2 REVIEW OF LITERATURE

2.1 Genotoxicity and its Principal Mechanisms

Genetic toxicology is gaining importance among the scientific fraternity across the globe since it affects not only the individual but also his progeny. As an effort to tackle this situation, attention to mutation research has increased and a significant pool of evidence has been accumulated towards the mutagenic potential of medicinal agents, environmental pollutants and industrial chemicals. Genotoxicity tests are a critical component of safety evaluation of drugs and chemicals like pesticides, food additives and packaging material. To obtain valid data on the mutagenic and carcinogenic properties of a chemical, information regarding the genotoxic effects of the chemical at different levels namely, the DNA, the chromosomes and the cellular machinery like spindle apparatus, necessary for chromosome segregation, is very much essential[36].

Genotoxicity is the ability of a chemical agent or radiation to cause damage to the genetic information within a cell by chemically interacting with DNA and/or non-DNA targets [37]. Such damage in germ cells affects reproduction or lead to heritable mutations. In case of somatic cells, it may lead to malignancy. All mutagens are genotoxic, while all genotoxins are not [37, 38].

Several mechanisms of genotoxicity have been proposed which can be broadly grouped as direct and indirect (Figure 2.1.1). Direct damage to DNA involves formation of oxidative DNA adducts, DNA strand breaks, nitrogen base (e.g., formation of 8-hydroxydeoxyguanosine adducts) or deoxyribose modifications, and cross-links [14, 15,39]. Continual DNA damage may result in genomic instability, replication errors, arrest or induction of transcription, and signal transduction pathways, which eventually lead to carcinogenesis [15].
Genotoxicity can also occur through indirect mechanisms where in the genotoxic agent interacts with non-DNA targets, leading to formation of protein adducts, oxidative stress, inflammation and abnormal signalling responses [40]. Target proteins include repair enzymes, DNA and RNA polymerases or other enzymes involved in DNA replication, factors involved in apoptosis (e.g. p53, bax, bcl-2), proteins that regulate cell cycle (e.g. p53, cyclins), and proteins such as glutathione that defend against oxidative damage [7,14, 41–43].

Genotoxins cause oxidative stress in the cellular environment and generate free radicals. Free radical-initiated damage to biomembranes results in peroxidation of lipids, which in turn causes production of such as reactive electrophiles like epoxides and aldehydes like Malondialdehyde (MDA) [15]. Reactive oxygen species (ROS) can also induce cell proliferation and apoptosis [44, 45], alter gene expression by altering
signalling pathways (e.g. cAMP-mediated cascades, calcium-calmodulin pathways), and activation of transcription factors. Different genotoxic agents employ different mechanisms of causing damage. They also may employ more than one mechanism and may vary with the type of tissue/cells [2, 5, 46, 47].

Genotoxic endpoints like incidence of micronuclei, chromosomal aberrations and DNA fragmentation are well known markers of genotoxicity and diminution in the occurrence of these genotoxic endpoints is indication of the antigenotoxicity of a particular compound [48].

2.2 Genotoxicity and Cytotoxicity Assays

2.2.1 Genotoxicity assays

Several in vivo and in vitro assay systems for assessing the genotoxic/antigenotoxic and cytotoxic/cytoprotective properties of substances have been developed. A test should be sensitive as well as specific [49]. Due to the diversity of the biomarkers for genotoxicity and/or mutagenicity of a compound, it is essential that multiple tests need to be conducted to evaluate a test substance. Different organisations and advisory bodies have formulated guidelines for such evaluation. The Organisation For Economic Cooperation and Development (OECD) [50] recommends that the battery of tests include short and long term tests, as well as in vitro and in vivo.

Assays are designed based on diverse endpoints of genotoxicity such as point mutations, deletions, insertions, gene amplifications, micronuclei (Mn), chromosomal aberrations (CA) and sister chromatid exchanges (SCE). The various test systems widely adopted by the scientific community for genotoxic assessment of compounds are described briefly here.

2.2.1.1 Bacterial reverse mutation test /Ames test (OECD TG 471)

This assay is based on identification of reverse mutations in Salmonella typhimurium strains with defined mutations in the histidine locus and hence, auxotrophic for histidine. Bacteria are exposed to the test compound, and the revertants,
able to survive in the absence of histidine are selected. Strains of *Escherichia coli* are also employed [51, 52].

### 2.2.1.2 In vitro mammalian chromosome aberration test (OECD TG 473)

The test identifies substances that can cause structural chromosome aberrations and numerical changes such as polyploidy and duplication in cells. It employs cultures of established mammalian cell lines or primary cell cultures. Most compounds that are positive in this test are mammalian carcinogens [38, 53].

### 2.2.1.3 Mammalian erythrocyte micronucleus test (OECD TG 474)

Erythrocytes are sampled from the bone marrow and/or peripheral blood cells of experimental animals (mice or rats), for analysis of damage to the chromosomes or the mitotic apparatus caused due to test compounds. The cytogenetic damage is depicted as micronuclei formed by lagging chromosome fragments or whole chromosomes [54].

### 2.2.1.4 Mammalian bone marrow chromosome aberration Test (OECD TG 475)

Structural chromosome aberrations induced in bone marrow cells of laboratory animals, usually rodents, treated with the test substance. Sometimes animals may be also treated with a metaphase-arresting agent. Bone marrow cells are collected, stained and analysed for chromosome aberrations [55].

### 2.2.1.5 In vitro mammalian cell gene mutation test (OECD TG 476) or the mouse lymphoma assay (MLA) assay

It detects mutations involving the thymidine kinase (Tk) locus in mutant mouse lymphoma cells. Other Suitable cell lines include the CHO and TK6 human lymphoblastoid cells. Mutations in hypoxanthine-guanine phosphoribosyl transferase (HPRT), and xanthine-guanine phosphoribosyl transferase (XPRT) loci are also commonly identified endpoints [56].

### 2.2.1.6 In vitro sister chromatid exchange assay in mammalian cells (OECD TG 479)

SCEs are interchanges of DNA replication products at homologous loci between the sister chromatids of a replicating chromosome. The cells *in vitro* are exposed to the
test chemical and grown for two generations in BrdU-containing medium, for differential labelling of the sister chromatids. Then they are subjected to treatment with a spindle inhibitor like colchicine to arrest the cells in a metaphase-like stage of mitosis (c-metaphase) and enumerated for SCE’s [56, 57].

2.2.1.7 In vitro mammalian cell micronucleus test (OECD TG 487)

Clastogenic (structural) and aneugenic (numerical) changes induced by test substances in cells that have undergone cell division during or after exposure to it are detected. Inclusion of the actin polymerisation inhibitor cytochalasin B, allows for the identification and analysis of Mn in cells that have completed one cycle of mitosis, because such cells are binucleate. The use of protocols without cytokinesis block is also allowed provided there is evidence that the cell population analysed has undergone mitosis [58].

2.2.1.8 Comet assay/ single cell electrophoresis test

This is single cell cytogenetic approach for detection of DNA crosslinking, single strand breaks and alkali-labile sites. This highly sensitive assay detects damage at individual cell level, even at alkaline pH and also provides for very specific damage detection using enzymes and antibodies [59].

2.2.1.9 Plant assays for assessing the genotoxicity

Higher plant genetic bioassays such as Allium root tip meristem analysis test and the Barley chlorophyll mutation assay are highly recommended to be relevant for the detection of chromosome aberrations and gene mutations induced by test substances. Other plants that are frequently used are Crepis capillaris, Lycopersicon esculentum, Hordeum vulgare, Pisum sativum, Tradescantia, V. faba and Zea mays [35, 60, 61]

2.2.2 Cytotoxicity assays

Cytotoxicity of substances plays a major factor in a number of pathological processes, like inflammation and carcinogenesis. Evaluating the cytotoxic nature of substances is essential in understanding the mechanisms of action of those substances
on living cells and tissues [7,26, 62–65]. Several cytotoxicity assays based on animal cell culture systems, which allow measurement of cell growth inhibition in response to chemicals tested, in a rapid and precise manner, are widely used in preliminary studies. They enable the select the suitable concentrations of the tested compound for further experiments and are also used in alternative in vitro methods for regulatory purposes. Assays are designed based on the measurement of various cytotoxicity biomarkers like cell membrane integrity, mitochondrial injury, lysosomal dysfunction, cell protein content, incorporation of radioisotopes, colorimetric and luminescence measurement tests.

Biological membranes can be damaged by cell disaggregation, cell separation or freeze–thaw process. This lose of integrity can be determined by uptake of dyes to which normally viable cells are impermeable (e.g. naphthalene black, erythrosine, trypan blue) or by the release of dyes normally taken in and retained by intact cells (e.g. neutral red, diacetyl fluorescein). Researchers across the world have evaluated and suggested the use of various in vitro assays in toxicology [66]–[68]. The most widely adopted testing procedures are given below.

2.2.2.1 Trypan blue (TB) staining

Viable or intact cells are impermeable to dyes such as trypan blue, nigrosin green, naphthalene black, and erythrocin B. As such, cells dead due to damaged plasma membrane allow entry of the stain, whereas live cells with intact plasma membrane do not stain. The number of intact (unstained) cells is a direct measure of the cell viability in the preparation.

2.2.2.2 Neutral red (NR) uptake test

Living cells stain with neutral red by active endocytosis through the cell membrane and its storage into lysosomes and Golgi apparatus. The number of stained cells gives the number of viable cells.
2.2.2.3 MTT, MTS and XTT assays

These assays are based on measuring the activity of mitochondrial dehydrogenases as an indicator of cell viability and cell number. A population of cells in culture are exposed to the tested compound drug during the log phase. The test compound is then removed and cells are allowed to proliferate for 2-3 population doubling times. They are then incubated with the tetrazolium salt substrate to allow its conversion into a coloured formazan product, by mitochondrial dehydrogenases. The commonly used tetrazolium compounds include MTT, MTS, XTT, and WST1. These are basically of two categories:

1) Positively charged salts like MTT and capable of penetration into viable eukaryotic cells

2) Negatively charged salts like MTS, XTT and WST1, which do not readily penetrate cells.

The number of surviving cells is directly proportional to the spectrophotometric measurement of the concentration of the colour intensity.

2.2.2.4 Sulforhodamine B (SR-B) assay

The SR-B dye binds to the proteins of cells fixed with Trichloroacetic acid. The amount of bound dye is a measure of cellular protein which indirectly determines the total number of cells.

2.3 Description of Genetic Toxicity Tests

2.3.1 Mammalian bone marrow micronucleus assay

The bone marrow micronucleus (Mn) assay system has been widely used for assessing genotoxicity and cytotoxicity [69]. Micronucleus is described as a small extra nuclear chromatin body originating from an acentric fragment or whole chromosome lost from the metaphase plate. They are also known as Howell–Jolly bodies, since their identification by the American scientist, William Howell, and Justin Jolly, a Frenchman. They appear as distinct membrane bound chromatin bodies in the cytoplasm of eukaryotic cells exposed to genotoxic agents. The significance of this technique was
emphasised by several studies as a consistent method for measuring of both chromosome breakage and chromosome loss caused by cytotoxic agents [41, 70]. Most preferred experimental animals are mice. Other rodents such as rats and hamsters are also used. Most suitable tissue for enumerating the micronuclei is the bone marrow as it is the major haematopoietic tissue, has rapidly dividing cells and is well-vascularized, in mature animals. Exposure to a test chemical during proliferation of erythropoietic cells can lead to chromosome breakage and damage to the mitotic apparatus. These broken chromosome fragments or lagging chromosomes form micronuclei. When the erythroblast develops into a erythrocyte, the main nucleus is extruded and the micronuclei are left in the cytoplasm and hence, well visualised by staining [71]. Moreover, the PCE still contains RNA, and stains blue grey with Giemsa, where as mature, haemoglobin-containing erythrocytes, stain orange with Giemsa. This aspect enables identification of the cells with micronuclei induced by the test substance. It is important to collect PCEs from the bone marrow or peripheral blood before their differentiation to mature erythrocytes. The induction of micronuclei may lead to cellular lethality and mutation, leading to a lower number of PCE. Erythropoietic safety assessment in new compounds is vital in drug discovery and development. Both structural (clastogenic) and numerical (aneugenic) chromosome changes can be detected. Since it is relatively simple and at the same time shows high sensitivity, the in vivo rodent erythrocyte micronucleus assay has gained extensive acceptance [54].

**Mechanisms of micronuclei formation**

Micronuclei harbouring chromatin bits are products of breakage of double-stranded DNA molecule, SSBs that get converted into DSBs following replication, or arrest of DNA synthesis. Fusion of two broken chromosomes could result in a dicentric chromosome and an acentric piece of chromatin. These acentric fragments in anaphase lag behind to form micronuclei. Mn with whole chromosomes appear due to defects in the genes of segregation machinery, spindle apparatus, mechanical or chemical disruption of centromeric DNA [72, 73]. Mn may also be generated through the breakage-fusion-bridge (BFB) cycles [74]. The fate of micronuclei is disappointingly
understood. The subsequent Figure 2.3.1-Figure 2.3.4 explains possible mechanisms for the formation of micronuclei.

a) Acentric chromosome/chromatid fragments resulting from SSB or DSB
b) Whole chromosomes/ chromatids that lag behind in anaphase
c) Depolymerization of tubulin
d) Defects in centromeric DNA, proteins or assembly
e) Late replication, peripheral location in the nucleus and epigenetic modifications of histones

f) Formation of nucleoplasmic bridges

1. Asymmetrical repair of two broken chromosomes producing a dicentric chromosome and an acentric fragment
2. Dicentric chromosomes formed by telomere end fusions.

NPB between the daughter nuclei are formed when kinetochores of the dicentric chromosomes are dragged to opposite poles at anaphase. Either the lagging acentric fragment or the broken NPB will form a micronucleus.

g) Breakage-fusion-bridge cycles leading to DNA amplification and its selective elimination via nuclear buds [73].
Figure 2.3.1 Mechanisms of micronuclei formation
Figure 2.3.2 Mechanisms of micronuclei formation
Figure 2.3.3 Mechanisms of micronuclei formation
2.3.2 Estimation of oxidative stress bio-markers in liver

2.3.2.1 Estimation of lipid peroxidation in the liver:

The metabolism of most endogenous compounds as well as exogenous substances in the human or animal body is primarily centred in the liver. Reactive oxygen species (ROS) or free radicals are generated during the metabolic processes in the liver tissues. They play an important role in modulating normal cellular functions, as secondary messengers. An imbalance between the production of ROS and their removal leads to a condition commonly termed as oxidative stress [75]. ROS can adversely affect nucleic acids, lipids, and proteins leading to disturbance in cellular homeostasis. ROS are relatively short-lived and the damage caused is predominantly local. But, they initiate oxidative deterioration of lipids in the cell, leading to production of aldehyde by-products. These secondary oxidative species have longer half-lives and can diffuse to distant intra- and extra-cellular targets, causing further oxidative damage. The production of ROS is amplified due to impairment of mitochondrial function by the aldehyde by-products [76]. Lipid peroxidation is a direct indication of oxidative stress. It is involved in aging and several pathological disorders [77]. The estimation of lipid
peroxidation by using the primary oxidative species is rendered impractical by their labile, short-lived nature. As a result, detection of LPO is mostly based on indirect methods based on analysis of secondary products like MDA [78].

The quantification of MDA in tissue or cell homogenates is widely done by the method of Okhawa et al., 1979 [77]. MDA is a volatile by product of enzymatic oxygenation of arachidonic acid and other lipids. It is a short-chain, low-molecular-weight (C₃H₆O₂; formula weight = 72.07), 1,3- dicarbonyl compound with a pKa of 4.46. This is a sensitive assay involving the reaction of Thiobarbituric acid (TBA) with Thiobarbituric acid reactive substance (TBARS or MDA) during aerobic incubation with tissue homogenates, forming a fluorescent red product (Figure 2.3.5) [78]. This method has been widely employed by researchers to quantify lipid peroxidation in a diversity of chemical and biological matrices as a biomarker of oxidative stress. The oxidant/anti-oxidant behaviour of substances subsequently is an indication of genotoxic/antigenotoxic abilities of the compounds being investigated [27, 79–83].

![Figure 2.3.5 Chromogenic reaction of MDA with TBA](image-url)
2.3.2.2 GSH estimation

An antioxidant is a substance that significantly lowers or inhibits oxidation of a substrate even when present at low concentrations, compared to that of the oxidizable substrate [84]. Reduced glutathione (GSH), is the most prevalent intracellular antioxidant. It is a low molecular weight soluble thiol synthesized in cells. GSH is a tripeptide, \( \gamma \)-L-glutamyl-L-cysteinyl-glycine. The sulfhydryl group (–SH) present in cysteine is crucial for the activity of GSH across a variety of metabolic processes, like synthesis of proteins and nucleic acids, transport of cellular molecules, enzyme activity, peroxide removal and cell-cycle regulation [85].

Cytosolic concentration of GSH is in the range of 1–10 mM. The hepatocytes export GSH, and have a high concentration of about 10 mM. It regulates apoptosis in mitochondria and cellular division in the nucleus. The ratio of reduced and oxidized forms of glutathione (GSH/GSSG) is an indicator of oxidative stress. Detoxification of \( \text{H}_2\text{O}_2 \) and lipid peroxides by GSH is catalysed by the enzyme GSH reductase (GSHPx) using NAD(P)H as electron donor. Under normal conditions, above 98% of cellular glutathione exists as GSH. GSH acts as proton donor to membrane lipids against oxidant attacks. It is a cofactor for enzymes, such as glutathione peroxidase and transferase, involved in cellular detoxification. It also is involved in onverting back other cellular antioxidants like vitC and vitE to their active forms and regulation of several transcription factors such as AP1,NFκB, and Sp1[2, 44, 85].

Due to this immensely important role of GSH in preservation of cellular redox balance, GSH has gained much importance in chemotherapy [86]. Measurement of GSH is based on its reaction with Dithionitrobenzoic acid (DTNB). DTNB reacts with GSH forming a conjugate and TNB anion that are quantified by absorbance.

2.3.3 Allium cepa root meristem cell analysis

The Allium cepa test has been widely accepted as a standard cytogenetic system for evaluation of potential genotoxic and cytotoxic effects of environmental pollutants as well as other synthetic and natural compounds of interest [87–90]. This plant is of
choice due to low raising costs, ease of handling, rapid execution, high sensitivity and appropriate chromosomal features. It has large chromosomes (2n = 16), facilitating good and convenient microscopic observation of chromosome damages and aneuploidy [91]. Available literature shows the data obtained from this system can be well correlated with that from prokaryotic and animal systems [29, 92, 93].

The two parameters that are evaluated as biomarkers of genotoxicity of substances, in the root tip meristems are mitotic index (MI) and the frequency of chromosomal aberrations (fCA). Mitotic index is obtained by counting all stages of mitotically active cells per 1000 cells. Lowering of mitotic index is an indicator of the mitodepressive effect of test substance [94, 95]. The mitotic index is given by

\[ MI = \frac{n_d}{n_t} \times 100\% \]

\[ n_d \text{ - number of dividing cells and } n_t \text{ - total number of cells}. \]

The number of cells showing mitotic abnormalities like lagging chromosomes, spindle abnormalities, adherent nucleus, anaphasic bridges and broken chromosomes, in anaphase or telophase, are counted per 1000 cells, in each root tip, by scanning the slides from right to left, up and down. The frequency of chromosomal aberrations is calculated as

\[ f\text{CA} = \frac{n_a}{n_t} \times 100\% \]

\[ n_a \text{ is the number of aberrant cells}. \]

2.3.4 MTT Assay

The MTT (3(4,5dimethylthiazol2yl) 2,5diphenyltetrazolium bromide) tetrazolium reduction assay is a high throughput screening (HTS) [96] assay developed for a 96well format [97]. It has been widely adopted in academic and research labs [98–102]. Only viable cells with active mitochondrial functioning convert yellow MTT into purple coloured formazan. The intensity of colour formation is directly proportional to the number of viable cells. The absorbance is measured at 570 -595 nm [103] in a plate reading spectrophotometer. The MTT- formazan product is insoluble and precipitates in
the cells and culture medium. This needs to be solubilized before reading the absorbance. A dose-response curve is prepared and used to calculate the IC50 value of the test agent [104]. Sensitivity of the MTT assay is influenced by factors like the physiological state of cells and variations in dehydrogenase activity.

\[
\text{% growth inhibition} = 100 - \% \text{ Cell viability}
\]

\[
\text{Cell viability (\%)} = \frac{\text{Mean OD}/\text{Control OD}}{\times 100}
\]

2.3.5 DNA fragmentation assay

Oxidative DNA damage is considered as carcinogenic and can actively lead to occurrence of pathological conditions like cancer and aging. Natural compounds offer protection against oxidative DNA damage by directly scavenging the free radicals or by reducing their production when incubated simultaneously with the genotoxic and antigenotoxic agents. In case of pretreatment, they may exert effects such as increasing the level of cellular enzymatic and non-enzymatic antioxidants, inhibition of phase I bioactivating enzymes and/or induction of phase II detoxifying enzymes [19,105]. DNA fragmentation is also a hallmark of apoptotic cell death. Several carcinogenic and chemotherapeutic agents, such as methylprednisolone, 4-hydroxytamoxifen, methotrexate, 5-fluorouracil, cisplatin, y-irradiation, and nitrogen mustard, are known to induce apoptosis. During apoptosis, caspase 3 activates ICAD to CAD by cleavage. CAD in turn cleaves the DNA at the linker sites between the nucleosomes. The degree of DNA damage is evaluated by extraction of DNA from the treated cells or tissues and resolving it on an agarose gel or by performing comet assay, along with DNA from suitable controls. The antigenotoxic effects of several natural substances has been confirmed by their ability to prevent DNA fragmentation. D. speciosum extracts wield protection against 4NQO induced genotoxicity, demonstrated by reduced DNA fragmentation, acting as a desmutagenic agent [106]. In yet another study, bixin, was found to decrease DNA fragmentation in cisplatin-treated PC12 cells [107].

2.3.6 Haemolysis assay

Haemolysis is the lysis of erythrocytes induced by the effect of varied substances like contact toxins, metal ions and drugs, on blood. It releases iron and other
components into the circulatory system and also leads to anaemia. Particularly during prolonged contact, haemolysis may cause adverse effects [108,109]. The biological haemolytic or antihaemolytic properties of compounds need to be assessed as an indication of their biological safety and protective efficiency [110–112].

2.4 Drugs and their Genotoxicity

Most of the chemotherapy agents cause DNA damage leading to growth inhibition and apoptosis. But most of the these are non-selective in their action with significant cytotoxicity and genotoxicity to normal cells. Incidence of secondary malignancies is a major constraint of chemotherapy [113]. Therefore it is vital to include agents that can avoid these side effects in chemotherapy regimen.

Cyclophosphamide (CP) is an alkylating chemotherapy drug, widely used for treating malignant and non-malignant disorders. The mutagenicity and genotoxicity of cyclophosphamide has been extensively studied in vitro and in vivo [114, 115]. The cytotoxic metabolites of this alkylating agent are acrolein and phosphoramidate mustards, capable of causing DNA crosslinking and strand lesions. It caused significantly higher frequency of MnPCEs in mice and other experimental organisms compared with controls in several studies [65, 80, 116].

Cisplatin has genotoxic effects like chromosomal aberrations, formation of Mn and granular chromatin condensation in cultured mammalian cells and in bone marrow and other types of cells and also sperm head abnormalities in experimental animals [86]. The induction of a Mn by Cycloplatum, a chemotherapy drug in vivo and in vitro indicates a potential risk for patients [117]. Studies by Vijaylaxmi et al., [118] showed induction of transplacental micronuclei in mice by Carboplatin, a widely used platinum anticancer drug. Oxaliplatin also causes CAs and SCEs in human lymphocytes [119].

Melphalan and Mitoxantrone are also anthracycline antibiotics used in cancer therapy capable of inducing genotoxic effects in human lymphocytes in vitro in a dose-dependent manner [120]. Doxorubicin, another anthracyclin antibiotic, used in the
treatment of leukaemia, lymphoma, solid tumours of the breast and ovaries has dose-limiting, cardiotoxic side effects caused by increased ROS production and genotoxic effects induced by inhibition of the enzyme topoiso merase II [121]. Investigations by YurtCu et al., [122] demonstrated significant genotoxic and cytotoxic effects of Doxorubicin and Silymarin, individually or in combination on HepG2 cells. Another semisynthetic anthracycline antibiotic Epirubicin, also causes micronucleation in mice bone marrow [123]. Direct strand breaks, oxidation and methylation of the DNA bases can underlie the DNA-damaging effect of anticancer drugs [124].

Taxanes are drugs effective against tumours in different organs, lead to development of nonfunctional microtubule bundles [125]. 5-Fluorouracil (5-FU) another widely used antimetabolite anti-neoplastic drug in cancer therapy, caused DNA damage in the liver and blood cells, and micronuclei in erythrocytes of zebra fish[126].

A vast number of drugs used to treat pathological conditions other than cancer, also possess genotoxic and cytotoxic abilities. A compendium on the genotoxicity information of more than four hundred structurally diverse pharmaceutical products by Snyder and Green [10] indicates that 28.7% of the studied compounds had positive results in at least one of the genotoxicity assays. Several marketed bronchodilators and asthmatics had tested positive for genotoxic effects [11]. A review by Brambille et al., [127] indicates that many antibacterial, antimalarial, antiviral and antifungal drugs showed genotoxic and carcinogenic abilities in vitro.

2.5 Significance of Antigenotoxic Agents

2.5.1 Role of ROS and antioxidants in genotoxicity

ROS are formed as a result of normal metabolism, but at high concentrations, they adversely affect cell components. A shift in the cellular redox balance towards oxidants is termed “oxidative stress.” This can occur either due to exhaustion of antioxidants or build up of ROS. Various forms of reactive oxygen species are singlet oxygen ($^1O_2$), superoxide anion (O$^2^-$), peroxyl radicals (ROO$^*$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (*OH). O$^2^-$combines with NO to yield peroxynitrite
anion (ONOO\(^{-}\)) and peroxynitrous acid, which are secondary oxidizing entities. This reduces NO bioavailability and is termed as nitrosative stress [16].

A sufficient level of intracellular antioxidants is crucial for maintaining normal cellular function. The inherent antioxidant defence system of human body is composed of several endogenous antioxidants, which act by different mechanisms such as scavenging of oxidative species, reduce them to non-toxic forms or chelation of the metal ions required for their activation. These can be grouped into 2 categories - enzymatic and non-enzymatic [8, 15].

- **Enzymatic antioxidants** – Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GTPx), Thioreductase (TRX), Peroxiredoxin (PRX) and Glutathione transferase (GST). Enzymatic antioxidants act on specific substrates and are responsible for converting ROS and reactive nitrogen species to harmless species.

- **Non-enzymatic antioxidants** (small molecules) – Vitamins A, C and E, β-carotene and Glutathione. These non-enzymatic antioxidants, act as oxidants and free radical scavengers.

Cells restore redox homeostasis by either activation or silencing of genes coding for defence enzymes, structural proteins and transcription factors. In addition to endogenous ones, exogenous antioxidants ingested through diet also play a role in maintaining the redox homeostasis. These act as chemopreventive agents by scavenging the ROS and enhancing host antioxidant defence systems against genotoxic carcinogens [65]. Antioxidants can protect DNA from oxidative damage, as indicated by a reduction in genotoxicity biomarkers such as micronuclei and chromosomal aberrations. The use of antioxidants from various natural and dietary sources, as antigenotoxic agents have been studied extensively.
2.5.2 Evidence of some natural products with genotoxic and antigenotoxic effects

Natural compounds can exhibit multiple biological activities such as antioxidant ability, metal ion chelation, modulation of DNA repair systems [128], inhibition of bioactivating enzymes and induction of detoxifying enzymes [3] and inhibition of cytochrome P 450 mediated activation of toxic compounds [7].

Ascorbic acid prevents oxidative DNA damage and Mn formation induced by heavy metals and radiation [129]. Flavonoids like quercetin, rutin and ursolic acid suppress the superoxide ion toxicity by scavenging it and enhance the production of DNA repair enzyme beta DNA polymerase, thereby increasing the cell survival [130]. Mozdarani & Kamali in 1998 [7] proposed that ROS scavenging ability is one of the mechanisms for the antigenotoxic activity of cimetidine against benzene.

Kilani-Jaziri et al., [131], reported the anti-microbial, antioxidant and antigenotoxic potential of Cyperus rotundus, could be due to compounds such as flavonoids and phenols. The antigenotoxic ability of Acai fruit pulp against doxorubicin was evidenced in studies conducted by Ribeiro et al., [132]. Flavonoids such as quercitin, rutin and terpenoids like ursolic acid have been reported to show antigenotoxic activities in different in vitro models [19]. Grape fruit juice is capable of reducing the concentration of blood cholesterol and also has efficient anticlastogenic properties [64]. Myrciaria dubia juice has antigenotoxic and antioxidant effects on mice blood cells [28]. Celiklar et al., in 2008 showed the antimutagenic activity of the green alga Ulva rigida[133]. Camel milk has shown anticlastogenic effects against the cisplatin induced micronucleation and anticytotoxic effects by restoring the mitotic index of bone marrow cells [134].

Hydroalcoholic extracts of Brassica leaves significantly reduced DNA damage inflicted by doxorubicin in mice [135]. Palmarosa and citronella essential oils [105] and Eicosapentanoic acid [65] show potential as adjuvants in chemotherapeutic regimen by virtue of their antigenotoxic, antioxidant and cytoprotective effects.
2.6 Review on Importance of Plant Products and Active Metabolites in Disease Therapy

Plants produce numerous secondary metabolites in response to environmental stimuli. These include phenols, carotenoids, alkaloids, amines, betalains, vitamins, terpenoids and other endogenous compounds. These compounds are also called phytochemicals. They protect the plants from diseases and grazing animals, as well as responsible for the colour and fragrance. Phytochemicals are stored in different parts of the plants. Epidemiological studies have revealed that many of these exhibit biological properties such as antioxidant, anti-inflammatory, antitumour, antimutagenic, antimicrobial and antiviral activities. They can modulate enzymes involved in detoxification and hormone metabolism and stimulate the immune system [28, 106, 133, 136]. Zuccagnia punctata [26], Cyperus rotundus [131], Acai (Euterpe oleracea) [132], Dendrobium species [106], Ocimum gratissimum [137], and several other plant extracts are bestowed with potential antihelminthic, antibacterial, antifungal, and antiviral activities, as well as immunomodulation and cancer chemoprevention [21]. Vanillin [138], Ginseng [139], Salvadora persica [27] have been reported to exhibit multifunctional effects, including antimutagenic, antiangiogenic, anticolitis, and antianalgesic effects. Some natural substances are used in cosmetic formulations due to prominent characters like UV absorption ability [81].

Inhibitors of mutagenesis are also inhibitors of carcinogenesis. The incorporation of antimutagens through everyday diet, either in the form of fruits and vegetables or supplements, will be a very effective stratagem for prevention of cancer and genetic diseases [140].

2.7 General Therapeutic Importance of Plant Phenols and Saponins

2.7.1 Phenols

Phenolics are secondary metabolites found practically in all higher plants. They are a large group comprising of thousands of individual entities. Phenolic compounds in plants serve in diverse purposes like growth, pigments, defence against pathogens and
environmental stresses and the list is still growing. They are of major importance concerned with human nutrition, ethnopharmacology as well as toxicology. Phenols are very good antioxidants and quench reactive radical intermediates in an array of molecular processes. They are considered as very good candidates for reverse pharmacology, a concept of studying activities of compounds from the state of proven safety and efficacy towards the molecular mechanism of their action [141].

2.7.1.1 Structure and Physicochemical properties

Phenolics, also termed as polyphenols consist of an aromatic ring with one or more hydroxyl groups. They exhibit great structural diversity ranging from monomers to polymer. They are formed from pentose phosphate and shikimate pathways. The number of hydroxyl ions the aromatic rings determines the antioxidant capacity [142]. They can be present as mono- and poly-saccharide conjugates or as esters and methyl esters. Their structure influences their absorption and metabolism, which in turn determines their biological properties. The phenolic compounds are further grouped into phenolic acids, quinones, tannins, stilbenes, flavonoids, lignans, coumarins and others.

2.7.1.2 Biological activities

Polyphenols have shown an array of important biological activities like antimicrobial activity, reduction of triglyceride deposition, protection against diabetes, cancer, cardiovascular inflammatory and allergic diseases [24, 25], majorly ascribed to their antioxidant capacity. Topical studies substantiate that the even very low concentrations of phenols or their metabolic products can wield modulatory effects on protein and lipid kinase cascades, crucial for cell cycle regulation [24, 142].

Phenolic acids and tannins are reported to have anticarcinogenic, antigenotoxic effects, as they protect DNA against free radicals, by blocking the sites of DNA vulnerable to electrophilic attack, inhibit enzymatic activation of pro-carcinogens and enhance enzymatic detoxification of xenobiotics [19, 23,24, 27, 133, 142]. They also influence the DNA repair pathway by modulation of gene expression and/or mRNA stabilization and inhibit DNA methylation and the formation of adducts. Phenolic substances may also protect biological systems through the reduction of absorption of
toxic compounds due to cytochromeP450 inhibition [116, 143] and increasing the expression of the enzyme beta DNA polymerase needed for the error-free DNA repair [141].

Their possible role in cancer therapy is indicated by studies that revealed their capacity to elicit apoptosis in leukaemia cells [24, 142]. A study of a large number of Chinese medicinal plants with antitumour properties indicated that these herbs are very rich in content and variety of phenolic antioxidant components [23].

2.7.2 Saponins

Saponins are glycosidic compounds present in plants and possess a steroidal or triterpenoid aglycone and one or more carbohydrate moieties in their structure. The structural diversity of this group of compounds is responsible for their diverse properties. Plant saponins are extensively used as surface active and foaming agents in food and industrial purposes. They are known to be the active constituents of many plant based medicines. Legumes such as Soya, Green gram, Red gram, Rajma are rich in dietary saponins. Saponins used in health and industrial applications are obtained from Soap nut tree, Fenugreek, Alfalfa, Horse chestnut, Licorice, and Ginseng [30], [144–146].

2.7.2.1 Structure and physicochemical properties

Saponins are a diverse collection of plant secondary metabolites spread across more than 100 families. They are found in some marine organisms also. They possess a characteristic structure which is responsible for their amphiphilic nature. They are usually composed of two major parts, one of which is a liposoluble aglycone (also called sapogenin) and the other being one or more water soluble sugar moieties. The oligosaccharides are linked to the aglycone by a glycosidic linkage. The aglycones mostly consist of unsaturated C–C bonds. The lyobipolar nature of their constituents explains their ability to interact with cellular membranes and their soap-like behaviour in aqueous solutions, due to which they are used as surface active agents, hair cleansers detergents, wetting agents and emulsifiers. Depending on the type of aglycone skeleton,
saponins are classified into triterpenoid saponins and steroidal saponins. Steroidal saponins are found in monocotyledonous plants and triterpenoid saponins, in dicotyledonous flowering plants. The number of oligosaccharide chains classifies them as mono, di-, or tridesmosidic. The common sugar moieties in saponins are pentoses, hexoses or uronic acids. Saponins, due to their diverse and complex structures, demonstrate a broad spectrum of physico-chemical and biological properties [30, 144].

2.7.2.2 Biological activity of saponins

Saponins display a wide array of activities, including adjuvant, antibacterial [147], antioxidant [148], cytotoxic [149], antiparasitic, chemopreventive, diuretic, antiallergic, antigenotoxic, antihepatotoxic, anti-inflammatory, antimicrobial, antimutagenic and hypocholesterolemic properties [30, 145]. Extracts of plants containing saponins have been shown to be effective in the prevention and treatment of inflammation, infection, alcoholism, pre- and post-menopausal symptoms, cardiovascular [150] and cancer [144, 146, 151]. Sapogenins present in Agave are employed as raw materials in production of steroid drugs. They have immune stimulatory properties and function as adjuvants in vaccine formulations. Some of them isolated from Mexican yam are precursors for progesterone and oral contraceptives [152].

2.8 Literature review on Apocynin and Diosgenin

2.8.1 Apocynin

2.8.1.1 Properties of apocynin

Apocynin was isolated first from the roots of Apocynum cannabinum (Canadian hemp) in 1883 and later in 1971, from Picrorhiza kurroa, a perennial plant growing in the Himalayan mountain ranges in India, Nepal, Tibet, and Pakistan [9]. Extracts from this plant are used widely in the herbal medicines in India, Sri Lanka, Nepal, China and other countries for treating diseases of heart, liver, joints, asthma and other lung problems [153].
Its IUPAC name is 1-(4-hydroxy-3-methoxyphenyl) ethanone and also known by the synonyms 4-hydroxy-3-methoxyacetophenone and acetovanillone (Figure 2.8.1 adapted from Wong et al. [154]). It is a methoxy-substituted catechol with MW 166.17 and melting point 115°C. It has a mild odour similar to vanilla [9]. It is a proven inhibitor of NAD(P)H oxidases and concomitant ROS production in several experimental systems [153, 155].

Apocynin is a prodrug, which was found to undergo dimerization into diapocynin on metabolically reacting with H$_2$O$_2$; myeloperoxidase acts as catalyst in formation of this dimer [156]. Another remarkable feature of this phenolic substance is that it has very low toxicity in experimental animals (LD50 value of 9g/kg) [157].

2.8.1.2 Pharmacological effects of apocynin in various experimental models

$_{P.scrophulariiflora}$ and $P. kurrooa$ are widely used in different ethnic medicinal systems in treatment of immune-related diseases [158]. Apocynin has been considered a miraculous molecule for the multitude of pharmacological effects it exhibits. This compound has shown promising effects in animal models of disease as well as cell culture studies where ROS are involved, including arthritis, septic shock, asthma, hypertension and other cardiovascular diseases, airway inflammation and endotoxin induced lung injury [17,159,160]. The anti-inflammatory properties of apocynin against
immune mediated disorders like rheumatoid arthritis are emphasized by inhibition of oxidative burst in neutrophils in vivo [161] and TNFα production in peripheral blood mononuclear cells in culture [162].

Its protective effects on various liver implications are well recognized, for example, in hepatic ischemia/reperfusion injury, hemorrhagic liver injury and hypercholesterolemia [76]. In certain experimental systems it extended antioxidative protection to the brain cells microglia and has shown promising results in alleviating the symptoms of neurodegenerative disorders, such as Multiple sclerosis, Parkinson’s disease, ALS and Alzheimer's disease [156, 161].

2.8.1.3 Mechanism of action of apocynin

Apocynin is an established inhibitor of Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX). NADPH oxidase is a multimeric enzyme responsible for conversion of oxygen molecules to superoxide anion [O$_2^-$] as well as capable of generation of H$_2$O$_2$. Apocynin blocks the expression of cytosolic components p47phox and gp91phox of this enzyme and also blocks their translocation to the membrane fraction, there by obstructing assembly of this enzyme complex [159].

Some researchers state that apocynin does not always act as NADPH oxidase inhibitor [163] and exhibits other modes of action such as blocking the production of thromboxaneA2 in pulmonary macrophages, inhibition of cytochromeP450 activity, interferes with cytoskeletal arrangement in PMN granulocytes [164], inhibits activation of NF-κB and AP-1[160], which are redox regulating transcription factors and reduces cytokine expression in lymphocytes [165]. It increases expression of antioxidant defence enzymes [76] and acts as a strong scavenger of radical oxygen species and reactive nitrogen species [154] and also non-radical oxidants like H$_2$O$_2$ and HOCl [159].

Pro-oxidant nature of apocynin is suggested by results of some studies where it enhances NO synthesis, perturbing the redox balance [166] and caused manifold increase in oxidation of GSH and coenzyme NADPH [167].
2.8.2 Diosgenin

2.8.2.1 Properties of diosgenin

Diosgenin is a plant secondary metabolite and its chief sources are Fenugreek (Trigonella foenum graecum), Wild yam (Dioscorea villosa), Solanum incaum, and S. xanthocarpum [168]. Diosgenin [(25R)-5- spirosten-3h-ol] or [(25R)-spirost-5-en-3b-ol] (Figure 2.8.2-adapted from Das and Bharali [169]) is a dioscin aglycone. It is a spirostanol or steroidal saponin with a hydrophilic sugar linked to the hydrophobic aglycone. It is structurally analogous to other steroids like cholesterol.

2.8.2.2 Pharmacological effects of diosgenin

Existing records prove that Fenugreek seeds and wild yam tubers are used to treat metabolic diseases like arthritis, cancer, diabetes, gastrointestinal disorders, inflammation and high cholesterol [34]. The tubers of Dioscorea species are well known conventional medicines for diarrhoea, diabetes, skin problems, and rheumatism, in China and Zimbabwe. Records of Aztec and Mayan civilizations mention use of wild yam tubers as relieve pain. These tubers are commercial sources of secondary metabolites used in the pharmaceutical industry and general medicine; the most important ones are steroidal saponins, including diosgenin. Extracts of Dioscorea tubers have shown major biological functions such as antitumour and anticancer properties, antimicrobial activity, hypoglycemia, anti-hypertension, antifatigue, antioxidant, and reno-hepato-protection, according to clinical supplement trials and animal/cell model system tests [170]. Fenugreek seeds were used to induce childbirth and as medicine for gynaecological inflammation. The anti-diabetic and hypocholesterolemic activity of Fenugreek are attributed to diosgenin [34,171].
Diosgenin exhibits anti-invasive and antiproliferative action against cancers of the breast, bone, colorectum, liver and also leukaemia mediated by a range of mechanisms [172]. It is used in oral contraceptive pills due to its oestrogenic effects on mammalian glands. It has been used in skin care formulations to reduce the signs of aging in relation with keratinocyte proliferation and has inhibitory effects against melanogenesis[173]. It is widely in use as precursor for various synthetic steroidal harmones like progesterone, and steroidal anti-inflammatory agents like cortisone [174]. Diosgenin enhanced the apoptotic effects of chemotherapy drugs Paclitaxel and Doxorubicin [172]. It significantly increases the hepatic GST and other detoxifying enzymes [169]. Oral pretreatment with diosgenin reduces the micronucleation, chromosomal aberrations, DNA damage and lipid peroxidation in hamsters treated with 7, 12-DMBA [175]. It was suggested to act as a unique chemotherapeutic modulator in the prevention and treatment of HER-2 overexpressing cancer [176].

2.8.2.3 Mechanism of action of diosgenin

The anti-cancer effects of diosgenin was shown to be due its structure which includes a hetero-sugar and a double bond between the carbon atoms at position 5 and 6 [34]. Diosgenin is said to target multiple stages like proliferation, angiogenesis, metastasis and immunosuppression in cancer cell cultures and in vivo tumour models. Some studies have indicated that it involves activation of p53, modulation of cell proliferation and apoptotic signalling, for example the STAT-3 pathway, and enhancement of certain cell death receptors [168]. It arrested the cell cycle in G2/M phase in HCC, Erythroleukaemia and Leukaemia cells but in Osteosarcoma and
hepatoma cells, arrested the cell cycle in G1 phase [177]. These studies put forward that the mechanism of action of diosgenin may vary with the cell type. In HER-2 over expressing cancer cells diosgenin suppressed the expression of FAS and modulate Akt, mTOR and JNK phosphorylation [176].

2.9 Cell lines – HepG2 and CHO-K1

2.9.1 HepG2 cell line

HepG2 is an immortalised human liver cancer cell line derived from a teenaged Caucasian American male. These cells have a modal chromosome number of 55. They do not grow into tumours in nude mice. They secrete a collection of major plasma proteins, such as albumin, transferrin, fibrinogen and plasminogen. This cancer cell line is acknowledged for their phase I and II biotransformation enzyme activities which regulate the metabolism related to activation and/or detoxification of xenobiotics and carcinogenesis. The cells morphologically are similar to the cells of liver parenchyma from which they originate. Hence the HepG2 cell line is taken to be an efficient model for in vitro investigations of the xenobiotic metabolism and toxicity in the liver, very close to the in vivo situation [178]. HepG2 cells have been extensively used in studying the genotoxic/antigenotoxic and cytotoxic/cytoprotective effects of several natural and synthetic compounds using various assay systems [170,179].

2.9.2 CHO-K1 cell line

CHO (Chinese Hamster Ovary) cells are a cell line derived from cells of the ovaries of Chinese hamsters (Cricetulus griseus). Due to their petite size and low chromosome number (2n=22) for a mammal, Chinese hamsters are popular as a convenient model for tissue culture and cytogenetics. The original CHO cell culture was derived from a Chinese hamster at the Boston Cancer Research Foundation by T. Puck. CHO cells are extensively used as experimental models for mammalian systems in medical and pharmaceutical research. Moreover, they are regularly used for commercial production of therapeutic recombinant proteins. Numerous lines with different characteristics have been developed from the first cell line. A frequently employed
CHO derivative is CHO-K1; these cells have less DNA than the original CHO cells [180] making them more suitable for studies involving gene expression, toxicity assessment, and virology, as well as Prion disease [181,182,183].