

**PROTECTIVE POTENTIAL OF BIOFLAVONOID:
HESPERIDIN AGAINST DIETHYLNITROSAMINE
INDUCED HEPATOCARCINOGENESIS IN RATS**

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*Dedicated to
My Beloved Parents
My Family
and My Friends*

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ABBREVIATIONS

ALP	=	Alkaline Phosphatase.
ALT	=	Alanine Aminotransferase
AST	=	Aspartate Aminotransferase
ATP	=	Adenosine Triphosphate
CAT	=	Catalase
CDK	=	Cyclin-Dependent Kinase
COX	=	Cyclooxygenase
DEN	=	Diethylnitrosamine
DNA	=	Deoxyribonucleic Acid
GI	=	Gastro Intestinal
GSH	=	Reduced Glutathione
GSSG	=	Oxidized Glutathione
GST	=	Glutathione S-Transferases
HBV	=	Hepatitis B Vaccine
HCC	=	Hepatocellular Carcinoma
HCV	=	Hepatitis C Vaccine
HIFU	=	High Intensity Focused Ultrasound
IL	=	Interleukin
LPO	=	Lipid Peroxidation
MDA	=	Malondialdehyde
MDM2	=	Murine Double Minute

NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
O4-Etdt	=	O4- Ethyldeoxythymidine
OS	=	Oxidative Stress
PB	=	Phenobarbital
PEI	=	Percutaneous Ethanol Injection
PUFA	=	Polyunsaturated Fatty acids
RFA	=	Radiofrequency Ablation
RNA	=	Ribonucleic Acid
ROS	=	Reactive Oxygen Species
SOD	=	Superoxide Dismutase
TACE	=	Transcatheter Arterial Chemoembolization
TGF- β	=	Tumor Growth Factor - Beta
TNF- α	=	Tumor Necrosis Factor - Alpha

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1. INTRODUCTION

Cancer or *malignant neoplasm* as it is termed medically is a class of diseases where a group of cells exhibit uncontrolled growth, invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize. The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology. Cancer affects people of all ages, even fetuses, but the risk for most varieties increases with age. Statistical data proves that cancer causes about 13% of all deaths. Cancer affects several organs of the body and cancers are thus named according to the organ it affects (**Figure 1**). The incidence of cancer development differs in males and females, however it has been reported that Males are more prone to cancer as compared to females.

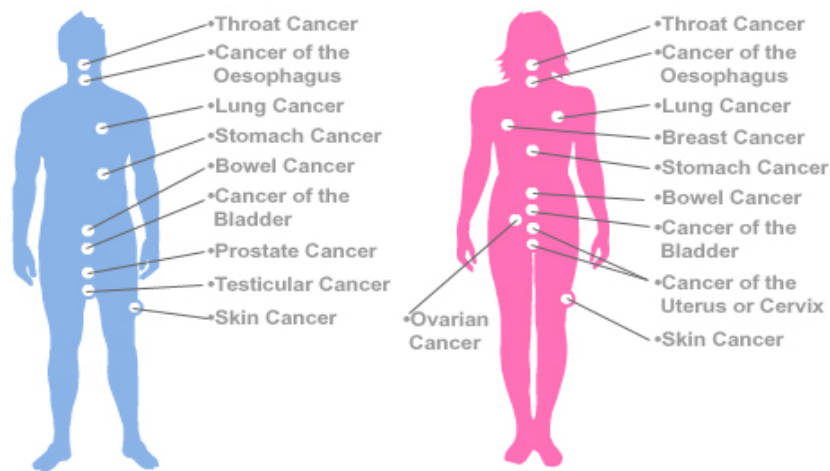


Figure 1: Types of Cancer (Men and Women)

1.1. Causes of cancer

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells, which may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers is usually affected by complex interactions between carcinogens and the host's genome.

New aspects of the genetics of cancer pathogenesis, such as DNA methylation, and micro RNAs are increasingly recognized as important. Research into the pathogenesis of cancer has been divided into three broad areas of focus. The first area of research focuses on the agents and events, which cause or facilitate genetic changes in cells destined to become cancer. Second, is to uncover the precise nature of the genetic damage, and the genes, which are affected by it. The third focus is on the consequences of those genetic changes on the biology of the cell, both in generating the hereditary defining properties of a cancer cell, and in facilitating additional genetic events, leading to further progression of the cancer.

1.2. Symptoms of cancer

Roughly, cancer symptoms can be divided into three groups: -

- ***Local symptoms:*** - unusual lumps or swelling (tumor), hemorrhage (bleeding), pain and/or ulceration. Compression of surrounding tissues may cause symptoms such as jaundice (yellowing the eyes and skin).
- ***Symptoms of metastasis (spreading):*** - Enlarged lymph nodes, cough and hemoptysis, hepatomegaly (enlarged liver), bone pain, fracture of affected bones and neurological symptoms. Although advanced cancer may cause pain, it is often not the first symptom.
- ***Systemic symptoms:*** - weight loss, poor appetite, fatigue and cachexia (wasting), excessive sweating (night sweats), anemia and specific paraneoplastic phenomena, i.e. specific conditions that are due to an active cancer, such as thrombosis or hormonal changes.

1.3. Pathophysiology of cancer

Cancers are caused by a series of mutations, which alters the behavior of the cells. Cancer is fundamentally a disease related to regulation of tissue growth. Transformation of a normal cell into a cancerous cell is due to the alteration of genes that deal with regulation of cell growth and differentiation, (Croce CM, 2008). There are two broad categories of genes, which are affected by the alteration – “Oncogenes” and “tumor suppressor genes”, (Knudson AG, 2001).

1.3.1. Oncogenes:

Oncogenes may be normal genes, which are expressed at inappropriately high levels, or altered genes, which have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Oncogenes primarily promote cell growth by several ways. Most produce hormones ("chemical messengers") between cells, which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissue, or cells. First oncogene that defined in cancer research was the ras oncogene. Mutations in the ras family of proto-oncogenes (comprising H-Ras, N-Ras, and K-Ras) are very common, being found in 20% to 30% of all human tumors, (Bos J, 1989).

Usually, oncogenes are dominant, as they contain gain-of-function mutations, while mutated tumor suppressors are recessive, as they contain loss-of-function mutations. Each cell has two copies of the same gene, one from each parent, and under most cases gain of function mutations in just one copy of a particular proto-oncogene is enough to make that gene a true oncogene. On the other hand, loss of function mutations needs to happen in both copies of a tumor suppressor gene to render that gene completely non-functional. However, cases exist in which one mutated copy of a tumor suppressor gene can render the other, wild-type copy non-functional. This phenomenon is called the dominant negative effect and is observed in many p53 mutations.

1.3.2. Tumor suppressor genes:

Tumor suppressor genes code for anti-proliferation signals and proteins that suppress mitosis and cell growth. These are often disabled by cancer-promoting genetic changes. Tumor suppressors are transcription factors that are activated by cellular stress or DNA damage. The functions of such genes is to arrest the progression of the cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells. The p53 protein, one of the most important studied tumor suppressor genes, is a transcription factor activated by many cellular stressors including hypoxia and ultraviolet radiation damage. p53 clearly has two functions: one a nuclear role as a transcription factor, and the other a cytoplasmic role in regulating the cell cycle, cell division, and apoptosis.

Development of cancer was proposed in 1971 to depend on at least two mutational events. In what became known as the Knudson two-hit hypothesis, an inherited, germ-line mutation in a tumor suppressor gene would only cause cancer if another mutation event occurred later in the organism's life, inactivating the other allele of that tumor suppressor gene, (Knudson A, 1971).

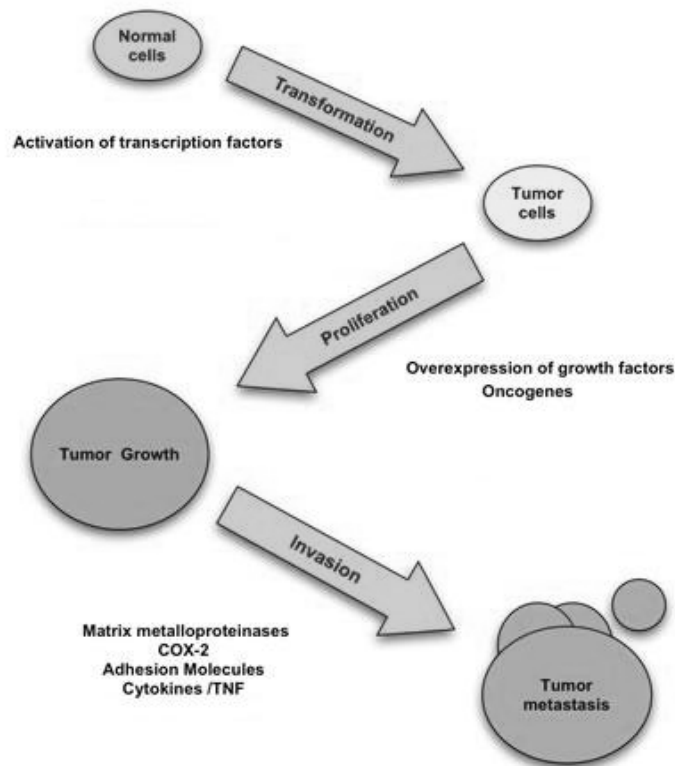


Figure 2: Pathogenesis of Cancer

1.4. Epigenetics of cancer

Epigenetics is the study of the regulation of gene expression through chemical, non-mutational changes in DNA structure. The theory of epigenetics in cancer pathogenesis is that non-mutational changes to DNA that lead to alterations in gene expression. Normally, oncogenes are silent, for example, because of DNA methylation. Loss of that methylation can induce the aberrant expression of oncogenes, leading to cancer pathogenesis. Known mechanisms of epigenetic change include DNA methylation, and methylation or acetylation of histone proteins bound to chromosomal DNA at specific locations.

1.5. Treatment of cancer

Surgery, immunotherapy, chemotherapy, monoclonal antibody therapy, and radiation therapy are methods generally used against various cancers as treatment. Choice however depends on the grade, size, and stage of the disease as well as the general state of the patient (performance status). Because “cancer” is said to be class of diseases, it is unlikely that there will be a single cure for cancer any more than there will be a single treatment for all infectious diseases.

1.6. Liver

Liver is an organ in the upper abdomen that aids in digestion and removes waste products and worn-out cells from the blood. The liver is the largest solid organ in the body. The liver generally weighs about three and a half pounds (1.6 kilograms) in adults. It measures about 8 inches (20 cm) horizontally (across) and 6.5 inches (17 cm) vertically (down) and is 4.5 inches (12 cm) thick. Liver is known to be one of the important organs of the biliary system.

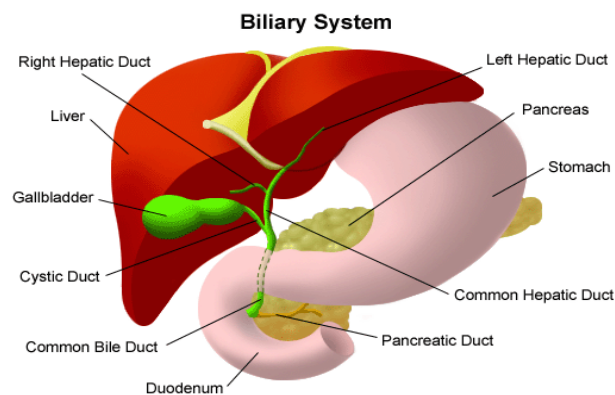


Figure 3: The Biliary System

Functions

- ✓ Manufacture (synthesize) proteins, including albumin (to help maintain the volume of blood) and blood clotting factors
- ✓ The liver stores a multitude of substances, including glucose (as glycogen), vitamin A (1-2 years' supply), vitamin D (1-4 months' supply), vitamin B12, iron, and copper.

- ✓ Synthesize, store, and process fats, including fatty acids and cholesterol.
- ✓ Metabolize and store carbohydrates, which are used as the source for the sugar (glucose) in blood that red blood cells and the brain use.
- ✓ Form and secrete bile that contains bile acids to aid in the intestinal absorption (taking in) of fats and the fat-soluble vitamins A, D, E, and K.
- ✓ Eliminate, by metabolizing and/or secreting, the potentially harmful biochemical products produced by the body, such as bilirubin from the breakdown of old red blood cells and ammonia from the breakdown of proteins.
- ✓ Detoxify, by metabolizing and/or secreting, drugs, alcohol, and environmental toxins.

1.7. Liver cancer

Liver cancer is characterized by the presence of malignant hepatic tumors -- tumors or growths on or in the liver (medical terms pertaining to the liver often start in hepato- or hepatic from the Greek word for liver, hēpar, stem hēpat-). They may be discovered on medical imaging (even for a different reason than the cancer itself), or may be present in patients as an abdominal mass, hepatomegaly, abdominal pain, jaundice, or some other liver dysfunction.

1.7.1. Classification:

Liver cancers can be classified as follows: -

- Most cases are metastases from other tumors, frequently of the GI tract (like colon cancer, carcinoid tumors mainly of the appendix, etc.), but also from breast cancer, ovarian cancer, lung cancer, renal cancer, prostate cancer, etc.
- The most frequent, malignant, primary liver cancer is hepatocellular carcinoma (also named hepatoma, which is a misnomer because adenomas are usually benign).
- More rare primary forms of liver cancer include cholangiocarcinoma, mixed tumors, tumors of mesenchymal tissue, sarcoma, and hepatoblastoma, and a rare malignant tumor in children.

1.7.2. Causes

- The most common cause for liver cancer is its cirrhosis in sixty to eighty percent of patients. Cirrhosis results from scar formation within the liver, most commonly due to chronic alcohol use.
- Chronic infection with hepatitis C virus (HCV) is also a common cause of liver cancer in the United States.
- Presence of the fungus on food in these regions leads to chronic exposure to the toxin, resulting in an increased risk of developing liver cancer.
- Worldwide, other risk factors, such as chronic infection with hepatitis B virus (HBV) and aflatoxin B1 food contamination are more common.
- Tobacco use has also been associated with increased risk; however, it is not as strong of a risk factor as alcohol-induced liver cirrhosis.
- Other environmental factors include exposure to thorium dioxide, vinyl chloride, arsenic exposure, and use of anabolic steroids.
- Several inherited diseases can increase the risk of liver cirrhosis, and therefore increase the risk of developing liver cancer. These diseases include hemochromatosis, Wilson's disease, and alpha-1-antitrypsin deficiency.
- Patients with a family history of liver cancer may also be at increased risk. Finally, males are about twice as likely to develop liver cancer as females.

1.7.3. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is the most common type of primary liver cancer and accounts for 70% of all liver cancers. Most cases of HCC are secondary to either a viral hepatitis infection or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis). Hepatocarcinogenesis is mediated by loss of heterozygosity, somatic mutation, de novo methylation, and/or functional inactivation.

1.7.4. Treatments for Hepatocellular carcinoma

- Liver transplantation
- Percutaneous ethanol injection (PEI)

- Transcatheter arterial chemoembolization (TACE)
- Sealed source radiotherapy
- Radiofrequency ablation (RFA)
- Intra-arterial iodine-131–lipiodol administration
- Combined PEI and TACE can be used for tumors larger than 4 cm in diameter
- High intensity focused ultrasound (HIFU)
- Hormonal therapy and Adjuvant chemotherapy
- Cryosurgery: Cryosurgery is a new technique that can destroy tumors in a variety of sites (brain, breast, kidney, and liver) where the destruction of abnormal tissue using sub-zero temperatures is performed.

1.7.5. Pathology of liver cancer

Macroscopically, liver cancer appears as a nodular or infiltrative tumor. The nodular type may be solitary (large mass) or multiple (when developed as a complication of cirrhosis). Tumor nodules are round to oval, grey or green (if the tumor produces bile), well circumscribed, but not encapsulated. The diffuse type is poorly circumscribed and infiltrates the portal veins, or the hepatic veins (rarely). Microscopically, there are four architectural and cytological types (patterns) of hepatocellular carcinoma: fibrolamellar, pseudoglandular (adenoid), pleomorphic (giant cell) and clear cell. In well-differentiated forms, tumor cells resemble hepatocytes, form trabeculae, cords and nests, and may contain bile pigment in cytoplasm. In poorly differentiated forms, malignant epithelial cells are discohesive, pleomorphic, anaplastic, giant. The tumor has a scant stroma and central necrosis because of the poor vascularization.

1.7.6. Pathogenesis of hepatocellular carcinoma

Hepatocellular carcinoma, like any other cancer, develops, when there is a mutation to the cellular machinery that causes the cell to replicate at a higher rate and/or results in the cell avoiding apoptosis. Accumulation of genetic alterations in

these preneoplastic lesions is believed to lead to the development of HCC. Hepatocarcinogenesis may begin in preneoplastic lesions such as macroregenerative nodules, low-grade and high-grade dysplastic nodules, (Takayama et al., 1990; Orsatti et al., 1993). Genomic alterations occur randomly, and they accumulate in dysplastic hepatocytes and HCC.

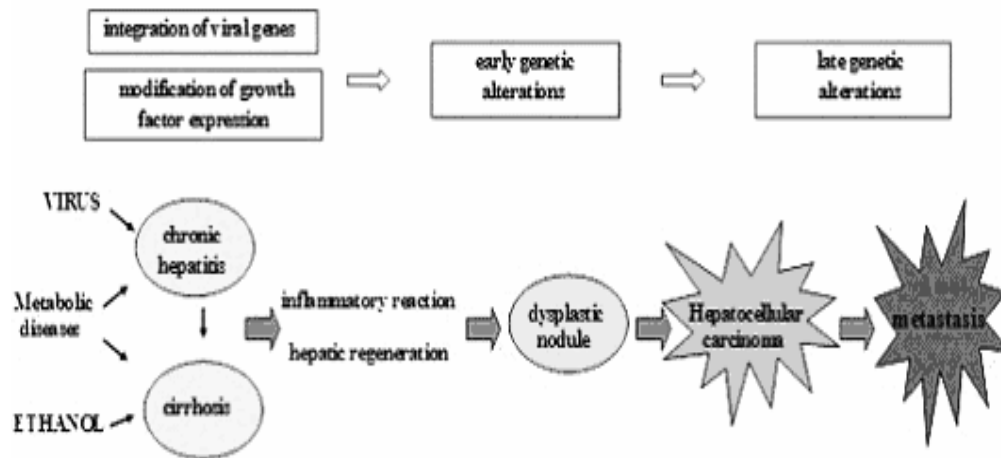


Figure 4: -Sequential changes in liver leading to HCC

HCC are believed to be caused by the accumulation of genetic changes resulting in altered expression of cancer-related genes, such as oncogenes or tumor suppressor genes, as well as genes involved in different regulatory pathways. The genetic changes involved can be divided in at least 4 different pathways, each pathway contributing to a limited number of tumors. These pathways are -

1. The *p53* pathway involved in response to DNA damage,
2. The *retinoblastoma* pathway involved in control of the cell cycle,
3. The *transforming growth factor-beta* (TGF-beta) pathway involved in growth inhibition, and
4. The *Wnt* pathway involved in cell-cell adhesion and signal transduction.

Several different genes have been implicated in the pathogenesis of HCC, and may be divided into four major groups: genes regulating DNA damage response; genes involved in cell cycle control; genes involved in growth inhibition and apoptosis, and genes responsible for cell-cell interaction and signal transduction, (Raphael Saffroy, 2006). Although genetic changes may occur independently of etiologic conditions, some molecular mechanisms have been more frequently related to a specific etiology.

For example, molecular pathways of HBV- and HCV-induced hepatocarcinogenesis involving above-mentioned pathways are different from those associated with alcoholism, (Wang et al., 2002; Suriawinata et al., 2004, Morgan et al., 2004).

p53

p53 has been described as "the guardian of the genome," "the guardian angel gene," and the "master watchman," referring to its role in conserving stability by preventing genome mutation, (Read, A. P, 1999). p53 clearly has two functions: one a nuclear role as a transcription factor, and the other a cytoplasmic role in regulating the cell cycle, cell division, and apoptosis. The Warburg hypothesis is the preferential use of glycolysis for energy to sustain cancer growth. p53 has been shown to regulate the shift from the respiratory to the glycolytic pathway. However, a mutation can damage the tumor suppressor gene itself, or the signal pathway, which activates it, "switching it off". The invariable consequence of this is that DNA repair is hindered or inhibited: DNA damage accumulates without repair, inevitably leading to cancer.

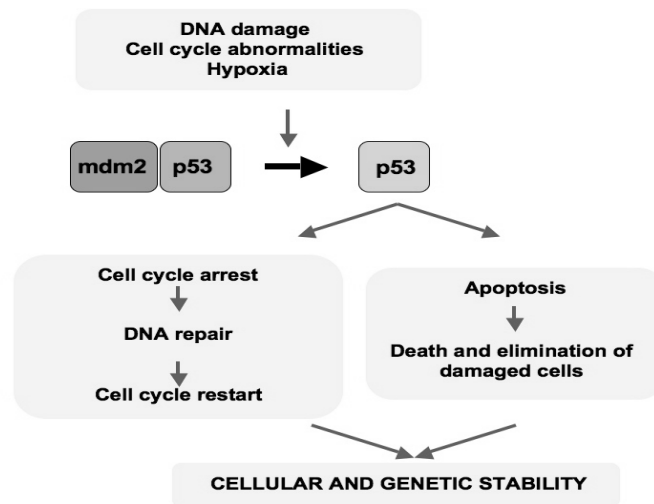


Figure 5 Action of mdm2 on p53

p53 is central to many of the cell's anti-cancer mechanisms. In normal cells, p53 is usually inactive, bound to the protein MDM2 (also called HDM2 in humans), which prevents its action and promotes its degradation by acting as ubiquitin ligase. Active p53 is induced after the effects of various cancer-causing agents (UV radiation, oncogenes, and some DNA-damaging drugs). This damage is sensed by 'checkpoints' in a cell's cycle, causing proteins such as ATM, CHK1, and CHK2 to phosphorylate p53

at sites that are close to or within the MDM2-binding region and p300-binding region of the protein. Oncogenes also stimulate p53 activation, mediated by the protein p14ARF. Some oncogenes can also stimulate the transcription of proteins, which bind to MDM2 and inhibit its activity. Once activated p53 activates expression of several genes including one encoding for p21. p21 binds to the G1-S/CDK and S/CDK complexes (molecules important for the G1/S transition in the cell cycle) inhibiting their activity.

p53 has been proved to play important role in apoptosis, genetic stability, and inhibition of angiogenesis. In humans, p53 is encoded by the *TP53* gene located on the short arm of chromosome 17. The p53 gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and therefore the p21 protein is not made available to act as the 'stop signal' for cell division. Thus, cells divide uncontrollably, and form tumors.

Functions

p53 has many anti-cancer mechanisms:

- ✓ It can activate DNA repair proteins when DNA has sustained damage.
- ✓ It can also hold the cell cycle at the G₁/S regulation point on DNA damage recognition.
- ✓ It can initiate *apoptosis*, the programmed cell death, if the DNA damage proves to be irreparable.

1.7.7. Apoptosis

Apoptosis is a form of programmed cell death in multi cellular organisms and involves a series of biochemical events leading to characteristic cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane. Loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation are the changes that occur to the

cell membrane. Research on apoptosis has increased substantially since the early 1990s. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer.

Functions

- ✓ **Cell termination:** Apoptosis occurs when a cell is damaged beyond repair, infected with a virus, or undergoing stressful conditions such as starvation. Damage to DNA induces apoptosis via the actions of the tumour-suppressing gene p53. Apoptosis also plays a role in preventing cancer. If a cell is unable to undergo apoptosis because of mutation or biochemical inhibition, it continues to divide and develop into a tumour.
- ✓ **Homeostasis:** In the adult organism, the number of cells is kept relatively constant through cell death and division. Cells must be replaced when they malfunction or become diseased, but proliferation must be offset by cell death, (Thompson, CB. 1995). Homeostasis is achieved when the rate of mitosis (cell division) in the tissue is balanced by cell death.
- ✓ **Development:** Development of an organ or tissue is often preceded by the extensive division and differentiation of a particular cell, the resultant mass is then "pruned" into the correct form by apoptosis.
- ✓ **Lymphocyte interactions:** The development of B-lymphocytes and T lymphocytes in a human body is a complex process that creates a large pool of diverse cells and subsequently eliminates those potentially damaging to the body. In T cells, apoptosis is initiated by the withdrawal of survival signals, (Werlin G et al, 2003). Cytotoxic T cells directly induce apoptosis by opening up pores in the target's membrane and releasing chemicals.

1.8. Apoptotic process

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic

inducers). Extra cellular signals may include toxins, hormones, growth factors, nitric oxide, or cytokines, and therefore must either cross the plasma membrane or transduce to affect a response (Popov SG et al, 2002; Brüne B, 2003). A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia, and increased intracellular calcium concentration, for example, by damage to the membrane can all trigger the release of intracellular apoptotic signals by a damaged cell. (Cotran et al; Mark P et al, 2003).

1.9. Oxidative Stress

Oxidative Stress (OS) is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species (ROS) (Davies, 2000). This damage affects a specific molecule or the entire organism. ROS, such as free radicals and peroxides, represent a class of molecules derived from the metabolism of oxygen and exist inherently in all aerobic organisms. There are many different sources by which the ROS are generated. Most come from the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria (Shouval & Elazar, 2007) or the detoxification reactions involving the liver cytochrome P-450 enzyme system.

Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacterial, fungal, or viral infections. The rate at which damage is caused is determined by how fast the reactive oxygen species are generated and then inactivated by endogenous defense agents called antioxidants. The rate at which damage is removed is dependent on the level of repair enzymes.

ROS are the root cause of oxidative stress where ROS are generated from normal metabolism, cytochrome P450, immunopotentiality, or reperfusion injury. These ROS are effectively scavenged by cellular antioxidant systems otherwise, their deleterious effect may lead to damage the lipids, proteins, and DNA, change in cellular functions or signal transductions and finally cell death or apoptosis (Shackelford et al., 2000).

However, severe oxidative stress may cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis (Lennon SV, 1991). Oxygen-derived species, including free radicals causes DNA damage in frequent type of aerobic cells. When this type of damage occurs to DNA, it is called oxidative DNA damage and it can produce a multiplicity of modifications in DNA including base and sugar lesions, strand breaks, DNA-protein cross-links, and base-free sites.

1.9.1. Free Radicals and Reactive Oxygen Species (Scandalios, 2002)

Free radicals, i.e. atoms, or molecules with lone-paired electron are generally reactive substances and have short life span except for few that are stable for long time. Examples of reactive free radicals are super oxide, hydroxyl radical, protein radical, nitric oxide, and peroxinitrite radical etc. ROS that are not free radicals (without lone-paired electrons) include hydrogen peroxide, singlet oxygen, and lipid hydroperoxide.

They play important roles in signal transduction (including gene expression, protein synthesis), sensing of oxygen tension and regulation of functions controlled by oxygen concentration and are essential in synthesis of energy (mitochondrial electron transport chain) and essential molecules. They also act as immune boosters. ROS reacts quickly with other compounds in order to capture the needed electrons to gain stability. Generally, free radicals attack the nearest stable molecule, which loses its electron; it becomes a free radical itself, beginning a chain reaction cascade resulting in disruption of a living cells.

Oxygen free radicals (Figure-5) are generated within the mitochondrial membrane where cytochrome oxidase catalyses the four electron reduction of oxygen (O_2) to water (H_2O). Intermediate compounds between reactions of O_2 to H_2O leads to three partially reduced species of oxygen, which are generated depending upon the number of electrons, transferred. These are:

- 1) Superoxide oxygen ($O_2^{\cdot-}$): one electron.
- 2) Hydrogen peroxide (H_2O_2): two electrons.
- 3) Hydroxyl radical (OH^{\cdot}): three electrons.

A few other oxygen radicals which may be generated in reactions other than those during O_2 to H_2O are hypochlorous acid (HOCl), peroxynitrate ion (ONOO⁻), nitric oxide (NO) generated by various body cells (endothelial cells, neurons, macrophages etc), and release of superoxide free radical in Fenton reaction.

Most common radical derivatives of oxygen like superoxide free radical anion ($O_2^{\cdot-}$), hydroxyl free radical (OH[•]), lipid peroxy (LO), lipid alkoxy (LOO) and lipid peroxide (LOOH) as well as non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) are collectively known as reactive oxygen species (ROS). These free radicals or reactive oxygen species are produced mainly from two important sources in the biological system. First source is from cellular metabolism like mitochondrial electron transport chain, endoplasmic reticulum oxidation, NADPH oxidase, xanthine oxidase, prostaglandin synthesis, reduced riboflavin, nitric oxide synthetase, reperfusion injury, cytochrome P_{450} , activated neutrophils, and phagocytic cells and secondly from environmental sources like drugs, pesticides, transition metals, tobacco smoke, alcohol, radiations and high temperature.

Superoxide anions ($O_2^{\cdot-}$): Superoxide anion is the first reduction product of O_2 . It is a base with the equilibrium with its conjugate acid, the hydroperoxy radical HO_2^{\cdot} , whose pKa is 4.8. In aqueous solution, at neutral or slightly acid pH, $O_2^{\cdot-}$ is a relatively non-reactive species and dismutates to H_2O_2 . This reaction may occur spontaneously or be catalyzed by intracellular enzyme, SOD.

Hydrogen peroxide (H_2O_2): Hydrogen peroxide is the most stable ROS. This is to say that it is the least reactive and the most readily detected. H_2O_2 may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of $O_2^{\cdot-}$. H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases, such as XO, uricase, D-amino acid oxidase and α -hydroxy acid oxidase localized in peroxisome.

Hydroxyl radical (OH[•]): This is a highly reactive that reacts with practically any molecule present in cells thus making it a short-lived radical. This insufficient stability does not allow it to diffuse through the cells. Therefore, it reacts with an organic substrate at the sites or near the sites of its formation. The life span of OH[•] at 37°C is 10^{-9} s. It does not survive for more than a few collisions after its formation. Due to such short lifetime, it is very difficult to investigate the OH[•] by conventional

methods. This OH[•] is produced following the reaction of O₂^{•-} and H₂O₂ in presence of metallic ions such as Fe²⁺/Cu⁺. Lipid is very susceptible to OH[•] attack and initiate LPO. Because of interaction of OH[•] with DNA, formation of many types of oxidized nucleoside has been reported.

1.9.2. Results of Oxidative Stress

Lipid peroxidation: Polyunsaturated fatty acids (PUFA) of membrane are attacked repeatedly and severely by oxygen-derived free radicals to yield highly destructive PUFA radicals-lipid hydroperoxy radicals and lipid hypoperoxides. The lipid peroxidase is decomposed by transition metals such as iron causes lipid peroxidation is propagated the widespread membrane damaged and destruction of organelles. Malondialdehyde is the major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acid (PUFA) present in the biological membrane. MDA, a secondary product of LPO, is used as an indicator of tissue damage by a series of chain reactions. MDA is also a by-product of prostaglandin biosynthesis. It reacts with thiobarbituric acid and produce red-colored products (Levine, 2002).

Oxidation of protein: Oxygen-derived free radicals cause cell injury by oxidation of protein macromolecules of the cells, cross-linking of labile amino acids as well as by fragmentation of polypeptides directly. At the end, the result is degradation of cytosolic neutral proteases and cell destruction (Poljak et al., 2003).

DNA damage: Free radicals cause breaks in the single strands of the nuclear and mitochondrial DNA. This result in cell injury; it may also cause malignant transformation of cells (Brennan et al., 1994).

Cytoskeletal damage: Reactive oxygen species are also known to interact with cytoskeletal elements and interfere in mitochondrial aerobic phosphorylation and thus cause ATP depletion (Buettner & Schafer, 2000).

1.9.3. Major Types of ROS Scavengers (Davies, 2000)

Superoxide dismutase: SODs belong to family of metalloenzymes. SOD is the most important enzyme found virtually in all aerobic organisms. There are found families of SOD: Cu-SOD, Cu-Zn-SOD (cytosolic), Mn-SOD (mitochondrial) and Fe-SOD. The transition metal of the enzyme reacts with O₂^{•-} taking its electron. O₂^{•-} is the

only known substrate for SOD. SOD is considered a stress protein, synthesized in response to oxidative stress. SOD has been detected in a large number of tissues and organisms, and is thought that it is present to protect the cell from damage caused by $O_2^{\cdot-}$.

Catalase: CAT is an enzyme, which is present in most cells and catalyses the decomposition of hydrogen peroxide to water and oxygen. CAT is a heme containing protein. CAT is found to act 10^4 times faster than peroxidase. It is localized mainly in mitochondria and in sub cellular respiratory organelles. CAT is present in peroxisome (80%) and cytosol (20%). The decreased CAT activity may be because of higher ROMs production, especially $O_2^{\cdot-}$ itself affected directly the CAT activity.

Glutathione: Glutathione plays a key role in the liver in detoxification reactions and in regulating the thiol/disulphide status of the cell. Oxidized glutathione (GSSG) can be enzymatically reduced by glutathione reductase and NADPH and simultaneously reduced glutathione (GSH) is oxidized to GSSG by glutathione peroxidase in the presence of H_2O_2 . Oxidized glutathione, usually exists in very small concentrations in the liver cells (5% of total glutathione equivalents in liver). The maintenance of low GSSG concentration is accounted for its rapid reactions with protein thiols, its reduction by NADPH, glutathione reductase and its active transport out of the cells. Under conditions of oxidative stress associated with increased formation of GSSG, the concentration of GSSG does not increase significantly in liver. Thus, the mechanisms handling GSSG ensure that it does not accumulate.

Glutathione S-transferases: GST's are a group of detoxifying enzymes that catalyze the conjugation of reduced glutathione with a variety of compounds bearing suitable electrophilic centers in them. These enzymes are widely distributed in higher animals and are known to perform several detoxification functions. This group of enzymes is analogous to serum albumin in the broad spectrum of compounds that serve as ligands, but differs significantly in that the transferases have an additional and specific site for GSH. Examples of the reactions catalyzed include conjugation to the compounds with a reactive electrophilic carbon to form thioethers, reactions with other electrophilic atoms including the nitrogen of organic nitrates and the sulfur of organic thiocyanates; isomerisation; disulfide interchange; glutathione peroxidase activity with organic peroxides and formation of thiol-esters.

1.10. N- Nitroso compounds: -

N-nitroso compounds result from reactions involving chemicals called alkyl amines and these are formed by side-reactions when other products are made. EPA however has studied nitroso compounds and classified it as probable carcinogens. N-nitroso compounds are highly toxic and carcinogenic. The characteristic toxic lesion produced by the nitrosamines is centrilobular necrosis of the liver, (Magee & Barnes, 1967). The N-nitroso compounds include some of the most powerful known carcinogens, several being capable of producing tumors when given as a single dose in spite of their rapid elimination from the body. They are thus valuable agents for the study of mechanisms of the induction of cancer. Industry makes small amounts of N-nitroso compounds for research purposes.

1.10.1. Exposure to humans:

Several ways of humans being exposed to nitroso compounds are listed below –

- **Eating:** - foods containing these compounds include fish, cheeses, bacon, and other cured meats.
- **Breathing:** - air containing these compounds near plants that use them, near a waste site containing N-nitroso compounds, or by breathing tobacco smoke or the air inside cars.
- **Drinking:** - some alcoholic beverages; including beer and other malt beverages. Infants drinking from a rubber bottle nipple could possibly be exposed.
- **Touching:** - these compounds if you work where they are used. Also by touching some cosmetics and toiletries, contain N-nitroso compounds.

<i>S. No</i>	<i>Nitroso compound</i>	<i>Abbreviation</i>	<i>Chemical formula</i>
1	<i>N-nitroso-diethylamine</i>	<i>NDEA</i>	$C_4H_{10}N_2O$
2	<i>N-nitroso-dimethylamine</i>	<i>NDMA</i>	$C_8H_{10}N_2O$
3	<i>N-nitroso-di-n-butylamine</i>	<i>NDBA</i>	$C_8H_{18}N_2O$
4	<i>N-nitroso-di-n-propylamine</i>	<i>NDPA</i>	$C_6H_{14}N_2O$
5	<i>N-nitroso-methylethylamine</i>	<i>NMEA</i>	$C_2H_6N_2O$
6	<i>N-nitroso-pyrrolidine</i>	<i>NPYR</i>	$C_4H_8N_2O$

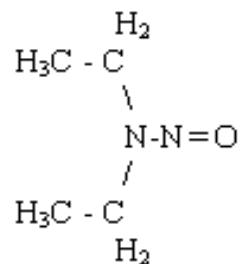
Figure 6:- Few examples of N Nitroso compounds.

1.10.2. N-nitroso-diethylamine

Synonyms: -

Diethylnitrosamine; diethylamine, N-nitroso; DEN; DENA; DANA.

Structure:



Physical properties: -

Appearance	:	a yellow, volatile liquid
Mol weight	:	102.1
Solubility	:	10% in water; soluble in organic solvents and in lipids
Boiling point	:	177°C (760 mm Hg) 64-65°C (17 mm Hg)
Density	:	0.9422 (20/4°C)

DEN has been shown to act as a carcinogenic to the mouse, the rat, the hamster, the guinea pig, the rabbit, the dog, the pig, the monkey, and to aquarium fish. Administration of DEN to animals produces cancer in liver and to other organs at a low incidence. The agent induces tumors primarily in the nasal cavity, trachea, esophagus, and liver. It causes cancer after different modes of exposure, which include ingestion, inhalation, and skin painting. It is carcinogenic in single doses and following prenatal exposure.

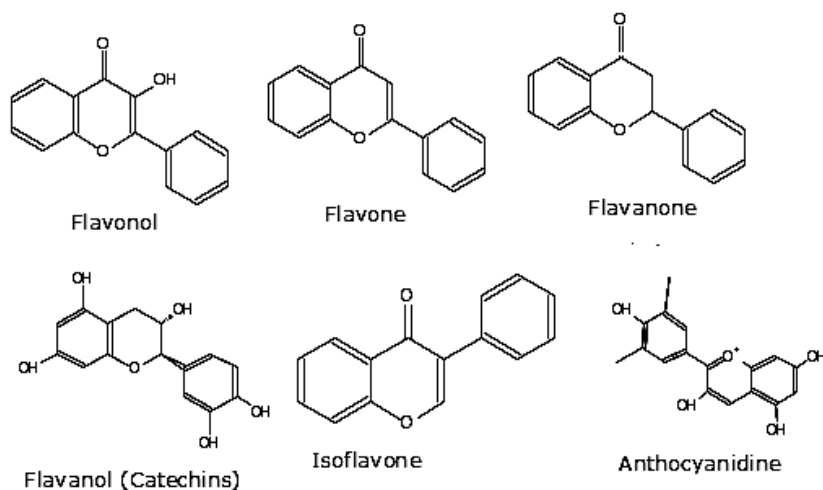
DEN is metabolized to its active ethyl radical metabolite, and this reactive product interacts with DNA causing mutation, which leads to carcinogenesis, (Anis et al., 2001; Chakraborty et al., 2007). Hence, O4- ethyldeoxythymidine adduct (O4-Etdt) accumulates in hepatocyte DNA following DEN administration which is thought to be important in tumour initiation, (Dyloff MC, 1986). The involvement of free radicals in DEN-induced liver cancer has been extensively studied.

Experimental, clinical, and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer. Diethylnitrosamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals, (Ramakrishnan et al., 2006; Valko et al., 2006). This was confirmed by over expression of 8-hydroxyguanine in DEN administered rat liver, (Nakae D, 1997). When the excess amount of ROS accumulates, numerous pathological effects may manifest in the cells including carcinogenesis (Dean RT, 1997).

Continuous interaction of the animal with these free radicals causes damage of proteins, lipid, DNA, carbohydrates and membrane, resulting in oxidative stress. It is widely accepted metabolic activation of nitrosamines by cytochrome P450 enzymes to reactive electrophiles is required for their cytotoxic, mutagenic, and carcinogenic activity. Because of its relatively simple metabolic pathway and potent carcinogenicity, DEN has been used as an effective experimental model. The relationship between the involvement of free radicals and DEN activation was also demonstrated by induction of lipid peroxidation, which may be responsible for tumor formation (Bartsch H, 1989).

1.11. Natural treatment against cancer: -

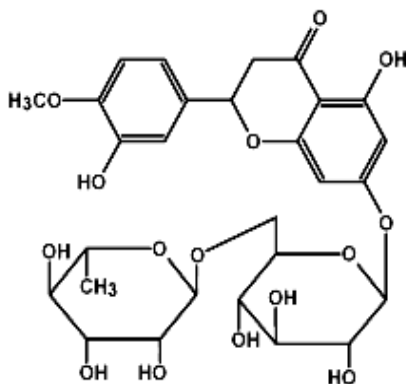
A large number of agents including natural and synthetic compounds have been reported which have some chemo preventive value. Among these compounds, most are present in plants. Few phytochemicals that play an imperative role against class of diseases include the flavonoids (Hesperidin, Rutin, Quercetin, Nobeletin); phytosterols (Beta-sitosterol); phenolic acids (Capsaicin, Ellagic acid, Gallic acid); triterpenoids (Ursolic acid) etc. Flavonoids have found to have a chemo preventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibit various activities of tumor promoters, which are involved in the process of carcinogenesis. Chemically, Flavonoids are polyphenolic compounds that are ubiquitous in nature and are grouped according to their chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities.



They are present in fruits, vegetables, and beverages (tea, coffee, beer, wine, and fruit drinks). They possess strong antioxidant and radical scavenging activities. This makes flavonoids to act as antiviral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor agents. Few examples of well-known flavonols include quercetin, rutin, and hesperidin, while well-known flavones include apigenin and luteolin. Flavonoids in some cases may also be named directly after the unique plant that contains them. Ginkgetin is a flavonoid from the ginkgo tree, and tangeretin is a flavonoid from the tangerine.

1.12. Hesperidin

Structure: -



Synonyms: - Hesperetin 7-rhamnoglucoside, hesperetin-7-rutinoside, Hesperidoside;

Molecular formula: - C₂₈H₃₄O₁₅

Molecular weight: - 610.57

Hesperidin is one among over 4000 flavonoids found in plants and is the abundant and inexpensive by-product that comes from citrus fruits thus making it a naturally occurring citrus bioflavonoid also known as a phytochemical. Hesperidin was first isolated in 1828 from the albedo (the spongy inner portion of the peel) of oranges, and has since been found in lemons and other citrus fruits. It is found to be present mainly in the peel of the ordinary orange *Citrus aurantium* L., (Kanes et al., 1993; Emim et al., 1994), *C. sinensis*, (Horowitz and Gentili, 1963), *C. unshiu*, (Kawaguchi et al., 1997) and other species of the genus Citrus (Family: Rutaceae).

1.13.1. Properties: -

Physical state: - White to yellow powder

Melting point: - 257 – 262°C

Solubility in water: - Soluble

pH: - 6.0 ~ 7.5

Stability: - Stable under ordinary conditions, hygroscopic, light sensitive.

1.13.2. Pharmacological Actions of hesperidin: -

Both hesperidin and its aglycone hesperetin have been reported to possess a wide range of pharmacological properties. In a combination with others or alone, hesperidin is most often used for vascular conditions such as hemorrhoids and varicose veins. Hesperidin seems to work by improving venous tone, reducing stasis, restoring normal capillary permeability, and improving lymphatic drainage.

Hesperidin has been reported to possess anti hypercholesterolemic activity (Son et al., 1999). Other than this it also possesses anti-inflammation, antioxidant, antifungal, anticarcinogenic, and analgesic effects, (Galati et al., 1994; Krolicki and Lamer-Zarawaska, 1984; Yang et al., 1997). The sugar causes hesperidin to be more soluble than hesperetin. The possible anti-cancer activity of hesperidin could be explained by the inhibition of polyamine synthesis.

1.13.3. Pharmacokinetics of Hesperidin: -

There is not much known about the pharmacokinetics of hesperidin in humans. It is unclear if hesperidin itself is absorbed from the intestine intact as a glycoside. The aglycone hesperetin is detected in the serum following ingestion and may be formed prior to or following absorption. Hesperetin may undergo glucuronidation in the wall of the intestine, as well as in the liver. Hesperetin is detected in the urine within three hours after ingestion of hesperidin. Urinary excretion appears to be the major route of excretion of the aglycone. Not much more is known about the metabolism of hesperidin.

2. LITERATURE REVIEW

Annida Balakrishnan *et al.*, (2007). Hesperidin is a polyphenol, has been observed to possess antioxidant activity and also quenches free radicals. We thought that this compound may prevent the toxic effects of nicotine, which manifest its action through the generation of free radicals.

Giuseppe Gattuso *et al.*, (2007). Flavonoids are aromatic secondary plant metabolites, which have been recognized as important due to their physiological and pharmacological role and their health benefits. The amount of interest they have attracted is demonstrated by more than 13,000 articles and books dealing with various aspects of flavonoids chemistry, biochemistry, pharmaceutical and nutritional role which have appeared since 1990. Flavonoids show a strong antioxidant and radical scavenging activity and appear to be associated with reduced risk for certain chronic diseases, the prevention of some cardiovascular disorders, and certain kinds of cancerous processes. Flavonoids exhibit also antiviral, antimicrobial, and anti-inflammatory activities, beneficial effects on capillary fragility, and an ability to inhibit human platelet aggregation, antiulcer and anti-allergenic properties.

Inge Lise F *et al.*, (2006). Bioavailability of hesperidin was modulated by enzymatic conversion to hesperetin-7-glucoside, thus changing the absorption site from the colon to the small intestine. This may affect future interventions concerning the health benefits of citrus flavonoids.

Balasubramanian Raj Kapoor *et al.*, (2005). *N*-Nitrosodiethylamine (DEN), one of the most important environmental carcinogens of this class, primarily induces liver tumor. It is widely accepted that metabolic activation of nitrosamines by cytochrome P450 enzymes to reactive electrophiles is required for their cytotoxic, mutagenic and carcinogenic activity. Because of its relatively simple metabolic pathway and potent carcinogenicity, DEN has been used as an effective experimental model in the field of carcinogenesis and chemoprevention.

Lin *et al.*, (2002). Nitrate and nitrite are added to meat and fish for the purpose of preservation, as colour fixatives and as flavouring. Ingestion of nitrite and nitrate can result in the endogenous formation of nitroso compounds, particularly in the presence

of nitrosatable precursors, such as primary amines, in the acidic condition of the stomach.

Thirunavukkarasu C, et al., (2001). *N*-Nitrosodiethylamine (DEN) is a representative chemical of a family of carcinogenic *N*-nitroso compounds. Administration of DEN to animals has been shown to cause cancer in liver and at low incidence in other organs also. The involvement of free radicals in DEN-induced liver cancer has been extensively studied.

Pattanayak D et al., (1999). Continuous interaction of the animal with these free radicals causes damage of proteins, lipid, DNA, carbohydrates and membrane, resulting in oxidative stress. In order to maintain cellular health, it is essential to have a specific and effective chemical scavenger to target multiple types of radicals. Most of the commercially based antioxidant supplements are single oxidant.

Hecht SS., (1997). In addition, exposure of man to preformed nitrosamines occurs due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals.

Cho KJ et al., (1993). The chronic CCl₄ administration produced complete or incomplete liver cirrhosis and exerted a strong promoting effect on the development of neoplastic nodules. Ethanol alone revealed no cirrhogenous or tumor-promoting effect, but enhanced both actions of CCl₄. Acetaldehyde increased only the cirrhogenous effect of CCl₄.

Beckman JS et al., (1990). Generally, oxygen free radicals are natural physiological products, but also extremely reactive oxygen species (ROS). They have been proved to cause numerous cellular anomalies, including but not limited to protein damage, deactivation of enzymatic activity, alteration of DNA and lipid peroxidation of membranes.

Dyroff MC et al., (1986). Initiation during or after DEN exposure is thought to be a rapid metabolism of DEN to reactive metabolites that interact with DNA, forming various DNA adducts that can lead to mutations. Hence, O⁴-ethyldeoxythymidine adduct (O⁴-Etdt) accumulates in hepatocytes DNA following DEN administration which is thought to be important in tumour initiation.

3. AIM AND SCOPE OF STUDY

Cancer or malignant neoplasm as it is termed is a class of diseases where a group of cells displays uncontrolled growth, invasion, and sometimes metastasis. Cancer is found to be responsible for partial or complete breakdown of the control mechanisms of cellular proliferation, including cell cycle regulatory proteins. Causing about 13% of all deaths worldwide it has gained itself massive importance thus making it a challenging area of research. In our study, liver was mainly concentrated being one of the deadly types of cancers. It has been reported that hepatocellular carcinoma alone causes 653,000 deaths worldwide per year, about half of them in China. Hepatocarcinogenesis is a multistage process, normally resulting in a highly malignant tumour; however, progressive growth of hepatocytes from their normal state to malignancy is not clear yet.

Several carcinogens can produce liver toxicity by initiating an abnormal process, which is followed by regeneration, growth, and clonal proliferation, eventually leading to cancer. Until today, several studies have been performed on cancer, its prevention; or its treatment; but the cause of many cancers are still unknown. Though numerous carcinogens are present, administration of nitrosamines to rats has proved to cause high incidence cancer in the liver and at low incidence in the other organs.

Nitrosamines are widely recognized as carcinogenic compounds but it requires microsomal activation before it can exert their cytotoxic and carcinogenic activity. DEN has been proved to be one of the effective agents to cause oxidative stress and cellular injury which is due to the enhanced formation of free radicals. It has been reported that DEN is present in tobacco and various processed foods, foodstuffs, such as milk and meat products, salted fish and alcoholic beverages and is formed in vivo in physiological conditions primarily inducing tumors of liver.

DEN being an oxidant can utilize several different avenues to alter cellular function including signal transduction pathways and expression of genes relevant to generation of mitogenic signals to the oxidant resistant carcinogen-initiated cells causing tumor promotion, apart from initiating a toxicity signal to normal cells. Reports suggest that the mechanism by which DEN produces its activity is by the oxidation of

the α -carbon by the cytochrome P450 monooxidase system to form unstable, intermediate, DEN radical and reactive oxygen species, which readily reacts with the DNA to form alkyl-DNA adduct or so called tumor promoters. This tumor promoter appears to induce a super oxide anion, which leads to the formation and thus the accumulation of super oxide and hydrogen peroxide in the cell. This exposure of DNA to super oxide-generating systems causes formation of reactive hydroxyl radical thereby causing extensive strand breakage and degradation of deoxyribose. This results in increased LPO, thus increasing membrane damage the extent of which is measured by the increased levels of MDA. Reports also proved that DEN induction led to increase in the liver markers (AST, ALP, and ALT) as well as the antioxidant enzymes (SOD, CAT, GPx, GR and GSH) which are helpful in knowing the extent of carcinoma.

Hesperidin (HDN) is a flavanone glycoside abundantly found in sweet orange and lemon and is an inexpensive by-product of citrus cultivation. Several studies prove that abnormal capillary leakiness as well as pain in the extremities causing aches, weakness, and night leg cramps. It has also been reported to possess significant anti-inflammatory, analgesic, antifungal, and antiviral activities. A number of studies have examined the antioxidant, anticancer activity, and free radical scavenging properties of hesperidin. Since Hesperidin has been proved to have antioxidant effect, an attempt to check the extent of free radicals scavenging activity over DEN induced conditions was made in our studies. To check this, significant changes that occurred in liver marker enzymes (AST, ALP, and ALT) along with serum bilirubin and antioxidant enzymes (SOD, CAT, GPx, GR and GSH) were studied under DEN induced and Hesperidin treated conditions. Several assay studies have been developed to examine the antioxidant activity and free radical scavenging activity of Hesperidin.

4. PLAN OF WORK

4.1. Estimation of liver marker enzymes

- Assay of Aspartate transaminase, (AST)
- Assay of Alanine transaminase, (ALT)
- Assay of Alkaline phosphatase, (ALP)

4.2. Determination of Lipid peroxides in Liver

4.3. Estimation of antioxidant status

- Assay of Superoxide dismutase (SOD)
- Assay of Catalase (CAT)
- Assay of Glutathione peroxidase (GPx)
- Assay of Glutathione reductase (GR)
- Assay of Glutathione (GSH)

4.4. Assay of Tumor Necrosis Factor - alpha (TNF- α)

4.5. Histopathological studies

4.6. Statistical Analysis

5. MATERIALS AND METHODS

5.1. ANIMAL MODEL

The study was conducted using Male albino Wistar rats (150 ± 10 gms). Animals were obtained from the animal house, Vel's College of Pharmacy; The Tamilnadu Dr MGR Medical University, Chennai, India. The animals were fed with commercially available standard rat pellet feed from M/s Hindustan Lever Ltd; Bangalore, India. The feed and water were provided *ad libitum*. The animals were deprived of food for 24 hr before experimentation but allowed free access to tap water through out. The rats were housed under conditions of controlled temperature ($25 \pm 2^{\circ}\text{C}$) and were acclimatized to 12-h light: and 12-h dark cycles. Experimental animals were used after obtaining prior permission and handled according to the Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment; Government of India.

5.2. EXPERIMENTAL PROTOCOL: -

Group I: Normal control rats were supplied with sufficient amount of food and water.

Group II: Rats were given hesperidin orally (100mg/kg/day) for 14 weeks.

Group III: Rats were given a single intraperitoneal injection of DEN (200mg/kg). After 1 week, the rats were given PB (0.05% in drinking water) for 13 weeks.

Group IV: Rats were given hesperidin one week prior to the injection of DEN. The oral administration was then continued throughout the experimental period (14 weeks) along with the administration of PB.

After the end of experimental period, all the animals were anesthetized and decapitated. Liver tissues were immediately excised and rinsed in ice-cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected and serum was separated for analysis of biochemical parameters.

5.3. BIOCHEMICAL ESTIMATIONS: -

5.3.1. Estimation of Liver Marker Enzymes

5.3.1.1. Assay of Aspartate transaminase, (AST)

The method of Reitman and Frankel (1957) was adopted for the assay of aspartate transaminase.

Reagents:

Phosphate buffer : 0.1 M, pH 7.4

Sodium hydroxide : 0.4 N solution

Substrate: 2.66 g of DL-aspartate and 38 mg of α -oxaloglutarate were dissolved in 20.5 ml of 1 N NaOH with gentle heating. This was made up to 100 ml with buffer.

DNPH : 1 mM 2, 4-dinitrophenyl hydrazine in 2 N HCl.

Standard pyruvate : 10 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer 0.1 M, pH 7.4.

Procedure

In different tubes, 1.0 ml of buffered substrate was added to 0.1 ml of serum or 0.1 ml of enzyme source and incubated at 37°C for 1 hour. Then 1.0 ml of DNPH reagent was added to arrest the reaction. To the blank tubes, 0.1 ml of distilled water was added instead of serum. The tubes were kept aside for 15 minutes. Then 10 ml of 0.4N sodium hydroxide was added and read at 520 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as IU/l for serum.

5.3.1.2. Assay of Alanine transaminase, (ALT)

The Reitman and Frankel (1957) methodology was adopted for the assay of alanine transaminase too.

The reagents and method used were the same as those used for the assay of aspartate transaminase except for the substrate solution and the incubation time was reduced to 30 minutes.

Substrate

1.78 g of DL-Alanine and 38 mg of α -oxaloglutarate were dissolved in buffer. 0.5 ml of sodium hydroxide was added and the volume was made up to 100 ml with buffer.

The enzyme activity was expressed as IU/l for serum.

Assay of Alkaline phosphatase, (ALP)

Alkaline phosphatase was assayed by the method of Kind and King (1954).

Reagents

1. Carbonate - bicarbonate buffer : 0.1 M, pH 10.0
2. Disodium phenyl phosphate solution : 0.01 M
3. Magnesium chloride solution : 0.1 M
4. Sodium carbonate solution : 15%
5. Folin's phenol reagent : Commercial reagent,
1:2 dilution
6. Standard phenol: 100 mg of recrystallized phenol in 100 ml of water was prepared. 100 μ g of phenol per ml was then prepared by proper dilution and used as the working standard.

Procedure

The reaction mixture of 3.0 ml containing 1.5 ml of buffer, 1.0 ml of substrate and requisite amount of the enzyme source was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. The control tubes received the enzyme after arresting the reaction. The contents were centrifuged and to the supernatant, 1.0 ml of 15% sodium carbonate solution, 1 ml of substrate and 0.1 ml of magnesium chloride were added and the mixture was incubated for 10 minutes at 37°C. The colour was read at 640 nm against a blank in a Shimadzu UV spectrophotometer. The standard solution of phenol of varying concentrations was also treated similarly.

The enzyme activity was expressed as IU/l for serum.

Determination of Lipid Peroxides in Liver

The levels of lipid peroxides were determined by 'Thio barbituric acid' reaction as described by Ohkawa *et al.* (1979).

Reagents

1. Sodium dodecyl sulphate (SDS) : 8.1%
2. Acetic acid : 20%, pH 3.5
3. Thiobarbituric acid (TBA) : 0.8%
4. n-butanol/pyridine mixture : 15:1 (v/v)
5. 1,1',3,3'-tetramethoxypropane

Procedure

To 0.2 ml of homogenate, 0.2 ml of SDS, 1.5 ml of acetic acid and 1.5 ml of TBA were added. The mixture was made upto 4 ml with water and then heated in a water bath at 95°C for 60 minutes using glass ball as a condenser. After cooling, 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4,000 rpm for 10 minutes, the organic layer was taken and its absorbance at 532 nm was measured. 1,1',3,3'-tetramethoxypropane was used as standard.

Tissue lipid peroxide levels were expressed as n moles of MDA/mg protein.

Estimation of Antioxidant Status

5.3.3.1. Assay of Superoxide dismutase, (SOD)

Superoxide dismutase was assayed following the method of Misra and Fridovich (1972).

Reagents

1. Carbonate bicarbonate buffer : 0.1 M, pH 10.2
2. EDTA solution : 0.6 mM
3. Epinephrine : 1.3 mM (prepared freshly)
4. Absolute ethanol
5. Chloroform

Procedure

0.1 ml of enzyme source was added to tubes containing 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance at 480 nm was measured in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as units/min/100 mg protein. One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

5.3.3.2. Assay of Catalase, (CAT)

Catalase was assayed according to the method of Beers and Sizer (1952).

Reagents

1. Phosphate buffer : 50 mM, pH 7.0
2. Hydrogen peroxide : 30 mM in phosphate buffer

Procedure

To 1.2 ml of phosphate buffer, 0.1 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm for 3 minutes in a Shimadzu UV spectrophotometer. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as $\mu\text{moles of H}_2\text{O}_2$ decomposed/min/mg protein.

5.3.3.3. Assay of Glutathione peroxidase, (GPx)

The activity of glutathione peroxidase was assayed by the method of Rotruck *et al.* (1973).

Reagents

1. Phosphate buffer : 0.4M, pH 7.0
2. EDTA : 0.8 mM
3. Sodium azide : 10 mM
4. Reduced glutathione : 4 mM
5. H_2O_2 : 2.5 mM

6. TCA : 10%
7. Disodium hydrogen phosphate : 0.3M
8. DTNB solution : 40 mg of DTNB in 100 ml of
1% sodium citrate
9. Standard reduced glutathione

Procedure

The reaction mixture consisted of 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.1 ml of H₂O₂, 0.2 ml of reduced glutathione, 0.4 ml of phosphate buffer and 0.2 ml homogenate was incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2,000 rpm. To the supernatant, 3 ml of disodium hydrogen phosphate and 1.0 ml DTNB were added and the colour developed was read at 420 nm immediately in a Shimadzu UV spectrometer. The enzyme activity was expressed as n moles of glutathione oxidized/min/mg protein.

5.3.3.4. Estimation of Glutathione Reductase (GR)

The activity of glutathione peroxidase was assayed by the method of (Smith et al., 1988)

Reagents

1. 5, 5'-dithiobis (2-nitrobenzoic acid)
2. NADPH

Procedure

GR activity was measured by monitoring the rate of production of 5-thio-2-nitrobenzoic acid (TNB) from 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm, which is coupled with the GR reaction, as described previously. Homogenates (5%) were centrifuged 10 min at 8400 3 g; assays were performed on crude supernatants as described, except that no glutathione disulfide was added to the reaction mixture. The blank with no NADPH in the mixture was subtracted from the rate with 0.1 mM NADPH (final concentration) for each sample. Activity was calculated using an extinction coefficient of 13,600 M⁻¹cm⁻¹, based on the change in absorbance at 412nm for 2.0–4.8 min after simultaneous mixing of control and experimental samples and blanks.

The enzyme activity was expressed as nmole TNB formed mg⁻¹protein min⁻¹.

5.3.3.5. Estimation of Glutathione (GSH)

The level of total reduced glutathione in the liver tissue was measured by the method of Moron *et al.* (1979).

Reagents

1. Phosphate buffer : 0.2 M, pH 8.0
2. DTNB : 0.6 mM in 0.2 M phosphate buffer, pH 8.0
3. TCA : 10%

Procedure

1.5 ml of liver homogenate was precipitated with 1 ml TCA. The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.5 ml of clear supernatant, 2.0 ml DTNB was added and the total volume was made upto 3 ml with phosphate buffer. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar way were also run to determine the glutathione content.

The amount of glutathione was expressed as n moles/g wet tissue.

Assay of Tumour Necrosis Factor – α , (TNF- α)

The level of TNF- α was measured by using enzyme linked immunosorbent assay as described earlier in section 2.26.6 using mouse monoclonal antibody against rat TNF- α (1:5000 dilution) and Goat-antimouse IgG-HRP conjugate.

The level of TNF- α was expressed as ng/mg protein using a calibration curve obtained from standard TNF- α .

HISTOPATHOLOGICAL STUDIES

Small pieces of liver tissues were collected in 10% formal saline for proper fixation. These tissues were processed, embedded in paraffin wax sections of 5-6 μ m thick, cut and stained with hematoxylin and eosin (Luna, 1966). The sections were examined under light microscope and photomicrographs were obtained.

STATISTICAL ANALYSIS

All the grouped data were statistically evaluated with Statistical Package for Social Sciences (SPSS), Version 7.5. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. A '*P*' value of less than 0.05 was considered to indicate statistical significance. All the results were expressed as mean \pm S.D. for six animals in each group.

6. RESULTS

➤ **Effect of HESP on DEN-induced alteration in body weight, liver weight and relative liver weight**

Table-1 represents the liver weights of DEN induced rats (Group III) significantly ($p < 0.05$) increases as compared to that of the control rats. Treatment with hesperidin (Group IV) however showed that weights are significantly ($p < 0.05$) restored as compared to rat of group III. This reduction showed that the liver relative weights are lowered near to normalization as in group II rats.

➤ **Effect of Hesperidin on the levels of diagnostic liver marker enzymes:**

In the present study, intraperitoneal administration of a single dose of DEN (200mg/kg body weight) induced severe biochemical changes as well as oxidative damage in hepatic tissues. There was a significant ($p < 0.05$) rise in the level of diagnostic marker enzymes (AST, ALT and ALP) in the serum of Group III DEN administered rats as compared to that of Group I control rats (Table 1). The administration of hesperidin restored the levels of these enzymes to near normalcy ($p < 0.05$) as compared to those group III DEN injected rats. In hesperidin alone administered rat's Group II vs. control, no significant changes were observed.

➤ **Effect of Hesperidin on the levels of GSH and MDA in hepatic tissue**

Injection of DEN induced a significant ($p < 0.05$) increase in the level of lipid peroxidation measured in terms of MDA (Figure-7) which was paralleled by significant ($p < 0.05$) reduction in the level of GSH in the liver tissue of Group III animals as compared to normal controls (Table 4). In this study the treatment with hesperidin group IV significantly counteracted the DEN induced LPO and restored the level of GSH to near normal level in group IV rats as compared to that of group III animals.

➤ **Effect of Hesperidin on the level of hepatic tissue antioxidants**

Activities of glutathione dependent antioxidant enzymes (GPx, and GR) and antiperoxidative enzymes (SOD and CAT) was significantly lower in liver tissue of Group III DEN injected rats as compared to that of Group I normal control rats (Table 3). The observed reduction in the activities of GPx, and GR in DEN induced hepatic

damage might be due to decreased availability of its substrate, reduced GSH. In the present study in the treatment of group IV rats of DEN with hesperidin significantly reversed all these DEN induced alteration in the activities of antioxidant enzymes (SOD, CAT, GPx, and GR) to a near normal status. The normal rats receiving alone hesperidin did not show any significant change when compared with control rats indicating that it does not per se have any adverse effects.

➤ **Effect of Hesperidin on the levels of serum TNF- α**

Figure-9 illustrates the serum levels of TNF- α respectively. There was a significant ($p < 0.05$) rise in the levels of TNF- α in the serum of DEN intoxicated rats (Group III) as compared to that of control rats (Group I). The administration of hesperidin to Group IV animals restored the levels of TNF- α to near normalcy ($p < 0.05$).

➤ **Effect of HESP on DEN-induced alteration in body weight, liver weight and relative liver weight in control and experimental rats**

MORPHOLOGICAL STUDIES

A. CONTROL

Normal untreated rat liver.

B. HESPERIDIN ALONE

Section shows normal cellular architecture

C. DIETHYLNITROSAMINE TREATED

Section shows area of aberrant hepatocellular phenotype with variation in nuclear size, hyperchromatism, and irregular sinusoids (400 \times);

D. HESPERIDIN + DIETHYLNITROSAMINETREATED

Section shows hepatocytes maintaining near-normal architecture.

HISTOPATHOLOGICAL STUDIES

The livers of normal animals (Group I) and HESP alone treated (Group II) revealed normal parenchymal cells with granulated cytoplasm and small uniform nuclei arranged (Figure-10, 11). Animals subjected to DEN regimen alone showed a loss of normal architecture with oval- or irregular-shaped hepatocytes (Figure-12). The transformed liver cells of foci and nodules were substantially enlarged, largely vesiculated and frequently binucleated, which were clearly distinguishable from the surrounding normal parenchyma. The nuclei were mostly found to be pleomorphic with fine granular chromatin and occasionally with small nucleoli. Several nuclei were large hyperchromatic (basophilic) with prominent and centrally located nucleoli. Additionally, extensive vacuolation was noticed in the cytoplasm encircling the nucleus with masses of acidophilic (eosinophilic) material. The cellular architecture of liver sections in rats that received HESP (100 mg/kg) + DEN (Group IV) seemed to be normal counterparts (Figure-13).

7. DISCUSSION

Diethylnitrosamine chemically belonging to the N-Nitrosamine family is proved to be one of the potent carcinogens that produced hepatotoxicity in around 30 species of animals. DEN is primarily metabolized by the cytochrome P-450 enzymes to reactive electrophiles (O6 alkyl-guanine and N7 alkyl-guanine) which are proved to be cytotoxic, carcinogenic and mutagenic (Archer MC, 1989; Swann PF and Magee PN, 1971). As proved, DEN grounds for producing oxidative stress by causing a redox imbalance between the production of ROS and its detoxification by the cellular enzymes, (James E *et al*; 1998). DEN through normal or futile mechanisms causes production of ROS, and continuous or excessive exposure of ROS on the cells causes DNA damage; oxidations of amino acids in proteins and polyunsaturated fatty acids in lipids; as well as oxidative inactivation of the specific cellular enzymes, thereby causing OS, (Ramakrishnan G *et al*; 2006; Dean RT *et al*; 1997).

Oxidative damage of DNA is however proved to exhibit long term effects, (Evans MD *et al*; 2004). Thus it becomes evident to select a scavenger that would reduce the ROS production, both evidently and effectively. Several evidences proved that induction of DEN leads to increased levels of LPO products, diagnostic liver marker enzymes (AST, ALP, and ALT) and bilirubin in serum and significantly decreased the levels of antioxidants (SOD, CAT, GPx, GR and GSH) proteins and uric acid, (Kumar RS *et al*; 2007). The restorative effect of hesperidin on the altered levels of LPO, liver markers, and antioxidants were reported in the study.

Polyunsaturated lipids (PUFA) in the hepatocellular membrane are deteriorated by the reaction of oxygen with the membrane lipids directly to form free radical intermediates and semi stable peroxides. This reaction referred to as LPO, causes damage to the structural integrity of the cell membrane, (Rice-Evans C and Burdon R, 1993). On administration of DEN, a significant elevation on the amount of malondialdehyde (MDA); considered to be the end product of LPO was observed which indicates increased levels of LPO, (Thirunavukkarasu C and Sakthisekaran D, 2001). This increase leads to failure of the antioxidant defense mechanism thereby causing tissue damage. Treating the rats with hesperidin however proved to modulate

the increased LPO level which is in consistent with the previous reports, (Balakrishnan A and Menon VP, 2007).

The serum levels of marker enzymes provide more information about the type and extent of hepatocellular damage. The levels of AST, ALT, ALP, and bilirubin in the serum were studied for the diagnosis of hepatic damage. This is because these are cytoplasmic enzymes and are released into circulation following cellular damage, (Ansari RA *et al*; 1991). The activities of marker enzymes in serum increased significantly following DEN induction, thus causing damage to the structural integrity of the cell membrane, (Pradeep K *et al*; 2007). Several evidences proved that treating the rats with hesperidin in a dose dependent manner reduced the level of the serum marker enzymes, thereby reducing the incidence of hepatotoxicity, (Pradeep K *et al*; 2008).

GSH a major tripeptide is a non enzymatic biological antioxidant mainly responsible for the regulation of cell functioning and protection against oxidative injury. Its levels are reduced automatically when the levels of LPO is increased which is because in increased LPO conditions there is more consumption of GSH, (Shanmugarajan TS *et al*; 2008). As reported that DEN induction causes the increase in levels of LPO and the GSH levels are declined significantly. The depletion of GSH in DEN induced rats leads to more exposure of oxidative stress on the cell, (Rajasekaran NS *et al*; 2002). However, hesperidin administration ameliorated the increased levels of GSH levels as compared to the levels of DEN intoxicated rats, (Kaur G *et al*; 2006).

SOD, CAT, GPx and GR are important anti per oxidative enzymes which effectively scavenge the superoxide and hydrogen peroxide levels, (Gupta M *et al*; 2003). These enzymes are responsible for the protection against oxidative damage. It has been strongly reported that the administration of DEN significantly lowered the enzymatic antioxidant defense mechanism which is attributed to the oxidative stress in liver, (Pradeep K *et al*; 2007). SOD is responsible for the dismutation of superoxide anions to hydrogen peroxide (Oberlay LW, 1979) and CAT decomposes the hydrogen peroxide to hydroxyl radicals (Chance B, 1952). These enzymes play a sequential antioxidant defense against the several oxidative stress models. In our present investigation the significant decrease in the levels of SOD and CAT were observed due

to the increase in the levels of superoxide anions thus, more consumption of SOD and the utilization of CAT in removal of H₂O₂ during DEN exposure. Apart from SOD and CAT, GPx is another important glutathione dependent antioxidant enzyme that is responsible for scavenging the H₂O₂ and hydroperoxide radicals (Gaetani GF, 1989; Huang Z, 2001). It has been suggested that the depletion of GSH causes decrease in glutathione dependent antioxidant enzyme levels in DEN provoked oxidative damage which is in line with the previous studies. Several studies suggested that the flavonoids possess potent antioxidant and free radical scavenging activity. Treatment with hesperidin thus restores the levels of the antioxidant enzymes and protects the hepatocytes against the DEN induced oxidative stress in rat liver which may be due to the several pharmacological activities taking place.

Mounting evidences also recommend that other than ROS generation, DEN is also involved in producing inflammatory responses. It has been suggested that DEN stimulates macrophages & neutrophils (Mitsuru Matsuda *et al*; 2005) which leads to release of certain pro-inflammatory cytokines such as the TNF- α which play an important role in amplification loop of immune responses, (Chia-Chou Yeh, 2007). In our study, administration of DEN increases the production of the circulating TNF- α . Hesperidin however has been strongly proved to suppress the expression of the TNF- α levels thereby behaving as a protective agent against the DEN.

SUMMARY AND CONCLUSION

The present study is an effort to evaluate the protective efficacy of Hesperidin by studying the morphological and biological alteration observed in DEN induced hepatocellular carcinoma in rats. The outcome of the investigation carried out was summarized below.

The liver weight and relative liver weight were found to be increased in DEN induced rats and restored upon treatment with the hesperidin indicates its protective effect.

The activities of serum marker enzymes namely AST, ALT, ALP were found to be elevated within rats induced with DEN. Hesperidin treatment against DEN brought back the activities of marker enzymes to near normal by protecting the cell membrane integrity.

An enhancement in lipid peroxide levels with simultaneous depression in the levels of antioxidants was observed in rats induced with DEN. Hesperidin treatment exerted a significant free radical quenching and antioxidant strengthening effects by decreasing the lipid peroxidation with consequent improvement in antioxidant status proving to be a potent antioxidant.

Histopathological observations in DEN induced and hesperidin treated rats revealed the protective nature of the extract against DEN induced hepatocellular carcinoma and further support the hepatoprotective potential of hesperidin.

Administration of DEN in rats resulted in increased levels of TNF- α . These elevated levels of TNF- α were inhibited and normal levels were attained by treatment with hesperidin.

The present findings explore that treatment shows promising protective effect of hesperidin and further studies are needed to understand the molecular mechanisms of the hesperidin against DEN induced liver cancer. Hence it is concluded that hesperidin as potential for further development as a therapeutic agent for liver cancer.

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HISTOPATHOLOGY REPORTS

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TABLES AND FIGURES

TABLE-1: Effect of HESP on DEN induced alteration in body weight, liver weight and relative liver weight in control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DEN)	Group-IV (HESP+DEN)
INITIAL BODY WEIGHT	163.16 ± 8.18	172.86±11.39 ^{NS}	176.83±7.38 ^{*a}	174.16±8.01 ^{*b}
FINAL BODY WEIGHT	274.50±8.66	269.50±16.4 ^{NS}	211.33±13.26 ^{*a}	264.16±15.62 ^{*b}
LIVER WEIGHT	7.63 ± 0.44	7.45±0.76 ^{NS}	13.16±0.81 ^{*a}	8.21±0.61 ^{*b}
RELATIVE WEIGHT	2.77±0.08	2.75±0.11 ^{NS}	6.23±0.38 ^{*a}	3.10±0.12 ^{*b}

Values are expressed as mean ± S.D. for six animals. Body weight and liver weight Unit: gm.

a=Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at p < 0.05.

NS represents non significance at p < 0.05.

TABLE-2: Effect of HESP on DEN induced liver marker enzymes in serum of control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DEN)	Group-IV (HESP+DEN)
AST	115.88±5.16	115.70±5.16 ^{NS}	365.52±12.10 ^{*a}	256.25±10.15 ^{*b}
ALT	42.04±1.28	42.56±1.25 ^{NS}	141.32±5.45 ^{*a}	96.21±3.10 ^{*b}
ALP	58.92±2.42	60.69±2.97 ^{NS}	134.48±4.24 ^{*a}	84.70±2.35 ^{*b}

Values are expressed as mean ± S.D. for six animals. Liver marker enzymes unit: IU L⁻¹ (AST, ALT and ALP).

a= Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at p < 0.05.

NS= represents non significance at p < 0.05.

TABLE-3: Effect of HESP on DEN induced MDA and GSH level in liver of control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DEN)	Group-IV (HESP=DEN)
LPO	1.83±0.08	1.79±0.07 ^{NS}	5.11±0.53 ^{*a}	2.79±0.28 ^{*b}
GSH	51.33±4.24	51.26±3.40 ^{NS}	14.91±1.69 ^{*a}	35.85±3.41 ^{*b}

Values are expressed as mean ± S.D. for six animals. Units: MDA (n mole mg⁻¹ protein); and GSH (mg dL⁻¹).

a= Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at p < 0.05.

NS represents non significance at p < 0.05.

Table-4: Effect of HESP on DEN induced antioxidant enzymes in liver of control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DEN)	Group-IV (HESP=DEN)
SOD	7.93±0.69	8.01±0.55 ^{NS}	4.03±0.27 ^{*a}	6.80±0.69 ^{*b}
CAT	69.34±4.65	68.89±3.67 ^{NS}	43.07±3.36 ^{*a}	58.37±3.48 ^{*b}
GPX	116.85±5.28	117.73±5.74 ^{NS}	67.52±4.79 ^{*a}	89.55±7.82 ^{*b}
GR	192.55±18.23	195.32±17.66 ^{NS}	102.31±8.07 ^{*a}	151.04±7.75 ^{*b}

Values are expressed as mean ± S.D. for six animals. Units: SOD (Units min⁻¹mg⁻¹ protein); CAT (μmole H₂O₂ consumed min⁻¹mg⁻¹ protein); GPx (μmole of GSH oxidized min⁻¹mg⁻¹ protein); and GR (nmole g⁻¹ tissue).

a= Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at p < 0.05.

NS represents non significance at p < 0.05.

Table-5: Effect of HESP on DEN induced TNF- α level in serum of control and experimental rats

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DEN)	Group-IV (HESP+DEN)
TNF- α	8.49 \pm 1.40	8.87 \pm 1.12 ^{NS}	18.96 \pm 1.37* ^a	13.69 \pm 1.56* ^b

Values are expressed as mean \pm S.D. for six animals. Units: TNF- α (ng/mg protein).

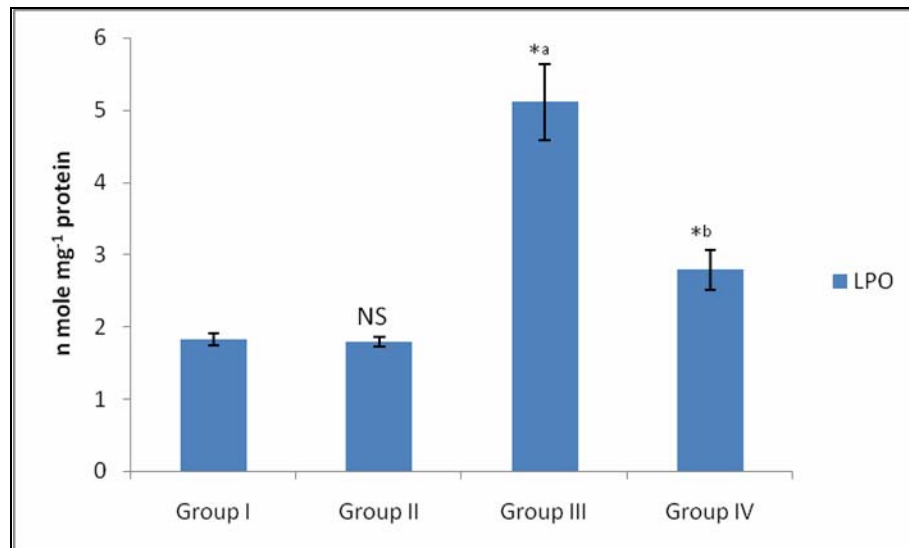
a= Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at $p < 0.05$.

NS = represents non significance at $p < 0.05$.

Figure-7: Effect of HESP on DEN induced LPO in liver of control and experimental rats



Values are expressed as mean \pm S.D. for six animals. Units: MDA (n mole mg⁻¹ protein);

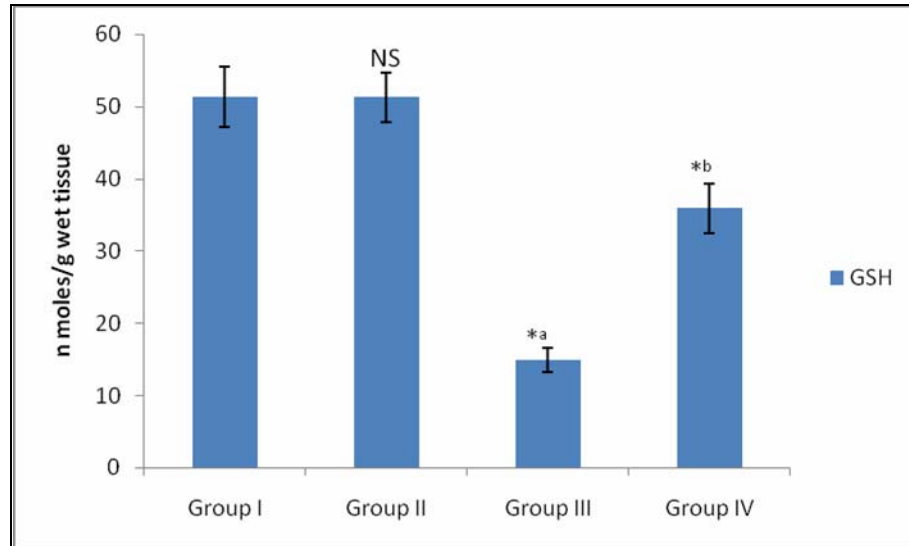
a=Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at $p < 0.05$.

NS represents non significance at $p < 0.05$.

Figure-8: Effect of HESP on DEN induced GSH in liver of control and experimental rats



Values are expressed as mean \pm S.D. for six animals. Units: GSH mg dL⁻¹.

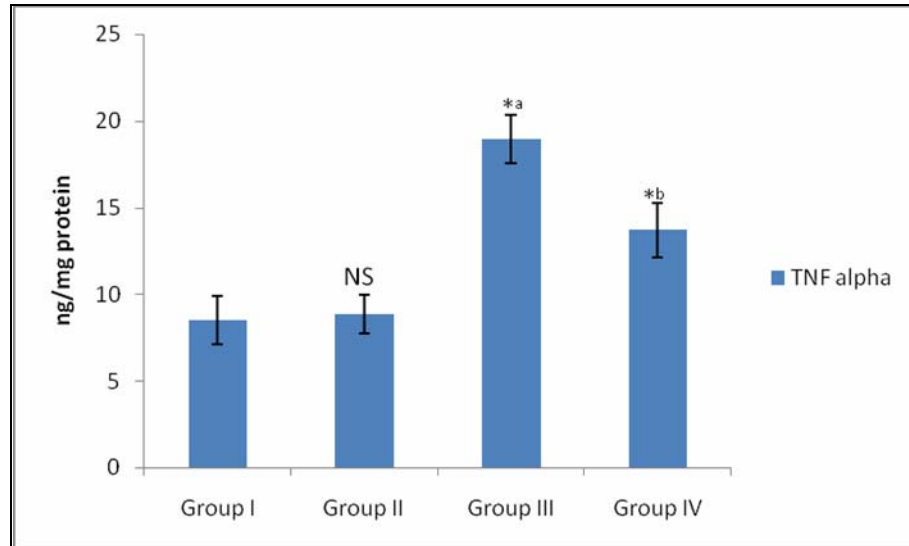
a=Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at $p < 0.05$.

NS represents non significance at $p < 0.05$.

Figure-9: Effect of HESP on DEN induced TNF- α in liver of control and experimental rats



Values are expressed as mean \pm S.D. for six animals. Units: TNF- α (ng/mg protein).

a=Comparisons are made between Group-I with Group-III.

b= Comparisons are made between Group-III with Group IV.

The * represent the statistical significance at $p < 0.05$.

NS represents non significance at $p < 0.05$.

HISTOPATHOLOGICAL EXAMINATION

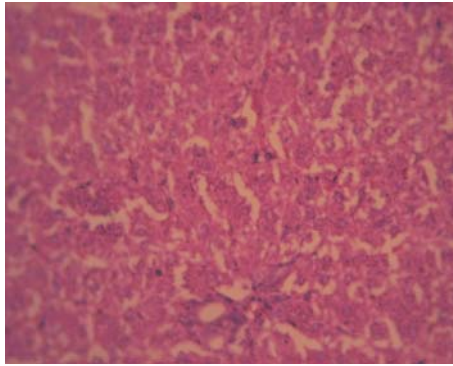


Figure 10 - (Group-I) Control rat (400x)

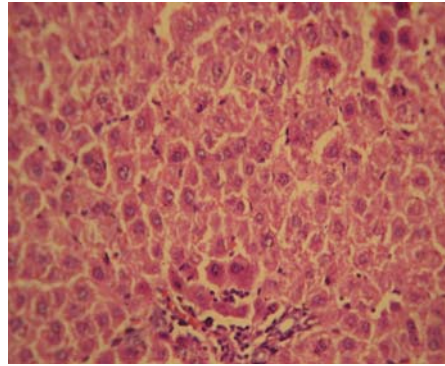
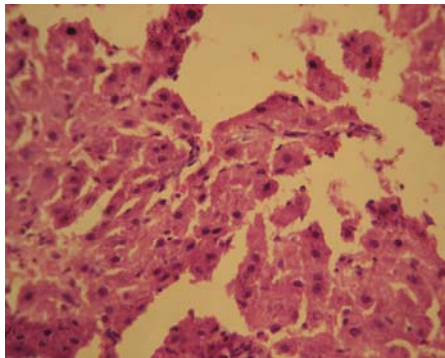
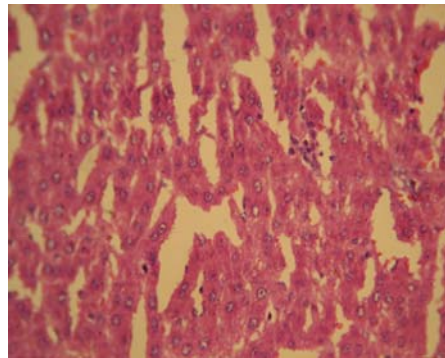


Figure 11 - (Group-II) HESP treated rat (400x)



**Figure 12 - (Group-III) DEN Induced rat
(400x)**



**Figure.13 - (Group-IV) HESP + DEN
treated rat (400x)**

MORPHOLOGICAL EXAMINATION



Figure 14 - (Group-I) Control rat

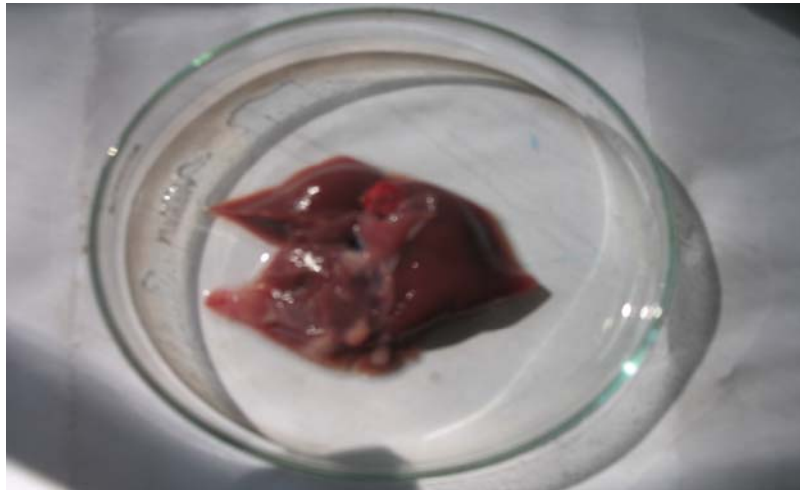


Figure 15 - (Group-III) DEN Induced rat



Figure.16 - (Group-IV) DEN+HESP treated rat

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