

The Foxgloves (*Digitalis*) Revisited*

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ABSTRACT

This review provides a renewed look at the genus *Digitalis*. Emphasis will be put on those issues that attracted the most attention or even went through paradigmatic changes since the turn of the millennium. PubMed and Google Scholar were used (“*Digitalis*” and “Foxglove” were the key words) to identify research from 2000 till 2017 containing data relevant enough to be presented here. Intriguing new results emerged from studies related to the phylogeny and taxonomy of the genus as well as to the biosynthesis and potential medicinal uses of the key active compounds, the cardiac glycosides. Several Eastern and Western Foxgloves were studied with respect to their propagation *in vitro*. In this context, molecular biology tools were applied and phytochemical analyses were conducted. Structure elucidation and analytical methods, which have experienced less exciting progress, will not be considered here in great detail.

Introduction

Despite advancements in synthetic chemistry, we still depend on biological sources to obtain various small natural products such as morphine, artemisinin, quinine, and cardenolides. It is estimated that 25% of prescribed drugs in industrialized nations contain natural plant products, whereas in the emerging nations, about 75% of the population relies on plant-borne remedies [1]. A trend can be seen towards the production of such compounds using transgenic fungi or bacteria. The commercial yeast process for artemisinin production [2] can be regarded as a breakthrough in this area, and the proof of concept given for morphinan production [3] is even more exciting considering the multitude of biosynthetic steps necessary to piece together morphine from its precursor amino acid tyrosine. Another approach for the alternative production of valuable plant compounds employs plant tissue cultures. The paclitaxel process using suspension-cultured cells of

Taxus species is a prime example [4]. The enzymes and genes regulating the morphine, artemisinin, and paclitaxel formation are well known, whereas the ones involved in the biosynthesis of cardenolides, tropane alkaloids, or most of the indole alkaloids are not yet identified.

Digoxin, a cardenolide, is still produced from dried leaves of *Digitalis lanata* [5] since its chemical synthesis is not economically viable. The *Digitalis* cardenolides do not play a major role in the current cardiovascular drug market. Their use in the treatment of other diseases, such as cancer or viral infections, may offer them a “second chance”. In 2000, Max Wichtl and the late Martin Luckner published their book “*Digitalis – Geschichte, Biologie, Chemie, Physiologie, Molekularbiologie, Pharmakologie, medizinische Anwendung*” [6], which summarized on more than 350 pages the scientists’ knowledge about this medicinally significant plant genus at the turn of the millennium. The authors have done a comprehensive literature review up to the year 1999 in their monograph, therefore these references will only be cited when it seems appropriate to provide the reader with immediate information. For further information and a full list of references, the reader is referred to the Luckner/Wichtl monograph [6], which reviews the following 18 topics: history, botany, cell and tissue culture, cryo-

* Dedicated to Professor Dr. Max Wichtl in recognition of his outstanding contribution to pharmacognosy research.

preservation, genetic engineering, molecular and cell biology, breeding and conservation, field cultivation, harvest and processing, cardenolide phytochemistry, biosynthesis, biotransformation, degradation, transport, biotechnology, biochemical ecology, general phytochemistry, pharmacopoeias, pharmacology and toxicology, medical uses, endogenous digitalis-like compounds, and digoxigenin antibodies. The monograph ends with a compilation of analytical protocols and a list of about 100 related Ph. D. theses, most of them executed and defended in the groups of Max Wichtl (Marburg), the late Martin Luckner (Halle), the late Ernst Reinhard (Tübingen), and Wolfgang Kreis (Erlangen).

History

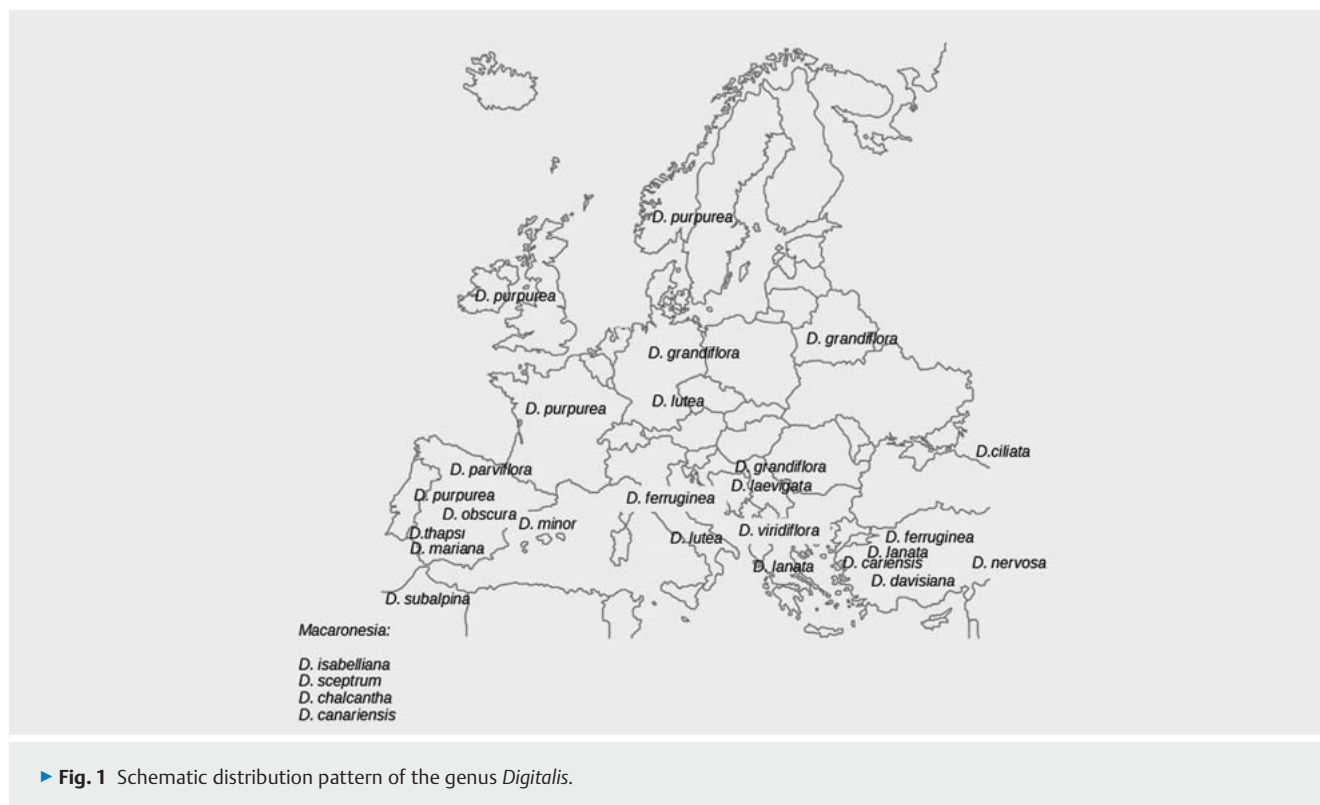
Though many of our medicinal plants have oriental origins, this may not be the case for foxgloves. For example, foxglove does not appear in the *Materia Medica* of Dioscorides. It was suggested that the ancients had little use for this plant, though Galen may have used it 'for cleaning the breath' [7]. More evidence for this assumption was provided by Groves and Bisset [6, 8]. It should be added that the Common Foxglove (*Digitalis purpurea* L.), which was the one investigated by William Withering [6, 9] in the 18th century (see below), is not native to Ancient Greece including, e.g., Sicily, Asia Minor, and Egypt. By the Middle Ages, the Common Foxglove was regarded as a panacea, a plant endowed with extensive healing properties [10]. The treatment of congestive heart failure (dropsy), among other ailments like tuberculosis, is mentioned in writings as early as 1250 A.D. when the Welsh *Physicians of Myddvai* [11] collected foxglove as one of their many herbs and included it in their remedies.

Erasmus Darwin, who wrote the verbose poem "Botanic Garden, Part II" [12], in which the Common Foxglove's use is described, urged Withering to accept an appointment at the Birmingham General Hospital and added a footnote concerning the medical uses of foxglove to his poem: *The effect of this plant in that kind of dropsy, which is termed anasarca, where the legs and thighs are much swelled, attended with great difficulty of breathing, is truly astonishing. In the ascites accompanied with anasarca of people past the meridian of life it will also sometimes succeed. The method of administering it requires some caution, as it is liable, in greater doses, to induce very violent and debilitating sickness, which continues one or two days, during which time the dropsical collection however disappears.* He finished this footnote with: *A theory of the effects of this medicine, with many successful cases, may be seen in a pamphlet, called, "Experiments on Mucilaginous and Purulent Matter" that was published in 1784, one year before Withering's "Account of the foxglove".* Hence, Erasmus Darwin [13] was the first who reported the use of foxglove in several cases. John Fulton [14] noted: *If priority of publication means anything he [Erasmus Darwin] has a legitimate claim over Withering. However, since he did not give Withering credit for having called the use of foxglove to his attention one may subscribe to the view that if one applies modern standards of ethics one must conclude that the grandfather of the celebrated naturalist was somewhat unscrupulous. Whatever the case, there is no doubt that it was Withering who convinced his medical contemporaries, and it was he who inaugurated the systematic use of the drug.*

In fact, William Withering deserves all the credit for a) his deduction that foxglove was the active ingredient in the old herbal formulations, and b) for carefully trying out different preparations of various parts of the *Digitalis* plant in 163 patients suffering from dropsy. His discussion of "effects, rules, cautions" and "inferences" still stands as our guide for clinical studies today. Only Withering's account of the foxglove [9] brought this plant and its genus into medical light, but it would take more than 200 years before its properties and potential uses could be harnessed in more depth. Details on the history of *Digitalis* and its use can be found in numerous reviews, e.g., [6, 8, 15].

Botany

Luckner and Wichtl [6] divided the genus *Digitalis* L. into five sections, namely *Frutescentes*, *Digitalis*, *Grandiflorae*, *Tubiflorae*, and *Globiflorae*, comprising 19 species. They included the genus in the Scrophulariaceae family, which has previously been put in the order of the Lamiales [16]. Chemotaxonomic considerations [6] and approaches were not fully convincing and a few studies focusing on chemotaxonomic markers have been published over the past 20 years, e.g., [17]. Rigorous approaches of assessing phylogenetic relationships using DNA sequences finally made it necessary to disperse the order Scrophulariales [18]. The classification of the genus *Digitalis* still followed the morphological analyses of Werner [6, 19, 20]. More recently, morphological, bio-geographical and molecular data were combined to challenge Werner's phylogenetic tree [21–23]. Moreover, the Scrophulariaceae were regarded as a collection of unrelated lineages and were dismembered. Olmstead et al. [24] used three plastid genes and revealed the existence of at least five distinct monophyletic groups and placed the tribus Digitaleae into the Veronicaceae. Albach and Chase [25] still found several incongruencies and proposed the "new" Plantaginaceae integrating the genus *Digitalis* and other members of the former Scrophulariaceae [26]. Currently, the genus *Digitalis* comprises 23 species including the four species of the former genus *Isoplexis* based on the molecular phylogeny of Bräuchler et al. [27] and Herl et al. [28]. The latter used the progesterone 5 β -reductase gene (P5 β R), a low-copy nuclear gene [29] encoding an enzyme supposed to be involved in the biosynthesis of 5 β -configured cardenolides (see below: Biosynthesis), to infer phylogenetic and biogeographic relationships. These studies revealed two major lineages that mark an early split in the genus. While the sections *Digitalis*, *Frutescentes*, and *Globiflorae* appear monophyletic, sect. *Tubiflorae* seems to be polyphyletic and sect. *Macranthae* should be expanded. Why *Digitalis lutea* L. subsp. *australis* was split into a Tuscan and a Corsican form, which were subsequently placed in different sections, is an issue yet to be resolved. Evidence was provided that all *Isoplexis* species have a common origin and that they should be embedded in *Digitalis*. Lindley [6, 30] already suggested reducing *Isoplexis* to sectional rank to avoid paraphyly. The *Isoplexis* species are now grouped in the section *Isoplexis* within the genus *Digitalis* and are referred to as *Digitalis isabelliana* (Webb.) Linding, *Digitalis canariensis* L., *Digitalis chalconantha* (Svent. & O'Shan.) Albach, Bräuchler & Heubl, and *Digitalis sceptrum* L. The members of the section *Isoplexis* are



notable for a number of reasons: 1) It was assumed that they represent the most primitive forms in the genus *Digitalis*, 2) they appear to contain not only of the therapeutically used 5β -configured cardenolides, but also 5α -cardenolides and $\Delta^{3,4}$ - and $\Delta^{4,5}$ -cardenolides, which can also be described as pregnenolone- and progesterone-type cardenolides, respectively (see below: Phytochemistry), 3) although 5β -reduction is not a step in *Isoplexis* biosynthesis, the respective progesterone 5β -reductase genes (see below: Biosynthesis) are present and they encode for active enzymes, and 4) birds but not insects are effective pollinators (see below: Ecology).

Only recently, the phylogenetic and morphological relationships regarding the section *Globiflorae* was investigated [31] and cDNA sequences of *P5 β R* isolated from *Digitalis trojana*, *Digitalis cariensis*, *Digitalis lamarckii*, *D. lanata* subsp. *lanata*, *Digitalis ferruginea* subsp. *ferruginea*, and *D. ferruginea* subsp. *schischkinii* were used to deduce relationships. The molecular and morphological considerations supported Davis' [32] system of the genus *Digitalis* in Turkey. Hence, four Turkish species and three subspecies were proposed with *D. lamarckii* separated from *D. trojana* and *D. lanata* (Table S1, Supporting Information).

The genus *Digitalis* has two centers of diversity: 1) The Western species found on the Iberian Peninsula and in Northwestern Africa and 2) the Eastern species found on the Balkan Peninsula and in Asia Minor. All members of the section *Isoplexis* represent species endemic to confined areas in the Macaronesian region but not the Azores [6, 33]. The areas in between do not have large numbers of species; in Germany, for example, there are only three (*D. purpurea*, *Digitalis grandiflora*, and *D. lutea*) and only the Common Foxglove is native to Ireland and Great Britain. A similar situation is described

for most Eastern regions where *D. grandiflora*, *Digitalis nervosa*, and *Digitalis ciliata* can be found. The geographical distribution (▶ Fig. 1) of the species nicely correlates with their taxonomic relationship. More detailed maps can be found in Luckner and Wichtl [6]. It should be noted that *D. purpurea* was introduced widely as an ornamental plant and can readily be found along the West Coast of the USA and Canada as well as in New Zealand. Three *Digitalis* species, namely *D. purpurea*, *D. grandiflora*, and *D. lanata*, were reported to grow in elevated montane habitats in Kashmir [34].

Biology and Physiology

Luckner and Wichtl [6] summarized studies that have shown that cardenolide contents in *Digitalis* species are linked to the seasonal variations of light intensity, the photoperiod, and the thermo-period. More recently, several studies focused on the relation between soil characteristics, plant nutrients and, cardenolide production. For example, cardenolide contents in *Digitalis obscura* were negatively correlated with soil nitrogen, phosphorous potassium, and copper, whereas iron and magnesium content of young leaves was positively correlated with cardenolide concentrations [35–37].

The time course of cardenolide accumulation during plant development was investigated in great detail in *D. lanata* and superficially in *D. purpurea* and *Digitalis mariana* ssp. *heywoodii* [6, 38, 39]. Roca-Pérez et al. [38] recorded the seasonal fluctuations of cardenolides in natural populations of *D. obscura*. Especially the analyses of Freier [6, 39] can be regarded as a landmark since the concept of “early glycosides” and “late glycosides” emerged. The

“early glycosides” comprise cardenolide disaccharides, such as glucodigifucoside and other rare glycosides, whereas the “late” ones are the more common cardenolide tetrasaccharides, such as the lanatosides and purpureaglycosides found in adult plants.

Cell suspension cultures established from different *Digitalis* species generating cardiac glycosides did not produce cardenolides [6,40–42], whereas somatic embryos, green shoot cultures as well as plants regenerated from tissue cultures contained cardenolides. A positive correlation between light and cardenolide production was assumed, e.g., [6,43], but it seems as if chloroplast development is not sufficient for expression of the cardenolide pathway, since photomixotrophic cell cultures did not produce cardenolides [6,44]. *Digitalis* roots cultivated *in vitro* are not capable of producing cardenolides, although they do contain these compounds *in planta*, indicating that the root is a sink organ for cardenolides [6,45]. Suspension-cultured *Digitalis* cells, e.g., [6,44,46], as well as roots or shoots cultivated *in vitro*, e.g., [6,47], are able to take up exogenous cardenolides and modify them. Biotransformation of appropriate precursors can also yield cardenolides that are not available commercially (see Munkert et al. [48], in this issue). Kreis et al. [6,46] described the cellular organization of cardenolide biotransformation.

Plant Cell, Tissue, and Organ Culture

Plant tissue culture comprises a set of *in vitro* techniques and methods applied in plant biotechnology. Tissue culture has been used to create genetic variability and also large-scale micropropagation of plants for the commercial market. Tissue culture protocols are available for most crop species, and *Digitalis* species have been tested for their eligibility for tissue culture methods, including cryopreservation. Most of the fundamental and trendsetting work was carried out before the turn of the millennium and is therefore well documented in the Luckner/Wichtl monograph [6]. Almost every explant of most of the *Digitalis* species studied, including cells, protoplasts, and anthers, have the potential to regenerate plants through organogenesis or embryogenesis, e.g., [5,49]. Methods for the micropropagation of the species of the section *Isoplexis* have also been described [50–52]. Especially the effects of phytohormones and growth regulators had their fair share of attention. In some cases the starting material (leaf, nodal, root explants) seems to be crucial for successful regeneration, but rooting is usually achieved in hormone-free media or media supplemented with auxins. Random amplification of polymorphic DNA (RAPD) analysis was used to assess genetic stability of long-term cultures. Sales et al. [53] found RAPD variation in long-term cultures of a high-yielding genotype in *D. obscura*. Somaclonal variation of cardenolide content was demonstrated in *D. mariana* ssp. *heywoodii*, an endangered endemite of the Iberian Peninsula. Plants were regenerated either from callus or shoot cultures analyzed for cardenolide content and pattern [54]. *D. mariana* ssp. *heywoodii* is a rich source of glucoevatromonoside (see below). The total cardenolide content of 2% is similar to what was previously reported, whereas the relative content of glucoevatromonoside (about 30% of total cardenolides) was about twice as high as that listed in the Luckner/Wichtl monograph [6].

Recent studies are questioning the finding that due to the fact that they produce no (suspension-cultured cells) or only low levels (shoot cultures) [6,55] of cardenolides, organ cultures are not suited to produce cardenolides in commercial quantities. For example, *D. purpurea* was used in a temporary immersion system (TIS) with the aim of producing cardenolides, albeit with limited success [56] only. In another study, multiple shoot formation was achieved in *D. purpurea* explants. These shoots were either regenerated to intact plants or used for the production of digitoxin and digoxin. It was reported that in the presence of progesterone (200 to 300 mg/L), digitoxin and digoxin accumulation was enhanced about 10-fold, but only reaching the lower $\mu\text{g/g}$ dry weight range. The large amounts of progesterone used in this experiment suggest that this compound is a precursor or an elicitor of cardenolide formation (see below: Biosynthesis) [57]. Elicitation as a method to enhance cardenolide production was used by Pérez-Alonso et al. [58], resulting in the production of about 0.1 mg cardenolides/g dry weight (similar to the figures reported by Hagimori et al. [6,59]) compared to about 5 to 10 mg/g in dried *D. purpurea* leaves [6].

Stuhlemmer et al. [6,60] and Greidziak et al. [6,61] reported the formation of about 1 mg cardenolides/g dry weight in partly submerged shoot cultures and somatic embryoids, respectively. In a more recent study, salicylic acid, exogenous yeast polysaccharides, and calcium chloride were tested for their capacity to enhance cardenolide formation in *D. lanata* shoot cultures. Cardenolide concentrations under the various conditions tested reached 0.5 to 1 mg/g dry weight [62], confirming the results obtained 20 years prior.

Another *Digitalis* species studied extensively in the tissue culture context is *D. obscura*, which was already addressed in the Luckner/Wichtl monograph [6]. The research reports concerning *in vitro* culture of *Digitalis* L. were compiled comprehensively with the aim of providing hands-on knowledge of the *Digitalis* genus, focusing on propagation and preservation [49]. This particular review presents the description of the important members of the genus *Digitalis* with respect to their growth, *in vitro* propagation and also give figures on cardenolide concentrations in the plants. The reports not already reviewed in the Luckner/Wichtl monograph [6] concern *D. thapsi*, *D. minor*, *D. davisiana*, *D. lamarckii*, *D. trojana*, *D. cariensis* and the two subspecies of *Digitalis ferruginea*. Especially protocols for the propagation of Eastern *Digitalis* species, some of which were considered as endangered, have been established recently [49].

D. thapsi was investigated by [63] who demonstrated effects of light and Ca^{2+} on plantlet growth and production of cardenolides. The authors also predicted a connection between H_2O_2 and cardenolide formation.

Studies with tissue cultures of *D. minor* had their focus on molecular biology and genetic transformation and are therefore described below (Genetic Engineering and Molecular Biology).

D. davisiana was propagated *in vitro* and cardenolide production was investigated. Plantlets regenerated from *in vitro* cultures contained 12 mg/kg dry weight digoxin. For natural populations, the highest amount of digoxin (246.58 mg/kg dry weight) was found in leaf samples collected in July during the flowering stage [64].

Verma et al. [65] studied direct shoot regeneration from leaf explants and also indirect somatic embryogenesis in *D. lamarckii*. Plants were regenerated from *in vitro* culture and it was reported that neo-odorobioside G and glucogitoroside were quite abundant (about 150 mg/kg dry weight) [66].

Leaf explants of *D. trojana* excised from axenic seedlings were used to induce shoots which after rooting were successfully transplanted in the greenhouse [67]. Besides cardenolide production other metabolites, such as proline, total phenolics and flavonoids content, were estimated under stress conditions (salicylic acid, temperature) [68].

D. cariensis plants were successfully regenerated from sterilized seeds germinated under aseptic conditions. Cardenolides were profiled from basal leaves of *D. cariensis* plants from natural populations and from *in vitro*-derived plantlets. There was no significant difference in lanatoside contents (A, B, and C) between the two sources. Digoxin and digitoxin were not detected in either source [69]. Efficient *in vitro* plant regeneration protocols for *Digitalis ferruginea* were also reported [70].

Haploids provide an important tool for crop improvement. Cultured anthers, microspores, and ovaries of a high number of plant species have been used to regenerate haploid plants via organogenesis or embryogenesis, e.g., [71]. Androgenic callus was obtained from cold-treated anthers and pollen of *D. lanata*. Mixoploid shoot cultures were derived from embryogenic haploid cell lines via somatic embryogenesis. Haploid shoots were selected and rooted. The haploids were smaller in size than diploid plants, showing morphological abnormalities and male sterility [72].

Cyropreservation

Methods for long-term conservation by freezing *Digitalis* cells or tissues in liquid nitrogen were developed in the 1980s and 1990s, and were summarized by Seitz [73]. The protocols employed are also summarized in Clemente et al. [5]. More recent studies focused on *D. obscura* and the preservation of elite genotypes [74]. Shoot-tips from several genotypes were successfully cryopreserved using the encapsulation-dehydration technique [75]. RAPD analyses demonstrated that cryopreservation is an efficient method to maintain genetic fidelity [53].

Genetic Engineering and Molecular Biology

Biotechnological approaches to improve cardenolide production have been widely reported, but establishing an economically viable process has not been successful as of yet. These approaches mainly focused on the biotransformation of suitable precursors using suspension-cultured cells or organ cultures (see above: Plant Tissue Culture). The biotransformation processes use the ability of selected cell lines of *D. lanata* to 12 β -hydroxylate digitoxin-type to digoxin-type cardenolides, e.g., [6,76]. However, the breeding of elite plants containing high levels of lanatoside C made these biotransformation processes obsolete. Metabolic engineering of plants and microorganisms has become a general approach to improve existing or establish novel processes for provid-

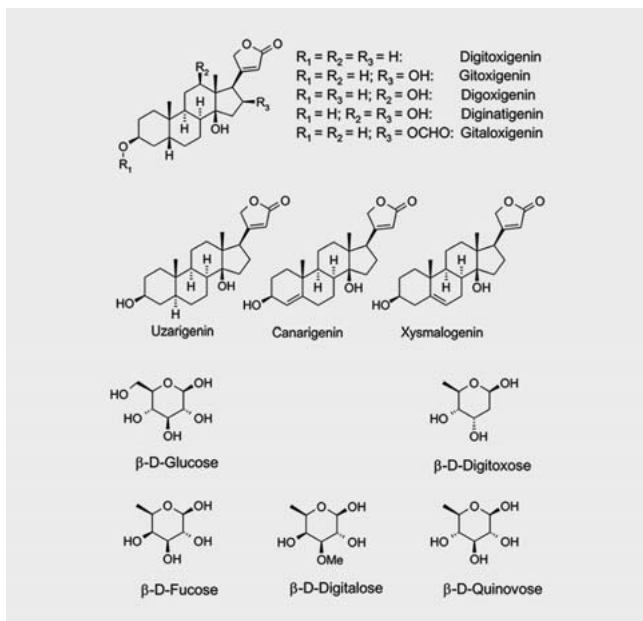
ing natural products (see [77], for a recent review). This requires biosynthetic genes that are introduced into microorganisms or appropriate genes that can be introduced in a given plant. Only limited studies have been published regarding cardenolides [78]. Transgenic *D. lanata* plants were obtained by Lehmann et al. [6, 79] using *Agrobacterium tumefaciens*-mediated transformation of protoplasts. A more efficient transformation protocol was developed by Sales et al. [80] for *Digitalis minor*. The *Arabidopsis thaliana* HMG1 cDNA, coding the catalytic domain of 3-hydroxy-3-methylglutaryl CoA reductase, was expressed in *D. minor*. This resulted in an increased sterol and cardenolide production in both *in vitro*- and greenhouse-grown plants [81]. A clear correlation between HMG1 expression and cardenolide accumulation in transgenic plants could not be established. Progress in this field is limited by the lack of sufficient information concerning the biosynthetic genes (see below: Biosynthesis).

Hybrids, Bastards, Populations

The number of naturally occurring bastards is limited. In 1777, Koelreuter [6,82] was the first to perform backcrossing experiments with *Digitalis* plants in order to generate hybrids. The number of artificially produced *Digitalis* bastards is very high. For a number of bastards, detailed analysis does not exist. Reciprocal crossings can yield different morphological and phytochemical phenotypes. The *D. grandiflora* \times *D. lanata* hybrid, not included in the comprehensive list of Luckner and Wichtl [6], showed amounts of lanatoside C in its leaves 1.5 times higher than in *D. lanata* [83], which was surprising since *D. grandiflora* was reported to contain only minor amounts of lanatosides. Some of the bastards are allopolyploid and others are fertile. This may cause problems in separating crossings from subspecies. *Digitalis sibirica*, for example, may be a bastard derived from *D. grandiflora* and *Digitalis laevigata*.

In Portugal, *D. purpurea* and *Digitalis thapsi* occur side by side. Little information is available concerning the genetic diversity of the natural populations of these species including the identification of spontaneous hybridization. The use of RAPD markers allowed the separation of three main groups, namely *D. thapsi*, *D. purpurea*, and *D. thapsi* \times *D. purpurea* [84, 85].

Genetic diversity within and between populations was also investigated in *D. obscura*, *D. minor*, and *D. grandiflora*. Molecular markers, mainly based on DNA polymorphisms, were used for the characterization. Utilizing RAPD markers, Nebauer et al. [23] detected interspecific variations among the species *D. obscura*, *D. lanata*, *D. grandiflora*, *D. purpurea*, *D. thapsi*, and *D. minor* and the hybrid *Digitalis excelsior* (*D. purpurea* \times *D. grandiflora*). The classification based on the RAPD analyses was fully consistent with the morphology-based classification (e.g., [6,20]). RAPD markers were also used to detect spatial genetic variations in wild populations of *D. obscura* and *D. minor* [53,69]. *D. minor* shows a high level of morphological variation, but RAPD analysis did not support the separation into two subspecies [53]. *D. grandiflora* populations growing in Russia were also analyzed by RAPD and ISSR (inter-simple sequence repeats) markers [86]. The indices characterizing polymorphism and genetic diversity pointed to a high level



► **Fig. 2** Cardenolide genins and examples of sugar moieties found in the cardenolides of *Digitalis* species.

of genetic variation. *D. grandiflora* and *D. purpurea* have a wide distribution and it could be interesting to further investigate the variability between and within different populations in order to assess the reliability of the use of sequence data to infer relationship and support germplasm conservation efforts.

Agriculture

Digitalis species are commonly propagated by seeds. Probert et al. [87] reviewed some of the threats to seed collection quality that arise during the period between collection, processing, and storage. Butler et al. [88] demonstrated that germination of *D. purpurea* seeds was improved, e.g., by rehydration. *D. cariensis* seeds germinate poorly after extensive dormancy. Seed germination was improved when seeds were pretreated with scarification followed by soaking in sterile distilled water overnight [89].

The main sources for cardenolides used in therapy are *D. lanata* and *D. purpurea*. Both species are cultivated for this purpose. Fochler et al. [90] described the cultivation of several *Digitalis* species. Predominantly, *D. lanata* is cultivated and used as the starting material for cardenolide isolation and production. The crop is sown in mid-April and the leaves are harvested from September to late November and subsequently dried at 60 °C. Drying of the leaf material is accompanied by partial hydrolysis of the so-called primary glycosides, such as the lanatosides. Both the terminal glucose and the acetyl group will be released and secondary glycosides such as digoxin appear as artefacts. In the dried material, which can be stored over long periods of time, glucosidase(s) and esterase(s) capable of hydrolyzing cardenolides are inactive, hence the conversion of primary to secondary glycosides is not yet completed at this stage. The enzymes mentioned can be reac-

tivated when the dried leaves are moistened during the industrial digoxin process. Complete deacetylation is usually achieved by alkali treatment. In this way, the target compounds digoxin and digitoxin will emerge as the main products. A new approach for post-harvest treatment of foxglove raw material was described, which includes storage of the freshly collected raw material in a silo, followed by air-sun drying after 8–9 months [91]. Under these conditions, digoxin and digitoxin will be released from their glucosylated and/or acetylated precursors.

The aim of numerous breeding trials was to improve leaf and cardenolide production as well as the resistance to *Septoria* leaf spots and bolting. Selection has only moderately improved the leaf yield. In the 1970s and 1980s, the selection of plants with a high resistance to *Septoria digitalis* infections produced a variety that did not need to be sprayed with fungicides and also showed a 50% increase in digoxin production [6, 92]. Another target was the breeding of plants containing high levels of digoxin-type cardenolides such as lanatoside C. Interspecific hybrids have also been established to elucidate genetic regulations of cardenolide production in *D. lanata* and *D. purpurea* [6, 93, 94], but the results are not fully conclusive since non-genetic factors affecting cardenolide accumulation in leaves were identified in previous studies [6]. Mutagenesis to induce genetic variation on cardenolide accumulation was also tried in *D. obscura* [95]. Only a few of the breeding efforts and successes have been published in detail and most of the programs have been terminated for various reasons [96].

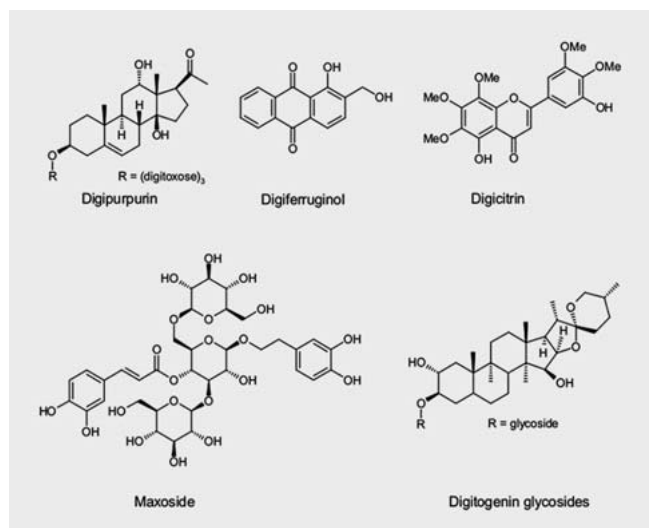
D. purpurea is mostly cultivated in small farms or agricultural companies and only limited matter is produced, e.g., for the production of homeopathic stocks from fresh leaves.

Phytochemistry

The most prominent compounds formed in the genus *Digitalis* are the cardenolides. They are present in all but one species, namely *D. sceptrum*, which is a member of the Macaronesian (section *Isoplexis*) species [6, 27]. Cardenolides have attracted more interest than other steroidal compounds (steroidal saponins, sterols) or the many phenolic compounds (anthraquinones, phenylethanoic acids, flavonoids) reported in *Digitalis* plants. The understanding of the occurrence of natural products in the various species has not been broadened significantly since the publication of the Luckner/Wichtl monograph [6]. The more recent comprehensive review of phytochemical studies of *Digitalis* species [97] focused on the presentation of data recorded by Eastern European research groups.

Cardiac glycosides

The *Digitalis* cardenolides can be grouped according to their steroidal genins, namely digitoxigenin, digoxigenin, gitoxigenin, gitaloxigenin, diginatigenin, oleandrigenin, uzarigenin, xysmalogenin, and canarigenin [6]. They all possess a 14 β -hydroxyl group, and the majority is 5 β -configured. 5 β -Cardenolides are assumed to be formed via progesterone (see below: Biosynthesis) and it is surprising that definitive proof for the presence of progesterone in vascular plants was only provided in 2010 [98]. At position 3 β of the genin, a sugar side chain with up to five carbohydrate units



► **Fig. 3** Examples of natural products other than cardenolides occurring in *Digitalis* species.

is attached that can be composed of glucose and various rare 6-deoxy, 2,6-dideoxy, and 6-deoxy-3-methoxy sugars, e.g., D-fucose, D-digitoxose, or D-digitalose (► **Fig. 2**). More than 100 different cardenolides have been isolated from *Digitalis* species. The members of the section *Isoplexis* are thought to be more primitive than other *Digitalis* species [27]. The *Isoplexis* cardenolides may also be regarded as more primitive since they only comprise mono- and diglycosides with gens that can be 5 α - or 5 β -configured. Δ^4 - or Δ^5 -unsaturated cardenolides also do occur [99, 100].

The observation that cardenolide content can differ dramatically in *D. purpurea* depending on the location of the plant analyzed [101] was confirmed in a more recent study with Sardinian accessions. HPLC analyses of 2-year-old plants revealed that the amounts of digitoxigenin and gitoxigenin in fresh leaves ranged between 11 and 241 mg · kg⁻¹ and 4 and 178 mg · kg⁻¹, respectively [102].

Digitanols

Digitanols are Δ^5 -pregnanes, some of which possess the 14 β -hydroxyl function and a sugar side chain typical for cardenolides, e.g., [6, 103]. Digitanols and cardenolides are therefore supposed to be formed by a common biosynthetic pathway. They can be highly oxidized and occur as tetracyclic or pentacyclic compounds (► **Fig. 3**). In the latter case, C-12 and C-20 are bridged with an oxygen to form a furanoid structure. Only *D. lanata* and *D. purpurea* have been examined sufficiently [6, 97].

Sterols and steroid saponins

Besides the common phytosterols, such as sitosterol or stigmasterol, *Digitalis* species also contain rare sterols, steryl esters, and steryl glycosides, e.g., [6, 104]. Steroid saponins are quite abundant in the monocots but rare in eudicots and magnoliids. As one of the exceptions among the dicots, *Digitalis* contains steroidal saponins that are derived from C₂₇ sterols. They bear a cholesterol scaffold, and indeed this “animal sterol” was detected in

Digitalis species [6, 105] and was discussed as a sterol precursor of cardenolides (see below: Biosynthesis). The *Digitalis* saponins identified so far comprise neutral monodesmosides (spirostanol type) or bisdesmosides (furostanol-type) possessing only a weak saponin character. Again, only *D. purpurea* and *D. lanata* were thoroughly examined.

Anthraquinones

About 40 different anthraquinone derivatives such as digiferruginol have been identified in the genus *Digitalis* (► **Fig. 3**). They share structural similarities with alizarin, which suggests that they are formed from building blocks provided by the shikimate and the methylerythritol phosphate pathways. Anthraquinones are also formed in tissue cultures that are not able to produce cardenolides [6, 106].

Phenylethanoids

Phenylethanoid glycosides were isolated from several *Digitalis* species [6]. Aqueous extracts of *D. thapsi*, *D. purpurea*, *D. chalcantia*, and *D. sceptrum* were tested for their content of main carbohydrates, iridoids, and caffeoyl phenylethanoid glycosides [17]. The *Digitalis* species contained cornoside and a number of other phenylethanoid glycosides, but lacked iridoid glucosides. Kirmizibekmez et al. [107] reported a new phenylethanoid bearing a quinovose. Maxoside, chosen here as a structural example (► **Fig. 3**), was reported for the first time by Max Wichtl's students in 1995 [6, 108].

Flavonoids

Digitalis species have also been investigated with respect to their flavonoid content and pattern. About 40 different flavonoids, mainly of the flavone and 3-methoxyflavone groups, have been described. Among them, digicitrin (► **Fig. 3**), the most highly oxygenated naturally occurring flavonoid substance [6, 109].

Other phenolic compounds

The phenolic composition of methanolic extracts of *D. ferruginea* and *D. lamarckii* was investigated and their antioxidant properties assessed. *D. lamarckii* was found richer in phenolic compounds than *D. ferruginea*, with chlorogenic acid as the dominant compound in both species [110].

Polysaccharides

Polysaccharides, mainly xyloglucans together with minor amounts of highly branched arabinogalactans, were isolated from the culture media of suspension-cultured cells from *D. lanata*. Polysaccharides were also isolated by sequential extraction from *D. purpurea* leaves. Main polysaccharides were neutral and acidic arabinogalactans, neutral and acidic glucomannans, and starch. Pectic material was identified as rhamnogalacturonan, and hemicellulosic cell wall polysaccharides consisted of a neutral, low substituted arabinoxyloglucan and several acidic xylans. Interestingly, 2,6-dideoxysugars, the typical carbohydrate components of several *Digitalis* glycosides, cannot be found in these polysaccharides [6, 111, 112].

Miscellaneous

In the course of recent studies with Eastern *Digitalis* species, several known compounds from various groups of natural products were identified. For example, the phytochemical investigation of *D. trojana* led to the isolation of two cardiac glycosides, one pregnane glycoside, and three furostanol-type saponins along with three cleroidicins, four phenylethanoid glycosides, two flavonoids, and two phenolic acid derivatives [113].

Biosynthesis of Cardenolides

The most prominent compounds formed in the genus *Digitalis* are the cardenolides. Hence, only their biosynthesis will be taken into consideration here.

Cardenolides are steroids and thus are supposed to be derived from mevalonic acid via triterpenoid and phytosterol intermediates. An important finding was that the carbon atoms C-22 and C-23 of the butenolide ring of the cardenolides are not derived from mevalonic acid. Possible biosynthetic routes leading to the cardenolides are outlined in ► Fig. 4. The early tracer and other precursor studies leading to the proposed pathway of cardenolide genin formation in plants have been summarized, e.g., [6, 114, 115].

Textbooks generally suggest cholesterol as the direct sterol precursor of cardenolides, converted to pregnenolone by a P450_{scc} not yet identified unambiguously in plants. Indirect evidence for a favored route not involving cholesterol was provided by studies using inhibitors of 24-alkyl sterol formation. The content of cholesterol increased, whereas the formation of 24-alkyl sterols was dramatically reduced, as was the content of cardenolides [6, 116], indicating a route via the more abundant 24-alkyl phytosterols.

The conversion of pregnenolone into progesterone is composed of two steps: Firstly, the NAD-dependent oxidation of the 3-hydroxy group, yielding $\Delta^{4,5}$ -pregnen-3-one catalyzed by the $\Delta^{4,5}$ -3 β -hydroxysteroid dehydrogenase. Early attempts to isolate the plant 3 β -HSD gene have been reported by Lindemann et al. [117]. Later on, Herl et al. [118] generated primers for PCR amplification of the *D. lanata* 3 β -HSD gene. No obvious similarities with the animal 3 β -HSD, which also possesses $\Delta^{4,5}$ -3-ketosteroid isomerase activity (3-KSI), were seen. In the presence of NAD, rD3 β -HSD converted pregnenolone and other 3-hydroxysteroids into the respective 3-ketosteroids. r3 β -HSD also possesses 17 β -dehydrogenase activity. Additionally, rD3 β -HSD was able to catalyze the reverse reaction, namely the reduction of 3-ketosteroids when NADH was used as a cosubstrate. It was presumed [6, 119, 120] that 3 β -HSD catalyzes at least two steps in cardenolide biosynthesis, namely the dehydrogenation of pregnenolone and the reduction of 5 β -pregnane-3,20-dione (► Fig. 4).

3 β -HSD activity could recently be separated from a 3-KSI catalyzing the allylic isomerization of the 5,6 double bond of $\Delta^{4,5}$ -3-ketosteroids to the $\Delta^{3,4}$ position [121, 122].

Gärtner and Seitz [6, 123] reported on the isolation and characterization of a progesterone 5 β -reductase (P5 β R) from *D. purpurea*. Soon after the first peptide [6, 124] and gene [38] sequences became available, P5 β R genes were identified in all *Digitalis* species, even in *D. scepstrum*, which is a cardenolide-free species [28]. The *Digitalis* P5 β Rs are members of the short-chain de-

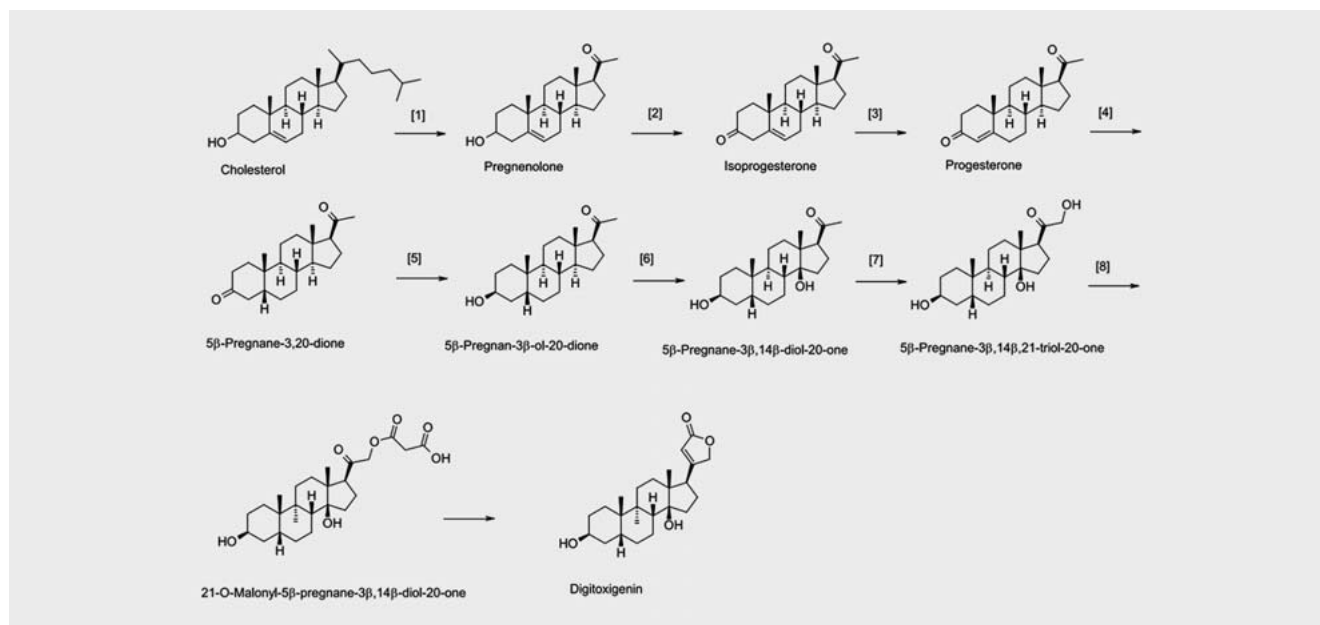
hydrogenase/reductase (SDR) superfamily of proteins. Herl et al. [125] found that a P5 β R from the cardenolide-free species *A. thaliana* was at least 10 times more active than the *Digitalis* enzymes. This finding challenged the importance of P5 β R in cardenolide biosynthesis and subsequent sequence analysis, and database search revealed that P5 β R genes and enzymes occur in many organisms, including cardenolide-free plants [126, 127]. This indicates that numerous genes so far annotated as P5 β R may have other physiological functions. Burda et al. [128] reported that P5 β Rs are capable of reducing a wide range of small molecules bearing an activated C=C double bond. The enzyme's substrate-promiscuous character was confirmed by Durchschein et al. [129]. More recently, a cDNA encoding for a second type of P5 β R (P5 β R2) was isolated from a *D. purpurea* cDNA library and further characterized [130]. It can be induced by various types of stress resulting in increased cardenolide production in *D. purpurea*. A gene from *Catharanthus roseus* showing high similarity to *D. purpurea* P5 β R2 was able to form *cis-trans*-nepetalactol from 10-oxo-geranial and was therefore named iridoid synthase [131]. The protein subfamily was recently termed PRISE (progesterone reductases and/or iridoid synthases) [132].

Progesterone 5 α -reductase (P5 α R), which competes with P5 β R for substrates and therefore redirects sterol precursors to 5 α -cardenolide formation, is located in the endoplasmic reticulum. The addition of finasteride, an inhibitor of animal and human testosterone-5 α -reductase, inhibited 5 α -POR of *D. lanata* completely but left P5 β R unaffected [133].

Other pregnane-modifying enzymes have been described mainly from crude or partially purified protein extracts from *D. lanata* and *D. purpurea* plants or tissue cultures. Several of the reactions described can also be catalyzed by 3 β -HSD, although they were assigned to other enzymes like 3 β -hydroxysteroid 5 α -oxidoreductase, 3 β -hydroxysteroid 5 β -oxidoreductase, or 3 α -hydroxysteroid 5 β -oxidoreductase (see [115] for a review).

The enzymes involved in pregnane 21-hydroxylation and pregnane 14 β -hydroxylation in the course of cardenolide formation have not been described as yet. Hence, it still remains unclear whether pregnane 14 β -hydroxylation precedes 21-hydroxylation or vice versa. It was communicated that baker's yeast transformed with a mouse P450 21-hydroxylase gene was also able to 21-hydroxylate cardenolide precursors *in vivo* [134].

An intra- or intermolecular nucleophilic attack of a malonyl-CoA at the C-20 carbonyl of a pregnane-20-one has been proposed as a mechanism of attaching C-22 and C-23 to the pregnane skeleton. The formation of the butenolide ring system can be accomplished by formal elimination of water and lactonization. Spontaneous butenolide ring formation of pregnane-21-*O*-malonyl hemiesters under mild reaction conditions was shown to be facilitated by the 14 β -hydroxy group present in all natural cardenolides [135, 136]. When 5 β -pregnane-14 β ,21-diol-20-ones were incubated with malonyl coenzyme A in a cell-free extract of *D. lanata* leaves, a product was formed which was identified as the malonyl hemiester of the substrate [137]. Luber [138] and Kuate et al. [139] reported the purification and characterization of malonyl-coenzymeA:21-hydroxypregnane 21-*O*-malonyltransferases (21MaT) from *D. lanata* and *D. purpurea*, respectively. At least two 21MaT isoforms exist in *D. lanata* (Meitinger, Fink, and Kreis, unpublished).



► **Fig. 4** Postulated biosynthetic pathway of cardenolide formation in *Digitalis*. 1 Sterol side chain cleaving enzyme (SCCE), 2 NAD:3 β -hydroxysteroid dehydrogenase (3 β -HSD), 3 $\Delta^{4,5}$ -3-ketosteroid-isomerase (3-KSI), 4 progesterone-5 β -reductase (P5 β R), 5 NADH:3 β -hydroxysteroid dehydrogenase (3 β -HSD), 6 steroid 14 β -hydroxylase (unknown), 7 steroid 21 β -hydroxylase (unknown), and 8 malonyl coenzyme A:21-hydroxypregnane 21-O-malonyltransferase (21MaT).

Other steps of the biosynthesis, including the formation of the sugar side chain, have not provided any new insights over the past 20 years. Although pregnane 2 β -hydroxylation was described [140] in addition to the 12 β - and 16 β -hydroxylations already known [6], no attempts to isolate the respective enzymes and/or genes were reported. When investigating the degradation of cardenolides, a cardenolide 16'-O-glucohydrolase (CGH I) was purified from young leaves [6, 141, 142] and partially sequenced. Nucleotide primers were deduced from these peptide fragments and a *cgh1* cDNA was synthesized. After genetic transformation, a recombinant CGH I protein was produced in *Escherichia coli* [143]. Cotyledon explants of *Cucumis sativus* were genetically transformed using *Agrobacterium rhizogenes* harbouring a *D. lanata cgh1* cDNA, and hairy roots formed at infected explants showed CGH I activity [144]. Another cardenolide glucohydrolase, termed CGH II, was isolated from *D. lanata* and *Digitalis heywoodii* leaves and cell cultures. This soluble enzyme hydrolyzes cardenolide disaccharides with a terminal glucose and appears to be quite specific for glucoevatromonoside, whereas the tetrasaccharides, e.g., purpureaglycoside A, which are rapidly hydrolyzed by CGH I, were only poor substrates for CGH II [6, 145].

Ecology

D. (Isoplexis) canariensis is one of the ornithophilous species of the Canary Islands. Though self-pollination is possible, cross-pollinated flowers produce a greater percentage of viable seeds. Flowers are visited by five nectar-feeding birds, whereas insect pollination is absent [146]. All other *Digitalis* species are pollinated by in-

sects, predominantly bumblebees. *Isoplexis*-like flowers may have developed in continental predecessors or after island colonization. As yet, it is not completely clear if *Digitalis* was solely insect-pollinated throughout all its evolutionary history. For example, the existence of some characteristics associated with bird visits in *D. obscura* [27] and the observation that *Digitalis* contains sucrose nectar, a preferred food resource for birds, leaves open the possibility that the pollination of the mainland *Digitalis-Isoplexis* ancestor was already assisted by nectarivorous birds.

Bumblebees did not avoid milkweed nectar spiked with digoxin, which might indicate that nectar cardenolides have little effect on pollination [147]. However, a primary effect of nectar cardenolides appears to be a reduction of monarch butterfly oviposition [148]. Whether coevolution and ornithophily had an impact on cardenolide evolution (and de-evolution in *D. sceptrum*) is still an open question. Interestingly, a recent study on cardenolide resistance in feeding insects indicated that major adaptations to plant toxins may be evolutionarily linked to sequestration. They may not necessarily be a means to eat toxic plants and develop metabolic resistance as was long assumed since it was shown that resistant Na⁺/K⁺-ATPases are not necessary to cope with dietary cardenolides in milkweed butterflies [149].

Pharmacology and Toxicology

The primary mechanism of action of *Digitalis* glycosides is their ability to inhibit membrane-bound Na⁺/K⁺-ATPase. As a consequence, Na⁺/Ca²⁺ exchange is promoted and more intracellular calcium becomes available for contractile proteins. This, in turn, in-

creases myocardial contraction. The various effects of cardenolides in systolic heart failure were summarized by Eichhorn and Gheorghide [150] (Table S2, Supporting Information). Several studies have demonstrated antiviral and antitumoral activities as well as potential beneficial effects on cystic fibrosis, e.g., [151–156]).

Digitoxin, at nanomolar concentrations, suppresses hypersecretion of IL-8 from cultured cystic fibrosis (CF) lung epithelial cells. Digitoxin could be considered a drug for suppressing IL-8-dependent lung inflammation in cystic fibrosis [157].

Digitoxin inhibited HSV-1 replication with an EC_{50} of 50 nM [158]. In another study, an anti-herpes screening was performed with 65 cardenolides (including cardenolide derivatives), and glucoevatromonoside was found to be the most active. It inhibited HSV-1 and HSV-2 replication at nanomolar concentrations. The CC_{50} value (a measure for cytotoxicity) was 50 times higher than that for digitoxin [151].

It was suggested that cardenolides could induce apoptosis in tumor cells at concentrations lower than those used for treating cardiac insufficiency in humans [159, 160]. These observations together with the a multitude of *in vitro* studies of cancer cells being treated with *Digitalis* cardenolides (Table S3, Supporting Information) led to the first clinical phase 1 trials of cardenolides in cancer patients [161–163]. Several comprehensive reviews focusing on the potential anticancer properties of cardenolides have recently been published, e.g., [164], and the reader is referred to them for more detailed information.

Medicinal Uses

A multitude of studies demonstrated the positive inotropic effect of *Digitalis* glycosides. However, in the 1970s and 1980s, several studies failed to show a clinical benefit of digoxin and some other cardenolides. These observations, together with a high incidence of digoxin intoxication, brought its use into question. As newer therapies emerged in the 1980s, the interest in digoxin and other cardenolides faded into the background [6, 165]. In a more recent study with over a thousand outpatients, it was demonstrated that digoxin had no effect on all-cause or cause-specific mortality or on all-cause or cardiovascular hospitalization [166]. On the other hand, digoxin had beneficial effects in patients who remained symptomatic after being treated with diuretics and/or angiotensin-converting enzyme inhibitors [167].

The knowledge to treat malignant diseases with extracts from plants containing cardiac glycosides could date back to Arab physicians in the eighth century [168]. The potential use of cardenolides in cancer therapy was evaluated about 50 years ago, but terminated soon after because of the toxicity of these compounds [169, 170]. In a 1980s study, cancer cells collected from women who were treated with cardenolides proved to be less malignant than cancer cells from control patients [171–173]. Other studies verified these findings [174–176] and they may lead to new clinical applications in the future (see above).

Endogenous Digitalis-like Factors

Endogenous „digitalis-like factors“ (EDLFs) were already described by Luckner and Wichtl [6], and Buckalev [177] gave a renewed overview. In 1991, Hamlyn et al. [6, 178] reported purification of a compound indistinguishable from ouabain, but Baecher et al. [179] using ultrasensitive UPLC-MS/MS found no endogenous ouabain in human plasma. Meanwhile, numerous likely structures have been suggested, including steroids, lipids, peptides, and a variety of other compounds. They were even regarded as a new class of (steroidal) hormones in view of their isolation from human blood plasma, adrenals, and hypothalamus. Despite all efforts their structure and indeed the existence of EDLFs remains controversial [180].

Cardenolide-inactivating Antibodies

Cardenolide intoxication is usually associated with an overdose in patients using *Digitalis*. Intoxication can occur when cardenolide excretion by the kidneys is impeded. The nonspecific signs and symptoms make the diagnosis of toxicity difficult. Digoxin-specific Fab fragments have revolutionized the treatment of *Digitalis* intoxication [6]. In a recent study, first-line therapy with Fab fragments in patients with *Digitalis* poisoning resulted in a decreased mortality rate [181]. Moreover, administration of digoxin-specific Fab antibody fragments in an otherwise healthy child after oleander intoxication was found to be safe and without adverse reactions [182]. Due to its bitter taste, it is highly unlikely that *D. purpurea* is ingested by humans. However, after an accidental contamination of comfrey leaves with leaves of *D. purpurea*, the clinical symptoms of cardenolide intoxication eased after the application of digoxin-specific antibody Fab fragments [183].

Analytical Protocols

One flaw in several studies is the identification of the cardenolides formed. Pérez-Alonso et al. [56] used HPLC-DAD to confirm cardenolide structures and scored data for digoxin and lanatoside C, which have never been reported to occur in *D. purpurea* before. Though it is tempting to use well-established HPLC procedures, one has to take into consideration that 1) cardenolide patterns can be very complex [6], 2) compounds of similar polarity will elute together, 3) individual cardenolides cannot be identified by their UV spectra, and 4) reference compounds may not be available for all cardenolides in a given extract. A minimal requirement for analysis should be the conclusive identification of the cardenolide genin (= aglycone) after hydrolysis [5]. HPLC-MS or NMR data are highly desirable. According to Nebauer et al. [74], the proportion of series A and B genins varied among the studied populations of *D. obscura*. Roca-Pérez et al. [35] reported that the predominance of A-series cardenolides does not depend on the population studied. The contradicting conclusions may be attributed to the different methods chosen for cardenolide determination. In other relevant literature, especially on the topic of plant tissue culture, various reports with

insufficient or inaccurate structural data can be found. To list and discuss them is beyond the scope of this review.

Meanwhile, highly sophisticated and validated techniques like HPLC-MS allow for the reliable determination of cardenolides as well as their corresponding genins, even in the pg/g range [184]. However, the HPLC protocols introduced by Max Wichtl's group [6, 185–187] are still relevant. They are valid and valuable starting points for cardenolide analysis and modifications thereof have been used ever since in a multitude of studies. Yet, an amazingly large number of HPLC protocols for the separation of *Digitalis* cardenolides have been published over the years, some of them coupled with MS, DAD, and/or pulsed amperometric detectors, e.g., [188, 189].

Outlook

Digitalis cardenolides are still used today for treating heart failure and atrial fibrillation despite the arrival of new medications. Cardenolides can play regulatory roles in several cellular processes, including proliferation. They can induce apoptosis in cancer cells. It seems as if malignant cells are more susceptible to this natural compound than healthy ones. Progress in biosynthesis, molecular biology, and plant regeneration opens up the possibility of recombinant production of designer cardenolides using various approaches combining chemical synthesis, biotransformation, and genetic engineering.

Supporting information

Tables showing the taxonomy of the genus *Digitalis*, the effects of cardenolides in systolic heart failure, and the effects of cardenolides on cancer cells are available as Supporting Information.

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Conflict of Interest

The author declares to have no conflicts of interest.

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