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REVIEW

Poisonous or non-poisonous plants? DNA-based tools and applications for accurate identification

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Abstract Plant exposures are among the most frequently reported cases to poison control centres worldwide. This is a growing condition due to recent societal trends oriented towards the consumption of wild plants as food, cosmetics, or medicine. At least three general causes of plant poisoning can be identified: plant misidentification, introduction of new plant-based supplements and medicines with no controls about their safety, and the lack of regulation for the trading of herbal and phytochemical products. Moreover, an efficient screening for the occurrence of plants poisonous to humans is also desirable at the different stages of the food supply chain: from the raw material to the final transformed product. A rapid diagnosis of intoxication cases is necessary in order to provide the most reliable treatment. However, a precise taxonomic characterization of the ingested species is often challenging. In this review, we provide an overview of the emerging DNA-based tools and technologies to address the issue of poisonous plant identification. Specifically, classic DNA barcoding and its applications using High Resolution Melting (Bar-HRM) ensure high universality and rapid response respectively, whereas High Throughput Sequencing techniques (HTS) provide a complete characterization of plant residues in complex matrices. The pros and cons of each approach have been evaluated with the final aim of proposing a general user's guide to molecular identification directed to different stakeholder categories interested in the diagnostics of poisonous plants.

Keywords Alkaloids · DNA barcoding · Food supply chain · Molecular identification · Poison centres · Secondary metabolites

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What are poisonous plants?

The relationship between plants and animals, including humans, has always been a difficult issue to define due to the high variability of organism interactions, even including their reciprocal effects on individual metabolisms. Being primary producers, plants have always been at the basis of human nutrition, even before domestication and the advent of agriculture. It is estimated that at least 7000 species of plants have been used for consumption in human history, yet fewer than 20 species and related cultivars and varieties now provide 90 % of global food demand (FAO data). Although there are several species with seeds and fruits rich in nutrients, most of these contain indigestible parts or secondary metabolites that could be poisonous to humans and animals. On the whole, plant biodiversity should not be intended as a food resource only, but also as a poison source deserving accurate investigations to prevent human health problems. A frequently asked question is: how we can recognize poisonous plants? There is no simple answer but one of the most adopted approaches is the characterization of toxic metabolites and of their effects on other organisms. The World Health Organization (WHO) identifies four toxicity classes based

on LD50 determination in rats: Class Ia, extremely hazardous (LD50 ≤5 mg/kg bodyweight); Class Ib, highly hazardous (5–50 mg/kg bodyweight); Class II, moderately hazardous (50–500 mg/kg bodyweight); Class III, slightly hazardous (>500 mg/kg bodyweight). Plants that fall into classes Ia and Ib are considered highly poisonous, those assigned to class II as poisonous, and the remaining plants belonging to class III are the least poisonous [1].

Unfortunately, a comprehensive list of worldwide poisonous plants is not available; however, some local institutions provide dedicated classifications. For example, the UK Horticultural Trades Association (HTA) proposed a labelling system for ornamental species to inform the consumers about their poisoning risk. Such system considers three risk categories, namely A, B, and C according to the severity of the potential hazard [2]. HTA's category A includes very dangerous species that are poisonous when eaten and/or commonly cause severe blistering by contact such as *Rhus* species (*Rhus diversiloba*, *Rhus radicans*, *Rhus rydbergii*, *Rhus striata*, *Rhus succedanea*, *Rhus toxicarium*, *Rhus verniciflua*, and *Rhus vernix*). HTA's category B comprises plants mainly provoking poisoning when ingested, such as genera *Aconitum*, *Colchicum*, *Veratrum*, *Taxus*, *Nerium*, *Digitalis*, and *Oenanthe*. Among these, the most dangerous are plants causing cardiovascular effects, like monkshood, yew, and foxglove. Several harmful Solanaceae containing tropane alkaloids are also present, such as *Atropa belladonna*, *Brugmansia*, *Datura*, *Hyoscyamus*, *Mandragora*, and *Solanum dulcamara*, containing different types of steroidal alkaloid glycosides, steroid saponins, and polyhydroxynortropane alkaloids. Finally, HTA's category C includes harmful species causing moderate poison risk depending on the dose and individual response. Indeed, this category includes edible species, such as *Capsicum annum* and *Ficus carica*, which are able to induce skin irritations.

Poisoning from plants is a frequently reported issue worldwide and the number of species involved is huge [3, 4]. However, in developed countries, severe cases of intoxication or death caused by plant exposure are rare. Considering 24,950 cases of plant poisoning reported by the Swiss Toxicological Information Centre between 1966 and 1994, a significant poisoning occurred only in 152 cases and fatal poisoning in 5 cases only, due to ingestion of *Colchicum* (two cases), *Oenanthe crocata*, *Taxus baccata*, and *Narcissus pseudonarcissus* (Jasperson-Schib et al. 1996). However, plant poisoning is a major clinical problem in many countries. Species like *Thevetia peruviana*, *Datura stramonium*, *Cerbera manghas*, and *Cleistanthus collinis* (a species of teak) cause a significant number of deaths each year in south Asia, most frequently resulting from suicide or homicide [5].

Data reported by poisoning centres worldwide suggest that plant exposures occur in a wide range of human age groups and social categories. In Europe, plants are responsible for about 5 % of paediatric poisonings [5]. Many plants or plant portions such as flowers, fruits, and seeds appear to be attractive for babies and young children who often eat coloured parts of ornamental plants in gardens and city parks [6–8]. Species belonging to Solanaceae are the most frequently reported cases with *C. annum* and other ornamental species (e.g. *Solanum pseudocapsicum*—christmas cherry) having ripened orange-red fruits, able to produce severe intoxication due to their high content of steroidal alkaloids. Other common intoxications are caused by ornamental flowers, such as species belonging to genera *Spathiphyllum*, *Anthurium*, *Tulipa*, *Delphinium*, and *Helleborus*. Most of the species belonging to HTA's category C do not usually provoke severe intoxication. However, a few paediatric cases of anticholinergic intoxication due to *D. stramonium* [8] and *Atropa belladonna* [6, 9] of B category have been reported.

During adolescence, the risk of poisoning is most frequently due to intentional recreational use of plant species containing psychotropic metabolites able to produce hallucinogenic and other effects. During the last decade, there has been an increase in the availability and use of "legal highs", which are novel psychoactive substances that include a wide range of products from plant-derived substances to synthetic compounds. These drugs can be easily purchased online or from high street retailers and are frequently used by adolescents and young adults as an alternative to marijuana and LSD. Four basic categories of such drugs can be distinguished: amphetamine- and ecstasy-like stimulants, synthetic cannabinoids, hallucinogenic/dissociative drugs, and opioid-like compounds [10]. Typical examples include traditional products, often with a long and culturally sanctioned history, such as herbs (e.g. *Salvia divinorum* and *Mitragyna speciosa*), seeds (e.g. *Argyreia nervosa*), fungi (e.g. magic mushrooms), and cacti (e.g. peyote). These ethnobotanicals have been recently put on the market mixed with a dizzying array of new synthetic compounds, including cannabinoid receptor agonists (such as JHW-018, JWH-250, and others), cathinones (such as mephedrone, 4-methylmethcathinone), and piperazines [11].

New drugs from plant sources are frequently abused, such as *Salvia divinorum*, a sage endemic to a small region of Mexico, where it is traditionally used by Mazatec Indians for spiritual healing. This species contains salvinorin A, probably the most potent hallucinogen of natural origin, which induces psychedelic-like changes in mood, hallucinations, and disorientation [12]. In addition, herbal mixtures called "Spice", smoked for euphoria, are increasingly used among adolescents and in the military. They are erroneously considered legal and are not detectable by urine drug screening [13]. These products often contain aromatic plant species that have the role of hiding synthetic cannabinoids, thus acting as a "green shuttle" rather than as real drugs. However, in some cases, the plant material in mixtures is made from psychoactive plants, such as the spice marked under the name "Kratom 10x", where leaf fragments from *M. speciosa*, containing the alkaloid mitragynine, were

found [14]. These new drugs include synthetic and naturally occurring substances that are not controlled under international law and are often produced with the intention of mimicking the effects of controlled substances [15].

Adult and elderly age classes are not exempt from accidental or intentional ingestion of poisonous plants or their metabolites. In such cases, the main reason is due to misidentification of dangerous species with edible congenerics or morphologically similar taxa [16–21]. This is the case for the substitution of spontaneous salad (*Lactuca alpine* (L.) Wallr.) with *Aconitum* spp. [3]. In such cases, the consequences of ingestion range from gastrointestinal temporary diseases to acute intoxication and even death [22]. Similarly, young leaves of *Digitalis* sp. containing cardiac glycosides are sometimes confused with *Borago officinalis*, and eaten as cooked vegetables causing toxicity in consumers [23].

Beyond causing health problems in humans, a wide panel of plant species is also responsible for many cases of livestock or pet poisonings. Animals can accidentally ingest plants containing alkaloids or other dangerous molecules [24, 25]. This is the case with *Colchicum autumnale* and *Datura stramonium* that cause dysphagia, toxic liver dystrophy, and even cardiac lesions and cardiorespiratory collapse in cattle and horses, respectively [26, 27]. Similarly, plant exposure cases occur in pets where the plant poisoning is mostly the result of ornamental house or garden plant ingestion. Frequently reported cases involve the occasional consumption of *Cycas revolute* or *Euphorbia pulcherrima* by dogs and cats, causing gastrointestinal symptoms and skin and mucous irritations [28, 29].

Tables 1 and 2 provide two lists of the most relevant cases of poisons in human and animals, with particular attention paid to European regions. Species and related metabolites responsible for poisoning are also indicated.

There are different reasons that explain the dangerous effects provoked by some plants on animal consumers. During 400 million years of evolution, plants developed efficient chemical signalling strategies to mediate mutualistic (e.g. with pollinators and seed dispersers) or deterrent (e.g. with herbivores or parasites) interactions with animals. Such strategies are based mainly on the production of different classes of secondary metabolites, such as flavonoids that protect against free radicals, terpenoids that attract pollinators and seed dispersers or inhibit competing plants, and alkaloids which usually ward off herbivore animals. Alkaloids, occurring in approximately 20 % of plant species, represent a diverse group of compounds characterized by a nitrogen atom in a heterocyclic ring, and are probably the most relevant category in terms of plant poisoning potentials. Among them indolizidine, piperidine, pyrrolizidine, tropane, and taxine alkaloids are of major interest to veterinary toxicology [25]. Another wide group of plant bioactive compounds involved in toxicity problems are glycosides, such as cardiac glycosides present in some Scrophulariaceae (e.g. *Digitalis purpurea*) and Convallariaceae (e.g. *Covallaria majalis*), cyanogenic glycosides present in Rosaceae (e.g. *Prunus* spp.), and saponins present in Liliaceae (e.g. *Narthesium ossifragum*, an important sheep toxic plant) as well as in other families. Hydrolysable tannins, which are polymers composed of a monosaccharide core with several catechin derivatives, have the potential to cause toxicity, while furocoumarines, frequently in Apiaceae, have photosensitising properties, small peptides, such as ricin in seeds of *Ricinus communis*, inhibit protein synthesis and induce systemic effects in animals and humans, with gastrointestinal symptoms dominating [30].

One could ask whether the metabolite composition of a plant is sufficient to categorize a species as poisonous. From an integrative point of view, the answer is negative since the poisoning risk depends upon several factors. A certain plant could be edible and poisonous at the same time for animals and humans respectively; this is the case for *Taxus baccata* and *Solanum dulcamara* [31–33]. Birds migrating across the Western Palearctic largely consume these fleshy-fruit species [34], but they are toxic to humans if ingested due to the high concentration of taxine and solanine, respectively, along with other alkaloids [35]. Alternatively, toxicity could vary depending on the plant portion or on stage of development. For example, the leaves of rhubarb (*Rheum rhabarbarum*) are highly dangerous, whereas stalks make delicious pies [36]. In addition, many species show different metabolic profiles during leaf, flower, or fruit maturation and change their condition from highly poisonous to highly appetizing to enhance pollination and fruit-seed dispersal. The toxicity of *Solanum* plants, due to the alkaloid solanine, varies depending upon maturity, environment, and the plant portion, with berries being generally the most toxic part. In *S. carolinense*, berries are more toxic when they have matured and turned yellow, whereas in *Sambucus nigrum*, toxicity is stronger in green, immature fruits [37].

Which are the causes of plant poisoning?

There are several causes and events generating poisonous cases. Most of these can be grouped in the following categories: spontaneous plant misidentification, the use of botanical products which taxonomy and safety have been not characterized yet, and the trading of phytochemical products for cosmetic, food, and medical purposes without defining proper control and regulations.

Plant misidentification

Nowadays, there is a positive trend in the harvesting and consumption of wild plants by people with poor or even no knowledge in systematic botany. Conventionally, the term “wild plant” refers to those species growing without being

Table 1 List of the most relevant plants (and related metabolites and toxic effects) involved in human poisoning episodes in Europe

Plant species	Toxic metabolites	Clinical features	References
Aconitum napellus	Aconitine	Neurological (paresthesia and muscle weakness); cardiovascular (hypotension, bradycardia, tachycardia, ventricular fibrillation); gastrointestinal (nausea, vomiting, diarrhea)	[124–126]
Atropa belladonna	Atropine, hyoscyamine, scopolamine	Neurological (anticholinergic symptoms such as blurred vision, confusion, hallucinations); cardiovascular (tachycardia); dermatological (skin rash, skin flushing, mouth dryness)	[3, 9, 127]
Colchicum autumnale	Colchicine	Neurological (ascending paralysis, respiratory failure, seizures, muscular weakness); gastrointestinal (nausea, vomiting, abdominal pain, and severe diarrhea); dermatological (alopecia, rash, dermatitis); other symptoms (anemia, kidney failure, liver failure)	[16, 128, 129]
Convallaria majalis	Convallarine	Neurological (drowsiness, blurred vision); cardiovascular (bradycardia); gastrointestinal (abdominal pain, vomiting); dermatological (rash)	[130]
Caulophyllum thalictroides	*n*-Methylcytisine	Neurological (dizziness, ataxia, seizures and other symptoms); cardiovascular (tachycardia, bradycardia and other symptoms); gastrointestinal (nausea, vomiting, diarrhea)	[131]
Conium maculatum	Conhydrine, coniine, γ-coniceine, n-methylconiine, and pseudoconhydrine		
Laburnum anagyroide	Cytisine, *n*-methylcytisine		
Nicotiana glauca and *N. tabacum*	Nicotine, anabasine		
Datura stramonium and *D. suaveolens*	Atropine, hyoscyamine, and scopolamine	Neurological (confusion, mydriasis, hallucinations and other symptoms); cardiovascular (tachycardia, bradychardia); gastrointestinal (paralytic ileus)	[132, 133]
Lupinus spp. (bitter lupin flour)	Lupinine	Neurological (anticholinergic symptoms such as blurred vision, confusion, hallucinations); cardiovascular (tachycardia); gastrointestinal (ileus); dermatological (skin rash, skin flushing, mouth dryness)	[52]
Mandragora autumnalis	Scopolamine	Neurological (mydriasis, delirium; hallucinations); cardiovascular (tachycardia); gastrointestinal (nausea, vomiting); dermatological (skin flushing)	[134]
Nerium oleander	Oleandrin	Neurological (blurred vision, confusion, dizziness and other symptoms); cardiovascular (arrythmia, bradycardia, hypotension); gastrointestinal (nausea, vomiting, diarrhea)	[135]
Ricinus communis	Ricin, ricinine	Neurological (seizures); cardiovascular (tachycardia, hypotension); gastrointestinal (nausea, abdominal pain, and severe diarrhea); Other symptoms (anemia, kidney failure, liver failure)	[136]
Robinia pseudoacacia	Robin, robinin	Neurological (muscle weakness, mydriasis, headache, dizziness); gastrointestinal (nausea, vomiting, abdominal pain, diarrhea)	[137]
Senecio vulgaris	Senecionine	Acute liver toxicity due to chronic exposure	[138]
Solanum nigrum	Solanine	Neurological (dizziness, mydriasis, seizures); cardiovascular (tachycardia); gastrointestinal (nausea, vomiting, diarrhea)	[139]

Table 1 (continued)

Plant species	Toxic metabolites	Clinical features	References
Taxus baccata	Taxine	Neurological (muscle weakness; clonic spasms); cardiovascular (tachycardia, cardiac arrest); gastrointestinal (nausea, vomiting)	[140–142]
Veratrum album	Veratridine, protoveratrine (A, B), jervine	Neurological (blurred vision, transitional blindness; dizziness); cardiovascular (bradycardia; hypotension); gastrointestinal (nausea, vomiting, abdominal pain)	[20]

cultivated. This definition is not always true, as in some cases this expression is improperly used to give a "positive marketing claim" to a certain species. In the context of the food supply chain, producers often support the claim that wild plants have additional nutritional properties and that they are healthier than the cultivated ones. In many cases, plants such as *Juglans regia*, *Corylus avellana*, *Prunus avium*, *Ficus carica*, and *Sambucus nigra* are passed off as wild. However based on the circumstances, the same species can be cultivated, wild, or semi-wild. Although these cases do not usually lead to poisoning, these commercial strategies incentivize the consumption of spontaneous plants as food items or for medicinal and esthetic purposes without evaluating the effects of their secondary metabolites on human health. For example, there are some case reports describing people looking for wild plants similar to salad or young shoots similar to asparagus who encountered morphologically similar species that were poisonous. Colombo and coworkers described several cases of substitutions of poisonous *Colchicum* spp. instead of *Allium ursinum* [3]. Another frequent intoxication is due to the confusion of edible *Pastinaca sativa*, *Daucus carota*, and *Foeniculum vulgare* (Apiaceae) with the poisonous species *Oenanthe crocata* and *Conium maculatum*, respectively [17, 38–40] (see also Table 1 for further cases).

The increasing incidence of such poisoning cases due to misidentification could be due to three main causes. The first one is the lack of botanical knowledge. This aspect is particularly relevant for consumers living in highly urbanized contexts with few or no contact with natural environments. This is a common situation occurring in developed countries where citizens have lost the heritage and traditions of rural areas regarding the uses of wild plants [41, 42]. The WHO report "Migration and health: key issues" (2016) [43] highlights that when people forage for food in an unfamiliar environment, they can fall victim to toxic plants and fungi that look similar to edible species in their countries of origin, as has happened in Germany when refugees ate poisonous mushrooms. Accidental ingestion of *Digitalis purpurea* is uncommon, due to its distinctive appearance and unpleasant taste. However, it has been reported that two men from Iraq ate boiled "cabbage" picked in Edinburgh, believing it to be the same plant that they were accustomed to eat in their home country. Both subjects had high digoxin blood levels and presented first-degree atrioventricular block due to *Digitalis* consumption [44]. A second issue of major concern is the scarce expertise about localized or peculiar floras. Even botanists can misidentify edible plants with poisonous ones, especially when they are dealing with species inhabiting regions out of their geographic area of expertise. The main reason for these mistakes is mainly due to the subtle morphological differences occurring sometimes between species, especially in the case of congenerics. In such cases, only local experts could have sufficient experience to differentiate close species. Finally, the third main cause for the increasing occurrence of plant poisoning is related to the introduction of new (e.g. alien) species. Similarly, to the previous point, the introduction of non-indigenous species in a certain area could cause misidentification by both experts and people without botanical knowledge, leading to the "experimental" ingestion and then to poisoning in humans and other animals.

New plant-based foods and supplements

New foodstuffs or supplements enhancing food taste that provide added value to nutritional properties and extend food shelf life are daily introduced into the market. Along with this trend, there is an increasing demand for botanical superfoods intended as plant-based foods declared to have outstanding nutritional and/or medicinal benefits for human health. Similarly, Plant Food Supplements (PFS) constituted of vegetal extracts are largely used to complement diets. Both categories are considered fashionable nutritional alternatives, and their consumption is growing and growing [45]. PFS have been harmonized by the European Directive 2002/46 [46] as products used to supplement the normal diet, as well as a source of nutrients or other substances with a nutritional or physiological effect. Most of the original plant species used for the production of PFS and superfoods are still unknown concerning their taxonomy, chemical composition, and potential effects on human health [45]. Moreover, in most Member States, the PFS are subjected to a notification procedure before

Table 2 List of the most relevant plants (and related metabolites and toxic effects) involved in animal poisoning episodes in Europe

Plant species	Toxic metabolites	Species poisoned	Clinical features	References
Allium spp.	Alk(en)ylcysteine sulfoxides	Dog, cat, cattle	Neurological (seizures, lethargy, polydipsia); gastrointestinal (vomiting, diarrhea, abdominal tenderness); cardiovascular (anemia); other symptoms (haematuria, icterus)	[143–145]
Astragalus spp. and *Oxytropis* spp.	Swainsonine	Horse	Neurological (abnormal behaviour, ataxia, hypermetria); other symptoms (kidney lesions)	[146]
Chimonanthus praecox	Calycanthine	Cattle	Neurological (limb rigidity, hyperesthesia, seizures)	[147]
Colchicum autumnale	Colchicine	Cattle	Cardiovascular (cardiorespiratory collapse); gastrointestinal (salivation, dysphagia, abdominal pain, diarrhea)	[27, 148]
Conium maculatum	Coniine, coniceine	Cattle	Neurological (nervousness, ataxia, trembling); cardiovascular (hyperpnea, tachycardia); other symptoms (teratogenic effects)	[149]
Convallaria majalis	Convallarine	Dog, cat	Cardiovascular (arrhythmias and tachycardia); gastrointestinal (vomiting and diarrhea)	[150, 151]
Cycas revoluta	Glycoside cycasin, bmethylamino-L-alanine	Dog	Neurological (lethargy; ataxia); gastrointestinal (vomiting, diarrhea); other symptoms (teratogenic effects; liver damage)	[28, 152]
Cynoglossum officinale	7-Angelylheliotridine, echinatine, acetylheliosupine and heliosupine	Horse	Neurological (depression, photosensitivity, aimless); gastrointestinal (diarrhea); other symptoms (liver dysfunction, necrosis and collapse)	[153, 154]
Datura stramonium	Hyoscyamine, scopolamine and atropine	Dog, cat, cattle, horse	Neurological (mydriasis; ataxia; seizures); cardiovascular (tachycardia); gastrointestinal (impaction colic)	[26, 151, 155–157]
Equisetum palustre	Thiaminase, nicotine	Cattle, horse	Neurological (weakness, motor incoordination); gastrointestinal (hemorrhagic enteritis); other symptoms (emaciation)	[158, 159]
Euphorbia pulcherrima	Diterpene esters	Dog, cat	Gastrointestinal (vomiting, diarrhea); dermatological (redness, swelling, itchiness)	[151, 160, 161]
Lilium tigrinum	Unknown	Cat	Neurological (lethargy, anorexia); gastrointestinal (vomiting); other symptoms (haematuria, anuria and renal failure)	[145, 151, 152]
Melia azedarach	Melianotoxins	Dog	Neurological (muscular seizures); gastrointestinal (vomiting, diarrhea)	[162]
Nerium oleander	Oleandrin	Dog, cat, cattle, horse	Cardiovascular (arrhythmia, bradycardia); gastrointestinal (vomiting, diarrhea)	[150–152, 163, 164]
Prunus spp.	Cyanide	Dog, cat, cattle, horse	Neurological (weakness, extreme changes in general behaviour); cardiovascular (bradycardia, hypotension, tachycardia); gastrointestinal (colic);	[152, 165, 166]
Pteridium aquilinum	Thiaminase; ptaquiloside	Cattle	Other symptoms (acute haemorrhagic disease, bone marrow aplasia, retinal atrophy, haematuria)	[148, 167]
Pyracantha spp.	Prunasin, amygadlin	Dog	Gastrointestinal (vomiting, diarrhea)	[152, 160]
Quercus spp.	Tannic acid and tannins	Cattle, sheep	Gastrointestinal (diarrhea); other symptoms (kidney damage)	[164, 167–169]
Rhododendron spp.	Grayanotoxins	Dog, cat, cattle, sheep	Neurological (ataxia); Cardiovascular (tachycardia); gastrointestinal (abdominal pain, vomiting); other symptoms (tachypnoea, pyrexia)	[152, 160, 170]

Table 2 (continued)

Plant species	Toxic metabolites	Species poisoned	Clinical features	References
Ricinus communis	Ricin, ricinine	Dog, cat	Neurological (weakness, trembling, incoordination); gastrointestinal (abdominal pain, vomiting, bloody diarrhea)	[151, 152, 171]
Robinia pseudoacacia	Robinin, robitin	Horse	Neurological (lethargy, weakness, posterior paralysis); gastrointestinal (colic)	[164, 172]
Senecio spp.	Senecionine	Cattle, horse	Neurological (extreme changes in general behaviour); gastrointestinal (diarrhea)	[164, 173–175]
Taxus baccata	Taxine	Dog, cattle, horse	Neurological (muscle trembling, ataxia); cardiovascular (bradycardia); other symptoms (difficult breathing)	[151, 152, 160, 163, 164, 176]

being placed on the market despite compositional criteria in relation to botanicals having not yet been harmonized.

The wide range of species used as botanical superfoods or PFS and the absence of a precise regulation for their trading impedes an efficient traceability system. Although almost all of these products are subjected to toxicological tests, Restani and co-workers [47] reported a list of more than 70 commonly used plants causing health problems to consumers. Of these, *Valeriana officinalis*, *Camelia sinensis*, *Ginkgo biloba*, and *Paullinia cupana* were among the most frequently found to have adverse effects on consumers. Most of the poisoning effects were related to the gastrointestinal tract, followed by problems involving the nervous system (e.g. insomnia) and the cardiovascular system (e.g. tachycardia). Surprisingly, in the consumer survey by Restani et al. [47], *V. officinalis*, a species usually recommended as sedative, induced the opposite effect in some consumers; similarly, *G. biloba* prescribed against anxiety was involved in three cases of insomnia and one of dizziness.

On the whole, the main problems related to these emerging plant product categories are due to different phenomena. The first one is species substitution and commercial frauds. Even though most botanical superfoods and PFS derive from cultivated species that are well known in their native countries, several cases of misidentification and substitution occur. In this context, Colombo and co-workers [17] reported the intoxication of human patients by *Conium maculatum* leaves that were confused with *Foeniculum vulgare* and used as PFS. The dangerous *Veratrum album* can be confused with *Gentiana lutea* in autumn when the flowers disappear and the leaves go brown. The root of white hellebore is then used to aromatize home-made distilled products, resulting in alcoholic extraction of alkaloids (veratrin) that render the drink very toxic [3]. Alcoholic drinks lead to faster absorption of toxic alkaloids with a more rapid onset of symptoms, including nausea, vomit, vertigo, and in some cases headache [48].

Another emerging problem related to the spread of botanical superfoods and PFS is the adulteration of traded plant food products. To increase the production yield of some PFS, the producer could add different botanical species in the expected pure extracts. A clear example is that of mustard (*Brassica nigra*) seeds or oil that are intentionally mixed with (*Argemone mexicana*) causing oxidative stress and posing a serious threat to human health [49, 50]. *Curcuma longa* is classically labelled as spice, dye, and cosmetic, but it is also becoming increasingly important as a medicinal herb. Marketed turmeric powder has been adulterated with *C. zedoaria*, cassava starch, wheat, rye, and barley. The lower price of *C. zedoaria* may induce turmeric manufactures to deliberately mix it with *Curcuma longa*, but the former is toxic and may cause health hazards or reduce the medicinal virtues of turmeric [51].

Finally, a further element of major concern refers to the incorrect processing of botanical superfoods and PFS. Different plant portions could contain nutritional elements along with poisonous molecules. For example, many fruits such as drupes show edible portions (exocarp and mesocarp) along with a toxic one (the endocarp). During the processing steps occurring along the food supply chain to transform raw material to the final PFS, it is sometimes necessary to treat or remove those portions potentially leading to poisoning. A typical case concerns the production of special flours made of plants belonging to fabaceae for people suffering from celiac disease. For example, the lupine (*Lupinus* spp.) provides alternative flours rich in protein, iron, and other minerals with no risks for celiac patients. However, the ingestion of lupines in the form of bitter lupine flour has been documented to provoke acute poisoning [52]. The bitter variety of lupines is rich in quinolizide alkaloids (e.g. lupanine) causing anticholinergic effects. Thus, an incomplete removal of such molecules during plant processing can result in severe health problems [53].

Lack of regulation of phytochemical products

Traditional medicine practices and food supplements based on herbs and other plants have been developed in different cultures, but without a parallel development of international standards and appropriate methods for evaluating their safety

for the consumer. Hence, many countries still face major challenges in the development and implementation of the regulation of herbal products. The current directives vary from country to country and are usually based on pharmacopoeia publication data or reports describing claims and therapeutic effects of a certain plant [54].

The main difficulties in defining national and international policies are related to regulatory status, assessment of safety and efficacy, quality control, and a lack of knowledge about the commercialized plant species within national regulatory authorities. The issue of major concern is the difference between countries in their definition and categorization of herbal products. A single herb may be introduced in local markets as food, functional food, dietary supplement, or herbal medicine depending on the national regulations. This makes it difficult to define the "role" of herbal products for the purposes of national drug regulation authorities, and also confuses patients and consumers. However, the use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly at the global scale [55] and most species have been not tested following pharmacopoeia criteria yet.

The current trend for the European Union (EU) is to harmonize the regulation of herbal medicine products to help the public in making choices about the use of such products. This necessity requires that all manufactured herbal products either gain a product license of the type needed for conventional products or become registered as traditional herbal medicinal products [56, 57]. To date, there are no European countries adopting shared implementation decrees to regulate the commerce of herbal products. For this reason, it is possible to find a certain plant product available on the market in a country and forbidden in another one. A well-known example concerns the trade and use of *Piper methysticum* (also referred to as "kava"), a perennial shrub native to some islands of the South Pacific used to treat anxiety disorders [58]. Although the sale of kava plant is regulated in Switzerland, France, and the Netherlands, other countries (e.g. the UK) prohibit the use, possession, and trade of this herb. This is mainly due to concerns raised about the safety of kava, related to some reports indicating potential cases of hepatotoxicity and other collateral effects [59].

Another key aspect to consider is controlling the consumption of a certain herbal product. In the absence of a clear regulation, everybody could buy a specific medicinal, cosmetic, or edible plant product with no indications concerning its dosage. In addition, the concentration of active ingredients could vary among different batches of commercialized products and the consumer could ingest small portions of plants rich in active compounds (including poisonous metabolites) or large amounts of plant material poor in active substances.

The lack of international shared regulation could also lead to illegal trade of plants or phytochemicals. A typical example is that of the so-called smart drugs. This term refers to a complex of herbal products, widely accessible on the Internet, able to produce psychotropic effects. The "smart" aspect of this products category is due to the fact that they are often sold as bath salts, potpourri, incense, and food additives despite potentially acting as potent drugs. For example, products marketed as incense (i.e. not suitable for human consumption) are conversely smoked much like cannabis [14]. These herbal products do not list ingredients; hence, it is difficult to understand whether an herbal mixture contains natural toxic metabolites, such as alkaloids, synthetic toxic compounds, such as synthetic cannabinoids, or aromatic plants.

The legislative systems of various developed countries are making efforts to comply with the diffusion of these products in the market. However, the continuous and growing emergence of new products with a borderline legal status is a severe challenge for the efforts of public authorities. Severe poisoning cases have been reported, for example due to the ingestion of *Mitragyna speciosa* that causes seizures and addiction, as well as liver toxicity and even death [60].

Independently from the cause of plant poisoning, recognizing the species producing health problems is essential for properly treating patients subjected to plant exposures. This requirement is generally devoted to specialized structures such as the poison centres. To accomplish their mission, poison centres need to maintain a comprehensive collection of information about poisonous plants and to develop analytical and treatment protocols to ensure that the information and advice given is rapid and reliable. The clinical diagnosis of intoxicated patients is typically based on the morphological analysis of plant fragments found in the stomach contents. This approach is very laborious and it requires a considerably broad experience from the operators. Moreover, identification success depends upon the availability and integrity of distinctive taxonomic elements of plant fragments. For these reasons, poison centres should host equipped laboratories to provide modern, fast, and accurate analytical toxicology services to assist with the diagnosis, assessment, and treatment of poisoning. To be rapid, such services should be independent from plant morphology and should exploit the universality and high-effectiveness of molecular markers.

Similarly to hospitals and poison centres, the actors involved in the food supply chain also need to demand rapid and reliable tools to identify food adulteration, species substitutions, and commercial frauds, especially if these involve plant materials posing risks to consumers' health. For both categories, chemical analyses represent effective tools; however in some cases, a chemical evaluation is not able to identify the plant responsible for poisoning [61]. Immunochemical screening, such as the dipstick tests assays, has been proposed to rapidly detect target substances in a given matrix. Dipsticks are easy-to-use low-cost lateral flow devices (LFDs) able to provide a fast response based on the visual inspection of the changes (e.g. colour) on a functionalized strip [62]. For example, this kind of test is

successfully used in detecting contamination of food matrices by tropane alkaloids [63], but the lack of universality and the possibility of obtaining false positives results limit its use to detect a broader range of substances.

On the contrary, DNA-based tools represent a valid solution for identifying the species leading to poisoning and then to properly evaluate the real risk and possible treatments.

DNA-based tools for toxic plant identification

In the last decade, more than 3000 eukaryote genomes have been sequenced and the number of nucleotide sequences deposited in the international GenBank archive passed from 50 to 200 million with a trend of available genetic information that is continuously growing. At the same time, DNA sequencing systems have been invested by a huge revolution passing from the conventional Sanger approach to the modern High Throughput Sequencing Techniques (HTS), and from pure research systems to rapid diagnostic tools. These technological advances supported transferring DNA-based technologies from a few equipped laboratories to a wide panel of structures and stakeholders, even including food supply chain control points and poison centres (Table 3).

Among the vast plethora of available diagnostic molecular tools, the DNA-based ones and, specifically, the DNA barcoding approach (and its technical variants) emerged as the most promising diagnostic techniques in this field [64].

DNA barcoding and sequence analysis

DNA barcoding is a popular molecular method for taxonomic identification and the characterization of species [65], and it

Table 3 List of diagnostic DNA-based methods used to identify poisonous plants. For each method, the pros and cons, the necessary analytical time (low, ≤ 3 h; medium, 3–6 h; high, ≥ 6 h) and equipment (i.e. in addition to basic instrumentation for DNA extraction, electrophoresis, and standard thermocyclers) are indicated. Moreover, the required skills of laboratory technicians and the potential stakeholders are also reported for each method

Method	Pros	Cons	Analytical time	Necessary equipment	Required skills	Potential stakeholders
DNA barcoding and sequence analysis	Standardized Reproducible Technically easy	Sensitive to DNA concentration and quality Absence of barcoding gap Paucity of reference databases for some plant groups	Medium	Genetic analyser	Basic molecular biology, DNA sequencing, basic bioinfomatics	Equipped poison centres and hospitals Agro-industrial and pharmaceutical companies Retailers and customers Researchers
Taqman probes, DNA microarray, LAMP and SCAR markers	Sequencing "free" Possibility of developing specific analysis kits for immediate detection Allow quantitative analysis High sensitivity	Require expensive equipment and consumables Demand species-specific set up Cannot be applied to unknown species or sporadic poisoning cases	Low	Real-time PCR, optical reading devices	Basic molecular biology	Equipped poison centres and hospitals Small and medium agricultural and pharmaceutical companies Food control laboratories Customs and forensics authorities
PCR-HRM and BAR-HRM	Sequencing "free" High precision Allow quantitative analysis Universality	Need for melting curve standard databases	Low	Real-time PCR, HRM facilities	Basic molecular biology, basic bioinformatics	Equipped Poison centres and hospitals Food control laboratories Researchers
HTS	High throughput Generate large amounts of sequence data Suitable for complex matrices Aid in developing new DNA markers	High equipment and consumables costs Need complex bioinformatics analyses Do not allow quantitative analysis	High	High throughput sequencing platforms, modern informatics facilities for data storage and analysis	Extensive knowledge in molecular biology and bioinformatics	Researchers Big pharmaceutical and food companies Specialized food control centres

has been extensively used in animal and plant biodiversity analysis, as well as the genetic traceability of livestock, crop species, and their related food products [66, 67]. The principal keystone of this approach is the use of sequence variability in one or a few universal genome regions (usually referred to as barcodes) shared by all the organisms as a marker to identify species. In 2010, Bruni and co-workers firstly proposed this approach as a support for forensic investigations used for identifying plant species involved in human poisoning cases [68]. The efficacy of DNA barcoding to identify plants potentially poisonous to humans was also documented by Newmaster and co-workers [69], where they used DNA barcoding to identify herbal products contaminated or substituted with alternative plants. In 2014, Xie and colleagues used DNA barcoding to detect poisonous plants among Chinese herbal accessions [70], whereas in 2016, Arunraj and co-workers assessed the efficacy of this method to trace and control the origin of Indian plant-derived raw drugs in various formulations [71]. In all cases, the analytical procedure was based on the amplification and sequencing of standard DNA barcodes. The principal constraint of this approach is the availability of reference DNA barcoding archives to successfully address taxonomic assignments of plants. However, the constantly growing information produced by research institutes provides a novel genomics perspective with broad applicative outcomes in the fields of medicine, veterinary, and food research.

A key theoretical advantage of DNA barcoding is its capability to identify cryptic species as well as different related species, even starting from degraded biological material or small portions of organisms [34, 72]. This is feasible by identifying genetic distance limit values within which two individuals can be considered to belong to the same or to different species.

In 2009, the CBoL (Consortium for the Barcode of Life) Plant Working Group [73] suggested the combination of two plastidial loci (*rbc*L and *mat*K) as core-barcode regions due to the straightforward recovery rate of *rbc*L and the high resolution of *mat*K. Among other potential barcodes, the trnH-psbA intergenic spacer is easily amplified and has a high genetic variability among closely related taxa [68, 74, 75]. The nuclear ITS region, and specifically the ITS2 portion, was also indicated as a supplementary DNA barcode region [76] due to its higher evolution rate [77, 78]. Along with universality, resolution, and standardization of the chosen marker regions, the strength of the DNA barcoding relies on the availability of well-populated and high-quality reference databases hosted by international platforms. For example, the International Barcode of Life Project (iBOL) coordinates BOLD (barcode of life database) that is a repository supporting the collection of DNA barcodes with the aim of creating a reference library for all living species [79, 80].

In 2010, a devoted DNA barcoding database was developed only for medicinal plant materials, which accepts all plastid DNA regions and nuclear ITS results. Medicinal Materials DNA Barcode (http://137.189.42.34/mherbsdb/index.php) is a website that contains DNA sequences with their information and important references of medicinal registers of the pharmacopoeia of the People's Republic of China, American Herbal Pharmacopoeia, and other related references. In addition, this database provides information on discriminating medicinal materials (plant, animal, and fungi) from their ordinary adulterants and substitutes [81].

As a diagnostic method, the DNA barcoding approach can be more or less fallacious, and it should be taken into account that failures are mainly in the essence of biological species rather than in the method itself. Furthermore, DNA barcoding could be used as a reliable tool for authenticating poisonous plants, but its application for processed plant-based dietary supplements requires fine tuning to deal with the possibly poor quality of total DNA obtained from such products [82].

The lack of strong biological support could generate different types of errors when the barcoding gap is used. If populations within one species show high rates of intraspecific variation, false positives could be generated. This occurs for instance in allopatric populations with interrupted gene flow. Furthermore, if there is little or no sequence variation in the barcoding region between different species, false negatives could be observed. Regarding genetic traceability purposes, the existence of a barcoding gap could be interdependent with the sampling of a given species. The individuals chosen to represent each taxon as a reference database should cover the vast majority of the existing diversity; otherwise, a barcoding gap could be generated by incomplete sampling and thus misrepresent reality [83].

Apart from theoretical limitations, DNA barcoding analysis relies on the success of two analytical steps: DNA amplification and sequencing. Although such procedures are greatly improved by continuously emerging technical advances, many poison centres and food laboratories cannot afford the necessary equipment to process plant DNA samples [84]. Moreover, DNA barcoding is typically an untargeted approach and it should be preferred when a putative poison centre deals with a large panel of plant species. Conversely, when the species leading to poisoning are well known, targeted approaches should be preferred to gain an advantage in terms of analytical time and the costs of the analysis. Among the target approaches, the most suitable for detecting poisonous plants are TaqMan probes, SCARs, and the recently developed PCR-HRM.

Smart detection systems based on target DNA-based methods

Starting from DNA polymorphism in nucleotide sequences (including the DNA barcoding regions) between poisonous and edible plants, species-specific genomic regions could be

identified to allow a simple and smart detection system. There are different strategies ranging from the use of probes targeting specific DNA regions, to methods based on species-specific primers.

The most famous target PCR method based on probes is the TaqMan. In this approach, a fluorogenic probe anneals exactly within a specific DNA region amplified by a set of primers (including universal DNA barcoding primers). As the Taq polymerase extends the primer and synthesizes the nascent strand, the exonuclease activity of the polymerase degrades the probe with the consequent release of a fluorophore detected by a quantitative PCR thermocycler [85]. Overall, TaqMan probes proved to be very sensitive and specific, yet the need for designing and synthesizing different dual-labelled probes for each target sequence increases assay setup time and costs thus limiting their use [85]. This method is largely used when accurate quantitative assays are required for supporting food-labelling procedures and preventing food contamination, misdescription, and fraud. For example, the TaqMan assay was used to identify and quantify bovine DNA in meats, milks, and cheeses [86] and to evaluate the presence of mandarin in commercial orange juice [87]. Given these premises, it is reasonable to assume that primers specifically designed to detect a poisonous plant species could be easily used in a TaqMan PCR context by poison centres or food control laboratories.

Another method based on species-specific probes is DNA microarray. This is a high throughput technology for simultaneous analysis of multiple loci characterizing a target species. The technique is based first on the identification of DNA oligonucleotide sequences that are unique to each species and then on the synthesis of corresponding probes that are immobilized in a regular pattern on an impermeable solid support (glass, silicon chips or nylon membrane). DNA extracted from the samples and labelled with a specific fluorescent molecule is then hybridized to the microarray DNA. A positive hybridization is detected and visualized with fluorescence scanning or imaging equipment [88, 89]. This technique has also been applied for the identification of toxic plants among traditional Chinese medicinal products [90]. The lack of universality and the expensive development of the array system are the main limiting factors to its widespread adoption in the context of plant exposure diagnostics.

A valid alternative based on species-specific PCR primers is the analysis of Sequence Characterized Amplified Regions (SCARs). The core of this technique is the identification of polymorphic DNA regions of poisonous plants where specific PCR primer/s can be designed. Such primers anneal only when the DNA of the target species occurs in the template. The SCAR approach was firstly developed starting from polymorphic fragments obtained by discontinuous molecular markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP). Specific SCAR sequence primers can be located at any locus within or flanking the RAPD/AFLP amplicon. The key point is that polymorphisms at the annealing region/s have to be conserved within the same species and be highly variable among congenerics or frequently substituted plant species. For this reason, a single SNP is not sufficient to identify a good SCAR region. Due to the large availability of plant genomic regions data in public databases (including DNA barcoding sequences), it is possible to identify in silico SCARs and design-specific primers targeting hypervariable DNA regions [91]. In their work from 2014, Federici and co-workers developed and tested SCAR regions based on the trnH-psbA barcode region to detect the occurrence of the toxic *Colchicum autumnale* and *Atropa belladonna* in processed food items. On the whole, SCAR marker analysis is fast, reliable, and less sensitive to reaction conditions. The results are interpreted by means of a presence/absence of the amplicon [91].

The loop-mediated isothermal amplification (LAMP) method is a technique involving stem-loop DNA constructs (DNA hairpins). This method is based on the use of a set of four specially designed primers that recognize a total of six distinct sequences of the target DNA [92]. The amplification is isothermal and based on an autocycling strand-displacement synthesis, catalysed by the thermo stable Bst DNA polymerase with no thermal cycling or any heat denaturation of the template DNA required. LAMP results could be visualized by colour change (e.g. using SYBR Green) or using agarose gel electrophoresis (i.e. presence/absence of bands). LAMP is a highly sensitive method that can be used without expensive equipment. LAMP has been used for the detection of different organisms including plants and specifically, traditional herbal medicine adulterants [93–95]. Recently, Wu and co-workers used LAMP to detect the toxic adulterant *Aristolochia manshuriensis* often substituted to the original Mu-Tong herbal medicine (*Akebia* spp.) [96].

TaqMan, DNA microarray, SCARs, and LAMP require an initial accurate setting, and when the method is perfectly tuned they can be used easily and with fast analytical times, avoiding the time and resource consuming steps of sequencing, sequence editing, and bioinformatics analysis.

The major shortcoming in using these methods occurs when the target species (e.g. a poisonous and an edible plant) are genetically similar. In such a condition, the selected genomic regions could differ for a few bases only, and the methods could decrease their discrimination power or even fail.

To overcome this limitation, more sensitive methods exist, such as those based on Real-Time PCR that exploit High Resolution Melting (HRM), as a tool to differentiate similar DNA traits.

PCR-HRM as suitable approach for the identification of closely related taxa

A typical DNA barcoding approach based on sequence analysis could reveal a low genetic divergence between

phylogenetically related species differing for one or a few nucleotide positions. If such differences clearly separate the species, it is possible to use them to recognize a poisonous plant from an edible one. A good solution could be that of amplifying the variable genomic regions with universal primers and to distinguish the two species by analysing the melting curves produced by a Real-Time PCR. This approach is usually referred to as PCR-HRM analysis and it uses the melting temperature of nucleic acid-specific products to detect sequence differences (including simple sequence repeats) in PCR-amplified products. For the detection of these differences, DNA-specific dyes, highly sensitive instruments, and high resolution software are required. Samples are distinguished depending on their sequence length, GC content, melting temperature, and nucleotide composition of the product [97]. Initially, the PCR amplification of the sequence of interest (DNA barcoding or other selected DNA markers) is conducted in the presence of a specialized double-stranded DNA (dsDNA) binding dye [98–100].

Changes in fluorescence of "release-on-demand" dyes allow real-time monitoring of DNA amplification during PCR [100]. In order to obtain a melting profile characteristic for each sample, a gradual heating from around 50 °C to around 95 °C is conducted. When the dsDNA denatures into single-stranded DNA, the dye gives a low fluorescence signal. A melting curve characteristic of each sample is obtained on the basis of fluorescence changes against temperature. The peak generated by the negative derivative of the fluorescence (F) over temperature (T) (-dF/dT) against the temperature (T) is the melting temperature (Tm) of the amplicon [101]. Every different DNA sequence melts at a specific temperature. Thus, using the characteristic melting curve, each amplicon can be viewed, compared, and detected. HRM analysis is a very sensitive method to the point of being able to detect a single base change between otherwise identical nucleotide sequences [102, 103]. For optimal HRM analysis, the experiment should be appropriately designed. Primer design, PCR reagents, cycling conditions as well as genomic DNA (gDNA) quality, amplicon length, and dye selection are crucial parameters and require special attention since differences in melting curves may occur due to other factors than the nucleotide sequence [101].

HRM analysis has several advantages over traditional methods for identifying poisonous plants in different matrices. First of all, this approach is conducted immediately after PCR amplification with no amplicon purification required, and this makes HRM suitable for high throughput amplification, and it is more cost effective than other similar techniques. Due to these advantages [104], the use of HRM has expanded in many scientific sectors including forensics, clinical settings (i.e. poison centres), and the authentication of plant-derived food products [105–108]. For example, PCR-HRM was recently used to identify herbal infusion compositions [109]. Species-specific primers were designed for nine different Mediterranean aromatic plant species and combined in a multiplex PCR resulting in fragments of different lengths and therefore different melting behaviours for each species. This multiplex HRM assay could be applied as routine tests for verifying the botanical origin of herbal teas and can also be extended to authenticate other herbal foodstuffs.

PCR-HRM can also be combined with DNA barcoding (Bar-HRM) to obtain a rapid detection of barcode differences in a complex matrix [106, 110, 111]. In the last few years, Bar-HRM has been proven to be an effective tool for determining the origin and quality of raw materials and detecting adulterations in the herbal processed supply chain [112]. For example, in 2014, Kalivas and co-workers developed a Bar-HRM approach, based on the barcode ITS2 region to identify *Sideritis* species in herbal infusions [113]. Similarly, other studies adopted the same approach to differentiate traditional Chinese medicinal herbs from adulterants, such as *Panax notoginseng* [114], *Akebia quinata*, *Clematis armandii*, and *Aristolochia manshuriensis* [115]. Moreover, recent studies supported the use of this approach to identify species substitutions and admixtures in herbal products with clear outcomes in the context of guaranteeing consumer's safety since poisonous plants could be involved in these cases. Specifically, Singtonat and Osathanunkul [116] demonstrated the use of Bar-HRM to detect the toxic adulterant *Crotalaria spectabilis* in *Thunbergia laurifolia* herbal products at as low as 1 % concentrations. On the other hand, Buddhachat and colleagues [117] reported a similar limit of detection using Bar-HRM in the case of the contamination of *Phyllanthus amarus* with other *Phyllanthus* species. Most importantly, the authors also highlighted the rapid detection power of this technique by verifying the occurrence of the contaminant within 2 h [117].

On the whole, Bar-HRM is a very promising technique due to its low cost and decreased time for analysis. Furthermore, the use of small amplicons allows the analysis of processed foods and complex matrices. One minor disadvantage is the need to obtain a melting curve database to serve as a standard when one needs to identify unknown samples. Thus, it is of great importance to develop methods to permit the curves to be shared among different laboratories [118].

The untargeted approach: HTS (High Throughput Sequencing)

In order to check for the occurrence of poisonous plants (even when present in low amounts) in herbal medicinal products, as well as in the food supply chain, it is necessary to overcome the limits of targeted approaches. With the ultimate goal of characterizing the complete spectrum of organisms in a certain biological matrix (e.g. a food, a mixture of herbs, or plant extracts), the traditional Sanger sequencing method is inadequate to uncover the huge diversity potentially occurring. This is the case of herbal mixtures or any food item including several

plant species in the same matrices. To date, several novel approaches, referred to as HTS, have been developed. HTS consists of devices able to perform a massive parallel sequencing on complex matrices and allows to analyse every single sequence separately. HTS techniques are able to provide sequence data around a hundred times faster and cheaper than the conventional Sanger approach [118]. Due to these practical advantages, it is possible to analyse a very high number of samples in parallel, and hence to lower the costs of analysis.

The principal disadvantages of the HTS approach rely on the complex steps following sequencing to analyse and interpret this large amount of data which requires an efficient bioinformatics platform and very specialized operators.

However, the reduction in cost and time for generating DNA sequence data has resulted in a range of new successful applications, ranging from food traceability to food safety assessment and even including the detection of potential contamination by poisonous plants. In this context, Coghlan and co-workers, in 2012 successfully applied the HTS methodology to analyse highly processed and degraded DNA from products of Traditional Chinese Medicine (TCM), which involved powders, crystals, capsules, tablets, and herbal tea [119]. The approach identified TCM plant composition, including species containing compounds poisonous to humans. Similarly, Cheng and colleagues [120] tested another TCM preparation known as Liuwei Dihuang Wan (LDW), and using HTS they found up to seven contaminant species among which *Senna obtusifolia*, which could potentially induce liver and kidneys damage.

Illegal and toxic biological ingredients have been found in various herbal products [119]. This illegal biological material is undesired and can be traced by combining DNA barcoding with HTS technologies. For example, HTS was used to analyse honey and to characterize the plant composition of other pollen-based products [121], as well as to verify their authenticity and potential adulteration. These topics are of current concern as pollen or other material from poisonous plants has been found within honey, for example *Atropa belladonna* [19], some Boraginaceae [122], and *Rhododendron* spp. [123].

A user's guide to DNA-based identification of poisonous plants

Based on the techniques described in the previous paragraph, we tried to schematize all the possible analytical scenarios occurring when a DNA-based identification of poisonous plants is required (Fig. 1). This scheme is directed to different stakeholders, ranging from physicians working at poison centres to the operators working at laboratories involved in the food supply chain.

To select the most suitable DNA-based tool, the first thing to consider is whether or not the candidate poisonous plant species generating the problem is/are known. For example, in the case of human intoxication, an accurate interview with the patient, the morphological screening of plant residues recovered from stomach content (i.e. seeds, leaves, or fruit portions), or information on the collection locality could help define a preliminary taxonomic hypothesis. If the operator (a doctor or a food control laboratory technician) is able to identify one or a few candidate species and these have been already characterized with a molecular approach, a rapid screening technology based on *target PCR* could be used. TaqMan or SCARs are the most suitable techniques when primers and/or probes have already been developed. This strategy could be applied only when patient symptoms clearly refer to one or a few well-known poisoning plant species. Usually, Target PCR methods demand short analytical times (see Table 3) and do not require complex laboratory equipment and operators' expertise.

Conversely, if the plant responsible for poisoning or adulteration is unknown and no hypotheses are available, the samples should be processed with a standard molecular identification approach. In such cases, DNA barcoding based on

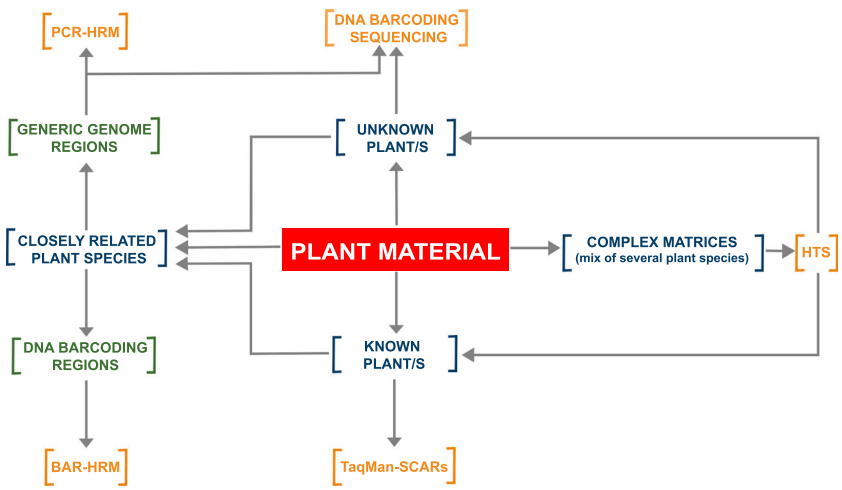

Fig. 1 Schematic workflow of the available DNA-based approaches used to identify poisonous plant species

sequence analysis is the simplest and most reliable strategy to adopt due to the universality of the system and the availability of the reference sequences databases. The entire process demands DNA amplification and sequencing with consequently longer analytical time and costs (Table 3). Moreover, the laboratories should be equipped with or have access to DNA sequencing facilities.

The initial investment in terms of time, cost, and equipment devoted to characterizing poisonous plants with a DNA barcoding strategy could be useful for developing specific target PCR systems for routine analyses. At the same time, poison centres working with standard DNA barcoding could contribute to populating dedicated public databases (such as BOLD) and to standardize the use of DNA barcoding and encourage its adoption worldwide.

Although it has great potential and applicative opportunities, the standard DNA barcoding regions could be not sufficient to reliably differentiate poisonous plants from other harmless species. Most plant genera encompass both edible and poisonous species, and in several cases, these are phylogenetically very close. In these situations, the diagnostic laboratories have to equip themselves with more sensitive systems able to distinguish two species based on few polymorphisms at a certain target genome region. In our schematization, the Real-Time PCR represents the most suitable alternative to differentiate plant species by means of two different opportunities, PCR-HRM or BAR-HRM, chosen depending on the selected DNA marker region.

The pipeline has become much more elaborate in terms of necessary technologies and resources, when complex matrices composed of different known and/or unknown plant species are considered. The HTS technologies offer the most suitable opportunity to screen the whole composition of matrices, where the analysis by PCR of universal markers (including the DNA barcoding ones) produces several amplicons corresponding to each species occurring in the sample. Bioinformatics analysis of the obtained data could reveal the occurrence of the known and unknown poisonous species. Also in this case, HTS data represents valuable information to develop target PCR strategies. In addition, it should also be noted that HTS techniques need more knowledge and laboratory facilities, especially regarding the bioinformatics analyses and the construction of DNA reference databases. Progresses in this field are growing and range from the increasing sequence coverage and quality of the genetic analyzers to the decreasing analytical times and processing costs. Third generation devices are now available and will be rapidly integrated in the field of molecular diagnostics. This is the case of the PacBio RS platform that can detect the incorporation of fluorescently labelled dNTPs in real-time mode or the very recent Oxford Nanopore technology, a USB sized sequencing device MinIon, in which detection is based on nano-sized pores. In the very next future, it will be possible to connect these instruments to smartphone-like devices and have direct access to online reference sequence matching systems, thus making the diagnostics of plant poisoning cases more rapid and portable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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