RAT IN VIVO EXPERIMENTS TO DEMONSTRATE THE EFFECTS OF CLEISTANTHIN B

A Dissertation submitted in partial fulfillment of the requirement for the

Degree of Doctor of Medicine in Physiology (Branch – V)

Of The Tamil Nadu Dr. M.G.R. Medical University,

Chennai - 600 032.

By

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CERTIFICATE

This is to certify that the thesis entitled "rat in vivo experiments to demonstrate the effects of cleistanthin B" is the bonafide, original work carried out by Dr. Rajam Krishna.S in partial fulfillment of the rules and regulations for the M.D - Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April 2011.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "rat in vivo experiments to demonstrate the effects of cleistanthin B" were carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

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Introduction

Poisoning is one of the preferred modes of committing suicide by both males and females in India. The mean age for committing suicide is around 40 years in males and 34 years in females (*Tanuj Kanchan, Ritesh G. Menezes*). Plant poisons are one of the common poisons, because they are easily available. One such plant is *Cleistanthus collinus*, which is a poisonous shrub belonging to the family Euphorbiaceae. It is found in many parts of India. It is popularly known as Oduvanthalai in Tamil.

Mortality associated with this poisoning is 28% (*Kurien T et al., 1987*). The mechanism of action of this poison is unknown and the treatment is symptomatic. Case reports published showed hypokalemia, metabolic acidosis, alkaline urine, respiratory failure, hypotension and cardiac arrhythmias as the major clinical findings in patients who had consumed this poison. (*Eswarappa et al., 2003*). *Cleistanthus collinus* injected intrapetineally in rats has shown to induce type I DRTA and type II respiratory failure in rats (*Maneksh et al., 2010*).

Cleitanthus collinus has many fractions of which the toxic ones are Cleistanthins A and B, Diphyllin and Collinusin. Cleistanthins A and B are the glycosides of Diphyllin and they have been shown to exhibit cytotoxicity on several cancer cell lines (*Paulo et al., 2007*). It was shown that Cleistanthin A causes DNA strand breaks induces apoptosis in cultured cells, whereas Cleistanthin B causes G1 arrest and induces apoptosis in mammalian cells (*Paulo et al., 2007*).

This study deals with the in vivo rat experiments with Cleistanthin B, one of the toxic fractions of *Cleistanthus collinus*. The aim of the study was to find out the molecular mechanism of action of this fraction. This study was started with the hypothesis that Type I DRTA and type II

respiratory failure are two isolated events caused by two different fractions. Hence we started working on the isolated fractions of *Cleistanthus collinus*. Previous studies in our department on cleistanthin A, another toxin fraction of *Cleistanthus collinus*, showed respiratory acidosis and sudden respiratory arrest, DRTA was not evident in those animals (*Anita M.D Thesis 2009*).

Review of Literature

Cleistanthus collinus commonly known as Oduvanthalai in Tamil, is a common suicidal plant poison, the leaves of which are crushed and consumed as a boiled decoction for suicidal purposes. Case reports which were published had shown different clinical pictures, but the consistent findings in all of them were hypokalemia, metabolic acidosis, alkaline urine (*Keshavan Nampoothiri et al., 2010, Benjamin et al., 2006).*

Hypotension, cardiac arrhythmias and adult respiratory distress syndrome have also been reported in patients (*Eswarappa et al., 2003; Benjamin et al.,*). A recent case report by *Keshavan Nampoothiri et al., 2010* has reported respiratory failure type I in patients who have died. They have not reported any kind of brady or tachyarrythmias in any of their patients, whereas cardiac arrhythmias have been reported in some of the case reports (*Kurien T et al., 1987; Eswarappa et al., 2003*). ARDS, DRTA and distributive shock secondary to in appropriate vasodilation have also been reported (*Benjamin et al., 2006*). A myasthenic crisis like syndrome has been reported in poisoning with *Cleistanthus collinus* (*Potikuri Damodaran et al., 2008*). However the exact mechanism of action of this plant is unknown.

In vivo experiments in rats with intraperitoneal injections of the whole aqueous extract of *Cleistanthus collinus* have shown a picture of type I DRTA and type II respiratory failure (*Maneksh et al., 2010*). Sudden respiratory arrest was shown as the cause of death in these animals. In vivo experiments in rats which were injected Cleistanthin A, a toxin fraction of *Cleistanthus collinus* was also shown to produce a respiratory acidosis picture with sudden

respiratory arrest, but the DRTA picture which was seen with the whole aqueous extract was not seen in these rats (*Anita MD Thesis 2009*). Cardiac toxicity was not evident in the animal model.

There are studies on isolated mouse phrenic nerve –diaphragm which have shown reduced neuromuscular transmission and reduced excitability of nerve and muscle membranes following exposure to the leaf extract (*Nandakumar et al., 1989*). An inhibition of thiol containing enzymes has been shown to cause toxicity in rats (*Sarathchandra et al., 1996*). Melatonin has been shown to have a protective effect against oxidative tissue damage induced by *Cleistanthus collinus* in rat brain (*Jayanthi et al., 2009*).

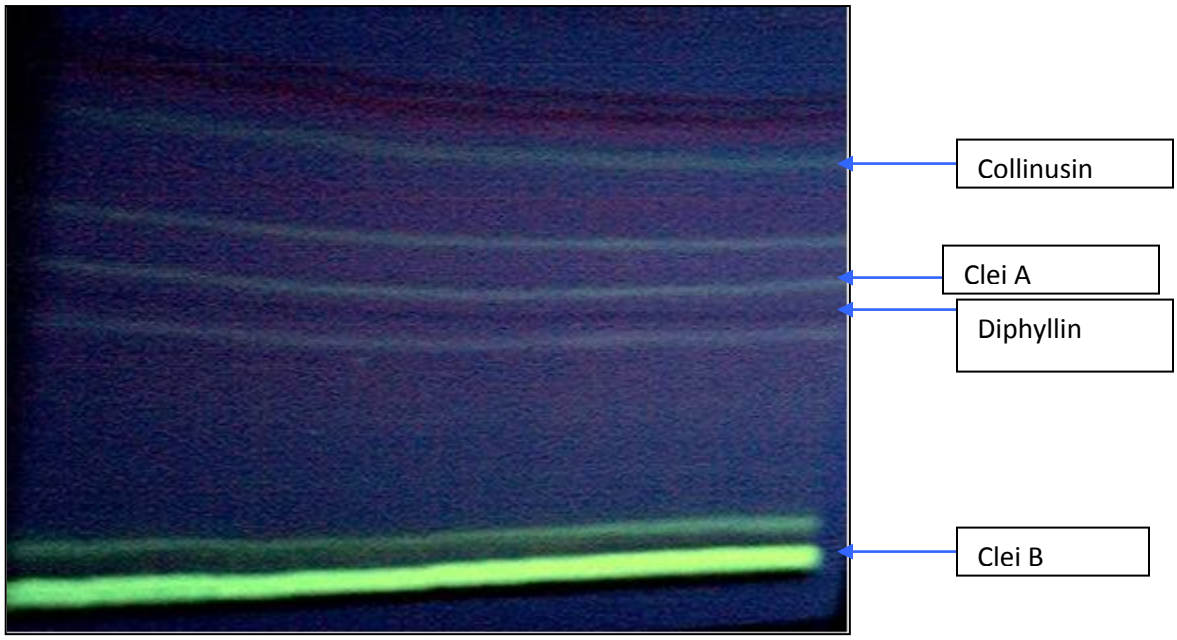
Cleistanthin A and B are being studied as potential anticancer agents. It was shown that Cleistanthin B was toxic to both normal and tumour cells with a higher sensitivity to lower doses for tumour cells (*Pradeep Kumar et al., 1996*). Cleistanthin B was shown to arrest the cells in G1 phase and induce apoptosis in mammalian cells (*Pradeep kumar et al., 1998*) and Cleistanthin A was shown to cause DNA strand breaks and induce apoptosis in cultured cells (*Pradeep Kumar et al., 2000*). Inhibitors of matrix metalloproteinases are potential therapeutic agents in the treatment of cancer. Cleistanthin A has been shown to inhibit MMP – 9 (*Jayaraman Meenakshi et al., 2000*). Diphyllin has been identified as a potent V-ATPase inhibitor and is being explored as a potential target molecule in the treatment of osteoclastic bone resorption.

Govindachari isolated Ellagic acid, Collinusin, Diphyllin and its lignan lactone Cleistanthin from the plant *Cleistanthus collinus* (*Govindachari et al., 1969*). Another Cleistanthin was isolated later on and named Cleistanthin B (*Subbarao et al., 1970*) after which the earlier one isolated by Govindachari was named Cleistanthin A. Flurodensitometric quantification of the lignan lactones of *Cleistanthus collinus* was done by *Annapoorani et al., 1984*. Thin layer chromatographic

measurements preceded the flurometric measurements. ELISA is also used for the estimation of Cleistanthin A and this has clinical applications in Forensic Science (*Raghupathy et al., 1992*).

Thin layer chromatographic estimation of lignan lactones was established and the compounds were identified with their characteristic fluorescence (*Subramaniyam et al., 1975; Annapoorani PhD Thesis*). Column chromatography was used to isolate and purify Cleistanthins A and B from the acetone extract of *Cleistanthus collinus* leaves (*Parasuraman et al., 2009*).

This is a TLC picture of acetone extract of *Cleistanthus collinus* from the Department of Physiology, CMC, Vellore.



Photograph-1: *Thin layer Chromatography picture from Delinda Maneksh MD Thesis 2008.*

Structure of Cleistanthin B

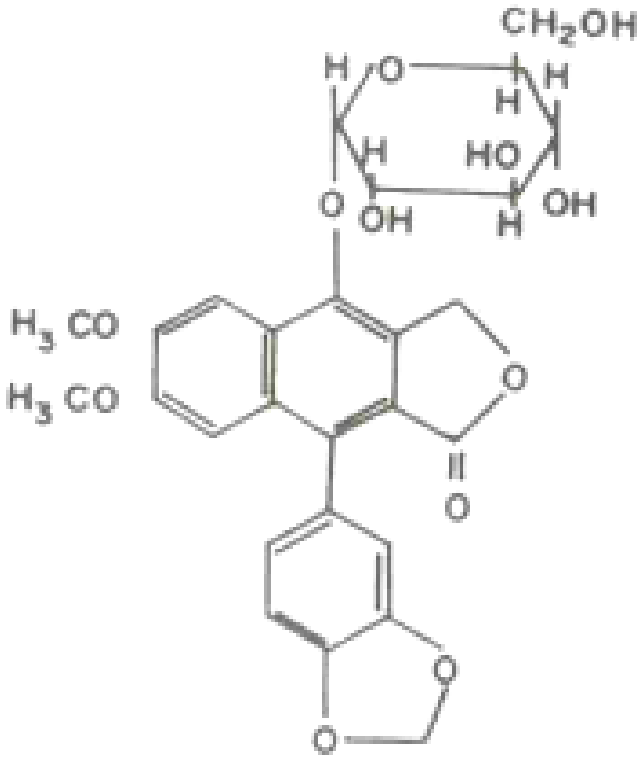


Fig 1*: Figure from Annapoorani KS et al, Enzyme-linked immunosorbent assay for the phytotoxin cleistanthin A. J Immunoassay: 13 (3):pg.331. 1992.)*

Aims and Objectives

Aim:

The aim of the experiment was to study the clinical picture in rats following the administration of Cleistanthin B enriched fraction.

Objectives:

To study the mechanism of action of Cleistanthin B the toxin enriched fraction was injected intraperitoneally into wistar rats and the following parameters were studied.

1. Arterial blood gases.
2. Respiration and ECG
3. Blood pressure
4. Urine pH

Two sets of experiments were done with two types of Cleistanthin B enriched fractions. Since the first Cleistanthin B fraction resolved into five fluorescent fractions, a another set of Cleistanthin B, a fairly pure compound, which was obtained using a different extraction

procedure was also studied. The vehicle used for the second set of cleistanthin B experiments was Ethanol.

Here in this book I would refer to the two sets of experiments as:

The first set of Cleistanthin B &

The second set of Cleistanthin B experiments.

Materials and methods:

Study design:

The study has two arms, the test arm and the control arm. The rats were anaesthetized with ketamine intraperitoneally and the carotid artery was cannulated on one side. In one of the test animals the femoral artery was cannulated. The first blood sample was taken for arterial blood gas measurements. If the initial parameters were within certain ranges as mentioned below in the inclusion criteria, the animals were injected either the Cleistanthin B enriched fraction or the control solution, which was ethanol for the second set of Cleistanthin B experiments and acetone for the first set of Cleistanthin B experiments. Both the toxin and the control solution were injected intraperitoneally. The dosage used for the first set of experiments was 5-7 mg/100gm body weight. The dosage used for the second set of Cleistanthin B experiments was 2-7 mg/100gm body weight.

Blood sample was taken at regular intervals for arterial blood gas measurements. ECG, respiration and BP were monitored continuously with appropriate transducers. Urine samples for pH measurements were taken at frequent intervals in these animals. Dextrose normal saline 0.5 ml was given through the intraperitoneal route after every blood sample was taken or after every hour. The animals were monitored continuously till death or for a period of 7-8 hours after which they were sacrificed.

Inclusion and Exclusion criteria:

All animals did not have good initial blood gas values. The reasons could be respiratory infections in the rats acidosis because of the procedure per se. Hence, only rats with the following initial blood gas values were included in the study.

- Arterial blood pH : 7.25-7.35
- Arterial PCO_2 : 35-55mmHg
- Arterial PO_2: 75-120mmHg

Other animals were excluded from the study.

Rat species used:

Both male and female Wistar rats weighing 100-200 gm. The rats were well bred and housed at animal housing facility, CMC, Vellore.

Materials used:

24 G IV cannula, 26 G needles, 3 way stopcocks, 1ml glass syringes, 24 G scalp vein set, Forceps, scissors, retractors, suture material, eppendorfs, Tuberculin syringes.

Data acquisition system:

1. CMC DAQ hardware and software developed by the department of Bioengineering CMC, Vellore.
2. ECG Preamplifiers and needle electrodes
3. Force transducer and preamplifier to record respiratory movements
4. Blood pressure transducer and preamplifier

Apparatus:

1. Thermo Orion pH meter and 8220BNWP Perphect Ross Micro pH electrode to measure urine pH.
2. i- STAT 200 Blood Gas Analyzer and EG7+ catridges to analyze arterial blood samples.

Drugs used:

1. Ketamine at a dose of 50-100mg/kg body weight to anaesthetise the animal

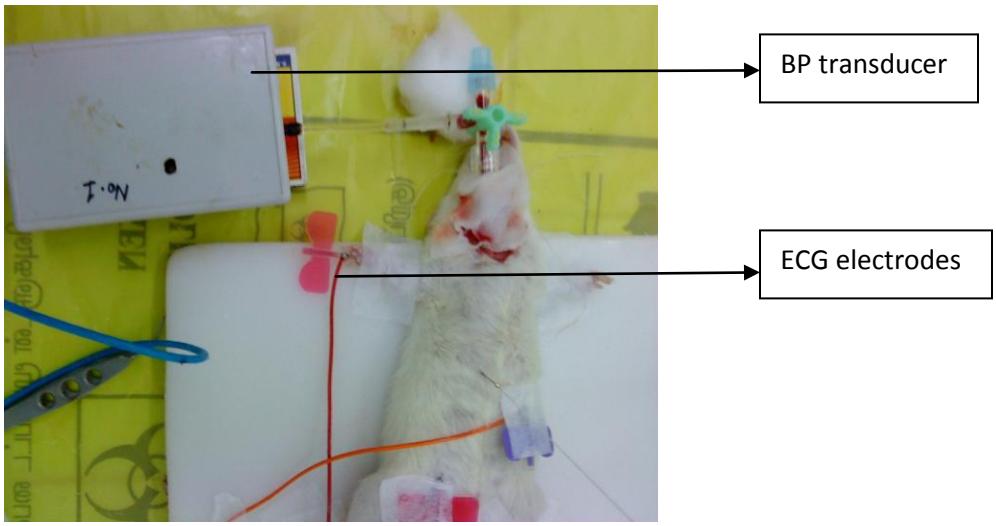
2. Midazolam at a dose of 0.1 mg/100gm body weight used along with ketamine in two of both test and control rats to sedate them, since the animals were hyperactive.

3. Heparin – used as an anticoagulant.5000 IU/ml was diluted five times, after which 0.25 ml of it was injected through the carotid artery after cannulation.

Method of carotid artery cannulation and the procedure:

The animal was anaesthetized with ketamine and was fixed to a board. A midline incision was made and the skin on the ventral area of the neck on one side was cut. The subcutaneous tissues and the submaxillary salivary gland were reflected. The sternohyoid and sternomastoid muscles were identified. Omohyoid mucle was identified after retracting the sternomastoid muscle and was hooked up using a forceps and was cut. The carotid artery was identified and hooked up with the carotid sheath and vagus nerve with a forceps. The carotid artery was separated from the vagus nerve and the sheath was cleared of completely. Now the artery was cannulated using a 24 G IV cannula fitted to a 3-way stop cock. 0.25 ml of 1000 IU/ml of Heparin was injected into the animal at the time of cannulation. The initial blood sample was taken for arterial blood gas analysis. Urine samples were also collected, if possible corresponding to the same time as that of

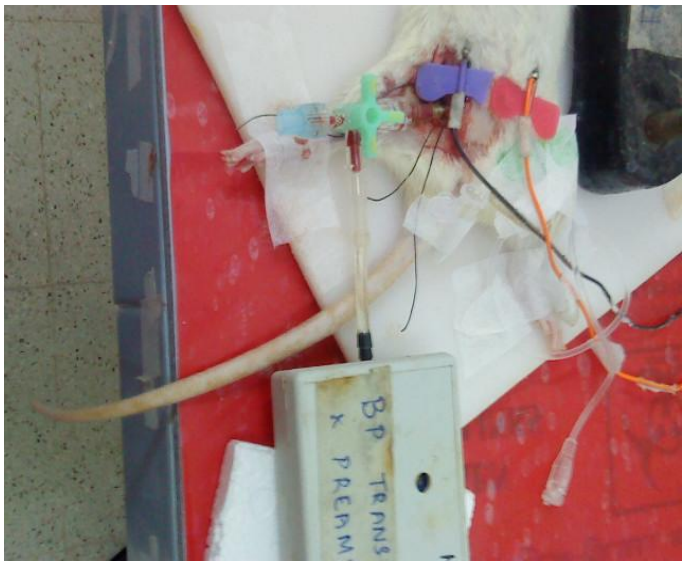
blood sample. BP transducer was fixed to one of the side ports of the 3 way stopcock. The ventral abdominal skin was hooked up with a curved needle and connected to a force transducer to record respiratory movements. Needle electrodes were fixed on to the animal in lead II configuration to record ECG. The active electrode was placed on the left abdomen, negative on the right arm and the reference electrode was placed on the abdomen. The recordings were acquired on to the computer using the CMC DAQ software. An initial normal recording was obtained after which the toxin or the control solution was injected intraperitoneally into the rats. A 24 G scalp vein needle was placed intraperitoneally and the top up doses of ketamine, DNS were given through it. Top up doses of Ketamine were given when required at one third the initial dose. 0.5 ml of DNS and NS were given alternately to the rat every hour or after every sample. The animals were continuously monitored till death or for 7-8 hours after which they were sacrificed.



Photograph-2: *Rat showing cannulated carotid artery and the ECG, respiration and BP transducers fixed*

Method of Femoral artery cannulation:

The skin on the ventral aspect of the animal between the anterior superior iliac spine and the pubic symphysis was incised. The femoral artery was identified in the groove along with the femoral vein and nerve. The femoral artery was separated from the vein and nerve and was cannulated. There after the procedure was the same as that of carotid artery cannulation. Femoral artery was cannulated in only one animal in the test group.



Photograph-3: *Rat after cannulation of femoral artery with the transducers fixed.*

Method of preparation of Cleistanthin B for the first set set of experiments:

Column chromatography with an acetone extract of *Cleistanthus collinus*:

250 grams of shade dried leaves of *Cleistanthus collinus* were taken and soaked in hexane for 5 days for delipidation. The leaves were taken and air dried, soaked in 3-4 litres of acetone for 5 days. The supernatant was collected after 5 days and the residue was disposed of. The

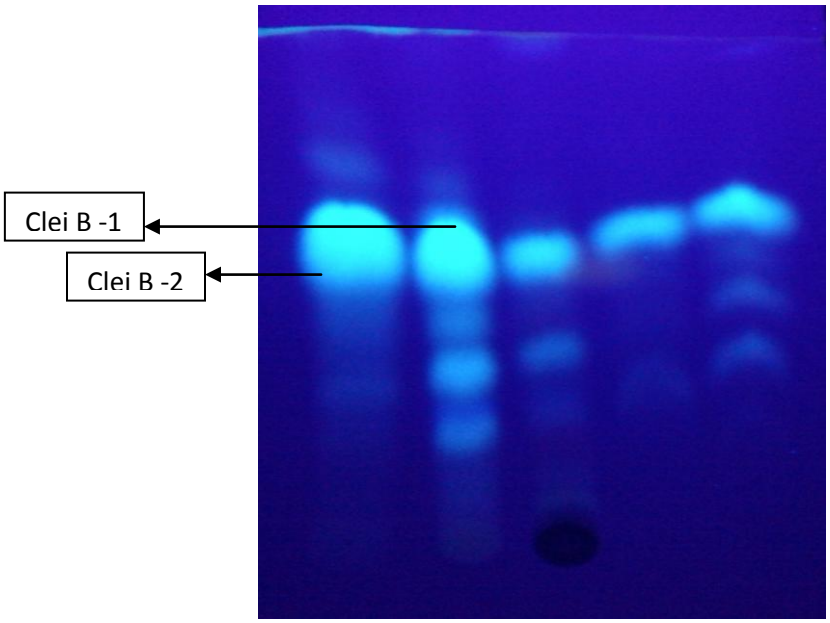
supernatant was rich in toxic components of *Cleistanthus collinus*. This was concentrated on a rotary evaporator to about 50 ml and subjected to neutral alumina column chromatography. Using different ratios of solvents the components were eluted separately and further subjected to thin layer chromatography for further purification. Cleistanthin B shows green fluorescence on TLC (Fig in Annexure).

Method of preparation of Cleistanthin B for the second set set of experiments:

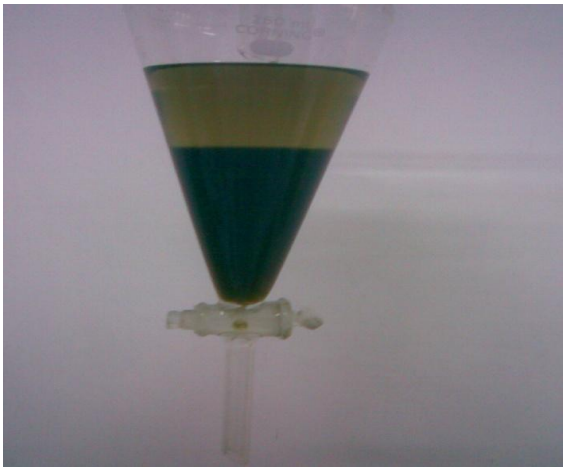
Liquid-liqiud partition chromatography:

The *cleistanthus collinus* leaves were soaked in Hexane for a week for delipidation.The supernatant was discarded and the leaves were then air dried and acidified ethanol was added to it. The supernatant was collected and ethyl acetate was added to this. The two immiscible compounds formed two different layers, the residue was collected and chloroform was added to it. The bottom layer was collected, poured into petridishes and allowed to dry, after which they were scraped and stored. Thin layer chromatography was done to identify the compounds at every step. After the final chloroform step, we got a compound enriched in Cleistanthin B. The figures are given below.

The following is a picture showing both the sets of Cleistanthin B on a thin layer chromatogram.



Photograph-4: *Thin layer chromatography of Cleistanthin B (CleiB-1---from column chromatography; cleiB-2---from liquid-liquid partition chromatography)*



Photograph-5: *Liquid-liquid partition chromatography. It shows two immiscible liquids which were partitioned into two layers.*

Analysis of data:

Analysis of data was done using the following:

- CMC DAQ software to obtain the heart rate plots.
- Igor pro software to analyse the ABG values in a graph form, also heart rate plots and respiration were plotted as graphs in igor.
- SPSS version 11 for statistical tests and box plots.

The following are the U-V Absorption and HPLC (High pressure liquid chromatography pictures of the first (clei B-1) and the second (clei B-2) sets of Cleistanthin B.

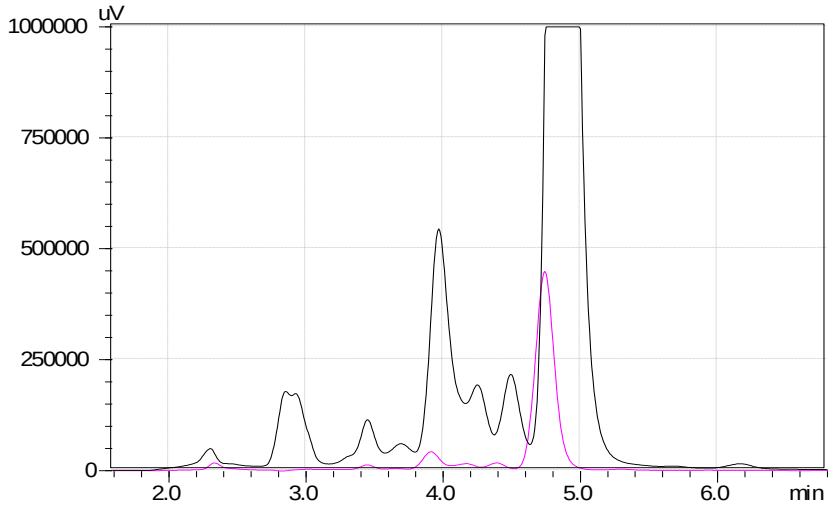


Fig 2: *HPLC and U-V spectrum of clei B-1(column chromatography).*

Black-HPLC Profile, pink-UV Absorption

The HPLC profile shows a lot of impurities with cleistanthin B as seen from the above graph.

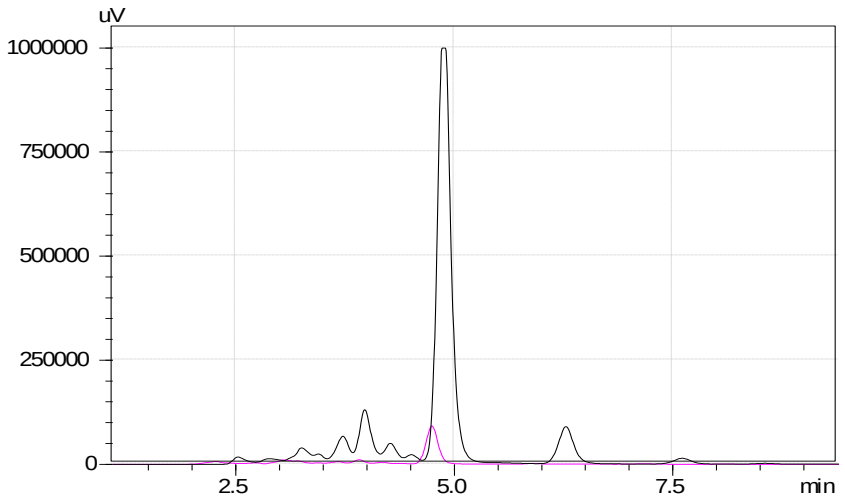


Fig 3: *HPLC and U-V spectrum of clei B-2 (Liquid-liquid partition chromatography).*

Black-HPLC Profile, pink-UV Absorption

A single HPLC peak is seen in the above graph, which tells us that the compound is fairly pure.

Results

Cleistanthin B from liquid-liquid partition chromatography

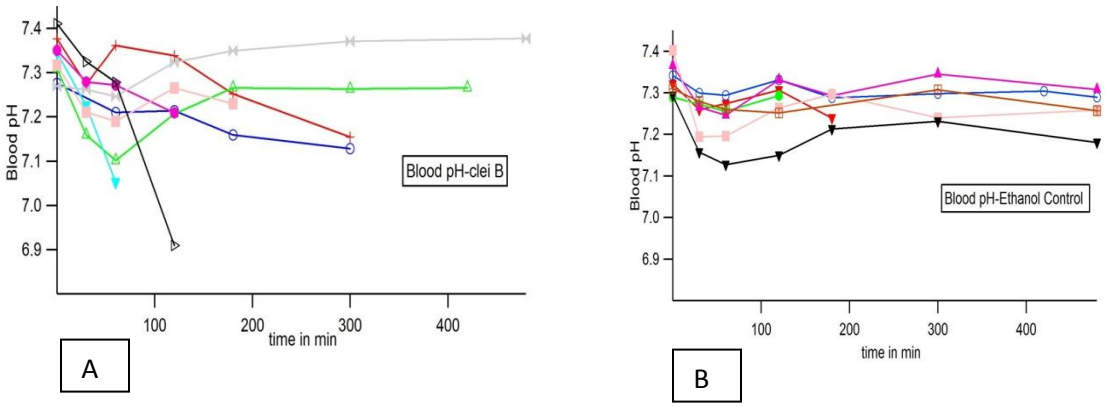
	Total no of animals	Animals died	Animals sacrificed	Mortality
Test animals	9	7	2	78%
Ethanol control	7	2	5	29%

Mortality with Cleistanthin B at a dose of 5-7 mg/100gm body weight is 78% in test animals, whereas in control animals it is only 29%.Of the two control animals one of animals died of suspected air embolism, the animal died immediately after the blood was injected back into the carotid. Of the animals which survived in the test group, in one animal during injection of the toxin there was some resistance in the syringe and half of the quantity spilled out, hence the animal did not receive the toxin at a dose of 7 mg/100 gm.

Arterial blood gases

Blood pH, PCO2, PO2, Bicarbonate and potassium values of both tests and controls are presented below. 0 hour values of the above parameters were compared with the corresponding 30, 60, 120, 180 and 300 minutes values using the non parametric statistical test, Wilcoxon signed rank test(WSR).

Blood pH



Graph-1: *Blood pH against time (A)-Tests; (B)-Controls.*

Values of blood pH of eight test and seven control animals are plotted against time. Significant acidosis is seen in both control and test animals.

The following are the p values of blood pH of test and control animals. P values of 0& 30, 60, 120, 180, 300 & terminal values of blood pH of test animals are given below.

Blood pH (Tests) - WSR

Time in minutes	P values –WSR
0 &30	0.016
0&60	0.008
0&120	0.078
0&180	0.118
0&300	0.375

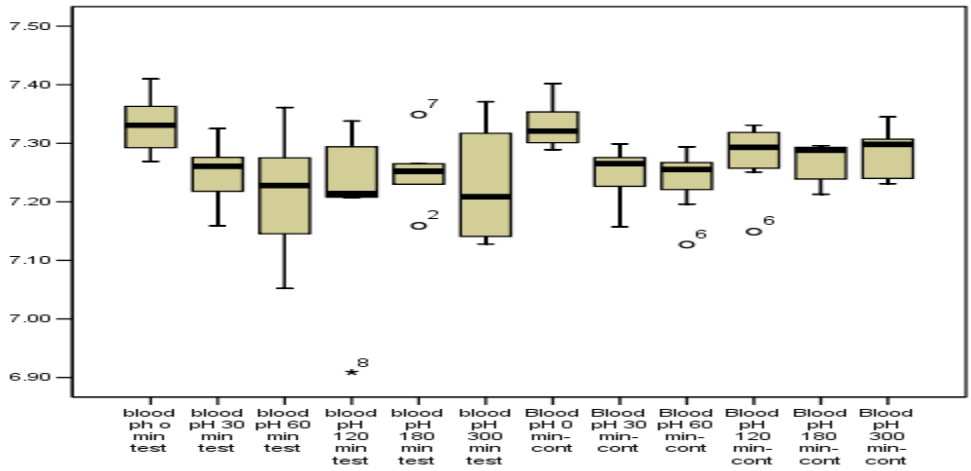
0 & Terminal	0.039

The P values of 0 & 30, 60, 120, 180, 300 and terminal values of blood pH of control animals are given below.

Blood pH (controls) - WSR

Time in minutes	P values - WSR
0 &30	0.016
0&60	0.016
0&120	0.031
0&180	0.063
0&300	0.063
0 & Terminal	0.031

The following are the box plots to show the distribution of the blood pH values in tests and control animals.



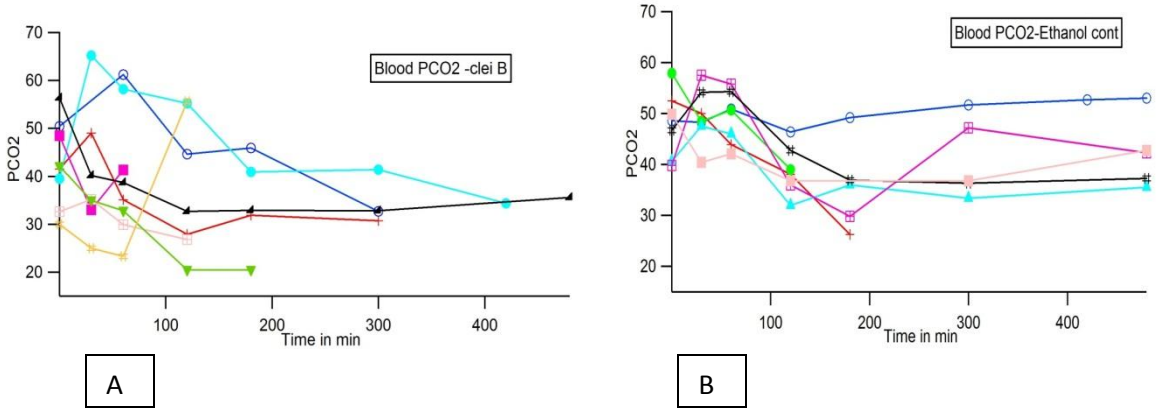
Box plot-1: *Blood pH test and control animals.*

0, 30 and 60 minute values of blood pH were compared in tests and controls using the non-parametric statistical test Mann Whitney U. No significant difference was seen between tests and controls. The p values are given below.

Tests vs. controls(Blood pH) – Mann Whitney U

Time in minutes	P values–Mann Whitney U
0	0.955
30	0.902
60	0.613

Blood PCO2



Graph-2: *Blood PCO2 against time (A)-Tests; (B)-Controls.*

Values of PCO2 in eight test and seven control animals are plotted against time in minutes. Hypocarbia is seen in both tests and controls as a respiratory compensation for acidosis.

Significant difference was not seen when 0 minute values of blood PCO2 were compared with the corresponding 30, 60, 120, 180, 300 minutes and the terminal values in tests. The following are the pvalues of blood PCO2 in test animals.

PCO2(test animals) - WSR

Time in minutes	P values – WSR
0 &30	0.813
0&60	0.641
0&120	0.656
0&180	0.125
0&300	0.25
0&Terminal	0.195

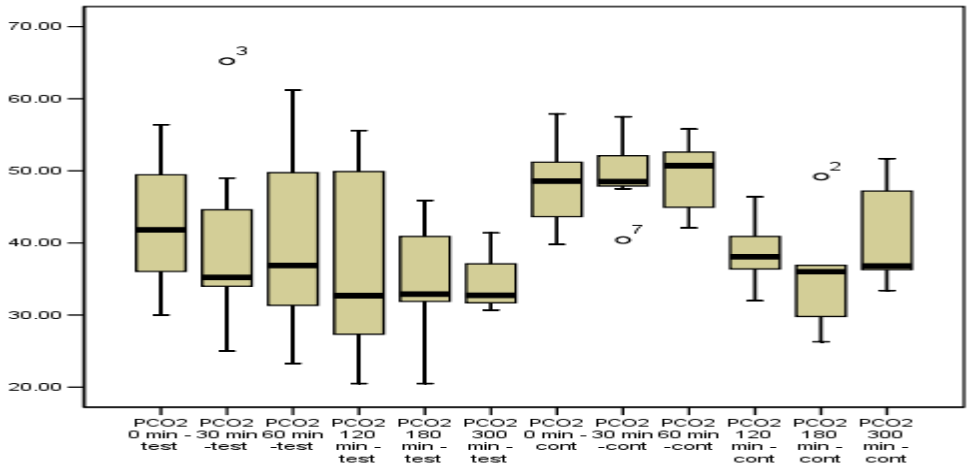
The following are the pvalues of blood PCO2 in control animals.

PCO2(control animals) - WSR

Time in minutes	P values – WSR
0 &30	1
0&60	1
0&120	0.016
0&180	0.125
0&300	0.438
0&Terminal	0.078

In controls, significant difference was seen between 0 and 120 minutes (120 minute values being lower than 0 hour values), whereas 30, 60, 180 and 300 minutes did not show any significant difference from the corresponding 0 minute values.

The following are the box plots to show the distribution of PCO2 values in test and control animals.



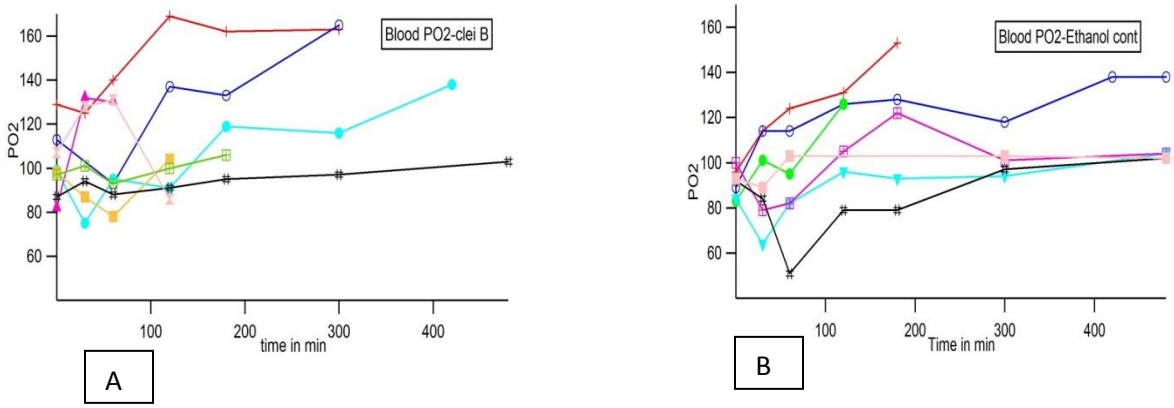
Box plot-2: *Blood PCO2 test and controls.*

Mann Whitney U for 0, 30 and 60 minutes PCO2 values showed no significant difference between tests and control. The following are the p values.

Tests vs. controls (PCO2) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.281
30	0.097
60	0.121

Blood PO2



Graph-3: *Blood PO2 against time (A)-Tests; (B)-Controls.*

Values of blood PO2 in eight test and seven control animals are plotted against time in minutes. Hyperventilation is seen in both test and control animals as a respiratory compensation for acidosis resulting in hyperoxia in these animals. There is no significant difference between the 0 minute PO2 values and the corresponding 30, 60, 120, 180, 300 minute values in both tests and controls. When the 0 and terminal values were compared significant difference was seen in both tests and controls. The following are the p values of 0, 30, 60, 120, 180, 300 minutes and terminal values of blood PO2 in test animals.

PO2 (Tests) - WSR

Time in minutes	P values – WSR
0 &30	0.719
0&60	0.844
0&120	0.469
0&180	0.063
0&300	0.125

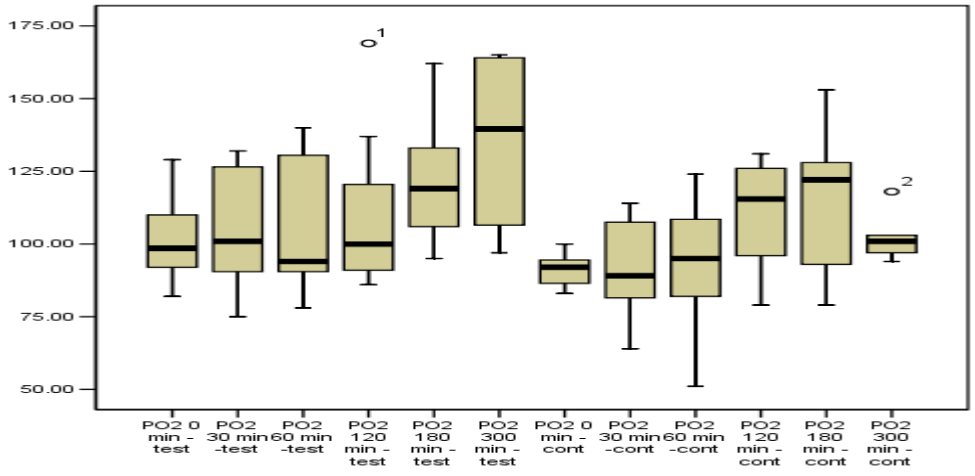
0 & Terminal	0.055

The following are the p values of 0, 30, 60, 120, 180, 300 minutes and terminal values of blood PO2 in control animals.

PO2 (Controls) - WSR

Time in minutes	P values – WSR
0 &30	1
0&60	0.813
0&120	0.156
0&180	0.188
0&300	0.063
0&Terminal	0.016

The following are the box plots showing the distribution of blood PO2 in test and control animals.



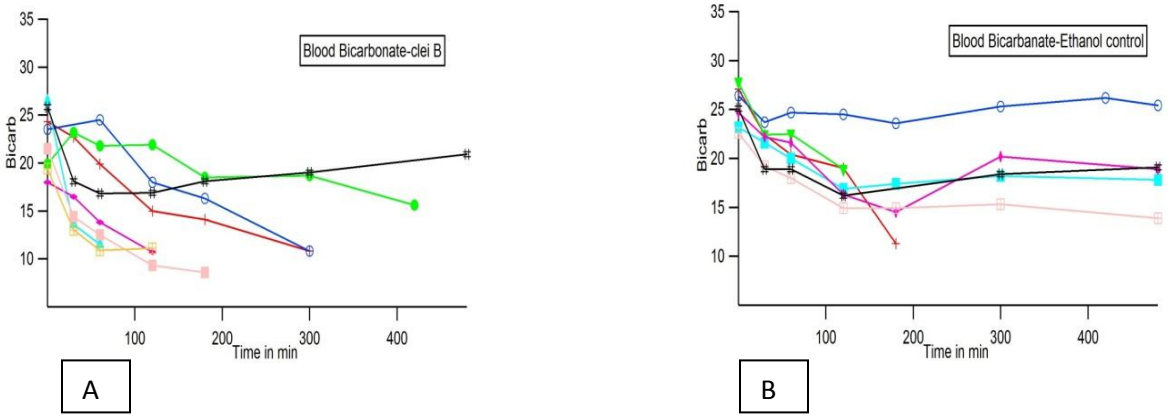
Box plot-3: *Blood PO2 test and controls.*

Mann Whitney U for 0, 30 and 60 minutes PO2 values showed no significant difference between tests and controls. The following are the p values.

Tests vs. controls(PO2) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.152
30	0.259
60	0.463

Blood Bicarbonate



Graph-4: *Blood Bicarbonate against time (A)-Tests; (B)-Controls.*

Values of blood bicarbonate of eight test and seven control animals are plotted against time. Bicarbonate has dropped in both tests as well as controls. Significant difference was seen when 0 minute values of bicarbonate were compared with the corresponding 60, 120 minutes and terminal values in tests.

The following are the p values in test animals.

Bicarbonate(Tests) - WSR

Time in minutes	P values – WSR
0 &30	0.078
0&60	0.039
0&120	0.031
0&180	0.063
0&300	0.125
0&Terminal	0.008

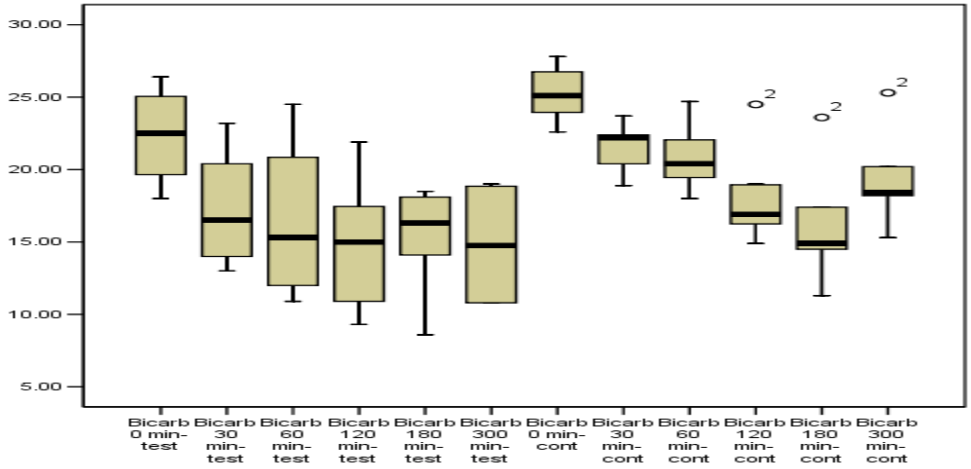
The following are the p values in control animals.

Bicarbonate(Controls) - WSR

Time in minutes	P values – WSR
0 &30	0.016
0&60	0.016
0&120	0.016
0&180	0.063
0&300	0.063
0&Terminal	0.016

Significant difference was seen when 0 minute values of bicarbonate were compared with the corresponding 30, 60, 120 minutes and terminal values in control animals.

The following are the box plots showing the distribution of the bicarbonate vaues in test and control animals.



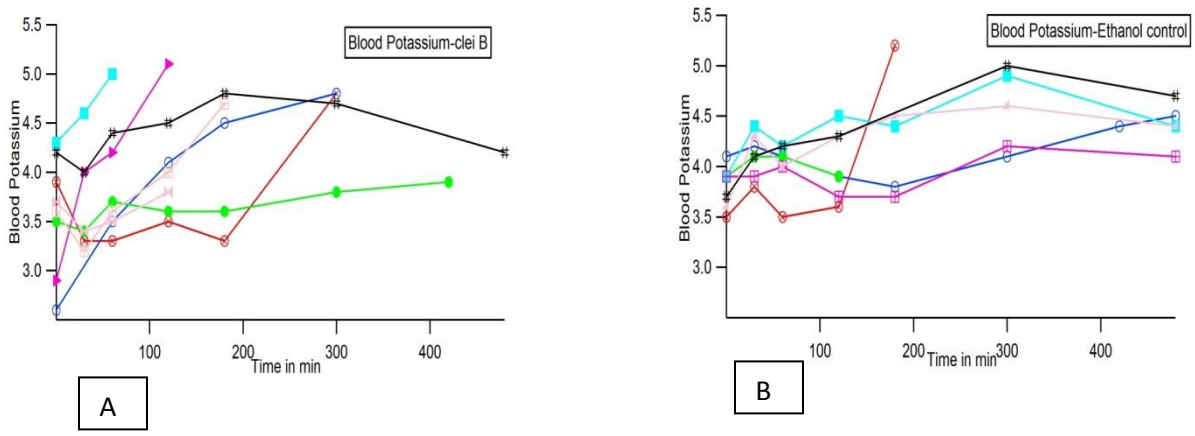
Box plot-4: *Blood Bicarbonate test and controls.*

Siginificant difference was not seen when the 0, 30 and 60 minute values of biacrbonate of both tests and control animals were compared. The following are the p values.

Tests vs. controls(Bicarbonate) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.072
30	0.128
60	0.094

Blood potassium



Graph-5: *Blood Potassium against time (A)-Tests; (B)-Controls.*

Values of blood potassium in eight test animals and seven control animals are plotted against time. Hyperkalemia is seen in both tests and controls as an appropriate response to acidosis. Significant hyperkalemia was seen only when the 0 and terminal values were compared in tests. P values of the 0 and 30, 60, 120, 180, 300 and terminal potassium values in test animals are given below.

Blood potassium (Tests) - WSR

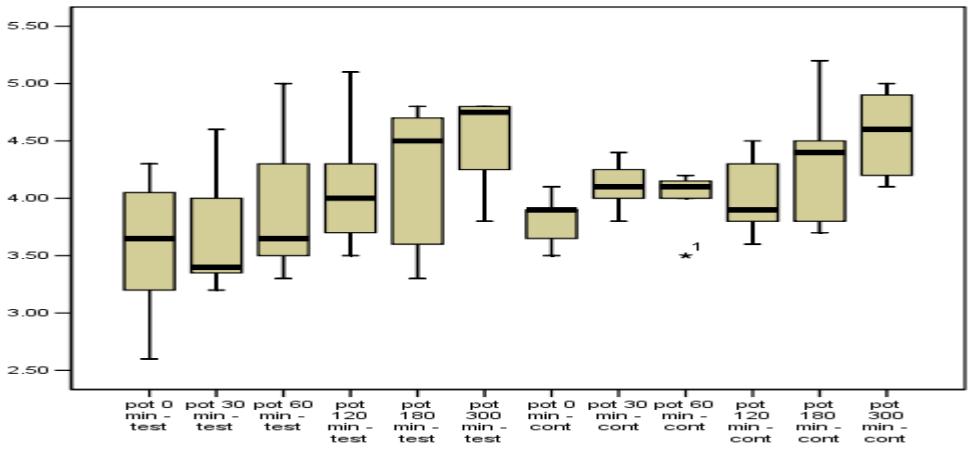
Time in minutes	P values – WSR
0 &30	0.578
0&60	0.219
0&120	0.141
0&180	0.188
0&300	0.125
0 & Terminal	0.016

Significant hyperkalemia was seen when the 0 minute potassium values were compared with the corresponding 30 minute and terminal values in controls. P values of the 0 and 30, 60, 120, 180, 300 and terminal potassium values in control animals are given below.

Blood potassium (Controls) - WSR

Time in minutes	P values – WSR
0&30	0.031
0&60	0.063
0&120	0.281
0&180	0.313
0&300	0.125
0 & Terminal	0.031

The following are the box plots showing the distribution of the potassium values in test and control animals.



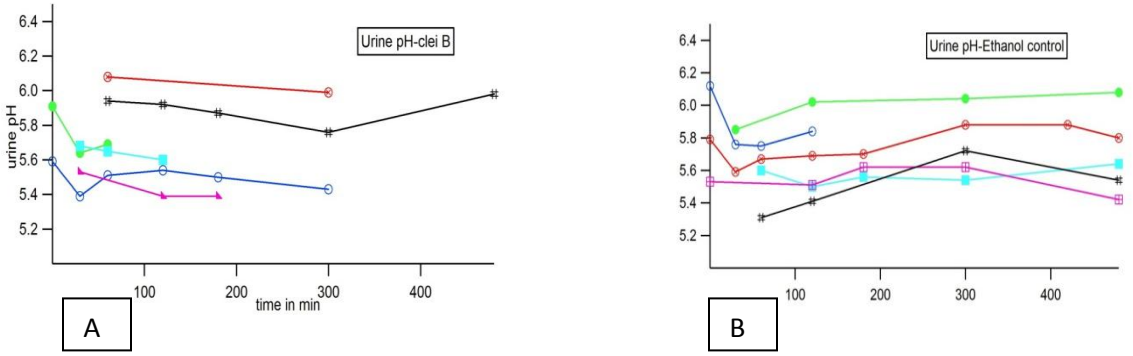
Box plot-5: *Blood Potassium test and controls.*

Significant difference between tests and controls was not seen when 0,30,60 minutes potassium values were compared using the Mann Whitney U test. The following are the p values.

Tests vs.Controls(Potassium)- Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.588
30	0.090
60	0.593

Urine pH



Graph-6: *Urine pH against time (A)-Tests; (B)-Controls.*

Urine pH values of six test animals and six control animals are plotted against time. Since 0 hour values of some experiments are missing it is difficult to interpret the urine pH results. However alkaline urine pH was not seen in both test and control animals. The urine pH was acidic in both tests and controls.

Tests of significance was not done within tests and controls, since there were only a few 0 hour values. When the 0, 30, 60 minute values of tests and controls were compared significant difference was not seen between them.

The following are the p values.

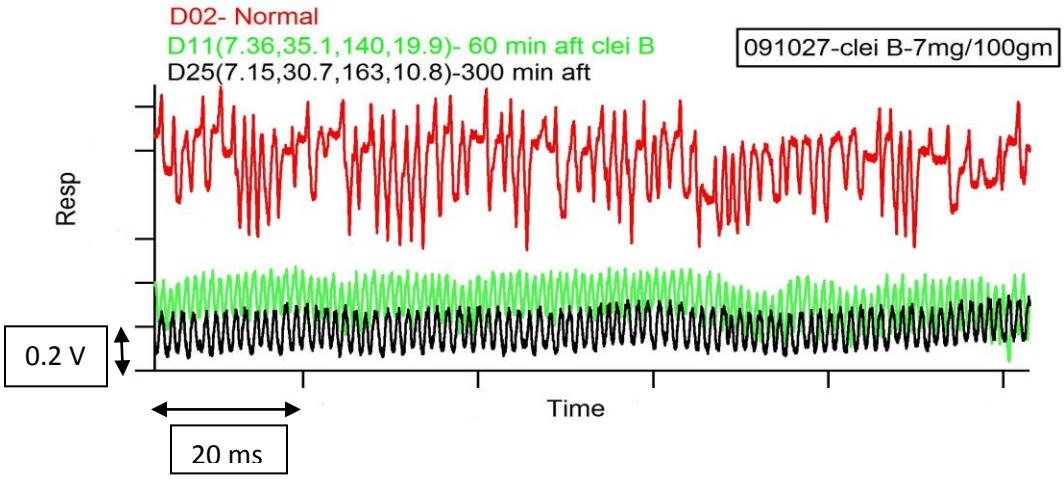
Tests vs.Controls(Urine pH)- Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	1
30	0.229
60	0.413

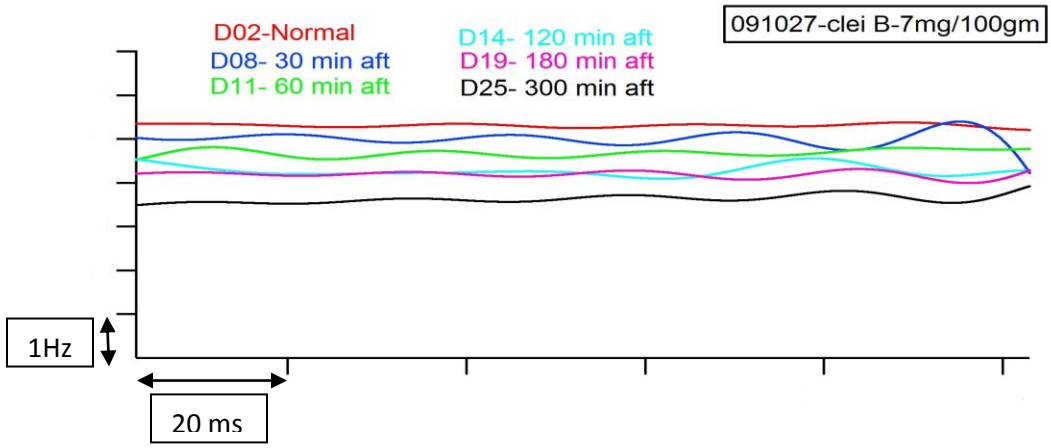
ECG and respiration

100 seconds of raw data from each respiration recording corresponding to the time of ABG sampling was exported to igor and plotted on the same graph to observe the trend in respiration. ECG tracings corresponding to the same time as that of respiration were analysed using CMC DAQ software and heart rate plots were exported to igor. Normal tracings of respiration and heart rate plots were compared with the tracings after the injection of the toxin or the control solution. In some of the test animals it was seen that the amplitude of respiration decreased after the injection of cleistanthin B, whereas in the control animals the amplitude increased. However,

this is not a good indicator of ventilation. The pattern of death in test animals was a sudden respiratory arrest and the ECG continued for some time even after that. The following are some of the respiration and heart rate plots of test animals.

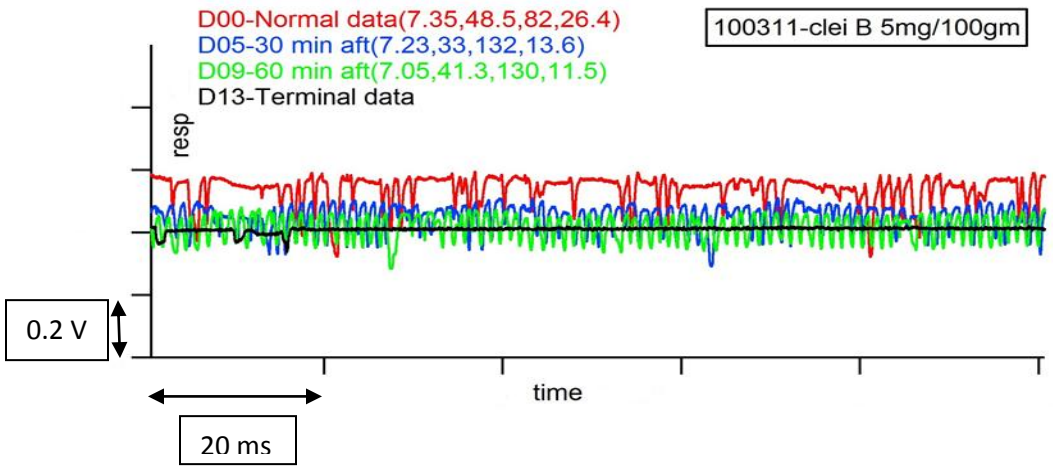


Graph-7: *Respiration tracings of test animal 1.*

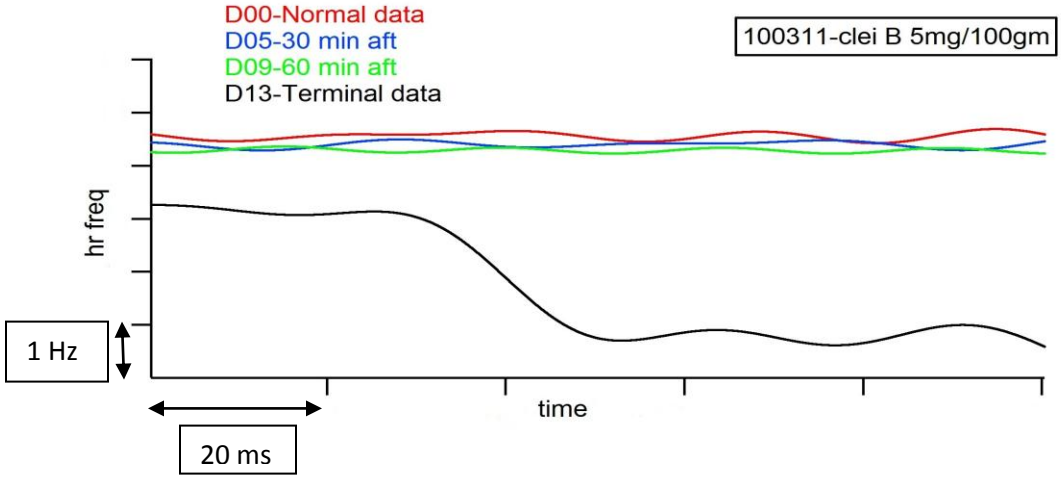


Graph-8: *Heart rate plots of test animal 1.*

In the tracings shown above it is seen that the amplitude of respiration has decreased 60 minutes after the injection of cleistanthin B, whereas there is no change in the heart rate of this animal.



Graph-9: *Respiration tracings of test animal 2.*



Graph-10: *Heart rate plots of test animal 2*

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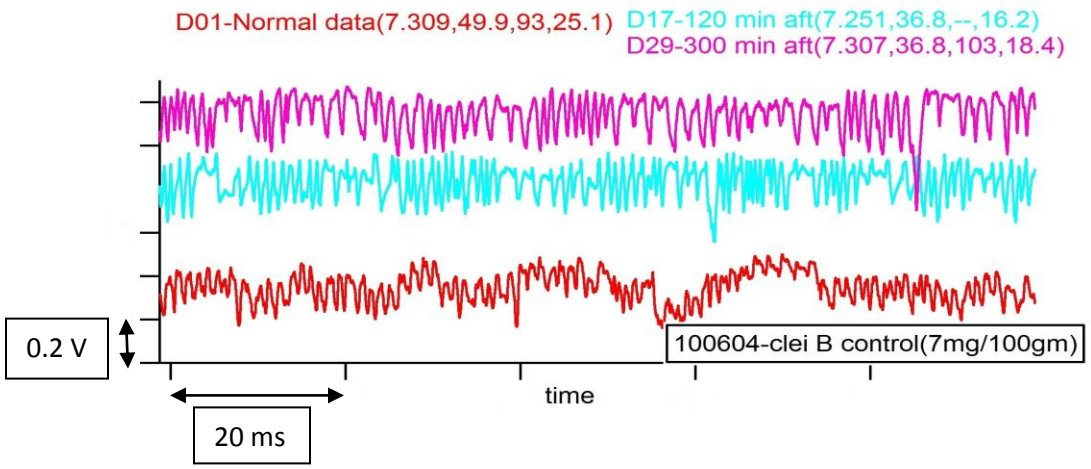
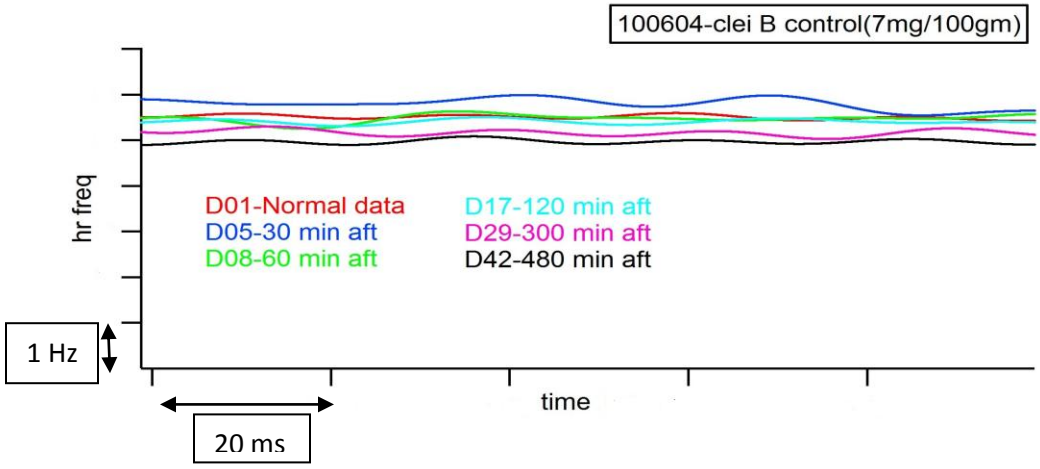
In the example shown above there is not much change in the amplitude or the rate of respiration as seen in the first test animal, but the respiratory arrest is the terminal event in this animal.

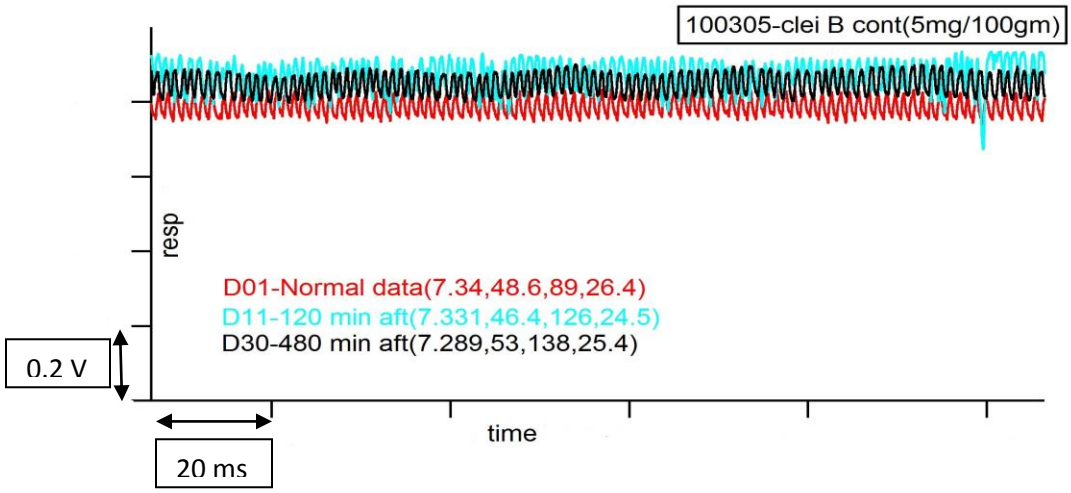
The following are the respiration and heart rate plots of ethanol control.



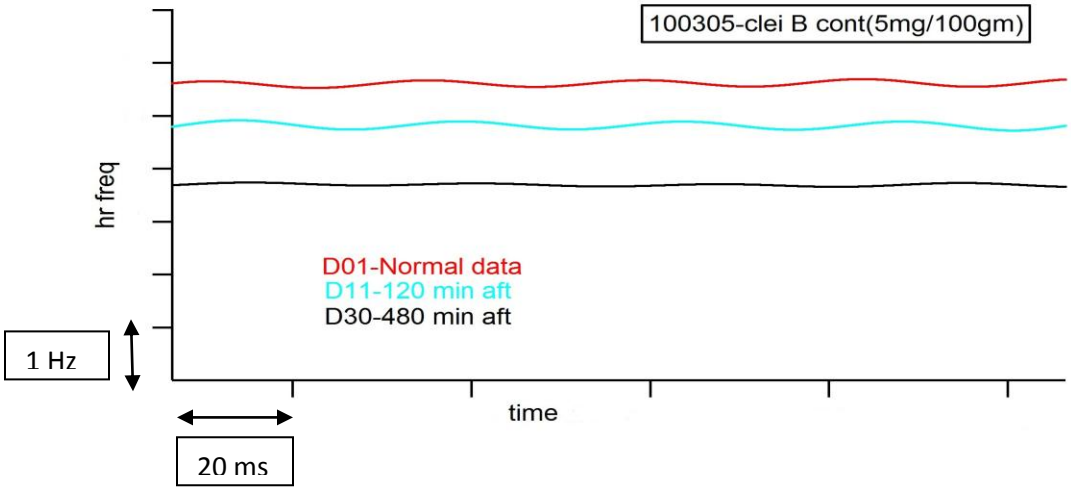
Graph-11: *Respiration tracings of Ethanol control animal 1.*



Graph-12: *Heart rate plots of Ethanol control animal 1.*



Graph-13: *Respiration tracings of Ethanol control animal 2.*



Graph-14: *Heart rate plots of Ethanol control animal 2.*

In the Ethanol controls there is no change in the amplitude or the rate of respiration as that of the test animals.

Cleistanthin B experiments from column chromatography(clei B -1)

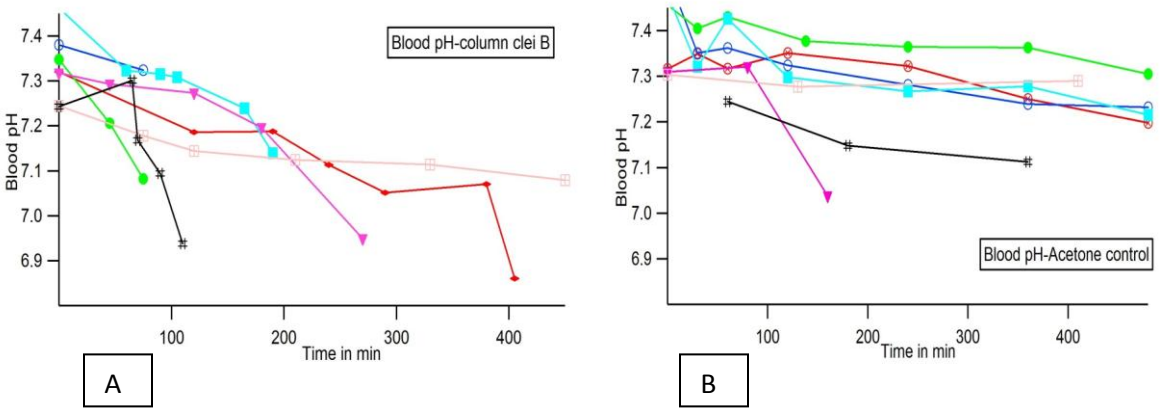
	Total no of animals	Animals died	Animals sacrificed	Mortality
Test animals	11	10	1	91%
Acetone control	7	1	6	14%

Mortality with clei B at a of dose 4-7 mg/100 gm body weight is 91% whereas mortality with acetone control is 14%. Four of the acetone controls were taken from Dr Anita's MD thesis 2009 and were analysed with the other three controls. This was done so as to avoid repetitive control experiments.

Arterial blood gases

Blood pH, PCO2, PO2, bicarbonate and potassium values of both tests and controls are presented below. 0 hour values of the above parameters were compared with the corresponding 30- 45 minutes and 60-90 minute values in both tests and controls using the non parametric statistical test, Wilcoxon signed rank test(WSR).

Blood pH



Graph-15: *Blood pH against time (A)-Tests; (B)-Controls.*

Values of blood pH of seven test and seven control animals are plotted against time. Significant acidosis is seen in test animals after 60 minutes of injection of the toxin. Siginificant acidosis is seen in controls only towards the end before the sacrifice of the animal. The following are the p values of blood pH of test and control animals.

P values of 0& 30 – 45, 60 – 90 & terminal values of blood pH of test animals are given below.

Blood pH (Tests) - WSR

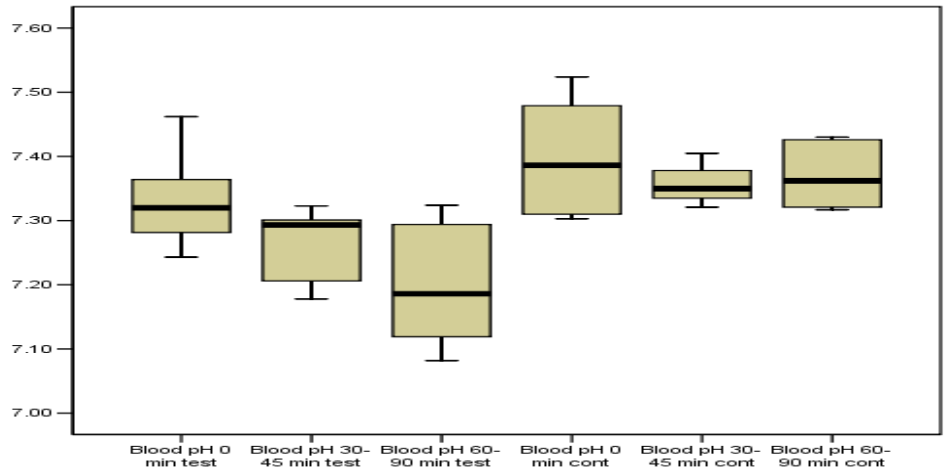
Time in minutes	P values –WSR
0 &30-45	0.188
0&60-90	0.016
0&Terminal	0.016

P values of 0 & 30, 60, 120, 180, 300 and terminal values of blood pH of control animals are given below.

Blood pH (controls) - WSR

Time in minutes	P values - WSR
0 & 30-45	0.250
0&60-90	0.313
0 & Terminal	0.031

The following are the box plots to show the distribution of the blood pH values in tests and control animals.



Box plot-6: *Blood pH test and controls.*

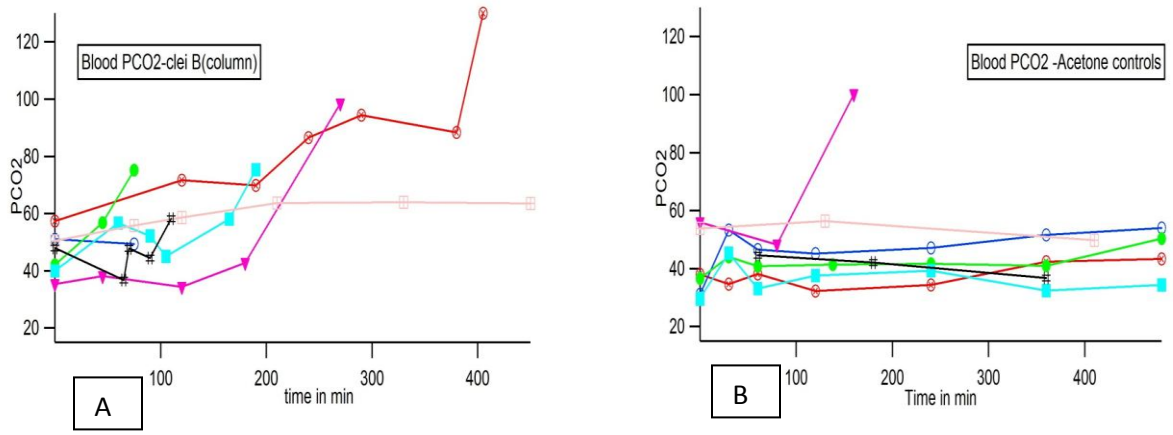
0, 30-45 and 60-90 minute values of blood pH were compared in tests and controls using the non-parametric statistical test Mann Whitney U. Significant difference was seen between tests and controls.

The p values are given below.

Tests vs. controls(Blood pH) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.534
30	0.032
60	0.01

PCO2



Graph-16: *Blood PCO2 against time (A)-Tests; (B)-Controls.*

Values of PCO2 in seven test and seven control animals are plotted against time in minutes. Hypercarbia is seen in most of the test animals, whereas only ane animal in the control group shows hypercarbia.That animal in the control group died after 2 and a half hours of clei B injection. Significant hypercarbia was seen when 0 minute values of blood PCO2 were compared with the corresponding terminal values in tests. The following are the pvalues of blood PCO2 in test and control animals.

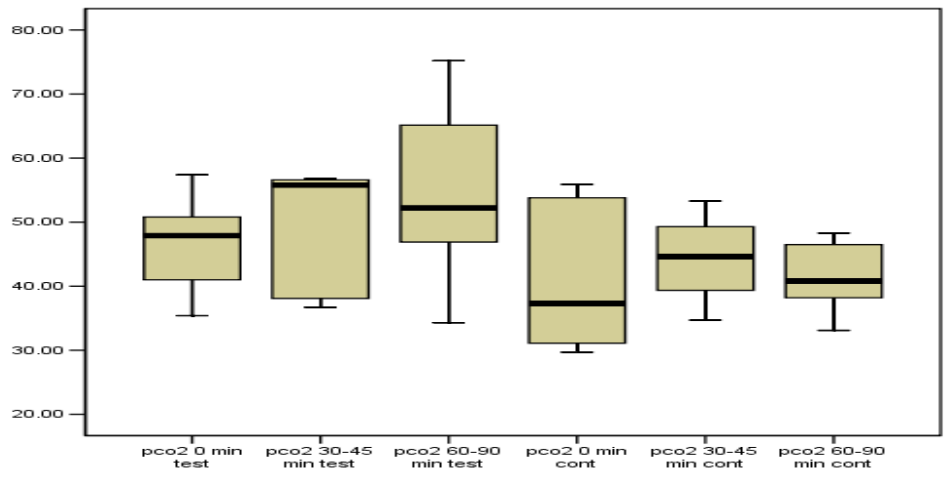
PCO2(test animals) - WSR

Time in minutes	P values – WSR
0 &30-45	0.313
0&60-90	0.219
0&Terminal	0.031

PCO2(control animals) - WSR

Time in minutes	P values – WSR
0 &30-45	0.250
0&60-90	0.438
0&Terminal	0.063

In controls, significant hypercarbia was not seen. The following are the box plots to show the distribution of PCO2 values in both test and control animals.



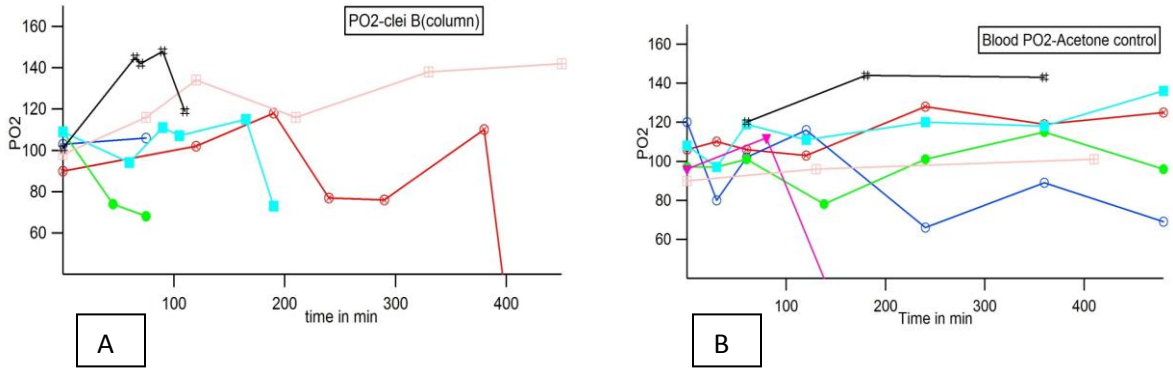
Box plot-7: *Blood PCO2 test and controls.*

0, 30-45 and 60-90 minute values of blood PCO2 were compared in tests and controls using the non-parametric statistical test Mann Whitney U. No significant difference was seen between tests and controls. The p values are given below.

Tests vs. controls(PCO2) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.366
30	0.413
60	0.073

PO2



Graph-17: *Blood PO2 against time (A)-Tests; (B)-Controls.*

Values of blood PO2 in six test and seven control animals are plotted against time in minutes. Hyperventilation is seen in both test and control animals as a respiratory compensation for acidosis resulting in hyperoxia in these animals. Significant hyperoxia is not seen in both tests and controls. The following are the p values of PO2 in test and control animals.

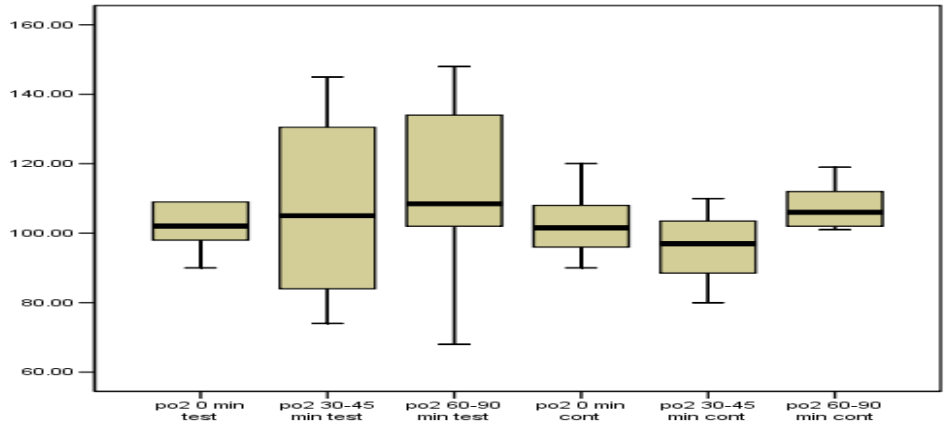
PO2(test animals) - WSR

Time in minutes	P values – WSR
0 &30-45	0.875
0&60-90	0.313
0&Terminal	0.844

PO2(control animals) - WSR

Time in minutes	P values – WSR
0 &30-45	0.5
0&60-90	0.875
0&Terminal	0.844

The following are the box plots to show the distribution of PO2 values in both tests and controls.



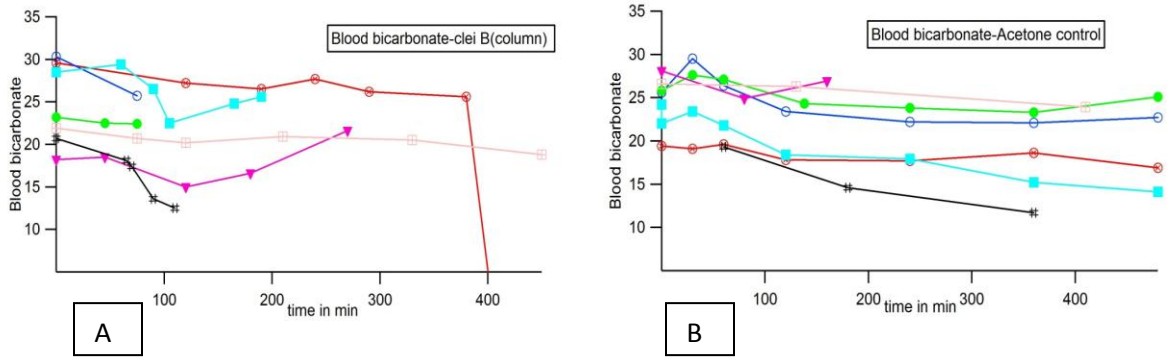
Box plot-8: *Blood PO2 test and controls.*

0, 30 and 60 minute values of blood PO2 were compared in tests and controls using the non-parametric statistical test Mann Whitney U. No significant difference was seen between tests and controls. The p values are given below.

Tests vs. controls(PO2) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.846
30	0.857
60	0.762

Blood Bicarbonate



Graph-18: *Blood Bicarbonate against time (A)-Tests; (B)-Controls.*

Values of blood bicarbonate of seven test and seven control animals are plotted against time. Bicarbonate has dropped in both tests as well as controls. Significant drop in bicarbonate was seen only in the terminal values of controls. The test animals did not show a significant drop in bicarbonate. The following are the p values of bicarbonate in test animals.

Bicarbonate(Tests) - WSR

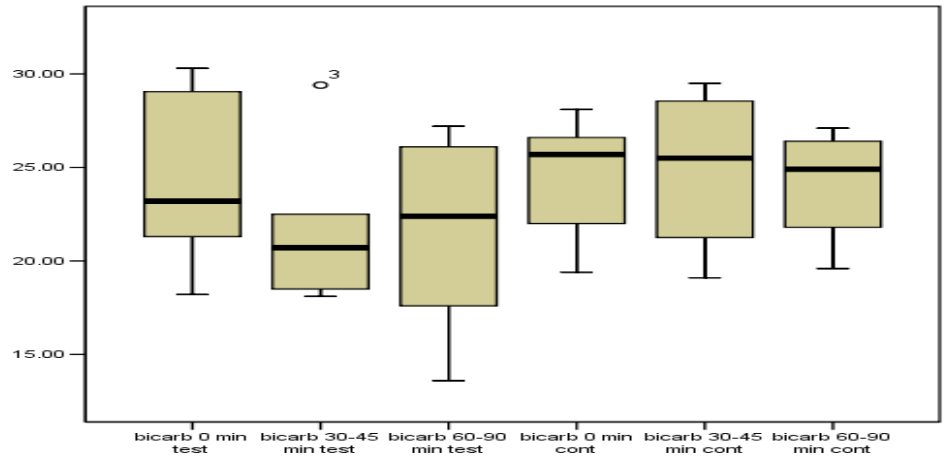
Time in minutes	P values – WSR
0 &30-45	0.438
0&60-90	0.061
0&Terminal	0.109

The following are the pvalues of bicarbonate in control animals.

Bicarbonate (control animals) - WSR

Time in minutes	P values – WSR
0 &30-45	0.25
0&60-90	0.875
0&Terminal	0.031

The following are the box plots to show the distribution of values in both tests and controls.



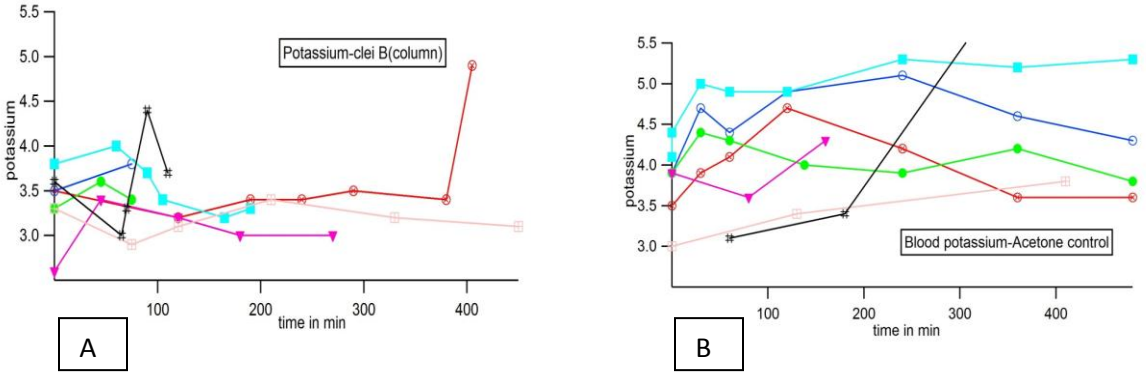
Box plot-9: *Blood Bicarbonate test and controls.*

0, 30 and 60 minute values of blood bicarbonate were compared in tests and controls using the non-parametric statistical test Mann Whitney U. No significant difference was seen between tests and controls. The p values are given below.

Tests vs. controls(Bicarbonate) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.945
30	0.286
60	0.755

Blood Potassium



Graph-19: *Blood Potassium against time (A)-Tests; (B)-Controls.*

Values of blood potassium in seven test animals and seven control animals are plotted against time. Hyperkalemia is seen in controls as an appropriate response to acidosis. There is a trend towards a decrease in potassium levels in the test animals except the one which survived for eight hours. Significant hyperkalemia was not seen in both tests and controls.

P values of the 0 and 30-45, 60-90 minutes and terminal potassium values in test and control animals are given below.

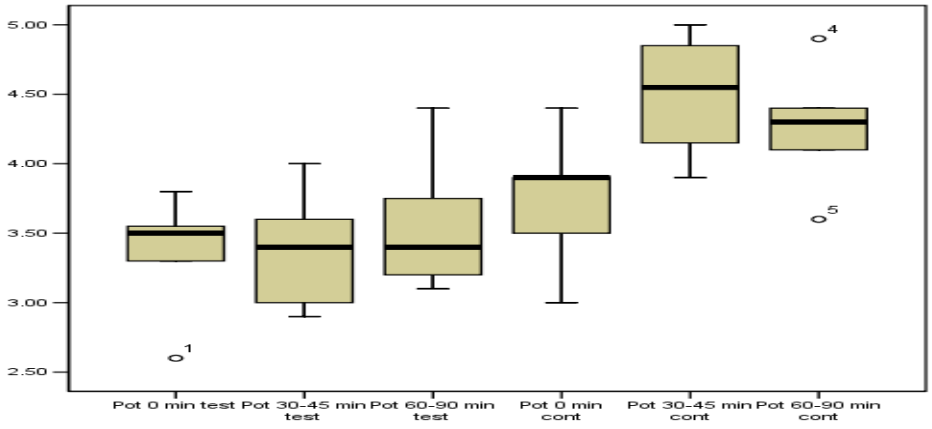
Blood potassium (Tests) - WSR

Time in minutes	P values – WSR
0 &30-45	1
0&60-90	0.391
0&Terminal	0.656

Blood potassium (Controls) - WSR

Time in minutes	P values – WSR
0 &30-45	0.125
0&60-90	0.125
0&Terminal	0.094

The following are the box plots of potassium values in both tests and control animals.



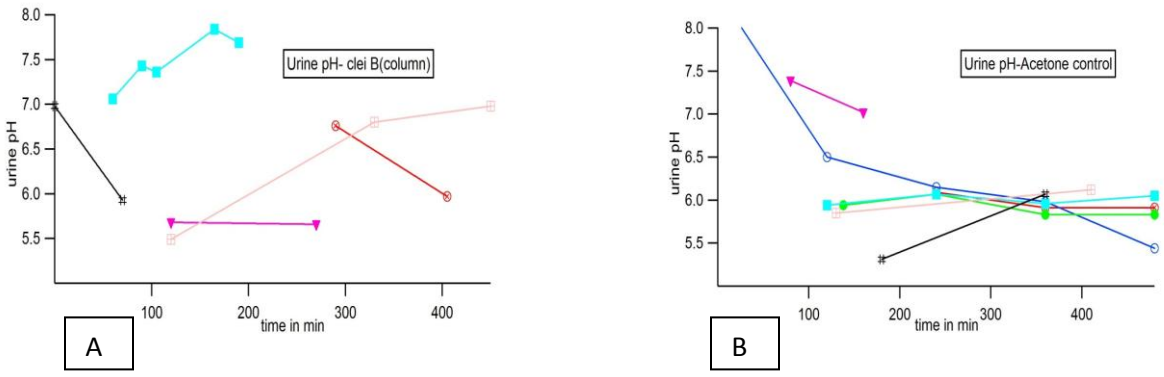
Box plot-10: *Blood Potassium test and controls.*

0, 30 and 60 minute values of blood Potassium were compared in tests and controls using the non-parametric statistical test Mann Whitney U. Significant difference was seen between tests and controls. The p values are given below.

Tests vs. controls(Potassium) – Mann Whitney U

Time in minutes	P values – Mann Whitney U
0	0.088
30	0.032
60	0.056

Urine pH



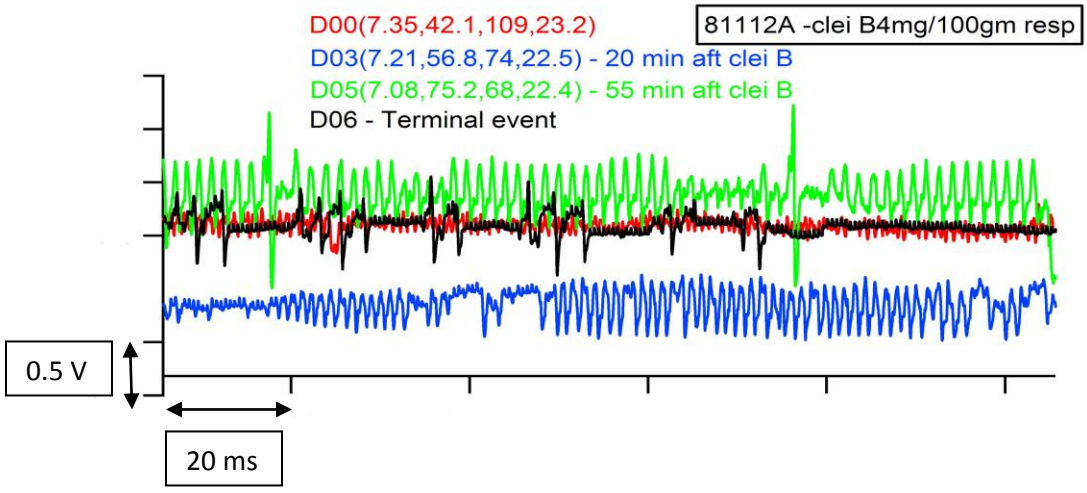
Graph-20: *Urine pH against time (A)-Tests; (B)-Controls.*

The values of urine pH are plotted against time in both tests and controls. Since the 0 hour values are missing it is difficult to interpret these graphs.

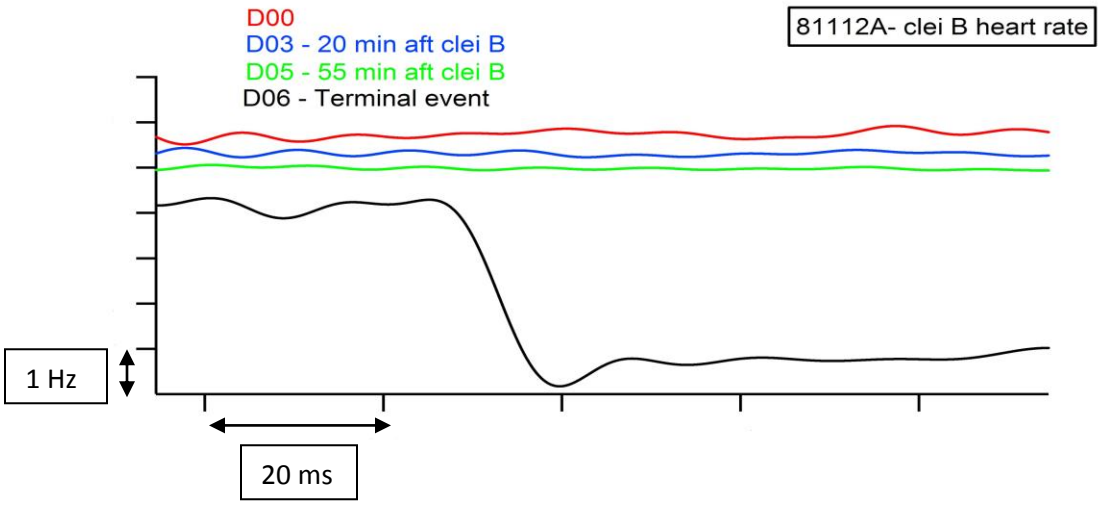
ECG and respiration

100 seconds of raw data from each respiration recording corresponding to the time of ABG sampling was exported to igor and plotted on the same graph to observe the trend in respiration. ECG tracings corresponding to the same time as that of respiration were analysed using CMC DAQ software and heart rate plots were exported to igor .Normal tracings of respiration and heart rate plots were compared with the tracings after the injection of the toxin or the control solution. In some of the test animals it was seen that the amplitude of respiration decreased after the injection of Cleistanthin B, whereas in the control animals the amplitude increased. However, this is not a good indicator of ventilation.

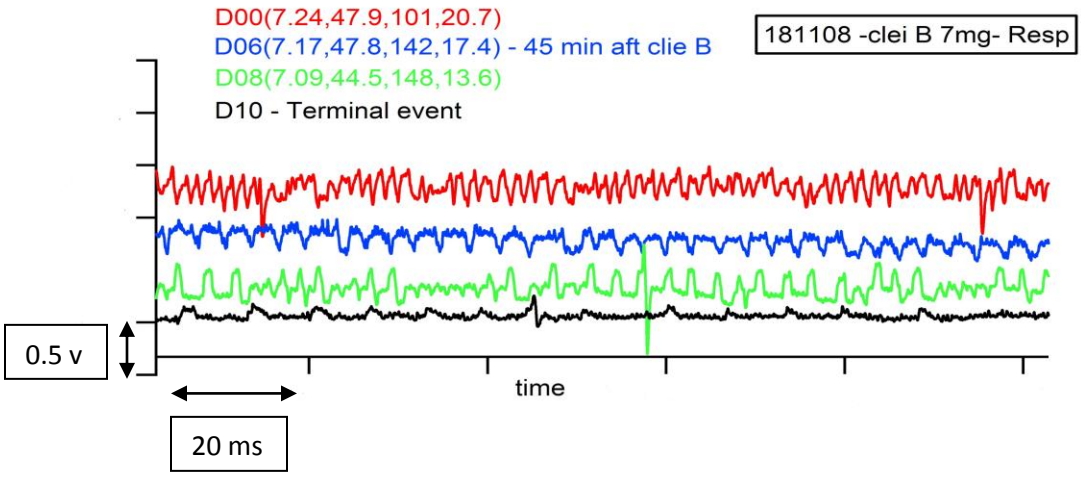
The pattern of death in the test animals was a sudden respiratory arrest and the ECG continued for some time even after that. The following are some of the respiration and heart rate plots of test animals.



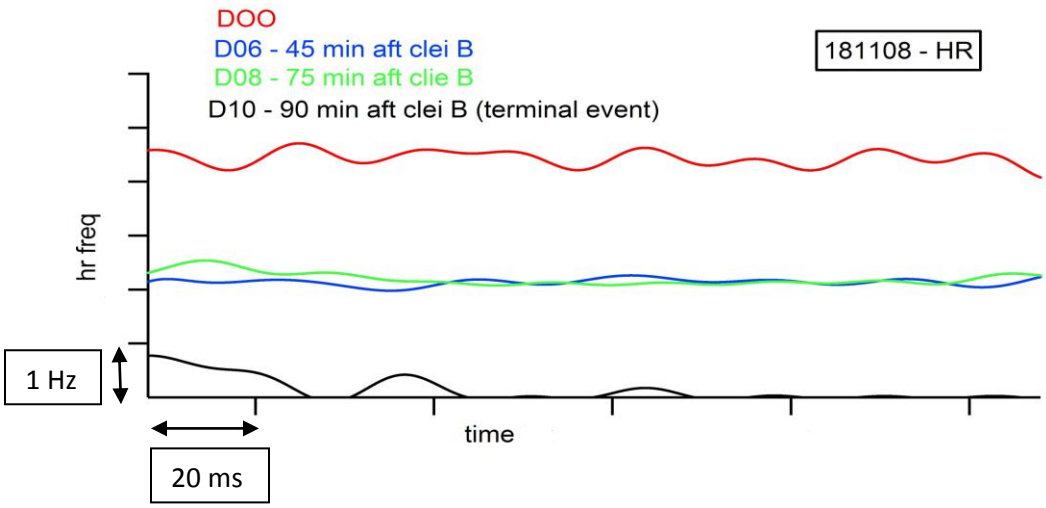
Graph-21: *Respiration tracings of test animal 1.*



Graph-22: *Heart rate plots of test animal 1.*



Graph-23: *Respiration tracings of test animal 2.*



Graph-24: *Heart rate plots of test animal 2.*

Discussion

The Cleistanthin B from column chromatography was used for the initial set of experiments, this had four to five fluorescent bands on thin layer chromatography. Hence, another set of Cleistanthin B was obtained using a different extraction procedure, this was fairly pure and used for the second set of experiments. The column Cleistanthin B was dissolved in acetone and the liquid-liquid Cleistanthin B was dissolved in Ethanol. Hence, two different sets of Cleistanthin B experiments were done.

It is evident from the results that significant acidosis was present in both the sets of Cleistanthin B experiments. When the control and the test blood pH values were compared, the column Cleistanthin B showed a significant difference from those of the control values, whereas the second Cleistanthin B from liquid-liquid partition chromatography did not differ from the controls significantly.

It is evident from the graphs and the box plots that hypercarbia was seen with the first set of Cleistanthin B experiments, but there was no significant difference between the 30 and 60 minute PCO2 values of tests and acetone controls. There was significant difference between the 0 hour and the terminal PCO2 values in tests. The lack of significant difference between the tests and controls in the first set of Cleistanthin B experiments can be attributed to the lack of standardisation in the timing of the bood samples and also to the less number of samples. The second set of Cleistanthin B showed more of a hypocarbia picture and the PCO2 and PO2 values

were not significantly different from the control values. The animals had shown a respiratory compensation for acidosis.

The bicarbonate values were significantly lower in the second set of experiments in both the tests and controls, pointing to metabolic acidosis in these animals. The bicarbonate values in the test animals were not significantly different from the control values. The bicarbonate values in the first set of Cleistanthin B experiments were not significantly different from the 0 hour values, where as in the acetone controls the terminal bicarbonate values were significantly different from the 0 hour values.

From the graph showing the potassium values against time, it is seen that there is a trend towards a decrease in potassium values in the first set of Cleistanthin B experiments, whereas the acetone controls have shown a trend towards an increase in potassium values. The statistical tests however did not show any significant difference within the tests or controls. The test potassium values are different from the control values significantly, because even the 0 hour values are different between controls and tests. In the second set of Cleistanthin B experiments there is a trend towards an increase in potassium values in tests and ethanol controls. Significant hyperkalemia is seen in both tests and controls.

The urine pH remained acidic in tests and controls in both the sets of experiments. However, the urine samples were not adequate to make a statistical comparison. Also, the 0 hour values were not present in most of the experiments. Hence, it is difficult to interpret the urine pH results. The triad of metabolic acidosis, alkaline urine and a trend towards hypokalemia which

was seen with the whole aqueous extract (Maneksh et al., 2010) was not seen in any of the two Cleistanthin B experiments.

Though acidosis is seen in both the sets of Cleistanthin B experiments, it is more likely due to a primary respiratory cause in the first set of Cleistanthin B experiments, whereas it is a metabolic acidosis in the second set of Cleistanthin B experiments. The mortality rate is lower in both the acetone and ethanol controls, inspite of the metabolic acidosis they had developed. This can tell us that acidosis is not the primary cause of mortality in the test animals. Looking at the ECG and the respiratory tracings it is seen that respiration stopped suddenly in the test animals, whereas the ECG continued for some time even after the stoppage of respiration. This pattern of death is common to both the sets of cleistanthin B experiments. In the second set of cleistanthin B experiments two patterns of death were observed. In some of the rats, the respiration wave showed a decrease in the amplitude and an increase in the rate after 30-60 minutes of injection of the toxin, but in some of the other rats the respiration continued with the same amplitude but stopped suddenly. The ethanol control animals had shown an increase in the amplitude of respiration. In the first case it looks like more of a neuromuscular blockade, whereas in the second case it looks like more of a central depression. With this kind of respiratory recordings, it is difficult to comment about ventilation, hence it is better to say that the cause of death could be either due to a central or a peripheral mechanism.

Cause for death in the control animals:

- Air embolism, which was the suspected cause in one of the ethanol controls (the rat died immediately after injection of the left out blood into the carotid artery).

- Repeated blood sampling in controls which could have caused a drop in the circulating blood volume.
- Anaethesia overdose- the control animals required frequent top up doses of ketamine as they were coming out of acidosis.

Limitations of the study:

- The timing of blood sample was not standardised when the first set of experiments were done, hence this resulted in less number of data for comparison and this could have been the cause for the lack of significance between test and control animals.
- Four of the acetone controls were taken from a another study to increase the number of rats for comparison.
- The column chromatography sample was not pure, it had four of the other unnamed fractions(Fig) and this might have been the cause for the difference in the results between the two sets of cleistanthin B experiments.
- We couldn't get 0 hour Urine pH values, which are very essential considering the fact that DRTA is seen in the whole aqueous extract(Maneksh et al., 2010).

Future course:

We plan to do more controlled experiments with purer compounds to minimise the errors.Also, we are trying to sort out ways to improve our respiration recordings to give us more information about ventilation. Our long term objective is to find out a treatment modality for the poison.

Summary and conclusions

Cleistanthin B is toxic to rats at dose of 2-7 mg/100gm body weight. The cause of death is more likely a respiratory event, the cause could be a central mechanism or a neuromuscular paralysis. Considering the more purer fraction it can be said that metabolic acidosis is seen in the rats. Acidosis per se is not the cause of death in these animals, since the control animals have also shown an equal amount of acidosis. The DRTA picture which was seen with the whole aqueous extract is not seen with Cleistanthin B, which is one of the toxic fractions of *cleistanthus collinus*.

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