

Review

Hypericum spp.—An Overview of the Extraction Methods and Analysis of Compounds

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Abstract: The *Hypericum* genus contains one of the few genera of flowering plants that contains a species with authorization for marketing as a traditional medicine, *H. perforatum*. Due to the fact that this is a large genus, comprising numerous species, a large amount of interest has been shown over the years in the study of its various pharmacological activities. The chemical composition of these species is quite similar, containing compounds belonging to the class of phloroglucinol derivatives, naphthodianthrones, phenols, flavonoids and essential oils. Taking all of this into consideration, the present study aims to offer an overview of the species of the genus from the point of view of their extraction techniques and analysis methods. An extensive study on the scientific literature was performed, and it revealed a wide range of solvents and extraction methods, among which ethanol and methanol, together with maceration and ultrasonication, are the most frequent. Regarding analysis methods, separation and spectral techniques are the most employed. Therefore, the present study provides necessary data for future studies on the species of the genus, offering a complete overview and a possible basis for their development.

Keywords: *Hypericum* spp.; solvents; extraction methods; separation methods; spectral methods



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1. Introduction

The genus *Hypericum*, belonging to the Hypericaceae family, comprises more than 500 species which are included in 36 sections [1–4]. These species can be found naturally in different areas, especially in the temperate climate, being absent in extreme environmental conditions [3,5,6]. They can be found as small shrubs and herbs, having opposite leaves, with transparent or black points, yellow flowers arranged in cymose inflorescences, with five sepals and five petals, numerous stamens, and an ovary with one to five ovarian cavities [7].

Despite the fact that there is a large number of *Hypericum* species, only *H. perforatum* L. (St. John's wort) has been intensively investigated, both chemically and pharmacologically, being considered as a model species for the genus [2,4,5]. The species has a monograph in the European Pharmacopoeia [8] and in the European Medicines Agency monographs [9], and it has been extensively studied over the years for its pharmacological activities [5]. The vegetal medicinal product consists of the aerial dried parts, harvested during the flowering period [2,10]. The chemical composition of different *Hypericum* species may be variable depending on pedoclimatic conditions [10].

Hypericum species represent widely known traditional healing agents, used for the treatment of numerous pathologies [5]. Several species are used for different dermatological issues such as burns or injuries, or for their antidepressant, antimicrobial or anti-

inflammatory properties [4,6]. Compounds that are responsible for the biological activities belong to the class of phloroglucinol derivatives (e.g., hyperforins), naphthodianthrone (e.g., hypericins), phenols (e.g., chlorogenic acid), flavonoids (e.g., hyperoside, rutoside, quercetin) and essential oils [3,4,10,11]. In addition, tannins, procyanidins, phenylpropanes, xanthenes and volatile compounds have been identified [11]. The chemical composition of the *Hypericum* genus can nevertheless be very different between species [4].

The officinal species, *H. perforatum*, has been used for centuries, being one of the most popular herbal remedies in traditional medicine. It has been used in different forms (e.g., ointments, tinctures, drops) [11] for the treatment of ulcers and other gastrointestinal disorders, diabetes mellitus, cold and flu, jaundice and other hepatic and biliary disorders, hemoptysis, hematemesis, metrorrhagia, traumatic hemorrhage, irregular menstruation, acute mastitis, swelling and pain of the eye, urinary tract infection, sore furuncle, burns, wounds and bruises and rheumatic arthritis [12,13]. Based on these traditional uses, among the most studied uses are the wound healing and anti-inflammatory activities related to skin conditions, which can be directly linked to antioxidant and anti-aging properties [12,14]. Other reported activities are the antibacterial [15], antimutagenic, antiviral, photodynamic, antitumor, antioxidant and analgesic effects [5,12,13,16]. It is especially known for its antidepressant activity [3,11]; mechanisms that are involved in this activity are related to the inhibition of monoamine oxidase, the inhibition of synaptosomal reuptake of amines, the effects on monoamine transporters and the effects on serotonin receptors, which are due to hypericin, hyperforin or to the polycyclic polyphenylated acylphloroglucinols [17,18]. Due to its therapeutic properties, the species and its derived pharmaceutical products are currently among the most used and consumed vegetal products in the world [10,11]. It is the only species of the genus that has marketing authorization as a traditional medicine in several European countries [19]. There are numerous phytotherapeutic products containing the aerial part of the species or its dry extracts [8,9] that are available on the market all over the world, some of them being protected by patents, such as Prosoft® 300 mg (from R&D Pharma company, Monaco, Europe), which is indicated for the treatment of moderate depression, Hyperoil® (from R.I.M.O.S. srl. Company, Mirandola, Italy), which is available in Europe and promotes wound healing of chronic and acute lesions, the Wound and 1-Primary Wound Dressing, indicated for the treatment of skin defects associated with skin disorders or some cosmetic formulations (from the Amore Pacific Corp., Seoul, South Korea) indicated as anti-aging agents or for skin exfoliation, maintenance of the barrier function of the skin, inhibition of inflammatory mediators, wound healing, protection against UV, epidermal regeneration and inhibition of tyrosinase, collagenase or elastase (e.g., Crodarom® ST John's Wort O produced by Crodarom S.A.S./CRODA, Chanac, France) [3,12].

The current taxonomical classification for the *Hypericum* genus is performed by comparing the morphology of flowers and other vegetative organs, but pedoclimatic conditions may lead to incorrect identification [1]. In order to establish the identity of these species and for avoiding uncertainties, it appears absolutely important to complement these at least with phytochemical analysis and sometimes even with molecular data [2]. Another reason for the correct analysis of herbal products belonging to the *Hypericum* genus is related to the establishment of their safety and toxicity. The vast majority of the existing studies concerning the toxicity of extracts or isolated compounds from these species are related to cell viability determination after exposure, and report significantly deficient data, due to the lack of relevant pharmacological or toxicological approaches [12]. However, *Hypericum* extracts' and phytotherapeutic remedies' side effects are considered to be mild and rare, when they are consumed in moderation [8,9]. Nevertheless, further toxicity studies are needed in order to establish the effects of administration or application of *Hypericum* vegetal products or extracts. Only the officinal species, *H. perforatum*, has been the subject of such toxicity studies to determine its safe use as vegetal medicinal product for different purposes [9,12], while the toxicological approaches of other species are yet to be studied and determined. Their lack in scientific literature represents an important matter to be considered in future studies of these species [12]. An important point regarding

the toxicity and pharmacological effects of *H. perforatum* involve induction of enzymes by its extracts. Plant–drug interactions or extract–drug interactions are highly reported in the literature. The molecules responsible for this kind of interactions are hyperforins and hypericins. Hyperforins have proven to be potent cytochrome (CYP) enzyme (members of the microsomal family) inducers, especially cytochrome P 450 and even P-glycoprotein inducers. Since many available drugs such as anticoagulants, anti-HIV drugs, antidepressants, antimicrobial drugs, anticonvulsants or oral contraceptives are substrates for CYP enzymes, the inductive effect may trigger the loss of the therapeutic effect of several active pharmaceutical ingredients [13].

Taking all of this into consideration, *Hypericum* spp. appear as important vegetal medicinal products and have significant potential for further studies that may bring evidence to support their traditional uses or may offer supplementary details on different biological activities and their correspondent mechanisms of action, which are studied but still need more scientific arguments. The high complexity of the genus is a strong reason for the lack of reviews. Existing studies offer specific evaluations on different aspects related to the genus, such as their cosmeceutical applications [12], their specific classes of compounds (e.g., flavonoids) [4], their genetic phytochemical profiling [2] or they offer an overview of their ethnobotany, phytochemical composition and pharmacological properties [6]. As the species of the genus represent important vegetal medicinal products, the interest towards their study is growing, especially as it presents a large number of species that may present similar activities to the officinal species [2,6]. To the best of our knowledge, there are no studies examining the methods of extraction and analysis of phytochemical compounds, which may represent the basis of developing future possible studies on the species of the genus.

Therefore, the main aim of the present study is to provide an overview of the main extraction and analysis methods used over the last decade for biologically active compounds of high interest from different species belonging to the genus *Hypericum*. Secondly, this study was also designed to be an important tool and to represent a starting point for researchers that wish to undertake further, new studies that may be focused on: extraction processes' optimizations; development, improvement and standardization of analytical methods; characterization of new or unstudied species; studies regarding the influence of external or internal factors on the composition of the secondary metabolic compounds. Thirdly, the study provides a synthesis of relevant information for the aforementioned aims, both in the form of an overview and also under a more specific and exhaustive form using Supplementary Materials, taking into consideration the relatively increased number of articles reviewed. In this way, by offering an evaluation and a summarization of these aspects, we may provide the necessary scientific arguments and the background for increasing the existing scientific information on these species. Finally, the novelty and originality of the present study rely on the fact that it represents one of the very few approaches in the literature capable to integrate relatively high quantity and updated data related to the extraction methods and analytical methods used for analysis of compounds derived from *Hypericum* genus, all due to the increased number of articles and the heterogeneity of the data.

2. Materials and Methods

2.1. Data Obtention

In order to construct the overview of the main extraction techniques and the main methods used for the analysis of compounds of *Hypericum* genus, a survey of the scientific databases (PubMed, Science Direct, Google Scholar, Web of Science) was performed. A period of search was established (2010–2021) and the keywords “*Hypericum*” and “*Hypericum* genus” were used. The keyword “*Hypericum*” returned more than 8000 results, while “*Hypericum* genus” more than 2000, “*Hypericum* extraction” more than 2500 and “*Hypericum* analysis” more than 6000. Narrowing the research for the selected interval resulted in 4500, 1200, 1600 and 3800 results. Among all these, only 102 were considered to be relevant for

the purpose of the study and analyzed further in order to construct the following sections of the present review.

2.2. Data Analysis

After obtaining the results of the performed searches, the articles were sorted by year and subject. Selection was evaluated after selection based on their content, using the following criteria:

- Methods of extraction;
- Analysis methods.

Articles discussing these aspects for the officinal species were taken into consideration, as it represents the model species. Regarding other *Hypericum* species, articles were taken into consideration if they evaluated at least two different species. For articles discussing individual species, only articles that were relevant in their content were considered.

3. Results and Discussion

3.1. Extraction Methods

Due to the fact that the chemical composition of *Hypericum* species is large and may be included in a wide range of compounds, it appears to be very difficult to obtain their profiling only by using a single extraction method and a single solvent [4]. The extraction of compounds is very different, depending on the extraction conditions (temperature, solvent concentration, duration), but also on the method. All these may impact the bioactive properties of the obtained extract [10]. Moreover, the content of bioactive compounds may also vary depending on the vegetal product (flowers, leaves, aerial parts), but also on the pedoclimatic conditions [4,10].

3.1.1. Extraction Solvents

The most frequently used solvents were pure ethanol and methanol, but it appeared that their mixing with water may be even more efficient [4]. Different extraction solvents together with compounds specific for these solvents can be found in Table 1.

Table 1. Different solvents used for extraction of compounds in the composition of *Hypericum* spp.

Vegetal Material	Solvent	Compounds	Reference
<i>H. perforatum</i> aerial parts	50% Aqueous ethanol	Quercetin derivatives (rutin, hyperoside, isoquercitrin, quercetrin), protocathechuic acid, (-)-epicatechin, I3, I18-biapigenin and chlorogenic acid derivatives, naphthodianthrones and phloroglucinols (hyperforin, adhyperforin, hypericin, pseudohypericin and furohyperforin)	[20]
<i>H. perforatum</i> , <i>H. annulatum</i> aerial parts	60% Aqueous ethanol	Hypericin, epicatechin, and rutin	[4]
<i>H. perforatum</i> leaves	80% Aqueous ethanol	Polysaccharides, hypericin, hyperforin, hyperoside, quercetin, quercitrin, rutin	[21,22]
<i>H. przewalskii</i> aerial parts	95% Aqueous ethanol, followed by partitioning between CH ₂ Cl ₂ , ethyl acetate and n-butanol	(±)-hyperzewalsins A-D	[23]
<i>H. perforatum</i> aerial parts	95% Aqueous ethanol, followed by partitioning between CH ₂ Cl ₂ , petroleum ether and acetone	Hyperforatins L-U	[24]
<i>H. perforatum</i> aerial parts	95% Aqueous ethanol, %, followed by partitioning between CH ₂ Cl ₂ , petroleum ether and acetone	Polycyclic polyphenylated acylphloroglucinols	[25]
<i>H. perforatum</i> aerial parts/ <i>Hypericum</i> spp. aerial parts	96% Aqueous ethanol	Flavonoids and polyphenols: chlorogenic acid, rutin, hyperoside, quercetin, quercitrin	[10,26,27]

Table 1. Cont.

Vegetal Material	Solvent	Compounds	Reference
<i>Hypericum</i> spp. aerial parts/flowers	Ethanol 100%	Naphthodianthrone, acylphloroglucinols, polyphenols and flavonoids: ex. hypericins (hypericin, pseudohypericin, protohypericin and protopseudohypericin), their presumed precursors (emodin and skyrin) and new skyrin derivatives (oxyskyrin, iridoskyrin, rubroskyrin and luteoskyrin)	[15,28–34]
<i>H. perforatum</i> aerial parts	Ethyl acetate	Polyphenols, flavonoids	[28]
<i>H. perforatum</i> aerial parts	Ethanol 100% followed by partitioning in a n-hexane-diethyl-ether gradient	Prenylated β -diketones: 2,6,9-trimethyl-8-decane-3,5-dione, 3,7,10-trimethyl-9-undecene-4,6-dione	[35]
<i>H. foliosum</i> , <i>H. androsaemum</i> and <i>H. perforatum</i> aerial parts	Methanol 100%, followed by partitioning in diethylether and methanol	Quinic acid 3,4-dimethylbenzoic acid, (+) catechin, chlorogenic acid, quercetin-3-O-sulphate, miquelianin, amentoflavone	[36]
<i>H. perforatum</i> aerial parts/ <i>Hypericum</i> spp. leaves and stems	70% Aqueous methanol	Phenolic acids (chlorogenic, caffeic, ferulic, cinnamic and gallic acid) and flavonoids (naringenin, apigenin, amentoflavone, kaempferol, kaempferol-3-O-glucoside, catechin, quercetin, rutin, hyperoside, quercitrin and isoquercetin)	[2,37,38]
<i>H. perforatum</i> and <i>H. annulatum</i> aerial parts	80% Aqueous methanol	Chlorogenic acid, hyperoside, rutin, quercitroside, quercetin, epicatechin, hyperforin, hypericin	[4,39]
<i>Hypericum</i> spp. aerial parts	Methanol 100%	Hypericin, epicatechin, and rutin Hypericin, pseudohypericin, hyperforin, adhyperforin, chlorogenic acid, neochlorogenic acid, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (-)-epicatechin, p-coumaric acid, gallic acid, quinic acid, chlorogenic acid, malic acid, protocatechuic acid, rutin, quercitrin, and isoquercitrin	[5,11,14,16,18,40–48]
<i>Hypericum</i> spp. aerial parts	Methanol—acetone 1:1	Chlorogenic acid, hyperoside, isoquercitrin, rutin, isoquercitrin, quercitrin, hypericin, pseudohypericin, hyperforin and quercetin	[19]
<i>H. perforatum</i> aerial parts	Ethanol—methanol 50:50, 30:70, and 70:30	Hypericin	[49]
<i>H. perforatum</i> aerial parts	Methanol 100%, followed by partitioning with petroleum ether-ethyl acetate	Hyperfols C-H	[50]
<i>H. hircinum</i> , <i>H. perforatum</i> , <i>H. scruglii</i> aerial parts	70% Aqueous methanol, MeOH-phosphate buffer, water	Shikimic acid, chlorogenic acid, quercetin and quercetin-7-O-glucoside, hyperoside, quercitrin, 3-geranyl-1-(2'-methylbutanoyl)-phloroglucinol, 3-geranyl-1-(2'-methylpropanoyl)-phloroglucinol	[51]
<i>Hypericum</i> spp. leaves and stems	Methanol: ethanol: acetone 1:1:1 (v/v/v)	Anthraquinones and phloroglucinols	[2,37,52,53]
<i>H. calycinum</i> cell suspension culture	Methanol 100%, followed by ethyl acetate	Xanthones	[54]
<i>H. perforatum</i> aerial parts	Water	Flavonoids, hypericin, pseudohypericin	[55,56]
<i>H. perforatum</i> aerial parts	10% Aqueous glycerol	Caffeoyl- and p-coumaroyl-quinic acid derivatives and quercetin glycosides	[57]
<i>Hypericum</i> spp. aerial parts	n-hexane	Phenolic compounds: uliginosin B, japonicin A, hyperbrasilol B, benzopyrans, cariphenone A, cariphenone B	[58–61]
<i>H. perforatum</i> adventitious roots from auxin-induced root cultures	Petroleum ether	Hyperforins	[62]

Table 1. Cont.

Vegetal Material	Solvent	Compounds	Reference
<i>H. perforatum</i> aerial parts	Fatty oils: medium-chain triglycerides, <i>Arachis</i> oil refined, soybean oil refined, almond oil, sunflower oil refined, sunflower oil refined, maize germ oil refined, macadamia nut oil refined, olive oil, sesame oil refined, <i>Simmondsia chinensis</i> seed oil	Quercetin, kaempferol, biapigenin, hyperforin, adhyperforin	[63]
<i>H. perforatum</i> aerial parts	Olive oil, followed by DMSO (dimethyl sulfoxide) extraction	Quercetin, 4-hydroxybenzoic acid, ferulic acid, kaempferol, p-coumaric acid, pinoselin, protocatechuic acid, vanillic acid, 2-hydroxycinnamic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxybenzoic acid, apigenin, luteolin, pyrocatechol, rosmarinic acid, sinapic acid, syringic acid and vanillin	[64,65]
<i>Hypericum</i> spp. aerial parts	Chloroform: methanol 2:1	Fatty acids	[66]
<i>H. perforatum</i> aerial parts	Water	Essential oils	[67]
<i>Hypericum</i> spp. aerial parts	Dichloromethane: methanol 1:1	Phenolic acids, flavonols, biflavones, naphthodianthrones and phloroglucinols	[68]

It can be clearly seen that polyphenols can be extracted in polar solvents, especially in hydroalcoholic ethanol or methanol, or even in hydroglycerolic medium, while hydrophobic compounds or may be extracted by changing the polarity of the extraction solvent, which may be obtained by increasing the alcoholic or glycerolic concentrations [57].

The most frequently used solvents are ethanol and methanol, either at 100% or in different dilutions, significantly improving the extraction of most of the types of compounds. Ethanol of different concentrations (decreasing concentrations—100%, 80%, 70% and 60%) appears to be the most efficient way to extract all types of compounds that may be found in the chemical composition of *Hypericum* species: anthraquinone derivatives (hypericin, pseudohypericin), prenylated phloroglucinol (hyperforin), chlorogenic acids (chlorogenic and neochlorogenic acid) and flavonoids (amentoflavone, catechins, quercetin, quercitrin, isoquercetin, hyperoside and rutin) [69].

Some extraction methods do not use solvents and are based on microwave technology for the extraction of essential oils [67]. The vegetal material consisted, in most of the cases, of the dried aerial parts of the species, which were extracted with the above-mentioned solvents and then completely evaporated until becoming a powdered extract [3,11,16,18,33,56,70,71]. Nevertheless, some studies use in vitro cultivated species [32,52,55,62,71,72].

3.1.2. Extraction Methods

The large variety of the species belonging to the *Hypericum* genus and the wide range of secondary metabolites that can be found in their composition indicate the fact that it is difficult to perform an adequate assessment just by using a single extraction solvent or a single extraction method [4]. This is the main reason for which numerous variants of extraction methods, using different extraction solvents and extraction parameters, have been developed over the years, allowing therefore to improve and increase the extraction rates and yields of the compounds that can be found in the composition of vegetal materials belonging to the *Hypericum* genus. The choice of the extraction method clearly depends on the compounds and their properties (e.g., polarity, solubility), and is performed following the further aims of the concerned study: either testing the biological activity of a species of the genus, establishing connections between species of the genus or testing the influence of different environmental parameters on the chemical composition and biological properties of the species of the genus.

Room-Temperature Extraction Techniques

- Room-temperature stirring

A simple cold stirring with solvents at room temperature may represent an extraction technique for different classes of compounds [27,29,30,34,39,55,57,60,61,66,70]. One of the most important conditions in this technique is to avoid light exposure, which may degrade some secondary metabolites which are photosensitive [32,34]. In some methods, extracts were evaporated to dryness under reduced pressure [48,70], especially if n-hexane was used as extraction solvent [60,61], but they can also be directly subjected to analysis after centrifugation [27]. Other techniques, especially those that concern the isolation of special classes of compounds, such as hyperzewalsins [23], polycyclic polyprenylated acylphloroglucinols [25] and hyperforatins [24], involve partitioning between different solvents (e.g., CH₂Cl₂, ethyl acetate, n-butanol, petroleum ether and acetone) and using different supports for separation (e.g., Sephadex LH-20, Pharmacia Biotech AB, Uppsala, Sweden) [23,25]. For the extraction of fatty acids, the chloroformic layer in the chloroform-methanol mixture was recovered, and solvent was removed in order to obtain methyl esters of fatty acids [66]. Water extracts for the analysis of different flavonoids, hypericin and pseudohypericin, were filtered and centrifuged [55], while a mixture of water-glycerol improved the extraction of polyphenols [57].

- Maceration

The most frequently found method of extraction is maceration, performed with different solvents [10,11,26,28,31,38,43,51,53,56,58]. In most of the cases, obtained macerates were evaporated to dryness [11,26,28,31,51,53,56,58], lyophilized [14,28] or centrifuged [38] before being subjected to analysis, or they were directly injected into the HPLC columns [10,43]. For particular methods, olive oil was used for extraction and DMSO was subsequently added [64]. Maceration with other types of oils may be used for extraction of flavonoids or other polyphenols [63].

Percolation may be used as an alternative extraction method, after maceration, using methanol for the extraction of a special class of compounds, polycyclic polyprenylated acylphloroglucinols (hyperfols) [50]. Similarly, after maceration in ethanol and fractionation by silica gel column chromatography in an n-hexane-Et₂O gradient, prenylated β-diketones were obtained [35]. In some methods, in order to separate polyphenols according to their polarity, after maceration with methanol and evaporation to dryness, dry extracts were further dissolved in water and partitioned between n-hexane, dichloromethane, ethyl acetate and n-butanol [16].

Heat Extraction Techniques

- Classical heating

The Soxhlet extraction method using heat may be used both for removing chlorophyll content, using chloroform, but also for extraction of different compounds, such as flavonoids [10,19,33,49], polyphenols and hypericins [33,46,65]. In order to obtain dry extracts, solvent was evaporated to dryness [33,49]. At the same time, classical heating may be used for the extraction of polysaccharides [21]. Decoctions may represent good extraction alternatives for the extraction of polyphenols and flavonoids [51].

- Ultrasonication

Besides maceration, ultrasonication is one of the most frequently used extraction techniques for different classes of compounds [2,4,5,18,22,32,45,47,51–53,59,68]. Most of the studies use temperatures of 40 °C [5,22,45,47,51,59,68] and 30 min as an extraction time [2,4,5,18,32,45,51–53,68], while the solvent-to-material ratio is maintained at 1:10. It may be followed by centrifugation [2,4,18,51,52], by drying under reduced pressure [22,32,53,59,68] or directly by the analysis after membrane filtration [5,44,45,47], before being subjected to chromatography. In some cases, ultrasonication was performed after the vegetal material was macerated and evaporated under nitrogen [15].

Hydrodistillation

For the *Hypericum* spp. aerial parts, hydrodistillation is used for the obtention of essential oils [19,48,67,73–76], which may be diluted in n-hexane before being subjected to analysis [54,73]. This extraction method concerns the use of a Clevenger-type apparatus, and the method is described in the European Pharmacopoeia, using water as extraction solvent [67]. Dehydration of essential oils was performed using dry sodium sulfate [67,74–76].

Microwave Extraction

Solvent-free methods concerning microwave-assisted extractions do not use solvents [20,41]. In fact, the vegetal material is moistened by spraying water on it to reach a fixed moisture content. The microwave heating of the water in the vegetal material releases the molecules in the essential oil, driven by the obtained vapors. Afterwards, the cooling system inside the extraction apparatus allows the continuous condensation of the distillate. The isolated essential oils are then dried with anhydrous sodium sulphate. The extraction rate of this method is accelerated in these types of methods by the fast increase in the temperature of extraction. When compared to hydrodistillation, which is also used for the extraction of essential oils, it seems that there is a difference in the extraction yield of different compounds, which may be due to the heat transfer and mass transfer, which occur on opposite sides, while for the microwave they occur in the same direction, facilitating diffusion [67]. Parameters used for microwave-assisted extraction of hypericin consisted of soaking the vegetal material for 30 min at 60 °C and extracting at 50 °C for 9 min [41], while for obtention of crude polysaccharide extracts from *H. perforatum*, the vegetal material was extracted for 60–180 min at 50–90 °C, before being thrice refluxed with 80% ethanol in water at 60 °C for 8 h [20].

Supercritical CO₂ Extraction

This method is an extraction technique that allows one to obtain high-quality vegetal products that may be used in the pharmaceutical, cosmetics or food industry, using environmentally friendly extraction conditions. It is especially useful for the analysis of essential oils, using high temperatures and pressures, and it is reported that the efficiency of essential oil extraction with liquid or supercritical CO₂ is higher than hydrodistillation [17]. The cosolvent used for extraction may be 10% aqueous ethanol, and extracts may subsequently be reduced using gaseous nitrogen under reduced pressure [77].

Preparative Chromatography for Extracts

Preparative chromatography is often used to isolate different fractions from the crude extracts, followed by analytical separations and identification of compounds through high-performance liquid chromatography. Crude extracts from *H. foliosum*, *H. androsaemum* and *H. perforatum* originated from Portugal were processed through low-pressure liquid chromatography using a reversed-phase stationary phase, with particles sizes between 43 to 60 µm. Moreover, separations of fractions or purification of some compounds from *Hypericum* species extracts can be achieved using permeation gel column chromatography and polar mobile phases such as mixtures between methanol and water [36].

New macroporous-resin column techniques coupled to preparative liquid chromatography are used for hypericin isolation from *H. perforatum* extracts. Five different macroporous resins were tested and evaluated, and the most suitable one turned out to be the Xad16 N weak-polarity microporous resin. This method, coupled further on with preparative liquid chromatography, using, as eluting solvents, acetonitrile and water, under gradient elution mode was able to improve the recovery rates of hypericin by up to 98% [78].

3.2. Analysis of Compounds

Quantitative analysis of compounds that can be found in the composition of different *Hypericum* species is most frequently performed by liquid chromatography, while qualitative approaches can be very different [4].

Hypericum species represent a valuable source of biologically active compounds that can positively favor the evolution of different types of pathologies. The chemical composition of multiple species belonging to the *Hypericum* genus, including the well-known *H. perforatum*, is often determined using different types of analytical methods, depending on the category or phytochemical class to which the secondary metabolites belong. Analysis of the chemical composition aims to better understand plant composition in terms of secondary metabolites, the influence of different environmental factors, variations of the chemical compounds between species and subspecies, antioxidant capacity, antibacterial, virucide, antifungal or other biological activities, and to better understand the pharmacological mechanisms for which *Hypericum* spp. secondary metabolites are responsible.

Hypericum species are widely investigated in terms of secondary metabolites and chemical composition, using different analytical techniques depending on the chemical classes of compounds. The main analytical techniques used for the qualitative and quantitative analysis of the chemical composition in all the articles that were taken into consideration for this review belong to the following categories:

- Separation methods and hyphenated separation methods: liquid chromatographic methods—high-performance liquid chromatography and ultra-high-pressure liquid chromatography (HPLC and UHPLC), liquid chromatography coupled to (tandem) mass spectrometry (LC-MS, LC-MS/MS), thin-layer chromatography and high-performance thin-layer chromatography (TLC and HPTLC), gas chromatographic methods—gas chromatography (GC), gas chromatography coupled to (tandem) mass spectrometry (GC-MS, GC-MS/MS); electrophoretic methods—capillary zone electrophoresis (CZE);
- Optical molecular spectroscopy-based methods and optical atomic spectroscopy-based methods: molecular electronic ultraviolet–visible spectroscopy (UV-VIS); near-infrared and medium-infrared molecular vibrational spectroscopy—near-infrared (NIR) and Fourier-transform infrared spectroscopy (FT-IR); atomic absorption spectroscopy (AAS); atomic emission spectroscopy (AES); inductively coupled plasma atomic emission spectroscopy (ICP-OES).

Finally, powerful analytical techniques for chemical structural elucidation such as nuclear magnetic resonance (NMR) are used in order to identify new compounds in *Hypericum* genus species.

Parameters and characteristics such as chemical structure, volatility of compounds, solubility, polarity, hydrophobicity, ionizable functional groups, pKa/pKb values, and number of chromophores, have a high impact on the types of the employed analytical methods. Lipophilic naphthodianthrones derivatives and phloroglucinols derivatives can be easily separated and retained using reversed-phase stationary phase while employing HPLC or UHPLC separation methods. Flavonoids are usually analyzed using separation methods (HPLC or UHPLC) next to phenolic acids. Due to ionizable groups, phenolic acids can also be analyzed using capillary electrophoresis. UV–VIS is often used to determine the total amount of phenolic acids and total content of flavonoids using specific reagents. *Hypericum* species also contain volatile compounds such as terpenes (monoterpenes, sesquiterpenes, terpenoids) and fatty acids. The elective analysis method for the volatile compounds of *Hypericum* species is GC or GC-MS.

The objective of this chapter is to extensively and explicitly present the main analytical methods, parameters and a performance evaluation for the applied and developed techniques used, in order to analyze, identify, quantify or elucidate the chemical structure of the specific compounds found in the species of the *Hypericum* genus.

3.2.1. Separation Methods and Hyphenated Separation Methods

Separation methods represent by far the largest category of analytical techniques used for the analysis of different chemical compounds in the composition of *Hypericum* species or for the analysis of different types extracts that are obtained from their vegetal material. Separation methods are used to solve particular applications in different contexts that involve *Hypericum* species, such as general chemical profiling; fingerprinting and species differentiation; quality assessment and quality control; phytochemical characterization of uninvestigated species from different parts of the world; assessment of newly developed extractive methods and procedures; the study of different exterior factors such as physical, chemical or biological factors that may impact the chemical composition; genetic variations; determination of the elicitation effect of different substances; in vivo and in vitro antioxidant, enzymatic and pharmacological activity; antibacterial and antifungal activity. For this plethora of applications, accurate separation methods have been developed in order to identify, quantify or to elucidate chemical structures of newly detected compounds.

An extensive literature review was performed regarding separation methods for different kinds of applications involving the *Hypericum* genus, with a special focus on chromatographic methods. Data comprising 76 chromatographic methods (HPLC, UHPLC, LC-MS(/MS), GC and GC-MS(/MS)) are summarized and displayed in three different supplementary data tables (Tables S1–S3). Summarized data tables cover information regarding the studied species, plant part, phytochemical class of compounds, specific compounds that were investigated, type of stationary phases and stationary phase characteristics, employed mobile phases, run time and type of detection. Data presented in Tables S1–S3 offer an overview regarding the possibility of analyzing different types of compounds. A general overview will be discussed further on, based on the type of the separation technique.

High-Pressure and Ultra-High-Pressure Liquid Chromatography

HPLC and UHPLC are the preferred techniques used to determine chemical composition of the *Hypericum* extracts. Multiple analytical methods have been developed depending on the phytochemical class of compounds or on the envisaged applications.

Naphthodianthrone are specific compounds for *Hypericum* species and are usually investigated and quantified in all types of *Hypericum* genus species. The main naphthodianthrone representatives are hypericin, pseudohypericin, protohypericin and protopseudohypericin. Precursors of naphthodianthrone are the anthraquinones emodin and skyrin and their derivatives—oxyskyrin, iridoskyrin, rubroskyrin [32]. Multiple studies have revealed that in order to separate and analyze naphthodianthrone, reversed-phase separation mode can be employed [2,46,52,71,79]. C18 (octadecylsilyl) is the most commonly used stationary phase [26,32,37,51,80]. However, other RP (reversed phase) chemistries with lower hydrophobicity are reported for hypericin analysis, such as C8 (octyl) [81]. Fully porous stationary phase can be successfully used for the analysis of naphthodianthrone, and monolith columns can allow for rapid analysis and lower retention of pseudohypericin and hypericin [20].

Mobile phases consisting of different mixtures between an aqueous component and an organic modifier such as acetonitrile and methanol are often used. The aqueous component is usually acidified with formic acid, trifluoroacetic acid, or phosphoric acid [41,44,46,62,69,71], or buffered with ammonium acetate [43,82]. Ethyl acetate can also be used in the composition of the mobile phase together with methanol and sodium dihydrogen phosphate (adjusted to pH = 2 with phosphoric acid) [45,83]. Retention of naphthodianthrone is improved significantly while using acidic mobile phase additives or buffer solutions that are able to create an acidic pH value of around 2.0 [41,43,44,46,82]. In order to detect naphthodianthrone such as hypericin or related compounds, UV–VIS detectors or photo diode array detectors/diode array detectors (PDA/DAD) are usually coupled at the end of the chromatographic column while scanning at a specific wavelength for this compounds at 590 nm or closer, such as 520 nm and 560 nm [5,34,48,80,84].

Hypericum genus species are also rich in phloroglucinols and their derivatives. The main classes of phloroglucinols that are investigated using HPLC and UHPLC techniques are the acylphloroglucinols, dimeric acylphloroglucinols and polycyclic polyprenylated acylphloroglucinols [85,86]. Phloroglucinols derivatives are frequently analyzed using RP stationary phases such as C18 [2,52,59,62,63] and phenyl stationary phase [36]. The mobile phases used for RP separations of phloroglucinols derivatives are based on mixtures that contain an aqueous component acidified with formic acid, trifluoroacetic acid or phosphoric acid, and an organic modifier such as acetonitrile or methanol [5,37,44,45,58,87]. Sometimes, the aqueous component can be a buffer such as acetic acid/ammonium acetate buffer [26]. PDA and DAD detectors were primarily used for the detection of the phloroglucinols in UV spectrum domains at different wavelengths, depending on the compounds. Compounds were commonly detected at 270, 290 or 370 nm [5,34,48,61]. Even though the selectivity of the method can be changed or improved for some phloroglucinols derivatives while using phenyl stationary phase [36], the preferred stationary phase remains C18 with acidic pH values of mobile phases.

Analysis of phenolic acids of the *Hypericum* species extracts by HPLC is made possible by using different analytical approaches that employ RP-C18 stationary phases coupled to UV-VIS or DAD detectors [20,39,79,80,88–91]. Mobile phases used for the analysis of phenolic acids consist mainly of acidified aqueous solvents mixed with acetonitrile or methanol. Phenolic acids are small molecules with phenol and carboxyl functional groups. Phenolic groups can induce an increase in polarity, while the dissociation of carboxyl groups at a high pH value can decrease the affinity for a hydrophobic stationary phase. Thus, mobile phases are usually acidified in order to induce the formation of the undissociated form while increasing hydrophobicity, and finally the retention of a RP hydrophobic stationary phase. Moreover, simultaneous analysis of phenolic acid compounds next to naphthodianthrones and phloroglucinol derivatives is feasible [36,39,44,48,69,84]. Flavonoids and flavonoids derivatives, either as aglycones or glycosylated flavonoids, are usually determined and separated through HPLC by using mainly RP mechanisms [39,51,79,84,88].

HPLC or UHPLC methods are also able to separate other compounds that are usually found in much lower concentrations, such as: tocopherols [63,66]; xanthenes [54,72,92,93]; skyrin derivatives—iridoskyrin, rubroskyrin, luteoskyrin, oxyskyrin [32]; benzophenones and benzopyrans [58]; bisanthrones [32]; cyclitols [51] and even amino acids. All these compounds are usually detected using DAD and UV-VIS detectors. However, fluorescence detectors were reported for the analysis of free amino acids, after a previous derivatization with o-phthalaldehyde and 9-fluorenylmethyl chloroformate [90].

C18 is the elective stationary phase due to the versatility of this phase in accordance with the studied compounds. The most used mobile phase mixtures consist mainly of water, methanol and acetonitrile. However, selectivity, retention and resolution can be further improved by employing different particle sizes; particle porosity; types of particles (fully porous, monolytic, fused-core particles), or by changing different chromatographic parameters such as elution mode, gradient types and gradient slopes.

Sometimes TLC techniques or HPTLC are used for quality control purposes or phytochemical screening for uninvestigated species, and may forego HPLC and UHPLC analysis [26,94].

Liquid Chromatography Coupled to Mass Spectrometry

The improved specificity, selectivity and sensibility of LC-MS and/or LC-MS/MS techniques represent an asset when these techniques are used for phytochemical profiling of particularly unstudied *Hypericum* species. Since a significant class of phytochemical compounds can be separated using RP-separation mechanisms and polar solvents as mobile phases, the coupling with mass spectral methods is easy and feasible. Different mass analyzers are usually employed for the targeted and untargeted analysis of *Hypericum* species, such as ion traps [18,19,27], single quadrupoles [38,57,59], orbitraps [32,68] and even hybrid mass analyzers such as triple quadrupoles (QQQ) [40,51,80,90], quadrupole-ion

traps (Qtraps) [62] and quadrupole time of flight QTOF [26,42,48]. Electrospray ionization sources (ESI) were used both in negative ionization mode (ESI-) and positive ionization mode (ESI+), depending on the targeted compounds. For the untargeted analysis, phytochemical profiling and fingerprinting, usually both ionization modes were used to cover as many compounds as possible [18,20,48]. The literature records multiple studies where targeted analysis was performed using particular MRM (multiple reaction monitoring) scan modes and triple quadrupoles were used as mass analyzers. Nevertheless, untargeted analysis applications were solved using scan and full-scan modes with the help of QTOFs orbitraps and Qtraps [51,80,90,95].

The phytochemical composition in terms of secondary metabolites for many species of the *Hypericum* genus, including *H. perforatum* from different parts of the world, were determined through LC-MS and LC-MS/MS. *H. perforatum*; *H. maculatum*; *H. Hirsutum* and *H. tetrapterum* extracts were screened for bioactive compounds, and antioxidant capacity was eventually determined based on the phytochemical composition. Three *Hypericum* species from Sardinia (*H. scruglii*, *H. hircinum*, *H. perforatum*) were screened with the help of an LC-ESI negative- QQQ , and the α -glucosidase inhibitory activity was determined. Phytochemical investigation using LC-MS techniques for species such as *H. caradonae*, *H. myricariifolium*, *H. laricifolium*, *H. humboldtianum*, *H. garciae*, *H. carinosum* and *H. cuatrecasii* from the remote Columbian Andes mountains was focused on flavonols, flavan-3-ols, stilbenoids, coumarins, cinnamic acids and flavones [40]. However, LC-MS and LC-MS/MS made possible the analysis of different *Hypericum* species from other particular parts of the world such as the Northern Republic of Macedonia, Canary Islands, Greece or the People's Republic of China [18,19,42,48].

Besides phytochemical profiling, LC-MS/MS has been involved in other special applications where the chemical composition of the *Hypericum* genus was needed, for example, the evaluation of acylphloroglucinols composition (uliginosin A, isouliginosin B, isohyperbrasilol, uliginosin B, hyperbrasilol B) on the antichemotactic activity of lipophilic extracts from Peruvian *Hypericum* species [59]. Other studies evaluate the influence of some external factors on the composition of *Hypericum* species. In order to increase the number of naphthodianthrone synthesized by *H. perforatum*, a study reports and confirms that nanoperlites and MnO_2 /perlite-NCs have a positive effect in terms of hypericin and pseudohypericin content [55]. Other external factors have been studied, such as the presence of lanthanum and cadmium. These two compounds, especially lanthanum, proved to produce modifications in the metabolic profile of *H. perforatum*. This conclusion was possible thanks to the MRM-targeted analysis while investigating different classes of compounds such as phenolic acids, flavonols, epicatechin, procyanidins and naphthodianthrone [96]. By using an LC-DAD-ESI ion trap system, and based on mass spectral data and UV-VIS, a comparison between the species *H. perforatum* and *H. maculatum* was performed based on phenolic acids, naphthodianthrone and flavonoids content, revealing distinct between-year and between-species diversity. The study also revealed that *H. maculatum* contains trace amounts of rutin, with a tendency to accumulate more phenolic compounds [27]. Applications regarding plant authenticity for *Hypericum* species are also of high interest. An original research study where compounds from different classes were analyzed through LC-MS full-scan mode proved that the ratio of hyperforin and furohyperforin allows the ranking the raw materials of *H. perforatum* depending on the place of growth, and the ratio between the rutin content and hyperforin content proved to be a good marker for the quality of the vegetal material belonging to *H. perforatum* [20]. Eventually, even antityrosinase activity and antiaging potential of three *Hypericum* species (*H. perforatum*; *H. calycinum*; *H. confertum*) was investigated through targeted MRM analysis by LC-ESI- QQQ [14].

Gas Chromatography and Gas Chromatography Coupled to Mass Spectrometry for Volatile Compounds

Even though *Hypericum* genus species are not renowned for volatile compounds, GC and GC-MS is an ideal tool for the analysis of some compounds such as fatty acids

or terpene derivatives from essential oils. These types of compounds analyzed from *Hypericum* species extracts are mainly separated and analyzed using film-coated capillary columns [21,47,54,67]. In order to improve retention and separation, derivatization was used for some types of compounds, either on column or before the injection [54,63]. Helium and nitrogen were the most encountered gas carriers used for the analysis of many of the compounds [66,76]. Due to the high content of carbon atoms of fatty acids and terpenes, a flame ionization detector can be used as the elective detection method [63]. However, mass spectrometry using electron impact ionization can provide better insight on the chemical composition, and can be of real help for fingerprinting, profiling or for special applications in which an external factor, e.g., altitude was studied [47], or when biochemical elucidation mechanisms are envisaged [54]. Identification of compounds can be performed using reference standards, however, for certain reported methods, when multiple compounds are analyzed, Kovats Index and other linear retention indices can be used [47,67,73]. Moreover, when MS detection is used, then mass spectra for the obtained compounds can be compared to reference spectra from different libraries [19,73,75].

Research based on the chemical content of *Hypericum* genus extracts revealed different classes of volatile compounds. *H. perforatum*, *H. perforiatum*, *H. tomentosum*, *H. ericoides*—four Tunisian *Hypericum* species—were investigated for their content in fatty acids and tocopherols [66]. Fatty acids were also analyzed as methyl esters obtained through on-column trimethylsulfonium hydroxide derivatization [63]. It was demonstrated that *H. perforatum* can contain also mono- and sesqui-terpenes [55] and oxygenated mono- and sesqui-terpenes for *H. perforatum* species coming from Iran [76]. New, interesting compounds such as prenylated β -diketones (2,6,9-trimethyl-8-decene-3,5-dione and 3,7,10-trimethyl-9-undecene-4,6-dione) were identified and confirmed using GC-MS and electron-impact ionization mode [35].

Unexplored species (e.g., *H. perforatum* ssp. *veronense*; *H. perforiatum*; *H. empetrifolium* ssp. *empetrifolium*; *H. triquetrifolium* from Greece) were analyzed using GC and GC-MS for their volatile compounds content [17,19,69,73,74,76], and 113 different compounds were identified [48]. *H. rochelii* and *H. umbellatum* were investigated through GC-FID and GC-MS, revealing a significant number of volatile compounds, out of which the terpene class is the best represented [74].

Capillary Electrophoresis

Capillary zone electrophoresis techniques represent a greener alternative to HPLC or UHPLC, due to its minimum consumption of solvents, requiring a small amount of sample to be injected into the capillary. Capillary electrophoresis makes no exception from the separative methods used for chemical fingerprinting. Phenolic acids and flavonoid glycosides were able to be identified using a fused silica capillary and a 50 mM sodium tetraborate pH = 9.1 separation buffer [97].

Capillary electrophoresis (CE) was found to be insufficiently sensitive for naphthodianthrones and phloroglucinol derivatives using a separation buffer with pH = 3.0, and was overall less reproducible [87]. Lipophilic compounds such as hypericin and hyperforin were able to be analyzed using nonaqueous medium containing methanol, dimethylsulfoxide N-methyl formamide (3:2:1) as solvent, and 50 mM ammonium acetate, 150 mM sodium acetate and 0.02% (*w/v*) of cationic polymer hexadimethrine bromide. Flavanoids and chlorogenic acid were analyzed using micellar electrokinetic electrophoresis, using sodium dodecyl sulphate as a surfactant. The results showed that while the methods for the analysis of flavonoids were considered to be suitable, quantification by HPLC and CE did not reveal significant differences in terms of analytical performances [4].

3.2.2. Molecular and Atomic Optical Spectroscopic Methods

Optical spectral methods, such as UV–VIS molecular spectroscopy, vibrational IR spectroscopy or ICP-OES, are usually used for screening purposes, and usually assist the specific applications for *Hypericum* extracts such as enzymatic activity, antioxidant activity

and correlations with total amount of flavonoids, phenols or even structural elucidation for unidentified compounds when FT-IR is employed.

UV–VIS molecular spectroscopy is an affordable and inexpensive technique, often used for quality assessment of medicinal plants and dietary supplements including those that contain *Hypericum* plant parts.

Naphthodianthrone, which can be considered as anthraquinone derivatives, are specific compounds biosynthesized in almost all species belonging to *Hypericum* genus [6,85,98]. Hypericin is the most encountered naphthodianthrone, alongside pseudohypericin, protohypericin, protopseudohypericin. In order to evaluate extraction efficiency of these compounds, or to assess the quality of plants and dietary supplements containing *Hypericum* species, total hypericin content is quantitatively evaluated using UV–VIS spectrometry, determining the absorbance values of processed extracts at a specific wavelength of 590 nm. The UV–VIS method for hypericin dosage of *H. perforatum* is also officialized by the current version (10.7) of the European pharmacopoeia [8]. This general method is used in order to assess and improve extraction procedures of biologically active compounds from *H. perforatum* plants [29], determine the authenticity and quality of medicinal plants [94] or for characterization of macerates with different fatty oils obtained based on *H. perforatum* plants parts [63]. By using the UV–VIS method, the total content in hypericin can be calculated based on the specific absorbance or based on calibration curves plotted using reference standards [8].

Phenolic compounds represent a major source of antioxidants in the *Hypericum* genus, and are usually investigated and determined as total phenolic content, eventually correlating with the antioxidant activity [99]. Usually, total phenolic content is assessed based on the well-known Folin–Ciocalteu reagent and expressed as gallic acid equivalents while reading the absorbance of samples and standard solutions at 760 nm. Assessment of total phenolic content of *Hypericum* species is performed in different contexts, such as temperature influence and temperature-dependent growth parameters of *Hypericum* species [81] or other chemical dependent factors [84], phytochemical profile of particular Sardinian *Hypericum* species, such as *H. scruglii* and *H. hircinum* [51], study of Canary Islands *H. reflexum*, *H. canariense* and *H. grandifolium*, evaluation of the chemoprotective effect of *H. perforatum* on rat oral squamous cell carcinoma [33], novel and improved extraction procedures based on microwave extraction [67], usage of special solvents such as glycerol [57], or by successive grinding and sieving [38]. Nevertheless, this analytical approach is often used for screening of total phenolic content for uninvestigated species belonging to *Hypericum* genus, such as *H. calycinum*, *H. confertum*, and *H. perforatum* [11] or *H. androsaemum*, *H. ericoides*, *H. x moserianum* and *H. olympicum* [1].

UV–VIS spectrometry can be successfully used to determine the total flavonoid content. Usually, total flavonoid content is determined as pyrocatechol or quercetin equivalents based on calibration curves. Total flavonoid content of *Hypericum* species was evaluated, especially for uninvestigated species [16] or in order to assess the quality of the plant products. External factors were researched in order to determine their influence over the secondary metabolites [81,84] or to determine the in vitro biological activities of *Hypericum* species extracts [11]. In order to assess the chemical composition of *Hypericum* species, UV–VIS spectrometry is also used to determine the total amount of anthocyanins based on molar extinction coefficient ($25,956 \text{ M}^{-1} \text{ cm}^{-1}$ using the molecular weight of 449 g mol^{-1}). Condensed tannins most often are determined in catechin equivalents [84].

Vibrational spectroscopic methods such as medium-infrared Fourier-transformation infrared spectroscopy and near-infrared spectroscopy represent powerful analytical techniques used for structural elucidation using FT-IR, or for quantitative analysis using NIR accordingly. FT-IR spectrometry was used in order to characterize and investigate functional groups of crude polysaccharides in *H. perforatum* extracts obtained after design-space surface methodology optimization, and they were analyzed further by FT-IR using KBr pellets and one milligram of extract [21]. Moreover, FT-IR, using an attenuated total reflectance approach, was used for metabolite profiling and species discrimination (between *H. annulatum*,

H. calycinum, *H. hirsutum*, *H. hookerianum*, *H. humifusum*, *H. maculatum*, *H. olympicum* and *H. pseudohenryi*) [69]. Crude and filtrated extracts in a study investigating the impact of the filtration process on bioactive extracts incorporated into carbohydrate-based hydrogels were directly investigated and characterized using FT-IR and KBr pelleting [56]. NIR techniques turned out to be of great value for the quantification of specific compounds such as hypericin and hyperforin from the *H. perforatum* extracts, with good determination coefficients ($R^2 > 0.99$ for hypericin and $R^2 > 0.98$) for the concentrations of the investigated compounds relative to a reference liquid chromatographic method [87].

As a complementary tool for *Hypericum* genus and *H. perforatum* analysis techniques, ICP-OES was successfully used for elemental analysis and fingerprinting of *H. perforatum* products such as plants, dry herbs, tablets and capsules. Vegetal products, capsules contents and tablet powders were dried and digested with highly pure nitric acid prior to ICP-OES analysis. The developed methods were able to investigate 25 different elements, while revealing through principal component analysis seven key elements: Ba, Ca, Cd, Mg, Mo, Ni, Y. The major constituents of *H. perforatum* were revealed to be Ca, Mg, Al, Fe, Mn, Sr, and Zn [100].

3.2.3. Spectral Methods for Structural Elucidation—Nuclear Magnetic Resonance

Nuclear magnetic resonance is an analytical technique that is used especially for chemical structure elucidation when *Hypericum* species applications are investigated. Different NMR techniques are reported in the literature for the analysis of new compounds of the *Hypericum* genus using ^1H -NMR, ^{13}C -NMR and even ^2D -NMR.

Chemical structure of new compounds such as (\pm)-hyperzewalsins A–D, representing the first nor-monocyclic, polyprenylated acylphloroglucinols bearing carbon chain constitutions based on diverse highly degraded phloroglucinols, were detected using both ^1H -NMR (600 MHz) and ^{13}C -NMR (150 MHz), from *H. przewalskii* Maxim. (Hypericaceae)-isolated fractions [23]. NMR represented an important tool when phytochemical profile of the *H. scruglii*, *H. hircinum* and *H. perforatum* was determined. ^1H -NMR revealed that the most abundant secondary metabolites for *H. hircinum* were shikimic acid, chlorogenic acid, quercetin and quercetin-7-glucoside. The same components were also identified in *H. perforatum*, which proved to contain also hyperoside and trigonelline. *H. scruglii* proved to contain, alongside shikimic acid and chlorogenic acid, 3-geranyl-1-(2'-methylbutanoyl)-phloroglucinol, 3-geranyl-1-(2'-methylpropanoyl)-phloroglucinol and quercitrin [51]. Hyperformitins A–I (9 compounds), which belong to the polycyclic polyprenylated acylphloroglucinols class, and new isomers such as hyperformitins J–M (four compounds), were identified and described in different fractions obtained based on *H. perforatum* extracts. Absolute chemical configurations for garcinielliptone L and garcinielliptone M next to garsubellins D and C were determined for the first time using NMR [25]. The chemical structures of hyperforatins L–U (10 compounds) belonging to the polycyclic polyprenylated acylphloroglucinols class were determined using NMR and were further studied in order to determine their neuroprotective activity, since it is known that this class of compounds can be important for their antidepressive effects and antineurodegenerative activity [2]. The hyperfols (particularly hyperfols C–H), which form another subclass of polycyclic polyprenylated acylphloroglucinols, were also identified in *H. perforatum* extracts, whereas the chemical structure was elucidated based on ^1H -NMR and ^{13}C -NMR [50]. Nevertheless, NMR can be used also as a tool for untargeted analysis coupled to principal component analysis in the context of authenticity assessment, adulteration discovery and quality control of different herbal medicinal products derived from the *Hypericum* genus [94].

Important demands in terms of increased market share and export rates of *Hypericum* plant-based medicinal products results in the appearance of counterfeit products adulterated with *H. perforatum* cultivars with distinct chemotypes [101,102].

4. Concluding Remarks and Future Perspectives

The necessity of standardized methods for the quality assessment of *Hypericum* herbal products has increased over the years, especially as herbal products may be altered due to misidentification or confusions, offering serious safety concerns. As the market share is expected to grow in the upcoming years, it appears important to study the species of the *Hypericum* genus in different ways, in order to offer significant scientific evidence to support their correct use in the therapy of several pathologies. It might be interesting to observe, in future studies, the evolution on the market of cosmetic products, supplementary foods and nutraceuticals that employ *Hypericum* extracts in their composition. Nevertheless, high difficulty of data gathering is envisaged due to the lack of harmonized worldwide legislation and regulation. These facts are responsible for the incorrect integration of this products in certain categories of consumer goods for health.

The *Hypericum* genus contains one of the most extensively studied and widely known species, *H. perforatum*, but also other species that may present similar properties, especially as this genus has numerous representatives and is one of the largest flowering plant genera. A significant number of species have not yet been studied. However, their important pharmaceutical potential remains to be determined and to be compared to the officinal species, starting from the extracts and then continuing with the phytochemical analysis and biological evaluation of their properties, having as a final purpose a significant relevance from the medical, cosmeceutical and nutraceutical points of view. In this regard, the gap for future studies on the genus becomes larger and larger. On the other hand, the mechanisms by which the compounds found in the composition of these species may exert their pharmacological properties are still unclear, which makes the study of the species of the genus even more interesting. All of these points may lead to the development of phytotherapeutical preparations that can be used by patients worldwide for the treatment of a large variety of pathological conditions. Study perspectives of the species of the genus are therefore numerous, and open possibilities for increasing the knowledge on one of the most important vegetal medicinal products.

The *Hypericum* genus comprises a large variety of secondary metabolites involved in many elucidated or yet undetermined biological mechanisms. Multiple studies have revealed that not only is the species *H. perforatum* of increased interest, but also other species may be of high potential regarding their chemical composition. The procedures used for obtaining extracts vary, from simpler ones up to those assisted by microwaves, ultrasonication or supercritical fluid CO₂ extraction. The solvents used for the extraction procedures are almost polar ones; however, organic, less polar solvents or other particular approaches such as the use of olive oil have proved to be suited for further chemical characterization or phytochemical profiling and optimal extraction. Extract analysis using separation methods such as liquid chromatographic methods, often hyphenated with mass spectrometry, cover a high range of phytochemical classes, such as naphthodianthrone, phloroglucinols, phenolic acids or flavonoids. Gas chromatographic methods are generally complementary to LC methods, while some spectral methods such as nuclear magnetic resonance, mass spectrometry and Fourier-transform infrared spectrometry were best suited for the characterization of new compounds. Usually, the newly investigated compounds relate to phloroglucinols phytochemical class and their secondary degrading products. The relevance of all the studies regarding the *Hypericum* genus are of high biological and medical interest due to its proven antioxidant, pharmacological and antibacterial activities. Finally, the plant diversity of the genus enhances the potential to discover new compounds with biological activity, which means that studies will continue, with direct benefits and improving overall the knowledge regarding the species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations9010017/s1>, Tables S1–S3. References [2,5,10,14,16–21,26,27,30,32,34–48,51,52,54,55,57–59,61–63,66–69,71–77,79–82,84,87–93,95–97] are cited in the Supplementary Materials.

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