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Review

Current Trends in Toxicity Assessment of Herbal Medicines: A Narrative Review

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Abstract: Even in modern times, the popularity level of medicinal plants and herbal medicines in therapy is still high. The World Health Organization estimates that 80% of the population in developing countries uses these types of remedies. Even though herbal medicine products are usually perceived as low risk, their potential health risks should be carefully assessed. Several factors can cause the toxicity of herbal medicine products: plant components or metabolites with a toxic potential, adulteration, environmental pollutants (heavy metals, pesticides), or contamination of microorganisms (toxigenic fungi). Their correct evaluation is essential for the patient's safety. The toxicity assessment of herbal medicine combines in vitro and in vivo methods, but in the past decades, several new techniques emerged besides conventional methods. The use of omics has become a valuable research tool for prediction and toxicity evaluation, while DNA sequencing can be used successfully to detect contaminants and adulteration. The use of invertebrate models (*Danio renio* or *Galleria mellonella*) became popular due to the ethical issues associated with vertebrate models. The aim of the present article is to provide an overview of the current trends and methods used to investigate the toxic potential of herbal medicinal products and the challenges in this research field.

Keywords: contamination; DNA sequencing; genotoxicity; omics; standardization

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1. Introduction

Herbal medicine has always represented an important component of primary health care. It is estimated that approximately 80% of the world's population uses herbal medicinal products for their therapeutic virtues [1–3]. The industry of phyto-preparations experienced a high growth rate during the last decades, leading to a wide variety of products circulating in the market, used in both alternative and complementary medicine. In the context of a high demand from consumers, there is an increased pressure to assess the efficacy of the products also to ensure their safety. The burgeoning demand was also accompanied by the escalation of the rate of fraudulent practices (e.g., the substitution of herbal material and the addition of synthetic compounds) [1,4], so standardization and quality control processes are of most importance. However, in some cases, the possible negative effects are not only attributed to adulteration, contamination, or misidentification of plant species but are related to the inherent toxicity of the plants. To address this aspect, an attentive toxicological assessment is required to eliminate potential safety concerns. Last, but not least, the adverse effects can be caused by foreign contaminants—chemicals (e.g., pesticide residues, heavy metals) or microbiological [5]. This review focuses on presenting the

current strategies and techniques used and recommended by regulatory authorities to investigate the authenticity and toxicity of medicinal herbal products. The data collection was performed using worldwide databases, including Scopus, PubMed, Web of Science, Google Scholar, and Science Direct. The analyzed literature sources included original articles, review articles, and books.

2. The Importance of Standardization for Safety and Toxicity Profiling of Herbal Medicine—Assuring the Herbs' Authenticity

Research performed during the last decades has pointed out the problems associated with the poor quality of the herbal medicinal products available on the market. Some of these issues are caused by species substitution and adulteration. Newmaster et al. tested several herbal products marketed in North America and found a considerable rate of product substitution, contamination, and use of fillers not listed on the label [6].

In some cases, contaminations and substitutions can cause important adverse reactions for consumers. For example, *Senna alexandrina* was detected in some *Hypericum perforatum* products, and the sennosides could determine a laxative effect, with diarrhea and abdominal pain [6]. *Juglans nigra* was also found in *Ginkgo* and *Echinacea* products. *Juglans nigra* contains a toxic compound (juglone), and furthermore, the contamination can be very dangerous for persons with nut allergies [6,7]. The substitution of *Stephania tetrandra* ("hang fang ji") with *Aristolochia fangchi* ("guang fang ji") in traditional Chinese medicine products may lead to renal toxicity and cancer, due to the presence of aristolochic acid, a carcinogenic and nephrotoxic agent [8,9]. *Datura stramonium* is a plant species frequently used in Ayurvedic medicine. In case of adulteration with *Brugmansia arborea*, an anticholinergic toxidrome can appear, with potentially serious outcomes for the patients [10,11].

In light of the above, the accurate identification of medicinal plant materials is essential for their safety profile. For consumer protection, it is imperative to authenticate the raw herbal material and also the final marketed products. Morphological, microscopic, and chemical identification are the traditional methods used for authentication, but other new and innovative techniques have emerged and gained popularity in the last decade [12].

2.1. Morphological Identification

For accurate morphological identification, the expertise of professional taxonomists is of utmost importance, but there are some significant limitations associated with morphological diagnosis. For example, morphological keys are often valid for plants in the flower or fruit stage. Furthermore, phenotypic and genetic variations can affect the morphology of the plants and cause misidentification [13].

2.2. The Metabolomics Approach in Herbal Medicinal Products Identification and Standardization-Chemical Fingerprinting

Metabolomics represents an omics-based approach for both qualitative and quantitative assessment of metabolites in a living system. The metabolome comprises only organic compounds with low molecular masses (called metabolites) and no polymerized structures (macromolecules) [14]. Plants produce a wide variety of phytometabolites (Figure 1). The secondary metabolites play different roles in the physiology of the plants (self-defense, environmental adaptation), but they also exhibit several pharmacological actions and are active compounds used in the treatment of human diseases. Secondary metabolites can be species-specific, and their investigation can be used in the identification and quality control of herbal medicinal products [14–16].

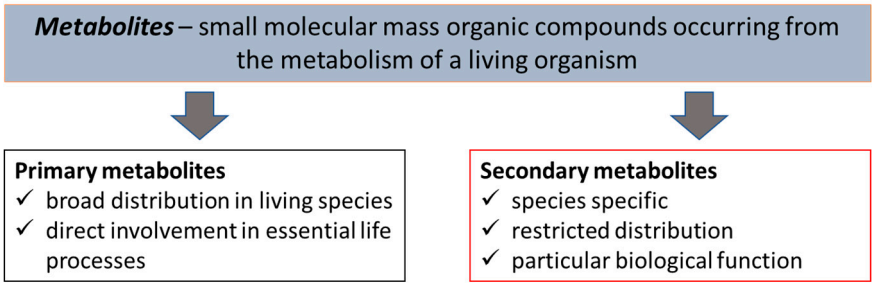

Figure 1. Classification and characteristics of metabolites in a living organism.

Metabolomics has become an important tool for assessing the quality and safety of herbal medicinal products, and it addresses the need for reliable methods that evaluate herbal products' authenticity and help discriminate between samples [17,18].

There are two approaches in metabolomics—targeted (only a specific group of known metabolites are investigated) and global (non-targeted—all metabolites are analyzed). Analytical chemistry and biostatistics play essential roles in modern metabolomics. The progress made in the field of analytical techniques had a remarkable impact on the profiling of secondary plant metabolites [14–16]. Several studies used metabolomics (alone or jointly with other methods) to investigate the identity and authenticity of plant species in different herbal products [19–22]. For example, Fukuda et al. recommend licochalcone A as a candidate biomarker to distinguish between *Glycyrrhiza* species [22].

Analytical techniques like high-performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC) with a diode-array detector (DAD), high performance liquid chromatography (HPLC)—high-resolution mass spectrometry (HRMS), capillary electrophoresis (CE), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), ultra-high-performance liquid chromatography (UHPLC) connected with OrbiTrap mass spectrometry (MS), rapid resolution liquid chromatography (RRLC)—quadrupole time-of-flight mass spectrometry (QTOF-MS), electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS), matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), supercritical fluid chromatography (SFC), liquid chromatography-nuclear magnetic resonance (LC-NMR), Fourier-transform infrared spectroscopy (FTIR), UV-Visible spectroscopy, NMR spectroscopy, Laser-induced breakdown spectroscopy (LIBS), and Raman spectroscopy have been successfully used for phytometabolites assessment (Table 1).

Table 1. Examples of metabolomic approaches for plant species authentication and differentiation.

Analyzed Material	Analytical Technique Used	Reference
Achillea millefolium	TLC HPLC-UV	[23]
Anemone spp.	HPLC-HR-ESI-MS/MS	[24]
Allium spp.	NMR HPLC–MS	[25]
Alisma plantago-aquatica and *A. orientale*	UHPLC/Orbitrap-MS	[26]
Aphanamixis polystachya	HPTLC	[27]
Asparagus officinalis	RP-HPLC-ESI-QTOF/MS/MS	[28]
Avicennia marina	HPTLC	[29]
Bacopa monnieri	HPLC-PAD HPLC-UV	[30] [31] [32]

Table 1. *Cont.*

Analyzed Material	Analytical Technique Used	Reference
Calendula officinalis	TLC HPLC-UV	[23]
Centella asiatica	HPLC-PAD	[30]
Cinnamomum spp.	UHPLC-HRMS	[33]
Crocus sativus	^{1}H-NMR	[34]
Curcuma spp.	TLC ^{1}H-NMR	[35–37]
Dendrobium spp.	2D NMR	[38]
Duboisia spp.	^{1}H-NMR HPLC-MS	[39]
Ephedra spp.	HPTLC GC–MS	[40]
	^{1}H-NMR	[41]
Ficus spp.	FTIR, GC-MS NMR	[42]
Fritillaria spp.	MALDI-MS	[43]
Gastrodia eleta	HPLC-DAD-MS	[44]
Ginkgo biloba	LC–MS	[45]
Glossostemon bruguier	GC-MS NMR	[46]
Glycyrrhiza spp.	HPTLC NMR	[47]
	GC-MS LC-MS ^{1}H NMR	[48]
	HPLC-HRMS	[49]
	HPTLC HPLC	[50]
	LC-UV LC-MS-MS	[51]
Hedera helix subspp.	HPTLC- image analysis HPTLC-MS	[52]
	HPLC CE	[53]
Hibiscus sabdariffa	TLC HPLC-UV	[23]
Iris domestica, *I. tectorum* and *I. dichotoma*	HPLC–DAD-CL ESI-QTOF-MS/MS	[54]
Lonicera spp.	RRLC-QTOF-MS	[55]
Matricaria recutita	TLC HPLC-UV	[23]
Mentha spp.	^{1}H-NMR	[56]
Ocimum sanctum	TLC FTIR ^{1}H-NMR	[57]

Table 1. *Cont.*

Analyzed Material	Analytical Technique Used	Reference
Panax ginseng	^{1}H NMR	[58]
Phyllanthus spp.	FTIR NMR	[59]
Uncaria spp.	UHPLC/QTOF-MS	[21]
Zingiber officinale	HPTLC (digitally-enhanced method)	[60]

Abbreviations: 1H-NMR, Proton nuclear magnetic resonance spectroscopy; 2D NMR, Two-dimensional nuclear magnetic resonance spectroscopy; CE, Capillary electrophoresis; ESI-QTOF-MS/MS, Electrospray ionization-Quadrupole time-of-flight-Mass spectrometry; FTIR, Fourier-transform infrared spectroscopy; GC-MS, Gas chromatography–mass spectrometry; HPLC-DAD-CL, High-performance liquid chromatography with diode array detection coupled with chemiluminescent detection; HPLC-DAD-MS, High-performance liquid chromatography coupled with diode array detector and tandem mass spectrometry; HPLC-HR-ESI-MS/MS, High-performance liquid chromatography-High resolution electrospray ionization-Mass spectrometry; HPLC-HRMS, High-performance liquid chromatography-High resolution mass spectrometry; HPLC-MS, High-performance liquid chromatography-Mass spectrometry; HPLC-PAD, High-performance liquid chromatography with Photodiode Array Detector; HPLC-UV, High-performance liquid chromatography with Ultraviolet detection; HPTLC, High-performance thin-layer chromatography; LC-MS, Liquid chromatography–mass spectrometry; LC-UV, Liquid chromatography with Ultraviolet detection; MALDI-MS, Matrix-assisted laser desorption/ionization-Mass spectrometry; NMR, Nuclear magnetic resonance spectroscopy; RP-HPLC-ESI-QTOF/MS/MS, Reverse phase High-performance liquid chromatography-Electrospray ionization- Quadrupole time-of-flight-Mass spectrometry; RRLC-QTOF-MS, Rapid resolution liquid chromatography-Quadrupole time-of-flight-Mass spectrometry; TLC, Thin layer chromatography; UHPLC/Orbitrap-MS, Ultra-high-performance liquid chromatography-Orbitrap-Mass spectrometry; UHPLC/QTOF-MS, Ultra-high-performance liquid chromatography-Quadrupole time-of-flight-Mass spectrometry; UHPLC-HRMS, Ultra-high-performance liquid chromatography-High resolution mass spectrometry.

2.3. DNA-Based Techniques for Herbal Products Authentication

Traditional methods used for the authentication of medicinal plants have limitations. In the case of plant species that are morphologically and phytochemically indistinguishable, DNA fingerprinting has become an indispensable and trustworthy tool for the authentication of herbal drugs for the reason that the genomic composition of each plant species is unique and specific. The DNA molecule is stable, and it is not affected by external factors (physiological and storage conditions). DNA sequencing methods allow the identification of the precise order of nucleotides in a DNA sample. These methods have good repeatability and high universality and are rapidly gaining popularity. The British, the United States, Japanese, and Chinese Pharmacopoeias have introduced guidelines for DNA barcoding and approved annotated DNA barcodes for the individual identification of some plant species [4,61,62]. In the USA, in 2016, the New York State Office of the Attorney General came to an agreement with herbal supplement manufacturers regarding the use of DNA barcoding during quality control stages [63].

DNA-based identification techniques can be applied for herbal material, but also for final herbal medicinal products [64]. However, the greater levels of gene tree paraphyly in plants make the task of distinguishing between species more challenging in comparison to animals [65].

DNA barcoding is a micro-genomic identification technique used for different biological samples. It analyzes the variability of a standard DNA region. After the amplification of a relatively short (<1000 bp) DNA sequence (from nuclear or organelle genomes), bar-code-like patterns are generated. The DNA sequence fragment is considered a molecular marker for species identification [16,66]. The standardization of the method can be achieved by creating unified databases and identification platforms [13].

Several barcode libraries are currently available: The barcode of life data system (BOLD), Consortium for the barcode of life (CBOL), Medicinal Materials DNA Barcode Database (MMDBD), International Barcode of Life project (iBOL), GenBank (NCBI), Traditional Chinese Medicine Database [66].

While in the animal kingdom, the mitochondrial genome (mitochondrial cytochrome c oxidase unit I—COI) proved to be a fine option for the generation of DNA barcodes [67],

for plant barcoding, the chloroplast and nuclear genomes were found to be suitable for identification, due to higher nucleotide substitution rates. Hypothetically, *matK* (maturase K gene) is the closest plant analogue of COI due to its high rate of evolution, but despite some successful experiments [68], no single locus can be used as a universal plant barcode. The choice of barcodes represents a challenge in the authentication process, and the evaluation of the efficiency of different candidate barcodes led to the conclusion that a combination of two or more loci is preferred to single-locus for plant barcoding. However, since the three (or more)-loci approach did not show significant improvement in the species discrimination process, to avoid additional expenses, the standard barcode for land plants is the two-loci barcode approach [65].

The ideal barcode should have a rapid enough evolution and sufficient nucleotide sequence variation to distinguish unambiguously between species but also limited intraspecific variation. The conserved regions will function as primers for PCR [4].

Several multi-loci combinations from chloroplast and nuclear genomes were assessed to find the best combination for plant barcoding [66]. The Consortium for the Barcode of Life adopted the combination of chloroplast regions *rbcL* (ribulose-1,5-bisphosphate carboxylase gene) and *matK* (maturase K gene) as DNA barcodes for plants, but several studies have also investigated over DNA barcodes. Tnah et al. used a two-tiered approach (*rbcL* and *trnH-psbA*—chloroplast intergenic spacer) for the authentication of common herbal plants in the tropics, with good species resolution [4].

Chen et al. [69] investigated *trnH-psbA*, *matK*, *rbcL*, *rpoC1*, *ycf5*, *ITS2*, and *ITS* as the best candidate DNA barcodes suitable for medicinal plant identification. They established that the rate of successful identification at the species level (92.7%) using *ITS2* was superior to that of *rbcL* and *matK*, therefore validating *ITS2* as a standard DNA barcode for plant identification [69]. Their findings were also supported by Miao et al., who identified *ITS2* as a universal barcode that can be used for the identification of poisonous medicinal plants found in the Chinese Pharmacopeia, with a similar rate of correct identification (92.59%) [13]. The findings of other studies also support the use of *ITS2* as a barcode for the identification of medicinal plant species and confirm its effectiveness. *ITS2* is a short sequence (200–230 bp on average) with rapid concerted evolution [70–73]. However, *ITS2* cannot be used for the global identification of plants due to potential intragenomic variations of *ITS* copies within individuals, which can cause incorrect identification of species [70,74].

At present, the best option for unequivocal plant species identification consists in an approach that combines noncoding intergenic spacers (e.g., *ITS*, *trnH-psbA*) and plastidial coding sequences (e.g., *matK*, *rbcL*) as barcodes [75].

Traditional DNA barcoding can be difficult to use in the case of highly processed medicinal plant products or on poorly preserved samples since the degradation of the DNA can affect the polymerase chain reaction [75,76].

Pawar et al. used the nuclear *ITS2* gene and the chloroplast gene *trnH-psbA* to investigate the authenticity of some herbal dietary supplements, and they pointed out the problems of using traditional DNA barcoding with complex finished herbal products [77].

DNA mini-barcoding can represent an alternative technique in such cases. DNA mini-barcoding uses shorter ($\leq$200 bp) DNA fragments from standardized *matK* and *rbcL* barcode regions [70]. The mini-barcodes have the advantage of being amplified more rapidly. Furthermore, they are more diversified and allow a more accurate identification. However, the method has some major limitations (e.g., it cannot be applied for the identification of unknown adulterants/ contaminants, and the reduced length of DNA fragments can cause the loss of relevant information) [76]. Several studies successfully used DNA mini-barcode for the identification of plant species in health products [75,78–81]. However, in the case of authentication of highly processed herbal products, the use of analytical techniques to identify key active compounds should come to complete the data collected from DNA barcoding [82].

There are different DNA sequencing methods—the early Sanger method (the chain termination method, developed in the 1970s) and the next-generation high-throughput methods. The applicability of the traditional Sanger sequencing method for the authentication of complex mixtures containing several plant species proved to be limited. Ivanova et al. pointed out that Sanger sequencing can lead to biased and misleading results during the analysis of samples containing multiple species, being unable to resolve the issue of mixed signals. The various stages covered during the production of standardized herbal products (e.g., extractions, fermentations with microorganisms) can determine the degradation of plant DNA or the contamination from foreign DNA sources. Other sources of foreign DNA can be represented by weed species or pollen from neighboring plants that can contaminate the herbal material during harvesting [8,83]. Furthermore, symbiotic, parasitic, and pathogenic plant-fungal interactions can also affect the DNA-based identification process. When the DNA template coming from the contaminant is preferentially amplified, false conclusions can be drawn because only the contaminant DNA is detected. These shortcomings were resolved by the development of next-generation sequencing (NGS) methods. Ivanova et al. elaborated an NGS workflow that enabled the simultaneous detection of plant and fungal DNA and can be used for quality and fungal contamination assessment [83].

The PCR-dependent DNA metabarcoding and PCR-free genome skimming/shotgun metagenomics are two important approaches used for herbal product identification [62].

Conventional DNA barcoding can be used exclusively for the authentication of herbal products containing a single herbal species. DNA metabarcoding (a combination of high-throughput sequencing and DNA barcoding) is applicable for the simultaneous identification of multiple taxa in a sample, suitable for quality control and contaminant species identification in various herbal products [82,84–87]. However, the discriminatory power and the success of the identification with DNA metabarcoding is still a challenge for highly processed herbal products because of the low quality of DNA samples. Metabarcoding of multiple mini-barcode loci can represent an option in such cases [62,88].

NGS can perform simultaneous sequencing of thousands of molecules. This method is appropriate for samples derived from multiple plant species, with varying levels of DNA degradation, even when fillers or contaminants are present, because, with NGS, numerous DNA sequences are determined at the same time, while the traditional Sanger sequencing identification requires only homogeneous DNA sequences. NGS allows the determination of genomic sequences and species identification even at concentrations of 1% in the complex mixture. For a successful analysis, comprehensive and well-developed sequence libraries are essential. On the other hand, the high sensitivity of the technique also requires extra precautions to ensure sterile work conditions and avoid potential contamination of the samples during analysis [8,16,83,89]. NGS was successfully used by Zhang et al. to unambiguously differentiate between *Echinacea* species [90].

To improve the performance of the method, DNA barcoding was integrated with HRM (high-resolution melting) analysis (Bar-HRM). Bar-HRM is a post-PCR detection method of sequence variation without the need for sequencing that uses a DNA-binding fluorescent dye. The dye specifically binds to double-stranded DNA (the previously amplified PCR products), and as the DNA fragments dissociate during the denaturation process with increasing temperatures, the dye is released, and the fluorescent signal diminishes. The DNA "melting" kinetics is monitored, and a melting curve is obtained. The discrimination between species can be performed based on the characteristics (shape and peak) of the distinct melting curves that are generated. In this way, specific species or adulterants that are present in herbal medicinal products can be identified [91–93].

There are several studies that successfully used Bar-HRM for medicinal plant authentication. Costa et al. investigated *ITS1* and *matK* as mini-barcode candidates for the distinction of *Hypericum* species using species-specific PCR and real-time PCR coupled with HRM analysis. Only *matK* was suitable for identification using the PCR-HRM assay due to some degree of intra-species variability displayed by the *ITS1* region [75]. Ounjai

et al. used the hybrid Bar-HRM method to distinguish between species in the Zingiberaceae family [93]. Zhao et al. successfully identified *Ardisia gigantifolia* and its adulterants (*Rhododendron molle* and *Clerodendrum cyrtophyllum*) using the Bar-HRM and a two-loci approach (*ITS2* and *trnH-psbA*) [94].

Other medicinal plant species for whose identification and quality control the Bar-HRM method was applied included *Phyllanthus* spp. [95], *Sideritis* spp. [96], *Paris polyphylla* [97], *Gentiana rhodantha* [98], *Hyoscyamus niger* [99], *Artemisia* spp. [100], *Acanthaceae* spp. [101], *Hippophae* spp. [102], *Panax* spp. [103], and *Eurycoma longifolia* [104].

Due to its effectiveness and reliability in authenticating medicinal herbal material, DNA barcoding represents an area of interest for many research teams. A selection of studies on this topic and their conclusions are summarized in Table 2.

Table 2. A selection of studies using DNA barcoding for herbal material authentication.

Analyzed Material	Analyzed Genomic Region	Conclusion of the Study	Reference
Angelica spp.	*matK*, *ITS*, *ITS2*, *rbcL*, *psbA-trnH*	• *ITS*—DNA barcoding to identify *A. anomala* and *A. dahurica*	[105]
Apiaceae family	*rbcL*, *matK*, *ITS*, *ITS2*, *psbA-trnH*	• *ITS* and *ITS2*—superior results in intra- and interspecific divergence assessments • *ITS*—the highest identification efficiency (73.3%) • *ITS* + *psbA-trnH* combination—82.2% identification efficiency • *ITS/ITS2* + *psbA-trnH*—potential standard DNA barcode for *Apiaceae* identification	[106]
Aquilaria spp.	*matK*, *rbcL*, *rpoB*, *rpoC1*, *psbA-trnH*, *trnL-trnF*, *ITS*, *ITS2*	• *trnL-trnF* + *ITS* / *trnL-trnF* + *ITS2*—the greatest species resolution using the least number of loci combination • *trnL-trnF* + *ITS2*—the best candidate barcode for *Aquilaria* spp. (*ITS2* has a shorter sequence length compared to *ITS* → easier PCR amplification of degraded DNA samples)	[107]
Astragalus spp.	*ITS*	• *ITS*—potential barcode marker for quality control of *Radix Astragali*	[108]
Boerhavia diffusa	*ITS*, *ITS1*, *ITS2*, *psbA-trnH*	• *ITS* and *ITS1*—potential candidate regions for identification and authentication of *B. diffusa* products	[109]
Canadian Arctic Flora (490 vascular plant species—half of the Canadian Arctic flora and 93% of the flora of the Canadian Arctic Archipelago)	*rbcL*, *matK*	• Higher sequence recovery for *rbcL* than *matK* (93% and 81%) • *rbcL*—easier to recover than *matK* from herbarium specimens (92% and 77%).	[110]

Table 2. *Cont.*

Analyzed Material	Analyzed Genomic Region	Conclusion of the Study	Reference
Caryophyllales	*ITS2*, *rbcL*, *matK*	• *ITS2*—the most successful in distinguishing between examined species to detect the contamination and adulteration	[111]
Codiaeum variegatum	*rbcL*, *matK*	• *rbcL*—more reliable for identification of *C. variegatum* than *matK*	[112]
Crocus spp.	*rbcL*, *matK*, *trnH-psbA*, *ITS*	• *ITS*—the most variable and informative region in *C. sativus* species	[113]
Curcuma spp.	*ITS*, *rbcL*, *matK*	• *rbcL* and *ITS*—100% PCR and sequencing success rate • *matK*—no amplification • *ITS*—ideal locus in discriminating the *Curcuma* species (showed greater variability than *rbcL*).	[114]
Dalbergia spp.	*ITS*, *matK*, *rbcL*	• *ITS* + *matK* + *rbcL*—a suitable barcode combination for identifying Dalbergia spp.	[115]
Dalbergia odorifera and *D. tonkinensis*	*trnH-psbA*	• *trnH*-psbA—proposed as DNA barcode for differentiation between *D. odorifera* and *D. tonkinensis*	[116]
Fabaceae family	*ITS2*	• *ITS2*—efficient and powerful marker and potential barcode to distinguish various species *in* Fabaceae family	[71]
Gentiana spp.	*rbcL*, *matK*, *ITS*, *5S rRNA*, *trnH-psbA*, *trnL-F*, *rpl36-rps8*	• All seven tested loci—able to differentiate medicinal *Gentiana* species from adulterants. • Only *5S rRNA* and *trnL-F*—able to discriminate the closely related species *G. triflora*, *G. scabra* and *G. manshurica*	[117]
Glehnia littoralis	*ITS2*	• *ITS2* distinguished commercial processed *Glehniae* Radix from common herbal adulterants	[118]
Hibiscus spp.	*matK*, *rbcL*, *trnH-psbA*, *ITS2*	• *matK*—differentiation of all the species of *Hibiscus* • Only the two-marker combinations with *matK* differentiated all the species • Better species resolution when the *matK* was in the three-marker combination • *matK*—more suitable than *rbcL*, *trnH-psbA*, *ITS2* for *Hibiscus* spp. identification	[119]

Table 2. *Cont.*

Analyzed Material	Analyzed Genomic Region	Conclusion of the Study	Reference
Hippophae spp.	*ITS2*	• Bar-HRM targeting *ITS2* assay—suitable to identify *Hippophae* species and authenticate commercial sea buckthorn products	[102]
Hypericum spp. *(H. perforatum* and *H. androsaemum)*	*ITS1*, *matK*	• Both regions (*ITS1*, *matK*)—successful in the species-specific PCR identification • Only *matK*—adequate for real-time PCR—HRM analysis differentiation	[75]
Lonicera spp.	*rbcl*, *matK*, *psbA-trnH*, *ITS2*, *ITS*, *trnL*, *trnL-F*	• *psbA-trnH*—the highest interspecific divergence intergenic spacer	[120]
Matricaria recutita	*matK*, *rbcL*, *psbA-trnH*, *ITS*, *ITS2*	• *ITS2*—the maximum genetic diversity • *ITS2*—adequate polymorphic sites to detect interspecific variation with high amplification and sequencing success.	[89]
Momordica spp.	*ITS*, *matK*	• Both regions (*matK*, *ITS*)—good PCR amplification and sequencing results	[121]
Nepenthes spp.	*ITS*, *rbcl*, *matK*	• The combination of *ITS* + *matK*—barcode for *Nepenthes* genus identification	[122]
Panax spp.	*ITS2*, *matK*, *psbA-trnH*	• ITS2 potential specific marker for the identification *Panax* species and their adulterants	[123]
	atpF-atpH, *rbcL*, *rpoB*, *and rpoC1*, *matK*, *psbK-I*, *psbM-trnD*, *rps16 and nad1*, *psbA-trnH*, *ITS*	• *psbA-trnH* and *ITS*—the most variable loci • The combination of *psbA-trnH* and *ITS*—sufficient for identifying all the species and clusters in the genus	[124]
Paris polyphylla	*ITS2*	• *ITS2*—successful for *P. polyphylla* authentication and quality control	[97]
Phyllanthus spp.	*rbcL*, *trnL*	• *trnL* derived primer pair (*PhylltrnL*)—higher specificity and power of discrimination for *Phyllanthus* species than *rbcL* derived primer pair (*PhyllrbcL*)	[95]
	psbA-trnH	• *psbA-trnH* can effectively discriminate *Phyllanthus* species	[125]

Table 2. *Cont.*

Analyzed Material	Analyzed Genomic Region	Conclusion of the Study	Reference
Piper nigrum	*trnL*, *psbA-trnH*	• *trnL*, *psbA-trnH*—suitable for specific detection of contaminants (*Carica papaya*, *Zea mays* and *Capsicum annuum*)	[126]
	psbA-trnH, *rbcL*, *rpoC1*	• *psbA-trnH*—ideal for detection of chilli adulteration in black pepper	[127]
Rhodiola spp.	*rbcL*, *matK*, *trnH-psbA*, *trnL-F*, *ITS*	• *ITS*—the best single-locus barcode (resolved 66% of the *Rhodiola* species) • *rbcL*, *matK*, *trnH-psbA*, *trnL-F*, *ITS* combination—the highest discrimination power, (resolved 80.9% of the species)	[128]
Rhododendron spp.	*rbcL*, *matK*, *psbA-trnH*, *ITS2*	• *psbA-trnH*—DNA marker for identifying the *Rhododendron* species	[129]
Ruta graveolens	*rpoB*, *rpoC1*, *ITS*	• *ITS*, *rpoB*, *rpoC1*—the best markers to differentiate *Ruta graveolens* and *Euphorbia dracunculoides* (adulterant)	[130]
Schisandraceae family	*ITS*, *trnH-psbA*, *matK*, *rbcL*	• *ITS* and *trnH-psbA*—higher species-resolving power than *matK* and *rbcL* • *ITS* + *trnH-psbA* + *matK* + *rbcL*—the most ideal DNA barcode for discriminating the medicinal plants of *Schisandra* and *Kadsura* • *ITS* + *trnH-psbA*—the most suitable barcode for *Illicium* species	[131]
Senna spp.	*trnH-psbA*	• *trnH-psbA*—effective at distinguishing *Senna* species	[132]
Sida cordifolia	*rbcL*, *matK*, *psbA-trnH*, *ITS2*	• *psbA-trnH* and *ITS2*—the best two-marker combination for *S. cordifolia* identification	[133]
Stephania spp.	*ITS*, *ITS2*, *psbA-trnH*, *matK*, *rbcL*, *trnL-F*	• *ITS*—the highest number of variable and informative sites, followed by *psbA-trnH* • *ITS* + *psbA-trnH*—the highest discrimination power	[134]

Table 2. *Cont.*

Analyzed Material	Analyzed Genomic Region	Conclusion of the Study	Reference
Trillium govanianum	*ITS*, *matK*, *trnH-psbA*, *rbcL*	• *ITS*, *matK* and *trnH-psbA*—ideal reference barcodes for *T. govanianum*, *ITS* and *trnH-psbA*—suitable for *Paris. polyphylla* (adulterant)	[2]
Verbenaceae family	*psbA-trnH*, *rbcL*, *matK*, *ITS2*, *ITS*	• *ITS2* and *psbA-trnH*—promising sequence for the identification of the species in Verbenaceae	[135]

Abbreviations: Bar-HRM, Barcode DNA-High Resolution Melting; PCR, Polymerase Chain Reaction; PCR—HRM, Polymerase Chain Reaction-High Resolution Melting.

Sometimes, the toxicity of herbal products can be caused by the replacement of the traditionally prescribed plant part with another part of the same plant species. In these cases, DNA-based techniques are not enough to assess the safety of the products, and other complementary analyses must be used (omics analyses) [65].

Sometimes, herbal dietary supplements have a complex composition, and in these cases, it is best to use an integrative approach for quality control. Thongkhao et al. used NGS, microscopic characterization, and HPTLC techniques to reveal the presence in a commercial product of an adulterant specie unlisted on the label [136].

To sum up, although DNA barcoding and chemical fingerprinting using complex analytical techniques exhibit some challenges and critical aspects, their implementation in the process of quality and safety assessment in the herbal supplements industry is essential for the discovery of fraud and adulteration. Ensuring the authenticity and correct identification of botanical species is a fundamental request for the security of the products.

3. Intrinsic Toxicity Evaluation

The toxicity of medicinal plants and herbal medicinal products is closely related to the presence of bioactive compounds in the plant material and their toxic potential [1]. The issue is even more complicated when it comes to heterogenous, complex mixtures of herbs which can cause unpredictable effects [137].

There are many examples of toxic endogenous compounds in the plant kingdom such as pyrrolizidine alkaloids (hepatotoxic, genotoxic, cytotoxic, phototoxic), furan derivatives (hepatotoxic, possible carcinogenic), epoxy-diterpenoids (hepatotoxic), anthraquinones (hepatotoxic), bis-benzylisoquinoline alkaloids (pulmonary toxicity), alkenylbenzenes (genotoxic, carcinogenic), ginkgolic acids (embryotoxic, cytotoxic, neurotoxic) [138]. In the case of phytometabolites with proven toxic potential (as the ones mentioned above), the regulatory authorities responsible for the quality and safety of herbal medicines have imposed concentration limits. In this context, efforts were made in the field of developing technologies to remove these compounds and efficient methods to detect and assess their concentration (for example, chromatographic methods and immunoassays using monoclonal antibodies) [139–141].

3.1. Acute/Sub-Acute/Chronic Toxicity Evaluation

Toxicological preclinical evaluation of herbal medicines uses both in vitro and in vivo models.

To evaluate acute and chronic toxicity (Figure 2), animal models are usually used. The Organization for Economic Cooperation and Development (OECD) elaborated guidelines for testing the toxicity of chemical compounds.

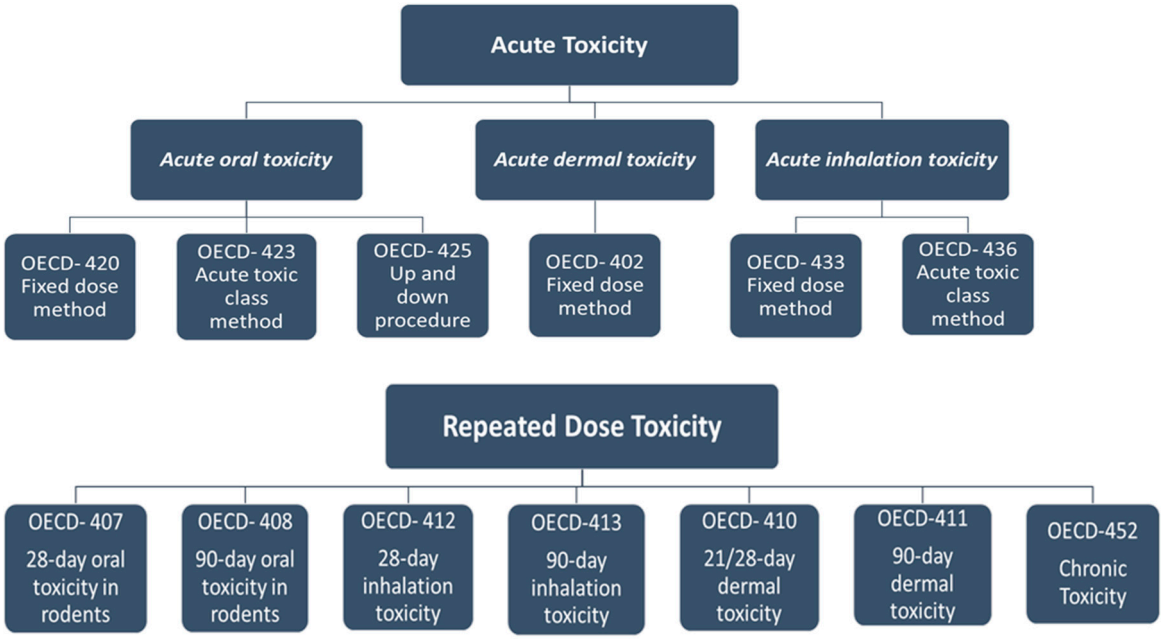

Figure 2. The Organization for Economic Cooperation and Development guidelines for toxicology studies.

Acute toxicity assessment implies the administration of a single dose from the tested product to each test animal. The signs of toxicity and the mortality of the animals are recorded for a period of 14 days in order to determine parameters like the maximally tolerated dose (MTD) and the half-lethal dose (LD50). For the sub-acute and chronic toxicity evaluation, the animal is exposed repeatedly to the tested products daily over a variable period (28 days—OECD 407 [142], 90 days—OECD 408 [143], 12 months—OECD 452 [144]). Toxicity parameters like NOAEL (no observed adverse effect level), NOEL (no observed effect level), LOAEL (lowest observed adverse effect level), and LOEL (lowest observed effect level) can be determined. In the case of medicinal plants and herbal medicine, the oral route of administration is used most frequently, but dermal, intraperitoneal, and inhalation are also available. Exposure via inhalation is usually applied to essential oils, while dermal toxicity is evaluated for herbal medicinal products used in the treatment of dermatological disorders [145–147]. The preferred animal species are rodents (mice or rats), but for dermal exposure, rabbits can also be used [148–154].

In vivo studies are the subject of many ethical issues, and the general tendency is to reduce to a minimum the number of experimental animals. In this context, preclinical toxicology was oriented toward other approaches.

Technological progress has led to the development of in vitro models that are superior in mimicking in vivo conditions than the original ones. These new models have evolved exponentially (Figure 3), from 2D cell cultures and 3D cell cultures to microfluidic devices and miniature artificial organs capable of imitating key elements of human physiology (organ-on-chip systems). By integrating and connecting more organ-on-chip devices, complex systems result in body-on-chip systems that can reproduce complex interactions that take place between different organs inside the organism [155,156].

In 3D-cell culturing systems, some of the disadvantages encountered in 2D cell cultures (e.g., altered cell morphology, modified division process, the lack of interactions between cells and the extracellular environment, homogenous distribution of resources, and unlimited access) have been overcome. Therefore, 3D cell culture models mimic better the in vivo conditions of tissues and offer improved prediction of bioactivity [157].

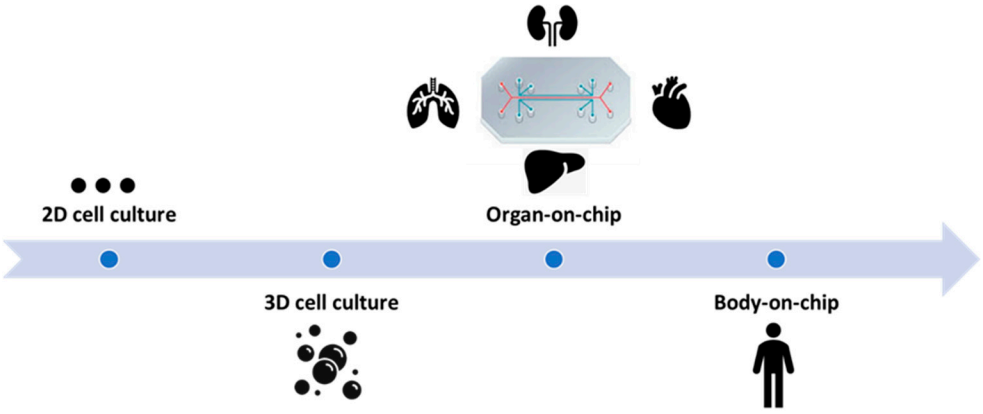

Figure 3. Evolution of in vitro models for toxicology assessment.

3D-tissue models have been used to study the activity of medicinal plants. The EpiAirway™ in vitro human airway model is a potential alternative for in vivo testing to assess inhalation toxicity [158,159]. This 3-D tissue model was previously used in medicinal plant studies and can represent a reliable method to evaluate the effect of herbal products on the integrity of the tissues, cell viability, and preservation of cilia [160].

Another in vitro tissue model is Episkin™ (reconstituted human epidermis), and it can be used as an alternative in dermal toxicity studies [161]. Ng'uni et al. used this method to evaluate the irritation potential of *Galenia africana* [151].

There are not many examples in the literature of studies on medicinal plants (or herbal medicinal products) that use organ-on-chip technology. Chang et al. linked two organ-on-chip systems (a kidney-on-a-chip and a liver-on-a-chip) and investigated the nephrotoxic and carcinogenic potential of aristolochic acid. The mechanisms of the hepatic biotransformation and transport into the kidney tissue were elucidated [162].

However, although these new technologies offer many advantages, and have the potential to revolutionize the field of medicinal products safety evaluation [163], the switch from conventional toxicity assessment methods is currently in a transition phase, with some obstacles to overcome [157].

Respecting the desiderate to restrict the use of higher animals in toxicity studies, alternative invertebrate animal models were explored. Zebrafish (*Danio renio*) assay is a highly used and appreciated toxicity screening tool, being simple and inexpensive. Adult zebrafish can be used, but also embryos, this animal model being suitable for various toxicity tests, including embryotoxicity (Figure 4). One advantage is the transparency of the embryos, which facilitates toxicity examinations during different developmental stages (the visualization of organogenesis is possible using fluorescence and transgenic strains) [164].

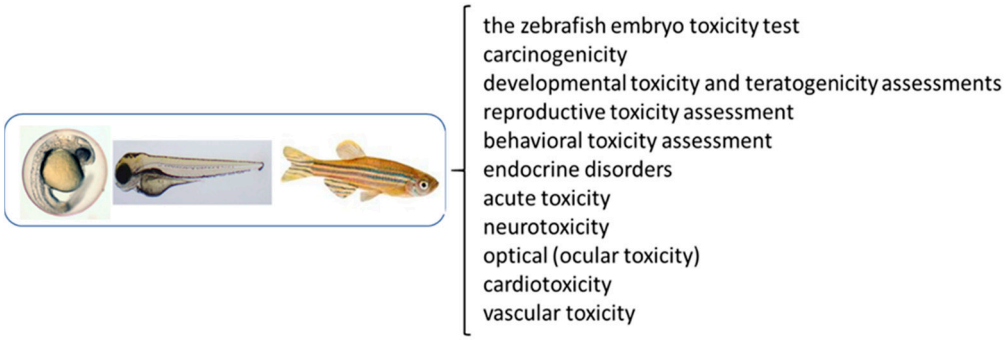

Figure 4. Zebrafish model—applicability for toxicity screening of xenobiotic agents.

The zebrafish model was used for the assessment of different medicinal plants, for example, *Hypericum lanceolatum* [165], *Enydra fluctuans* [166], *Piper sarmentosum* [167], *Grap-*

tophyllum pictum [168], *Murraya koenigii* [169], *Urena lobata* [170] and *Endopleura uchi* [171]. In their review, Modarresi Chahardehi et al. make an up-to-date presentation of studies that used zebrafish for toxicity screening of medicinal plants [164]. Falcão et al. also reviewed this method as an alternative to the embryotoxicity assessment of plant products [172].

Another eco-friendly approach uses an insect model—*Galleria mellonella* (greater wax moth) as a test organism. Mbarga et al. evaluated the acute toxicity of some traditional medicinal plants from Cameroon (*Cymbopogon citratus*, *Moringa oleifera*, *Vernonia amygdalina*, *Cinchona officinalis*, *Enantia chlorantha*, *Garcinia lucida*, and *Azadirachta indica*). Their results pointed out that *Galleria mellonella* can be a potential invertebrate model for toxicity assessment, but further studies are needed [173].

Although much progress has been made in the field of toxicity evaluation, for a reliable prediction of potential risks for humans, in vivo vertebrate toxicity studies remain the gold standard.

3.2. Genotoxicity and Carcinogenicity Evaluation

Genotoxicity is defined as "the property of a compound to induce genetic damage by various mechanisms" [174], and genotoxic agents can be carcinogens and/or mutagens for humans. Therefore, genotoxicity testing is very important for safety assessment. The International Conference on Harmonisation (Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use) recommends a battery of tests for genotoxicity testing (Figure 5), emphasizing that a singular test is not conclusive. There are several indicators of genotoxicity that can be analyzed: mutagenic potential (the ability to induce gene mutations), primary DNA damage, and chromosomal damage (structural and numerical aberrations) [175].

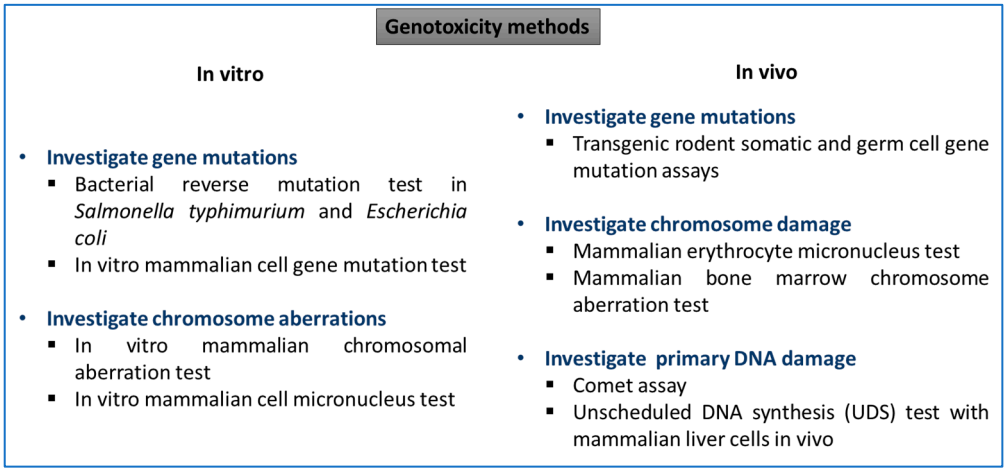

Figure 5. Genotoxicity assessment methods—examples and mechanisms.

Genotoxicity assessment is important for the safety evaluation of herbal medicinal products because there are several examples of plants with phytometabolites that present genotoxic/carcinogenic properties. Da Silva Dantas et al. screened the electronic databases for articles published between 1975 and 2020 that investigated the genotoxic/mutagenic effects of medicinal plants. A significant percentage of medicinal plants presented mutagenic properties, emphasizing the importance of accurate testing for natural products [176]. Plants belonging to the genus of *Symphytum*, *Senecio*, *Crotalaria*, and *Heliotropium* biosynthesize pyrrolizidine alkaloids. The compounds that contain a double bond in the molecule (e.g., retronecine, heliotridine, otonecine) can form 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine-derived DNA adducts, responsible for genotoxicity, explaining tumor induction. Other categories of genotoxic phytometabolites are represented by the alkenylbenzenes from essential oils (e.g., safrole, myristicin, estragole, asarones, and eugenol), anthraquinones (from *Rubia tinctorum* and *Morinda officinalis*), and aristolochic acids [177].

Plant-derived alkylating agents are considered pro-carcinogenic substances; they interact with DNA bases, and if the affected cells do not undergo apoptosis and continue to proliferate, carcinogenesis is initiated [178].

There are different assays (in vitro or in vivo) available to assess genotoxicity, and they cover the different mechanisms of genotoxicity mentioned above (gene mutations, primary DNA damage, and numerical and structural chromosomal damage).

To get a more reliable evaluation, more than one assay is required. The typical assessment is performed using a battery of tests that includes a gene mutation test (on bacteria), another in vitro assay, and an in vivo assay (on mammals). EMEA recommends in its Guideline on the assessment of genotoxicity of herbal substances/preparations the following combination of tests: the Ames test, mouse lymphoma assay (or other mammalian cell assays), and rodent micronucleus test (or other in vivo genotoxicity tests) [176,179]. The EFSA Scientific Committee recommends for the initial screening step a combination of two in vitro tests: the bacterial reverse mutation assay, which covers gene mutations, and the in vitro micronucleus test, which covers chromosome aberrations. If in vitro testing identifies positive genotoxic endpoints, then further in vivo testing is required. The recommended in vivo tests by EFSA are the mammalian erythrocyte micronucleus test, transgenic rodent somatic and germ cell gene mutation assays, and an in vivo Comet assay [180].

Table 3 presents a selection of genotoxicity assessment studies performed on herbal medicinal products.

Table 3. Genotoxicity assessment studies for herbal medicinal products—a selection.

Genotoxic Endpoints	Test	Reference
Gene mutations	Ames (Bacterial Reverse Mutation Test)	[176,181–187]
	Mouse lymphoma assay	[185,187,188]
	In Vitro Mammalian Cell Gene Mutation Tests	[189]
DNA damage	Comet assay	[181,188,190–196]
Chromosomal damage (structural and numerical aberrations)	Chromosome aberration test	[184,197–200]
	Micronucleus assay	[181,185,187,188,192,197,198,201,202]
	Allium cepa test	[182,203–206]

Genotoxicity evaluation using in vitro and in vivo test systems can sometimes generate false positive or false negative results and is not in accordance with the carcinogenic potential identified using in vivo rodent carcinogenicity studies. These differences can be linked to the limited metabolization capability of in vitro systems (in the case of xenobiotics with genotoxic metabolites) but also to the fact that genotoxicity assessment cannot reveal the nongenotoxic carcinogens. The S9 fraction of liver homogenate can be used to simulate mammalian metabolism [207]. On the other hand, a significant percentage of non-carcinogenic compounds gave false positive results in in vitro chromosomal damage assays due to the low specificity of the methods [208,209]. Based on these considerations, Kirkland et al. divided the chemicals into three groups—the first group ("true positives") includes the known mutagenic carcinogens that should give positive results in in vitro genotoxicity tests; the second group ("true negatives") contains non-DNA reactive compounds and should give negative results, while the substances in the third group ("false positive") give positive results in in vitro testing, but the in vivo genotoxicity results are negative. However, some of the compounds in groups two and three can pose risks for humans, being non-genotoxic carcinogens [210].

The refinement of in vitro genotoxicity methods is necessary to avoid excessive in vivo testing and to improve predictive capacity. New evaluation tools were developed (e.g., ToxTracker®, Vitotox®, the TGx-DDI (DDI = DNA damage-inducing) transcriptomic

biomarker assay, in vitro MicroFlow® micronucleus assay, the in vitro CometChip® assay), and a combination of these tools has proven efficient in differentiating between relevant and irrelevant positive results in in vitro genotoxicity assays [208,209,211].

Some of these new genotoxicity tools were also used in the study of medicinal plants. Vitotox®, a test that evaluates the bacterial SOS-response triggered by exposure to genotoxic agents [212], was used to investigate the mutagenic potential of extracts from different medicinal plants [213–215]. The combination of CometChip® and the TGx-DDI biomarker assay was used to evaluate eugenol's ability to induce DNA damage and classified the compound as a DNA damage-inducing (DDI) agent at high concentrations [216].

When it comes to genotoxicity testing for herbal products, there are some specific challenges due to the complexity of the mixtures, usually containing a large number of constituents [177]. Furthermore, the characteristics of plants can also have an important influence on the results. For example, the Bacterial Reverse Mutation Test (Ames assay) is one of the most popular genotoxicity studies used in herbal product research. The test uses histidine-deficient bacteria (*Salmonella*) strains which can be converted into histidine-independent strains by mutagen compounds. But sometimes, the accuracy of these tests is questioned. Verschaeve pointed out that the high histidine content of some plants can influence the outcome of the test, generating false-positive results (the growth of the bacterial colonies can be sustained by the higher concentration of histidine available in the medium and not by histidine reverse mutation). Furthermore, some plants have antibacterial properties, which can affect bacterial growth and influence the results of the Ames test [217].

At present, there are no specific guidelines for the carcinogenicity assessment of herbal medicinal products. The existing OECD 451—Carcinogenicity studies and OECD 453—Combined chronic toxicity/carcinogenicity studies designed for pharmaceutical compounds could be extrapolated to herbal products as well when this is required [177]. There are some examples of older studies that used animal models to investigate the carcinogenicity potential of herbal medicinal products [218,219], but currently, long-term rodent carcinogenicity testing is not frequently used for herbal products [220]. A more recent study is the 2-year bioassay performed by Hoenerhoff et al., where they evaluated the hepatocellular carcinogenicity risk in mice associated with long-term consumption of *Ginkgo biloba* leaf extract, and found considerable evidence connecting ginkgo extracts to oncogenic events [221]. Their findings were further supported by other studies [222,223]. The National Toxicology Program within the US Department of Health and Human Services also conducted several in vivo carcinogenicity studies on animal models (rodents) for herbal medicines (*Aloe vera*, *Panax ginseng*, *Hydrastis canadensis*, *Piper methysticum*, *Silybum marianum*), and for components isolated from different essential oils (e.g., pulegone, αβ-thujone) [224–226]. The issue of herbal medicine with genotoxic and carcinogenic potential is also emphasized by International Agency for Research on Cancer (IARC). *Aloe vera*, *Hydrastis canadensis*, *Ginkgo biloba*, *Piper methysticum*, and pulegone have monographs in Volume 108 of IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (that focuses on the carcinogenicity of herbal products). They have all been classified as possibly carcinogenic to humans (Group 2B) [227].

In summary, as there is clear evidence of genotoxicity and carcinogenicity associated with herbal medicine, this aspect needs to be carefully investigated to ensure the safety of the consumers.

3.3. Omics-Based Toxicology

The increasing demand for the toxicological evaluation of herbal medicinal products has led to the development of various methods for toxicity testing [1]. The *"Omics"* approach (from the Latin "ome"—many, a totality) relies on comprehensive, integrated evaluations [228].

Omics-based toxicology is based on the reflection at the cellular level of the interaction between toxic xenobiotics and biological systems. Toxico-transcriptomics, toxico-

proteomics, and toxico-metabonomics are powerful tools for the toxicity assessment of herbal products (Figure 6). These technologies evaluate the outcome of the interactions between xenobiotics and living cells at different levels. The final goal is to discover the mechanisms behind the effects of a compound [137].

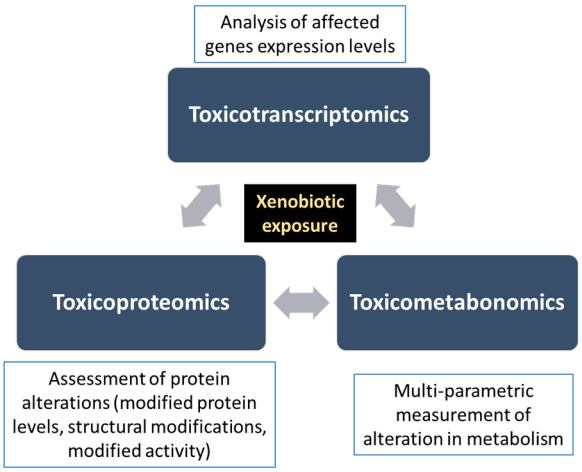

Figure 6. Omics-based approaches in toxicology—evaluation of the interactions between xenobiotics and living cells at different levels.

In some cases, the toxic effects of medicinal plants can be closely related to the metabolic activation inside the exposed organism of the phytometabolites. In these situations, omics technologies can be successfully used to evaluate the toxicity of natural products, identify reactive metabolites of natural molecules, and identify the targeted peptides modified by interaction with these metabolites [138].

3.3.1. Toxico-Transcriptomics

Toxico-transcriptomics investigates the effects of exposure to xenobiotics on genes transcription, toxico-proteomics identifies protein alterations, while toxic-metabonomics analyzes the physiological changes (perturbation of metabolic profile) that appear as a response to the chemical stressors [1].

Transcriptomics studies the RNA transcripts produced by cells under specific circumstances. The transcriptome is very dynamic and sensitive to xenobiotic agents, and the investigation of the genes with significantly affected expression levels after exposure can give insights into the toxicity of the tested agent. Therefore, transcriptome analysis can be considered an early indicator of toxicity [229]. There are different transcriptome technologies: serial analysis of gene expression (SAGE), microarray technologies, and sequencing technologies [230]. One of the most popular techniques used is DNA microarray because it allows the simultaneous monitoring of the expression of thousands of genes, and it is accurate, specific, sensitive, and reproducible. Transcriptomics (toxico-transcriptomics) has been successfully used to elucidate the toxicological effects and mechanisms of natural compounds [231]. For example, the aristolochic acid from *the Aristolochia* species inhibits the nuclear factor-kappa B (NF-κB) signaling in kidney cells, explaining the nephrotoxicity [232]. Emodin, a bioactive compound from *Reum palmatum*, alters the expression of genes involved in the apoptosis of cancer cells, exhibiting anti-cancer properties [233,234]. But, at the same time, with the help of transcriptomics, the testicular toxicity of the compound was explained via the IGF-1 (insulin-like growth factor-1) receptor signaling pathway [235]. Another category of toxic phytometabolites is pyrrolizidine alkaloids, found in species belonging to Boraginaceae, Asteraceae, and Fabaceae families. They exhibit cytotoxic, genotoxic, and carcinogenic potential. Several research teams investigated the liver and lung toxicity of these alkaloids in rats, and transcriptomics analysis revealed altered expression patterns for genes involved in DNA damage response and cell-cycle regulation [236–239].

3.3.2. Toxico-Proteomics

The purpose of this approach is to assess protein alterations as a consequence of exposure to xenobiotics. The changes can consist of modified protein levels, increased/decreased activation of key proteins (for example, apoptosis-related proteins), structural modifications, and post-translational protein modifications (e.g., phosphorylation, glycosylation, acetylation, proteolysis). The proteomics research has different areas of interest: protein profiling proteomics (quantitative evaluation), structural proteomics, functional proteomics, and protein-protein interactions. The identification of biomarkers that are closely connected to the toxicity signature of xenobiotic compounds represents an important issue for the use of proteomics in toxicology [1,240–242].

Several techniques can be used for proteomics studies. The traditional approach is two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry (2D-PAGE-MS) protocol, but, at present, there are also available improved methods, like Matrix-assisted laser desorption/ionization coupled to time-of-flight mass spectrometry (MALDI-TOF-MS) or Surface-enhanced laser desorption/ionization coupled to time-of-flight mass spectrometry (SELDI-TOF-MS) [1,240].

The proteome analysis was used to investigate both the therapeutic and toxicity mechanisms of herbal medicinal products [242–245].

As we previously mentioned, pyrrolizidine alkaloids are a group of highly hepatotoxic phytometabolites. The proteomic approach was used in several studies to investigate the molecular mechanism of hepatotoxicity for these compounds. Among the affected structures are proteins with importance for cellular energy metabolism and whose activity was also related to the oxidative stress process. Carbamoyl-phosphate synthase (CPS1), ATP synthase subunit beta (ATP5B), and thrombospondin 1 (TSP1) were among the affected protein structures [246–248].

The toxicity of aristolochic acids is a subject of interest, and Liu et al. analyzed the changes in proteome profiles in liver and kidney tissues from rats exposed to aristolochic acid. The most downregulated proteins were SEC14-like protein 2 and synaptic vesicle membrane protein VAT-1 homolog [249].

Tripterygium wilfordii is frequently used in Chinese traditional medicine for its immunosuppressant properties, but it is associated with male reproductive toxicity. Dai et al. performed a proteomics analysis of testis tissue samples collected from rats exposed to *Tripterygium* glycosides, and their results confirmed the alteration of proteins involved in sperm production and differentiation. The glycosides exhibited an inhibitory effect on the expression of proteins related to the PI3K-Akt signaling pathway, which can explain reproductive dysfunction [250].

Phosphorylation is one of the most important post-translational modifications suffered by proteins, and it influences many biological processes. Phosphoproteomics (the study of protein phosphorylation) has become a popular tool to evaluate phosphorylation-mediated signaling [251], also used in the field of medicinal plants [252]. Gelsenicine is a neurotoxic alkaloid from *Gelsemium elegans* (Gelsemiaceae). Huang et al. used phosphoproteomics to assess the changes in brain protein phosphorylation. Different brain regions were investigated, but the protein phosphorylation was mainly affected in the hippocampus area [253].

3.3.3. Toxico-Metabonomics

Both metabolomics and metabonomics are used in the scientific community. However, there is still some disagreement regarding the exact differences between the two terms. Metabolomics was defined as a "comprehensive and quantitative analysis of all metabolites". Metabonomics is considered a subset of metabolomics and represents "the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [254–256]. Toxicometabonomics allows the detection of alterations in the homeostasis and metabolic control of the organism secondary to exposure to a toxic compound. The modifications in the

pattern of endogenous metabolites can provide important insight into the targettissues and the mechanism of the toxic action [255]. To sum up, toxico-metabonomics has several applications: to evaluate the levels of endogenous biochemicals potentially affected by the interaction with the toxic agent, to identify the metabolites of the toxic in order to reveal the molecular mechanisms of action and to identify novel biomarkers of toxicity. NMR and MS-coupled with chromatographic techniques are used for metabolic profiling [1,137,256].

Toxico-metabonomics can also be used in the field of herbal medicinal products to evaluate herb/host interactions. Most of the studies use experimental animal models to evaluate the metabolic response of the interaction (Table 4).

Table 4. A selection of toxico-metabonomics studies applied for the potential toxic assessment of herbal medicinal products and bioactive phytometabolites.

Analyzed Sample	Analytical Methods	Aim of the Study	Reference
Aconitum spp. alkaloids	GC-TOF/MS	Identification of altered metabolites associated with induced metabolic disorders	[257]
Aconitum carmichaelii	LC-QTOF-MS	Identification of serum biomarkers of toxicity	[258]
Aurantio-obtusin (anthraquinone from *Cassia* seed)	UPLC-QTOF-MS	Identification of urinary biomarkers associated with hepatotoxicity	[259]
Coptidis rhizoma	^{1}H NMR GC-MS	Investigation of gastro-intestinal toxicity via the alteration of gut microbiota	[260]
Dioscorea bulbifera rhizome	^{1}H NMR GC-MS	Assessment of hepatotoxicity and identification of altered metabolites in blood and tissue samples	[261]
	GC-MS	Identification of altered metabolites from rat plasma, urine, and feces associated with hepatotoxicity	[262]
Dioscorea bulifera root	^{1}H NMR	Prediction of hepatotoxicity based on urinary metabolic perturbations	[263]
Mesaconitine (diterpenoid alkaloid from *Aconitum* spp.)	UPLC-Q-Exactive Orbitrap-MS	Investigation of the hepatotoxicity mechanism	[264]
Pharbitis nil seeds	UPLC-MS	Investigation of changes of urine metabolite biomarkers associated with nephrotoxicity	[265]
Pinellia ternata	UPLC Q-TOF-MS	Investigation of general toxicity (liver, kidney, heart) via profiling serum metabolic alterations	[266]
Polygonum multiflorum	UHPLC-MS	Identification of hepatotoxicity biomarkers and pathways	[267]
	HPLC-MS		[268]
	UPLC-QTOF-MS		[269]
Realgar (Traditional Chinese medicine)	^{1}H NMR	Assessment of hepatotoxicity using plasma and urine samples metabolic profiling	[270]
	^{1}H NMR	Assessment of hepato- and nephrotoxicity via metabonomic analysis of urine, serum, and liver tissue	[271]
Sophora alopecuroides	^{1}H NMR	Evaluation of metabonomic profiling alterations associated with hepatic and renal toxicity	[272]

Table 4. *Cont.*

Analyzed Sample	Analytical Methods	Aim of the Study	Reference
Tripterygium wilfordii	GC-MS	Identification of urine biomarkers to elucidate *Tripterygium wilfordii* poisoning	[273]
Xanthii fructus	UPLC-QTOF-MS	Identification of urinary toxicity biomarkers associated with lipid metabolism alteration	[274]

Abbreviations: 1H-NMR, Proton nuclear magnetic resonance spectroscopy; GC-MS, Gas chromatography–Mass spectrometry; GC-QTOF-MS, Gas chromatography- Time-of-flight-Mass spectrometry; HPLC-MS, High-performance liquid chromatography-Mass spectrometry; LC-QTOF-MS, Liquid chromatography–Time-of-flight-mass spectrometry; UHPLC-MS, Ultra-high-performance liquid chromatography-Mass spectrometry; UPLC-Q-Exactive Orbitrap MS, Ultra-performance liquid chromatography-Quadrupole-Electrostatic field Orbitrap-Mass spectrometry; UPLC-QTOF-MS, Ultra- performance liquid chromatography-Quadrupole time-of-flight-Mass spectrometry.

The evidence presented in the scientific literature supports the fact that omics-based approaches have changed the field of herbal medicine research and are essential tools in toxicity assessment. They allow the identification of toxicity biomarkers and target tissues and reveal relevant details regarding the mechanism of toxicity.

4. Evaluation of Toxicity Arising from Extrinsic Sources

Several external factors can represent a source of toxicity enhancement for herbal medicine (microbial and mycotoxins contamination, pesticides, and fumigation agent residues, radioactive contamination, residual solvents, or toxic heavy metal content—Figure 7).

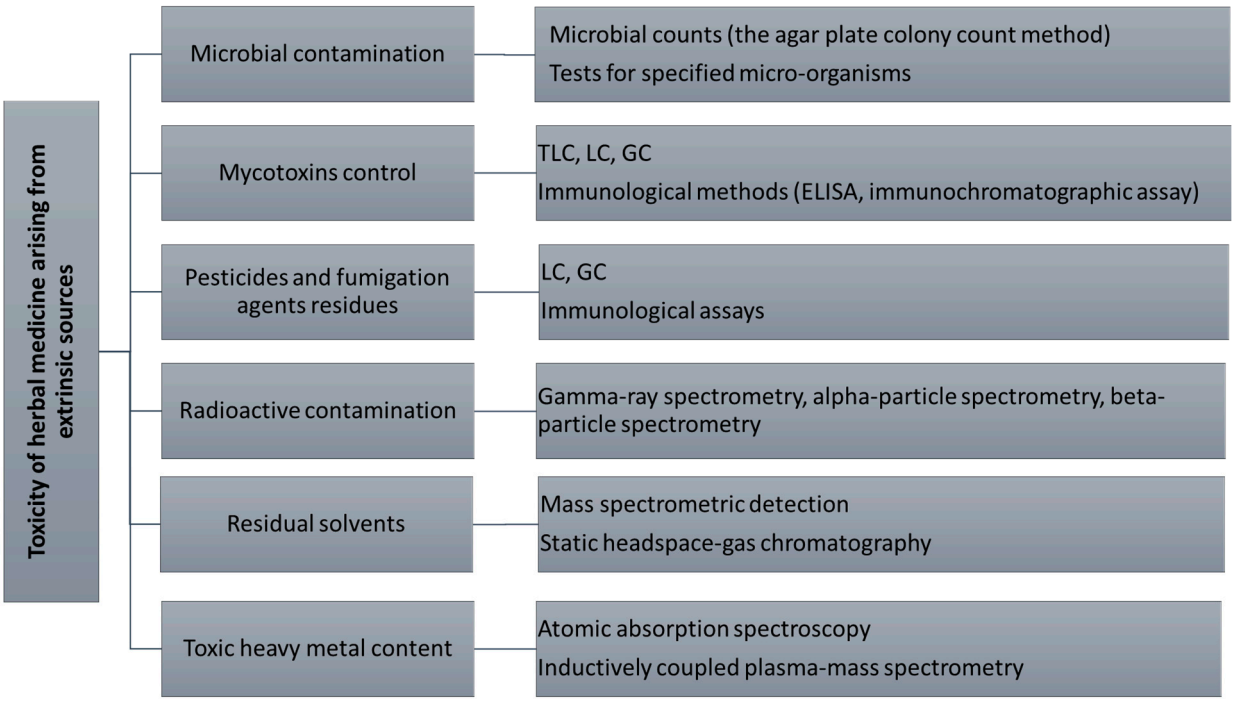

Figure 7. Evaluation of toxicity arising from extrinsic sources—potential contaminants and methods of quantification. Abbreviations: GC, Gas-Chromatography; LC, Liquid-Chromatography; TLC, Thin Layer Chromatography.

Mycotoxins, pesticides, and heavy metals are the most common aspects of concern when it comes to the toxicity of herbal medicine arising from extrinsic sources.

4.1. Mycotoxins Control

Mycotoxins (aflatoxins, ochratoxin A, fumonisins, zearalenone, deoxynivalenol) are secondary metabolites produced by fungal species (e.g., *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, *Claviceps*). Their occurrence in food and medicinal products represents a research subject of interest, due to their highly toxic potential (carcinogenic, mutagenic, neurotoxic, hepatotoxic, nephrotoxic, teratogenic, and immunotoxic). Aflatoxin B1 was included in the Group-1 carcinogen by the IARC and is considered the most toxic mycotoxin [275]. To ensure the safety of consumers, regulations for mycotoxins in herbal medicinal products were established by international organizations and agencies (World Health Organization, European Food Standards Agency, European Medicine Agency, United States Food and Drug Administration). Many research groups investigated the contamination of medicinal herbal products with mycotoxins and revealed that this subject could represent a potentially serious issue of concern because several studies found significant levels of mycotoxins above the maximum admitted limits [276–278].

The detection and quantification of mycotoxins are essential for assuring the safety of herbal medicinal products. Most methods used for mycotoxins assessment rely on chromatographic techniques. In the 1960s, after the discovery of aflatoxins, the traditional method of analysis was TLC, but at present, its usage is limited. However, this technique is still recommended as a screening tool when a large number of samples are investigated, being simple, inexpensive, and rapid [279–282].

The need for the separation of various mycotoxins and accurate qualitative and quantitative determination has shifted the interest towards more performant methods. There are several chromatographic techniques available (HPLC, GC/MS, LC/MS, LC-MS/MS, LC-FLD) that can perform both quantitative and qualitative analyses with high sensitivity, recommended in international pharmacopeias [280,283–288]. Liquid-chromatography techniques are preferred to gas-chromatography because no complex derivatization procedure is required during the analysis, as most mycotoxins are nonvolatile. Chromatographic methods have high specificity and sensitivity and can be used for the simultaneous determination of multiple mycotoxins [280,284,285,289]. However, these methods are more laborious and require several sample preparation steps (extraction and clean-up), and rapid detection technologies were developed for simpler and more convenient monitoring of the mycotoxins' contamination [290].

Immunological techniques developed on the specific affinity of an antibody for an antigen are very popular for rapid mycotoxins screening. Mycotoxins detection using enzyme-linked immunosorbent assay (ELISA) is based on the interaction between the antigen (mycotoxin) and an antibody, and various test kits for the determination of mycotoxins are currently available on the market [280,289]. Shim et al. used ELISA and investigated the aflatoxins levels in 70 types of herbal medicine distributed in South Korea and confirmed the potential risk to consumers [291].

Another alternative is represented by immunochromatographic assays (ICA). Lateral Flow Immunoassay (LFIA) has been successfully used for the detection of a single mycotoxin but also for the simultaneous determination of multiple mycotoxins (e.g., multicomponent assay for aflatoxin B1, ochratoxin A, and zearalenone) [292].

Colloidal gold-based immunochromatographic assay (GICA) uses colored colloidal gold-antibody conjugates, and it is currently a popular method used for mycotoxins detection [280,293–295].

The aptamer-based lateral flow assay is an alternative that presents the advantage of using nucleic acid-based aptamers instead of antibodies. The aptamers showed better resistance to aggressive medium conditions (pH, solvents) and had improved sensitivity and specificity [280,288].

Cytometric bead array is a new flow cytometry technique based on the quantification of the signal of fluorescence-encoded microspheres for the assessment of the desired analytes. It allows a rapid determination of multiple analytes in reduced sample volumes. It has many applications in various biological and environmental samples [296], and it

was also applied to the investigation of mycotoxins. Chang-Bin et al. were the first to use this technique for rapid mycotoxins analysis in the field of medicinal herbal products, investigating the Chinese herb 'Mai Ya' (barley sprout, Malt) [280,297].

The development of simple, rapid, and effective methods to determine and quantify mycotoxins in herbal products is needed to lower the risks to human health.

4.2. Pesticides and Fumigation Agent Residues Control

The presence of pesticide residues in medicinal herbs is another issue of concern. There are different classes of pesticides: organochlorine, organophosphorus, nitrogen-containing pesticides, and pesticides of plant origin [298]. Several studies revealed the presence of these contaminants in herbal products. The most studied types of products are Asian herbal medicines. To ensure the safety of consumers, Maximum Residual Levels (MRLs) were established for pesticides [299]. Wang et al. analyzed 1017 samples of 10 Chinese herbal medicines and determined 168 pesticides. Of the analyzed samples, 76.0% contained multiple residues, and only 10.8% were residue-free. However, they did not classify the potential health risk associated with the consumption of these products as high [300].

Chromatographic methods (GC and HPLC coupled with different detectors) are usually used to carry out the identification and quantification of pesticides from herbal medicinal products. One of the most challenging aspects of the analysis is the preparation of the samples (using extraction and clean-up procedures) because of the complex nature of the herbal matrix, which contains pigments (carotenoids, chlorophyll), phenolic compounds, and essential oils—Table 5 [301–303].

Table 5. A selection of techniques used for the identification and quantification of pesticides in herbal medicinal products.

Analyzed Sample	Purification and Analysis Methods	Reference
Brazilian medicinal plants	SFE HRGC–ECD/FPD	[304]
Cassia angustifolia	QuEChERS GC–MS	[305]
China herbal tea	UPLC-MS/MS coupled with vortex-assisted DLLME	[306]
Commercial market medicinal plant samples in India	QuEChERS GC–MS	[307]
Codonopsis Radix (in China)	QuEChERS-GC–MS/MS QuEChERS-LC–MS/MS	[308]
Fritillaria spp.	QuEChERS LC-MS/MS	[309]
Herba epimedii	SPE GC-MS	[310]
Herbal Decoctions in Traditional Korean Medicine	GC/ECD GC/MSD	[311]
Mentha piperita	QuEChERS GC/ECD/NPD	[312]
Polish herbal raw materials	QuEChERS HPLC–MS/MS	[313]
Polish products of plant origin	QuEChERS HPLC–MS/MS	[314]
A selection of medicine and food herbs	SPLE GC-MS/MS	[315]

Table 5. *Cont.*

Analyzed Sample	Purification and Analysis Methods	Reference
A selection of medicinal plants—*Matricaria chamomilla*, *Tilia* spp., *Pulmonaria* spp., *Melissa* spp., *Mentha piperita*, *Thymus vulgaris*.	MSPD LSE GC	[303]
Traditional Chinese herbal medicines	GC/MS/MS	[302]
	QuEChERS GC-MS with PTV-LVI-SV	[316]
	HPLC-MS/MS GC/MS/MS	[300]
	QuEChERS UHPLC–MS/MS	[301]
	SPE GC-MS	[317]
	GPC GC-MS	[318]

Abbreviations: DLLME, Dispersive liquid-liquid microextraction; GC-ECD-NPD, Gas chromatography coupled to electron capture and nitrogen phosphorus detectors; GC-ECD, Gas chromatography with electron capture detection; GC-MSD, Gas chromatography with mass selective detection; GC–MS, Gas chromatography–Mass spectrometry, GPC, Gel Permeation Chromatography; HPLC–MS-MS, High-performance-liquid chromatography–Mass spectrometry; HRGC–ECD-FPD, High-resolution gas chromatography with electron-capture and flame photometric detection; LC-MS, Liquid chromatography–Mass spectrometry; LSE, Liquid–solid extraction; MSPD, Matrix solid phase dispersion; PTV-LVI-SV, Programmed temperature vaporizer in solvent vent mode; QuEChERS, Quick, Easy, Cheap, Effective, Rugged, and Safe extraction method; SPLE, Selective pressurized liquid extraction; SPE, Solid-phase extraction; SFE, Supercritical fluid extraction; UHPLC–MS-MS, Ultra-high-performance-liquid chromatography–Mass spectrometry; UPLC-MS/MS, Ultra-performance-liquid chromatography–Mass spectrometry.

The chromatographic methods are a sensitive, reliable tool for pesticide analysis, but the development of rapid detection techniques is needed for the efficient screening of herbal medicinal products. Immunological assays were also developed for pesticide residues (for example, IC-ELISA (indirect competitive enzyme-linked immunosorbent assay), and colloidal gold ICA for the detection of organophosphorus pesticides [319,320], ELISA for the monitoring of pyrethroids [321,322], immunosensors and immuno-loop-mediated isothermal amplification assays (iLAMP) for the determination of organophosphates) [323,324]. To expand the number of compounds analyzed simultaneously in a single step, pesticide biochips were also designed [325]. These technologies are widely used in environmental analyses, but they can be successfully applied to medicinal plants or other herbal medicines.

4.3. Heavy Metals Control

Heavy metal contamination can be another safety issue for some herbal medicines, especially Asian traditional medicines. Sometimes, metals are voluntarily incorporated in the formulas for their therapeutic virtues, but in other cases, the contamination occurs via the plants that accumulated heavy metals from the soil, or during the manufacturing process [326].

The presence of heavy metals like Pb, Cd, As, and Hg in herbal medicine in quantities exceeding the maximum permissible limits can cause negative health effects (abdominal discomfort, liver damage, damage to the nervous, renal, and respiratory systems, and skin conditions) [327–329].

The quantification of heavy metal levels in herbal medicine is a subject of interest, approached by many research groups (Table 6). Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), and atomic absorption spectroscopy (AAS) are used for the quantification of heavy metal contamination [330–335]. Luo et al. used ICP-MS and determined cadmium, lead, arsenic, mercury, and copper in 1773 extract samples from 86 different kinds of herbal medicines.

Most products were within acceptable risks, but there were samples where at least one heavy metal was detected to be over the maximum acceptable limit. The authors also pointed out the higher risks associated with As contamination and recommended improved monitoring activity [328].

Table 6. Heavy metals quantification in herbal medicinal products—a selection of studies.

Analyzed Material	Analysis Method	Conclusion of the Study	Reference
Emblica officinalis *Terminalia chebula* *Terminalia belerica* *Withania somnifera*	AAS	• Pb, Cd, Hg, and As were within permissible limits	[327]
A selection of herbal medicines	ICP-MS	• 70.93% of the herbal plants were within the acceptable risks • As posed the highest risk • *Tetradium ruticarpum*, *Plantago asiatica*, and *Desmodium styracifolium*—the highest risk	[328]
A selection of herbal products in Nigeria	AAS	• As and Hg were present in all the samples at concentrations below the USP limits • Cd—above the USP limits in 55% of the samples	[336]
A selection of medicinal herbs from Ghana	AAS	• Fe, Zn, Pb levels were within the WHO maximum permissible limits • For some samples, Cd levels were above the WHO limits	[331]
Petroselinum crispum *Ocimum basilicum* *Salvia officinalis* *Origanum vulgare* *Mentha spicata* *Thymus vulgaris* *Matricaria chamomilla*	AAS	• Most herbs contained levels of heavy metals (Cd, Pb, Cu, Fe, Zn) that exceeded the WHO permissible limits	[332]
A selection of medicinal herbs from India	AAS	• Except for the Cr content in three plants, the levels of As, Pb, Cd, Hg, Ni were below the permissible limits	[333]
A selection of health supplement products	ICP-OES	• The majority of products—low levels of heavy metals • Exception—higher Cd levels for 3 products	[334]
A selection of medicinal plants	AAS	• Pb was present in all plant species, with one exception • 40% of the plant species exceeded the limit for Cd	[335]

Abbreviations: AAS, Atomic Absorption Spectroscopy; ICP-MS, Inductively Coupled Plasma Mass Spectrometry, ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry, USP, United States Pharmacopeia; WHO, World Health Organization.

In the context of environmental pollution, the contamination of herbal medicinal products with different agents can represent a potential health safety concern, and the

development of sensitive, simple methods to identify and quantify contaminants is needed to avoid negative consequences for consumers.

5. Conclusions

In the context of the growing popularity of herbal medicinal products, ensuring the safety of consumers must become a priority for producers and regulatory authorities. The assessment of the toxicologic potential is a complex process, as many factors (both plants -related and environmental) can contribute to the toxicity of medicinal herbal products. The scientific community is making a continuous, joined effort to improve the techniques used for the authentication of herbal species, the detection of harmful phytochemicals, the elucidation of toxicity mechanisms, and the quantification of environmental pollutants in herbal materials.

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