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Hypericin: Source, Determination, Separation, and Properties

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ABSTRACT

Hypericin is a naturally occurring compound synthesized by certain species of the genus *Hypericum*, with various pharmacological effects. It is used as a natural photosensitizing agent with great potential in photodynamic therapy. This review discusses the latest results about the biosynthetic pathways and chemical synthetic routes to obtain hypericin. Although many analysis methods can be used for the determination of hypericin purity, HPLC has become the method of choice due to its fast and sensitive analyses. The extraction and purification of hypericin are also described. Hypericin can be used as a photosensitizer due to a large and active π-electron conjugated system in its structure. Medical applications of hypericin are not easy due to several unsolved practical problems, which include hypericin phototoxicity, poor solubility in water, and extreme sensitivity to light, heat, and pH.

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Hypericin; synthesis; extraction; photosensitivity; solubility; stability

INTRODUCTION

Hypericum or Saint John's wort, is one of the nine genera belonging to the *Clusiaceae Lindl* family widely spread throughout the world. A large number of *Hypericum* species, including *Hypericum perforatum* L., *Hypericum perfoliatum* L., *Hypericum ascyron* L., *Hypericum androsaemum* L., and *Hypericum chinense* L., have been identified in Europe, Asia, North Africa, and North America.[1] In China, there are 55 species of *Hypericum*, 18 of which have been used as local resources for medicinal purposes in traditional Chinese medicine.[2,3] The plants of the genus *Hypericum* contain numerous bioactive substances, such as naphthodianthrones, flavonoids, phloroglucinols, and polyphenols.[1,4] Naphthodianthrones are considered as characteristic constituents for the identification of *Hypericum* species[5] and one of the most important kinds of compounds, which includes hypericin and its biosynthetic precursors: protohypericin, pseudohypericin, and protopseudohypericin (Figure 1). Hypericin (4,5,7,4′,5′,7′-hexahydroxy-2,2′- dimethylnaphtodianthrone, $C_{30}H_{16}O_8$, m.w. 504) is a brownish-black powder with a unique bitter taste that is mainly found in *Hypericum* plants.[6] Bucher first discovered that hypericin was an active ingredient of *Hypericum perforatum*, and it was renamed hypericin by Cerny in 1911.[7]

Hypericin is one out of the most biologically active substances in the genus *Hypericum*,[1,4,8] and has drawn much interest in recent years. Evidence of antidepressant properties has been reported.[9] Hypericin was active against chronic unpredictable mild stress-induced depression and metabolic dysfunction by affecting excitatory amino acids and monoamine neurotransmitters.[10] It also exhibits antitumor activity as an antineoplastic and photocytotoxic agent, a property attributed to its photosensitivity.[11–14] Studies demonstrated that hypericin possesses immunomodulatory properties and can induce the production of interferon.[15,16] It was found to be particularly effective as an antiviral agent against the herpes virus,[17] infectious bronchitis virus,[18] hepatitis C virus,[19] human immunodeficiency virus,[20] and novel duck reovirus.[21] Finally, hypericin was considered as an antimicrobial agent, antioxidant, and as a promising candidate for photodynamic diagnosis.[22,23]

Recently, investigations on the pharmaceutical and clinical purposes of hypericin surged, and *Hypericum perforatum* as a source of hypericin has gradually become one of the three most popular Chinese herbal medicines.[24] The aim of this review is to describe the recent advances on hypericin research, focusing on biosynthesis, chemical synthesis, analysis, extraction, purification, photosensitivity, solubility in water and stability.

HYPERICIN SOURCES

Natural Sources

Hypericin as a natural bioactive compound can be obtained from plants, insects, and protozoa.[3] It is found in the integument of Australian Lac insects of the Coccoidea family,[25,26] and the blue-green ciliate, *Stentor coerulus*, which is a form of protozoa.[27] However, the *Hypericum* genus has spread throughout the temperate and tropical regions worldwide, and is therefore the leading natural source of hypericin. The genus contains 484 species divided into 36 subgroups.[28] The *Hypericum* genus has 30 species in Italy[29] and 89 in Turkey.[5,30] In China, the 55 various *Hypericum* species are widely spread across the country, but the main *Hypericum* containing areas are concentrated in southwest China. An early

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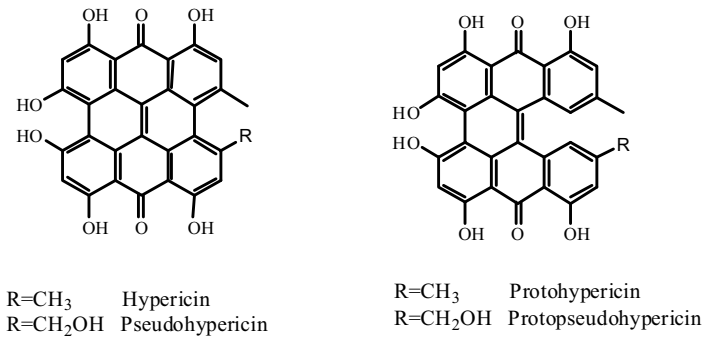

Figure 1. Chemical structures of hypericin, protohypericin, pseudohypericin, and protopseudohypericin.

survey of circa 200 species of *Hypericum* indicated that almost all hypericin-containing species belong to the sections *Euhypericum* and *Campylosporus* of Keller's classification.[31] The most important and well-known species is *Hypericum perforatum* which is commonly known as St. John's wort.[32] *Hypericum perforatum* is a perennial herbaceous plant widely distributed in the world and it has been included in numerous pharmacopeia. Hypericin is produced in specialized minute glands on all aerial parts of the plant, predominantly in flowers and leaves. The hypericin concentration varies depending on the species, the geographical locations of *Hypericum*,[1,8] and the part of the plant,[30] as shown in Table 1. Additionally, the developmental stage of the plant and seasonal variations also influences the hypericin concentration.[30,33] Although there are numerous other *Hypericum* species known to contain approximately similar amounts of hypericin as *Hypericum perforatum*,[34] information from the literature on these species is scarce.

Biosynthesis of Hypericin

The biosynthesis of hypericin in *Hypericum* is more complicated than known chemical synthetic routes and involves the

Table 1. The hypericin concentration in some species of the genus *Hypericum*.

Hypericum species	Provenance [ref]	Plant part	Hypericin ($mg \cdot g^{-1}$)
Hypericum perforatum	Italy (Sicily)[1]	Flowering tops 15–20 cm	3.69
	Italy (Latium)[9]	Flowering tops	0.27
	Italy (Trentino)[9]	Flowering tops	0.22
	Italy (Tuscany)[9]	Flowering tops	0.16
	Italy (Molise)[9]	Flowering tops	0.13
	Turkey (Samsun)[5]	Top 1/3 of the crown	2.82
	Japan (Tokyo)[4]	Flowering tops	1.20
	China (Hubei)[89]	Above the ground	1.50
	China (Guizhou)[90]	Above the ground	0.25
Hypericum avicularifolium	Turkey (Gumus)[5]	Top 1/3 of the crown	2.14
Hypericum aegypticum	Italy (Sicily)[1]	Flowering tops 15–20 cm	0.03
Hypericum enshiense	China (Hubei)[89]	Above the ground	3.00
Hypericum empetrifolium	Greece (Crete)[6]	Above the ground	0.09
Hypericum faberi	China (Guizhou)[90]	Above the ground	0.05
Hypericum hirsutum	Italy (Bulgaria)[6]	Flowers	0.43
	Italy (Sicily)[1]	Flowering tops 15–20 cm	0.15
	Italy (Siena)[9]	Flowering tops	0.02
	China (Xinjiang)[91]	Above the ground	0.06
	Serbia (Rudina Planina)[92]	Above the ground	0.024
Hypericum linarioides	Serbia (Rudina Planina)[92]	Above the ground	0.02
Hypericum lydium	Turkey (Havza)[5]	Top 1/3 of the crown	0.18
Hypericum maculatum	Serbia (Rudina Planina)[92]	Above the ground	0.03
Hypericum montanum	Italy (Sicily)[1]	Flowering tops 15–20 cm	1.42
Hypericum montbretii	Turkey (Samsun)[5]	Top 1/3 of the crown	1.39
Hypericum origanifolium	Turkey (Samsun)[5]	Top 1/3 of the crown	1.43
Hypericum patulum	Italy (Sicily)[1]	Flowering tops 15–20 cm	0.02
Hypericum perfoliatum	Turkey (Samsun)[30]	Top 1/3 of the crown, floral budding	1.06
	Turkey (Samsun)[30]	Top 1/3 of the crown, full flowering	0.96
	Turkey (Samsun)[30]	Top 1/3 of the crown, fresh fruiting	0.41
	Italy (Sicily)[1]	Flowering tops 15–20 cm	0.93
Hypericum pruinatum	Turkey (Cumus)[5]	The top 1/3 of the crown	0.79
Hypericum rumeliacum	Serbia (Rudina Planina)[92]	Above the ground	0.18
Hypericum sampsonii	China (Jiangxi)[90]	Flowering tops 20 cm	0.04
Hypericum scabrum	China (Xinjiang)[91]	Above the ground	0.06
Hypericum tetrapterum	Italy (Stia)[9]	Flowering tops	0.84
	Italy (Sicily)[8]	Flowering tops 15–20 cm	0.40
	Serbia (Rudina Planina)[92]	Above the ground	0.09
Hypericum wightianum	China (Guizhou)[90]	Above the ground	0.023

expression of multiple genes. The precise regulation of hypericin biosynthesis remains uncertain until today. The generally accepted biosynthetic hypericin pathway can be divided into two main parts: the formation of emodin anthrone and the conversion of emodin anthrone to hypericin (Figure 2).[35] Emodin anthrone is most likely the immediate precursor of hypericin. Early studies presumed that emodin anthrone synthesis followed the polyketide pathway.[36,37] The cyclization of a linear polyketide starting with the condensation of acetyl-CoA and malonyl-CoA results in the formation of emodin anthrone catalyzed by polyketide synthase (PKS). Two cDNAs encoding for PKS designated as HpPKS1 and HpPKS2 were cloned and identified from *Hypericum perforatum*.[38] Although the expression of HpPKS2 was correlated with the concentrations of hypericin, the recombinant HpPKS2 protein failed to convert the acetylated substrates to emodin or hypericin under *in vitro* conditions.[39] Pillai and Nair[40] provided direct biochemical and molecular evidence in support of the PKS hypothesis of hypericin biosynthesis in 2014. Auxin inducible culture systems of *Hypericum hookerianum* were applied as a model system to study the metabolic pathway of hypericin synthesis. The results demonstrated the presence of additional protein components besides PKS activity.

In later steps of hypericin biosynthesis, emodin anthrone is oxidized to emodin by emodin anthrone oxygenase.[35] Emodin dianthrone can be produced by a condensation reaction with emodin and emodin anthrone. This subsequently undergoes oxidation to form protohypericin, then protohypericin produces hypericin on irradiation. Bais, et al.[41] discovered that hypericin biosynthesis is related to a gene termed *hyp-1*. Based on an *in vitro* study, the phenolic oxidative coupling protein (Hyp-1) was shown to catalyze the dimerization of emodin and emodin anthrone, the dehydration of the intermediate to emodin dianthrone, and further phenolic oxidation to protohypericin and hypericin. A high-resolution crystal structure of the Hyp-1 protein indicated that it has a pathogenesis-related class 10 protein structure.[42] However, it is unable to dimerize emodin to hypericin using Hyp-1 protein as biocatalyst. Kosuth, et al.[43] also found that the *hyp-1* gene is not a limiting factor for hypericin biosynthesis. The low expression of the genes in the early stages of the hypericin biosynthetic route may be the potential key factors in the accumulation and biosynthesis of hypericin.[44] The role of the *hyp-1* gene should be further verified by functional validation experimental approaches. In addition, Kimakova et al.[45] identified new compounds present in the genus *Hypericum* and proposed that the anthraquinone skyrin is the key intermediate in hypericin biosynthesis. Further research on the role of anthraquinone derivatives in plant metabolism should be performed.

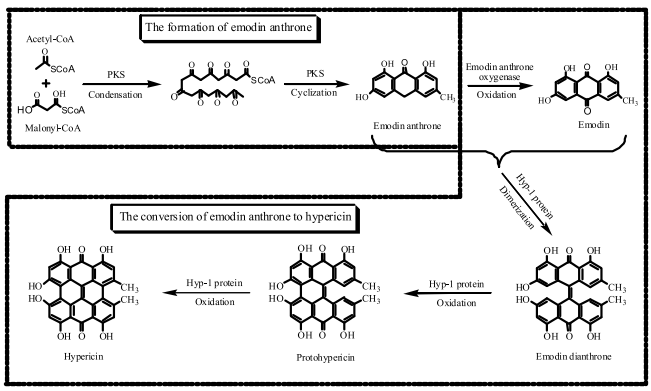

Figure 2. The proposed biosynthesis of hypericin.

Chemical Synthesis of Hypericin

In 1957, Brockmann, et al.[46] published the first multistep chemical synthesis method for the production of hypericin. This synthesis route starts with the reaction of 3,5-dimethoxybenzoic acid methyl ester and chloral hydrate. The procedure with 12 steps is complicated, and the overall yield is 6% - 9%. Such a low yield and complex synthetic route are no longer acceptable for the industrial production of hypericin, and new synthetic pathways have been studied to increase the synthesis yield and simplify the route. Hypericin can be obtained from 2-methyl anthraquinone in eight steps .[47] The key step in this process is to obtain emodin by steps such as nitration, reduction, bromination, deamination, and substitution. Emodin is condensed in the presence of hydroquinone under alkaline conditions to give protohypericin, which is subsequently photochemically converted to hypericin by irradiation with a halogen lamp. Although the synthetic route was optimized to perform under mild conditions, it involves multiple synthesis steps resulting in a low yield.

In 2007, Motoyoshiya, et al.[48] proposed a six-step method for synthesizing hypericin (Figure 3). The regioselective twofold Diels-Alder reaction of 1,4-benzoquinone with (1-methoxy-3- methylbuta-1,3-dienyloxy)trimethylsilane results in 7-methyljuglone in the first step. Emodin and its *O*-methylated derivative are subsequently produced from 7-methyljuglone and (1,3-dimethoxybuta- 1,3-dienyloxy)trimethylsilane. The reduction of both compounds with $SnCl_2$ in acidic media was accompanied by an acid hydrolysis that produced emodin anthrone, which after oxidative dimerization with $FeCl_3$ hydrate gave bianthrone in high yield. Bianthrone is oxidized by N-ethyldiisopropylamine (i-Pr_2EtN) to produce protohypericin, which is then converted to hypericin by irradiation. This shorter route realizes the straightforward synthesis of hypericin from a simple compound. However, the yield in the final step is low and should be improved.

In general, the synthesis of emodin is a necessary step in the synthesis of hypericin. The synthesis methods for emodin are

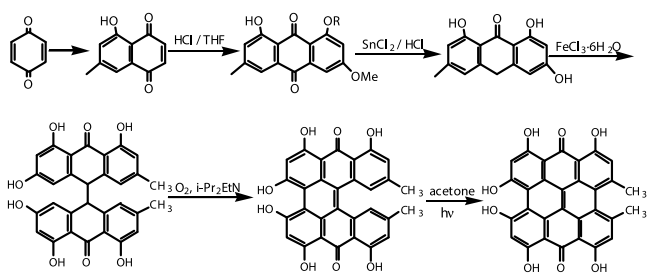

Figure 3. Synthesis of hypericin from 1,4-benzoquinone.

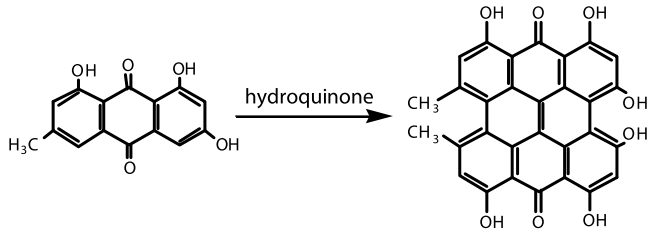

Figure 4. The direct synthesis of hypericin with emodin.

numerous and well optimized. Therefore, it has also been reported that emodin can be used as the starting material.[49,50] After interaction with $SnCl_2$, emodin is first converted into emodin anthrone. Then, emodin anthrone is used as an intermediate reactant. It is reacted with pyridine, piperidine, pyridine *N*-oxide and $FeSO_4 \cdot 7H_2O$ to form hypericin.[51] Some scholars have developed an even more direct method to synthesize hypericin. Emodin was converted to hypericin[52,53] using hydroquinone as catalyst under nitrogen and light illumination after 2 weeks (Figure 4). A large number of scientists have focused their attention on the synthesis of hypericin, and the synthesis technology of hypericin has gradually matured. However, the methods can still be improved further in terms of atom economy and environmental impact (E-factor).

DETERMINATION AND SEPARATION OF HYPERICIN

Analysis of Hypericin

Various hypericin determination methods have been developed including: ultraviolet-visible spectroscopy (UV-VIS), chemiluminescence-flow injection analysis (CL-FIA), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC).

UV-VIS spectroscopy was applied to determine hypericin and pseudohypericin in the extracts of *Hypericum perforatum*, and the solution of the compounds in methanol was measured at 588 nm.[54] The molar extinction coefficient was difficult to establish because none of the routine purity criteria can be applied to hypericin and pseudohypericin. However, specific reference spectra can successfully be used to analyze hypericin and pseudohypericin.

Shi[55] combined chemiluminescence and flow injection analysis to establish a CL-FIA method to detect the hypericin content in *Hypericum perforatum*. Hypericin has a sensitizing effect on the chemiluminescence intensity of the Luminol-$KMnO_4$ system in an alkaline medium. Under optimized experimental conditions, the hypericin mass concentration is linearly related to the luminescence intensity in the range of 1.9×10^{-5} - 3.8×10^{-4} g·L^{-1} with a limit of detection (LOD) of 3.8 μg·L^{-1}. The content of hypericin in *Hypericum perforatum* was detected as 0.492 mg·g^{-1}. The experimental results also indicated that CL-FIA and UV methods are equally sensitive. Moreover, CL-FIA shows some advantages in terms of larger linear range, higher sensitivity, and higher speed of analysis.

Mulinacci, et al.[56] established a TLC-densitometry method with fluorescence detection to detect the hypericin content in *Hypericum perforatum*, and this method was compared with reversed-phase HPLC-DAD (diode array detection). The mobile phase was optimized by adjusting the ratio of toluene, ethyl acetate, and formic acid. The TLC densitometry was performed without the use of spray or dipping reagents which improved the speed of the analytical procedure. The method is cost-effective because of the short analysis time and the low solvent consumption. The accuracy and reproducibility of TLC densitometry were comparable with those obtained by HPLC-DAD. However, HPLC-DAD does provide much more information than TLC densitometry.

Currently, various HPLC methods for hypericin analysis are gradually emerging. Wang, et al.[57] established an HPLC-visible spectroscopy method for hypericin determination in the extracts of *Hypericum perforatum*. The flow rate of mobile phase was 1.0 mL·min^{-1} (Table 2, A). The linear relationship of hypericin was good in the range of 6–36 mg·L^{-1} (r = 0.9996), the average recovery rate was 98.86% (n = 6), the relative standard deviation of the peak area was 3.15%. This HPLC detection method has a good reproducibility and accuracy and is suitable to determine hypericin in complex samples. In 2006, Ruckert, et al.[58] presented an HPLC method for the quantitation of hypericin using a new and sensitive amperometric detection. Using Ag/AgCl as a reference electrode in the detector, hypericin was eluted isocratically using a mobile phase consisting of ammonium acetate, methanol, and acetonitrile (Table 2, B). Hypericin eluted at a retention time of 12 min. Linearity was obtained over the range 0.035–1.30 mg·L^{-1} (r = 0.9994). The LOD of hypericin was 0.010 ng injected on-column. These parameters show that the method is selective, simple, rapid, and accurate. Zhang, et al.[59] established a high-resolution HPLC method for determining hypericin by comparing different chromatographic conditions. The different methods showed different detection efficiencies depending on the mobile phase and detection wavelength. A purchased crude extract of *Hypericum perforatum* was applied to optimize the chromatographic conditions for the determination of hypericin in a complex sample. The results demonstrated that the best resolution was obtained at 590 nm and with the mobile phase composition: methanol/acetonitrile/0.1 mol·L^{-1} sodium dihydrogen phosphate 200/300/100 v/v/v (Table 2, C). The hypericin calibration curve showed a good linearity in the range of 4–14 mg·L^{-1} (r = 0.9986), and the established method is fast and easy to use.[59]

In addition, the stationary phase in the column has a significant effect on the resolution of HPLC. Dolezal, et al.[60] selected four stationary phases modified by the pentafluorophenyl group to investigate the contribution of π-π interactions to the improvement of hypericin separation in comparison to the separation obtained with a conventional C18 reversed-phase column. The best analytical method employing the pentafluorophenyl stationary phase (Table 2, E) showed sufficient linearity, accuracy, and precision and was used for the determination of hypericin in *Hypericum perforatum*. Besides the HPLC methods discussed above, other reported HPLC methods with different mobile and stationary phases are presented in Table 2 (F-I). For routine hypericin analyzes, UV detection at 278 and 284 nm, and VIS detection at 579–590 nm, have been proposed. However, the VIS detection has a better sensitivity and selectivity than the UV detection.[58,59,61] HPLC

Table 2. Different chromatographic conditions for determining hypericin.

Methods [ref]	Mobile phase v:v:v	Stationary phase	Temperature (°C)	Detection wavelength (nm)
A[57]	Acetonitrile, 0.02 M sodium dihydrogen phosphate, volume ratio of 85: 15 v:v	ODS-A	25	588
B[58]	Ammonium acetate, methanol and acetonitrile, volume ratio of 10: 40: 50	LiChroCart Purospher RP18e	22	254
C[59]	Methanol, acetonitrile, sodium dihydrogen phosphate solution, volume ratio of 200: 300: 100 v:v:v	Kromasil C18	40	590
D[59]	Methanol, acetonitrile, sodium dihydrogen phosphate solution, volume ratio of 200: 300: 100 v:v:v	Kromasil C18	40	284
E[60]	Solvent A: H_2O, 0.1 M acetic acid, 0.1 M trimethylamine, solvent B: acetonitrile, 0.1 M acetic acid, 0.1 M trimethylamine 0–1 min (A: B = 15: 85), 1–8 min (A: B = 0: 100), 8.0–10.0 min (A: B = 15: 85).	Pentafluorophenyl	25	278
F[93]	Methanol, ethyl acetate, 0.1 M Na_2PO_4, volume ratio of 72: 23: 5 v:v:v	Ultracarb 7 ODS	23	579
G[94]	Ethylacetate, 15.6 g L^{-1} sodium dihydrogen phosphate (pH = 2 with phosphoric acid) and methanol, volume ratio of 39: 41: 160 v:v:v	ACE C18	40	590
H[95]	Methanol, acetonitrile, water and 3% aqueous phosphoric acid, volume ratio of 45: 50: 4.5: 0.5 with triethylamine adjusted to pH = 6	Supelcosil ODS	25	590
I[96]	Methanol, ethyl acetate and phosphate buffer (pH = 2, 0.1 M), volume ratio of 60: 20: 20 v:v:v	VP-ODS C18	25	590

analyzes with various detection modes will replace the other method for hypericin analysis because it is fast, accurate, and sensitive.[58,60]

Separation of Hypericin

The hypericin content in *Hypericum* is extremely low, in most cases below 3 mg·per gram of dry weight of plant material. Therefore, effective hypericin extraction and purification methods are needed. The most common method is solvent extraction using methanol, ethanol, and polar alcohols. In addition, microwave-assisted extraction has been used to reduce the extraction time and enzyme-assisted extraction to increase the extraction yield. The lye (sodium hydroxide) extraction method has been rarely used because of its higher energy consumption and longer extraction time. Separation and purification of hypericin has been performed by using macroporous resin column chromatography (MRCC) and molecular imprinting techniques. Additionally, counter-current chromatography (CCC) has been used widely due to its high purification rate.

Extraction of Hypericin

Cossuta et al.[62] used Soxhlet extraction to extract hypericin from *Hypericum perforatum* with four different solvents (*n*-hexane, ethyl acetate, 2-propanol, and ethanol), and the extracts were analyzed by UV-HPLC. Ethanol was the best solvent to extract hypericin producing a maximum of 0.060 mg·g^{-1}. However, the 16 h Soxhlet extraction time was discouraging needing to be optimized.

Xing[63] took a two-step impurity removal method to extract hypericin. Ether was first used to remove apolar impurities including chlorophyll, and water-soluble impurities including sugars were removed by suspension in warm water. After that, ethyl acetate was utilized to extract hypericin. Using 40% and 80% ethanol as eluents, respectively, the extracts were separated by MRCC. The red MRCC effluent was concentrated under reduced pressure to obtain a hypericin paste. Finally, the content of hypericin in the paste, determined by UV-HPLC, was 1.2%, which far exceeds the 0.3% hypericin content required for the international market of medicinal products. This hypericin optimized extraction method has a high extraction rate and is simple and effective. Moreover, the MRCC product has the characteristics of low hygroscopicity and low tackiness.

Punegov, et al.[64] studied the extraction of hypericin and pseudohypericin from raw *Hypericum perforatum* using microwave activation. The maximal extraction efficiency was achieved when 55% ethanol or isopropanol was used as extractant at 0.0205 W·cm^{-3} microwave irradiation power density and a microwave frequency of 2450 MHz for 60 s. Microwave activation was found to improve 10-fold the extraction efficiency, reducing the time necessary to fully extract hypericin and pseudohypericin compared to classical extraction methods. Zhang, Feng, Xu, Tan, Hagedoorn, and Ding[59] used a xylanase-assisted associated with a microwave-assisted extraction to improve the hypericin extraction. This method was found to improve the extraction yield of hypericin significantly compared to unassisted extraction. Microwave-assisted extraction after xylanase-assisted extraction was found to be the most efficient strategy for extracting hypericin. The yield was 0.32 ± 0.006 mg·g^{-1}, which was a 210% increase over unassisted extraction. Compared to conventional solvent extraction, enzyme-assisted extraction can be accomplished using a low enzyme concentration. It reduces energy consumption due to less wastewater generation and lower temperatures. In addition, the unique microwave heating method decreases the extraction time and increases the yield even further. In conclusion, enzyme and microwave assistance are effective strategies to improve the mass transfer rate of hypericin during the extraction process. Scalability of the equipment has to be addressed in order to use microwave assistance on an industrial scale.

Purification of Hypericin

The purity of hypericin in the extracts is not sufficient to meet the requirements for pharmaceutical products. Further research on how to purify hypericin is necessary. Xue, et al.[65] selected six macroporous adsorption resins (D101, AB-8, HZ-801, HZ-818, HZ-806, X-5) with different specific surface areas and pore sizes to separate hypericin. The crude extracts of *Hypericum perforatum* were used to investigate the hypericin separation and purification performance of six resins. Optimal conditions were determined using a medium operating

pressure separation method. The results showed that HZ-801 resin is the preferred separation material with a high adsorption rate and a high elution rate, and the purity of hypericin after elution with ethanol solution was 79%. Hypericin separation using MRCC is feasible on industrial scale due to its easy operation and recyclability. However, contaminating substances which are similar in structure to hypericin affect the results of hypericin separation due to the physical adsorption principle of the resins.

Molecular imprinting is a newly established method for chemical separation and purification in recent years. The synthetic molecularly imprinted polymer used for separation shows high selectivity and specificity for the template (target) molecule. A core-shell structure molecularly imprinted magnetic nanospheres of hypericin (Fe_3O_4@MIPs) were prepared by mercapto-alkyne click polymerization.[66] The Fe_3O_4@MIPs showed a good adsorption capacity of 3.43 $mg{\cdot}g^{-1}$, high fast mass transfer rates, and good reusability. Hypericin, acrylamide, and pentaerythritol triacrylate were used as a template molecule, functional monomer, and molecular imprint pre-assembly cross-linker, respectively.[67] A cooperative hydrogen-bonding complex between hypericin and acrylamide was formed at the ratio of 1:6 in the prepolymerized system. A high recovery of 82.3% was achieved by molecular-imprinted polymers to extract hypericin from *Hypericum perforatum* extracts. Molecular imprinting is simple, rapid, accurate, and reliable. The main disadvantage is the amount of pure hypericin which has to be used to prepare the molecular-imprinted polymers.

Cao, et al.[68] found that CCC combined with pre-separation by ultrasonic solvent extraction was successful for the separation of series of bioactive compounds from the crude extracts of *Hypericum perforatum*. The ethyl acetate extract was separated by using the solvent system hexane-ethylacetate- methanol-water (1:1: 1:1 and 1:3: 1:3) in gradient through both reverse phase and normal phase elution mode. The hypericin purity was determined to be 95% by HPLC-DAD. CCC does not require a solid carrier, and the hypericin obtained in less than 5 hours by this method has high purity. However, CCC is difficult to realize on an industrial scale and it is not environmentally friendly due to the amount of organic solvents used.

The extraction and purification technologies that have been developed provide an indispensable foundation to prepare hypericin products with different purities. Further studies are required to achieve a method that combines low cost and a green process. Furthermore, preparative liquid chromatography is a feasible technology to prepare a high purity product, can be utilized to separate hypericin.[69–71] This technology is also amenable to industrial scale-up.

PROPERTIES OF HYPERICIN

Photosensitivity

Hypericin is one of the most effective natural photosensitizers, and shows a good photosensitivity due to the extensive electron-conjugated system in its structure (Figure 1). Hypericin is extremely sensitive to light and it is photoactivated to produce peroxides. The photosensitive mechanism of hypericin has been described as follows: in the photodynamic action, light quanta are absorbed by the sensitizer, generating the excited singlet state. This excited singlet state may undergo intersystem crossing to the triplet excited state. The triplet sensitizer will excite singlet oxygen that is produced when the energy is transferred to ground state triplet oxygen, and the singlet oxygen forms a peroxide subsequently.[72] Moreover, it has been confirmed that the photosensitivity of hypericin induces cell apoptosis and inhibits the growth of cancer cells. This optical activity of hypericin has been widely used in optical diagnostics.[73]

Hypericin can produce superoxide-free radicals under the irradiation of visible light and in the presence of oxygen.[74] Hypericin shows an electron paramagnetic resonance (EPR) signal caused by a semiquinone-like radical formed by intermolecular electron transfer in the absence of light and electron donors. The amplitude of the radical EPR signal for the water-dispersed lysine salt of hypericin is significantly increased under visible light irradiation. This finding indicates that the free hypericin radicals and the superoxide radicals are formed during light irradiation, and may also be implicated in the biological activities.

The photosensitivity of hypericin is commonly used in photodynamic therapy, providing an effective treatment of cancer. Rabbits and mice xenografted with P3 human squamous cell carcinoma were used to assess the usefulness of hypericin for laser photoinactivation of solid tumors.[75] The tissue uptake and distribution of hypericin in rabbits and mice were measured. The degree of absorption of hypericin by intravenous injection at 4 and 24 hours in both animal tissues was determined by ethanol extraction and quantitative fluorescence spectrophotometry. Experimental results show that elimination of hypericin was rapid in most animal organs with residual hypericin under 10% of the maximum after 7 days. The retention rate of squamous cell tumors is only 25% to 30%. It indicated that photodynamic therapy using hypericin can, to a certain extent, eliminate some cancer cells.[76] In addition, hypericin and laser irradiation induced cell death mediated by the intracellular reactive oxygen species and mitochondrial damage. These data demonstrate that hypericin is an effective photosensitizer with potential for human cancer therapy.

Hypericin can absorb light in the ultraviolet and visible range. Although hypericin has potential for cancer treatment, some studies have shown that hypericin is phototoxic to the skin and human eye. Ingestion of hypericin containing drugs is potentially phototoxic to the retina, which may lead to retinal or early macular degeneration.[77] In addition, hypericin is not cytotoxic in the dark.[78] Further research into the practical application of hypericin in photodynamic therapy should be performed.

Water Solubility of Hypericin

Hypericin is readily dissolved in dimethylsulfoxide, methanol, ethanol, and alkaline aqueous solution, and it is red-colored at pH <11.5 and green-colored above pH 11.5.[79] However, it is a serious drawback that hypericin exhibits low level of solubility in neutral water because of its hydrophobicity.[80,81] Hypericin forms nonsoluble aggregates in an aqueous environment.

Fluorescence spectroscopy and diffusion coefficient measurements were used to investigate the self-association of hypericin molecules in DMSO/water mixtures.[82] Fluorescence measurements revealed that hypericin remained in its monomeric form in DMSO/water mixtures containing up to 20% - 30% water. As the proportion of water passes 30%, hypericin gradually formed non-fluorescent aggregates, and the size of the aggregates increased with increasing water concentrations. Hypericin presumably produces large molecular weight stacked aggregates in a neutral aqueous environment. In addition, molecules of hypericin remain in the monomeric state in an aqueous environment at alkaline pH.

The insoluble hypericin aggregates in aqueous solutions do not possess biological activity. This characteristic restricts hypericin applications in medicine. Polymeric micelles made with polyethylene glycol (PEG) have been utilized to improve the solubility of hypericin.[83] PEGs with low molecular weight (<1000 $g{\cdot}mol^{-1}$) did not significantly contribute to the hypericin solubilization. However, PEGs with molecular weight >2000 $g{\cdot}mol^{-1}$ efficiently transformed hypericin aggregates to the monomeric state. The solubility of hypericin in water increased significantly by adding cromolyn disodium salt (DSCG).[81] The monomerization of hypericin under these conditions can be explained as a result of the hydrotropic effect of DSCG. This hydrotropic effect is most likely a result of interactions between the two relative rigid aromatic rings of DSCG and a delocalized charge on the surface of the hypericin molecule. Kubin, et al.[84] prepared a non-covalently bound hypericin-polyvinylpyrrolidone (PVP) complex to enhance the water solubility of hypericin. The hypericin-PVP complex bound more than 1000 mg of hypericin in presence of 100 g PVP and the resulting complex was soluble in 1 L of pure water. The proposed methods provide strategies to improve the solubility of hypericin in water, which will facilitate medical applications. The effects of these additives on the biological activities of the resulting hypericin preparations have to be investigated.

Hypericin Stability

Due to its photosensitivity, light inevitably affects the stability of hypericin. Therefore, exposure to light has a significant impact on the biological activities of hypericin. The stability of hypericin in extract solutions of *Hypericum perforatum* and standard solutions has been evaluated under different light conditions monitored by HPLC-VIS.[85] Hypericin was extremely unstable after exposure to light, and light was the main factor reducing the effective hypericin concentration. Additionally, temperature was a factor affecting hypericin stability. Wang, et al.[86] investigated the effects of light and temperature on the long-term stability of hypericin extracts. After 8 weeks, the content of hypericin in the extracts decreased by 49% under constant light at room temperature, and it declined by only 8.5% in the dark. The hypericin content of the extracts was unchanged under dark and low temperature (−24°C) conditions. Long-term storage is possible for hypericin dissolved in a polar solvent, under a nitrogen atmosphere at freezing temperature (<-30°C).[87] Wang and Zhang[88] studied the effects of visible light, temperature, pH, Na_2SO_3 and ascorbic acid on the stability of hypericin by UV-VIS spectroscopy. The results indicated that light is the major factor influencing the stability of hypericin. Light and temperature were found to have a greater effect on stability under alkaline conditions than acidic conditions. Therefore, alkaline solvents should be avoided during the extraction of hypericin. However, the stability of hypericin improved when ascorbic acid or Na_2SO_3 was added. The instability of hypericin has always been a major challenge in the separation and purification process.

CONCLUSIONS

Hypericin is one of the effective bioactive substances primarily extracted from the *Hypericum* plants, and has various pharmacological activities such as anti-depressive, anti-tumor, and anti-viral. The biosynthetic pathways in the plants are known, but the precise regulation of these pathways remains uncertain. Chemical synthesis routes for hypericin from different starting compounds have been developed. However, a novel synthesis route combining a high overall yield, low cost, and less environmental pollution is still desired. HPLC is widely used for hypericin analysis, and it may replace all other analytical methods due to its fast analysis times and high sensitivity. Extraction of hypericin from *Hypericum* can provide low purity products or extracts. Microwave-assisted extraction and enzyme-assisted extraction contribute to a higher hypericin yield. Macroporous adsorption resin, molecular imprinting techniques, CCC, and preparative liquid chromatography systems were used to prepare high purity hypericin. Especially, preparative liquid chromatography system is a feasible strategy to realize the industrial production of high purity hypericin.

Hypericin is photosensitive due to its extensive system of conjugated C = C double bonds. It can be utilized for photodynamic therapy. However, the phototoxicity of hypericin to the skin and human lens should be considered. Hydrophobic groups in hypericin account for its low solubility in water. PEG, DSCG, and PVP can significantly improve the solubility. In addition, the storage and operation conditions, such as light, temperature, and pH will affect the hypericin stability. Without a doubt, the disadvantages of hypericin can be overcome with technical solutions, which are worthwhile of investigation because its great medicinal value.

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