# Chapter 1

# Introduction and Literature review

## 1.1 The human eye:

The Human eye is hollow, fluid filled spherical structure which is about 2.5 cm in diameter which is enclosed by three layers of tissue. The outer most layer is composed of white fibrous tissue called sclera, transparent layer called cornea through which light rays enters in to the eye and limbus which is interdigitation zone of sclera and cornea. The middle layer is central vascular layer of eye globe and is composed of iris, ciliary body and choroids. The iris is coloured portion of the eye which consists of two sets of muscles with opposite actions. With the help of these muscles pupil adjust the size under neuronal control. Ciliary body is ring of tissue which encircles the lens. Choroid consist of capillary bed which supply blood to photoreceptors of retina. Retina is the innermost layer of the eye which contains neurons which are light sensitive and transmit visual signals to central targets. For light to reach to the retina, it passes through cornea, lens and two fluid environments (Purves *et al.*, 2001). There are three chambers of fluids, anterior chamber located between cornea and iris; posterior chamber located between iris, zonule fiber and lens, and vitreous chamber located between lens and retina. The anterior and posterior chambers are filled with aqueous humor and vitreous chamber is filled with more viscous vitreous humor (Kolb *et al*., 1995). Aqueous humor is produced in posterior chamber which enters in anterior chamber via pupil (Purves *et al.*, 2001). Figure 1.1 shows anatomical structure of the human eye.

The light rays that pass through retina, are absorbed by rhodopsin which is present on outer membrane of rods or by opsin which are present in cones where they generate potentials (impulses) (Kaplan, 2007). Human eyes are such that they focus on only one set of the object which is called convergence in which two eye balls move in such a way that they are directed towards the object being viewed. After formation of the image on retina, it is converted in to nerve impulse by action of rods and cones. The impulse generated by rods and cones, passes through optic nerve and then to thalamus where the fibres synapse with other neurons whose axons pass to visual areas of cerebral cortex located in occipital lobe (Goyal *et al*., 2007).

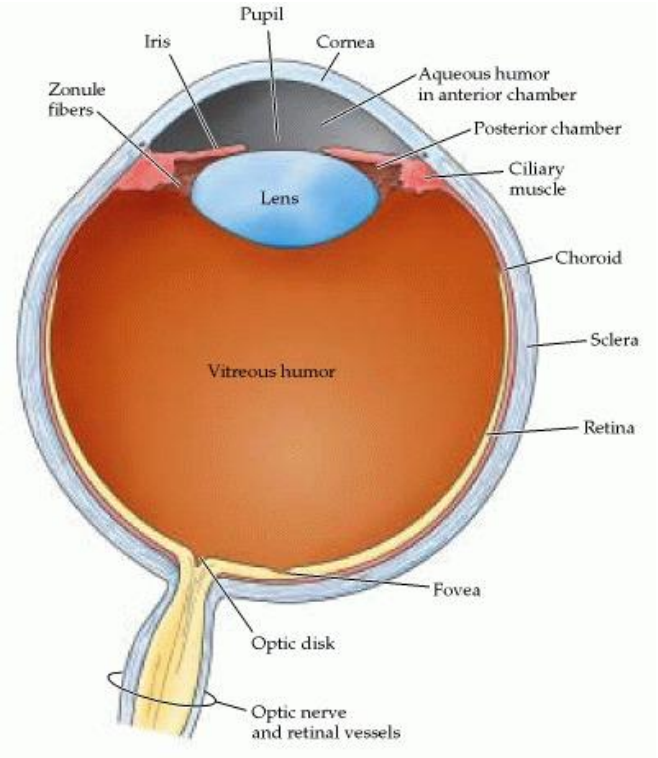



**Figure 1.1: Anatomy of human eye** (Purves *et al.*, 2001).

### 1.2 Cornea:

The cornea is outermost transparent avascular tissue of the eye which protects the structures inside of the eye and covers iris, pupil and anterior chamber. It is a structural barrier which provides protection against infections (DelMonte and Kim, 2011). Cornea contributes to two-third of the refractive power of the eye. The refractive power of the cornea is 40-45 D. 70% of total refraction is contributed by cornea. The refractive index of cornea is 1.376 (Sridhar, 2018). The cornea is horizontally oval, measuring 11–12 mm horizontally and 9–11 mm vertically. The thickness of cornea increases gradually from central region to peripheral region which is due to increased amount of collagen in the peripheral stroma (DelMonte and Kim, 2011).

The central corneal thickness in normal eyes is found to range from 551-565 μm and the peripheral corneal thickness from 612-640 μm. The corneal thickness is found to decrease with age. Anterior corneal stromal rigidity appears to be particularly important in maintaining the corneal curvature (Feizi *et al.*, 2014). Transparency, avascularity, the presence of immature resident immune cells, and immunologic privilege makes the cornea a very special tissue. The human cornea has mainly five layers. Corneal epithelial layer,

Bowman's Layer, Corneal stroma, Descemet's membrane and corneal endothelial layer (Figure 1.2).

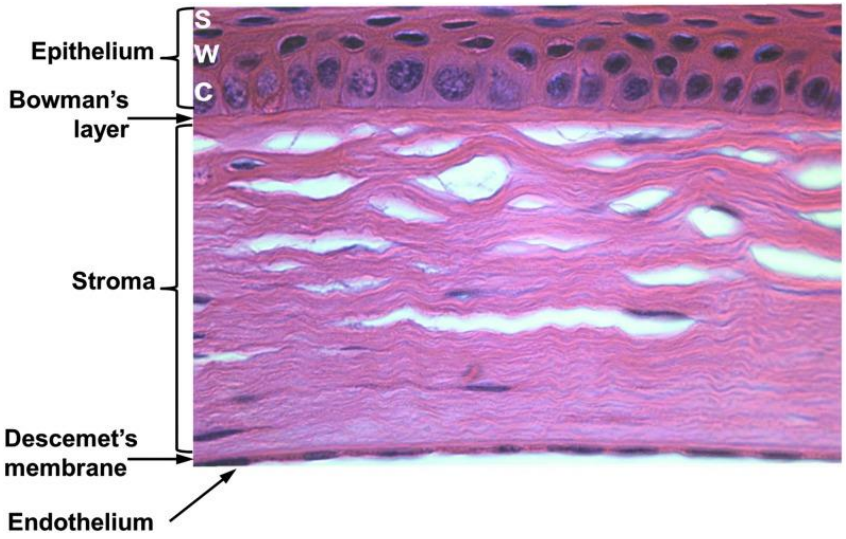



**Figure 1.2: Structure of cornea** (Griffith *et al.*, 2017).

**Epithelial Layer:** Epithelial layer is having 5-7 layers of uniformly distributed cells which provides smooth regular surface and is made up of non-keratinised stratified squamous epithelium which is about 40 μm - 50 μm in thickness. The lifespan of epithelial cells is of 7 to 10 days. The epithelium is filled with thousands of tiny nerve endings that make the cornea extremely sensitive to pain when rubbed or scratched. The part of the epithelium that serves as the foundation on which the epithelial cells anchor and organize themselves is called the basement membrane (Farjo *et al.*, 2008; DelMonte and Kim, 2011; Sridhar, 2018).

**Bowman's Layer:** Bowman's membrane 15 μm thick and is made up of Type I and Type V collagen and proteoglycans. It has no regenerative ability so upon injury it may lead to formation of scar. It helps the cornea to maintain its shape (DelMonte and Kim, 2011; Sridhar, 2018).

**Stroma:** Corneal stroma comprises approximately 80-85% of cornea's thickness. The transparency of stroma is due to proper organization of stromal fibres and extracellular matrix (ECM). It contains mainly collagen type I, Type VI and type XII (Farjo *et al.*, 2008; DelMonte and Kim, 2011; Sridhar, 2018).

**Descemet's Membrane:** The Descemet's membrane is 7 μm thick and can be increased up to 10 μm with age. It serves as a protective barrier against infection and injuries. Descemet's membrane is regenerated readily after injury (DelMonte and Kim, 2011; Sridhar, 2018).

**Endothelial Layer:** The endothelium is a single layer of cuboidal cells. The thickness is 5 μm. The cells are metabolically active. Increase in age, trauma, inflammation or any other disease affect the endothelial cell number. The remaining endothelial cells can stretch and fill the space created by degeneration of endothelial cells (DelMonte and Kim, 2011; Sridhar, 2018).

### 1.3 Keratitis:

Keratitis is the term applied for inflammation of the cornea. It is caused by invasion of the corneal epithelium, stroma and in more severe disease by invasion in endothelium and anterior chamber of the eye (Sara *et al.*, 2016). Keratitis occurs in both children and adults. Corneal infections are known to be the second most significant cause of monocular blindness rated after un-operated cataract in some developing nations in particular and in the tropics in general. WHO (World Health Organization) estimated a total of 285 million people suffering from visual impairment worldwide (Mariotti and Pascolini, 2010). Microbial Keratitis is a particular type of corneal infiltrative event (CIE) characterized by the fact that replicating microorganisms are the cause. In the early stages, pathogen may be confined primarily to the epithelium. As the disease progresses, the stroma becomes hazy and the epithelium above the infiltration begins to break down, leading to staining of the ulcer and surrounding cornea. Microbial keratitis can be 'self-limiting', whereby it develops to a certain level of severity and then subsides and resulting in mild discomfort at the peak of its development. If not treated early, microbial keratitis can be progressive and potentially devastating to the cornea. In very severe cases, the patient may suffer from a partial or complete loss of sight. The terms 'infectious keratitis' and 'microbial keratitis' are essentially synonymous. The term 'ulcerative keratitis' has also been used as a synonym for microbial keratitis (Efron, 2018). Emphasizing the importance of corneal ulceration as an important cause of visual loss, many studies have reported the prevalence of microbial pathogens and identified the risk factors predisposing

a population to corneal infection in India and abroad (Bharathi *et al.*, 2003). Microbial keratitis is a common, potentially vision-threatening ocular infection that may be caused by bacteria, fungi, viruses or parasites. Symptoms are tearing**,** pain**,** infiltration in the cornea, hypopyone, sensitivity to light**,** inflammation of the eyelid**,** decrease in vision**,** redness, watery eye, difficulty in keeping eyelids open, foreign body sensation in eye and increased lacrimation.

**Risk factors:**

- Poor contact lens care; overuse of contact lenses.
- Illnesses or other factors that reduce the body's ability to overcome infection.
- Cold sores, genital herpes and other viral infections.
- Crowded, dirty living conditions; poor hygiene.
- Dry eye

### 1.3.1 Bacterial Keratitis:

It can result from infection from contact lens use or from injury to the eye. Bacterial keratitis can have a rapid and devastating infection. The initial infection symptoms includes, formation of milky white infiltrate. The infection gets worsen upon the formation of a creamy, pussy ulcer, hypopyon and iritis. Initial symptons will appear in 4 to 6 hours. A serous or mucopurulent discharge could be observed. If not treated properly, the stroma can melt and lead to perforation of corneal. If such condition is not treated then it can cause blindness (Efron, 2018). Bacteria that cause keratitis are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* (including *Klebsiella, enterobacter, Serratia* and *Proteus*).

### 1.3.2 Viral Keratitis:

This infection generally begins with inflammation of the membrane lining the eyelid (conjunctiva) and the portion of the eyeball that comes into contact with it. It usually occurs in one eye. Viruses that causes keratitis are *Adenovirus*, *Herpes simplex type 1*and *Varicella zoster.*

### 1.3.3 *Acanthamoeba* Keratitis:

Amoebic protozoan keratitis is not developed as fast as rapid as bacterial keratitis; typical signs include corneal staining, pseudodendrites, epithelial and anterior stromal infiltrates which may be focal or diffuse, and a classic radial keratoneuritis. A fully developed corneal ulcer may take weeks to form.

### 1.3.4 Fungal Keratitis:

Fungal keratitis also seems to develop over a more prolonged time course. Vision can be deteriorated over a period of days. Fungal infiltrate can cause complete loss of the overlying epithelium. Upon further progression of infection, the infiltrate would appear fluffy. Fungi that cause keratitis: *Aspergillus*: *A. flavus*, *A. niger*, *A. fumigatus*; *Fusarium*: *F. solani*, *F. verticillioides*, *F. oxysporum* (*F. moliniformae*), *F. polyphialidicum*; *Candida*: *C. albicans*, *C. lunata*, *Sagenomella keratitidis*, *Sarcopodium oculorum*, *Dematiatous spp*. *Curvularia spp*., *Alternaria*, *Papulaspora* etc.

Keratitis due to filamentous fungi is reported worldwide including Asia, Africa, America and European countries (Bharathi *et al.*, 2003; Ritterband *et al.*, 2006; Xie *et al.*, 2006; Tuft and Tullo, 2009). The principal routes of inoculation are scratch and wound caused by either mechanical injury or surgery, and introduction through an epithelial defect. The fungus grows slowly in the cornea and proliferates to involve the anterior and posterior stromal layers. The fungus can break through the descemet's membrane and pass into the anterior chamber. The patient presents with fungal keratitis, a few days or weeks later. Some of the possible causes of keratitis due to fungi are the frequent use of topical corticosteroids, overuse of antibacterial agents, and the use of contact lenses. The most common risk factor for keratitis is ocular trauma through vegetative or any other material (Rosa *et al.*, 1994; Gopinathan *et al.*, 2002; Bharathi *et al.*, 2003). Other predisposing factors for mycotic keratitis can be systemic diseases such as diabetes mellitus, preexisting ocular diseases and use of contact lenses (Srinivasan *et al.*, 1997; Mselle, 1999; Choi *et al.*, 2001; Ahearn *et al.*, 2008).

Mycotic keratitis may have severe complications, especially in individuals who wear contact lenses or work in outdoor fields. Among severe infective

corneal ulcers, fungal keratitis is most common in many developing countries (Upadhyay *et al.*, 1991; Hagan *et al.*, 1995; Leck *et al.*, 2002; Chowdhary and Singh, 2005). Fungal corneal infections are the major cause of ocular morbidity throughout the world and are most prevalent (Whitcher *et al.*, 2001) and the incidences had been increased significantly over the last two-three decades (Rosa Jr *et al.*, 1994; Prajna *et al.*, 2017; Jain *et al.*, 2018; Kuo *et al.*, 2019). Fungi causing keratitis have gained importance in Asian countries and contribute nearly half of the world's fungal keratitis cases (Ansari *et al.*, 2013; Thomas and Kaliamurthy, 2013). Fungal keratitis is significantly more common in tropical and subtropical regions (Paty *et al.*, 2018; Jain *et al.*, 2018; Kuo *et al.*, 2019). Fungal organisms are the etiologic agents in 1.2% in New York (Ritterband *et al.*, 2006) and 62% in North China (Xie *et al.*, 2006).

Fungal keratitis is a refractory and potentially blinding fungal infection, with corneal ulceration and suppurative infection. Fungal Keratitis is associated with risk factors like trauma, ocular surface disease like dry eyes, systemic disease such as diabetes mellitus, improper usage of contact lenses, use of topical corticosteroids, prolonged use of antibiotics, Ocular surgery or transplantation (Toshida *et al.*, 2007; Thomas and Kaliamurthy, 2013; Acharya *et al.*, 2017; Venkatesh *et al.*, 2018). Fungal keratitis is an insidious, rapidly progressive disease that is difficult to diagnose, and it can be resistant to treatment (Al-Hatmi *et al.*, 2016; Sara *et al.*, 2016). Sometimes it can lead to blindness or loss of the affected eye (Acharya *et al.*, 2017).

#### 1.3.4.1 Epidemiology:

The epidemiology of fungal keratitis varies at different geographical locations. The outcome of fungal keratitis is poor due to delay in diagnosis and difficulty in treatment. Fungal keratitis is a common cause of corneal ulcers in developing nations, accounting for 59.09 % of corneal ulcers in India (Mohod *et al.*, 2019), 32.9% in Bangladesh (Ahmed *et al.*, 2010; Gugnani *et al.*, 2017), 51.7% in Ghana (Gyanfosu *et al.*, 2016), 78% in London (Ong *et al.*, 2016) and 78.1% in Nepal (Puri and Shrestha, 2015). Prevalence varies by region and is highest in South India (97.8%) (Manikandan *et al.*, 2019) but is also common in West India (35.9%) (Gajjar *et al*., 2013) and East India (26.4%)

(Rautaraya *et al.*, 2011). *Fusarium* and *Aspergillus* species are the most common isolates. Males are affected more commonly than females (Rautaraya *et al.*, 2011; Gajjar *et al.*, 2013; Manikandan *et al.*, 2019). Many studies report the prevalence of *Candida* spp., followed by *Aspergillus* spp. and *Fusarium* spp. (Rondeau *et al.*, 2002). However, there is a shift in this prevalence in recent studies where *Fusarium* is now reported as the most common fungal pathogen for keratitis (Ranjini and Waddepally, 2016; Verma *et al.*, 2016). A 5-Year Retrospective Review of Fungal Keratitis in Malaysia showed that that the incidence of fungal keratitis has increased each year from 2007 to 2011 by 12.50%, 17.65%, 21.21%, 26.83%, and 28.57%, respectively. *Fusarium* species were the most common fungal isolated, followed by *Candida* species (Mohd-Tahir *et al.*, 2012). In Tehran, prevalence of fungal keratitis was studied and *Fusarium* spp. accounts for 49.59% of cases and *Aspergillus* spp. accounts for 26.44% of cases. (Ebadollahi-Natanzi *et al.*, 2016).

*Fusarium* spp. have emerged as one of the leading causes of human keratomycosis (Van Diepeningen and de Hoog, 2016; Kulkarni *et al.*, 2017; Boral *et al.*, 2018) and are along with *Aspergillus* and *Candida* spp., among the most common fungal corneal isolates in the southern United States, South America, the Middle East, sub-Saharan Africa, India, and southeast Asia (Mayayo *et al.*, 1999; Gupta *et al.*, 2000; Leck *et al.*, 2002; Thomas, 2003).

In India, high incidence of fungal keratitis is reported from every corner of the country (Tewari *et al.*, 2012; Manikandan *et al.*, 2013). In West Bengal, 289 patients of microbial keratitis were studied and there were 110 (38.06%) patients diagnosed with fungal keratitis. The predominant fungal species isolated was *Aspergillus* spp. (55.40%) followed by *C. albicans* 14 cases (18.91%) and *Fusarium* spp. in 8 cases (10.81%) (Saha *et al.*, 2009). Agricultural activity related ocular trauma was the principal cause of mycotic keratitis and males were more commonly affected. In Kerala, out of 1503 patients, 224 (69.78%) were of fungal keratitis. The most common fungal pathogen isolated was *Fusarium* spp. (37.05%) followed by *Aspergillus* spp. (26.34%) (Geethakumari *et al.*, 2011). *Fusarium* species have been found to be the principal pathogen in other studies from South India. In Gujarat,

microbiological profile of infective keratitis was studied. Out of 150 patients 31 patients had fungal infection. *Aspergillus* species was found to be most common (35.4%), followed by *Fusarium* species (22.5%) (Tewari *et al.*, 2012). In one more study on keratitis in Gujarat, *Fusarium* spp. was most common (26.6%) followed by *Aspergillus* spp. (21.6%) and Dematiaceous spp. (11.6%) (Gajjar *et al.*, 2013). *Fusarium* species are implicated in fungal keratitis cases primarily from South India, while *Aspergillus* species are reported from other parts of the country (Ghosh *et al.*, 2016).

**1.3.4.2 Risk factors:**

In developed countries the common risk factor is pre-existing ocular disease and contact lens usage while in developing countries the most common pre disposing factor is trauma (Gopinathan *et al.*, 2002; Ahmed *et al.*, 2010). Often ocular injury is vegetative in nature which includes trauma with plant twigs, cotton plant and rice husk. Trauma causes destruction of epithelial layer as well as bowman's layer of cornea and leads to formation of excessively hydrated stromal layer which leads to fungal infection by forming most suitable environment for their growth. Sometimes ocular trauma can be because of insects. (Ahmed *et al.*, 2010; Kulkarni *et al.*, 2017) has reported case where patient has suffered from fungal keratitis because of trauma caused by insects. Several reports are there which document major risk factor for keratitis is trauma. The percent of patient affected with keratitis because of ocular trauma varies depending upon geographical location, environmental condition as well as occupation of patient and their socio-economic status. Trauma as common predisposing factor has been reported in 82.08 % cases (Venkatesh *et al.*, 2018), 61.6 % cases (Cheikhrouhou *et al.*, 2014), 25.5 % cases (Das *et al.*, 2015), 89.36 % cases (Roy *et al.*, 2017), 65.21 % cases (Verma *et al.*, 2016), 46 % cases (Ranjini and Waddepally, 2016), 78 % cases (Reddy *et al.*, 2017), 72 % cases (Chidambaram *et al.*, 2018), 37 % cases (Pereira and Foschini, 2018). *Aspergillus*, *Fusarium* and *Candida* are mainly associated with reported ocular trauma cases of keratitis where *Fusarium* is most prevalent pathogen.

Keratitis can occur to person of any age but most common age group found to be 41- 60 years followed by 21- 40 years and 0-20 years. There are several cases where range is very wide but average or mean age also fall in 40-60 years age group. Males are majorly affected then females. People who are engaged in agricultural activity, labourer activity and any other outdoor activity are affected the most. The workers do not use eye protection while working in the field which might lead to eye damage and followed by keratitis (Das and Konar, 2013; Cheikhrouhou *et al.*, 2014; Das *et al.*, 2015; Ranjini and Waddepally, 2016; Verma *et al.*, 2016; Chidambaram *et al.*, 2018; Pereira *et al*., 2019). There are reports where mean age fall in age group of 21- 40 years (Reddy *et al.*, 2017; Roy *et al.*, 2017). Aruljyothi and group has reported keratitis incidences in children where they found that 53.4 % children were affected by ocular trauma as pre disposing factor and mean age was 9.3 years and age of children fall in range of 8 days to 16 years (Aruljyothi *et al.*, 2016).

Excessive or prolonged use of antibiotics and corticosteroids make eye susceptible for infection. Steroids are used widely for inflammatory disease but excessive usage causes reduced host defence and provide suitable environment for fungal inoculation and growth. It might disturb the normal microbial flora of eye and provide opportunity to saprophytic fungi to cause disease and become pathogenic. Systemic disease like diabetes mellitus, chronic granulomatous disease, ocular surgery, organ transplantation, chemotherapy, AIDS and many other diseases leads to severe impairment of host defence and opportunity for fungal keratitis. In case of diabetes there is high level of glucose in body which provides suitable growth medium for microbial growth (Bharathi *et al.*, 2003; Dóczi *et al.*, 2004; Acharya *et al.*, 2017).

Use of contact lens has been increases there days in general community which is also leading cause of fungal keratitis. Factors associated with contact lens induced fungal keratitis includes usage of contact lenses during sleep, smoking history, unhygienic contact lens behaviour. There are higher chances of adherence of microbes to cornea because of contact lens and also sometimes it leads to formation of biofilms. Hypoxia and hypercapnia are pathogenic

changes associated with contact lens induced microbial keratitis (Liesegang, 1997a; Liesegang, 1997b; Acharya *et al.*, 2017).

### 1.4 *Fusarium*:

*Fusarium* spp. are important plant pathogens causing various diseases such as crown rot, head blight, and scab on cereal grains. *Fusarium* is filamentous fungi present abundantly in soil, decaying plant material, water and organic matter. It has ability to grow in different environmental condition and on wide range of substrates (Tupaki-Sreepurna and Kindo, 2018). The widespread distribution of *Fusarium* spp. may be attributed to their ability to grow on a wide range of substrates and their efficient mechanisms for dispersal. They may occasionally cause infection in animals (Evans *et al.*, 2004). *Fusarium* spp., rarely cause disease but are considered emerging pathogens. *Fusarium* spp. have become one of the important causes of mold infections in humans in whom it is second only to *Aspergillus* spp.

Infection caused by *Fusarium* spp. fall in wide range, including superficial infection like keratitis and onchomycosis; locally invasive infections like cellulitis and sinusitis; and disseminated infection. Superficial and locally invasive infections occur mainly in immunocompetent patients while disseminated infections occur mainly in immunocompromised patients (Nelson *et al.*, 1994; Gupta *et al.*, 2000; Nucci and Anaissie, 2007). Other infections caused by *Fusarium* spp. includes, cutaneous infections (Nucci and Anaissie, 2002), catheter associated fungemia (Velasco *et al.*, 1995), osteomyelitis, brain abscess, otitis and septic arthritis (Dignani and Anaissie, 2004). *Fusarium* infection are increasingly occurring in patients with haematological malignancies, haematopoietic stem cell transplantation (HSCT), neutropenia and diabetes (Tupaki-Sreepurna and Kindo, 2018). Fusarial infection has a tendency for seasonal variation. In some countries the infection is most prevalent in autumn, e.g. France and in some other countries during summer, e.g. Texas, Italy, Israel. Several species of *Fusarium* have been identified and majority of them are plant and animal pathogen but only few are human pathogen (Nucci and Anaissie, 2007).

In recent years, there has been an increasing number of reports of human infection due to *Fusarium* species, mostly involving immunocompromised hosts. *Fusarium* keratitis is a serious ocular disorder that may lead to vision loss. Because of its risk of permanent loss of vision, corneal infections are considered an ophthalmologic emergency (Tupaki-Sreepurna *et al.*, 2017). Historically, the genus *Fusarium* has developed into a large genus with multiple species complexes, some of which can cause opportunistic infections in humans. Especially members of the *Fusarium solani, F. oxysporum, F. fujikuroi, F. dimerum, F. chlamydosporum,* and *F. incarnatum-equiseti* species complexes have been involved in human and animal infections. (Nucci and Anaissie, 2007) has reported that among the *Fusarium* spp. associated with infections, *F. solani* was the most frequent followed by *F. oxysporum, F. verticilloidis* and *F. moliniforme*. Other infecting species included *F. dimerum, F. proliferatum, F. sacchari, F. nygamai, F. napiforme, F. antophilum,* and *F. vasinfectum.* (Dóczi *et al.*, 2004) has also reported *F. solani* as most frequent pathogen causing *Fusarium* keratitis. In immunocompetent murine model of fusariosis, 13 *Fusarium* isolates were tested for their virulence and it was found that five strains of *F. solani* cause death of animals while the mouse infected with *F. oxysporum*, *F. verticillioides* and *F. proliferatum* survived. Thus, *F. solani* was considered as most virulent spp. (Mayayo *et al.*, 1999). *Fusarium* species are among the most resistant fungi; infections are commonly refractory against treatment with the most known systemic and conventional antifungal agents (Guarro, 2013; Van Diepeningen *et al.*, 2014). Estimated mortality rates of 50–75% in disseminated infections may arise, especially in immunosuppressed patients (Muhammed *et al.,* 2013). *Fusarium* pathogens typically show broad *in vitro* resistance to antifungal agents with a high variability being present within each species (Al-Hatmi *et al.,* 2014; Araujo *et al.*, 2015; Taj-Aldeen *et al.,* 2016). In vitro susceptibility testing may represent a tool for the selection of an appropriate therapy. In general, members of the *Fusarium solani* species complex (FSSC) are most commonly observed in all clinical infections and show the highest minimum inhibitory concentrations (MICs) against various antifungal drugs (Taj-Aldeen, 2017).

### 1.5 Virulence factor:

Virulence factors are molecules expressed and secreted by pathogens (bacteria, viruses, fungi, protozoa) that enable them to achieve the colonization of niche in to the host, immunoevasion (evasion of host's immune response), immunosupression (inhibition of host's immune response), entry in to and exit out of the cells and to obtain nutrition from the host. They are determinants of pathogenicity. Pathogenic microbes often possess number of virulence factors and mechanisms. These factors determine whether the organism (host) lives or dies during host-microbe interactions. The damage-response framework defines a virulence factor as a microbial component that damages the host (Casadevall and Pirofski, 2001).

Pathogenic fungi retain several factors which allow their growth in adverse conditions provided by the host and contribute to disease development. Virulence factors are of great interest in microbial pathogenesis because they are often the target of the immune response and the responses that neutralize the action of virulence factors are often protective. The fungal pathogenesis is enabled by the expression of different virulence factors. In response to host defence mechanisms, fungi has evolved several virulence elements which includes host recognition by cell surface adhesins and morphogenetic switch from unicellular to hyphae form. The virulence factors play role in disease development on to the host by acting as toxins or by over stimulation of host defence (Hogan *et al.*, 1996; Van Burik and Magee, 2001; Santos, 2011). Secreted proteases (Rodríguez *et al.*, 2017; Costa *et al.*, 2019), lipases (Voigt *et al.*, 2005), phospholipases (Barman *et al.*, 2018), pigments, mycotoxins (Pitt and Miller, 2017) are reported as virulence factors of pathogenic *Fusarium* spp.

#### 1.5.1 Protease:

During infection, fungi secrete degradative enzymes like protease, collagenases which promote its invasion into the host and are also required to obtain nutrient from the host (Gauthier and Keller, 2013). Fungi produce several types of secreted and surface-bound proteases (Santos, 2011). The

proteases cleave the peptide bonds in protein and polypeptides and generate free amino acids (Monod *et al.*, 2002).

#### 1.5.1.1 Classification of Proteases (Figure 1.3):

a. Based on pH: Proteases are active at specific pH and can be classified as acidic, neutral and alkaline proteases (Monod *et al*., 2002; Santos, 2011).
b. Based on site of action: Proteases can be classified based on site of action as endopeptidase and exopeptidase. Endopeptidase hydrolyse peptide bond internally within polypeptide chain. Exopeptidase cleaves peptide bond at N & C terminus of polypeptide chain and are further classified in to aminopeptidases and carboxypeptidases. Both of these proteases cooperate very efficiently in protein digestion, in which the main function of the endoproteases is to produce a large number of free ends on which the exoprotease may act (Monod *et al*., 2002; Santos, 2011; Menon and Rao, 2012).
c. Based upon functional group present at active site: Serine protease, metallo protease, cysteine protease and aspartic protease. There are two newly established families: glutamic acid protease & threonine protease. There are a few miscellaneous proteases which do not fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Dash *et al*., 2003; Monod *et al*., 2002; Santos, 2011; Menon and Rao, 2012).

#### 1.5.1.2 Serine protease:

Serine proteases contain a serine group in their active site, which is essential for substrate binding and cleavage. Serine proteases are distinguished by their broad substrate specificity and have peptidase, esterase and amidase activities. Serine proteases are of various types like exoprotease, endoprotease, omega protease and oligoprotease. Chymotrypsins, subtilisins, carboxyprotease C and Escherichia D-Ala-D-Ala protease A are important serine proteases. Serine proteases have a conserved glycine-containing peptide, Gly-Xaa-Ser-Yaa-Gly. The serine proteases secreted by pathogenic fungi belong to the subtilisin subfamily (S8A) and are inhibited by phenylmethylsulfonyl fluoride (PMSF),

antipain and chymostatin. However, some fungal proteases are not inhibited by PMSF. Optimum pH of serine proteases fall in range 7-11 and isoelectric pH in range 4–6. (Menon and Rao, 2012; Monod *et al*., 2002**).**

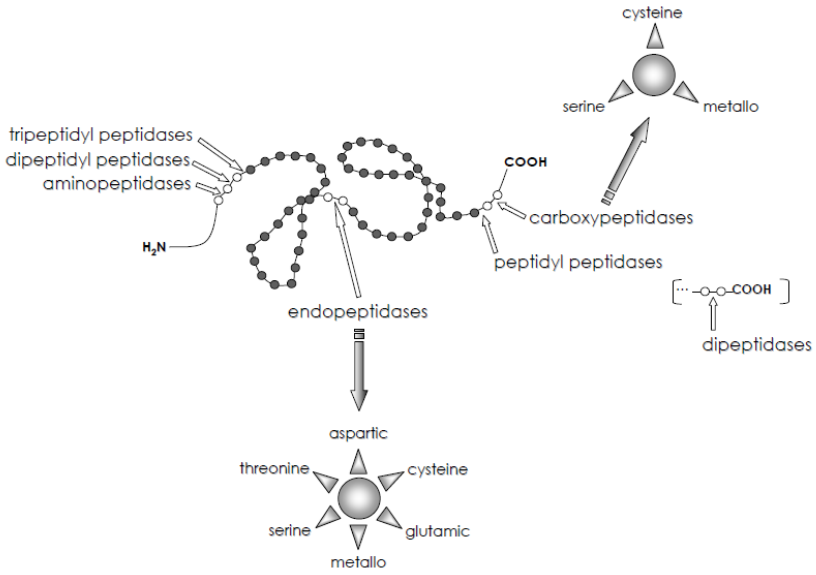



**Figure 1.3: Classification of proteolytic enzymes.** Open circles represent amino acids and grey circles indicate the amino acid sequence that is bound to the protease. White arrows point to the site of cleavage. The grey arrows indicate the classes of endopeptidases and carboxypeptidases according to the chemical group present in their catalytic sites (Santos, 2011).

### 1.5.1.3 Metalloproteases:

These enzymes require divalent metal ion for activity. Metalloproteases are a very distinctive group of proteases, which include both endoproteases and exoproteases. The metalloproteases are inhibited by ethylenediaminetetraacetic acid (EDTA) but not by sulfhydryl agents. Motif His-Glu-Xaa-Xaa-His is present in the metal-binding site. Fungal Metallo-endoproteases fungi belong to two families, the deuterolysins (M35) and the fungalysins (M36). (Menon and Rao, 2012; Monod *et al*., 2002).

### 1.5.1.4 Aspartic proteases:

These enzymes require aspartic acid amino acid for their catalytic activity. They have been grouped into different families, pepsin (A1), retropepsin (A2) and enzymes from pararetroviruses (A3). The members of families, pepsin and

retropepsin are related to each other. The aspartic proteases secreted by fungi are generally similar to pepsin or belong to another family which contains only fungal secreted enzymes (A4). The peptidases of the pepsin family are totally inhibited by pepstatin, whereas the enzymes of the A4 family to which the *A. niger* PepB belongs, are insensitive to this inhibitor. (Menon and Rao, 2012; Monod *et al*., 2002).

#### 1.5.1.5 Synthesis and secretion of proteases:

Proteases are synthesised and secreted in many ways. The prepeptide or signal peptide is required for entering the secretory pathway and are transported across the endoplasmic reticulum (Pfeffer and Rothman, 1987). Once the protein folding has been completed, the propeptide is cleaved and removed to create an active form of enzyme through autoproteolytic reaction. In *Candida* spp., Kex2 membrane bound protease cleaves peptides to generate secreted aspartic proteases (Sap) (Newport and Agabian, 1997). The secretory exopeptidase of pathogenic fungi have no propeptide but have signal peptide only. However, there are exceptions to this rule, secretory carboxypepptidase of *A. niger* have preproproteins (Monod *et al.*, 2002).

#### 1.5.1.6 *Aspergillus* proteases:

*A. fumigatus* secrete aspergillopepsin A which is able to hydrolyse structural protein components of basement membrane, elastin, collagen and laminin. The optimum pH of this protein is pH 5.0 and is inhibited by pepstatin A, DAN (diazoacetyl-norleucine methyl ester) and EPNP (1,2-epoxy-3-(nitrophenoxy propane) (Lee and Kolattukudy, 1995). Expression analysis of Cts D protease which is secretory aspartic protrease from *A. fumigatus* was carried out in an *in vitro* in nutrient rich media and in an *in vivo Galleria mellonella* insect model. The gene expression was not detected in an *in vitro* condition but was expressed in an *in vivo* infection model of *A. fumigatus*. This indicates that certain genes and proteases were activated and expressed at time of infection (Vickers *et al.*, 2007). Alp1 from *A. fumigatus* is alkaline protease and is able to cleave complement component C3, C4, C5 and Cq1 and Alp1 mutant strain showed reduced degradation activity. However, Alp1 deletion mutant strain

did not show reduced virulence compared to wild type strain in murine model of pulmonary infection (Behnsen *et al.*, 2010).

#### 1.5.1.7 *Candida* proteases:

The Sap family of proteases secreted by *C. albicans* are very important virulence factors which makes it more virulent and more adapted than other *Candida* spp. in overcoming host defensive barriers and helps in development of mucosal candidiasis and deep mycoses. In mice model of infection the *C. albican* single mutant strain of *SAP1*, *SAP2*, *SAP3* as well as triple mutant strain of *SAP4*, *SAP5* and *SAP6*, showed attenuated virulence compared to wild type strain which indicate their importance in progression of infection (Hube *et al.*, 1997; Sanglard *et al.*, 1997). In rat model of *C. vaginitis*, deletion mutant strain of either *SAP1*, *SAP2* or *SAP3* showed less virulence while mutant strain of *SAP4-SAP6* did not exhibit detectable virulence defects (De Bernardis *et al.*, 1999). *C. glabrata* produces yepsin family of proteases which are required for survival in cell wall stress condition, for survival in macrophage, for adherence to mammalian cells as well as for virulence in mouse model of disseminated candidiasis (Kaur *et al.*, 2007).

#### 1.5.1.8 *Fusarium* proteases:

There are several reports of protease production and characterization from *Fusarium* spp. pathogenic to plants and animals. A 45 Kilodalton (KDa) metallopeptidase with optimum pH of 7.2 has been characterized from *F. moliniforme* (Rodier *et al.*, 1997). Kolaczkowska and group has reported purification and characterization of 38 Kda aspartic protease from *F. moliniforme* culture filtrate with optimum pH of 3.2 (Kolaczkowska *et al.*, 1983). Destruction of collagen bundles by *F. moliniforme* culture filtrate in rabbit model has been reported (Dudley and Chick, 1964). 41 Kda trypsin like alkaline serine proteases has been characterized in *F. oxysporum* with optimum pH of 8.0 (Barata *et al.*, 2002). 20kD protease has been purified and showed optimum pH of 8.0 and characterised as serine protease. Also there are reports of *F. solani* proteases production with optimum activity at pH 8.0 & pH 9.0 (Bhuvaneswari and Balasubramanian, 1999; Rodarte *et al.*, 2011; Al-Askar *et al.*, 2014). To study the mode of invasion of *F. solani* in to the

corneal stroma, electron microscopy studies were carried out in rabbit model of keratitis. When fungal hyphae penetrated in to the corneal stroma, irregularities were observed into collagen fibril arrangements. Collagen fibres were dissolved by hyphae and vacuoles were observed in stroma. This degraded collagen was utilised by fungi for nutrition (Kiryu *et al*., 1991). Protease characterization from *A. flavus, F. solani* was carried out in an *in vivo* rabbit model of keratitis. Researchers found out that 200kD, 92kD and 58kD gelatinase were found in infected cornea with both *A. flavus* and *F. solani*. Also, 65kD protease was found in all infected and non-infected eyes. These gelatinases showed inhibition with EDTA and were considered as Metalloproteases (Gopinathan *et al*., 2001; Matsubara *et al*., 1991).

**1.5.1.9 Host proteases:**

Apart from the proteases produced by pathogen, there are several host proteases which are also involved in pathogenesis. One of these proteases are matrix metalloproteases (MMP). MMP are Zinc-dependent enzymes which degrade extracellular matrix and proteins involved in signalling pathway (Pardo *et al.*, 2016; Paumier and Thinakaran, 2019). MMPs are also responsible for cleavage and activation of growth factors, chemokines, cytokines and cell surface receptors and affect cell functions (Pardo *et al.*, 2016).

Role of MMPs in keratitis has been studied well. It has been reported that MMP2 and MMP9 are present in cornea in an inactive, proenzyme form. Upon infection, these MMPs becomes active in the area of ulcer and necrosis which leads to polymorphonuclear (PMN) cell infiltration and cause tissue destruction (Gopinathan *et al.*, 2001; Yang *et al.*, 2003). Human neutrophils are also reported for MMP production which hydrolyse gelatine and collagen (Hibbs *et al.*, 1985). MMP-2 is produced by corneal ketarocytes and MMP-9 is produced by lacrimal gland, corneal and conjuctival epithelium, which are involved in ulcerative keratitis (Smith *et al.*, 1999). MMP-1 and MMP-8 are necessary for type 1 fibrillar collagen and MMP-2 and MMP-9 degrade type IV collagen of basement membrane (Smith *et al.*, 2001). Also, MMP-8 cause collagen breakdown in corneal stroma and generate chemotactic peptides which leads to migration of neutrophils through cornea (Lin *et al.*, 2008).

Overexpression of MMP is related to corneal ulceration (Brejchova *et al.*, 2009). In *F. solani* keratitis, expression of MMP-3, MMP-8 and MMP-9 was increased during mid-phase of infection while in later stage expression of MMP-2 and MMP-7 was increased (Li *et al.*, 2016). Kalkanci and group has also reported expression of MMP-9 and MMP-13 in *Fusarium* and *Candida* keratitis (Kalkanci *et al.*, 2018). Increased expression of MMP-2, MMP-8 and MMP-9 along with increased infiltration of PMNs in cornea during fungal Keratitis leads to tissue degeneration (Mitchell *et al.*, 2007; Rohini *et al.*, 2007; Ruban *et al.*, 2018). Similary in *C. albicans* keratitis, increased expression of MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13 and MMP-19 has been reported (Yuan *et al.*, 2009).In HSV-1 keratitis, expression of MMP-1 and MMP-9 increased which increased development of keratitis (Ke *et al.*, 2019).

MMP plays role in several other disorders. In Alzheimer's disease, MT1 and MT5-MMP stimulate production of amyloid β in alzimer's disease (Baranger *et al.*, 2017; Paumier *et al.*, 2018). In ML-1 thyroid cancer, sphinosine-1 Phosphate lipid induces activity of MMP-2 and MMP-9 (Kalhori and Törnquist, 2015). In lung carcinoma, increased expression of MMP-7 leads to increased metastasis (Han *et al.*, 2015). In breast cancer, MMP-9 promotes tumour invasion and metastasis (Mon *et al.*, 2017). Severity of Huntington's disease is increased with increased level of MMP-9 and MMP-2 (Connolly *et al.*, 2016; Naphade *et al.*, 2018). In murine model of pulmonary fibrosis (PF), MMPs promotes PF development by reducing antifibrotic mediator, increasing epithelial cell migration and interference with repair process (Craig *et al.*, 2015).

### 1.5.2 Fungal cell wall:

Fungal cell wall (Figure 1.4) is most important for fungal virulence and pathogenicity. The fungal cell wall enable fungus with adhesion properties which are pivotal for invasion of host tissue and for protection from host defence mechanism (Free, 2013). The fungal cell wall is polysaccharide-rich, which envelopes the fungal cell & is a dynamic organelle that functions in several important processes. The cell wall maintains the viability of fungal cells, protecting the cell against external injuries & aggressors such as

environmental fluxes (Bowman and Free, 2006; Arana *et al.*, 2009). It the outermost layer of pathogen and hence it contains several antigenic determinants which are important and required for adhesion to the host tissue. These antigenic determinants also helps the pathogen to colonise on host surface and ultimately leads to diseased condition. Adhesins like Ala1 and Ala5, Epa1 of *C. albicans* and *C. glabrata* help the fungus to colonise on host surface (Sundstrom, 2002; Arana *et al.*, 2009). The mechanical strength is provided to pathogen by its cell wall which allows the cell to hold out against the changes in osmotic pressure inflicted by environment (Bowman and Free, 2006; Geoghegan *et al.*, 2017). It is very important for pathogenic fungi to maintain cell wall elasticity at requisite level which is important for cell growth, cell division, for formation of various cell types during fungal life cycle (Sundstrom, 2002; Bowman and Free, 2006; Geoghegan *et al.*, 2017). Fungal cell walls are multi-layered structure comprised of glycoproteins and polysaccharides which are chitin, chitosan, mannans, galactomannan and glucan. The glucan present in the fungal cell wall is of various types which includes, β-1,3-glucan, β-1,3-1,4-glucan, β-1,3-1,6-glucan, β-1,6-glucan and α-1,3-glucans (Free, 2013). The glucan content in the fungal cell walls account for approximately 50–60% of the total cell wall (Arana *et al.*, 2009). Chitin, a β -1,4 *N*-acetylglucosamine polymer comprise in 1–2% of fungal cell wall but is very important for it. Mannan (also called phosphopeptidomannan) is composed of mannoproteins and accounts for about 35-40% of fungal cell wall (Bowman and Free, 2006; Arana *et al.*, 2009).

The β-1,3-glucan and chitin are mainly responsible for providing the strength to fungal cell wall and appear as a dense inner layer by transmission electron microscopy (Arana *et al*., 2009). As the amount of β-1,3-glucan is higher in fungal cell wall, it works as the main structural component and building block to which other cell wall components attaches covalently. Hence, β-1,3-glucan synthesis is pivotal for fungal cell and also it is require for appropriate cell wall formation and for normal fungal development (Bowman and Free, 2006). β-1,3-Glucan is well characterised fungal cell wall constituent. In the *S. cerevisiae* cell wall, β-1,3-Glucan is present as a branched polymer and branches are attached to the core polymer by b-1,6-branches (Klis *et al.*, 2006;

Free, 2013). Polymers of glucan are composed of repeating units of glucose which are assembled into chains through different chemical linkages. 65% and 90% of the cell wall glucan is found to be beta-1,3-glucan (Bowman and Free, 2006). Chemical analysis of β-1,3-Glucan has been carried out by several reporters and it was found that β-1,3-Glucan in solution can exist as single stranded helics and able to form triple stranded helical structure as well. It provides elasticity and tensile strength to fungal cell wall by functioning as coiled spring. Thus, disruption of fungal cell wall has extreme consequences on fungal growth and morphology and leads to cell lysis and death (Klis *et al.*, 2006; Free, 2013; Geoghegan *et al.*, 2017).

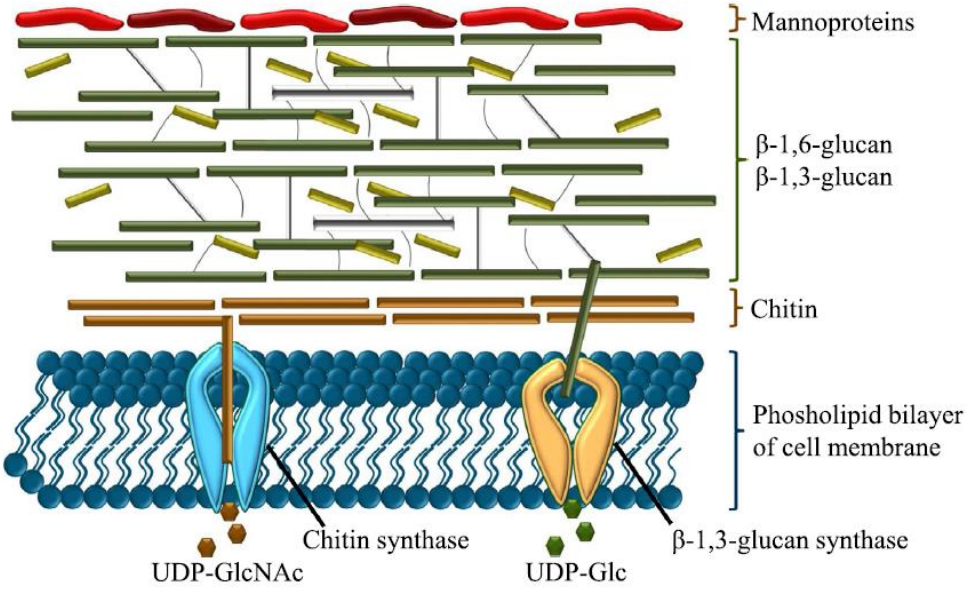



**Figure 1.4: Schematic overview of fungal cell wall composition** (Fesel and Zuccaro, 2016).

### 1.5.2.1 β-1,3-Glucan synthase:

Polymers of β-1,3-Glucan are generated by enzyme complexes associated with the plasma membrane and extruded into the extracellular space by means of vectorial synthesis.(Douglas, 2001; Shematek *et al.*, 1980; Manners *et al.*, 1973). The main enzyme responsible for β-1,3-Glucan synthesis is β-1,3-Glucan synthase. It has several transmembrane domain. Cytoplasmic UDP-Glucose residues are taken up as a substrate by enzyme and added in to chain one after another to the non-reducing end and leads to formation of a long

linear growing chain of β-1,3-Glucan polymer (Frost *et al.*, 1994) which is composed of approximately 1500 glucose residues and linked via β linkage to each other, after approximately 40-50 glucose residues in the chain, at C-6 position another glucan polymer chain is being attached via 1,6 β- linkage and leads to formation of branched structure (Bowman and Free, 2006). To this branched structure chitin, mannan and other cell wall components bind and increase the mechanical strength and tensile strength of the cell wall (Kollár *et al.*, 1997; Arana *et al.*, 2009). However, the enzyme responsible for formation of 1,6-β-linkage is not yet identified. During β-1,3-Glucan synthesis the transmembrane domains of enzyme forms a channel which releases the growing polymer chains in to cell wall space (Free, 2013). The enzyme complex FKS-RHO are localised predominantly in the areas of active cell growth and division like budding point and branching point in *S. cerevisiae* (Qadota *et al.*, 1996; Delley and Hall, 1999). The gene which code for β-1,3-Glucan synthase is *FKS*. Different fungal species have several different types of *FKS* (Free, 2013). *FKS1* is regulated by Rho1 G protein encoded by *RHO1*. Both of these genes are highly conserved in fungal kingdom. Rho1 is involved in signal transduction system and controls MAP kinase pathway in *S. cerevisiae* and regulate growth and cell wall integrity of fungi (Klis *et al.*, 2006; Free, 2013). Rho1 activates glucan synthesis by regulating *FKS1* when there is need to produce β-1,3-Glucan to strengthen the cell wall against environmental stress, osmotic shocks. And it also shuts down the glucan production when there is no requirement (Free, 2013).

In *S. cerevisiae*, three *FKS* are reported, *FKS1*, *FKS2* and *FKS3*. *FKS1* acts as a major β-1,3-Glucan synthase during vegetative growth. Both *FKS1* and *FKS2* are part of glucan synthase complex and their disruption cause cell wall defects and slow growth rate, and *FKS3* is not important for S. cerevisiae (Douglas *et al.*, 1994; Bowman and Free, 2006). *FKS2* is required for sporulation during nutrition starvation (Mazur *et al.*, 1995). *A. fumigatus*, *C. neoformans* and *N. crassa* have a single β-1,3-glucan synthase gene *FKS1* (Tentler *et al.*, 1997; Thompson *et al.*, 1999; Beauvais *et al.*, 2001). In *S. pombe*, four β-1,3-Glucan synthase genes are reported of which only one is

involved in spore formation and germination, septa formation, in mating and in growth (Cortés *et al.*, 2002).

As beta glucan are important building block of fungal cell wall architecture, a minor defect can impact fungal virulence. Several reports are there where *FKS* genes and *RHO* genes have been disrupted or deleted individually as well as simultaneously and as a result of which cell wall formation and morphology is greatly affected which ultimately affect fungal growth and virulence (Free, 2013). The *A. fumigatus FKS1* and *RHO1* genes have been cloned and individually disrupted. Both genes are required for cell viability (Beauvais *et al.*, 2001; Bowman and Free, 2006). RNAi-mediated knockdown mutants of Glucan synthase gene of *C. graminicola* resulted in reduced levels of β-1,3-Glucan in the cell wall. The appressoria differentiation was also affected. There was reduced adhesiveness of appressoria of mutant strain compared to wild type and increase cell wall elasticity as well as appressoria was not able to resist the turgor pressur (Oliveira-Garcia and Deising, 2013).

There are very few reports on study of *Fusarium* glucan synthase. Knockdown mutation of *FsFKS* gene in *F. solani* resulted in lysis of spores and hyphae (Ha *et al.*, 2006). Sequence analysis of *FKS1* gene revealed mutation in gene which is responsible for Echinocandin resistance *Fusarium* and *Scedosporium species* (Katiyar and Edlind, 2009). The glucan synthase reported in *F. oxysporum* is FOS_14431T0 which shows up-regulation during conidial germination (Deng *et al.*, 2015) and *fks1* and *gas1* are required for cell wall biosynthesis, and Sho1, Msb2 and Fmk1 are required for their up-regulation (Perez-Nadales and Pietro, 2015). In *F. culmorum,* β-1,3-Glucan synthase gene (Fc*Gls1*) silencing leads to abnormal cell morphology, swollen hyphae and cells were not able to resist turgor pressure because of reduced levels of β-Glucan (Chen *et al.*, 2016). In *F. solani*, three β-1,3-glucan synthase genes has been reported in UniProtKB (https://www.uniprot.org/) which are FKS1 (Q2L7J5) code for β-1,3-Glucan synthase, FKS1 (C7ZLR4) code for glycosyl transferase family 48 and NECHADRAFT_88969 (C7ZN13) code for FKS1_dom1 domain-containing protein.

### 1.5.2.2 β-1,3-Glucanases:

These enzymes are also known as glycosyl hydrolases, glucanosyl tranferases. Different families of these enzymes have been reported. These enzymes are capable of cleaving β-1,3-Glucan polymer as well as creating new 1,3 beta bonds and hence are able to shorten and/or lengthen the β-1,3-Glucan polymers. Fungi have multiple glucanases in their genomes (Free, 2013; Moghaddam *et al.*, 2017).

#### 1.5.2.2.1 The *Gas1p*/*GEL1*/*Phr1p* family of β-1,3-Glucanases:

In ascomycetes family, members of this family are found as major cell wall protein (Free, 2013). *PHR1P* & *PHR2P* are required for cross-linking between β-1,3-Glucan and β-1,6-Glucan as well as for normal morphology and virulence in *C. albicans* (Fonzi, 1999). In *A. fumigatus*, deletions of *GEL2* leads to an altered cell wall and a reduction in virulence (Mouyna *et al.*, 2005). In *S. cerevisiae* deletion of *GAS1* leads to reduced glucan level in cell wall and an increased glucan level in extracellular medium as well as swollen cells have also been observed (Ram *et al.*, 1998). In *F. oxysporum*, Gel1 (*Fg*BGT) which is β-1,3-glycosyl transferase is required to maintain cell wall integrity (Moghaddam *et al.*, 2017).

#### 1.5.2.2.2 The *Crh*/*Utr* family of β-1,3-Glucanase:

In the mature fungal cell wall stability of the cell wall is increased by crosslinking between glucans and chitin. *Chr*/*Utr* family of glucanases are known to create crosslinking between cell wall components (Free, 2013; Geoghegan *et al.*, 2017). These enzymes have transglycosidase activity. In *S. cerevisiae* it forms cross link between chitin and β-1,6-Glucan (Cabib *et al.*, 2007) while in *C. albicans* it is required for attachment of chitin with β-1,3-Glucan (Pardini *et al.*, 2006) and deletion of these enzymes leads to formation of defective cell wall. These enzymes are important for controlling morphogenesis in *S. cerevisiae* as well as for cell wall biogenesis (Hadwiger, 2013; Arroyo *et al.*, 2016).

#### 1.5.2.2.3 The *Bgl2p*/*Scw4p*/*Scw10p*/*Scw11p* family of β-1,3-Glucanases:

These enzymes function by formation of kinked polymer. For the formation of kinked polymer, it cleaves disaccharide from one end of β-1,3-Glucan

polymer and form activated intermediate which goes and binds to non-reducing end of another β-1,3-Glucan polymer (Free, 2013). *Scw10* is important for cell wall stability in *S. cerevisiae* (Sestak *et al.*, 2004). In *C. albicans*, *BGL2* mutant strain was having altered phenotype and attenuated virulence (Sarthy *et al.*, 1997). In *A. fumigatus Afbgt1* and *Afbgt2* have been characterised which also belongs to Bgl2 family. Gastebois and group reported that, *Afbgt1* and *Afbgt2* are not important for cell wall formation because *Afbgt1/Afbgt2* double mutant did not have any mutant cell wall and it might indicate that *A. fumigatus* require additional enzymes for formation of cross-linked β-1,3-Glucan cell wall matrix (Gastebois *et al.*, 2010).

**1.5.2.3 β-1,6-Glucan:**

β-1,6-Glucan are important cell wall components of fungi. In *S. cerevisiae* it forms crosslinks with β-1,3-Glucan, chitin and other GPI anchored disaccharides (Kollár *et al.*, 1997). β-1,6-Glucan synthase is not yet definitively characterised in any fungal species, but there are certain number of genes are characterised which affect the synthesis of β-1,6-Glucan (Free, 2013). In *S. cerevisiae KRE6* and *SKN1* are required for normal β-1,6-Glucan synthesis. Sequence and structural analysis suggested that these enzymes have glyosylhydrolse and transglycosidase activity which might be required for crosslinking β-1,6-Glucan to cell wall (Lesage and Bussey, 2006; Free, 2013). *KRE1* mutant strain of S. cerevisiae had shorter β-1,6-Glucan which indicate that KRE1 is required to elongate β-1,6-Glucan and mutation in *KRE5* and *KRE6* also caused reduced production of β-1,6-Glucan and had defect in cell wall (Boone *et al.*, 1990). *KRE9* and *KHN1* are involved in cross linking β-1,6-Glucan in cell wall and their loss is lethal to cells of *S. cerevisiae* (Lesage and Bussey, 2006).

In *C. neoformans*, *KRE5*, *KRE6* and *SKN* mutant strain was reduced amounts of β-1,6-Glucan with complex phenotype, altered morphologies and sensitivity to environmental stress. In mouse model of inhalation these this mutant strain was found to be avirulent and this indicates that β-1,6-Glucan is important and essential for cell wall of *C. neoformans* (Gilbert *et al.*, 2010). In *N. crassa*, *A. fumigatus* and *S. pombe* similar set of genes were not found which indicates

that these genes are restricted to limited number of species and those fungi which does not have similar set of genes might have some other genes which are involved in cross linking their cell wall components (Free, 2013).

### 1.5.3 Mycotoxins:

Mycotoxins are low molecular weight secondary metabolite product of fungi impacting human, plant and animal health. Mycotoxicosis is called toxic effect of mycotoxin on human and animal health (Bennett and Klich, 2003; Adeyeye, 2016). The first incidence of mycotoxicosis came in to light in 1960 in England. In that case 100.000 turkeys died because of contaminated peanut meal and it was found that peanut was contaminated with aflatoxin produced by *A. flavus* and disease was called turkey X disease (Blount, 1961; Forgacs, 1962). Subsequently aflatoxins were reported to cause haepatocellular carcinoma in human and animals. The periods between 1960 -1975 was referred as mycotoxin gold rush as several scientist started research on toxin being produced by filamentous fungi and molds (Maggon *et al.*, 1977). Till now nearly 400 mycotoxins have been characterised (Bennett and Klich, 2003) but from all of these toxins aflatoxins, zearalenone, fumonisins, trichothecenes and ochratoxins are of public significance because of their hazardous toxic effects on humans and animals (Peraica *et al.*, 1999; Bennett and Klich, 2003; Antonissen *et al.*, 2014; Adeyeye, 2016).

Mycotoxins are produced by several fungal genera including, *Fusarium, Aspergillus, Peniciilium, Alternaria, Clavicep, Epichloe* and *Neotyphodium* but *Fusarium* and *Aspergillus* have gained special attention as the toxins produced by these two genera have hazardous effect on human and animal health (Heidtmann-Bemvenuti *et al.*, 2011). Mycotoxicosis can be acute or chronic. Acute mycotoxicosis is rapid toxic response while chronic mycotoxicosis developed by low exposure over a long period of time resulting in cancer development, immunosuppression, kidney toxicity and other irreversible effects (Bennett and Klich, 2003). Mycotoxicoses is frequently not recognised by medical professional however, when some symptoms which are obvious and can be found in several patients and has no direct correlation or connection with pathogenic microorganism then it is taken in to consideration (Liew and Mohd-Redzwan, 2018).

*Fusarium* spp. are known to produce several hazardous mycotoxins; fumonisin, zearalenone, nivalenol, deoxynivalenol, T2 toxin, HT-2 toxin, beauvericin and enniatin (Asam *et al.*, 2017; Bertero *et al.*, 2018) are produced by *Fusarium* spp. Toxins produced by *F. solani* are DON, neosolaniol, nivalenol, T-2, toxin, Ht-2 toxin, fusaron-X (Ueno, 1977), DON, fumonisisn, fusaric acid (Shi *et al.*, 2017), beauvericin (Azliza *et al.*, 2014) and ZEA (Najim, 2013).

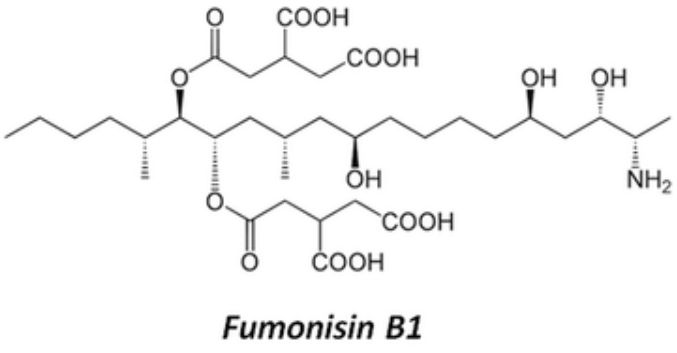
**1.5.3.1 Fumonisin:**

Fumonisin have long hydroxylated hydrocarbon chin in their structure (Figure 1.5). They are derived from condensation of alanine in to acetate derived precursor. They were first characterised in 1988 (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988). Fumonisins are mainly produced by *Fusarium* spp. which includes *F. vericillioides*, *F. moliniforme*, *F. solani*, *F. fujikuroi*, *F. proliferatum* and *F. nygamai*. However *F. vertcillioides* is of major economic significance (Heidtmann-Bemvenuti *et al.*, 2011; Shi *et al.*, 2016; Asam *et al.*, 2017; Bertero *et al.*, 2018). Sixteen different fumonisins have been characterised which are $FB_1$, $FB_2$, $FB_3$, $FB_4$, A1, A2, A3, AK1, C1, C3, C4, P1, P2, P3, PH1A and PH1B Among them fumonisin $B_1$ and fumonisin $B_2$ are of major importance as they constitute up to 70% of the fumonisins found as natural contaminant (Seo *et al.*, 2001; Niderkorn *et al.*, 2009; Heidtmann-Bemvenuti *et al.*, 2011).

**1.5.3.1.1 Mechanism of action:**

FB1 shares structural similarity with cellular sphingoloipids. Sphingolipids are important for protein and membrane structure. It is important secondary messenger for TNF alpha, IL-1. Sphingolipids are involved in interaction between cells and extra cellular matrix (ECM) (Soriano *et al.*, 2005; Heidtmann-Bemvenuti *et al.*, 2011). Sphingolipid accumulation is related to several diseases like cancer, immunological as well as neurological disease (Masching *et al.*, 2016; Asam *et al.*, 2017). Sphinolipid is converted to ceramide by action of ceramidase and sphingomylinase. As FB1 has structural similarity with sphingosine, it inhibits action of ceramide synthase on sphingosine and leads to sphingosine accumulation in cells which leads to

cytotoxicity, oxidative stress and apoptosis in cells. Such phenomenon has been detected in kidney, liver, intestine, urine and serum (Enongene *et al.*, 2000; Soriano *et al.*, 2005; Masching *et al.*, 2016; Asam *et al.*, 2017).




***Fumonisin B1***

**Figure 1.5: Chemical structure of Fumonisin** (Pierron *et al.*, 2016).

**1.5.3.1.2 Toxicity:**

Fumonisin is listed as group 2B human carcinogen by IARC (IARC, 2002). Fumonisin affect humans and animals by interference in sphigolipid metabolism. Oesophageal cancer is mainly related to FB1 toxicity (Bennett and Klich, 2003; Liew and Mohd-Redzwan, 2018). Several cases have been recorded worldwide for FB1 toxicity and oesophageal cancer. The epidemiological studies revealed that in China, South Africa and Italy high incidences of oesophageal cancer is related to consumption of food contaminated with FB1 (Chilaka *et al.*, 2017). In India case of acute FB1 toxicity has been recorded where consumption of bread prepared from infected corn and shorgum caused diarrhoea, borborygmus and abdominal pain (Bhat *et al.*, 1997). In rabbit when fumonisin was gavaged, it caused haemorrhage in cerebral white matter and hippocampus, malacia, degeneration of hepatocytes and renal tubule epithelium, apoptosis in cells in liver and kidney (Bucci *et al.*, 1996). Similar results were also seen when studies were carried in rats and equine (Pozzi *et al.*, 2001; Bennett and Klich, 2003). Harrison and group reported the death of pig and swine due to consumption of fumonisin contaminated corn and pulmonary edema and hypothorax was observed in dead animals (Harrison *et al.*, 1990). Fumonisin can cause neural tube defect which includes defect in spine and brain. Epidemiological studies of neural tube defect has been reported in southern Texas, South Africa and China (Bennett and Klich, 2003; Liew and Mohd-Redzwan, 2018). Intestinal

epithelial cell viability as well as cell proliferation is affected by $FB_1$. It was found that $FB_1$ leads to accumulation of sphingosine which block the G0/G1 phase in cells and hence apoptosis occur. Tight junction proteins are getting supressed by $FB_1$ and intestinal permeability is affected which leads to bacterial translocation in cells (Angius *et al.*, 2015; Kelly *et al.*, 2015; Romero *et al.*, 2016; Yamazoe *et al.*, 2017).

### 1.5.3.2 Trichothecene:

Trichothecene (TC) are sesquiterpanoid metabolites. It is produced by several fungal genera including *Fusarium, Myrothecium, phomopsis, Stachybotrys, trichoderma, trichothecium* and many other genera (Ueno, 1977; Scott, 1989). In *Fusarium*, TC are produced by *F. langsethiae*, *F. sporotrichioides*, *F. polyphialidicum, F. culmorum*, *F. poae*, *F. meridionale, F. solani* and *F. graminearum* (Shi *et al.*, 2016). *F. sporotrichiella* and *F. poae are mainly reported to produce trichothecene* (Heidtmann-Bemvenuti *et al.*, 2011; Adhikari *et al.*, 2017). Thrichothecene have 12,13-epoxide in common structure (Figure 1.6) which is responsible for their toxicity feature (Bennett and Klich, 2003; Heidtmann-Bemvenuti *et al.*, 2011). There are mainly two types of trichothecene, Type A and Type B. Type A TC have hydrogen or ester type side chain at c8 position and include mainly T2, HT2 toxins and diacetoxyscirpenol (DAS). Type B TC have ketone ring and mainly include fusarenon-x, nivalenol, and deoxynivalenol (Bennett and Klich, 2003; Shi *et al.*, 2016; Asam *et al.*, 2017) .

#### 1.5.3.2.1 Mechanism of action:

Trichothecene act by inhibiting protein synthesis. They bind to active polysome and ribosome and interfere with peptide linkage at initiation, elongation and termination stage and disrupt the ribosomal protein synthesis cycle. They inhibit peptidyl transferase by binding to ribosome binding site (Bennett and Klich, 2003). They can also cause polyribosomal disaggregation (Heidtmann-Bemvenuti *et al.*, 2011). They are also known to interfere with membrane transport amino acids, nucleotides and glucose. They interfere with calcium potassium channel (Bunner and Morris, 1988). They supresses

succinate dehydrogenase and hence leads to interference in mitochondrial electron transport chain (Khachatourians, 1990).

**1.5.3.2.2 Toxicity:**

LD50 value via oral administration for T2 toxin is 10.5 mg/kg bodyweight, for HT2 toxin is 9.2 mg/kg bodyweight (Asam *et al.*, 2017).

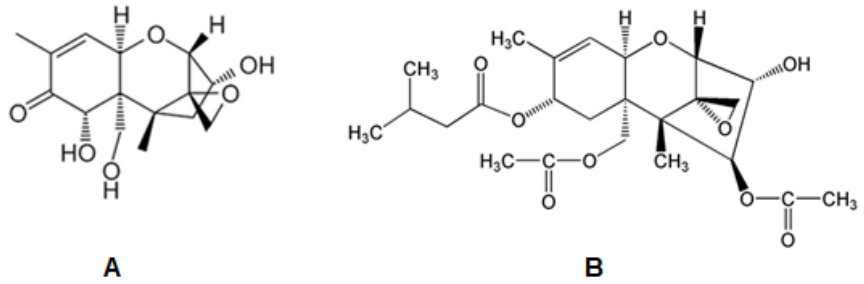



**Figure 1.6: Chemical structure of Trichothecene: (A)** DON and **(B)** T-2 toxin (Yang *et al.*, 2016; Pierron *et al.*, 2016).

Trichothecene are able to cause neurological, reproductive, developmental, gastrointestinal, hematological and dermatological disease. T2, toxin, DAS and DON are majorly studied trichothecene. It affects both humans and animal health. In animals it has been reported that DON when ingested in large amount it can cause nausea, vomit and diarrhoea while at lower dose it causes weight loss and food refusal (Bennett and Klich, 2003). TC are known to cause alimentary toxic aleukia in humans in which TC damages hematopoietic tissue, causes skin inflammation, vomiting, oral cavity necrosis, nose bleeding, mouth and vaginal bleeding, dystrophy in kidney, liver, heart, brain and nervous system and ulcer in digestive track. (Bennett and Klich, 2003; Adhikari *et al.*, 2017). In 1940, Soviet scientist have reported stachybotryotoxicosis and symptoms were nose bleeding, sore throat, cough, dyspnea and fever. It occurred by inhalation of the *Stachybotrys* mycotoxin. Similar effects to alimentary toxic aleukia during World War II have been reported in Russia. T2 toxin was used as biological weapon as civilians have consumed *Fusarium* contaminated wheat which caused lethal illness, the effects were nose bleeding, sore throat, cough, dyspnea and fever. After 20 years, the trichothecenes were discovered (Bennett and Klich, 2003; Afsah-Hejri *et al.*, 2013; Adhikari *et al.*, 2017). Wang and group had reported T2

toxin outbreak in china where large population was affected by consuming mold contaminated rice and major symptoms were vomiting and diarrhoea (Wang *et al.*, 1993). Similar DON outbreaks have also been reported from India (Bhat *et al.*, 1997), China (Luo, 1988) and USA (Steinberg *et al.*, 2006). In rats T2 toxin causes alteration in metabolic pathways of different organs like, kidney, liver, spleen, thymus, stomach (Wan *et al.*, 2015). It leads to promotion of oxidative stress by generating ROS which in terms are elevated by increased level of glutathione disulphide and 3-hydroxy butyrate. It also reduces the speed of TCA cycle which was proved in rats by reduced level of succinate and citrate in urine and fumarate in liver (Adhikari *et al.*, 2017). In pigs toxicity of T2 toxin is exerted by multiple hemorrhages of the serosa of the liver, stomach, intestinal tract and esophagus, blood in abdominal cavity and intestine (Devreese *et al.*, 2013). It also promotes leukocyte apoptosis by activating c-Jun N-terminal kinase 1 (JNK1) and/or p38MAPK (SAPK2) and by stimulating MAP kinases involved in cell proliferation regulation (Li and Pestka, 2008).

#### 1.5.3.3 Zearalenone:

Zearalenone (ZEA) (Figure 1.7) is secondary metabolite synthesised through polyketide pathway by gene polyketide synthase. It is non-steroidal estrogenic mycotoxin which act mainly on reproductive function of human and animal and is known as mycoestrogen (Kotowicz *et al.*, 2014; Adhikari *et al.*, 2017; Liew and Mohd-Redzwan, 2018). It is produced mainly by *Fusarium* species which includes *F. graminerum* (Gibrella zeae), *F. culmorum*, *F. equiseti*, *F. pseudograminerum*, *F. verticilliodes*, *F. sporotrichioides*, *F. semitectum* and *F. crookwellense* (Zhang *et al.*, 2014; Gupta *et al.*, 2018). Its toxicological effects includes enlarged uterus, decreased fertility, abnormal levels of hormones, estradiol and progesterone and altered reproductive tract (Zhang *et al.*, 2014).

##### 1.5.3.3.1 Mechanism of action:

It has structural resemblance with 17-beta-oestradiol which is produced by ovary in mammals and hence it interferes with binding of 17-beta-oestradiol to oestrogen receptors. ZEA is converted to its different metabolites by action of

hydroxysteroid hydrogenase. These different metabolites includes α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). All these metabolites are endocrine disruptor and have different potential of toxicity. ZEA can be identified on thin layer chromatography under ultra violet light as blue fluorescent spot (Kuiper-Goodman *et al.*, 1987; Eze *et al.*, 2018).

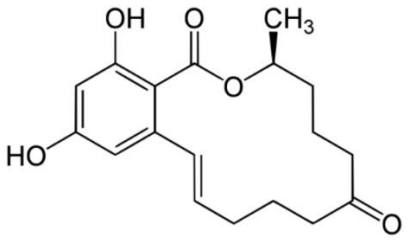



**Figure 1.7: Chemical structure of Zearalenone (ZEA)** (Erdem *et al.*, 2019).

**1.5.3.3.2 Toxicity:**

LD50 for ZEA is 2,000-20,000 μg/kg bodyweight (Zinedine *et al.*, 2007). Zearalenone and its metabolites are known to affect mainly reproductive organs and functions in males and females in humans as well as in animals. In addition to that, it can also affect kidney, liver, adipose tissue and GI tract (Gupta *et al.*, 2018; Liew and Mohd-Redzwan, 2018). In one study on pigs, it was reported that once ZEA fed to pigs, ZEA and its metabolites were detected in plasma before 30 minutes of administration and then transported and localised in reproductive organs as well as adipose tissue. ZEA and its metabolites induce production of uterine proteins and leads to increased uterine weight (Gajęcka *et al.*, 2012; Gupta *et al.*, 2018). Zearalenone and its metabolite affects ovarian follicles development and maturation process by activating apoptotic like process in germ cells which decreases healthy follicles count and ultimately cause premature oocyte depletion and mRNA expression estrogen receptor beta has been increased after ZEA exposure (Skorska-Wyszyńska *et al.*, 2005; Schoevers *et al.*, 2012). ZEA can also affect early pregnancy stages by affecting fertilization, embryo development, transport and implantation in uterus (Zhao *et al.*, 2013). In male rats when ZEA administered orally, the serum prolactin concentration increase was observed as well as it affected sertoli cell function by disrupting tubulin

filament and actin which are key cytoskeleton structures of these cells and also affect nucleus of these cells and ultimately secretory function is compromised in male rats (Milano *et al.*, 1995; Zheng *et al.*, 2016).

Marin and group have reported the toxicity of ZEA and its metabolite α- ZOL and β-ZOL on intestinal procaine epithelial cell line (IPEC-1). It was found that ZEA did not affect transepithelial resistance (TER) while α- ZOL and β-ZOL redused the TER which leads to increased epithelial permeability (Marin *et al.*, 2015). It has also been reported that ZEA increase permeability in Caco-2 cell line (Pfeiffer *et al.*, 2011). TER reduction occur because of alteration in tight junction protein. mRNA expression level of claudin-4, occludin and connexion-43 has been decreased after exposure of mice to ZEA (Liu *et al.*, 2014). ZEA increase expression of Toll like receptors (TLR) and cytokines TNF-α, IL-1β, IL-6, IL-8, MCP-1, IL-12p40, CCL20 which recruits inflammatory cells and are responsible for inflammation and caused down-regulation of DKK-1, PCDH11X and TC531386 genes which are tumour suppressor genes and hence ZEA might have carcinogenic potential (Taranu *et al.*, 2015).

### 1.5.4 Fungal pigments:

Fungi naturally produce secondary metabolites as a by-product of their metabolism. These metabolites are low molecular weight compound, not required for growth and development of fungi and produced during stationary growth phase (Calvo *et al.*, 2002). These secondary metabolites can be toxic to other microorganisms, plants, animals and humans. The production is dependent upon surrounding microbial community, stress, fungal species and availability of nutrient (Frisvad *et al.*, 2008; Cheli *et al.*, 2013). Secondary metabolites contributes to pathogenicity by invading immune system of host and are also considered as virulence factors (Casadevall and Pirofski, 2001; Saharan *et al.*, 2016).

Secondary metabolites mainly include mycotoxins and pigments. Pigments produced by fungi are of various types but widely distributed pigment types among fungal kingdom are napthoquinones, anthroquinones and melanine (Calvo *et al.*, 2002). On the basis of structural properties, secondary

metabolites can be divided in four categories which are polyketides, terpens, nonribosomal peptides and amino-acid derived compounds (Studt *et al.*, 2012). Polyketide which includes red and green pigments, is the most abundant among secondary metabolite. These pigments belongs to naphthoquinone group. The production of secondary metabolite is regulated at chromosome level. The genes required for secondary metabolite production are highly clustered in filamentous fungi. These clusters contain up to 20 genes (Keller and Hohn, 1997; Shwab and Keller, 2008).

Naphthoquinones are organic derivatives of napthelene. The napthoquinones are naturally produced by plant and fungi but are also synthesised chemically. They have antibacterial, phytotoxic and cytotoxic effects (Fowler *et al.*, 2018). Structurally napthoquinones are very diverse group. More than 300 napthoquinones are known and upto 100 napthoquinones are structurally characterised. The genus *Fusarium* is widely known to produce napthoquinone pigments but structural characterization is done for few napthoquinones and new naphthoquinones are continuously being discovered (Medentsev and Akimenko, 1998; Studt *et al.*, 2012). Naphthoquinone pigments reported in *Fusarium* spp. are Javanicin, Fusarubin (Kumar *et al.*, 2017), aurofusarin (Cambaza, 2018), solaninaphthoquinone (Tadpetch *et al.*, 2015), bostricoidin (Medentsev and Akimenko, 1992), anhydrofusarubin (Medentsev and Akimenko, 1992) and modified forms of these napthoquinones. The basic backbone structure of napthoquinones are presented in Figure 1.8. Structures of Fusarubin, anhydrofusarubin, javanicin, bostricoidin and bikaverin are presented in Figure 1.9.

*Fusarium* spp. reported to produce naphthoquinones are *F. decemcellulare* (Medentsev *et al.*, 2001), *F. bulbigenum* (Medentsev *et al.*, 2005), *F. moniliforme* (Premalatha *et al.*, 2012; Pradeep *et al.*, 2013), *F. fujikuroi* (Studt *et al.*, 2012), *F. solani* (Tadpetch *et al.*, 2015), *F. vericillioides* (Boonyapranai *et al.*, 2008), *F. culmorum* (Baker and Roberts, 1966), *F. langsethiae*, *F. proliferatum*, *F. graminerum*, *F. sambucinum*, *F. poae*, *F. acuminatum*, *F. avenaceum*, *F. pseudograminearum*, *F. sporotrichioides*, *F. crookwellens*, and *F. tricinctum* (Frandsen *et al.*, 2006; Cambaza, 2018; Beccari *et al.*, 2018).


**Figure 1.8: The basic structures of 1,4-naphthoquinone and 1,2-naphthoquinones** (Medentsev *et al.*, 2005).

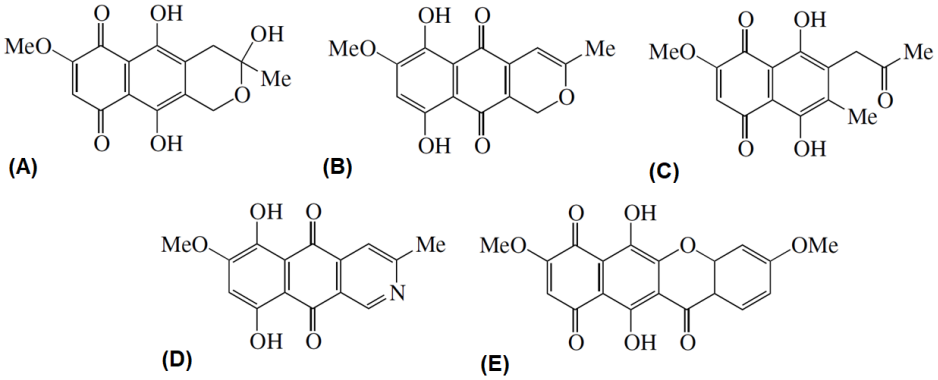



**Figure 1.9: Structure of Napthoquinones: (A)** Fusarubin, **(B)** anhydrofusarubin, **(C)** javanicin, **(D)** bostricoidin and **(E)** bikaverin (Medentsev *et al.*, 2005).

The mechanism of action of napthoquinone is not completely understood. It has been reported that naphthoquinone acts by interfering with electron transport chain. Napthoquinone has structural resemblance with ubiquinones and it binds with bc1 site in complex III which consequently results in ATP and nucleic acid synthesis inhibition (Riffel *et al.*, 2002). Another mechanism reported is arylation of nuclephillic sulfodryl group (Fowler *et al.*, 2018) and formation of ROS and $H_2O_2$ during redox cycling (Shang *et al.*, 2012) which causes oxidative stress. Napthoquinones have genotoxic and mutagenic activity and causes DNA intercalation (Da Costa Medina *et al.*, 2008).

In fungi, several polyketide synthase are reported. They are of two types, reducing PKS and non-reducing PKS. Non-reducing polyketide synthase (NR-PKS) is responsible for napthoquinone synthesis. The size ranges from 2000-2300 AAs. NRPKS contains, beta ketoacyl synthase (KT or KS) domain, malonyl coenzyme A: acyl carrier protein (MAT) domain, acyl carrier protein

(ACP) domain, starter unit ACP transacylase (SAT) domain, thioesterase (TH or TE) domain and product template (PT) domain (Studt *et al.*, 2012; Brown *et al.*, 2012).

### 1.6 *In vivo* model for fungal keratitis:

#### 1.6.1 Murine model:

Murine models are used widely because of the genome similarity, biochemical similarity, organ similarity and similarity of pathological condition with humans. Quantification and understanding of immunological changes occuring during infection which have some similarity with human infection can be studied well (Capilla *et al.*, 2007).

In mouse model of *C. albicans* Keratitis, it was shown that immunocompromised mice as well as non-immunocompromised mice when treated with methylprednisolone and cyclophosphamide, disease severity and fungal persistence was increased (Wu *et al.*, 2003). Similar report of increased disease severity and reduced fungal clearance by treatment with cyclophosphamide in mouse model of *F. solani* keratitis has been reported (Wu *et al.*, 2004). Development of mouse model of *F. solani* was reported by Zhu and group, where they have reported standardization of infection dose for development of infection and found that $10^8$ CFU/ml induce moderate keratitis which was consistent with human infection (Zhu *et al.*, 2011). Important role of neutrophils in fungal clearance during fungal keratitis has been shown in mouse model of *Fusarium* and *Aspergillus* keratitis where neutrophil depletion leads to excessive fungal growth (Leal Jr and Pearlman, 2012). Taylor and group had reported use of mice model to find out role of Th-17 cells and IL-17 producing neutrophils during keratitis caused by *F. oxysporum* and *A. fumigatus*. They found that when mice was induced by heat killed conidia, there was increased production of IL-17 and INF- γ compared to uninfected corneas which leads to produce elevated levels of ROS and leads to fungal clearance by these cells (Taylor *et al.*, 2014). Similarly, role of ISG15 in controlling fungal keratitis has also been reported in mouse model of *Candida* keratitis (Dong *et al.*, 2017). However in one study it has been reported that increased ROS leads to corneal destruction in fungal keratitis by activation of

MAP Kinase pathway (Hua *et al.*, 2017). In mouse model of *A. fumigatus* keratitis, macrophage induced c-type lectin (mincle) expression was up-regulated with progression of infection along with several other cytokines compared to uninfected mice which indicates the role of mincle in early innate immune response of cornea against infecting fungi (Zhao *et al.*, 2017). It has also been reported that mast cells play important role in corneal protection during fungal keratitis through degranulation, increased limbal vascular dialation and increased permeability which leads to neutrophil infiltration by ICAM-1 stimulation in mouse model of *F. solani* keratitis. (Xie *et al.*, 2018)

### 1.6.2 Rabbit model:

After mouse models, rabbit as an animal model for fungal keratitis being used very often. It has advantage over murine model as rabbits have large eyes and hence large area of cornea is available to conduct and understand the pathogenesis, and to study the elements involved in pathogenesis. Rabbits have similarity in anatomy and physiology with humans. Several reports are available for development of rabbit model of fungal keratitis (Capilla *et al.*, 2007).

Rabbit model of *F. solani* keratitis was developed by (Kiryu *et al.*, 1991). It was reported that in dexamethasone treated corneas, the fungus was able to survive, it penetrated in to corneal stroma and digestion of collagen fibrils of cornea was also observed, which was absent in control or untreated group where fungus was entrapped in pseudopodia and destructed. *F. moloniforme* extract when applied to rabbit corneas, it produced lesions in the cornea and collagen bundles were fragmented which indicated the enzymatic (proteolytic nature of the extract) nature of extract. However, the boiled extract was not able to create such lesions (Dudley and Chick, 1964). In rabbit model of fungal keratitis by *A. flavus* and *F. solani*, proteolytic activity was reported and it was found that the protease being produced by fungi during corneal infection was metalloprotease and identified as Matrix Metalloproteases 2 (MMP2) and Matrix Metalloproteases 9 (MMP9) with molecular weight (M.W.) of 65 kD and 92 kD respectively. Also they have reported 200 kD gelatinase in infected corneas. It was suggested that resident corneal cells

and/or inflammatory cells are involved in matrix degradation through increased proteolytic activity in fungal infected corneas as neutrophils are also known to produce MMP (Gopinathan *et al.*, 2001). Rabbit model of fungal keratitis was used to compare the pathogenicities of different fungal pathogens which included *F. solani*, *Cylindrocarpon tonkinense* and *C. albicans*. It was found that *F. solani* produced more severe infection than *C. albicans* while *C. tonkinense* pathogenicity was similar to *F. solani* (Ishibashi *et al*., 1986a, 1986b). Rabbit model of *F. solani* was also developed by (Forster and Rebell, 1975) where different methods of inoculation were developed for proper infection model development. To understand the distribution of Fungus causing keratitis in the different environment in Columbia, a study was carried out where different samples were taken from different location and then infected in rabbits, and *F. solani* was prevalent in the environment (Cuero, 1980). O'Day and group had reported that use of corticosteroids at the start of fungal keratitis in patient which supresses the fungal growth for initial days but later on it increases the fungal growth, which was done is rabbit model of *F. solani*. They also reported that, when the rabbit which was not treated with corticosteroids the fungus was not able to grow further after day 5 (O'Day *et al.*, 1991). Recently, one study has reported use of cDPC (cross-linked decellularized porcine corneal) graft for treatment of fungal keratitis. In rabbit model, the cDPC graft was implanted which resulted in reduced corneal perforation and restoration of corneal transparency (Lin *et al.*, 2017).

### 1.6.3 Other animal models:

Other animals which were used to develop infection model of keratitis are rats and monkeys (Burda and Fisher, 1959; Forster and Rebell, 1975).

## 1.7 *Ex vivo* model for fungal keratitis:

*In vivo* studies require large number of animals, their maintenance is also tedious and costly, and animals suffer from stress. They require proper monitoring, in case of understanding and treating the disease, the treatment is costly for animals. Special animal care personnel are require. Also ethical issues regarding animals have increased and hence the number of animals to

conduct a research has been reduced. The use of *ex vivo* models became alternative option to this situation.

*Ex vivo* rabbit cornea model as well as human cornea model for bacterial and fungal keratitis has been developed by (Pinnock *et al.*, 2017). They have used several methods of fungal inoculation for disease development in excised cornea, for eg., the use of scalpel to form a scar, use of intra-stromal injection to deliver the pathogen. The organisms tested were *S. aureus*, *Pseudomonas aeruginosa*, *C. albicans* and *F. solani* for infection and disease development. *P. aeruginosa* was found to be the most pathogenic organism which penetrated deep in to the corneal stroma compared to other pathogens. To study the mechanism of fungal adherence to the corneal surface an *ex vivo* murine corneal model was developed (Zhou *et al.*, 2011). They showed that adherence is dependent on inoculum concentration and incubation time. Human *ex vivo* corneal model for *Fusarium* keratitis was developed and used by Hua and group to understand the pathogenicity of *F. oxysporum* keratitis. They have constructed *pacC* loss of function mutant and overexpression strain to investigate the role of *pacC* in virulence. They were able to discover that *pacC* pathway regulates fungal gene transcription and enable fungi to adapt on ocular surface help in invading deep in to corneal stroma. The *pacC* overexpression mutant and wild type strain showed hyphal penetration in to stroma rapidly compared to *pacC* loss of function mutant strain (Hua *et al.*, 2010).