Introduction
General Introduction:

Fungi are a large group with about 250,000 species which are major pathogens of agricultural plants and important opportunistic pathogens of humans. Recently ranking as the seventh most common cause of infectious disease-related deaths (Odds, 2000). More than 300 species have been reported to be potentially pathogenic to humans (Guarro and Barradell 1997). The infections caused by fungi are generally referred to as Mycoses. These can further be classified into four groups viz. superficial, subcutaneous, systemic and opportunistic. Fungal infections in humans are generally confined to superficial areas such as skin infections; however, in immunocompromised people mild or nonpathogenic fungi can prove to be fatal. Opportunistic fungal pathogens are the causes of variety of invasive or systemic fungal infections in immunocompromised or immunosuppressed individuals.

Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients. Fungi were previously thought to be non-pathogenic for humans or sporadically associated with human diseases. The prevalence of invasive or systemic fungal infections has increased significantly during the past decades (Walsh et al., 1996). These have become one of leading cause of death among patients due to greater use of broad spectrum antibiotics, immunosuppressive agents, intensive care of low birth weight infants, organ transplantation and the acquired immunodeficiency syndrome (AIDS) epidemic (Graybill, 1996).

1.0 DISEASES CAUSED BY FUNGI:

Fungi affecting humans can be divided into different groups based on the level of penetration into the body tissues:

(i). Superficial mycoses are caused by fungi that grow only on the surface of the skin or hair.

(ii). Cutaneous mycoses or dermatomycoses include such infections as athlete's foot and ringworm, in which growth occurs only in the superficial layers of skin, nails, or hair.

(iii). Subcutaneous mycoses penetrate below the skin to involve the subcutaneous, connective, and bone tissue.

(iv). Systemic or deep mycoses are able to infect internal organs and become widely disseminated throughout the body. This type is often fatal.
Opportunistic ones cause infection only in the immunocompromised.

1.1 Superficial Mycoses:

Infection is localized in to the skin, hair and nails. An example is ring worm or tinea, an infection of the skin by a dermatophyte. Ringworm refers to the characteristic central clearing that often occurs in dermatophyte infections of the skin. *Candida albicans* causes candidiasis or thrush in humans. Candidiasis typically infects and is termed as a “commensal”. During times of ill-health or impaired immunity, the balance can alter and the organism multiplies to cause diseases. Antibiotic treatment can also alter the normal bacterial flora allowing *C. albicans* to flourish.

1.2 Cutaneous Mycoses:

These diseases are restricted to the keratinized layers of the skin, hair, and nails. Unlike the superficial mycoses, host immune responses may be evoked, resulting in pathologic changes expressed in the deeper layers of the skin. They extend deeper into the epidermis, as well as invasive hair and nail diseases.

1.3 Subcutaneous Mycoses:

Infections are confined to dermis, subcutaneous tissue or adjacent structure. Infection may arise following the wounding of the skin and the introduction of vegetable matter. These mycoses are rare and confined mainly to tropical regions. They tend to be slow onset and chronic in duration. The most common symptom is an ulcerative lesion that may develop into lymphangitis.

1.4 Systemic Mycoses:

These are invasive infections of the internal organs with the organs gaining entry by the lungs, gastrointestinal tract or through intravenous lines. They may be caused by: (i) primary pathogenic fungi (ii) opportunistic fungi that are of marginal pathogenicity but can infect the immunocompromised host.

1.4.1 Primary pathogenic fungi:

Infections occur in previously healthy persons and arise through the respiratory route. Examples of primary pathogenic fungi include histoplasmosis, coccidiomycosis and paracoccidioidomycosis.

1.4.1.1 Histoplasmosis:

This is caused by *Histoplasma capsulatum*. The organism is dimorphic, found in soil and growth is enhanced by the presence of bird and bat excreta. The lungs are
the main site of infection but dissemination to the liver, hear and central nervous system. Pulmonary infection can resemble symptoms seen in tuberculosis.

1.4.2 Opportunistic fungi:

The disease includes aspergillosis, systemic candidosis and cryptococciosis. Exceptionally, other fungi that are normally not pathogenic such as Trichospora, Fusarium or Penicillus may cause systemic infections. These are normally seen in patients suffering from AIDS, who are undergone surgery. metabolic defect.

1.5 List of Diseases Caused By Fungi:

Candidiasis (Candida spp.); Aspergillosis (Aspergillus spp); Cryptococciosis (Cryptococcus neoformans); Blastomycosis (Blastomyces dermatitidis); Coccioidoindymycosis (Coccioides immitis); Sporotrichosis (Sporothrix schenckii); Zygomycosis (Zygomycetes); Histoplasmosis (Histoplasma capsulatum var. duboisii and Histoplasma capsulatum var. capsulatum) (Table 1).

Today fungal infections are a real problem, having doubled in number from the 1980's to the 1990's, and with bloodstream infections increasing five-fold with an observed mortality of 55% (Torres et al., 1995). Candidiasis, Cryptococciosis and Aspergillosis have been frequently reported in persons suffering from acquired immune deficiency syndrome (AIDS) (White, 1997). Systemic candidiasis has been reported to occur in up to 10% of infants weighing less than 1kg; the greatest increases in surgical patients and 78% of fungal infections are due to Candida spp.

**Table 1:** List of diseases caused by different fungi.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Casual agent</th>
<th>Organs affected</th>
<th>Description of agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptococciosis</td>
<td>Cryptococcus neoformans</td>
<td>Lungs, Spinal Cord</td>
<td>Air</td>
</tr>
<tr>
<td>Candidiasis, Vaginitis, Thrush, Onychia</td>
<td>Candida albicans</td>
<td>Intestine, Vagina, Skin, Mouth</td>
<td>Air, Sexual Contact</td>
</tr>
<tr>
<td>Tinea Pedis</td>
<td>Trichophyton spp.</td>
<td>Skin</td>
<td>Contact</td>
</tr>
<tr>
<td>Tinea Captis</td>
<td>Microsporum spp.</td>
<td>Skin</td>
<td>Contact</td>
</tr>
<tr>
<td>Tinea Corporis, Tinea Barae</td>
<td>Epidermophyton spp.</td>
<td>Skin</td>
<td>Contact</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>Histoplasma capsulatum</td>
<td>Lungs, other organs</td>
<td>Air</td>
</tr>
<tr>
<td>Blastomycosis</td>
<td>Blastomyces dermatitidis</td>
<td>Lungs, other organs</td>
<td>Air</td>
</tr>
<tr>
<td>Coccioidoindymycosis</td>
<td>Coccioides immitis</td>
<td>Lungs, Ears</td>
<td>Air</td>
</tr>
<tr>
<td>Aspergillosis (Otomycosis)</td>
<td>Aspergillus</td>
<td>Lungs, Ears</td>
<td>Air</td>
</tr>
</tbody>
</table>
Human fungal infections in all parts of the world are uncommon in normally healthy persons, being confined to conditions such as candidiasis (thrush) and dermatophyte skin infections such as athlete's foot. However in immunocompromised patients, a variety of normally mild or non-pathogenic fungi can cause potentially fatal infections.

1.5.1 Candidiasis:

In severely immunocompromised patients, it can proliferate and disseminate throughout the body.

Fig. 1: Hyphae form of C. albicans.

The genus Candida is composed of an extremely heterogeneous group of organisms that grow as yeasts. Most members of the genus also produce a filamentous type of growth (pseudohyphae). In addition to pseudohyphae, Candida albicans (Fig. 1) and C. dubliniensis form true hyphae (germ tubes) and thick-walled cells referred to as chlamydospores. Although C. albicans is the predominant etiologic agent of candidiasis, other Candida species that tend to be less susceptible to the commonly used antifungal drugs such as C. krusei, C. glabrata, C. lusitaniae, and the newest Candida species, C. dubliniensis, have emerged as substantial opportunistic pathogens. Candida dubliniensis shares with C. albicans many virulence factors, such as germ tube formation, exoenzyme production, and phenotypic switching (Sullivan and Coleman, 1997). This species, however, unlike C. albicans, has been shown to readily develop stable resistance to fluconazole in vitro and in infected patients, strongly suggesting that C. dubliniensis possesses a readily inducible fluconazole resistance mechanism (Jabra-Rizk et al., 2000). In 1990, 16.1 fungal infections per 1,000 discharges were seen in burns and trauma patients, 10.1 per 1,000 in cardiac surgery patients, and 7.3 per 1,000 in general surgery patients; the vast majority (78%) of which were due to Candida species (Beck-Sagué and Jarvis, 1993).
*Candida albicans* is frequently present as part of the microflora of the gastrointestinal tract or the oropharynx in the normal human host. Between 10 to 40% of healthy people carry yeasts in throat and gut in low concentration of $10^3$ CFU/ml of saliva or gram of faeces. Changed host defense can lead to an overgrowth of *C. albicans* (Vincent et al., 1999; 1998).

An estimated 75% of women will experience at least one episode of vulvovaginal candidiasis (VVC) during their lifetime. VVC is often associated with conditions such as diabetes mellitus, antibiotic therapy, and pregnancy, but many women have no predisposing factors (Edwards, 1995). High degrees of anti-fungal drug resistance have been reported in *Candida* species and these have exhibited primary resistance patterns towards 5-Flucytosine (5-FU), Fluconazole and Ketoconazole (Mellado et al., 2007; 2001). In India, the occurrence of *Candida* in vaginal swabs of infertile women is 13% of which 40.7% are isolates of *Candida albicans* (Verghese et al., 2001). These developments and the associated increase in fungal infections intensified the search for newer, safer and more efficacious agents to combat serious fungal infections. So to overcome the problem now-a-days, medicinal plants are used as an alternative strategy for treating fungal infections.

1.5.2 *Aspergillosis*:

This is the name given to a number of different diseases caused by the mould *Aspergillus*. It produces large number of spores and occurs world-wide. It can infect the lungs, inner ears, sinuses and rarely the eye of previously healthy persons. In the immunocompromised patients, it can disseminate throughout the body.

1.5.3 *Cryptococcosis*:

This is a systemic infection caused by the yeast *Cryptococcus neoformans*. The common manifestation is a sub acute or chronic form of meningitis resulting from the inhalation of the organism. Pulmonary infection can also occur. The disease affects both healthy and immunocompromised individuals world-wide.

Diseases like Candidiasis (*Candida albicans*), Aspergillosis (*Aspergillus niger*) and Cryptococcosis (*Cryptococcus species*) are the forerunners in all major fungal infections. The frequency of candidiasis has increased ten-fold, so that *Candida albicans* has become the fourth most common culture isolate. *Candida* species are the second most frequent isolates from blood cultures in hospitals with
large population of immunocompromised patients, second only to coagulase-negative *Staphylococcus aureus* (Banerjee *et al.*, 1991). Invasive pulmonary aspergillosis is a leading cause of attributed mortality in bone-marrow transplant recipients (Beck-Sague and Jarvis, 1993).

2.0 TREATMENT

2.1 Existing Antifungal Drugs:

There has been extensive research on the development of antifungal drugs, but only few of the antifungal agents were licensed for use. These include polyene, amphotericin-B, miconazole, ketoconazole, fluconazole, itraconazole and one pyrimidine synthesis inhibitor flucytosine (Espinel-ingroff and Pfaller, 1995). However, it has been found that treatment with these drugs, especially for extended periods can lead to problems with toxicity to the patients (Amphotericin-B) or with development of resistant pathogenic organisms during the course of therapy (5-fluorocystine) (Boonchird and Flegel, 1982). Incidence of these opportunistic infections is on the increase, attempts are made to develop new chemotherapeutic agents or a combination of agents to inhibit the causative fungus (Table 2 and Fig.2).

Table 2: An overview of antifungal agents (Didomenico, 1999).

<table>
<thead>
<tr>
<th>Compound/Class</th>
<th>Mode of action</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin/ Polyene</td>
<td>Selective binding to ergosterol, major sterol of fungal membranes</td>
<td>Fungicidal Broad spectrum, Intravenous, little resistance observed significant nephrotoxicity.</td>
</tr>
<tr>
<td>Ablcet/polyne Ambisom</td>
<td>Selective binding to ergosterol, major sterol of fungal membranes</td>
<td>Liposomal function of AMB. Similar efficacy as AMB, reduced toxicity observed.</td>
</tr>
<tr>
<td>Amphote</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nyotran/Nystatin</td>
<td>Selective binding to ergosterol, major sterol of fungal membranes</td>
<td>Liposomal formulation of nystatin, lower toxicity compared to nystatin.</td>
</tr>
<tr>
<td>5-Fluorocytosine (5 FC)/nucleoside analog</td>
<td>Selective conversion to toxic intermediate</td>
<td>Most often given in combination with AMB for Cryptococcal meningitis, poor activity against</td>
</tr>
</tbody>
</table>

7
<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of Action</th>
<th>Clinical Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miconazoles/azoles</td>
<td>Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-demethylase</td>
<td>Static activity against yeast, dimorphic fungi, dermatophytes. General fungistatic activity.</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazoles/triazoles</td>
<td>Selective inhibition of fungal cytochrome P450 dependent lanosterol-14-demethylase</td>
<td>Broad spectrum including Aspergillus spp. FLU-resistant C. albicans strains and non-albicans strains increasing. Efficacious in immune compromised models.</td>
</tr>
<tr>
<td>Itraconazole, Voriconazole, Posaconazole, UR-9825, SYN-2869, BMS-207147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspofungin FK-463</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMS181184/ pradimicins</td>
<td>Calcium-dependent binding to manno-proteins in cell wall</td>
<td>Broad spectrum except for Fusarium. Oral hepatotoxicity led to discontinuation.</td>
</tr>
<tr>
<td>Nikkomycin's</td>
<td>Chitin synthase inhibitors</td>
<td>Liposomal formulation of nikkomycin. Limited spectrum for fungi. Effective against cells with high chitin content.</td>
</tr>
<tr>
<td>Terbinafine/allylamines</td>
<td>Squalene epoxidase inhibitors</td>
<td>Fungicidal, Active against dermatophytes, Topical and oral formulations.</td>
</tr>
<tr>
<td>Basifungin/aureobasidins</td>
<td>Inositol-P ceramide synthase inhibitor</td>
<td>Fungicidal broad spectrum.</td>
</tr>
<tr>
<td>Soradarin/Sordarins</td>
<td>Selective binds to fungal EF2/ribosomal stalk proteins</td>
<td>Fungicidal broad spectrum.</td>
</tr>
</tbody>
</table>
Fig. 2: Chemical structures of some systemic licensed antifungal drugs (a) Amphotericin-B (b) 5-fluorocytosine (c) miconazole (d) ketoconazole (e) fluconazole (f) itraconazole.
2.2 Inhibitors of Fungal Cell Membranes:

2.2.1 Polyenes:

The only polyene approved for systemic use is Amphotericin B (AMB). Its primary advantages include its fungicidal activity against most clinically relevant pathogens, and the low occurrence of resistance. The primary disadvantage of AMB is its nephrotoxicity. Ambisome, Abelcet and Amphocil/Amphotech (Fig. 3) all exert relatively similar efficacies with fewer side effects than AMB (Walsh et al., 1998). Composition of the lipid bilayer containing the polyenes appears to contribute to slight differences in efficacy as a result of both redistribution of the antifungal drug to tissues and the selective release of active AMB from the bilayer (Boswell et al., 1998).

**Lipid Amphotericin B formulations**

![Diagram of lipid formulations](image)

**Fig 3: Lipid formulations of Amphotericin-B.**

2.2.2 Azoles:

There is a wide variety of azoles that have *in vitro* efficacy, but only a few have had significant utility. Azoles inhibit cytochrome P-450-dependent lanosterol 14-α-demethylase, causing accumulation of methylated sterols, depletion of ergosterol and inhibition of cell growth (Koltin and Hitchcock, 1997). Sensitivity of other P-450-dependent enzymes accounts for their primary mode of toxicity. Although azoles
demonstrate a broad spectrum of activity with less toxicity than AMB, they are not generally fungicidal but rather fungistatic.

2.3 Inhibitors of Fungal Cell Wall:

Fungal cell wall (Fig. 4) is an ideal target for the search for novel, fungicidal compounds. Several of the enzymes involved in the biosynthesis of the cell wall are unique to fungi, including chitin and glucan synthases (Georgopapadakou, 1997).

Fig 4: Fungal cell wall (Adopted from www.doctorfungus.com).

2.3.1 Echinocandins and Pneumocandins:

β-1,3-Glucan synthase is the target of both the echinocandins and pneumocandins. 1,3Y203366 compound is both orally and parenterally active and more potent. It has in vitro and in vivo activity against numerous clinical isolates of C. albicans, H. capsulatum, A. fumigatus (Espingel-Ingoff et al., 2002; 1998). Caspofungin has partly fungicidal activity in vitro against some Candida spp. and some dimorphic fungi.
2.3.2 Nikkomycins:

These appear to act competitively as substrate analogs of UDP-N-glucosamine preventing the synthesis of chitin. Although chitin synthesis is an essential function, multiple isozymes present in fungi add a level of complexity. It has a narrow spectrum as a solo agent but has been shown to have either additive or synergistic effects in combination with azoles against a number of pathogens (Li and Rinaldi, 1999).

2.3.3 Pradimicins:

These exert their action by selectively binding to calcium-dependent binding of cell surface mannoproteins leading to cell membrane leakages and loss of viability (Watanable et al., 1996). These exhibit in vitro and in vivo activity (Oki et al., 1992). In a direct comparison with AMB, the compound is 40-to 50 fold less active, but also 130-fold less toxic.

2.4 Inhibitors of Protein Synthesis

2.4.1 Sordarins:

Sordarins are highly specific inhibitors of fungal translation. Several derivatives are active against C. albicans (Aviles et al., 1998). The ability of sordarins to selectively inhibit fungal translation underscores the possibility that other essential proteins, as well as EF2, may be important targets in antifungals.

3.0 NEW POTENTIAL TARGETS IN DEVELOPING ANTIFUNGAL DRUGS:

3.1 Fungal Cell Wall:

Fungal cell wall acts as the interface between the fungus and environment. It has several roles, which include providing the fungus with its shape and supporting it against osmotic forces. It acts as a filter, controlling the secretion and uptake of molecules into the cell. Some enzymes are also responsible for enzymatic conversion of nutrients into metabolizable form, prior to their entry in to the protoplast (Peberby, 1990). This structure is not only important to viability of the fungal cell, but also unique to fungi as it is not present in mammalian cells. These features make it an ideal target for study of antifungal agents.
3.1.1 β-1, 3-D-Glucan Synthase:

These are an abundant class of polysaccharides that are involved in structural and functional and certain morphological roles on the fungal cell surface (Fig.5). Membrane bound enzyme β-1,3-D-glucan synthase catalyses the synthesis of β-1,3-glucan, an essential glucose polymer found in fungi. It forms a fibril composed of three helically entwined linear polysaccharides, which provide rigidity and integrity to the cell structure. Since the β-(1, 3) glucan structure is not found in mammalian cells, the glucan synthase has become a target for research into antifungal agent development (Inoue et al., 1995).

3.1.2 Chitin Synthase:

Chitin (Fig.5) is a major structural component of the cell wall of many fungi. It is a (1, 4)-β-linked homopolymer of N-acetyl-D-glucosamine, and is produced by chitin synthase from the nucleotide UDP-GlcNAc and follows the reaction

\[ 2n \text{UDP-GlcNAc} \rightarrow (\text{GlcNAc-β-(1,4)-GlcNAc})n + 2n \text{UDP} \]

Chitin synthesis is cell cycle regulated; and the amount and distribution of chitin in the cell wall changes as the cell proceeds from vegetative growth to diploid.
formation and then sporulation. Since chitin is not present in mammalian cells, it has the potential to be a highly selective target for development of therapeutics.

3.1.3 Mannoproteins:
Mannose constitutes a major portion of the cell wall of many fungi (Fig.5), and the glycoproteins form the protective capsule. Mannoproteins are formed by O-linkages joining mannose and small oligosaccharides to the hydroxyl groups of the amino acids serine or threonine. A second type of linkage connects high molecular weight and highly branched mannoproteins to the proteins by N-acetylglucosamine and asparagines. Dolichol phosphate mannose synthase transfers mannose, from GDP-mannose to dolichol phosphate forming Dol-P-mannose, a key intermediate in protein glycosylation. The glycosylation of protein occurs in the rough endoplasmic reticulum, after which they are transported to the cell wall. From all these steps, mannoproteins are also highly potential targets for drug development (Kapteyn et al., 1995a; 1995b)

3.1.4 Extracellular Proteins:
Proteinases, Phosphlipases and hydrolases are present in the extracellular membrane. Proteinases play an important role in adhering to the host tissue thereby causing pathogenicity. SAP (Secreted Aspartyl Proteinases) is also thought to be produced as precursor’s forms containing a signal peptide. As SAP are important virulence factors for several types of fungal infections and the inhibition of these proteinases having a protective effect for the host (De Bernardis et al., 2001; Hube and Naglik, 2001). Antifungal drugs may have broad mode of actions and antifungal components may also influence the activity of SAP, which in turn may enhance the antifungal activity of a particular drug. So the extra cellular proteins could also be antifungal drug target.

3.2 Fungal Cytoplasmic Membrane:
Fungal plasmamembrane is similar to mammalian, containing phospholipids, sphingolipids, sterols and proteins. The main functions of the plasmamembrane are its fluidity, rigidity and transport mechanisms.

3.2.1 Sphingolipids:
These are essential components of plasmamembrane and modulation of them exerts a deep impact on cell viability (Hannun and Luberto, 2000). The presence and function of sphingolipids are common in both fungi and mammalian cells but their
pathways are different. So this represents a new strategy, suitable for the development of antifungal agents. The synthesis and metabolism appears to be conserved among non-pathogenic and pathogenic fungi (Zong et al., 2000).

3.2.2 Ergosterol Synthesis:

Ergosterol biosynthesis pathway and its targets sites for antifungal agents are known. Azole antifungal agents present the synthesis of ergosterol by inhibition of the cytochrome P-450-dependent enzyme, lanosterol demethylase. Azoles possess a much greater affinity for the fungal enzyme than mammalian cells and as such are currently the most widely used antifungal agents.

3.2.3 Plasma Membrane ATPase:

Plasma membrane ATPase is encoded by the PMA1 gene and controls both efflux and influx of cations across the plasma membranes. The fungal PMA1 enzyme differs considerably from the mammalian and plant enzymes, especially in transmembrane segments 1, 2, 3, and 4 (Monk et al., 1995). Site-directed mutagenesis of these regions frequently results in lethal mutations. These observations suggest that P-ATPase pumps can be considered potential targets for the development of new antifungal agents.

4.0 ANTIFUNGAL DRUG RESISTANCE:

Pathogenic fungi present a threat not only to immunocompromised patients with immune systems weakened by AIDS, aggressive cancer chemotherapy, or drugs aimed at foiling rejection of transplanted organs but also to others, particularly when microbes are resistant to antifungal agents (McGinnis et al., 1997; Odds et al., 2000). For instance, 33% of late-stage AIDS patients in one study had drug-resistant strains of Candida albicans in their oral cavities (Denning et al., 1997). The rise in the incidence fungal infections has exacerbated the need for next generation of antifungal agents, since many of currently available drugs (Fig. 6 and 7) have undesirable side effects, are ineffective against new or reemerging fungi or lead to rapid development of resistance (Vanden Bossche et al., 1994; Graybill 1988). This drug resistance has resulted in drastic increase in incidence of opportunistic and systemic fungal infections. Resistance is considered as primary when an organism is resistant to drug before exposure and whereas, in secondary resistant is that which develops in response to the drug. The latter mechanism of resistance accounts for the emergence of resistant fungi to azoles and polyenes seen over last few years (White, 1997).
Fig 6: Schematic representation of clinical resistance.

The prevalence of resistant strains is due to:

- An increased reliance on antimicrobial medication, giving resistant strains a selective advantage.
- The recent trend towards aggressive resuscitation and invasive surgery, favoring infection.
- The treatment of more immunocompromised patients such as very elderly, the HIV positive and intentionally immunodepressed.

There are only few available drugs for treatment of fungal infections in immunocompromised patients or in severe systemic pathology i.e, therapeutic choices for treatment of fungal infections are limited. Search for new compound with low toxicity and stability is a priority in field of anti-fungal therapy.

Antifungal drugs basically belong to two broad categories:

(a) Those made synthetically.
(b) Those produced by various organisms.

Most people become interested in synthetic drugs because of their quick action as compared to traditional medicines and secondly because of their bulk production in industries. New microbes and their products are discovered for medicinal uses. Their
products were extracted and then synthesized in the laboratory. Since 1970, almost 75% of such medicines are of synthetic origin or products of fermentation (Brewer, 2000).

Fig 7: Medical mycology of the last 50 years (Source: WHO 2000).

4.1 Occurrence of Resistance in Fungi

The incident of fungal infections, including resistant infections, has increased during the last ten years, reflecting increased incidence of immunodeficiency associated with cancer chemotherapy, organ and bone marrow transplantation and HIV epidemic (Mary et al., 2004). More than 450,000 patients annually suffer from serious, systemic infections caused by fungi and number is expected to increase significantly. Existing drugs are not adequately addressing the problem and mortality rate remains high. Recently it was reported that 30% of patients with advanced AIDS have developed azole resistance to Candida infections (Revankar et al., 1996). Nearly 60% of patients infected with Candidiasis have developed fluconazole-resistant Candida isolates (Cartledge et al., 1997). Amphotericin B is used in the treatment of serious disseminated dimorphic fungal and yeast infections, caused by Blastomyces, Candida, Cryptococcus and Histoplasma sps. However, it causes nephrotoxicity, reduction of renal blood flow, nausea, vomiting and anorexia. Nystatin, although too toxic for systemic use, is mainly applied topically in cases of mucous membrane Candidiasis.

Griseofulvin causes hepatotoxicity and gastrointestinal distress but is used for the treatment of certain dermatophyte infections, caused by Epidermophyton, and
Trichophyton species. 5-Fluorocytosine interferes with DNA synthesis and causes bone-marrow toxicity, leukopenia and liver enzyme elevations. The predominant factors that affect and limit the use of some of the existing antifungal antibiotics, are low potency, poor solubility, limited or inconvenient dosage forms, narrow clinical spectrum, rapid emergence of resistant strains and drug toxicity (McGinnis and Rinaldi, 1991; Odds, 2000).

The possible mechanisms of drug resistance are: (1) changes in membrane permeases (Kurtz, 1998), (2) changes in cellular efflux mechanism (Clark et al., 1996), (3) changes to a particular fungal “activase” whose action is required before that agent becomes metabolically active, and (4) mutations that render the target enzyme less sensitive or insensitive to the antimycotic agents. In addition, regulatory mutations that increase cellular levels of an essential enzyme can also provide means of resistance to a particular agent. For instance, increased production of lanosterol demethylase enables Candida albicans to withstand antimycotic agents such as amphotericin B (Kurtz, 1998; Wills et al., 2000).

5.0 MEDICINAL PLANTS:

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs” (Sofowora, 1982). This definition of medicinal plant has been formulated by WHO (World Health Organization). The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as “Medicinal Plants”. Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medically important. It has now been established that the plants which naturally synthesize and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties (http://www.mapbd.com.).

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries. In this way indigenous medicinal plants play significant role of an economy of a country.
5.1 Medicinal plants – Their Secondary Metabolites:

Natural products are typically secondary metabolites, produced by organisms in response to external stimuli such as nutritional changes, infection and competition (Cotton, 1996; Strohl, 2000). Natural products produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. Approximately one-third of the top-selling drugs in the world are natural products or their derivatives often with ethno pharmacological background. Moreover, natural products are widely recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. Natural products are organic compounds that are formed by living systems. The elucidation of their structures and their chemistry, synthesis and biosynthesis are major areas of organic chemistry. Naturally occurring compounds may be divided into three broad categories. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars. They are known as primary metabolites. Secondly, there are the high molecular weight polymeric materials such as cellulose, the lignins and the proteins which form the cellular structures. Finally those compounds are characteristic of a limited range of species. These are the secondary metabolites. Secondary metabolites, otherwise called as phytopharmaceutical’s help the plants for survival helping in many ways as antimicrobials to resist pathogens toxins, to deter the large insects and large herbivores, pollination guide to guarantee effective pollination, allelochemics to control the plants of the neighbour hood and to protect the plant from a highly oxidizing environment. They have decisive role in dormancy, vegetation patterning, pollination and seed dispersal. Time is not a off when we have to accept that the higher plants owe their survival and success to their secondary products. The established fact is that those plants which produce better chemicals proliferated best becoming dominant on earth, while those plants having less efficient chemicals evolved slowly as failed to evolve further.

Plants during the course of their life encounter infection by a variety of bacteria, fungi, viruses and parasites specific to them. They are expected to synthesize a variety of secondary metabolites capable of providing them protection against the infecting agents. These may include a wide variety of antibacterial and antifungal compounds. These compounds protect the plant from seedling stage to the fruiting
stage. The water soluble antimicrobial compounds leach out in to the soil at the time of seed germination and sterilize the surrounding environment so that the thunders radical and plumose are not affected. The young leaves, flowers, and fruits possess various compounds which protect the plants from microbes, so also the roots, bark and wood.

The main classes of antimicrobial compounds are the following phenolics, sulphur containing compounds, iridoides and phytoalexins. The components of the volatile oils, the monoterpenes and sesquiterpenes are markedly antimicrobial in nature. These increase the resistance of plants to microbial attack. But the volatile oils are found to be less efficient in a tropical climate where they evaporate. Therefore, only a few angiosperms develop volatile oils in leaves or stems. They are the Rutaceae, Geraniaceae, Apiaceae, Lamiaceae and Asteraceae. The Asteraceae volatile oils are peculiar in containing sesquiterpenes lactones, which act as alio chemics too. It is widely reported that 150 species of forest and road side trees (including shrubs) in India produce oilseeds which are popularly known as minor or non-edible oils. Most of the oils contain valuable active principles and chemical compounds useful as medicines and pesticides.

5.2 Antimicrobial Activity of Some Plant Constituents- An Overview:

Some plant secondary metabolites which are synthesized by photosynthesis via various pathways have antimicrobial activities.

5.2.1 Tannins:

Tannins are water soluble polyphenols, which differ from other natural phenolic compounds in their ability to precipitate proteins such as gelatin from solution (Bruneton, 1995). They differ in that respect from simpler phenols such as catechol, pyrogallol, gallic acid, catechin, epicatechin. They are commonly found in large array of higher plant species of both herbaceous and woody types. They accumulate in large amounts (often more than 10% of the dry weight) in particular organs or tissues, which can be almost any plant: bark, wood, leaves, fruits, or roots (Haslam, 1989). They are classified in two groups according to their structures proanthocyanidins (condensed tannins) and hydrolysable tannins. One of their molecular actions is to complex with polymers such as proteins and polysaccharides through so called non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996). Thus their mode of
antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport protein and complex with cell wall (Cowan, 1999). Scalbert (1991) reviewed the antimicrobial properties of Tannins in which he listed 33 studies which had documented the inhibitory activities of tannins. Tannins can be toxic to filamentous fungi, yeasts and bacteria (Taylor et al., 1995).

5.2.2 Flavones, Flavonoids and Flavonols:

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-OH group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. They are found in fruits and vegetables essential for processing vitamin-C and needed to maintain capillary walls. They may aid in protecting against infections (Mosby Medical Encyclopedia, 1997). These are water soluble, responsible for colour of flowers and fruits and some times, biflavonoids are dimmers of flavonoids. The majority of natural biflavonoids are dimers of flavones and flavonones. These are known to be synthesizing in plants, in response to microbial infections, they have been found in vitro to be effective antimicrobial substances against a wide range of microorganisms (Cowan, 1999). Their activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell walls.

Flavonoids isolated from the leaves of C. micranthum have been shown to have antimicrobial activity against Gram positive and Gram negative microorganisms (Rogers and Verrotta, 1996).
5.2.3 Terpenoids and Essential oils:

The fragrance of plants is carried in the so-called quintessential or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is C_{10}H_{16}, and they occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30}, and C_{40}) as well as hemiterpenes (C_{3}) and sesquiterpenes (C_{15}). If the compound contains additional elements, usually oxygen, they are termed as Terpenoids. These are synthesized from acetate units, and as such, they share origins with fatty acids. They differ from fatty acids in that they obtain extensive branching and are cycled. These are active against bacteria and fungi (Taylor et al., 1995). The mechanism of action of terpenes is not fully understood but it is speculated to involve membrane disruption by the lipophilic compounds.

5.2.4 Alkaloids:

Heterocyclic nitrogen compounds are called alkaloids. Indo quinine alkaloids, the active principles in Cryptolepis sanguinolenta has been shown to inhibit Gram
negative bacteria, yeast (Silva et al., 1996). Additional studies have shown this plant to have bactericidal property. Recent in vitro studies have shown the activity against bacteria specifically enteric pathogens and some activity against Candida sps (Sawer, 1995).

![Structure of Colchicine.](image)

5.2.5 Saponins:

The saponins are mostly non-nitrogenous water soluble glycosides which reduce surface tension and produce lather on shaking. They are hydrolyzed by acids to yield genins on one hand and sugars on the other. Saponins have been found to possess various pharmacological and biological activities. They have been reported to possess property of asphyxiation of lice and other skin parasites and, therefore, find excessive applications in shampoos and other cosmetic preparations. Recently, saponins have been found to possess spermicidal, cardiovascular, fungicidal, spasmolytic, expectorant, and antihistaminic and anti-tussive activities, and economic importance lies in their facile conversion to medicinally used steroidal hormones. Most of the saponins having triterpenoidal base showed better activity than the saponins having steroidal base. (www.wikipedia.org/wiki/saponin).

6.0 ANTIOXIDANTS:

Antioxidant is defined as any substance that, when present at low concentration, compared with those of oxidizable substrate, considerably delays or inhibits oxidation of the substrate (Gutteridge, 1995). Antioxidants can act at different stages in an oxidative sequence. They show their action by:

- Removing oxygen or decreasing local oxygen concentration.
- Removing catalytic metal ions.
- Removing key ROS such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$. 

23
Scavenging by initiating free radicals such as hydroxyl, alkoxyl and peroxyl species.

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species (ROS), which are continuously produced in vivo, result in cell death and tissue damage. ROS play important roles in some pathogenesis of serious diseases, such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular diseases, atherosclerosis, cataracts, diabetes and inflammation (Halliwell et al., 1995). Compounds that can scavenge free radicals have great potential in ameliorating these diseases (Kirakosyan et al., 2004). Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), perhydroxy radical (HOO') and hydroxyl radical (HO•). These radicals are formed by a one electron reduction process of molecular oxygen (O$_2$). ROS can easily initiate the lipid peroxidation of the membrane lipids, causing damage of the cell membrane of phospholipids, lipoprotein by propagating a chain reaction cycle. Thus, antioxidant defense systems have coevolved with aerobic metabolism to counteract oxidative damage from ROS. Most living species have efficient defense systems to prevent themselves against oxidative stress induced by ROS. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases like aging process. In this respect flavonoids and other polyphenolic compounds have received the greatest attention.

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. Due to the presence of the conjugated ring structures and hydroxyl groups, many phenolic compounds have the potential to function as antioxidants by scavenging superoxide anion (Robak and Dryglewski, 1988), singlet oxygen (Husain et al., 1987), and lipid peroxy radicals (Torel et al., 1986), and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Shahidi et al., 1994). There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage (Silva, et al., 2006). Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants in vitro than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol
derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1996).

Plants are a major source of phenolic compounds, which are synthesized as secondary metabolites during normal development in response to stress conditions, such as wounding and UV radiation among others (Stahl and Sies, 2003; Close et al., 2005). Plants may contain simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignins and lignans (Naczk and Shahidi, 2006). Distribution of phenolics in plants at the tissue, cellular and subcellular levels is not uniform. Insoluble phenolics are found in cell walls, while soluble phenolics are present within the plant cell vacuoles (Randhir and Shetty, 2005; Bengoechea et al., 1997). Cell wall phenolics may be linked to various cell components such as sugars (Chaoui and El Ferjani, 2005). Therefore, the nature of polyphenol compounds in plants is complex. The biosynthesis of phenolic compounds and related substances is derived from some proteins, including tyrosine and tryptophan, in the shikimic acid pathway. In addition, the phenolics usually occur in bound form such as flavonoid glycosides and phenolic acid derivatives, which are synthesized from sugars. An interesting aspect is the complex relation between chemical components and phenolic compounds in plants.

6.1 Oxidative Stress:

Oxidative stress has been defined as "a disturbance in the pro-oxidant/antioxidant balance in favour of the former, leading to potential damage" (Sies, 1991). Mammalian cells generate reactive oxygen species (ROS) during normal metabolic processes. The cell has several ways to respond to ROS. It can either repair and remove the damaged nucleotides and lipid peroxidation by-products or directly reduce the ROS via enzymatic and non-enzymatic antioxidants.

6.1.1 Reactive Oxygen Species:

The incomplete reduction of molecular oxygen during cellular metabolism or spontaneously by auto-oxidation reactions in the environment can result in formation of reactive oxygen intermediates (ROIs) such as superoxide radicals (O2•−), hydrogen peroxide (H2O2), hydroxyl radicals (OH•) and singlet oxygen (1O2) (Fridovich, 1978). These ROI's can damage cell components such as DNA, RNA, protein and lipids (Fig.8).
6.2 Molecular Oxygen:

Theoretically, O₂ should be an excellent terminal electron acceptor because the E’o of the O₂/H₂O half-cell system is very high (+0.8 V at pH 7.0). Oxygen in its ground state is a non-toxic triplet inorganic molecule which has one unpaired electron in each of its two π* outer antibonding orbitals. However, due to the parallel directions of spin of these electrons, molecular oxygen cannot always accept two electrons readily from a reduced molecule. O₂ must accept a pair of electrons having a spin direction opposite to that of the two unpaired electrons of the O₂ molecule, thus obeying the Pauli Exclusion Principle (Martinez-Cayuela, 1995). This requirement restricts the range of compounds oxidized by oxygen (Farr and Kogoma, 1991). The alternative to spontaneous two-electron reduction is a one-electron reduction that leads to formation of O₂⁻. The reduction of O₂ to H₂O as the terminal reaction of an electron transport system requires four electrons (O₂ + 4e⁻ + 4H⁺ → 2 H₂O) and does not generate O₂⁻. However, partial reduction of O₂ can generate ROIs, as indicated below (Salin and Brown-Peterson, 1993):

\[
O₂ + 1e^- \rightarrow O₂^-.
\]
6.3 Singlet Oxygen ($^1\text{O}_2$):

Singlet oxygen is an energized form of $\text{O}_2$ in which the direction of spin of one unpaired electron of ground-state dioxygen is reversed by an input of energy. This can give rise to either of two forms of singlet oxygen: $\text{O}_2 (^1\Sigma_g)$, in which the two electrons continue to occupy separate orbitals and $\text{O}_2 (^1\Delta_g)$, in which the two electrons occupy one orbital and neither occupies the other orbital. Singlet oxygen is not a radical therefore does not possess unpaired electrons. Singlet oxygen is highly reactive because the spin restriction associated with ground state $\text{O}_2$ has been removed. It can subsequently oxidize a large variety of biological molecules such as lipids, proteins, and DNA and is responsible for cell destruction caused by light and some photosensitizers (Weters, 1987; Sies and Menck, 1992). $^1\text{O}_2$ can be formed in a number of chemical, photochemical, and biochemical systems involving photo oxidations, free radicals and lipid peroxides (Murray, 1979).

6.4 The Superoxide Radical ($\text{O}_2^-$):

The univalent reduction of molecular oxygen produces the superoxide radical, which has one unpaired electron. Superoxide radicals exhibit moderate reactivity towards biomolecules in an aqueous environment compared to other ROIs and are capable of acting as either a reductant or oxidant. This moderate activity allows $\text{O}_2^-$ to diffuse for relatively long distances in biologic systems and thus can be generated at sites distant to the site at which it eventually causes toxicity (Miller and Britigan, 1995). Superoxide can be generated enzymatically by certain flavoprotein dehydrogenases or non-enzymatically through the auto oxidation of molecules such as ferredoxins, hydroquinones, and thiols (Fridovich, 1978; Salin and Brown-Peterson, 1993). The superoxide radical has been reported to exert a direct effect on certain enzymes such as catalase (Kono and Fridovich, 1982), aconitase (Gardner and Fridovich, 1992) and glutathione peroxidase (Blum and Fridovich, 1985). However its main role in oxygen toxicity is probably due to its dismutation to form $\text{H}_2\text{O}_2$ or its interaction with $\text{H}_2\text{O}_2$ in an iron catalyzed Haber-Weiss reaction which can produce

\[
\text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 \\
\text{O}_2 + 3\text{e}^- + 3\text{H}^+ \rightarrow \text{OH}^- + \text{H}_2\text{O}
\]
reactive hydroxyl radicals (Salin and Brown-Peterson, 1993). The dismutation to \( \text{H}_2\text{O}_2 \) occurs when one \( \text{O}_2^- \) gives up its electron to another \( \text{O}_2^- \) as follows:

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

Superoxide radicals will dismutate spontaneously but the reaction is limited by the electrostatic repulsion of the two anions (Fridovich, 1978). At pH 13.0 superoxide radicals have a half-life of about 160 min whereas at pH 7.0 it is approximately a millisecond. The half-life is about 100 times less at pH 4.8, which is the pK value, where equal concentrations of the ionized and nonionized forms are present (\( \text{O}_2^- \) and \( \text{HO}_2^- \)).

\[
\text{HO}_2^- + \text{O}_2^- \rightarrow \text{HO}_2^- + \text{O}_2
\]

At pH 4.8 there is no charge repulsion, and dismutation takes place faster. The rate actually decreases from pH 4.8 to pH 2.0 and then remains constant below pH 2.0. The decrease occurs because the reaction

\[
\text{HO}_2^- + \text{HO}_2^- \rightarrow \text{HO}_2^- + \text{O}_2
\]

is slower than

\[
\text{HO}_2^- + \text{O}_2^- \rightarrow \text{HO}_2^- + \text{O}_2
\]

The iron catalyzed Haber-Weiss reaction occurs in two steps as follows:

\[
\text{O}_2^- + \text{Fe}^{3+} \text{chelate} \rightarrow \text{O}_2 + \text{Fe}^{2+}
\]

\[
\text{Fe}^{2+} \text{chelate} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} \text{chelate} + \text{OH}^- + \text{OH}^-
\]

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2
\]

In this series of reactions, \( \text{O}_2^- \) acts as a reducing agent for the iron in \( \text{Fe}^{3+} \) chelate. Other reducing agents can accomplish the same reduction and thus superoxide radicals are not absolutely necessary for the generation of hydroxyl radicals. The second step, in which ferrous ions (produced from ferric ions by whatever mechanism) reduce \( \text{H}_2\text{O}_2 \) is called the Fenton reaction (Goldstein et al., 1993)

6.5 Hydrogen Peroxide (\( \text{H}_2\text{O}_2 \)):

The most stable of the oxygen intermediates is hydrogen peroxide, which is not a free radical. It results from the addition of two electrons to \( \text{O}_2 \) or as a product of dismutation of superoxide radicals. \( \text{H}_2\text{O}_2 \) is a more reactive oxidant than \( \text{O}_2^- \) and, being uncharged and soluble in organic solvents, it readily crosses biological
membranes. The reactions of H₂O₂ with organic molecules remain unclear, partly because it reacts quickly in the presence of contaminating metals to form other ROIs which obscure its own role in oxidation reactions (Farr and Kogoma, 1991). It can act as a weak oxidizing agent and can damage DNA (Steiner et al., 1984), lipids (Kellogg and Fridovich, 1977) and can attack thiol groups of proteins or reduced glutathione. It can also react directly with some keto acids (Halliwell and Gutteridge, 1990; Wefers and Sies, 1983). Most importantly, H₂O₂ will react with reduced iron or copper ions to generate hydroxyl radicals (OH') in the Fenton reaction (Cadenas, 1989).

Certain reactions catalyzed by flavoproteins such as xanthine oxidase or NADPH oxidase generates H₂O₂ by forming O₂⁻ as an intermediate, which can then dismutate. The O₂⁻ is generated when the reduced prosthetic group, FADH₂ reacts spontaneously with two molecules of O₂ (Fig. 9).

\[ \text{FADH}_2 + 2 \text{O}_2 \rightarrow 2 \text{O}_2^- + \text{FAD} \]

The 2O₂⁻ then undergo dismutation to yield O₂ and H₂O₂. In other oxidase reactions, however, H₂O₂ can be generated directly by a two-electron reduction of O₂ without formation of O₂⁻ as an intermediate (Salin and Brown-Peterson, 1993).

\[ \text{FADH}_2 + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{FAD} \]

Both O₂⁻ and H₂O₂ can also be generated nonenzymatically during the auto oxidation of various reduced flavins, quinones, thiols, and iron/sulfur proteins (Fridovich, 1978; Misra and Fridovich, 1971).

6.6 The Hydroxyl Radical (OH'):

Hydroxyl radicals result from the univalent reduction of H₂O₂. Hydroxyl radicals are extremely powerful oxidants (the E°; pH 7.0) of the reaction OH⁺ + e⁻ → OH⁻ is +2.33 V) and have the potential to cause oxidative damage to almost any cell component. Hydroxyl radicals have a short half-life in solution since they react with other molecules at nearly diffusion controlled rates. The main source of hydroxyl radicals is the metal catalyzed Haber-Weiss reaction as described above (Martinez-Cayuela 1995).

6.7 Sources of Reactive Oxygen Species:

Free radicals and various reactive oxygen species are continuously produced in the body (Halliwell and Chirico, 1993; Rice-Evans and Burdon, 1993; Halliwell,
1997; Halliwell et al., 1995). They can be formed as a by-product in the mitochondrial respiratory chain due to leakage of electrons from the electron transport chain or by reactions catalysed by transition metal ions such as iron and copper ions. They may also be derived from external sources such as cigarette smoke, radiation, UV light, pollution and from the metabolism of certain drugs. The free radicals formed can react with DNA, proteins and lipids in the body and cause extensive oxidative damage (Halliwell and Chirico, 1993; Rice-Evans and Burdon, 1993; Halliwell, 1994; Halliwell et al., 1995).

Free radicals (Table 3) are not only produced as an unwanted product; they are also formed deliberately in the body for useful purposes and have important physiological functions (Halliwell and Chirico, 1993; Rice-Evans and Burdon, 1993; Halliwell, 1994; Halliwell et al., 1995). A well-defined role for free radicals is when activated phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) produce superoxide anion radicals and hydrogen peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses (Curnutte and Babior, 1987). In addition, free radicals are also produced by an array of enzymes e.g. pyruvate metabolising enzymes, oxidases, carboxylases, hydroxylases, peroxidases, fruit ripening enzymes and radical enzymes (Halliwell and Gutteridge, 1999).

Table 3: Types of reactive oxygen species.

<table>
<thead>
<tr>
<th>ROS</th>
<th>Types</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radicals</td>
<td>Superoxide</td>
<td>O_2^-</td>
</tr>
<tr>
<td></td>
<td>Hydroxyl</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td>Alkoxy</td>
<td>LO/RO'</td>
</tr>
<tr>
<td></td>
<td>Peroxyl</td>
<td>LOO' /ROO'</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide</td>
<td>NO'</td>
</tr>
<tr>
<td></td>
<td>Thyl radical</td>
<td>R-S'</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide</td>
<td>H_2O_2</td>
</tr>
<tr>
<td></td>
<td>Hypochlorous acid</td>
<td>HOCl</td>
</tr>
<tr>
<td></td>
<td>Ozone</td>
<td>O_3</td>
</tr>
<tr>
<td></td>
<td>Singlet oxygen</td>
<td>^O_2</td>
</tr>
<tr>
<td></td>
<td>Peroxynitrite</td>
<td>ONOO'</td>
</tr>
<tr>
<td></td>
<td>Lipid peroxide</td>
<td>LOOH</td>
</tr>
</tbody>
</table>
6.8 Oxidative-Damage Targets and Types:

The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components (Comporti, 1989) and alters many cellular functions (Gracy et al., 1999). Among the biological targets most vulnerable to oxidative damage are proteinaceous enzymes (Halliwell and Gutteridge, 1999, Levine and Stadtman, 2001), lipidic membranes (Davis, 1987; Halliwell and Gutteridge, 1999), and DNA (Beckman and Ames, 1998; Halliwell and Gutteridge, 1999).

6.9 Lipid Peroxidation:

The process of lipid peroxidation involves a set of chain reactions that are initiated by the abstraction of a hydrogen atom from the carbon in an unsaturated acyl chain by ·OH (Fig. 10). Then the carbon-centered lipid radical (·L) is oxidized by oxygen and forms lipid peroxyl radical (LOO·). Lipid peroxyl radical can propagate the peroxidation chain reaction and cause another abstraction of hydrogen from other vicinal unsaturated fatty acids. The series of chain reactions can spread into remote sites (Southorn and Powis, 1988), resulting in the production of several alkanes, hydroxyl, epoxy derivatives, alcohols, ketones and aldehydes (e.g. malondialdehyde).

Lipid peroxidation can alter the fluidity, selective permeability and under extreme circumstances, the integrity of cell membranes, thus affecting the viability of...
cells. The end products of lipid peroxidation, such as water-soluble aldehydes can act as cross linking agents causing protein to aggregate; one example is the formation of age pigment, lipofuscin (Davis, 1987). Lipid peroxidation products also cause the inhibition of protein synthesis (Fraga et al., 1989), alterations in enzyme functions and they can react with nitrogenated bases of DNA, giving rise to mutations and altered patterns of gene expression (Demple et al., 1986).

Fig 10: Lipid peroxidation pathway (Source: Ho-Yan Yiu, 2000).
6.10 DNA Damage:

Nucleic acids are also targets of ROS. It is shown that mitochondrial DNA (mtDNA) is more susceptible to ROS attack than nucleic DNA (Agarwal and Sohal, 1994; Yakes and van Houten, 1997) because ROS are mainly generated in mitochondria and mtDNA is not shielded by histone proteins as in the case for nuclear DNA (Fig.11 and 12). The nucleotide bases in DNA molecules, especially the pyrimidines, are vulnerable to free radical attack. Alterations in the pyrimidine ring results in local distortions of the double helix structure (Tice et al., 1985) and consequently causes DNA breaks, sister chromatid exchange, DNA-DNA and DNA-protein cross-linking and base modifications. The replication process can be affected by DNA damages. This occurs when the DNA polymerase encounters strand lesions and the enzyme misreads the modified genetic sequences, as a result a faulty DNA daughter strand will be generated.

8-Hydroxydeoxyguanosine (8-OHdG), one product of oxidized nucleotide is used as an indicator of DNA damages. For e.g. it has been demonstrated that both 8-OHdG level and protein carbonyl content increase with age (Sohal et al., 1995, Sohal, 1997), showing that DNA damage and protein oxidation are biochemical processes associated with aging.

![Diagram](attachment:image.png)

**Fig 11:** ROS-induced DNA damage activates PARP and modifies GAPDH (Brownlee, 2005).
ROS-induced oxidative damage

Release from cellular and non-cellular storage

\[ \text{NO}^+ \quad (\text{Nitric oxide radical}) \]
\[ \text{O}_2^- \quad (\text{Hydroxyl ion radical}) \]
\[ \text{O}_2 \quad (\text{Oxygen}) \]

Spontaneous and/or enzymatic dismutation

\[ \text{Mn}^{3+} \quad (\text{Transition metals}) \]
\[ \text{Fe}^{3+}, \text{Cu}^{2+} \]

Reduction of the metals

\[ \text{O}_2 \quad (\text{Oxygen}) \]

Fenton-like

\[ \text{H}_2\text{O}_2 \quad (\text{Hydrogen peroxide}) \]

Myeloperoxidase

\[ \text{ONO}^- \quad (\text{ Peroxynitrite}) \]

CT

\[ \text{ClO}^- \quad (\text{Hypochlorous acid}) \]

\[ \text{OH}^- \quad (\text{Hydroxyl radical}) \]

\[ \text{DNA} \quad (\text{e.g., base modification, single- and double-strand breaks}) \]

\[ \text{Lipids} \quad (\text{e.g., peroxidation, fatty acid loss}) \]

\[ \text{Proteins} \quad (\text{e.g., degradation, fragmentation, carbonyl, peroxidation, modification and inactivation}) \]

\[ \text{RO}^- \quad (\text{Alkoxyl radical}) \]

\[ \text{ROO}^- \quad (\text{Peroxyl radical}) \]

\[ \text{ROOH} \quad (\text{Oxy radical}) \]

Fig 12: Reactive oxygen species (ROS)-induced oxidative damage (Kohen and Nyska, 2002).