Prof. Mrs. R. THARABAI, M.Pharm.,
PRINCIPAL I/C,
College of Pharmacy,
Madurai Medical College
Madurai – 625 020.

CERTIFICATE

This is to certify that the dissertation entitled **"STUDIES ON LEAVES AND ITS VOLATILE OIL OF *Citrus aurantium* L - A PROMISING ANTI INFECTIVE WITH ANTIPLASMID, INNATE IMMUNITY STIMULATION AGENT"** submitted by **MS. S. DHANALAKSHMI (Reg No : 26092033)** 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 her during the academic year 2009-2011 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020.

(R. THARABAI)

Station :

Date :

Dr. Mrs. AJITHADAS ARUNA, M. Pharm., Ph. D.,
Professor & Head,
Department of Pharmacognosy
College of Pharmacy,
Madurai Medical College
Madurai – 625 020.

CERTIFICATE

This is to certify that the dissertation entitled **"STUDIES ON LEAVES AND ITS VOLATILE OIL OF *Citrus aurantium* L - A PROMISING ANTI INFECTIVE WITH ANTIPLASMID, INNATE IMMUNITY STIMULATION AGENT"** submitted by **MS. S. DHANALAKSHMI (Reg No : 26092033)** 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 her during the academic year 2009-2011 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020.

(Dr. Mrs. AJITHADAS ARUNA)

Station :

Date :

Dr. Mr. K. PERIYANAYAGAM, M. Pharm., Ph. D.,
Assistant Reader,
Department of Pharmacognosy
College of Pharmacy,
Madurai Medical College
Madurai – 625 020.

CERTIFICATE

This is to certify that the dissertation entitled **"STUDIES ON LEAVES AND ITS VOLATILE OIL OF *Citrus aurantium* L - A PROMISING ANTI INFECTIVE WITH ANTIPLASMID, INNATE IMMUNITY STIMULATION AGENT"** submitted by **MS. S. DHANALAKSHMI (Reg No : 26092033)** 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 her under my guidance during the academic year 2009-2011 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020.

(Dr. Mr. K. PERIYANAYAGAM)

Station :

Date :

ACKOWLEDGEMENT

“To take medicine only when you are sick is like

digging a well only when you are thirsty – is it not already too late”

Ch’I Po,c.2500 BC

Words are not just enough to express my gratitude to the lord Almighty who directed me throughout the work.

It is my privilege and honour to extend my profound gratitude and express my indebtness to **Mr. Edwin Joe, M.S., Dean**, Madurai Medical College, Madurai.

My profound thanks to **Mrs. R. Tharabai., M.Pharm., Principal i/c**, College of Pharmacy, Madurai Medical College, Madurai for her help.

With immense pleasure I record here my indebtness and hearty thanks to **Dr. Mrs. Ajitha Das Aruna, M. Pharm., Ph.D**., Professor and Head, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai, for her excellent encouragement, for the successful completion of this work.

I owe a great debt of gratitude and heartfelt thanks to **Dr. Mr. K. Periyanayagam, M. Pharm., Ph.D., P.G. Diploma in Clinical Pharmacy (Australia)**, Assistant reader in Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai, for his enthusiastic co-operation as my project guide and all the constant encouragement, suggestions, contribution and support rendered during the project work. As a gesture of respect I would like to extend my thanks for his patience in listening and answering to my questions.

I express my thanks and honourable regards to **Ms. R. Gowri, M. Pharm**., Assistant reader in Pharmacognosy, **Mr. T. Venkatarathinakumar, M.Pharm., (Ph.D)**., Assistant reader in Pharmacognosy and College of Pharmacy, Madurai Medical College, Madurai

I extend my sincere thanks to **Mrs. A. Sethuramani, M.Pharm., (Ph.D)**., and **Mrs. A. Krishnaveni, M. Pharm., (Ph.D)**., Tutors in Pharmacy, Department of Pharmacognosy, for their contribution and support rendered during the project work.

I am thankful to **Mr. P. Sivakumar, M.Sc., DMLT**., Lab Supervisor, **Mr. L.Rathinam, DMLT**., Lab Technician, **Mrs. Athisakthi, DMLT**., Lab Technician and **Mrs. P. Ellayee** for their support during my study of this work.

I take this opportunity to express my thanks to teaching and non-teaching staffs of Department of Pharmaceutics and also Department of Pharmaceutical Chemistry, College of Pharmacy, Madurai Medical College, Madurai for their co-operation during the course of dissertation work.

I submit my sincere thanks to **Prof. P.Jayaraman, Ph.D.,** Director of Plant Anatomy Research Institute, Tambaram, Chennai for microscopical studies and identification of this plant.

I wish to place a record here my indebtedness and heartfelt thanks to **Mr. M. Muthiya, Junior Inspector of Sericulture, Tirupur,** for their timely help and suggestions.

I especially thank my class mates **Ms. S. Sakthipriya, Ms. S. Padma Thanga Parameswari, Ms. S. Lakshmi and Mrs. R. Vimala** for their co-operation in carrying out my dissertation work.

I would like to thank my **Juniors of April-2010 & April 2011** for their direct and indirect help in my project

I am grateful to **Juniors** and **P.G's of other Departments** who have indirectly helped in completion of my work.

I thank **Mr. Ranganathan, B.Pharm.,** and **Mr. Senthil Ram, B.Pharm.,** especially for their co-operation in carrying out my dissertation work.

CHAPTER -1

INTRODUCTION

Natural products as a source of drugs:

Plants have been the source of most of the active ingredients of medicines which is widely accepted to be true when applied to drug discovery in olden times before the advent of high-throughput screening and the post genomic era. Above 80% of drug substance were natural products or inspired by a natural compound. Comparisons of the information presented on sources of novel drugs from 1981 to 2007 indicate that 50% of the drugs approved since 1994 are based on plant source. Thirteen herbal-products-related drugs were approved from 2005 to 2007 which is pointed out by Butler. Five of which represented by first members of new classes of drugs; the peptides exenatide, ziconotide and the small molecules. Ixabepilone, retapamulin and trabectedin (Fig. 1)

The recently accepted natural-product-based drugs have been described extensively in previous reviews. This include compounds from natural sources (including elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (exenatide and ziconotide), and synthetic or semi-synthetic compounds used on plant sources. (eg.tigecycline, everolimus, telithromycin, micafungin and caspofungin) (Fig 1). It covers a range of therapeutic indications, anti infective, anti-cancer, anti-diabetic. The chemical nature of the small molecule herbal products that have recently been developed into drugs have been

analyzed. Most of them were found to be closely compliant with Lipinski's rule of five for orally available compounds, and the remainder had higher molecular weight, highly rotable bonds and more stereogenic centre, and they retained relatively declined log P value. Finally the natural products are more readily absorbed than synthetic drugs.

Perceived demerits of natural products:

Many pharmaceutical companies have decreased the usage of natural products in drug discovery screening. It is due to the perceived disadvantages of natural products chemistry (more difficulties in access and supply, complexities of natural products chemistry, inherent slowness of working with natural products, and also concern about intellectual property rights) and the belief associated along with the use of collections of compounds prepared by combinatorial chemistry methods.

Natural products under clinical trials and preclinical development:

Around 100 natural-products- derived compounds are currently in clinical trials and also similar projects are in preclinical development. Mostly are from plants and microbial sources. These projects are predominantly being carried out for use in cancer or as anti infective, in 312 projects about 30% in natural-product-based development between 2001 and 2008. There is a growing interest in developing products which contain mixtures of natural compounds from traditional medicines and a mixture of compounds extracted.

Green tea (Veregen ^{TM}) has been approved by FDA has newly come on the market.

Increase in access to natural products:

Sorafenib reached the market from the source of high-throughput screening of combinatorial chemistry libraries. With the increasing realization, chemical diversity of natural products is a better match to that of successful drugs than the diversity of collections of synthetic compounds, the research in applying natural chemical diversity to drug discovery appears to be increasing once again.

Most of the compounds from natural sources that are currently in development have come from either plant (or) microbial sources. Previous publications have been mentioned that relatively little of the world's plant biodiversity has been extensively screened for bioactivity and that very little of the estimated microbial biodiversity has been available for screening. So, more extensive collections of plants or further advances in the ability to culture microbes could provide many novel chemicals for use in drug discovery assays.

Fungai and terrestrial Actinomycetes are the main sources for microbial area. There is increasing interest in marine Actinomycetes specifically with the discovery of species unique to the marine environment and the demonstration that they can produce chemically novel bioactive metabolites.

For example, salinosporamide A (fig 2) from *salinospora tropica* highly inhibits the 20S proteasome and also it has anti-cancer activity in experimental models, now it is undergoing clinical trials. Most of the technical hurdles still have to be overcome before large-scale bio-processing in marine bacteria is a realistic activity.

Another source is which is receiving increasing attention is cyanobacteria. It produces curacin A and dolastatin 10 which are evaluated as anti-cancer agents. About 120 cyanobacterial alkaloids were published in 2001 and 2006. About 120 cyanobacterial alkaloids were published in 2001 and 2006 and it have high structural diversity and also a variety of biological activities like cytotoxicity, sodium channel modulation anti-fungal and inhibition of proteases.

Although the highly untapped microbial diversity of marine environment is recognized continued productive use of terrestrial bacteria has been restricted by difficulties in culturing the vast majority of species. It has led to the interest in different genetic manipulation technique such as combinatorial genetics.

Most of the research in this field focuses on polyketide pathways in bacteria or fungi and it has led, for example, to the creation of rapamycin analogue along with a range of activities. There are; however concerns about the application of the approach and also most of the specialist biotech companies that were discovered to exploit the technology have not survived.

Molecular biology-bioinformatics in the natural product drug discovery

Molecular biology techniques have also been applied to use bacteria to produce drug like isoprenoid compounds originally seperated from plants and also produce newer flavanones and dihydroflavonols.

A bioinformatic appproach has also been applied to predict which microbes will produce newer chemicals on the basis of the gene sequences encoding polyketide synthesis. It's led to the discovery of novel compounds with potential anti-fungal and anti-cancer activities.

In recently a metagenomics technique has also been to access a wider range of synthetic capabilities from bacteria. It involves sampling all bacterial DNA derived from an environmental sample and cloning the DNA in the host organisms like *E.coli*. Recombinant bacteria could be cultured and also tested for the expression of biologically active metabolites. Same work has explored the peptide synthetase genes and also polyketide synthase genes of cyanobacteria. The metagenomics approach has led to the discovery of novel compounds with antibiotic activity, the turbomycins. (Fig. 2)

One more approach is to adapt the strategy widely used to increase activity of naturally available antibiotics by applying combinations of synthetic and enzymatic methods to produce complex natural products. Mutasynthetic procedures have been used to produce macrocyclic compounds related to the antibiotic daptomycin and the anti-cancer drug cryptophicin, vancomycin

analogues have been made with the help of oxidative modifications carried out by Cytochrome P_{450} enzymes.

Plan sources have inspired so many developments in organic chemistry leads to advances in synthetic methodologies and also possibility of producing analogues of the original lead compound along with improved pharmacological or pharmaceutical properties. Natural products Scoffolds have also been well established as being privileged structures in terms of their ability to be the basis for successful drugs. Such scaffolds are being used as cores of compound libraries prepared by combinatorial techniques. There are many examples of library based on alkaloids and flavanoids, and also a description of computational methods to compare the natural products likeness of compound libraries with the application of various techniques to produce analogues and derivatives of natural products. It becomes possible to produce newer compounds that can be patented, even though the orginal structure was previously disclosed.

New approaches to the value of natural sources:

With improvements in seperation techniques to isolate and purity natural products Eg: counter-current chromatography and in analytical techniques to determine structures of compounds, screening of natural product mixtures is nowadays highly compatible with the expected timescale of high-through put screening campaigns. Singh and Barrett concluded that pure bioactive compound can be seperated from fermentation broths in less than 2 weeks and

that the chemical structures of more than 90% of new compounds can be elucidated within 2 weeks. With improvements in NMR techniques, complex chemical structures can be solved with much less than 1 mg of compound. (Quinn et al). It is recently demonstrated that it can be prepare a screening library of highly diverse compounds from plants with the compounds being pre-selected from an analysis of the dictionary of natural compounds to be drug-like in their physicochemical properties. It would 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 enhance the speed and efficiency with which natural products can be applied to drug discovery.

Activity profiling of extracts by binding potency on receptors:

Because it is extremely time-consuming and more expensive to create extensive collections of isolated and structurally characterized natural products (the example mentioned above had only 814 different compounds), there is an attraction to screen the mixtures of compounds isolated from extracts of plant material or from microbial broths. But it is not always easy to select extracts from primary screens that are likely to contain novel compounds with the selective biological activity. The concept of 'differential smart screens' approached this by screening extracts of unknown biological activity against pairs of related receptor sites. By the comparison of the ratios of the binding potencies at the two receptor sites for a well known selective ligand and for an

extract, it was shown that it is possible to predict which extract was likely to contain components with the appropriate pharmacological activity. The technique, as described which is limited to a relatively small number of target sites, but the concept has been extended in a various therapeutic area by the use of antisense RNA against specific therapeutic targets to improve the sensitivity of the modified cells to compounds acting at those targets. It was used to screen fermentation broths for antibacterial activity following inhibition of fatty acid synthesis. The use of whole-cell screen along with the antisense silencing technology was more productive and has led to the discovery and characterization of the novel antibiotics platensimycin and platencin (fig.2)

Chemical genetic profiling and molecular effects of unrelated drugs:

A different approach to the similar area is the 'chemical-genetics profiling' carried out in yeasts. This exploits a panel of yeast strains along with selective mutations that highlight sensitivities to particular drugs. By building up a database of the effects of a wide range of well known compounds, it can be possible to interrogate drugs with unknown mechanisms or mixtures of compounds such as natural product mixtures. The technique highlighted unexpected similarities in the molecular effects of unrelated drugs. (Eg. Amiodarone and tamoxifen) and also revealed that potential anti-fungal activity of crude extracts, this activity was confirmed by seperation and testing of defined compounds, stichloroside and theopalauamide (fig. 2). Because these

compounds are not structurally similar, they would not have been expected to act via at the same biological target.

Exploiting leads from traditional medicines

Although there are key examples of traditional medicines that have led to the modern drugs, the traditional approach of bioassay guided fractionation could be laborious, and it is also dependent on the availability of a convenient assay. There are many publications suggesting the value of data-mining approaches to pinpoint the active ingredients in traditional Chinese medicines (TCM). These are based on assuming that the biological effects of a mixture of the herbal extracts would still vary in a quantitative manner with the relative amounts of the constituents, leading to the concept of quantitative composition-activity relationships. They also seem to be making the assumption that the activity of complex TCM preparations could be associated with a relatively small number of compounds or even with a single compound. Many traditionally used preparations were spilt into 6 fractions by chromatography and the relative amounts of each fraction by chromatography and the relative amounts of each fraction measured. Activity under test was the ability of the TCM to reduce blood cholesterol in rats. Different computational procedures (including multiple linear regression, artificial neural networks and a stepwise the confirmed through in vivo tests. The comparisons are, however, performed with only a single dose and it may be difficult to conclude that there was true synergism. Another example concerned the ability of various versions of the

same TCM to reduce the consequences of myocardial ischemia and reperfusion injury twenty-four variuos samples were analyzed chemically and pharmacologically: the computer–based analysis of causal relationship highlighted two peaks that were then analyzed by using HPLC and LC-MS and found to contain two known active ingredients. In a further example, many preparations of ginseng (*Panax ginsen*g) were analyzed for their component to nine chemicals previously proposed to be associated with the biological activity of the plant extract. The preparations were analysed for effects on the proliferation of the breast cancer cell line MCF -7. Depending on the level of the significance set for the computer analysis, either one or a group of two to three components were predicated to be associated along with anti-proliferent effects. Unfortunately, the bioassay appeared not to be sensitive enough to give data to confirm the predications. In a different approach, random forest proceduress were used to examine possible links between identified constituents of TCM preparations and biological targets. This allowed linkages to be suggested in between broad classes of traditional preparations to be made, including preparations that may be have anti-viral activities.

There has been a various approach to make use of a database of active structures from TCM preparations. By a 'reverse docking' method, the active ingredients are screened in Silico for the ability to bind to sites on proteins in the structural database. Although this as suggested to be a means to predict the biological mechanism for the seperated natural products, it assumes that the

componentss are entering cells (most of the proteins being tested being intracellular) and it ignores the fact that most of the well known drug targets are proteins, such as G-protein-coupled receptors, whose structures have not been determined. A somewhat same approach has been used with other plant chemical including those from western herbal remedies.

Biology-oriented synthesis

As mentioned earlier, natural products have been utilised to inspire the synthesis of small but highly diverse chemical libraries for screening. This still have to be used in a random screening technique. Structural information from natural products has been used differently to make a way to focus on the most relevant part of chemical space for a particular assay through what has been called biology-oriented synthesis. All of the ~ 150,000 structures from the CRC dictionary of natural products are reduced to ~25,000 simple two-dimensional scaffolds that were then clustered in a 'parent-child' relationship. It allowed easy selection of related structures to base library creation for focused screening. It also facilitated the selection of chemically simpler scaffolds that can be expected to maintain the ability to support the desired biological activity. When combined along with the author's complementary approach of clustering biological targets through similarities in binding sites, this method was demonstrated to result in the quite selective and potent enzyme inhibitors being found from testing of a very less number of compounds: selective inhibitors of 11β-hydrp-xysteroid dehydrogenase, potent, selective

and novel inhibitors of various protein phosphatases, and anticholinesterase compounds which is derived from lead structures that inhibited CDC25A phospatase .

Integration of in silico screening and natural products

Facilities for high-throughput screening are now available in the academic labs as well as in drug companies; however, the cost of random screening of very wide collections of compounds can be prohibitive, and it makes sense to utilise in silico or virtual screening where possible to filter down the number of compounds used in real screens. Whereas the dictionary of natural products gives structural information on ~ 150,000 various compounds would still have to be physically available for any predicated activity to be confirmed through testing in the relevant assay. As mentioned above, clustering of chemically similar scaffolds can be very useful in guiding the synthesis of novel compounds, but obviously there will be a delay and expense in the synthesis. In an attempt to combine the merits of virtual screening of chemically diverse natural products and their synthetic analogues with the rapid availability of physical samples for testing, an academic collaboration has established drug discovery portal. This brings together a large variety of compounds from academic laboratories in several different institutions in a database that could be used for virtual screening. Academic biology groups can also be propose structures as targets for virtual screening with the portal's database (and with conventional commercially available database). When hits

were predicated from the in silico screening, they can be sourced from the originating chemist for confirmatory tests. Often, there will be an immediate link to expertise for the preparation of analogues to help start a lead optimization programme. Access to the portal is free for the academic groups and the conditioned expansion of the chemical database means that there is a valuable and growing coverage of chemical space through many novel chemical compounds. Although the compounds the portal's database would generally have already been disclosed in a thesis or in a chemistry journal, very few of them have been previously analyzed for biological activity. This is a common feature ofwell known natural products: of the ~ 150,000 structures in the CRC dictionary of natural products only ~ 1% of them have any biological test results ascribed to them the MDL drug data report database.

Natural products as pharmacological tools in novel aspects of physiology:

There is many historical examples in which the natural product has not just been the medicinal product but has also helped reveal a novel aspect of physiology. For example, digitalis from the foxglove showed the role of sodium-potassium-ATPase; morphine pointed the way to the receptors affected by the endogenous opioids; muscarine, nicotine and tubocurarine helped explore the different types of acetylcholine receptors and so on . More recently, there has been interest in the systematically searching for small molecule inhibitors of the key steps in biochemical processes (chemical genetics) given

that so many assays involve identifying phenotypic changes in living cells (as opposed to binding interactions with isolated proteins), it is probable that natural products would provide useful probes for such studies. Moving beyond observations of phenotypic changes to defining the modifications in gene expression or protein function that are responsible will be require advances in transcriptomic and proteomic methods.

So it is obvious conclusively that despite a period in which pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the recent development pipeline that are of natural origin. Technical drawbacks associated along with natural product research have been lessened, and there are better opportunities to explore the biological activity of previously inaccessible plant sources of natural products. With the increasing acceptance that the chemical structural diversity of natural products is well suited to the provide the core scaffolds for future drugs, there would be further developments in the use of novel natural products and chemical libraries based on natural products in the drug discovery campaigns.

Bioactivity of Volatile Oils

V.O are valuable natural substances used in many areas like perfumery, cosmetics, spices, herbal theraphy, aromatheraphy etc.

Recently it has attracted many investigators 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. V.O's are complex mixtures contains many single compounds. The constituent of V.O contributes to the therapeutic or adverse effects of these oils. Hence the knowledge of composition of V.O is necessary for a better and specially directed application. Such a detailed knowledge of constituents of V.O's can be obtained by means of GC experiments [19]. By investigating the bioactivity of V.O's we can meet the situation of unsettling facts of modern pharmaceutical industry which facing lately its pipeline of new drug delivery discovery seems to have almost empty. Now the R& D 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.

Vertebrate animal model have been used for evaluation of the therapeutic effects of antibiotics for decades. The use of animal for the development of antibiotics,however,creates a number of problems. First one is cost; Injection experiments in specific-pathogen-free facilities,which are essential for the maintanance of experimental animal,are expensive. Another problem is the ethical issues surrounding the use of mammalian animals for the development of medicine, which is regulated by laws in European countries. To overcome these problems, evaluation of the therapeutic effects of chemicals in an alternative animal model is desirable.

Invertebrates have been used as an animal model of infectious disease. Mammals such as Mouse, Rat, Marmot, Rabbit, Dog and Monkeys are commonly used as drug screening models. The development of invertebrate animals as drug screening models will overcome these problems.

Silkworms are sufficiently large enough to be used in injection experiments, large enough for preparations of the hemolymph to be made, and large enough for organs such as the midgut to be isolated; these are important methods for studying the pharmacodynamics of drugs in individual bodies. The

less cost and little space required for the maintanance of silkworms compared to other animal model allow us to handle a larger number of animals in limited facilities,because of the history of the silk industry, the method for taking care of silkworms is already well established.

Reason for the selection of this plant:

Citrus aurantium L is a widely and easily available plant belonging to the family Rutaceae. The leaves of *C.aurantium* really do not have any match as a cheap natural and easily available plant. It is traditionally known to be useful for the treatment of wide panel of diseases like stomach ache, vomiting etc. Leaf is used fro emmenagogue, blood pressure, cough, cold, bronchitis, ear ache, dysentery, diarrhea, abdominal pain, UI ailments, dysmenorrhea, diuretic during pregnancy, influenza, insomnia, fever, sedative, digestive. Bark used for UIT ailments. Infusion of dried flower is orally used for influenza, insomnia, as a cardiovascular analeptic, anti spasmodic, for cold, sedative, digestive. Fruit as a cardiovascular analeptic, as a febrifuge, cold, flu, abortifacient and to speed birth in pregnant women, as a laxative, to treat fishbone caught in the throat, influenza, insomnia, to stimulate appetite, liver troubles, externally cutaneous infection, irritation, dermatitis, inflammation, wounds, bruises, sores, head ache, antidiabetic, dysentery, diarrhea, hypertension etc. This plant is much more popular in India and widely cultivated. In India fruits used for pickles. The economic aspect of this crop evidently proved that as commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored well. By a

well coordinated effort, we can 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 and exploitation.

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.

CHAPTER – 2

REVIEW OF LITERATURE

The review of literature comprises information on taxonomy, pharmacognosy, phytochemistry and pharmacological studies on various parts of *Citrus aurantium L*.

2.1. TAXONOMY

Kingdom	:	Plantae
Division	:	Eudicots
Class	:	Rosids
Order	:	Sapindales
Family	:	Rutaceae
Genus	:	*Citrus*
Species	:	*aurantium*
Binomial name	:	*Citrus aurantium* L. **(www.wikipedia.com)**

2.2. BOTANICAL INFORMATION

Name of the plant	:	*Citrus aurantium* L.
Synonym	:	Bitter orange,Sour orange, Seville orange,Bigarade orange . **(www.wikipedia.com)**

Vernacular names

Sans.	:	Brihatjambhira
Hindi.	:	Khatta
Uri	:	Nagorongo
Tel.	:	Mallikanarangi
Tam.	**:**	**Narangam, Narattai**
Kan.	:	Heralay
Mal.	:	Karna **(www.wikipedia.com & wealth of India)**

2.3. PLANT DESCRIPTION

A tree, rarely a shrub; young shoots glabrous, greenish white. Leaves foliolate; Leaflets 7.5-15cm. Long, elliptic or ovate, obtuse, acute or acuminate; petioles naked or winged, the wing often obovate and nearly as large as the blade. Flower bisexual, pure white. Stamens 20-30. fruit globose, generally oblate, not mamillate, usually orange-coloured; rind loose or adherent; pulp sweet, yellow, rarely red. (Kirtikar K.R & Basu.B.D)

2.4. HISTORY AND GEOGRAPHICAL DISTRIBUTION

Widely cultivated in India.- said to be indigenous in the Mothronwala Swamp Dehradun,Garhwal,Kumaon,Sikkim, Khasia hills, Manipur mountain forests of the Peninsula. It is growing in semi-wild state particularly in the Naga and Khasi hills.

It is regularly cultivated in Khasi hills and Cachar. Two varieties are met with in Assam " Karun-jamir" which can withstand extreme conditions of soil and climate and "Gondh-huntra" a vigorous grower with juicy fruits.

The sour orange is native of south eastern Asia. Natives of the South Sea Islands, especially Fiji, Samoa, and Guam, believe the tree to have been brought to their shores in the prehistoric period. Arabs are thought to have been carried it to Arabia in the 9th Century. It was reported that to be growing in the Sicily in 1002 A.D., and it was cultivated around Seville, Spain, in the end of 12th Century. For 500 years, it was the only orange in Europe, and it was the first orange to reach to the New World. It was naturalized in Mexico by 1568 and in the Brazil by 1587, and not long after it was running wild in Cape Verde Islands, Bermuda, Jamaica, Puerto Rico and Barbados. Sir Walter Raleigh have took sour orange seeds to England; they were planted in the Surrey and the trees began bearing regular crops in 1595, but were killed by cold in 1739.

Spaniards wer introduced the sour orange into St. Augustine, Florida. It was quickly acceptedted by the early settlers and local Indians in the 1763, sour oranges were being exported from the St. Augustine to England. Sour orange trees could still be found in Ever glades hammocks in the sites of former Indian dwellings. The first sweet orange budwood were grafted onto sour orange trees in pioneer dooryards and, from that time on, the sour orange became more widely grown as a rootstock in all the citrus-producing areas of the world than

for its fruit or other features. Today, the sour orange is found to be growing wild even in southern Georgia and from Mexico to Argentina.

It is grown in orchards or groves only in the Orient and the various other parts of the world where its special products are of commercial importance, including southern Europe and offshore islands, North Africa, the Middle East, Madras, India, West Tropical Africa, Haiti, the Dominican Republic, Brazil and Paraguay. (Wealth of India and www.wikipedia.com)

2.5. CULTIVATION

Area and production

Citrus 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 mandarin 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:

Citrus 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. Notwithstanding 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:

Harvesting; in most of he 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:

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. (Wealth of India)

2.7. WHOLE PLANT REVIEW

Ethnomedical Information

Plant orally used for fever, epilepsy, emotional shock, cold, rheumatism, digestive and gall bladder problems, hepatic disorder, in food preparation and externally used for skin blemishes, externally and internally for bruising in Haiti. (Paul, A. 1995)

Hot aqueous extract of dried plants orally used for malaria in Sudan. (Khalid, S.A.1986)

Hot aqueous extract of entire plants orally used for menorrhogia in India. (Jain,S.K. 1970)

Pharmacological studies

Laxative Effect

Decoction of mixture of rheum species, *Mangolia officinalis* and *Citrus aurantium* screened for laxative effect in China. It is active effect described are from a multi-component prescription. (Gu,W.*et.al*.,1985)

Antiulcer Activity

Aqueous extract of plant screened for antiulcer activity in rat and found inactive against HCL/ethanol induced gastric ulcers at 500mg/kg dose.

(Jeong,C.S.*et.al*.,2002)

Neuraminidase Inhibition Activity

Methanol extract of whole plant screened for neuraminidase inhibition activity and found active at 1ppm concentration. (Lee,C.H.*et.al*.,2003)

Phytochemical studies

1	Whole plant	Alkaloid-Diphenylamine	-	-	Karawva,M.S.1986
2	Dried entire plant	Isoquinoline alkaloid-Synephrine, 5-methyl tyramine.	-	China	Guo,2G.1983
3	Dried entire plant	Triterpene-Limonin, Nomilin.	-	Sudan	Khalid,S.A.*et.al*., 1986

ESSENTIAL OIL REVIEW

Pharmacological studies

Antifungal Acitivty

Essential oil screened in Paraguay for antifungal activity (plant pathogens) by agar plate method. It is active against *Polyporus versicolor, Lentinus lepideus and Lenzites trabea.*

(Maruzzella,J.C. *et.al*.,1960)

Essential oil screened in Egypt for antifungal activity in agar plate method and found inactive against *Trichoderma viride, P.cyclopium*.

(Ross,S.A.*et.al*.,1980)

Antibacterial Activity

Essential oil screened for antibacterial activity in Egypt by agar plate method and found active against *S. aureus, P.aeruginosa* and inactive against *E.coli, B.areus.* (Ross,S.A.*et.al*.,19

Essential oil screened in Thailand for antibacterial activity in agar plate method and found that active against *S. pyogenes* and *S. aureus*.

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

Antiyeast activity

Commercial sample of essential oil screened in Australia for antiyeast activity in agar plate method 0.25% found active against *C. albicans.*

(Hammer,K.A.*et.al*.,1998)

Smooth Muscle Relaxant activity

Essential oil of plant screened for smooth muscle relaxant activity and found active in guinea pig trachea at ED_{50} 64mg/liter dose and also found inactive in guinea pig ileum at 100mg/liter. (Reiter, M.1985)

Insect Repellent Activity

Essential oil screened in India for insect repellent activity in Apis florae. It is active in 0.0125% by Olfactometer test. (Gupta, M.1987)

Antiulcer Activity

Essential oil screened for glutathione-S-transferase induction in mouse liver. Dose of 30mg/animal given by intragastric route every 2 days for total of 3 doses is inactive in ulcer in liver, stomach and small intestine. (Lam,L.K.T.1991)

Anti tumor Activity

Essential oil screened for tumor promotion inhibition in rat. Dose 1% of diet is active in CA-mammary-DMBA. (Russin,W.A.1988)

Essential oil of plant screened for Glutathione-S-transferase induction activity in mouse stomach, small intestine, liver by intragastric route at dose 30mg/animal and find inactive.

Doses given every 2 days for a total of 3 doses. (Lam,L.K.T.1991)

Action on CNS

Essential oil of plant screened for tranquilizing effect in mouse by inhibition (1.897mg/L) and found active. Greatest activity seen within 30 minutes of dosing. Air concentration of compound at end of exposure of 1hour given. (Jager,W.*et.al*.,1992)

Essential oil screened in Yugoslavia for CNS depressant activity by using gold fish externally used and found that it is inactive. (Wesley-Hadzija, B.1956)

Phytochemical studies

1	Essential oil	Monoterpene-Limonene	-	-	Russin,W.A. 1988
2	Essential oil	Monoterpene-Limonene	86.2	-	Pino,J.A.2000
3	Essential oil	Sesquiterpene- α-Bergamotene, β-Bisabolene, β-Caryophyllene.	-	-	Burk,L.A. 1992
4	Essential oil	Monoterpene-Linalool, Linalool acetate.	-	Brazil	Hanneguelle,S.*et.al*,1992
5	Commercial sample of essential oil	Alkaloid- 3-(but-cis-1-enyl) pyridine.	-	-	Maurer,B.1992

DRIED BRAIN

Pharmacological studies

Antibacterial Activity

95% Ethanolic extract of dried brain (2.8mg/disc) screened in Mexico for antibacterial activity in agar plate method and found equivocal against *S.aureus, B. subtilis* and inactive against *Streptococcus faecalis, E.coli.*

(Hou,Y.C.*et.al.*,1999)

Antiyeast activity

95% Ethanolic extract of dried brain (2.8mg/disc) screened for antiyeast activity and found inactive against *C. albicans*.

(Hou,Y.C.*et.al*.,1999)

BRANCHES

Ethnomedical information

Juice of branches orally used to treat convulsions in children in Cook Island, the scraped branches of the *Citrus* and *Psidium guajava* are squeezed through cloth in to water to treat convulsion in children, the medicine is considered best prepared at the time of a full or new moon, but not between a breast feeding baby and mother drink a sweetened solution once. Other fits and convulsions use the scraped bark of new shoots with the Guava bark. The solution is given daily for three days and repeated in a month.

Effects described are from a multi-component remedy.

(Holdsworth,D.K.1990)

Decoction of fresh branches used orally to treat Gonorrhea in Cook Island. Gonorrhea may be treated with the following medicine scraped bark with two ripe and unripe *morinda citrifolia* fruits are pouned a tablespoon measure of powdered piper methysticum are mixed, squeezed through a cloth into half a gallon of water and boiled. The cooled solution is drunk daily for two days.

Effects described are from a multi-component remedy.

(Holdsworth,D.K.1990)

Branches used for stomach ache by oral route in Mexico.

(Dimayuga, RE.*et al.,* 1998)

Branches used for vomiting by oral route in Mexico.

(Dimayuga,RE.*et.al* 1998)

STEM PITH

Pharmacological studies

Immunosuppressant Activity

Aqueous extract of dried stem pith screened for immunosuppressant activity in mouse by intra gastric route (500mg/kg) and found active against con-A induced proliferation in thymocytes, also active against LPS induced proliferation in splenocytes,

Statistical data in report indicating significant results.

(Yul,J.Y.1998)

Nitric Oxid Synthesis activity

Aqueous extract of dried stem pith screened for nitric oxide synthesis stimulation activity and found inactive in macrophages and inactive against LPS induced proliferation in macrophages with gamma interferon.

Statistical data in report indicating significant results.

(Yul,J.Y.1998)

LEAF REVIEW

Ethnomedical Information

Decoction of leaf orally taken as an emmenagogue in West Indies and used for high blood pressure in Curacao.

Hot aqueous extract of leaf orally taken with leaf decoction of *Annona muricata* for gall bladder trouble in Curacao.

Effect described are from a multi-component remedy.

(Morton,JF.1968)

Decoction of leaf orally used for cough, colds, bronchitis in Rodrigues Islands. (Gurib-Fakim,A.1996)

Hot aqueous extract of *C.auranticum* var. Tachibana leaf used to treat inflammation in traditional medicine as early as the year 1613 in South Korea.

(Cha,S.1977)

Juice of fresh leaf used for earaches in East Africa.

Aqueous extract of *C.auranticum* aff fresh leaf orally used for dysentric, diarrhea in East Africa cold water extract of leaves of *deinbollia borbonica* is mixed with juice of *C. auranticum* and heated with red hot charcoal.

Effects described are from a multi-component prescription.

(Hedberg,I. *et.al.*,1983)

Fresh leaves orally used for abdominal pain in Cook Islands. Leaves of *C.aurantium* and *psidium guajava* are crushed together and taken for pain centered around the navel.

Effects described are from a multi-component prescription.

Fresh leaves orally used for urinary tract ailments in Cook Islands leaves are boiled.

(Whistler, W.A.1985)

Decoction of fresh leaves externally used for urinary infection in Mexico.

Decoction is made with water and lime is used in a poultice.

Decoction of fresh leaves orally used to speed recovery of postpartum, dysmenorrhea, hemorrhage between menstrual periods in female in Mexico. (Browner, C.H.1985)

Aqueous extract of dried leaf externally used for head ache in Haiti. (Weniger,B.*et.al.*,1986)

Decoction of dried leaf orally used as a diuretic during pregnancy in Sierra leone, *C.auranticum* and *Trema guineensis* are used in decoction. Effects described are from a multi-component prescription. (Kargbo,T.K.1984)

Infusion of *C.auranticum* var amara leaf orally used for influenza, insomnia and as a tranguilizer in Canary Islands. (Darias, v.1986)

Juice of leaf orally used for fever in Guatemala.

Decoction of leaf orally used for vomiting, nervousness, cardia weakness, diarrhea in Guatemala. (Giron,L.M. *et.al.*,1991)

Infusion of leaf and flower are used orally as a sedative, digestive in Italy. (De feo,v.1993)

Decoction of leaf and fruit are used for fever, never, colds in Nicaragua. (Barrett,B.1994)

Decoction of dried fruit, leaf, and root are orally used for fever, hypertension, diarrhea, digestive for stomach ache and ulcers.

(Coe, F.G.1996)

Decoction of leaf externally used as a bath for high fever in Guatemala. Decoction of leaf orally used for vomiting, blood pressure, stomach aches and blood circulation improvement in Guatemala.

(Comerford, S.C.1996)

Leaves orally used as a tranguilizer, hypoglycaemic, carminative in Canary Islands. (Darias,V.1996)

Hot aqueous extract of leaf orally take with leaf decoction of *Annona muricata* every morning to relieve nervousness and in every night to induce sound sleep in Curacao.

Effects described are from a multi-component prescription.

(Morton,J.F.1968)

Pharmacological studies

Cytotoxic Activity

Aqueous extract of dried leaf screened for cytotoxic activity and antiviral activity (10%) by cell culture method and found inactive against *Hela cells, Herpes Type-, Influenza A2 (manheim 57), Vaccinia, Poliovirus II* type of viruses.

(May, G.1978)

Antiyeast, Antibacterial and Antifulgal Activity

95% ethanolic extract of dried leaf screened for antiyeast activity, antibacterial, antifungal activity by agar plate method (50mg/ml) and found active against *Bacillus subtilis* and inactive against *C.albicans, S.aureus, E.coli, P.aeruginosa, A.niger.* Extract of 10ml/g plant material, 0.1ml extract placed in well on plate in Surinam.

(Verpoorte, R.1987)

Phytochemical studies

1	Leaf	Gentisic acid-Benzenoid.	-	Trinidad	Griffiths,L.A. 1959
2	Leaf immature	Flavone- Neodiosmin, Rhoifolin. Flavanone-Neohesperidin, Naringin.	-	-	Rio,J.A.D.*et.al*., 1992
3	leaf	Flavone- Neodiosmin	-	-	Benavente-garcia,O.1993
4	leaf	Proteid-Proline.	-	India	Giri, K.V.*et.al*.,1952
5	leaf	Alkaloid- Stachydrine.	-	West Indies	Morton, J.F.1968
6	Dried leaf	'O'alkaloid	-	Cuba	Magadan figuerua,R.*et.al*.,1979
7	Dried fruit + leaf + root	'O'alkaloid	-	Nicarague	Coe, F.G.1996

LEAF ESSENTIAL OIL REVIEW

Pharmacological studies

CNS Depressant Activity

Essential oil screened in Brazil for Amuolytic effect (1mg/kg) CNS depressant activity (1mg/kg) by intragastric route in male mouse and found active.

Hexane and dichloromethane extract screened in Brazil for CNS depressant activity by intragastric route in male mouse and found active in 1mg/kg dose level. (Carvalho freitas,M.I.R.2002)

Antibacterial and Antifungal Activity

Essential oil of leaf screened in Egypt for antibacterial and antifungal activity by agar plate method and found active against *S.aureus, B.cereus* and inactive against *E.coli,P.aeruginosa* and also having strong antifungal activity against *A.aegyptiacus, P.cyclopium, T.viride.* (EL-eltawi,N.E.M.*et.al*.,1980)

Antiamoebic Activity

Essential oil of leaf screened for antiamebic activity by broth culture method and found equivocal against *Entamoeba histolytuica* at 4µl/ml concentration. (De blasi,V.*et.al*.,1990)

Toxicity Studies

Essential oil of leaf externally screened for toxicity assessment (quantitative) in rabbit and found active (LD_{50} 2gm/kg). (Anon, 1992)

Essential oil of leaf externally screened for photo toxicity activity in mouse and found inactive. (Anon, 1992)

Sensitization and Irritant Activity

Essential oil of leaf externally screened for irritant activity in rabbit (2gm/kg dose) found weak activity. (Anon, 1992)

Essential oil of leaf externally screened for irritant activity (8%) found inactive in human. (Anon, 1992)

Essential oil of leaf externally screened for sensitization (skin) activity in human (8%) and found inactive. (Anon, 1992)

Pharmacognostical studies

Plant Growth Inhibition activity

Essential oil of leaf screened in Irag for plant germination inhibition and plant growth inhibitor (unspecified) activity and found activity on Amaranthus retroflaxus. (Alsaadawi, H.S.*et.al*.,1985)

Phytochemical studies

1	Leaf essential oil	Monoterpene-Citronellol, (DL) Limonene, α-Pinene, β –Pinene, Octan-1-ol alkanol C5 or more.	-	-	Alsaadawi,H.S.1985
2	Leaf essential oil	Monoterpene-Linalool, Myrcene, Sabinene.	-	-	Lin,Z.K.1992

BARK REVIEW

Ethnomedical information

Dried bark is boiled and used for urinary tract ailments by oral route in Cook Islands. (Whistler,W.A. 1985)

Pharmacological studies

Cytotoxic Activity

Aqueous and methanolic extract of dried bark screened in Japan for cytotoxic activity(5%) by cylinder plate method and found equivocal against CA-*ehrlich-ascites* and inhibition is found to be 28 mm and 27 mm for respective extracts. (Veki,H.*et.al*.,1961)

Methanolic and Acetone extract of dried bark screened in Japan for cytotoxic activity by cylinder plate method and found equivocal in CA-*Ehrlich-ascites* and inhibition is found to be 27 mm and 30 mm for respective extracts.

(Veki,H.*et.al*.,1961)

Phytochemical studies

1	Fungus infected bark	Coumarin-Scoparone.	0.01625	Israel (cult)	Afek,U.1986
2	Fungus infected bark	Coumarin-Scoparone.	-	Israel	Afek,U.1988

FLOWER REVIEW

Ethnomedical information

Infusion of dried flower of *C.aurantium* var amara orally used for influenza, insomnia and as a tranguilizer in Canary Islands.(Darias,V. *et.al.*,1986)

Extract of dried flowers of *C.aurantium* var amara orally used as a cardiovascular analeptic in Tunisia. (Buukef.K. *et.al.*,1982)

Infusion of dried flower orally used for cold and antispasmodic in Brazil

(Elisabetsky, E.1990)

Infusion of flower and leaf used orally as a sedative and digestive in Italy (De feo, V.1993)

Infusion of dried flowers orally used as a digestive in Spain.

(Vazquez,F.M. *et.al.*,1997)

Phytochemical studies

1	Dried flower	Steroid- Desmosterol, Ergosterol, β-Sitosterol, Stigmasterol and 'O' saponins (unspecified type or hemolytic absent)	-	-	Moursi,S.A.H. 1980
2	Flower	Alkaloid- caffeine	31mcg/g	-	Stewart,I.1985

ESSENTIAL OIL OF FLOWER REVIEW

Pharmacological studies

Antiamoebic Activity

Essential oil of flower screened (1.0µl/ml)for antiamebic activity in Broth culture method and found active against *Entamoeba histolytica.*

(Deblasi.V.*et.al*., 1990)

Phytochemical studies

1	Flower essential oil	Monoterpene- β-limonene, Linalool, Linalool acetate.	 45.9 06.87 16.69	China	Ma,L.A.*et.al*., 1988

FRUIT REVIEW

Ethnomedical information

Hot aqueous extract of fruit orally used to reduce gas pain in China.

(Li,C.P.1974)

Extract of dried fruit of *C.aurantium* var.dulcis orally used as a cardiovascular analeptic in Tunisia. (Boukef. K.1982)

Extract of dried fruit and root of *C.aurantium* AFF orally used for polio in Tanzania. (Hedberg, I. 1983)

Dried fruit externally used as a febrifuge. Fruits are boiled and used as foot baths in Mexico. (Martinez, M.A. *et.al*.,1984)

Decoction of fresh fruit orally used for colds, flu and as an abortifacient and to speed birth in pregnant women, externally for mange. Prepared with sulfa, fat or pine sap and used as a poultice in Mexico. (Browner, C.H.1985)

Fresh fruit juice orally used as laxative in Cook islands. Oil of *ricinus communis* or *cocos mucifera* is mixed with the fruit juice of citrus aurantium and crushed leaves of *ordyline terminalis*.

Effects described are from a multi component prescription.

(Whistler, W. A. 1985)

Fresh fruit orally used to treat people with a fishbone caught in the throat in Cook Islands. (Whistler, W.A. 1985)

Infusion of dried fruit *citrus aurantium* var.amara orally used for Influenza, Insomnia and as a tranquilizer in Canary Islands.

(Darias, V. *et.al*., 1986)

Fresh fruit juice orally used for cough, stimulate appetite, flu, indigestion, liver troubles and externally cutaneous infections in Haiti.

(Weniger, B. *et.al*., 1986)

Hot aqueous extract of dried fruit externally used to skin disease, irritation, dermatitis, inflammation, wounds, ulcer, bruises and sores in Guatemala. (Caceres, A. 1987)

Hot aqueous extract of dried fruit orally used for headache, antidiabetic, antispasmodic in Peru. (Ramirez, V.R. *et.al*., 1988)

Infusion of dried fruit orally used for cold and antispasmodic in Brazil.

(Elisabetsky,E.1990)

Decoction of fruit and leaf are used for fever, nerves, colds and orally for diarrhea in Nicaragua. (Barrett, B.1994).

Fruit juice is orally used for dysentery in India. It is mixed with *Emblica officinalis.*

Effects described are from a multi component prescription.

(Bajpai, A.*et.al*., 1995)

Decoction of fruit used for cough, fever, cold, diarrhea, hypertension and also as a digestive in Nicaragua. (Coe, F.G.1996)

Decoction of dried fruit, leaf and root are orally used for fever, hypertension, diarrhea, ulcers and digestive for stomachache in Nicaragua.

. (Coe, F.G.1996)

Fresh fruit juice is orally used for diabetes in Mexico.

(Alarcon-Aguilara,F.J.*et.al*.,1998)

Pharmacological studies

Toxicity Study

Extract of dried fruit screened in China for quantitative toxicity assessment in mouse by IV route and its LD50 is found to be 71.8gm/kg.

(Anon 1978)

Cytotoxic Activity

Aqueous extract of dried fruit screened in China (500mcg/ml) for cytotoxic activity by cell culture method and found inactive in cell-human-embryonic HE-1 and weak activity in CA-mammary. (Sato,A.1989)

Antiallergenic Activity

Methanol:water (1:1) and aqueous extract of dried unripe fruit screened for antiallergenic activity in rat by gastric intubation and found active against induction of passive cutaneous anaphylaxis in china. (Nishiyori.T.1981)

Extract of dried unripe fruit screened for antimutagenic activity in rat by externally biological activity reported has been patented in Japan.

(Shirota.A.1994)

Action on CNS

80% ethanolic extract screened in S.Korea for 5HT uptake inhibition in rat brainstem neurons by cell culture method and it is active in 100mcg.

(Cho, H.M. *et.al.*,1995)

Anticonvulsant Activity

Aqueous extract of dried unripe fruit screened for anticonvulsant activity (800 mg/kg) by gastric intubation and found inactive against strychnine, picrotoxin induced convulsion in mouse.

Result is significant at $P<0.05$ level. (Hung,N.D.1983)

Anti alzeimer activity

Methanolic extract of immature fruit screened for acetylcholinesterase inhibition activity and found weak activity in South Korea. (Lee, B.H.1997)

Antiinflammatory Activity

Aqueous extract of fruit screened for anti-inflammatory activity by albumin stabilizing assay method and found inactive in South Korea. (Han,B.H.1972)

Antipyretic Activity

Aqueous extract of dried unripe fruit screened for antipyretic activity (200mg/kg) and found active against typhoid vaccine induced pyrexia by gastric intubation in rabbit and mouse in South Korea.

This preparation contains *Bupleurum falcatum, Scutellaria Baicalensis, Paconia Alba*, *Rheum tanguticum, C.aurantium,Pineceia ternatea, Zingiber officiniale* and *Zizyphus inermis*. (Hung,N.D.1983).

Analgesic Activity

Aqueous extract of dried unripe fruit screened for analgesic activity (200mg/kg) by gastric intubation method in mouse and found active against acetic acid induced writhing.

Result is significant at P<0.01 level and also active against pressure pain threshold test. Hung,N.D.1983)

Cyclooxygenase Inhibition activity

Methanolic extract of dried fruit screened for cyclo oxygenase inhibition activity (100mcg/ml) and found inactive in South Korea. (Min, K.R. 1986)

Fruit screened for cyclooxygenase inhibition activity in rat platelets and found weak activity in IC_{50} 0.36mg/ml and incase of lipoxygenase inhibition activity in rat platelets found active in IC_{50} 0.056mg/ml in Japan. (Nogata,Y.1996)

Immunomodulatoy Activity

Aqueous extract of dried fruit screened for immunoglobulin-G induction activity and found weak activity in 0.5mg/ml concentration.

(Bae, E.A. *et.al.*,2000)

Antithyroid Activity

Juice of fresh fruit screened for antithyroid activity by oral route and found weak activity in 1179gm/person dose. In that iodine uptake by thyroid was measured. (Greer,M.A 1948)

Action on CVS

Hot aqueous extract screened for cardiac depressant and cardiotonic activity (3mg/ml) and found active in guinea pig atrium. (Kato.M.*et.al*.,1982)

Chronotropic and Inotropic effect

Extract of dried fruit screened in China, administered by IV route in guinea pig found active in chronotropic effect negative and inotropic effective positive.

Synephrine and methyltyramine were active ingredients. (Anon.1978)

PAF Binding Inhibition

Hot aqueous extract of dried fruit screened in South Korea for platelet activating factor binding inhibition activity in rabbit and found inactive in platelets at 10mg/ml concentration. (Han, B.H.1994)

Antihypertensive Activity

Hot aqueous extract of dried unripe fruit screened in Taiwan for antihypertensive activity in rat and found active in 0.07mcg/kg dose against acute study. PVP was lowered and MAP was elevated and also active in 0.027g/kg dose against chronic study. Elevated the M.A pin PVL. Rats, but had no effect on P.V.P. (Huang,Y.T.1994)

Hepato Protective Activity

Aqueous extract of dried unripe fruit screened for antihepatotoxic activity (1mg/ml) by cell culture method and found inactive in hepatocytes against complement mediated cytotoxicity.

(1:1) ethanol-water extract of dried unripe fruit screened in China for antihepatotoxic activity by cell culture method and found active in hepatocytes against complement mediated cytotoxicity at 1mg/ml concentration.

(Kiso,Y.1990)

Antidiabetic Activity

Aqueous extract of dried fruit equivocal in cholecystokinin receptor binding effect, HGPRT inhibition and HMG-CO-A reductase inhibition effect and weak activity in platelet activating factor binding inhibition and also found

strong activity in adrenergic receptor blocker (α-2) effect. In necrotic doses its inactive in Angiotensin-II inhibition in China. (Han,G.Q.1991)

Fruit juice of fresh fruit was screened in Mexico for anti hyperglycemic activity in male rabbit by intra gastric route and found inactive against glucose induced hyperglycemia at 4mg/Kg dose level.

(Alarcon-Aguilara,F.J. *et.al.*, 1998).

Antiobesity Activity

Extract of dried unripe fruit screened for weight loss activity in human by oral route and found active in obese adult. Anti obesity compounds contain *Citrus* or *Evodia* extracts as fat degradation promoters and >1 substance selected from xanthine derivatives .beta-adrenaline agonists, A_2 -adrenaline antagonist and pipyridine derivatives as an eg: Tablets were formulated containing *C.aurantium* extract 25g. Theophyllin 10g, lactose 30g, Dextrin 25g, Glucose 10g and colouring materials to 100%. The active ingredients also can be incorporated in to cosmetics or bath preparation.

Activity reported has been patented. (Mort,S.1996)

Action on Liver

Juice of fresh fruit screened for cytochrome P450 inhibition activity by cell culture method and found active in microsome of rat liver at 300µl/well concentration. (Edwards,D.J.1996)

Hot aqueous and (40%) ethanolic extract of commercial sample of fruit screened (2.5%) for cytochrome 450 inhibition activity and found inactive and weak activity in China. (Guo,L.Q.2001)

70% Ethanol and ethyl acetate extract of fresh unripe fruit juice screened for cytochrome P450 inhibition activity and found inactive at various concentration in CA-colon-CACO-2.

70%ethanol and ethyl acetate extract of fresh unripe fruit juice screened for glycoprotein secretion inhibition activity and found active at various concentration in CA-colon-CACO-2. (Takanaga,H.*et.at.*,2000)

Hot aqueous extract and (40%) ethanolic extracts are screened for cytochrome p450 inhibition activity (2.5% conc) and found inactive for hot aqueous and weak activity for ethanolic extracts. (Guo,L.Q. *et.al.*,2001)

Enzyme Inhibition activity

Methanolic extract of dried fruit screened in South Korea for multi drug resistance inhibition by cell culture method and found active in CA-KB-V1 at IC_{50} 155.8mcg/ml concentration and also active in CA-human cervical KB-3-1 at IC50 150.2mcg/ml concentration. (Kim, S.E. *et.al.*,1997).

80% methanolic extract of dried fruit screened in S. Korea for tyrosinase inhibition activity (100mcg/ml) and found weak activity (Shin,N.H. *et.al.*,1997).

Intestinal Motility Activity

Aqueous extract of dried unripe fruit screened for intestinal motility stimulation activity (200mg/kg) by gastric intubation method and found active in mouse small intestine.

Result issignificant at P<0.05 level. (Hung,N.D.1983)

Antidiarrheal Activity

Extract of dried fruit screened in S.Korea for anti diarrheal activity in mouse by intragastricroute and found active against 5HT induced diarrhea at 2Gm/Kg. (Yoo, J.S. *et.al.*,1995).

GIT Absorption activity

Juice of fresh fruit screened in USA-FL.for pharmacokinetic alteration activity orally used to ale human adult at 1250ml/dose level. Study to evaluate the oral absorption of Naringin and hesperidin from the citrus products. Results show that they are absorbed from the GI tract after oral administration of pure compound citrus juice or whole grape fruit. (Ameer,B. 1996)

Fruit juice of dried fruit screened for pharmacokinetic alteration activity by oral route in Taiwan and found active in 1.0L/day dose level.

Naringin is the major constituent of grape fruit and is suspected to be one of the causative agents for grape fruit juice interaction. The flavanone absorption of grape fruit juice with znikiao and huajuhong in healthy individuals was compared. Results showed that flavanone absorption of these

herbal decoctions are faster in rate higher in extent, higher in absorption percentage than that of grape fruit juice. (Hou,Y.C. *et.al.*,1999.)

Decoction of dried fruit screened for pharmacokinetic alteration activity by intragastric route in pig and found it is active administration of the decoction with cyclosporine significantly increased the 'C' max and AUC of cylosporin.

Decoction of dried fruit screened for drug interaction activity by intragastric route in pig and found active in blood. Co-administration of cyclosporine with a decoction of the fruits or pericarp significantly increased the blood concentration of cyclosporine.

Decoction of dried fruit screened for general toxic effect by intragastric route in pig and found active in various dose. Co-administration of the decoction with cyclosporine increased blood concentrations and acute toxic reactions due to interaction. (Hou,Y.C. *et.al.*,2000)

Juice of fresh fruit screened for drug interaction activity orally given to both sexes of human adult and found active in 11 healthy volunteers (5 female and males), age ranged from 14 to 43 years long lasting and irreversible inhibitors of gut CYP3A/P-glycoprotein. (Di marco, M.P. *et.al.,* 2002)

Antiulcer activity

Decoction and hot aqueous extract of the dried fruit screened in Japan for antiulcer activity (100mg/Kg, 250mg/Kg respectively) in both sexes of rats by intragastric route and found active against HCl/Ethanol induced gastric ulcer

(69.9, 28.2% inhibition respectively) and weak activity against aspirin induced ulcer (46.6, 36.3% inhibition respectively).($p<0.05$). (Hirano,H. *et.al.*,1997).

Action on Smooth Muscles

Antispasmodic Activity

Hot aqueous extract of dried unripe fruit screened for antispasmodic activity (unspecified type) (1.7mcg/ml) in female rat and found active in uterus (non-pregnancy) in female rat at 1.7mcg/ml concentration.

Hot aqueous extract screened for antispasmodic activity (unspecified type) in female rat and found active in uterus (non-pregnancy) at 10 mcg/ml concentration.

Result is significant at $p<0.02$ level. (Kinoshita.T.*et.al*., 979)

Hot aqueous extract of dried unripe fruit screened for antispasmodic activity (unspecified type) in guinea pig (3mg/ml).It was found inactive against nor epinephrine induced contraction of vas deferens and also found active against histamine induced contraction of ileum and it was active against barium, acetylcholine induced contraction of ileum at 1mg/ml.

(Kato.M.*et.al*.,1982)

Hot aqueous extract screened for antispasmodic activity (unspecified type) (3mg/ml) and found weak activity against nor epinephrine induced contractions in guinea pig vas deferens in Japan.

Effects described are from a multicomponent remedy.

(Kato.M.*et.al*.,1982)

Aqueous extract of dried unripe fruit screened for antispasmodic activity (unspecified type) (10mg/ml), found inactive against acetylcholine induced contraction and active against barium, histamine (1-5 mg/ml) induced contractions in mouse ileum. (Hung, N.D.1983)

Uterine Relaxation activity

Hot aqueous extract of dried unripe fruit screened for uterine stimulant effect and uterine relaxation effect in female rat at 0.1mg/ml concentration in uterus (unspecified condition) and found active in uterine relaxation effect and inactive in uterine stimulant effect. (Kato.M.*et.al*.,1982)

Oestrogenic Activity

Aqueous extract of dried fruit screened for estrogenic effect by cell culture method and found active in various concentration in CA-mammary-MCF-7 in Japan

By placing Oestrogen responsive elements upstream to the reporter gene, and have used this assay to determine the estrogen activity in herbal medicine. (Shizaki,K. *et.al.*,1999)

Antimicrobial Activities

Antibacterial activity:

Tincture of dried fruit screened for antibacterial activity by Agar plate method. It is active against *S. aureus* and inactive against *P. aeruginosa* in 30µL/Disc concentration in Gutemala.

Extract of 10gm plant material in 100ml ethanol. (Caceres.A.*et.al*.,1987)

Aqueous extract of fruit screened in South Korea for (5mg/ml) antibacterial activity by agar plate method and found inactive against *H. pylori* and in case of urease inhibition activity (0.3mg/ml). (Bae,E.A.1998)

Antiviral activity:

Decoction of fruit screened in South Korea (0.05mg/ml) for antiviral activity and found active against virus-*Rotavirus* by cell culture method.

(Kim.D.H.2000)

Decoction of dried fruit screened in South Africa for anti viral activity and found that its activity against virus-*Rotavirus*-WA by cell culture method.

(Song, M.J.1998)

Antiyeast activity:

Fruit juice of dried fruit screened in Thailand for antiyeast activity by agar plate method and found inactive against *Saccharomyces cerevisiae, C. albicans, Cryptococcus neoforman*. (Achararit,C.1983)

*Antifungal Activity***:**

Fruit juice of dried fruit screened in Thailand for antifungal activity by agar plate method and found inactive against *Microsporum gupseum, Trichophyton rubrum, Epidermophyton floccosum*. (Achararit, C.1983)

Phytochemical studies

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
1	Dried fruit	**Alkaloid**-Synephrine-Isoquinoline, N-methyl-tyramine.	-	China	Zneng.H.J.*et.al*., 1983
2	Fresh fruit juice	**Flavanone**-Hesperidin, Neo hesperidin, Narirutin, Naringin.	-	USA-FL (Cult)	Rousett,R.L.1987
3	Fruit	**Flavanone**-Hesperidin, Neohesperidin, Naringin **Coumarin**-Umbelliferone.	-	Taiwan	Lee,Y.C.1989
4	Dried fruit (unripe)	**Flavanone**-Hesperidin	-	China	Chang,M.*et.al*., 1990
5	Fruit juice	**Flavanone**-Neohesperidin, Naringin	-	Spain	Arriaga,F.J.*et.al*., 1990
6	Dried fruit (unripe)	**Flavanone**-Hesperidin, Neohesperidin,	-	Taiwan	Wu,F.J.1992

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
		Naringin, **Coumarin-** umbelliferone			
9	Dried fruit (unripe)	**Flavanone-** Hesperetin-7-o-β-D-glucoside. Prunin	-	Spain	Castillo,J.*et.al*., 1993
10	Fresh fruit juice	**Flavanone-** Eriocitrin, Neoeriocitrin, Neonesperidin, Naringin, Narirutin.	-	France (Cult)	Mouly,P.P.*et.al*., 1994
11	Fruit	**Coumarin** Aurapten, Marmin, 5-[6'-7'-dihydroxy-3'-7'-dimethyl-oct-2-enyl-oxy psoralen] **Flavone-** Nobiletin, Tangeretin flavone.	0.0813 0.0244 0.00036 0.0198 0.0244	China	Satoh,Y.S.*et.al*.,1996
12	Immature fruit	**Flavanone-** Hesperetin,	-	-	He,X.G.*et.al*., 1997

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
		Hesperidin, Neohesperidin, Naringenin, Naringin, Isonaringin, Nobiletin, Tangeritin.			
13	Fruit	**Triterpene-** Limonin, **Coumarin-** (+)Marmin (R), **Flavone-** Nobiletin.	0.00533 0.00297 0.00167	Japan	Hirano,H.*et.al*., 1997
14	Dried fruit	**Flavonol-** Heptamethoxy flavone, Quercetagetin. **Flavone-** Nobiletin, Sinensetin, Tangeretin.	-	-	Del Rio,J.A.*et.al*., 1998
16	Commercial sample of fruit	**Flavanone-** Hesperidin, Neohesperidine.	0.32 0.11	South Korea	Kim,D.H.*et.al*., 2000

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
17	Fresh fruit juice (unripe)	**Flavonol-** 3-3'-4'-5-6-7-8-Heptamethoxy flavone, **Flavone-** Tangeretin, Nobiletin.	-	-	Takanaga,H.*et.al*., 2000
18	Dried fruit	**Isoquinoline alkaloid-Synephrine, N-methyl tyramine.**	-	China	Anon.1978
19	Dried fruit unripe	**Isoquinoline alkaloid-Synephrine**	01.3-2.6	Japan	Kinoshita,T.*et.al*., 1979
20	Dried fruit unripe	**Flavanone-Naringin, Narirutin, Hesperidin, neohesperidin**	-	Japan	Hosoda,K.1988
21	Dried fruit unripe	**Isoquinoline alkaloid-Synephrine**	0.69	Japan	Hosoda,K.*et.al*., 1990
22	Fruit juice	**Triterpene-Limonin, Nomilin.**	-	-	Widmer,W.W. 1991

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
23	Dried fruit unripe	**Isoquinoline alkaloid-Synephrine, N-methyl tyramine.**	-	Japan	Kusu,F.1992
24	Dried fruit	**Isoquinoline alkaloid-Synephrine.**	-	Japan	Obta,I.1994
25	Fresh fruit	**Coumarin-Scoparone.**	-	Spain	Ortuno,A.*et.al*. 1997
26	Dried fruit + leaf + root	'O'alkaloid	-	Nicarague	Coe,F.G.1996

FRUIT ESSENTIAL OIL REVIEW

Pharmacological studies

Antifungal Activity

Essential oil of fruit screened in Paraguay for antifungal activity (Plant pathogens) by agar plate method and found active against several plant pathogenic fungi. (Maruzzela, J.C.1959)

Fruit essential oil screened in Spain for antifungal activity (500mg/) by agar plate method and found active against Phytophthora citrophthora, Penicillium digitatum, Geotrichum species. (Del.Rio, J.A.*et.al*.,1998)

Carcinogenesis Activity

Fruit essential oil screened in USA for carcinogenesis inhibition activity (1% of diet) in female mouse in ration and found active against Bengo (A) pyrene induced carcinogenesis. (Wattenberg,L.1995)

Phytochemical studies

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
1	Fruit essential oil	**Flavonol-** **3-3'-4'-5-6-7-8-Heptamethoxy flavone,** **Quercetagetin.** **Flavone- Nobiletin,** **Sinensetin,** **Tangeretin.**	 0.05 0.16 0.54 0.002 0.14	Spain	Del Rio, J.A.1998
2	Fruit essential oil	**Monoterpene-** **Limonene,** **Limonene,** **Myrcine,** **Myrcine.**	 94.34 92.43 02.075 01.81	 Spain Italy Italy Spain	Boelens, M.H. 1989

PERICARP REVIEW

Ethnomedical information

Pericarp of fruit orally used as a digestive and stomachic in Italy.

(De fee, V.1993)

Dried pericarp of fruit orally used as a carminative, digestive in Spain.

(Vazquez, F.M.*et.al*., 1997)

Pharmacological studies

Toxicity Studies

70% methanolic extract of commercial sample of pericarp screened in China for toxicity assessment (quantitative) in mouse. (MLD >2gm/kg), Abortifacient effect in pregnant rat (500mg/kg dose), Teratogenic activity in pregnant rat (500mg/kg) by gastric intubaion method and found inactive. Dose on day 13 of pregnancy. (Lee,E.B.1982)

Mutagenic Activity

Commercial sample of pericarp screened for mutagenic activity by agar plate method (80µl/disc) and found inactive against *S. typhimurium* TA98 and TA100. Metabolic activation has no effect on the results.

(Schimmer.O.*et.al*.,1994)

Action on CNS

80% ethanolic extract of immature pericarp screened in South Korea for miscellaneous effect by cell culture method (10mcg/ml) and found active against 5HT uptake inhibition in rat brain stem neurons.

(Cho, H.M.*et.al*., 1995)

CNS Depressant Activity

75% Methanolic extract of pericarp screened in South Korea for barbiturate potentiation and barbiturate sleeping time decreased activity by IP route in mouse and found inactive at 500mg/ml dose.

(Woo,W.S. *et.al*.,1978)

Methanolic extract of dried pericarp screened for barbiturate potentiation (500mg/kg), barbiturate sleeping time decreased activity (250mg/kg), antitoxic activity (250mg/kg) in mouse by intraperitoneal route and found active results. Significant at $P<0.001$ level, Inactive. Dose on days 1-3 also found weak activity against strychnine poisoning. (Shin,K.H.,1980)

Action on CVS

Hot aqueous extract of dried pericarp screened in Japan for platelet aggregation inhibition and stimulation against ADP induced aggregation in human and found active in platelets.

PA inhibition effects described are from a multi-component remedy.

A mixture of *Zingiber officinale, Panax ginseng, Citrus aurantium SSP. Nobilis, Glycyrrhiza glabra* and *Atractylodes japonica* was used.

(Okuyama.T.*et.al*., 1987)

Aqueous extract of immature pericarp screened in South Korea for immunosuppressant activity in mouse by intragastric route (500mg/kg) and found active against Con-A-induced proliferation in thymocytes and also against LPS- induced proliferation in splenocytes, screened for nitric oxide

synthesis stimulation and found active in macrophages and also active against LPS induced proliferation in macrophages with gamma interferon.

Statistical data in report indicating significant results. (Yul.J.Y.1998)

Aqueous extract of dried pericarp screened in South Korea for Immunoglobulin-A and G induction (0.5mg/ml) and found weak activity and eguivocal activity respectively. (Bae.E.A.2000)

Methanolic extract of dried pericarp screened in S. Korea for lyrosinase inhibition activity and found active at IC 90. 0.33 mg/ml concentration.

(Choi,B.W.1998)

Action on GIT

Cholerectic Activity

Methanolic extract of dried screened for choleretic activity (1.5gm/kg) in rat by intragastric route and found active in Japan. (Miura,M.*et.al*.,1987)

Intestinal Motility activity

Extract of immature pericrp screened in South Korea for antidiarrheal activity (2gm/kg) by intragastric route in mouse and found active against 5-HT induced diarrhea. (Yoo,J.S.*et.al*.,1995)

Intestinal motility inhibition in mouse by IP route (2gm/kg) found active. Gastric secretory inhibition in rat by IP route (5gm/kg) found active. Pepsin inhibition in rat stomach found active.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp mobilis, Pachyma hoelen, Elettaria*

cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and *Ziziphus vulgaris.* (Hong, N.D.1989)

Antiulcer Activity

Antiulcer activity –rat-IP route (500mg/kg) active against pylorus ligation induced ulcers, mouse-IP route (1gm/kg) active against stress-induced ulcer (water-immersion), rat-intragastric route (1gm/kg) active against histamine induced ulcers, rat-intra duodenal route (1gm/kg) active against aspirin induced ulcers.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp mobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale* and *Ziziphus vulgaris.* (Hong, N.D.1989)

Antiemetic Activity

Antiemetic activity- in frog (2mg/gm) and found active against cupric sulfate induced vomiting.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp mobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale* and *Ziziphus vulgaris.* (Hong,N.D.1989)

Action on Smooth Muscles

Antispasmodic Activity

95% ethanol and aqueous extract of dried pericarp screened for antispasmodic activity (unspecified type) (200mcg/ml) in guinea pig ileum and

found weak activity against histamine induced contractions, active against barium induced contraction, 95% ethanol equivocal against histamine induced contractions, weak activity against barium induced contractions (aqueous extract) in Japan. (Itokawa,H.1983)

Extract of dried pericarp antispasmodic activity (unspecified) in rat ileum (0.005mg/ml) and rat uterus (unspecified condition) (0.001mg/ml) and found active against ACH induced (rat ileum) and (rat uterus) ACH, barium induced contractions.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp nobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and Ziziphus vulgaris.* (Hong, N.D.1989)

Antispasmodic activity (unspecified type) in rat ileum and found (0.001mg/ml) active against barium induced contractions.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp nobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and Ziziphus vulgaris.* (Hong,N.D.1989)

Smooth Muscle Relaxant activity

Extract of dried pericarp screened for smooth muscle relaxant activity (0.001mg/ml) in rat ileum and found active.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium ssp nobilis, Pachyma hoelen, Elettaria*

cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and Ziziphus vulgaris. (Hong,N.D.1989)

Utrine Stimulant Activity

Methanolic extract of pericarp screened in South Korea in rat (1mg/ml) concentration and found inactive in rat ileum for smooth muscle stimulant activity in uterus of rat for uterine stimulant effect. Having strong activity in uterus (estrogen) of rat against oxytocin-induced contractions active in rat ileum against acetylcholine induced contractions for antispasmodic (activity unspecified). Inactive in rat ileum for smooth muscle relaxant activity.

(Woo,W.S.1976)

Uterine relaxation effect in female rat uterus (unspecified condition) found active.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium, Ssp mobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and Ziziphus vulgaris*. (Hong,N.D.1989

Antihistamine Activity

Antihistamine activity in guinea pig ileum (0.001mg/ml) found active against histamine induced contractions.

Preparation contained *Atractylodes macrocephala, Amomum sp, Magnolia officinalis, Citrus aurantium, Ssp mobilis, Pachyma hoelen, Elettaria cardamomum, Panax ginseng, Saussurea lappa, Glycyrrhiza sp, Zingiber officinale and Ziziphus vulgaris*. (Hong, N.D.1989)

Antibacterial Activity

Aqueous extract of commercial sample of pericarp screened in South Korea for antibacterial activity (5mg/ml) by agar plate method and found inactive against *H. pylori*. (Bae, E.A.*et.al*., 1998)

Antioxidant Activity

Methanolic extract of dried pericarp screened for antioxidant activity by intra gastric route in mouse (1.6gm/kg) and found active against ethanol induced lipid peroxidation in mouse liver.

Dose expressed as dry weight of plant. (Han,B.H.*et.al*.,1984)

Antitumor Activity

Ethanolic extract of dried pericarp screened in South Korea for antitumor activity (extract is defatted with petroleum ether) in mouse by IP route (250 mg/kg) and found inactive in sarcoma 180 (ASC), Leuk-SN36, CA-ehrlich-ascites and also screened (quantitative) toxicity assessment and found L_{D50} >0.5 gm/kg. (Woo.W.S.*et.al*, 1977)

Chloroform extract of pericarp screened in South Korea for cytotoxic activity by cell culture method ($IC_{50.}$ 300mcg/ml) and found inactive in cells-human-SNV-I and human-SNV-C4 and Leuk (shay). (Park.J.G.*et.al*, 1993)

Antiradiation Activity

Methanolic extract of dried pericarp screened in Japan for antiradiation effect in mouse by IP route (1000mg/kg) and found inactive against soft X-ray irradiation at lethal dose. (Ohta.S.*et.al*, 1987)

Phytochemical studies

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
1.	Dried pericarp	Flavonol-Rutin.	-	Brazil	Krebs,D. 1972
2.	Pericarp	Flavanone-Neohesperidin, Narngin.	-	-	Wagner,H.*et. al*.,1975
3.	Dried pericarp	Isoquinoline alkaloid- N-methyl tyramine.	-	China	Yun,Y.F. 1981
4.	Pericarp	'O' saponins (unspecified type or hemolytic) present.	-	South Korea	Han,B.H.*et.al* 1981
5.	Dried pericarp	'O' Iridoids absent.	-	South Korea	Chi,H.J.1981
6.	Dried pericarp	Isoquinoline alkaloid-Synephrine.	0.112	Japan	Hashimoto,K .*et.al*.,1992
7.	Pericarp	'O' saponins (foam test) absent, 'O' saponins (hemolysis)	-	-	Han, B.H. 1981

S.No	Parts of plant	Name of the constituents	% yield	Country	Reference
		present, 'O' saponins (unspecified) type or hemolytic absent, 'O' sterols and /or triterpenes.			

FRUITPEEL REVIEW

Ethnomedical information

Hot aqueous extract of fresh fruit peel orally used as an antipyretic, analgesic, tonic, carminative and as an anti rheumatic in Nigeria.

(Adesina, S.K. 1982)

Hot aqueous extract of dried fruit peel orally used for liver disease in China. (Chang, I.M.1984)

Infusion of dried fruit peel orally used as a tonic-eupeptic in Italy.

(Antonone, R. *et.al*., 1988)

Pharmacological studies

Hepato Protective Activity

Extract of dried fruit peel screened for antihepatotoxic activity in mouse and found inactive against chloroform induced hepatotoxicity. Duration of hexobarbital sleeping time was used as a measurement for this activity.

(Yun, H.S.1980)

Methanol: water (1:1) extract of dried fruitpeel screened in South Korea for antihepatotoxic activity in mouse and found active against CCL_4 induced hepatotoxicity. (Chang, I.M.1980)

Methanolic extract of dried fruitpeel screened for antihepatotoxicity in rat and found inactive against CCL_4 Induced hepatotoxicity.

Activity was measured in terms of the elongation of hexobarbital sleeping time after CCL_4 treatment. Elongation of sleeping time indicated negative results. (Choi, S.Y.1982)

Methanolic extract of dried fruitpeel screened for antihepatotoxic activity (670mg/kg) by gastric intubation in mouse and found inactive against CCL_4 induced hepatotoxicity. (Chang,I.M.1984)

Antioxidant Activity

Methanolic extract of dried fruitpeel screened in Japan for antioxidant activity (IC_{50} 26.8 mcg/ml) and found active in microsomes of rat liver against NADPH-ADP induced lipid peroxidation. (Tanizawa,H.*et.al*.,1992)

Anti Viral Activity

Hexane, 95% ethanol extracts of *Citrus aurantium* and *C.aurantium*,var.cri spa are screened for *Epstein-Barr virus* early antigen activation inhibition activity by cell culture method (10mcg/ml) and found inactive in lymphoblasts in human (Raji) and it is inactive against 12-o-tetradecanoyl phorbol-13-acetate (TPA) induced caranogenesis.

(Iwase,Y.*et.al*.,1999)

Anti Obesity Activity

Dried fruitpeel screened in S. Korea for weight loss, cholesterol level decrease, hypotriglyceridemia activity by oral route and found active in obese women and obese women blood. (Choi,J.*et.al*.,2002)

Extract of fruitpeel screened in Italy for antiobesity activity and general toxic effect activity by intragastric route in rat and found active in 2.5mg/kg dose level.

In toxic effect it showed cardiovascular toxicity. (Calapal, G.*et.al*., 1999)

Toxicity Studies

Ethanol: water (1:1) extract screened for toxicity assessment (quantitative) by intragastric route in mouse and the LD_{50} was found to be 476.9mg/kg. (Parra, A.L.*et.al*.,2001)

Anti tumor activity

Ethanol: water (1:1) extract of dried fruitpeel screened in Cuba for anticrustacean activity and found active at IC_{50} 3.99 mcg/ml in *Artemia salina*. Assay system is intended to predict for antitumor activity.

(Parra, A.L.*et.al*.,2001)

Enzyme Induction Activity

Extract of dried fruitpeel screened for cytochrome P_{450} induction activity (700mg/day) by oral route and found inactive against CYP1A2, CYP2D6, CYP2E1, CTP3AA activity. Activity screened in both sexes of human adult.

(Gurley,B.J.2004)

Anti Mutagenic Activity

Methyl chloride extract of dried fruitpeel screened for antimutagenic activity by agar plate method and found active against in furylfuramide (AF-2) induced mutagenesis in *S. typhimurium* TA1535. Suppressed sos response in the UMV test. (Miyazawa, M.*et.al*.,1999)

Phytochemical studies

S. No	Parts of plant	Name of the constituents	% yield	Country	Reference
1	Fruitpeel, (var CV deliciosa) Fruit peel (Var sinensis)	**Alkaloid-** (mis)-Diphenylamine **Alkaloid-** Diphenylamine	0.14, 0.27 0.10, 0.10	Egypt	Karaway, M.S.1986
2	Dried fruit peel	**Alkaloid-** Synephrine-Isoquinoline, N-methyl-tyramine.	-	China	Zneng, H.J.*et.al*., 1983
3	Dried fruit peel	Flavanone-Hesperidin, Naringenin.	10 3.26	-	Moustafa,S. M.I.1984
4	Dried fruitpeel	Flavanone-Hesperidin	0.024	-	Han,D.I.*et.al* .,2001
5	Fruitpeell	Flavonid-Cirantin	0.015	-	Ghosh,B.P.*et .al*.,1955
6	Fruitpeell	Flavonid-Cirantin	-	-	Ghosh,B.P.1 958
7	Fruitpeel	Flavanone-Hesperindin	-	-	Manwaring, D.G.1968
8	Fruitpeel	Flavanone-Hesperidin, Neohesperidin, Naringin,	0.19-0.73 3.01-4.84 2.83-4.25	-	Kim, T.J.*et.al*., 1989

S. No	Parts of plant	Name of the constituents	% yield	Country	Reference
		Poncirin.	0.15-0.25		
9	Ripe fruitpeel Unripe dried fruitpeel	Flavanone-Neohesperidin, Naringin. Flavanone-Neohesperidin, Naringin	3.56 5.59 10.91 11.97	-	Ishhara,S.*et. al.*,1990
10	Fruit peel	Flavone-Apigenin Trimethylether, 4'-5-7-8-tetramethoxy flavone, Nobiletin, Sinensetin, Isosinensetin, Tangeretin.	-	-	Mizuno,M.*et .al.*,1991
11	Fruit peel	Flavanone-Hesperidin	-	-	Wang,J.Z.*et. al.*,1994
12	Fruit peel	Cyclo(gly-leu-leu-leu-pro-pro-phe)-proteid, Cyclo(gly-leu-val-leu-pro-ser)-proteid.	0.0014 0.011	-	Mat sumoto,T.*et. al.*,2002
13	Fruit peel	Coumarin-	-	-	Tatum,

S. No	Parts of plant	Name of the constituents	% yield	Country	Reference
		Scoparone.			J.H.1977
14	Dried fruit peel	Flavanol- Rutin, Rutin.	 0.64 01.2	Pakistan	Shaft,N.1982
15	Fruit peel	Monoterpene- +Limonene	-	-	Letcher,R.M. 1983
16	Dried fruit peel	Isoquinoline alkaloid- Synephrine.	-	Japan	Namba,T.19 85
17	Dried fruit peel	Isoquinoline alkaloid- Synephrine.	-	China	Zu,Z,1987
18	Dried ripe fruit peel Dried unripe fruit peel	Isoquinoline alkaloid- Synephrine. Isoquinoline alkaloid- Synephrine	0.073 0.109	Japan	Shi,L.*et.al*.,1 992
19	Dried fruit peel	Isoquinoline alkaloid- Synephrine	-	Japan	Hashimoto,K .1993
20	Fruit peel	Isoquinoline alkaloid- Synephrine	-	Italy	Calapai,G.*et.* *al*.,1999
21	Dried fruit peel	Flavone- Nobiletin, Tetra-o-methyl- Scutellarein, Sinensetin.	0.0116 0.00116 0.0068	China	Miyazawa,M .*et.al*.,1999

FRUITPEEL ESSENTIAL OIL REVIEW

Pharmacological studies

Antispasmodic Activity

Essential oil of fruitpeel screened in Japan for antispasmodic activity (unspecified type) in mouse intestine and found inactive and found inactive and 2% as active as papaverine. (Haginiwa, J.*et.al*.,1963)

Anti Yeast Activity

Essential oil of fruitpeel screened for antibacterial and antiyeast activity by agar plate method and found inactive against *pseudomonas aeruginosa,* active against *Candida albicans, Bacillus subtius, S.aureus, E-coli.*

(Janssen,A.M.*et.al*.,1986)

Phytochemical studies

S. No	Parts of plant	Name of the constituients	% yield	Country	Reference
1	Fruit peel essential oil	Umonene (mono), Myrcene (-mono), Gamma-Terpinene (mono), α-Pinene (mono), α-Terpineol (mono)	73.8 24.3 0.78 0.30 0.14	-	Kusunose, H.1980

S. No	Parts of plant	Name of the constituients	% yield	Country	Reference
2	Fruit peel essential oil	2-H-1.8(3- ß-D-Glucopyranosyl-oxy-2-hydroxy-3-methyl-butyl)-7-methoxy benzopyran-2-one-coumarin, Columbianetin-O-ß-D-Glcopyranoside-coumarin, Meranzin-coumarin, Meranzinhydrate-coumarin, Isomeranzin-coumarin	0.03271 0.01301 0.00429 0.01714 0.00429	-	Mc Hale,D.*et.al.*,1987
3	Unripe fruit peel essential oil Ripe fruitpeel essential oil	**Monoterpene**-Limonene, Linalool, Linalool acetate, **Monoterpene**-Limonene, Linalool, Linalool acetate, **Sesquiterpene**-Nootkatone, α-Selinenone.	-	-	Boelens,M. H.1989

S. No	Parts of plant	Name of the constituients	% yield	Country	Reference
4	Fruitpeel essential oil	**Monoterpene-** Limonene, Linalool, Linalool acetate. **Sesquiterpene-** Farnesol, Nootkatone.	 80.1 5.5 0.8 0.5 0.7	-	Chialva, F.1990
5	Fruitpeel essential oil	**Monoterpene-** Camphene,Car-3-ene, Terpinolene	-	-	Dugo, G. 1993
6	Fruitpeel essential oil	**Monoterpene-** +Limonene	90.4	Brazil	Carvalho freitas,M.I. R.2002
7	Fruit peel essential oil	**Monoterpene-** **Limonene,** **A-Pinene.**	97 -	Japan	Haginiwa,J *.et.al.*,1963
8	Fruit peel essential oil	**Triterpene-** **Friedelin**	-	-	Chandler, R.F. 1979

SEED REVIEW

Pharmacological studies

Cytotoxic Activity

Aqueous extract of dried seed screened in China for cytotoxic activity by cell culture method (500mcg/ml) and found inactive in human embryonic cells HE-1 and weak activity in CA-mammary micro alveolar. (Sato.A.1989)

Antigen Activation Activity

Hexane and 95% ethanolic extract of dried seed screened in Japan for Epstein-Barr virus early antigen activation inhibition activity (10mcg/ml) by cell culture method and found inactive against 12-O-tetradecanoylphorbol-13-acetate (TPA) induced carcinogenesis in lymphoblasts human-Raji.

(Iwase,Y.*et.al*.,1999)

Phytochemical studies

Parts of plant	Name of the constituients	% yield	Country	Reference
Dried seed	Triterpene- Limonin, Nomilin, Deacetyl nomilin, Obacunone	-	USA-FL (cult)	Rousett,R.L. 1982
Dried seed	Triterpene – Ichangin-17- β-D-glucoside,Isolimonic acid, β-D-Glucoside, Limonin-17-o- β- D-glucoside, Deacetyl nomilin-17-o- β-D-glucoside, Nomilin-17- β-D-glucoside, Deacetyl nomilinic acid-17- β-D-glucoside, Deacetyl nomilinic acid-19-(OH)-17- β-D-glucoside, Nomilinic acid -17- β-D-	-	-	Benneh, R.D.1991

Parts of plant	Name of the constituients	% yield	Country	Reference
	glucoside, Obacunone-17- β-D-glucoside.			
Dried seed	Triterpene- Ichangin, Isolimonic acid, Limonin, Nomilin, Nomilin glycoside, Deacetyl nomilin, Nomilinic acid, 19-(OH) deacetyl nomilinic acid aglycone, Deacetyl nomilinic acid, Obacunone Nomilinic acid,	 130ppm 110ppm 2470ppm 178ppm 360ppm 840ppm 20ppm - 120ppm 32ppm	-	Miyake, M.*et.al*.,1992
Fresh seed	Triterpene- Ichangin, Isolimonic acid, Nomilinic acid, Deacetyl nomilinic acid.	0.0093 0.0066 0.0008 0.0072	-	Bennett,R.D. 1980
Seed	Triterpene-Limonin, Deacetyl nomilin.	-	USA-CA	Dreyer, D.L.1966.

SEED ESSENTIAL OIL REVIEW

Pharmacological studies

Antimicrobial Activity

Oil of dried seed screened for antibacterial, antifungal, antiyeast activity by agar plate method (10mg/ml) and found inactive against *S. viridans, Diplococcus pneumoniae, C. diphtheriae, S. aureus, Streptococcus pyogenes,*

Piedraia hortae, Microsporum canis, Microsporus gypseum, Trichophyton mentagrophytes, Phialophora jeanselmei, Candida albicans, Candida tropicalis and also screened for anthelmintic activity and found inactive against Anthelmintic parasite. (Naovi,S.A.H. *et.al.*,1991)

ROOT REVIEW

Ethnomedical information

Extract of dried root and fruit of *C aurantium* AFF orally used for polio in Tanzania. (Hedberg,I. *et.al.*, 1983)

Aqueous extract of dried root of *C.aurantium* aff orally used for stomach upset in Tanzania. (Hedberg, I. *et. al.*, 1983)

Decoction of dried root, fruit and leaf are orally used for fever, hypertension, diarrhea, ulcers and digestive for stomach ache in Nicaragua.

(Coe,F.G.1996)

Pharmacological studies

Protein Binding Activity

Dichloromethane extract of dried root screened in France for P-glycoprotein binding activity and found active in erythroleukemia (K562).

Mutagenesis

Dried root screened in France for multi drug resistance efflux pump inhibition by cell culture method (017.5mcg/ml) and found active in erythroleukemia (K562) against DMBA induced mutagenesis.

(Simon,P.N.*et.al.*,2003)

Phytochemical studies

S. No.	Parts of plant	Name of the constituients	% yield	Country	Reference
1	Root	Coumarin- Braylin, Geranyl-oxy pyranocoumarin, Seselin, Suberosin, Xanthoxyletin, Xanthyletin.	-	-	Nordhy, H.E. 1981
2	Fresh root	Coumarin- Seselin.	-	Isravel	Tomer,E. *et.al.*,1969
3	Dried fruit + leaf + root	'O'alkaloid	-	Nicarague	Coe,F.G.1996

CALLUS TISSUE REVIEW

Phytochemical studies

S. No.	Parts of plant	Name of the constituents	% yield	Country	Reference
1	Callus tissue	Sesquiterpene- Nootkatone, Valencene.	-	Spain	Del rio,J.A.1991

CHAPTER -3

AIM AND OBJECTIVE

Volatile oils (V.O) are valuable natural products find applications in many areas, including pharmaceuticals, cosmetics, perfumes, Aroma therapy, phytotherapy, spices 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
- Sesquiterpenes

- Oxygenated compounds
- Phenols
- Alcohols
- Monoterpene alcohols
- Sesquiterpene alcohols
- Aldehydes
- Ketones
- Esters
- Lactones
- Coumarins
- Ethers
- Oxides
- **Terpenes Hydrocarbons:**
- **Monoterpene** - anti-inflammatory, antiseptic, antiviral and antibacterial therapeutic properties
- **Sesquiterpenes -** It has anti-inflammatory and anti-allergy properties.
- **Oxygenated compounds:**
- **Phenols** - great antiseptic, anti-bacterial and disinfectant qualities
- **Alcohols**
- **Monoterpene alcohols** - good antiseptic, anti-viral and anti-fungal properties
- **Sesquiterpene alcohols** - liver and glandular stimulant, anti-allergen and anti-inflammatory property.

- **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.
- **Lactones and coumarins** - great mucus moving and expectorant 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 aurantium.* To study in detail one of its constituents. Further to evaluate quantitatively the therapeutic effect and tio determine innate immunity *in vivo* using invertebrate model organism infected with human pathogenic micro organism.

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. Scanning Electron Microscopic study (SEM).

4. 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.

- Determination of trace elements present in the leaves by Energy Dispersive X-ray analysis (EDAX).

- Isolation of V.O from the leaves of *C.aurantium.*

- 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:-

- Collection of 5^{th} instar larvae of Bombyx mori

- To evaluate the toxicity of the isolated VO of the leaves of *C.aurantium.*
- To screen the in vitro antibacterialeffect of the VO and synthetic drug of choice using micro dilution method against *S.aureue* and MRSA.
- To examine the surface morphological changes of VO treated *S.aureus* using SEM.
- To determine MIC of VO and drug of choice against MRSA.
- To study the intra hemolymph (Equivalent to IV in human) intra midgut (Equivalent to PO in human) route in the silkworm larvae.
- To evaluate quantitatively the therapeutic effect of the VO, drug of choice invivo using silkworm model infected with human pathogenic microorganism MRSA both intra hemolymph (ih) and intra midgut (im) route after determination of theoretical minimal effective dose (tED_{mini}) using MIC.
- To determine ED_{50} / MIC ratio to evaluate pharmacokinetic problems.
- To evaluate the innate immunity by muscle contraction method in silkworm larvae
- To determine the antiplasmid activity of VO, drug of choice using *E.coli* F'*lac* strain *in vitro*

CHAPTER – 4

MATERIALS AND METHODS

4.1. PLANT COLLECTION AND AUTHENTIFICATION

The leaves of the plant *Citrus aurantium* L. selected for our study was collected from **Chinthamani, Villupuram District**, Tamil Nadu, India during the month of January 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 aurantium* L.

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

4.2.2. Microscopical studies on the leaf of *C.aurantium*

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 [108]. 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 potassium iodide (for starch).

For studying the stomatal morphology, venation patttern and trichome distribution, **paradermal sections** (sections taken parallel to the surface of leaf as well as **clearing** of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid (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 labphot 2 Microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light were employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifigations of the figures are indicated by the scalebars. (Johansen.D.A, (1940),Purvis.M.J.et.al 1966)

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 (Johnsen, 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. MICROSCOPICAL STUDY OF LEAF USING SCANNING ELECTRON MICROSCOPE

Scanning Electron Microscope (SEM)

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

Usage

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

pattern to a few simple characters [Heywood.V.H]. SEM plays a vital role when a specimen need to be satisfactorily defined in terms of characters. For most biological materials, maximum information is obtained by employing light and electron microscopy jointly and an attempt was made by applying SEM to the leaf of *C.aurantium*, to pinpoint the positions of specific characters with in the cell, which can be easily seen in final image.

SEM sample preparation

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

4.2.6. 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 Values (Individual Solvent)

- **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°C and weighed. The percentage of the petroleum ether soluble extractive value was calculated with reference to the air dried powder.

- **Ethyl Acetate Soluble Extractive Value**

Five gram of the coarsely powder was macerated separately with 100ml of ethyl acetate 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 ethyl acetate. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethyl acetate 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.

EXTRACTIVE VALUES (Successive Solvents)

❁ ***By using solvents successively with increasing order of polarity***

Five grams of the coarsely powder was extracted continuously in soxhlet apparatus for six hours individually, separately with solvents of increasing order of polarity. After six hours, the solvents was removed and evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}c and weighed. The percentage of the individual solvent 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 acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20ml filtrate; 1ml concentrated ammonia was then added. The mixture was then shaken with two portions of 10ml diethyl ether. The ether was dried over anhydrous sodium sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.

- Powdered drug was mixed with one gram of calcium hydroxide and 5ml of water, made into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. 20ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.

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

1. Mayer's reagent

2. Dragendorff's reagent

3. Hager's reagent

4. Wagner's reagent

Test for 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.

Anthraquinones

- ***Borntrager's test***

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

❖ ***Modified Borntrager's test***

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

Test for cyanogenetic glycosides

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

Test for cardiac glycosides

❖ ***Keller Killiani 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.

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.

(WHO,1998.Chaudhri.R.D.1999,,Kokate.C.K,2005,Agarwal.2007,Horbone.JB. 1973)

4.3.2. FLUORESCENCE ANALYSIS

Powdered leaf material of *C.aurantium* was subjected to analysis under UV light after treatment with various chemical and organic reagents like Ethanol, Ethyl acetate, Chloroform, Water, 50% sulphuric acid, 10% sodium hydroxide, 50% nitric acid and dried leaf powder. (Horbone. JB.1973)

4.3.3. DETERMINATION OF TRACE ELEMENTS IN LEAF OF *C.aurantium* BY ENERGY DISPERSIVE X-RAY ANALYSIS (EDAX)

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

EDAX analytical capabilities

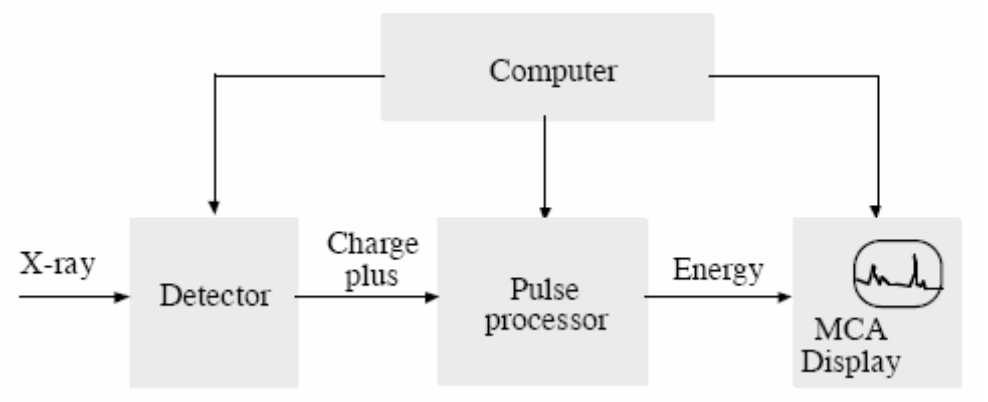
Back scattered electron images in the SEM display compositional contrast that results from different atomic number elements and their distribution. EDAX is used to find particular elements and their Atomic %. The Y-axis shows the counts (number of X-rays received and processed by the detector) and the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

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

EDAX system comprises of 3 basic components

- An X-ray Detector - detects and converts X-ray into electronic signals.

- A Pulse Processor - measures the electronic signals to find out energy of each X-ray detected.
- A Multiple Channel Analyzer - interprets and displays analytical data.



EDAX is an analytical technique in which the specimen emits X-rays due to the bombardment of electron beam on it which is used to identify the elemental composition of the specimen due to the ejection of electrons from the atoms on the specimen surface. To explain further, when the sample is bombarded by the electron beam of the SEM, electrons a re ejected from the atoms on the specimens surface. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The EDS X-ray detector measures the number of emitted X-rays emitted versus their energy.

The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy versus relative counts of the detected x-rays is obtained and evaluated for the determinations of the elements.

4.3.4. ISOLATION OF VOLATILE OIL (V.O) FROM THE LEAVES OF *C.aurantium* L.:

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)

4.3.5 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, 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 physical constant 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.

4.3.6. 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 impinge upon the collector; the signal received is amplified and recorded. The height or intensity of the resulting peak is proportional to the ion abundance.

COMBINATION OF GC WITH MASS SPECTROMETRY

The identification of fractions in gas chromatography is essentially comparative, in that the characteristic of the unknown are compared with those of known library compound. By correct choice of column, the fraction consists of single substance only, so that, if each is examined by other methods for identification, a powerful analytical tools becomes available. This may be done in several ways and GC is now used in 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 produce a wealth of data rapidly. To process and interpret all of this data manually would be excessively time consuming.

[Comment]

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

[GC-2010]

Column Oven Temp.	:70.0 ℃	
Injection Temp.	:200.00 ℃	
Injection Mode	:Split	
Flow Control Mode	:Linear Velocity	
Pressure	:98.8 kPa	
Total Flow	:19.6 mL/min	
Column Flow	:1.51 mL/min	
Linear Velocity	:45.1 cm/sec	
Purge Flow	:3.0 mL/min	
Split Ratio	:10.0	
High Pressure Injection	:OFF	
Carrier Gas Saver	:OFF	
Splitter Hold	:OFF	
Oven Temp. Program		
Rate	Temperature(℃)	Hold Time(min)
-	70.0	2.00
10.00	300.0	7.00

< 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]

IonSourceTemp	:200.00 ℃
Interface Temp.	:240.00 ℃
Solvent Cut Time	:3.00 min
Detector Gain Mode	:Relative
Detector Gain	:0.00 kV
Threshold	:1000

[MS Table]

--Group 1 - Event 1--	
Start Time	:3.00min

End Time	:32.00min
ACQ Mode	:Scan
Event Time	:0.50sec
Scan Speed	:2000
Start m/z	:40.00
End m/z	:1000.00
Sample Inlet Unit	:GC
[MS Program]	
Use MS Program	:OFF

(Horbone.J.B.1973)

4.4. PHARMACOLOGICAL STUDIES

Introduction

Resistance to anti microbial agent has resulted in treatment failures and enhancement in health care costs. Now there is only a little doubt that emerging antibiotic resistance is a serious global problem.

Suitable antibiotics use has unquestionable benefits but the patients use these agents inappropriately. Physician provides antimicrobials to treat viral infections also results in an inappropriate usage and using insufficient criteria for diagnosis of infections that have a bacterial etiology, unnecessarily prescribing costly, broad spectrum agents and not considering established recommendations for using chemoprophylaxis also results inappropriate usage.

This inappropriate use of antibiotic exerts a pressure that drives in the development of antibiotic resistance. The association between increased rates of antimicrobial use along with the resistance has been documented for nosocomial infections and resistant community acquired infections.

Principle:

The principles of determination of effectiveness of antibacterial agents to a bacterium were well enumerated by Rideal, Walker and many scientists at the turn of the century. The discovery of antibiotics made these tests too difficult for the large numbers of tests necessary to be put up as a routine. The ditch plate method of agar diffusion used by Alexander Fleming introduced variety of agar diffusion methods. The Oxford Group used these initially to evaluate the antibiotic contained in blood by allowing the antibiotics to diffuse out in the medium in containers placed on the surface.

With the advent of numerous antimicrobials, it is necessary to perform the antibacterial susceptibility test as a routine. In this method the antimicrobials contained in a reservoir was allowed to diffuse out into the medium and act in a plate freshly inoculated with the test organisms. Though different antimicrobials containing reservoirs are used, but the antimicrobial impregnated absorbent paper disc is by for the commonest type followed. The diffusion method is the mostly the method of choice for the average laboratory in this country as well as smaller laboratories of even advanced countries and it will certainly be the commonest microbiological test for many years to come..

4.4.1 Antimicrobial susceptibility testing methods:

The methods are divided into types based on the principle applied in each system. They include:

Diffusion	Dilution	Diffusion and dilution
Stokes method	Minimum inhibitory Concentration	E test method
Kirby-Bauer method	i) Broth dilution ii) Agar Dilution	

Disk Diffusion:

Reagents:

1. Mueller-Hinton Agar Medium: It is considered to be the best for routine susceptibility testing for the following reasons:
   - It shows acceptable batch to batch reproducible results
   - It gives satisfactory growth of most pathogens
   - A large body of data and experience available concerning susceptibility tests performed with this medium

We used formulations that have been tested according to and that meet the acceptance limits described in NCCLS document M62-A7- protocols for evaluating Dehydrated Muller-Hinton Agar.

Preparation of MH Agar:

It includes the following steps:

1. It was prepared from commercially available (HI-Media) dehydrated base according to the manufacturer's instructions.
2. It was allowed to cool in a 45-50ºC water bath.

The freshly prepared cooled medium was poured into glass flat bottomed Petri dishes on a level, horizontal surface to give a uniform

depth of approximately 4mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30ml for plates with a diameter of 100mm.

3. This medium was allowed to cool to room temperature and if it was not used in the same day, stored in a refrigerator (2ºC to 8ºC) and used within 7days and wrapping in plastic was undertaken to minimize drying of the agar.
4. Sample of each batch of plates was examined for sterility by incubating at 30-35º C for 24hrs or longer.

Antimicrobial discs:

Commercially available cartridges containing paper discs (HiMedia) specifically for susceptibility testing were used and maintained according to the manufacturer's instructions.

Turbidity standard for inoculum preparation:

To standardize the inoculum density for the susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland Standard was prepared.

1. A 0.5ml aliquot of 0.048ml/L$BaCl_2$(1.175%w/v$BaCl_2$,2H_2O) was added to 99.5ml of 0.18 ml/L H2SO_4(1%v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard was verified by using a spectrophotometer (Shimatzu UV1800). The absorbance at 625nm was adjusted to be 0.008 to 0.01 for the 0.5McFarland standard,

3. The barium sulphate suspension was transferred in to 4 to 6 ml aliquots in to screw cap tubes of the same size as those used in growing or diluting bacterial inoculum, tightly sealed and stored in the dark at room temperature.
4. It is vigorously agitated on mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

Disc diffusion method:

The Kirby-Bauer and Stokes' methods are usually used for AST. Kirby-Bauer method recommended by NCCLS is usually recommended. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedure described here. NCCLS is approved by FDA-USA and recommended by **WHO.**

Procedure for performing the Disc Diffusion Test:

Inoculum preparation:

Growth method: At least 3 to 5 isolated colonies of MTCC No,260 strain of *S.aureus* were selected from an agar plate culture. The top of each colony is touched with a loop and the growth is transferred in to a tube containing 4 to 5ml of broth medium. Broth culture was incubated at 35°C until it achieves turbidity of the 0.5 McFarland standard (usually 2 to 6hrs). The turbidity of the actively growing culture was adjusted with sterile saline or broth to obtain a turbidity optically compared to that of the 0.5McFarland standard. This results in a suspension containing approximately1to 2 X 108

CFU/ml for *E.Coli* ATCC 25922. To perform this step properly, visually adequate light is needed to compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

Inoculation of Test Plates:

1. Optimally, within 15mts after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

2. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60^{0} each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

Application of Discs to inoculated Agar plates:

The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated plates distributed evenly so that they are no closer than 24mm from centre to centre. Each disc was pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 35^{o} C within 15mts after the discs were applied.

Reading Plates and Interpreting Results:

After 24 hours (16 to 18 for some organisms) of incubation, each plate was examined for resulting zones of inhibition uniformly circular and confluent lawn of growth. The diameters of the zones of complete inhibition as judged by unaided eye were measured, including the diameter of the disc, using ruler held on the back of the inverted Petri plate, to the nearest whole millimeters. Transmitted light (plate held up to light) was used to examine the zones for light growth of methicillin resistant colonies, within apparent zones of inhibition.

DILUTION METHOD:

Dilution susceptibility testing methods are used to determine the MIC (Minimum inhibitory concentration- The minimal concentration of antimicrobial to inhibit or kill the microorganism) this can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials were tested in $\log_2$ serial dilutions (two fold).

Procedure:

Sterile graduated pipettes of 10, 5, 2 and 1ml sterile capped 7.5 x 1.3 cm tubes /small screw capped bottles, Pasteur pipettes, overnight broth culture of *S.aureus*, antibiotic powder form and a suitable rack to hold tubes, DMSO

Sterile tubes were arranged in rack in two rows. 2ml of broth containing the specified quantity of VO was transferred using a pipette to the first tube in each row. Using a fresh pipette 4ml of broth was added and mixed well and

transfer 2ml to the second tube in each row and dilution was continued.2ml Control broth containing vehicle without test drug or standard drug was placed in the last tube in each row. Inoculation was done with one drop of an over night broth culture of the test organism (10^6 organism/ml) Then incubated for 24 hrs at 37^o C. A tube containing 2ml broth with the organism was kept at 4^o C in a refrigerator overnight to be used as standard for the determination of complete inhibition.

Micro-broth dilution test:

MTCC strain No. 260 of *S.aureus* was cultured in Luria-Bertani 10(LB10) medicum (10g bactotryptone, 5g yeast extract, 0g NaCl per litre) at 37 ^{o} C for 24 h. The full growth culture was diluted to 1/1000 in Mueller-Hinton (MH) medium and then a 100µl aliquot was added to each well 96 well plate. V.O of the leaf of *C.aurantium* was serially diluted 2 fold and 100µl of each dilution was added to the bacteria solution and cultured at 37 ^{o} C for 24h. Bacterial growth was visually determined. The lowest concentration showing inhibition of growth was considered the MIC. Same test was carriedout for the standard antibiotic Linezolid.

Reading of the result:

MIC is expressed as the highest dilution which inhibited growth judged by the lack of turbidity in the tube. As very faint turbidity may be given by the inoculum itself, the inoculated plate kept in the refrigerator overnight was used as the standard for the determination of complete inhibition. Control run with the test was used as the control to check the reagents and conditions.

4.4.2 In vivo Screening process:

To better understand bacterial pathogenisity, the use of animal models is essential. But various problems are inherent during the use of animal model such as high cost of housing animals and ethical issues which has become more serious in recent years. Laws strictly regulate the use of mammals for the development of medicine. To overcome these problems, the use of invertebrates such as *D.melanogaster* has been suggested.

These are very powerful tools for identifying host proteins involved in immune systems because they are genetically tractable and many mutant lines have been constructed. But some of these animals are too small to handle and they are not suitable for injecting precise volumes of samples into the body fluid, a technique that is important for quantitative evaluation of bacterial pathogenisity and the therapeutic effects of antimicrobial compounds.

Advantages of the silkworm infection test system:

Silk worms have many advantages for studying bacterial pathogenicity and the therapeutic effects of antibiotic. Silkworm, Lepidoptera ZS: *Bombyx mori* was domesticated over the past 5000 years for obtaining silk fibers from its cocoons. It can be raised from fertilized eggs to 5^{th} instar larvae for three weeks. (Plate 15)

There are no ethical problems and biohazards associated in the use of a large number of silkworms. The body size of silk worm 5^{th} instar larvae (5cm) is large enough to handle, sample solution of pathogens and drug samples can be injected into the hemolymph or gut of the larvae using syringe and needles.

It was also shown that the results of whether antibiotics can be absorbed or not were similar in silkworms and mammals. Metabolic pathways with cytochrome P_{450s} and conjugated enzymes were also similar. But in other small organism it should be performed under microscope. Hence low cost, no ethical issues, available to accurate injection into hemolymph and gut, available to pharmacological test with isolated organs and no biohazards are favorable features.

Injection into hemolymph and midgut:

Previous studies indicated that doses of chemicals in silkworm were consistent with those in mammals when normalized by body weight. Silk worms share conserved mechanisms for the pharmacokinetics of chemicals absorption, distribution, metabolism and excretion (ADME) with mammals.

When red coloured beetroot water extract was injected into the hemolymph of silkworms, whole body colour immediately changes to red, because the insect has an open circulatory system. If the needle is introduced deep enough to reach into the midgut, the red solution disperses throughout the midgut without changing the body colour. (Plate) These features allow for the evaluation of bacterial pathogenisity and the therapeutic effects of antibacterial agents. Thus it is a basic system of study of "Infection and therapy". M

Recently, the silkworm genome project was completed by Japanese and Chinese groups by reverse genetic methods, RNA interference etc were established in silkworms. Moreover the information obtained based on the

genome and RNA interference method will facilitate the study of host factors involved in infectious screening. (Kaito,et *al*., 2007).

When *S. aureus* was injected into the hemolymph of silkworm larvae, all of the larvae were died within 2 days. All the control group which were injected with DMSO and saline. Chloramphenicol has therapeutic effect as all of the worms injected with 100µg (Plate) were alive. Previous studies also reported the antibiotics used for clinical purposes have therapeutic effects on silkworm infected with pathogens (Hamomoto *et.al* 2004).

4.4.3 EVALUATION OF THERAPEUTIC EFFECT OF VO OF THE LEAF OF *C.aurantium* USING SILK WORM MODEL *in vivo*:

Determination of LD_{50} in silk worm:

5^{th} instar larvae were reared for one day at room temperature with normal feeding. The larvae (n=6) were injected with 2fold serial dilution of the VO (50µl) solution in to the hemolymph and reared at room temperature. The number of surviving larvae was counted 2 days later. The LD_{50} was determined by the survival curve as the dose that killed half of the larvae (LD_{50}). For calculating LD_{50} the least tolerated (smallest) dose (100% mortality) and most tolerated (highest) dose (0% mortality) was found out by hit and trial method.

5 doses in between these doses were selected and the mortality due to this doses were observed (n=6).

Calculation of the theoretically minimal effective dose of VO required for the treatment of infected silkworms:

The fifth instar larva (2g) used for therapeutic assay has a hemolymph with volume of approximately 500µl. Therefore injection of 50µl of VO into the silkworm hemolymph results in a 1:10 dilution. The putative minimal effective dose of VO minimally required to achieve therapeutic effect on the infected silkworm, tED_{mini} (µl/g larva) was theoretically defined based on the following formula using the MIC.

Method:

Fully grown *S.aureus* culture was diluted 10 fold with normal saline. A 50µl aliquot was injected into the hemolymph of the silkworm. VO (50µl) Diluted with PBS was further injected into the hemolymph and midgut. The silkworms were reared at room temperature and the number of surviving silkworms was counted 2days later. Linezolid (30µg/ml) was administered as a positive control along with normal control. To determine the number of viable bacteria in the hemolymph, the hemolymph was collected 1 day after the injection of VO and diluted in normal saline before spreading on mannitol-salt agar plate. The plates were incubated at 37 ^{o} C for overnight and the colonies were counted. Percentage curve was plotted against dose administered. The value of ED_{50} were foundout from the graph.

4.4.4 Assay for stimulants of natural immunity:

We undertake screening for stimulants of innate immunity using the silk worms. There are two types of protection in mammals. It is known that acquired immunity which depends on antibodies and innate immunity which is independent on them. A silkworm lacks acquired immunity but instead protects

itself through innate immunity. It was previously reported that muscle of silkworms is contracted by stimulation of innate immunity. For this purpose, we use silkworm muscle contraction as an index. A unique phenomenon, in which the muscle of the silkworm contracts and silkworm's length gradually decrease.

When stimulants activate immune cells, reactive oxygen species (ROS) are released. These act on serine proteases, which activate BmPP (*Bombyx* paralytic peptide), which in turn induces muscle contraction. Therefore, by observing the circumstances of muscle contraction valuable stimulants of innate immunity can be detected.

Method:

Two sets of three groups of (n=6) 5^{th} instar healthy larvae fed normally were selected. First three groups were injected µl/g intra hemolymph and another three groups midgut. Initially head and tail portion of the first group were removed and the muscle specimen was hung in between metal claws. Then the second group was treated similarly. Muscle contraction was measured after 10mts using a scale.

4.4.5 ASSAY FOR F' lac PLASMID ELIMINATION ACTIVITY:

A major problem in antimicrobial chemotherapy is the increasing occurrence of resistance to antibiotics, which leads to the insufficiency of antimicrobial treatment. The misuse of antibiotics and consequent antibiotic selection pressure may be the most important factor contributing to the

appearance of different kinds of resistant microbes. The genetical basis of resistance is often the R-plasmid, which can be transferred to other bacteria in the environment of the recipient and these extra chromosomal DNA sequence can be responsible for the emergence of multiple resistance to antibiotics. Plasmid may be lost spontaneously in a very low frequency, but certain effects can increase the probability of plasmid loss, which is the basis of artificial plasmid elimination. In early studies acridine orange, ethidium bromide, sodium dodecylsulphate were found to be powerful plasmid eliminators. But their toxicity did not allow their in vivo testing for their antiplasmid effect. The possible mechanism of action is complex formation with the guanine-cystine-rich regions of the plasmid DNA which is necessary for normal plasmid replication in an uncomplexed form.

In the present study besides the antibacterial effect their anti plasmid activities on an *E.Coli* F'lac strain were determined.

Method:

An overnight preculture of E. coli F'lac K_{12} was diluted 10^4 fold in physiological saline solution and inoculated in 0.05µl aliquots (approximately 5 X 10^3 cells) into 5ml of MTY nutrient broth.

Low dilution of VO (0.125µl/ml), linezolid 1µg/ml and 0.3mg/ml menthol (positive control) were used for the qualitative observation of plasmid elimination. The tubes were incubated at 37° C for 24h without shaking.

A 10^5 fold concentrations were prepared from the tubes showing growth and plated in 100µl amounts on EMB agar. The plates were incubated at 37°C for 24h and were observed for *lac*$^-$ plasmidless (pink) and *lac*$^+$ plasmid containing (deep violet) colonies

CHAPTER - 5

RESULTS

5.1 PHARMACOGNOSY

5.1.1 Morphological features of *C.aurantium*

It is a tree with greenish white, glabrous shoots. (Plate-1, Fig -3).

Stem :

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

Shape	:	Foliate, elliptic
Colour	:	Whitish green
Margin	:	Serrated margin
Apex	:	Acuminate
Base	:	Symmetrical
Petiole	:	winged
Inflorescence	**:**	Larger more strongly scented
Fruits	**:**	Bisexual

5.1.2 MICROSCOPY OF LEAF:

The leaf is dorsiventral with prominent midrib. Lamina is thick dorsiventral differentiated.

LEAF MIDRIB (Plate 6)

In transectional view,

Shape	: Circular broad
Adaxial side	: 1.Prominent elevated round in shape,thick
	2. 630µm thick, 300µm wide
Abaxial side	: 1. less thick
	2. 400µm wide

Epidermis of midrib (Plate 7,,10,11)

Adaxial Epiermis	:	Squarish thick walled with smooth cuticle
		Polygonal in surface view with thick straight wall Apostomatic
Abaxial Epiermis	:	Thick with papillate surface due to cuticular outgrowth larger cells with slightly wavy
walls in surface view	:	Cyclocytic stomata are present, where the stoma is encircled by three inner whorls, 5 or 6 outer whorl of subsidiary cells (Plate No9)
		The guard cells are 40-50 µm in size
Collenchyma	:	3 or 4 layers of cells inner to the both epidermis are present
Vascular system	:	Large and double stranded. (Adaxial and Abaxial)
Adaxial strand	:	Comparatively narrow, horizontal
Abaxial strand	:	Wide, shallow arc
Xylem elements	:	Both strands have several short, compact, parallel rows of xylem (both vessel and fibre)
	:	The vessels are angular to circular and thick walled 1 µm wide (Plate .8)

Phloem	:	Present as thick are beneth the abaxial bundle and outside the xylem of abaxial strand
Sclerenchyma	:	Both abaxial and adaxial bundles have thick sclerenchymatous caps lying outside the phloem composed of thick lignified fibres.
Ground tissue	:	Large thin walled compact parenchyma cells

LAMINA (Plate 10, 11,12,13): It is 160 µm thick. The mesophill is differentiated into adaxial zone of three layers of short palisade cells and wider abaxial zone of compact layers of spongy parenchyma with wide air chamers in the middle part vascular strnds of lasteral veins are present

Secretary cavity	:	Wide circular secretary cavities surrounded by fairly thick spindle shaped epithelial cells (200 µm diameter) with amorphous inclusion.
Ctystals	:	Exclusively abundent prismatic calcium oxalate crystals. The distribution pattern is characteristic. They are located in sub epidermal layers of adaxial epidermis. The cell bearing crystals are wide circular filled with mucilage and called as crystal Idioblast
Venation pattern	:	The lateral veins and vein islets are thick and prominent. They are straight and form distinct of polygonal vein-islets with thick vein boundaries.

The vein terminations are distinct. They are thick and simple (or) branched. Branching is once or repeated resulting in dendroid vein-termination.

PETIOLE (Plate 14)

Shape	:	Almost round. Flat on the abaxial side. Semicircular on the abaxial side.
Size	:	1.7mm thick, 1.8mm wide
Epidermis	:	Thin with cubicle cells
Ground tissue	:	Homogenous parenchymatous
Secretary cavities	:	Specially more in adaxial part and similar as in lamina
Vascular strand	:	Closed hollow cylinder 1mm in diameter
Xylem	:	Long uniserriate parallel xylem elements containing both vessels and fibres
Vessels	:	Solitary narrow and thick walled 20 µm wide
Fibres	:	Thick walled and lignified
Phloem	:	Continuous like thick sheath all around the xylem cylinder containing phloem elements and phloem parenchyma
Ground tissue	:	Parenchymatous
Crystals	:	Calcium oxalate truses and prismatic crystals present in normal cells not modified into Idioblast unlike in Lamina.

5.1.3 SEM study of leaf (Plate 8)

Scanning Electron Microscopy of midrib showed many folded appearance.

5.1.4 POWDER MICROSCOPY (Fig -6)

Organoleptic characters

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

We have observed the following microscopical cell structures,

- ♦ Upper epidermis with underlying palisade cells
- ♦ Xylem vessels
- ♦ Epidermal cells with anticlinal wall
- ♦ Palisade cells with multiple epidermal cells
- ♦ Parenchyma
- ♦ Epidermal cells with cyclocytic stomata
- ♦ Secretory cavity
- ♦ Stomata
- ♦ Crystal Idioblast
- ♦ Fibres , Xylem, Phloem.

5.1.5 MICROSCOPIC SCHEDULE

As per the methods described in materials and methhods, microscopic schedule was carried out and the results were tabulated from the Tables 1- 4. The following evaluation were carried out.

Table – 1

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

Observation number	Vein Islet number	Vein termination number
1	3	8
2	3	5
3	3	4
4	5	9
5	2	4
6	4	6
7	3	6
8	3	5
9	4	7
10	4	8

Range	Minimum	Average	Maximum
Vein islet number	2	3.38	5
Vein termination	4	6.13	9

Table – 2

STOMATAL NUMBER OF *C.aurantium*

Observation number	Lower epidermis
1	60.75
2	54.25
3	67.50
4	63.00
5	49.50
6	62.25
7	56.00
8	64.50
9	65.50
10	66.25

Range	Minimum	Average	Maximum
Lower epidermis	49.5	67.75	67.50

Table – 3

STOMATAL INDEX OF C.*aurantium*

Observation number	Lower epidermis
1	16.66
2	15.94
3	16.66
4	16.46
5	16.02
6	16.48
7	16.40
8	16.54
9	16.66
10	16.18

Range	Minimum	Average	Maximum
Lower epidermis	15.94	16.42	16.66

TABLE – 4

PALISADE RATIO OF *C.aurantium*

Observation number	Upper Epidermis
1	3
2	4
3	4
4	2
5	3
6	3
7	2
8	4
9	3
10	3

Range	Minimum	Average	Maximum
Lower epidermis	2	3.125	4

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 5-8. The following evaluations were carried out.

Table – 5

ASH VALUE FOR THE LEAVES OF *C.aurantium*

Observation Number	Total Ash (%)	Acid Insoluble Ash (%)	Water soluble Ash (%)
1	10.286	2.05	5.80
2	9.345	1.6	6.05
3	9.821	2.15	5.5
4	9.047	1.65	5.7
5	10.802	1.55	5.45
6	9.298	1.8	5.10
7	9.713	1.75	5.30
8	10.212	1.95	5.65
9	10.028	2.2	4.7
10	10.110	1.5	4.5
Minimum	9.047	1.50	4.5
Average	9.852	1.813	5.4
Maximum	10.802	2.20	6.05

Table – 6

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

Observation Number	Loss on Drying (%) W/W
1	1.8
2	3.03
3	1.29
4	2.28
5	1.6

Material	Minimum	Average	Maximum
Leaves powder	1.29	1.90	3.03

Table – 7

EXTRACTIVE VALUES (INDIVIDUAL SOLVENTS) BY COLD MACERATION

Solvents	Extractive Value (%)
Petroleum ether	0.48
Ethyl acetate	0.68
Ethanol	0.72
Water	7.58

Table – 8

EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)

Solvents	**Extractive Value (%)**
Petroleum ether	0.53
Ethyl acetate	0.44
Ethanol	0.77
Water	7.13

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 9, 10)

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 : No brick red colour on paper shows the

glycosides **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	:	No appearance of reddish pink colour shows the **absence** 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 – 9

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

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	-
	Cyanogenetic glycosides	

	Coumarin glycosides	**-**
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**	-

The above described tests were also performed on the different extracts of leaves of ***C.aurantium*** and the results were as follows,

TABLE – 10

Tests	**Petroleum Ether extract**	**Ethyl Acetate 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	-	-	-	-
Cyanogenetic glycosides	-	-	-	-
Coumarin glycosides	+	+	+	+
STEROLS				
Salkowski test	-	-	-	-
Liberman Burchard's test	-	-	-	-

RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENINGFOR THE DIFFERENT EXTRACTS OF LEAF OF *C.aurantium*

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.aurantium* was studied. The results were as follows (Table -11**)**

Table -11

FLUORESCENCE ANALYSIS

No	**Treatment**	**Visible light**	**UV 254**	**UV 365**

			nm	**nm**
1	Powder	Pale green	Slightly Emerald green	Dark brown
2	Powder + Ethanol	Pale green	Slightly Greenish yellow	Slightly brown
3	Powder + Ethyl acetate	Straw yellow	Pale green	Greenish brown
4	Powder + Chloroform	Straw yellow	Pale green	Pale brown
5	Powder + water	Straw yellow	Pale green	Dark brownish black
6	Powder + 50%H2SO4	Pale green	Slightly Green	Dark brown
7	Powder + 10% NaOH	Pale leaf green	Green	Dark brown
8	Powder + 50% HNO3	Pale brown	Light green	Dark brown

5.2.3 IDENTIFICATION OF INORGANIC MINERALS OF THE LEAVES OF *C.aurantium* by Energy Dispersive X-ray Analysis (EDAX)

Estimation of the elements like C, O, Na, Mg, K, Ca, showed the following mg / weight percentage and atomic percentage.

5.2.4 PHYSICO-CHEMICAL EVALUATION OF ISOLATED VOLATILE OIL

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

1. Percentage oil obtained: 0.3 to 0.4 %
2. Colour: Greenish yellow
3. Odour: Aromatic characteristic
4. Taste: Bitter Aromatic
5. Characteristic feel: Slightly viscous
6. Solubility: Soluble in petroleum ether, toluene, chloroform and ethanol. It is immisible with water.
7. Refractive index:
8. Specific gravity: 0.8811 at 20ºC
9. Optical rotation: 1.4654 at 20ºC

5.2.5 GC-MS PROFILE OF V.O Fig 7

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

5.3 PHARMACOLOGICAL STUDIES

5.3.1 ANTIMICROBIAL SUSCEPTIBILITY TEST:

Screening of volatile oil of the leaves of *C.aurantium* was carried out to determine the antibacterial activity against the pathogenic organism of both *S.aureus* and MRSA. Disc diffusion method was used. Zone of inhibition in mm were 18 ± 0.2, 23± 0.13, 26 ± 0.1 in the concentration of 0.25, 0.5, 0.75 µl/disc for *S.aureus* respectively. Linezolid was used as standard, showed 24±0.2 zone of inhibition in mm.

Zone of inhibition in mm were 14±0.3, 16±0.2, 20±0.1 in the concentration of 0.25, 0.5, 0.75 µl/disc for MRSA respectively.

Linezolid showed 21±0.2 zone of inhibition in mm. (Table-12)

MINIMUM INHIBITORY CONCENTRATION BY MICRODILUTION METHOD:

- MIC was determined for VO against *S.aureus* and MRSA.
- It was 4µg/ml and 6µg/ml respectively.
- MIC for the standard drug Linezolid was 2, 4µg/ml respectively.

TABLE - 12

ANTIBACTERIAL ACTIVITY OF VO OF LEAF OF *C.aurantium* AGAINST *S.aureus* and MRSA.

Organism	Conc	Zone of inhibtion
S.aureus	0.25	18 ± 0.2
	0.5	23± 0.13
	0.75	26 ± 0.1
	STD	24±0.2
MRSA	0.25	14±0.3
	0.5	16±0.2
	0.75	20±0.1
	STD	21±0.2

Observation VO induced alterations on the surface morphology of MRSA under SEM:

We used SEM to see the effect of the VO on the surface morphology of MRSA. The VO has different levels of activity against bacteria as measured by the minimum inhibitory concentration. Although the mode of action of VO is unclear, the SEM examination showed that it had some activity on the cell surface that resulted in morphologic abnormalities. The result suggests biochemical activities occurring in several layers of the cell wall. Appearance of blast like bleb structures on the surface, irregular spherical structures and

eventual cell ruptures of the bacterial cell wall in other words surface destruction of the MRSA with intervening spaces have been resulted.

5.3.2 EVALUATION OF THERAPEUTIC EFFECT OF VO OF THE LEAF OF *C.aurantium* USING SILK WORM MODEL *IN VIVO*:

Determination of toxicity of VO on silk worm larvae:

To determine the toxicity various concentration of VO and Linezolid both were injected through intrahemolymph and intra midgut. It was observed that the larvae were alive > 100µg/larva and showed no toxicity sign like change in movement, rolling, change in feeding habit etc.

Calculation of the theoretically minimal effective dose of VO and Linezolid required for the treatment of infected larvae of silkworm:

For antibacterial agents to show therapeutic effects their concentration in the hemolymph after injection should be higher than their MIC values and at the same time, they should not be toxic to the host animals. As the MIC value enables us to calculate the minimal required dose for the therapeutic effect in infected silkworm larvae, we defined the value as the theoretical minimal effective for the therapeutic effect ($tED_{\mathbf{mini}}$) and compared with the toxic dose.

From our study it was known that the MIC of linezolid and VO were 4. 6 µg/ml against MRSA. Considering the approximate hemolymph volume and body weight of 5^{th} instar larvae the calculated tED_{mini} were 7 and 10 µg/g of larva for intra hemolymph route. For the dose consideration of intra midgut route the absorption and other factors were taking in to the consideration. Then the survival percentage was calculated. It was plotted against the dose

administered. ED_{50} was determined from the graph. (Fig). There were no viable bacteria in the hemolymph of the hundred percent protected group but colonies of viable bacteria were observed in the other groups.

TABLE – 13

THERAPEUTIC EFFECT OF LINEZOLID AGAINST MRSA INFECTED LARVAE (INTRA HEMOLYMPH) (n=10)

DOSE µg/g larva	NUMBER OF LARVAE ALIVE	SURVIVAL PERCENTAGE
4	0	0
6	2	20
7	3	30
9	5	50
11	7	70
12	10	100

TABLE - 14

THERAPEUTIC EFFECT OF LINEZOLID AGAINST MRSA INFECTED LARVAE (INTRA MIDGUT) (n=10)

DOSE µg/g larva	NUMBER OF LARVAE ALIVE	SURVIVAL PERCENTAGE
4	0	0
6	1	10
7	2	20
9	3	30
11	5	50
12	9	90

TABLE - 15

THERAPEUTIC EFFECT OF VO AGAINST MRSA INFECTED LARVAE (INTRA HEMOLYMPH) (n=10)

DOSE µg/g larva	**NUMBER OF LARVAE ALIVE**	**SURVIVAL PERCENTAGE**
4	0	0
6	1	10
8	3	30
10	5	50
12	7	70
14	10	100

TABLE - 16

THERAPEUTIC EFFECT OF VO AGAINST MRSA INFECTED LARVAE (INTRA MIDGUT) (n=10)

DOSE µg/g larva	NUMBER OF LARVAE ALIVE	SURVIVAL PERCENTAGE
5	0	0
7	2	20
9	4	40
11	5	50
13	7	70
15	10	100

5.3.3 Assay for stimulants of natural immunity:

We undertake screening for stimulant of innate immunity using the silk worms. Muscle contraction was used as an index. A unique phenomenon, in which the muscle of the silkworms contracts and silkworm's length gradually decrease. So by observing the muscle contraction valuable stimulants of innate immunity can be detected.

We investigated 14, 12 µg/g larva intra hemolymph, intra midgut route dose protecting the hundred percent from the MRSA infection was tested for innate immunity. There was a gradual contraction of muscle from 5± 0.2 to 3.5 ± 0.4 cm in relative length in 10 mts (Plate 21, Fig - 12) (Table-17)

TABLE 17

ASSAY FOR STIMULANTS OF NATURAL IMMUNITY

Time mts	**Relative length cm ± SEM**		
	VO	**Linezolid**	**Control**
1	5±0.2	4.9± 0.1	5± 0.3
2	4.6±0.1	4.9± 0.1	
3	4.5±0.3	-	
4	4.3±0.2	-	
5	4.2±0.1	-	
6	4.0±0.2	-	
7	3.8±0.4	-	
8	3.7±0.6	-	
9	3.6±0.7	-	
10	3.5±0.4	4.8±0.1	5±0.3

5.3.4 ASSAY FOR F' *lac* Plasmid elimination activity:

The genetical basis of resistance is often the R-plasmid, which can be transferred to other bacteria in the environment of the recipient and these extra chromosomal DNA sequence can be responsible for the emergence of multiple resistance to antibiotics. Plasmid may be lost spontaneously in a very low frequency, but certain effects can increase the probability of plasmid loss, which is the basis of artificial plasmid elimination. We examined the ability of plasmid elimination of VO, linezolid and menthol qualitatively. Significant difference was observed in the culture.

More plasmid less pink colonies were observed in menthol and VO treated plates. But only plasmid containing colonies were observed in control and linezolid treated plates. This result showed that VO has considerable antiplasmid activity (Plate 22).

CHAPTER - 6

DISCUSSION

The dissertation covers a study on the widely available a member of the family Rutaceae is known botanically as *Citrus aurantium* L commonly called as bitter orange. The leaves of *C.aurantium* really do not have any match as a cheap natural and easily available plant. It is traditionally known to be useful for the treatment of wide panel of diseases like stomach ache, vomiting etc. Leaf is used fro emmenagogue, blood pressure, cough, cold, bronchitis, ear ache, dysentery, diarrhea, abdominal pain, UI ailments, dysmenorrhea, diuretic during pregnancy, influenza, insomnia, fever, sedative, digestive. Bark used for UIT ailments. Infusion of dried flower is orally used for influenza, insomnia, as a cardiovascular analeptic, anti spasmodic, for cold, sedative, digestive. Fruit as a cardiovascular analeptic, as a febrifuge, cold, flu, abortifacient and to speed birth in pregnant women, as a laxative, to treat fishbone caught in the throat, influenza, insomnia, to stimulate appetite, liver troubles, externally cutaneous infection, irritation, dermatitis, inflammation, wounds, bruises, sores, head ache, antidiabetic, dysentery, diarrhea, hypertension etc. This plant is much more popular in India and widely cultivated. In India fruits used for pickles. The economic aspect of this crop evidently proved that as commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored well. By a well coordinated effort, we can 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. 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 exist in the pharmacological, phytochemical, and pharmacological studies in the leaves of *C. aurantium*.

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 with **wide air chambers** in the

middle part were observed. **Wide circular secretary cavities surrounded by fairly thick spindle shaped epithelial cells with amorphous inclusion were present. A characteristic exclusively abundant prismatic calcium oxalate 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. Calcium oxalate druses and prismatic 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, prismatic cells druses, 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** 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.

Palisade Ratio is another criteria for identification and evaluation of herbal drugs. This value remains constant within a range for a given plant species and is of diagnostic value in differentiating the species. This value does not alter based on geographical variation and differs from species to species and that is why it is a very useful diagnostic feature for characterization and identification of different plant species. (Table 1-4)

The ash content of the crude drug is generally taken to be the residue remaining after incineration. If usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also involve the inorganic matter added for the purpose of adulteration. There is a considerable difference within narrow limits in the case of individual drug. Hence ash determination furnishes a basis for judging the 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 -5).

Extractive values of crude drugs determine the amount of active constituents in a given amount of medicinal plant material when exhausted with solvents. It is employed for that material for which no chemical or biological assay method exist. As mentioned in different official books (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 -7, 8).

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 -6)

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 9, 10)

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 - 11) (Harborne JB 1973).

Identification of inorganic minerals of the leaves of *C.aurantium* by Energy Dispersive X ray Analysis (EDAX) showed the presence of minerals Na (0.09%), Mg (0.09%) K (0.72) Ca (0.42) listed in the (Plate 18)

We have isolated one of the most important constituent of the leaf volatile oil (0.3-0.4%). 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 7). 35 peaks were identified. The important known major constituents were α Pinene, β Pinene, Sabinene, β Myrcene, O cymene, D-Limonene, Eucalyptol (1,8 Cineole) β Linalool, 4 terpineol, α Terpineol etc.

Resistance to existing antimicrobial agent has resulted in treatment failures and enhancement in health care costs. Now there is only a little 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.aurantium* a widely available and menacingly 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 menacing wastage and to maximize the revenue generated by this crop to boost up our national economy as well as the proper exploitation of this plant for therapeutic purpose. By investigating its bioactivity of its VO we can meet the situation of unsettling facts of modern pharmaceutical industry which facing lately its pipeline of new drug discovery seems to be almost empty.

Most of the screening of plant products on antimicrobials is restricted to *in vitro* studies because of problems in vivo studies especially in animal usage.

We planned to carry out both *invitro* and in vivo study without using mammalian system in this drug discovery process and also to help cut cost and save time.

In this dissertation we planned to investigate the in vitro and in vivo antimicrobial activity of the isolated VO as we have detailed knowledge of its constituents.

As the complicated physiological environment is not found in the *in vitro* systems, hence *in vitro* results poorly correlates with *in vivo* results.

Even though cell based assays provide reliable results, cultured cells do not give physiological environment and interaction between different cell types and tissues. Human tissues provide an isolated *ex vivo* condition, it is not complete representative of *in vivo* response as the action of drug

involves metabolism and they interplay among different tissues. For example, drug acting on muscle may involve intestinal absorption and metabolism by the liver. Therefore results in animal studies are important to validate. But various problems are inherent during the use of animal models such as high cost of housing animals and ethical issues which has become more serious in recent years. Law strictly regulates the use of mammals for the development of medicine. As the small animal models are emerging, it is now possible to perform *in vivo* testing. So researchers have developed animal model system using both vertebrates like zebrafish and invertebrate models like fly, *Drosophilla melanogaster* for drug screening. These are very powerful tools for identifying host protein involved in immune system because they are genetically tractable and many mutant lines have been constructed. But some of the animals are too small to handle and they are not suitable for injecting precise volumes of samples into the body fluid, a technique that is important for quantitative evaluation of bacterial pathogneisity and therapeutic effects of antimicrobial compounds. So we have preferred silk worms for studying bacterial pathogenicity and the therapeutic effects of antibiotic along with VO. There are no ethical problems and biohazards associated in the use of a large number of silkworms. It was also shown that the results of whether antibiotics can be absorbed or not were similar in silk worms and mammals. Metabolic pathways with cytochrome P 450 and conjugated enzymes were also similar. But in other small animals it should be performed under microscope. Hence low cost, no ethical issues, available to accurate injection into hemolymph and

gut with no biohazards were attracted us to undertake this study in vivo using silk worms to screen antimicrobial studies.

We selected established insect models of human pathogenic microbial infection using the silkworm *Bombyx mori*. It shares conserved mechanisms for the pharmacokinetics of chemical absorption, distribution, metabolism, and excretion (ADME) with mammals when we review the previous reports. **Therefore, the silkworm infection model is potentially useful for the evaluation of toxicity and ADME of candidate compounds in therapeutic drug screening for infectious diseases. Furthermore, silkworm larvae are large enough to handle for the injection of reagent solution and for the collection of hemolymph for analysis. It is highly advantageous for studies of pharmacokinetics and this model can likely be extended to various human diseases. It will be helpful to exclude candidate therapeutic agents that are not effective at an early stage of drug development.**

As it was previously reported that silk worms were killed by injection of *S.aureus* or other human pathogenic bacteria and that clinically used antibiotics are effective in the silkworm systems, we preferred MRSA infected model of this organism to screen the therapeutic effect of the VO of the leaves of *C.aurantium.*

Initially we screened the inhibitory effect of VO against *S.aureus* and MRSA using agar plate disc diffusion method. The results were encouraging that it is highly effective than that of the standard drug linezolid which is

effective than vancomycin in MRSA infection both orally and parentarily (Tripathi, KD 2010).

Hence we determined MIC for both VO and linezolid by microdilution technique.

The MIC for linezolid 2 and 4µg/ml against *S.aureus* and MRSA respectively.

But for VO MIC were 4 and 6µg/ml respectively.

To understand the VO induced alterations on the surface morphology of MRSA we examined the VO treated MRSA under SEM. Although the mode of action of VO is unclear, the SEM examination showed that it had some activity on the cell surface that resulted in morphologic abnormalities. The result suggests biochemical activities occurring in several layers of the cell wall. Appearance of blast like bleb structures on the surface, irregular spherical structures and eventual cell ruptures of the bacterial cell wall, in other words surface destruction of the MRSA with intervening spaces have been resulted. This shows bactericidal activity

We further evaluated the effect of VO in vivo using silkworm model both intrahemolymph and intra midgut which are equivalent to IV and per oral route in mammals. The uniform immediate distribution of aqueous extract of beet root dye injected intrahemolymph and difference in midgut injection was studied which were consistent with previous report where trypan blue was injected.

We performed toxicity evaluation of VO and linezolid using silk worm 5^{th} instar larvae. The result showed that they were alive in the dose > 100µg /larva and showed no toxicity signs.

Next we determine the tED_{mini} i.e theoretical minimal effective dose for the therapeutic effect using the MIC and body weight and compared with the toxic dose. From this we selected six doses of equal proportions which were less than 1/3 of toxic dose for screening and extra increment was added for intra midgut route considering absorption and other factors in this route. Then the drugs and antibiotic screened in separate groups of different doses on MRSA infected 5^{th} instar larvae. The survival percentage was calculated.

ED_{50} was drawn from the graph plotted using survival percentage and dose administered for ih and im. ED_{50} for the standard drug linezolid were 9, 11µg/g larva for ih and im respectively. In the case of VO they were 10, 11µg/g larva for ih and im respectively. The ED_{50} value of linezolid is consistent with the previous report. Further there were no viable bacteria in the hemolymph of the hundred percent protected group but colonies of viable bacteria were observed in the poorly protected groups when the infected hemolymph were cultured on agar plates.

The ED_{50}/MIC ratios were below 10 for both linezolid and VO protected group. They were 2.25, 1.67 for ih, to standard drug and VO respectively and 2.75, 1.83 respectively for im. It was previously proposed than an antibacterial agent whose ED_{50}/MIC values were lower than 10 had proper pharmacokinetics characteristics. Further this reported value of linezolid is

consistence with the previous value. (Hamamoto.H 2004). In addition to that this ratio is almost near to the value obtained in mouse model 1.5 in an investigation (Ford C.W 1996).

Therefore the above studies proved that the volatile oil of the leaves of C.aurantium is therapeutically effective agent in the silk worm MRSA infection model without toxicity in mammals and effective than the synthetic oxazolidinone, a new class of antibiotic available for the treatment of resistant gram positive coccal and bacillary infections. This antibiotic has the side effect such as mild abdominal pain and bowel upset occasionally rashes, pruritis, head ache, oral/vaginal candidiasis have been reported. Neutropenia and thrombocytopenia are infrequent and usually mild. Because linezolid is a MAO inhibitor, interacts with adrenergic/seretonergic drugs and excess dietary tyramine are expected. But the VO of our study will not produce stomach upset as it is used for that ailment and we can expect safe antibacterial formulation without side effects.

We further investigated the stimulation of innate immunity in the silk worm. The effective unique phenomenon of contraction of silkworm muscle by the stimulation of innate immunity is useful in defense mechanism additionally. Linezolid, normal saline treated larvae showed no contraction. The stimulation of innate immunity may be due to action on serine proteases which activate BmPP (Bombyx mori paralytic peptide) which in turn induce muscle contraction. (Fujiyuki. T, *et al*., 2010).

A major problem in antimicrobial chemotherapy is the increasing occurrence of resistance to antibiotics, which leads to the insufficiency of antimicrobial treatment. The misuse of antibiotics and consequent antibiotic selection pressure may be the most important factor contributing to the appearance of different kinds of resistant microbes. The genetical basis of resistance is often the R-plasmid, which can be transferred to other bacteria in the environment of the recipient and these extra chromosomal DNA sequence can be responsible for the emergence of multiple resistance to antibiotics. Plasmid may be lost spontaneously in a very low frequency, but certain effects can increase the probability of plasmid loss, which is the basis of artificial plasmid elimination. In early studies acridine orange, ethidium bromide, sodium dodecylsulphate were found to be powerful plasmid eliminators. But their toxicity did not allow their in vivo testing for their antiplasmid effect. The possible mechanism of action is complex formation with the guanine-cystine-rich regions of the plasmid DNA which is necessary for normal plasmid replication in an uncomplexed form.

In the present study besides the antibacterial effect their anti plasmid activities on an *E.Coli* F'lac strain were determined. The results showed significant plasmid elimination activity of both VO and menthol (positive control).

But the plate of linezolid and control showed no plasmid elimination activity.

This showed the VO has significant resistance modifying activity *in vitro*.

CONCLUSION

The present investigation highlights the pharmacognostical, phytochemical and potential antimicrobial action against pathogenic drug resistance MRSA infection with innate immunity stimulation, more effective therapeutically than the synthetic counterpart, without toxicity of the VO of the leaves of *Citrus aurnatium* L a widely, easily available plant. Ethnomedical information revealed that it was used in various ailments for long time all over the world.

The tremendous economic potentiality of this cash crop remains neglected by the scientists, technologists, physician, traders, administrators, policy makers, farmers etc.

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

Detailed microscopical characters of the leaves showed the presence of secretary cells, crystal idioblasts specifically under the upper epidermis,three layers of well defined palisade cells, big air chambers in the spongy mesophyll, double stranded vascular bundles in the midrib region and calcium oxalate druses in the petiole

Quantitative microscopic parameters were presented.

Preliminary phytochemical screening showed the presence of carbohydrates, proteins and amino acids, flavonoids, saponin, terpenoids,

tannins, phytosterols, and volatile oil. Fixed oil, alkaloids, were found to be absent.

Inorganic analysis by EDAX showed the presence of Na, Mg, K, Ca.

Volatile oil was isolated from the fresh leaves

The physical parameters were studied and presented.

The GC-MS profile of the isolated VO was studied and the presence of thirty five compounds were identified. The known constituents were

1. α Pinene
2. Sabinene
3. β Pinene
4. β Myrcene
5. O-Cymene
6. D-Limonene
7. Eucalyptol (1,8 cineole)
8. β-Linalool
9. 4-Terpineol
10. α Terpineol

In this eucalyptol, sabinene, β-Linalool were found to be predominant.

The **3 R's** ethical principle (**R**eduction, **R**efinement, **R**eplacement) was implemented that help to minimize harms to vertebrate animals used in science. In our study we used silk worm 5^{th} larvae which are a preclinical novel emerging *in vivo* model that support rapid decision making in the early phases of drug discovery process. The properties of its genome have established as an

excellent model system that is relevant to studies of human infectious diseases and on stimulation on innate immunity.

We investigate the in vitro anti bacterial activity of the VO of the leaves and determined MIC against human pathogens *S.aureus* and drug resistant MRSA.

Though the mode of action of VO is unclear, the SEM examination of the VO showed that it had some activity on the cell surface that resulted in morphologic abnormalities. The result suggests biochemical activities occurring in several layers of the cell wall. Membrane disruption by the lipophilic constituents may be involved in this mechanism. Appearance of blast like bleb structures on the surface, irregular spherical structures and eventual cell ruptures of the bacterial cell wall, in other words surface destruction of the MRSA with intervening spaces have been resulted

We further determine the toxicity and ED_{50} for antibacterial activity for VO *in vivo* silk worm model. **The therapeutic efficacy of the VO proved to be more than the synthetic drug of choice oxazolidinone, Linezolid, by its lower ED_{50}/MIC ratio and stimulation of innate immunity mechanism of silk worm larvae against the human pathogenic drug resistant organism MRSA and a preferable cheap alternative from the plant source.**

In addition to that it was observed that VO has significant antiplasmid activity on an *E.coli* F' *lac* strain *in vitro*.

It can be concluded that the VO isolated from the leaves of *Citrus aurantium* L. has promising anti infective with innate immunity and

antiplasmid (resistance modifier) activity against drug resistant MRSA. It has to be further evaluated as "**safe herbal formulation for the treatment of human pathogenic infection without side effects and with immuno stimulation with resistance modifier activity". Moreover the vast economic potentiality of this crop can be adequately exploited and can create employment opportunity to an agricultural worker through out the year.**

REFERECNES

1. Achararit C, Panyayong W, Ruchatakomut E, "Inhibitory action of some Thai herbs to fungi" Undergraduate Special Project Report 1983, 1983. 13

2. Adesina .S.K, (1982) "Studies on some plants used as anticonvulsants in Amerindian and African traditional medicine" *Fitoterapia* 53, 147-162.

3. Afek U, Sztejnberg A, (1988)"Accumulation OS Scoparone, a phytoalexin associated with resistance of citrus to phytophthora citrophthora", *Phytopathology* , 78 (12) 1678-1682.

4. Afek U, Sztejnberg A, Crmely S, "6,7-Dimethoxy coumarin, a citrus phyto alexin conferring resistance against phytophthora gummosis" Phytochemistry 1986. 25 (8) 1855-1856.

5. Agarwal SS, Paridhav, "Herbal Drug Technology", 1st edn Universities Press (India) Private Ltd, Hyderabad, (2007).

6. Alarcon-Aguilara F.J, Roman-Ramos. R, Perez-Gutirrez S, Aguilar-Contrrras.A, Contreras-weber.C.C, Flores-Saenz.J.L, (1998) "Study of the Anti-hyperglycemic effects of plants used as antidiabetics" *J Ethnopharmacol* 61:(2) 101-110.

7. Alsaadawi HS Arif MB Alrubeaa AJ, "Allelopathic effect of citrus aurantium L." Isolation, characterization and virological activities of phytotoxins", J Chem. Ecol (1985),11 (11): 1527-1534.

8. Ameer b, weintraub RA johnson jV, yost Ra Rouseff RL "flavanone absorption after naringin hesperidin and citrus administration" Clin pharmacol ther (1996) 60(1) 34-40

9. Anon "Petitgrain Bigarade oil" Food Chem Toxicology" 1992,30,101s

10. Anon "Pharmacological inestigation of the pressor action of citrus aurantium L" Ko Huseh Tung Pao 1978 23(1) 58-62

11. Anon "Traditional-Western combined treatment of 217 cases of tetanus" Chung-hua, H-such TSA Chih (Beijing) (1973) 53, 683-684

12. Anonymous (1998) "*Quality control Methods for Medicinal Plant Materials*" WHO, 28-35.

13. Anonymous "*Wealth of India*" Raw materials, Ca-Ci" Vol III, New Delhi, National Institute of Science Communication and Information Resources (NISCAIR), CSIR, (2005).

14. Anonymous(1996) "*Indian Pharmacopoeia*" New Delhi, Ministry of Health and Family Welfare, Vol II, A-53: 54,89.

15. Anonymous,"The Ayurvedic Pharmacopoeia of India", Part I, Ist edn, Vol 1, Government of India, Ministry of Health and Family Welfare, Indian Systems of Medicine and Homeopathy, 2001: 140-145.

16. Antonone.R, De Simone.F, Morrica P, Ramundo.E, (1988), "Traditional phytotheraphy in the roccamonfina volcanic group Campania Southern Italy" J Ethnopharmacol, 22:(3) 295-306.

17. Arriaga FJ, Rubero A, "naringin, hesperidin and neo hesperidin content in juices from thirteen citrus SSP", Fitoterapia (1990), 51(1): 31-37.

18. Avirutnant w Ponpan a "The antimicrobial activity of some Thai flowers and plants" Mahidol Univ J Pharm sci (1983) 10(3) 81-86

19. Azuma S Yada Y Imokawa g tazaki S shinho T "Skin-lightening cosmetics containing plant extracts and ascorbic ascid or placenta extracts" patent Japan kokai tokkyo koho -08 208, 451 (1996) 9, Patent chemical abstracts 125 25678 FR kao corp Japan

20. Bae EA han MJ Kim DH, "Effect of herbal medicine on the IgA, Production in payer patches" Korean J Pharmacog (2000) 31 (3) 259-263

21. Bae EA Han MJ Kim Nj Kim DH "Anto-helicobacter pylori activity of herbal medicines" biol pharmbull (1998) 21(9) 990-992

22. Bajpai.A, Ojha.J.K, Sant.H.R, (1995) "Medicobotany of the Varanasi District" *Int J Pharmacog* 33 : (2) 172-176

23. Barrett.B, (1994) "Medicinal plants of Nicaragua's Atlantic coast". *Econ bot.* 48 : (1) 8-20.

24. Benavente-Garcia.O, Castillo J Del Rio Conesa JA "Changes in neodiosmin levels duringthe development of citrus aurantium leaves and fruits. Postulation of a neodiosmin biosynthetic pathway" J Agr food chem (1993) 41(11): 1916-1919.

25. Benneh RD, Hasegawa S, "Isolimonic acid, A new citrus limonoid" Phytochemistry (1980) 19; 2417-2419.

26. Benneh RD, Miyake M Ozaki Y Hasegawa.S "Limonoid Glucosides in citrus aurantium" Phytochemistry (1991) 30 (11) 3803-3805.

27. Bob Hafner , "Energy Dispersive Spectroscopy on the SEM: A Primer'':1,15

28. Boelens MH, Jimeney, "The chemical composition of the peel oils from unripe and ripe fruit of Bitter orange citrus aurantium L ssp amara", Eng Flavour Fragrance J (1989) 4 (3) 139-142

29. Boelens MH, Jimenez R "The chemical composition of some Mediterranean Citrus Oils" J Essent Oil Res (1989) 1, 151-159.

30. Boukef.K, Souissi.H.R, Balansard.G, (1982) "Contribution to the study of plants used in traditional medicine in Tunisia". *Plant med phytother*. 16 : (4) 260-279.

31. Browner.C.H,(1985) "Plants used for reproductive health in Oaxaca, Mexico". *Econ bot*. 39 : (4) 482-504.

32. Burk LA, Chung LY "The stereo structure of Bisabolene tri hydro chloride" J Nat prod (1992). 55 (9) 1336-1338.

33. Caceres A, Giron LM, Alvarado SR, Torres MF, "Screening of antimicrobial activity of plants popularly used in Guatemala for the treatment of dermato mucosal diseases" J Ethnopharmacol 1987 20 (3) 223-237

34. Calapai.G, Firenzuoli F, Saitta A, Aquadrito F, Arlotta MR, Costantino G, Inferrera G, "Anti obesity and cardiovascular toxic effects of citrus aurantium extracts in the rat. A preliminary report",. Fitoterapia (1999) 70(6), 586-592.

35. Carvalho Freitas MIR, Costa M, "Anxiolytic and sedative effects of extracts and essential oil from citrus aurantium L" Biol pharm bull (2002), 25(12) 1629-1633

36. Castillo J, Benavente O, Del Rio JA, "Hesperidin-7-O-Glucoside and prunin in citrus species (C.aurantium and C.paradisi). A study of their quantitative distribution in immature".

37. Cha.S, (1977) "Potential anticancer medicinal plants. A statistical evaluation of their frequencies of appearance in oriental medicine formulation". *Korean J pharmacog*. 8: 1.

38. Chandler Rf, Hooper SN "Friedelin and associated triterpenoids" Phyto chemistry (1979) 18, 711-724.

39. Chang Im Yun HS "Evaluation of medicinal plants wth potential hepatonic activities and study on hepatonic activities of plantago semen"abstr 4th Asian symp med plants spices Bangkok Thailand September 15-19, 1980, (1980) 69

40. Chang Im Yun HS "Plants with lliverprotective activities, pharmacology and toxicology of aucubin' Advances in Chinese medicinal materials research HM Chang HW Yeung WW TSO and A Koo (EDS) world scientific press philadelphia PA (1984) 269-285

41. Chang M, Jiang M, Li AJ, "Fluorescence TLC-densitometric determination of hesperidin in fructus aurantii immaturus and its three Chinese traditional patent formulations" Yaowu Fenxi Zazhi (1990) 10 (2); 94-97.

42. Chang.I.M, Yum.H.S, (1984) "Plants with liver-protective activities pharmacology and toxicology of Aucubin" Advances in Chinese medicinal materials Research HM chang HW Young WW Tso and A koo (EDS) world scientific press philadelphia PA. 269-285

43. Chaudhri RD., "Herbal Drug Industry", Eastern Publishers, New Delhi, (1999) :489-501.

44. Chen X, Heang QX Zhou TJ Dai Hy, "Studies of citrus aurantium and its hypertensive ingredients on the cardiac functions and hemodynamics in comparison with dopamine and Dobutamine" Yao Hsueh Pao (1980) 15, 71-77.

45. Chi HJ Kim HS Lee SY, "Iridoid-containing korean medicinal plants I", Korean J Pharmacog (1981), 12 : 19-22.

46. Chialva F, Doglia G:Essential oil constituents of chinotto (citrus aurantium L var myristifolia guill) J Essent Oil Res (1990) 2 (1) 33-35.

47. Cho HM Jung JS Lee Th Son KH Suh HW Song DK Kim YH " Inhibitory effects of extracts from traditional herbal drugs on 5-HT uptake in primary cultured rat brainsten neurons" Korean J pharmacog (1995), 26(4) 349-354

48. Choi BW Lee Bh Kang KJ Lee ES, Lee NH "Screening of the tyrosinase inhibitors from marine algae and medicinal plants" Korean J Pharmacog (1998) 29 (3) 237-242.

49. Choi J, LeeKM, Kim HJ Kim SH Kim HS Ro JS OH KM LEE KS "The effects of dried orange peel compound intaking and exercise training on the physique body fat and concentration of blood lipid in obese women" korean j Pharmacog (2002) 33(1) 57-63

50. Choi SY chang Im "Plants with liver protective activities" Ann rep nat prod res inst seoul Nati Univ (1982) 21, 49-53

51. Coe.F.G, Amderson.GJ, (1996) "Screening of medicinal plants used by the Garifuna of Eastern Nicaragua for Bioactive compounds" *J Ethnopharmacol* 53, 29-50.

52. Coee.F.G, Anderson.G.J, (1996) "Ethnobotany of the garifuna of Eastern Vicaragua". *Econ bot.* 50 : (1) 71-107.

53. Comerford.S.C, (1996) "Medicinal plants of two mayan healers from San andres, Peten, Guatemala". *Econ bot.* 50 : (3) 327-336.

54. Darias .V, Bravo.L, Barquin.E, Herrera.D.M, Fraile.C (1986) "Contribution to the ethnopharmacological study of the Canary Islands "*J Ethno pharmacol* 15: (2) 169-193

55. Darias.V, Abdala.S, Martin.D, Ramos.F, (1996) "Hypoglycaemic plants from the Canary Islands". *Phytother res suppl.* 10: S3-S5.

56. De blasi V, Debrot S, Menoud PA, Gendre L. Schowing J "Amoebicidal effect of essential oils in vitro" J toxicol clin exp (1990) 10(6) 361-373

57. De-feo.V, Senatore.F, (1993) “Medicinal plants and phytotheraphy in the Amalfitan coast, Salerno province, Campania, Southern Italy”. *J Ethano pharmacol*. 39 : (1) 39-51.

58. Dei Rio JA Ortuno A, Garcia PD, Iborra JL, Sabater F, “Accumulation of the sesquiterpenes nootkatone and valencene by callus cultures of citrus paradisi, citrus limonia and citrus aurantium” Plant cell Rep (1991) 10(8), 410-413.

59. Del Rio JA, Arcas MC, Benavente O, Sabatr F, Ortuno A, “Change of polymethoxylated flavones levels during development of citrus aurantium (CV. Sevillano) fruits” Planta Med (1998) 64 (6) 575-576.

60. Del.Rio JA, Arcas MC, Benavente-Garcio O, Ortuno A, “Citrus polymethoxylated flavones can confer resistance against phytophthora citrophthora penicillium digitatum and geotrichum species” J Agr food chem (1998), 46 (10), 4423-4428.

61. Di Marco MP Edwards DJ Wainer IW Ducharme MP” Theeffects of grape fruit and seville orange juice on the pharmacokinetic of dextromethorphan. The role of gut CYP 2A and P-Glycoprotein” Life Sci (2002) 71(9) 1149-1160

62. Dimayuga.R.E, Urgen.M, Ochoa.N,(1998) “Antimicrobial activity of medicinal plants from Baja California sur (Mexico)”. *Journal of pharmaceutical biol*. 36: (1) 33-43.

63. Dreyer DL, “Citrus bitter principles V. Botanical distribution and chemotaxonomy in the rutaceae” Phytochemistry (1966), 5, 367

64. Dugo G, Verzera A, Stegno D, Alcontres I, Cotroneo A Ficarra R, “On the genuineness of citrus essential oils parts VLI. Italian bitter orange essential oil: composition and detection of contamination and additions of oils and terpenes of sweet orange and of lemon” Flavour fragrance J (1993) 8 (1): 25-33.

65. Edwards DJ Bernier SM” Naringin and naringenin are not the primary CYP 3A inhibitors in grapefruit juice” life sci (1996) 59(3) 1025-1030

66. Elisabetsky.E, Eastilhos.Z.C, (1990) ”Plants used as Analgesics by Amazonian carboclos as a basis for selecting plants for investigation”. *Int J crude drug res.* 28 : (4) 309-320.

67. EL-Keltawi NEM, Megalla Se, Ross SA ‘Antimicrobial activity of some Egyptian aromatic plants” Herbo pol (1980) 26(4) 245-250

68. Forster HB Nikias H Lutz s “Antisapsmodic effects of some medicinal plants” Planta med (1980) 40 (4) 309-319.

69. Fujiyuki.T, Imamura.K, (2010) “Evaluation of therapeutic effects and pharmacokinetics of antibacterial chromogenic agents in a silkworm model of *Staphylococcus aureus* infection” *Drug discoveries and therapeutics*, 4:(5) 349-354.

70. Ghosh BP, Mukherjue AK, Banerjee S, “A new pyrone derivative cirantin as an oral contraceptive” Naturwissenschaften (1955), 42. 77.

71. Ghosh.BP “Antihyaluronidase activity of cirantin an oral contraceptive” Indian J med. Sci (1958), 12, 991.

72. Giri KV, Gopalakrishnan KS, Radhakrishnan AN, Vaidyanathan CS, “Proune and hydroxy proline in leaves” nature (London) 1952, 170. 529

73. Glron.L.M, Freire.V, Alonzo.A, Carceres.A, (1991) “Ethanobotanical survey of the medicinal flora used by the Caribs of Guatemala”. *J Ethno pharmacol*. 34 : (2/3) 173-187.

74. Greer MA Astwood EB “The antithyroid effect of certain foods in man as determined with radioactive iodine” endocrinology 1948 43, 105-119

75. Griffiths.LA “On the distributon of gentisic acid in green plants” J Exp Boil (1959), 10: 437.

76. Gu W, Bai Y Li Z Zhang Y Tian LI Tian F Yang Y Shi S “Effect of different decocting methods on the extract contents of rhebarb anthraquinone in DA cheng QI Tang and its pharmacological effect” chung TSAO Yao (1985) 16(1) 8-11

77. Guo 2G, “Effect of Zhi-shi “(citrus aurantium) and its active pronciples on the contractility and automaticity of CAT papillary muscle in endotoxin shock”, Hu-nan I Hsueh Yuan Hsueh Pao (1983) 8 (3), 267-271.

78. Guo LQ. Taniguchi n, Chen QY Baba K Yamazoe Y ‘ Inhibitory potential of herbal medicines on human cytochrome P450 – mediated oxidation: Properties of umbelliferous or citrus crude drugs and their relative prescriptions” Jap J pharmcol (2001) 85(4) 399-408

79. Gupta M "Essential oils: A new source of bee repellents" Chem Ind (London) (1987) . (1987) (5), 161-163

80. Gurib-Fakim.A, Sweraj.M.D, Gueho.J, Dullo.E, (1996) "Medicinal plants of Rodrigues". *Int J pharmocog.* 34 : (1) 2-14.

81. Gurley BJ "In vivo assessment of botanical supplementation on human cytochrome P450 phenotypes citrus aurantium, echinacea purpurea milk thistle and saw palmetto

82. Haginiwa J harada m Morishita I, "Pharma cological studies on crude drugs Vii. Properties of essential oil components of Aromatics and their pharmacological effect on mouse intestine' yakugaku zasshi (1963) 83, 624

83. Hamamoto.H, Kurokawa.K, Kaito.C, Kamura.K, Razanajatovo.I.M, Santa.T, Sekimizu.T (2004) "Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms" *American Society for microbiology* 48: (30) 774-779.

84. Hammer KA carson CF riley TV "In –vitro activity of essential oils, in particular nelaleuca alternifolia (Tea-tree) oil and tea tree oil products, Against canida SPP" J antimicrob chem ther (1998) 42(5) 591-595

85. Han BH Chi HJ Han YN Ryu KS "Screening on the anti-inflammatory activity of crude drugs" Korean J pharmacog (1972) 4(3) 205-209

86. Han BH Han YN Park MH" Chemical and biochemical studies on antioxidants compounds of ginseng" Advances in Chinese medicinal materials research H.M.Chang H.W. Yeung W.W.Tso and A Koo (EDS) world scientific press Philadelphia PA (1984) 485-498

87. Han BH, Lee EB, Woo WS, "Screening of saponins in the plants" Korean J pharmacog (1981) 12 (2) 88-93.

88. Han BH, Lo EB, Woo WS, "Screening of saponins in the plants" An rep nat prod Res Inst Seoul Natl Univ (1981) 20: 49-54.

89. Han BH, Yang OK Kim YC, Han YN "Screening of the platelet activating factor (PAF) antagonistic activities on herbal medicines" Yakhak Hoe Chi (1994) 38(4) 462-468

90. Han DI, Hwang By, Hwang SY, Part JH SON KH, Kee SH Chang SY, Kang SJ, Rojs Lee.KS, "Isolation and quantitative analysis of hesperidin from aurantii fructus", Korean J Pharmacog (2001),32 (2)93-97.

91. Han GQ, Pan JX, Li Cl, Tu.F, "The screening of Chinese Traditional drugs by biological assay and the isolation of some active components" Int J Chin med 1991, 16(1): 1-17

92. Hanneguelle s Thibault Jn naulet N Martin GJ " authentication of essential oils containing linalool and linalyl acetate by isotopic methods" J Agr food chem (1992) 40(1) 81-87.

93. Harborne JB, Phytochemical methods,London – Chapman Hall, (1973):54

94. Harborne JB.,"Phytochemical Analysis- A guide to modern techniques of plant analysis" London, Chapman & Hall; (1973)

95. Hashimoto K, Uchimura H, Seki Y, Watanabe M, Yasuda T, Ohsawa K "Determination of synephrine in oriental pharmaceutical decoctions containing aurantii nobilis percarpium by ion-pair high-performance liquid chromatography", Yakugaku Zasshi 1993, 113 (8), 600-603.

96. Hashimoto K, Yauda T, Ohsawa K, "Determination of synephrine from chinese nedicinal drugs originating from citrus species by ion-pair High-performance liquid chromatography" j chromatogr (1992) 623, 386-389

97. He XG Lian HZ Lin LZ Bernart MW "High-performance liquid chromatography-electrospray mass spectrometry in phytochemical analysis of sour orange (citrus aurantium L) "J Chromatogr A (1997) 791 (½) 127- 134.

98. Hedberg.I, Hedberg.O, Madati.P.J, Mshigeni.K.E, Mshiu. E.N, Samuwlssom.G, (1983) "Inventory of plants used in traditional mediine in Tanzania. Part III. Plants of the families papilionaceae-vitaceae". *J Ethno pharma col*, 9 : (2/3) 237-260.

99. Heywood V H, "The Systemics Associations, "Scanning Electron Microscopy, Systemics and evolutionary appplications", Proceedings of an International Symposium held at the Department of Botany, Spl vol.4, University of Reading, Academic Press, London, (1971): 1-16.

100. Hharma.R.K, Arora.R, (2006) eds, "Herbal drugs A twenty first century perspective" 1st edn., Jaypee Publishers New Delhi.

101. Hirano H Takase H Yamamoto K Yanase T Abe K saito Y " Anti-ulcer effect od aurantii fructus immaturus aurantii fructus and the principles in aurantii fructus immaturus" Natural med (1997) 51(3) 190-193

102. Holdsworth.D.K. (1990) "Traditional medicinal plants of Rarotonga, Cook islands part I". *Int crude drug Res*. 28 : (3) 209-218.

103. Hong ND Chang IK, Kim NJ Lee SI "Studies on the efficacy of combined preparation of crude drugs (XXXIX). Effects of 'Hyangsayangwee – Tang' on the stomach and intestinal disorder." Korean J pharmacog 1989 20 (3) 188-195.

104. Hosoda K Noguchi M, "Studies on the preparation and evaluation of kijitsu the immature citrus fruits I. Evaluation of the new dryimh method", Yakugaku Zasshi (1988), 108 (10), 1008-1011.

105. Hosoda K Noguchi M, Kanaya J, Hiquchi M, "Studies on the preparation and evaluation of kijitsu the immature citrus fruits III. Relation between diameter o kijitsu and synephrine content" Yakugaku Zasshi (1990), 110 (1), 82-84.

106. Hou YC Hsiu SL Tsao CW Wang YH Chao PDL "Acute intoxication of cyclosporin caused by coadministration of decoctions of the fruits of citrus aurantium and the percarps of citrus grandis" Planta med (2000) 66(7) 653-655

107. Hou YC Yen HF Hsiu SL Chen CC Chao PDL "Comparison of flavanone absorption of grape fruit juice with herbal decoctions of zhigiao and huajuhong in healthy chinese" J Chin medf (1999) 10(1) 65-73

108. Huang YT, Lo JW, Hong CY, Chem CF, Yu PC, Yang MEM, "Hemodynamic effecs of fructus aurantium on the portal hypertensive rats" J Chin Med 1994 5(4) 164-165

109. Hung ND, Chang IK, Jung HC, Kim NJ "Studies on the efficacy of combined prescriptions of crude drugs XII" Korean J Pharmacog (1983) 14(1) 9-16

110. Ishhara S, Yoshida.S, Tosa.S, Nakayawa.H,Tomimatsu T, "Study of flavanoids in citrus changes in content of flavonone glycosides in the peel of citrus" Shoyakugaku Yasshi (1990) UU 127-130

111. Itokawa H, Mihashi S, Watanabe K Natsumoto H hamanaka T "Studies on the constituents of crude drugs having inhibitory activity against contraction of the ileum caused by histamine or barium chloride (1). Screening test for the activity of commercially available crude drugs and the related plant materials" Shoyakugaku Zasshi (1983) 37 (3) 223-228

112. Iwase Y, Takemura Y, Motoharu J Kawaiis. Yano M Okuda Y Mukainaka Ttsuruta A, Okuda M, Takayasu J, Tokuda H, Nishino H "Inhibitory effect of Epstein-Barr virus activation by citrus fruits, A cancer chemopreventor'Cancer lett (1999) 139(2) 227-236

113. Iyengar MA, Nayak SGK, "Pharmacognasy Lab Manual", (1994): 78-87.

114. Jager W Buchbarer g jirovetz l Dietrich H Plank C " Evidence of the sedative effect of neroli oil, citronellal and phenyethyl acetate on mice" j essent oil res (1992) 4(4) 387-394

115. Jain.S.K, Tarafder.C.R, (1970) "Medicinal plant-lore of the santals". *Econ Bot.* 24: 241-278.

116. Janssen AM Chin NLJ Schetter JJC baerheim-svendsen A" screening for antimicrobial activity of some essential oils by the agar overlay technique" pharm weekbl (Sci ed) (1986) 8(6) 289-292

117. Jeong CS Kyun JE Kang MH Kim HP Park JM Lee SY"Anti gastric and anti-ulderative effect of P020701" Korean J pharmacog (2002) 33(4) 389-394

118. Johansen DA, "Plant Micro technique", McGraw Hill Book Company (1940): 27-93, 126-154.

119. Kanes K, Tisserat B, Berhow M, Vandercook C, "Phenolic composition of various tissues of Rutaceae species" Phytochemistry (1993),32 (4): 967-974.

120. Karawva MS, Khayyal SE, Farrag NM, Ayad MM, "Screening of diphenylamine as an antihyperglycemic in certain edible plant organs" Egypt J Pharm Sci (1986), 25 (1/2/3): 21-25.

121. Karawy MS, Ehayyal ASE, Farrag NM, Ayad MM, "Screening of diphenylamine as an antihyperglycaemic against in certain edible plant organs" Acta pharm Hung (1986), 56, 55-58.

122. Kargbo.T.K, (1984) "Traditional practices affecting the health of women and children in Africa". Unpublished manuscript.

123. Kato M, Nagao J, Hayashi M, Hayashi E, 'Pharmacological studies on saiko prescriptions 1. Effects of saiko prescriptions on the isolated smooth muscles" Yakugaku Zasshi 1982 102, 371-380

124. Khalid.S.D, Farouk.A, Geary.T.G, Jensen.J.B. (1986) "Potential antimalarial candidates from African plants an in vitro approach using *Plasmodium Falciparum*". *J ethno pharma col*. 15 : (2) 201-209.

125. Kim Dh, Song MJ, Bae EA, Han MJ "Inhibitory effect of herbal medicines on rotavirus infectivity" Biol pharm bull 2000 23(3) 356-358

126. Kim SE Hwang BY Kim KH Kim YC Lee KS Lee JJ " Multidrug-resistance reservinf activity of medicinal plants" korean J pharmacog (1997) 28(4) 174-178

127. Kim TI, No JYKO, Rhee JS, "The separation and determination of flavanoid glycoside from poncirus Trifoliate Rafia and citrus" Aurantum L Punsok Kwahak (1989).2 (2)301-307

128. Kim.DH, Song MJ, Bae Ea, Han MJ, "Inhibitory effect of herbal medicines on rotavirus infectivity" Biol pharm bull (2000), 23 (3), 356-358.

129. Kinoshita T, Sameshima M, Sankawa V, "Isolation of a sympathomimetic substance from chinese medicinal drugs originated from citrus species" shoyakugaku zasshi (1979), 33, 146-149.

130. Kirtikar K.R. Basu B.D., "Indian Medicinal Plants", 2nd ed. Vol.1, Publishers: Bishen Singh Mahendra Pal Singh, Dehradun, 1998.

131. Kiso Y, Suzuki M, Yasuda M, Watanabe H, Minegishi T, Hikino H, Yang LL, Yen KY, "Antihepatotoxic actions of traditiomal chinese medicines 2. The phatmacological interaction of components of a dai-saiko-to employing complement mediated cytotoxiciyt in primary cultured mouse hepatocytes" Phytother Res (1990) 4 (1) 36-38

132. Kokate CK, Purohit AP, Gokhale SB, "Pharmacognosy", 32th edition, Pure, Nirali Praksahan, (2005): 99-100, 111-112.

133. Krebs D, Weigert E, "Flavomoid compounds especially rutin found in Santa Maria Flowers" rev cent cienc biomed univ fed st maria (1972) 1. 77-82.

134. Kusu F, Li XD, Takamura K, "Determination of synephrine and n-methyl tyramine in Zhishi and Zhike (immature citrus fruits) by high-performance liuid chromatography with electrechemical detection" Chem Pharm Bull (1992) 40 (12) 3284-3286

135. Kusunose H, Sauamura M, "Aroma constituents of some sour citrus oils", Nippon Shokuhin Kogyo Gakkaishi, (1980) 27, 517-521.

136. Lam LKT zheng BL" Effects of essential oils on Glutathione s-tranferase activity in mice" J Agr food chem (1991) 39(4) 660-662

137. Lee CH Kim SI Lee KB Yoo YC Ryu SY Song KS "Neurominidase inhibitors from reynoutria elliptica" Arch pharma res (2003) 26(5) 367-374

138. Lee EB" Teratogenecity of te extracts of crde drugs" Korean J pharmacog (1982) 13, 116-121

139. Lee YC Sheu SJ, "Analysis and processing of Chinese herbs (IX). The study of Aurantii fructus" Chung-Hua Yao Hsueh TSA Chih (1989) 41(3): 247-254.

140. Leo BH Cho BW Ryu GS Leo ES Kang KJ Hwang DY Hong ND " Screening of the acetylcholinesterase inhibitors from medicinal plants" Korean J pharmacog (1997) 28(4) 167-173

141. Letcher. RM, "Structure Elucidation of a Natural Products" J Chem Ed (1983) 60(1) 79-81.

142. Lin ZK Hua YF, "Systematic evolution relation of chemical components of the essential oils from 'Taxa of citrus leaves' Chih wu hsueh pao (1992) 34(2); 133-139.

143. Ma LA, zheng YQ, Sun Y, Liu MX, Wu. ZP " Aroma volatile constituents of citrus aurantium var anara Engl" Beijing daxue xuebao ziran kexueban 1988, 24 (6) 687-694

144. Magadan Figueroa R, Rojas Carballosa L, “Rutaceae, phytochemical possibilities of Cuban flora” Rev Cubana farm 1979,13, 101-107.

145. Malhotra s Bailey DG Paine MF Watkins PB “ Seville orange juice felodipine interaction. Comparison with dilute grape fruit juie and involvement of furocoumarins” Clin phasrmacol ther (2001) 69(1) 14-23

146. Manwaring Dg, Rickards RW, “The identity of cirantin A reported antifertility against with hesperidin” phytochemistry (1968), 7, 1881-1882.

147. Martinez M.A, (1984) “Medicinal plants used in a totanac community of the sierra norte De Puebla: Tuzamapan de galeana, Puebla Mexico” *J Ethno pharmacol* 11:(2) 203-221.

148. Maruzzella JC Balter J “The action of essential oils on phytopathogenic fungi” Plant dis rept (1959) 43, 1143-1147

149. Maruzzella JC scrandis D Scrandis JB Grabon G “Action of odoriferous organic chemicals and essential oils on wood-destroying fungi” Plant dis rept (1960) 44 789

150. Matsumotu T, Tashiro N, Nishimura K, Takeya K, “New cyclicpeptides from citrus aurantium”, Heterocycles (2002), 56 (3) 477-482

151. Maurcer B Hauser A, “New pyridine derivatives from essential oils” Chimia (1992) 46 (4) 93-95.

152. May G, Willuhn G, “Anti viral activity of aqueous extracts from medicinal plants in tissue cultures” Arzneim-Forscb (1978)28(1) 1-7

153. Mc Hale D, Khopkar PP, Sheridan JB, “Coumarin glycosides from citrus flavedo”, Phytochemistry (1987), 26 (9), 2547-2549

154. Me.E 140 C, Huang G, Liu F, “The treatment of severe hemorrhage of the gastrointestinal tract in burn children by combined traditional chinese and western medicine” Chung 1, TSA Chin (Engl ed) 1983, 3(1): 59-61

155. Min KR Kim Y Kang SH Masr WC Leo KS Ro JS Leo SH Kim Y “Inhibitory effects of herbal extracts on cyclooxygenase activity of prostaglandin H2 systhase from sheep senimal vesicle’ Nat prod sci 91996), 2 (10) 56-74

156. Mitscher LA Leu RP bathala MS Wu W Boal JL "Antimicrobial agents from higher plants 1. Introduction rationale and methodology" lloydia (1972) 35(2) 157-166

157. Miura M Ohta s kamogawa A Shinoda M " Basic study of assay method of cholerectic effect and the screening of crude drugs" yakugaku zasshi (1987) 107(12) 992-1000.

158. Miyake M Ayano S Ozaki Y Herman Z, Hasegawa S, "Limonoids in seeds of sour orange (citrus aurantium)" Nippon Nogei Kagatu Kaishi (1992) 66(1): 31-34.

159. Miyazawa M, Okuno Fukuyama M, Nakamura SI, Kosaka H "Anti mutagenic activity of polymeyhoxy flavanoids from citrus aurantium" J Agr food chem (1999) 47 (12), 5239-5244

160. Miyuno M, Inuma M, Ohara M, Tanaka T, Iwamasa.M, "Chemotaxonomy of the genus citrus based on polymethoxy flavones", Chempharm Bull (1991) 39 (4) 945-949

161. Mori S, Tanaka N, Ichii J, Yorozu H, "Antiobesity compositions containing citrus or evodia extracts and other active ingredients" Patent-Japan kokai tokkyo koho-08 81, 382 (1996) 8 patent Chemical abstracts 125 19013 C kao corp Japan

162. Moromoto I Watanabe f Osawa T, Okitsu T Kada T, "Mutageneciy screening of crude drugs with bacillus subtilis Rec assay and salmonella/microsome reversion assay"Mutat Res (1982) 97 , 81-102

163. Morton.J.F, (1968) "A survey of medicinal plants of curacao". *Econ bot*. 22: 87.

164. Mouly PP, Arzouyan CR, Gaydou EM, Estienne Jm, "Differentiation of citrus juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides" J Agr food chem. (1994),42(1): 70-79.

165. Moursi SAH, Al-Khatib MH, Al-Shabibi MM, "Phytochemical investigation of the flowers of citrus aurantium", Fitoterapia (1980) 51, 207-209.

166. Moustafa SMI, Attia IA, Hassan NA, "Determination of flavonids" Fitoterapia (1984) 55(5) 295-296.

167. Namba T, Arak I, Mikage M, Hattori M, “Fundamental studies on the evaluation of crude drugs. VIII, Monthly variations in anatomical characteristic and chemical components of the dried fruit peels of citrus unshiu, c.aurantium and C. Matsudaidai” Shoyakugaku Zasshi (1985) 39 (1), 52-62.

168. Naovi SAH, Khan MSY, Vohora SB “Anti-bacterial, Anti-fungal and Anthelmintic investigations on Indian medicinal plants” Fitoterapia (1991) 62(3) 221-228

169. Nishiyori T, Matsuura N, Nagai H, Koda A, “Anti-allergic action of chinese drugs” Jap J Pharmacol Suppl 1981) 31, 115

170. Nogata Y, Yoza KI, Kusumoto KI, Kohyama N, sekiya K, Ohta h,” Screening for inhibitory activity of citrus fruit extracts against platelet cyclooxygenase and lipoxygenase” J agr food chem (19960 44(3) 725-729

171. Nordbyhe, Nagy S, “Chemotaxonomic study of neutral coumarins in roots of citrus and poncirus by Thin-layer, Gas-liquid and High performance liquid chromatographic analysis” J Chromatogr (1981, 207; 21-28.

172. Ohta I, Ogawa K, Saito H, Ohsawa K “Determination of synephrine in oriental pharmaceutical decoctions containing aurantii fructus immaturus by ion-pair high-performance liquid chromatography” yakugaku zasshi (1994), 114 (9), 691-696.

173. Ohta S, Sakurai N Inoue T Shinoda M “Studies on chemical protectors against radiation XXV. Radioprotective activities of various crude drugs” yakugatu zasshi (1987) 107(1) 70-75.

174. Okuyama T, Takata M, Shibata S, Hoson M, Kawada J Masaki H, Noguchi T, “Effect of Sino-Japanese Medicine on Platelet Aggregation (IV). Chinese medicinal prescriptions employed for angina pectoris like symptom” hoyukugaku zasshi (1987) 41 (2) 147-152.

175. Ortuno A, Botia JM, Fuster MD, Porras I garcia-Lidon A, del Rio JA, “Effect of scoparone (6,7 Dimethoxy coumarin) biosynthesis on the resistance of tangelo nova, citrus paradisi, and citrus aurantium fruits against phytophhora parasitica” J Agr food chem (1997), 45(7) 2740-2743.

176. Park JG hyun JW Lim KH Shin JE Won YJ Yi YD Shin KH Chang Im Woo WS, "Antineoplastic effect of extracts from traditional medicinal plants" Korean J pharmacog (1993) 24(3) 223-230.

177. Parra AL yhebra RS Sardinas IG Buela LI "Comparative study of the assay of Artemia salina L and the estimate of the medium lethal dose (LD50 value) in mice to determine oral acute toxicity of plant extracts" Phytomedicine (2001) 8 (5) 395-400

178. Paul .A, Cox.P.A, (1995) "An ethnobotanical survey of the uses for Citrus aurantiun (Rutaceae) in Haiti" *Econ Bot* 49: (3) 249-256

179. Pino JA, Rosado A, "Composition of cold-pressed bitter orange oil from Cuba" J essent oil Res (2000) 12 (6) 675-676

180. Pino JA, Rosado. A, "Composition of cold-pressed bitter orange oil from Cuba" J essent oil Res (2000) 12(6); 675-676.

181. Purvis MJ, Collier DC, Wallis D, "Laboratory Techniques in Botany", 2nd edn, Butter worth & Co Ltd, (1966): 82 -169.

182. Ramirez. V.K, Mostacera. L.J, Garcia .A.E, Mejia. C.F, Pelaez. P.F, Medina.C.D, Miranda.C.H, (1988) "Vegetables empleaodos En medicina traditinal norperuana" Banao Agrario Del Peru & NACL Univ trujillo, Trujillo, Peru, June 1988, 54.

183. Reiter M Branat W "Relaxant effevts on tracheal and Ileal smooth muscles of the guinea pig" Arzneim-forsch (1985) 35(1) 408-414

184. Rio JAD, Benavente O, Castillo J Borrego F, "Neodiosmin, a flavone glycoside of citrus aurantium" Phytochemistry (1992), 31 (2): 723-724.

185. Robards "Electron micrcoscopy and plant ultra structure", McGraw Hill, London, (1970): 14-15, 36-59.

186. Roengsumran s Petsom a Thanivanvarn S Pornpakakul S, khantahiran S "antibacterial activity of some essential oils" J sci res chulalongkorn univ (1997) 22(1) 13-19

187. Ross SA El-keltawi NE , Megalla Se "Antimicrobial activity of some egyptian aromatic plants" Fitoterapia (1980) 51 201-205

188. Rouseff RL Nagy S, "Distribution of limonoids in citrus seeds" Phytochemistry (1982) 21,85-90.

189. Rousett RL Martin SF, Youtsey CO, "Quantitative survey of narirutin, Naringin, Hesperidin and Neo hesperidin in citrus" J Agr food chem. (1987), 35 (6): 1027-1030.

190. Russin WA Gould MN "Cancer chemopreventive effects of terpene components of orange oil" Amer J Bot (1988),75 (6): 133.

191. Sata A "Studies on anti-tumor activity of crude drugs 1. The effects of aqueous extracts of some crude drugs in shorterm screening test" Yakugaku zasshi (1989) 109(6) 407-423

192. Satoh YS, Tashiro SI, Satoh MT, Fcyimoto YS, Xu JY Ikekawa TS, "Studies on the bioactive constituents of auranti fructus immaturus" Yakugaku gasshi (1996), 116 (3), 244-250.

193. Schelz Z, Molnar.J, Hohmann.J, (2006) "Antimicrobial and antiplasmid activities of essential oils", *Fitoterapia* 77, 279-285.

194. Schimmer O Kruger A Paulini H Haefeke F "An evaluation of 55 commercial plant extract in the ames mutagenecity test" Pharmazie (1994) 49(6) 448-451

195. Shaft N, Ikram M, "Quantitative survey of rutin- containing plants. Part.I" Int J Crude Drugs Res (1982) 20 (9). 183-186.

196. Shi l, Gotou Y, Shindo K, Ogawa K, Shida Y Sashida Y, Shimomura H, Araki C, Yashida T, "Synephrine contents and their seasonal variation in peels of citrus plants" Shoyakugaku Zasshi (1992). 46 (2) 150-155.

197. Shin KH Woo WS 'A survey of the response of medicinal plants on drug metabolism" Jorean J pharmacog (1980) 11, 109-122

198. Shin Nh Lee KS Kang SH Min KR Lee SH Kim YS "Inhibitory effects of herbal extracts on Dopa oxidase activity of tyrosinase" Nat prod sci (1997) 3(2) 111-121

199. Shirota A, Miura M, "Extraction of antimutagenic substances from unripened oranges" patent Japan Kokai Tokkyo Koho-06 211, 624 (1994) 3, chenical abstracts 121 286580 C Kisho Kk Japan

200. Shizaki K Goto K Ishige A Komatsu Y "Bioassay of phyto estrogen in herbal medicine used for post menopausal disorder using transformed MCF-7 cells" Phyto ther Res (1999) 13(1) 498-503

201. Simon PW Chaboud A Darbour N Dipletro A Dumonter C Raynaud J Barron D "The two-complementary method assay fro screening new reversal agents of cancer cell multidrug resistance" Pharmaceutical biol (2003) 41(1) 72-77

202. Sokmen A, Gulluce M, Akpulat HA, Dafarera D, Tepe B, Pollissiou M, *et al*. Food Control, 2004, 15:627.

203. Song MJ Kim DH "Onhibitory effect of herbal medicines on Rotavirus infection' Korean J pharmacog (1998) 29(2) 125-128

204. Stewart I "Identification of caffeine in citrus flowers and leaves" J Agr food chem. (1985), 33(6): 1163-1165.

205. Takanaga H, Obnishi A, Yamada S, Matsuo H, Morimoto S, Shoyama Y, Obtani H, Sawada Y, "Polymrthoxylated flavones in orange juice are inhibitors of P-Glycoprotein but not cytochrome P450 3A 4" J pharmacol Exp ther (2000) 293 (1) 230-236

206. Tanizawa H Ohkawa Y Takino Y Miyase T, Veno A Kageyama T Hara S " Studies on natural Antioxidants in citrus species.1.Determination of antioxidative activities of citrus fruits'Chem pharm Bull (1992) 40(7): 1940-1942

207. Tatum Jh, berry RE "6, 7 Domethoxy coumarin in the peels of citrus" Phytochemistry (1977) 16, 1091-1092.

208. Tomer E, Goren R, Monselise SP,"Isolation and Identification of seselin in citrus roots" Phytochemistry 1969, 8, 1315-1316.

209. Vazquez .F.M, Sauarez.M.A, Perez.A (1997) "Medicinal plants used in the barros area, Badajoz province (Spain)". *J Ethno pharmacol*. 44 : (2) 81-85.

210. Veki H, Kaibara M, Sakagawa m, Hayashi S" Anti-tumor activity of plant constituents I" Yakugaku zasshi (1961) 81, 1641-1644

211. Verpoorte R, Dihal PP 'Medicinal plants of Surinam IV. Antimicrobial activity of some medicinal plants"J Ethnopharmacol (1987), 21(3) 315-318

212. Wagner H, Bladt S, Munzing-Vasitian K, "Thin-layer chromatography of bitter principle drugs" Pharm-ZTG (1975), 120, 1262. Chemical abstracts 83

213. Wallis TE , "Analytical Microscopy", 3rd edn, J&A Churchill Ltd, (1965): 180, 183, 184.

214. Wallis TE, "Practical Pharmacognosy" 6th edn, J& A Churchill Ltd, (1953): 170-175

215. Wang JZ, Chem DY, Wang .J, "Determination of Hesperidin in pericarpium citri", by HPLC Khonggu Yhongyao Yayhi (1994), 19 424-425

216. Wattenberg L "Chalcones, Myo-inositol and other novel inhibiotors of pulmonary carcinogenesis" j cell biochem (1995) 22, 162-168

217. Weniger B rouzier. M, Daguilh .R, Henrys.D, Henrys .J.H, Anton.R, (1986) "Popular medicine of the central plateau of Haiti. 2. Ethnopharmacological Inventory" *J Ethno pharmacol* 17: (1) 13-30

218. Wesley-Hadzija b Bohing P" Influence of some essential oils on the central nervous system of fish" Ann Pharm FR (1956) 14, 283

219. Whistler W.A, (1985)" Traditional and herbal medicine in the Cook Islands" J *Ethnopharmacol* , 13 : (3) 239-280.

220. Widmer WW "Improvements in the quantitation of limonin in citrus juice by reversed-phase high performance liquid chromatography" J agr food chem (1991) 39(8) 1472-1476

221. Woo WS Lee EB "The screening of biological active plants in Korea using isolated oragan preparations 1. Anticholinergic and oxytocic actions in the ileum and uterus" Ann Rep Nat prod Res inst s eoul natl Univ (1976) 15.138

222. Woo WS Lee EB Chang I "Biological evaluation of korean medicinal plants II." Yakhak Hoe chi (1977) 21,177-183.

223. Woo Ws Shin Km Kim IC Lee CK "A survey of the response of Korean medicinal plants on drug metabolism" Arch pharm Res (1978) 1, 13-19

224. Wu. FJ Sheu SJ, "Analysis and processing of Chinese drugs (XII), The study of fructus aurantii immaturus" Zhonghua Yaoxue Zazhi (1992), 44 (3): 257-263.

225. www.essentialoils.co.za/components.html *The chemistry of essential oils, and their chemical components.* accessed on 27.12.2010

226. Yen YF chung WP, "Preliminary study of the effect on receptors and of the positive inotropic effects of N-methyl tyramine, an active principle of zhishi (citrus aurantium)", Hu- Nan I Hsueh Yuan Hsueh Pao (1981) , 6. 131-134.

227. Yoo JS Jung JS Lee TH Son KH Suh HW Song DK Kim YH " Inhibitory effects of extracts from traditional herbal drugs on 5-Hydroxy tryptophan induced diarrhea in mice" korean J Pharmacog (1995) 26(4) 355-359

228. Yul Jy Eun JS, "Effect of Aurantii Nobilis Pericarpium and Aurantii Immaturi Pericarpium on Immunocytes in Mice" Korean J Pharmacy (1998) 29 (3) 173-178.

229. Yun Hs Chang Im "Liver protective activities of Korean medicinal plants" Korean J pharmacog (1980) 11, 149-152

230. Zheng HJ, Liu YF, Zhou YY, "Determination of synephrine and N-Methyl tramine in citrus crude drugs and zhi shi (immature citrus fruit) inhections by reversed phase Ion pairing HPLC", Chung TS'AO Yao (1983) 14 (5): 200-204.

231. Zu Z, "Determination of the synephrine content in Zazhishi by first derivative spectrometry" Yaowu Fenxi Zazhi (1987), 7 (2): 100-103.

PLATE - 1
HABIT AND HABITAT OF *Citrus aurantium* L.

PLATE - 2

A TWIG SHOWING LEAF ARRANGEMENT OF *Citrus aurantium* L.

PLATE - 3

LEAF OF *Citrus aurantium* L. DORSAL AND VENTRAL

PLATE - 4

FLOWERS OF *Citrus aurantium* L.

PLATE - 5

FRUITS OF *Citrus aurantium* L.

Fig.
T.S OF PETIOLE (GROUND PLAN)

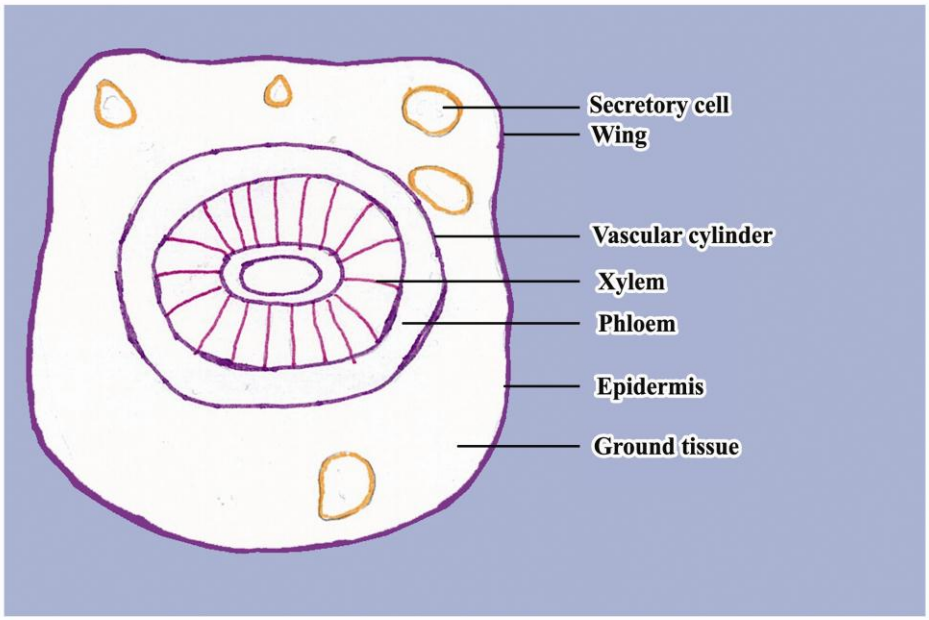


Fig.
T.S OF PETIOLE - DETAILED DIAGRAM

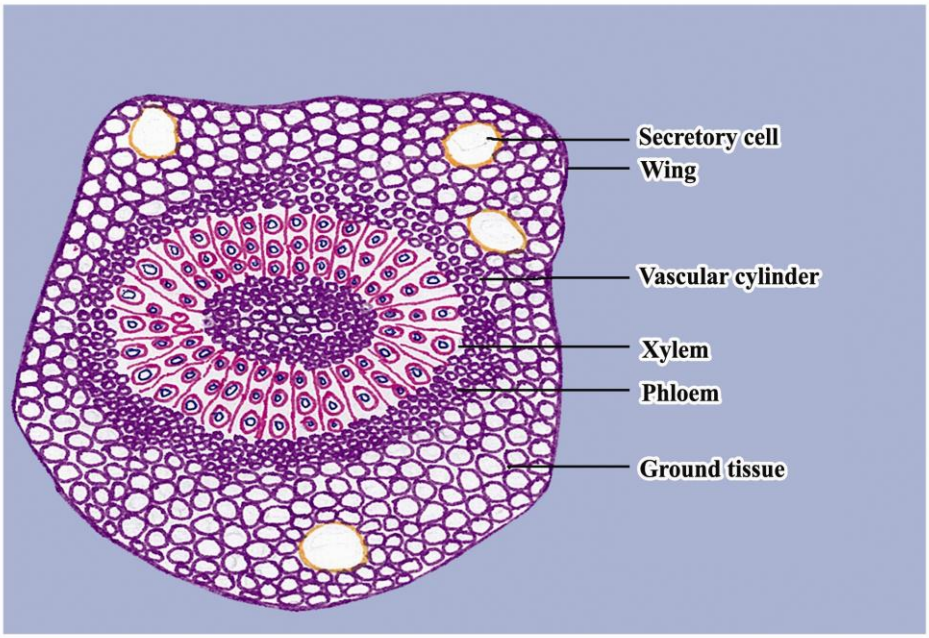


Fig.1 DIAGRAMATIC REPRESENTATION OF *Citrus aurantium* L.

Flower Parts

Fig – 6

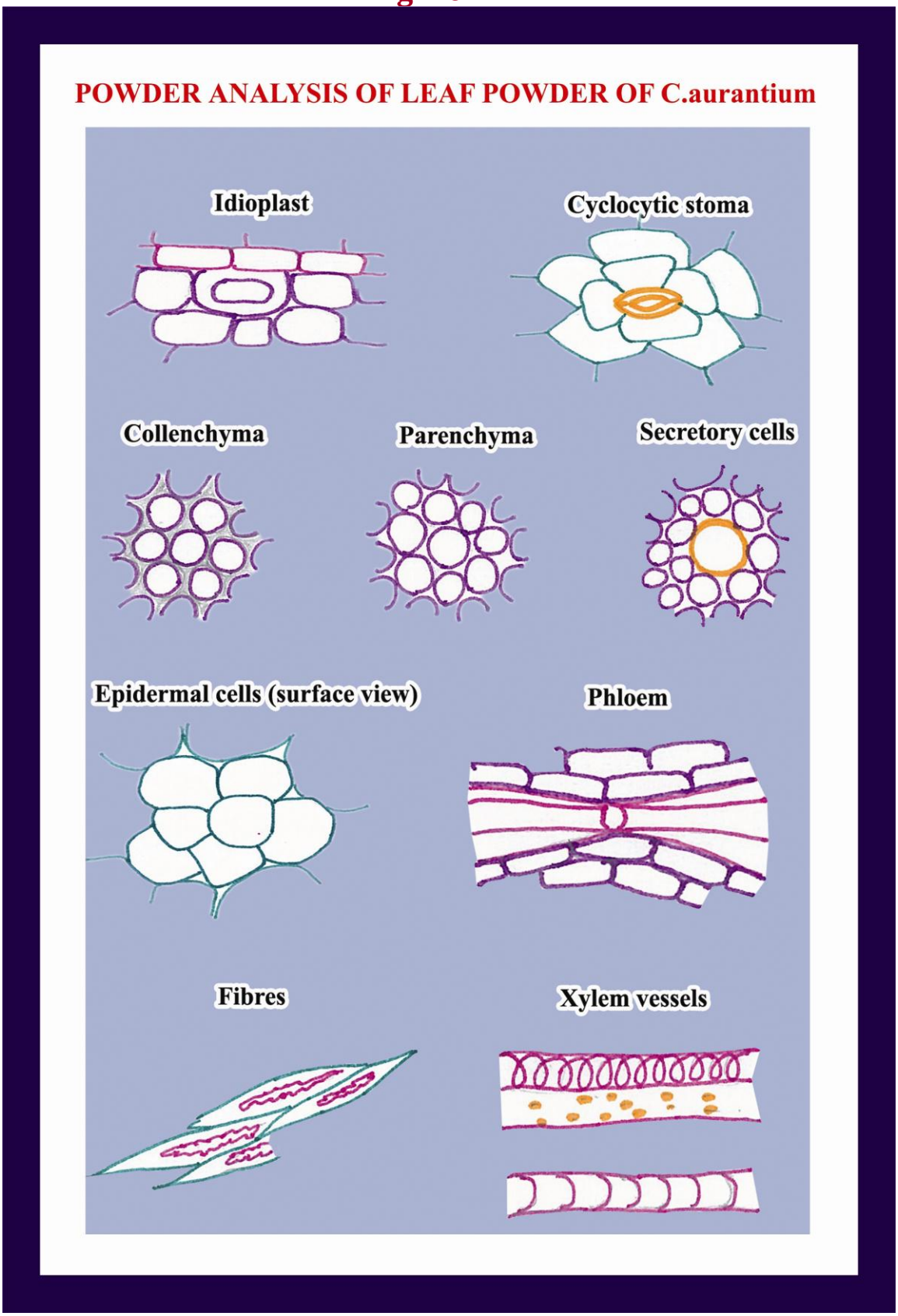
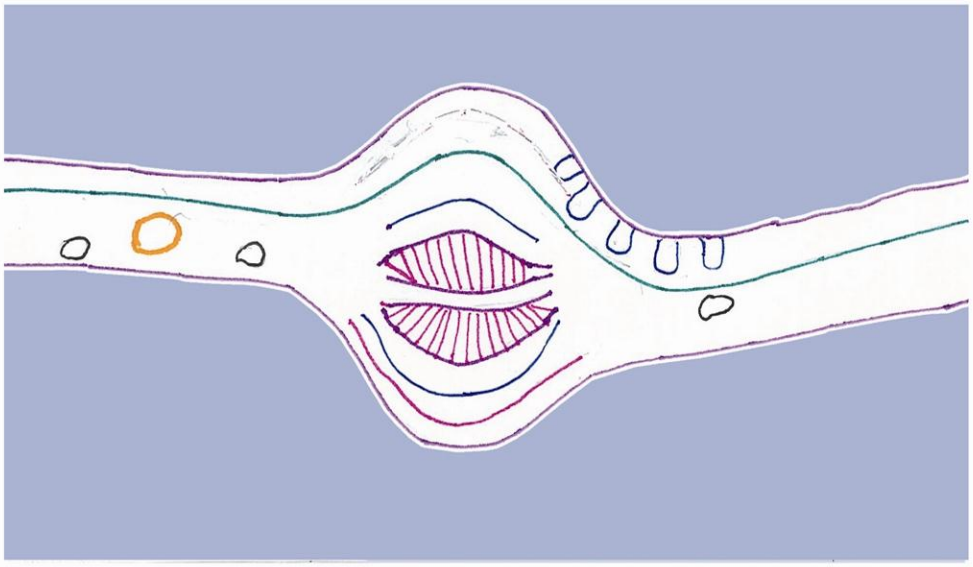

POWDER ANALYSIS OF LEAF POWDER OF C.aurantium
Idioplast
Cyclocytic stoma
Collenchyma
Parenchyma
Secretory cells
Epidermal cells (surface view)
Phloem
Fibres
Xylem vessels

Fig. 4

T.S OF LEAF THROUGH MIDRIB (GROUND PLAN)



T.S OF LEAF THROUGH MIDRIB (DETAILED DIAGRAM)

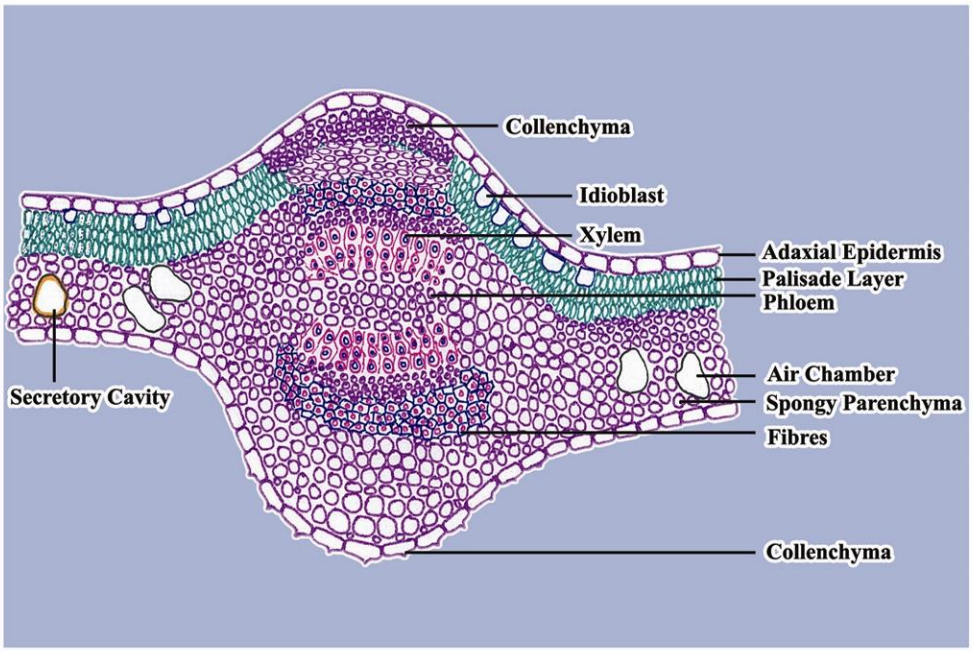
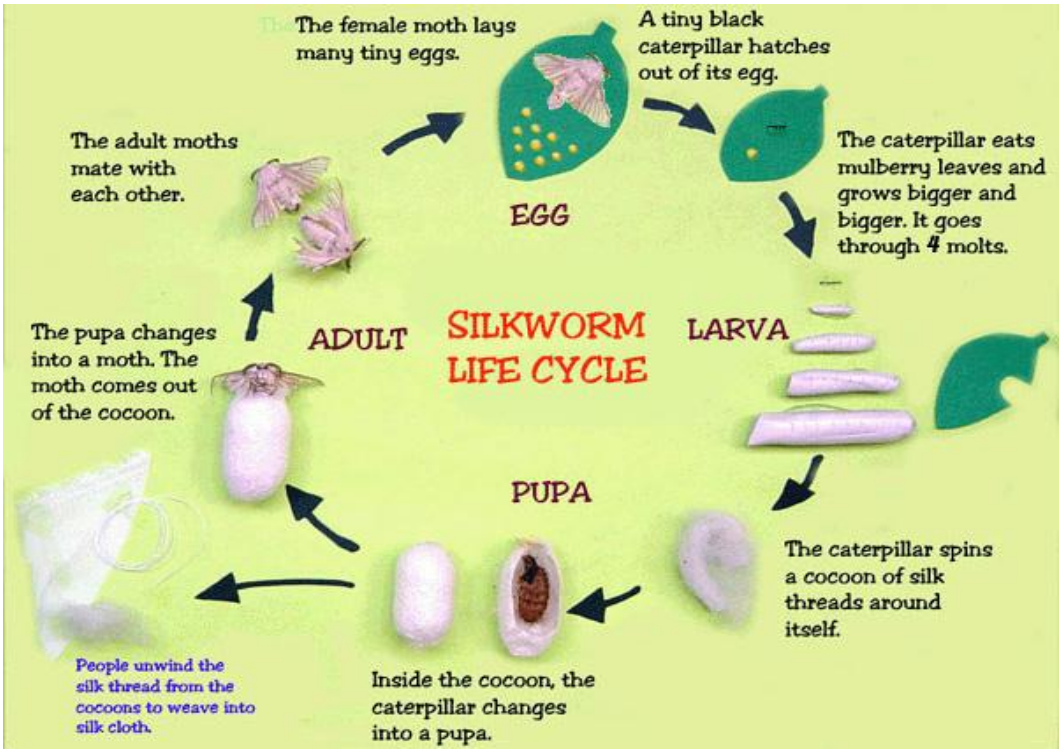


PLATE - 15



REARING OF *Bombyx mori* – 5TH INSTAR LARVAE

PLATE - 16
INJECTION OF DYE INTO INTRA HEMOLYMPH OF LARVA

INJECTION OF DYE INTO MIDGUT

PLATE - 17

DYE INJECTED *Bombyx mori* SHOWING COLOUR DIFFERENCE FROM NORMAL (In day light)

DYE INJECTED *Bombyx mori* SHOWINCOLOUR DIFFERENCE FROM NORMAL(Under Illumination)

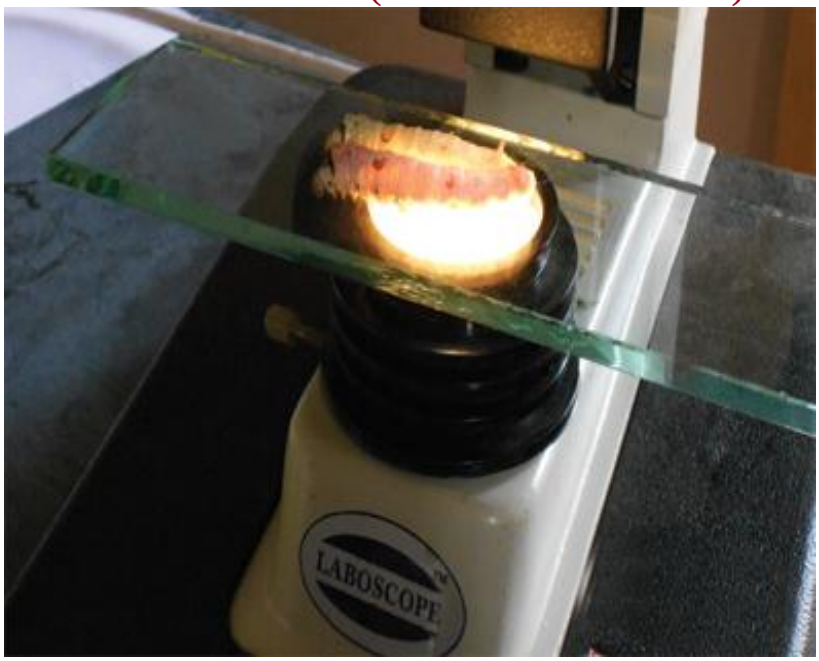


PLATE - 18

S.aureus INFECTED *Bombyx mori* 5^{TH} INSTAR LARVAE

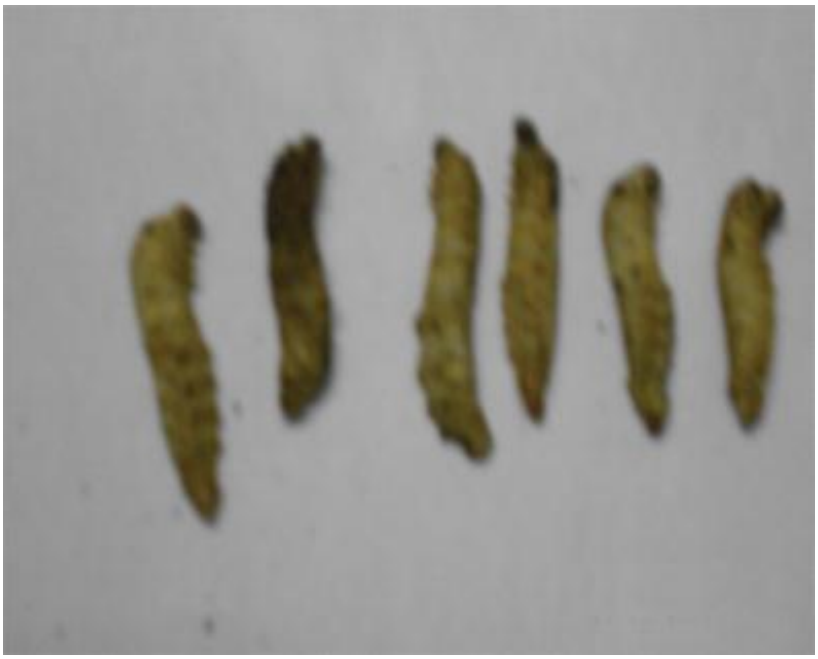


PLATE - 20

COLLECTION OF HEMOLYMPH

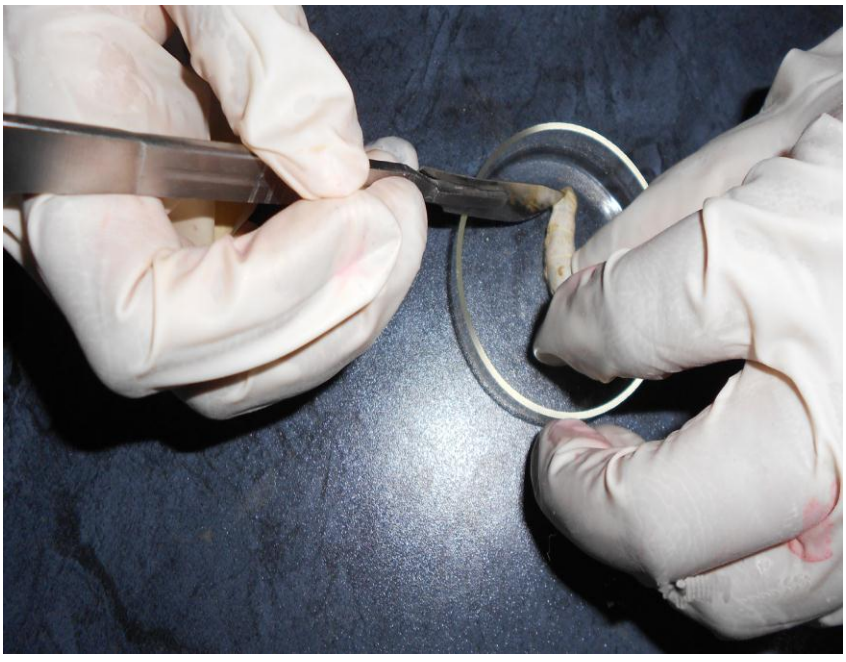
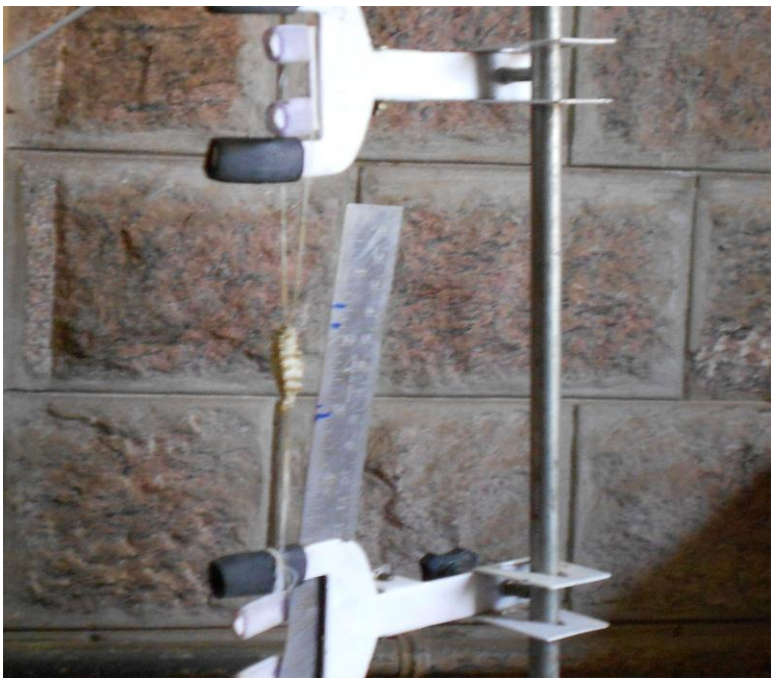
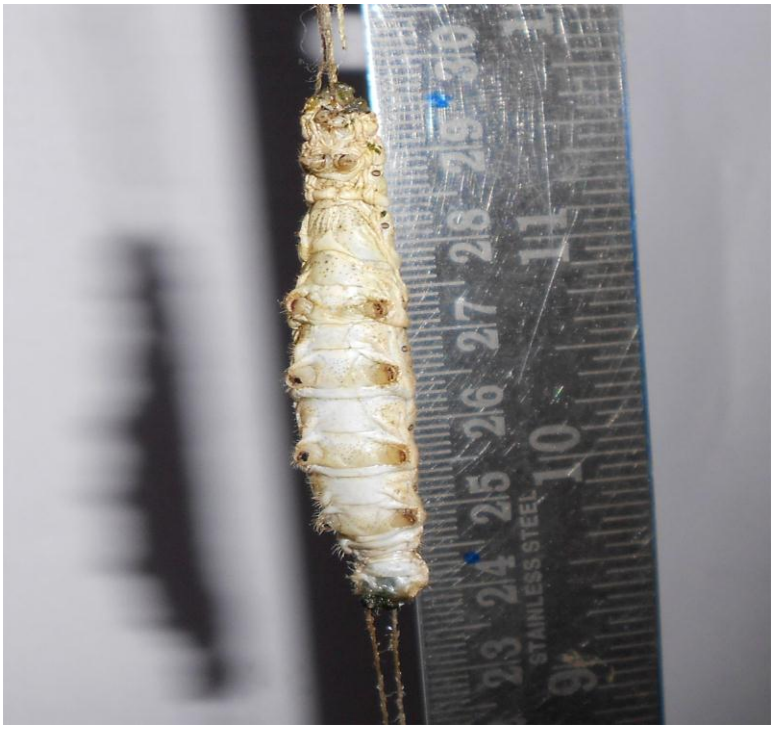


PLATE - 21

MEASUREMENT OF BODY LENGTH OF *Bombyx mori* 5TH INSTAR LARVAE STIMULATION OF INNATE IMMUNITY BY VO



INITIAL LENGTH 0 MIN



AFTER 6 MIN

FINAL LENGTH AFTER 10 MIN

PLATE -19


ANTI BACTERIAL ACTIVITY OF VOLATILE OIL OF LEAF OF C.aurantium on S.aureus
0.25
S
0.5
C
0.75


ANTI BACTERIAL ACTIVITY OF VOLATILE OIL OF LEAF OF C.aurantium on MRSA
0.25
C
S
0.5
0.75