Elusive toxin in *Cleistanthus collinus* causing vasoconstriction and myocardial depression: Detailed NMR analyses and biological studies of Cleistanthoside A

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1. Instruments and experimental information.

General information:

All chemicals were purchased from Merck, S.D. Fine, Fisher scientific, Sisco Research Laboratories, Hayman and Sigma Aldrich and were used without further purification. FT-IR spectra were recorded on a Perkin Elmer Spectrum 2 spectrophotometer. NMR spectra of Fraction 3 (Cleistanthoside A) were recorded on a Bruker AVANCE III HD 600 MHz
spectrometer equipped with a 5 mm BBI probe at 298 K. The $^1$H and $^{13}$C chemical shifts were referenced to solvent signals at $\delta_{\text{H/C}}$ 2.49/39.5 (DMSO-$d_6$) and 3.35/49.0 (MeOH-$d_4$) relative to TMS. 1D and 2D homo- and heteronuclear NMR spectra were measured with standard Bruker pulse sequences. Super long range HMBC was recorded by a in house modified Bruker pulse sequence according to Abdel-Mohsen et al., 2013 and Furihata and Seto, 1995. PIP HSQMB (Castañar et al., 2014) was used and implemented for determination of $J_{\text{CH}}$ long range coupling constants. NOAH4-BSCN and NOAH4-BSCN-NUS sequences and the processing program MDD-NMR (Orekhov et al., 2003) were implemented from the on line Bruker user library. Topspin 4.0.8 (Copyright 2019, Bruker Biospin GmbH) and SpinWorks 4.2.10 (Copyright 2019, K. Marat, University of Manitoba, CA) were used for processing of NMR spectra.

For Fraction 6 (Cleistanthin A), FT-IR spectra were recorded on a Perkin Elmer Spectrum 2 spectrophotometer. NMR spectra in Acetone-$d_6$ were recorded on Jeol-ECX-500 MHz spectrometer. Temperature studies were performed on a Bruker 500 MHz spectrometer AVANCE III with a 5 mm BBO probe. The $^1$H and $^{13}$C chemical shifts were referenced to solvent signals at $\delta_{\text{H/C}}$ 2.49/39.5 (DMSO-$d_6$) and 2.05/29.8 (Acetone-$d_6$) relative to TMS.

LCMS-ESI spectra were recorded on a Bruker impact-HD spectrometer. X-ray diffraction analysis patterns were measured on X-ray diffractometer system (Agilent Technologies) using Cu $\alpha$ $\lambda$ radiation ($\lambda=1.5406$ Å).
2. Isolation of Cleistanthin A and Cleistanthoside A

**Figure S1.** (A) Pie-chart representing proportions of fractions isolated from chloroform fraction of boiled aqueous extract of fresh *C. collinus* leaves (FLB-CF) with preparative HPLC. Fraction 3 is the major fraction and is identified as Cleistanthoside A. (B) Overlay of HPLC chromatograms of individual fractions isolated from FLB-CF. The analyte concentration in all cases was 10 μg/ml. (C) Overlay of analytical chromatograms of fractions 0, 2, 4 and 7 from FLB-CF at 1 mg/ml concentration. (D) Thin layer chromatogram of prep HPLC fractions 0 – 8 (lanes F0 to F8), Cleistanthin A (Lane A), Cleistanthoside A (Lane C), water fraction of FLB after chloroform partitioning (Lane WF). (E) TLC demonstrating that water fraction of FLB after chloroform partitioning (Lane 4) does not have fluorescence even at 10 mg/ml concentration. (Lane 1, Cleistanthin A; Lane 2, Cleistanthoside A; Lane 3, Chloroform fraction of FLB). The major fluorescent Fraction 3 of FLB-CF and Fraction 6 were characterized extensively. Fraction 3 warranted analysis, as it formed the major fraction and caused death in rats in small doses. Fraction 3 was determined to be Cleistanthoside A and Fraction 6 was confirmed to be Cleistanthin A.
3. $^1$H NMR spectrum of Cleistanthoside A in DMSO-$d_6$ (A) and MeOH-$d_4$ (B)

A: Expansion shows anomeric protons 1'''-H and 1''''-H

B: Expansion shows anomeric protons 1'''-H and 1''''-H

Figure S2. $^1$H NMR spectrum of Cleistanthoside A in DMSO-$d_6$ (A) and MeOH-$d_4$ (B) at 600 MHz.
4. $^{13}$C NMR spectrum of Cleistanthoside A in DMSO-$d_6$ and MeOH-$d_4$

A: Expansion shows multiple resonances for carbons C-1", C-1"', C-5 and C-7'

B: Expansion shows single resonances for carbons C-1", C-1"', C-5 and C-7'

Figure S3. $^{13}$C NMR spectrum of Cleistanthoside A in DMSO-$d_6$ (A) and MeOH-$d_4$ (B) at 150 MHz.
5. Super long range HMBC of Cleistanthoside A in MeOH-\textit{d}4

![Figure S4.](image)

Figure S4. Super long range HMBC of Cleistanthoside A in MeOH-\textit{d}4.

6. PIP HSQMB of Cleistanthoside A in MeOH-\textit{d}4

![Figure S5.](image)

Figure S5. Part of PIP HSQMB of Cleistanthoside A in MeOH-\textit{d}4 displaying the $J_{CH}$ long-range coupling constants of 5-H and 8-H.
7. Selective 1D TOCSYs on anomeric protons $1''$-H and $1'''$-H of Cleistanthoside A

![Figure S6. Selective 1D TOCSYs on anomeric protons $1''$-H (blue) and $1'''$-H of (black) Cleistanthoside A.]

8. Structure elucidation of Cleistanthoside A

The aglycone moiety of Cleistanthoside A was established as diphyllin by evaluation of the $^1$H NMR, COSY and HSQC spectra for the $^1$H spin systems and their carbons ($2'$-H/$5'$-H/$6'$-H; $7'$-H; 3a-H; 5-H/8-H; 6'-OMe; 7'-OMe) and HMBC for the assignment of the remaining 12 quaternary carbons as shown in Fig S7 and experimental section. Each of both aromatic protons 5-H at $\delta$ 7.95 ppm and 8-H at $\delta$ 7.09 ppm showed four $J_{CH}$ long-range couplings with quaternary carbons at $\delta$ 128.34, 131.89, 151.84 and 153.35 ppm. Since in common HMBC $^2J_{CH}$ and $^3J_{CH}$ correlations cannot be differentiated the respective positions C-1a, C-4a, C-6 and C-7 could not be unambiguously assigned. A combination of super long-range HMBC$^{1,2}$ (Figure S4) and evaluation of $^1$H-$^{13}$C long-range coupling constants measured by PIP-
HSQMBC (SI Figure S5) enabled the complete assignment of the above carbons. Thus a $^4J_{CH}$ super long-range coupling between 2’H and 6’-H and carbon at δ 131.89 ppm and a $^3J_{5-H,C1-a}$ = 6.9 Hz established the C-1a position. A second coupling of $^3J$ = 8.3 Hz between 5-H and carbon at δ 151.84 ppm fixed the C-7 position whereas a significant smaller $^2J_{5-H,C6}$ = 2.8 Hz coupling constant along with a $^4J_{C-H}$ super long-range coupling between the methoxy group at C-7 at δ 3.77 ppm and carbon at δ 153.35 ppm determined the C-6 position. C-4a at δ 128.34 ppm exhibited a $^3J_{CH}$ = 6.7 Hz with 8-H and $^2J_{CH}$ = 2.6 Hz with 5-H (Figure S7).

**Figure S7.** Important HMBC (blue) and super long-range HMBC (pink) correlations, and $J_{CH}$ long range couplings derived from PIP HSQMBC (green) of Cleistanthoside A.

Further analysis of the $^1$H NMR and $^{13}$C NMR spectrum revealed two sugar moieties as indicated by two anomeric methine groups at δH 5.26 (1’’-H)/ δC 103.71 ppm (C-1’’) and δH 4.84 (1’’’-H)/ δC 105.01 ppm (C-1’’’) along with eleven protons in the region δ 3.30 - 4.21 ppm and two methoxy groups. Selective 1D-TOCSYs on both anomeric protons displayed a six-proton spin system for 1’’-H and a seven-proton spin system for 1’’’-H (Figure S6). Evaluation of the COSY, NOESY, HSQC, HMBC spectra and analysis of the coupling
constants established a 3,4-Di-\textit{O}-methyl-\textit{\beta}-xylopyranosyl (1'''-H) and a \textit{\beta}-glucopyranosyl moiety (1''''-H) with a $^4\text{C}_1$ conformation in the case of a D-configurated pyranose.\textsuperscript{5} Coupling constants of strongly overlapped signals were resolved by sel 1D TOCSY (Figure S6). The 1 → 2 interglycosidic linkage and the linkage of the carbohydrate chain with the aglycone was unambiguously derived indicated by HMBC and NOESY as indicated in Figure S7 and Figure S8.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure8}
\caption{Important NOESY correlations of Cleistanthoside A.}
\end{figure}
9. Figure S9. Comparison of conventional HMBC (A) and HMBC subspectrum (B) of NOAH4-BSCN with traditional and NUS sampling in F1. TD 2048 (F2) x 512 (F1), ns 16.

A: conv. HMBC; Traditional sampling; 4h 1min  
B: HMBC-NOAH4; Trad. sampling; 1h 48min

A: conv. HMBC; NUS sampling 50%; 2h 50s  
B: HMBC-NOAH4; NUS sampling 50%; 54 min

A: conv. HMBC; NUS sampling 25%; 1h 45s  
B: HMBC-NOAH4; NUS sampling 50%; 27 min

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10. Figure S10. Comparison of conventional HSQC (A) and HSQC subspectrum (B) of NOAH4-BSCN with traditional and NUS sampling in F1. TD 2048 (F2) x 512 (F1), ns 16.

A: conv. HSQC; Traditional sampling; 3h 54min
B: HSQC-NOAH4; Trad. sampling; 1h 48min

A: conv. HSQC; NUS sampling 50%; 1h 57min
B: HSQC-NOAH4; NUS sampling 50%; 54min

A: conv. HSQC; NUS sampling 25%; 58 min 48 s
B: HSQC-NOAH4; NUS sampling 25%; 27min
Figure S11. Comparison of conventional COSY (A) and COSY subspectrum (B) of NOAH4-BSCN with traditional and NUS sampling in F1. TD 2048 (F2) x 512 (F1), ns 16.

A: conv. COSY; Traditional sampling; 4h 1min

B: COSY-NOAH4; Trad. sampling; 1h 48min

A: conv. COSY; NUS sampling 50%; 2h 1min

B: COSY-NOAH4; NUS sampling 50%; 54min

A: conv. COSY; NUS sampling 25%; 1h 45s

B: COSY-NOAH4; NUS sampling 25%; 27min
12. Figure S12. Comparison of conventional NOESY (A) and NOESY subspectrum (B) of NOAH4-BSCN with traditional and NUS sampling in F1. TD 2048 (F2) x 512 (F1), ns 16.

A: conv. NOESY; Traditional sampling; 5h 35min         B: NOESY-NOAH4; Trad. sampling; 1h 48min

A: conv. NOESY; NUS sampling 50%; 2h 48min             B: NOESY-NOAH4; NUS sampl. 50%; 54min

A: conv. NOESY; NUS sampling 25%; 1h 24min             B: NOESY-NOAH4; NUS sampl. 50%; 27min
13. Spectral data for Cleistanthoside A

Yellowish white powder;

**Melting point:** 162-166 °C

**FT-IR** (KBr, $\tilde{\nu}_{\text{max}}$ in cm$^{-1}$): 3446, 1753, 1507, 1479, 1434, 1228, 1263, 1169, 1074, 1036.

**$^1$H NMR** (600 MHz, MeOH-$d_4$): $\delta$ 3.33 (1H, $ddd$, $J = 2.3$, 5.5, 9.4 Hz, 5‴′-H), 3.35 (1H, $dd$, $J = 7.9$, 9.2 Hz, 2‴′-H), 3.38 (1H, $ddd$, $J = 0.8$, 8.5, 9.5 Hz, 4‴′-H), 3.39 (1H, $dd$, $J = 7.7$, 11.9 Hz, 5‴-H)$_{ax}$, 3.47 (1H, $dd$, $J = 8.6$, 9.1 Hz, 3‴′-H), 3.51 (3H, s, CH$_3$-O-C4‴′), 3.52 (1H, $dd$, $J = 7.7$, 11.9 Hz, 5‴-H)$_{ax}$, 3.60 (1H, $t$-like, $J = 7.2$ Hz, 3‴-H), 3.67 (1H, $ddd$, $J = 1.3$, 5.6, 11.8 Hz, 6‴′-H$_a$), 3.72 (3H, s, CH$_3$-O-C3‴′), 3.77 (3H, s, CH$_3$-O-C7), 3.81 (1H, $ddd$, $J = 1.3$, 2.3, 11.8 Hz, 6‴′-H$_b$), 4.09 (3H, s, CH$_3$-O-C6), 4.14 (1H, $dd$, $J = 4.2$, 11.8 Hz, 5‴-H)$_{eq}$, 4.18 (1H, $dd$, $J = 5.9$, 7.3 Hz, 2‴-H), 4.84 (1H, $d$, $J = 7.8$ Hz, 1‴′′-H), 5.26 (1H, $d$, $J = 5.9$ Hz, 1‴-H), 5.55 (1H, $dd$, $J = 2.9$, 14.6 Hz, 3a-H$_a$), 5.64 (1H, $dd$, $J = 2.6$, 14.6 Hz, 3a-H$_b$), 6.08 (1H, $d$, $J = 1.1$ Hz, 7′-H$_a$), 6.09 (1H, $d$, $J = 0.8$, 1.1 Hz, 7′-H$_b$), 6.78-6.81 (1H, higher order m, 6′-H), 6.80-6.83 (1H, higher order m, 2′-H), 6.99 (1H, br d, $J = 7.8$ Hz, 5′-H), 7.09 (1H, $d$, $J = 2.6$ Hz, 8-H), 7.95 (1H, $d$, $J = 1.1$ Hz, 5-H); coupling constants are directly taken from the spectra and are not averaged.

**$^{13}$C NMR** (150 MHz, MeOH-$d_4$): $\delta$ 56.03 (CH$_3$-O-C7), 57.02 (CH$_3$-O-C6), 58.26 (CH$_3$-O-C4‴′), 60.43 (CH$_3$-O-C3‴′), 62.67 (C-6‴′), 62.90 (C-5‴′), 69.09 (C-3a), 71.69 (C-4‴′), 75.61 (C-2‴′), 77.97 (C-3‴′′), 78.28 (C-5‴′′), 78.61 (C-2‴′′), 80.22 (C-4‴′′), 84.17 (C-3‴′′), 102.62 (C-7′), 110.24 (C-5), 103.71 (C-1‴′), 105.01 (C-1‴′′), 106.99 (C-8), 109.01 (C-5′), 111.72 (C-2′), 120.12 (C-3), 124.70 (C-6′), 128.34 (C-4a), 130.05 (C-1′), 130.13 (C-2), 131.89 (C-1a), 136.97 (C-1), 145.66 (C-4), 148.99 (C-4′), 149.04 (C-3′), 151.84 (C-7), 153.35 (C-6), 172.06 (C-2a).

**MS** (HRMS): m/z calculated for [M+Na]$^+$ 725.2058, found 725.2477.
14. Structure elucidation of Cleistanthin A

**Figure S13.** Important HMBC (blue) and NOESY (orange) correlations of Cleistanthin A
15. $^1$H NMR spectrum of Cleistanthin A in Acetone-$d_6$

Figure S14. $^1$H NMR spectrum of Cleistanthin A in Acetone-$d_6$ at 500 MHz.

16. $^{13}$C NMR spectrum of Cleistanthin A in Acetone-$d_6$

Figure S15. $^{13}$C NMR spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz.
17. COSY spectrum of Cleistanthin A in Acetone-$d_6$

Figure S16. COSY spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz.

18. HSQC spectrum of Cleistanthin A in Acetone-$d_6$

Figure S17. HSQC spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz.
19. HMBC spectrum of Cleistanthin A in Acetone-$d_6$

![HMBC spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz](image)

**Figure S18.** HMBC spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz.

20. NOESY spectrum of Cleistanthin A in Acetone-$d_6$

![NOESY spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz](image)

**Figure S19.** NOESY spectrum of Cleistanthin A in Acetone-$d_6$ at 125 MHz
21. NMR Temperature study of Cleistanthin A in DMSO-\textit{d6}

\textbf{Figure S20.} $^1$H NMR (A) and $^{13}$C NMR (B) spectra of Cleistanthin A at different T in DMSO-\textit{d6} at 500 MHz showing coalescence of the signals of the anomeric proton and beginning of collapsing of the aromatic protons and carbons at 85°C.
22. ESI-MS spectrum of Cleistanthin A

Figure S21. ESI-MS spectrum of Cleistanthin A.

23. Spectral data for Cleistanthin A

Melting point: 134-136°C

FT-IR (KBr, $\tilde{\nu}_{\text{max}}$ in cm$^{-1}$): 3451, 2917, 1745, 1625, 1506, 1429, 1218, 1169, 1071, 769.

$^1$H NMR (500 MHz, Acetone-$d_6$): $\delta$ 3.23 (1H, dt-like, $J = 1.8, 9.9$ Hz, 5’’-H$_{\text{ax}}$), 3.24 (1H, t-like, $J = 8.8$ Hz, 3’’-H), 3.37 (1H, dt-like, $J = 4.7, 8.9$ Hz, 4’’-H), 3.42 (3H, s, CH$_3$-O-C4’’), 3.60 (3H, s, CH$_3$-O-C3’’), 3.68 (3H, s, CH$_3$-O-C7), 3.74 (1H, dd, $J = 7.4, 9.0$ Hz, 2’’-H), 3.97 (3H, s, CH$_3$-O-C6), 4.11 (1H, ddd, $J = 2.5, 4.7, 11.1$ Hz, 5’’-H$_{\text{eq}}$), 4.87 (1H, d, $J = 7.6$ Hz, 1’’-H), 5.38 (1H, dd, $J = 1.9, 15.0$ Hz, 3a-H$_a$), 5.48 (1H, d, $J = 15.0$ Hz, 3a-H$_b$), 6.05 (1H, br s, 7’-H$_a$), 6.06 (1H, br s, 7’-H$_b$), 6.74-6.80 (1H, higher order m, 6’-H), 6.79-6.83 (1H, higher order m, 2’-H), 6.94 (1H, br d, $J = 7.6$ Hz, 5’-H), 7.02 & 7.03 (1H, br s, 8-H), 8.07 (1H, d, $J = 2.0$ Hz, 5-H); coupling constants are directly taken from the spectra and are not averaged.
\(^{13}\)C NMR (125 MHz, Acetone-\(d_6\)): \(\delta\) 55.59 (CH\(_3\)-O-C7), 56.29 (CH\(_3\)-O-C6), 58.30 (CH\(_3\)-O-C4’’), 60.58 (CH\(_3\)-O-C3’’), 63.56 (C-5’’), 67.75 (C-3a), 73.82 (C-2’’), 79.89 (C-4’’), 85.68 (C-3’’), 101.98 (C-7’), 102.18 (C-5), 106.23 (C-1’’), 106.31 (C-8), 108.54 & 108.56 (C-5’), 111.35 & 111.42 (C-2’), 119.54 (C-3), 124.33 (C-6’), 127.90 (C-4a), 129.30 (C-1’), 130.84 & 130.87 (C-2), 131.99 (C-1a), 136.20 & 136.30 (C-1), 145.54 (C-4), 148.00 (C-4’), 148.06 (C-3’), 151.06 (C-7), 152.60 (C-6), 170.12 (C-2a).

24. X-ray Crystal Structure of Cleistanthin A

Colourless needle shaped crystals of Cleistanthin A, suitable for single-crystal X-ray diffraction analysis were obtained from slow evaporation of diethyl ether solution after 2 days. Single-crystal X-ray diffraction analysis patterns of Cleistanthin A were measured on X-ray diffractometer system (Agilent Technologies) using CuK\(\alpha\) radiation (\(\lambda=1.5406\) Å) source, which reveals that Cleistanthin A crystallizes in triclinic P1 space group with \(Z = 1\) (Table S1). Data collection was done using CrysalisPro Software and reduction was undertaken with CrysalisPro Software. Structure was solved by direct methods using olex2,\(^6\) SHELXS-97,\(^7\) and refined by full-matrix least squares on F\(^2\) using SHELXL-97. The positions of all the atoms were obtained by direct methods. Crystallographic refinement details and data collection parameters of Cleistanthin A are summarized in Table S1. The ORTEP diagram for the crystal structure of Cleistanthin A is given in Figure S22a.
Figure S22: (a) ORTEP diagram of the X-ray crystal structure of Cleistanthin A with 50% probability and (b) structure in dimeric form

The formula weight for the same in X-ray crystallography is 1081, because Cleistanthin A in its crystal structure forms a dimer (crystals possess triclinic crystal system, thereby behave in dimeric form). The data have been submitted to the Cambridge Crystallographic Data Centre and were assigned the following deposition number: CCDC 2093163.

Table S1. Crystal data and structure refinement for Cleistanthin A

|   | Identification code | Clei-A |
|---|---------------------|--------|
| 2 | Empirical formula   | C_{28}H_{28}O_{11} |
| 3 | Formula weight      | 540.50 |
| 4 | Temperature         | 150(2) K |
| 5 | Wavelength          | 1.54184 Å |
| 6 | Crystal system, space group | Triclinic, P1 |
|   | a = 9.4812(6) Å alpha = 76.434(4) deg. |
| 7 | Unit cell dimensions | b = 11.0371(6) Å beta = 74.676(5) deg. |
|   | c = 13.0913(6) Å gamma = 73.104(5) deg. |
| 8 | Volume              | 1245.59(12) Å³ |
| 9 | Z, Calculated density | 1, 1.441 Mg/m³ |
| 10| Absorption coefficient | 0.944 mm⁻¹ |
11 F(000) 568
12 Crystal size 0.462 x 0.235 x 0.112 mm³
13 Theta range for data collection 3.553 to 66.813°.
14 Limiting indices -10<=h<=11, -13<=k<=10, -15<=l<=14
15 Reflections collected / unique 7408 / 4931 [R(int) = 0.0193]
16 Completeness to theta = 67.684° 96.6 %
17 Absorption correction Gaussian
18 Max. and min. transmission 0.920 and 0.773
19 Refinement method Full-matrix least-squares on F²
20 Data / restraints / parameters 4931 / 3 / 713
21 Goodness-of-fit on F² 1.029
22 Final R indices [I>2sigma(I)] R1 = 0.0467, wR2 = 0.1336
23 R indices (all data) R1 = 0.0470, wR2 = 0.1340
24 Absolute structure parameter -0.17(15)
25 Largest diff. peak and hole 0.560 and -0.270 e.Å⁻³

**CIF Check Alerts and Discussion:**

**PLAT089_ALERT_3_C:** Poor Data / Parameter Ratio (Zmax < 18) ....... 6.21 Note

**Response:** Diffuse scatter beyond 1.06 Å was omitted which limited the number of unique reflection available during refinement.

**PLAT094_ALERT_2_C:** Ratio of Maximum / Minimum Residual Density .... 2.07 Report

**Response:** All the crystal is showing strong diffuse scattering in the form of diffuse Bragg peaks and scattering between them. As the model does not account for this type of disorder this results in a Ratio of Maximum / Minimum Residual Density 2.07

**PLAT230_ALERT_2_C:** Hirshfeld Test Diff for C43 --C44 .... 5.7 s.u.

**Response:** The Hirshfeld difference between C43 and C44 is likely due to slight disorder of the aromatic ring.

**PLAT340_ALERT_3_C:** Low Bond Precision on C-C Bonds .............. 0.00519 Ang.

**Response:** At 150 K, and as a result of the single crystal transformation to this phase, the X-ray data quality is poor resulting in low bond precession accuracy.
25. References

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