INVESTIGATION ON NON-FLUORESCENT FRACTION (NFF) OF AQUEOUS EXTRACT OF *CLEISTANTHUS COLLINUS* LEAVES AS AN ANTIDOTE TO *CLEISTANTHUS* POISONING

A Dissertation submitted in partial fulfilment 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

Department of Physiology

Christian Medical College, Vellore,

Tamilnadu

April 2015

CHRISTIAN MEDICAL COLLEGE
Post Office : THORAPADI
VELLORE – 632 002. S.India

Telephone : 2222102: 228+Extn.
Telegrams : MEDICOL
Telefax : India : 0416-2262788, 2262268
Telefax : Abroad: 0091-0416- 2262788,
2262268

Ref:

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CERTIFICATE

This is to certify that the thesis entitled "**Investigation on non-fluorescent fraction (NFF) of aqueous extract of *Cleistanthus collinus* leaves as an antidote to *Cleistanthus* poisoning** " is a bonafide, original work carried out by Dr. Neetu Prince, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamilnadu Dr. M.G.R. Medical University, Chennai to be held in April- 2015.

Dr. Sathya Subramani
Professor and Head
Department of Physiology,
Christian Medical College,
Vellore – 632 002.

Professor & Head
Department of Physiology
Christian Medical College,
Vellore - 632 002, Tamilnadu, India.

DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Investigation on non-fluorescent fraction (NFF) of aqueous extract of *Cleistanthus collinus* leaves as an antidote to *Cleistanthus* poisoning** " 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.

Dr. Neetu Prince
Department of Physiology,
Christian Medical College,
Vellore – 632002.

ACKNOWLEDGEMENTS

Thank you God for blessing me much more than I deserve.

I thank my dear parents and sisters, for what I am now.

I thank my husband, for his unconditional support. Thank you so much.

I like to thank my mam, Dr. Sathya Subramani for introducing me to the field of research and for guiding me through my project and for all I learnt from her. Your dedication to work always made me admire and look up to you. Thank you mam for everything.

I like to thank Soosai for his support and helping me in all ways to complete my project.Without your help this project would not have been possible.

I like to thank Dr. Renu, Dr. Rashmi, Dr. Solomon, Dr. Snekhalatha and Dr. Silviya for their guidance and moral support.

I like to thank Dr. Anand, Dr.Vinay and Dr. Praghalathan for being supportive and giving their valuable inputs,which helped me to improve myself.

I like to thank Geetha, Selvam sir, Nataraj Boss for their constant support and love.

I thank Samuel, Ramesh, Henry, Prasad and Nalina for their help at all times.

I would like to thank Dr.Arul Das for his spiritual guidance.

Special thanks to all my dear friends, Aneesh, Kamalakannan, Lathadi, Elsydi, Swetha, Ramya, Kavitha, Abirami, Benjamin, Anandith, Prasanth, Sajjal, Renu and Teena for being with me.

Three cheers to my best friends, Pijushda, Jesi and Upasana for making my postgraduate life memorable forever.

I thank Fluid Research Grant Committee, Christian Medical College, for funding this study.

I would like to thank Clinical Epidemiology Unit,for organizing Research Methodology workshops, research and PG office staff for all their secretarial help.

Thanks to my Physiology department for making my dream come true and being a second family away from my home.

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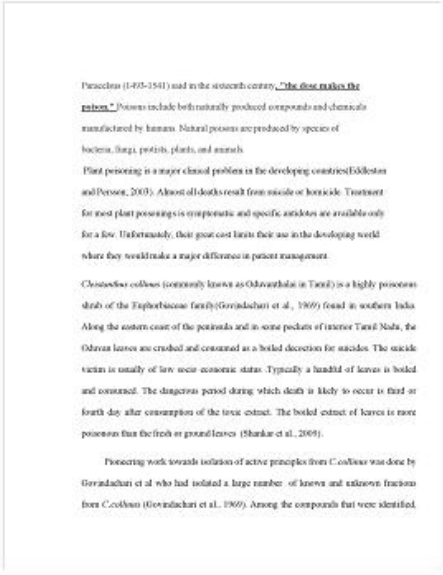

Paracelsus (1493-1541) said in the sixteenth century, "the dose makes the poison." Poisons include both naturally produced compounds and chemicals manufactured by humans. Natural poisons are produced by species of bacteria, fungi, protists, plants, and animals.

Plant poisoning is a major clinical problem in the developing countries(Eddleston and Persson, 2003). Almost all deaths result from suicide or homicide. Treatment for most plant poisonings is symptomatic and specific antidotes are available only for a few. Unfortunately, their great cost limits their use in the developing world where they would make a major difference in patient management.

Cleistanthus collinus (commonly known as Oduvanthalai in Tamil) is a highly poisonous shrub of the Euphorbiaceae family(Govindachari et al., 1969) found in southern India. Along the eastern coast of the peninsula and in some pockets of interior Tamil Nadu, the Oduvan leaves are crushed and consumed as a boiled decoction for suicides. The suicide victim is usually of low socio economic status. Typically a handful of leaves is boiled and consumed. The dangerous period during which death is likely to occur is third or fourth day after consumption of the toxic extract. The boiled extract of leaves is more poisonous than the fresh or ground leaves (Shankar et al., 2004).

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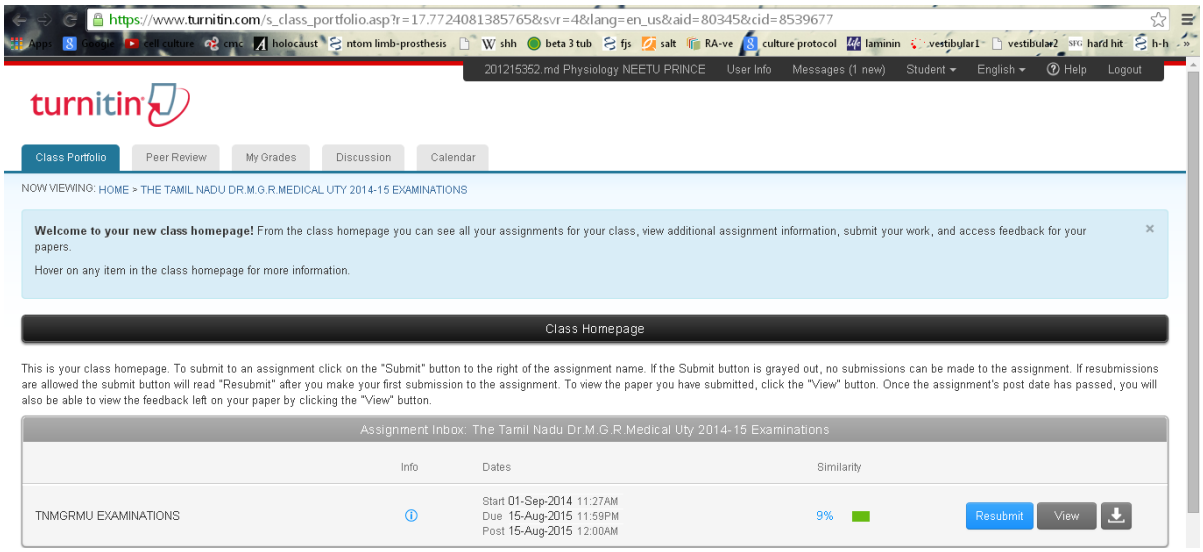
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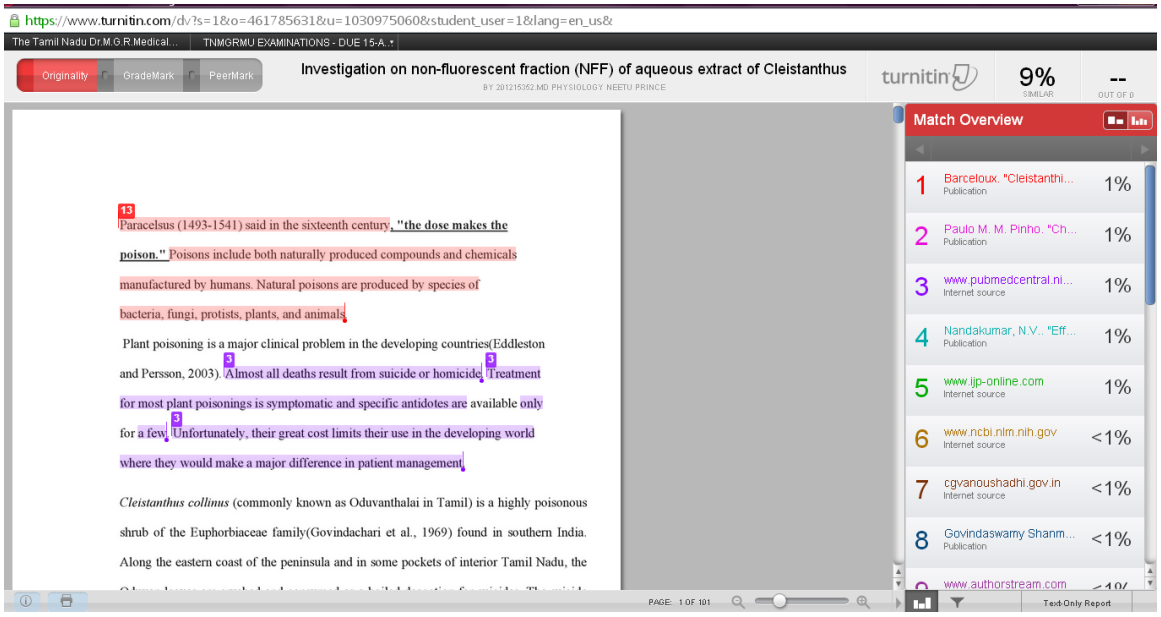
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ABSTRACT

Cleistanthus collinus poisoning is common in rural south India. Water extract of *Cleistanthus collinus* leaves is a common suicidal poison. Victims consume either a boiled decoction (FLB, fresh leaves boiled) or fresh leaves blended and filtered. Mortality is about 28% and is stated to be higher with boiled decoction.

Aim

The aim of the present study was to develop an antidote for *Cleistanthus collinus* poisoning

Objectives

(1) To get the minimum lethal dose (MLD100) of FLB, FLG, purified Cleistanthin A and C .

(2) To see if a particular fraction of *Cleistanthus collinus* extract (non fluorescent fraction of cold water extract, NFF) can prevent death in rats when administered after administering the lethal dose of boiled water extract / cleistanthin C.

Methods

Boiled decoction and fresh leaf ground extract was prepared and minimal lethal dose for the same was estimated. Toxic principles, Cleistanthin A and C was isolated to its single peak purity for experiments. Non fluorescent fraction (NFF)

to try as an antidote was prepared from fresh leaf ice cold extract of *C.collinus* leaves. NFF was administered to the rats poisoned with boiled extract or cleistanthin C. Toxins were administered via oral gavage.

Results

Minimum lethal dose (MLD100) of FLB, FLG, purified Cleistanthin A and C in rats was determined. There is not much difference in MLD100 for FLB (75mg/100gm) and FLG (50mg/100gm) in rats. The concentration of Cleistanthin C in the MLD100 of FLB is sufficient to account for lethality of FLB. However, the concentrations of both Cleistanthin C and Cleistanthin A are insufficient to account for lethality in the case of FLG. Therefore we conclude that, the toxicity of fresh leaf ground is neither due to cleistanthin A or C , but due to a yet unidentified compound. Experiments with NFF as an antidote in *C.collinus* poisoning are yet to be standardized.

Keywords:

Cleistanthus collinus , Cleistanthin A, Cleistanthin C, Plant poisoning, TLC, HPLC

INTRODUCTION

Paracelsus (1493-1541) said in the sixteenth century**, "the dose makes the poison."** Poisons include both naturally produced compounds and chemicals manufactured by humans. Natural poisons are produced by species of bacteria, fungi, protists, plants, and animals.

Plant poisoning is a major clinical problem in the developing countries(Eddleston and Persson, 2003). Almost all deaths result from suicide or homicide. Treatment for most plant poisonings is symptomatic and specific antidotes are available only for a few. Unfortunately, their great cost limits their use in the developing world where they would make a major difference in patient management.

Cleistanthus collinus (commonly known as Oduvanthalai in Tamil) is a highly poisonous shrub of the Euphorbiaceae family(Govindachari et al., 1969) found in southern India. Along the eastern coast of the peninsula and in some pockets of interior Tamil Nadu, the Oduvan leaves are crushed and consumed as a boiled decoction for suicides. The suicide victim is usually of low socio economic status .Typically a handful of leaves is boiled and consumed. The dangerous period during which death is likely to occur is third or fourth day after consumption of the toxic extract. The boiled extract of leaves is more poisonous than the fresh or ground leaves (Shankar et al., 2009) .

Pioneering work towards isolation of active principles from *C.collinus* was done by Govindachari et al who had isolated a large number of known and unknown fractions from *C.collinus* (Govindachari et al., 1969). Among the compounds that were identified,

the important ones were Diphyllin, collinusin and cleistanthin A which are lignan lactones. Cleistanthin B was identified by Subba Rao in 1960 (N.V.Subba rao, 1960). Thin layer chromatograms reveal that all the above four compounds are intensely fluorescent under UV light, each fluorescing with a characteristic colour. In fact, almost 21 different compounds have been identified (Pinho and Kijjoa, 2007). K.S.Annapoorani has shown that of the four tested fluorescent compounds, Diphyllin and Collinusin were nontoxic while Cleistanthin A and B were toxic in rats (K.S.Annapoorani,Ph.D Thesis.).

Mortality rate in patients is as high as 28% and death usually occurs 3-7 days after ingestion of the poison (Thomas et al., 1991). Molecular mechanism of oduvan poisoning is still an unsolved problem. The patients develop life threatening complications such as hypokalemia(56.5%),hypotension(17.5%), cardiac arrhythmias, neuromuscular weakness, reapiratory distress(15.8%) and renal failure(25.3%) following a transient quiescent period of upto four days (Eswarappa et al., 2003).

Retrospective chart review of 97 patients admitted with *C.collinus* poisoning to Medicine department at Christian Medical College, Vellore during the period of 1997-2004 reveals that mortality was 26% even with treatment(Zachariah A et al.) At admission, laboratory abnormalities were leucocytosis (64%), hypokalemia (56%), acidosis(86%) and renal failure (25%). Complications occurring during hospitalization were hypotension (17%), bradycardia (11%), ARDS (4%) and new onset acute renal failure (10%). Patients were treated with gastric lavage (68%), potassium supplementation (87%), temporary pacing

(93%), continous cardiac monitoring (83%), mechanical ventilation (24%) and dialysis (2%). Factors associated with mortality were hypokalemia, renal failure, shock, metabolic acidosis and ARDS. None of the above interventions were found to improve survival. Case reports are there to show patients developed features of distal ranal tubular acidosis.(Benjamin et al., 2006; Eswarappa et al., 2003)

Animal studies have been done to identify the mechanism of toxicity of *C.collinus* from various angles: Proposed mechanisms of actions are, distal renal tubular acidosis, electrolyte imbalance, neuromuscular blockade resulting in respiratory paralysis (Nandakumar et al., 1989), inhibition of LDH isoenzymes (Kanthasamy A et al,1986.), glutathione and ATP depletion (Sarathchandra G et al,1997), K^+ channel blocker (Jose et al., 2004). Cytotoxic nature of Cleistanthin A and B was demonstrated against several cell lines. Irrespective of all these extensive work, the exact cause of death is still a mystery.

No specific antidote discovered till date. A study on the use of multiple doses of charcoal on patients within 24 hrs of presentation demonstrated a mortality benefit, but further studies are required (Raja G et al,2007.) . N-acetylcysteine, L-cysteine, melatonin and thiol compounds have all been suggested as possible antidotes in *C.collinus* poisoning (Jayanthi et al., 2009,Benjamin et al., 2006) . In one case, use of N-acetylcysteine as treatment has been reported, but beneficial effect is still not clear. Neostigmine was also tried in a patient who presented with a myasthenic crisis-like syndrome (Damodaran et al., 2008).

Research in our department is focused on the mechanism of action of oduvanthalai poisoning and to find a specific antidote for the same. In our lab, animal experiments with whole aqueous extract of oduvanthalai leaf showed type I DRTA (hypokalemia, alkaline urine and metabolic acidosis) (Maneksh et al., 2010) as in human cases, but type II respiratory failure too, which is not seen in humans. The molecular mechanism of DRTA was determined to be inhibition of the proton pumps in the brush border membrane of distal renal tubule. However, this may not be the cause of death in oduvan poisoning, because Cleistanthin A and Cleistanthin C, when administered in pure form, resulted in death, without evidences of DRTA or type 2 respiratory failure. Experiments with Diphyllin showed that it is non-toxic and neither did it produce DRTA . Patch clamp studies showed that the aqueous extracts blocked the voltage gated channels, but not seen with the individual compounds.

Till date, our lab has performed whole animal experiments with intraperitoneal administration of the toxins. That may be the reason why the clinical picture does not match the human condition exactly. However, the experiments have helped us determine the electrolyte and ABG profiles during the course of the toxicity.

Some studies have reported that Cleistanthin A is safe upto 800mg/kg orally,(Parasuraman and Raveendran, 2012) but, even 20mg/kg intraperitoneally resulted in 100% mortality in the studies done in our lab. However, it is also

reported that Cleistanthin A and B are not present in the aqueous extracts of *C.collinus* leaves (Subramani, 2014.) Discrepancies spurred us on to design and carry out experiments to determine oral toxic dose of clinically relevant extract and toxic constituent of *C.collinus* .

Therefore, experiments were designed to answer these questions to our best abilities.

Minimal lethal dose which cause 100 % mortality has been estimated for fresh leaf boiled extract , fresh leaf ground extract, Cleistanthin A and Cleistanthin C of *Cleistanthus collinus* .

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The World Health Organization (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs.Also, modern pharmacopoeia still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants.

There are many plants containing poisonous parts that pose a serious risk of illness, injury, or death to humans or animals. Many of these poisonous compounds also have important medicinal benefits.

What is a poison?

To be precise, a poison is a foreign chemical (xenobiotic) that is capable of producing a harmful effect on a biologic system.

What is a toxin?

It originally referred to a poison of animal or plant origin

Toxicant is the currently preferred scientific term for all poisons.

What is a toxidrome?

It is the association of several clinically recognizable features, signs, symptoms, phenomena or characteristics which often occur together, so that the presence of one feature alerts the physician to the presence of the others.

Recognition of poisoning

May be difficult because of non-specific symptoms.

High index of suspicion is required, especially in acute poisoning.

- History may be unreliable
- Look for corroborative history
- Course that a poison runs (toxidromes)

Toxicology screening - helpful only in a few cases because of very diverse and varied presentation.

Goals of treatment

- Reduce absorption of the toxin (xenobiotic)
- Enhance elimination
- Neutralize toxin

Virtually all plants contain poisons as a defense mechanism.In most cases, their toxins are removed through cooking or digestion.Many of these natural toxins, acts to disrupt the biological machinery at the level of signal transduction mechanisms or the essential metabolic pathway. Some poisons are powerful enough to a grinding halt with just a trifling amount, while others are slower in their effect.

Poisons found in plants are of four primary kinds, namely, alkaloids, glycosides, resins and essential oils. It is a major clinical dilemma as antidotes are not available for most of the plant poisons. Therapy for some other plant poisonings might also benefit from the development of antitoxins. Antitoxin antibodies are available for only two plant families and for just one of these are antitoxin antibodies in current clinical practice. However, until issues of cost and supply are worked out, plant anti-toxins are going to remain a dream in many of the areas where they are now urgently required.

This study is concerned with a poisonous plant called *Cleistanthus collinus* which is native to southern India.It is commonly used as a suicidal poison in this part of world(Chopra, 1965). Availability and easy accessibility are probably the reason for the consumption of this poison (Chrispal, 2012a). Even though the toxic constituents of the plant are capable of causing 28% mortality, their exact mechanism of action still evades science. Treatment is only symptomatic. No

specific antidote is available against this toxin(Subrahmanyam et al., 2003). Let alone the quest of designing an antidote.

Oduvanthazhai is a highly poisonous shrub of the Euphorbiaceae family (Govindachari et al., 1969). Euphorbiaceae is a highly diversified family with substantial difference in morphology, chemical and ecological characteristics. This family is widely distributed throughout with approximately 8000 species of plants.

The genus cleistanthus comprises about more than hundred species ranging from shrubs to trees. *Cleistanthus collinus* is a plant poison also called "Oduvan" in Tamil, "Vadise" in Telugu, Nilappala or *Oduku* in Malayalam, Garari in Hindi. The name derives from Euphorbus, the physician to King Juba of Mauritania (50 BC-23 AD) who discovered the therapeutic properties of Euphorbiaceae plants. Euphorbiaceae family includes large desert succulent to small herbaceous plants and trees in forest. The presence of latex (milky sap) is characteristic of plants from spurge family. Some species of the spurge family contain potent systemic toxins, including *Ricinus communis* L. (ricin),*Cleistanthus collinus* (Roxb) Hook. F. The extracts of these two species are ancient homicidal agents in India.

Common Name : Oduvan, Garari, Karrada, Pasu, Karlajuri

Scientific Name : *Cleistanthus collinus* (Roxb.) Hook .f.

Botanical Family : Euphorbiaceae (spurge)

Distribution and Ecology : *Cleistanthus collinus* is a native plant of India, Malyasia, Africa.

Physical description : Cleistanthus collinus is a small deciduous tree or shrub with spreading rigid twiggy branches. Leathery and glaborous leaves about 3.5 to 10 cm long and arranged alternately. They are orbicular, obovate or elliptic. Five to six pairs of main lateral leaves are present. Fruits are generally dark brown and woody. Seeds are globose. Almost all parts of the plant contain toxic compounds.

Figure 1 : *Cleistanthus collinus* plant

The hard wood of the plant is durable and used in making agricultural implements. Posts, poles, fence, etc. are made from this wood as it is not attacked by white ants. Leaves are used as manure.Tannins isolated from the bark of the plant are used in leather industry.

The bark of the plant is considered to have useful application in cutaneous disease.Water in which leaves are steeped are used as a treatment option for headache . *Cleistanthus collinus* has been investigated for its antibacterial, antifungal, antiprotozoal, antiviral, anticancer, diuretic and hypoglycemic effects(Suman.T, 2013), (Pradheepkumar and Shanmugam, 1999), (Vishal L. Bagde, 2013).

The leaves of this plant have been known to be used for suicidal and homicidal purposes . Almost all parts of the plant are reported to be highly toxic. FRoots and leaves are used as cattle poison, bark and seeds of the plant are used as fish poison. The leaves are mainly exploited for suicidal, homicidal, cattle poisoning and for doing criminal abortion (Devaprabhu S, 2007).

This poison is consumed in two ways. Either the fresh leaves are crushed and the filtered juice is consumed or a boiled decoction (aqueous extract) or consumes as a paste made with these leaves or swallows the crushed leaves (Thomas et al., 1987). The boiled extract of the leaves is more poisonous than the fresh or ground leaves (Thomas et al., 1987). This might be due to release of toxic components from leaves after boiling.

Clinical presentation of cleistanthus collinus poisoning varies from nausea to death.Patients may be asymptomatic too.

The usual presenting symptoms are gastrointestinal symptoms of nausea, vomiting, diarrhea, abdominal pain, dysphagia and decreased bowel movements.(Chrispal, 2012).

Some patients complains of chest pain, breathlessness, dizziness (Nagaraj S, 1987).

Documented clinical neurological abnormalities are visual disturbances, muscle cramps and weakness, altered sensorium, giddiness, headache, altered speech, tremors (Subrahmanyam et al., 2003a)(Eswarappa et al., 2003)(Benjamin et al.,2006).

Fever within the first 96 hours of admission, and dehydration have also been recorded (Shankar et al., 2009).

Clinical features reported repeatedly are

1. Distal renal tubular acidosis which is a triad of hypokalemia, metabolic acidosis and alkaline urine .

2. Intractable hypotension(5).

Though cardiac arrhythmias were reported earlier, current opinion says that there is no cardio toxicity .We have demonstrated an animal model with intraperitoneal injection of water decoction of *C.collinus* leaves(4). In this model DRTA occurred repeatedly similar to human cases. However, type II respiratory failure also occurred which is never seen in patients.What is reported in patient is in fact a type I respiratory failure(6). The discrepancy in our model versus the patient findings could be due to differences in the route of toxin administration. While patients consume the toxin orally we had resorted to intraperitoneal

administration, because of the ease of using the anaesthetized animal for recording ECG, respiration and blood pressure.

Reported laboratory abnormalities includes

Hypokalemia,

Hyperchloremic high anion gap acidosis,

Leucocytosis,

Coagulopathy,

Elevated enzyme levels of alkaline phosphatase, transaminases,

Hyponatremia,

Hyperbilirubinemia,

Type 1 distal renal tubular acidosis,

ECG changes like QTc prolongation and ST changes

(Subrahmanyam et al., 2003) (Eswarappa et al., 2003) (Nampoothiri et al., 2010).

The mainstay of *C. collinus* poisoning management is close monitoring and correction of electrolyte imbalances.

Aggressive correction of potassium and, acidosis is crucial.

Shock management with crystalloids and inotropes (both epinephrine and dobutamine) is indicated. Cardiogenic cause for shock yet to ruled out.

Renal function with strict intake-output monitoring, central venous access and serum creatinine levels (Nampoothiri et al., 2010) .

The exact role of cardiac pacing in the setting of *C. collinus* poisoning is a matter of conjecture as the evidence for reducing mortality rate through its use is inadequate. Indicated in the setting of rhythm disturbances such as bradycardia and QTc prolongation, and may be placed electively in patients with severe poisoning. It is unlikely that elective pacemaker insertions for all patients with *C. collinus* poisoning will be of benefit, and studies on its indications and its duration of use are needed .(Thomas et al., 1991) . Cardiac monitoring may be required up to 5 days in symptomatic patients.

Mechanical ventilation is indicated in the setting of respiratory failure.

N-acetylcysteine has been suggested as possible antidote for management of *C. collinus* toxicity.(Annapoorani KS et al, 2003). N-acetylcysteine has been reported to be used, but the benefit of this intervention is unclear (Benjamin et al., 2006). Neostigmine was used for a patient who presented with a myasthenic crisis–like syndrome (Damodaram et al., 2008).

Other compounds suggested as possible antidotes for treatment of *Cleistanthus collinus* poisoning are L-cysteine, melatonin and thiol-containing compounds, but the benefit is unclear (Jayanthi et al., 2009) (Sarathchandra G et al, 2000) (Sarathchandra G et al, 1997) .

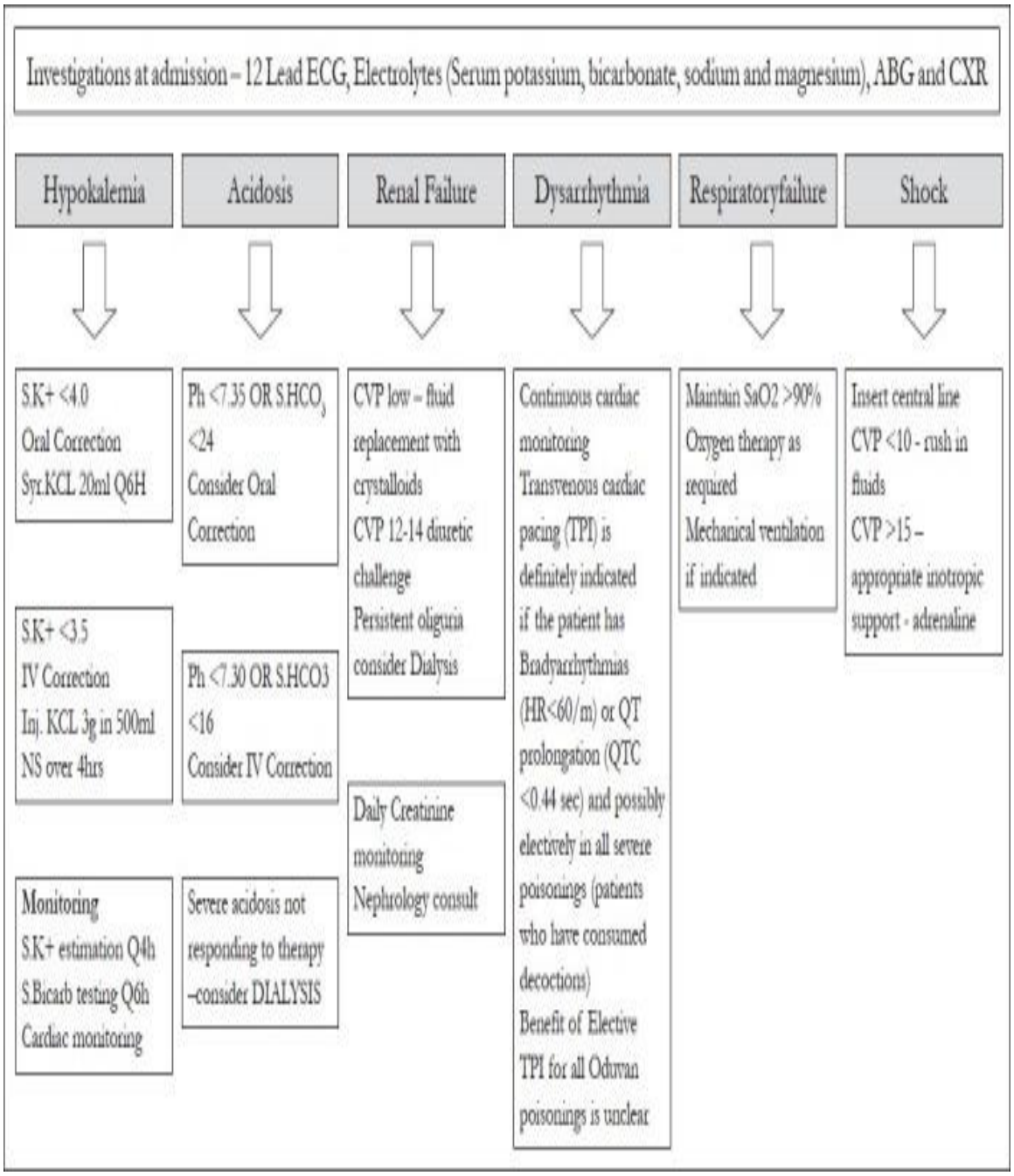


Figure 2

Management protocol based on current evidence (Chrispal, 2012)

PHYTOCHEMISTRY OF CLEISTANTHUS COLLINUS

The genus cleistanthus comprises about 140 species among which only four species have been investigated in detail. Study of chemical nature of cleistanthus collinus Roxb., demonstrates the presence of arylnaphthalide lignans and their glycosides as well as some furofuranoid lignans.

The compounds so far isolated from the plants of genus cleistanthus are lignans , terpenoids, ellagic acid, gracicleistanthoside. Lignans include arylnaphthalide lignans,furofuranoid lignans and dibenzylbutanone lignans. Terpenoids comprises triterpenes, diterpenes.

Cleistanthus collinus is composed of many toxic and non toxic constituents . Naidu et al isolated " Oduvin '' from the leaves to the toxicity of *C.collinus* was attributed(Subramanyam CA RNS, 1944). Oduvin was reported to be slow poison.

Subsequent investigation of oduvin by Irudayasamy and Natarajan showed it to contain Principle A and Principle B. chemical investigation of leaves of cleistanthus collinus led to isolation of lignin lactones like cleistanthin A, collinusin and diphyllin besides β sitosterol and ellagic acid.

Arylnaphthalide lignans are the major constituents and they were isolated both as free and as glycosides. Diphyllin was isolated from the leaves and the heartwood

of the plant *cleistanthus collinus* (Govindachari et al., 1967) (Anjaneyulu et al., 1981).

Collinusin and cleistanone were reported to be present in aerial parts of the plant (Govindachari et al., 1967) (Chinmai Ramesh, 2003).D iphyllin glycosides have the sugar moiety on C-4 of diphyllin. Govindachari et al isolated ellagic acid, Diphylllin and lignan lactones namely cleistanthin and collinusin from Cleistanthus collinus leaves (Govindachari et al., 1967).

Subba Rao reported the presence of another compound which had the structure diphyllin- O-glycoside and named it as Cleistanthin B and the earlier identified cleistanthin as Cleistanthin A. Cleistanthin A and Cleistanthin B have been shown to be glycosides of diphyllin (Annapoorani et al., 1984).

Cleistanthin E is the only triglycoside isolated from the plant of this genus (Anjaneyulu et al., 1981).

Cleistanthin C contained 2,3-di-o-methyl-β-D –xylopyranosyl9—40-β-D-glucopyranose,the sugar moiety of cleistanthoside A was also isolated from the heartwood of *C.collinus*(Anjaneyulu et al., 1981) (Chinmai Ramesh et al., 2003). Furafuranoid lignans isolated from the genus *cleistanthus* are sesamin, 4-hydroxysesamin, paulownin and wodeshio were isolated only from *C.collinus* (Anjaneyulu et al., 1981). Dibenzylbutane ligan present in C.collinus is

dihydrocubebin(Anjaneyulu et al., 1981). Ellagic acid was also reported to be present in C.collinus (Govindachari et al., 1967). Cleistanthin ($C_{28}H_{28}O_{11}$), a grey crystalline compound, with a melting point of 135-136 degree, was the main compound. On methanolysis in the presence of acid, it gave rise to diphyllin.

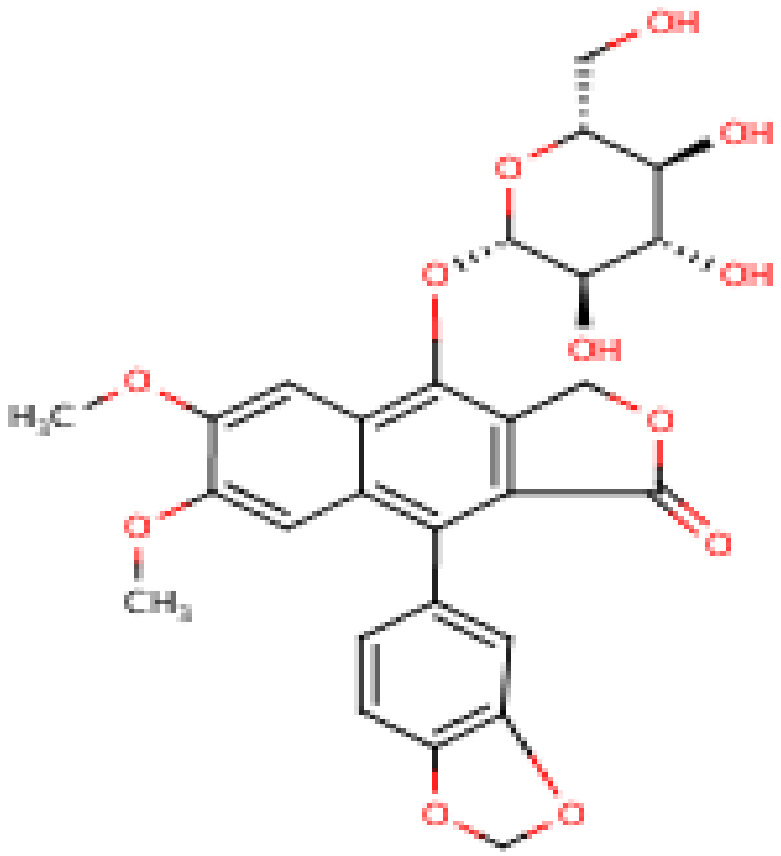


Figure 3 :

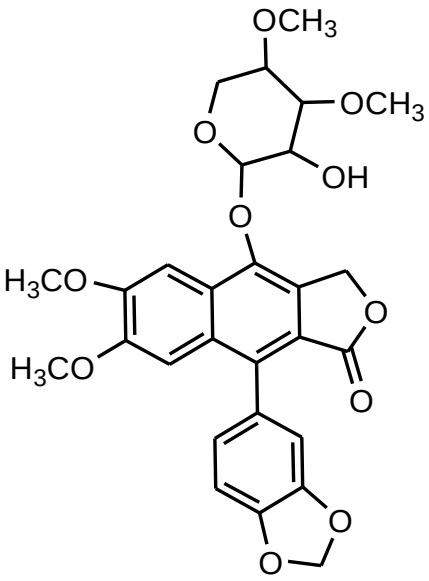
CLEISTANTHIN B

Molecular Formula

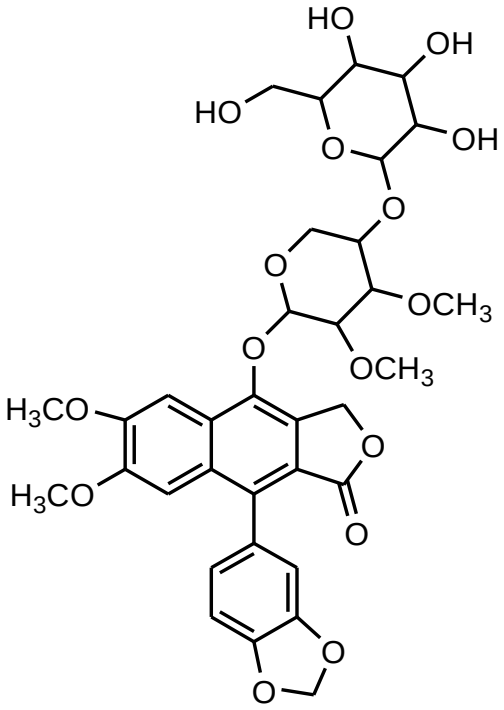
- C27-H26-O12

Molecular Weight

- 542.4904



Cleistanhin A



Cleistanthin C

Figure 4: CLEISTANTHIN A CAS No: 25047-48-7

Molecular Formula C28 H28O11

Molecular weight 540.52

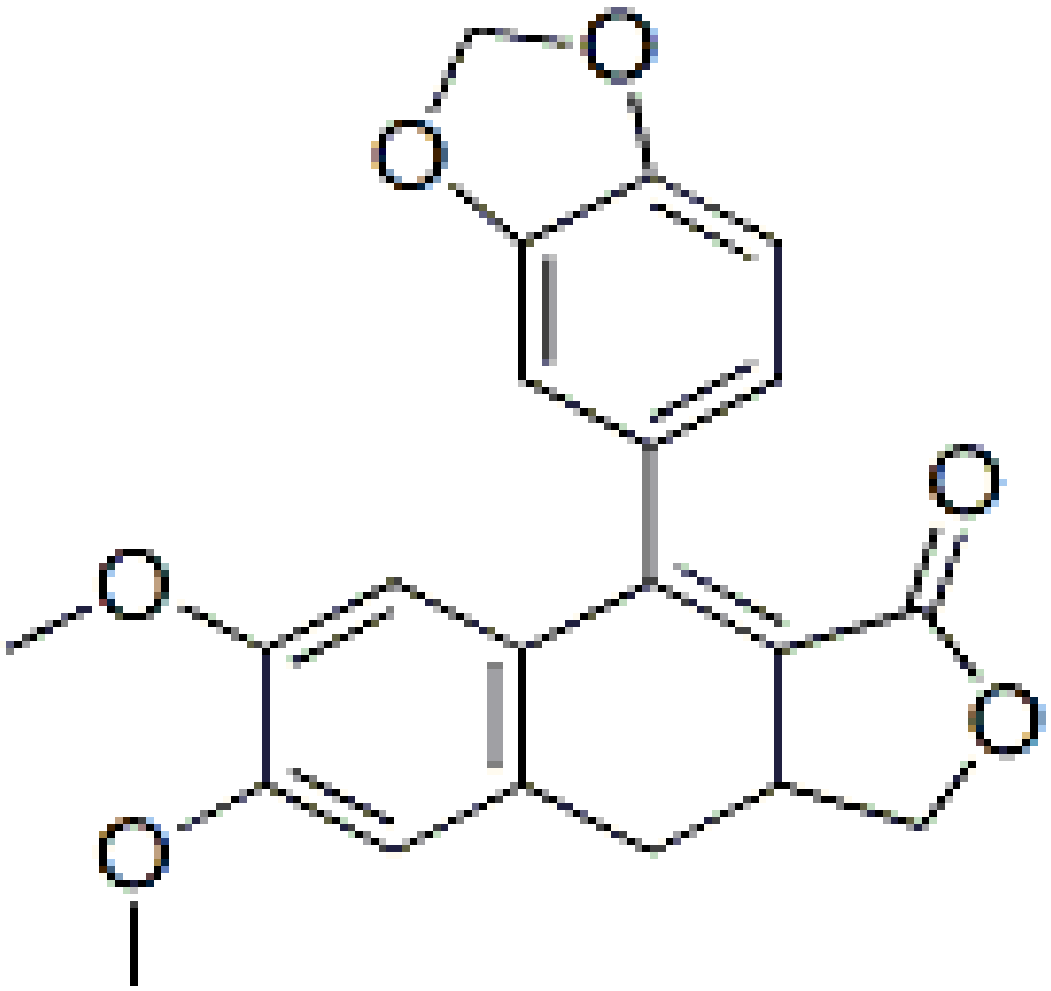


Figure 5 : COLLINUSIN

Molecular Formula C21 H18 O6

PROPOSED MECHANISM OF ACTION

As the exact mechanism of action of Oduvanthazhai is still unknown, many possible mechanisms have been suggested by many research groups. Most of them could be the downstream effect of a single still undetected main action. The suggested actions of Oduvanthazhai are:

Sodium Potassium Pump Inhibition

Inhibition of LDH isoenzymes

Depletion of Glutathione and Adenosine Tri phosphatase

Potassium channel blocker

Blocker of Neuromuscular Junction

Neutrophilic granulocytosis

Inhibition of DNA synthesis by Cleistanthin A

Cytotoxic and genotoxic effect of Cleistanthin B

Sodium Potassium Pump Inhibition

In 1997, Sarathchandra et al., showed inhibition of ATPase activity (Sodium Potassium ATPase and Magnesium ATPase) in both rats and rabbits. The clinical picture of patients with oduvanthazhai poisoning showed hypokalemia, kaliuresis and diuresis (Kurien et al.,1991). Cardiac glycosides are known blockers of sodium potassium pump. Oduvanthazhai being a glycoside was also expected to act in a same way.

Inhibition of LDH isoenzymes

Aqueous leaf extract was given intravenously to the test rabbits while distilled water was given to control rabbits. After three days, the test rabbits were sacrificed along with their controls.Blood samples were collected and tissue samples were taken from heart, liver, kidney. They were homogenized and used for assay.In the seum and tissue of test animals, the LDH activity was markedly reduced as compared to the controls. Among the tissues , kidney shwed the maximum decrease in the LDH levels (Kanthasamy et al.,1986)

Inhibition of Enzymes

The boiled extract was given to rats and rabbits followed by which they were sacrificed after 2hours. Tissues like brain ,heart , liver,kidney, skeletal muscle were used for glutathione assay and ATPase estimation. Glutathione profile revealed depletion in organs of rats and in rabbits as compared to that of controls in the respective species. A similar trend of inhibition of ATPase activity is observed in the vital organs of rats as well as in case of rabbits. *Cleistanthus collinus* during its assault causes a definite depletion of thiol containing enzymes which is responsible for the manifestation of toxicity and this finding could pave way for the selection of thiol compounds as probable antidotes to combat *C.collinus* toxicity (Sarathchandra et al., 1997)

Potassium channel Blocker

Minoxidil and Glibenclamide are known potassium channel blockers. Influence of minoxidil and glibenclamide on *cleistanthus collinus* induced toxicity was studied ED 50 for arrhythmia, changes in contractility and heart rate were recorded . Cleistanthus at low doses caused a transient tachycardia and increase in contractility and at a high dose caused arrhythmia and cardiac arrest (Jose et al.,2003)

Neutrophilic Granulocytosis

Rao *et al* observed neutrophilic granulocytosis in rats injected with cleistanthin. Hence, the toxicity of the extract was tested in different species.

Cleistanthin was isolated from acetone extract of *cleistanthus collinus* leaves .Solution was administered viaoral and parenteral modes (IV, SC, IM) respectively. The study was conducted on albino rats, swiss mice, rhesus monkeys and mongrel cats. Post administration, leucocyte count was done with improved neubauer haemocytometer. Neutrophil count was done using leishman's staining and haemoglobin was assessed by Sahli-Helinge method . There was a significant dose dependent and species dependent neutrophilic granulocytosis. It also depended on the route of administration. It was seen that intravenous and intraperitoneal were more toxic compared to oral. The exact role of cleistanthin induced neutrophilic granulocytosis is yet to be investigated (Rao et al.,1970)

NEUROMUSCULAR BLOCKADE

Toxic symptoms of cleistanthus collinus include muscle cramps and weakness, the effect of the leaf extract on the electrical and mechanical responses to nerve and muscle stimulation was studied in the isolated phrenic nerve diaphragm preparation of the mouse (Nandakumar et al.,1996). Following an hour's exposure to 0.015% extract, the response compound nerve action potential to supramaximal nerve stimulation was reduced by 38%. The compound muscle action potential of muscle reduced by 97% and isometric tension was reduced by 38%. Ther was only 11%

reduction in resting membrane potential.The tension and compound action potentials were monitored using an *in vitro* electromyography chamber. The intracellular recordings were done for resting potentials, miniature end plate potentials and end plate potentials. The amplitude of miniature end plate potentials reduced by 72% and shortened fall time by 53% indicating a post junctional inhibition similar to curare. The irreversible effects was attributed to the structural alterations in the neuromuscular junction which in turn caused symptoms such as respiratory failure, muscle weakness and cramps (48).

Albino rat model suggests that cleistanthus collinus can result in neuromuscular junction blockade, particularly at the post synaptic acetylcholine receptors without affecting excitation contraction coupling of the muscle fibres.

INHIBITION OF DNA SYNTHESIS

Cleistanthus collinus has been screened for its anti protozoal, anti fungal, anti bacterial, anti viral , anti- inflammatory, anti cancer, or any other pharmacological activities in Central Drug Research Institute in 1969(54).

Anti cancer property of *Cleistanthus collinus* has been observed against human epidermal carcinoma of nasopharynx in tissue culture (Bhakuni et al.,1969), Walker carcinosarcoma 256 and L-1210 lymphoid leukemia in mice (Pradheepkumar et al.,2000). In Various studies the lignans have been tested for its anti cancer property. Tumorigenic effects of Cleistanthin A and B on K-562 tumour cell lines showed a reduction of nucleotides incorporation into the DNA

and RNA. Replication and transcription steps are affected sparing the translation process (Rajkumar S et al., 2001). MT2 cell lines incubated with cleistanone ,diphyllin product exhibited cytotoxicity (Ramesh et al.,2003). Cleistanthin B was found to be toxic against normal and tumour cel line. Cleistanthin B appears to have less affinity to bone marrow,fibroblasts, peripheral blood lymphocytes and oral fibroblasts. Human cancer cell lines showed higher sensitivity to this toxin than the three human normal cell types examined(Pradheepkumar et al.,1996) Detailed studies in Cleistanthin B showed that it affected the DNA synthesis by G1 phase arrest of mitosis and caused subsequent apoptosis (Pradheepkumar et al.,1996). Time lapse video microscopic recordings of cleistanthin A treated cells showed vigorous membrane blebbing, characteristic of apoptosis. Cleistanthin A resulted in DNA strand breaks and cell apoptosis at higher concentrations (Meenakshi et al., 2000).Matrix Metalloproteinase 9,(MMP-9) which is involved in extracellular matrix remodelling was inhibited by cleistanthin A , thereby viability of proliferating cells was reduced (Pinho et al., 2007).

Pinho and Kijjo compared the Cleistanthin A cytotoxicity with that of 5 known anti cancer drugs. Cleistanthin A cytotoxicity was prominent in cancer cell lines and the tumor mass was reduced. But the higher concentrations of Cleistanthin B exhibited cytotoxicity against the normal cells along with the tumorigenic cells (Pinho et al., 2007)..Hence, its cytotoxicity was tested against normal and

tumorigenic cell lines. At higher doses, the DNA breaks and chromatid aberrations made by cleistanthin B in the normal cells were irreparable. Prabhakaran et al., in 1996 found that it can break chromatids and isochromatids in Chinese hamster ovary(CHO)cells. It also induces micronucleus formation in cultured lymphocytes.

Distal Renal Tubular Acidosis & Type II Respiratory Failure:

Renal tubular acidosis (RTA) is applied to a group of transport defects in the reabsorption of bicarbonate (HCO3-), the excretion of hydrogen *relatively normal GFR* and ion (H+), or both.

The RTA syndromes are characterized by a a metabolic acidosis accompanied by hyperchloremia and a normal plasma anion gap.

Distal RTA (type 1) has been defined as a disorder of renal tubular function characterized by hypokalemia, hyperchloremic acidosis and the inability to lower urine pH below 5.5. In the subcohort , six patients fulfilled the criteria for dRTA.

In our lab ,a series of *in vivo* experiments was performed to address this problem. We developed an animal model to study the molecular mechanisms of the toxin (Maneksh et al., 2010). The boiled aqueous and acetone extract of *Cleistanthus collinus* were used. The intraperitoneal route was chosen. The respiratory rate, heart rate, pressure, arterial blood gases and electrolytes were monitored over a period of 8 hours. The test rats showed severe respiratory acidosis with alkaline

urine. This indicated that the acid secreting mechanism was affected, that is, a type I Distal renal tubular acidosis. The renal brush border membrane was isolated to measure the total ATPase activity by measuring the amount of inorganic phosphate formed. Venous blood samples, where the RBC have the same ATPases, were incubated with the acetone extract and plasma potassium levels were measured. *In vitro* experiments on the rat kidney demonstrate that there is ATPase inhibition in the brush border as well as the basolateral membrane of the distal renal tubules ,sparing of the sodium potassium ATPases. The *in vivo* experiments indicated that the respiratory failure may be of central origin which caused the death of the rats. In addition to the DRTA seen in rat in vivo experiments, the severe respiratory acidosis that was also seen must be contributing to the mortality due to toxic extract and has to be explored further. Vacuolar ATPase (proton pump) inhibition was further confirmed on rat renal basolateral membrane and brush border membrane by incubating them with boiled extract(Kettimuthu et al., 2011).

Suggested Treatment Modalities:

Jose *et al* observed the effects of potassium channel modulators such as Minoxidil, Glibenclamide on Cleistanthin's boiled extract induced toxicity. The pharmacological modulators tried in treatment did not improve this scenario. The study conclusion was these drugs cannot be used in acute cardiac toxicity .

It is known that Oduvan poisoning causes oxidative stress in tissues, especially heart and brain. Jayanthi *et al* proposed the **use of melatonin and cysteine** to reverse oxidative stress (53). The toxin was administered orally along with melatonin in wistar rats. Following their death, blood was collected for malondialdehyde, glutathione, catalase and glutathione peroxidase assays. Brain & heart tissues was processed for histopathological examination. After oduvan administration, the antioxidants significantly reduced the malondialdehyde levels. But, there was no depletion of glutathione levels contradictory to the study done by Sarathchandra *et al*. Hence, the beneficiary effects of the antioxidants on glutathione could not be assessed. Histopathological examination done after antioxidant treatment showed reversal of brain damage but not the heart damage. The damage control was pronounced with melatonin. However, it did not improve the survival. Hence, melatonin can be used to control oxidative stress in *Cleistanthus collinus* poisoning but cannot be used as an antidote .

DIAGNOSTICS

An enzyme linked immunosorbent assay (ELISA) was reported for the quantification of the active principlesof *C,collinus* (Raghupathi et al.,1992).This method was found to be very sensitive as it detects 2ng/ml of cleistanthin B and

3ng/ml of cleistanthin A.ELISA can be applied for oduvan identification in clinical and forensic toxicology.

Spectrofluorometric quantitation of diphyllin and its glycosides has been mentioned by K.S A nnapoorani.This method is highly reproducible and sensitive to 0.1µg/ ml of cleistanthins in blood.

Cleistanthin B was also estimated by a solid state fluorodensitometric method(Ragupathi et al.,1992). It can measure the hapten at nanogram level.

Cleistanthin B was conjugated with bovine serum albumin followed by a TLC on silica gel.. The concentration of Cleistanthin B was obtained by a standard graph plotting the peak area against the concentration. This was chromatographed with a control, that is, a known amount of conjugate. The number of molecules was also determined from UV spectral data of the three components known as the UV method. Cleistanthin B was determined by determining the number of free amino groups in the protein, called the TNBS method.

Thin layer chromatographic detection of the lignan lactones has been developed by Subramanian et al in 1975. The lignan lactones could be distinguished by their characteristic fluorescent colour. Diphyllin (violetish blue), Cleistanthin A was (blue), Cleistanthin B (greenish blue)and collinusin (green)(K.S Annapoorani, PhD Thesis). All colours were stable for many days except diphyllin. Also, it was

observed that the sequence of appearance of the colours was different in different solvent systems. This method is highly sensitive.

High performance liquid chromatography was used to confirm the purity of the compound. The fractions can be characterized with Liquid chromatography/Gas chromatography – Mass Spectrometry (LC-MS/ GC-MS). Structural characterization can be done using various spectroscopic tools that include 1H/13C-NMR, 2D NMR, FT-IR and high resolution mass spectroscopy.

INTERESTING FACTS ABOUT ODUVAN FROM OUR LAB

Though many of the active constituents in *C.collinus* have been identified and purified and chemical structures known, they are not available commercially. Though some companies sell synthesized compounds, the costs are excessive (about $500 for 20 mg). The Dept of Physiology at CMC, Vellore has many research questions being pursued regarding the toxicity of *C.collinus* and even to meet the demands of our department, we have not been able to procure either Cleistanthin A or other toxins commercially. When Cleistanthin B was procured from a Chinese company which was the only one to respond to a request placed on the internet, the item turned to be a hoax. Extensive research regarding Oduvanthazhai is happening in our department for the last ten years. As part of a Ph.D Thesis (unpublished data),

1. Different types of aqueous extracts were prepared from 100 grams of fresh or dried leaves and compound profiling was done. They are as follows:

a) Hexane delipidated leaves

i.Boiled extract

ii.Room temperature extract (24hours of soaking)

iii.Room temperature extract(24 hours of soaking) boiled later

b) Shade dried leaves

i.Boiled extract

ii.Room temperature extract (24hours soaking)

iii.Room temperature extract(48 hours soaking)

iii.Room temperature extract (24 hours) boiled later

c) Fresh leaves

i.Boiled extract

ii.Room temperature extract (24 hours of soaking)

iii.Room temperature extract (48 hours of soaking)

iii.Room temperature extract boiled later

iv.Fresh leaf ground extract

2.Simple method for isolation and quantification of cleistanthins and Diphyllin from cleistanthus collinus leaves was established. Liquid/Liquid partition chromatography of aqueous extracts with chloroform to isolate fluorescent compounds was started**.** Traditionally, these compounds were isolated from acetone extracts of dried and delipidated leaves with column chromatography. The methods required extensive use of solvents such as n-hexane, benzene (known to be highly toxic, and therefore banned from countries like Australia) and ethyl acetate We have developed easy methods of purification of these

compounds from the aqueous extract with thin layer chromatography, without the use of Benzene or ethyl acetate (As part of a Ph. D Thesis).

3. Quantification of fluorescent compounds in different extracts was done by High performance liquid chromatography in collaboration with clinical Pharmacology Unit of our own institute. (As part of a Ph. D Thesis).

4. After extensive quantification experiments, we short-listed the best type of aqueous extract that provides large quantities of a given fluorescent compound (As part of a Ph. D Thesis).

5. Short-listed aqueous extracts are used to isolate a particular fluorescent compound (identified in HPLC) in large amounts for mechanistic studies on animals. (As part of a Ph. D Thesis).

6. Structure of the isolated compounds with Thin layer chromatography was characterized with Mass Spectrometry, NMR spectrum (NOSEY, COSEY and HMPC), Infrared spectrum, melting point analysis in collaboration with Chemistry department of IIT, Mandi. (As part of a Ph. D Thesis).

7. Established an animal model of *C.collinus* poisoning:

Boiled whole aqueous extract was administered to the rats, there was a type I distal renal tubular acidosis due to a possible proton pump inhibition, and there

was type II respiratory failure. *In vitro* studies on the renal brushborder membrane and basolateral membrane showed V-type ATPase blockade, causing DRTA (Maneksh et al.,2010)

We have determined the clinical profile of toxicity by administering following extracts intraperitoneally into the rats.

8. Cleistanthin A from hexane delipidated acetone derived boiled extract was tested *in vivo* by injecting it intraperitoneally in rats. Rats died due to sudden respiratory arrest followed by cardiac arrest (Dr. S. Anitha, M.D Thesis, 2010).

9.Cleistanthin B when tested *in vivo* showed toxicity. The rats died with respiratory arrest followed by cardiac arrest administration (Dr. Rajam, M.D. Thesis, 2011)**.**

10. Diphyllin**,** when tested *in vivo,* did not kill the rats upto 8 hours after administration (Dr. Latha, M.D. Thesis, 2011).

Hence, Diphyllin is the only known non toxic fluorescent component of *cleistanthus collinus*.

11. NFF (non-fluorescent fraction) was found to be non toxic when administered intraperitoneally (Dr.Swetha,MDThesis.,2014)

12. Proton channel modulating activity of whole aqueous extract, Cleistanthin A, Cleistanthin B have been tested by patch clamp technique on neutrophils.

It was found that the whole aqueous extract blocked proton channels, while Cleistanthin A and Cleistanthin B did not affect the conductance. (This issue of proton channel blockade is very important because there is no known proton channel blocker except zinc). It blocked the voltage gated sodium, calcium and delayed rectifier potassium channels, hence proved to be **a** non specific cation channel blocker (Dr. Praghalathan Kanthakumar, M.D Thesis 2010).

However it was found later that the whole aqueous extract contains significant amount of zinc, which is a known proton channel blocker. Drum stick leaf extract which contains equal amount of zinc also blocks the proton channel. Therefore this line of investigation has been discontinued temporarily. Hence, the active principles were extracted by adsorption chromatography to test them individually.

Patch clamp studies on proton channels in neutrophils showed that, the proton current densities increased due to Cleistanthin A. (Dr. R.Rajalakshmi, M.D Thesis, 2012). Cleistanthin B was tested in patch experiments and it was seen that it did not block proton channels of neutrophils (G.Gnanasenthil, M.D Thesis, 2012).

AIMS AND

OBJECTIVES

Aim

The aim of the present study was to develop an antidote for *Cleistanthus collinus* poisoning.

Objectives

(1) To determine the minimum lethal dose of boiled water extract of *C.collinus* when administered orally.

(2) To see if a particular fraction of *Cleistanthus collinus* extract (non fluorescent fraction of cold water extract, NFF) can prevent death in rats when administered after administering the lethal dose of boiled water extract.

(3) To determine the minimum lethal dose of pure Cleistanthin C when given orally**.**

(4) To determine if non fluorescent fraction of cold water extract of *C.collinus* can prevent death in rats poisoned with lethal dose of Cleistanthin C.

(5) To determine the minimum lethal dose of fresh leaf ground extract when given orally**.**

(6) To determine the minimum lethal dose of pure Cleistanthin A when given orally**.**

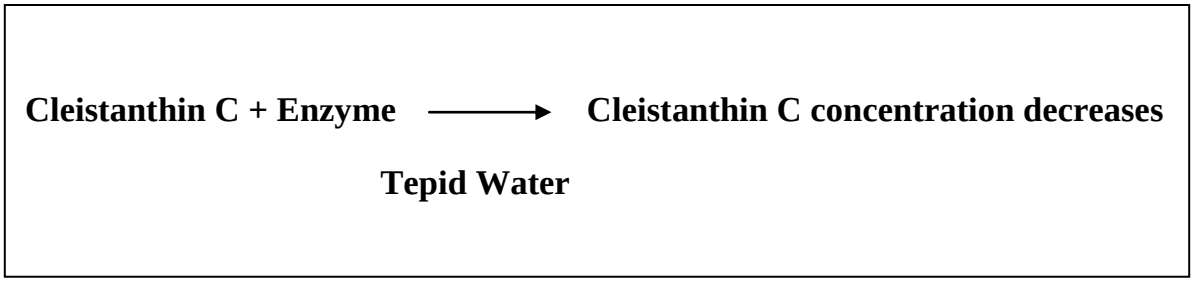
STUDY

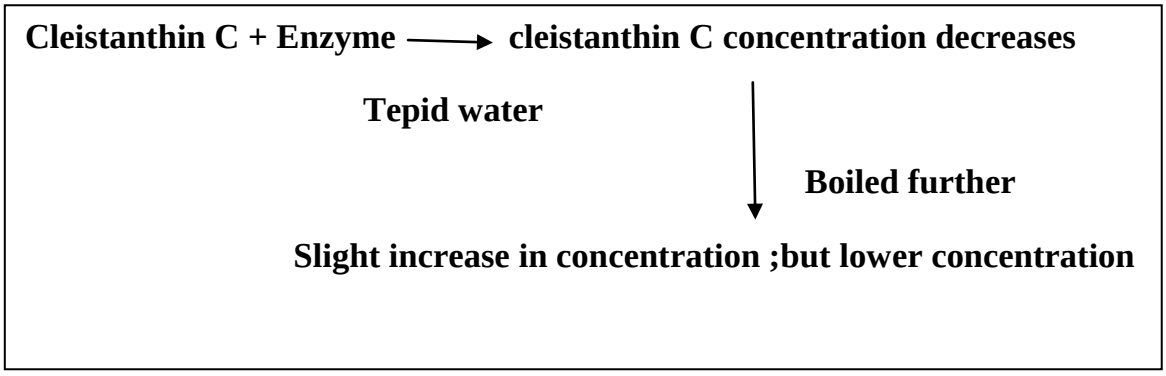
RATIONALE

It is a known fact that mortality is higher when boiled decoction of the leaves is consumed, as against crushed leaves. Parallel work in our lab led to the observation that the boiled extract contains cleistanthin C while the room temperature extract does not. The logical assumption at first was that cleistanthin C can be extracted only by boiling. However, when the leaves were soaked in water in room temperature for 24 hours were then boiled, Cleistanthin C was still not found in the extract. The hypothesis, then was that exposure to tepid water destroys Cleistanthin C while boiling without exposure to tepid water preserves Cleistanthin C. This led us to hypothesize that there may be a heat labile substance in cleistanthus collinus leaves which can destroy Cleistanthin C. This may be probably an enzyme.

Cleistanthin C + Putative Enzyme ⟶ **Cleistanthin C concentration Increases**

Boiling destroys the Enzyme


Cleistanthin C + Enzyme
Cleistanthin C concentration decreases
Tepid Water


Cleistanthin C + Enzyme
cleistanthin C concentration decreases
Tepid water
Boiled further
Slight increase in concentration ;but lower concentration

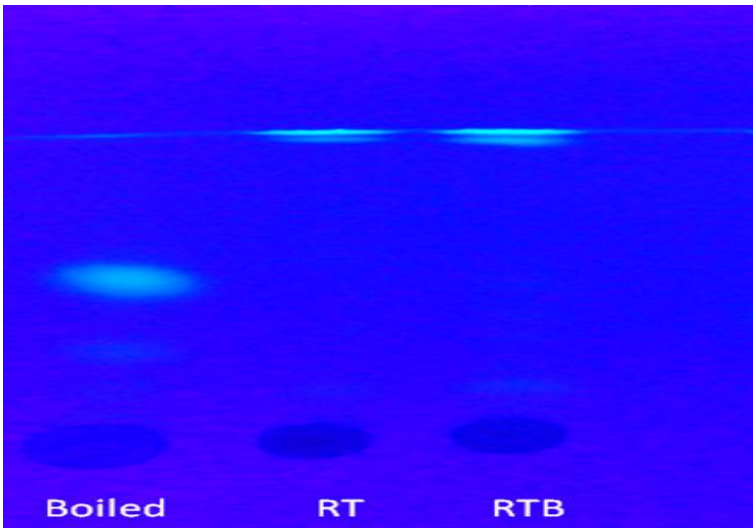


Figure 6 : Thin layer chromatogram of boiled, room temperature (RT) and room temperature boiled extracts (RTB) of Fresh leaves of *C.collinus.*

Lane1 : Boiled extract

Lane 2 : Room temperature extract

Lane 3 : Room temperature boiled extract.

Notice that in lanes 2 and 3, Cleistanthin C fluorescence is absent where as in lane 1 shows Cleistanthin C fluorescence (Boiled).

If indeed there was neutralization of Cleistanthin C by an enzyme during room temperature extraction, accounting for the observations discussed so far, then we reasoned that ice cold extraction may disallow enzyme action and Cleistanthin C

would be present in the ice cold extract. As expected, Cleistanthin C is detected in aqueous extracts with ice cold water.

The enzyme being water soluble, would not dissolve in organic solvents. Any extract made with organic solvents should therefore contain Cleistanthin C.

We expected that this putative enzyme will elute only in aqueous extracts and that when the aqueous extract is partitioned with chloroform (see method section), the putative enzyme will remain in the water fraction. Therefore we took ice cold water extract (ICWE) which would contain the enzyme intact (but without optimal temperature for its activity) and removed all the fluorescent compounds (which include toxins) with chloroform partitioning. The toxins went into chloroform. The remaining water part of the ice cold extract, we hypothesized, would contain the enzyme which degrades the Cleistanthin C and be devoid of toxins.

Our research question was if this fraction would serve as an antidote for *Cleistanthus colllinus* poisoning. A good part of thesis was devoted to preparation of extracts, standardization of toxic dose of Fresh leaf boiled extract (FLB), standardization of toxic dose of cleistanthin C and determination of non toxicity of the non-fluorescent fraction of the ice cold water extract (NFF OF ICWE) which was to serve as the antidote. Corroborative evidence that NFF of ICWE destroys Cleistanthin C is provided as chromatograms. Pilot studies were performed to test

effect of the probable antidote in fresh leaf boiled extract poisoning and cleistanthin C poisoning.

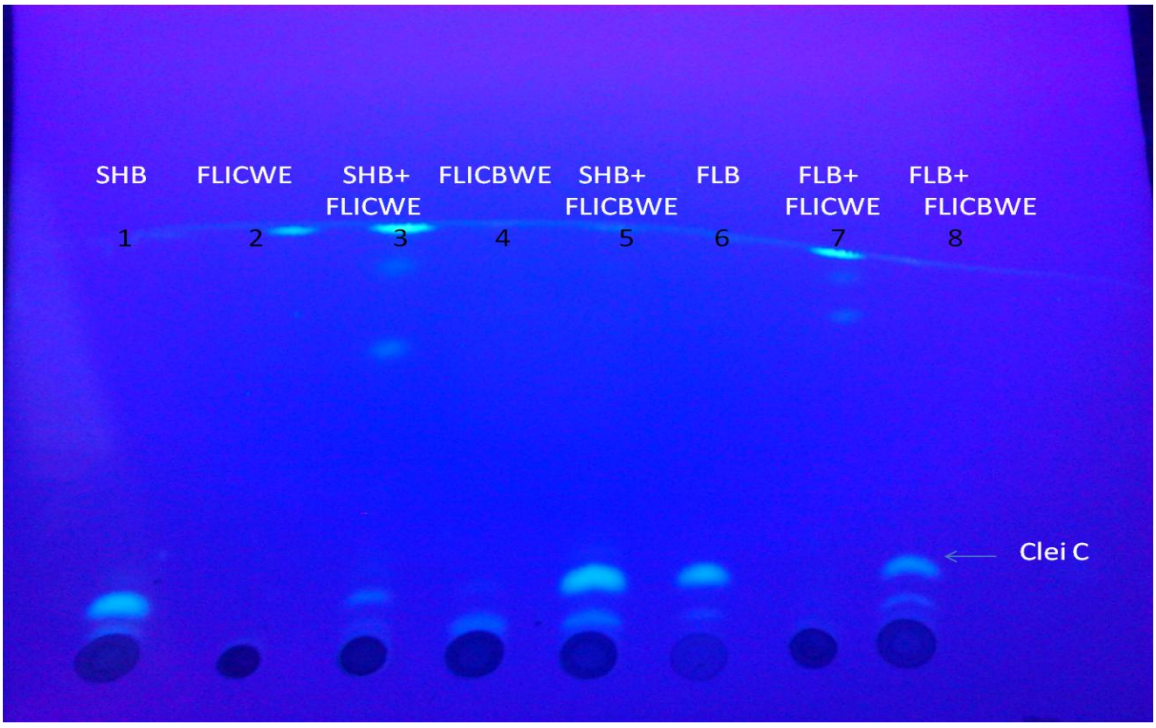


Figure 7 : Thin layer chromatogram of boiled extracts prepared from shade dried and fresh leaves incubated with two different type of non fluorescent fractions (FLICWE & FLICBWE).

Lane 1 :SHB- shade dried boiled, Lane 6 :FLB- Fresh leaf boiled extract.

Lane 2 :FLICWE-Fresh leaf ice cold water extract (NFF of FLICWE)

Lane 4 :FLICBWE- fresh leaf ice cold boiled extract (NFF of FLICBWE)

As we see, in lane 3 and 7 boiled extracts when incubated with non fluorescent fraction of ice cold water extract, cleistanthin C fluorescence disappears. On the contrary, boiled extracts incubated with non fluorescent fraction of ice cold boiled extract , which is devoid of enzymatic activity due to boiling ,there is no quenching of Cleistanthin C fluorescence.

The conclusion therefore is that Cleistanthin C destroyed by an enzyme, also present in the leaves, when the two (Cleistanthin C and the enzyme) come into contact with each other, which happens when the leaves are soaked in water at room temperature. This may be the reason why crushed leaves or room temperature extracts are not as lethal as boiled extracts in humans. Toxicity studies may now focus on Cleistanthin C because it seems to be the major lignan in fresh leaves. It remains to be seen if the putative enzyme in *C.collinus* leaves that can destroy Cleistanthin C could serve as an antidote to *C.collinus* poisoning.

Quantification studies showed that boiled water extract contains a large amount of Cleistanthin C in addition to Cleistanthin A and at low concentration in fresh leaf water ground extract which further supports our hypothesis.

In previous experiments done in our lab, toxins were administerd intraperitoneally which may be the reason why the clinical picture does not match the human condition exactly. However, the experiments have helped us to understand the

electrolyte and ABG profiles during the course of the toxicity. However, we now realize that it is important to develop an animal model of the poisoning which mimics human cases so as to study treatment options.

MATERIALS

AND

METHODS

EXPERIMENTAL DESIGN

A boiled decoction of *C.collinus* was made in a way patients normally prepare it. Leaves were added to water and then boiled for 10 minutes. The decoction was filtered and dried. To isolate cleistanthin C and A, cooled decoction was subjected to liquid liquid partition chromatography with chloroform. The top water fraction was devoid of fluorescence and therefore termed as the non fluorescent fraction or NFF. The bottom chloroform fraction (CF) had sequestered all fluorescent compounds, concentrated and dried to a powder. The three major active priniciples Cleistanthin A, C and diphyllin was separated by TLC on silica gel plates. Cleistanthin C and A was purified further for animal studies.Fresh leaf ground extract was also prepared and minimal lethal dose for the same was estimated to see if any gross difference in lethal dose from fresh leaf boiled extract of *C.collinus* .

Toxicity studies were performed with oral administration of boiled decoction and Cleistanthin C. The minimum toxic dose (causing 100 % mortality) of the water decoction of fresh leaves of *C.collinus* and toxic principle, Cleistanthin C was determined. After estimating lethal dose for the above mentioned extracts, non fluorescent fraction considered to have antidote action was administered to the poisoned rats. The mortality rate was assessed.

Materials Required For Extract Preparation

Leaves: *Oduvanthazhai* leaves

Solvents Used

i. n-Heptane (SRL)

ii. Chloroform (Fisher scientific)

iii. Methanol (ExcelaR)

iv. Ethanol AR 99.9% (Hayman)

For Isolation And Quantification Of Compounds

1. **Liquid/Liquid chromatography**

Separating funnel, standard flask, Glass beakers, petri dishes, funnels, borosilicate- proccured from science house.

2. **Thin layer chromatography**

Silica gel-G (contains 13% calcium sulfate as a binder) from Fisher Scientific. Glass plates, TLC tank with lid, and spreader from Science house.

3. **Solvent recovery**

Rotary evaporator (Evator from Science house) connected to vaccum pump, circulating water bath (equitron from Science house).

4. **Exhaust fume hood**
5. **Hot air oven for drying (Science house)**
6. **UV illuminator (Toshiba)**
7. **High-performance liquid chromatography (HPLC)**

    a. Instrument Name : Shimadzu (HPLC)

    Column : Supelco HPLC column, Discovery® HS C(18)

    b. Size : 25cm x 4.6mm, 5µm

    c. Detector : RF10 AXL- Fluorescence detector

8. **Lyophylizer for freeze drying**

Materials Required For Animal Experiments

For Oral gavaging:

Oral feeding tube of size F-15

Miscellaneous:

1cc and 5cc syringes

Hand gloves and apron

Eppendorffs-.5ml, 1ml

Extracts used

1. Fresh leaf boiled aqueous extract
2. Fresh leaf ground extract

3. Cleistanthin A
4. Cleistanthin C
5. NFF of C.collinus

Preparation of Fresh leaf boiled extract of *C.collinus* (FLB extract)

Cleistanthus collinus leaves were collected from Amirthi hills, Tiruvannamalai district, Tamilnadu, India. 100 grams of fresh leaves was added to 3 litres of boiling water , then boiled for 10 minutes. The crude solution was filtered and the filtrate was dried at 37ºdegree celsius to get a dry powder and residue was preserved. The extract was scraped out using a scalpel and the powder was stored in bottles at -20 degree Celsius. The boiled decoction was referred to as FLB (fresh leaves, boiled). Desired doses of dried powder of *C.collinus* extract dissolved in distilled water were administered to the rats by oral gavage for toxicity studies.

Figure 8 : Preparation of fresh leaf boiled extract

Identification, characterization and quantification of fluorescent compounds in the boiled aqueous extract of *Cleistanthus Collinus* leaves was done using different established techniques, namely, thin layer chromatography (TLC), nuclear magnetic resonance spectroscopy (NMR) and high performance liquid chromatography (HPLC). Details of the above mentioned techniques will be discussed later.

Preparation of fresh leaf ground extract (FLG) of *Cleistanthus collinus*

For fresh leaf juice, 100 gm of fresh leaves were ground in 1 litre of water using an electric blender and the blend filtered. The filtrates were centrifuged and supernatant was collected. The crude solution was filtered and the filtrate was dried to get a dry powder and residue was preserved. The extract was scraped out and stored in bottles at -20 degree Celsius. The extract was referred to as FLG extract.

Isolation and Characterization of Cleistanthin C and A

Isolation of the toxic principles to purity is important for mechanistic studies on toxicity and for development of antidotes.

Liquid/Liquid partition chromatography

500 ml of chloroform was added to the filtered boiled decoction for liquid liquid partitioning (LLP). The immiscible water and chloroform fractions were separated using a separating funnel. LLP was repeated twice with smaller volumes of chloroform.

All the fluorescent compounds in the aqueous extract sequestered in the chloroform fraction, henceforth called the chloroform fraction (CF). The water fraction after LLP was devoid of fluorescence and was termed as non fluorescent

fraction (NFF). Chloroform was removed from CF with a rotary evaporator, the concentrate dried to a powder in an oven or on a hot plate and weighed. The chloroform recovered during the process was used for further extractions.

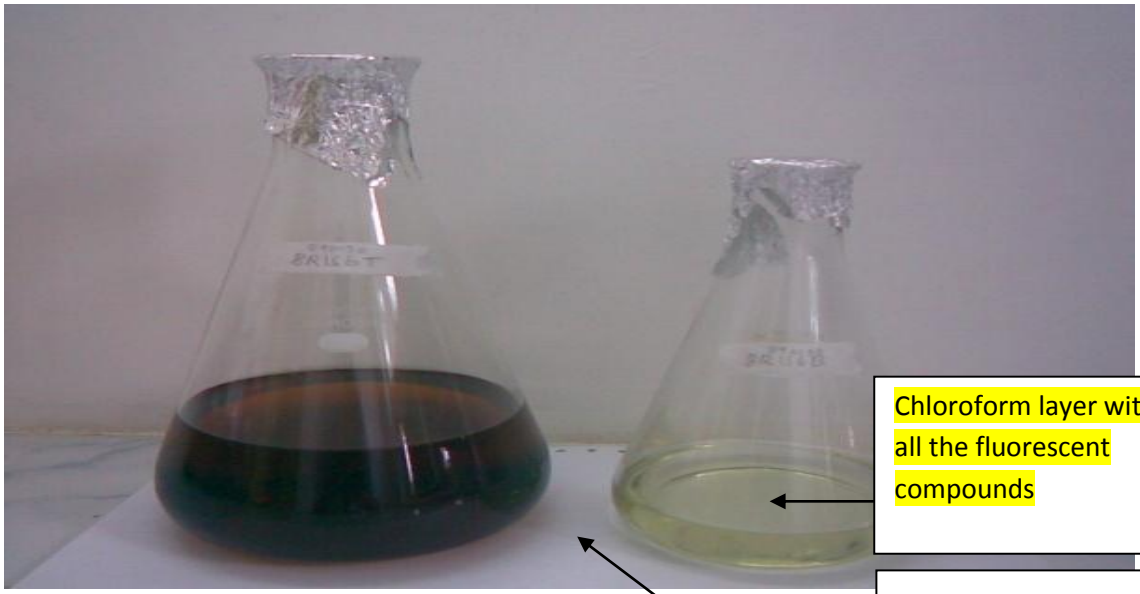


Figure 9 : Liquid liquid partitioning

Figure-9 Liquid/Liquid Chromatography (a) Separating funnel showing two immiscible layers. Top aqueous and bottom Chloroform layer.(b) Separated aqueous and Chloroform fractions of boiled aqueous extract.

Figure 10 : Shows aqueous extract kept for drying on hot plate.

Figure 11 : Whole extract and dry powder of the same extract.

Figure 12 : Rotary evator was used to recover chloroform from chloroform fraction.

Preparatory Thin Layer Chromatography

100 mgs of CF powder was dissolved in chloroform and chromatographed on silica gel layered plates. These plates were then placed in a solvent system containing mixture of n-heptane-chloroform-ethanol in the ratio 50:50:10 (preparatory TLC). Solvent migration was allowed upto a convenient height (about 10 cms) in an ascending manner. The plates were then air dried and viewed under short wave ultraviolet light (352 nm). The band corresponding to cleistanthin A & C were scraped off separately and crude cleistanthin C was eluted from silica with methanol and the eluted material was dried to obtain Cleistanthin C. Cleistanthin C was purified further by a second thin layer chromatogram with chloroform: methanol in the ratio 90:10 and processed further. Cleistanthin A was also purified further for toxicological studies. The compounds obtained were weighed for quantification.The compound isolated was characterized with mass spectra, 2D NMR spectra and melting point analysis. After the confirmation of structure with spectral methods, reverse phase HPLC was performed to test the purity of the isolated compound.

Techniques in detail

Thin Layer Chromatography

Thin layer chromatography (TLC) method was used to analyze the compounds present in the extract and to isolate them for further purification steps. The sample to be chromatographed was spotted on a TLC plate made with silica gel as the stationary phase. 35gms of Silica gel was mixed with 70ml of distilled water (1:2 ratio) to form a mixture. Then the slurry was poured in the spreader and the silica gel was applied over the glass plates (0.5mm thickness).After natural drying the plates were activated by keeping in an oven at 110°C for 30 minutes.

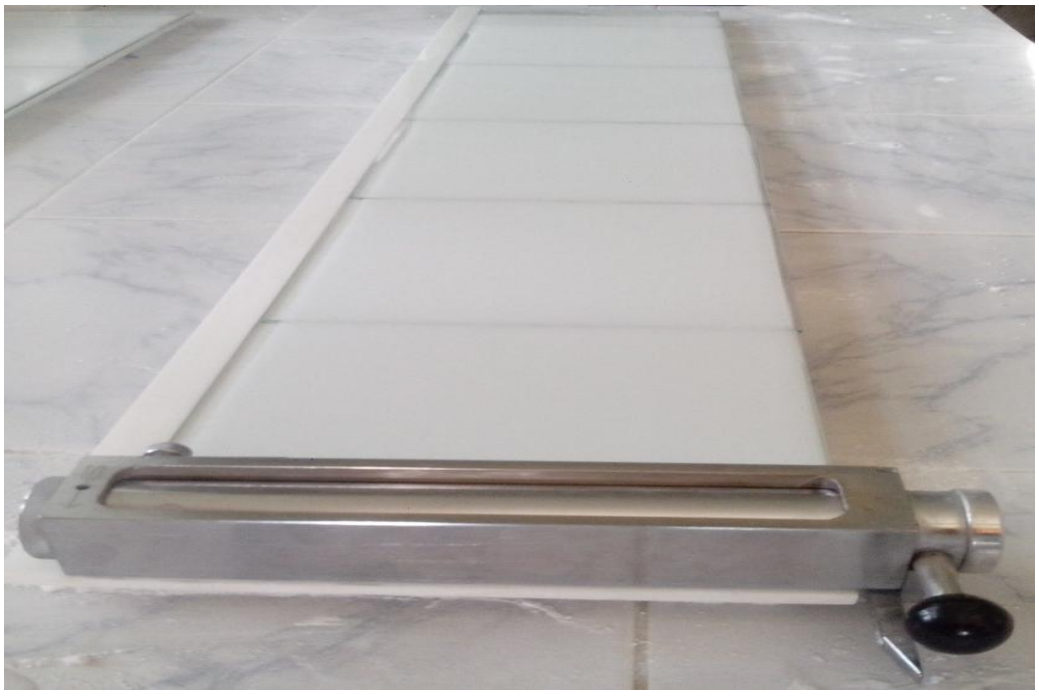


Figure-13 TLC glass plates were coated with the silica gel using spreader

Whole aqueous extracts, chloroform and aqueous fractions of the aqueous extracts were chromatographed. Powder form of both whole aqueous extract and aqueous fraction after partitioning were dissolved in water whereas chloroform extract was dissolved in chloroform at a concentration of 1mg /10µL and 5 µl of these samples were spotted with the help of capillary tubes on the TLC plates. The TLC plates were placed in a TLC tank, containing a mixture of solvents namely n-heptane , chloroform and ethanol in the ratio 50:50:10 (Fig-2). When the mobile solvent front rises up by capillary action, highly polar solutes remains at the initial spot whereas, the less polar solutes were carried upwards with the mobile phase. Once solvent front reaches the top of the plate, the TLC was stopped.

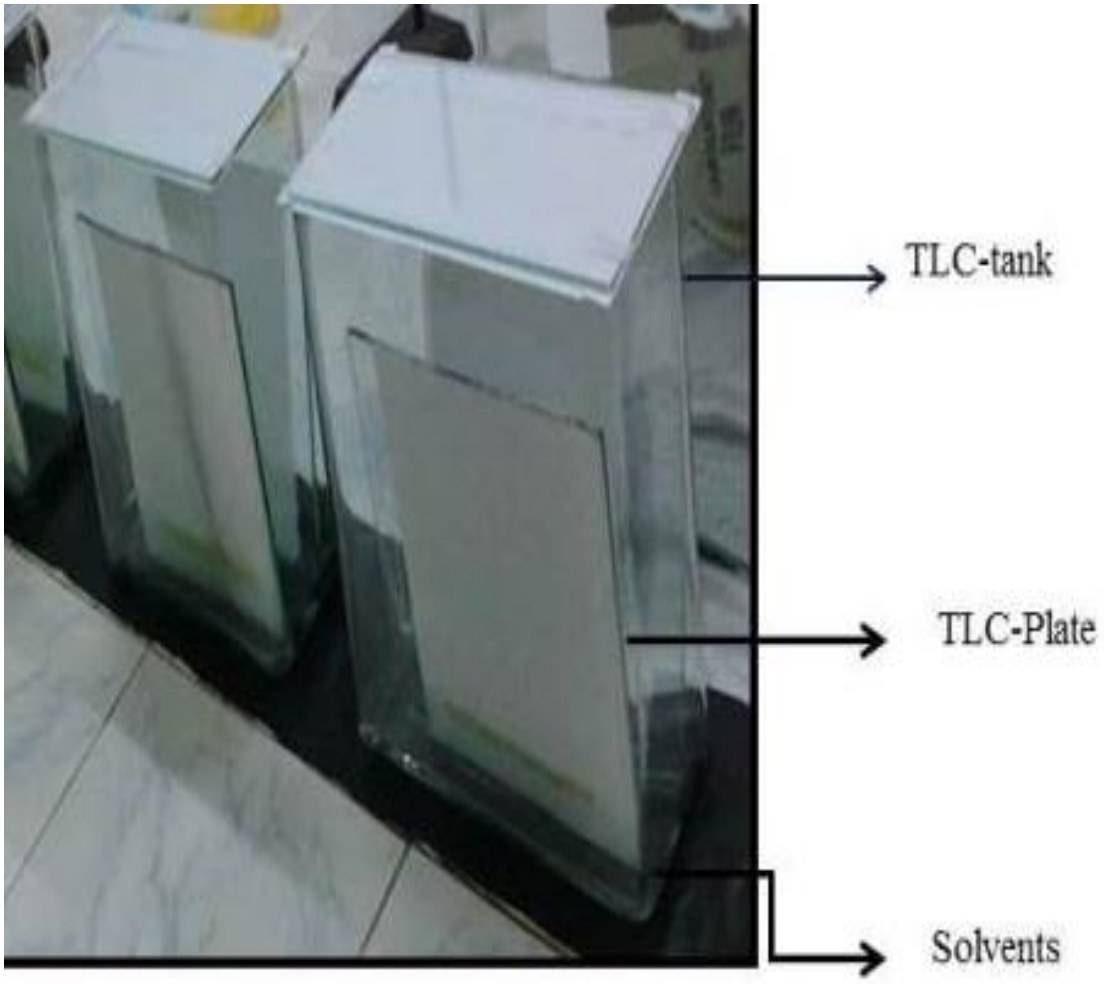


Figure 14 : Demonstrates TLC-tank with plates spotted with sample placed in solvent tank .

The plates were then visualized under Ultraviolet light in 352nm(Fig-15). The fluorescent compounds were seen as bands under the UV light. The non fluorescent compounds were not visible under UV light.

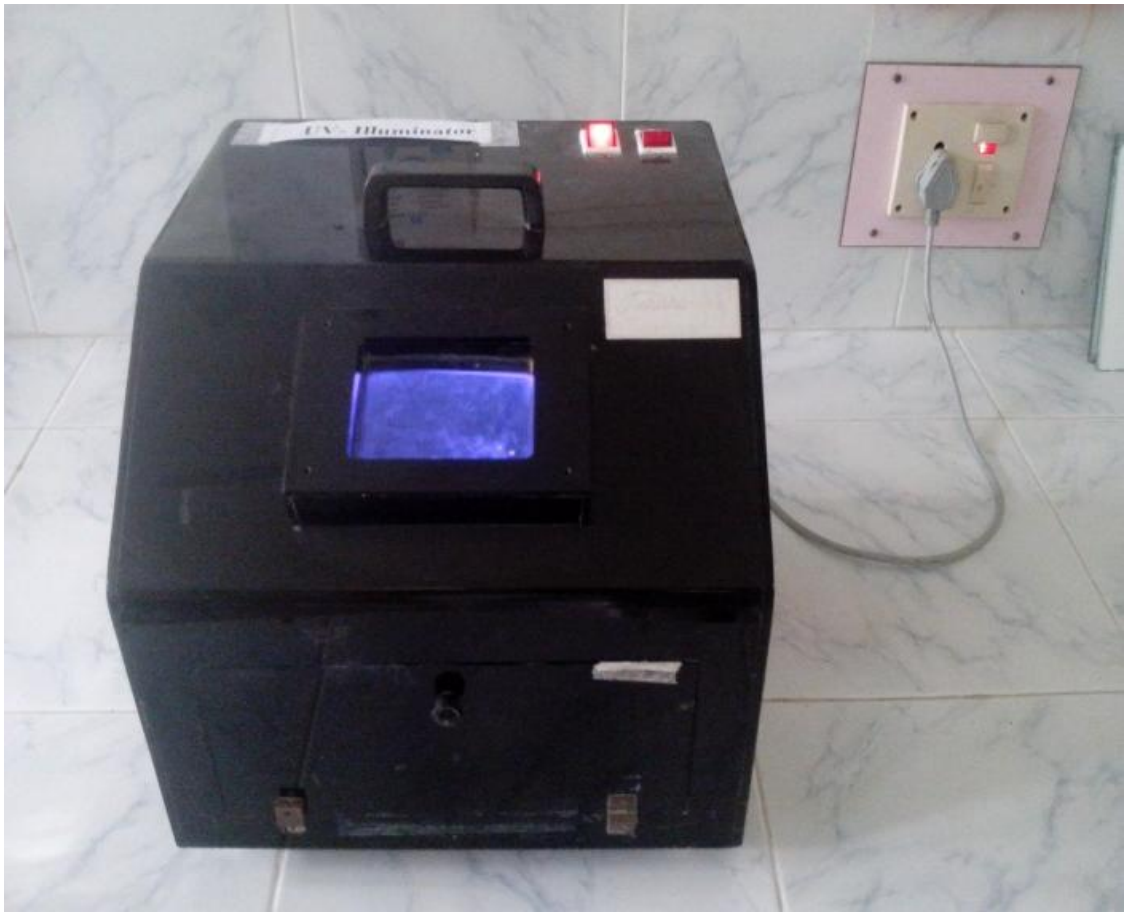


Figure -15: UV box

TLC results were interpreted in terms of Retardation factor (Rf value) which is the ratio of distance moved by the compound from the point of application to the distance moved by the solvent front from the point of application .The bands corresponding to Cleistanthins A & C and Diphyllin were marked and collected

based on the known Rf values for each of these compounds. The eluted material was dried and the compounds obtained were weighed for quantification.

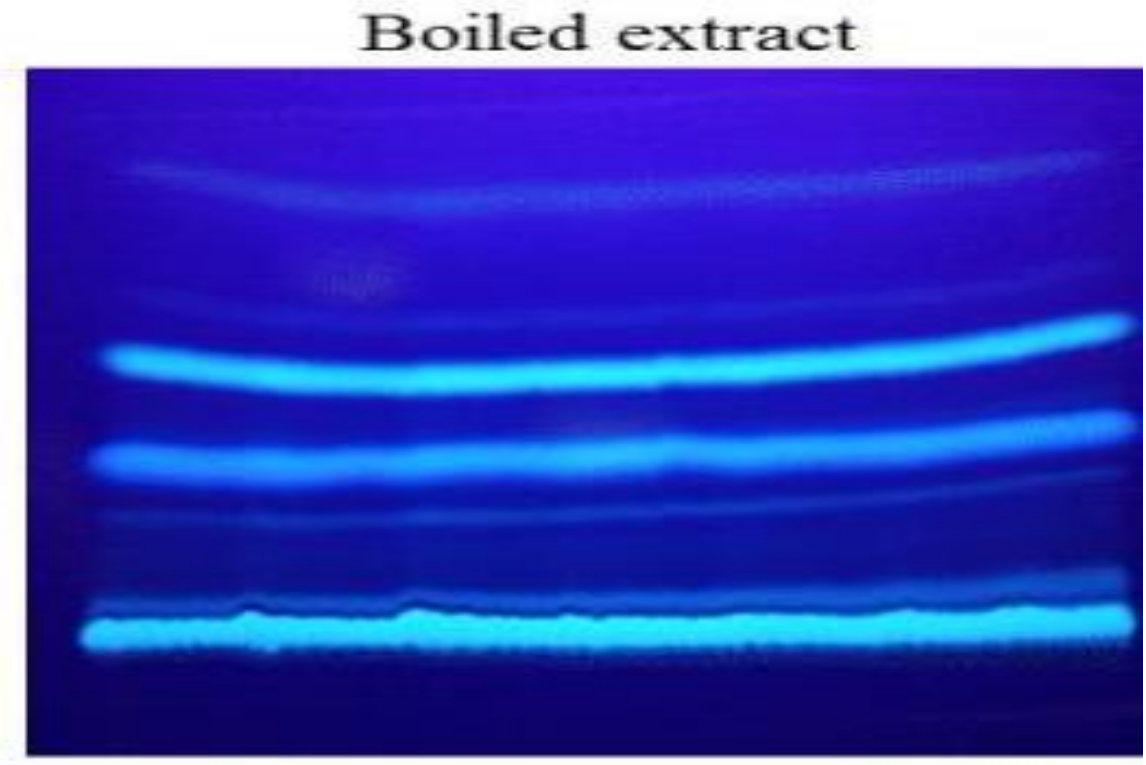


Figure-16 Thin layer chromatogram of fresh leaf boiled extract shows three prominent fluorescent bands of Cleistanthin-A, Diphyllin and Cleistanthin C in sequence from top to bottom.

In TLC, the compound with Rf value 0.34 as same as reported by K.S Annapoorani migrated similar to an authenticated sample of Cleistanthin A obtained from Godavari .

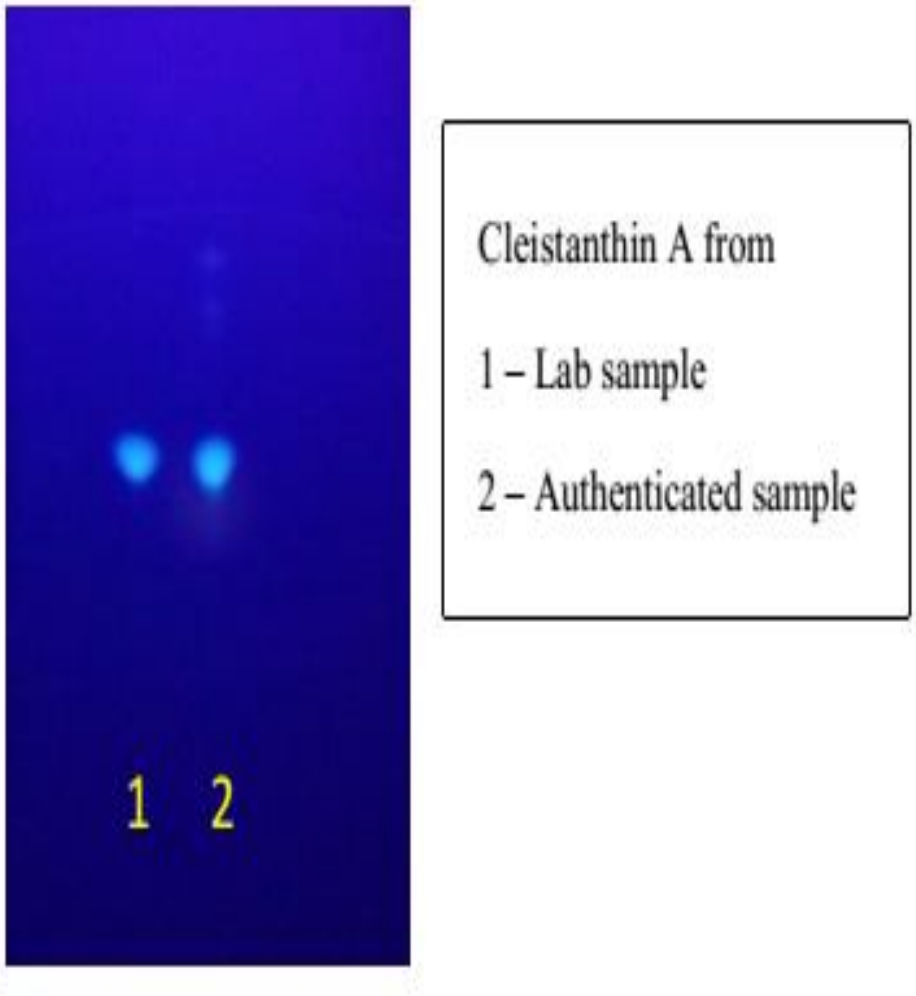


Figure 17: demonstrates that the fluorescent compound with Rf value 0.34 obtained by us migrates similar to an authenticated sample of Cleistanthin A provided by Godavari Biorefineries in TLC performed with Chloroform-n-heptane-ethanol (50:50:5). Rf stands for retardation factor .

High Performance Liquid Chromatography (Clinical Pharmacology Unit, CMC Vellore).

In preparatory thin layer chromatography, fluorescent bands corresponding to the reported Rf values were collected and purified further. The purity of the

compounds were checked with HPLC .

Reverse phase HPLC using C18 columns (Supelco HPLC column, Discovery® HS C(18) of size 25cm x 4.6mm, 5µm particle size) were used in a Shimadzu HPLC system with a fluorescent detector. Excitation and emission wavelenghts were λ_{exc} 320 nm and λ_{emis} 450 nm respectively.

The major fluorescent compounds which were isolated by preparatory TLC (Cleistanthin C and Cleistanthin A) were dissolved in ethanol at different concentrations (10, 20, 30 and 40 µg/ml for the cleistanthins) and loaded in the column to elicit HPLC chromatograms. The mobile phase was 70 % methanol. With "Area Under Curve" (AUC) for each concentration, standard curves were constructed (AUC versus concentration).

Chloroform fraction (CF) obtained from FLB was subjected to HPLC to (i) identify and (ii) quantify the major peaks detected by a fluorescence detector as these peaks were likely to be the major active principles in the respective extracts. The concentration of CF of the extracts used was 50 or 100 µg/ml.

The concentrations of the compounds in CF of fresh leaf boiled extract was calculated using the HPLC standard curves. Then, the amount of compound present in the extract from 100 gram leaves was calculated from the total weight of CF obtained from the boiled extract. In addition, the compounds separated by preparatory TLC from FLB was weighed to see if the weights matched the HPLC quantification, after allowing for reasonable losses during the TLC.

Chemical characterization of Cleistanthins A and C was performed at IIT Mandi.

Cleistanthin C and Cleistanthin A were thoroughly characterized using various spectroscopic tools that include 1H/13C-NMR, 2D NMR, FT-IR and high resolution mass spectroscopy. Though some characterization data for these compounds were available, they were not in detail. The 2D NMR spectra for Cle C were too complicated and therefore acetate derivative of Clei C was prepared which indeed showed much simpler spectra than its parent molecule.

X-ray Crystal Structure Analysis of Cleistanthin A (IIT Mandi)

Colourless needle shaped crystals of Cleistanthin A suitable for single-crystal X-ray diffraction analysis were obtained from slow evaporation of diethyl ether solution after 2 days. Data collection was done using CrysalisPro Software and reduction was undertaken with CrysalisPro Software. The structure was solved by direct methods using olex^2, SHELXS-97 and refined by full-matrix least squares

on F^2 using SHELXL-97. The positions of all the atoms were obtained by direct methods.

TOXICITY EXPERIMENTS

The primary goal was to find out the minimum dose of the following extracts which results in 100 % mortality. Experiments were designed in such a way that it mimics the patient scenario who comes with history of oduvanthazhai poisoning.

Extracts used : Fresh leaf boiled extract, cleistanthin C

Route of administration : Oral and Intraperitoneal routes

Methods used : Oral gavage with feeding tube

Mode of Anaesthesia : Inhalational, chloroform was used

Observational Period : 2 weeks

Parameter assessed : Mortality rate

Animals Used

Wistar rats weighing between 120 and 150grams were used. The rats were housed at the institutional animal housing facility in Christian medical college, Vellore under adequate conditions. Animals were fed with food and water ad libitum.Experimental protocol was approved by the Institutional Animal Ethics Committee. All animal experiments were done in accordance with the guidelines of the CPSCEA,India.

DETERMINATION OF LETHAL DOSE OF FRESH LEAF BOILED EXTRACT OF *C.COLLINUS*

Wistar rats were starved for 4 – 6 hours prior to the experiment.They were housed in different cages in groups of four. For toxicity studies, anesthetized rats were administered different doses of FLB extract dissolved in water by oral gavage and returned to their cages. Afterwards rats had access o food and water ad libitum. The animals were closely monitored for any kind of distress or any abnormal behaviour after intervention. Four rats in a group were used per dose and the different concentrations used were 100, 75, 50 and 25mg/100 gram bodyweight of the rat. Toxic doses killed the rats in 14 -18 hours duration. In the case of survivals, were observed for 2 weeks to decide if the administered fraction caused death or not. Approximate minimum lethal doses were determined for the whole extract using minimum number of animals. The procedures were approved by the institutional animal ethics committee.

DETERMINATION OF LETHAL DOSE OF FRESH LEAF GROUND EXTRACT OF *C.COLLINUS*

Different concentrations used were 25 and 50mg/100 gram body weight of the rat. The animals were closely monitored for any kind of distress or any abnormal behaviour after intervention and they will be housed for a duration of two weeks time.

DETERMINATION OF LETHAL DOSE OF CLEISTANTHIN C ISOLATED FROM *C.COLLINUS* EXTRACT

Cleistanthin C of various concentrations was administered and observed for a specified period.Oral gavage was employed to administer the toxin and looked for any immediate post interventional complications.Dose concentrations of 1, 2, 3 and 4 mg/100grm body weight were given. Another set of four rats was used for each dose to determine the minimal lethal dose of cleistanthin C which results in 100% mortality.

DETERMINATION OF LETHAL DOSE OF CLEISTANTHIN A ISOLATED FROM *C.COLLINUS* EXTRACT

As mentioned above, the same method was followed for Cleistanthin A also. Dose concentrations of 1, 3 and 4 mg/100grm body weight were given via oral gavage. Animals were observed for a period of two weeks.

"Investigation on non-fluorescent fraction (NFF) of cold water extract of *Cleistanthus collinus* leaves as an antidote to *Cleistanthus collinus*"

Preparation of non fluorescent fraction (NFF) extract of C.collinus

NFF to be tested as an antidote was prepared from fresh leaf ground water extract. Ice cold water was used instead of tepid water to preserve the action of the enzyme (putative anti C enzyme).100 grams of fresh leaves was ground in 1 litre of ice cold water using an electric blender to get a thick concentrate. To be precise, NFF was prepared from fresh leaf ground ice cold water extract(FLGICWE) so as to preserve its enzymatic nature if at all present. The crude material was centrifuged at 2400rpm for 20mins, filtered and transferred to glass beaker . Filtered extract was partitioned with chloroform immediately. As explained earlier, two immiscible layers were separated using separating funnel and fraction of concern, top water fraction called as non fluorescent fraction was collected in glass vials. Clearing step with chloroform was repeated many times to make sure that, the extract collected is devoid of all fluorescent compounds. During all the processing steps, care was taken to maintain cold temperature. 10

ml glass vials were filled with 5 ml filtrate and stored in -20 degree Celsius deep freezer prior to lyophilization .

Figure 18 : Fresh leaf ice cold ground extract before and after centrifugation

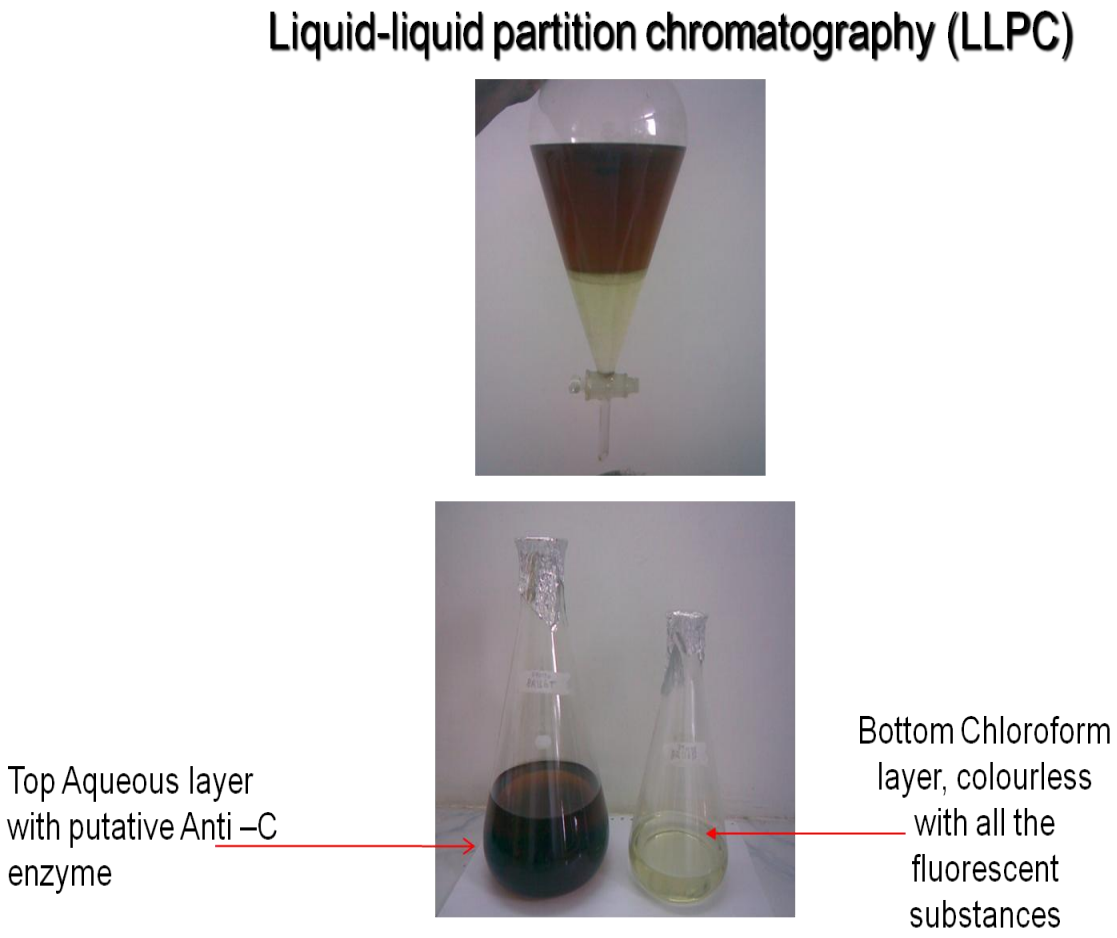


Figure 19 : LLP

Once samples are frozen, they were transferred to lyophilizing machine, kept aside for 24 hours to get fine powder of the extract. Leaf extract was weighed and stored in refrigerator for future use.

Figure 20: Freeze dryer / Lyophylizer

Figure 21 : Lyophylized powder

Thin layer chromatography was done to confirm that, the non fluorescent fraction of cold water extract was free from all known toxic cleistanthins.

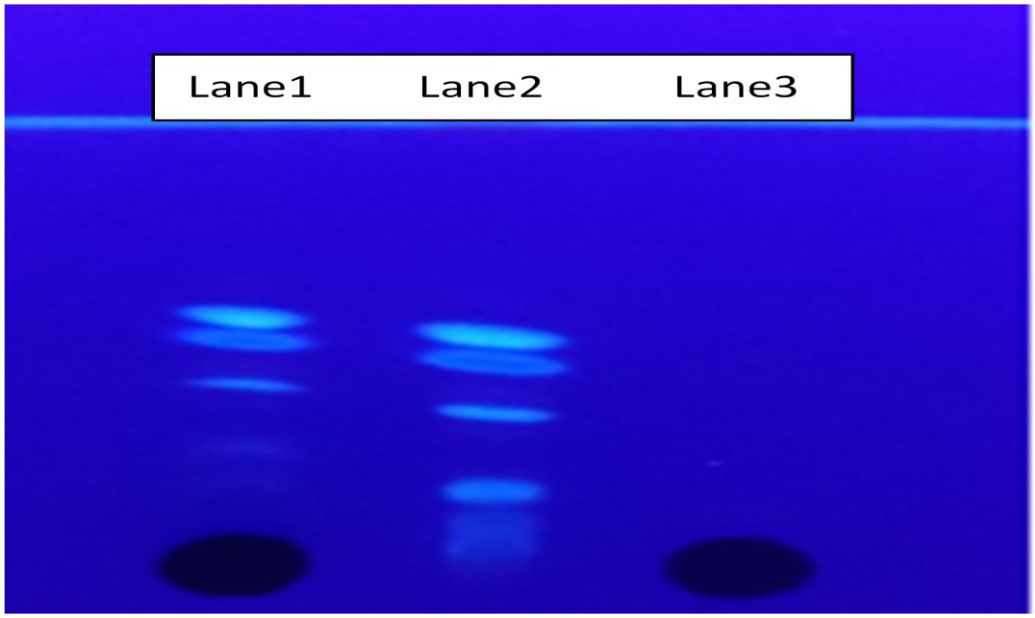


Figure 22: Thin layer chromatogram of fresh leaf ground extract of *C.collinus* .

From left to right-

Lane 1: Fresh leaf ground whole extract before partitioning (FLG)

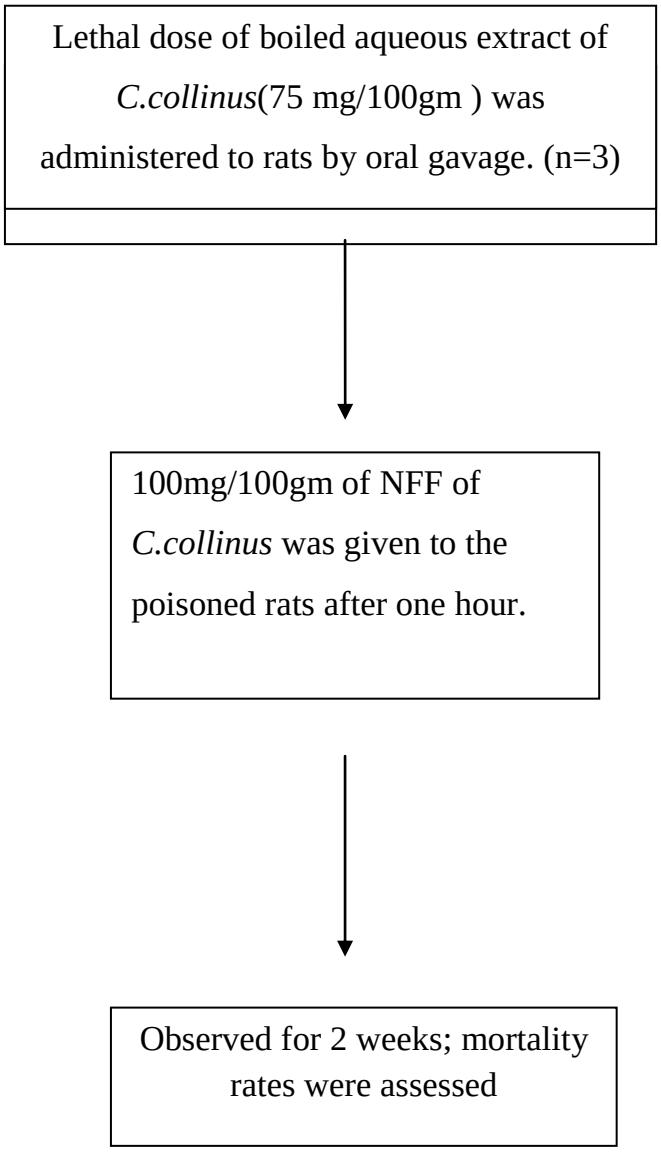
Lane 2: chloroform fraction of ground extract (FLG-CF)

Lane 3: water fraction of ground extract after chloroform partitioning (FLG-NFF).

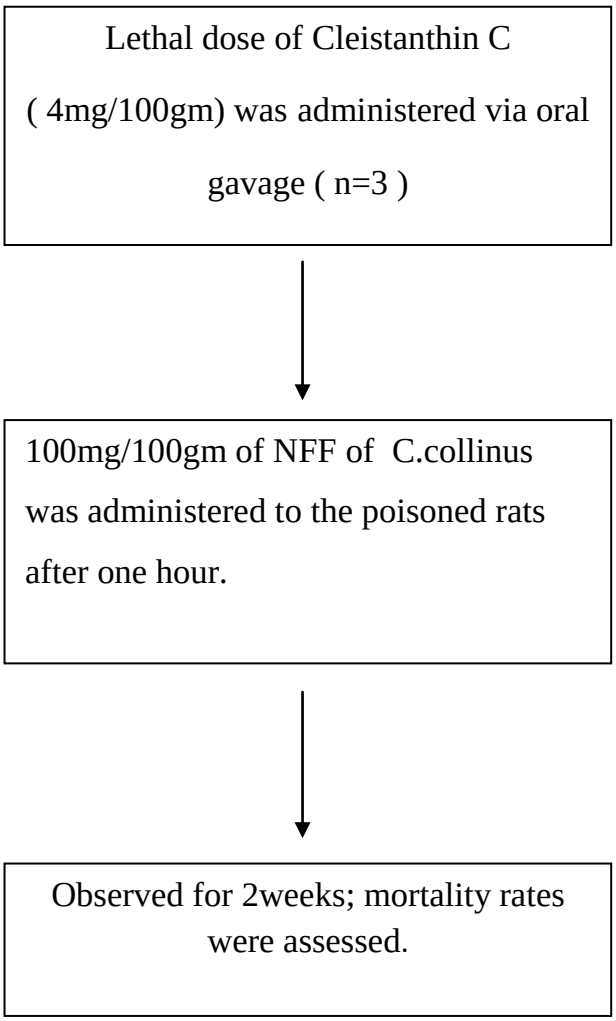
Water fraction after chloroform partitioning(lane 3) do not have fluorescence.

EXPERIMENTAL PROTOCOL TO TEST IF NON FLUORESCENT FRACTION CAN BE AN ANTIDOTE IN C.COLLINUS POISONING

SET 1 EXPERIMENTS - FRESH LEAF BOILED EXTRACT + NFF



SET 2 EXPERIMENTS – CLEISTANTHIN C + NFF



To prove the non lethality of non fluorescent fraction itself, rats were administered orally 200 mg of NFF per 100 gram body weight (n=2) and 5mg/100 GM (n = 2) intraperitoneally and observed for a period of 2 weeks.

In order to study the role of NFF, the same was administered to the rats poisoned with a lethal dose of boiled extract (75 mg /100 gm) or cleistanthin C (4 mg/ 100gm). More than 95 % dose of NFF was administered orally and animals were returned to the cages, closely monitored for a period of two weeks (n = 3 in each group). Maximum volume administered intraperitoneally was 0.3 ml at a time. Mortality rate and time to death was recorded.

The dose required to prove the antidote effect of non fluorescent fraction was not standardized due to various reasons. Main reason being no previous studies based on this concept. Secondly, intraperitoneal route was the chosen method of administration, but the final calculated dose was dissolvable only in relatively a large volume of solvent. The high volume after reconstitution was difficult to inject.

RESULTS

RESULTS OF LETHAL DOSE ESTIMATION OF DIFFERENT EXTRACTS AND COMPOUNDS ISOLATED FROM CLEISTANTHUS COLLINUS

1. Minimal Lethal dose Estimation of Fresh Leaf Boiled Extract of *Cleistanthus collinus*

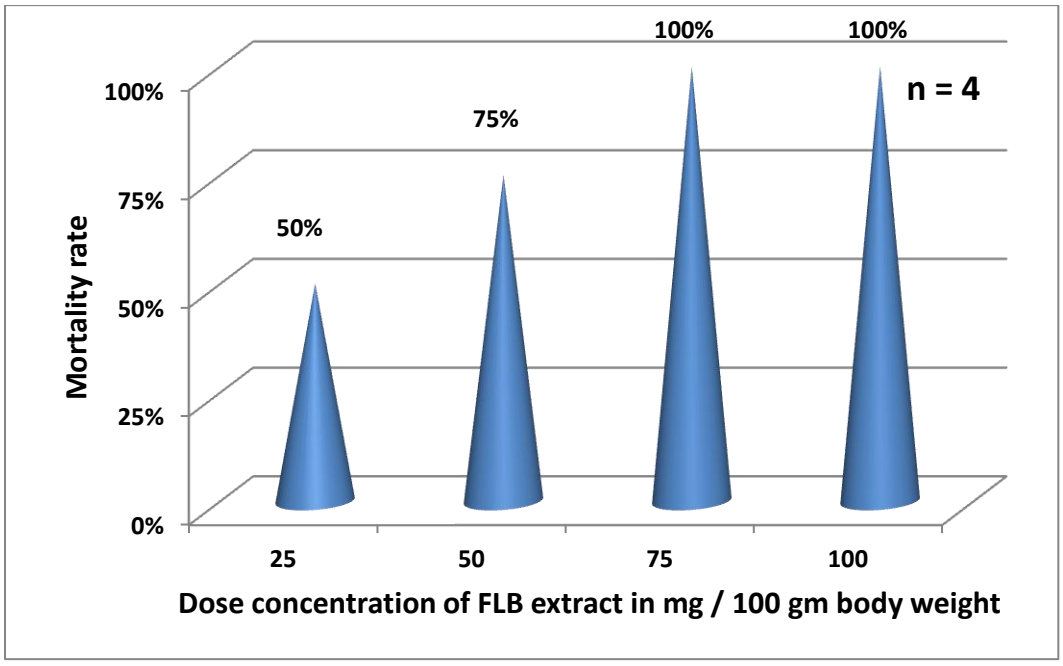


Figure 23: Graph shows minimal lethal dose (LD 100) of fresh leaf boiled extract of *cleistanthus collinus* (n = 4).

(x axis – dose concentration in mg /100 gm, y axis – mortality rate) .

2. Minimal Lethal dose Estimation of Fresh Leaf Ground Extract of *Cleistanthus collinus*

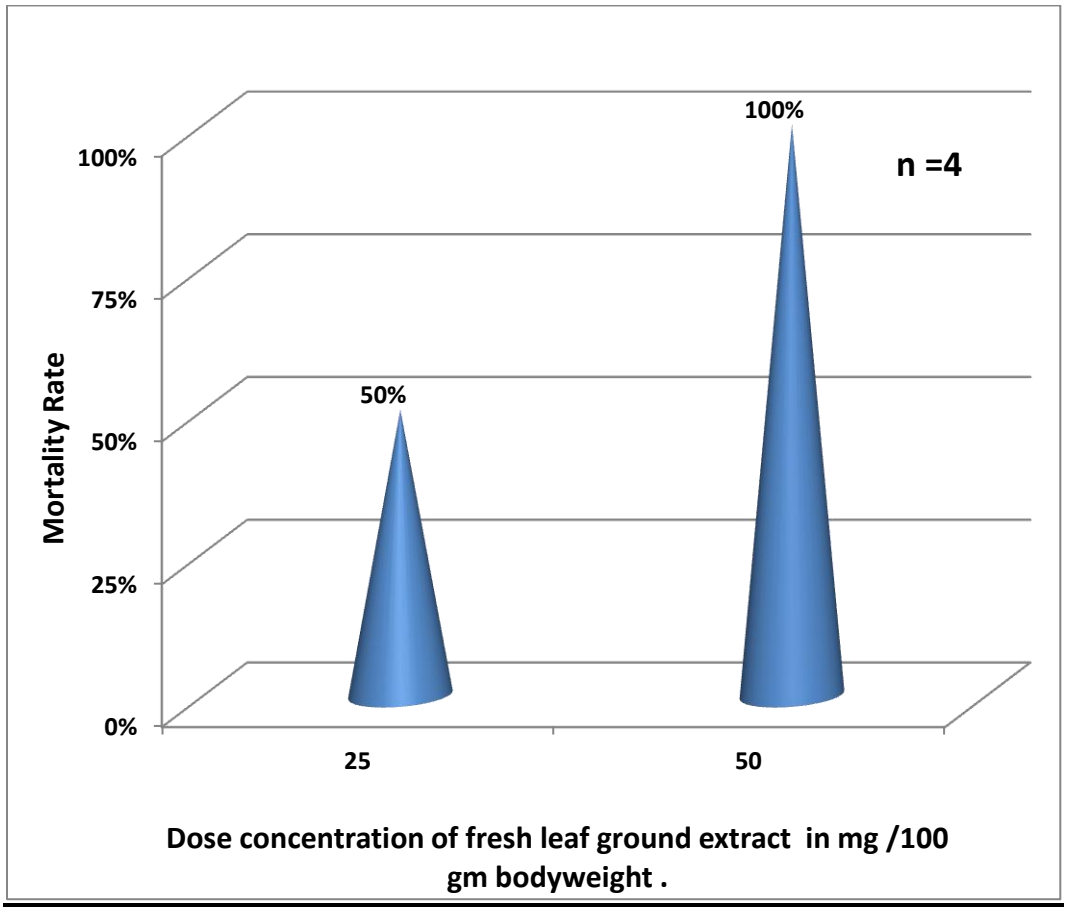


Figure 24 : Graph shows minimal lethal dose (LD 100) of fresh leaf ground extract of *cleistanthus collinus* (n = 4).

(x axis – dose concentration in mg /100 gm, y axis – mortality rate) .

3. Minimal Lethal dose Estimation of Cleistanthin C

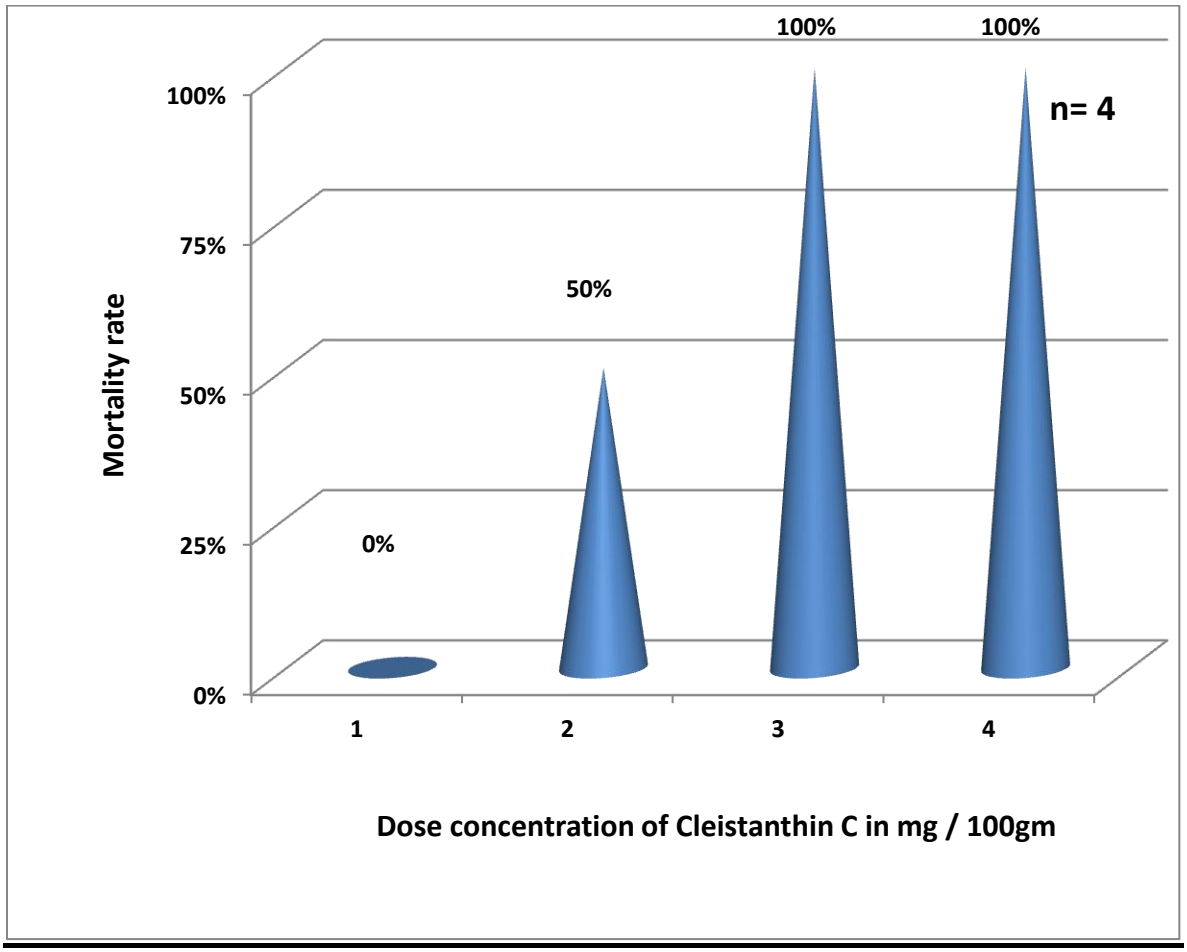


Figure 25: Graph shows minimal lethal dose (LD 100)of Cleistanthin C (n = 4).

(x axis – dose concentration in mg/100gm, y axis – mortality rate) .

4. Minimal Lethal dose Estimation of Cleistanthin A

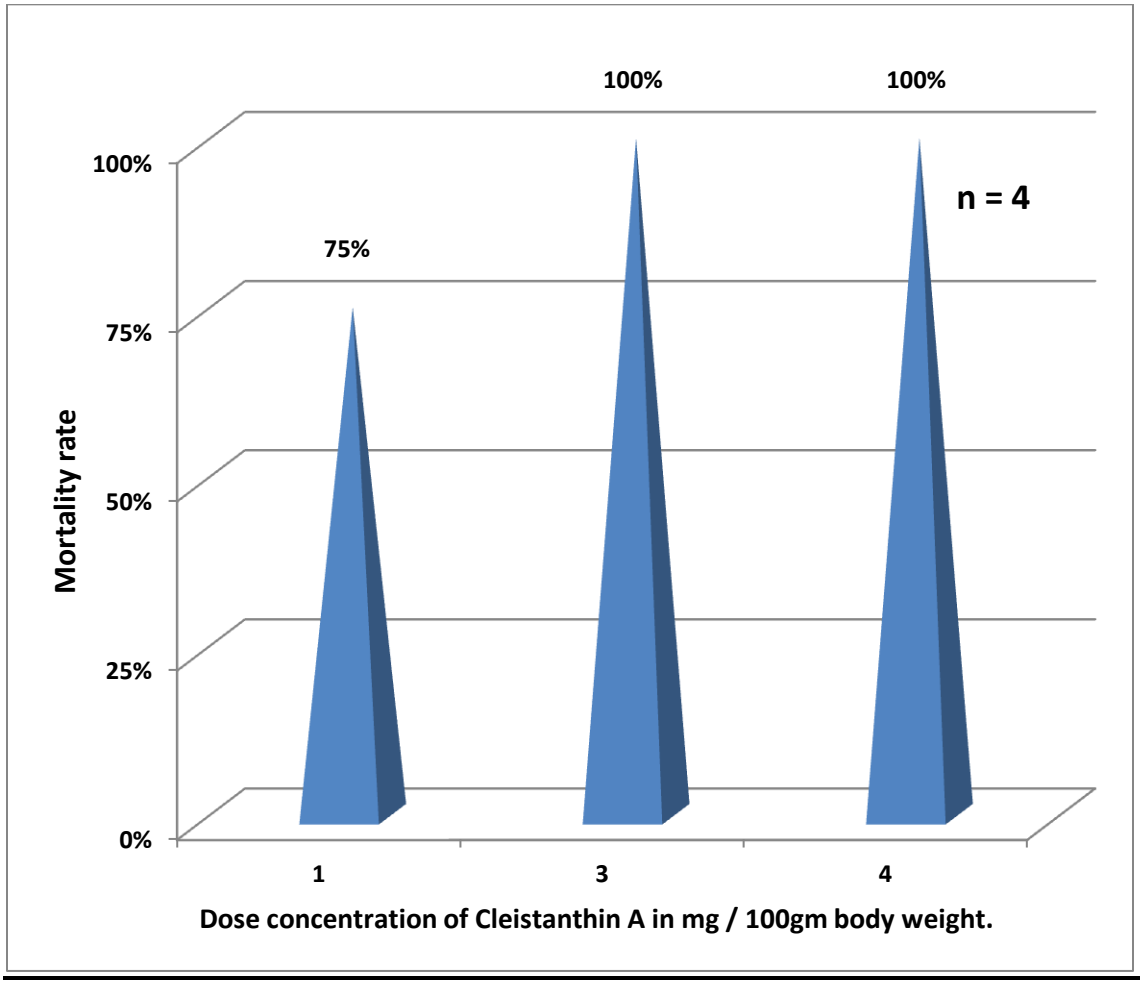


Figure 26 : Graph shows minimal lethal dose (LD100) of Cleistanthin A (n = 4).

(x axis – dose concentration in mg/100gm, y axis – mortality rate) .

The minimum lethal dose causing death in all animals for the toxic fractions tested is given in table 1.

Table 1: Lethal doses in rats per 100 gm body weight is summarized below:

Fraction tested	LD 100 Dose (n = 4)	LD 75 Dose (n = 4)	LD 50 Dose (n = 4)	Non lethal dose (n = 4)
Fresh leaf boiled extract (FLB)	75 mg	50 mg	25 mg	-
Fresh leaf ground extract (FLG)	50 mg	-	25 mg	-
Cleistanthin C	3 mg	-	2 mg	1 mg
Cleistanthin A	3 mg	1 mg	-	-

Results of the study on the antidote effect of non fluorescent fraction of cold water extract of *cleistanthus collinus*

1. **Cleistanthin C + Non fluorescent fraction of *cleistanthus collinus***

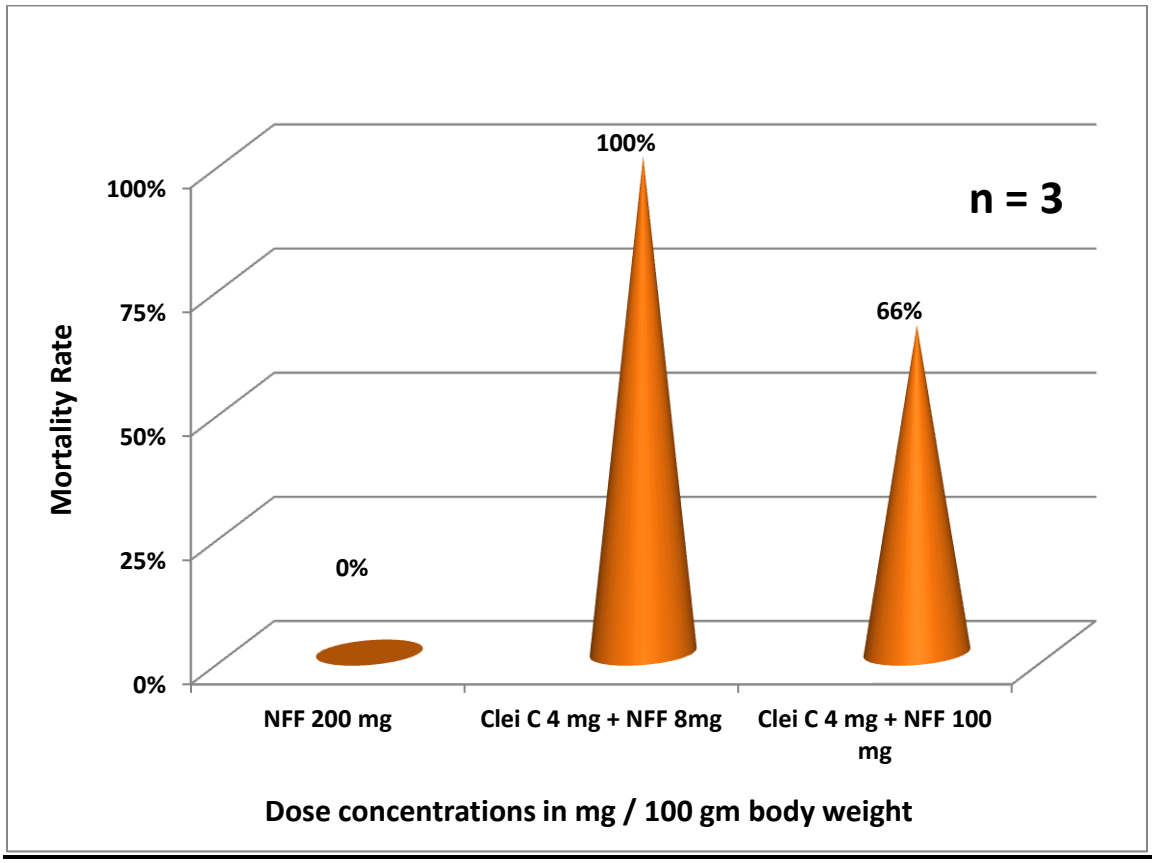


Figure 27: Graph shows the effect of NFF on Cleistanthin C poisoned rats (n = 3).(x axis – dose concentrations in mg /100gm body weight, y axis – mortality rate) .

2. **Fresh leaf boiled extract + Non fluorescent fraction of *Cleistanthus collinus***

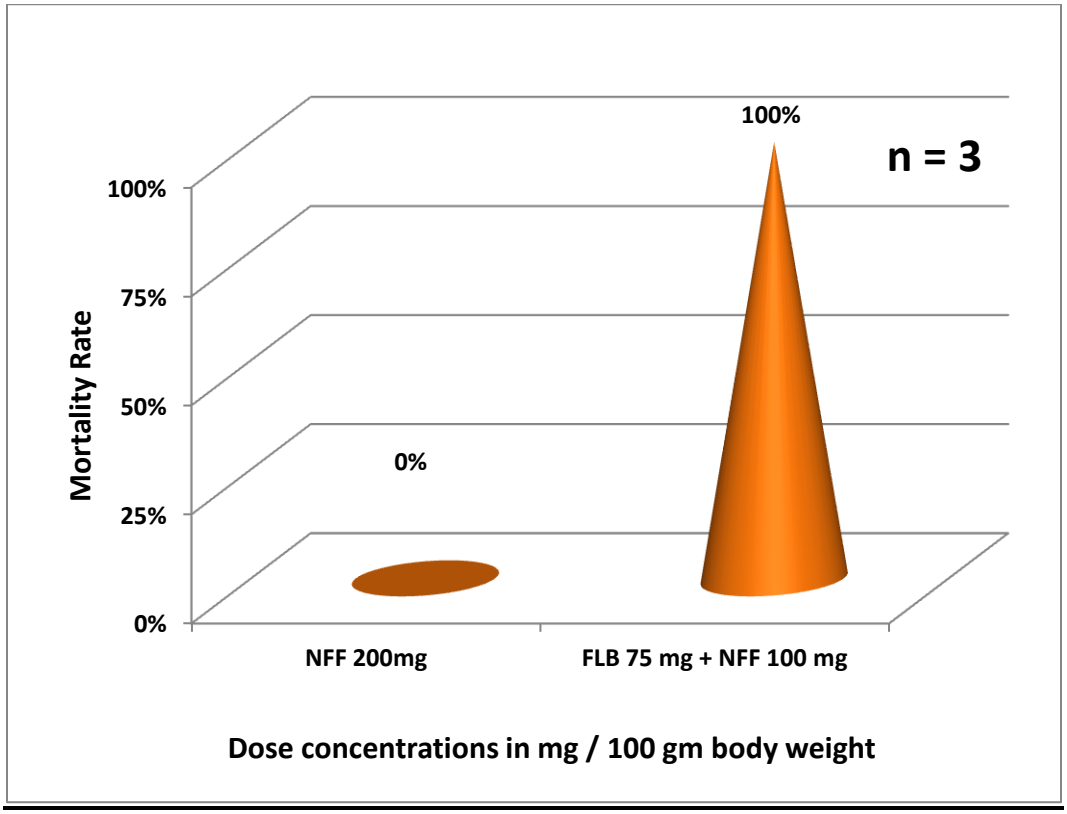


Figure 28: Graph shows the effect of NFF in rats poisoned with fresh leaf boiled extract of *Cleistanthus collinus* (n = 3).

(x axis – dose concentrations in mg /100gm body weight, y axis – mortality rate)

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Doses upto 200mg/100gm body weight of NFF orally (n=3) was nonlethal.

100 mg /100 gm body weight of NFF was not adequate enough to save the animal.

In fresh leaf boiled extract group , even though the mortality rate remains unaltered, time to death was found to be delayed in one animal (took around 40 hrs to die as against 14-16 hrs in other rats poisoned with FLB).

In Cleistanthin C administered group, one rat survived out of four rats.

As shown in the figures 27 and 28 non fluorescent fraction was found not to have any protective effect on mortality at the dose discussed previously. Therefore, it becomes critical to perform dose standardization for non fluorescent fraction (NFF) in order to consider it for a better outcome.

Pattern of Death

Animals that received acute dose of extracts were found to be restless, moving around the cage; with progress of time convulsions started which became intense and the animals died subsequently.

THIN LAYER CHROMATOGRAPHY: COMPOUND PROFILE

Thin layer chromatography was performed in order to demonstrate the difference between fresh leaf boiled and fresh leaf ground extracts of *Cleistanthus collinus* based on cleistanthin C fluorescence. As compared to the cleistanthin C fluorescence in fresh leaf boiled extract (Lane 2), it was observed that the Cleistanthin C fluorescence was almost nil in fresh leaf ground extract (Lane 4). Intensity of the fluorescence gives us a clue about concentration of different compounds. Chromatogram also provides us an overview about the compound profile in the different extracts of cleistanthus collinus .

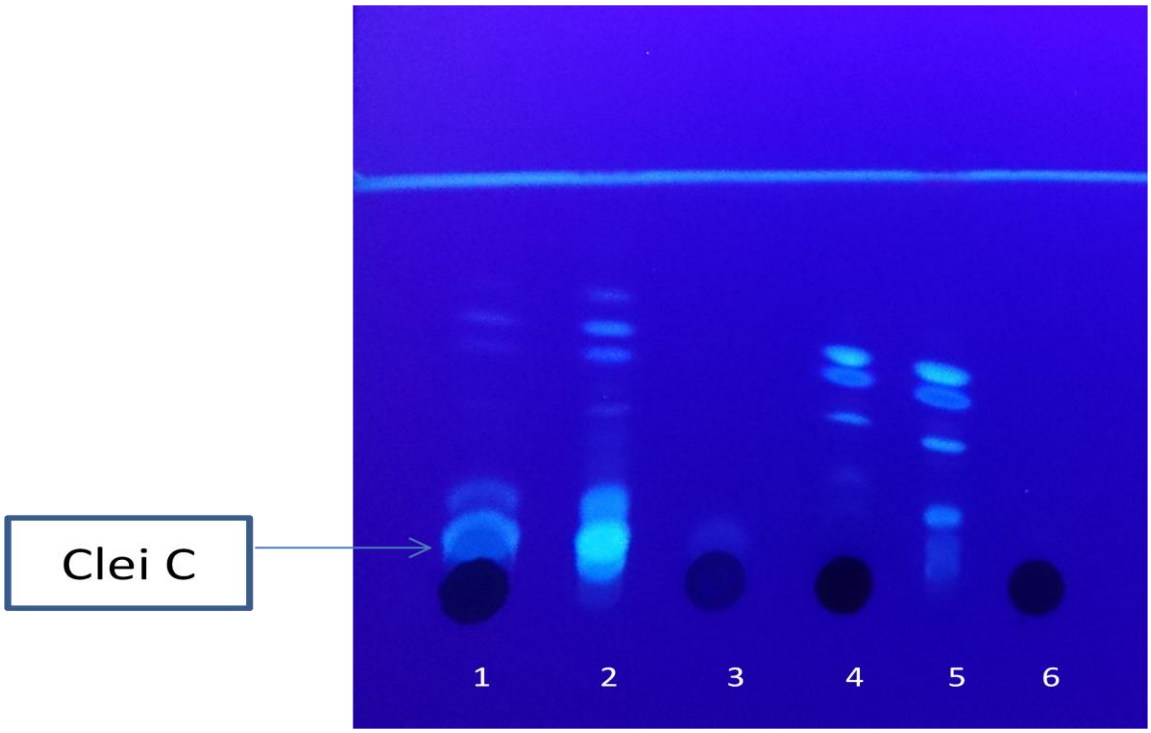
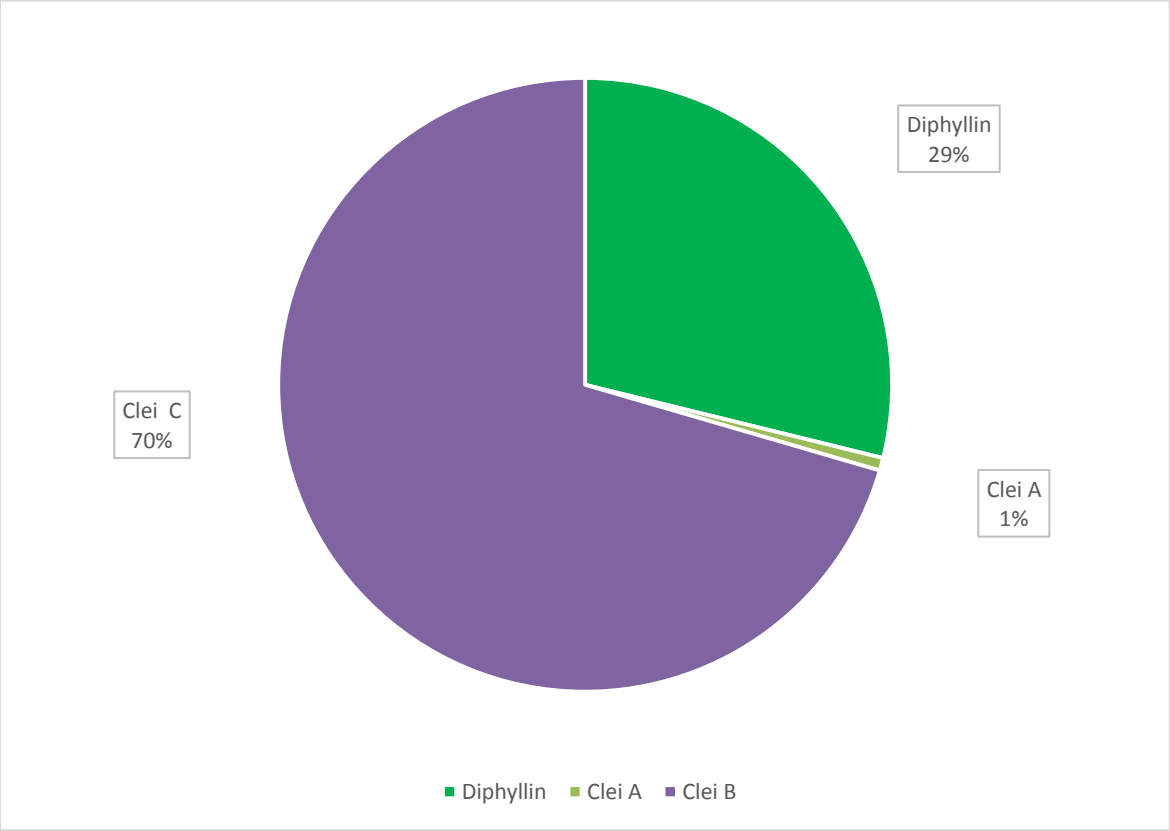


Figure 29 : Thin layer chromatogram of boiled extract (FLB, lane 1), chloroform fraction from boiled decoction (FLB-CF, lane 2), water fraction of boiled decoction after chloroform partitioning (FLB-NFF, lane 3), fresh leaf ground (FLG, lane 4), chloroform fraction from fresh leaf ground (FLG-CF, lane 5), and water fraction of fresh leaf ground extract after chloroform partitioning (FLG-NFF, lane 6). All the fluorescent compounds partition into chloroform (lanes 2 and 5). Water fractions after chloroform partitioning (lanes 3 and 6) do not have fluorescence.

Figure 30 : Percent distribution of fluorescent compounds in fresh leaf boiled extract prepared from 100 gms leaves from TLC data .



The amount of compound present in the extract from 100 gram leaves was calculated from the total weight of CF obtained from the boiled extract. Cleistanthin C constitutes the major part of the toxic fraction of fresh leaf boiled extract which can be accounted for the toxicity of the extract.

DISCUSSION

Cleistanthus collinus poisoning is common in rural south India. Water extract of *Cleistanthus collinus* leaves is a common suicidal poison. Victims consume either a boiled decoction (FLB, fresh leaves boiled) or fresh leaves blended and filtered. Mortality is about 28% and is stated to be higher with FLB than fresh leaf ground extract (FLG).

The leaves contain more than 20 fluorescent compounds(Pinho and Kijjoa, 2007). It has been documented that Cleistanthin A and B are toxic and cause death in rats (Annapoorani, Ph.D Thesis). Two other lignan lactones in aqueous extract of *C.collinus* namely collinusin and diphyllin were shown to be nontoxic (Annapoorani, Ph. D Thesis). It is therefore suggested that toxicity of *C.collinus* extract is primarily due to Cleistanthins A and B . We have also made similar observations in studies on rats (yet unpublished). However, it is also reported that Cleistanthin A and B are not present in the aqueous extracts of *C.collinus* leaves (Subramani et al., 2014.) and that purified Cleistanthin A and B do not cause death up to concentrations as high as 800 mg/kg bodyweight in rats (Parasuraman and Raveendran, 2012).

It is considered thus far that Cleistanthins A and B are the toxic principles responsible for death. But we found that (a) Cleistanthin B is not present in either extract. (b) Cleistanthin C is present in aqueous extract. Concentration of Cleistanthin C was very high in FLB, but was negligible in FLG (Unpublished

observation from a concurrent Ph. D Thesis). Whether this difference accounts for the higher toxicity of FLB in patients remains to be seen.

In the present study, toxins present in the clinically relevant *C.collinus* leaf extract, namely the boiled extract was investigated. Apart from boiled extract, purified cleistanthin C was also studied after confirming the identity of the compound. Toxicity profiles were also investigated.

The boiled aqueous extract of fresh leaves contains more than 7 fluorescent compounds. The identities of the two major fluorescent compounds are confirmed as Cleistanthin A, Cleistanthin C undoubtedly with the help of NMR. Apart from these known fluorescent fractions ,the identity of other unknown compounds are to be explored. NMR was performed in collaboration with chemistry department ,IIT Mandi to confirm the compound identity.

This is the first time that we report cleistanthin C as a clinically relevant toxin in *C. collinus* extract. All clinical studies published thus far mention Cleistanthin A & B as clinically important toxins. However we were unable to isolate cleistanthin C from the aqueous extract. Cleistanthin A & B are monoglycosides while cleistanthin C is a diglycoside. In studies where cleistanthin B has been studied for toxicity, the compound was isolated from an acetone extract. However we believe that, only toxins present in the aqueous extract are clinically relevant as this is the

form that patient consume. So we have focused on only the toxins which elute with water extract. The success of our approach in studying the toxins present only in water extract is due to a chance finding that chloroform partitioning of the water extract sequesters all the fluorescent compounds into chloroform. A previous MD thesis from the department (Dr. Swetha, MD.Thesis., 2014), found the water fraction after chloroform partitoning of aqueous extract to be non toxic. Therefore the toxin must occur in chloroform fraction.

An observation that is stated repeatedly is that fresh leaf boiled extract is more toxic than the fresh leaf ground. One objective in our lab was to show fresh leaf boiled extract differs from fresh leaf ground extract in terms of fluorescent compounds present in the chloroform fraction of water extract. In thin layer chromatography, fresh leaf ground does not contain cleistanthin C while fresh leaf boiled has it. This could be the reason why fresh leaf boiled is more toxic than fresh leaf ground.

In humans, it is probable that boiled decoction is more poisonous the fresh leaf ground extract, because first contains high amount of cleistanthin C.

However as the results of this study shows, the minimum lethal dose for fresh leaf boiled and fresh leaf ground are not very different in rats .

Quantification experiments done by a Ph.D student in our department (Synopsis submitted) show that cleistanthin C is the predominant lignan present in boiled

aqueous extract of *Cleistanthus.collinus*. Weights of cleistanthin A in Chloroform fraction of fresh leaf boiled extract were negligible. Based on the quantification done as part of the Ph.D thesis, I have estimated the probable weights of pure compounds present in minimum lethal doses of FLB and FLG. The estimated weights are given in table 2.

Table 2: Calculations for probable amounts of each of the three studied compounds in minimum lethal doses of aqueous extracts from HPLC data:

Minimum lethal dose for whole leaf extracts for 100 g BW	**Compound 1**	**Cleistanthin C**	**Cleistanthin A**
FLB 75 mg	**< 0.03 mg**	**9.6 – 10.3 mg**	**0.16 – 0.26 mg**
FLG 50 mg	**0.02 – 0.08 mg**	**0.23 – 0.61 mg**	**0.23 – 0.6 mg**

The major observations in this table are that the fresh leaf juice is as poisonous as the boiled decoction in rats. Even if species differences can be attributed to difference in humans and rats it still remains to be investigated as to why fresh leaf ground extract is toxic in rats. As shown in table 2,,the amount of cleistanthin C present in minimum lethal dose of fresh leaf boiled extract is considerable. It is infact double that of minimal lethal dose of cleistanthin C. Therefore all toxicity of fresh leaf boiled extract in rat can be attributed to cleistanthin C. However the amount of cleistanthin C in minimal lethal dose of fresh leaf ground extract is lower than non lethal dose of cleistanthin C.therefore cleistanthin C cannot be the toxin in FLG. Even the amount of cleistanthin A in minimal lethal dose of of FLG is extremely low and cannot account for the high toxicity of FLG in rats.
Therefore we conclude that, the toxicity of fresh leaf ground is neither due to cleistanthin A or C , but due to a yet unidentified compound.

In this study, we hypothesized that this water fraction (NFF) from the room temperature extract may contain an enzyme that destroys cleistanthin C (See study rationale). The experimental scheme was to use this fraction as an antidote in pure cleistanthin C poisoning and in poisoning with fresh leaf boiled extract which contains large amount of cleistanthin C.
The table below summarizes the LD_{50} and LD_{100} data for various fractions of *C.collinus* extracts as reported by K.S.Annapoorani (Annapoorani, Ph.D Thesis.)

Table 3:

Extract or fraction	Species	Route of administration	LD_{50} gm/kg	LD_{100} gm/kg
Whole aqueous extract (Boiled)	**Wistar rats**	**Oral**	**10.5 ± 1.25**	**13**
		Intraperitoneal	**2.5 ± 0.75**	**4.5**
		Intravenous	**2 ± 0.5**	**3.3**
Cleistanthin B	**Wistar rats**		**mg/kg**	**mg/kg**
		Oral	**10.0 ± 1.25**	**12.5**
		Intraperitoneal	**1.8 ± 0.48**	**4**
		Intravenous	**1.5 ± 0.6**	**3**

Sarathchandra et al. also report that 24 hr LD_{50} for boiled aqueous extract given orally was 7.5 gm /kg body weight for rat.

An alternate view found in literature is that the toxicity of C.collinus leaves is not attributable to Cleistanthins A and B, as these are not present in the aqueous extract of the leaves, and pure forms of the compounds do not cause death in rats upto doses as high as 800 mg/kg body weight (Parasuraman and Raveendran, 2012). LD_{50} for Cleistanthin A and B are 1200 and 1000 mg/kg respectively (Parasuraman and Raveendran, 2012) as per this report.

In comparison, we report that mld100 for both A and C is 3 mg/100 g BW or 30 mg/kg. It remains to be seen why Raveendran et al found even 800 mg/kg of Cleistanthin A non-toxic.

Animal experiments play a major role in the toxicological evaluation of drugs/ chemical substances. Acute toxicity studies constitute an essential part of such investigations. Rodents are mostly used as model system. Controversies still exist in the existing methodology for the determination of LD 100. The species and number of animals to be used vary with different groups of scientists. Use of large number of animals is not advocated since the results are not always reproducible even when experiments are repeated under controlled conditions. Hence the use of minimum number of animals and determination of probable range for LD100 rather than an accurate value are recommended. This point was taken into consideration in the present study.

Methods section provides information on the protocol for the toxicity experiments with the extract of *C.collinus*. Since chronic toxicity is not reported, all toxicity studies were carried out after administration of single dose of the extract / compound.

Minimum lethal dose (LD min) is the least amount of drug that can produce death in a given animal species under controlled conditions.

While determining the minimal lethal dose, it was seen that a dose of 30 mg/kg body weight of cleistanthin C, when administered by means of oral gavage caused 100% mortality in all test rats (n = 4). On the other hand, a dose of 750 mg/kg body weight of fresh leaf boiled extract gave similar results and caused death in all test animals (n = 4). Similar experiments conducted for minimal lethal dose estimation for Fresh leaf ground extract and cleistanthin A yielded the following results. For fresh leaf ground extract , 500 mg/ kg body weight achieved 100% mortality (n = 4), whereas a dose of 30 mg/ kg body weight was required for Cleistanthin A (n = 4).

As discussed earlier, non fluorescent fraction was believed to contain an antidote that could potentially attenuate toxicity mediated by Cleistanthin C. But before any experiments could be performed to confirm its antidotal property, non fluorescent fraction needed to be analyzed to prove that it was non toxic in

isolation. Non fluorescent fraction when given orally at a dose of 200mg/100gm (n=3) was seen to be nontoxic. (For conducting experiments using NFF as the antidote and fresh leaf boiled extract as the toxin, a dose of 100 mg / 100 gm body weight was used. For experiments where cleistanthin C was the toxin in question, NFF at the doses of 8mg/ 100 gm and 100 mg / 100gm body weight was used).

When experiments were conducted using fresh leaf boiled extract of C. collinus as the toxic agent, it was observed that the mortality rate remained unchanged even after administration of 100mg/100gm NFF. One observation from this group is worth mentioning: of the 3 animals used for the experiment, delayed death was observed in one. Reasons for 100% mortality can only be speculated. One cause may be the additional presence of Cleistanthin A in fresh leaf boiled extract that confers additional toxicity to the isolate over and above the ill effects of Cleistanthin C.

Mortality rate did not show any improvement when NFF was used as an antidote at a dose of 8 mg/ 100gm body weight to rats poisoned with cleistanthin C. But when the dose of NFF was increased to 100 mg/ 100 gm body weight , a reduction in mortality was seen as one out of three rats survived.

Prolongation of time of death in fresh leaf boiled group and survival of one rat in Cleistanthin C administered group after administering NFF, may be due to

neutralization of the toxins in the gastrointestinal tract by the “unknown compound- putative anti C enzyme” present in the NFF or by an inherent detoxification mechanism present in the experimental animal.

A bigger sample size and a more elaborate study in this area will open new avenues in toxicology. This will aid in unravalling mechanisms by which plant poisons act and the discovery of their potential antidotes.

CONCLUSION

CONCLUSIONS

Lethal dose estimation of different clinically relevant extracts of *C.collinus* were done by using minimum number of animals in an iterative manner. Minimal lethal dose which caused 100 % mortality for fresh leaf boiled and fresh leaf ground extract of *C.collinus* was found to be 75mg and 50 mg per 100 gram body weight of the rat respectively. The major observations are that the fresh leaf juice is as poisonous as the boiled decoction in rats. Even if species differences can be attributed to difference in humans and rats it still remains to be investigated as to why fresh leaf ground extract is toxic in rats. MLD 100 of both Cleistanthin A and C was as low as 3mg / 100 gram body weight. The amount of cleistanthin C present in minimum lethal dose of fresh leaf boiled extract is considerable. It is infact double that of minimal lethal dose of cleistanthin C. Therefore all toxicity of fresh leaf boiled extract in rat can be attributed to cleistanthin C. However the amount of cleistanthin C in minimal lethal dose of fresh leaf ground extract is lower than non lethal dose of cleistanthin C.therefore cleistanthin C cannot be the toxin in FLG. Even the amount of cleistanthin A in minimal lethal dose of of FLG is extremely low and cannot account for the high toxicity of FLG in rats. Therefore we conclude that, the toxicity of fresh leaf ground is neither due to cleistanthin A or C , but due to a yet unidentified compound.

Non fluorescent fraction studies as an antidote was not able to standardize due to multiple reasons. Any how ,we were able to do a set of animal experiments as per our protocol and we show NFF of 200mg/ 100gm bodyweight when given orally is nontoxic. As an antidote, its role has to explored.

LIMITATIONS

LIMITATIONS

1.The number of study rats have to be increased.

2. Dose of NFF, timing of administration, route of administration has to be standardized.

3. Non lethal dose of Cleistanthin A has to be determined.

FUTURE

PROSPECTS

FUTURE PROSPECTIVES

1. Determination of mechanism of toxicity of Cleistanthin C .
2. Isolation and characterization of two other unknown compounds causing prominent fluorescent bands on thin layer chromatogram of fresh leaf ground extract.
3. Study of toxicity profile of newly identified compounds.
4. Development of antidote and treatment strategies for *Cleistanthus.collinus* poisoning.

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INSTITUTIONAL ANIMAL ETHICS COMMITTEE (IAEC)

CHRISTIAN MEDICAL COLLEGE

VELLORE, INDIA

Dr. Alfred Job Daniel
Principal & Chairman

Dr. Solomon Sathishkumar
Secretary

23rd December 2013

Dr. Neetu Prince,
PG Demonstrator,
Department of Physiology.

Dear Dr. Neetu,

Your research proposal titled "Investigation on non-fluorescent fraction (NFF) of cold water extract of Cleistanthuscollinus leaves as an antidote to Cleistanthuscollinus" has been approved by the Institutional Animal Ethics Committee (IAEC).

After discussion, 44wistar rats have been approved for the study.

The IAEC approval number for the study is 23/2013.

You are required to maintain all records as per form D, ensure humane treatment of animals and submit a final report to the IAEC.

With best wishes,

Yours sincerely,

Dr.Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc: Dr. Solomon Sathishkumar
Secretary, IAEC

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDIN)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

November 03, 2013

Dr. Neetu Prince
PG Demonstrator
Department of Physiology
Christian Medical College
Vellore 632 002

Sub: **Fluid Research grant project**: (Animal Study)
Investigation on non-fluorescent fraction (NFF) of cold water extract of Cleistanthus collinus leaves as an antidote to Cleistanthus collinus?
Dr. Neetu Prince, PG Demonstrator, Physiology, Dr. Sathya Subramani, Physiology, A. Soosai Manickam, Physiology.

Ref: IRB Min. No. 8442 dated 10.09.2013

Dear Dr. Neetu Prince,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Investigation on non-fluorescent fraction (NFF) of cold water extract of Cleistanthus collinus leaves as an antidote to Cleistanthus collinus?" on September 10, 2013.

The Committees reviewed the following documents:

1. Format for IRB application
2. CV's of Drs. Neetu Prince, Satya Subramani, A.Soosai Manickam.
3. No of documents 1-2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on September 10, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Name	Qualification	Designation	Other Affiliations
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Colorectal Surgery, CMCH.	Internal, Clinician
Dr. Anup Ramachandran	Ph. D	The Wellcome Trust Research Laboratory Gastrointestinal Sciences, CMCH.	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMCH.	Internal, Clinician
Dr. Rajesh Kannangai	MD, Ph D.	Professor & In-charge Retrovirus Laboratory (NRL under NACO), Clinical Virology, CMCH.	Internal, Clinician
Dr. Paul Ravindran	PhD, Dip RP, FCCPM	Professor, Radiotherapy, CMCH.	Internal, Clinician
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerology & Leprosy, CMCH.	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), FCCP (USA)	Professor, Pulmonary Medicine, CMCH.	Internal, Clinician
Dr. Anil Kuruvilla	MBBS, MD, DCH	Professor, Child Health, CMCH.	Internal, Clinician
Dr. Simon Rajaratnam	MBBS, MD, DNB (Endo), MNAMS (Endo), PhD (Endo), FRACP	Professor, Endocrinology, CMCH.	Internal, Clinician
Dr. Bobby John	MBBS, MD, DM, Ph D, MAMS	Professor, Cardiology, CMCH.	Internal, Clinician
Dr. Ashok Chacko	MD, DM, FRCP, FRCPG,FIMSA, FAMS	Director, Institute of Gastroenterology and Liver Disease, Madras Medical Mission, Chennai	External, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMCH.	Internal, Legal Expert
Dr. Vathsala Sadan	M.Sc, PhD	Professor, Community Health Nursing, CMCH.	Internal, Nurse
Mr. Joseph Devaraj	B. Sc, BD	Chaplaincy Department, CMCH.	Internal, Social Scientist
Mr. C. Sampath	B. Sc, BL	Legal Expert, Vellore	External, Legal Expert
Mrs. Amala Ranjan	M Sc, RN, RM	Professor, Medical Nursing, CMC	Internal, Nurse
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Nihal Thomas	MD.MNAMS DNB (Endo)FRACP (Endo) FRCP(Edin) (Glasg)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	Internal, Clinician

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 76,500/- INR (Rupees Seventy Six Thousand Five Hundred only) will be granted for 2 years.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in