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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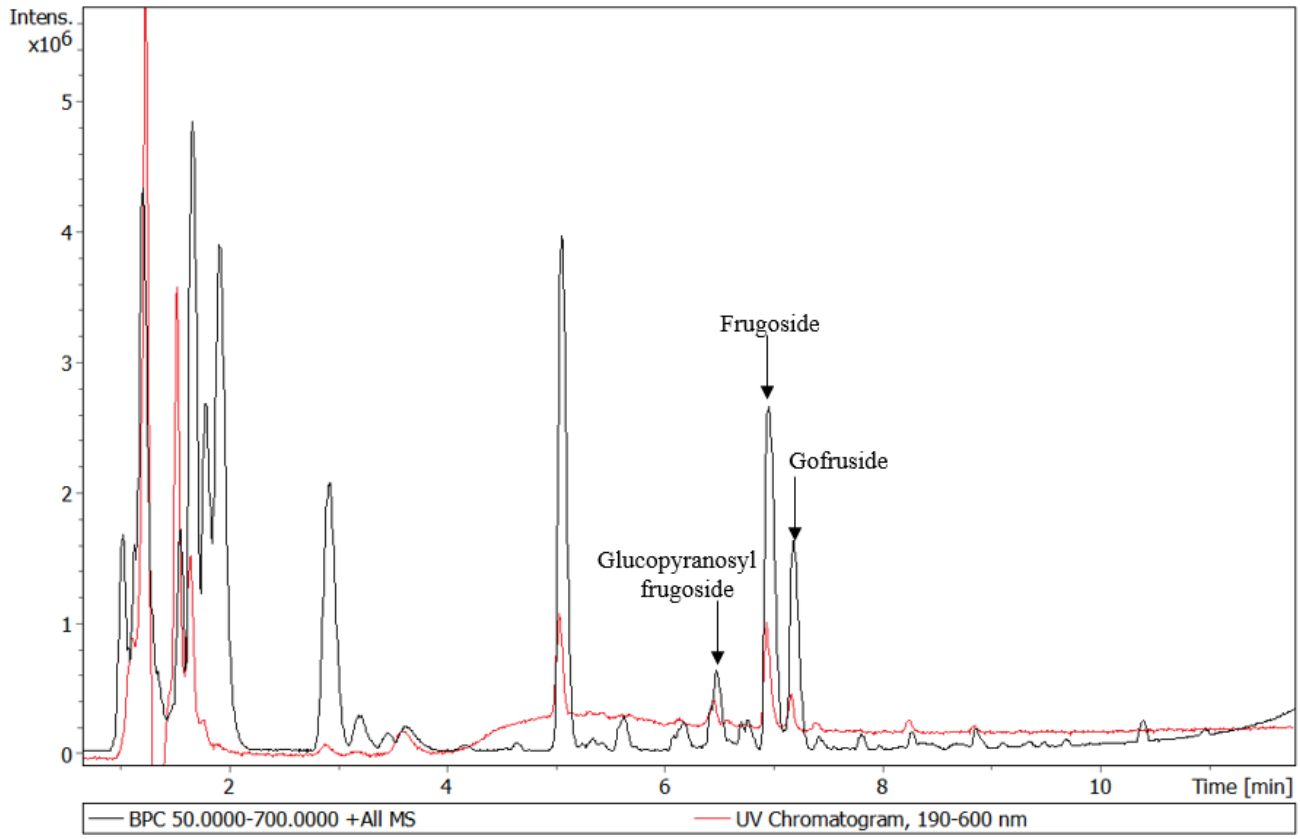
1. **Supplementary Figure S1.** UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* adults. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.
2. **Supplementary Figure S2.** UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* nymphs. Cardenolides found 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.

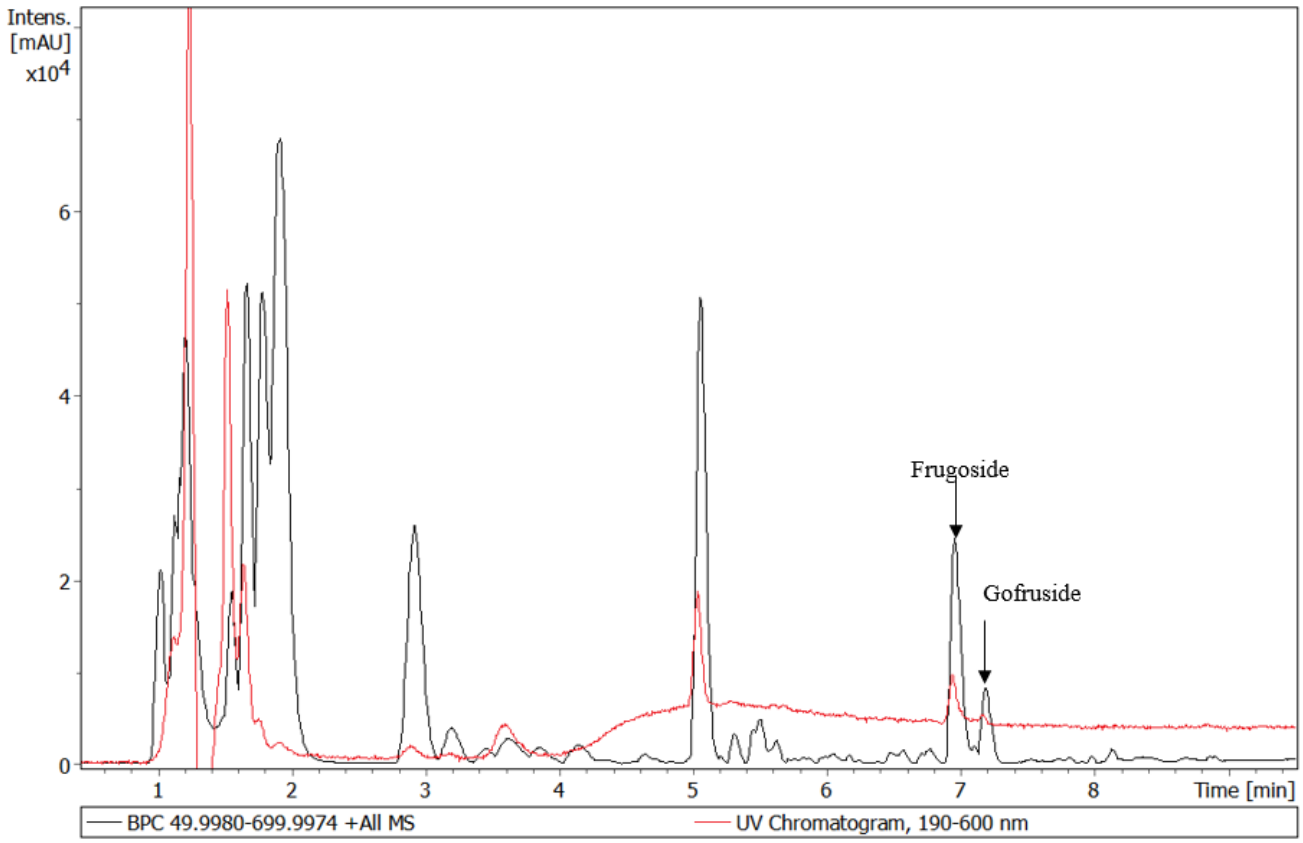
Supplementary Material

1.



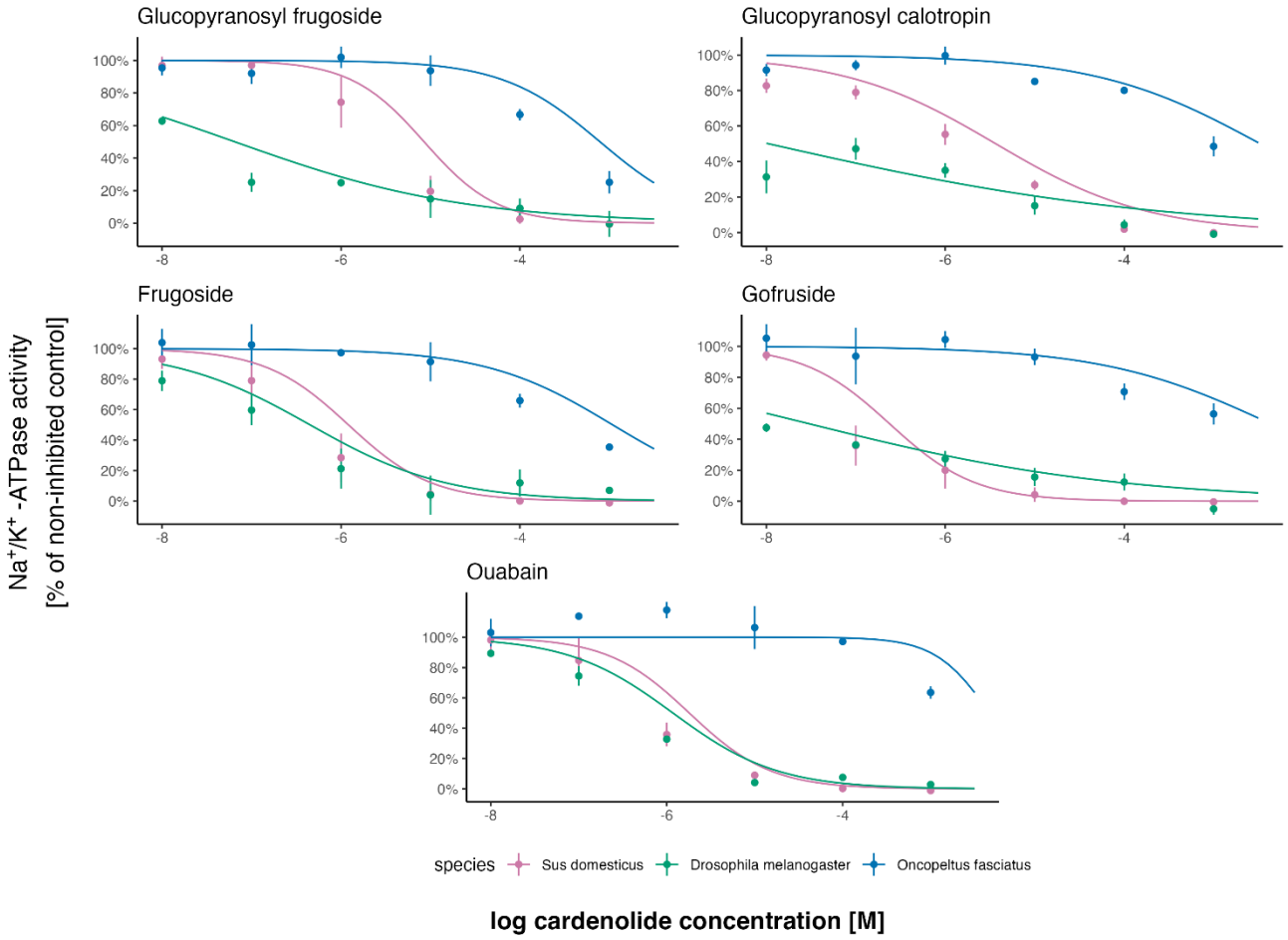
Supplementary Figure S1. UV chromatogram and LC-HRMS trace of extract of *O. fasciatus* adults. Cardenolides found in the sample appear from 5.6 to 7.9 min retention time.

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.

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

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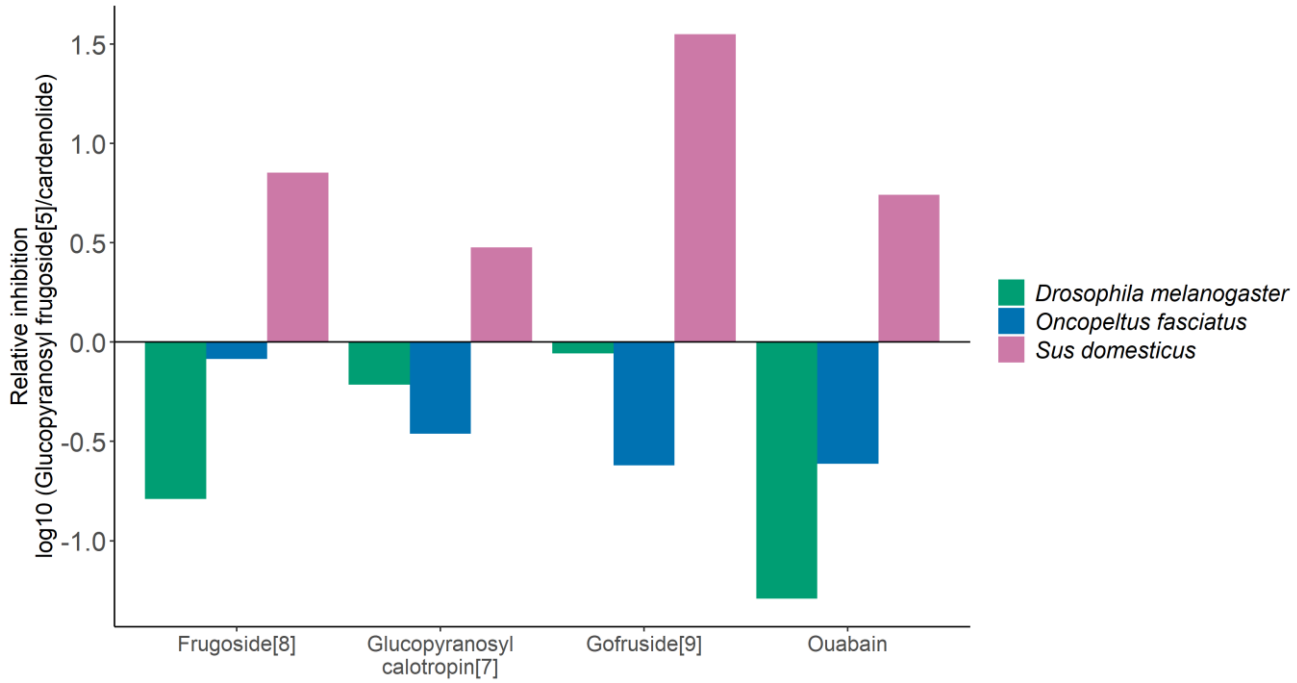
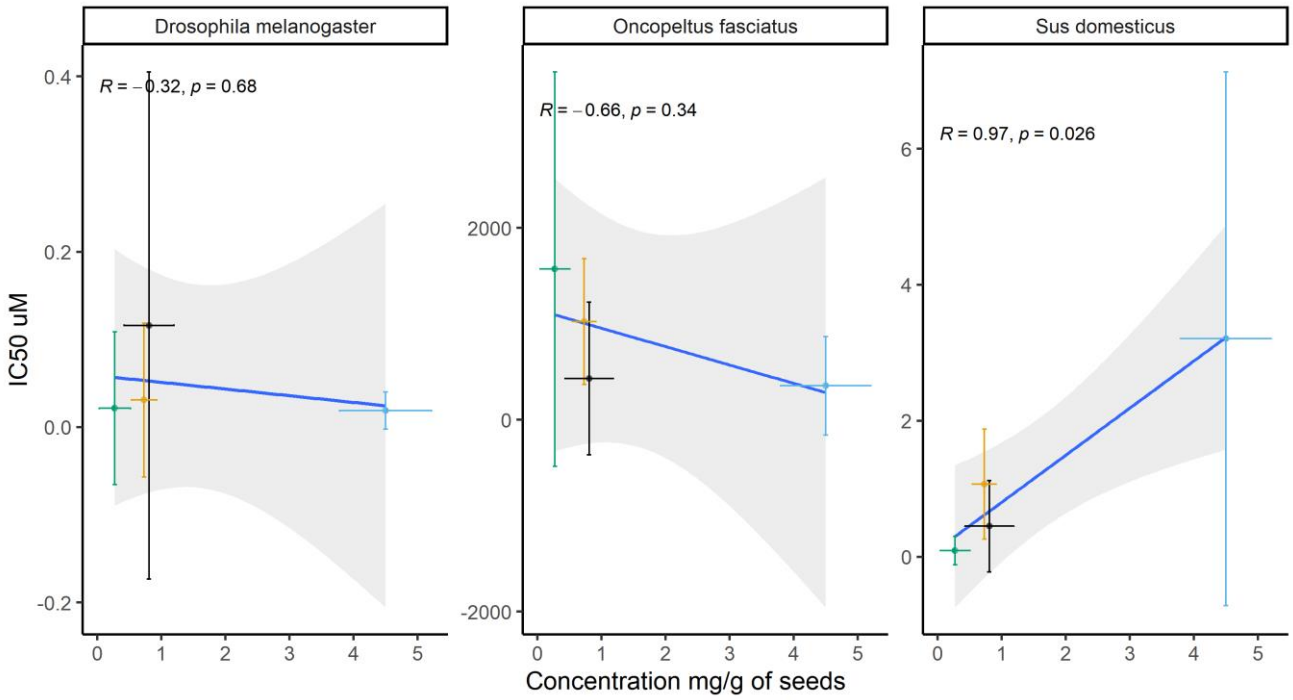


Supplementary Figure S4: Selective effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (*O. fasciatus*, blue; non-adapted *D. melanogaster*, green, and *S. domesticus*, pink). Relative inhibition values are based on IC_{50} values compared with the standard to the abundant 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

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

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)