BIOPROSPECTIVE OF *Citrus sinensis* Linn. LEAVES -- ITS VOLATILE OIL AS A WEAPON ON CONVULSION IN ZEBRA FISH LARVAE SEIZURE MODEL

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MASTER OF PHARMACY
IN
BRANCH III- PHARMACOGNOSY

Submitted by

Mr.T.PRABAHAR

(Reg.No: 261620705)

UNDER THE GUIDANCE OF

Dr. K.PERIYANAYAGAM, M.Pharm, PhD.,

DEPARTMENT OF PHARMACOGNOSY

COLLEGE OF PHARMACY
MADURAI MEDICAL COLLEGE
MADURAI - 625 020

MAY 2018

CERTIFICATE

This is to certify that the dissertation entitled **BIOPROSPECTIVE OF *Citrus sinensis* Linn. LEAVES -- ITS VOLATILE OIL AS A WEAPON ON CONVULSION IN ZEBRA FISH SEIZURE LARVAE MODEL** is a bonafide workdone by **Mr.T.PRABAHAR (Reg.No:261620705),** Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625020 in partial fulfilment of the Tamil Nadu Dr. M.G.R. Medical University rules and regulations for award of **MASTER OF PHARMACY IN PHARMACOGNOSY** under my guidance and supervision during the academic year 2017-2018.

Name & Signature of the Guide:

Name & Signature of the Head of Department:

Name & Signature of the Dean/Principal:

TAMIL NADU AGRICULTURAL UNIVERSITY
DEPERTMANE OF HORTICULTURE
AGRICULTURAL COLLEGE AND RESEARCH INSTITUTE
MADURAI– 625 104

Dr. T.N. BALAMOHAN, Ph.D., PDF.,
Professor and Head (Hort.)

Phone : 0452-2424956
E-mail : hortimac@tnau.ac.in

Date: 06.09.2017

CERTIFICATE

This is to certify that leaves of *Citrus sinensis* belongs to the Family – Rutaceae and called as *Sathukudi* in tamil were collected from the Department of Horticulture, Agricultural College and Research Institute by the Department of Pharmacognosy, Madurai Medical College, Madurai for research purpose.

Professor and Head
Dept. of Horticulture
PROFESSOR AND HEAD
Department of Horticulture,
Agrl. College & Research Institute,
Madurai – 625 104.

Place: AC&RI, Madurai

Dr. D.STEPHEN, M.Sc.,Ph.D.,
ASSISTANT PROFESSOR
MADURAI-625002

DEPARTMENT OF BOTANY
THE AMERICAN COLLEGE

CERTIFICATE

This is to certify that the specimen brought by Mr.T.Prabahar,II M.Pharm, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai is identified as *Citrus Sinensis* belonging to the family Rutaceae.

Station : Madurai.

Date : 06.09.2017.

(Dr.D.STEPHEN)

Dr. D. STEPHEN, Ph.D.,
ASST. PROFESSOR IN BOTANY
THE AMERICAN COLLEGE
MADURAI - 625 002
TAMILNADU-INDIA

HERBARIUM

NAME T. PRABAHAR.

Reg.No. 261620705

COLLEGE/SCHOOL COLLEGE OF PHARMACY
MADURAI MEDICAL COLLEGE

NAME : CITRUS SINENSIS

FAMILY : RUTACEAE

GENUS : CITRUS

SPECIES : SINENSIS

LOCALITY : AGRICULTURAL COLLEGE, MADURAI

DATE : 06.09.2017.

Date :

Dr. D. STEPHEN, Ph.D.,
ASST. PROFESSOR IN BOTANY
THE AMERICAN COLLEGE
MADURAI - 625 002
TAMILNADU-INDIA

Professor/Teacher-in-charge

Dr.K.PERIYANAYAGAM M.Pharm.,Ph.D.
Professor & Head
Department of Pharmacognosy
College of Pharmacy
Madurai Medical College
MADURAI - 625 020

Manonmaniam Sundarannar University
Constituent Model College,
NAGALAPURAM TK,
Thoothukudi TK. 628904

Sub:

Our ref:

Your Ref:

Date:19.09.2017

Prof. (Major) P.Chandra Sekaran,
Principal,MSU Constituent Model College
Nagalapuram 628 904.
Formerly Faculty of PG & Research
Department of Zoology and Biotechnology
Vivekananda College (Autonomous)
Tiruvedakam west 625217.

CERTIFICATE

This is to certify that the specimen brought by **Mr.T. PRABAHAR**, II M.Pharm. Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai has been identified as, *"Danio rerio"*, family: Cypriniade

Station: Madurai

Date:

Prof (Major) P.CHANDRASEKARAN,
PRINCIPAL,
MS University Constituent Model College,
NAGALAPURAM,
Vilathikulam Taluk. Thoothukudi Dt 628 904
majorpcs@yahoo.co.in

E-Mail id:

Phone: 04638-242666

Fax No:

Dedicated to my Guru, Family

&Friends

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INTRODUCTION

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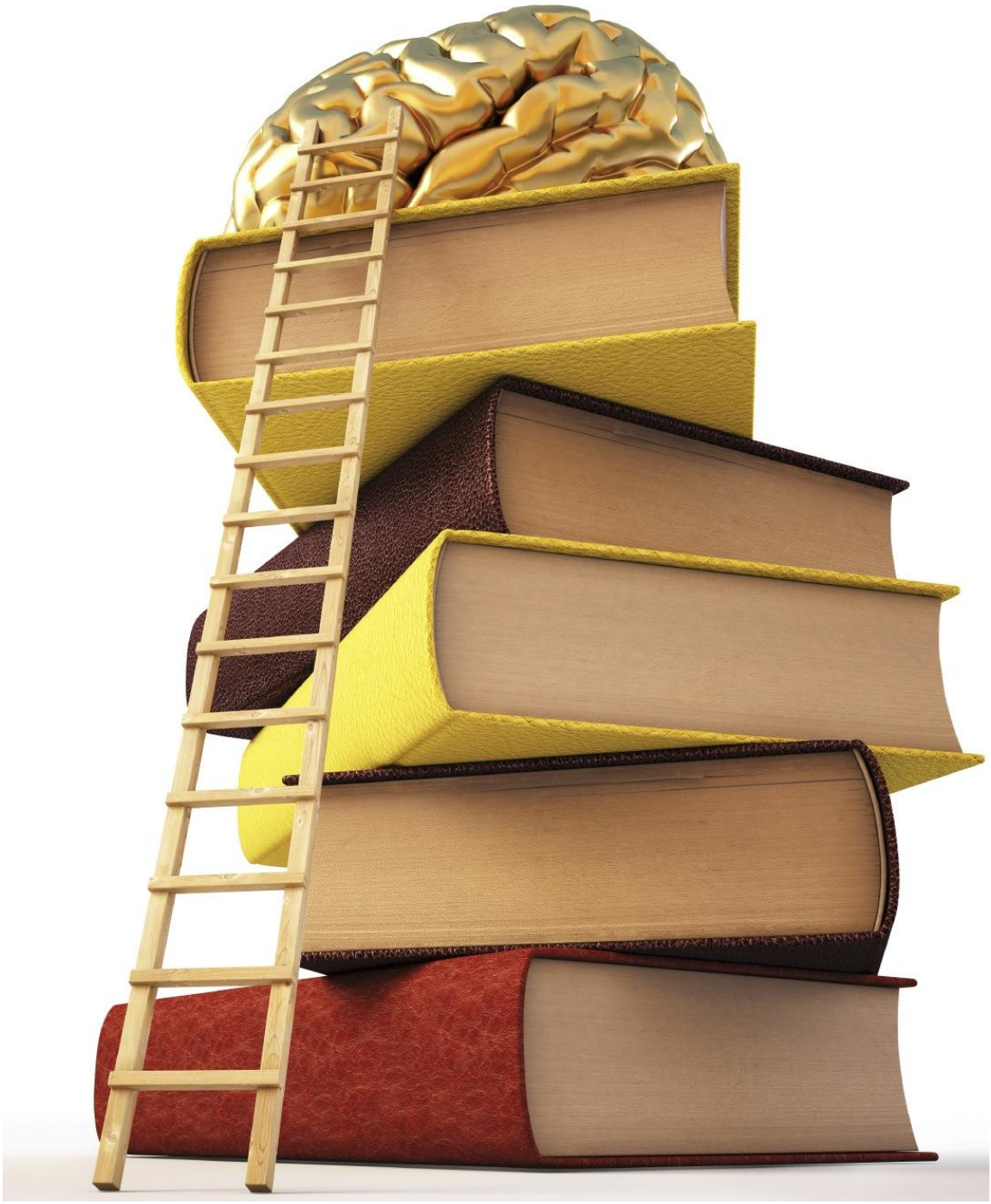

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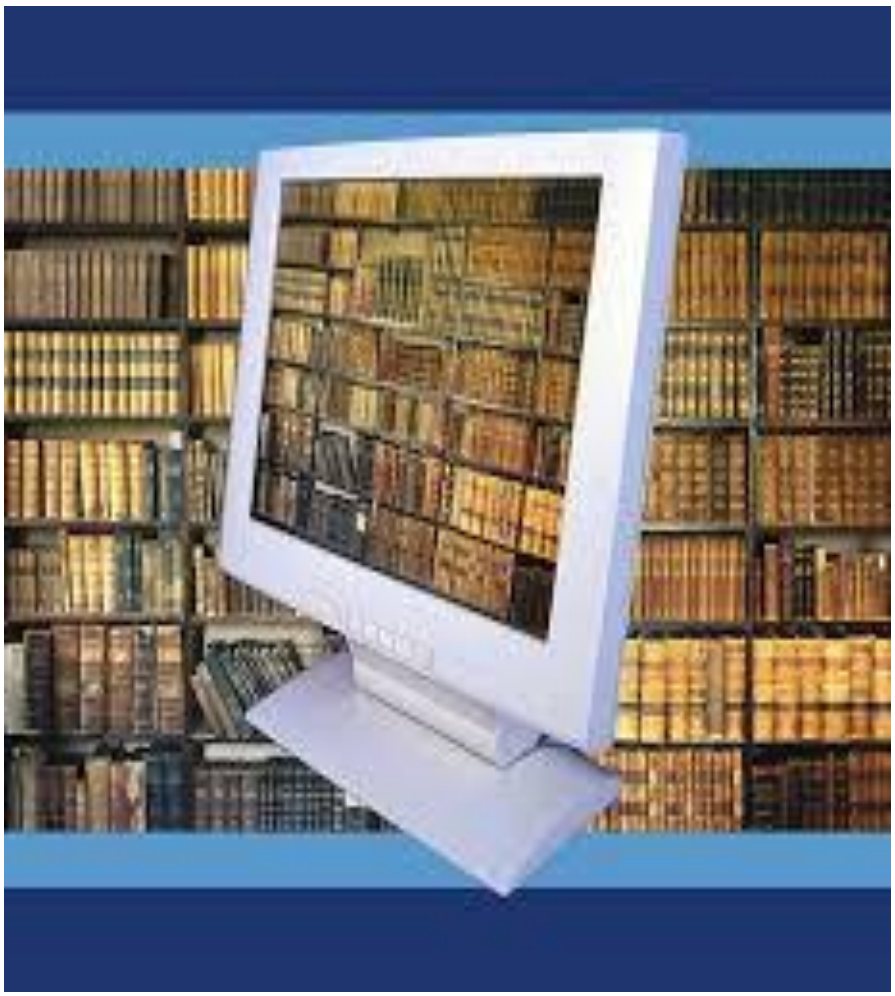



Thank you!

INTRODUCTION

Traditional medicine has been commonly known as folk medicine consists of medical knowledge that has been improved over generations within various societies starting the era of modern medicine. The herbal industry distributes about US $100 billion with good growth potential. The European community botanical medicine introduces an import share of the pharmaceutical market. Natural products compounds introduces from medicinal plants (and their analogues thereof) have proved numerous clinically useful drugs in the treatment of chronic and or acute disease and mainly used as an essential component in the search for new medicines. So, these traditionally used plants can be explored effectively in order to find New Chemical Entity for the treatment of chronic and acute disease. Hence the small but having greater future perspectives review was performed systematically.

HISTORY OF HERBAL MEDICINE

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Siddha, Ayurveda, Unani) in which herbal therapies were used. The consumption of plant-based medicines and other botanicals in the West has increased manifold in recent years. About two centuries ago, our medicinal practices were largely dominated by plant-based medicines. However, the medicinal use of herbs went into a rapid decline in the West when more

predictable synthetic drugs were made commonly available. In contrast, many developing nations continued to benefit from the rich knowledge of medical herbalism.

Herbal medicine was also an effective method, but was viewed less enthusiastically. Herbal products were discarded from conventional medical use in the mid-20th century, not necessarily because they were ineffective but because they were not as economically profitable as the newer synthetic drugs.

In the early 19th century, scientific methods become more advanced and preferred, and the practice of botanical healing was dismissed as quackery. In the 1960s, with concerns over the important effects of conventional medicine and desire for more self-reliance, interest in “natural health” and the use of herbal products increased. Recognition of the rising use of herbal medicines and other non-traditional remedies led to the establishment of the office of Alternative Medicine by the National Institute of Health USA, in 1992. Worldwide, herbal medicine received a boost when the WHO encouraged developing countries to use traditional plant medicine to fulfil needs unmet by modern systems.

TRENDS IN HERBAL MEDICINE USE

Worldwide it is estimated that 80% of the population uses herbs in the developing world rates could be as high as 95%. The U.S. continues to see an increase in the use of herbs. The most recent national survey conducted in 2007 by the National Center for Complementary and Alternative Medicine (NCCAM) showed that 17.7 % of adults have used natural products (primarily herbs) in a one year period. Complementary and alternative medicine (CAM) was used most

commonly by whites (43.1%) followed by Hispanics (23.7%).. Another recent trend in Western countries involves adding herbs to energy drinks and weight loss and nutritional products.

MEDICINAL PLANTS

The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities. These medicinal plants consider as a rich resources of ingredients which can be used in drug development and synthesis. Besides that these plants play a critical role in the development of human cultures around the whole world. Moreover, some plants consider as important source of nutrition and as a result of that these plants recommended for their therapeutic values. These plants include ginger, green tea, walnuts and some others plants. Other plants their derivatives consider as important source for active ingredients which are used in aspirin and toothpaste.

FUTURE OF MEDICINAL PLANTS

Medicinal plants have a promising future because there are about half million plants around the world, and most of them their medical activities have not investigate yet, and their medical activities could be decisive in the treatment of present or future studies.

CHARACTERISTICS OF MEDICINAL PLANTS

Medicinal plants have many characteristics when used as a treatment, as follow:

• **Synergic medicine**- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.

• **Support of official medicine**- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.

• **Preventive medicine**- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment. Therefore it is a very important point for the open access journals to encourage researchers and clinicians to work hard in order to clarify the main active ingredients which can be extracted from medicinal plants. Moreover, to clarify their role in the treatment of present diseases, and how they can be used to produce or synthesis more effective drugs. (Bassam Abdul Rasool Hassan, 2012)

PRESENT STATUS OF HERBAL MEDICINE

The wide spread use of herbal medicine is not restricted to developing countries, as it has been estimated that 70% of all medical doctors in France and German regularly prescribe herbal medicine. The number of patients seeking herbal approaches for therapy is also growing exponentially. With the US Food & Drug Administration (FDA) relaxing guidelines for the sale of herbal supplement.In the last few decades, a curious thing has happened to botanical medicine. Instead of being killed off by medical science and pharmaceutical

chemistry, it has made come back. Herbal medicine has benefited from the objective analysis of the medical science, while fanciful and emotional claims for herbal cures have been thrown out, herbal treatments and plant medicine that works have been acknowledge. And herbal medicine has been found to have some impressive credentials. The penicillin that replaced mercury in the treatment of syphilis and put an end to so many of the deadly epidemics comes from plant mould. Belladona still provides the chemical used in ophthalmological preparations and in antiseptics used to treat gastrointestinal disorders. *Rauvolfia serpentina* (The Indian snake root) which has active ingredient, reserpine, was the basic constituent of a variety of tranquilizer first used in the 1950‟s to treat certain types of emotional and mental problems. Though reserpine is seldom used today for this purpose, its discovery was a breakthrough in the treatment of mental illness. It is also the principal ingredient in a number of modern pharmaceutical preparations for treating hypertension.(Manish *et al*., 2015*)*

HERBAL MEDICINE AND EPILEPSY

Herbal medicine is one of the most common forms of complementary and alternative medicine and patients generally consider this form of treatment to be both safe and effective.

The use of complementary and alternative medicine is on the rise, including among patients with epilepsy. Herbal medicine, one of the most popular forms of CAM, is considered to be both safe and effective by most consumers. Yet many herbs may increase the risk for seizures, through intrinsic proconvulsant properties or contamination by heavy metals, as well as via

effects on the cytochrome P450 enzymes and P-glycoproteins, altering antiepileptic drug (AED) disposition.

Herb–drug interactions may be difficult to predict, especially since the quality and quantity of active ingredients are often unknown. Since most patients do not inform their physicians that they are taking herbal medicines, health care professionals must initiate a dialogue in order to prevent complications with the combined regimen. At the same time, AED treatment. (N Samuels *et al*., 2008)

EPILEPTIC ACTIVITY

Epilepsy is a common neurological disorder marked by abnormal electrical discharges in the brain, which typically involves sudden brief episodes of altered or diminished consciousness.

About 65 million people worldwide are estimated to have epilepsy, and about 1% of people in the United States have had an unprovoked seizure at least once. There are many known epileptic syndromes. While each affects the nervous system in different ways, seizures are the most common phenotypes. To date, a wide range of antiepileptic drugs have been developed with anticonvulsant activity, each of which acts via a different mechanism. For instance, benzodiazepines make GABA receptors more effective, whereas carbamazepine and lamotrigine block sodium and calcium channels. Despite the wide range of discoveries, 30% of people suffering from epilepsy fail to respond to antiepileptic drugs (AEDs). Also, many marketed AEDs are associated with substantial side effects, including seizure liability. This is called the seizure liability of a drug and is a substantial adverse side effect. Unfortunately, seizure

liability can only be identified in later stages of pre-clinical safety studies on higher vertebrates, which does not allow sufficient time to reduce this risk by chemical modification. More effective antiepileptic therapeutics with fewer side effects are therefore needed.

Seizures are induced by different mechanisms. Therefore, a broad understanding of mechanisms of seizures is essential for AED discovery. For this, a large-scale model of different seizure mechanisms would be desirable. There are many drugs associated with inducing seizures (convulsants), which could help construct this large-scale seizure model.

A high-throughput screening of different seizure mechanisms of these convulsants would be even more desirable. If these different controlled epileptic states could be screened, we would be able to test many candidate AEDs on these controlled epileptic states. Additionally, this could also help chemically modify a drug on the market, which is reported as causing seizures as a side effect. For instance, if we identify a drug as a potassium channel blocker, it can be chemically modified with phenytoin.

ZEBRA FISH: A POWERFUL ANIMAL MODEL

The zebra fish (*Danio rerio*) has become a widely used vertebrate **model** organism for drug discovery because of its high availability, transparent embryos and larvae, morphological and physiological similarity to mammals, allowing *in vivo* analysis of embryogenesis and organogenesis combined with ease of use and low cost. Zebra fish as model organism, fields of applications, advantages

and limitations of this vertebrate model system and then cited the utility of this model in the drug discovery and toxicity studies.

Zebra fish are omnivorous, primarily eating zooplankton, insects, insect larvae and phytoplankton. The approximate generation time for *Danio rerio* is three to four months. Adult females are able to laying 200-300 eggs in each clutch. Upon release, embryonic development begins after the first few cell divisions immediately become transparent, a characteristic that makes *Danio rerio* a convenient research model species . Development progresses very rapidly. Precursors to all major organs appear within 36 hours of fertilization and hatching takes place 12-36 hours later, depending on the embryo's internal conditions and the external temperature, swimming and feeding behavior begin about 36 hours later.

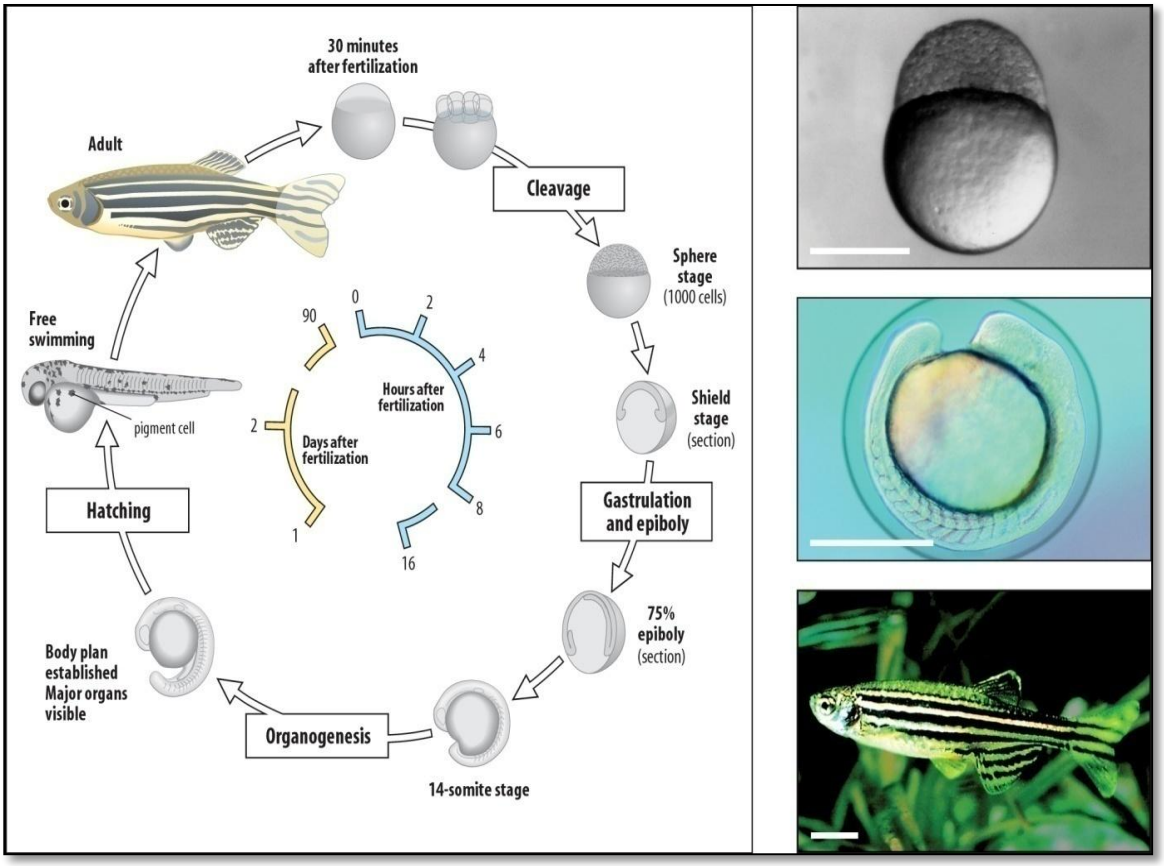
The zebra fish promises to contribute to several aspects of the drug development process, including target identification, disease modelling, lead discovery and toxicology.

The numerous advantages of this whole animal approach provide new promise for the discovery of safe, specific and powerful new drugs. The zebrafish as model organism provides vast new opportunities in future Model organisms are chosen on the basis of characteristics such as short life-cycle, techniques for genetic manipulation (inbred strains, stem cell lines and methods of transformation) and non-specialist living requirement *In vivo* studies represent an essential step in drug development and rely largely on mice. But there are several limitations of mammalian models which motivated the search for

complementary, vertebrate model systems. So now it is focused on Zebra fish (*Danio rerio*) the facile model system to study human and animal disease and drug responses.

The Zebrafish is a tropical freshwater fish and is an important vertebrate model organism in scientific research. It is particularly notable for its regenerative abilities and has been modified by researchers to produce several transgenic strains.

ZEBRA FISH: AN EXCELLENT ANIMAL MODEL



REASON FOR THE STUDY AND SELECTION OF THE PLANT:

Epilepsy is a serious and complex set of neurological conditions, which affects 65 million individuals worldwide, and more than 85% of these patients live in developing countries.

Although the majority of patients with epilepsy will achieve remission, seizures in up to 30% will become drug-resistant despite treatment with adequate doses of appropriate antiepileptic drugs (AEDs).

Approximately 30–40% of patients with epilepsy suffer from numerous side effects from the standard AEDs. In the context of this predicament, there is a constant search for novel modes of treatment. Over thousands of years, individuals with epilepsy have used a variety of botanicals and herbs, which are considered natural and are generally regarded as safe in many instances. Botanical therapies may potentially yield new treatment options for patients whose seizures are uncontrolled despite available AEDs and may also represent inexpensive, culturally acceptable treatments for the millions of individuals worldwide with untreated epilepsy. (Fenglai Xiao, *et al*., 2015).

We still need better treatments and increased understanding of mechanisms of epilepsy in humans. The lack of novel antiepileptic drugs (AEDs) represents a challenge, requiring screening of multiple new compounds and pathways relevant to epilepsy, new compounds and pathways relevant to epilepsy.

Several plants used for the treatment of epilepsy in different systems of traditional medicine have shown activity when tested in modern bioassays for the detection of anticonvulsant activity and many such plants are yet to be scientifically investigated. *Citrus sinensis* common name sweet orange belong to Rutaceae family widely cultivated all over the world. It bears sweet smelling rounded fruits which are deep yellow to orange in colour. Many substances have been isolated from *C.sinensis* leaves like glycosides, ruteosides, flavonoids, hesperidin, diosmin, triterpene, limonene etc . Leaf extracts of *C.sinesis* have been used in Nigerian local folk medicine to treat neurological diseases. It also reported that methanolic extract posseses sedative activity. (Nagula JB and Reddy NL 2017). *C.sinensis* plant is used for treating wide panel of diseases.

Though there is traditional and experimental evidences to support various claims and benefits of this plant still it needs proper evaluation and exploitation.

A study aims to scientifically explore its important medicinal uses which have not been fully studied is inevitable. These initiated us to investigate the leaves of this plant with strict scientific protocols so that the vast economic potentiality of this crop can be properly exploited.

Based on the above discussion we focus our study to utilize the vast economic potentiality, which was fully established by its vast consumption. It is evident that there is a good level of experimental evidence to support claims and advantages of various medicinal herbs used in our traditional diet and medicines. In this view we selected the widely available plant *Citrus sinensis L.* for our study.

Review of literature showed some lacunae exist in the pharmacognostic, phytochemical and pharmacological studies of *C.sinensis.*

WHOLE PLANT

TAXONOMICAL CLASSIFICATION (P Milind P and Dev C 2012).

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Dicotyledons
Subclass	:	Sapindales
Order	:	Rosidae
Family	:	Rutaceae
Subfamily	:	Aurantoideae
Genus	:	Citrus
Species	:	Sinensis

INDIAN SYNONYMS OF *CITRUS SINENSIS*

Language	:	Name
Hindi	:	Orange
Bengali	:	Kamla, nembu, Musambi
Tamil	:	Sathgudi
Malayalam	:	Nagaranga
Punjabi	:	Malta
Marathi	:	Mosambi
Telugu	:	Sathgudi, mosambi
Gujarathi	:	Naringi, santra,
Oriya	:	Naranga

ETHANOMEDICAL USE

Citrus sinensis is consumed all over the world as an excellent source of vitamin C, which is a powerful natural antioxidant that builds the body's immune system.

It has been used traditionally to treat ailments like constipation, cramps, colic, diarrhoea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress. (Juan Manuel J *et al*., 2016).

Citrus sinensis is used to manage malaria as well as skin diseases (Morton 1987).

HISTORY

Citrus sinensis tree is mostly cultivated and rarely found in the forests. It was primarily grown for its medicinal purposes. Italian traders might have introduced it to the Mediterranean area after 1450 AD. It was quickly adopted as an edible fruit. It was so highly regarded that wealthy persons grew oranges in private conservatories, called orangeries. Spaniards introduced the sweet orange into South America and Mexico in the mid-1500s. In 1646, orange was well-known in Europe. They were introduced in Florida by Spanish explorer Juan Ponce de León, in 1753 and were introduced to Hawaii in 1792. The first record of citrus, *Citrus medica* L, was done by Theophrastus, in 350 BC, and was introduced as a fruit by Alexander. In early European history, writers wrote about Persian citrus, that it had a wonderful fragrance and was thought to be a remedy for poisoning, a breath sweetener, and a repellent to moths. The citron was the first of the citruses to be known in Europe.

Alexander the Great used orange fruit as a perfume first and then as a food. Orange trees had existed on American soil, years before the declaration of independence, and commercial cultivation in Florida (in 1820s) and California (in 1870s). (Milind P and Dev C 2012). *Citrus sinensis* is commonly known as sweet orange (Mosambi) (Anonymous 1992) **.** Sweet orange (*Citrus sinensis* L. Osbeck) commonly called orange is a member of this family. (Angew 2007). where it was referred to as ‘’Chinese’’ apple (Ehler 2011).

GEOGRAPHICAL DISTRIBUTION

C. sinensis is native to Asia and is now widespread throughout the Pacific and warm areas of the world . (Juan Manuel *J et al.,* 2016).

The main production regions of oranges are found in United States of America (led by Brazil, Mexico, and Argentina), the Mediterranean basin (led by Spain, Italy, Egypt, and Turkey), and the South and East Asian regions (led by China, India, and Japan). (Milind P and Dev C 2012).

Oranges probably originated from south East Asia, and were cultivated in China by 2500 BC (Nicolosi *et al.,*2008).

BOTANICAL DESCRIPTION

Sweet Orange is an evergreen flowering tree. Height of orange tree is generally 9–10 m (although very old specimens have reached 15 m). The leaves 4-10 cm long arranged alternately, are ovate in shape with crenulated margins. Trees have thin smooth, and grey-brown to greenish bark. Most species are single-trunked with very hard wood. Canopy widths range from slender to broad, depending on species .The orange fruit is a hesperidium. It is type of berry that ranges widely in size, colour, shape and juice quality. Fruits are globose to ovoid

in shape. Wild orange fruit has a smooth skin, and the petiole of sour orange leaves is much larger than that of sweet orange. The word “orange” is derived from Sanskrit term narang. Sathukudi are round citrus fruits with finely textured skins. It’s colour just like their pulpy flesh. Sweet orange seeds are a greenish to pale whitish, flattened, and angular .Seeds are greenish to pale whitish, flattened, and angular. The seed is generally poly embryonic. The embryos are either “zygotic” or “nuclear.” The zygotic embryos are derived from pollination of the ovary, i.e., sexual reproduction, and therefore are not always similar in horticultural qualities to the parent tree. Diameter of orange flowers is 2–4 cm (0.8–1.6 in). Flowers are axillary, fragrant, single, few or cymose, and often perfect (having both functional stamens and pistils) or staminate. The calyx is 4–5 lobed and there are generally five petals and contain some oil glands Leaves are smooth, oval, 5-15cm x 2-8 cm, dark green, and glossy possessing a distinctive smell often similar to the fruit. Petioles are generally winged. Leaves are unifoliate, ovate, ovular elliptical, with acute to obtuse tips, and contain some oil glands, which are released when crushed. Young twigs are green and angled in cross-section, and axillary single spine, while older twigs and branches are spineless and circular in cross section. (Milind P and Dev C 2012). Stomata in species of the genus Citrus are confined to the ventral surface of the leaves. Along the margin of the leaf the stomata have their long axes parallel to the margin; between lateral veins they are parallel to the veins along the midrib they are parallel to the midrib. (Reed H.S 1931).

CULTIVATION

C. sinensis trees are widely cultivated in tropical and subtropical climates for its tasty juice and medicinal value. (Milind P and Dev C 2012).

ALTITUDE : up to 1500 m, Mean annual temperature: 10-35 ˚C.

RAIN FALL: 100-120cm.

SOIL TYPE:

Trees will grow in almost any soil type if well aerated. The optimum conditions for citrus orchards are fertile, light to medium, well-drained, deep, loose loams soils with a high water table should be avoided. The species is sensitive to excess salts.

pH RANGE :

5-8 pH is preferred. (Orwa *et al.,*2009).

PROPAGATION

Propagation through seed is associated with problems like poor pollen production, self-incompatibility and muscular embryo (Mortton 1987).

POLLINATION

Orange blossoms yield very little pollen and orange growers do not practice artificial pollination. However, there is evidence of self-incompatibility and need for cross- pollination in the TANGOR and TANGELO. Self-pollination is facilitated by citrus flowers having both sexes present on the same blossom. Cross-pollination is used only by some cultivars, occurs in tangerines and tangerine hybrids, mandarins. Honeybees are mostly used for cross pollination.

The most important period for pollination was the morning in the studied crops. The beginning of fructification in sweet orange flowers depends on the number of honeybee visits. Honey bee pollination influenced quantity and quality of fruit production. The flowers frequently visited by bees produced heavier, less acid fruit, with fewer seeds per bud. (Milind P and Dev C 2012).

CHEMICAL COMPOSITION

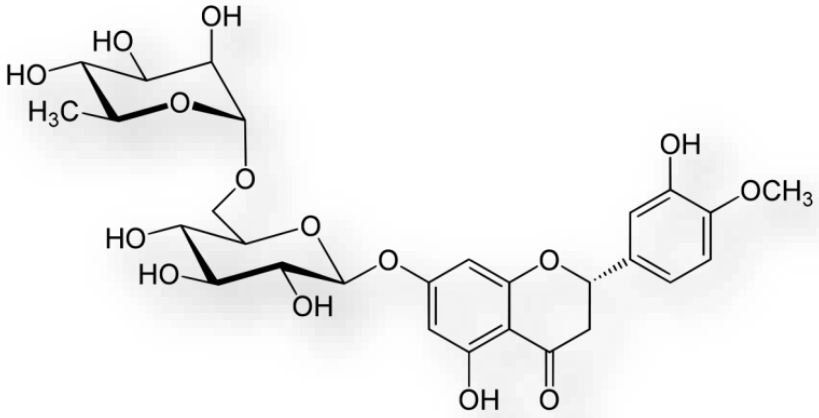
It was reported that *C. sinensis* is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits, peel, leaves, juice and roots of *C. sinensis*, which include the following groups: flavonoids, steroids, hydroxyamides, alkanes and fatty acids, coumarins, peptides, carbohydrates , carbamates and alkyl amines , carotenoids ,volatile compounds , and nutritional elements such as potassium, magnesium, calcium and sodium . (Juan Manuel *J et al.,*2016).

Citrus sinensis has been found to be a valuable source of essential oil. The components include bioflavonoids, carbohydrates, terpenoids. (Morton 1987).

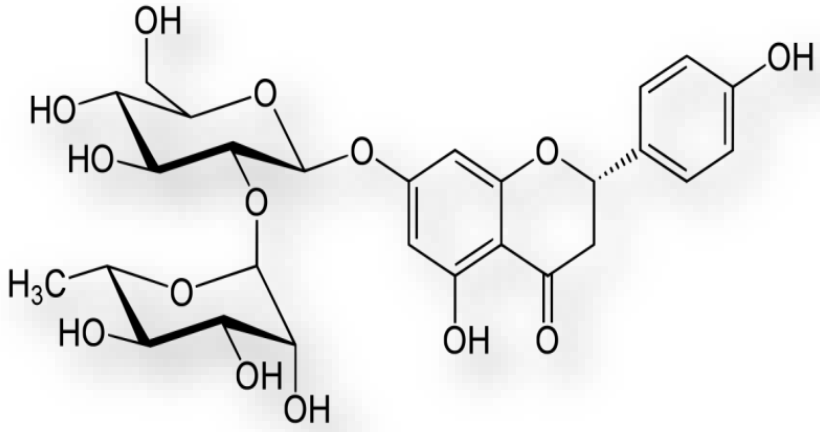
It was reported that to contain major source of vitamins, especially vitamin C, sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium.(Angew 2007).

Citrus sinensis reported that most prevalent flavanones present in *Citrus sinensis* are hesperidin, naringin, tangeretin and nobiletin. (Intekhab J and Aslam M,2009).

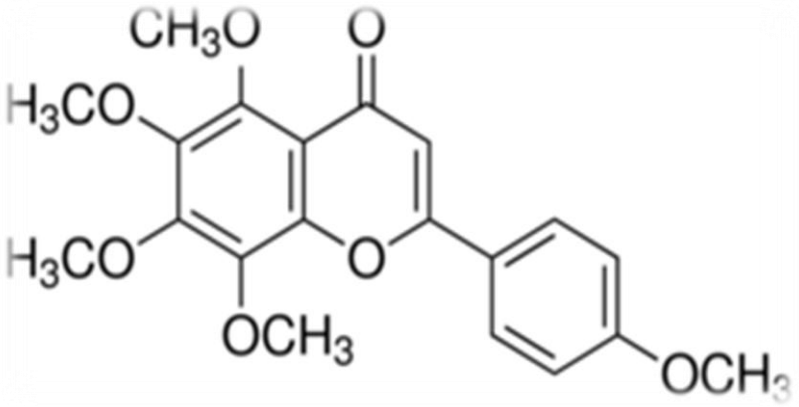
HESPERIDIN



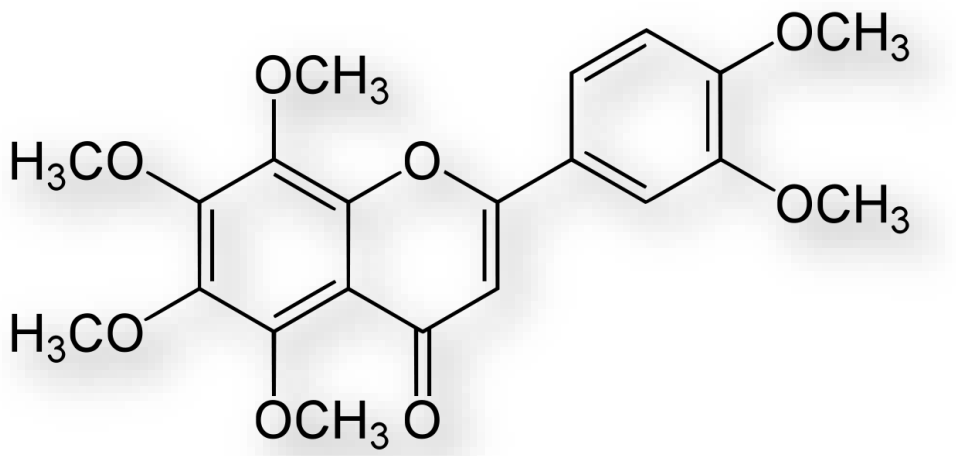
NARINGIN



TANGERETIN



NOBILETIN



PHARMACOLOGICAL ACTION

It was reported that *Citrus sinensis* possess antioxidant, antibacterial, antifungal, anti-carcinogenic, anti-ulcer, anti-anxiety, anti-diabetic and anti-inflammatory properties .(Milind P, Dev C 2012).

ANTI-INFLAMMATORY ACTIVITY

It was reported that Citrus flavonoids contain compounds with anti-inflammatory activity due to the presence of regulatory enzymes (protein kinase C, phosphodiesterase, phospholipase, lipoxygenase, and cyclooxygenase) that control the formation of the biological mediators, responsible for the activation of endothelial cells and specialized cells involved in inflammation. Flavonoid inhibition of the immune and inflammation responses can be associated with their inhibition of these enzymes.(Tripoli *et al.,* 2007).

Citrus flavonoids are able to inhibit the kinases and phosphodiesterase's essential for cellular signal transduction and activation. They also affect the activation of a number of cells involved in the immune response, including T and B lymphocytes (Manthey *et al*., 2001).

It was showed that Citrus flavonoids also prevented atherosclerosis, inhibiting the formation of atheroma (Hertog *et al*., 1993). Tripoli *et al,* (2007) reported that hesperidin obtained from citrus cultures may have a potential therapeutically use as a mild anti-inflammatory agent, being also useful as a precursor of new flavonoids endowed with this activity (Da Silva *et al.*, 1994). Studies using mouse macrophage cells also show that hesperidin has an inhibitory effect on lipopolysaccharide (LPS)-induced over expression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), over-production of prostaglandin E2 and nitric oxide (NO) (Sakata *et al.*, 2003).

ANTI-CANCER AND ANTI-ARTERIOSCLEROSIS ACTIVITY:

Citrus flavonoids can prevent cancer through selective cytotoxicity, antiproliferative actions and apoptosis. Flavonoids are ant mutagenic, thus protects the DNA from damage by their ability to absorb ultraviolet light. They neutralize free radicals that promote mutations when they are generated near DNA. This has been shown in mice body irradiated with c-ray. Flavonoids can also protect the DNA by interacting directly with the tumoral agents, as in the induced chromosomal aberrations by bleomycin. The inhibitory effect of citrus flavonoids on tumoral development and cell proliferation by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats have been reported. The ability to

function as such by citrus flavonoids are based on cell mobility inhibition. Oranges are also rich in iron, chlorine, manganese, zinc, sodium, phosphorous, iodine, calcium, folic acid, potassium, pectin, beta-carotene and amino acids and fibre. A single orange is said to have about 170 phytonutrients and over 60 flavonoids with anti-tumour, anti-inflammatory blood clot inhibiting and antioxidant properties. All these properties help to promote overall health. (Etebu, *et al.*, 2014)

ANTI-OBESITY:

Sweet oranges contain low calories and no saturated fats or cholesterol, but they are rich in dietary fibre, pectin which is very effective in persons with obesity. Pectin as bulk laxative protects the mucous membrane from exposure to toxic substances, as well as by binding to cancer causing chemicals in the colon. Pectin has also been shown to reduce blood cholesterol levels by decreasing its re-absorption in the colon by binding to bile acids in the colon. Orange peels contain the alkaloid synephrine, which reduces the production of cholesterol in the liver. The antioxidant elements in oranges combat oxidative stress that oxidizes the LDL (low-density lipoprotein) in the blood. (Etebu, *et al*.,2014).

LEAF

ETHANOMEDICAL USE

Leaf extracts of *C. sinensis* have been used in folk medicine to treat **neurological disorders** and to facilitate the digestion of food. (Intekhab J and Aslam M 2009).

PHARMACOGNOSY:

Leaves are smooth, oval, 5-15cm x 2-8 cm, dark green, and glossy possessing a distinctive smell often similar to the fruit. Petioles are generally winged. Leaves are unifoliate, ovate, ovular elliptical, with acute to obtuse tips, and contain some oil glands, which are released when crushed. Young twigs are green and angled in cross-section, and axillary single spine, while older twigs and branches are spineless and circular in cross section. (Milind P and Dev C 2012).

PHYTOCHEMISTRY

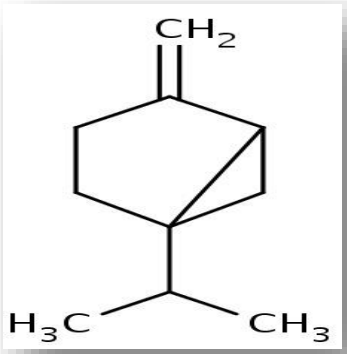
It was reported that the ethanol extract to contain leucoanthocyanins, Gallic tannins catechin tannins and saponins. (Yovo M *et al.,* 2016)**.**

The hydro distilled fresh leaves of VOCSL to contain α – Pinene , l – Limonene , cis-Sabinene hydrate , Citral , Lavandulol, Perillaldehyde, Perillyl alcohol, tert-Butyl benzoate (Singh V *et al.,* 2015).

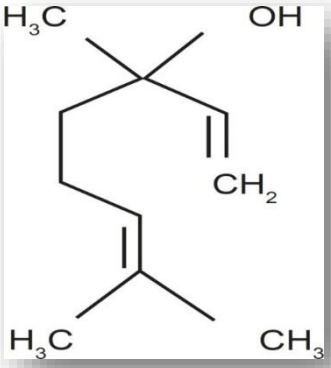
The leaf ethanolic extract was reported that FT-IR and UV showed the presence of various kinds of terpenoids, alkaloids, tannins, saponins and flavonoids in it**.**(Yamuna J, Anthony N, 2015).

It was reported that leaf oils of three major commercial varieties of sweet orange growing in Israel were investigated using GC/MS. The study revealed that all three varieties belong to the same chemical type. Each of the forty-five identified constituents was found in the three essential oils examined. Major components were found to be sabinene **(15.81-32.58%)** linalool **(5.13-20.92%)** and Gamma terpinene **(7.41-10.50%).** (Fleisher and Fleisher A 2015).

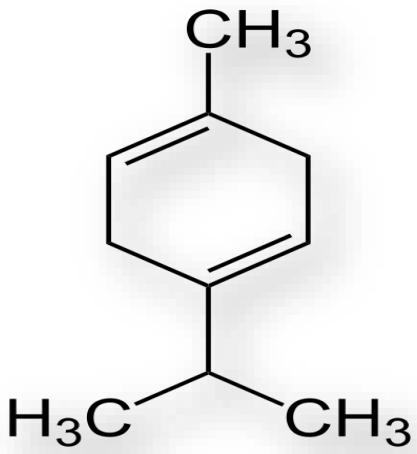
SABINENE



LINALOOL



Gamma.-Terpinene



PHARMACOLOGICAL ACTION

It was reported that leaf extract of this family was evaluated for its antagonistic activity on the hypertensive action of angiotensin (Reddy NL *et al* .,2016).

It was reported used to kill mosquito larvae and mites. There are strong evidences showing that the essential oil of *Citrus sinensis* have larvicidal, repellent and fumigant activities against *Aedes aegypti L* (Intekhab J and Aslam M 2009).

Citrus sinensis reported that most prevalent flavanones present in *Citrus sinensis* are hesperidin, naringin, tangeretin and nobiletin. Hesperidin was shown to have anti-inflammatory, antihypertensive, diuretic, analgesic and hypolipidemic properties. It also has antioxidant, anti-allergic, vasoprotective and

anti-carcinogenic actions. Nobiletin is a novel anti-inflammatory and immune modulatory drug. It has been shown to have an inhibitory effect on phorbol ester induced skin inflammation, oxidative stress and tumour promotion in mice. (Intekhab J and Aslam M 2009).

It has also been used as an antidiabetic, antibacterial, antifungal, hypotensive, antioxidant, insect repellent, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent due to the presence of copious oil**.** (Muhammad N.O *et al* ., 2013).

ANTIMICROBIAL ACTIVITY

It was reported that the antimicrobial activities of volatile oil of fresh leaves Citrus *sinensis* were performed in various oil concentrations were studied by the cup plate method. Chloramphenicol and Ketoconazole were used as standard and the activity of each concentration was compared with the corresponding concentration of standard drugs. Different concentrations of oil of *Citrus sinensis* were compared by measuring the diameter of the zone of inhibition. The control DMSO showed no inhibition of growth, while all the concentrations of oil were effective against *Escherichia coli* & *Staphylococcus aureus* when compared to chloramphenicol and Ketoconazole (Singh V *et al*.,2015).

It was reported that leaf aqueous extract of *Citrus sinensis* had conspicuous zone of inhibition on *Escherichia coli* that is 7.00mm in diameter while on the other test organisms, it had little or no zone of inhibition as well as the effect of the ethanol extracts on all the test organisms whose zones of inhibition was not greater than 3.00mm.On the contrary, 2mg/ml of gentamycin(positive control) showed wide zones of inhibition on all the test

organisms which is incomparable to the 100mg/ml concentration of the plant extracts. Dimethyl sulphoxide (DMSO) negative control shows no zone of inhibition. It was completely resistant to all the test organisms.(Ekwenye U N and Edeha O.V 2010).

ANTICONVULSANT ACTIVITY

The hydro ethanolic leaf extract was concentrated in a rotary evaporator. Standard Drug Sodium valproate: (Encorate Inj.). The dose usually used in animal is 150 mg/kg given Intraperitoneally 60 minutes before the induction of epilepsy. The anticonvulsant property of hydro-ethanolic leaf extract was evaluated in albino mice by the following method. Maximal Electric Shock (MES) induced convulsions in mice Maximal electric shock (MES) induced convulsions in mice: Twenty four male albino mice with bodyweight between 21 and 36g were divided into 4 groups with six mice each. Each group is treated as follows:

Group I: CONTROL; received distilled water0.5ml p.o.

Group II: STANDARD; Sodium valproate in a dose of 150mg/kg i.p.

Group III: TEST 1; *Citrus sinensis* (hydro ethanolic leaf extract) 50mg/kg p.o.

Group IV: TEST 2; *Citrus sinensis* (hydro ethanolic leaf extract) 100mg/kg p.o.

Percentage of inhibition of seizures with standard drug and test drug was calculated compared with control group. (Reddy NL *et al*.,2016).

ANTI EPILEPTIC ACTIVITY

It was reported that leaf extract of *Citrus sinensis* have been used to treat neurological disease to evaluate the anti-epileptic property of hydro-ethalonic leaf extract of *Citrus sinensis*. The method utilized was the ability of *Citrus*

sinensis to inhibit Pentylenetetrazole (PTZ) induced convulsions in mice. The mice were divided into 4 groups having 6 mice each with Group I receiving distilled water, Group II receiving Sodium valproate 150mg/kg i.p. Group III receiving *Citrus sinensis* hydro-ethanolic extracts of 50mg/Kg p.o. and Group IV *Citrus sinensis* 100mg/kg p.o. The mice were closely observed for 30 minutes and the following parameters were recorded, Onset of convulsion, Duration of convulsion and Time of recovery Percentage of inhibition of seizures compared with standard drug Sodium valproate. Antiepileptic properties are lesser when compared to sodium valproate. It may be used as an adjuvant therapy along with standard anticonvulsant drugs in seizure prevention.(Nagula1 J B, Reddy N L 2017).

ANTI-INFLAMMATORY ACTIVITY

It was reviewed that anti-inflammatory activity of ethanolic extracts of *Citrus sinensis* leaf. The crude extract and aspirin were separately administered in IP route. Group A was used as negative control thus, received neither aspirin nor the crude extract group B received 250 mg/ml of the extract. Group C received 200mg/ml, group D received 100 mg/ ml while group E received 50 mg/ml of the extract. Group F was used as positive control, thus, received 100mg/ml of aspirin. The animals were left for 30mins after which 1 ml of fresh egg albumen was injected into the sub-plantar of the right hind paw of each of the rats. Using a Vernier calliper, the diameter of the paw was measured and recorded at 30mins intervals for 3hrs. Aspirin (100mg/ml) was used as standard. Percentage inflammation and inhibition of inflammation were calculated. (Omodamiro O. D. and Umekwe J. C 2013).

ANTI-BACTERIAL ACTIVITY

It was showed that Antibacterial activity properties of ethanolic extracts of *Citrus sinensis* leaves .The disc agar diffusion method was used in this study. The test organisms (1:100 dilution of an 18h broth cultures) were inoculated onto nutrient agar plates with sterile cotton swabs soaked in the Inoculate. Disc of different extract concentrations were placed firmly on the surface of the inoculated agar plates and incubated at 37oc for 18hrs under aerobic conditions. Zones of inhibition were measured and recorded in millimetres. . (Omodamiro O. D. and Umekwe J. C 2013).

ANTITUBERCULAR ACTIVITY:

Methanolic Extract of *Citrus Sinensis* Leaf was reported 200µl of sterile de ionized water was added to all outer perimeter wells of sterile 96 well plates to minimized evaporation of medium in the test wells during incubation. The 96 well plates received 100 µl of the Middle brook 7H9 broth and serial dilution of compound were made directly on plate. The final drug concentration tested were 100µgm/ml to 0.8µgm/ml. Plates were covered and sealed with paraffin and incubated at 37˚C for five days. 25 µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hours various concentrations 100 gm/ml,50 µgm/ml,25 µgm/ml, 12.5 µgm/ml, 6.25 µgm/ml,3.125 µgm/ml,1.6 µgm/ml, 0.8 µgm/ml in different solvents like water, methanol, ethanol, chloroform and diethyl ether . Here MIC is defined as the lowest drug concentration which prevents the colour change from blue to pink. Higher concentration of 100µgm/ml all the solvent showed sensitivity

against Mycobacterium tuberculosis while lowering the concentration shows resistance by the bacteria. Water soluble portion showed significant activity also at 50µg/ml. (Manish K *et.al.,*2013).

CORROSION INHIBITOR ACTIVITY

It was reviewed that values of corrosion rates and inhibition efficiencies obtained from mass loss measurements using *Citrus sinensis* extract (CSE) and of different concentrations of biocides (*Ocimum sanctum*). When the concentration of biocides was increased, the inhibition efficiency was also increased .

It was reviewed that values of corrosion rates and inhibition efficiencies obtained from mass loss measurements of using *Citrus sinensis* extract (CSE) and of different pH value. When the pH was increased, the inhibition efficiency was decreased.

It was reported that values of corrosion rates and inhibition efficiencies obtained from mass loss measurements of different concentrations of *Citrus sinensis* extract (CSE). 3 ml of the CSE offered 92.1% corrosion inhibition efficiency to carbon steel immersed in 100 ml solution. When the concentration of CSE was increased, the inhibition efficiency was decreased. This is due to the fact that when higher concentrations of *Citrus sinensis* are added the protective film (Fe2+– *Citrus sinensis* complex) formed on the metal surface goes into the solution and thus destroying the protective film. It may be considered that the protective film formed may go into transpassive state, where the film is broken. (Yamuna J, Anthony N 2015).

FLOWER

PHARMACOGNOSY

Diameter of orange flowers is 2–4 cm (0.8–1.6 in). Flowers are axillary, fragrant, single, few or cymose, and often perfect (having both functional stamens and pistils) or staminate. The calyx is 4–5 lobed and there are generally five petals and contain some oil glands. Number of stamens range from 20 to 40. The sub globose ovary is superior, with 8–18 locules (cavities), with 4–8 ovules per locule in two rows. Flowers are small, waxy greenish-white. (P Milind P and Dev C,2012).

Flowers are axillary borne singly or in whorls of 6 (5 cm wide) with five white petals and 20–25 yellow stamens (Juan Manuel *J et al.,* 2016).

FRUIT

ETHNOMEDICAL USES:

- Orange juice helps to eliminate toxins from the body.
- Orange juice helps to maintain hydration.
- It is used as a general tonic.
- Orange juice is useful in cases of anxiety disorder and stress.
- It is used as a Mexican traditional medicine for the treatment of tuberculosis.
- It is used in stomach upsets; it improves appetite and prevents constipation.
- The humble Orange has a long history in Chinese Medicine as a cooling agent for coughs, colds and respiratory disorder.

- It is a traditional Chinese symbol of good luck and prosperity.
- It is used in the treatment of obesity.
- Orange symbolizes innocence and fertility.
- In France, it is used for the treatment of angina, hypertension, constipation, diarrhea, menstrual disorder and Palpitation. (Milind P and Dev C,2012).

PHARMACOGNOSY

The fruit may be globose to oval (6.5 to 9.5 cm wide) and ripens to orange or yellow. Anatomically, the fruit consists of two distinct regions, the pericarp, also called the peel, skin or rind, and the endocarp or pulp with juice sac glands. The skin consists of an epidermis of epicuticular wax with numerous small aromatic oil glands that give its particular smell. The pericarp consists of the outer flavedo or epicarp, largely made of parenchymatous cells and cuticle. The fruit usually contains a sweet pulp and several to numerous seeds within . The fruit pulp is typically formed of eleven segments of juice filled with flavour that goes from sour to sweet. In orchards it is sensitive to frost. The fruit is perennial and it has adapted to a variety of climates. (Juan Manuel *J et al.,* 2016).

PHYTO CONSTITUENTS

It was reported that *Citrus sinensis* fruit contains phytochemical compounds like polyphenols, anthocyanine and hydroxyl cinnamic acid (Ahmed N e*t a* .,2016).

NUTRITIONAL VALUE

A single orange provides 12.5% of the daily need for fiber, which has been shown to reduce high cholesterol levels thereby helping to prevent atherosclerosis. Fibers also help in keeping blood sugar levels under control, which may explain why oranges can be a very healthy snack for people with diabetes. In addition, the natural fruit sugar present in oranges, viz., fructose can help to prevent blood sugar levels from rising too high after eating. The fiber in oranges can grab cancer-causing chemicals and keep them away from cells of the colon, providing yet another line of protection from colon cancer. Furthermore, the oranges may be helpful in reducing the constipation or diarrhea in those suffering from irritable bowel syndrome (Milind P and Dev C 2012).

ORANGES, HUMAN HEALTH AND NUTRITION

The human diet contains important micronutrients namely vitamins C and E, carotenoids and flavonoids, essential for maintenance of human health. Multiple dietary sources of these compounds are present virtually in all plant material (Di Majo *et al*, 2005).

The nutritional importance of foods is due to the presence of these functional food ingredients and antioxidant nutraceuticals or phytochemicals. Phytochemicals are present in edible fruits and vegetables and when eaten potentially modulate human metabolism in a favourable manner, thereby prevent chronic and degenerative diseases (Tripoli *et al.,* 2007).

Increase in fruits and vegetables consumption protects against degenerative pathologies such as cancer and atherosclerosis (Keys, 1995); as epidemiological surveys had shown an inverse relationship between dietary flavonoid intake from citrus and cardiovascular diseases (Hertog *et al*.,1993).

Citrus fruits are the main source of important phytochemical nutrients and for long have been valued for their wholesome nutritious and antioxidant properties. It is scientifically proven that oranges being rich in vitamins and minerals have many health benefits. Moreover, it is now appreciated that other biologically active, non-nutrient compounds found in citrus fruits such as phytochemical antioxidants, soluble and insoluble dietary fibres are known to be helpful in reducing the risk for cancers, many chronic diseases like arthritis, obesity and coronary heart diseases (Crowell 1999).

PHARMACOLOGICAL USE

Fruits are an essential part of our diet and are known to reduce the risk of several chronic diseases, including cancer.(Madhuri S *et al*., 2014).

It was reported that the consumption of citrus fruits is also believed to confer some protection against diseases such as cardiovascular disease and cancer (Baghurst, 2003; Guimaraes *et al*., 2010; Atolani *et al*., 2012).

Anti-inflammatory and antithrombotic actions were reported. (Reddy NL *et al*., 2016).

It was reported that it was used for decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorders, and so forth as well as blood pressure modulator (Vinodhini.M *et al*.,2017).

PEEL

EHANOMEDICAL USES

It was reported used to kill mosquito larvae and mites. (Intekhab J and Aslam M 2009).

PHYTOCONSTITUENTS

The ethanolic extract of peels was reported to contain citral, aldehyde and 90% d-limonene in volatile oil**. (**Omodamiro O. D. and Umekwe J. C .2013).

The peel was confirmed that the presence of alkaloids, phytosterols, phenols, flavonoids, quinines, proteins and amino acid.(Hany *et al*.,2015).

The hydro-distilled Citrus peel essential oils had been reported to be a rich sources of bioactive compounds such as mono- and sesqui-terpenes, coumarins, flavonoids, carotenes, etc. (Mondello L *et a*l .,2005).

The citrus peels are rich in nutrients and contain many phytochemicals with strong potential for use in drug production or as food supplements (Mathew *et al*., 2012).

It was reported that the same study also revealed a weaker activity for *C. sinensis* with a higher limonene content of over 77%. In fact bergamot oil (*Citrus aurantium*) with less than 3% limonene was more active. (Djenane . D 2015).

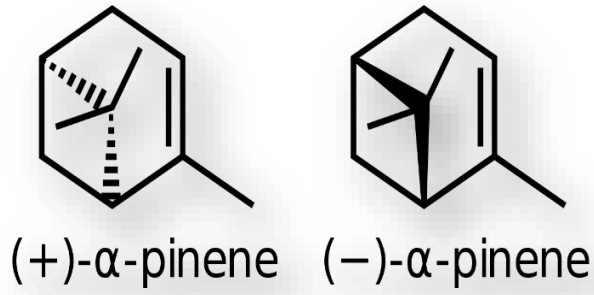
The ethanolic extract of *C. sinensis* to contain presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids

(Lawal D *et al.,* 2013).

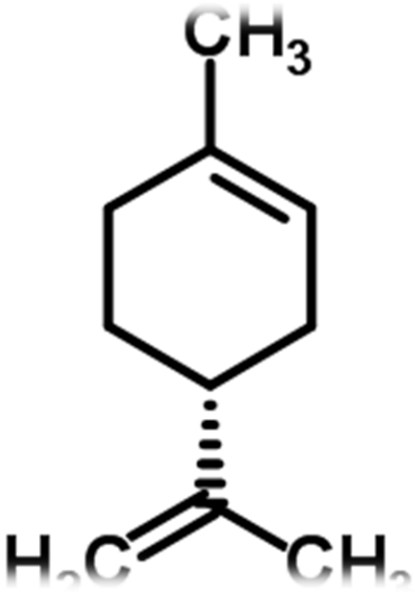
It was reported the essential oil of citrus family to contain α-pinene, β-pinene, sabinene, β-myrcene, p-cymene, limonene, γ-terpinene, neryl acetate, β-bisabolene, and α-bergamotene. **.**(Vinodhini.M *et al.,* 2017).

The hydro distilled fresh peels was reported that volatile phytoconstituents constituents of *Citrus sinensis* Linn include citral, limonene, γ-terpine, α- and β- pinene, camphene, linalool, α- and β-carotene, methyl anthranilate, cryptoxanthin, limocitrin, nerol and sabinene. It also contains flavone glycosides (neohesperidin, naringin, hesperidin, narirutin), pigments (anthocyanin, beta-cryptoxanthin, zeaxanthin) (Singh V *et al.,* 2015) , vitamins (B1, B2, B3, B5, B6 and vitamin C) and minerals such as calcium, iron, magnesium, zinc, phosphorus, potassium.(Parle M,Dev C,2012).

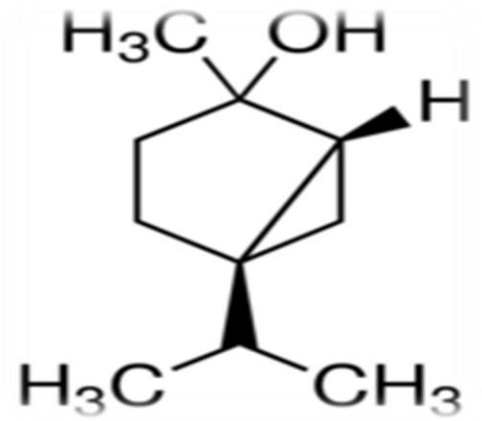
α-PINENE



L – LIMONENE

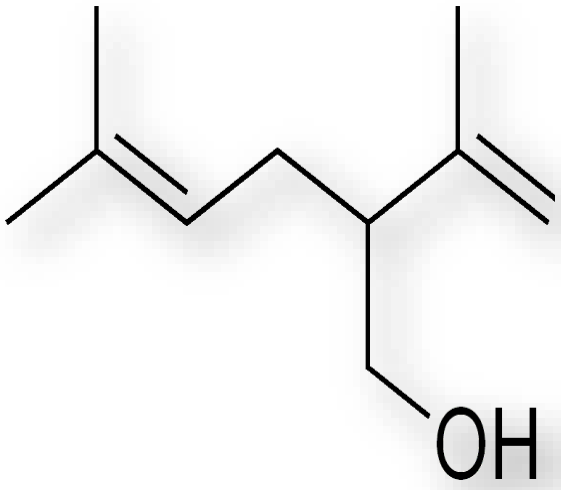


CIS- SABINENE HYDRATE

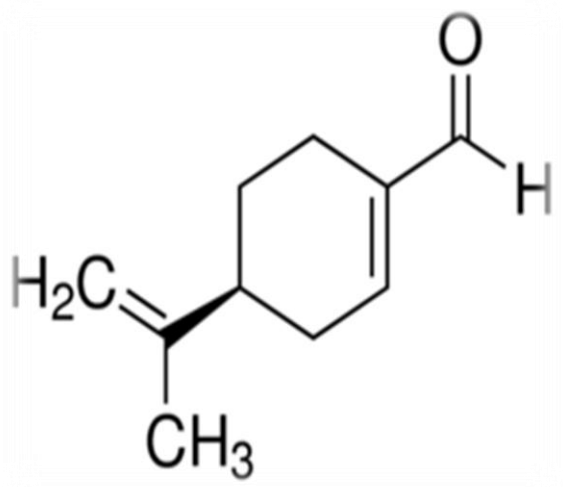


CITRAL

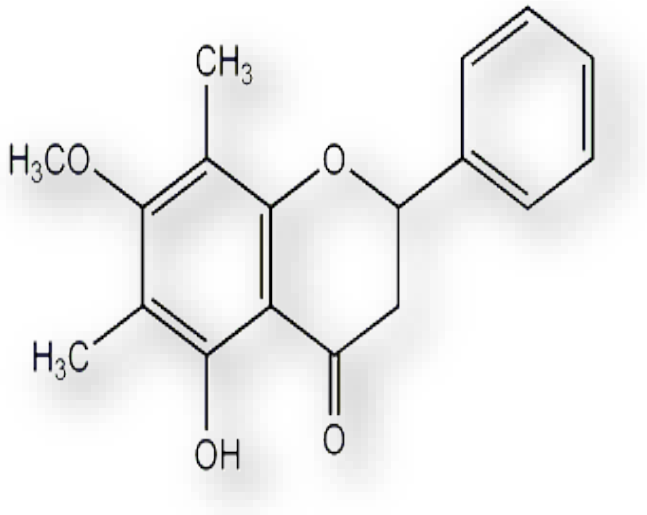
LAVANDULOL



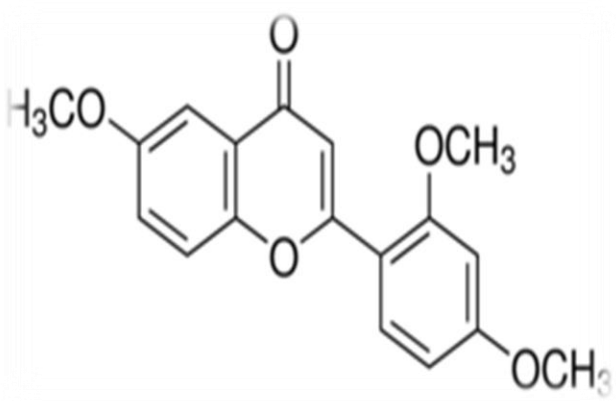
PERILLALDEHYDE



5, 8-DIHYDROXY-6



7, 4-TRIMETHOXYFLAVONE



PHARMACOLOGICAL ACTION

The Peel extract of *C. sinensis* has been reported for a wide range of biological activities such as anti-typhoid, anti-diabetic, anti-carcinogenic, sedative action, anti-hypercholestremic, antibiotic, antifungal, antioxidant, anxiolytic effect, anti-lipidperoxidative effect, anti-inflammatory, lipoxygenase inhibitor, amylase inhibition urease inhibition, Anti-hyperthyroidism, trypsin inhibition, inotropic effect, anti-arthritic, cardio-protective, anti-asthmatic, depression treatment, hepatotoxicity treatment, thrombosis treatment, rheumatic disease treatment (Manish K *et.al.,* 2013).

The hydro distilled fresh peels was reported some cardio protective constituents-vitamin C, flavonoids & carotenoids. (Singh V *et al.*, 2015). Limonene is responsible for its cholesterol lowering property (Kurowska E.M., Manthly J. 2004). and anti-carcinogenic activity of sweet orange. It also possesses anti-ulcer (Sima J.A.,*et al.,* 2003) anti-anxiety, anti-typhoid (Kumar V *et al* 2010), anti-bacterial (Strange R.R., *et al*.,1993), larvicidal (Weisman Z,

Chapagain B.P, 2005) ,anti-diabetic, anti-fungal and anti-inflammatory activities (Haiquing J *et al.,*2004).

The hydrodistilled *C. sinensis* peel essential oil was reported to evaluate the *in vitro* thrombolytic, anti-haemolytic and anti-inflammatory activities (Vinodhini.M *et al.,* 2017)

Ethanol extract of *C. sinensis* peel showed hypoglycemic effects in mice as well as larvicidal, pupicidal, and insecticidal activities . (Patricia C *et al*.,2014).

Hexane extract of *C. sinensis* peel showed activity against chloroquine (CQ)-sensitive (3D7) strain of *Plasmodium falciparum*. (Patricia C *et al.*,2014).

Hexane extract of *C. sinensis* peels exhibited activity against one sensitive (MIC200 µg/mL) and two mono resistant strains (MIC 25-50 µg/ml)of *M. tuberculosis* H37Rv . (Patricia C *et al.*,2014).

Citrus sinensis peel has reported many medicinal properties and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, carminative, immune-enhancing, stomachic, tonic to digestive system, immune system and skin.(Hussain K.A., Tarakji, B.P.P., *et al.,* 2015).

Citrus peel essential oils have also been scientifically reported to possess antioxidant, insect-repellent and antimicrobial activities(Viuda-Martos M *et al*.,2008). Polymethoxyflavones, hydroxylated polymethoxyflavones and hydroxylated polymethoxychalcones from *C. sinensis* had been reported to exhibit anticancer and antioxidant activities (Esquivel-Ferriño P.C *et al*., 2004)

ANTI-OXIDANT ACTION

The hydrodistilled fresh peels was reported that it was excellent anti-oxidant agent due to its flavonoid content. (Singh V *et al.,*2015).

The ethanolic extract of peels was reviewed that it was leads to oxidative stress and damage of cells and tissues. There is evidence that oxidative damage is an important contributor to aging and various chronic diseases such as cancer and neurodegeneration . Both dietary anti oxidant and those endogenous to the body are involved in controlling oxidative damage. In the context of nervous system, antioxidants have been shown to improve motor and cognitive functions in experimental animals and prevent ROS medicated neuronal death.

(Omodamiro O. D.and Umekwe J.C.2013)

ANTI-BACTERIAL ACTIVITY

It was showed that Antibacterial activity properties of ethanolic extracts of *Citrus sinensis* peel The disc agar diffusion method was used in this study. The test organisms (1:100 dilution of an 18h broth cultures)were inoculated onto nutrient agar plates with sterile cotton swabs soaked in the Inocula. Disc of different extract concentrations were placed firmly on the surface of the inoculated agar plates and incubated at 37oc for 18hrs under aerobic conditions. Zones of inhibition were measured and recorded in millimetres. **.** (Omodamiro O. D. and Umekwe J.C 2013).

Extracts of peels showed moderate antibacterial activity against *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa*. Results clearly indicate that free radical

scavenging, antibacterial and anti-inflammatory properties of ethanolic extracts of *Citrus sinensis* are comparable to ascorbic acid, ciprofloxacin and aspirin used as standards respectively.(Omodamiro O. D. and Umekwe J. C. 2013).

ANTI-FUNGAL ACTIVITY

Citrus sinensis peel essential oil is an effective inhibitor of bio degrading and storage-contaminating fungus *A. Niger*. Major antifungal constituents of orange are limonene, linalol and myrcene (Neeta S and Abhishek T,2008).

CHOLESTEROL LOWERING EFFECT

Cholesterol lowering effect of orange was produced by Limonene. Polymethoxylated flavones (PMFs) are present in citrus fruit peel, which can lower cholesterol more effectively than some prescription drugs, without showing any side effect. Although, a variety of citrus fruits contain PMFs, the most common PMFs are tangeretin and nobiletin, which are found in the peels of oranges. PMFs work like statin drugs that inhibit the synthesis of cholesterol and triglycerides inside the liver. However, grating a tablespoon or so of the peel of orange each day and using it to flavour tea, salads, yogurt, soups, snacks or rice may be a practical way of achieving some cholesterol-lowering benefits. (P Milind P and Dev C,2012)

ANTI-ANXIETY EFFECT

Aroma-therapists use orange oil as a tranquilizer. Researchers have found evidence that sweet orange oil is an anxiolytic activity include flavonoids like citacridone, citbrasine and saponins. (Milind Pand Dev C,2012).

ANTI-INFLAMMATORY ACTIVITY

It was reviewed that anti-inflammatory activity of ethanolic extracts of *Citrus sinensis* peel .The crude extract and aspirin were separately administered in IP route. Group A was used as negative control thus, received neither aspirin nor the crude extract group B received 250 mg/ml of the extract. Group C received 200mg/ml, group D received 100 mg/ ml while group E received 50 mg/ml of the extract. Group F was used as positive control, thus, received 100mg/ml of aspirin. The animals were left for 30mins after which 1 ml of fresh egg albumen was injected into the sub-plantar of the right hind paw of each of the rats. Using a Vernier calliper, the diameter of the paw was measured and recorded at 30mins intervals for 3hrs. Aspirin (100mg/ml) was used as standard. Percentage inflammation and inhibition of inflammation were calculate.(Omodamiro O. D. and Umekwe J. C. 2013).

SKIN DISEASE USE

The roasted pulp is prepared as a poultice for skin diseases.(P Milind P and Dev C,2012)

ANTI ACNE AND ANTI FUNGI EFFECT

The fresh peel is rubbed on acne .Orange peel oil produces lethal effect on fleas, fire ants, and houseflies due to its 90-95% limonene. Orange peel is medicinally used against fungi. (PMilind Pand Dev C,2012).

LARVICIDAL ACTIVITY

The saponins present in the peel possess larvicidal activity. (Milind P and Dev C,2012).

ANTI-DIABETIC ACTIVITY

Anti-diabetic activity of orange is due to bioflavonoids such as hesperidin and naringin present in citrus fruit peels. These peels play an anti-diabetic role in C57BL/Ks J-db/db mice via regulation of glucose regulatory enzymes. They decrease the activity of glucose-6-phosphatase and phosphoenolpyruvate. The anti-diabetic potential of orange peel and juice appear to be mediated via anti peroxidation, inhibition of α-amylase enzyme activity that is responsible for the conversion of complex carbohydrates to glucose, increased hepatic glycogen content, stimulation of insulin secretion, and repair of secretory defects of pancreatic β-cells.(Milind P and Dev C 2012).

SEED

PHARMACOGNOSY

Seeds are greenish to pale whitish, flattened, and angular. The seed is generally poly embryonic. The embryos are either “zygotic” or “nuclear.” The zygotic embryos are derived from pollination of the ovary, i.e., sexual reproduction, and therefore are not always similar in horticultural qualities to the parent tree. The nuclear embryos are derived wholly from the mother plant and show very similar characteristics to the parent plant**.** (MilindP and DevC 2012).

PHYTOCHEMISTRY

The seeds of fruits such as oranges (*Citrus sinensis*) are shown to be promising sources of oils, rich in carotenoids, phenolic compounds, tocopherols, and phytosterols.(Jorge N *et al*.,2015).

PHARMACOLOGY

ANTIOXIDANT ACTIVITY

To determine the antioxidant activity of the oils,1 g of oil was diluted with 10 ml of ethyl acetate. From this solution, 1 ml was added to 4 ml of a DPPH• solution, in 10-4 mol /l ethyl acetate, and was vigorously shaken in vortex, for 10 sec. After30 min in the dark, the mixture absorbance was measured at 517 nm. A control sample (without oil) was prepared and the absorbance was equally measured. The levels of absorbance obtained were converted to percentage of antioxidant activity (AA).The efficient concentration (EC), defined as the sufficient concentration to obtain 50% of the maximum effect estimated in 100% (expressed in kilograms of oil per kilogram of DPPH•), was graphically determined. To do so, oil samples were diluted with ethyl acetate in concentrations of 10, 25, 50, 75, and 100 mg/ml. Measurements of the absorbance of reaction mixtures (1 ml of solution sample and 4 ml of DPPH• in ethyl acetate)were performed at 517 nm at 0 and 30 min. The antiradical efficiency (AE) of oils was determined according to Equation $AE = 1/EC50$.(Jorge N *et al.*,2015).

ROOT

CHEMISTRY

The ethyl acetate extract of the roots of *Citrus sinensis* to contain flavonoid. The compound was characterized as 5, 8-dihydroxy-6, 7, 4-trimethoxyflavone on the basis of UV, I.R, mass and N.M.R (1H, 13C) spectral studies. (Intekhab J and Aslam M,2009).

PHARMACOLOGY

It reported that used as an anti-diabetic, antimicrobial, antifungal, hypotensive agent, antioxidant, carminative, insect repellent, antibacterial, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent. (Intekhab J and Aslam M,2009).

ZEBRAFISH AS MODEL ORGANISM FOR DRUG DISCOVERY

The zebrafish (*Danio rerio*) has become a widely used vertebrate model organism for drug discovery because of its, transparent embryos and larvae**, morphological and physiological similarity** to mammals, allowing *in vivo* analysis of embryogenesis and organogenesis combined with ease of use and low cost.

Zebra fish embryo and larvae are relatively tolerant to dimethylsulphoxide, a commonly used solvents in *vitro* assays. Another advantage of this species is its high fecundity. One pair of adult fish is capable laying 200 eggs a day and depending on the conditions of maintenance,. Zebrafish can also be useful model

organism to study the mechanisms enabling regenerative neurogenesis.

(V.N. Sarvaiya,2014)

Studies has been carried out to assess pharmacological effect of drugs on the potic nerves , motor neurons, dopominergic neurons performed whole mount immune staining were performed and visualize different neuronal cell types in vivo, the compound which shows neuro toxicity in humans shows similar neurotoxicity in zebra fish.(Parng,C *et,al*.,2007)

Work has been carried out to evaluate the teratogenic effects of the human **anti- epileptic drug** phenytoin which generates malformations such as oedema, brain deformities a shortened and bent tail and bipartite axiation of the posterior trunk on zebra fish larvae.(Herrmann,K 1993)

A data on behavioural responses of zebra fish to a wide spectrum of putative anxiolytic and anxiogenic agents was carried out. Using the novel tank test as a sensitive and efficient behavioural assay , zebra fish anxiety like behaviour, can be bidirectionally modulated by drugs affecting the gamma-aminobutyric acid, monoaminergic, cholinergic glutamatergic and opioidergic systems.(Stewart,A *et al.,*2010).

Zebrafish (*Danio rerio*) are rapidly emerging as a promising model organism to study various brain disorders. Seizure-like behavioural and neurophysiological responses can be evoked in larval and adult zebrafish by various pharmacological and genetic manipulations, collectively emphasizing the growing utility of this model for studying epilepsy. Here, we discuss recent developments in using zebrafish models to study the seizure-like behaviour involved in epilepsy, outlining current challenges and strategies for further translational research in this field.

The availability of both larval and adult zebrafish is also beneficial, enabling the investigation of a wider spectrum of epilepsy-related phenomena throughout the ontogenesis. zebrafish also possess a tight junction based blood brain barrier that is similar to higher vertebrates, with substantial macromolecule permeability yielding a high sensitivity to drugs

(Adam Michael Stewart,*et al*,2014)

AIM AND OBJECTIVE

Epilepsy is a common neurological disorder marked by abnormal electrical discharges in the brain, which typically involves sudden brief episodes of altered or diminished consciousness.

There are many known epileptic syndromes. While each affects the nervous system in different ways, seizures are the most common phenotypes.

A wide range of antiepileptic drugs have been developed with anticonvulsant activity, each of which acts with different mechanism Benzodiazepines make GABA receptors more effective, whereas carbamazepine and lamotrigine block sodium and calcium channels Despite the wide range of discoveries, 30% of people suffering from epilepsy fail to respond to antiepileptic drugs (AEDs). AEDs are associated with substantial side effects, including seizure liability. This is called the seizure liability of a drug and is a substantial adverse side effect. Unfortunately, seizure liability can only be identified in later stages of pre-clinical safety studies on higher vertebrates, which does not allow sufficient time to reduce this risk by chemical modification. More effective antiepileptic therapeutics with fewer side effects are therefore needed.

Seizures are induced by different mechanisms. Therefore, a broad understanding of mechanisms of seizures is essential for AED discovery.

There are many drugs associated with inducing seizures (convulsants), which could help construct this large-scale seizure model.(Mehmet Tugrul Savran, 2013)

AIM

To study the pharmacognostic, preliminary phytochemistry *in vivo* effect on the seizure induced zebra fish larvae of the volatile oil of the leaves *of Citrus sinensis* FAM: Rutaceae, to evaluate its anti-epileptic activity.

OBJECTIVE

The objective of the study was divided into three parts.

PART 1: PHRAMACOGNOSTICAL STUDY

- Collection and authentication of plant
- Morphological study of the plant
- Microscopy of the leaves
- Anatomical study using light microscope
- SEM analysis
- Powder microscopy
- Microscopic schedules

PHYSIO-CHEMICAL PARAMETERS

- Ash values
- Loss on drying
- Extractive values

PART 2: PRELIMINARY PHYTOCHEMICAL SCREENING

- Qualitative analysis of the leaves for the presence of various phyto constituents
- Determination of flavonoid content, total phenolic content of the leaves of ***Citrus sinensis***.

- Determination of trace elements present in the leaves by Energy Dispersive spectrum analysis(EDS)
- Isolation of Volatile oil of the leaves of ***Citrus sinensis*** **(VOCSL).**
- GCMS Profile of the **VOCSL** to identify and quantify flavonoids and triterpenoids.

PART 3: PHARMACOLOGICAL STUDIES:

The **3R's** ethical principle (**R**eduction, **R**efinement, and **R**eplacement) was implemented that help to minimize harms to vertebrate animals used in science.

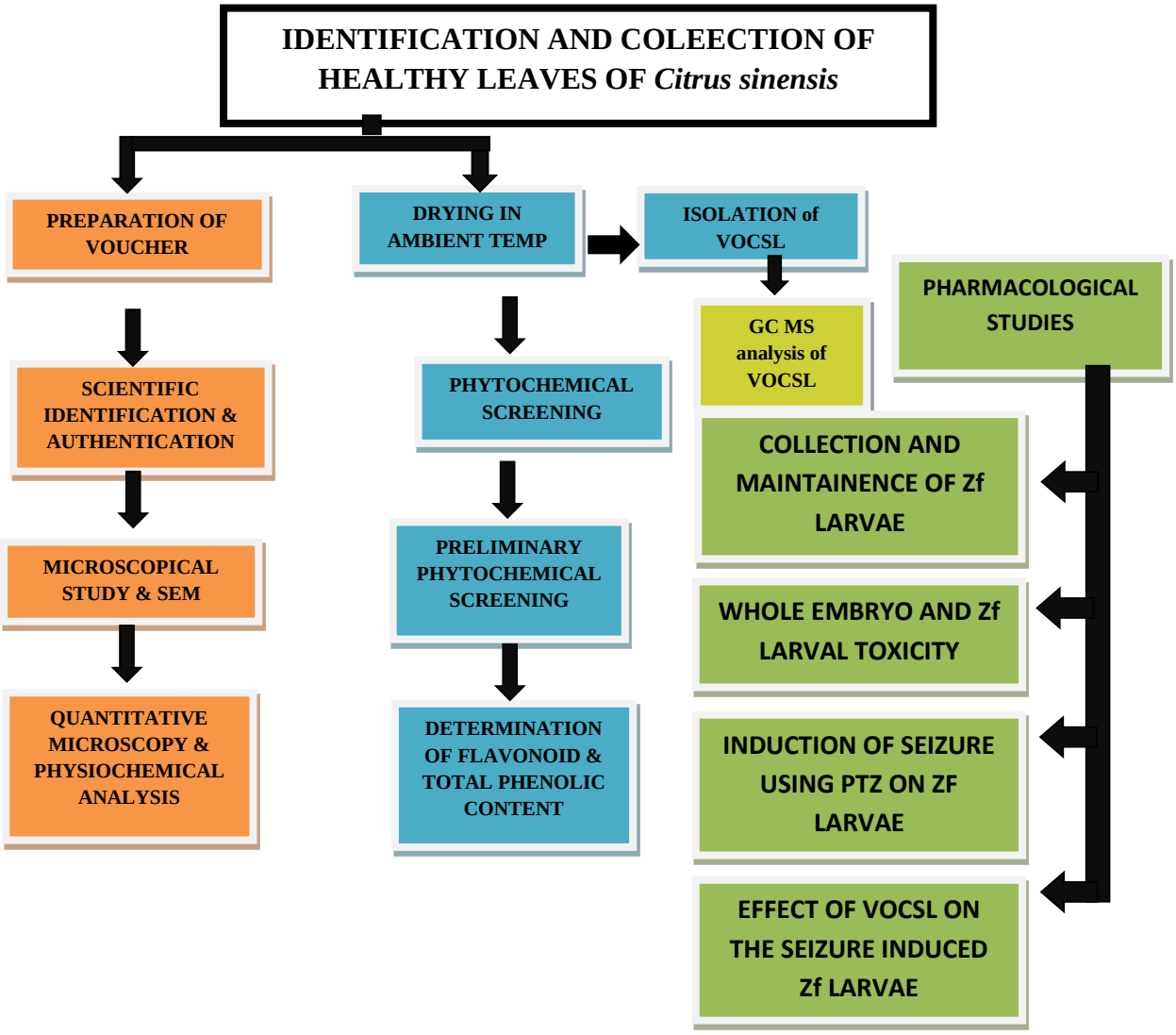
- Collection of *Danio rerio* or zebra fish (Zf) larvae.
- To study the preliminary toxicological studies of the **VOCSL** on the early development.

1. Whole Embryo Culture Toxicity Studies
2. Larval Toxicity Study

- To study the effect of **VOCSL** on seizure induced zebra fish larvae.
- To determine the effect **VOCSL** on the CNS (seizure induction, Behavioural assay, locomotor distance) of Zf larvae.

MATERIALS AND METHODS

RESEARCH DESIGN



4. 1. PLANT COLLECTION AND AUTHENTIFICATION

Leaves of the plant ***Citrus sinensis*** selected for our study was collected from **Tamil Nadu Agricultural University, Department of Horticulture, Agricultural College And Research Institute, Madurai**, Tamilnadu India during the month of September 2017 and was authenticated by **Dr.BALAMOHAN, Ph.D., PDF.,** Professor and Head (Hort.) DEPARTMENT OF HORTICULTURE,

AGRICULTURAL COLLEGE AND RESEARCH INSTITUTE, MADURAI and was supported by **Dr.Stephen**, Department of Botany, American college, Madurai.

LEAF DRYING AND PULVERIZING

The leaves were collected and shade dried. It was powdered in a mixer. The powder was sieved in a No.60 sieve and kept in a well closed container in a dry place.

4.2. PHARMACOGNOSTICAL STUDIES

Morphological and micro morphological examination and characterization of medicinal plants have always been accorded due credentials in the Pharmacognostical studies. Botanical identity of the plants is an essential prerequisite for undertaking the analysis of medicinal properties of any plant.

A researcher may succeed in getting a new compound or may find many useful pharmacological active properties in the plant. If the botanical identity of the plant happens to be dubious or erratic, the entire work on the plant becomes invalid. Thus it is needless to stress the botanical identity of the crude drug is the threshold in the processes of pharmacological investigations. The researcher should be equipped with all possible diagnostic parameters of the plant on which the researchers plan to work.

4.2.1. MORPHOLOGICAL STUDIES OF *C.sinensis*

Aerial part, leaf and petiole, flower, fruits and root were studied individually for its morphological characters by organoleptic test.

4.2.2. MICROSCOPICAL STUDIES ON THE LEAF OF *Citrus sinensis*

COLLECTION OF SPECIMEN

Care was taken to select healthy plants and for normal organs. Leaf, Petiole specimens were collected from a healthy plant by making a cut with

petioles.The materials were cut into pieces and immediately immersed in fixative fluid FAA (Formalin – 5ml + Acetic acid – 5ml +70% Ethyl alcohol – 90ml).

DEHYDRATION

After 24 hours of fixing , the specimens were dehydrated with graded series of ethyl alcohol and tertiary-butyl alcohol (Sass, 1940). The specimen is kept is in each grade of the fluid for about 6 hrs.Every time the fluid is decanted and immediately the specimen were flooded with next grade of fluid.

INFILTRATION WITH PARAFFIN WAX

After dehydration, the shavings of paraffin wax were added to the vial containing the plant material with pure TBA. The paraffin shavings are added every 30mts at about 40-45ºC four or five times. Then the vials were filled with wax without damaging the tissues. The vial filled with wax is kept open in warm condition to evaporate all TBA, leaving the specimen in pure molten wax. The specimen filled with pure molten wax for 2 or 3 times by decanting the old wax every time.

CASTING TO MOLD

A boat made out of chart board, by folding the margin, is used to prepare a mold of wax containing specimens. The paraffin along with the leaf and petiole specimen was poured into the boat. With the help of heated needles, the specimen were arranged in parallel rows with enough space in between the specimens. The block was then immersed in chilled water and allowed to cool for few hours.

SECTIONING

The paraffin embedded specimens were sectioned with the help of microtome**.** The thickness of the sections was 10-12µm. Dewaxing of the sections was by customary procedure. The sections were stained with **Toluidine blue** as per the method published by O'Brien *et al.,*1964. Since toluidine blue is a poly chromatic strain, the straining results were remarkably good and some **cytochemical reactions** were also obtained. The dye rendered pink colour to the **cellulose** walls, blue to the lignified cells, dark green to **suberin**, violet to the **mucilage**, blue to the **protein** bodies etc. Where ever necessary sections were also stained with **safranin** and **fast-green** and potassium iodide (for starch). For studying the stomatal morphology, venation patttern and trichome distribution, **paradermal sections** (sections taken parallel to the surface of leaf as well as **clearing** of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid) were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of differentparts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured (Sass, JE,1940).

PHOTOMICROGRAPHS

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken.with Nikon labphot 2 Microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light were employed. Since these structures have birefringent property, under polarized light

they appear bright against dark background. Magnifigations of the figures are indicated by the scalebars. (Johansen DA, 1940, Purvis MJ *et al*., 1966).

4.2.3. MICROSCOPICAL STUDY OF LEAF USING SCANNING ELECTRON MICROSCOPE (SEM):

Movement of beam of foccussed electrons across an object forms a 3D image on a cathode - ray tube in a Scanning Eectron Microscope and it reads both the electrons scattered by the object and the secondary electrons produced by it. The electromagnetic lenses are used in SEM and focussing is done by the current. On photographic plate of screen the image is projected which gives comprehensive, quasi 3-D representation of the objects gives the ultra structure of plant cells. In addition , shows the unsuspected details and any undescribed characters. In other words the micrograph from SEM, shows the best possible structural details of the specimens. (Robards, 1970)

USAGE

SEM info was handled as conventional character (or) character complexes as “pure” information without being broken down (or) interpreted as individual character using computer processing. The SEM information can be used some what at the superficial level just described to assist in solving taxonomic problem by confirming, changing (or) other grounds. It is also used often as diagnostic feature to avoid misleading by over simplified descriptions and one may find new kinds of microstructures not previously recognised and apprently simple structures may be extremely complex. Remarkably, poor conventional descriptions enabling taxonomic process of reducing a complex pattern to a few simple characters (Heywood VH, 1971). SEM plays a vital role when a specimen need to be satisfactorily defined in terms of characters. For most biological

materials, maximum information is obtained by employing light and electron microscopy jointly and an attempt was made by applying SEM to the leaf of *S.torvum*, to pinpoint the positions of specific characters with in the cell, which can be easily seen in final image.

SEM SAMPLE PREPARATION

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with I sq. cm glass slide and kept in carbon adhesive sheet.. Samples were coated with gold to a thickness of 100 AO using hitachi vacuum evaporator. Coated sample were analysed in a Hitachi Scanning electron Microscope 3000 H model.

4.2.4. POWDER MICROSCOPY

MACERATION TECHNIQUE

Maceration is the process of separation of individual cells by selectively dissolving the pectic middle lamella between the cells. The middle lamella binds the cells with each other forming different tissues. The middle lamella is dissolved by employing a chemical that dissolves the lamella to free the cells to obtain their three dimensional view.

MACERATION FLUID

Jaffrey's maceration fluid is one that is commonly used for maceration (Johnsen DA, 1940). The fluid consists of equal volumes of 5% chromic acid and 5% nitric acid. The plant material is cut into small pieces and immersed in the maceration fluid. The fluid with the materials is kept at 55ºC for 3-5 hrs. Then the material is washed thoroughly with water and placed on a glass slide in a drop of Safranin (0.5%) for 15-20 min. The stain is drained carefully and mounted with a drop of dilute glycerine. The cells are spread well with a needle and the material

is covered with cover slip. The slide so prepared is examined under the microscope to study different components of the macerate.

4.2.5. MICROSCOPIC SCHEDULES (Wallis TE. 1953, Wallis TE, 1965, Iyengar MA, 1994, Anonymous, 2001)

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaves using standard procedures.

A. VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER

The term vein islet in used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein islets per sq.mm. Area is called vein islet number.

Vein terminal number may be defined as the number of vein terminals present in one sq., mm. area of the photosynthetic tissue.

B. DETERMINATION OF VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER

Small square portion from the lamina region of the leaf was cleared in chloral hydrate, stained and mounted on a slide. A camera Lucida is set up and by means of a stage micro meter the paper is divided into squares of $1mm^2$ using a 16mm objective. The stage micro meter is then replaced by the cleared preparation and the veins are raced in four continuous squares, either in a square 2mm x 2mm (or) rectangle 1mm x 4 mm.

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left-hand sides of the square (or) rectangle but included if cut by the other two sides. Ten readings for vein islet and vein termination number were recorded.

C. STOMATAL INDEX

It is the percentage, which the numbers of stomata from the total number of epidermal cells, each stoma being counted as one cell.

Stomatal index = S/S+E x 100

Where, S = Number of stomata per unit area

E = Number of epidermal cells in the same unit area

D. DETERMINATION OF STOMATAL INDEX

The procedure adopted in the determinations of stomatal number observed under high power (45 X).The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above formula.

4.3 PHYSICOCHEMICAL PARAMETERS (Anonymous, 1996, 1998, 2001)

DETERMINATION OF ASH VALUES

ASH VALUE

The ash values were determined by using air dried powder of the leaf as per the official method.

TOTAL ASH

Two grams of the air dried leaf powder was accurately weighed in a silica crucible separately. The powder was scattered into a fine even layer on the bottom of the crucible and incinerated by gradually increasing the temperature not exceeding 450^{o}c, until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

WATER SOLUBLE ASH

The ash obtained from the total ash procedure was boiled with 25ml of water for 5 minutes and the insoluble matter was collected on an ash less filter paper and washed with hot water. Then it was ignited for 15 minutes at a temperature not exceeding 450^{o}c. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

ACID INSOLUBLE ASH

The ash obtained from the total ash was boiled for five minutes with 25ml of dilute hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, dried and weighed. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

DETERMINATION OF LOSS ON DRYING

For the determination of loss on drying, the method described by Wallis was followed. One gram of dried powdered leaf was accurately weighed in a tarred Petri dish, previously dried under the conditions specified in IP'96. The powder was distributed as evenly as practicable, by gentle sidewise shaking. The dish was dried in an oven at 100 – 105°C for 1 hour. It was cooled in desiccators and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

EXTRACTIVE VALUES

PETROLEUM ETHER SOLUBLE EXTRACTIVE VALUE

Five gram of the coarsely powder was macerated separately with 100ml of petroleum ether in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of petroleum ether. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the petroleum ether soluble extractive value was calculated with reference to the air dried powder.

ETHANOL SOLUBLE EXTRACTIVE VALUE

Five gram of the coarsely powder was macerated separately with 100ml of ethanol in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethanol. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the ethanol soluble extractive value was calculated with reference to the air dried powder.

WATER SOLUBLE EXTRACTIVE VALUE

Five gram of the coarsely powder was macerated separately with 100ml of chloroform water in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform water. 25ml of the filtrate was evaporated to0020dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the chloroform water soluble extractive value was calculated with reference to the air dried powder.

4.3 PHYTOCHEMICAL STUDIES [Anonymous, 1998, Chaudhri RD, 1999, Kokate CK, 2005, Agarwal, 2007, Horbone JB, 1973].

4.3.1 PRELIMINARY PHYTOCHEMICAL SCREENING

TEST FOR ALKALOIDS:

VARIOUS PROCEDURES TO LIBERATE ALKALOIDS

- Powdered drug was mixed thoroughly with 1ml of 10% ammonia solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was mixed thoroughly with 1ml of 10% sodium carbonate solution and then extracted for 10 minutes with 5ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was ground in a mortar for about 1 minute with 2ml of 10% ammonia solution and then thoroughly mixed with 7 gram basic Aluminium oxide. The mixture was then loosely packed into a glass column and 10ml chloroform was added, eluted, dried and methanol was added.
- Powdered drug was shaken for 15 minutes with 15ml of 0.1 N sulphuric acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20ml filtrate; 1ml concentrated ammonia was then added. The mixture was then shaken with two portions of 10ml diethyl ether. The ether was dried over anhydrous sodium
- Sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.
- Powdered drug was mixed with one gram of calcium hydroxide and 5ml of water, made into a smooth paste and set aside for 5 minutes. It was

then evaporated to dryness in a porcelain dish on a water bath. 20ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.

The above made extracts were tested with various alkaloid reagents as follows.

1. MAYER'S REAGENT

2. DRAGENDORFF'S REAGENT

3. HAGER'S REAGENT

4. WAGNER'S REAGENT

TEST FOR CARBOHYDRATES

MOLISCH'S TEST

The aqueous extract of the powdered material was treated with alcoholic solution of α - naphthol in the presence of sulphuric acid**.**

FEHLING'S TEST

The aqueous extract of the powdered material was treated with Fehling's I and II solution and heated on a boiling water bath.

BENEDICT'S TEST

The aqueous extract of the powdered drug was treated with Benedict's reagent and heated over a water bath.

TEST FOR GLYCOSIDES

GENERAL TEST

TEST A

200 mg of the powdered drug was extracted with 5ml of dilute sulphuric acid by warming on a water bath, filtered and neutralized with 5% sodium

hydroxide solution. Then 0.1ml of Fehling's solution I and II were added, until it becomes alkaline and heated on a water bath for 2 minutes.

TEST B

200 mg of the powdered drug was extracted with 5ml of water instead of sulphuric acid. Boiled and equal amount of water was added instead of sodium hydroxide solution. Then 0.1ml of Fehling's solution I and II were added, until it becomes alkaline and heated on a water bath for 2 minutes.

ANTHRAQUINONES

BORNTRAGER'S TEST

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The inorganic layer was separated and ammonia solution was added slowly.

MODIFIED BORNTRAGER'S TEST

About 0.1gram of the powdered leaf was boiled for two minutes with dilute hydrochloric acid and few drops of ferric chloride solution was added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well.

TEST FOR CYANOGENETIC GLYCOSIDES

Small quantity of the powdered leaf was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

TEST FOR CARDIAC GLYCOSIDES

KELLER KILLIANI'S TEST

About 1gram of the powdered leaf was boiled with 10ml of 70% alcohol for two minutes, cooled and filtered. To the filtrate 10ml of water and 5 drops of solution of lead sub acetate were added and filtered. The filtrate was then extracted with chloroform and the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 3ml of glacial acetic acid containing a trace of ferric chloride. To this 3ml of concentrated sulphuric acid was added along the sides of the test tube carefully.

RAYMOND TEST

To the alcoholic extract of the leaf, hot methanolic alkali was added

LEGAL'S TEST

To the alcoholic extract of the powdered drug, pyridine and alkaline sodium nitropruside solution were added.

COUMARIN GLYCOSIDES

A small amount of powdered leaf was placed in test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The covered test tube was placed on water bath for several minutes. Then the paper was removed and exposed to UV light.

TEST FOR PHYTOSTEROLS

The powdered leaf was first extracted with petroleum ether and evaporated. The residue obtained was dissolved in chloroform and tested for sterols.

SALKOWSKI TEST

Few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside.

LIBERMANN – BURCHARD'S TEST

To the chloroform solution few drops of acetic anhydride was added and mixed well. 1ml of concentrated sulphuric acid was added through the sides of the test tube and set aside for a while.

TEST FOR SAPONINS

About 0.5gram of the powdered leaf was boiled gently for 2 minute with 20 ml of water and filtered while hot and allowed to cool. 5ml of the filtrate was then diluted with water and shaken vigorously**.**

DETERMINATION OF FOAMING INDEX

One gram of the coarsely powdered leaf was weighed and transferred to 500 ml conical flask containing 100 ml of boiling water. The flask was maintained at moderate boiling, at 80-90^{o}C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100 ml (V_1).

Ten Stoppard test tubes were cleaned (height 16 cm, diameter 1.6 cm) and marked from 1 to 10. Measured and transferred the successive portions of 1, 2, 3ml up to 10ml and adjusted the volume of the liquid in each tube with water to 10ml. Then the tubes were Stoppard and shaken lengthwise for 15 seconds, uniformly and allowed to stand for 15 minutes and measured the length of the foam in every tube.

TEST FOR TANNINS

To the aqueous extract of the powdered leaf, few drops of ferric chloride solution were added.

GOLD BEATER'S SKIN TEST

2% hydrochloric acid was added to a small piece of gold beater skin and rinsed with distilled water and placed in the solution to be tested for five minutes. Then washed with distilled water and transferred to a 1% ferrous sulphate solution.

TEST FOR PROTEINS AND FREE AMINOACIDS

MILLON'S TEST

The acidulous alcoholic extract of the powdered leaf was heated with Millon's reagent.

BIURET TEST

To the alcoholic extract of the powdered leaf 1ml of dilute sodium hydroxide was added. Followed by this one drop of very dilute copper sulphate solution was added.

NINHYDRIN TEST

To the extract of the powdered drug, Ninhydrin solution was added, and boiled.

TEST FOR MUCILAGE

To the aqueous extract of the powdered leaf, Ruthenium red solution was added.

TEST FOR FLAVONOIDS

SHINODA TEST

A little amount of the powdered leaf was heated with alcohol and filtered. To the alcoholic solution a few magnesium turnings and few drops of concentrated hydrochloric acid were added, and boiled for 5 minutes.

ALKALINE REAGENT TEST

To the alcoholic extract of the powdered leaf, few drop of sodium hydroxide solution was added.

ZINC HYDROCHLORIDE TEST

To the alcoholic extract, mixture of zinc dust and concentrated Hydrochloric acid was added.

TEST FOR TERPENOIDS

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue was dissolved in small amount of chloroform. To the chloroform solution tin and Thionyl chloride were added.

TEST FOR VOLATILE OIL

About 100gram of fresh leaves, were taken in a volatile oil Clevenger apparatus and subjected to hydro distillation for four hours.

TEST FOR FIXED OIL

A small amount of the powdered leaf was pressed in between in the filter paper and the paper was heated in an oven at 105^{o}C for 10 minutes.

4.3.2. FLUORESCENCE ANALYSIS

Powdered leaf material of C.sinensis *was* subjected to analysis under UV light after treatment with various chemical and organic reagents like Ethanol,

Ethyl acetate, Chloroform, Water, 50% sulphuric acid, 10% sodium hydroxide, 50% nitric acid and dried leaf powder**.** (Horbone JB, 1973).

4.3.3 ISOLATION OF VOLATILE OIL FROM THE LEAVES OF *C.sinensis* (VOCSL)

Weighed quantity of fresh leaves was subjected to hydro distillation using Clevenger apparatus used for the determination of VO in which Clevenger oil arm fitted with condenser through which cooled water was circulated to prevent low volatiles from escaping. The oil sample was dried over anhydrous sodium sulphate and kept in scaled glass bottles and stored in refrigerator.

PHYSIOCHEMICAL ANALYSIS

ORGANOLEPTIC PROPERTIES

The VO was placed in a transparent bottle over a white background and the colour and clarity were observed; the characteristic odour was determined by sniffing and to determine its characteristic feel to the touch, it was rubbed between the fingers.

SOLUBILITY

The solubility of the VO was determined by mixing increment volumes of the VO in specified volumes of water, chloroform, ethanol, and toluene.

SPECIFIC GRAVITY

The specific gravity is an important criterion of the quality and purity of volatile oils. It was determined by using pycnometer. It was filled with water and weighed. The procedure was repeated using VO in place of water. The specific gravity of the oil is expressed as the ratio of the weight of the volume of the oil to that of an equal volume of pure water when both are determined at 250 C.

REFRACTIVE INDEX

The index of refraction is physical constant made use to determine the identity and purity of the volatile oils. It was determined using Atago DR abbes refractometer. The test plate was attached to the refracting prism of the instrument by applying with the VO and pressing against the refractive prism. The light was focused on the test plate. The instrument was adjusted until the borderline of the between the light and dark halves of the field of view exactly coincides with the cross wires of the telescope and the reading was taken pressing against the refractive prism. The light was focused on the test plate. The instrument was adjusted until the borderline of the between the light and dark halves of the field of view exactly coincides with the cross wires of the telescope and the reading was taken.

SPECIFIC ROTATION

Both the degree and of rotation its direction are important criteria of purity. The extent of optical activity of VO was determined by a polarimeter (Polax 2L). The zero point of the polarimeter was adjusted and determined. The previously cleaned and dried polarimeter tube was filled with the VO. The analyzer was rotated until equal illumination of light of the two halves of the visual field was achieved and reading was taken. Determination of acid value, saponification value and phenol content serves to detect adulteration and to establish the quality and purity of the VO.

4.3.4. ESTIMATION OF FLAVONOID CONTENT

[Chang CC *et al*., 2002, Mabry TJ*et al*., 1970 and Siddiquie MA *et al.,* 2010].

The flavonoid content of plant extract was estimated by aluminium chloride method. In this method, aluminium chloride complexes with flavonoids of C3-C5

hydroxyl group and to produce intense colour in acidic medium. The intensity of the colour is proportional to the amount of flavonoids and can be estimated as quercetin equivalent at wavelength of 415nm.

MATERIALS REQUIRED

- Ethyl acetate Extract of leaves of *Citrus sinensis*
- 10%w/v aluminium chloride
- 1M Potassium acetate
- 95%v/v ethanol

PROCEDURE

0.5ml of the extract was transferred to a test tube. To this solution, 0.1ml of aluminium chloride, 0.1ml of potassium acetate, 1.5ml ethanol were added and made up to 5ml with distilled water. The mixture was allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 415nm. The calibration curve was generated using quercetin as a standard at different concentrations (5-50µg/ml). The reaction mixture without aluminium chloride was used as a blank. The flavonoids content was expressed as mg of quercetin equivalent per gram of extract.

4.3.5 ESTIMATION OF TOTAL PHENOLIC CONTENT (Singleto VL *et al*., 1979, Gouthamchandra K *et al.,* 2010)

PRINCIPLE

The total phenolic content of the extract was determined by Folin & Cio-calteu's phenol reagent. This reagent consists of phosphotungstate and phospho molybdate mixture which is reduced to mixture of blue molybdenum and tungsten oxides while phenolic content of the extract was oxidized. The intensity of colour is proportional to the amount of phenolic content of the extract and which was

measured at 765nm. The total phenolic content in the extract was expressed as milligrams of Gallic acid equivalent (GAE) per gm. of extract.

MATERIALS

- Ethanolic extract of the leaves of *Citrus sinensis*
- 10%w/v sodium carbonate solution
- Gallic acid
- Folin&Cio-21021calcateu's phenol reagent

PROCEDURE

0.5ml and 1ml of VO was transferred into separate test tube. To this solution, FCR 0.5ml and 1ml of sodium carbonate were added and final volume made up to 10ml with distilled water. The mixture was allowed to stand for 1hr with intermittent shaking. The absorbance was measured at 765nm. A calibration curve was generated using Gallic acid as a standard at different concentrations (2, 4, 6, 8, 10µg/ml). The reaction mixture without sample was used as a blank. The total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per g of extract.

4.3.6 DETERMINATION OF TRACE ELEMENTS IN THE LEAF OF *Citrus sinensis* BY ENERGY DISPERSIVE X-RAY SPECTROMETER (EDS)

The SEM allows the observation of materials in macro and submicron ranges. SEM is capable of generating 3-D images for analysis of topographic features. When SEM is used along with EDS the analyst can perform an elemental analysis on specimens of microscopic sections or contaminants that may be present.

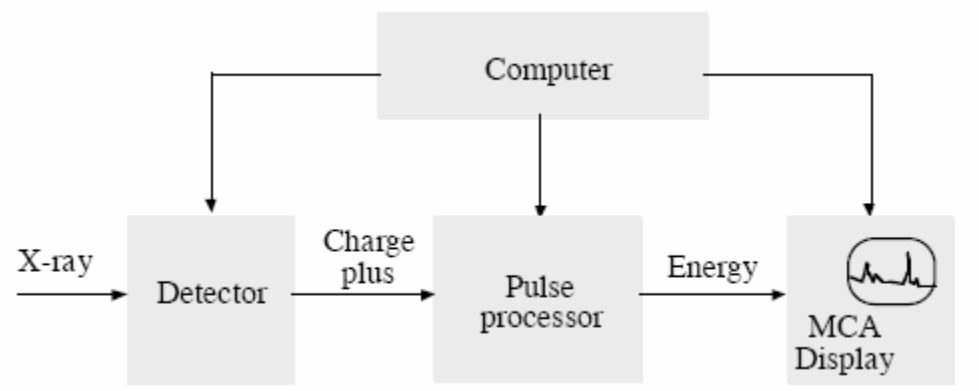
EDS ANALYTICAL CAPABILITIES

Backscattered electron images in the SEM display compositional contrast that results from different atomic number elements and their distribution. EDS is used to find particular elements are and their Atomic %. The Y-axis shows the counts (number of X-rays received and processed by the detector) and the X-axis shows the energy level of those counts [Bob Hofner].

By Viewing 3-D images of specimens solves some of the problem in an analysis and it is also necessary to detect different elements associated with the specimen. This is accomplished by using the “built-in” spectrometer called an Energy Dispersive X-ray Spectrometer.

EDS SYSTEM COMPRISES OF 3 BASIC COMPONENTS

- An X-ray Detector - detects and converts X-ray into electronic signals.
- A Pulse Processor - measures the electronic signals to find out energy of each X-ray detected; and
- A Multiple Channel Analyser - interprets and displays analytical data.



EDS is an analytical technique in which the specimen emits X-rays due to the bombardment of electron beam on it which is used to identify the elemental composition of the specimen due to the ejection of electrons from the atoms on the specimen surface. To explain further, when the sample is bombarded by the

electron beam of the SEM, electrons are ejected from the atoms on the specimen's surface. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The EDS X-ray detector measures the number of emitted X-rays emitted versus their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the *energy versus relative counts of the detected x-rays is obtained and evaluated for the determinations of the elements.*

4.3.7 IDENTIFICATION OF COMPOUNDS PRESENT IN THE VOLATILE OIL OF LEAVES BY GC-MS ANALYSIS.

GAS CHROMATOGRAPHY

GC now ranks as the most important technique in analytical chemistry because of its several advances in its instrumentation. GC requires the vapourization of sample at the injection point which is carried by carrier gas (mobile phase) at a suitable temperature and pressure. The carrier gas which passes through the injection point is heated to the temperature of stationary phase (column) or heated injection block or if flash heater is used to about 50ºC above that of the column. The sample must be stable when vaporized and also its passage through the packed column, in order to avoid the production of complex chromatogram (carrier gas elutes the product from the column) and also when vaporized. The instantaneous vapourisation of sample and the detector produces an electrical output proportional to the amount of compound emerging from the column.

MASS SPECTROMETRY

Wien, in 1898, produced the first crude mass spectra when he demonstrated that positive ions could be deflected according to their masses in electric or magnetic fields. This observation was developed by Thompson (1910) who used combined electrostatic and a magnetic field to observe the mass spectrum of mixture of rare gases.

In single focusing mass spectrometer the sample is introduce into the instrument in such a way that its vapour is bombarded by electrons having an energy of about 70eV. Positive ions formed in ion source are accelerated between two plates by potential difference of a few thousand volts (V). The ions pass through the source slit and are deflected by magnetic field (H) according to their mass/charge ratios. They then pass through the exit or collector slit and impinge upon the collector; the signal received is amplified and recorded. The height or intensity of the resulting peak is proportional to the ion abundance.

COMBINATION OF GC WITH MASS SPECTROMETRY

The identification of fractions in gas chromatography is essentially comparative, in that the characteristic of the unknown are compared with those of known library compound. By correct choice of column, the fraction consists of single substance only, so that, if each is examined by other methods for identification, a powerful analytical tools becomes available. This may be done in several ways and GC is now used in conjuction with IR spectra and mass spectra.

Gas liquid chromatography is a very effective method for separating a complex mixture into its individual components. The high sensitivity of mass spectrometry provides the necessary information for either identification of compounds by comparison with available spectra or structural elucidation of small quantities of compounds. Gases and volatile liquids are admitted to the source through a small leak from the gas reservoir. Hence GC-MS is the introduction of GC effluents without most of carrier gas into a mass spectrometer has its increasing utility in structural organic chemistry, pharmaceutical analysis and biochemistry. Here the fractions which elutes from GC column is condensed into a capillary or onto a small metal surface and this fractions are introduced into the MS source. For multicomponent mixture many operations are involved and losses may occur during the collection of fractions; however, the mass spectrometer may be operated at high resolution and GC carrier gas is admitted to the instrument.

GC/MS combination produce a wealth of data rapidly. To process and interpret all of this data manually would be excessively time consuming.

Instrument Name : JEOL GC MATE 11

Front inlet temp : 220 degree c

Column : HP 5 Ms

Carrier gas : High Pure Helium Gas

Flow Rate : 1 ml/ min

Oven Tem : 50 to 250 @ 10 °C /min

Ion chamber temp : 250 ° C

GC interface Tem : 250 °C

Mass analyser : Quadrupole with double focusing mass **analyser**
Detector : Photon Multiplier Tube
Scan : 50 to 600 amu 70 eV

4.4.1. PRELIMINARY TOXICOLOGICAL STUDIES OF VOCSL ON THE EMBRYO AND LARVAE OF ZEBRAFISH

Toxicology through intensive studies has traditionally focused on the effects of chemicals on living organisms which was done by one chemical at a time. Such approaches show the mode of action of many chemicals and provide a detailed mechanistic understanding of the molecular target of toxicity for some as the cost of this approach is high. Toxicology studies rely on the utility of vertebrate animals which is an expensive undertaking in both time and cost with debatable predictive power in case of safety aspects for human. (Bucher, JR 2002).

Role of zebra fish in high throughput screening:

In the last two decades safety pharmacology has become a most important part of the non- clinical safety assessment in finding new chemical entities(Bass, A *et al*., 2004).

The relative novelty of this discipline has granted it the flexibility to incorporate new experimental tools (Claude, JR and Claude, N 2004).

In addition to requiring small amount of compounds, the time and cost effectiveness of invitro assays have led to their use by the pharmaceutical industry for high or medium throughput safety screens (Suter, W 2006).

One of the limitations encountered with *in vitro* studies is that they are not fully representative of *in vivo* models. Therefore, safety pharmacology needs an

in vivo model with the capacity for higher throughput screening.The zebrafish model system is done from medium to high throughput because of many advantages, as they are small in size, cheap to maintain and fecundity as a single spawning produces 100 – 200 eggs. Larvae, which are only 1-4 mm long, can live for seven days in a single well of a standard 96 or 386 – well microtitre plate by the support of nutrients stored in the yolk sac.

ADMINISTRATION OF DRUGS IN ZEBRA FISH

Larvae of zf can absorb small molecules diluted in the surrounding water through their gills and skin. Drugs can be given orally after this stage because zebra fish begin to swallow at 72(hpf).Drugs can also be delivered by oral intubation in case of adult zf. Compared to testing in other animals models, statistically significant numbers of zebra fish can be used for each assay and small amounts (mg) of drugs are required.in addition the transparency of zebra fish larvae for several days of post fertilization (dpf) enables in vivo observation of live or whole fixed specimens, including the visualization of vital dyes, fluorescent tracers, antibodies and riboprobes. By 120 hpf, zebra fish develop discrete organs and tissues, including brain, heart, liver, pancreas, intestines, bone, muscles, nerve systems and sensory organs. These organs and tissues have been shown to be similar to their mammalian counter parts at the anatomical, physiological and molecular levels. Although conventional in vitro assays using cultured cells can be used to evaluate potential drug toxicity effects, results are frequently not predictive .Results in vivo will involve drug absorption, distribution, metabolism excretion (ADME). To streamline the drug development timeline prioritize drug candidates for animal testing and reduce unnecessary costs for

mammalian studies, drug screening assays using zf are becoming increasing popular (MC Grath P and Chun-Qi Li 2008).

MORPHOLOGY OF ZEBRA FISH LARVAE

Fig-1

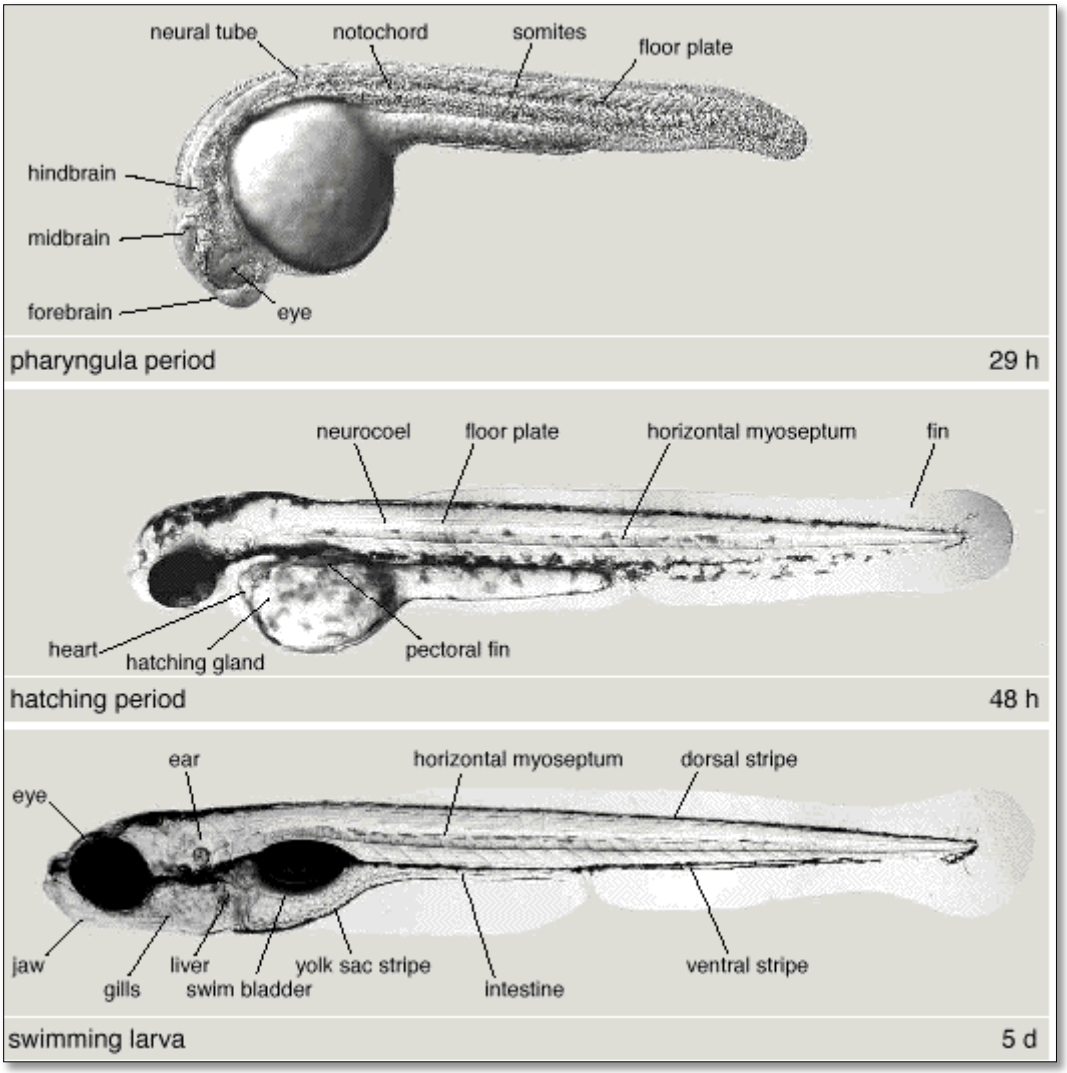


Fig-2

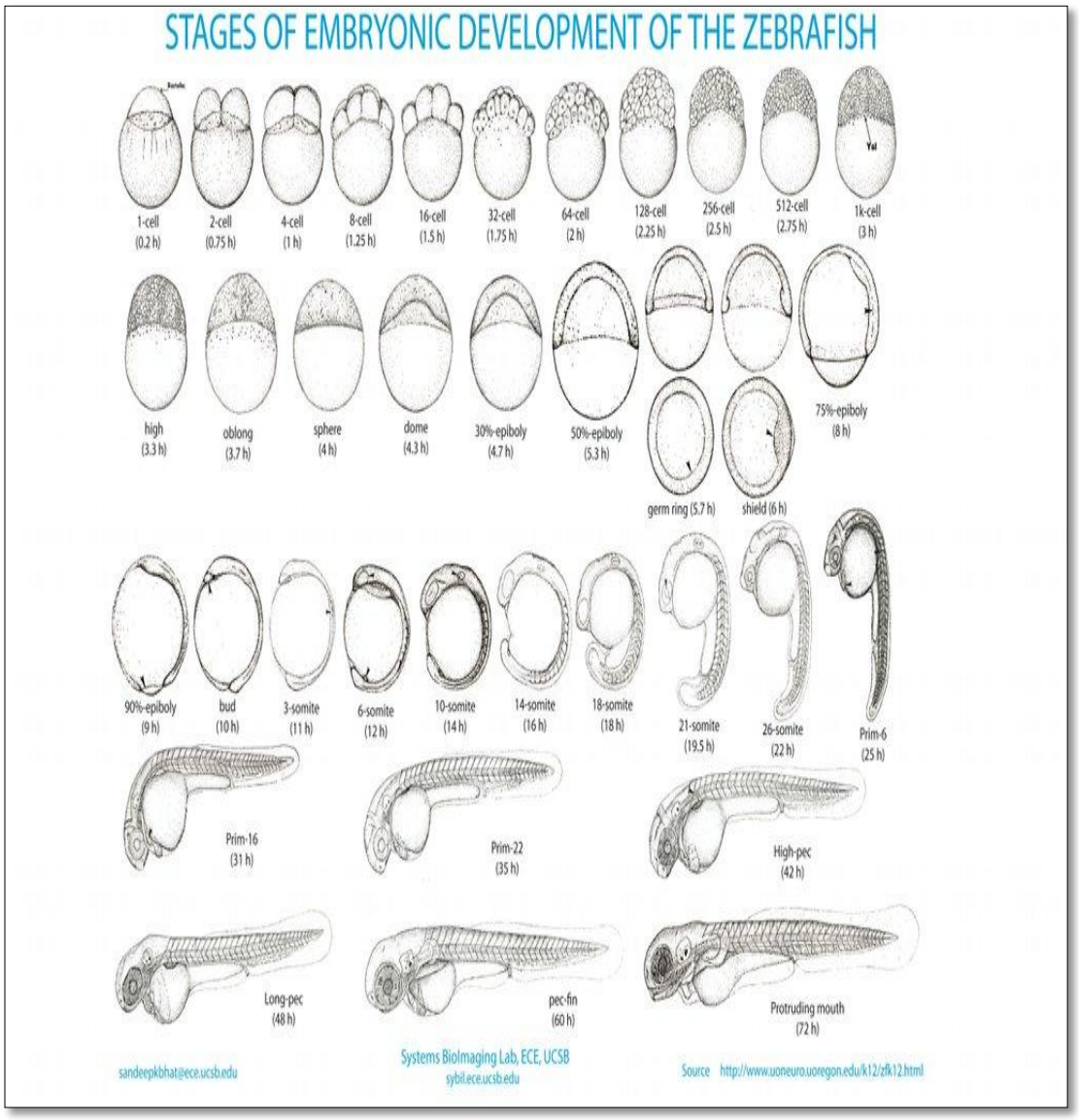


Fig-2 Schematic diagram of the early development of the zebra fish. The stages and the stage specific structures are given according to hisaoka and battle (1958) with the corresponding time after fertilization at 26 abbrevations B.blastomere, C.Chorion, DC.deep cells, EL.enveloping layer, ES.embryonic shielded head enlarge, MY.myomere, N. notochord, O. Oocyte, OP.optic placode, OT. Otic placode, PF.perivitteline fluid, S.somites, T. Tail, Y.yolk, YSL. Yolk syncitial layer.

ZEBRA FISH CENTRAL NERVOUS SYSTEM DEVELOPMENT

Zebrafish have become an important model organism to study the genetic control of vertebrate nervous system development.

We discuss the formation of dopaminergic neuronal groups in zebra fish and compare the positions of DA neurons in fish and mammals using the neuromere model of the vertebrate brain. The novel genes involved in dopaminergic development through forward genetics mutagenesis screens.

An important avenue for biomedical research for Parkinson's disease involves differentiation and circuit formation of the dopaminergic system in animal models.

Several features predispose the zebra fish *(Danio rerio)* as an excellent model organism to study neural development of vertebrates. Zebrafish embryos develop rapidly, and a functional larval nervous system is established within four days of development.

The small embryos and larvae are particularly popular among developmental neurobiologists since genetic analysis, cell biological manipulations, and pharmacological interference can easily be combined in a single embryo.

The transparent embryos allow direct visualization of the results in vivo using transgenic marking of cells with fluorescent proteins. These features make zebra fish an attractive model system to study dopaminergic system development. (S. Ryu, *et al*, 2006)

Epilepsy may be defined as a complex brain disorder which produces spontaneous epileptic seizures. This may also produces typical neurobiological and behavioural alterations. Epilepsy is a complex brain disorder with multiple

underlying causes and poorly understood pathogenetic mechanisms. Animal models have been an indispensable tool in experimental epilepsy research. Zebrafish (*Danio rerio*) are rapidly emerging as a promising model organism to study various brain disorders. Seizure-like behavioural and neurophysiological responses can be evoked in larval and adult zebra fish by various pharmacological and genetic manipulations, collectively emphasizing the growing utility of this model for studying epilepsy. Here, we discuss recent developments in using zebra fish models to study the seizure-like behaviour involved in epilepsy.

Zebra fish larvae was most commonly used new model for chemo convulsant models of epilepsy.

Because of genetic similarity between zebra fish and humans zebra fish larvae offer significant advantages for high through put drug screening.

Pentylene tetrazole [PTZ] induced zebra fish larvae epileptic seizure model for anti convulsant drug screening.

PTZ induced seizure model has most commonly used to introduce anti convulsant from plant extract.

COLLECTION OF ZF LARVAE:

Zf spawn in the morning and the embryos are collected from the bottom of the tank and transferred into a petridish and incubated in vitro using standard medium . Embryo hatch from the chorion at around 48 hour post fertilization (hpf) and the larvae are incubated at 28^0c by 5-6 dpf the yolk has been depleted and larvae must now eat to acquire nutrients (Munoz.G *et al.*, 1990).

WHOLE EMBRYO CULTURE TOXICITY STUDY

Materials

Fertile eggs, E_3embryo medium (standard medium), dimethyl sulphoxide (DMSO), glass petridishes, research microscope (laproscopic microscope with microphotography), incubator, micropipette, VOCSL and standard podophyllotoxin.

Collection of eggs

Eggs were collected from natural spawning and reared in embryo medium at pH 7.2, and kept in an incubator at 28±0.5^{0}C for our assay. The developmental assay stage of the embryos was determined using microscope. (Hisaoka, Kand Battle, HI (1958) (fig-2)

At around 2-4h post fertilization, only the fertilized eggs (blastula stage) were selected. The fertilized eggs were collected and rinsed several times with tap water.

EXPERIMENTAL DESIGN:

The eggs were transferred to each of the glass petri dishes (3 per dish) containing different concentration of VOCSL (0.5, 0.75, 1 and 2µg/ml) dissolved in 1% DMSO at 28^{0}C as well as DMSO control. Embryo medium served as an over-all control. Standard podophyllotoxin of concentration 10µg/ml was taken as positive control .occasional stirring was done to ensure even distribution of the chemical. The maximal acceptable toxicant concentration (MATC) was calculated accordingly by scoring the malformations. (Dave, G *et al*., 1987)

The development of blastula eggs was monitored at specified time points (12, 36, 60, &80 hrs.) under microscope. Endpoints were used for assessing the effect of drug during the major organ is visible included edema, eye,

malformations, bent tail, undulated notochord, twisted notochord and death. Malformations were also noted and described among the juveniles from the control 1% DMSO treated and standard podophyllotoxin.

LARVAL TOXICITY STUDY

Materials

Zf larvae of 5 dpf ,E3 embryo medium ,1% DMSO, glass petri dishes ,research microscope (laboscope microscope with microphotography) , incubator, micropipette VOCSL , and standard podophyllotoxin.

Experimental design;

Healthy 5 dpf larvae was selected and used for larval toxicity study. About 5 larvae were released in the embryonic medium (10ml) taken in a petridish, in triplicate. Various concentration of EERM (0.5, 0.75, 1 and 2mg/ml) dissolved in 1% DMSO in the embryonic medium were tested. Two controls were used DMSO and embryonic medium respectively. Podophyllotoxin (10µg/ml) was used as standard toxin.

LETHALITY CONCENTRATION DETERMINATION

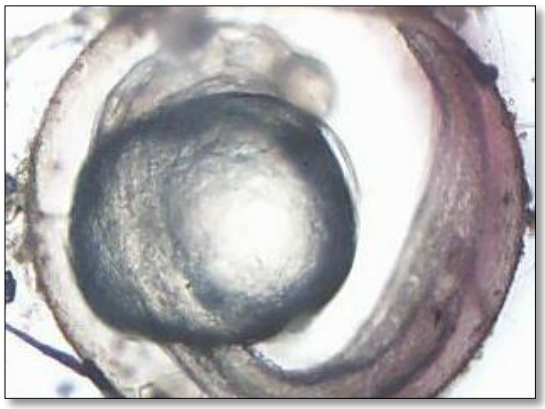
The percentage lethality was determined by comparing the mean surviving larvae of the test and control vials. Graph was plotted, concentration versus percentage lethality. Podophyllotoxin was used as a positive control in the bioassay. The percentage lethality was calculated from the mean survival larvae of various concentrations of VOCSL treated dishes and control. The corrected (%) mortality was calculated by using schneider-Orelli's formula.

$$\text{Corrected\%} = \frac{\text{Mortality (\%) in treated} - \text{Mortality (\%) in control}}{\text{100-Mortality (\%) in control}} \times 100$$

This study was focused on the induction of seizure after the administration of convulsant drugs and the distance travelled was measured and the behavioural changes in the CNS was determined and also the inhibition of seizure was focussed in the CNS of zf that provides an *in vivo* experiment as well as the potential for h high throughput drug screening.

EMBRYO AND LARVAE OF ZEBRA FISH

EMBRYO OF ZEBRA FISH **LARVAE OF ZEBRA FISH 5 dpf**



ZEBRA FISH LARVAE 7dpf

ZEBRA FISH HANDLING (Zhou, J *et al*., 2015)

200-300 embryos were maintained at 28^{o}c in fish water (0.2% salt in demonized water, pH 6.9-7.2) and the embryos were collected.

DETERMINATION OF MAXIMUM NON LETHAL CONCENTRATION (MNLC)

To determine MNLC of a test drug, 5 dpf Zf larvae were treated with a testing drug for 48 hrs. and mortality was recorded at the end of the treatment . Dead Zf larvae were defined as the loss of memory or brain actions under a dissecting microscope. In the initial tests various concentration (100-900µg/ml) were used.

BEHAVIOURAL ASSSAY USING PENTYLENE TETRAZOLE (PTZ) ON ZEBRA FISH SEIZURE MODEL :(Chongbin Liu *et al*, 2016)

Epilepsy is a complex brain disorder marked by recurrent spontaneous epileptic seizures associated with typical neurobiological and behavioural alterations in the past few decades, rodent seizure models induced by a broad spectrum of mechanisms have contributed significantly to our knowledge on epilepsy and anti-epileptic drugs (AEDs) discovery .

Zebrafish larvae have recently emerged as a new species for chemoconvulsant-based models of epilepsy. Because of the genetic similarity between zebra fish and humans, zebra fish larvae offer significant advantages for high-throughput drug screening.

The availability of zebra-fish larvae model of epileptic seizure provides advantages to identify novel anticonvulsants for treatment of people with epilepsy.

To induce seizures, PTZ (1.25 - 30 mM) was added to fish water. PTZ added to fish reliably elicited distinct seizure-like behaviours in a concentration-

dependent manner. Zebra-fish larvae exhibited signs of agitation within seconds after exposed to PTZ. The larvae swam along the periphery of the multi-well plate, thus displaying stage I seizure-like behaviour as described previously. This was succeeded by rapid 'whirlpool-like' movement, and then followed by a short pause before swimming in a rapid, jerky manner with occasional body-stiffening and loss of posture (larva turning onto its side or back).

It was quantified by high speed (V> 20mm/s) moved distance. PTZ 10mM induced most significance increase in locomotor activity within 1hr. All compounds were dissolved in DMSO and diluted in embryo medium to achieve the final concentration in zebra-fish larvae model exposed to 10 mM pentylene tetrazole. The anti-epileptic drug, phenytoin, showed the same efficacy patterns in zebra-fish seizure model as in mammalian epileptic models. Concentration-dependent inhibition of locomotor activity, confirming their anticonvulsant characteristics. Hence this zebra-fish larvae model could be useful for assessing anti-epileptic drug efficacy, facilitating the primary drug screening and evaluating of effective components in medicinal plants.

4.4.2. Evaluation of VOCSL on PTZ induced seizure model

Seizure induced zf larvae of 5dpf were placed in 48 well plates at 1 larvae per well which different concentration of VOCSL (0.5, 1, 1.5, 2mg/ml) (test). The larvae was treated with normal diet in embryonic medium serve as the control. Seizure like behaviour was assessed both for normal diet and PTZ administered larvae. All zf larvae were exposed for 1 hour. After 1 hours using digital video camera, the distance travelled per minute for each larva was recorded. Seizure like behaviour and distance travelled. The test was performed in duplicate. (n=6).

After the pre-incubation, 100 µl of embryo medium or 100 µl of PTZ solution was added. Larvae were allowed to habituate for 10 min in a dark chamber of an automated tracking device (ZebraBoxTM apparatus; Viewpoint, Lyon, France). Control groups were embryos of the same stages without any chemical treatment. The locomotor activity was then quantified using ZebraLab TM software (Viewpoint, Lyon, France) (Afrikanova *et al.,* 2013). The system consists of an infrared light source, a high-resolution digital video camera to capture larval movements within a defined time period (60 min in our experimental set-up) and the software to analyze larval locomotor activity.

BEHAVIOURAL ASSAY

5 dpf (days post fertilization) zebra-fish larvae were pre-incubated in 100 µl of AED, or vehicle for 1 hr. in individual wells of a multi-well plate .For AEDs primary screening from plant extract, we performed in individual wells of a 48-well plate. At least eight larvae were used for each group and every experiment was performed in duplicate.

After the pre incubation, 100 µl of embryo medium or 100 µl of PTZ solution was added. Larvae were allowed to habituate for 10 min. Control groups were embryos of the same stages without any chemical treatment. The locomotor activity was then quantified. The total and high-speed moved distances were chosen for subsequent efficacy assay. The total and high-speed moved distances were chosen for subsequent efficacy assay.

Efficacy of a test drug on epilepsy was calculated based on the formula below:

Drug effect on = [1- Distance (vehicle) Distance (drug)] × 100%

Epileptic locomotor activity (%)

Statistical Analysis

One-way ANOVA was used to compare differences among groups. All statistical analysis was performed using SPSS 16.0 software (SPSS, USA) and $P < 0.05$ was considered statistically significant. For quantitative analysis, all data were presented as mean ± SEM and results were statistically compared between drug-treated and vehicle-treated zebra fish groups.

RESULTS

5.1 PHARMACOGNOSY

5.1.1 MORPHOLOGICAL FEATURES OF *Citrus sinensis* (Plate1-6)

Citrus sinensis is an evergreen flowering tree growing to a height of about 9 to 10 metres and although very old specimens have reached 15 meters with large spines on branches.

Arrangement and shape	:	The leaves 4-10 cm long arranged alternately. The shape of blades ranges from elliptical, oblong to oval, bluntly toothed
Characters	:	Leaves having dark green above, glossy, with a distinctive smell often similar to the fruit, bluntly toothed and they emit a strong characteristic citrus odour due to the presence of copious oil.
Size	:	About 4-12 cm long and 6 cm wide,, with visible veins
Colour:	:	Dark green above
Petiole	:	Petiole narrowly winged-petioles (3-5 cm long)
Apex	:	Acute to obtuse
Margins	:	crenulate margins.

FLOWERS (Plate-4)

Arrangement : Flowers are small, waxy greenish-white. Flowers are axillary ,fragrant, single, few or cymose and often perfect (having both functional stamens and pistils.

Calyx : 4-5 lobed ,calyx broad saucer-shaped.

Bell-shaped hypanthium with 4 pale yellow opposite sepals.

Sepals : pointed leathery.

There are generally five petals, white elliptic, 1.3-2.2 cm long

Petals : and contain some oil glands.

Stamens : Number of range from 20 to 40 yellow stamens

Anthers : 6–8 mm long, and 4 opposite petals.

Peduncles : 2.5-5 cm long; flowers with 4-8 mm long pedicels and united, cup-shaped bracteoles at; calyx deeply lobed, 13-19 mm long,

Base : pale yellow or almost white;

Style : Flowers are axillary borne singly or in whorls

FRUIT (Hypocotyl) (PLATE-5)

Shape : Fruits are globose to ovoid in shape.

Size : 4-12 cm Long , 4-6 cm Wide with consist of a leathery peel 6mm thick, tightly adherent, protecting the juicy inner pulp, which is divided into segments that may not contain seeds depending on the cultivar,

Colour : Reddish-green to yellowish- green colour .colour may be typical of the variety.

Seed origin : Seeds are greenish to pale whitish, flattened, and angular. It is generally poly embryonic.

Shape : Angular and flattened

STEM

Shape : Cylindrical, or developing a straggling or semi-prostrate

Colour : Dull brown colour

Bark colour : Black or reddish,

Shape : Rough or sometimes scaly, with prominent, Horizontal crack almost encircling the stem.

T.S.OF MIDRIB (Plate-7, 8)

Transverse section of midrib shows a small hump on the adaxial surface and very slight convexity on the abaxial surface. Epidermis is single layered and covered by a very thick cuticle. Palisade tissue is not continuous along the midrib region. Secretory cavities are present near the epidermis.

Vessels are circular and arranged as a row. Ground tissue is parenchymatous and most of the parenchyma cells contain tannin.

EPIDERMIS IN SURFACE VIEW:

Adaxial and abaxial epidermal cells are polygonal in shape with straight wall. Stomata are anamocytic and confined to the abaxial surface.

Vascular bundle: Large vascular bundle present in the centre. It is surrounded by sclerenchyma fibres outside.

T.S.OF LAMINA (Plate-9)

Transverse section of lamina is dorsiventral. No trichomatous out growth. Adaxial and abaxial epidermal cells are more or less similar in structure. Both the epidermis are made up of rectangular cells covered by a very thick, smooth cuticle.

Mesophyll is differentiated into outer palisade tissue made up of two layer of small, columnar closely packed cells. It is followed by spongy tissue made up of 3 to 5 layers of loosely arranged parenchyma cells forming large intercellular spaces. Vascular bundles of the vein is small and mostly embedded in the mesophyll. Secretory cavities (Lysigenous) are present near to epidermis.

T.S.OF PETIOLE: (Plate-11)

Transverse section of petiole presents a circular outline with lateral wings. The outermost epidermis is single layered and covered by a thick cuticle. Trichomes are absent. Ground tissue is made up of loosely arranged parenchyma cells. Most of the parenchyma cells contain tannin. Vascular bundle is present in the centre.

SCANNING ELECTRON MICROSCOPY (SEM) (Plate-14)

The surface study of leaf was studied using SEM. It revealed the presence of **anamocytic stomata** in the abaxial side of leaf. Stomata aperture and epidermal cells are clearly seen.

POWDER MICROSCOPY (FIG-3)

ORGANOLEPTIC CHARACTERS

1. Nature : Coarse fibre powder
2. Colour : Yellowish Green ash
3. Odour : Aromatic characteristics odour
4. Taste : Characteristic sour pungent taste
5. Shaking with water: Frothing occurs in water

6. Pressed in between two filter paper: No oil mark on the paper we have observed the following microscopically cell structures, anamocytic stomata.

We have observed the following microscopically cell structure,

- ❖ Upper epidermis with palisade cells
- ❖ Xylem vessels
- ❖ Epidermal cells with anticlinal wall
- ❖ Subsidiary cells with multiple epidermal cells
- ❖ Epidermal cells with stomata
- ❖ Secretory cavity
- ❖ Stomata are **anamocytic type**
- ❖ Crystals
- ❖ Fibers
- ❖ Xylem, Phloem

5.1.5 MICROSCOPIC SCHEDULES

As per the methods described in materials and methods, microscopic schedule was carried out and the results were tabulated from the Tables 1.

TABLE-1

VEIN ISLET AND VEIN TERMINATION NUMBER OF *Citrus sinensis*

OBSERVATION NUMBER	VEIN ISLET NUMBER	VEIN TERMINATION NUMBER
1	4	6
2	6	5
3	6	7
4	8	8
5	5	6
6	6	6
7	5	4
8	6	8
9	5	5
10	6	6
Minimum	**4**	**6**
Average	**5.7**	**6**
Maximum	**8**	**8**

TABLE-2

STOMATAL NUMBER OF *Citrus sinensis*

OBSERVATION NUMBER	LOWER EPIDERMIS
1	37
2	37
3	36
4	39
5	36
6	30
7	31
8	30
9	38
10	32
Minimum	29
Average	34.6
Maximum	39

Table-3

STOMATAL INDEX OF *Citrus sinensis*

OBSERVATION NUMBER	LOWER EPIDERMIS
1	37
2	38
3	35
4	39
5	36
6	29
7	32
8	30
9	38
10	32
Minimum	**29**
Average	**34.6**
Maximum	**39**

Table-4

PALISADE RATIO OF *Citrus sinensis*

OBSERVATION NUMBER	PALISADE RATIO
1	3
2	4
3	3
4	5
5	4
6	3
7	4
8	3
9	4
10	4
Minimum	**3**
Average	**3.7**
Maximum	**5**

5.1.6 PHYSIOCHEMICAL PARAMETERS

As per the methods described in materials and methods, physic chemical parameters was carried out of quantitative microscopy and the results were tabulated from the tables 5-7.

TABLE: 5

ASHVALUE FOR THE LEAVES OF *Citrus sinensis*

OBSERVATION	TOTAL ASH (%)	ACID INSOLUBLE ASH (%)	WATER SOLUBLE ASH (%)
1	12.79	0.75	-
2	11	0.57	-
3	12.98	0.65	-
4	13.32	0.6	-
5	12.05	0.7	-
6	12.86	-	4.85
7	12.12	-	5.08
8	13.49	-	5.04
9	13.25	-	4.96
10	12.23	-	5.13
Minimum	**11**	**0.57**	**4.85**
Average	**12.6**	**0.65**	**5.01**
Maximum	**12.98**	**0.75**	**5.3**

TABLE-6

TABLE: 6

PERCENTAGE OF LOSS ON DRYING FOR THE LEAVES OF *Citrus sinensis*

OBSERVATION NUMBER	LOD %w/w
1	5.35
2	6.02
3	5.25
4	5.85
5	5.9
Minimum	**5.25**
Average	**5.67**
Maximum	**6.02**

TABLE: 7

EXTRACTIVE VALUE FOR THE LEAVES OF *Citrus sinensis*

SOLVENTS	EXTRACTIVE VALUE (%)
Petroleum ether	2.39
Ethanol	9.75
Water	8.68

5.2 PHYTOCHEMICAL STUDIES

5.2.1 PRELIMINARY PHYTOCHEMICAL SCREENING

QUALITATIVE PHYTOCHEMICAL TEST

Preliminary phytochemical screening of the powdered mature leaves were carried out and the results are as follows (Table 8)

TEST FOR ALKALOIDS

Mayer's test	:	Cream precipitate shows the **Presence** of alkaloids
Dragendorff's test	:	Reddish brown precipitate shows the **Presence** of alkaloids
Hager's test	:	Yellow precipitate shows the **Presence** of alkaloids

TEST FOR CARBOHYDRATES

Molisch's test	:	Purple colour shows the presence of carbohydrates.
Fehling's test	:	Reddish brown precipitate shows the presence of free reducing sugars.
Benedict's test	:	Reddish brown precipitate shows the presence of free reducing sugars.

TEST FOR GLYCOSIDE

Keller Killani' s test	:	No reddish brown colour ring at the junction shows the absence of cardiac glycoside
Borntrager's test	:	No appearance of pink colour Shows the absence of anthraquinone glycosides
Modified Borntrager's test	:	No pink colour in ammonical layer shows the absence of anthraquinone glycoside

TEST FOR PHYTOSTEROL

Salkowski's test	:	Appearance of red colour in lower layer shows the **Presence** of sterol
Liebermann – Burchard's test	:	Brown ring at the junction of two layers and green colour in the upper layer shows the **Presence** of sterols

TEST FOR SAPONINS:

Frothing not occurs indicates the absence of Saponins

TEST FOR TANNINS

Ferric chloride test	:	Appearance of bluish black colour shows the **presence** of tannins
Gold beater skin test	:	Appearance of brown colour shows the **presence** of tannins.

TEST FOR PROTEINS AND FREE AMINOACIDS

Millon's test	:	Appearance of red colour on heating shows the **presence** of proteins
Biuret test	:	Appearance of violet colour shows the **presence** of proteins
Ninhydrin test	:	Formation of violet colour shows the **presence** of amino acids

TEST FOR TERPENOIDS

Appearance of pink colour shows the presence of terpenoids

TEST FOR FLAVONOIDS

Shinoda test	:	Purple colour shows the **presence** of flavonoids
Alkaline reagent test	:	Yellow - orange colour shows the **presence** of flavonoids
Acid test	:	Yellow – orange colour shows the **presence** of flavonoids
Zinc hydrochloride	:	Red colour shows the **presence** of flavonoids test

TEST FOR VOLATILE OIL: Volatile oil is obtained. It shows the presence of VO

TEST FOR FIXED OIL: No translucent greasy spot shows the absence of fixed oils

TABLE-8 RESULTS FOR THE PRELIMINARY PHYTOCHEMICAL SCREENING OF LEAVES OF *Citrus sinensis*

S.NO	TEST	OBSERVATION
I	**ALKALOIDS**	
	Mayer's reagent	+
	Dragondroff's reagent	+
	Hager's reagent	+
II	**CARBOHYDRATES**	
	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
III	**GLYCOSIDES**	
	Anthroquinone glycosides	-
	Borntrager's test	-
	Modified Borntrager's test	-
	Cardiac Glycosides	
	Keller killiani test	-
	Raymond test	-
	Legal test	-
IV	**STEROLS**	
	Salkowski test	+
	Libberman burchard's test	+
V	**SAPONINS**	**-**
VI	**TANNINS**	**+**
	Ferric chloride test	+
	Gold beater's skin test	+
VII	**PROTEINS AND FREE AMINO ACIDS**	
	Millon's test	+
	Biuret test	+
	Ninhydrin test	+
VIII	**MUCILAGE**	-
IX	**TERPENOIDS**	+
X	**PROTEINS AND FREE AMINO ACIDS**	
	Millon's test	+
	Biuret test	+
	Ninhydrin test	+
	Acid test	+
	Zinc/Hcl test	+
XI	**VOLATILE OIL**	**+**
XII	**FIXED OIL**	**-**

5.2.2 FLUORESCENCE ANALYSIS OF POWDERED LEAF

The fluorescence analysis of the leaf powder of *Citrus* ***sinensis*** was studied. The results were as follows (Table-9).

TABLE-9

FLUORESCENCE ANALYSIS

S.NO	TREATMENT	VISIBLE LIGHT	UV 254nm	UV 365nm
1	POWDER	Light brown	Green	Purple
2	POWDER+ETHANOL	Dark brown	Dark green	Greenish brown
3	POWDER+ETHYL ACETATE	Brown	Brown	Purple
4	POWDER+CHLOROFORM	Black	Dark green	Fluorescent red
5	POWDER+WATER	Brown	Brown	Dark brown
6	POWDER+50%H_2SO_4	Dark brown	Greenish black	Brown
7	POWDER+10%NAOH	Blackish brown	Greenish black	Violet
8	POWDER+50%NITRIC ACID	Yellowish brown	Green	Violet

5.2.3 ESTIMATION OF FLAVONOID CONTENT

Flavonoid content of Ethyl acetate Extract of leaves of *Citrus sinensis* in terms of Rutin by aluminium chloride was found to be **26.73 ± 1.10µg/g.**

5.2.4 ESTIMATION OF TOTAL PHENOLIC CONTENT

Total phenolic content of Ethyl acetate Extract of leaves of *Citrus sinensis* in terms of Gallic acid was found to be **879.13 ± 0.92 µg /g**

5.2.5 DETERMINATION OF TRACE ELEMENTS IN THE LEAF OF *Citrus sinensis* BY ENERGY DISPERSIVE X-RAY SPECTROMETER (EDS)

Estimation of the elements like C, O, Na, Mg, Al, Si, P, S, Cl, K, and Ca showed the following mg weight percentage and atomic percentage.

TABLE-10

***Citrus sinensis* LEAVES ELEMENTS WEIGHT AND ATOMIC PERCENTAGE**

Element	Weight%	Atomic%
C K	73.17	78.85
O K	25.6	20.71
Al K	0.34	0.16
Si K	0.2	0.09
K K	0.29	0.1
Fe K	0.4	0.09
Totals	100	

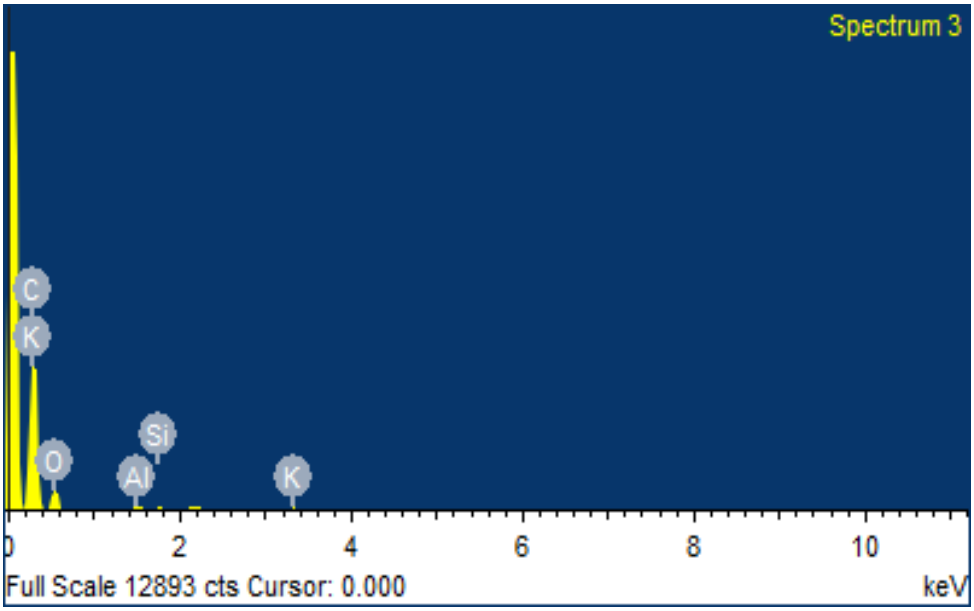


FIG-4

ENERGY DISPERSIVE X-RAY SPECTRUM FOR *Citrus Sinensis* LEAVES

5.4.6 IDENTIFICATION OF COMPOUNDS PRESENT IN THE VOLATILE OIL OF LEAVES BY GC-MS ANALYSIS

The GC-MS analyscis of the isolated V.O indicated the presence of following constituents by comparing with the instrument library.

1) α-Pinene and β-Pinene
2) Sabinene
3) Myrcene
4) Limonine
5) Terpinolene
6) Linalool
7) β-Elemene
8) Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
9) Gamma.-Terpinene
10) 1-Pentanol, 5-cyclopropylidene
11) 2,6,10-Dodecatrienal, 3,7,11-trimethyl-
12) (R)-lavandulyl acetate
13) Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-
14) Caryophyllene
15) Humulene
16) 1,5,9-Cyclododecatriene, 1,5,9-trimethyl
17) 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)
18) Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)
19) Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-,
20) Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta

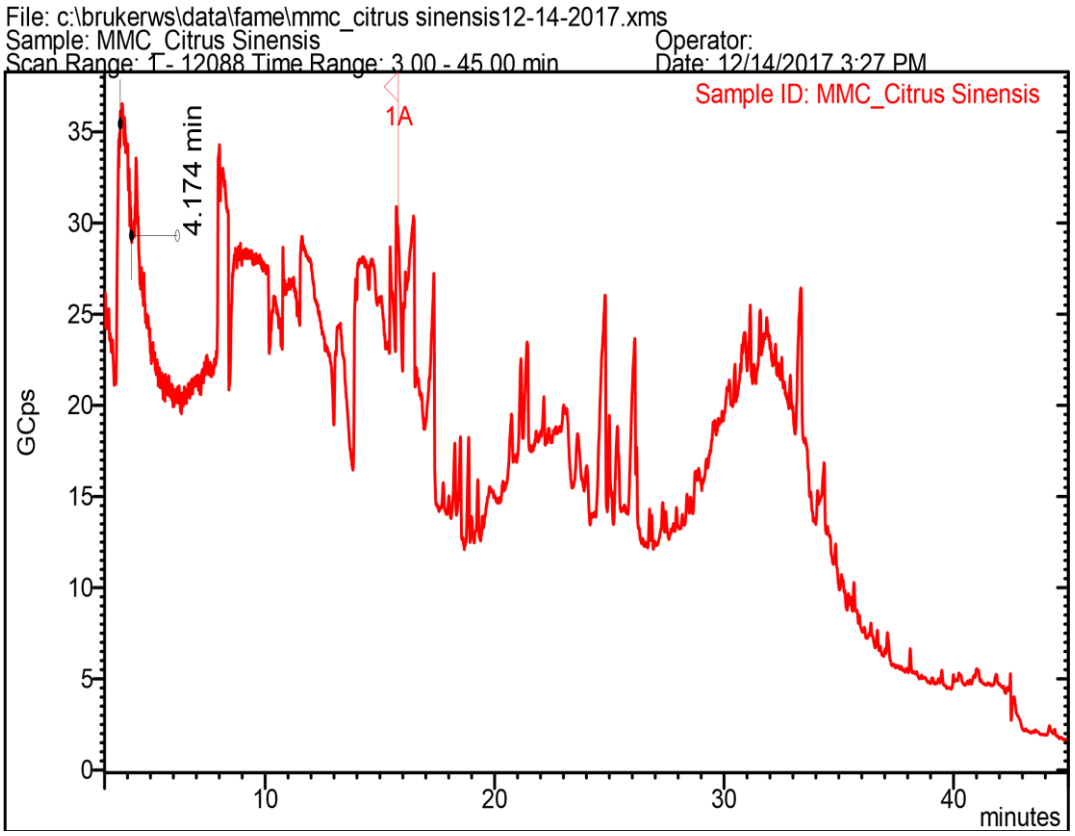
21) 1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-,

22) Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)

23) 3,7-Cyclodecadiene-1-methanol, .alpha.,.alpha.,4,8-tetramethyl-,

24) 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-

25) Caryophyllene oxide



5.3 PHARMACOLOGICALSTUDIES

5.3.1 WHOLE EMBRYO CULTURE TOXICITYSTUDY

Effect of VOCSL on the developmental stages of Zf embryo was carried out. The eggs were cultured in the embryonic medium. The maximal acceptable toxicant concentration (MATC) was calculated by scoring the malformation 1 % DMSO and podophyllotoxin (0.010µg/ml) were used as control and standard toxin. No malformations and incidence of mortality was observed up to the 0.5 – 1.5µl/ml concentration level (score 0). But medium to strong edema, eye malformation, bent tail, weak undulated notochord and twisted notochord were observed from above 2.0 to 4 µl/ml concentration up to 80 hpf. No mortality was observed in this concentration level. Total mortality was observed in the standard podophyllotoxin at 0.01µg/ml concentration (score40).

TABLE 11

SCORES FOR THE WHOLE EMBRYO TOXICITY

Conc. µl/ml	Hours	score
0.5 – 1.5	**All**	**Nil**
2	12	1
	36	1
	60	2
	80	2
4	12	5
	36	5
	60	10
	80	13

5.3.2 ZEBRA FISH LARVAL TOXICITYSTUDY

Larval toxicity study was carried out on zf larvae of 5 dpf cultured in E3 embryonic medium. Five larvae per group was taken and treated with 0.5, 0.75, 1 and 2 µl was taken as a test trail and mortality percentage was calculated.

From the experiment it was observed that there was no mortality in 0.5 and 0.75µl/ml concentrations. But 5% and 10% mortality was observed at 1 and 2 µl/ml concentrations respectively. No mortality was observed in the control. 100% mortality was observed in the standard podophyllotoxin at 0.5 µg/ml concentration.

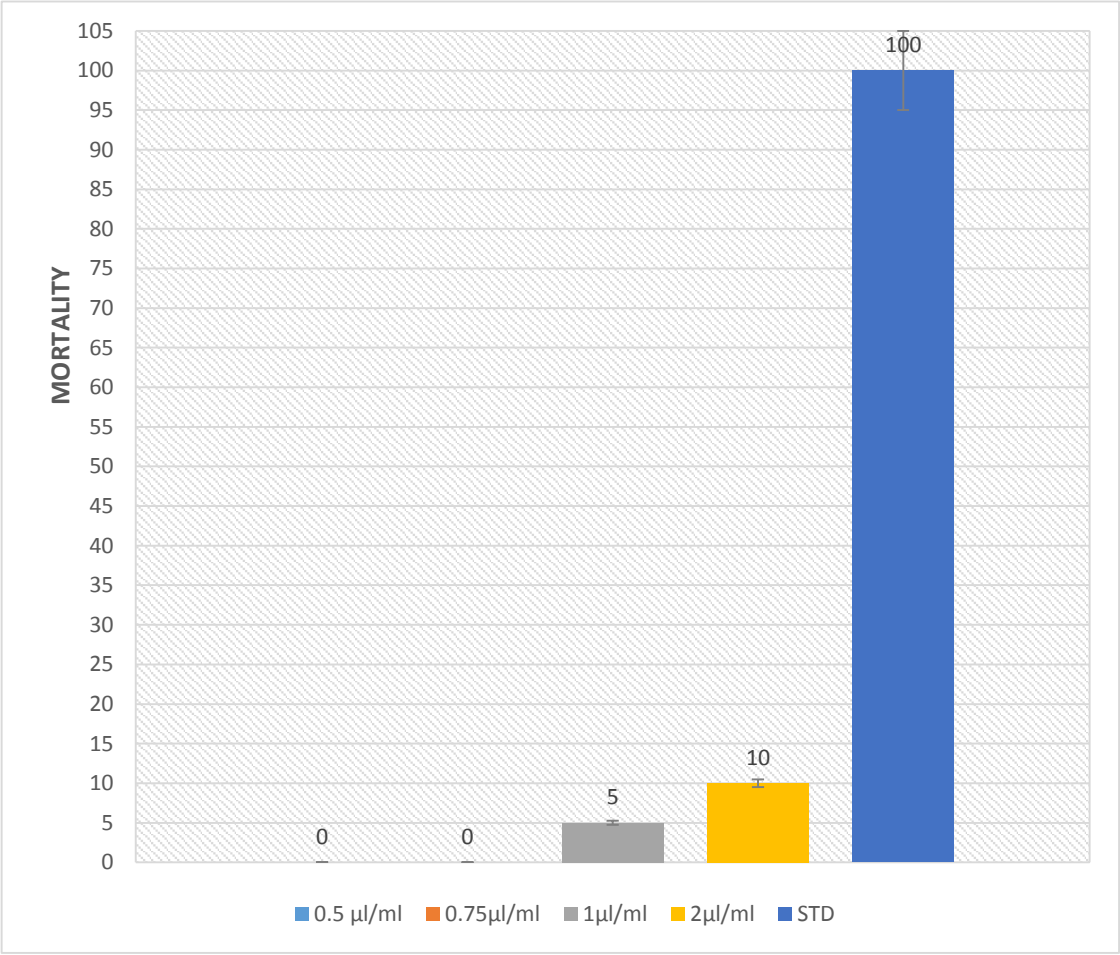
TABLE12

ZEBRA FISH LARVAL TOXICITY STUDY

CONCENTRATION (µl/ml)	NUMBER OF LARVAE	AFTER 24h	MORTALITY	CORRECTED MORTALITY
0.5	20	NIL	NIL	NIL
	20			
	20			
0.75	20	NIL	NIL	NIL
	20			
	20			
1	20	NIL	NIL	5
	20	NIL	NIL	
	20	1	5	
2	20	1	10	10
	20	1	10	
	20	NIL	NIL	
Control (vehicle)	20	NIL	NIL	NIL
	20			
	20			
	20			
Standard Podophyllotoxin	20	20	100	100
	20	20	100	
	20	20	100	

FIG. 5

ZEBRA FISH LARVAL TOXICITY



5.3.3 BEHAVIOURAL ASSSAY USING PENTYLENE TETRAZOLE ON ZEBRA FISH LARVAE SEIZURE MODELS

The capacity of the VOCSL to reduce PTZ-induced convulsions in zebra fish larvae was assessed through quantification of the high-speed moved distance. A sample movement plot was given. Phenytoin suppressed zebra fish larvae locomotion (477mm/hr.) when comparing with PTZ treated alone group (4016mm/hr.).

5.3.4 EVALUATION OF ANTI- CONVULSANT ACTIVITY OF VOCSL ON ZEBRA FISH LARVAE SEIZURE MODEL

PTZ induced seizure zf model showed increased high speed distance moved (4016±2.24). But there was a capacity of decrease in the high speed movement per mm were observed in phenytoin (1000µm), for VOCSL 0.5,1, 1.5µl/ml as follows 477±1.2, 790±1.54, 609±.88, 498±1.15 respectively. The values were statistically significant ($p \leq 0.001$).This result showed potential protection of PTZ induced convulsion (i.e.) anti- convulsant activity. The results also showed that seizure like swimming pattern was alleviated by the addition of the VOCSL. It exhibited concentration dependent inhibition of locomotors activity confirming their anticonvulsant activity.

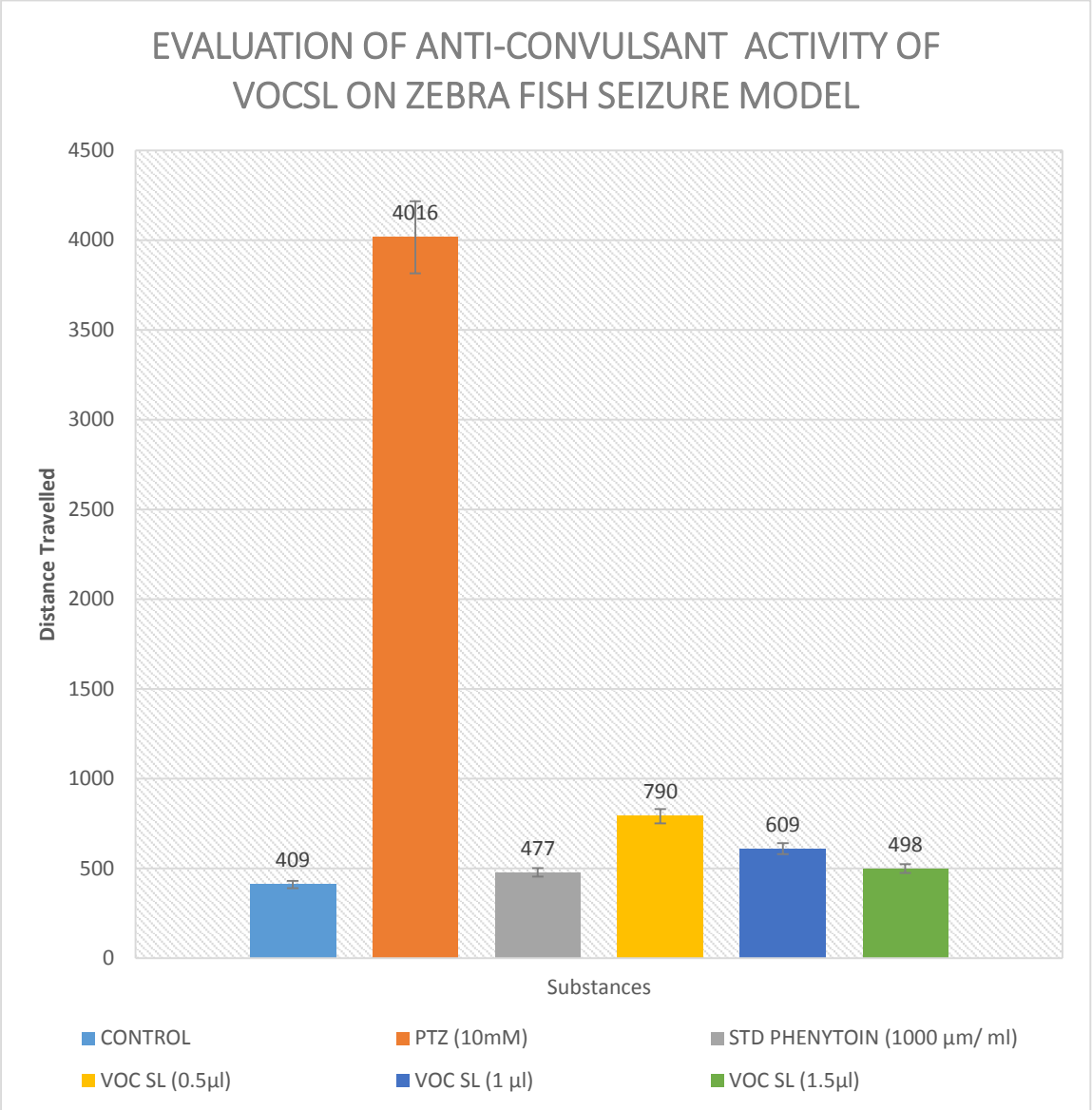
TABLE-13

EVALUATION OF ANTI- CONVULSANT ACTIVITY OF VOCSL ON ZEBRA FISH LARVAE SEIZURE MODEL

NAME OF THE SUBSTANCE	CONCENTRATION	SEIZURE INDUCTION (DISTANCE TRAVELLED)
CONTROL	_	409±1.32
PENTYLENE TETRAZOLE(PTZ)	10mM	4016 ±2.24
STANDARD DRUG PHENYTOIN	1000µm/ml	477±1.2
VOCSL	0.5µG/ml	790±1.54
VOCSL	1µl/ml	609±1.88
VOCSL	1.5µl/ml	498±1.15

FIG. 6

EVALUATION OF ANTI- CONVULSANT ACTIVITY OF VOCSL ON ZEBRA FISH LARVAE SEIZURE MODEL



DISCUSSION

The dissertation covers a study on widely available member of the family Rutaceae known botanically as *Citrus sinenesis* L commonly called as '**Sweet orange**' in English and sathukudi in Tamil. The leaves of *C. sinenesis* really do not have any match as a cheap natural and easily available plant. It was reported that Rutaceae family members have phytoconstituents useful in the treatment of various diseases and was also claimed that these plants merit detailed study which can prove useful in the discovery of lead compounds leading to novel and more efficacious drugs. Leaves of *C. sinenesis* of this family, is traditionally known to be useful for the treatment of wide panel of diseases. *C. sinensis* is consumed all over the world as an excellent source of vitamin C, which is a powerful natural antioxidant that builds the body's immune system. It has been used traditionally to treat ailments like constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress. **Leaf extracts of *C. sinensis* have been used in Nigerian local folk medicine to treat neurological disorders and to facilitate the digestion of food** ((Intekhab J and Aslam M 2009). It has also been used as an antidiabetic, antibacterial, antifungal, hypotensive, antioxidant, insect repellent, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent due to the presence of copious oil.

The oils are also generally in use in many foods, confectionary, drug, cosmetic and flavoring products. Various scientific investigation of the leaves showed Leaf essential oil was reported to possess anti-ulcer, anti-anxiety, anti-typhoid, anti-bacterial, larvicidal, anti-diabetic, anti-fungal and anti-inflammatory activities.

C. sinensis is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits, peel, leaves, juice and roots of *C. sinensis*, which include the following groups: flavonoids**.** steroids , hydroxyamides, alkanes and fatty acids, coumarins, peptides , carbohydrates, carbamates and alkylamines , carotenoids , volatile compounds , and nutritional elements such as potassium, magnesium, calcium and sodium. Fruit helps to eliminate toxins from the body. Fruit juice helps to maintain hydration. 3 It is used as a general tonic. Orange juice is useful in cases of anxiety disorder and stress. It is used as a Mexican traditional medicine for the treatment of tuberculosis. It is used in stomach upsets; it improves appetite and prevents constipation. The humble fruit has a long history in Chinese Medicine as a cooling agent for coughs, colds and respiratory disorder. It is a traditional Chinese symbol of good luck and prosperity .It is used in the treatment of obesity.

It's fruit symbolizes innocence and fertility. In France, it is used for the treatment of angina, hypertension, constipation, diarrhoea, menstrual disorder. Pharmacologically fruits was proved to possess antioxidant, protection against cardiovascular diseases, analgesic, antiarthritic, antidiabetic, anticarcinogenic, antiulcer, antianxiety, antifungal and larvicidal activity.

The survey of literature and preliminary phytochemical screening reveals that leaves contain various phytoconstituents like volatile oil, flavonoids, and terpenoids. Many substances have been isolated from the leaves like glycosides, flavonoids like ruteosides, hesperidin, and diosmin. Leaf extracts of *C. sinensis* have been used in Nigerian local folk medicine to treat neurological disorders.

The GC-MS analyscis of the isolated VOCSL indicated the presence of following constituents α-Pinene , β-Pinene, Sabinene, Myrcene, Limonine, Terpinolene, Linalool and β-Elemene

Methanolic extract and hydroethanolic extract (both 100mg/kg/*P.O*) of the leaves showed anticonvulsant activities induced by maximal electric shock and PTZ in mice respectively (Reddy, NL *et al*., 2016 Nagula, JB *et al.,* 2017). In this investigation the phytoconstituents responsible for the anticonvulsant activity was not found out. Hence we decided to investigate the anticonvulsant property of the VOCSL which is the one of the important constituent present in the leaves of *C.sinensis*.

The economic aspect of this crop evidently proved that as commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored well. By a well-coordinated effort, we can properly exploit this plant. Therefore research on development of herbal products from this plant is required to be initiated immediately for exploring the unique potential of this crop which would also minimize the menacing wastage especially the leaves. It may be further envisaged that the revenue generated by this plant would easily exceed that generated by any major crop of the country even with a present level of traditional agro economic practices. Therefore a well-coordinated effort by the farmers, traders, scientist, technologists, extension workers, physician, administrators, and policy makers is required to be initiated to boost up the national economy as well as the proper exploitation of this for proper therapeutic purpose. The review of literature showed some lacuna exists in the pharmacological, phytochemical, and pharmacological studies in the leaves.

PHARMACOGNOSTICAL STUDIES:

Morphological and micromorphological examination and characterization of medicinal plants have always been accorded due credentials in the pharmacological studies. There was no detailed pharmacognostical work has been carried out including botanical identity based on micro morphology in this leaves of this plant.

The application of morphological studies in drug analysis is pertinent in the field of crude drug authentication. It was studied for the leaf. Interpretation of the morphological characteristics based on different parameters, for the plant organs give a guideline for the diagnosis of the original plant and its adulterants. Colour, size, shape, margin, texture, arrangement were observed and compared with previous data.

Microscopic techniques help to magnify the fine structure of minute objects and there by confirm the structural details of the plant drug. Though the microscopical evaluation cannot provide complete profile, still it can offer supporting evidences which when combined with other analytical parameters can be used to obtain full evidence for standardization and evaluation of herbal drugs. Consideration must therefore be given to the types of cells and cell inclusions and the manner in which they are distributed in different organ of the plants. The habit and habitat and the various morphological characters of the various parts have been studied after proper identification and authentication.

PHARMACOGNOSTICAL STUDIES (Plate: 7-11)

The leaves 4-10 cm long arranged alternately. The shape of blades ranges from elliptical, oblong to oval, bluntly toothed. Leaves having dark green above, glossy, with a distinctive smell often similar to the fruit, bluntly toothed and they

emit a strong characteristic citrus odour due to the presence of copious oil. About 4-12 cm long and 6 cm wide, with visible veins, Acute to obtuse apex and crenulate margin.

Transverse section of midrib shows a small hump on the adaxial surface and very slight convexity on the abaxial surface. Epidermis is single layered and covered by a very thick cuticle. Palisade tissue is not continuous along the midrib region. Secretory cavities are present near the epidermis. Vessels are circular and arranged as a row. Ground tissue is parenchymatous and most of the parenchyma cells contain tannin.

Adaxial and abaxial epidermal cells are polygonal in shape with straight wall. Stomata are anamocytic and confined to the abaxial surface. Large vascular bundle present in the centre. It is surrounded by sclerenchyma fibres outside.

Transverse section of lamina is dorsiventral. No trichomatous out growth. Adaxial and abaxial epidermal cells are more or less similar in structure. Both the epidermis are made up of rectangular cells covered by a very thick, smooth cuticle.

Mesophyll is differentiated into outer palisade tissue made up of two layer of small, columnar closely packed cells. It is followed by spongy tissue made up of 3 to 5 layers of loosely arranged parenchyma cells forming large intercellular spaces. Vascular bundles of the vein is small and mostly embedded in the mesophyll. Secretory cavities (Lysigenous) are present near to epidermis.

Transverse section of petiole presents a circular outline with lateral wings. The outermost epidermis is single layered and covered by a thick cuticle. Trichomes are absent. Ground tissue is made up of loosely arranged parenchyma cells. Most of the parenchyma cells contain tannin. Vascular bundle is present in the centre.

Scanning electron microscopy (SEM) study revealed the presence of **anamocytic stomata** in the abaxial side of leaf. Stomata aperture and epidermal cells are clearly seen. **(Plate :12)**

The plant drugs are generally used in the powdered form where the macro morphology is generally destroyed, so the diagnosis of the plant through the microscopical character is essential. The powdered crude drugs can be identified based on the presence or absence of different cell types.

In powdered microscopy the following characteristics are observed upper epidermis with palisade cells, xylem vessels, epidermal cells with anticlinal wall, subsidiary cells with multiple epidermal cells, epidermal cells with stomata, secretory cavity, stomata are anamocytic type, crystal, fibres, xylem and phloem..**(Fig-3)**

Quantitative microscopy includes certain measurements to distinguish some closely related species which are not easily differentiated by general microscopy. The **stomatal number** is the oldest technique but a simple method of diagnosis of fragmentary leaf parts. The **stomatal index** is the percentage of stomata in relation to the epidermal cells. Both are very specific criteria for the identification and characterization of leafy drugs. **Vein islet and vein termination number** are another simple technique for distinguishing fragmentary specimens at specific levels. It is used as the distinguishing character for the leaf of the same species or different one.

Palisade Ratio is another criterion for identification and evaluation of herbal drugs. This value remains constant within a range for a given plant species and is of diagnostic value in differentiating the species. This value does not alter based on geographical variation and differs from species to species and that is why it is a

very useful diagnostic feature for characterization and identification of different plant species. **(Table 1-4)**

The ash content of the crude drug is generally taken to be the residue remaining after incineration. If usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also involve the inorganic matter added for the purpose of adulteration. There is a considerable difference within narrow limits in the case of individual drug. Hence ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information related to its adulteration with inorganic matter. The ash or residue yielded by an organic chemical compound is a rule to measure the amount of inorganic matter, which is present as impurity. In most cases the inorganic matter is present in small amounts which are difficult to remove in the purification process and which are not objectionable if only traces are present. Ash values are helpful in determining the quality and purity of the crude drug in especially in powdered from. The **acid insoluble ash** is of more value to detect the earthy matter adhering to the drug. In this way one can obtain evidence of the presence of foreign matter, which likely to occur with root, rhizomes and also in pubescent leaves. **The water soluble ash** is used to detect the presence of matter exhausted by water. Insufficient drying favours spoilage by moulds and bacteria and makes possible the enzymatic destruction of active principles **(Table -5).** **Extractive values** of crude drugs determine the amount of active constituents in a given amount of medicinal plant material when exhausted with solvents. It is employed for that material for which no chemical or biological assay method exist. As mentioned in different official books [Anonymous, 1996, Anonymous, 2006, Horborne JB., 1973] the determination of water-soluble and alcohol soluble extractive, is used as means of evaluating crude drugs which are not readily estimated by other means. The

extraction of any crude with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The use of single solvent can be the means of providing preliminary information on the quality of a particular drug sample. The **water soluble extractive** values play an important role for the evaluation of crude drugs. It can be used to indicate poor quality, adulteration with any unwanted material or incorrect processing of the crude drug during the drying, storage etc. The **alcohol soluble extractive** is also indicative for the same purpose as water soluble extractive values **(Table -7).**

Loss on drying at 105^{o}C is determined as the presence of excess moisture is conductive to the promotion of mould and bacterial growth, and subsequently to deterioration and spoilage of the drug **(Table -6)**

THE PRELIMINARY PHYTOCHEMICAL STUDIES:

The preliminary phytochemical screening reveals the presence of carbohydrates, proteins and amino acids, alkaloids, flavonoids, terpenoids, tannins, saponins, sterols, volatile oil. Fixed oil was found to be absent. **(Table -8)**

The reaction of drugs in powdered form in ordinary light and with filtered UV light is of importance in several cases by the luminosity in UV light by **fluorescent analysis**. Many flavonoids showed distinctive colours under UV light: Bright yellow (6-hydroxy flavonoids and flavones and some chalcones), dark brown (most flavone glycosides, dark may be (isoflavones and flavonols). Hence this parameter can also be used as a diagnostic tool for the standardization of herbal drugs for the detection of adulterants in crude drugs **(Table-9)** (Horborne JB 1973).

Determination of total flavonoid content was found to be **26.73 ± 1.10µg/g** .Determination of total phenolic content was found to be **879.13 ± 0.92 µg /g.**

Identification of inorganic minerals of the leaves of *C.Sinensis* by energy dispersive X-ray spectrometer (EDS) showed the presence of minerals carbon (73.17%), oxygen (25.6 %), aluminium (0.34%), potassium (0.29%) and silica (0.2%) **(Table-10)**. No special characters were observed in SEM analysis.

The GC-MS analyscis of the isolated V.O indicated the presence of following twenty five constituents by comparing with the instrument library.

α-Pinene,β-Pinene,Sabinene,Myrcene,Limonine,Terpinolene,Linalool,β-Elemene,Bicyclo[3.1.0]hexane,4-methylene-1-(1-methylethyl)-,ϒ.-Terpinene,1-Pentanol, 5-cyclopropylidene,2,6,10-Dodecatrienal, 3,7,11-trimethyl-(R)-lavandulyl acetate,Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-Caryophyllene,Humulene,1,5,9-Cyclododecatriene,1,5,9-trimethyl, 1, 6-Cyclo decadiene,1-methyl-5-methylene-8-(1-ethylethyl),Naphthalene, decahydro-4a-met -hyl-1-methylene-7-(1-ethylethenyl),Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-,Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta1,3,6,10-Dodecatetraene,3,7,11-trimethyl-,Naphthalene,1,2,3,5,6,8 a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis) 3,7-Cyclodecadiene-1-methanol, alpha.,.alpha.,4,8-tetramethyl-,1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-Caryophyllene oxide.

PHARMACOLOGICAL STUDIES:

ACUTE TOXICOLOGICAL STUDY:

We performed preliminary toxicological studies of VOCSL on the embryo and larvae of the zebra fish. The zf appeared to be suitable for evaluating maximum non- lethality concentration (MNLC).Effects of compounds any possible retardation of development due to the substances be clearly and distinctly observed. Either mortality or incidence of malformation was not observed with 0.5

– 1.5µl/ml. Podophyllotoxin was used as standard toxin which showed total mortality a t 0.01µg/ml concentration. This observation showed no pronounced retardation in zf embryo development when exposed to normal concentration, which **showed that VOCSL would pose no hazard to early life stages of *Danio rerio.***

Larval toxicity study was carried out on zf larvae of 5 dpf cultured in E3 embryonic medium. Five larvae per group was taken and treated with 0.5, 0.75, 1 and 2 µl was taken as a test trail and mortality percentage was calculated.

From the experiment it was observed that there was no mortality in 0.5 and 0.75µl/ml concentrations. But 5% and 10% mortality was observed at 1 and 2 µl/ml concentrations respectively. No mortality was observed in the control. 100% mortality was observed in the standard podophyllotoxin at 0.5 µg/ml concentration.

This study was aimed with the objective of developing an ideal method for epilepsy. The developing larval zebra fish are small and translucent, enabling visualization of organs and biological processes *in vivo* with high resolution. We treated zf larvae with PTZ to induce seizure like behavioural assay, and distance travelled by zf were quantified using high speed camera. In our model VOCSL significantly decrease seizure like behaviour and highspeed distance travelled were also decreased for dose levels 0.5,1,1.5mg/ml for 1 hr after treatment ($p \leq 0.001$). Anti-epileptic drug (AEDS) phenytoin significantly decreases the seizure like behaviour and the highspeed distance travelled by the zf larvae, after treatment. 5 dpf zf larvae were treated with PTZ for 1 hour to induce seizure like behavioural assay. PTZ exhibit increased locomotor activity, seizure like behaviour and reported epileptiform electrographic activity which have good correlation between zf and rodent data.(Liu, C *et al*.,2016) After 1 hour the zf

larvae shows seizure like behaviour which was quantified by image analysis. After 1 hr zf larvae was treated with the standard drug phenytoin , for 2 hours, seizure like behavioural twitching, can be controlled slowly. Also the VOCSL was treated for 2 hours to quantify the anti- epileptic activity.

PTZ induced seizure zf model showed increased high speed distance moved (4016±2.24). But there was a capacity of decrease in the high speed movement per mm were observed in phenytoin (1000µm), for VOCSL 0.5,1, 1.5µl/ml as follows 477±1.2, 790±1.54, 609±0.88, 498±1.15 respectively. The values were statistically significant ($p \leq 0.001$).This result showed potential protection of PTZ induced convulsion (i.e.) anti- convulsant activity. The results also showed that seizure like swimming pattern was alleviated by the addition of the VOCSL. It exhibited concentration dependent inhibition of locomotors activity confirming their anticonvulsant activity. Zebrafish (*Danio rerio*) has emerged over the last decade as an attractive model for genetic studies and drug screening. The high-speed moved distance is more typical than the total distance as an index for anticonvulsant efficacy assessment. Moreover it was already reported that both the behavioural and RT-PCR analysis data showed drugs were absorbed well by zebra-fish larvae within a defined time period (1 hr pre-treated and 1 hr co-treated with PTZ, 2 hrs totally), and most false negative results could be avoided if maximum tolerant concentration was used.

Hydro ethanolic leaf extract has been reported to possess anticonvulsant activity against maximal electric shock induced seizures and PTZ induced convulsions in mice and recommended for further study to isolate the active principle responsible for the activity.(Nagula JB and Reddy NL 2017) (Reddy NL et al.,2016). In our study we have proved that the volatile of the leaf which is one

of the important constituent possesses the anticonvulsant activity. **It provides scientific background for its ethnomedical use in neurological disorders.** Further investigation required to prove the mechanism of activity and other details.

CONCLUSION

The dissertation highlights the pharmacognostical phytochemical and the anti-epileptic activity of the leaves of *Citrus sinensis L* which belonging to the family Rutaceae which should easily available variety in South Asian places , It has been used traditionally to treat ailments like constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress. **Leaf extracts of *C. sinensis* have been used in Nigerian local folk medicine to treat neurological disorders** and to facilitate the digestion of food. It has also been used as an antidiabetic, antibacterial, antifungal, hypotensive, antioxidant, insect repellent, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent due to the presence of copious oil.

The morphological evaluvation showed the adherence of general characters to the family.

- Detailed microscopical studies reveals the usual leaf characters like vascular bundles showing both xylem and phloem, **anamocytic stomata**, are seen only in lower epidermis. Two layers of palisade cells and secretory cavities are present.
- Scanning electron microscope (SEM) shows presence of **anamocytic stomata** in the abaxial side of leaf. Stomata aperture and epidermal cells are clearly seen.
- Petiole is winged, showing vascular bundle in the centre and secretory cavities.

- Powder microcopy, microscopical analysis, vein islet number, vein termination number, stomatal number, stomatal index ,palisade ratio, physiochemical parameters, ash values, extractive values, loss on drying were determined and represented.
- Preliminary Phytochemical screening showed the presence of carbohytrates, proteins,amino acids, saponins, flavaonoids , triterpenes and volatile oils are present.
- Identification of inorganic minerals of the leaves of *C.sinensis* by energy dispersive X-ray spectrometer(EDS) showed the presence of minerals
- Total flavanoids and total phenolic contents were determined and presented as **26.73 ± 1.10µg/g, 879.13 ± 0.92 µg /g.**
- **GC-MS** profile of the **VOCSL** showed the presence of α-Pinene, β-Pinene, Sabinene, Myrcene, Limonine, Terpinolene, Linalool and β-Elemene as major components along with many minor terpenes.
- The 3 Rs (**Reduction, Refinement, Replacement**) of ethical principle was implemented that help to minimize harms to animals used in science.
- In our investigation we used zebra fish larvae which is an emerging novel preclinical *in vivo* model that support rapid decision making in the early phases of drug discovery process.
- We performed preliminary toxicological studies of **VOCSL** on the embryo and larvae of zebra fish and found no pronounced retardation in zf embryo development when exposed to normal concentrations (0.5 to 1.5µl/ml) which showed that **VOCSL** of leaf would produce no hazard to early life stages

Danio rerio but standard toxin podophyllotoxin showed 100% mortality at 0.01µg/ml.

- Larval toxicity study was carried out on zf larvae of 5dpf. From the experiment it was observed that there was no mortality in 0.5 and 0.75µl/ml concentrations. But 5% and 10% mortality was observed at 1 and 2 µl/ml concentrations respectively. No mortality was observed in the control. 100% mortality was observed in the standard podophyllotoxin at 0.5 µg/ml concentration.
- We used **Pentylene tetrazole (PTZ)** to induce seizure like behaviour and increase in high speed moved distance travelled by **PTZ** treated zf larvae.
- In our model **VOCSL** significantly decreases seizure like behaviour and also decrease in highspeed distance travelled at 0.5,1, 1.5µl/ml after treatment ($p \leq 0.001$) which is encouraging and comparable to the standard drug phenytoin.
- The result exhibited concentration dependent inhibition of locomotor activity confirming its anticonvulsant characteristics.
- We recommend further investigations in animal model and clinical trials to confirm the potential therapeutic effect.

REFERENCES

- Agarwal S Sand Paridhavi. 2007, 'Herbal Drug Technology', *1st edn Universities Press (India) Private Ltd, Hyderabad*.
- Ahmed N ,Ahmed T, Akbar N, Andnahmed. S 2016. Phytochemical screening, cytotoxic and hypoglycemic activity of methanolic extract of *citrus sinensis peel. Inter res j of pharma.*
- Allan V. Kalueff,, Stewart AM, and Gerlai R 2013.Zebrafish as an emerging model for studying complex brain disorders **35(2):** 63–75.
- Angew, O. N.2007. Functional foods, *Trends in Food Sci and Tech*, **30**: 19-21.
- Anonymous.1992 The Wealth of India - A Dictionary of Indian Raw Materials and Industrial Products, B, *Council of Sci and industrial Res , New Delhi*, 625-644
- Anonymous.1996, Indian Pharmacopoeia', Vol II, *Ministry of Health and Family Welfare, New Delhi*, A-53: 54, 89.
- Anonymous.1998, 'Quality control Methods for Medicinal Plant Materials', *WHO*, 28-35.
- Anonymous, 2001 140-145. "*The Ayurvedic Pharmacopoeia of India*", Part I, edn, Vol 1, Government of India, Ministry of Health and Family Welfare, Indian Systems.

- Arsingrin, P. S.1999. *Citrus Sinensis* Information, In: Bennie and Simpson (Eds), *Fruits.* 2nd Edition, *Welford Publi* ,258–261.
- Atolani, O., Omere, J., Otuechere, C.A.and Adewuyi, A. 2012.Antioxidant and cytotoxicity effects of seed oils from edible fruits. *J of Acute Disease*: 130-134
- Baghurst, K. 2003. The health benefits of citrus fruits. CSIRO Health Sci.
- BassamAbdul Rasool Hassan 2012**,** *Medicinal Plants (Importance and Uses)Pharmaceut Anal Acta.*
- Chang CC, Yang MH, Wen HM and Chern JC. 2002, 'Estimation of total flavonoid content in propolis by two complementary colorimetric method' *J Food DrugAnalysis.***10(3)**: 178- 182.
- Chaudhri, R D, 1999, '*Herbal Drug Industry* 'Eastern publishers', New Delhi 489-501
- Chede, P.S. 2013. Phytochemical analysis of *Citrus sinensis* peel.*Inter J of Pharma and Bio Sci,* **4(1)**: 339 – 343.
- Chemical composition and biological activities of essential oil from *citrus sinensis peel world j of pharm and medi res* **3(8)**, 164-168
- Chemistry and Pharmacology of *Citrus sinensis Molecules* **21**, 247.
- Chongbin Liu, Juan Zhou, Yong Zhang, Taotao Zhu, Rui Wang and Chunqi Li, 2016, Validation of the Zebra-fish PentyleneTetrazol Seizure Model: Behaviour Assay for Assessing Anti-Epileptic Drug Efficacy, Anal Biochem, **5(2)** doi.org/10.4172/2161-1009.1000270

Citrus limonum pulp and peel.*Inter J Pharma and Pharma Sci*, **4(2)** 269-371

- Claude, JR, & Claude, N, 2004, "Safety Pharmacology: An essential interface of Pharmacology and toxicology in the non-clinical assessment of new pharmace", *Toxi letters*, **151**: 25 – 28.
- Crowell, P. L .1999. Prevention and therapy of cancer by dietary monoterpenes. *The J of Nutri* **129 (3)**: 775S–778S, PMID 10082788.
- Da Silva, E. J. A., Oliveiraand, A. S., Lapa, A. J. (1994). Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartinand clause quinone, in rats and mice, *J Pharma and Pharmacol*,. **46,**. 118–122
- Dave, G, Damgaard, B, Grande, M., Martelin, JE, Rosander, B and Viktor, T, 1987, "Ring test of an embryo – larval toxicity test with zebrafish (*brachydanioreiro*) using chromium and zinc as toxicants", *Env, tox.chem.,* **6:** 61 – 71.
- Di Majo, D., Giammanco, M., La Guardia, M., Tripoli, E., Giammanco, S., Finotti, E. 2005. Flavanones in Citrus fruit: Structure antioxidant activity relationships, *Food Res Inter*, **38**, 1161–1166.
- Denane D. 2015 Chemical Profile, Antibacterial and Antioxidant Activity of Algerian Citrus Essential Oils and Their Application in *Sardina pilchardus* Foods **4,** 208-228.
- Dooley, K, Zon, LI, 2000, "Zebrafish:a model system for the study of human disease"*, curr opin genet dev*.,**10(3)**:252-256

- Ehler, S. A. (2011) Citrus and its benefits, *Journal of Botany*, **5**, 201-207.
- Esquivel-Ferriño P.C., Clemente-Soto A. F., Ramírez-Cabriales M. Y., Garza-González E., Álvarez L., Camacho-Corona M. R. 2004.Volatile Constituents Identified in Hexane Extract of *Citrus sinensis* peel and anti-*Mycobacterium tuberculosis* Activity of some of its constituents. *J. Mex. Chem. Soc.* **, 58(4)**, 431-434.
- Etebu E. and Nwauzoma A. B.2014 A Review On Sweet Orange (*Citrus Sinensis* L Osbeck): Health, Diseases And Management *American J Res Commun* **2(2)** 33-70
- Fatma A. Ahmed and 2Rehab A. Lotfy 2015 .Phytochemical Evaluation of Some Selected Medicinal Plants Growing Wildly in Southeastern Egypt, *Mid East J of Appli Sci,* **5 (4)** ,1239-1246.
- Fenglai Xiao , Bo Yan, Lei Chen, Dong ZhouXiao, 2015, Review of the use of botanicals for epilepsy in complementary medical systems — *Traditional Chinese Medicine, Epilepsy &Behaviorvol*: **52** -281–289.
- Fleisher Z & Fleisher A 2015. Sweet Orange Leaf Oil (*Citrus sinensis* (L.) Osbeck). *J Essential Oil Res* **2(4)***,* 203-205
- Gouthamchandra K, Mahmood R and Manjunatha H. 2010, 'Free radical scavenging antioxidant enzymes and from *Clerodendrumin fortunatum* L' *Env Toxic Pharmacol* **30:** 11- 18.
- Guimaraes, R., Barros, L., Barreira, J.C.,Sousa, M.J., Carvalho, A.M. and Ferreira, I.C.F.R.2010. Targeting excessive free radicals with peels

and juices of citrus fruits grapefruit,lemon, lime and orange. *Food and Chemi Toxi,* **48(1)**: 99 –106.

- Haiquing J, Robert JR & Chitang H, 2004. Anti-inflammatory activity of polymethoxy flavones in sweet orange (*Citrus sinensis*) peel. Metabolites Study of Nobiletin. 337-344.
- Hany O E, Neelam A Alamgir A.2015 Screening Of Phytochemical Constituted, Antimicrobial And Antioxidant Activities Of Orange Peel (*Citrus Sinensis*) Extract. *Bull. Env.Pharmacol. Life Sci.,* **4[6]** *102-108*
- Harborne JB 1973 Phytochemical Analysis- A guide to modern techniques of plant analysis" London, Chapman & Hall.
- Herrmann, K,1993,, "Effects of the anti convulsant drug phenytoin and related substance on the early development of zebrafish(Brachydaniorerio)"Toxic, in Vitro, **7(1**):41-54.
- Hertog, M. G., Hollman, P. C. H., Katan, M. B., Kromhout, D.1993. Dietary antioxidant flavonoids and risk of coronary heart disease, *Lancet*,. **342**, 1007–1011.
- Heywood VH.1971,'The Systemic Association, Scanning Electron Microscopy, Systemics and evolutionary applications', Proceedings of an International Symposium held at the Department of Botany, University of Reading, Academic Press, London. **4(1)** -16.
- Hisaoka,KK and Battle, HI, 1958, " The normal developmental stages of the *zebrafish, brachydanioreiro* (Hamilton – Buchanan*)", J Morphology*, 102: 311

- Hussain K.A, Tarakji B, Kandy B.P.P, John J , Mathews J , Ramphul V ,Divakar D.D 2015. Antimicrobial effects of citrus sinensis peel extracts against periodontopathic bacteria: an in vitro study. *Nat inst of pub health .* **66(2)**:173-178
- Iyengar MA, Nayak SG K.1994, '*Pharmacognosy Lab Manual'*, *Manipal press,* Manipal, **78-87**.
- Johansan, DA, 1940, "Plant micro technique" McGraw Hill Book Co, **27(93)**: 126 – 154.
- Jorge N, silva A and caroline P.M. Aranha .2015.Antioxidant activity of oils extracted from orange (*citrus sinensis*) seeds. *An acad bras cienc* **88 (2)**.
- Juan Manuel J. Hernández F ,Santiago O. G , Mónica A. Cabrera R ,Kokate C K, Purohit A P and Gokhale S B.2005, '*Pharmacog' 32th* edition, *Nirali Praksahan, 99*, 100, 111, 112.
- Keys, A. 1995. Mediterranean diet and public health: Personal reflections, *Ame J of Clini Nut*, **61**,.1321–1323.
- Kumar V, Wandeni R, Shashidhara S, Anitha S. 2010.Antityphoid activity of aqueous extract of fruit peels *Citrus sinensis*.**2:** 217-219
- Kurowska E.M., Manthly J.A., 2004. Hypolipidemic effects & absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia. *J Agric Food Chem.* 2879-2886.
- Lawal, D., Bala, J. A., Aliyu, S. Y. and Huguma, M. A.2013.Phytochemical screening and in vitro anti-bacterial studies of the ethanolic extract of *Citrus Sinensis* (Linn.) peel against some

clinical bacterial isolates. *Inter J of Innovation and Appli Studies,* **2(2)**: 138 -145.

- Mabry TJ, Markham KR. and Thomas MB. 1970, 'The systemic identification of flavonoids' Springer verlay. Newyork.
- Madhuri S, Ashwini U. Hedge, Srilakshmi N.S., Parshith Kekude T.R. 2014 Antimicrobial activity of *Citrus sinensis* and *Citrus aurantium* peel extracts. *J Pharma. & Sci Innovation.;* **3(9)***:366-368.*
- Manish *et al. World J of Pharma Res www.wjpr.netl* **4(135)**:269- 371.
- Manish K, *Mahesh AR, M Somashekhar 2013 Evaluation of Antitubercular Activity of Methanolic Extract of *Citrus sinensis* *Inter Jof Pharma Res & Review* **2(8)***:18-22*
- Manthey, J. A., Guthrie, N., Grohmann, K. 2001. Biological properties of citrus flavonoids pertaining to cancer and inflammation, *Curr Med Chem* ,**(8)**, . 135–153.
- McGrath, P and Chun – Qi Li, 2008, "Zebrafish a preclinical model in assessing drug induced toxicity" *rev drug dis today*, **13 (9)**.
- Mehmet TugrulSavran, , 2013 Zebrafishbehavioral assays as a high-throughput and large-scale model for screening drugs, Res Sci Institute.
- Mondello L., Casilli A., Tranchida P. Q., Dugo P.,2005 Dugo G. Comprehensive two-dimensional GC for the analysis of Citrus essential oils. *Flavour and Fragrance J,* **20:** 136-140.
- Morton J 1987 orange .In: Fruits of warm climates. *J* 134-142 .

- Muhammad N. O. , Soji-Omoniw O. , Usman L. A. and Omoniwa B. P. 2013 Antihyperglycemic Activity of Leaf Essential Oil of *Citrus sinensis* (L.) Osbeck on Alloxan-Induced Diabetic Rats. *Annual Rev& Res Biology* **3(4)***: 825-834.*
- Nagula J B, Reddy N L 2017. Evaluation of Antiepileptic Property of Citrus Sinensis (Leaf Extract) ByPentylenetetrazol (PTZ) Induced Convulsions in Mice . *Sch. J. App. Med. Sci.,* **5(7)***:2830-2835.*
- Nicolosi, E., Deng, Z. N., Gentile, A., La Malfa, S., Continella, G. Tribulato, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers, *Theoretical and Applied Genetics*, **100(8)**, . 1155–1166.
- Noah Samuels, Yoram Finkelstein, Shepherd Roee Singer, andMenachem Oberbaum,2008, Herbal medicine and epilepsy: Proconvulsive effects and interactions with antiepileptic drugs,*Epilepsia,* **49(3)**:373–380, 2008 doi: 10.1111/j.1528-1167.2007.01379.
- O' Brien, TP, Feder, N and MC Cull, ME, 1964, "Polychromatic staining of plant cell walls by toluidine Blue – O' *Protoplasma*, **59**: 364 – 373.
- Omodamiro O. D. and Umekwe J. C. 2013 .Evaluation of anti-inflammatory, antibacterial and antioxidant properties of ethanolic extracts of *Citrus sinensis* peel and leaves *J of Chem and Pharm Res,* **5(5)***:56-66*

- Orwa C, A Mutua, Kindt R , Jamnadass R, S Anthony. 2009 Agroforestree Database:a tree reference and selection guide version 4.0
- Parle M, Chaturvedi D ,2012. Orange: Range of benefits. I*nt. Res. J of Pharma.* **3(7)**:59-63.
- Parng. C, Roy, NM, Ton, C, Yingxin Lin, MCGrath, P, 2007, Neurotoxicity assessment using zebra fish *,J pharmacol. Toxi Met.,* **55:**103-11
- Patricia C. Esquivel-Ferriño, Aldo F. Clemente-Soto, Mayela Y. Ramírez-Cabriales, Elvira Garza-González, Laura Álvarez, and María del Rayo Camacho-Corona.2014 . Volatile Constituents Identified in Hexane Extract of *Citrus sinensis* Peeland Anti-Mycobacterial Tuberculosis Activity of Some of its Constituents. *J. Mex. Chem. Soc.* , **58(4)**, 431-434
- Patricia C. Ferriño E and Corona M R C .2016 Chemistry and Pharmacology of *Citrus sinensis Mol*, **21**, 247
- Purvis, MJ, Collier, DC and Wallis, D. 1966, “Laboratory techniques in Botany” Butterworth & Co Ltd. 2nd edn, 82 -169.
- Reddy NL,Pushpalatha C, Sharan kumar KP 2016 Eeperimental Evaluation of Anti convulsant Property of *Citrus sinensis(*Leaf Extract) in Mice.Sch.Acrd .J.Pharma.**5(8)**:337-41.DOI:10.21276/sajp.2016.5.8.5
- Reed, h. S.1931 the density of stomata in citrus leaves. *J agri res,* . **43**
- Robards 1970, ‘Electron microscopy and plant ultra-structure’ *McGraw Hill, London,* **14(15):** 36- 59

- Ryu.S, J. Holzschuh, J. Mahler, and W. Driever, Genetic analysis of dopaminergic system development in zebrafish,2006, *J Neural Transm [Suppl*] **70**: 61–66
- Sakata, K., Hirose, Y., Qiao, Z., Tanaka, T. Mori, H. (2003). Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line, *Cancer Letters*, **199**, . 139–145.
- Sarvaiya.V.N, K.A. Sadariya, M.P. Rana and A.M. Thaker 2014. Zebrafish as model organism for drug discovery and toxicity testing: A review, 31-38
- Sass, JE, 1940, "Elements of botanical microtechnique" McGraw Hill Book Co, New York, 222
- Sharma Neeta and Tripathi Abhishek 2008. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis *of Aspergillus niger (L.). Micro res* **163**: 337-344.
- Sima J.A., Hudes E.S., Perez G.I. 2003. Relation of serum ascorbic acid to Helicobacter pylori serology in US adults: The third National Health & Nutritio Examination Survey*. J. Am Coll Nutr.* 605-609.
- Singleton VL, Orthofer R, Raventos R ML. 1979, ''Analysis of total phenols and other oxidation substrates and anti- oxidant by folinciocalteu reagent'' *Methods InEnzymology*. **299:** 152- 178.
- Strange R.R., Miland S.L., Eckert J.W., Sims J.J., 1993 .An antifungal compound produced by grapefruit & Valencia orange after wounding of the peel. *J of Natural Products*. ***56***: 1627-1629.

- Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D. Giammanco, M. (2007). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review, *Food Chemistry*,**104.** *466–479*.
- Uchechi n. Ekwenye and oghenerobo v. Edeha 2010.the antibacterial activity of crude leaf extract of *citrus* sinensis (sweet orange) *inter j of pharma and bio sci* .**1(4)**.
- Vijender Singh , Deepti Katiyar, Mohammed Ali 2015 Comparative study of volatile constituents and antimicrobial activities of leaves and fruit peels of *Citrus sinensis Linn.* The *J Phyto pharma ;* **4(2)**: 102-105*.*
- Vinodhini.M, Kalaiselvi.M, Amsaveni.R, and Bhuvaneshwari.V .2017. Viuda-Martos M., Ruiz-Navajas Y., Ferna´ndez-Lo´pez J., Pe´rez-A´lvarez J. 2008 Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulate* L.), grapefruit (Citrus paradisi L.) and orange (*Citrus sinensis* L) essential oils. Food Control , **19 (12)**: 1130-1138.
- Wallis TE. 1953. *Analytical Microscopy*,3rdedn, J&A Churchill Ltd, London*, 180,183, 184*
- Wallis TE.1965. *Analytical Microscopy*,3rdedn, J&A Churchill Ltd, London*, 180,183, 184.*
- Weisman Z, Chapagain B.P., 2005.Larvicidal aqueous extract of *Balanites aegyptiaca* against *Culex pipens* mosq. Afr. Biotec. 1351-1354*.*

- Yamuna J, Anthony N 2015 Citrus sinensis L. leaf extract as an efficient green corrosion inhibitor for mild steel in aqueous medium. *Int J ChemTech Res .***7,(1)***,* 37-43
- Yovo M, Alitonou G A, Sessou1 P, Dedome L, Tchobo F, Avléssi F, Menut C, and Sohounhloué D 2016. Phytochemistry and Antibacterial Activity of *Citrus sinensis* Extracts against Three Pathogenic Bacteria in Benin. *Res J Pharma, Bio and Chemi Sci* 1344*.*

PLATE:1 *Citrus sinensis* – HABIT AND HABITAT

PLATE:2 *C.sinensis* –LEAF ARRANGEMENT

PLATE:3 *C.sinensis* – DORSAL AND VENTRAL VIEW

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PLATE: 4 *C.sinensis* –FLOWERS ARRANGEMENT

PLATE: 5 *C.sinensis*–FRUITS

PLATE: 6 *C.sinensis* –SEEDS

PLATE:12 *C.sinensis* – STOMATA (ANAMOCYTIC)

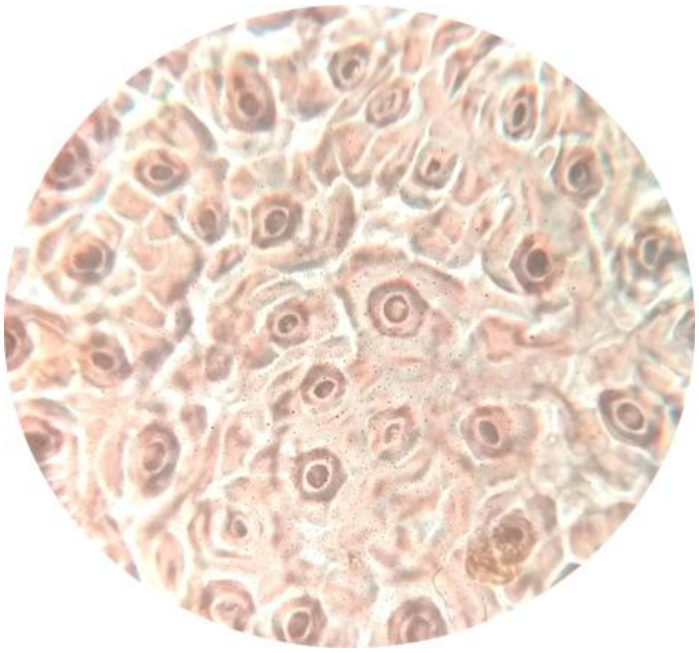


PLATE:13 *C.sinensis* – VENATION PATTERN

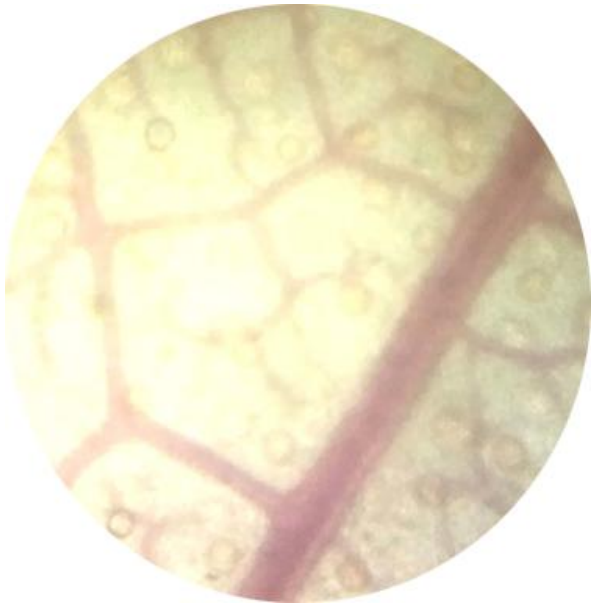


PLATE:8 *C.sinensis* -T.S OF LEAF THROUGH MIDRIB

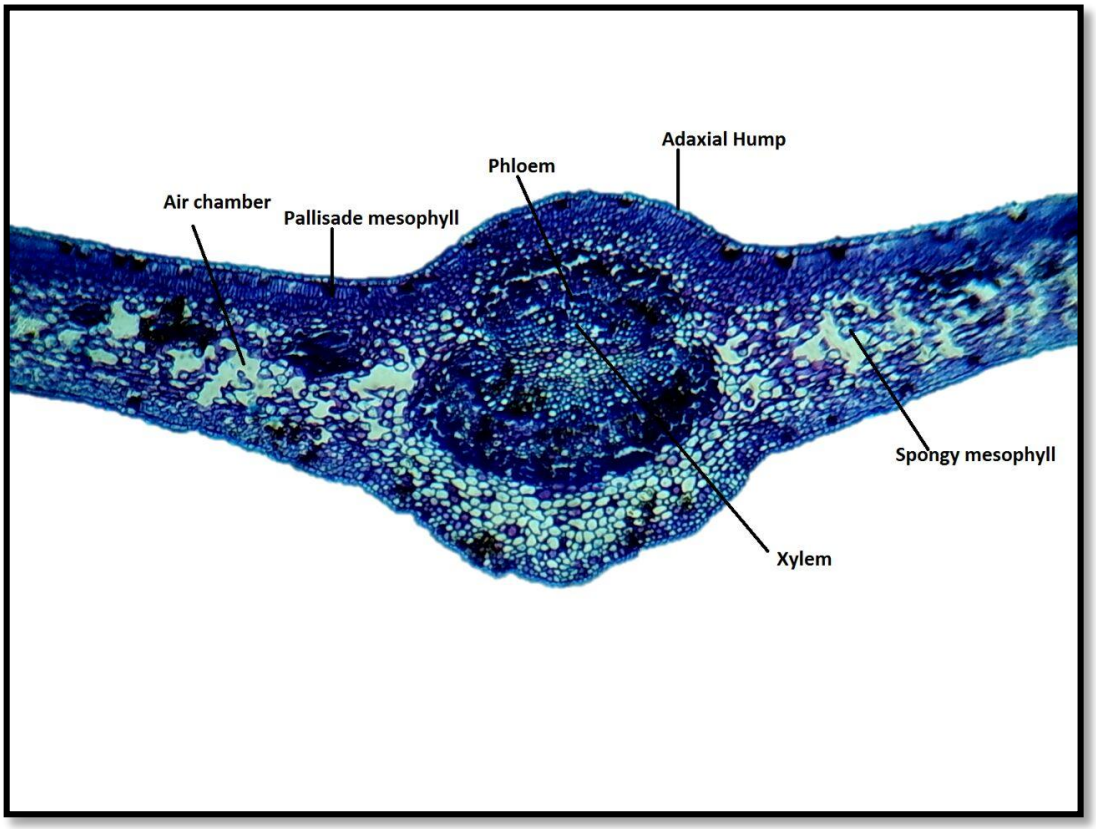


PLATE:9 *C.sinensis* -T.S OF MIDRIB ENLARGED

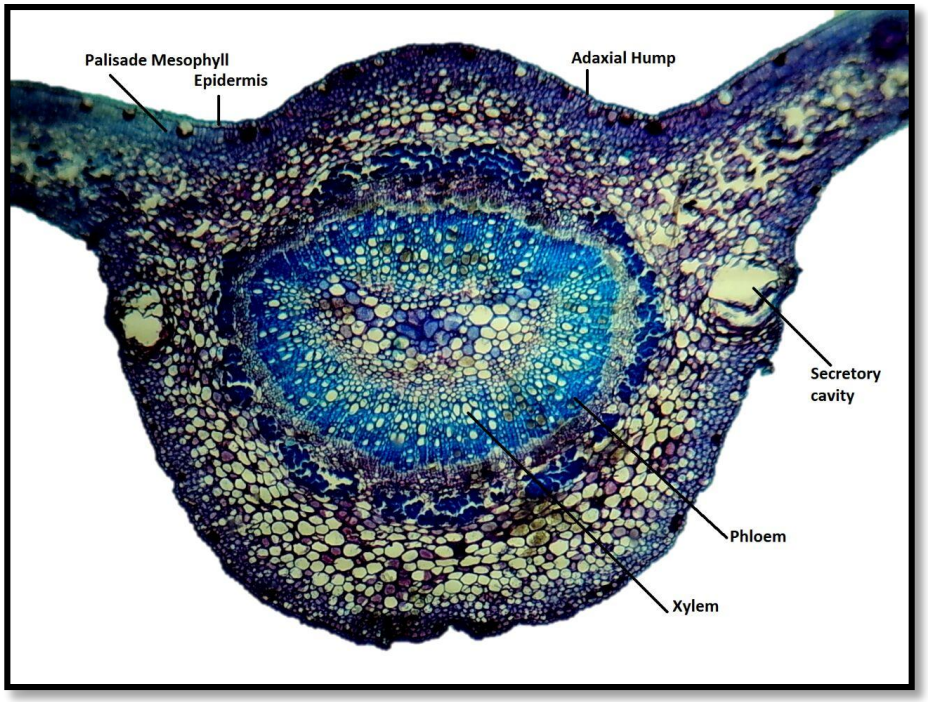


PLATE:10 *C.sinensis*- T.S OF LAMINA ENLARGED

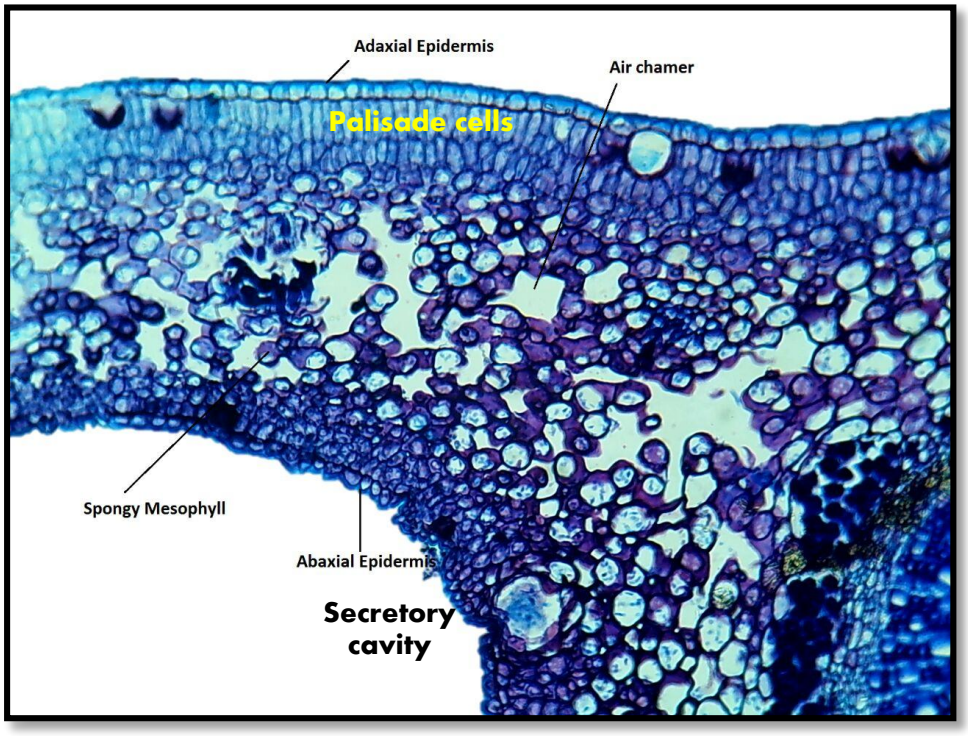


PLATE:11 *C.sinensis*- SECRETORY CAVITY ENLARGED

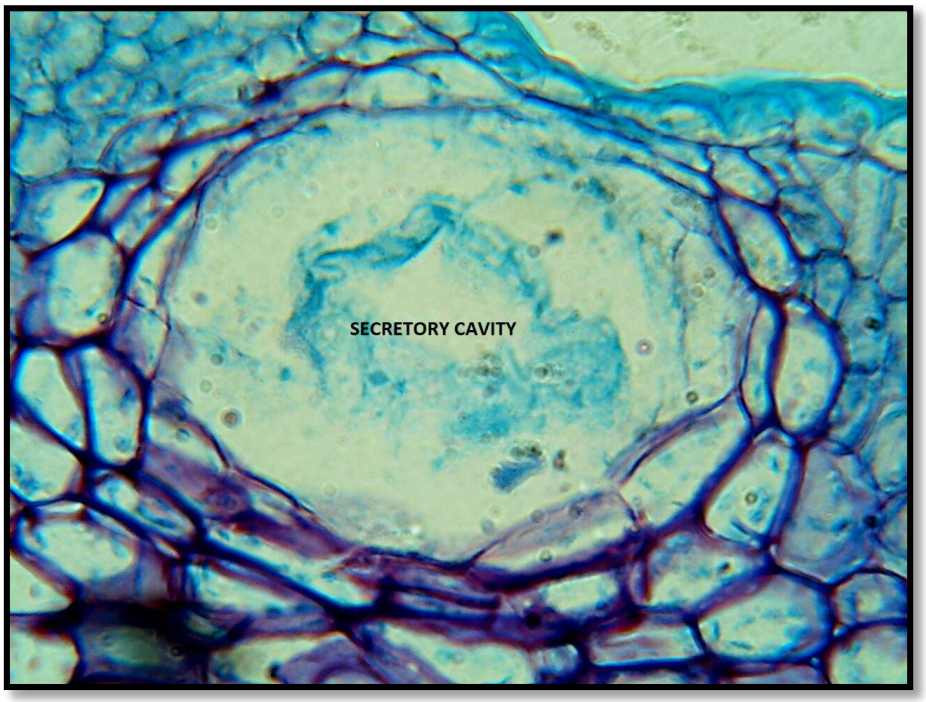
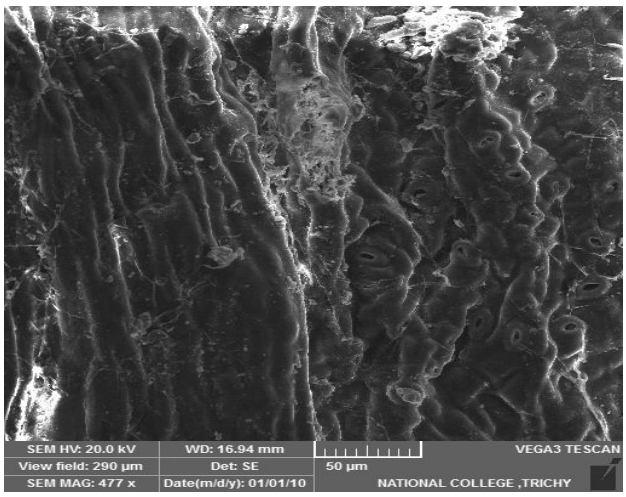
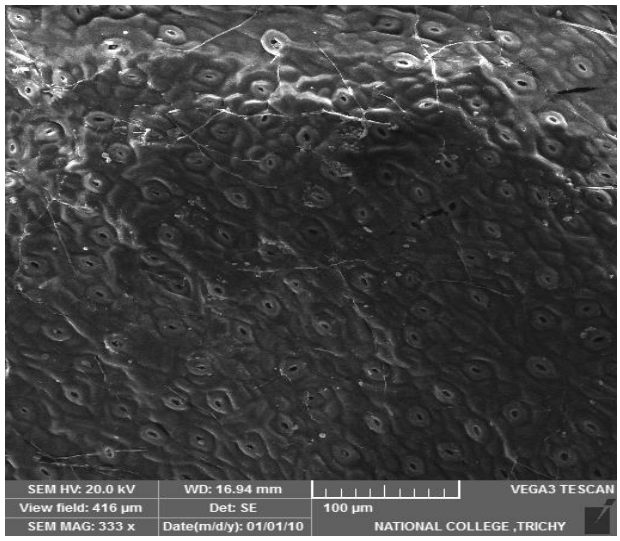


PLATE:14 SEM OF *C.sinensis*





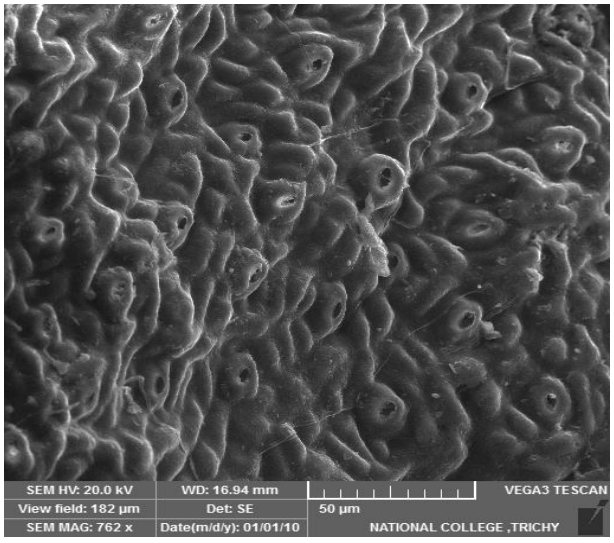


PLATE: 11 *C.sinensis*-T.S OF PETIOLE

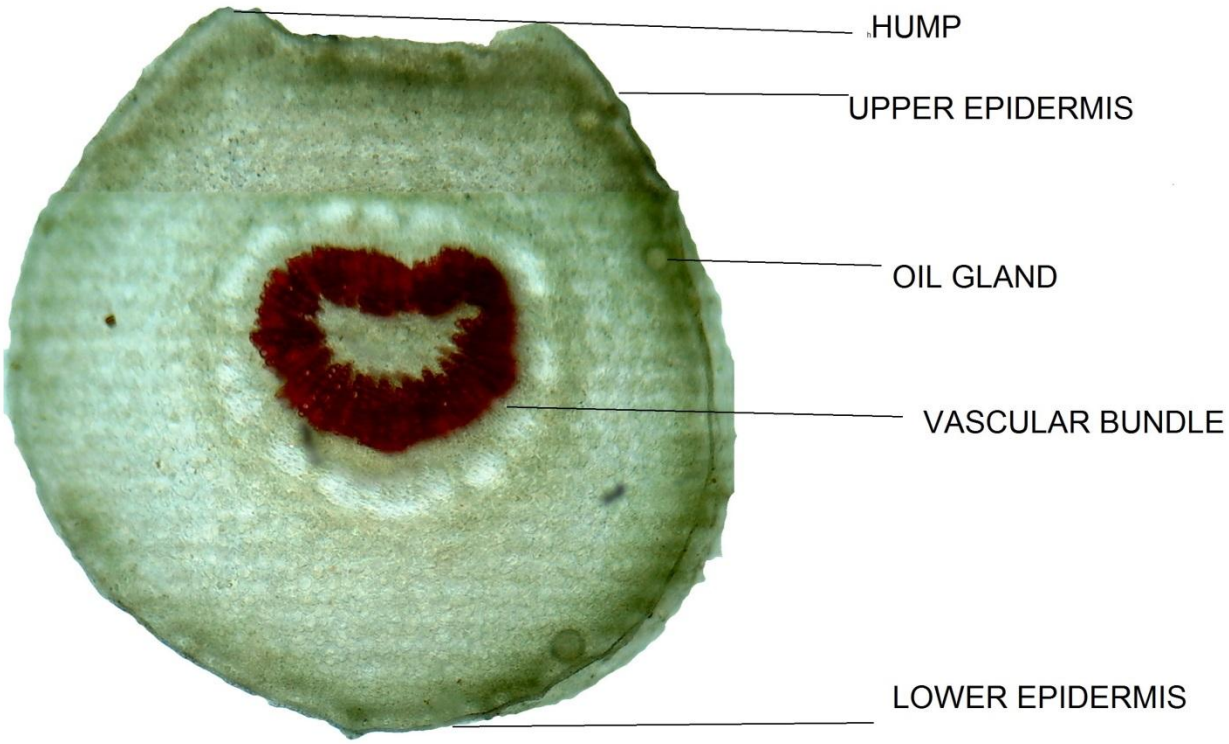
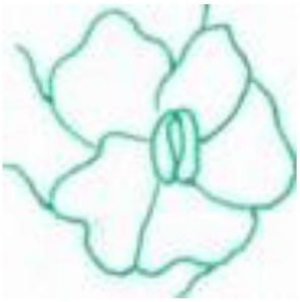


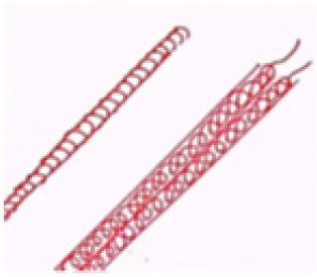
Figure- 3

POWDER MICROSCOPY OF *Citrus sinensis Leaf*

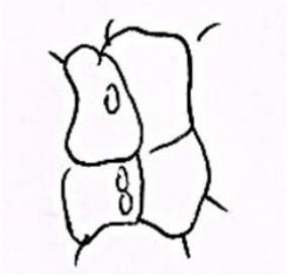
Anamocytic stomata



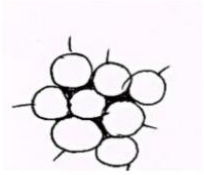
Xylem vessel



Fibers



Epidermal Cell



Collenchyma

Pholem

Parenchyma