# CHEMICAL CHARACTERIZATION AND TOXICITY ANALYSIS OF EXTRAFLORAL NECTAR IN *Nepenthes khasiana* Hook.f.

**A DISSERTATION SUBMITTED TO MAHATMA GANDHI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF**

**MASTER OF PHILOSOPHY IN BOTANY**

**BY**

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I hereby declare that the dissertation entitled **"Chemical Characterization and Toxicity Analysis of Extrafloral Nectar in *Nepenthes khasiana* Hook.f."** is an authentic record of the research work carried out by myself under the guidance of Dr. B. Sabulal, Senior Principal Scientist and Head, Phytochemistry and Phytopharmacology Division, KSCSTE- Jawaharlal Nehru Tropical Botanic Garden and Research Institute (KSCSTE-JNTBGRI), Thiruvananthapuram and under the supervision of Dr. Lizzy Mathew, Principal and Professor, St. Teresa's College (Autonomous), Ernakulam in partial fulfillment of the requirements for the degree of Master of Philosophy in Botany. The present work has not been submitted to any other University or Institute for the award of any degree or diploma.

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Place: Ernakulam

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# ABBREVIATIONS

ACh - Acetylcholine

AChE - Acetylcholinesterase

APCI - Atmospheric Pressure Chemical Ionization

DTNB - 5,5′-Dithiobis (2-nitrobenzoic acid)

FNs - Floral Nectars

GC - Gas Chromatography

HPLC - High Performance Liquid Chromatography

HPTLC - High Performance Thin Layer Chromatography

ICP-MS - Inductively Coupled Plasma-Mass-Spectrometry

Kcat - Catalytic constant

Km - Michaelis constant

LC-MS - Liquid Chromatography-Mass Spectrometry

Rf – Retention factor

S.E.M – Standard error of the mean

TIC - Total Ion Chromatogram

TLC – Thin Layer Chromatography

Vmax - Maximum reaction velocity

# ABSTRACT

*Nepenthes* is a genus of carnivorous plants, and they have unique ways of attracting preys into their leaf-evolved biological traps, known as pitchers. Sugar rich nectar, pitcher colour, UV induced fluorescence emissions, slippery wax crystals, $CO_2$ emissions, narcotic alkaloid secretions, volatile metabolites and other biochemicals play various roles in attracting and capturing preys into the pitchers. Nectar produced by the pitcher plays an important role in prey capture. *Nepenthes khasiana* Hook.f., the only pitcher plant native to India, is largely endemic to the Khasi Hills in North East India. Nectar extracted from the peristome and lid of *N. khasiana* pitchers was tested for its sugar content, and its glucose, fructose and sucrose were quantified by HPTLC-densitometry. The results showed that fructose was the dominant sugar in the extrafloral nectar of *N. khasiana* pitchers. Metal content analysis by ICP-MS revealed that *N. khasiana* pitcher peristome and lid nectar was rich in magnesium, calcium and iron. Toxic metals like arsenic, cadmium, lead and mercury were also detected in the nectar. In LC-MS analysis of nectar shows some common peaks in peristome and lid, so it shows the presence of similar secondary metabolites. This is the first report of systematic chemical characterization of the nectar of *N. khasiana*. Toxicity study of nectar shows that, the increase in the Vmax of AChE and subsequent inhibition of AChE activity resulting in accumulation of ACh suggests a brain milieu imbalance paving way to neuronal excitability or seizures in the test ants which consecutively result in their mortality.

# CHAPTER 1

# INTRODUCTION

Plant animal relations are usually recognized as the scenario where herbivorous insects/animals feed on the plants. However, the opposite is also known, where plants consume insects and are commonly called as Carnivorous plants. The genus *Nepenthes* and several other carnivorous plants obtain nutrients, especially nitrogen from the insects trapped by special mechanisms. Once captured, the prey is digested by the enzymes in the digestive fluid to release nutrients and make it available for the plants. Special glands are localized on the inner surface of the pitchers to taken up nutrients.

The carnivorous syndrome in plants have intrigued men at least since mid of the 19$^{th}$ century, and not just because of Charles Darwin's pioneer studies published in his book 'Insectivorous Plants'. The insectivorous, or more broadly carnivorous plants, usually grow in nutrient poor environments and carnivory has evolved as a supplementary pathway to get nutrients such as nitrogen and phosphorus (Adamec, 1997). Carnivory in angiosperms has been developed on an independent basis at least six times and carnivorous plants are polyphyletic (Albert et al., 1992; Ellison and Gotelli, 2009). Whenever carnivorous syndrome has been established in higher plants it represents the striking example for a comprehensive interaction of design and function in nature. Remarkably, structural homologies do not essentially relate with trap form, and similar trap forms may not be architecturally homologous (Albert et al., 1992). Carnivory includes morphological and anatomical features related to the (i) attraction, (ii) trapping and killing of prey, followed by (iii) its digestion and absorption of the nutrients.

The plant species in several genera show some of them, but not all these characteristics. *Dionaea, Drosera, Drosophyllum, Darlingtonia, Nepenthes, Cephalotus, Heliamphora,*

*Sarracenia, Byblis, Pinguicula, Genlisea, Utricularia* etc., are some of the genus belonging to carnivorous plants.

Carnivorous plants have five basic trapping methods.

1. Pitfall traps (pitcher plants) catch prey in a curled leaf with a puddle of digesting enzymes or bacteria (*Heliamphora, Sarracenia, Darlingtonia, Nepenthes, Cephalotus*).
2. Flypaper traps use sticky mucilage (*Drosophyllum, Byblis, Drosera, Pinguicula*).
3. Snap traps utilize rapid leaf movements (*Dionaea*).
4. Bladder traps catches prey using an internal suction created by bladder (*Utricularia*).
5. Lobster traps, also known as eel traps, use inward-pointing hairs forcing preys to migrate towards the digestive organ *(Genlisea)*.

**1.1 *Nepenthes***

The genus *Nepenthes* is a carnivorous plant belongs to the family Nepenthaceae and they also known as tropical pitcher plants or monkey cups. Around 170 species, as well as numerous natural and cultivated hybrids, are found in South China, Indonesia, Malaysia, and the Philippines; westward to Madagascar (two species) and the Seychelles (one); southward to Australia (three species) and New Caledonia (one); and north-eastern parts to India (one species) and Sri Lanka (one). Borneo, Sumatra, and the Philippines have the most diverse ecosystems, with numerous endemic species.

A shallow root system and prostrate or ascending stem are the typical features of *Nepenthes* species. These plants are recognized to have a super changed leaf that includes a tube-formed cup (pitcher) on the cease of leaf tendril. A protrusion of the

midrib (the tendril), that is from the tip of the leaf which aids in climbing of some species, actually the pitcher forms at the end of the leaf tendril. The pitcher begins as a tiny blossom and grows into a globe- or tube-shaped trap over time. Each pitcher's bottom half is filled with a digestive fluid in which the trapped prey drowns and decomposes. The nutrients supplied by the pitcher are absorbed by multicellular glands on the inner wall. The utilization of this is in nutrient- poor habitats where other plants struggle to survive.

The pitcher may be divided into three distinct zones, which are:

(a) Peristome, which is thought to be related in attracting and trapping the prey

(b) Slippery and waxy internal zone, which is used to entice and hold the prey from escaping

(c) Digestive zone, that is completely blanketed with glands and full of digestive fluids

The pitcher trap consists of several specialised structures it includes lower glandular and an upper waxy zone split the inner pitcher wall. The pitcher rim (peristome) is often conspicuously coloured and characterised by a regular pattern of radical ridges. It has an overhanging inner edge that is densely packed with extra floral nectaries. The rim of the pitcher is frequently prolonged upwards to the lid, giving a unique "neck." The lid covers the pitcher aperture in most species and protects it from heavy rain; however it is decreased or curved backward in some species. A pitfall mechanism captures prey; no moving plant elements are involved in the trapping operation. Insects lose their footing on the specialised, anti-adhesive surfaces of the peristome and the inner pitcher wall. *Nepenthes* species have epicuticular wax crystals on the upper section of their inner pitcher wall.

*Nepenthes khasiana* Hook f., belongs to the Nepenthaceae family and having polyploidy $2n = 80^{12}$ chromosomal number. The species has a relatively limited range and is quite

rare in the wild. Isolated populations exist in the Jarain area of the Jaintia Hills, the Baghmara area of the Garo Hills, adjacent to the Khasi Hills region of Meghalaya and in the Upper Kharthong area of Dima Hasao district Assam. It is an endangered tropical pitcher plant and the only representative of the genus *Nepenthes* found in India. The Khasi people call the plant 'tiew-rakot', which means demon-flower or devouring-plant. The plant has long, oblong-lance shaped leaves. Some unusual leaves first look like normal leaves, then develop a tendril at their top. As the pitcher matures, the lid turns to a reddish hue. Prey capture and digestion in *Nepenthes* species through their leaf and evolved biological traps involve a sequence of exciting events. Different components of the pitcher are involved in attracting and collecting insects, as well as digesting and absorbing nutrients. Sugar rich nectar, aroma chemicals, narcotic alkaloid secretions, slippery wax crystals, fluorescent light in the peristome, $CO_2$ emission and other biochemicals take part in attracting, capturing and digesting preys in *Nepenthes* pitchers (Kurup et al., 2013). The pitchers features are unusual construction with exceptional anti-adhesive characteristics for catching insects, according to ultrastructure studies. The high levels of $CO_2$ dissolved in *N. khasiana*'s pitchers are acidic fluid; it acts as a trigger to attract insects. The dissolved $CO_2$ in pitcher fluid has also been shown to influence the most unique aspects of *Nepenthes* pitchers, such as improved growth rate, high carbohydrate content, low protein content, low photosynthetic capacity, higher respiration rate, and developed stomata (Baby et al., 2017). Pitcher peristome in *N. khasiana* exhibits blue fluorescence, which attracts insects because they see the pitcher peristome's blue light ring as a source of food (Kurup et al., 2013). The fluid of the unopened pitcher of *N. khasiana* is used by local Khasi's, as an eye drop for redness, itching, to cure cataract and night blindness and they are also taken for stomach troubles, diabetes and for female diseases. The unopened pitcher and its contents were

made into a paste and applied for various skin related diseases. Hence the species is an important ethno medical plant and needs to be conserved. The plant is also characterized by the synthesis of bioactive antifungal naphthoquinones.

*N. khasiana* is a key source of naphthoquinones, which has a variety of medicinal properties and anti-fungal activities are possible against broad spectrum of human fungal pathogens (Raj et al., 2011). Additional investigation into existing data is required in order to develop new clinical trials for advanced therapeutics. Further, successful strategies must be implemented in order to ensure its long-term survival and conservation. The variety of human-caused actions continually, are causing problems for the wild populations of *N. khasiana* that result in the verge of insectivorous plant extinction from India.

**1.2 Phytochemistry**

The study of phytochemicals, or plant-derived substances, is known as Phytochemistry. Such studies strive to describe the structures of the large number of secondary metabolic compounds found in plants, the functions of these compounds in human and plant biology. It is closely interrelated to taxonomy, ethno botany, pharmacology and plant biochemistry. Ethno botany can be used to lead activities in botanical gardens or in the wild. Extraction, isolation, and structural elucidation of natural products, as well as other chromatographic techniques, are extensively utilized in the field of Phytochemistry (Torsell, 1997).

Phytochemicals can be classified as primary metabolites and secondary metabolites. Primary metabolites are low molecular weight carboxylic acids, alpha amino acids, carbohydrates, fats, proteins, nucleic acids, etc. involved in the life processes of plants. The role of primary metabolites is vital in the plant life, mainly as a source of energy,

storage food material, important physiological regulators, etc. and plants cannot survive without the primary metabolites. It is believed that ageing of the plant is a result of blockage of any of the metabolic cycles for the primary metabolites. All other compounds are produced from intermediates of primary metabolism. Secondary metabolites are derived from primary metabolites through various biosynthetic pathways. Secondary metabolites are in principle non-essential to plant life, but they definitely contribute to the survival of the species (Thomson, 1993).

### 1.3 Nectar

Nectars are the fluids which are secreted by plants to entice and recompense animal mutualists. The two important functional groups of mutualists include the pollinators like insects, birds and bats, enticed to floral nectar and safeguarding from arthropods such as ants and parasitoids, which are enticed to extra floral nectar. Floral nectar is most common in animal pollinated plants for proper pollination and normally extra floral nectars are not involved in pollination, but in direct defence towards herbivores.

The soluble solids that present in nectars mainly include mono- and disaccharides and amino acids. The other minor group of compounds include phenols, alkaloids, lipids, proteins and volatile organic compounds, reported from various nectars. The main function of nectar compounds is related for the action or power of evoking interest towards mutualistic organisms. Due to high content of nutritionally rich compounds, nectar is also enticed to other organisms who do not offer any mutualistic act of assistance to plant. They act as exploiters which may be nectar robbers or nectar-infecting microorganisms; these can predominantly reduce the defending efficiency of extra floral nectar and pollinating capability of floral nectar. So, disease causing microorganisms may use nectar and there by nectaries would be an introduction for the

state of infection to plants. Nectar components serve both in attraction and protection of nectar. The techniques used for nectar analysis require only small liquid volume, initial scenario only identifications was carried out but new enabled with quantification of different substances. It involves chromatographic techniques for sugars and amino acids – initially measured with thin-layer and paper chromatography, currently with Gas Chromatography (GC), High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) (Kearns and Inouge, 1993).

The importance of sugar and amino acid composition of nectar is often less clear than that of its concentration and volume, they together determine the energetic recompense available to foragers. Correlation between nectar concentration and sugar composition can arise very early in floral development. In nectar chemistry which contains sugar and some other components but incase of sugar - dominated by three simple sugars, mainly the disaccharide sucrose and the monosaccharides glucose and fructose. They usually originate from sucrose-rich phloem sap or from sucrose synthesized in the nectary tissue, and the production of monosaccharides depends on the presence and activity of various nectary enzyme systems. The distinctions between extrafloral and floral nectar complements are thought to be related to the latter's role in feeding ant or wasp "guards," which may have different nutritional needs than pollinators. Extrafloral nectars have more non-protein amino acids, presumably indicating chemical protection of exposed nectar from "thieves." Early in flower development, a link between sugar content and nectar concentration may emerge. The amount of monosaccharides in nectar is determined by the presence and activity of numerous nectary enzyme systems, including invertase, and comes from sucrose-rich phloem sap or sucrose produced in the nectary tissue (Baker et al., 1978).

Sucrose hydrolysis raises the osmolality of nectar, which draws water into it. Many nectars contain enough additional solutes in addition to sugars to disguise the true sugar concentration in nectar. Corbet et al., (1979) compared the refractive index of several nectars versus either ambient relative humidity or nectar osmolality: departures from the corresponding curves for pure sugar solutions revealed microclimatic and chemical impacts, respectively. Sucrose is translocated in phloem sap or produced in the nectary for all of them. Nectar invertase, which hydrolyses sucrose to glucose and fructose before or during nectar release, determines the relative amounts of each (Nicolson and Thornburg, 2007).

The production of extrafloral nectar is also known to be influenced by the environment. Smith et al., (1990) investigated the production and content of extrafloral nectar after simulated *Impatiens sultani* herbivory. Although the volume and carbohydrate content of nectar did not differ between intact and defoliated plants, HPLC analysis revealed a significant rise in amino acid contents in extrafloral nectars even after 24 hours of defoliation. Plant nectars also accumulate a wide range of other biochemicals. Some of them are supposed to enrich nectar and provide a healthier diet for pollinators, while others are thought to make nectar less appealing to unwelcome floral visitors. Some animals may find nectar harmful or repulsive due to alkaloids, coumarins, saponins, and non-protein amino acids (Nicolson and Thornburg, 2007).

Plentiful data on sugar composition of nectar have led to the idea for consideration that there are co-evolutionary relationships between the sugar proportions in nectar and the types of floral visitors.

### 1.4 Characterisation Techniques

Separation of mixture into individual compounds is a critical step in natural product chemistry, synthetic organic chemistry, molecular biology and other related research fields. The most frequently used technique in the separation stage is 'Chromatography'. In phytochemical analysis, chromatographic techniques are widely used for the detection and isolation of individual compounds from extracts. Chromatography was first introduced by the Russian Botanist, Mikhail S. Tsvet, in the beginning of the 20$^{th}$ century by combining two Greek terms-'chroma' (colours) and 'graphein' (write). It is a separation technique where the separation is affected by the differential migration of solute molecules between the stationary and mobile phases.

#### 1.4.1 Thin Layer Chromatography (TLC)

TLC is widely used in natural product chemistry and synthetic organic chemistry. In this, a uniformly thin layer of solid powder held on a glass plate forms the stationary phase and a mobile phase runs over, separating the components of mixture. Detection of the compounds on a TLC plate is normally carried out by spraying the suitable reagent, the commonly used spray reagents are ceric sulphate, concentrated sulphuric acid, vanillin-sulphuric acid reagent, anisaldehyde-sulphuric acid reagent etc. the compounds separated can also be detected under UV light at 254 or 365nm, provided the compounds show fluorescence under UV light (Stahl, 1983). The movement of a chemical compound relative to the solvent front in each chromatographic system is constant and characteristic of the compound under identical experimental conditions. This relative movement ($R_f$ value), is defined as,

$R_f$ = (distance traveled by the component)/ (distance traveled by the solvent front).

TLC is used for quick profiling of extracts.

### 1.4.2 High Performance Thin Layer Chromatography (HPTLC)

High Performance Thin Layer Chromatography (HPTLC) is a major advancement of the principles of TLC. It is very essential for the qualitative as well as quantitative analysis of plant extracts, essential oils and their constituents. The basic differences between conventional TLC and HPTLC are in particle and pore size of the sorbents. Pre-coated plates are available for HPTLC in which silica gel of very fine particle size is used. The smaller particle size of the stationary phase helps in better resolution, sensitivity and only requires shorter time duration. HPTLC needs very less amount of concentrated sample and the size of the sample spot is in the range of 1mm in diameter. About 3-6 cm solvent front migration is enough to effect proper separation. Linear development is the most used technique in HPTLC. HPTLC techniques are very useful in obtaining fingerprint patterns of herbal formulations, extracts, essential oils and in detection of adulteration.

One important feature of HPTLC is that densitometry scanning; the amount of a constituent can be detected quantitatively by using appropriate standards (or) we can get a profile, which gives number of compounds present in the extracts. These are achieved by means of UV detection mainly at short (254 nm), long UV (366 nm) and by derivatization using suitable spray reagents.

### 1.4.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a very effective automated method for the separation of complex mixture of molecules. The major advantages of HPLC are high resolution, rapid separation, requirement of only small amount of sample and detection of fractionated molecules by passive detection methods such as UV absorbance measurements. Hyphenated instrumental techniques, in which Liquid Chromatography-Mass Spectrometry (LC-MS) and Liquid Chromatography-Nuclear Magnetic

Resonance Spectroscopy (LC-NMR) are very useful new generation techniques in phytochemical analysis.

The HPLC instrument normally includes degasser, sampler, pumps and detector, in addition there is a pattern of combination for separating and analyzing also introduced. The movements of cellular sections are percolating through the column in a discrete small volume. The additives of the pattern circulate through the column at specific velocities, which can be a feature of unique bodily interactions with the adsorbent (additionally known as desk bound section). The speed of every aspect relies upon its chemical nature, on the character of the desk bound section and at the composition of the cellular section. The time at which a particular analyte elutes is known as its retention time. The retention time measured below precise situations is figuring out function of a given pattern. The selected composition of the cellular section relies upon at the depth of interactions among diverse pattern additives and desk bound section. Depending on their affinity for the desk bound and cellular stages, analytes partition among two and throughout the separation system taking location with inside the column. The separation in which cellular section composition stays regular, during the process named as isocratic. Otherwise the mobile phase composition does not remain constant. The separation in which composition changed is described as a gradient elution.

### 1.4.4 Liquid chromatography – Mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC- MS) is an effective analytical chemistry technique and it is a hyphenated technique in which High Performance Liquid Chromatography connected to a mass spectrometer. It is used for plenty applications, that mixes the bodily separation competencies of excessive overall performance liquid

chromatography with the mass evaluation competencies of mass spectrometry. Mass spectrometry gives most important blessings because of its excessive sensitivity and mass selectivity. The generation includes use of an HPLC, in with which an inside character constituents and a combination are first separated primarily, based on totally their affinity with the cell section and desk bound section. They are mostly of silica and it is primarily based on totally column accompanied via a way of means of ionization and separation of ions (in MS) mainly based on their mass/charge ratio. The ion supplys are likewise relying on nature of analyte i.e., polar or nonpolar. The outcomes could be the mass numbers available withinside the sample.

The LC-MS generation entails use of HPLC and the character additives in combination are first separated, accompanied with the aid of using ionization and separation of the ions on premise in their mass/charge ratio. The separated ions are then directed to an image or electron multiplier tube detector, which identifies and quantifies every ion. The ion supply is an essential issue in any MS evaluation, as this essentially aids in green era of ions for evaluation. To ionize intact molecules, the ion supply might be APCI (Atmospheric Pressure Chemical Ionization), ESI (Electronspray Ionization), etc. are to call some famous ones. The preference of ion supply additionally relies upon at the chemical nature of the analyte of interest i.e. polar or non-polar. The most important blessings of this generation encompass sensitivity, specificity and precision as evaluation is achieved at the molecular level.

### 1.4.5 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

ICP-MS (inductively coupled plasma-mass-spectrometry) is a technique to determine low-concentrations (range: ppb = parts per billion = µg/l) and ultra-low-concentrations of elements (range: ppt = parts per trillion = ng/l). Atomic elements become ionized

when they pass through a plasma source and these ions are sorted according to their mass. ICP-MS more advanced than that of Atomic Absorption Spectroscopy (AAS) or Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) and some of the advantages are:

- Extremely low detection limits
- A large linear range
- Possibilities to detect isotope composition of elements

ICP-MS technique has multi-element character and high sample throughput that is, similar to ICP-OES but it allows to perform more sensitive measurements.

### 1.4.6 Objectives of the study

1. Collection and identification of *Nepenthes khasiana* Hook.f.
2. Extraction of nectar from *N. khasiana* pitcher peristome and lid.
3. Estimation of sugar in nectar extracts by High Performance Thin Layer Chromatography (HPTLC).
4. Estimation of amino acids in nectar extracts by High Performance Thin Layer Chromatography (HPTLC).
5. Chemical characterization of nectar using LC-MS and ICP-MS.
6. Toxicity studies of the nectar.

# CHAPTER 2

# REVIEW OF LITERATURE

Caryophyllales, Oxalidales, Ericales, Lamiales, and Poales are five orders of carnivorous plants that include both monocotyledons and eudicotyledons. There are around 600 species of carnivorous plants worldwide, divided into nine families. Droseraceae, Nepenthaceae, and Lentibulariaceae are the three principal groups of insectivorous plants in India. They have specific structural organs for trapping insects, such as pitcher-like leaves or comparable leaf groupings (Juniper et al., 1989 and Barthlott et al., 2007).

The genus *Nepenthes*, sometimes known as tropical pitcher plants, is one of the largest among insectivorous plants, belonging to the monotypic family Nepenthaceae. There are approximately 170 species in all, including numerous natural and artificial hybrids. *Nepenthes* species actively produce beautiful colors, sugary nectar, and even delicious odours to attract and kill their prey. These plants grow in soils were minerals are often deficient; the plants primarily obtain nitrogen and phosphorus from the captured insects to fulfill their nutrient requirements. Ants and other insects are the most common prey, which belong to an abundant and diversified group of arthropods (McPherson, 2009, Cheek and Jebb, 2009 and Giusto et al., 2009).

In Meghalaya, *N. khasiana* is one of the most harvested medicinal plant species and different indigenous people in Meghalaya have long utilized it to treat a variety of diseases. Local Khasis and Garos utilize the unopened pitcher's fluid as an eye drop for redness, irritation, cataract, and night blindness. The fluid is also used to treat gastrointestinal problems and feminine illnesses. The contents of the unopened pitcher

are ground into a paste and used to treat a variety of skin disorders, including leprosy. The pitcher's fluid is prescribed by indigenous herbalists in the Khasi and Janita Hills for the treatment of diabetes and painful urination (Devi et al., 2019).

Local people in Meghalaya drink the liquid in the *N. khasiana* pitcher in the morning as a digestive tonic, and the fine powder of pitchers mixed with water taken as orally for the treatment of cholera. Ear drops are made from the liquid within the closed pitcher. Pitcher extract of *N. khasiana* has been shown to drastically lower glucose and lipid levels in rats after oral administration of the pitchers' methanolic abstract, supporting the species' traditional use in the treatment of diabetes. The methanolic extract of *N. khasiana* leaves demonstrated substantial hepatoprotective properties, suggesting that it could be employed as a natural protective against liver injury. Plumbagin, a type of chemical naphthoquinone found in leaves of the genus *Nepenthes*, has staining effects (Raj et al., 2011).

*N. khasiana* is known for producing bioactive chemicals known as naphthoquinones, which have anti-malarial, antibacterial, antifungal, and anticancer properties. Droserone and its methylated derivative 2-methyl-3-hydroxy-5-methoxy-1, 4- naphthoquinone (5-O-methyldroserone) were discovered as the natural naphthoquinones generated in *N. khasiana* closed pitchers following chitin injection, which mimicked the prey capturing circumstances in the insect trap. In addition to these naphthoquinones, plumbagin was found in the root of *N. khasiana* and plumbagin content in potted *N. khasiana* plants with limited growth had higher levels of plumbagin accumulation than field plants, which was the highest found in any natural source (Sandur et al., 2006, Eilenberg et al., 2010, Eilenberg et al., 2006 and Raj et al., 2011).

In vitro propagation has emerged as an effective approach for large-scale propagation and conservation of germplasm of numerous plant species. Multiplication of this endangered insectivorous plant is tough beneath herbal situations because the seeds take 223 days to germinate and the proportion of germination is quite low. Factors affecting in vitro regeneration of *N. khasiana* from nodal segments of in vitro raised seedlings and the improvement of pitchers had been studied. The observers found out that a couple of shoots had been triggered on MS (Murashige and Skoog) medium supplemented with 0.1 mg/L IAA (Indole-3- acetic acid) and 2.0 mg/L BAP (6-Benzyl aminopurine). The range of shoot changed into expanded through incorporation of ascorbic acid, citric acid, arginine and adenine sulphate to the subculture medium (Bordoloi, 1977 and Rathore et al., 1991).

Different components of pitcher play distinct roles in attracting and shooting bugs, digesting the prey and soaking up the nutrients. The ultrastructure research has showed that the pitchers own splendid structure with wonderful anti-adhesive houses for trapping bugs. High degree of $CO_2$ dissolved in acidic pitcher fluids of *N. khasiana* act as a cue for attracting bugs. It has additionally been verified that the maximum particular capabilities of *Nepenthes* pitchers along with improved boom price, excessive degree of carbohydrate, low degree of protein, low photosynthetic capacity, improved breathing price and advanced stomata, are motivated via a way means of the dissolved $CO_2$ in pitcher fluid. Pitcher peristome in *N. khasiana* emits blue fluorescence attracting the bugs as they could understand the blue mild ring withinside the pitcher peristome as a touchdown pad indicating that the blue emission performs an essential function in guiding the traffic to the pitcher traps (Baby et al., 2017).

Sugars and amino acids form the nectar traveler assemblage in step with its composition. Thus, their feature especially seems associated with the various

physiological suitability of consumers, and, consequently, to their attractiveness to the specific traveler guilds. By contrast, secondary metabolites which are usually related to herbivore defense, including alkaloids and phenols, were additionally defined as nectar additives and in floral nectars (FNs) of at the least 21 plant families. These compounds are commonly seemed as “poisonous compounds” that flowers secrete into floral nectar to reap a safety from nectar robbers. For example, FN of *Catalpa speciosa* consists of iridoid glycosides that fended off nectar robbers however now no longer valid pollinators. Similarly, phenols in FN of *Aloe vryheidensis* decreased its palatability to generalist bugs and, as a result, the enchantment of non-powerful pollinators to the plant life of this species. By contrast, Adler and Irwin manipulated contents of gelsemine, which represents the predominant FN alkaloid of *Gelsemium sempervirens*, and determined that the supplementation of gelsemine to FN deterred now no longer simplest nectar robbers however additionally powerful plant pollinators, as a result reducing the variety of plant life probed and the time spent in step with flower via way of means of both, pollinators and nectar robbers (González-Teuber and Heil, 2009).

Nectar chemistry is largely dominated by sucrose, a disaccharide, and its monosaccharaides, fructose and glucose. Sucrose is translocated in phloem sap or produced in the nectaries. The enzyme, nectar invertase, which hydrolyzes sucrose to glucose and fructose before or during nectar release, determines the relative amounts of each. Within genera like *Erica* and *Leucospermum*, the stark dichotomy between sucrose and hexose nectars, with nectars at both ends of the sucrose–hexose continuum indicates the absence or presence of invertase activity. The heterogeneous sugar composition appears to be due to partial hydrolysis (Nicolson et al., 2007).

Since the mid-1950s, when Ziegler (1956) compared sieve element fluid to other plant fluids, including floral nectar, and found ninhydrin-reactive material in nectar, the

presence of amino acids in nectar has been known. Following that, Lüttge found glutamine, asparagine, methionine, serine, tyrosine, cysteine, proline, and alanine in the nectar of plantain bananas and later in the nectars of five other species using descending paper chromatography. They also concluded that amino acids have been a constituent of nectars since the earliest stages of angiosperm evolution, based on the phylogeny of the plant species studied. Various plant nectars have been discovered to contain all 20 of the typical amino acids found in protein, suggesting that necessary amino acids may be an important nitrogen supply for nectarivorous pollinators (Nicolson et al., 2007).

The production of extra floral nectar is also known to be related to the environment. Following imitated herbivory of *Impatiens sultanii*, Smith et al investigated the production and content of extra floral nectar. Although the volume and carbohydrate content of nectar did not differ between intact and defoliated plants, HPLC analysis revealed a significant rise in amino acid contents in extra floral nectars 24 hours after defoliation. This could be a plant's good way of attracting more defense insects. The plants appear to be return back in homeostasis quickly, since the amino acids in extra floral nectar were restored to normal levels ie, after a 72 hours of defoliation. Extra floral nectaries on the inflorescence of the Cowpea (*Vigna unguiculata*) have substantially higher quantities of amino acids than those on the leaf stalks. Pitcher plants produce extra floral nectar to entice insect prey, and Dress et al., (1997) hypothesizes that the amino acid composition of this nectar may be linked to previous capture success (Nicolson et al., 2007).

Luttge (1961) reported on the extra floral nectar of *Sansevieria zeylanica* (L.) Willd. (Asparagaceae) with clearly an incomplete list of three amino acids. In paper chromatograms of extra floral nectar from *G. barbadense* L., Mound (1962) detected two ninhydrin-positive compounds, but Hanny and Elmore (1974) found 24 amino acids

in *G. hirsutum* extra floral nectar using gas chromatography. 20 amino acids were found in extra floral nectar of *Helianthella quinquenervis* (Hook.) A. Gray (Compositae) according to D. W. Inouye and R. S. Inouye. Baker (1978) found a difference in nectar amino acid complements between floral and extra floral nectars of *Inga vera* subsp. spuria (Willd.) J. Leon (Mimosaceae). It should be emphasized that a less refined (paper chromatography) method of analysis was used instead of gas chromatography (Baker et al., 1978).

Bennett and Ellison experimentally displayed that prey of the carnivorous plant *Sarracenia purpurea* are drawn to sugar, now no longer to colour. Prey seize became now no longer related to overall crimson location or patterning on pitchers of dwelling pitcher flowers. The difference in coloration and nectar effect was separately studied by the usage of painted 'pseudo pitchers', 1/2 of which had been covered with sugar and other with no sugar. Unsugar pseudo pitchers captured honestly no prey, while pseudo pitchers with sugar were captured the identical quantity of prey as dwelling pitchers. The study infer nectar, not colour, in principal way by which pitcher plants attract animals and contrast to a recent study that linked red coloration with prey capture but lacked controls for nectar availability (Bennett and Ellison, 2009).

Extrafloral nectar produced through *Sarracenia purpurea* draws bugs no longer for safety from herbivores however as prey for a water-stuffed pitfall trap. Nectar from pitchers of *Sarracenia purpurea* contained amino acids, while amino acid composition becomes variable amongst pitchers. The commonly found nine amino acids are aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, methionine, serine, valine. Extrafloral nectar shows more variations than that of floral nectar. Prey capturing in the same pitcher or old pitchers depends on the varying composition of amino acid in pitchers. Many bugs, however, depends upon amino acids in nectar, and

pitchers won't achieve success in attracting prey without them. Ants are the maximum not unusual place traveller to pitchers and may be a vital prey component. The small quantity of amino acids found in pitcher nectar might also notably influence insect behavior, inflicting bugs to feed on pitchers for an extended time and growing capacity seize of prey through the pitcher. The variable composition of amino acids withinside the extrafloral nectar of the pitchers can be a characteristic of the prey seize through the identical or in advance pitchers at the plant. The newly opened pitchers had constrained numbers and portions of amino acids present, the nectar of the older pitcher contained 19 of the 20 amino acids tested (no proline become detected) and the full amino content material become over 300%, better than that suggested for the newly opened pitchers. Such a boom in amino acids with pitcher age is steady with contamination, even though the shortage of proline might imply that pollen isn't always the supply of contamination (Dress et al., 1997).

Honey is taken into consideration as an acceptable aspect in a selection of various foodstuffs, because of its nutrient and healing effect. The honey trait specially relies upon at the form of plant life visited through the bees and the climatic situations wherein the flowers are growing. Here the attention variety version of eighteen elements (Al, As, Ba, Ca, Cd, Co, Cr, Cu, Mg, Mn, Na, Ni, K, Pb, Sr, Ti, V, and Zn) turned into investigated in four kinds of honey (linden, acacia, rape and sunflower) originating from Romania, because the elemental profile of honey might also additionally provide vital facts to distinguish its geographical and varietal beginning for authenticity purpose. All the determinations have been performed through ICP-Q-MS. The maximum considerable minerals reduced with inside the following order: K > Ca > Mg > Na, having the suggested values of 248.70 mg $kg^{-1}$, 59.97 mg $kg^{-1}$, 20.54 mg $kg^{-1}$ and 11.91 mg $kg^{-1}$ respectively. The mineral content material marks the variations in honey

samples from specific botanical beginning and may be used as device for authentication purposes, and additionally amplify its applicability to evaluate the traceability of honey. Analysis of variance confirmed the initial relationships among the factors and samples. Further, the discrimination among different studied honeys samples became done through Principal Component Analysis (PCA). The multivariate evaluation of the statistics allowed us to split the honey samples into wonderful agencies in line with their macro-and micro-factors composition, emphasizing the starting place version of factors concentrations through honey type. Therefore, this technique is probably doubtlessly beneficial for managing honey quality, starting place or authenticity, or even to apply the honey as environmental tracer (Voica et al., 2020).

For the determination of atropine and scopolamine in *Datura* flower nectar, a liquid chromatography method with electrospray mass spectrometric detection was devised. The separation does not require any sample preparation prior to analysis with this approach. The findings revealed that the approach is extremely sensitive, repeatable, and precise. For atropine, the LOD utilising ESI-MS detection is 75.08 pg $mL^{-1}$, whereas for scopolamine, it is 150.15 pg $mL^{-1}$. Our findings show that the alkaloids found in the vegetative and reproductive sections of *Datura* plants are also found in the nectar secreted by the flowers, though at lesser concentrations. The alkaloid content increases correspondingly in large flowered *Datura* species, where the volume of nectar can reach 100–150 L per flower, and hence the nectar could be a source of intoxication (Boros et al., 2010).

# CHAPTER – 3

# MATERIALS AND METHODS

## 3.1. Plant profile

**Figure 3.1: *Nepenthes khasiana* Hook.f.**

Botanical name : *Nepenthes khasiana* Hook.f.

Common names : Monkey cups, Pitcher plant

Vernacular names : Ksete-phare, Memang koksi, Tiewrakot

Trade name : Indian Pitcher plant, Tiew rakot

### 3.1.1. Taxonomical Classification

Kingdom - Plantae

Phylum - Tracheophytes

Class - Magnoliopsida

Order - Caryophyllales

Family - Nepenthaceae

Genus - *Nepenthes*

Species - *N. khasiana*

### 3.1.2. Description of the Family

**Habit:** Herbaceous or shrubby plants.

**Root:** Tap, tetrarch to hexarch.

**Stem:** Rhizome with a ring of collateral open, endarch vascular bundles.

**Leaves:** Simple, alternate, stipules absent, stalked (petiolate), tendrillar leaf apex and tendril terminating into a pitcher with apical lid or operculum.

**Leaf highly metamorphosed into prey catching organ:**

**The fully developed leaf of *Nepenthes* has four parts,**

(i) Lamina or winged portion seated on stem,

(ii) Tendril coming out from lamina as midrib prolongation,

(iii) Pitcher or acidium (tendril terminal) and

(iv) Operculam or lid (apex of pitcher).

**Inflorescence:** Racemose to paniculate.

**Flower:** Actinomorphic, unisexual, greenish, small, hypogynous.

**Perianth:** Haplochlamydeous, simple dimerous and rarely tepals 3 or 5-6, polyphyllus, imbricate, very rarely connate at the base, two whorls arranged in crosswise (2 + 2). Tepals are elliptic, round or linear, outside hairy and glandular inside.

**Staminate flower**

**Androecium:** Stamens 4-24, the filaments with anthers are united together in a column or tube – monadelphous in columns; anthers distinct bithecous, extrorse, dehiscing longitudinally; pollen grains in tetrad.

**Pistillate flower**

**Gynoecium:** Carpels 4 (rarely 3), opposite to tepals, superior; 3-4 locular ovary, axile placentation; style very small or absent; stigma persistent, discoid, flat or concave, ovules many, anatropous, bitegmic with dorsal raphe.

**Fruit:** Loculicidal capsule, elongated, leathery.

**Seed:** Numerous, saw-dusk like, long, narrow, winged with fleshy endospermic.

**Pollination:** Entomophilous.

**Functions of Nepenthaceae:**

The pitcher serves as a digestion as well as an insect trap; the inside edge is bent, the lid is apical, the entry or hole of the pitcher includes the base of the lid with honey or nectar glands, and the lid serves as a landing stage as well as an enticing object for the prey. Attracted by the honey and the bright colour of the pitcher's cover, insects and flies descend among the glands, eventually arriving at the prey section near the entrance and falling into the pitcher, where they arse sucked into the liquid filling the pitcher's base. The enclosed prey dies and decays and the plant survives by absorbing the putrefying body of the prey's fluid (Cheek and Jebb, 2001).

**Distribution of Nepenthaceae:**

Nepenthaceae is a unigeneric monotypic own circle of relatives comprising simplest one genus *Nepenthes* with approximately 50 species. It is particularlly restricted to Indo-Malayan vicinity of the globe and *Nepenthes khasiana* Hook.f. located in Khasi hills of Assam.

**Economic Importance of Nepenthaceae:**

- **Ornamentals:** Many species and hybrids of *Nepenthes* are cultivated regionally in glass houses as novelties.
- **Cordage:** The stems of *Nepenthes distillatoria* (Sri Lanka) and *N. reinwardtiana* (Malaysia) are used as a type of cordage in addition for making baskets.

### 3.1.3. Description of *Nepenthes khasiana*

**Distribution:** Meghalaya (Khasi and Jaintia hills) - Endemic.

**Habitat:** This is a carnivorous plant species found in Meghalaya at an altitude about 1000-1500 m. This plant prefers acidic and nitrogen deficient soil, high rainfall and warm climate. Deficiency of nitrogen in soil compensated by the insects trapped in pitchers.

**Habit:** Shrubs, staminate and pistilate flowers borne on different individuals, clinging with tendrils from leaves.

**Leaves:** Alternate, an expanded lamina with tendril at the tip and modified by hanging downwards, coloured cylindrical pitcher with a bent ridged peristome and a lid.

**Inflorescence:** Racemes or panicles.

**Flowers:** Unisexual, actinomorphic, hypogynous.

**Tepals:** 3 – 4 teapals in 2 whorls, nectariferous.

**Male flowers:** Stamens 2 – 24, connate filaments, bilocular anthers.

**Female flowers:** Carpels 3 – 4, ovary hypogynous, ovules many, single style, stigma discoid.

**Fruits:** Elongated, leathery capsule.

**Seeds:** Numerous, filiform.

**Importance:** The fluid of unopened pitcher are used by Khasi and Garo tribes as eye drops to cure cataract and night blindness, and in treating stomach troubles, diabetes and gynaecological problems. The pitcher and its contents are made into paste and applied on the affected areass of leprosy patients.

## 3.2.METHODOLOGY

### 3.2.1 Collection and taxonomical identification of plant material

Fresh plant materials were collected from the campus sites of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram. The collected plant materials were authenticated by Dr. A.A. Hussain, Taxonomist, JNTBGRI, and a voucher specimen (39201) has been deposited at the JNTBRI Herbarium (TBGT).

### 3.2.2 Herbarium specimen preparation

The fresh plant material collected was processed using standard methods. The plant material was pressed between newspapers and the dried specimen was pasted on a herbarium sheet of standard size 29×42 cm. After mounting, the herbarium number,

family, genus, species, local name, habit, habitat, date of collection and name of collector of the specimen was entered in the sheet.

### 3.2.3 Nectar Extraction

*N. khasiana* pitchers of uniform age group (5-8 days after opening) was collected (at two-time intervals, early morning and afternoon) and the physical parameters of pitchers was noted (Table 4.1). For nectar extraction, peristome and lid from the pitcher was separated, washed with water (5 mL, 5× 15 min) individually and evaporated to dryness using a rotary evaporator at 37°C under reduced pressure.

### 3.2.4 Chemical characterization of Nectar

The nectar extracted from both peristome and lids was chemically tested for detecting the presence/quantification of sugars and amino acids using TLC and HPTLC. Secondary metabolites in the nectar were checked by LC-MS, and the mineral contents were tested by ICP-MS.

#### 3.2.4.1 Quantification of Sugars in the Nectar

Quantitative estimation of glucose-fructose-sucrose content in the water extracts of both peristome and lid were carried out by HPTLC-densitometry (CAMAG, Switzerland) comprising an automatic Linomat V sample applicator, twin trough plate development chamber, TLC Scanner 3, Reprostar 3 photo documentation system and WinCATS software 4.03, using pre-treated silica gel 60 $F_{254,}$ 20 x 10 cm, 0.2 mm thickness (E. Merck, Germany). The silica gel TLC plates were modified by dipping in 0.2 M potassium dihydrogen orthophosphate solution (aqueous), wet plates were dried at 90°C for 10 minutes. The dried plates were then allowed to cool to room temperature in a desiccator. Stock solution (10 mg/mL) of the standard glucose, fructose and sucrose (Sigma Aldrich, USA) was prepared in methanol-water (8.5:1.5, v/v; 1mL) and applied

onto the pre-treated silica gel TLC plate with the Linomat V sample applicator, fitted with a micro syringe under nitrogen flow (band width 8 mm, application rate 150 nL/s, space between two bands 12.1 mm). Plates were developed three times, each up to a distance of 80 mm in a filter paper-lined twin trough plate development chamber, equilibrated with the mobile phase, acetonitrile: water (8.5: 1.5, v/v; 20 mL). For each run mobile phase was changed and fresh solvent was used. The plate was thoroughly dried with a hair drier in between runs. After the third run the plate was dried and derivatized using anisaldehyde-sulphuric acid reagent, heated at 110°C for 5 min. The plate was then scanned densitometrically at 570 nm using TLC Scanner 3 equipped with WinCats software, slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s. Concentrations of the standard sugars (sucrose 0.25, 0.5, 1.0, 2.0 μg), (glucose 0.25, 0.5, 1.0, 2.0, 3.0 μg), (fructose 0.25, 0.5, 1.0, 2.0 μg) were plotted against peak area(s) to obtain the calibration plots (glucose: $y = 1161x + 169.53$, $r^2 = 0.989$) (fructose: $y = 1660.6x + 270.82$, $r^2 = 0.987$) (sucrose: $y = 1713x + 148.11$, $r^2 = 0.995$) ( Figure 4.2, Figure 4.3 and Figure 4.4 respectively).

Water extracts of *N. khasiana* pitcher peristome and lid were dissolved in methanol-water mixture (3.2:0.8, v/v; 4 mL), 3 and 1 μL of these solutions were applied to a modified Silica gel TLC plate (E. Merck, Germany) (60 $F_{254,}$ 20 x 10 cm, 0.2 mm thickness) as 8 mm wide bands with the Linomat V sample applicator, fitted with a micro syringe under nitrogen flow (application rate is 150 nL/s, space between two bands is 12.1 mm). The plates were developed for three times and each time a distance of 80 mm in a twin trough glass chamber saturated with the mobile phase acetonitrile: water (8.5: 1.5, v/v; 20 mL). For each run fresh solvent was used and after each run, the plate was thoroughly dried with a hair drier. The plate was dried after the third development and derivatized using anisaldehyde-sulphuric acid reagent, heated at 110°C

for 5 min. Plates were scanned densitometrically at 570 nm using TLC Scanner 3 equipped with WinCats software, slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s.

### 3.2.4.2 Amino Acid analysis

The presence of amino acids in the water extracts of peristome and lid of *N. khasiana* pitchers was tested by HPTLC-densitometry (CAMAG, Switzerland) made of an automatic Linomat V sample applicator, twin trough plate development chamber, TLC Scanner 3, Reprostar 3 photo documentation system and WinCATS software 4.03. Stock solution (1 mg/mL) of twenty standard amino acids (Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) (Fluka Analytical, USA) were individually prepared in methanol- water mixture (0.9:0.1, v/v; 1 mL) and applied (2 µL) onto a silica gel TLC plate (60 $F_{254,}$ 20 x 10 cm, 0.2 mm thickness) as 8 mm wide bands with the Linomat V sample applicator, fitted with a micro syringe under nitrogen flow (application rate 150 nL/s, space between two bands 12.1 mm. Plate was developed up to 80 mm in the twin trough plate development chamber saturated with Butanol: Acetic acid: Water (12:3:5, v/v; 20 mL). The developed plate was dried and derivatized using ninhydrin reagent, heated at 110°C for 5 min. Plates were scanned densitometrically at 570 nm using TLC Scanner 3 equipped with WinCats software, slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s.

*N. khasiana* peristome and lid water extracts/nectar were dissolved in methanol- water mixture (3.2:0.8, v/v; 4 mL), 5 and 10 µL of these solutions were applied to a Silica gel TLC plate (E. Merck, Germany) (60 $F_{254,}$ 20 x 10 cm, 0.2 mm thickness) as 8 mm wide bands with the Linomat V sample applicator, micro syringe was fitted with under nitrogen flow; the application rate was 150 nL/s and the space between two bands 12.1 mm. The plate was developed to a distance of 80 mm in a twin trough glass

chamber saturated with the mobile phase Butanol: Acetic acid: Water (12:3:5, v/v; 20 mL). After development the plate was dried and derivatized using ninhydrin reagent, heated at 110°C for 5 min. Plate was scanned densitometrically at 570 nm using TLC Scanner 3 equipped with WinCats software, slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s.

**3.2.4.3 Liquid Chromatography- Mass Spectrometry (LC-MS)**

The LC-MS/MS method was performed at CLIF, University of Kerala, Thiruvananthapuram with a SHIMADZU LC MS-8045 mass spectrometer with electrospray ionization in positive ion mode equipped with a Shimadzu ultra-high-performance liquid chromatography system. The standard elution was carried out on a Shim-pack GISS C18 5µm, 4.6 x 250 mm column (Shimadzu) maintained at 30°C. The mobile phase was a mixture of A 10:90 acetonitrile: 0.1% ammonium formate in water and B 90:10 acetonitrile: 0.1% ammonium formate in water at a flow rate of 0.2 ml/min in a 35 min linear gradient as follows: 0 – 30 min, 100-70% B; 30 - 30.1 min, 70 - 60% B; 30.1 - 33 min, 60 - 80% B; 33 - 35 min, 80 -100 % B. The injection volume was 10 µL, the column was set at 35°C, and the sample manager temperature was set at 40°C. The mass spectrometer detector conditions were set as follows: Capillary voltage - 2.80 kV, Interphase temperature - 300°C, DL temperature - 250°C, drying gas flow - 10 L/minute, and cone gas flow - 10 L/minute.

**3.2.4.4 Mineral content in Nectar**

The mineral contents were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (iCAP RQ ICP- MS, Thermo Scientific, USA) at CSIR-NIIST, Thiruvananthapuram. The samples were acid digested before analysis. For the sample digestion, 0.1 g of the sample (water extract from peristome and lid separately) were added with 3 mL $HNO_3$ and 2 mL $H_2O_2$ (both ICP MS grade) and transferred to the

microwave digestion tube, done the digestion at 180°C in the microwave digestion system. The samples were made up to 50 mL after the digestion, using ultrapure water for the analysis.

### 3.2.5 Toxicity Studies of Nectar

#### 3.2.5.1 Chemicals and reagents

Acetyl choline Iodide and 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), quinidine sulphate was procured from Sigma-Aldrich, MO, USA.

The test and control samples (ants, 20 each) were collected from the *N. khasiana* field. The control ants were captured from the pitcher surface, outer surface of lid, outer region of peristome, tendril, leaves of the plant etc. The test ants were collected from the pitcher fluid of mature opened pitchers. For this, the pitcher fluid from the pitchers were separated and transferred to a petri dish. Using micropipettes, the fluid volume was reduced, and the same species of ants taken as control were collected with the help of a handheld lens and a light source. Control ants along with the test ants (lack of pitcher fluid) were carefully transferred to a suitable container and stored under refrigeration for the experiment.

#### 3.2.5.2 Estimation of mode of action of acetyl choline esterase kinetics and inhibition activity on nectar fed ants.

Cholinergic neurotransmitters catalytic enzyme Acetylcholinesterase (AChE) kinetics and inhibition activity were analyzed with colorimetric methods using specific substrates (acetyl choline iodide) for the enzyme. Enzyme activity measurements were conducted at 30°C based on changes in absorbance using the microplate reader. Michaelis constant (Km), Catalytic constant (Kcat) and maximum reaction velocity (Vmax) were calculated using non-linear regression. The Hill equation was used

throughout for variable cooperativity and non-cooperative analysis. Accordingly, % inhibition was calculated (van Asperen., 1962; Pour et al., 2014; Guengerich et al., 2009; Ellman et al., 1961; Timea and Scott., 2014 and Pietsch et al., 2009) and the estimation of protein was done by Lowry method (Lowry et al., 1951).

Briefly, twenty numbers of adult ants were used for each control and test experiments independently. For the enzyme activity, crude homogenate of the ants was prepared in sodium phosphate buffer (50 mM, pH 7). Homogenization was performed in an eppendorf tube using a manual homogenizer at approximately 4°C. Extracts were centrifuged at 12,000× g for 15 min at 4°C to separate the supernatant. The supernatant sample from both control and test was collected and frozen at −20°C until further analysis (Onyeogaziri and Papaneophytou, 2019).

The experimental procedures for AChE inhibitory activity assay was carried out by the method of Ellman et al., 1961 with slight modifications. Purified AChE was prepared to a concentration of (400 Units/L /10µL) of enzyme from the whole ant (control and test independently) homogenate as described elsewhere. A reaction mix for each well of reaction was prepared by mixing 100 µL of substrate (Acetylcholine iodide; 40mM), 60 µL of DTNB, 10 µL of enzyme ( from the insect homogenate) and made up to 500 µL of phosphate buffer (pH 8). The plate was incubated for 15 min. Control (no enzyme), and the no inhibitor control wells were also read to determine the specificity of the experiment. The plate was tapped to mix. 0.1% quinidine sulphate was added to inhibit the reaction and the absorbance was measured at 412 nm, at 0 min and at 10 min. The acetylcholinesterase activity was calculated as the % of inhibition. All samples were assayed in triplicate. For the generation of standard curve, reaction mixture consists of 25 µL of Acetylcholine iodide solution (1 mM) as substrate, 50 µL (5 units/mL) of bovine erythrocyte cholinesterase as enzyme source and

0.4 mL (0.4 mg/mL) of DTNB (1 mM) for determination, and final volume made up to 500 µL of phosphate buffer (pH 8).

# CHAPTER - 4

# RESULTS AND DISCUSSIONS

Nectar produced by the plants is the main reward offered to insects that visit the plants. Floral nectar is a sugary aqueous liquid produced by specialized glandular tissue nectaries. Sucrose is found to be the major sugar present in the floral nectar. The amino acid composition as well as the quantity of amino acids in the floral nectar helps in determining the taste of the nectar in addition to whatsoever nutritional meaning it may have for the visiting insects. The chemical composition of floral nectar varies depending up on the kinds of visitors attracted to the flowers. In addition to floral nectars, there are extrafloral nectars present in plants, where nectars are produced like that in flowers. The extrafloral nectars may be situated on the inflorescence or flower stalks or, more commonly, on the laminae of leaves, on petioles, or on stipules. This extrafloral nectars near flowers feeds ants that prevent large bees from sucking floral nectar. The extrafloral nectar is very much different in characteristics of the normal floral nectars (Baker et al., 1978). In the current study we have focused on the chemical analysis of the extrafloral nectar produced by the Indian pitcher plant, *Nepenthes khasiana*.

*Nepenthes khasiana* pitchers were collected from JNTBGRI garden and the specimens were authenticated. A herbarium specimen was deposited in JNTBGRI herbarium with voucher specimen number TBGT39021. The nectar secreted by the pitchers, the insect trapping organ of *N. khasiana* is the major attractant to the visiting insects in *Nepenthes*. Nectars are produced in the extrafloral nectaries present in the peristome and lid of the pitcher. In the study, we have extracted the extrafloral nectar from the

peristome and lid separately and characterized it to quantify the sugars, checked for the presence of amino acids, heavy metals and secondary metabolites.

*N. khasiana* pitchers of uniform age group (5-8 days after opening) were collected (at two-time intervals, early morning and afternoon) and the basic morphological parameters of pitchers were noted (Table 4.1).

**Table 4.1. Morphological parameters of *N. khasiana* Pitchers**

| Sample collection date and time | Pitcher length (cm) | Peristome diameter (cm) | Diameter of the liquid zone (cm) | Lid diameter (cm) | Pitcher fluid (mL) |
|---|---|---|---|---|---|
| 04/01/2022 5:30am | 19.9 Pitcher1 | 4.6 | 3.5 | 5.7 | 8.0 |
| | 20.2 Pitcher2 | 4.8 | 3.5 | 5.4 | 5.6 |
| | 17.2 Pitcher3 | 3.4 | 2.5 | 4.9 | 6.6 |
| | 16.7 Pitcher4 | 3.3 | 2.5 | 5.0 | 5.3 |
| 03/02/2022 | 14.3 Pitcher1 | 3.7 | 2.3 | 4.3 | 1.7 |
| | 15.7 Pitcher2 | 4.1 | 3.0 | 4.5 | 3.8 |

| | | | | | |
|---|---|---|---|---|---|
| 5:30am | 16.8<br>Pitcher3 | 4.0 | 3.3 | 5.3 | 4.0 |
| | 15.1<br>Pitcher4 | 3.9 | 2.6 | 4.4 | 2.4 |
| 03/02/2022<br>2:30pm | 16.3<br>Pitcher1 | 3.8 | 2.9 | 4.5 | 2.4 |
| | 13.6<br>Pitcher2 | 3.5 | 2.5 | 4.1 | 2.2 |
| | 18.0<br>Pitcher3 | 4.2 | 2.8 | 5.0 | 2.3 |
| | 18.0<br>Pitcher4 | 4.2 | 3.0 | 5.1 | 5.8 |
| 17/02/2022<br>5:30am | 18.5<br>Pitcher1 | 4.5 | 3.0 | 5.0 | 7.5 |
| | 15.6<br>Pitcher2 | 4.2 | 2.6 | 4.5 | 5.2 |
| | 17.3<br>Pitcher3 | 3.7 | 2.4 | 4.6 | 5.6 |
| | 15.6<br>Pitcher4 | 3.1 | 2.3 | 4.2 | 4.6 |
| | 16.9<br>Pitcher5 | 3.7 | 2.9 | 4.1 | 6.4 |
| | 19.3<br>Pitcher1 | 4.4 | 3.0 | 5.0 | 7.0 |

| | | | | | |
|---|---|---|---|---|---|
| 17/02/2022 2:30pm | 19.5 Pitcher2 | 4.9 | 3.2 | 5.6 | 6.5 |
| | 19.0 Pitcher3 | 4.3 | 3.1 | 5.5 | 8.3 |
| | 19.4 Pitcher4 | 4.4 | 3.1 | 5.3 | 6.8 |
| | 18.0 Pitcher5 | 4.0 | 2.7 | 4.8 | 6.6 |
| 10/03/2022 5:30am | 16.8 Pitcher1 | 3.7 | 3.0 | 4.5 | 3.9 |
| | 14.9 Pitcher2 | 3.3 | 2.6 | 4.1 | 6.4 |
| | 18.3 Pitcher3 | 3.6 | 3.0 | 4.5 | 6.6 |
| 10/03/2022 2:30pm | 18.7 Pitcher1 | 4.1 | 3.4 | 4.7 | 6.4 |
| | 14.7 Pitcher2 | 3.0 | 2.6 | 3.9 | 2.8 |
| | 15.1 Pitcher3 | 3.4 | 2.2 | 4.2 | 3.6 |

Nectar was extracted from both the peristome and lid of the pitcher separately by washing with water (5 mL, 5× 15 min). The water wash (nectar) were evaporated to dryness using a rotary evaporator at 37°C under reduced pressure. The nectar yield of both peristome and lid were recorded (Table 4.2), from Table 4.2. it is clear that lid of

the pitcher secretes more nectar than the peristome. Moreover, the results suggest that there is not much difference in the nectar yield between the morning and afternoon collections.

**Table 4.2. Yield of Nectar in Peristome and Lid**

| Sample | Morning (n = 16) | Afternoon (n = 12) |
|---|---|---|
| **Peristome** | **0.0085 ± 0.0065 g** | **0.0095± 0.0036 g** |
| **Lid** | **0.0099± 0.0074 g** | **0.0109 ± 0.0065 g** |

### 4.1 Sugar analysis in the Extrafloral Nectar

In general, floral nectar is dominated by mainly three simple sugars: the disaccharide sucrose and its component monosaccharides- fructose and glucose. Quantitative estimation of the major sugars present in the nectar was carried out by HPTLC-densitometry. The sugar analysis of the extrafloral nectar of pitcher peristome and lid also showed sucrose-glucose-fructose pattern in both time intervals.

In the HPTLC plate showing sugar analysis (Figure 4.1.), the first thirteen tracks were of various concentrations of lid and peristome and the last three were the standard sugars of various concentrations.

**Figure 4.1: HPTLC plate showing Sugar analysis**

Concentrations of standard sucrose-glucose-fructose were plotted against corresponding peak area(s) to obtain calibration plots and using these standard curves the sugar content in nectar was quantified.

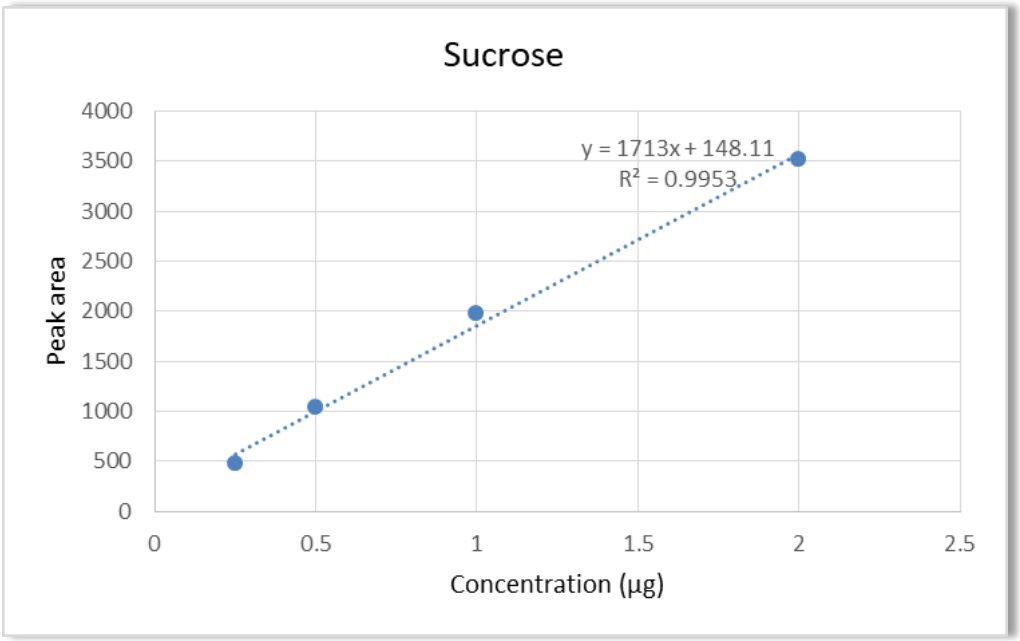



**Figure 4.2: Peak area vs Concentration chart of Sucrose**

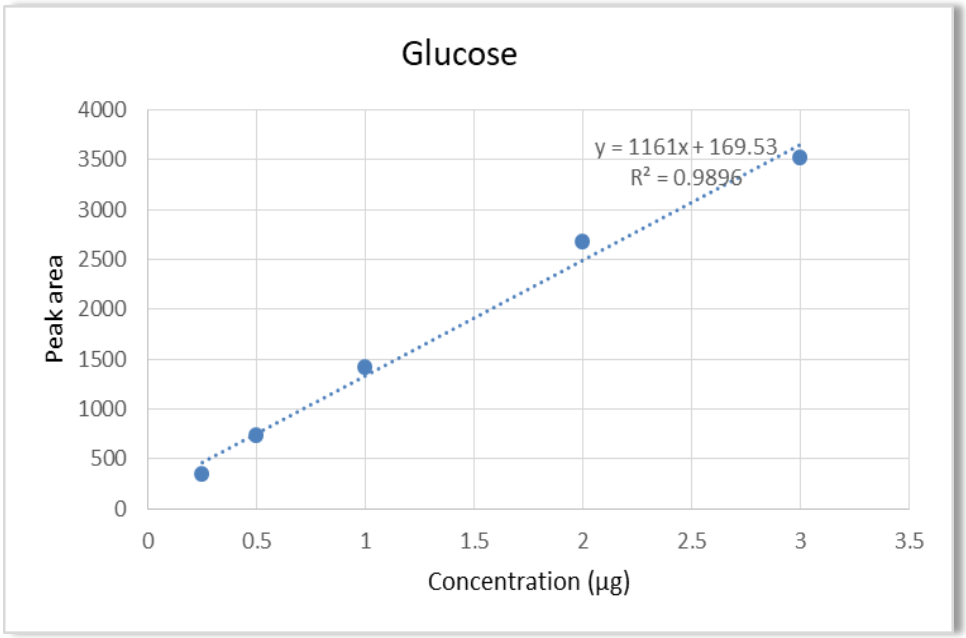



**Figure 4.3: Peak area vs Concentration chart of Glucose**

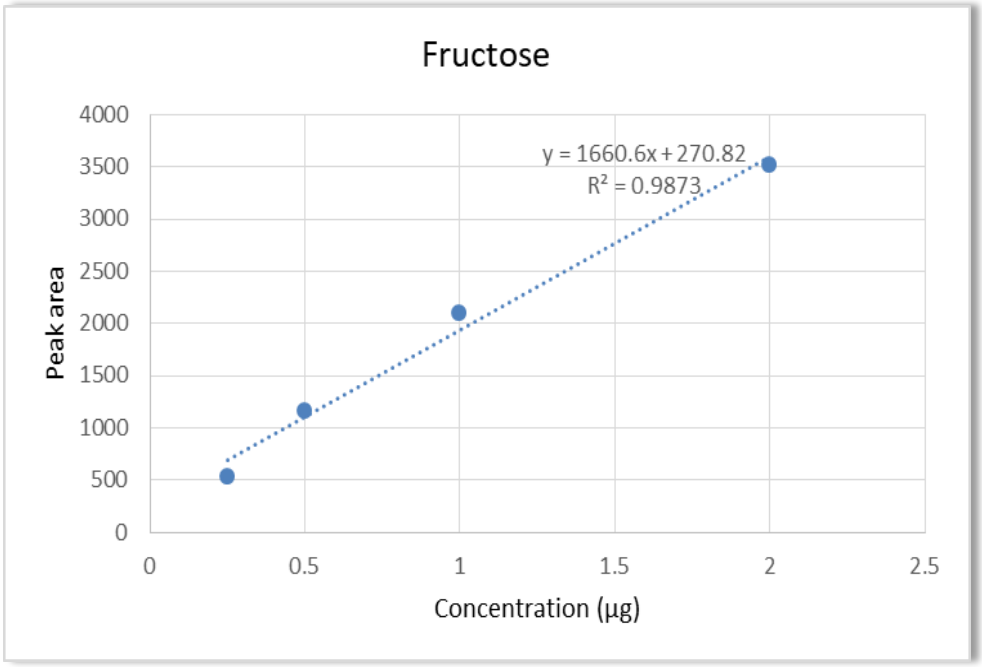



**Figure 4.4: Peak area vs Concentration chart of Fructose**

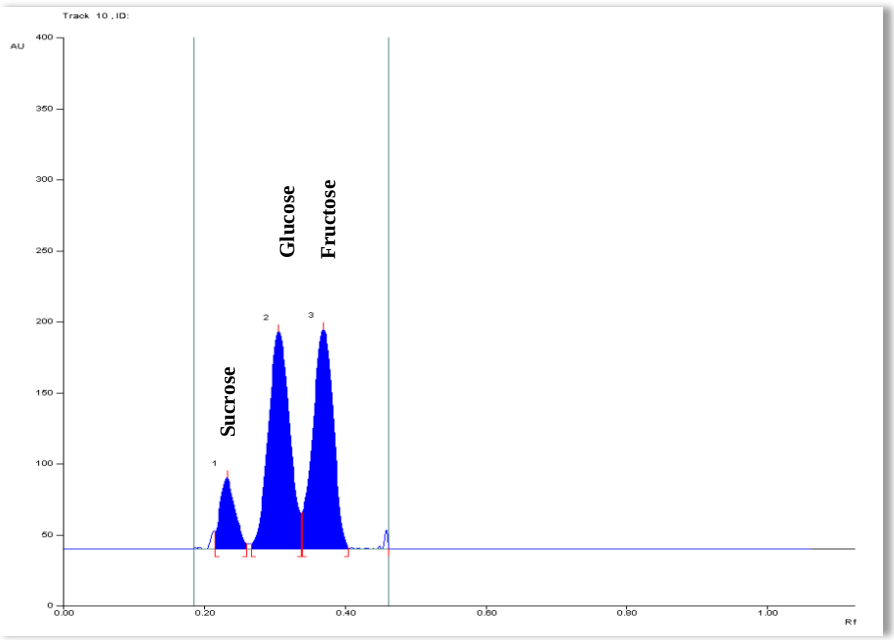
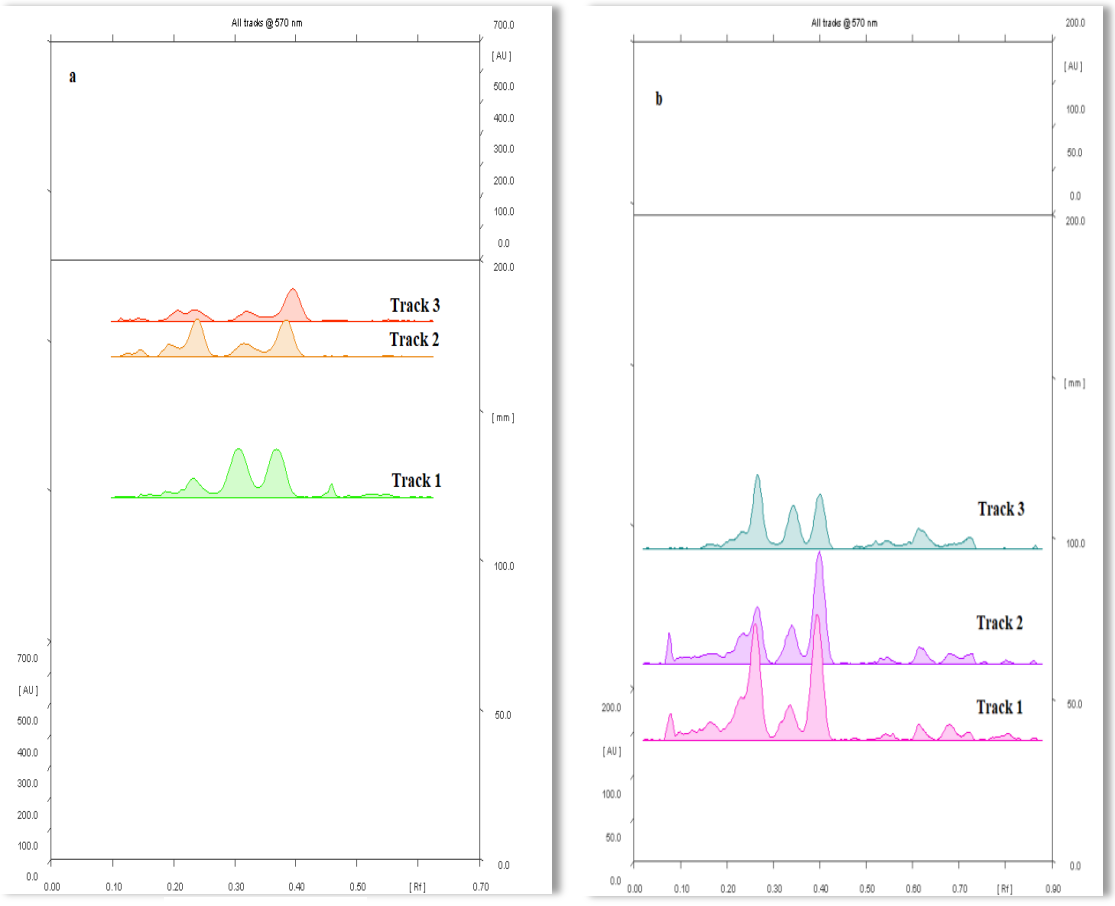



**Figure 4.5: Chromatogram of Standard Sugars (Sucrose, Glucose & Fructose)**




**Peristome** **Lid**

**Figure 4.6: Densitogram of Sugar analysis in Nectar**

**a:** Densitogram of nectar of peristome, Track 1 – standard sugars (sucrose, glucose and fructose), Track 2 – nectar collected during morning and Track 3 – nectar collected during afternoon.

**b:** Densitogram of nectar of lid, Track 1 – nectar collected during morning, Track 2 – nectar collected during afternoon and Track 3 – standard sugars (sucrose, glucose and fructose).

In the peristome nectar collected during morning, sucrose-glucose-fructose contents were, 7.12, 1.31 and 11.87 % respectively and in the afternoon collections sucrose-glucose-fructose contents were, 3.49, 3.83 and 25.12 % respectively, on the opened pitchers (5-8 days after opening). In case, pitcher lid of *Nepenthes khasiana* sucrose-glucose-fructose contents collected during morning were, 6.99, 12.77 and 45.91 % respectively and during afternoon sucrose-glucose-fructose contents were, 5.16, 14.06 and 45.99% respectively, on the opened pitchers (5-8 days after opening).

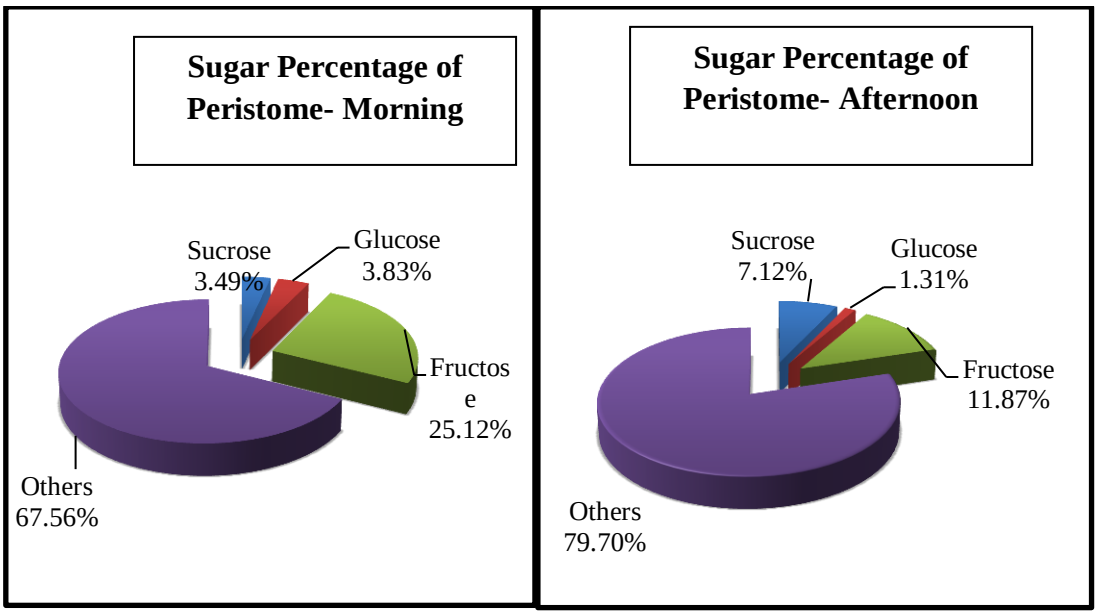



**Figure 4.7: Sugar percentage of Peristome during morning and afternoon**

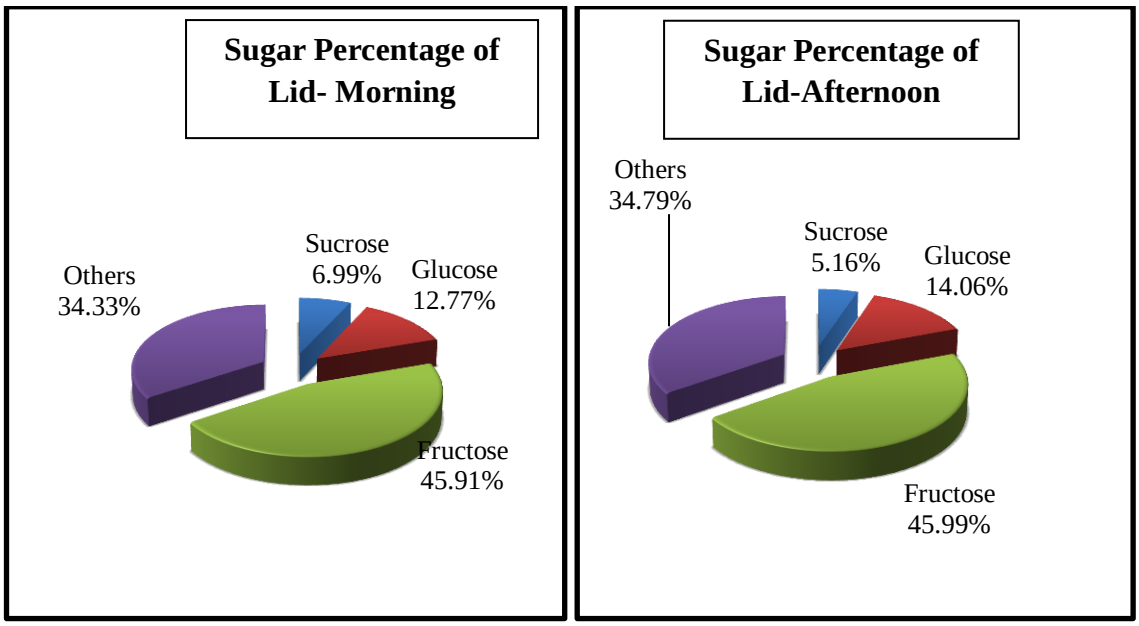



**Figure 4.8: Sugar percentage of Lid during morning and afternoon**

The sucrose content in the peristome nectar collected during early morning ranged from 1.08 to 20.25 percentages with an average value of 7.12 ± 8.35. Similarly, the glucose and fructose contents ranged from 3.29 to 5.94 percentages (average: 1.31 ± 2.21) and 3.65 to 26.16 percentages (average: 11.87 ± 10.97) respectively.

The sucrose content in the peristome nectar collected during afternoon ranged from 1.92 to 11.57 percentages with an average value of 3.49 ± 4.91. Similarly, the glucose and fructose contents ranged from 0.83 to 8.97 percentages (average: 3.83 ± 3.99) and 3.42 to 35.87 percentages (average: 25.12 ± 12.89) respectively.

The sucrose content in the lid nectar collected during morning ranged from 2.15 to 20.25 percentages with an average value of 6.99 ± 0.08. Similarly, the glucose and fructose contents ranged from 5.84 to 36.28 percentages (average: 12.77 ± 0.12) and 30.11 to 82.21 percentages (average: 45.91 ± 0.17) respectively.

The sucrose content in the lid nectar collected during afternoon ranged from 2.52 to 24.44 percentages with an average value of 5.16 ± 8.27. Similarly, the glucose

and fructose contents ranged from 1.76 to 25.89 percentages (average: 14.06 ± 9.97) and 19.28 to 72.62 percentages (average: 45.99 ± 18.26) respectively.

The nectar analysis of pitcher peristome collected in the morning showed a high content of fructose and very low glucose content. The sucrose content was nearly half of that of the fructose. Peristome nectar collected in the afternoon also showed a high content of fructose. But the glucose content was higher than sucrose by 0.46 % in afternoon. A similar study was conducted for lid as well. Nectar from lid collected during morning and afternoon contained about 46 % of fructose, which is found to be the main sugar in the extrafloral nectar. In both cases, sucrose was found to be very low. The glucose content was comparatively higher than sucrose and lower as compared to fructose.

### 4.2 Amino Acid analysis in the Extrafloral Nectar

Free amino acids in the *N. khasiana* peristome and lid nectar were analyzed by HPTLC-densitometry. Twenty standard amino acids at 2 µg concentration (Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) (Fluka Analytical, USA) were applied individually along with the water extracts at 5 and 10 µL concentrations. From the analysis, it was clear that even at high concentration of the samples no amino acids were present/detected in the extrafloral nectar of *N. khasiana* unlike the normal floral nectars which are rich in amino acids.

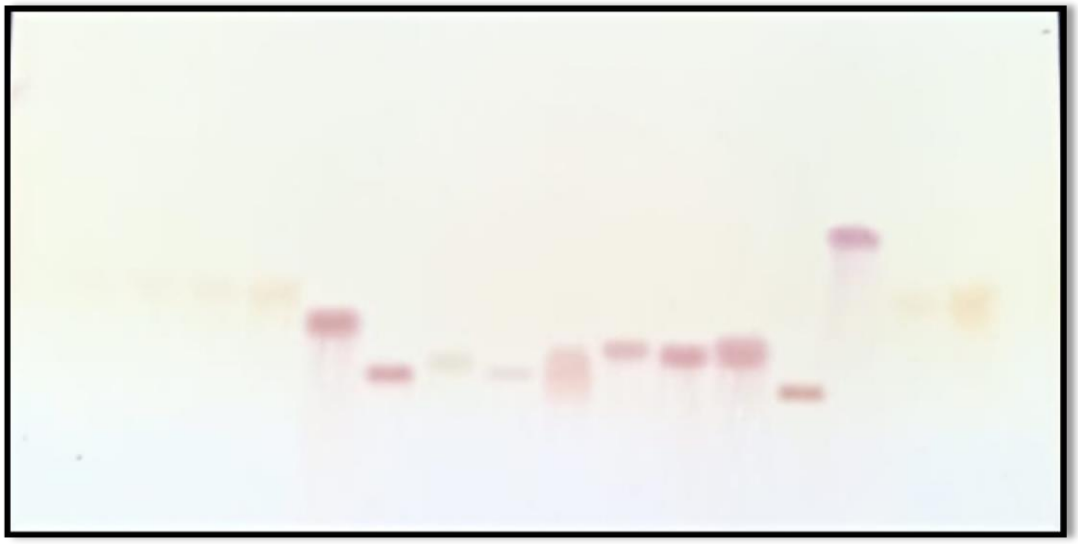


**Figure 4.9: HPTLC plate showing Amino Acid analysis**

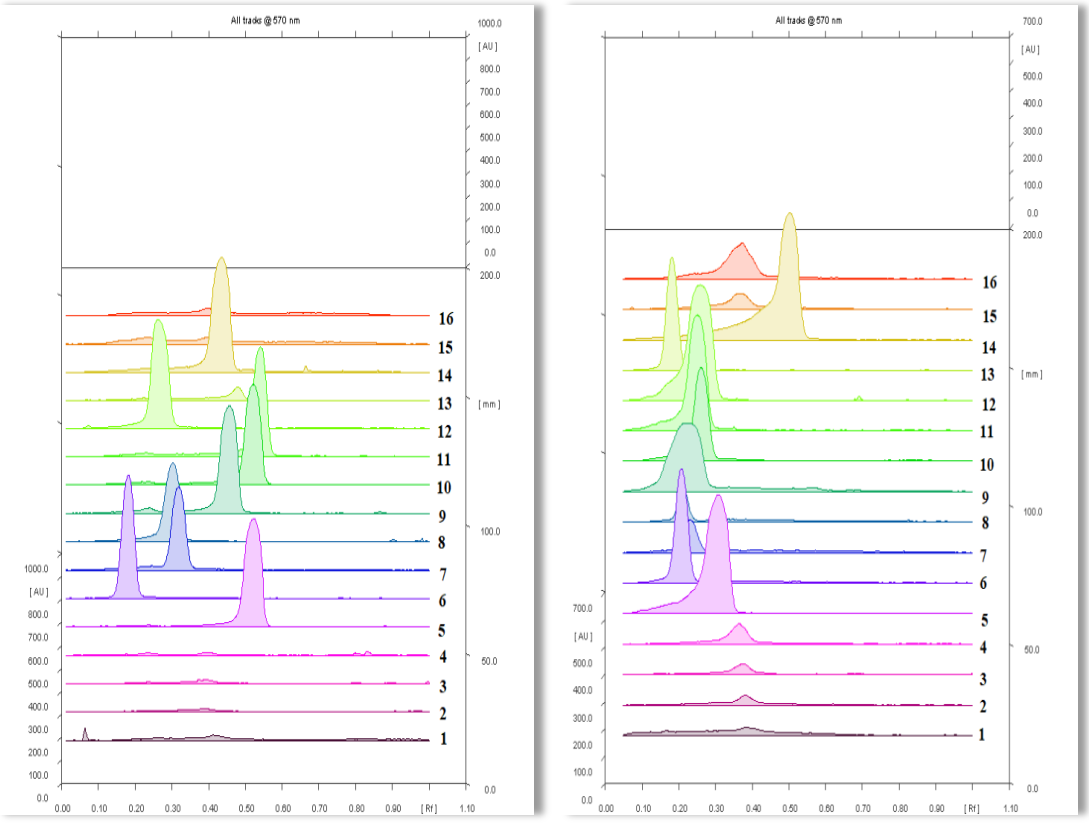



**Figure 4.10: Densitogram of Amino Acid analysis in Nectar**

**Note:** In both densitogram first four tracks and last two tracks represent the nectar of peristome and lid. The ten tracks between them represent the standard amino acids.

### 4.3 Metal content analysis in the Extrafloral Nectar

Metal content of *N. khasiana* peristome and lid nectar were analyzed by ICP-MS and the results of the analysis are presented in Table 4.3. The results show that the nectar is rich in magnesium, calcium and iron. Toxic metals like arsenic, cadmium, lead and mercury were also detected in the nectar. These heavy metals are commonly absent in the normal floral nectars (Spiric et al., 2019 and Voica et al., 2020).

**Table 4.3. Metal content analysis by ICP-MS**

| Element (ppb) | Peristome Nectar (ppb) | Lid Nectar (ppb) |
|---|---|---|
| **Li** | 14.1 | 11.9 |
| **Be** | 0.01 | BDL |
| **Mg** | 1254.8 | 1149.4 |
| **Ca** | 384.3 | 378.9 |
| **V** | 0.41 | 0.31 |
| **Cr** | 3.7 | 5.8 |
| **Mn** | 14.8 | 10.5 |
| **Fe** | 235.3 | 278.5 |
| **Co** | 0.44 | 0.76 |
| **Ni** | 14.1 | 13.7 |
| **Cu** | 29.4 | 23.1 |
| **Zn** | 61.1 | 30.6 |
| **As** | 0.11 | 0.11 |
| **Se** | 0.25 | 0.22 |

| | | |
|---|---|---|
| **Cd** | 0.17 | 0.11 |
| **Pb** | 7.90 | 2.70 |
| **Hg** | 0.23 | 0.20 |

### 4.4 LC-MS/MS analysis of the Extrafloral Nectar

The presence of secondary metabolites in nectar extracted was analyzed by LC-MS/MS and the chromatograms are presented in Figure 4.11 and Figure 4.12. From the TIC of the nectars, it is seen that both the nectars have a common peak at retention time 15.5 min. In addition to this peak a clear peak at retention time 7.5 min was seen in the lid nectar. Further studies are needed to find out and to characterize these compounds present in the nectar.

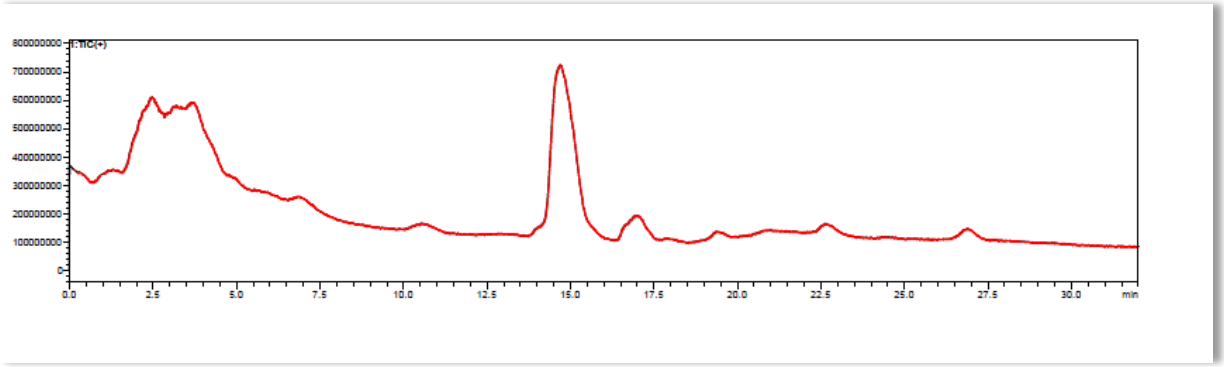


**Figure 4.11: Total Ion Chromatogram (TIC) of Peristome Nectar**

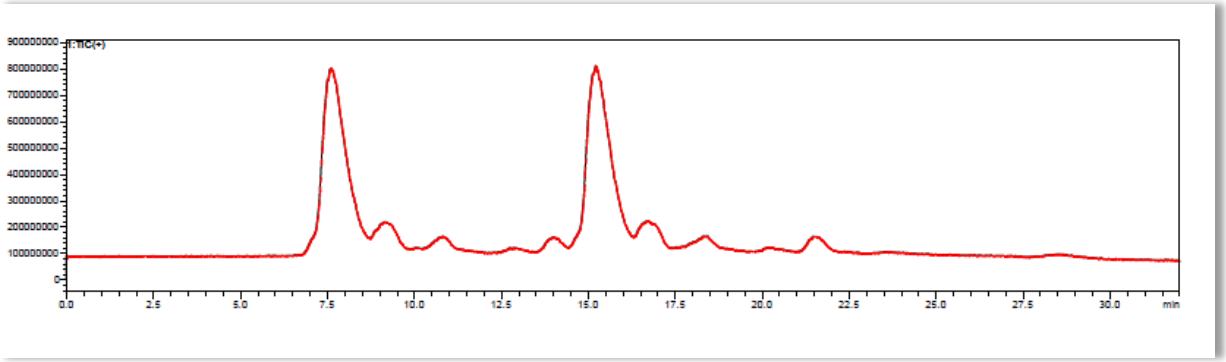


**Figure 4.12: Total Ion Chromatogram (TIC) of Lid Nectar**

### 4.5 Measurement of AChE Activity and *In Vitro* Inhibition of Acetylcholinesterase

**Table 4.4. AChE Activity**

| **Experimental Group** | Vmax (mmoles/min/mg protein) | Km (mM) |
|---|---|---|
| **Control** | 3.3 ± 1.2 | 9.8 ±2 |
| **Test** | 15 ± 0.9* | 10.2 ± 1.6 |

Values are mean ± S.E.M of 4-6 separate experiments, * P<0.01 compared with control group

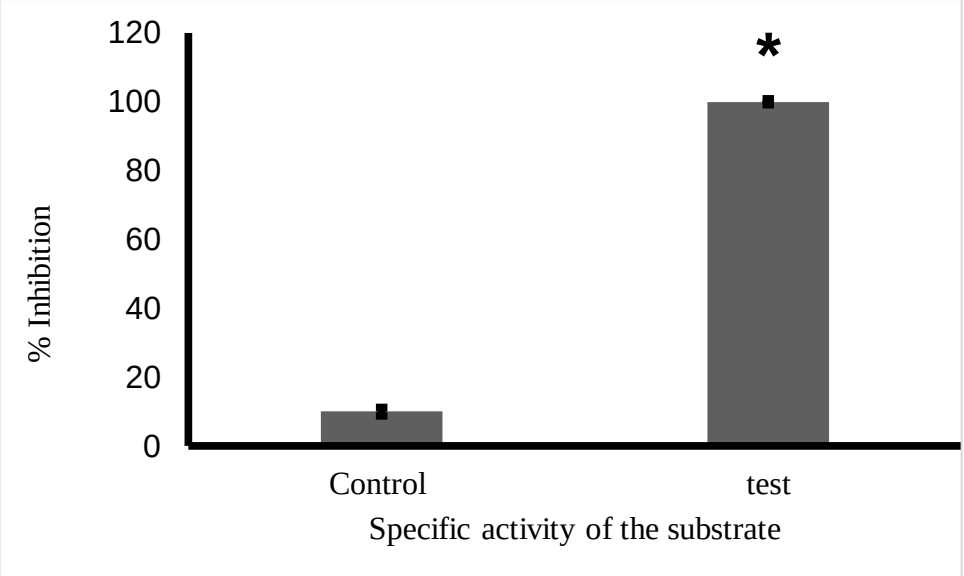



**Figure 4.13: Percentage of Inhibition AChE**

Acetylcholine esterase kinetics and inhibitory studies showed that Vmax significantly increased in ($p < 0.01$) in the nectar fed ants with no significant difference in the Km value when compared with the control group. The components in the nectar significantly affected the Vmax ($p < 0.01$) as well as the inhibition of acetylcholine esterase activity (100% inhibition in the test group; $p < 0.01$) and 10% inhibition in control group. However, the Km showed no change in both the groups.

AChE is the enzyme in charge of executing acetylcholine (ACh) breakdown and is a vital component of cholinergic signaling. AChE levels are controlled through transcriptional and post-transcriptional mechanisms and are radically increased under toxicological, physical and psychological stress. Interestingly in our study we observed a drastic increase in the kinetics of the AChE however the product formation was inhibited to 100%.

Studies suggest that rapid and vigorous increase in AChE levels is a retort to neuronal activation and has been suggested to act as a homeostatic control to reduce neuronal excitability which is also associated with augmented neural network excitability (Gnatek et al., 2012). Reports suggest that the inhibition of AChE by soman (pinacoly methyl phosphonofluoridate), a highly potent irreversible inhibitor of acetylcholinesterase (AChE) leads to increased acetylcholine (ACh) and neuronal excitability resulting in seizures and gliosis (Sterling et al., 1988 and Wang et al., 2021). In connection with above studies, our finding, would postulate that the increase in the Vmax of AChE and subsequent inhibition of AChE activity resulting in accumulation of ACh suggests a brain milieu imbalance paving way to neuronal excitability or seizures in the test ants which consecutively result in their mortality.

Further studies are crucial to demarcate the impact of cholinergic signaling in neurological network of ants fed on nectar in addition to nectar component toxicity on insects.

# SUMMARY AND CONCLUSIONS

The nectar secreted by the pitchers of *Nepenthes khasiana* is the major reward to the visiting insects. Peristome and lid of the pitcher contains extrafloral nectaries, which secretes the nectar. Here, the chemical characteristics and toxicity studies of the extralfloral nectar in *N. khasiana* has been carried out. *N. khasiana* pitchers were collected from JNTBGRI garden and the specimens were authenticated. The extrafloral nectar from the peristome and lid were separately extracted with water and characterized. For the extraction pitchers of uniform age group (5-8 days after opening) were collected at two-time intervals. From the yield of the nectar, it is clear that the lid of the pitcher secretes more nectar in *N. khasiana.* The sugar content of the extrafloral nectar of pitcher peristome and lid were analysed and it showed sucrose-glucose-fructose pattern in both time intervals. The sugar contents were quantitatively estimated using HPTLC-densitometry. Fructose is found to be the major sugar in both peristome and lid water extracts (nectar), unlike the taste giving sucrose normally found in floral nectars. The glucose and sucrose contents were found to be very low in comparison with floral nectars. The presence of free amino acids in the peristome and lid nectar were analysed by HPTLC-densitometry. The floral nectars are rich in amino acids but even at high concentration of the samples no amino acids were present/detected in the extrafloral nectar of *N. khasiana.* Metal content analysis by ICP-MS showed that both the extra floral nectars are rich in magnesium, calcium and iron. The pitcher nectar shows the presence of toxic metals like arsenic, cadmium, lead and mercury normally they are absent in floral nectars. The presence of secondary metabolites in the extra floral nectar extracted were analysed by LC-MS/MS. The presence of a common peak in the TIC of the nectars indicates the presence of a common component in both the nectars. Further studies are required for identifying the

components present in the nectar. The toxicity of the nectar from the pitcher were tested using the nectar fed ants collected from the inside of pitcher. The measurement of AChE Activity and *in vitro* inhibition of acetylcholinesterase suggests a brain milieu imbalance paving way to neuronal excitability or seizures in the test ants. Further studies are important to establish the impact of cholinergic signalling in the neurological network of ants fed on nectar in addition to nectar component toxicity on insects.

The main attractant for the prey is the extrafloral nectar produced by the *N. khasiana* pitcher peristome and lid. Based on the findings it is clear that, the chemical composition of the extrafloral nectar is very much different in comparison with floral nectar. This difference in chemical composition of extra floral nectar is one of the reasons behind the trapping and killing of the visiting insects.

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