A Comparative, Pharmacognostic, Phytochemical and Pharmacological Analysis of *Abhava Pratinidhi* (substitutes) Plant Drugs: *Pistacia integerrima* (*Karkatshringi*) and *Terminalia chebula* (*Haritaki*)

A THESIS SUBMITTED TO

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M.Sc. LIFE SCIENCES (AYURVEDA BIOLOGY)

BY

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UNDER THE GUIDANCE OF

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DECLARATION BY THE CANDIDATE

I declare that this thesis, **"A Comparative, Pharmacognostic, Phytochemical and Pharmacological Analysis of *Abhava Pratinidhi* (substitutes) Plant Drugs: *Pistacia integerrima* (*Karkatshringi*) and *Terminalia chebula* (*Haritaki*)",** submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr Subrahmanya Kumar. K and co-supervision, Dr Noorunnisa Begum S., I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and Material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarized.

Place: Bangalore

Signature of the Candidate

Date: 15th June 2023

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

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CERTIFICATE FROM THESIS SUPERVISOR/S

This is to certify that the work incorporated in this thesis. **"A Comparative, Pharmacognostic, Phytochemical and Pharmacological Analysis of *Abhava Pratinidhi* (substitutes) Plant Drugs: *Pistacia integerrima* (*Karkatshringi*) and *Terminalia chebula* (*Haritaki*)"** submitted by Ms. Hrishita Srinivas Gorityala was carried out under our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and Material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

Dr Subrahmanya Kumar, Associate Professor,	Guide	Signature
Dr Noorunnisa Begum S Assistant professor,	Co-guide	Signature

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr Subrahmanya Kumar. K and Dr Noorunnisa Begum for their dedicated guidance and invaluable support for my project work. I would also like to thank TDU for its constant support and encouragement to undertake such thesis work, which helped me learn a lot and achieve a new perspective on this subject. I am grateful for their valued guidance during the entire duration of the project. I thank Dr. Megha, Dr. CN.Vishnuprasad a, Dr.Sanket Sharma, Dr. Arun Bhanu,Mr. Patturaj. R, Mr. Ashutosh.S, Dr. Dhatchanamoorthy, Dr Ganesh Babu, Dr. Sridevi.G, Ms.Saniya, Ms. Priyanka G.Pinto, Ms.Chaya Bharati, Mr. Debashis Rout and Ms. Pallavi P from TDU and Mr. Ranjith, A.P., Research Associate Atree, Dr. Laurence Alfred Mound for helping me succeed in this work. Towards the end, I would like to thank my family, friends and loved ones for supporting me during my studies.

SUMMARY

This thesis is a systematic study on the development of galls in *Terminalia chebula* galls and to check the similarities between the galls of *Pistacia integerrima* and *Terminalia chebula* through a pharmacognosy, phytochemistry, and pharmacological activity study. This study intends to reduce the misuse of medicinal plants and reduce the human-induced pressure on endangered native species. Highlights of this thesis study are Day-to-day Photo-Documentation of the infestation and growth of leaf galls in *Terminalia chebula*. The study found that the galls reach full maturity within 65-70 days, and the presence of insect thrips inside the galls are *Liothrips*. Pharmacognosy microscopic study revealed that *Pistacia integerrima* and *Terminalia chebula* galls had different cell arrangements and histochemical characters. Qualitative phytochemistry studies were done using HPTLC, which revealed that *Pistacia integerrima* galls, *Terminalia chebula* galls and *Terminalia chebula* fruits have presences of similar alkaloids, flavonoids, terpenes and glycosides with references to Rf values. *Drosophila melanogaster* -based Pharmacology studies were done to check anti-fertility and anti-inflammation activities, and it revealed that the *Kashaya* of galls of *Terminalia chebula* significantly reduced fecundity compared to the control and flies fed on Kashaya of *Pistacia integerrima* galls and *Terminalia chebula* fruits. However, the gene expression study for inflammation showed significant anti-inflammatory activity.

Further studies demonstrating similar properties and activities may help establish whether Terminalia chebula could be a viable substitute for Pistacia integerrima.

PERSONAL REFLECTION

I learned a great deal in my project, both in terms of technical and social skills. From a technical standpoint, I gained valuable experience in as follows-

Dealing with literature surveys, scientific writing, understating of Ayurveda and Sanskrit literature, data maintenance presenting scientific data and calculating data using different software, including R language, has helped me improve my software and data handling knowledge. Working and standardizing protocols, working with the *D.melanogaster* animal model, Working in the molecular biology lab and phytochemistry lab gave me exposure to the use of PCR, and *RT-qPCR* and HPTLC kinds of high-tech machines which gave me experience working with machines and gene expression studies. The photo documentation and critical observation on the development of a plant part helped me learn about photography and editing. Using a microscope in pharmacognosy gave me an idea about software use and the plant section. In terms of social skills, I gained insights into how to work in a team effectively. Working under the supervision of multiple seniors allowed me to learn about communication and negotiation. Overall, my project was a great opportunity to improve my skills and learn valuable lessons on working in a lab and field. I am confident that the skills that I have acquired will be very useful in the future.

ABBREVIATIONS

P.int	*Pistacia integerrima*
T.che	*Terminalia chebula*
TLRs	Toll-like receptors
NOD	Nucleotide-binding oligomerization-domain protein
ECM	The extracellular matrix
PAMPs	Pathogen-associated molecular pattern molecules
Imd	Immuno Deficiency
TNFRs	Tumour necrosis factor receptors
Rp49	Ribosomal Protein 49
NF-κB	Nuclear factor kappa B
AMP	Adenosine monophosphate
FRLHT	Foundation for Revitalisation of Local Health Traditions
HPTLC	High-Performance Thin Layer Chromatography
SDS	sodium dodecyl sulfate
DEPC	Diethyl Pyrocarbonate
rpm	Revolutions per minute
EtBr	Ethidium Bromide
TAE	Tris-acetate-EDTA
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
qRt-PCR	Real-Time Quantitative PCR
X	Xylem
Ph	Pholem
VB	Vascular bundle
SXV	Spiral type Xylem
sXV	Scalariform type xylem
S.D.	Secretory duct
Cr	Crystal
Tan	tanniferous
Pa	Paranchayama
Pro	Protein
Sg	Starch grain
Lig	Lignin

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1. Introduction

1.1 Introduction to Ayurveda

- ***Ayur-veda*** is made up of two Sanskrit words: ***Ayur*** means life or longevity, and ***Veda*** means science or knowledge
- Ayurveda is a system of medicine that was originated in India several thousand years ago.
- *हिताहितं सुखं दुःखमायुस्तस्य हिताहितम्|*

  मानं च तच्च यत्रोक्तमायुर्वेदः स उच्यते|| (Ca.Su.1/41)

  Science deals with or describes the favourable or unfavourable happy or unhappy measures for a life span. What is conductive or non-conductive for such a life is known as Ayurveda. (Maharishi Charaka & Dr P.V. Tewari, n.d.)

- *समदोषः समाग्निश्च समधातुमलक्रियः* |

  प्रसन्नात्मेन्द्रियमनाः स्वस्थ इत्यभिधीयते|| (Su. Su.15/41)

  An organism is called perfectly healthy when the Three *doshas (Vata, Pitta, Kapha)* are balanced*(sama)*, Digestive Fire *(Agni)* (digestion and metabolism) is balanced, and all the body tissues *(Dhatus)* (physical body), all excretory functions *(mala kriya)* (the physiological functions of urination and defecation) are in perfect order with an equilibrium state of mind *(Mana)*, senses*(Indriyas)* and spirit *(Atma)*. (Susruta & P.V Sharma, n.d).

- *प्रयोजनं चास्य स्वस्थस्य स्वास्थ्यरक्षणमातुरस्य विकारप्रशमनं च*|| (Ca.Su.30/26)

  The purpose of Ayurveda is to maintain the health of the healthy individual and to relieve the disorders of the sick.(Charak & P.V.Shrama, n.d.).

- The World Health Organisation reported that 80% of the population in this field relies more heavily on conventional medicine for health care. Moreover, with approximately 45,000 plant species, the Indian subcontinent is regarded as one of the world's largest biodiversity centres. Approximately 15,000 medicinal plants have been identified in India, and local populations use 7,000–7,500 plant species to treat various illnesses. One or more herbs (polyherbal) are utilized for medical purposes in Ayurveda(Parasuraman et al., 2014; *WHO Establishes the Global Centre for Traditional Medicine in India*, 2022).

- The Vedas and Samhitas, as well as other ancient texts, describe plants as some of the most potent medicinal agents. The *rasapanchaka* are the five components of dravya called guna a dravya which are as follows*: rasa, guna, veerya, vipaka, prabhava, and karma*(Dr. Sonal Bhola & Dr. M. Paramkussha Rao, 2016).

- ***Rasa*** (the flavour or sensation that herbs give off when they come into touch with the tongue) Six flavours (*Madhura-Sweet, Amla-Sour, Lavana-Salty, Katu-Pungent, Tikta-Bitter, and Kashaya-Astringent)* are present, and each is composed of two of the five elements. Each of the tastes affects *dosha.*
- ***Guna*** (the substance's quality, use, and characteristic) According to Ayurveda, a guna is a substance naturally contained in *dravya* (a drug), functions as a causal agent, and lacks both property and action. The three main classifications are *Vaisheshika Guna*, *Samanya Gunas, and Atma Gunas*.
- ***Veerya*** (the energy that a herb releases when consumed) It might be *sheeta* (cooling) or *ushna* (heating). The former is found in sweet, astringent, and bitter herbs that cool the body and reduce inflammation; the latter is found in sour, salty, and pungent spices, which enhance circulation, aid digestion, and encourage sweating.
- ***Vipaka*** (Post-digestive effect) There are three varieties of *vipaka:* sweet (*Madhura*), sour (*amla*), and pungent (*katu*), each of which affects the dosha differently.
- ***Prabhava*** (special and unique power of a herb that has variable action) These plants are not comparable to other plants that exhibit the same *rasa, veerya, or vipaka*.
- ***Karma*** (therapeutic action), *Deepana* (stimulant), *Pachana* (digestive*), Shodhana* (purification), *Anuloman* (carminative), and *Virechana* (purgative) are the different categories for these(Dr. Sonal Bhola & Dr. M. Paramkussha Rao, 2016).

1.2 *Abhava Pratinidhi Dravya*

The ancient science of life known as Ayurveda states that four fundamental pillars are essential to treat a disease: the doctor (*Bhishak*), the Drug (*Dravya*), the paramedics (*Upasthatha*), and the patient (*Rogi*) (Shukla VD, 2006.) Among these, the following physician, medicine, or Drug is considered to be the most critical pillar.

Due to the rise in popularity of herbal medicine in recent years, an increase in its use has been seen globally. Drug shortages are frequently reported due to rising drug demand, concurrent industrialization, and civilizational growth. Formulation (*Kalpa*), a mixture of several herbal medicines, is thought to be the foundation of Ayurvedic treatment.

A perfect medication should be of high quality (*Sampannata*), capable of curing the disease (*Yogyata*), abundant in nature (*Bahuta*), and able to be processed into a variety of dosage forms (*Anekavidha Kalpana*). (Tripathi B, 2014.) Ancient seers were aware of the need for substitutes whenever there was a shortage of medicines. As a result, the concept of "*Abhava Pratinidhi Dravya*" was emphasized in commentaries on the *Sharangdhara Samhita*, including those by *Adhmalla* (14th century), *Kashirama Vaidya* (18th century), *Bhavaprakasha* (16th century), *Yogratnakara* (18th century), and

Bhaishajya Ratnavali (16th century). In *Bhavaprakasha*, under the heading "*Anekartha naam Varga,"* the term "*Abhava Dravya*" is explained. (Sastri BS,2015.; Shastri R,2016.)

Under the heading "*Abhava varga," Yogratnakara* wrote a treatise, as well as by Govinddas Sen in *Bhaishajya Ratnavali* under the title "*Abhava Prakarana*" before the book's treatment section. Following Govinddas Sen, alternatives of *Pratinidhi Dravya* should have a comparable *rasa* (taste), *guna* (property), *veerya* (potency), and *vipaka (*the effect on digestion) to the *Pratinidhi Dravya*(Shastri R, n.d.) (Venkatasubramanian et. el, 2015)

- *मुस्ता चातिविषाभावे देया तत्र शिवा मता । अभावे च हरीतक्या मता कर्कटशृङ्गिका* (Yogaratnakara.Pu. 26)

Yogratnakar mentions that if *T.chebula* is unvailable, *P.integerrima* can be used as a substitute(Dr. Madham Shetty Suresh Babu, n.d.).

1.2 *Pistacia integerrima* J.L.Stewart
(Anacardiaceae – Dicotyledonae)

- **Botanical description**- Pistacia chinensis ssp. *integerrima* is a multibranched, single-stemmed deciduous tree of P.*integerrima* which belongs to Amrakula. This dioecious tree is with well-developed vertical resin canals. Leaves are alternate, deciduous, pinnately compound but sometimes trifoliolate and leathery. Inflorescences are determinate and terminal. Flowers are unisexual, radial, and small, with well-developed carpel lodes. Fruits are purple to blue, rounded with a 4-6 mm diameter. A typical type of worm (Dasia aedificator) makes horn-shaped galls on the branches and leaves. These galls are pale greenish brown elongated horn-shaped, twisted.
- **Habitat**:- it is a dioecious shedding tree widely distributed in Nepal, China, Afghanistan, Pakistan, Armenia, Northwest and West Himalayas.
- **Vernacular Name**:- (Sharma, 1981) Karkarshringi, Shringi, Kuliravishanika, Ajay Shringi, Chakara, Karkatkhya, Vakra, Visanika. It is commonly known as chakra, shikari in Sanskrit, kakra in Hindi, Crabs claw in English, kaakadashringi in Kannada, and kakarsingi in Telugu (Grover, 2021).
- **Phytochemicals**:- P.*integerrima* contains various important phytoconstituents for commercial value and therapeutic potential. The pistacia plant contains two isomeric triterpene acids (pistacienoic acids A and B), tannins, triterpene alcohol (zircalloy), beta-sitosterol, tetracyclic triterpenes, and pistacigerrimones A.B, C (galls). discovered three new phytoconstituents from galls of a plant, in addition to the previously known compound b-sitosterol. The compounds were n-decan-30 -ol-y-eicosanoate, n-octadecan9,11-diol-7-one and 3-oxo-9b-lanost1,20(22)-dien-26-oic acid6(Uddin et al., 2011).

- **Pharmacological activities:-** Antioxidant- Eshwarappa et al. studied the antioxidant activity of aqueous and ethanol extract of leaf galls using diphenylpicrylhydrazyl (DPPH), hydroxyl scavenging and ferric reducing power (FRAP) assays. The study revealed that high total phenolic and flavonoid content in the ethanol extract was associated with potent antioxidant activity compared to the aqueous extract[vol10]. Rauf et al. (2018) demonstrated the promising anti-inflammatory effects of Pistacia *integerrima* in a carrageenan-induced paw oedema model in mice, indicating its potential to be used as an anti-inflammatory agent. It was revealed from the study that the compounds isolated from the chloroform fraction of the galls, i.e. flavonoids (1-4), exhibited potent anti-inflammatory actions during various assessment times (1-5h). In a novel in-vivo study, researchers from Rauf et al. sought to evaluate the gastroprotective properties of pistagremic acid derived from galls of Pistacia *integerrima* in mice models. The pretreatment of the models with the extract at the dosage of 500 mg/kg p.o. In the charcoal meal G.I. transit test, the compound significantly reduced the GIT motility and increased the intestinal transit time. These results were comparable to atropine(Uddin et al., 2011).
- **Folk view**:- Galls are also used in traditional medicine in Pakistan in various ways unique to the region. These uses include a remedy for snake bites, eye infections, skin disorders, and a laxative and an antacid. Additionally, galls have been used to treat liver disorders, headaches, and even fever. Galls are also said to have anti-inflammatory and anti-bacterial properties and can be used to reduce inflammation and treat skin conditions such as eczema—and scorpion stings. Additionally, the bark of this plant is said to help treat jaundice and hepatitis.[vol10] The tribal communities of Lesser Himalayas-Pakistan, orally use leaf gall powder to treat cough and asthma [vol10]. In North India, galls treat inflammatory conditions, diabetes, liver infection, pain, and fever. The Gall and leaves of the plant are used to treat cough, asthma, common fever, jaundice, diarrhoea, and snake bites in many regions. The local vaidyas (Hakims) used galls for the treatment of pulmonary infections, vomiting, and diarrhoea [vol10](Grover, 2021)

❖ **Ayurveda**

- *Rasa* (taste):-*kashaya* (Astringent*), Tikta* (bitter)
- G*una* (qualities):- *laghu* (light to digest), *Ruksha* (dry)
- *Veerya* :- hot potency
- *Vipaka* :- *katu* (bitter)
- Pharmacological action:-Astringent, tonic, expectorant, stimulant.
- Therapeutic effects:- *kapha vata hara* (balance kapha and vata), *kshayahara*(used in chronic respiratory disorders), *jwarahara* (used for fever), *shwasa* (bronchitis and asthma), *kasa* (cough), *hikka* (hiccups), *vamana*(vomiting).

- Ayurvedic Formulation:- It is used in polyherbal Ayurvedic formulations like *dasamularista*, *chyavanaprasam*, *shringyadi leha*, and *shringyadi curna*, which are used to manage *swasa* (asthma), *yakshma* (tuberculosis), *ajeerna* (indigestion), *hridayaraga* (heart disease), *jwara* (fever) and *yakrit roga* (liver disorder).

1.3 Terminalia chebula Retz
(Combretaceae- Dicotyledonae)

- **Botanical description-** *T. chebula* is a deciduous tree growing up to 30 meters in height with spreading branches and a broad, roundish crown. The tree is distinguished by its lush green foliage and the distinctively bumpy, dark brown fruit it bears, making it a unique and striking addition to any landscape. The leaves are elliptic-oblong, with an acute tip, cordate at the base, and margins entire, glabrous above with a yellowish pubescence below. The flowers are light white to yellow and have a strong, musty odour. They are monoecious and grow in terminal spikes or short panicles. The fruits are rough, ellipsoid to ovoid drupes, yellow to orange-brown, containing a single angled seed.
- **Habitat:-** This species is commonly found scattered in teak forests, mixed evanescent forests, and low elfin-wood forests in the Indian key, growing on dry pitches up to 900m in elevation. It's also standard on the Deccan trap and Mahabaleshwar hilly terrain at 1,370m.(Muhammad et al., 2012)
- **Vernacular Name**:- Eng.- Chebulic myrobalan. Hindi-Harara, Harad, Beng.-Haritaki. Kan Harra, Karakkayi Aalekayi. Tel-Karakkaya, Karitaki. Urdu-Hazard.(Singh S, 2019)
- **Phytochemicals:-** Haritaki comprises several phytochemicals such as tannins, flavonoids, sterols, amino acids, fructose, resin, and fixed oil. It contains 33% hydrolyzable tannin, which is responsible for its pharmacological action. The primary components of tannin in Haritaki are chebulic acid, chebulinic acid, chebulagic acid, Gallic acid, corilagin, and ellagic acid(Shankara et al., 2012).
- **Pharmacological activities**:- Anti-carcinogenic activity, antifungal activity, anti-bacterial activity, antioxidant activity, antiplasmodial activity, Hepatoprotective activity, Laxative property, anti-typhoidal activity, Wound healing, Anti-ulcer activity, Cytotoxic activity and antidiabetic activity(Ravi et al., 2011).
- **Folk view**:-It is traditionally used in *Kushta* (skin diseases), *Gulma* (Abdominal tumour), *Udavarta* (bloating of the abdomen), *Shotha* (inflammation), *Pandu (*Anemia, initial stages of liver diseases), *Mada* (delirium*), Arsha* (Hemorrhoids), *Shiroroga* (Head and Headache Diseases), *Atisara* (Diarrhea and Dysentery), A*rochaka* (Anorexia), *Kasa* (Cough and Cold), *Kaphapraseka* (Increased Salivation due to *Kapha Dosha*), *Kaamala* (Jaundice), *Krimi* (Worm Infestation and Infection), *Chardi* (Vomiting), *Sroto Bandha* (Obstruction of Body Channels), *Pralepa, Hrudayoraso* (Stiffness and Heaviness of Chest))(Ravi et al., 2011).

❖ **Ayurveda (FRUITS)**

- *Rasa* (Taste) -*Pancharasatmaka* except *Lawan* rasa (All 5 tastes except salt)
- *Guna* (Quality)-*Laghu*(Light), *Ruksha* (Rough)
- *Veerya* (Potency) - *Ushna*(Hot)
- *Vipaka – Madhur*(Sweet) Because of its sweet, bitter, and astringent tastes, it balances *Pitta.* Because of its pungent, painful, and astringent flavours, it balances *Kapha.* And because of its sour taste balances *Vata.*
- Pharmacological action:- antioxidant, anti-microbial, antidiabetic, hepatoprotective, anti-inflammatory and antiarthritic,
- Therapeutic effects*:-* *jwarahara* (used for fever), *shwasa* (bronchitis and asthma), *kasa* (cough), *hikka* (hiccups), *vamana*(vomiting), *verechana* (Laxative).
- Ayurvedic Formulation:- It is used in polyherbal Ayurvedic formulations like *Abhayarishta, Agastya Haritaki Rasayana, Agastya Haritaki Rasayana, and Triphala Curna* which are used to manage *swasa* (asthma), *yakshma* (tuberculosis), *ajeerna* (indigestion), *hridayaraga* (heart disease), *jwara* (fever) and *yakrit roga* (liver disorder).
- There is a concept of *ritu haritaki* mentioned by Aacharya Bhavprakash

Varsha Ritu- Haritaki is given along with *saindhava* (salt)

Sharad Ritu- It is given along with *Sharkara*(sugar)

Hemanta Ritu-It is given along with *Shunti* (dry ginger)

Shishir Ritu- It is given with *Pippali* (long pepper)

Vasant Ritu- It is given with *Madhu*(sweet)

Greeshma Ritu- It is given along with *Guda*. (jaggery)(C & A, 2017)

1.5 What is inflammation

Inflammation is a process our bodies use to protect us from infection, illness, and injury. It involves the recruitment of leukocytes and plasma proteins to the area that has been affected. This is triggered by white blood cells that combat outside invaders like bacteria and viruses.

Inflammation may be classified as acute or chronic, depending on the speed and duration of the reaction. Acute inflammation is short-lived, while chronic inflammation is long-lasting.

Acute inflammation is an immediate immune response to an injury or insult that involves increased infiltration of leukocytes (especially granulocytes) and plasma from the bloodstream into the affected tissue.

The Acute inflammation process is initiated by several immune cells, including Dendritic cells, Kupffer cells, Histiocytes, Resistant macrophages, and Mast cells(Ha et al., 2017).

When infections, burns or injuries occur, Toll-like receptors (TLRs) and NOD (Nucleotide-binding oligomerization-domain protein) like receptors of the innate immune system trigger the activation and release of inflammatory mediators. This results in vasodilation and increased blood flow, which causes redness and a temperature rise. The increased permeability of the blood vessels leads to the exudation of plasma proteins into the tissue, causing swelling. Pain, heat, redness and swelling are the cardinal signs of acute inflammation.

Chronic inflammation is an inflammation that lasts for months or years. It typically follows a period of acute inflammation. It is usually caused by prolonged irritation from chemicals, foreign particles such as dust or surgical thread, or long-term infections from microorganisms like tuberculosis and syphilis. The immune cells involved in chronic inflammation are macrophages, neutrophils, and lymphocytes (Emily Shacter, n.d.).

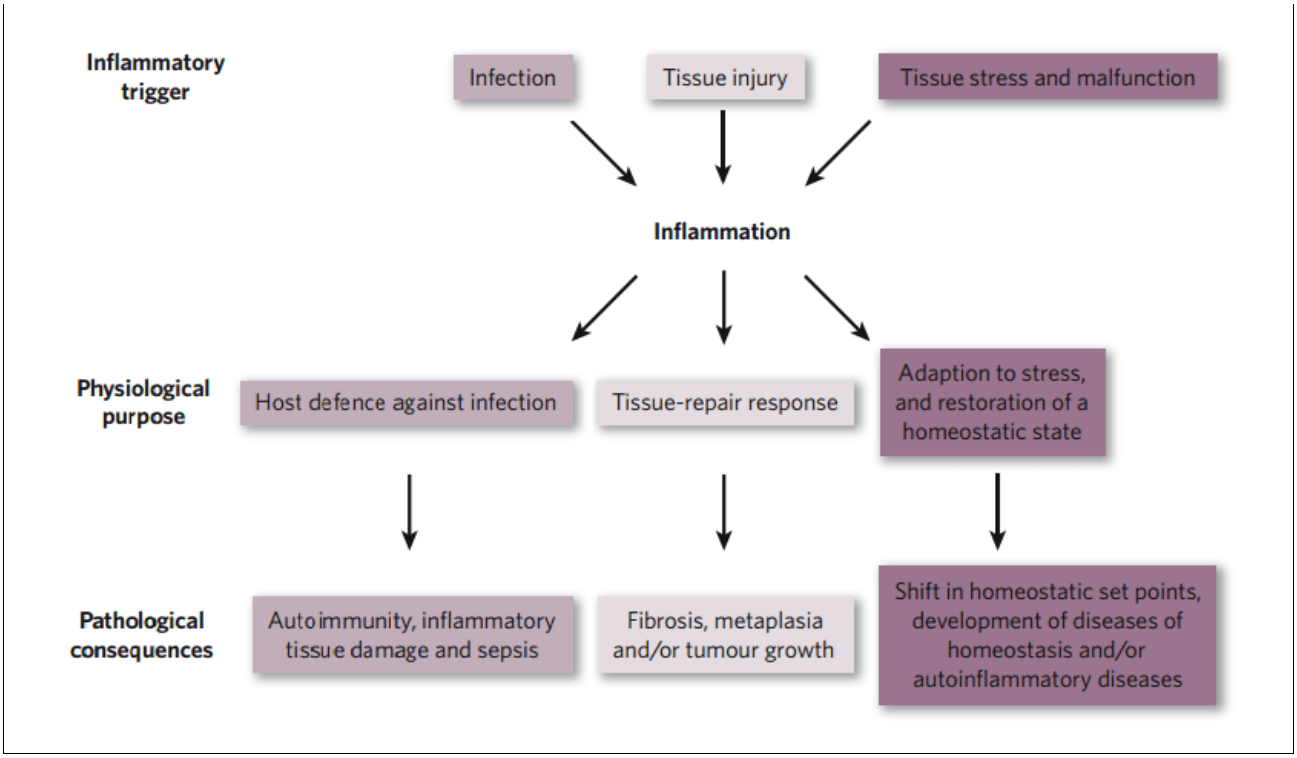


Figure:-.1 Causes and physiological and pathological outcomes of inflammation(Medzhitov, 2008)

The inflammation pathway is triggered by exogenous sources such as microbial and non-microbial mediators or by endogenous sources such as cell-derived, tissue-derived, plasma-derived, and extracellular tissue-derived mediators due to infection or injury.

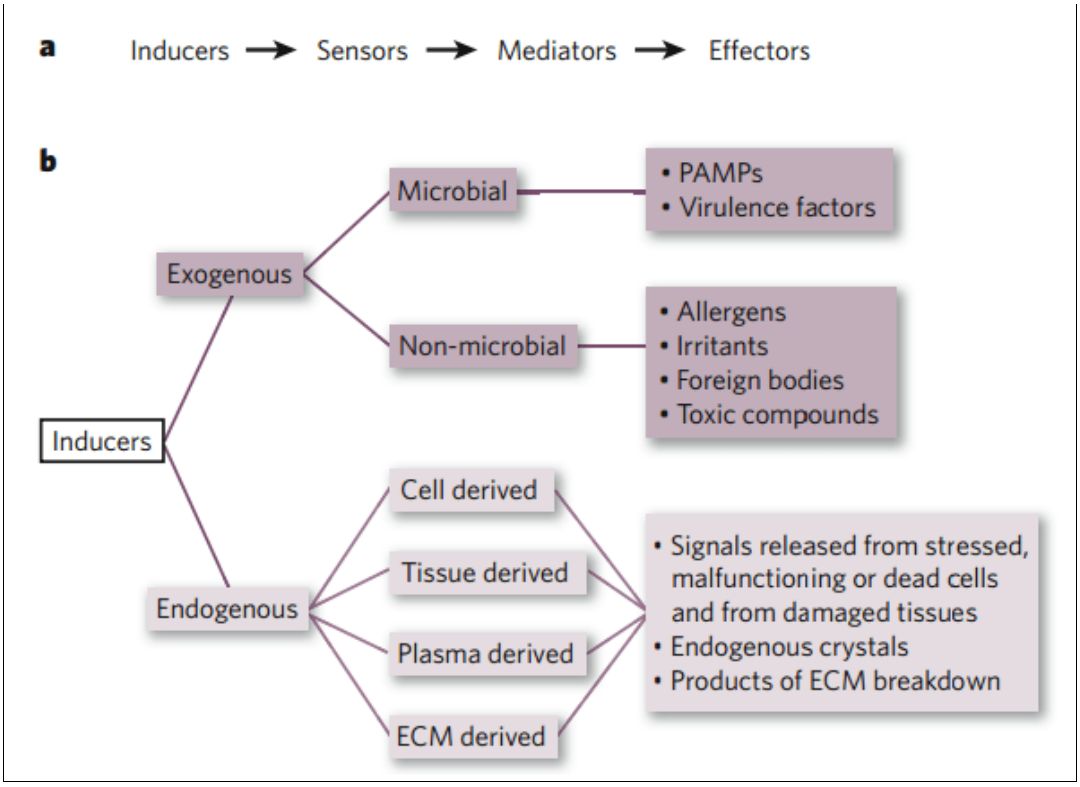


Figure:-.2 The inflammatory pathway(Medzhitov, 2008)

1.6 Pharmacology model used in this study, *Drosophila melanogaster*

Kingdom: Animalia

Class: Insecta

Family: Drosophilidae

Genus: *Drosophila*

Species: *melanogaster*

The fruit fly *Drosophila melanogaster* is used as a versatile model organism to investigate a wide range of subjects, including fundamental genetics and the development of tissues and organs. With much less genome redundancy and 60% genetic similarity between the human and *Drosophila* genomes, approximately 75% of the genes causing human disease are homologous in flies. These characteristics allow the fruit fly to study complex pathways pertinent to biomedical research, and they work in conjunction with the fruit fly's rapid technological advancement, low maintenance requirements, and accessibility to practical genetic tools.

1.6.1 Physical appearance

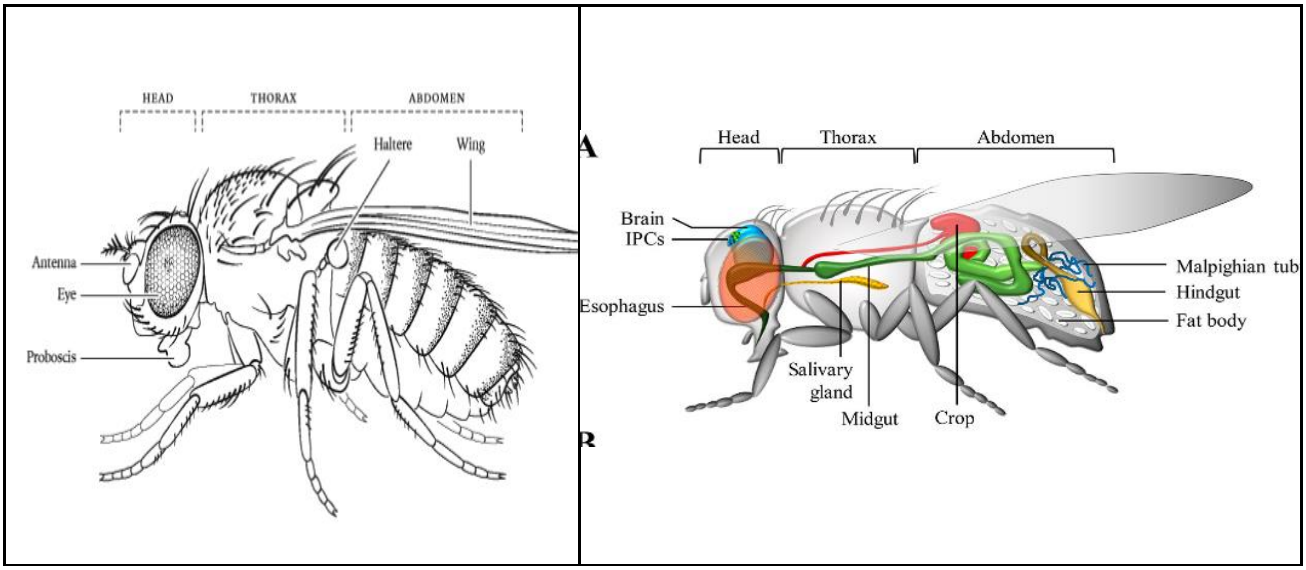


Figure:-.3 An adult *Drosophila melanogaster* (stephanie elizabeth mohr, 2018)

- Fruit flies of the wild variety have yellow-brown bodies, brick-red eyes, and transverse black bands running the length of the abdomen. The black spot on the abdomen, which is smaller in newly emerged flies, and the intercourse combs make male and female flies easily distinguishable (a row of darkish bristles on the tarsus of the first leg).

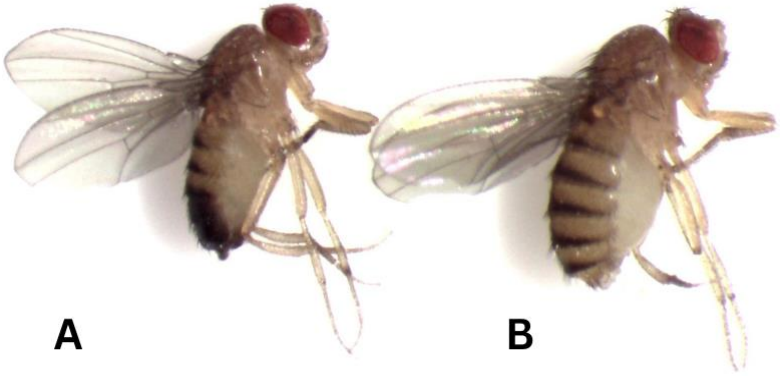


Figure:-*.4* Adult *Drosophila* (A) male and (B) female flies.

1.6.2 Life Cycle of *Drosophila melanogaster*

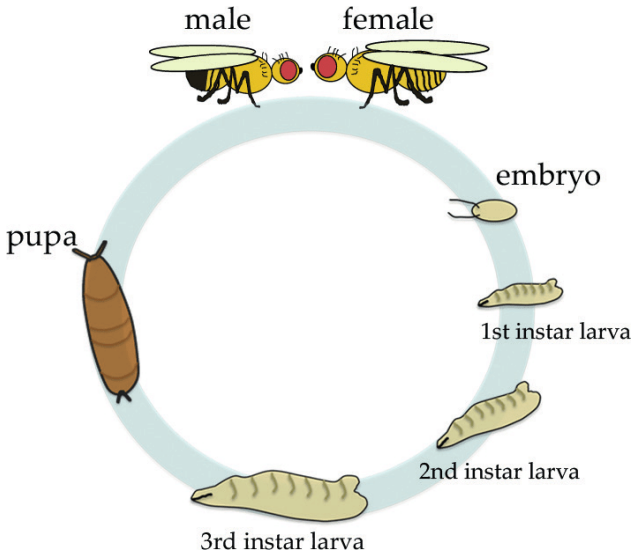


Figure:-.5 *Drosophila melanogaster* life cycle. The life cycle lasts approximately ten days at 25°C(Mariateresa A, 2018).

- The life cycle of the *Drosophila melanogaster* involves an egg, larval (worm-like) form, pupa, and ultimately emergence (enclosure) as a flying adult. Temperature affects how quickly *D.melanogaster* reproduces. The *D.melanogaster* lifespan is approximately 50 days from egg to death under ideal growth circumstances at 25 °(77 °F). At 28 °C (82 °F), the quickest development period (from egg to adult) is seven days. Lower-temperature raised flies (to 18°C, or 64°F) will take twice as long to mature. A female can produce up to 100 embryos per day.

1.6.3 Inflammation studies *D.melanogaster*

Due to the evolutionary preservation of the innate host defence mechanism, *D.melanogaster* has evolved as an excellent insect to investigate the genetic control of immune recognition and responses. In humans and other mammals, comparable immune signalling networks and transcriptional regulators are present(Henna Myllymäki 1, 2014)

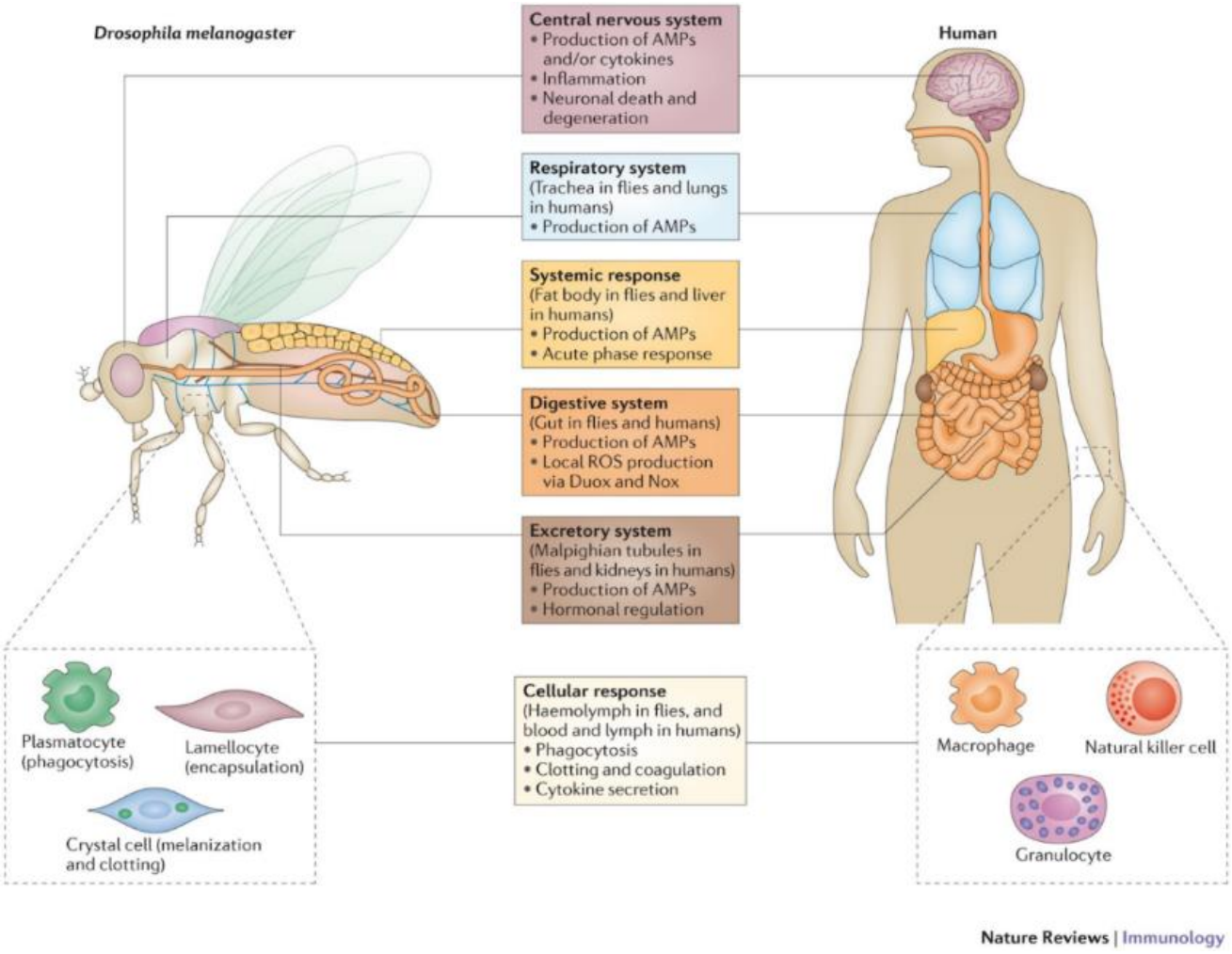


Figure:- 6 The organ systems of *Drosophila Melanogaster* are analogous to those in vertebrates (Taeil Kim 1, 2020).

The remarkable resemblance between many organic processes and systems in flies and humans makes *Drosophila* a successful immunological model. Most importantly, the fruit fly's innate immune system has been preserved throughout development. Additionally, unlike other insects, *Drosophila* has no adaptive immunity mechanisms, so it relies entirely on its natural immune system. Physical barriers like the chitin exoskeleton, tracheal epithelium, and intestinal epithelium act as the fruit fly's main line of defence against the entrance of invasive pathogens. One of the distinguishing characteristics of the *Drosophila* humoral reaction is the systemic production of amps into the surrounding hemolymph, which is controlled by the toll and Imd pathways(Hoffmann, 2003; panelLeena-Maija Vanha-aho a, 2015)

Imd pathway

The *Drosophila* Imd pathway is similar to the TNFR signalling pathways in mammals and the TLR signalling pathways. This pathway is primarily associated with defence against Gram-negative bacteria, regulating the production of various anti-microbial peptides, including Diptheric in, Drosocin, Cecropins, and Attacins.

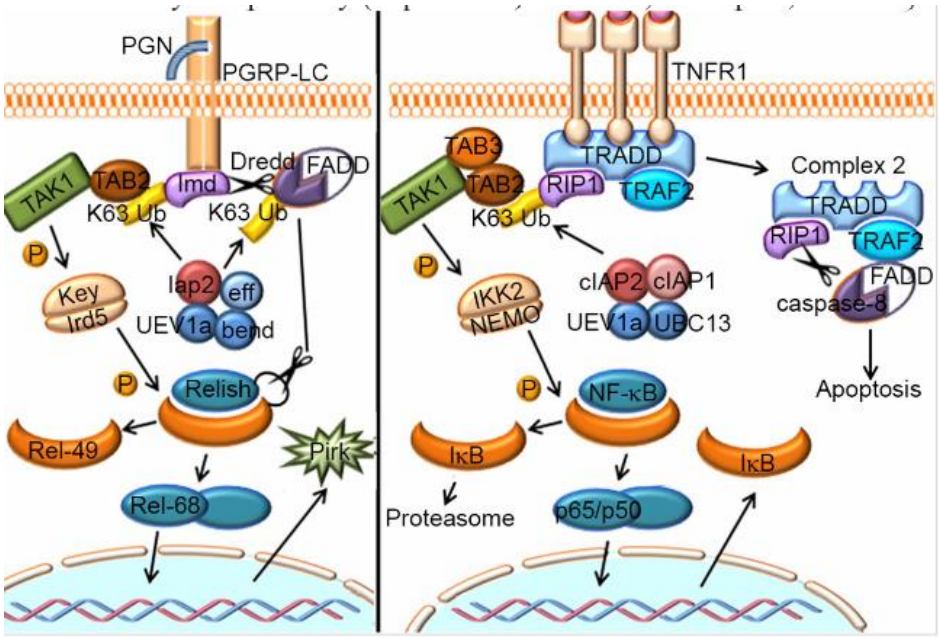


Figure:-.7 *Drosophila Melanogaster* Imd pathway and Human TNFR signaling(Myllymäki et al., 2014)

Inflammatory Genes

- *Relish*

- The *Drosophila* NF-kB, transcription factor *Relish*, is an essential regulator of anti-microbial peptide gene induction after Gram-negative bacterial infection.
- It is a bipartite NF-kB precursor protein with an N-terminal Rel homology domain and a C-terminal IkB-like domain, similar to mammalian homologs.
- Two pathways control the activation of *Drosophila* Rel/NF-kB homologs and the induction of the AMP genes.
- The Toll pathway, which is stimulated by fungi and many Gram-positive bacteria
- The Imd pathway, triggered by Gram-negative bacteria, induces anti-microbial peptide genes, including Diptericin (K C Johansson 1, 2006; Puja Verma 1, 2012).
- Unlike the mammalian NF-kB proteins, *Relish* processing does not require the ubiquitin/proteasome pathway but is an endoproteolytic, caspase-dependent event.

- Stimulation of the Imd pathway by Gram-negative bacteria leads to cleavage of *relish* and activates immune genes(Puja Verma 1, 2012)

House keeping genes

- R.P. 49

- RP49 is a Ribosomal protein49
- It is a housekeeping gene, typically a constitutive gene that is required for the maintenance of basic cellular functions essential for a cell's existence, regardless of its specific role in the tissue or organism.
- These genes are always expressed because they code for proteins that are constantly required by the cell, and their expression is unaffected by experimental conditions.
- The proteins they code are generally involved in the essential functions necessary for the sustenance or maintenance of the cell.

2. Material and Methodology

2.1 Plant Material

- The Material was collected from the Repository of Raw Drugs (FRLHT) Galls of *Pistacia chinensis ssp. integerrima* Coll No: 4434 and Galls of *Terminalia chebula* Coll No: 4432 were collected in Andheri West, Mumbai, Maharashtra raw drug market. Senior field botanists and taxonomists examined all the plant materials to confirm their identity.

2.2 Development study of *Terminalia chebula*

2.2.1 Leaf Gall documentation

- The *Terminalia chebula* tree's habitat was located with I-AIM gardeners' help.
- Senior field botanists from FRLHT examined the tree, and galls and taxonomists confirmed the identity.
- A herbarium of *T.chebula* was made and submitted to National Herbarium and Raw Drug Repository, Bangalore (FRLHT)
- The newly formed galls were identified and tagged
- The photo documentation was done using the GPS map Camera App.
- The observation was done in different Seasons and on a daily basis.
- The development of galls was observed continuously from day 1 to day 30, and then a seven days gap was given until the day of maturity.

2.2.2 Identification of the insects found in the Galls

- The insects inside the galls were collected and identified by entomologists, kept at -20 for 5 hours, mounted with glycerin on a glass slide, and observed under Stereomicroscope (Olympus SZX10 Japan). Then the images were sent to entomologists.

2.2 Pharmacognosy anatomy

2.3.1 Sample processing

The sample was collected and underwent some steps

- **Soaking:-** The galls of *P.integerrima* and Galls of *T.chebula* were soaked overnight in water.
- **Sectioning**:- The sectioning is done with a sharp blade.
- **Staining:-** Safranin and Toluidine Blue was used for staining the section.
- **Observation:-** Observed under the microscope at 4X, 10X, and 40X (Olympus-CX33), and pictures were captured in Mag Vision software.

2.3.2 Histochemistry

- For histochemical detection, the sections of *P.integerrima* and Galls of *T.chebula* were dipped in the chemicals by which the chemical reaction occurs, and the colour change was observed under the microscope(Raj et al., 2021).

Table:- 1- Histochemistry tests protocol

SR.No	TEST FOR	METHOD USED	PROCEDURE	OBSERVATION
1	Starch	iodine-Potassium Iodide reaction	Mounted sections in I2KI for 30 seconds.	Blue to black in a few minutes, and newly formed starch may appear red to purple.
2	Lignin	Potassium Iodide-iodine-sulphuric acid	Stain the sections in potassium iodide-iodine solution (Lugol's Iodine). Transfer. the sections to 60-70% sulphuric acid solution	Lignin becomes yellow, yellowish-orange, or brown
3	Polyphenols	Toluidine blue	Stain sections for 1-5 min and wash in running water for 30 to 40 seconds until most of the stain has washed out.	Polyphenols stain turquoise green or blue-green.
4	Proteins	Fast Green	Stain the sections in Fast Green stain solution by gently heating (over a Bunsen burner) until the stain begins to evaporate (30-60 seconds) Remove the stain by gently rinsing it with water.	Proteins appear bright green.

2.4 Phytochemistry (Qualitative analysis)

2.4.1 Extraction of the plant material

- Successive soxhlet extraction
- A thimble was prepared with thick filter paper with 20g of plant sample in it and kept in the soxhlet extractor, and 200mL solvent was poured from non-polar to polar

respectively.

- And the set of apparatus was kept in the water bath at a specific temperature according to the solvents boiling point.
- After a specific time (7 to 8 hr), the apparatus was taken out, and the solvent was stored in a conical flask.
- The same thimble was dried and used for the subsequent solvent extraction (Successive Extraction).

Table:- 2 Successive extraction method

SOLVENT	WATER BATH TEMP °C	DURATION	CYCLE
Hexane	85	7 to 8 hrs.	For all samples, an average of 8 cycles were performed.
Chloroform	75		
Ethyl acetate	95		
Ethanol	95.5		
Water	50-55 frequency in a heating mantle		

- Preparation of extracts
- After successive Extraction, the extracted samples are kept for evaporation. The china dishes were weighed before adding the solvent. The solvent extracts from the soxhlet were poured into a china dish and left in a water bath for evaporation. The temperature of the Water Bath is set according to the solvent's boiling point. After evaporation, the china dish was weighed again to get the extract yield. The samples were scraped with the help of blades and stored in clean test tubes. These samples were used for further test

2.4.2 Preliminary Phytochemical Detection

- The test was carried out as described below from Table-3 to Table- 10.

Table:- 3 Alkaloids test

Solvent-free extract of 50 mg is stored with a few mL of H_2SO_4 acid, and a filter Filter is used in various alkaloids tests.

SR.No	TEST	PROCEDURE	OBSERVATION
i	Mayer's test	A drop of Mayer's reagent was added along the sides of the test tube	A white or creamy precipitate indicated the presence of alkaloids. (Evans,1997)
ii	Wanger test	A drop of Wagner's reagent was added along the sides of the test tube	A reddish-brown precipitate indicated the presence of Alkaloids. (Wanger,1993)
iii	Hager's test	A drop of Hager's reagent was added along the sides of the test tube	A yellow precipitate indicated the presence of alkaloids.
iv	Dragendorff's test	A drop of Dragendorff's reagent was added along the sides of the test tube	A prominent yellow precipitate indicated the presence of Alkaloids. (Waldi,1965)

Table:- 4 Carbohydrates and glycosides test

Extract (100mg) dissolved in 5 mL of water and filtered (Ramakrishna et al.,1994)

SR.No	TEST	PROCEDURE	OBSERVATION
i	Molisch's test	Filtrates were treated with two drops of alcoholic α-naphthol solution in a test tube. To this mixture, 2 mL of concentrated sulphuric acid was added along the sides of the test tube.	A violet ring at the junction of two solutions indicates the presence of carbohydrates.
ii	Benedict's test	1 mL of filtrate was treated with 1 mL of Benedict's reagent and heated in a boiling water bath.	Orange-red precipitate indicates the presence of carbohydrates.
iii	Barfoed's test	To 1 mL of filtrate, 1 mL of Barfoed's reagent was added and heated on a boiling water bath.	A red precipitate indicates the presence of reducing sugars.

iv	Fehling's test	1 mL of filtrate was boiled in the water bath with 1 mL of each of Fehling's solutions A and B.	The formation of a red precipitate indicates the presence of reducing sugars.

Table:- 5 Glycosides test

- 50mg of the extract is hydrolyzed in conc H_2SO_4 for 2 hours in a water bath, then filtered, and the hydrolysate is subjected to the following tests.

SR.No	TEST	PROCEDURE	OBSERVATION
i	Borntrager's test	To 2 mL of filtrate, 3 mL of chloroform+shaken. The chloroform layer was separated, and an equal amount of 10% ammonia was added to it.	A pink colour indicates the presence of anthraquinone glycosides.
ii	Legal's test	To 2 mL of filtrate, one drop of pyridine, and 1 mL of sodium nitroprusside solution were added. This mixture was made alkaline using 10% sodium hydroxide.	The pink to blood-red colour indicates the presence of glycosides.
iii	Liebermann Burchard's test	To 2 mL filtrate, 3 mL of chloroform and a few drops of acetic anhydride were added. This mixture was boiled and cooled in an ice bath. To this, 2 mL concentrated acetic anhydride was added along the sides of the test tube.	brown ring at the junction of the two layers, along with the formation of violet/green/ blue the colour indicates the presence of steroidal glycosides.

Table:- 6 Detection of saponins test(Kokate,1999)

SR.No	TEST	PROCEDURE	OBSERVATION
i	Foam test	50mg extract is diluted in distilled water and made upto 20 mL The suspension is shaken in a graduated cylinder for 15 mins	A 2 cm layer of form

Table:- 7 Proteins and amino acids test

- The extract(100m) is dissolved in 10 mL of distilled water. And filtered through Whatman No.1 filter paper.
- The filtrate is subjected to a test for protein and amino acids

SR.No	TEST	PROCEDURE	OBSERVATION
i	Millon's test	To 2 mL of extracts, a few drops of Millon's reagent	white precipitate indicates the presence of proteins.(Rasch and Swit,1960)
ii	Biuret test	2 mL of extracts were treated with one drop of 2% copper sulfate solution and 1 mL of 95% ethanol, and an excess of potassium hydroxide (KOH) pellets.	The pink colour in the ethanolic layer indicates the presence of proteins.
iii	Ninhydrin test	To 2 mL of filtrate, two drops of ninhydrin solution were added.	purple the colour indicates the presence of amino acids.(Yasuma and Ichikawa,1953)

Table:- 8 Phenol and Tannins test

SR.No	TEST	PROCEDURE	OBSERVATION
i	Ferric chloride test	To 2 mL of filtrate, a few drops of neutral 5% ferric chloride	, the dark green colour indicates the presence of tannins. (Mac,1963)
ii	Gelatin test	To 2 mL of filtrate, 2 mL of a 1% gelatin solution containing 10% sodium chloride	A white precipitate indicates the presence of tannins. (Evans,1997)
iii	Lead acetate test	To 2 mL of filtrate, 3 mL of 10% lead acetate solution	white precipitate indicates the presence of phenolic compounds.

Table:- 9 Flavanoids test

SR.No	TEST	PROCEDURE	OBSERVATION
i	Alkaline reagent test	2 mL of filtrate was treated with 2 mL of 10% ammonia.	Yellow fluorescence indicates the presence of flavonoids.
ii	Magnesium and HCl reduction (Shinoda test)	To 2 mL of filtrate, 1 mL of Methanol a few fragments of magnesium ribbon and concentrated hydrochloric acid	Pink to crimson colour indicates the presence of flavanols. (Harborne,1998)
iii	Lead acetate Test	2 mL of extract was treated with a few drops of lead acetate solution.	Yellow precipitate indicates the presence of flavonoids.

Table:- 10 Gums and mucilage test

SR.No	TEST	PROCEDURE	OBSERVATION	SR.No
I	Alcoholic precipitation test	Absolute alcohol	25mL of absolute alcohol was slowly added to the extracts with constant stirring.	The formation of a precipitate indicates the presence of gums & mucilages.

2.4.3 High-performance thin-layer chromatography HPTLC

HPTLC (High-Performance Thin Layer Chromatography): HPTLC is one of the sophisticated instrumental techniques based on TLC, comparatively increased resolution of the compounds to be separated and allow quantitative analysis of botanicals and herbal drugs. In TDU, HPTLC is equipped with CAMAG (Muttenz, Switzerland) components (Linomat 5 applicator, Visualizer 2, and Scanner 3). The software VisionCATS organizes the workflow of the HPTLC analysis controls the CAMAG instruments and manages data evaluation.

- Reconstitution of the sample: - 5mL of respective solvent was added to 1g of extract yield.

- TLC Silica gel 60G F_{254} 25 Glass plates 20 x 20 cm was used.
- The solvent system was used according to the phytochemical groups.

- The sample was loaded on the TLC plate with the help of a *Linomat 5 applicator* and then kept in the TLC chamber (CAMAG) until the solvent ran to ¾ of the plate.
- Then the plate was observed in the TLC Visualizer (CAMAG)

Table:- 11 The solvent system used

PHYTOCONSTITUENTS	SOLVENT SYSTEM	RATIO
Alkaloids	Toluene: Ethyl acetate: Methanol: 25% Ammonia	(30: 30: 15: 1)
Glycosides	Ethyl Acetate: Methanol: water	(20: 2.8: 2)
Flavonoids	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water	(10: 0.5: 0.5: 1.3)
Terpenes	n-hexane: Ethyl Acetate	(1: 1)
Polyphenols	Tetra Hydro Furan : Toluene: Ethyl Acetate: Water	(16: 8: 2: 1)

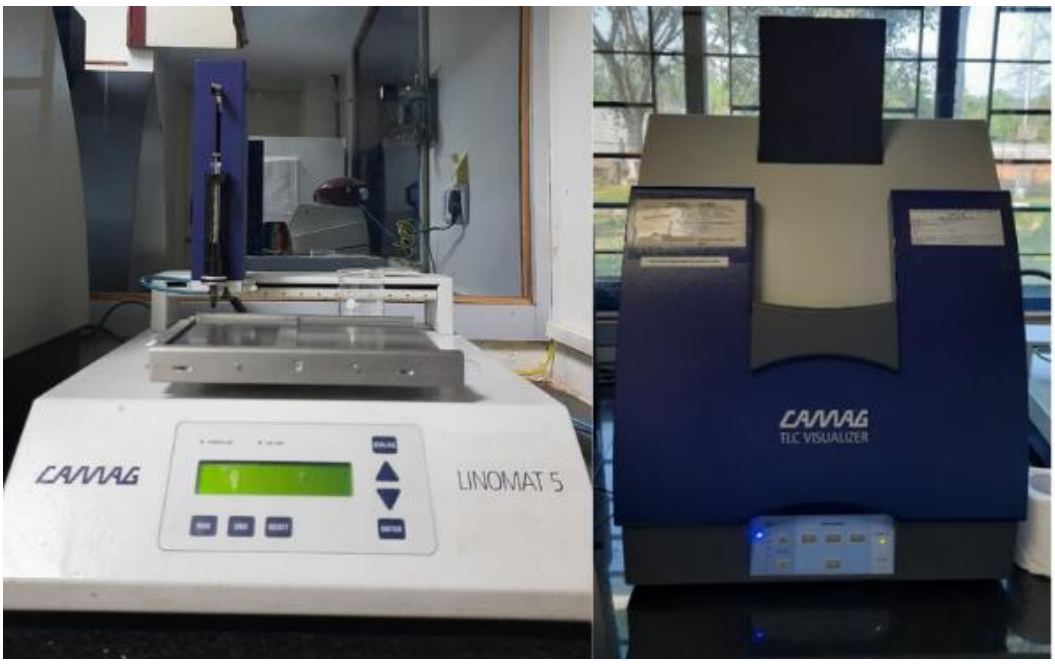


Figure:- 8 HPTLC Linomat 5 applicator and Visualizer 2,

2.5 Pharmacology (*Drosophila melanogaster* as an in vivo model organism)

2.5.1 Media Preparation and Maintenance of *Drosophila melanogaster*

Table:- 12 Media prepared for maintaining the flies - Corn flour media(100mL)

SR. NO.	COMPOSITION	QUANTITY
1	Corn flour	8g
2	D- Glucose	4g
3	Sucrose	2g
4	Yeast extract	5g
5	Agar	0.8g
6	Propionic acid	0.4mL
7	Benzoic acid	0.7mL
8	Orthophosphoric acid	0.6mL

.5.2 Preparation of kashaya from raw drug material

- Raw Drug Used

i) *P. integerrima* dried leaf gall.

ii) *T. chebula* dried leaf gall.

iii) *T. chebula* dried fruit pulp.

- The kashaya was prepared by weighing raw drugs 10g and boiling them in 160 mL of potable-water on low flame until it was reduced to 40 mL.

Figure:-.9 Kashaya prepared from the drugs

2.5.3 Fecundity assay by long-term feeding.

Table:-13 Different media and concentrations for fecundity assay.

MEDIA	AMOUNT OF CONTROL DIET (FOR 100mL)	AMOUNT OF DRUG *(KASHAYA)* (FOR 100mL)
Control	100mL	----
P. integerrima gall (5%)	95 mL	5 mL
T. chebula galls (5%)	95 mL	5 mL
T. chebula fruits (5%)	95 mL	5 mL

- The flies were collected on day zero, flipped in the media as mentioned above, and continued for five days.
- On the sixth day flies were sorted using ice and transferred into cut bottles.
- Four females and two males were taken in each cut bottle, and three biological replicates were kept.
- After 24hr, the flies were transferred to new cut bottles with respective media and repeated for four days.
- The cut bottles were opened, and egg counting was done using a Stereomicroscope (Olympus SZX10 Japan).
- The data was collected, and a graph was plotted.

2.5.4 Experimental setup for inflammation study

- **SDS standardization using 0.6% and 1.5%**
- **Inflammation assay (inducing inflammation with sodium dodecyl sulfate)**

Table:- 14 Different experimental media.

MEDIA	AMOUNT OF CONTROL DIET (FOR 100mL)	AMOUNT OF DRUGS (FOR 100mL)
1% Agar media	----	1g of agarose
0.6% SDS	100mL	0.6g of SDS
1.5% SDS	98mL	1.5g of SDS
Control	100mL	----

Table:- 15 Co-treatment media.

MEDIA	AMOUNT OF 1.5% SDS DIET (FOR 100mL)	AMOUNT OF DRUGS (*KASHAYA)* (FOR 100mL)
1.5% SDS + *P. integerrima* gall (5%)	95 mL	5mL
1.5% SDS +*T. chebula* galls (5%)	95 mL	5mL
1.5% SDS + *T. chebula* fruits (5%)	95 mL	5 mL

1. 1% Agarose media for Starvation (100mL)

In 100mL of distilled water, 1g of agar was added, boiled, and then poured into food bottles.

2. 1.5%SDS media (100mL)

In the control media, 1.5g of SDS was added and then cooked for 2 mins.

3. Treatment media 1.5% SDS+ 5% Drug (100mL)

In 95mL of 1.5% SDS media, 5mL of respective Kashaya was added.

- Day 4 flies were taken and starved for 2 hours in 1% agar media and then were fed on 1.5 % SDS, 1.5 % SDS+ 5% *P.integerrima*, and 1.5 % SDS+ 5% *T.chebula* (galls),

1.5 % SDS+ 5% *T. chebula* (fruits) respectively, for three days; then the flies were collected, and further molecular studies were done as shown in (Figure:- *10).* Then the flies were collected for gene expression(Ma et al., 2021).

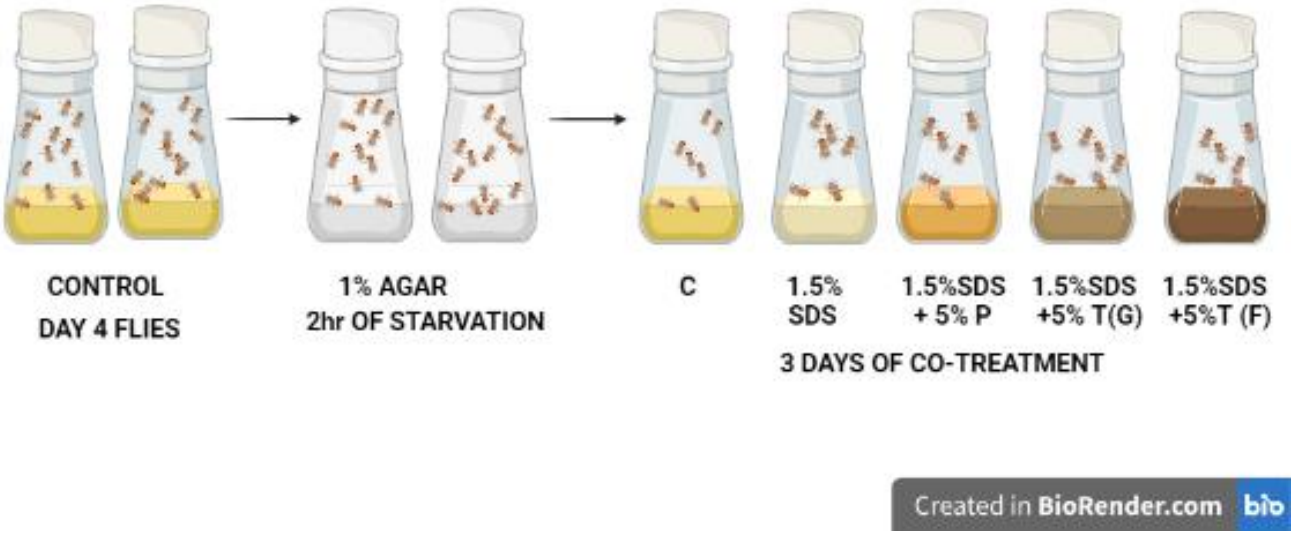


Figure:-.10 *D. melanogaster* experimental setup for Anti-inflammatory action (C)-control, SDS- sodium dodecyl sulfate (P) *P.integerrima* galls,(T.G.)*T.chebula* galls and (T.F.)*T.chebula* fruits

2.5.5 RNA isolation

- The flies were collected in DEPC-treated 1.5mL Eppendorf tubes, and 200µL of triazole was added.
- The sample was homogenized with the micro pestle to the homogenized sample, and 200µL of Trizol reagent was added.
- The 1.5mL tubes containing samples were thawed, 200µL of chloroform was added, and the vortex was until the solution turned pink.
- Centrifuged the above tubes at 4^0C 14000 rpm for 20 minutes
- The supernatant was collected in DEPC-treated 1.5mL fresh tubes, added an equal volume of chilled isopropanol, and inverted gently.
- It was incubated for 20 minutes at -20^0C and then for 10 minutes at R.T.
- The tubes were centrifuged at 4^0C 14000 rpm for 30 minutes
- The supernatant was discarded.
- The pellet was washed with 70% DEPC-treated ethanol. 200µL 70% DEPC treated ethanol was added, Then centrifuged the tubes were at 4^0C 14000 rpm for 10 minutes (2X)
- Discarded the supernatant and semi-dried the pellet (till the pellet became translucent)
- 10µL of DEPC-treated water was added and stored at -20^0C (Green & Sambrook, 2020).

2.5.6 Gel electrophoresis

- 1g of agarose was dissolved in 50 mL of 1X TAE buffer by heating to make 1% agarose gel, and 2.5 µL of EtBr (Ethidium Bromide) was added to the liquid agar.
- 50X TAE buffer
    - 242g Tris base.
    - 57.1mL glacial acetic acid.
    - 100mL 0.5M EDTA (pH 8.0)
    - dH_2O volume up to 1 litre.
    - Dilute 20 mL of 50X stock into 980 mL of distilled water.
- The gel tray was cleaned with ethanol and checked for leakage, and then the agarose gel was poured and left for solidification.
- After the gel solidified, the gel tray was placed in a tank containing a TAE buffer, and the gel comb was taken out.
- The dye (bromophenol blue/ xylene cyanol) 5µL was taken on a parafilm, and 2µL of the sample was added and mixed well.
- Then the samples with dye were added into wells of the gel tray.

- 90 volts (electric current) was applied to pull them through the gel.
- Nucleotide is negatively charged, so they move toward the positive electrode (anode).
- When the dye reached ¾ the gel, it was carefully removed from the gel tank.
- The gel was placed in the Gel doc machine to check the.

2.5.7 Quantification of RNA using Nanodrop Spectrophotometer

- The purity of RNA was measured at the 260/280 ratio.
- Concentration was measured in ng /mL.
- The protein contamination in the sample will be shown in a 260/230 ratio column.
- The nanodrop was started and initialized to quantify the RNA sample.
- DEPC-treated water (1 µL) was measured for BLANK.
- The isolated RNA samples were thawed and kept on ice.
- 1µL of the sample was placed on the nanodrop, and the absorbance was read at A280/260 and A260/230.

Figure:-.11 Nanodrop spectrophotometer from Thermo Scientific measures the purity of nucleic acids.

2.5.8 cDNA synthesis

- For 500 ng of cDNA synthesis, the volume to be aliquot was calculated from the above-obtained amount of RNA / µL
- The master mix was prepared
    - Buffer - 2µL
    - Primer – 0.5µL
    - Enzyme - 05µL
    - Hexamer – 0.5µL (note: volume to be added per sample)

- X volume of RNA sample was taken, and 3.5µL of master mix the volume was made up to 10µL with Rnase free water into PCR tubes (give a shot spin)
- PCR tubes were transferred into the PCR machine, and set the program as follows (RR037A)
- Step 1- 37^0C for 15 minutes
- Step 2 – 85^0C for 5 seconds
- Step 3 – 4^0C at infinite
- Lid temperature – 105^0C
- Total reaction volume - 10µL
- After completing the program, the sample was stored at -20^0C.

Figure:- 12 PCR machine amplifies RNA into cDNA.

2.5.9 RT-PCR

- Real-time PCR is a quantitative polymerase chain reaction used to measure amplified DNA.
- The total volume of the well should be 10 µL.
- Master mix contains
    - SYBR green - 5 µL
    - Forward primer - 0.4 µL
    - Reverse primer - 0.4 µL
    - Nice water - 2.2 µL, give a short spin and start adding.

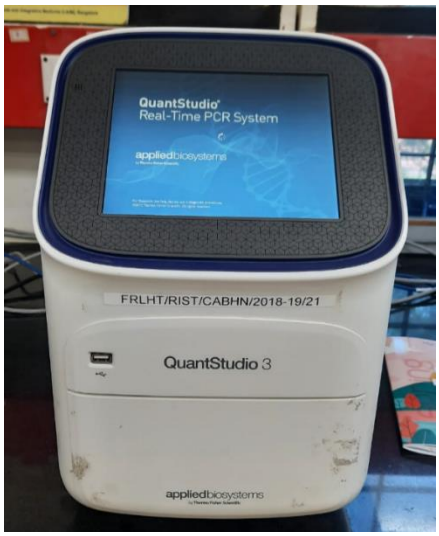


Figure:- 13 Real-time polymerase chain reaction machine

- A 2 µL sample was added to the PCR 96 well plate.
- Forward and reverse primers were added according to the genes we took in a sample.
- After adding the sample and master mix, the plate was covered with a PCR strip and tightly sealed with a film applicator to protect against evaporation for high-efficiency PCR.
- The PCR plate was Centrifuged for 1 min at room temperature, 1000 rpm.
- Conditions

    - Volume 10 µL
    - Cover 105.0^0C
    - Hold stage - 50^0C – 02.00 mins
      90^0C – 03.00 mins
    - PCR stage (40 cycles) - 95^0C – 00.10 sec
      60^0C – 00.30 sec

- Melt curve stage - 95^0C – 00.15 sec
  60^0C – 01.00 mins
  95^0C – 00.15 sec

- The program was set up initially, and the plate was transferred into the machine, allowing the program to run.
- After the program, the amplification curve obtained was saved, and raw data was exported into an Excel sheet.
- The gene expression was calculated against the housekeeping gene versus the functional gene expressed.

Table:- 15 The genes for inflammatory response RP49 and, *Relish*

	GENE NAME	**PRIMERS**	**GENE SEQUENCE**
1.	**RP49**	Forward primer	GAGTTCTTGTAACGTGGTCGG
		Reverse primer	GTGCTGCTATCCCAATCTCAG
2.	***Relish***	Forward primer	AAGATAAGGACGTGGACCGC
		Reverse primer	CAATACGTCCGTGGCTTG

2.6 Statistical Analysis

- For statistical analysis One way ANOVA was performed.

3. Result and Discussion

3.1 Identification of *Terminalia chebula* habitat.

The galls of *T.chebula* are not as much explored as *P.integerrima*. The development of the *T.chebula* galls were observed on the trees located in Mylappanahalli village, Bangalore (lat 13.133508°, long 77.539401°, altitude 920m), and senior botanists and taxonomists from FRLHT did the authentication of the tree.

Figure:- 14 Habitat of *T.chebula* and Herbarium

It is a moderate to large deciduous tree, typically growing to 15-24 m tall. Its leaves are ovate or elliptic in shape, with a pair of large glands at the top of the petiole. The flowers are hermaphroditic, 4mm across, sessile, dull-white, or yellow, and have an offensive odour. The flowers usually form spikes, either simple or short panicles, terminal, and the axils of the uppermost leaves. The fruit is a drupe, ellipsoidal, obovoid, or ovoid, yellow to orange-brown, hard when ripe, 3-5 cm long, and with five ribs when dried. The seeds are hard and pale yellow. The galls are non-uniform and irregular in shape, immature galls are 10mm to 20mm, and mature galls are 1cm to 1.5 cm in width. On maturation, the galls get brown patches indicating they are ready to harvest.

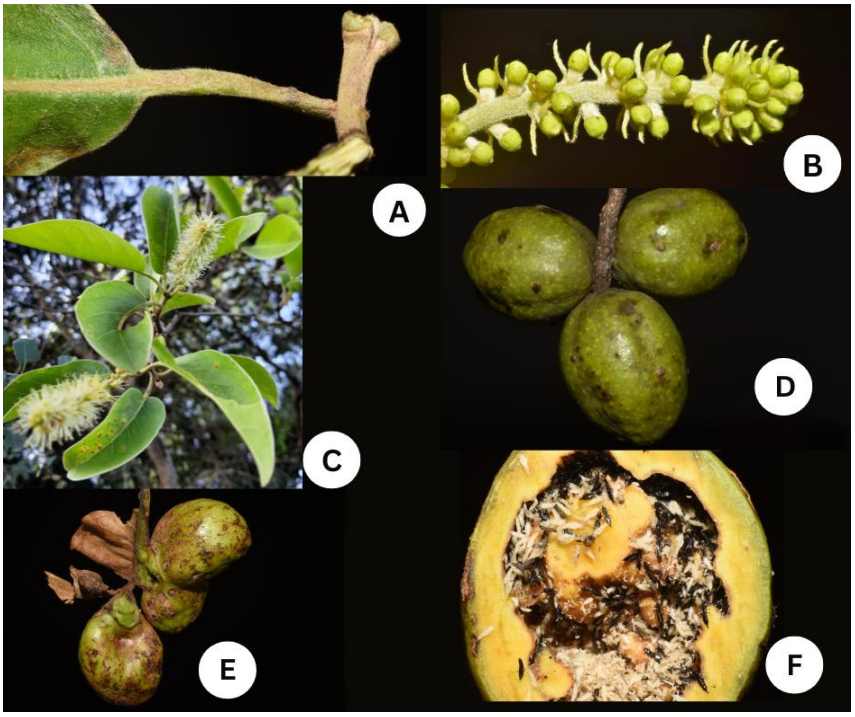


Figure:-15 (A) leaf petiole with a pair of large glands. (B) flower inflorescences showing terminal spikes (C) sessile, dull-white or yellow (D) mature fruits (E) mature leaf galls (F) L.S of leaf galls showing the population of a specific insect.

3.2 *Terminalia chebula* Leaf Gall documentation

The main observation was to know in which season the galls would start developing and how many days it takes to get a gall fully mature. From November to June, It was observed that new Galls were beginning to develop on the young leaflets in different months, as shown in (Figure: 16).

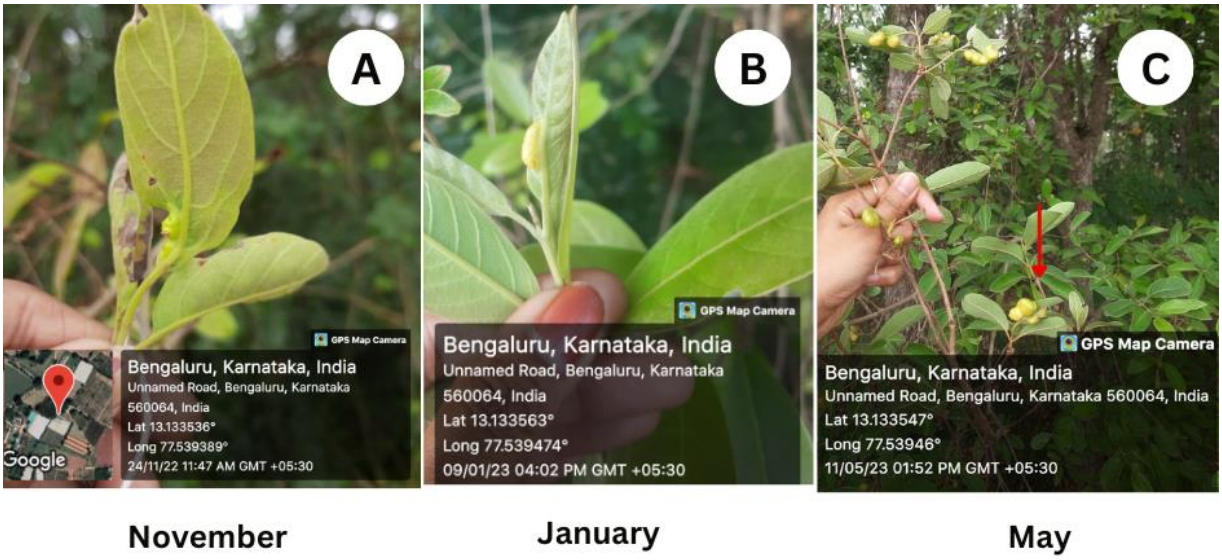


Figure:-16 Gall initiating in various seasons (A) November (B) January (C) May.

Table:-- 16 Organoleptic characters of *T.chebula* galls

ORGANOLEPTIC CHARACTERISTICS OF T.CHEBULA GALL (FRESH)	MESOCARP OF THE LEAF GALL
Colour	Yellowish
Odour	Characteristics smell
Touch	Soft
Taste	Astringent in the beginning and sweet in the end

- It was observed that The Development of galls of *T.chebula* happens within 65 -70 days. The Same was photo-documented. Development studies were done on around 20 galls; however, most of the galls were affected due to pest infestation. The observation continued on the remaining galls till full maturation. One galls case study is shown in Figure- 17, where Figure 17 (1) is day 1 of the Gall and (29) is day 68.

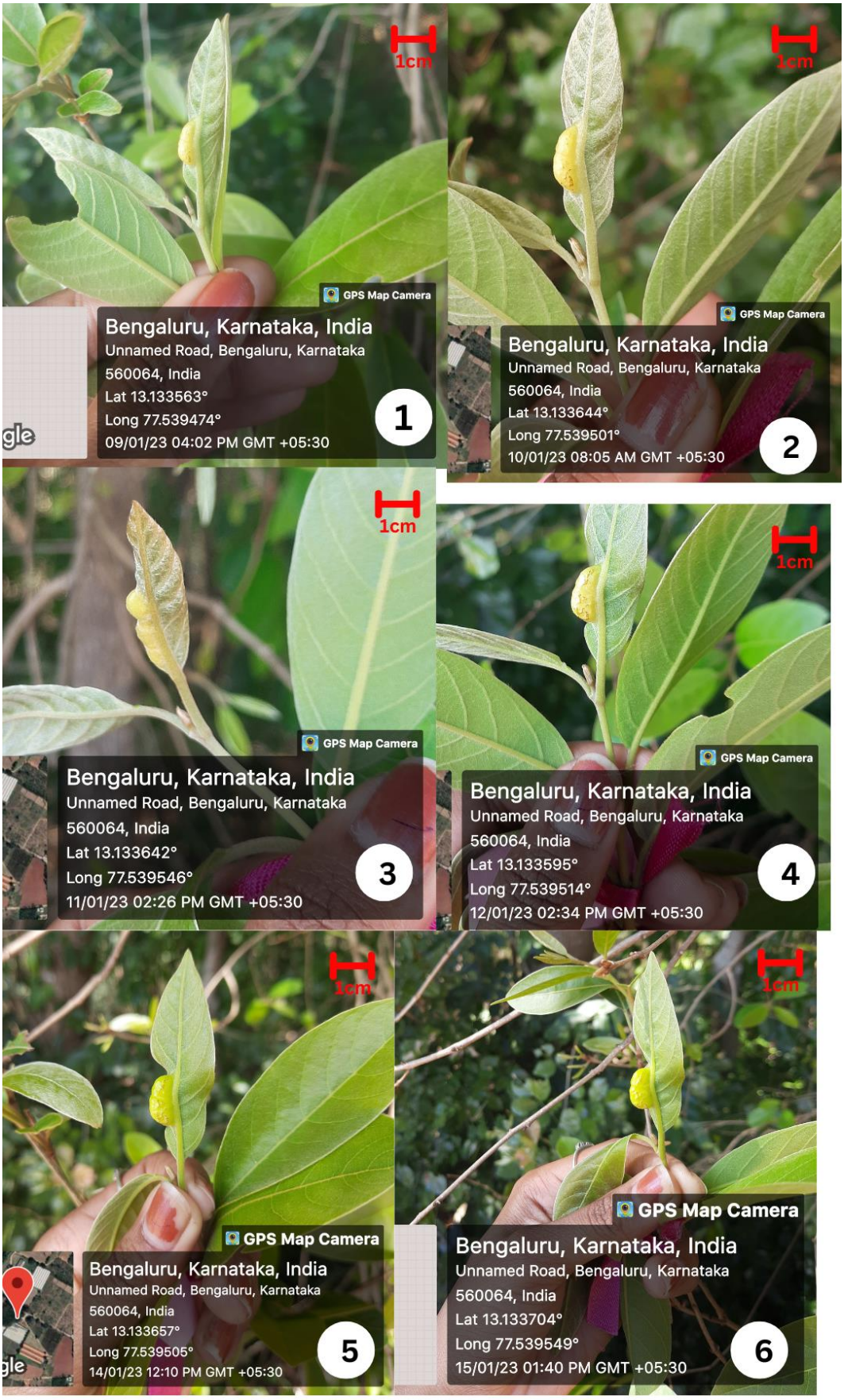


Figure:-17 Development of *T.chebula* leaf gall photo-documented for 68 days(part1/5)

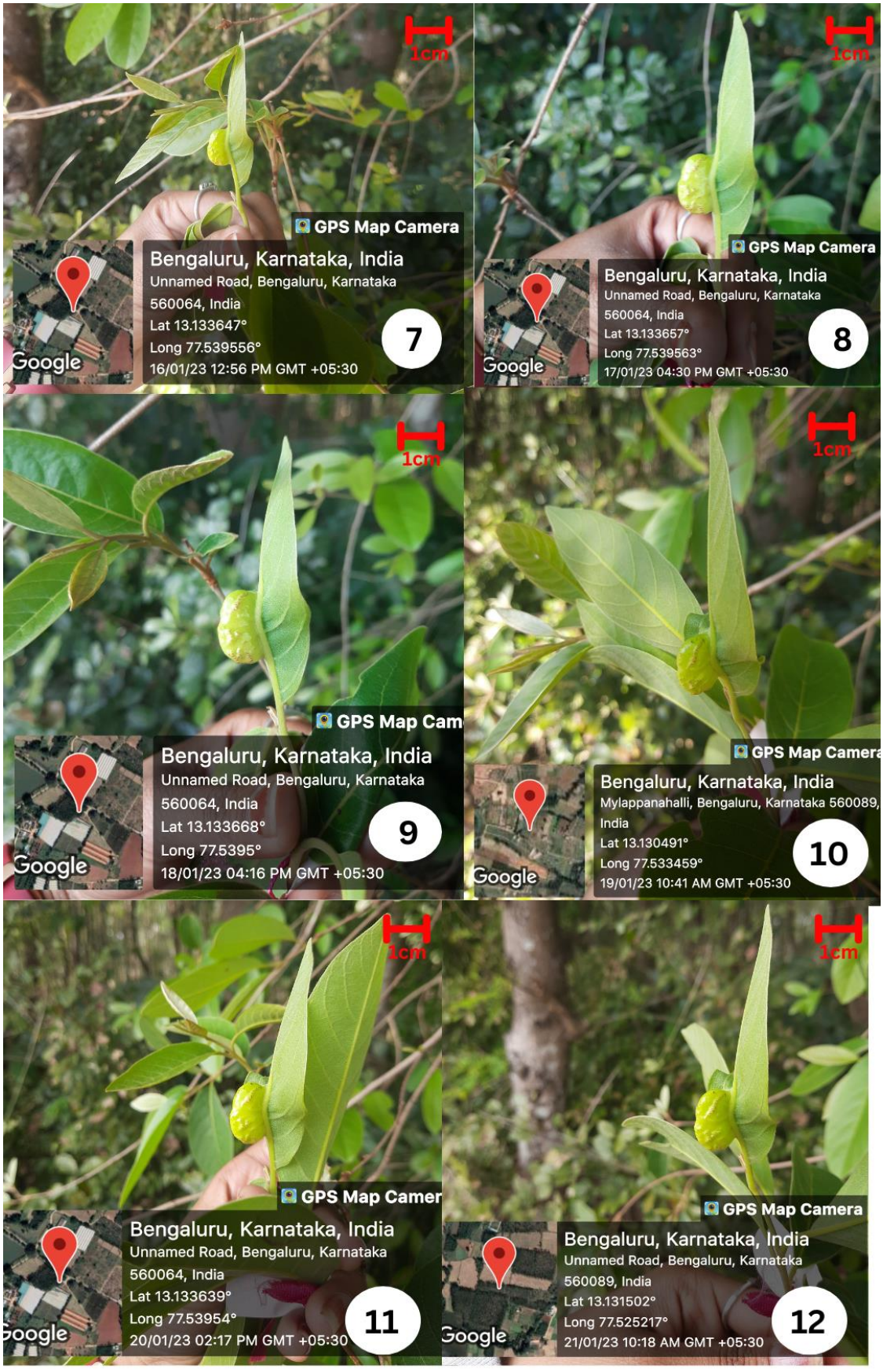


Figure:-17 Development of *T.chebula* leaf gall photo-documented for 68 days(part2/5)

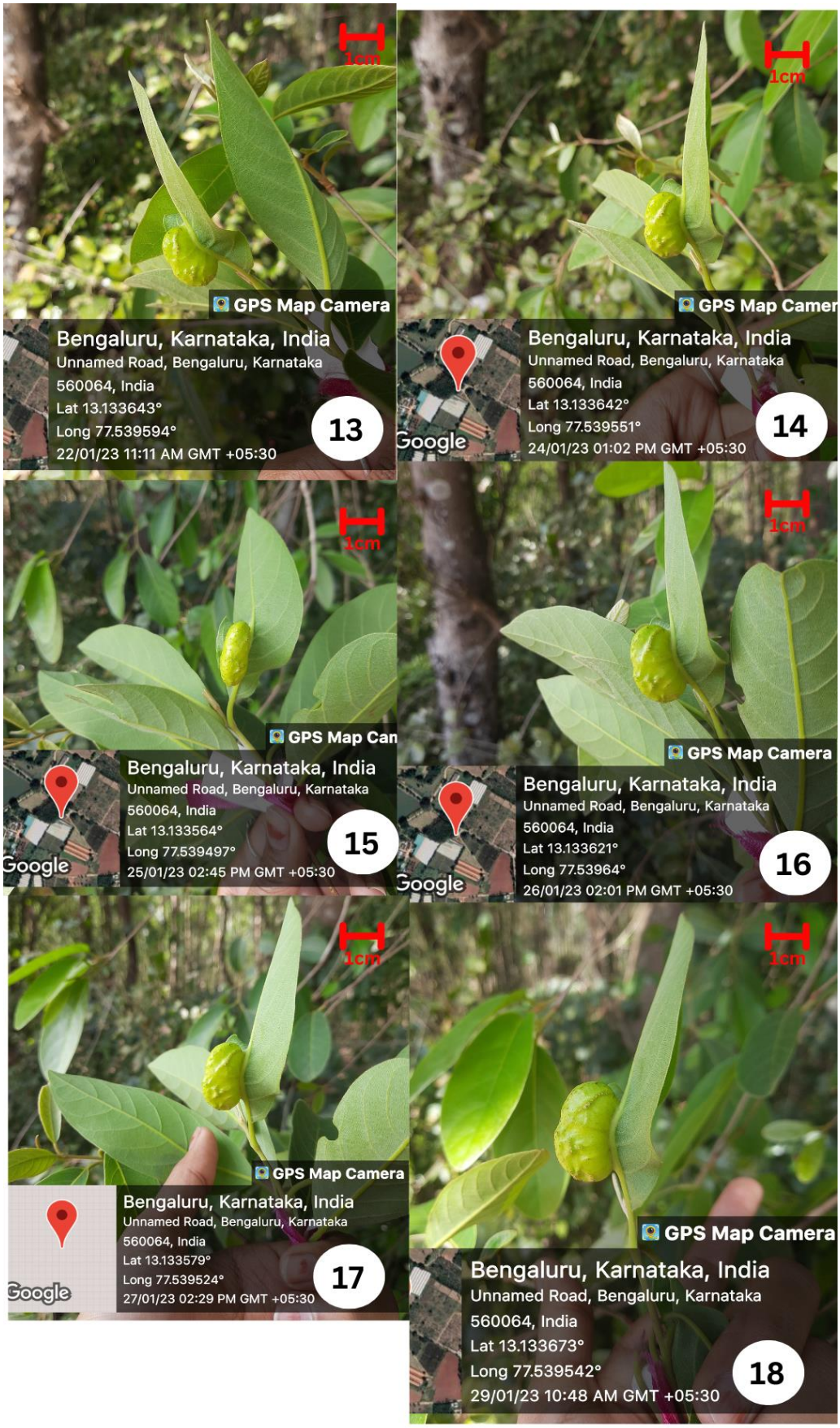


Figure:-17 Development of *T.chebula* leaf gall photo-documented for 68 days(part 3/5)

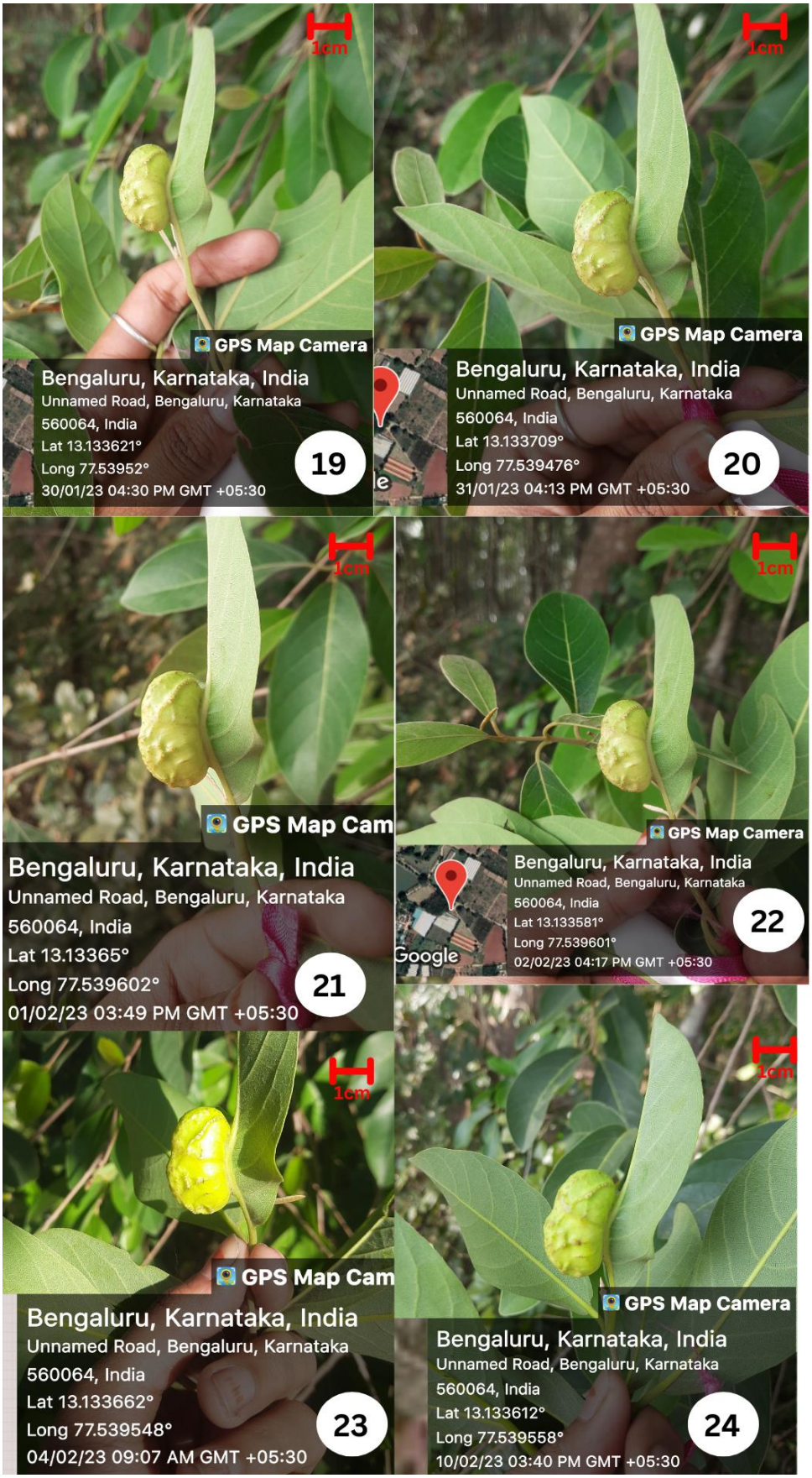


Figure:-17 Development of *T.chebula* leaf gall photo-documented for 68 days(part 4/5)

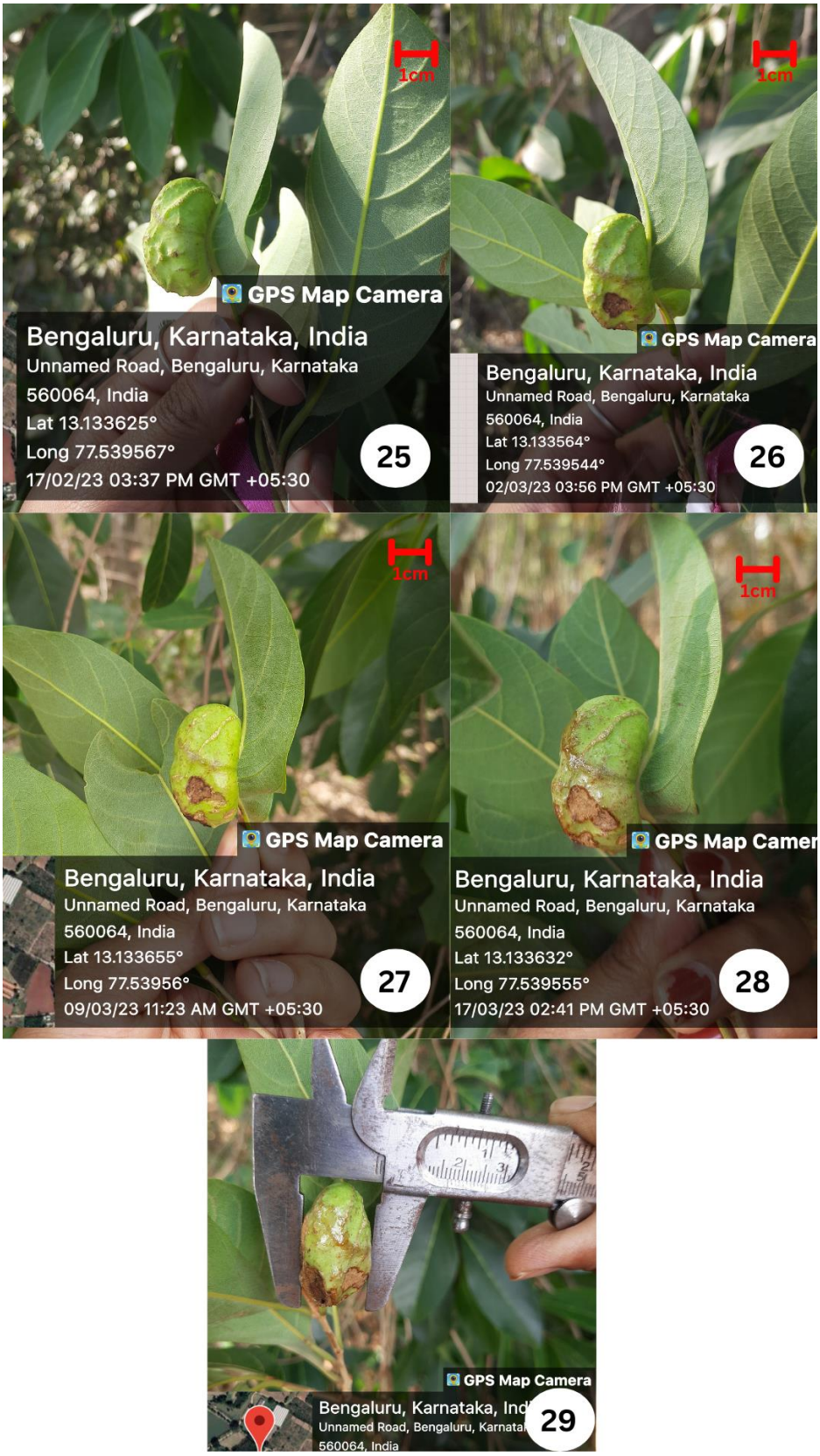


Figure:-17 Development of *T.chebula* leaf gall photo-documented for 68 days(part 5/5)

3.2.1 identification of the insects found in the Galls

The L.S. of the leaf gall showed the presence of insects identified as the *Liothrips* and Rhipiphorothrips genera. Thrips are the smallest winged insects with tube-like abdomens, hence coming in Division Tubulifera. And the wings are fringe wings, meaning needle-like; therefore, they come under the Order Thysanoptera.

Taxonomy

Kingdom – Animalia

Phylum – Arthropoda

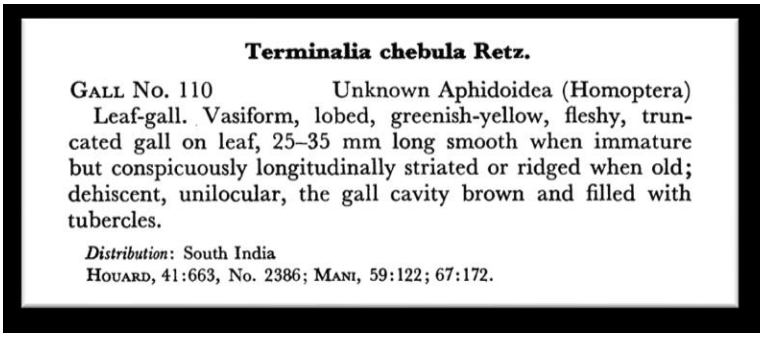
Class – Insecta

Division - Tubulifera

Order - Thysanoptera

Genus – *Liothrips and Rhipiphorothrips*

Only one Gall is reported from *T. chebula* (Mani 1974) induced by homopteran. The thrips are inquilines in the gall system. It seems that the *cecidomyiids* of some homopterans induce the gall*s.*



Terminalia chebula Retz.

GALL NO. 110 Unknown Aphidoidea (Homoptera)
Leaf-gall. Vasiform, lobed, greenish-yellow, fleshy, truncated gall on leaf, 25–35 mm long smooth when immature but conspicuously longitudinally striated or ridged when old; dehiscent, unilocular, the gall cavity brown and filled with tubercles.

Distribution: South India
HOUARD, 41:663, No. 2386; MANI, 59:122; 67:172.

Further, an entomologist who works mainly on the biology and systematics of *Thysanoptera* (thrips), an area in which he is considered a world authority, says Some thrips are highly characteristic. However, others are members of the impossible genus *Liothrips*, and it looks like one of them.

Figure:-18 Thrips insect showing tube-like abdomen and fringe wings

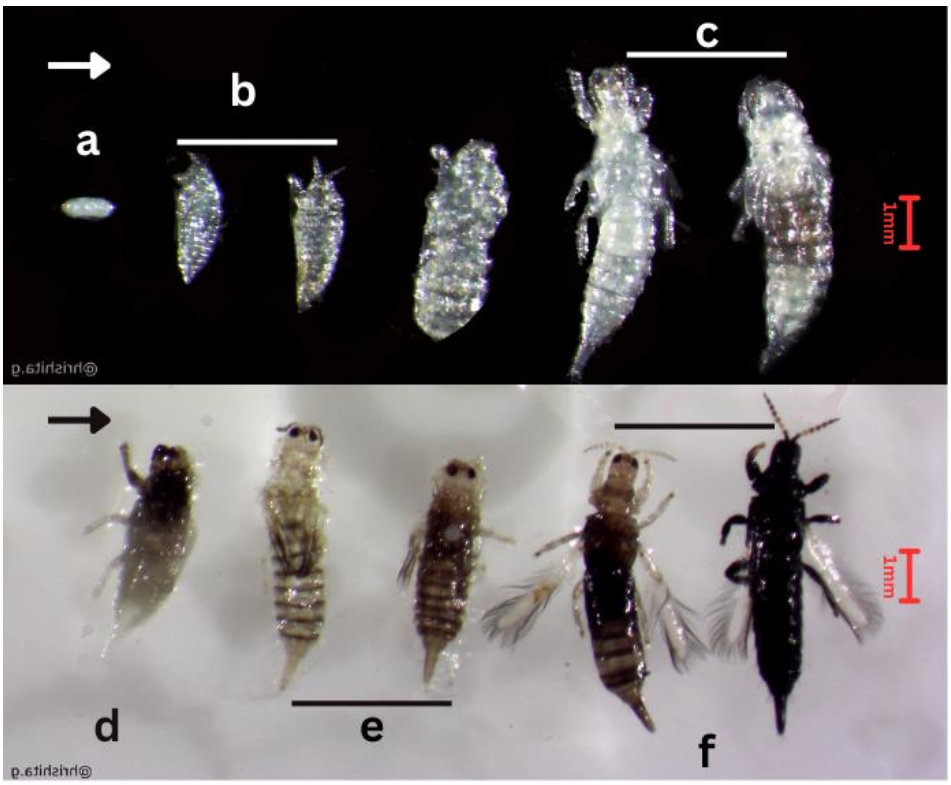


Figure:-19 Developmental stages of Liothrips genus thrips (a) Egg 0.5mm (b) 1st instar larva 1-1.5mm (c)2nd instar larva 1-2mm (d) Propupa 2mm (e) Pupa 2-3mm (f) Adult 3-4mm.

3.3 Pharmacognosy anatomy

3.3.1 Macroscopic characters

- The galls of Pistacia *integerrima* are dried pale greenish-brown, horn-shaped, rigid, rugose, hollow, and generally cylindrical with a length ranging from 3.8 cm which can go up to 20 cm or more. Upon breaking open the galls, one would observe a reddish inner surface covered with whitish particles of dust(Shankara et al., 2012).
- The galls of *Terminalia chebula* that are dried are greenish-yellow, fleshy, ovate, and laterally compressed with an irregular outer surface in the mesocarp that is yellow and slightly soft compared to the outer layer in nature.

Figure:- 20 (A) Habitat of P.int tree and (B) Habitat of tree

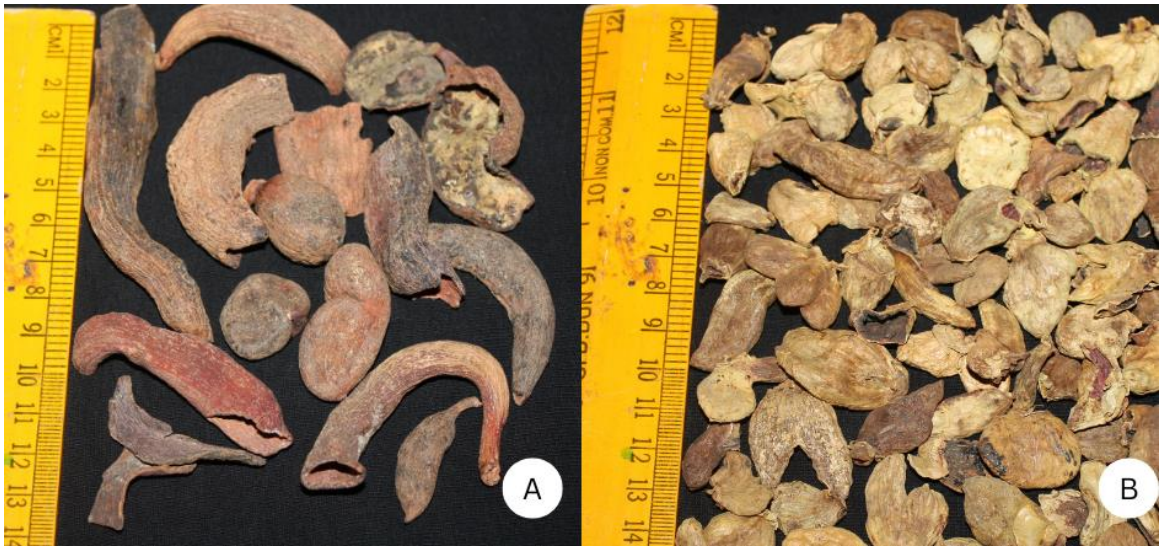


Figure:- 21 (A) *P.integerrima* dried leave galls and (B) *T.chebula* dried leave gall.

3.3.2 Microscopic characters

- The transverse section of the Gall of *Pistacia integerrima* exhibits an outermost layer of single-layered cuboidal epidermal cells. Brown idioblasts are present about 3-4 layers below the epidermis. Further down, two rows of resin canals encircled by 2-3 layers of phloem cells can be observed. These cavities are invariably associated with the conducting elements arranged in the shape of a fan, mainly composed of spirally thickened vessels. The remaining portion of the transverse section is densely packed with simple parenchymatous cells that are rounded, square, and elongated.
- The transverse section of the galls of *Terminalia chebula* shows the upper and lower epidermis, which is followed by hypodermis; hypodermis on the upper side is followed by large parenchyma cells. Vascular bundles composed of Xylem and phloem were seen with a layer of sclerenchyma cells(Krishnamurthy, 1988).

Table:- 17 Comparative microscopic characters

MICROSCOPIC CHARACTERS	GALLS OF PISTACIA INTEGERRIMA	GALLS OF TERMINALIA CHEBULA
Epidermis	Single-layer sharp teeth like	Single layer square shape
Parenchyma	Large rounded with space	Large Hexagon shaped without space
Vascular bundle	Fan-shaped in two rows, the V.B. is arranged	Fan-shaped Xylem towards the outer and phloem towards the inner
Xylem	Spiral type	Scalariform type
Secretory ducts and Crystal	Resin canals are present and absent	Absent and rosette shape
Starch grains	Present	Small scratch grains
Idioblast	Absent	Present in the shape of a parenchyma cell
Tannins	Orange to red tanniferous	Blue to black tanniferous
Crystal	Present	Present (Druse crystal)

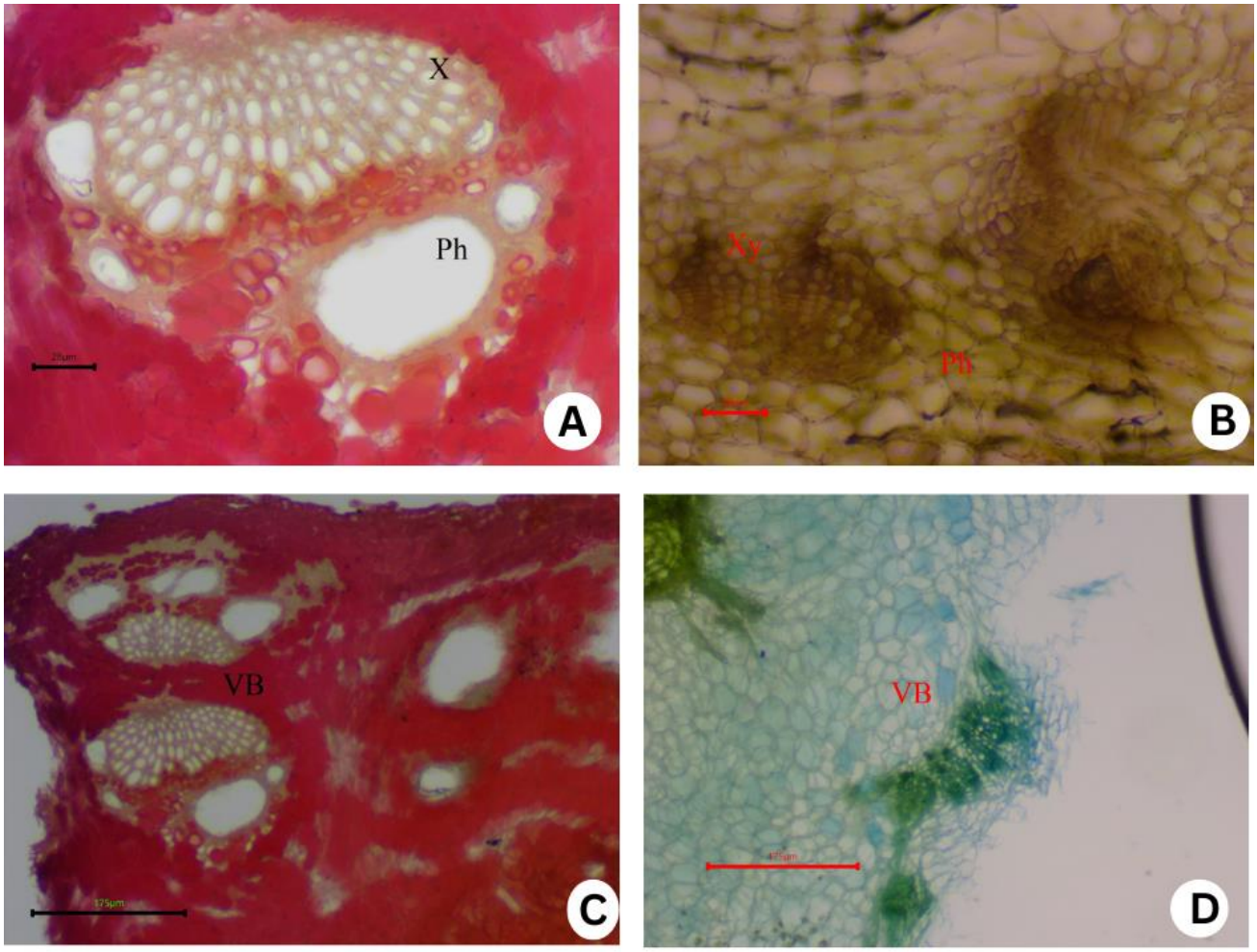


Figure:- 22 T.S of leaf galls (A, C) *P.integerrima* showing fan-like vascular bundle (B, D) *T.chebula* showing Fan shaped Xylem towards outer and phloem towards the inner side.

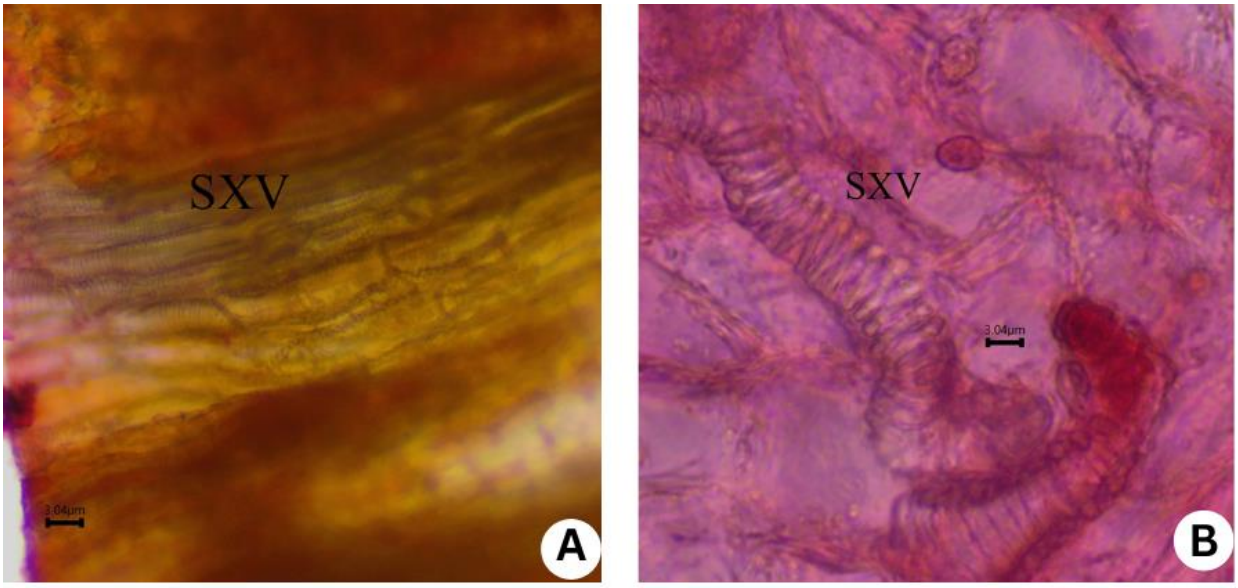


Figure:- 23 T.S. of galls showing (A) *P.integerrima* Spiral type Xylem (B) *T.chebula* Scalariform type xylem

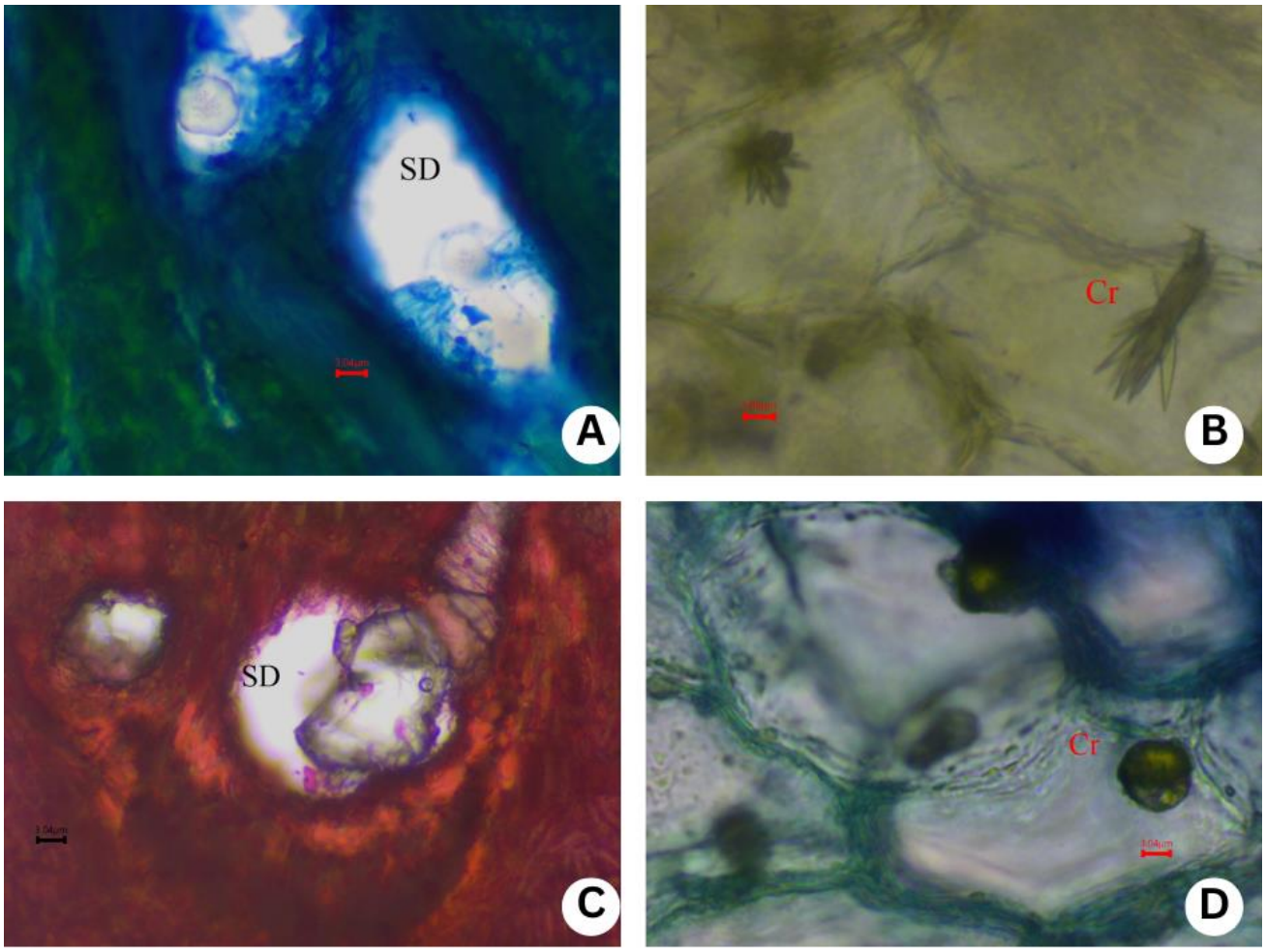


Figure:- 24 T.S. of galls showing (A, C) Secretory duct (Resin canals) (B, D) *T.chebula* Druse crystal absences of secretory ducts

- **Histochemistry**

Histochemistry of *P.integerrima galls* and *T.chebula galls* shows the presence of Tannins (Figure:- 25), protein (Figure:- 26) and lignin (Figure:- 28), although Starch grains are only present in *P.integerrima* galls (Figure:-27)

Table:-- 18 Comparative Histochemical Analysis

PHYTO COMPONENTS	REAGENTS USED	P. INTEGERRIMA	T. CHEBULA
Polyphenols (Tannins)	Toluidine Blue	Present	Present
Protein	Fast green	Present	Present
Starch	I_2KI(Lugol's iodine)	Present	Absent
Lignin	$I_2KI + H_2SO_4$	Present	Present

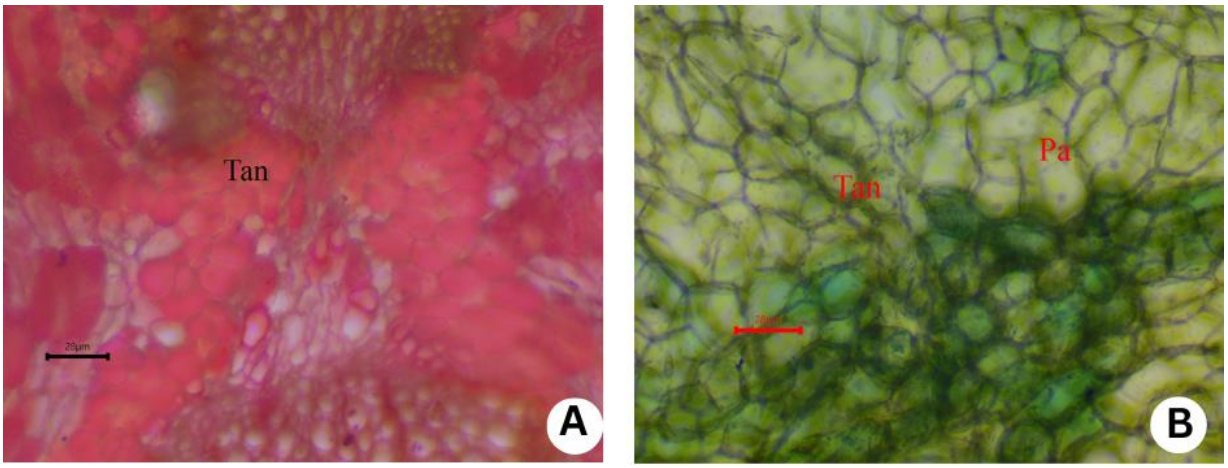


Figure:- 25 T.S. of galls showing tanniferous (A) *P.integerrima* red tanniferous (B) *T.chebula* Blue tanniferous

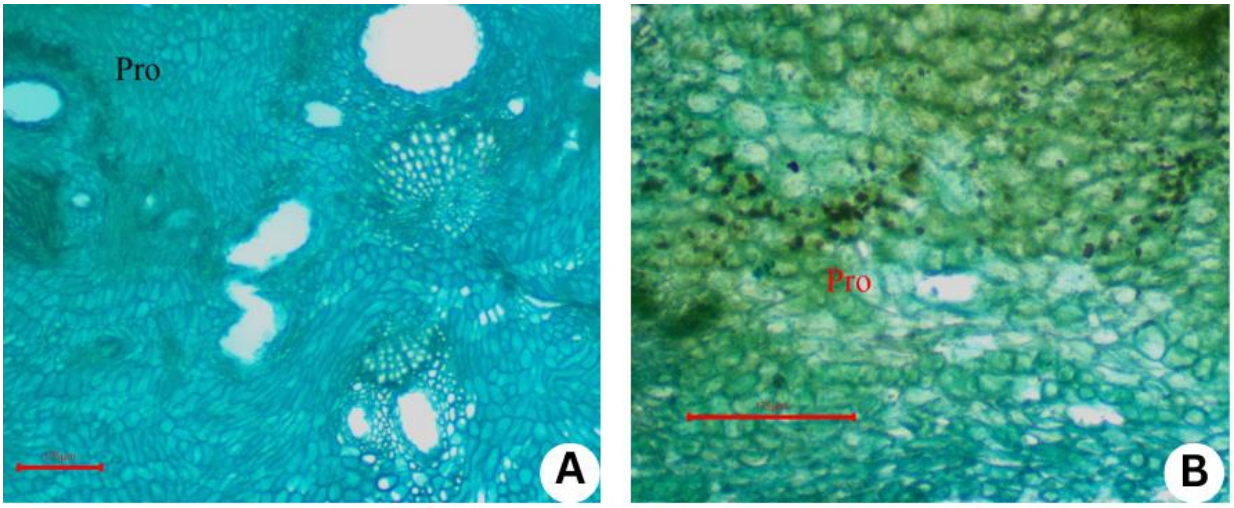


Figure:- 26- T.S. of galls showing (A) *P.integerrima* presence of protein (B) *T.chebula* presence of protein.

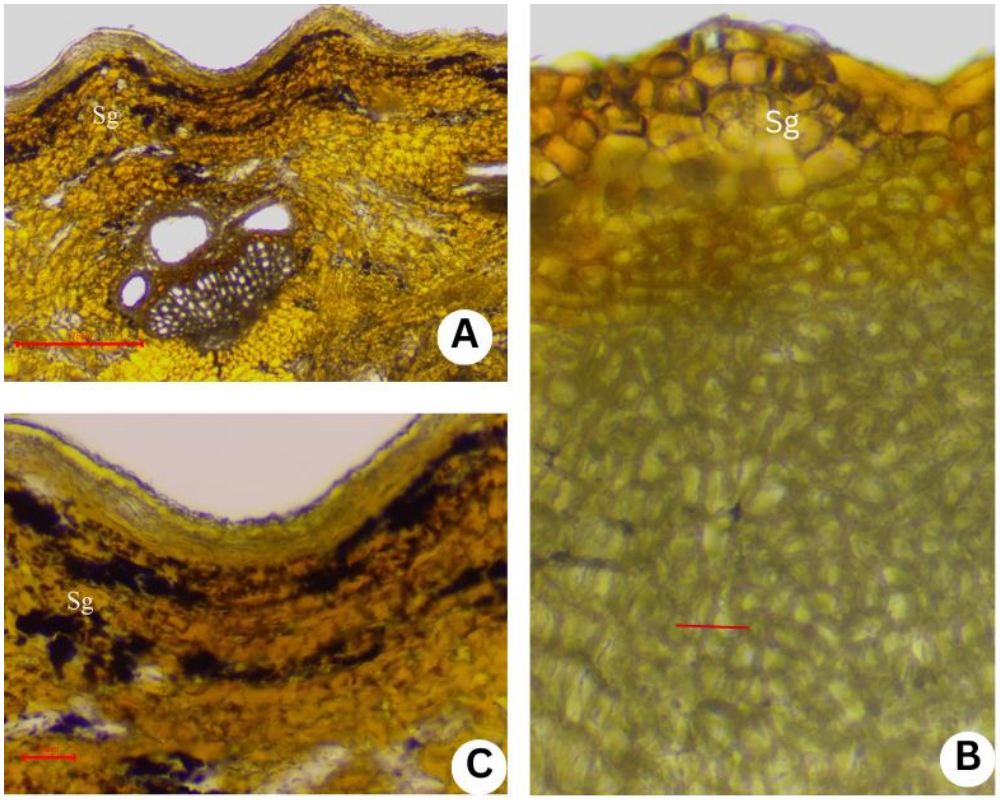


Figure:- 27 T.S. of galls showing (A) *P.integerrima* presences of starch grains in high amounts (B) *T.chebula* small starch grains in small quantities.

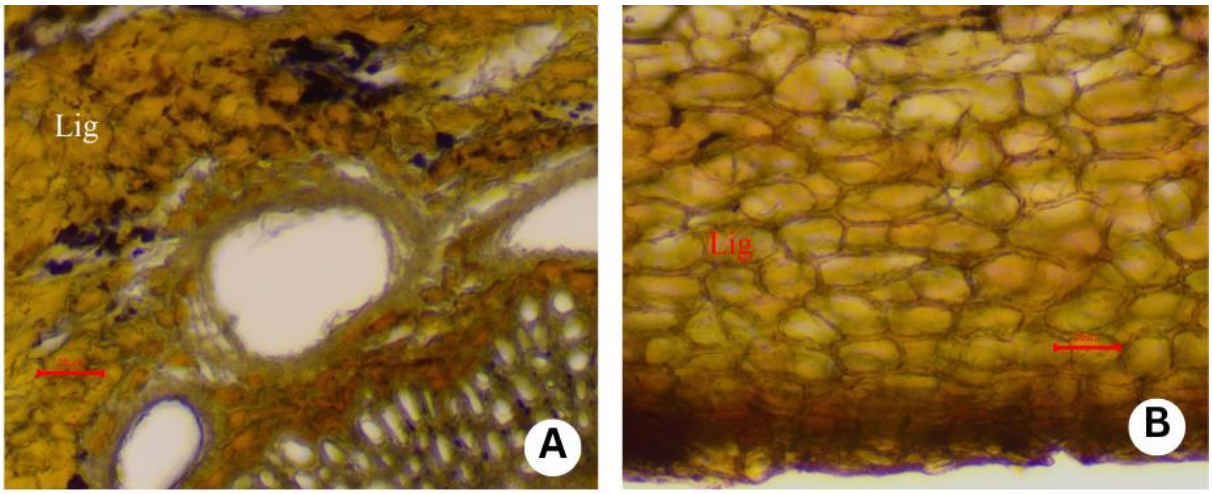


Figure:- 28 T.S. of galls showing (A) *P.integerrima* presence of lignin (B) *T.chebula* presence of lignin.

3.4 Phytochemistry (Qualitative analysis)

3.4.1 Extraction of the plant material

All plant sample crude extracts were done using n-hexane, chloroform, ethyl acetate, ethanol, and water of *Pistacia integerrima* galls and *Terminalia chebula* galls and fruit.

P.integerrima have a high amount of extractive value in hexane, as non-polar compounds like oils get extracted, and it has a characteristic smell which indicates it is high in aromatic compounds or flavonoids. Chloroform, ethyl acetate, and ethanol were used as mid-polar, and water extracts high polar compounds like tannins, etc.

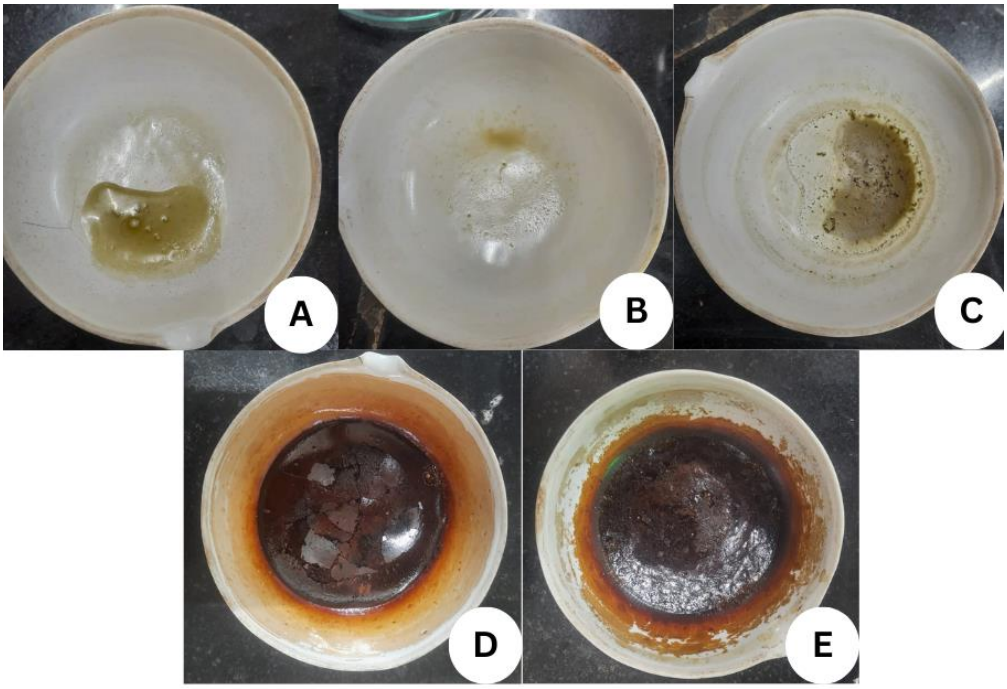


Figure:-29 Extract yield of *P.integerrima* plant material (A) Hexane (B) Chloroform (C) Ethyl acetate (D) Ethanol (E)Water.

3.4.2 Preliminary Phytochemical Detection

The phytochemical screening of n-hexane, chloroform, ethyl acetate ethanol, and water extracts of *Pistacia integerrima* galls, and *Terminalia chebula* galls were done (Gul et al., 2017). The test gave positive and negative results in all the phytoconstituents (Table:-19).

Table:- 19 Comparative Preliminary Phytochemical Detection

SR.NO.	PHYTOCHEMICAL CONSTITUENTS	SUCCESSIVE EXTRACTION (P.INTEGERRIMA)					SUCCESSIVE EXTRACTION (T.CHEBULA)				
		HEXANE	CHLOROFORM	ETHYL ACETATE	ETHANOL	WATER	HEXANE	CHLOROFORM	ETHYL ACETATE	ETHANOL	WATER
1.	Phenol and Tannins										
	i) Ferric chloride test	-	-	+	+	+	-	-	+	+	+
	ii)Gelatin test	-	-	+	+	-	-	-	-	+	-
	iii) Lead acetate test	-	-	+	+	-	-	-	+	+	+
2.	Flavonoids										
	i) Alkaline reagent test	-	-	+	+	-	-	-	+	+	+
	ii)Magnesium and HCl reduction (Shinoda test)	-	-	+	+	-	-	-	+	+	-
	iii) Lead acetate Test	-	-	+	+	-	-	-	+	+	-
3.	Glycosides										
	i) Borntrager's test	+	+	-	-	-	+	-	+	+	-
	ii) Legal test	-	-	+	+	+	+	-	+	+	-
	iii)Liebermann Burchard's test	-	-	+	+	+	+	-	+	+	-

4.	Alkaloids i) Mayer's test	+	+	+	-	+	+	+	-	-	-
	ii) Wanger test	+	+	+	+	-	+	+	-	-	-
	iii) Hager's test	-	-	-	-	-	-	-	-	-	-
	iv) Dragendorff's test	+	+	+	-	+	+	+	+	-	+
5.	Carbohydrates										
	i) Molisch's test	-	-	-	-	-	-	-	-	-	-
	ii) Benedict's test	-	+	+	+	+	-	+	+	+	+
	iii) Barfoed's test	-	-	+	+	+	-	-	+	+	+
	iv) Fehling's test	+	-	+	+	+	-	+	+	+	+
6.	Saponins i) Foam test	-	-	+	+	+	-	-	-	+	+
7.	Proteins and amino acids i) Millon's test	-	-	-	-	-	-	-	-	-	-
	ii) Biuret test	-	-	-	-	-	-	-	-	-	-
	iii) Ninhydrin test	-	-	-	-	-	-	-	-	-	-

Note :- *(+) indicates presence of phytoconstituenst and (–) indicates absences of Phytoconstituents*

3.4.3 HPTLC Fingerprinting (Qualitative Analysis)

High-performance thin layer chromatography (HPTLC) was used to determine the presence of phytochemicals in *P.integerrima* galls, *T.chebula* galls, and *T.chebula* fruits. Each solvent extract was tested for Alkaloids, flavonoids, terpins, polyphenols, and glycosides.

The stationary phase used was Merk, HPTLC silica gel 60 F_{254}. The plate format was 200 X100 and 100 X 100, and the sample loaded was 2µL. The sample position is given in (Figure:-30*)*

Plate layout:

Stationary phase	Merck, HPTLC Silica gel 60 F_{254}
Plate format	200 x 100 mm
Application type	User
Application	Position Y: 8.0 mm, length: 9.0 mm, width: 0.0 mm
Track	First position X: 30.0 mm, distance: 15.0 mm
Solvent front position	80 mm

Plate layout:

Stationary phase	Merck, HPTLC Silica gel 60 F_{254}
Plate format	100 x 100 mm
Application type	User
Application	Position Y: 8.0 mm, length: 10.0 mm, width: 0.0 mm
Track	First position X: 19.2 mm, distance: 15.4 mm
Solvent front position	80 mm

Track Assignment

Track	Vial ID	Description	Volume	Position	Type
1	1	P.HEX	2.0 µl	N/A	Sample
2	2	P.CHL	2.0 µl	N/A	Sample
3	3	P.EA	2.0 µl	N/A	Sample
4	4	P.ETH	2.0 µl	N/A	Sample
5	5	P.WATER	2.0 µl	N/A	Sample
6	6	TG. HEX	2.0 µl	N/A	Sample
7	7	TG.CHL	2.0 µl	N/A	Sample
8	8	TG.EA	2.0 µl	N/A	Sample
9	9	TG.ETH	2.0 µl	N/A	Sample
10	10	TG.WATER	2.0 µl	N/A	Sample

Track Assignment

Track	Vial ID	Description	Volume	Position	Type
1	1	TF.HEX	2.0 µl	N/A	Sample
2	2	TF.CHL	2.0 µl	N/A	Sample
3	3	TF.EA	2.0 µl	N/A	Sample
4	4	TF.ETH	2.0 µl	N/A	Sample
5	5	TF. WATER	2.0 µl	N/A	Sample

Track Assignment notes

Figure:- 30 HPTLC page layout and Track assignment details

1. **Alkaloid** - Toluene: Ethyl Acetate: Methanol: 25% Ammonia (30: 30: 15: 1)

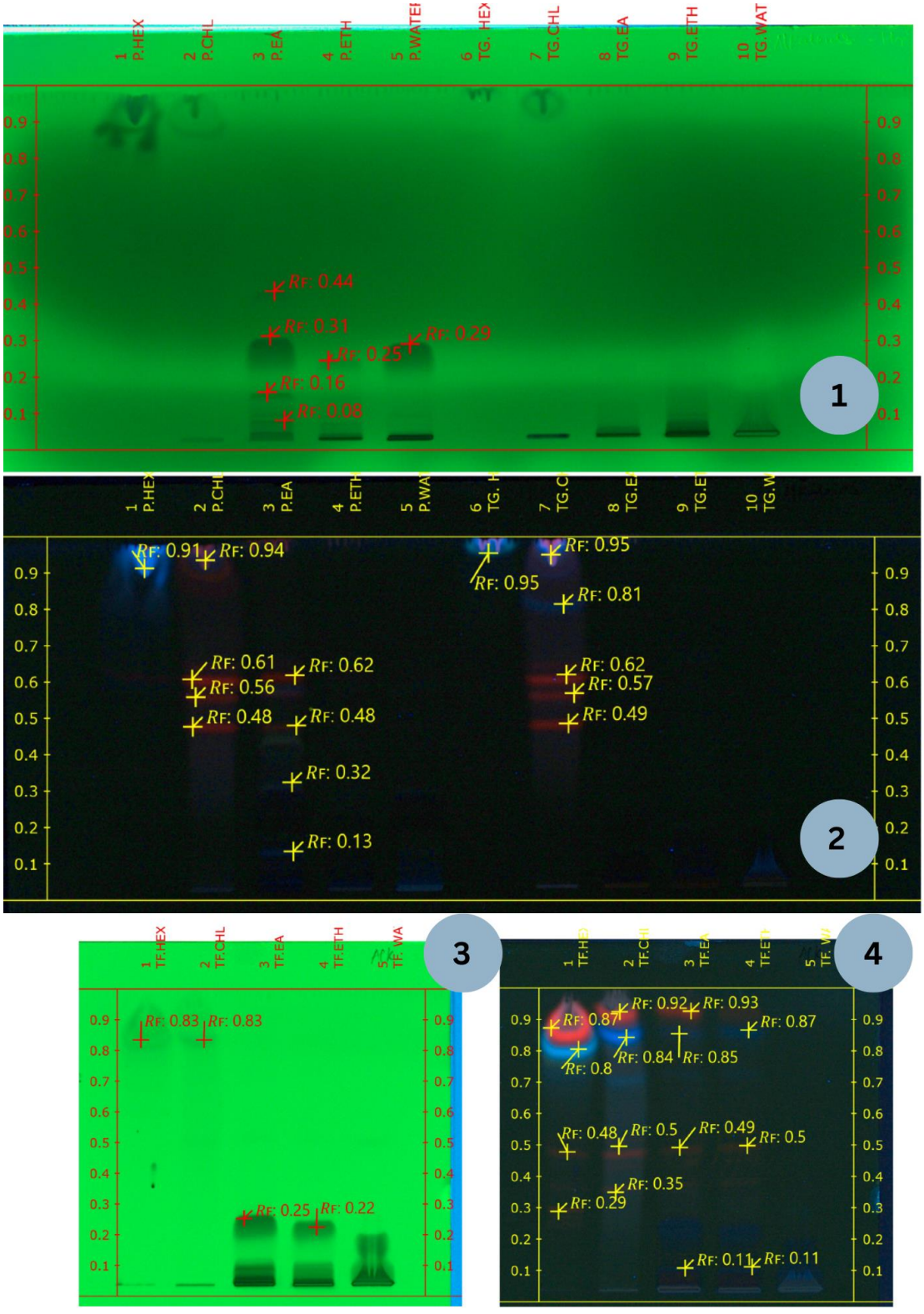


Figure:- 31 HPTLC plates Alkaloids test (1) *P.integerrima* and *T.chebula* galls under 254nm (2) *P.integerrima* and *T.chebula* galls under 366nm (3) *T.chebula* fruit under 254nm and (4) *T.chebula* fruit under 366nm

Table:- 20 Comparative Rf values for Alkaloids

EXTRACT	SOLVENT SYSTEM	WAVE LENGTH	RF VALUES (*P.integerrima)*	RF VALUES (*T. chebula) Gall*	RF VALUES *(T.chebula) fruit*
Hexane	**Alkaloid** 1 Toluene: Ethyl Acetate: Methanol: 25% Ammonia (30: 30: 15: 1)	254nm			0.8
		366nm	0.91	0.95	0.29 0.48 0.8
Chloroform		254nm			0.83
		366nm	0.13 0.32 0.48 0.62	0.49 *0.57* 0.62 0.81	0.35 *0.5* 0.84 0.92
Ethyl acetate		254nm	0.08 0.16 0.31 0.44		0.25
		366nm	0.13 0.32 0.48 0.62		0.10 0.49 0.85 0.93
Ethanol		254nm	0.25		0.22
		366nm			0.11 0.5 0.87
Water		254nm	0.29		
		366nm			

2. **Flavonoids** - Ethyl Acetate: formic acid: glacial acetic acid: water (10: 0.5: 0.5: 1.3)

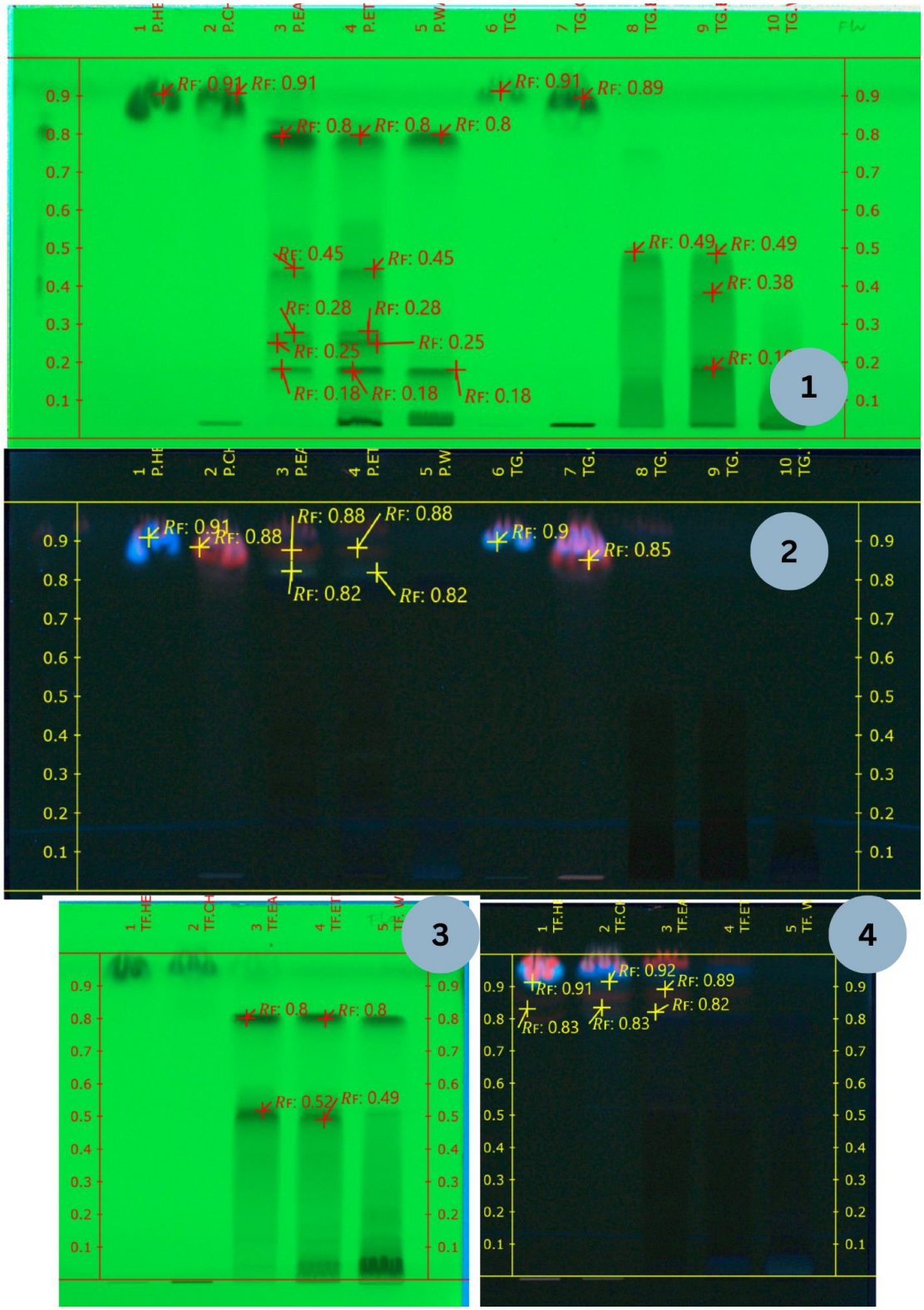


Figure:- 32 HPTLC plates Flavonoids test (1) *P.integerrima* and *T.chebula* galls under 254nm (2) *P.integerrima* and *T.chebula* galls under 366nm (3) *T.chebula* fruit under 254nm and (4) *T.chebula* fruit under 366nm.

Table:- 21 Comparative Rf values for Flavanoids

EXTRACT	SOLVENT SYSTEM	WAVE LENGTH	RF VALUES (*P.integerrima*)	RF VALUES (*T. chebula*) *Gall*	RF VALUES *(T.chebula) fruit*
Hexane	**Flavonoids** Ethyl Acetate: formic acid: glacial acetic acid: water (10: 0.5: 0.5: 1.3)	254nm	0.91	0.91	
		366nm	0.91	0.88	0.83 0.91
Chloroform		254nm	0.91	0.89	
		366nm	0.88	0.85	0.83 0.92
Ethyl acetate		254nm	0.18 0.25 0.28 0.45 0.8	0.49	0.52 0.8
		366nm	0.82 0.88		0.82 0.89
Ethanol		254nm	0.18 0.25 0.28 0.45 0.8	0.19 0.38 0.49	0.49 0.8
		366nm			
Water		254nm	0.18		0.8
		366nm			

3. Glycosides - Ethyl Acetate: Methanol: water (10: 1.5: 1)

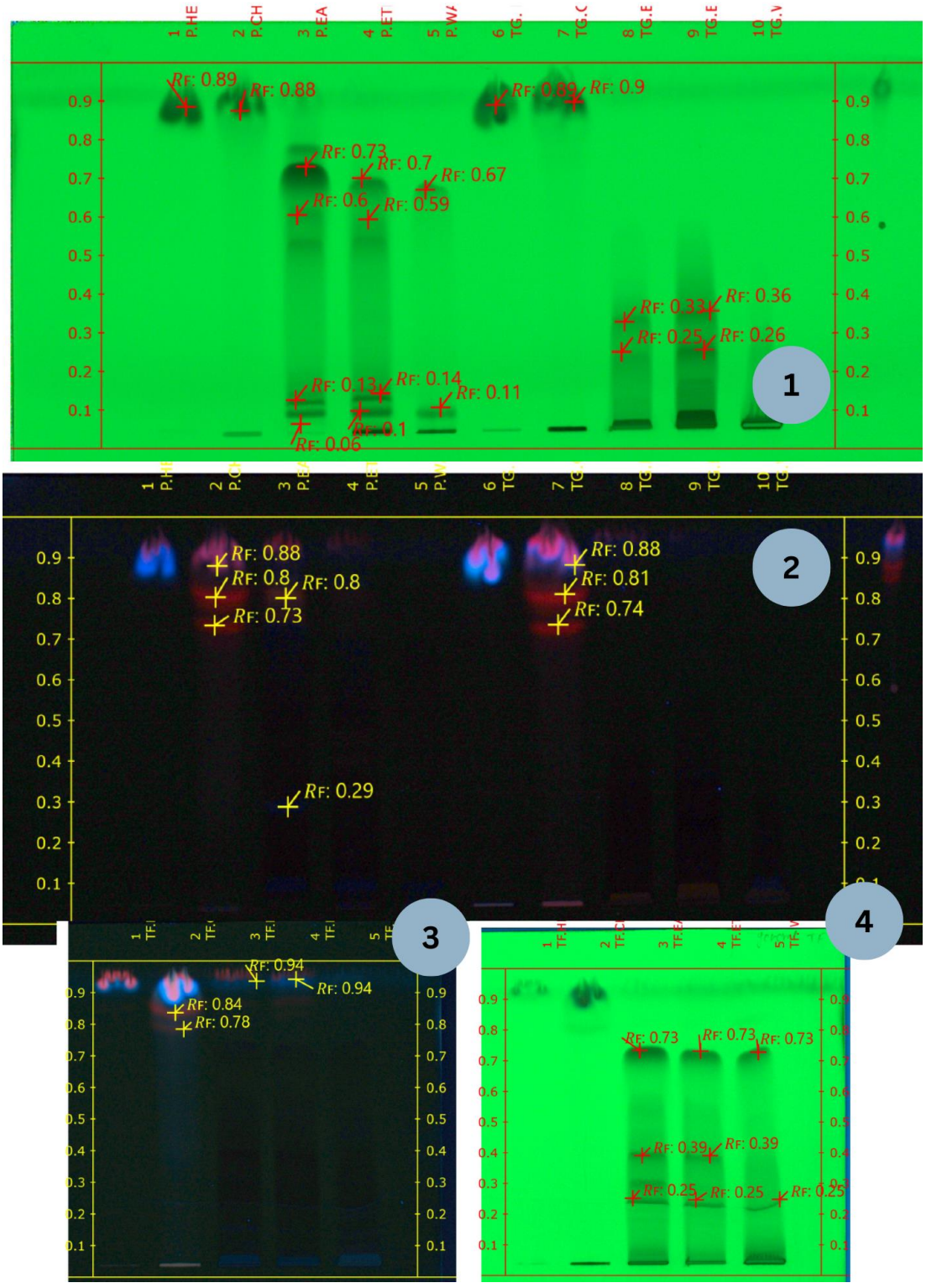


Figure:- 33 HPTLC plates Glycosides test (1) *P.integerrima* and *T.chebula* galls under 254nm (2) *P.integerrima* and *T.chebula* galls under 366nm (3) *T.chebula* fruit under 366nm and (4) *T.chebula* fruit under 254nm.

Table:- 22 Comparative Rf values for glycosides

EXTRACT	SOLVENT SYSTEM	WAVE LENGTH	RF VALUES (*P.integerrima)*	RF VALUES (*T. chebula) Gall*	RF VALUES *(T.chebula) fruit*
Hexane	**Glycosides** Ethyl Acetate: Methanol: water (10: 1.5: 1)	254nm	0.89	0.89	
		366nm			
Chloroform		254nm	0.88	0.9	
		366nm	0.73 0.8 0.88	0.74 0.81 0.88	0.78 0.84
Ethyl acetate		254nm	0.6 0.13 0.6 0.73	0.25 0.33	0.25 0.39 0.73
		366nm	0.29 0.8		
Ethanol		254nm	0.1 0.14 0.6 0.7	0.26 0.36	0.25 0.39 0.73 0.94
		366nm			
Water		254nm	0.11 0.67		0.25 0.73
		366nm			

4. **Terpenes** - n-hexane: Ethyl Acetate (1: 1)

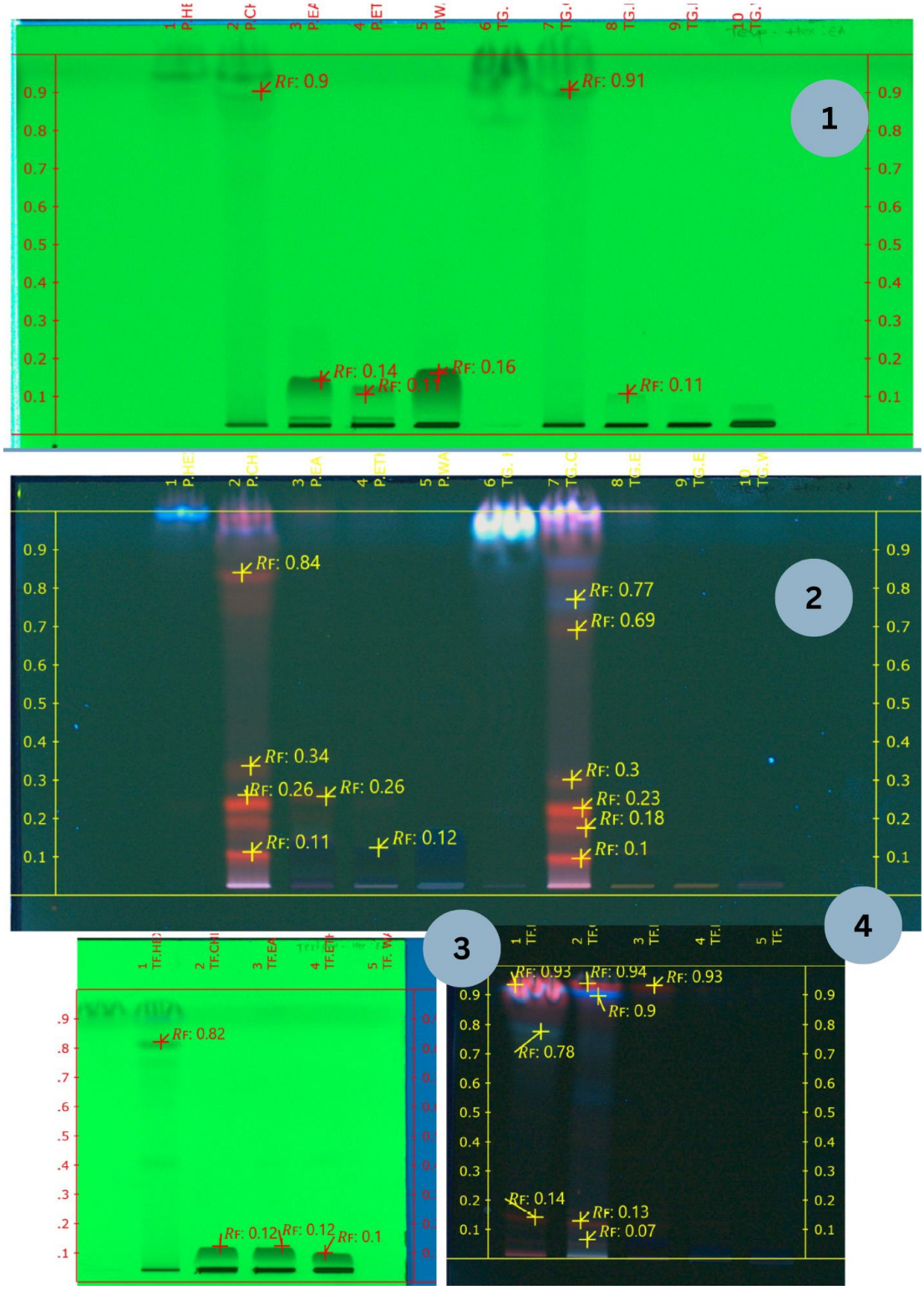


Figure:- 34 HPTLC plates Terpenes test (1) *P.integerrima* and *T.chebula* galls under 254nm (2) *P.integerrima* and *T.chebula* galls under 366nm (3) *T.chebula* fruit under 254nm and (4) *T.chebula* fruit under 366nm.

Table:- 23 – Comparative Rf values for Terpenes

EXTRACT	SOLVENT SYSTEM	WAVE LENGTH	RF VALUES (*P.integerrima)*	RF VALUES (*T. chebula) Gall*	RF VALUES *(T.chebula) fruit*
Hexane	**Terpenes** n-hexane: Ethyl Acetate (1: 1)	254nm			
		366nm			0.14 0.78 0.93
Chloroform		254nm	0.9	0.91	0.12
		366nm	0.11 0.26 0.34 0.84	0.1 0.18 0.23 0.3 0.69 0.77	0.17 0.13 0.9 0.93
Ethyl acetate		254nm	0.14	0.11	0.1
		366nm	0.26		0.93
Ethanol		254nm 366nm	0.11 0.12		
Water		254nm	0.16		
		366nm			

5. **Polyphenols** (mobile phase) – Tetra HydroFuran: Toluene: Ethyl Acetate: Water (16: 8: 2: 1)

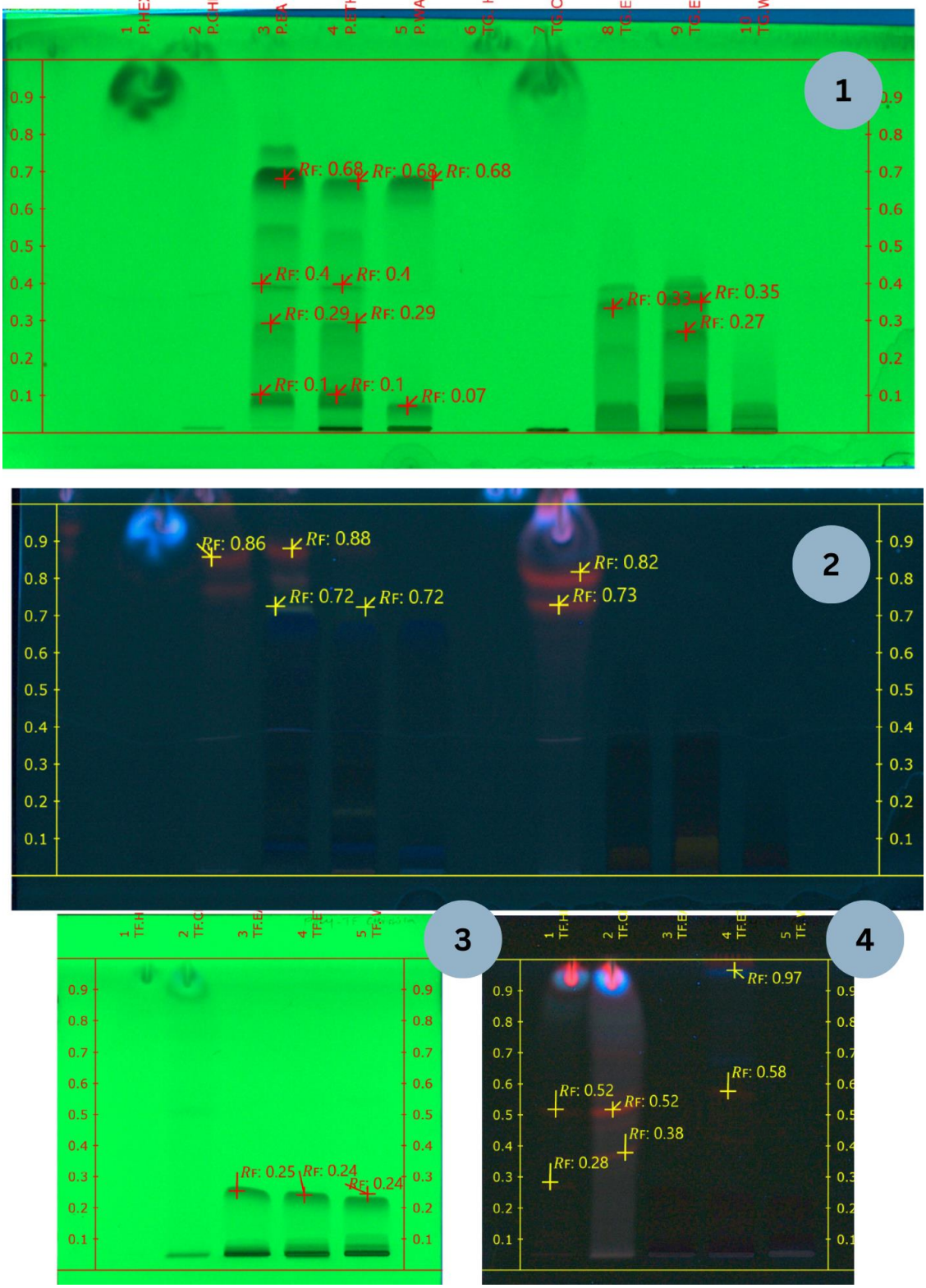


Figure:- 35 HPTLC plates Polyphenols test (1) *P.integerrima* and *T.chebula* galls under 254nm (2) *P.integerrima* and *T.chebula* galls under 366nm (3) *T.chebula* fruit under 254nm and (4) *T.chebula* fruit under 366nm.

Table:- 23– Comparative Rf values for Polyphenols

EXTRACT	SOLVENT SYSTEM	WAVE LENGTH	RF VALUES (*P.integerrima)*	RF VALUES (*T. chebula) gall*	RF VALUES *(T.chebula) fruit*
Hexane	**Polyphenols** Tetra HydroFuran: Toluene: Ethyl Acetate: Water (16: 8: 2: 1)	254nm			
		366nm			0.28 0.52
Chloroform		254nm			
		366nm	0.86	0.73 0.82	0.38 0.52
Ethyl acetate		254nm	0.1 0.29 0.4 0.68	0.33	0.25
		366nm	0.72 0.88		
Ethanol		254nm	0.1 0.29 0.4 0.68	0.27 0.35	0.24
		366nm	0.72		0.58 0.97
Water		254nm	0.7 0.68		0.24
		366nm			

❖ The HPTLC fingerprinting shows the presence of

- Alkaloids' most common bands were seen in *P.integerrima, T.chebula* galls *and T.chebula* fruits chloroform and ethyl acetate extract.

- Flavonoids' most common band were seen in *P.integerrima, T.chebula* galls *and T.chebula* fruits ethyl acetate and ethanol extracts.

- Glycosides' most common bands were seen in *P.integerrima, T.chebula galls and T.chebula Fruits* chloroform, ethyl acetate and ethanol extract.

- Terpenes' most common band were seen in *P.integerrima, T.chebula* galls *and T.chebula* fruits chloroform extract.

- Polyphenols' most common band were seen in *P.integerrima, T.chebula* galls *and T.chebula* fruits chloroform, ethyl acetate and ethanol extract.

3.5 Pharmacology (*Drosophila melanogaster*)

3.5.1 Fecundity

Haritaki is mentioned in Ayurveda with potential Rasayana values (Dr Chakraborty Subhrajyoti, n.d.; Narendra, n.d.; Santosh Poudel, n.d.). However, the same fruits have been mentioned as drying up the *Shukra dhatu* (reproductive tissues, especially the semen) (Sarangadaharacarya & Dr. P. Himasagara Chandra Murthy, n.d.). Reports also mention the anti-fertility properties of *T.*chebula (Ghosh et al., 2015, 2017; Narendra, 2021).

प्रवर्तनी स्त्री शुक्रस्य रेचनं बृहतीफलम् ।। १७ ।। जातीफलं स्तम्भकं च शोषणी च हरीतकी । (Sa.Pu.4/18)

Therefore it was planned to examine and compare the fertility OR anti-fertility activities of *Kashaya* of *T.chebula* fruit, *T.chebula* galls and *P.integerrima.* One-way ANOVA was performed, and statistical differences were calculated between data sets by a posthoc Tukey analysis which showed P- value as 0.0083(**) and *T.chebula* galls have a significant reduction in egg laying compared to Control and *P.integerrim* galls but not significant to *T.chebula* galls.

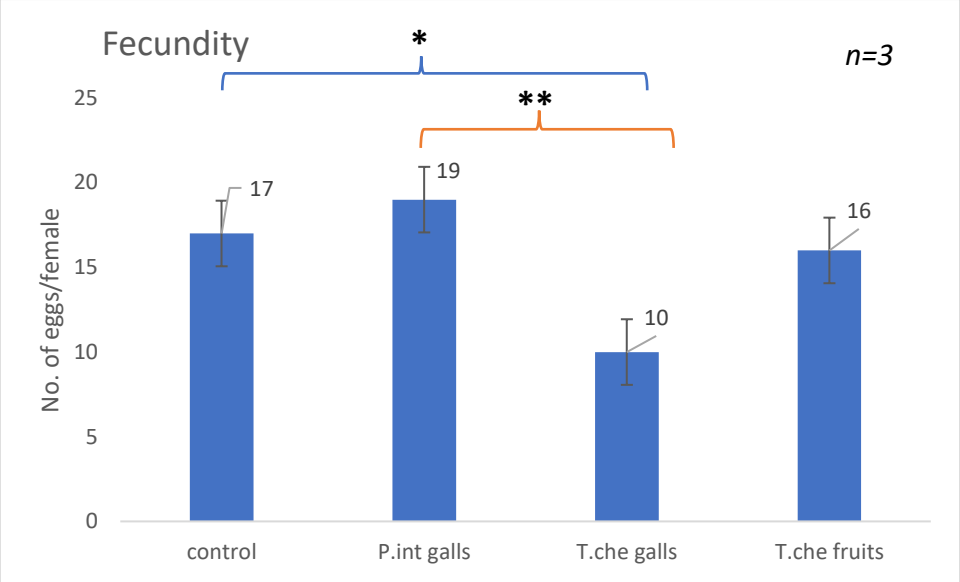


Figure:- 36 Fecundity assay graph (P<0.05*, P<0.01**, P<0.001***)

3.5.2 RT-PCR Gene expression of *relish.*

- The quantitative RT-PCR method starts with the RNA transcribed into Complementary. DNA (cDNA) by reverse transcriptase from messenger RNA (mRNA).
- The cDNA was then used as the template for the *RT-qPCR* reaction. It allows the sensitive, specific, and reproducible quantification of nucleic acids.
- Standardization was done on some percentages of SDS for induction of inflammation
- 0.6% SDS was used to induce inflammation. After three days and six days of treatment, *RT-qPCR* was performed to check *Relish* m-RNA expression levels and found a decrease in gene expression on both days, as seen in the graph (Figures: - *37 and 38).*

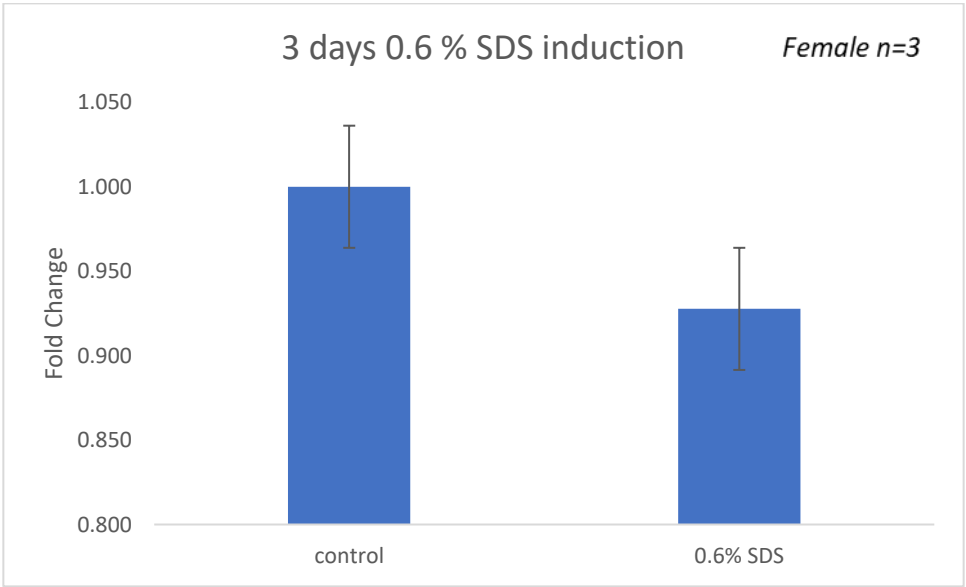


Figure:- 37 Gene expression of *relish* for 0.6% SDS induction three days

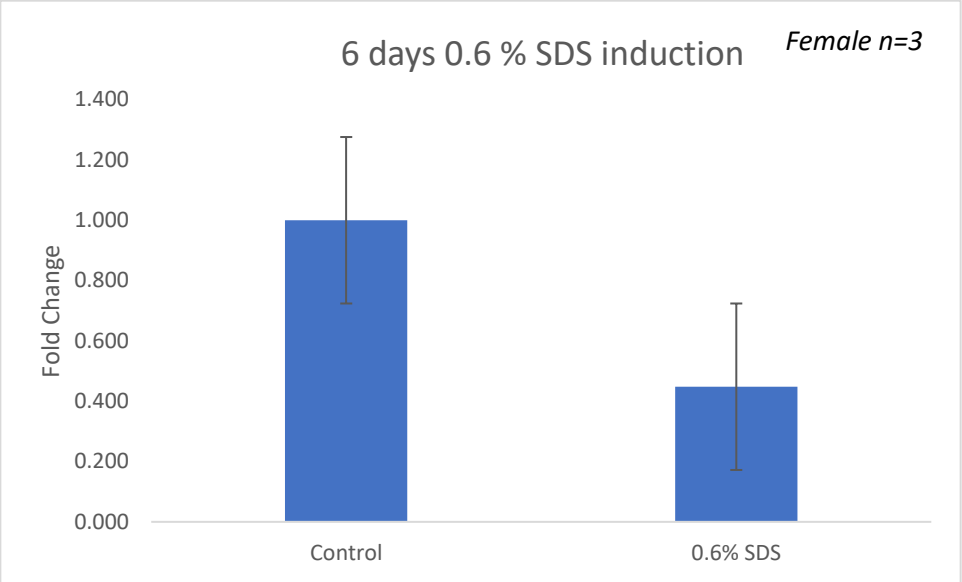


Figure:- 38 Gene expression of *relish* for 0.6% SDS induction six days

- 1.5% SDS was used to induce inflammation three days of treatment was given. Then *RT-qPCR* has performed on three days induced flies as flies did not survive in 1.5% of media for six days. *Relish* m-RNA expression levels were checked, and a decrease in gene expression was seen, as shown in the graph (Figure:- *39).*

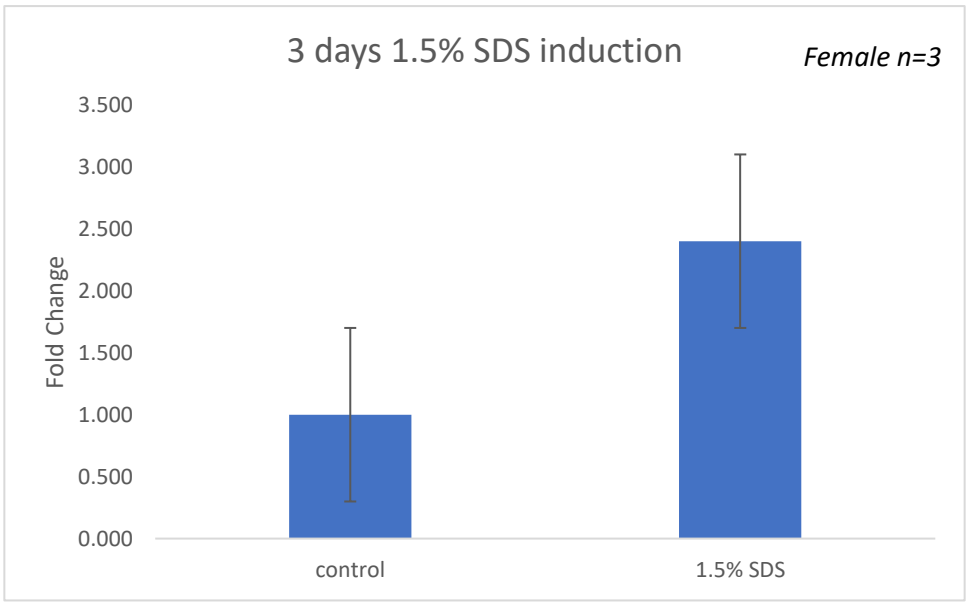


Figure:- 39 Gene expression of *relish* for 1.5 % SDS induction three days

- *शृङ्गी कर्कटशृङ्गी च स्यात्कुलीरविषाणिका ।*
  अजशृङ्गी च चक्रा च कर्कटाख्या च कीर्त्तिता ॥ (Bha.pra.ni.purv.khand.Haritakyadi varga.178)
  शृङ्गी कषाया तिक्तोष्णा कफवातक्षयज्वरान् ।
  श्वासोर्ध्ववाततृकासहिक्काऽरुचिर्वमीन्हरेत्॥ (Bha.pra.ni.purv.khand.Haritakyadi varga.179)
  Śṛṅgī (P. integerrim) is astringent, bitter, hot potency, mitigates *Kpha, Vata*, consumption, fever, dyspnoea (*Swasa hara*), hiccups, loss of taste, appetite and vomiting(Bhavaprakasha& Prof. K.R. Srikantha Murthy, n.d.).
  P.integerrim is used for *Swasa* (respiratory) problems; therefore Anti- Inflammation studies were planned.
- Flies were co-treated with 1.5% SDS and 5% concentration of the respective Drug (*Kashaya*) for three days, one group was subjected to SDS treatments for three days, and the other group was fed on normal food for three days post-treatment RNA was isolated, and *RT-qPCR* was performed to check m-RNA expression levels of *relish*.
  - In the SDS group, the mRNA expression levels of *relish* increased by 2.635 fold.
  SDS+ 5%*P.integerrima* galls showed a 2.1-fold decrease compared to the SDS group, and S
  DS+ 5% *T.chebula* galls showed a 2.44 fold decrease compared to the SDS group,
  SDS+ 5% *T.chebula* Fruit showed a 2.74-fold reduction as compared to the SDS group. The graph is shown in (Figure:- *40).* One-way ANOVA was performed, and statistical differences were calculated between data sets by a post hoc Dunnett's analysis which showed a P value of 0.046(*) and *kashaya* of galls of *P.integerrim* showing significantly less induction as compared to 1.5% SDS. However, *Kashaya* of galls and fruits of *T.chebula* also showed less induction compared to 1.5% SDS; the values were insignificant.

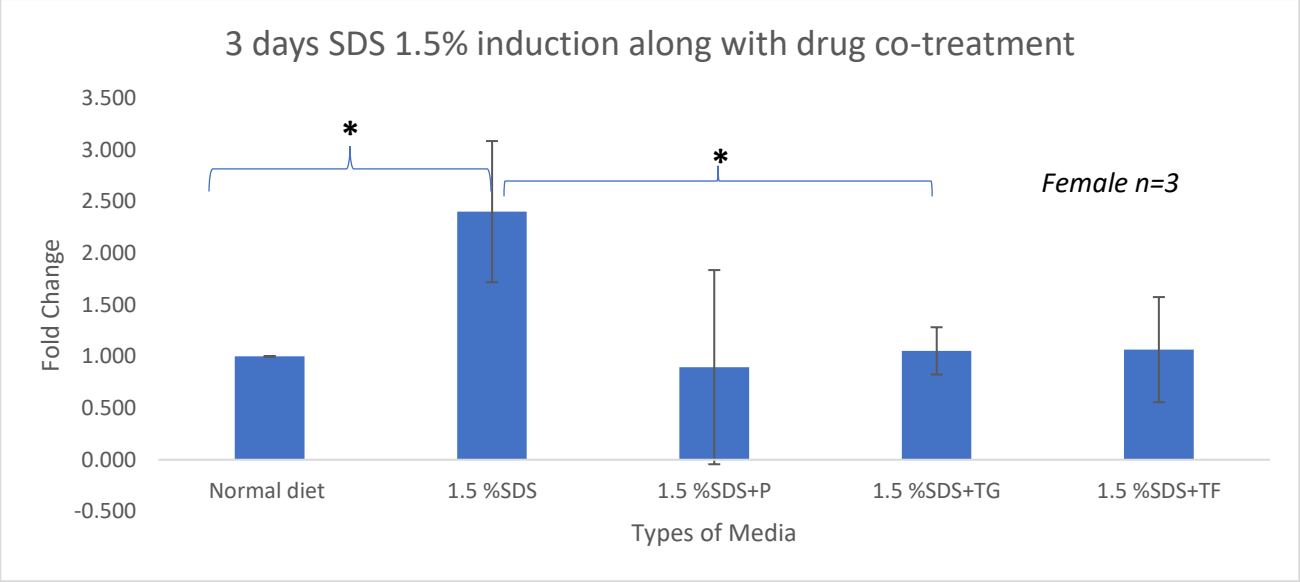


Figure:- 40 Gene expression of *Relish* for Co-treatment of the respected drugs. (P) *Kashaya* of P.integerrima galls,(T.G.) *Kashaya* of T.chebula galls and (T.F.) *Kashaya* of T.chebula fruits (P<0.05*, P<0.01**, P<0.001***).

3.6 Discussion

The results of this study indicate that *P.integerrima* and *T.chebula* galls have a variety of potential medicinal properties. A pharmacognosy study showed the presence of different phytochemicals and types of cell arrangement in both species of leaf galls. A phytochemistry study (HPTLC) revealed the presence of terpenes, polyphenols, and flavonoids with most bands in successive extracts of galls of *P.*integerrima, *T.chebula,* and *T.chebula* fruits. These compounds are known to have a variety of pharmacological activities, including antioxidant, anti-inflammatory, and anti-microbial effects (Ahmad et al., 2010; Shankara et al., 2012; Uddin et al., 2011; Uddin & Rauf, 2012). The pharmacology study on *D. melanogaster* suggests that the fruits and galls of *T.chebula* could potentially reduce fecundity. This is important for people planning to use *T.chebula* for medicinal purposes, as it could impact fertility(C & A, 2017; Ghosh et al., 2017). The inflammation induced by SDS with drug co-treatment was reduced compared to only SDS treatment. This suggests that galls of *P.integerrima*, *T.chebula* and *T.chebula* fruits may have anti-inflammatory properties.

The results of this study suggest that there is potential for further research on the medicinal properties of *P.integerrima* and *T.chebula* galls. Some specific areas for future research could include:

- A more detailed study on leaf gall development and species-level Identification of insects causing the infestation on leaves of *T.chebula.*
- A more detailed study on the phytochemical test or quantification of *P.integerrima* and *T.chebula* galls.

- A study of the pharmacological effects of *P.integerrima* and *T.chebula* on other animal models, such as rats or mice, can include an anti-histaminic study.
- As *P.integerrima* is not used directly as a single drug, it is used in polyherbal formulations, for example, Shringyadi churna (Rawat et al., 2019) *P.integerrima* can be substituted with *T.chebula* and then checked for the pharmacology and phytochemical properties.
- Further research can help to confirm these findings and determine the optimal dosage and administration route for *T.chebula* to treat various conditions.
- This type of research on substitutes can reduce the misuse of medicinal plants and the human-induced pressure on endangered original species and benefit conservation.

4. Conclusion

- The galls of *T.chebula* get mature within 65 – 70 days.
- A pharmacognosy study showed the presence of different phytochemicals and differences in cell arrangement in *P.integerrima, and T.chebula* of leaf galls.
- Phytochemistry study (HPTLC) revealed the presence of alkaloids, terpenes, glycosides and flavonoids by showing standard bands in successive extract yield of *P.integerrima, T.chebula galls and T.chebula* fruits.
- The pharmacology study on *D. melanogaster* suggests that the fruits and galls of *T.chebula* could potentially reduce fecundity.
- The inflammation induced by SDS compared with drug co-treatment groups showed less *relish* gene expression.
- These results indicate that *T.chebula* galls can be used in the absence of *P.integerrima* galls; however, the use of the galls of *T.chebula* for an extended period can cause infertility in the long term.
- Further, a systematic study to explore the anti-histamine activity of *P.integerrima and T.chebula* galls may be required to establish the mode of action of this species.
- This study intends to find the similarities between substitutes and the original, which will reduce the misuse of the substitutes and the anthropogenic pressure on the original endangered species and thus benefit its conservation.

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