ANTIMICROBIAL AND CYTOTOXIC EVALUATION OF METHANOLIC EXTRACT OF *THESPESIA POPULNEA* (MALVACEAE) FLOWERS

A Dissertation Submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY, CHENNAI

In Partial fulfillment of the requirement for the

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Submitted by

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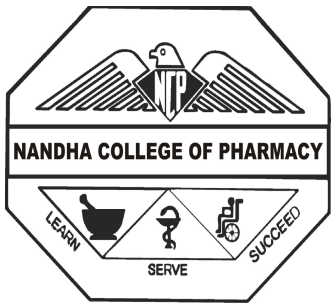

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MARCH - 2008

DECLARATION

I declare that I have carried out the work presented in this thesis entitled, **"Antimicrobial and cytotoxic evaluation of methanolic extract of *Thespesia populnea* (Malvaceae) flowers"** in the Department of Pharmaceutical Bio-technology, Nandha College of Pharmacy, Erode-52. This work is original and has not been previously formed the basic for the award of other degree, diploma, associate ship, fellowship or any other similar title of any other University.

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled**. "ANTIMICROBIAL AND CYTOTOXIC EVALUATION OF METHANOLIC EXTRACT OF *Thespesia populnea* (MALVACEAE) FLOWERS"** in the Department of Pharmaceutical Bio-technology"Submitted to the Tamilnadu, Dr. M.G.R. Medical University, Chennai, was carried out by K.VENKATESHWARAN, Department of Pharmaceutical Bio-technology, Nandha College of Pharmacy, Erode-52. in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Biotechnology under my supervision and guidance.

This work was original and has not been submitted in part or full for any other degree, diploma, associateship, fellowship or any other similar title and the dissertation represent entirely and the dissertation represent entirely and independent work on the part of the candidate.

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CONTENTS

ABBREVIATIONS

%	:	percentage
±	:	Positive or negative
mm	:	millimeter
Temp	:	Temperature
$TCID_{50}$	:	Tissue culture infective dose at 50%
IC_{50}	:	Inhibitory concentration at 50%
CTC_{50}	:	Cytotoxic concentration at 50%
Rf	:	Retention factor
m/z	:	mass to charge ratio
gram-ve	:	gram negative
gram+ve	:	gram positive
min	:	minute
mg/ml	:	milligram per milliliter
µg/ml	:	microgram per milliliter
Hr	:	hour
Kg	:	kilogram
gm	:	gram
µl	:	microliter
℃	:	degree Celsius

1. INTRODUCTION

Herbal medicines are the oldest remedies known to mankind herbs had been used by all cultures throughout history. Plant extracts and phytochemicals are becoming popular as potential source of antivirals, and several reviews have been written.[1] But India has one of the oldest, richest and most diverse cultural living traditions associated with use of medicinal plants. Experimentation with plants and passage of knowledge from one generation to next resulted in the development of a vast knowledge about plants to use as medicines and narcotics.[2] In the present scenario, the demand for herbal products is growing exponentially throughout the world and pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicine value. Viral infections are an important health problem all over the world, both in developed and developing countries, due to morbidity and mortality[3] . In many journals, national and international we find an interesting number of research publications based on herbal drugs and their formulations.

These research works are carried out worldwide by peoples from various departments like veterinary, biotechnology, pharmacy, medicine etc. Plants are known to offer excellent perspectives for the discovery of new therapeutic products including anti-infectious agents[4].Indigenous knowledge is vital information. At present it is diminishing at an alarming rate. There is an urgent need to collect it before it is irretrievably lost. So, the ethno botanical surveys are carried out among tribals or people of particular community or rural areas of a particular district. The researchers collected information regarding their medical practices for the treatment of human aliments. Plants

have a long evolution of resistance against viral agents and lead to alternative direction in drug development[5].

The collected medicinal plant was identified by local people of that area and authenticated by person working in the botanical survey of India. A voucher specimen is also preserved. From these study vernacular names, family, morphology, preparation of medicine and therapeutic combinations were enumerated. Plant preparation are used in coted jvoire to treat fevers associated to or identified as malaria without any available scientific study of efficacy[6].Apoptosis which is a major way of programmed cell death plays an important role in tissue development and homeostasis. Disruption of cellular balance can leads to cancer[7].

1.1. Polyphenolic compounds

Phenolic compounds embrases a wide range of plant substances which possess in common an aromatic ring bearing one or more hydroxyl substituents. Phenolic substances tend to be water soluble, since they most frequently occur combined with sugar as glycoside. Among the natural phenolic compounds, of which thousand structures are known, flavonoids forms the largest group but simple monocyclic phenols, phenyl propanoids and phenolic quinones all exist in considerable numbers. Several important groups of polymeric materials in plants the lignins, melanins, and tannins are polyphenolics and occasional phenolic units are encountered in proteins, alkaloids and among terpenoids.

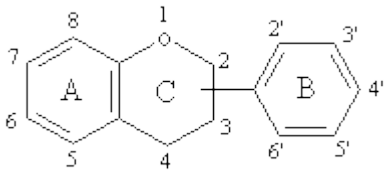
Plant phenols have the ability to complex with protein by hydrogen bonding. The phenols rapidly complex with protein as a result, there is often inhibition of enzyme activity in crude extracts. On the other hand, phenols are themselves very susceptible to

enzymic oxidation and phenolic material may be lost during isolation procedures, due to the action of specific phenolase enzyme present in all plants. Extraction of phenols from plants with boiling alcohol normally prevents enzymic oxidation occurring and adopted routinely.

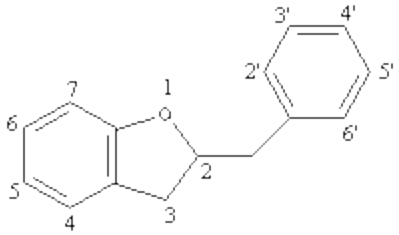
1.2. Flavonoids

Flavonols are widely distributed in plants as co-pigments to anthocyanins. Although two or three are at all common kaempferol, quercetin and myricetin. In case of quercetin a number of o-methylated derivatives are known, the 3'-methyl ether (isorhamnetin) and the 5-methyl ether (azaleatin) being but two examples. Addition of a hydroxyl in the 8 thposition to the structure of quercetin gives gossiped.

The general chemical structure of flavonoids are depicted as given below with a chromane ring

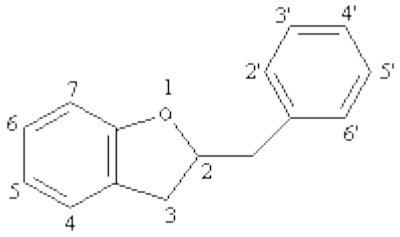


In some cases six membered heterocyclic ring may occurs called as aurone



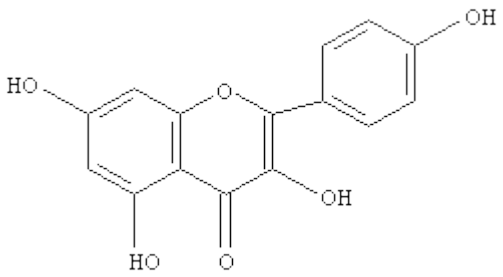
The various sub groups of flavonoids were classified as given below

1) The chemical structure of chalcones is given as

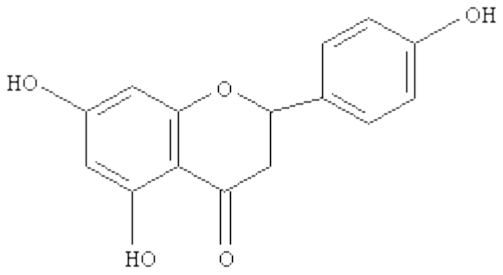


There is a considerable range of flavonol glycosides present in plants. Flavones only differ from flavonols in lacking 3-hydroxy substitution this affects their UV absorption, chromatographic mobility and colour reactions. Simple flavones can be distinguished from flavonols on these bases. There are only two common flavones apigenin and luteolin, corresponding in hydroxylation pattern to kaempferol and quercetin. Flavones occur as glycosides. A common type is 7-glucoside, exemplified by luteolin-7-glucoside. Flavones unlike flavonols, also occurs by sugar bound by a carbon carbon bond. One structural variant in the flavone series is the biflavonyl, these dimeric compounds are formed by carbon carbon or carbon-oxygen coupling between two flavones[8]. The chalcones, aurones, flavanones, dihydrochalcones and isoflavones are designated as minor Flavonoids. Chalcones and aurones together comprise anthochlor's. Most flavones carry o-methyl substituents a typical example is kayaflavone. The different types of flavonoids are tricin, jaceosidine, eupafolin, diosmetin, chrysoeriol, isorhamnetin, apigenin, kaempferol, luteolin and rutin. Natural products have proven to be rich source of biologically active materials and potentially useful lead for drug development[9]. Many plants derived natural products possess antimicrobial and insecticidal activities[10].

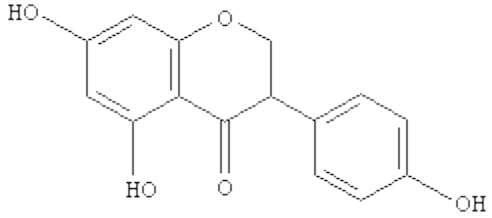
2) The chemical structure of flavonol was given as



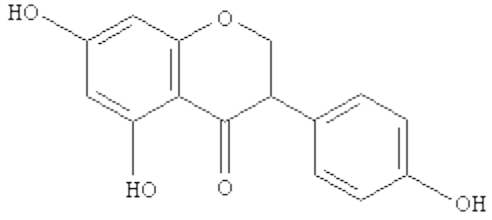
3) The chemical structure of flavonone is given as below



4) The chemical structure of anthocyanin is given as



5) The chemical structure of isoflavonoid was shown as



Polyphenols like flavonoids are well known antioxidant agents, some of which exerts therapeutic antiviral, antiallergic, antiplatelet and anti-inflammatory activities. The World Health Organization (WHO) is pursuance of its goal of providing affordable, accessible and culturally acceptable health care to the global population has encouraged the rational use of traditional medicines, to facilitate this process, the guidelines for assessment of quality, safety and efficacy of herbal medicines have been drawn up. The increase in infectious diseases caused by bacteria, viruses or fungi and the prevalent resistance of microorganisms to antibiotics, antiviral agents, urges us to search for new medicinal compounds which are novel and more efficient. Traditional medicinal plants have sometimes been found to be source of valuable potential medicines. The need for some collection strategy in order to explore the large pool of plants with pharmacologically active compounds of potential therapeutic interest. This principle has been shown to work efficiently in screening of antivirals, where a high percentage of positive results have been obtained. During the last few years efforts were made to increase the number of substances with antiviral activity[11].

Biological methods due to reliability, simplicity and sensitivity have been extensively used for evaluation of natural products in past three decades. Moreover flavonoid exhibits a broad spectrum of biological activity, including anti- thrombogenic and anticarcinogenic properties. Polymethoxyflavonoids have been shown to block adhesion molecule biosynthesis by cytokine induced endothelial cells, to block activation-induced degranulation of neutrophils and mast cells, to inhibit expression of tumor necrosis factor–alpha, to reduce the invasiveness of tumors in animal models, to

induce the differentiation of myeloid leukemic cells, so as to suppress proliferation while promoting apoptosis, to reduce lymphocyte proliferation and platelet aggregation.

1.3. Secondary chemical profile

Although it may be desirable to undertake a detailed analysis of secondary chemistry of a medicinal herb for the purpose of identifying bioactive compounds, constraints of budget and time may render this impractical. Furthermore it may not be possible to link bioactivity of a single compound or a group of related compounds; indeed it appears that demonstrated pharmacological activity may often be the result of many secondary metabolites working in concert. Nevertheless, evidence of the presence of classes of secondary metabolite may be useful indicator of both efficacy and potential toxicity. The approach adopted here was to test for the presence of phytochemical classes with known bioactivity.

1.4. Herbal products

Herbal products are made from different parts of medicinal plant such as leaf, stem, root, flower, seed or bark. The use of medicinal plants has increased in recent years in North America, Europe, Australia and South East Asia. There is a mythical yet predominant view that herbal medicines are harmless because they are natural. The safety of several commercially available herbs has come in to question due to reports of adverse reactions. Therefore some countries have implemented regulatory procedures on the safety and quality of herbal medicines. The WHO classified herbal drugs with new combination of ingredients as herbal medicine of uncertain safety and also regards this products as new substances[12].

1.5. Tissue culture technique

Tissue culture techniques have been used to evaluate the cytotoxicity of toxic plant extracts. Tissue culture techniques using cultured human cells have been developed and have shown good correlation with data obtained using animal studies. Evaluation of binding activity of plant extract to DNA would provide useful information on the safety of herbal products. The development of tissue culture as modern sophisticated techniques owes much to the needs of two major branches of medical research. The production of antiviral vaccine and the understanding of neoplasia. The Standardization of conditions and cell lines for the production and assay of viruses. In addition to cancer research and virology other areas of research also have come to depend heavily on tissue culture technique. The introduction of cell fusion technique and genetic manipulation established somatic cell genetics in the genetic analysis of higher animals including man and contributed greatly, via the monoclonal antibody technique to study immunology, already dependent on cell culture for assay technique and production of haemopoetic cell lines.

Bioprospecting is described as tapping the potential of bioresources for human welfare of humanity and to improve the quality of life in a sustainable manner. It is an environment friendly exploitation of biological resources and also described as the search for valuable compounds. Much recent attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine, several records are available on studies of folk medicine.

In India herbal medicines have been the basis of treatment and cure for various diseases or physiological conditions in traditional methods practiced such as ayurveda, unnani and siddha. Although reports of antibacterial activity of indigenous plants have

been evaluated. Phenolics like flavonoids and tannins are widely distributed in plant kingdom, vegetables, flowers etc. For centuries preparations containing flavonoids as active constituents have been used to treat human diseases and in anti-infective research. With this concept the antimicrobial screening results of *Thespesia populnea* (Family-Malvaceae) was selected for our study along with references to their traditional use was emphazised, what interesting is quiet often a particular plant may be used for different diseases for example the decoctions of *Thespesia populnea* is considered to be used in the treatment of cutaneous infections, skin and liver diseases.

The development of antimicrobial agents for clinical use has bought unquestionable benefit to individuals and society. Infectious diseases that were formerly often fatal became curable. However, mankind is now confronted with new re-emerging infections for which no effective treatments are available. In contrast, to other types of medication, antibiotics ultimately lose their effectiveness as they are used overtime and resistant strains of Bacteria develop. One example is that of gram positive, methicillin resistant *Staphylococcus aureus* strains. Incidence figures in some hospitals have shown that more than 40% of *Staphylococcus aureus* strains are now resistant to methicillin. There is thus an urgent need to identify novel, active chemo types as lead for drug development. Natural products could play a crucial role in meeting this demand of drugs approved between 1983 and 1994 by either the united states Food and Drug Administration (FDA) or comparable entities in other countries, Drugs of natural origin predominated (78%) in the area of antibacterial research.

Many plants derived from nature possess antimicrobial and insecticidal activities. The interest in these plants is increasing because of finding safer microbicides in

combination with the need of preventing environmental degradation. For centuries preparations containing flavonoids as the principal physiologically active constituents have been used to treat Human Diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research and many groups have isolated and identified the structures of flavonoids possessing antimicrobial and cytotoxic activities.

Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to their inter and intra assay variation in susceptibility testing. However, several high quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. *Thespesia populnea* is a large tree found in tropical regions and coastal forests of India The bark, leaves, and flowers are useful in cutaneous infections such as scabies, psoriasis, eczema, ringworm and guinea worm. The bark and flowers possess astringent, hepatoprotective and antioxidant activities. The flowers contain kaempferol, kaempferol-7-glucoside and gossypetin. *Thespesia populnea* is closely related to cotton (Gossypium). Infectious diseases are usually characterized by symptoms, so it is likely that traditional healers have able to recognize such diseases, and have developed effective therapies. Moreover as antibiotics mostly have clear effects, the chance of finding antimicrobial active traditional medicine is considered high. Most of the medicinal plants have identified and used for treatment of human diseases are well documented. *Thespesia populnea* reduced signicantly the central (brain) cholinesterase activity in mice. *Thespesia populnea* exhibited a remarkable cholesterol lowering property comparable to simvastatin (a standard drug) in the present study. Furthermore, we observed that, *Thespesia populnea* bark possessed a powerful memory enhancing activity in mice. Since

diminished cholinergic transmission and increased cholesterol levels appear to be responsible for development of amyloid plaques and dementia in alzheimer patients, *Thespesia populnea* may prove to be a useful medicine on account of its multifarious beneficial effects, such as memory improving property, cholesterol lowering, anticholinesterase and anti-inflammatory activity affected by dementia caused due to degeneration. *Thespesia populnea* (Malvaceae) is a large tree found in the tropical regions and coastal forests of India. Various parts of *Thespesia populnea* are found to possess useful medicinal properties, such as antifertility, antibacterial, anti-inflammatory, antioxidant, purgative and hepatoprotective activity.

Polyphenolic compounds are well known antioxidant, antiallergic, cytotoxic, antiplatelet, anti-inflammatory agents. Numerous pathological events, such as carcinogenesis are associated with the generation of reactive oxygen species. Reactive oxygen species (ROS)[13] results from cell metabolism as well as from extra cellular processes. ROS exerts some functions necessary for cell homeostasis. When produced in excess they play a role in pathogenesis of cancer.

Bioprospecting[14] is described as tapping the potential of bioresources for the welfare of humanity and to improve the quality of life in a sustainable manner. Plant derived medicines have been part of traditional health care in most parts of world for thousand of years. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern day medicine with many studies showing significant increase in the incidence of bacterial resistance to several antibiotics.

Due to increased resistance of many microorganisms towards established antibiotics, much recent attention has been paid to extracts and biologically active compounds isolated from plant species.

The screening of plants for antiviral growth inhibitors *in- vitro* and the use of ethno botanical approach enhances the probability of identifying new bioactive compounds. Biological methods due to reliability, simplicity and sensitivity have been extensively used for evaluation of natural products in past three decades. The increase in infectious diseases caused by bacteria, viruses or fungi and the prevalent resistance of microorganisms to antibiotics and antiviral agents urges us to search for new medicinal compounds which are novel and more efficient.

1.6. Viruses

Viruses are ultimate expression of parasitism; they not only take nutrition from host cell but also direct its metabolic machinery to synthesize new virus particles. Viral chemotherapy therefore is difficult as it would require interferences with cellular metabolism in the host. However virus directed enzymes have been identified in the infected cell and some viruses have few enzymes of their own may have higher affinities for some antimetabolites or inhibitors. Drugs could also targets virus specific steps like

- Cell penetration
- Un coating
- Reverse transcription

To be effective, therefore therapy has to be started in the incubation period ie has to be prophylactic. DNA viruses, i.e. viruses with DNA genomes, have always been the most important model systems for eukaryotic and DNA replication. About 99%

of population of world is infected with (Hepatitis B virus, Epstein-Barr virus or herpes simplex virus).

Until well in to the 19 th century, harmful agents were often grouped together and sometimes called viruses (Latin virus-poison or venom). The development in 1884 of the porcelain bacterial filter by charlers chamberland one of Pasteur's collaborator's and inventor of autoclave made possible the discovery of what are now called viruses. Viruses can exists in two phases extra cellular and intracellular virions the extra cellular phase possess few if any enzymes and cannot reproduce independent of living cells. In the intracellular phase, virus exists primarily as replicating nucleic acids that induce host metabolism to synthesize virion components. Resistant to acyclovir and related nucleosides analogues can occur mutation. Therefore new antiviral agents exhibiting different mechanisms are urgently needed[15].

1.7. Stages of virus replication

Some of the target sites are as follows

- Adsorption of virus to cell surface
- Transport of virus across cell membrane
- Uncoating of virus
- Release of viral genome in to cytoplasm or transport in to nucleus
- Replication of viral genome
- Transcription
- Posttranscriptional modification of viral DNA methylation, polyadenylation, capping and splicing.
- Protein synthesis and processing

- Virus encoded enzymes and regulatory protein
- Assembly of macromolecules in to the virion
- Release of virus from the cell.

1.8. Hepatitis B Virus

Hepatitis B Virus is the causative agent of acute and chronic hepatitis B in humans. Chronic infections in particular possess a serious problem to public health. According to WHO, about 5% of the world population are HBV carriers, with a greatly increased risk for developing liver cirrhosis and eventually primary liver cell carcinoma. It is estimated that more than one million deaths per year are due to liver diseases associated with chronic HBV infection. While an effective prophylactic vaccination based on recombinant virus envelope protein is available, current treatment is the high dose systemic administration of interferon-α but virus elimination is achieved in less than one third of the patients.

1.9. Papiloma Virus

Certain members of the human papilloma virus genus are the etiological agents of lower genital tract cancers, in particular cervical cancer, and so an understanding of protein-protein interactions involved in viral replication would be important if antiviral therapy were to become a reality. Viral DNA replication occurs in the upper half of the stratified epithelial cells. Human Papilloma Virus replicate in either cutaneous surface or mucosal surfaces and it is at the latter sites that most of the cancers arise. The virus codes for three early proteins $E_{6,}$ E_7 and E_5 which are responsible for altering the normal program of keratinocyte differentiation to facilitate replication of viral DNA.

However regarding antiviral activities there has been no comprehensive analysis of such activities, and furthermore it is not clear whether these are due to direct or indirect effects on specific virus infection, whether the effects are due specific classes of Phytochemical or which type of formulation might be present antiviral activity. We therefore decided to study antiviral effects of phytochemically characterized extracts.

The local flora has been traditionally used in many cultures as a source of medicine. Many workers have therefore chosen the ethno botanical approach as a means of focusing on plants with pharmacologically active compounds of potential therapeutic interest. This principle has been shown to work efficiently in screening for antiviral, where a high percentage of positive results have been obtained. All the antiviral tests were carried out with non-cytotoxic concentrations of the extracts, since we were interested in potent antiviral activities without deleterious effects on the host cells. Viruses represent a broad spectrum of potential targets (DNA/RNA, membrane/no membrane and viral proteins) for antiviral compounds.

1.10. Herpes Simplex Virus

The family Herpes viridae comprises a diverse and interesting group of viruses characterized by their distinctive virion morphology and the possession of large linear double stranded DNA genomes. The family has historically been subdivided in to alpha, beta and gamma herpesvirinae subfamilies on the basis of biological properties. Two excellent reviews of HSV-1 DNA replication have recently been published which describe in detail the advances leading to our present understanding of the viral origins of DNA replication and the functions, biological properties of the virus encoded proteins involved in DNA synthesis.

1.11. Epstein-Barr Virus

The discovery in 1964 of herpes virus in tumour cell lines derived from patients with Burkitt's lymphoma was a crucial observation that subsequently led so called Epstein-Barr Virus (EBV) being the first virus to be implicated in the pathogenesis of human cancer. This gamma herpes virus has since been associated with several other malignant diseases, including nasopharyngeal carcinoma, B cell lymphomas in immunosupressed patients, certain T cell lymphomas and Hodgkin's diseases.

1.12. CytomegaloVirus

Human cytomegalovirus (HCMV) is a member of herpes virus family whose seroprevalence generally depends on socio-economic status but can vary from 50-90% depending on the population. HCMV maintains a latent infection throughout lifetime of infected host.[20]

1.13. AdenoVirus

Human adenovirus induces productive infection in their natural host, but in rodent cells they usually cause only abortive infection. Some abortively infected rodent cells will undergo transformation, in which case adenovirus DNA is integrated in to host genome and atleast some of the Ad genes are expressed. Early region 1(E1) region consists of two transcriptional units E1A and E1B. E1A alters the transcription of genes by binding to proteins of the cellular transcription regulation process.

1.14. RNA Viruses

1.14.1. RiboVirus

The ordinary non-retro virus RNA viruses that replicate their RNA genomes entirely via RNA templates are called riboviruses. Their ultimate origins are uncertain and probably quiet varied. Some may be directly descended from early primordial RNA replicons, whereas others may have originated more recently by recombination, reassortment or mutation of cellular RNAS.

1. 14.2. RetroVirus

Retroviruses, hepadnaviruses and calimoviruses have a unique capacity for their genomes to participate regularly as part of both the RNA and the DNA worlds. It is probable that they have evolved from retroid transposable elements as originally proposed in Temin's protovirus hypothesis. Retrovirus of course has a potential to recombine at the proviral DNA level. However they have the additional capacity to undergo recombination as a result of the tendency for reverse transcriptase to switch from one template to another during DNA chain elongation. This occurs at an extremely high frequency is responsible for most retrovirus recombination events and can play a major role in retrovirus

Inhibition of antiviral effects of Interferon's INFS stimulates transcription of genes that limit virus replication. Both INF-α/β and INF-γ induces signal transduction from the cell surface to promoters via the JAK-STAT pathways. E1A prevents transcription of IFN- inducible genes and VA-RNA, prevents the IFN/PKR mediated shut-off of protein synthesis.

1. 15. Cancer

Cancer in various forms is one of the major causes of death of human population. Several chemotherapeutic agents are commonly[17]. employed to reduce the human mortality due to cancer. The use of many cytostatic agents has been restricted because of severe side effects and their inability to prevent many forms of malignancies. Several natural products of plant origin have potential value as chemotherapeutic agents. A screening test using human cancer cell lines is commonly performed as a preliminary step to evaluate the efficacy of chemotherapeutic agents for the treatment of cancer. The compounds that are found to be potent in killing the cancer cells or of tumor in animal models. However the compounds produced by *in vitro* cell culture techniques do not always conform to those produced by whole plant, and thus is not always biologically active. A wide variety of flavonoids shows estrogenic activity as well as anti-allergic, anti-inflammatory, anti-viral and anti-microbial activities. More over flavonoids in diet may have a role in reducing the risk of cancer and some flavonoids are well known to possess anti-oxidant and radical scavenging properties.

1.16. Tumor Virology

The incidence of tumours in animals and humans often shows geographical variation. This clustering sometimes reflects genetic differences in the populations at a risk but more often results from environmental factors that vary between populations. A correlation between the ability of viruses readily to cause solid mesenchymal tumours in animals and their induction of morphological transformation of cultured cells. The development of a fertilized egg in to a multicellular organism and the subsequent maintenance of its form and function require complex control over cell proliferation, cell

death and expression of differentiated cell functions. These attributes of cells result from an interplay between stimuli from outside the cell and its current program of activity. A wide variety of flavonoids show estrogen type activity as well as antiallergic, anti-inflammatory, antiviral and antimicrobial activities[10].

1. 17. Antiviral Therapy

Because of the intimate association between viruses and their host cells, the development of antiviral agents lagged behind that of compounds active against other microbes. Since the replication of viruses was not well understood, its basis was at first purely empirical and depended largely on random screening of many potentially useful compounds, the devising of compounds directed against specific target activities such as attachment to the host cell, nucleic acid synthesis and release of mature virus is of more recent origin. An early development of antiviral therapy was the discovery by Isaacs and Lindermann of Interferon, (IFN) a low molecular weight protein produced by cells in response to infection with viruses, some bacteria, double stranded nucleic acids and other biological compounds.

There is a growing body of evidence suggesting that the genetic properties of viruses may reside largely in their nucleic acids and a number of recent observation shows that virus multiplication can be delayed by compounds which interfere with nucleic acid metabolism. As a class, analogues of purines and pyrimidines bases may inhibit growth by variety of mechanisms.

This prediction was fulfilled the greatest achievements have in fact been the therapy and prophylaxis of herpes virus infection with nucleoside analogues at first Idoxyuvidine and latterly Acyclovir. Another such compound zidovudine had a limited

measure of success against infection with human immunodeficiency virus. It is however clear that our successors will regard this era as still part of the antiviral therapy.

1.18. Anti-RetroViral Drugs

These are drugs active against HIV which is a retrovirus. They are useful in prolonging and improving the quality of life and postponing complication of AIDS or AIDS related complex but do not cure the infection. The clinical efficacy of antiretrovirus drugs is monitored primarily by plasma HIV-RNA assays and CD4 lymphocyte count carried out at regular intervals

The first antiretrovirus drug zidovudine was developed in 1987. Over the past 20 years > 20 drugs belonging to 3 classes have been introduced and a large number of others are under development. The various target sites of anti retro viral drugs are

- Nucleoside reverse transcriptase inhibitors (NRTIS)
- Non Nucleoside reverse transcriptase inhibitors (NNRTIS)
- Retroviral protease inhibitors (PIS)

1.19. Choice of an antimicrobial agent

The choice of an antimicrobial agent in a patient by assessing that the condition is due to a treatable infection, and that it is not likely to resolve by itself or local measures only, one has to choose a drug from the large number available. The choice depends on various factors such as.

- Age
- Renal and hepatic functions
- Local factors
- Drug allergy

- Impaired host defence
- Pregnancy
- Genetic factors
- Organism related considerations
- Drug factors

1.20. Postantibiotic effect

After a brief exposure if the organism is placed in antibiotic free medium, it starts multiplying again, but after a lag period which depends on the antibiotics as well as organisms. This lag period in growth resumption is known as post antibiotic effect and is the time required for reattainment of logarithmic growth. A long PAE has been noted with fluroquinolones, amino glycosides and β-lactum antibiotics

1.21. Combined use of antimicrobials[19]

More than one antimicrobial agent is frequently used concurrently. This should be done only with a specific purpose and not blinding in the hope that if one is good, two should be better and three should cure almost any infection. The objectives of using antimicrobial combinations are.

- To achieve synergism

  Example - Rifampin + Isoniazid in Tuberculosis

  Penicillin/Ampicillin +Streptomycin/Gentamycin for SABE.
- To reduce severity or incidence of adverse effects
- To prevent emergence of resistance
- To broaden the spectrum of antimicrobial action.

1.22. Disadvantages of antimicrobial combinations

- Choice of antimicrobial agents
- Produces exaggerated kidney failure
- Increased chances of super infection
- Emergence of resistance
- Increased cost of therapy.

1.23. Prophylatic use of antimicrobial agents

- Prophylactic against specific microorganisms
- Prevention of infection in high risk situations.

1.24. Antiviral study methods

a) XTT Assay.

b) MTT Assay[21].

c) Anti-HIV-assay

d) Cytopathic effect reduction assay

e) Cytotoxicity assay

f) Protease inhibition assay

g) Plaque reduction assay

h) Viral yield assay

2. BOTANICAL INFORMATION

Family : Malvaceae

Scientific name : *Thespesia populnea*

Common name : Seaside Mahoe

Tamil name : Poovarasan

2.1. Description

Height : 40 to 50 feets

Spread : 30 to 40 feets

Propagation : By seeds and cuttings

Cultivation : Tropical and subtropical areas

2.2. Flower

Colour : Yellow

Characteristics : Showy

2.3. Leaf

Shape : Heart shaped

Characteristics : Leaf blades are slightly fleashy, hairless, narrower, papery and toothed leaf blades

Five dark dots at center of flowers are present. Sepals are edged cup stigma was yellow to deep crimson purple coloured.

2.4. Propagation

This species is easily propagated from seed. The seed pods are indehiscent, that is the seed pods do not open when mature. The capsules can be opened by hand and the seeds removed. The seeds do not require soaking, but soaking them overnight in warm water may hasten germination. The seeds should be planted in sterile potting mix at a depth of about twice the diameter of the seed. Germination takes 14 to 28 days.

2.5. Cultivation

Its blooms are attractive both when they are pale yellow and also when they age to a deep pink. The plant also performs well in a pot. This species grows well by the sea and grows in USDA zone.

2.6. Location

Thespesia populnea is included in the category 1 list as a species that is invading and disrupting native plant communities in Florida.

2.7. Products

The various products of the plant are fibres, mats, paper and tapa cloth.

2.8. Chemical constituents

The flower contains kaempferol, kaempferol-7-glucoside and gossypetin.

2.9. Uses

The seeds are applied to scabies and other skin diseases, and are rubbed on swollen joints. The yellowish juice extracted from young fruits is used to treat insect bites, gonorrhoea, ringworm and migraine and is also used for fistula, psoriasis, scabies, sprains and wart removal. The plant also produces rope and dye.

3. LITERATURE REVIEW

1) V.Kott *et al.,* (1990) performed the antiviral activity in Argentine medicinal plants for the treatment of infectious diseases; aqueous extracts of 5 species were assayed in vitro to detect antiviral activity HSV-1, RSV ADV-7. *Polygonum punctatum, Lithraea molleoides, Sebastiania brasiliensis and Sebastiania klotzschiana* but not *Myrcianthes cisplatensis* showed *invitro* antiherpetic activity with 50%effective dose ranging from 39 to 169 μg/ml Polygonum *punctatum, Lithraea molleoides*, showed antiviral activity against RSV with 50% effective dose 78 to 120 μg/ml.

2) R.S.L.Taylor *et al.,* (1996) performed the antiviral activities of Nepalese medicinal plants to treat diseases that could be caused by viruses, methanol extract from 21 species were assayed for activity against three mammalian viruses herpes simplex virus, sindbis virus and polio virus. Assays were performed in UV-A or visible light as well as dark. Individual species of *Hypercium Lygodium* and *Maesa* exhibited impressive antiviral activities.

3) Gordana Rusak *et al.,* (1997) performed inhibition of tomato bushy stunt virus infection using a quercetagetin flavonoid isolated from *Centaurea rupestris L*, the extracts revealed a strong antiviral activity when inoculated simultaneously with tomato bushy stunt virus in two Nicotiana species. Almost complete reduction of local lesion number resulted from these inoculations in *N.glutinosa*. A similar effect was observed in inoculated leaves of *N.megalosiphon* causing the absence of systemic infection in 40% of treated plants. Results presented in this paper suggest that the flavonoid may interfere with the initation of virus infection.

4) S.J.Semple *et al.,* (1999) performed the antiviral flavonoids from *Pterocaulon sphacelatum*, an Australian Aboriginal medicine. Antiviral activity guided fractionisation of the extract of *Pterocaulon Sphacelatum* using an inhibition of poliovirus induced cytopathic effect assay, has yielded the antiviral flavonoid chrysosphenol. These compounds inhibited the replication of rhinoviruses, the most frequent causative agent of cold.

5) L.Pieters *et al.,* (1999) performed the screening of seven Rwandan medicinal plants for antimicrobial and antiviral activities only two of selected plants showed a true antiviral activity against herpes simplex virus type 1, while all of them exhibited virucidal properties against several enveloped viruses. Four plants were diversely active against gram-positive bacteria, two of these shows bactericidal effects against acid fast. None of the selected plant was active against gram negative bacteria's.

6) Satoshi Tahara *et al.,* (2000) studied the HPLC analysis of white lupin isoflavonoids. Here an investigation of the HPLC analytical conditions for simple isoflavones, prenylated isoflavones and some of their glucosyl derivatives resulted in reasonable separation and total elution in 35 min when using a reverse phase C_{18} Lichrospher column. This method was successfully applied to quantify the changes in isoflavonoid constituents in white lupin tissues young legumes during maturation and soaked germinating seeds. In the developing legumes, genistein and 2'-hydroxygenistein, as well as their prenylated derivaties, were present in the pods as the major components, together with minor amount of glycosides.

7) F.Lohezic-Le Devehat *et al.,* (2002) performed the antiviral and cytotoxic activities of some Indonesian plants, they were phytochemically screened and evaluated for antiviral

(HSV-1 and Poliovirus) and cytotoxic activities of murine and human cancer cell lines. *Elytranthe tubaeflora, Elytranthe maingayi, Elytranthe globosa and Scrrula ferruginea* exhibited attractive antiviral and cytotoxic activities. *Piper aduncum* was found active on poliovirus.

8) M.J.Deena *et al.,* (2002) performed antimicrobial screening of essential oils of *Coleus Aromaticus* and *Coleus Zeylanicus* by in vitro microbicidal test against seven bacteria and eight fungi. Of the two oils tested the oil was found to have slight higher inhibitory activity against a wide spectrum of bacteria and fungi.

9) M.M.F.S.Miranda *et al.,* (2002) performed anti-herpes simplex virus effect of seed extract from tropical plant *Licania tomentosa (Benth)* incubation of acyclovir-resistant herpes simplex virus type 1, during infection of HEp-2 cell culture, with an extract prepared from *Licania tomentosa (Benth)* impaired the productive replication of the virus in a concentration dependent manner. The extract was able to possess virucidal effect with a very early event of cell infection, at a non-cytotoxic concentration.

10) Robert B. Bates *et al.,* (2002) performed antiviral saponins from *Trieghemella heckelii* Arganine C and a new saponin, tieghemelin were isolated from *Trieghemella heckelii* fruits. Arganine strongly inhibited HIV entry in to the cells in a cell fusion assay. The less potent tieghemelin was converted in to arganine C by reduction of its ethyl ester with sodium borohydride. The removal of the four sugar chains from arganine C and tieghemelin to give 16α-hydroxyprotobassicacid 3-O-β-D-glucopyranoside caused total loss of activity. Arganine was not significantly cytotoxic to HeLa-CD4 cells and the level required to reduce the synctium count to zero, suggesting it to be a promising candidate for further study as an antiviral drug.

11) A.K.Srivastava *et al.,* (2002) studied the cytotoxicity of *invitro* produced podophyllotoxin from *podophyllum hexandrum* on human cancer cell lines by in vitro culture conditions. A maximum of 42.6 mg/l of podophyllotoxin was produced when *podophyllum hexandrum* was cultivated in 3L stirred tank bioreactor. The compound extracted from the cell culture was applied to human breast cancer cell line (MCF-7) and 1nM podophyllotoxin was able to inhibit the growth of cancer cells by 50%. The most profound inhibitory effect of podophyllotoxin was observed when it was applied in the beginning of cell growth.

12) A.M.Madureipa *et al.*, (2003) evaluated the antiviral and antimicrobial activities of triterpenes isolated by *Euphorbia severalities* which yield five tetra cyclic triterpenes and were evaluated for their antiviral activities against herpes simplex virus (HSV) and African swine fever virus (ASFV). Lupeone exhibited strong viral plaque inhibitory effect against HSV-1 and HSV-2. The *in-vitro* antifungal and antibacterial activities of cycloart-23-ene-3, 25-diol, 3-acetate were also investigated.

13) A.A. Shahat *et al.,* (2004) studied the flavonoids from *cressa cretica*, the aerial parts of *cressa cretica* yielded five flavonoids that were identified as quercetin (1), quercetin-3-O-glucoside (2), kaemoferol-3-O-glucoside (3), kaemoferol-3-O-rhamnoglucoside (4) and rutin. All of the isolated flavonoids were identified by spectroscopic methods by UV, FAB-MS, ^{1}H NMR and ^{13}C NMR).

14) Nongluksna Sribolmas *et al.,* (2004) performed endophytic fungi with antimicrobial, anticancer and antimalarial activities isolated from Thai medicinal plants. Of the 582 pure isolates obtained, 360 morphologically distinct fungi were selected for cultivation on malt czapek broth and yeast extract sucrose broth from which extracts were

tested for biological activity. Extracts of 92 isolates could inhibit *Mycobacterium tuberculosis* (MIC 0.0625-200 µg/ml) when tested by micro plate alamar blue assay. The sulphorhodamine B assay for activity against cancer cell lines revealed that 60 were active against human oral epidermoid carcinoma cells (EC_{50} 0.42-20 µg/ml) and 48 against breast cancer cells (EC_{50} 0.18-20 µg/ml). Bioactivity profile was affected by type of culture medium.

15) Scott *et al.*, (2004) performed the antibacterial study of 26 South African plant species used as traditional medicines. Quality standard of identification of these species by using HPLC, TLC and traditional microscopy are given. The results of investigations using disk assay method of their efficacy as antimicrobial agents are reported. In this study the 24 species belongs to the families Asteraceae, Geraniaceae and Lamiaceae well represented in indigenous traditional medicine practices.

16) Julia Serkedjieva *et al.,* (2005) suggested the in vitro antioxidant activity of polyphenol extracts with antiviral properties from *Geranium Sanguineum L* by three separate methods DPPH assay, ß-carotene–linoleic acid assay and NBT reduction assay. For comparative reasons caffeic acid and synthetic antioxidant BHT were used. Total phenolic constituents measured by folin's-ciocalteu reagent were found as 34.60 % (w/w). The O_2^- scavenging activity of all preparations correlated with the rate of protective effect shown in murine model of experimental influenza virus infection.

17) Pannarat Akanitapichat *et al.,* (2005) performed anti-herpes virus activity of *Dunbaria bella prain* using a bioassay guided fractinisation procedure; tertiary fractinisation of a dichloromethane methanol plant extract afforded a partially purified

fraction equally active against replication of herpes simplex virus. Furthermore the antiviral activity was dependent on multiplicity of infection and the type of host cell.

18) Jolanta Nazaruk *et al.,* (2005) performed the flavonoid composition and antimicrobial activity of *Cirsium rivulare* (jacq) flowers here a new flavonoid isokaempferide 7-o-β-D-(6”-methylglucuronide) was isolated from flowers of *Cirsium rivulare*. The antimicrobial activity of methanolic and ethanolic extracts of *Cirsium rivulare* flowers and leaves were evaluated.

19) Alexis Valentin *et al*.,(2005) performed the antiplasmodial activity and cytotoxicity of plants used in west African traditional medicine for the treatment of Malaria extracts of these plants were tested against two strains of *Plasmodium falciparum* chloroquine sensitive strain and Nigerian chloroquine sensitive strain. A radioactive micro method allowed the evaluation of antiplasmodial activity of extracts on *Plasmodium falciparum*

20) Barasan Dulger *et al.,* (2005) evaluated the antimicrobial activity of some endemic *Scrophulariaceae* members from Turkey, the methanolic extract obtained from endemic *Scrophulariaceae* members *Verbascum bellum Hub-Mar- Verbascum dalamanicum Hub-Mor, scrophularia cryptophila Boiss and Veronica lycica E. Lehm* have been investigated for their antimicrobial activity. Antimicrobial activity was determined with *Escherichia coli* ATCC 11230, *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* UC57, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427, *Bacillus cereus* ATCC 7064, *Mycobacterium smegmatis* CCM 2067, *Listeria monocytogenes* ATCC 15313 by disk diffusion method. The extracts of all *Scrophulariaceae* members used in this study has strong antimicrobial activity against gram-positive bacteria and yeast cultures.

21) Z.Ulukanli *et al.,* (2005) suggested the antimicrobial activities of some plants from the eastern Anatolia region of Turkey. Crude extracts obtained from the roots and aerial parts of *Rumex crispus L* and *Acinos rotundifolius* were evaluated for *in vitro* antimicrobial activity against five gram-positive bacteria including *staphylococcus aureus, Micrococcus luteus, Mycobacterium smegmatis, Bacillus subtilis, Bacillus subtilis var niger* and three gram negative bacteria including *Aeromonas hydrophila, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans*. The inhibition zone diameter was determined for each extract using agar well diffusion method at a concentration of 12.5 mg/ml. The acetone extracts of roots of o *Rumex crispus L and Acinos rotundifolius* demonstrated significant inhibitory effects against most microorganisms used.

22) Thomas lanaras *et al.,* (2005) studied the antimicrobial, cytotoxic and antiviral activities of *salvia fruticosa* essential oil by GC-MS showed high content of 1,8-cineole,thujone and camphor, representing 47.48%, 11.93% and 9.04% of total oil respectively. The essential oil and its isolated components thujone and 1, 8-cineole exhibited antimicrobial activity against 8 bacterial strains, while camphor was almost inactive against all bacteria tested. The essential oil was bactericidal at1/4000 dilution and dilutions up to 1/10000 caused considerable decrease in bacterial growth rates. The essential oil of *S. fructicosa* and the three main components exhibited cytotoxicity against African green monkey kidney cells and high level of virucidal action against herpes simplex virus1 a ubiquitous human virus.

23) Minas arsenakis *et al.,* (2005) performed the antimicrobial and cytotoxic activities of origanum essential oils, *Origanum vulgare ssp.hirtum, Origanum dictamnus* and a

commercially available Origanum oil were analysed by gas chromatography-mass spectrometry and showed a high content of carvacol, thymol, γ-terpinene and p-cymene representing 73.7%,92.8% and 87.78% of the total oil, respectively. The three essential oils exhibited high levels of antimicrobial activity, while their biosynthesic precursor's γ-terpinene and p-cymene were inactive. The essential oil of *Origanum vulgare ssp.hirtum* was extremely bactericidal at 1/4000 dilution and even at dilutions as high as 1/5000 caused considerable decrease in bacterial growth rates. The same essential oil also exhibited high levels of cytotoxicity against four permanent animal cell lines including two derived from human cancers.

24) K.E.Aidoo *et al.,* (2005) performed the cytotoxicity and DNA interaction of polyherbal products from Malaysia. Here the extracts were tested for mycoflora and analysed for aflatoxins and ochratoxins.The herbal extracts were also tested for cytotoxicity on human cell lines Hep2 and HFL1 using the MTT assay. All extracts were cytotoxic to both cell lines at a concentration of 500µg/ml but showed varying effects at 5µg/ml. In vitro cytotoxicity tests showed that half of the products were cytotoxic and interacted with DNA.

25) Hojjat sadeghi-aliabadi *et al.,* (2005) performed the cytotoxic evaluation of Iranian Conifers on cancer cells. In this study branches or fruits of *Taxus baccata L*. as well as other species of Iranian conifers were collected, identified and cytotoxic effects of hydroalcoholic extracts on three human tumor cell lines was evaluated using MTT assay, extracts from fruits of *P. orientalis* showed inhibitory activities against MDA-MB-468 cells. In conclusion obtained extract from bark of *Taxus baccata* showed comparable cytotoxic effect to doxorubicin against Hela cells.

26) Jim Hudson *et al.,* (2005) studied the antiviral activities in *Echinacea* root preparations he used the taxa of *Echinacea purpurea, Echinacea pallida var.pallida and Echinacea pallida var angustifolia* the fractions were analysed by HPLC for their content of caffeic acid derivatives and alkamides and antiviral activities against three viruses. The ethylacetate fraction contains significant but weak activity against both HSV and FV and contained significant levels of cichoric acid. In contrast, *Echinacea pallida var angustifoli*a gave no water soluble antiviral activity, but the ethanolic and ethylacetate fractions contained significant activity against all three viruses, and this activity correlated with the presence of alkamines.

27) Chi- Tang Ho *et al.,* (2006) studied the hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*citrus sinensis*) peel because of their broad spectrum of biological activities, including anti-inflammatory, anticarcinogenic and antiatherogenic properties. They have been increasing interests in the exploration of health beneficial properties of PMFS in citrus fruits. Therefore the isolation and characterization of PMFS from sweet orange peel will lead to new application of the byproducts from orange juice processes and other consumption, one polymethoxyflavone, one hydroxylated polymethoxyflavone and two hydroxylated polymethoxy chalcones were elucidated by various MASS, UV and different NMR techniques.

28) Supinya Tewtrakul *et a.,* (2006) performed the anti-HIV-1 integrase activities of Thai medicinal plants known as Hua-Khao-Yen ethanolic and water extracts were tested for their activities. The results revealed that the ethanolic extract *of Smilax corbularia* exhibits anti-HIV activity with an IC_{50} value of 1.9 μg/ml. Increasingly only the ETOH

extract of *Dioscorea membranacea* showed appreciable activity againstHIV-1 PR, while others possess mild activity. These results supported the basis for use of *Smilax corbularia* and *Dioscorea membranacea* for AIDS treatment by Thai traditional doctors.

29) F.Conforti *et al.,* (2006) suggested cytotoxic activity of antioxidant constituents from *Hypericum triquetrifolium Turra* by sulforodamine assay to test cytotoxicity against 4 human cancer cell lines and one normal cell line of antioxidant constituents isolated from *Hypericum triquetrifolium turra*. Methanolic extract and pure compounds were tested against the large lung carcinoma cell line COR-L23, the hepatocellular carcinoma cell line HepG-2, renal cell adenocarcinoma ACHN, the amelanotic melanoma cell line C32 and normal foetal lung MRC5. The result shows strong cytotoxicity against different cell types.

30*)* S.J. Semple *et al.,* (2006) performed the screening of Australian medicinal plants for antiviral activity at non cytotoxic concentrations. Here the extracts of *Euphorbia australis* and Scaevola spinescens were more active against HCMV. Extracts of *Eremophila subsp.glabra and pittosporum phylliraeoides var.microspora latrobei* exhibited antiviral activity against RRV.

4. SCOPE AND PLAN OF THE WORK

Many synthetic Drugs which causes serious side effects and drug resistance in case of acyclovir for recurrent HSV episodes and the development of acyclovir resistant strains has been observed and may be problematic. However chemotherapy is an essential strategy for treatment of disseminated cancers however its efficiency is restricted by both intrinsic and acquired cell resistances to drugs. Anticancer compounds with new cellular targets are needed. To overcome this above circumstances the main objective of my project is to investigate and isolate the naturally originating antimicrobial compounds without any side effects. Early studies shows antiviral activity against FIV infections assayed by synctia formation using feline kidney crandell cells, several plant parts have been described as potential antiviral agent's special attention to Retrovirus (HIV) and anti-HIV-1 integrase activities have been reported. The main objective of my study is the isolation and purification of the active compound present in the selected south Indian plant *Thespesia populnea* belongs to family Malvaceae and its antimicrobial screening.

So, keeping the above point in mind myself decided to work on plants used in traditional system of medicine for bioactive compounds that may lead to the identification of novel compounds, which may have anti-infective property against microbes like bacteria, fungi and viruses. Higher plants have provided many antiviral compounds and these may serve as a relatively easy source. So attempts are made clearly to discuss previous test methods together with some additional methods to simplify test procedures and assay methods.

Aim and Objectives

The main objective of my Project is to investigate the natural antimicrobial and cytotoxic activities of Plant extract. Cancer is a widespread diseases responsible for millions of death Worldwide. To circumvent chimioresistance to conventional anticancer drugs, anticancer compounds with new cellular targets are needed. This observation stimulates the search for new anticancer agents, and in this regard, the investigation of naturally originating compounds could be very valuable. However in case of Acyclovir, an acyclic guanosine analog, is widely used to treat HSV infection. However, this gold standard drug is less effective in recurrent HSV episodes and the development of Acyclovir resistant strains has been observed and can be problematic. Thus the development of novel anti-HSV agents is still an important area of research. In addition to the development of synthetic drugs as mentioned above, another approach is based on screening of plant derived substances for antiviral activity. With this main concept I selected flavanoid containing plant *Thespesia populnea* their antimicrobial and cytotoxic activities and their isolation techniques to know the particular principle necessary for these activities.

The work has been divided in to following

1) Selection and collection of plant materials
2) Preparation of crude extract
3) Preliminary phytochemical investigation
4) Total phenolic constituents study
5) Antimicrobial studies
    - 5.1) Antibacterial study
    - 5.2) Synergistic activity study
    - 5.3) Antifungal study
    - 5.4) Antiviral and cytotoxic study
6. Isolation of active constituents
    - 6.1) Column chromatography
    - 6.2) Preparative TLC
7. Confirmational study of the isolated compound
    - 7.1) Physicochemical parameters of isolated compound
    - 7.2) Thin Layer Chromatography
    - 7.3) Paper Chromatography
    - 7.4) Lamda max Study
    - 7.5) IR Spectrum study
    - 7.6) NMR Spectrum study
    - 7.7) MASS Spectrum study
8. MIC of the isolated compoud

MATERIALS AND INSTRUMENTS

1. Materials

S. NO.	Name of Chemical	Company
1	n- hexane	LOBA Chemical, Mumbai
2	Petroleum ether	LOBA Chemical, Mumbai
3	Ethyl acetate	LOBA Chemical, Mumbai
4	Methanol	LOBA Chemical, Mumbai
5	Ethanol	LOBA Chemical, Mumbai
6	Sodium carbonate	LOBA Chemical, Mumbai
7	Ferric chloride	LOBA Chemical, Mumbai
8	Folin's colcateus reagent	LOBA Chemical, Mumbai
9	Gallic acid	LOBA Chemical, Mumbai
10	n- butanol	LOBA Chemical, Mumbai
11	Acetic acid	LOBA Chemical, Mumbai
12	Formic acid	LOBA Chemical, Mumbai
13	Benzene	LOBA Chemical, Mumbai
14	Silica Gel.G.	HIMEDIA, Mumbai
15	Nutrient broth	HIMEDIA, Mumbai
16	Nutrient agar	HIMEDIA, Mumbai
17	Sabouraud's-Dextrose Broth	HIMEDIA, Mumbai
18	Sabouraud's-Dextrose Agar	HIMEDIA, Mumbai
19	Dimethyl sulphoxide	HIMEDIA, Mumbai
20	Sulphanilic acid reagent	HIMEDIA, Mumbai
21	Phosphate buffer saline	HIMEDIA, Mumbai
22	Dulbecco's modified eagle	HIMEDIA, Mumbai
23	Toluene	LOBA Chemical, Mumbai
24	Cyclohexane	LOBA Chemical, Mumbai
25	Acetone	LOBA Chemical, Mumbai
26	Petroleum ether	LOBA Chemical, Mumbai
27	Ethyl acetate	LOBA Chemical, Mumbai
28	Dragendorffs reagent	LOBA Chemical, Mumbai
29	Ninhydrin reagent	LOBA Chemical, Mumbai
30	Lead acetate	LOBA Chemical, Mumbai
31	Iodine	LOBA Chemical, Mumbai
32	Mayers reagent	LOBA Chemical, Mumbai

2. Instruments

S.NO.	Name of Equipment	Company
1	Autoclave	New Lab
2	Laminar air flow	Scientec genuine
3	Glass ware	Borosil
4	Incubator	Genuine
5	Refrigerator	Godrej

6	Electronic Balance	Shimadzu
7	IR Spectrophotometer	Perkin-elmer
8	NMR Spectrophotometer	Bruker
9	MASS Spectrophotometer	Schimadzu GC-MS
10	Water bath	Genuine
11	Deep freezer	Blue star
12	Cooling centrifuge	Remi C24-BL
14	Colony counter	Neo Lab
15	BOD Incubator	Rotek

3. Test microorganisms

The test microorganisms used were *Shigella sonei (ATCC29930), Escherichiae coli* (ATCC11229), *Streptococcus faecalis* (ATCC8043), *Shigella boydi* (ATCC8700), *Rhodococcus terrae* (NCIM 5126), *Micrococcus flavum* (NCIM 2984), *Flavobacterium devorans* (NCIM 2581), *Proteus mirabilis* (NCIB 8268), *Brevibacterium leuteum* (ATCC 15830), *Bacillus lichenformis* (NCIM 2468), *Shigella dysentriae* (ATCC 13313), *Klebsiella pneumoniae* (ATCC 11229), *Micrococcus leuteus* (ATCC 9341) and *Shigella flexneri* (NCIM 4924).

The various fungi used for my study are *Aspergillus niger* (NCIM 1207), *Sacchromyces cerevisae* (ATCC 204508), *Candida albicans* (NCIM 3484), *Monilinia fruticola* (NCIM 1011), *Auricularia polytricha* (NCIM 1303), *Chaetomella raphigera* (NCIM 1231) and *Arthrobotrys oligospora* (NCIM 1246).

4. Antibiotics

Oxytetracycline, Kanamycin, Nystatin, Amphotericin-B, Acyclovir, Ganciclovir, Ribavirin, Bivudin.

5. METHODOLOGY

5.1 Selection and collection of plant materials

Flavonoids are reported as having many pharmacological activities such as antioxidant, anticancer, antimicrobial, and enzymatic inhibition properties. Based on this I have selected the flower parts of *Thespesia populnea* plant containing high amount of flavonoids. The plant materials were collected during April-May 2006 from tropical areas of Western Ghats regions of Erode and Nagercoil and then shade dried at room temperature. The Plant material were identified by G.S.R.Murthy, joint Director at Botanical survey of India (BSI) Coimbatore, India and a voucher specimen (sc/23) was deposited in Herbarium of Laboratory of Botany ,Coimbatore, Tamilnadu, India.

5. 2 Preparation of crude extract[1]

The powdered plant materials (10gms) were extracted with 100ml of methanol for 1hr on an ultrasonic bath. The extract was filtered the filtrate was evaporated in vacuo at 45 ˚C and then lyophilized. The extracts were prepared according to the polarity starting from n- hexane to methanol.The residue thus obtained was thus dried in vacuum desicator to remove the final traces of solvents completely and dried in vacuum desiccator for solvent elimination. So, that the activity due to solvent wont interfere the result.

5.3 Preliminary phytochemical screening

The preliminary phytochemical screening of *Thespesia populnea* was carried out for the decotion of various phytoconstituents using standard procedure of Harbone.The following solvents were used for the study, petroleum ether, chloroform, ethyl acetate, methanol, ethanol and water. The methanolic extract was found to contain more

Flavonoids. The preliminary phytochemical screening of methanolic extract reveals the presence of alkaloids, flavonoids tannins, triterpenes, gums and mucilage's.

5.4 Total phenolic constituents study[22].

The amount of polyphenols present in *Thespesia populnea* methanolic extract was quantified by Gallic acid as standard and further addition of folin-ciocalteu reagent,2% sodium carbonate with intermittent shaking then absorbance measured at 760nm and standard standard graph was plotted the same procedure used for methanolic extract and the amount of total phenolic constituents was thus evaluated. The formula used to calculate the total phenolic constituents was given as

Absorbance= 0.0012× concentration of ascorbic acid (µg) +0.0033

5.5 Antimicrobial study

5. 5.1. Antibacterial study (plate hole difussion method) [18].

The plate hole diffusion assay was used to determine the growth inhibition of bacteria by plant extracts. Bacteria were maintained at 4 ˚C on nutrient agar plates before use. Nutrient agar was prepared and 25ml of each was poured in to sterile universals. The universals with the broth were inoculated with different species of bacteria's and incubated at 37˚c overnight. A total of 25ml of molten Muller Hinton (MH) agar (OXOID) held at 40˚c was poured in to sterile universals maintained at 40˚C in a water bath. Each universal was inoculated with 0.2ml of different bacterial species mixed well with the HM in to sterile Petri dishes agar and allows to set. Using a sterile cork-borer 6mm diameter, four holes per plate were made in to the set agar containing the bacterial culture. A total of 0.2ml of plant extracts were poured in to the wells and one containing

distilled water, the plates were kept in incubator overnight and the zone diameter was then recorded if greater than 6mm.

5.5.2. Synergistic activity study[59]

The synergistic activity study was calculated by combining with the standard antibiotics oxytrtracycline by means of cup plate method (Kirbauy bauer technique) using two wells in a plate methanolic plant extract of *Thespesia populnea* (500µg/ml) was used in combination with oxytetracycline (500µg/ml) the distance between the two wells was maintained as standard of about 0.8 cm then incubated at the standard conditions and the zone diameters was measured in the second data.

5.5.3. Antifungal study[10]

Sauboard dextrose agar medium was prepared and 25ml of each was poured in to sterile universals. The universals with the broth were inoculated with different species of fungus and incubated at 28±0.1˚c overnight. A total of 25ml of medium was poured in to sterile universals. Each universal was inoculated with 200 µl of different fungal species spreaded well and allows to set. Using a sterile cork-borer 6mm diameter, four holes per plate were made in to the set medium containing the fungal culture. A total of 0.2ml of plant extracts were poured in to the wells and one containing distilled water, the plates were incubated overnight for 36 to 48 hours and the zone diameter was then recorded if greater than 6mm.

5.5.4 Antiviral and cytotoxic study

5. 5.4.1 Materials Required

- Hel cell culture (human embryonic lung) cells
- HeLa cell culture
- Crandell-Rees feline kidney cells
- Vero cell culture- African green monkey kidney cells

5.5.4.2 Viruses

Herpes simplex virus-1

Herpes simplex virus-2

Vaccinia virus

Vesicular stomatitis virus

Coxsackie virus

Respiratory syncytical virus

Feline corona virus

Feline herpes virus

Para influenza virus

Reo virus-1

Sindbis virus

Punta toro virus

All viruses were grown at 37ºC in a humidified atmosphere of 5% CO_2 in air. The Herpes simplex virus-1 was identified as acyclovir resistant strain.

5.5.4.3. Maintenance of human cell lines[16]

Cells were grown in RPMI-1640, was supplemented with 10% fetal calf serum, 5ml penicillin/streptomycin, 5ml of L-glutamine, 5ml of sodium pyruvate (1 Mm), sodium bicarbonate (1gm). Completed media was sterilized by 0.22 µm microbiological filters after preparation and kept at 4 ºC before using. Cell lines were maintained and grown in RPMI-1640 up to 15 subcultures.

5.5.4.4. Materials

Confluent monolayer cell culture

96 well plate

Pipettes

Titertek multidrop

Automatic plate reader

Triton x-100

Isopropanol

5.5.4.5. MTT Assay[16]

Principle

The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial dehydrogenases of metabolically active cells to a blue formazan which can be measured spectrophotometrically.

Procedure

a) To each well, 20 µl of 7.5 mg/ml MTT solution in phosphate buffered saline was added using tritertek multidrop

b) After incubation for I hr at 37ºC , a fixed volume of 150 µl medium was removed from each well using an M96 washer (ICN flow).

c) Solubilisation of formazan crystals was achieved by addition of 100 µl 10% (v/v) Triton-x-100 in acidified iso-propanol (2 ml concentrated Hcl per 500 ml solvent).

d) Complete dissolution of the formazan crystals was achieved by shaking the plates for 10 minutes on a plate shaker.

e) Finally optical density of each well was measured using an automatic plate reader with a test wavelength of 540 nm and a reference wavelength of 690 nm. Blanking was carried out with wells containing all reagents except cells, virus and plant extracts.

f) The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduces the absorbance (OD_{540}) of the infected control sample by 50%.

6. Isolation of active constituents

6.1. Column chromatography

The active component present in the methanolic extract can be isolated by using column chromatography using silica gel of mesh size 100-200 and eluted using methanol and finally using the TLC solvent system n-butanol: acetic acid: water (4: 1: 5).

Individual bands were collected and the brownish green band was collected separately and Rf value was checked side by using TLC using the same solvent systems.

6.2. Preparative thin layer chromatography[23]

The Preparative thin layer chromatography was carried out for the methanolic extract of *Thespesia populnea* using silica gel by using n-butanol: acetic acid: water for isolation of the active components by using streaks of spots and runned by usual procedure the similar spots were collected then dissolved in methanol further filtered to remove the silica gel the filtrate was then evaporated to dryness and the individual components was then collected.

7. Preliminary confirmation of the isolated compound

7.1. Physicochemical parameters of the isolated compound

Melting point	-	123
Nature	-	solid state
Test for heavy metals	-	NIL
Solibility	-	Soluble in DMSO, Methanol
		Partially soluble in water

7.2. Thin layer chromatography[23]

The mobile phases were selected according to their polarity all solvents were of analytical grade .The plates were developed at room temperature in vertical separating chamber to the height of approximately 16 cm from the start. The chamber was previously saturated with appropriate mobile phase saturation time was 1 hour .After drying visualization was performed in short UV light (254 nm).

7.3. Paper chromatography[23]

The paper chromatography was also carried out using the same solvent system used in Thin Layer Chromatography (n-butanol:aceticacid:water) in the ratio of 4:1:5 using what man's filter paper and allowed to run in the chamber after full saturation for 1 hour then it was allowed to run up to ¾ th of the paper. The spots were then identified using UV at 254 nm.The Rf value was then calculated.

7.4. Lamda max

The Lamda max of the isolated compound was thus calculated by using methanol as the solvent and using UV Spectrophotometer wavelength ranges from 215 nm to 390nm and their Lamda max was thus calculated by plotting the wavelength against absorbance

7.5. IR spectrum study

The IR spectrum of the isolated compound is performed and a calibration curve of absorbance against concentration may be constructed, strictly the integrated areas of the absorption bands should be compared but with sharp bands, peak heights can be used in the calculations. Here little or no interference occurs with each others.

7.6. NMR spectrum study

The NMR spectrum of the isolated compound is performed, here the chemical shift values indicates the electronic environment of the groups of equivalent protons. Correlation charts should be constructed to establish the probable nature of each group. Resonance line overlap occurs frequently, particularly at high fields and spectrum simplification by spin-spin decoupling may be necessary. Examination of the spectrum in

the presence of D_2O or CD_3OD will establish the identity of bands due to –OH or-NH_2. Spin spin coupling gives the number of protons in the interacting equivalent groups.

7.7. MASS spectrum study

The MASS Spectrum of the isolated compound is performed; The MASS Spectrum is the line spectrum corresponding to the positive ions of specific mass. The mode of fragmentation can be identified by MASS Spectrum by their mass to charge ratios. By a process known as peak matching the molecular weight of an ion may be determined to six decimal points on double focusing MASS Spectrometer. The base peak is the most intense peak

Relative abundances of ions are given as percentages of the base peak which is given the value 100%. The relative abundance of molecular ions helps in the ready interpretation of the datas.

8. Minimum inhibitory concentration of isolated compound.

1) A series of culture tubes were prepared all containing the same volume of medium inoculated with test microorganisms. The lowest concentration of sample at which the subculture from test dilution yielded no viable organisms was recorded as minimum inhibitory concentration[10].
2) Decreasing concentration of drug was added to the tubes usually a step wise dilution (two fold serial dilutions) was used starting from 500µg/ml to 4.25µg/ml. One tube was left without drug to serve as positive control and other without drug and inoculum to serve as negative control.

3) The cultures were incubated at a temperature optimal for growth of the test organism and a period of time sufficient for growth for atleast 10-15 generators (usually 24hrs for bacteria at 37℃ and 48 hrs for fungi at 28℃).
4) The tubes were inspected visually to determine the growth of organisms by the presence of turbidity and the tubes in which antibiotic is present in minimum concentration sufficient to inhibit the microbial growth which remains clear was noted as MIC of the extract.
5) In experimental terms MIC is the concentration of the drug present in the last clear tube, i.e.the tube having the lowest antibiotic concentration in which growth is not observed.

6. RESULTS AND DISCUSSION

6. 1. Preliminary phytochemical screening

The preliminary phytochemical screening reveals the presence of Flavonoids Alkaloids, Tannins and Anthraquinone glycosides. The results were tabulated in table no.1.

Table.1 Preliminary phytochemical screening

Plant name	Extract fractions	Flavonoids	Tannins	Alkaloids	Anthroquinone glycosides	Resins	Steroids
Thespesia populnea	a	+	+	+	+	_	_
	b	+	+	+	+	_	_
	c	+	++	+++	++	_	_
	d	+++	+	+	+++	_	_
	e	++	+	+	+	_	-
	f	+	+	-	+	_	_

a- Petroleum ether
b- Chloroform
c- Ethyl acetate
d- Methanol
e- Ethanol
f- Water

+++ = Excess amounts
++ = Presence
+ = Trace amounts
- = Absent

6.2. Total phenolic constituents study[23].

Table.2 Total phenolic constituents study

s no	Concentration (µg/ml)	Absorbance (nm)	
1	1	0.010	
2	10	0.003	
3	100	0.007	
4	1000	0.115	
5	Sample (*Thespesia populnea*) (10, 100)	0.06	0.07

Absorbance= 0.0012× concentration of ascorbic acid (µg) +0.0033

a) For 1 µg

0.010= 0.0012× 1+0.0033

0.010= 0.0045

b) For 10 µg

0.003= 0.0012× 10+0.0033

0.003= 0.012+ 0.0033

0.003= 0.0153

c) For 100 µg

0.007= 0.0012 ×100+0.0033

0.007= 0.12+0.0033

0.007= 0.1233

d) For 1000 µg

0.115= 0.0012×1000 + 0.0033

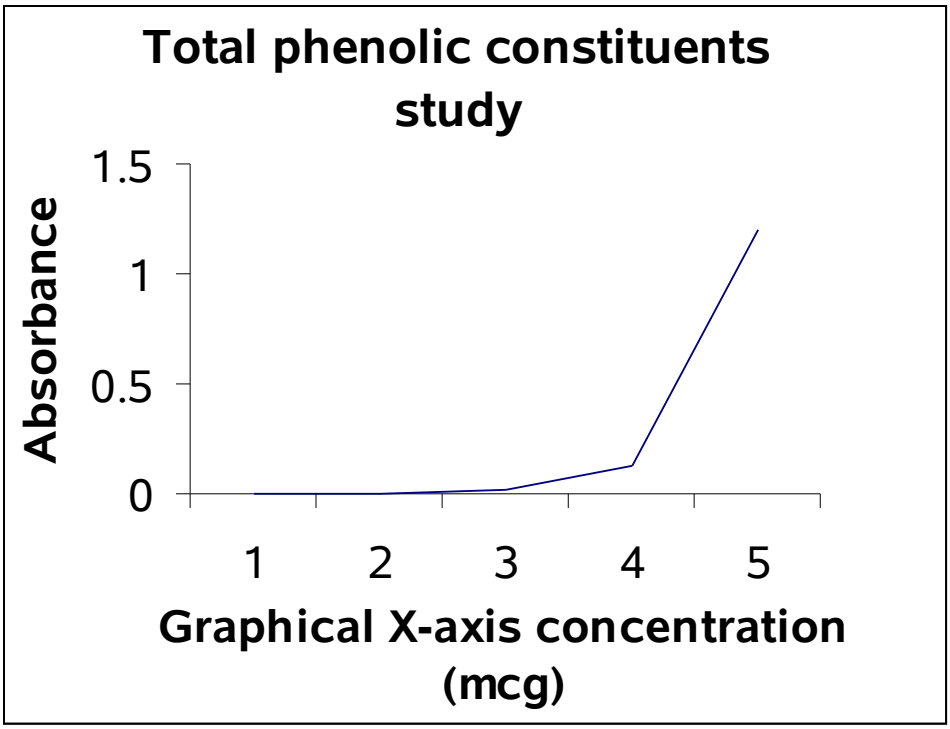
0.115= 1.2 + 0.0033

0.115= 1.2033

Table.3. Graphical method

S.no	Graphical x axis concentration (µg)	Absorbance
1	0.0045	-0.010
2	0.0153	0.003
3	0.1233	0.007
4	1.2033	0.115
5	0.1233 (sample)	0.07

The total phenolic constituents present in the methanolic extract of *Thespesia populnea* was by graphical method was found to be 0.04 µg



6.3. Antibacterial study

From the phytochemical screening, the methanolic extract showed high amount of flavonoids when compared to others, so we selected the methanolic extract of *Thespesi*a *populnea* flowers for antibacterial screening. The fourteen Bacteria's were used for antibacterial screening. Various concentrations of methanolic extract were used (1000µg/ml, 500µg/ml, 250µg/ml, and 62.5µg/ml) to test the antibacterial activity. From the results of Antibacterial screening, 7.2% of methanolic extract were active in the lowest tested concentration of 62.5µg/ml, 5% active in a concentration of 250µg/ml, 75.7% active in a concentration of 500µg/ml, and 92.8% active in a concentration of1000µg/ml. Amikacin and oxytetracycline was used as standard drug. The results were shown in the Table. 4.

Table. 4 Antibacterial study of methanolic extract of *Thespesia populnea* flowers

Microorganisms	**Zone of inhibition (mm)**					
	1000µg /ml	**500µg /ml**	**250µg /ml**	**125µg /ml**	**62.5µg /ml**	**Oxytetracycline (1 mg/ml)**
Rhodococcus terrae (NCIM 5126)	23	18	9	0	0	26
Micrococcus flavum (NCIM 2376)	17	0	0	0	0	25
Brevibacterium leuteum (NCIM 2923)	12	10	9	0	0	28
Flavobacterium devorans (NCIM 2581)	15	10	9	0	0	32
Shigella sonei (ATCC 29930)	18	10	0	0	0	34
Shigella flexneri (NCIM 4924)	21	18	17	16	14	30
Shigella boydii (ATCC 8700)	16	13	12	0	0	36
Streptococcus faecalis (NCIB 2406)	0	0	0	0	0	35
Shigella dysenteria (ATCC 13313)	15	12	20	0	0	31

Escherechiae coli (ATCC 11775)	18	16	10	0	0	32
Bacillus lichenformis (NCIM 2468)	20	17	14	0	0	38
Proteus mirabilis (NCIB 8268)	16	13	11	0	0	31
Klebsiella pneumoniae (ATCC 13883)	21	18	17	16	14	28
Micrococcus leuteum (ATCC 2984)	17	14	12	0	0	26

6.4. Synergistic activity study

The results of synergistic activity study were reported in the given table and shows that the methanolic extract of the plant show good synergistic activity when combined with the standard antibiotic oxytetracycline. The results are discussed in Table.5.

Table.5 Synergistic activity of methanolic extract of *Thespesia populnea* flowers

s.no	Microorganisms	Zonediameter (mm)
1	*Rhodococcus terrae* (NCIM 5126)	22
2	*Shigella sonnei* (ATCC 29930)	34
3	*Salmonella typhi* (ATCC NCIM 2479)	32
4	*Flavobacterium devorans* (NCIM 2581)	32
5	*Micrococcus flavus* (NCIM 2376)	28
6	*Brevibacterium leuteus* (NCIM 2923)	27
7	*Shigella flexneri* (NCIM 4924)	34
8	*Shigella boydii (*ATCC 8700)	36
9	*Escherichia coli* (ATCC 11775)	26
10	*Bacillus licheniformis* (NCIM 2468)	24
11	*Klebsiella pneumonia* (ATCC 13883)	22
12	*Micrococcus leuteus* (ATCC 2984)	21

6.5. Antifungal study

In the lowest tested concentration of 62.5 µg/ml, 14.28% of the plant extract was active, 42.85% active in the concentration of 125 µg/ml, 100% activity was seen in 1000 µg/ml in a dose dependent manner. The results of the antifungal study were reported in Table.6.

Table.6 Antifungal study of methanolic extract of *Thespesia populnea* flowers

S.no	Microorganisms	Zone of inhibition (mm)					
		1000 µg/ml	**500 µg/ml**	**250 µg/ml**	**125 µg/ml**	**62.5 µg/ml**	**Standard Amphotericin-B (1000 µg/ml)**
1	*Aspergillus niger* (NCIM 1207)	18	17	15	11	9	17
2	*Sacchromyces cerevisae* (ATCC 204508)	16	14	12	0	0	19
3	*Candida albicans* (NCIM 3484)	17	15	11	0	0	15
4	*Monilinia fruticola* (NCIM 1011)	19	15	12	9	0	14
5	*Auricularia polytricha* (NCIM 1303)	18	13	12	8	0	16
6	*Chaetomella raphigera* (NCIM 1231)	19	15	10	0	0	17
7	*Arthrobotrys oligospora* (NCIM 1246)	17	14	13	0	0	18

6.6. Antiviral and Cytotoxic study

The results of antiviral and cytotoxic screening were reported in the given tables

Table. 7. Antiviral and cytotoxic study in Human Embryonic Lung Cell culture

S. No	Plant name	Minimum cytotoxic concentration (µg/ml) [a]	Herpes simplex virus-1 (KOS)[b]	Herpes simplex virus-2 [b]	Vaccina virus [b]	Vesicular stomatitis virus [b]	Herpes simplex virus-2 [b]
1	*Thespesia populnea* methanolic extract	>100	>100	>100	>100	>100	>100
2	Acyclovir	>250	2	2	7	>250	2
3	Ganciclovir	>100	0.06	0.1	>100	>100	12

[a] Required to cause microscopically detectable alteration of normal cell morphology

[b] Required to reduce virus induced cytopathogenicity by 50%

Table.8. Antiviral and Cytotoxic study in HeLa cell cultures

s.no	Plant name	Minimum cytototoxic concentration (µg/ml) [a]	Vesicular stomatitis virus [b]	Coxsackie virus B4 [b]	Respiratory syncytical virus [b]
1	*Thespesia populnea* methanolic extract	100	>20	>20	>20
2	Ribavirin	>250	12	146	10

[a] Required to cause microscopically detectable alteration of normal cell morphology

[b] Required to reduce virus induced cytopathogenicity by 50%

Table.9. Antiviral and Cytotoxic study in Crandell Reus Feline Kidney Cells

s.no	Plant name	CC_{50} (µg/ml)[a]	Feline corona virus (FIPV) [b]	Feline herpes virus [b]
1	*Thespesia populnea* methanolic extract	>100	>100	>100
2	Ganciclovir	>100	>100	6.1

[a] 50% cytotoxic concentration as determined by measuring cell viability with colorimetric based MTT assay

[b] 50% effective concentration or concentration producing 50% inhibition of virus induced cytopathic effect as determined by measuring cell viability with colorimetric formazan based MTT assay

Table.10. Antiviral and Cytotoxic study in Vero cell cultures

s.no	Plant name	Minimum cytototoxic concentration (µg/ml)[a]	Para-Influenza-3 virus[b]	Reo-1 virus[b]	Sindbis virus[b]	Coxsackie virus B4[b]	Punta Toro virus[b]
1	*Thespesia populnea* methanolic extract	>100	>100	>100	>100	>100	>100
2	Ribavirin	112	146	>250	>250	>250	50

[a] Required to cause microscopically detectable alteration of normal cell morphology

[b] Required to reduce virus induced cytopathogenicity by 50%

The results of the study demonstrated a significant in-vitro antiviral activity of the methanolic extract derived from flowers of Thespesia populnea and these effects might be due to their content of flavonoids. It should be pointed out, however different cell lines were used; there may be some variation in way of plant compounds behave in different cell types. The extracts found to be active each showed activity against all viruses tested.

6.7. Thin layer chromatography of methanolic extract of flowers

Table.11 Rf value of the isolated compound using various solvent systems..

S no	Solvent system	Rf Values
1	n-butanol: acetic acid: water (4 : 1 : 5)	0.88
2	Benzene:pyridine:formic acid (3.6:9:5)	0.42
3	Hexane: ethyl acetate (3:1)	0.54
4	Chloroform: methanol (9:1)	0.86
5	Chloroform:methanol:water (7:3:0.2)	0.94
6	Toluene:ethylacetate:formicacid (36:12:5)	NIL
7	Cyclohexane:ethylacetate:formicacid (30:15:5)	O.96
8	Petroleum ether:ethyl acetate: formic acid (30:15:5)	NIL
9	Carbontetrachloride:acetone:formic acid (35:10:5)	NIL
10	n hexane: ethyl acetate: acetic acid (31:14:5)	O.34
11	Ethylacetate:formicacid:water (6.8:0.8:0.8)	0.65
12	Acetic acid:chloroform (1:9)	0.72
13	Ethyl acetate:Benzene (9:11)	0.58
14	Ethylacetate:methanol:formicacid:water (10:1.3:0.2:1)	NIL
15	Ethylacetate:methanol:water (10:1.3:1)	NIL
16	Carbon tetra chloride:acetone:formicacid (3.5:10:5)	0.62
17	Cyclohexane:ethylacetate:formicacid (3.1:1.4:0.5)	0.82
18	Toluene:acetone:formicacid (3.8:1:0.5)	NIL
19	Cyclohexane:ethylacetate:aceticacid (3.1:1.4:0.5)	0.32
20	Ethylacetate:1.propanol:water:formicacid (4:4:2.8:0.2)	0.84
21	Ethylacetate:methyl ethyl ketone:formic acid:water (5:3:1:1)	NIL
22	n-hexane:ethyl acetate:methanol:water[5]. (10:11:11:8)	NIL
23	Ethylaetate:aceticacid:formic acid:water[43]. (300.8:1.2:8)	0.78

Standard Rf values of flavonoids

The Rf value of the plant extract was compared with the standard values of the flavonoids which were listed below

1) Quercetin-0.36

2) Apigenin-0.38

3) Rhamnetin-0.41

4) Kaempferol-0.49

5) Chrysin-0.55

6) Galagin-0.61

The clear spots are seen in n-butanol: acetic acid: water (4:1:5) solvent system and that solvent ratio was used to elute the compound while running column chromatography.

6.8. Column chromatography

The greenish-brown coloured bands were collected in case of column chromatography with the TLC solvent system for each time of collection TLC was runned with the same solvent system which coincides with already existing Rf value so the same coloured bands were collected and left others. The column was packed so that the elution time was around 1 hr and 30 minutes.

6.9. Preparative Thin Layer Chromatography

The yield was very less while using Preparative Thin Layer Chromatography by scrapping the silica gel and isolating the constituents from the silica gel.

6.10. Paper Chromatography

The Rf value of the isolated compound was found by means of paper chromatography using the solvent system n-butanol: aceticacid: water (4:1:5) as 0.89.

6.11. Lamda max of isolated compound

Table.12 Lamda max determination

s.no	Wavelength (nm)	Absorbance
1	215	0.12
2	230	0.28
3	245	0.41
4	260	0.48
5	275	0.58
6	290	0.54
7	310	0.36
8	315	0.28
9	330	0.24
10	345	0.11

The standard λ_{max} values of some flavonoids are listed below which was compared with the isolated compound

a) Scutellarein - 285

b) Luteolin - 255

c) Cirsiliol - 252

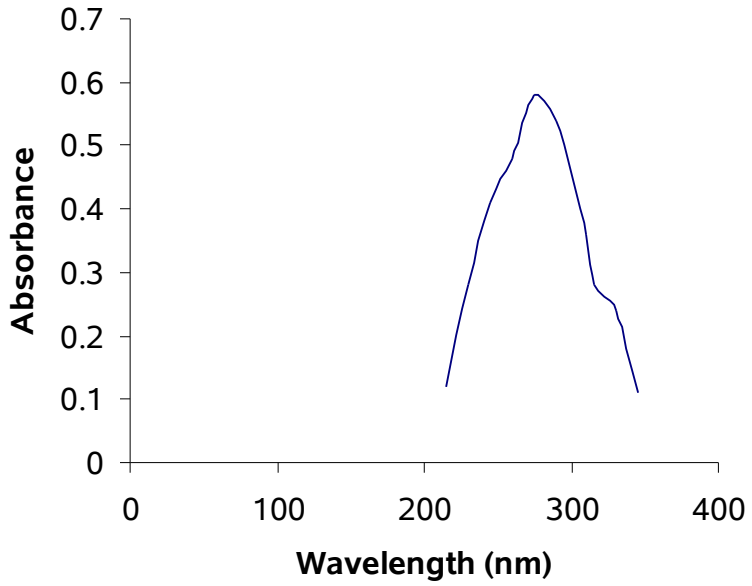
d) Apigenin - 268

e) Pilosin - 287

f) Salvigenin - 275

g) Genkwanin - 268

h) Nevadensin - 281



The Lamda max of the isolated compound was found to be 275 nm

6.12. Table.13. Minimum inhibitory concentration of isolated compound.

S.No	Microorganisms	500µg /ml	250µg /ml	125µg /ml	62.5µg /ml	31.2µg /ml	17.5µg /ml	8.5µg /ml
1	*Rhodococcus terrae* (NCIM 5126)	–	–	–	–	+	+	+
2	*Micrococcus flavum* (NCIM 2376)	+	+	+	+	+	+	+
3	*Brevibacterium leuteum* (NCIM 2923)	–	–	–	+	+	+	+
4	*Flavobacterium devorans* (NCIM 2581)	–	–	+	+	+	+	+
5	*Shigella sonei* (ATCC 29930)	–	–	+	+	+	+	+
6	*Shigella flexneri* (NCIM 4924)	–	–	–	+	+	+	+
7	*Shigella boydii* (ATCC 8700)	–	–	–	+	+	+	+
8	*Streptococcus faecalis* (NCIB 2406)	+	+	+	+	+	+	+
9	*Shigella dysenteriae* (ATCC 13313)	–	–	–	+	+	+	+
10	*Escherechiae coli* (ATCC 11775)	–	–	+	+	+	+	+
11	*Bacillus lichenformis* (NCIM 2468)	–	–	–	+	+	+	+
12	*Proteus mirabilis* (NCIB 8268)	–	–	–	+	+	+	+
13	*Klebsiella pneumoniae* (ATCC 13883)	–	–	+	+	+	+	+
14	*Micrococcus leuteum* (ATCC 2984)	–	–	–	–	–	–	+
15	*Bacillus subtilis* (ATCC 9372)	–	–	+	+	+	+	+

+ = Turbid, - = Clear solution

Fig. 1 Shows Antibacterial activity of methanolic extract of *Thespesia populnea* flowers against *Rhodococcus terrae*

Fig. 1

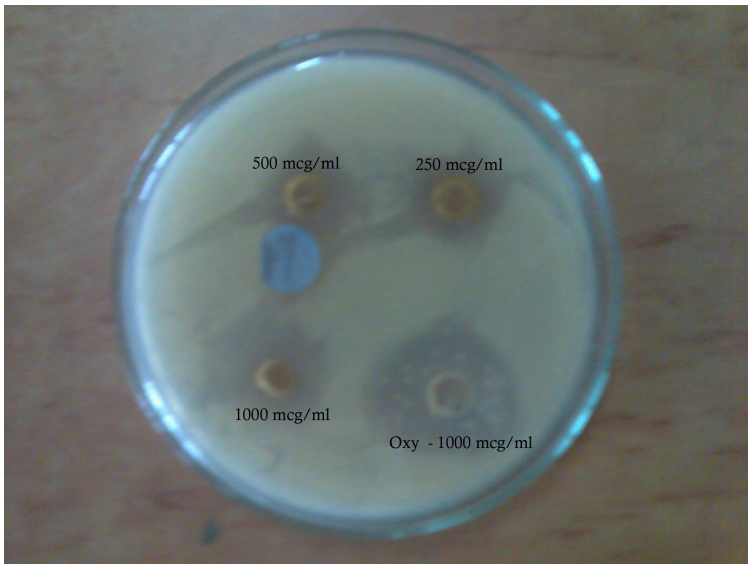


Fig. 2 Shows Antibacterial activity of methanolic extract of *Thespesia populnea* flowers against *Flavobacterium devorans*

Fig. 2

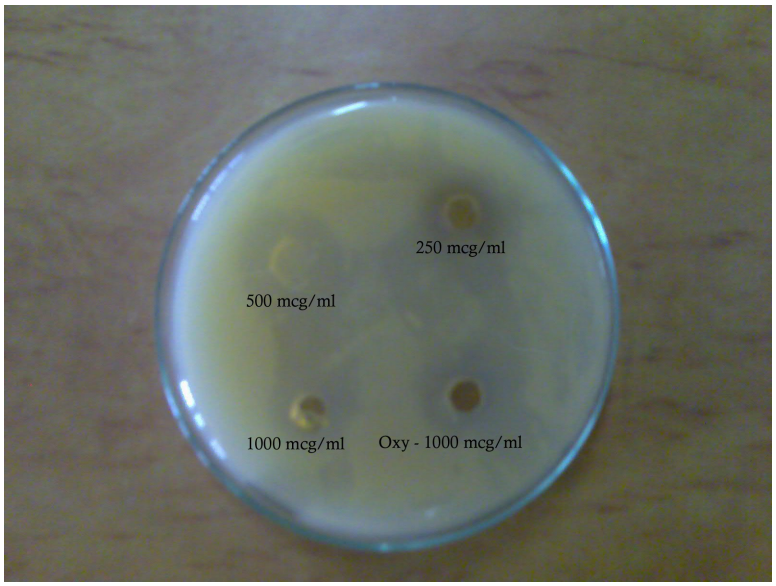


Fig. 3 Shows Synergism activity of methanolic extract of *Thespesia populnea* flowers against *Micrococcus flavus.*

Fig. 3

Fig. 4 Shows Synergism activity of methanolic extract of *Thespesia populnea* flowers against *Shigella sonei*

Fig. 4

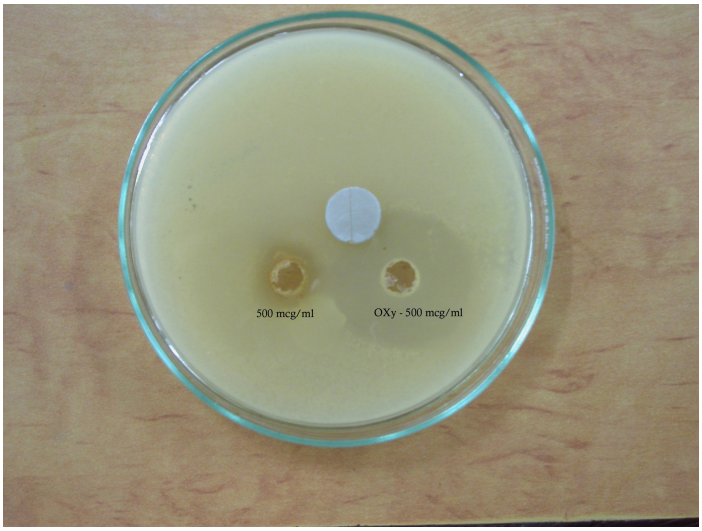


Fig. 5 Shows Synergism activity of methanolic extract of *Thespesia populnea* flowers against *Brevibacterium leuteum*

Fig. 5

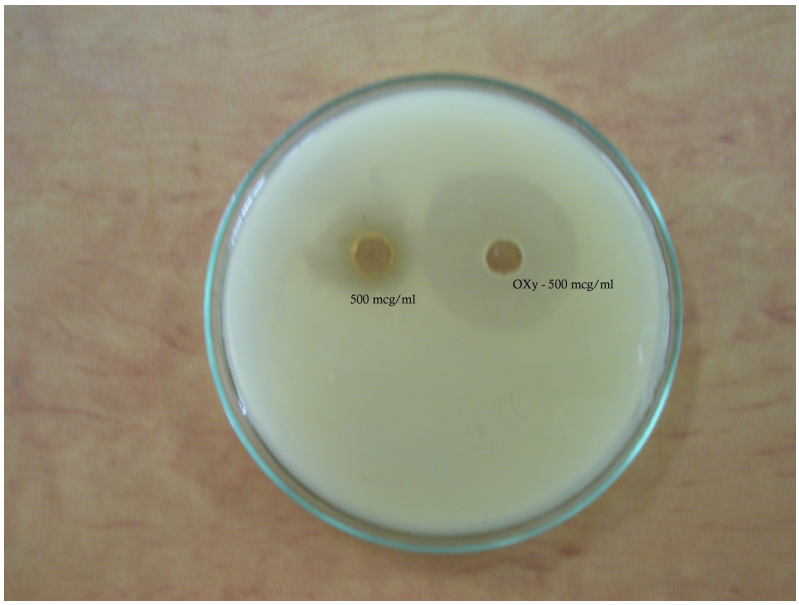


Fig. 6 Shows Synergism activity of methanolic extract of *Thespesia populnea* flowers against *Rhodococcus terrae*

Fig. 6

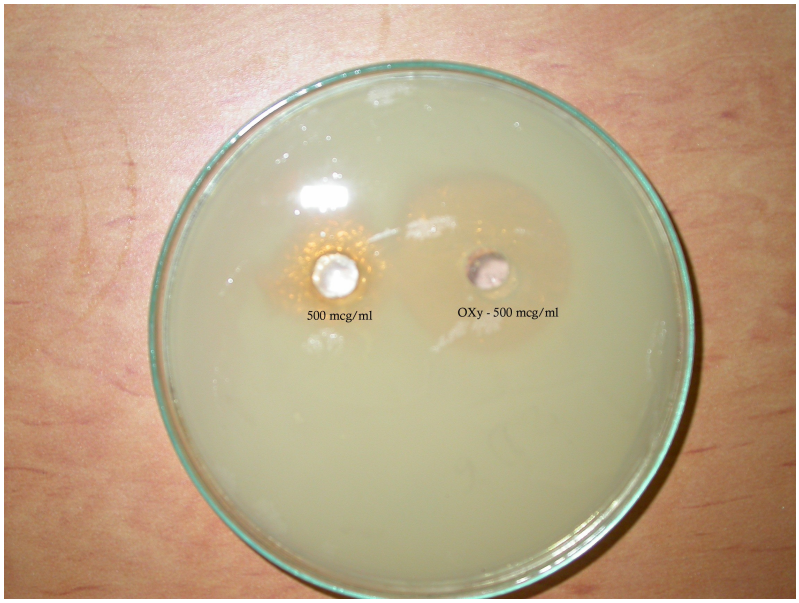


Fig. 7 Shows Synergism activity of methanolic extract of *Thespesia populnea* flowers against *Salmonella typhii.*

Fig. 7

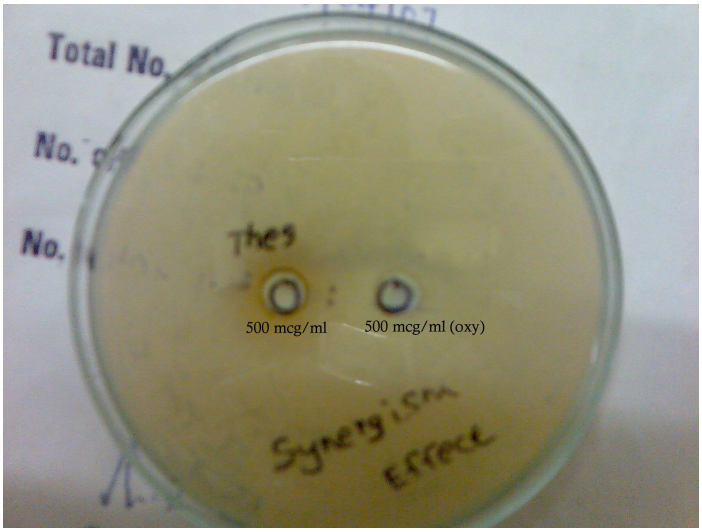


Fig. 8 Shows Antifungal activity of methanolic extract of *Thespesia populnea* flowers against *Molinia fruticola*

Fig. 8

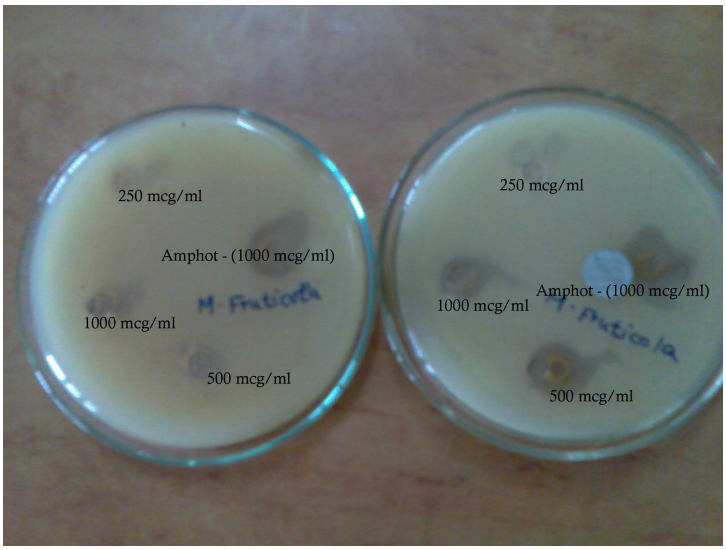


Fig. 9 Shows Antifungal activity of methanolic extract of *Thespesia populnea* flowers against *Chaetomella raphigera*

Fig. 9

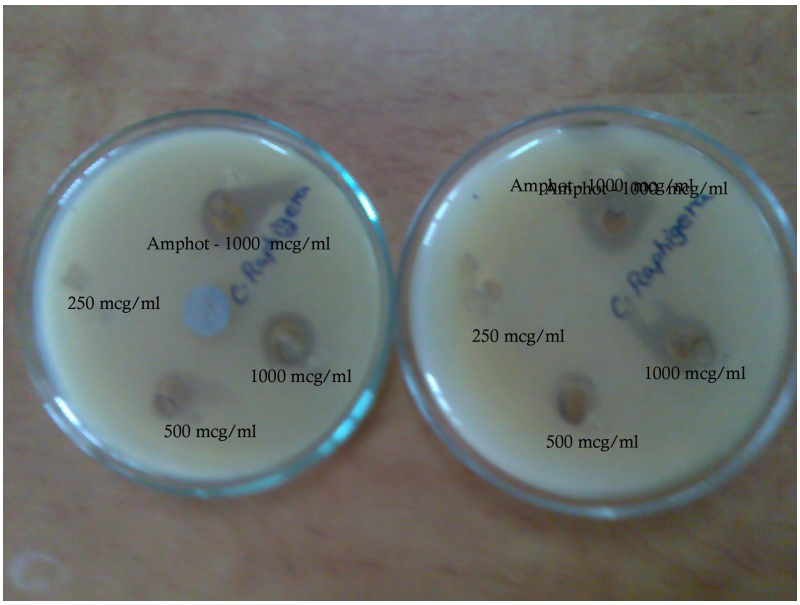


Fig. 10 Shows Thin Layer Chromatography of methanolic extract of *Thespesia populnea* flowers

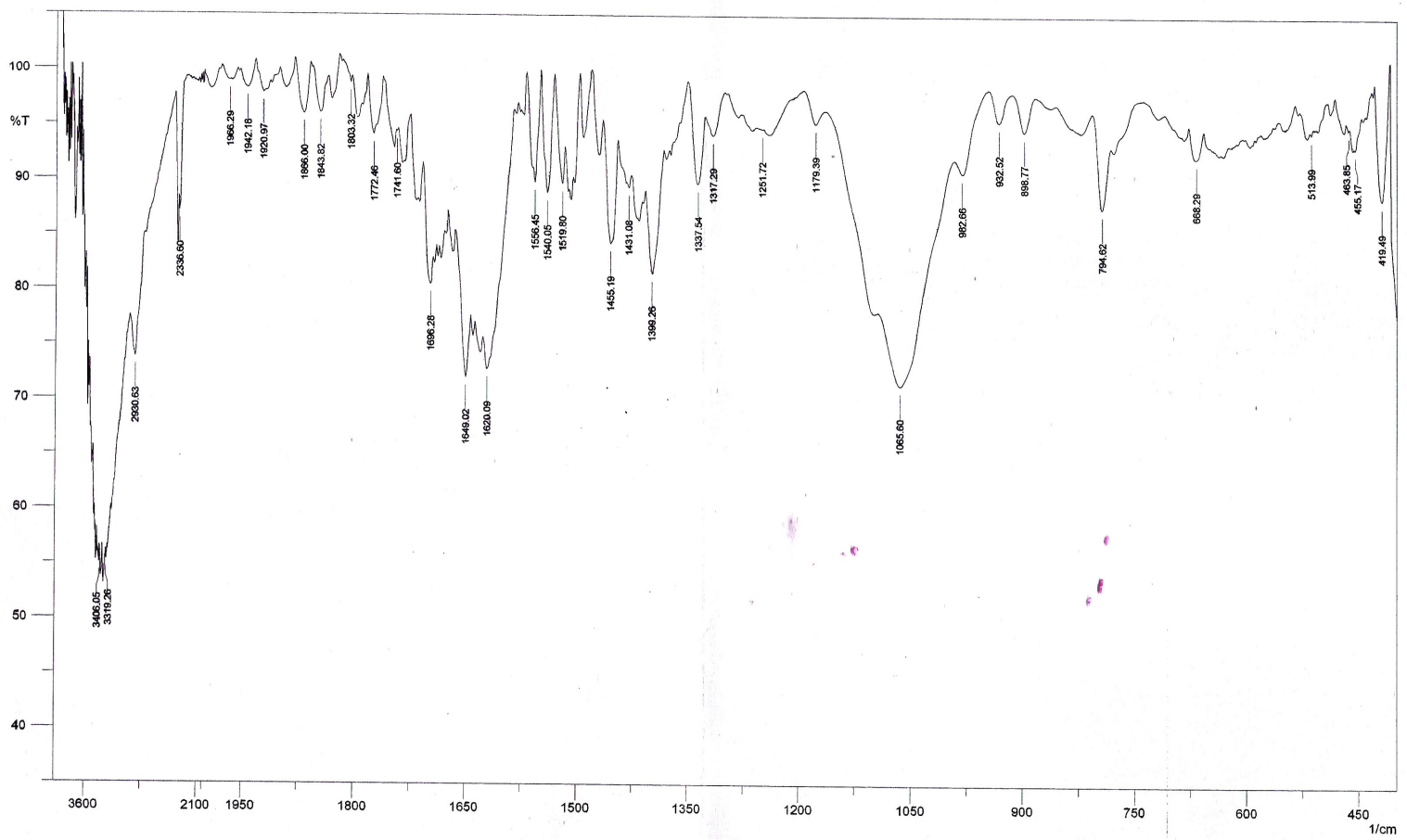
Fig. 10

Fig. 11 Shows Thin Layer Chromatography of the isolated fraction.

Fig. 11

NANDHA COLLEGE OF PHARMACY, ERODE.

SHIMADZU



Sample ID : SAM V 1

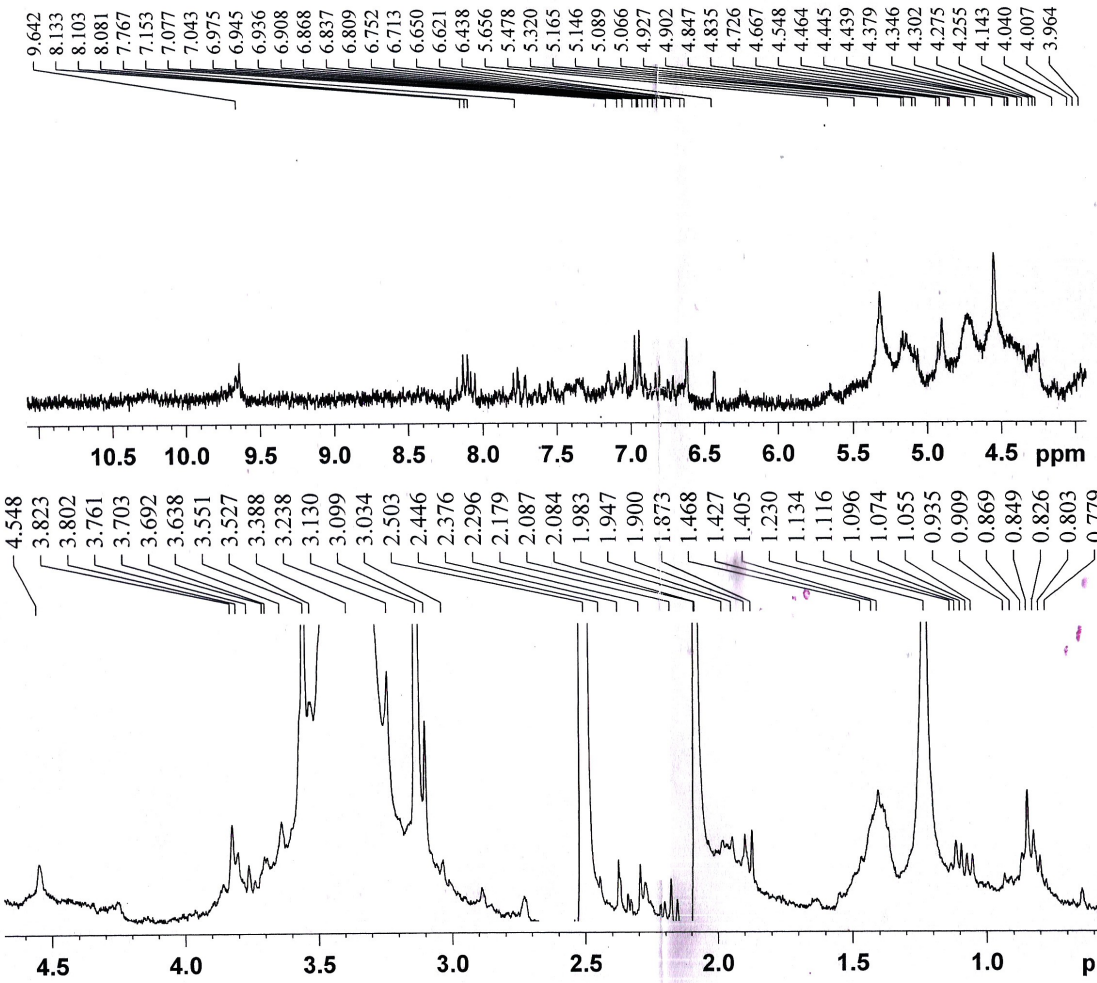
Resolution : 4 cm^{-1}

Apodization : Happ-Genzel

No.of Scans : 20

Analyst : M.Jagadeeswaran

Date : 15/12/07



BRUKER

Current Data Parameters

NAME	V1
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20071226
Time	16.16
INSTRUM	spect
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PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	16
DS	2
SWH	6188.119 Hz
FIDRES	0.094423 Hz
AQ	5.2953587 sec
RG	256
DW	80.800 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec
TD0	1

======== CHANNEL f1 ========

NUC1	1H
P1	8.60 usec
PL1	-2.00 dB
SFO1	300.1318534 MHz

F2 - Processing parameters

SI	32768
SF	300.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

File: V-1
Sample:
Instrument: JEOL GCmate
Inlet: Direct Probe

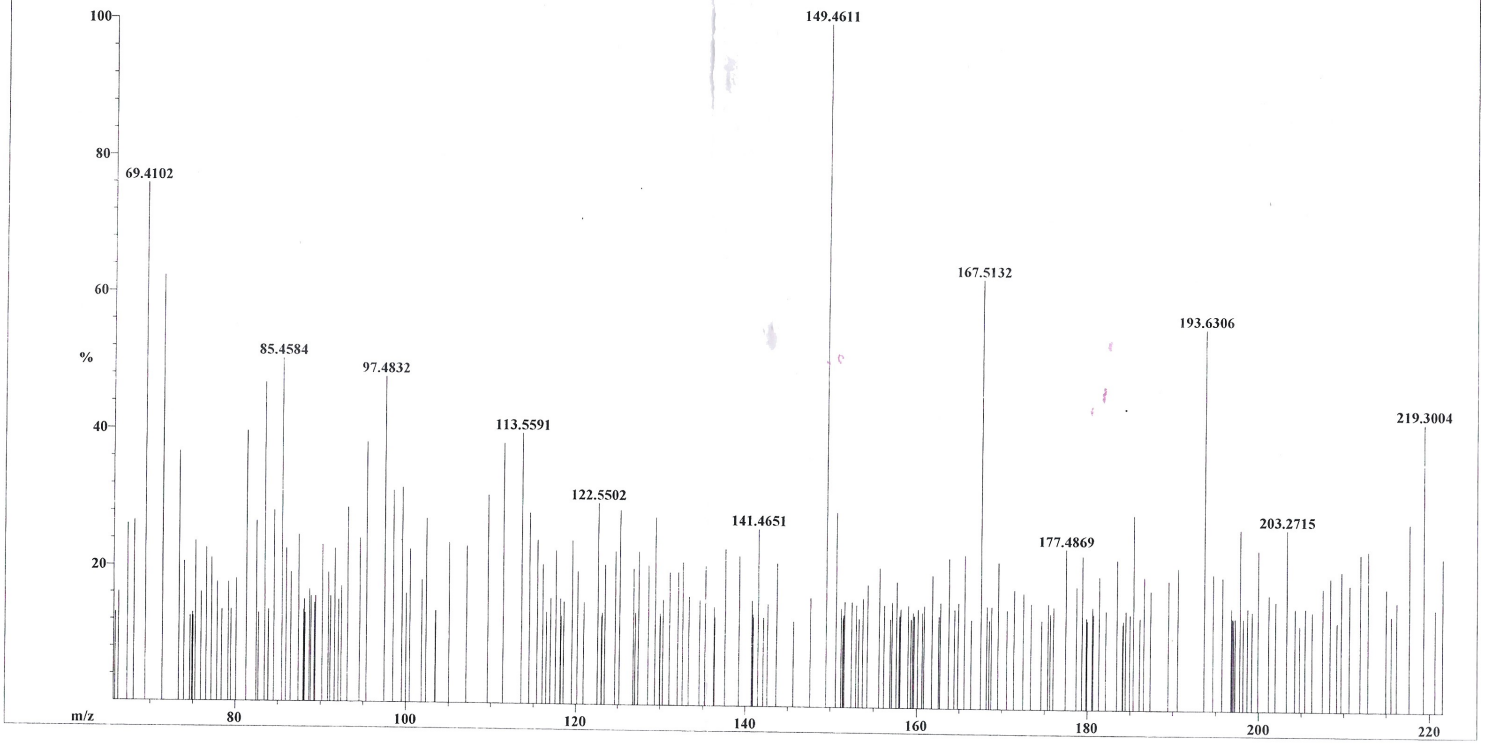
Date Run: 11-30-2007 (Time Run: 12:34:45)

Ionization mode: EI+

Scan: 37
Base: m/z 149; .3%FS TIC: 389584

R.T.: .72

#Ions: 681



7. CONCLUSION

In the current approach of *Thespesia populnea* which have been used therapeutically for various medicinal purpouses, however there had been no reports regarding the methanolic extract of the plant. We therefore decided to study the cytotoxic and antimicrobial properties of the same plant the *in-vitro* antibacterial activity of *Thespesia populnea* used by Indian peoples to show that therapeutic properties. The antibacterial activity was expressed at varying degrees with the activity being both strain and dose dependent. Fourteen Bacteria's were used for antibacterial studies.

Medicinal plants are being used by large proportion of Indian population. The reasons for this include a) True improvement of diseases conditions after Herbal treatment b) harmful side effects and high cost of the other forms of treatment. In the present study, the results were encouraging, as *the Thespesia populnea* appeared to contain substances that had antimicrobial properties because of, the methanolic extract of *Thespesia populnea* flowers were active against 13 out of 14 Bacteria's. Five concentrations of the extract were used (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and 62.5µg/ml). It is estimated that if an inhibition is obtained by 250µg/ml-1000µg/ml) of test solution, the extract can be considered worthy of further investigations. Compared to other authors it is relatively low concentration. Regarding antifungal activity the methanolic extract of the plant shows positive results for all fungus.

The aqueous and organic extracts from the plants showed different activities. There are no common rules for this, but in most cases, organic extracts showed the same (or) greater activity than aqueous extracts. The plate hole diffusion method showed larger

activity than disc diffusion method. Most likely; this is because large amount of solvents may influence Bacterial growth, there by demonstrating that larger doses are required.

Since the medicinal plants studied appear to have a broad antimicrobial activity spectrum, they could be useful in antiseptic and disinfectant formulations as well as in chemotherapy. The optimal effectiveness of a medicinal plant may not be due to one main active constituent, but to the combined action of different compounds originally in the plant.

In Literature, it has been indicated that the antibacterial activity is due to different chemical agents in the extract, including essential oils (especially thymol), flavonoids and triterpenoids and other compounds of phenolic nature or free hydroxyl group, which are classified as active antimicrobial compounds. A complete study conducted with the purpose of finding these chemicals is worthwhile. These findings can form the basis of further studies to isolate active compounds, elucidate them against wider range of Bacterial strains with the goal to find new therapeutic principles.

Under this experimental study the extract was active for Bactericidal action. The findings revealed that the extract capability to penetrate the cell walls with hydrophobic environment (gram negative) and hydrophilic environment (gram positive) Bacteria's responsible for the Bactericidal action which can be isolated and identified by some analytical techniques. The results of the study supports to a certain degree, traditional medicinal uses of the plants evaluated both for human and animal diseases therapy and reinforce the concept that the concept that ethno botanical approach to screening plants as potential sources of bioactive substances is successful. Plants showing significant activity may be due to the presence of alkaloids, flavonoids, tannins and polyphenols. Among the

various microorganisms, the methanolic extract was more active against *Rhodococcus terrae*. The aqueous extract generally exhibits a high degree of antibacterial activity this seems to confirm the traditional therapeutic claims of this plant. These results suggest the presence of either good antibacterial potency or high concentration of an active principle in the extract. This antibacterial activity would support the folk therapy of infections whose symptoms might involve Bacteria.

Plant extracts and phytochemical are becoming popular as potential sources of antibacterial and several reviews have been written. By this ethno botanical knowledge of indigenous as a means to select potentially antibacterial plants, this screening potentially to be more successful than a random screen of plants. Moreover the discriminary effect against specific microorganisms suggests the presence of different chemical compounds. According to many Journals plant extract showing activity between the ranges of 1000 µg/ml to 100µg/ml, for isolated compound 100µg/ml to1µg/ml is an accepted range the values varying this range could not accepted as a good antimicrobial activity. But according to my study the values of these activities are within in these limits, so accepted for good activity.

Also, we isolated the particular active principle necessary for the activities by Column chromatography, Preparative thin layer chromatography, and then the preliminary confirmation of the isolated compound was carried out by thin layer chromatography here myself selected and performed for more than 70 solvent systems by changing the ratios of the solvent systems according to the polarity before going for column chromatography but only 60% of solvent systems shows the spots clearly among this only one solvent system n-butanol:aceticacid:water shows clear spots under UV so, I

used this solvent for column and the isolated compound was runned in the same solvent system and the same Rf value as that of the extract was thus obtained the samples of same Rf value was collected separately. Then the minimum inhibitory concentration of the isolated compound was carried out which gives the positive results.

a. IR Spectrum

1) The absorption spectrum consists of few bands and the substances are therefore relatively simple. The presence of -OH is indicated by the broad absorption at 3319 cm^{-1} and at 1065 cm^{-1}

2) The unsaturation is of an aromatic nature is evident from the characteristic absorption in the regions 1620 cm^{-1} and 794 cm^{-1}

3) An aromatic system is indicated by the absorption at 1600 cm^{-1}, 1519 cm^{-1}, and 668 cm^{-1}

4) The strong absorption at 1620 cm^{-1} is of aromatic absorption.

5) Strong carbonyl absorption at 1696 cm^{-1} can be interpreted as C=O.

b. NMR Spectrum

1) A chemical shift value at 1.20 indicates the alkane group protons in $–CH_3$ usually appears at δ = 0.9-1.0. This singlet may be due to CH-C-OH (OR)
2) The other signal at chemical shift value of 2.10 (singlet) may be due to the keto group bonded to aliphatic alkane group CH- (C=O)R
3) The shift value at 2.50 may due to keto group attached to an aromatic ring CH-(C=O) Ar.
4) A small singlet at 4.5 indicates HO-Ar hydroxyl group attached to aromatic ring

5) A triplet is found at the chemical shift value between 3-4 (1:2:1). Proton proton coupling between a single and double bond in a five membered ring system.

c. MASS Spectrum

1) The mass spectrum is the line spectrum

2) It exhibits m/z at 219 at 45% of relative abundance

3) The fragmentation patterns are 203 (CH_3 -15)

4) Here the peak at 149.46 is the base peak of most intense having 100% of relative abundance.

Plant extracts are complex mixtures of many compounds. This study clearly shows that the cytotoxicity and antiviral activity of a extract are not necessary to the same compounds and that the cytotoxicity of some plant compounds may mask the antiviral properties of other plant substances. The result presented here indicates that the cited above methanolic extract of *Thespesia populnea* possess moderate antiviral and cytotoxic activities, and these effects might be due to their content of flavonoids, specially biflavones and C-glycosyl flavonoids for which antiviral activity was already described.

In this work I presented results concerning the antiviral activity of methanolic extract of *Thespesia populnea* flowers. The final conclusion is that extracts from plants employed in ethno medicine can exhibit antiviral activity. Accordingly medicinal plants can be a source for isolation of pure compounds against HSV-1 and2. Despite the fact that the amount of information on anti-HSV plant extract is very relevant, not all the bioactive anti-HSV molecules responsible for the activity of plant extracts have been identified, isolated, synthesized and tested. This is a crucial point, and is highly advisable

that all promising plant extracts should undergo further analysis and purification steps in order to identify active principles and clarify the chemical nature.

Synthetic approaches should also be developed for production of larger amounts of bioactive molecules and their analogues for all required preclinical and clinical studies. Finally gene expression profile studies employing microarray technology could help to identify molecular targets of biological activity of anti-viral molecules from natural products, allowing moving for gene-based drugs to be used for anti-viral therapy.

8. PAPER PRESENTATIONS

8.1) Accepted Papers

8.1.1) One Paper accepted and presented in Poster session in Indian Pharmaceutical Congress held at Varanasi entitled " Antibacterial activities of metabolic extract of *Thespesia populnea* flowers" (C 82) under the session Pharmacognosy Indigenous Drugs and Herbal Formulations.

8.1.2) One Paper Published in the Pharmacognosy Magazine entitled "Evaluation of Antioxidant activity, phenol and flavonoid contents of some selected Indian medicinal plants". It is to be published in April-June issue of 2008.

8.2) Communicated Papers

8.2.1) One Paper communicated in International Journal of Pharmacology entitled "Antibacterial and antifungal activities of methanolic extract of *Thespesia populnea* (Malvaceae) flowers"

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