# PHARMACOGNOSTICAL, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION ON LEAVES AND VOLATILE OIL OF *Citrus limon*.(Linn.)

*A dissertation submitted to*

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*for the award of the degree of*

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**IN**
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*Submitted by*

**26108666**

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# CERTIFICATE

This is to certify that the dissertation entitled "**PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION ON LEAVES AND ITS VOLATILE OIL OF *Citrus limon* (Linn.)"** submitted by **MR. RAGHURAMAN V. (Reg No : 26108666)** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University, is a bonafide work done by him under my guidance during the academic year 2011-2012 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020.

**(AJITHADAS ARUNA)**

Date:

Place: Madurai

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### Food as Medicine and Medicine as a Food

*(ÁÕóS¾ ¯½× ¯½SÅ ÁÕóÐ)*

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# Contents

# CHAPTER -1

# INTRODUCTION

## Plants are used in Traditional Medicine for Drug Discovery systems

Early mankind depended upon the nature for both health and illness. The Fossil evidence for the use of herbal remedies dates back to 60,000 years ago. From that point of view, to promote the Traditional Medical Systems (TMS) was combined with plants as a means of therapy can be traced back.As far as recorded, documented was similar. However, the important of these systems is the science of whole nature of man and mankind in the ancient times. According to the World Health Organization (WHO), almost 80% of the world population have been combined into their methodology value for primary modality of their health care sector. The main aim of using the plants as a source of therapeutic agents, and to isolate the bioactive compounds for directly used as a drug.(e.g., Digoxin, Digitoxin, Morphine, Reserpine, Taxol, Vinblastin, Vincritine etc). The known or unknown isolated bioactive compounds are semisynthesised and to produce the patentable things of their higher activity/and or lower toxicity (e.g., Metformin, Nabilone and other narcotic analgesics, Oxycodon, Taxotere, Teniposide, Verapamil, Amiodarone, galegine, tetrahydrocannabinol, morphine, taxol, podophyllotoxin,khellin, and khellin). The bioactive compounds were used as pharmacological tools. (e.g., lysergic acid diethylamide, mescaline, yohimbine). The whole plants or parts used as a herbal remedy (e.g., cranberry, Echinacea, feverfew, garlic, ginkgo biloba, st.john's wort, saw palmetto).

However, we are consider the past, present, and future information value of plants, employed in traditional medical practices (ethnomedicine) for the discovering of new bioactive compounds.

The large number of higher plant speices (angiosperms and gymnosperms) on this planet was estimated at different levels. The biological activity of compounds were screened by 6% only, and reported 15% have been evaluated phytochemically. Since advanced screening methods are available, these number will be change, but some differences will be there in the evaluating of plant species versus another plant. There are some broad starting points to selection and collecting the plant material of potential therapeutic uses. However, the main aim was try to put efforts and honestly.

The advantages of the plants in this area based on their long term used by human beings. The bioactive compounds obtained from such plants to have low human toxicity, and some plants may be toxic with in the some particular affected area only. There is no reporting system to documentation of these effects. However, that acute toxic effects following the use of a plant in this area would not be noticed, and the plant would then be used cautiously. The chronic toxic effects of plants are not to be used. In addition to the chemical changes of the plant evaluation may be equal or superior to that found in synthetic combined chemical libraries.

In 1991, the estimation was executed in the United States, for every 10,000 pure compounds (mostly based on synthesis) are biologically evaluated by *in vitro*. The 20 compounds would be tested in animal models, 10 of these

would be clinically evaluated, and only one should be reached U.S. Food and Drug Administration (US-FDA) approval for trading. The time required for this process was estimated in 10 years at cost of 231 million US dollar.

Most of the pharmaceutical companies and some small biotechnology firms were doing screening nearly 1000 substances per week, by using High Throughput Screening (HTS*) in vitro* assays. In addition to synthetic compounds from their own progress order. Some of the companies are screen the plant, microbial, and marine organisms.

In this manner, challenges facing these companies in acquiring organisms and extracts (*vide infra*), are usally the results will be failure to consider the collection of plants especially if the acquirisitions are based on ethnomedical uses. Despite of these problems one cannot avoid the past importance of plants as source of structurally novel drugs.

Ethnomedicine may be defined elaborately as the use of plants by humans as medicine( exactly ethnobotanical medicine). Traditional medicine is broad trem used to define any non-Western medical practice. Ethnopharmacology is highly different method to drug discovery systems, and involuing the observation, description, and experimental investigation of indigenous drugs and their biological studies. It is based on botany, Chemistry, biochemistry, pharmacology, and many other areas that contribute to the discovery of natural products with biological evaluation.

**Methods of Drug Discovery system by using Higher Plants**

The selection methods of plants are used for drug discovery systems. However, the concern was screening the plants for anti-cancer and anti-HIV activity. Here the methods was briefly concentration on the ethnomedical approaches in the major topics are discussed.

Random selection followed by chemical screening or phytochemical screening methods (for the presence of cardenolides/bufadenolides, alkaloids, triterpenes, flavonoids, isothiocynates, iridoids etc.) have been used in the past and are presently followed in the developing countries. The tests were very simply performed, the result will be false-postive and false-negative tests were obtained, and difficult to assess. It is impossible to relate one type of phytochemicals to another biological targets(the alkaloids or flavonoids produce, and it is not a predictable one to arrange the huge numbers of random orders of biological effects.

Random selection followed by one or more biological assays. In the olden times, plant extracts was evaluated mainly in experimental animals, primarily mice and rats. The Central Drug Research Institute(CDRI) in India more than 35,000 species was screened *in vitro* and later *in vivo*. Taxol and camptothecin was discovered in this program as well as several other plant-derived compounds that was unsuccessful in human studies.

The CDRI evaluated nearly 2,000 plants species for several biological activities, including antibacterials, antdiabetic, antifertility, antifungal, antitumor,cardiovascular, central nervous –systems depressant, cytotoxicity,

dirutic, and others. There is no biologically active drugs for human use. The huge number of known and novel bioactive compounds was isolated from the active plants.

According to the biological activity reports showed that the plant extracts having interesting biological activities, but the extracts was not studied for their active principles.

Thousands of years plants are used in organized traditional medical systems like Siddha, Ayurveda, Unani, and traditional Chines medicine etc. Even though Western medical science systems was more trusted , and used widly by most of the people in this planet. Adverse effects of these plants are not well documented,and efficacy of these plants and plant mixers is more difficult to assess by Western scientific methods.

In ancient time of india, herbs played an important role in Ayurvedic Medical Systems(AMS). The basic ayurvedic writings are at least 2,000 years old; it can be divided into three major and three minor parts. The three major parts are *Charaka Samhita, Susruta Samhita* and *Astanga Hrdayam Samhita* and three minor parts are *Sarngadhara samhita, Bhava Prakasa Samhita* and *Madhava Nidanam Samhita. Charaka Samhita* is the oldest and the authoritative writing in *Ayurveda* dates back to $6^{th}$ to $7^{th}$ century B.C. It describes about 582 herbs, while *Sushruta Samhita*, lists nearly 600 herbal remedies.(Text Book of Pharmacogonosy & Phytochemistry).

The value of ethnomedical information was documented, and is valuable one, to initiated the drug discovery systems. The WHO Traditional Medicine

Programme was provided some useful evidence of ethnomedical studies for the drug discovery systems in several years ago. The WHO-TRM centers throughout the world asking their help to identifying all plant-derived pure compounds used as drugs in their respective countries, and they are surveyed pharmacopoeias of developed and developing countries to identify all such useful drugs.

**Latest trend to added value of Natural sources**

The improvements in seperation techniques to isolate and purity of natural products e.g., counter-current chromatography and in analytical techniques to determine structures of compounds, screening of natural product mixtures is corrently highly compatible with the expected timescale of high-through put screening campaigns. Singh and Barrett point out that pure bioactive compound can be isolated from fermentation broths in less than 2 weeks and that the structures ( more than80- 90%) of new compounds can be elucidated within 2 weeks. With advances in NMR techniques, complex structures can be solved with very less than 1 mg of compound. It is recently demonstrated that it is possible to prepare a screening library of highly diverse compounds from plants with the compounds being pre-selected from an analysis of the dictionary of natural products to be drug-like in their physicochemical properties. It will be interesting to see if such a collection proves to be enriched in bioactive molecules.

So many alternative techniques are also being explored in efforts to increase the speed and efficiency with which natural products can be applied to drug discovery.

## Bioactivity of Volatile Oils (Essential Oil)

Volatile Oils are very important role in the natural substances, used in many areas like Pharmaceuticals industries(additives), Food industries(preservatives), perfumery(scent), cosmetics(powders, soaps, creams etc), spices, herbal theraphy, aromatheraphy etc.

Currently it has been attracted many industrialist and researchers to screen plants to study the biological activities of their oils both in phytochemical, pharmacological & therapeutic aspect. Many research papers on the biological activity of Volatile oils have been published. Volatile Oils are complex mixtures contains many single compounds. The constituent of Volatile Oils contributes to the therapeutic or adverse effects of these oils. Hence the knowledge of composition of Volatile Oils is necessary for a better and specially directed application. Such a detailed knowledge of constituents of Volatiles Oils can be obtained by means of GC experiments. By investigating the bioactivity of Volatile Oils we can face the situation of unsettling facts of modern pharmaceutical industry which facing lately its pipeline of new drug discovery systems to have almost empty. Now the Research & Development has attained saturation point. On the other hand prevalence of disease economy

and IPR regulations in the developing countries have deviated so much from the developed world which have set up a vicious spiral of no win situation for the pharma industry. A Bangalore declaration (workshop on medicinal herbs and plants: Scope for Diversified and sustainable Extraction, funded by Common Fund for Commodities (CFC) Amsterdam in collaboration with Biocentre and Food and Agriculture Organisation, Rome held at Bangaluru, India (during July 22 & 26, 2004) deliberated in detailed issues concerned with herb, R&D, IPR, TM etc. This workshop document a good vision statement that emphasize importance to provide sustainable livelihood chances to farmers and poor in the region through organic cultivation system and managed collection and to give affordable health care options in the form of quality traditional medicines and to build gradually regional brands in the global market. Hence to tape the remunerative and fastly growing markets for herbal products and services in the health care, neutraceuticals health foods, perfumes and cosmetic business sectors.

## Reason for the selection of this plant:

*Citrus limon (L)* is a nearly house hold plant (or) Domestic plant. It is very widely and easily available plant belonging to the Family Rutaceae. The leaves of *C.limon* really do not have any match as a cheapist natural and easily available plant. It is traditionally known to be useful for the treatment of wide panel of diseases like stomach ache, vomiting, carminative, excellent refrigerant drink and culinary uses etc.In acute rheumatism and rheumatic gout,

in some forms of acute tropical dysentery and diarrhoea,etc. it has been successfully employed. As an antidote to some acro-norcotic poisons, it often proves effectual. *Lemon* juice and gun powder used topically for scabies.The bark and root has been used in the West Indies as a febrifuge and the seeds as a vermifuge. Rind of ripe fruit used for stomach, carminative jucie of ripe fruit used for refrigerant in scurvy. And used for culinary purpose for food items and cosmetic purpose (essention oil, perfume soap, powder etc.) This plant is very popular in India and widely cultivated. Fruits used for jucies in cool drinks and pickles. The economic aspect of this corp 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 exploit properly 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 of this plant 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 agroeconomic practices. Though there is good level of traditional and experimental evidences to support various claims and advantages of this plant, still it needs proper evaluation.

In India, *Lemon* is used in day to day life for various purposes. It is used in all Indian traditional medicines mainly is Siddha Medicin and Ayurveda. It is the one of the main ingredients in many of the Indian cuisines. Either *lemon*

pickle or Mango pickle is part of everyday lunch meals in southern India. In Hindu Pooja, *Lemon* takes a very important place.

An investigation aims to scientifically explore its important medicinal use especially the anti microbial activity in vivo have not been studied, is inevitable.

This initiated us to investigate the leaves of this plant with strict scientific protocols so that the vast economic potentiality of this crop can be adequately exploited. (Wealth of India, www.citruspages.com)

# CHAPTER – 2

## REVIEW OF LITERATURE

The review of literature comprises information on taxonomical studies, pharmacognostical studies, phytochemical studies and pharmacological studies of different parts of *Citrus limon (L) Burm.f.*

### 2.1. TAXONOMY

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Division | : | Eudicots |
| Class | : | Rosids |
| Order | : | Sapindales |
| Family | : | Rutaceae |
| Genus | : | *Citrus* |
| Species | : | *limon* (*C x limon*) *Limonum* |
| Binomial name | : | *Citrus limon(*L*) Burm.f.* |

### 2.2. BOTANICAL INFORMATION

Name of the plant : *Citrus limon(Linn).f.*

Synonym : Sour orange, *C.medica*, Sub spices.*Limonum(*Risso), *C.Limonia* osbeck,CortexLimonis,Limone,Neemoo,Citronnier,Limoun Zitroneschale,Limonenschale(Ger), Ecorce de citron(Fr)

(http;//users.kymp.net/citrus pages/lemon.html)

**Vernacular names**

| | | |
|---|---|---|
| Tamil/siddha | : | Periya yelumichhai,Malai elumichhai, Kodai elumuchhai**.** |
| Ayurvedic | : | Jambira, Jamba |
| Hindi | : | Nimbu,Baranimbu,pahari nimbu |
| Sanskrit | : | Maha Nimbu, Nimbuka, Jambira, Jambaka |
| English | : | Lemon, Lime Tree |
| Telugu | : | Bijapuram, Nimbu pundu |
| Malayalam | : | Cherunarakam, Cherunaranga |
| Kanada | : | Bijapura, Bijuri |
| Gujarat | : | Meta limbu |
| Marathi | : | Idalimbu, Thoralimbu |
| Bengali | : | Baranebu, Goranebu, Karnanebu |
| Unani | : | Utraj |
| Arabic | : | Qalambak |
| Ceylon | : | Kidanarathankai |
| Oriya | : | Bijapura, Gojjonimbo |

(Kirtikar K.R & Basu B.D)

## 2.3. PLANT DESCRIPTION

A *lemon* tree can grow upto 6-10m (33ft) in height, of spreading habit, but they are usually smaller the branches are thorny and form an open crown.Spines small, sout; The leaves are yellowish green, shiny and elliptical-acuminate (or) oblong to elliptic ovate, lancedate, sharp-pointed, sub-serrate, petioles narrowly winged; Flowers are white on the outside with a violet streaked interior and have a strong fragrance. Some plant flowers are purple in the bud, large; Fruits are oval, ovoid or oblong 7.5-12.5cm long with a terminal nipple, very acid. The fruits are used primarily for their juice, though the pulp and rind (zest) are also used, primery in cooking and baking. Lemon jucie has about 5% acids, which gives lemons a sour taste and a pH of 2-3. This makes lemon jucie a cheap, readily available acid for use in educationl science experiments. When ripe, they have a bright yellow nose, a layer of pith underneath and paler yellow segmented interior. (Kirtikar K.R & Basu.B.D, Wealth of India)

## 2.4. HISTORY AND GEOGRAPHICAL DISTRIBUTION

The origin of the *lemon* is unknown. Some think it originated in north-western India, others think it came from India's north-eastern parts thought to be native to India but not found growing wild anywhere. From plantation in the Mediterranean region, especially southern Italy and Spain. The drug is mainly imported from Spain. It is reported to have been grown in southern Italy in the Third century AD and in Iraq and Egypt after 700 AD. The first reliable

information is from Sicily where it is known to have grown around 1000 AD. The Arab conquerors took the *lemon* with them around the Mediterranean basin all the way to southern Spain where we know it has been cultivated since 1150 AD. We know that they were growing lemon in 1297 when Marco Polo arrived. *Lemon* was among the first new fruits to arrive to the New World on 'Christopher Columbus' second voyage in 1493. He brought *lemon* seeds to the island of Hispandiola (Haiti and Dominican Republic). The *lemon* slowly spread to other islands and the continent. Large-scale commercial cultivation of *lemon* began in Florida and California in the early days of the 19$^{th}$ century. In Europe, the island of Sicily and other parts of southern Italy have exported *lemon* for several centuries (www.citruspages.com).

The *lemon* presents a number of diver's forms. The common *lemon* in India are probably indigenous citron-*lemon* hybrids. Ten forms are recorded in Assam alone. Of these, 'Assam *lemon*' has originated as a chance seedling at Burnihat and has proved superior in both quality of fruits and yield. It is called 'Patnimbu' in western Maharashtra, 'Seville *lemon*' in Andhra Pradesh and 'Sivakasi *lemon*' and 'Nepali Oblong' in other parts of South India. It is gaining popularity due to its prolific bearing nature and the big-sized, abundantly juicy fruits. It has been propagated vegetatively. The fruits are oblong, medium large, highly juicy and seedless. (Wealth of India)

'Pati-lebu' distributed throught Assam is valued for its flavour and jucie which makes a refreshing sherbet. The fruits are harvested when still green.

The variety has wide adaptability for soil and climate and is propagated through seeds and marcottage. It fruits throughout the year.

'Jora-tenga' is found throughout Assam. The fruit has abundant jucie which makes a fine refreshing drink. It is not suited to low-lying situations as it is susceptible to gummosis and scab.

'Godha-pati-lebu' can withstand a certain amount of water-logging. It is intermediate between Citron and *lemon* and is referred to as CITRON-LEMON.

'Kata-jamuri' resembles 'Eureka'. It has a fair amount of juice but is difficult to squeeze as the vesicles are tough. It is prolific bearer.

'Elaichi-lebu' (CARDAMOM-LEMON) is grown as a novelty in home gardens especially in Karimganj (Cachar district) for household use. The rind rough and thick with the fragrance of cardamom. (Wealth of India)

'Soh-long' similar to 'Pani-jamir' (SWEET LEMON) is common in Khasi hills. The fruit appears suitable for lemon squash as it is rich in jucie. It is susceptible to gummosis, scab and canker.

'Soh-synteng' is met with in the sub-montance tracts of Assam. It is similar to the sweet lime except in the acidity of the fruits. It is prolific bearer. The fruit is very juicy and can be profitably used for making *lemon* squash. Some authors treat it as *C.limettioides* var.*latifolia* Tanaka (Wealth of India)

'Pani-jamir' is grown in home gardens as a novelty. The fruit is sweet or insipid.

'Pani-muri' or 'Muri-tenga' has long been in existence in Assam. The fruit is sweet or rather insipid but with a distinct flavour and juice is plentiful. It makes a refreshing drink (Wealth of India. //users.kymp.net/citruspages/lemon.html)

## 2.5. CULTIVATION

### Area and production

*Lemon* is a very demanding plant to grow totally intolerant of frost. The fruit and flowers are destroyed at minus 1-2ºC degrees. and does it do well in extreme heat. It thrives in areas which are too cool for oranges and grape fruit. The *lemon* belt is narrow area on the cooler side of orange growing district in both the northen and southern hemispheres. In best conditions the *lemon* tree produces flowers and fruits almost around the year. The immature fruit is green and during the cool night of autmn and winter the colours turns to yellow. The harvests can be controlled by regulating the irrigation. The main harvesting period is winter and new flowering starts in spring. In dry areas the trees can be left to dry in the summer for 6-8 weeks until they look shriveled and sickly. When they are then waterted and fertilized heavily a new flowering appears in August – September. The fruit of this flowering will mature in the following summer when lemons are in short supply. The length of the harvest period can be prolonged in this way to increase productivity. In Italy some trees produce four crops a year.

The biggest *lemon* produces countries are India tops the production list with about 16% (2,060,000 tonnes) of the world's overall *lemon* & *lime* output, followed by Mexico 14% (1,880,000 tonnes),Argentina 10%(1,260,000 tonnes), Brazil 8% (1,060,000 tonnes) and spain 7% (880,000 tonnes) and other countries are China, US, Iran, Italy, Greece, Turkey, Cyprus, Lebanan, South Africa and Australia etc (www.citruspages.org.).

With the recent introduction of several exotic cultivars, *lemon* cultivation is becoming popular. *Lemons* are grown commercially in Assam, Bihar, West Bengal, Punjab, Orissa, Rajasthan and Tripura. *Lemon* occupy about 5,663 hectare which accounts for 5.4 per cent of total area under citrus with an annual production of 64,406 tonnes. Their cultivation poses less problems as they are prolific bears and resistant to major insect pests and adverse soil and climatic conditions (Wealth of India)

The important *lemon* varieties grown in India are : 'Nepali Round' and 'Nepali Oblong', 'Seedless *lemon*' or 'Baramasia', 'Eureka', 'Lisbon', 'Villafranca', 'Meyer' and 'Malta'.

*Lemon* is the 3rd most important fruit crop in India and the area under its cultivation is estimated at 2.4 lakh hectares with a production of 19 lakh tones. it occupies about 9% of the area under fruit cultivation. The most important commercial *Citrus* fruits in India are the sour orange followed by sweet orange and the acid limes.

With regard to production of *Citrus* fruits, India occupies a significant position in the world productivity. India's orange (sweet, mandarin and sour)

production amounts to15% of the orange production in Asia, next only to China. The annual production of oranges in India ranged from 1,300 thousand tones in 1984 to 1,370 thousand tones in 1988. 30-32 thousand tones of other *Citrus* fruits annually.

The major *Citrus* producing states, viz. Andhra Pradesh, Bihar, Gujarat, Maharashtra, Punjab, Tamil Nadu and Madhya Pradesh contribute 82 % of the total *Citrus* production in India with Andhra Pradesh leading with a contribution of 39%.

## Climate

In countries with warmer climate *lemon* can grow at altitudes of 800-1200 meters, where nights are cool but without frost.

*Lemon* crop thrives well in frost-free sub-tropical to semi-tropical climate. However, most of the species tolerate light frost. Being evergreen, it has no specific requirement of winter chilling but cessation of growth during winter helps in flower bud induction resulting in spring flowering. Young trees are injured by temperatures below freezing while temperatures above 46° cause sunburn and granulation fruits. Best growth occurs within 29-35°.

An annual rainfall of 700 mm is sufficient if it is well distributed. Unevenly distributed rainfall can be supplemented by irrigation and best quality fruits are obtained in semi-arid, sub-tropical regions.

## Soil

*Citrus* thrives well in deep, loose, well-aerated soils devoid of any hard pan of calcium carbonate in the rooting zone. The ideal soil $P^H$ is 5.5 to 7.5 but with proper management it can grow with success even in highly acidic soils up to 4.5 and those containing free *lime* with ph 8. The crop is highly sensitive to water-logging in the root zone and is susceptible to salt injury; hence it does not thrive in saline and alkaline soils. Loamy soils with comparatively heavier sub-soils or even heavy soils with good drainage are ideal for the crop.

## Propagation

*Citrus* trees are propagated both by seed and vegetative means. Vegetative propagation is preferred because it ensures true to type plants, uniform quality, regular bearing, etc. not with standing the merits of vegetative propagation, seed propagation is still in vogue in the case of certain *Citrus* species .many citrus species can be raised from cutting when they are desired to be clonally propagated on their own roots. A large number of graft compatible species and varieties are available in India. Some of the commonly used root stocks are sour orange, rough *lemon*, karna khatta sweet lime Rangpur *lime* and trifoliate orange and its hybrids.

Sour orange used extensively in the past for sweet orange, mandarin, grape fruit and *lemon*. Due to its susceptibility to tristeza, its use is being abanded even though it is resistant to cold, adapts to heavy soils, resists many soil-borne diseases and improves the fruit quality in general.

## Seed Propagation

It requires collection of seed from fruits of healthy, virus-free old trees which have a good pedigree performance. The extracted seeds are mixed with ash and dried in the shade. The nursery is located away from old existing orchards to reduce the hazards of pests. Soil which is free of soil-borne pathogens and nematodes is selected and properly fertilized with adequate organic manures and laid out into beds of convenient size. Bold seeds collected from desired trees are treated with fungicides before sowing to prevent seed-borne infections. The seeds have no dormancy and are sown immediately after extraction; treating them with IAA (100ppm) improves germination. Sowing is done in May-June or September-October in Southern and Western India, in spring or rainy season in Northern India and in July-August in Assam. The seeds are sown 2-3cm deep in lines 8-25 cm apart.

## Planting

Planting of seedlings or budlings in orchards is usually done after the initial rains. Pits of 50-75 cm are dug in a square system 5-8 m apart in summer. The soil is replaced after adding to it about 40k of farmyard manure.

## Irrigation

*Citrus* requires irrigation in places where the annual rainfall is below 890 mm. 60-80% of the root activity of a citrus tree is confined to the first 60cm of top soil: the roots spread far beyond the vegetative spread of the trees. The trees are sensitive to excessive moisture and water-logging, and moisture

stress is avoided during growth period and in flowering and fruiting as it reduces the fruit size. The soil is allowed to dry out only during spring and summer months but wilting is avoided. Irrigations absolutely essential tlll the fruits attain 2 cm diameter.

## Manures and Fertilizers

A crop of 18 tones of *Citrus* fruits removes nitrongen,21; phosphorus,5; potassium, 41; calcium, 19; magnesium,3.6; and sulphur,.3kg; and born,0; copper,9; iron,50; manganese and zinc,13 g per hectare.

## Interculture

Interculture is chiefly done to remove weeds which compete with the trees for moisture and nutrients, and for incorporating manure.

## Harvesting and yield

Most of the citrus species the fruits remain fresh for several weeks in the tree without any deterioration after attaining fruit maturity. Facilitate harvesting according to one's choice and market demand. They do not improve their quality after harvest, and are, therefore, harvested when fully riped *Citrus* fruits are considered fully mature for harvesting when hey turn from green to golden yellow even tough colour break is not reliable index of fruit maturity.

## Storage

*Lemon* suffer less from disease than other *Citrus* types and the picked fruits are not easily damaged by transportation or storage. *Citrus* fruits can be

stored well for a few days at room temperature and in cold storage for several months without any appreciable depreciation in quality.

### Grading and packing

The harvested fruits are graded according to size and appearance mainly by the wholesalers near the centres of production. Grading is done manually; recently a cheap and simple hand-grading machine has been designed at the Punjabrao krishividyapeeth, Akola, Maharashtra. The government of India has passed legislation for the grading and marketing of *Citrus* fruits.

Rapid packing after harvesting is recommended to avoid loss of moisture from the fruits. The fruits are packed in wooden boxes for transporting to distant markets while for near markets, they are packed in bamboo baskets. Chopped straw and dry grasses are used for padding. Hundred fruits are packed in baskets and112-330 in boxes of mango wood, depending on the size of fruits. (Anon'ymous author)

## 2.6. WHOLE PLANT

### 2.6.1 Ethnomedical Information

In this review, it was identified 122 compounds (including Hespridin and Rutin) of defined structrure, obtained from only 97 species (like *Citrus* spp..etc.) of plants, that were used globally as drugs demonstrate that 80% of these have had an ethnomedical use identical and elements of the plant.

(Daniel S.*et.al*., 2001)

The plant extract was reported by Dominican healers in New York city, used for women's health conditions. To treat abund hot flashes, menorrhagia and uterine fibroids.

(Andreana L.*et.al*., 2002)

The study reported the indigenous ethnomedical knowledge of the (one of the trible group in Assam) formulation containing *Citrus* species as one of its ingredients taken daily relieves high blood pressur.

(Ripunjoy S. & Iindra B., 2011)

An ethnomedical survey of medicinal plants species belonging to 27 families was identified in the fight against malaria. One among them was the decoction of fruits and leaves of *C.limon*.

(Saotoing P.*et.al*., 2011)

**2.6.2 Phytochemical studies**

The volatiles emitted *in vivo* by different parts of *Citrus limon*, have been identified by (HS-SPME) and (GC-MS) analyses, The particular profiles of flower buds, mature flowers, petals, stamens, gynaecium, pericorp of unripe and ripe fruits, young and adult leaves and pollen have been examined, and the essential oil obtained from expression of ripe pericarp was studies. The highest amount of limonene (62.5%) was emitted by gynaecium, followed by stamens (22.9%) and petals (3.1%). The same sompounts is contained in higher amounts in the young leaves than in old one (65.3% vs 30.1%). The possible defensive role *limone* and other V.O(terpene,aldehydes) produce by young leaves has been hypothesized.

(Flamini G.*et.al.,*1985)

Radioactive traced work showed that obacunone was converted to at least four metabolites in *Citrus limon*. Two were identified as obacunoate and limonin. When methyl obacunoate was fed, limonin was found to be one of the metabolites. Based on these results and data accumulated, the biosynthetic pathways of limonoids in *Citrus* were proposed (parts are not mentioned).

(Herman Z & Hasegawa S.1986)

One of the phytochemical constituents nomilinate was found in the major acidic limonoid present in seedlings of *Citrus limon*. Nomilin was converted to at least six acidic metabolites in *C.limon*, one of which was identified as nomilinate. The metabolism via nomilinate is the fifth metabolic pathway of nomilin shown to be present in nature(parts are not mentioned).

(Shin H. *et.al*.,1986)

| S.No | Parts of plant | Name of the constituents | % yield | Country | References |
|---|---|---|---|---|---|
| 1 | Fruits | Flavonoids<br><br>Hesperidin<br><br>Vitamin-c<br>Eriocitrin<br>(Multivitaminic complex) | <br><br>15.5 | Spain | (Del Ri Oa *et.al*.,2003)<br>(Fuster.*et.al*.,1997)<br><br>(Benarente.1997)<br>(Fuster.1997) |
| 2. | Fruit jucie | | | Japan | (Osawa.1998) |
| 3 | Leaves | | 18 | | |
| 4 | Young stems | Bioflavonoids | 1.02 | Eureka | (Hosoda &Noguchi.1998) |
| 5. | Flower | Flavonoids<br>Diosmin<br>Eriocitrin | 0.18 | Lisbon | |

### 2.6.3 Pharmacological studies

*Antidiabetic activity*

Hesperidine is the main constituents of the *Citrus* plant. The investigation carried out on the hypoglycemic and hypolipidermic effects of two hesperidine glycosides, namely hesperidin, and *Citrus* bioflavonoid cyclodextrin, clathrated hesperctin by using Goto-Kakizaki meanling rats and demonstrated the glucose level was normalized.

(Akiyama.S.*et.al.* 2009)

*Neuroinflammation Activity*

The study revealed that flavonoids found in cocoa, tea, berries and *citrus* have been shown to be highly effective in inhibition of neuro inflammation through an attenuation of microglial activation and associated cytokine release, INOS expression, nitric oxide production and NADPH oxidase activity.

(Spencer J.P.*et.al*., 2011)

*Anti viral Assay*

The maximun non toxic dose (MNTD) of the methanolic extract along with other six plants was determined using *vero E6* cell *in vitro*. More over methanolic extrct *C.limon* did not prevent cytopathic effect (or) cells in a cytopathic *in vitro* antiviral assay (plant not mentioned).

(Tang Li.*et.al*. 2010)

### 2.6.4 Pharmacognostical studies

***Insecticidal & pisicicidal activity***

The antifeedant activity was carried out for the *Citrus* derived limonoids, limonin, nomilin and obacunone and their semisynthetic derivatives were evaluated against commercially inporttant pest, *Spodoptera frugiperda.* Simple chemical conversions were carried out on the natural limonoids obtained from seeds of *C.limon.* The known antifeedant properties of the *Citrus* limonoids are confirmed. Comparison with previsouly reported data was shown.

(Ruberto,G.*et.al*.,2002)

The characterization of wild medicinal plants (*Citrus limon*) provides valuble information on anthentication of plants through their RAPD pattern. The molecular characterization of certain plants was carried out using RAPD primers. The plant was subjected to the preparation of DNA fingerprint profile for authentic documentation and characterization of Germplasm for their conservation. It is used on the specificity of Genotype of a plant, a particular DNA profile can be described to a particular plant.

(Vaishali.*et.al*., 2009)

Here the author has described about the standard operating procedures developed in a systemic way including procurement of raw drug, authentication, removal of the adulterants if any powdering to required sieve size, method of preparation, ash determination, extractable matter

determination, identification, raw data, microbial test, heavy metals test, aflatoxin level and estimation of pesticide residue,etc..

(Meena R. *et.al*., 2010)

In this article, the various aspects of *Lemon* plants has been described. *Lemon* has been recognized as an important fruit for the world wide. The origin and history, nomenclature and brief description of the plant with its distribution, its local, trade, vernacular names, parts used, and various uses and chemical constituents, pharmacological studies etc..

(Shah, N.C.2010)

The study dealt with the salt damage to plants . It has been attributed to a comination of several factor including mainly osmatic stress and the accumulation of toxic ions.

(Yardena G. *et.al*.,1997)

The work investigated the fingerprinting and phenotyping of *Citrus* germplasm, and its supposed ancestors, along with some other species of the *Citrus* genus. Morphological trait analysis and statistical of DNA content and markers were useful for reconstructing a *Citrus* phylogeny. The experiment estimated the genetic variation within and the genetic relatedness among *C.limon, C.limoni-medica* and *C.medica(L)*. The results of the multidisciplinary analyses was allowed to confirm a remarkable differention between poncitrus and these investigated *Citrus* species.

(Diego P. *et.al*.,2011)

Statisfactory experimental values of the soil-to-fruit transfer factor[F(v)] was obtained at the initial time of fruit growth and decrease as the fruit develops being lowest at the matutation period.

(Velasco H. *et.al*., 2011)

## 2.7 ESSENTIAL OIL

### 2.7.1 Phytochemical studies

The paper dealt with use of SPME/GC-MS. The *Citrus* oils cultivarswere collected from Jammu region. The SPME and followed by gas chromatography on capillary column. It was quicker and has been found to very useful in the analysis of volatile compounds, essentionl oils/aroma principles.

(Malhotra, S.*et.al.,* 2003)

Steam distilled oils of some species of the Genus *Citrus* were analyzed by GC-MS. It was observed that Citral B was the most common constituent of the oils, which could be a good inhibitor of beta-glucuronidase as all the tested essential oils showed significant inhibition of beta-glucuridase. The oils also exhibited positive response against tested microbes.

(Saleem, M.*et.al*. 2004)

The article described about the locality, quality and species for essential oil. The multivariate analysis was carried out by MS fragment method. The ratio of monoterpene hydrocarbons clearly distinguished three *Citrus* species

(*yuze, lemon & lime*). The carbonyl fractions were also extracted from *Citrus* essential oils by the sodium hydrogensulfite method. The isotope ratio of M.S fragments of octanal, nonanal, and decanal was also examined. And ther was no significant difference in the individual fragment isotope ratio of three aldehydes.

(Satake, A.*et.al*. 2004)

**2.7.2 Pharmacological studies**

The aromatheraphy was investigated under four conditions by compositional changes in commercial *lemon* essential oil for applications.

(Sawamura M. *et.al.,*2000)

*Toxicity effect*

Here the article discussed about the toxicity, such as reddening of the skin, inflammation of mucous, nausea, headache and as harmful as development of cancer. Some of the oils have toxic effects on the brain, liver and kidney.Toxicity may be seen in the presence of sunlight (few oils). These oils known as phototoxic oils and some of the oils having possible toxic ingredients.

(Ranade, G.G. 2002)

A number of essential oil compounds has been identified as effective anti-bacterials and preservatives, foodborne pathogens (Citral 50%). *In vitrio* studies have demonstrated anti-bacterial activity of essential oils against *E.coli, Listeria Monocytogenes, Salmallella, Typhimurium.*etc.

(Sara B., 2004)

*Antibacterial Activity*

The essential oil have been widely used for antibactericidal activity (*Steptococcus Pnemoniae* and *Candida*) and fungicidal virucidal etc. and also cytotoxicity, genotoxicity, Antigenotoxicity, prooxidant activity.

(Bakkali F. *et.al*. 2006)

*Anti-oxidant Activity*

Anti-oxidant activity, such as vitamins, enzymes, or $Fe^{2+}$ etc. are able to neutralize free radicals they excert a health enhancing effect on the human organisum because they protect cells from oxidant damage. The essential oil of *Citrus limon* was studied by capillary gas-liquid chromatography. The antioxidant activity was evaluated by oxidation of the alphatic aldehyde hexanal to the carboxylic acid.

(Dr.Gerhard B.,2010)

*Anti-oxidant Activity (Chemotaxonomy)*

The commercially available herbs*(Citrus limon)* essential oil were analysed by GC-MS and their anti-oxidant activities were determined by testing free radicals scavenging capcity and lipid peroxidantion in the linoleic acid system. The major compounds of the essential oil are limonene (64.5 and 94.2% and p-menth 2-en-ol). The highest DPPH radical scavenging activity was obtained by *Citrus* essential oil (limonene).

(Yang, S.A.*et.al.,* 2010)

The essential oil of *Citrus limon* (EOCL) sub-inhibitory concentration in the growth medium led to MIC decrease for amikacin, imipenem and

meropenem. In the combination of antibiotics and essential oils had shown as a synergistic effect with both essential oils/amikacin combinations. The study reveals that essential oils *C.limon* may supperss the growth of Acinetobacter species and could be a source of metabolities with antibacterial modifying activity.

(Guerra F.Q. *et.al*. 2011)

The efficacy of *Citrus*-vaporized blend of *Citrus*(E.O) at removing *Enterococcus sp*. And *Aureus*, to investigate its effect on the formation of bacterial biofilm *Citrus* vapours has potential for application in the clinical environment, for instance as a secondary disinfectant to reduce surface contamination by VRE and MRSA.

(Laird K. *et.al*., 2011)

## 2.8 LEAF

### 2.8.1 Ethnomedical Information

Four teaspoonful of leaf extract of *C.limon* mixed with small amount of common salt is given to taken orally thrice daily for the treatment of amebiasis.

(Dilip Kalita & Bikask Deb.2006)

Decoction of leveas is given during cough and colds, usually taken in the morning and evening in the kalanguya tribles in Tinoc, Ifugao, Luzon Philippines.

(Teodora D.*et.al*., 2009)

### 2.8.2 Phytochemical studies

The studies was carried out phytoconstituents of the leaves content esp. ascorbic acid, it has been proved that due to low temperature, it loses 70% ascorbic acid.

(Alvarez M.R. *et.al.,*1986)

The analysis of glycosyltransferease activity in various *Citrus* plant tissue during the plant development was investigated.The separation of flavonoid glucosyltransferase substances and products using HPLC was developed to allow their accurate assay in extracts from plant tissues down to levels of less than 0.5µg. per injection. The flavanonoes(naringenin and hesperetin) and the flavonols(morin and kaempferol) were shown to be glycosylated by the adding the enzyme. The activity was slightly changed by the adding the metal ions. Hesperetin 7-0-glucosyltransferase activity was evaluated in young, developing flowers and leaves of *lemon* trees.

(Berhow M.A. & Smolensky,1997)

Both 2-(4-ethyl phenoxy) triethylamine and 2-(3,4-dimethyl phenoxy) triethylamine marketedly inhibited by the biosynthesis of limonoids in *lemon* leaves.

(Hasegawa S. *et.al*.,1997)

The main secondary compounds are analysed and to identify the flavonoids(Hesperidin) from mature fruits and leaves.

(Del Rio J.A. *et.al*.,2003)

The extraction of *Citrus l*eaf proteins for analysis by two-dimentional electrophoresis (2-DE) were developed through the evaluation and modification of existing methods. In this methods are evaluated, and obtained best results. When Tris-Hcl, Kcl and phenol extraction were followed by precipitation with organic solvents to purity and concentration the samples. The method was demonstrated on the leaves of *Citrus* species. The methods decribed are versatile and result in high resolution 2-DE gels *Citrus* leaf proteins.

(Audrius A.Z and Andrew P.B.2005)

Dietary supplements containing sour orange unripe leaf and fruit extracts/*p*-synephrine were consumed world wide for lose weight. The study were conducted to determine the concentration of *p*-synephrine in unripe fruits and leaves from *C.limon*. In southern Brazil, and to evaluate the acute toxicity of *Citrus* species extract and p-synephrine. A HPLC-DAD : was optimized and validated for determination of p-synephrine. The results indicate that all of analyzed sample present p-synephrine in amounts that range from 0.012% to 0.099% in the unripe fruits and 0.029% to 0.438% in the leaves.

(Arbo M.D. *et.al.,*2008)

| S. No | Parts of plant | Name of constituents | % yield | country | Referance |
|---|---|---|---|---|---|
| 1. | Leaves | -Linalool<br>-Myrcene<br>-Citronellal | 56.37%<br>7.21%<br>24.54% | Taiwan<br>”<br>” | (Chen.*et.al*.,2010)<br>”<br>” |

### 2.8.3 Pharmacological studies

The research was performed to experimentally evaluate the anti-oxidant capacity of different plant products by herbalists. The method was using a superoxide dismutase (SOD) Biosenser. Measurements were carried out by comparing biosensor response to the superoxide radical's prouduced in solution using the xanthine oxidase system, both in the present and absence of the antioxidant sample is considered.

(Campanella, L. *et.al*. 2003)

Aqueous extraction of dried leaf was estimated by nebulisation and fraction to produce a product high in hesperidine, eriodictyol and narigenin which is then enscapsulated in nanopheres to stability the activities during delivery to the skin.

(Dr.Tiedtke J.*et.al*., 2004)

### 2.8.4 Pharmacognostical studies

The study of various nutrient of the *Lemon* leaves. The nutrient like phosphourus, the leaves are affected by due to deficience of phosphourus.

(Miriam A. *et.al.,*1978)

The cybrid possessed nuclear and chloroplast genomes of Eureka *lemon* plus mitochondria from willow leaf mandarin. The levels of organic acids were slightly higher in the cybrid fruit pulp than in Eureka *lemon*. There is no significant difference in sugar and carotenoid.

(Jean-Baptiste Bassene. *et.al*.,2008)

## 2.9 FRUIT

### 2.9.1Phytochemical studies

One of the phytocontituents of pectins from *Citrus* fruits were identified in this report. Evaluated the effects of soluble fibers on beta-carotene and lutein Micellization during simulated digestion *in vitro*. The carotenoid uptake from mixed micells by caco-2cells. The (M.H) viscosity aliginate and pectins inhibited carotenoid micellezation and celluar uptake relative to the fibre-free control. Alginates, carboxy-methyl cellulose, and methylcellulose inhibited beta-cartotene uptake mainly by increase the medium viscosity, but pectins might inhibit carotenoid uptake by additional mechanisms.

(Yonekura L. and Nagao.2009)

The presence of total flavonoids and five subclasses of flavonoids including flavanols, flavones, flavonones flavan-3-ols and anthocynidines in present in the *Citrus* fruits in this above study reveals that flavonoids protections against aging.

(Maras J.E. *et.al*., 2011)

| S.No | Nutritional value of Raw Lemon (without peel) | Value per 100 gm |
|---|---|---|
| 1. | Energy | 121 kj (29 kal) |
| 2. | Carbohydrates | 9.32 gm |
| 3. | Sugars | 2.50 gm |
| 4. | Dietary Fiber | 2.8 gm |
| 5. | Fat | 0.30 gm |
| 6. | Protein | 1.10 gm |
| | **Vitamins** | |
| 1. | Thaimine (vit B1) | 0.040 gm (3%) |
| 2. | Riboflovin (vit B2) | 0.020 gm (2%) |
| 3. | Niacin (vit B3) | 0.100 mg (1%) |
| 4. | Panthothenic Acid (B5) | 0.190 mg (4%) |
| 5. | Pyridoxine (vit B6) | 0.080 mg (6%) |
| 6. | Folate (vit B9) | 11µg (3%) |
| 7. | Ascarbic Acid (vit C) | 53.0 gm (64%) |
| | **Minerals** | |
| 1. | Calcium | 26 mg (3%) |
| 2. | Magnesium | 8 mg (2%) |
| 3. | Phosphorus | 16 mg (16%) |
| 4. | Potassium | 138 mg (3%) |
| 5. | Zinc | 0.06 mg (1%) |

(% is relative to US recommended adults)

Source: USDA Nutrienal data base .www.citruspages.org

## 2.9.2 Pharmacological studies

Among the various species and geneses studied for treatment of urinary, cardiovascular aliments, Flavanoids isolated from *Citrus* fruits do not have the traditional uses in the treatment of urinary or cardiovascular aliments.

(Riberio *et.al*.,1988)

The flavonoids improved the permeability of vascular vessels, they shows anti-phologestic effects and Diuretic properties, *Citrus* flavonoids inhibit bacterial mutagenesis.

(Calomme M. *et.al*.,1996)

The characterization of anti-oxidative activity was studied about in the *Citrus* fruits flavonoids glysosides.

(Miyake Y. *et.al*.,1998)

The methodology carried out for the antibody production, the labeling of furananocoumarin derivaties with beta-D-Galactosidase (beta-cell) to act as a tracer, the characterization of antibody specificity and the technique developed for the screening of furavocoumarin derivatives in *Citrus sp*. Fruits by ELISA has been described.

(Saita, T. *et.al*.,2004)

The constituents of *Citrus* flavonoids hesperidin (HES) was administration to the mice before LPS challenge significantly reduced tumor necrosis factor-α-production in a dose-dependant manner. Treatment of hesperidin is 3h before inraperitoneal infection with 10.8 CFU typhimurium

aro A resulted in rescue from lethal shock as similar to LPS-non responder mice. Pretreatment of LPS with HES suppressed the chromogenic Limulus reation.

(Kawaguchi K. *et.al.,*2004)

The isolated essential oil and their volatile constituents from *Citrus* fruits are used widely to prevent and treatment of human diseases like cancer, cardiovascular disease, including atherosclerosis and thrombosis as well as their bioactivity as antibacterial, antiviral, anti-oxidants and antidiabetic agent and the therapeutic properties of essential oil in aroma and massage therapy will also be outlined.

(Amr E. Edris, 2007)

The aqueous fermented preparation of *Citrus* fruits extract was used for the investigation of the immunomodulatory and anti-allergic properties by *in vitro* method. The analysis with respect to their impact on the degranulation capcity from basophilic cells as well as mediator release from activated human must cells *in vitro*, including IL-8 and TNF-α secretions. *Citrus* has inhibited production of IL-8 and TNF-α from human mast cells, and at low concentration additive effects were observed. The positive inhibition control Dexamethasone was used. LC-MS analysis showed that the major phenolic compounds in extract from C*itrus limon* is erioctrin. These compounds do not show biological effects at concentration levels detected in their extract of fruits.

(Huber R. *et.al.,* 2011)

The alcoholic extract of *Citrus limon* juice sacs was evaluated separately for Anthelamintic activity on adult Indian earthworm, various concentration of all extracts were tested and results were expressed in terms of time for paralysis and time for death of worms. The standard drug piperzine citrate(10mg/ml) was used.

(Munne S.*et.al.,* 2011)

The methanolic extract of fruit juice was investigated in the anti-tumor activity on the MUF-7 breast cancer cell line by *in vitro*. The expression of a pro-apoptotic gene, bax was increased and the expression of an anti-apoptotic gene, bel-2 was decreased by LE extract treatment, resulting in a shift in the Box; Bel-2 ratio to one that favored apoptosis.

(Alshatwi A.A. *et.al*.,2011)

### 2.9.3 Pharmacognostical studies

The evaluation of ontogency and ultrastructure of sceretory oil glands was studies the fruits of C*itrus limon*. Under the light and electron microscopy(TEM). The oil glands were initiated by cell division in globular/oval clusters of young meristmatic cells. The separation of the cell walls, in *Citrus* species was accompanied by strong structural disorganization of the cytoplasm, loss of nuclei, plasmolysis and followed by autolysis process. In *C.limon* oil droplets appeared only in the cytoplasm and no connection with the plastids was observed. The process of schizogency and lysigeny occurred in

a centrifugal direction. These cell modification were proceded by an abundant essential synthesis. These cells, probably, had a protective function to produce and secrete oil compounds in the *Citrus* species.

(Andrea B. and Corrado T. *et.al*.,1995)

The study deals about the cell walls and separation of cell walls. Accompained by strong structural disorganization of the cytoplasm cell modification study of ontogency and secretory cavities and ultrastrcture.

(Turner . 1998)

The accumulation of *Citric* acid and its decline toward fruits maturation in typical of *Citrus* fruits. The study was deals with NADP isocitrate dehydrogenese (NADP-IDH), an enzyme involved in citrate metabolisim. A cDNA encoding the enzymes was done from *lemon* jucie sac cells and is the first reported NADP-IDH from fruits. The mRNA levels in the jucie sac cells was induced during *lemon* fruit growth and increased by about 15 fold to a peak as the fruit neared maturation. The activity associated with the mitochondrial prepation and that, as the fruit grew, the activity shifted to the soluble fraction. The possible role of form of the enzyme in citric acid catabolism in the pulp is discussed.

(Avi sadlka *et.al*.,2000)

One of the main aim of the genetic improvement of *lemon* and its fruits quality from the Mediterranean area is certainly the obtainent of new genotypes tolerant or to protect the *mal seacco* disease by using some techniques. *Lemon*

were used as mother plants in backcress with adiploid alone fermillnello. *lemon* tolerant to the *mal seacco* diseases(CTMS) to improve tolerance and fruit quality.

(Maria. T.S. *et.al.,* 2003)

*Plant growth Hormone*

The susceptibility of Fortune of *Citrus limon* fruits to *Alternaria alternate pv. Citrus* was investigated using different artificial inculation methods. The results obtained reveals that the *C.limon* fruits are less susceptible to *A.alternate pv. Citri* than Fortune fruits, although all showed symptoms of *Alternaria* brown spot when the cuticle was broken and the flavedo+albedo was removed. Further, it was seen that susceptibility to the fungus decreased as the age of the fruit increased. There was a correlation between the two susceptibility of the *Citrus* fruits. The most of susceptible fruit(Fortune) producing more ethylene during growth then the less susceptible *C.limon.* This suggest that ethylene will be consider as a possible marker of *citrus* fruits suspectility to *A.alternata pv. Citrus.* The role of ethylene as a factore involved in diseases development is discussed.

(Otruno A. *et.al*.,2008)

## 2.10 FRUIT JUICE

### 2.10.1 Ethnomedical information

The study was done in Khamti tribels area(Arunachal Pradesh) used for the treatment for Leg paralysis.

(Das A.K. and Hui Tag.,2006)

### 2.10.2 Phytochemical studies

Aqueous extracts of *C.limon* were presently studied, the silver nanoparticals were rapidly synthesized at room temperature by treating with silver ions. The standardized process, 10-2M silver nitrat solution was interacted for 4 hrs with *lemon* juice(2% citric acid concentration and 0.5% ascorbic acid concentration) in the ratio of 1:4 (vol:vol). The formation of silver nanoparticals was confirmed. We found that citric acid was the principle reducing agent for the nanosynthesis process. The theoretical particals size corresponding to 2% citric acid con. Was compared to those obtained by various experimental techniques like X-rays analysis, atomic force microscopy and transmission electron microscopy.

(Prathna T.C. *et.al*.,2011)

### 2.10.3 Pharmacological studies

Evaluation on human cytochrome P450 3A(CYP3A) inhibiting the activity of miderzolam-1-hydroxylase.

(Fujita K.I. *et.al*.,2004)

The main objective of this study was to determine the effect of fruit juice on CYP2C9-mediated drug metabolism (Nine *Citrus* fruits(*C.limon*) and Eight tropical fruits were chosen). There is limited information on the effect of fruits on human cytochrome P450(CYP) 2C9 activity. The investigated effects of the fruits on (standard drug) 4-hydroxylation and tolbutamide hydroxylation by human liver microsomes.

(Hidaka M. *et.al*.,2008)

The study was discussed about the juice contain high amount of β-cryptoxanthin and hesperdin β-cryptoxanthin, a carotenoid and hesperdin a flavonoid possess inhibitory effects on carcinogenesis on several tissuses. These constituents are inhibit chemically induced rat colon, rat tongue and mous lung tumerigensis. Gavage with CHRP resulted in an increase of activities of detoxifying enzymes in the liver, colon and tongue rats. It also able to supperss the expression of proinflammatory cytokines and inflammatory enzymes in the target tissue.

(Takuji Tanaka *et.al*., 2011)

## 2.11 PEEL

### 2.11.1 Phytochemical studies

The peel oil (*C.limon*) samples have been investigated by GC and GC-MS. The oil was extracted by the cold-processing method, Hydrocarbons, followed by aldehydes and alcohols, were the most abundant compounds. The terpinolene was detected in small amounts. The sequiterpene hydrocarbon fraction of this oil is qualitatively more complex and quantitatively more abundant than in the other oils.

(Minn Tu N.T. *et.al*., 2002)

The phytochemical study was decribed in this review and revealed the presence of (*Citrus* peels) bioactive compounds, comprising Alkaloids (0.22-1.60%), Saponin (0.30-.98%), Flavonoids (0.30-0.89%), Phenols (0.02-0.64%) and Tannins (0.23-1.45%). The growth of Furarium oxysporm which causes

damping off diseases of Okra (*Hibiscus esculents*) was inhibited *in vitro* by the extract of *Citrus* species. The extract from the peels of *Citrus* species showed (83.55%, 71.10% and 68.14%) inhibition activity.

(Okwu D.E. *et.al*., 2007)

**2.11.2Pharmacological studies**

The highest antibacterial potentiality exhibited by the acetone peel extract of *C.limon* as very potent as the antibiotics such as metacillin and penicillin. The phytochemical analysis of the *Citrus* peel extract showed the presence of flavonoids, saponins, steroids, terpenoids, tannins and alkaloids.

(Ashok kumar K. *et.al*., 1987)

**2.12 PEEL AND LEAF**

**2.12.1 Pharmacological studies**

Inhibitory effect of 10% concentration of peel and leaf extract of *Citrus* and benomyl on *in vitro* growth of Furarium oxysporum.

| Treatment | - | % of inhibition |
|---|---|---|
| Benomyl | - | 83.64±0.11 |
| Control | - | 0.00 |

| Citrus example ---- | Leaf extract --- | Peel extract |
|---|---|---|
| *C.limon* | 27.75±0.13 | 48.48±0.07 |

(Okwu D.E. *et.al*., 2007)

## 2.13 PEEL AND FRUIT

### 2.13.1 Pharmacological(cosmetic skin properties) studies

The aqueous extraction of *Citrus* bioflavonoids are used as a *in vitro* evaluation of Depigmentation factor, such as hesperidin eriodictyol and naringenin, which is then encapsulated in nanospheres. It helps in designing of skin care products to prevent age spots and irregular skin pigmentation.

(Dr.Jane Tiedtk *et.al*.,2004)

Antibactrial activity of five different solvent extract of *Citrus* fruit peel against five pathogenic bacteria.

(Ashok kumar K. *et.al*., 2008)

## 2.14 PEEL AND LEAF OIL

### 2.14.1 Phytochemical studies

The study was discussed about the peel and leaf oil of 43 texa of *Lemon* and limes. Were investigated by capillary GC,GC-MS, and $^{13}C$ NMR, The result were submitted to principle component analysis to check for chemical variability.

(Marie-Laure Lota *et.al*.,2002)

The phytochemical studies was carried out and to identify the volatile compounds of peels and leaf by using GC-MS & $^{13}NMR$. The constituents are α-thujene, α-pinene, α-terpinene, β-pinene, camphene, sabinene, myrcene, limonene, octanol and 3-carene.

(Marie-Laure Lota *et.al.,* 2002)

Aconitase, which catalysis the comarssion of citrate into iso citrate, requires Ferrous ion for its activity which is used for investigation of *C.limon* fruits.

(Avi sadka *et.al*., 2007)

The study was identified 2 new 4'-substituted flavons are isolated from the fresh fruits peels. One is *limon* flavonyl lactone-A, and another one limonflavonyl lactone –B on the basis of spectral data and chemical analysis.

(Sultana S. *et.al*., 2008)

**2.15 FLOWER**

**2.15.1 Phytochemical studies**

The experimental study was carried out the flower extract was used by genetic modification of tobacco *cv petit* Havana SRI using three different Monoterpene synthesis from *lemon.*

(Joost L. 2004)

**2.16 SEED**

**2.16.1 Pharmacognostical studies**

The problem occur in the cultivation of *lemon* seedling, in presence of herbicide like Norfluorazon(NFZ). The seedling were obtained by cultivation under continueous dark condition. When NFZ trated with *lemon* seedlings showed altered chloroplasts and etiolated seedlings only proplastituds was observed.

(Prieto H. *et.al*., 2007)

Polyembryony, in which multiple somatic nucellar cell-derive embryos develop in addition to the zygotic embryo in a seed, is common in the geneus *Citrus*. By sequencing three BAC clones aligned on the polyembroyony haplo type, a single contigueous draft sequencing consisting of 380kb containg to predicted open reading frames (ORFS) was reconstructed. The synthetic relationship among these species is conserved even through *v. vinifera* and *p.trichoparpa* are non.apomictic species.

(Nakano M. *et.al*., 2012)

## 2.17 SEED AND BUDWOOD

### 2.17.1 Pharmacognostical studies

The paper dealt in this manner that, there was no difference in blight incidence or tree growth between trees propagated by budding valencia buds from a health tree or a blight affected tree. Tree height and trunk circumference varied with rootstock cultivar and seed source.

(Pelosi R.R. *et.al*., 1987)

## 2.18 STEM AND ROOT

### 2.18.1 Phytochemical studies

The investigation was carried out the anti-inflammatory principle from the stem and root barks of *C.medica* has led to the isolation of a New known compounds(coumarin, namely citrumedin-B) are guided by Bio assay. The active components were xenthyletin, nordentatin, atalanto flavon and

lonchocarpol are which displayed potent nitric acid(NO) reducing activity in microgial alucidated by using combination of 2D NMR techniques.

(Chan Y.Y. *et.al*., 2010)

## 2.19 ROOT AND BARK

### 2.19.1 Phytochemical studies

The isolated compounds are obtained from root and bark was investigated to developing a useful agents that are effective in cancer Chemoprevention. Their structures were determined using spectroscopic methods(2D-NMR). And they were found so many compounds like Anrapten and Nobiletin are very useful constituents for the cancer Chemoprevention and also synthesized pentallyl queercetin(QPA) is very useful anti-tumor compounds cyclosporine A and verapamil have anti-tumor promotion activity are potention candidates for effective multidrug resistance agents in cancer Chemotherpy.

(Motoharu J.U., 2005)

## 2.20 CITRUS MOLASSES

### 2.20.1 Phytochemical studies

The study was discussed about the recovery the limonoid glucosides from *Citrus* molasses by using ion-exchanges method and styrene/divinylbenzene resins was used for development. A cation exchange resin is used to decolorize the molasses. An analysis of the efficiency of the

methods shows that less than 10% of the total limonoid glucosids are lost during recovery.

(Schoch T.K. *et.al*., 2002)

## 2.21 CHROMOBACTERIUM

The Microorganism was isolated from six blood cultures and two suppurated skin lesions. It is first such described in Argentina, and it reinforces the need for prolonged treatment and careful clinical evaluation to ensure complete remission of human infection caused by this bacterium.

(Sara C. *et.al*., 1986)

# CHAPTER -3

## AIM AND OBJECTIVE

Volatile oils (V.O) are valuable natural products and find applications in many areas including pharmaceuticals, cosmetics, perfumes, Aroma therapy, phytotherapy, spices (culniary) etc. Attention of many scientists was attracted towards the screening of plants to study the biological activities of their oils from phyto chemical and pharmacological to therapeutic aspects. This may be hopefully lead to new directions on plant applications and new perspectives on the potential therapeutic use of these natural products. V.O's are complex mixtures comprising many single compounds. The knowledge of its composition permits for a better and specially directed application. (Sokmen,*et,al* 2004)

Esssential oils consist of monoterpenes and sesquiterpenes which are the lipophilic secondary metabolites of plants derived from mevalonate and isopentenyl pyrophosphate. It is found widely in nature with the diversity of **terpenoid structures in plants**. Essential oil is generally responsible for plant's distinctive scent and taste. Various organic components are identified when essential oils are analyzed with a GC chromatography which are as follows

- Terpene hydrocarbons
- Monoterpene hydrocarbons
- Phenols

- Alcohols
- Monoterpene alcohols
- Aldehydes
- Ketones
- Esters
- Ethers
- Oxides
- **Terpenes Hydrocarbons:**
- **Monoterpene** - anti-inflammatory, antiseptic, antiviral and antibacterial therapeutic properties
- **Phenols** - great antiseptic, anti-bacterial and disinfectant qualities
- **Alcohols** –also great antiseptic antibacterial and disinfectants
- **Monoterpene alcohols** - good antiseptic, anti-viral and anti-fungal properties
- **Aldehydes** - anti-fungal, anti-inflammatory, disinfectant, sedative yet uplifting therapeutic qualities.
- **Ketones** - mucolytic properties, but is also useful in skin regeneration, wound healing and reducing old scar.
- **Esters** - sedative, antispasmodic, anti-fungal and anti-microbial properties.
- **Ethers** - Phenolic ethers are the most widely found ethers in essential oils with anethol found in aniseed, the only real ether of importance together with methyl chavicol found in basil and tarragon.

- **Oxides** – Expectorents (www.essentialoils.co.za)

## AIM

- Pharmacognostical, preliminary phytochemical studies on the leaves of *Citrus limon.* To study in detail one of its constituents. Further to evaluate quantitatively the therapeutic effect and to determine the Antioxidant activity and Antibacterial activity by *in vitro* method infected human pathogens.

## OBJECTIVE

The objective of the study was divided into 3 parts.

**Part 1: Pharmacognostic study:-**

- Collection and authentification of plant.
- Macroscopy of the leaf.
- Microscopy :-

  1.Anatomical studies.

  2. Microscopic schedules.

  3. Powder microscopy of the leaf
- Physico-chemical parameters:-

  1.Ash values.

  2. Loss on drying.

  3. Extractive values.

**Part 2: Preliminary phytochemical screening:-**

- Qualitative analysis of the leaves for the presence of various phytoconstituents.
- Isolation of V.O from the leaves of *C.limon.*
- Physico-Chemical evaluation of the isolated V.O.

Nature, Colour, Odour, Taste, Solubility, Refractive index, Wt/ml, Optical rotation.

- Identification of compounds present in the volatile oil of the leaves by GC – MS profile.

**Part 3: Pharmacological screening:-**

*In vitro* Antioxidant activity

Antibacterial activity

# CHAPTER – 4

## MATERIALS AND METHODS

### 4.1. PLANT COLLECTION AND AUTHENTIFICATION

The leaves of the plant *Citrus limon* (L) selected for our study was collected from **K. Pudur, Madurai**, Tamil Nadu, India during the month of septamber 2011 and was authenticated by Dr. P. Jayaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamil Nadu, India.

### 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. PHARMACOGNOSTIC 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

researchers 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*itrus limon(L)*

Leaf, petiole,flower,fruit and seed were studied individually for its morphological characters by organoleptic test.

### 4.2.2. Microscopical studies on the leaf of *C.limon(L)*

### Collection of specimens

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 weare dehydrated with graded series of ethyl alcohol and tertiary-butyl alcohol as per the schedule given by 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 parafin 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 **rotary 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. Whereever necessary sections were also stained with **safranin** and **fast-green** and (IKI) potassium iodide (for starch ).

For studying the stomatal morphology, venation pattern 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 (Sass, 1940) were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured.

## Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary.photographs of different magnifications were taken.with **Nikon labphoto 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. Descriptive terms of the anatomical features are as given in the standard Anatomy books(Esau, 1964).

## 4.2.3. 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 (Johnson, 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 glycerin. 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.

## 4.2.4. MICROSCOPIC SCHEDULES

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 terminal 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.

## Determination of Vein Islet Number and Vein Termination Number

Leaf lets were cleared in chloral hydrate, stained and mounted on a slide. A camera Lucida is set up and by means of a stage micrometer the paper is divided into squares of $1mm^2$ using a 16mm objective. The stage micrometer 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.

## 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.

I, 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

## Determination of Stomatal Index

The procedure adopted in the determinations of stomatal number was 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. (Wallis.T.E, 1953, Wallis.T.E.1965, Iyengar.M.A. 1994, Ayurvedic PC 2001)

## 4.2.5 PHYSICOCHEMICAL PARAMETERS

### 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 the 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 a desiccators and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

### Extractive Value:

### 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 to dryness 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.

(IP. 1996, WHO.1998, Ayurvedic PC.2001)

## 4.3. PHYTOCHEMICAL STUDIES

### 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 acids and then filtered. The filter was washed with 0.1 N sulphuric acids 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 purine group (Murexide test)***

The residue obtained after the evaporation of chloroform was treated with 1ml of hydrochloric acid in a porcelain dish and 0.1g of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapors of dilute ammonia solution.

**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.

**TEST FOR ANTHRAQUINONES GLYCOSIDES**

- ***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 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 nitro pruside solution were added.

**TEST FOR 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 (V1).

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 aciduous 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. ISOLATION OF VOLATILE OIL (V.O) FROM THE LEAVES OF *Citrus limon.*

Weighed quantity of fresh leaves was subjected to hydro distillation using Clevenger apparatus used for the determination of V.O 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. (WHO, 1998, The Ayurvedic PC, 2001)

### 4.3.3 PHYSICOCHEMICAL 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, organic solvents like methanol, 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 25° C.

## Refractive Index

The index of refraction is a physical constant that is made use to determine the identity and purity of the volatile oils. It was determined using Atago DR abbes refracto meter.

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.

## Specific rotation

Both the degree of rotation and 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. 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 vaporization 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 vaporization 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 vapor 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 impinges 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 combination 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 fraction 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 multi component 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 produces a wealth of data rapidly**. To process and interpret all of this data manually would be excessively time consuming. The data were compared with the Wiley library version.

[Comment]

===== Analytical Line 1 =====

| | | |
|---|---|---|
| [GC-2010] | :50.0 °C | |
| Column | :240.00 °C | |
| Oven Temp. | :Split | |
| Injection | :Linear | |
| Temp. | Velocity | |
| Injection | :91.1 kPa | |
| Mode Flow | :20.0 | |
| Control | mL/min | |
| Mode | :1.55 | |
| Pressure | mL/min | |
| Total Flow | :45.1 cm/sec | |
| Column | :3.0 mL/min | |
| Flow Linear | :10.0 :OFF | |
| Velocity | :OFF :OFF | Hold |
| Purge Flow | Temperature | Time(min) |
| Split Ratio | (°C) 50.0 | 3.00 10.00 |

High Pressure 300.0
Injection
Carrier Gas
Saver
Splitter Hold
Oven Temp.
Program
Rate -10.00

< Ready Check Heat Unit >
Column Oven : Yes SPL1 :
Yes MS : Yes < Ready
Check Detector(FTD) > <
Ready Check Baseline Drift
> < Ready Check Injection
Flow > SPL1 Carrier : Yes
SPL1 Purge : Yes < Ready
Check APC Flow > <
Ready Check Detector APC
Flow > External Wait :No
Equilibrium Time :0.1 min
[GC Program]
[GCMS-
QP2010]
IonSource
Temp
Interface
Temp.
Solvent
Cut Time
Detector :200.00 °C
Gain Mode :240.00 °C
Detector :3.00 min
Gain :Relative :0.00
Threshold kV :1000
[MS
Table] --
Group 1 -
Event 1--
Start Time
End Time
ACQ :3.00min
Mode :35.00min
Event :Scan :0.50sec
Time Scan :2000 :40.00
Speed Start :1000.00

m/z End
m/z

Sample Inlet Unit [MS Program] :GC

Use MS Program :OFF

## 4.4 PHARMACOLOGICAL STUDIES

### *IN VITRO* ANTIOXIDANT ACTIVITY

### Introduction

Antioxidants play an major role in the treatment of various diseases. It has been reported in many studies that the antioxidant property of the plants may due to the presence of phenolic compounds. The natural antioxidants are ascorbic acid, vitamin E, phenolic acids etc. The antioxidants scavenge the free radical generated, due to oxidative stress and exhibit a protective effect against many diseases like cancer, cardiovascular disease, diabetes and ageing. Some of the *in vitro* models for the evaluation of antioxidant activity are listed below

- DPPH radical scavenging activity
- Superoxide radical scavenging activity
- Hydroxyl radical scavenging activity
- Nitric oxide radical inhibition assay
- Reducing power method

- Phosphomolybdenum method
- Peroxy nitrile radical scavenging activity
- Xanthine oxidase method
- Ferric reducing ability of Plasma
- Thiobarbituric acid assay etc.
- Conjugated diene assay.
- Thiobarbituric acid assy.
- Erthrocyte ghost system.
- Cytochrome C.test.
- B-carotene linoleate method.
- Oxygen radical absorbance capacity.
- N,N dimethyl P-phenylene diamine dihydrochloride method.
- 2,2 –azobis(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt method etc.

**4.4.1 DPPH (Diphenyl picryl hydrazyl) RADICAL SCAVENGING ACTIVITY METHOD**

## Principle:

DPPH is a stable free radical with a distinctive ESR signal. Its reaction with antioxidants can be followed by the loss of absorbance at 517nm. It is widely accepted that DPPH accept an electron or hydrogen radical and become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of DPPH (purple colour solution) shows a strong absorption at 517nm. DPPH

radicals react with suitable reducing agents where the pairing of electrons takes place and the solution loses colour stochiometrically with the number of electrons taken up(Williams BW *et al.,* 1995, Halliwell B *et al*., 1999, Blois 1958).

$$DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}$$

## Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

## Reagents

0.1mM diphenyl picryl hydrazyl(DPPH) in ethanol/methnol

## Procedure

A stock solution of DPPH was prepared in ethanol (4mg/100ml). To the 1mL of test samples of different concentrations (VOCL), 4mL of DPPH was added. Control without test compound was prepared in an identical manner. Blank was prepared in the similar way, where DPPH was replaced by ethanol. The reaction was allowed to be completed in the dark place for about 30min. Then the absorbance of test mixtures was read at 517nm. The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical. Vitamin C was used as standard.

The percentage scavenging was calculated using the formula

**%inhibition = [(Control-Test)/Control] x 100**

The concentration of the sample required for 50% reduction in absorbance ($IC_{50}$) was calculated using linear regression analysis. The results obtained are presented in **Table 11** and F**ig. 4**

### 4.4.2. NITRIC OXIDE SCAVENGING ACTIVITY ASSAY

## Principle

Nitric oxide scavenging activity was determined according to the method reported ( Green *et al.,* 1982). Sodium nitropruside(SNP) in aqueous solution at physiological pH spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions. These nitrite ions can be determined by Griess Illosvoy reaction. The nitrite ions produced diazotizes sulphanilamide and the diazonium salt thus obtained reacts with NN naphthyl ethylene diamine dihydrochloride(NEDD) to give a pink colour chromophore which has a maximum absorption at 546nm(Sreejayan N, Rao MNA 1997).

## Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

## Reagents

10mM sodium nitroprusside(SNP)

Phosphate buffered saline pH 7.4(PBS)

2% sulphanilamide in ortho phosphoric acid

0.1% naphthyl ethylene diamine dihydrochloride(NEDD)

## Procedure

To 1mL of sodium nitroprusside, 2.5mL phosphate buffered saline pH 7.4 was added. 1mL of VOCL at various concentrations were added to the above solution and the mixture was incubated at 25$^{o}$C for 30min. To 1.5mL of

the incubated mixture add 1mL of sulphanilamide in phosphoric acid and 0.5mL of naphthyl ethylene diamine dihydrochloride. The absorbance was measured at 546nm. Ascorbic acid was used as a standard. The percentage inhibition of nitric oxide radical generated was calculated using the following formula:

**% inhibition = [(Control-Test)/Control] x 100.**

The $IC_{50}$ was calculated using linear regression analysis. The results wewe presented in **Table 12** and **Fig.**.**5**

#### 4.4.3.REDUCING POWER ASSAY

## Principle

This is a spectrophotometric method and is based on the principle that increases in absorbance of the reaction mixture as concentration increases showing an increased antioxidant activity. The assay is based on the reduction of ferric in potassium ferricyanide to potassium ferrocyanide by the sample and the subsequent formation of Prussian blue colour with ferric chloride. The absorbance of the blue complex is measured at 700nm(Oyaizu M 1986, Chang LW *et al*., 2002).

## Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

## Reagents

1% potassium ferric cyanide

10% trichloro acetic acid

0.2M, pH 6.6 phosphate buffer

0.1% ferric chloride

## Procedure

The reducing power ability of the volatile oil was screened by assessing the ability of the test sample to reduce FeCl3 solution as mentioned by Oyaizu *et al*. (1986). 0.1-0.5mL of volatile oil in ethanol was mixed with 0.75mL phosphate buffer and 0.75mL of 1% potassium ferricyanide [$K_3Fe(CN_6)$], then mixture was incubated at 50$^{o}$C for 20min. 0.75mL of 1% trichloro acetic acid was added to the mixture, allowed to stand for 10 minutes. The whole mixture was then centrifuged at 3000rpm for 10min. Finally, 1.5mL of the supernatant was removed and mixed with 1.5mL of distilled water and 0.1mL of 0.1% ferric chloride solution and the absorbance was measured at 700nm in UV-Visible spectrophotometer. Higher the absorbance observed in test mixture indicates the stronger reducing power of the test solution. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean ± standard error of the mean. The antioxidant activity was expressed as equivalents of Vitamin C (µg/g). The results obtained are presented in **Table 13** and **Fig.6.**

## 2. *IN VITRO* ANTIBACTERIAL ACTIVITY

## Introduction

Antibacterial activity of isolated volatile oil of *Citrus limon* has been studied. The primary screening by Disc diffusion method against Gram-positive (*Steptococcus pyogens and Staphylococcus aureus*) and Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Shigella and Chromobacterium violaceum*) test bacteria. It was observed that 6 isolated bacteria were Gram-negative bacteria, 2 were Gram-positive bacteria.

An antibacterial is a compound or substance that kills or slows down the growth of bacteria. The term antibacterial is otherwise called as "antibiotic" . It was coined by Selmon Waksman in 1942. Even though pharmacological industries have produced a number of new antibiotics in the last three decades, a resistance to these drugs by microorganisms has increased. In general bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. The problem of microbial resistance is growing and the outlook for the antimicrobial drugs in the feature is still uncertain. For a long period of time, plants have been a valuable source of natural products for maintaining human health. The use of isolated volatile oil and phytoconstituents with known antimicrobial properties can be of great significance in therapeutic treatments.

Historically pharmacological screening of material for many herbal industries. Although compounds of natural or synthetic origin of the plant

species have been tested for source of innumerable therapeutic agents. Considering the vast molecules has been most productive in the area of potentiality of plants as sources for antimicrobial drugs . Some of the *in vitro* models for the evaluation of antioxidant activity are listed below

1. **Diffusion methods**

   Disc diffusion method

   Hole plate diffusion method

2. **Dilution methods**

   Solid or agar dilution method

   Liquid dilution method

3. **Bioautographic methods**

These microbiological assays are based upon a comparison of the inhibition of growth of microorganism by measuring concentration of the antibiotics that are examined with that produced by known concentration of standard preparation of the antibiotic having the known activity.

The volatile of *Citrus limon* was screened for antibacterial activity by disc diffusion method.

**Minimum Inhibitory Concentration (MIC)**

**Bacteria**

The various organisms used in the present study include *Staphylococcus aureus, streptococcus pyogens, Escherichia coli, Klebsiella pneumoniae,Proteus mirabilis, Pseudomonas aeruginosa,Shigella Flexneri, Chromobacterium violaceium*. These organisms were confirmed by biochemical tests.

**Preparation of media**

Muller Hinton Agar (MH, Hi media) was used. The formula (gm/litre) Beef - 2 gm, caesin acid hydrolysate 3.5gm, starch 300mg and agar 4gm, distelled water 200ml pH 7.0 ± 0.2.

MH agar (38g) was weighed and dissolved in 200ml of distilled water and adjusted to pH 7.3 ± 0.2, sterilized by autoclaving at 121$^{o}$C for 15 minutes at 15psi pressure and was used for sensitivity tests.

**Preparation of bacterial cultures**

Few colonies of the bacterial strains selected for study were picked from the agar slopes and inoculated into 4ml peptone water in a test tube. These tubes were incubated for 2-4 hours to produce suspensions. The suspensions were then diluted, if necessary with saline to a density visually equivalent to that of standard prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1% sulphuric acid. These suspensions were used for seeding.

**Preparation of the solution of volatile oil**

The volatile oil of *Citrus limon* (VOCL) was dissolved in DMSO to get the desired concentration.

**Preparation of agar plates**

The media (20mL) was introduced aseptically into sterilized petridishes and the petridishes were swirled(rotate) until the agar begins to set.

**Disc Diffusion technique**

The pathogenic strains were then seeded on the MH agar media in a petridish by streaking the plate with the help of a sterile cotton swab. Care was taken for the even distribution of culture all over the plate. The seeded plates were allowed to dry.

**Test Procedure**

The plain sterile discs of 6mm diameter were obtained from( Hi Media) the market. The discs were then impregnated with different concentrations of the volatile oil of *Citrus limon* and solvent DMSO. Amikacin was used as a standard. Each disc contained 30μg. The standard, VO and DMSO discs were then placed on the seeded medium plates. The plates were then incubated at 37$^{o}$C for 24h. The results were read by the presence or absence of zone of inhibition. The zone of inhibition was then measured. The results are tabulated in **Table 15**.

# CHAPTER – 5

## RESULTS

## 5.1 PHARMACOGNOSY

### 5.1.1 Morphological features of *C.limon*

It is a tree with yellowish green, Spreading habit. (Plate-1)

**Leaves** : (Plate -2,3,4,5, Fig- 1)

| | | |
|---|---|---|
| Shape | : | Light green, oblong to elliptic ovate |
| | | Scarcely winged, lanceolate, sharp-pointed |
| Colour | : | Yellowish green |
| Margin | : | Subserrated margin |
| Petiole | : | Narrowly winged |

**Flowers** **:** Axillary single or in small clusters

**Fruits** **:** Oblong ovoid berry (7.5 -12.5cm)

### 5.1.2 MICROSCOPY OF LEAF:

### T.S. of Leaf of *C.limon(L)* through the Midrib

### LEAF MIDRIB (Plate-6,7)

**Shape** : Prominent, uniformly thick(800µm) consists of wide short adaxial hump, wide semicircular abaxial side.

**Epidermis (Plate-8,9) :** Comprising small sprindle shaped thick walled cells, and polyhedral in outline in surface view with straight wall. The adaxial epidermis is apostomatic the walls have dense simple pits giving beaded in appearance. **The abaxial epidermis is stomatiferous**. The stomata are

**amplicyclocytic type** surrounded by two rings of cylindrical subsidiary cells. The lower epidermis is comparatively of thin walls with dense simple pits some of the epidermals cells of the Adaxial side dilated into wide circular cells(30µm in diameter) having dense mucilage content.

## Ground Tissue

## Thin walled parenchyma cells

**Vascular system :** Multi stranded single wide arc shaped strand in the lower side but, small to lateral stand on upper side.Collateral with uniseriat xylem elements and discrete masses of phloem elements. Thick sclerenchyma bands present between them. Small prismatic crystals are sparingly present in the phloem sclerenchyma.

**LAMINA (plate-10)**

400µm thick, distintctly dorsiventral with compact dark zone of adaxial palisade cells below that loosely arranged spongy paraenchyma cells which are spherical and lobed. Lateral veins and veinlets are present in the medium portion.

**Secretory Cavity:**

Wide circular, 200µm in diameter lysigenous cavity are fairly common both in lamina and midrib.

**Venation Pattern:(plate-11)**

The vein straight, diffuse in orientation, reticulate, well defined vein-islets of various shapes of veinlets and once or twice forced vein termination forming dendroid outlined.

**PETIOLE (plate-12,13)**

**Shape :**

Planoconvex(1.3mm) upper semicircular part with two short thick lateral wings (300µm) thick and (500µm) long.

**Epidermal Cells :**

Small and thick walled ground tissue, Homogeneous small compact paraenchyma cells.

**Vascular Systems :**

It contains elliptical cylinder of xylem and phloem, xylem stands have short, narrow, radial, paralled lines of lignified fibers. External to the xylem continous wide zone of phloem is present with thick blocks of sclerenchyma on the Adoxial side of the segments.

### 5.1.4 POWDER MICROSCOPY (Fig -2)

### Organoleptic characters

1. Nature : Coarse fiber powder
2. Colour : Yellowish Green ash
3. Odour : Aromatic characterictic odour
4. Taste : Characteristic Sour Pungent Taste
5. Shaken with water : Froathing occurs in water
6. Pressed in between two filter paper : No oil mark on the paper

We have observed the following microscopical cell structures,

- 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 amplicyclocytic type
- Crystals
- Fibers , Xylem, Phloem.

## 5.1.5 MICROSCOPIC SCHEDULE

As per the methods described in materials and methods, microscopic schedule was carried out and the results were tabulated from the Tables 1- 3. The following evaluation were carried out.

**Table – 1**

**VEIN ISLET AND VEIN TERMINATION NUMBER OF *C.limon***

| Observation number | Vein Islet number | Vein termination number |
|---|---|---|
| 1 | 3 | 6 |
| 2 | 4 | 4 |
| 3 | 5 | 5 |
| 4 | 7 | 7 |
| 5 | 4 | 6 |
| 6 | 6 | 5 |
| 7 | 4 | 4 |
| 8 | 4 | 5 |
| 9 | 6 | 6 |

| Range | Minimum | Average | Maximum |
|---|---|---|---|
| **Vein islet number** | 3 | 4.60 | 7 |
| **Vein termination** | 4 | 5.40 | 7 |

**Table – 2**

**STOMATAL NUMBER OF *C.limon***

| Observation number | Lower epidermis |
|---|---|
| 1 | 62.75 |
| 2 | 55.25 |
| 3 | 66.50 |
| 4 | 64.00 |
| 5 | 50.50 |
| 6 | 63.25 |
| 7 | 55.00 |
| 8 | 65.50 |
| 9 | 64.50 |
| 10 | 65.25 |

| Range | Minimum | Average | Maximum |
|---|---|---|---|
| **Lower epidermis** | 50.50 | 61.25 | 66.50 |

**Table -3**

**STOMATAL INDEX OF C.*limon***

| Observation number | Lower epidermis |
|---|---|
| 1 | 16.65 |
| 2 | 14.95 |
| 3 | 15.65 |
| 4 | 16.45 |
| 5 | 15.00 |
| 6 | 16.45 |
| 7 | 15.40 |
| 8 | 15.55 |
| 9 | 16.65 |
| 10 | 16.25 |

| Range | Minimum | Average | Maximum |
|---|---|---|---|
| **Lower epidermis** | 14.95 | 15.90 | 16.65 |

## 5.1.6 PHYSICO CHEMICAL PARAMETERS

As per the methods described in materials and methods, physicochemical parameter was carried out of quantitative microscopy and the results were tabulated from the Tables 4-7. The following evaluations were carried out.

**Table – 4**

**ASH VALUE FOR THE LEAVES OF *C.limon***

| **Observation Number** | **Total Ash (%)** | **Acid Insoluble Ash (%)** | **Water soluble Ash (%)** |
|---|---|---|---|
| 1 | 10.256 | 2.15 | 5.60 |
| 2 | 9.245 | 1.70 | 6.25 |
| 3 | 9.720 | 2.25 | 5.35 |
| 4 | 9.050 | 1.75 | 5.75 |
| 5 | 10.705 | 1.60 | 5.55 |
| 6 | 9.188 | 1.85 | 5.25 |
| 7 | 9.693 | 1.55 | 5.60 |
| 8 | 10.172 | 1.10 | 5.55 |
| 9 | 10.030 | 2.50 | 4.65 |
| 10 | 10.140 | 1.70 | 4.75 |
| **Minimum** | 9.050 | 1.10 | 4.65 |
| **Average** | 9.819 | 1.815 | 5.43 |
| **Maximum** | 10.702 | 2.50 | 6.25 |

**Table – 5**

**PERCENTAGE LOSS ON DRYING FOR THE LEAVES OF *C.limon***

| Observation Number | Loss on Drying (%) W/W |
|---|---|
| 1 | 1.60 |
| 2 | 2.90 |
| 3 | 1.30 |
| 4 | 2.50 |
| 5 | 1.80 |

| Material | Minimum | Average | Maximum |
|---|---|---|---|
| **Leaves powder** | 1.30 | 2.02 | 2.90 |

**Table – 6**

**EXTRACTIVE VALUES (INDIVIDUAL SOLVENTS) BY COLD MACERATION**

| Solvents | Extractive Value (%) |
|---|---|
| Petroleum ether | 0.45 |
| Ethanol | 0.78 |
| Water | 6.50 |

**Table – 7**

**EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)**

| **Solvents** | **Extractive Value (%)** |
|---|---|
| | |
| Petroleum ether | 0.58 |
| Ethanol | 0.75 |
| Water | 7.15 |

## 5.2 PRELIMINARY PHYTOCHEMICAL SCREENING

### 5.2.1 Qualitative Phytochemical Test

Preliminary phytochemical screening of the powdered mature leaves were carried out and the results are as follows (Table 8,9)

**TEST FOR ALKALOIDS**

Mayer's test : No cream precipitate shows the **absence** of alkaloids

Dragendorff's test : No reddish brown precipitate shows the **absence** of alkaloids

Hager's test : No yellow precipitate shows the **absence** of alkaloids

Wagner's test : No reddish brown precipitate shows the **absence** of alkaloids

Murexide test : No appearance of purple colour shows the **absence** of purine alkaloids

**TEST FOR CARBOHYDRATES**

Molish's test : Appearance of purple colour shows the **presence** of carbohydrates.

Fehling's test : Formation of reddish brown precipitate shows the **presence** of free reducing sugars.

Benedict's test : Formation of reddish brown precipitate shows the **presence** of free reducing sugars.

**TEST FOR GLYCOSIDES**

Test A : No red colour precipitate shows the **absence** of glycosides.

Test B : Appearance of red colour precipitate less than test A shows the **absence** of glycosides.

## Anthraquinone glycosides

Borntrager test : No pink colour in ammonical layer shows the **absence** of anthraquinone glycosides.

Modified Borntrager's test : No pink colour in ammonical layer shows the **absence** of anthraquinone glycosides.

Test for cyanogenetic glycosides : No brick red colour on paper shows the **absence** of cyanogenetic glycosides

## Test for cardiac glycosides (for deoxy sugar)

Keller killiani's test : No reddish brown colour ring at the junction shows the **absence** of cardiac glycosides.

Raymond's test : No violet colour shows the **absence** of cardiac glycosides

Legal's test : No blood red colour shows the **absence** of cardiac glycosides

Test for coumarin glycosides : Appearance of green fluorescence shows the **presence** of coumarin glycosides

## TEST FOR PHYTO STEROLS

Salkowski's test : Appearance of red colour in lower layer shows the **Presence** of sterol

Libermann – 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 :** Froathing occurs indicates the **presence** of saponins

## TEST FOR TANNINS

Ferric chloride test : No appearance of bluish black colour shows the **absence** of tannins

Gold beater's skin test : No appearance of brown colour shows the **absence** 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 MUCILAGE** | : | Appearance of reddish pink colour shows the **presence** of mucilage |
| **TEST FOR TERPENOIDS** | **:** | Appearance of pink colour shows the **presence** of terpenoids |

**TEST FOR FLAVONOIDS**

| | | |
|---|---|---|
| Shinoda test | : | Appearance of purple colour shows the **presence** of flavonoids |
| Alkaline reagent test | : | Appearance of yellow - orange colour shows the **presence** of flavonoids |
| Acid test | : | Appearance of yellow – orange colour shows the **presence** of flavonoids |
| Zinc hydrochloride test | : | Appearance of red colour shows the **presence** of flavonoids |

**TEST FOR VOLATILE OIL** : Volatile oil obtained shows the **presence** of volatile oil

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

**Table – 8**

**RESULTS FOR THE PRELIMINARY PHYTOCHEMICAL SCREENING OF LEAVES OF *C.limon***

| S.NO | TEST | OBSERVATION |
|---|---|---|
| **I.** | **ALKALOIDS** | |
| | Mayer's reagent | - |
| | Dragondroff's reagent | - |
| | Hager's reagent | - |
| | Wagner's reagent | - |
| | **Test for purine Group(Muroxide test)** | |
| **II.** | **CARBOHYDRATES** | |
| | Molisch's test | + |
| | Fehling's test | + |
| | Benedict's test | + |
| **III.** | **GLYCOSIDES** | |
| | Anthroquinone glycosides | - |
| | Borntrager's test | - |
| | Modified Borndrager's test | - |
| | **Cardiac glycosides** | |
| | Keller Killiani test | - |
| | Raymond test | - |
| | Legal test | - |
| **IV.** | **STEROLS** | |
| | Salkowski test | + |
| | Liberman Burchard's test | + |
| **V.** | **SAPONINS** | + |
| **VI.** | **TANNINS** | _ |
| | Ferric chloride | - |
| | Gold Beater's skin test | - |
| **VII.** | **PROTEINS AND FREE AMINO ACIDS** | |
| | Millon's test | + |
| | Biuret test | + |
| | Ninhydrin test | + |
| **VIII.** | **MUCILAGE** | + |
| **IX.** | **TERPENOIDS** | + |
| **X.** | **FLAVONOIDS** | |
| | Shinoda test | + |
| | Alkali test | + |
| | Acid test | + |
| | Zn/Hcl test | + |
| **XI.** | **VOLATILE OIL** | + |
| **XII.** | **FIXED OIL** | - |

**Table No-9**

**RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE DIFFERENT EXTRACTS OF LEAF *C.limon***

| TEST | Petroleum Ether Extract | Ethanolic Extract | Aqueous Extract |
|---|---|---|---|
| **ALKALOIDS** | - | - | - |
| Mayer's reagent | - | - | - |
| Dragondroff's reagent | - | - | - |
| Hager's reagent | - | - | - |
| Wagner's reagent | - | - | - |
| **Test for purine Group(Muroxide test)** | - | - | - |
| **CARBOHYDRATES** | | | |
| Molisch's test | - | + | + |
| Fehling's test | - | + | + |
| Benedict's test | - | + | + |
| **GLYCOSIDES** | | | |
| Anthroquinone glycosides | - | - | - |
| Borntrager's test | - | - | - |
| Modified Borndrager's test | - | - | - |
| **Cardiac glycosides** | - | - | - |
| Keller Killiani test | - | - | - |
| Raymond test | - | - | - |
| Legal test | - | - | - |
| **STEROLS** | | | |
| Salkowski test | + | + | - |
| Liberman Burchard's test | + | + | - |
| **SAPONINS** | - | + | + |
| **TANNINS** | | | |
| Ferric chloride | - | + | + |
| Gold Beater's skin test | - | + | + |
| **PROTEINS AND FREE AMINO ACIDS** | - | - | - |
| Millon's test | - | + | + |
| Biuret test | - | + | + |
| Ninhydrin test | - | + | + |
| **MUCILAGE** | - | + | + |
| **TERPENOIDS** | + | + | - |
| **FLAVONOIDS** | | | |
| Shinoda test | - | + | - |
| Alkali test | - | + | - |
| Acid test | - | + | - |
| Zn/Hcl test | - | + | |
| **FIXED OIL** | - | - | - |

(+)Indicative Positive Reaction (-) Indicative Negative Reaction

## 5.2.2 FLUORESCENCE ANALYSIS OF POWDERED LEAF

The fluorescence analysis of the leaf powder of *C.limon* was studied. The results were as follows (Table -10**)**

**Table -10**
**FLUORESCENCE ANALYSIS**

| **No** | **Treatment** | **Visible light** | **UV 254 nm** | **UV 365 nm** |
|---|---|---|---|---|
| 1 | Powder | Yellowish Green | Slightly Emerald green | Dark brown |
| 2 | Powder + Ethanol | Yellowish Green | Slightly Greenish yellow | Slightly brown |
| 3 | Powder + Ethyl acetate | Greenish yellow | yellowish green | Greenish brown |
| 4 | Powder + Chloroform | Greenish yellow | Light yellowish green | Light brown |
| 5 | Powder + water | Light Greenish Yellow | Light Yellowish green | Bright brownish black |
| 6 | Powder + 50%H2SO4 | Yellowish Green | Slightly Green | Dark brown |
| 7 | Powder + 10% NaOH | Pale leaf Green | Green | Light brown |
| 8 | Powder + 50% HNO3 | Light Brown | Light green | Dark brown |

## 5.2.3 PHYSICO-CHEMICAL EVALUATION OF ISOLATED VOLATILE OIL

The results of physicochemical analysis, GC-MS analysis were as follows.

1. Percentage oil obtained : 0.5 to 0.8 %
2. Colour : Pale to deep yellow or greenish yellow
3. Odour : Strong, fragnant, aromatic characteristic
4. Taste : Spicy, sour aromatic taste
5. Characteristic feel : Slightly viscous
6. Solubility : Soluble in petroleum ether, toluene, chloroform and ethanol. It is immisible with water.
7. Refractive index at 20ºC : 1,4740 – 1,4755
8. Specific gravity at 20ºC : 0,8560 - 08570
9. Optical rotation at 20ºC : +57 - +65

## 5.2.4 GC-MS PROFILE OF V.O ( Fig.3)

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

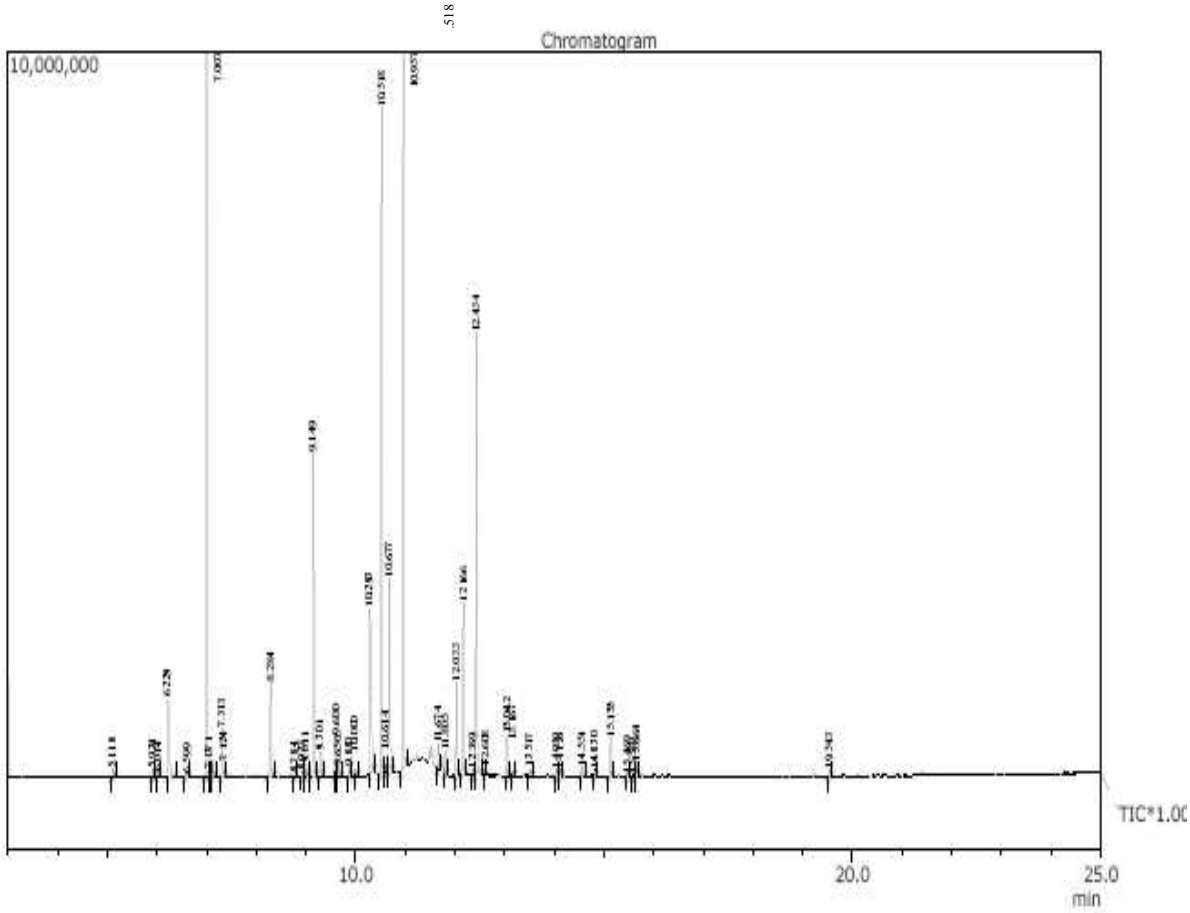
# Fig. 3 GC-MS Profile of VO of *Citrus limon* (L)

Sample Information

Sample Name : NO1201-1482-01

Sample ID : CITRUS-LIMON (L) BURM OF LEAF OIL

Data File : D:\MSDATA\year 2012\Jan-12\19-01-12\NO1201- 1482- 01.QGD



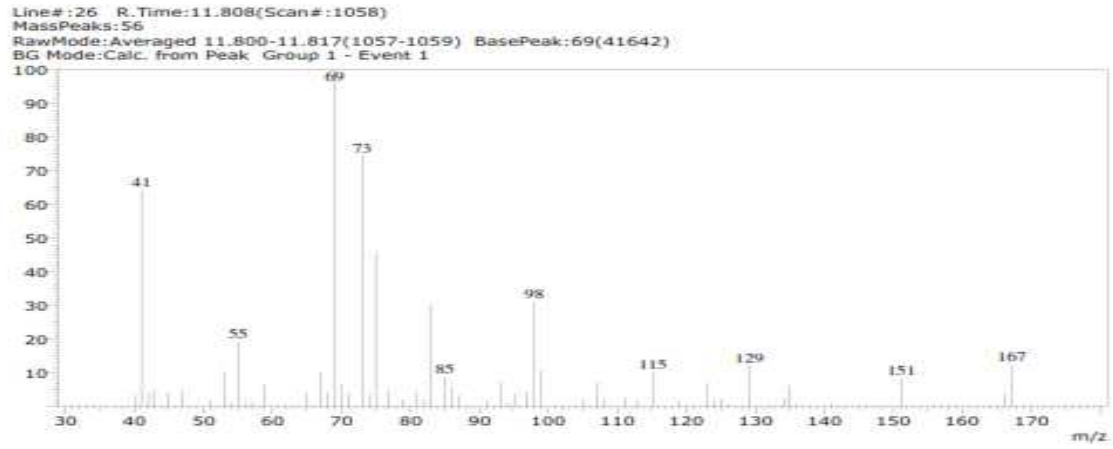
Sample Information

Sample Name : NO1201-1482-01
Sample ID : CITRUS-LIMON (L) BURM OF LEAF OIL
Data File : D:\MSDATA\year 2012\Jan-12\19-01-12\NO1201- 1482- 01.QGD

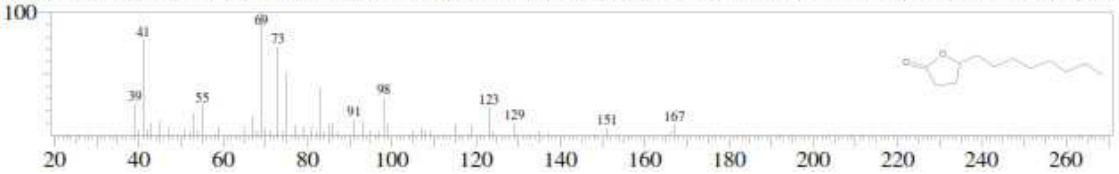
Peak Report TIC

| PEAK# | R.TIME | AREA | AREA% | NAME |
|---|---|---|---|---|
| 1 | 5.118 | 180192 | 0.17 | ALPHA.-(+)-PINENE |
| 2 | 5.924 | 153521 | 0.14 | beta.-Thujene |
| 3 | 6.014 | 119888 | 0.11 | (-)-.beta.-Pinene |
| 4 | 6.229 | 2393317 | 2.25 | 6-METHYLHEPT-5-EN-2-ONE |
| 5 | 6.599 | 84042 | 0.08 | 4-Carene |
| 6 | 7.007 | 35115455 | 33.08 | D-Limonene |
| 7 | 7.071 | 167993 | 0.16 | Eucalyptol |
| 8 | 7.124 | 306733 | 0.29 | trans-.beta.-Ocimene |
| 9 | 7.313 | 1095183 | 1.03 | beta.-cis-Ocimene |
| 10 | 8.284 | 1865585 | 1.76 | beta.-Linalool |
| 11 | 8.784 | 63271 | 0.06 | 3,7-Dimethyl-1,6-octadiene |
| 12 | 8.927 | 139336 | 0.13 | Limonene oxide, trans- |
| 13 | 9.011 | 255985 | 0.24 | 2-Ethyl-6-methyl-1,5-heptadiene |
| 14 | 9.149 | 6188944 | 5.83 | (R)-(+)-Citronellal |
| 15 | 9.301 | 545414 | 0.51 | cis-Verbenol |
| 16 | 9.600 | 927169 | 0.87 | Carane, 4,5-epoxy-, trans |
| 17 | 9.650 | 228352 | 0.22 | Benzyl isonitrile |
| 18 | 9.880 | 189916 | 0.18 | alpha.-Terpineol |
| 19 | 10.000 | 120462 | 0.11 | Capraldehyde |
| 20 | 10.293 | 4437897 | 4.18 | cis-Geraniol |
| 21 | 10.518 | 13378339 | 12.60 | beta.-Citral |
| 22 | 10.614 | 143432 | 0.14 | 2,7-DIMETHYL-2,7-OCTADIENE |
| 23 | 10.677 | 4045402 | 3.81 | trans-Geranial |
| 24 | 10.957 | 15222227 | 14.34 | alpha.-Citral |
| 25 | 11.674 | 673190 | 0.63 | Methyl nerolate |
| 26 | 11.805 | 406466 | 0.38 | GAMMA.-DODECALACTONE |
| 27 | 12.033 | 1824214 | 1.72 | Citronellol acetate |
| 28 | 12.166 | 3537057 | 3.33 | Nerol acetate |
| 29 | 12.369 | 146197 | 0.14 | alpha.-Terpinyl acetate |
| 30 | 12.434 | 8808140 | 8.30 | Geraniol acetate |
| 31 | 12.608 | 157998 | 0.15 | BETA.-ELEMENE |
| 32 | 13.042 | 832438 | 0.78 | Caryophyllene |
| 33 | 13.167 | 115066 | 0.11 | .alpha.-Bergamotene |
| 34 | 13.517 | 154646 | 0.15 | alpha.-Caryophyllene |
| 35 | 14.050 | 172944 | 0.16 | alpha.-Farnesene |
| 36 | 14.126 | 146630 | 0.14 | beta.-Bisabolene |
| 37 | 14.551 | 222800 | 0.21 | 5,12-Dihydroxyergost-25(27)-ene-3,6-dione |
| 38 | 14.830 | 102649 | 0.10 | gamma.-Elemene |
| 39 | 15.133 | 876062 | 0.83 | Caryophyllene oxide |
| 40 | 15.469 | 82269 | 0.08 | 1,3-Hexadiene, 3-ethyl-2,5-dimethyl- |
| 41 | 15.580 | 43355 | 0.04 | Germacrene D-4-ol |
| 42 | 15.664 | 331708 | 0.31 | Spathulenol |
| 43 | 19.543 | 146088 | 0.14 | 2-Decyldecahydronaphthalene |

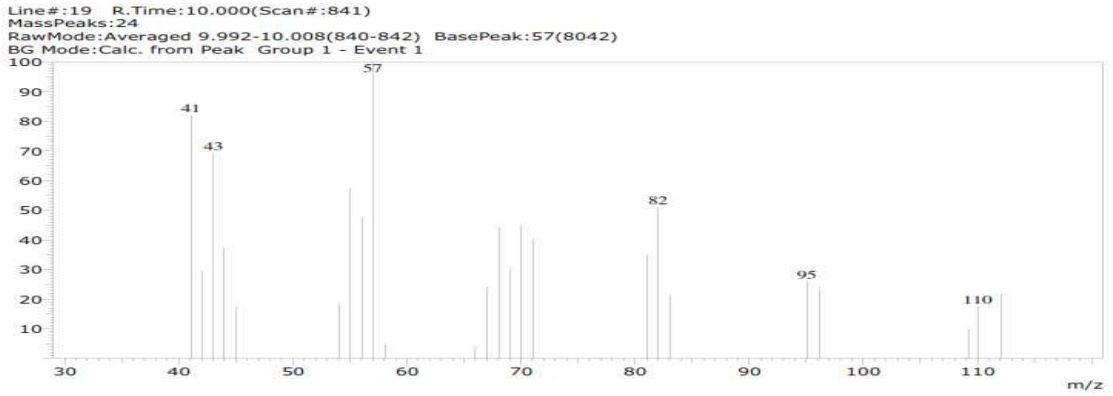
## 1) .Gamma Dodecalactone ( 11.805)



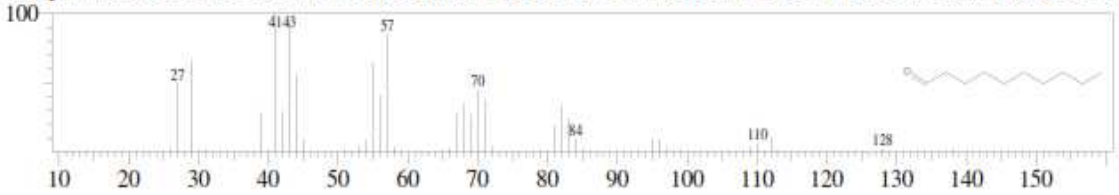

Hit#:1 Entry:101449 Library:WILEY8.LIB
SI:92 Formula:C12H22O2 CAS:2305-05-7 MolWeight:198 RetIndex:0
CompName:5-OCTYLDIHYDRO-2(3H)-FURANONE # $$ .GAMMA. DODECALACTONE $$ .GAMMA.-DODECA



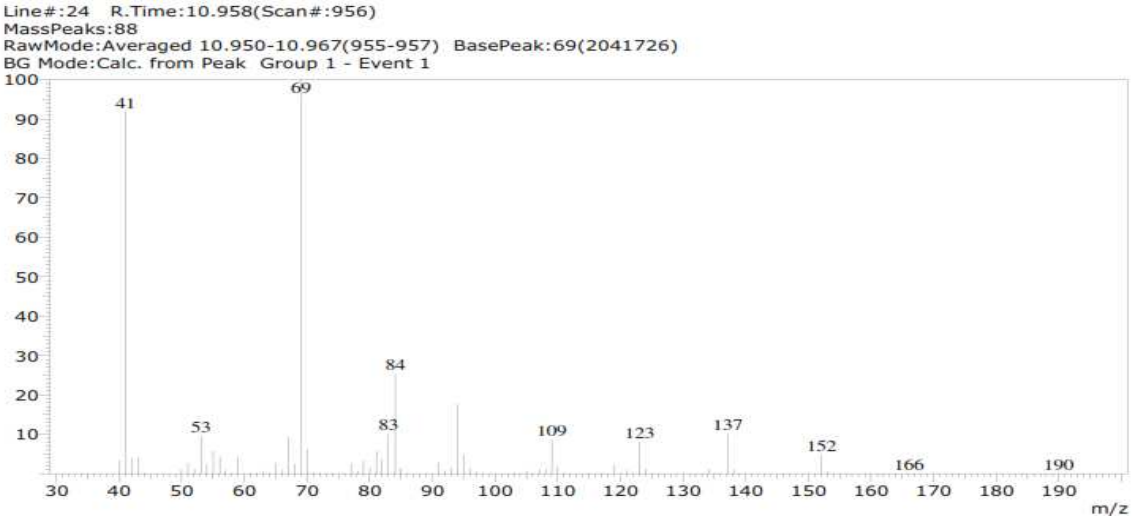
## 2) Capraldehyde (10.00)

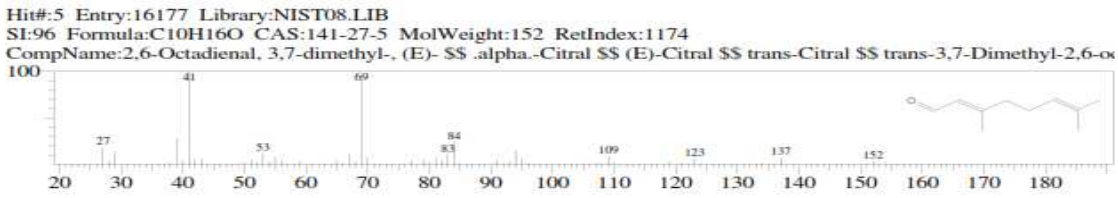



Hit#:5 Entry:49406 Library:WILEY8.LIB
SI:91 Formula:C10H20O CAS:112-31-2 MolWeight:156 RetIndex:0
CompName:DECANAL $$ CAPRALDEHYDE $$ 1-DECANAL $$ 1-DECANAL (MIXED ISOMERS) $$ 1-DECAN

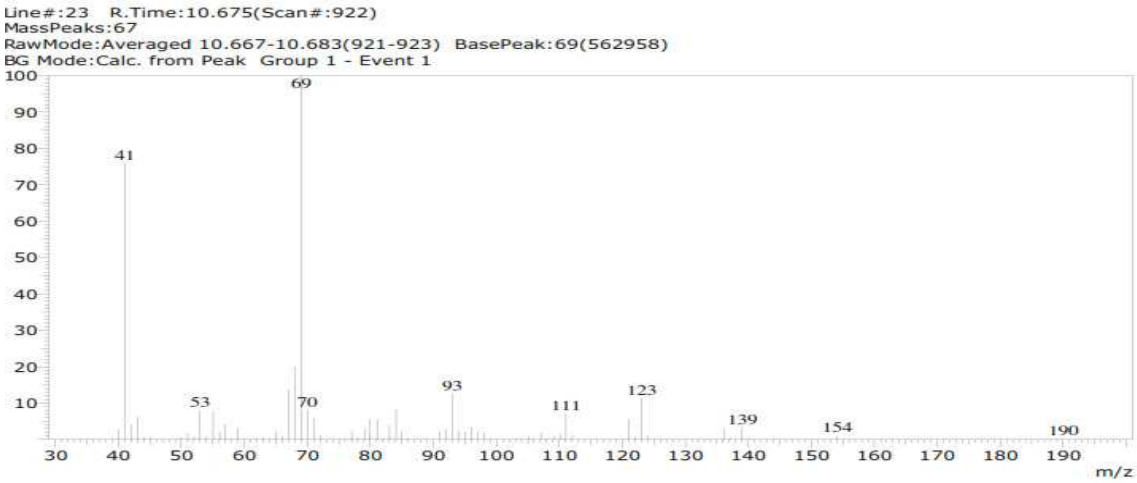


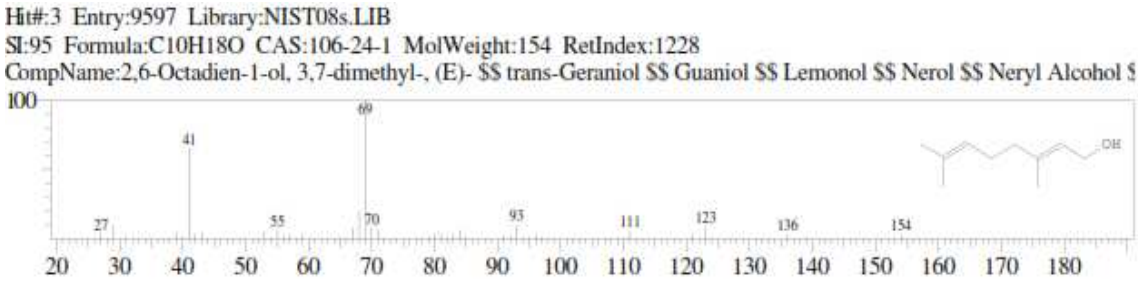
## 3).Alpha Citral (10.957)



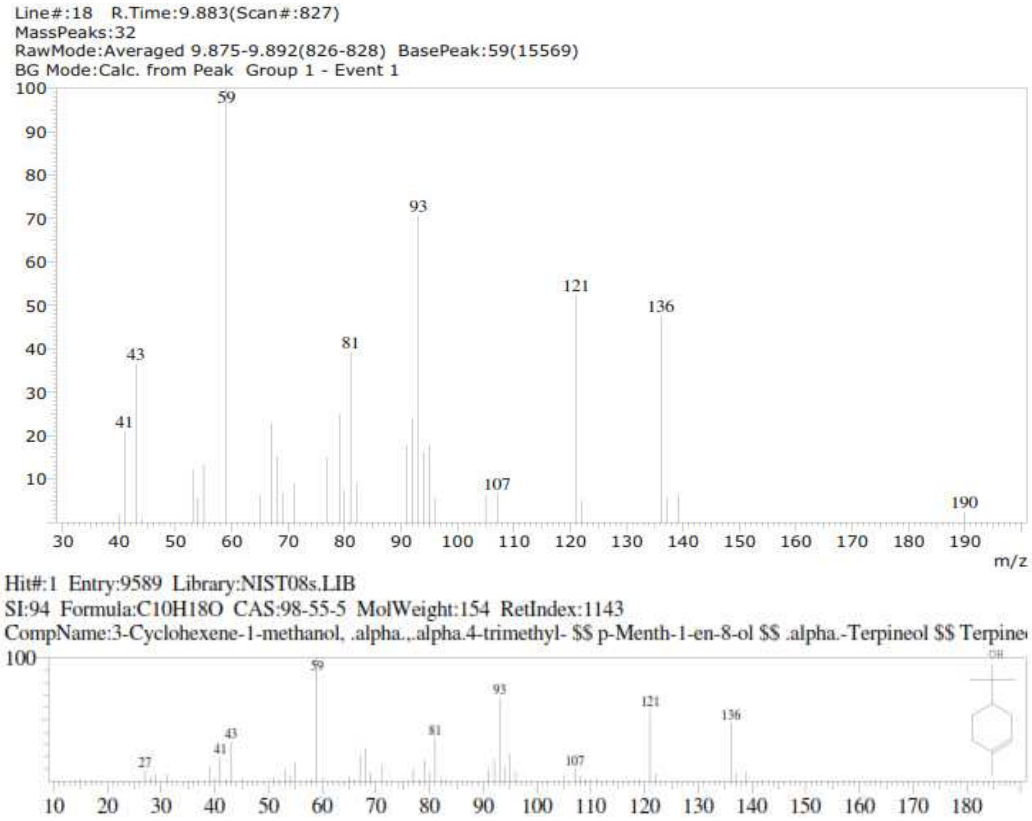




## 4). Trans Geraniol (10.677)

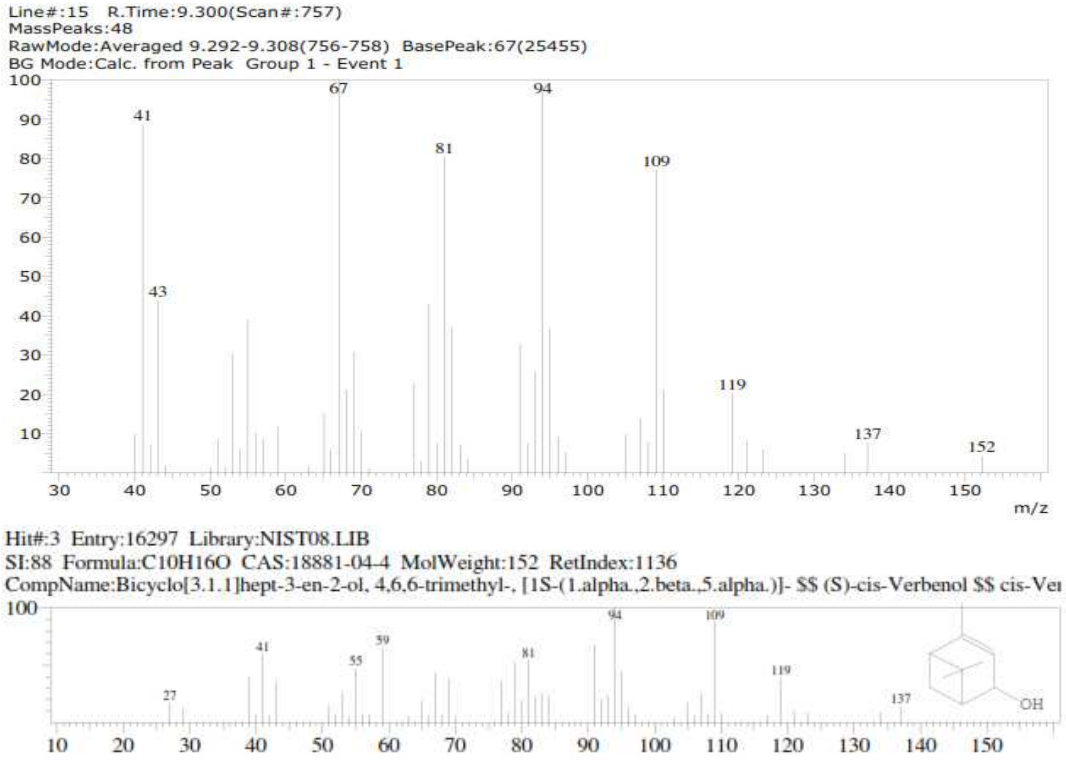


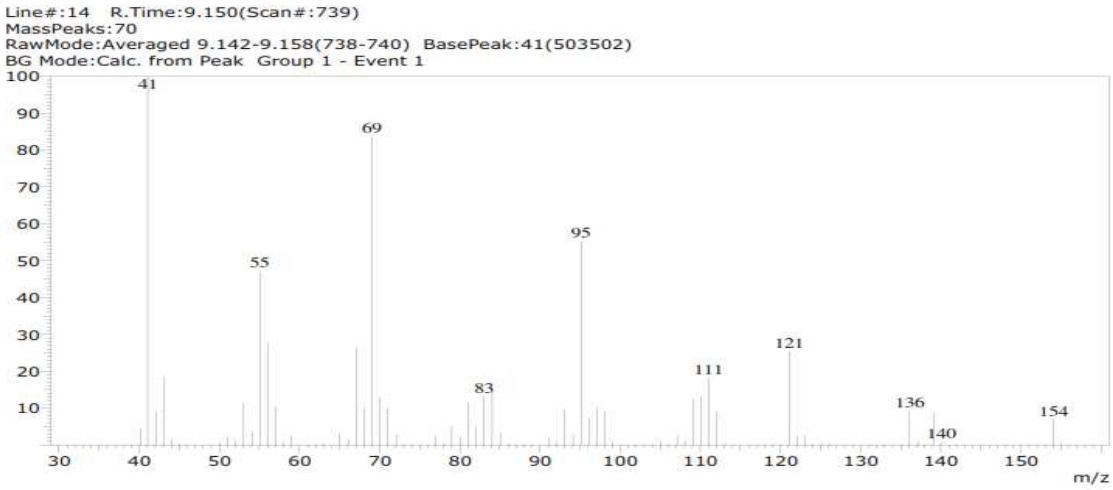
## 5). Alpha Terpineol (9.880)



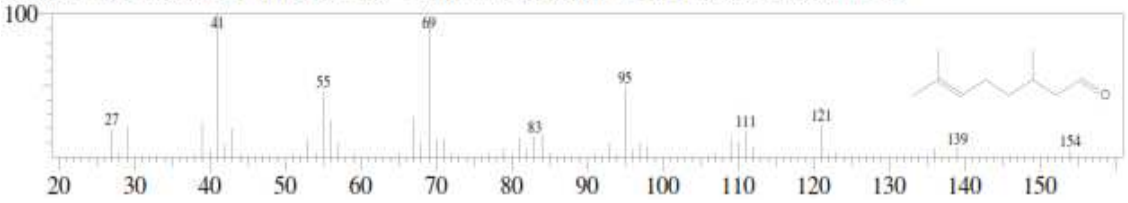

## 6). Cis Verbenol (9.301)



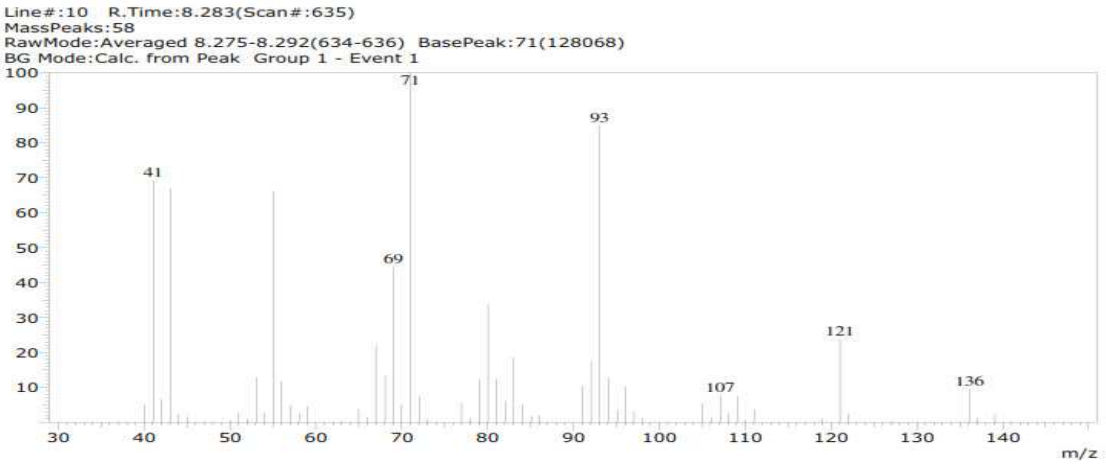
## 7). (R) – (+) Citronellal (9.149)



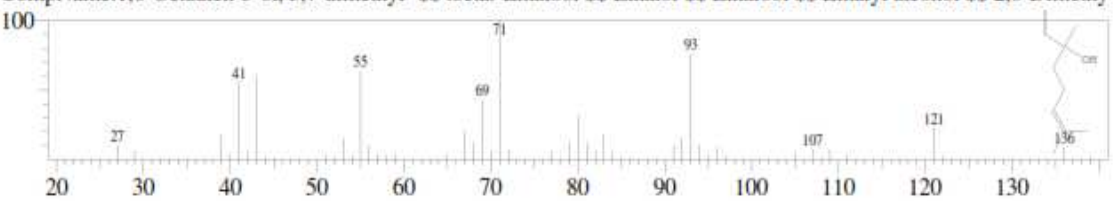

Hit#:1 Entry:17267 Library:NIST08.LIB
SI:98 Formula:C10H18O CAS:2385-77-5 MolWeight:154 RetIndex:1125
CompName:6-Octenal, 3,7-dimethyl-, (R)- $$ (R)-(+)-Citronellal $$ 3,7-Dimethyl-6-octenal # $$



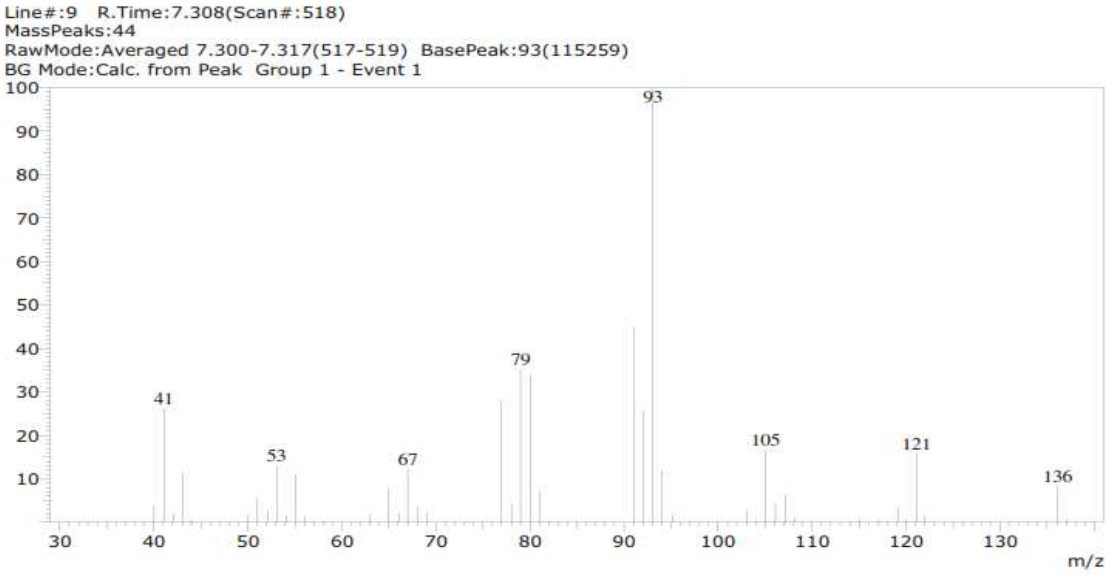
## 8). Beta linalool (8.284)



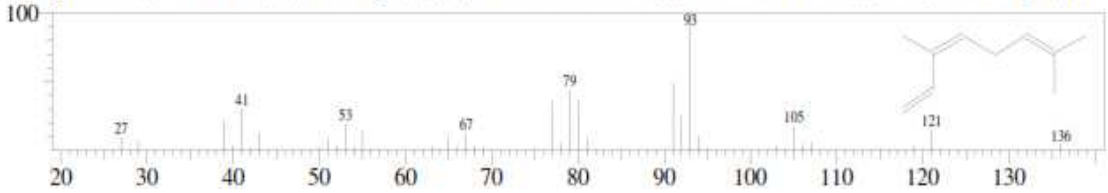

Hit#:1 Entry:17386 Library:NIST08.LIB
SI:98 Formula:C10H18O CAS:78-70-6 MolWeight:154 RetIndex:1082
CompName:1,6-Octadien-3-ol, 3,7-dimethyl- $$ .beta.-Linalool $$ Linalol $$ Linalool $$ Linalyl alcohol $$ 2,6-Dimethy



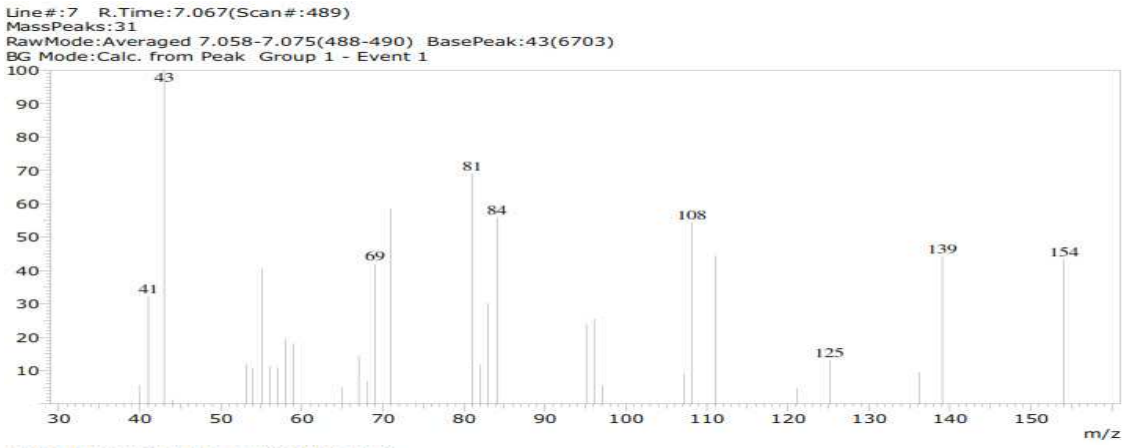
## 9). Beta cis Ocimene (7.313)



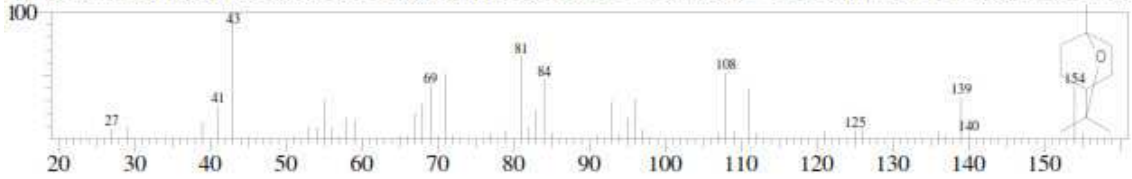

Hit#:2 Entry:6453 Library:NIST08s.LIB
SI:96 Formula:C10H16 CAS:3338-55-4 MolWeight:136 RetIndex:976
CompName:1,3,6-Octatriene, 3,7-dimethyl-, (Z)- $$ .beta.-cis-Ocimene $$ cis-.beta.-Ocimene $$ cis-3,7-Dimethyl-1,3,6-(



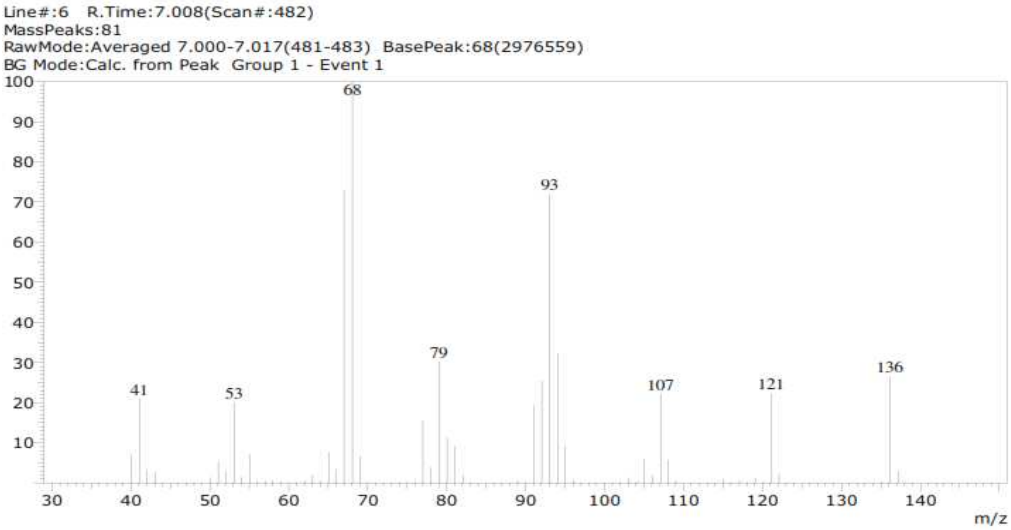
## 10). Eucalyptol (7.071)



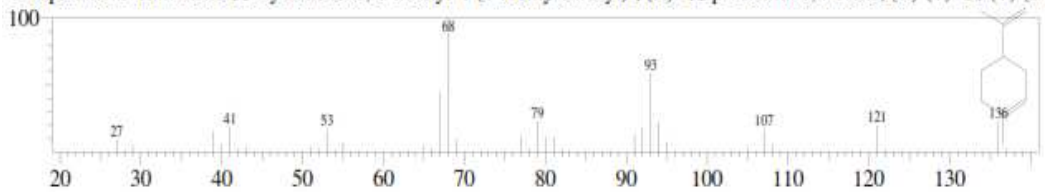

Hit#:4 Entry:9571 Library:NIST08s.LIB
SI:89 Formula:C10H18O CAS:470-82-6 MolWeight:154 RetIndex:1059
CompName:Eucalyptol $$ Cineole $$ 2-Oxabicyclo[2.2.2]octane, 1,3,3-trimethyl- $$ p-Menthane, 1,8-epoxy- $$ p-Cineo



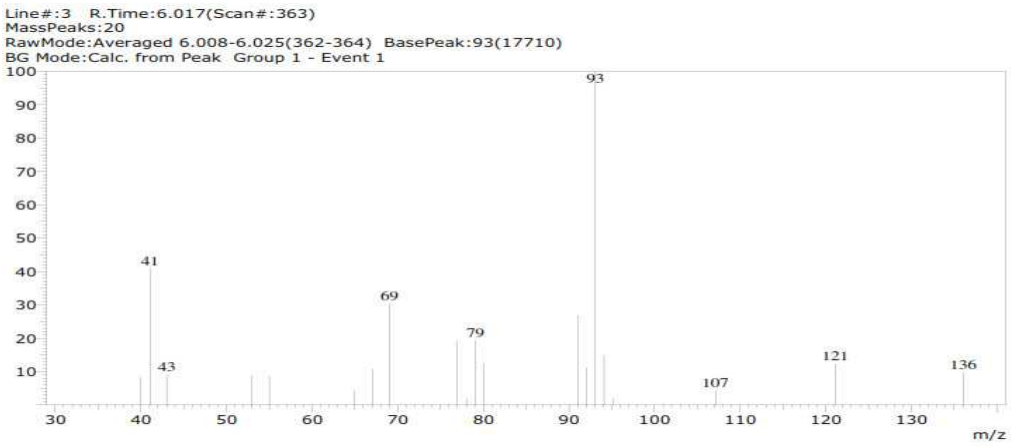
## 11). D limonene (7.007)



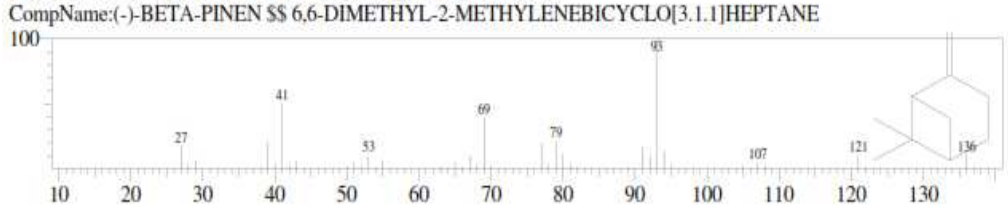

Hit#:3 Entry:9719 Library:NIST08.LIB
SI:95 Formula:C10H16 CAS:5989-27-5 MolWeight:136 RetIndex:1018
CompName:D-Limonene $$ Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (R)- $$ p-Mentha-1,8-diene, (R)-(+)- $$ (+)-(R



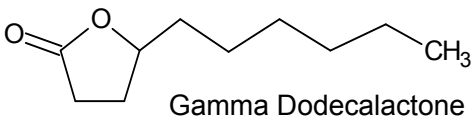
## 12). Beta – (+) – Pinene (6.014)




Hit#:5 Entry:28601 Library:WILEY8.LIB
SI:92 Formula:C10H16 CAS:0-00-0 MolWeight:136 RetIndex:0
CompName:(-)-BETA-PINEN $$ 6,6-DIMETHYL-2-METHYLENEBICYCLO[3.1.1]HEPTANE

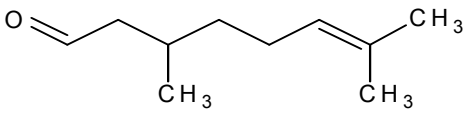


## Structure of compounds identified by GC-MS in V O of *Citrus limon*

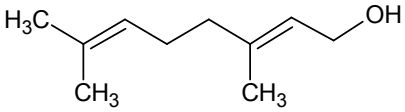


Gamma Dodecalactone

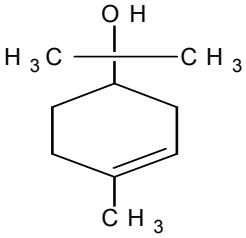
Capraldehyde



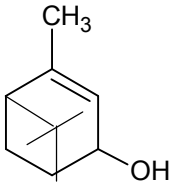
Alpha Citral



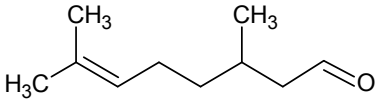
Trans Geraniol



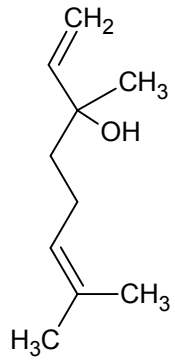
Alpha Terpineol



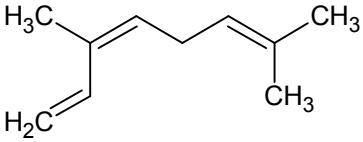
Cis Verbenol



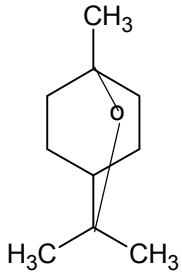
(R)-(+) Citronellal



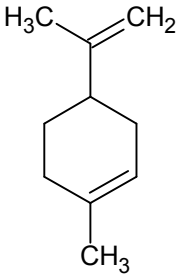
Beta - Linalool



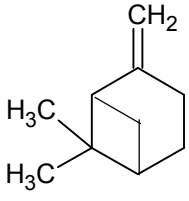
Beta - cis - Ocimene



Eucalyptol



D - Limonene



Beta - (+) - Pinene

## 5.3 PHARMACOLOGICAL STUDIES

### 5.3.1 *IN VITRO* ANTIOXIDANT ACTIVITY

### 1.DPPH Scavenging Activity

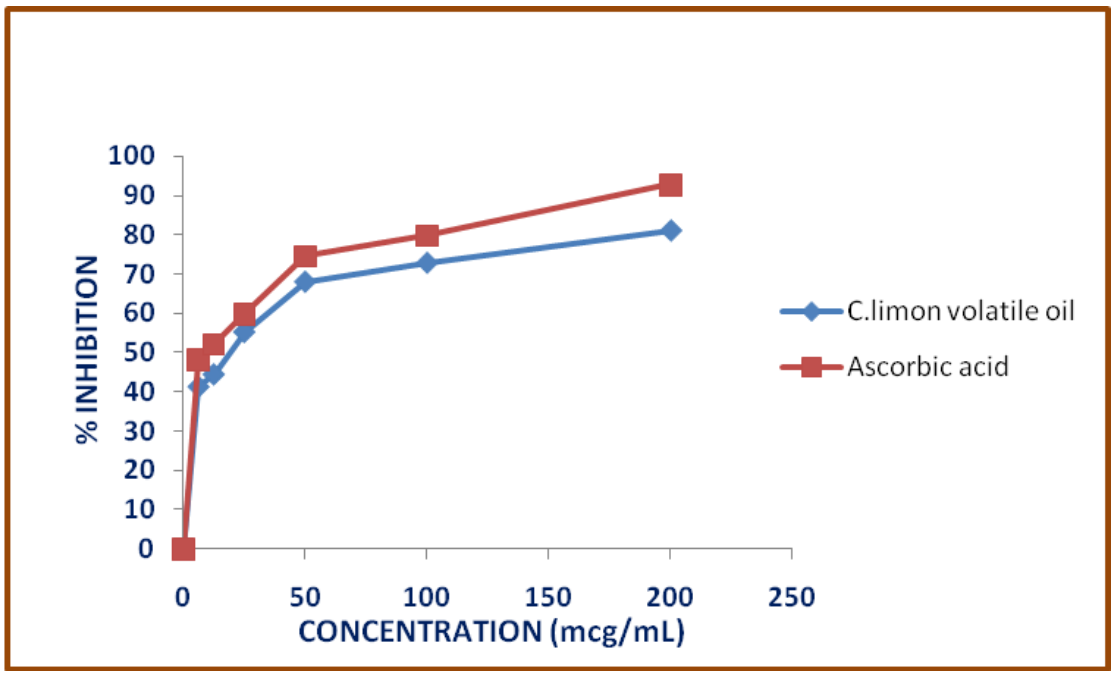
The percentage scavenging of DPPH free radical and $IC_{50}$ value obtained for the Volatile Oil of *Citrus limon* and ascorbic acid are tabulated in the Table 11 and Fig.4. The DPPH Scavening Activity of the volatile Oil was comparable with that the standard ascorbic Acid.

**Table 11: Percentage Inhibition of Volatile Oil of *Citrus limon* and Standard Ascorbic Acid against DPPH at 517nm**

| S. No. | Conc. in µg/mL | Percentage inhibition by standard Ascorbic acid | Percentage inhibition by VOCL |
|---|---|---|---|
| 1 | 6.25 | 48.21 ± 0.27 | 41.36 ± 0.43 |
| 2 | 12.5 | 51.18 ± 0.78 | 44.51 ± 0.69 |
| 3 | 25 | 59.97 ± 1.12 | 55.29 ± 0.83 |
| 4 | 50 | 74.65 ± 0.34 | 68.02 ± 0.82 |
| 5 | 100 | 79.84 ± 0.71 | 72.86 ± 0.63 |
| 6 | 200 | 92.82 ± 1.28 | 81.04 ± 0.76 |
| | **$IC_{50}$** | **30.09 µg/mL** | **49.57 µg/mL** |

*mean of three readings ± SEM

**Fig.4: Free Radical Scavenging of Volatile Oil of *Citrus limon* and Ascorbic Acid against DPPH**




## 2. Nitric Oxide Scavenging Activity Assay

The results obtained for Nitric oxide Scavening assay for Volatile Oil of *Citrus limon* and Standard Ascorbic acid are Tabulated in **Table 12 and Fig 5**

**Table 12: Percentage Inhibition of Ascorbic Acid and Volatile Oil of *C. limon* Against Nitric Oxide at 546nm**

| S. No. | Ascorbic acid | | *C. limon* | |
|---|---|---|---|---|
| | Conc. in µg/mL | Percentage inhibition by standard Ascorbic acid | Conc. in µg/mL | Percentage inhibition by Volatile Oil |
| 1 | 22.22 | 47.19 ± 0.53 | 2.78 | 41.57 ± 0.92 |
| 2 | 44.44 | 54.31 ± 0.81 | 5.56 | 52.43 ± 2.01 |
| 3 | 88.89 | 60.67 ± 1.06 | 11.11 | 65.92 ± 2.61 |
| 4 | 177.78 | 65.92 ± 1.33 | 22.22 | 71.16 ± 2.91 |
| 5 | 222.22 | 84.64 ± 1.33 | 44.44 | 94.01 ± 2.14 |
| | $IC_{50}$ | **84.56 µg/mL** | | **11.74 µg/mL** |

***mean of three readings ± SEM**

The $IC_{50}$ value for volatile oil was found to be **11.74 µg/mL** while for ascorbic acid it was **84.56 µg/mL** which indicates that the volatile oil had a very potent nitric oxide scavenging activity.

**Fig. 5: Nitric Oxide Radical Scavenging By Ascorbic Acid**

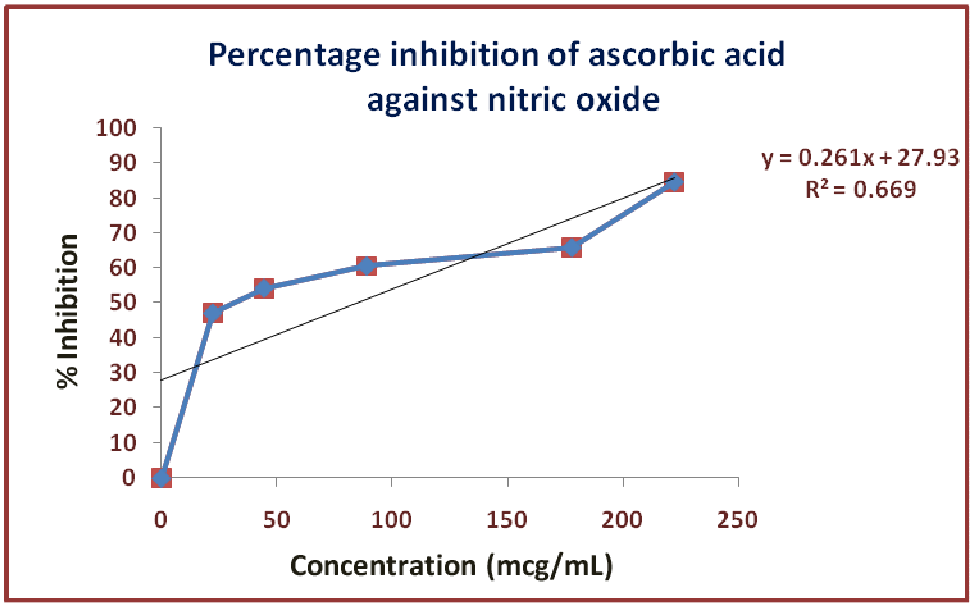
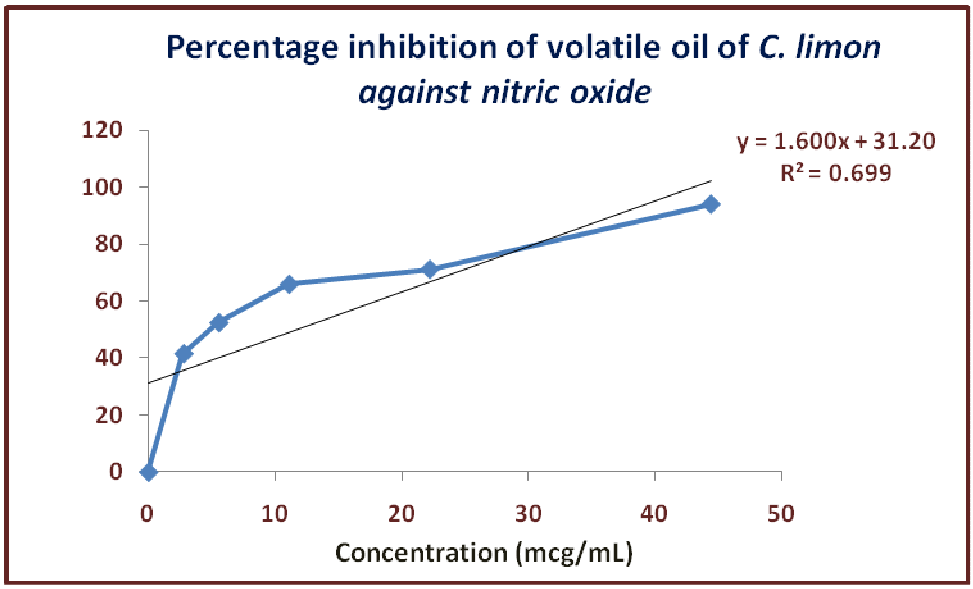



**Fig. 5: Nitric Oxide Radical Scavenging By Volatile Oil of *C. limon***



### 3.Ferric Reducing Power Antioxidant Assay

The result of the Ferric Reducing Power Assay for Volatile Oil of *Citrus limon* and Standard Ascorbic Acid are presented in **Table 13 and Fig.6.**

**Table – 13 : Total Ferric Reducing Power Assay of Ascorbic Acid and VOCL**

| Ascorbic acid | | VOCL | |
|---|---|---|---|
| **Concentration (µg/mL)** | **Absorbance*** | **Concentration (µg/mL)** | **Absorbance*** |
| 10 | **0.243 ± 0.003** | 12.5 | **0.252 ± 0.003** |
| 20 | **0.278 ± 0.005** | 25 | **0.266 ± 0.002** |
| 30 | **0.362 ± 0.008** | 50 | **0.280 ± 0.003** |
| 40 | **0.413 ± 0.003** | 100 | **0.296 ± 0.003** |
| 50 | **0.436 ± 0.006** | 200 | **0.317 ± 0.004** |
| | | 400 | **0.410 ± 0.010** |

*** Mean of three readings ± SEM**

**Fig. 6: Reducing Power Assay of Ascorbic Acid on Potassium Ferricyanide**

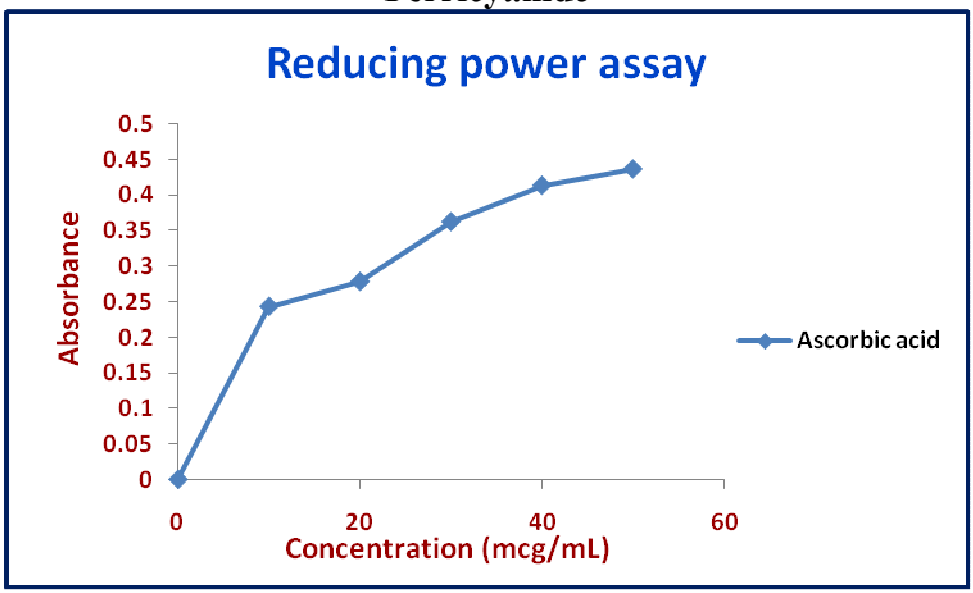
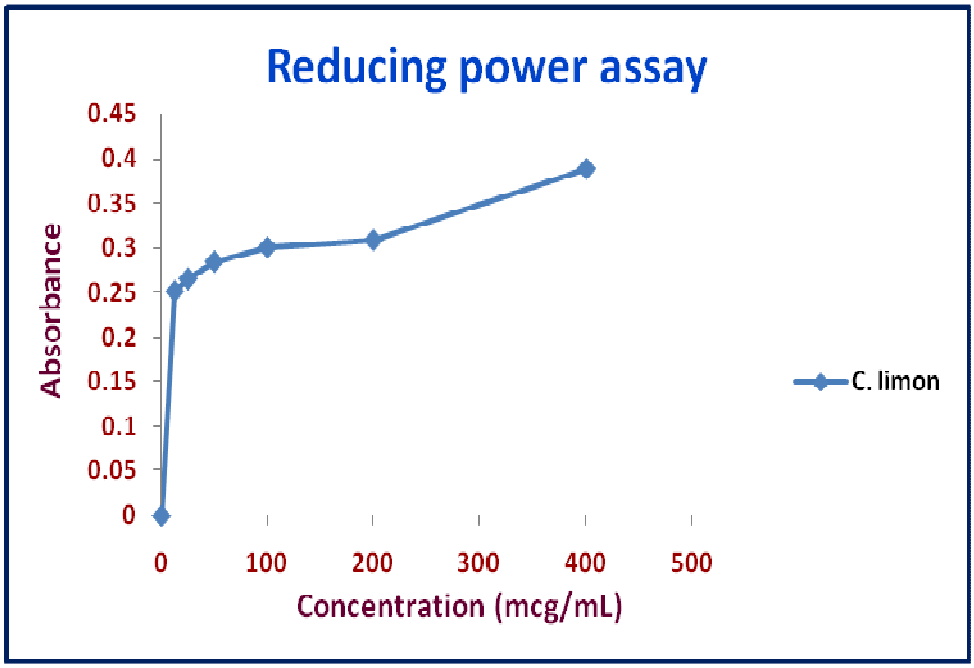


**Fig. 6: Reducing Power Assay Volatile Oil of *C. limon* on Potassium Ferricyanide**




The absorbance for volatile oil was found to be **0.410 ± 0.010** for a concentration of **400µg/mL** while for ascorbic acid it was **0.436 ± 0.006** at a concentration **of 50µg/mL.** An increase in absorbance indicates an increase in reducing power of the volatile oil.

### 5.3.2 ANTIBACTERIAL ACTIVITY

The results obtained for the susceptibility tests of the volatile oil against various Microorganisms are presented in **Tables 14 to 16** and photographic documentation pertaining to this are presented in **Figs.7 to 10**. From the **Table 14,** it can be seen that there was no growth against all the tested micro-organisms at a concentration of 15µL/disc.

**Table 14: Susceptility tests of Volatile Oil of *C.limon* for various Microorganisms**

| S.No. | Name of the drug | Concentration (µL/disc) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | **Control (DMSO)** | **------** | + | + | + | + | + | + | + | + |
| ***2*** | **Standard (Amikacin)** | **--------** | + | + | + | + | + | + | + | + |
| ***3*** | **Volatile Oil of *Citrus Limon*** | **5µL** | _ | _ | _ | _ | + | + | + | _ |
| | | **10µL** | _ | + | _ | + | + | + | + | + |
| | | **15µL** | + | + | _ | + | + | + | + | + |

**NOTE:- (+)** indicates growth**; (-)** indicates no growth

1.*Chromobacterium violaceum*, 2.*Escherichia coli***,** 3.*Klebsiella pneumonia*, 4.*Proteus Mirabilis*, 5.*Shigella Flexneri*, 6.*Streptocoocus pyogenes*, 7.*Staphyolococcus aureus*, 8.*Pseudomonas aeruginosa*

**Minimum Inhibitory Concentration (MIC)**

Minimum Inhibitory Concentration was defined as the lowest concentration of the volatile oil that allows no more than 20% growth of microbes after incubation on agar at 37ºC for 18-48 hrs.

**Table 15: MIC of VOCL against various microorganisms**

| S. No. | Name of the microorganism | Minimum inhibitory concentration (µL/disc) |
|---|---|---|
| 1 | *Chromobacterium violaceum* | 15 |
| 2 | *Escherichia coli* | 15 |
| 3 | *Klebsiella pneumonia* | - |
| 4 | *Proteus Mirabilis* | 15 |
| 5 | *Shigella Flexneri* | 15 |
| 6 | *Streptococcus pyogenes* | 15 |
| 7 | *Staphyolococcus aureus* | 15 |
| 8 | *Pseudomonas aeruginosa* | 15 |

From the **Table 15,** it can be observed that the MIC for *Chromobacterium violaceum*, *Escherichia coli***,** *Klebsiella pneumonia*, *Proteus Mirabilis Shigella Flexneri*, *Streptocoocus pyogenes*, *Staphyolococcus aureus* and *Pseudomonas aeruginosa* MIC was15µL /disc.

**Table 16: Antibiotic disc diffusion assay against various microorganisms**

| S. No. | Name of the microorganism | Zone of inhibition (mm)* | |
|---|---|---|---|
| | | **Standard** | **VOCL** |
| **1** | *Chromobacterium violaceum* | 20 ± 0.01 | 12 ± 0.02 |
| **2** | *Escherichia coli* | 22 ± 0.05 | 26 ± 0.06 |
| **3** | *Klebsiella pneumonia* | 24 ± 0.04 | -- |
| **4** | *Proteus Mirabilis* | 26 ± 0.02 | 26 ± 0.04 |
| **5** | *Shigella Flexneri* | 24 ± 0.02 | 12 ± 0.02 |
| **6** | *Streptococcus pyogenes* | 24 ± 0.06 | 24 ± 0.05 |
| **7** | *Staphyolococcus aureus* | 21 ± 0.00 | 24 ± 0.03 |
| **8** | *Pseudomonas aeruginosa* | 22 ± 0.08 | 22 ± 0.06 |

***means of 2 readings ± SEM**

The results obtained for the antibiotic disc diffusion technique are presented in **Table 16** and **Fig 7 to 10**. From the **Table 16**, it can be observed that the zones of inhibition of the volatile oil of *Citrus limon* for the tested organisms was less than that produced by the standard Amikacin.

From the above study, the volatile oil of *Citrus limon* inhibited the growth of above tested organism at a concentration of 15µL/disc and also the volatile oil of *Citrus limon* were more potant activity against the above microorganisms and standard drug Amikacin.

# CHAPTER – 6

# DISCUSSION

The dissertation covers a study on the easily available Rutaceae family plant botanically known as *Citrus limon(* L) and commonly called as sour orange. The leaves of *C.limon ,* there is no comparetion and cheapist natural and easily available plant. It is traditionally known to be useful for the treatment of wide panel of diseases like stomach ache, vomiting, carminative, excellent refrigerant drink and culinary uses etc.In acute rheumatism and rheumatic gout, in some forms of acute tropical dysentery and diarrhoea,etc. it has been successfully employed. As an antidote to some acro-norcotic poisons, it often proves effectual. *Lemon* juice and gun powder used topically for scabies.The bark and root has been used in the West Indies as a febrifuge and the seeds as a vermifuge. Rind of ripe fruit used for stomach, carminative jucie of ripe fruit used for refrigerant in scurvy. And used for culinary purpose for food items and cosmetic purpose (essention oil, perfume soap, powder etc.) This plant is very popular in India and widely cultivated. Fruits used for jucies in cool drinks and pickles and Hindu poojas. The economic aspect of this crop evidently proved that it is a commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored as well. By a well coordinated effort, we can exploit properly for 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 harmful wastage of this plant 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 agroeconomic 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 of *C. limon*.

**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 micromorphology 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. Leaves are dorsiventral with prominent midrib, long elliptic or ovate obtuse, acute or acuminate and petioles naked or winged. In transactional view it is broad circular shape with prominent elevated round adaxial side and comparatively less thick abaxial side. The upper epidermis is apostomatic, polygonal in surface view with smooth cuticle. The lower epidermis was thick papillate surface due to cuticular outgrowth. Cyclocytic stomata were present where the stoma was encircled by three inner whorl, 5 or 6 outer whorl of subsidiary cells. 3 or 4 layers of collenchyma were present beneath both the epidermis. Large double stranded vascular bundles were seen. Both strands have several xylem and phloem surrounded by lignified sclerenchyma fibres. Large smooth compact parenchyma cells were present in ground tissue.

A characteristic three layers of short palisade cells and wide abaxial zone of compact layers of spongy parenchyma in the middle part were observed. Wide circular secretory cavities surrounded by fairly thick spindle shaped epithelial cells with amorphous inclusion were present. A exclusively abundant crystals distributed in sub epidermal layers of adaxial epidermis were

noticed. The veins and vein islets were thick and prominent. Polygonal vein islets with thick vein boundaries and distinct dendroid vein termination were present. The outline of petiole was almost round. Flat on abaxial side and semicircular on the abaxial side. Secretory cavities, more in adaxial side. Crystals were present in normal cells not modified into idioblast unlike in lamina was characteristic feature. The scanning electron microscopy study showed the above structures in 3D view.

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, we have observed parenchyma cells, epidermal cells with cyclocytic stomata, secretary cavities, crystal idioblast, collenchyma, fibes, xylem, phloem were noticed.

Quantitative microscopy includes certain measurements to distinguish some closely related species which are not easily differentiated by general microscopy. The **stomatal number** (Table No.2) 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 numbers** 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(Table No.1).

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 identify 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 favors spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles (Table -4).

**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 (IP

1996 and BP 1980, BHP 1990 etc.), 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 -6, 7).

**Loss on drying** at 105$^{o}$C is determined as the presence of excess moisture is conductive to the promotion of mold and bacterial growth, and subsequently to deterioration and spoilage of the drug (Table -5)

The preliminary phytochemical screening reveals the presence of carbohydrates, proteins and amino acids, flavonoids, terpenoids, tannins, saponin, volatile oil and phytosterols and coumarin glycosides. Alkaloids, fixed oil were found to be absent. (Table -8,9)

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 flavanoids and flavones and some

chalcones), dark brown (most flavanol glycosides, dark mauve (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 - 10) (Harborne JB 1973).

We have isolated one of the most important constituent of the leaf volatile oil (0.5-0.8%). The yield of the oil depends upon the type, situation of the place in the stem, time of collection, method of isolation, nature of the material distilled, fresh or dried, bleached or unbleached etc. The physical characters like solubility, refractive index, specific gravity, optical rotation were determined and presented.

The isolated VO was subjected to GC-MS analysis and the presence of the following constituents was found (Fig 3). 43 peaks were identified. The important known major constituents were Gamma Dodecalactone, α Citral, trans Geraniol, Capraldehyde, α Terpineol, cis Verbenol, (R)(+)Citronellal, β Linalool, β cis Ocimene, Eucalyptol,D Limonene, β Pinene etc.

Resistance to existing antimicrobial agent has resulted in treatment failures and enhancement in health care costs. Now there is no doubt that emerging antibiotic resistance is a serious problem. The association between increased rates of antimicrobial use along with the resistance has been documented for nosocomial infections and resistant community acquired infections. The study of medicinal properties of plants and their extracts are as old as medicine itself. Curative properties of plant extracts have been studied from the ancient time for various ailments. But it is only recently that a really

scientific mode of action based upon in depth study on the efficacy of various plant molecules has been investigated. (Sharma R.K, 2006). Essential oils are widely used in medicine, in the food industry as flavouring additives and also in cosmetics as fragrances. Many of the volatile oils are known to exert antimicrobial activity, but the mechanism of action is often not entirely understood. Membrane disruption by the lipophilic constituents may be involved in the mechanism. It was already proved that increase of hydrophiliicity of kaurene diterpenoids by the addition of a methyl group reduced their antimicrobial effect. (Schelz *et al*., 2006).

Plants have an almost limitless ability to produce aromatic substances, most of which are phenols or their oxygen substituted derivatives. In many case these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some like terpenoids give plants their odors other are responsible for plant pigments. The phenolics and its derivatives were reported to be effective against viruses, bacteria and fungi. For e.g. catechol and pyrogalllol both are hydroxylated phenols, shown to be toxic to the microorganisms. The site, number of hydroxyl group on the phenol groups are thought to be related to their relative toxicity to the microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity include enzyme inhibition, possibly through reaction with sulfhydryl groups or through more non specific interactions with the proteins. (Sharma.R.K 2006).

The above points prompted us to investigate the volatile oil isolated from the leaves of *C.limon* is a easily and nearly available and harmful wasted part of it. Its medicinal applications are still to be explored well. So we have initiated a research for exploring the unique potential or the VO of the leaves to minimize the harmful wastage and to maximize the revenue generated by this crop to boost up our Indian economy as well as the proper exploitation of this plant for therapeutic purpose. By investigating its bioactivity of its VO we can meet the senorio of unsettling facts of modern pharmaceutical industry which facing lately its pipeline of new drug discovery seems to be almost empty.

The *in vitro* antibacterial activity on the volatile oil was carried and the volatile oil was found to be active all the organisms tested at a very low concentration. It was effective against both gram positive and gram negative bacteria. The volatile oil was also possessed a very potent antioxidant property and the combination of the antioxidant and antibacterial activity throws light the use of the volatile as an antibacterial agent.

# CHAPTER-7

# CONCLUSION

This dissertation is entitled "**Pharmacognostic, phytochemical and Pharmacological evaluation of *Citrus limon* (Linn.) Burm f.** *Citrus limon(*L)Burm.f. is a easilyavailable plant. The ethnomedical information revealed that it was used in various diseases for long period in the world.

Now a days economic potentiality of this cash crop remains neglected by the researchers, physician, traders, farmers etc.

The morphological evaluation showed the adherence of general character to the family.

Detailed microscopical characters of the leaves showed the presence of secretory cells, crystals specifically under the upper epidermis,three layers of well defined palisade cells, spongy mesophyll, vascular bundles in the midrib region and calcium oxalate druses in the petiole. Quantitative microscopic parameters were studied which will help in the identity and purity of a plant.

Preliminary phytochemical screening and quantitative estimation of phytoconstituents throws light on the type of secondary metabolites present in the plant and the quantification helps in determining the amount of certain type of secondary metabolities which may be responsible for the pharmacological activity. The preliminary phytochemical screening showed the presence of carbohydrates, proteins and amino acids, flavonoids, saponin, terpenoids, tannins, phytosterols, musilage and volatile oil. There is no fixed oil.

The volatile oil was isolated from the fresh leaves of *Citrus limon.*The physical parameters were studied and presented. The GC-MS profile of the isolated VO was studied and the presence of forty three compounds were identified. The known constituents were Gamma Dodecalactone, α Citral, Trans Geraniol, Capraldehyde, α Terpineol, cis Verbenol, (R)(+) Citronellal, β Linalool, β cis Ocimene, Eucalyptol, D Limonene, β Pinene etc.

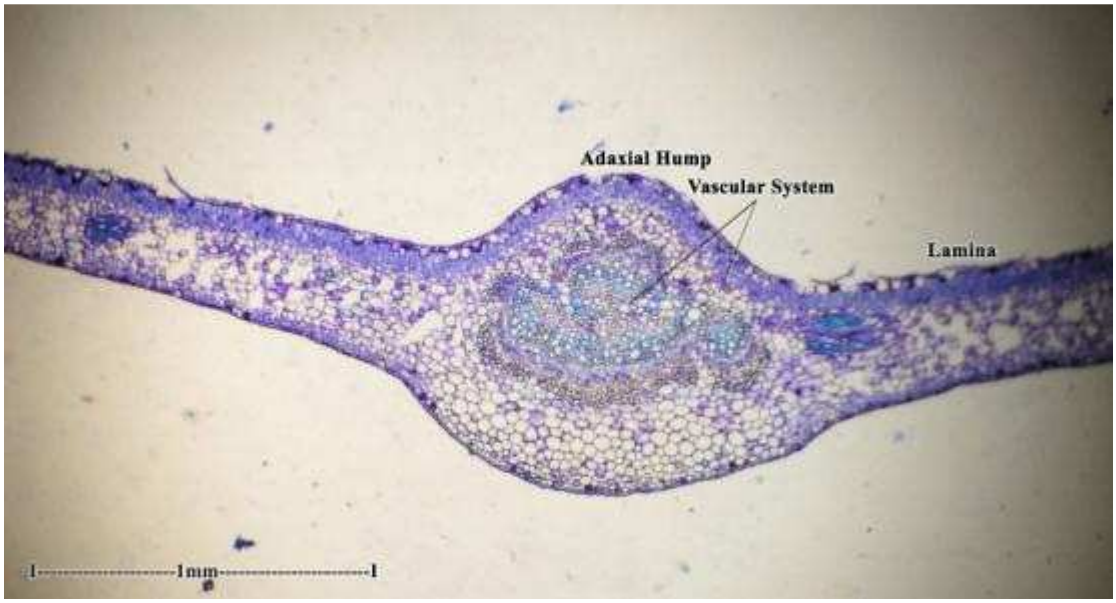
Pharmacological activity of the plant has showed that the volatile oil of *Citrus limon* possessed good antioxidant properties. A very good *in vitro* antioxidant activity against DPPH and nitric oxide Scavenging etc and reducing power assay may be attributed to the presence of polyphenolic and flavonoid compounds. The antibacterial activity has shown that the volatile oil has a broad spectrum of activity against the tested gram positive and gram negative organisms(human pathogens)like *E.coli, Steptococcai*,

We have **3 R's** ethical principle (**R**eduction, **R**efinement, **R**eplacement) implemented that will help to minimize harm to vertebrate animals used in pharmacological screening activities.

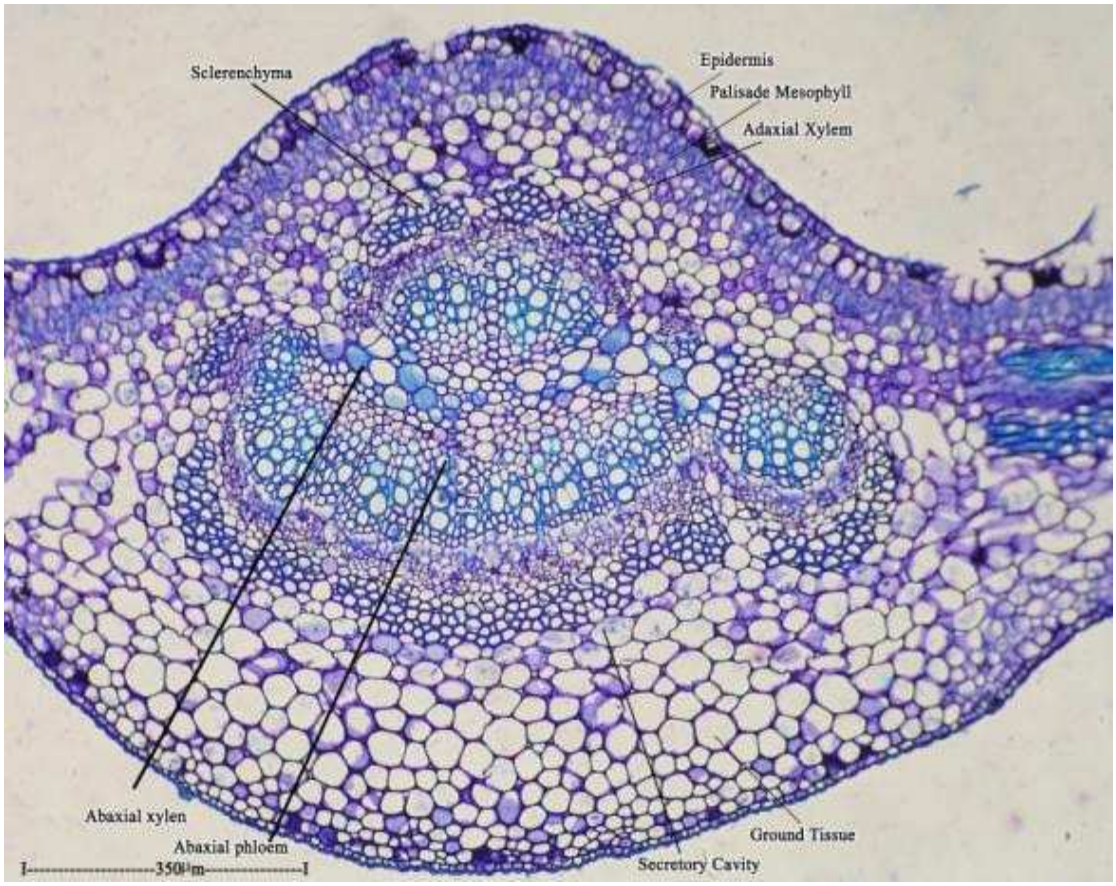
**-JAI HIND-**

# PLATE NO: 6

## T.S. OF THE LEAF OF *C.limon* (L) THROUGH THE MIDRIB (4X)

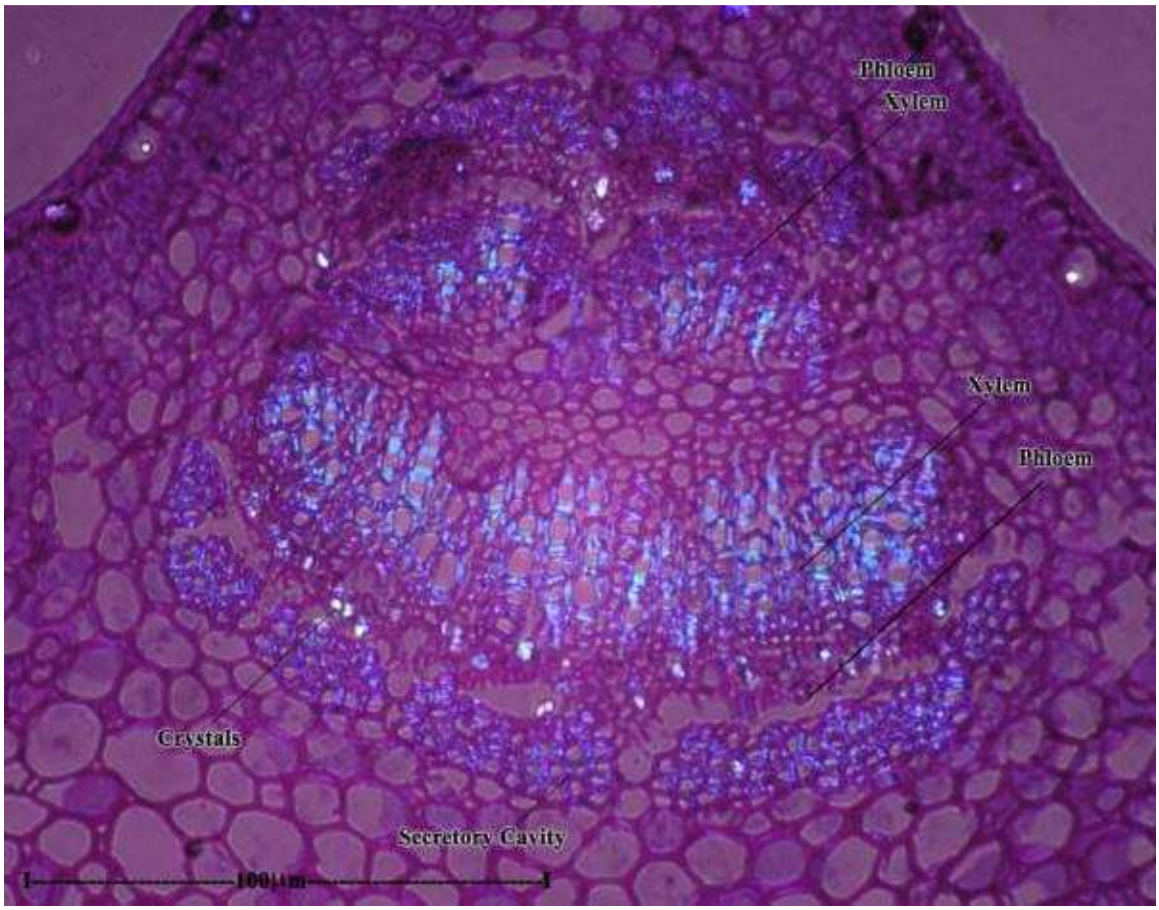



## MIDRIB ENLARGED (10X)



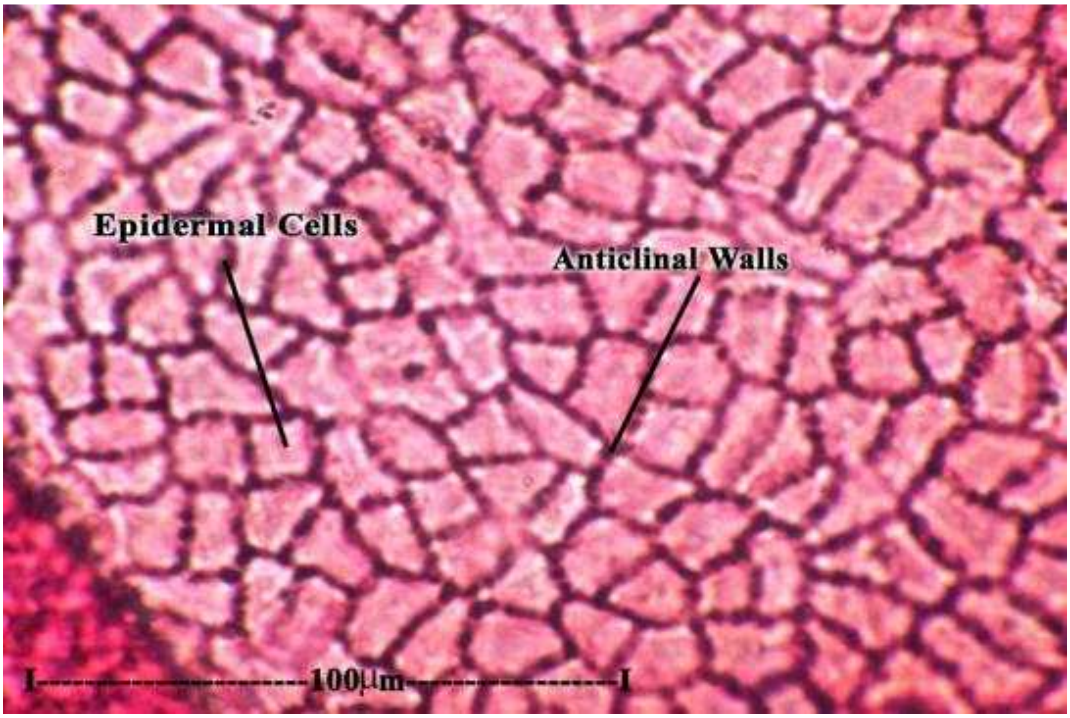
# PLATE NO: 7

## MIDRIB UNDER POLARISED LIGHT (15X)

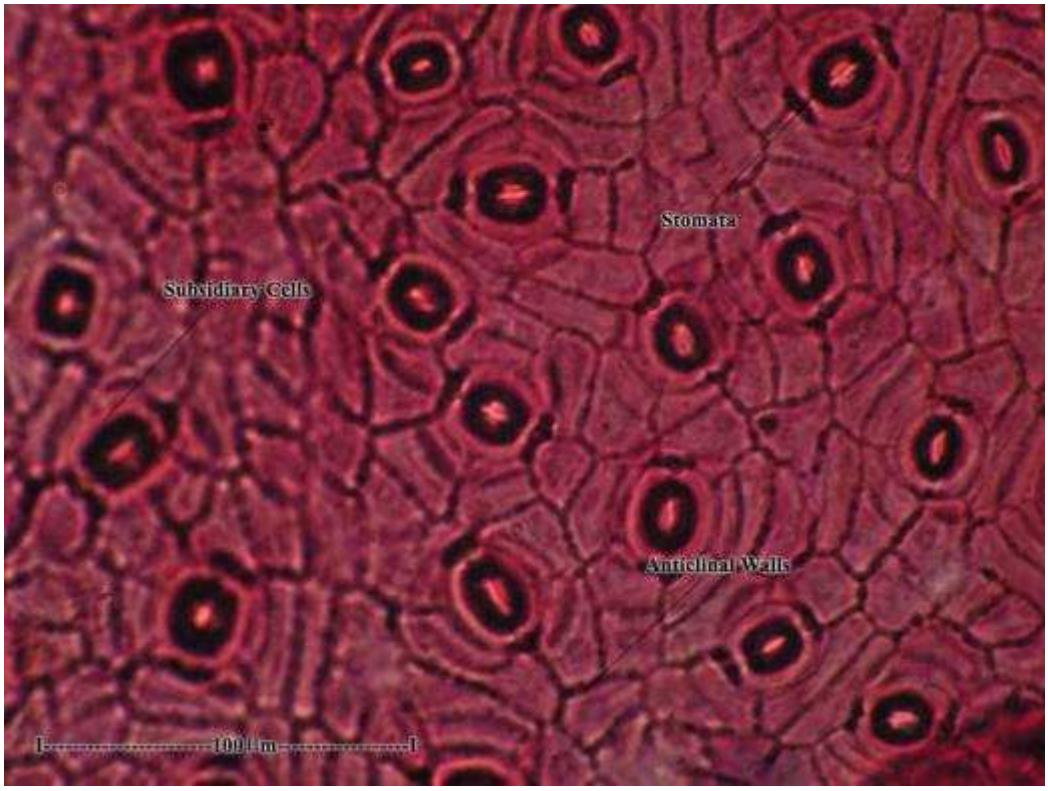


# PLATE NO: 8

## ADAXIAL EPIDERMIS (SURFACE VIEW) (40 X)

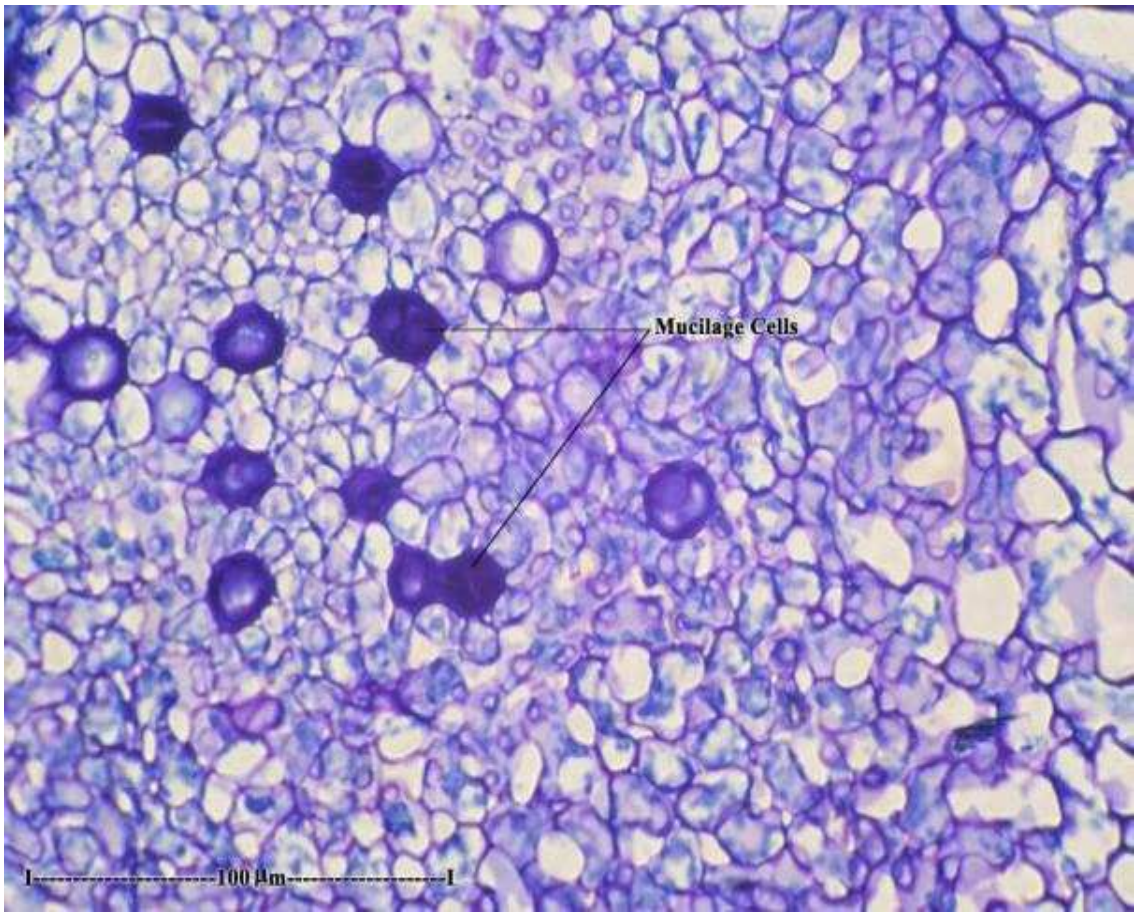



## ABAXIAL EPIDERMIS (SURFACE VIEW) (40 X)



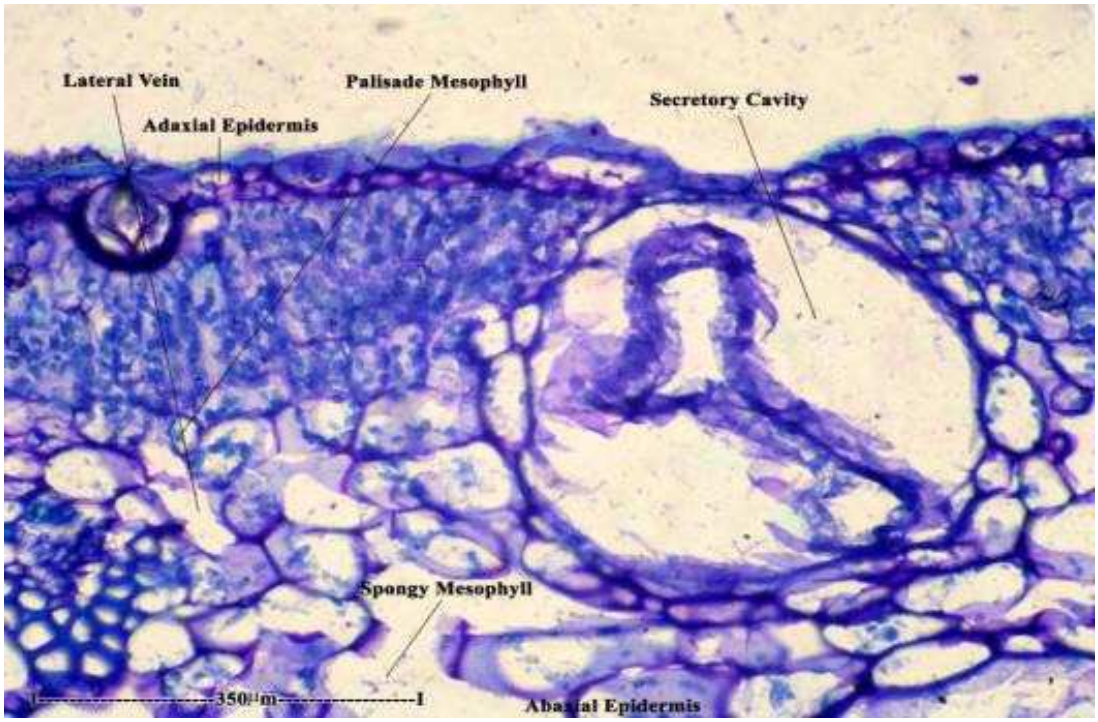
# PLATE NO: 9

## ADAXIAL SUBEPIDERMAL MUCILAGE CELLS IN PARADERMAL SECTION (40X)

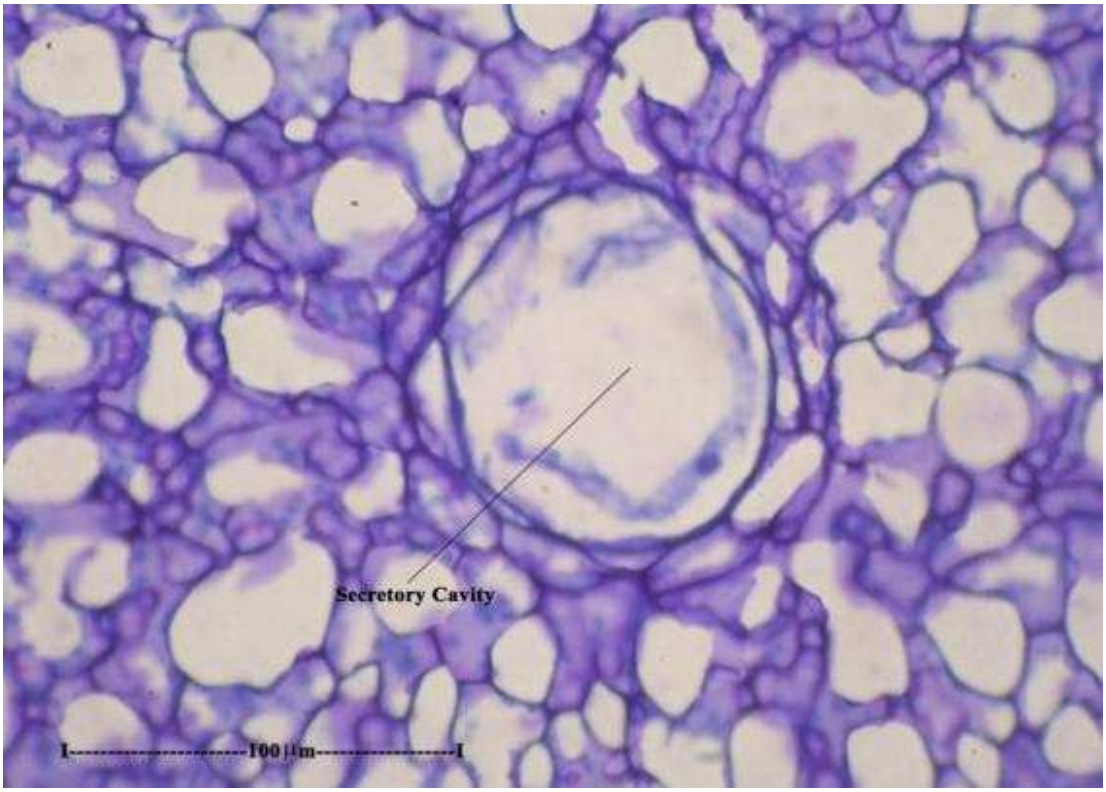


# PLATE NO: 10

## T.S. OF LAMINA (10 X)

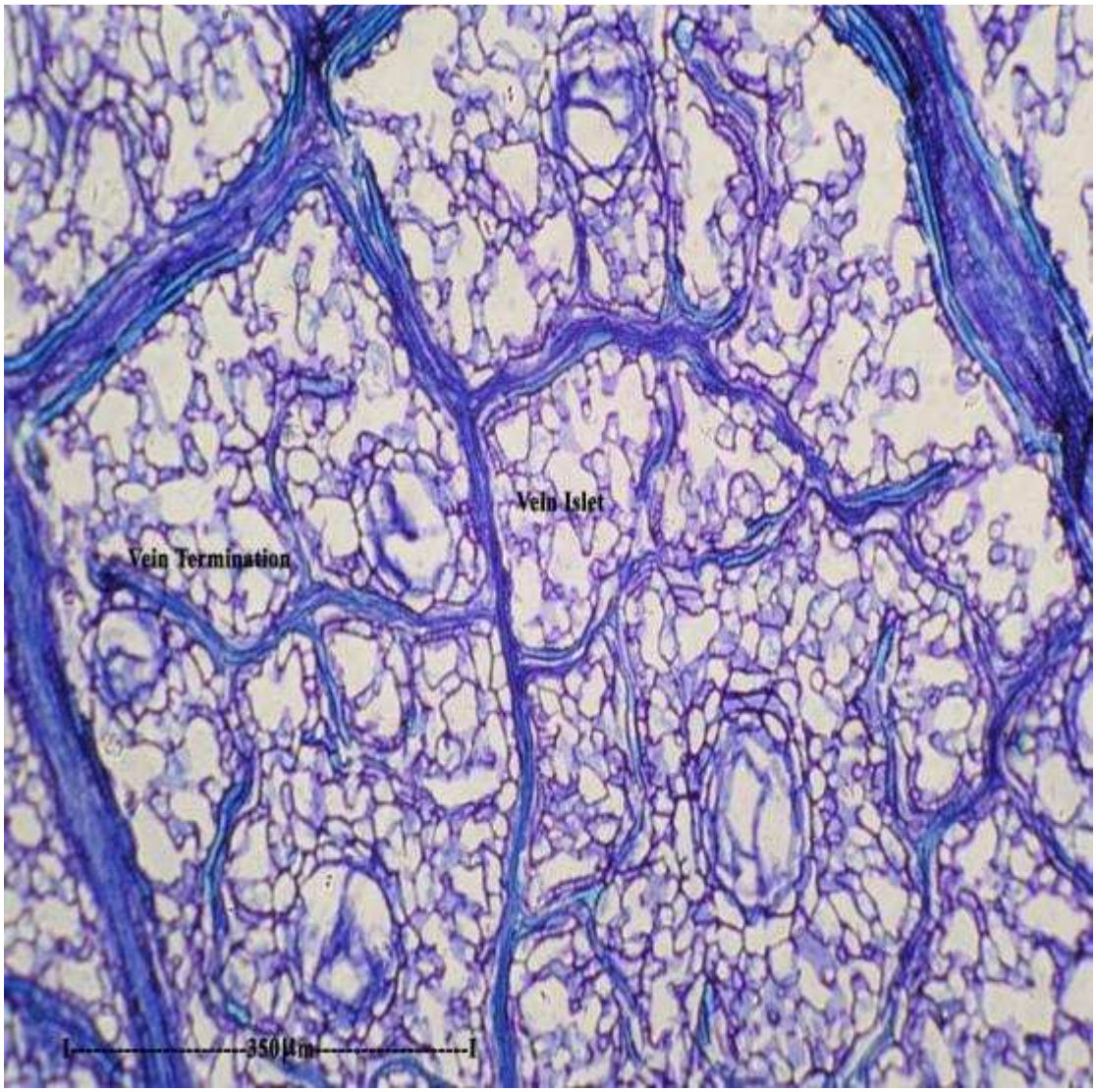



## SECRETORY CAVITY (40X)



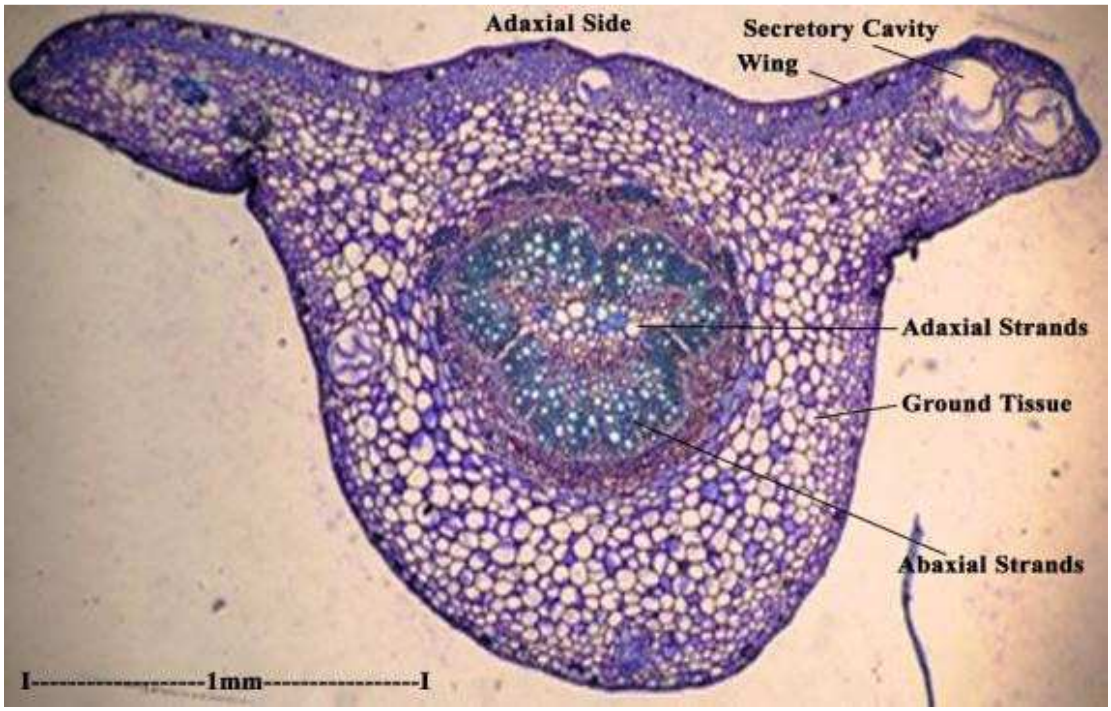
# PLATE NO: 11

## VENATION PATTERN (10X)

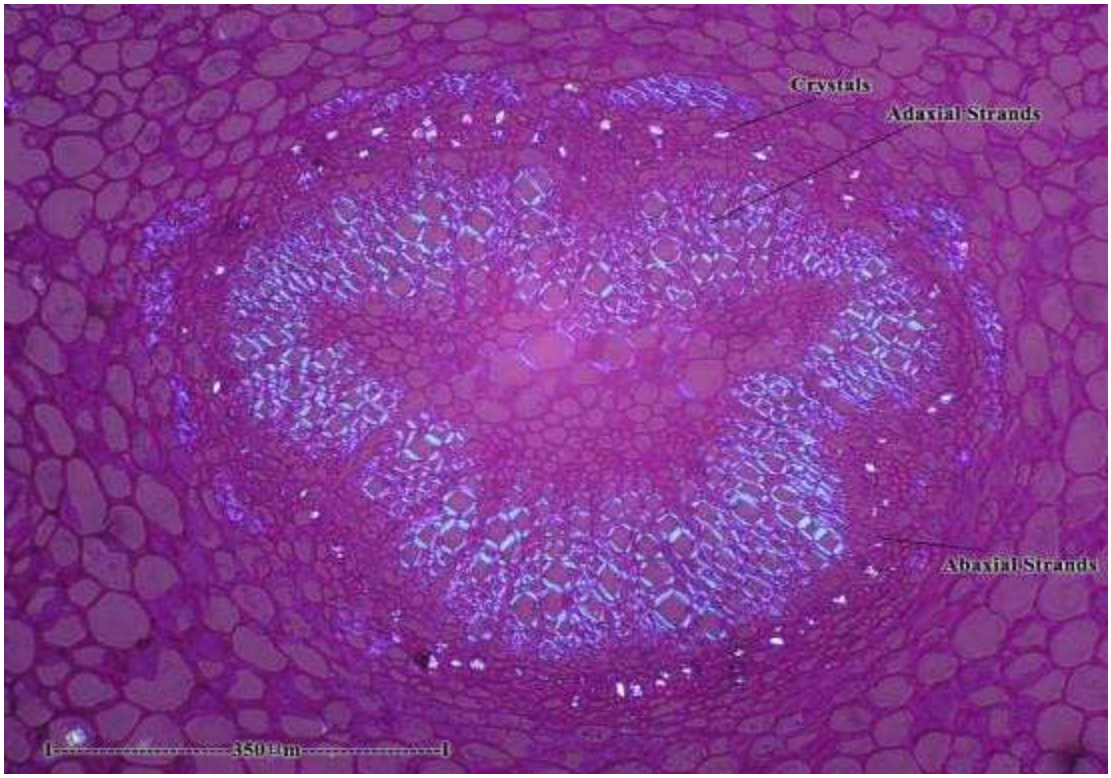


# PLATE NO: 12

## T.S.OF PETIOLE (4X)

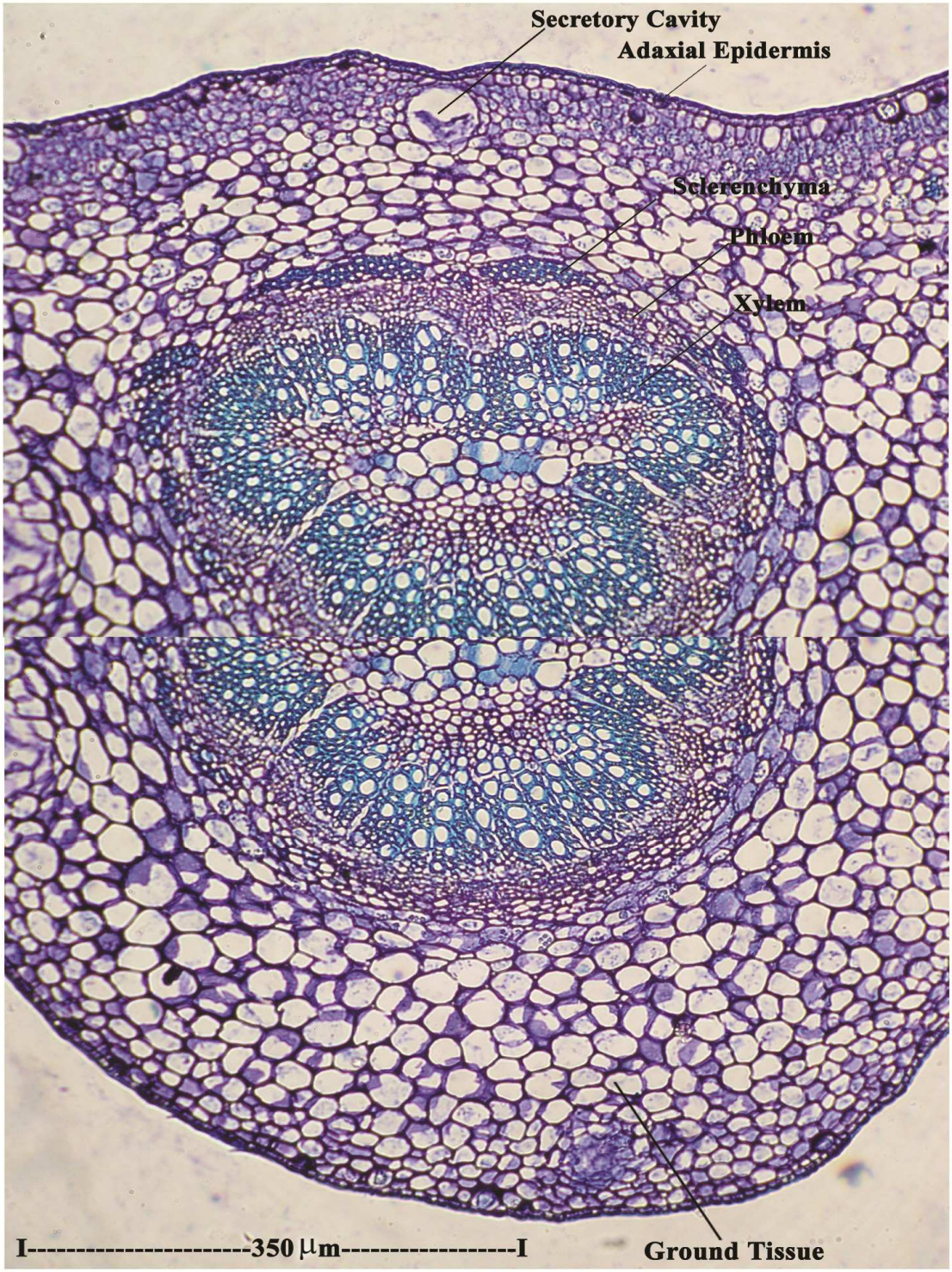



## T.S. OF PETIOLE UNDER POLARIZED LIGHT (10X)

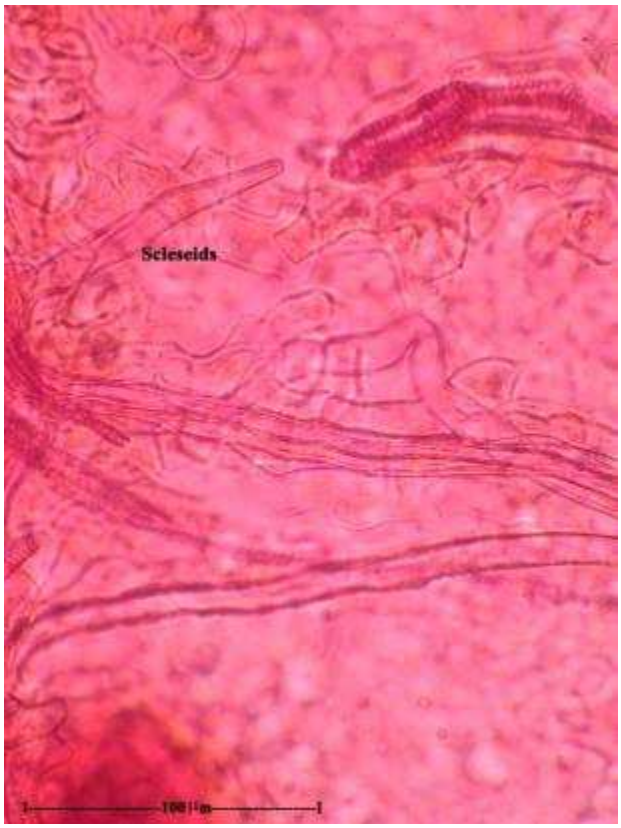


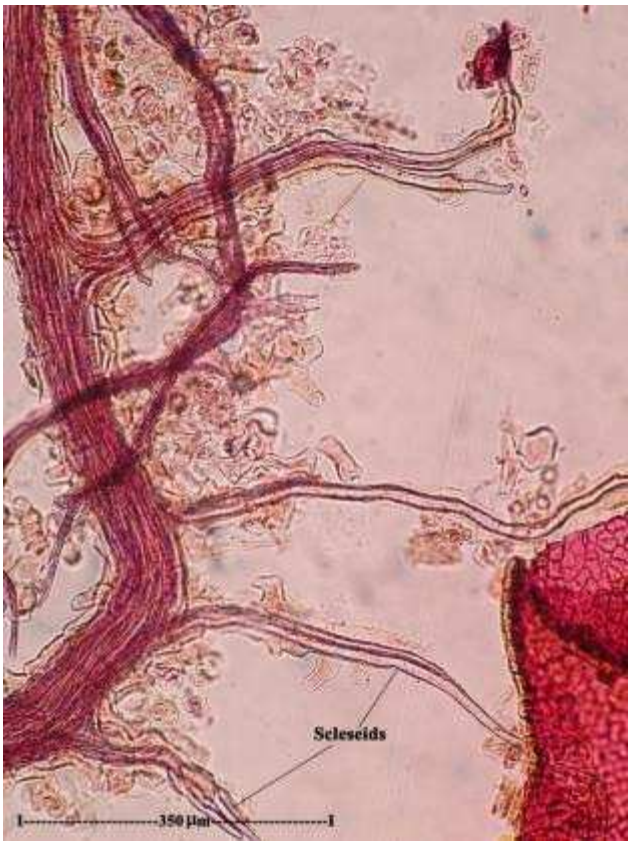
# PLATE NO: 13

## T.S.OF PETIOLE ENLARGED (10 X)



## Fig.2 POWDER ANALYSIS OF *Citrus limon*(L)



# PLATE – 1

## HABIT AND HABITAT OF *Citrus limon* (L)

# PLATE – 2

## LEAF ARRANGEMENT OF *Citrus limon* (L)

# PLATE – 3

## LEAF OF *Citrus limon* (L) DORSAL AND VENTRAL

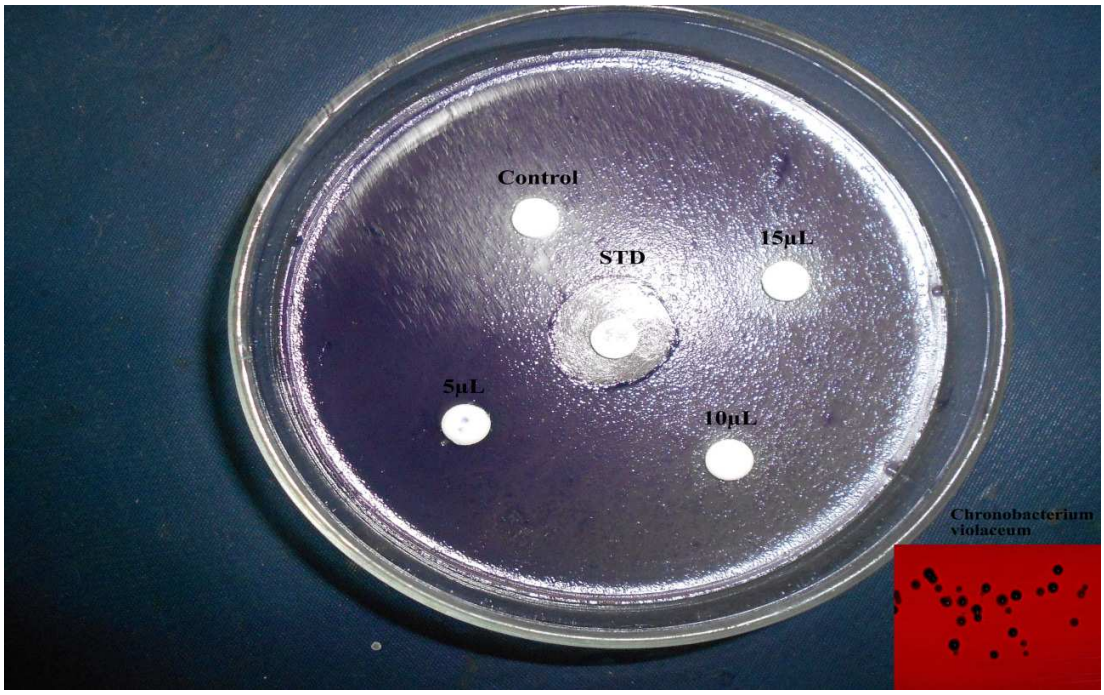
## PLATE – 4

## FLOWERS OF *Citrus limon* (L)

## PLATE – 5

## FRUITS OF *Citrus limon* (L)

**Fig.7 Antibacterial Activity of VO of *Citrus limon* against *Chromobacterium violaceum* and *E.coli***



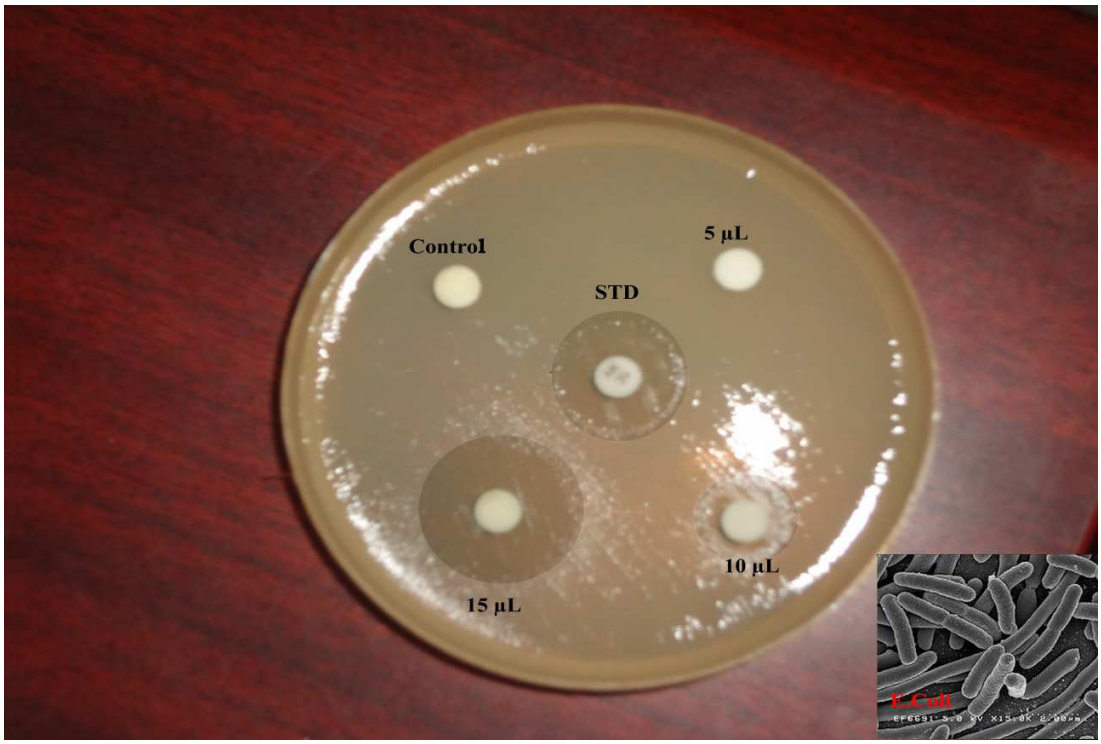
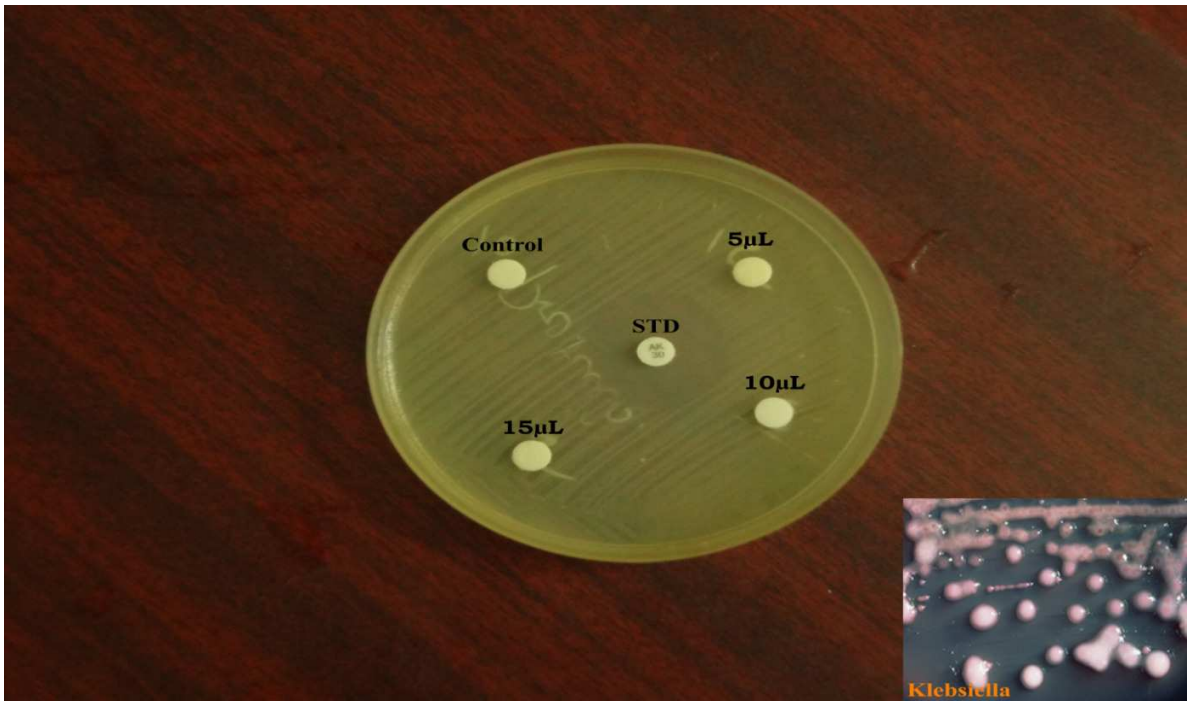



**Fig.8 Antibacterial Activity of VO of *Citrus limon* against *Klebsiella pneumoniae* and *Proteus Mirabilis***



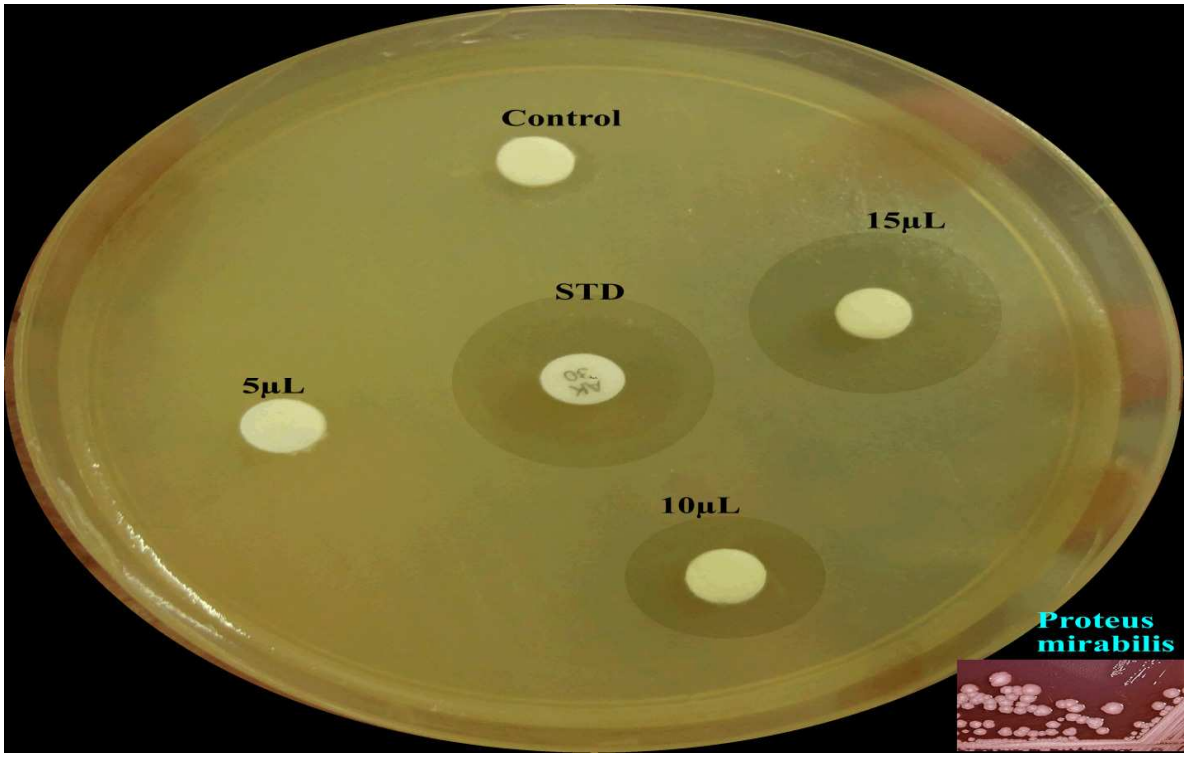
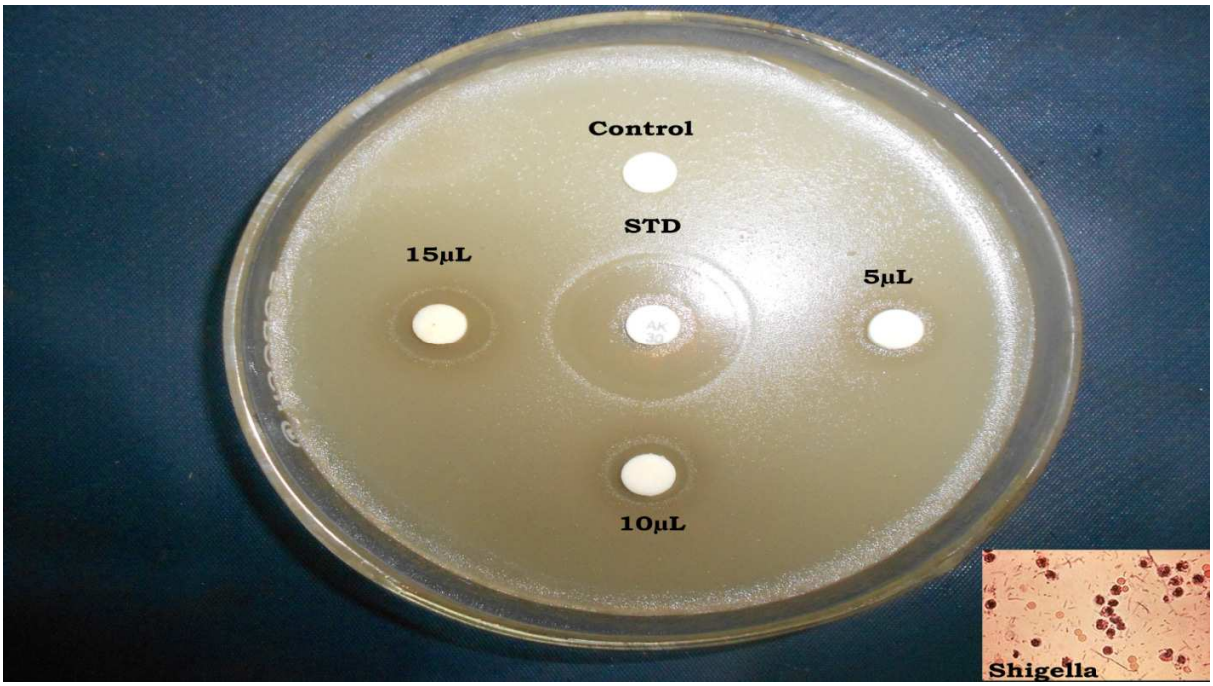



**Fig.9 Antibacterial Activity of VO of *Citrus limon* against *Shigella Flexneri* and *Streptococcus pyogens***



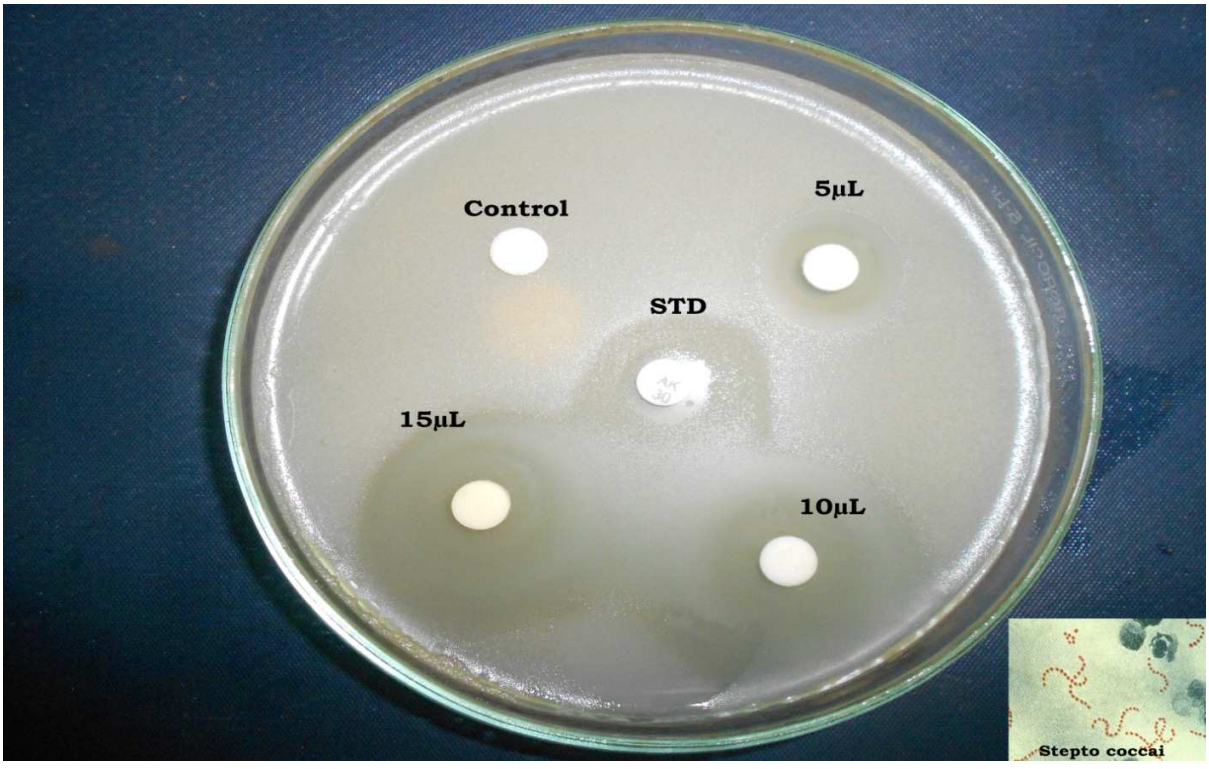
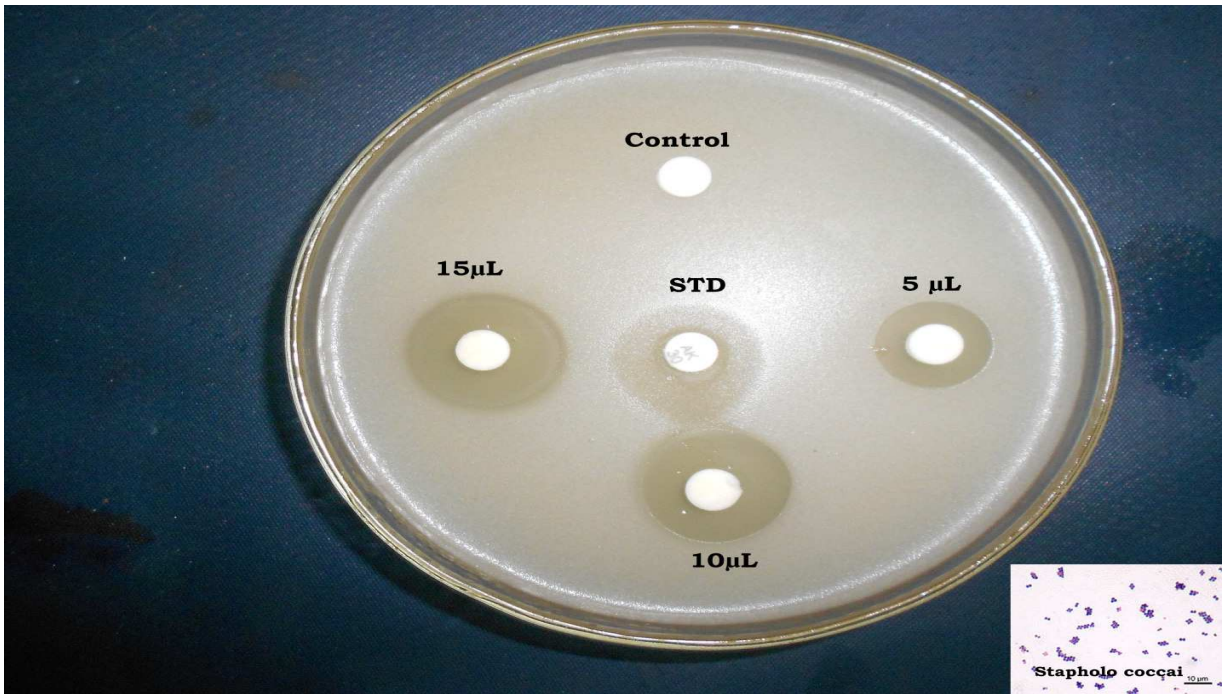
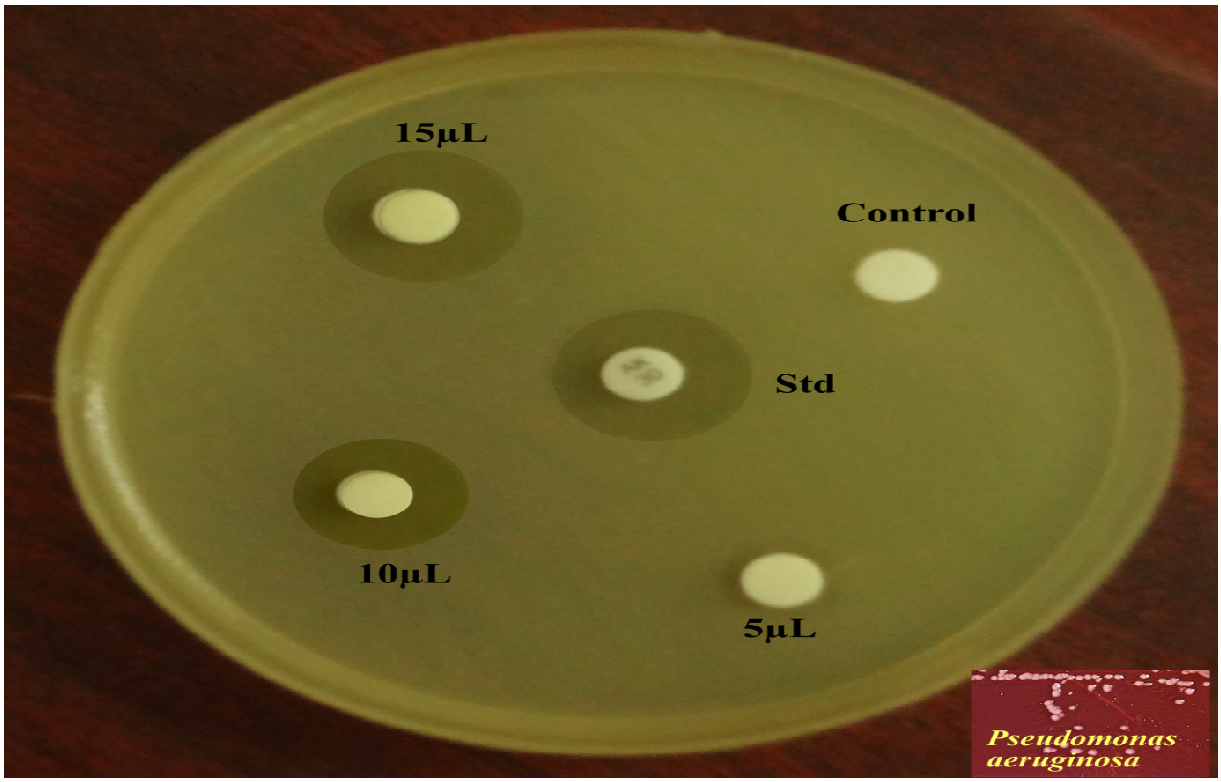



**Fig.10 Antibacterial Activity of VO of *Citrus limon* against *Staphylococcus aureus* and *Pseudomonas aeruginosa***



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