CHAPTER I

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Fungi are ubiquitous to the environment and are considered to be primarily saprophytic in nature. During the digestion process fungi secrete enzymes into the nutrient source to breakdown complex compounds into simpler compounds, which are taken up by the fungi and digested. These digested nutrients are classified into two categories primary and secondary metabolites. The primary metabolites consist of cellulose and other compounds that are used for energy to grow and reproduce.

The secondary metabolites are commonly known as antibiotics and mycotoxins (Pelczar et al., 1993). They are produced to give fungi a competitive edge against other microorganisms including other fungi. Antibiotics are special kind of chemotherapeutic agents usually obtained from fungi and used for the treatment of various human diseases. Whereas, the other form of secondary metabolites includes mycotoxins, which are well known for causing variety of short-term, as well as, long-term health effects, ranging from immediate toxic response to potential long-term carcinogenic and teratogenic effects in human beings and other animals. Illnesses caused by consuming contaminated food/feed stuffs are conveniently referred as primary mycotoxicosis. Sometimes toxicosis may occur as a result of ingestion of animal products such as milk, milk products or meat which themselves are not contaminated by moldy growth. Illnesses arising from such sources are referred as secondary mycotoxicosis (Fig. 1.1).

There are over 200 recognized mycotoxins, however the study of mycotoxins and their health effects on human is in its infancy and many more are waiting to be
Fig: 1.1 Primary and secondary mycotoxicosis
discovered (FAO, 1977; 97). Generally mycotoxins are a group of low molecular weight organic compounds characterized by their diversity, their frequent specificity with regard to the taxonomy of the producing organisms and their production during the stationary phase of batch cultures.

Mycotoxins are produced in cereals either during pre or post-harvest conditions by the growth of toxigenic fungi (Langseth et al., 1993). The most significant mycotoxins are contaminants of agricultural commodities, food and feeds. The Academy of Grain Technology (Webley and Jackson, 1998) has been analyzing cereals and other food samples for mycotoxins for over a decade. The mycotoxins most frequently found in cereals are shown in Table 1.1.

Among all mycotoxins identified so far, ochratoxins have received considerable attention because of its toxicological characteristics and intend ability to affect kidneys alongwith other organs.

**OCHRATOXINS**

Ochratoxins are one of the natural mycotoxins which are secondary toxic fungal metabolites produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum* (Krogh et al., 1974; Pitt, 1987). The ochratoxins were initially discovered in a laboratory during the screening of a large number of fungal cultures for toxicity by South African workers (Van der Merwe et al., 1965 a, b). They attributed the toxicity of a strain of *Aspergillus ochraceus* Wilhelm reported by Scott et al. (1972) to ochratoxin A the main component in the culture extracts.

Ochratoxin A was originally described as a metabolite of *Aspergillus ochraceus*. Apart from *Aspergillus ochraceus*, ochratoxin A is also produced by *Penicillium*
Table: 1.1 Common mycotoxins in grains.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Mold source</th>
<th>Commodities</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Pre-harvest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aflatoxin</td>
<td><em>Aspergillus flavus</em></td>
<td>Maize</td>
</tr>
<tr>
<td>2. Deoxynivalenol (DON)</td>
<td><em>Fusarium graminearum</em></td>
<td>Cereals</td>
</tr>
<tr>
<td>3. Zeralenone</td>
<td><em>Fusarium spp</em></td>
<td>Corn, maize</td>
</tr>
<tr>
<td>4. Fumonisin</td>
<td><em>Fusarium moniliforme</em></td>
<td>Maize, sorghum</td>
</tr>
<tr>
<td>5. Tenuazonic acid</td>
<td><em>Alternaria tenius</em></td>
<td>Cereals, oil seeds</td>
</tr>
<tr>
<td>6. Atenariol</td>
<td><em>Alternaria spp</em></td>
<td>Cereals</td>
</tr>
<tr>
<td>B. Post-harvest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aflatoxin</td>
<td><em>Aspergillus flavus</em></td>
<td>Cereals, pulses</td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus parasiticus</em></td>
<td></td>
</tr>
<tr>
<td>2. Ochratoxin</td>
<td><em>Aspergillus ochraceus</em></td>
<td>Cereals</td>
</tr>
<tr>
<td></td>
<td><em>Penicillium verrucosum</em></td>
<td></td>
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</table>
verrucosum. Aspergillus carbonarius has been recognized recently as another ochratoxin producing species (Wicklow et al., 1996).

1. *Aspergillus ochraceus*: It is a mesophillic xerophile. Growth occurs between 8 and 37°C, with optimum temperature 31°C. *Aspergillus ochraceus* has been isolated from salted dry fish, dried beans, soya beans, pepper, dried fruits, peanuts and walnuts. *Aspergillus ochraceus* has also been reported in cheese, spices, black olives and processed meat (Pitt and Hocking, 1997).

2. *Penicillium verrucosum*: It is a slow growing species and normally grows at lower temperature (0-31°C). It is normally found in cold temperate regions mainly Northern and Central Europe and Canada. It has been found in bread, cereal products, pig meat and especially flour-based food (Pitt and Hocking, 1997).

3. *Aspergillus carbonarius*: It grows at higher temperature (32-35°C) and these are the black Aspergilli and normally found in major grape growing regions in surrounding Mildura, Victoria and Australia. *Aspergillus carbonarius* is the major source of ochratoxin A in grapes and grape products, including wine and dried wine fruits (Varga et al., 1996).

**CHEMICAL STRUCTURE OF OCHRATOXIN A AND RELATED METABOLITES**

The structure was proposed on the basis of infrared, ultraviolet, nuclear magnetic resonance and optical rotatory dispersion spectra. The toxin was found to be composed of a dihydroisocoumarin moiety linked over its 7-carboxy group to L-β phenylalanine. The dihydroisocoumarin closely resembles mellein (3-methyl-8-hydroxy-3, 4, dihydroisocoumarin) and 4-hydroxymellein, known metabolites of some *Aspergillus ochraceus* strain and possible precursors of ochratoxin A (Cole et al., 1971; Hutchinson
et al., 1981). The other related metabolites have also got the dihydroisocoumarin moiety as the parental structure as mentioned below:

**Ochratoxin A** had $\lambda_{\text{max}}$ at 213 and 332 nm, with $\varepsilon$ (extinction coefficient) of 36800 and 6400 respectively. Ochratoxin A is formulated as $C_{20}H_{18}ClNO_6$, N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-oxo-1-$H_2$ benzopyran 7-yl cabonyl] L-phenylalanine. It yielded L-β phenylalanine and an optically active lactone-acid having molecular formula 7-carboxy-5-chloro-3,4-dihydro-8-hydroxy-3-methyl isocoumarin (ochratoxin α) ($C_{11}H_{9}O_5Cl$).

**Ochratoxin B** is the dechloro analogue of ochratoxin A and is also a colourless crystalline compound. The $\lambda_{\text{max}}$ is 218 and 318 nm and its $\varepsilon$ (extinction coefficient) is 37200 and 6900 respectively. The molecular formula is $C_{20}H_{19}NO_6$.

**Ochratoxin C** is the amorphous ethyl ester of ochratoxin A, with the molecular formula $C_{22}H_{22}ClNO_6$ and has $\lambda_{\text{max}}$ at 213, 331 and 378 nm and its $\varepsilon$ (extinction coefficient) is 32700, 4100 and 2050 respectively (Fig. 1.2).

Only ochratoxin A and very rarely ochratoxin B have been encountered as natural contaminant of food stuffs. On acid hydrolysis ochratoxin A yields phenylalanine and an optically active lactone-acid. Ochratoxin α is a metabolite which has been found in the kidney, liver, intestine, faeces and urine of test animals ingesting ochratoxin A contaminated-feed (Galtier et al., 1981).

Although natural occurrence of ochratoxin in agricultural products has been reported from various parts of the world and on vast array of crops, the levels of ochratoxins detected vary greatly from area to area. In general more quantities of ochratoxins occur in commodities from tropical and subtropical countries where
<table>
<thead>
<tr>
<th>BOX</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ochratoxin A</strong></td>
<td>Phenylalanine</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td><strong>Ochratoxin B</strong></td>
<td>Phenylalanine</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td><strong>Ochratoxin C</strong></td>
<td>Phenylalanine ethyl ester</td>
<td>Cl</td>
<td>H</td>
</tr>
</tbody>
</table>

Fig: 1.2 Chemical structures of ochratoxins (O’Brien and Dietrich, 2005).
environmental conditions are congenial for moldy growth and toxin production. In India ochratoxin A has been detected in the broken rice from Andhra Pradesh (Reddy et al., 1985) and in sorghum from Tamil Nadu (Manickam et al., 1985). Verma and Mathew (2000) have reported the presence of ochratoxin in different varieties of oil seeds, oil cakes and cereals in Bhavnagar district of Gujarat, India. Ochratoxin A contaminations of black pepper, coriander seeds, powdered ginger and turmeric powder have been reported. In 126 samples obtained from retail shops, ochratoxin A was found in 14/26 black pepper (15-69 μg/kg), 20/50 coriander seeds (10-51 μg/kg), 2/25 ginger (23-80 μg/kg) and 9/25 turmeric samples (11-102 μg/kg) (Thirumala et al., 2001). Apart from India and European countries, it is also found in Brazil, Canada, Dubai, Japan and USA (Pittet et al., 1996). It was reported in oat and rye samples in Denmark and Norway and in Maize samples in United Kingdom (Scudamore and Patel, 2000). Ochratoxin A contamination was also found in food stuffs in Africa (Jonsyn et al., 1995) and also in wheat, barley, mixed cereals, dried vegetables and olives collected from Tunisia (Maaroufi et al., 1995). Several countries have limits for ochratoxin A in different food products, which are shown in Table 1.2.

DISTRIBUTION OF OCHRATOXIN IN BODY

The small intestine is found to be the major site of absorption with maximal absorption from the proximal jejunum (Kumagai, 1988). Once the toxin reaches to the blood, it binds readily to serum albumin (Galtier et al., 1980) and other macromolecules (Hult and Fuchs, 1986). Once it binds to serum albumin the elimination is limited from blood stream to hepatic and renal cells (Kumagai, 1985). The rate of disappearance of ochratoxin A was slower from blood than from kidney, liver and other tissues in pigs.
Table: 1.2 Concentration limits for ochratoxin A in food products.

<table>
<thead>
<tr>
<th>Food Product</th>
<th>Concentration limit (μg/kg)</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereals, non-processed</td>
<td>5</td>
<td>Europe</td>
<td>Byrne (2002)</td>
</tr>
<tr>
<td>Cereals, processed</td>
<td>3</td>
<td>Europe</td>
<td>Byrne (2002)</td>
</tr>
<tr>
<td>Cereals</td>
<td>20-50</td>
<td>Brazil, Israel</td>
<td>FAO (1977)</td>
</tr>
<tr>
<td>Cereals</td>
<td>2</td>
<td>Switzerland</td>
<td>FAO (1977)</td>
</tr>
<tr>
<td>Dried vine fruits</td>
<td>10</td>
<td>Europe</td>
<td>Byrne (2002)</td>
</tr>
<tr>
<td>Milk products</td>
<td>0.5</td>
<td>Cyprus</td>
<td>FAO (1977)</td>
</tr>
<tr>
<td>Raw coffee beans</td>
<td>20</td>
<td>Greece</td>
<td>FAO (1977)</td>
</tr>
<tr>
<td>Pig kidneys</td>
<td>10</td>
<td>Denmark</td>
<td>FAO (1977)</td>
</tr>
</tbody>
</table>
(Hult et al., 1979). The tissue distribution in pigs, rats, mice, chickens and goat generally followed the order kidney > liver > other vital organs (Harwig et al., 1983). Several proteins from rat intestine, liver, spleen and kidney were found to bind with ochratoxin A. The binding contributes to intracellular accumulation of ochratoxin A, thus leading to prolonged half-life in mammalian body (Schwerdt et al., 1999). The biological half-life of ochratoxin A in different rats is 9-12 days (Zepnik et al., 2003), human beings is 35-55 days (Studuer-Rohr et al., 2000), 21-25 days in monkeys (Hagelberg et al., 1989) and 3-5 days in pigs (Galtier et al., 1981).

**BIOTRANSFORMATION**

Omar et al. (1996) has shown that a small percentage of absorbed ochratoxin A is converted to hydroxyochratoxin A (OH-OA) by liver microsomes. Ochratoxin A is metabolized by the main cytochrome P<sub>450</sub> isoforms IA1/IA2, IIB1 and IIIA1/IIIA2 of rats mainly to 4R and to smaller extent to 4S-hydroxyochratoxin A, leading to its detoxification. The minor metabolites of ochratoxin A are 4-OH-(4R-4S) epimers produced in rat and rabbit liver and rat kidney under the influence of cytochrome P<sub>450</sub> (Stormer et al., 1981). The 4R-OH-OA epimer is considered to be less toxic than ochratoxin A which is the major of these metabolites formed from ochratoxin A in human and rat liver microsomal systems, whereas the 4S-OH-OA epimer is more prevalent with pig liver microsome.

Another mechanism for detoxification of ochratoxin A involves hydrolysis of ochratoxin A to the non-toxic ochratoxin a at various sites. In rats detoxification by hydrolysis to ochratoxin a is a function of the bacterial microflora of the caecum (Galtier, 1978). The enzyme responsible for hydrolysis to ochratoxin a in cows and rodents are
carboxypeptidase A and chymotrypsin (Pitout and Nel, 1969). Studies in mice suggest that ochratoxin A circulates from the liver into the bile and into the intestine, where it is hydrolysed to ochratoxin a (Moroi et al., 1985) (Fig. 1.3).

**OCHRATOXICOSIS**

Human exposure to ochratoxin is a widespread problem in certain European countries and in India too. The contamination of various food products by this group of mycotoxins has lead to ochratoxicosis in both human beings and animals (IARC, 1993). Ochratoxin A primarily is a nephrotoxic and has been classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC), Lyon, France. Ochratoxin A has been shown to induce renal adenomas and carcinomas in mice and rats (IARC, 1993).

The putative cause of Balkan endemic nephropathy (BEN) was found to be ochratoxins. Balkan endemic nephropathy is a chronic renal disease which is found in geographically close areas of Bulgaria, Romania, Serbia, Croatia, Bosnia and Herzegovina, Slovenia and the Former Yugoslav Republic of Macedonia (Krogh, 1974; Petkova-Bocharova and Castegnaro, 1991; Abouzied et al., 2002). The kidney disease was initially recognized in 1950s (Tancev et al., 1956). The disease was qualified by WHO experts and explained as “progressive and very gradually developing renal failures with insidious onset of the disease and the last stage shows marked fibrosis”. It is also characterized by tubular degeneration, interstitial fibrosis alongwith hyalinization of glomeruli, enzymura and impaired renal function without nephritic syndrome (Vukelic et al., 1991). The kidneys and associated urinary tract were found to be infested with
tumors. So it is not only considered to be nephrological but also oncological. For this the term “endemic uropathy” is used (Pfohl – Leszkowicz et al., 2002).

Ochratoxin A is primarily a nephrotoxin, but is also found to be hepatotoxic, cytotoxic and carcinogenic in various laboratory animals and in human beings (Verma and Mathew, 1997 a). Clinical features of ochratoxicosis involve progressive renal failure, anemia, and decrease in lymphocyte count, microproteinuria of tubular type, small and shrunken kidneys, and associated urothelial tumors. Several major mechanisms have been characterized showing involvement of ochratoxin A in cellular toxicity. Inhibition of protein synthesis, promotion of membrane peroxidation, disruption in calcium homeostasis, inhibition of mitochondrial respiration and DNA damage are the major ones.

Ochratoxin A is regarded as an analogue of phenylalanine. Ochratoxin A directly affects the transcription step in protein synthesis, which involves competitive inhibition of phenylalanine tRNA phe synthetase, so that aminoacylation and peptide elongation are stopped. The level of key enzyme like phosphoenolpyruvate (PEP) carboxykinase activity is inhibited because of specific degradation of mRNA that codes for the enzyme (Dirheimer and Creppy, 1991). The most dramatic effects of ochratoxin A on metabolite synthesis was its in vivo and in vitro inhibition of protein synthesis. An intraperitoneal dose of 1 mg/kg bw given to mice produced a 50% inhibition in protein synthesis in the liver (Creppy et al., 1984).

Subcutaneous doses of ochratoxin A at 10 mg/kg bw for 5 days decreased the activity of muramidase alongwith the activities of lactate dehydrogenase, alkaline phosphatase, glutamate dehydrogenase and acid phosphatase in the kidney (Ngaha,
Fig: 1.3 Metabolites of ochratoxin
(O’Brien and Dietrich, 2005).
The activities of alanine peptidase, leucine amino peptidase and alkaline phosphatase were decreased by 60, 50 and 35\% respectively in isolated kidney tubules in the presence of 0.1 mmol/l ochratoxin A (Endou et al., 1986). When porcines were given ochratoxin A at 0.1-2.0 mg/kg bw/day orally for 2-5 days, the PEP carboxykinase activity decreased by 50 - 70\% at the highest dose (Krogh, 1988). When kidney cortex slices from rats fed with grain and peanut contaminated with 2.0 mg of ochratoxin A/kg feed for 2 days, PEP carboxykinase activity was decreased by 55\% and overall gluconeogenesis process was affected (Meisner and Selanik, 1979). In rats given ochratoxin A by gavage at a dose of 0.14 mg/kg bw every 48 h for 8-12 weeks, the activities of lactate dehydrogenase, alkaline phosphatase, leucine aminopeptidase and \( \gamma \)-glutamyl transferase decreased significantly. The last three enzymes are located in the brush border of the proximal convoluted tubules indicating damage at that site (Endou et al., 1986). The appearance of N-acetyl beta-D-glucosidase, a lysosomal enzyme indicated active regeneration and exfoliation of necrotic proximal convoluted tubular cells (Stonard et al., 1987). Ochratoxin A caused leakage of cytosolic enzymes in monkey kidney cells (Baudrimont et al., 1997). When male Wistar rats were given 289 \( \mu \)g/kg bw for every 48 h for 2, 4, 6 and 8 weeks by gastric intubations, the treatment resulted in increase in testicular enzyme activities particularly \( \alpha \)-amylase, alkaline phosphatase and \( \gamma \)-glutamyl transferase. The increase in \( \gamma \)-glutamyl transferase was thought to be because of impairment in spermatogenesis and accumulation of premeiotic germinal cells induced by ochratoxin A (Gharbi et al., 1993). Significant effects due to ochratoxin interaction were also observed in cytochrome P450 content, NADPH- dependent dehydrogenase and NADPH- cytochrome C reductase activities (Siraj et al., 1981).
Though the kidney is the target organ, but changes in liver, skeletal muscles, heart, spleen and brain have also been noted during studies on acute effects (Galtier, 1978; Verma and Mathew, 1997 b). Recently the toxin was found as a neurotoxic agent. The ventral mesencephalon, hippocampus, striatum and cerebellum were the main target of cytotoxicity in rat brain (Belmadani et al., 1998 a). The toxin also caused changes in the concentration of amino acids tyrosine and phenantherene and damage tissues in the hippocampus (Belmadani et al., 1998 b).

Carcinogenicity

Ochratoxin A is a potent carcinogen. When diet containing ochratoxin A at 5.6 mg/kg bw/day was fed to rats, it causes hepatic cell tumors, renal cystic adenomas and solid renal cell tumors (Kanizawa and Suzuki, 1978). Two types of renal tumors were distinguished by Kanizawa (1984), papillary cyst adenomas (benign) and solid renal cell tumors which contained typical cells, displayed infiltration growth and were interpreted as malignant. Renal carcinomas were found in 16 out of 51 male rats at a dose of 70 μg/kg bw/day and in 30 out of 50 at a dose of 210 μg/kg bw/day. No carcinomas were found at lower doses. In female rats, renal carcinomas were less common with 0 out of 50, 1 out of 50 and 3 out of 50 animals showing carcinomas at the low, intermediate and high doses respectively. Renal adenomas were found in all groups of male rats. When male mice were treated with ochratoxin A, liver apoptosis was seen and presence of intracellular apoptotic bodies were detected in all. Presence of eosinophilic globules containing apoptotic bodies were found within the cytoplasm of intact hepatic cells (Atroshi et al., 2000). Weanling male rats given comparable doses of the toxins
developed widespread hyaline degeneration of the liver cells with focal necrosis. The ultrastructural lesions suggested that endoplasmic reticulum is the primary site of action of the toxin in experimental rats (Theron et al., 1966). In another experiment when crude toxin of *Aspergillus ochraceus* was treated with monocytic cell lines THP-1, leads to secretion of tumour necrosis factor (TNF). The toxin also impaired the mitochondrial activity of THP-1 cells marginally (Heller et al., 2002).

**Immunotoxicity**

Bone marrow depression and decrease in the size of thymus was seen alongwith decrease in erythropoiesis and increased phagocytosis by macrophages (Boorman et al., 1984). The spleen was discovered to be the most sensitive immune tissue to ochratoxin A. Differences in the proportions of mature and immature CD4⁺ and CD8⁺ population suggests that ochratoxin A may affect the last stage differentiation of T cells (Thuvander et al., 1995). Several studies have shown that ochratoxin A affects both humoral and cell mediated immunity in poultry (Dwivedi, 1984). Serum Ig levels particularly IgM, IgG and IgA in fowls were reduced due to 2 to 4 ppm ochratoxin A (Dwivedi and Burns, 1984 a). In electron microscopic studies ochratoxin A was found to cause degeneration and necrosis of lymphoid cells including plasma cells in the lymphoid organs (Dwivedi, 1984 b). Chicken fed with diet containing ochratoxin A at 5 mg/kg for 56 days showed reduced level of α₁, α₂, β and γ globulins in plasma (Rupic et al., 1978). Ochratoxin A also inhibited the proliferative response of bovine peripheral blood mononuclear cells cultured in 10% fetal calf serum.

It has been further shown that 4(R)-hydroxy-ochratoxin A, a metabolite of ochratoxin A, was as strongly immunosuppressive as ochratoxin A in mice in doses of 1
mg/kg bw and resulted in 80 to 93% suppression of IgM and IgG synthesis (Creppy et al., 1983).

Teratogenicity

Ochratoxin A is teratogenic to mice, rat and hamsters (Shirai et al., 1985). When rats were treated per-orally with ochratoxin A at 0.75 and 1.0 mg/kg bw on gestation days 6-15, fetuses taken out on day 20 showed decrease in weight and various abnormalities (Brown et al., 1976). Ochratoxin A administered subcutaneously to rats (1.75 mg/kg bw) on gestation days 5-7 resulted in the highest number of malformations, including hydrocephaly, omphalocele and anophthalmia and shift in position of the oesophagus (Mayura et al., 1985). In mice, ochratoxin A administration for 1-2 days is known to cause cell death in the developing brain of embryos. Microcephaly was observed with high frequency. The deficits in synapse to neuron ratios were seen in ochratoxin A-induced microcephalic brain seems to result from a dendritic growth (Fukui et al., 1992). When primary cultures of neurons and astrocytes isolated from embryo or newborn rat brain were treated with ochratoxin, inhibition in protein and DNA synthesis was observed along with increase in lactate dehydrogenase leakage and lipid peroxidation (Bruinink et al., 1998) (Fig. 1.4).

Genotoxicity

The presence of a chlorine moiety at C-5 position in ochratoxin A and in other analogues of ochratoxin A appears to be one determinant of their genotoxicity (Malaveille et al., 1994). Ochratoxin (1 μg/kg bw/day) when administered orally daily to
Fig: 1.4 Interactions between ochratoxin, man and animals
albino Swiss mice for 14 continuous days, increase in the incidence of abnormalities in mitotic and meiotic metaphase chromosomes were seen. It was also concluded that the electrophilic metabolites of ochratoxin form adducts with DNA or produce replacement type mutations (Kumari and Sinha, 1994). Single strand breaks induced by ochratoxin A in DNA was also observed in liver, spleen and kidney (Creppy et al., 1985). The role of critical biotransforming enzymes involved in genotoxicity and carcinogenicity that is cytochrome P450 (CYPs) IA, 2A, 2B, 2C, 2D and 3A in the kidney and liver microsomes were studied on untreated and ochratoxin A-treated dark agouti and Lewis rats of both the sexes. Large numbers of DNA adducts were found in these organs (Pfohl-Leszkowicz et al., 1993).

Cytotoxicity

Several investigators (Bondy and Armstrong, 1998; Schaeff et al., 2002) have reported cytotoxic effects of ochratoxin on different cell lines particularly kidney derived primary rat proximal tubular cells, LLC-PK1 cells and OK cell lines. When RBC suspension was treated with ochratoxin in vitro, a concentration-dependent lysis was observed indicating permeability alterations and membrane destabilization (Zofair et al., 1996). Exact mechanism of ochratoxin-induced cytotoxicity is not clearly understood. It could be due to its effect on mitochondria, increased lipid peroxidation, increased adduct formation with DNA, RNA and protein or all the three (Fig. 1.5).

Action of ochratoxin A on mitochondria have been demonstrated by Meisner (1976). He suggested that ochratoxin A uptake by mitochondria is an energy-dependent process resulting in depletion of intramitochondrial ATP and inhibition of inorganic
Severe oxidative stress

Excess reactive oxygen species and low antioxidant defense

Damage to biomolecules

Lipid peroxidation (Damage to membrane ion channel and ion transporter, raised intracellular Ca^{+2})

DNA damage (DNA damage, strand breakage)

Protein damage (Damage to receptor, enzyme, ion channel, raised base modification)

Cellular damage with release of more radicals

Cell death and tissue damage

Carcinogenesis, atherosclerosis, aging etc.

Fig: 1.5 Reactive oxygen species
phosphate transport. These deteriorative changes in mitochondria cause large amplitude swelling. When ochratoxin A was added to isolated rat renal proximal tubule cells in suspension, mitochondrial dysfunction was seen as an early event in the process of nephrotoxicity. Mitochondrial impairment apparently occurred at sites I and II of the respiratory chain (Aleo et al., 1991). The middle and terminal segments of proximal tubule of isolated nephron were found to be most sensitive to the toxic effects of ochratoxin A, where decrease in cellular ATP and dose-related decrease in mitochondrial ATP were observed (Jung and Endou, 1989). When ochratoxin A was added in rat liver mitochondrial suspension, there was uncoupling of mitochondrial respiration and the oxidative phosphorylation was affected at higher concentrations. Ochratoxin A also inhibited the electron transfer function of mitochondria (Wei et al., 1985).

The improper balance between reactive oxygen species (ROS) production and anti-oxidant defenses results in “oxidative stress” which deregulates the cellular function leading to various pathological conditions including cancer.

Oxidative stress is believed to play an important role in ochratoxin A-induced toxicity and carcinogenicity (Rahimtula and Chang, 1991; Verma and Mathew, 1997 a). Ochratoxin A (1 mg/kg) treatment, results in 5 fold increases in the expression of the oxidative stress responsive protein haem oxygenase-1 specifically in the kidney (Gautier et al., 2001). The lipid peroxidation induced by ochratoxin A has been found to induce lesions which are related to oxidative damage. This was assayed in monkey kidney cells (vero cells) treated by ochratoxin A (5-50 µM) (Baudrimont et al., 1997). Ochratoxin A also increased the formation of malondialdehyde and very low concentration of ochratoxin A was shown to induce apoptosis and oxidative damage to kidney cells (Petrik
et al., 2003). Oxidative stress is the most important event which is seen normally in case of ochratoxicosis. Oxidative stress can cause mutagenicity, cytotoxicity and stimulate changes in gene expression (Bashir et al., 1993). Mutation induced by oxidants may initiate carcinogenesis along with oxidative modification of the genetic material and may also participate in the progression of benign to malignant neoplasm (Guyton and Kensler, 1993; Ray and Hussain, 2002). Reactive oxygen species can cause lipid peroxidation of plasma membrane and the membrane of cytoplasm leading to disturbances in structural integrity and capacity for cell transport and energy production especially in proximal tubule segment (Baud and Ardailliou, 1993). Furthermore, all these effects lead to development of cancer.

Ochratoxin A exposure to various cell lines resulted in concentration-dependent elevation of ROS levels, depletion of glutathione and increased formation of 8-deoxyguanosine. It was also concluded that oxidative stress contributes to the tubular toxicity of ochratoxin A (Schaef et al., 2002). Induction of free radicals generation in bacteria Bacillus brevis has also been studied (Hoehler et al., 1996). Lipid peroxidation is considered to be the most important mode of action in ochratoxin A-induced toxicity in rats and chicks (Hoehler et al., 1997).

Under normal circumstances the major source of free radicals in cells is electron leakage from electron transport chain, such as those in mitochondria and in endoplasmic reticulum to molecular oxygen resulting in formation of superoxide radicals. Other enzymes can also produce superoxide or H₂O₂, e.g., flavin oxidases located in peroxisomes. Other sources are autooxidation of ascorbic acid and thiols (glutathione, cysteine, flavin coenzyme) which can also produce superoxide. Further the process of
autooxidation increases in the presence of transition of metals like iron/copper. The damage is seen maximum in those where the proteins (mainly enzymes) binds a transition metal ion at the particular site eg: copper by histidine residue. Here the autooxidation of transition metal and reaction with $\text{H}_2\text{O}_2$ generates hydroxyl radical that will react with metal binding site (Marx and Chevion, 1986). The mechanism of free radicals attack on proteins and formation of peroxides and carbonyls have been explained (Simpson et al., 1992). DNA is also readily attacked by oxidizing radicals inducing strand break or may elude the repair system before replication occurs leading to mutation. The detection of oxidised nucleobases in human urine has been taken as evidence for a continual oxidative attack on DNA (Kasai and Nishimura, 1991). Sugars including glucose, mannitol and deoxy sugars react readily with hydroxyl radicals (Greenwald and May, 1980).

Several studies (Nicotera et al., 1992) have suggested that calcium activated catabolic processes are involved in cytotoxicity. The involvement of calcium activated catabolic process in cytotoxicity has been suggested by Verma and Mathew (1998 a). Khan et al. (1989) have reported that ochratoxin A inhibited the rate of ATP-dependent calcium uptake both in vivo and in vitro in rats. This effect was associated with an enhanced rate of lipid peroxidation. Lipid peroxidation enhanced by ochratoxin A was also accompanied by leakage of calcium from calcium loaded microsomes. Turner et al. (1988) have demonstrated that the accumulation of calcium by mitochondria mobilized iron which, in turn could stimulate the production of OH$^-$ from $\text{H}_2\text{O}_2$ which leads to lipid peroxidation further.

As ochratoxin A increases the permeability of the cell to $\text{Ca}^{2+}$, it enhances cellular concentration of $\text{Ca}^{2+}$, and uncouples oxidative phosphorylation resulting in an increased
leakage of electron from the respiratory chain producing \( \text{O}_2^- \) and hence \( \text{H}_2\text{O}_2 \). A lack of adequate supply of NADPH and GSH to permit \( \text{H}_2\text{O}_2 \) consumption by the dependent glutathione peroxidase and glutathione reductases together with an increased concentration of free iron within the cells stimulates the production of \( \text{OH}^- \) via Fenton reaction due to mobilization of Fe\(^{2+}\) by Ca\(^{2+}\). This is one of the mechanisms by which ochratoxin A causes its cytotoxic effect (Hoehler et al., 1996: 1997) (Fig. 1.6).

Calcium homeostasis was studied in rats treated intra-peritoneally with a single dose of 10 mg/kg bw of ochratoxin A or multiple doses of 0.5 to 2 mg/kg ochratoxin A. An increase in renal endoplasmic Ca\(^{2+}\) pump activity was observed suggesting an association with ochratoxin A-induced renal cytotoxicity (Rahimtula and Chang, 1991).

Ochratoxin A also acts on the synthesis of RNA and DNA and is considered to be one of the major factors of its toxicity (Creppy, 1984). The toxin was reported to form DNA adducts which were detected by \( ^{32}\text{P} \) post-labelling after exposure of ochratoxin A (Castegnaro et al., 1998). It was also concluded that adducts might have formed due to ochratoxin A-induced cytotoxic effect that generates reactive oxygen species (Grosse et al., 1997). High level of DNA adducts were found in kidney in males and liver of female mice after administration of a single dose of ochratoxin A (Petkova-Bocharova et al., 1998).

**SOURCES OF OXYGEN FREE RADICALS**

Reactive oxygen species (ROS) are generated by specialized phagocytic cells (neutrophils and macrophages) as cytotoxic agents, when they fight to invade microorganisms, this process is also known as respiratory burst or oxidative burst. For
Fig. 1.6 Proposed mechanism for ochratoxin A induced hydroxyl production.
this, phagocyte normally uses the membrane bound NADPH oxidase complex which catalyzes the one electron reduction $O_2$ to $O_2^{-}$ (Morel et al., 1991).

The ROS are generated in biological system via several enzymatic and non-enzymatic pathways. They are also produced by electron leakage from the transport chain in mitochondria and endoplasmic reticulum where molecular $O_2$ is sequentially reduced to $O_2^{-}$ and $H_2O_2$. The five possible reactive oxygen metabolites are superoxide anions ($O_2^{-}$), hydroperoxyl radical ($HO_2^{·}$), peroxide ion ($HO_2^{-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{·}$) (Chessmen and Slater, 1993). Hydroxyl radicals have got the half life of $10^{-5}$ second and considered to be the most damaging one which are produced from $O_2^{-}$ and $H_2O_2$ by Haber - Weiss reaction (Beauchamp and Fridovich, 1970). Reactive oxygen species are directly or indirectly involved in multistage process of carcinogenesis. They also lead to mutation, deletion, gene amplification and rearrangement (Marnett, 2000). These free radicals are the species with very short half-life, high reactivity and damaging activity towards other macromolecules like proteins, carbohydrates and lipids too. Oxygen radicals are produced as a consequence of the normal process of reduction of oxygen to water and represent byproducts of oxidative cellular metabolism. Series of reactions are involved in the conversion of oxygen to water. Figure 1.7 demonstrate the site of formation of the superoxide ($O_2^{-}$) and the hydroxyl ($OH^{·}$) free radicals in the series (Dickerson and Williams, 1990).

Out of all biomolecules, lipids are probably the most susceptible one. Cell membranes are the rich sources of polyunsaturated fatty acid (PUFAs) which are normally attacked by oxidizing radicals. Oxidative destruction of PUFAs known as lipid peroxidation proceeds a self perpetuating chain reaction (Cheeseman and Slater, 1993).
Peroxyl radicals are the carrier of chain reaction. They can oxidize further PUFA molecule and initiate new chains, producing lipid hydroperoxides (LOOH) that can break down to more radical species mainly aldehydes. Breakdown of lipid hydroperoxides often involves lipid peroxy, lipid alkoxy radicals and hydroxyl alkenals derivatives (Esterbauer et al., 1991). These free radicals interact with DNA and bring about the mutation which may initiate carcinogenesis and oxidative modification of genetic material and can also participate in the progression of benign to malignant neoplasm.

These free radicals are highly reactive species and may attack the double bonds of polyunsaturated fatty acid (PUFA) chains of membrane phospholipids. The lipoperoxyl free radicals thus formed can attack adjacent PUFA residues and thereby initiate a chain free radical reaction with widespread harmful consequences to membrane structure. Peroxidation chain reactions are characterized by initiation, propagation and termination stages with lipid hydroperoxides as the primary products (Fig. 1.8). The generally accepted mechanism of peroxide formation involves the formation of a free radical (R") by a PUFA (RH) molecule, followed by the addition of oxygen to form peroxide (ROO'). The peroxide can react with another PUFA molecule to produce another free radical, thus the reaction is propagated.

Chemical compounds capable of generating potential toxic oxygen species or free radicals are normally known as prooxidants. On the other hand, compounds which scavenge out these radicals are known as antioxidants. In a normal cell there is an appropriate balance between prooxidants and antioxidants. This balance shifts towards the prooxidant when production of oxygen species increases or levels of antioxidants decreases. This state is called oxidative stress which can result in serious cell damage.
\[
\begin{align*}
O_2 & \xrightarrow{e^-} O_2^- & \text{Superoxide anion} \\
O_2^- & \xrightarrow{e^-} O_2^{2-} & \text{Peroxy anion} \\
O_2^{2-} & \xrightarrow{H^+} [O_2H^-] & \text{Hydrated Peroxy anion} \\
[O_3H] & \xrightarrow{H^+} H_2O_2 & \text{Hydrogen Peroxide} \\
[H_2O_2] & \xrightarrow{e^-} OH^- + OH^- \quad \left\{ \begin{array}{c}
2 OH^- \xrightarrow{2H^+} 2H_2O
\end{array} \right.
\end{align*}
\]

Fig. 1.7 Site of formation of free radical in the reduction of oxygen to water
(Source: Dickerson and Williams, 1990)
Fig: 1.8 Attack of reactive oxygen species on the double bonds of polyunsaturated fatty acid (PUFA) chain of membrane phospholipids
Oxidative stress can lead to various human disorders (Frei, 1994). Several studies have shown the role of oxidative stress in the causation and progression of different diseases including atherosclerosis, carcinogenesis, neurodegenerative diseases, chronic inflammatory diseases, radiation damage and other pathobiological effects (Mc Cord, 1985; Ames et al., 1996; Evans and Halliwell, 1999; Li et al., 1999). Free radical injury and oxidative stress have been lead to various renal diseases like acute renal failure (ARF), nephropathy, and anemia of chronic renal failure (CRF) and ischemic kidney. Plasma glutathione peroxidases have been suggested as an index of renal antioxidant defense which is reported to be markedly decreased in persons suffering from CRF (Galli and Ranco, 2000; Hasselwander and Young, 1998). The reactive oxygen species also play role in the pathogenesis of hepatic fibrosis with the loss of normal liver architecture (Poli, 1993).

**ANTIOXIDANTS**

The term antioxidant has been defined by Halliwell et al. (1992) as “any substance that delays or inhibits the oxidative damage to a target molecule”. That means they mainly counteract the lethal effects of oxidative damage. Normal living cells have developed antioxidative defense system induced by various enzymes like superoxide dismutase, catalase, glutathione peroxidase as well as by non-essential endogenous antioxidants like glutathione, protein, ubiquinol and by essential radical scavengers like vitamin C (ascorbic acid), vitamin-E (α-tocopherol) and carotenoids (Mascio et al., 1991). If free radicals are aetiologically involved in the major causes of death, optimization of antioxidant status should offer preventive prospects for “optimum health” as defined by World Health Organisation (Silva, 1999). Various studies have revealed
that deficiencies in antioxidant levels are likely to be important risk factor for cancer (Preston-Martin et al., 1990). The status of vitamin antioxidant (in contrast to that of other antioxidative defense lines) can easily, and without side effects is modified by dietary measures.

The first line of defense comprises preventive antioxidants that act by quenching of \( O_2^- \), decomposition of \( H_2O_2 \) and sequestration of metal ion. The antioxidants belonging to this category are enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase as well as non-enzymatic molecules like minerals and some proteins (Halliwell and Gutteridge, 1999).

The antioxidants belonging to second line of defense include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E, carotenoids, flavonoids and ubiquinol. Glutathione is the most abundant non-protein thiol, synthesized in the liver and acts as a substrate for glutathione peroxidase. This also serves as a scavenger of different free radicals (Halliwell et al., 1992) (Fig. 1.9).

Repair and \textit{de novo} enzyme comprises the third line of defense. Third line antioxidants are a complex group of enzymes for repair of damaged DNA, protein, oxidized lipids and peroxides and also to stop the chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damaged cell membrane for example lipases, proteases, DNA repair enzymes such as tranferases, and methionine sulfoxide reductases etc (Irshad and Chaudhari, 2002).

The oxidative stress caused by ochratoxin A was found to be reduced in various animals when treated with combined antioxidants such as coenzyme Q\(_{10}\), L-carnitine, Zn\(^{+2}\), Mg\(^{+2}\), N-acetyl cysteine, vitamin C, vitamin E and selenium/tamoxifen. These
Superoxide dismutase (Zn-containing) converts superoxide radicals to hydrogen peroxide.

\[ 2H^+ + O_2^- + O_2^- \rightarrow H_2O_2 + O_2 \]

Glutathione peroxidase (Se-containing) converts hydrogen peroxide to water

\[ H_2O_2 + \text{Reduced glutathione} \rightarrow 2H_2O + O_2 + \text{Oxidised glutathione} \]

Glutathione reductase (Vitamin B_{12}-containing) regenerates reduced glutathione

\[ \text{Oxidised glutathione} \rightarrow \text{Reduced glutathione} \]

Catalase (Fe-containing) converts hydrogen peroxide to water and oxygen

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]

Vitamin C can quench free radicals

\[ \text{Oxidised ascorbic acid} \rightarrow \text{Reduced ascorbic acid} \]

Fig: 1.9 Some mechanisms for disposal of free radicals
Antioxidants were also able to intervene in apoptosis induced in liver of mice by ochratoxin A (Atroshi et al., 2000). Antioxidative effect of melatonin against ochratoxin A–induced oxidative stress in liver and kidney in rats was evaluated where melatonin was effective in improving feed intake, body weight gain, serum total protein, albumin and in the activities of glutathione peroxidase, superoxide dismutase and malondialdehyde (Abdel-Wahhab et al., 2005).

During the last two decades scientists are in search for new plant products having antioxidative properties and their role in the prevention of various diseases associated with oxidative stress such as cancer, cardiovascular, reproductive and neurodegenerative diseases. So far 119 secondary metabolites have been isolated from higher plants, which are globally used as drugs. These secondary metabolites are normally involved in the defensive mechanism in plants. It has been estimated that 80% of the world’s population still use traditional medicine for their primary health care needs (Farnsworth, 1988). Many important modern plant drugs such as vinblastine and vincristine, have been discovered by following leads from traditional medicines (Carter and Livingston, 1976). Table 1.3 lists examples of certain bioactive compounds showing antioxidative activity.

**EMBLICA OFFICINALIS (AMLA)**

*Emblia officinalis* commonly known as amla (Synonym - Indian gooseberry) is one of the fruit which contains array of bioactive components showing antioxidative property and is widely used in India as a traditional medicine (Ghosal et al., 1996; Bhattacharya et al., 1999), ayurvedic herbal formulation and also in Unani medicines.
TABLE: 1.3 Different types of polyphenols and their sources

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Structure</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Flavonols</td>
<td>Glycosylated form</td>
<td>Fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vegetables</td>
</tr>
<tr>
<td>2. Flavones</td>
<td>Glycosides, luteolin and epigenin</td>
<td>Millet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wheat</td>
</tr>
<tr>
<td>3. Isoflavones</td>
<td>Analogues to estradiol</td>
<td>Soya</td>
</tr>
<tr>
<td>4. Flavanones</td>
<td>Glycosides</td>
<td>Tomatoes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citrus fruits</td>
</tr>
<tr>
<td>5. Anthocyanins</td>
<td>Glycosides</td>
<td>Fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flowers</td>
</tr>
<tr>
<td>6. Flavanols</td>
<td>Monomer-Catechins</td>
<td>Flowers</td>
</tr>
<tr>
<td></td>
<td>Polymer-Proanthocyanidins (tannins)</td>
<td>Fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citrus fruit</td>
</tr>
</tbody>
</table>
*Emblica officinalis* belongs to family *Euphorbiaceae*, normally found in tropical parts of India, Pakistan, Bangladesh, China, Sri Lanka and Malayan peninsula. It is also found in woodland Savanna and dry deciduous forests of other parts of the world. The branches and branchlets are mostly weak and drooping. Branchlets are alternate, superposed and they all face one plane. The length of the branchlet is around 40 cm and more than 100 leaves are found on one branchlets. Leaf measures approximately 1.8 cm X 0.5 cm. The diameter of the fruit ranges between 1.8 – 2.5 cm. The fruits are globular in shape and have depressed grooves, with shinning yellowish green colour when ripens (Fig. 1.10).

The fruits of *Emblica officinalis* has been used in treatment of vomiting, haemorrhage, fever, cough, dyspnea, eye inflammation, ulceration, anorexia, emaciation, scurvy, diabetes, jaundice, menorrhagia, leucorrhoea, common cold, heart diseases, cancer, hepatotoxicosis and renotoxicosis (Roy *et al*., 1991; Sivarajan, 1994; Mathur *et al*., 1996; Vani *et al*., 1997; Jose *et al*., 2001). The fruit contains high concentration of glutamic acid (29.6%), proline (14.6%), aspartic acid (8.1%), alanine (5.4%) and lysine (5.3%) of the total amino acid. The fruit also contains Cr (2.5 ppm), Zn (4 ppm) and Cu (3 ppm) along with gallic acid (1.32%).

*Emblica officinalis* is highly nutritious and is an important dietary source of vitamin C, minerals and amino acids. The edible fruit tissue contains protein concentration 3 fold and ascorbic acid concentration 160 fold to apple. *Emblica officinalis* contains a range of polyphenols especially tannins and other phenolic compounds. These include hydrolysable tannins (10-12%) with a molecular weight of less than 1000, including emblicanin A, emblicanin B, punigluconin and pedunculagin.
Fig: 1.10 EMBLICA OFFICINALIS (AMLA)
(Ghosal et al., 1996; Bhattacharya et al., 1999). It also contains quercetin (Gulati et al., 1995). They show cytoprotective activity and possible inhibitory effects on carcinogenesis, mutagenesis and tumor genesis (Kumar and Muller, 1999). Table 1.4 shows different kinds of bioactive compounds present in fruit of *Emblica officinalis*.

Several flavonoids from other plant sources have been reported to inhibit either enzymatic or non-enzymatic lipid peroxidation (Pierpoint, 1986). Maridonneau Parini et al. (1986) have reported a heterogeneous effect of flavonoids on K⁺ loss and lipid peroxidation induced by oxygen free radicals in human RBC. A number of flavonoids are known to possess antitumour promoter activity in the two stage model of carcinogenesis. Mookerjee et al. (1986) have observed in studies with human peripheral blood lymphocytes that several flavonoids reversibly inhibit lymphocyte proliferative response. Naturally occurring and synthetic flavonoids are reported to have striking effects on cyt P₄₅₀ - dependent monoxygenase system (Sato and Omana, 1978). Flavonoids have been associated with several biochemical reactions. The addition of quercetin and flavanones to liver homogenate or adipose tissue extracts play an important role in biological reduction-oxidation reactions by retarding the oxidation of lipids (Letan, 1967). The flavonoids produce a clear increase in vitamin-A and a moderate carotenoids in the liver (Shipochliev et al., 1970). Quercetin like flavonoids are able to reduce the cytotoxic effect of T-2 mycotoxin and reduce mortality in mice (Markhaem et al., 1987). Flavonoids are thought to exert protective effects against cell damage, produced by lipid peroxidation and related disorders that is stimulated by variety of toxic substances. The protective effect is due to antioxidative property of the compounds. These flavonoids
TABLE: 1.4 Different types of bioactive phytochemical components reported in *Emblica officinalis*.

<table>
<thead>
<tr>
<th>CLASS</th>
<th>COMPOUNDS</th>
<th>STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TANNINS</td>
<td>Emblicanin A</td>
<td><img src="image1" alt="Emblicanin A structure" /></td>
</tr>
<tr>
<td></td>
<td>Emblicanin B</td>
<td><img src="image2" alt="Emblicanin B structure" /></td>
</tr>
<tr>
<td></td>
<td>Punigluconin</td>
<td><img src="image3" alt="Punigluconin structure" /></td>
</tr>
</tbody>
</table>
Pedunculagin

Ellagitannin

Gallotannin
FLAVONOID

Ellagic acid

Gallic acid

Quercetin
FURANOLACTONE

Ascorbic acid

Ascorbic acid (reduced form)  Dehydro-ascorbic acid (oxidized form)

OTHER IMPORTANT STRUCTURES INCLUDES:

BENZENOID

Amlaic acid
Corilagin
1,6-di-O-galloyl β-D-Glucose
Phyllembic acid

ALKALOID

Phyllantine
Phyllantidine
present in *Emblica officinalis* could prove to be promising agents for protection against free radicals mediated cell injury.

Tannins are the class of polyphenols which are soluble in water, alkali, alcohol, glycerol and acetone but are only slightly soluble in other organic solvents. Tannins exert an astringent effect in the duodenum that is opposite to that produced by laxative or purgative drugs, due to combination and precipitation of proteins that inhibit the absorption of substances. Most of the polyphenols are probably too hydrophilic to penetrate the gut wall by passive diffusion, and the membrane carriers are thought to be involved in the absorption (Craker and Simon, 2002).

Ascorbic acid is an important water soluble antioxidant found in *Emblica officinalis*. Human have no ability to synthesize vitamin C due to mutation in the gene coding for L-gulanolactone oxidase, an enzyme required for biosynthesis of vitamin C via the glucuronic acid pathway. So vitamin C is normally obtained from diet mainly from citrus fruit, other fresh fruits and vegetables.

The concentration of ascorbic acid varies from 3.25-4.5% for the dried fruit and 0.1-0.7% for the fresh fruit (Khopde *et al.*, 2001). The addition of ascorbic acid increases the inhibitory activity of flavonoid and both ascorbic acid and flavonoid appear to have synergistic properties in a mixture (Damodaran and Nair, 1936; Zloch, 1969). The aerobic oxidation of ascorbic acid in neutral or alkaline solution is catalyzed by copper (II) ions and thought to proceed through a free radical mechanism. The ability of flavonoids to inhibit aerobic oxidation has been attributed to their ability to act as free radical acceptors and to remove catalytic metal ions through formation of complexes with the metals (Thompson and Williams, 1976).
BIOLOGICAL EFFECTS OF *EMBLICA OFFICINALIS*

Earlier it was thought that antioxidative activity of *Emblica officinalis* is mainly because of the presence of vitamin C but current scientific interest explains that tannins and flavonoids also show powerful antioxidative property. Recent studies also suggest that *Emblica officinalis* has the ability to stimulate our natural antioxidant enzyme systems including catalase, superoxide dismutase (SOD) and glutathione peroxidase (Bhattacharya *et al.*, 2000; Rajak *et al.*, 2004). Research has also shown that this is a potent-stimulator of natural killer (NK) cells activity (Suresh and Vasudevan, 1994) and is found to increase tolerance to stress. An extract of fresh *Emblica officinalis* fruit was found to inhibit lipid peroxidation induced by gamma radiation in rat liver microsomes (Khopde *et al.*, 2001). The same extract also inhibited radiation-induced damage to the antioxidant enzyme superoxide dismutase in rat liver mitochondria. In another *in vitro* study, two low molecular weight hydrolysable tannins, emblicanin A and B were shown to provide dose-dependent protection against rat peripheral blood erythrocyte haemolysis induced by oxygen radicals (Ghosal *et al.*, 1996). An extract containing hydrolysable tannins (emblicanin A-37%, emblicanin B- 33%, punigluconin-12% and pedunculagin-14%) has demonstrated antioxidative activity in various animal models (Bhattacharya *et al.*, 1999).

The fruit offers a number of protections from heavy metals. It completely prevents DNA and cell damage from arsenic poisoning (Anila and Vijayalakshmi, 2002). The fruit was also shown to prevent cellular damage resulting from lead, aluminium, nickel, cadmium and chromium (Dhir *et al.*, 1990; 1991; 1993; Roy *et al.*, 1992; Khandelwal *et al.*, 2002; Sai Ram *et al.*, 2003). Fresh *Emblica officinalis* fruit juice was
found to protect the animals from liver toxicity due to overload of iron (Bhattacharya et al., 2000).

The lipid lowering and anti-atherosclerotic effects of the fresh fruit juices of *Emblica officinalis* were studied in rabbits (Mathur et al., 1996). Rabbits were hyperlipidaemic following an atherogenic diet. The fruit juice was administered at a dose of 5 ml/kg bw for 60 days. Serum cholesterol, triglycerides, phospholipids and LDL levels were reduced by 82, 66, 77 and 90% respectively. The tissue lipid level was found to be decreased. The animals also excreted high contents of cholesterol and phospholipids. The most exciting effects are its ability to decrease cholesterol and LDL (bad cholesterol). The tannins in *Emblica officinalis* were found to inhibit the production of aldose reductase, an enzyme found in diabetic cataracts (Suryanarayan et al., 2004). The fruit has ability to lower cholesterol by inhibiting cholesterol production and enhancing its degradation (Jacob et al., 1998). Several animal studies have shown that flavonoids in *Emblica officinalis* reduce serum cholesterol levels and aortic plaques (Thakur, 1985; Mathur et al., 1996).

*Emblica officinalis* has been used largely in traditional Indian medicine for the treatment of stomach disorders like heart burn, ulcers and indigestion. This also decreases gastric acid and pepsin output and increases protective mucus secretion in stomach (Al-Rehaily et al., 2002). The fruit's antioxidative property is thought to be responsible for protecting the stomach wall in laboratory animals from the harmful effects of drugs specifically known to cause ulcers (Bandhopadhyay et al., 2000).

The extract of *Emblica officinalis* fruit and the flavonoid constituent quercetin were shown to provide protection against liver toxicity caused by ethanol and
paracetamol in vivo (Gulati et al., 1995). Quercetin was found to be more potent than the plant extract and was considered to be the hepatoprotective constituent in the fruit. The mechanism for hepatoprotection was thought to be decrease in glutathione depletion and prevention of cytochrome P450 stimulation by other hepatotoxic agents. The aqueous extract of *Emblica officinalis* fruit protected mice from hepatotoxic and nephrotoxic effects of lead and aluminium salts (Roy et al., 1991). The fresh juice was found to have protective action from liver toxicity (Bhattacharya et al., 2000).

The fruit of *Emblica officinalis* contain compounds showing potent inhibitory activity against human immunodeficiency virus (HIV) reverse transcriptase (El-Mekkawy et al., 1995). The most potent compounds in terms of inhibition was found to be ellagitannin and putranjivain. Other potent compound showing inhibitory activity included several phenolic compounds derived from gallic acid and two flavonoid glycosides.

*Emblica officinalis* extract can inhibit the growth of human breast cancer cells in vitro (Lambertini et al., 2004). The extract was found to contain 18 compounds that inhibit the growth of gastric and uterine cancer cells (Zhang et al., 2004). The aqueous extract of *Emblica officinalis* fruit was shown to protect mice against the chromosome-damaging effects caused by carcinogen 3, 4 (a) benzo-pyrene (Nandi et al., 1997). This carcinogen causes formation of oxygen derived free radicals with DNA damaging potential. The protective effect against this type of damage mainly involves antioxidative property of *Emblica officinalis* fruit extract. An antitumor activity of the fruit aqueous extract was demonstrated in tumor bearing mice, resulting in a 35% increase in life span.
(Suresh and Vasudevan, 1994). Here the natural killer cell activity and antibody-dependent cellular toxicity was found to be enhanced.

In another study the aqueous extract of *Emblica officinalis* significantly reduced solid tumors in mice suggesting interaction with the cell cycle regulation (Jose et al., 2001). Extract of *Emblica officinalis* was found to inhibit the proliferation of 4 human tumor cell lines *in vitro* (Khan, 2002). Here pyrogallol was identified as an active component in the extracts.

In India, ochratoxin is found as a major food contaminant in spices, groundnut and other food-stuffs. It mainly acts on kidney, liver and other vital organs. The aim of the present study was to assess detailed biochemical changes in liver, kidney and testis. We have selected testis as a part of our study, as no detailed study has been done on it. Male reproductive health has been deteriorated in many countries during the last few decades. A number of toxins in environment have been suspected to affect reproductive system in male, and ochratoxin is one of them.

Lipid peroxidation and oxidative stress is believed to play an important role in ochratoxin A-induced toxicity. Free radicals with other reactive oxygen species (ROS) are normal by-products of the oxidative processes in cells and large numbers of naturally occurring components in various fruits, vegetables and spices have ability to dispose off free radicals and limit their tissue damaging effects. Recently plant-derived products have gained much attention in different pharmaceutical preparations due to their pharmacological properties and *Emblica officinalis* (amla) is one of them. In the present study we have also evaluated the possible ameliorative effect of *Emblica officinalis*
extracts on ochratoxin-induced toxicity. It has wide range of bioactive compounds showing antioxidative properties and is easily available in local markets of India.

Researchers have worked with different fractions of the fruit extracts (*Emblica officinalis*) showing its potency. However, pharmacological effects by administration of whole fruit extract can be quite different from the effects of administering a single active component. It is believed that the bioactive component present in the extract may act together leading to increase in potency and decrease in toxicity. So, in our present study we have used crude aqueous extract of the fruit which is believed to contain large numbers of bioactive compounds. A commonly recognized fact of drug treatments is that “the presence of inert substances may modify or prevent the absorbability or potency of the active constituents” (Claus and Tyler, 1967). More than one active constituent may be present in drug/plant extracts and more than one active constituent could compete for the same site of action or act at different sites that are both connected with a larger physiological system. Such interactions are shown by tannins which influences solubility and absorbability of other polyphenols. The present extract was also found to be rich in vitamin C which is an important antioxidant, which once again increases the potency of the extract.