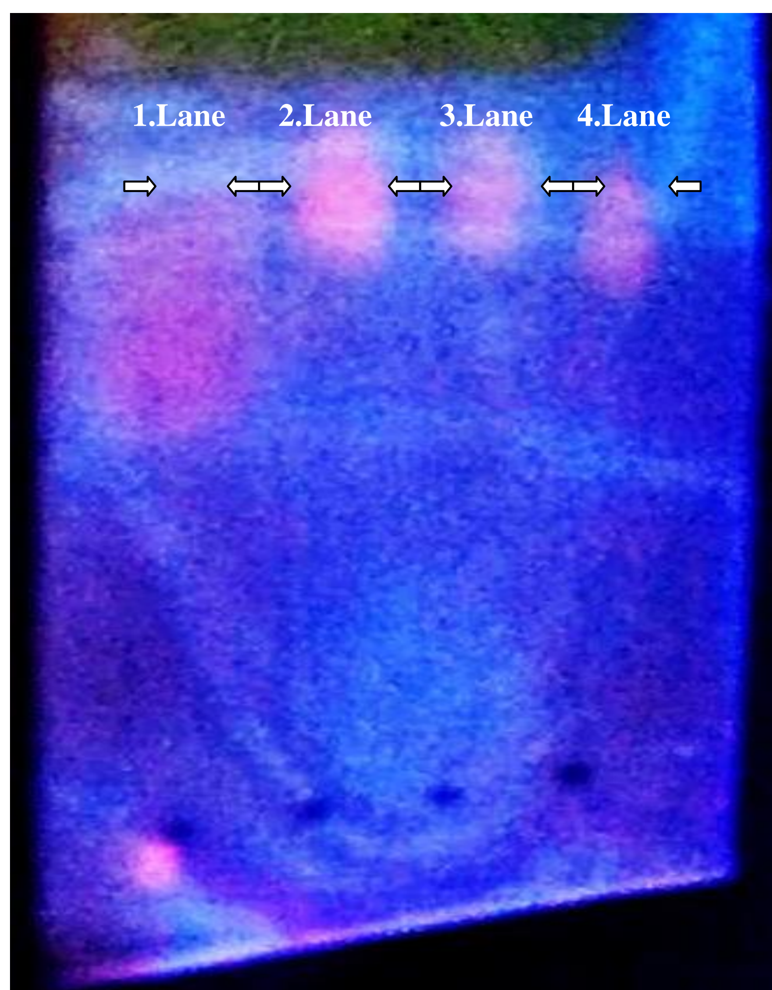


Figure 27. TLC results of Optimum Conditions of *H.perforatum*

(1.Lane: 20μM hypericin standard, 2.Lane: the optimum condition of ultrasonication, 3.Lane: the optimum condition of sonication, 4.Lane: the extraction with only incubation on stirrer)

In the first lane, 20 μM hypericin standard is loaded, in the second lane the optimum condition of ultrasonication is loaded. Third lane belongs to the optimum condition of sonication and the last lane represents the extraction with only incubation on stirrer. When the brigthnesses of samples are observed, it's clear that optimum condition of ultrasonication has the brightest result. Therefore, it is proved that the

optimum condition for extraction of *H.perforatum* is the 20 minute exposure to ultrasonication which is mentioned in Section 1.3.3. On the other hand, samples are not exactly at the same level with hypericin standard. It shows that extracts do not include pure hypericins but other ring structures as predicted.

3.4.3. Analyses of Hypericin with Paper Chromotography

For paper chromatography, first of all chromatography papers were cut in suitable dimensions (length: 10cm, width: 4cm). As solvent systems, same solvent-dye systems with TLC were tried. All procedure was applied same with TLC. When all solvent-dye systems were tried, the optimum one was the same with TLC which is given below;

Optimum Solvent-Dye System:

Ethylacetate: Formic Acid: Glacial Acetic Acid: Water

+

10% KOH

By using optimum solvent-dye system, in the first line hypericin standard was loaded, then the optimum condition of ultrasonication, the optimum condition of sonication and extraction with incubation on stirrer were loaded, respectively. After approximately half an hour, 10% KOH was sprayed on them and they were heated to be dried. Afterwards, they were analyzed under UV light. The result is given below;

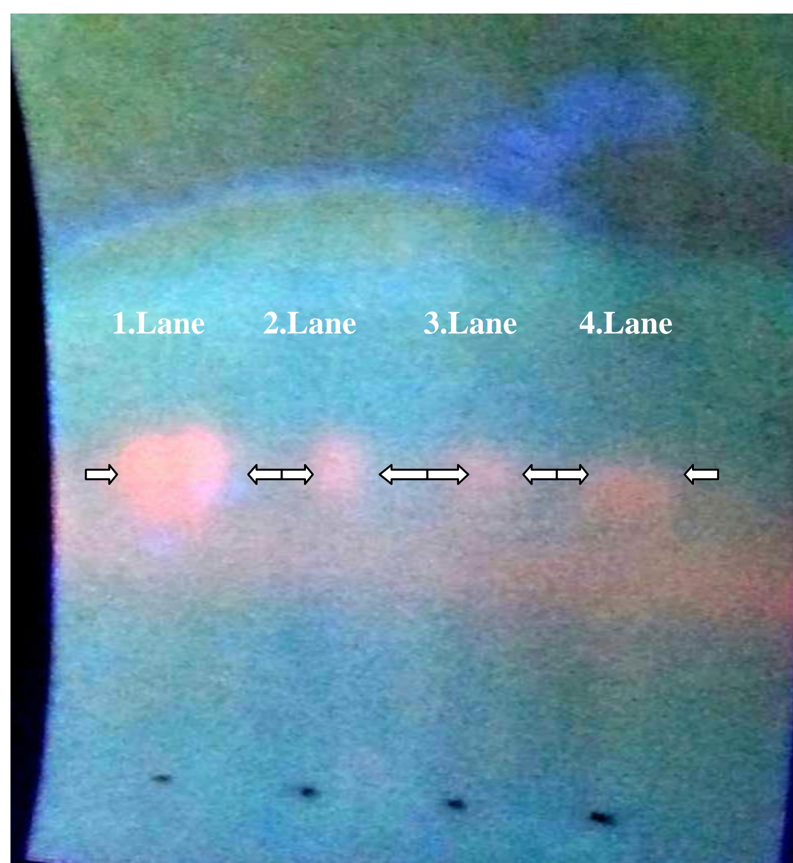


Figure 28. Paper Chromotography Results of Optimum Conditions of *H.perforatum* (1.Lane: 20µM hypericin standard, 2.Lane: the optimum condition of ultrasonication, 3.Lane: the optimum condition of sonication, 4.Lane: the extraction with only incubation on stirrer)

From the results, it is clear that the brightest one is the optimum condition for ultrasonication which is determined as the optimum condition for the extraction of *H.perforatum*.

3.4.4. Analyses of Hypericin with LC-MS

In order to analyze the purity of hypericin from *H.perforatum* extract, 2 mM hypericin standard, sample from optimum condition of ultrasonication (1 mg extract

powder dissolved in 1 mL 99.5% GC methanol) and sample from extraction by using strirrer (1mg extract powder dissolved in 1 mL 99.5% GC methanol) were given to Central Laboratory in Middle East Technical University. The samples were compared with hypericin standard. Hypericin standard was given as 1000 ppm and its peak was constituted by using 0.125 ppm. During analyses, samples were ¼ diluted. The results of the samples are given below in **Table 9**;

Table 9. LC-MS Results of Hypericin.

(Results were given by regarding the dilutions of samples and results show the hypericin concentrations in 1000 ppm samples.)

Extraction Method	Hypericin (ppm)
Optimum condition of extraction (20 min time to exposure to ultrasonication)	0.2020 ± 0.0009
Extraction by incubation on stirrer	0.1403 ± 0.0001

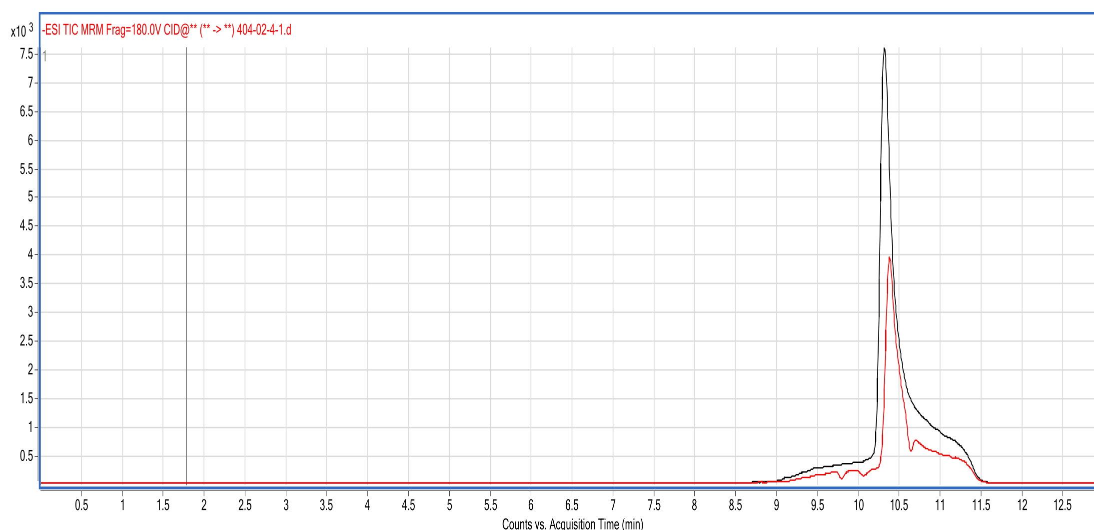


Figure 29. LC-MS Results of Extraction of *H.perforatum* by the Optimum Condition for Ultrasonication (Black peak shows 0.125 ppm hypericin standard, red peak shows ¼ diluted sample)

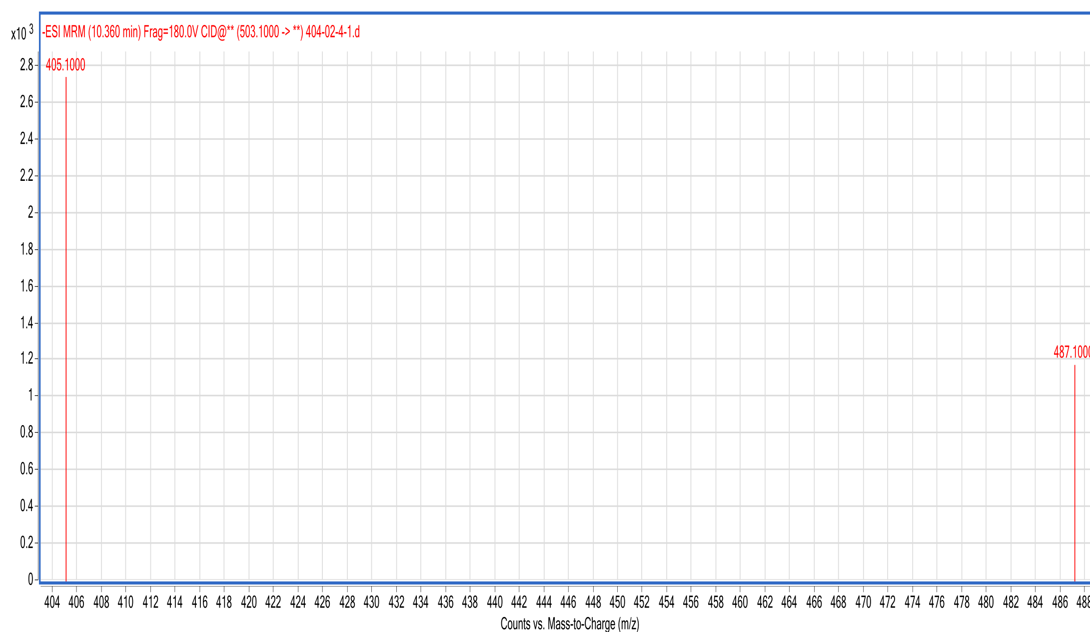


Figure 30. Product ions of Hypericin Standard (Quantifier ion: 405.1m/z, Qualifier ion: 487.1m/z)

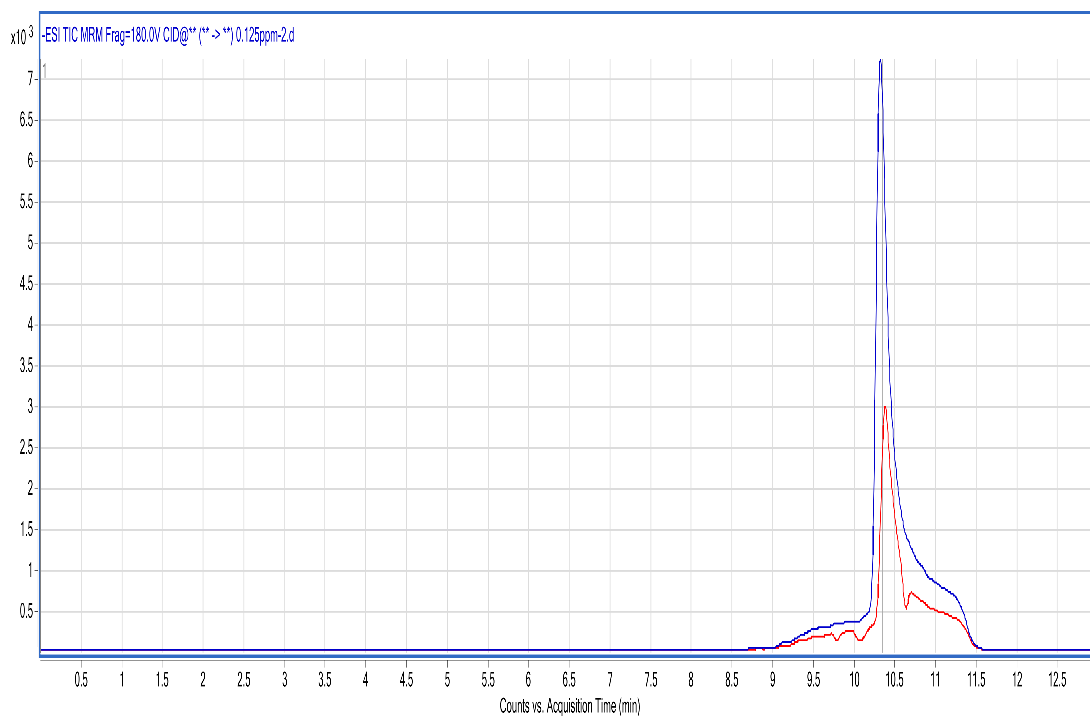


Figure 31. LC-MS Results of Extraction of *H.perforatum* by Incubation on Stirrer (Blue peak shows 0.125 ppm hypericin standard, red peak shows ¼ diluted sample)

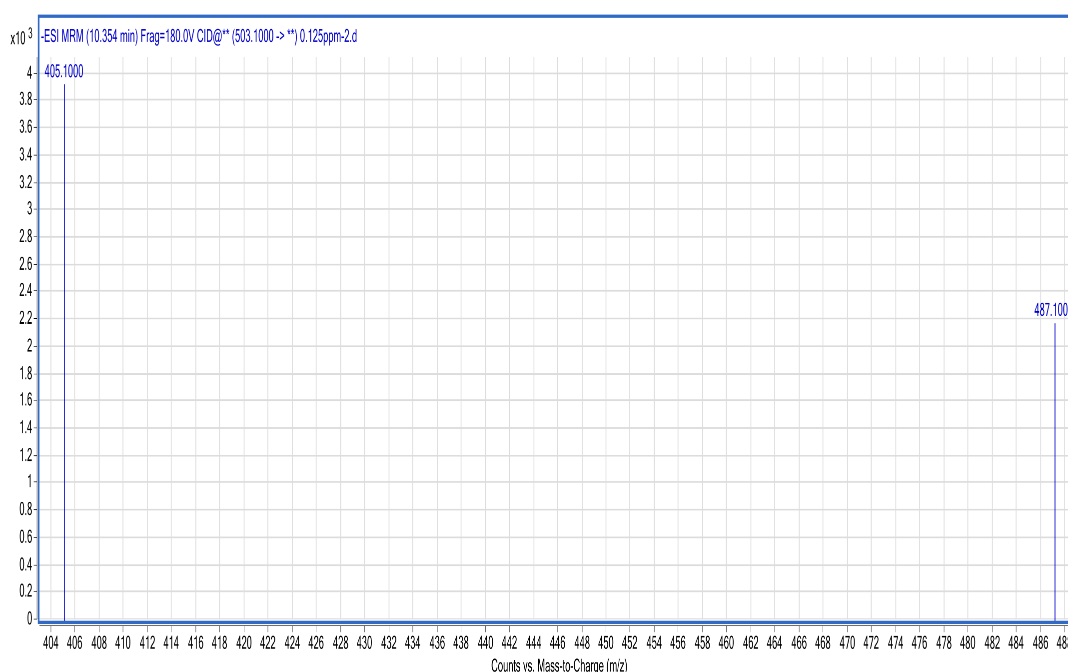


Figure 32. Product ions of hypericin standard (Quantifier ion: 405.1m/z, Qualifier ion: 487.1m/z)

As results of LC-MS of two samples are observed, it is clear that the optimum condition for ultrasonication has more hypericin than extraction of *H.perforatum* by incubation on stirrer as expected, because OD values in 590 nm of both samples gave parallel results which are given in **Table 6**. Both optimum condition for ultrasonication's and extraction by incubation on stirrer's peaks were overlapped with the peak of hypericin standard. These results show that extracted samples include hypericin as their components.

3.5. Designing of Solar Panels by Using Hypericin as Dye-Sensitized Solar Cell (DSSC)

In this study the aim was to extract hypericin from *H.perforatum* and use the hypericin in dye-sensitized solar cells (DSSC). This part of the study was conducted in cooperation with Metallurgical and Materials Engineering in Middle East

Technical University. Because hypericin has photodynamic action, it is thought to be suitable for designing DSSC. Therefore, an organic dye would be used in solar panels to reduce the fabrication costs.

In order to conduct the experiment, sample from optimum condition of hypericin extraction which is 20 min time exposure to ultrasonication and sample from hypericin standard were given to Metallurgical and Materials Engineering Department. Hypericin based solar cells were formed by imbuing 0.5 mM hypericin sample dissolved in methanol to thick films for 24 hours. The results are given in **Table 10**;

Table 10. Solar Cell Parameters by Using Hypericin based DSSC

Sample of Hypericin	V_{oc} (V)	J_{sc} (mA/cm^2)	FF	% Efficiency
Hypericin Extract	0,41	0,3	0,47	0,057
Hypericin Standard	0,51	0,4	0,58	0,118

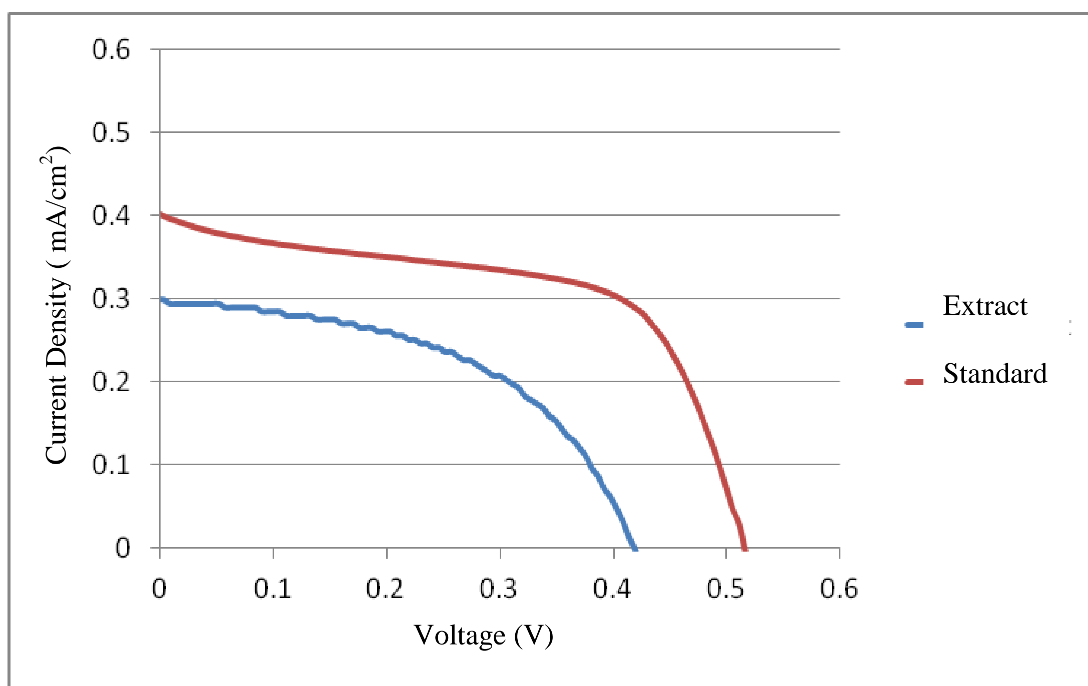


Figure 33. Curves of hypericin based solar cells

In the curve, blue line represents the hypericin extract by using optimum condition of ultrasonication, red line represents the hypericin standard. It is clear that efficiency of hypericin standard is more than the extracted one. It may show that the extracted hypericin is not as pure as the standard. Although their efficiencies are less, it looks promising for the future. By improving the purity of extracted hypericin, hypericin can be used in solar panels as DSSC.

CHAPTER 4

CONCLUSION

To isolate hypericin from *H.perforatum* three different plant sources; namely, *H.perforatum* grown from seed, callus from tissue culture and dried *H.perforatum* obtained from herbalist were used. Firstly, in order to germinate *H.perforatum*'s seeds, viols were filled with turfs, seeds were placed on the top of the turfs. The key point was that the seeds were not covered by a thin layer of turf. Next, the viols were covered with stretch film. After a week, germination of seeds was seen and plants were grown until the plant reached enough length for tissue culture.

From the fresh plants, tissue culture was conducted to form callus structures. To do this, three different MS medium were tested. The only difference in these mediums was the concentration of 2-4-D hormone. The best results were observed at 3 mg/L 2-4-D hormone, where 13 leaves out of 25 were formed callus. Therefore, these calluses were used in the extraction as plant source.

In order to optimize the extraction method, dried *H.perforatum* from herbalist was used. Firstly, dried plants were blended to make them powder. Afterwards, lipid soluble carotenoid pigments were removed from the plant since carotenoid pigments behave like hypericin under ultra-violet light. Chloroform successfully removed the carotenoid pigments from *H.perforatum*.

For extraction, ultrasonication and sonication were compared. Exposure time to sonication, incubation time on stirrer and temperature of stirrer were the variables. As extraction solvent, 99.5% GC grade methanol was used for whole optimization studies. Results show that under all conditions, extraction with ultrasonication was more efficient than extraction with sonication. Under optimum conditions OD value

of 0.78 was obtained as extracted hypericin which is equivalent to 10.06 μM hypericin as calculated from "Standard Curve of Hypericin".

TLC analyses and paper chromatography results given in **Figure 27** and **Figure 28** also supported the spectrophotometric results. The optimum solvent-dye system was determined as Ethylacetate: Formic Acid: Glacial Acetic Acid: Water (100: 11: 11: 26 v/v) with 10% KOH.

After analyzing hypericin by TLC and paper chromatography, LC-MS was conducted to determine the purity of hypericin. Comparison was made with hypericin standard. 0.2020 ppm pure hypericin was detected in the extract of the optimum condition of ultrasonication.

The extracted hypericin was used as an organic dye in dye-sensitized solar cells. Although it is not as efficient as the commercially available pure hypericin, the extracts exhibit a potential to be used in the solar panels which brings the possibility to reduce the cost of production.

REFERENCES

Adamski, R., E. Styp-Rekowska,. 1971. "Stability of hypericin in juice, dry extract, and tablets from Hypericum perforatum plants," Farm. Pol. Vol. 27, pp. 237-41.

Agostinis, 2002. “Hypericin in cancer treatment more light on the way”, The international journanl of Biochemistry& Cell biology, Vol. 34, pp, 221-24.

Araya, O.S., Ford, E.J.H., 1981. "An investigation of the type of photosensitization caused by the ingestion of St. John's wort (Hypericum perforatum) by calves", J. Comp. Path., Vol. 91. pp. 135-41.

Ayan & Çırak, C, 2008. “Pseudohypericin and hyperforin in Hypericum perforatum from Northern Turkey: variation among populations, plant parts and phenological stages”, Department of Agronomy, Faculty of Agriculture, University of Ondokuz Mayis

Bestic, L.,1999. “The suddenly fashionable wort”. The Independent, p. 11, 6 April.Bilia, A, 2010. “Hypericins as potential leads for new therapeutics”. International Journal of Molecular Science 11: 562–594

Blank, 2001. “Anti-Cancer activities of Hypericin in the dark”, Photochemistry and Photobiology, Vol. 74, pp. 120-125.

Bombardelli E & Morazzoni P, 1995 “Hypericum perforatum”. Fitoterapia 66: 43−68

Brolis, M., 1998. “Identification by high performance liquid chromatography-diode arry detection- mass spectrometry and quantification by high-performance liquid

chromatography- UV absorbance detection active constituents of Hypericum Perforatum L", Journal of Chromatograohy A, Vol. 825, pp. 9-16.

Bruni R & Sacchetti G, 2009. "Factors affecting polyphenol biosynthesis in wild and field grown St. John's Wort (Hypericum perforatum L. Hypericaceae/Guttiferae)". Molecules 14: 682−725.

Butterweck, V, 2003. "Mechanism of action of St John´s wort in depression: what is known?"
CNS Drugs 17: 539−562

Cellarova E.,Davey M.R.,Pover J.B. and Lowe K.C., 1994. "Stimulation of multiple shoot regeneration from seedling leaves of Hypericum perforatum L. by Pluronic F-68". Acta Biotechnol. 14: 347-353.

Clare, N.T., 1952. "Photosensitization in diseases of domestic animals", Review Sweries No. 3 of the Commonwealth Bureau of Animal Health, Commonwealth Agricultural Bureaux, Bucks, England.

Costes, D. Daelemans, E.H. Cho, Z. Dobbin, G. Pavlakis, S. Lockett, 2004. "Automatic and quantitative measurement of protein–protein colocalization in live cells", Biophys. J. 86 (2004) 3993–4003.

Çırak, 2006. **"**Farklı Doku Kültürü Uygulamalarının iki Kantaron Türünde *(Hypericum perforatum* ve *H. bupleuroides)* Mikroçoğaltım Yeteneği ve Hiperisin ile Toplam Fenolik Birikimi Üzerine Etkileri" University of On Dokuz Mayıs.

Duran, N, Song PS, 2008. "Hypericin and Its Photodynamic Action" Photochemistry and Photobiology Vol. 43. No. 6. pp. 617 - 680, 1986

Garrett, B.J., et al. 1982. "Consumption of poisonous plants (Senecio jacobaea, Symphytum officinale, Pteridium aquilinum, Hypericum perforatum) by rats: chronic

toxicity, mineral metabolism, and hepatic drug-metabolizing enzymes”, Toxicology Letters, Vol. 10, pp. 183-88.

Gerard, J., 1633. “The Herbal. Revised and enlarged by T. Johnson, reprinted by Dover Publications”, NY (1975).

Giese, A. C.,1971 “Photosensitization of natural pigments”.Photophysiology 6, 77-129.

Grebe, 2011. “LC-MS: Why use it and what is it?” Metabolic Services at JC.

Greeson J, 2001. “St. John’s wort (Hypericum perforatum): a review of the current pharmacological, toxicological and clinical literatüre”. Psychopharmacology 153: 402–414

Hudson, J. B., Lopez, Bazzochi I., Towers, G. H., 1991. “Antiviral activities of hypericin”, Antiviral Research, Vol. 15, pp. 101-12.

Hunt, E. J., Lester, C. E., Lester, E. A., Tackett, R. L., 2001. “ Effect of St John’s wort on free radical production”, Life Sciences, Vol. 69, pp. 181-90.

Jaeger, E.C. 1972. “A Source-Book of Biological Names and Terms”. Charles C. Thomas, Springfield

James, L.F., Johnson, A.E., 1976. "Some major plant toxicities of the western United States," J. of Range Manag. Vol. 29, pp. 356-63.

Karakaş, Ö; Toker, Z; Onay, A, 2005. “Effects of different concentrations of benzylaminopurine on shoot regeneration and hypericin content in Hypericum triquetrifolium Turra”. pp 23(16):1459-65

Karioti, A; Bilia, A. 2010. “Hypericins as Potential Leads for New Therapeutics “ Int. J. Mol. Sci. 2010, 11, 562-594

Kirakosyan A, Sirvent TM, Gibson DM & Kaufman PB (2004) The production ofhypericins and hyperforin by in vitro cultures of St. John’s wort (Hypericum perforatum). Biotechnol Appl Biochem 39: 71−81

Kitanov, 2001. “Hypericin and psuedohypericin in some Hypericum species”, Biochemical Sytstematics in Ecology, Vol. 29, pp. 171-178.

Lima, A; Pizzol, C; Monteiro, F; Andrade, G, 2013. “Hypericin encapsulated in solid lipid nanoparticles: phototoxicity and photodynamic efficiency” pp 125:146-54.

Maidment, I, 2000. “The use of St John's Wort in the treatment of depression”Psychiatric Bulletin2000, 24:232-234.

Maleš Ž, Brantner AH, Sović K, Pilepić KH &Plazibat M, 2006 “Comparative phytochemical and antimicrobial investigations of Hypericum perforatum L. subsp. perforatum and H. perforatum subsp. angustifolium (DC.) Gaudin”. Acta Pharm 56: 359−367.

Matzk, F, Meister A, Brtutovska R, Schubert I, 2001.”Reconstruction of reproductive diversity in Hypericum perforatum L. opens novel strategies to manage apomixis”. Plant Journal. 26: 275-282.

Medina MA, 2006.” Hyperforin. More than an antidepressant bioactive compound” Life Sci 79, 105–111.

Mitich LW, 1994 “Intriguing world of weeds: common St. Johnswort”. Weed Technol 8: 658−661.

Mosaleeyanon K, Zobayed S.M.A, Afreen F, Kozai T, 2005.”Relationships between net photosynthetic rate and secondary metabolite contents in St. John’s wort”. Plant Sci. 169: 523–531.

Nathan, PJ, 2001. “Hypericum perforatum (St John's Wort): “A non-selective reuptake inhibitor” A review of the recent advances in its pharmacology”, pp 47-54, Brain Sciences Institute, Swinburne University of Technology, Hawthorn Victoria, Australia

Piette J, Volanti C, Vantieqhem A, Matroule JY, Habraken Y, Agostinis P, 2003. “Cell death and growth arrest in response to photdynamic therapy with membrane-bound photosensitizers”, Biochemical Pharmachology, Vol. 66, pp. 1651-1659.

Poole, K. (2001). “Overcoming antimicrobial resistance by targeting resistance mechanisms. Journal of Pharmacy and Pharmacology”, 53,283–284.

Poutaraud, A, 2001. “Effect of light on hypericin contents in fresh flowring top parts and in extract of St John’s wort (Hypericum Peforatum)”, Planta Medica, Vol. 67,pp. 254-259.

Pretto FR, Santarém ER ,2000 “Callus formation and plant regeneration from *Hypericum perforatum* leaves”. Plant Cell Tis. Org. Cult. 62:107-113.

Reid, W., Laird, V., , Meyer, S. A., , Gamez C. A., Sittenfeld, R., Janzen, A., D. H., Gollin, M. A., , Juma, C. , 1993. “Biodiversity prospecting: Using genetic resources for sustainable development”, World resources InstituteUSA

Santarem, E.R. and L.V. Astarita. 2003.” Multiple shoot formation in Hypericum perforatum L. and hypericin production”. Braz. J. Plant Physiol. 15: 43-47.

Suhaimi, S.; Shahimin, M; Mohamad, I , 2013. “Comparative Study of Natural Anthocyanins Compound as Photovoltaic Sensitizer” Advances in Environmental Biology, 7(12) October Special Issue 2013, Pages: 3617-3620.

Vattikuti U.M.R., Ciddi, V. 2005: “An overview on Hypericum perforatum Linn. Natural Product Radiance”, 4 (5): 368-381

Verpoorte, R., 1989. “Some phytochemical aspects of medicinal plant research”, Journal of Ethnopharmacology, Vol. 25, pp. 43-59

Vickery, A.R., 1981. "Traditional uses and flolklore of Hypericum in the British Iles", Economic Botany, Vol. 35, pp. 289-295.

Vulto, A.G., Smet, P.A.G.M., 1988. In: Dukes, M.M.G. (Ed.) Meyler’s Side Effects of Drugs, 11th Ed.. Elsevier, Amsterdam, pp. 999–1005

Williamson, E., Okpako, D. T., Evans, F.J., 1996. “Selection preparation and pharmacological evaluation of plant material.” Wiley, Chichester

Wirz, A., Meier B, Sticher O, 2001. “Stability of hypericin and psuedohypericin in extract soluions of Hypericum Perforatum and in standart solutions”, Pharmazeutische Industrie, Vol. 63, pp. 410-415.

Yow, C; Tang, H; Chu, E; Huang, Z, 2012. "Hypericin-mediated Photodynamic Antimicrobial Effect on Clinically Isolated Pathogens" Photochemistry andPhotobiology, 2012, 88: 626–63

Zhou, H; Wu, L; Gao, Y; Ma, T, 2011. "Dye-sensitized solar cells using 20 natural dyes as sensitizers" Journal of Photochemistry and Photobiology A: Chemistry 219 (2011) 188–194.

Zobayed, S.M.A., S.J., Murch, H.P.V., 2004. "In vitro production and chemical characterization of St.John's wort (Hypericum perforatum L. cv 'New Stem')". Plant Sci.
166: 333–340

Zobayed SMA, Afreen F, Goto E & Kozai T (2006) Plant–environment interactions: accumulation of hypericin in dark glands of Hypericum perforatum. Ann Bot 98: 793−804