TO DEMONSTRATE ON RATS, THE EFFECTS OF DIPHYLLIN, EXTRACTED FROM THE TOXIC PLANT CLEISTANTHUS COLLINUS

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 "To demonstrate on rats, the effects of Diphyllin, extracted from the toxic plant *Cleistanthus collinus*" is the bonafide original work carried out by Dr. R.Latha in partial fulfillment of the rules and regulations for the MD- Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in March 2011.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "To demonstrate on rats, the effects of diphyllin, extracted from the toxic plant *Cleistanthus collinus*." 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

INTRODUCTION

Plants are our basic food, plants can also serve as medicines, but there are some plants which could be potentially fatal. Worldwide suicidal rate is on the rising phase. People commit suicide by various ways. Consuming poisonous plants is one of the modes of committing suicide. Poisoning by plants can be through any of the modes like ingestion, inhalation or by contact.

Especially in countries like India where we have plenty of vegetation, suicide due to plant poisoning is common. This is still more common among the villagers. India's suicide figures have risen starkly over the past decade, even worse than the west. India has the highest suicide rate in the world. 95-100 people die every day out of suicide. Most of the poisoning due to plants produces acute symptoms. It can lead onto multiple organ failure and produce immediate death. But still the revolutionary evolution of medicine field has saved many lives even after such deadly poisoning. We have various treatment modalities such as gastric lavage, antidotes for that particular poison, various life saving drugs and artificial life support procedures.

For most of the common plant poisoning in India, the specific modes of treatment have been laid out. But still, for many others, the specific antidote and specific protocol for treatment are not available. One of such case is the *Cleistanthus collinus* poisoning.

Cleistanthus collinus is abundant in many parts of India, Africa and Malaysia (*Sarathchandra et al, 1997*). *Cleistanthus collinus* is commonly called as oduvanthalai in Tamil Nadu in India *(Sarathchandra et al, 1997). Cleistanthus collinus* is an extremely toxic poison *(Benjamin et al, 2006).* Oduvan poisoning is one of the common suicidal poisoning in the rural

parts of southern India *(Viswanathan et al, 2005). Cleistanthus collinus* is used as a suicidal or homicidal poisoning, and it is also used to induce abortions *(Sarathchandra et al, 1997).*

Cleistanthus collinus poisoning causes various disorders like hypokalemia, hypotension, cardiac arrhythmias, renal failure, neuromuscular weakness and respiratory failure *(Eswarappa et al, 2003).* Diphyllin, two new lignan lactones, cleistanthin and collinusin and ellagic acid have been isolated from *Cleistanthus collinus (Govindachari et al, 1969).*

One of the research works in our department is mainly focused on the details of mechanism of action of oduvanthalai poisoning and to find out a specific antidote for the same. *Cleistanthus collinus* is known to produce type 1 distal renal tubular acidosis and type II respiratory failure in rats and the mortality is 100% *(Delinda Maneksh et al, 2010).*

This study is focused on diphyllin, which is one of the fractions of *Cleistanthus collinus.* Experiments were done in rat models to identify if this fraction is the cause of type 1 distal renal tubular acidosis that is developing with the whole extract of the *Cleistanthus collinus* and also to delineate the toxicity profile of diphyllin.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Epidemiology:

Suicide due to plant poisoning is a major problem in the developing world. But only few of them have specific antidotes *(M.Eddleston, 2000). Cleistanthus* is a genus of the family Euphorbiaceae. The genus comprises 140 species, found from Africa to the Pacific Islands *(wikipedia.com). Cleistanthus collinus* is a toxic plant found in the regions of South East Asia. Water decoction of the leaves is used for suicidal purposes. Death occurs within 1 to 3 days *(Parasuraman et al, 2009)*. The plant is very lethal especially when consumed as an extract *(Shankar Viswanathan et al, 2005).* "Patients average age was 29.1, 62% were female, 76% were married and 49% were housewives. The cumulative case fatality was 30%. The probability of survival was 0.88, 0.75, 0.64 and 0.57 at 1, 2, 3 and 5 days respectively" *(Shankar Viswanathan et al, 2005).*

Clinical features:

The toxins of *Cleistanthus collinus* such as cleistanthin A and cleistanthin B are diphyllin glycosides which produce hypoxia, metabolic acidosis, hypokalemia, cardiac arrhythmias and hypotension *(Benjamin et al, 2006).* Other findings in *Cleistanthus collinus* poisoning includes leucocytosis, alkaline urine pH, elevated liver enzymes, coagulopathy, hyperchloremia *(Eswarappa et al, 2003).* There are reports of hyponatremia, raised AST/LDH/CPK/CPK-MB, prolonged QTc and nonspecific ST-T changes on ECG and hypoxia with increased alveolar-arterial oxygen difference (A-aDO2) *(Subrahmanyam et al, 2003).* In a case of oduvanthalai poisoning, ARDS, distal renal tubular acidosis and distributive shock due to inappropriate vasodilatation were also reported *(Benjamin et al, 2007).* Hypokalemia has been found to be an

important risk factor for death espciallly in elderly people and patients with existing chronic diseases *(Shankar Viswanathan et al, 2005).* Poisoning with *Cleistanthus collinus* frequently causes cardiac manifestations such as rhythm disturbances *(Pokuri Damodaran et al, 2008).* A lower dose causes transient tachycardia and increased contractility and at higher doses it causes cardiac arrhythmias and cardiac arrest *(Jose et al, 2004).*

Mechanism of action:

The patients develop neuromuscular failure. The hypokalemia can cause rhabdomyolysis and can induce myoglobinuric renal failure *(Eswarappa et al, 2007).* Electromyographic and electrodiagnostic studies were made on sciatic nerve–anterior tibialis muscle preparations in rat *in vivo* to confirm the neuromuscular junctional (NMJ) blocking action of *Cleistanthus collinus* leaf extract. The metabolic picture is probably mediated by injury to the distal renal tubules, pulmonary epithelium and peripheral blood vessels due to glutathione depletion *(Saratchandra G et al, 1998). Cleistanthus collinus* induces type 1 distal renal tubular acidosis in rats and the cause of death is usually type II respiratory failure *(Delinda Maneksh et al, 2010).* There are also reports of proximal tubular injury with *Cleistanthus collinus (Keshavan et al, 2010).* Recently, oxidative mechanisms have also been found to have an important role in *Cleistanthus collinus* induced tissue damage *(M.Jayanthi et al, 2008).*

Isolation of components of Cleistanthus collinus:

Spectrofluorometric quantitation of the active principles of *Cleistanthus collinus* such as diphyllin, cleistanthin A and cleistanthin B has been done by the chemist *Annapoorani et al* in the year 1984. The dried and fresh leaves have similar constituents. A total of 17 compounds

were identified by GC-MS analysis *(Parasuraman et al, 2009)*. Chemical examination of the aerial parts of *Cleistanthus collinus* afforded the arylnaphthalide lignans, cleistanone), diphyllin, Cleistanthin A, C and D, and 4-O-(3"-O-methyl-.BETA.-D-glucopyranosyl)-diphyllin *(Das et al, 2003).* "The isolation and characterisation of five new compounds and nine known compounds from the heartwood of *Cleistanthus collinus* are reported. The new compounds are wodeshiol **16**,3,4-dihydrotaiwanin C **20**, and three new glycosides **24**, **25** and **26** of diphyllin and taiwanin E" *(Anjaneyulu et al, 2001).* Cleistanthin A and B are isolated from the leaves of *Cleistanthus collinus* leaves using chromatographic methods. The structures of the isolated fractions were confirmed by spectroscopic methods *(Parasuraman et al, 2009).* A potent neuromuscular junctional blocking agent has been isolated by chromatographic—cholinesterase inhibition technique from chloroform extracts of *Cleistanthus collinus (Vijayalakshmi et al, 2006).*

Treatment review:

Treatment of some plant poisonings might benefit from the development of antitoxins, for example, anti-digoxin Fab fragments against yellow oleander poisoning. But most of them are expensive and the supply is also limited. Thus, plant anti-toxins remains as a dream in many of the areas where they are now urgently required *(Michael Edleston et al, 2003)*. Animal studies have shown benefit with N-acetyl cysteine therapy for *C.collinus* poisoning. *(Annapoorani et al, 1986).* Neuromuscular junctional blockade by *Cleistanthus collinus* can be reversed with neostigmine *(Vijayalakshmi et al, 1998)*. "Melatonin, by balancing oxidant-antioxidant status ameliorates oxidative organ injury in brain due to *C. collinus* toxicity" *(M.Jayanthi et al, 2008).*

Therapeutic uses:

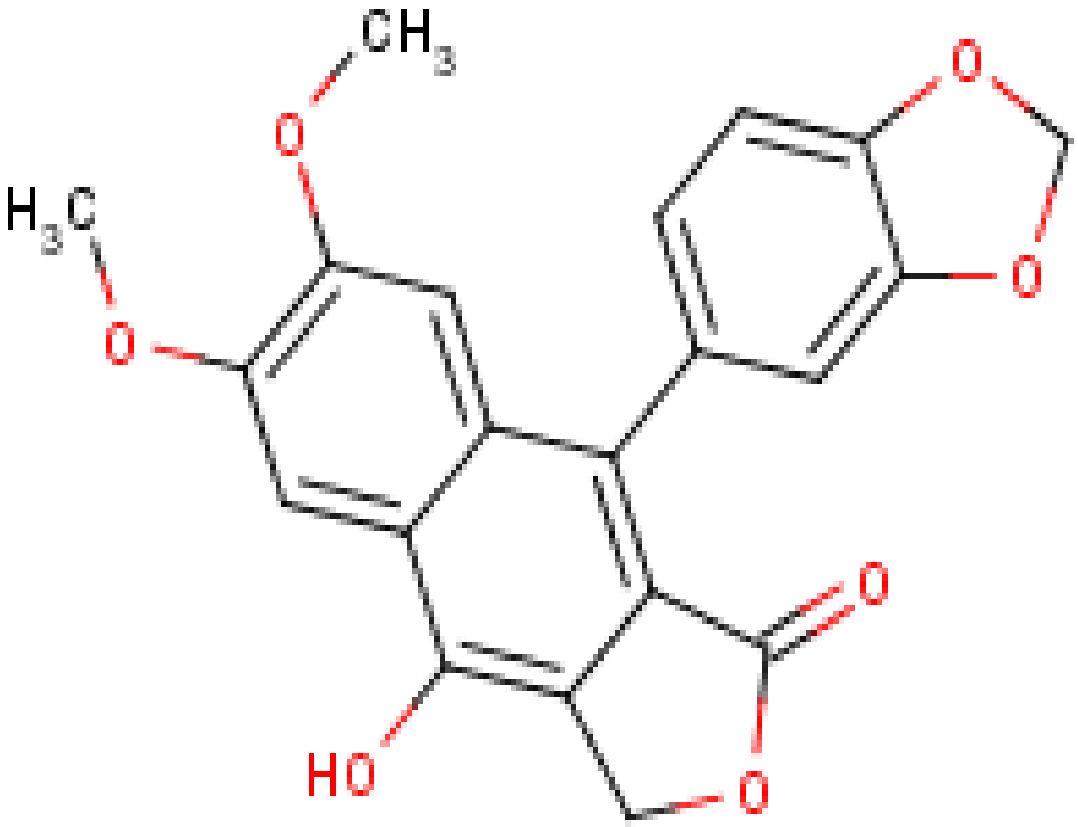
Cleistanthin A and cleistanthin B, which are the diphyllin glycosides isolated from *C. collinus* were reported to exhibit cytotoxicity on several cancer cell lines *(Paulo et al, 2007).* Cleistanthin B was known to be toxic to normal and tumour cells. But it is toxic to tumour cells even at lower doses *(Chaaliyil et al, 1996).* Cleistanthin A was found to possess cytotoxic and tumour regressing properties *(Meenakshi Jayaraman et al, 2000).*

Diphyllin isolation and its application:

The active principles can be isolated by thin layer chromatographic methods. Spectroflurometric quantitation is done for comfirmation of the principles including diphyllin *(Annapoorani et al, 1984).* Cleistanthin A and B are isolated from the leaves of *Cleistanthus collinus* leaves using chromatographic methods. The structures of the isolated fractions were confirmed by spectroscopic methods *(Parasuraman et al, 2009*

Diphyllin is found to be a V-ATPase inhibitor useful in treatment of osteoclastic bone resorption (*Sorenson et al, 2007).* The V-ATPase blocking activity of diphyllin is also useful in treatment of gastric acidification *(Sorenson et al, patented, 2008).* Diphyllin was found to be effective against vesicular stomatitis virus and low cytotoxicity against cultured rabbit lung cells *(J Assano et al, 1996).* Diphyllin, isolated from Haplophyllum bucharium had shown anti proliferative activity against monocytes and Leishmaniasis *(Giorgio et al, 2005).* Diphyllin acetylapioside proved to be the main lignan endowed with 5-LOX inhibitory activity, thus playing a role in inflammation *(Jose Ma Preito et al, 2002).).*

CHEMICAL STRUCUTURE OF DIPHYLLIN:



CAS TYPE 1 NAME: 9-(13-benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho(2,3-c)furan-1(3H)-one *(Min Gui et al, 2010).*

AIMS AND OBJECTIVES

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AIMS:

To demonstrate on rats, the effects of diphyllin, extracted from the toxic plant *Cleistanthus collinus.*

OBJECTIVES:

1. To inject diphyllin enriched fraction extracted from *Cleistanthus collinus* into anaesthetized rats and
    a) Make serial measurements of
        i. blood electrolytes,
        ii. blood gases and
        iii. urine pH
    b) Monitor clinical parameters such as
        i. ECG,
        ii. Blood Pressure and
        iii. Respiration

    till the animal dies spontaneously or for a period of 8 hours.

2. To do control experiments and monitor the above parameters till the animal dies spontaneously or for a period of 8 hours.

MATERIALS AND METHODS

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MATERIALS:

RATS:

- Sex: male and female rats
- Species: Wistar
- Body weight: 150 to 250 grams
- Preconditions: healthy rats without any previous interventions done. No overnight starvation required. Non pregnant and non lactating female rats are selected. These conditions are considered so that the pre physiological conditions are the same in all the selected rats.

APPARATUS REQUIRED:

i. Arterial blood gas analyzer for arterial blood gases analysis
ii. CMC Daq (CMC data acquisition system) for acquiring ECG, respiration and blood pressure
iii. ECG needle electrodes with preamplifier for acquiring ECG
iv. Respiration transducer for recording respiration
v. Blood pressure transducer for recording blood pressure
vi. Computer for displaying the acquired data
vii. Micro pH meter for measuring urine pH

MATERIALS REQUIRED:

Drugs:

- ketamine for anesthesia
- heparin for anticoagulation
- normal saline (NS) and dextrose normal saline (DNS) for fluid and electrolyte replacement

Others:

EG7+ cartridges for blood gas analysis, 25G iv cannula, 26G needles, scalp iv sets, 3 way stop cocks, tuberculin syringes, glass syringes, suture materials, scissors and forceps for dissection, spatula, eppendorfs, gloves and cotton.

For recording and analysis:

CMC Daq software, IGOR Pro software, SPSS software, Microsoft excel software.

METHODS:

DIPHYLLIN ISOLATION:

- **Processing of leaves:**

Oduvanthalai plant leaves are obtained from Amriti forest in Vellore. These leaves are dried under shade. The shade dried leaves are soaked in n-hexane overnight for delipidation. The delipidated leaves are again left for complete drying under shade.

- **Preparation of aqueous extract of oduvan**:

About 500g of the delipidated leaves of oduvanthalai are soaked in water and left overnight. Next day, it is filtered through cotton and gauze. The leaves are discarded. The filtrate is called the aqueous extract. This aqueous extract contains the dissolved pigments, and the toxic and non-toxic constituents of the plant *Cleistanthus collinus.* The aqueous extract appears dark

greenish in color. The thin layer chromatography done with this extract shows an array of various florescent compounds (figure 1). The aqueous extract is rich in mainly two constituents' viz., cleistanthin A and diphyllin and collinusin and with some other unknown fractions. Cleistanthin B is present only in trace amounts in aqueous extract because it is not a highly polar compound.

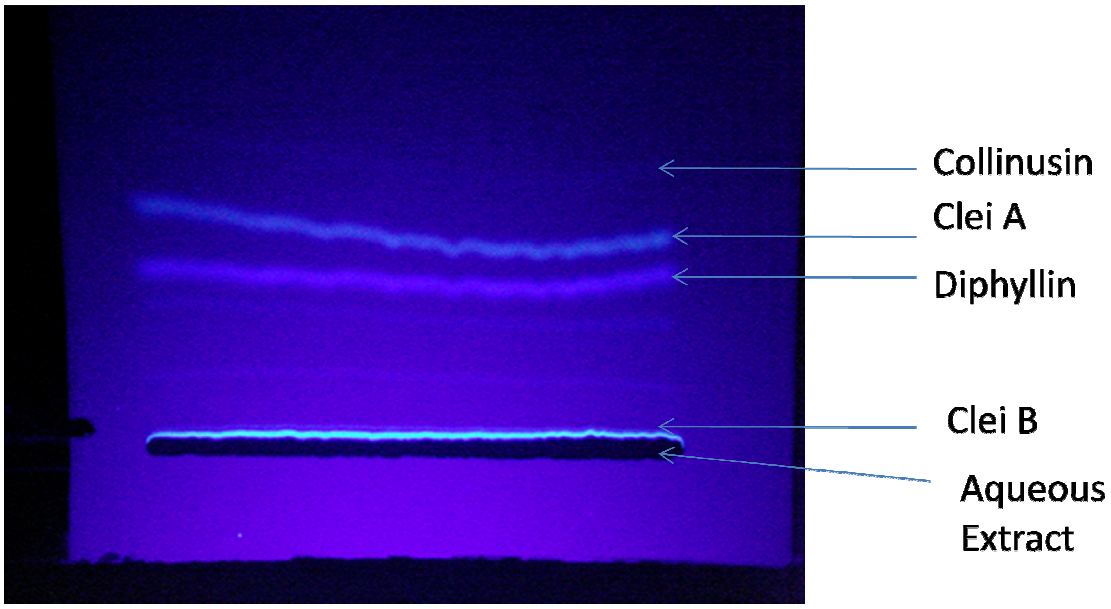


Figure 1: TLC of aqueous extract. Diphyllin is present beneath Cleistanthin A and it shows a bright purple florescence.

- **Removal of pigments:**

Before the TLC is done, Chloroform is added to the aqueous extract in the ratio of 1:5 to remove the pigments and separate the constituents of *Cleistanthus collinus* especially cleistanthin A and diphyllin. The dissolved pigments and the fractions dissolved separate in two layers. The pigments are present in the top layer which appears brown in color and the liquid with dissolved fractions appear light greenish in color. This is separated through a separator funnel. The slightly greenish liquid is collected in a separate flask (Figure 2).

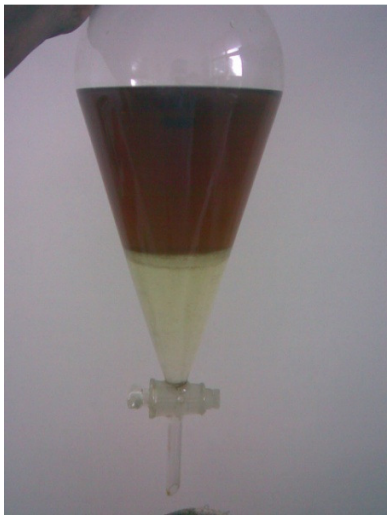


Figure 2: The separator funnel showing two distinct layers. Brown color is the dissolved pigments from the leaves and slightly greenish color has the florescent compounds from the plant

- **Preparation of aqueous powder:**

The greenish liquid is poured into petridishes and the chloroform allowed to evaporate in an oven or kept outside for drying overnight. After complete drying the powder is scraped off the petridish with a scalpel blade. This is the aqueous powder of *Cleistanthus collinus.*

- **Thin layer chromatography (TLC):**

About 500g of aqueous powder is prepared from 500g of oduvanthalai leaves. Thin layer chromatography is done with this aqueous powder dissolved again in chloroform in an amount just required to dissolve the powder. TLC plates are prepared by plating silica dissolved in water onto glass plates and drying them. The TLC plates are activated half an hour before layering of the aqueous extract. The aqueous extract is layered with capillary tubes on the TLC plates. Three solvents namely n-heptane, chloroform and ethanol in the ratio of 50:50:5 are taken and poured into TLC tanks. The TLC plates layered with aqueous extract are placed inside the tanks. The solvents start rising over the plates thereby separating the fractions of *Cleistanthus*

collinus. It takes half an hour to one hour, depending on climatic conditions, for the solvent to rise and fractionate the aqueous extract of oduvanthalai.

- **Scraping of diphyllin**:

After one hour, the plates are taken out and allowed to dry. The plates are examined under UV chamber for florescence. Cleistanthin A and diphyllin with some other unknown florescent compounds are observed. Diphyllin produces a bright purple colored florescence, whereas cleistanthin A produces bluish green florescence (figure 1). Under UV chamber the band of diphyllin below cleistanthin A is marked. This is done in all the TLC plates. The diphyllin band looks brownish in color with naked eye. The marked diphyllin band is scraped off along with silica with gloved finger or small wooden sticks, taking at most care to avoid scraping the neighboring compounds to minimize contamination as much as possible. The diphyllin compound along with silica is collected in a tube.

- **Centrifugation :**

To separate the diphyllin from silica and obtain a diphyllin enriched fraction, the mixture is centrifuged. Distilled water is added to the diphyllin and silica mixture. Diphyllin dissolves in water whereas silica mixes in water but is insoluble. The tubes are centrifuged at 2500rpm for 15 minutes with temperature set at 15 degree Celsius in a cooling centrifuge. The insoluble silica settles at the bottom and the dissolved diphyllin remains in the supernatant. The supernatant is pipetted out into another tube. The supernatant is again centrifuged to remove any remnant of silica in it. The supernatant appears brown in color.

- **Freeze drying:**

The diphyllin dissolved in water is obtained in a powder form by a process called freeze drying. The supernatant is poured into small bottles in a volume of about 3 ml in each of them. A cork with 2 side ports is just placed over the bottles. The bottles are then kept for pre-freeze in a cryofreezer for one hour. After pre-freezing the bottles are immediately transferred to the freeze drying system (figure 3A). The freeze drier is started immediately for operation. The freeze drier is run for 36 hours. The diphyllin enriched fraction is obtained in a fluffy, brownish powdery form (figure 3B)

A B

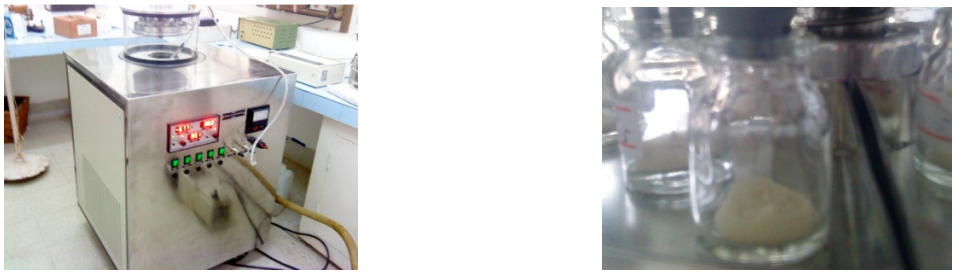


Figure 3: A) freeze drying system under operation, B) the diphyllin as lyophilized powder.

- **Confirmation with TLC and HPLC**:

To confirm that the isolated fraction is diphyllin enriched, some amount of the freeze dried powder is dissolved in water and a TLC done. The fractions show bright purple florescence (figure 4A). Aqueous extract of Cleistanthus collinus is also plated by the side. The diphyllin band corresponds with the diphyllin band seen with aqueous extract. The bright purple florescent color is also the same as that seen in the aqueous extract (figure 4B). The isolated sample is analyzed with HPLC also (figure 4C). Diphyllin shows a long peak with florescent detector with a small peak occurring later implying minimal contamination. Thus, this is a diphyllin enrinched fraction

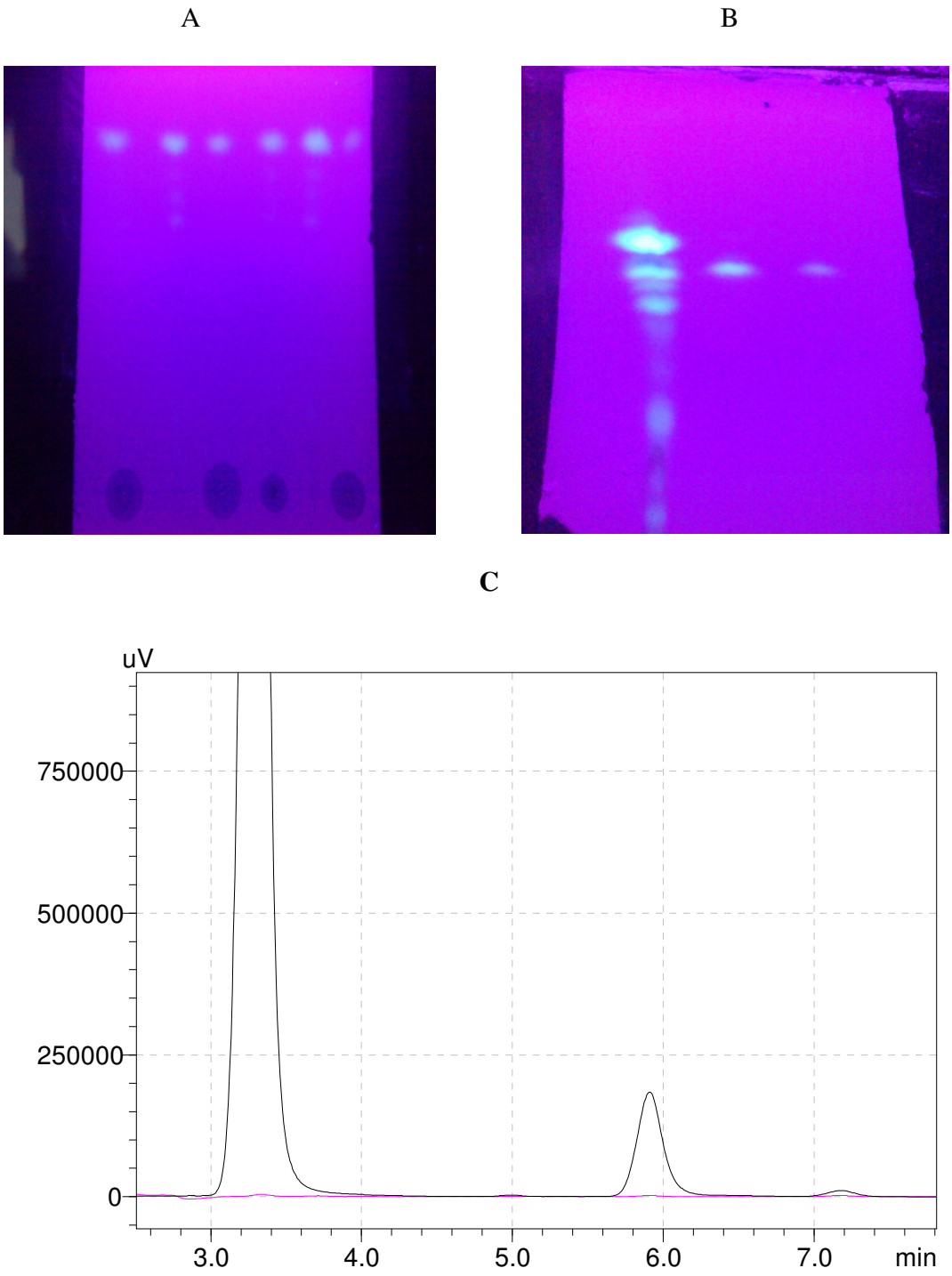


Figure 4: A) TLC picture of the isolated freeze dried powder showin single band of bright purple florescence, B) aqueous extract spotted by the side. The arrow shows that the freeze dried diphyllin band corresponds with that seen in the aqueous extract, confirming that it is diphyllin, C) HPLC data.

ANIMAL EXPERIMENTS:

- **Anesthesia:**

Male or female wistar rat of body weight ranging from 150g to 250 g is obtained from animal house. The rat is anesthetized with ketamine intraperitoneally using a tuberculin syringe. The dosage for ketamine is 75 to 100g / kg body weight of the rat. Full dose is given initially. Rats usually get anaesthetized within 15 minutes. Anesthesia is checked by pinching the skin with forceps or observing for conjunctival reflex.

Sometimes male rats do not get completely anaesthetized with the initial full dose. In such cases 1/3rd or ½ of the initial dose of ketamine is given as top up anesthesia. Then during the course of the experiment, 1/3rd top up doses are given when the rat is moving the tail or lifting up the head, which are some signs of recovery from anesthesia. Over dosage of anesthesia is risky because it might itself, sometimes, be the cause of death.

An intraperitoneal line with 24G scalp IV set is fixed for administration of top up doses of anesthesia.

- **Femoral artery dissection:**

Once the rat is completely anesthetized, it is strapped onto a plastic board with the abdomen facing upwards. Femoral artery has to be cannulated for obtaining blood samples for arterial blood gas analysis. Either the right or left femoral artery is cannulated. The skin on the junction between the abdomen and thigh is wet with cotton. A small flap of skin is removed. The underlying subcutaneous tissues are stripped off. Once the subcutaneous tissues are removed the femoral vein, femoral artery and the femoral nerve enclosed within the femoral sheath are seen clearly. The covering sheath is stripped off. The femoral vein lies medially, femoral artery in the middle and the femoral nerve lies laterally. The femoral artery is separated from the femoral vein

and nerve by careful blunt dissection. The femoral nerve is handled carefully to avoid the reflex response in the animal. The femoral artery is dissected proximally and distally to obtain a length of about 7 to 10 mm for easy cannulation. Any branches of femoral artery seen are left intact. The femoral artery is completely cleared off its covering sheath, which otherwise may cause counter-puncture during cannulation.

- **Femoral artery cannulation:**

Once the femoral artery is dissected, two silk threads are placed underneath the vessel, one towards the proximal end and the other towards the distal end. The distal suture is ligated. A thin spatula is placed underneath and the artery is cannulated with a 24G IV cannula. After cannulation, a 3way stop cock is fixed to the cannula (figure 5). The cannula is flushed with 0.2 ml of heparinised saline (heparin: saline is 1:4). The cannula is secured with ethicon sutures.

If the femoral artery cannulation is failed, the artery is tied with the proximal ligature. The skin over the abdomen is also sutured with ethicon materials. The femoral artery on the other side is dissected and cannulated.

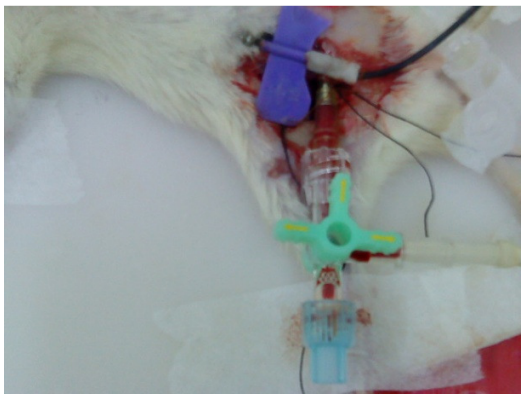


Figure 5: cannulated right femoral artery with the 3 way stop cock. The blue color needle seen above is the ECG needle electrode fixed over the abdomen.

- **Arterial blood gas analysis:**

After the cannulation about 0.2 to 0.5 ml of blood is drawn through the cannula with a glass syringe for arterial blood gas analysis. This is the 0 hour sample. The nozzle of the syringe is immediately closed with the capped 26G needle. The blood sample is immediately transferred to the EG7+ cartridges. The cartridges are fed to the arterial blood gas analyzer and the results are obtained.

If the arterial blood gas and the electrolyte values are within the normal range, the rat is considered to be normal and the experiment proceeds. The inclusion criteria followed is

  - Arterial PO2 – 75 to 120 mmHg
  - Arterial PCO2 – 35 to 55 mmHg
  - Blood pH – 7.25 to 7.4

- **Acquiring clinical data:**

If the rat fits the inclusion criteria for the arterial blood gas values, then is the acquisition of clinical data. ECG needle electrodes with preamplifier are placed in lead II configuration (figure 6). Negative electrode is placed on the right hand, positive electrode over the left side of the abdomen and the ground or the reference electrode on the right side of the abdomen. The ECG preamplifier is then plugged onto to the CMC data acquisition system. The recordings are obtained on the computer through CMC data acquisition software.

Respiration is recorded by using a respiration transducer which is nothing but a force transducer. It has a curved needle hook which is anchored onto the abdominal wall. The movements of respiration are picked up by the hook, transmitted through a thread tied to the transducer (figure6). The inspiratory and expiratory tracings are obtained on the computer.

Blood pressure is recorded with the help of a pressure transducer. The transducer is connected to the side port of the 3 way stop cock. The 3 way is adjusted so that the blood in the femoral artery contacts the oil in the transducer. The pressure of the artery is transmitted through the oil in the tube to the transducer and the blood pressure recordings are obtained on the computer.

The data are recorded for 5 to 10 minutes. The instrumentation for acquiring ECG, respiration and blood pressure and the CMC Daq software are made by our bioengineering department.

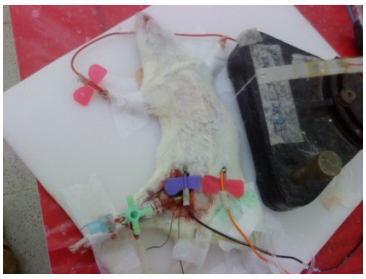


Figure 6: ECG needle electrodes in lead II configuration and respiration transducer hooked onto the abdomen seen

- **Estimating urine pH:**

A small eppendorf is placed near the urethra for the collection of urine. As the urine is getting collected the urine pH is immediately checked with the micro pH meter.

- **Replacement of fluids:**

0.25ml to 0.5 ml of NS or DNS is given intraperitoneally every half an hour to one hour and after sample collection through the intaperitoneal line already fixed.

- **Administration of diphyllin:**

The normal recordings of ECG, respiration and blood pressure are obtained. Diphyllin is administered to the rat at a dose of 7mg/100g body weight. The required

lyophilized diphyllin is weighed according to the body weight of the rat. Each 1mg of diphyllin is dissolved in 20µL of distilled water. The diphyllin is administered intraperitoneally.

- **Sample collection and monitoring:**

After the administration of diphyllin, arterial blood samples are obtained at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours or up to the time of survival of animal. The values of the arterial blood gas analysis are noted down in the log book. The corresponding clinical data are also recorded and the data number noted down. Urine pH is checked, if urine is collected. If urine is not collected, slight abdominal pressure is given to collect urine. The clinical data are recorded continuously in between sample times. This is done to keep a track of the changes in the clinical events, if happens, after the administration of diphyllin.

- **Control experiments:**

As diphyllin is dissolved in water and administered to the rat, the control experiments are done with water injection intraperitoneally. Distilled water is administered at a dose of 140µL/100g body weight (diphyllin dosage is 7mg/100g BW and 1 mg of diphyllin is dissolved in 20µL of water) of the rat. The arterial blood gas samples, urine samples and clinical data are obtained at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours, or up to the time of survival of the animal.

ANALYSIS OF RESULTS:

- **Analysis of ABG values**:

The arterial blood sample analyzer gives the values of blood pH, arterial PO2, arterial PCO2, serum electrolytes like sodium, potassium, bicarbonate and ionized calcium, base excess, hemoglobin and hematocrit values. Out of these, bicarbonate and hemoglobin are

calculated values. These values are noted in the Microsoft excel sheets indicating the corresponding values at each time points viz.0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours.

The ABG values are analyzed using IGOR Pro software. Graphs are plotted. The trend of the ABG values in various rats is plotted against time. The corresponding hour ABG values of diphyllin and control group rats are compiled together and plotted in the same graph, with different markers for diphyllin and control group and observed for any difference between the two groups.

Non-parametrical tests like Wilcoxon sign rank test (for comparison within the groups) and Mann Whitney U test (for comparison between test and control groups) are done using SPSS software. P values are obtained from these tests. P value ≤ 0.05 is considered significant.

- **Analysis of clinical data:**

Heart rate changes with time are observed by observing the raw data for any bradycardia or tachycardia over time. Heart rate over the period of time is also manually counted by counting the R peaks from the raw ECG tracing. Heart rate plot is done using CMC Daq software. The heart rate plot is shifted to IGOR Pro software. Graphs are done and the changes in heart rate over time are observed.

Respiration is analyzed from the raw data, and also by counting the respiratory rate manually and plotting in IGOR. Respiratory wave pattern at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours are plotted in a single graph with Igor Pro software and changes over time are compared. The analysis of blood pressure needs to be standardized. The graphical plots are compared between the diphyllin and the water control groups.

RESULTS

RESULTS

MORTALITY:

In the test group, i.e. diphyllin administered rats, 6 experiments were performed, 4 female rats and 2 male rats. In the control group, i.e. water administered rats, 5 experiments were performed, 4 female rats and 1 male rat. Out of them, 1 rat died in both the groups. Thus the mortality in the diphyllin group is 16.66% and the mortality in the control group is 20%

Groups	n=	survival	Mortality
Test-Diphyllin	6	83.33%	16.66%
Control-water	5	80%	20%

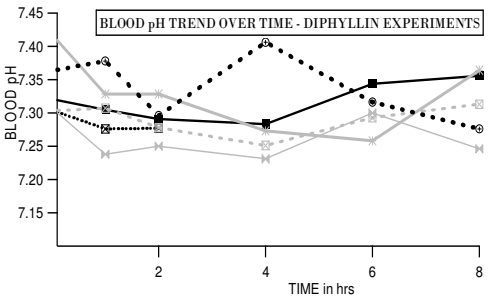
- ✓ *Inference from mortality data:*

The mortality in the diphyllin group is similar to that of the control group.

ANALYSIS OF ARTERIAL BLOOD SAMPLE PARAMETERS:

Blood pH:

A



B

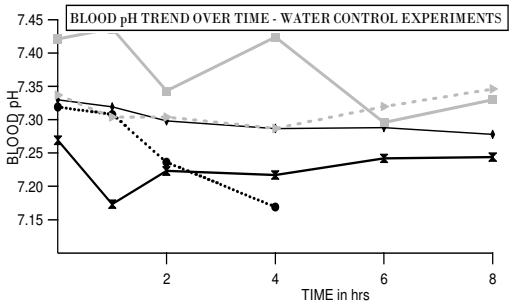


Figure 7: A) Blood pH trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8 hours in diphyllin experiments, n=6, B) blood pH trend over time in control experiments, n=5

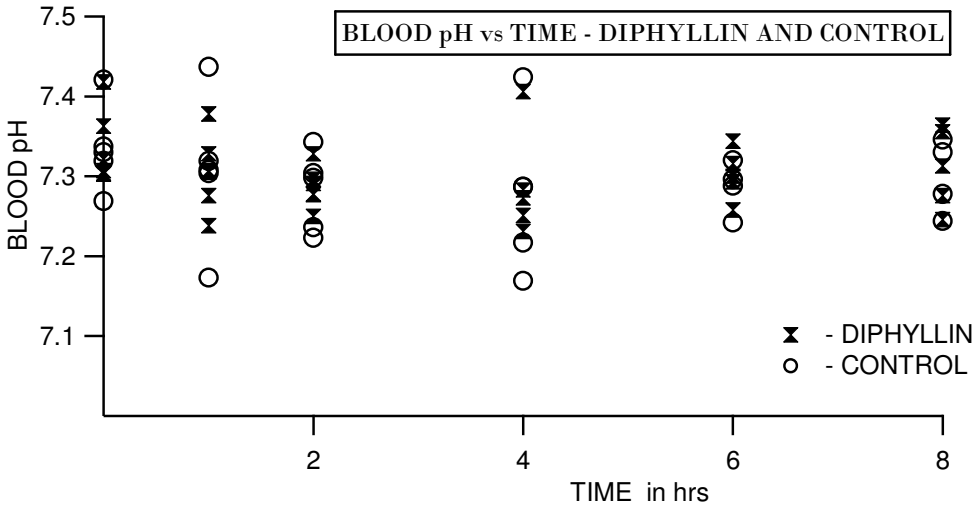


Figure 8: blood pH over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from blood pH:*

Blood pH is within normal limits at 0 hour in both the groups. Blood pH fluctuates over the 8 hours in the diphyllin experiments (figure 7A). Blood pH decreases in the initial hours, then compensates and returns near the baseline value. In one experiment of diphyllin, there is increase in blood pH in the 1st hour, and then it decreases. This cycle seems to continue over the period of 8 hours. Diphyllin causes acidosis in the initial hours. In the control experiments (figure 7B) also, we observe a similar type of picture. Most of the rats tend to develop acidosis in the initial hours and then tries to compensate. In the control group also, in one experiment, there is initial increase in pH in the 1st hour and then it decreases. The figure 8 shows that most of the blood pH tends to decrease in the initial hours after diphyllin when compared with the 0 hour values in both the groups.

To find out if there is a significant difference between the test and control groups, nonparametric tests like Wilcoxon's sign rank test (comparison within groups) and Mann Whitney U test (comparison between test and control groups)

P values by Wilcoxon's sign rank test are

Between	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	0.156	**0.031**	0.188	0.313	0.313	0.313	0.063	0.125	0.125	0.250

From the table, we infer that there is a significant difference in the acidosis only in the 2nd hour in the test group.

P values by Mann Whitney U test are

Between	Test & control-0hour	Test & control-1hour	Test & control-2hours	Test & control-4hours	Test & control-6hours	Test & control-8hours
P value	0.792	0.931	0.931	0.952	0.556	0.730

This table shows us that there is no significant difference in the blood pH between the diphyllin and the control groups. The decrease in blood pH though present in the initial hours is not significantly different from the zero hour value. That is the acidosis is not that severe except in the 2nd hour of the test group. The acidosis could be metabolic or respiratory.

Serum bicarbonate:

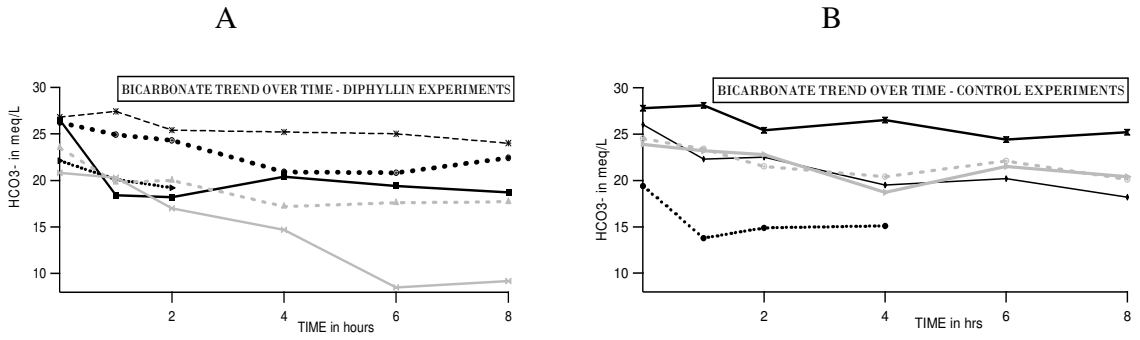


Figure 9: A) the serum bicarbonate trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8 hours in diphyllin experiments, n=6, B) the serum bicarbonate trend over time in control experiments, n=5

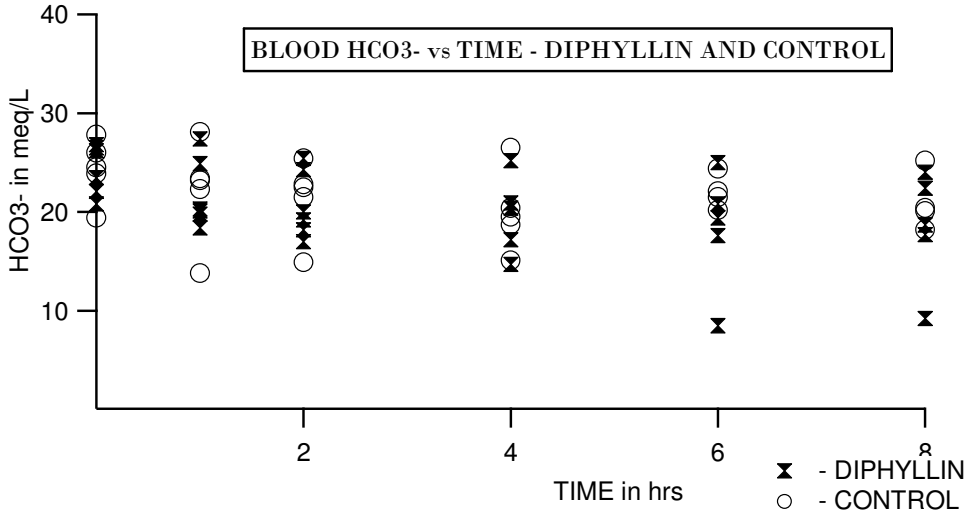


Figure 10: serum bicarbonate values over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from serum bicarbonate:*

From the figures 9A and 9B, the serum bicarbonate values tend to decrease with time when compared to the 0 hour value in both the groups. But the values still seem to be within the normal range for bicarbonate, except one experiment under test group where there is much decrease in serum bicarbonate over the 8 hours. Figure 10 infers that the bicarbonate values over

time are not much different in the diphyllin and control groups except few values towards the later hours under test group.

The p values can clarify if these values are going to be significantly differing.

P values by wilcoxon's sign rank test are

Between	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	0.094	**0.031**	0.063	0.063	0.063	0.125	0.063	0.063	.125	0.125

There is a significant difference in the values of bicarbonate in the 2nd hour in the test group, correlating with the significant acidosis developed in the test groups during the same time.

P values by Mann whitney U test are

Between	Test & control-0hour	Test & control-1hour	Test & control-2hours	Test & control-4hours	Test & control-6hours	Test & control-8hours
P value	0.651	0.662	0.706	0.413	0.286	0.556

P values do not show significance between test and control groups.

However, the decreasing bicarbonate trend points towards acidosis developing in the test and control groups. The decrease in bicarbonate with time might be due to a primary metabolic acidosis or an uncompensated respiratory alkalosis. The trend of base excess can give us an extra clue regarding the acidosis development in the rats.

Base excess:

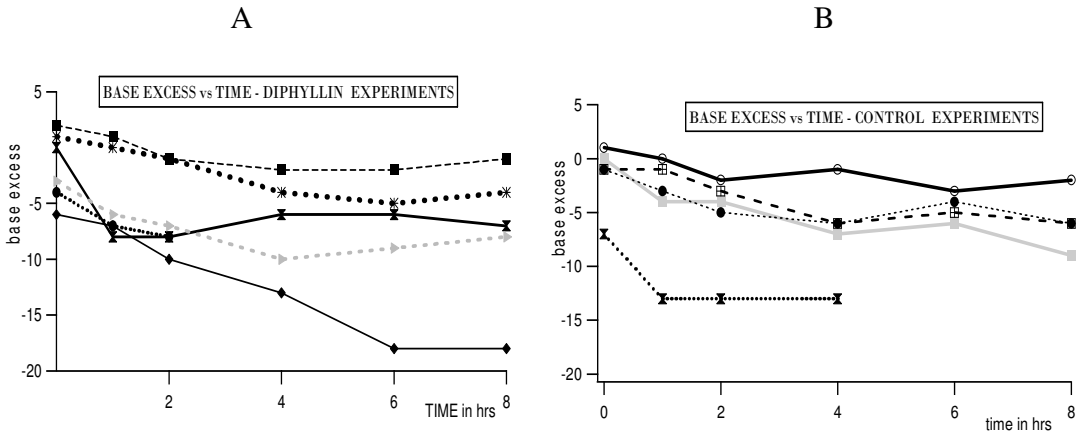


Figure 11: A) base excess trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8 hours in diphyllin experiments, n=6, B) base excess trend over time in control experiments, n=5

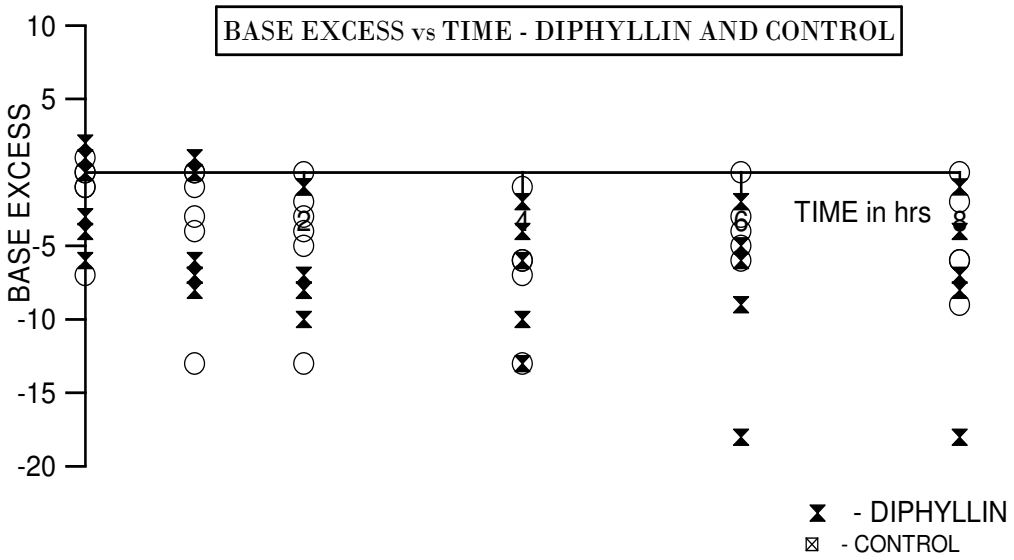


Figure 12: base excess over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from base excess:*

The base excess graph of diphyllin experiments (figure 11A) implies that the base excess becomes more and more negative with time. This infers that there is decrease in bicarbonate over time and development of acidosis. Control experiments also show negative base excess values

(figure 11B). One of the experiments in the diphyllin group shows a more negative base excess (figure 12). Significance tests clarify this issue as follows.

P values by Wilcoxon's sign rank test are

Between	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	**0.031**	**0.031**	0.063	0.063	0.063	0.125	0.063	0.063	0.125	0.125

There is a significant difference in the base excess values in the 1st and 2nd hours in the test groups. This again correlates with the significant acidosis and a decrease in bicarbonate that develops in the test groups at the 2nd hour. All these features point towards development of acidosis in the initial hours, resolving with time.

P values by Mann Whitney U test are

Between	Test & control-0hour	Test & control-1hour	Test & control-2hours	Test & control-4hours	Test & control-6hours	Test & control-8hours
P value	0.459	0.827	0.903	1	0.373	0.857

Though there is significant difference within test groups in the initial hours, there is no significant difference in the base excess values in the initial hours also in between the test and control groups.

The acidosis could be a primary metabolic acidosis or an uncompensated respiratory alkalosis. To find out the type of acidosis we go for further analysis of blood gases.

Blood PCO2:

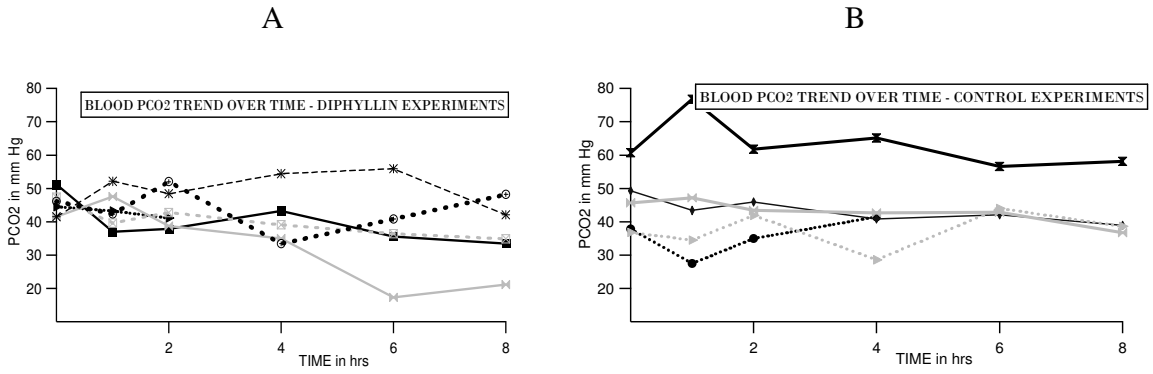


Figure 13: A) Arterial PCO2 trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8 hours in diphyllin experiments, n=6, B) Arterial PCO2 trend over time in control experiments, n=5

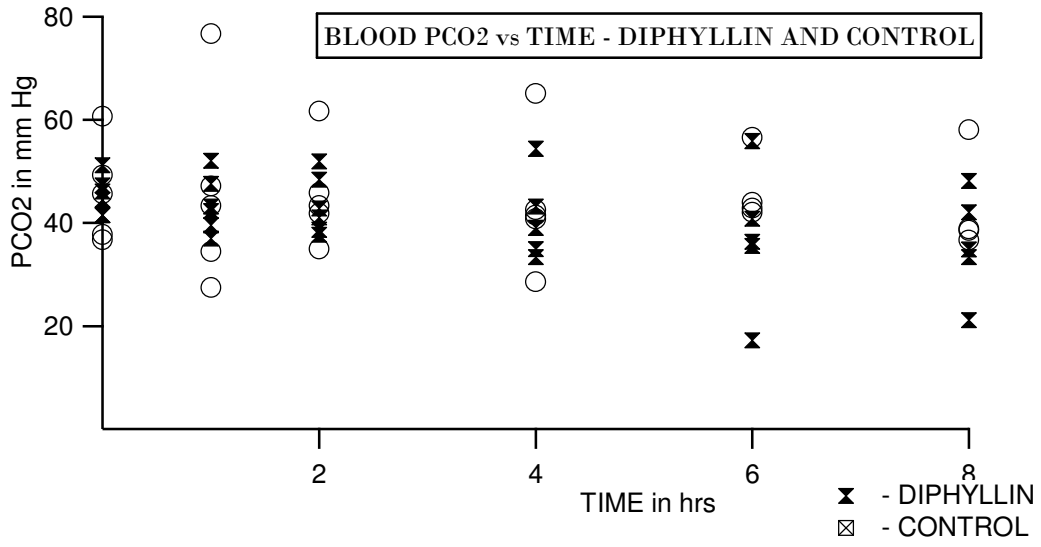


Figure 14: Arterial PCO2 over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from blood PCO2*:

In the initial hours, there is hypercarbia in few rats and hypocarbia in few others in the test group (figure 13A). In one of the control experiment, there is a high PCO2 in the 2nd hour, but other experiments show tendency towards hypocarbia (figure 13B). There seems to be a

tendency of hypocarbia in the later hours in the test group (figure 14). Between 2 to 4 hours, there is overlap of the values in both the groups and most of the values of blood PCO2 fall within normal limits.

P values by Wilcoxon's sign rank test are

Between	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	0.688	0.844	0.625	0.313	0.313	0.813	0.813	0.625	0.750	0.250

There is no significant difference between the 0 hour and 1, 2, 4, 6 and 8 hours in both the tests and control groups.

P values by Mann Whitney U test are

Between	Test & control-0hour	Test & control-1hour	Test & control-2hours	Test & control-4hours	Test & control-6hours	Test & control-8hours
P value	0.890	0.931	0.792	0.841	0.111	0.413

Even between groups blood PCO2 is not significantly different. So there is significant hypocarbia. But we observe a tendency towards hypocarbia in both the groups from the trend of blood PCO2 over time. Development of hypocarbia implies that the animals have hyperventilated to blow out CO2 as a respiratory compensation for metabolic acidosis

Blood PO2:

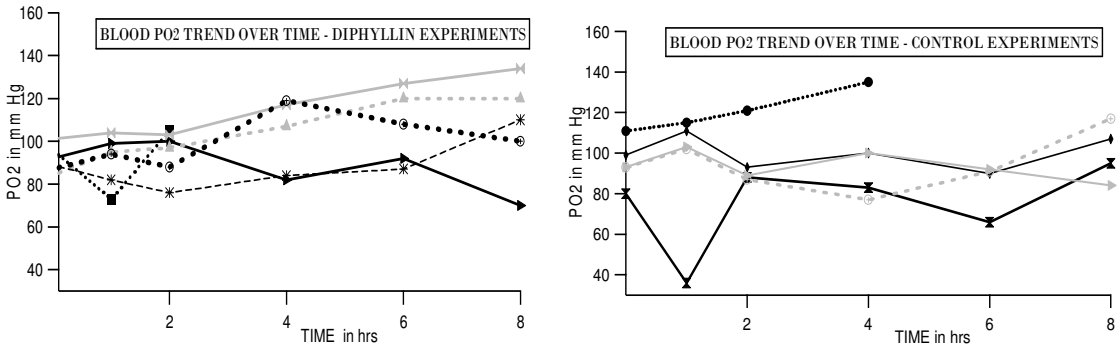


Figure 15: A) Arterial PO2 trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8 hours in diphyllin experiments, n=6, B) Arterial PO2 trend over time in control experiments, n=5.

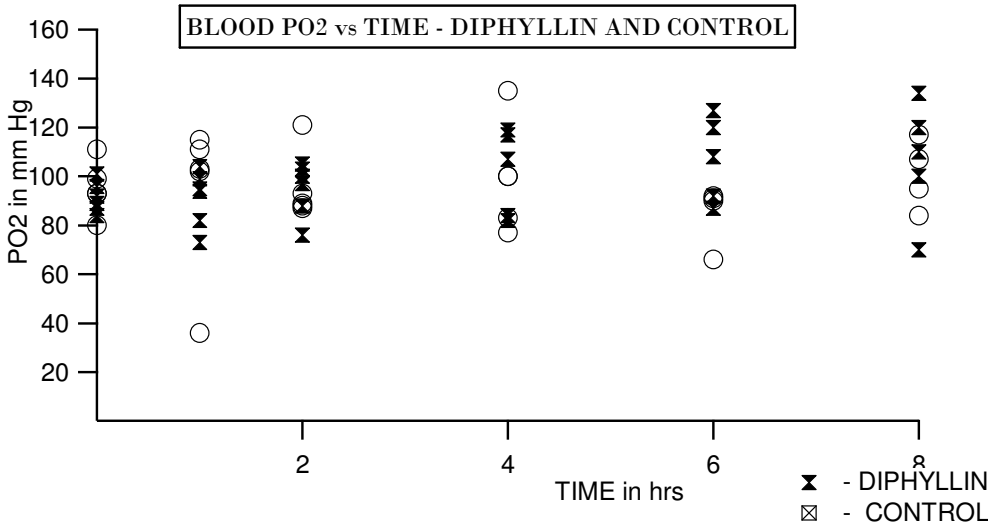


Figure 16: Arterial PO2 over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from blood PO2:*

From the figures 15A and 15B, we observe that there is development of hyperoxia in most of the experiments in the diphyllin and the control group. The hyperoxia is especially prominent in the later hours. In one of the control experiment, there is severe hypoxia in the initial hours

P values by Wilcoxon's sign rank test are

Btween	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	0.875	0.344	0.313	0.250	0.313	0.625	0.750	0.438	0.125	0.375

P values by Mann Whitney U test are

Between	Test & control- 0hour	Test & control- 1hour	Test & control- 2hours	Test & control- 4hours	Test & control- 6hours	Test & control- 8hours
P value	0.890	0.931	0.792	0.841	0.111	0.413

There is no significant difference in the blood PO2 values within the groups or between the groups. But the trend of hyperoxia in the later hours in both the groups correlates with the hypocarbia observed during the same time though they are not significantly different. This reflects the feature of hyperventilation which happens in acidosis. This observation confirms the presence of metabolic acidosis with respiratory compensation. The blood pH restores around normal value in the later hours because of this compensatory mechanisms.

Blood K+:

A B

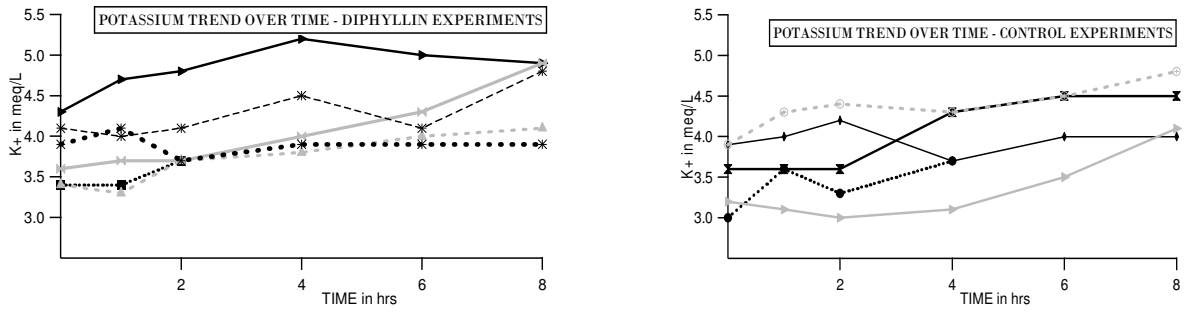


Figure 18: A)serum potassium trend over time i.e. at 0 hour, 1hour, 2 hours, 4 hours, 6 hours, 8

hours in diphyllin experiments, n=6, B serum potassium trend over time in control experiments, n=5

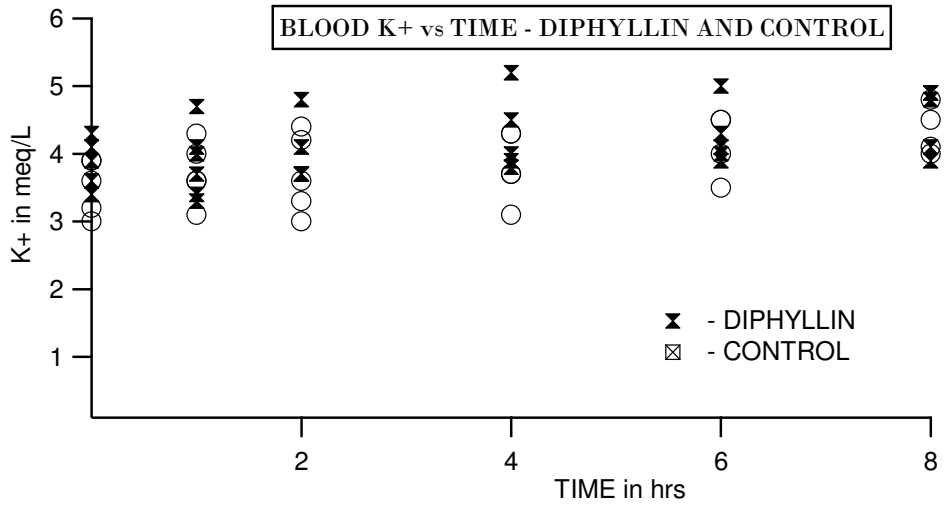


Figure 19: serum K+ over time in diphyllin (n=6) and control group (n=5)

✓ *Inference from blood K+:*

There is a trend towards increase in K+ levels with time observed in both diphyllin and control groups (figures 18A and B), especially more prominent in the later hours of the experiments. But the increase in levels is not too high. From the figure19, the potassium has maximum gone up to 5. The increase in K+ correlates with the acidosis developing in the initial hours with diphyllin. But whether there is definite hyperkalemia or not can be assessed by looking at the P values.

P values by Wilcoxon's sign rank test are

Between	Test 0 & 1 hr	Test 0 & 2hr	Test 0 & 4hr	Test 0&6 hr	Test 0&8 hr	Control 0 & 1 hr	Control 0 & 2 hr	Control 0 & 4 hr	Control 0 & 6 hr	Control 0 & 8 hr
P value	0.375	0.188	0.125	0.250	0.125	0.375	0.250	0.313	0.125	0.125

P values by Mann Whitney U test are

Between	Test & control- 0hour	Test & control- 1hour	Test & control- 2hours	Test & control- 4hours	Test & control- 6hours	Test & control- 8hours
P value	0.359	0.697	0.4	0.206	1	0.508

No values are significant within the groups or between the groups. Though there seems to be an increasing trend, it is not significantly higher than the initial values for potassium.

Urine pH:

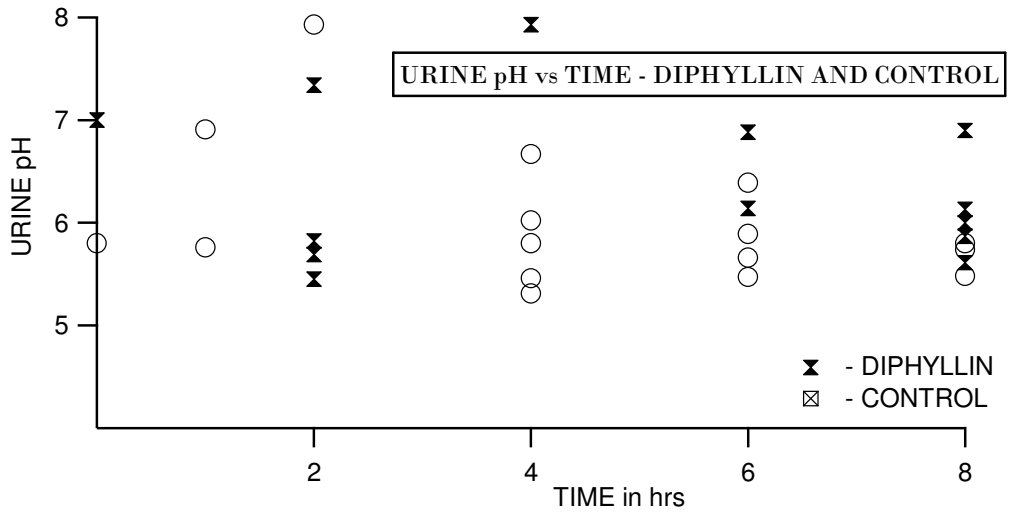


Figure 20: Urine pH over time in diphyllin (n=6) and control group (n=5)

- ✓ *Inference from urine pH:*

Urine pH data are less compared to others because in a single experiment, the animal may not give frequent urine samples. With the values available urine pH values for both diphyllin and control groups are plotted. The 0 hour value is 5.5 to 7. The urine pH almost remains in the normal range for the rest of the experiment period except one value in both diphyllin and control

group where it shows a tendency towards alkalinity (figure 20). But the 0 hour urine pH was itself around 7 in these experiments and especially they were male rats.

P values by Mann Whitney U test are

Between	Test & control-0hour	Test & control-1hour	Test & control-2hours	Test & control-4hours	Test & control-6hours	Test & control-8hours
P value	1	1	0.857	0.190	0.2	0.486

This infers that the urine pH is not significantly differing between the diphyllin and control groups.

Blood pH vs urine pH:

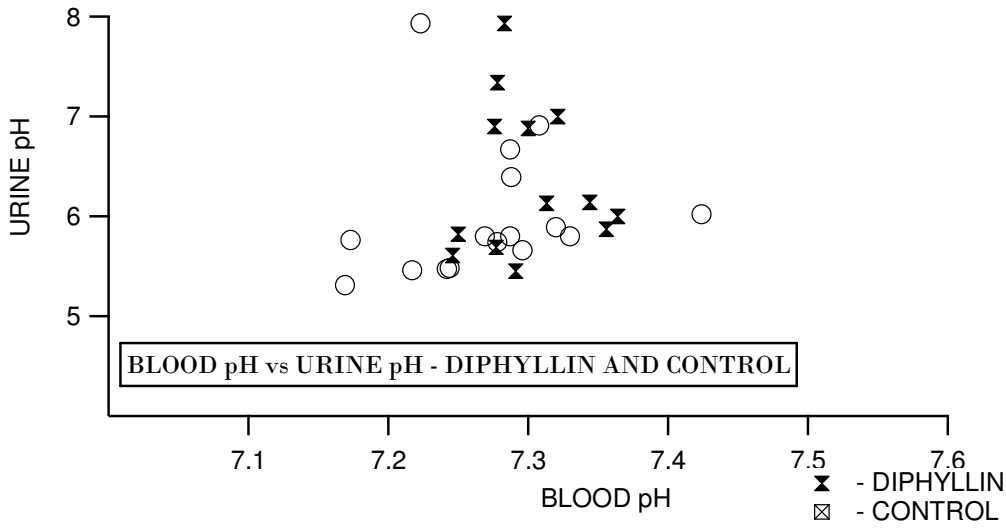


Figure 21: Blood pH vs. urine pH over time in diphyllin (n=6) and control group (n=5)

The urine pH is also acidic when the blood pH is acidic (figure 21). But when the blood pH is around 7.25 to 7.4, which we have considered as the normal pH, some values under test group and 1 value under control group is showing alkaline pH.

ANALYSIS OF CLINICAL DATA:

The arterial blood sample analysis in both the diphyllin and control groups suggests some features of hyperventilation seen over time, settling off later. The analysis of the respiration and ECG recordings can add on the information what we have obtained from the blood parameters.

Normal recordings-Raw data:

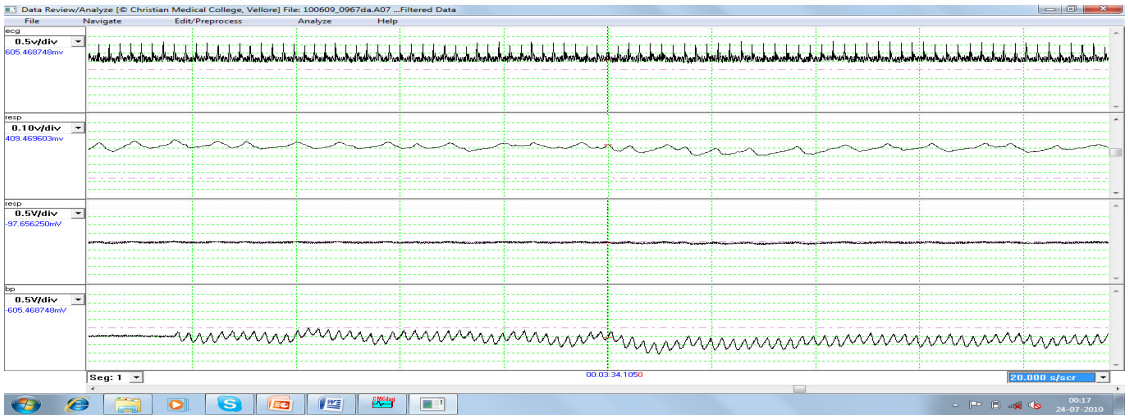


Figure 22: This picture shows the raw recording of ECG in the first panel, respiration in the second panel, and blood pressure tracings in the fourth panel

The raw recording of the clinical parameters are shown in figure 22. The first panel shows the recording of ECG, second panel is the recording of respiration and blood pressure recording is in the fourth panel. The third panel is just the duplication of any of the wave.

Recordings in the survived animals:

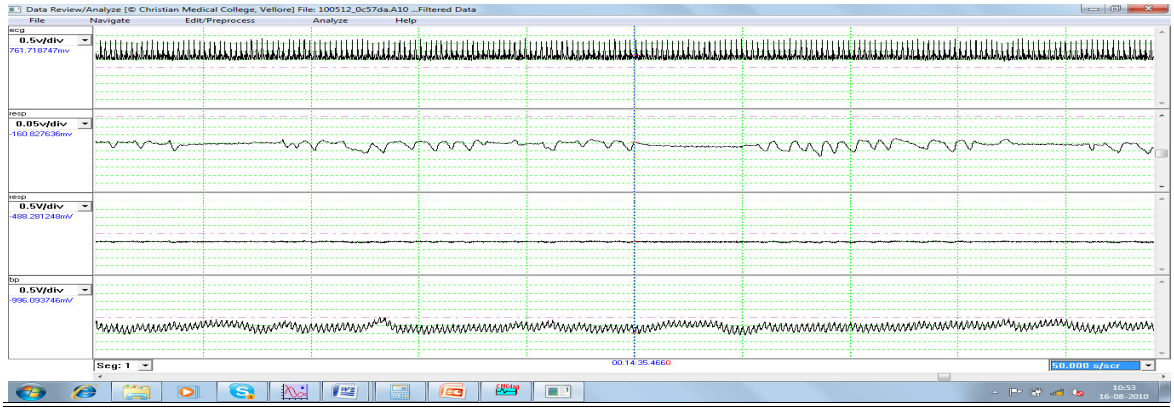


Figure 23: raw recording on one of the diphyllin rat showing features of hyperventilation followed by apnea in the second panel.

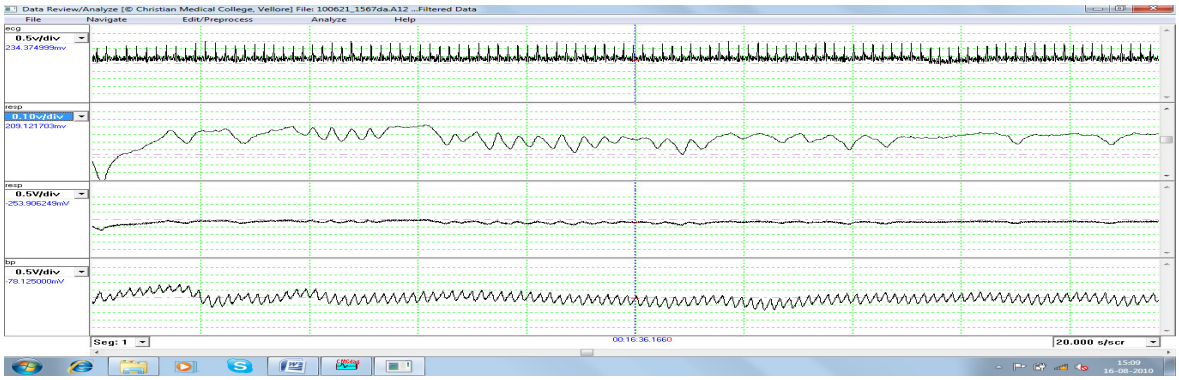


Figure 24: raw recording on one of the control rat showing features of hyperventilation followed by apnea in the second panel.

- ✓ *Inference from raw data:*

The heart rate is usually in the range of 250 to 350beats/min and Respiratory rate in the range of 30 to 50/min (figure 22). The recordings are obtained continuously to monitor any change in the ECG or respiratory tracings during the course of the experiments. If the animal has survived for 8 hours, there is usually not much change in the recordings. There is slight tachycardia or bradycardia and hyperventilation or apnea (figures 23, 24) over the duration of the experiments in both the groups according to the changes in blood pH.

- Heart rate plot graphs in Igor:

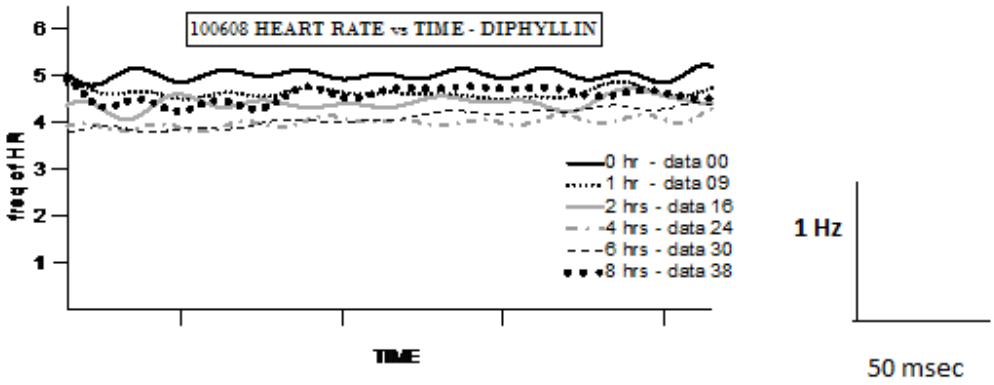


Figure 25: heart rate plot over time – diphyllin experiment. The plot is done at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours of the same experiment.

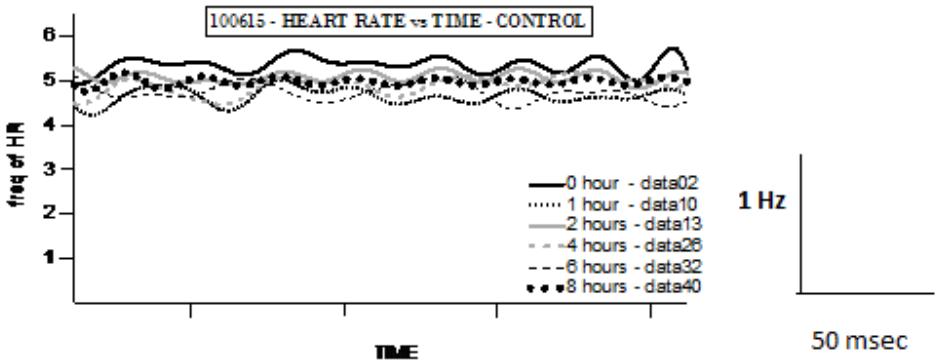


Figure 26: heart rate plot over time – control experiment. The plot is done at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours.

- ✓ *Inference from heart rate plots:*

The heart rate plot in one of the diphyllin rat shows that there is no much change in the heart rate of the animal over the survival period of 8 hours (figure 25). The heart rate is between 240 to

300beats/min. Similarly in the control group animal also there is no much change in the heart rate over time (figure 26). The heart rate is between 220 to 320beats/min.

- Respiratory tracings in Igor:

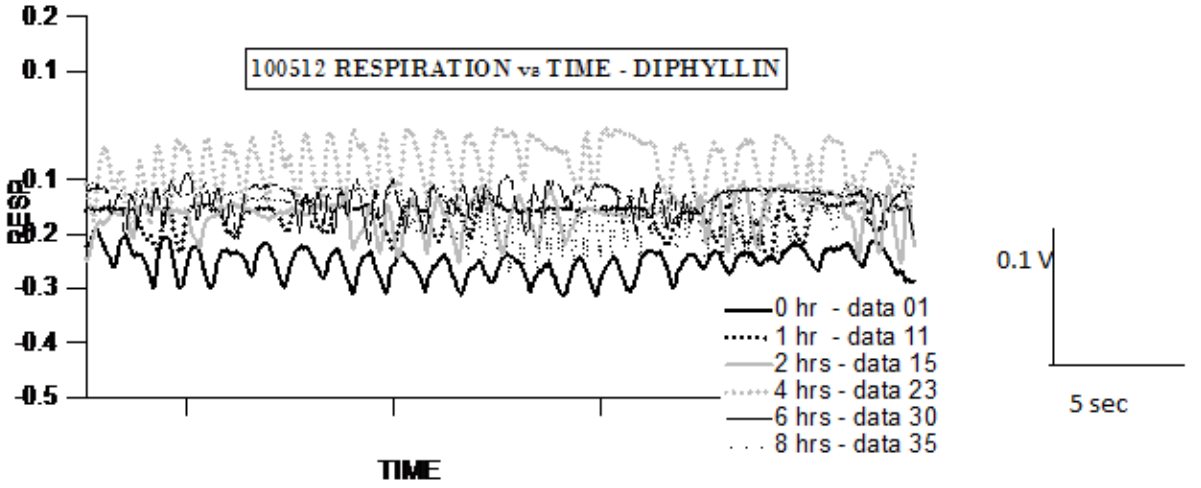


Figure 27: respiration tracings over time – diphyllin experiment from one rat at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours.

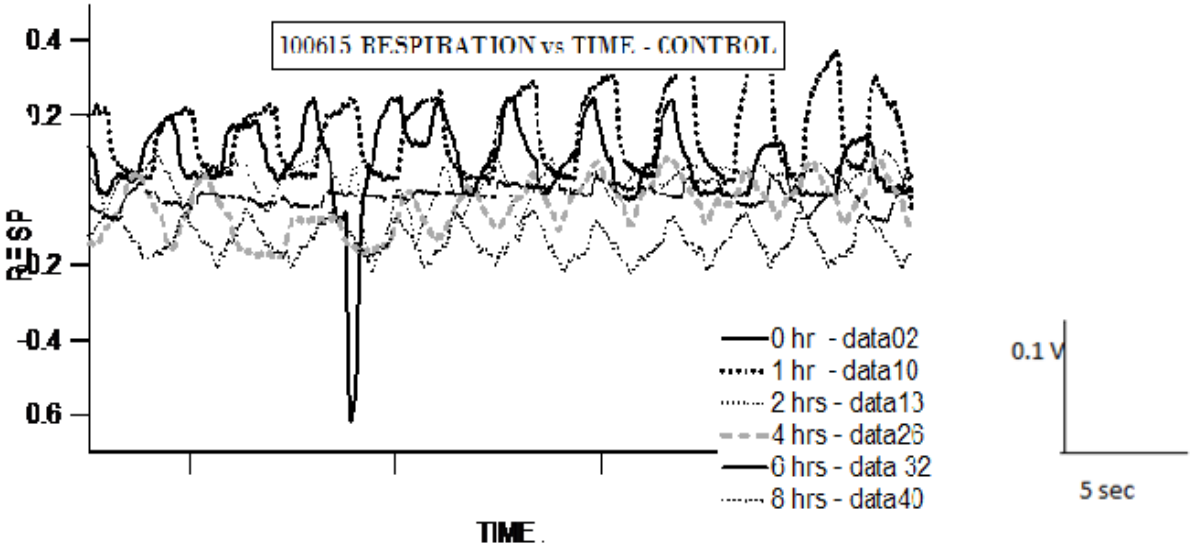


Figure 28: respiratory tracings – control experiment from one rat – at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours.

✓ *Inference from respiratory plots:*

The respiratory pattern in both the diphyllin (figure 27) and control (figure 28) is normal at 0 hour. The downward stroke is inspiration and the upward stoke is expiration. As the experiment is progressing, we can see the features of hyperventilation seen at 4 hours and 8 hours in the diphyllin group and at 1 hour and 6 hours in the control group. With time the hyperventilation settles off and the respiratory pattern resumes to normal.

➢ Quantitative data of heart rate and respiratory rate:

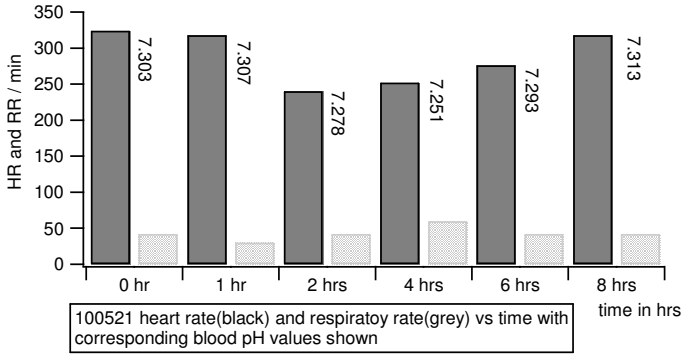


Figure 29: absolute values of heart rate and respiratory rate at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours in a diphyllin rat experiment. The blood pH values at the corresponding time interval are also shown.

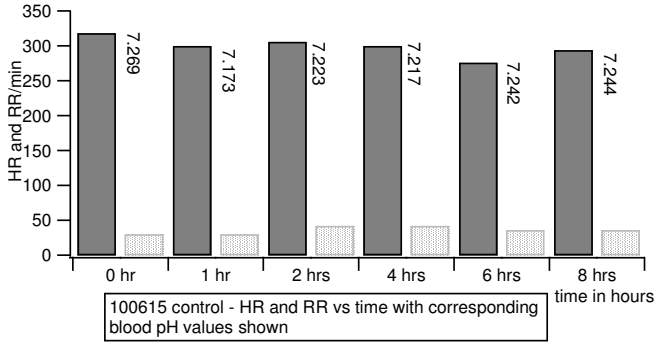


Figure 30: absolute values of heart rate and respiratory rate at 0 hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours in a control rat experiment. The blood pH values at the corresponding time interval are also shown.

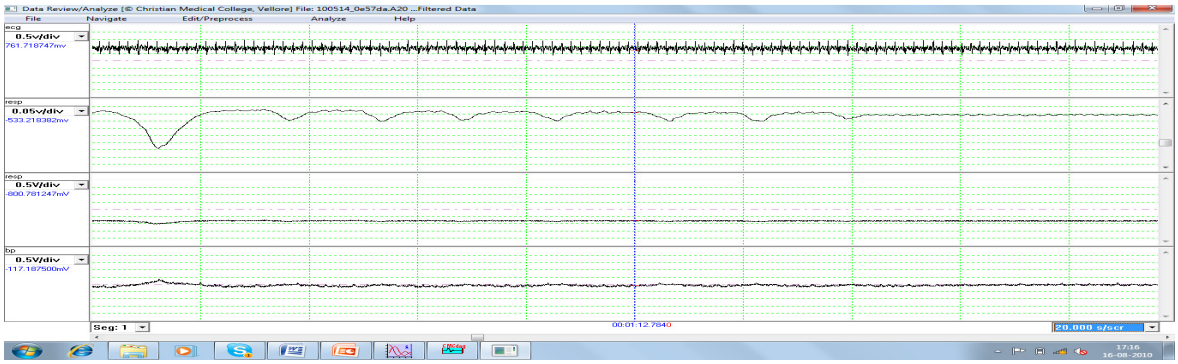
✓ *Inference from quantitative data:*

In the survived animals in both the diphyllin and control group the heart rate and respiratory rate shows fluctuations over time (figures 29 and 30). The changes in respiratory rate especially can be correlated with the changes in the blood pH values. We can see an increase in respiratory rate with decreasing blood pH and a decrease when the blood pH is returning to the original value.

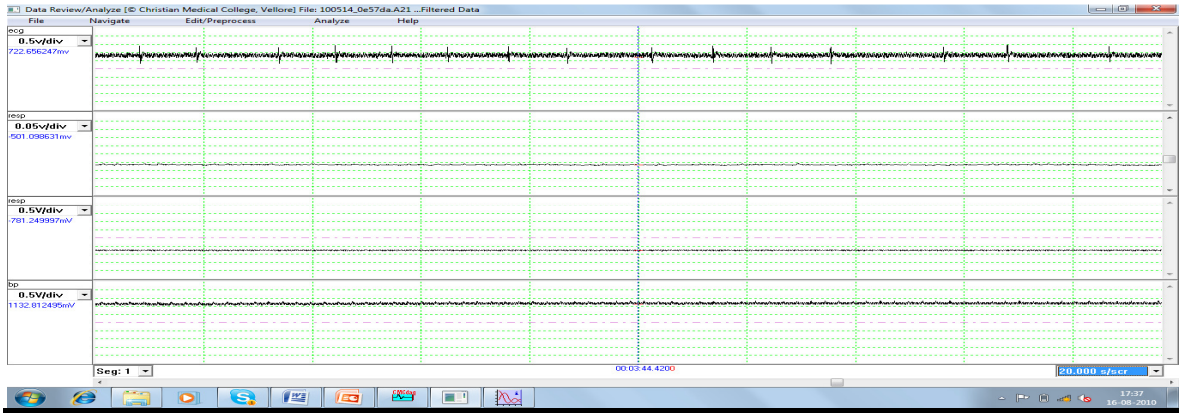
Clinical data from the dead animals:

Diphyllin:

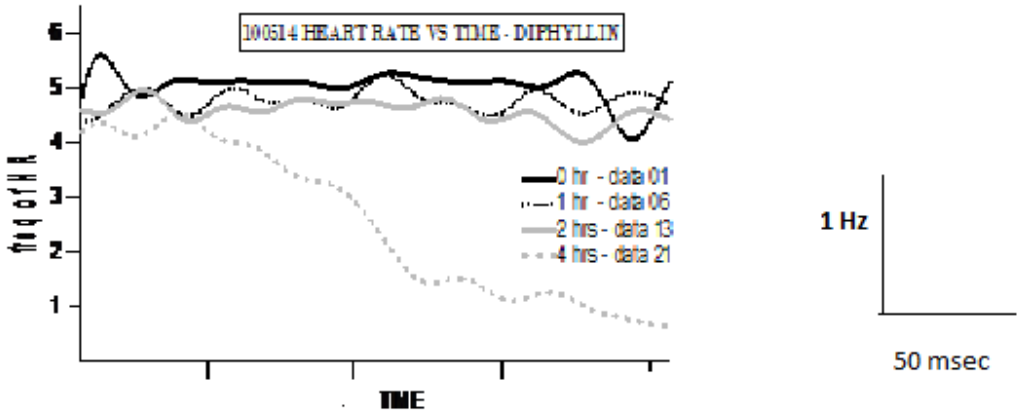
A



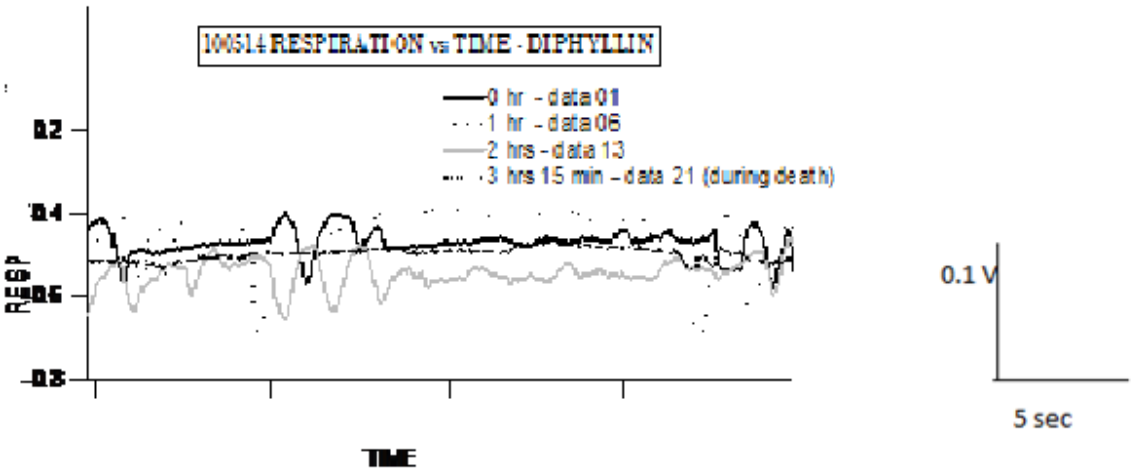
B



C


100514 HEART RATE VS TIME - DIPHYLLIN
6
5
4
3
2
1
0 hr - data 01
1 hr - data 06
2 hrs - data 13
4 hrs - data 21
TIME
1 Hz
50 msec

D


100514 RESPIRATION vs TIME - DIPHYLLIN
0 hr - data 01
1 hr - data 06
2 hrs - data 13
3 hrs 15 min - data 21 (during death)
0.2
0.4
0.6
RESP
TIME
0.1 V
5 sec

E

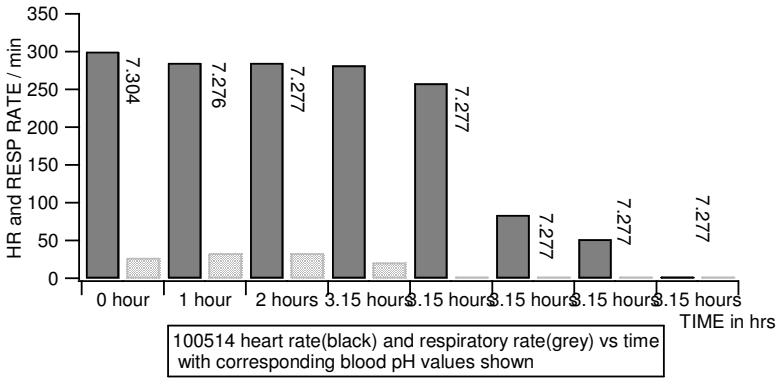
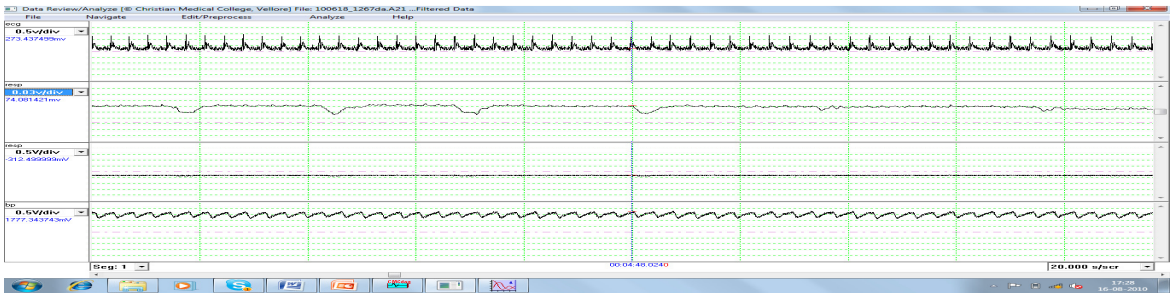


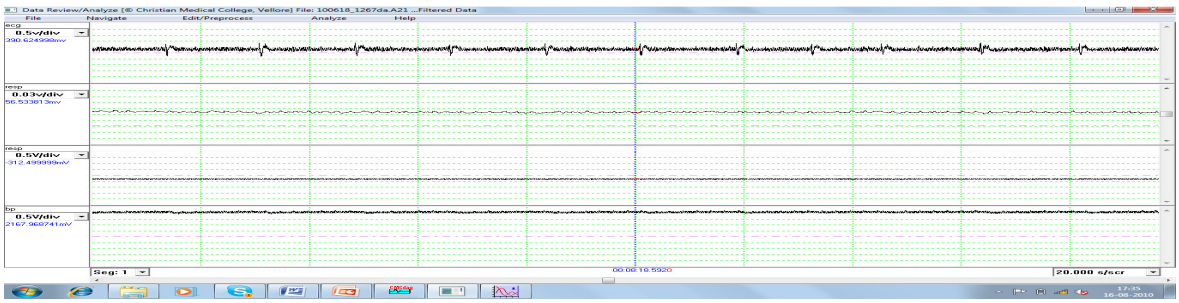
Figure 31: A and B represents the raw recording from the diphyllin administered rat which expired after 3 hours and 15 min. C and D are the heart rate plot and respiratory tracings and E represents the quantitative heart rate and respiratory plot from the same experiment

Control:

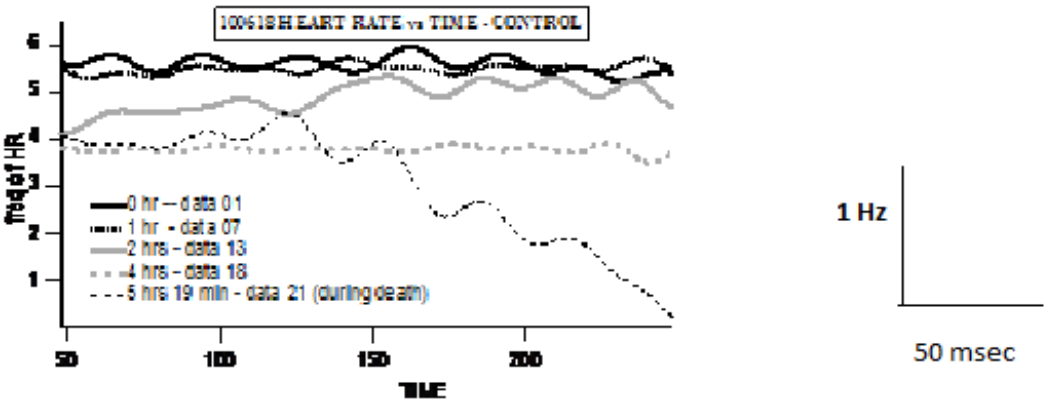
A



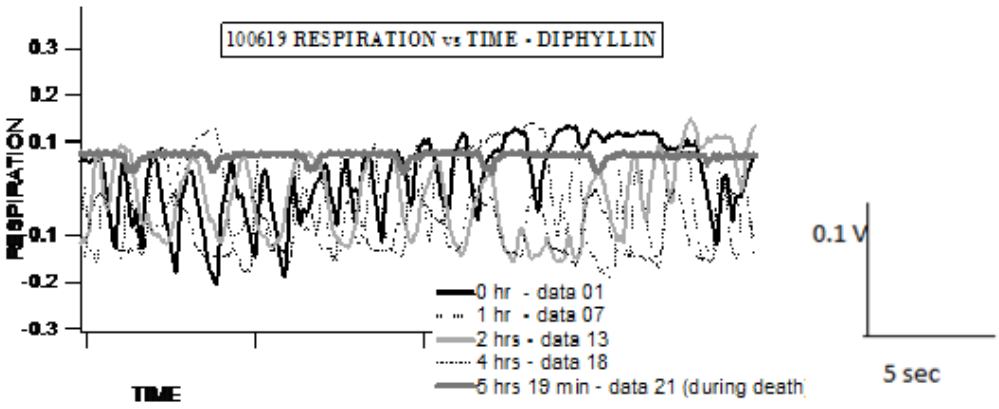
B



C


100619 HEART RATE vs TIME - CONTROL
freq of HR
TIME
0 hr – data 01
1 hr - data 07
2 hrs - data 13
4 hrs - data 18
5 hrs 19 min - data 21 (during death)
1 Hz
50 msec

D


100619 RESPIRATION vs TIME - DIPHYLLIN
RESPIRATION
TIME
0 hr - data 01
1 hr - data 07
2 hrs - data 13
4 hrs - data 18
5 hrs 19 min - data 21 (during death
0.1 V
5 sec

E

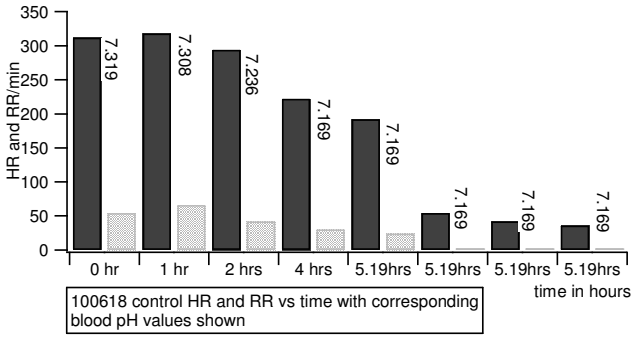


Figure 32: A and B represents the raw recording from the water administered rat which expired after 3 hours and 15 min. C and D are the heart rate plot and respiratory tracings and E represents the quantitative heart rate and respiratory plot from the same experiment

- ✓ *Inference:*

The tracings obtained from the dead rats in both the diphyllin and control group show similar pattern of death. There is respiratory arrest followed by bradycardia in both the groups. The changes are sudden as is evident from the heart rate plots and the respiratory tracings over time. The quantitative data implies that even after the respiration has stopped the bradycardia is slow to happen and then the heart beat ceases.

DISCUSSION

DISCUSSION

Cleistanthus collinus, one of the commonest consumed plant for suicidal purposes in rural southern India. The boiled extract of the leaves is consumed. "All parts of this plant are potentially toxic. The primary toxins in the leaf have been identified as aryl-naphthalene lignin lactones: Collinusin, the glycosides Cleistanthin A and B, and their genin Diphyllin" (*Prabhakaran C et al, 1996*). The mortality is 28% (*Thomas et al, 1987*). The mortality is usually between 1 to 3 days. There are arrays of symptoms with which the patients present with. Cleistanthin A and B, the toxins of *Cleistanthus collinus,* are diphyllin glycosides which produce cardiac arrhythmias, urinary potassium wasting, hypoxia, metabolic acidosis and hypotension. Also there is a report of ARDS, **distal renal tubular acidosis** and distributive shock secondary to inappropriate vasodilatation in a case following ingestion of its leaves (*Benjamin et al. 2006*). It is type 1 dRTA which consists of the triad of metabolic acidosis, hypokalemia and alkaline urine (*Delinda Maneksh et al, 2010*).

There is 100% mortality seen in rat models which are given intraperitoneal injection of aqueous extract of *Cleistanthus collinus.* The mortality is primarily due to respiratory arrest. (*Delinda Maneksh et al, 2010*). "Cause of death appears to be mainly due to its cardiac and respiratory effects. Metabolic disturbances especially hypokalaemia was a prominent feature" (*Subrahmanyam et al, 2003).*

There are about 17 compounds that are analyzed in the plant *Cleistanthus collinus (Parasuraman et al, 2009)*. Cleistanthin A and B are known to be toxic. The toxicity profile of diphyllin is unknown.

In our department of Physiology, experiments have been done with whole aqueous extract and powder. The clinical triad viz...

1. METABOLIC ACIDOSIS
2. HYPOKALEMIA
3. ALKALINE URINE

has been demonstrated in rat models. The whole aqueous extract of Oduvanthalai produces 100% mortality. The mortality is primarily due to sudden respiratory arrest. Type 1 dRTA, though observed, does not seem to be the cause of death. Experiments with Cleistanthin A and Cleistanthin B have also been done in our department. The mortality with Cleistanthin A is 90% and the mortality with Cleistanthin B is around 80%. But the picture of dRTA is not observed with Cleistanthin A and B. Thus both the fractions are proven to be toxic in rats. The other common fraction of *Cleistanthus collinus* is diphyllin. The experiments with diphyllin on rats are done in this study to find out its toxicity profile and to find out if this fraction is the cause of dRTA.

6 experiments were done under test groups with intraperitoneal injection of freeze dried diphyllin enriched fraction and 5 experiments under control group with intraperitoneal injection of distilled water. The dosage of diphyllin was 7mg/100g body weight of the rat.

Mortality with diphyllin is 16.66% (n=6) and in the control group, it is 20% (n=5). One rat in both the groups has died. The difference in percentages is due to the difference in the n value. Thus there is no difference in the mortality profile in both the test and control groups. Diphyllin is non-toxic to the rats.

Acidosis develops both in tests and controls. Significant acidosis develops in the 2^{nd} hour in the test group. The acidosis is not severe as well as not progressing. It seems that there could be compensatory mechanisms which are trying to restore the blood pH to normal. Controls

also show decreasing pH in the initial hours but are not differing significantly. But the whole aqueous extract of oduvan produces severe acidosis.

The significant decrease in the serum bicarbonate in the 2nd hour in the test group also support the acidosis developing at the same time, as is evident from the blood pH values. There is also a significant negative base excess seen in the diphyllin group in the initial hours. Thus there is development of significant acidosis within 2 hours after administration of diphyllin to the rats. The acidosis could be metabolic or respiratory. The blood pH, however tends to increase after 2 hours, and restores around the normal value. This implies that there are compensatory mechanisms happening with time.

The analysis of the blood gases clarifies this issue. There is no significant difference in the arterial PO2 and PCO2 values in both the groups. But the trend of the PO2 seems to be on the increasing side and PCO2 values on the decreasing slope, especially in the later hours. Also, the raw recording of respiration shows the features of hyperventilation in both the groups. This gives a clue that, there is a respiratory compensation happening in the animal to blow out the acids in the form of carbon dioxide. This is the usual response to metabolic acidosis. Thus, the acidosis, the trend towards increasing PO2 and decreasing PCO2 and the features of hyperventilation suggest that there is development of metabolic acidosis with respiratory compensation.

Hyperkalemia is seen in both the test and control groups. There is no significant difference between them. Hyperkalemia is the usual response to acidosis. But, the data definitely says that there is no hypokalemia which is the usual presentation with *Cleistanthus collinus* poisoning.

Urine pH remains acidic, except two values in the test group and one value in the control group. But in these experiments, the zero hour urine pH values itself is around 7. Also the

difference in the urine output in various experiments has limited the availability of data for urine pH. With the values obtained, urine seems to remain acidic over the 8 hour period in both the test and control groups. For the same blood pH some of the urine pH values are higher than in the control group. The alkaline urine cannot be excluded because of the outliers in the test group. But even in the control group there is an outlier. More experiments may be done in future to sort this issue.

One of the rats in both the groups has died. In both the groups, there is sudden cessation of respiration followed by bradycardia. Even with the whole aqueous extract of oduvanthalai, the mortality in control group is 17% (*Delinda Maneksh et al. 2010*). The death occurring could be due to many confounding factors occurring during the course of the experiment. They are blood loss due to counter puncture during cannulation, frequent anaesthesia top ups, dehydration, hypoglycaemia, accidental introduction of air bubble into the artery during sampling, internal bleeding due to heparinisation or idiopathic.

The metabolic acidosis developing in the diphyllin group may not be entirely due its direct effect because controls also develop acidosis. The reasons that could be attributed to the development of acidosis in both the groups are anesthesia, tissue injury due to dissection, blood loss during dissection and cannulation, dehydration, starvation, blood loss due to frequent blood sampling.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Mortality is similar in both diphyllin and water control group. One of the triad which is metabolic acidosis does develop in diphyllin group. But it is seen in controls also. There is significant acidosis in diphyllin group in the initial hours. And the responses to acidosis like hyperventilation, hyperkalemia and acidic urine are similar in both the test and control groups. But because there is significant acidosis in the initial hours with diphyllin, Diphyllin may contribute a part to the development of severe metabolic acidosis which develops with whole aqueous extract.

Diphyllin produces metabolic acidosis, less severe than the whole aqueous extract. Diphyllin do not show hypokalemia or 100% alkaline urine as seen in the whole aqueous extract .Mortality is only 16.66% as compared to 100% mortality in whole aqueous extract of *Cleistanthus collinus*.

Conclusive to say that diphyllin may not be the sole cause of distal renal tubular acidosis which is developing in poisoning with *Cleistanthus collinus* and DIPHYLLIN IS NON-TOXIC.

FUTURE PLANS

- Double the dosage of diphyllin can be administered to the rats and checked, if it is toxic at higher doses.
- To do more experiments to sort out the issue of alkaline urine with diphyllin.
- To standardize some procedures for collection of urine samples at the specific hour of requirement.
- To try out various treatment modalities for oduvanthalai poisoning.
- To develop a specific antidote for *Cleistanthus collinus.*

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ANNEXURES

PHOTOGRAPH 1: Oduvanthalai plant (*Cleistanthus collinus*)

PHOTOGRAPH 2: Femoral artery cannulation with the ECG amplifier and Respiration transducer.