Introduction
Toxicology is the study of the adverse effects of naturally occurring or man-made (anthropogenic) substances on living systems. It is largely concerned with the development and use of toxicity information as a means of predicting safe or threshold amounts of toxicants, which may be encountered in the workplace, home or the general environment (Aldridge, 1981).

Toxicity is typically mediated by a reaction of the ultimate toxicant with a target molecule. Subsequently, a series of secondary biochemical events occur, leading to dysfunction or injury that is manifested at various levels of biological organization, such as the target molecule itself, cell organelles, cells, tissues and organs and even the whole organism. The amounts of toxicant required to produce these changes is dependent on the chemical and physical properties. Toxicity may be classified as acute toxicity which refers to the exposure within a short time of administration of dose of substance or multiple doses given within 24 hrs and chronic toxicity which refers to exposure of long duration i.e., repeated or prolonged exposure. Subchronic toxicity is defined as adverse effects appearing on repeated chemical exposure for 1-3 months.

Toxicity is objectively evaluated on the basis of test doses, made on experimental animals (like rats, mice and guinea pigs) under controlled conditions. The tests include exposure of the animals through (a) oral ingestion (b) extended skin contact and inhalation of the material under test. LD$_{50}$ is one of the tests used in the evaluation of toxic substances. LD$_{50}$ is the mean lethal dose when administered to animals kills 50% of them. Usually, the exposure
is in terms of mg toxicant / kg body weight either orally, intravenously or subcutaneously (Eaton and Klassen, 1996).

There are many diverse examples of xenobiotics whose toxicity is directly dependent on the activity of the biotransformation enzymes. The product of xenobiotic metabolism is more toxic than the parent compound. For example, the majority of genotoxic chemical required metabolic activation before initiating genotoxicity. The enzymes that protect animal from the toxicity of certain compounds may be responsible for toxicity of others. An organism’s susceptibility to the toxicity of a particular chemical is dependent, on the delicate balance between detoxication and metabolic activation that exists during exposure to the xenobiotic and this balance differs among individuals at different points (Bethizy and Hayes, 1994).

**Dye - stuffs**

To meet the needs of growing population in modern society, a great number and variety of chemicals have been manufactured. A large number of synthetic chemicals are extensively used and their use is expanding so rapidly, so that human exposure to them, through manufacture, handling or consumption has become almost inevitable. With the development of textile, leather, and paper industries, the requirement of synthetic dyes are constantly increasing. Today, more than 95% of the dye stuffs consumed in India are produced indigenously and this gives an idea regarding the possible extent of human exposure to them (Khanna and Das, 1991).
Dye stuffs are manufactured from basic chemicals such as benzene, naphthalene, anthracene, etc. Dyes are classified on the basis of chemical structure namely, azo, anthraquinone, azine, oxazine, thiazine, stilbene, diphenylmethane, triphenylmethane, etc. (Shenai, 1998).

A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to a benzene ring. The auxochrome by itself does not produce colour but deepens the colour of a chromogen. Auxochromes may be either acidic or basic, like hydroxy (-OH) or amino (-NH₂) groups respectively. Other auxochromes include carboxy (-COOH), sulphonic acid (-SO₃H), sulphato (-O-SO₃H), dialkylamino (-NR₂), monoalkyl amino (-NHR) groups etc. These groups form salts with alkalies or acids. They also form hydrogen bonds with certain groups such as hydroxyl group of cellulose, amino group of wool, silk and nylon, and form the basis for affinity of dyes for the fibres (Shenai, 1998).

Dyes have many beneficial applications (for example: as anticancer drugs; adriamycin and daunomycin, in liquid crystal displays, lasers and solar cells), however some exhibit many adverse effects on usage of which the most threatening one is the carcinogenicity. Hepatoma, bladder cancer and skin cancer are the main types. Suspected carcinogenic free amines such as β-naphthylamine, benzidine 3-3'-dichlorobenzidine, 4-amino-biphenyl and 2,4-diamino azobenzene, hydrazines, heterocyclics, azides and imides have been shown to be carcinogenic and mutagenic by Khanna and Das (1991).
Toxicity of phenylmethane dyes

Phenylmethane dyes include malachite green, crystal violet, ethyl violet, gentian violet and auramine O. These dyes pose health hazards and are of great environmental concern.

Exposure to triphenylmethane dyes in human beings show symptoms of eczema and the probable determinant groups for this contact sensitization are -N(CH₃)₂ or -N(C₂H₅)₂ in the para position of the benzene ring structure (Bielicky et al., 1969). The toxic effects observed by Clemmensen et al., (1984) in rats exposed to malachite green, a triphenylmethane dye are diarrhoea, piloerection and dilatation of the gastro-intestinal tract.

Malachite green is used for dyeing silk, wool, leather, jute, cotton, as a laboratory agent and also as a topical antiseptic. It is also used as a non-permitted food colouring agent. Malachite green is extremely cytotoxic to mammalian cells and also acts as a potent liver tumor promoter. Cytotoxicity of malachite green is associated with production of oxyradicals as well as membrane damage. Exposure of Syrian hamster embryo cells to malachite green resulted in the oxidative DNA damage due to the free radical formation which may be responsible for the induction of morphological transformation (Panandiker et al., 1994).

Gentian violet has been known to cause bladder carcinoma, renal, hepatic, lung and mammary gland tumours. Crystal violet induce detrimental changes in DNA molecules. It acts primarily on the nucleic acids in cell nuclei
and cytoplasmic RNA with secondary effects on cytoplasmic protein synthesis, which in turn will reduce the requirement for energy and thus limit mitochondrial respiration (Mobacken et al., 1974).

**Auramine O - an account**

Auramine O (Basic Yellow, Colour Index, CI-41,000) is a cationic fluorescent dye belonging to the category of diphenylmethane colouring substances (ketone amine) having >C = NH group as the chromophore and was first synthesised by Kern and Caro in 1883. Its molecular weight is 267.36 Da. Auramine O is synthesised from dimethylaniline and formaldehyde which react to form Michler's base (Tetramethylidamino diphenyl methane). This base is subsequently converted to auramine O by heating with sulfur and ammonium chloride in the presence of ammonia (IARC Monograph, 1972). It has been manufactured world-wide singly or along with its base or its derivatives by about 24 firms and is marketed under 17 different product formats (Colour Index, 1976).

Despite its low fastness and ease of hydrolysis, it has been used for dyeing wool, silk, leather, acrylic fibre, cotton, paper and jute as well as for the manufacture of pigments (Venkataraman, 1952). In the 1970's auramine O was listed as extensively applied food colourant in Europe (Bigwood et al., 1975). In India, it is detected in food stuffs though not permitted (Singh and Khanna, 1992). Auramine O has also been used as an antiseptic during nose and ear surgery and in the treatment of gonorrhea. Due to its brilliant fluorescence,
it is used in light microscopy for the identification of many microorganisms like *Trypanosoma* and *Mycobacteria* (Gurr, 1963).

Auramine O decomposes at a temperature above 70°C and is a weak base that forms salts with HCl, \( \text{H}_2\text{SO}_4 \) etc. Auramine O is moderately soluble in water, soluble in ethyl ether and very soluble in ethanol. The structure of auramine O which exists in two resonance forms is shown in the Figure No.1.

**Biological properties of auramine O**

Auramine O binds specifically to several proteins / enzymes and forms fluorescent complexes, for example: with horse liver alcohol dehydrogenase (Sigman and Glazer 1972); with lactate dehydrogenase from pig skeletal muscle (Ivanov *et al.*, 1982); with actin (Tellam and Turner 1984); with human \( \alpha \)-1 acid glycoprotein (Sugiyama *et al.*, 1985); with glyceraldehyde-3-phosphate dehydrogenase (Klichko *et al.*, 1986); to calcium liganded calmodulin (Steiner *et al.*, 1992) and with A-T rich regions of DNA where it binds to the outside of the helix (Muller and Gautier, 1975). The anionic polyelectrolyte heparin electrostatically binds auramine O and hydrolyses it into Michler’s Ketone with the liberation of \( \text{NH}_4^+ \) ions in acidic medium. Such binding studies are important in ascertaining the ligand binding site / active site of proteins / enzymes.

In aqueous solutions, auramine O (5 x10\(^{-5}\) M concentration) is weakly fluorescent but in viscous solutions and in the complexed form as in the case of binding with above macro molecules and in the presence of cyclodextrins and
Fig. 1  Shows resonance forms of Auramine O.
surfactants, its fluorescence is enhanced. This enhancement in fluorescence is due to the loss of intramolecular quenching modes resulting from the highly flexible structure of auramine O. So recent interest in auramine O is therefore on using it as a fluoroprobe in basic biochemistry (Mwalupindi et al., 1994).

**Occupational exposure of auramine O**

Human exposure to auramine O may occur during its manufacture by direct and prolonged contact of the skin with the dye. It may enter the environment from industrial discharges or spills. Auramine O has been classified by the International Agency for Research on Cancer (IARC) in Group I dyes on the basis of 'Sufficient' evidence of carcinogenicity in humans. There is increasing evidence of human bladder cancer due to occupational exposure of this dye. Auramine O may also cause reproductive damage in humans (Vineis and Pirastus, 1997). Chronic exposure to auramine O as in the case of workers involved in its manufacture, has led to urinary bladder carcinoma, prostatic carcinoma and papilloma (Kotake, 1989).

**Auramine O - a food dye**

Auramine O has been incorporated in a number of food products due to its brilliant yellow colour. About 70% of the examined coloured food products contained non-permitted colouring substances, mostly industrial dyes. Among these, auramine O is one of the non-permitted food colours which constitutes hazard to public health (Singh and Khanna, 1992). The means of exposure of human beings to this dye is very widespread and common. Public concern over
the use of this food colour may be a question of need and safety. Khanna et al., (1973) reported 3%, 8%, 9% and 10% incorporation of auramine O in milk, non-milk products, pulses and miscellaneous products respectively. This non-permitted food dye has thrived in the market for being less expensive.

**Animal studies on auramine O**

Williams and Bonser (1962) illustrated that administration of auramine O in the concentration of 0.1% of feed to rats for 87 weeks produced hepatoma, mild cirrhosis and minimum bile duct proliferation. Subcutaneous injection of 2.5% suspension (0.1 ml / 100 g body weight for 21 weeks, 5 days per week, totally 110-120 mg) produced hepatoma, fibrosarcoma and intestinal carcinoma. Kidney also showed well differentiated adenocarcinoma and early transitional cell carcinoma of the bladder. Oral administration of auramine O to rabbits to the limit of tolerance has resulted in metaplasia of urinary tract epithelium suggestive of precancerous change and subcutaneous administration has led to fibrosarcoma, hepatoma and in a less extent to intestinal carcinoma (IARC Monograph, 1972).

**Genotoxicity of auramine O**

Rats exposed to auramine O for 4 hr in the concentrations of 125, 250 and 500 mg/kg body weight showed a statistically significant increase in the frequency of DNA single strand breaks detected by the comet assay in both the liver and the urinary bladder mucosa (Martelli et al., 1998). The carcinogenicity/mutagenicity of auramine has been illustrated by various
studies such as: morphological transformation of early passage golden Syrian hamster cells (Pienta et al., 1977); DNA modification in DNA polymerase I deficient Escherichia coli (Rosenkranz and Poirier 1979); mutation induced in histidine strains of Salmonella typhimurium strains TA 98 and TA 100 in the presence of mammalian microsomal mix (S9 mix obtained from rat liver); such an assay detects the base pair substitution or frame shift mutation (Simmon 1979; Parodi et al., 1981).

**Auramine O - a scenario in South India**

Of late, auramine O is used particularly by women as a substitute for cow dung for swabbing floors in Coimbatore, Nilgiris, Periyar, Salem and Dharmapuri districts of Tamil Nadu, India. For ages it has been a tradition for women folk in the rural and emerging urban areas of South India to use aqueous slurry of cow dung for hand swabbing the living area as well as the yards of the house-hold. Factors like mechanisation of agriculture with the resulting reduction in cattle population (and so the availability of cow dung), and modern living without compromise on tradition have culminated in the sporadic use of the so-called "cow dung powder".

The ingenious abuse of "cow dung powder" came to light in 1988 when the Regional Forensic Science Laboratory, Coimbatore (Tamil Nadu, India), received the first forensic toxicology case of suicidal poisoning of a female; ever since the trend of such "ethnotoxicological" abuse has assumed alarming proportion (54 forensic case inputs in the year 1995 alone). Requirements for
forensic determination as well as clinical toxicology of the misnomer "Yellow Cow Dung Powder" therefore, prompted the laboratory to identify its chemical constituents. The scientific exercise that followed established the "Yellow Cow Dung Powder" (YCDP) as the synthetic basic dye of diphenylmethane type - auramine O. The visceral extracts obtained from a suicidal victim were also found to contain auramine O (Damodaran et al., 1995 unpublished data).

Toxic symptoms mimick gastroenteritis and include vomiting, diarrhoea, hypotension, impairment of peripheral circulation, fits/convulsion and finally death. The ignorant local South Indian girls and women face the danger of chronic toxicity from auramine because it is they who for years do the traditional time-and labour-intensive exercise of "cow dung swabbing" at home periodically and thereby exposed to the toxicity of auramine O.

Free radical toxicity

A free radical is a molecular entity that contains one or more unpaired electrons in its outer orbital. In the energy producing processes of cells, molecular oxygen is reduced by four electrons to yield two molecules of water as the end product. Apart from this, partially reduced oxygen species or its excited form (Singlet oxygen) can also be produced. Univalent reduction of oxygen produces the superoxide anion (O$_2^{•-}$) whereas bivalent reduction generates hydrogen peroxide (H$_2$O$_2$) and trivalent reduction generates hydroxyl radical (•OH). Most of these are highly reactive and cytotoxic, but •OH is
considered to be the most reactive species. The generation of $^\cdot$OH is accelerated by transition metals, especially iron (Nakazawa et al., 1996).

The free radicals and other reactive oxygen species (ROS) induce cellular damages. Highly reactive free radical or alkylating species exhibit some specificity in terms of the molecules that are modified and this modification impairs cell function (Bandyopahyay, 1999).

Free radical addition reactions can ultimately lead to covalent binding of xenobiotics to proteins and disrupt their function. Specific amino acid residues within proteins are modified by free radical induced oxidation reactions and are much more susceptible to proteolysis (Davies, 1987). Free radicals react with membrane lipids and cause lipid peroxidation, eventually leading to cell death (Janero, 1990). The interaction of ROS with DNA may involve direct modifications of DNA (i.e., the oxidation of DNA bases or sugars, and strand breaks), or they may be mediated through changes in transcription factors or enzymes involved in regulating gene expression (Cerutti and Trump, 1991).

Free radicals have been implicated in the genotoxicity of the dyes (Combes and Smith, 1982). Free radicals are produced as a part of normal cellular metabolism as well as under conditions stimulated by xenobiotic compounds which have the potential to cause tissue damages associated with a variety of toxicities (Kehrer, 1993). Food dyes, malachite green and metanil yellow, induce lipid peroxidation in Syrian hamster embryo cells and rats
respectively (Panandiker et al., 1992; Ramachandani et al., 1996). Brennan and Schiestl (1998) observed production of free radical species in *Saccharomyces cerevisiae* following exposure to auramine O and suggested a possible role of oxidative stress induced by this carcinogen. The present study has therefore been designed to understand the biochemical alterations in experimental rats on auramine O exposure.

**Defense system against oxidative stress**

Substances that neutralise the potential ill-effects of free radicals are called antioxidants or free radical scavengers. Both enzymatic (superoxide dismutase, catalase, glutathione peroxidase etc.) and non-enzymatic antioxidants (glutathione, ascorbic acid, α-tocopherol, etc.) have been evolved and provide sufficient cellular protection against oxidative stress (Yu, 1994).

Dismutation of \(O_2^{*-}\) to \(H_2O_2\) by superoxide dismutase is called the primary defence, because this enzyme prevents further generation of free radicals (Marklund, 1984). Catalase is a major primary antioxidant defense component which catalyses the decomposition of \(H_2O_2\) to \(H_2O\) (Thomas et al., 1987). Glutathione peroxidase (GPx) catalyses the reduction of \(H_2O_2\) and organic hydroperoxides (Wendel and Cükkryt, 1980).

Glutathione (GSH), a non-enzymatic antioxidant, is a cysteine containing tripeptide and is the most abundant non-protein thiol in mammalian cells. GSH acts a substrates for GST and GPx enzymes which catalyse the reactions for the detoxification of xenobiotic compounds and for the autooxidation of
reactive oxygen species and free radicals (Bray and Taylor, 1993). Vitamin C is a water soluble vitamin which is found in the cytosol and plasma. It scavenges superoxide, hydroxyl and peroxyl radicals, hydrogen peroxide, hypochlorite and singlet oxygen (Sies et al., 1992). The antioxidant action of lipophilic vitamin E is highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxy radicals (McCay, 1985).

**Xenobiotic - biotransforming enzymes**

The process of elimination of xenobiotic compounds by the conversion of water soluble chemicals is called as biotransformation. Physical properties of the xenobiotics are generally changed from those favouring absorption (lipophilicity) to those favouring excretion (hydrophilicity). Xenobiotic - biotransforming enzymes are called drug - metabolising enzymes and are generally divided into two groups, phase I and phase II. Phase I reactions involve hydrolysis, reduction and oxidation. These reactions expose or introduce a functional group (-OH, NH₂, -SH, -COOH) and usually result in only a small increase in hydrophilicity. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione and conjugation with amino acids (such as glycine, taurine and glutamic acid). Drug metabolising enzymes are located primarily in the endoplasmic reticulum (microsomes) or the soluble fraction of the cytoplasm (cytosol) with lesser amount in mitochondria, nuclei and lysosomes.
Biotransformation of some xenobiotic compounds begins in the liver, where it is oxidised by cytochrome P\textsubscript{450} and conjugated with glucuronic acid. This glucuronide is excreted in bile and biotransformed by intestinal microflora. The glucuronide is hydrolysed by β-glucuronidase. Metanil yellow, a monoazo dye contains quinone group which is reduced by quinone NADH reductase, a cytosolic flavoprotein (Ramachandani et al., 1996).

Ruddick et al. (1979) reported that microsomal detoxification system is involved in metabolising synthetic dyes. The highest concentration of cytochrome P\textsubscript{450} are found in liver microsomes and also present virtually in all tissues. Liver microsomal cytochrome P\textsubscript{450}, a heme-containing protein plays a very important role in determining the intensity and duration of action of drugs, and also plays a key role in the detoxification of xenobiotics. The heme iron in cytochrome P\textsubscript{450} is usually in the ferric (Fe\textsuperscript{3+}) state. When reduced to ferrous (Fe\textsuperscript{2+}) state, cytochrome P\textsubscript{450} can bind ligands such as O\textsubscript{2} and carbon monoxide (CO). The complex between ferrous cytochrome P\textsubscript{450} and CO absorbs light maximally at 450 nm (Guengerich, 1994).

The basic reaction catalysed by cytochrome P\textsubscript{450} is monoxygenation, in which one atom of oxygen is incorporated into a substrate (RH) and the other is reduced to water with reducing equivalents derived from NADPH, as follows:

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\text{Substrate (RH)} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Product (ROH)} + \text{H}_2\text{O} + \text{NADP}^+
\]
Liver microsomes contain polymorphic forms of cytochrome $P_{450}$ but contain a single form of NADPH - cytochrome $P_{450}$ reductase and cytochrome $b_5$. For each molecule of NADPH - cytochrome $P_{450}$ reductase in rat liver there are 5-10 molecules of cytochrome $b_5$ and 10-20 molecules of cytochrome $P_{450}$. NADPH cytochrome $P_{450}$ reductase will reduce electron acceptors other than cytochrome $P_{450}$, which enables this enzyme to be measured based on its ability to reduce cytochrome c (Guengerich, 1987).

Glutathione can also conjugate xenobiotics containing electrophilic heteroatoms (O, N and S) which is catalysed by glutathione S-transferase and is present in high concentrations in liver, kidney and intestine.