CHAPTER - I

Introduction
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1.1 INTRODUCTION

Sight, more than any of our senses, helps us to navigate the world around us. In a single glance, lasting a fraction of a second, our eyes work with our brains to tell us the size, shape, color and texture of an object.

The surface of the eye is rich in nutrients and consequently supports a diverse range of microorganisms that constitute the normal ocular flora (Armstrong, 2000). These microorganisms interact with each other and with the immune system of the host. The consequence of these interactions is that, under normal conditions, the growth of individual microorganisms is regulated and eye infection is prevented. Infection of the eye results from either the acquisition of a virulent microorganism or uncontrolled growth of an existing organism because of lowered host resistance.

The human eye responds differently to infection than other organs. The immune privileged environment of the eye often prevents a vigorous inflammatory response to infection - a response that may otherwise damage delicate tissues and cells responsible for vision. The corneal epithelium is the first cellular line of defense for the eye and although protected by the eyelids and tear film, can be exposed to all manner of environmental insult. Corneal, infection is a major cause of blindness in developing countries. A previous study reported that the incidence of corneal infections in India is almost ten times than reported in the United States (Gonzales et al., 1996)

The amount of bacteria present on the healthy conjunctiva is usually less than ten colonies, which causes acute bacterial conjunctivitis yield a more confluent growth (Kowalski et al., 2005). In addition to the sweeping and flushing action of eyelids and
tear fluid and the barrier function of the conjunctiva and corneal epithelium, lysozyme, lactoferrin, beta-lysis, IgA and other immunoglobulins and complement system, components in the lacrimal fluid help to prevent the normal bacterial flora from causing conjunctivitis or keratitis (Mc Cellan, 1997; Kowalski and Rout, 2005; Mannis and Plotnik, 2005). In addition metabolic products made by the normal conjunctival bacterial flora also inhibit colonization and multiplication of more virulent bacterial species (Mc Cellan, 1997). The greatest risk factor for infectious keratitis with *Pseudomonas aeruginosa* and other bacterial pathogens is the extended or overnight use of soft contact lenses (Callaghan et al., 1996). The integrity of the corneal defense are dependent on an oxygenated tear film regularly bathing the corneal surface.

*Staphylococcus aureus* is the leading cause of microbial keratitis in humans (Ashell et al., 1982 and Tuffs et al., 2000). It is the major cause of ocular morbidity and avoidable visual impairment worldwide. Since 1980’s, quinolones have been used as a broad spectrum action against pathogens (Baum et al., 2000). *S.aureus* infections may be difficult to treat due to the ability of the bacterium to acquire resistance to many antibiotics. In Japan, about 60 percent of *S.aureus* strains isolated from all infections are multidrug resistant (WHO, 1999). *S.aureus* is generally considered to be an opportunistic pathogen. Ocular infections are generally community-acquired and not the result of a hospital stay.

Defense in the open eye environment is dependent upon maintenance of a highly efficient multi-layer passive barrier (Selinger, 1979; Smolin, 1985; Pleyer, 1997; Gachon, 1998). This is designed to minimize the microbial colonization following a trauma. Critical components of this system include an outer barrier consisting of the lid, the lashes, the blink and the outermost lipid layer of the Pre-ocular tear film.
A secondary barrier is the aqueous tear layer. It is supplied with a wide array of anti-inflammatory and anti-microbial factors. The conjunctiva is provided with an immune-based defense system.

A wide range of anti-inflammatory proteins has been identified in the tear fluid. These include anti-proteases (Sathe et al., 1998), cytokines (Pflugfelder, 1999), Vitronectin (Sack et al., 1993) as well as neutrophil gelatinase and lipocalin (Beaton, 2000). The last layer of the pre-ocular barrier that the microorganism must penetrate is the thick mucous blanket that is thought to encompass the entire epithelium (Price Schiari, 1998). The glycoproteins present in the apical epithelium serve as a foil by tying up binding sites on bacteria thus decreasing their capacity to adhere to the ocular epithelial surface (Mc Namara et al., 2000).

The presence of normal flora is also believed to be an important factor in the ocular defense mechanism. The normal flora depletes the tear fluid of nutrients, sops up binding sites that might otherwise be available for pathogens, secrete bacteriocidens that make the environment less hospitable for the growth of potential pathogens (Beaton, 2000).

The host defense in the open eye environment losses much of its validity in the closed environment. Prolonged eye closure results in a loss of the cleaning, mixing and pumping action of the blink, limiting the capacity to remove microorganisms by means of fluid turn over. The risk of dessication and exposure to new potential pathogens and risk due to trauma are greatly reduced. Instead, the defense mechanisms of the ocular surfaces face new set of challenges. These include the problem of the potential proliferation of entrapped micro-organism, protection from ocular and microbial waste and toxic products that build up. Studies have revealed that prolonged eye closure
results in corneal hypoxic stress (Madigan et al., 1987). Identified changes include a
decrease in corneal epithelial pit, a shift to a more anaerobic mode of glucose
metabolism, the build-up of lactic acid and CO$_2$ (Thakur et al., 1998). The ocular
surface defense systems are extraordinarily complex with many levels of feedback.

Many opportunistic pathogens break open these complex, extra ordinary
defense system of the eye and make their presence in the form of diseases. It includes
pink eye or conjunctivitis in conjunctiva, uvetis (redness) in the middle layer of the eye,
corneal ulcer (Keratitis) in cornea, cataract in the lens (not due to pathogens), sty
(red bump), Blepheritis (lid inflammation) and chalazion (gland inflammation) in the
lids. Ocular therapeutics such as antibiotics are found to give relief from these
pathogens, but with some side effects. Plate 1.3 explains various eye infections.

1.2 OCULAR THERAPEUTICS

Various chemotherapeutic agents and antibiotics are widely used in prophylaxis
and treatment of ocular infections. Trachoma, keratitis, corneal ulcer and ocular
infections following intraocular surgery are being efficiently managed with the help of
therapeutic agents. However, with indiscriminate use and abuse of these drugs, ocular
toxic allergic reactions and super infections have cropped up. Each drug has one or
more limitations in terms of efficiency and toxicity. Selection of antimicrobial agents
for treatment is a complex procedure. An antibiotic may either be bactericidal or
bacteriostatic. These agents can hit several targets in the bacteria namely, the cell wall,
the cytoplasmic membrane, the ribosomes and the molecules involved in the
transcription of genetic information. The following are a few to mention:
Penicillin is the most important antibiotic, obtained from the mould *Penicillium notatum*. It is widely used in the control of infection because of wide range of bactericidal action. It is effective against cocci, gram-positive organisms, but gram-negative bacilli are insensitive. The drug is quite safe but some individuals are so sensitive to it. A number of organisms become resistant to it over a period of time.

Cephalosporin is derived from the mould *Cephalosporium acremonium*. They resemble penicillin and have high potency against gram positive and gram negative bacteria, penicillin sensitive and penicillin resistant *Staphylococci* and *Pneumococci*.

Macrolide antibiotics include erythromycin, azithromycin, moxithromycin and clanthromycin are effective against few bacteria but they cause gastrointestinal disturbance.

To treat gram-negative infections streptomycin, kanamycin, gentamycin, tobramycin, amikacin and neomycin are used. Streptomycin is a toxic drug and can damage the VIII cranial nerve. Gentamycin may produce vestibular damage and ototoxicity. It should be avoided in pregnancy and patients with renal impairment. Tobramycin have toxicity similar to Gentamycin, Amikacin a semi synthetic antibiotic have adverse reactions similar to those of Gentamycin. Neomycin is a polybasic water soluble antibiotic. It is poorly absorbed on oral administration and is highly toxic.

Tetracyclines are broad spectrum antibiotics used to treat gram-positive and gram-negative organisms. They inhibit the growth of certain *Actinomyces*, *Chlamydia* etc.

Fluoroquinolones are a family of antibiotics that selectively inhibit DNA gyrase. Ciprofloxacin, norfloxacin, ofloxacin, gatifloxacin and moxifloxacin are other
commonly used antibiotics. Several studies have shown that the frequency of fluoroquinolone resistance in bacterial isolates is increasing (Graves et al., 2001, Goldstein et al., 1999, Alexandrakis 2000, Schaefer et al., 2001). The newer generation fluoroquinolones such as levofloxacin, gatifloxacin and moxifloxacin have gained widespread use as prophylaxis. This prophylaxis measures have been a concomitant rise in frequency of fluoroquinolone resistance among ocular bacterial isolates (John et al., 2007). Adverse reactions of fluoroquinolones include gastrointestinal disturbances, head ache, dizziness, insomnia, confusion, tremors, rashes and photosensitivity. The use of multiple antibiotics simultaneously and with frequent dosing may result in toxicity (Judson, 1989). With these adverse side effects it is not advisable to continue with antibiotics. Alternate therapy techniques from natural plant products may serve better results. Our search in siddha medicine gave promising idea regarding alternative methods of ocular therapeutics.

1.3 THE SIDDHA SYSTEM OF MEDICINE

Eye diseases are classified as 96 types in siddha medicine viz., Corneal disease-45, conjunctiva-20, lid infection-16, retinal diseases-15 etc. For all eye ailments, there is remedy from plant products. A mixture of flowers, leaves and roots from various plants is used widely in all types of siddha medicine. Most of the medicines are prepared with a combination of flowers of *M.oleifera* (murungai) and *T.divaricata* (nadiyavattam). The following verse proves the efficiency of both these flowers to treat eye ailments.
The verse depicts the importance of moringa and nadiyavattam flowers. Moringa flowers (Drumstick) are used in almost all medicines and is found to cure all 96 types of eye diseases. Nadiyavattam is considered as ‘The best herb for all eye diseases’ as well as ‘a rejuvenator for eyes’. Based on the use of traditional medicine and records it is planned to test the antimicrobial activity of these two flowers (*M.oleifera* and *T.divaricata*) on microbial pathogens isolated from human eyes. Also, the antioxidant potential and phytochemical characterization of the compounds present in these selected plants are studied.

1.4 DETECTION OF ANTIBACTERIAL ACTIVITY IN THE FLORAL PARTS-PETALS OF PLANTS

Resistance towards prevailing antibiotics among bacteria and fungi leads into a search of new class of antimicrobial substances urgently. It is well known that plants, although lacking the typical immune response, have an in-built system for protection against biotic and abiotic stress conditions. Since plants have co-evolved with
pathogens, they understandably have also developed the chemical protection pathways against the parasitic organisms. Therefore it is reasonable to expect a variety of plant compounds with antimicrobial activity and antibiotic potential (Sakarkar and Deshmukh, 2011).

The bioactive substances in plants are produced as secondary metabolites (William et al., 1989). While plant leaf, stem and root extracts have been widely evaluated for bioactive compounds, screening of plant flower has not been extensive.

The petals of flowers, which provide physical protection to the reproductive components and developing embryos in the pollinated gynoecium, can be expected to synthesize potent bioactive compounds. Interestingly, the symptoms of most plant diseases of bacterial and fungal origin have been reported mostly on the leaves, stems, roots and seldom on petals (Darokar et al., 1998).

Hypothesizing that petals must possess some protective mechanism against microbial attack in most of the plants, and the usage of flower extract in treating eye ailments in siddha medicine, the petals of M.oleifera and T.divaricata were selected for a detailed research in the present study.

Natural antioxidants that are present in plants are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. Natural antioxidants are preferred in allopathic drugs to overcome their side effects. Most of the polar compounds such as phenolic and flavonoid substances are potent inhibitors of reactive oxygen species attack (Owen et al., 2003). The biological properties including cytotoxic and antioxidant property of flavonoids are considered in an evaluation of the medicinal and nutritional values of these compounds (Harborne, 2000). Flavonoids are
amazing array of over 6000 different substances found in all plants, which are responsible for many of the plant colors that dazzle us with their jubilant shades of yellow, orange and red. In the present study the two selected flowers are pure white in color. Their antioxidant potential is ascertained to reveal the prevailing gaps.

*Moringa* is a plant genus whose various species have not been explored fully despite the enormous reports concerning their medicinal use viz., Cardiac and circulatory stimulants, anti-tumour; anti-pyretic; anti-inflammatory; anti-ulcer, antioxidant, antidiabetic, antibacterial and anti-fungal activities. The indigenous knowledge and use of *Moringa* is referenced in more than 80 countries and it is known over in 200 local languages. The phytochemistry and a number of chemical constituents from the leaves, stems and roots of *T. divaricata* (Family Apocynaceae) have been previously reported. Constituents studied include alkaloids and non-alkaloids (Akhtar, 1995). The members of the family Apocynaceae are the richest source of drugs in the plant kingdom. The growing scientific evidence supports that this plant has medicinal benefits and its extracts could possibly be used as pharmacological interventions in various diseases (Rumjhum et al., 2012). In this study, the main focus is to evaluate the bio medical potency of *M.oleifera* and *T.divaricata* flowers which is relatively less explored. The outcome of the study will help to find natural remedy for eye ailments. For ocular infection, the traditional physicians and tribal people use plant products but the science behind the action is less explored.

**EYE - STERILE ZONE**

The ocular surface of healthy individuals inherently supports a small population of bacteria, typically Staphylococci which are believed to exist as commensals on the mucosa and lid margins (McCulley et al., 2003). Under ideal conditions, there is little
or no opportunistic bacterial colonization of the conjunctiva or cornea, because of the washing effect of the tears (Armstrong, 2000), in conjunction with the action of antibacterial proteins and enzymes within the tear film (Haynes et al., 1999). Dry eye, due to tear deficiency is often associated with alternations in the concentration and type of bacteria present (Callaghan et al., 2003). Such disorders, among others have been associated with several Gram-positive and negative bacteria including *S. aureus, B. subtilis, P. aeruginosa, H.influenzae*, etc., (Pinna et al, 2005).

Hence, in the present study it is planned to find out the efficacy of the extracts of flowers of *M.oleifera* and *T.divaricata*, to inhibit the ocular microbial pathogens. The ocular pathogens are to be identified using rDNA techniques. Further the bioactive compounds in the flowers of these plants are to be identified using modern techniques.

1.5 DESCRIPTION OF SELECTED PLANTS

*A. Moringa oleifera*

<table>
<thead>
<tr>
<th>TAXONOMIC CLASSIFICATION</th>
<th>BOTANICAL DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom - Plantae</td>
<td>Latin - Moringa oleifera</td>
</tr>
<tr>
<td>Subkingdom - Tracheobionta</td>
<td>Sanskrit - Subhanjana</td>
</tr>
<tr>
<td>Super Division - Spermatophyta</td>
<td>Hindi - Saguna, Sainjna</td>
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<tr>
<td>Division - Magnoliophyta</td>
<td>Gujarati - Suragaro</td>
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<tr>
<td>Class - Magnoliopsida</td>
<td>Tamil - Moringkai</td>
</tr>
<tr>
<td>Subclass - Dileniidae</td>
<td>Telugu - Mulaga, Munaga</td>
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<tr>
<td>Order - Capparales</td>
<td>Malayalam - Murinna, Sigru</td>
</tr>
<tr>
<td>Family - Moringaceae</td>
<td>Punjabi - Sainjan, Soanjna</td>
</tr>
<tr>
<td>Genus - Moringa</td>
<td>Ayurvedic - Akshiva, Haritashaaka</td>
</tr>
<tr>
<td>Species - oleifera</td>
<td>French - Moringe agraine ailee</td>
</tr>
<tr>
<td>English - Drumstick tree</td>
<td>Portugese - Moringa, Moringueiro</td>
</tr>
</tbody>
</table>
MORPHOLOGY

*Moringa oleifera* is a medium or large, fast-growing evergreen or deciduous tree that usually grows as high as 9m, with a soft and white wood and corky and gummy bark. Roots have the taste of horse-radish. Leaves are longitudinally cracked leaves, 30-35 cm long main axis and its branch jointed. The twigs are finely hairy and green. Flowers are white, scented in large axillary down panicles, pods are pendulous, ribbed, seeds are 3-angled (Gupta, 1999). (Plate 1.1)

**B. Tabernaemontana divaricata**

<table>
<thead>
<tr>
<th>TAXONOMIC CLASSIFICATION</th>
<th>BOTANICAL DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom - Plantae</td>
<td>Tribal name - Boyamaa baajaa (marma)</td>
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<tr>
<td>Subkingdom - Angiosperms</td>
<td>Bengali - Tagar, Dudhphul</td>
</tr>
<tr>
<td>Class - Eudicots</td>
<td>Chinese - Gou Ya Hua</td>
</tr>
<tr>
<td>Subclass - Asterids</td>
<td>Sinhalese - Wathusudda (gardenwhite)</td>
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<tr>
<td>Order - Gentianales</td>
<td>Kannada - Nandi Battalu</td>
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<tr>
<td>Family - Apocynaceae</td>
<td>Tamil - Nadiyavattam</td>
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<tr>
<td>Sub family - Rauvolfioideae</td>
<td>Gujarati - Sagar</td>
</tr>
<tr>
<td>Genus - Tabernaemontana</td>
<td>Marathi - Anata, Tagar</td>
</tr>
<tr>
<td>Species - divaricata</td>
<td>Hindi - Chandni</td>
</tr>
</tbody>
</table>

Common name - Crepe Jasmine, Moonbeam, Carnation of India.

English - Crape Jasmine, Pin wheel flower, wax flower.

MORPHOLOGY

*Tabernaemontana* is a genus of 100-110 species of flowering plants in the family Apocynaceae. It has a pan-tropical distribution. These plants are shrubs and small trees
growing to 1-15m tall. The leaves are evergreen, opposite, 3-25cm long, with milky sap, hence it is one of the diverse plant genera commonly called ‘milk wood’. The flowers are fragrant, white, 1-5cm in diameter, found in small clusters on the stem tips. The (single) flowers have the characteristic ‘Pin Wheel’ shape. Crepe Jasmine blooms in spring, but flowers may appear sporadically all year. (Plate 1.2)
PLATE 1.2
TABERNAEMONTANA DIVARICATA
(NADIYAVATTAM)

SHRUB

FLOWER
1.6 OBJECTIVES OF THE WORK

1. To characterize the nature and degree of ocular disorders in South Tamil Nadu Population and to identify the ocular pathogens from ophthalmic infected cases. Also, testing the antibiotic resistance in the isolated.

2. To cure ocular infections by application of natural remedy using the extracts of two flowers of plants (*M. oleifera* and *T. divaricata*) that are traditionally and indigenously used by traditional healers and kani Tribes.

3. Extraction of bioactive compounds from the flowers used for eye-cure flower therapy and characterization and structural elucidation of active compounds.

4. *Invitro* testing of the efficacy of the extracts of flowers on microbial isolates from ocular infection.

5. Antioxidant potential evaluation of extracts of flowers.

1.7 FLOW CHART OF MICROBIAL ISOLATES

SCREENING OF PATIENTS VISITING EYE CARE HOSPITAL

ISOLATION OF BACTERIA AND FUNGAL AGENTS CAUSING EYE INFECTIONS-ANTI BIOGRAM ASSAY USING STANDARD ANTIBIOTICS

IDENTIFICATION OF BACTERIA AND FUNGI USING
a) Conventional Cultural Characteristics
b) Bio chemical markers
c) Molecular techniques

PARTIAL GENE SEQUENCING OF MICROBIAL ISOLATES AND DOCUMENTATION IN GEN BANK
1.8 FLOW CHART OF FLOWER EXTRACTION AND EFFICACY TESTING

Identification and Collection of Flowers
(M. oleifera and T. divaricata)

COLD EXTRACTION
5Kg petals, soaked in 10 L of solvent for 7 days
(Methanol, DCM, EA)

Filter (Whatmann No.1)

Evaporated using vacuum rotator extractor (120 rotations / minute)

CRUDE EXTRACT

FRACTIONATION
(Column Chromatography)

Phytochemical Characterization

- TLC Analysis
- U.V Spectrophotometer Analysis
- FTIR analysis
- GC-MS analysis

Biomedical Studies

- Anti bacterial Testing of Crude extract
  (Methanol, Ethyl acetate and DCM)
- Antibacterial Testing of Column fraction
  (Methanol and DCM)
- Anti fungal Assay of Crude extract
  (Methanol, Ethyl acetate, DCM)
- Anti fungal Column fraction
  (DCM and Ethyl acetate)
- Antioxidant Studies (DCM extract)
PLATE 1.3
EYE INFECTIONS

CHALAZION

CORNEAL ULCER

CONJUNCTIVITIS

UVETIS

STY

BLEPHARITIS