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Romanian Aromatic and Medicinal Plants: From Tradition to Science

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Abstract

From ancient times, plants have been used by humans for food, fodder, fibre and medicinal purposes. Several plants were empirically considered as treatments for a large array of illness and medical conditions. Each community had specific natural remedies, based on the geographical area, environmental conditions and other factors. Thus, the use of plants can be considered as part of the intangible cultural heritage of each community. In the geographical area of today's Romania, the ancient inhabitants, Dacians, had very good knowledge regarding the use of plants for medicinal purposes, as presented by several historical sources. The present work describes protocols for the extraction and purification of natural extracts, analytical characterisation, *in vitro* and *in vivo* evaluation of their potential applications as well as some practical examples of their application on selected Romanian native medicinal and aromatic plants. The presented results offer scientific support to their traditional use, suggesting in the same time some modern applications, for example in the nanotechnology field.

Keywords: aromatic and medicinal plants, Romanian, traditional remedy, scientific evidence

1. Introduction

1.1. Traditional use of medicinal and aromatic plants

The first human's tries to treat diseases were aiming for the environmental plants, so natural products could be considered the main mean of diseases treatment across the globe until the

advent of scientific medicine. During their evolution on Earth, plants have developed the ability to synthesize certain chemical compounds for protection in the fight against world's predators, such as insects, fungi, herbivorous mammals, etc.

Although some of these compounds are toxic to predators, they proved to have beneficial effects in the treatment of human diseases. In the past centuries, the practice of empirical use of plants for therapeutic purposes was passed either in writing or orally, from generation to generation. Thus, the oldest way of treatment, phytotherapy, whose beginnings lie in Palaeolithic and whose traces are preserved in folk medicine up to our days, was developed on an empirical base, but also in the context of a magic vision of the world, where the symbol, the analogy and the correspondences principle have played and are playing an important role in the choice of useful plants [1–3].

People always appealed to nature, mainly to plants, for treating and curing various diseases; harvesting plants with everything they prove useful and their complete exploitation gave humans the opportunity to familiarize with their curative properties [4]. The first more precise data about the use of plants for healing in what is today Romania were given by the Greek doctor Discorides (doctor in Nero's army) in the five-volume treatise on plants "De Materia Medica", the precursor of all modern Pharmacopoeia and one of the most important botanical atlases in history. He pointed out that in Dacia numerous plant species were used on a large scale, making a vague and incomplete description of them. Published in 77, the book was describing 600 species of medicinal plants, of which 40 species were specific to Dacia's territory. Among these species, 27 plants have Daco-Thracian names, 8-Latin and 5-Greek, which is a confirmation of the age of phytotherapy in Romania [2, 3, 5, 6].

Over the time, the inhabitants of Romania's lands kept the continuity of rich traditions in the use of plants from spontaneous flora, which proved to be effective in curing physical or psychological suffering (injuries, fractures, bleeding, poisoning, animal bites, sunstroke, frostbite, infectious diseases, etc.).

Due to its geographic position, with a varied landscape and a climate favourable for rich vegetation, Romania is the meeting place of the Eurasian and Mediterranean flora. Here, grow more than 3600 species of higher plants, of which over 700 have become medicines, thanks to the long experience of the Romanian people in their use to cure diseases. In the Romanian plant heritage, there are numerous wild and cultivated plants that have different uses [1–9].

1.2. Short presentation of plants selected for the study

Among the Romanian traditional plants, *Heracleum sphondylium* L. (hogweed) occupies a special role due to its extremely intense vitalizing effects, therefore being called "Romanian Ginseng". The plant is known since antiquity, from the time of Plinius the Elder, under the name of *Heracleum*, derived from Heros, Heracles, Hercules, who had discovered its medical effect. It is an herbaceous plant, perennial or biannual that belongs to the family of *Apiaceae*, frequently found in meadows, scrublands, rarely in forests, from the plain region up to the mountains. The flowers are grouped in rich clusters, white or pink pal. The Romanian name of the plant comes from the leaves' shape which resembles a bear's paw. The herbal medicine uses the whole plant (especially leaves, buds, roots and seeds). The leaves and the buds are

harvested from April to October; the whole plant is harvested during flowering, while the roots are harvested in spring and autumn. It is dryed in the shadow, in a thin layer. Heracleum's root and seeds quickly refresh and rejuvenate the body, increase exercise capacity and psychological tone, eliminate sterility, impotence and frigidity. It is a remedy indicated to the third age, relieving the discomforts of the premature physical decline, restoring tone and appetite for life [2, 3, 5, 6].

Anethum graveolens L. (dill) is an herbaceous, annual, terofite, vegetable, spicy, aromatic, cultivated and sub-spontaneous plant, which belongs to the *Apiaceae* family. It is known and cultivated since antiquity by Egyptians, Greeks, Romans and Gauls. In Romania, it is grown throughout the country, especially in assorted cultures. The stem, leaves and fruits have therapeutic uses in the traditional human medicine and in cult or traditional veterinary medicine. The harvesting of the stems and leaves is made when inflorescence is formed, while the harvesting of fruits is made when they are mature. From ancient times, culinary and medicinal uses were attributed to the dill. Decoction of the dill's seeds is used to treat stomach aches; the tea obtained by boiling the floriferous stems and dried inflorescences of dill is used to treat kidney diseases, hearts diseases and atherosclerosis and to increase lactation at women. Also, crushed dill's seed are used to treat intestinal worms at children. The dill is recommended in anorexia, indigestions, difficult digestion, heart diseases, urinary diseases, haemorrhoids, flatulence, various internal and external inflammations and menstrual pains [2, 5].

Taraxacum officinalis (L.) Weber ex F.H. Wigg (dandelion) is an herbaceous and perennial plant, which belongs to the *Asteraceae* family, common throughout Romania, in sunny places or in semidarkness, forests, meadows, pastures, on the roadside, from plains until subalpine area. The whole plant is harvested in spring, before and after the flowering, aerial parts or just the leaves are harvested in spring and the root is harvested in autumn or in early spring. Drying is made in the shadow, in a thin layer, in very airy places. The root and aerial parts of the plant have therapeutic uses in human and veterinary medicine, cult and traditional. Since Dacian times, the flowers and the juice resulting from squeezing dandelion's leaves were used in various skin disorders, the root was used in rheumatic diseases, the decoction of the leaves was used to treat liver diseases, while the decoction from the root was used to treat kidney diseases and against bleeding. The dandelion is recommended in liver diseases, urinary disorders, circulatory disorders, obesity, constipation, haemorrhoids, anaemia, cataracts, acne and endocrine diseases. In addition, the traditional medicine uses the plant to stimulate the pancreatic secretion, in the treatment of cancer and as an easy purgative in preventing constipations [2, 6].

Arctium lappa L. (burdock) is an herbaceous and biannual plant, which belongs to the *Asteraceae* family, common throughout Romania, from plain to mountain areas, wild lands, roadsides, along fences and flood groves. Known since antiquity by Geto-Dacians, the burdock was one of the most important and the oldest medicine practiced by our human folk medicine. The uses of the plant passed under the name of empirically remedies. Raw leaves were put on wounds and swellings; they were used to relieve the fever, to treat corns and to remove back pains. Tackling the lost hair and the stimulation of their growth was made by washing the head with broth resulting from boiling the burdock's leaves and stems. The harvesting of burdock's roots is made in spring for the 2 years plants and in autumn for the 1 year plants. After washing, the

aerial plants are cleaned and dried at the sun, in thin layer. The leaves are harvested without petiole, in May–June, before flowering and dry in the shadow in one place. The roots show the therapeutically importance for the human and veterinary medicine. They have stronger and laster detoxifying effects, with an impressive range of uses, from food poisoning, skin diseases or rheumatism to glandular or metabolism disorders. In the long term, they have beneficial effects on the activity of the liver and gallbladder and they prevent diabetes and tumour diseases. In case of respiratory diseases and flu, the burdock is a valuable remedy, helping to stop the growth of the microorganisms, reducing fever and preventing complications. It is used the decoction, infusion, hot steeping, tincture and powder of burdock's root [6].

Anthriscus sylvestris (L.) Hoffm. (wild chervil) and *Anthriscus cerefolium* (L.) Hoffm. (the garden species) are herbaceous, biannual (respectively annual), terofite plant, belonging to the *Apiaceae* family, with pleasant smelling, white flowers arranged in small umbrellas, related with parsley and carrot. It is common in temperate regions, on the roadsides and forests, in pastures and hay fields. In the Romanian folk medicine, the aerial flowered parts of the plant are used, harvested in May-June, dried in a thin layer in well ventilated spaces. It has been used traditionally as a stimulant, diuretic, anti-haemorrhoidal, antipyretic, anti-inflammatory and analgesic. In the human folk medicine, it is also used in digestive disorders, having slightly laxative action, in bronchitis and chronic lung diseases, as an antitussive. Externally, it is used in inflammations of the eyelid, dermatitis and eczemas [4].

2. Obtaining and characterisation of natural extracts with biomedical applications

2.1. Extraction and separation of active compounds

The concentration of biologically active compounds of natural extracts directly depends on a series of factors, such as genomic composition, biological value of the cultivar, maturity stage of the plant, climatic zone, environmental conditions, post-harvest and storage conditions, as well as *the extraction methods applied* [10]. Processing of medicinal plants can be divided into two stages: *primary processing*, which consists of plants drying, conditioning and packaging, and *advanced processing* that consists of transforming raw materials obtained from primary processing into the desired final product. Extracts may be categorized according to several parameters, as follows [10]: considering the *nature of the solvent* extracts may be: aqueous extracts (infusion or maceration-type), hydroalcoholic extracts (tincture-type), oily extracts, medicinal vinegars (macerated in vinegar) or medicinal wines (macerated in wine); considering the *obtaining method*: selective extract (targeting the compounds of interest), non-selective extracts (tincture, macerated type, etc.), extracts obtained by pressing (fruit and vegetables juice); considering the *parts of the plant* subject to extraction: partial extract (only certain anatomical parts of the plant) or total extract (whole plant); considering the *water content* of the plant (humidity): extract obtained from dried plant or fresh plant extract; considering the *method of preparation*: simple, successive or multiple extracts.

Specific biologically active compounds are obtained from the vegetal products (different parts of plants or various mixtures of aromatic and medicinal plants) using appropriate solvents. To

obtain water-soluble active substance at a pH close to neutral one (such as acids, bases, salts, sugars, phenols and polyphenols, amino acids, glycosides, gums, tannins, enzymes) water is used as solvent. Given that water is not a good solvent for resins, alkaloid, and oils - type compounds, etc., for obtaining them, alkaline or acidified water can be used. By-products as volatile oils, pigments, lecithin, resins, etc., are obtained using alcohol as a solvent (alcoholic or hydroalcoholic extracts). To prepare extractive solutions, different concentrations are used, ensuring the best yield, but also different solvents, depending on the nature of the substance to be extracted or the nature of raw material. When preparing extracts, it must be taken into account the influence of the following factors: *nature of the solvent* (water for salts of alkaloids, glycosides, sugars, proteins, enzymes, tannins, etc.; alcohol 50 or 70% for volatile oils, hydrocarbons, tannins, alkaloids bases and their salts, glycosides, resins, chlorophyll, etc.; ethyl ether for alkaloid bases, resins, volatile oils, etc.; ethanol for polyphenols, reducing compounds, alkaloids, salts, amino acids, polyphenolic glycosides, sterol glycosides), *the shredding degree of plant*, *the ratio between the amount of plant and solvent, time and temperature*. The extraction techniques can be *batch processes* (maceration, percolation, infusion, decoction, accelerated solvent extraction, microwave-assisted extraction, supercritical fluid extraction) or *continuous processes* (organic solvents continuous extraction, continuous percolation, Soxhlet extraction) [10].

Maceration is the extraction process for slightly soluble and thermosensitive principles, which consists of treating the vegetal product with a solvent, for well-established time periods, followed by separation by filtration or decantation; maceration time depends on the type of used solvent. *Percolation* is the cold extraction process using solvents in counterflow. *Infusion* is used for the extraction of compounds that are not affected by high temperature and consists of wetting the shredded vegetal material with water, followed by the addition of boiling water and leaving them in contact for a specific period of time (usually 30 min). This process is not used for plants containing volatile oils; in this case, the shredded vegetal product is wetted with a diluted alcohol solution, followed by the method described above. *Decoction* is a technique commonly used for roots, rhizomes and bark, and it is similar with infusion process: the plant is soaked in cold water, macerated, heated water is added and then brought near boiling temperature (this method is also not suitable for plants containing high content of volatile oils). *Microwave extraction* is the technique that uses microwave energy to heat the solvent and sample, in order to increase the rate of mass transfer between the substances dissolved in sample matrix and solvent, contributing to their easier passage into the solvent [11]. *Ultrasonic extraction* involves the use of ultrasounds with varying frequencies, increasing the permeability of cell walls, thus favouring the extraction of biologically active compounds with considerably yields [12, 13]. *Supercritical fluid extraction* (alternative to classical solvent extraction) is performed with supercritical fluids (CO_2), at critical pressure (74 bar) and low temperature [14, 15]. *Continuous extraction* in special equipments (Soxhlet type) is based on a large difference between the boiling points of the solvent and the targeted compounds, representing a highly efficient method [16]. *Extraction via alcoholic fermentation* is the extraction technology based on fermentation processes and *in situ* generation of alcohol. *Continuous extraction with organic solvents* is based on the continuous solvent recycling in the mass of vegetal material. *Accelerated solvent extraction* is a method based on the use of high temperature and pressure in order to accelerate the kinetics of dissolution and to break the bonds of analyte/matrix interaction. It has the advantage of using smaller amounts of raw materials, resulting in high extraction yields [17]. *Cold extraction* methods is used for plants

with thermosensitive biologically active compounds (maceration, percolation and counterflow extraction); for thermostable active principles, other methods (Soxhlet extraction, decoction, microwave or accelerated solvent extraction, etc.) are selected.

At the basis of extraction of *volatile oils* from vegetal products are their physico-chemical properties, especially high vapour pressure and solubility in non-aqueous volatile solvents and fatty substances. Vegetal materials subjected to extraction can be both fresh and dried, as a whole or fragmented (leaves, grass), crushed (groundwater bodies) or as sawdust (wood). Choosing appropriate extraction method is based on the amount of oil in vegetal product, disposing and physico-chemical properties. For example, by pressing may be processed vegetal materials with high content of volatile oil, for the ones with medium content is preferably to use steam distillation and for the ones with low content—volatile solvents or lipophilic substances extraction [18, 19]. The main methods for *obtaining* volatile oils are as follows: *hydrodistillation* has several disadvantages: esters hydrolysis caused by the high temperature, obtaining other compounds (such as coumarins), oxidation of alcohols, aldehydes and ketones; the main advantage is the direct obtaining of volatile oil; s*team distillation*—oils having superior quality are obtained, compared with the hydrodistillation; *organic solvents extraction*—especially used for obtaining thermosensitive natural compounds that cannot be obtained by steam distillation; the active principles are isolated and then the solvent is removed by distillation; *animal fat extraction* is a technique exclusively used in cosmetics, and it is applied for fresh flowers. This process enables the extraction of natural fragrances without altering the composition, and it is suitable even for small amounts of plant material [20]; *extraction with liquefied gases*—applies especially for flowers, in the cosmetics industry, using high pressure and supercritical fluids. Using this method are obtained better quality and less colourful extracts than those obtained with organic solvents, rich in waxes and fatty components [21–23]. *Pressing extraction* is applied to aromatic and medicinal plants with high content of volatile oils. It is a suitable method to obtain essential oils from citrus fruits and consists of mechanical pressing, obtaining volatile oils but also mucilage, pectin, proteins, liposoluble colorants, etc. *Adsorption on an adsorbent material* is applied for the extraction of odorants from flowers by their adsorption on a substrate such as activated charcoal, alumina, etc. *Purification and separation* of biologically active compounds can be achieved by chromatographic techniques, membrane techniques, as well as liquid-liquid extraction. *Chromatographic techniques* are effective methods of separation and purification of organic compounds, based on the components distribution of a mixture between two phases: one fixed and one mobile; *liquid-liquid extraction* is applied for the separation of compounds of interest from impurities and is based on solubility difference of the extracted component in one or more solvents immiscible or partially miscible between them. *Membrane techniques* are categorized according to the size of particles to be filtered as follows: microfiltration—the process used for the separation of particles ranging in size from 0.1 to 10 µm; ultrafiltration—used for the separation at low pressure of colloidal substances and compounds with molecular weight between 500 and 500,000 Da, such as viruses, bacteria, colloidal substances biomolecules; nanofiltration—process at low pressure through which the molecules with the size of approximately 0.001 µm are removed; reverse osmosis (hyperfiltration)—process where an important factor is the osmotic pressure [24].

2.2. Analytical characterisation of extracts/active compounds

The plants are considered in our days as established sources of pharmaceutical, aromatic and industrial compounds. Various biocompounds gives the colour, odour or therapeutic actions. Used as pure compounds [25], impregnated in different supports [26–28] or used as intermediaries (for example for nanoparticle phytosynthesis) [29], bioactive compounds offers a natural health source. The potential applications of medicinal plants are determined by their compositional profile and possible synergies between those compounds. Nevertheless, as previous stated, the composition of the natural extracts varies with a series of factors. So, a variation in the phytochemical profiles of extract of the same plant, harvested from different areas, in different seasons or using different techniques is inherent [30]. In the following paragraphs, we will present the main methods used for the analytical characterisation of natural extracts.

2.2.1. Phytochemical assays

The phytochemical assays are currently used for the preliminary assessment of the extracts composition, following some major type of compounds, such as sesqui- and monoterpenoids, phenolics, anthocyanins, flavonoids, saponins, oligomeric proanthocyanidins, flavan-3-ols, tannins, o-quinone or other parameters, such as the polyphenol index or the potential browning. *The terpenoids* (especially simpler mono- and sesquiterpenoids) represents the main constituents of the essential oils. The non-volatile di- and tri-terpenoids are usually obtained from plants, tree gums and resins [31]. *Sesquiterpene alcohols* represent substances consisting of 15 carbon atoms and contain an alcohol group. They usually accompany other compounds that are in excess in some oils, having specific antidepressant and sedative action [32]. *Sesquiterpene hydrocarbons* are compounds containing 15 carbon atoms, usually accompanying monoterpenes compounds in the volatile fraction. They possess anti-inflammatory, anti-allergic and emmenagogue action [32]. *Polyphenolic carboxylic acids* belong to the heteroside group, having an acetal structure. To this group belong a series of well-known phenolic heterosides such as rosmarinic, caffeic, gentisic, vanillic, siringic acid [32]. *Triterpenic compounds* are actually triterpenic saponozides with a pentacyclic chemical structure, with immunostimulatory, pharmacodynamic, antimicrobial, anti-inflammatory and hypoglycaemic action [32]. *Saponins* are natural occurring substances with sterol or triterpenic structure that, in colloidal solution with water, foams under stirring [32]. *Flavonoids* are natural occurring phenolic substances containing plant pigments. Their anti-inflammatory, anti-allergic and antioxidant properties are well-established [32]. *Phenolics* represent the naturally occurring compounds with one or more aromatic rings and one or more hydroxyl groups. They are the most abundant secondary metabolites of plants. Their widespread in the plant kingdom makes them also one of the most studied classes of natural occurring compounds. They are of special interest not only due to their antioxidant activity, but also due to their presence in plant foods and beverages [33]. The *anthocyanins* are pigments found in flowers, fruits, leaves, roots that change colour depending on the cell pH. The most known anthocyanins are: peonin, malvin, cyanin, rutin, etc. [32]. *Tannins* are naturally occurring plant compounds with a very complex chemical structure (comprising of several phenolic hydroxyl and carboxyl groups). They possess antidiarrheal, antifungal, antiviral and antiseptic properties [32].

The phytochemical assays usually involve a specific reaction, standard substances and spectrophotometric measurements at specific wavelengths [34]. In the following paragraphs will be presented the most common photochemical assays; it must be mentioned that other particular recipes and standards are also used in the literature. The presented recipes could also be applied for the study of essential oils, with proper dilution in alcohols.

The total sesquiterpenoids are determined through the reaction between 98% acetyl chloride, 70% perchloric acid and the extract. The determinations are performed by scanning the 350–800 nm region and measuring at 608 nm. The standard used for calibration is α-santalol, and the results are presented as μg/mg of extract [34, 35]. *The total monoterpenoids* determination involves the reaction of the extract with 2% vanillin-H_2SO_4 reagent, the heating and cooling of the mixture and reading the absorbance at 608 nm. The most common standard is linalool [34, 36]. The results are presented as μg linalool equivalents. One of the earliest phytochemical assays, the *total phenolics content*, is also the most encountered in the literature. The determination involves the reaction between extract, Folin-Ciocalteu reagent and sodium carbonate. The absorbance is measured at 765 nm with a gallic acid calibration curve [34, 37–39]. The results are presented as gallic acid equivalents (GAE) or as percent [38]. *The total anthocyanins* are determined through the reaction of the extract, n-butanol:HCl (95:5) and 2% $NH_4Fe(SO_4)_2$ solution in 2 M HCl, with the absorbance reading at 550 nm. The standard used is cyanidin chloride [34, 40]. *The total flavonoids* content is determined by the reaction of the extract with ethanol, aluminium chloride (10%), 1 M potassium acetate in bidistilled water [39, 41]. The absorbance is recorded at 415 nm and rutin is used as standard. The results are presented as rutin equivalent. *The total saponins* are determined by the reaction between the extract, 8% vanillin in ethanol and 72% H_2SO_4; the absorbance is recorded at 544 nm, using as standard sapogenin [34]. The results are presented as mg saponins/g dry weight. The determination of *oligomeric proanthocyanidins* involves the reaction of the extract 0.5% vanillin reagent and 4% HCl in methanol. The absorbance is recorded at 500 nm, and catechin is used as standard [34]. The results are presented as catechin equivalents. *Determination of total polyphenols and casein/ BSA(bovine serum albumin)/PVPP (polyvinylpolypyrrolidone)-bound tannins*—the extract diluted with water reacts with BSA or casein or PVPP; polyphenols are determined as above for total phenolics; absorbance is recorded at 720 nm on a catechin calibration curve and the *bound tannins* = (total polyphenols) − (unbound polyphenols); the result are presented as mg catechin/g dry plant extract [34]. *Total flavan-3-ols* are determined from the reaction between the extract and p-dimethylaminocinnamaldehyde (DMACA) (0.1% in 1N HCl in methanol); the absorbance is measured at 640 nm; catechin is used as standard and the results are presented as mg catechin/g dry plant extract [34, 42]. *The polyphenol index, potential browning and soluble o-quinone content* is determined following the same recipe as presented for the total polyphenols and casein/BSA/PVPP-bound tannins, reading the absorbance at 280 nm (for polyphenol index), 320 nm (for potential browning) and 437 nm (soluble *o*-quinone) [34].

2.2.2. Chromatographic methods

The main objective of the analytical methods is to determine both quantitatively and qualitatively the target compounds from plant extract. It is a difficult task since generally an extract contain numerous compounds (some of them being highly labile) with a broad range of polarities,

volatility, molecular weight and quantities. Therefore, it is unrealistic to believe that a complete evaluation of an extract can be performed using a single method. Several aspects must be taken into consideration for appropriate selection of analytical methods: what type of information we need from the sample, amount of sample available for analysis, relative quantities of different components present in the sample. Due to the extract complexity, the analytical tools fitted for this task fall mainly in the chromatographic methods area: thin-layer chromatography (TLC) [43], gas chromatography (GC) [44], high-performance liquid chromatography and capillary electrophoresis (CE) [45]. These techniques show a good performance in plant extract analysis due to simple treatment required for samples and diversity of detectors corresponding to different molecules properties (**Table 1**).

Chromatographic methods represent a suitable choice for nearly all biomolecules that come across in plant extracts. These methods have the advantages of high specificity and also allow us to determine a large number of compounds in a single analysis and to benefit from a high dynamic range. Impact of gas chromatography in biomolecules analysis is somewhat hindered by low thermostability of some of these species which involve the necessity of time consuming and expensive pretreatment of samples, like derivatisation [46, 47].

TLC has been improved in the last years and has a large potential to determine some group of compounds using different detection methods and colouring reagents. Although high-performance thin-layer chromatography (HPTLC) has a good efficiency, the limiting factors in its use remain the low resolution and reproducibility [48, 49]. In contrast, *HPLC* use increase tremendously in the last years due to high efficiency and flexibility. Some certain advantages over other methods are as follows: different separation techniques, large range of HPLC columns suitable for all type of extracts, optimization of measurement using various mobile phases, gradient and isocratic approach extend the options during analysis, reverse phase techniques allow simultaneous analysis of compounds with large differences in properties. In addition, HPLC can be used as preparative or purification method, most analysis require ambient temperature, in most cases analysis are performed in short time interval, large number of detectors and the possibility to be connected in series are available.

Counter-current chromatography (CCC) is a special form of chromatography using only liquid-liquid partition without solid support [50]. This approach give the possibility to overcome some of the classic techniques drawbacks such us: column deactivation, samples contamination, etc. CCC was constantly improved during the years in terms of efficient separation in a short period of time hence the recent development in this field are focused on high-speed

Methods	Target biomolecules	Sample preparation	Detector
GC	Non-polar, thermostable	Difficult, derivatisation	FID, TCD, NPD, MS, HePD
TLC	Large range	Simple	Colour reagents
HPLC	Polar	Simple	UV-VIS, RI, MS, ELSD, NMR, fluorescence
CE	Ionic	Simple	UV, MS

Table 1. Characteristics of the main chromatographic techniques used for the study of natural extracts.

CCC (HSCCC) with two main technology high performance centrifugal partition chromatography (HPCPC), and fast centrifugal partition chromatography (FCPC). The performance of these methods allows the separation of plant extracts with complex matrix such us: separation of non-polar compounds, isolation of vitamin B12, chiral resolution of carboxylic acids, complete resolution of isoleucine, DCCC of anthocyanins, etc.

A further improvement of analytical methods in the biological samples is represented by hyphenation techniques, which are a combination or coupling of two or more analytical techniques using an appropriate interface. Most common link between techniques is represented by separation methods (chromatography) with an online spectroscopic detection technology (mass spectrometry or NMR). Also hyphenation techniques can be extended in both ways: in the separation parts (two separation methods) or in the spectrometry zone (two or more spectrometry methods) such us: SPE-LC-MS, LC-PDA-MS, LC-MS-MS, LC-NMR-MS. Albeit these techniques are more expensive than former methods the advantages overcome the capital costs: fast analysis, better automation, large number of sample processed in a period of time, higher reproducibility, less contamination, etc.

Most common and established hyphenation technique is *GC-MS* with a large number of improvements comparing with the components techniques used separately: direct transfer of components from GC to MS, accurate chemical identification of target compounds, possibility to expand analytical capability using the same equipment (GC-MS-MS) or even GC-MS^n). However, the same restrictions apply for biomolecules as in the case of using GC combined with classic detectors: cannot detect non-volatile, polar and thermally labile compounds, requires time consuming sample preparation (hydrolysis/derivatisation).

LC-MS is another relatively frequently used method that became more and more used in biological samples analysis. LC-MS advantages compared to GC-MS are evident: higher sensitivity and specificity, easy sample preparation (aqueous matrix is frequent but forbidden in GC or GC-MS), co-eluting compounds can be more easily separated, can be applied to detect non-volatile, polar and thermally labile compounds, several mass analysers can be used: quadrupole ion traps, time of flight (TOF), time of flight reflection (TOFR) and ion cyclotron resonance (ICR). Among major disadvantages there are lack of mass spectral libraries, hindering of the analyses by matrix effect, high capital costs and the need for qualified operators [51].

A novel hyphenation technique is represented by *LC-HMRS* (liquid chromatography-high resolution mass spectrometry) with sensitivity sub pictogram level that distinguishes compounds with the same nominal mass but different exact mass, avoids development of time-consuming protocols like in LC-MS/MS and requires operators with less experience. The main advantage of *continuous-flow HPLC-NMR* [52] is its simplicity, although the technique is less sensitive than other hyphenated methods previously described. This is mostly due to the limited residence time of the analytes in the flow cell, but also because the volume of most HPLC peaks exceeds the volume of the NMR flow-cell, thereby placing much of the analyte outside the cell during data acquisition. But still these methods remain an important direction in the development of methods for biological samples characterization.

2.2.3. Other methods

Besides the above presented analytical methods, other techniques are currently applied for the characterisation of natural extracts.

The *UV-Vis absorption spectroscopy* is one of the oldest methods of molecular spectroscopy very adequate in characterizing chemical extracts. This technique can be used both to obtain a general spectra of the extracts, but also for other types of measurements such phytochemicals assays. In the UV-Vis spectrophotometric measurements, the biologically active compounds present specific wavelengths (for phenolic acids adsorption shows between 220 and 280 nm, flavonoids, quinones and furanocoumarins between 290 and 420 nm, chlorophylls 600–660 nm, carotenoids between 400 and 500 nm, sesquiterpenoids at 608 nm and so on) [39, 41, 53]. *Fourier transform infrared spectroscopy* (*FTIR*) is based on the detection of molecular vibrations and is a technique particularly suitable for samples that may contain many pigments, being able to detect both organic functional groups and inorganics [54]. With a spectra library, FTIR can provide considerable information useful to the analysis, due to the presence of peaks in specific regions [29, 39, 55–57]. Very often used complementary with FTIR, *Raman spectroscopy* offers information on the molecular vibrations of a system and can be used for qualitative and quantitative determinations [58, 59]. *Nuclear magnetic resonance spectroscopy* (*NMR*) is used to determine the properties of atoms or molecules by exploiting the magnetic properties of the atomic nuclei. It is currently used for qualitative and quantitative determination on natural extracts, either by itself or combined with other techniques [60, 61]. *Matrix-assisted laser desorption/ionisation* (*MALDI*) represents a technique used in mass spectrometry that allows the analysis of thermally labile and high molecular weight compounds (synthetic polymers, biopolymers, macromolecules, etc.). Most often, for biochemistry applications, MALDI is associated with TOF (time-of-flight mass spectrometer) [62]. Less encountered, the *X-ray diffraction (XRD)* can be used for the identification and quantification of natural crystalline compounds, either from dried extracts or in liquid matrixes [63, 64].

By determining the concentrations of carbon, hydrogen, nitrogen, sulphur or oxygen, the *elemental analysis* technique can be an important step in characterizing chemical extracts, given that it can provide information that can be corroborated with other analyse results to get a complex image of the sample [65]. Very often, the elemental composition of an extract is needed, as the mineral components can enhance the therapeutic applications of plants, while other elements can be hazardous for human health [66]; also, the trace element profile can very well be used to characterize a specific soil-plant system [67]. The mineral composition of the extracts is most often quantified using *inductively coupled plasma* (*ICP*) techniques [68] (with variants *atomic/optic emission spectrometry—ICP-AES/OES* and *mass spectrometry—ICP-MS*) or *atomic absorption spectroscopy* (*AAS*) (also with several variants). Lately, with the development of the technique, *X-ray fluorescence* is also used for the elemental characterisation of extracts, having the advantages of rapid and multi-element analysis, lower analysis costs and the possibility of portable instruments [29].

2.3. *In vitro* protocols for the evaluation of natural extracts

The *in vitro* characterisation methods cover a very large area of applications. The most common application is the evaluation of the *antioxidant activity*. Among the methods for evaluating the antioxidant potential, the most encountered in the literature data are as follows: *DPPH assay*—a method based on the reduction of DPPH-free radical, quantified by absorbance reading at 517 nm [39]. The *Trolox equivalent antioxidant capacity* (*TEAC*) measures the antioxidant capacity of a given substance compared with the standard Trolox. Commonly, the antioxidant

capacity is determined using the ABTS decolourisation assay [69]. *The ferric-reducing ability of plasma* (FRAP) assay measures reduction of the ferric iron induced by antioxidants. The method measures the absorption changes at 593 nm generated by the reduction of it is based on the reduction of the complex of ferric iron and TPTZ to the ferrous form at low pH [70]. *The ferrous oxide-xylenol orange* (FOX) assay directly measures the peroxide-dependent oxidation of Fe(II) to Fe(III) in acidic environment. The absorbance is measured at 560 nm [71]. The *ORAC assay* (oxygen radical absorbance capacity) measures the oxidative degradation of a fluorescent molecule (β-phycoerythrin or fluorescein) by a radical source (AAPH). The antioxidants protect the fluorescent molecule by quenching, thus inhibiting the fluorescence decay [72]. The *HORAC assay* (hydroxyl radical antioxidant capacity) is based on the oxidation of fluorescein by hydroxyl radicals, free radicals being generated by hydrogen peroxide. The hydroxyl radical mediated oxidation of fluorescein is blocked by the antioxidants present in the samples [70]. *TRAP method* (total radical antioxidant potential) is based on the influence of antioxidants on the fluorescence decay of R-phycoerythrin (R-PE) during a controlled peroxidation reaction, using as radical generator ABAP [70]. The total oxyradical scavenging capacity (TOSC) assay measures the decrease in ethylene production caused by antioxidants. In the presence of antioxidants, KMBA inhibits the thermal hydrolysis of ABAP. The determinations are carried out through gas-chromatography. In the last decades, new electrochemical methods emerged having several advantages when compared with the classical methods: sensitivity, fastness, simple and inexpensive instrumentation, small volumes of samples. The electrochemical methods cover a large area of electrochemical techniques [73].

Another very important group of *in vitro* methods is represented by the *antimicrobial assays*. All the antimicrobial techniques involve the determination of the effect of the sample against relevant strains, compared with a negative control (the solvent used for extraction) and a positive control (an appropriate antibiotic). *The agar diffusion test* (or the Kirby-Bauer antibiotic testing) represents a highly standardized assay [74]. The media used is Mueller-Hinton, at a pH between 7.2 and 7.4. The inoculation is made with a 0.5 McFarland broth culture. *The plate-hole diffusion assay* is commonly used to evaluate the antibacterial potential of natural products. The test material diffuses from pre-cut wells through agar seeded with bacteria. The antibacterial effect is evaluated by the clear zone surrounding the well [75]. *The well diffusion assay* is performed in order to establish the antimicrobial potential of extracts both on bacteria and fungi. Few drops of extract are placed in the appropriate wells and, after incubation, the zones of inhibition in millimetres are measured [75]. *The agar dilution method* involves the incorporation of extracts with varying concentrations into an agar medium (using serial two-fold dilutions), followed by inoculation with the test microbial onto the surface of the plate. The minimum inhibitory concentration is determined as the lowest at which the sample completely inhibits the microbial growth [75]. *The broth micro- or macro-dilution* represents one of the basic techniques for establishing the antimicrobial potential of natural extracts. The procedure uses two-fold dilutions of the sample in a liquid growth medium dispensed in tubes of minimum 2 mL (macrodilution) or in 96-well microtitration plate (microdilution). After inoculation, the tubes or plates are incubated under suitable conditions. The MIC determinations for this method are more difficult, so often viewing devices are used for reading microdilution tests. *The liquid-dilution method* can determine whether a sample has a cidal or static action at a certain concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) can be determined using completely inhibited dilution cultures and assessing

growth (static) or no-growth (cidal) after incubation [76]. *The disc diffusion technique* represents a modified version of the Kirby-Bauer assay. It involves the use of discs impregnated with the test substance for the determination of inhibition area, by comparison with a standard substance [75]. *The agar slant scheme* is particularly useful for the storage of bacteria over extended periods. Agar slants also aids at the identification of bacteria by characteristic patterns of movement [77]. In *the cup plate method*, the test samples diffuse from a cup through an agar layer in a Petri dish; after incubation, around the cup appears zones of inhibition. Similarly, *the agar well procedure* involves the formation of wells in the agar media and the addition of samples and standard in the respective wells [77].

The *antiviral potential* of extracts can be evaluated using some cell-based assays. The most encountered techniques are plaque inhibition assay, plaque reduction assay, inhibition of virus-induced cytopathic effect (CPE), virus yield reduction assay and endpoint titration technique (EPTT). *The plaque inhibition assay* is based on the infection of a monolayer of host cells (pre-incubated with a solution containing the sample) with the targeted virus at various concentrations (expressed as plaque forming units—*pfu*); the surface of the layer is also covered with a sample-containing solution. After incubation, the plaques are stained (for example, with neutral red) and counted; the percent of inhibition is calculated by reference to negative control (without sample) [76]. *The plaque reduction assay* implies the mixing of the sample (or controls) with the viral suspension, incubation and subsequently addition of the mixture to host cells. The overlay used in this case is composed of agar or carboxymethyl cellulose. After specific periods of time, the *pfu* are measured by microscopy, fluorescent antibodies or specific dyes [78]. *The virus-induced cytopathic effect* (*CPE*) *assay* is particularly useful for the evaluation of the effect on viruses that induce CPE, but not form plaques. It is based on the detection of structural changes in host cells that are caused by viral invasion, with and without treatment with a non-toxic dose of tested substance [76]. *The virus yield reduction assay* is a two-step procedure. The cells (incubated with the tested materials) are infected with specific viruses. After incubation, the supernatants are collected and the virus titers are determined. *The endpoint titration technique* involves the determination of virus titer reduction, using 2-fold dilutions of the tested substance [76]. For the quantification of viruses, modern methods developed can complete the presented assays: tuneable resistive pulse sensing, flow cytometry, quantitative polymerase chain reaction (*qPCR*), enzyme-linked immunosorbent assay (ELISA), etc. [76].

Unlike the previous presented assays, the *antiparasitic assays* are highly species-specific. Several studies were published, especially covering the tropical infectious diseases, such as leishmaniasis, malaria, African sleeping sickness or Chagas disease [76]. In order to perform the *in vitro* assays, the models used for each illness (*Leishmania donovani*, *Plasmodium falciparum*, *Trypanosoma brucei*, respectively, *Trypanosoma cruzi*) are incubated in specific growth medium (with or without host cells) with the tested materials. By evaluating the parasite multiplication and total parasite burdens (using methods specific for each species), the results can be expressed as % reduction (compared to control), IC_{50} and IC_{90} (50% and 90% inhibitory concentration).

The *cytotoxicity assays* are commonly used in *in vitro* studies. The assays study the effect of tested substances on specific cellular lines. The cytotoxic effect can be manifested in a series of cell fates: the cells may undergo *necrosis* (premature death of cells by lysis) can stop growing and dividing (decrease in cell viability) or they can undergo *apoptosis* (controlled

cell death). As the cytotoxic compounds often compromise the cell membrane integrity, *the cellular membrane integrity assays* are the most common ways to determine the cytotoxicity of specific compounds. The cellular integrity can be determined either by determining the effect produced inside the cells of specific vital dyes (for example, *trypan blue* or *propidium iodide*, dyes that are excluded from the healthy cells) [79] or by monitoring the passing to the outside of substances normally sequestered inside the cells (such as *lactate dehydrogenase—LDH*). Other methods of monitoring the cytotoxicity are the colorimetric methods (MTT assay, XTT assay, MTS assay, sulforhodamine B (SRB) assay, other water-soluble tetrazolium salts—WSTs assay, etc.) [80]. *The neutral red assay* can be also used to measure cell viability, as living cells take it up and concentrate it in the lysosomes. *The protein assay* represented an indirect measurement of cell viability, measuring the protein content of viable cells after washing of the treated plates [80].

The genotoxicity assays represents a very important parameter when proposing new drugs (either of natural origin or synthetic ones), determining aberrations in the affected cells (chromatid and chromosome gaps, chromosome breaks, chromatid deletions, fragmentation, translocation, complex rearrangements, etc.). The most used assays are the *Ames test*, *in vitro chromosome aberration assay*, *in vitro micronucleus test* and *comet assay*. The *Ames test* represents an *in vitro* assay for bacterial gene mutations using strains of *Salmonella typhimurium* developed by Ames et al. [81]. The general procedure involves the spreading of bacteria on agar plate containing small histidine amounts, allowing it to develop for a short time and have the opportunity to mutate. The surviving bacteria after histidine depletion are those that gain the histidine-producing ability trough mutation. The mutagenicity potential of the tested substance will be proportional to the number of colonies observed. In the *in vitro chromosome aberration assay*, cell cultures are exposed to the test substance, with and without an exogenous source of metabolic activation. At specific intervals, the cells are treated with a metaphase-arresting substance, harvested, stained and analysed in order to determine the presence of chromosome aberrations [82]. For the *in vitro micronucleus test*, cell cultures are exposed to the test substance, with and without an exogenous source of metabolic activation. The cells are grown to allow the formation of micronuclei in interphase cells, harvested and analysed for the presence of micronuclei. For the *comet assay* or single cell gel electrophoresis, cells are embedded in agarose on a microscope slide and are lysed, followed by electrophoresis staining and observation by fluorescence microscopy. A particularly interesting application of the cytotoxicity assays is the evaluation of *anticancer potential* of natural products. In order to establish the anticancer effect, the previously presented assays for cell viability are applied on a series of specific cancerous cells, like bladder cancer (i.e. UM-UC-3), breast cancer (MCF-7), colon cancer (HCT116), cervical cancer (HeLa), gastric cancer (BGC823), kidney cancer (M-1), leukaemia (Jurkat), lung cancer (SHP-77), melanoma (A375), pancreatic adenocarcinoma (SW-1990), ovarian cancer (A2780) and others. Many studies present the anti-cancer effects of various natural extracts [83].

2.4. *In vivo* protocols for the evaluation of natural extracts

When exploring crude natural extracts, the use of *in vivo* assays can prove to be problematic, as many of those bioassays can be expensive, time-consuming (turnaround time approximately 2 months) and require high-level expertise. Also, the screening of crude extracts, consisting of

multiple components, can be questionable. Thus, many scientists uses the *in vitro* assays for screening the natural extracts and reserve the *in vivo* assays for purified compounds [84]. The *in vivo* methods currently used can be divided by the biological entities subjected to test into assays using plant systems and assays using animals. Several types of *plant-using assays* are available, i.e. specific-locus tests (using maize, corn, etc.) [85] or *Arabidopsis* multilocus assay [86], appropriate for genetic risk assessment. Cytogenetic and chromosomal aberration tests were developed using *Tradescantia* [87] and, respectively, onions or beans [29]. Also, DNA adducts analysis is applicable to somatic and germinal plant cell systems [88] and cucumber seeds can be used for the study of the germination inhibitory effect [39]. What all those tests have in common is the recognized quality of plants to be very goof indicators of genotoxic and cytotoxic effects.

The *animal-using assays*, in contrast, are much more regulated by the national and international legislation. In Europe, studies involving animals for experimental and other scientific purposes have to be carried out in compliance with EU legislation (Directive 2010/63/EU) [89]. For all *in vivo* methods, the tested samples are administered to the test animals (fishes, mice, rats, etc.) at a definite dosage, described by the specific method. After a period of time, the animals are usually sacrificed and blood or tissues are used for the assay [70]. Practically, for most of the already presented *in vitro* assays, there is an *in vivo* correspondent. However, due to the legislation and moral issues raised by the use of animal *in vivo* models, the current focus is on the development of viable *in vitro* alternatives. Before any *in vivo* study can be performed, *the acute toxicity* of any tested material should be established. This is expressed as the concentration that is lethal to 50% of the test organisms in a specific time interval. The test organism could be vertebrate organisms, but for obvious reasons, very often testing on invertebrate is preferred (for example, the *Daphnia magna assay*) [41]. The animal models can be used for the evaluation of the *antioxidant potential*, in assays such as ferric reducing ability of plasma, reduced glutathione (*GSH*) estimation, glutathione peroxidase (*GSHPx*) estimation, glutathione S-transferase (*GSt*), superoxide dismutase (*SOD*) method, γ-glutamyl transpeptidase activity (*GGT*) assay, glutathione reductase (*GR*) assay, lipid peroxidation (*LPO*) assay, *LDL* assay or catalase assay [70]. For the evaluation of *anti-diabetic potential* of natural extracts, the test animals (usually rodents) are induced diabetes (i.e. with *streptozotocin*) and the variation of peak blood glucose is recorded [90]. The *anti-inflammatory potential* can be evaluated using paw-oedema assay (induced by carrageenin, dextran, kaolin, etc.) and observation of the effect of the tested material on the evolution of the oedema [91]. The *gastroprotective effect* can be determined using animals to which gastric ulcer is induced (usually using absolute ethanol). After treatment, the animals are sacrificed, and gastric tissues are collected to evaluate the ulcers and to measure enzymatic activity [92]. For the evaluation of *anti-microbial* and *anti-viral potential*, the microbial strain or virus is induced to the test animals and the effectiveness of the natural extract is subsequently evaluated [93, 94]. The *antitumor activity* can be determined on tumour bearing animals (i.e. Ehrlich ascites carcinoma-induced or Dalton's ascites lymphoma induced animals). The effect is determined using survival time, haematological studies, lipid peroxidation, solid tumour mass, etc. [95]. The *antipyretic activity* can be evaluated using animals with induced pyrexia (i.e. by Brewer's yeast). The animals' temperatures are measured at specific time intervals after treatment [96]. The *analgesic effect* can be determined using either the hot plate test (pain reflex in response to an external thermal stimulus is observed after treatment) or the acetic acid induced writhing test (after treatment, 0.7% acetic acid is administered and the constriction of abdomen,

turning of trunk and extension of hind legs are observed) [96]. Other several assays can be performed using *in vivo* models, such as *subacute/subchronic oral/dermal toxicity*, *eye/dermal irritation*, *reproduction toxicity*, *chromosome aberration*, *micronucleus assay*, *carcinogenicity*, *aquatic toxicology*, *bioaccumulation*, very often provided by specific companies [97].

3. Evaluation of some traditionally used plants

The following paragraphs will shortly present selected results published by the authors, regarding the obtaining, characterisation and application of natural extracts from selected medicinal and aromatic plants, presented in Section 1.2.

Recently, our group presented the preliminary evaluation of the crude hydroalcoholic extract (50% ethanol) obtained from the upper aerial part of *Heracleum sphondylium* L. subsp. *Sphondylium* [39]. The evaluation consisted of chemical evaluation, as well as antifungal, antioxidant and germination inhibitory properties. The chemical evaluation consisted of UV-Vis, FTIR and GC-MS analyses, as well as phytochemical assays—total phenolics, total terpenoids and total flavonoids. The tested extract revealed good antioxidant potential (determined by the DPPH assay) and good antifungal properties (evaluated by *in vitro* disc diffusion testing, against *Aspergillus niger* and *Penicillium hirsutum*, using miconazole nitrate as positive control). These findings support the traditional use of hogweed as a natural antifungal and antioxidant. The *germination inhibitory effect*, determined on seeds of *Cucumis sativus* Cornichon Wisconsin, recommends its use for the development of environmentally friendly and safe bio-herbicide. The described antifungal and germination inhibitory effects are most probably due to the content in furanocoumarins and total phenolics, as found by analytic determinations.

The essential oils extracted from various parts of *Anethum graveolens* L. (fruits, flowers and leaves), characterised by gas chromatography [98], TLC or phytochemical assays [99], were the subject of several pharamacognostic and applications studies previously published, revealing not only their composition [98, 99], consisting of flavonoids (aglycones and glycosides), hydroxycinnamic acid derivatives, coumarins, sterols, terpenoids (e.g. carvone) and mucilages, but also the antifungal activity (against *Candida albicans* [98]) determined by disk diffusion method, using as positive standard miconazole impregnated disc) and the possibilities of incorporating the essential oils in liposomal delivery vehicles [100–102]. The entrapment of *A. graveolens* essential oils in such delivery vehicles represents a very important step in developing topical pharmaceuticals capitalizing their good antimicrobial properties.

The hydroalcoholic extracts (40% alcohol) obtained from the leaves of *Taraxacum officinalis* (L.) Weber ex F.H. Wigg and roots of *Arctium lappa* L. were characterised using HPLC, phytochemical assays and absorption emission atomic spectrometry (to determine the mineral content) [7]. The content in total polyphenols and total flavone derivatives was found to be higher in the burdock extract compared with the dandelion extract. Several polyphenol carboxylic acids (caffeic, chlorogenic, ferulic, cichoric, cinaric) and flavone derivatives (rutin, quercetrin, quercetin, luteolin, apigenin) were identified in the two extracts by means of HPLC. Also, several minerals (Ca, Mg, Na, K, Mn, Fe, Zn, Cu) were quantified, minerals that could offer the extracts antioxidant properties [7]. The extracts exhibited bactericidal effects against *Escherichia coli* and *Salmonella abony enterica* [8].

Anthriscus with two variations (wild chervil—*A. sylvestris* (L.) Hoffm. and garden chervil—*A. cerefolium* (L.) Hoffm.) represented the subject of an ethnomedicinal and a phytochemical and pharmacological study [4] and of a paper dealing with the obtaining, characterisation and applications of hydroalcoholic extracts (50% ethanol) [41]. The previously mentioned review paper [4] contained the botanical and ethnomedicinal presentation of *A. sylvestris*, the phytochemical profile of its roots, aerial parts and fruits, and its possible applications, such as antitumor, anti-microbial, anti-inflammatory, antioxidant and biotechnology applications. *A. cerefolium* extracts were obtained from the aerial parts, via classical hydroalcoholic method and microwave extraction [41]. The extracts were characterised using UV-Vis, GC-MS and phytochemical assays. The sample obtained by microwave extraction was not only richer in terpenoids, flavonoids and phenolic compounds, but also had a better antifungal (against *A. niger* and *P. hirsutum* fungal lines) and antioxidant activity (as determined by the DPPH assay and a chemiluminescence method). Finally, the paper described the applications of the extracts in nanotechnology, for the phytosynthesis of silver nanoparticles. The nanoparticles obtained using the microwaves-extract had smaller dimensions (evaluated by UV-Vis, SEM and TEM) and enhanced antifungal and antioxidant properties. The phytosynthesised nanoparticles presented a relatively good stability in time; also, no evidence of toxicity exhibited by the silver nanoparticles was shown by an *in vivo* toxicity assay (*Daphnia magna* bioassay) [41].

The present work aims not to exhaustively present the obtaining and characterisation methods of natural extracts, but to bring its contribution to the field of phytochemistry, by addressing the most common methods for obtaining/characterisation of natural products, supported by some examples from our previously published works.

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