Elusive Toxin in *Cleistanthus collinus* Causing Vasoconstriction and Myocardial Depression: Detailed NMR Analyses and Biological Studies of Cleistanthoside A

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ABSTRACT: *Cleistanthus collinus* leaf extracts are consumed for suicidal purposes in southern India. The boiled decoction is known to be more toxic than the fresh leaf juice. Although several compounds have been isolated and their toxicity tested, controversy remains as to which compounds are responsible for the high level of toxicity of *C. collinus*. We report herein that cleistanthoside A is the major toxin in the boiled aqueous extract of fresh leaves and causes death in rats in small doses. The toxicity of the boiled extract prepared in the manner described can be attributed entirely to cleistanthoside A. Cleistanthin A could also be isolated from the boiled extract, albeit in trace amounts. As hypotension not responding to vasoconstrictors is the cause of death in patients who have consumed the boiled extract, effects of

cleistanthoside A on the determinants of blood pressure, namely, force of cardiac contraction and vascular resistance, were tested in isolated organ experiments. Cleistanthoside A has a direct vasoconstrictor effect; however, it inhibits ventricular contractility. Therefore, the notion that the shock in *C. collinus* poisoning is of vascular origin must be considered carefully, and the possibility of cardiogenic shock must be studied. We present the crystal structure of cleistanthin A and show the potency of fast NMR methods (NOAH4-BSCN-NUS) in the full spectral assignment of cleistanthoside A as a real-world sample of a natural product. We also compare the results of the NOAH4-BSCN-NUS NMR experiments with conventional NMR methods.
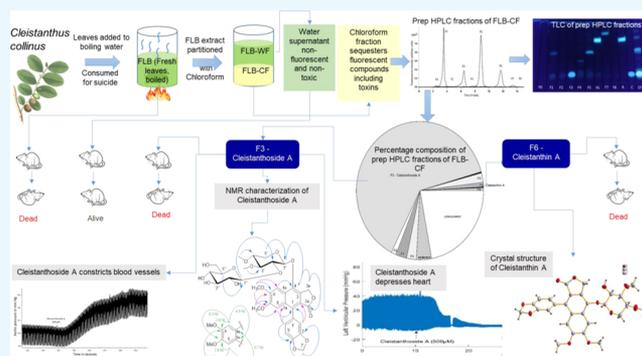
1. INTRODUCTION

Cleistanthus collinus is a small deciduous shrub native to India, Malaysia, and Africa. Almost all parts of the plant are poisonous. Suicidal poisoning by consumption of aqueous extracts of the leaves of *C. collinus* is common in rural south India.[1] Either a boiled decoction or a fresh juice of the leaves is consumed for suicidal purposes. The boiled decoction is reported to be associated with a higher mortality than the fresh leaf juice.[2] Death occurs between third to seventh day after consumption.[3]

While the mechanism of toxicity is still under research, it is important to analyze the chemical composition of the plant extract and isolate the bioactive constituents to enable mechanistic studies. The leaves contain more than 20 fluorescent compounds.[4] Of these, the lignan lactones cleistanthin A and B are considered to be the toxic principles in the leaf extracts, while two other compounds, diphyllin and collinusin, have been reported to be non-toxic.[5] However, it is also reported that cleistanthins A and B found in acetone

extracts are not present in the aqueous extracts of *C. collinus* leaves,[6] and therefore, they may not be clinically relevant toxins as patients only consume aqueous extracts of the leaves.[6] It is also reported that purified cleistanthins A and B do not cause death up to concentrations as high as 800 mg/kg body weight (BW) in rats.[7] In view of such controversy as to what compound is responsible for the mortality in patients who consume aqueous extracts of *C. collinus* leaves, we sought to reinvestigate the constituents of the boiled extract.

Since many of the plant constituents can only be isolated in the small sample amount in the range of several 100 μg to 1−3 mg, recording of 1D and 2D NMR spectra needed for
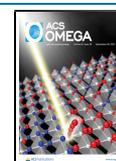
unambiguous structure elucidation requires very long acquisition times (up to 48 h or more). Due to the unknown chemical stability of those compounds, degradation/rearrangement reactions may take place in such long time precluding any analysis. Therefore, technologies reducing NMR analysis time are highly required. One cost-intensive possibility relies on hardware improvements, for example, use of higher magnetic fields, N_2/He cryo-cooled NMR probes, multi receivers, and so on. A second approach lies in the development of time-saving fast NMR methods such as Hadamard encoding, computer-optimized folding, covariance processing, projection reconstruction, ultrafast NMR spectroscopy, and non-uniform sampling (NUS) with or without the above hardware.[8−15] Only recent developments combine different 2D techniques like heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum coherence (HSQC), correlation spectroscopy (COSY), and nuclear Overhauser enhancement spectroscopy (NOESY) in nested super sequences (NOAH-4) to accelerate data collection.[16,17]

In this report, we demonstrate our investigation on the chemical composition of the boiled extract to mainly study what compound is responsible for the mortality in patients who consume aqueous extracts of *C. collinus* leaves. Moreover, we present the full structural assignment of the major compound cleistanthoside A by fast NOAH-4-BSCN-NUS NMR experiments and demonstrate their efficiency in reducing NMR acquisition time compared to conventional NMR methods. We also report the single-crystal structure and detailed NMR analyses of cleistanthin A present in trace amounts in the boiled extracts. Studies determining the lethal dose for some fractions and mechanism of toxicity of the major toxic principle in the boiled extract, namely, cleistanthoside A, are also reported here.

2. RESULTS

2.1. Isolation and Quantification of Cleistanthoside A and Cleistanthin A.

The dry weight of the boiled extract of fresh leaves (FLB) obtained from 10 g of leaves was 1.32 ± 0.1 g (n = three different leaf samples). The dry weight of the chloroform fraction of the aqueous extract (FLB-CF) from 10 g of fresh leaves was 0.09 ± 0.03 g (n = 4).

Preparative chromatography of FLB-CF (the chloroform fraction after partitioning FLB with chloroform) dissolved in methanol was performed, and nine fractions (F0 − 8) were collected. The fractions collected from 100 mg of FLB-CF were dried and weighed, and the weight of each fraction is given in Table 1. Proportions of each fraction as a percentage

Table 1. Dry Weight of Fractions Collected from Prep HPLC of 100 mg of the Chloroform Fraction of the Boiled Extract of *C. collinus* Leaves (FLB-CF)

| fraction number	weight in mg (mean ± SD)	identity of compound
fraction 0	2.6 ± 2	
fraction 1	2.9 ± 1.1	
fraction 2	1.3 ± 0.4	
fraction 3	65.8 ± 10	*Cleistanthoside A*
fraction 4	0.3 ± 0.3	
fraction 5	2.3 ± 1.7	
fraction 6	1.1 ± 0.5	*Cleistanthin A*
fraction 7	0.2 ± 0.3	
fraction 8	0.7 ± 0.2	

of FLB-CF are shown as a pie chart in Figure S1 (Supporting Information). The pie chart also includes the methanol-insoluble sediment of FLB-CF, as stated in Experimental Section 5.4.

From Table 1 and Figure S1 (Supporting Information), it can be seen that fraction 3, consisting of cleistanthoside A, forms the major component of the chloroform fraction of the boiled decoction of *C. collinus* leaves, FLB-CF. Chemical characterization of fraction 6 afforded cleistanthin A as the minor constituent. As an alternative to the time and solvent consuming method based on prep high-performance liquid chromatography (HPLC) and weighing of the fractions, an analytical quantification technique consisting of analytical HPLC in combination with standard curves prepared with purified compounds was established. Retention time for cleistanthoside A was 5.5 min and that for cleistanthin A was 9 min. 1 mg of FLB contained 77.4 ± 11.4 μg of cleistanthoside A and 0.5 ± 0.01 μg of cleistanthin A. These values are in agreement with the quantification carried out with actual isolation with prep HPLC and weighing of the fractions.

2.2. Chemical Characterization of Fraction 3 as Cleistanthoside A and Fraction 6 as Cleistanthin A.

Cleistanthoside A, a yellowish white powder, is insoluble in water at room temperature but dissolves on boiling (tested up to 10 mg/mL concentration). Its chemical formula is $C_{34}H_{38}O_{16}$. MS (HRMS): m/z calcd for $[M + Na]^+$, 725.2058; found, 725.2477. Cleistanthoside A was first reported by Sastry and Rao in 1983.[19] However, to our knowledge, there has been no report on the complete NMR assignment of natural product isolated cleistanthoside A. Previously, NMR data of its acetyl derivative or NMR data of chemically synthesized cleistanthoside A have been reported.[19−23] The ^{1}H and ^{13}C NMR spectra of cleistanthoside A recorded in dimethyl sulfoxide (DMSO)-d_6 displayed duplicated signals for anomeric and aromatic protons and carbons due to hindered rotation. Even at slightly higher and lower temperature (data not shown), only a small change in the intensities of the two sets of signals could be observed. Methanol-d_4 showing only one set of signals was selected for further NMR studies (Supporting Information, Figures S2 and S3). The super sequence NOAH4 BSCN comprising HMBC, HSQC, COSY, and NOESY nested in one pulse sequence[14] was applied for structure elucidation and spectral assignment of the ^{1}H and ^{13}C NMR data (details in the Supporting Information).

A combination of super long-range HMBC (Supporting Information, Figure S4)[24,25] and evaluation of ^{1}H−^{13}C long-range coupling constants measured by PIP-HSQMBC (Supporting Information, Figure S5; Castañar et al., 2014) enabled the complete assignment of carbons C-1a, 2a, 5, 6, 7, 8 not assignable otherwise (Figure 1). To show the potency of time saving of recent NMR methods on a natural product such as cleistanthoside A at a concentration of 2.8 mg in 600 μL, we compared the spectra obtained with the super sequence NOAH4-BSCN with the conventional spectra once and show how NUS sampling applied in both methods can additionally reduce time by a factor of 2 or even 4. Comparison of the acquisition times of the conventional 2D spectra (HMBC, HSQC, and COSY, 4 h each) with the NOAH4-BSCN sub spectra (1 h 48 min each) afforded up to 45% time-saving effect of the latter with similar spectral quality for HMBC, HSQC, and COSY (Supporting Information, Figures S9−11). Compared to the conventional NOESY (5 h 35 min), the
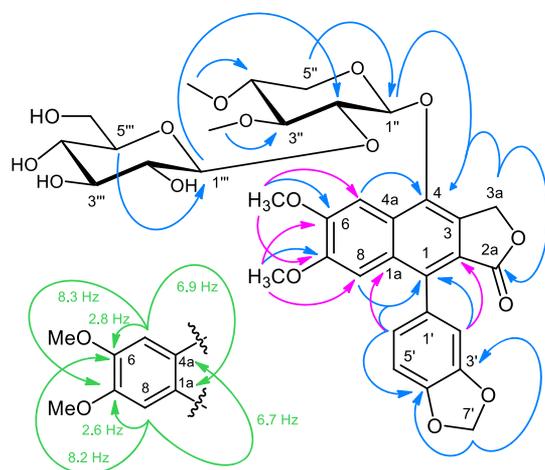
Figure 1. Important HMBC (blue) and super long-range HMBC (pink) correlations and J_{CH} long-range couplings derived from PIP HSQMBC (green) of cleistanthoside A.

NOESY obtained by NOAH 4 (1 h 48 min) was of poorer quality but still contained all relevant information (Supporting Information, Figure S12). The weaker NOESY spectrum in NOAH4-BSCN is generally expected because the HMBC leaves the bulk magnetization depleted, and it only has time to recover during the two previous FID-s (HMBC and HSQC). Unless the relaxation time is really long, one would expect about 25−40% signal of the stand-alone NOESY module.[14] Applying NUS in lieu of conventional sampling in the indirect dimension further reduces the acquisition time by a factor of 2 for 50% NUS and a factor of 4 for 25% NUS without any significant loss of spectral quality (Supporting Information, Figures S9−S12). In summary, application of, for example, NOAH4-BSCN plus 25% NUS compared to conventional methods with traditional sampling in F1 may save spectrometer time up to a factor of 8 with similar spectral quality.

Cleistanthin A (Figure 2), a reported toxin in *C. collinus* (Govindachari et al., in 1969), was isolated from fraction 6 of
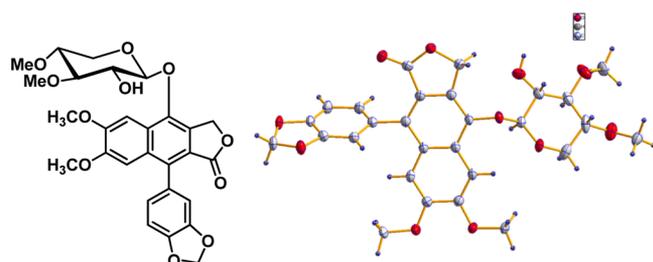
Figure 2. Chemical structure of cleistanthin A (left) and ORTEP figure of cleistanthin A (right).

the prep HPLC as pale yellow colored powder. The chemical formula for cleistanthin A is $C_{28}H_{28}O_{11}$. MS (HRMS): m/z calcd for $C_{28}H_{29}O_{11}$ $[M + H]^+$, 541.1710; found, 541.1594 and $[M + Na]^+$, 563.1529; found, 563.1412 and $[M + K]^+$, 579.1269; found, 579.1149 (Figure S21). Similar to cleistanthoside A, the ^1H and ^{13}C NMR spectra of cleistanthin A exhibited duplicated or more signals in all the solvents tested ($CDCl_3$, DMSO-d_6, and acetone-d_6; only rarely soluble in MeOD). A NMR temperature study in DMSO up to 85 °C revealed coalescence of the sugar protons and carbons at 85 °C

and beginning of collapsing of the aromatic protons and carbons in question (Supporting Information, Figure S20). Higher temperature was not applied to avoid damaging of the sample and probe. The ^1H and ^{13}C NMR data in acetone-d_6 were completely assigned by extensive 2D NMR and are presented in Supporting Information (Figures S14−S19). The single-crystal structure and allied data are presented in Figure 2 and Supporting Information (Figure S22 and Table S1).

2.3. Toxicity Studies. The boiled aqueous extract of *C. collinus* (FLB) was lethal in rats at a dose of 75 mg/100 g BW. Three fractions of FLB were also tested for toxicity, namely, FLB-WF (water fraction after chloroform partitioning of the boiled aqueous extract of FLB), cleistanthoside A, and cleistanthin A. FLB-WF was not lethal even at an oral dose of 200 mg per 100 g of BW (Table 2). Therefore, it was considered non-toxic and was not pursued further.

Table 2. Lethal Doses of Boiled Aqueous Extract of *C. collinus* Leaves (FLB) and Fractions Isolated from It

fraction tested	minimum lethal dose (MLD100) mg/100 g BW ($n = 4$)	LD$_{75}$ mg/100 g BW ($n = 4$)	LD$_{50}$ mg/100 g BW ($n = 4$)	non-lethal dose ($n = 4$)
FLB	75 mg		25 mg	
Cleistanthoside A	3 mg	2 mg		
Cleistanthin A	4 mg	3 mg		
FLB-WF				200 mg

It was found that cleistanthin A and cleistanthoside A were lethal at very small doses. It is seen (from the concentration of cleistanthoside A in FLB reported in Section 2.1 and MLD$_{100}$ values reported in Table 2) that the amount of cleistanthoside A that is present in the minimum lethal dose of FLB is equal to or more than the MLD$_{100}$ of pure cleistanthoside A. Therefore, all the toxicity of FLB can be attributed to cleistanthoside A.

2.4. Rat Hind Limb Preparation to Study the Effect of Cleistanthoside A on Vascular Resistance. Rat hind limb preparation is described in experimental Section 5.9. Descending aorta was cannulated and perfused with standard extracellular solution using a pulsatile pump. Aortic pressure was measured with a pressure transducer connected to the cannula. Any change in the pressure is entirely due to changes in vascular characteristics as the heart is replaced with a pulsatile pump. Mean aortic pressure (MAP) values were computed for every pulse (from area under the curve for aortic pressure), using MATLAB. The average of MAP values from all pressure pulses recorded over a 50 s time window before and after addition of cleistanthoside A was calculated and compared. Raw tracings of experiments with low- and high-dose cleistanthoside A are shown in Figure 3. Thus, a concentration of 100 μM of cleistanthoside A does not affect vascular resistance (Figure 3A), whereas at 500 μM concentration, it acts as a vasoconstrictor (Figure 3B).

The average MAP values after addition of cleistanthoside A 100 μM were not significantly different from those before addition of the toxin [Wilcoxon's signed rank (WSR) test; $P = 0.070$, $n = 16$]. Average MAP values after ethanol were also not significantly different from those before addition of ethanol (WSR test; $P = 0.17$, $n = 9$). When post-intervention MAP was expressed as a percentage of the pre-intervention value, and the percent post-intervention MAP values were compared between cleistanthoside A (100 μM) and ethanol groups with the
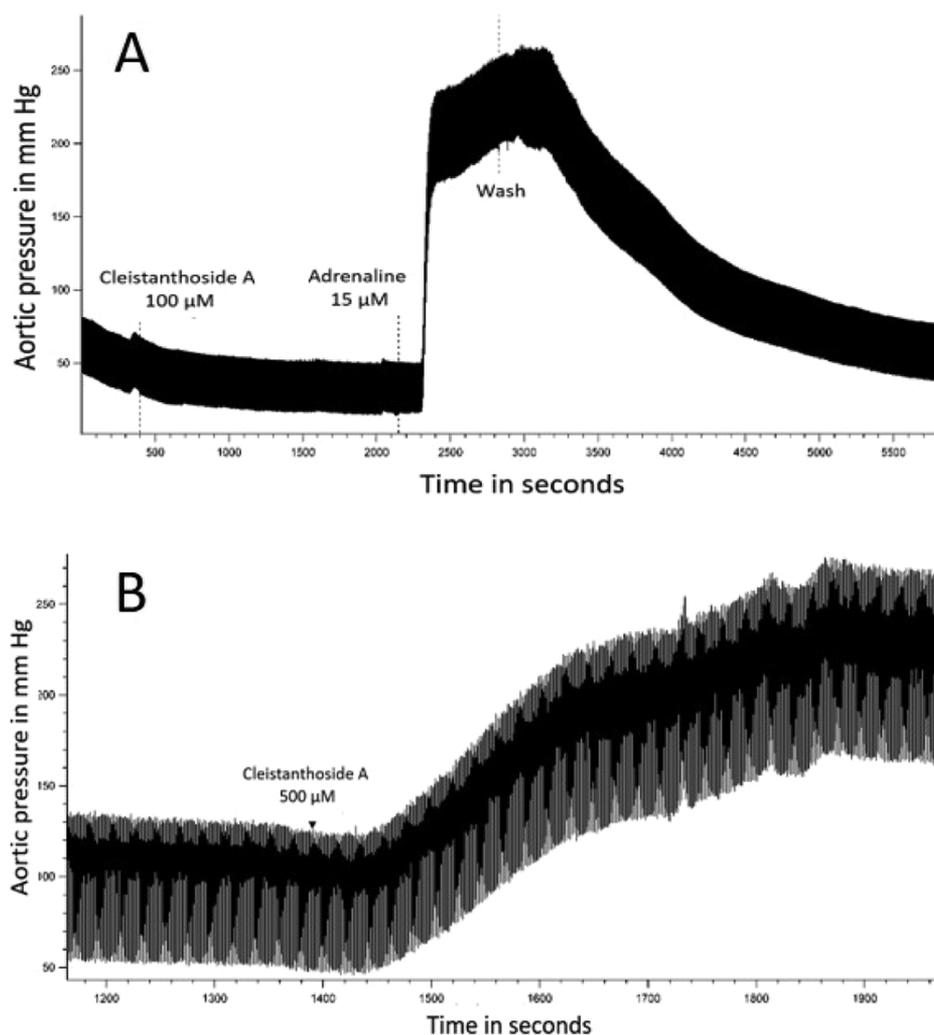
Figure 3. Effect of cleistanthoside A on blood vessels. Pressure tracings from descending aorta in rat hind limb preparations. (A) Cleistanthoside A 100 μM does not affect vascular resistance, and adrenalin can induce vasoconstriction subsequent to cleistanthoside A. (B) Cleistanthoside A 500 μM increases aortic pressure implying a vasoconstrictor effect on the peripheral vessels. Average MAP from all experiments performed for each group is given in Table 3.

Table 3. Average MAP in mm Hg (mean $\pm$ SD) in the Test and Control Groups, before and after Respective Interventions

interventions	average MAP in mm Hg (mean $\pm$ SD)
before cleistanthoside A 100 μM (n = 16)	55.4 $\pm$ 20.4
after cleistanthoside A 100 μM (n = 16)	49.4 $\pm$ 13.3
before ethanol (n = 9)	52.6 $\pm$ 23.6
after ethanol (n = 9)	37.4 $\pm$ 8.4

Mann–Whitney U test, there was no significant difference between the test and control groups (P = 0.78).

Addition of adrenalin subsequent to low-dose cleistanthoside A led to an increase in MAP (Figure 3A), suggesting that the vasculature is responsive to vasoconstrictors in the presence of the toxin.

Cleistanthoside A at 500 μM concentration increased MAP (n = 6) from 90 $\pm$ 47 mmHg (mean $\pm$ SD) to 187 $\pm$ 41 mmHg (Figure 3B). The increase was statistically significant (P = 0.028, WSR test, n = 6).

2.5. Isolated Heart Studies in the Langendorff Perfusion Mode.

In five out of seven preparations, ventricular systolic pressure decreased upon addition of cleistanthoside A to the extracellular solution perfusing the coronary vessels through a cannula placed in the aorta (Figure 4A). Pulse pressure diminished to very small values. In two out of seven preparations (Figure 4B), diastolic pressure increased to higher values, thereby diminishing pulse pressure. Since the consistent observation was reduction in pulse pressure due to cleistanthoside A in all seven preparations, that parameter alone was quantified. It was noted that the effect of cleistanthoside A was reversible upon perfusion with normal extracellular solution.

Ventricular pulse pressure (VPP, the difference between systolic and diastolic pressures in the ventricle) was taken as the index of ventricular contractility. VPP before the intervention (cleistanthoside A in tests and ethanol in controls) was estimated by averaging over 30 s just prior to the intervention. VPP after intervention was estimated at the end of the experiment, which for test preparations was 15–30 min and for controls, 30–40 min. Results are shown in Figure 4C as a scatter plot of ventricular pressures before and after cleistanthoside A or ethanol (vehicle). There was no significant
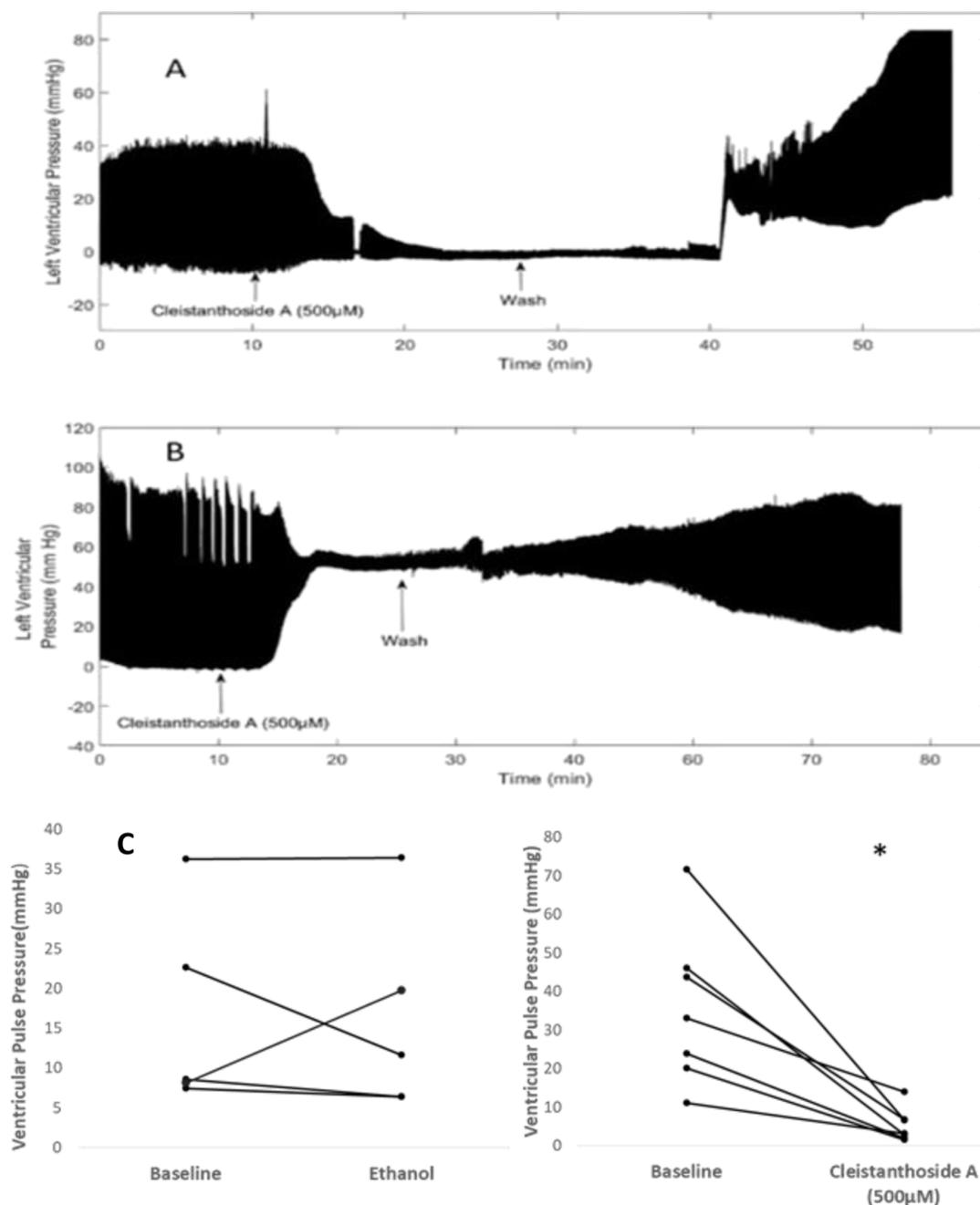
Figure 4. Left ventricular pressure recording in isolated rat heart. (A,B) Raw tracings from two experiments showing different response patterns to cleistanthoside A (500 μM). (C) Scatter plots for the effect of cleistanthoside A (500 μM), and its vehicle ethanol—cleistanthoside A reduces ventricular contractility in isolated rat hearts perfused in the Langendorff mode.

difference in VPP before and after ethanol ($n = 5$, WSR test, and P value = 0.5), while there was a significant decrease in VPP after addition of cleistanthoside A ($n = 7$, WSR test, and P value = 0.043).

3. DISCUSSION

The toxins in *C. collinus* leaf extracts have thus far been thought to be cleistanthin A and cleistanthin B which are aryl naphthalide lignans. Reports from physicians also quote these two substances as toxins.[1,3,26−29] While most reports on chemical constituents have had a non-aqueous solvent as the extracting solvent,[5,18,30,31] there exists one on the characterization of the aqueous extract of *C. collinus*.[6] As patients would

typically consume a water extract of the leaves, the aqueous extract is considered to be the clinically relevant extract. We report here for the first time that the arylnaphthalide lignan glycoside cleistanthoside A is the major constituent of the boiled aqueous extract of *C. collinus*. Cleistanthin A is also found in the boiled aqueous extract in trace amounts.

Cleistanthoside A, a diphyllin diglycoside, was first isolated from the heartwood of *Cleistanthus patulus*, and its structure was elucidated by Sastry and Rao in 1983.[19] Cleistanthin A was first isolated by Govindachari et al. in 1969.[18] They called it just cleistanthin as it was the first of the cleistanthins to be discovered. Lakshmi et al. in 1970 isolated another compound

and named it cleistanthin B and reassigned the name of Govindachari's isolate to cleistanthin A.[32]

We report here the single-crystal structure of cleistanthin A and detailed NMR analyses of cleistanthin A. Also, the present work reports the detailed NMR analyses for structural characterization of cleistanthoside A with fast NMR techniques (published only recently). Both these compounds were isolated from the aqueous extracts of *C. collinus* leaves.

The focus of this study was to identify the toxin responsible for causing death in 30% of patients who have consumed an aqueous extract of *C. collinus* leaves. The minimum lethal dose (MLD_{100}) of the FLB is 75 mg/100 g BW in rats. MLD_{100} for cleistanthoside A and cleistanthin A are 3 mg and 4 mg/100 g BW, respectively. As per the quantification data in Section 2.1, 75 mg of FLB should contain a minimum of 5 mg of cleistanthoside A. Therefore, the toxicity of the boiled aqueous extract can be attributed entirely to cleistanthoside A. On the other hand, the concentration of cleistanthin A in 75 mg of FLB would be 0.038 mg, a value far less than (less than 1% of) 4 mg, which is the MLD_{100} for cleistanthin A.

Determination of MLD_{100} for cleistanthin A as 4 mg/100 g BW is particularly significant because in an earlier report, it has been observed that cleistanthin A does not lead to mortality in rats in doses up to 800 mg/kg (i.e., 80 mg/100 g BW).[7]

Given that cleistanthoside A is the major toxin in the boiled extract of *C. collinus* leaves, experiments were performed to understand its mechanism of toxicity. As physicians have reported that intractable hypotension, not responding to vasoconstrictors, is the cause of death, the mechanism of hypotension was investigated. Hypotension is a reduction in the arterial pressure, and a sudden reduction is referred to as shock. Arterial pressure is a function of stroke volume (volume pumped by heart per beat), heart rate (beats per minute), and vascular resistance. In the rat hind limb preparation, as the heart is removed and its function is replaced with a pump (which pumps at a constant stroke volume and rate), any change in aortic pressure is due to change in vascular resistance. The results from rat hind limb preparation reveal that cleistanthoside A does not affect vascular resistance at a dose of 100 μM, but it increases vascular resistance at a dose of 500 μM. It may be safely stated that cleistanthoside A does not cause direct vasorelaxation.

If shock does not occur due to vasorelaxation, the next possibility is cardiogenic shock. Isolated heart experiments were performed to investigate whether there was a depressant effect on force of ventricular contraction which could result in cardiogenic shock. The results demonstrate that there is indeed a reduction in ventricular contractility due to cleistanthoside A. Whether this is due to a direct depressant effect on the myocardium, or a vasoconstrictor effect on the coronary vasculature, just as in the case of peripheral vasculature, remains to be determined. Coronary vasospasm can lead to hypoperfusion of myocardium and consequent depression.

The observations warrant a reconsideration of the therapeutic strategy in *C. collinus* poisoning. While vaso-constrictors are used currently, it must be considered if they can worsen the hypotension, by increasing the afterload for a failing myocardium. Alternate strategies could involve use of dopamine, or the use of adrenergic vasoconstrictors combined with alpha adrenergic blockers like phentolamine, to avail the benefits of cardiac inotropy, while preventing peripheral vasoconstriction and a consequent increase in the afterload.

If coronary vasospasm is involved, then vasodilator therapy may prove beneficial.

4. CONCLUSIONS

Cleistanthoside A is reported for the first time as the clinically relevant toxin in the boiled aqueous extract of *C. collinus*. A trace amount of cleistanthin A was also identified in the extract. Detailed NMR characterization of *C. collinus* leaf-extracted cleistanthoside A with fast NMR techniques -NOAH4-BSCN- (with and without NUS, published only recently) is reported for the first time. A single crystal of cleistanthin A was successfully obtained, and its single-crystal structure and detailed NMR analysis are presented here. Minimum lethal doses of the toxins cleistanthoside A and cleistanthin A in rats are reported. Isolated tissue studies revealed that cleistantho-side A causes peripheral vasoconstriction and depresses myocardial contractility. While the current clinical under-standing of the cause of death in *C. collinus* poisoning is distributive shock which is attributed to vasodilation, it is seen that the direct effect of cleistanthoside A on vasculature is not vasodilation but indeed vasoconstriction. Isolated rat heart studies reveal that cleistanthoside A inhibits myocardial performance. It is worthwhile therefore to consider whether the circulatory shock leading to death in patients could be cardiogenic. A limitation of this study is that the heart and vessel experiments were performed with cleistanthoside A acting directly on the tissues. Whether cleistanthoside A is the toxin that is being absorbed after oral administration of the boiled extract or if it undergoes modification in the gastrointestinal system or after absorption are issues that require consideration in future studies.

5. EXPERIMENTAL SECTION

5.1. General Experimental Procedures. The study was cleared by the Institutional Review Board and Institutional Animal Ethics Committee, Christian Medical College, Vellore (IAEC 25/2002-2003; 23/2013; 15/2016). The animals were procured from the institutional animal house. *C. collinus* leaves were collected from Amirthi hills (located in Tiruvannamalai district, Tamilnadu, India). No specific permission was required for the collection of these leaves as this is not an endangered species. All chemicals were purchased from Merck, S.D. Fine, Fisher Scientific, Sisco Research Laboratories, Hayman and Sigma-Aldrich and used without further purification.

5.2. Preparation of Boiled Decoction. Ten grams of fresh leaves was added to 300 mL of boiling water and boiled for 10 min. This method of extraction is important as addition of leaves to water first and then boiling will not yield as much cleistanthoside A as adding the leaves to water which is boiling already (unpublished observation). The decoction was filtered and cooled and is referred to as FLB. For toxicological studies and quantification of cleistanthin A and cleistanthoside A, the FLB extract was filtered and dried in an oven (without chloroform partitioning) to give a powder. Three different extractions were made in triplicate, and the final dry weight of the powder obtained from 10 g of fresh leaves was quantified.

5.3. LLP Chromatography. To isolate fluorescent compounds, the filtered FLB extract prepared as above was subjected to liquid−liquid partition chromatography (LLP) with chloroform (chloroform/FLB; 2:1) repeatedly for four times. 400 mL of chloroform was added to 200 mL of the FLB

extract each time. The chloroform fractions (FLB-CF) from the four partitions were pooled and concentrated to dryness in a rotary evaporator. Since the aqueous layer (FLB-WF) was non-toxic (did not cause death in rats), we focused on the chloroform fraction containing most of the fluorescent compounds in FLB for toxin identification.

5.4. Preparative HPLC for Fractionation of FLB-CF. Prep-HPLC (Shimadzu, Japan) was employed to fractionate FLB-CF. Chromatography was performed on Shim-pack VP-ODS (5 μm C18 column, 20 mm × 250 mm). 25 mg of FLB-CF was dissolved in 1 mL of methanol and centrifuged at 4472g for 5 min. A foamy non-fluorescent sediment was obtained on centrifugation (dry weight − 1 mg). The supernatant was chromatographed on a semi prep column with 70% methanol as the mobile phase. The flow rate was set at 10 mL/min. A fluorescence detector was used to identify peaks for fractionation. Excitation was at 320 nm, and emission was read at 450 nm. Nine fractions (fractions 0−8) were collected. The individual fractions were dried and weighed.

5.5. Analytical HPLC and Thin-Layer Chromatography for Monitoring Purity of Prep HPLC Fractions. Each of the nine fractions obtained with prep HPLC was dissolved in methanol at two different concentrations, 10 μg/mL and 1 mg/mL. 20 μL of the sample with each concentration was chromatographed on an analytical HPLC column (shim-pack, Material: 5 μm C18, Dimensions 4.6 × 250 mm) (Figure S1B,C).

The fractions were also spotted on a thin-layer chromatography (TLC) plate to check for purity of the isolate and to compare with previously reported TLC profiles. Silica gel plates of 0.5 mm thickness were used. The plates were activated for 30 min at 100 °C. Prep HPLC fractions 0−8 (dissolved in methanol, at a concentration of 1 mg/mL), purified cleistanthin A (1 mg/mL in methanol), cleistanthoside A (1 mg/mL in methanol), FLB-CF (10 mg/mL methanol), and FLB-WF (1 mg/mL in 50% ethanol and 10 mg/mL) were chromatographed using the mobile phase n-heptane/chloroform/ethanol in the ratio 50:50:10. This ratio gave better separation of compounds. However, for comparison with earlier TLC data reported in the literature, TLC was also performed with the ratio of mobile phase as n-heptane/chloroform/ethanol 50:50:5. The developed TLC plates were observed under a UV illuminator and photographed (Figure S1D,E).

5.6. Quantification of Cleistanthin A and Cleistanthoside A in FLB Extract with Analytical HPLC. Standard curves (area under the curve from HPLC peaks vs concentration) were generated by chromatographing different concentrations of cleistanthoside A (derived from prep HPLC) dissolved in absolute methanol on an analytical column. The mobile phase was 70% methanol. The test sample in which cleistanthoside A had to be estimated was FLB. It was dissolved in 50% ethanol at 0.5 mg/mL concentration and chromatographed on the same column under similar conditions as standards. The concentration of cleistanthoside A in the whole extract was then calculated from the standard curves. Quantification of cleistanthin A in FLB was also performed in a similar fashion as it is a reported toxin in C. collinus.

5.7. Chemical Characterization of Fractions 3 and 6 Isolated with Prep HPLC of FLB-CF. Fraction 3 from prep HPLC of FLB-CF was from a single HPLC peak and constituted about 65% of the sample dry weight. This compound was purified by repeated HPLC and characterized using various spectroscopic tools that include high-resolution mass spectroscopy (HRMS), NMR spectroscopy, and FT-IR.

NMR spectra of fraction 3 (cleistanthoside A) were recorded on a Bruker AVANCE III HD 600 MHz spectrometer equipped with a 5 mm broadband inverse (BBI) probe at 298 K. The ^{1}H and ^{13}C chemical shifts were referenced to solvent signals at $\delta_{H/C}$ 2.49/39.5 (DMSO-d_6) and 3.35/49.0 (MeOH-d_4) relative to tetramethylsilane (TMS). 1D and 2D homo- and heteronuclear NMR spectra were measured with standard Bruker pulse sequences. Super long-range HMBC was recorded by an in-house modified Bruker pulse sequence according to a study by Abdel-Mohsen et al. and Furihata and Seto.[24,25] PIP HSQMBC was used and implemented for determination of J_{CH} long-range coupling constants.[33] NOAH4-BSCN and NOAH4-BSCN-NUS sequences and the processing program (MDD-NMR) were implemented from the online Bruker user library.[34] Topspin 4.0.8 (Copyright 2019, Bruker Biospin GmbH) and Spin-Works 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 PerkinElmer Spectrum 2 spectrophotometer. NMR spectra in acetone-d_6 were recorded on a JEOL-ECX-500 MHz spectrometer. Temperature studies were performed on a Bruker 500 MHz spectrometer AVANCE III with a 5 mm broadband observe (BBO) probe. The ^{1}H and ^{13}C chemical shifts were referenced to solvent signals at $\delta_{H/C}$ 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. Single crystals of cleistanthin A were isolated, and the crystal structure was determined. Details are given in the Supporting Information with results.

5.8. Toxicity and Mechanistic Studies. *5.8.1. Rat Studies for Lethal Dose Estimation.* Wistar rats between 8 and 10 weeks old were used for lethal dose estimation. Rats were maintained under optimal conditions as required and fasted for 4−6 h prior to experimentation. Following the period of fasting, test compounds were administered in a single dose by oral gavage under mild chloroform anaesthesia (to prevent stress to the animals during the gavage; the animals quickly came out of anaesthesia). The animals were then returned to their cages. The animals were monitored for 2 weeks to confirm whether a particular compound or dose was non-lethal. In most cases, death occurred within a day or 2 if the dose was lethal. The animals that survived were then sacrificed humanely under anaesthesia. The fractions tested for toxicity were FLB, FLB-WF, cleistanthoside A, and cleistanthin A. The whole extract FLB and FLB-WF, obtained after chloroform partitioning, were dissolved in water and cleistanthoside A and cleistanthin A in ethanol.

5.9. Rat-Hind Limb Preparation to Assess Effects on Vascular Tone. Wistar rats weighing between 270 and 300 g were anaesthetized with ketamine (100 mg/kg BW) and xylazine (1.87 mg/kg BW) intraperitoneally. A vertical incision was made to expose abdominal cavity. Abdominal aorta was identified, and a site distal to renal artery and proximal to iliac artery bifurcation was chosen for cannulation. Prior to cannulation, the abdominal aorta and inferior vena cava (IVC) were ligated proximal to the chosen site, to avoid bleeding during transection. The trunk was then transected proximal to ligatures, and the upper half of the body was removed. Abdominal aorta was then cannulated and flushed

with heparinized extracellular solution. IVC was opened for the effluent to drain. Once the IVC effluent become clear, the aortic cannula was connected to a peristaltic pump and a pressure transducer through a three-way connector. The peristaltic pump was used to set up pulsatile flow with an extracellular solution containing (in mM) NaCl 100, KCl 3, $CaCl_2$ 1.3, $MgCl_2$ 2, glucose 5, NaH_2PO_4 0.5, Na_2HPO_4 2, $NaHCO_3$ 25, HEPES 10, and dextran 3% with a pH of 7.4 with NaOH. Dextran was used to maintain colloid osmotic pressure in the microcirculation. The perfusate was maintained at 37 °C and was gassed with carbogen (95% oxygen and 5% carbon dioxide). The signals from the pressure transducer were acquired with CMCdaq, an in-house data acquisition system.

Isolated hind limb preparation was perfused at a flow rate of 4 mL/min until a stable pressure recording was obtained. Test preparations were then given cleistanthoside A 100 μM for 20–30 min, and control preparations were given an equal volume of 30% ethanol. As cleistanthoside A at 100 μM did not change arterial pressure, and since a drop in arterial pressure not responding to vasoconstrictors is stated to be the major cause of death, it was tested whether the vasculature was rendered unresponsive to vasoconstrictors even if the toxin did not have any direct effect. Therefore, subsequent to cleistanthoside A, adrenalin (15 μM) was added to test whether the tissue was capable of vasoconstriction. In another set of experiments, a higher dose of cleistanthoside A (500 μM) was used. Aortic pressure recordings were made throughout the experiment. Since the flow rate of the peristaltic pump was constant, any change in aortic pressure (especially mean pressure) would occur only due to changes in vascular resistance. This experimental set up was therefore designed to study the effect of cleistanthoside A, the major toxin in the boiled aqueous extract, on vascular resistance.

5.10. Isolated Rat Heart Preparation to Assess Effects on Ventricular Performance. Rats were anaesthetized with a combination of ketamine (80 mg/kg BW) and midazolam (1.5 mg/kg BW) given intraperitoneally. Abdomen was opened and the IVC cannulated. Heparinized extracellular solution (Heparin 12500 IU/100 mL of ECF) containing (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 3.5, glucose 10, NaH_2PO_4 0.4, HEPES 10 with a pH of 7.4, and dextran 1% w/v was perfused through IVC to remove blood so as to prevent clotting. Blood was let out by opening the abdominal aorta. Once the fluid draining from abdominal aorta was fairly clear, thorax was opened; descending thoracic aorta was cannulated, and the branches from arch of the aorta were ligated. The heart with the cannula in thoracic aorta was then removed from the chest cavity. The cannula in the thoracic aorta was connected to a fluid-filled reservoir (at a height of 4–5 cm) to maintain constant afterload. Fluid from the reservoir perfused the coronary arteries as the cannula was kept above the coronary artery openings at the base of the aorta.

A cannula was then placed in the left ventricle and connected to a pressure transducer. The signals from the pressure transducer were acquired with CMCdaq, an in-house data acquisition system at a sampling rate of 1 KHz. A low pass filter of 30 Hz was then applied. Pressure recordings were made before and after perfusion of cleistanthoside A..

◼ ASSOCIATED CONTENT

Ⓢ Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03138.

NMR spectra, details about structure elucidation, FT-IR data, experimental details, spectra, and single-crystal XRD data (PDF)

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■ ABBREVIATIONS

FLB, fresh leaf boiled extract; WF, water fraction; CF, chloroform fraction; MLD, minimum lethal dose; MAP, mean aortic pressure

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