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Cardenolide diversity and toxicity in tri-trophic interactions

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PSM: plant specialized (or secondary) metabolites (PSM)

DLS: Dorsolateral space

e.g.: exempli gratia (Lat) “for example”

i.e.: id est (Lat) “that is” or “in other words”

ADME: absorption, distribution, metabolism, and excretion

HPLC-DAD-MS, LC-MS, or HPLC-HRMS: Refers to High-Performance Liquid Chromatography coupled with Diode Array Detection and High Resolution Electrospray Ionization Mass Spectrometry

HRESIMS: High Resolution Electrospray Ionization Mass Spectrometry

NMR spectroscopy: Nuclear magnetic resonance spectroscopy

UV: ultraviolet

1 INTRODUCTION

1.1 Chemical diversity: Plant —prey —predator systems

Secondary or specialized metabolites (SM) such as phenolics, terpenes, and nitrogen-containing compounds have evolved in many animals, plants and micro-organisms in response to natural enemies (e.g., pathogens, parasites, herbivores and predators) (Mauricio and Rausher, 1997). An important characteristic of these chemical defences is within population diversity both in the quantity and the profile of specific SM (Speed et al., 2012). Defensive diversity can be thought of in three ways: (1) the absence of toxicity in a proportion of individuals in an otherwise toxic prey population (auto-mimicry); (2) broad variation in quantities of toxin within individuals in the same population; (3) variation in the chemical constituents of chemical defence (Speed et al., 2012). Explaining the factors that contribute to and maintain this variability is a primary goal of chemical and evolutionary ecology and the focus of this thesis.

In plant–herbivore interactions, plants produce SM (PSM) in response to herbivore pressure (Mithöfer and Boland, 2012; Dyer et al., 2018). Adapted herbivores avoid PSMs by evolving feeding behaviours to consume only plant parts which contain minimal amounts of the chemicals (Hesbacher et al., 1995); evolving physiological adaptations like impermeable gut membranes that permit fast excretion of the toxins (Scudder and Meredith, 1982b); detoxification enzymes (Yu, 2006); the acquisition of endosymbiotic microorganisms (Shen and Dowd, 1991); and resistance mechanisms that allow them to tolerate, accumulate or modify the PSM for their own benefit, i.e., sequestration (Opitz and Müller, 2009). The resulting biochemical co-evolutionary arms race (Ehrlich and Raven, 1964) is thought to drive the diversity of PSMs and insect herbivores (Futuyma and Agrawal, 2009; Erb and Kliebenstein, 2020)

Herbivores that accumulate PSMs as chemical defences via sequestration are often unprofitable to predators (Paradise and Stamp, 1991), and demonstrate their protection by conspicuous warning signals, often combined with gregarious behaviour, i.e., aposematism (Opitz and Müller, 2009). PSM sequestered by herbivores can also have damaging effects on the herbivore's parasitoids (Pasteels et al., 1983). In prey that sequester PSMs, variability in the quantity and biochemical profile of chemical defences

is common, both within and between species (e.g., in poison frogs, *Dendrobates tinctorius*, (Lawrence et al., 2019); *Heliconius* butterflies (Arias et al., 2016; Sculfort et al., 2020); ladybirds, (Arenas et al., 2015); and nudibranchs (Winters et al., 2019)).

There are a number of explanations for the variability and complexity of defensive chemicals, including the variable nature of the environments within which prey organisms exist and develop, selective PSM sequestration, life history, and variation in the selection pressures exerted by predators/other natural enemies (Speed et al., 2012). In tri-trophic interactions between plants, herbivores, and predators, SMs are central to top-down and bottom-up control of herbivores, and can contribute to enemy-free space, and diversification (Ode, 2006; Jeckel et al., 2022).

1.1.1 Phytochemical diversity

Phytochemical diversity, in broad terms, describes the diversity of SM from single plant tissues to entire plant communities (see Thon et al 2023; Wetzel & Whitehead, 2020). There are an estimated 200,000 secondary metabolites that have been identified across the plant kingdom (Recorded by the Dictionary of Natural Products, 2015) (Harvey et al., 2015). This ‘chemodiversity’ can be quantified by focusing on particular compounds or all types of metabolites, both composition and variation across spatial and temporal scales (Wetzel and Whitehead, 2020; Thon et al., 2023).

A specific PSM is often confined to a particular systematic unit, but isolated occurrences can occur in widely unrelated taxonomic group (Wink, 2008; Mithöfer and Boland, 2012; Dyer et al., 2018; Zhang et al., 2021). In general, plants tend to have a major SM, and several minor components, which are often biosynthetically related to the main constituents (Wink, 2008). For example, *Tanacetum vulgare* L. (common tansy; Asteraceae) has more than 30 ‘chemotypes’ that vary in their dominant terpenoids (Keskitalo et al., 2001; Wolf et al., 2011; Clancy et al., 2016)

While no single metric can summarise phytochemical diversity alone, several concepts and metrics can be applied to this data including: richness, evenness, structural complexity, compositional and functional diversity, and alpha, gamma and beta diversity (Wetzel and Whitehead, 2020). Richness is the count of unique PSM present in a plant sample or group of samples (Winters et al., 2019; Wetzel and Whitehead, 2020). Richness

can be defined at many scales (e.g., organ, individual, genotype, species, or community). Evenness describes the distribution of total PSM production among all the compounds within a sample (Glassmire et al., 2020; Wetzel and Whitehead, 2020). A sample where all compounds have equal concentration is perfectly even, whereas a sample composed of one abundant compound and multiple low-concentration compounds has low evenness (Wetzel and Whitehead, 2020).

Understanding what maintains this phytochemical diversity is important, because natural selection is expected to lead to the loss or fixation of some variants (Speed et al. 2012). Among genetic processes (Hamberger and Bak, 2013), the abiotic environment (Monson et al., 2022), metabolic pathways (Shoji, 2019), and phenotypic plasticity (Defossez et al., 2021), interactions with animals play a role in most hypotheses and models for the evolution of phytochemical diversity (Whitehead et al., 2021). For example, Calf et al. (2018) found that the composition and total amounts of glycoalkaloids in bittersweet nightshade *Solanum dulcamara* varied depending on slug (*Deroceras reticulatum*) density (Calf et al., 2018).

1.1.2 Herbivores as drivers of phytochemical diversity

The trophic interactions described in 1.1 can lead to biochemical co-evolution, which is hypothesised to lead to rapid adaptation by both plants and insect herbivores, and the production of novel SM in an evolutionary arms race (Ehrlich and Raven, 1964). But given that most PSMs have unknown functions or may not have a biological effect, a number of alternative hypotheses have been proposed including apparency, synergy, moving target, interaction diversity, and the “screening hypothesis” (Firn and Jones, 2003).

1.1.2.1 Apparency

Feeney’s plant apparency hypothesis was developed to understand how plants in a community produce diverse secondary metabolites and this depends on how easy it is for herbivores to discover them in comparison to other plants. In practice, this hypothesis has been difficult to test and there is very little empirical support for it (Smilanich et al., 2016).

1.1.2.2 Synergy

The synergy hypothesis describes how individual PSMs can function additively or even super-additively. That is mixtures of PSMs can have an effect that is greater than the sum of the effects of the individual secondary metabolites. Those mixtures affect generalist herbivores more than specialists (reviewed by Richards et al., 2016) and interclass synergy between compounds can occur (Berenbaum and Neal, 1985; Steppuhn and Baldwin, 2007). In the pharmaceutical industry, synergies are more likely to occur among structurally dissimilar compounds (Liu and Zhao, 2016). Most studies, however, have only tested this hypothesis with only a small number of compounds.

1.1.2.3 Moving target

The moving target hypothesis was proposed by Adler & Karban (Adler and Karban, 1994) in regard to inducible defences, and describes the phenomenon of when a plant in a population changes its defence in response to an attack by a herbivore. This variability could benefit plants if it requires herbivores to adjust detoxification and metabolic pathways when they move between plants, which is expected to be costly to the insect in terms of energy and opportunity (Pearse et al., 2018).

1.1.2.4 Interaction diversity

The interaction diversity hypothesis posits that plants produce numerous PSM because they interact simultaneously with numerous organisms, also known as the ‘common sense scenario’ (Berenbaum and Zangerl, 1996). If different compounds are active in interactions with different antagonists or mutualists, and there is independent selection on each compound, this could lead to chemical diversity. Whitehead et al (2021) tested the richness and structural diversity of phenolic metabolites on four species of insect herbivores, and found that more diverse mixtures were effective against a larger range of consumers (Whitehead et al., 2021).

1.1.2.5 Screening

The screening hypothesis suggests that for plants to develop an effective defence, they must sample a wide range of metabolites. As a result, while some PSMs may not offer immediate adaptive advantages, they are retained to enhance the likelihood that the plant will eventually produce a PSM effective against specific threats (Dyer et al., 2018; Kessler

and Kalske, 2018; Whitehead et al., 2021). A major assumption of the screening hypothesis is that most compounds are not bioactive (Firn and Jones, 2003).

1.1.2.6 Co-evolutionary arms race

Coevolution describes reciprocal evolutionary change in interacting species driven by natural selection(Thompson, 2005). The co-evolutionary arms race hypothesis proposes that plants have accumulated phytochemical diversity in a stepwise process (Wetzel and Whitehead, 2020). Plants evolve novel defences and herbivores, in turn, evolve counter-adaptations. For example, the evolution of novel genes for glucosinolate detoxification in Pieridae family of butterflies has been accompanied by duplication and neofunctionalization of defensive glucosinolate genes in Brassicaceae host plant species (Wheat et al., 2007; Sønderby et al., 2010; Edger et al., 2015; Blažević et al., 2020). Another aspect of the co-evolutionary arms race hypothesis is phenotype matching across space and time – the geographical mosaic of coevolution (Thompson, 2005). This describes populations of plants that have different concentrations of secondary metabolites that is related to herbivore abundance (Zangerl and Berenbaum, 2003).

These six hypotheses are not mutually exclusive. They can be used to address different phytochemical patterns at different scales (Mithöfer and Boland, 2012; Dyer et al., 2018; Wetzel and Whitehead, 2020). Herbivores face not only a range of PSMs that are heterogeneously distributed through time and space, but also selective pressures from predators, parasitoids and competitors. As a result, the degree and nature of PSM resistance by the plants´ enemies varies depending on the geographical distribution of plant phenotypes, the specificity of the plant–insect association and the local community composition (Després et al., 2007).

1.1.3 Chemically defended prey

All animals are at risk from predation. Which defences against predation are used depends upon their relative costs and benefits, the evolutionary history of a prey, and the stage of the predation sequence that the defence is deployed (Kikuchi et al., 2023). It is to the advantage of the prey to interrupt the predation sequence as early as possible, which can be divided into six stages: encounter, detection, identification, approach,

subjugation (Endler, 1991). Different defences have evolved for these different stages. Prey reduce the chance of *encounter* by avoiding habitats where predators are more common, *detection* through reduced movement and cryptic appearance (Merilaita et al., 2017; Stevens and Ruxton, 2019), *identification* through mimicry or masquerade (Skelhorn et al., 2009), and the chance of being *subjugated* and *consumed* with physical and chemical defences (Blum, 1981; Ruxton et al., 2013).

Chemical defences are widespread across in the animal kingdom (e.g., poison frogs (Saporito et al., 2006; Lawrence et al., 2023), nudibranchs (Rogers and Paul, 1991; Winters et al., 2019, 2022), newts (Brodie et al., 2002)). In arthropods chemical defences are prevalent, but unevenly distributed across taxa (Zvereva and Kozlov, 2016). For example, the metathoracic glands of the Heteroptera, that contain secretions rich on chemical defences, are considered characteristic of the taxon (Aldrich et al., 1997; Krajicek et al., 2016; Raška et al., 2020). On the other hand, chemical defence seems to be absent or rare in the Ephemeroptera or Diptera (Dettner, 2014). Within the Coleoptera, some families are chemically defended, (e.g. Carabidae (Sugiura, 2021) or Tenebrionidae), whereas in some other families, SMs have not been reported so far. Therefore, phylogeny alone does not determine insect chemical defence (Pasteels et al., 1983; Dettner, 2014). Ecological conditions are thought to be a substantial factor, with SMs positively correlated with the probability of discovery by predators (Maan and Cummings, 2012; Arenas et al., 2015; Blount et al., 2023), and negatively correlated with the existence of alternative defence mechanisms (Feeny, 1976; Pasteels et al., 1983) .

One trait some chemically defended prey have is the ability to release sequestered PSMs. This is done by secretions from glands (e.g. leaf beetles), cuticular cavities (e.g. burnet moths), or from special body spaces (dorsolateral space-DLS) via segmental orifices (e.g. Lygaeinae). Ejecting sequestered PSM upon predator threat could protect the individual insect from being eaten, and toxin-ejecting structures also allow for compartmentalization which could be an important mode of resistance to reduce internal exposure to PSM. In contrast, mere incorporation of PSM in the body (i.e., monarch caterpillars) may not save the individual from being killed (Petschenka and Agrawal, 2016). Compartmentalization can imply variation in the storage of PSM across tissues, creating intra-individual diversity

in the PSM profile inside one individual (Scudder et al., 1986; Winters et al., 2019; Agrawal et al., 2021).

Defensive secretions can be roughly classified in three categories according to their mode of action. First, sticky, slimy, or entangling secretions, acting mechanically. These defences are mainly efficient against arthropod predators, and sometimes against parasitoids. Second, nonspecific irritants acting as repellents, those appear to be aimed mainly at arthropods, and possibly at mammalian predators, and seem to be less of a deterrent to birds. The third category is toxic secretions (this includes PSM-sequestering prey) acting at specific sites or interfering with specific physiological processes. Toxic secretions seem to be more efficient against birds and probably other vertebrate predators. The three categories are not necessarily mutually exclusive (Pasteels et al., 1983).

Colourful warning signals are often correlated with chemical defence which is termed aposematism (Eisner *et al.* 2005; Speed *et al.* 2012). Aposematic animals acquire their chemical defences by sequestering PSMs (Beran and Petschenka, 2022), and/or by de novo synthesis (Burdfield-Steel et al., 2018; Pinheiro de Castro et al., 2019). Chemical defence in prey populations can differ in the abundance, compound type and richness. High individual variation between specimens can also occur in the same population (Winters et al., 2019).

The concentration of sequestered-PSM in insects can vary between sexes. In some lepidopteran species, males sequester more than females, and later, the sequestered metabolites can serve as nuptial gifts (Pokharel et al., 2020). In contrast, levels of sequestered-PSM are higher in females of some Lygaeid bugs and monarchs than in males feeding on Apocynaceae (Brower et al., 1972, 2009; Isman et al., 1977; Lynch and Martin, 1987). In many cases, sequestered PSM can be used by females to protect their offspring with these compounds (Pokharel et al., 2020).

1.1.3.1 Sequestration

Sequestration is defined as ‘the selective uptake, transport, modification, storage and deployment of PSM for the insect’s own defence’ (Petschenka and Agrawal, 2016). In total, more than 250 herbivorous insect species of at least six orders sequester PSM from

plant species in at least 40 plant families (Opitz and Müller, 2009). Insects can accumulate the ingested PSM compounds in their haemolymph, in specialised defence glands, or in their integument (Frick and Wink, 1995; Opitz and Müller, 2009; Kowalski et al., 2020). The accumulation is achieved by different mechanisms that can be selective in different points of the PSM metabolism, including absorption across the gut, transport within the body, endogenous transformations, and excretion via the Malpighian tubules (Lindstedt et al., 2010; Chahine and O'Donnell, 2011; Pokharel, 2023).

The first step in the sequestration processes is the absorption of PSMs from the gut lumen, across the peritrophic matrix and gut epithelium, and into the hemocoel. This can be achieved either by passive diffusion or carrier-mediated transport (Beran and Petschenka, 2022). There are several insect gut transporters that have been characterised (Zagrobelny et al., 2014; Holtof et al., 2019). After absorption, the next step is for the PSMs to be either stored in the hemolymph or moved into specific tissues. In the horseradish flea beetle, *Phyllotreta armoraciae*, multiple glucosinolate-specific transporters control the sequestered glucosinolate levels and the composition of glucosinolates in the body, by reabsorption in the Malpighian tubules (Yang et al., 2021). The sequestered PSMs can be stored in the integument, e.g., in cuticular cavities (Pentzold et al., 2016), in subcuticular compartments (Scudder and Meredith, 1982a; Bramer et al., 2017), or in exocrine defence glands (Giglio et al., 2011).

Many insects appear to regulate the concentration and/or the composition of defence SMs (Beran et al., 2014; Yang et al., 2020, 2021). For example, the seed bug *Oncopeltus fasciatus* often sequesters intermediate and more polar PSM, even if reared on different host plants that have distinct chemical profiles (Moore and Scudder, 1985). The bug's chemical profiles often differ from the profile of the host plants (Duffey and Scudder, 1974; Isman et al., 1977; Scudder et al., 1986), which can be explained by the metabolism of some PSM into distinct products (Agrawal et al., 2022). One other mechanism for the compound selectivity are transporters of the ATP-binding cassette (ABC) subfamily C (Beran and Petschenka, 2022). In poplar leaf beetles, *Chrysomela populi*, this transporter is highly expressed in the defence glands and is required for salicin sequestration (Strauss et al., 2013). ABC transporters in the subfamily B have also been implicated in the

accumulation of PSMs in the defence glands of dogbane beetles (*Chrysochus auratus*; Kowalski et al., 2020).

1.1.3.2 Insect resistance to sequestered PSM

PSM sequestration is also often associated with specific tolerance strategies that allow the herbivores to maintain the functionality of the PSM and tolerate their presence in the body (Dobler et al., 2012; Ujvari et al., 2015; Mohammadi et al., 2018). For example, insects from at least seven orders (Heteroptera, Sternorrhyncha, Caelifera, Hymenoptera, Coleoptera, Diptera, Lepidoptera) have evolved resistant Na^+/K^+–ATPases, the biological target of the PSMs called cardiac glycosides (CG). They acquire the resistance through key amino acid substitutions that decrease binding of the CG to the enzyme (i.e., target site insensitivity) Emery et al., (1998). Some examples of this phenomena include the Lygaeinae, the milkweed butterflies, pyrgomorphid grasshoppers and chrysomelid leaf beetles. Remarkably, the Na^+/K^+–ATPase mutations often involve the same amino acids at homologous positions of the protein, providing a striking example of molecular convergence (Beran and Petschenka, 2022).

Though sequestration needs specific tolerance strategies, not all tolerance strategies that are compatible with sequestration are necessarily associated with this phenomena (Erb and Robert, 2016; Beran and Petschenka, 2022). For example, many, but not all, species of milkweed butterflies are associated with CG-containing host plants. When analysed 16 species, representing all phylogenetic lineages for the occurrence of target site insensitivity, not all butterflies consuming CGs showed the genetic evidence for toxin tolerance, rather the target site insensitivity was strongly associated with sequestration of the CG (Petschenka et al., 2013).

Glucosinolates, iridoid glycosides, cyanogenic glycosides, benzoxazinoids, and salicinoids in contrast to CG, are two-component defences that occur in plants as glycosylated inactive storage forms which require activation by β-glucosidases coming from the plant, the insect, or both (Pentzold et al., 2014b). These two component defences are circumvented by phloem-feeding insects that ingest intact glucosinolates and sequester them (Aliabadi et al., 2002; Pentzold et al., 2014b; Sporer et al., 2021). In many Lepidoptera and other Mecopterida, having an alkaline midgut, is another method of

coping with two-component plant defences (Pentzold et al., 2014a). For insects that tolerate hydrocyanic acid (HCN) when sequestering cyanogenic glycosides, the enzymes rhodanese and β-cyano-L-alanine synthase have been implicated (Herfurth et al., 2017; Steiner et al., 2018).

Resistance to pyrrolizidine alkaloids (PAs) is achieved by several mechanisms (Hartmann et al., 1999). The best studied are the flavin-dependent monooxygenases (FMO) Naumann et al., (2002). PAs occur in plants in nontoxic N-oxide form, which are reduced to a pro-toxic free base in the insect gut and become toxic by metabolic activation via cytochrome P450 monooxygenases forming pyrrolic metabolites (Sehlmeyer et al., 2010). Within Lepidoptera, and a pyrgomorphid grasshopper (*Zonocerus variegatus*) a gene family of FMOs have undergone duplications (Sehlmeyer et al., 2010; Wang et al., 2012)

1.1.4 Trophic interactions: insects and natural enemies

PSMs-sequestrating herbivores turn plant defences against their own enemies (Zhang et al., 2019). However, plants can modify the interactions between herbivorous insects and their natural enemies in various ways. For example, volatile chemicals produced by the plant in response to herbivory can attract natural enemies, thereby strengthening the interaction (Figure 1, see War et al., 2011; Zhou and Jander, 2022). These effects can affect the population dynamics, the co-existence of species and even the trophic network structure of ecological communities (Van Veen, 2015).

Specialist natural enemies are more likely to evolve tolerances or defences against the sequestered PSM. From the plants' perspective, sequestration of PSM by specialist herbivores represents a backfiring of the defence, potentially increasing the insects' fitness if the sequestered toxins prevent their predation. Consequently, sequestering insects can exert selection on plants to reduce the defences available for sequestration, as predicted by the defence de-escalation hypothesis (Beran and Petschenka, 2022).

The *Passiflora*- heliconiine system is an example of PSM diversity associated with selective pressure by a specialist herbivore and interactions with predators. The colourful heliconiine butterflies are distasteful to predators due to their content of defence compounds called cyanogenic glucosides (CNglcs), which they acquire mostly as larvae through their *Passiflora* diet where ~30 kinds of CNglcs have been reported.

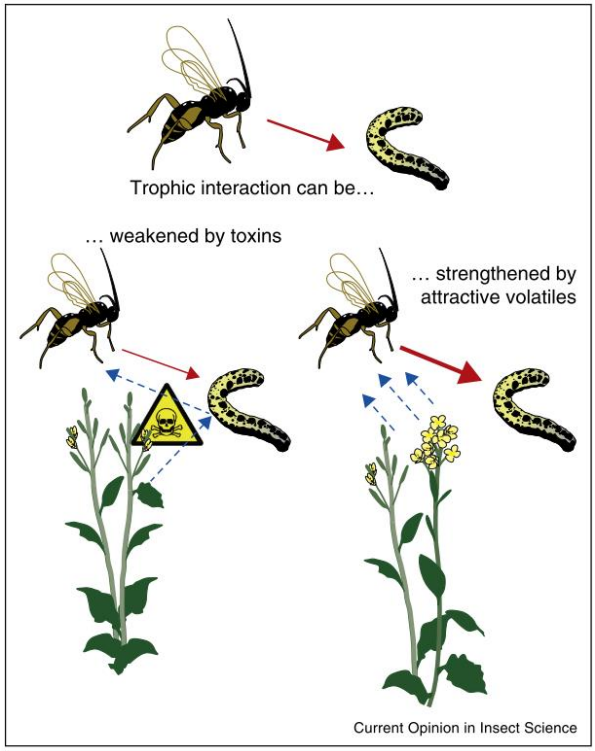

Figure 1. Two ways in which plants can modify the strength of trophic interactions between herbivores and their enemies: the interaction may be weakened when the plant's chemical anti-herbivore defences harm the natural enemy when they feed on the herbivore; the interaction may be strengthened when volatile chemicals emitted by the plant attract the natural enemy, allowing it to discover prey at a higher rate (Figure from Van Veen, 2015).

In a study that included 19 butterfly species and CNglc profile of 42 *Passiflora* species, *Passiflora* plants diversified their cyanogenic profile to escape heliconiine herbivory. And species that have become acyanogenic did so, presumably, as a way to avoid the sequestering specialists (Pinheiro de Castro et al., 2019).

1.1.4.1 Predators of chemically defended herbivores

Predators are an important evolutionary force selecting for host plant (and defence chemical) specialisation in herbivores. Specialists are often better protected from predators than generalists (Dyer, 1995). Predators are therefore predicted to explain why specialist herbivores sequester higher concentrations of PSMs than generalists. For example, four aphid species that feed on common milkweed (*Asclepias syriaca*) differ in the concentration of cardenolides from the lowest in the generalist *Myzus persicae*, to the highest in the monophagous *Myzocallis asclepiadis* (Züst and Agrawal, 2016). Likewise,

specialised *Heliconius* butterflies sequester sevenfold higher amounts of monoglycoside cyclopentenyl cyanogens compared to generalists (Engler-Chaouat and Gilbert, 2007). Sequestration, however, does not protect herbivorous insects equally well against all kinds of natural enemies, and no universal anti-predator defence have been found (Sugiura, 2020).

In the study of chemically defended herbivores, the invertebrate model predators utilized include Hymenoptera, Coleoptera, Hemiptera and Diptera, and spiders (Skow and Jakob, 2006; Petschenka et al., 2011; Burdfield-Steel et al., 2020; Raška et al., 2020; Sugiura, 2020). Praying mantises (Mantodea) are also significant predators of herbivores (Moran et al., 1996). These sit-and-wait predators have been used to test the effectiveness of chemical and morphological defences in beetles, bugs, bees and butterflies (Paradise and Stamp, 1991; Rafter et al., 2017b; Sugiura, 2021). Vertebrate model predators such as fish, amphibians, reptiles, birds, and mammals are also used to examine the effectiveness of insect antipredator defences (Sugiura, 2020). Insectivorous birds hunt visually, and thus they are commonly accepted to have strong pressure on the evolution of visual and chemical defences such as aposematism, crypsis, masquerade and mimicry (Sugiura, 2020).

The existence of predator-specific defences in prey suggests that predators are not functionally identical entities that can be lumped together into a single mortality factor for prey (Hoverman and Relyea, 2007). Different degrees of resistance to PSMs sequestered by prey have been observed in predators and parasitoids (Zhang et al., 2019; Mohammadi et al., 2022). A 2019 study analysed nematodes as the natural enemy of the western corn rootworm, a chemically defended prey that sequesters maize´s PSM. Researchers found resistance by the nematodes to the sequestered maize´s PSM. The nematodes PSM resistance allowed the nematodes to infect and kill the rootworm more efficiently (Zhang et al., 2019). The impact of multiple predators on chemically defended prey and the loose target specificity of the defences could explain apparent redundancies in anti-predator strategies of some insects. For example, *Oncopeltus fasciatus* and possibly other lygaeid bugs secrete both volatile defensive aldehydes and release sequestered CG (Pasteels et al., 1983)

1.2 Focal PSM: Cardiotonic steroids—cardenolides

Cardiac glycosides (CG) or cardiotonic steroids (CTS) are a PSM that exhibit considerable structural diversity (Seiber et al., 1983; El-Seedi et al., 2019). However, all compounds share a common structural scaffold that differentiates them from other steroidal metabolites. They consist of two parts: an aglycone fragment (steroidal genin) and a sugar fragment. The genin contains an unsaturated lactone moiety and at the C-17 position which divides cardiac glycosides into two sub-groups, cardenolides (five-membered lactone), and bufadienolides (doubly unsaturated six-membered lactone; El-Mallakh et al., 2019). A wide range of cardenolides has been isolated from many different plant species (El-Seedi et al., 2019). Most of the plants that provide the best-known sources of medicinally important cardenolides are in the genera *Digitalis*, *Acokanthera*, and *Strophanthus*. In contrast, cardenolide-rich species of the Asclepiadaceae family hold the main interest in cardenolide-mediated interactions between plants and herbivores (Malcolm, 1991).

Cardenolides have a steroidal moiety with a unique ring system. Most cardenolides found in nature have *cis–trans–cis* configuration of their A/B, B/C, and C/D rings, however, cardenolides from *Asclepias* genus (aka. milkweed) have *trans–trans–cis* ring configuration (See Figure 2) Ren et al., (2022). Most cardenolides have between one and four sugars attached to the 3*β*-OH group of the genin. Sugars range from typical hexoses and pentoses, including D-glucose, L-rhamnose, L-arabinose and D-xylose, to deoxy sugars, which are unique to CG (Petschenka et al., 2018; El-Seedi et al., 2019).

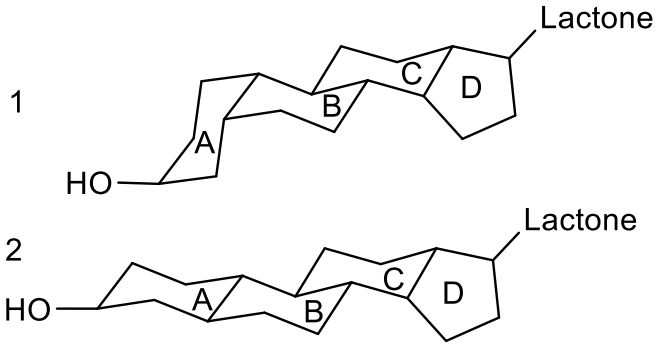

Figure 2 Spatial configuration of CG. Ring A/B and ring C/D of compound 1 are cis-junction, while ring B/C is trans-junction; Ring A/B of compound 2 is transfused which is less common, mostly occurring in cardenolides from the *Asclepias* genus (Ren et al., 2022).

Plants of the Asclepiadoideae subfamily produce some cardenolides that are resistant to acid hydrolysis (Seiber et al., 1983). This property is explained by the double attachment of the carbohydrate group through acetal and hemiacetal bonds at positions 3β and 2α, respectively, to the cardenolide aglycone (Figure 3). This dioxane ring substructure is present in calactin, calotropin, proceroside, syriobioside, desglucosyrioside and eriocarpine. The same carbohydrate, but in an altered form, is also found in uscharidin, calotoxin, uscharin, voruscharin, syrioside, asclepin and other cardenolides. Such specific PSM (markers) are actively produced by plants from the genera *Asclepias, Calotropis, Gomphocarpus* and *Pergularia* (Tomilova et al., 2022). In general, structures of representative cardenolide genins in Asclepiadaceae are shown in Figure 4 (Seiber et al., 1983).

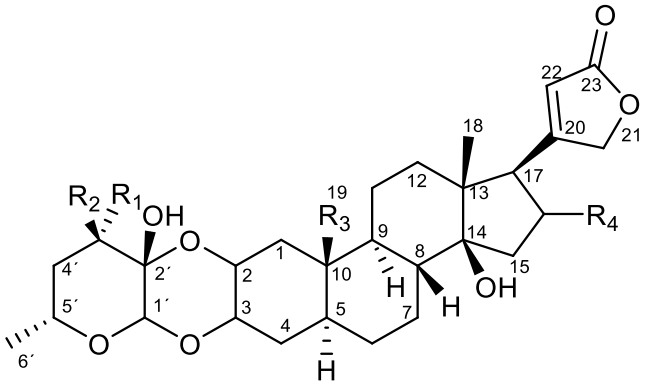

Figure 3. Chemical structure of double-linked cardenolides

1.2.1 Biological activity

CGs have a long history of medicinal applications, even though some are highly toxic to humans and animals (El-Seedi et al., 2019). Extracts of plants containing CGs, can be prepared from various parts, including roots, stems, leaves, barks, seeds, fruits and flowers. During heart failure, the heart becomes more sensitive to the effects of CGs. Plant extracts rich in CGs were first used in antiquity by the Egyptians as cardio-protective drugs by ca. 1500 BCE (El-Seedi et al., 2019). The activity of CG against cancer has been known for more than 50 years, but it was not initially developed due to concerns associated with their high toxicity.

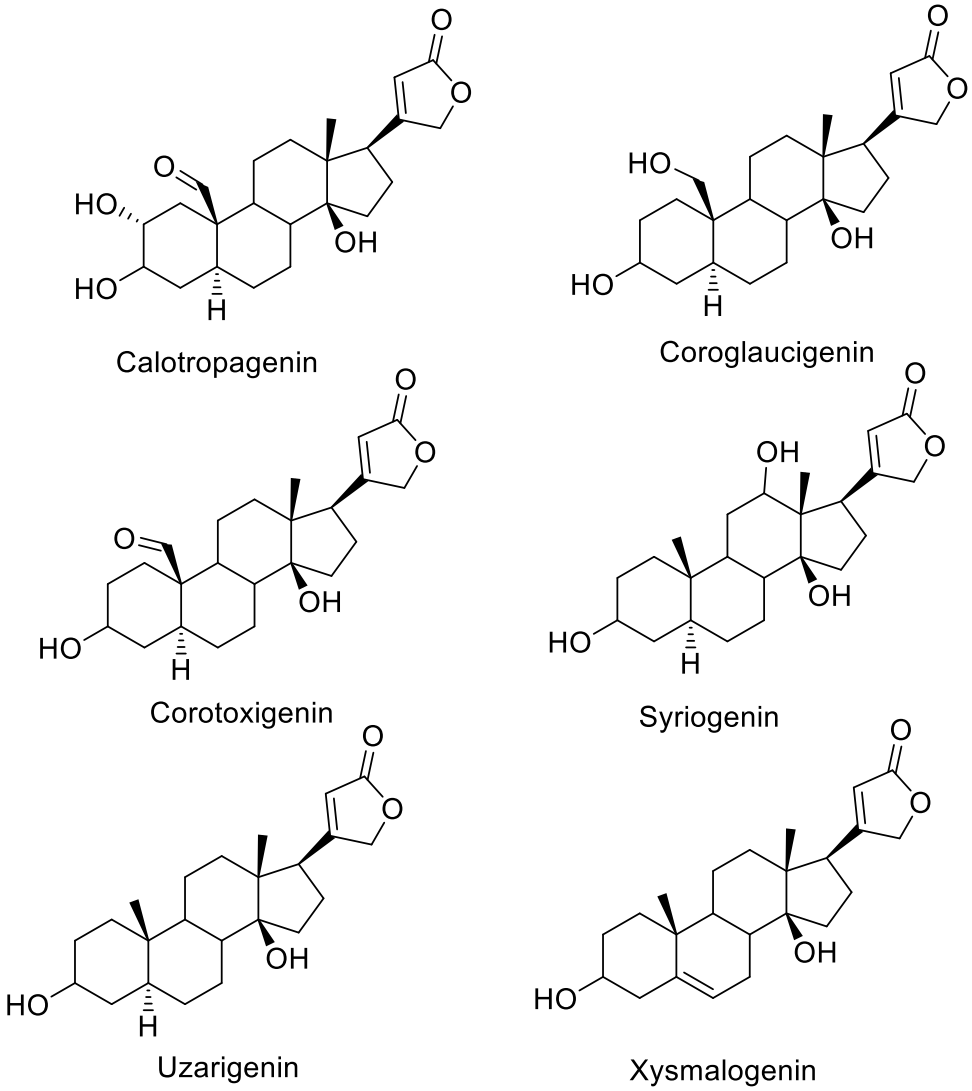

Figure 4. Representative cardenolide genins in Asclepiadaceae

Currently, the use of CGs in oncology is actively developing (Tomilova et al., 2022) and they are expected to be novel therapeutic drugs in the treatment of diseases in the respiratory system, in the nervous system and immune inflammation-related illnesses (Ren et al., 2022).

The mode of action of CG resides on its specific inhibitory properties of the target site, the Na^+/K^+–ATPase pump. CGs bind to the extracellular surface of the enzyme (Köksoy, 2002). This pump main function is to maintain the electric potential in animal cells and other essential physiological functions (Mithöfer and Boland, 2012). During each cycle of the Na^+/K^+–ATPase pump, one molecule of ATP is hydrolysed and the energy is used to

translocate three Na^+ ions to the extracellular side in exchange for two K^+-ions that are shuffled into the cytosol (Dobler et al., 2011). Binding with CG causes a change in the Na^+/K^+–ATPases conformational state, and results in an inactive complex until dissociation occurs, at which point the pump resumes its activity. The CG or the genin alone can react with the pump although the interaction with the genin is often less stable and therefore less effective (Emery et al., 1998; Mithöfer and Boland, 2012). In insects, although the enzyme is abundant in the nerve tissue, their expression in other tissues varies phylogenetically and physiologically (Petschenka et al., 2012; Bramer et al., 2015; Lohr et al., 2017). For tissues with multiple isoforms that differ in their CG sensitivity there is a characteristic biphasic shoulder in the log-linear part of the dose–response curve for selected CGs (Emery et al., 1998).

1.2.2 Ecological relevance of cardenolides

CGs are strong target site inhibitors and potentially bitter-tasting defences (Brower, 1969; Agrawal et al., 2012). Cardenolides have a deterrent effect in non-adapted herbivorous insects, suggesting that the compounds induce negative chemo-perception (Huang and Renwick, 1994). In mammals, cardenolides trigger a chemoreceptor responsible for emesis at concentrations below toxic doses (Dobler et al., 2011). That explains why in birds, the first reaction after oral ingestion of cardenolides is to vomit (Brower and Fink, 1985). Cardenolides are more or less bitter to humans (Malcolm, 1991). They can also cause headaches, altered vision, psychosis and hormonal effects (Janssen et al., 2016). The exact mechanisms leading to these symptoms are hard to trace, but the immediate toxic effect of ingested cardenolides is simple and well understood (Dobler et al., 2011). Although the sensitivity of the Na^+/K^+–ATPase to cardenolides is such a prevalent characteristic in the majority of animals, some insects have colonized cardenolide-containing plants and use them as food apparently without suffering ill effects (Malcolm, 1991; Dobler et al., 2011).

1.3 Milkweed-herbivore system

Asclepius was the physician son of Apollo in Greek mythology who infuriated Zeus. Asclepius had the temerity to resurrect a dead man condemned by Zeus. His mythical skills in surgery and the use of drugs are revered as the inspiration for modern medicine.

The milkweed family Asclepiadaceae and its dominant genus, *Asclepias* were named after the Asclepius, the god of medicine in ancient Greek and Roman mythology (Malcolm, 1991). Cardenolides from milkweed species have been the focus of several studies in chemical ecology. Milkweed plants have provided clear examples of plasticity in PSM, particularly with respect to changes in chemistry following herbivory (Dyer et al., 2018). Milkweeds have been instrumental to understand specialised herbivore adaptations such as physiological target site insensitivity and sequestration (Bramer et al., 2015). The co-evolution between milkweed species and their community of herbivores is often described as diffuse (Birnbaum and Abbot, 2018).

1.3.1 Milkweed

The milkweed family (Asclepiadaceae) comprises some 200 genera and 2500 species of perennial shrubs, herbs, and vines distributed throughout the tropics and extending to temperate areas of the world. They include some highly prized ornamentals and economically significant weeds. They are generally characterized by the milky latex they exude when a leaf or other organ is ruptured. The milkweed family in North America, including Mexico, is represented by several genera. Only the genus *Asclepias* contains above a hundred of described species (Roeske et al., 1976).

While co-evolutionary theory predicts escalation of plant defences, research in the last decade demonstrated a phylogenetic decline of cardenolide concentrations and diversity in milkweeds. However, a decline in concentration could be compensated by increasing potency of individual PSM. This strategy may be more efficient and less costly to cope with increasingly resistant herbivores (see section 1.1.3.2 and Züst et al., 2019).

Previous studies analysed 49 *Asclepias* species for latitudinal clines in the production of cardenolides. They found that milkweeds from tropical regions are better defended by cardenolides than more temperate species. The inducibility of cardenolides (i.e. the amount increased in response to insect herbivory) was significantly correlated with latitude, with higher inducibility evolving in the tropics. Milkweed species from lower latitudes appear better defended with higher inducibility, greater phytochemical diversity, and lower polarity of cardenolides (Agrawal et al., 2012).

1.3.2 Milkweed herbivores

Milkweed herbivores, including the famous monarch butterfly (*Danaus* spp.), are model organisms in plant–insect coevolution for more than 60 years (Birnbaum and Abbot, 2018). In 1932, Jones found that various brightly coloured insect herbivores of milkweed were rejected by a variety of bird predators (Jones, 1932). Later, Brower and Brower showed that scrub jays and blue-jays refused to eat monarch butterflies (Brower and Brower, 1964). Then in 1965, Parsons discovered that the unacceptability of monarch butterflies by bird predators was attributable to cardenolides. However, it was 1966 when Rothschild and co-workers first reported that the monarch butterfly and a large aposematic grasshopper, *Poekilocerus buionius*, obtained cardenolides from their host plants (Malcolm, 1991). Cardenolide sequestration by milkweed herbivores was corroborated after that by laboratories across Europe and in the United States (Malcolm, 1991). They reported variation in cardenolide content within various tissues of a given milkweed species. Thus, a single plant species can provide a spectrum of PSMs in its tissue upon which insects can feed (Duffey and Scudder, 1972). Most of the available information on cardenolide sequestration by herbivores concerns the monarch butterfly, the oleander aphid, and the large milkweed bug (Malcolm, 1991).

A recent study described the defence-offence interactions between the tropical milkweed (*Asclepias curassavica*) and multiple herbivores (López-Goldar et al., 2022). They found that the roots, leaves, flower buds and seeds of the *A. curassavica* show increasing concentrations of cardenolide toxins acropetally, with latex showing the highest concentration. In vitro assays of the Na^+/K^+–ATPase pump, of three specialized milkweed herbivores (root- feeding cerambycid beetle, leaf- feeding monarch butterfly, and as seed-feeding the large milkweed bug) show that they are proportionally tolerant to the cardenolide concentrations in the tissues of their diet. Molecular substitutions in the insects' sodium pumps explain their tolerance to cardenolides from their target tissues. However, when comparing the response of the insects´ enzymes to cardenolide content of target versus non-target plant tissues, there are varying degrees of adaptation to tissue-specific cardenolides in these specialists (López-Goldar et al., 2022).

1.4 The large milkweed bug - *Oncopeltus fasciatus*

Oncopeltus fasciatus is a member of the Lygaeinae family, who are typically small-to-medium-sized insects, ranging in size from 1 to 12 mm. The majority of Lygaeinae species are cryptically coloured, however the subfamily Lygaeidae, which *Oncopeltus fasciatus* belongs, is widespread aposematic (Burdfield-Steel and Shuker, 2014).

In the United States, *O. fasciatus'* habitat ranges from Massachusetts, westward to the Rocky Mountains, and south and southwest to Florida, Texas and California (Malcolm et al., 1989). It is also present in Mexico, and Brazil (Feir, 1974). The usual host plants are different members of the milkweed family, mainly *Asclepias* (Feir, 1974). Reproduction takes place on only those host plants that are producing seed pods (Miller and Dingle, 1982). The bugs have been successfully reared in the laboratory, and strains have been adapted to sunflowers, cashews, and almonds (Feir, 1974). Information on the predators of the large milkweed bug is scarce, though lacewing larvae have been reported (Sauer and Feir, 1972). Some other reports of predation include the ambush bug, nabids, reduviids, spiders, and a katydid (Feir, 1974).

1.4.1 Anti-predator defence of *Oncopeltus fasciatus*

The large milkweed bug utilises endogenous volatiles and sequestered cardenolides for its defence (Duffey and Scudder, 1972; Everton and Staddon, 1979; Lohr et al., 2017). In nymphalid stages, the scent glands are situated in abdominal segments four and five. When a nymph is seized it may respond by ejecting the liquid contents of both glands simultaneously. Along with the ejection from the scent glands, the nymph ejects a copious liquid from the rectum. The rectal fluid usually flows onto the back where it mixes with the secretion (Games and Staddon, 1973; Everton and Staddon, 1979; Aldrich et al., 1997). The dorsal scent glands of *O. fasciatus* nymphs cease to function in the adults. From instar IV there is development of the adult metathoracic scent gland (Staddon, 1995).

The integument of the large milkweed bug is made up of a vacuolated and a pigmented epidermal cell layer, the dorsolateral space (DLS). The DLS is present from late embryo to adult in both sexes and independent of their diet. When reared on milkweed, *O. fasciatus* concentrates cardenolides in it throughout its life cycle. In the adult, droplets of cardenolide-rich fluid appear at precise points along the DLS margins when external

pressure is applied to the thorax and abdomen. The DLS fluid spreads onto the animal's surface and remains as discrete droplets due to the cuticular morphology. There are no specialized muscles involved with fluid release (Scudder and Meredith, 1982a). The DLS margins are repaired after the fluid discharge by an elastic snap-back mechanism which possibly, in combination with coagulating haemolymph, seals the release site again. The adaptations for the storage and release of cardiac glycosides have evolved in a stepwise manner. The DLS originated simultaneously along with the ability to sequester cardenolides (Bramer et al., 2017).

1.4.2 Cardenolides in *Oncopeltus fasciatus*

Experiments carried out in 1986, found that injecting 200 nmol of the cardenolide ouabain into *O. fasciatus* resulted in no mortality. This dose is higher than the cardenolide lethal doses recorded for any vertebrates and invertebrates (Moore and Scudder, 1986). Since then, many studies have come forward unveiling the reasons behind the high resistance of *O. fasciatus*. The alpha subunit of the sodium pump, Na^+/K^+–ATPase (ATPα1) modulates cardenolide sensitivity. *O. fasciatus* along with many insects that feed from Apocynaceae evolved adaptations by repeated duplication of ATPα1, and share parallel changes in gene expression, and parallel amino acid substitutions (Zhen et al., 2012; Bramer et al., 2015). *O. fasciatus* has three known copies of the Na^+/K^+–ATPase α: α1A, α1B and α1C. Of the three, an ancestral copy of the pump remains comparatively sensitive, but acts as a more efficient ion carrier. The α1A and α1B are required for cardenolide handling (Lohr et al., 2017).

In early studies with *O. fasciatus* reared on *Asclepias syriaca* seeds, milkweed bug adults concentrate 60 to 95% of the cardenolide content in the DLS fluid. Very little cardenolide was detectable in the haemolymph of adults and nymphs, but cardenolides do appear in the adult metathoracic gland secretion and in the nymph abdominal gland fluid. The cardenolide content acquired throughout the development is not related simply to increase in body weight (Duffey and Scudder, 1974).

Milkweed bugs sequester polar but not non-polar cardenolides into the DLS fluid (Duffey and Scudder, 1974). However, a decade later, researchers found that cardenolides are not sequestered in the insect simply based on polarity and that metabolism and differential

excretion of cardenolides are key in the sequestration process (Moore and Scudder, 1985). The similarities in the cardenolide profiles of *O. fasciatus* reared on different milkweeds in laboratory conditions, and analysis of different tissues of *O. fasciatus* reared on a single food source indicated a regulation of the cardenolide composition of the bug (Moore and Scudder, 1985). The accumulation of the cardenolides in both the nymph and adult appears to be, at least in part, associated with the ability of the insect to metabolize the cardenolides (Duffey and Scudder, 1974). Field studies found that a source of high variability in the chemical defence of *O. fasciatus,* include temporal and geographical intraspecific differences in the cardenolide content of milkweed seed. However, it showed that the cardenolide content of the host species cannot alone account for the variability in the cardenolide content of the bugs, even between populations feeding on the same plant organ (Isman et al., 1977).

Cardenolides have been reported to be present in the eggs of *O. fasciatus* (Duffey and Scudder, 1974). Regardless of male diet, eggs were afforded better protection when females had been raised on milkweed. Maternal contribution of cardenolides is significantly greater than paternal contribution of cardenolides to the eggs (Newcombe et al., 2013).

A recent study focused at molecular level on the milkweed-herbivore coevolution. *Oncopeltus fasciatus* reared on common milkweed seeds (*Asclepias syriaca*) metabolised two major seed cardenolides into distinct compounds that were sequestered. Through *in vitro* analysis of Na^+/K^+–ATPase enzymes, they found little variation among cardenolides in their inhibition of an unadapted Na^+/K^+–ATPase. They found high variation in inhibition on the adapted monarch butterflies and *O. fasciatus*. Labriformin was the most inhibitive cardenolide tested for both milkweed herbivores. Three metabolized (and stored) cardenolides by *O. fasciatus* were less toxic than their parent compounds found in seeds. These results suggest that a potent cardenolide in milkweed (i.e. labriformin and similar) has evolved by natural selection and targets specialist herbivores, but is potentially counteracted by insect tolerance, detoxification, and sequestration (Agrawal et al., 2022).

1.5 Predators and cardenolide-sequestering prey

There are few analyses on the effectiveness of cardenolides as insect chemical defence against predators. Generalist predators of cardenolide-containing insects (including spiders and mice) are deterred by the compounds despite high degrees of structural and concentration variability. A wide range of animals show pre-ingestive (gustatory) sensitivity to cardenolides (Agrawal et al., 2012). Petschenka, et al, found that milkweed bugs fed on Apocynaceae did not improve growth or speed of development, but sequestration did mediate protection against two natural predators: lacewing larvae and passerine birds (Petschenka et al., 2022). In another study with the same model system, the milkweed bug's survival to lacewing attacks strongly depends on the source of the sequestered cardenolides, even when the two analysed diets (*Adonis vernalis* and *Digitalis purpurea*) were rich in cardenolides. Sequestration analysis showed that it is very likely that specific structural features of one or more of the *Digitalis* cardenolides, rather than quantitative differences between plant species, were the reason behind high survival rates of milkweed bug fed on *Digitalis*. The prey rejection (survival) was based on taste, as researchers observed aversive behaviour by the predator (Pokharel et al., 2020).

Records show how some birds have succeeded in breaking through the cardenolide defence of the monarch butterfly. Flocks of birds including the black-headed grosbeak (*Pheucticus melanocephulus*) and the black-headed oriole (*Oriolus larvatus*), feed from thousands of butterflies in the large overwintering aggregations in Mexico (Mohammadi et al., 2022). Emetic studies by Fink and Brower in 1981, concluded that grosbeak birds can be resistant to cardenolide poisoning up to a certain extent (Fink and Brower, 1981). These birds have amino acid substitutions in their Na^+/K^+–ATPases pumps, which may confer target-site insensitivity, the same ones that convergently evolved in the herbivores of milkweed (Groen and Whiteman, 2021). Co-evolution between cardenolide-sequestering prey and their predators is receiving more attention in the last years. The recurring emergence of predators that can feed on and exploit cardenolide-defended prey involves remarkable convergence in the behaviours, physiology and molecular mechanisms by which they achieve this adaptation (Mohammadi et al., 2022).

1.6 Research objectives and thesis outline

Chemical defence is one of the most taxonomically and ecologically widespread defences in animals, plants and micro-organisms (Rojas et al., 2017). One of the most universal findings in chemical ecology is that chemical profiles are often variable within populations, both in terms of the total quantity and the structures of the chemical constituents (Speed et al., 2012). A variety of biological processes have been hypothesised to explain this 'chemodiversity' including, genetic processes, abiotic environmental conditions, metabolic pathways, phenotypic plasticity, and interactions with animals (Speed et al., 2012); section 1.1.2).

Among plants in the genus *Asclepias* there is a high diversity of cardenolides along a polarity gradient. There are multiple specialised herbivores of *Asclepias*, each having genetically based tolerance to toxic cardenolides via substitutions in the herbivore's Na^+/K^+—ATPase. Specialist lygaeid bugs such as *Oncopeltus fasciatus* feed on seeds of milkweed plants, and sequester the cardenolides as a defence against predators. These insects are hypothesised to impose natural selection for chemical defences in the plant tissues on which they eat. This led me to propose three aims:

1. To conduct a phytochemical profiling of the cardenolides in the seeds of *Asclepias curassavica.*
2. To test the phenotypic match between cardenolide composition in the seeds and those sequestered by *O. fasciatus*, with an assessment of the inhibitory capacity of cardenolides to the bugs' target site.
3. To test the effectiveness of the cardenolides sequestered by *O. fasciatus* on the target sites of different predators.

To achieve these three aims, I conducted a phytochemical profiling of the cardenolides in the seeds of *Asclepias curassavica*, by means of isolation of cardenolides and examination by means of HRMS and NMR spectroscopy. The concentration of all cardenolide in the seeds were quantified by using each compound as a standard for their corresponding calibration curve. I tested the in vitro inhibitory capacity of each cardenolide on the porcine protein Na^+/K^+—ATPase. To test whether the cardenolide defences could represent the plant's response to natural selection by *O. fasciatus* I measured the

phenotypic match of the cardenolide composition of seeds of *Asclepias curassavica* to those sequestered by nymphs and adults of *O. fasciatus.* I combined this assessment with tests of the inhibitory capacity of a subset of the seed cardenolides against the Na^+/K^+—ATPase of *O. fasciatus* and a non-adapted insect (*Drosophila melanogaster*). To test whether the sequestered cardenolides by *O. fasciatus* effective against predators, I tested the inhibitory properties cardenolides of the insect's defence against the target sites of a potential invertebrate predator, the giant Asian mantid (*Hierodula membranacea*), and representatives of resistant and sensitive bird predators: the black-headed grosbeak (*Pheucticus melanocephalus*) and Australian zebra finche (*Taeniopygia castanotis*).

Chapter 2.1 describes the composition and structural diversity of the cardenolides in the seeds of *Asclepias curassavica*. The structures of three new compounds, as well as seven previously reported cardenolides are reported. The range of inhibitory potency of eight milkweed cardenolides in the biological target is tested in vitro on the sensitive porcine Na^+/K^+–ATPase.

In chapter 2.2 the cardenolide composition of *O. fasciatus* was analysed across two life stages and compared to the cardenolide composition of the seeds of *A. curassavica*. The inhibitory capacity of a subset of seed cardenolides were tested against the Na^+/K^+–ATPase of *O. fasciatus* and a non-adapted insect (*Drosophila melanogaster*).

In Chapter 2.3 the composition and diversity of cardenolides in the defensive fluid that *O. fasciatus* was measured along with what remains in the body tissues after the defensive fluid is deployed. This was paired with tests of the cardenolides in the insects' defence on the Na^+/K^+–ATPase pump of potential vertebrate and invertebrate predators, one of whom has empirical and genetic evidence of cardenolide resistance.

2 Publications

2.1 New structures, spectrometric quantification, and inhibitory properties of cardenolides from *Asclepias curassavica* seeds

Rubiano-Buitrago, P.; Pradhan, S.; Paetz, C.; Rowland, H.M. *Molecules* 2023, 28, 105. https://doi.org/ 10.3390/molecules28010105

Erratum: We described in the manuscript 3-O-β-allopyranosyl coroglaucigenin (**1**) incorrectly as novel. The structure was previously published by Hamida Ghorab et al, Nat. Prod. Comm. 2017, 12, 3-5; where they reported 3-O-β-allopyranosyl coroglaucigenin as salsotetragonin. A corrigendum was submitted.

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Article

New Structures, Spectrometric Quantification, and Inhibitory Properties of Cardenolides from *Asclepias curassavica* Seeds

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Abstract: Cardiac glycosides are a large class of secondary metabolites found in plants. In the genus *Asclepias*, cardenolides in milkweed plants have an established role in plant–herbivore and predator–prey interactions, based on their ability to inhibit the membrane-bound Na^+/K^+-ATPase enzyme. Milkweed seeds are eaten by specialist lygaeid bugs, which are the most cardenolide-tolerant insects known. These insects likely impose natural selection for the repeated derivatisation of cardenolides. A first step in investigating this hypothesis is to conduct a phytochemical profiling of the cardenolides in the seeds. Here, we report the concentrations of 10 purified cardenolides from the seeds of *Asclepias curassavica*. We report the structures of new compounds: 3-*O*-*β*-allopyranosyl coroglaucigenin (**1**), 3-[4′-*O*-*β*-glucopyranosyl-*β*-allopyranosyl] coroglaucigenin (**2**), 3′-*O*-*β*-glucopyranosyl-15-*β*-hydroxycalotropin (**3**), and 3-*O*-*β*-glucopyranosyl-12-*β*-hydroxyl coroglaucigenin (**4**), as well as six previously reported cardenolides (**5**–**10**). We test the in vitro inhibition of these compounds on the sensitive porcine Na^+/K^+-ATPase. The least inhibitory compound was also the most abundant in the seeds—4′-*O*-*β*-glucopyranosyl frugoside (**5**). Gofruside (**9**) was the most inhibitory. We found no direct correlation between the number of glycosides/sugar moieties in a cardenolide and its inhibitory effect. Our results enhance the literature on cardenolide diversity and concentration among tissues eaten by insects and provide an opportunity to uncover potential evolutionary relationships between tissue-specific defense expression and insect adaptations in plant–herbivore interactions.

Keywords: Na^+/K^+ ATPase activity; Apocynaceae; toxicity; secondary metabolites; phytochemistry

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1. Introduction

Plants produce a range of low molecular weight organic compounds, some of which are not involved in the 'primary' functions of plants but which mediate plant–environment interactions—known as secondary metabolites (or natural products) [1]. Most plant secondary metabolites have evolved to defend plants against insects and other natural enemies [2,3]. That does not mean, however, that all secondary compounds have a defensive function, and criteria for determining this are still not fully developed [1,4]. Even in some of the most well-studied systems, the structures and functions of these metabolites are still undescribed [5]. Therefore, testing the biological activity of secondary metabolites in a plant and, if they are active, whether they are of evolutionary and ecological significance is important for understanding the mechanisms and function of chemodiversity [5].

Milkweed plants in the genus *Asclepias* (Apocynaceae) are optimal candidates to investigate chemodiversity and secondary metabolite activity because their defenses of cardenolides and latex [6–8] vary among species [9,10] and among plant parts [11,12] and because cardenolides have a specific physiological target, the transmembrane protein Na^+, K^+−ATPase (NKA [3–6,9,11,13,14]), which can be tested in vitro [15]. Cardenolides are toxic because they bind to the extracellular surface of the NKA and, when bound, block

this ion pump, leading to the breakdown of membrane potentials and cell homeostasis with potentially fatal effects [7,16–18]. Specialist herbivores, such as the monarch butterfly (*Danaus plexippus*), which feeds on the foliage of milkweed plants [19–23], and the large milkweed bug (*Oncopeltus fasciatus*), which feeds on the seeds [24–27], have reduced sensitivity to cardenolides through the evolution of amino acid substitutions in the NKA, preventing the cardenolides from binding [15,28–33]. The resulting arms race is thought to drive the diversity of plant secondary metabolites and insect herbivores [4].

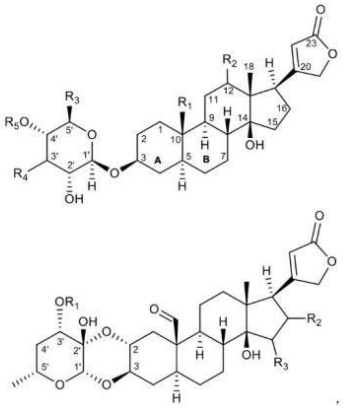
Cardenolide diversity ranges up to 30 compounds in a single plant [7,34]. Plants show longitudinal trends in these natural products, with implications for the survival of herbivores [35]. Cardenolides vary in polarity [9], resulting in different rates of absorption through the gut of animals [36], with non-polar compounds being more readily absorbed than polar ones [37]. Cardenolides also vary in the structural sugar groups (the 'glycoside' of cardiac glycosides) that conjugate to the core aglycone steroidal structures of cardenolides, and this can alter the chemical properties of the molecule [38,39]. Cardenolides can also have reactive moieties, such as aldehydes that form H-bonds between the molecule and the NKA [39,40]. Sugars in cyclic bridges, such as a dioxane ring, are also highly resistant to acid hydrolysis [39]. Not all cardenolides, then, are predicted or shown to be equally toxic to herbivores [11,39].

Recently, Agrawal et al. (2022) tested the inhibitory capacity of a subset of purified cardenolides from *Asclepias syriaca* on the Na^+/K^+-ATPase. There was little variation among compounds in inhibition of an unadapted Na^+/K^+-ATPase, but impacts on that of monarchs and *Oncopeltus* varied significantly [41]. Here, we focus instead on *Asclepias curassavica* which, although native to the Caribbean area, is cultivated widely as an ornamental plant, and it is now found in numerous semitropical areas [6]. *A. curassavica* is understudied in its interactions with specialist herbivores in comparison with other species, despite being a critical hostplant worldwide [19]. The seeds of *A. curassavica* have received less attention than its foliage [40,42–44] but are subject to selection by seed herbivores [11]. The seeds of *A. curassavica* have one detailed phytochemical profiling by Abe et al [45] (though, see [11,46]). In that study, fourteen compounds were isolated, several of which have only been found in the seeds of *A. curassavica*, in contrast to some cardenolides that are present in foliage, latex, and roots [45]. Our goal was to describe the structures, concentrations, and activity of these metabolites.

2. Results and Discussion

2.1. Isolation and Structure Elucidation

For the isolation of cardenolides, 264 g of vacuum-dried *A. curassavica* seeds were ground and extracted exhaustively using water and MeOH as solvents. The crude extracts were subjected to HPLC-HRMS analyses. (Supporting Information, Figure S1). Extracts containing chromatographic peaks with a cardenolide-like spectrum were selected for further purification. The cardenolide characteristics in the spectrometric data involved identifying values of a neutral loss that correspond to sugars in cardenolides (e.g., 162.05 Da-possible glucose and 146.05 Da-possible methyl allose). The spectrum must also contain high intensity fragments between m/z 353.20–391.25, tentatively corresponding to an unsaturated triterpene [47,48]. After the putative structural assignment based on HRMS data, the isolated compounds were examined by means of NMR spectroscopy. We identified six known cardenolides: 4′-*O*-*β*-glucopyranosyl frugoside (**5**) [49], 4′-*O*-*β*-glucopyranosyl gofruside (**6**) [50], 3′-*O*-*β*-glucopyranosyl calotropin (**7**) [45], frugoside (**8**) [50], gofruside (**9**) [51], and 16*α*-hydroxycalotropin (**10**) [45] (Figure 1). In addition, we isolated four new cardenolides (Figure 1): 3-*O*-*β*-allopyranosyl coroglaucigenin (**1**), 3-(4′-*O*-*β*-glucopyranosyl-*β*-allopyranosyl) coroglaucigenin (**2**), 3′-*O*-*β*-glucopyranosyl-15-*β*-hydroxycalotropin (**3**), and 3-*O*-*β*-glucopyranosyl-12-*β*-hydroxy-coroglaucigenin (**4**). All compounds were isolated as white solids. We quantified the concentration of all cardenolide in the seeds and tested the in vitro inhibitory capacity of each purified cardenolide on porcine NKA [15].

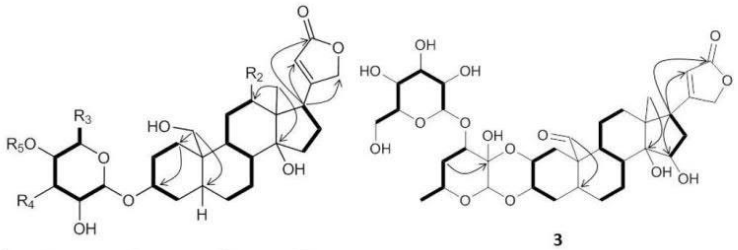


	R_1	R_2	R_3	R_4	R_5
1	-CH_2-OH	H	-CH_2-OH	α-OH	H
2	-CH_2-OH	H	-CH_2-OH	α-OH	β-glc
4	-CH_2-OH	β-OH	-CH_2-OH	β-OH	H
5	-CH_2-OH	H	-CH_3	α-OH	β-glc
6	-CHO	H	-CH_3	α-OH	β-glc
8	-CH_2-OH	H	-CH_3	α-OH	H
9	-CHO	H	-CH_3	α-OH	H

	R_1	R_2	R_3
3	β-glc	H	β-OH
7	β-glc	H	H
10	H	α-OH	H

Figure 1. Structures of compounds **1–10** (glc, glucosyl).

The molecular structures of *Asclepias* cardenolides have been intensively studied in the past [52–54]. On the basis of X-ray analysis, their principle structures have been determined: the triterpene scaffold of *Asclepias* cardenolides has the common feature of an α-orientation of the methine proton at C-5; therefore, the rings A and B of the scaffold are trans-fused [55]. Analysis of the NMR data led us to the conclusion that **1**, **2,** and **4** are coroglaucigenin-type molecules with a hydroxylation at C-19, while compound **3** is a calotropin derivative (Figure 2). We found compounds with one or two glycosylations, whereas in the previous study of the seeds, four cardenolides containing cellobiosyl units were reported [45].



	R_2	R_3	R_4	R_5
1	H	-CH_2-OH	α-OH	H
2	H	-CH_2-OH	α-OH	β-glc
4	β-OH	-CH_2-OH	β-OH	H

3

Figure 2. Important ^{1}H-^{1}H COSY (bold lines) and ^{1}H-^{13}C HMBC long-range correlations (arrows) for compounds **1–4** (glc, glucosyl).

Compound **1** has the molecular formula of $C_{29}H_{44}O_{10}$, determined by the ion peak at m/z 553.3015 $[M + H]^+$ (calculated for $C_{29}H_{45}O_{10}$, m/z 553.3013). It is a coroglaucigenin derivative with one glycosylation, analogous to 4′-*O*-*β*-glucopyranosyl frugoside (**5**) [45]. However, the sugar moiety bound to the sterol at position C-3 shows, unlike for many cardenolides reported in *Asclepias*, an oxidized methylene at C-6′. (Supporting Information, Figures S3–S11)

Further analysis of the glycosyl relative configuration revealed that the carbinolic protons H-1′and H-3′ are both in equatorial position. Proton H-3′ is furthermore in syn-periplanar position to H-2′ and H-4′. We therefore assumed an *α*-oriented hydroxyl function in position C-3′ (Figure 3). This stereochemistry is characteristic for allose, the C-3′ epimer

of glucose [56]. Compound **1** is, accordingly, 3-*O*-*β*-allopyranosyl coroglaucigenin (see Supporting Information, Figure S5 for the IUPAC name).

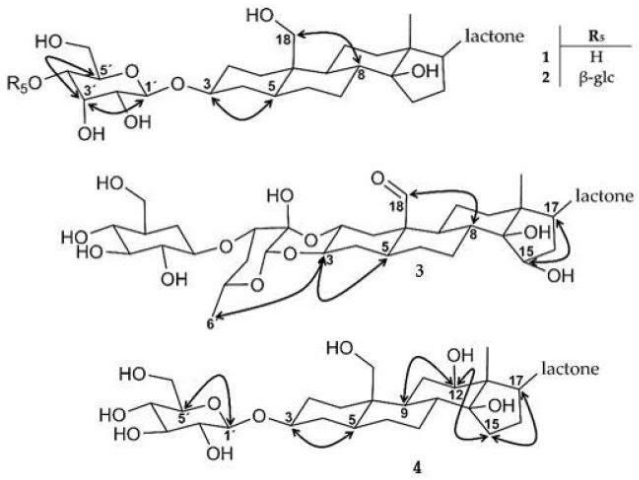


Figure 3. Important ^{1}H-^{1}H ROESY correlations (arrows) for compounds **1–4** (glc, glucosyl).

Compound **2** has the molecular formula $C_{35}H_{54}O_{15}$, determined by the ion peak at m/z 715.3549 $[M + H]^+$ (calcd for $C_{35}H_{55}O_{15}$, 715.3535) (Supporting Information, Figures S12–S24). The structure is similar to compound **1** but shows two signals in the ^{1}H NMR spectrum at δ_H 4.87 (δ_C 97.8, H-1′) and δ_H 4.56 (δ_C 103.6, H-1″) that we assigned to anomeric glycosyl positions. It suggested a glycosyl chain, where the first sugar was again an allose. For the second glycosyl moiety, however, the multiplicity of H-2′ and H-3′, both dd multiplicities with a large coupling constant ($^{3}J_{HH}$ > 9 Hz), revealed an anti-periplanar arrangement, consistent with the *β*-OH orientation at C-3′. This is characteristic for a glucosyl rest and, accordingly, compound **2** is 4′-*O*-*β*-glucopyranosyl-3-*O*-*β*-allopyranosyl coroglaucigenin (see Supporting Information, Figure S17 for the IUPAC name).

Compound **3** is a calotropin-type cardenolide with a molecular formula $C_{35}H_{48}O_{14}$, determined by the ion peak at m/z 693.3113 $[M - H_2O + H]^+$ (calcd for $C_{35}H_{49}O_{14}$, 693.3117) (Supporting Information, Figures S25–S33). This compound is similar to compound **7**, with the only difference being a hydroxylation at C-15. Analysis of the ^{1}H-^{1}H ROESY data showed that H-17 and H-15 are in syn-periplanar orientation. Given the absolute configuration of H-17 according to biosynthetic considerations, we assign H-15 as 15R [54]. We determine compound **3** as 3′-*O*-*β*-glucopyranosyl-15*β*-hydroxycalotropin (see Supporting Information, Figure S29 for the IUPAC name).

Compound **4** has a molecular formula of $C_{29}H_{44}O_{11}$, determined by the ion peak at m/z 569.2970 $[M + H]^+$ (calcd for $C_{29}H_{45}O_{11}$, 569.2956). It is of the coroglaucigenin type but, in this case, with a carbinolic proton resonating at δ_H 3.32 (δ_C 74,7, H-12). We defined the stereocenter of this oxydized methine as (R) from ^{1}H-^{1}H ROESY correlations between H-1*α*↔H-9↔H-12↔H-15*α*↔H-17. Furthermore, the large coupling between H-12 H_2-11*β* ($^{3}J_{HH}$ = 12.2 Hz) was consistent with their trans-periplanar orientation. The sugar moiety was identified as glucose by the large coupling constants and the ^{1}H-^{1}H ROESY correlations between H-1′ and H-5′. We therefore describe the compound as 3-*O*-*β*-glucopyranosyl-12*β*-hydroxy coroglaucigenin (see Supporting Information, Figure S39 for the IUPAC name).

2.2. Quantification of Cardenolides

We quantified compounds **1–10** in mg of compound per gram of seeds (dry weight; Figure 4 and Supporting Information Table S2). We used each compound as a standard for their corresponding calibration curve (Supporting Information Figure S2). Compound **5**, 4′-*O*-*β*-glucopyranosyl frugoside, is the most abundant cardenolide, with 4.5 mg/g of seeds, approximately two times more than 4′-*O*-*β*-glucopyranosyl gofruside **6** (2.06 mg/g). The other eight cardenolides are present in amounts below 1 mg/g. Compounds **1** and **4** are the least abundant at 0.01 and 0.004 mg/g, respectively.

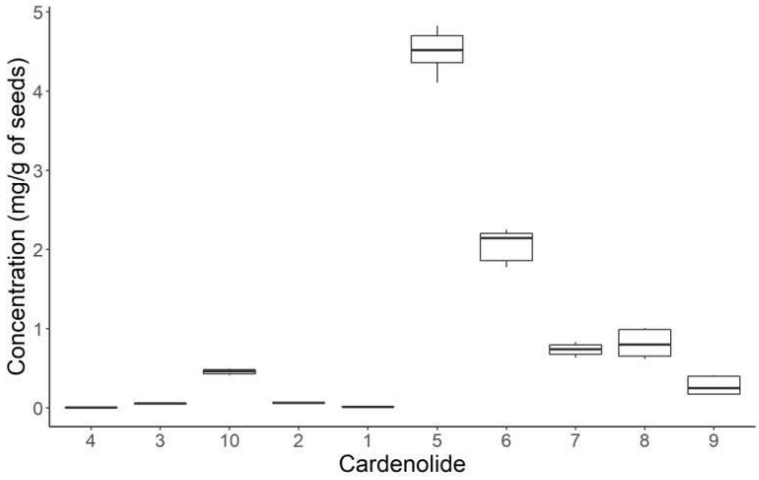


Figure 4. Quantification of the isolated cardenolides **1–10** from polar to non-polar (left to right) according to their retention time in reversed-phase chromatography. Boxplots show the median, interquartile range, and the whiskers represent the largest and smallest value within 1.5 times the 25^{th} and 75^{th} percentile.

The range of retention times of the isolated compounds was 19 to 30 min (Supporting Information Figure S1, for chromatography conditions see Section 3.1). The minor compounds **1–4** and **10** have a higher polarity than the more abundant compounds (Figure 4). Recently, López-Goldar et al. [11] reported a predominance of more polar compounds in the seed extracts of *A. curassavica*. Allomethylose and deoxy-allomethylose are present as sugar moieties in the abundant cardenolides **5–10**, whereas allose and glucose were found in the minor compounds **1**, **2**, and **4**. This finding may provide clues regarding the biosynthetic pathways of the rare sugars in *Asclepias*, where allosyl cardenolides could be intermediates of interest.

2.3. Na^+/K^+ ATPase (NKA) Inhibitory Activity

We tested the inhibitory capacity of the new compounds **1** and **2** and the known cardenolides 5–10 against porcine NKA. We used an in vitro assay to determine the IC_{50} of each compound and used ouabain as a reference.

There was a significant variation in inhibition of an unadapted NKA from *Sus domesticus* (Supporting Information, Tables S3 and S4). The IC_{50} values ranged from 10^{-6} to 10^{-8} M; this is consistent with the sensitivity for cardenolide inhibition expected from *S. domesticus* NKA [19,39,57]. Gofruside **9** was the most inhibitory compound tested ($IC_{50} = 9.653 \times 10^{-8}$ M). The least inhibitory was 16*α*-hydroxycalotropin 10, with an IC_{50} of 3.667×10^{-6} M (Figure 5).

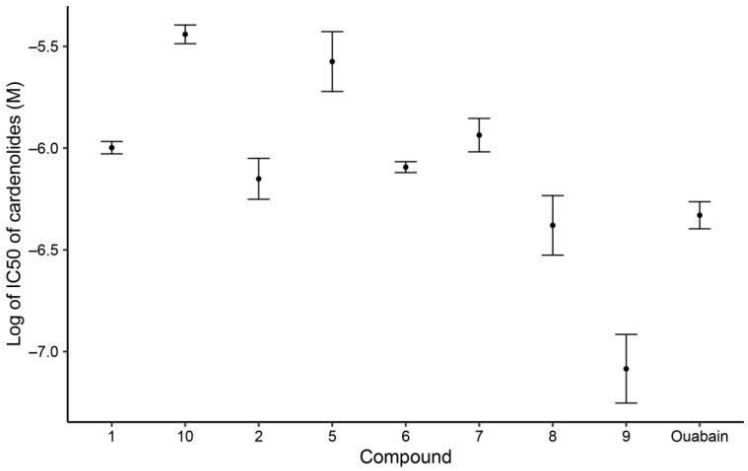


Figure 5. Mean + se IC_{50} values of isolated cardenolides 1, 2, and 5–10 and ouabain as reference.

Compounds **1** and **8** are coroglaucigenin derivatives with allosyl and allomethylosyl substitution, respectively, but they did not differ in their inhibition properties (Supporting Information, Tables S3 and S4). Compounds **8** and **9** share the same glycosylation, but **9** shows a different oxidation state of C-18 (alcohol vs. aldehyde). The higher inhibition of **9** compared with **8** suggests that the aldehyde is the main reason for the difference. This can be due to the high reactivity of **9** towards biomolecules. However, 16*α*-hydroxycalotropin **10** also contains an aldehyde but has the lowest inhibitory capacity. It has been reported that calotropin, the 16-deoxy aglycone of **10**, has an IC_{50} of 2.7×10^{-7} M ($\log_{10}$: −6.56) against porcine NKA [19]. This difference in inhibition may also be attributed to the fact that in **10,** the 16*α*-hydroxylation interferes with binding to the biological target. Molecular docking analyses are required for a better understanding.

We found a reduced inhibitory potential when a glucosylation of cardenolides occurred. The compounds **8** and **5** (coroglaucigenin-type) and **9** and **6** (corotoxigenin-type) differ only in the glucosylation, the aglycones having the higher inhibition potential. However, we also found that compounds **1** and **2**, which differ in the presence of glucose as a second sugar unit, have no significant difference in their inhibitory potential when compared with one another. An explanation for this could be the increased bulkiness of the molecule caused by the higher degree of glycosylation in both cases, which hinders the access to the active site of the NKA. In addition, in this case, molecular docking analyses would be needed to unravel the impact of the glycone moieties in the inhibition of NKAs.

Overall, our Na^+/K^+ ATPase inhibition results by cardenolides from *Asclepias* seeds are in line with a previous comparison of structural characteristics and NKA inhibition by Petschenka et al. [39], who described a differential response in the inhibition of vertebrate NKA by cardenolides with the same aglycons but different glycosylations.

Phytochemical diversity, like that described here, is linked to herbivore community in several systems [58–60], but disentangling concentration and inhibitory potency of phytochemicals is challenging and may be related to the costs of producing phytochemicals for the plant and differences in toxicity against herbivores with specific tolerance mechanisms [11]. In our future work we will seek to identify the selective pressures that lead to the differential investment in the compounds, given their different inhibitory effects against porcine NKA, and determine whether these effects vary depending on different cardenolide tolerances.

3. Materials and Methods

3.1. General Experimental Procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III HD spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a cryoplatform and a 5 mm TCI CryoProbe, field strengths of ^{1}H (500.13 MHz)/^{13}C (125.76 MHz): 11.747 T. Spectrometer control and data processing was accomplished using Bruker TopSpin 3.6.1, and standard pulse programs as implemented in Bruker TopSpin 3.6.1. were used. Samples were measured in MeOH-d_3 (99.5%) or D_2O (99.9%), depending on solubility of the compounds. For compounds measured in MeOH- d_3, the residual solvent signals were δ_H 3.31/δ_C 49.15. For measurements in D_2O, all chemical shifts were left uncorrected after carefully tuning and matching the NMR instrument. High performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-HRMS) analyses were performed on an Agilent 1260 Infinity, using a reversed-phase column Agilent Zorbax RP-18e (3.5 µm particle size, 3 × 150 mm). The mobile phase consisted of acetonitrile (ACN, supplied with 0.1% formic acid, FA, Carl Roth GmbH, Karlsruhe, Germany) and water (HPLC grade, 0.1% FA, deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany). An elution gradient was used as follows: starting with ACN/H_2O (5:95) for 5 min, then to ACN/H_2O (45:55) for 25 min and maintained for 10 additional min. Later, it was set back to ACN/H_2O (5:95) for 2 min and maintained for another 3 min, a total time of 45 min. For calibration curves and quantification of each compound, an Agilent Poroshell 120 column (2.7 µm particle size, 4.6 × 50 mm) was used. The elution gradient started with ACN/H_2O (5:95) for 1 min, then to ACN/H_2O (95:5) for 8 min, which was maintained for 2 min. Later, it was set back to ACN/H_2O (5:95) for 1 min. The gradient had a total length of 12 min.

High resolution mass spectra were recorded on a Bruker Compact OTOF spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Electrospray ionization (ESI) in positive ion mode was used for the analysis in full scan and auto MS/MS modes, scanning masses from m/z 50–1300. Capillary voltage was set at 4500 V, charging electrode at 2000 V, and corona current at 0 nA; nebulizer pressure gas was set at 1.8 bar, drying gas temperature at 220 °C, and drying gas flow at 9.0 L/min. Sodium formate adducts were used for internal calibration with a Quadratic + HPC mode. Bruker Compass ver.1.9 (OTOF Control ver.5.1.107 and HyStar 4.1.31.1) was used for data acquisition and instrument control, and Bruker DataAnalysis ver. 5.1.201 was used for data processing.

Reversed-phase MPLC separations were carried out on a Biotage Isolera One (Biotage SB, Uppsala, Sweden) using a Biotage Sfär C18 D—Duo 100 Å 30 µm 120 g column. A linear gradient, using a mobile phase consisting of MeOH (supplied with 0.1% FA, Carl Roth GmbH) and water (0.1% FA) was used, with a flow rate of 50 mL/min, and UV detection was carried out at 218 nm. For separations on Sephadex LH-20 (VWR GmbH, Dresden, Germany), a column containing 44 g of sorbent was used and eluted with the Isolera MPLC equipment, using water as the mobile phase. MPLC separations on MCI gel CHP20P (Merck KgA, Darmstadt, Germany) were carried out using a linear MeOH-water gradient on the Biotage Isolera equipment. Semi-preparative HPLC separations were carried out on a Shimadzu Prominence HPLC System, consisting of an autosampler SIL-20AC, gradient pump LC-20AT, UV-Vis detector SPD-20A programed for detection at 220 nm and a fraction collector FRC-10A. For separations, isocratic elutions with MeOH-water mixtures were performed at a flow rate of 0.8 mL/min. A C-18 Nucleodur Isis column (4.6 × 250 mm, 5 µm particle size, from Macherey-Nagel, Düren, Germany) was used. The purity of the isolated compounds was calculated by ^{1}H qNMR experiments, with ouabain as external standard (ERETIC).

3.2. Extraction and Isolation

A. curassavica seeds were purchased from Jelitto Perennial Seeds (Art. No.: AA974). We ground 264 g of desiccated seeds to powder and extracted with 100% water, water/MeOH (1:1), and later 100% MeOH. Each of the extracts was dried in vacuum, then re-suspended

in MeOH, left at −20 °C overnight, and filtered through paper (Whatman grade 50, 185 mm diameter). We centrifuged the filtrate for 10 min at 13,200 rpm to remove remaining particles. The filtrates were pooled together, and the solvent was removed in vacuum by rotary evaporation at 40 °C. We obtained 11.0 g of crude extract. We separated the crude extract using reversed-phase (RP-18) silica gel by MPLC, obtaining 13 fractions (See Section 3.1). We first targeted the masses of the cardenolides previously isolated from *A. curassavica* seeds by HPLC-HRMS and analyzed fragmentation patterns that indicated the presence of steroidal glycosides [45,47,48]. We detected the presence of cardenolides in fractions 6 to 11. We used fraction 10 (F10) to isolate the cardenolides described here.

F10 (506.5 mg) was subjected to an MCI CHP20P gel column eluted with MeOH, giving five fractions. F10.2 and F10.3 were further separated on Sephadex LH-20 using water as eluent, giving the fractions F10.2.1-4 and F10.3.1-5. Sub-fractions that contained the same cardenolides were pooled together according to the HPLC-HRMS analyses. The combined fractions were further separated by reversed-phase HPLC with isocratic elution (MN C-18 Isis, ACN/H_2O (2:8)). From F10, we isolated glucopyranosyl frugoside (**5**, 88 mg, 98% purity), glucopyranosyl gofruside (**6**, 4.2 mg, 72% purity), and glucopyranosyl calotropin (**7**, 6.5 mg, 97% purity). Similarly, F11 (2.3 g) was separated (ACN/H_2O, 25:75). We obtained frugoside (**8**, 153 mg, 98% purity) and gofruside (**9**, 49 mg, 98% purity).

F9 (77.7 mg), separated with a mobile phase of ACN/H_2O (16:84), yielded compound **1** (3 mg, 75% purity). F6-8 (86.7, 39.6, and 48.6 mg, respectively) were separated in a similar way (ACN/H_2O isocratic mobile phase: 12:88, 14:86, and 15:85, respectively) and produced compounds **2** (3.7 mg, 91% purity), **3** (1.1 mg, 58% purity), **4** (2.5 mg, 48% purity), and 16*α*-hydroxycalotropin (**10**, 4.4 mg, 58% purity). The lower purity levels of compounds 1-4 were due to the persistent co-elution with several minor compounds. The isolated cardenolides, together with their chemical data, are listed below. For ^{1}H NMR and ^{13}C NMR data, see Table 1 and Supporting Information, Table S1.

3-*O*-*β*-allopyranosyl coroglaucigenin (**1**) $[\alpha]_D^{25}$ +3.4510 +/− 5.0554 S.D. (*c* 0.14, H_2O), UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 553.3015 $[M + H]^+$ (calcd for $C_{29}H_{45}O_{10}$, 553.3007, Δ 1.4 ppm).

4′-*O*-*β*-glucopyranosyl-3-*O*-*β*-D-allopyranosyl coroglaucigenin (**2**) $[\alpha]_D^{25}$ +112.5669 +/− 5.8027 S.D. (*c* 0.12, H_2O), UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 715.3549 $[M + H]^+$ (calcd for $C_{35}H_{55}O_{15}$, 715.3535 Δ 1.9 ppm).

3′-*O*-*β*-glucopyranosyl 16*β*-hydroxycalotropin (**3**) $[\alpha]_D^{25}$ +33.6123 +/− 9.5351 (*c* 0.08, H_2O), UV (ACN/H_2O): 218 nm; HRESIMS *m*/*z* 693.3113 $[M –H_2O + H]^+$ (calcd for $C_{35}H_{49}O_{14}$, 693.3117, Δ 0.6 ppm).

3-*O*-*β*-glucopyranosyl 12*β*-hydroxy coroglaucigenin (**4**) $[\alpha]_D^{25}$ +55.5112 +/− 5.8428 (*c* 0.11, H_2O), UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 569.2970 $[M + H]^+$ (calcd for $C_{29}H_{45}O_{11}$, 569.2956, Δ 2.5 ppm).

4′-*O*-*β*-glucopyranosyl frugoside (**5**) white powder, UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 699.3593 $[M + H]^+$ (calculated for $C_{35}H_{55}O_{14}$, 699,.3586 Δ 1 ppm).

4′-*O*-*β*-glucopyranosyl gofruside (**6**) white powder, UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 697.3427 $[M + H]^+$ (calcd for $C_{35}H_{53}O_{14}$, 697.3430, Δ 0.4 ppm).

3′-*O*-*β*-glucopyranosyl calotropin (**7**) white powder, UV (ACN/H_2O): 220 nm; HRESIMS 67.3161 *m*/*z* $[M –H_2O + H]^+$ (calcd for $C_{35}H_{49}O_{13}$, 677.3168, Δ 1 ppm).

Frugoside (**8**) white powder, UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 537.3059 $[M + H]^+$ (calcd for $C_{29}H_{49}O_9$, 537.3058, Δ 0.2 ppm).

Gofruside (**9**) white powder, UV (ACN/H_2O): 222 nm; HRESIMS *m*/*z* 535.2894 $[M + H]^+$ (calcd for $C_{29}H_{43}O_9$, 535.2902, Δ 1.5 ppm).

16*α*-hydroxycalotropin (**10**) white powder, UV (ACN/H_2O): 220 nm; HRESIMS *m*/*z* 549.2706 $[M + H]^+$ (calcd for $C_{29}H_{41}O_{10}$ 549.2694, Δ 2 ppm).

Table 1. ^{1}H- and (500.13 MHz) ^{13}C-NMR data (125.76 MHz) of compounds **1-4** in D_2O (δ ppm, rel. orientation, multiplicity*, (*J* [Hz])).

Position	1		2		3		4	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.15 β brd (13.4) 0.81 α dd (13.4, 11.1)	30.7 CH_2	2.23 br, 0.89 br	30.6 CH_2	2.45 β dd (12.6, 4.4) 1.19 α brt (12.6)	34.4 CH_2	2.15 α dt (13.5, 3.1) 0.83 α brt (13.4)	30.5 CH_2
2	1.85 brd, (11.1), 1.35 br	29.1 CH_2	1.92 br, 1.43 br	29.2 CH_2	3.91 β br	69.1 CH	1.86 br, 1.37 br	28.9 CH_2
3	3.77 m	79.1 CH	3.85 m	79.1 CH	4.02 α td (10.6, 4.0)	72.1 CH	3.78 α m	79.0 CH
4	1.72 brd (12.2), 1.31 br	34.0 CH_2	1.81 br, 1.41 br	34.0 CH_2	1.76 α br, 1.31 β br	32.6 CH_2	1.72 α br, 1.31 β q (12.2)	33.8 CH_2
5	1.19 brt (11.5)	43.7 CH	1.28 br	43.8 CH	1.70 α br	42.2 CH	1.20 α br	43.8 CH
6	1.26 m, 1.26 m	27.3 CH_2	1.34 m, 1.34 m	27.4 CH_2	2.16 m, 1.57 m	26.1 CH_2	1.25 m, 1.25 m	27.3 CH_2
7	1.88 m, 1.07 m	26.9 CH_2	1.96 m, 1.16 m	26.9 CH_2	1.90 β m, 1.74 α m	26.9 CH_2	1.86 m, 1.06 m	27.1 CH_2
8	1.63 br	41.2 CH	1.71 br	41.3 CH	1.72 β br	41.8 CH	1.63 β br	40.6 CH
9	1.00 br	49.1 CH	1.08 br	48.9 CH	1.64 α br	46.9 CH	1.02 α brt (13.8)	45.3 CH
10	-	38.7 C	-	38.7 C	-	53.3 C	-	38.6 C
11	1.55 m, 1.33 m	22.5 CH_2	1.64 m, 1.42 m	22.5 CH_2	1.73 α m, 1.12 β m	21.5 CH_2	1.76 α m, 1.46 β q (12.5)	30.6 CH_2
12	1.44 m, 1.33 m	39.7 CH_2	1.53 m, 1.42 m	39.7 CH_2	1.53 m, 1.46 m	37.2 CH_2	3.32 α dd (12.2, 1.7)	74.7 CH
13	-	49.8 C	-	49.5 C	-	48.3 C	-	55.8 C
14	-	86.3 C	-	85.7 C	-	82.1 C	-	86.4 C
15	2.09 m, 1.63 m	31.7 CH_2	2.17 m, 1.72 m	31.8 CH_2	4.66 α brd (8.4)	71.8 CH	1.88 α m, 1.67 β m	31.7 CH_2
16	2.09 m, 1.73 m	26.5 CH_2	2.19 m, 1.82 m	26.6 CH_2	2.68 m, 1.66 m	36.0 CH_2	2.10 m, 1.79 m	26.8 CH_2
17	2.81 α br	50.2 CH	2.89 α br	50.2 CH	2.77 α dd (9.8, 4.9)	47.3 CH	3.20 br	45.4 CH
18	0.82 β s	15.2 CH_3	0.91 β s	15.3 CH_3	0.85 β s	16.1 CH_3	0.73 β s	8.9 CH_3
19	3.82, br 3.67 br	59.0 CH_2	3.90 br, 3.76 br	59.0 CH_2	10.10 s	213.1 CH	3.81 d (12.2) 3.68 d (12.2)	58.6 CH_2
20	-	178.5 C	-	178.2 C	-	177.6 C	-	178.4 C
21	4.99 d (18.8) 4.93 d (18.8)	75.2 CH_2	5.06 d (18.7) 5.00 d (18.7)	75.1 CH_2	5.09 d (18.3) 5.02 d (18.3)	75.0 CH_2	4.95 br, 4.95 br	75.1 CH_2
22	5.89 s	115.9 CH	5.98 s	115.7 CH	6.02 s	116.2 CH	5.92 s	116.2 CH
23	-	179.3 C	-	178.9 C	-	178.2 C	-	178.5 C
1′	4.77 d (8.3)	98.0 CH	4.87 d (8.2)	97.8 CH	4.63 s	94.8 CH	4.51 α d (7.8)	100.3 CH
2′	3.32 dd (8.3, 3)	70.2 CH	3.43 dd (8.7, 2.7)	70.0 CH	-	91.6 C	3.14 β brt (8.7)	73.1 CH
3′	4.09 t (3)	71.3 CH	4.45 t (3.1)	70.9 CH	3.95 br	81.5 CH	3.39 α t (9.3)	75.8 CH
4′	3.53 dd (10, 3)	66.9 CH	3.78 dd (10, 2.7)	76.2 CH	2.13 β br, 1.71 α br	37.1 CH_2	3.29 β t (9.3)	69.6 CH
5′	3.68 brddd (10.6, 1.5)	73.6 CH	3.88 brd (10.3, 2.2)	72.4 CH	3.82 β q (5.8)	68.7 CH	3.36 α ddd (9.3, 5.8, 1.7)	75.9 CH
6′	3.82 dd (12.0, 1.5) 3.61 dd, (12.0, 6.0)	61.2 CH_2	3.90 brd (12.1) 3.76 dd (12.5, 4.2)	60.6 CH_2	1.29 α d (6.3)	19.8 CH_3	3.82 dd (1.7, 12.2) 3.63 dd (5.8, 12.2)	60.7 CH_2
1″			4.56 d (7.9)	103.6 CH	4.64 d (7.7)	104 CH		
2″			3.32 brt (8.9)	73.2 CH	3.37 t (9.1)	73.4 CH		
3″			3.48 t (9.2)	75.7 CH	3.51 t (9.1)	75.5 CH		
4″			3.42 br	69.3 CH	3.43 br	69.4 CH		
5″			3.44 br	75.7 CH	3.44 br	75.8 CH		
6″			3.90,br, 3.76 br	60.6 CH_2	3.90 dd (12.2, 1.5) 3.74 dd (12.2, 5.7)	60.2 CH_2		

3.3. Quantification of Cardenolides

We quantified the cardenolides isolated from the *A. curassavica* seeds using HPLC-HRMS with a linear calibration method. Each compound was diluted in order to obtain ten data points in a concentration range from 0 to 1 mg/mL, and each value was corrected according to the measured purity of the compound. The spectrometric data were obtained using the method described in Section 3.1. We extracted the ion chromatograms on the basis of the m/z of the most abundant peak for each cardenolide ($[M+H]^+$ for compounds **1**–**6** and **8**–**10** and $[M+H\text{-}H_2O]^+$ for compound **7**). The area of the corresponding peak was calculated and used as a quantification parameter. The data points that were out of the linear range, especially at high concentration, were excluded. Linear regression for compounds **1**–**10** was calculated with concentration and peak area as variables (Supporting Information, Figure S2).

Analysis of the content of cardenolides **1**–**10** in the seeds was performed as follows: 6 g of *A. curassavica* seeds were collected from plants grown in the greenhouse of the MPI for Chemical Ecology in Jena, Germany (seeds purchased from Jelitto Perennial Seeds, Art. No.: AA974). Seed samples (three technical replicates, each of 2 g) were freeze-dried, ground, and exhaustively extracted with $MeOH/H_2O$ (1:1) three times and then twice more with MeOH. The extracts were pooled and filtered using grade 50 Whatman paper filter to remove particles. Lipophilic substances were removed by passing the filtrate through a MN HR-X 500 mg cartridge. The samples were then dried using N_2 gas at 36 °C, obtaining 214.9 mg, 230.6 mg, and 218.9 mg, respectively, of raw extract. The HPLC-HRMS measurements were conducted as described above. From each technical replicate a solution of 2.6 mg/mL was prepared, and from each of these solutions, three injections of 5 µL were used for analysis, resulting in nine data points per compound. Data processing was accomplished as for the calibration curve, by extracting the ion chromatogram per compound and measuring the corresponding peak area. The concentration per injection was later expressed in mg of cardenolide per g of seed (dry weight).

3.4. Na^+/K^+ ATPase (NKA) Inhibitory Activity Assay

We quantified the NKA inhibitory activity of the isolated cardenolides using purified NKA from *S. domesticus* cerebral cortex (Sigma-Aldrich-A7510-5UN, Steinheim, Germany), following protocols standardized by Petschenka et al. [15]. Briefly, porcine NKA was diluted in di-water to a final assay concentration of 0.01 U/mL. The ATPase was exposed to exponentially decreasing concentrations from 10^{-3} to 10^{-8} M of ouabain (Sigma-Aldrich, O3125-1G, Steinheim, Germany) or the cardenolides **1**–**2** and **5**–**10**. There was an insufficient amount of compounds **3** and **4** to allow testing. We prepared a stock solution of each cardenolide in 100% DMSO, which was used for preparing the concentrations for the assay. The concentration of DMSO in the final solutions did not exceed 2% per well. The reaction mixture with NKA and cardenolides was incubated at 37 °C for 10 min, followed by addition of ATP (Sigma Aldrich, A9062-1G, Steinheim, Germany) and another incubation for 20 min. The NKA activity after cardenolide exposure was determined by quantification of inorganic phosphate released from enzymatically hydrolysed ATP. The ATP levels were measured by photometric determination, reading the absorbance at 700 nm with a microplate reader (BMG Clariostar, BMG Labtech, Germany). The absorbance of each reaction was corrected with the respective background, and the inhibition curves were plotted in R studio [61] with $\log_{10}$-transformed cardenolide concentration versus percent of uninhibited control, with top and bottom asymptotes set to 1 and 0, respectively [30,32,62]. For each cardenolide, we carried out three biological replicates, with two technical replicates, resulting in each data point being an average of six measurements. IC_{50} values for each cardenolide and ouabain were also calculated from the inhibition curves using R studio. We employed Bonferroni-adjusted significance tests for pairwise comparisons between the IC_{50} values for cardenolides **1**–**2** and **5**–**10** and ouabain. The isolated cardenolide purity was addressed in the preparation of the stock solutions for compounds **5** and **7**–**9**. For compounds **1, 2, 6**, and **10**, stock solutions were prepared without this correction, given

the low amounts available and/or low purity; instead, the final IC_{50} concentrations values were adjusted with purity percent as the factor.

4. Conclusions

The re-examination of the seeds of *A. curassavica* allowed us to describe and quantify four new cardenolides (**1**–**4**). We also isolated six known compounds (**5**–**10**) and confirmed by spectrometric quantification that 4′-*O*-*β*-glucopyranosyl frugoside **5** is the most abundant cardenolide in the seeds. The examination of the isolated compounds against NKA enzymes from a sensitive vertebrate (*S. domesticus*) revealed that the most inhibitory cardenolide is gofruside **9,** and the least inhibitory are 4′-*O*-*β*-glucopyranosyl frugoside **5** and 16*α*-hydroxycalotropin **10**. Comparison of the IC_{50} values obtained with the structural characteristics of each cardenolide confirmed that glycosylation, when leading to higher polarity, corresponds directly to a decrease in toxic potential of the cardenolides. However, the structure of the glycosyl substituents and the degree of oxidation at position 19 (alcohol vs. aldehyde) in coroglaucigenin-type cardenolides can also influence inhibitory capacity. The chemical defense of *A. curassavica* seeds varies in quantity and NKA inhibition potential. The cardenolide profile described here may be the result of biosynthetic constraints that result in a high diversity of bioactive compounds, which increases the chances of achieving an effective defense against several herbivore pressures.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28010105/s1, NMR data of compounds **5**-**10,** NMR data, UV, and HR-ESI-MS data of compounds **1**–**4**.

Author Contributions: Conceptualization, H.M.R., C.P. and P.R.-B.; isolation and structural elucidation, C.P. and P.R.-B.; biological activity assays, S.P. and P.R.-B.; statistical analysis, H.M.R. and P.R.-B.; resources, C.P. and H.M.R.; writing—original draft preparation, P.R.-B.; writing—review and editing, S.P., C.P. and H.M.R.; supervision, C.P. and H.M.R.; project administration, H.M.R.; funding acquisition, H.M.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds **5**, **7**, **8**, and **9** are available from the authors.

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Supporting Information

New structures, spectrometric quantification and Na^+/K^+ inhibitory properties from cardenolides of *Asclepias curassavica* seeds

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Contents

Figure S1: UV and MS chromatograms of *Asclepias curassavica* extract (compounds **1-10** above their correspondent peak)

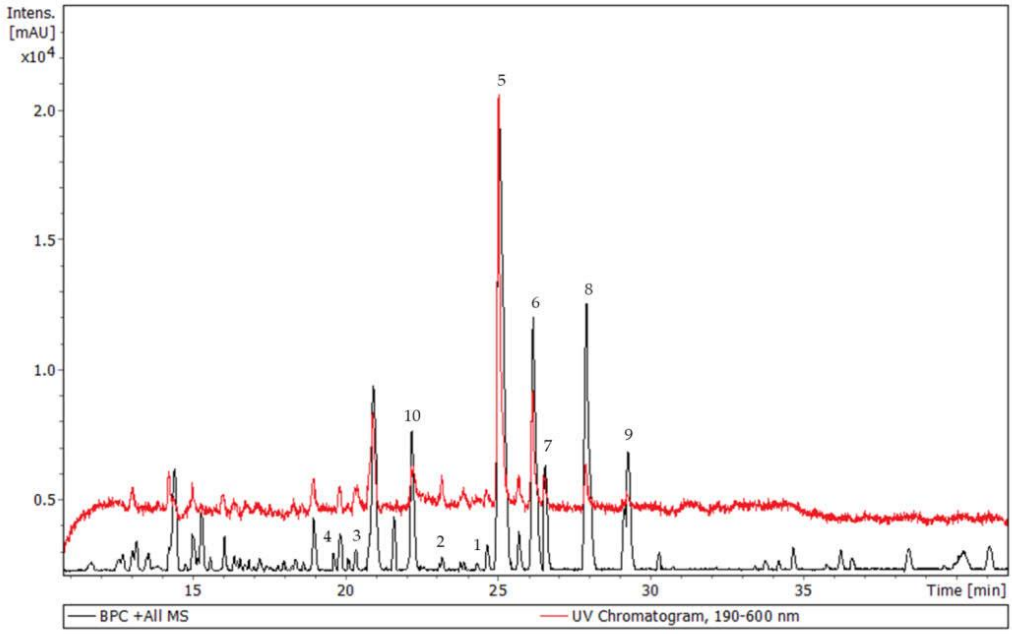


Figure S2: Calibration curves of compounds 1-10

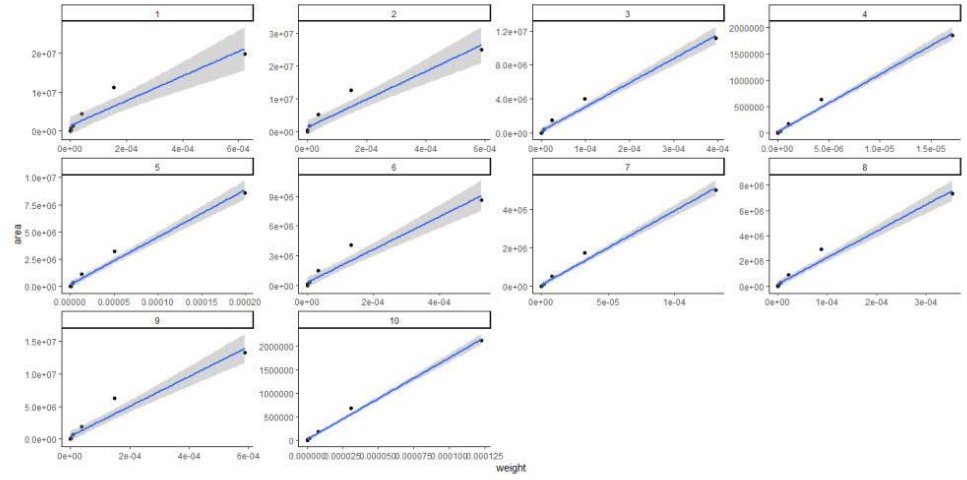


Table S1: ^{1}H NMR (500 MHz) and ^{13}C (125 MHz) data of compounds **5**-**10** [δ in ppm, configuration, multiplicity (*J* in HZ)]

Position	**5***		**6***		**7***		**8****		**9***		**10***	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.26 β, 0.92 α	30.8	2.39 bd (13.4) 1.06 bt (13.4)	30.1	2.46 β dd (12.6,4.3) 1,19 α t (12.6)	34.4	2.39 β dt (13.6,2.9) 1.08 bt (13.6)	30	2.31 β dt (13.1, 3.3) 0.78 td (13.1, 3.3)	32.5	2.37 β dd (12.6,4.4) 1.12 α t (12.6)	34.3
2	1.44, 1.94	29.1	1.97, 1.24	29.8	3.90 β	69.1	1.98, 1.24	29.8	1.88, 1.53	30.7	3.74 β ddd (12.6,10.7, 4.4)	69
3	3.85 α	78.8	3.79	78.4	4.03 α t (3.0)	72	3.83 α	78.3	3.67 α	79.2	3.89 α td (10.7, 4.0)	72.1
4	1.41, 1.83	34	1.99, 1.21	35.4	1.78, 1.35 β	30.8	1.99 α, 1.2 β	35.7	1.76, 1.42	35.8	1.67, 1.24 q (12.6)	32.7
5	1.3 α	43.8	1.51	42.1	1.71	42.3	1.6 td (12.2, 3.1)	42.3	1.21	45.6	1.6 α	42.3
6	1.36, 1.36	26.7	1.94, 1.68	27.7	2.19, 1.77	26.7	2.16, 1.35	26.7	1.31, 1.21	29.3	1.86 bdd (13.1, 3.3), 1.26	26.8
7	1.98, 1.18	27.1	2.16 bd (12.3) 1.32	26.8	1.98 β qd (13,1,3.1) 1.39	26.8	1.95 β, 1.69	27.8	2.06, 1.13	28.6	2.08, 1.66	26.7
8	1.74 β	41.3	1.6 t (12.4)	42.3	1.65β	41.6	1.53 β	42	1.77	42.8	1.53 β	41.6
9	1.12 α	49.2	1.41	47.8	1.58	47.5	1.44 α	47.7	1 α m	51.3	1.52 α	47.4
10		39.1		51.9		53.2		52.1		40.3		53.2
11	1.66, 1.44	22.5	1.71, 1.12	21.5	1.73 1.16 β	21.5	1.73, 1.13 β	21.5	1.62, 1.62	23.7	1.68, 1.09	21.8
12	1.44, 1.55 α	39.7	1.53, 1.44	38.7	1.56, 1.47, bt (13.6)	38.4	1.52, 1.44	38.9	1.48, 1.39	41.3	1.63, 1.57	39.6
13		49.2		49.4		49.4		49.5		50.9		48.9
14		86.3		85.7		85.6		85.8		86.4		85.4
15	2.20, 1.73	31.7	2.2, 1.71	31	2.16, 1.74	30.8	2.14, 1.71	31.1	2.14, 1.69 dd (11.5;9.5)	33.3	2.06, 2.06	39.1
16	2.22, 1.84	26.5	2.12, 1.83	26.5	2.21, 1.82	26.4	2.21, 1.83	26.5	2.16, 1.86	27.8	4.48 β td (8.2,4.1)	76.1
17	2.91 α	50.1	2.88	49.9	2.92 dd (9.5, 5.6)	49.7	2.9 α dd (9.5, 5.4)	50.2	2.82 α	52.1	2.62 α d (5.1)	59.2
18	0.95 s β	15.2	0.81 β s	15.1	0.83 β s	14.9	0.82 β s	15.1	0.92 β s	16.2	0.71 β s	14.9
19	3.92, 3.77	60.1	10 s	214.9	10.09 β s	212.8	9.95 β s	214.9	3.86 dd (11.7, 4.2) 3.73	60	9.96 s	212.9
20		178.9		178.7		178.4		178.5		176.9		175.4
21	5.06 dd (18.2, 1.3) 5.08 dd (18.2, 1.3)	75	5.06 bd (18.5) 5.01 bd (18.5)	75	5.07 dd (18.2, 1.3), 5.04 dd (18.2, 1.3)	75	5.08, 5.03	75.1	5.03, 4.90	75.2	4.92, 4.92	75.1
22	6.00 s	116.1	5.98 s	115.8	6.00 s	116	5.97 s	116	5.89 s	117.5	5.96 s	116.7
23		179.4		178.9		178.8		178.9		178.1		177.9
1′	4.87α d (8.3)	97.9	4.82 α d (7.9)	97.8	4.65 β s	94.8	4.81α d (8.2)	98	4.72 d (8.0)	99.5	4.53 β s	95.2
2′	3.46 β	69.7	3.4 β dd (7.7,1.8)	70		91.6	3.38 dd (8.2,3.1)	70.5	3.26 dd (7.9, 2.9)	72.6		91.8

3′	4.4 β t (2.8)	70.7	4.37 β bd	70.5	3.96 β dd (11.6, 4.5) 2.15 β bdd (12.0, 4.5)	81.5	4.13 α t (3.1)	71.2	4.01 α t (2.9)	73	3.68 β dd (12.1,5.0) 1.76 β ddd (12.6,4.8,1.3),	71.6
4′	3.53 β	82	3.49β d (9.4) 3.9 α bdt	81.6	1.73 q (11.3, 12.0)	36.9	3.35 dd(9.8,3.1)	72.4	3.14 dd (9.5,2.7)	74.6	1.54 α	37.2
5′	3.94 α	68.2	(6.2, 6.2, 3.2)	68.4	3.84 β dd (11.3,5.9)	68.6	3.8 α m	69.4	3.71 α	70.4	3.72 β	68.9
6′	1.34 β d (6.2)	16.8	1.31 β d (6.2)	16.8	1.3 α d (5.9)	19.7	1.25 β d (5.9)	16.7	1.22 β d (6.3)	18	1.19 α d (5.7)	19.7
1"	4.6 α d (7.8)	104	4.58 α d (7.8)	104	4.66 α d (7.9)	104						
2"	3.35 β bt (8.3)	73.1	3.32 β bt (8.8)	73.2	3.39 bt (8.7)	73.3						
3"	3.5 α	75.7	3.43 α	75.6	3.53 α t (9.1)	75.5						
4"	3.46 β	75.7	3.42 β	69.4	3.44 m	69.3						
5"	3.46 α	75.8	3.48 α t (9.4)	75.6	3.46 α m	75.8						
6"	3.92, 3.77	60.1	3.89 bd (12.1) 3.75 dd (12.1,3.3)	60.2	3.92 bd (12.2) 3.76 dd (12.2, 5.1)	60.4						

*-in D_2O, **-in MeOD-d3

Figure S3: HR-ESI-MS spectrum of compound **1.**

Compound Spectrum List Report

Analysis Info

Acquisition Date 08/03/2022 11:25:26

Analysis Name \\filer\bird-lab\Users\Paola R-B\LCMS data\Asclepias extracts\553 calibration\allosyl-corog 8.3.22 dilu 2_P1-E-2_01_172.d

Method paola_lcms_pos_checkuplc040221.m

Operator MPI Jena

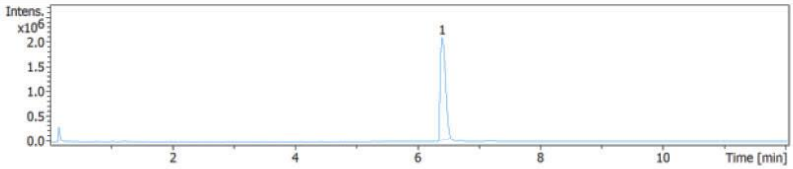
Sample Name allosyl-corog 8.3.22 dilu 2

Instrument compact 8255754.20124

Comment

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



#	RT [min]	Area	Int. Type	I	S/N	Trace	Max. m/z	FWHM [min]
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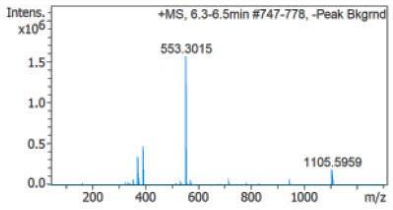
Cmpd 1, 6.4 min



#	m/z	Res.	S/N	I	I %	FWHM
1	373.2377	11047	8971.6	347780	22.2	0.0338
2	374.2419	11063	2629.5	102053	6.5	0.0338
3	391.2483	11304	11511.1	468366	29.9	0.0346
4	392.2521	11232	3403.2	138660	8.9	0.0349
5	553.3015	11452	20743.1	1565642	100.0	0.0483
6	554.3050	11599	7294.6	551036	35.2	0.0478
7	555.3088	11604	1947.4	147316	9.4	0.0479
8	943.5433	12025	866.9	66771	4.3	0.0785
9	1105.5959	11978	3710.1	189855	12.1	0.0923
10	1106.5992	11915	2676.7	136792	8.7	0.0929

Figure S4: UV spectrum of compound **1**

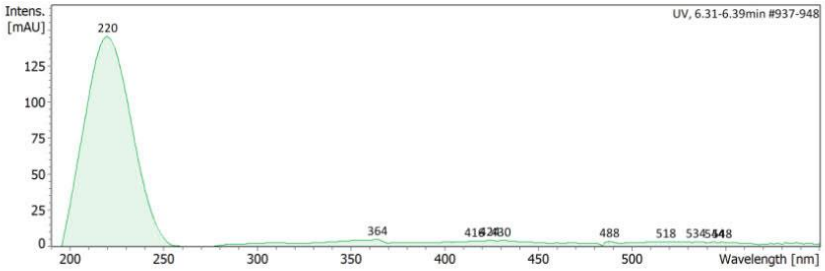
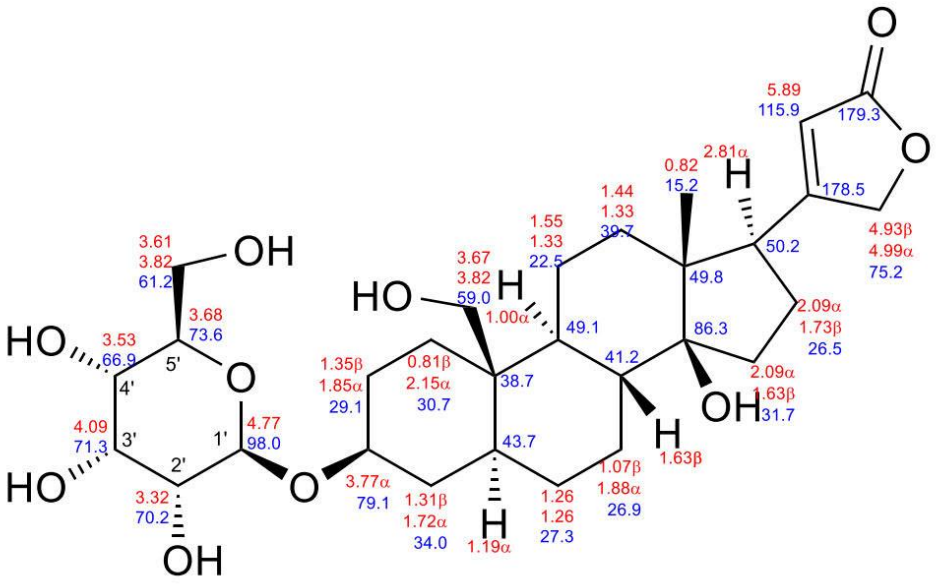


Figure S5: Compound **1**-NMR shift assignment



3β-(β-D-allopyranosyloxy)-14,19-dihydroxy-5α-card-20(22)-enolide

Figure S6. ^{1}H-NMR spectrum (500 MHz, D_2O) of compound **1**.

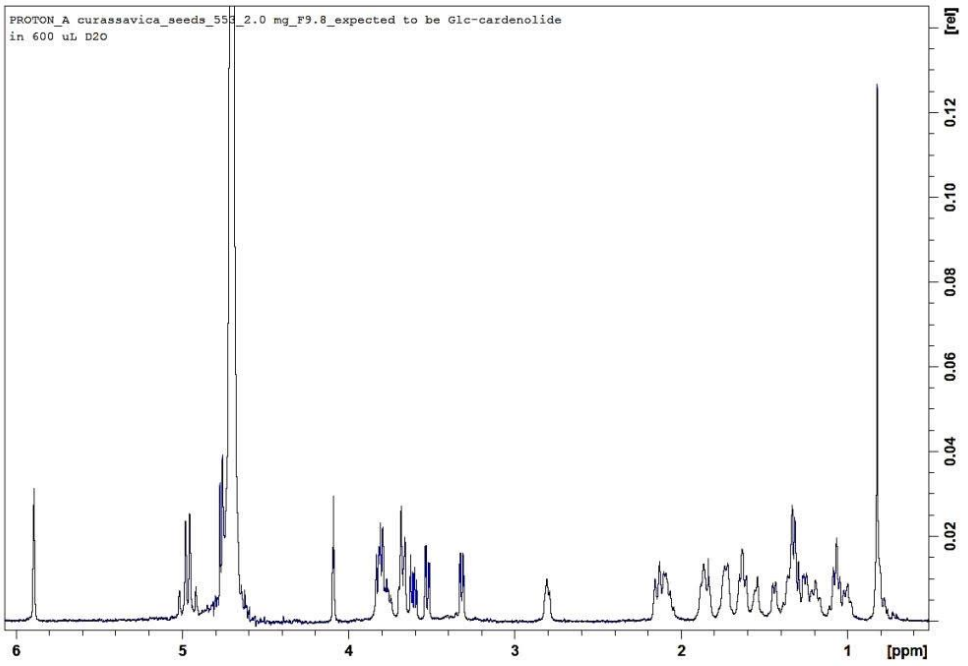


Figure S7. DEPTQ spectrum (125 MHz, D_2O) of compound **1**.

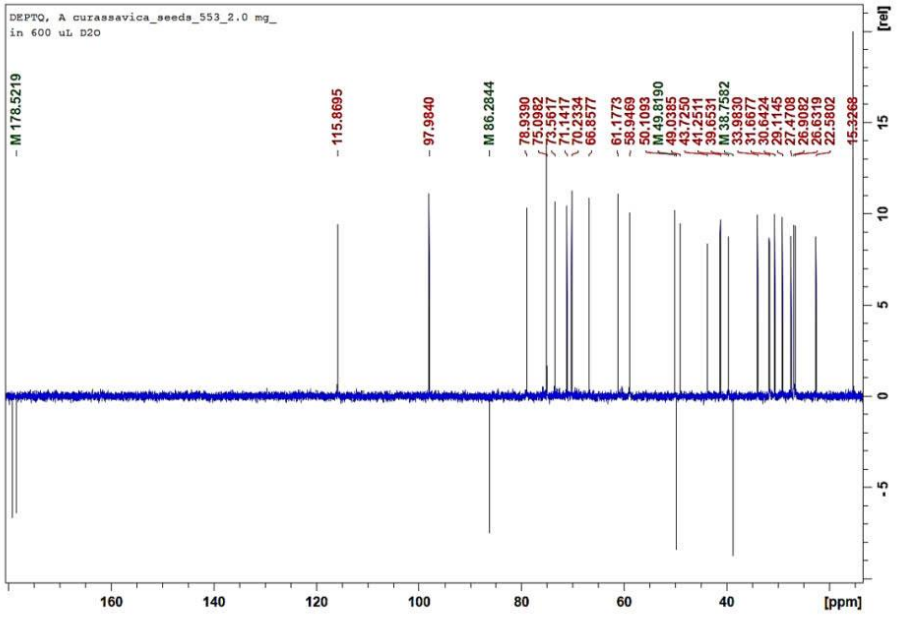


Figure S8. HSQC spectrum (500 MHz, D_2O) of compound **1.**

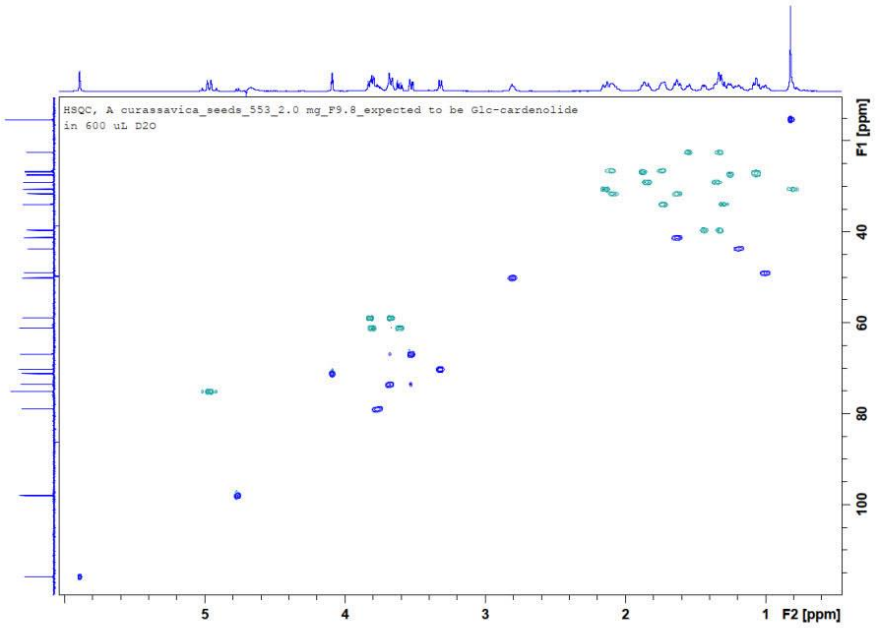


Figure S9. ^{1}H-^{1}H COSY spectrum (500 MHz, D_2O) of compound **1.**

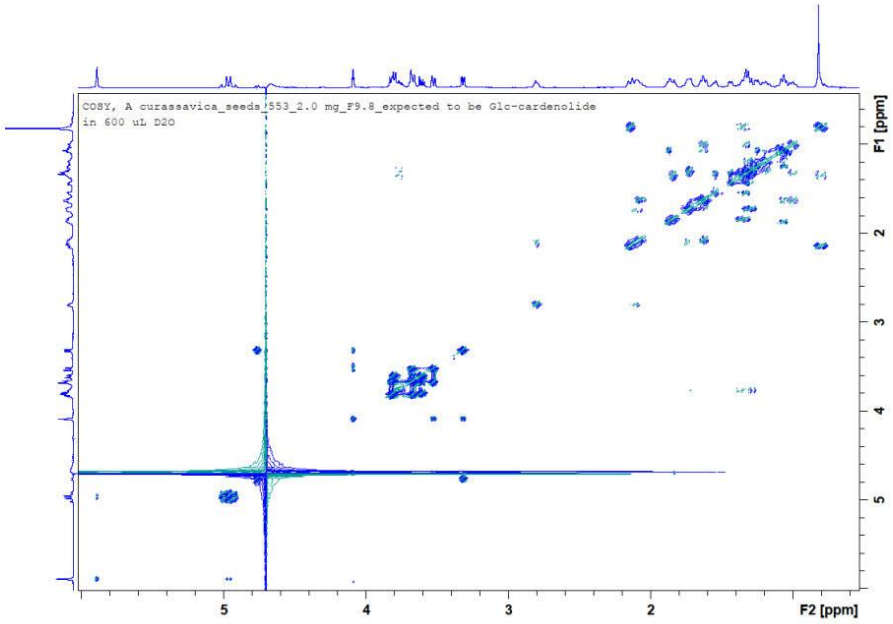


Figure S10. ^{1}H-^{13}C HMBC spectrum (500 MHz, D_2O) of compound **1.**

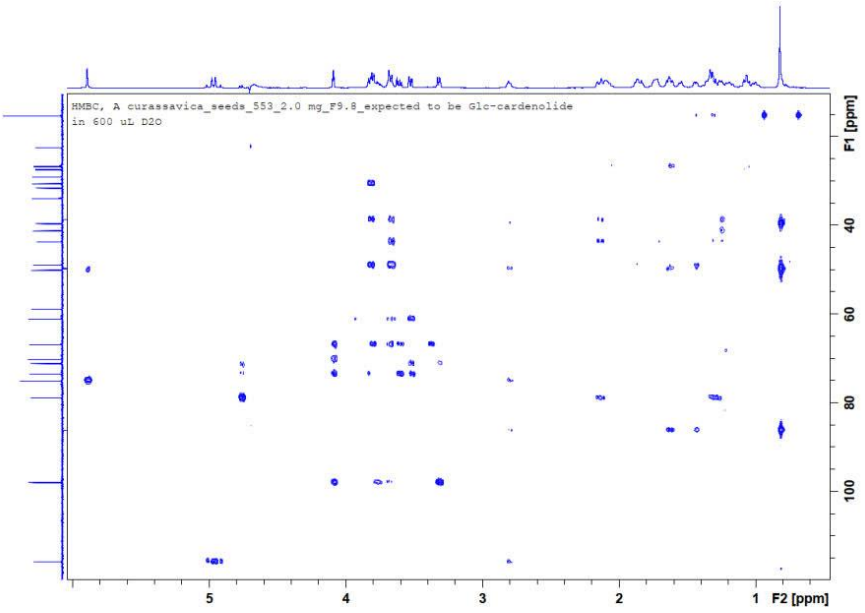


Figure S11. ROESY spectrum (500 MHz, D_2O) of compound **1.**

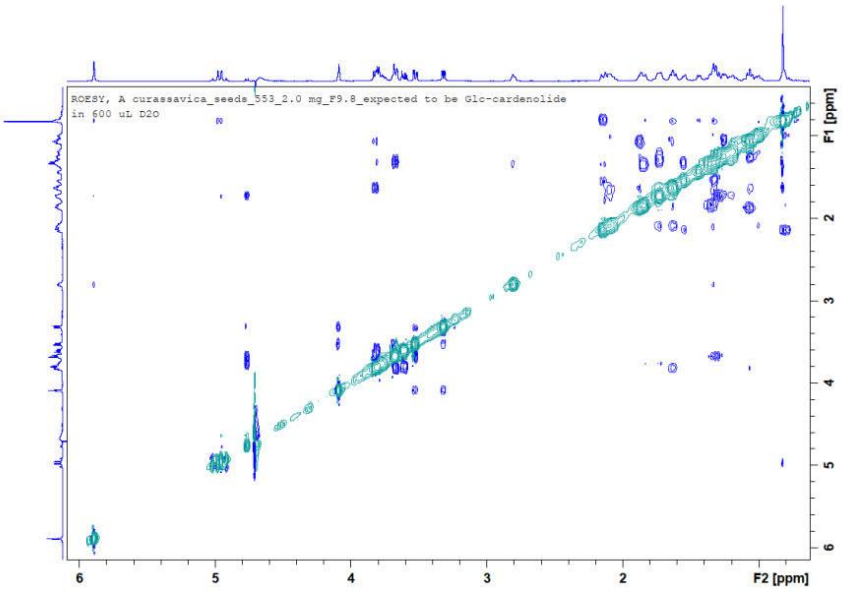


Figure S12. HSQC-TOCSY spectrum (500 MHz, D_2O) of compound **1.**

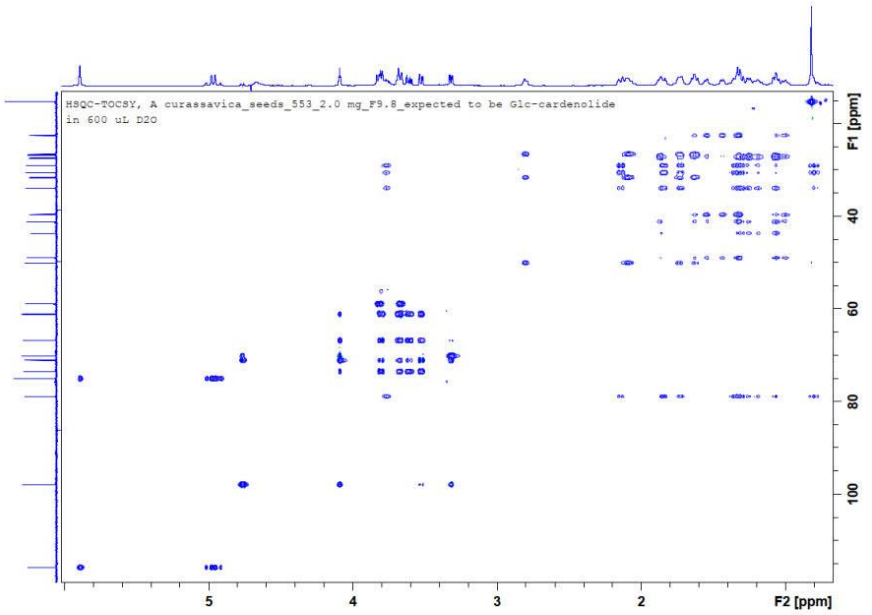


Figure S13. SELTOCSY spectrum (500 MHz, transmitter frequency at 3.609 ppm (H-6′), D_2O) of compound **1.**

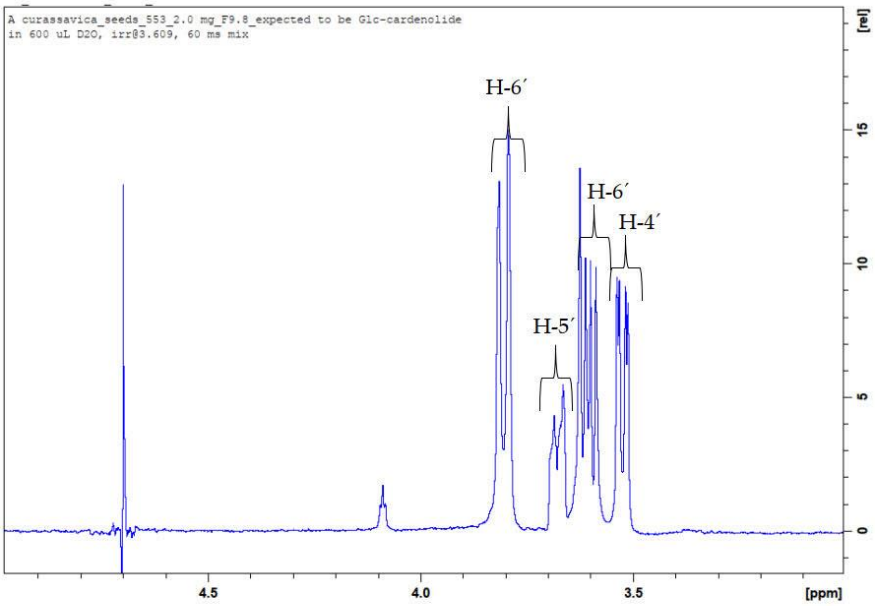


Figure S14. SELTOCSY spectrum (500 MHz, transmitter frequency at 3.765 ppm (H-3), D_2O) of compound **1.**

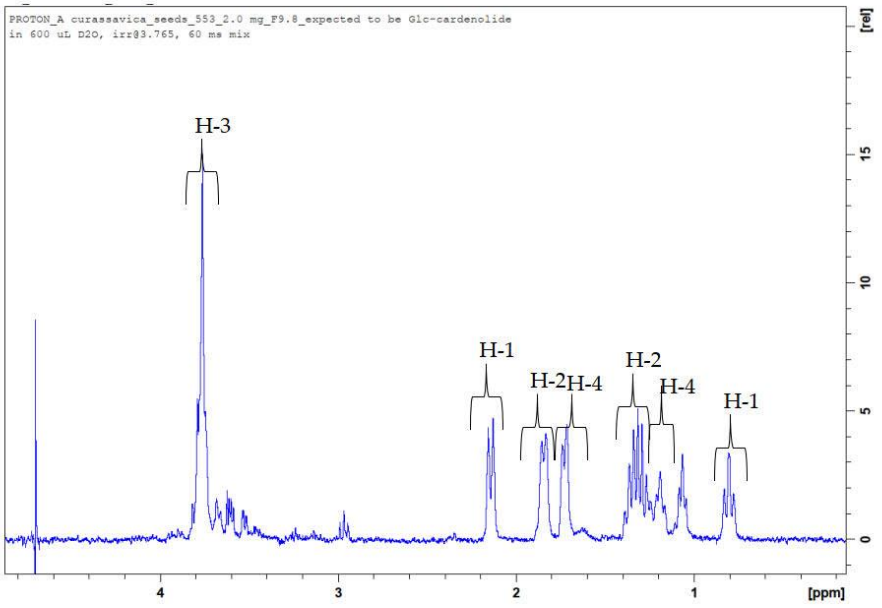


Figure S15: HR-ESI-MS spectrum of compound **2.**

Compound Spectrum List Report

Analysis Info

Acquisition Date 17/03/2022 23:50:28

Analysis Name \\filer\bird-lab\Users\Paola R-B\LCMS data\Asclepias extracts\715\alloglucoroglau 17.3.22 dilu 2_P1-D-4_01_239.d

Method paola_lcms_pos_checkuplc040221.m

Operator MPI Jena

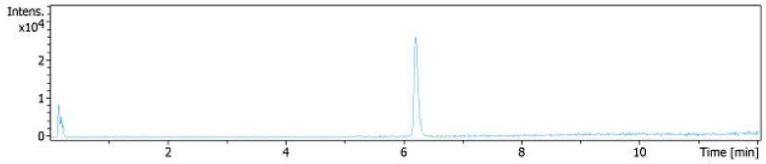
Sample Name alloglucoroglau 17.3.22 dilu 2

Instrument compact 8255754.20124

Comment

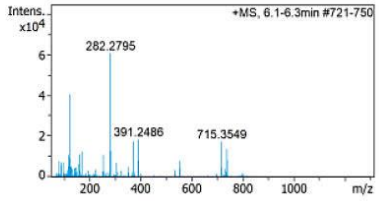
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



#	RT [min]	Area	Int. Type	I	S/N	Trace	Max. m/z	FWHM [min]
5	6.2	0		60745		EIC 715.3553 +All MS	282.2795	

Cmpd 5, 6.2 min



#	m/z	Res.	S/N	I	I %	FWHM
1	123.0556	9465	576383.1	40302	66.3	0.0130
2	163.1235	9869	159008.9	11119	18.3	0.0165
3	173.0790	9853	178719.4	12497	20.6	0.0176
4	282.2795	10774	868709.9	60745	100.0	0.0262
5	283.2826	10561	177397.7	12405	20.4	0.0268
6	284.2947	10235	183544.4	12834	21.1	0.0278
7	373.2380	10655	249696.2	17460	28.7	0.0350
8	391.2486	10880	264242.6	18477	30.4	0.0360
9	715.3549	11565	250492.6	17516	28.8	0.0619
10	737.3366	11616	196734.8	13757	22.6	0.0635

Figure S16. UV spectrum of compound **2.**

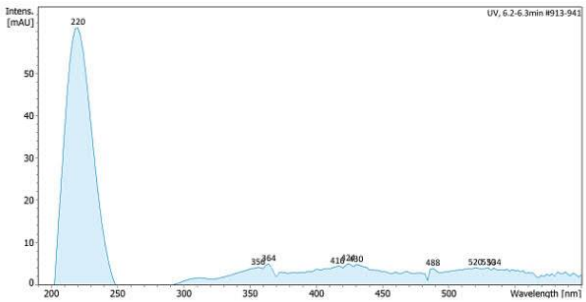
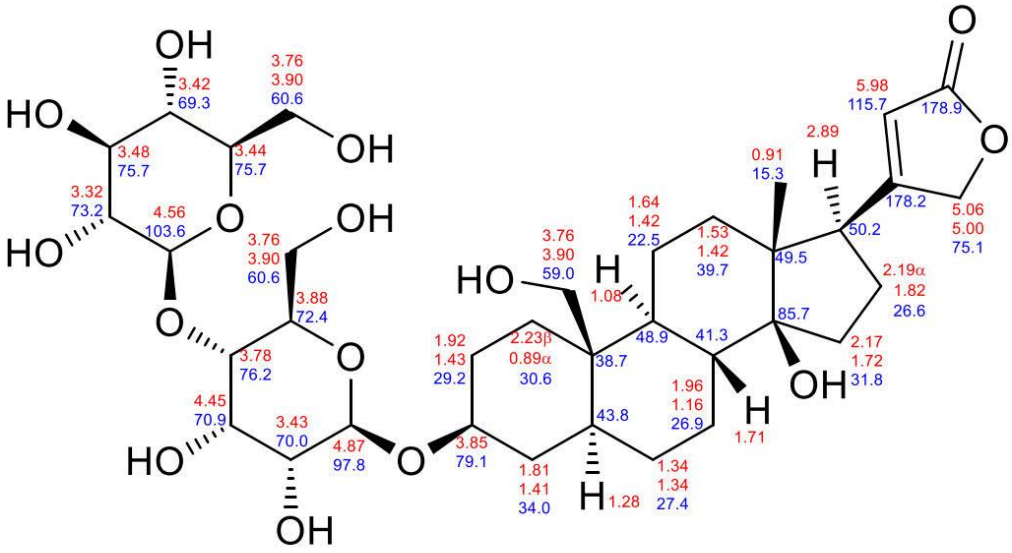


Figure S17: Compound **2**-NMR shift assignment



3β-[(4-O-β-D-glucopyranosyl-β-D-allopyranosyl)oxy]-14,19-dihydroxy-5α-card-20(22)-enolide

Figure S18. ^{1}H-NMR spectrum (500 MHz, D_2O) of compound **2**.

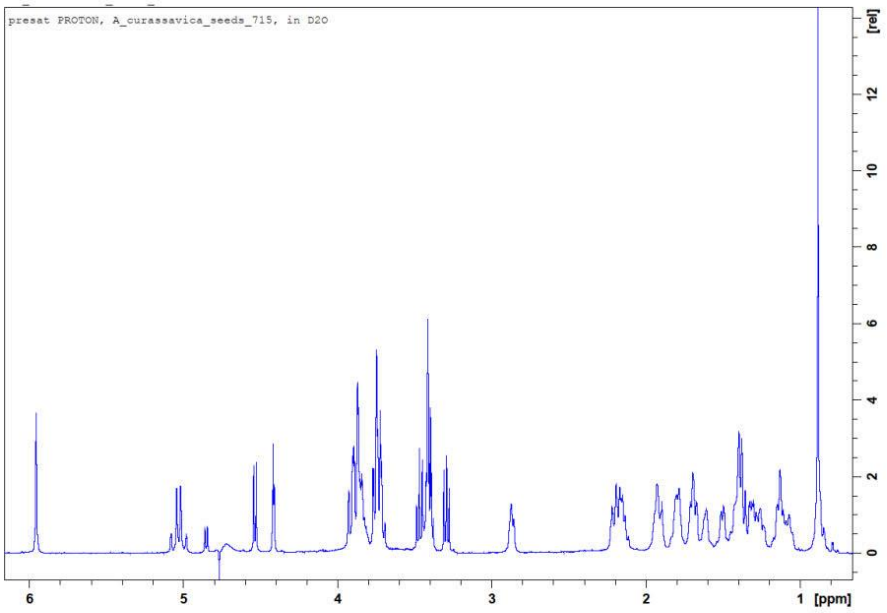


Figure S19. DEPTQ spectrum (125 MHz, D_2O) of compound **2**.

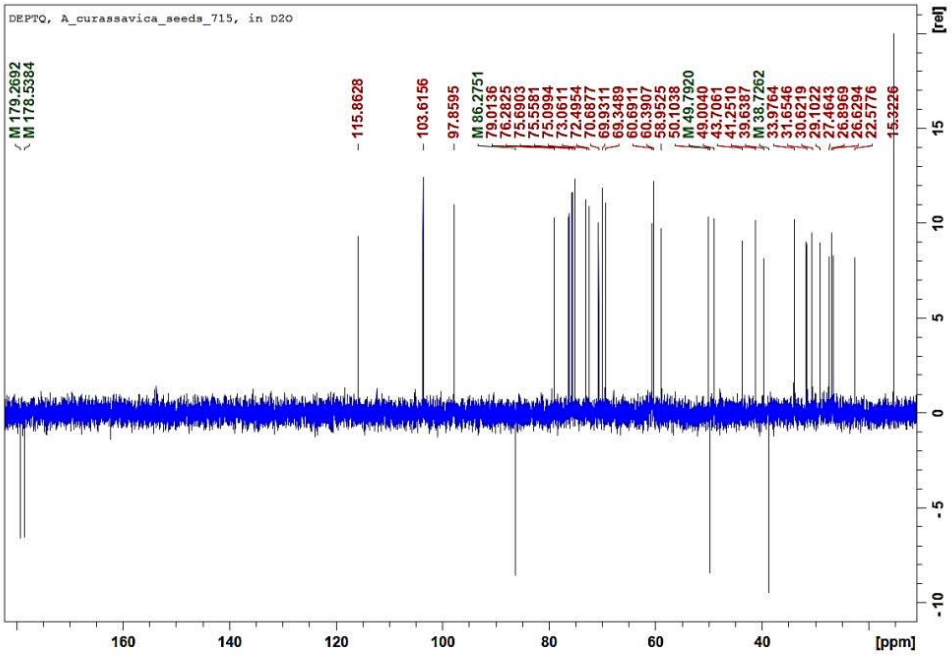


Figure S20. HSQC spectrum (500 MHz, D_2O) of compound **2.**

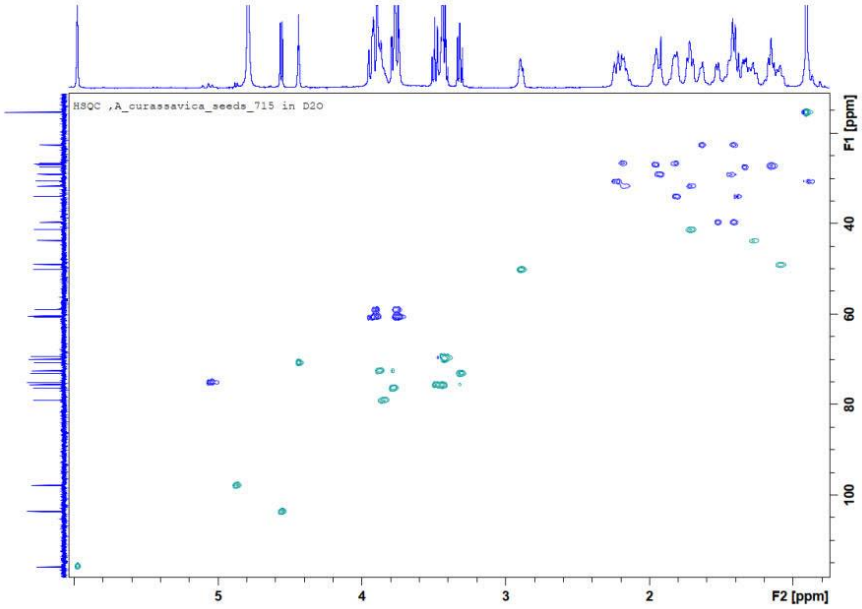


Figure S21. ^1H-^1H COSY spectrum (500 MHz, D_2O) of compound **2.**

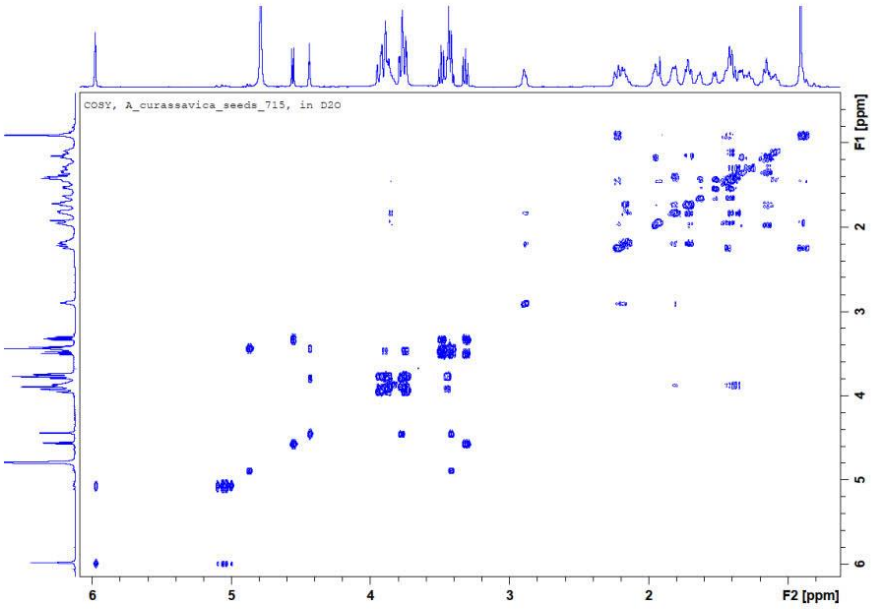


Figure S22. ^{1}H-^{13}C HMBC spectrum (500 MHz, D_2O) of compound **2.**

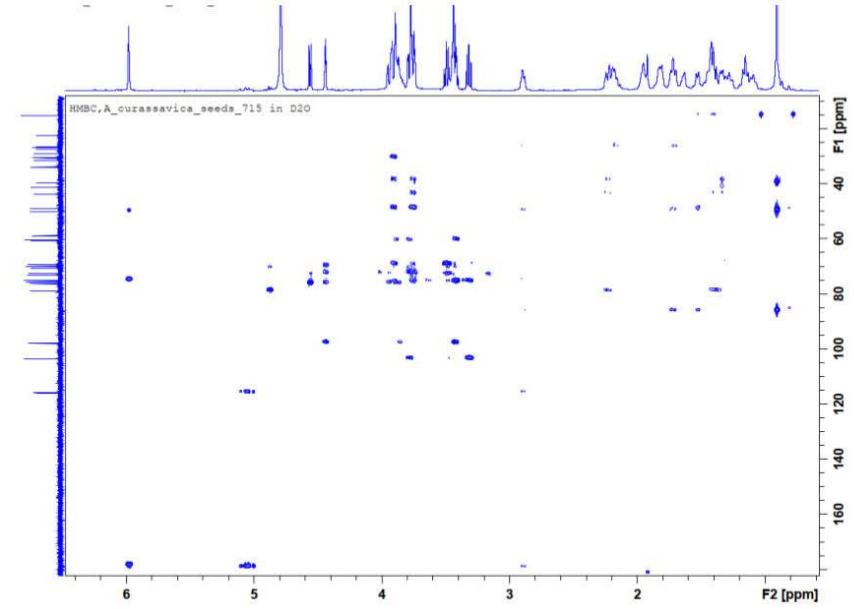


Figure S23. ROESY spectrum (500 MHz, D_2O) of compound **2.**

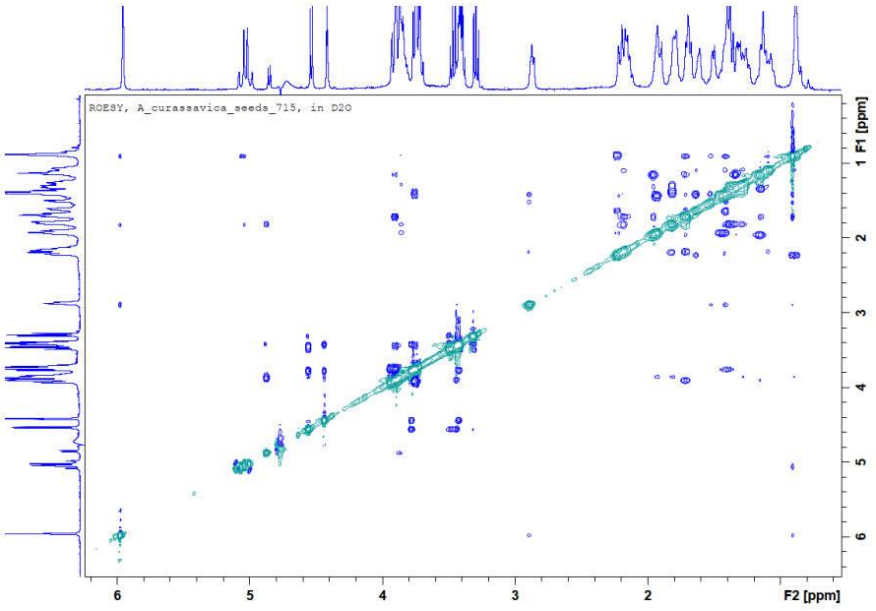


Figure S24. HSQC-TOCSY spectrum (500 MHz, D_2O) of compound **2.**

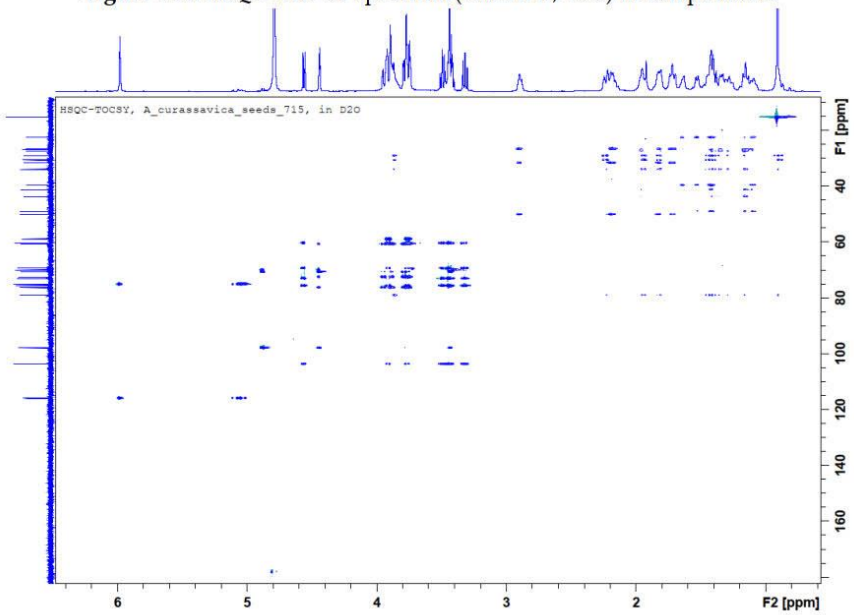


Figure S25. SELTOCSY spectrum (500 MHz, transmitter frequency at 4.87 ppm (H-1′), D_2O) of compound **2.**

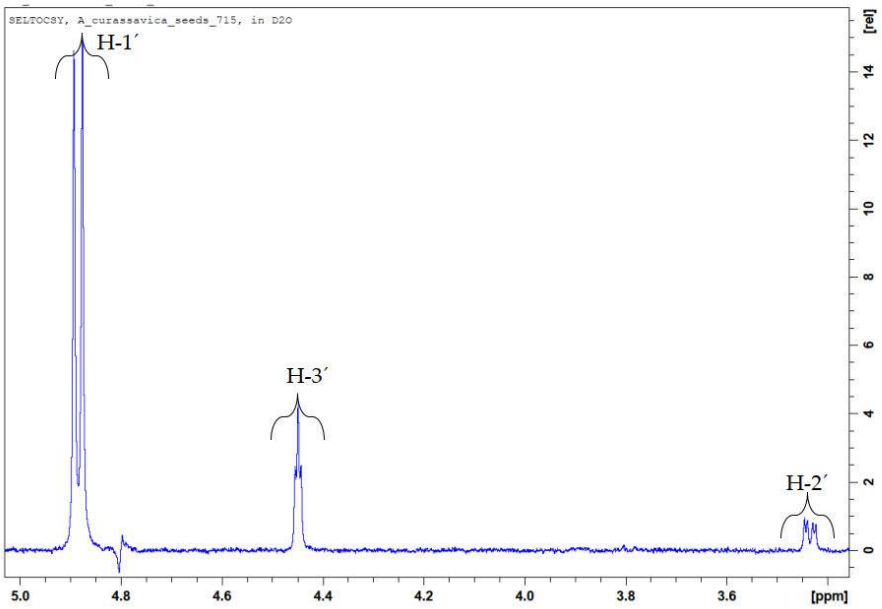


Figure S26. SELTOCSY spectrum (500 MHz, transmitter frequency at 4.45 ppm (H-1′′), D_2O) of compound **2.**

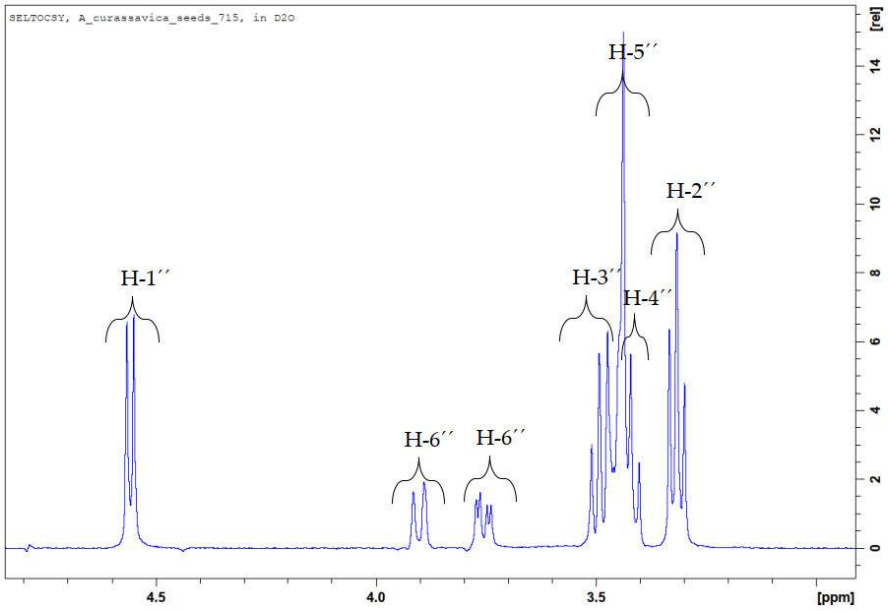


Figure S27: HR-ESI-MS spectrum of compound **3.**

Compound Spectrum List Report

Analysis Info

Acquisition Date 18/03/2022 03:06:11

Analysis Name \\filer\bird-lab\Users\Paola R-B\LCMS data\Asclepias extracts\693\OHglucocalotrop 17.3.22 dilu 2_P1-E-5_01_250.d

Method paola_lcms_pos_checkuplc040221.m

Operator MPI Jena

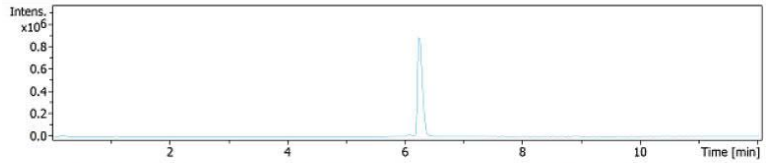
Sample Name OHglucocalotrop 17.3.22 dilu 2

Instrument compact 8255754.20124

Comment

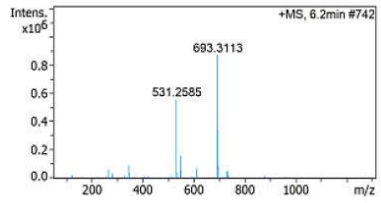
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



#	RT [min]	Area	Int. Type	I	S/N	Trace	Max. m/z	FWHM [min]
1	6.2	0		870479		EIC 693.3114 +All MS	693.3113	

Cmpd 1, 6.2 min



#	m/z	Res.	S/N	I	I %	FWHM
1	266.1326	11730	491.6	60963	7.0	0.0227
2	347.1585	12481	741.5	91945	10.6	0.0278
3	531.2585	11453	4492.6	557083	64.0	0.0464
4	532.2620	11322	1450.9	179915	20.7	0.0470
5	549.2693	11554	1278.0	158466	18.2	0.0475
6	612.2844	12184	550.4	68246	7.8	0.0503
7	693.3113	11752	7020.0	870479	100.0	0.0590
8	694.3145	11731	2766.1	342992	39.4	0.0592
9	695.3174	11857	728.1	90286	10.4	0.0586
10	733.3048	11744	426.0	52824	6.1	0.0624

Figure S28: UV spectrum of compound **3**.

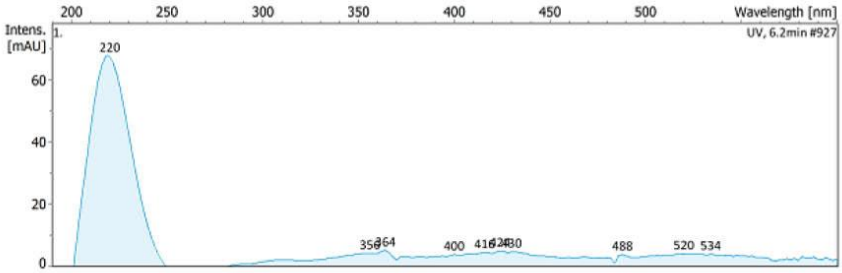
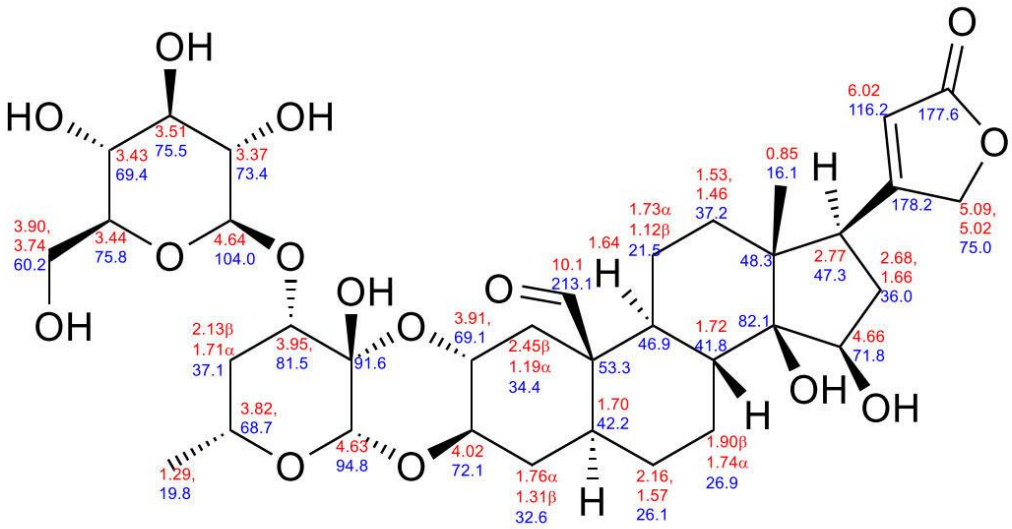


Figure S29: Compound **3**-NMR shift assignment



(1*R*,3*R*,3a*S*,3b*R*,6a*R*,7a*S*,9*R*,11*S*,11a*S*,12a*R*,13a*R*,13b*S*,15a*R*)-13a-formyl-3,3a,11a-trihydroxy-9,15a-dimethyl-1-(5-oxo-2,5-dihydrofuran-3-yl)icosahydro-1*H*,7a*H*-cyclopenta[7,8]phenanthro[2,3*b*]pyrano[3,2-*e*][1,4]dioxin-11-yl β-L-glucopyranoside

Figure S30. ^{1}H-NMR spectrum (500 MHz, D_2O) of compound **3**.

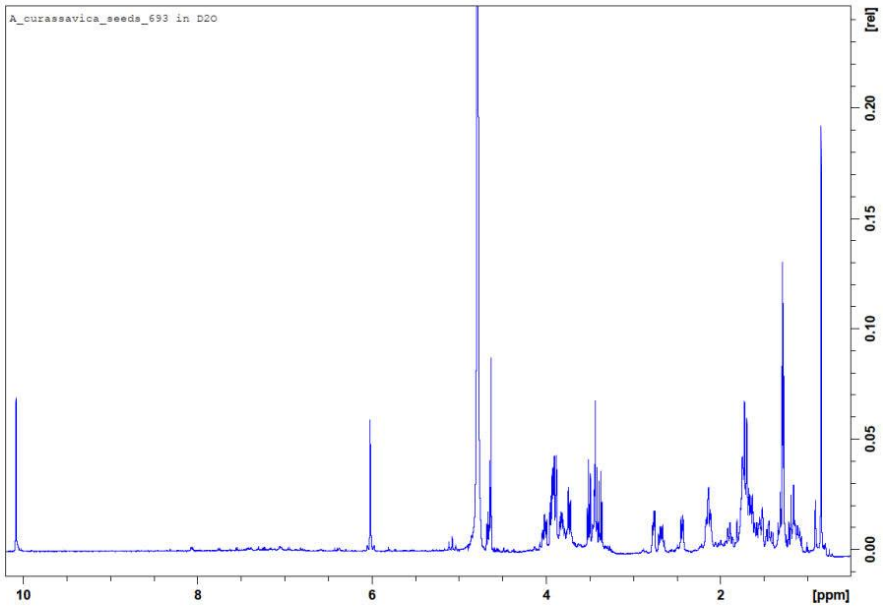


Figure S31. DEPTQ spectrum (125 MHz, D_2O) of compound **3**.

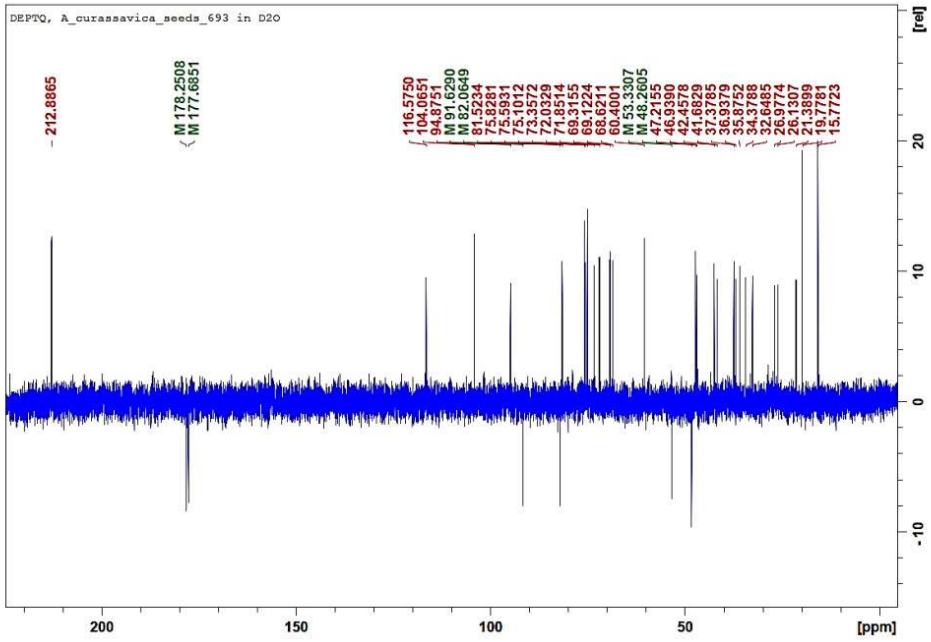


Figure S32. HSQC spectrum (500 MHz, D_2O) of compound **3.**

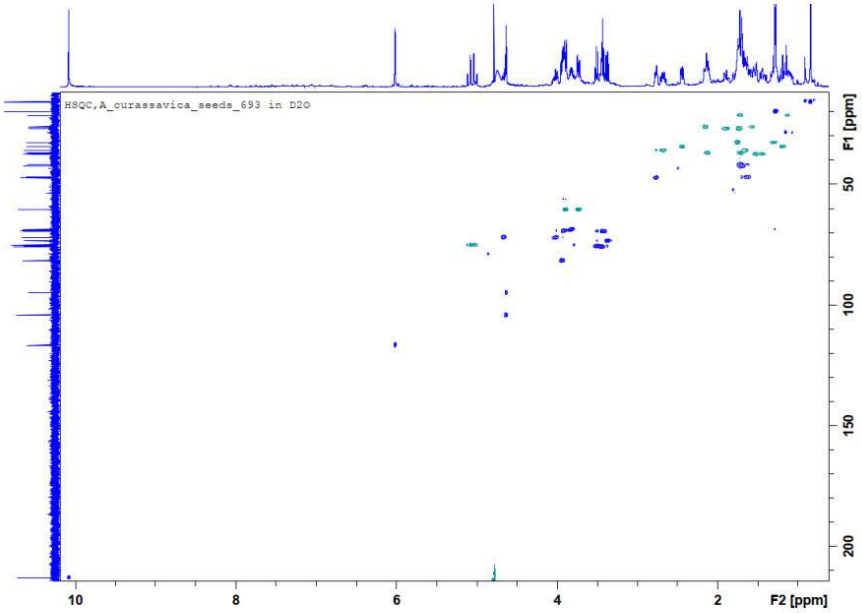


Figure S33. ^1H-^1H COSY spectrum (500 MHz, D_2O) of compound **3.**

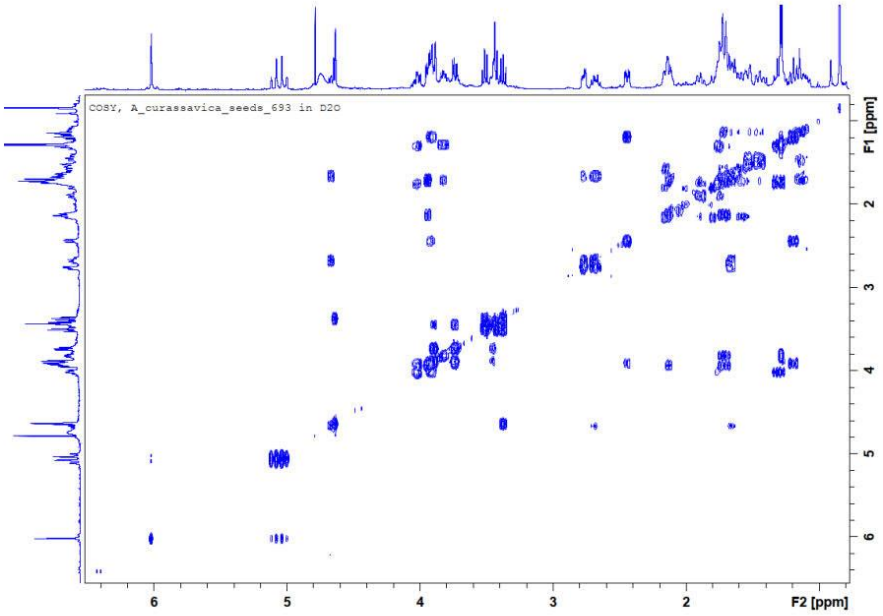


Figure S34. ^{1}H-^{13}C HMBC spectrum (500 MHz, D_2O) of compound **3.**

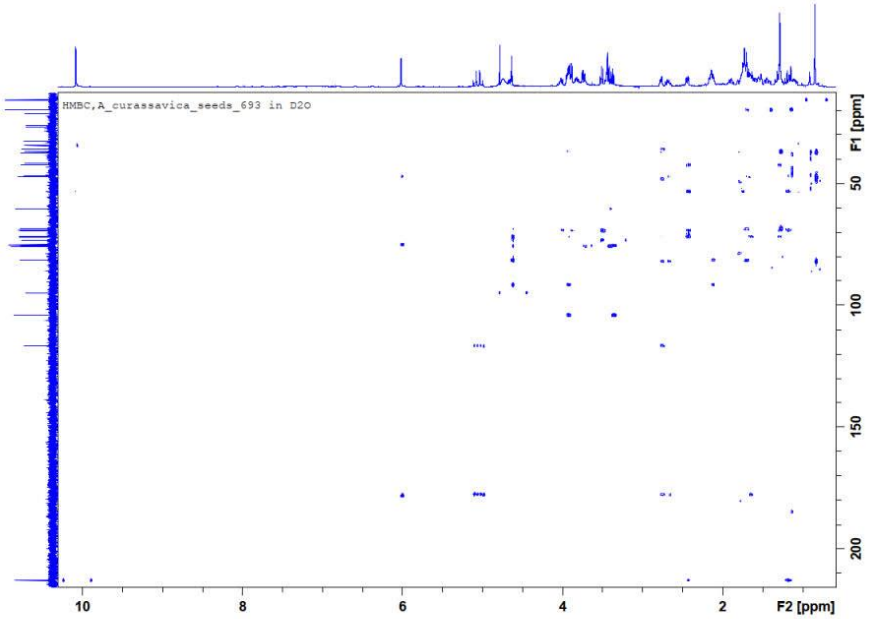


Figure S35. ROESY spectrum (500 MHz, D_2O) of compound **3.**

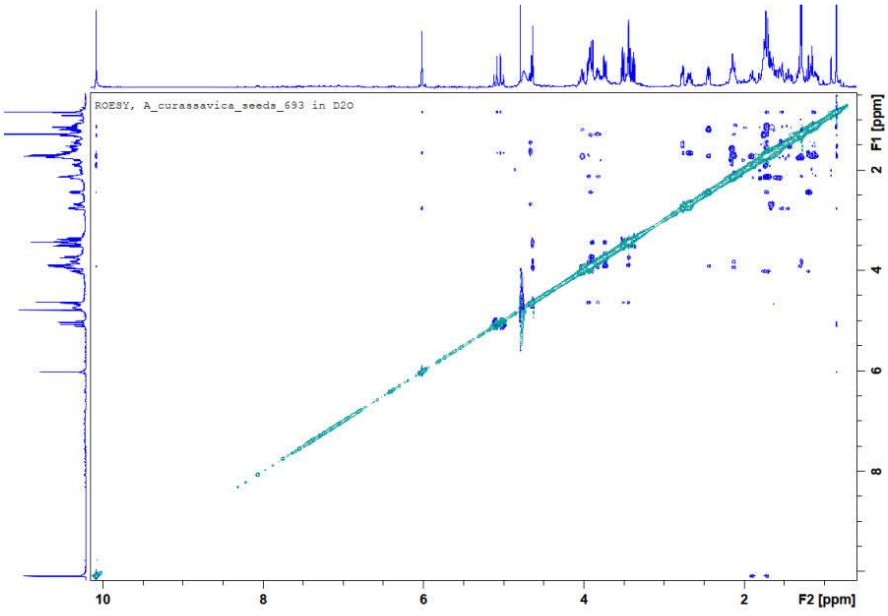


Figure S36. HSQC-TOCSY spectrum (500 MHz, D_2O) of compound **3.**

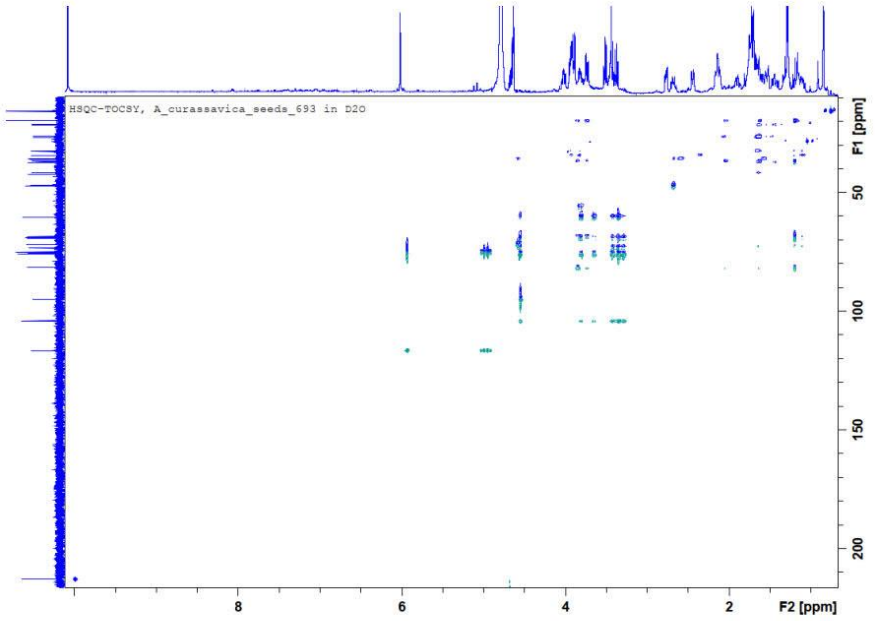
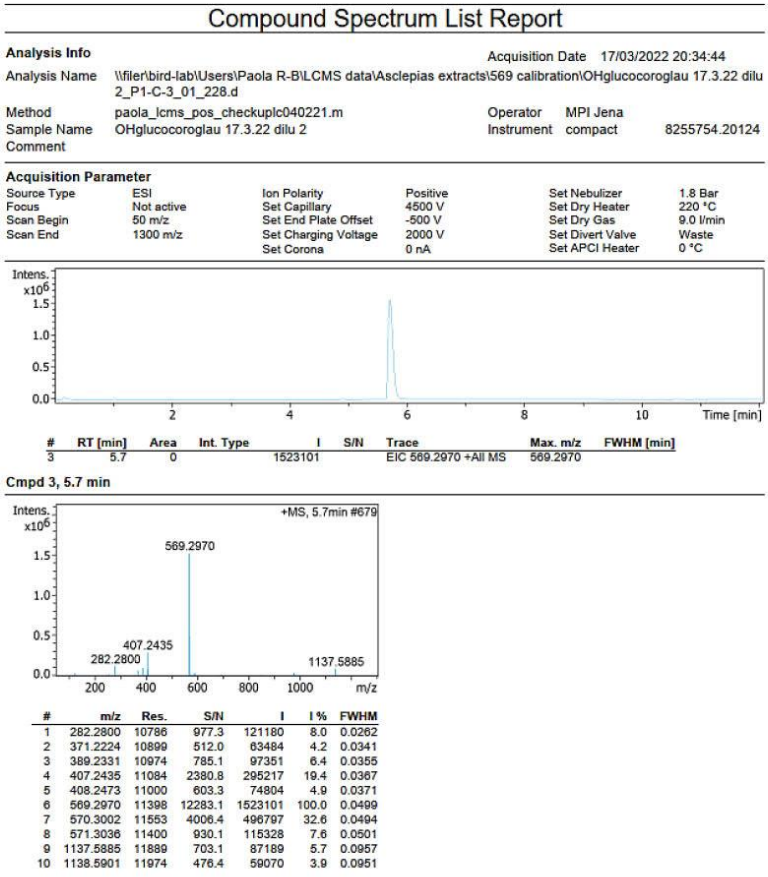


Figure S37: HR-ESI-MS spectrum of compound **4**.



Compound Spectrum List Report

Analysis Info

Acquisition Date 17/03/2022 20:34:44

Analysis Name \\filer\bird-lab\Users\Paola R-B\LCMS data\Asclepias extracts\569 calibration\OHglucocoroglau 17.3.22 dilu 2_P1-C-3_01_228.d

Method paola_lcms_pos_checkuplc040221.m

Operator MPI Jena

Sample Name OHglucocoroglau 17.3.22 dilu 2

Instrument compact 8255754.20124

Comment

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C

#	RT [min]	Area	Int. Type	I	S/N	Trace	Max. m/z	FWHM [min]
3	5.7	0		1523101		EIC 569.2970 +All MS	569.2970	

Cmpd 3, 5.7 min

#	m/z	Res.	S/N	I	I %	FWHM
1	282.2800	10786	977.3	121180	8.0	0.0262
2	371.2224	10899	512.0	63484	4.2	0.0341
3	389.2331	10974	785.1	97351	6.4	0.0355
4	407.2435	11084	2380.8	295217	19.4	0.0367
5	408.2473	11000	603.3	74804	4.9	0.0371
6	569.2970	11398	12283.1	1523101	100.0	0.0499
7	570.3002	11553	4006.4	496797	32.6	0.0494
8	571.3036	11400	930.1	115328	7.6	0.0501
9	1137.5885	11889	703.1	87189	5.7	0.0957
10	1138.5901	11974	476.4	59070	3.9	0.0951

Figure S38: UV spectrum of compound **4.**

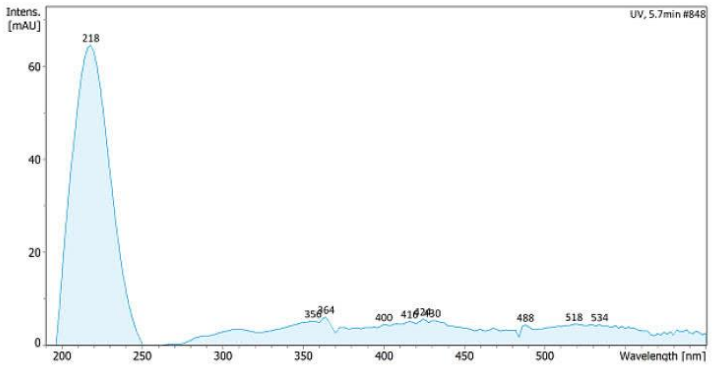
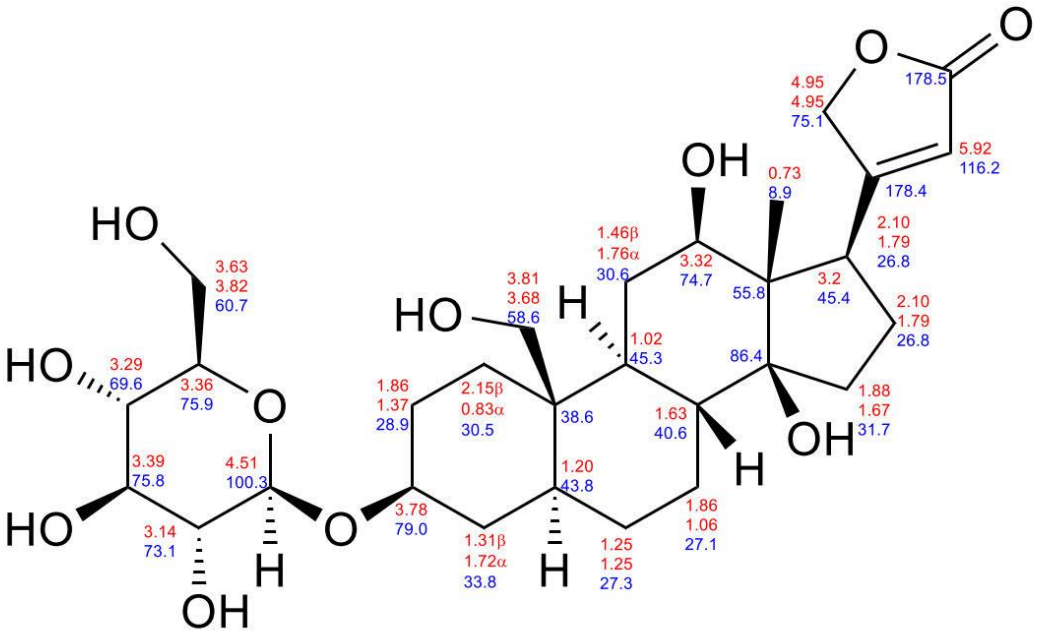


Figure S39: Compound **4**-NMR shift assignment



3β-(β-D-glucopyranosyloxy)-12β,14,19-trihydroxy-5α-card-20(22)-enolide

Figure S40. ^{1}H-NMR spectrum (500 MHz, D_2O) of compound **4**.

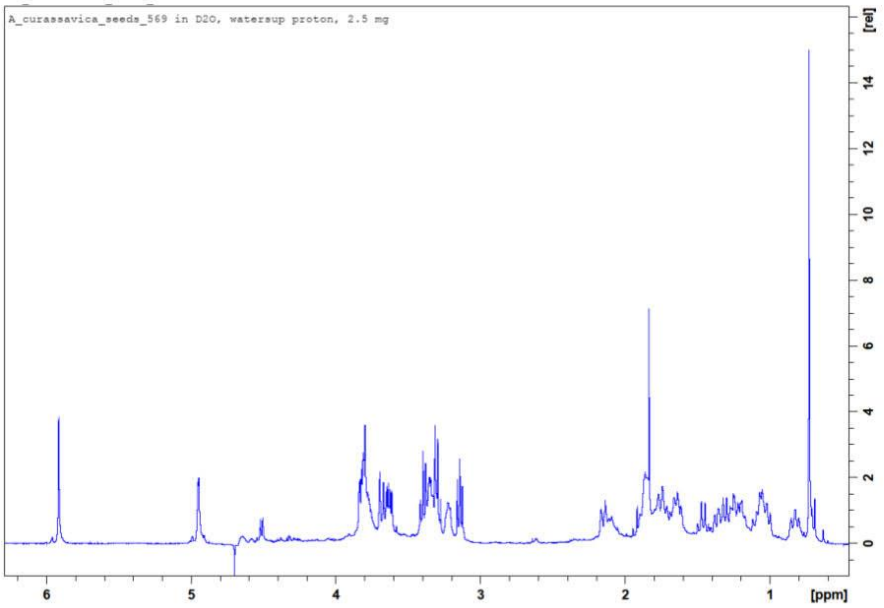


Figure S41. APT spectrum (125 MHz, D_2O) of compound **4**.

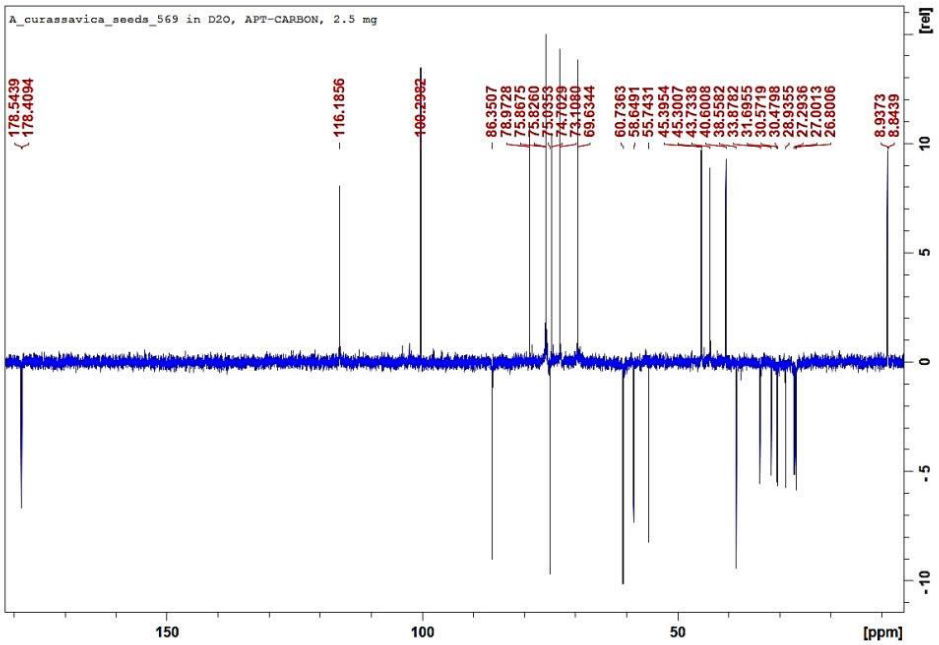


Figure S42. HSQC spectrum (500 MHz, D_2O) of compound **4**.

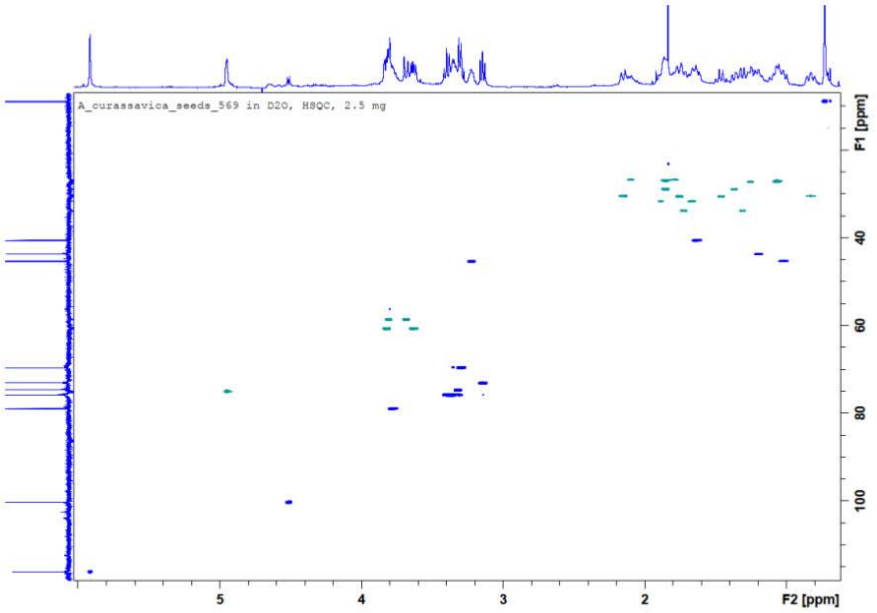


Figure S43. ^1H-^1H COSY spectrum (500 MHz, D_2O) of compound **4**.

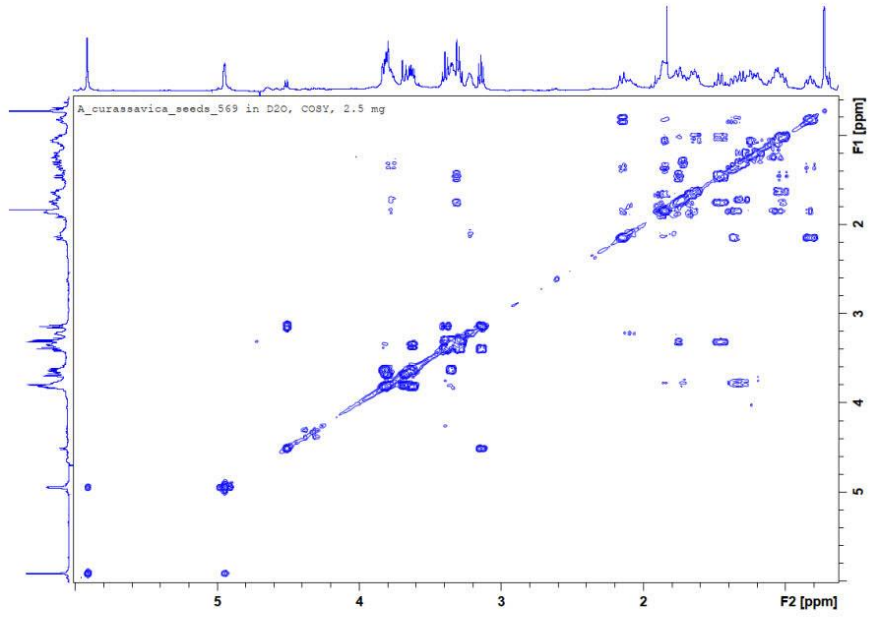


Figure S44. ^{1}H-^{13}C HMBC spectrum (500 MHz, D_2O) of compound **4**.

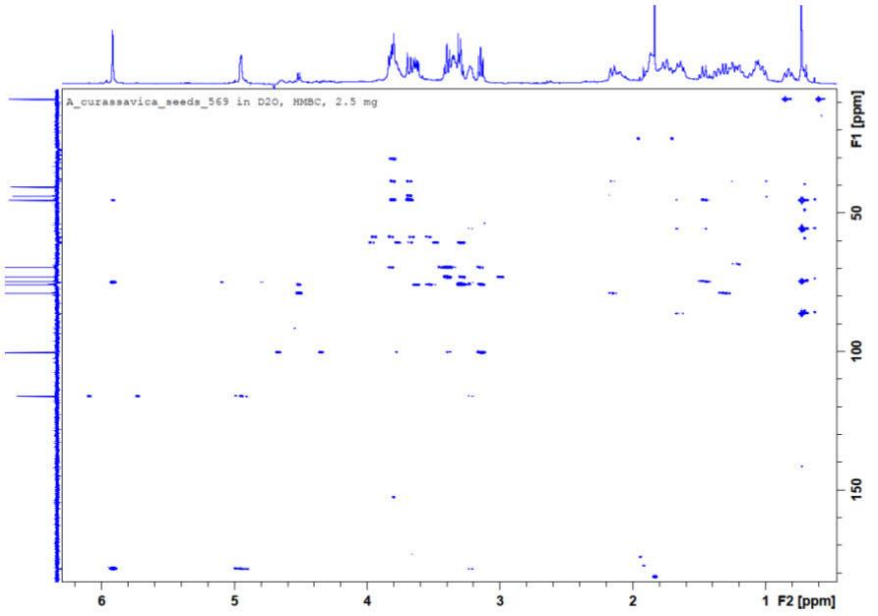


Figure S45. ROESY spectrum (500 MHz, D_2O) of compound **4**.

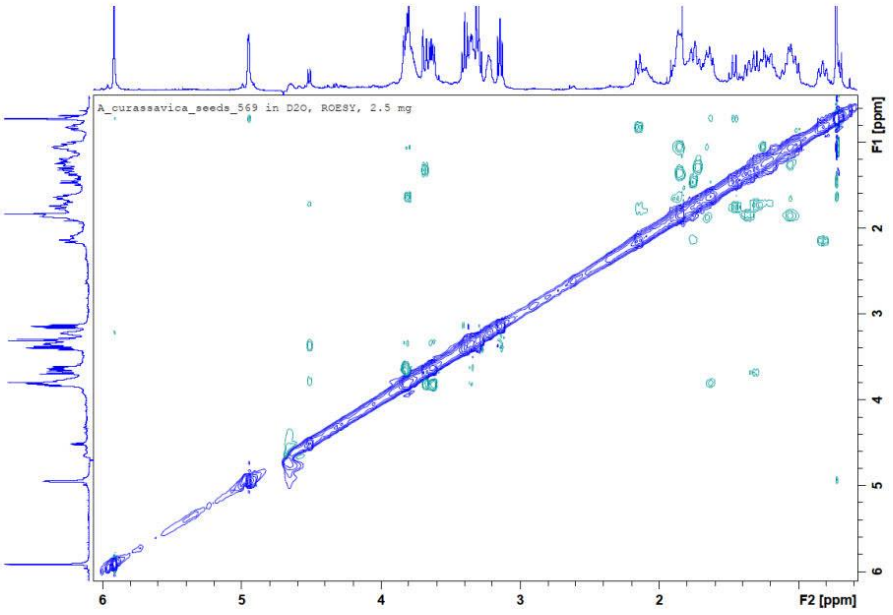


Figure S46. HSQC-TOCSY spectrum (500 MHz, D_2O) of compound **4.**

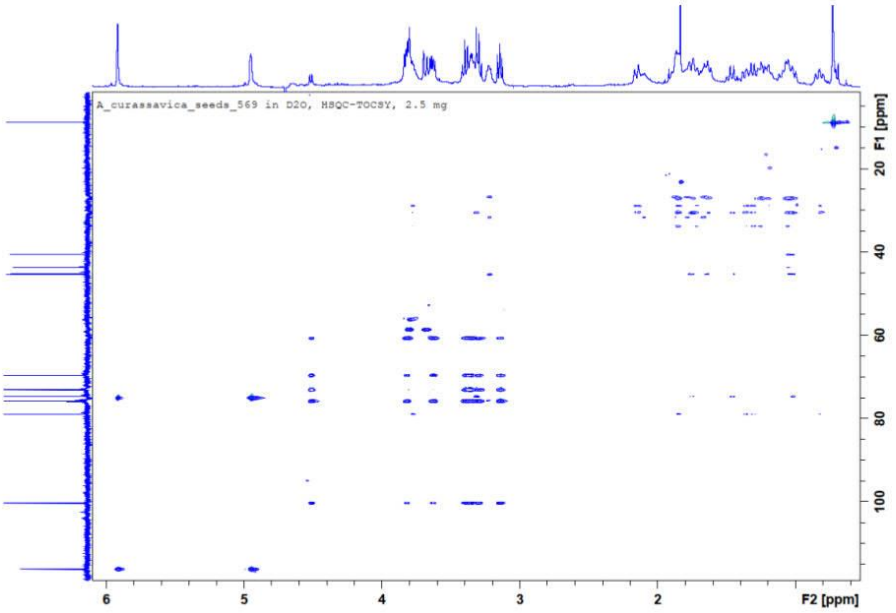


Figure S47. SELTOCSY spectrum (500 MHz, transmitter frequency at 4.514 ppm, D_2O) of compound **4.**

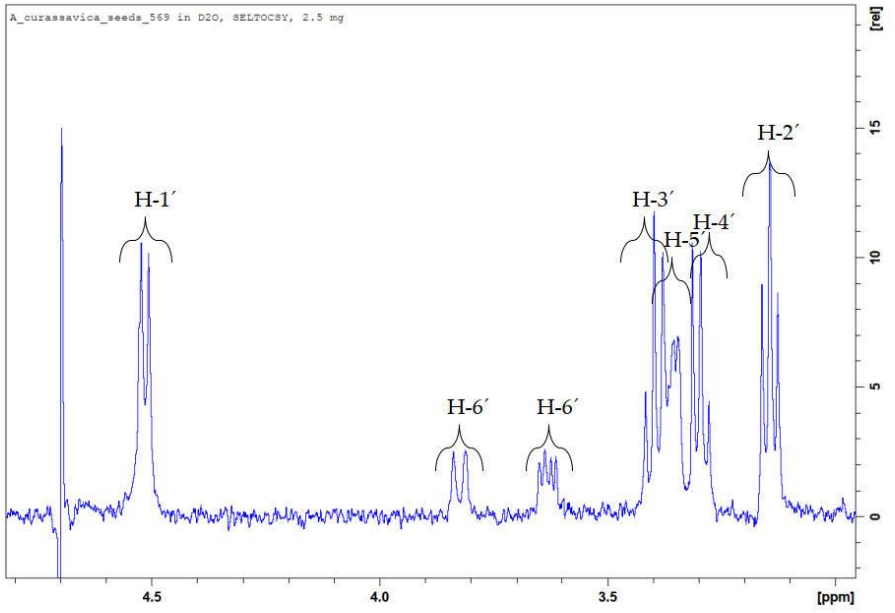


Figure S48. SELNOESY spectrum (500 MHz, transmitter frequency at 3.764 ppm (H-3), D_2O) of compound **4**.

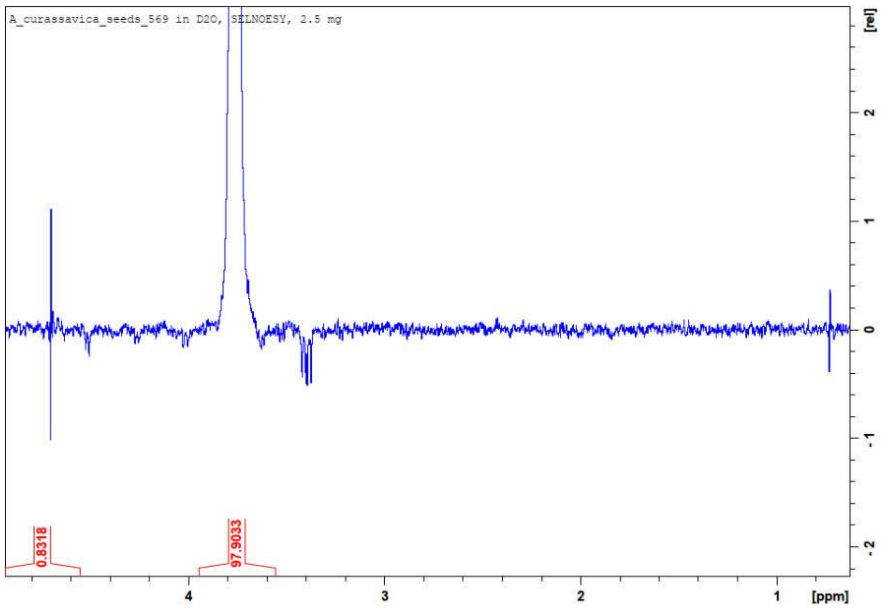


Figure S49. SELNOESY spectrum (500 MHz, transmitter frequency at 3.223 ppm (H-17), D_2O) of compound **4**.

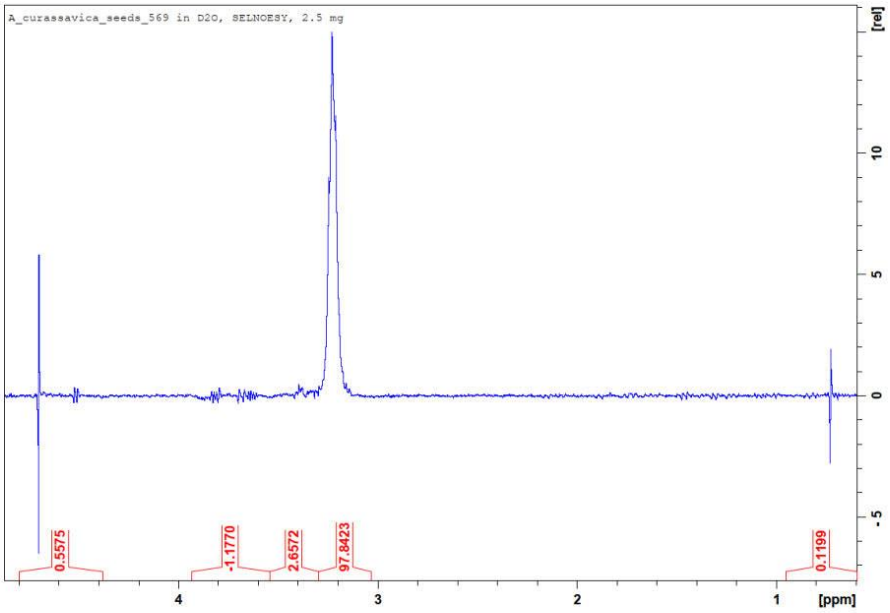


Table S2: Cardenolides quantification (mg/g of seeds)

Compound	Concentration (mg /g of seeds)
1	0.014 (+/- 0.0062)
2	0.063 (+/- 0.0038)
3	0.053 (+/- 0.0029)
4	0.004 (+/- 0.0009)
5	4.503 (+/- 0.2630)
6	2.065 (+/- 0.1930)
7	0.733 (+/- 0.0724)
8	0.810 (+/- 0.1570)
9	0.275 (+/- 0.1040)
10	0.457 (+/- 0.3020)

Figure S50: Inhibition curves of *Sus domesticus* Na^+/K^+ ATPase by compounds **1**,**2**, **5**—**10** and ouabain

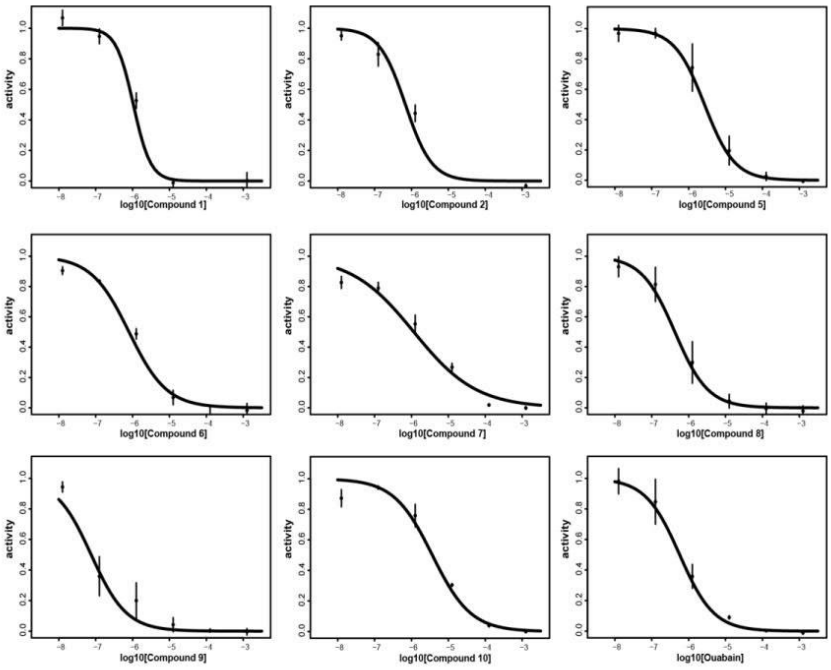


Table S3: IC_{50} values (μM) of compounds **1**,**2**,**5**—**10** and ouabain against porcine Na^+/K^+ ATPase.

Compound	IC_{50} [μM]
1	1.01×10^{-6} (+/- 1.20×10^{-8})
2	7.42×10^{-7} (+/- 2.60×10^{-8})
5	2.98×10^{-6} (+/- 1.69×10^{-7})
6	8.09×10^{-7} (+/- 8.00×10^{-9})
7	1.20×10^{-6} (+/- 3.6×10^{-8})
8	4.72×10^{-7} (+/- 3.00×10^{-8})
9	9.65×10^{-8} (+/- 6.00×10^{-9})
10	3.67×10^{-6} (+/- 7.00×10^{-8})
Ouabain	4.48×10^{-7} (+/- 1.1×10^{-8})

Table S4: Comparison of IC_{50} values between compounds **1**,**2**,**5**—**10** along with ouabain, p-values adjusted with Bonferroni method.

	1	**2**	**5**	**6**	**7**	**8**	**9**	**10**	**Ouabain**
1		1.000 ns	0.347 ns	1.000 ns	1.000 ns	0.651 ns	**<0.001** ****	**0.046** *	1.000 ns
2			**0.035** *	1.000 ns	1.000 ns	1.000 ns	**<0.001** ***	**0.005** **	1.000 ns
5				0.083 ns	0.858 ns	**0.001** **	**<0.001** ****	1.000 ns	**0.002** **
6					1.000 ns	1.000 ns	**<0.001** ****	**0.011** *	1.000 ns
7						0.260 ns	**<0.001** ****	0.119 ns	0.537 ns
8							**0.005** **	**<0.001** ****	1.000 ns
9								**<0.001** ****	**0.002** **
10									**<0.001** ***

2.2 Differential accumulation of cardenolides from *Asclepias curassavica* by large milkweed bugs does not correspond to availability in seeds or biological activity on the bug Na^+/K^+—ATPase

Rubiano-Buitrago P, Pradhan S, Grabe V, Aceves-Aparicio A, Paetz C and Rowland HM. (2023) *Front. Ecol. Evol.* 11:1175205. doi: 10.3389/fevo.2023.1175205

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doi: 10.3389/fevo.2023.1175205

Differential accumulation of cardenolides from *Asclepias curassavica* by large milkweed bugs does not correspond to availability in seeds or biological activity on the bug Na^+/K^+-ATPase

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[1]Research Group Predators and Toxic Prey, Max Planck Institute for Chemical Ecology, Jena, Germany, [2]Research Group Biosynthesis/NMR, Max Planck Institute for Chemical Ecology, Jena, Germany, [3]Microscopic Imaging Service Group, Max Planck Institute for Chemical Ecology, Jena, Germany

Milkweed–herbivore systems are characterized by cardenolide chemical defenses and specialized herbivore adaptations such as physiological target site insensitivity. Cardenolide defenses in milkweeds can vary in terms of the total concentration, differences in the polarity of individual cardenolides, and the substitution of the steroidal structures that can contribute to the molecule's reactivity. The variability in cardenolide defenses could represent the plant's response to natural selection and adaptation of resistant herbivores and is a characteristic of phenotype-matching between defensive and offensive traits resulting from coevolution. Here, we test the phenotypic match of the cardenolide composition of seeds of *Asclepias curassavica* and those sequestered by nymphs and adults of the specialized seed herbivore *Oncopeltus fasciatus*, combined with tests of the inhibitory capacity of a subset of seed cardenolides against the Na^+/K^+-ATPase of *O. fasciatus* and a non-adapted insect (*Drosophila melanogaster*). We compare this with the inhibitory capacity against the highly sensitive porcine Na^+/K^+-ATPase. Among the five most abundant cardenolides present in milkweed seeds, glucopyranosyl frugoside, glucopyranosyl gofruside, and glucopyranosyl calotropin were significantly more abundant in the seeds than in the adults and nymphs; the bugs contained higher concentrations of the deglucosylated compounds. The most abundant compound, glucopyranosyl frugoside, was also the most inhibitory for *O. fasciatus*, but *O. fasciatus* was significantly more tolerant to all compounds compared to *D. melanogaster* and the highly sensitive porcine enzyme. Our results add to the evidence that *O. fasciatus* sequesters specific individual cardenolides from its *Asclepias* host plants that are not directly linked to the concentration and inhibitory potency.

KEYWORDS

***Oncopeltus fasciatus*, cardiac glycoside, phytochemical diversity, structure–activity relationship, toxin–receptor interactions, resistance**

1. Introduction

Insects can impose natural selection for chemical defenses in the plant tissues on which they feed, leading to phenotype-matching between defensive and offensive traits that may have resulted from coevolution (Futuyma and Agrawal, 2009). Such biochemical coevolutionary arms races (Ehrlich and Raven, 1964) can drive evolutionary innovation and diversification (Mauricio and Rausher, 1997; Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003; Agrawal, 2005; Benderoth et al., 2006; Prasad et al., 2012). For example, the evolution of novel genes for glucosinolate detoxification in Pierid butterflies has been accompanied by duplication and neofunctionalization of defensive glucosinolate genes in Brassicaceae host plant species (Wheat et al., 2007; Sønderby et al., 2010; Edger et al., 2015; BlaŽević et al., 2020). Explaining the diversity of plant secondary metabolites and insect herbivore tolerance mechanisms observed in nature is of interest to evolutionary and chemical ecologists who seek to understand the origins of phytochemical diversity and the scope for coevolution in a community context (Berenbaum et al., 1991; Jones and Firn, 1991; Berenbaum and Zangerl, 1996; Richards et al., 2015).

Many plants simultaneously produce multiple secondary metabolites of the same class (Dyer et al., 2018). One explanation for this is that across populations, different herbivores can select for specific defensive traits (Ayres et al., 1997; Züst et al., 2012), and individual plant compounds are targeted at distinct herbivores (i.e., compound selectivity). For example, multiple specialized herbivores of milkweed plants (*Asclepias* spp.) each have well-characterized genetically based physiological differences in tolerance to the overall concentration of the plant's toxic cardenolides (Dobler et al., 2012; Karageorgi et al., 2019). Cardenolides are a structurally diverse group of compounds that are found in at least 12 plant families (Agrawal et al., 2012). They consist of a steroid backbone, an unsaturated lactone, and glycoside moieties (Malcolm, 1991). Single species of *Asclepias* can contain up to 21 different cardenolides, and both compound diversity and concentration are variable among tissues eaten by insects (Abe et al., 1992; Warashina et al., 2008; Opitz and Müller, 2009; Zhang et al., 2014). These host plant cardenolides are selectively sequestered by insect herbivores. For example, the seed bug *Oncopeltus fasciatus* often sequesters intermediate and more polar cardenolides, even if reared on different host plants that have distinct chemical profiles (Moore and Scudder, 1985). The bug's cardenolide profiles often differ from the cardenolide profile of the host plants (Duffey and Scudder, 1974; Isman et al., 1977; Scudder et al., 1986), which can be explained by the metabolism of some cardenolides into distinct products (Agrawal et al., 2022). Studies that investigate the functional link between the specific defense compounds and their impacts on herbivores will advance our understanding of how the diversity of the same secondary metabolite compound arises, and why plants repeatedly derivatize the same class of compounds (Whitehead et al., 2022).

Recently, the interactions between particular toxic cardenolide heart poisons and insect target sites have begun to be identified (Petschenka et al., 2018; Agrawal et al., 2022; López-Goldar et al., 2022). Agrawal et al. (2022) found that the dominant seed cardenolide in *Asclepias syriaca* (glycosylated aspecioside) is more inhibitory to the target site of the seed herbivore than its sequestered conversion product (aspecioside A) and that the subdominant seed cardenolides—diglycosylated syriogenin and labriformin—were significantly more inhibitory than the more abundant compounds. The differential accumulation of the compounds could be evidence that they are under selection, especially if this is correlated to geographical areas that vary in insect diversity and herbivory. Further studies on the specificity in chemical defense toward particular resistant enzymes will increase our understanding of milkweed–herbivore coevolution and more broadly phytochemical diversity.

Here, we study the specific interaction of cardenolide toxins from *Asclepias curassavica* plants and insect Na^+/K^+-ATPases. We compare the cardenolide composition of seeds of *Asclepias curassavica* to those sequestered by nymphs and adults of the specialized seed herbivore *Oncopeltus fasciatus*. We combine this with tests of the inhibitory capacity of a subset of the seed cardenolides against the Na^+/K^+-ATPase of *Oncopeltus* and a non-adapted insect (*Drosophila melanogaster*). The cardenolides can be differentiated by the presence of a hydroxyl group (coroglaucigenin) or a carbonyl group (corotoxigenin/calotropagenin) at the C-19 position of the steroidal structure (see Figure 1 and Rubiano-Buitrago et al., 2023). The native range of *A. curassavica* is South America and Mexico, and it has spread to California, Florida, Tennessee, and Texas. *A. curassavica* is eaten by several specialist herbivores including true bugs, from the family Lygaeidae. *A. curassavica* is present in the migration range of milkweed bugs (Miller and Dingle, 1982; United States Department of Agriculture, 2020). The bugs feed on the seeds (Burdfield-Steel and Shuker, 2014) and sequester cardenolides present in the seeds for their own defense (Scudder and Meredith, 1982; Moore and Scudder, 1985). *O. fasciatus* is adapted to feed on *Asclepias* due to specific amino acid substitutions in the physiological target—the Na^+/K^+-ATPases—which reduce the insect's sensitivity (Bramer et al., 2015). We aimed to answer the following question in the context of milkweed-herbivore interactions: Do milkweed bugs sequester cardenolides according to the availability in the seeds or according to the inhibitory capacity of the cardenolides on the bugs' Na^+/K^+-ATPase?

2. Materials and methods

2.1. Cardenolides from *Asclepias curassavica* seeds and sequestration

Here, we compared the quantification of 10 seed cardenolides sequestered by nymphs and adults of the large milkweed bug *Oncopeltus fasciatus* (see methods in Rubiano-Buitrago et al., 2023). These cardenolides are: 3-O-β-allopyranosyl coroglaucigenin, 3-[4′-O-β-glucopyranosyl-β-allopyranosyl] coroglaucigenin, 3′-O-β-glucopyranosyl-15- β-hydroxycalotropin, 3-O-β-glucopyranosyl-12-β-hydroxyl coroglaucigenin, 4′-O-β-glucopyranosyl frugoside, 4′-O-β-glucopyranosyl gofruside, 3′-O-β-glucopyranosyl calotropin, frugoside, gofruside, and 16α-hydroxycalotropin.

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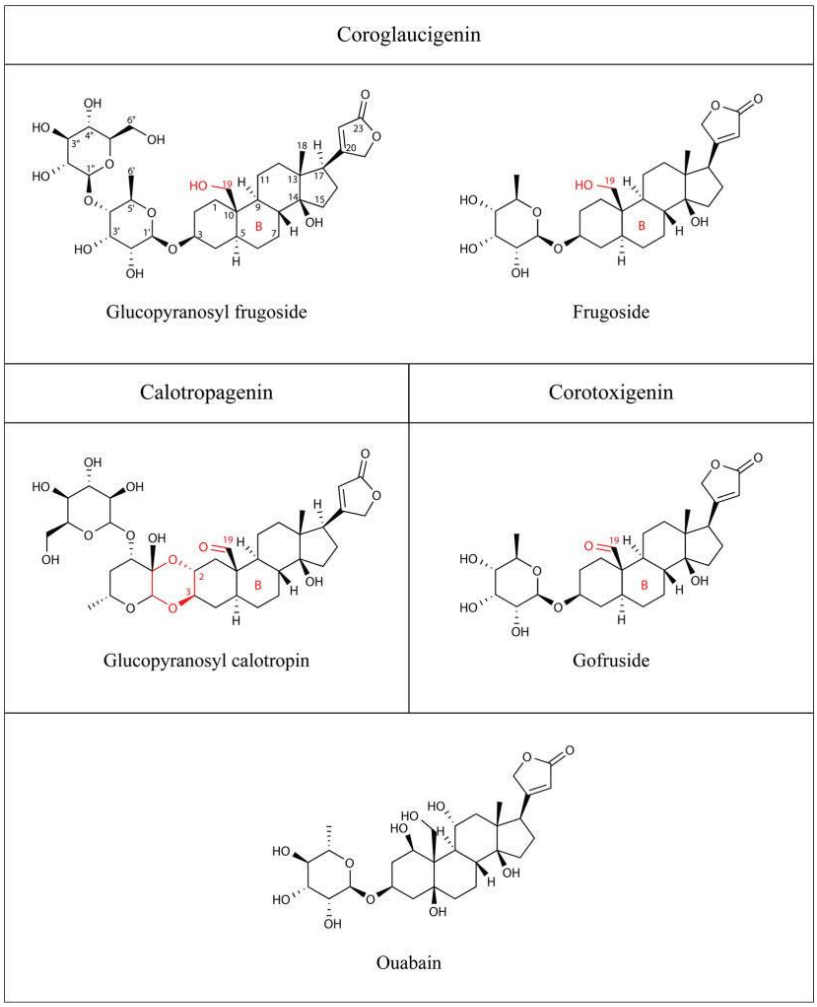

FIGURE 1
Structures of the cardiac glycosides used in this study: glucopyranosyl frugoside, glucopyranosyl calotropin, frugoside, gofruside, and ouabain. Structural features that differentiate the three types of steroidal structure are drawn in red and include functional groups at C-19, a dioxane ring in C-2, C-3, and the ring B of the steroidal core.

To assess the sequestration behavior of *O. fasciatus*, we used bugs that originated from a long-term laboratory colony (originally from the United States) maintained on sunflower seeds. We reared the bugs in terrarium boxes (37 cm × 22 cm × 25 cm) lined with tissue paper and provided them with *ad libitum* water in Eppendorf tubes plugged with dental cotton, and *ad libitum* *Asclepias curassavica* seeds as a food source. The colonies of *O. fasciatus* were kept in an incubator (Polyklima PK 520-LED) at 70% humidity with a day/night cycle of (18:6) and temperatures of 28°C during the day and 18°C at night. *O. fasciatus* nymphs develop through five instars (N1–N5), after which they molt into adults. We collected three groups of adults and three groups of N5 nymphs from the experimental colonies and froze them at −80°C.

We extracted cardenolides from the three pools of adults and three pools of N5 nymphs. Each pool weighed ~700 mg. We ground the frozen material and exhaustively extracted cardenolides using a 1:1 MeOH/H_2O three times (MeOH ROTISOLV 99.9%, Carl Roth GmbH, Karlsruhe, Germany, water was HPLC grade,

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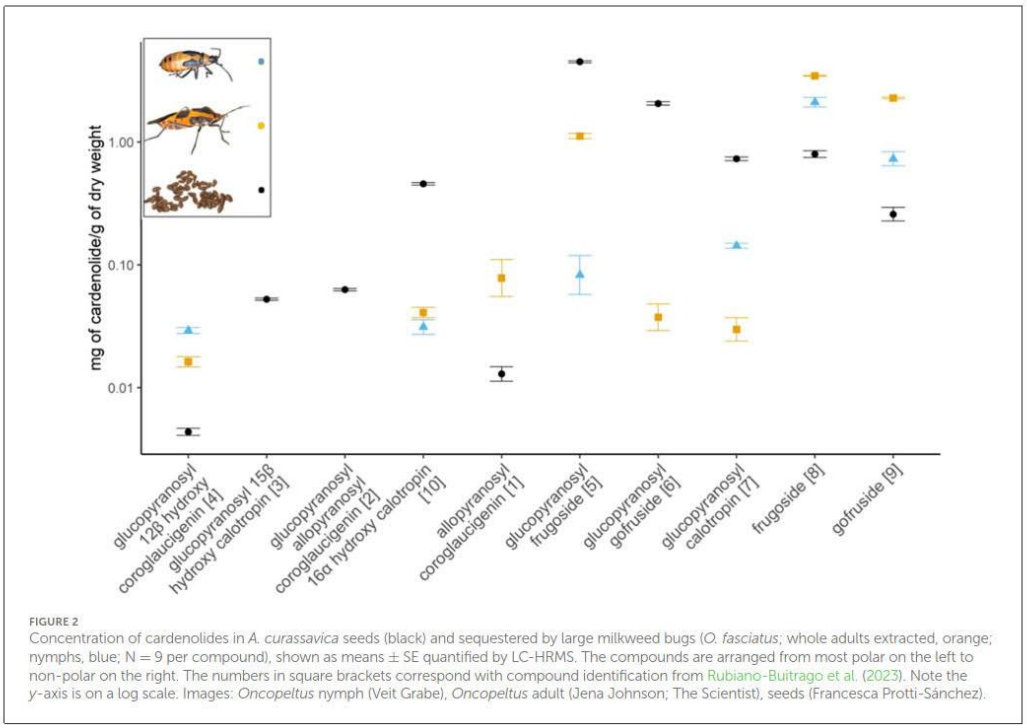

FIGURE 2
Concentration of cardenolides in *A. curassavica* seeds (black) and sequestered by large milkweed bugs (*O. fasciatus*; whole adults extracted, orange; nymphs, blue; N = 9 per compound), shown as means ± SE quantified by LC-HRMS. The compounds are arranged from most polar on the left to non-polar on the right. The numbers in square brackets correspond with compound identification from Rubiano-Buitrago et al. (2023). Note the *y*-axis is on a log scale. Images: *Oncopeltus* nymph (Veit Grabe), *Oncopeltus* adult (Jena Johnson; The Scientist), seeds (Francesca Protti-Sánchez).

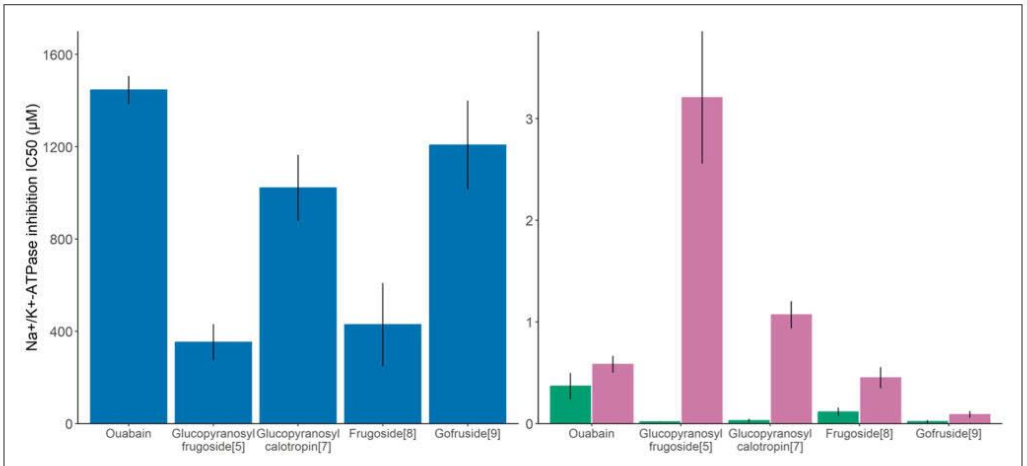

FIGURE 3
Molar concentration of plant toxin necessary to cause 50% inhibition of the *O. fasciatus* enzyme (blue), non-adapted insect (*D.melanogaster*, green), and a vertebrate reference (*S. domesticus*, purple) Na^+/K^+-ATPases, or IC_{50} of the dominant seed cardenolide (glucopyranosyl frugoside) and the subdominant compounds, all of which are sequestered, shown as means ± SE, *N* = 4–6 per compound). Higher values on the *y*-axis indicate that the enzyme is more tolerant to the cardenolide.

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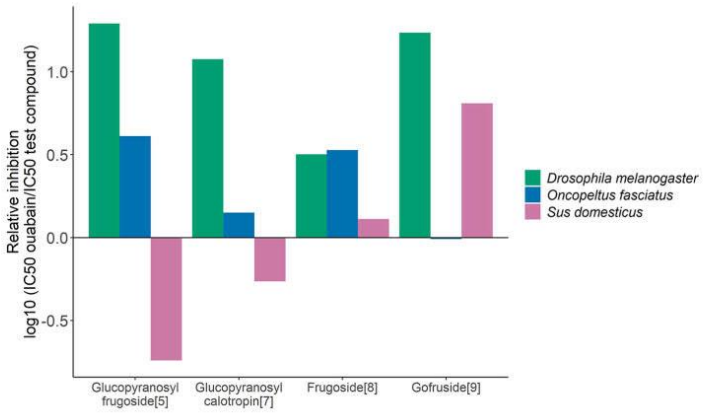

FIGURE 4
Selective effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (*O. fasciatus*, blue; non-adapted *D. melanogaster*, green, and *S. domesticus*, pink). Relative inhibition values are based on IC_{50} values compared with the standard ouabain (computed as the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). Inhibition weaker than ouabain is indicated by negative values, while inhibition greater than ouabain is indicated by positive values.

deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany), followed by two more times with 100% MeOH. The extracts were pooled and filtered through an MN HR-X 500 mg cartridge and washed with MeOH to remove lipophilic substances. The samples were dried using N_2 gas at 36°C.

We obtained three nymph extracts of 85.2, 115.1, and 121.0 mg, and three adult extracts of 86.0, 92.0, and 81.9 mg. From each replicate, we prepared a 2.6 mg/mL solution for high-resolution mass spectrometry (HPLC-HRMS). We injected 5 µL of each replicate three times, resulting in nine data points per compound. The high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) was achieved using an Agilent 1,260 Infinity, with a reversed-phase Agilent Poroshell 120 column (2.7 µm particle size, 4.6 × 50 mm). The mobile phase consisted of acetonitrile (ACN, supplied with 0.1% formic acid, FA, Carl Roth GmbH, Karlsruhe, Germany) and water (HPLC grade, 0.1% FA, deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany). The elution gradient started with ACN/H_2O (5:95) for 1 min, then to ACN/H_2O (95:5) for 8 min, which was maintained for 2 min. Later, it was set back to ACN/H_2O (5:95) for 1 min. High-resolution mass spectra were recorded on a Bruker Compact OTOF spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Electrospray ionization (ESI) in positive ion mode was used for the analysis in full scan and auto MS/MS modes, scanning masses from m/z 50–1,300. Sodium formate adducts were used for internal calibration HPC mode. Bruker Compass ver.1.9 (OTOF Control ver.5.1.107 and HyStar 4.1.31.1) was used for data acquisition and instrument control, and Bruker DataAnalysis ver. 5.1.201 was used for data processing.

We processed the data by extracting the ion chromatogram per compound and measuring the corresponding peak area. The concentration per injection was calculated as mg of cardenolide per g of dry weight.

2.2. Cardenolide toxicity tested by functional Na^+/K^+-ATPase assays

Na^+/K^+-ATPase extractions from the cardenolide-resistant seed bug (*Oncopeltus fasciatus;* Bramer et al., 2015; Agrawal et al., 2022; López-Goldar et al., 2022) and one non-adapted insect (*Drosophila melanogaster* wild type Canton S) (Dalla and Dobler, 2016; Dobler et al., 2019) were prepared according to the methods described in Petschenka et al. (2013, 2018, 2022). Fruit flies were reared under controlled conditions, and *O. fasciatus* were reared in terrarium boxes as described in section Cardenolides from *Asclepias curassavica* seeds and sequestration except that for this analysis, *O. fasciatus* adults were reared on sunflower seeds (*Helianthus annuus*).

Briefly, the brains and thoracic ganglia of individual *O. fasciatus* (killed and stored at −80°C) were dissected under deionized water. We pooled the brains of six individuals (following Bramer et al., 2015) and homogenized them in 500 µL of deionized water using an all glass 1 mL Wheaton grinder. For *D. melanogaster*, we dissected the heads of 90 individuals and homogenized them in 900 µL of deionized water and then split them into three 300 µL aliquots (following Taverner et al., 2019). All extracts were frozen, lyophilized, and stored at −80°C until use. Prior to the assays, the insect lyophilisates were reconstituted by adding 200 µL water (*O. fasciatus)* or 600 µL (*D. melanogaster*) followed by vortex and incubation for 10 min in a chilled ultrasonic bath. Porcine lyophilized Na^+/K^+-ATPase was dissolved in water to a concentration of 1 U/ml, stored at −80°C in single-use aliquots, and diluted with H_2O to 0.05 U/ml for use in the *in vitro* assay (final concentration in the assay, 0.01 U/ml).

All cardiac glycosides (Figure 1) were tested with at least two biological replicates of Na^+/K^+-ATPase (i.e., Na^+/K^+-ATPase

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from genetically different specimens or pools of specimens). Reactions were performed in 96-well microplates to which the Na^+/K^+-ATPase protein was added in an eight-well row containing stabilizing buffers (see formulas in Petschenka et al., 2023). The first six wells in the eight-well row were exposed to exponentially decreasing concentrations of cardenolide (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M). The seventh well was 2% DMSO (as an experimental control), and the eighth well was exposed to a combination of inhibition buffers lacking KCl but supplemented with 10^{-3} M cardenolide. This allows the measurement of background ATP hydrolysis. The proteins were incubated on a BioShake Iq microplate shaker (Quantifoil Instruments) at 200 rpm and 37°C for 10 min after which ATP (Sigma Aldrich, A9062-1G) was added to each well and then incubated at 200 rpm and 37°C for further 20 min. The Na^+/K^+-ATPase activity was measured as the amount of inorganic phosphate enzymatically released from ATP in the presence of K^+ (Na^+/K^+-ATPase active) minus the amount of phosphate released in the absence of K^+ (Na^+/K^+-ATPase inactive). The activity was quantified photometrically at 700 nm with a microplate reader (BMG Clariostar) following (Taussky and Shorr, 1953). Background phosphate absorbance levels from the reactions with inhibiting factors were used to calibrate phosphate absorbance in wells measuring cardenolide inhibition and in the control wells (Petschenka et al., 2013). For each cardenolide, we carried out two technical replicates, making each data point an average of four to six measurements (Petschenka et al., 2012, 2013; Taverner et al., 2019).

2.3. Data evaluation

To compare the concentration of individual cardenolides available in the seeds to those sequestered by adults and nymphs we log-transformed the concentration data and analyzed the transformed data using analysis of variance. For glucopyranosyl frugoside and glucopyranosyl calotropin, the transformation did not improve the fit of the data for parametric tests, thus we compared them using the Wilcoxon rank test with a continuity correction. We did not analyze data where the bugs had not sequestered compounds (glucopyranosyl hydroxycalotropin and glucopyranosyl allopyranosyl coroglaucigenin) or where only the adult or the nymph (not both) had sequestered (allopyranosyl coroglaucigenin and glucopyranosyl gofruside).

For cardenolide sensitivity measurements, calibrated absorbance values were converted to the percentage of non-inhibited Na^+/K^+-ATPases activity based on measurements from the control wells. These data were plotted, and $\log_{10}$ of the IC_{50} values was calculated for each biological replicate from non-linear fitting using a four-parameter logistic curve, with the top and bottom asymptotes set to 100 and zero, respectively. We fitted the curves using the nlsLM function of the minipack.lm library in R (R Core Team, 2022) (Supplementary Figure 3 and Supplementary Table 1). We compared the $\log_{10}$ IC_{50} values of individual cardenolides for each Na^+/K^+-ATPase enzyme using the analysis of variance, with Tukey's HSD pairwise contrasts. We calculated the fold differences between the IC_{50} values of individual cardenolides for non-adapted and adapted insect Na^+/K^+-ATPase and the porcine Na^+/K^+-ATPase. To calculate the effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (adapted or non-adapted), we compared the inhibition to a reference cardenolide by calculating the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). We used ouabain (Sigma Aldrich, O3125-1G) as a reference as it is the most widely used cardenolide in research on Na^+/K^+-ATPase (Petschenka et al., 2018). Here, negative values represent compounds that inhibit the Na^+/K^+-ATPase more weakly than ouabain, and positive values represent compounds that inhibit the Na^+/K^+-ATPasemore strongly than ouabain. We also calculated this ratio using glucopyranosyl frugoside as the reference, because it is the most abundant cardenolide in the seeds (Rubiano-Buitrago et al., 2023).

All analyses were conducted in R (version 1.4.1717).

3. Results

3.1. Seed cardenolides and sequestration

We found significant differences among the concentration of cardenolides available in the seeds of *A. curassavica* and those sequestered by nymphs and adults of *O. fasciatus* (see Supplementary Table 2). Among the five most abundant cardenolides present in milkweed seeds (accounting for 91% of the total), glucopyranosyl frugoside, glucopyranosyl gofruside, and glucopyranosyl calotropin were significantly more abundant in the seeds than the adults and nymphs. Frugoside and gofruside were significantly more dominant in the adults and nymphs than in the seeds (Figure 2 and Supplementary Figures 1, 2). Of the minor compounds, the adults and nymphs sequestered significantly more glucopyranosyl-12β-hydroxyl coroglaucigenin than was present in the seeds, but significantly less 16α-hydroxycalotropin. Neither adults nor nymphs sequestered glucopyranosyl-15β-hydroxycalotropin or glucopyranosyl-allopyranosyl coroglaucigenin that were present in the seeds. Adult bugs, but not nymphs, sequestered allopyranosyl coroglaucigenin and glucopyranosyl gofruside from the seeds. There were eight other cardenolides in the seeds that we did not identify and that the bugs did not sequester. There were also 14 cardenolides in the LC-HRMS data of *O. fasciatus* that did not have the retention time or the fragmentation pattern of our purified cardenolide reference obtained from the seeds, and that were also absent from the spectrometric data of the seeds. Of these 14 compounds, seven were present in both adults and nymphs, five were only found in the nymphs, and two were only found in the adults (see Supplementary Table 3).

3.2. Cardenolide toxicity

The compounds tested (glucopyranosyl frugoside, glucopyranosyl calotropin, frugoside, gofruside, and ouabain, Figure 3) had significantly different effects on the *O. fasciatus* Na^+/K^+-ATPase enzyme (ANOVA: $F_{(4,17)} = 9.70$, $p = 0.0003$). Glucopyranosyl frugoside was four times more inhibitory than ouabain (Tukey's HSD: estimate $= -0.68 \pm 1.19$, $p = 0.002$). Frugoside was 3.5 times more inhibitory than ouabain (Tukey's HSD: estimate $= -0.64 \pm 1.13$, $p = 0.007$). Glucopyranosyl

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calotropin and gofruside did not significantly differ from ouabain in inhibitory capacity (Tukey's HSD: estimate = −0.165 ± 0.65, $p = 0.84$; estimate = −0.01 ± 0.51, $p = 0.99$).

For the sensitive invertebrate *D. melanogaster*, we also found a significant difference between the compounds on the IC_{50} values (ANOVA: $F_{(4,23)} = 8.04$, $p = 0.0003$). Glucopyranosyl frugoside was 20 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.21 ± 2.14, $p = 0.007$). Gofruside was 17 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.43 ± 2.36, $p = 0.001$), and glucocalotropin was 12 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.40 ± 2.33, $p = 0.002$). Frugoside did not differ significantly from ouabain (fold difference = 3; Tukey's HSD: estimate = −0.51 ± 1.46, $p = 0.5$). We also found a significant variation in the inhibitory capacity of the compounds on the highly sensitive porcine enzyme (ANOVA: $F_{(4,25)} = 49.63$, $p < 0.0001$). Glucopyranosyl frugoside was five times less inhibitory than ouabain (Tukey's HSD: estimate = 0.72 ± 0.37, $p = 0.00002$), and glucopyranosyl calotropin was 1.5 times less inhibitory (Tukey's HSD: estimate = 0.27 ± 0.08, $p = 0.18$), whereas gofruside was 6.5 times more inhibitory (Tukey's HSD: estimate = −0.88 ± 1.23, $p = 0.00007$). Frugoside was not significantly different from ouabain in inhibitory activity (fold difference = 1.3, Tukey's HSD: estimate = −0.13 ± 0.48, $p = 0.78$).

Frugoside caused a unidirectional effect on the three enzymes compared to ouabain, whereas glucopyranosyl calotropin, glucopyranosyl frugoside, and gofruside produced countervailing effects (Figure 4). Glucopyranosyl calotropin and glucopyranosyl frugoside were stronger than ouabain on the insect enzymes but weaker than ouabain on the porcine Na^+/K^+-ATPases. Gofruside had almost equal to lower effect than ouabain for *O. fasciatus* but was more inhibitory for the sensitive species. Using glucopyranosyl frugoside as a reference in place of ouabain shifted the direction of the effects (Supplementary Figure 4).

We found no significant relationship between concentration in the seeds and inhibitory effects of cardenolides across the insect enzymes (Pearson's correlations: *D. melanogaster* $R = -0.32$, $p = 0.68$; *O. fasciatus* $R = -0.66$, $p = 0.34$), but there was a trend toward a decrease in inhibitory capacity with increasing concentration for the porcine enzyme ($R = 0.97$, $p = 0.026$) (Supplementary Figure 5).

4. Discussion

We quantified the cardenolides sequestered by the specialized milkweed herbivore *Oncopeltus fasciatus* across two stages of its life cycle when reared on seeds of *Asclepias curassavica*. We combined the identification and quantification of sequestered cardenolides with tests of their inhibitory capacity of the Na^+/K^+-ATPase. Among the five most abundant cardenolides present in milkweed seeds, frugoside and gofruside were more concentrated in the bugs than the seeds. These two compounds had contrasting potency toward the *O. fasciatus* enzyme–frugoside (and its glucosylated counterpart), which was the most inhibitory, whereas gofruside was among the weakest inhibitors. When comparing the inhibitory capacity of all the compounds to a non-adapted insect *Drosophila melanogaster* and the highly sensitive porcine Na^+/K^+-ATPase, we found that the compounds varied less in their inhibition of the *O. fasciatus* enzyme compared to the non-adapted insect enzyme. The finding that there is differential accumulation of *A. curassavica* seed cardenolides by *O. fasciatus* adds to the evidence that the bug sequesters particular, but not all, cardenolides from its *Asclepias* hostplants (Duffey et al., 1978; Scudder and Meredith, 1982; Meredith et al., 1984).

Milkweed bugs reared on seeds of *A. curassavica* sequestered two of the more non-polar cardenolides available in the seeds (frugoside and gofruside), which is in line with the preferential sequestration of the non-polar cardenolide digitoxin in the fat body of adult *O. fasciatus* reported by Duffey et al. (1978). The fat body often accumulates non-polar compounds owing to the lipophilic nature of such compounds (Kilby, 1963). *O. fasciatus* in our study did not sequester several compounds that are present in the seeds, which is also in line with the results of Moore and Scudder (1985) who showed that *O. fasciatus* sequestered most, but not all, of the cardenolides in seeds of *A. speciosa* (9 of 13 cardenolides sequestered) and *A. syriaca* (6 of 8 cardenolides sequestered). We found 14 compounds in the bugs that did not have the retention time or the fragmentation pattern of cardenolides from the seeds. A similar sequestration behavior has also been reported by Moore and Scudder (1985) who found that *O. fasciatus* contained 12 cardenolides that were not present in *A. speciosa* seeds, and 8 cardenolides that were not present in *A. syriaca* seeds. Our results could indicate that *O. fasciatus* concentrated some compounds that have such low abundance in the seeds that they were not detectable by spectrometric measurements or could indicate the metabolism of cardenolides, which is known to occur when *O. fasciatus* is reared on *A. syriaca* (Agrawal et al., 2022). The higher concentration of frugoside and gofruside in the bugs than in seeds could indicate that *O. fasciatus* cleaves the sugar from the glucosylated compounds during sequestration. To verify this, the insects could be reared on artificial diets containing these isolated cardenolides and the sequestration products could then be quantified (e.g., Pokharel et al., 2021; Agrawal et al., 2022).

Until recently the Na^+/K^+-ATPase of *O. fasciatus* has been studied almost exclusively with reference to ouabain (though see: Herbertz et al., 2020; Agrawal et al., 2022; López-Goldar et al., 2022). The main result from our tests of four compounds of *A. curassavica* is that glucopyranosyl frugoside and frugoside have potency against *O. fasciatus*, which has a highly adapted sodium pump characterized by gene duplications and substitutions (Bramer et al., 2015; Lohr et al., 2017). Phytochemical profiles of the aerial parts and roots of *A. curassavica* have no reports of glucopyranosyl frugoside being isolated, which could suggest it is a compound specific to the seeds and, given its inhibitory capacity, under selection by the seed bugs. Frugoside, on the other hand, is well distributed across the plant's tissues (Roy et al., 2005; Warashina and Noro, 2008; Warashina et al., 2008; Li et al., 2009; Al-Snafi, 2015; Ji et al., 2022). We did not test these compounds on other adapted herbivores, such as Monarch butterflies (*Danaus plexippus*), and so our data do not allow us to draw specific conclusions about *Oncopeltus*–milkweed coevolution (Berenbaum and Zangerl, 1996), but open up a path for future research on toxin–receptor interactions.

Our results are in contrast with those of Agrawal et al. (2022), who found that the dominant cardenolide in seeds of *Asclepias syriaca* was a less potent inhibitor of the *O. fasciatus* Na^+/K^+-ATPase than a minor compound, labriformin, that

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was the most potent inhibitor. Labriformin is characterized by nitrogen- and sulfur-containing thiazolidine ring moiety, which was not present in any of the major cardenolides isolated in *A. curassavica* seeds, but is known to be present in the leaves (Agrawal et al., 2022). Cardenolides with a thiazolidine ring are predicted to interact with the binding site of *O. fasciatus* Na^+/K^+-ATPase in *in silico* molecular docking simulations (Ramos et al., 2021), which likely explains why *O. fasciatus* sequesters the less toxic nitrogen-containing oxidized labriformin (Agrawal et al., 2022). Of our isolated compounds, we might have expected gofruside (which contains an aldehyde moiety as part of their corotoxigenin steroidal structure, Figure 1) and glucopyranosyl calotropin (which contains the same aldehyde group as gofruside and also has a dioxane ring bond in C-2 and C-3, Figure 1) to have been more inhibitory to the *Oncopeltus* enzyme (Zhang et al., 2014). For example, corotoxigenin cardenolides, like gofruside, are predicted to form a strong hydrophobic pi-sigma interaction between amino acid residue 783 and the steroidal ring B, which is hypothesized to assist the cardenolide-sensitive Na^+/K^+-ATPase complex (see the porcine model in Meneses-Sagrero et al., 2022). However, gofruside and glucopyranosyl calotropin did not differ in their inhibitory properties compared to ouabain to the *O. fasciatus* Na^+/K^+-ATPase. Both cardenolides were less inhibitory than the major compound in the seeds, which have hydroxyl moiety at C-19 (Figure 1). Gofruside and glucopyranosyl calotropin were, however, highly inhibitory to the *D. melanogaster* enzyme.

Different degrees of oxidation in the C-19 position are predicted to generate a key hydrogen bond between the cardenolide and the enzyme amino acid residue 111, a site that is strongly implicated in cardenolide resistance (Bramer et al., 2015). The hydrogen bonds between amino acid residue 111 and the aldehyde or hydroxyl moieties are predicted to influence the stability of the cardenolide-protein complex (Meneses-Sagrero et al., 2022). Further *in silico* molecular docking simulations of these compounds against both resistant and sensitive enzymes are needed to clarify the importance of the oxidation in the steroidal C-19 in relation to the amino acid residues that have been implicated in resistance.

Glycosylated cardenolides are generally considered to be more toxic than corresponding genins in whole organism vertebrate assays (Hoch, 1961). Petschenka et al. (2018) found that glycosides were on average 6-fold more potent in their inhibition of insect Na^+/K^+-ATPases, including the specialized monarch butterfly Na^+/K^+-ATPase, compared to genins. Agrawal et al. (2022) also found that diglycosylated compounds were highly inhibitory. We found similar inhibition of glucopyranosyl frugoside and frugoside against *O. fasciatus,* but the deglycosylated version was more inhibitory for the porcine enzyme. Given that we have only one comparison of a glycoside vs. its aglycone, we can tentatively suggest that the effect of the cardiac glycoside sugar moiety on inhibition depends on the specific biochemical properties of the target, the Na^+/K^+-ATPase enzyme. It is also suggested that the toxicity of cardenolides can increase from polar to non-polar compounds (Petschenka and Dobler, 2009; Rasmann and Agrawal, 2011; Agrawal et al., 2012; López-Goldar et al., 2022). In our analysis of five cardenolides with different polarities and Na^+/K^+-ATPase pumps from three species, we do not observe a clear trend where polarity is a key factor for the inhibitory response.

4.1. Synthesis

Our study, along with Agrawal et al. (2022) and Petschenka et al. (2018), focuses on individual compounds, rather than natural mixtures in which those compounds are encountered in nature (though see López-Goldar et al., 2022). Mixtures of cardenolides may be maintained because of synergistic or additive interactions among compounds (Richards et al., 2016; Whitehead et al., 2021). Mixtures may also be maintained because of antagonism, where the effects of active constituents are masked by other compounds in a complex mixture (Caesar and Cech, 2019). Comparison of the IC_{50} values for single cardenolides vs. mixtures of cardenolides would be useful for uncovering the combined effects of the compounds and could provide a functional explanation for the repeated derivatization of cardenolides in milkweeds (Whitehead et al., 2021) and the differences in the abundance and inhibitory properties of cardenolides in different species of *Asclepias* (Agrawal et al., 2022; López-Goldar et al., 2022). Such research could also shed more light on whether there are costs associated with the biosynthesis of different cardenolides (e.g., those with extra glycosylation, or further modifications to the sugar moiety), and the cost-benefit of producing specific cardenolides in relation to differences in insect diversity, herbivore specialization, and the constraints on those herbivores. Although Agrawal et al. (2022) found no significant effects of the most potent inhibitors on *O. fasciatus* growth and performance, we have recently shown that sequestration can be costly in terms of oxidative state (Blount et al., 2023; Heyworth et al., 2023) which is visible in the wing colors that milkweed herbivores use as anti-predator defenses. More tests on the costs of sequestration will also be important for understanding the evolutionary and ecological interactions between the plant and the herbivore.

Data availability statement

The original contributions presented in the study are publicly available. The raw data and R scripts for statistical analysis and data visualisation can be found at: https://edmond.mpdl.mpg.de/dataset.xhtml?persistentId=doi:10.17617/3.SQGWHL.

Author contributions

Conceptualization: HMR, CP, and PR-B. Methodology: PR-B, SP, VG, CP, and HMR. Formal analysis and writing—original draft preparation: HMR and PR-B. Investigation: PR-B and SP. Resources and supervision: HMR and CP. Data curation: PR-B. Writing—review and editing: SP, CP, AA-A, and VG. Visualization: AA-A and PR-B. Project administration and funding acquisition: HMR. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1175205/full#supplementary-material

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Supplementary Material

Differential accumulation of cardenolides from *Asclepias curassavica* by large milkweed bugs does not correspond to availability in seeds or biological activity on the bug Na^+/K^+-ATPase

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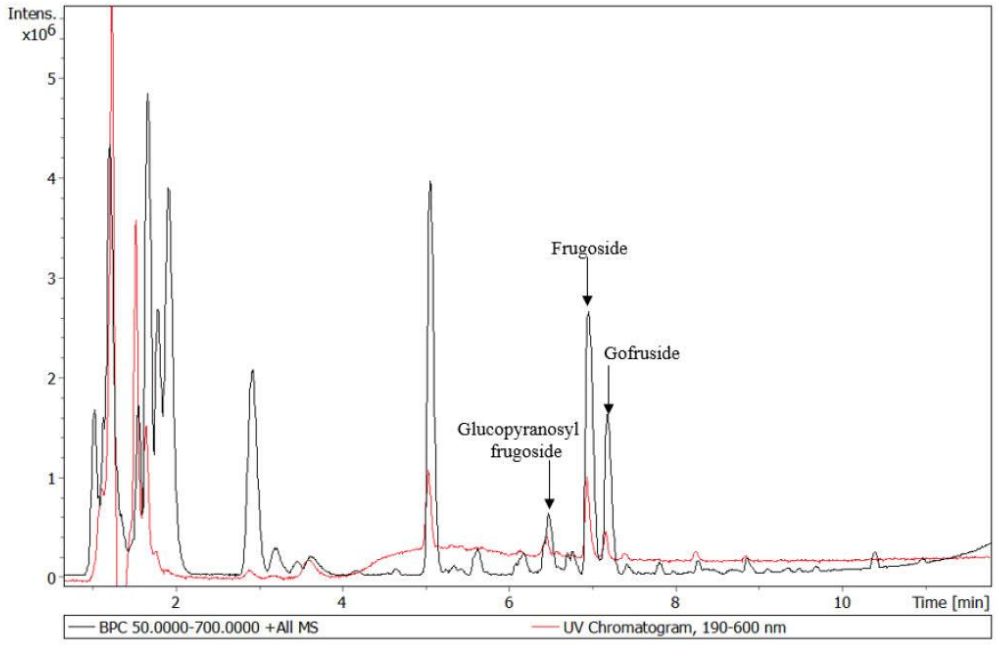
1. **Supplementary Figure S1.** UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* adults. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.
2. **Supplementary Figure S2.** UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* nymphs. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.
3. **Supplementary Figure S3:** Inhibition curves of *Sus domesticus*, *Drosophila melanogaster* and *Oncopeltus fasciatus* Na^+/K^+ ATPases by compounds from *Asclepias curassavica* seeds and ouabain
4. **Supplementary Table S1:** IC_{50} values of ouabain and *Asclepias curassavica* cardenolides on the three analyzed enzymes N= number of replicates.
5. **Supplementary Figure S4:** Analysis on the effects of cardenolides on the adapted insect (*O.fasciatus*, blue), non-adapted insect (*D.melanogaster*, green) and a vertebrate reference (*S. domesticus*, purple) Na^+/K^+-ATPases. The IC_{50} values (Supplementary Table xx) are compared to the abundant compound, glucopyranosyl frugoside. Log_{10} transformation of the ratio of IC_{50} values (glucopyranosyl frugoside/test compound) results in inhibition weaker than glucopyranosyl frugoside indicated by negative values, while inhibition greater than glucopyranosyl frugoside is indicated by positive values. Specific interactions between Na^+/K^+-ATPases and cardenolides are called "countervailing effects", where cardenolides are more potent than glucopyranosyl frugoside on some Na^+/K^+-ATPases and less potent than glucopyranosyl frugoside on others. Glucopyranosyl frugoside is the most potent of all compounds analyzed for the adapted Na^+/K^+-ATPase.
6. **Supplementary Figure S5:** Relationship between concentration and inhibitory effects of cardenolides (mg per g of seeds) across the three analyzed enzymes. Black= Frugoside, Yellow= Glucopyranosyl calotropin, Blue= Glucopyranosyl frugoside, Green=Gofruside.
7. **Supplementary Table S2**: Comparison of the concentration of individual cardenolides available in the *Asclepias curassavica* seeds to those sequestered by adults and nymphs (log transformed data) using analysis of variance.
8. **Supplementary Table S3:** HRMS data of the cardenolides and compounds putatively assigned as cardenolide metabolites found in samples of *Asclepias curassavica* seeds, *Oncopeltus fasciatus* nymphs and adults.

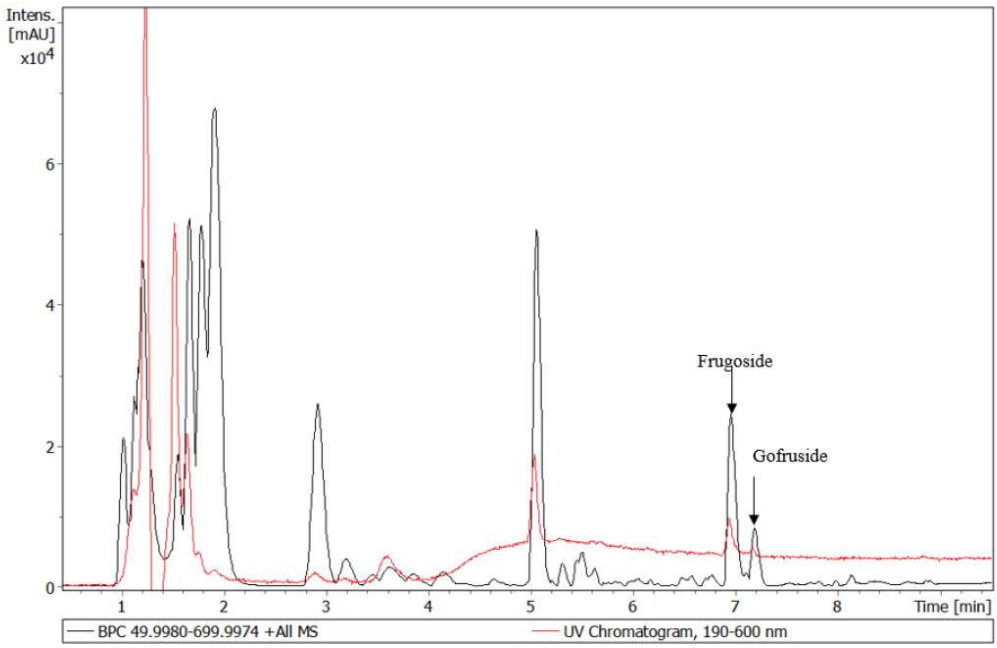
Supplementary Material

1.



Supplementary Figure S1. UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* adults. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.

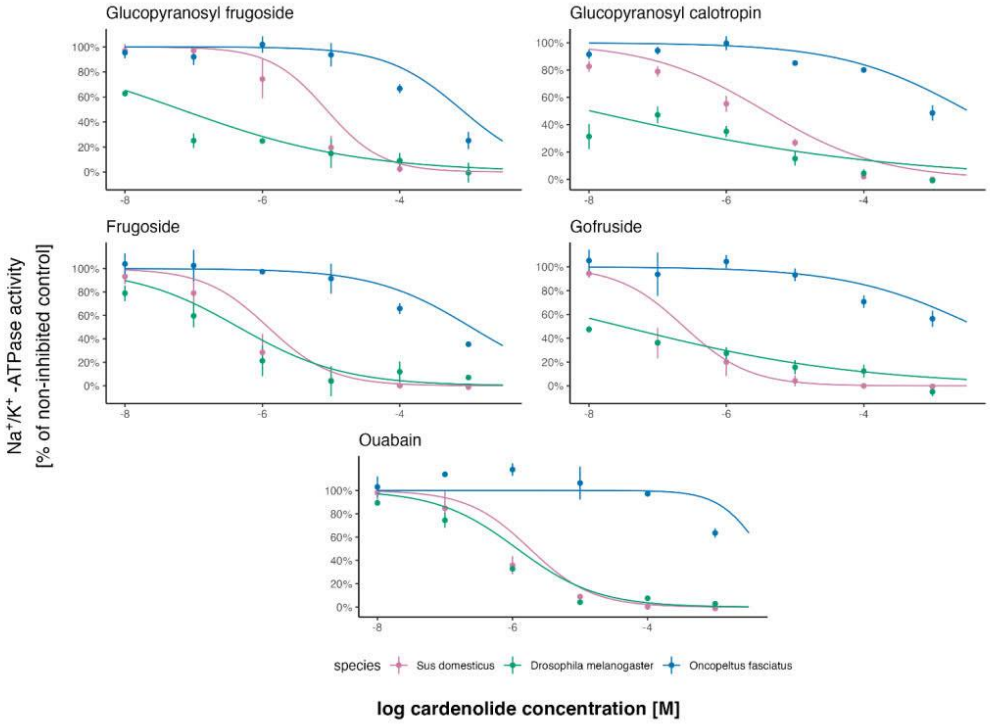
2.



Supplementary Figure S2. UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* nymphs. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.

Supplementary Material

3.



Supplementary Figure S3: Inhibition curves of *Sus domesticus*, *Drosophila melanogaster* and *Oncopeltus fasciatus* Na^+/K^+ ATPases by compounds from *Asclepias curassavica* seeds and ouabain (N between 4 to 6 replicates)**.** log10 IC_{50} values were estimated using a four-parameter logistic curve, with the top asymptote set to 100 and the bottom asymptote set to zero, using the nlsLM function of the minipack.lm library in R.

4.

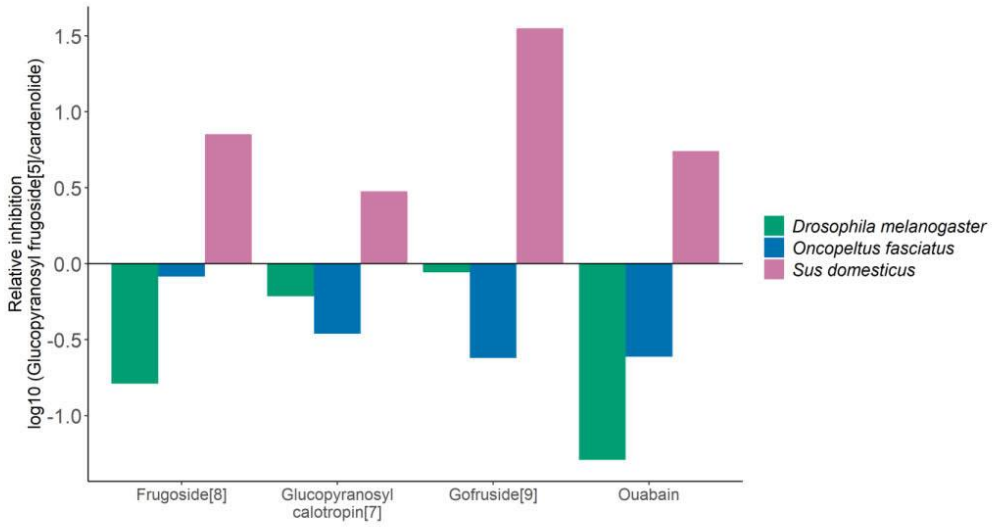
Supplementary Table S1: IC_{50} values of ouabain and *Asclepias curassavica* cardenolides on the three analyzed enzymes N= number of replicates.

Species	Compound	N	Log_{10} IC_{50} (M)	IC_{50} (M)
Drosophila melanogaster	Ouabain	4	-6.540 (±0.19)	$3.69x10^{-7}$ ($\pm1.30x10^{-7}$)
Drosophila melanogaster	Glucopyranosyl frugoside [5]	6	-7.748 (±0.06)	$1.90x10^{-8}$ ($\pm3.0x10^{-9}$)
Drosophila melanogaster	Glucopyranosyl calotropin [7]	6	-7.937 (±0.33)	$3.10x10^{-8}$ ($\pm1.5x10^{-8}$)
Drosophila melanogaster	Frugoside [8]	6	-7.052 (±0.14)	$1.16x10^{-7}$ ($\pm4.1x10^{-8}$)
Drosophila melanogaster	Gofruside [9]	6	-7.970 (±0.20)	$2.10x10^{-8}$ ($\pm1.4\ x10^{-8}$)
Oncopeltus fasciatus	Ouabain	5	-2.829 (±0.02)	$1.49x10^{-3}$ ($\pm6.5x10^{-5}$)
Oncopeltus fasciatus	Glucopyranosyl frugoside [5]	6	-3.512 (±0.11)	$3.53x10^{-4}$ ($\pm7.8x10^{-5}$)
Oncopeltus fasciatus	Glucopyranosyl calotropin [7]	4	-3.006 (±0.07)	$1.02x10^{-3}$ ($\pm1.4x10^{-4}$)
Oncopeltus fasciatus	Frugoside [8]	4	-3.481 (±0.18)	$4.29x10^{-4}$ ($\pm1.8\ x10^{-4}$)
Oncopeltus fasciatus	Gofruside [9]	4	-2.857 (±0.24)	$1.47x10^{-3}$ ($\pm4.8x10^{-4}$)
Sus domesticus	Ouabain	6	-6.256 (±0.06)	$5.80x10^{-7}$ ($\pm8.3x10^{-8}$)
Sus domesticus	Glucopyranosyl frugoside [5]	6	-5.541 (±0.09)	$3.21x10^{-6}$ ($\pm0.7x10^{-6}$)
Sus domesticus	Glucopyranosyl calotropin [7]	6	-5.987 (±0.05)	$1.07x10^{-6}$ ($\pm0.1x10^{-6}$)
Sus domesticus	Frugoside [8]	6	-6.390 (±0.08)	$4.50x10^{-7}$ ($\pm1.0x10^{-7}$)
Sus domesticus	Gofruside [9]	6	-7.140 (±0.12)	$9.0x10^{-8}$ ($\pm3.3x10^{-8}$)

*Number is brackets correspond to Rubiano-Buitrago et al. (2023).

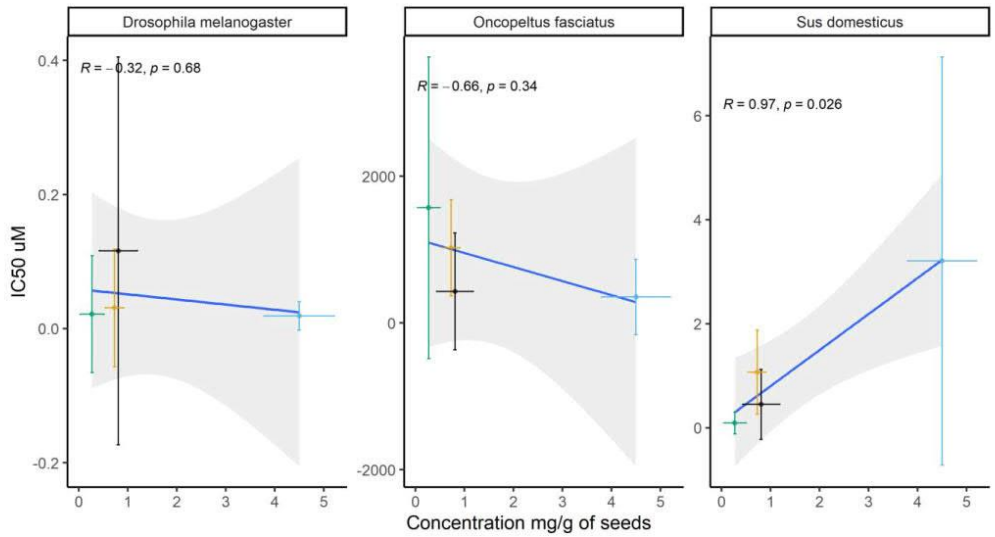
Supplementary Material

5.



Supplementary Figure S4: Selective effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (*O. fasciatus*, blue; non-adapted *D. melanogaster*, green, and *S. domesticus*, pink). Relative inhibition values are based on IC_{50} values compared with the standard to the abundant compound, glucopyranosyl frugoside (computed as the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound; number of replicates between 4 and 6). Log_{10} transformation of the ratio of IC_{50} values (glucopyranosyl frugoside/test compound) results in inhibition weaker than glucopyranosyl frugoside indicated by negative values, while inhibition greater than glucopyranosyl frugoside is indicated by positive values. Specific interactions between Na^+/K^+-ATPases and cardenolides are called "countervailing effects", where cardenolides are more potent than glucopyranosyl frugoside on some Na^+/K^+-ATPases and less potent than glucopyranosyl frugoside on others. Glucopyranosyl frugoside is the most potent of all compounds analyzed for the adapted Na^+/K^+-ATPase.

6.



Supplementary Figure S5. Relationship between concentration and inhibitory effects of cardenolides (mg per g of seeds) across the three analyzed enzymes. Black= Frugoside, Yellow= Glucopyranosyl calotropin, Blue= Glucopyranosyl frugoside, Green=Gofruside.

Supplementary Material

7. Supplementary Table S2: Comparison of the concentration of individual cardenolides available in the *Asclepias curassavica* seeds to those sequestered by adults and nymphs (log transformed data) using analysis of variance.

*Parametric:

Cardenolide	comparison	df	sumsq	meansq	statistic	p.value	adj.p.value
glucopyranosyl 12ß hydroxy coroglaucigenin	seed_nymph	1	16.2359	16.2359	481.82147	2.27×10^{-13}	9.07×10^{-13}
16a hydroxy calotropin	seed_nymph	1	32.3483	32.3483	376.3054	1.53×10^{-12}	3.06×10^{-12}
frugoside	seed_nymph	1	4.260992	4.260992	74.79661	1.99×10^{-7}	2.65×10^{-7}
gofruside	seed_nymph	1	4.866219	4.866219	33.02439	3.00×10^{-5}	3.00×10^{-5}
glucopyranosyl 12ß hydroxy coroglaucigenin	seed_adult	1	7.771768	7.771768	129.09996	4.52×10^{-9}	4.52×10^{-9}
16a hydroxy calotropin	seed_adult	1	26.1799	26.1799	586.729	4.90×10^{-14}	1.96×10^{-13}
frugoside	seed_adult	1	9.669131	9.669131	494.95496	1.84×10^{-13}	3.68×10^{-13}
gofruside	seed_adult	1	21.34477	21.34477	290.63016	1.11×10^{-11}	1.47×10^{-11}

*Non parametric: Wilcoxon rank sum test with continuity correction

Cardenolide	comparison	statistic	p.value	alternative	adj.p.value
glucopyranosyl frugoside	seed_nymph	81	0.00041	two.sided	0.000412
glucopyranosyl calotropin	seed_nymph	81	0.000412	two.sided	0.000412
glucopyranosyl frugoside	seed_adult	81	0.00041	two.sided	0.000412
glucopyranosyl calotropin	seed_adult	81	0.000412	two.sided	0.000412

frontiers

8. Supplementary Table S3: HRMS data of the cardenolides and compounds putatively assigned as cardenolide metabolites found in samples of *Asclepias curassavica* seeds, *Oncopeltus fasciatus* nymphs and adults.

compound *	RT	precursor ion	observed m/z	smart formula	calculated m/z	error	observed m/z	genin fragment formula	calculated m/z	error	Sample**
A	5.6	$[M+H]^+$	663.3001	$C_{34}H_{47}O_{13}$	663.3011	1.5					S
B	5.7	$[M+H]^+$	707.3614	$C_{37}H_{55}O_{13}$	707.3637	3.3					S
3-O-β-glucopyranosyl 12β-hydroxy coroglaucigenin (4)	5.7	$[M+H]^+$	569.2967	$C_{29}H_{45}O_{11}$	569.2956	-1.9	407.2437	$C_{23}H_{35}O_6$	407.2428	-2.3	S-A-N
C	5.8	$[M+H]^+$	715.3516	$C_{35}H_{55}O_{15}$	715.3535	1.9	407.2411	$C_{23}H_{35}O_6$	407.24281	1.7	S-A
D	5.8	$[M+H]^+$	665.2458	$C_{32}H_{41}O_{15}$	665.244	-2.8					S
E	5.9	$[M+H]^+$	693.3127	$C_{35}H_{49}O_{14}$	693.3117	-1.5					S
F	6.1	$[M+H]^+$	553.2998	$C_{29}H_{45}O_{10}$	553.3007	0.9	389.2324	$C_{23}H_{33}O_5$	389.2322	-0.2	A-N
4-O-β-glucopyranosyl-3-O-β-D-allopyranosyl coroglaucigenin (2)	6.2	$[M+H]^+$	715.3553	$C_{35}H_{55}O_{15}$	715.3535	-2.5	391.2494	$C_{23}H_{35}O_5$	391.2479	-3.8	S
16α-hydroxycalotropin (10)	6.2	$[M+H]^+$	549.2703	$C_{29}H_{41}O_{10}$	549.2694	-1.6	407.2459	$C_{23}H_{35}O_6$	407.2428	-7.5	S-A-N

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3-O-β-allopyranosyl coroglaucigenin (1)	6.2	$[M+H]^+$	553.3016	$C_{29}H_{45}O_{10}$	553.3007	-1.8	391.2488	$C_{23}H_{35}O_5$	391.2479	-2.2	S
G	6.2	$[M+H]^+$	636.2463	$C_{31}H_{42}NO_{11}S$	636.2473	1	407.2429	$C_{23}H_{35}O_6$	407.2428	0	N
3-O-β-glucopyranosyl 16β-hydroxycalotropin (3)	6.3	$[M+H]^+$	693.3127	$C_{35}H_{49}O_{14}$	693.3117	-1.5	421.223	$C_{23}H_{33}O_7$	421.2221	-2.2	S
H	6.4	$[M+H]^+$	861.4132	$C_{41}H_{65}O_{19}$	861.4115	-2	391.2488	$C_{23}H_{35}O_5$	391.2479	-2.2	S-A
I	6.4	$[M+H]^+$	553.2982	$C_{29}H_{45}O_{10}$	553.3007	2.5	371.2214	$C_{23}H_{31}O_4$	371.2217	0.3	A-N
J	6.4	$[M+H]^+$	617.2698	$C_{29}H_{46}O_{12}P$	617.2721	2.3	391.2459	$C_{23}H_{35}O_5$	391.2479	2	A-N
4-O-β-glucopyranosyl frugoside (5)	6.5	$[M+H]^+$	699.3592	$C_{35}H_{55}O_{14}$	699.3586	-0.8	391.2484	$C_{23}H_{35}O_5$	391.2479	-1.3	S-A-N
K	6.6	$[M+H]^+$	859.3963	$C_{41}H_{63}O_{19}$	859.3958	-0.6	353.2115	$C_{23}H_{29}O_3$	353.2111	-1	S
L	6.6	$[M+H]^+$	615.2461	$C_{29}H_{43}O_{12}S$	615.247	0.9	371.2199	$C_{23}H_{31}O_4$	371.2217	1.8	A
4-O-β-glucopyranosyl gofruside (6)	6.7	$[M+H]^+$	697.3426	$C_{35}H_{53}O_{14}$	697.343	0.6	371.222	$C_{23}H_{31}O_4$	371.2217	-0.8	S-A
3-O-β-glucopyranosyl calotropin (7)	6.8	$[M-H_2O+H]^+$	677.3163	$C_{35}H_{49}O_{13}$	677.3168	0.6	399.1811	$C_{23}H_{27}O_6$	399.1802	-2.3	S-N
M	6.8	$[M+H]^+$	595.2299	$C_{29}H_{40}O_{11}P$	595.2303	0.4	391.248	$C_{23}H_{35}O_5$	391.2479	0.1	A
N	6.8	$[M+H]^+$	617.2611	$C_{32}H_{41}O_{12}$	617.2593	-1.9					N

O	6.9	$[M+H]^+$	695.3279	$C_{35}H_{51}O_{14}$	695.3273	-0.8	389.2329	$C_{23}H_{33}O_5$	389.2323	-1.7	S
Frugoside (8)	7	$[M+H]^+$	537.3056	$C_{29}H_{49}O_9$	537.3058	0.5	391.2483	$C_{23}H_{35}O_5$	391.2479	-1	S-A-N
P	7.0	$[M+H]^+$	615.2433	$C_{32}H_{39}O_{12}$	615.2436	0.3	405.2266	$C_{23}H_{33}O_6$	405.2272	0.6	A-N
Q	7.1	$[M+H]^+$	547.2545	$C_{29}H_{39}O_{10}$	547.2538	-1.3	371.2224	$C_{23}H_{31}O_4$	371.2217	-2	S
R	7.1	$[M+H]^+$	683.3639	$C_{35}H_{55}O_{13}$	683.3637	-0.2	375.2536	$C_{23}H_{35}O_4$	375.253	-1.8	S
S	7.1	$[M+H]^+$	620.2517	$C_{31}H_{42}NO_{10}S$	620.2524	0.7	391.2468	$C_{23}H_{35}O_5$	391.2479	1.1	A-N
Gofruside (9)	7.2	$[M+H]^+$	535.2897	$C_{29}H_{43}O_9$	535.2902	0.8	371.2218	$C_{23}H_{31}O_4$	371.2217	-0.4	S-A-N
T	7.3	$[M+H]^+$	620.2502	$C_{31}H_{42}NO_{10}S$	620.2524	2.2					N
V	7.4	$[M+H]^+$	543.2468	$C_{23}H_{43}O_{12}S$	543.2469	0.1					N
W	7.5	$[M+H]^+$	533.2748	$C_{29}H_{41}O_9$	533.2745	-0.6	389.2364	$C_{23}H_{33}O_5$	389.2328	-7.19	S-A-N
X	7.6	$[M+H]^+$	579.3159	$C_{31}H_{47}O_{10}$	579.3164	0.4	373.2355	$C_{23}H_{33}O_4$	373.2373	1.8	A-N
Y	7.7	$[M+H]^+$	653.3865	$C_{39}H_{57}O_6S$	653.3870	0.6	389.2318	$C_{23}H_{33}O_5$	389.2322	0.5	N
Z	7.9	$[M+H]^+$	593.3294	$C_{32}H_{49}O_{10}$	593.3320	2.6	355.2265	$C_{23}H_{31}O_3$	355.2268	0.3	A-N

*numbers correspond to the cardenolides in P. Rubiano-Buitrago, S. Pradhan, C. Paetz, H. M. Rowland, New Structures, Spectrometric Quantification, and Inhibitory Properties of Cardenolides from *Asclepias curassavica* Seeds. Molecules 28, 105 (2023).

**(S=seeds, A=*O. fasciatus* adults, N=*O. fasciatus* nymphs)

2.3 Cardenolides in the dorsolateral defensive fluid of adult large milkweed bugs have differential potency on vertebrate and invertebrate predator Na^+/K^+—ATPase

Rubiano-Buitrago, P., Pradhan, S., Aceves, A. A., Mohammadi, S., Paetz, C., Rowland, H.M. Submitted to Ecology letters: Manuscript number ELE-00990-2023.

Cardenolides in the defensive fluid of adult large milkweed bugs have differential potency on vertebrate and invertebrate predator Na^+/K^+–ATPases

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Running title: cardenolide toxicity to predators

Keywords: *Oncopeltus fasciatus*, cardiac glycoside, black-headed grosbeak, toxin–receptor interaction, predator-prey

Type of article: **Letter**

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- number of figures **3**

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Statement of authorship: Conceptualization: PRB, CP, HMR; Methodology: PRB, CP, HMR; Formal analysis: PRB, AAA, HMR; Investigation: PRB, SP, SM; Resources: CP, HMR; Data Curation: PRB, SP, AAA; Writing - Original Draft: PRB, HMR; Writing - Review & Editing:

SP, AAA, SM, CP; Visualization: PRB, AAA; Supervision: CP, HMR; Project administration: CP, HMR; Funding acquisition: HMR

Data accessibility statement: The data supporting the results will be archived in the Max Planck open access data repository Edmond.

Abstract

The defences of aposematic animals are characterised by diversity and variability of secondary metabolites. Here we examine the nature and function of chemical defence diversity in large milkweed bugs, *Oncopeltus fasciatus*, testing the hypothesis that different chemical defence compounds have evolved in response to different enemies. We profiled and quantified the cardenolides sequestered by large milkweed bugs in their defensive secretions and their bodies, and measured the inhibitory properties of a subset of isolated milkweed cardenolides in the insect's defence against the Na^+/K^+—ATPase target site of vertebrate and invertebrate predators, using porcine Na^+/K^+—ATPase data as a reference. We show that highly concentrated coroglaucigenin cardenolides in the insect's defence (glucopyranosyl frugoside and frugoside) are toxic for both resistant and sensitive predators, whereas corotoxigenin and calotropagenin cardenolides have varying degrees of enzyme inhibition among various predators. Overall, *O. fasciatus* is well defended against a range of enemies due to the differential effect of these compounds´ target sites. Our results suggest that the compounds the insect sequester have evolved in response to predation pressure.

INTRODUCTION

Toxins and colourful warning signals characterise the defences used by aposematic species against predators (Eisner *et al.* 2005; Speed *et al.* 2012). Aposematic animals acquire toxins by sequestering plant specialised metabolites (Beran & Petschenka 2022), or by de novo synthesis (Burdfield-Steel *et al.* 2018; Pinheiro de Castro *et al.* 2019). In prey that sequester toxins, variability in the quantity and biochemical profile of chemical defences is common, both within and between species (e.g., in poison frogs, *Dendrobates tinctorius*, (Lawrence *et al.* 2019); *Heliconius* butterflies (Arias *et al.* 2016; Sculfort *et al.* 2020); ladybirds, (Arenas *et al.* 2015); and nudibranchs (Winters *et al.* 2019). Providing and evaluating evolutionary explanations for this variation, as well as alternative explanations, can shed light on the ecological relevance of chemical defence composition, and how natural selection constrains or encourages toxin diversity (Speed *et al.* 2012).

There are a number of explanations for the variability and complexity of defensive chemicals, including the stochastic nature of the environments within which prey organisms exist and develop, selective sequestration of compounds, life history effects on defences, and variation in the selection pressures exerted by predators and other natural enemies (Speed *et al.* 2012). For example, the concentration of sequestered cardenolides in monarch butterflies (*Danaus plexippus*) varies depending on host plant chemistry (Brower *et al.* 1982; Jones *et al.* 2019), and host plant chemistry is impacted by environmental conditions (Agrawal *et al.* 2012). Chemical diversity can also be explained by selective sequestration (Duffey & Scudder 1974; Meredith *et al.* 1984; Lindstedt *et al.* 2010). For example large milkweed bugs (*Oncopeltus fasciatus*) sequester intermediate and more polar cardenolides from milkweeds (Apocynaceae: Asclepiadoideae), even if reared on different host plants that have distinct chemical profiles (Moore & Scudder 1985). The cardenolide profiles of the bugs frequently diverge from those of their host plants (Isman *et al.* 1977). This variance can be attributed to

the metabolic conversion of certain cardenolides into unique compounds, mitigating self-toxicity and minimising the costs of sequestration (Agrawal et al. 2022).

Rubiano-Buitrago *et al.* (2023a) have found higher concentrations of the cardenolides frugoside and gofruside in *O. fasciatus* than is available in the seeds on which they feed. These two compounds differ in potency toward the *O. fasciatus* target site Na^+/K^+—ATPase. Frugoside is the most inhibitory, whereas gofruside is among the weakest inhibitors. The greater sequestration of these compounds could not therefore be explained by their availability in seeds or the biological activity on the bug's target site. An alternative hypothesis is that the sequestration patterns are explained by the effect different cardenolides have on the target sites of natural enemies (Speed *et al.* 2012). For example, just as individual plant compounds are targeted at distinct herbivores (i.e., compound selectivity hypothesis: Ayres *et al.* 1997; Züst *et al.* 2012), the defensive neck fluids of the wood tiger moth, *Arctia plantaginis*, defend against bird predators (but not invertebrates) and abdominal fluids defend against invertebrates (but not birds: Rojas *et al.* 2017). Whether the milkweed bug's chemical profiles are explained by the effectiveness of chemical defences against different natural enemies that vary in resistance to chemical defences is untested (Sih *et al.* 1998; Hoverman & Relyea 2007; Speed *et al.* 2012; Mohammadi *et al.* 2022b).

Direct tests of chemical defences with relevant predators are still rare (Ottocento *et al.* 2022; Lawrence *et al.* 2023). Toxicity has been tested via injecting frog alkaloids into mice (e.g.,Darst *et al.* 2006). Another approach is to test the strength of chemical defence is assessing the mortality of water fleas or brine shrimp (*Daphnia* sp and *Artemia* sp.) after mixing chemical compounds into their water (e.g., Arenas *et al.* 2015; Chan *et al.* 2021; Winters *et al.* 2022). Because cardenolides inhibit the ubiquitous animal enzyme Na^+/K^+ — ATPase (Emery *et al.* 1998; Fedosova *et al.* 2022), and the inhibitory properties of

cardenolides can be tested in vitro (Petschenka *et al.* 2023), functional tests of cardenolide defences on ecologically relevant predators are possible.

In the context of the cardenolide chemical defences, several species of predators have succeeded in breaking through the cardenolide defences of milkweed herbivores (Mohammadi *et al.* 2022b). Flocks of birds including the black-headed grosbeak (*Pheucticus melanocephalus*) and the black-headed oriole (*Oriolus larvatus*), feed on thousands of butterflies in the large overwintering aggregations in Mexico (Fink & Brower 1981; Fink *et al.* 2006). Grosbeaks have evolved amino acid substitutions in their Na^+/K^+ —ATPases, which may confer target-site insensitivity (Groen & Whiteman 2021). Many invertebrate predators are also able to feed on milkweed herbivores (Feir 1974; Mohammadi *et al.* 2022b).

Here, we investigate the identity and quantity of cardenolides sequestered by individual large milkweed bugs adults reared on tropical milkweed seeds in the lab (*Asclepias curassavica*; Rubiano-Buitrago *et al.* 2023a,b). The large milkweed bug has evolved a vacuolated double-layered integument i.e., dorsolateral space (DLS) where it accumulates the cardenolides sequestered from seeds. Upon mechanical stress, adults bugs release a complex mixture of cardenolide-rich fluid throughout exit points in the thin cuticle of the DLS (Scudder *et al.* 1986; Bramer *et al.* 2017). After its release, the fluid is held in droplets which increases the likelihood that predators contact the fluid during subjugation of prey. We analysed the chemical composition of the defensive secretion and examined the inhibitory capacity of the main compounds in the fluid on the Na^+/K^+—ATPases of three biologically relevant predators, a putatively resistant bird (black-headed grosbeak, *Pheucticus melanocephalus*) a generalist bird predator (zebra finch, *Taeniopygia castanotis*), and generalist invertebrate predator (giant Asian mantis, *Hierodula membranacea*). Porcine Na^+/K^+—ATPase enzyme was used as reference. We show that cardenolide content in the bug's defensive fluid and tissues is dominated by non-polar compounds, and that the compounds have differential

effects on the target sites of birds and invertebrate predators. We advance the compound selectivity hypothesis suggesting that specialist herbivores sequester compounds that are toxic to a range of potential enemies. Support for this hypothesis provides an explanation for the diversity of cardenolides found in large milkweed bugs.

MATERIALS AND METHODS

Study species

Oncopeltus fasciatus were obtained from a long-term laboratory colony at the University of Giessen in 2019. This colony originates from the USA and was acquired by the University of Hamburg in 2015. We reared the bugs on sunflower seeds in terrarium boxes (37 × 22 × 25 cm) lined with tissue paper and provided them with ad libitum water in Eppendorf tubes plugged with dental cotton. The boxes were equipped with pieces of cotton wool for oviposition. The colonies were maintained in an incubator at 28 °C and 70% humidity with a day/night cycle of (18:6), with a temperature of 18 °C at night (Polyklima PK 520-LED).

We purchased 25 individual giant Asian mantis in L4 stage (M&M Wust- Mantids and more, Muhlheim am Main, Germany). We reared them individually in double-ventilated boxes (19 × 19 × 19 cm). We provided them with green-bottle fly pupae as a food source, twice per week (two pupae during the nymph stages, and three when they reached the adult stage). Mantids were sprayed with water every two days for hydration. The mantids were kept in an incubator (Snijders Scientific premium, Tilburg, Netherlands with an Imago 500 JUMO controller, Fulda, Germany) at 28 °C and 70% humidity with a day/night cycle of (18:6), with a temperature of 18 °C at night.

Sequestration behaviour, collection of DLS fluid, and extraction of cardenolides from whole bodies

We formed two new colonies of milkweed bugs by selecting 20 mating pairs, and an additional 12 adults and 12 L5 larvae that had been reared on sunflower seeds. The new colonies were provided *Asclepias curassavica* seeds ad libitum. These colonies and their resulting offspring were maintained on the milkweed seeds for five generations.

From the fifth-generation colony, we randomly selected 66 adults (33 males and 33 females, without controlling for reproductive stage), weighed them, and then PRB manually stressed them by squeezing between forceps to elicit the release of the defensive fluids from the dorsolateral space (Bramer *et al.* 2015). PRB collected the defensive fluids from each individual separately, in disposable 1–5 µL micropipettes made of Duran glass with a ringmark (Hirschmann Laborgeräte GmbH, Eberstadt, Germany). The micropipettes with the fluid were washed thoroughly with 100 µL MeOH immediately after collection. The solvent was evaporated in ambient temperature under N_2 gas. Later the fluid was re-suspended in 50 µL of MeOH for LC-MS measurements.

After manual stress, bugs were weighed again, frozen at −80c, and then freeze dried overnight at −85 °C and 0.014 mbar (Martin Christ Alpha 1-2 LD Freeze Dryer, Osterode am Harz, Germany). The dried tissues of the adults were extracted following a method standardised by Pokharel *et al.* 2021. The freeze-dried bugs were placed into a fast prep matrix tube (MP Biomedicals Germany GmbH, Eschwege, Germany) with approximately 450 mg of 2.3 mm zirconium/glass-pellets (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 1 mL of MeOH (Rotisolv 99.9%, Carl Roth GmbH, Karlsruhe, Germany). The sample was homogenised in the FastPrep 24-5G Tissue Homogenizer (MP Biomedicals Germany GmbH, Eschwege, Germany) in two cycles of 45 s at 6.5 m/s, with a pause time of 100 s between cycles. The homogenate was then centrifuged at 16 000 RCF for 3 min, and 700 µL of supernatant collected. We repeated the homogenisation with the addition of 1 mL of MeOH other two times. The three collected supernatants per bug were pooled and washed

with MeOH through a Chromabond HR-X 86 µM cartridge 200 mg (Macherey-Nagel GmbH, Düren, Germany). The extracts obtained were dried under N_2 gas and weighed. They were then diluted in 200 µL of MeOH for LC-MS analysis.

LC-MS spectrometric quantification of *Asclepias* cardenolides in DLS fluid and bodies

We analysed the concentration of eight cardenolides in the DLS fluid and dried tissues of *O. fasciatus* using a linear calibration method for high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-HRMS. See Rubiano-Buitrago *et al.* 2023a; cardenolide standards: glucopyranosyl-12-β-hydroxyl coroglaucigenin, 16α-hydroxycalotropin, allopyranosyl coroglaucigenin, glucopyranosyl frugoside, glucopyranosyl gofruside, glucopyranosyl calotropin, frugoside and gofruside). We injected 1 µL of the re-suspended DLS fluid and 4 µL of the dried tissue extracts of *O. fasciatus* into the HPLC-HRMS and followed the chromatography conditions and quantification parameters described in Rubiano-Buitrago *et al.* 2023a (see also supplementary methods S1).

Functional Na^+/K^+ – ATPase assays

Preparations of Na^+/K^+—ATPases were obtained by homogenisation of dissected brains (*T. castanotis* -zebra finch and *H. membranacea*-giant Asian mantis), from commercially obtained enzyme (*S. domesticus*-domestic pig), or Na^+/K^+—ATPase expressed in cell culture (*P. melanocephalus* -black-headed grosbeak). Zebra finch brain tissues were obtained from a breeding colony at the University of St Andrews, UK, under Home Office license 70/8159. Individual brains were dissected and flash frozen on dry ice before being shipped from St Andrews to the Max Planck Institute for Chemical Ecology, Jena, Germany, where they were kept at −80°C until used in the assays.

Preparation of lysates of zebra finch and giant Asian mantis

We sliced and weighed 5 mg of zebra finch brain tissue, and dissected the head capsule of female giant Asian mantis to obtain the central body with intact optic lobes. Neural tissues were transferred it to a 1mL glass grinder (Wheaton dounce tissue grinder, 1 mL, #357538), and homogenised in 500 µL distilled water. Zebra finch homogenates were transferred to a 50 mL falcon tube on ice and resuspended with 15 mL resuspension buffer (0.25 M sucrose, 2 mM ethylenediaminetetraacetic acid, and 25 mM HEPES/Tris; pH 7.0). Samples were sonicated at 85 W (Fisherbrand Model 120 Sonic Dismembrator, # 12337338) for three 45 s intervals at 0 °C, followed by centrifugation for 30 min at 10,000×g (Sigma 3-18K, #10290) at 4 °C to remove debris. The supernatant was collected and further centrifuged for 60 min at 100,000 × g at 4 °C (Optima Max-XP tabletop ultracentrifuge, #393315) to isolate the membrane fraction. The pelleted membranes were washed twice and resuspended in 1mL MilliQ water (MilliQ- Direct water purification system, Merk, #C85358) and stored at −20 °C. Giant Asian mantis homogenates were frozen in −80 °C and then freeze dried overnight and then resuspended in 1800µl distilled water. Each sample was divided into aliquots of 600µl and sonicated twice in ice water bath (Bandalin Sonorex, #Z659584) for 5 min. Samples were centrifuged at 3000 rpm for 5 min and used to perform functional assays.

Expression of black-headed grosbeak Na+/K+ —ATPase

ATPA1 and ATPB1 genes of black-headed grosbeak were synthesized (Invitrogen GeneArt) and codon optimized for *Spodoptera frugiperda*, and cloned by GeneArt (Invitrogen) in pFastBac Dual (PFBD) plasmid with ATP1B1 under p10 promoter and ATP1A1 under P_{PH} promoter and confirmed by sequencing (accession number 196465). Recombinant Na^+/K^+ —ATPases were expressed after infection of Sf9 cells with P0 virus stock following the optimised baculovirus expression system described by Scholz & Suppmann 2017. The cells were pelleted by centrifugation; resuspended and sonicated to disrupt membranes; and further centrifuged to remove cell debris. Cell membranes were pelleted by ultracentrifugation of the

supernatant and finally resuspended in MilliQ water (see Mohammadi *et al.* 2021) and supplementary methods S2). Prior to the Na^+/K^+ —ATPase inhibition assay, the black headed grosbeak protein were verified by SDS-PAGE/western blotting following the methods of Mohammadi *et al.* 2022a (see supplementary figure S1) and quantified by ELISA (see supplementary methods S2d).

Na^+/K^+ —ATPase inhibition assay

The inhibitory effects of increasing concentrations of four cardenolides (ouabain, glucopyranosyl frugoside, frugoside, and gofruside) to the black headed grosbeak, zebra finch, and giant Asian mantid were determined by photometric measurement of inorganic phosphate released from enzymatic ATP hydrolysis while subtracting the background ATPase activity following Petschenka *et al.,* (2013; see also supplementary methods s2e). The inhibitory effect of glucopyranosyl calotropin was tested for only black-headed grosbeak and zebra finch. The inhibitory effects of the five compounds for the porcine ATPase was taken from Rubiano-Buitrago *et al.* 2023b for comparison. All assays were run in three biological replicates and the average of the two technical replicates of each biological replicate was used for subsequent statistical analyses. Raw data are available in the Max Planck data repository (https://doi.org/10.17617/3.CVRRWV).

Data analysis

Sequestration

To determine the percentage of cardenolide content measured by the available standards and the linear calibration method we summed all areas in the MS trace that we recognised as cardenolides based on the fragmentation patterns and masses, and calculated the percentage of the samples that were not represented by the eight known cardenolides (Table S1, Figure S2).

To compare the concentration of individual cardenolides in the DLS fluid and in the bodies of milkweed bugs, we first log transformed the data for 16α-hydroxycalotropin and glucopyranosyl calotropin, which improved the model fit and allowed analysis by Tukey HSD pairwise contrasts. Transformation of the other six cardenolides did not improve the model fit and we compared the concentrations in the DLS fluid and body using Wilcoxon rank test with a continuity correction. We used a Levene's test to assess the homogeneity of variance of the cardenolides from the different sites of collection.

To determine the concentration of cardenolides in µg per µL of DLS fluid, we calculated the sum of the concentrations of the eight cardenolides present in the DLS fluid. This sum was subsequently divided by the volume ascertained for each individual. We compared the cardenolide content of the DLS fluid between sexes using Wilcoxon rank sum test with continuity correction for compounds exhibiting non-parametric data characteristics, and Tukey multiple comparisons of means for compounds demonstrating parametric data properties. To calculate the µg cardenolide/mg of dry weight of tissue, we divided the sum per dried weight (Brower & Moffitt 1974).

Na^+/K^+ —ATPase inhibition

For cardenolide inhibition we converted the calibrated absorbance values to percentage non-inhibited Na^+/K^+—ATPase activity based on measurements from the control wells. We fitted inhibition curves by nonlinear fitting using a four-parameter logistic curve, with the top and bottom asymptotes set to 100 and zero, respectively, using the nlsLM function of the minipack.lm library in R (R Core Team, 2022). From this we calculated the IC_{50} values for each biological replicate. We compared the log10 IC_{50} values of individual cardenolides for each Na^+/K^+—ATPase enzyme using a linear model (LM) with the reference value set as the inhibitory capacity of each compound against the porcine enzyme. We calculated the fold

differences between the IC_{50} values of individual cardenolides for black-headed grosbeak, zebra finch and giant Asian mantis versus the IC_{50} values on the porcine enzyme.

All analyses were conducted in R (version 1.4.1717).

RESULTS

Defensive secretion volume and sequestered cardenolides in *Oncopeltus fasciatus*

Adults of *Oncopeltus fasciatus* released between 0.2 and 2.0 µL of fluid from the dorsolateral space (DLS) after manual stress (mean ± SE: 0.98 ± 0.06 µL, N=66). Heavier bugs secreted a significantly larger volume of DLS fluid than lighter bugs ($R = 0.6$, $p < 0.0001$; Figure S3). For the DLS fluid, the eight cardenolides that we quantified though linear calibration corresponded to 88.4 ± 0.53% of the total cardenolide area in the samples (Figure S2). For the extract of the bug's tissue, the eight cardenolides represented 72.3 ± 0.7% of the cardenolide area in the LC-MS trace (Figure S2). Two undescribed cardenolides (compounds D and E) were consistently more abundant in the tissue samples compared to the DLS fluid (Figure S4).

Frugoside and gofruside were the most abundant components in the DLS fluid (Figure 1). We did not find glucopyranosyl calotropin in 60% of samples from bug's DLS fluid. The main cardenolides in the dried tissues after depletion of the fluid were frugoside, glucopyranosyl frugoside, and gofruside (Figure 1, and Table S2). The variance in concentration of cardenolides was higher in the DLS fluid compared to the dried tissue samples for all compounds, except 16α-hydroxycalotropin and glucopyranosyl calotropin (Table S3). Seven of the eight cardenolides were significantly higher in concentration in the tissues compared to the DLS fluid (Table S4), with the exception of 16α-hydroxycalotropin that was not significantly different in concentration between the DLS fluid and body tissues (Tukey HSD: estimate = 0.09 ± 0.066, p = 0.25).

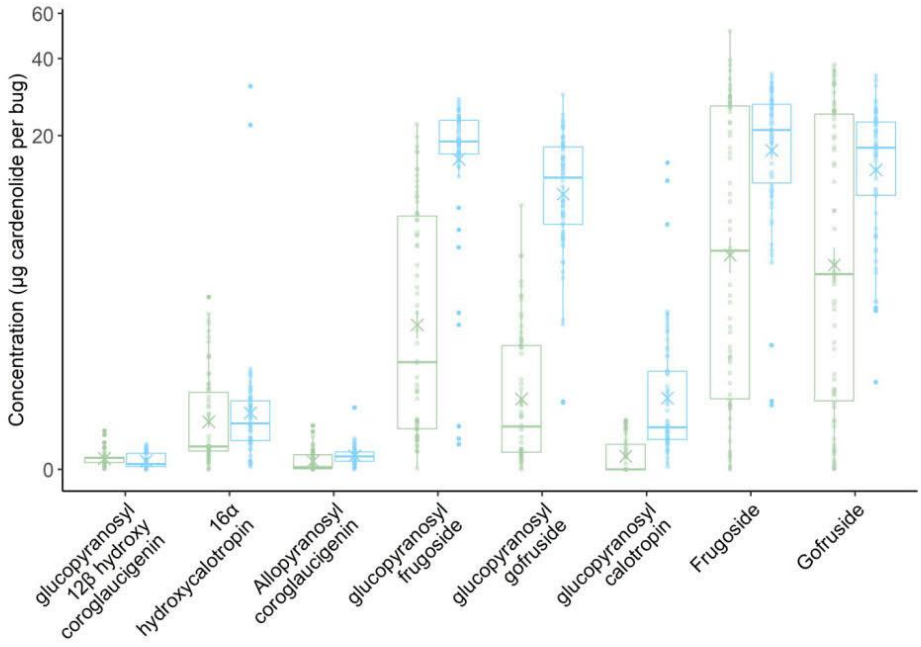

Figure 1. Concentration of cardenolides (ug per bug) in *O. fasciatus* adults in the DLS fluid (green) and in the body tissue after depletion of the fluid (blue; x indicates the mean, N=66). Note that the *y* axis is in pseudo log scale.

Cardenolide toxicity to predators tested by functional Na^+/K^+ - ATPase assay

According to the criteria defined by Agrawal *et al.* (2022), we found that the coroglaucigenin cardenolides (glucopyranosyl frugoside and frugoside) are toxic (IC_{50}<10 µM for black-headed grosbeak, and highly toxic (IC_{50}<1 µM) for zebra finch. For the invertebrate predator, the giant Asian mantis, the monoglycosidic cardenolides (frugoside and gofruside) were highly toxic (IC_{50}<1 µM, Figure 2, Table S5).

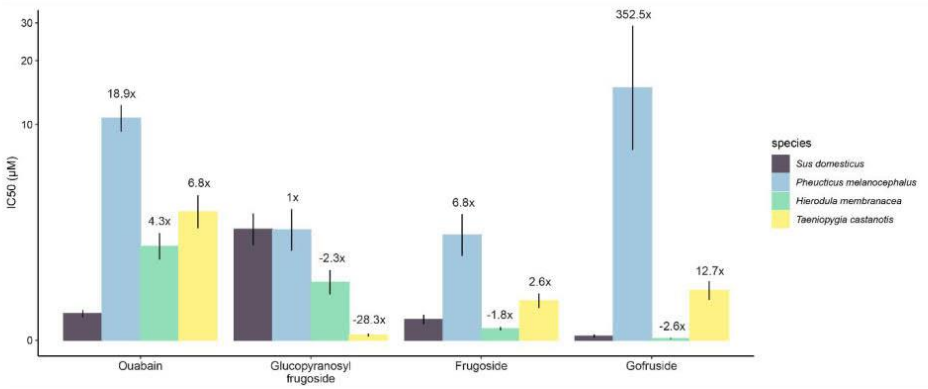

Figure 2 Mean molar concentration of sequestered milkweed toxin necessary to cause 50% inhibition of the predator Na^+/K^+—ATPase enzyme (IC_{50}± SE, N = 4–6 per compound). Higher values on the *y* axis indicate that the enzyme is more resistant to the cardenolide. Note that the *y* axis is in pseudo log scale.

We found a significant interaction between cardenolide and predator Na^+/K^+—ATPase ($F_{9,70}$ = 30.06, p <0.0001) and therefore analysed how different species were affected by each cardenolide, splitting the dataset by compound. We found a significant difference between the species responses to ouabain ($F_{3,18}$ = 41.76, p <0.0001). Ouabain was 18.9 times less inhibitory for black-headed grosbeak than for pig (t (18) =10.75, p < .001), 4.3 times less inhibitory for the giant mantis (t (18) =5.76, p < .001), and 6.8 times less for zebra finch enzyme (t (18) =7.60, p < .001).

Glucopyranosyl frugoside had significantly different effects on the predator Na^+/K^+—ATPase ($F_{3,16}$ = 40.52, p <0.0001; figure S6). It was 28.3 times more inhibitory to zebra finch than to the domestic pig (t (16) =-9.95, p < .001), and 2.3 times more inhibitory to giant mantis (t (16) =-2.58, p=0.02). It was not significantly different in potency for black-headed grosbeak (t (16) =-0.03, p =0.98).

Frugoside had significantly different effects on the predators' sodium pumps ($F_{3,18}$ = 23.43, p <0.0001). It was 6.8 times less inhibitory for black-headed grosbeak than for pig (t (18) =0.80, p < .001), and 2.6 times less inhibitory for zebra finch (t (18) =2.52, p=0.02). For the giant mantis, frugoside inhibited 1.8 times more strongly than for the porcine enzyme, but this is not significant at the alpha 0.05 level (t (18) =2.52, p =0.09).

Gofruside also had significantly different effects on the pumps ($F_{3,18}$= 48.80, p <0.00001). It was 352.5 times less inhibitory for black-headed grosbeak than the porcine enzyme (t (18) =9.58, p < .001). Gofruside was 12.7 times less inhibitory to zebra finch than the porcine enzyme (t (18) =5.02, p< .001) and did not differ in inhibitory potential between the porcine enzyme and the giant mantis (t (18) =-1.68, p =0.11).

The IC_{50} values also differed significantly for glucopyranosyl calotropin (Figure 3; $F_{2, 13}$= 10.55, p= 0.002). Glucopyranosyl calotropin was 15.1 times less inhibitory for black-headed grosbeak than for pig (t(13) = 4.52, p < .001), and 3.4 times less inhibitory to zebra finch than the porcine enzyme, but this was not statistically significant at the alpha 0.05 level (t(13) = 1.32, p = 0.186) (Figure 3).

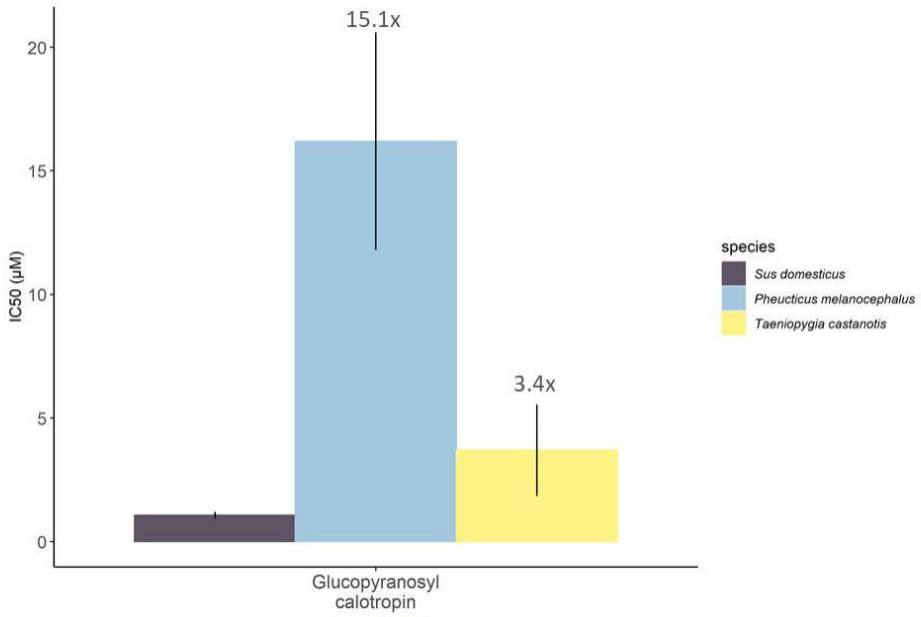

Figure 3. Mean molar concentration of glucopyranosyl calotropin necessary to cause 50% inhibition of the predators' enzyme (IC_{50}) shown as means ± SE, N = 5. Higher values on the *y* axis indicate that the enzyme is more tolerant to the cardenolide.

Discussion

We investigate the nature and function of chemical defence diversity in large milkweed bugs, testing the hypothesis that compound diversity has evolved as a response to multiple enemies. We observed an order of magnitude difference in the variability of cardenolide concentrations in the defensive secretions and body tissues of milkweed bugs. This within-population variation in the quantity of toxins is a typical feature of aposematic animals (Speed *et al.* 2012) but is puzzling if prey survival is dependent on the strength of the defence (Ottocento *et al.* 2023). We also identified variability in the biochemical profile between individuals. Among the eight compounds that we quantified, which collectively constitute 88% of the cardenolides present in the defensive fluid, a specific compound (glucopyranosyl

calotropin) was detectable only in a subset of individuals. Several hypotheses have been put forward to explain the variability observed in defensive profiles, including competition, life history, and additional functions of defensive substances (Speed *et al.,* 2012). Our tests of the multiple enemy hypothesis involved measuring the inhibitory properties of a subset of the individual components against the target sites of several predators. We found that glucopyranosyl calotropin functions as a less potent inhibitor of target sites for both resistant and sensitive bird predators. In contrast, frugoside acts as a robust defence against both categories of predators. Gofruside exhibited varying degrees of enzyme inhibition among various predators, exerting its strongest inhibitory effects on invertebrate predators, while demonstrating comparatively weaker inhibition towards the target site of the resistant predator. Whilst defence against predators is perhaps the function most often associated with sequestration and defensive secretions, our results add to the growing literature showing the ecological relevance of the chemical composition of sequestered defences (Rojas *et al* 2017; Lawrence *et al* 2023; Ottocento *et al* 2023), and the evolutionary explanations for toxin diversity (Speed *et al* 2012).

The total cardenolide concentration of the bugs measured in this study ranged from 25.4 µg to 208.7 µg per bug. This range is similar to Isman (1977) who reported that some milkweed bugs lack cardenolides (or have levels of cardenolides below the detectable limit of the measuring equipment), whereas others contained up to 375 µg. The variability in the bug's sequestration behaviour in our study cannot alone be accounted for by the cardenolide content of the host plant, because the bugs in our study were provided with an ad libitum supply of seeds of *Asclepias curassavica*. This within population variation may reflect genetic differences in the individuals' capacity to sequester (Freedman *et al.* 2020), differences in individual physiological state (Blount *et al.* 2023; Heyworth *et al.* 2023), or the absence of predation pressure and the relaxed selection in the lab (Ottocento *et al.* 2022). The

higher variance in the concentration of cardenolides in the defensive fluid could also be because we were only able to collect a fraction of the DLS content during manual stress. Duffey and Scudder (1974) suggested that only half of the DLS content is collectable or released upon manual stress, and the total vacuolar volume is difficult to determine (Duffey & Scudder 1974; Scudder *et al.* 1986). The concentration that we measured does, however, take into account the volume collected. The variance could also reflect sex differences in sequestration, but we found no differences between male and female cardenolide concentration in the DLS fluid (see supplementary Figure S5, Table S6, and also Moore & Scudder 1985). But we did not control for the insect's reproductive stage, or age, which might contribute to variation (Heyworth *et al.* 2023). Testing whether predators are able to detect the variation present in the chemical defence will be important for understanding how intraspecific variation in chemical defence concentration is maintained(Lawrence *et al.* 2019).

The diversity of defence compounds that we report in the defensive secretion and bodies of large milkweed bugs are characteristic of many aposematic animals including poison frogs, lepidoptera (Rothschild *et al.* 1979; Trigo 2000; Pentzold *et al.* 2016; Rojas *et al.* 2017), nudibranchs (Faulkner *et al.* 1990; Winters *et al.* 2019), coleoptera (Vogler & Kelley 1998; Triponez *et al.* 2007), and orthoptera (Jones *et al.* 1986). That specialist herbivores concentrate some toxins while not sequestering others has long been known (Seiber *et al.* 1980; Malcolm 1994). A common question about defensive variability is whether it represents 'ecological noise', variation caused by the stochastic nature of prey environments, or is of no adaptive evolutionary significance (Speed *et al.* 2012; Whitehead *et al.* 2022). We previously reported higher concentrations of the cardenolides frugoside and gofruside in the bugs than is available in the seeds on which they feed (Rubiano Buitrago *et al* 2023). These two compounds have contrasting potency toward the *O. fasciatus* target site Na^+/K^+—

ATPase: frugoside is the most inhibitory, whereas gofruside is among the weakest inhibitors. Our present results, which demonstrate the effectiveness of frugoside against diverse predator classes, offer insight into why the bugs sequester a more potent inhibitor specific to their own target site. Our results are consistent with those reported by Lawrence *et al* (2023) who found core alkaloids in poison frog defences that may provide the consistency in protection necessary for aposematic warning signals to be maintained. Our results indicate that gofruside, characterised as a modest inhibitor of the bug's target site, varied in levels of enzyme inhibition among different predators. This implies that relying solely on this cardenolide might not suffice to deter the diverse array of predator types that milkweed bugs encounter. While gofruside does prove effective against invertebrate predators, an optimal defence strategy might necessitate a blend of compounds for maximal effectiveness. Future research could compare the dose–effect curves of the single compounds to the relative ratios of the compounds in the defensive mixture, to determine if the compounds act synergistically, additively, or antagonistically (Richards *et al.* 2016). These types of functional assay could allow new predictions about which cardenolides or combinations of cardenolides influence predator aversive responses (Lawrence *et al* 2023). Understanding how *Oncopeltus* accumulated its enemy fauna over the course of evolutionary time would also help to reveal the assembly of its defence arsenal (e.g., Cavender-Bares *et al.* 2009; Vencl & Srygley 2013). The differential effect of cardenolides on the different predator enzymes gives support for the idea that natural enemies can foster defence diversification and that the assembly of sequestered defensives might depend on which selection pressure that predators impose (Vencl & Srygley 2013; Rojas *et al* 2017). We did not, test non-sequestered cardenolides on predator enzymes because we were unable to isolate them in sufficient concentration or purity (Rubiano-Buitrago *et al.* 2023b). Testing the effects of the non-sequestered compounds on predators and the bugs' performance would also provide more information on the costs or

other constraints on sequestration. For example, particular cardenolides can be detrimental to insect growth (Agrawal *et al* 2021), as well as redox state (Heyworth *et al* 2023). Sequestration might also be constrained by transport and modification of specific compounds (Kowalski *et al.* 2020; Agrawal *et al.* 2022). Testing the uptake, transport, modification, and accumulation of the cardenolides that we found in similar concentrations in the body and defensive fluid (i.e., 16α-hydroxycalotropin) and comparing to those that were more abundant in the bugs than the seeds (frugoside and gofruside, Rubiano-Buitrago *et al.* 2023a) would be useful for establishing whether the bugs use deglycosylation as a key metabolic process when feeding on *Asclepias* (see Agrawal *et al.* 2022).

Conclusion

Previous studies have not revealed whether the accumulation pattern of cardenolides by *O. fasciatus* when consuming *A. curassavica* seeds stems from dietary availability or the potential toxicity to the bug's Na^+/K^+–ATPase enzyme. The results of the present study suggest that the cardenolide sequestered of *O. fasciatus* is shaped by the forces of predation pressure. While cardenolides have long been known for their role in shaping predator-prey interactions, this is the first test in vitro of specific cardenolide on the target site of predators. Such investigations are important for understanding the coevolution of plants and consumers, along with the myriad factors that contribute to the diversification of phytochemicals (Dyer & Jeffrey 2021).

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Supporting information to accompany: Cardenolides in the dorsolateral defensive fluid of adult large milkweed bugs have differential potency on vertebrate and invertebrate predator NA^+/K^+— ATPases

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Contents

1. Methods: HPLC-HRMS quantification

We injected 1 µL of the re-suspended DLS fluid and 4 µL of the dried tissue extracts of O. fasciatus into an HPLC-HRMS Agilent 1260 Infinity, fitted with a reversed-phase column Agilent Poroshell 120 column (2.7 µm particle size, 4.6 × 50 mm). The mobile phase consisted of acetonitrile (ACN, supplied with 0.1% formic acid, FA, Carl Roth GmbH, Karlsruhe, Germany) and water (HPLC grade, 0.1% FA, deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany). The elution gradient started with ACN/H2O (5:95) for 1 min, then to ACN/H2O (95:5) for 8 min, which was maintained for 2 min. Later, it was set back to ACN/H2O (5:95) for 1 min. The gradient had a total length of 12 min. HRMS data was recorded on a Bruker Compact OTOF spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Electrospray ionization (ESI) in positive ion mode was used for the analysis in full scan and auto MS/MS modes, scanning masses from m/z 50–1300. Sodium formate adducts were used for internal calibration with a Quadratic + HPC mode. Bruker Compass ver.1.9 (OTOF Control ver.5.1.107 and HyStar 4.1.31.1) was used for data acquisition and instrument control, and Bruker DataAnalysis version 5.1.201 was used for data processing.

We extracted the ion chromatogram of each cardenolide and measured the corresponding peak area. Using the concentration per injection, we calculated each cardenolide in the DLS fluid and the dried tissue samples as µg of cardenolide per bug. For the content in the DLS fluid, values were adjusted using the volume of fluid per bug, to have it as µg of cardenolide per µL of fluid. For the analysis of the tissues content adjusted by weight, we divided the concentration obtained per bug over the lyophilized weight, to have it as µg of cardenolide per mg of dried weight (d.w) tissue.

2. Methods on expression of black-headed grosbeak Na,K-ATPase

a) Synthesis of ATPA1 and ATPB1 genes of black-headed grosbeak. ATPA1 and ATPB1 genes of black-headed grosbeak were synthesized (Invitrogen GeneArt) and codon optimized for *Spodoptera frugiperda.* The genes were cloned by GeneArt in pFastBac Dual (PFBD) plasmid with ATP1B1 under p10 promoter and ATP1A1 under P_{PH} promoter and confirmed by sequencing. The plasmid construct was deposited at Addgene repository under accession numbers 196465.

b) Construction of recombinant baculoviruses. The PFBD vector was used to transform *Escherichia coli* DH10Bac cells harbouring the baculovirus genome (bacmid) and a transposition helper vector (Thermo Fisher Scientific; #10361012) according to the manufacturer's protocol. Recombinant bacmids were selected by blue-white screening method in agar plates containing kanamycin (50 µg/mL), gentamycin (7 µg/mL), tetracycline (10 µg/mL), X-gal (100 µg/mL) and IPTG (40 µg/mL). Positive (white) colonies were confirmed by PCR using the M13 primers (Forward primer: 5'-CCCAGTCACGACGTTGTAAAACG-3', Reverse primer: 5'-AGCGGATAACAATTTCACACAGG-3') as recommended by manufacturer. Production of the recombinant Na^+/K^+ —ATPase α-β subunit proteins followed a modified protocol described by Scholz & Suppmann 2017. The transfection mix was prepared in 100 µL PBS with 10 µg recombinant bacmid and 20 µl PEI-MAX (1mg/mL) per biological replicate. After 30 min of incubation, the transfection mix was added to actively growing Sf9 cells (8× 106 cells/mL) in 10 mL of Insect-Xpress medium (Lonza; #BE12-730P10) with 30 µg/ml gentamycin. Transfected cells were incubated at 27°C and 120rpm (VWR 3500I Incubating Orbital Shaker, #12620-946), and amplified viruses were harvested after 5 days by pelleting the cells (Eppendorf centrifuge 5810 R) and isolating the supernatant (P0 virus stock).

c) Production of recombinant proteins. For production of black-headed grosbeak Na^+/K^+ —ATPase, Sf9 cells were infected with 500 µl of P0 virus stock at multiplicity of infection of 1-3. Sf9 cells ($5x10^6$ cells/mL) were cultured in 50 mL Insect-Xpress media with 30 µg/mL gentamycin at 27°C and 120rpm. 72h following infection, Sf9 cells were harvested by centrifugation at 3000rpm for 10 min. Cell pellets were immediately stored in -80°C. Frozen pellets were homogenized in 15 mL resuspension buffer on ice.

The cell suspension was sonicated at 85W (Fisherbrand Model 120 Sonic Dismembrator, # 12337338) for three 45 s intervals on ice. The cell suspension was then centrifuged for 30 min at 10,000×g at 4 °C to remove debris (Sigma 3-18K, #10290). The supernatant was collected and further centrifuged for 60 min at 100,000 × g at 4 °C) to pellet the cell membranes (Optima Max-XP tabletop ultracentrifuge, #393315). The pelleted membranes were washed twice and resuspended in 1mL MiliQ water and stored at −20 °C. Protein concentrations of Na^+/K^+ —ATPase was determined by ELISA. Three biological replicates of black-headed grosbeak Na^+/K^+ —ATPase were used in the functional assays.

d) Quantification of proteins by Enzyme Linked Immunosorbent Assay (ELISA). We followed the protocol as described by Löptien *et al*. (unpublished). First, 100 µL of diluted samples (brain tissue extracts of zebra finch, and membrane isolates of black-headed grosbeak Na^+/K^+ —ATPase) and relative standards were added to a 96-well polystyrene, slightly hydrophilic flat-well plate (Immulon™ 2 HB, Thermo Fisher Scientific, Massachusetts, USA; #6302) in duplicate (two technical replicates). The standards were prepared from an aliquot of lyophilized black-headed grosbeak membrane isolate as described by Löptien *et al*. (unpublished). The plate was sealed and incubated overnight at 4 °C (12-18 h). The next day, non-coated leftovers were removed from the wells and washed five times with washing buffer (Phosphate-buffered saline – 137 mM NaCl, 1.47 mM KH_2PO_4, 7.749 mM Na_2HPO_4, 2.683 mM KCl with 0.05 % Tween® 20). Next, non-specific binding sites were blocked by adding 200 µL of blocking buffer (1% BSA in PBS + 0.02% Tween® 20) and incubating at room temperature for 2 h. Following the blocking, the wells were washed another five times with washing buffer. Na^+/K^+—ATPases were detected by adding 50 µL of primary antibody solution containing 2 µg/mL antibody (α5-antibody; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; RRID:AB 2166869) in blocking buffer and incubating for 1 h at room temperature, then washed five times with washing buffer. To detect the primary antibody, 50 µL of secondary antibody solution containing 10 µg/mL HRP-conjugated goat-anti-mouse antibody (HRP-antibody; Dianova, Hamburg, Germany; RRID: AB_2617176) in blocking buffer was added and incubated for 1 h at room temperature. Following secondary antibody incubation, wells were washed seven times with washing buffer. The secondary antibody was stained by adding 100 µL 3,3',5,5' tetramethylbenzidine (TMB; Merck/Sigma-Aldrich; Cat#T4444) to each well, and then left to incubate at room temperature for 10 mins. Staining was stopped by adding 100 µL 0.5 M sulfuric acid to each well. Lastly, absorbance was measured at 495 nm using a plate reader (BMG CLARIOstar Plus).

e) Na^+/K^+ —ATPase activity and inhibition assay. For each compound, 200 ug of zebra finch and black-headed grosbeak protein, 30 µL of mantis sample, and 0.05 U/mL of purified porcine Na^+/K^+—ATPase was pipetted into an eight-well row of a 96-well flat-bottom microplate containing stabilizing buffers (see buffer formulas in Petschenka *et al.* 2013, 2023). The first six wells contained exponentially decreasing concentrations of the compound (10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M, 10^{-8} M, dissolved in a final concentration of 2% DMSO). The seventh well was exposed to 2% DMSO only (experimental control). The eighth well contained a combination of an inhibition buffer lacking KCl and 10^{-3} M of the respective compound or ouabain (Sigma Aldrich; CAS #11018-89-6) that completely inhibit the Na^+/K^+—ATPase activity and allow us to measure background ATPase activity (see Petschenka *et al.* 2013). The proteins were incubated at 37°C and 200 rpms for 10 minutes on a microplate shaker (BioShake iQ; Quantifoil Instruments, Jena, Germany; #1808-0506). Next, ATP (Adenosin-5-triphosphat Bis-(Tris)-salt hydrate; Merck/Sigma-Aldrich; CAS#102047-34-7) was added to each well and the proteins were incubated again at 37°C and 200 rpms for 20 minutes. The activity of Na^+/K^+—ATPases following compounds/ouabain exposure was determined by quantification of inorganic phosphate (Pi) released from enzymatically hydrolysed ATP. Released Pi levels were measured according to the procedure described by Taussky *et al.* 1953. Absorbance for each well was measured at 700 nm with a plate absorbance reader (BMG CLARIOstar Plus)(Taussky *et al.* 1953). All assays were run in 3 biological replicates and the average of the two technical replicates of each biological replicate was used for subsequent statistical analyses. Raw data are available in the Max Planck Data repository (https://doi.org/10.17617/3.CVRRWV).

3. Western blot

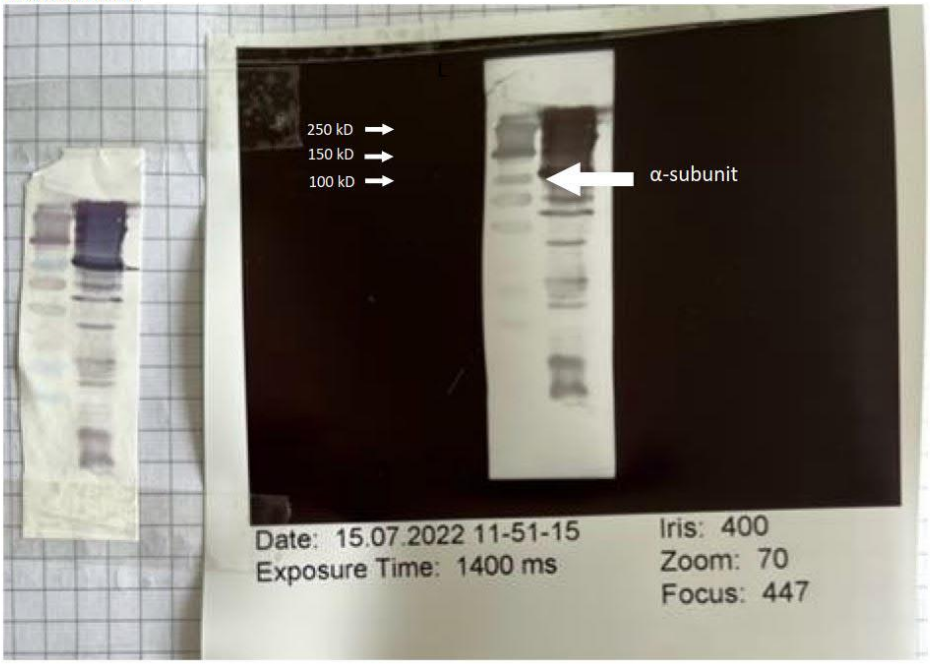

Figure S1. Western blot analysis of Na^+, K^+-ATPase with engineered *Pheucticus melanocephalus* ATP1A1 (α-subunit) aliquot used to produce the standards for the ELISA in this study. The 110 kDa α-subunits are stained with the α5 monoclonal antibody followed by a horseradish peroxidase conjugated goat anti-mouse antibody. In total, 5 µl of undiluted membrane isolate was run on the western blot. The letter "L" indicates the protein ladder.

4. The percentage of samples that were not represented by the eight known cardenolides

Table S1. Non-elucidated cardenolides present in the samples of *O. fasciatus* adults

Retention time	Cardenolide	Reference*	observed m/z
5.8	A	C	715.3516
6.1	B	F	553.2998
6.4	C	H	861.4132
6.4	D	J	617.2698
6.6	E	L	615.2461
6.8	F	M	595.2299
7.0	G	P	615.2433
7.1	H	S	620.2517

7.5	I	W	533.2748
7.6	J	X	579.3159
7.9	K	Z	593.3294

*(Rubiano-Buitrago *et al.* 2023)

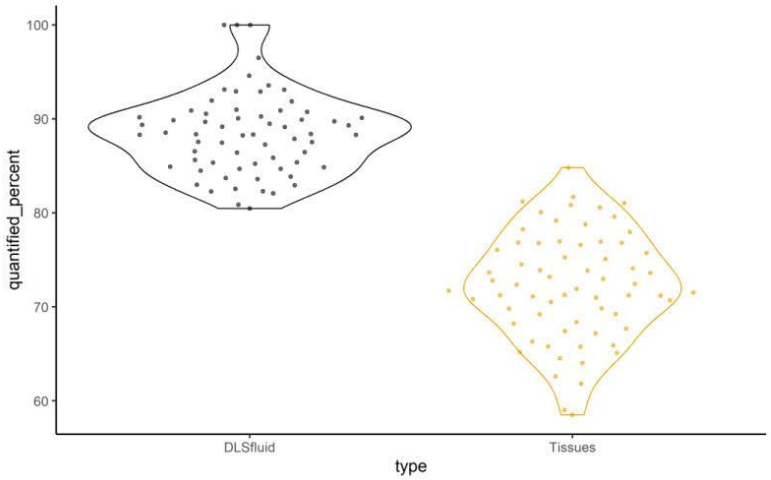

Figure S2. Percent of cardenolide content covered by quantification of the eight isolated *Asclepias* cardenolides.

5. DLS fluid correlation with body mass

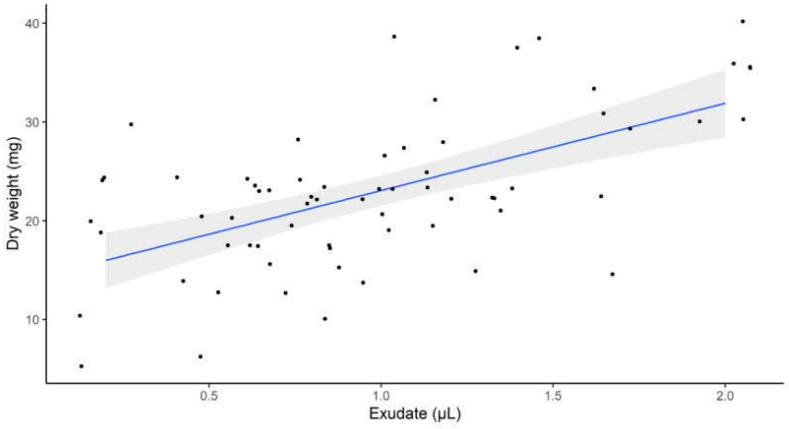

Figure S3. Pearson´s correlation between the volume of DLS fluid per bug and their dry weight (d.w) tissue

6. Distribution of cardenolides

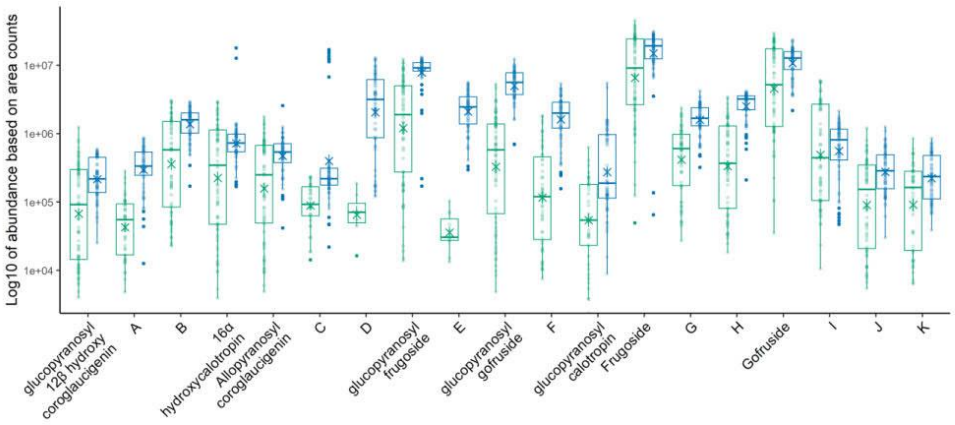

Figure S4. Distribution of abundance of cardenolides-like peaks in *O. fasciatus* adults both in the DLS fluid of the insect (green) and in the body tissue after depletion of the fluid (blue) (x indicates the mean values, N=66). Note the y axis is in $\log_{10}$ scale.

7. Cardenolides in the tissue and DLS fluid

Table S2. Cardenolide concentrations in dried tissue-DLS fluid µg per bug

type	compound	mean	sd	se
DLS fluid	glucopyranosyl 12β hydroxy coroglaucigenin	0.20	0.12	0.01
DLS fluid	16α hydroxycalotropin	0.99	1.07	0.13
DLS fluid	Allopyranosyl coroglaucigenin	0.15	0.19	0.02
DLS fluid	glucopyranosyl frugoside	5.50	5.92	0.73
DLS fluid	glucopyranosyl gofruside	1.69	2.03	0.25
DLS fluid	glucopyranosyl calotropin	0.24	0.35	0.04
DLS fluid	Frugoside	13.24	13.39	1.65
DLS fluid	Gofruside	12.20	12.59	1.55
dried tissue	glucopyranosyl 12β hydroxy coroglaucigenin	0.15	0.13	0.02
dried tissue	glucopyranosyl allopyranosyl coroglaucigenin	0.10	0.15	0.02
dried tissue	16α hydroxycalotropin	1.67	4.54	0.56
dried tissue	Allopyranosyl coroglaucigenin	0.25	0.17	0.02
dried tissue	glucopyranosyl frugoside	18.33	6.23	0.77
dried tissue	glucopyranosyl gofruside	13.29	5.90	0.73
dried tissue	glucopyranosyl calotropin	1.79	2.68	0.33
dried tissue	Frugoside	20.15	8.55	1.05
dried tissue	Gofruside	16.93	7.80	0.96

8. **Table S3** Levene´ test of homogeny of variance, cardenolide concentrations (log10 corrected) in dried tissue-DLS fluid µg per bug

compound	statistic	p.value	df	df.residual
Allopyranosyl coroglaucigenin	58.00	<0.0001	1	128
glucopyranosyl 12ß hydroxy coroglaucigenin	11.27	0.001	1	130
glucopyranosyl frugoside	48.88	<0.0001	1	130
glucopyranosyl gofruside	50.87	<0.0001	1	129
glucopyranosyl calotropin	4.43	0.038	1	90
Frugoside	42.69	<0.0001	1	130
Gofruside	49.22	<0.0001	1	130
16α hydroxycalotropin	0.37	0.544	1	128

9. **Table S4.** Comparison of the cardenolide concentrations (log_{10} corrected) in dried tissue-DLS fluid per bug
*Parametric data – Method: Tukey multiple comparisons of means. 95% family-wise confidence level

Compound	estimate	conf.low	conf.high	adj.p.value
16α hydroxycalotropin	0.0931	-0.0664	0.2527	0.2503
glucopyranosyl calotropin	0.2775	0.0913	0.4637	0.0039

* Non-Parametric data – Method: Wilcoxon rank sum test with continuity correction

compound	statistic	p.value
Allopyranosyl coroglaucigenin	1292	0.0001
glucopyranosyl 12β hydroxy coroglaucigenin	2823	0.0033
glucopyranosyl frugoside	395	<0.0001
glucopyranosyl gofruside	101	<0.0001
Frugoside	1417.5	0.0005
Gofruside	1526.5	0.0030

10. **Table S5**. Log_{10} data of IC_{50} of different model predators

Species	compound	N	Log_{10} IC_{50}	IC_{50} (M)	Toxicity
Pheucticus melanocephalus	Ouabain	4	-4.971(±0.06)	1.10×10^{-5} ($\pm 1.5 \times 10^{-6}$)	Moderately toxic
Pheucticus melanocephalus	Glucopyranosyl frugoside	4	-5.545(±0.12)	3.20×10^{-6} ($\pm 8.0 \times 10^{-7}$)	Toxic
Pheucticus melanocephalus	Glucopyranosyl calotropin	5	-4.908(±0.19)	1.62×10^{-5} ($\pm 4.4 \times 10^{-6}$)	Moderately toxic
Pheucticus melanocephalus	Frugoside	5	-5.588(±0.13)	3.10×10^{-6} ($\pm 8.0 \times 10^{-7}$)	Toxic
Pheucticus melanocephalus	Gofruside	5	-4.843(±0.30)	3.18×10^{-5} ($\pm 1.8 \times 10^{5}$)	Moderately toxic
Hierodula membranacea	Ouabain	6	-5.640(±0.08)	2.50×10^{-6} ($\pm 5.0 \times 10^{-7}$)	Toxic
Hierodula membranacea	Glucopyranosyl frugoside	5	-5.920(±0.12)	1.40×10^{-6} ($\pm 3.0 \times 10^{-7}$)	Toxic
Hierodula membranacea	Frugoside	6	-6.616(±0.05)	3.00×10^{-7} ($\pm 3.3 \times 10^{-8}$)	Highly toxic
Hierodula membranacea	Gofruside	5	-7.544(±0.12)	2.86×10^{-8} ($\pm 1.3 \times 10^{-8}$)	Highly toxic
Taeniopygia castanotis	Ouabain	6	-5.443(±0.09)	4.00×10^{-6} ($\pm 7.0 \times 10^{-7}$)	Toxic
Taeniopygia castanotis	Glucopyranosyl frugoside	5	-7.003(±0.11)	1.00×10^{-7} ($\pm 2.9 \times 10^{-8}$)	Highly toxic
Taeniopygia castanotis	Glucopyranosyl calotropin	5	-5.671(±0.25)	3.70×10^{-6} ($\pm 1.8 \times 10^{-6}$)	Toxic
Taeniopygia castanotis	Frugoside	6	-6.088(±0.08)	9.00×10^{-7} ($\pm 2.0 \times 10^{-7}$)	Highly toxic
Taeniopygia castanotis	Gofruside	6	-5.993(±0.10)	1.20×10^{-6} ($\pm 2.0 \times 10^{-7}$)	Toxic
Sus domesticus	Ouabain	6			Highly toxic
Sus domesticus	Glucopyranosyl frugoside	6			Toxic
Sus domesticus	Glucopyranosyl calotropin	6			Toxic
Sus domesticus	Frugoside	6			Highly toxic

11. Sex differences in *Oncopeltus fasciatus* defence

We found no significant differences in male and female cardenolide concentration in the DLS fluid (ANOVA: $F_{(1,64)}$ =0.301, p = 0.585). The cardenolide content in DLS fluid of large milkweed bugs reared in *A. curassavica* ranged from 1.09 to 113.02 µg per µL, with an average of 36.89 (±3.69) µg cardenolide per µL. We found significant differences in the cardenolide content between the dried tissues of females and males (ANOVA: $F_{(1,64)}$ =6.129, p = 0.016). The cardenolides in dried tissues in females range from 0.505 to 4.42 µg per mg d.w, with an average of 2.98 (±0.13) µg per mg d.w. In males, cardenolides in dried tissues range from 1.70 to 8.98 µg per mg d.w, with an average of 3.66 (±0.24) µg per mg d.w. (Figure S5).

The Levene's test for homogeneity of variance showed that the concentration significantly differed between sexes for, glucopyranosyl 12β hydroxy coroglaucigenin, glucopyranosyl frugoside, glucopyranosyl gofruside, frugoside, 16α hydroxycalotropin and glucopyranosyl calotropin while it did not for allopyranosyl coroglaucigenin and gofruside. Based on these results, we analysed the variance between females and males. We found significant differences in the concentration of allopyranosyl coroglaucigenin, glucopyranosyl frugoside, glucopyranosyl calotropin and gofruside. Concentration of glucopyranosyl 12β hydroxy coroglaucigenin, glucopyranosyl gofruside, frugoside and 16α hydroxycalotropin concentration did not differ between sexes (Figure S5 and Table S6).

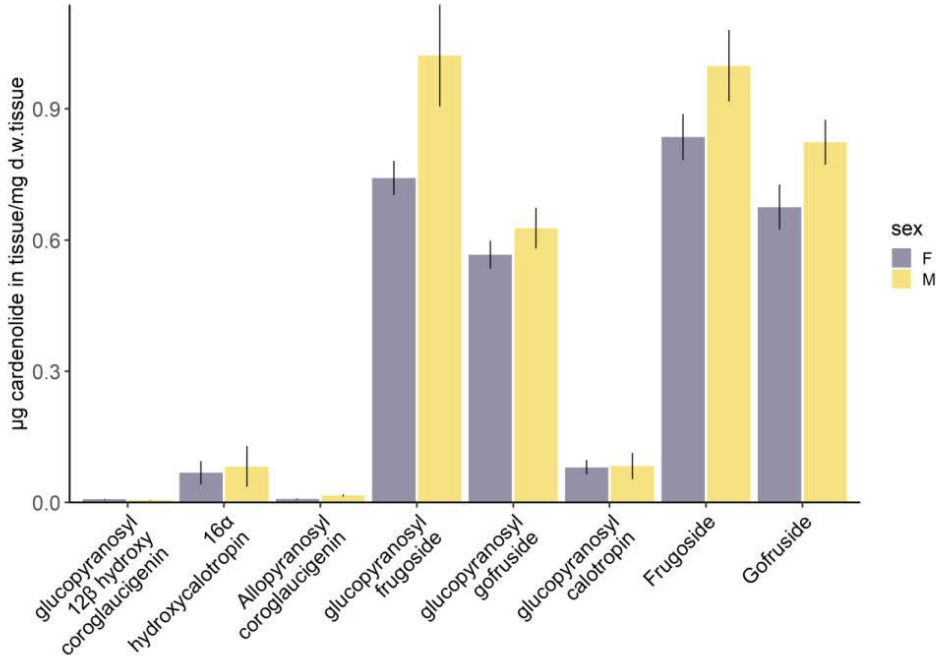

Figure S5. Concentration of the seed cardenolides in the dried tissue of *O. fasciatus* males and females, after depletion of the DLS fluid, values adjusted by the dry weight of each individual (shown as means ± SE, N=33 females, 33 males).

Table S6. Comparison of the cardenolide concentrations (log_{10} corrected) between males and females µg per mg of dried weight

*Parametric data – Method: Tukey multiple comparisons of means. 95% family-wise confidence level

Compound	contrast	estimate	conf.low	conf.high	adj.p.value
Allopyranosyl coroglaucigenin	M-F	0.3523	0.1808	0.5239	0.0001
gofruside	M-F	0.1090	0.0059	0.2120	0.0385

* Non-Parametric data – Method: Wilcoxon rank sum test with continuity correction

compound	statistic	p.value
glucopyranosyl 12β hydroxy coroglaucigenin	670	0.1093
glucopyranosyl frugoside	312	0.0025
glucopyranosyl gofruside	464	0.3071
glucopyranosyl calotropin	769	0.0036
Frugoside	445	0.2057
16α hydroxycalotropin	633	0.2610

12. Directional effects of cardenolides

To calculate the effects of individual cardiac glycosides on different forms of Na^+/K^+—ATPase (adapted or non-adapted), we compared the inhibition to a reference cardenolide by calculating the log_{10} of the ratio (IC_{50} reference compound)/(IC_{50} test compound). We used ouabain (Sigma Aldrich, O3125-1G) as a reference as it is the most widely used cardenolide in research on Na^+/K^+ —ATPase (Petschenka *et al.* 2018). Here, negative values represent compounds that inhibit the Na^+/K^+ —ATPase weaker than ouabain, and positive values represent compounds that inhibit the Na^+/K^+ —ATPase stronger than ouabain.

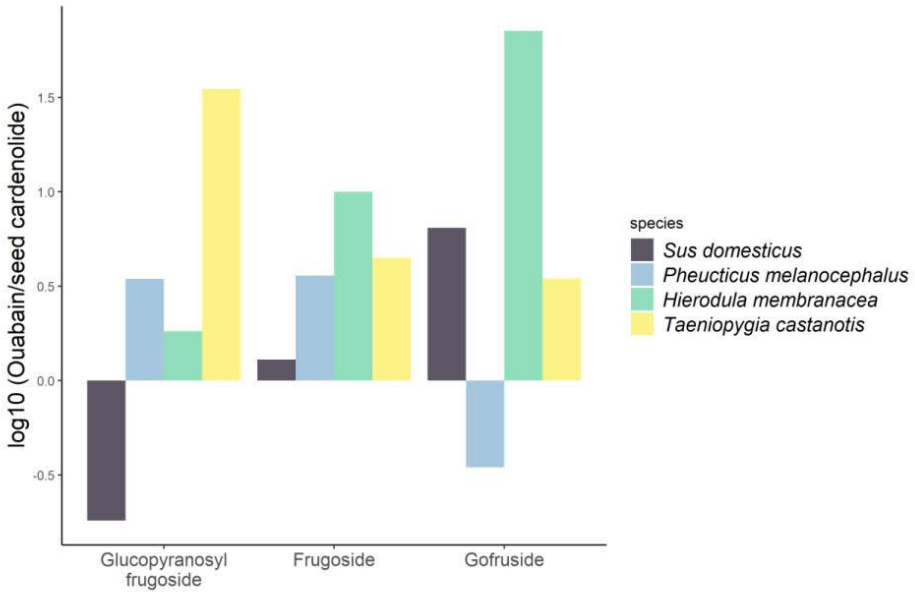

Figure S6 Selective effects of individual cardiac glycosides on different forms of Na^+/K^+ —ATPase. Relative inhibition values are based on IC_{50} values compared with the standard ouabain (computed as the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). Inhibition weaker than ouabain is indicated by negative values, while inhibition greater than ouabain is indicated by positive values.

Frugoside had a unidirectional effect on the four enzymes, and was more inhibitory than ouabain across all enzymes tested. Glucopyranosyl frugoside and gofruside produced countervailing effects. Gofruside was weaker than ouabain for the *P. melanocephalus* enzyme but was the stronger inhibitor for the invertebrate predator Na^+/K^+ —ATPase enzyme.

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3 Discussion

Cardiac glycosides represent a diverse group of secondary metabolites commonly found in plants (Seiber et al., 1983; Agrawal et al., 2012). Within the genus *Asclepias*, milkweed plants are known for their cardenolides, which play a well-established role in plant-herbivore and predator-prey interactions (Duffey and Scudder, 1972; Berenbaum and Miliczky, 1984; Agrawal et al., 2021; Beran and Petschenka, 2022). This role stems from their ability to inhibit the membrane-bound Na^+/K^+—ATPase enzyme (Dobler et al., 2011; Agrawal et al., 2012; Krishna, 2015; El-Seedi et al., 2019).

Milkweed seeds serve as a primary food source for specialist lygaeid bugs, which happen to be among the most cardenolide-tolerant insects in existence (Bramer et al., 2015; Lohr et al., 2017; Agrawal et al., 2022). It is highly likely that these insects exert natural selection pressures that drive the repeated modification of cardenolides (Ehrlich and Raven, 1964; Salazar et al., 2018; Massad et al., 2022). To explore this hypothesis, the first essential step was to conduct a comprehensive phytochemical analysis focused on the cardenolides present in the seeds of a milkweed species (*Asclepias curassavica*). This analysis showed that the cardenolide defence within the seeds exhibited several facets of variation, including differences in their overall concentration, the polarity of individual PSMs, and structural substitutions that could potentially influence the molecule's reactivity (see section 3.1).

The cardenolide diversity in the seeds may well represent the plant's adaptive response to natural selection, driven by herbivores' resistance, which is a hallmark of phenotype-matching—a result of the ongoing coevolution between defensive and offensive traits (Berenbaum and Zangerl, 1998; Brodie et al., 2002; Zangerl and Berenbaum, 2003). To test this, the second step was to evaluate the degree of phenotypic alignment between the cardenolide composition in *A. curassavica* seeds and the cardenolides sequestered by both nymphs and adults of the specialised seed herbivore, *Oncopeltus fasciatus* (Lygaeidae). Additionally, the inhibitory capabilities of a selected subset of seed cardenolides were tested on the Na^+/K^+—enzyme of *O. fasciatus*, as well as on a non-adapted insect, *Drosophila melanogaster*. To provide a broader context, this was also compared to the inhibitory effects on the highly sensitive porcine Na^+/K^+—ATPase. This

analysis showed that there was no clear trend where concentration, polarity, or chemical structure could explain the cardenolide composition in the seeds and the specialist insect.

An alternative hypothesis for the observed sequestration patterns may be attributed to the varying impacts different cardenolides have on the specific target sites of natural enemies, as suggested by Speed et al. (2012). The third step in this thesis was to test whether, just as individual cardenolides exhibit selectivity towards distinct herbivores (referred to as compound selectivity, as shown in studies by Ayres et al., 1997; Züst et al., 2012), the chemical profiles of milkweed bugs (and in turn the compounds produced by the plant) could be explained by the efficacy of chemical defences against various predators that exhibit differing levels of resistance to cardenolides, as discussed by Sih et al., (1998); Hoverman and Relyea (2007); Speed et al., (2012) and Mohammadi et al., (2022).

Thus, this step involved an analysis of the chemical composition of the defensive secretion in milkweed bug adults (DLS fluid), along with an assessment of the inhibitory capacity of the main cardenolides within the fluid against three biologically relevant predators: a bird species presumed to be cardenolide-resistant (black-headed grosbeak, *Pheucticus melanocephalus*), a generalist bird (zebra finch, *Taeniopygia castanotis*), and a generalist invertebrate (giant Asian mantis, *Hierodula membranacea*). The results indicate that the cardenolide content in both the bug's DLS fluid and its tissues is primarily composed of non-polar cardenolides. Additionally, these compounds exhibit distinct effects on the target sites of birds and invertebrate predators. These observations give support to the compound selectivity hypothesis, suggesting that specialist herbivores sequester PSMs that possess toxicity against a broad spectrum of potential enemies. Altogether, the three parts of this thesis offer some explanations for the diverse array of cardenolides found within tropical milkweed seeds and large milkweed bugs, shedding light on the mechanisms governing their defensive strategies.

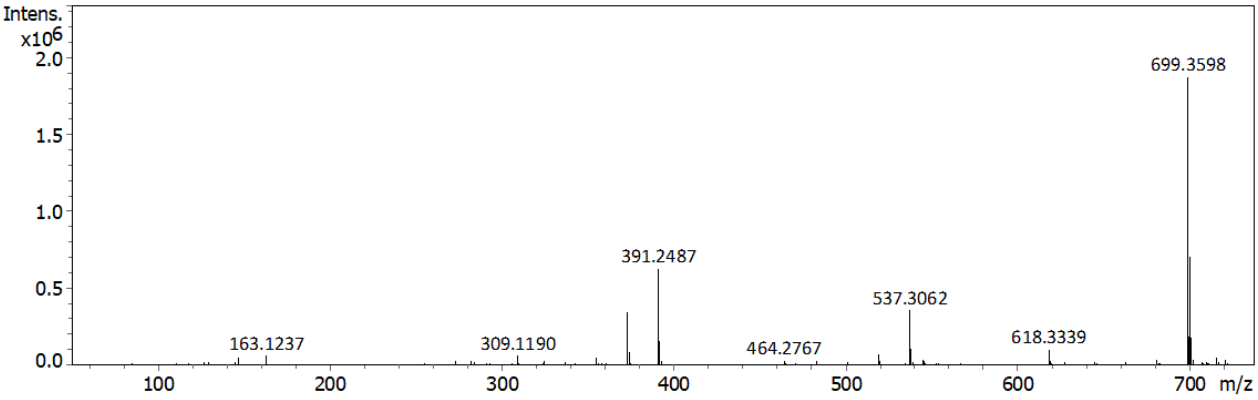
3.1 Phytochemical diversity in tropical milkweed seeds

This thesis investigated chemical diversity in the seeds of the tropical milkweed (*Asclepias curassavica*) (Chapter 2.1-2.2). *A. curassavica* is a relevant species in the analysis of milkweed-herbivore systems, due its presence in areas occupied by southern populations of large milkweed bugs (United States Department of Agriculture; Miller and Dingle, 1982).

The tropical milkweed serves as a model species due to its abundant and diverse composition of cardenolides (Züst et al., 2019; Agrawal et al., 2021; Blount et al., 2023).

Using HPLC-DAD-MS data of the crude extract of this *A. curassavica* seeds, cardenolide-like peaks were traced by *m/z* fragmentation patterns analysis (Figure 5) and their characteristic UV spectra-i.e. maximum absorbance between 216-222 nm(Petschenka et al., 2023). 21 compounds could be assigned as cardenolides (see Supplementary Information chapter 2.2). In a targeted chemical study of the tropical milkweed seeds, 10 of the 21 tentative cardenolides were purified, elucidated by HRESIMS and 1D and 2D NMR data, and quantified by mass spectrometry. The characterised cardenolides included three new compounds: 3-[4'-*O*-β-glucopyranosyl-β-allopyranosyl] coroglaucigenin (**2**), 3'-*O*-β-glucopyranosyl-15- β-hydroxycalotropin (**3**), and 3-*O*-β-glucopyranosyl-12-β-hydroxyl coroglaucigenin (**4**), as well as six previously reported cardenolides (4'-*O*-β-glucopyranosyl frugoside (**5**), 4'-*O*-β-glucopyranosyl gofruside (**6**), 3'-*O*-β-glucopyranosyl calotropin (**7**), frugoside (**8**), gofruside (**9**), and 16α-hydroxycalotropin (**10**). 3-*O*-β-allopyranosyl coroglaucigenin (**1**) although described as new in chapter 2.1, this had been previously reported by Ghoraba et al., (2017).

The results reported in chapter 2.1 are in line with previous research which found up to 21 cardenolides across all plant material of *A. curassavica* (Abe et al., 1992; Li et al., 2008, 2009; Warashina and Noro, 2008; Warashina et al., 2008; Zhang et al., 2014; Ji et al., 2022). The isolated cardenolides have either of these three steroidal cores: coroglaucigenin, corotoxigenin and calotropagenin (Seiber et al., 1983). Cardenolides in *Asclepias* seeds varied widely in quantity. Quantifiable cardenolides range from 0.004 (compound **4**) to 4.5 (compound **5**) mg/g of seeds, and several in trace amounts (Wetzel and Whitehead, 2020; Rubiano-Buitrago et al., 2023).



Ion	Fragment	*m/z* observed	Formula	*m/z* theoretical	Error (ppm)
A		699.3598	$C_{35}H_{55}O_{14}^{+}$	699.3586	1.8
B	A-[$C_6H_{10}O_3$]	537.3062	$C_{29}H_{45}O_9^{+}$	537.3058	0.7
C	B-[$C_6H_{10}O_4$]	391.2487	$C_{23}H_{35}O_5^{+}$	391.2479	2.0
D	C-[H_2O]	373.2374	$C_{23}H_{33}O_4^{+}$	373.2373	0.3
E	D-[H_2O]	355.2268	$C_{23}H_{31}O_3^{+}$	355.2266	0.6

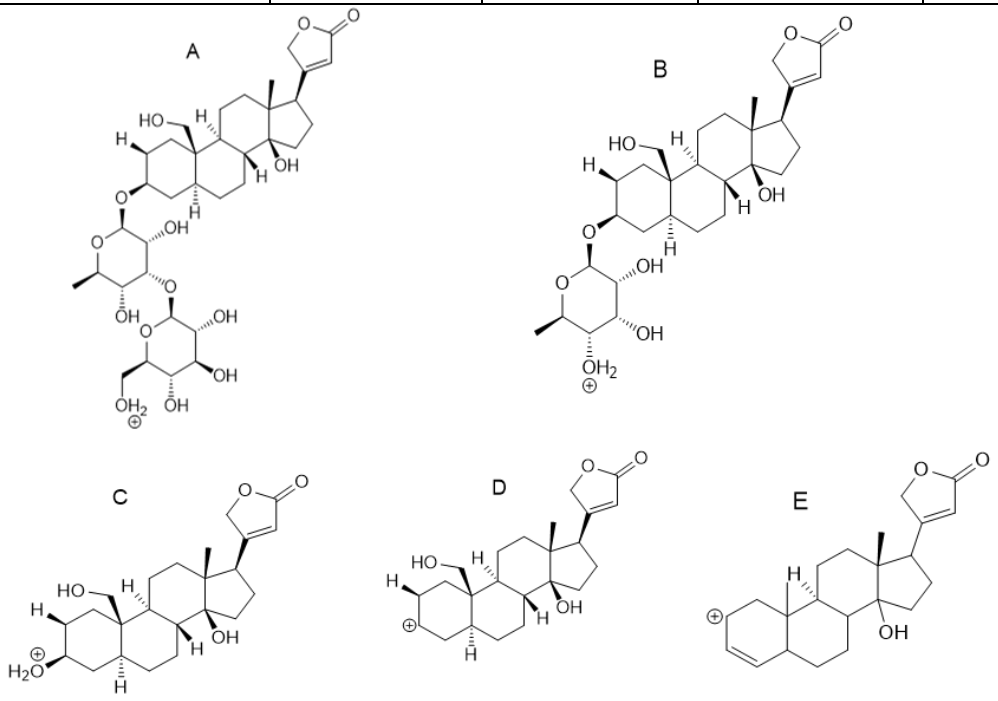


Figure 5. Scheme of one of the fragmentation pattern analysis in the mass spectra of *Asclepias* compounds. In this case, the major chromatogram peak, later identified as glucopyranosyl frugoside (**5**). We determined the list of *m/z* of interest through the study of mass spectra of commercial cardenolides, theoretical fragmentation of reported *Asclepias* cardenolides and previous studies of PSM of other Apocynaceae. (Ravi et al., 2020) (*m/z* 271.09, 273.09, 351.2, 353.2, 355.2, 357.2, 359.2, 369.2, 371.2, 373.2, 375.2, 377.2 389.2).

Chapter 2.1 focused on the cardenolides of just one milkweed tissue, the seeds. Although the tropical milkweed seeds have been the subject of chemical study by Abe et al., (1992), the results reported in this thesis include new compounds that were not previously described, and expanded on that research by testing the functional capacity of cardenolides **1**–**2** and **5**–**10** on inhibitory potency against the sensitive porcine Na^+/K^+—ATPase. There was a significant variation in inhibition of this unadapted enzyme, with IC_{50} values ranging from 3.67 x 10^{-6} to 9.65 x 10^{-8} M. The most abundant compound in the seeds, 4'-*O*-β-glucopyranosyl frugoside (**5**), was the least inhibitory compound, along with the minor compound 16α-hydroxycalotropin (**10**). Gofruside (**9**) was the most inhibitory seed cardenolide for the porcine enzyme.

Compounds **1** and **8** are both derivatives of coroglaucigenin, featuring allosyl and allomethylosyl substitutions, respectively. Interestingly, despite their structural differences, they exhibited similar inhibition properties. Conversely, compounds **8** and **9** share identical glycosylation patterns, with the only distinction being the oxidation state of C-19 (alcohol vs. aldehyde). The fact that compound **9** exhibited higher inhibition compared to **8** strongly suggests that the aldehyde group plays a pivotal role in this difference. This discrepancy could be attributed to the heightened reactivity of compound **9** towards biomolecules. However, it's worth noting that 16α-hydroxycalotropin **10** also contains an aldehyde, yet it displayed the lowest inhibitory capacity. This cardenolide also has a highly substituted sterol and multiple chiral centers. Interestingly, prior research reported that calotropin, the 16-deoxy counterpart of **10**, exhibited an IC_{50} of 2.7×10^{-7} M ($\log_{10}$: −6.56) against porcine Na^+/K^+—ATPase (Meneses-Sagrero et al., 2022). This disparity in inhibition may be linked to the 16α-hydroxylation, potentially interfering with binding to the biological target.

The results in chapter 2.1 also revealed reduced inhibitory potential when glycosylation of cardenolides occurred. Specifically, compounds **8** and **5**, both of the coroglaucigenin type, and **9** and **6**, of the corotoxigenin type, only differed in terms of glycosylation, with the aglycones exhibiting higher inhibition potential. Conversely, compounds **1** and **2**, which differ in the presence of glucose as a second sugar unit, exhibited no significant difference in their inhibitory potential when compared to each other. One plausible explanation for this phenomenon could be the increased bulkiness of the molecules due to the higher

degree of glycosylation in both cases, potentially hindering access to the active site of the Na^+/K^+—ATPase. These results regarding Na^+/K^+—ATPase inhibition by milkweed cardenolides align with previous findings by Petschenka et al., (2018), who conducted a comparative study of cardenolide structural characteristics versus Na^+/K^+—ATPase inhibition. They observed varying responses in the inhibition of vertebrate Na^+/K^+—ATPase by cardenolides with similar aglycones but different glycosylation patterns.

Given the different concentrations that I found in the seeds and the range of inhibitory potency of these compounds in the sensitive pump, I moved forward analysing the functional capacity of a subset of major seed cardenolides against the potential selection imposed by herbivory from the large milkweed bug-*Oncopeltus fasciatus*.

3.2 Oncopeltus fasciatus as driver of phytochemical diversity in *Asclepias curassavica*

To understand the ecological pressures that might explain the cardenolide content of *A. curassavica* seeds, I conducted a comprehensive analysis of the cardenolides sequestered by the specialised milkweed herbivore, *Oncopeltus fasciatus*, at two distinct stages of its life cycle while feeding on *A. curassavica* seeds. I compared the sequestration to the cardenolides available in the seeds and combined this with evaluations of their inhibitory potential on the Na^+/K^+—ATPase enzyme of the milkweed bug. To see the inhibitory capacity of the subset of milkweed cardenolides against a sensitive invertebrate, we used *Drosophila melanogaster* as reference.

The large milkweed bug (as described in chapter 1.4) is an cardenolide-sequestering herbivore that feeds almost exclusively on seeds of Apocynaceae family (Burdfield-Steel and Shuker, 2014). *Asclepias curassavica* is native and abundant across the migration range of the bug´s populations (United States Department of Agriculture; Miller and Dingle, 1982).

Among the five most prevalent cardenolides found in milkweed seeds, glucopyranosyl frugoside (**5**), glucopyranosyl gofruside (**6**), glucopyranosyl calotropin (**7**), frugoside (**8**) and gofruside (**9**), frugoside and gofruside exhibited higher concentrations within the bugs than in the seeds themselves. Notably, these two compounds displayed contrasting levels of potency towards the *O. fasciatus* enzyme. Frugoside, and its glucosylated counterpart,

emerged as the most potent inhibitors, while gofruside exhibited weaker inhibitory potential.

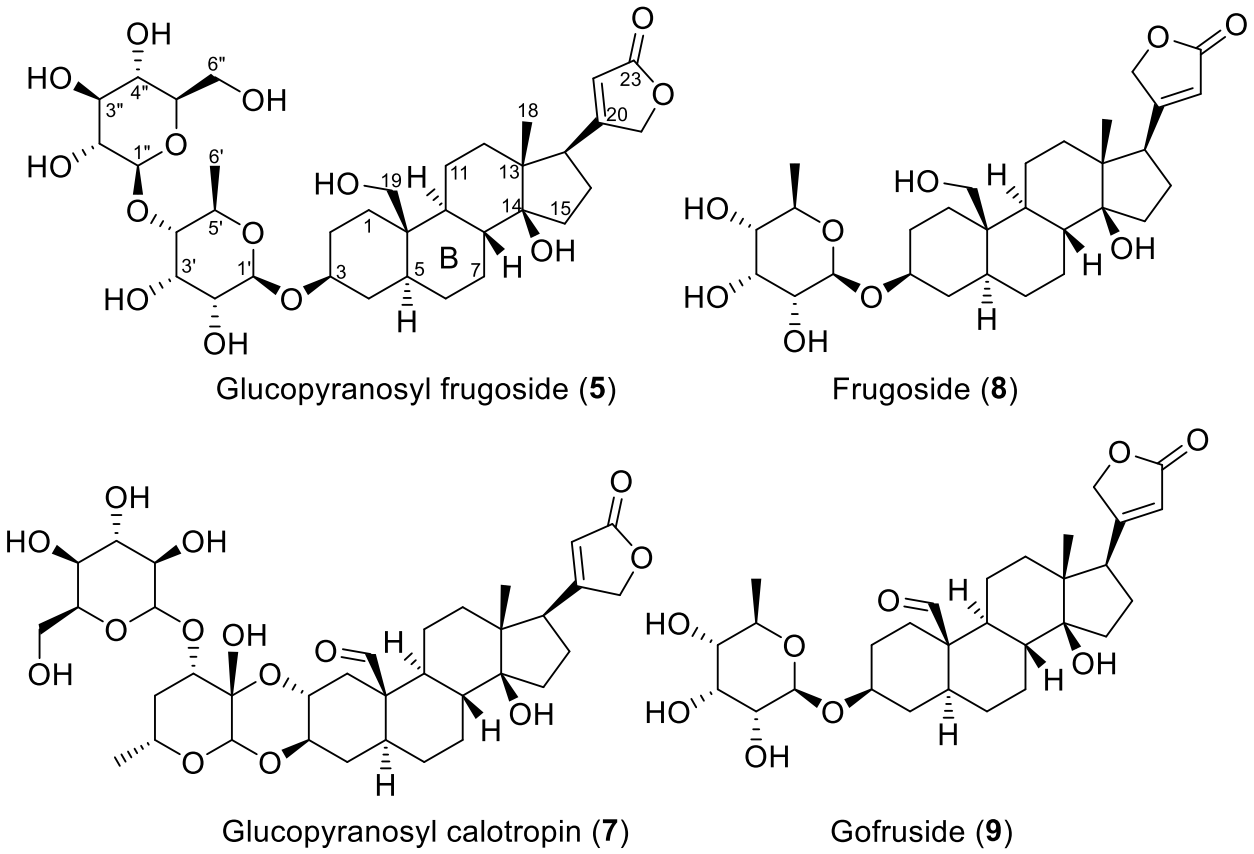


Figure 6. Subset of milkweed cardenolides used in the in vitro analysis against multiple Na+/K+—ATPase enzymes in chapter 2.2 and 2.3.

In line with previous research, the compounds exhibited consistent low inhibitory effects on the *O. fasciatus* enzyme compared to the non-adapted insect enzyme- *Drosophila melanogaster* (Bramer et al., 2015; Dalla and Dobler, 2016; Agrawal et al., 2022; López-Goldar et al., 2022). *O. fasciatus* was highly resistant to the array of cardenolides tested, particularly to glucopyranosyl calotropin (**7**) and gofruside (**9**). In contrast, these two cardenolides where highly toxic to the sensitive invertebrate *D. melanogaster.* The most abundant cardenolide, glucopyranosyl frugoside (**5**), had the lowest IC_{50} value for *O. fasciatus* pump ($3.53x10^{-4}$ M), along with its aglycone (**8**). The result differs with the inhibitory analysis of Agrawal et al., with *A. syriaca* seeds cardenolides, where the minor compound, labriformin, inhibited *O. fasciatus* pump with the lowest IC_{50} value reported for this bug ($9.09x10^{-5}$ M) see Agrawal et al., (2022).

In the literature of chemodiversity of *A. curassavica*, glucopyranosyl frugoside (**5**) has only been described in the seeds (Abe et al., 1992; Rubiano-Buitrago et al., 2023), with no report to be found in the several isolations carried out with the whole plant material or individual parts (Roy et al., 2005; Warashina et al., 2008; Li et al., 2009; Zhang et al., 2014; Al-Snafi, 2015; Nakano et al., 2020). The wide variation of individual cardenolide concentration in *A. curassavica* seeds, most notably the high accumulation of glucopyranosyl frugoside (**5**) in this plant organ, could be the result of the pressure exerted by specialist seed bugs, in the framework of a co-evolutionary arms race. This result gives a path in which the profile of milkweed seeds can have phenotypic matching with their specialist herbivores, however, the greater sequestration of gofruside and frugoside by the bugs than what is available in the seeds, and the opposite inhibitory properties of those two compounds leaves this hypothesis hard to accept.

More experiments are needed to decipher the role of glucopyranosyl frugoside (**5**) in the defence of *A. curassavica* seeds. Tests of this compound on other milkweed herbivores would be worthwhile for comparing the inhibitory effects linked to the degree of herbivory. Furthermore, other tests of the effects of this compound on other physiological parameters of *O. fasciatus,* i.e., using an artificial diet enriched with this compound and tracking potential costs and negative effects in the insect, would be worthwhile for determining whether the sequestration of cardenolides by *O. fasciatus* is selective, and whether it also involves metabolism, or rapid elimination of metabolised products. This is where experiments with diet manipulation, and artificial diets are an excellent tool to control insect nutrition qualitatively and quantitatively (Jeckel et al., 2022). To ensure that the metabolites in the insects are the ones sequestered from the host plants, feeding experiments could also label compounds, which generally give clear evidence and facilitate further quantifications and traceable signals throughout the ADME (absorption, distribution, metabolism, and excretion) process (Meredith et al., 1984; Jeckel et al., 2022).

The results reported here could be evidence that glucopyranosyl frugoside (**5**) is part of the co-evolution between milkweed and seed bugs. But the differential inhibition of both high and low accumulated cardenolides in the bug does not give a clear answer. What the

cardenolide content of the bug revealed however is that the bug can modify its sequestered compounds.

3.3 Evidence for cardenolide metabolism/modification in *O. fasciatus*

Extracts of *O. fasciatus* nymphs (L5) contained 19 cardenolide-like signals, five are unique to the insect in larval stage. Seven of the 19 compounds can be traced back to the seed extract and six are part of the ten seed cardenolides described (**4**, **5**, **7**—**10**). Adult´s extracts had 18 cardenolide-like peaks, two of the compounds are exclusive to the adult stage. Nine compounds can be correlated to the seed extract trace and of those nine, **4**—**6**, **8**—**10** are part of the isolated cardenolides (see chapter 2.2). Almost a quarter of the nymph´s cardenolide profile do not correspond directly to seed cardenolides. One of the potential explanations behind the dissimilarities in cardenolide content of the two life stages can be different predation pressures over the life cycle of the bug (Raška et al., 2023).

The cardenolides present in the bugs that could not been traced to the diet suggest they could be products of modifications during ADME process in the bugs, changing their original structures for example by enzymatic modification or degradation. A possibility is that bugs can enrich cardenolides that are actually present in trace amounts in the seeds, making it feasible to be detected in the chromatographic analysis of the insect´s extracts. To test this, experiments require modified diets and labelled compounds to track potential degradation/modification products (discussion chapter 2.2 and 2.3).

It is clear from my experiments that the cardenolide content in the seeds do not match the cardenolide composition of the bug, and the bug's composition cannot be explained by the seed cardenolide's toxicity to the insect. To explore other explanations for the individual cardenolides that compose the chemical defence of the insect and host plant, I next tested how predators might exert selection in the milkweed-herbivore system.

3.4 Role of predators on the sequestration behaviour of *O. fasciatus*

Predator-prey interactions serve as crucial mediators in the intricate world of insect defences (Brower and Brower, 1964; Brower and Fink, 1985; Rojas et al., 2017; Sporer et al., 2021; Ottocento et al., 2022). In the ecological framework of the milkweed-herbivore system which is the focus of my research, one of the potential interaction that predators

can have with the milkweed cardenolides is through the secretion rich in sequestered compounds that *Oncopeltus fasciatus* evolved to release during an attack (Duffey et al., 1978; Scudder and Meredith, 1982a; Moore and Scudder, 1985; Bramer et al., 2017). This defensive fluid is accumulated in reservoirs called dorsolateral spaces (DLS). Having analysed the overall accumulation of seed cardenolides by the whole bug in chapter 2.2, in chapter 2.3 the focus was shifted to the chemical content that the bugs gather in the DLS fluid and the effect the cardenolides within can have in potential predators. Chapter 2.3 tested two hypotheses: i) the cardenolide content would vary between individual bugs, but overall the DLS fluid would be dominated by non-polar compounds that would be inhibitory to potential predators, and ii) the accumulated cardenolides in the DLS fluid have different potency on different predators´ biological target.

3.4.1 Chemical diversity in the DLS fluid of *O. fasciatus*

In order to understand the cardenolide content that a potential predator encounters while feeding on *O. fasciatus.* I manually stressed the bugs and collected their DLS fluid, following with quantifications of milkweed cardenolides in the fluid sampling and in the dried tissues after depletion. The chemical diversity found in the DLS fluid and tissues post-depletion encompasses a total of 19 compounds, including all eight seed cardenolides as detailed in chapters 2.1 and 2.2. The cardenolide concentrations within the defensive secretions and body tissues of milkweed bugs varied by an order of magnitude. Seven out of the eight milkweed cardenolides exhibited higher concentrations in the dried tissues compared to the DLS fluid. Such variability in the quantity of toxins within a population is a characteristic trait often associated with aposematic animals, as previously highlighted in Speed et al. (2012). However, this phenomenon becomes perplexing when we consider that prey survival is believed to rely on the strength of their defence mechanisms (Ottocento et al., 2022). Some compounds, for example,16α-hydroxycalotropin (**10**) showed similar concentrations in both tissue and DLS fluid, which could indicate that the bugs are selectively allocating this compound to its defensive fluid. This could be tested by feeding the bugs this compound in an artificial diet and tracking it's uptake (Burdfield-Steel et al., 2018; Heyworth et al., 2023). The major cardenolides of *O. fasciatus* adults (**5**-**6**,**8**-**9**) are in a narrow range of concentration in the dried tissues while being highly variable in the DLS fluid. Meaning that, glucopyranosyl frugoside (**5**),

glucopyranosyl gofruside (**6**), and their aglycones (**8** and **9**) sustain a balanced concentration in the bug's tissues after fluid depletion.

There were also biochemical profile differences among individuals. Among the eight quantified compounds, which collectively make up 88% of the cardenolides found in the fluid, one specific compound, glucopyranosyl calotropin, was detectable only in a subset of individuals. To account for such variability in defensive profiles, various hypotheses have been proposed, including factors related to competition, life history, and potential additional functions of these defensive substances, as previously explored by Speed et al. (2012). A starting point into understanding the lack of glucopyranosyl calotropin (**7**) present in the bug, could include experiments with artificial diets enriched with only this compound. This could provide information regarding modified compounds, and effects of this cardenolide in the insect performance.

3.4.2 Do multiple predators select for multiple cardenolides?

Tests of the multiple enemy hypothesis (Rojas et al., 2017; Ottocento et al., 2022; Lawrence et al., 2023) have revealed the ecological relevance of the chemical composition of sequestered PSMs in other herbivores and chemically defended animals.

Through MS quantification I found that the three major compounds in the DLS fluid of *O. fasciatus* are frugoside (**8**), gofruside (**9**) and glucopyranosyl frugoside (**5**). I tested these compounds against the Na^+/K^+—ATPase of three predators. The three species were: the invertebrate generalist *Hierodula membranacea*, which is a model predator in toxic prey studies (Gelperin, 1968; Berenbaum and Miliczky, 1984; Mebs et al., 2017; Rafter et al., 2017a), and the avian predators, *Taeniopygia castanotis* and *Pheucticus melanocephalus*. The latter has reports of eating cardenolide-sequestering prey without significant ill effects (Fink and Brower, 1981). The use of a group of predators diverse both in complexity and in differential response to cardenolides, gives us an idea of the functional diversity of the chemical defence secreted by the insect. The data of *Sus domesticus* Na^+/K^+–ATPase inhibition by ouabain was used as the reference.

Frugoside (**8**) was particularly toxic to both the resistant and sensitive bird. It has the same inhibitory potency against the invertebrate arthropod as for the reference porcine enzyme. Gofruside (**9**) inhibited the mantid's pump at one of the lowest IC_{50} values seen in the

literature (Petschenka et al., 2018; Agrawal et al., 2022; López-Goldar et al., 2022). In general, gofruside did not impact the avian predators. Gofruside (**9**) had the lowest potency among the compounds tested against the black-headed grosbeak.

These results suggest that the accumulation of frugoside (**8**) and gofruside (**9**) is influenced by both invertebrate and vertebrate predators. Notably, the consistent toxic potential of frugoside (**8**) leads me to likening this compound to a "silver bullet", because it possesses the capacity to effectively deter a range of predators [11,100,101], but test on more predators are required. The presence of gofruside (**9**) in the fluid probably adds to their protection against predators, particularly against invertebrate generalists. To gain deeper insights, the next steps should involve in vitro analyses or feeding assays with predators, employing varying proportions of frugoside and gofruside mixtures. This would test whether these compounds exhibit additive, synergistic, or antagonistic relationships when serving as the primary components of the DLS fluid in *O. fasciatus* populations fed on *A. curassavica*.

More research is needed to understand in detail the contrast between the inhibitory properties of gofruside (**9**) against the resistant bird predator compared to frugoside (**8**), its coroglaucigenin counterpart. This could expand the knowledge of which structure features in cardenolides are relevant for the intermolecular interaction between sensitive/resistant enzymes and the PSM (Zhang et al., 2014; Laursen et al., 2015; Fedosova et al., 2022; Meneses-Sagrero et al., 2022). Future research can focus on the crystallization of different Na^+/K^+–ATPases and the two compounds, or in silico modelling using frugoside (**8**) and gofruside (**9**), and predator enzymes with different mutations in the target site.

Tests involving glucopyranosyl calotropin (**7**), which is a non-accumulated compound in the insect but is notably abundant in the seeds showed a similar activity pattern to that of ouabain when exposed to vertebrate predators and the porcine enzyme. Interestingly, when compared chapter 2.3 results to chapter 2.2 glucopyranosyl calotropin does not pose significant harm to *O. fasciatus*, and it ranks as the third most abundant element in their diet (0.733 mg/g of seeds, as presented in chapter 2.1). It appears that the relatively low levels of glucopyranosyl calotropin in the chemically defended prey may be

attributable to the redundancy of its effects, which are essentially parallel to those already accomplished by compounds **5**, **8**, and **9** present in the DLS fluid.

As discussed in the chapter 2.3, one plausible scenario explaining the absence of glucopyranosyl calotropin (**7**) is that it undergoes deglycosylation as a crucial metabolic process within the insect. It's conceivable that modified derivatives of this compound could be present among the cardenolides in the insect that we have not yet characterised. The uniform impact of glucopyranosyl calotropin (**7**) across trophic levels, and the potential for this being a substrate from which other cardenolides might derive akin to a screening hypothesis, may be factors behind its low concentrations within the insect.

Glucopyranosyl gofruside (**6**) while being the second most abundant compound in the seeds (2.065 mg/g of seeds), is present in low content within the insect. This might be due to the bug cleaving the glucose moiety, as discussed in chapter 2.3. Nevertheless, the isolated quantities and lack of purity of glucopyranosyl gofruside (**6**) prevented further testing, as elaborated in chapter 2.1.

The results presented here, based on effects of individual cardenolides, are the groundwork future research on the effect of mixtures of these compounds on different enzymes.

3.5 Conclusion

In the realm of insect defences, many species have evolved chemical defences to protect themselves against both predatory vertebrates and invertebrates. However, our understanding of the evolution and diversity of these insect defences remains somewhat limited, primarily because previous research has predominantly concentrated on the visual signalling of defences aimed at deterring birds (Arenas et al., 2015). Only recently have tests of chemical defences started to emerge (Rojas et al., 2017; Winters et al., 2018; Lawrence et al., 2023). This thesis harnessed the system of cardenolides, which are toxic due to their inhibitory potency on the Na^+/K^+–ATPase function in animal cells and, which can be functionally tested in vitro on ecologically relevant herbivores and predators. I found differential effect of cardenolides on the different herbivore and predator enzymes, which gives support for the idea that natural enemies can act as selection pressure that leads to SMs diversification in the insect prey and their host plants.

4 Summary

Cardiotonic steroids are a diverse group of compounds that derive from triterpenoids and are found primarily in plants but also in animals. There are two classes of cardiotonic steroid: cardenolides and bufadienolides. Cardenolides contain a single five-membered lactone ring in the β position at C17, and bufadienolides contain a six-membered lactone ring at this position and, in most cases, both have sugar moieties. Both compounds are produced de novo in plants and animals, and some animals also sequester cardiotonic steroids from their host plants or prey. Cardiotonic steroids have a specific physiological target; they bind to and inhibit the transmembrane protein Na^+/K^+—ATPase. The Na^+/K^+—ATPase is involved in essential physiological processes, so when it is inhibited by cardiotonic steroids many physiological systems become dysregulated. Cardenolides have convergently evolved in at least 12 botanical families and are found most abundantly in plants belonging to Apocynaceae.

Among plants in the genus *Asclepias* there is a high diversity of cardenolides along a polarity gradient. From an evolutionary perspective the existence of such 'chemodiversity' is puzzling because cardenolides are synthesised via a complex metabolic pathway that involves multiple enzymes modifying a sterol precursor over several steps. Natural selection is expected to drive evolution towards a small number of the most beneficial cardenolides. Despite this, a high diversity of cardenolides have been found within and between plant populations.

A variety of biological processes have been hypothesised to explain chemodiversity including, genetic processes, abiotic environmental conditions, metabolic pathways, and phenotypic plasticity. Interactions with animals form many hypotheses for the evolution of chemodiversity, with broad empirical support.

There are multiple specialised herbivores of *Asclepias*, each having genetically based tolerance to toxic cardenolides via substitutions in the herbivore's Na^+/K^+—ATPase. Specialist lygaeid bugs such as *Oncopeltus fasciatus* feed on seeds of milkweed plants, and the caterpillar of the monarch butterfly (*Danaus plexippus*) feeds on the leaves of milkweed. These insects sequester the cardenolides as a defence against predators.

These insects are hypothesised to impose natural selection for chemical defences in the plant tissues that they eat.

One major objective of this thesis was to test whether cardenolides in the seeds of Asclepias curassavica defend against the specialist bug, *Oncopeltus fasciatus.* The first step in investigating this hypothesis was to conduct a phytochemical profiling of the cardenolides in the seeds. Cardenolides were isolated based on the putative structural assignment based on HRMS. The isolated compounds were examined by means of NMR spectroscopy. Six known cardenolides were identified: 4′-O-β-glucopyranosyl frugoside (**5**), 4′-O-β-glucopyranosyl gofruside (**6**), 3′-O-β-glucopyranosyl calotropin (**7**), frugoside (**8**), gofruside (**9**), and 16α-hydroxycalotropin (**10**). In addition, three new cardenolides were isolated: 3-(4′-O-β-glucopyranosyl- β-allopyranosyl) coroglaucigenin (**2**), 3′-O-β-glucopyranosyl-15-β-hydroxycalotropin (**3**), and 3-O-β-glucopyranosyl-12-β-hydroxy-coroglaucigenin (**4**). 3-O-β-allopyranosyl coroglaucigenin (**1**) although described as new in chapter 2.1, a publication a report in 2017 by Ghorab, et al. was found characterising this molecule as salsotetragonin, isolated from aerial parts of *Salsola tetragona*. The concentration of all cardenolide in the seeds was quantified by using each compound as a standard for their corresponding calibration curve, and the in vitro inhibitory capacity tested on the porcine protein Na^+/K^+—ATPase. This study showed that the seeds had a higher concentration of non-polar compounds, and compound **5**, 4′-O-β-glucopyranosyl frugoside, is the most abundant cardenolide, approximately two times more abundant than 4′-O-β-glucopyranosyl gofruside (**6**). The other eight cardenolides are present in amounts below 1 mg/g of seeds. There was a significant variation in the compounds' inhibition of the unadapted Na^+/K^+—ATPase.

Phytochemical diversity, like that described in this thesis, is linked to herbivore community in several systems. To test whether the diversity and variability in cardenolide defences could represent the plant's response to natural selection by *O. fasciatus* the phenotypic match of the cardenolide composition of seeds of *Asclepias curassavica* and those sequestered by nymphs and adults of *O. fasciatus* was combined with tests of the inhibitory capacity of a subset of the seed cardenolides against the Na^+/K^+—ATPase of *O. fasciatus* and a non-adapted insect (*Drosophila melanogaster*). Among the five most

abundant cardenolides present in milkweed seeds, glucopyranosyl frugoside, glucopyranosyl gofruside, and glucopyranosyl calotropin were significantly more abundant in the seeds than in the adults and nymphs. Frugoside and gofruside were more concentrated in the bugs than is available in the seeds on which they feed. *O. fasciatus* was significantly more tolerant to all compounds compared to *D. melanogaster* and the highly sensitive porcine enzyme. Frugoside and gofruside had contrasting potency toward the *O. fasciatus* target site Na^+/K^+—ATPase – frugoside was the most inhibitory, whereas gofruside was among the weakest inhibitors. The greater sequestration of these compounds could not be explained by their availability in seeds or the biological activity on the bug's target site. Glucopyranosyl frugoside is only reported in the seeds of *A. curassavica*, which could suggest it is under selection by the seed bugs.

To test the alternative hypothesis that the plant chemodiversity and sequestration patterns of *O. fasciatus* are explained by natural selection by the third trophic level. The inhibitory properties cardenolides of the insect's defence was tested against several predator target sites. Coroglaucigenin cardenolides (glucopyranosyl frugoside and frugoside) were toxic for both resistant and sensitive predators, whereas corotoxigenin and calotropagenin cardenolides had varying degrees of enzyme inhibition. The different effect of cardenolides on the predator enzymes suggests that predators can contribute to defence diversification in both milkweed plants and milkweed herbivores.

5 Zusammenfassung

Herzglykoside sind eine vielfältige Gruppe von Verbindungen, die von Triterpenen abstammen und hauptsächlich in Pflanzen, aber auch in Tieren vorkommen. Es gibt zwei Klassen von Herzglykosiden: Cardenolide und Bufadienolide. Cardenolide enthalten einen einfach ungesättigten, fünfgliedrigen Lactonring in der 17β-Position, während Bufadienolide an dieser Position einen doppelt ungesättigten, sechsgliedrigen Lactonring besitzen. Beide Verbindungen werden *de novo* in Pflanzen und Tieren produziert, und einige Tiere sind in der Lage, solche Steroide speichern, wenn sie diese zuvor über ihre Wirtspflanzen oder Beutetiere aufgenommen haben. Herzglykoside haben ein spezifisches physiologisches Ziel; sie binden an das Transmembranprotein Na^+/K^+–ATPase und hemmen dieses in seiner Funktion. Die Na^+/K^+–ATPase ist an essenziellen physiologischen Prozessen beteiligt, und viele physiologische Systeme werden fehlreguliert, wenn sie durch Herzglykoside beeinflusst werden. Cardenolide sind in mindestens 12 botanischen Familien vorhanden und kommen am häufigsten in Pflanzen der Familie der Hundsgiftgewächse (Apocynaceae) vor.

In der Gattung der Seidenpflanzen (*Asclepias*) ist eine Vielzahl an Cardenoliden unterschiedlicher Polarität vertreten. Aus evolutionärer Sicht ist die Existenz einer solchen Chemodiversität rätselhaft, da Cardenolide über einen komplexen Stoffwechselweg synthetisiert werden, bei dem mehrere Enzyme einen Sterolvorläufer über mehrere Schritte modifizieren. Die natürliche Anpassung sollte die Evolution hin zu einer geringeren Anzahl der vorteilhaftesten Cardenolide lenken. Trotzdem wurde eine hohe Vielfalt an Cardenoliden gefunden. Es wurden verschiedene biologische Prozesse zur Erklärung dieser Chemodiversität vorgeschlagen, darunter genetische Prozesse, abiotische Umweltbedingungen, verschieden geartete Stoffwechselwege und phänotypische Plastizität. Auch die Wechselwirkung mit Tieren ist eine Hypothese zur Evolution der Chemodiversität, die breite empirische Unterstützung findet.

Es gibt mehrere auf *Asclepias* spezialisierte Herbivoren, in denen eine genetisch bedingte Toleranz gegenüber giftigen Cardenoliden durch Veränderungen in der Na^+/K^+–ATPase vorhanden ist. Spezialisierte Bodenwanzen (Lygaeidae) wie *Oncopeltus fasciatus* fressen die Samen von Milchpflanzen, und die Raupe des Monarchfalters (*Danaus plexippus*)

frisst die Blätter von Seidenpflanzen. Diese Insekten sequestrieren dann die aufgenommenen Cardenolide als Verteidigung gegen ihre Fressfeinde. Es wird vermutet, dass dabei die Insekten die chemischen Abwehrstoffe in den Pflanzengeweben gezielt selektieren.

Die Beantwortung der Frage, ob Cardenolide aus Samen von *Asclepias curassavica* für *Oncopeltus fasciatus* toxisch sind, war ein Hauptziel dieser Arbeit. Der erste Schritt dazu war die Durchführung einer phytochemischen Analyse der Cardenolide in den Samen. Dazu wurden die Cardenolide, basierend auf ihrer Fragmentierung in der hochaufgelösten Massenspektroskopie (HRMS), chromatographisch selektiert und isoliert. Die gereinigten Verbindungen wurden dann mittels NMR-Spektroskopie untersucht. Sechs bekannte Cardenolide wurden so identifiziert: 4'-*O*-β-Glucopyranosylfrugosid (**5**), 4'-*O*-β-Glucopyranosylgofrusid (**6**), 3'-*O*-β-Glucopyranosylcalotropin (**7**), Frugosid (**8**), Gofrusid (**9**) und 16α-Hydroxycalotropin (**10**). Darüber hinaus wurden drei neue Cardenolide isoliert: 3-(4'-*O*-β-Glucopyranosyl-β-allopyranosyl) -coroglaucigenin (**2**), 3'-*O*-β-Glucopyranosyl-15-β-hydroxycalotropin (**3**) und 3-*O*-β-Glucopyranosyl-12-β-hydroxycoroglaucigenin (**4**). 3-*O*-β-Allopyranosyl-coroglaucigenin (**1**) wurde fälschlicherweise als neuer Naturstoff beschrieben. Die Verbindung wurde jedoch zuvor in einer Arbeit von Ghorab et al. aus dem Jahr 2017 als Salsotetragonin veröffentlicht. Die Konzentration der in den Samen vorhandenen Cardenolide wurde quantitativ bestimmt, und deren hemmende Wirkung auf eine nicht an die Seidenpflanzencardenolide angepasste Na^+/K^+–ATPase aus Schweinen (*Sus scrofa domesticus*) wurde ermittelt. Die Ergebnisse dieser Studie zeigten, dass die analysierten Samen eine höhere Konzentration an apolaren Verbindungen aufweisen, wobei 4'-*O*-β-Glucopyranosylfrugosid (**5**) das Cardenolid mit der höchsten Konzentration ist. Letzteres ist etwa doppelt so hoch konzentriert in den Samen vorhanden wie 4'-*O*-β-Glucopyranosylgofrusid (**6**). Die anderen acht Cardenolide sind nur in Mengen von unter einem Milligramm pro Gramm Trockenmasse der Samen vorhanden. Bei den Biotests war eine signifikante Variation der Hemmung der untersuchten Na^+/K^+–ATPase zu verzeichnen.

Phytochemische Vielfalt, wie sie in dieser Arbeit beschrieben wird, ist im Zusammenhang mit der entsprechenden Herbivorengemeinschaft zu interpretieren. Um zu testen, ob die strukturelle Vielfalt und mengenmäßige Variabilität der Cardenolidabwehr in den Samen eine Reaktion der Pflanze auf die natürliche Selektion durch *O. fasciatus* darstellen könnte, wurden die Cardenolide der Samen von *Asclepias curassavica* mit den durch Nymphen und Adulten von *O. fasciatus* sequestrierten Cardenoliden verglichen. Weiterhin wurden *in vitro* Tests mit den Na^+/K^+-ATPasen von *O. fasciatus* und *Drosophila melanogaster* durchgeführt.

In *Asclepias*-Samen waren Glucopyranosylfrugosid, Glucopyranosylgofrusid und Glucopyranosylcalotropin signifikant höher konzentriert als in Adulten und Nymphen von *O. fasciatus*. Frugosid und Gofrusid waren hingegen stärker in den Wanzen konzentriert als in den Samen. Die Na+/K+-ATPase von *O. fasciatus* war gegenüber allen Verbindungen signifikant toleranter als die von *D. melanogaster;* am empfindlichsten war das Schweineenzym. Frugosid war das am stärksten hemmende Cardenolid, während Gofrusid zu den schwächsten Inhibitoren der Na^+/K^+-ATPase gehörte. Die stärkere Sequestrierung dieser Verbindungen konnte nicht durch deren Verfügbarkeit in den Samen oder die biologische Aktivität gegenüber der Na^+/K^+-ATPase in den Wanzen erklärt werden. Glucopyranosylfrugosid wurde nur als Inhaltsstoff der Samen von *A. curassavica* beschrieben, was darauf hindeuten könnte, dass diese Verbindung von den Wanzen selektiv sequestriert wird.

Um die alternative Hypothese zu testen, wonach die chemische Vielfalt der Cardenolide in der Pflanze und das Sequestrierungsmuster in *O. fasciatus* durch natürliche Selektion auf der dritten trophischen Ebene erklärt werden kann, wurde die hemmende Wirkung der Cardenolide auf die Na^+/K^+-ATPase verschiedener Fressfeinde ermittelt. Coroglaucigenin-Cardenolide (Glucopyranosylfrugosid und Frugosid) waren für sowohl resistente als auch für empfindliche Raubtiere giftig, während Corotoxigenin- und Calotropagenin-Cardenolide unterschiedliche Grade der Enzymhemmung zeigten. Die unterschiedliche Wirkung der Cardenolide auf die Raubtierenzyme legt nahe, dass Raubtiere sowohl bei Seidenpflanzen als auch bei Bodenwanzen zur Verteidigungsdiversifizierung beitragen können.

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Appendices

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I am deeply grateful to all my friends and family. This thesis is dedicated to my mom, brother and grandma; who will forever be my biggest blessing and pride.

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II. Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Place Date Paola Andrea Rubiano Buitrago

III. Declaration on authorship and copyright in a cumulative doctoral thesis

Publication #1: Rubiano-Buitrago P, Pradhan S, Paetz C, Rowland HM. New Structures, Spectrometric Quantification, and Inhibitory Properties of Cardenolides from *Asclepias curassavica* Seeds. *Molecules.* 2023 Dec 23;28(1):105.	
Involved in	
	Paola Andrea Rubiano Buitrago
Conceptual research design	X
Planning of research activities	X
Data collection	X
Data analysis and interpretation	X
Manuscript writing	X
Suggested publication equivalence value	**1.0**

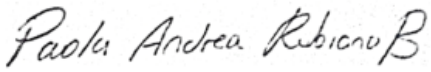
Publication #2: Rubiano-Buitrago P, Pradhan S, Grabe V, Aceves-Aparicio A, Paetz C, Rowland HM. Differential accumulation of cardenolides from *Asclepias curassavica* by large milkweed bugs does not correspond to availability in seeds or biological activity on the bug Na^+/K^+-ATPase. *Front Ecol Evol*. 2023 Jun 8;11.	
Involved in	
	Paola Andrea Rubiano Buitrago
Conceptual research design	X
Planning of research activities	X
Data collection	X
Data analysis and interpretation	X
Manuscript writing	X
Suggested publication equivalence value	**1.0**

Publication #3:	
Involved in	
	Paola Andrea Rubiano Buitrago
Conceptual research design	X
Planning of research activities	X
Data collection	X
Data analysis and interpretation	X
Manuscript writing	X
Suggested publication equivalence value	**1.0**

IV. Declaration of the doctoral candidate's own contribution

Reprint permissions have been obtained for all manuscripts used as part of the doctoral thesis.

The co-authors of the manuscripts used in the present cumulative doctoral thesis have been informed about the use of the manuscripts and about the declared individual contributions; they have given their consent. The declared individual contributions of the doctoral candidate in the publications are listed in the attachment III.

06.10.2023 Jena 

Paola Andrea Rubiano Buitrago Date Place Signature