ISOLATION OF A BIOACTIVE COMPOUND HYPERICIN FROM A MEDICINAL PLANT HYPERICUM PERFORATUM L. USING BASIC CHROMATOGRAPHY METHODS

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

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Medicinal plants which have been widely used in folk medicine are known to contain important biologically active compounds. Most of today's synthetic drug raw materials are to be prepared by using plant originated compounds as the starting material.

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.

From the time of the ancient Greeks 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. As a practical folk-remedy, it has been used widely to heal wounds, remedy kidney troubles, and alleviate nervous disorders, even insanity.

In the last thirty years, Hypericum perforatum has undergone extensive clinical and laboratory testing.

The extract of the flower is a red liquid that contains many biologically active compounds such as: naphtodianthrones (hypericin, pseudohypericin), phloroglucinols (hyperforin, adhyperforin), flavonoids (quercetin, hyperoside, quercitrin, isoquercitrin, rutin, campferol, myricetin, amentofloavone), procyanidins (procyanidin, catechin, epicatechin polymers) , tannins (tannic acid), essential oils (terpenes, alcohols), amino acids (GABA, Cysteine, glutamine, leucine, lysine, ornithine, praline, threonine), phenyl propanes (caffeic acid, chlorogenic acid), xanthones (keilcorin, norathriol), organic acids peptides and polysaccharides (other water soluble compounds).

These compounds have previously been isolated using HPLC method.

The aim of this study is to isolate the main biologically active compound groups of Hypericum Perforatum and simply characterize the compounds with TLC, UV-VIS spectroscopy, NMR spectroscopy using standard compounds as references.

Keywords: Hypericum perforatum, hypericin, sephadex LH-20, NMR spectra.

ÖZ

BIYOAKIF MADDE HIPERISININ TIBBİ BIR BİTKİ OLAN HYPERICUM PERFORATUM (SARI KANTARON) DAN TEMEL KROMATOGRAFIK YÖNTEMLERLE IZOLASYONU

Duru, Betül

Yüksek Lisans, Kimya Bölümü

Danışman: Doç. Dr. Nursen Çoruh

Aralık 2003, 56 sayfa

Tıbbi bitkiler, günümüze kadar halk arasında ilaç olarak kullanılmış biyolojik aktiviteye sahip olan birçok madde içerimektedirler. Günümüzde kullanılan birçok sentetik drug 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 St. John's Wort'tür.

Eski Yunan'dan Orta Çağa kadar bu bitkinin sihirli güçlere sahip olduğuna, şeytanı kovduğuna ve hastalıklara karşı koruduğuna inanılırdı. Bir halk ilacı olarak bitki yaraları iyileştirmede, böbrek rahatsızlıklarında ve delilik de dahil olmak üzere sinir hastalıklarının tedavisinde kullanıldı.

Geçtiğimiz otuz yıl içerisinde Hypericum perforatum üzerinde yoğun klinik ve laboratuvar araştırmaları yapılmıştır.

Bitki günümüzde hafif ve şiddetli depresyon tedavisinde, yanıklarda ve kanser tedavisinde kullanılmaktadır.

Bitkinin ekstresi bir çok biyolojik aktif madde içeren kırmızı bir sıvıdır. Naftodiantronlar (hiperisin, psuedohiperisin), floroglukinoller (hiperforin, adhiperforin), flavonoidler (kuersetin, hiperozit, kuersitrin, izokuersitrin, rutin, kampferol, mirisetin, amentoflavon), prosiyanidiler (prosiyanidin, katekin, epikatekin polimerleri), tanenler (tannik asit), sabit yağlar (terpenler, alkoller), aminoasitler (GABA, sistein, glutamin, lözin, lizin, ornitin, prolin, treonin), fenilpropanoidler (kafeik asit, klorojenik asit), ksantonlar (kalkorin, noratriol), organik asitler, peptidler ve polisakaritler (diğer suda çözünür bileşenler), bitkinin bileşenlerindendir.

Bitkinin bileşenlerinin izolasyonu daha önce HPLC metoduyla yapılmıştır.

Bu çalışmanın amacı bitkinin Hypericum Perforatum'dan biyoljik aktiviteye sahip madde gruplarını izole edilmesi ve bunların İTK, UV-VIS spektroskopisi, NMR spektroskopisi ile standart maddeler referans alınarak karekterize edilmesidir.

Anahtar Kelimeler: Hypericum perforatum, hiperisin, sephadex LH-20, NMR spektroskopisi.

DEDICATED TO MY HUSBAND AND MY PARENTS

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NOMENCLATURE

TLC : Thin Layer Chromatograpy

CHAPTER I

INTRODUCTION

1.1 Medicinal Plants

A medicinal plant is any plant used in order to relieve, prevent or cure a disease or to alter physiological and pathological process, or any plant employed as a source of drugs or their precursors.

In recent years, there has been growing interest in the usage of medicinal plants (Vulto and Smet, 1988). This is due to several reasons, namely, conventional medicine can have side effects, abusive or incorrect usage of synthetic drugs result in complications, and the large percentage of world's population do not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that natural products are harmless.

Nowadays, about 25 percent of the drugs prescribed worldwide come from plants and 252 of them are considered as basic and essential by the World Health Organization. The World Health Organization considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs in developing countries (Vulto and Smet, 1998)

Large pharmaceutical companies such as Merk, CIBA, Glaxo, Boehringer and Syntex, now have specific departments dedicated to the study of drugs from natural resources (Reid et al., 1993).

Of the estimated 250,000-500,000 plant species; only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties. It is estimated that 5,000 species have been studied for medical use (Payne et. al., 1991).

Research into, and development of therapeutic materials from plant origin is a hard and expensive task (Boris, 1996). Each new drug requires a large amount of investment and minimum ten years of work.

The process of medicinal plant research is multi-disciplinary (De Pasquale, 1984; Verpoorte, 1989). The basic sciences involved are the botany, chemistry and pharmacology, including toxicology. Any research into pharmacological active natural compounds depends on the integration of these sciences.

The selection of a suitable plant for a study is very important. This can be done considering the traditional use of plant as a medicine, the chemical content of the plant, and toxicology (Williamson et. al., 1996).

The most common strategy of selecting a plant is careful observation of the use of natural resources in folk medicine in different cultures. This is known as ethno botany or etnopharmacology. Information on how the plant is used by an ethnic group is extremely important. The preparation procedure of the plant material may give an indication of the best extraction method.

Once the plant is chosen, the next step is its collection and botanical identification and then the plant should be submitted to a stabilization process. It is important that plant collection involves a professional botanist; who is able to correctly identify the species and prepare part of the material for herbarium preservation, in order to have a reference material.

Stabilization is usually made by drying the material in a shady place in a controlled airflow and temperature. Freezing, lyophilisation, and alcohol vapor are used for stabilization (Williamson et. al., 1996).

The dried materials should then be powdered and subjected to a suitable extraction process. Extraction methods should be directed at obtaining these compounds as high yield and purity as possible. When the chemical composition is unknown, the best procedure is to extract with the method that the plant is used in the folk medicine.

The active components are then separated by chromatographic methods, the structure of the components are determined by spectroscopic methods (UV, IR, NMR, mass spectra); and the biological activity tests are performed (Verpoorte, 1989).

A method for obtaining biologically active components is bioactivity-guided fractionation. Each fraction obtained by the chromatographic method is then subjected to bioassays, using microorganisms, insects, cellular enzymes, cell culture and isolated organs; and the target component that has biological activity is determined by obtaining a pure compound from that fraction (Hamburger and Hostettman, 1991; Souza, 1996).

Plant extracts contain low concentrations of active compounds and a large number of promising compounds (Souza, 1996).

After the structure determination, the compounds can be synthesized by chemists to be used as drugs.

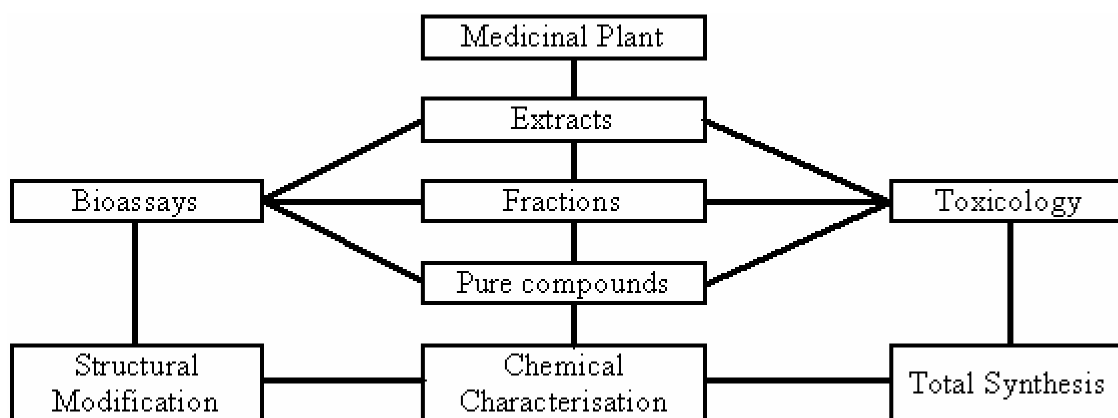


Figure 1. Methods for obtaining bioactive components from plants

1.2 Hypericum Perforatum

Among the many medicinal herbs used throughout the long history of Occidental culture, *Hypericum perforatum* L., has always been and still is of great interest. From the time of the ancient Greeks 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. As a practical folk-remedy, it has been used widely to heal wounds, remedy kidney troubles, and alleviate nervous disorders, even insanity.

Hypericum Perforatum is a very strong antidepressant and because of the great effort devoted to identify the anti-depressant component from a phytochemical point of view Hypericum perforatum is one of the best investigaed medicinal plants (Butterweck, 2003).

1.2.1 Habitat of Hypericum Perforatum

Hypericum Perforatum is a member of the genus Hypericum that has 400 species worldwide. The plant is native to Europe, West Asia, North Africa, Madeira, and the Azores and is naturalized in many parts of the world, notably North America and Australia (Hickey and King, 1981) Hypericum Perforatum spreads rapidly by

means of runners or from the prodigious seed production and can invade pastures, disturbed sites, dirt roads, the sides of roads and highways, and sparse woods.

In western United States, northern California and Southern Oregon the plant is known as “Klamath weed”; because it is toxic for cows and sheep. The phototoxicity caused by the plant is hypericism (Giese, 1980).

Prior to 1949, it was estimated to inhabit 2.34 million acres of range land in northern California. For years an attempt was made to control the plant with herbicides, but with little success. The solution to the problem was of biological. In 1946, the leaf-beetles *Chrysolina quadrigemina* Rossi, and to a lesser extent *C. hyperici* Forst, were introduced from Australia, where it had been observed that they had a voracious appetite for *Hypericum* and by 1957 northern California's stands of St. John's wort were reduced to only 1% of their original number (Campbell et. al. 1979).

1.2.2 Nomenclature of Hypericum Perforatum

The plant was placed over religious icons as a symbol of protection, for that reason Linneus who described the genus thought that Hypericum came from yper (upper) and eikon (an image) (Jaeger, 1972). The specific epithet perforatum comes from Latin perfor to pierce through and refers to the translucent glands which are evident when one holds the leaf up to the light.

For the golden flowers of the plant represent the sun and the summer the church declared the plant sacred to St John, who was born in a midsummer’s day (Vickery, 1981). St John had blessed the plant with healing power. The common name for Hypericum Perforatum is St John’s wort. The "wort" implies that the plant is used as a medicinal plant.

1.2.3 The early uses of Hypericum Perforatum as a medicinal plant

Dioscorides, the foremost herbalist of the ancient Greeks, used Hypericum Perforatum for choleric excrement and burns (Gunther, 1933).

Several noted English herbalists, reflecting their beliefs wrote that Hypericum Perforatum was the most precious herbal remedy for deeper wounds and added to their notes that there is no better natural balsam to cure and such wound (Gerard, 1633).

Other early uses of *Hypericum* include as an oil (made by macerating the flowering tops of the plant in oil and then placing them in the sun for two or three weeks), which was "esteemed as one of the most popular and curative applications in Europe for excoriations, wounds, and bruises". This preparation was even used by the surgeons to clean foul wounds, and was official in the first London Pharmacopeia as *Oleum Hyperici* (State Historical Society of Wisconsin, 1944).

Other popular folk-uses for St. John's wort have included: as a decoction for gravel and ulcerations of the ureter (Hill, 1808); for ulcerations of the kidneys, febrifuge, vermifuge, jaundice, gout, and rheumatism (Greene, 1824).

1.2.4 Constituents of Hypericum Perforatum

The constituents of Hypericum perforatum have been isolated by HPLC method and were charecterized by NMR and mass spectroscopic measrements (Brolis et. al., 1998)

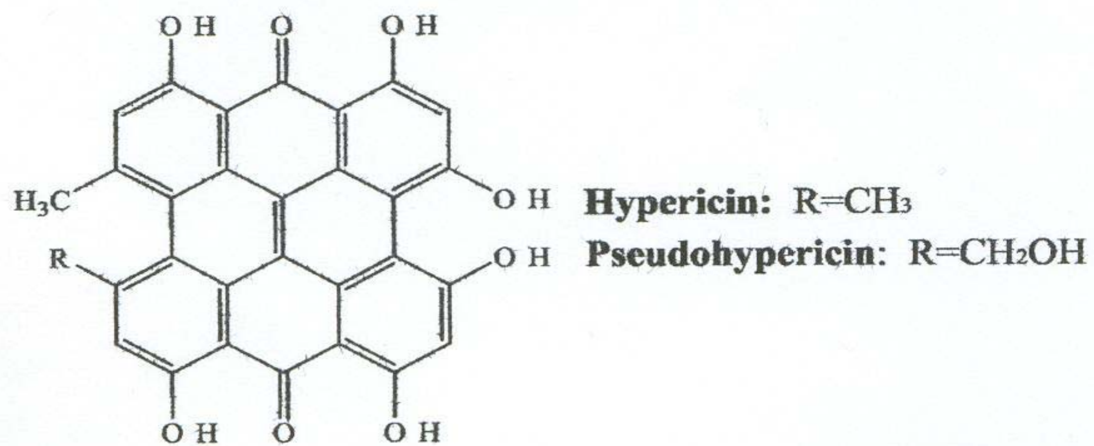
Table 1. Constituents of Hypericum Perforatum L. with main biological interest.

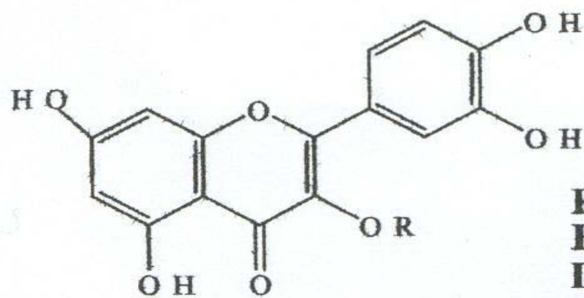
Constituent groups	Names of consituents
Naphtodianhrones	Hypericin Psuedohypericin Protohypericin Protopsuedohypericin
Phloroglucinols	Hyperforin Adhyperforin
Phenolic carboxylic acids	Caffeic acid Chlorogenic acid Genistic acid Ferrulic acid
Flavonoids	Quercetin Hyperoside Quercitrin Isoquercitrin Rutin 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

Hypericum Perforatum contains wide variety of flavonoids (Tota Dorossiev, 1985). The flavonoid levels of the plant were found to be around %3.2. The flavonoid levels were found to be higher in the younger flowering tops (Girzu-Amblard et. al., 2000). Flavonoids are located on the leaves, stalk and buds of the plant. The localization of flavonoids in Hypericum Perforatum flower was studied (Repcak and Martonti, 1997).

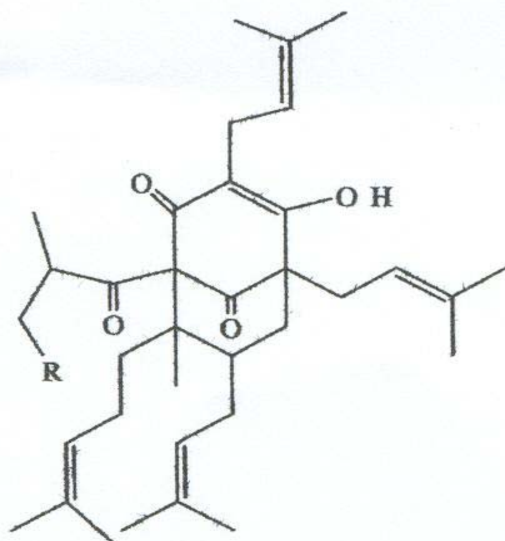
Table 2. The flavonoid content of Hypericum Perforatum L.

Flavonoid	Percentage (%)
Quercetin	2.0
Hyperoside	0.7
Quercitrin	0.5
Isoquercitrin	0.3
Rutin	0.3
Kampferol	Not applicable
Myricetin	Not applicable
Amentoflavone	0.01-0.05
I3,II8-Biapigenin	0.10-0.50





Rutin: R=Glc-Rha
Hyperoside: R=Gal
Isoquercitin: R=Glc
Quercitrin: R=Rha
Quercetin: R=H



Hyperforin: R=H
Adhyperforin: R=CH_3

Figure 2. Constituents of Hypericum Perforatum L. with main biological interest

1.2.5 Hyperforin the antidepressant component of the plant

Hyperforin, the most abundant lipophilic compound is likely to account for the antidepressant action of the plant. Hyperforin has significant effects on serotonergic, noradrenergic, dopaminergic, cholinergic and opioid system activities in vitro, as well as in animal models (Greeson et. al., 2001). Hyperforin also was found to have anti-bacterial activity (Guevi et. al. 1971).

1.2.6 Hypericin the photodynamic component of the plant

The discovery of hypericin was made with hypericism, a skin photosensitivity seen in cattle that ingested large amounts of Hypericum Perforatum (Giese, 1980).

27 of 36 Hypericum species are observed to contain hypericin. Hypericin is found in the plant with its derivative psuedohypericin. Most of the species contain both of the constituents hypericin and psuedohypericin and usually the amount of psuedohypericin exceeds hypericin. Some species such as Hypericum Hirsutum and Hypericum Empetrifolium contains only hypericin, whereas Hypericum Formosissimum contains only pseudohypericin. The total amount of hypericin varied from %0.009 in Hypericum Empetrifolium to %0.512 in Hypericum Boisseri (Kitanov, 2001).

Hypericin is found to be localized in dark granules located on stem, leaves, petals, stemans and ovules of the fresh plant with psuedohypericin and the protoforms, protohypericin and protopsuedohypericin. The function of this compound in plant is not well known (Agonstinis et. al., 2002).

Hypericin was proved to be synthesized frangula-emodin-anthranol. Frangula-emoidin-anthranol one step further is biochemically converted to protohypericin that has orange crystalls (Brocmann et. al., 1953). It is thought that in the presence of light protohypericin is converted to hypericin. The optimal wavelenght for the

transformation of the protopigments in the extract and the flowertops of the plant was found to be 515 nm (Poutaraud, 2001).

1.2.7 Hypericin in cancer treatment

Hypericin is a strong photosentisizer fluoescent red plant pigment (Piette, et. al., 2003). A photo sensitizer is a molecule that generates free super oxide radicals that might form peroxide and hydroxyl radicals or non radical singlet oxygen molecules. Hypericin induces free radical production in the prescence of light and the producion of this free radicals leads to apoptosis in tumor cells (Hunt et. al., 2001). Thus it is used in antitumoral photodynamic therapy; where delivery of visible light is made to the tumor lesion consequently to the systemic oral administration of a photosensitizer leading to tumor destruction. Changes in tumor vasculature are observed in tumor tissues applied with light and a photo sensitizer. In addition to direct tumor cell killing PDT causes the release of cytokines and other anti-inflammatory mediators from treated cells and this can produce an inflammatory response.

PDT offers an obvious advantage over conventional therapeutic drugs or radiation cancer treatments, for it has minimum systemic toxicity and a highly selective photo destruction of tumor cells. PDT is the best way to treat skin cancer and is becoming increasingly accepted for many types of tumors such as esophageal cancer, early lung cancer, cancers of the bladder, brain, head neck eye ovary and lung, as well as skin cancer. The development of powerful lenses combined with flexible optical fibers has supported the clinical applications (Agostinis et. al., 2002).

Anticancer activities of hypericin in the dark has also been studied. Studies showed that hypericin inhibed growth of tumor cells; but there was no evidance that apoposis was induced by hypericin (Blank, 2002).

2.8 Hypericin as an anti-viral agent

Hypericin is a trong antiviral reagent and was shown to inhibit murinecyomegalovirus (MCMV), Sindbis virus, and human immunodefficiency virus type I especially in the presence of fluorescent light (Hudson et. al., 1991).

1.2.9 The toxicity caused by hypericin

In the case of Hypericum toxicity, the compound hypericin is absorbed from the intestine and concentrates near the skin. When the skin of the animal is exposed to sunlight, an allergic reaction takes place. Oxygen is necessary for the photodynamic hemolysis, leading to tissue damage. 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 secondary photosensitization, where the liver and other internal organs can be damaged (James and Johnson, 1976).

1.2.10 Extraction of hypericin from the plant Hypericum Perforatum L.

Hypercin was first isolated from Hypericum Perforatum in 1912, by alcoholic extraction of the plant as a dark violet-red powder and was thought to belong to the flavone group of pigments (Cerny, 1912).

Later the extraction of the pigment from the plant was performed with methanol (Brockmann, Hans et al. 1942) and acetone (Pace, 1941).

Vegetable oil or other oils were suitable for the extraction of the compound. The isolation of hypericin was more effective with glycol and sunflower seed oil when the moisture content of the herb was between %50 and %70, and 2-7 times higher at 70˚C than at 20˚C. The total extraction of hypericin was not depended on the water content of the plant. According to the literature, ethanol is the most studied

extraction solvent for the plant and extraction of the plant with ethanol was found to be the best method to isolate the compound (Georgiev et. al., 1985)

Sephadex column chromatography was found to be the best method among high speed coutercurrent chromatography, flash column chromatography, XAD solid phase extraction in isolating hypericins from Hypericum Perforatum L. (Smelcerovic et. al., 2002). Hypericin and psuedohypericin are very much alike each other with their colors, fluorescent and UV spectral properties. This has been a disadvantage in isolating hypericin. Later hypericin and psuedohypericin could be isolated individually by HPLC that is a very fast and acurate method for the seperation of compounds (Sirvent and Gibson, 2000).

Psuedohypericin was first extracted with paper chromatography in 1954 (Brockmann et. al. 1954). This compounds could be chemically distinguished from each other by the sulphuric acid reaction advanced by Hans Brockmann and it was shown that in the presence of sulphuric acid the fluorescent color of psuedohypericin increased and there has been a shift in the absorbence of psuedohypericin to 651 nm (Brockmann and Sanne, 1957).

1.2.11 The Stability of Hypericin

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).

1.3 The description of chromatography

Chromatography is a separative technique, that has arisen from the chemists interest to be able to separate a mixture of compounds into its constituents. The name chromatography was firstly used in 1906 by a botanist called Tswett; who worked to separate colored plant pigments. Chromatography means color-writing in Greek. Tswett made his experiments by allowing a solution of mixed pigments to pass through a column of crushed chalk and than he observed the separated color zones on the column (Sewell, 1987).

The international Union of Pure and Applied Chemistry (IUPAC) defined chromatography as follows:

Methods used primarily for the separation of components of a sample, in which components are distributed between two phases, one of which is stationary while other moves. The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. In these definitions “chromatographic bed” is used as a general term to denote any of the forms in which the stationary phase may be used. The mobile phase may be a gas or a liquid (Sewell, 1987).

1.3.1Classical column chromatography

Column chromatography is a technique in which the components of a mixture are separated by passing through a stationary phase that is packed in a column that is made up of glass. The glass column has a frit at the bottom to provide a support for the stationary phase. The tube is also fitted with a tab to control the flow of the mobile phase.

Various column dimensions are used that have the optimum ratio of height to diameter is between four and ten. The overall size of the column is governed by the amount of sample to be separated since the amount of sample to be applied on the column depends on the amount of the stationary phase (Sewell, 1987).

The modes of separation in classical column chromatography can be exclusion, affinity, ion-exchange, bonded phase, partition, and adsorption.

The quality of isolation depends on the length of the column, the flow-rate of mobile phase, the type of the adsorbent, the activity level and particle-size of the adsorbent, temperature and the choice of the mobile phase. As the particle size of the adsorbent decreases, the separation capacity of the column increases; because the surface-area of the adsorbent increases (Sewell, 1987).

Usage of very long columns can lead to band-broadening and long elution time. The effective column length can be determined by trial and error.

1.3.1.1 Adsorption chromatography

Adsorption is the ability of a solute to attract other molecules to its surface and to hold them on its surface. There are sites on the adsorbent molecule that the components and the mobile phase are competing to be adsorbed. The difference in separation rises from the changes in polarity of the components. The most polar

components are held onto the surface of a polar adsorbent and are not willing to move with the mobile phase so the least polar compounds are washed away faster with the mobile phase (Sewell, 1987).

1.3.1.1.1 Silicagel

Silica gel is an amorphous porous adsorbent variously referred to as silica, silisilic acid or porous glass. It is prepared by the hydrolysis of sodium silicate fallowed by further condensation or polymerization. Silica gels activity is due to the hydroxyl groups on the surface. The adsorbent is highly acidic in nature (Hamilton, 1987).

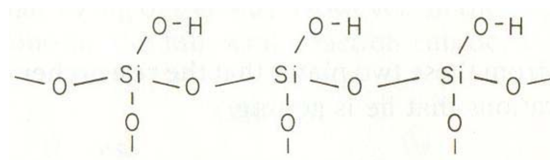


Figure 3. Silica gel structure

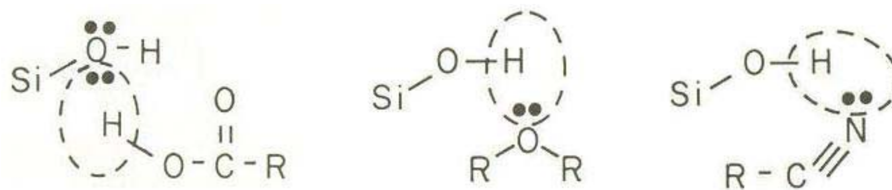


Figure 4. Association between molecules and silica

The types of Silica gel are:

Silica gel G with %13 calcium sulphate binder

Silica gel H without binder

Silica gel F254 with fluorescent indicator

Silica gel UV254 with fluorescent indicator

Solutes are adsorbed on the surface by hydrogen bonding surface hydroxyls serve as hydrogen donors. Silica has its middle activity when the water content is %11-12 because the most reactive sites are bound with water molecules.

1.3.1.2 Size Exclusion Chromatography

Size exclusion chromatography is referred as, gel filtration chromatography and is used to separate the macromolecules of biological origin.

Solutes are separated according to their molecular sizes through a gel matrix. The porous gel matrix separates the molecules in order of decreasing molecular sizes. Any solute that is larger than the pores of the support material are excluded and carried with the eluent through the column (Hosttetman, 1998).

The support matrix is nuetral and hydropilic to minimize the interactions between solutes and the support material.

The most widely used support materials are cross linked dextran or agarose (Sephadex), polyacrylamide beads (Biogel) and dextran derivatives (Sephacryl).

1.3.2 Thin Layer Chromatography

Thin layer chromatography is a rapid, simple, versatile, sensitive and inexpensive analytical technique for the separation molecules. The stationary phase in TLC is a thin layer of adsorbent that is spread uniformly over a plate All the adsorbents used is simple column chromatography can be used in TLC but the particle sizes should be very small compared to the column chromatographic method for a good yield of

separation. The plate can be prepared by spreading the adsorbent on glass in the lab or commercial pre-coated plates can be used (Richard Hamilton 1987).

The movement of molecules on a thin layer plate is a result of opposing forces the driving force of the mobile phase and the retarding force of the sorbent at the end of the development, each molecule has migrated a certain distance substances that move slowly are more retained by the adsorbent. The differential migration of molecules let the separation. The ability to achieve differential migration (resolution or separation) among the mixture components is the result of the selectivity, efficiency, and the capacity of the chromatographic system (Hamilton, 1987).

$$R_F = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent font}}$$

TLC is used mostly for preparative separations of molecules to decide the most suitable mobile-stationary phase composition for a specific mixture to be separated. And is a good technique for the separation of very small amounts of substances in the milligram (mg), nanogram (ng) and even picogram (pg) range.

Following separation spots can be excluded from the plate for qualitative analysis and densitometric plates are used for quantitative analysis.

1.3.2.1 Visualization of the spots

The colored spots can be detected directly. The colorless spots can be visualized by using different spray reagents or under ultraviolet radiation. Spray reagents can be fluorogenic (producing fluorescent zones) or chromogenic (producing colored zones). Fluorogenic and chromogenic spray reagents are destructive reagents; because they give reaction with the molecules. Commercial glass sprayers are used for spraying. Heating is often required for producing detectable zones after spraying

because certain reactions require higher temperatures and a long period of time. Different functional groups can be detected by different reagents (Hamilton, 1987).

1.4 Methods for obtaining components from medicinal plants

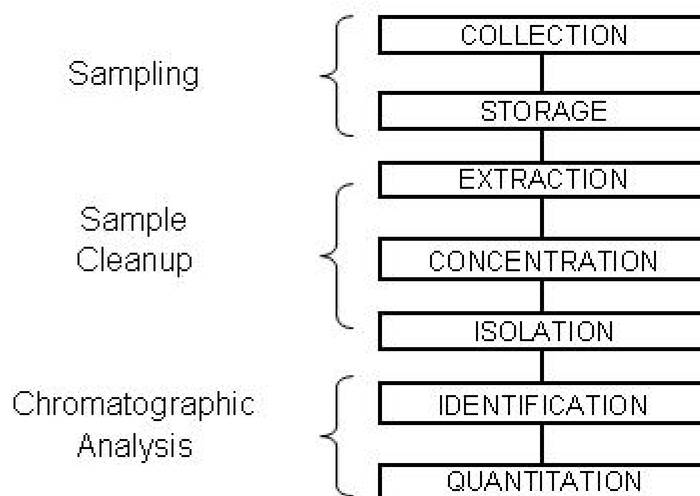


Figure 5. Extraction of compounds from medicinal plants

1.4.1 Collection and storage of the plants

After the plant to be analyzed is collected it is dried to prevent the enzymatic hydrolysis of biologically active components. Drying can be made in an oven under vacuum or by lyophilization. It is important that the plant should be avoided from the sunlight during the drying process (Poole 1991).

The plant should be stored in a dark place with little or no humidity.

1.4.2 Extraction of the plant material

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.

For thin layer chromatography is a very effective method for visualization of the components in a sample, the choice of the solvent used for extraction should be made by thin layer chromatography.

Extraction can be made in a soxhlet extractor or a shake flask extractor. During the extraction process the plant should be avoided form light for some compounds in the presence of light can be decomposed. Soxhlet extractor is a better model than the shake flask extractor model (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 diethyl-ether or petroleum ether, filtered and dried in an oven before the main extraction step (Tanker, 1991).

1.4.3 Preliminary fractionation of the components of the plant

The active compounds are found %0.01-2 in plants and samples from biological origin are very complex to be analyzed at one step so preliminary fractionation of the components can be required (Poole, 1991).

Preliminary fractionation can be made by solid-liquid extraction or liquid-liquid extraction. Solid liquid extraction can be made is a Soxhlet extractor or in a shake-flask extractor. Liquid-liquid extraction can be made in a simple separatory funnel. By these methods components of the plants will be separated from each other according to their solubilities in different solvents. The solvents required for the effective fractionation can be detected by thin layer chromatography (Poole, 1991).

Volatile components can be separated by distillation. The effectiveness of the distillation depends on the nature of the components and the method used for distillation. The most common method for the analysis is steam distillation. By steam distillation essential oils can be separated from the extract (Poole, 1991).

1.4.4 Column chromatography techniques

The separation of the desired components or the fractionation of the components can be made by column chromatography.

The choice of the column material is made according to the components of the plant or the molecules desired to be separated (Tanker, 1991). Data form the literature is often reliable in the choice of the column material.
Solvent systems required for the best separations can be found by trial on preparative TLC plates if; a silica column is to be used.

The size of the column, the flow rate of the mobile phase, column material and the composition of the mobile phase is vital for the separation of components (Tanker, 1991).

The extract can be loaded into the column by being dissolved in a few milliliters of the mobile phase or directly dry extract (Tanker, 1991). The eluting solvent is collected as equal fractions from the column. The fractions are subsequently analyzed by UV spectroscopy and TLC.

The choice of the mobile phase and the spray reagent in thin layer chromatographic analysis of the fractions, is important for the fractionation of components.

The obtained fractions may be subjected to new column procedures for obtaining pure components.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Hypericum perforatum was obtained from Arifoğlu, İstanbul. Ethanol, acetone, ethyl acetate, formic acid, were analytical grade, diethyl-ether, petroleum ether, sulphuric acid from Fluka, vanillin, rutin, quercetin, myricetin, Sephadex LH-20, d-DMSO, hypericin from Sigma from, acetic acid from Merck, methanol from Merck were purchased for the experimental method. For TLC 20x20 silica gel 60 F_{254} aluminum sheets were used.

2.2 Methods

2.2.1 Reagents:

Vanillin-sulphuric acid spray reagent:
Vanillin in ethanol:1% vanillin in ethanol was prepared.
Sulphuric acid in ethanol: 5% sulphuric acid in ethanol was prepared.
Vanillin and suphuric acid ethanolic solutions were combined in 1:1 ratios.

2.2.2 Separation of carotenoid pigments:

2.2.2.1 Pre-extraction of the plant with petroleum ether:

10 grams of plant material was blended and extracted with 100 mililiter of petroleum ether in a shaker at 50˚C for 10 hours. The plants were filtered out from petroleum ether and left in an oven at 30˚C overnight for the removal petroleum ether from the plant.

The second extraction was made with the dried plants by ethanol. The dried plants were extracted with 100 mililiters of ethanol at 50˚C for 10 hours. The extract was filtered. 20 µl of each petroleum ether and ethanol extracts were loaded on a TLC plate. TLC was performed with ethyl acetate: formic acid: glacial acetic acid: water solvent system in ratios of 10:1.1:1.1:.2.6.

TLC plates were dried and sprayed with vanillin-sulphuric acid spray reagent and heated to 120˚C on a hot plate for 5 minutes for the visualization of the spots.

2.2.2.2 Eluting the dried extract with diethyl ether

100 grams of Hypericum Perforatum was blended and extracted with 500 ml of ethanol twice in a soxhlet extractor at 50˚C for 10 hours. The two extracts were combined and left in a rotary evaporator until dryness.

The dried extract was washed 200 ml of diethyl-ether for five times in a beaker at room temperature, each for half an hour on a magnetic stirrer until no fluorescent pink color was observed in the ether phase in the presence of ultraviolet radiation. Ether phases were combined.

For the observation of the efficiency of the separation; 20µl of each diethyl ether and ethanol phases were loaded on a TLC plate and TLC was performed in a chamber with ethyl acetate: glacial acetic acid: formic acid: water, 10: 1.1: 1.1 :2.6. The plates were dried and sprayed with vanillin-sulphuric acid spray reagent and left to be heated on a hot plate for 5 minutes at 120˚C.

2.3 Isolation of hypericin by Sephadex LH-20 gel filtartion method:

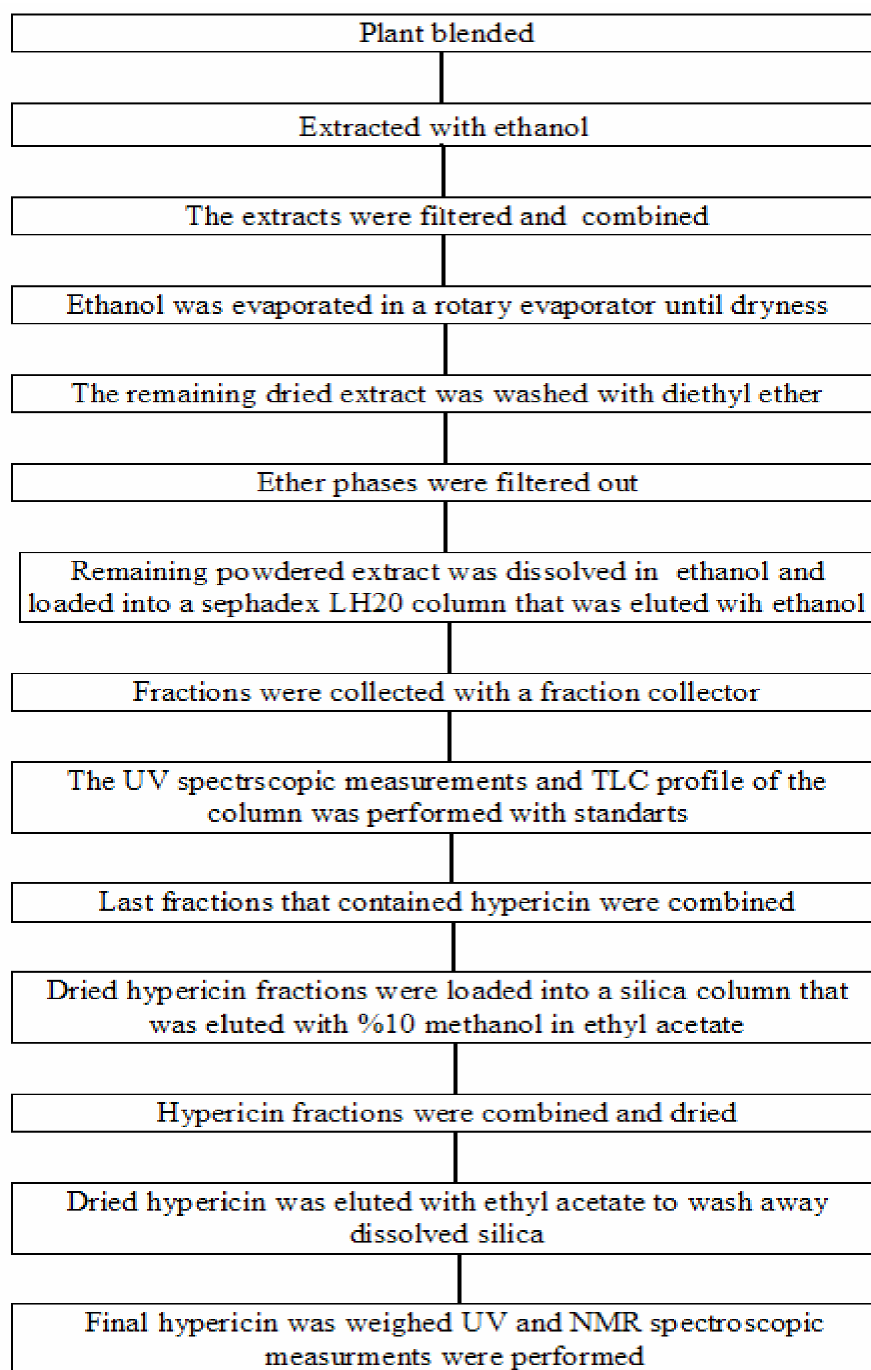


Figure 6. The flow chart of hypericin isolation by Sephadex LH-20 gel filtration method

2.3.1 Extraction:

100 g of the dried plant material was powdered in a blender. The blended plant material was extracted with 500 ml of ethanol twice in a soxhlet extractor at 50 ^{o}C for 10 hours. The two filtered extracts were combined and ethanol was evaporated in a rotary evaporator until dryness. The dried extract was weighed.

2.3.2 Removal of the carotenoid pigments:

The dried extract was weighted and washed with 200 ml of diethyl-ether for five times in a beaker at room temperature, each for half an hour on a magnetic stirrer until no fluorescent pink color was observed in the ether phase in the presence of ultraviolet radiation. Ether phases were filtered out and the remaining brown powder was weighed.

2.3.3 Isolation of hypericin

100 grams of sephadex LH-20 was weighed and left in 900 ml of ethanol overnight. The column material was then packed into a 3.5 cm diametered and 90 cm length glass column with a Teflon tap.

0.987 grams of the carotenoid pigment free extract was dissolved in 5 ml of ethanol and loaded into the sephadex LH-20 column.

The column was eluted with ethanol. The flow rate of the column was 1.2 ml/min.

First 270 ml's of the eluent was taken as void volume. The fractions were collected with a fraction collector. First fractions were collected 25 drops per tube after 140th fraction fractions were collected 50 drops per tube.

UV spectroscopy measurements were performed each four fraction by diluting 20 µl in 2 ml of ethanol. The maximum absorbances and the wavelength of the absorptions were recorded in terms of observing the elution profile of the column.

TLC was performed each 5 fractions by loading 20 µl of the fractions on a TLC plate. The mobile phase was ethyl acetate: glacial acetic acid: formic acid: water, 10: 1.1: 1.1: 2.6 solvent system. After the TLC plates were dried and were spayed with vanillin-sulphuric acid spray reagent for visualization of the spots. The plates were heated on a hot plate for 5 minutes at 120˚C.

The fractions that were observed to contain the same components by vanillin-sulphuric acid visualizing reagent were combined and final UV-VIS spectroscopy measurements and TLC was performed by using the standards.

Rutin, quercetin, myricetin, coumarin and hypericin were used as standards. Standard solutions were prepared by dissolving 1 mg's of each compound in 1 ml of ethanol. Each standard was loaded on the TLC plate in volumes of 5 µl's.

The last fractions that were observed to contain hypericin were collected in 100 ml volume beakers. TLC and UV-VIS spectroscopy measurements were performed on the 100 ml volume fractions. The last fractions were combined and ethanol was evaporated. Dried hypericin was weighed and subjected to TLC.

2.3.4 Final purification of hypericin:

1 mg of the isolated hypericin fraction was dissolved in 1 ml of ethanol. The isolated hypericin and the standard hypericin were loaded on a TLC plate together to test the purity of the isolated hypericin in volumes of 5µl's. The mobile phase was ethyl acetate: glacial acetic acid: formic acid: water solvent system in ratios of 10: 1.1: 1.1: 2.6.

The plate was dried and sprayed with vanillin-sulphuric acid spray reagent for visualization of hypericin and other components. The plate was heated to 120˚C on a hot plate for five minutes.

5 µl of the isolated hypericin solution was loaded on a TLC plate and TLC was performed with ethyl acetate: methanol solvent system in ratios of 9: 1. The plate was dried and sprayed with vanillin- sulphuric acid reagent and the plate was heated to 120˚C for 5 minutes on hot plate.

15 grams of silica was weighed and 50 ml of the solvent system ethyl acetate: methanol 9:1 was added on the silica in a 250 ml beaker and finally stirred with a glass rod. Silica was loaded in a glass tapped 30 cm heighed 1 cm diametred glass column. The hypericin fraction that was isolated by Sephadex LH-20 gel filtration method was dissolved in 2 ml of the ethyl acetate: methanol solvent system. First 30 ml was taken as the void volume. The flow rate of the column was 1.5 ml/min. The fractions prior to the void volume were taken as 30 drops per tube. 90 tubes were collected until the fluorescent red color of hypericin could not be observed in the fractions.

Every fifth fraction was subjected to TLC applying the spots as 10 µl of the fractions on the plate. The plates were dried and sprayed with vanillin-sulphuric acid spray reagent or the visualization of the impurity and hypericin. The plates were heated to 120˚C for 5 minutes on a hot plate after being sprayed.

The fractions that contained only hypericin were collected. The solvent was evaporated in a rotary evaporator. The final dried hypericin was eluted with ethyl acetate for the removal of silica by filtering by filter paper.

Ethyl acetate was evaporated in a rotary evaporator and the remaining hypericin was weighed.

CHAPTER III

RESULTS AND DISCUSSION

The aim of this study was to isolate hypericin, the main photoactive constituent of Hypericum Perforatum L. by using a simple chromatography methods (Agostinis et. al. 2002). For this purpose Sephadex LH-20 gel filtration method was used. Sephadex LH-20 was used in literature and was found to be a good method for isolation of hypericin compared with high speed couter-current chromatography, flash column chromatography, XAD solid phase extraction (Brockmann and Walter, 1957).

First the plant was blended in a Waring blender to obtain the highest surface interaction between the plant material and the extracting solvent. For the extraction of the plant material ethanol was the solvent of choice; because according to the literature ethanol was the most studied extraction solvent for this plant and extraction of the plant with ethanol was found to be the best method to isolate the hypericin (Georgiev, et. al. 1985).

Extraction of compounds from biological origin is a hard task; because there are so many complex molecules in the solid matrix. For this reason preliminary fractionation of the plant has a vital importance for effectiveness of column separation (Poole 1991).

According to the previous experiments made on the separation of components of Hypericum Perforatum L. carotenoid pigments were quite troublesome, because

under the ulraviolet radiation, red fluorescence of the carotenoid pigments resemble the florescence of hypericin (Costes et. al. 1967).

Carotenoid pigments are lipid soluble molecules and can be extracted from the plant by non-polar solvents (Robinson, 1963). For the extraction of flavonoids and other phenolic compounds, alcohols are the solvent of choice unfortunately carotenoid pigments can also be extracted with alcohols, and it is often required to make a preliminary extraction with a non-polar solvent especially petroleum-ether, before the alcohol extraction (Goodwin, 1965).

For this reason two methods were tried for the separation of lipid soluble carotenoid pigments from the other phenolic constituents of Hypericum Perfratum L. One the methods were to apply a preliminary extraction step on the plant material with petroleum-ether then continue with alcohol extraction. Another method to wash the dreid ethanol extract with diethyl ether.

3.1 Effect of pre-extraction of the plant with petroleum ether:

To determine the effectiveness of preliminary extraction method, on the removal of carotenoid pigments, 10 grams of plant was blended into a powder and extracted with 100 ml of petroleum ether at 50˚C for 10 hours in a shaker. Then petroleum ether was filtered out and the plant was dried in an oven at 30˚C overnight. The dried plant was then extracted with 100 ml's of ethanol for 10 hours at 50˚C in a shaker.

To see the difference between before and after preliminary extraction step, onto a silica gel TLC plate 20µl's of each extract was applied with the mobile phase of ethyl acetate: glacial acetic acid: formic acid: water in 10**:** 1.1**:** 1.1**:** 2.6 ratios. The spots were visualized by vanillin-sulphuric acid spray reagent.

Figure 7. Effect of pre-extraction of the plant with petroleum ether

The first sample loaded on the TLC plate is the ethanol extracted without any ether preextraction step, the second sample is the ethanol extract with preliminary extraction with petroleum ether and the third sample is the ether phase after extraction and filtration (**Figure 7**).

Pre-extraction of the plant with petroleum ether for 10 hours at 50˚C was not very effective in separating the carotenoid pigments; which are easily visible at the top of the solvent front (**Figure 7**). The method was also time consuming and the removal of petroleum ether from the plant was difficult that the plant weighed 1.3 times of its weight after drying in an oven overnight at 30˚C.

3.2 Effect of washing the dried extract with diethyl ether

For this method 100 grams of Hypericum Perforatum L. was blended in a blender and was extracted twice with 500 ml of ethanol in a Soxhlet extractor for 10 hours at 50˚C. The extract was filtered and dried. The dried extract was weighed and washed

5 times with 200 ml's of diethyl-ether, until there would be no fluorescent pink color in the ether phase observable under ultraviolet radiation. During these washing steps the sticky structure of the ethanol extract has been changed into a brown powdered structure, this was weighed of and continued with the rest of the analysis.

1 mg of the remaining powdered extract, untreated ethanol extract and the extract that was removed with diethyl ether were dissolved in 1 ml of ethanol, the solutions were diluted 1/10 and 20 µl's of each diluted solution was loaded on a TLC plate. The TLC performed with ethyl acetate: glacial acetic acid: formic acid: water 10**:** 1.1**:** 1.1**:** 2.6 solvent system as the mobile phase. The spots were visualized with vanillin-sulphuric acid spray reagent.

Figure 8. The effect of eluting the dried extract with diethyl ether

The first spot belongs to the ethnol extract, before any ether washing steps, the second spot represents the carotenoid pigment free ethanol extract after the ether washing steps and the third spot represents the ditehyl ether phase from the washes that has a fluorescent pink color under UV radiation. According to the TLC results

shown in **Figure 8** there are no carotenoid pigments observable in the powdered extract (the sample in the middle) .

This method is much faster (lasts only a few hours) method , when compared with other fractionation methods for the removal of lipid soluble compounds from plant matrix such as liquid-liquid extraction method.

Subsequent extractions showed that, the important point for this method was the removal of the extraction solvent ethanol completely before the ether washing step, otherwise the washing step of the extact with dietyl ether was not possible.

10 grams of extarct was obtained from 100 grams of Hypericum Perforatum L. by 500 ml ethanol extraction in a Soxhlet exractor two times at 50˚C. The yield was %10.

From the 10 g of dry ethanol extract obtained above, 6 grams of powdered carotenoid free extract was obtained through the washes with 200ml's of diethyl ether for half an hour five times repeatedly at room temperatures.

In the literature the carotenoid pigments found in Hypericum Perforatum L. are luteolin,violaxanthin, luteoxanthin, cis-trollixanthin, trollichrome and an unidentified pigment (Costes and Claude, 1967). By the experiment performed in this study, about 4% of the dry alcohol extract of Hypericum Perforatom L. Was foun to be conisting of carotenoid pigments.

3.3 Isolation of hypericin by Sephadex LH-20 gel filtartion method

3.3.1 The elution profile of the column and the analysis of the fractions

For the isolation of hypericin by Sephadex LH-20 gel filtration method, 100 g of the dried plant material was powdered in a blender and extracted with 500 ml of ethanol twice in a soxhlet extractor at 50 ^{o}C for 10 hours. The filtered extracts were combined and ethanol was evaporated in a rotary evaporator until dryness. The dried extract was weighed. The lipid soluble carotenoid pigments were separated from the ethanol extract by washing the extract with 200 ml of diethyl-ether for five times in a beaker at room temperature, each for half an hour on a magnetic stirrer, until no fluorescent pink color was observed in the ether phase in the presence of ultraviolet radiation. Ether phases were filtered out and the remaining brown powder was weighed as 6 g.

0.987 grams of the powdered plant extract was dissolved in 5 ml of ethanol and was loaded into a 3.5 cm in diameter and 90 cm in length glass column, which was filled with 100 g of Sephadex LH-20 as support material was. The column was eluted with ethanol and the flow rate of the eluting solvent was 1.2 ml/min.

After collecting the first 270 ml as void volume; elution started with the collection of fractions with 25 drops per tube until the 140th fraction; then the number of drops per fractions doubled to 50 drops.

UV-VIS spectroscopy measurements were performed once in every fourth fraction, by diluting the fractions into a ratio of 1/10. The maximum absorbances and wavelengths obtained by scanning from 200-700 nm. Then the results were plotted as the elution profile of the column. The following figure shows the elution profile of carotenoid pigment free ethanol extract using ethanol as the eluent. Maximum absorbances for varying wavelengths are given versus eluent volume (**Figure 9**).

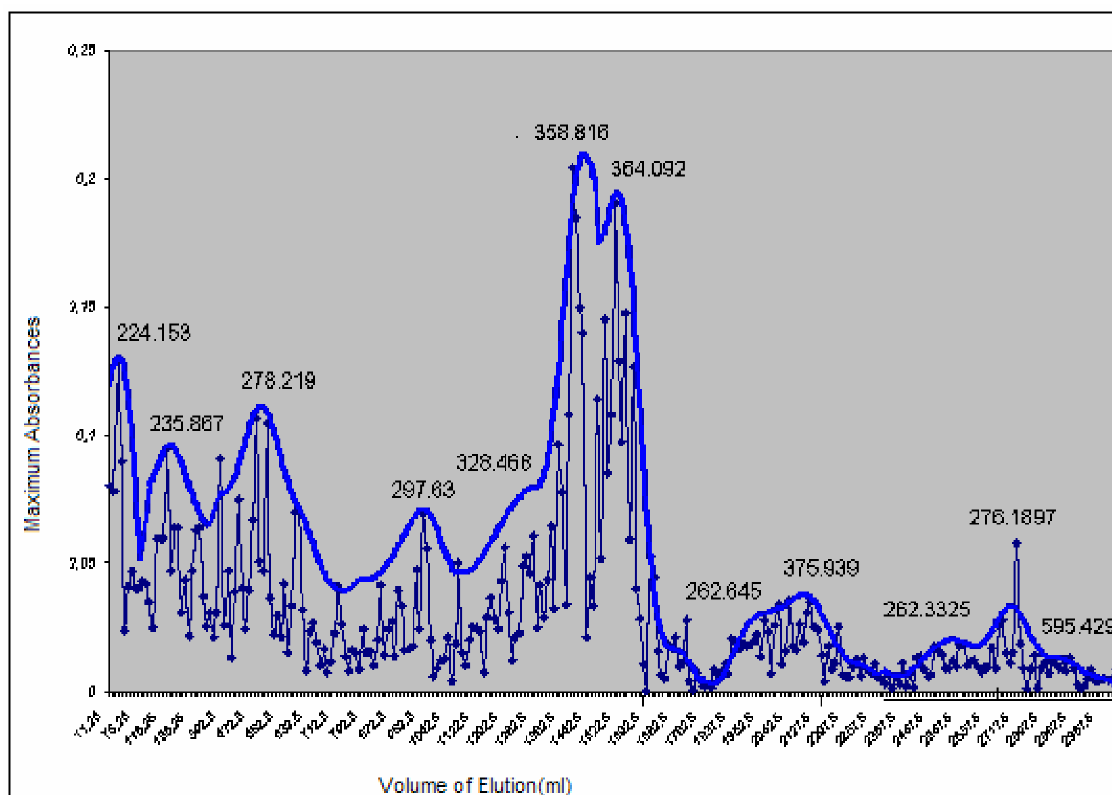


Figure 9. Elution profile of the sephadex column

According to the wavelenghts of the maximum absorbtion points, this can be clearly seen that, compounds with different UV spectral properties are eluted from the colum at different rates of elution. And the fractionation was succesfull.

According to the UV-VIS spectroscopy measurements it is often not valid that a compound or a compound group can be identified truly; because even a single substitution in the molecules or the change of solvent can cause shifts in the spectrum.

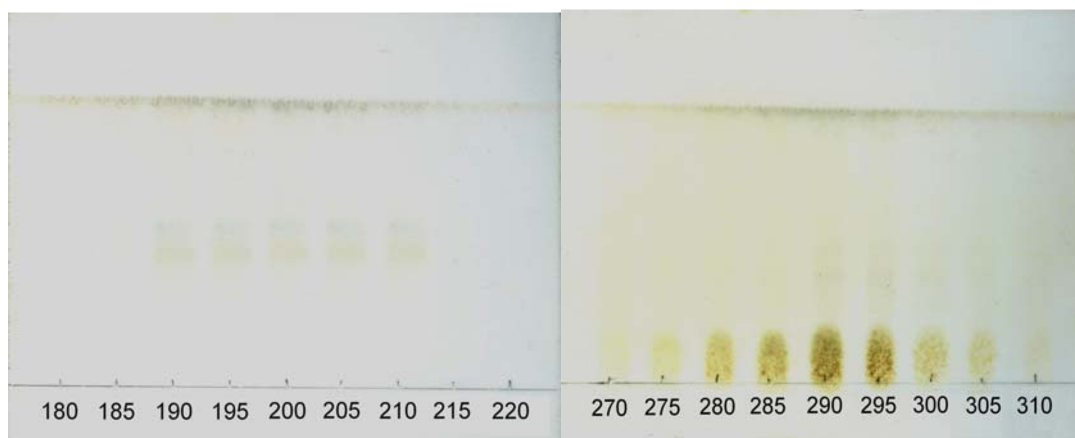
The fractions between 1400 and 1600 ml of the eluting volume may be thought as flavonoids; because these fractions have high absorbances. Hypericum Perforatum has a very high flavonoid content, the plant was shown to be containing the largest fraction of flavonoids among 223 species tested **(**Tsisina**)**. The other reason that the compounds eluted in this integral may can be thought of flavonoids is that, rutin and

other flavonoids of Hypericum Perforatum quercetin, myricetin, kaempfrol are flavonol group of pigments, and all flavone and flavonol group of pigments have a more or less intense absortion bands at about 220-270 nm and another strong band at about 330-375 nm (Robinson, 1963) . These factions have bands with maximum at 230 and about 358 - 364 nm's.

The last fractions are observed must be hypercin with the maxium absorbance at 595 nm (**Figure 18**).

To support the UV-VIS spectrum, TLC run with the standarts was performed by loading 20 µl of every fifth fraction on the silica sheets with the mobile phase composition ethyl acetate: glacial acetic acid: formic acid: water, 10: 1.1: 1.1: 2.6 solvent system, spots for each sample were visualized with vanillin-sulphuric acid spray reagent.

Following TLC plates show the components in the fractions that were detectable with vanillin-sulphuric acid reagent. The results of TLC data are given (**Figure 10**).



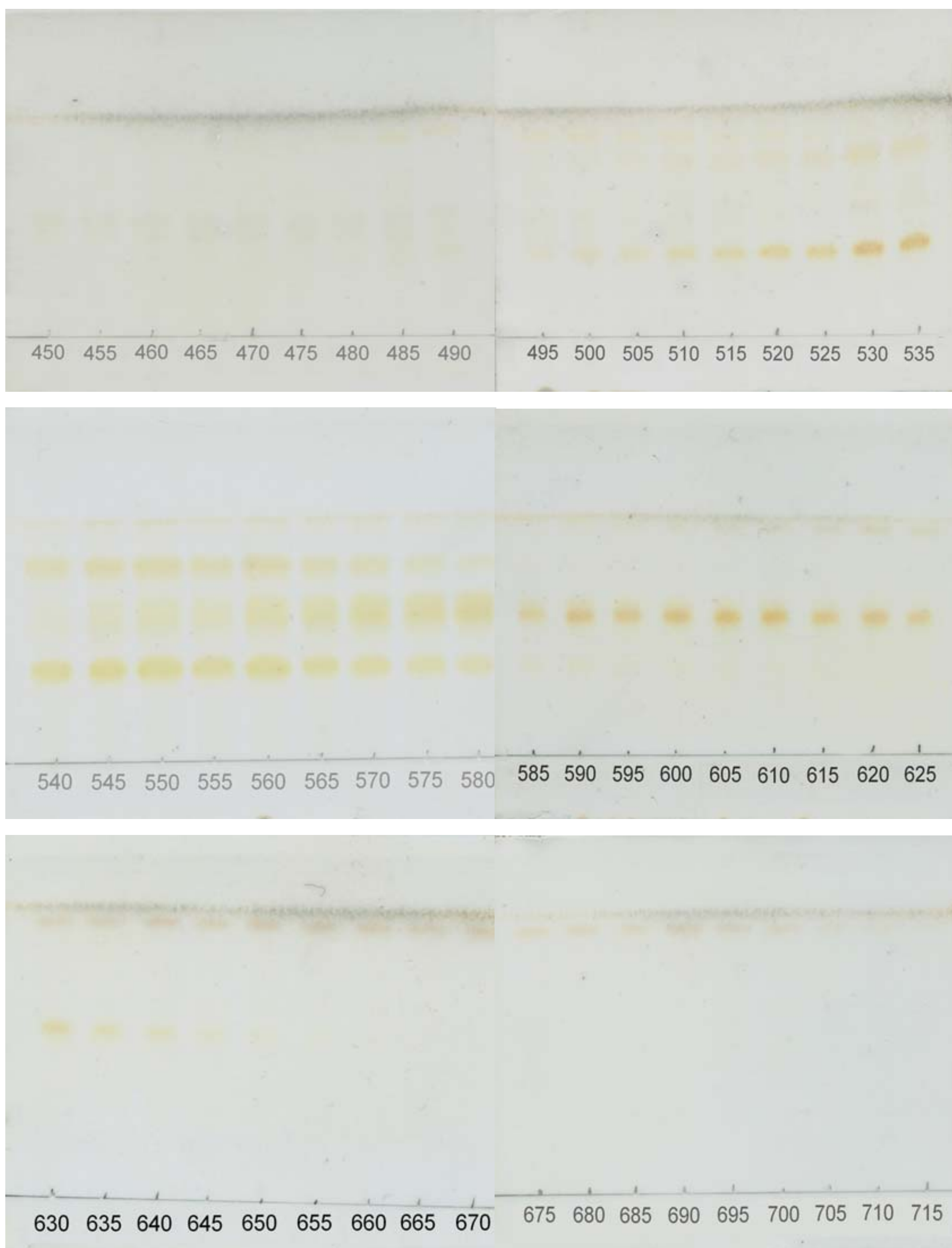


Figure 10. TLC data of the elution profile visualized with vanillin-sulphuric acid reagent.

According to the results obtained by spraying the plates by vanillin-sulphuric acid spray reagent and the elution profile it can be concluded that trial of other spray reagents is required for the detection of spots that could not be visualized by vanillin-sulphuric acid spray reagent. This can lead us to the fractionation and isolation of other compound groups from the plant.

3.3.2 Analysis of the collected fractions:

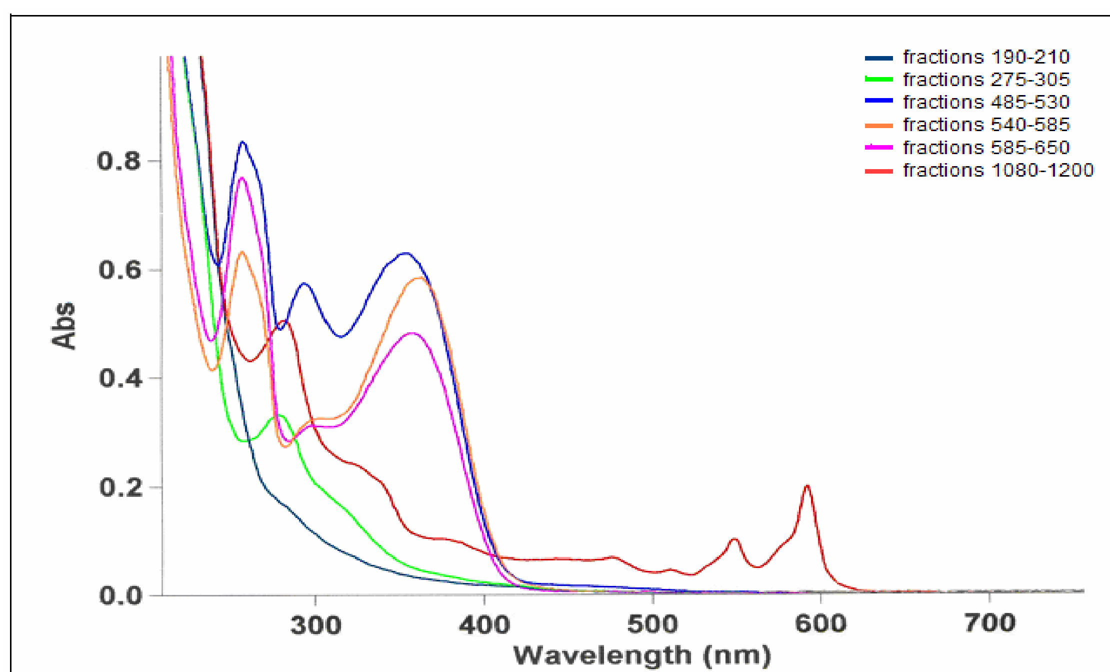


Figure 11. The UV-VIS spectrum of collected fractions

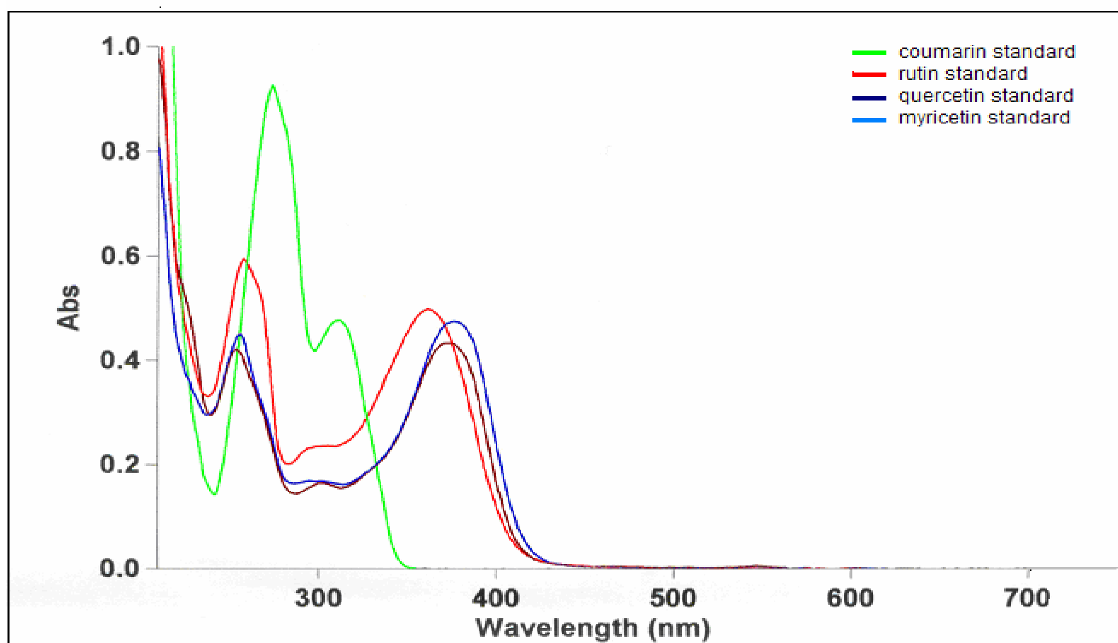


Figure 12. The UV-VIS spectrum of standards 0.01mg/ml in ethanol

The fractions that contained the same components were collected according to the TLC data in **Figure 10**. **Figure 11** in the previous page shows the UV-VIS spectrum of the collected fractions from 190 to 210, 275 to 305, 485 to 505, 540 to 580, 585 to 650, and 650 to 710 in en ethanol. Figure 12 shows the UV-VIS spectrum of the standards in ethanol.

According to the UV-VIS spectrum comparisons of standard compounds and of the fractions collected, it can be concluded that the fractions between 485 to 530th tubes contains both rutin and coumarins. For Sephadex LH-20 separates the molecules according to their sizes, subsequent elution of the compounds from the column leads us to a conclusion that their sizes are close to each other.

The absorption spectra of the fourth and fifth fractions (**Figure 11**) are very similar and according to absorption bands they seem to be belonging to the flavonol group of pigments such as myricetin and quercetin.

When to the UV spectrum of the fractions collected after 1080th fraction is compared with the UV spectrum of the standard hypericin (**Figure 18**), this can be clearly seen that the 6th fraction from figure 11 contains hypericin.

The fractions that were collected were loaded onto TLC plates, in volumes of 20 µl's with 5 µl of 1mg/ml rutin, quercetin, myricetin, coumarin and hypericin standards. The mobile phase was ethyl acetate: glacial acetic acid: formic acid: water with the ratio of 10: 1.1: 1.1: 2.6 solvent system. The spots were visualized with vanillin-sulphuric acid spray reagent.

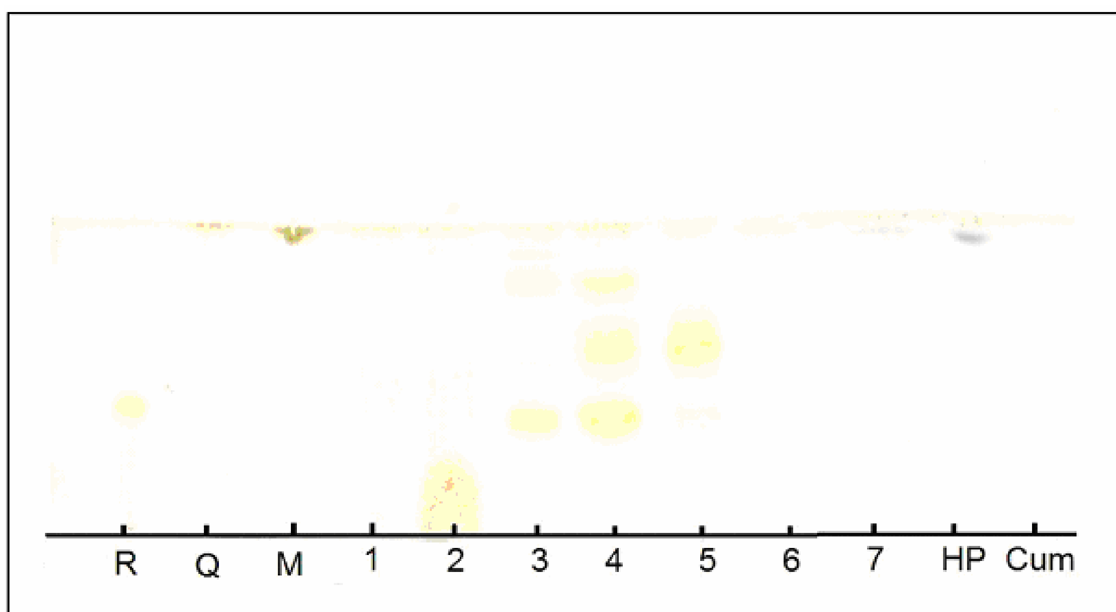


Figure 13. TLC data of the collected fractions in comparison with the standards

Spots 1, 2, ,3 ,4 , 5, 6, 7 represent fractions 190 to 210, 275 to 305, 485 to 505, 540 to 580, 585 to 650, and 650 to 710 respectively and spots M, Q, R, H, C represent myricetin, quercetin, rutin, hypericin and coumarin standards respectively.

According to the data obtained from the TLC separations The 3rd and 4th collected fractions contained rutin; but for the fact that myricetin and quercetin ran to the solvent front, their presence in fractions were not observable.

For this reason the improvement of this step in the experiment must be to use other solvent systems: if further separation of flavonoids is desired to be made.

Coumarin standards could not be visualized by vanillin-sulphuric acid spray reagent; but the UV spectral measurement made in comparison with the coumarin standard led us to the presence of coumarins in the 3rd collected fraction.

In the experiment the reason for the choice of ethyl acetate: glacial acetic acid: formic acid : water 10: 1.1:1.1 :2.6 solvent system was due to the fact that this solvent system is the best solvent system for isolation of flavonoids by silica columns or plates (Tanker, 1991) and Hypericum Perforatum has a very high flavonoid content among the other species tested.

No bands were observed with absorption wavelength greater than 600 nm, in any of the eluted fractions. This is a good indication that the carotenoid pigments were totally separated from the ethanol extract with the ether washes of the extract.

The presence of only the bands that belong to the flavonoid group of pigments in the UV-VIS spectrum of collected flavonoid fractions show that the method is also a good method for the fractionation of flavonoids in Hypericum Perforatum L.

Groups of compounds were collected consistent with their molecular weight similarities. Coumarins and rutin quercetin may be separated through another column with silica gel. Further isolation steps can be carried out with the related fractions to obtain pure flavonoids. For the detection of the flavonoid pigment content of the fractions other spectroscopic measurements should be made with the other flavonoid standards that the plant contain; but for the reason that the compounds are same group of pigments and their UV-VIS spectroscopy measurements can be so much alike each other, TLC by the fractions in compared with the standards will give more accurate results about the contents

3.3.3 Analysis of hypericin fractions

The TLC data compared with the standards, the elution profile of the column and the UV-VIS spectrum of the last fractions compared with the UV-VIS spectrum of standard hypericin shows that the last fractions contain hypericin.

After the collection of the last 100 ml fractions the fractions were collected and subjected to TLC with in comparison with 1mg/ml hypericin standard with the solvent system Ethyl acetate: Formic acid: Glacial acetic acid : water 10: 1.1: 1.1: 2.6 solvent system. The spots were visualized by vanillin-sulphuric acid spray reagent. The TLC data of isolated hypericin compared with standard hypericin is given in **Figure 14.**

Figure 14. TLC data of standard and isolated hypericin

A second compound right below hypericin was observed in the isolated hypericin fraction. This impurity was dilute in the 100 ml hypericin fractions, that it could not be observed in the TLC separations made previously on the 100 ml fractions.

3.3.4 Final purification of hypericin

For the separation of this impurity from hypericin, hypericin was subjected to TLC with ethyl acetate: methanol, 9:1 solvent system. And the plate was sprayed with vanillin-sulphuric acid spray reagent.

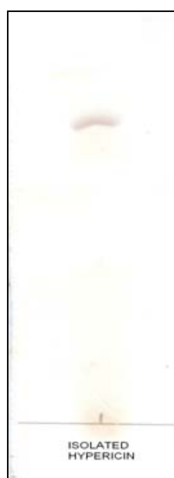


Figure 15. Isolated hypericin with mobile phase composition ethyl actate: methanol

The result of the TLC with mobile phase composition is as ethyl acetate: methanol in ratios of 9:1. This mobile phase composition seemed to separate the impurity from hypericin.

A silica column that has dimensions 30 cm height and 1 cm diameter was used for the final purification of hypericin with the mobile phase composition of ethyl acetate: Methanol, 9:1. 18 mg of hypericin fraction was loaded into the column.

The isolated hypericin was washed with ethyl acetate to remove silica particle that were carried away with methanol. The silica particles were not soluble in ethyl acetate so they were easily separated by filtration from hypericin.

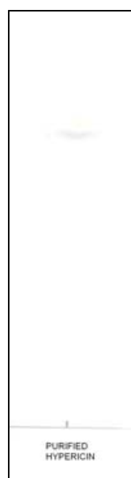


Figure 16. Hypericin fraction after purification

The final as seen in **Figure 16** hypericin was dried and weighed to be 12 mg. The UV-VIS data of the isolated hypericin is given in **Figure 17.**

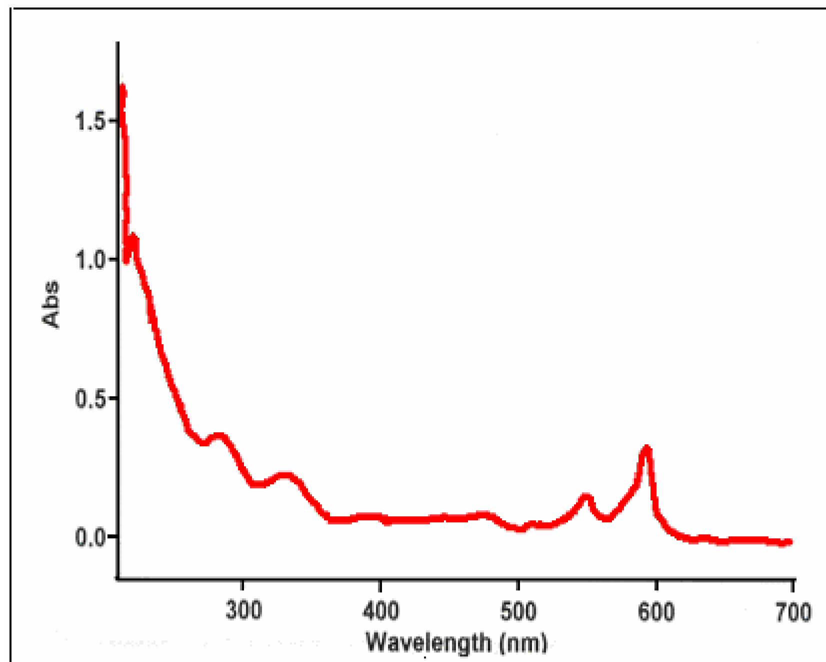


Figure 17. The UV-VIS spectrum of isolated hypericin

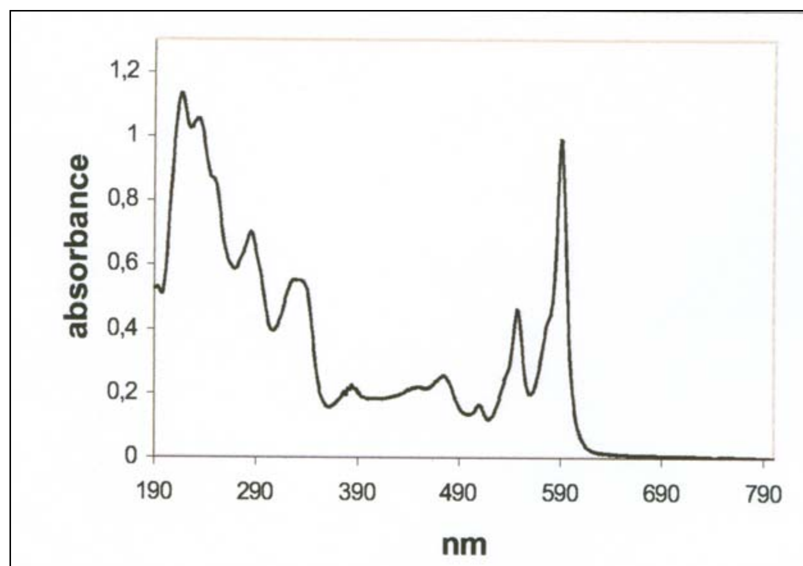


Figure 18. The UV-VIS spectrum of hypericin from the literature (Brockmann and Sanne, 1957)

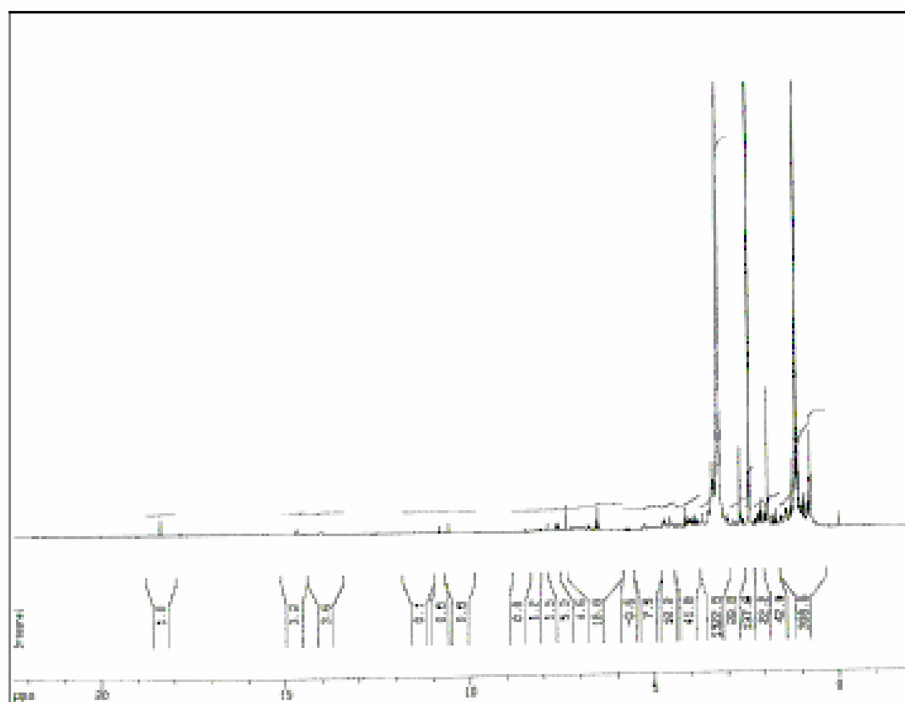


Figure 19. NMR Spectrum of Isolated Hypericin

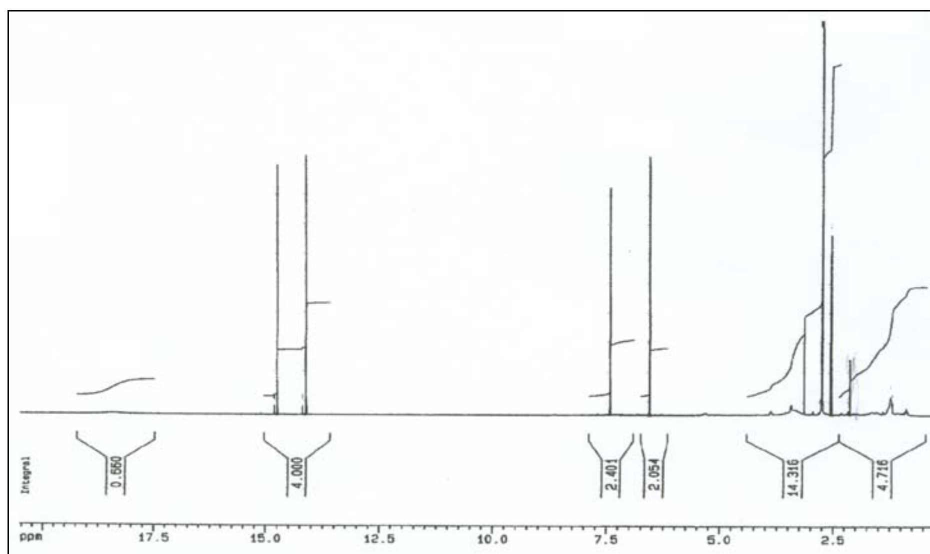


Figure 20. NMR spectrum of hypericin form the literatre

The NMR spectroscopy data of 6 mg of isolated hypericin in d-DMSO is given above in **Figure 19**. **Figure 20** shows the NMR spectrum of standard hypericin in d-DMSO.

For stuctural determination of the isolated components UV-VIS spectroscopy and TLC are not sufficient, additional structure determination with NMR is required to identify the isolated compound.

In the NMR spetrum the isolated spectrum the signals for hypericin can be clearly observed with the signals for dDMSO, that was used as the solvent for hypericin and aceton that was used to clean the glass material.

CHAPTER IV

CONCLUSION

To isolate the main photoactive component Hypericin of the medicinal plant Hypericum Perforatum L. Sephadex LH-20 gel filtration method was performed on the ethanolic extract of Hypericum Perforatum L. Biological samples are very complex and it is often required a preliminary fractionation for an effective chromatographic separation. Due to their behviour under ultraviolet radiation the lipid soluble carotenoid pigments are very much like Hypericin.

Carotenoid pigments although are lipophilic molecules are soluble in alcohols that are often used for the extraction of many biologically active plant constituents. In this study a new method was applied for the removal of carotenoid pigments which includes a repeated steps of washing of the dried ethanol extract with diethyl ether. It was found to be an efficient and faster method for removing the lipid soluble carotenoid pigments. This can also be seen from the TLC and the UV spectroscopic data **Figure 11** and **Figure 13** from which there are no indications of carotenoid pigments. However it was important to apply the washing step with diethyl-ether to totally dried ethanol extract. From the results 4% of the dried ethanolic extract was consistent of carotenoid pigments.

The method used in this study, with the ethanol extraction of the plant Hypericum Perforatum L. and petroleum ether washes of the extract and followed with the basic column chromatography using sephadex LH-20 and eluting with ethanol, has given quite efficient results in terms of fractionation of bioactive and photoactive groups

from the plant extract. The sephadex column chromatography procedure was also succesfull in fractionating flavonoids of Hypericum Perforatum L. and the spray reagent, vanillin-sulphuric acid, was succesfull for the visualization of flavonoids and hypericin.

Hypericin is found in the plant with its psuedo and protoforms and their molecular structures are similar to each other so they can be eluted from the column at the same rates and the conversion of the pigments into each other makes the task harder.

The second column has a silica gel 60 preperative column support, and the elution solvent was chosen as the ethylacetate :methanol in 1:9 solvent ratio, which was succesfull for the purification of the impurities.

10 gram dried extract was obtained from 100 g of the plant. Of this dried extract 4 gram was washed away with diethyl ether, 0.987 g of the remaining brown powder was dissolved in 5 ml of ethanol and loaded on column, 22 mg hypericin was isolated and further purification on silica lead 14.3 mg pure hypericin. The amunt of hypericin isolated form the plant also was compatible with literature.

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