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Supplementary Information for

Cardenolides, toxicity and the costs of sequestration in the coevolutionary interaction between monarchs and milkweeds

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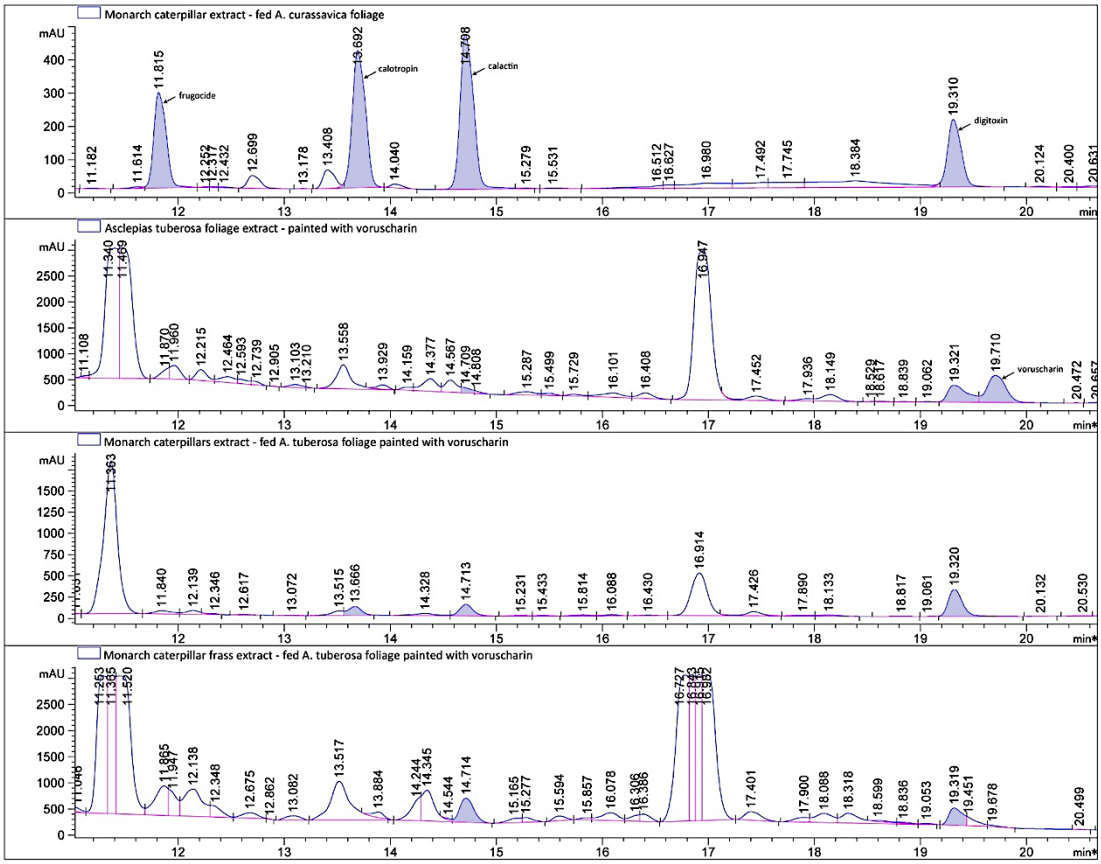

Fig. S1. Sample HPLC chromatograms.

(A) Monarch caterpillar feeding on *Asclepias curassavica*, (B) *A. tuberosa* foliage painted with voruscharin, (C) monarch caterpillars fed *A. tuberosa* painted with voruscharin, and D) monarch frass from feeding on *A. tuberosa* painted with voruscharin. Digitoxin (RT 19.31-19.32) was added as the internal standard.

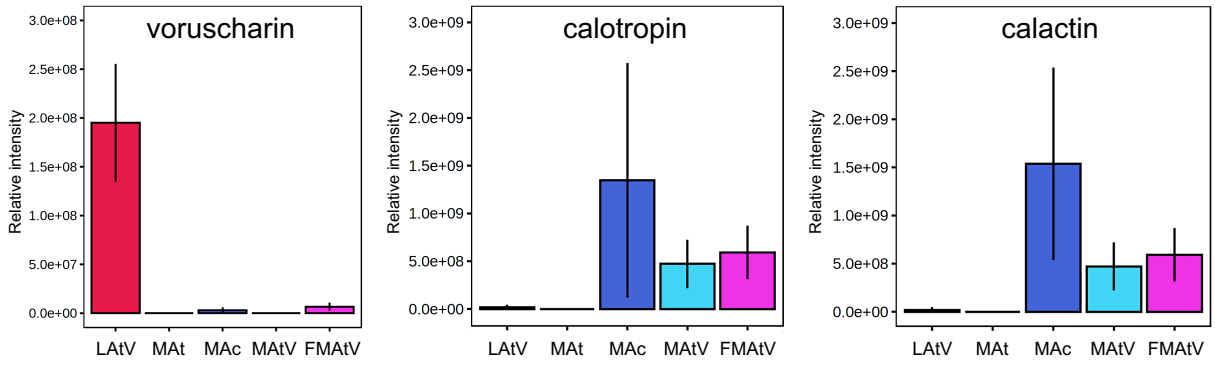

Fig. S2. Voruscharin painted on cardenolide-free milkweed leaves of *A. tuberosa* are converted to calactin and calotropin as revealed by LC-ESI- HRMS (n=4).

Ion mass of voruscharin, calactin and calotropin respectively detected at 590.2775 ($[M+H]^+$), 533.2745 ($[M+H]^+$), and 590.2741 ($[M+H]^+$) in positive ionization mode. Shown are means ± SE relative concentrations (normalized mass spec ion abundance). LAtV: leaf of *A. tuberosa* painted with voruscharin; MAt: monarchs on *A. tuberosa*; MAc: monarchs on *A. curassavica*; MAtV: monarchs on *A. tuberosa* painted with voruscharin; FMAtV: frass from monarchs fed *A. tuberosa* painted with voruscharin.

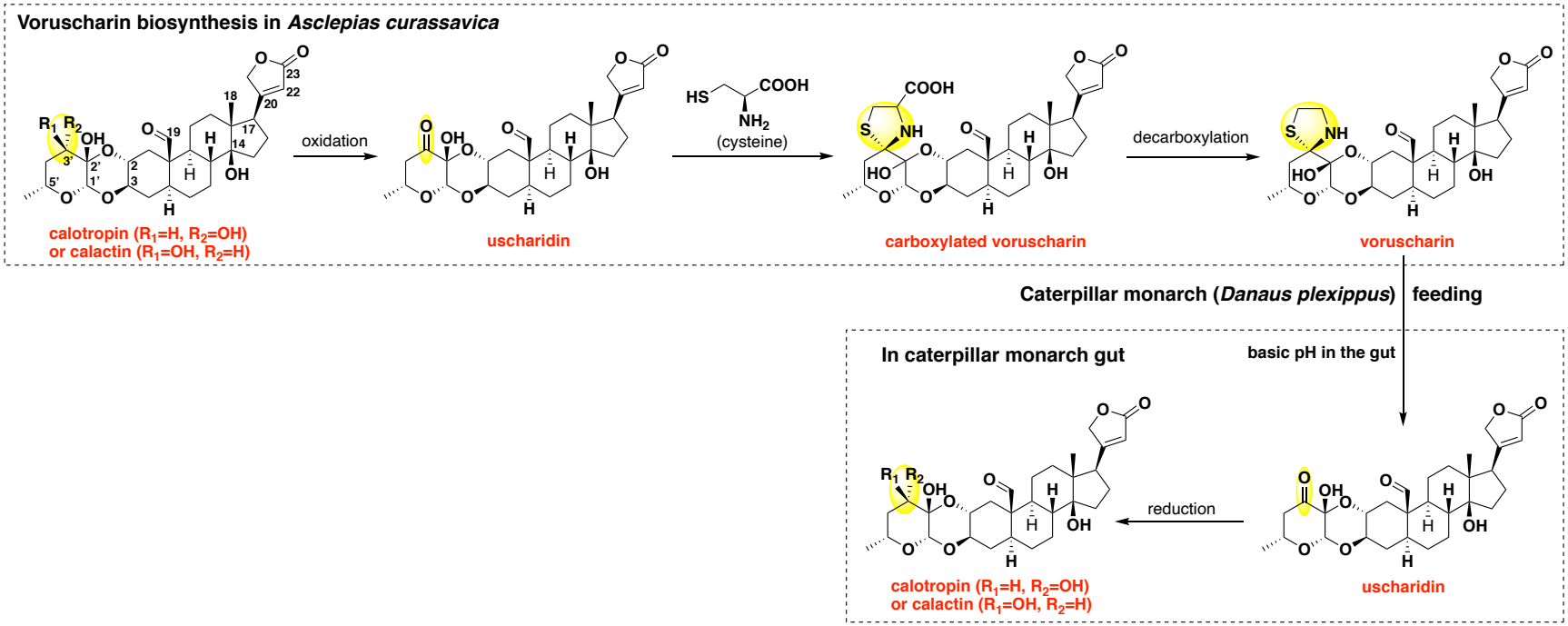

Fig. S3. Voruscharin putative biosynthesis in *Asclepias curassavica* and its degradation pathway in monarch gut.
The functional group in position C3' is highlighted in yellow to show the successive steps leading from calactin/calotropin to voruscharin.

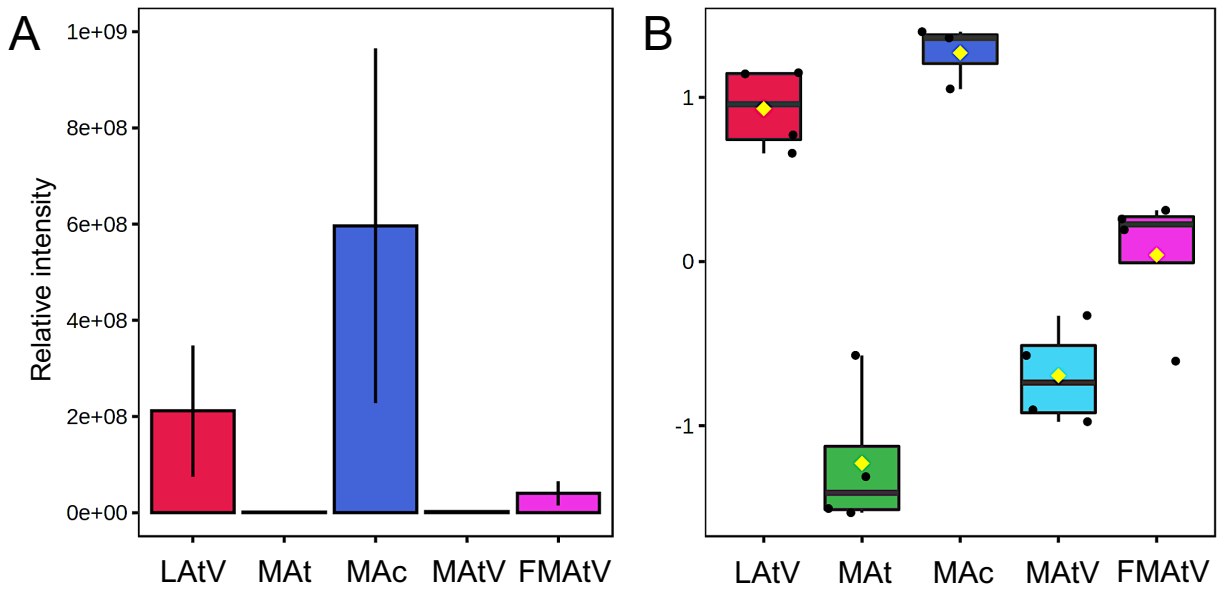

Fig. S4. Uscharidin relative concentrations in samples.

The bar plots show (A) the original values (mean +/- SD). The box and whisker plots (B) summarize the normalized values. The mean concentration of each group is indicated with a yellow diamond. Ion mass detected at 531.2588 ($[M+H]^+$) in positive ionization mode. LAtV: leaf of *A. tuberosa* painted with voruscharin; MAt: monarchs on *A. tuberosa*; MAc: monarchs on *A. curassavica*; MAtV: monarchs on *A. tuberosa* painted with voruscharin; FMAtV: frass from monarchs fed *A. tuberosa* painted with voruscharin.

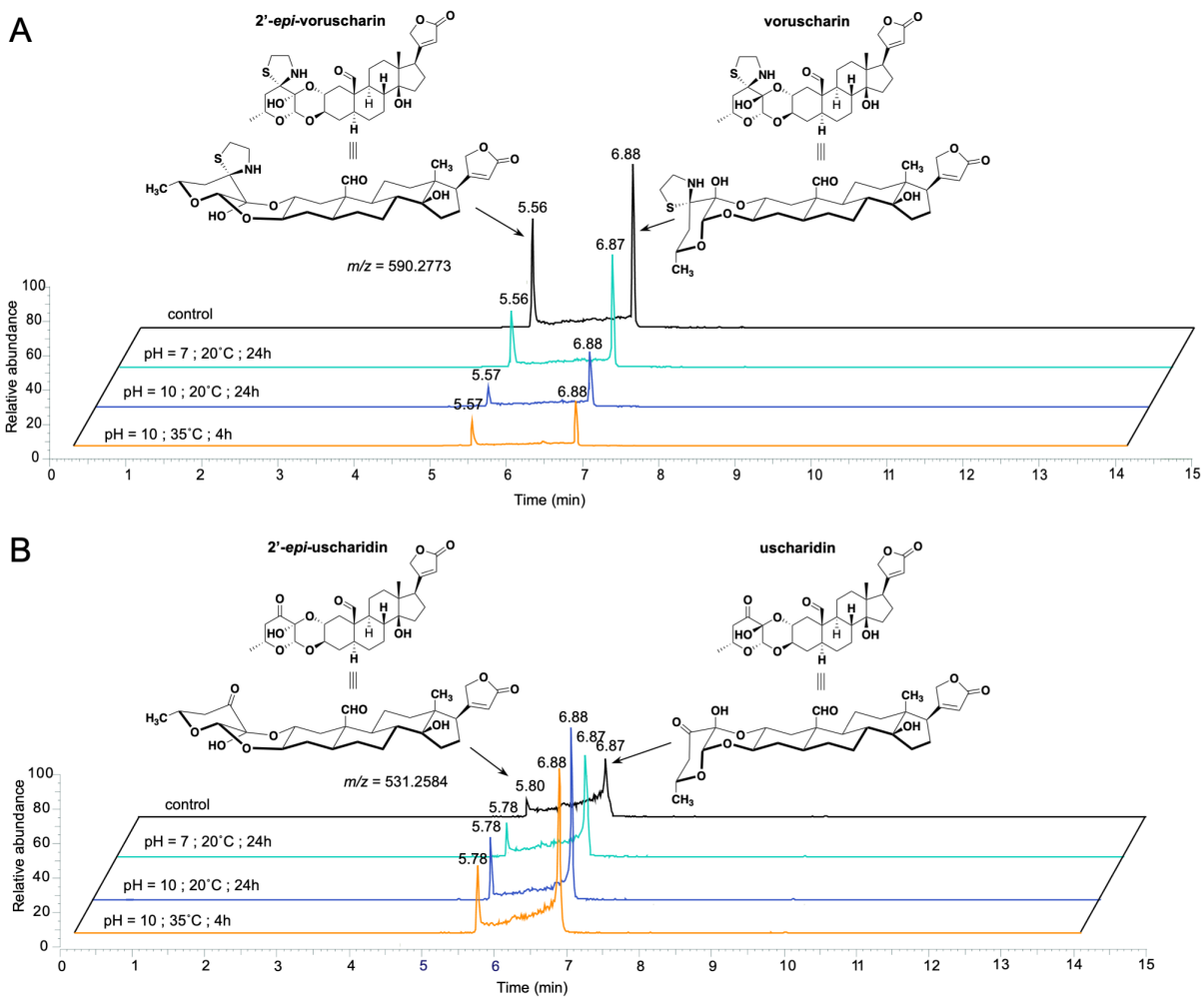

Fig. S5. Voruscharin stability assay at different pH values.

Chromatograms of voruscharin (A) and uscharidin (B), and corresponding epimers, after being stored at different temperatures and pH values. The control was stored at -20°C for 24h. The epimer's retention times was anticipated based on previous results of 2'-*epi*-uscharin (1).

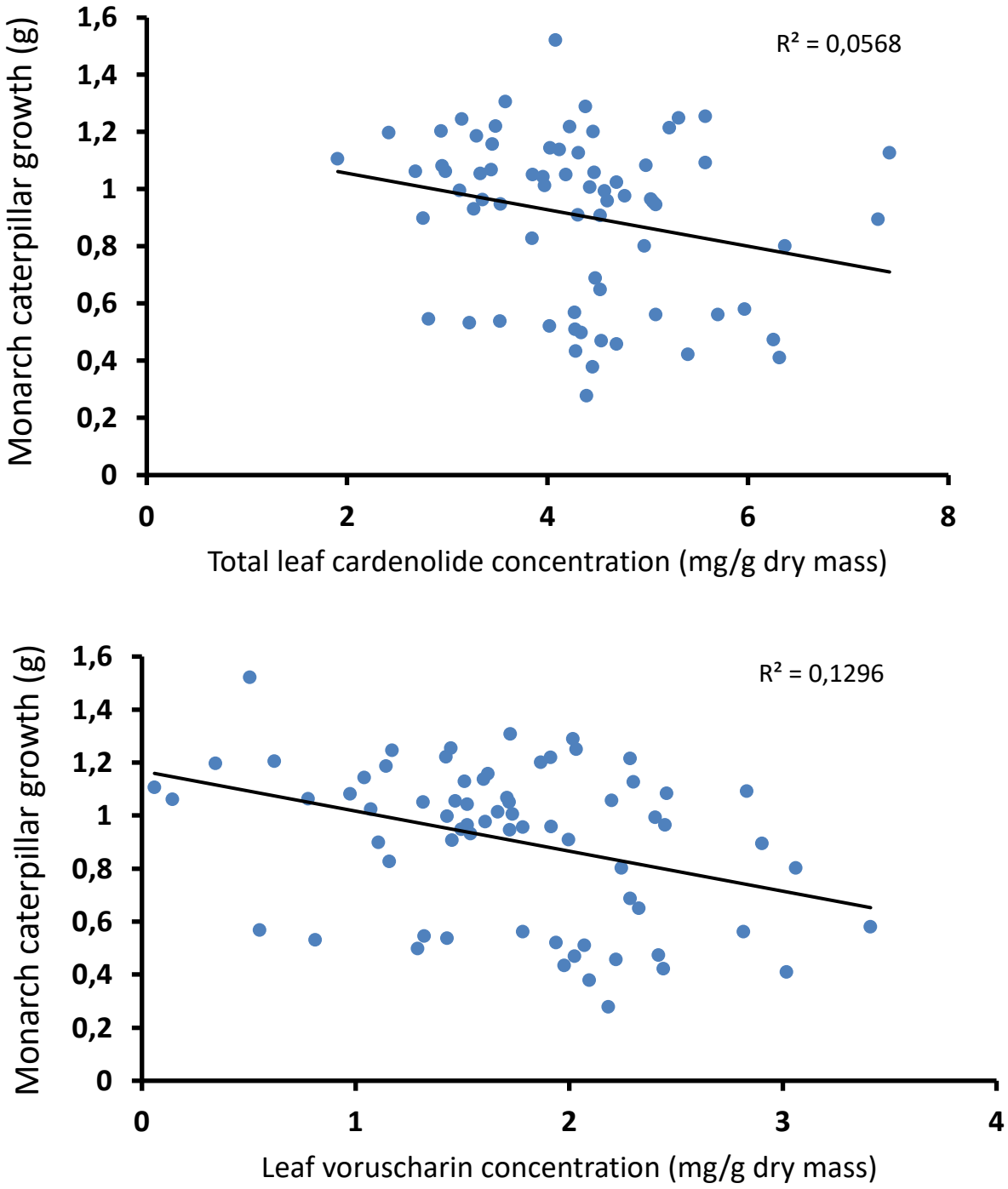

Fig. S6. Monarch caterpillar growth predicted by A) total leaf cardenolide and B) leaf voruscharin concentrations.

Note the weak relationships compared to sequestered cardenolides presented in Fig. 2 (and main text, which explained >60% of the variation in caterpillar growth).

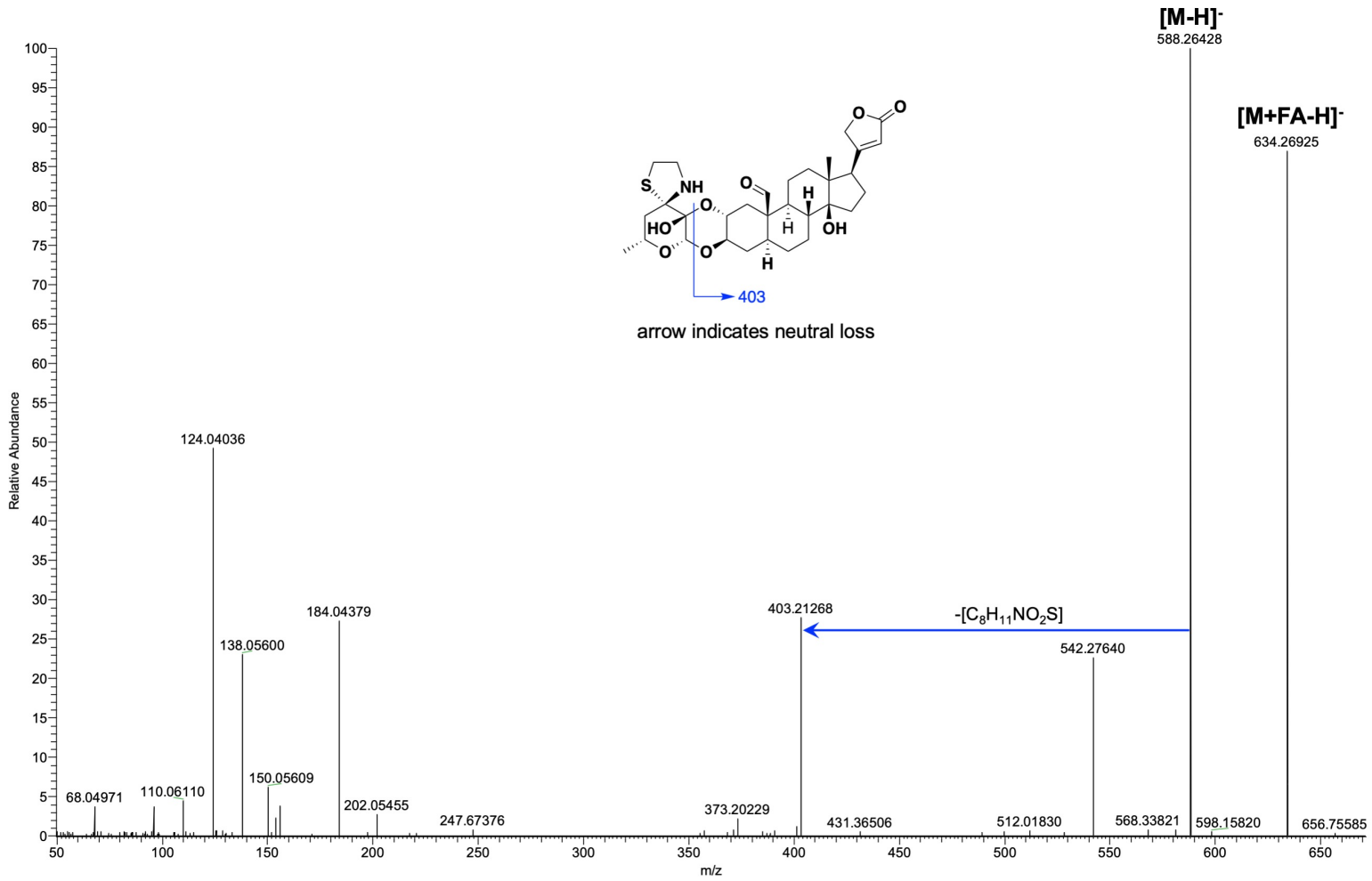

Fig. S7. MS/MS product ion mass spectrum from $[M+FA-H]^-$ adduct of voruscharin.

The MS^2 spectrum of voruscharin was deposited to the GNPS database.
https://gnps.ucsd.edu/ProteoSAFe/gnpslibraryspectrum.jsp?SpectrumID=CCMSLIB00005724352#%7B%7D

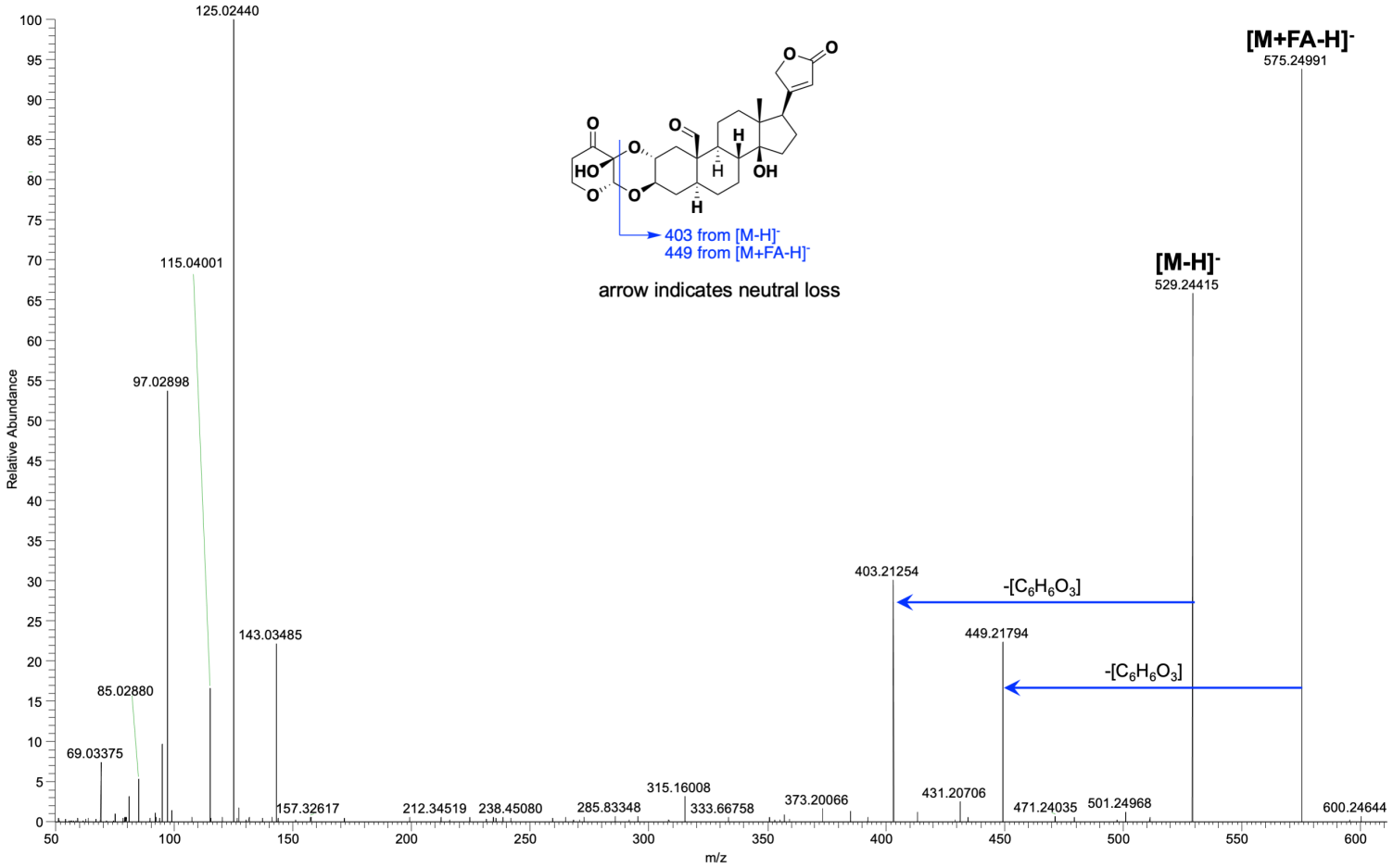

Figure S8. MS/MS product ion mass spectrum from $[M+FA-H]^-$ adduct of uscharidin.

The MS^2 spectrum of uscharidin was deposited to the GNPS database.
https://gnps.ucsd.edu/ProteoSAFe/gnpslibraryspectrum.jsp?SpectrumID=CCMSLIB00005724353#%7B%7D

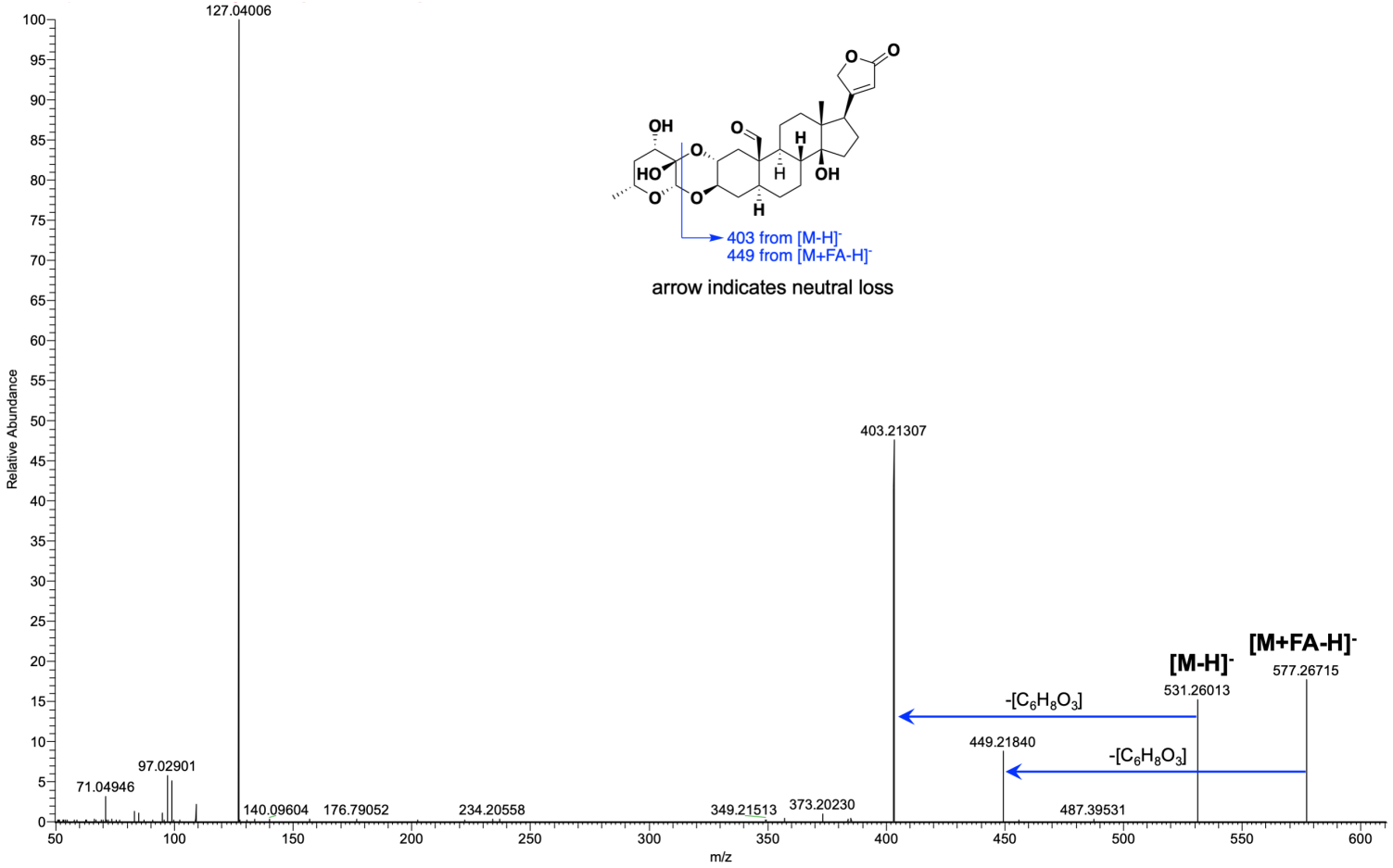

Figure S9. MS/MS product ion mass spectrum from $[M+FA-H]^-$ adduct of calotropin.

The MS^2 spectrum of calactin/calotropin was deposited to the GNPS database.
https://gnps.ucsd.edu/ProteoSAFe/gnpslibraryspectrum.jsp?SpectrumID=CCMSLIB00005724354#%7B%7D

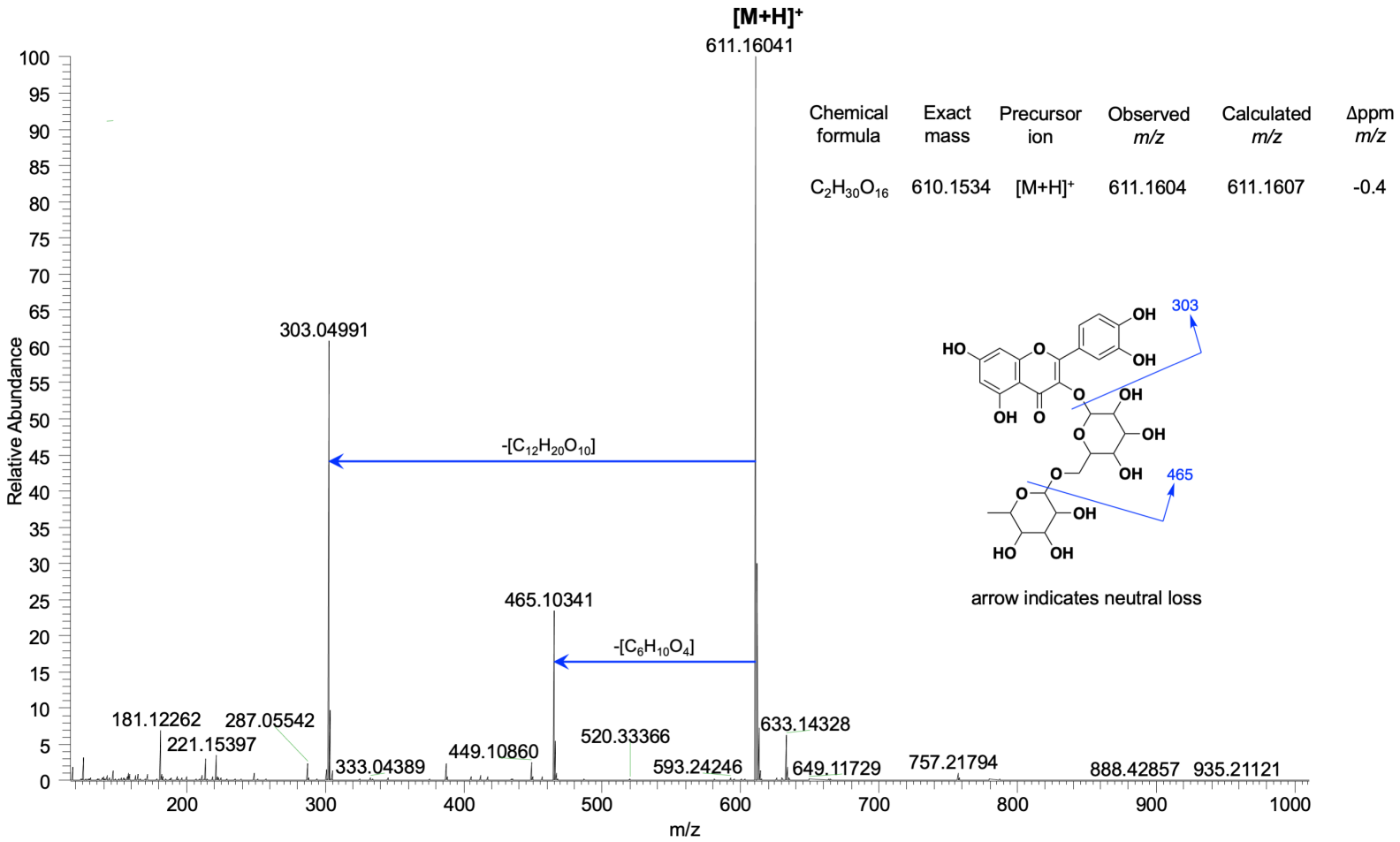

Figure S10. MS/MS product ion mass spectrum from $[M+H]^+$ adduct of quercetin-rhamno-hexoside.

Based on previous phytochemical studies of characterized flavonols in *A. curassavica* (2) the structure is either quercetin 3-*O*-α-(2"-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside or quercetin 3-*O*-α-(2"-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranosyl (rutin).

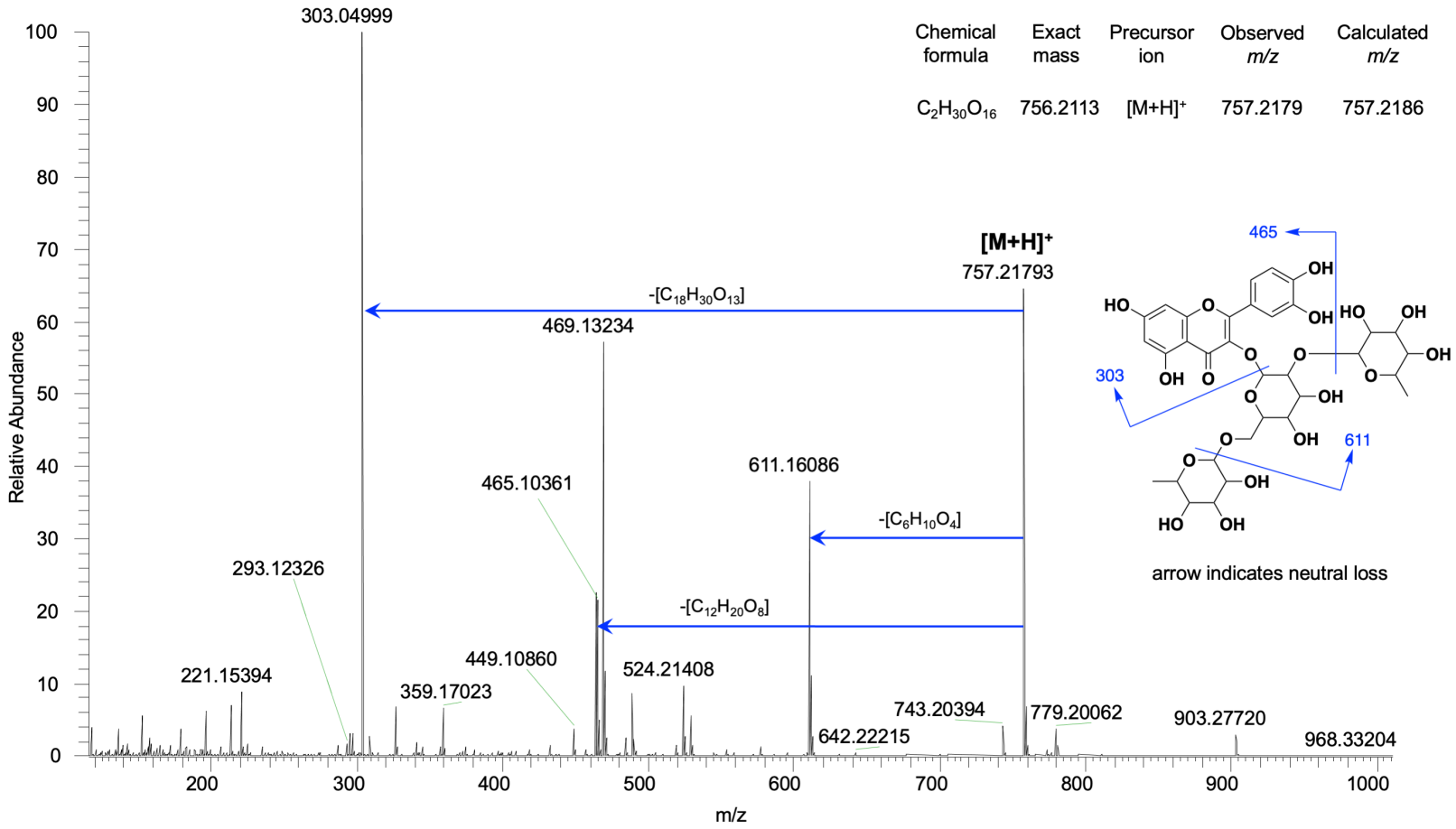

Figure S11. MS/MS product ion mass spectrum from $[M+H]^+$ adduct of quercetin-rhamno-di-hexoside.

Based on previous phytochemical studies of characterized flavonols in *A. curassavica* (2) the structure is either quercetin 3-*O*-(2",6"-di-α-L-rhamnopyranosyl)-β-D-galactopyranoside or quercetin 3-*O*-(2",6"-*O*-di-α-L-rhamnopyranosyl)-β-D-glucopyranoside.

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Table S1. Pairwise phenotypic Pearson correlations between the nine major cardenolides quantified from *Asclepias curassavica* leaves grown in a common environment (n=212).

Where compounds were not identified their retention time is given. Four non-significant correlations are highlighted in grey.

		r	P
Frugoside	9.95	0.609	<.0001
12.54	9.95	0.666	<.0001
12.54	Frugoside	0.642	<.0001
14.66	9.95	0.757	<.0001
14.66	Frugoside	0.722	<.0001
14.66	12.54	0.905	<.0001
Calactin	9.95	0.727	<.0001
Calactin	Frugoside	0.747	<.0001
Calactin	12.54	0.820	<.0001
Calactin	14.66	0.950	<.0001
asclepin	9.95	0.405	<.0001
asclepin	Frugoside	0.795	<.0001
asclepin	12.54	0.426	<.0001
asclepin	14.66	0.521	<.0001
asclepin	Calactin	0.577	<.0001
18.18	9.95	0.693	<.0001
18.18	Frugoside	0.636	<.0001
18.18	12.54	0.913	<.0001
18.18	14.66	0.933	<.0001
18.18	Calactin	0.858	<.0001
18.18	asclepin	0.418	<.0001
Uscharin	9.95	0.145	0.0351
Uscharin	Frugoside	0.581	<.0001
Uscharin	12.54	0.238	0.0005
Uscharin	14.66	0.288	<.0001
Uscharin	Calactin	0.377	<.0001
Uscharin	asclepin	0.785	<.0001
Uscharin	18.18	0.228	0.0008
Voruscharin	9.95	-0.018	0.7917
Voruscharin	Frugoside	0.422	<.0001
Voruscharin	12.54	-0.035	0.6154
Voruscharin	14.66	0.063	0.3654
Voruscharin	Calactin	0.161	0.0191
Voruscharin	asclepin	0.569	<.0001
Voruscharin	18.18	-0.037	0.5885
Voruscharin	Uscharin	0.675	<.0001

Table S2. Principal component analysis loadings for the nine individual cardenolides on the two main principal components (Eigen values >1) for plant (85% variation explained) and monarch (72% variation explained) tissues.

Also shown is the average percentage of that each compound relative to the total cardenolide concentrations. Note that cardenolide compounds are not identical for the two tissues and the PC analyses are completely independent. Compounds are ordered by retention time (RT).

	Cardenolide	% of the total	PC axis 1	PC axis 2
Plant tissue[1] (n=212)				
	RT9.95	6.3	0.767	-0.312
	Frugoside	6.0	0.876	0.249
	RT12.54	4.0	0.865	-0.346
	RT14.66	11.4	0.939	-0.278
	Calactin	5.1	0.934	-0.153
	Asclepin	12.2	0.739	0.555
	RT18.18	4.1	0.877	-0.365
	Uscharin	10.9	0.533	0.741
	Voruscharin	39.9	0.283	0.843
Insect tissue[2] (n=70)				
	RT5.6	3.3	0.651	-0.476
	RT8	6.5	0.792	-0.316
	RT8.3	1.5	0.498	0.709
	RT9.5	5.9	0.643	0.282
	Frugoside	22.5	0.869	0.119
	RT12.8	2.7	0.901	-0.136
	RT13.5	2.7	0.805	0.077
	Calotropin	22.8	0.878	0.185
	Calactin	32.1	0.866	-0.207

[1] PC axes are orthogonal and both are highly significant in explaining variation in total plant cardenolides (PC1 R^2=0.50, PC2 R^2=0.43).

[2] PC axes are orthogonal and only PC1 was significant in explaining variation in total cardenolides sequestered by monarchs (PC1 R^2=0.97).

Table S3. PERMANOVA results analyzing metabolic data based on Bray-Curtis dissimilarities.

Source of variation	DF	SumSq	MeanSqs	F. model	R^2	P
All chemical extracts	4	0.067220	0.0168050	60.8942	0.6477	**0.001**
Residuals	15	0.036563	0.0024376		0.3523	
Total	19	0.103783			1.0000	
Pairwise						
LAtV × MAt	1	0.039476	0.039476	3.429	0.36367	**0.037**
LAtV × MAc	1	0.045634	0.045634	2.1744	0.266	0.123
LAtV × MAtV	1	0.026582	0.026582	1.7521	0.22601	0.143
LAtV × FMAtV	1	0.020933	0.020932	1.3962	0.18877	0.181
MAt × MAc	1	0.024287	0.024287	2.2136	0.26951	0.121
MAt × MAtV	1	0.026924	0.0269240	6.3835	0.51548	**0.002**
MAt × FMAtV	1	0.033727	0.033727	4.3275	0.41902	**0.036**
MAc × MAtV	1	0.028296	0.0282957	3.4997	0.3684	**0.024**
MAc × FMAtV	1	0.046877	0.046877	3.2347	0.35028	**0.033**
MAtV × FMAtV	1	0.021856	0.0218561	2.2519	0.27289	**0.038**

LAtV: Leaf of *A. tuberosa* painted with voruscharin; MAt: Monarchs on *A. tuberosa*;
MAc: Monarchs on *A. curassavica*; MAtV: Monarchs on *A. tuberosa* painted with voruscharin;
FMAtV: Frass from monarchs fed *A. tuberosa* painted with voruscharin.
DF - degrees of freedom; SumSq - sum of squares; MeanSqr - mean squares.
P-values based on 999 permutations (lowest P-value possible 0.001).

Table S4. The relative inhibitory potential of seven isolated cardenolides on the sensitive porcine and typically-resistant monarch butterfly Na-K-ATPase (See also Figure 3).

IC_{50} is the concentration (μM) at which the enzyme is inhibited by 50%. Sequestered+ indicates that these compounds are sequestered intact from consumed leaves as well as converted products from consumed voruscharin and uscharin.

	Porcine ATPase (IC_{50})	Monarch ATPase (IC_{50})	IC_{50} ratio	Total n
Ouabain (standard)	0.81	122.09	151	11
Digitoxin (standard)	0.49	16.25	33	12
Frugoside (sequestered)	0.37	27.80	76	8
Calotropin (sequestered+)	0.27	10.90	40	6
Calactin (sequestered+)	0.24	3.21	13	6
Uscharin (unsequestered)	0.78	1.00	1	12
Voruscharin (unsequestered)	0.46	2.02	4	26

Table S5. Poisson GLM model fits for predicting monarch oviposition on 212 *Asclepias curassavica* plants in a common greenhouse environment.

Breaking down the predictors by cardenolide principal components, the four of nine significant individual cardenolides, and two quercetin glycosides (there was no indication of a quadratic fit for the quercetin glycosides). Voruscharin concentration dominates PC2 and was not a significant predictor in individual analyses.

Plant compound	Factor	L-R χ^2	p
PC1	PC1	4.687	0.030
	PC1 squared	5.317	0.021
	Plant height	14.628	<0.001
PC2	PC2	0.2249	0.617
	PC2 squared	0.135	0.713
	Plant height	13.746	<0.001
Frugoside	Frugoside	6.156	0.013
	Frugoside squared	6.161	0.013
	Plant height	12.677	<0.001
Calactin	Calactin	6.228	0.013
	Calactin squared	5.282	0.022
	Plant height	16.529	<0.001
RT14.66	RT14.66	7.386	0.007
	RT14.66 squared	6.978	0.008
	Plant height	14.907	<0.001
RT18.18	RT18.18	7.388	<0.001
	RT18.18 squared	8.053	0.004
	Plant height	14.657	<0.001
Quercetin glycosides	quercetin-rhamno-di-hexoside[1]	0.115	0.731
	quercetin-rhamno-hexoside[2]	0.002	0.960
	Plant height	14.285	<0.001

[1] Molecular mass 756 g/mol
[2] Molecular mass 610 g/mol

Table S6. Effects of two quercetin glycosides on monarch butterfly oviposition, tested with pure compounds in cages. >1 gram fresh leaf equivalent of each compound was added to each sponge.

A mixed-model ANOVA was used (random effect was blocking cage): overall effect of treatment $F_{2,27.98}=9.901$, $p<0.001$.

Compound	Eggs laid (LS mean)	Standard error	Tukey comparison
Control	39.6	12.2	A
quercetin-rhamno-di-hexoside[1]	44.8	13.7	A
quercetin-rhamno-hexoside[2]	84.9	14.2	B

[1] Molecular mass 756 g/mol
[2] Molecular mass 610 g/mol

Table S7. HRMS data of the cardenolides ions detected in samples.

To simplify the table, only MS data from one replicate is listed for each precursor ion.

Cardenolide	Formula	Precursor ion	Observed *m/z*	Calculated *m/z*	Δ*m/z* (ppm)	Sample (replicate number)
Voruscharin	$C_{31}H_{43}NO_8S$	$[M+H]^+$	590.2781	590.2775	-1.0	*A. tuberosa* vor. Treated foliage (1)
				590.2782	0.1	Frass from cats (1)
		$[M+Na]^+$	612.2601	612.2592	-1.4	*A. tuberosa* vor. Treated foliage (1)
				612.2605	0.6	Frass from cats (1)
		$[M-H]^-$	588.2636	588.2634	-0.3	*A. tuberosa* vor. Treated foliage (1)
				588.2648	2.0	Frass from cats (1)
		$[M+FA-H]^-$	634.2691	634.2689	-0.3	*A. tuberosa* vor. Treated foliage (1)
				634.2689	-0.3	Frass from cats (1)
Uscharidin	$C_{29}H_{38}O_9$	$[M+H]^+$	531.2588	531.2585	-0.5	*A. tuberosa* vor. Treated foliage (1)
				531.2588	0.0	Frass from cats (1)
				531.2592	0.7	*A. curassavica* feds cats (1)
		$[M+Na]^+$	553.2408	553.2403	-0.9	*A. tuberosa* vor. Treated foliage (1)
				553.2402	-1.0	Frass from cats (1)
				553.2416	1.4	*A. curassavica* feds cats (1)
		$[M-H]^-$	529.2443	529.2440	-0.5	*A. tuberosa* vor. Treated foliage (1)
				529.2443	0.0	Frass from cats (1)
				529.2443	0.0	*A. curassavica* feds cats (1)
		$[M+FA-H]^-$	575.2498	575.2498	0.0	*A. tuberosa* vor. Treated foliage (1)
				575.2501	0.5	Frass from cats (1)
				575.2500	0.3	*A. curassavica* feds cats (1)
Calotropin	$C_{29}H_{40}O_9$	$[M+H]^+$	533.2744	533.2737	-1.3	*A. tuberosa* fed cats w/ voruscharin (1)
				533.2745	0.1	Frass from cats (1)
				533.2740	-0.7	*A. curassavica* feds cats (1)
		$[M+Na]^+$	555.2564	555.2549	-2.7	*A. tuberosa* fed cats w/ voruscharin (1)
				555.2546	-3.2	Frass from cats (1)
				555.2553	-1.9	*A. curassavica* feds cats (1)
		$[M-H]^-$	531.2599	531.2590	-1.6	*A. tuberosa* fed cats w/ voruscharin (1)
				531.2612	2.4	Frass from cats (1)
				531.2597	-0.3	*A. curassavica* feds cats (1)
		$[M+FA-H]^-$	577.2654	577.2655	0.1	*A. tuberosa* fed cats w/ voruscharin (1)
				577.2659	0.8	Frass from cats (1)
				577.2665	1.9	*A. curassavica* feds cats (1)

Calactin	$C_{29}H_{40}O_9$	$[M+H]^+$	533.2744	533.2733	-2.0	*A. tuberosa* fed cats w/ voruscharin (1)
				533.2741	-0.5	Frass from cats (1)
				533.2742	-0.3	*A. curassavica* feds cats (1)
		$[M+Na]^+$	555.2564	555.2552	-2.1	*A. tuberosa* fed cats w/ voruscharin (1)
				not detected	-	Frass from cats (1)
				555.2554	-1.8	*A. curassavica* feds cats (1)
		$[M-H]^-$	531.2599	531.2596	-0.5	*A. tuberosa* fed cats w/ voruscharin (1)
				531.2593	-1.1	Frass from cats (1)
				531.2596	-0.5	*A. curassavica* feds cats (1)
		$[M+FA-H]^-$	577.2654	577.2654	0.0	*A. tuberosa* fed cats w/ voruscharin (1)
				577.2659	0.8	Frass from cats (1)
				577.2664	1.7	*A. curassavica* feds cats (1)

References

1. Parhira S, Zhu G-Y, Jiang R-W, Liu L, Bai L-P, & Jiang Z-H (2014) 2'-*Epi*-uscharin from the latex of *Calotropis gigantea* with HIF-1 inhibitory activity. *Sci. Rep.* 4:4748.

2. Haribal M & Renwick JA (1996) Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica*. *Phytochemistry* 41:139-144.