
**EFFECT OF BIOTIC ELICITOR ON SECONDARY METABOLITES
PRODUCTION OF *KALANCHOE PINNATA* (RANAKALLI) AND
CHAMAECOSTUS CUSPIDATUS (INSULIN)**

PROJECT WORK

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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**SCHOOL OF BIO AND CHEMICAL ENGINEERING
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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

CATEGORY -1 UNIVERSITY BY UGC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **NAVEEN.B (41300024)**, **UDHAYAN.T (41300052)** who carried out the project "**EFFECT OF BIOTIC ELICITOR ON SECONDARY METABOLITES PRODUCTION OF KALANCHOE PINNATA (RANAKALLI) AND CHAMAECOSTUS CUSPIDATUS (INSULIN)**" under my supervision from January 2024 to April 2024.

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We, **B.NAVEEN (41300024)** and **T.UDHAYAN(41300052)** hereby declare that the Project Report entitled "**EFFECT OF BIOTIC ELICITOR ON SECONDARY METABOLITES PRODUCTION OF *KALANCHOE PINNATA* (RANAKALLI) AND *CHAMAECOSTUS CUSPIDATUS* (INSULIN)**" is done by us under the guidance of **Dr.J.THEBORAL** at **Sathyabama Institute of Science and Technology, Chennai,Tamil Nadu, India**, is submitted in partial fulfillment of requirements for the award of **Bachelor of Science in Microbiology**.

DATE: 24.04.2024

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATES

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Abstract

Secondary metabolites are essential compounds produced by plants that confer various advantages in their natural environments, including defense against stresses and regulation of physiological processes. *Kalanchoe pinnata* and *Chamaecostus cuspidatus* are the medicinal plants with significant pharmacological potential due to their rich secondary metabolite content. *K. pinnata*, widely used in traditional medicine, exhibits diverse pharmacological activities such as wound healing, antioxidant, antimicrobial, antiviral, chemo protective, and antilithiatic effects. Similarly, *C. cuspidatus* known as the insulin plant, possesses various bioactive compounds such as flavonoids, chlorophylls, and alkaloids, which contribute to its anti-diabetic, anti-inflammatory, antioxidant, and neuroprotective activities. Research on these plants highlights their potential as sources of novel pharmaceutical agents for treating various diseases. The primary aim of this study is to enhance the biosynthesis of secondary metabolites in medicinal plants through the utilization of biotic elicitors. Our investigation focuses on two specific medicinal plants, *K.pinnata* and *C.cuspidatus*. Methodologically, we developed biotic elicitors by isolating pathogenic bacteria from healthy soil in rice fields and pathogenic fungi from the leaves of afflicted plants. These isolated microorganisms were then cultured on a large scale to generate bio elicitors, which were subsequently administered to the target plants. Subsequent to this elicitation process, plant samples underwent analysis *via* Gas Chromatography-Mass Spectrometry (GC-MS), enabling a comparative evaluation between untreated control specimens and those subjected to elicitor treatment. Further investigation into the specific bioactive compounds and their mechanisms of action could lead to the development of safer and more effective phyto-derived drugs with broader therapeutic applications. Overall, *K.pinnata* and *C.cuspidatus* represent promising candidates for pharmaceutical development, offering opportunities to address unmet medical needs and improve patient outcomes.

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LIST OF ABBREVIATIONS AND SYMBOLS:

GC-MS	-	Gas Chromatography -Mass Spectrometry
HR	-	Hypersensitive Response
PAs	-	Pyrrolizidine Alkaloids
ROS	-	Reactive Oxygen Species
HAP	-	Hydroxyapatite
CHPD	-	Calcium Phosphate Di-Hydrate
LPS	-	Lipopolysaccharide
MEC	-	Methanol Extract
HRCs	-	Hairy Root Cultures
SDA	-	Sabouraud Dextrose Agar
Mg/kg	-	Milligrams Per Kilogram
°C	-	Degree Celsius
LPCB	-	Lactophenol Cotton Blue

CHAPTER 1

1. INTRODUCTION:

1.1 SECONDARY METABOLITES OF PLANTS:

Secondary metabolites are compounds produced by plants that confer competitive advantages in their native habitats and regulate processes such as flowering, fruit development, abscission, perennial growth maintenance, or the initiation of deciduous behavior. These compounds frequently participate in the defence mechanisms of plants against both biotic and abiotic stresses. Secondary metabolites originate from diverse families of metabolites and can exhibit significant inducibility in reaction to stressors. In the plant kingdom, more than 50,000 secondary metabolites have been identified, many of which serve as crucial components in medicinal herbs and contemporary pharmaceuticals, relying on their biological activities for therapeutic effects (Teoh and Teoh 2016). In plants, according to the source of their biosynthesis, secondary metabolites are classifiable into three major groups-terpenoids, Phenolics, N (Nitrogen), and S (sulphur) containing compounds.

1.2 TYPES OF SECONDARY METABOLITES:

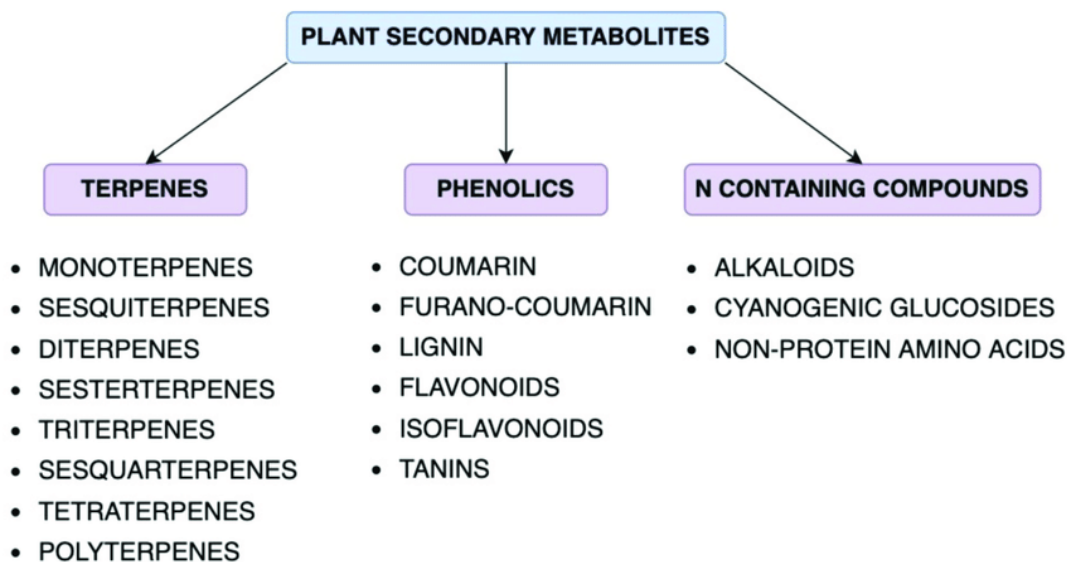


Fig1.1: Types of plant secondary metabolites

1.2.1 Terpenes:

Terpenes represent the largest category of secondary metabolites, distinguished by their shared biosynthetic origin either from acetyl-CoA or glycolytic intermediates. All 30,000 identified terpenes exhibit a shared attribute: they consist of repetitive five-carbon isopropene units. They are further classified into monoterpenes, sesquiterpenes, diterpenes, triterpenes, and polyterpenes. Monoterpenes are commonly found in the leaves and flowers of *Chrysanthemum* species, exhibiting potent insecticidal properties against various insects including beetles and wasps. They are frequently utilized as components in commercial insecticides due to their minimal persistence in the environment and low toxicity to mammals. Several sesquiterpenes have been identified thus far for their involvement in plant defense mechanisms, including compounds like costunolides.

Abietic acid, which is a diterpene, is discovered in pine trees and leguminous plants. It is commonly found within the resin canals of the tree trunk, either independently or in association with resin. Phorbol, a diterpene compound, is present in plants of the Euphorbiaceae family and functions as both a skin irritant and an internal toxin to mammals. Milkweeds produce various glycosides with preferable taste, serving as protection against herbivores, including insects and cattle. Polyterpenes, consisting of numerous isoprene units, represent a class of organic compounds with extended chain lengths, differentiating them from simpler terpenes characterized by fewer isoprene units. Notably, specific polyterpenes demonstrate anti-inflammatory, antimicrobial, and anticancer properties (Saurabh *et al.*, 2016).

1.2.2 Phenolics:

Plants synthesize a diverse array of secondary compounds incorporating a phenol group—a hydroxyl functional group attached to an aromatic ring, known as phenol—constituting a chemically varied group. Coumarins, which are simple phenolic compounds, are abundant in vascular plants and seem to play diverse roles in multiple plant defense mechanisms against insect herbivores and fungi.

Tannins, categorized among plant phenolic polymers with defensive attributes, act as general toxins, markedly diminishing the growth and survival rates of numerous herbivores. Additionally, they serve as feeding repellents to a wide range of animals Teoh and Teoh (2016).

1.2.3 Sulphur Containing Secondary Metabolites :

These compounds encompass GSH, GSL, phytoalexins, thionins, defensins, and allinin, which are directly or indirectly associated with plant defense against microbial pathogens. GSL, or glucosinolates, constitute a class of plant glucosides with low molecular weight, containing both nitrogen and sulphur. They are synthesized by higher plants as a means to enhance their resilience against the adverse impacts of predators, competitors, and parasites.

Phytoalexins are synthesized as a response to bacterial or fungal infection, as well as other forms of stress, serving to restrict the dissemination of invading pathogens. This defense mechanism involves the accumulation of phytoalexins around the infection site, which appears to be a widespread means of resistance against pathogenic microbes across various plant species. Many of these defense responses are associated with a rapid apoptotic reaction, leading to the demise of an individual or a few invaded plant cells, a phenomenon known as the hypersensitive response (HR) (Gowin *et al.*, 2020).

1.2.4 Nitrogen - Containing Compounds:

These encompass alkaloids, cyanogenic glucosides, and non-protein amino acids, many of which are synthesized from common amino acids. Alkaloids are present in about 20% of vascular plant species, predominantly in herbaceous dicots, with fewer occurrences in monocots and gymnosperms. Generally, alkaloids, such as pyrrolizidine alkaloids (PAs), exhibit varying degrees of toxicity and primarily function in defense against microbial infections and herbivore attacks. Cyanogenic glucosides, a separate group of nitrogen-containing protective compounds, release the poison hydrogen cyanide and while not inherently toxic, they decompose upon plant damage, releasing volatile toxic substances like HCN and hydrogen sulfide (Gowin *et al.*, 2020).

1.3 STRATEGIES TO ENHANCE PLANT SECONDARY METABOLITES PRODUCTION:

Biotechnology aims to increase the production of secondary metabolites, often present in small amounts within cells. While enhancing gene expression is one approach, limited information can hinder this method. Alternatively, treating plants with environmental micro-stresses can boost specific metabolite production. A cross-talk between the plant cell wall and the external environment, monitored by cell wall integrity signaling, plays a crucial role. This signaling can trigger responses like increased plasticity or stiffening of the cell wall. Similar mechanisms exist in yeast and fungi. Alterations in the cell wall composition can affect secondary metabolite production.

Enhancing plant secondary metabolites production can be achieved through various strategies. Genetic engineering introduces genes encoding enzymes involved in secondary metabolite biosynthesis to enhance production. This can be done through techniques like gene stacking or CRISPR-Cas9 editing. Optimized Growing Conditions can ensure plants are grown in optimal conditions such as appropriate temperature, humidity, light intensity, and nutrient availability. These factors can significantly influence metabolite production. Stimuli and Elicitors are used as chemical or physical stimuli to induce secondary metabolite production. For example, applying elicitors like methyl jasmonate or salicylic acid can trigger defense mechanisms in plants, leading to increased metabolite synthesis. Microbial Symbiosis establishes symbiotic relationships with beneficial microbes like mycorrhizae or endophytic fungi that can enhance nutrient uptake and stimulate metabolite production. Bioreactor cultivation aids to scale up production using bioreactors that provide controlled environments for plant growth and metabolite extraction. This can improve efficiency and yield consistency. Metabolic engineering modifies metabolic pathways in plants to prioritize secondary metabolite synthesis over primary metabolites. This can be achieved through targeted gene manipulation. Plant hormones and growth regulators like auxins, cytokinins, and gibberellins to regulate metabolic pathways and enhance metabolite production. Each of these strategies can be tailored to specific plant species and target

metabolites to maximize production efficiency and quality (Murthy *et al.*, 2014; Guerriero *et al.*, 2018; Fazili *et al.*, 2022).

1.4 ELICITORS:

Elicitation refers to the stimulated or augmented biosynthesis of metabolites resulting from the introduction of minute quantities of elicitors. An 'elicitor' can be described as a substance that, when added in low concentrations to a living cell system, triggers or enhances the production of particular compounds through biosynthesis. Multiple variables such as the concentration and specificity of elicitors, the duration of elicitor exposure, the developmental stage of the culture, the cell type, growth regulation, nutrient composition, the quality of cell wall constituents, and substantial enhancement of product accumulation have been observed. Thus, the increased synthesis of secondary metabolites in plant cell cultures due to elicitation has opened up a new area of potential research with potential significant economic advantages for the pharmaceutical sector.

1.4.1 Types of Elicitation:

Elicitors can be categorized based on their 'nature' into abiotic elicitors or biotic elicitors, or based on their 'origin' as exogenous elicitors and endogenous elicitors. The first biotic elicitors were described in the early 1970s. Elicitors typically can trigger diverse plant defense mechanisms, such as the generation of ROS (reactive oxygen species), the hypersensitive response, and the synthesis of phytoalexins, which are secondary compounds with antimicrobial properties.

Plants consist of stationary cells enclosed within rigid cell walls, rendering the migration of specialized defense cells to microbial invasion sites impossible, unlike in animals. Instead, plant defense mechanisms have evolved such that each cell possesses the ability to detect attempted infections and mount a defensive reaction. Consequently, plants exhibit resistance to the majority of potential pathogens in their surroundings. Fungal elicitors of a general nature seem to be inherently present in the cell wall, possibly serving as structural constituents (Alvarado *et al.*, 2019).

1.5 MECHANISM OF SECONDARY METABOLITES IN PLANTS:

A generalized process of biotic elicitation in plants can be summarized based on the interaction between elicitors and receptors. When a plant or plant cell culture encounters an elicitor, these events are initiated:

When plants detect pathogens, a cascade of defenses is initiated, starting with elicitor binding to plasma membrane receptors. This triggers ion fluxes, G-protein activation, and ROS production. Subsequent responses include cytoskeletal changes, protein accumulation, and cell wall modifications, culminating in the hypersensitive response and gene activation. Plants also synthesize secondary messengers like jasmonic acid and salicylic acid, inducing systemic resistance. These mechanisms illustrate plants' intricate defense strategies.

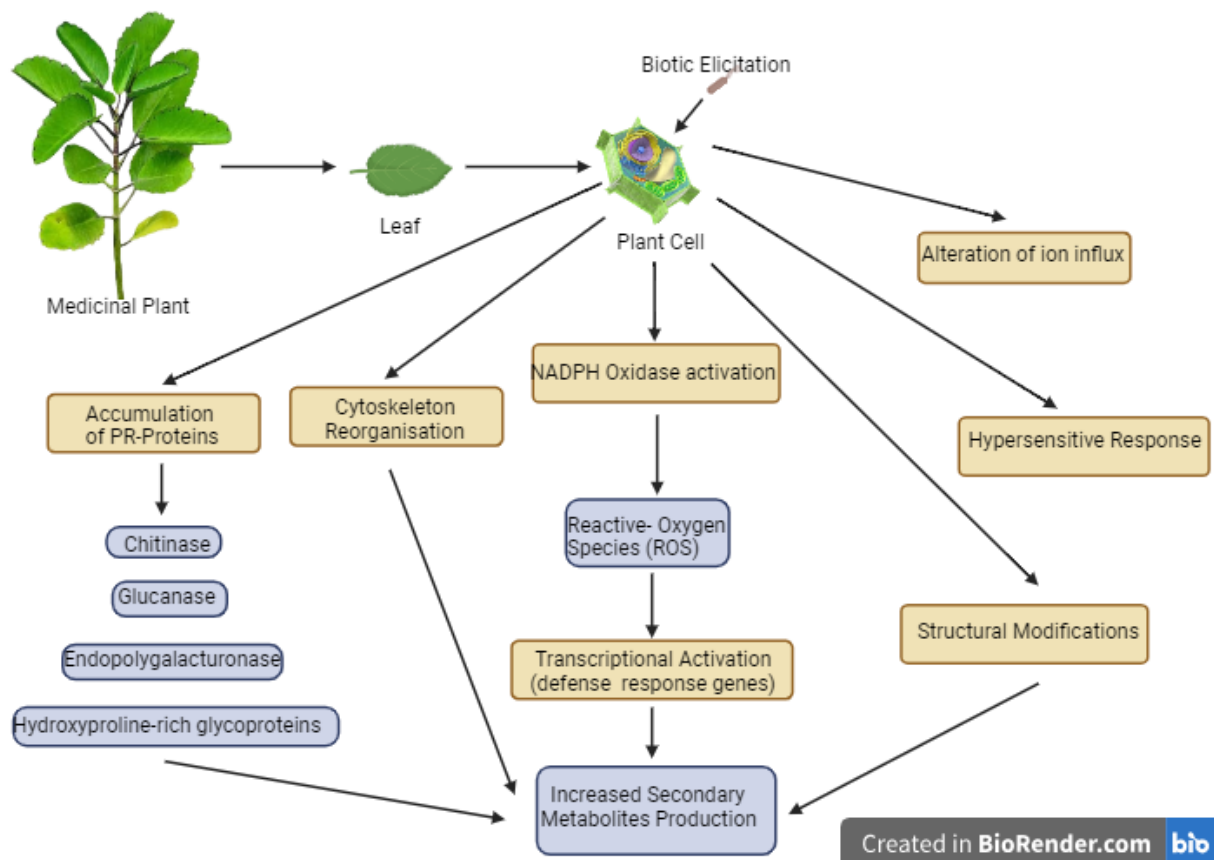


Fig 1.2: Molecular cues and mechanisms of plant secondary metabolites production in response to elicitation.

1.6 EXPERIMENTAL CROPS *KALANCHOE PINNATA*:

Kalanchoe pinnata, a medicinal plant extensively employed in traditional medicine, is utilized for various therapeutic purposes such as kidney stones, gastric ulcers, pulmonary infections, and rheumatoid arthritis. It has established itself in temperate areas across Asia, Australia, New Zealand, the West Indies, Macaronesia, the Mascarene Islands, the Galapagos Islands, Melanesia, Polynesia, and Hawaii through naturalization processes. In India, it is cultivated in gardens and wild on the hills of North-Western India, Deccan, and Bengal.



Fig 1.3 Physical appearance of *K. pinnata* in habitat

1.6.1. Plant description:

K. pinnata, an herbaceous plant ranges from 0.3 to 1.2 meters in height. Its stems are four-angled, initially light-colored but turning reddish with white speckles as they mature. The leaves, arranged in a decussate pattern, vary in complexity, with lower leaves typically simple and larger, while upper leaves tend to have 3-5 (sometimes 7) leaflets, pointed tips, and united petioles forming a ridge around the stem. The leaflets are ovate or elliptic, with toothed margins. Notably, the leaves often generate buds along their edges, equipped with roots, stems, and leaves, which detach to form new plants. The flowers are reddish-purple, drooping in large, widely spreading clusters with

stout, opposite branches. The calyx is red and green at the base, striated, and greenish above, with triangular teeth. The corolla is swollen and octagonal at the base, reddish-purple, and constricted in the middle. Filaments are green at the base and pinkish below the anthers, which are black and hastate in shape.

The fruits are enclosed within the persistent papery calyx and corolla. Seeds are small, smooth, oblong-ellipsoid, scarcely striated, and likewise smooth. *K.pinnata* has medicinal properties and is commonly used in traditional medicine across India. It is found throughout India and cultivated in gardens, as well as growing wild in the hills of North-Western India, Deccan, and Bengal.

1.6.2 Scientific classification

Kingdom	Plantae
Clade	<i>Tracheophytes</i>
Clade	<i>Angiosperms</i>
Clade	<i>eudicots</i>
Order	Saxifragales
Family	Crassulaceae
Genus	<i>Kalancho</i>
Species	<i>K.pinnata</i>

1.6.3 Pharmacological Review:

1.6.3.1 Wound - healing activity

The ethanol extract of *K. pinnata* exhibited notable wound-healing properties, evidenced by its ability to reduce the size of the wound area and alleviate oedema at the site of injury (Khan *et al.*, 2004).

1.6.3.2 Anti-oxidant activity

Anti-oxidative agents play a crucial role in shielding cells from the detrimental impacts of reactive oxygen species (ROS) like singlet oxygen, superoxide, peroxy radicals, hydroxyl radicals, and peroxynitrite. In the case of *K. pinnata*, it has been observed that

the leaves demonstrate superior scavenging effects compared to the stems, and the ethanol extract displays higher concentrations of total phenolic and flavonoid compounds compared to other extracts. This abundance of phenols and flavonoids in the extract likely accounts for their potent anti-oxidative activity (Bhatti *et al.*, 2012).

1.6.3.3 Antimicrobial activity

Ethanol extract showed the antimicrobial properties of the plant, with these compounds demonstrating the ability to hinder the growth of various gram-negative and gram-positive bacteria as well as fungi commonly encountered in nature (Okwu *et al.*, 2011).

1.6.3.4 Antiviral activity

Mahata *et al.*, (2012) investigated the potential anticancer and anti-HPV properties of the chloroform extract derived from the plant. Their study involved subjecting extract fractions to cancer cell lines, resulting in the suppression of viral protein expression. This suppression consequently inhibited both viral proliferation and tumor growth.

1.6.3.5 Haemoprotective activity

The raw methanol extract obtained from *Kalanchoe pinnata* demonstrated notable protection against the lysis of human erythrocyte membrane induced by hypotonic solution and heat, effectively preventing the hemolysis of red blood cells (Akanda *et al.*, 2014).

1.6.3.6 Antilithiatic activity

The decrease in urinary oxalate excretion leads to the formation of calcium oxalate stones. Patients with stones in their bodies received fresh juice extracted from *Kalanchoe pinnata* leaves as part of medical prophylaxis. Consistent consumption of the juice successfully dissolved stones irrespective of their location, composition, or prior treatments. Additionally, there was an elevation in urine volume, indicating the diuretic properties of the juice. Furthermore, it aided in reducing oxalate excretion while promoting citrate excretion. This plant contains a variety of bioactive compounds like

alkaloids, triterpenes, glycosides, flavonoids, steroids, and bufadienolides, which exhibit diverse pharmacological effects such as antimicrobial, anticancer, anti-inflammatory, cardio protective, and diuretic properties. Studies have shown that extracts of *Kalanchoe pinnata* have efficacy in wound healing, infectious diseases, cancer, cardiovascular issues, and urinary stone dissolution. Further investigation into specific bioactive compounds from *Kalanchoe pinnata* could lead to the development of novel drugs with better efficacy and safety (Gilhotra *et al.*,2013).

1.7 CHAMAECOSTUS CUSPIDATUS:

Chamaecostus cuspidatus, commonly known as the insulin plant, is a traditional medicinal herb originating from Southeast Asia. Recently introduced to India, it is primarily cultivated as an ornamental plant in the southern region. The plant is rich in various phytochemicals including steroids, alkaloids, flavonoids, triterpenes, glycosides, and saponins. Its leaves are utilized as a dietary supplement for managing diabetes mellitus. Consumption of these leaves is believed to reduce blood glucose levels, with reported decreases in blood glucose levels among diabetic individuals who consume them.



Fig .1.4 : *Chamaecostus cuspidatus* in its habitat

1.7.1 Plant Description:

Chamaecostus cuspidatus, a member of the Costaceae family, is a tropical evergreen plant characterized by its perennial, upright growth habit. It's simple, oblong leaves, measuring 4-8 inches in length, feature parallel venation and possess dark green upper surfaces with light purple undersides, arranged spirally around the stems, forming

attractive, arching clusters emerging from underground rootstocks. With stems reaching heights of approximately 60cm, some may bend and rest on the ground. During warm months, the plant produces striking orange flowers, ranging from 2.5 to 12.5cm in diameter, appearing in cone-like heads at the tips of branches. Propagation typically occurs through stem cutting.

1.7.2 Scientific Taxonomy:

Kingdom	Plantae
Clade	Tracheophytes
Clade	Angiosperms
1Clade	Monocots
Clade	commelinids
Order	Zingiberales
family	Costaceae
Genus	<i>Chamaecostus</i>
Species	<i>C.cuspidatus</i>

1.7.3 Pharmacological Review:

1.7.3.1 Anti-Diabetic Effects:

The leaves of the insulin plant play a crucial role in exerting significant anti-diabetic effects, reducing both fasting and postprandial blood glucose levels. However, the precise mechanism underlying its anti-diabetic activity remains unclear. In addition to its anti-diabetic effects, the insulin plant also mitigates diabetic complications, regulates renal and hepatic parameters, reduces glycosylated hemoglobin levels, improves lipid profiles, increases body weight and insulin levels, and demonstrates notable improvements in histopathological examination results (Mathew et al.,2009).

1.7.3.2 Antiurolithiatic Property

The anti-urolithiatic potential of the insulin plant was investigated by utilizing its aqueous extract derived from the stem and rhizome. The study revealed that the plant extract facilitated the formation of hydroxyapatite (HAP) crystals while decreasing the

nucleation rate of calcium hydrogen phosphate di-hydrate (CHPD) crystals, which are a significant constituent of calcium urinary stones (Manjula *et al.*,2017).

1.7.3.3 Anti-Inflammatory Potential

The anti-inflammatory potential was investigated through the isolation of β -amyrin from *Chamaecostus cuspidatus* leaves. This study utilized a rat model induced with carrageenan and human peripheral blood mononuclear cells (hPBMCs) stimulated with lipopolysaccharide (LPS) in vitro. Fractionation of the methanol extract (MEC) of *C. cuspidatus* leaves revealed the highest percentage inhibition of paw edema at a dose of 100 mg/kg body weight (Krishnan *et al.*,2014).

1.7.3.4 Neuroprotective Role

Research has shown that the combination of plant extract and melatonin led to a notable reduction in the activity of anti-oxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in the brain. Both melatonin and the plant extract have been shown to exhibit significant efficacy in mitigating the adverse effects on the brain induced by hyperglycemia associated with diabetes. They effectively restored brain function by replenishing the population of astrocytes and glial cells, thus ameliorating the damage caused. This plant contains a variety of bioactive compounds like flavonoids, chlorophylls, and alkaloids which have shown beneficial effects including anti-diabetic, anti-microbial, anti-inflammatory, and antioxidant properties. Further research is needed to confirm these benefits and identify the specific compounds responsible for each effect. This knowledge could lead to the development of safer and more reliable treatments in the future (Hajam *et al.*,2018).

1.7.3.5 Learning and Memory:

In a study conducted by Adiga *et al.*, (2014), the impact of *C. cuspidatus* on learning and memory was investigated using passive avoidance tests in both normal and diabetic-induced rats. The rats were administered ethanol extracts of *C. cuspidatus* doses of 250 and 500mg/kg. Diabetes was induced in the rats through a single intraperitoneal injection of streptozotocin (35 mg/kg). After a 30-day study period, blood glucose levels were measured, and the rats underwent passive avoidance tests.

Results indicated that treatment with *C.cuspidatus* led to a significant dose-dependent reduction in blood glucose levels in diabetic rats, with a notable 75.70% reduction observed at the 500mg/kg dose.

Furthermore, during retention testing at 24 hours and 48 hours post-shock, *C. cuspidatus*-treated diabetic rats demonstrated increased entrance latency and decreased time spent in the dark room, suggesting enhanced memory retention. In summary, the ethanol extract of *C. cuspidatus* exhibited significant effects on learning and memory in diabetic rats, particularly at a dose of 500mg/kg.

1.8 BIOTIC ELICITATION STUDIES:

While numerous traditional medicinal plants hold immense therapeutic promise, their widespread utilization remains limited, primarily due to the inherent chemical variability of plant-derived therapeutics. The production of medically beneficial secondary metabolites in plants often relies on their response to stress. However, when cultivated under optimal conditions, the natural stressors are removed, leading to reduced secondary metabolite content and therapeutic efficacy. Elicitation, involving the use of biotic and abiotic elicitors to induce stress responses in plants, presents a promising solution to this challenge. Although elicitation has shown success in enhancing disease resistance in plants and metabolic studies in cell culture, its full potential in the production of medicinal plants remains largely untapped. Future developments in elicitation techniques hold great promise for advancing the characterization and development of various medicinal plants. Despite the evidence supporting its benefits, elicitation has yet to be widely implemented in agricultural settings. Further research and innovation in elicitation methods are necessary to bridge this gap and unlock the full therapeutic potential of medicinal plants on a larger scale.

CHAPTER 2

2. REVIEW OF LITERATURE:

2.1 PLANT SECONDARY METABOLITES:

Saurab *et al.*, (2015) talks about secondary metabolites which are produced by plants that interact with their environment for the defence against both biotic and abiotic stress and have economic value, due to their diverse chemical structures. Metabolites such as terpenoids, polyketides, phenyl propanoids, and alkaloids are synthesized in specific locations but can be transported throughout the plant. Secondary metabolites play crucial ecological roles in plants by defending them from microbial pathogen infection and herbivore consumption. They operate as attractants (taste, color, and smell) for seed-dispersing animals and pollinators. Thus, the plants' secondary metabolites have a significant impact on their capacity to compete and endure. The generation of secondary metabolites by genetic engineering is also facilitated by biotechnological methods.

Nobukazu (2016) discusses the developments of plant secondary metabolites. Plants produce various secondary metabolites with diverse biological functions, some of which can be harmful to eukaryotic cells. To cope with these compounds, plants employ strategies like transporting them to specific compartments such as the apoplast or vacuole, or employing self-tolerance mechanisms. To optimize the production of these bioactive compounds in plants or microbes, transport, and self-tolerance mechanisms must work in tandem with the biosynthetic enzymes. Through extensive research, scientists have identified and characterized the proteins responsible for these processes. Many transporters have been isolated and their roles in plant physiology have been proposed.

Teoh (2016) states that Secondary metabolites are small molecules produced by plants that have a broad spectrum of effects on both the plant itself and other organisms. They play roles in processes such as flowering, fruit development, and leaf shedding. Additionally, they serve as antimicrobial agents and can attract or repel other organisms. The plant kingdom contains a vast array of these compounds, with over

50,000 identified so far. Many medicinal herbs and modern pharmaceuticals rely on these secondary metabolites for their therapeutic actions. The ongoing search for new secondary products in plants aims to uncover novel treatments for diseases, involving collaborations between academic and pharmaceutical institutions.

Namdeo (2007) termed secondary metabolites are the essence of a medicinal plant's therapeutic power. These compounds not only determine a plant's medicinal value but also serve as markers for quality control. However, understanding their production is challenging due to the intricate interplay of internal (genetics) and external (environment) factors. Ultimately, manipulating these internal and external factors paves the way for advanced biotechnologies for high-quality medicinal plant production.

2.2 FUNCTIONS OF PLANT SECONDARY METABOLITES:

Richards *et al.*, (1994) discusses the functions of plant secondary metabolites. Secondary metabolites play a crucial role in plant defense mechanisms, which can include acting as deterrents or anti-feedants, exhibiting toxicity, or serving as precursors to physical defense structures. While some herbivores and pathogens may attempt to bypass the deterrent or toxic effects of these compounds, others utilize them as cues for host recognition or as sources of nutrients. This phenomenon is observed in compounds like cyanogenic-glucosides and glucosinolates, which are extensively discussed as examples of defensive compounds. Some classes of secondary products are induced in response to infection, wounding, or herbivory. This induction serves to enhance the plant's defense capabilities against potential threats.

Erb *et al.*, (2020). discusses how the plant's secondary metabolites act as regulators in defense. Research has shown that various classes of secondary metabolites, including glucosinolates, benzoxazinoids, terpenes, aromatics, and green-leaf volatiles, can act as regulators of plant defense responses. For instance, glucosinolates and benzoxazinoids have been found to regulate callose deposition, a key defense response, in both *Arabidopsis* and maize. Additionally, these compounds seem to influence the accumulation of other secondary metabolites, suggesting a broader regulatory role. Volatile secondary metabolites like terpenoids and green-leaf volatiles are released by plants upon herbivore or pathogen attack and can directly induce or

prime hormonal defense signaling pathways. These compounds can also influence systemic acquired resistance in plants. The mechanisms by which secondary metabolites regulate defense responses are still not fully understood. They may act indirectly by affecting detoxification enzymes or directly by binding to specific receptor proteins. Further research is needed to elucidate the precise targets and mechanisms of action of secondary metabolites in plant defense regulation.

The same review also talks about how secondary metabolites, traditionally known for their roles in defense, are now recognized as key regulators of plant growth and development. These compounds dynamically modulate growth patterns in response to environmental cues such as herbivores or pathogen attacks. Glucosinolates, for instance, have been found to inhibit root meristematic growth and regulate stomatal closure. Their breakdown products interfere with auxin signaling pathways, thereby affecting various aspects of plant growth.

2.3 *KALANCHOE PINNATA*:

Walter *et al.*, (2016) discusses about the medicinal plant *Kalanchoe pinnata*. *K. pinnata* is well known to display a broad spectrum of pharmacological actions, including the management of the most severe human diseases. This plant has been used for a wide range of purposes in pharmacological research, including wound healing, antioxidant, anticancer, anti-proliferative, antimicrobial, antiviral, antiprotozoal, antileishmanial, anthelmintic, insecticidal, anti-allergic, analgesic, antinociceptive, anti-hematogenic, anti-inflammatory, muscle-relaxant, antipyretic, anticonvulsant, antidepressant, sedative, anti-lithiatric, hepatoprotective, gastroprotective, anti-diabetic, nephroprotective, hepatoprotective, antihistamine, antihypertensive, immunosuppressive, tocolytic, and anthroposophic effects. Analyses of the plant's extracted biomolecules and extracts' potential for medicinal use have been conducted.

Quazi- Majaz *et al.*, (2011) stated that alkaloids, triterpenes, glycosides, flavonoids, steroids, and lipids are abundant in the *Kalanchoe* plant. Bufadienolides include a class of highly active and have aroused scientists' curiosity. They share many structural and functional similarities with digoxin and digitoxin, two additional cardiac glycosides that are used for the therapeutic management of congestive heart failure and associated

illnesses. Clinical studies have shown that the bufadienolides found have antibacterial, antitumor, cancer-preventive, and insecticidal properties. Leaf, leaf juice, and the entire plant are typically employed; the root portion has not yet been investigated, therefore more research is needed to assess its pharmacological and chemical action.

2.4 CHAMAECOSTUS CUSPIDATUS :

Mathew *et al.*, (2019), reviewed about the *Chamaecostus cuspidatus* plant and its pharmaceutical properties. *C.cuspidatus* (insulin plant), a Southeast Asian herb, contains various bioactive compounds like steroids, alkaloids, and flavonoids. Traditionally used for diabetes, its leaves are explored as a dietary supplement for managing blood sugar. Studies suggest the plant possesses anti-diabetic, anti-cancer, anti-microbial, and anti-inflammatory properties. Additionally, it might improve memory, act as an antioxidant, and protect nerves. This review explores the potential of *C. cuspidatus* for the future development of therapeutic formulations.

Padule *et al.*, (2022) talks about the chemical nature of the *C.cuspidatus* plant. Various chemical constituents found in the plant contribute to its pharmacological properties. Triterpenoids, composed of three terpene units or six isoprene units, include compounds like β -carotene and lupeol, while steroids, characterized by four rings arranged in a specific molecular configuration, encompass molecules such as stigmasterol and β -sitosterol. Alkaloids, nitrogen-containing phytoconstituents with diverse chemical structures, are notably absent in *C. cuspidatus*. Phenols, featuring a hydroxyl group attached to an aromatic hydrocarbon group, comprise compounds like catechin and strychnine. Flavonoids, hydroxyl polyphenolic phytoconstituents recognized for their antioxidant properties, include derivatives such as epigallocatechin gallate and quercetin. Proteins, essential biomolecules composed of amino acid chains, encompass substances like insulin-like proteins. Fatty acids, long aliphatic chains with saturated or unsaturated bonds, include compounds like hexadecanoic acid and oleic acid. These diverse chemical constituents play vital roles in plant physiology and have garnered interest for their potential therapeutic applications, particularly in managing conditions like diabetes mellitus.

2.5 ELICITATION:

Namdeo (2007) investigated elicitation as a strategy to augment secondary metabolite production in medicinal plants. It emphasizes the definition, classification, and mechanism of action, key characteristics of elicitors, and their application in enhancing the production of medicinally valuable compounds within plant systems. Elicitation refers to the phenomenon where minute quantities of elicitors trigger or elevate the biosynthesis of specific metabolites within the plant. Elicitor application or incompatible pathogen encounters induce a cascade of plant defense reactions, including the accumulation of valuable secondary metabolites. Despite extensive research, the precise mechanism of elicitation remains unclear, with hypotheses centered on calcium ion signaling, cell membrane integrity changes, and modulation of intracellular pathways.

Angelova *et al.*, (2006) gives insight into plant pathogen resistance and elicitor recognition in plants. Plant immobility necessitates unique defense strategies. Unlike animals with mobile immune cells, plants have evolved a system where individual cells can independently recognize and respond to potential microbial threats. This intrinsic response allows plants to resist most pathogens in their environment. The initial recognition of a pathogen is crucial for a resistant plant's defense. Elicitor molecules, presumably detected by specific receptors located either on the cell surface or internally, trigger signalling cascades that activate plant defense mechanisms. It also explains that the biotic elicitation in plants likely functions through a fundamental mechanism centered on the interaction between elicitors and their corresponding receptors.

Vasconsuelo *et al.*, (2007) give a brief about the elicitation depending on complex interactions between the elicitor and plant cells. Several factors influence this, including elicitor specificity (plants respond differently to the same elicitor), concentration (affects the intensity and optimal dose varies by species), and treatment interval (time of exposure). Additionally, culture conditions like growth stage, medium composition

(presence of growth regulators), and light can significantly impact the elicitation response. Optimizing these factors is crucial for designing successful elicitation protocols.

Nisha et al., (2020) explained the use of elicitors. Plants lack mobile immune systems, so they rely on sophisticated strategies to defend themselves. Elicitors offer a promising approach to enhancing plant health and disease resistance. These compounds act as signals, triggering the plant's inherent immune system genes. This ecologically safe method essentially primes the plant's defenses, putting it on high alert before potential threats arrive. Elicitor application not only strengthens the plant's overall defense system but also induces a broad-spectrum resistance against a wide range of pathogens like fungi, bacteria, and viruses. This multi-layered defense activation makes elicitors a valuable tool for sustainable plant protection.

Zehra et al.,(2021) discusses the efficiency of microbial bio-agents as elicitors in plant defense mechanisms under biotic stress. Microbial bio-control agents (MBCAs) are emerging as a promising alternative to harmful chemical pesticides. These beneficial microbes, including bacteria, fungi, and viruses, can combat plant pathogens through various mechanisms. MBCAs can directly attack pathogens (e.g., producing antimicrobial compounds) or indirectly stimulate the plant's defense mechanisms (elicitors). Elicitor MBCAs trigger a complex signaling cascade within the plant, leading to increased production of defensive proteins and activation of defense pathways. While the exact mechanisms are still being elucidated, research suggests MBCAs can enhance plant defences against a broad range of pathogens in an eco-friendly manner. Further research on microbe-plant interactions at the cellular and molecular level will facilitate the development of even more effective and targeted bio-control agents.

Li et al.,(2020) specifies the applications of elicitors in pharmacological industries. Plants are a treasure trove of bioactive secondary metabolites (SMs), with over 100,000 identified from just half that number of plant species. However, obtaining sufficient quantities of these valuable SMs for pharmaceutical use from field-grown plants is often

challenging. While tissue culture techniques have emerged, limitations remain due to incomplete knowledge of SM pathways and regulation within plants. Elicitation offers a promising solution. Elicitors are diverse molecules, either from living organisms (biotic) or non-living sources (abiotic). Applied at minute concentrations, they trigger or enhance SM production in plant systems by activating defense responses. This approach has been successfully used in both hairy root cultures (HRCs) and plant cell cultures of various species.

Kuzel et al.,(2009) talks about the quality of medicinal plants used in the pharmacological industry. The quality of medicinal plants is heavily dependent on the content of their bioactive metabolites. However, these levels are highly susceptible to environmental factors like weather and pest damage, as well as inherent genetic variation within the plant population. This variability poses a significant challenge for the pharmaceutical industry, hindering reliable and consistent production of desired medicinal compounds. This study specifically addresses the potential of in vivo elicitation as a strategy to enhance the production of these valuable metabolites in field-grown plants. The concern lies in the potential barrier of the plant's cuticle, a waxy layer, limiting the uptake of elicitors applied in a soil environment.

CHAPTER 3

3. AIM AND OBJECTIVES:

3.1. AIM:

To study the influence of biotic elicitors in the production of secondary metabolites in *K. pinnata* (Ranakalli) and *C. cuspidatus* (Insulin).

3.2. OBJECTIVES:

- Isolation of bacteria species from the soil samples collected from a healthy rice field
- Isolation of plant pathogenic fungal samples from affected plant species
- Identification and characterization of the bacterial and fungal isolates through staining studies
- To study the effect of biotic elicitors (bacterial and fungal samples) on production of secondary metabolites of Ranakalli and the Insulin plant *via* GC-MS analysis.

CHAPTER 4

4.MATERIALS AND METHODS

4.1. CHEMICALS AND REAGENTS:

This study utilized media components such as Nutrient Agar, Nutrient Broth, Sabouraud Dextrose Agar, and Sabouraud Broth, obtained from SRL Ltd. in India. Chemical reagents were dissolved in distilled water, while solvents like Ethanol, Tween-20, and Hexane were sourced from SRL Ltd. India. Stains for bacterial and fungal characterization, including crystal violet, Gram's iodine solution, acetone, 0.1% basic fuchsin solution, and lactophenol cotton blue, were acquired from Loba Chemie Pvt Ltd. in India.

4.2. SAMPLE COLLECTION:

Pathogenic bacteria and fungal samples were gathered for this study. Soil samples from multiple healthy rice fields were collected to obtain pathogenic bacteria, while affected leaf samples were gathered for fungal isolation. All samples were carefully collected and stored in airtight containers to prevent contamination. The leaf samples were then pulverized using a mortar, rendering them suitable for fungal isolation procedures.

4.2.1 Area of Sample Collection :

4.2.2 Bacterial Sample Collection:

The primary objective of soil sample collection is to isolate these pathogenic bacteria, thereby utilizing the soil from these areas. These bacteria are isolated from soil samples of Kalpakkam, Tamil Nadu with the intention of generating biotic elicitors.



Fig.4.1 Bacterial sample collection area

4.2.3 Pathogenic Fungal Sample Collection:

Samples for isolating pathogenic fungi are commonly sourced from diseased plant foliage. Leaves from a range of botanical species, including *Annona squamosa* (custard apple), *Mangifera indica* (mango), *Artocarpus heterophyllus* (jackfruit), and other affected plants like cabbage, cauliflower are collected from various locations of kalpakkam, Tamil Nadu. These selections are made based on observable symptoms of fungal infection or disease.



Fig. 4.2 Fungal pathogens were collected from infected leaves

4.3 ISOLATION OF BACTERIA AND FUNGI:

The isolation of bacteria constitutes a crucial step in this project. Various equipment were employed for the isolation of bacteria and fungi, including inoculation loops, spirit lamps, test tube holders, micropipettes, cotton, and glassware such as L-rods, petri plates, and test tubes. The isolation procedure was conducted exclusively within a laminar airflow hood to prevent any potential contamination.

4.3.1 Isolation of Bacteria :

Sterile nutrient agar plates were prepared and autoclaved.

4.3.2 Serial dilution technique:

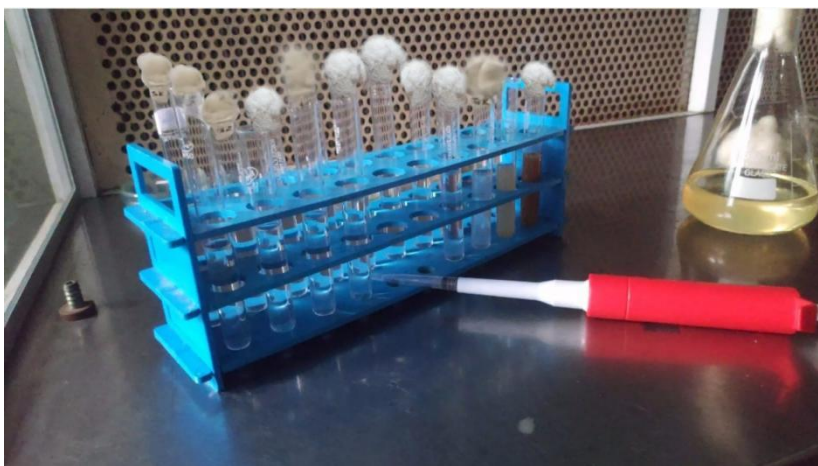


Fig.4.3 Serial dilution technique

Initially, bacteria were isolated using the serial dilution method, a technique aimed at obtaining pure bacterial colonies from soil samples containing high microbe concentrations. Each soil sample underwent serial dilution up to a 10^{-7} dilution, with 1 ml from both the 10^{-5} and 10^{-7} dilutions added to sterilized petri plates in duplicate. The concentration factor for each test tube remained consistent at 10 ml. Seven dilutions of 10 ml were prepared in each test tube for the experiment. Subsequently, the 5th and 7th dilutions were chosen for culturing onto media plates. This serial dilution process successfully yielded distinct bacterial colonies, preparing the soil samples for subsequent plating.

4.3.3 Isolation of fungi pathogens:

The isolation of pathogenic fungi also employs the spread plate technique. Initially, fungal samples collected are homogenized using a mortar. Subsequently, Sabouraud Dextrose Agar (SDA) is prepared at the required concentration and autoclaved for sterilization. Under laminar airflow conditions, the autoclaved SDA agar is poured into petri plates and allowed to solidify. Then, one milliliter of the fungal sample is aseptically transferred onto each SDA plate using a micropipette. The sample is evenly spread across the surface of the agar using an L-rod, ensuring uniform distribution throughout each plate. Subsequently, the samples are placed in the incubator, with the temperature set according to the optimal growth conditions for fungi (20°C to 30°C).

4.4 SUBCULTURING OF BACTERIAL AND FUNGAL ISOLATES:

After isolating bacteria and fungi, they undergo subculture to obtain different microbial strains. Bacteria are subcultured based on their colony morphology, plated onto nutrient agar plates using the streak plate technique, and then incubated. Fungal samples, on the other hand, are subcultured according to their color pigments, which may include green, yellow, white, black, etc. Each distinct color pigment is subcultured onto separate Sabouraud Dextrose Agar (SDA) plates and then incubated at optimized temperature

4.5 CHARACTERISATION OF BACTERIA AND FUNGI ISOLATED:

Following staining procedures, bacterial and fungal specimens are subjected to Gram staining for bacteria and Lactophenol cotton blue staining for fungi. Subsequently, the stained specimens are examined under a microscope for further analysis. bacteria and fungi cultures are subsequently cultivated in large quantities for further study.

4.6 MASS CULTIVATION OF BACTERIA AND FUNGI:

The isolated bacteria and fungi are cultured in significant quantities for their use as biotic elicitors. This procedure necessitates a range of glassware, including conical flasks (also known as Erlenmeyer flasks), as well as tools like inoculation loops and cotton plugs, alongside the original cultures and their corresponding culture media.

Nutrient broth is used for bacterial cultivation, while Sabouraud dextrose broth is employed for fungal culture.

The bacterial cultures have been inoculated into flasks containing nutrient broth under laminar airflow conditions. The flasks are sealed with cotton plugs and placed in an incubator shaker set at 37°C, with agitation at 250 rpm. The fungal cultures are inoculated into flasks containing Sabouraud dextrose broth, and the flasks are sealed with cotton plugs. This procedure occurs within a laminar airflow hood to prevent contamination. Subsequently, the flasks are placed in an incubator shaker set at 28°C, with agitation at 200 rpm.

4.7 ELICITOR PREPARATION:

Various equipment is utilized in this procedure, including large-scale cultured bacteria and fungi, a 100ml beaker, Tween 20 solution (10ml), distilled water, gauze cloth, centrifuge, hot air oven, and a sprinkler. Therefore, the preparation of biotic elicitors involves several steps:



Fig 4.4 Bioelicitor preparation

Step 1: Equipment was sterilized by autoclaving at 121°C for 15 minutes.

Step 2: 10 ml of bacterial growth culture from the conical flask was transferred to each Eppendorf tube and centrifuged at 2000 rpm. This process was repeated until all the mass-cultivated bacterial culture had been processed. The pellet from each Eppendorf tube was collected and placed separately in aluminum foil.

Step 3: Fungi were extracted from the growth media, combining different colors to create a mixed biotic elicitor, and individually wrapped in aluminum foil.

Step 4: The fungal and bacterial extractions, wrapped in aluminum foil, were placed in a hot air oven set at 60°C for one hour.

Step 5: Upon removal from the hot air oven, the extractions were allowed to air dry and then ground into powders for biotic elicitor formulation.

Step 6: The elicitor powder was transferred into a test tube, a few drops of Tween twenty solution were added, and the test tube was filled with distilled water up to a total volume of 10ml.

Step 7: Gauze cloth was added on top of the beaker to act as a filter, the mixture was poured through the filter to separate any solid particles, and the filtered solution was transferred into the sprinkler to obtain a finely dispersed elicitor

4.8 MAINTANANCE OF PLANT SPIECIES:

Growing plants in pots and maintaining them under suitable conditions are crucial steps in this project, as it revolves around the plants' ability to produce secondary metabolites with the assistance of biotic elicitors. Specifically, the project focuses on Ranakalli (*K. pinnata*) and Insulin (*C. cuspidatus*) plants. We selected these plants for their medicinal properties, and they are still under investigation. The effects of elicitation on these two plants have yet to be studied. To conduct an elicitation test, two sets of plants are required: one for the elicitation treatment and another for the control. Subsequently, both treated and control plants undergo GC-MS analysis, and their results are

compared. It is essential to ensure uniform conditions and consistent nutrient provision while growing the plants.

Both Ranakalli and Insulin plants possess various medicinal properties. They synthesize organic compounds known as secondary metabolites, which offer diverse medicinal benefits, making them highly valuable in pharmaceutical applications. Ranakalli thrives in moderate temperatures ranging from 15°C to 25°C (59°F to 77°F) and prefers full sunlight to partial shade. It grows best in well-draining, slightly acidic to neutral soil with consistent moisture levels. On the other hand, Insulin plants prefer warm to hot temperatures between 20°C to 35°C (68°F to 95°F) and require regular watering to maintain soil moisture. They also thrive in partial shade to full sunlight and prefer well-draining soil to prevent root rot.



Fig 4.5 Experimental crops maintenance

4.9 ELICITATION STUDIES ON SELECTED PLANT SPECIES :

After completing all the preceding steps, the focal point of this project emerges: the inoculation of the biotic elicitors we have prepared, which involves spraying them onto the plants. The inoculation process is straightforward: spraying the elicitors onto the leaves of the plants and then tying threads around the treated leaves. All the control and treated plants are then placed together in the same location, ensuring they are exposed to days, then reapply the elicitors by spraying them onto the marked plant leaves. Afterwards, return the plants to the same location for the continuation of the

procedure. After the second round of elicitor spraying, it's imperative to promptly prepare the leaves for GC-MS analysis. Any delay in this process could result in plant death.



Fig 4.6 Elicitation studies

4.10 SAMPLE PREPARATION FOR GC-MS STUDIES:

Before conducting GC-MS studies, sample preparation is essential. This involves the dissolution of samples in a specific solvent such as methanol or ethanol. In this case, hexane solvent was utilized. The process also requires various equipment including a water bath, hexane solvent, leaves from both control and treated plants, a mortar, and

Eppendorf tubes. A mortar was utilized to crush the treated leaves of the Ranakalli plant thoroughly, ensuring complete pulverization. This procedure was repeated separately for the insulin plant. The same process was replicated for both the treated plants and the control plants. Following the smashing of the plants in the mortar, the pulverized leaves were combined with hexane solvent. Subsequently, the resulting mixture was transferred into a test tube.

The sample was submerged in a water bath maintained at a temperature of 70 degrees Celsius for a duration of one hour. Following this, the sample was filtered using a Whatman filter paper to separate any solid particles or impurities from the solution. This filtration step aided in obtaining a clean and purified sample suitable for subsequent analysis. The filtered sample was transferred into an airtight container, such as an Eppendorf tube, to ensure its preservation and prevent any contamination or evaporation. Proper storage in such containers maintained the integrity of the sample for further analysis, allowing for accurate and reliable results in subsequent experimental procedures.



Fig 4.7 Sample preparation for GC-MS analysis

4.11 GC-MS ANALYSIS:

Proceed with further analysis of the provided sample to assess whether there has been any enhancement in the production of secondary metabolites in the treated plants. This entails conducting comprehensive examinations, such as chromatographic techniques or spectroscopic methods, to quantify and identify specific compounds indicative of

increased metabolite synthesis. By scrutinizing the sample in detail, we aim to ascertain the effectiveness of the treatment in stimulating the biosynthesis of valuable secondary metabolites. This evaluation serves to elucidate any potential improvements in the treated plants' biochemical composition, thereby contributing to a deeper understanding of their medicinal or industrial relevance. The sample was submitted for GC-MS analysis at the International Research Center (IRC) of Sathyabama Institute of Science and Technology.

CHAPTER 5

5.RESULT AND DISCUSSIONS

5.1 ISOLATION OF FUNGI AND BACTERIA:

Soil samples from various rice fields were collected to isolate bacteria, while affected leaf samples were obtained to isolate pathogenic fungi. The samples were cultured using specific techniques: the spread plate method for bacteria and Sabouraud dextrose agar for fungi. From the soil samples, three distinct bacterial colonies were obtained, which were further subcultured. From the fungal samples, five pigmented colonies were observed and subculture based on their pigments. These procedures are crucial for identifying and characterizing microorganisms present in agricultural ecosystems. In this context, the presence of pathogenic bacteria in healthy rice fields is concerning due to its implications for crop yield and food safety. Several pathogenic bacteria commonly found in rice fields include *Burkholderia glumae*, *Lactobacilli*, *Xanthomonas campestris*, *Agrobacterium tumefaciens*, and *Pseudomonas syringae*. These bacteria have the potential to infect rice plants, leading to reduced yields. The use of specific culturing techniques allows researchers to study the diversity and prevalence of microorganisms in agricultural environments, providing insights into disease management strategies and promoting sustainable farming practices



Fig 5.1: Bacteria and fungi isolated from collected samples

5.2 CHARACTERIZATION OF FUNGI AND BACTERIA:

The isolated bacteria and fungi underwent staining procedures for characterization. Gram staining was performed on the bacteria, while Lactophenol cotton blue (LPCB) staining was applied to the fungi samples. Consequently, the bacteria samples exhibited characteristics of gram-negative bacilli, whereas the morphology of the fungal samples was observed under a microscope. Microscopic observations reveal that all isolated bacteria appear as gram-negative bacilli, which are anticipated to include *Ralstonia solanacearum*, *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Xanthomonas campestris*, and *Erwinia amylovora*. For fungi, microscopic examination after LPCB staining indicates the presence of expected species such as *Aspergillus*, *Puccinia spp.*, *Colletotrichum gloeosporioides*, and *Peronospora parasitica*. The bacteria and fungi cultivated in large quantities possess a diverse range of molecular mechanisms to interact with their environment. Bacteria release various molecules such as lipopolysaccharides, flagellin, and exopolysaccharides, while fungi produce chitin, glucans, and glycoproteins. These compounds act as signals that trigger plant defense pathways, resulting in the production of defense-related proteins, phytoalexins, and other secondary metabolites

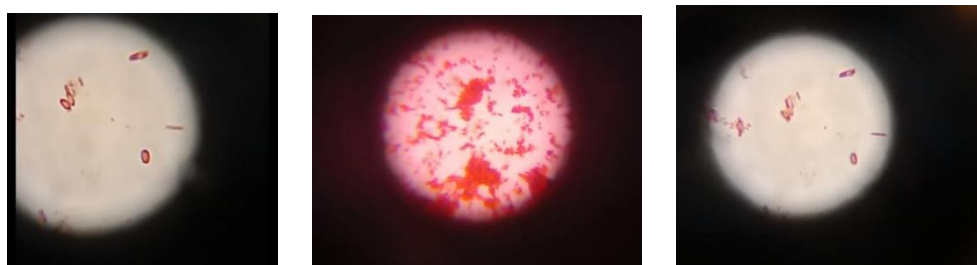


FIG 5.2: Bacterial characterization (Gram staining)

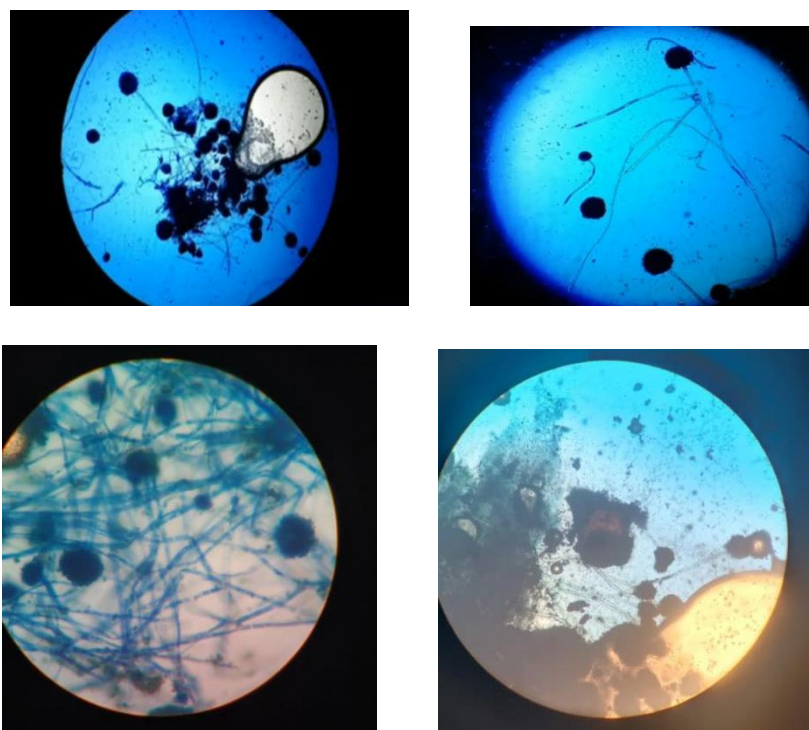


Fig 5.3: Staining of fungi (lactophenol cotton blue)

Table 5.1: Bacterial gram staining results

Bacterial colony	Cellular structure	Shape
Colony 1	Gram negative	Bacillus
Colony 2	Gram negative	Bacillus
Colony 3	Gram negative	Bacillus

5.3 GC-MS ANALYSIS:

The analysis of samples through GC-MS was performed, revealing a significant increase in the production of secondary metabolites following the introduction of biotic elicitors. Secondary metabolites serve as a defense mechanism in plants, synthesized in response to external stressors, and are crucial components utilized in pharmaceutical industries for medicinal purposes. The application of biotic elicitors, acting as sudden stressors, has effectively stimulated the plants to produce secondary metabolites in substantial quantities. The GC-MS analysis clearly demonstrates that the concentration of secondary metabolites in the treated plants surpasses that of the

control plants. This outcome validates the success of the project, as the elicitors have successfully augmented the production of secondary metabolites. This finding holds promise for the pharmaceutical industry, indicating a potential avenue for enhancing the yield of valuable medicinal compounds through targeted elicitation strategies.

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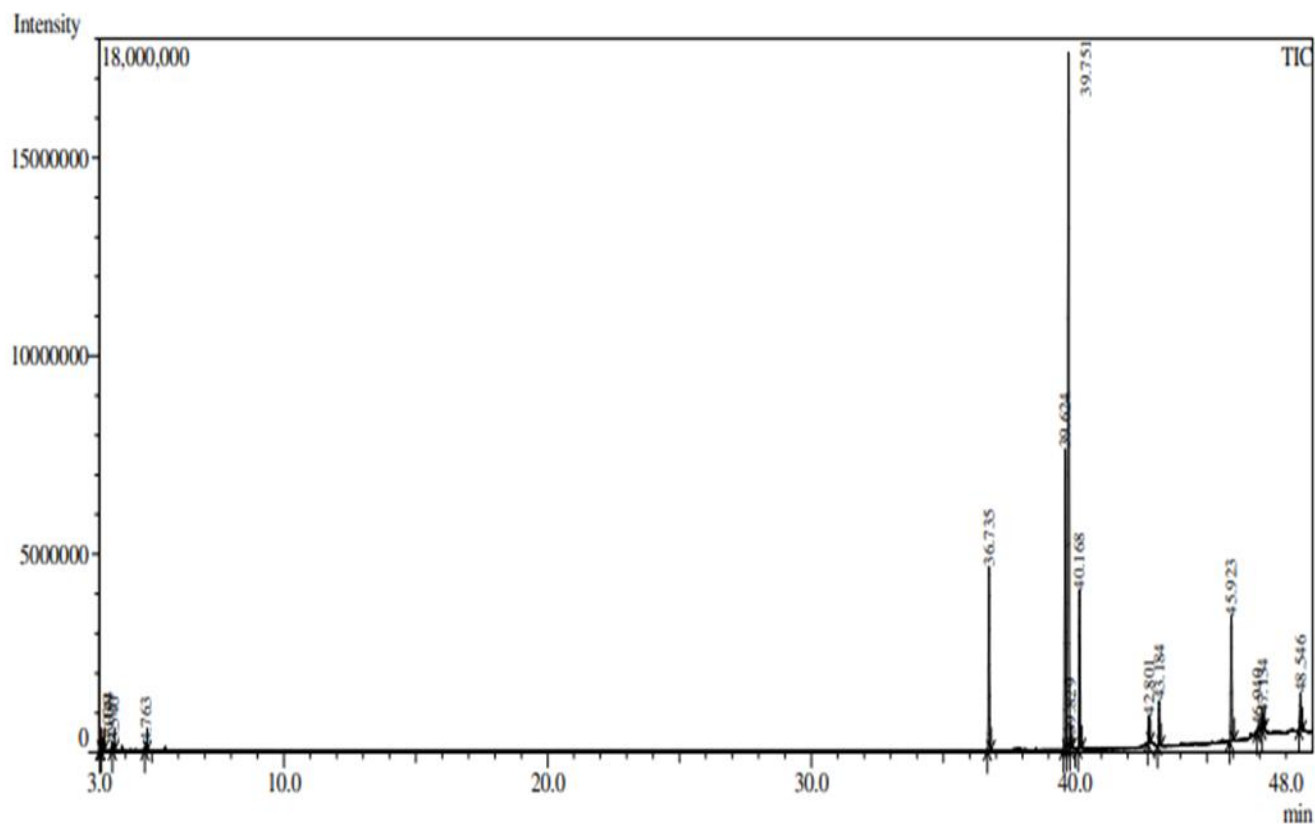


Fig: 5.4: GC-MS Analysis of untreated control plant, Ranakalli (*K. pinnata*).

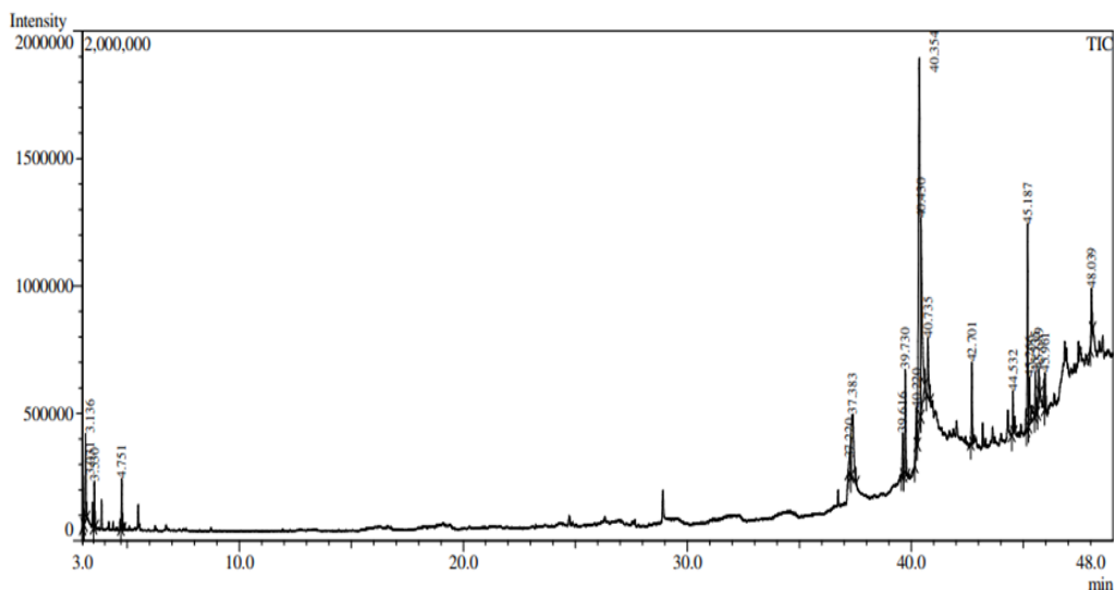


Fig: 5.5: GC-MS Analysis of treated plant Ranakaalli (*K.pinnata*)

Table 5.2: Peak report control plant Ranakalli (*K.pinnata*)

Peak Report TIC				
Peak#	R.Time	Area	Area%	Name
1	3.039	384812	0.35	BENZENE, ETHYL-
2	3.144	663104	0.61	BENZENE, 1,2-DIMETHYL-
3	3.540	343633	0.31	Pentane, 3-ethyl-2,4-dimethyl-
4	4.763	401376	0.37	3-HEXEN-2-ONE
5	36.735	11198717	10.24	HEXADECANOIC ACID, METHYL ESTER
6	39.624	18263998	16.70	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
7	39.751	51210901	46.82	9-Octadecenoic acid, methyl ester, (E)-
8	39.829	732750	0.67	9-Octadecenoic acid, methyl ester, (E)-
9	40.168	9814941	8.97	Methyl stearate
10	42.801	1552879	1.42	9-OCTADECENOIC ACID (Z)-, METHYL ESTER
11	43.184	2537200	2.32	EICOSANOIC ACID, METHYL ESTER
12	45.923	7840693	7.17	DOCOSANOIC ACID, METHYL ESTER
13	46.940	856363	0.78	2-PHENYL-FURO[B]BENZOPYRAN-4(4H)-ONE
14	47.134	1029752	0.94	2-[5-(2-Methyl-benzooxazol-7-yl)-1H-pyrazol-4-yl]ethanol
15	48.546	2546876	2.33	TETRACOSANOIC ACID, METHYL ESTER
		109377995	100.00	

Table 5.3: Peak report treated Ranakalli (K. pinnata)

Peak#	R.Time	Area	Area%	Name
1	3.031	339994	1.39	BENZENE, ETHYL-
2	3.136	736552	3.02	BENZENE, 1,2-DIMETHYL-
3	3.530	349915	1.43	Pentane, 3-ethyl-2,4-dimethyl-
4	4.751	437499	1.79	3-Hexen-2-one
5	37.220	562680	2.31	BUTANEDIOIC ACID, 2,3-DIETHYL-, DIMETHYL ESTER
6	37.383	1945618	7.98	n-Hexadecanoic acid
7	39.616	480954	1.97	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
8	39.730	1096321	4.49	9-Octadecenoic acid, methyl ester, (E)-
9	40.220	794054	3.26	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
10	40.354	8147416	33.40	Oleic Acid
11	40.430	3125414	12.81	CYCLOHEXANE, [6-CYCLOPENTYL-3-oxo-1-octadecylidene]-
12	40.735	927127	3.80	Octadecanoic acid
13	42.701	829647	3.40	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-epoxy-3-oxo-
14	44.532	379755	1.56	Oleoyl chloride
15	45.187	2054659	8.42	9-Octadecenoic acid, 1,2,3-propanetriyl ester
16	45.266	615728	2.52	9-Octadecenoic acid, 1,2,3-propanetriyl ester
17	45.535	390913	1.60	Glycidol stearate
18	45.689	332241	1.36	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)-2-oxo-
19	45.961	318858	1.31	Pentadecanal-
20	48.039	527012	2.16	Oleoyl chloride
		24392357	100.00	

K. pinnata sample was subjected to GC-MS analysis. The provided chromatography data comprises two sets of peaks, control and test samples. In the control sample, notable compounds include Benzene, Ethyl- and Benzene, 1,2-Dimethyl-, indicating the presence of aromatic hydrocarbons. Additionally, fatty acid methyl esters such as Hexadecenoic Acid, Methyl Ester and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester are observed, suggesting the presence of lipid components. Other compounds like Methyl stearate and Docosanoic acid, methyl ester are also identified, potentially originating from fatty acid derivatives. Comparatively, the test sample reveals a broader range of compounds. Alongside aromatic hydrocarbons and fatty acid methyl esters, it includes diverse compounds such as Butanedioic Acid, 2,3-diethyl-, dimethyl ester and Cyclohexane derivatives, indicating a more complex mixture. Notably, Oleic Acid and its derivatives are prevalent, suggesting a significant presence of unsaturated fatty

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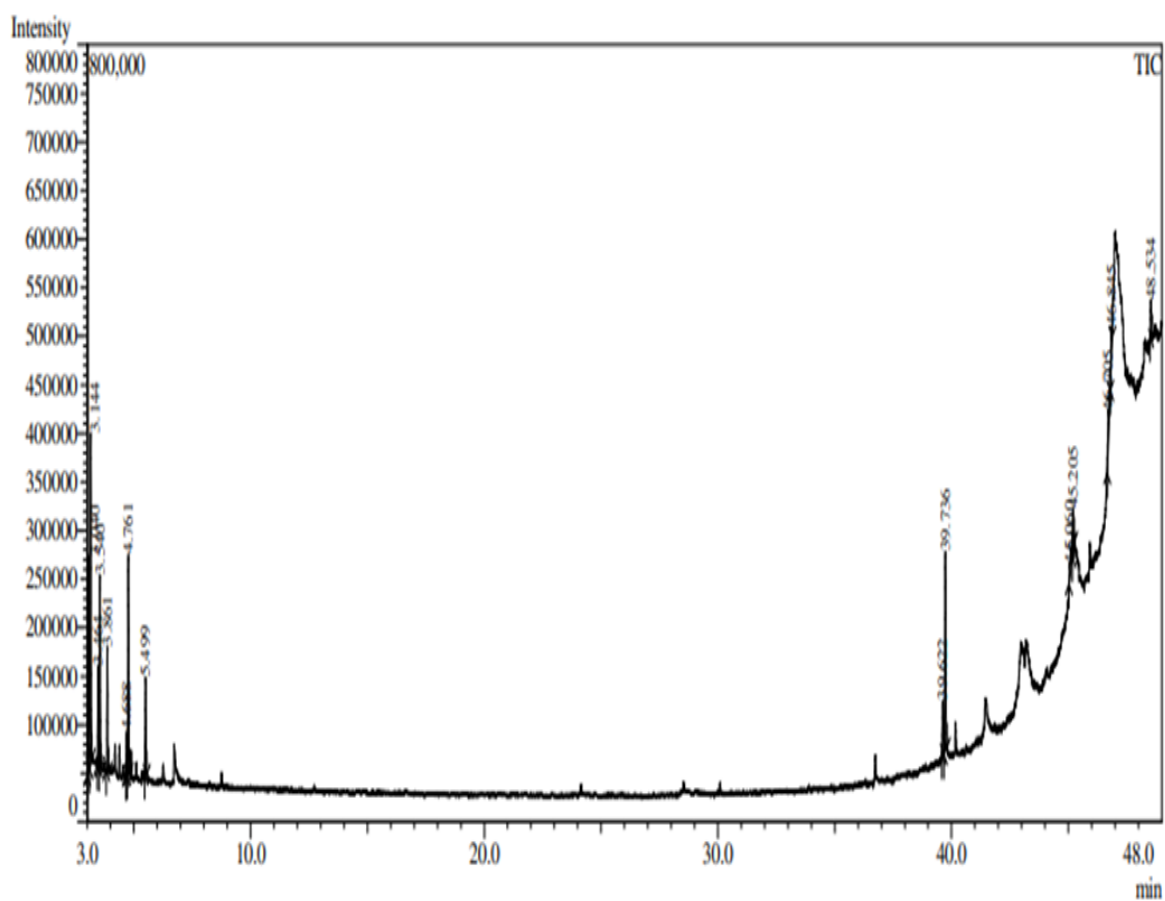


Fig: 5.6 GC-MS Analysis of untreated control insulin plant

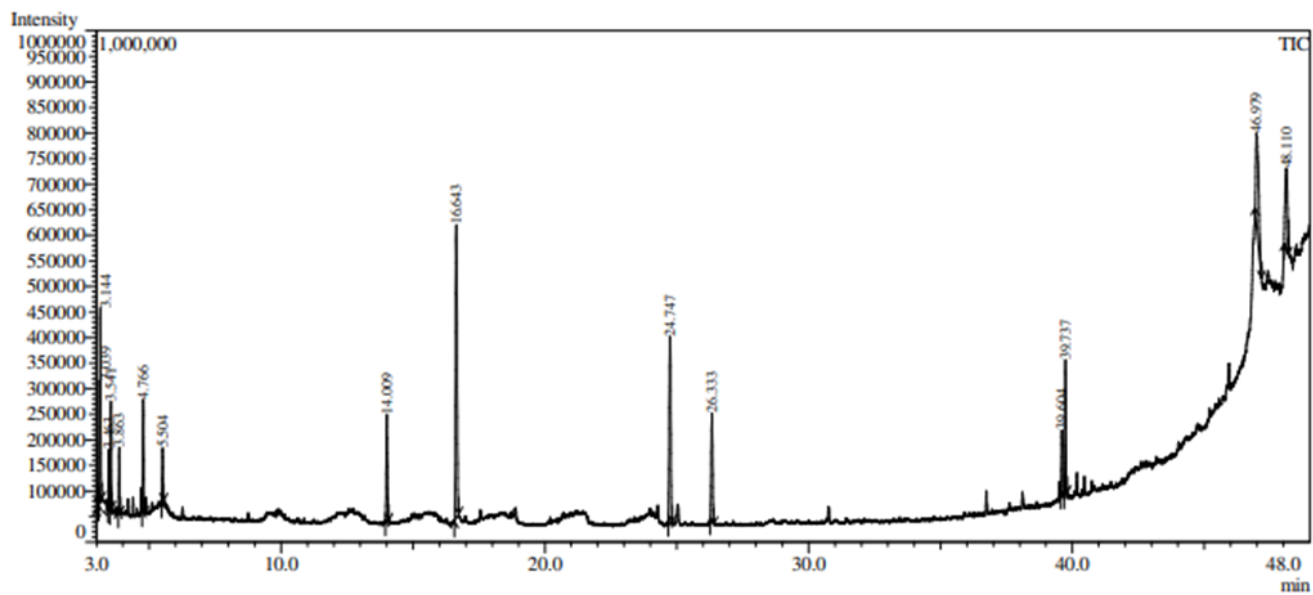


Fig 5.7: GC-MS Analysis of biotic elicitor treated insulin plant

Table 5.4: GC-MS Peak report of treated plant insulin

Peak Report TIC				
Peak#	R.Time	Area	Area%	Name
1	3.040	445647	10.98	BENZENE, ETHYL-
2	3.144	719200	17.72	BENZENE, 1,2-DIMETHYL-
3	3.464	200385	4.94	BENZENE, 1,2-DIMETHYL-
4	3.540	406592	10.02	Pentane, 3-ethyl-2,4-dimethyl-
5	3.861	258293	6.36	2-Aminoethanol, N,O-diacetyl-
6	4.688	93087	2.29	Benzene, 1-ethyl-2-methyl-
7	4.761	513673	12.65	3-HEXEN-2-ONE
8	5.499	228312	5.62	BENZENE, 1-ETHYL-3-METHYL-
9	39.622	127843	3.15	9,12-Octadecadienoic acid (Z,Z)-, methyl est
10	39.736	528569	13.02	9-OCTADECENOIC ACID (Z)-, METHYL
11	45.060	123512	3.04	OLEAN-12-EN-28-OIC ACID, 2.BETA.,3.B
12	45.205	106639	2.63	LUP-20(29)-ENE-3,28-DIOL, (3.BETA.)-
13	46.705	111624	2.75	3-(4-P-TOLYL-THIAZOL-2-YL)-4,5,6,7-TE
14	46.845	92985	2.29	SILICONE OIL
15	48.534	103321	2.55	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,1
		4059682	100.00	

Table 5.5: Peak report of untreated control plant insulin

Peak Report TIC				
Peak#	R.Time	Area	Area%	Name
1	3.039	480233	4.31	BENZENE, ETHYL-
2	3.144	812091	7.30	BENZENE, 1,2-DIMETHYL-
3	3.463	199256	1.79	BENZENE, 1,2-DIMETHYL-
4	3.541	400054	3.59	Pentane, 3-ethyl-2,4-dimethyl-
5	3.863	246852	2.22	3-NONANONE
6	4.766	491758	4.42	3-Hexen-2-one
7	5.504	229437	2.06	Benzene, 1-ethyl-2-methyl-
8	14.009	687338	6.18	ETHANONE, 1-(1,4-DIMETHYL-3-CYCL
9	16.643	2107130	18.93	PHENOL, 5-METHYL-2-(1-METHYLETH
10	24.747	1353188	12.16	NAPHTHALENE, DECAHYDRO-4A-MET
11	26.333	769801	6.92	Androstan-3-ol, 9-methyl-, acetate, (3.beta.,5
12	39.604	413122	3.71	1,2,5,5,8a-Pentamethyl-1,2,3,5,6,7,8,8a-octa
13	39.737	604456	5.43	9-OCTADECENOIC ACID (Z)-, METHYL
14	46.979	1324207	11.90	ERGOST-5-EN-3-OL, (3.BETA.,24R)-
15	48.110	1011138	9.08	STIGMASTA-5,20(22)-DIEN-3-OL
		11130061	100.00	

Comparing the two sets of chromatography data with the first set as the control sample and the second set as the test sample for *Chamaecostus cuspidatus*, we observe several differences in compound composition. In the control sample, significant compounds include Benzene, Ethyl- and Benzene, 1,2-Dimethyl-, indicating the presence of aromatic hydrocarbons. Pentane, 3-ethyl-2,4-dimethyl- is also notable, along with various fatty acid methyl esters like 9,12-Octadecadienoic acid (Z,Z)-, methyl ester and 9-OCTADECENOIC ACID (Z)-, METHYL ESTER, suggesting lipid components. Additionally, minor compounds such as OLEAN-12-EN-28-OIC ACID, 2.BETA.,3.BETA.,23-TRIHYDROXY-, METHYL ESTER and LUP-20(29)-ENE-3,28-DIOL, (3.BETA.)- are present. In contrast, the test sample exhibits differences in compound presence and abundance. While Benzene derivatives and fatty acid methyl esters are still detected, there are notable variations. Notable additions include PHENOL, 5-METHYL-2-(1-METHYLETHYL and ERGOST-5-EN-3-OL, (3.BETA. 24R)-. Furthermore, compounds like 3-NONANONE and ETHANONE, 1-(1, 4-DIMETHYL-3-CYCLOHEXEN-1-YL) - are found exclusively in the test sample, suggesting potential changes in chemical composition or experimental conditions. Overall, the comparison indicates differences in compound composition between the control and test samples of *C. cuspidatus*, with notable variations in aromatic hydrocarbons, fatty acid methyl esters, and additional compounds, implying potential changes in the chemical profile under different conditions. Gizawy *et al.*, (2022) also characterized *S. Costus* with same components under GC_MS analysis .

Exploring biotic elicitors to enhance secondary metabolite production in plants like insulin and ranakalli shows promise. These elicitors, derived from microbes, activate plant defense mechanisms, boosting the synthesis of crucial compounds for plant protection. Plants like insulin and ranakalli benefit from these metabolites, which include alkaloids, flavonoids, terpenoids, and phenolics, known for their diverse health benefits. For instance, *Kalanchoe pinnata* shows increased levels of bufadienolides, known for their anti-inflammatory properties. Similarly, *Chamaecostus cuspidatus* may see elevated levels of flavonoids like kaempferol and quercetin, with antioxidant effects. Understanding metabolic pathways is key for optimizing elicitor use. Stress influences plant chemistry, and deliberately inducing stress responses can alter phytochemical

profiles. Biotic elicitors stimulate specific compound production, aiding plant adaptation to stress. Leveraging them to enhance secondary metabolite production holds promise for improving medicinal properties and discovering new bioactive compounds.

CHAPTER 6

6. SUMMARY AND CONCLUSION:

In conclusion, the investigation into the impact of biotic elicitors on the production of secondary metabolites in *Kalanchoe pinnata* (Ranakalli) and *Chamaecostus cuspidatus* (Insulin) offers promising avenues for enhancing the medicinal properties of these plants. Utilizing biotic elicitors, such as bacterial and fungal extracts, has demonstrated notable increases in secondary metabolite production in both species, particularly flavonoids, alkaloids, and phenolic compounds, which possess varied medicinal benefits including antimicrobial, antitoxic, and anticancer properties. The effectiveness of biotic elicitation in stimulating secondary metabolite production is influenced by various factors, including the specific plant species involved, the conditions of the tissue culture environment, the desired target metabolites, and the characteristics of the elicitor used. For instance, fungal elicitors have been observed to induce higher and more significant increases in metabolite production in certain plant species compared to bacterial elicitation. This variation underscores the importance of understanding the complex interactions between elicitors and plant biochemical pathways to optimize the elicitation process for maximum yield. Understanding the precise mechanisms through which biotic elicitors stimulate secondary metabolite production is imperative for optimizing yields and evaluating the suitability of enhanced extracts for medicinal and nutritional applications. Despite ongoing research and advancements in plant tissue culture techniques, widespread large-scale production of secondary metabolites remains limited. However, the utilization of elicitors in plant tissue culture systems holds significant potential for enhancing secondary metabolite production and meeting the growing demand for natural medicinal compounds in healthcare and industry. The efficacy of elicitation depends on various factors, including plant species, culture conditions, target metabolites, and the nature of the elicitor employed. Further exploration and refinement of elicitation strategies are essential for realizing the full potential of biotic elicitors in maximizing the medicinal value of *K. pinnata* and *C. cuspidatus*.

REFERENCES:

A medicinal potency of anti diabetic insulin plant (Costus igneus). (2023). *International Journal of Biology, Pharmacy and Allied Sciences*, 12(10). <https://doi.org/10.31032/ijbpas/2023/12.10.7462>

BENNETT, R. N., & WALLSGROVE, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytologist*, 127(4), 617-633. <https://doi.org/10.1111/j.1469-8137.1994.tb02968.x>

Bhaskar, R., Xavier, L. S., Udayakumaran, G., Kumar, D. S., Venkatesh, R., & Nagella, P. (2021). Biotic elicitors: A boon for the in-vitro production of plant secondary metabolites. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 149(1-2), 7-24. <https://doi.org/10.1007/s11240-021-02131-1>

Biswas, K. (2011). Literature review on pharmacological potentials of kalanchoe pinnata (Crassulaceae). *African Journal of Pharmacy and Pharmacology*, 5(10), 1258-1262. <https://doi.org/10.5897/ajpp11.273>

Coste, A., Vlase, L., Halmagyi, A., Deliu, C., & Coldea, G. (2011). Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of hypericum hirsutum and hypericum maculatum. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 106(2), 279-288. <https://doi.org/10.1007/s11240-011-9919-5>

Dal Bosco Ducatti, R. (2022). Plant elicitation: The generation of misleading and biased information. *Journal of Plant Growth Regulation*, 42(6), 3785-3788. <https://doi.org/10.1007/s00344-022-10838-4>

Erb, M., & Kliebenstein, D. J. (2020). Plant secondary metabolites as defenses, regulators and primary metabolites- The blurred functional trichotomy. <https://doi.org/10.31219/osf.io/6ba7r>

Georgiev, S., & Roos, W. (2006). Elicitation of plants. *Biotechnology & Biotechnological Equipment*, 20(2), 72-83. <https://doi.org/10.1080/13102818.2006.10817345>

Hatcher, C. R., Ryves, D. B., & Millett, J. (2019). The function of secondary metabolites in plant carnivory. *Annals of Botany*, 125(3), 399-411. <https://doi.org/10.1093/aob/mcz191>

Jaafar, H. Z. (2015). Whole plant elicitation: A new approach toward enhanced production of plant secondary metabolites harvest index. *Medicinal & Aromatic Plants*, 04(04). <https://doi.org/10.4172/2167-0412.c1.002>

Kalanchoe pinnata (Lam.) Pers. (n.d.). *SpringerReference*. https://doi.org/10.1007/springerreference_68789

Kuzel, S., Vydra, J., Triska, J., Vrchotova, N., Hruby, M., & Cigler, P. (2009). Elicitation of pharmacologically active substances in an intact medical plant. *Journal of Agricultural and Food Chemistry*, 57(17), 7907-7911. <https://doi.org/10.1021/jf9011246>

Li, Y., Kong, D., Fu, Y., Sussman, M. R., & Wu, H. (2020). The effect of developmental and environmental factors on secondary metabolites in medicinal plants. *Plant Physiology and Biochemistry*, 148, 80-89. <https://doi.org/10.1016/j.plaphy.2020.01.006>

Pattewar, S. V. (2012). Kalanchoe pinnata : Phytochemical and pharmacological profile. *International Journal of Phytopharmacy*, 2(1). <https://doi.org/10.7439/ijpp.v2i1.223>

Rajsekhar, P., Bharani, R., Ramachandran, M., Angel, K., & Rajsekhar, S. (2016). The and ldquo;Wonder plant and rdquo; Kalanchoe pinnata (Linn.) Pers.: A review. *Journal of Applied Pharmaceutical Science*, 151-158. <https://doi.org/10.7324/japs.2016.60326>

Sharangi, A. (2016). Chapter 4 secondary metabolites in spices and medicinal plants: An overview. *Plant Secondary Metabolites, 3 Volume Set*, 143-168. <https://doi.org/10.1201/9781315207506-5>

Shitan, N. (2016). Secondary metabolites in plants: Transport and self-tolerance mechanisms. *Bioscience, Biotechnology, and Biochemistry*, 80(7), 1283-1293. <https://doi.org/10.1080/09168451.2016.1151344>

Sonia, T. A., & Sharma, C. P. (2014). Diabetes mellitus – an overview. *Oral Delivery of Insulin*, 1-57. <https://doi.org/10.1533/9781908818683.1>

Teoh, E. S. (2016). Sources of medicinal orchids and conservation. *Medicinal Orchids of Asia*, 691-727. https://doi.org/10.1007/978-3-319-24274-3_23

Vasconsuelo, A., & Boland, R. (2007). Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Science*, 172(5), 861-875. <https://doi.org/10.1016/j.plantsci.2007.01.006>

Yoshikawa, M., & Takeuchi, Y. (1991). Molecular aspects of elicitation of host defense reactions. *Molecular Strategies of Pathogens and Host Plants*, 165-176. https://doi.org/10.1007/978-1-4612-3084-7_14

Zehra, A., Raytekar, N. A., Meena, M., & Swapnil, P. (2021). Efficiency of microbial bio-agents as elicitors in plant defense mechanism under biotic stress: A review. *Current Research in Microbial Sciences*, 2, 100054. <https://doi.org/10.1016/j.crmicr.2021.100054>

