ABSTRACT

Cleistanthus collinus is a poisonous plant, the leaves of which are consumed for suicide in many parts of southern India. Exact mechanism of toxicity remains unclear. Previously, two toxic principles present in this plant namely, Cleistanthin A and Cleistanthin C were tested for proton channel blockade in human neutrophils. While neither compound blocked proton channels, the impression was that Cleistanthin A may actually enhance proton currents, though the increase was not statistically significant. Since ATP was not present in the pipette in the earlier experiments, we hypothesized that in the presence of ATP, Cleistanthin A may enhance proton currents significantly. This data is important because it will help us establish this effect as the mechanism of toxicity leading to respiratory arrest in cases of *Cleistanthus collinus* poisoning.

AIM: To study the effect of Cleistanthin A on voltage gated proton channels of human neutrophils, with ATP included in the patch pipette.

OBJECTIVES

1. To record proton currents in freshly isolated human neutrophils using Patch clamp technique.

2. To compare the percentage of proton currents remaining after the addition of the test (Cleistanthin A) and control solutions (ethanol), as compared to the currents recorded before the addition, while ATP was included in the patch pipette.

METHODS:

- Isolation of fresh human neutrophils
- Isolation of Cleistanthin A
- Recording proton currents in isolated neutrophils by Patch Clamp technique
- Analysis of the percentage of proton currents remaining after the addition of the test and control solutions in comparison to currents recorded before addition

RESULTS: When the proton currents recorded at different voltage-clamp levels, after the addition of either Cleistanthin A (test) or ethanol (control), were expressed as percentages of the pre-intervention currents at the same voltages, there was no statistically significant difference in the percentage current remaining after intervention in both groups.

CONCLUSION: The results indicate that even in the presence of ATP, Cleistanthin A does not show any significant effect on voltage gated proton channels of human neutrophils.

INTRODUCTION

TOXICOLOGY

Toxicology, commonly described as "*the science of poisons*" is a multidisciplinary field that focuses on the adverse effects of toxic substances on living organisms. It includes the study of symptoms that occur on ingestion of the toxin, the mechanism by which the toxin affects the body, detection of the toxin in the body, and appropriate treatment to be given. The relationship between dose of the toxin and its effects on exposure is of high significance in this field. The historical development of this field started with the ancient cave dwellers who identified poisonous animals and plants and made use of their extracts for hunting and in warfare. With time, poisons became biological weapons. Famous poisoning victims include Cleopatra and Socrates.

A growing incidence of deaths due to suicide has been recorded in many parts of India. About 800,000 suicidal deaths have been reported worldwide every year(1). Of these, 135,000 (nearly 17%) are Indians(2). Reports from the National Crime Records Bureau show that Tamil Nadu, Andhra Pradesh and West Bengal have consistently reported a higher number of deaths due to suicide(3). As reported by Bose et al, in India 19% of the suicidal deaths were due to consumption of plant poisons(4). Among the plant poisons, yellow oleander (*Thevetia peruviava*) and *Cleistanthus*

collinus (*Oduvanthalai in tamil*) were the most commonly consumed plants(4).

Plant toxins are varied and complicated, so many varieties exist. Some plants toxins are fatal, while many are not. Some plant toxins are destroyed by cooking, while others are not. Ingestion of most of the common and identified plants does not result in fatal illness. Humans have been poisoned only by ingestion of misidentified plants, or by accidental ingestion of known poisonous plants. People also consume poisonous plants for suicidal purposes.

The biggest hassle with plant poisoning is that their toxicity depends on which parts of the plant one consumes. Another pitfall is that there are so many plant toxins for which there are no available antidotes. The lack of antidotes is a reflection of lack of knowledge about the mechanism of action of these plant toxins. The present study is a step towards finding the mechanism of toxicity of a most commonly consumed poisonous plant named ***Cleistanthus collinus.***

Cleistanthus collinus, is a toxic shrub. It belongs to the genus Cleistanthus and family Euphorbiaceae(5). This plant is commonly seen in the hilly forests of South India, Africa and Malaysia(6)(7). The leaves of the plant are usually boiled and consumed as decoction for suicidal purposes. On admission to hospital, patients present with vomiting,

headache, dyspnoea, tachypnoea, hypotension and weakness. Laboratory findings are as follows, hypokalemia, hyponatremia, hyperbilirubinemia, elevated AST/LDH/CPK/CPK-MB and metabolic acidosis. Life threatening complications such as cardiac arrhythmias, neuromuscular weakness, respiratory and renal failure were also reported(8).

Although there are a variety of clinical presentations of the poisoning, the changes the toxin produced at cellular level, propelled us to design and carry out this experiment. This study was designed to study the effects of one of the toxic compounds present in the plant on voltage gated proton currents of human neutrophils. The study was done using patch clamp technique.

What is patch clamp technique?

The word “patch” denotes a small piece or patch of cell membrane and the word “clamp” has an electro-technical significance. The patch clamp technique is a widely accepted standard method for fundamental studies of various ion channels. The technique requires expensive equipment and also intensive technical skills.

Why voltage gated proton channels?

In previous studies done in our department, proton channel modulating activities of whole aqueous extract, Cleistanthin A, and Cleistanthin C have been tested by patch clamp experiments on neutrophils. It was found that the whole aqueous extract blocked proton channels. Later, two toxic principles Cleistanthin A and C were tested for proton channel blockade in human neutrophils. While neither compound blocked proton channels, the impression was that Cleistanthin A may actually enhance proton currents, though the increase was not statistically significant. Since ATP was not present in the pipette in the earlier experiments, we hypothesized that in the presence of ATP, Cleistanthin A may enhance proton currents significantly. This data is important because it will help us establish this effect as the mechanism of toxicity leading to respiratory arrest in cases of *Cleistanthus collinus* poisoning.

Why human neutrophils?

We have chosen human neutrophil as a study cell for the following reasons,

a) Neutrophils express the proton channels on their membrane

b) Neutrophils can be isolated easily from blood

REVIEW OF LITERATURE

INTRODUCTION TO PATCH CLAMP TECHNIQUE

The patch clamp technique is a sophisticated technology for studying single or multiple ion channels. Patch clamping is a laborious process, and requires a skilled experimenter to handle the micropipettes and the cell in a single field under a microscope and to make recordings from cells.

APPLICATIONS OF PATCH CLAMP TECHNIQUE

Patch clamp studies can answer a huge variety of unanswered physiological questions. It is an important technique in medical research, because there are so many diseases related to malfunction of ion channels. In pharmacological research, this technique can be used to screen drugs for specific ion channel modifications.

HISTORICAL DEVELOPMENT OF PATCH CLAMP TECHNIQUE

The earliest foundations of electrophysiology were laid in late 1660s, when Jan Swammerdam who was a dutch scientist, discovered a neuromuscular preparation. He observed muscle contraction on stimulation of the nerve. He improvised on this method by insertion of muscle into a small glass tube and also attached needles to the muscle ends.

80 yrs later, in 1791, Luigi Galvani published his work on animal electricity. This publication was a compilation of his 10 yrs of experiments on isolated nerve-muscle preparations in frog. As a result of these experiments Galvani was able to identify the electrical excitation and derive the relationship between muscle contraction and intensity of stimulus applied. Electro-physiological experiments became easier when John Z.Young reported that squid axons could be used for studying the electrical properties of cells. Later, Cole and Curtis along with Alan Hodgkin and Andrew Huxley discovered intracellular electrodes(9). They were the first to record action potentials in squid axons.

In 1949, it was Cole and Marmont who designed the voltage-clamp technique. Hodgkin and Huxley proposed that the ion fluxes determined the membrane excitability. In the same year Gilbert and Ralf Gerard made microelectrodes from glass pipettes. Microelectrodes then became the universally accepted choice of technique for recording from all types of cells.

Frederick Pratt and Eisenberger developed a fine-pointed electrode with 4-8 micrometer diameter. These were used for demonstrating the all or none law in skeletal muscle. In cellular membranes the first extracellular recordings were performed by Alfred Strickholm. It was Peter Baker,

Trevor and Hodgkin who performed experiments with replacement of inside of cell with salt solutions for the first time.

Meech and Standen in 1975, used the two-electrode mode method while Wilson and Goldner used the single – electrode mode method of voltage clamping using micropipettes for recording intracellular potentials.

Noble prize for Patch clamp discovery

In the year 1976, Erwin Neher and Bert Sakmann were the first to introduce the technique of Patch clamp. They used this technique to record currents through single acetylcholine – activated channels of frog skeletal muscle in the cell attached mode. In 1981 Neher and Sakmann along with Hamill and Martey showed the importance of achieving Giga ohm seal during patch clamp recording(10). In 1991, the Noble prize in Physiology or Medicine was awarded to Erwin Neher and Bert Sakmann for their contribution to electrophysiology.

PATCH CLAMP CONFIGURATIONS

1) Cell attached patch

2) Whole cell patch

3) Inside out patch

4) Outside out patch

5) Perforated patch

Each of the above configurations has its own limitations. So, depending upon the desired experiment, one of the above configurations is chosen.

Cell attached patch

Cell attached patch allows recording of single-channel currents. Here the pipette is attached to a small patch of cell membrane. This procedure causes tight sealing of the patch of cell membrane against the tip of the pipette.

Whole cell configuration

This is the method of choice for almost all tissue preparations and cultured cells. By applying a pulse of suction, the patch that was created in the cell-attached configuration is ruptured. This procedure exposed the whole cell to the pipette solution. Hence there is a low resistance electrical continuity between the pipette lumen and the cell. The limitation of this method is that the cellular content can move into the pipette resulting in dialysis. But this can be prevented by recording within few minutes of patch removal.

Inside out patch

Inside out patch is achieved by withdrawing the pipette from the cell-attached configuration, thus exposing the inside of the cell to the bath solution. This can be useful to study the ion channels that are particularly regulated by intracellular ligands.

Outside out patch

Outside out patch is achieved by withdrawing the pipette tip from whole cell configuration. This generally results in resealing of the membrane, so that the outside of the cell membrane is exposed to the bath solution. This is desirable for studying ion channels that are regulated by extracellular ligands.

Perforated patch

During whole cell recordings, washout of intracellular constituents like ATP and intracellular Ca^{2+} into the pipette significantly affects the properties of ion channels. This can cause reduction of ionic current flow through them over time. The perforated patch clamp technique addresses this problem by avoiding seal rupture and inserting pores in the cell membrane. This is achieved by using antibiotics like Amphotericin-B or Nystatin that can create pores in the patch, thus facilitating whole cell

recording. The disadvantage of this method is that it takes 10 – 40 minutes to perforate cell.

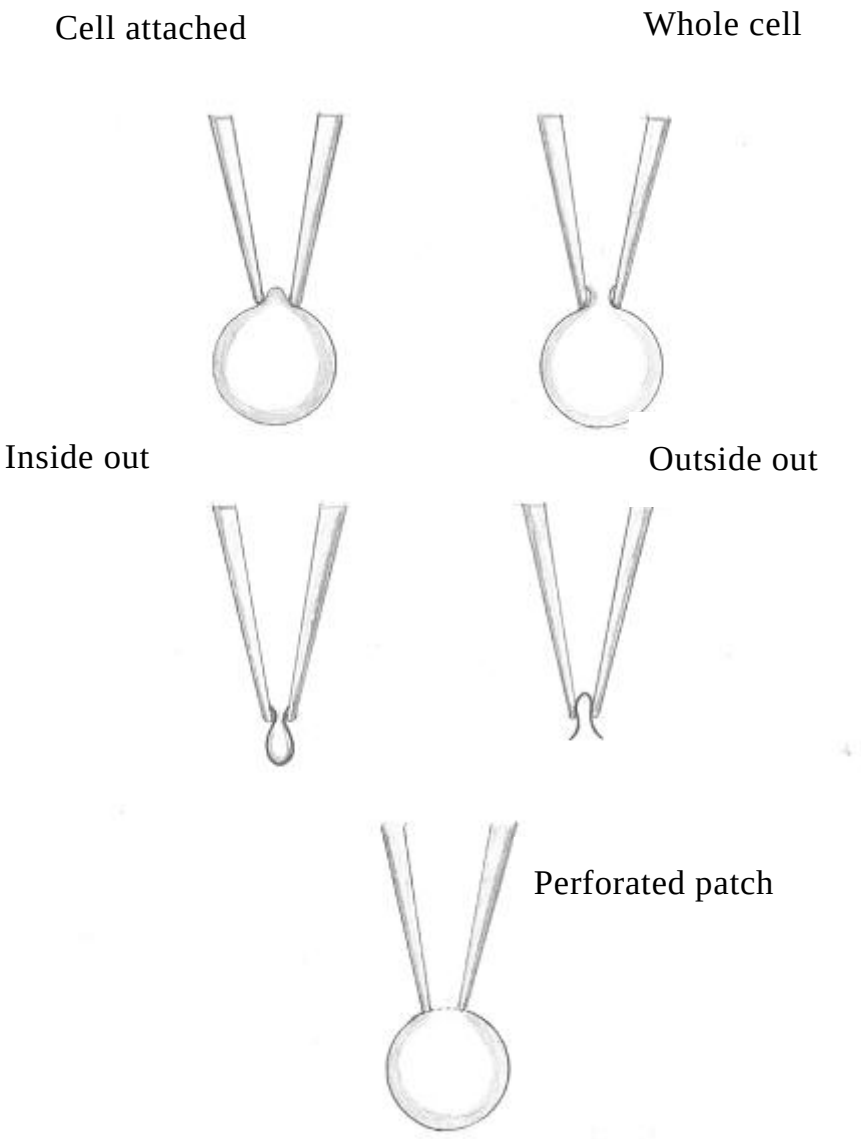


Figure 1 : Types of Patch clamp configurations

INTRODUCTION TO PLANT POISONING

Plant poisoning in animals is almost always accidental. Humans have been poisoned by ingestion of misidentified plants, or by accidental ingestion of known poisonous plants. People also consume poisonous plants for suicidal purposes. Poisonous plants can pose damage to the entire spectrum of organ systems, while some plants contain certain toxic principles that affect different individual organs. The toxicity profile also depends on the growth stage of the plant, part of the plant being consumed, the amount of toxin consumed, the species and also the susceptibility of the victim.

For ages unknown, consumption of poisonous plant extracts has been a common mode of suicidal poisoning. In south Asia, the commonly consumed plant toxins are, ***Cleistanthus collinus*** **(oduvan),** ***Thevetia peruviana*** **(yellow oleander),** ***Datura stramonium***. The plant toxin of interest in the present study is Cleistanthin A which is a deadly toxic principle present in the plant *Cleistanthus collinus*.

INTRODUCTION TO CLEISTANTHUS COLLINUS

The plant genus *Cleistanthus* is a poisonous shrub that belongs to the family Euphorbiaceae(8). This is a highly diversified family with substantial difference in morphology, chemical and ecological characteristics. Euphorbiaceae is widely distributed throughout the world

with approximately 8000 species of plants. The family includes large desert succulent to small herbaceous plants. The genus *Cleistanthus* comprises of approximately 134 species. Of all species, the toxic constituents of *Cleistanthus collinus, Cleistanthus patalus, Cleistanthus Schlechteri* and *Cleistanthus gracilis* have been studied.

Cleistanthus collinus is a poisonous plant found in forests native to India, Malaysia and Africa. It is called as Indrayava in sankrit, Garari in Hindi/Marathi , Oduvanthazhai / Odaichi in Tamil, Badadarige in Kannada, Kandishe in telugu, Nilappala in Malayalam, and Karada in oriya(5). Almost every part of the plant is potentially toxic to humans as well as animals. Consumption of leaf extract from this plant is a common mode of suicidal and homicidal poisoning in various parts of southern India. There are also evidences of this plant being used as a cattle and fish poison. The leaves from this plant have also been used as an abortifacient(13). Availability and easy accessibility are probably the reasons for the consumption of this plant poison. Consumption of boiled extract of leaves from the plant is the most common mode of poisoning as reported in southern parts of India.

CLEISTANTHUS COLLINUS - BOTANICAL PROFILE	
Common names	Oduvan, Garari, Karada, Pasu, Karlajuri
Botanical name	*Cleistanthus collinus*
Kingdom	Plantae
Phylum	Tracheophyta
Subphylum	Euphyllophytina
Class	Magnoliopsida
Subclass	Rosidae
Super order	Euphorbianae
Order	Euphorbiales
Botanical family	Euphorbiaceae
Subfamily	Phyllanthoideae
Genus	Cleistanthus
Species	Collinus

CLEISTANTHUS COLLINUS – PLANT MORPHOLOGY

Cleistanthus collinus grows as a small deciduous tree or shrub with spreading and rigid branches. The leaves are leathery measuring about 3.5 to 10 cm long and arranged alternately. They are 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 2 *Cleistanthus collinus* plant leaves

CLEISTANTHUS COLLINUS - CHEMICAL PROFILE

Chemical examination of *Cleistanthus collinus* by various researchers has resulted in the identification of many toxic and non toxic constituents. Oduvin which was reported for the first time by Naidu et al was found to be a slow poison. Further investigation of oduvin was done by Irudayasamy and Natarajan. The results showed that it contains Principle A and Principle B. Govindachari et al isolated ellagic acid, Diphylllin and lignan lactones namely cleistanthin and collinusin from Cleistanthus collinus leaves (14). Diphyllin was isolated from the leaves and the heartwood of the plant *cleistanthus collinus* (14) (15). Collinusin

and cleistanone were reported to be present in aerial parts of the plant by Anjaneyulu ASR et al(14) (16).

Subba Rao et al 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 (17). Cleistanthin E is the only triglycoside isolated from the plant of this genus (15).

Few other compounds were isolated from the heartwood of the plant. This includes wodeshiol 16,3,4-dihydrotaiwanin C 20, taiwanin E and glycosides 24, 25 and 26 of diphyllin and (15).

STRUCTURE OF CLEISTANTHIN A

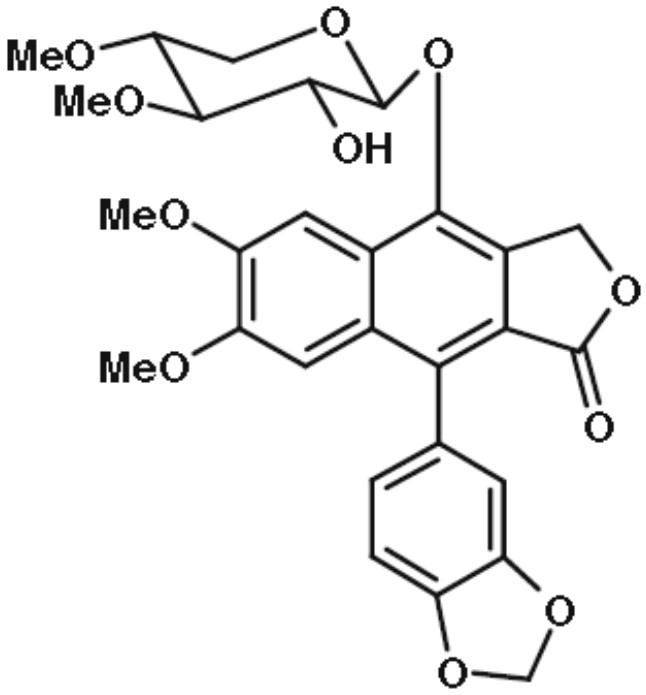


Figure 3 Structure of Cleistanthin A

CLEISTANTHUS COLLINUS - BENEFICIAL EFFECTS

Most of the drugs in practice have originated from plants. *Cleistanthus collinus* has been investigated for its antibacterial, antifungal, antiprotozoal, antiviral, anticancer, diuretic and hypoglycemic effect(18)(19). The bark of the plant is considered to have useful application in cutaneous diseases. Water in which leaves are steeped is used as a treatment option for headache. The alcoholic extracts of this plant, are used to treat gastro intestinal disorders. The hard wood of the plant is durable and is used for 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. It was found to cause tumor regression in mice by inducing apoptosis. Studies done by Kalimuthu Kovendan et al proved that C. collinus leaf extracts had larvicidal property particularly against *Culex quinquefasciatus* species. This effect is important because it can be evolved as an ecofriendly method for vector control programmes(20).

CLEISTANTHUS COLLINUS – COMMON MODES OF CONSUMPTION

All parts of this plant are poisonous; but leaves are more commonly consumed for suicidal purposes. Rural women commonly crush the leaves and consume the filtered juice or prepare a boiled decoction from the leaves (aqueous extract)(21). There are studies that show that boiled extract of the leaves is more poisonous than the fresh or ground leaves(22).

CLEISTANTHUS COLLINUS POISONING - MORTALITY REPORTS

28% mortality has been reported in cases of *Cleistanthus collinus* poisoning. Death usually occurs 3-7 days after ingestion of the poison. Patients develop a series of life threatening complications such as hypokalemia, hypotension, cardiac arrhythmias, neuromuscular weakness,

and renal failure(5). Human toxicity has also resulted in renal tubular dysfunction, most commonly distal renal tubular acidosis(23). Acute respiratory distress syndrome (ARDS) has been observed in severe cases. Aggressive treatment of these derangements hence becomes crucial. The mystery behind the mechanism of toxicity of this plant has persuaded many researchers to do extensive research in this field since 3 decades.

THEORIES PROPOSED FOR MECHANISM OF TOXICITY

1. Damage at neuromuscular junction
2. Inhibition of sodium potassium pump
3. Reduced LDL activity
4. Depletion of Thiol containing enzymes
5. Effect on neutrophils
6. Distal renal tubular acidosis and type 2 respiratory failure

Damage at neuromuscular junction

Studies done on isolated phrenic nerve diaphragm preparation of mouse by Nandakumar et al.,1996, reported muscle cramps and weakness in Cleistanthus collinus poisoning. Following an hour's exposure to 0.015% extract, the response of 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%. There was only 11% reduction in resting membrane

potential. An *in vitro* electromyography was used to measure the tension and compound action potentials. 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 were attributed to the alterations at the muscle nerve junction which caused symptoms such as respiratory failure, muscle weakness and cramps(24).

Inhibition of sodium potassium pump

Inhibition of ATPase activity (Sodium Potassium ATPase and Magnesium ATPase) was reported by, Sarathchandra et al in 197. Patients presented with hypokalemia, kaliuresis and diuresis (Kurien et al., 1991). Cardiac glycosides are known for their sodium potassium pump blocking activity. *Cleistanthus collinus* being a glycoside was also expected to act in a same way by **inhibiting sodium potassium pump**.

Reduced LDL activity

Studies done by Kanthasamy et al., 1986 reported inhibition of LDH activity as mechanism of toxicity in *Cleistanthus collinus* poisoning. 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 serum and tissue of test animals, the LDH activity was markedly reduced as compared to the controls. Among the tissues, kidneys showed the maximum decrease in the LDH levels.

Reduction of Thiol containing enzymes

Studies done by Sarathchandra et al., 1997 reported a reduction 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.

Effect on neutrophils

Neutrophilic granulocytosis was reported by Rao *et al* in rats injected with cleistanthin. 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 observed that intravenous and intraperitoneal doses

were more toxic compared to oral doses. The exact role of Cleistanthin induced neutrophilic granulocytosis is yet to be investigated(25)

Distal renal tubular acidosis and type 2 respiratory failure

Administration of *Cleistanthus collinus* aqueous extract was done by intraperitoneal routes. A measurement of electrolytes, urinary pH and blood gases were done. Brush border and basolateral membranes were isolated from rat kidney and ATPase activity was studied. Sodium potassium pump activity was also estimated in venous blood samples incubated with *Cleistanthus collinus* acetone extract. The results showed 100 % mortality in tests and 17% mortality in controls. The important finding was inhibition of renal brush border proton pumps with active principles of *Cleistanthus collinus*. It was also reported that the *Cleistanthus collinus* extracts did not have any effect on sodium potassium pump activity. Respiratory arrest was found to be the immediate cause of death(23).

CLEISTANTHUS COLLINUS – CYTOTOXIC EFFECTS

Cytotoxic assays done on *Cleistanthus collinus* by Bhakuni et al in 2007 reported a cytotoxicity level of 10µg/ml within an incubation period of 28 hrs. Pinho et al in 2007 have confirmed the cytotoxicity effect of *Cleistanthus collinus* on mouse normal cell lines(12). Pradeep kumar et al

in 2000 reported that high concentrations and longer exposure to Cleistanthin A enhance DNA strand breaks and resulted in apoptosis in CHO cells (Chinese hamster ovary cells) and p53 deficient cell line(26). Ramesh *et al* in 2003, found that Cleistanone (diphyllin derivative) and its acetyl derivative showed cytotoxicity against MT2 cell lines(16). Tumorigenic effects of Cleistanthin A and B on K-562 tumour cell lines was demonstrated by Rajkumar S *et al*. They observed a reduction in the incorporation of nucleotides into the DNA and RNA, hence affecting the replication and transcription, but not translation.

The anticancer activity of Cleistanthin A was studied by Pradeep kumar et al in 1999(19). They had compared the cytotoxicity of Cleistanthin A with 5 other anticancer drugs. It was found that Cleistanthin A was most effective among all drugs tested for oral carcinoma cell line and cervical carcinoma cell line. The effect of Cleistanthin A in arresting the growth of tumor in mice affected with Dalton's ascites lymphoma and sarcoma was studied. The results showed a drastic reduction in tumor size in samples treated with Cleistanthin A. There was also an increase in life span in mice with sarcoma. This increase in life span was almost similar to that observed with cisplatin. There are studies that reported anticancer activity of *Cleistanthus collinus* against nasopharyngeal epidermoid carcinoma.

In 2014, Thamburaj Suman et al reported the response of normal cell line to selected fractions of *Cleistanthus collunus*(27). The investigators performed cytotoxicity assays for *Cleistanthus collinus* aqueous and ethyl acetate solvent based fractions. They were able to show significant anti proliferative effects of *Cleistanthus collinus.*

Although the exact molecular mechanisms that contribute to the anticancer properties of *Cleistanthus collinus* are yet to be explored, it is clearly evident that fractions of this plant may emerge as potent anticancer drugs in near future.

CLEISTANTHUS COLLINUS TOXICITY - CLINICAL PROFILE

Clinical studies done thus far on *Cleistanthus collinus* poisoning demonstrates a wide spectrum of clinical presentations. Few important studies have been discussed below.

It was Thomas et al in 1987, who first reported 32 cases of *Cleistanthus collinus* poisoning. Of the 32 cases studied, 9 patients had died. The cause of death was sudden cardiac arrest in 8 cases and the remaining by progressive respiratory failure. The survived patients were found to develop hypokalemia. In 1991 the same group reported a prospective study done on 11 cases to interpret the reason for hypokalemia(21). A measurement of Serum, urine and RBC potassium,

serum and urine osmolality, serum creatinine and bicarbonate levels were made. Continuous cardiac monitoring reported that ventricular premature contraction was the most common type of arrhythmia in these patients. Irrespective of hypokalemia, there was high potassium loss in urine. Hence, the electrolyte imbalance was attributed to renal tubular potassium leak.

Case study done by Eshwarappa et al reported 2 cases who died with ingestion of the liquid extract of boiled crushed leaves (28). The patients were observed to suffer from various known clinical features along with hypokalemia, neuromuscular weakness, cardiac toxicity, respiratory failure and renal failure. In 2003 Subrahmanyam *et al* recruited 46 cases to study the clinical features and consequences of oduvanthazhai poisoning, of whom 32% died (29). Mortality was reported mainly due to cardiac or respiratory complications. Majority of the deaths were observed on the 3rd day after the poisoning and the rest occurred within a week. The remaining survivors were treated symptomatically due to unavailability of a specific antidote.

Benjamin *et al* in 2006 reported a case report of a 24 year old male admitted with *Cleistanthus collinus* poisoning(30). In addition to the known symptoms and signs, they reported distal renal tubular acidosis, distributive shock and acute respiratory distress syndrome (ARDS). For the

first time, Myasthenic crisis like syndrome due to *Cleistanthus collinus* poisoning was reported by Mohan A et in 2008(31).

A prospective study on 32 patients was done over a period of two years by Nampoothiri *et al* in 2010. This study reported 60% incidence of hypokalemia and 15% incidence of renal failure. Of all patients with renal failure, 4 died. There were no signs of cardiotoxicity.

CLEISTANTHUS COLLINUS TOXICITY – TREATMENT MODALITIES AVAILABE THUS FAR

The exact mechanism of toxicity of *Cleistanthus collinus* poisoning remains unclear. Hence the management of patients becomes a challenge to the treating physician. But the mainstay of treating these patients is to closely monitor them for development of any complications. Correction of electrolyte imbalance and acidosis is also important.

Patients who go in for shock can be managed with crystalloids and ionotropes. Cardiogenic cause for shock is not known. Studies by Nampoothiri et al reported that monitoring of renal function with strict intake-output charts, and a central venous access are crucial in saving the life of victims(32).

Cardiac pacing may be indicated in the setting of rhythm disturbances such as bradycardia and QTc prolongation, and may be placed electively in patients with severe poisoning. Further studies are required to

confirm the need for cardiac pacing. Cardiac monitoring may be required up to 5 days in symptomatic patients. Mechanical ventilation is indicated in the setting of respiratory failure.

There are studies that report N-acetylcysteine as a possible antidote for management of *Cleistanthus collinus* toxicity(33).But the benefit of this intervention is unclear. Neostigmine was used for a patient who presented with a myasthenic crisis–like syndrome(34).

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(33).

NEUTROPHILS

The name Neutrophil originated from the cell's eminent neutral staining properties with eosin and hematoxylin stains. Neutrophils are the major effector cells of the body's immune system. They are produced in the bone marrow, and have a life time of 6-8 hrs in the blood after which they undergo apoptosis. After death, neutrophils are cleared by the liver, spleen and bone marrow(35). The discovery of neutrophils dates back to the Nobel laureates Ilya Ilyich Mechnikov and Paul Ehrlich, who won the Nobel Prize for their research on immunity. They are best known for their pioneering research into the immune system. As a reflection of their cytological appearance, neutrophils have been rightly called as polymorphonuclear cells or granulocytes. They posses multilobed nucleus that greatly facilitates their emigration through the narrow junctions present between endothelial cells. Another identifiable property of neutrophils is the presence of large number of granules in their cytoplasm. Their granules contain potent antimicrobial molecules used for killing the microbes. By releasing their granule proteins, neutrophils can communicate with the endothelium, monocytes and lymphocytes, thereby playing a major role in the host defence mechanism.

FUNCTIONS OF NEUTROPHILS

Neutrophils kill the invading pathogens by following mechanisms(36)

- ✓ Phagocytosis
- ✓ Production of reactive oxygen species
- ✓ Neutrophil extracellular traps

The phagocytic activity of neutrophil is very rapid. They can uptake IgG opsonised particles in less than 20s. This is followed by rapid fusion of the phagocytic vacuole with neutrophil granules to form the phagosome. This process is called phagosomal maturation. Neutrophil granules contain NADPH oxidase and other enzymes that initiate the killing mechanisms.

Co-incident with phagocytosis is the enormous increase in the respiratory burst along with the ROS (reactive oxygen species) generation by the activation of the enzyme NADPH oxidase. This enzyme plays an important role in protecting the body against harmful microorganisms. Studies show that the activation of NADPH oxidase in neutrophils is coupled to the generation of neutrophil extracellular traps (NET). NET products can bind to and destroy bacteria(37)(36).

ION CHANNELS EXPRESSED IN NEUTROPHILS

Other than proton channels, three distinct ion channel currents in human neutrophils were identified by Karl-Heinz Krause et al; in 1990 using whole cell patch clamp technique. The channels identified were,

a) A voltage dependent K^+ current

The voltage dependent K^+ current was found in the cells even at rest. However, the channel was activated at potentials less negative than -60mV and it showed an inwardly rectifying property. This is suggestive of the voltage dependent K^+ current being the determinant of resting membrane potential of neutrophils.

b) A Ca^{2+} activated K^+ current

The Ca^{2+} activated K^+ channel was insensitive to voltage, improved with the Ca^{2+} ionophore ionomycin and rectified outwardly.

c) A Ca^{2+} activated Cl^- current

The Ca^{2+} activated Cl^- current was also voltage independent with a tendency for outward rectification. These two currents may play a role in cell volume regulation and/or cellular activation(38).

VOLTAGE GATED PROTON CHANNELS

Discovery of Proton channels

The existence of voltage gated proton channels was first proposed by Fogel and Hastings in 1972. However, the biophysical characterization of these channels was first done by Thomas and Meech in 1982. Thomas and Meech performed patch-clamp studies on snail neurons(39). They observed that, upon depolarization, the permeability of H^+ greatly increased. Subsequently, H_v channels were discovered in various cell types from various organisms. It was in 2006, Clapham and Okamura groups who first published the genes for H_v channels(40).

Structure of voltage gated proton channels

Voltage gated proton channels have 4 membrane-spanning regions, S1-S4, which are similar to the S1-S4 regionsf of voltage gated sodium, potassium and calcium channels. The pore forming segments namely S5 and S6 are absent in voltage gated proton channels(41)(40)(42). The human analogue was recently cloned in 2006(40) and was also found to exist as dimers, whose pores are gated separately by two different voltage sensors(42).

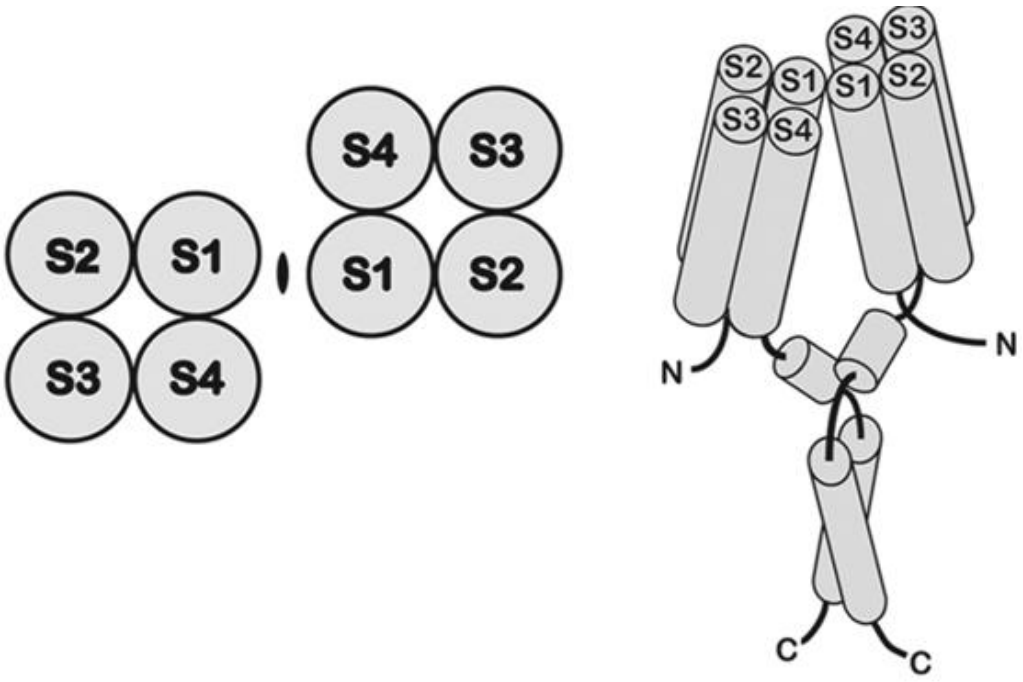


Figure 4 : Structure of a voltage gated proton channel. The S1-S4 are the four trans-membrane segments. Picture taken from (43)

The voltage gated proton channel is called a channel for various reasons. It consumes no energy. Their reversal potential follows the Nernst equation and also these channels are electrogenic. Compared to other ion channels, the proton conductance through these channels is considerably small. They have the smallest turn-over rate. There are few properties of proton channels which could be studied by patch clamp methods. This includes the maximal proton current at steady state, conductance voltage plots, time constant, voltage threshold of activation, the channel closure kinetics and reversal potential of these channels.

Voltage gated proton channels are known for their high temperature dependence. This feature of voltage gated proton channels seems to play an important role in the ion channel field.

Unique features of Voltage gated Proton channels

The unique biophysical features of voltage gated proton channels include

- ✓ ΔpH dependence of gating
- ✓ Inhibition by Zn^{2+}

ΔpH dependence of gating

H_v channels are the chief acid extruders allowing protons to move out of the cell. This biophysical property is responsible for the various physiological roles played by these channels. The gating of H_v channels depends not only on the trans-membrane potential, but also strongly depends upon the difference in pH across the cell membrane.

Inhibition by Zn^{2+}

The defining characteristic of H_v channels is their inhibition by polyvalent cations (zinc and cadmium being the most important ones). It was Thomas E. DeCoursey who first studied the mechanism of this inhibition in rat alveolar epithelial cells using voltage clamp techniques. Thomas E. DeCoursey observed the reduction in H_v channels with externally applied $ZnCl_2$. The study also showed that the external receptor for zinc could be the same modulatory site at which the external pH regulates proton channel gating(44).

Diverse physiological roles of Voltage gated proton channels

In humans, the proton channels are mainly present on the cells of immune system. They are also found in sperm cells. In addition, proton channels also have a significant role in cancer cells.

Role in phagocytes

In phagocytes, proton channels play an important role during oxidative bursts. Proton channels execute their role by optimizing NADPH oxidase activity. This is accomplished by compensating charge, minimizing the changes in pH and providing substrate protons for production of reactive oxygen species in the phagosome(45)(46).

Role in human sperm cells

Sperm intracellular pH is the keystone that controls the efficiency of sperm. Studies by Lishko et al proves that the proton channel is vital for maintaining the internal pH of the human sperm cells, and also for the regulation of essential processes required for the fertilization of the ovum. (47)(48).

Role in cancer cells

The expression of proton channels have been demonstrated in highly metastatic breast and colorectal cancer cells. Additionally, Wang et al

demonstrated that proton channels are over-expressed in glioma cells and that inhibition of proton channels by zinc results in significant reduction in tumor size(49). These findings show that proton channels might be the targets for anticancer drugs.

Proton channels - Contributions of DeCoursey

Rat alveolar epithelial cells were studied by TE DeCoursey et al using patch-clamp technique. By replacement of permeant ions with N-methyl-D-glucamine methanesulfonate, they were able to eliminate other ionic conductances and observed only large voltage-activated hydrogen-selective currents. These proton currents in alveolar epithelium were activated by depolarization and deactivated on repolarization. The currents were also blocked by Cd^{2+} and Zn^{2+}, copper, beryllium, nickel, aluminium, lanthanum, cobalt and mercury. These studies indicate the existence of voltage-dependent H^{+} currents in mammalian cells(50).

Thomas DeCoursey et al studied proton channels in basophils. Basophils release histamine in response to allergic reactions. This study reports that basophils have voltage gated proton channels in their membrane. these channels resemble those that are present in human eosinophils in amplitude and properties(51)

Studies were done on Dimerization in proton channels by Thomas DeCoursey et al. These studies show that dimerization in proton channel enables cooperative gating mechanism which may be the reason for its voltage dependent conductance. This property of proton channel is important for its phagocyte function.

DeCoursey et al studied the effects of replacing deuterium oxide for water, on the pH dependence of proton currents and also their conductance. The focus of this study was to identify the effect of isotope on proton conductance, which might help to arrive at the mechanism of permeation. They reported that deuterium oxide had stronger effect on activation than deactivation, which in turn suggests that the internal and external regulatory sites are completely different.

Studies were done to determine the effect of concentration of buffer on proton channels. The results of the study showed that the overall effect of varying buffer concentration on proton channel conductance was negligible.

PREVIOUS WORK DONE IN OUR DEPARTMENT

1. Isolation and characterization of major fluorescent compounds in *C.collinus* extract

-Cleistanthin A

-Cleistanthin C (erroneously identified as Cleistanthin B earlier)

-Another compound which was at that time thought to be a Diphyllin (current experiments suggest that this compound may be Taiwanin E)

2. The clinical profile of toxicity was determined by administering different types of *Cleistanthus collinus* plant extracts intraperitoneally into the rats. (52)(53)

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).

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

? Taiwanin E (erroneously identified as Diphyllin earlier)**,** when tested *in vivo,* did not result in mortality (Dr. Latha, M.D. Thesis, 2011).

3. Animal experiments done in our lab with whole aqueous extract showed 100% mortality, type 2 respiratory failure and type 1 DRTA(23)

4. We found that, partitioning the whole aqueous extract of *C.collinus* with chloroform causes all fluorescent compounds to sequester into bottom chloroform fraction while top water fraction is free of all fluorescent compounds including toxins.

5. We have succeeded in establishing a Simple method for isolation and quantification of Cleistanthins from *Cleistanthus collinus* leaves. Liquid/Liquid partition chromatography of aqueous extracts with chloroform to isolate fluorescent compounds was also initiated. Traditionally, these compounds were isolated from acetone extracts of dried and delipidated leaves by column chromatography. These methods required extensive use of solvents such as n-hexane, benzene (known to be highly toxic) and ethyl acetate. We have developed easy methods of purification of these compounds from aqueous extract with thin layer chromatography, without the use of Benzene or ethyl acetate. (Paper yet to be published)

5. We have quantified the fluorescent compounds in different extracts by High performance liquid chromatography in collaboration with clinical Pharmacology department of our hospital.

6. We were able clearly identify the best type of aqueous extract that provides large quantities of a given fluorescent compound.

7. Characterization of the isolated compounds using Mass Spectrometry, NMR spectrum (NOSEY, COSEY and HMPC), Infrared spectrum, melting point analysis in collaboration with Chemistry department of IIT, Mandi.

8. Patch clamp studies were done to study the effect of aqueous extract of *Cleistanthus collinus* on voltage gated sodium channels and delayed-rectifier potassium channels of rat ventricular myocytes(54). Results showed that the voltage-gated sodium channels and delayed- rectifier potassium channels present on the rat ventricular myocytes were blocked by aqueous extract of *Cleistanthus collinus.*

9. Proton channel modulating activities of whole aqueous extract and few other toxic compounds have been tested by patch clamp technique on neutrophils(55)(56). Studies done by Pragalathan et al on proton currents of human neutrophils show that the activation voltage for these currents was approximately -40mV, and their reversal potential was around -67mV. These values were close to the calculated values for proton currents.

AIMS AND OBJECTIVES

AIM

To study the effect of Cleistanthin A on voltage gated proton channels of human neutrophils (with ATP included in the patch pipette)

OBJECTIVES

1. To record proton currents in freshly isolated human neutrophils using patch clamp technique.
2. To identify the effect of Cleistanthin A on proton currents in freshly isolated human neutrophils with ATP included in the patch pipette.

MATERIALS AND METHODS

Materials Required For Isolation Of Fresh Neutrophils	
1	5 ml of venous blood
2	20 µL of Heparin
3	3 ml of 6% Dextran
4	0.6M Potassium Chloride solution
5	Deionised water
6	Phosphate buffered saline
7	Ficoll – (Histopaque : Density = 1.077)
8	15 ml centrifuge tubes
9	1 ml Pipettes with appropriate tips

Materials required for isolation of Cleistanthin A	
1	Shade dried leaves of *C.collinus*
2	Distilled water
3	Hot plate
4	Chloroform
5	Rotary evaporator
6	Glass petridishes
7	Thin Layer Chromatography-Tank and plates
8	Solvents – N-Heptane, Chloroform, Ethanol

Apparatus used For Patch Clamp Experiment	
1	Kimble chase Borosilicate glass capillary tubes
2	Narishige Model PP-830 Pipette Puller
3	GlasswoRX F-500 Fine Point Microforge
4	Sylgard- Silicone Polymer
5	A patch clamp setup – this includes Axopatch 200B patch-clamp amplifier, Axon instruments Digidata 1322A analogue-digital converter and a computer with a data acquisition software
6	Nikon Eclipse TE2000-U Inverted phase contrast microscope
7	Octaflow II Perfusion system
8	-20 °C Mini cooler
9	Glass petridishes
10	Appropriate bath and pipette solutions
12	Thermo Orion Micro pH meter
13	Fiske Micro- Osmometer
14	Faraday cage
15	Micromanipulator

ISOLATION OF HUMAN NEUTROPHILS

Method – Dextran gradient centrifugation method

Collection of blood - 5 ml of venous blood was collected from 12 healthy volunteers, who fit into the following criteria,

Inclusion criteria

- Volunteers more than 18 yrs

Exclusion criteria

- History of chronic infectious diseases
- History of diabetes mellitus, hypertension
- Pregnant women

The procedure was done after getting consent from the volunteers.

Steps involved in isolation of human neutrophils

1. The blood was transferred to a 15ml centrifuge tube (prefilled with 20 µL of anticoagulant heparin to prevent clotting of blood inside the tube).

2. 3 ml of 6% Dextran was added to the blood in the tube and mixed well.

3. The centrifuge tube containing blood was left undisturbed for 1 hour at 25 °C. This step was done to sediment the Red Blood Cells in the mixture.

4. After an hour, the supernatant including the buffy coat (which contains the white blood cells) was carefully pipetted out and transferred to another centrifuge tube.

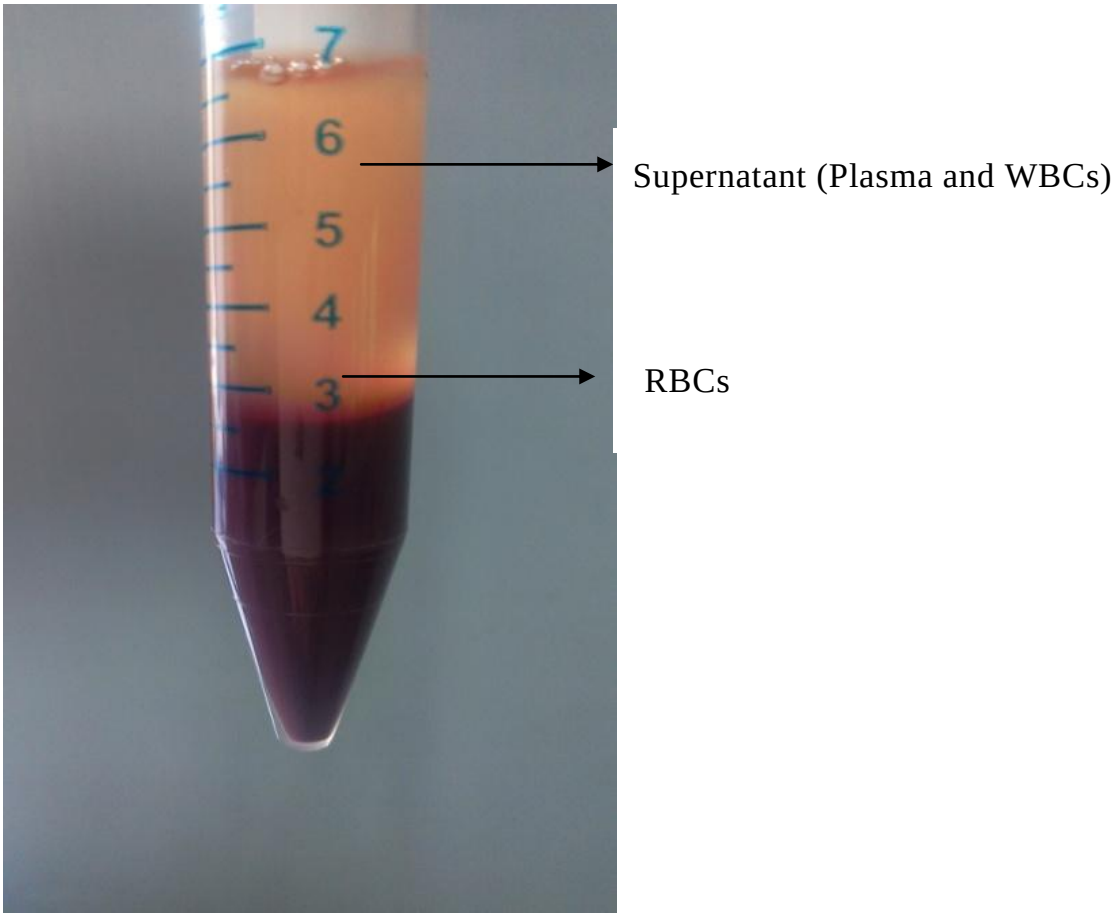


Figure 5 : Centrifuge tube containing plasma, WBCs and RBCs

5. This was centrifuged at 1000 rpm for the duration of 12 minutes at 6 °**C**.

6. The supernatant was discarded. To the remaining pellet, 3ml of distilled water was added and mixed gently. (This step was done to cause hypotonic lysis of Red Blood Cells)

7. 1 ml of 0.6M Potassium chloride was added to this within 25 seconds. (This step was done to restore the tonicity of the solution)

8. The tube was then filled with Phosphate Buffered Saline and centrifuged at 1000 rpm for 8 minutes at 4 °C

9. At the end of 8 minutes, the supernatant was removed and the pellet was visualized for any red tinge (which represents Red Blood Cells). If Red Blood Cells were still present, then the steps involving hypotonic lysis of Red Blood Cells were repeated until a white pellet containing only White Blood Cells was seen.

10. To this white pellet, 1ml of Phosphate Buffered saline was added and mixed gently

11. This cell suspension was layered carefully on 1ml of Ficoll (Histopaque) in another test tube and centrifuged at 1000 rpm for 30 minutes at 4 °**C.** (Ficoll is a hydrophilic polysaccharide that dissolves

readily in aqueous solutions. It is used here because it acts as a density gradient and separates the mononuclear cells from the rest of the white blood cells.

12. After 30 minutes, Ficoll had separated peripheral blood mononuclear cell layer (PBMC) from the polymorphonuclear cell layer.

13. Supernatant was discarded. 0.5 ml of Phosphate buffered saline was added to the pellet containing the neutrophils, and mixed gently.

14. The cell suspension was stored in refrigerator until taken for patching.

ISOLATION OF CLEISTANTHIN A FOR TEST SOLUTION

Collection and processing of leaves

Leaves of the plant *Cleistanthus collinus* were collected from Palamathi hills in Vellore. The freshly collected leaves were shade dried for one week. 100 grams of leaves were soaked in 3 litres of distilled water and left for 24 hours. The next day the supernatant was collected. The supernatant obtained from the above process was subjected to liquid-liquid partition chromatography.

Liquid/Liquid partition chromatography

The supernatant was mixed with equal amount of chloroform. As these two liquids were immiscible, a clear junction was seen, with

the coloured solution on top and chloroform at the bottom (Fig 6.). The active constituents preferentially dissolved in the chloroform fraction of the mixture. The bottom layer was collected using a separating funnel, and was concentrated in a rotary evaporator. The concentrated chloroform fraction was poured into petridishes and dried in hot air oven at 37º C for 24 hrs. The dried extract was scraped out and collected. The dried extract was subjected to thin layer chromatography.

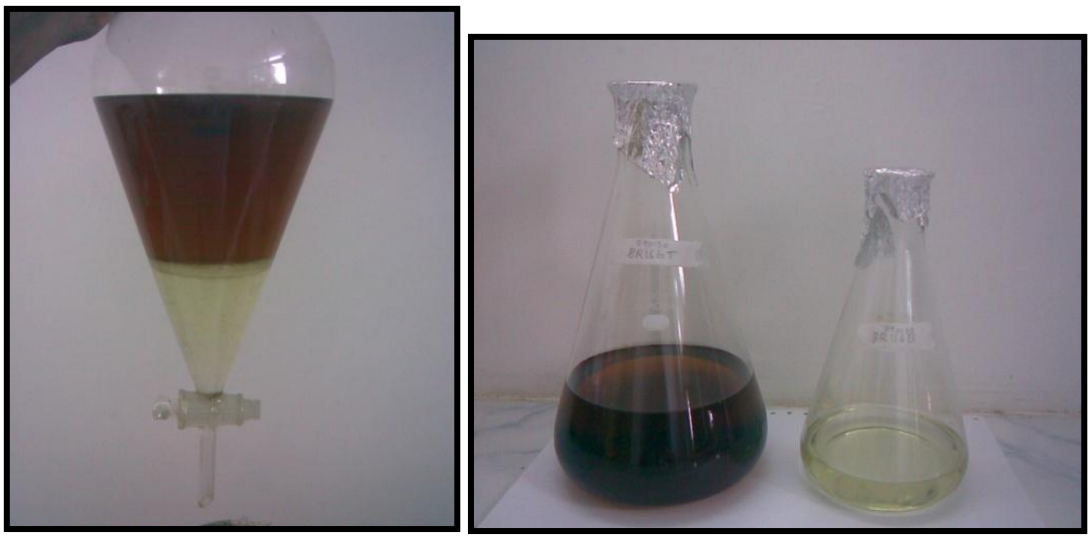


Figure 6: Liquid/Liquid partition chromatography

Thin Layer Chromatography

Glass plates were layered with Silica gel G (0.5 mm). The powdered chloroform fraction was dissolved in chloroform at a concentration of 1mg/10µl and was spotted on the plates. The plates were run to a height of

10 cm and visualized with UV illuminator. The band presumed to be containing Cleistanthin A, was scraped from the chromatography plates and stored at room temperature.

Confirmation of the identity of Cleistanthin A

To confirm that the compound used for study was a Cleistanthin A enriched fraction, the following procedures were performed,

1) TLC comparison with an authenticated sample of Cleistanthin A
2) High performance Liquid chromatography

TLC comparison with an authenticated sample of Cleistanthin A

To confirm the identity of Cleistanthin A, a thin layer chromatography was done comparing the isolated fraction with an authenticated sample of Cleistanthin A obtained from Godavari bio refineries, Mumbai. Except for a very minor contamination, it was seen that the isolated Cleistanthin A matched perfectly with the authenticated sample in its TLC profile.

High performance liquid chromatography

The chloroform fraction was subjected to High performance liquid chromatography to identify the major peaks detected by a fluorescence detector.

Reverse phase HPLC using C18 columns (Supelco HPLC column, Discovery® HS C(18) of size 25cm x 4.6mm, 5µm particle size) was performed in a Shimadzu HPLC system with a fluorescence detector(Fig-8a,b). The mobile phase was 70 % methanol. Excitation and emission wavelengths were λexc=322nm and λemis=440nm respectively. The flow rate was maintained 1ml/min and the run time was set at 15 minutes.

Figure 7 : Shimadzu HPLC system with a fluorescence detector.

A single peak was obtained on High performance liquid chromatography which confirmed the purity of Cleistanthin A. the identity of Cleistanthin A was further confirmed by chemists from IIT Mandi.

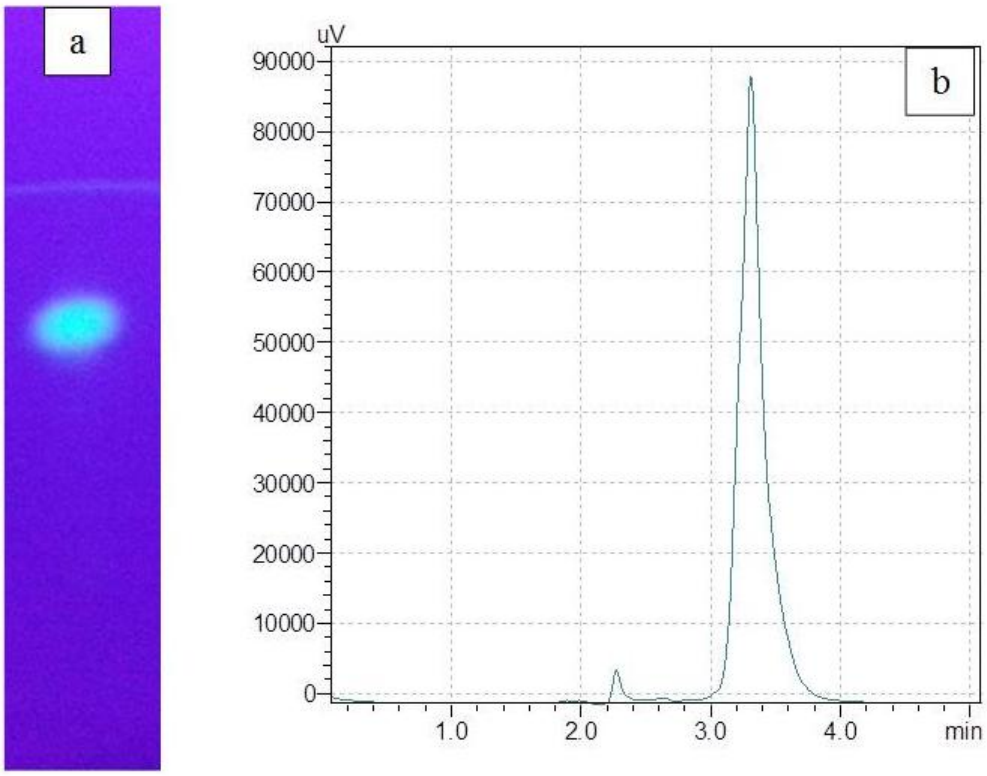


Figure 8 : a) Thin layer chromatography showing purity of Cleistanthin A

b) HPLC chromatogram showing purity of Cleistanthin A (1microgram/ml) in fluorescence detection (λexc/λemis=322/440nm), mobile phase - 70% methanol

PATCH CLAMP EXPERIMENTS

Patch clamp setup

A

B

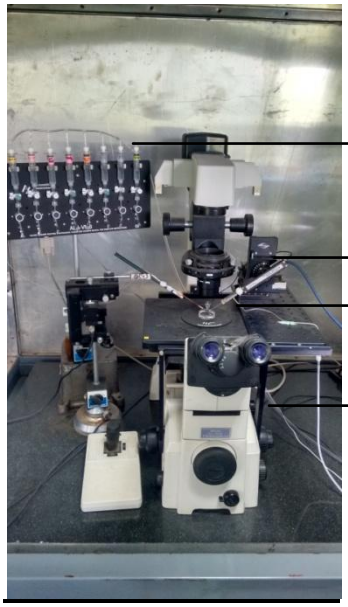


Figure 9 A and B: Patch clamp set up in our laboratory

Making of pipettes

Patch pipettes of 2-5 Mohm resistance were fabricated from Kimble Chase borosilicate glass capillary tubes. Dimensions of the glass tubes were 1.8 mm outer diameter and 1.5mm inner diameter. A Narishige Model PP 830 (Gravity assisted) pipette puller (fig 10) was used to pull these pipettes.

A

B

Figure 10 A and B : Making of pipettes using A Narishige Model PP 830 (Gravity assisted) pipette puller

Polishing of pipettes

The Pipette tips were polished using the Glassworx F-500 Fine Point Microforge filament. This step was done to smoothen the tips, and to prevent them from damaging the cell membrane. The weight settings and the temperature adjustments of the pipette puller and the microforge were standardised such that they yielded pipettes of resistance 2-5 MΩ.

Figure 11 : Polishing of pipette tips using Glassworx F-500 Fine Point Microforge filament

Sylgard coating of pipettes

Sylgard is a silicone elastomer which solidifies on exposure to high temperature. The polished pipettes were coated with this sylgard material under naked eye vision and were dried using a hot air blower. This solidified the sylgard. This helped to decrease the pipette capacitance.

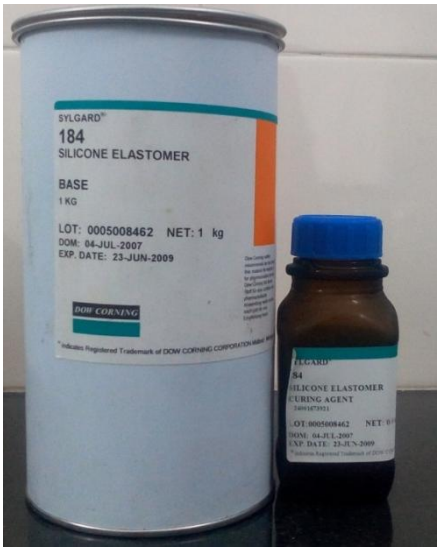


Figure 12 : Sylgard (silicone elastomer) used for coating the pipette tips

The polished and sylgard coated micropipettes were stored in a clean and spacious box to prevent the tips from dust contamination or breaking.

PREPARATION OF SOLUTIONS FOR RECORDING PROTON CURRENTS

Two different solutions were prepared.

1. For filling the pipette (internal or pipette solution)
2. For suspension of cells (external or bath solution)

Preparation Of Internal Solution	
Magnesium Chloride	2 mM
MES	100 mM
EGTA	1 mM
Caesium Chloride	35 mM
Glucose	10 mM
Ph	6
Osmolarity	300 mOsm

Table 1 : Composition of internal solution

Preparation Of External Solution	
Calcium Chloride	3mM
HEPES	100mM
EGTA	1mM
Caesium Chloride	60mM
Glucose	10mM
pH	7.5
Osmolarity	300mOsm

Table 2 : Composition of external solution

In order to prepare the cell suspension bath, 100 µL of the isolated neutrophil suspension was added to a petridish. After 1-2 minutes, 1900 µL of external solution was added. The final bath volume of 2ml was maintained for all the experiments to maintain a constant dilution factor even after the addition of the Cleistanthin A enriched fraction or the control solution.

Loading of pipette with internal solution

2 ml of internal solution was added to 400mM ATP and 30mM GTP. This was done to obtain an internal solution containing 4mM ATP and 0.3mM GTP. The tip of the micropipette was dipped in this internal solution and backfilled with the same. Care was taken to avoid air bubbles inside the pipette.

Positioning of electrodes

A silver pellet coated with chloride was used as the bath electrode. A silver wire coated with AgCl was placed inside the pipette. The micropipette containing the internal solution when dipped into the bath solution completed the electrical connection.

Focussing of cells

The cells were viewed under 40X magnification. Healthy cells were chosen for patching. Healthy cells were identified by their non distorted round shape and refractile nature.

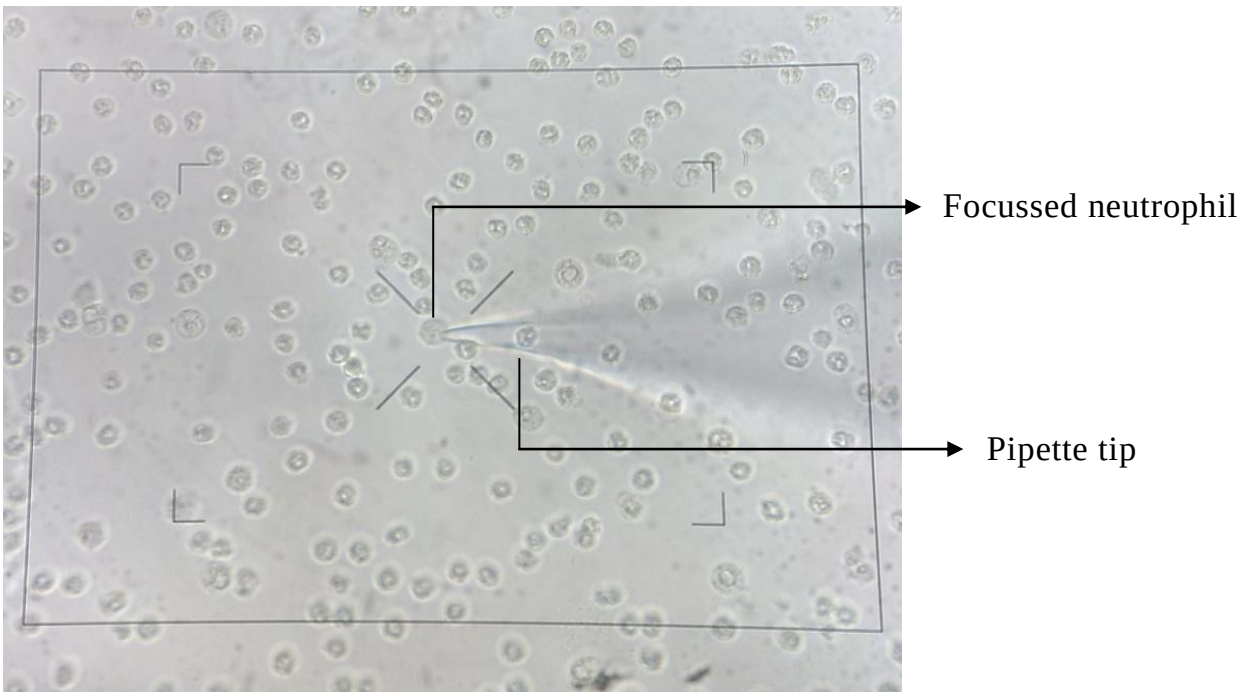


Figure 13 : Image of Freshly isolated human neutrophils as visualized under 40 X using NIKON inverted microscope

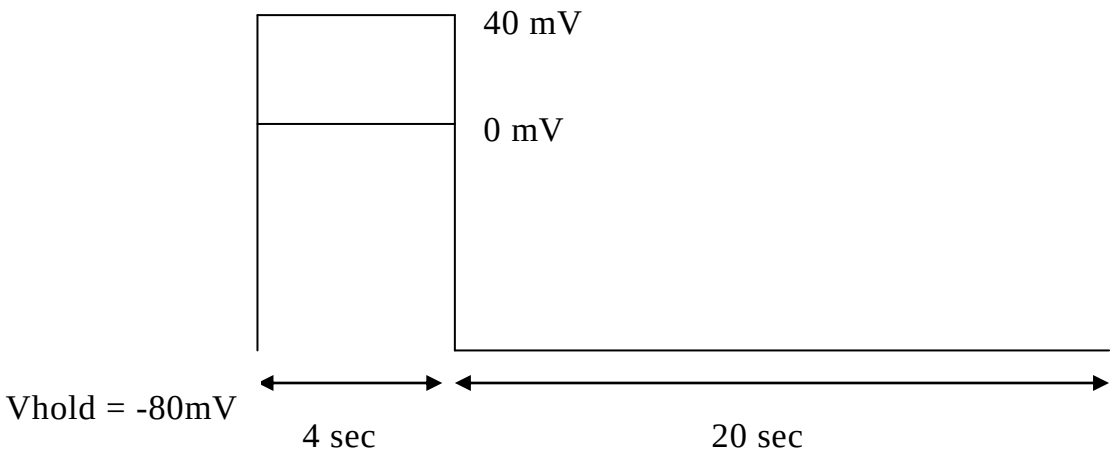
A micromanipulator was used to lower the tip of the pipette into the bath. The resistance of the pipette was displayed on the computer screen. The tip is lowered further to touch the cell membrane. The pipette resistance was found to increase slowly as the contact between the tip and the cell membrane was established. Giga ohm seal was achieved by introducing negative pressure through a syringe connected to the pipette. The holding voltage was changed from 0mV to -80mV. In order to gain access into the cell, a sharp suction was applied to break the membrane. Cell capacitance was measured using the Membrane Test function provided by Clampex 9.2 software. This provided information about cell

capacitance, membrane resistance, access resistance and time constant. The cell capacitance was cancelled electronically before proceeding to make a recording. The Octaflow perfusion system was used to perfuse the test and control solutions. Different protocols were used to make specific recordings. (Protocol has been described below).

Data acquisition was done using Axopatch 200B patch-clamp amplifier and digitization with Axon instruments Digidata 1322A analogue-digital converter. pClamp 9.2 software was used for data acquisition and analysis. Igor pro 5.04B software was used for offline analysis. Series resistance compensation of 40-60% was done.

Description of Protocol

Protocol for recording voltage gated proton currents



- ✓ The V_{hold} (holding potential) for the cell was -80mV
- ✓ Two depolarizing voltage pulses were applied, at 0 mV and 40 mV
- ✓ The duration of each pulse was 4 seconds
- ✓ After each test pulse, the membrane was brought back to the holding potential and held for 20 seconds before the commencement of the next pulse

Statistical analysis

For comparison of the peak currents within the groups (control and test) before and after intervention (control solution or Cleistanthin A), Wilcoxon Sign Rank Test was used. Similarly, for comparing the percentage current remaining across the groups (control and test) non-parametric Mann Whitney – U Test was applied. P value <0.05 was considered significant.

RESULTS

NORMAL PROFILE OF PROTON CURRENTS IN HUMAN NEUTROPHILS

Using a depolarizing protocol during patch clamp technique, a family of proton currents were recorded in freshly isolated human neutrophils.

Protocol: Holding voltage= -80mv, Command voltage= -20 to +80mv at 20mV increments, Duration of each command voltage= 4 sec; interval between each command voltage= 20 sec

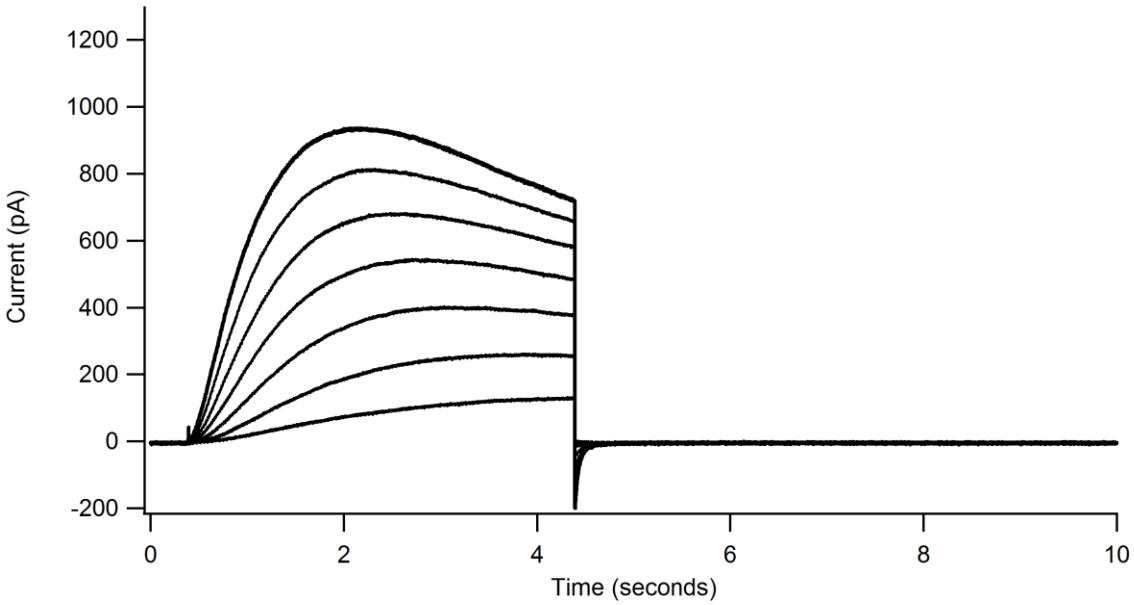


Figure 14 : Raw proton current tracing from a freshly isolated human neutrophil. Data filtered using Bessel (8 pole) low pass 100Hz.

REPRESENTATIVE I-V PLOT OF VOLTAGE GATED PROTON CURRENTS FROM HUMAN NEUTROPHILS

The recorded currents were plotted against voltages and an I-V graph was generated.

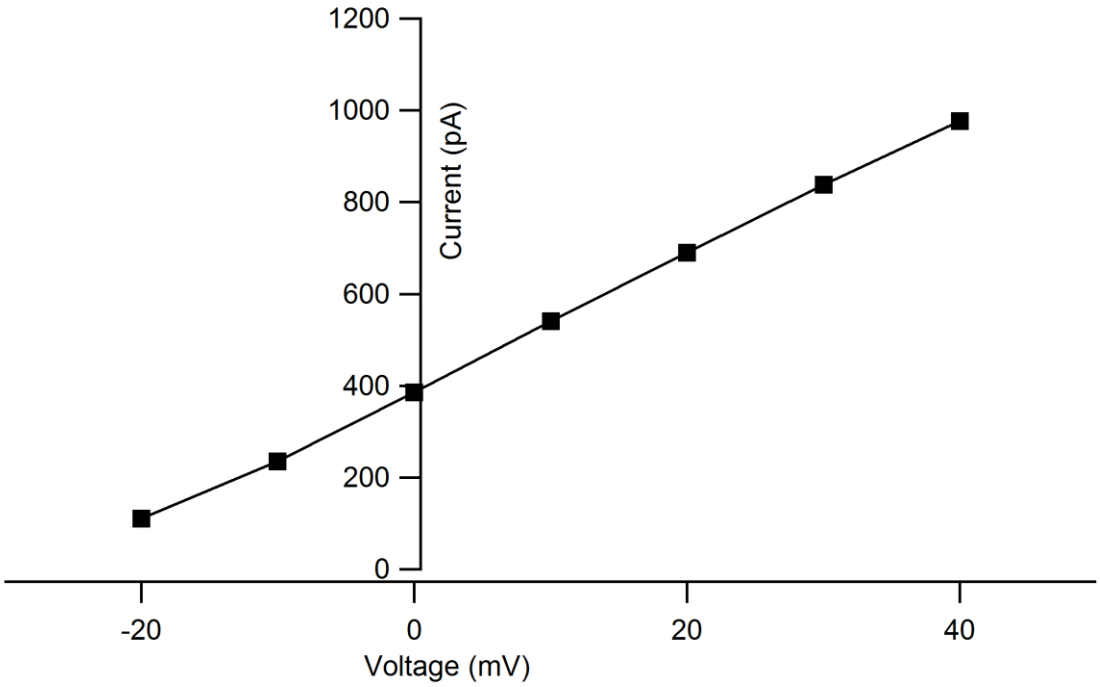


Figure 15 : Representative I-V plot of voltage-gated proton currents from freshly isolated human neutrophils. The plot shows the amplitude of current (pA) at different voltages.

I-V plot shows that with increasing depolarizing voltages, the open probability of proton channel and the electromotive force for proton currents increases. Hence, this explains the reason for the increase in current amplitude observed with increasing positive voltages.

REPRESENTATIVE CAPACITANCE TRACING

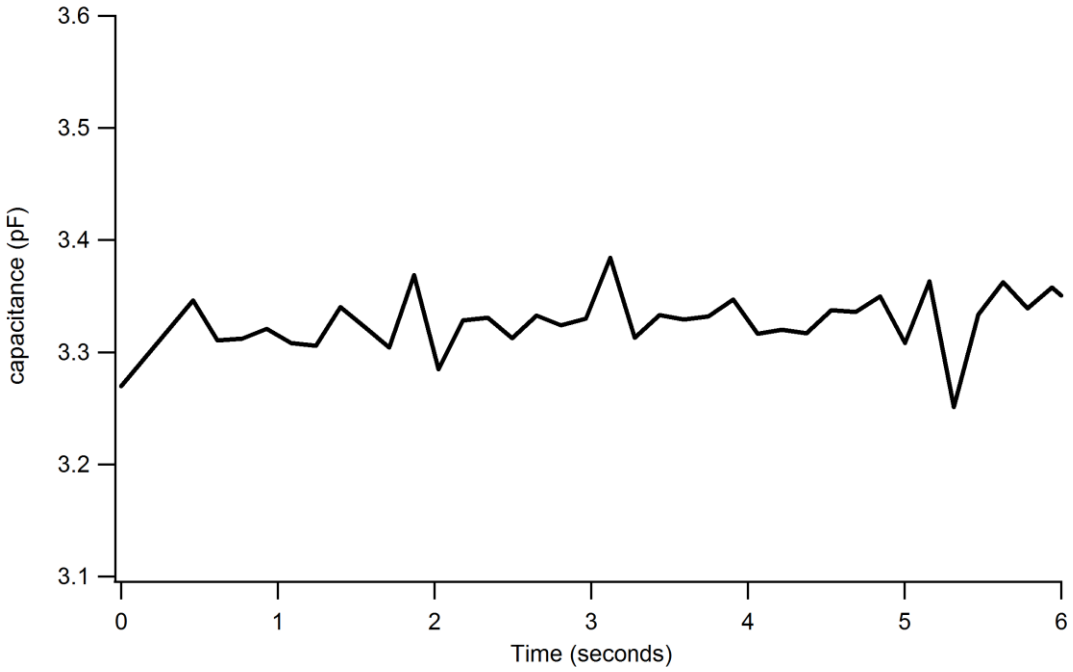


Figure 16 : Representative cell capacitance tracing recorded from freshly isolated human neutrophil. The cell capacitance was recorded immediately after going whole cell.

EFFECT OF ETHANOL (CONTROL SOLUTION) ON PROTON CURRENTS OF HUMAN NEUTOPHILS (with ATP included in the patch pipette)

To study the effect of control solution (ethanol) on proton currents, the pre-intervention currents were compared with the post-intervention currents at corresponding voltages.

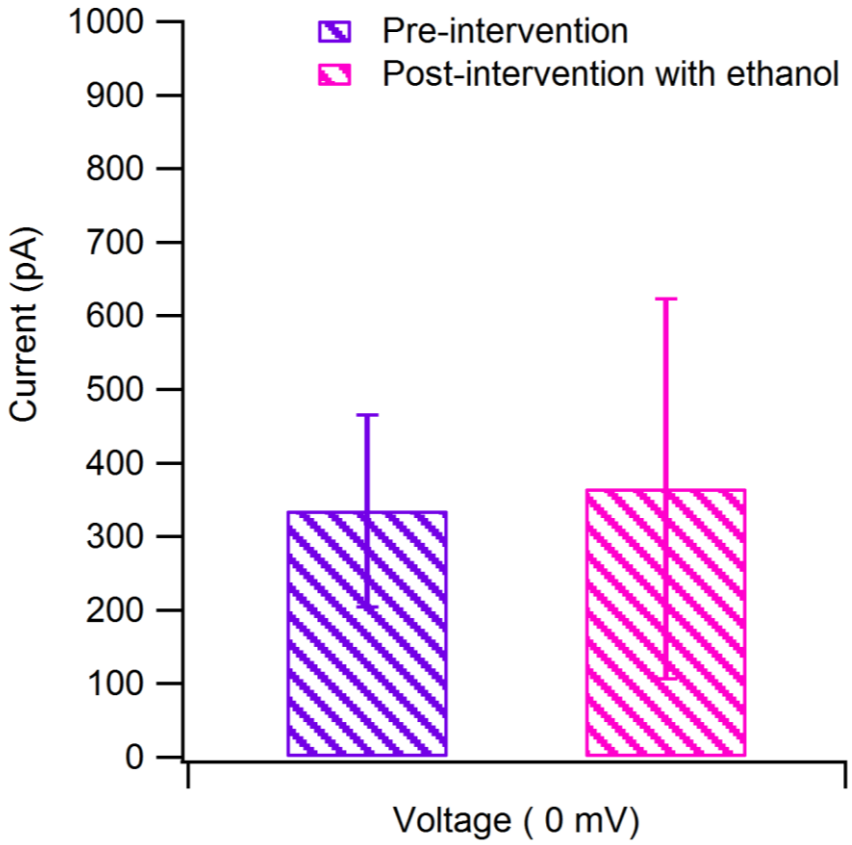


Figure 17 : Bar graph depicting the mean peak currents after intervention with ethanol as compared to currents recorded before intervention at 0mV (Mean ± SD, N=6 control and N=6 for test). P-values obtained from Wilcoxon Sign Rank test, P-value at 0mV = 0.753

Comparison of currents recorded before and after addition of ethanol at 40 mV

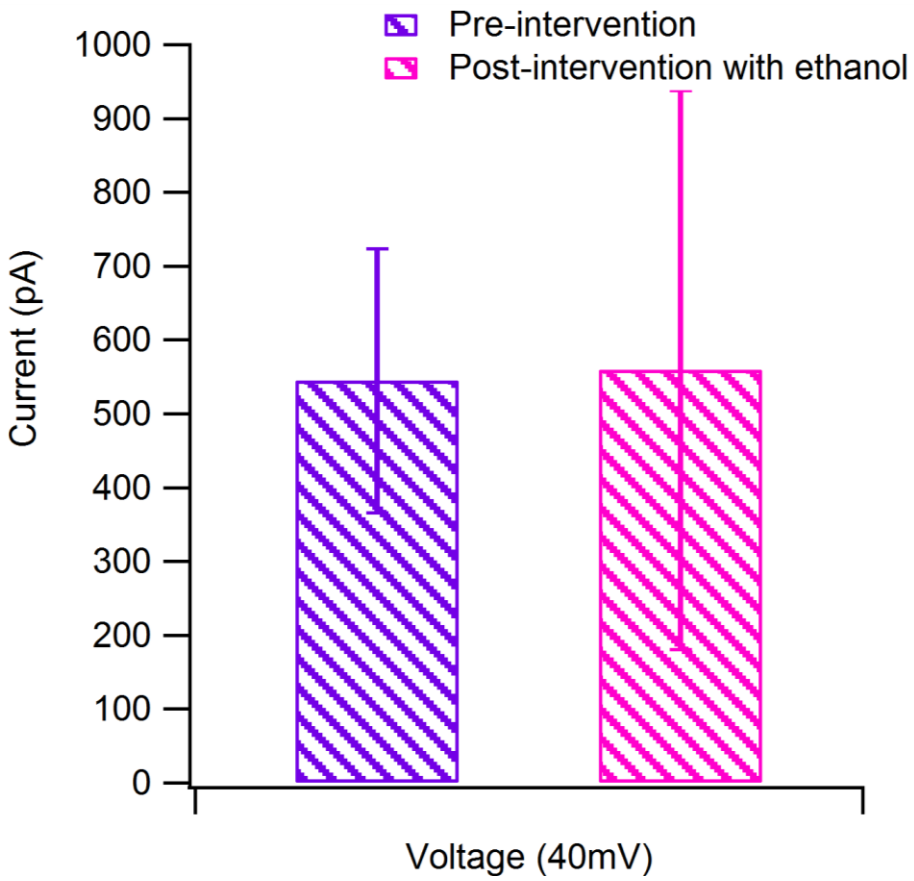


Figure 18 : Bar graph depicting the mean peak currents after intervention with ethanol as compared to currents recorded before intervention at 40mV (Mean ± SD, N=6 control and N=6 for test). P-values obtained from Wilcoxon Sign Rank test, P-value at 40mV = 0.463

EFFECT OF CLEISTANTHIN A (TEST SOLUTION) ON PROTON CURRENTS (with ATP included in the patch pipette)

Comparison of currents recorded before and after addition of Cleistanthin A at 0 mV

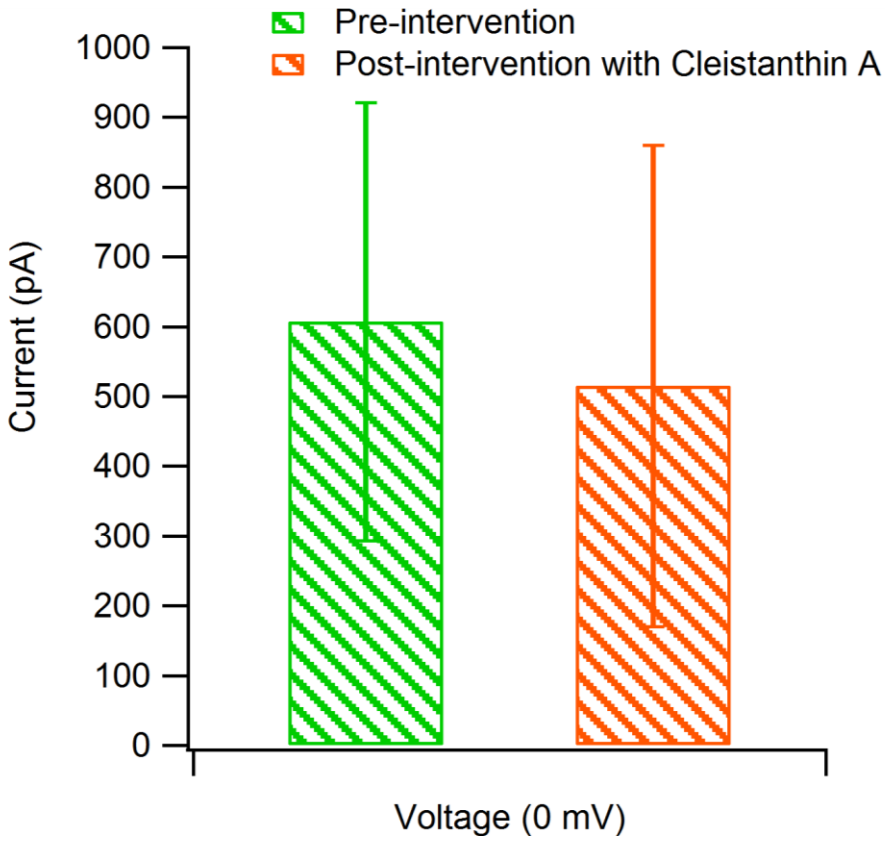


Figure 19 : Bar graph depicting the mean peak currents after intervention with ethanol as compared to currents recorded before intervention at 0mV(Mean±SD, N=6 control and N=6 for test) P values obtained from Wilcoxon Sign Rank test = 0.917)

Comparison of currents recorded before and after addition of Cleistanthin A at 40 mV

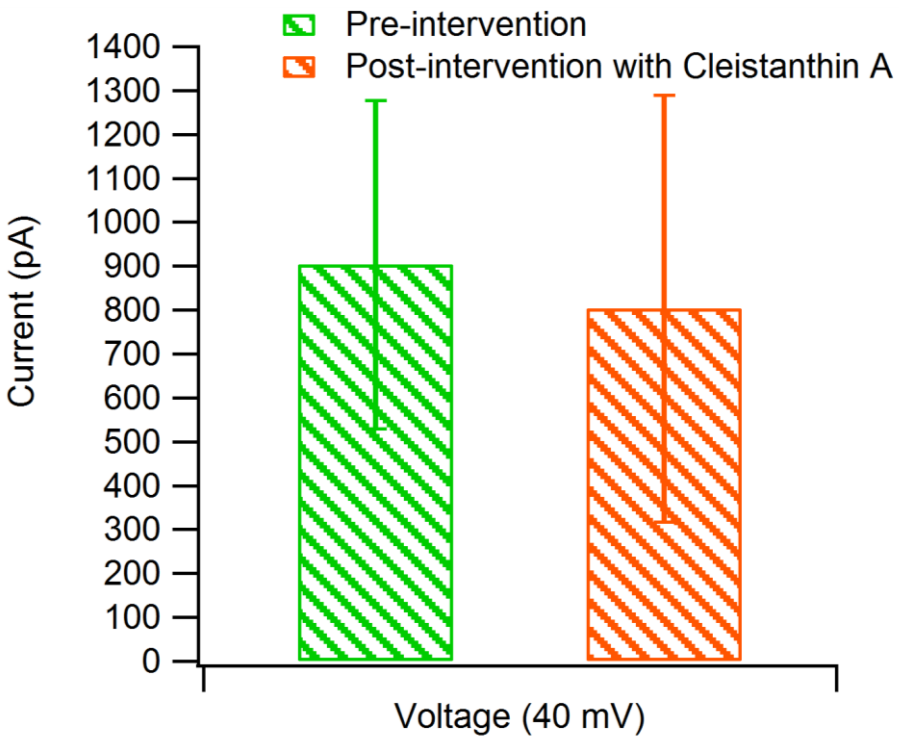


Figure 20 : Bar graph depicting the mean peak currents after intervention with Cleistanthin A as compared to currents recorded before intervention at 40mV (Mean ± SD, N=6 control and N=6 for test). P-values obtained from Wilcoxon Sign Rank test, P-value at 40mV = 0.916

COMPARISON OF PERCENTAGE OF CURRENT REMAINING IN THE CONTROL AND TEST GROUPS

The percentage of current remaining in the control and test group was calculated using the formula,

$$\% \text{ current remaining} = \frac{\text{Post-intervention current for a voltage x 100}}{\text{Pre-intervention current for the same voltage}}$$

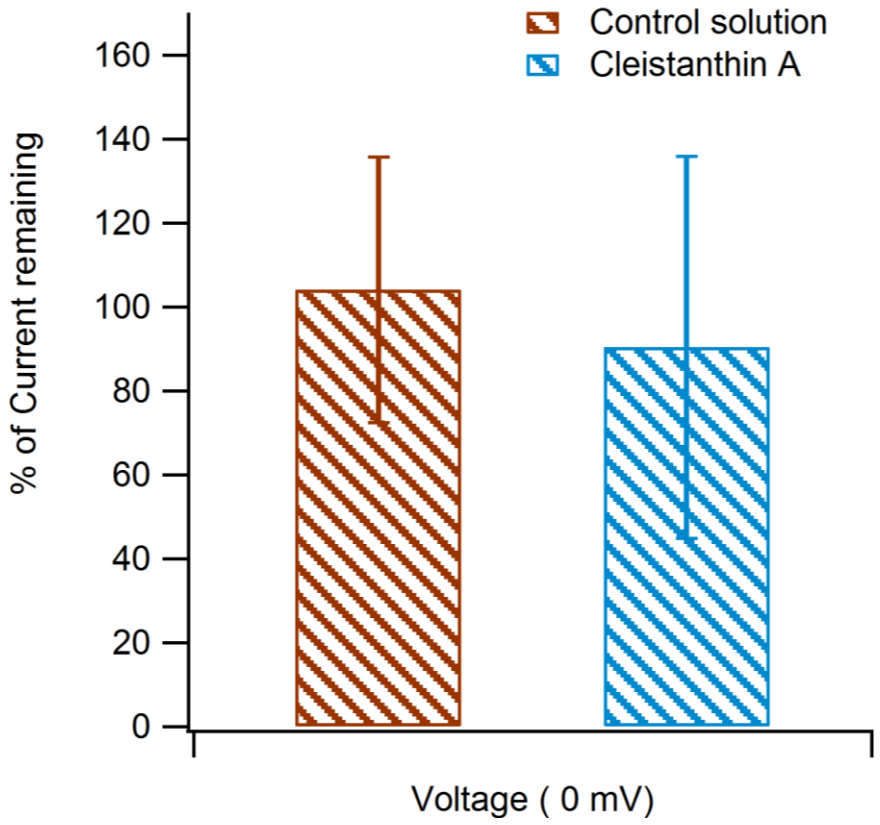


Figure 21: Bar chart depicting the percentage of current remaining after intervention with control solution (ethanol) and test solution (Cleistanthin A). The currents were recorded at 0mV (Mean ± SD, N=6 control and N=6 for test). P-values obtained using Mann Whitney U test =0.522)

Comparison of percentage current remaining in the control and test group at 40 mV

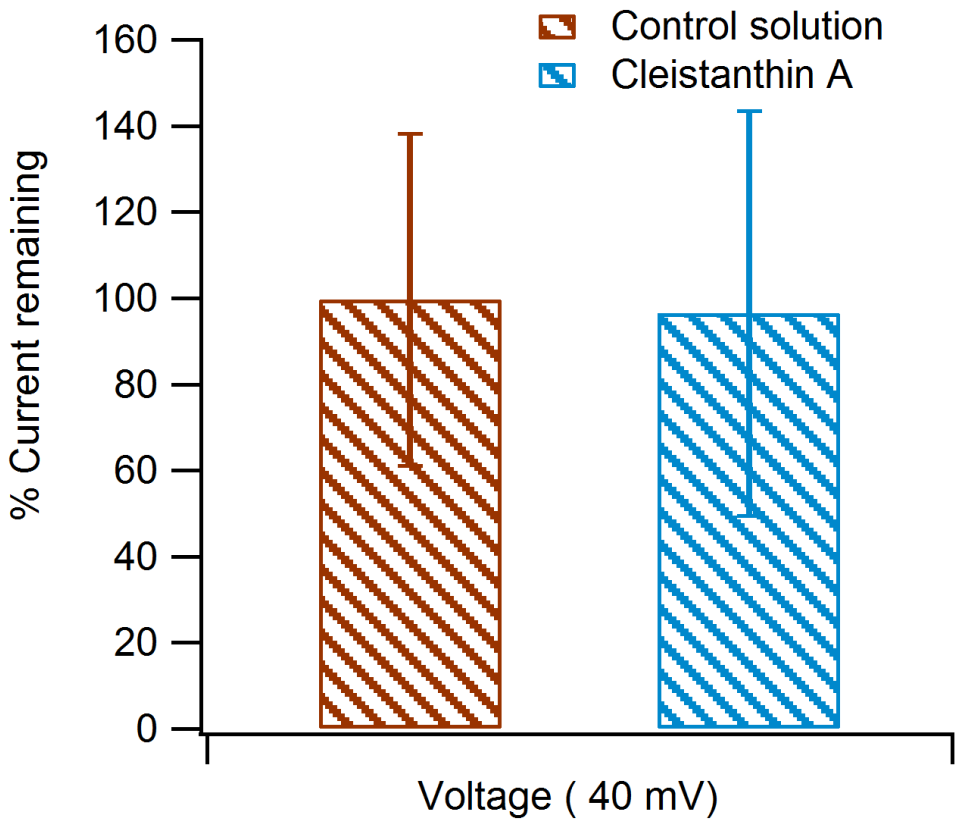


Figure 22 : Bar chart depicting the percentage of current remaining after intervention with control solution (ethanol) and test solution (Cleistanthin A). The currents were recorded at 40mV (Mean ± SD, N=6 control and N=6 for test). P-values obtained using Mann Whitney U test =0.873)

DISCUSSION

The plant *Cleistanthus collinus* contains many toxic compounds of which Cleistanthin A and Cleistanthin C are the most toxic compounds. Our main focus was to determine the mechanisms that lead to respiratory arrest in cases of *Cleistanthus collinus* poisoning.

In previous studies done in our department, proton channel modulating activities of whole aqueous extract, Cleistanthin A, and Cleistanthin C have been tested by patch clamp experiments in neutrophils. It was found that the whole aqueous extract blocked proton channels. Later, two toxic principles Cleistanthin A and C were tested for proton channel blockade in human neutrophils. While neither compound blocked proton channels, the impression was that Cleistanthin A may actually enhance proton currents, though the increase was not statistically significant. Since ATP was not present in the pipette in the earlier experiments, we hypothesized that in the presence of ATP, Cleistanthin A may enhance proton currents significantly.

Therefore objective of the present study was to test our hypothesis that Cleistanthin A enhances proton currents in the presence of ATP. This was achieved by analysing the percentage of proton currents remaining after the

addition of the test or control solutions in comparison to currents recorded before addition.

Using a depolarizing protocol during patch clamp technique, a family of outward proton currents was recorded in freshly isolated human neutrophils. The pipette and bath solutions were designed in such a way that protons were the only charge carriers. Potassium currents were ruled out because the pipette and bath solutions contained caesium chloride which is a potassium channel blocker. Calcium currents were also ruled out because they are a family of inward currents unlike proton currents which are outward currents.

Proton currents recorded in human neutrophils had a similar profile to that described in literature. The currents showed slow activation kinetics and increase in magnitude with increasing depolarizing voltages.

The effect of ethanol (control solution) on proton currents of human neutrophils was studied by recording proton currents before and after the addition of ethanol into the external bath. Ethanol was chosen as the control solution because, the test compound Cleistanthin A dissolved well in this solution. The peak current at each voltage within the control group was analyzed using Wilcoxon signed rank test. P-values obtained from Wilcoxon Sign Rank test are as follows;

- P-value at 0mV = 0.753
- P-value at 40mV = 0.463

Obtained P-values indicate that ethanol itself may have no significant effect on proton currents of human neutrophils.

Similarly, the effect of Cleistanthin A (test solution) on proton currents of human neutrophils was studied by recording proton currents before and after the addition of Cleistanthin A into the external bath. The peak current at each voltage within the test group was analyzed using Wilcoxon signed rank test. P-values obtained from Wilcoxon Sign Rank test are as follows;

- P-value at 0mV = 0.917
- P-value at 40mV = 0.916

Obtained P-values indicate that Cleistanthin A also does not have any effect on proton currents of human neutrophils.

COMPARISON OF PERCENTAGE OF CURRENT REMAINING IN THE CONTROL AND TEST GROUPS

The percentage current remaining after addition of Cleistanthin A solution and control solution (ethanol) was analyzed using Mann Whitney U test. The results show that there was no statistically significant difference in %

current remaining after addition of Cleistanthin A as compared to control solution.

CONCLUSION

Our aim was to see if Cleistanthin A modulates the activity of proton currents in the presence of ATP in the internal solution. Patch clamp experiments were performed to record normal proton currents and to study the effect of control and test solutions on them.

When the proton currents recorded at different voltage-clamp levels, after the addition of either Cliestanthin A (test) or ethanol (control), were expressed as percentages of the pre-intervention currents at the same voltages, there was no statistically significant difference in the percentage current remaining after intervention in both groups.

The results indicate that even in the presence of ATP, Cleistanthin A does not show any significant effect on voltage gated proton channels of human neutrophils.

LIMITATION OF THE STUDY

- ✓ Lack of temperature controlled systems to maintain the temperature of the bath solution containing neutrophils

FUTURE COURSE OF THE STUDY

- ✓ The effect of Cleistanthin A on other voltage gated ion channels can be pursued further.
- ✓ To find other possible mechanisms that lead to respiratory arrest in *Cleistanthus collinus* poisoning.

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ANNEXURES