ANTICARCINOGENIC POTENTIAL OF HESPERIDIN IN A CHEMICAL CARCINOGENESIS MOUSE MODEL AND IN CULTURED HUMAN A431 CELLS

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Dedicated to the my beloved Parents

CHAPTER 1 General introduction

Chapter 1

It is usually believed that cancer is the disease of modern world however, it is not the case as humans and other animals have shown the presence of cancer throughout recorded history. The earliest evidence of cancer is found among fossilized bones and human mummies preserved in ancient Egypt, and also the ancient manuscripts, which has recorded the occurrence of cancer (Grmek, 1975-76; Cassileth, 1983). The earliest known descriptions of cancer appeared in several Papyri from ancient Egypt. The Edwin Smith Papyrus was written around 1600 BC (possibly a fragmentary copy of a text from 2500 BC) and contains a description of cancer, as well as a procedure to remove breast tumours by cauterization. It wryly observed that the disease has no treatment (*American Cancer Society, 2009).*

Hippocrates (460-370 BC), is considered as the "Father of Medicine and he used the terms *karkinos* and carcinoma to describe non-ulcer forming and ulcer-forming tumors for the first time. In Greek, these words refer to a crab, most likely applied to the disease because like crab the cancer has a central core and the finger-like spreading projections from a cancer resembling the legs of a crab. The Roman physician, Celsus (28-50 BC), later translated the Greek term into cancer, the Latin word for crab. Galen (130-200 AD), another Greek physician, used the word *oncos* (Greek for swelling) to describe tumors. Although the crab analogy of Hippocrates and Celsus is still used to describe malignant tumors, Galen's term is now used as a part of the name for cancer specialists – oncologists (Woelfer, 1881; Breasted, 1930; Ebbell, 1937).

Ancient Theories about Cancer

Humoral theory

Hippocrates understood that the body contained 4 humors (body fluids), (a) blood, (b) phlegm, (c) yellow bile and (d) black bile. Any imbalance of these fluids will result in disease and excess of black bile in a particular organ site was thought to cause cancer. This theory of cancer was standard through the Middle Ages for over 1300 years. During this period autopsies were prohibited for religious reasons, thus limiting knowledge about cancer.

Lymph theory

According to this theory cancer formation was by fluid called the lymph. Life was believed to consist of continuous movement of the fluids similar to that of blood

and lymph in the body. The lymph theory was supported in 17th century that tumors grow from lymph constantly thrown out by the blood.

Blastema theory

Muller demonstrated that cancer is made up of cells but not with lymph in 1838. His student, Virchow (1821-1902) determined that all cells including cancer cells were derived from other cells.

Chronic irritation theory

Virchow proposed that chronic irritation was the cause of cancer. Later Thiersch had shown that cancers metastasize through the spread of malignant cells and not through some unidentified fluid.

Trauma theory

From the late 1800s until the 1920s, cancer was thought to be caused by trauma.

Parasite theory

Till 18th century, scientists believed that cancer was contagious and spreads through parasite.

Modern Theory

In the modern context, the cancer is a disease of unrestricted cell proliferation. Cancer or neoplastic cells are a distinguished exception, where they have lost their usual growth control and they usually arise as variants of normal cells. The cells of a malignant (cancerous) tumor express abnormal phenotype and continue to divide without control or order. Such cells with constant division develop into a mass of tissue called tumor. The cancer cells can invade and damage nearby tissues and can also break away from a malignant tumor and spread into the distant parts of the body by a process called metastasis. Genetic alterations into two types of genes can contribute to the cancer process-the ***neoplastic transformation***.

1. Proto-oncogenes are normal genes that are involved in cell growth and division. The alteration in the regulation of these genes leads to their conversion into oncogenes that subsequently cause excessive cell proliferation and growth.
2. Tumor suppressor genes are normal genes that tightly control cell division. The mutation/s in the both alleles of a tumor suppressor gene results in its dysfunction, leading to dysregulation of cell division, as a result the cells undergo a continuous division and growth, causing development of neoplasia

or cancer. Dysregulation of gene expression or changes in genome that are not corrected by the cell can lead to the expression of abnormal proteins as a consequence the cells either fail to respond to normal signals, or may over-respond to normal signals. The altered gene expression cause malfunctioning of proteins and disruption of normal crosstalk between the signaling components of cell division machinery, which is largely under the control of a network of chemical and molecular signals. The disruption of normal signaling process results in the abnormal cell growth and division leading to a condition called as cancer or malignant transformation. Human species had never been completely shielded from some form of dysregulation of biological control of cell growth and division that may have finally led to the formation of malignant tumors (Oberling, 1952; National Cancer Institute, 1987; Greaves and Evans, 2000).

The ***cancer*** is one of the major causes of death worldwide including India. The IARC 2012 reports diagnosis of 14.1 million new cancer cases, 8.2 million cancer deaths and approximately 32.6 million people living with cancer (within 5 years of diagnosis) worldwide. The number of new cases is expected to rise by about 70% over the next 2 decades (Figure 2). The cancer is a group of more than 100 diseases and there are many different types of cancers, however, they are grouped into five major categories: ***carcinoma, sarcoma, myeloma, leukemia and lymphoma***. In addition, there are also some cancers of mixed types. The, breast cancer has emerged as one of the most common types of cancer among females, whereas oral and lung cancer are more common in males (Figure 1 and 2).

Carcinoma refers to a malignant neoplasm of epithelial origin or cancer of the internal or external lining of the body. Carcinomas, the malignancies of epithelial tissue, account for 80 to 90 percent of all cancer cases diagnosed.

Sarcoma is a condition of cancer that originates in the supportive and connective tissues such as bones, tendons, cartilage, muscle, and fat and they generally occur in young adults. The most common sarcoma often develops into a painful mass on the bone and usually resembles the tissue in which they grow.

Myeloma is a form of cancer that arises in the plasma cells of bone marrow whereas *Leukemias* ("liquid cancers" or "blood cancers") are cancers of the bone marrow (the site of blood cell production).

Lymphomas develop in the glands or nodes of the lymphatic system. Unlike leukemias, which are sometimes called "liquid cancers," lymphomas are "solid cancers." Lymphomas may also occur in specific organs such as the stomach, breast, or brain.

The phenomenon of development of cancer is known as carcinogenesis. The carcinogenesis is a multistep process that begins with a normal cell population. The normal cells are transformed into a preneoplastic cell population and subsequently form a highly malignant tumor (Sugimura, 1992). Each step during carcinogenesis involves a varying degree of stability and reversibility. This multistep process of experimental carcinogenesis can be divided into initiation, promotion and progression (Farber, 1984; Pitot and Riegel, 1987; Shields and Harris, 1991).

Multistage Carcinogenesis

The process by which a normal cell is transformed into a cell that has acquired the property of uncontrolled proliferation is called **carcinogenesis**. Nearly almost a century ago, Haaland (1911) first recognized the successive stages in the development of tumors. It was not until 1947, when Berenblum and Shubik (1947) defined the multistep process of carcinogenesis and categorized it into two stages namely Initiation, and Promotion.. Epidemiological studies in humans also indicate the multistage nature of development of malignant neoplasia in a variety of organ systems. The earliest studies of multistage carcinogenesis in experimental animals were based on studies by Rous and Friedewald (1941) on mouse skin indicating the presence of two stages during carcinogenesis namely: initiation and promotion. Foulds (1954) proposed the concept of tumor progression describing the characteristics of malignant neoplasia and its evolution to higher degrees of autonomy and malignancy, which is now regarded as the third stage of tumor development. Thus it is widely accepted that cancer development is a multistep event proceeding through discrete morphological and biochemically altered stages from normal to preneoplastic lesions to highly malignant tumors (Sugimura *et. al.,* 1991; Rundhaug and Fischer, 2010) involving dysfunction of genes involved in cell growth, differentiation and cell cycle control, which includes proto-oncogenes and tumor suppressor genes (Harris, 1991; Coleman and Tsongalis, 2006).

In the traditional view of carcinogenesis derived from animal models, the mechanisms in carcinogenesis can be interpreted as:

Multistep process beginning with **normal cells** then **preneoplastic cells** and ending with a **highly malignant tumor** (Sugimura *et. al.,* 1992). Each step involves varying degree of stability and reversibility (Figure 3).

The modern view of carcinogenesis is the multistep process of experimental carcinogenesis can be divided mainly into three stages namely, ***"initiation"***, ***"promotion"*** and ***"progression"*** (Pitot *et al.*, 1987; Shields and Harris, 1991; Farber, 1984; Rundhaug and Fischer, 2010).

Initiation

The process by which a normal cell is converted into a neoplastic phenotype by administration of a carcinogen is called as "***initiation***" and it is the first stage in the process of chemical carcinogenesis. Initiation is an irreversible, normally rapid phenomenon, where the chemical/s produces permanent changes in DNA of the target cell (Figure 3) that provides the cell with both an altered responsiveness to their microenvironment, and advantage of a selective clonal expansion when compared to the surrounding normal cells. This is usually of a short duration and occurs within 1-2 days of carcinogen treatment. Initiation occurs after limited exposure and direct action of the carcinogen on the target cell leading to biochemical lesions in the initiated cells. It is an irreversible change that predisposes the target cell to neoplastic transformation. This completes the initiation stage.

Promotion

The second stage of carcinogenesis is the process whereby tumor formation is stimulated in tissues that have been exposed to an initiating agent and is called "**promotion**". The promotion allows the acquisition of those changes that result in continuous cell proliferation and/or survival of the initiated cell to a greater extent than normal cells. It also enhances the probability of additional genetic damage including endogenous mutations accumulating in the expanding population of these cells. It is a reversible process wherein multiple exposures to agents that are considered weak carcinogens or non- carcinogens lead to focal proliferation or clonal expansion of initiated cells into benign lesions, which may either remodel to normal tissue or removed by cell death or apoptosis. This clonal growth reflects a reversible alteration of genetic expression during promotion. The co-carcinogens chemicals cause alterations in the DNA, leading to mutations, translocations or conformational and functional changes (Afshari and Barrett, 1993; Melnick *et. al.*, 1993). In case, of non-genotoxic carcinogen

(chemically non DNA reactive carcinogen) such as peroxisome proliferators, an oxidative DNA damage has been suggested as one of the possible mechanisms of tumor progression (Clayson *et. al.,* 1994). The stage of promotion differs from initiation by virtue of its reversible nature and its modulation by environmental factors (Pitot and Dragan, 1991; Pitot *et. al.,* 1991). Promotion can be basically divided into two phases; ***conversion phase***, where the initiated cell becomes a dormant neoplastic cell and ***propagation phase***, where these dormant cells begin to proliferate leading to the development of neoplastic nodule (Owens *et. al.,* 1999). The continued presence of at least a threshold level of promoting agent has been shown to be necessary for promotion in skin model of carcinogenesis (Boutwell, 1974). The dose response characteristics of a promoting agent include a threshold dose below which there is no promotional effect, a linear portion of the response curve, and a maximal effect (Goldsworthy *et. al.,* 1984). The maximal effect of a promoting agent is dependent on duration and format (Pitot *et. al.,* 1991) of its administration (Boutwell, 1974), as well as the finite number of initiated cell's response to that specific promoting agent. The initiated cells responding to the promoting stimulus are heterogeneous in population and possess a variety of genetic lesions (Goldsworthy *et. al.,* 1984).

Progression

The third stage of tumor development is called "***progression***", which involves the stepwise evaluation of cancer cells progressively towards more malignant phenotype. This is the final step in the process of carcinogenesis in which benign tumor develops into an irreversible malignant neoplasm. It represents a series of heritable changes in subpopulation of initiated cells resulting in malignancy. During progression, neoplasms develop progressively and acquire characters that help in increased invasiveness and the ability to metastasize with altered biochemical, metabolic and morphological changes (Farber, 1984; Pitot and Dragan, 1991). Cytogenetic studies have provided evidence supporting the hypothesis that genomic instability is the potential mechanism during tumor progression (Sargent *et. al.,* 1996). Genomic instability is manifested by the abnormal number and structure of chromosomes, gene amplification, and altered gene expression (Gray and Collins, 2000).

Molecular Mechanism of Carcinogenesis

Carcinogenesis is the mechanism of formation of a cancer, whereby normal cells are transformed into cancer cells. Majority of the chemical carcinogens are not capable

of causing hazardous effects but the metabolism of these compounds play a crucial role in the initial host response to the environmental exposure. Disturbance in the balance between capacity of activation and detoxification may thus explain the individual variations in response to exposures to carcinogens. The amount of ultimate carcinogen produced depends on the action of competing activation and detoxifying pathways involving phase I and phase II enzymes (Kensler and Cooney, 1981; Cooney, 1982; Guengrich, 1988). There are two theories of carcinogenesis: somatic mutation theory, where a carcinogen causes mutation and confers selective advantage of cell proliferation leading to neoplastic transformation and thus the neoplasia is monoclonal in origin and that the default state of a metazoan cells is quiescence. This theory was proposed by Boveri in the year 1914. According to this theory cancer is irreversible. The other theory of carcinogenesis is the tissue organization field theory, which states that carcinogenesis is primarily a problem of tissue organization, where a carcinogen destroys the normal tissue architecture and disrupts cell-to-cell signaling, and compromising genomic integrity (Sonnenschein and Soto, 2000). Hence, in this theory the DNA mutations are the effect, and not the cause, of the tissue-level events (Rosenfeld *et al*., 2013). According to this theory cancer is reversible and curable. It is well known that carcinogens cause a point mutation leading to a change in the single base pair or change several base pairs encoding an abnormal protein. These genetic changes may lead in the breakage of chromosomes or duplication or loss of chromosomes during or after DNA replication. This results in genomic instability, where the cancer cells have unstable genome including aneuploidy. The other way is that carcinogen may change the way of DNA packaging, the epigenetic change. Several forms of genetic changes have been reported including gene amplifications, deletions, insertions, rearrangements, and point mutations in the neoplastic cells indicating that these process are fundamental to carcinogenesis (Lengauer *et al.,* 1998)

Chemical Protection

The major mechanisms of chemical protection against mutagenesis, carcinogenesis and other forms of toxicity is the induction of phase II metabolizing enzymes. The phase II metabolizing enzymes include various transferases such as UDP-glucuronosyl transferase, glutathione S-transferase and NADPH quinone reductase. The phase II enzymes act on the electrophilic products generated in the phase I reaction by incorporating them into endogenous moieties (glucuronide, glutathione, sulphate) to

produce extremely electrophilic products that are excreted from the cell (Cooney, 1982; Guengrich, 1988).

There is a substantial evidence of involvement of free radicals during the enzymatic activation of various carcinogens such as benzo(a)pyrene (BaP), dimethylbenz(a)anthracene (DMBA), aromatic amines and N-nitrasocompounds (Clemens, 1991). All these compounds induce free radicals through normal metabolic pathways, which interact with DNA to form DNA adducts (Klaunig *et. al.*, 1998). The reactive oxygen species generated during tumor promotion interact with a wide variety of cellular biomolecules resulting in altered phenotypic expression, which may be mediated through direct modification of the genome or epigenetic pathways. The pharmacological intervention may inhibit the generation of free radicals by various sources and offer protection to the cellular genome.

Need for chemoprevention

Chemoprevention is a strategy to prevent the development of cancer by various means. Despite of the immense efforts to improve treatment of cancer and find its cure, the overall mortality rates for most form of cancer have not significantly declined in the past 50 years (Jemal *et. al.*, 2008). Conventional therapeutic (chemotherapy and radiation) and surgical approaches have not been able to control the incidence of most of cancer types. The major treatment strategies of cancer cause damage to the cellular genome of not only the neoplastic cells but also the normal cells, which in turn become neoplastic in due course of time. Therefore, strategies that could inhibit the occurrence of cancer could be most desirable than those that allows the development of cancer and treat it thereafter. **Chemoprevention** is a term used frequently to describe the paradigm that can block the occurrence of cancer.

The old age saying that *"**prevention is better than cure**"* could be an important strategy to reduce the risk of cancer in human population and the most important imaginative approach to reduce the cancer cases worldwide, could be to inhibit the induction of carcinogenesis or cancer by pharmacological intervention, which will not allow the cellular DNA to undergo mutagenic changes, in other words the cellular DNA will be preserved in its native form despite the onslaught from various physical and chemical sources (Liu, 2004; Liu *et. al.*, 2007). This new pharmacological approach to arrest or reverse the process of carcinogenesis, and thus prevent cancer, is called "***chemoprevention"***. Although still in its initial stages, the new science of

chemoprevention has been established as an important approach to control malignancy. For the first time, it has been shown convincingly that the use of chemopreventive agents in men and women with premalignant lesions can substantially reduce the subsequent development of truly invasive cancer. Chemoprevention is now recognized as an important strategy to prevent the cancer both in clinical and basic science research (Chemoprevention Working Group, 1999).

It is believed that dietary factors may contribute to the reduction of as much as one-third of potentially preventable cancers. The long-term preventive effect of plant-based agents for chemoprevention of cancer and several other chronic diseases is well documented (Jang and Pezzuto, 1999; Link *et al.*, 2010). The development of a malignant tumor involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, and immunological). In addition, carcinogenesis often proceeds through multiple discernible but often overlapping stages. The overall process can occupy a major portion of the life span of an individual (Figure 6). The transitions between successive stages can be enhanced or inhibited by various agents. Several types of evidences indicate that 50–80% of human cancers are potentially preventable, because its causation, i.e., the factors that determine the incidence, is largely exogenous. Exogenous causative agents or factors that have been identified in humans include cigarette smoking, exposure to occupational and environmental chemicals, radiation, carcinogenic factors present in the diets, lifestyle, socioeconomic factors, specific viruses, bacteria, and/or parasites. Apart from these factors hereditary factors play a critical role in influencing individuals' susceptibility to cancer and that in certain rare forms of human cancer; it is the hereditary factors that determine the development of cancer in an individual. However, in the majority of human cancers, the exogenous factors present the most likely opportunities for interventions targeted to primary prevention of cancer.

There are three sequential levels of disease prevention depending on whether the intervention is addressed to healthy individuals (primary prevention) or patients in preclinical or early stage (secondary prevention) or patients after therapy (tertiary prevention) (De Flora *et al.,* 2001). Preventing the occurrence of disease is "primary prevention", "secondary prevention is an early detection and intervention, preferably before the condition is clinically apparent, and has the aim of reversing, halting, or at least retarding the progress of a condition", whereas minimizing the effect of disease

by preventing complications and premature deterioration is "tertiary prevention" (Figure 4).

Chemopreventive compounds have been classified into blocking agents or suppressing agents according to the carcinogenic stages they interrupt (Wattenberg, 1997). The blocking agents are compounds, which discourage the metabolic activation of procarcinogens and subsequent formation of reactive carcinogens or prevent active carcinogens from reaching or reacting with critical cellular targets such as DNA, RNA and proteins, whereas the suppressing agents are those pharmacological agents that deter malignant transformation of initiated cells after reaction of carcinogens with important cellular targets during promotion or progression.

Dietary polyphenols having antioxidant, antimutagenic activities and modulating effects on certain cytochrome P-450 enzymes and may play an important role in chemoprevention strategies. Because of the expected safety following long-term administration to human, the diet has been considered as a rich source of potential chemopreventive agents. In fact, a number of natural compounds with inhibitory effects on tumorogenesis have been identified from human diet or sources of diet. These compounds include isothiocyanates from cruciferous vegetables, catechins from green tea, resveratrol from grape seeds, red wine, curcuminoids from turmeric, procyanidins from various fruits and nuts, isoflavones from soybean, and antioxidant vitamins in various foods. With a significant advancement in our understanding of the cellular events leading to cancer, synthetic chemopreventive agents have been also developed, which include selective inhibitors of ornithine decarboxylase (ODC), selective estrogen receptor modulators (SERM), selective inhibitors of retinoid X receptors (rexinoids), and inhibitors of inducible cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS).

Skin cancer chemoprevention is theoretically similar to chemotherapeutics, but focuses on pre-invasive rather than invasive lesions. Accessibility of the skin allows for easier detection and removal of neoplastic or preneoplastic lesions, using noninvasive or minimally invasive techniques. The skin, therefore, is a model organ for investigating cancer prevention processes that may be relevant to other organs as well. The incidence of skin cancer has been increasing at an alarming rate with an estimated 3.6 million cases in 2005; accounting for 40% of all cancer diagnoses in

Western world. The majority of skin cancers are nonmelanomas (NMSCs) and include epidermal keratinocyte derived squamous cell (SCC) and basal cell carcinomas (BCC) both of which are closely associated with chronic exposure to ultraviolet light (UV). A pre-malignant lesion or actinic keratosis (AK) has been identified for SCC, but not for BCC. AKs are far more common in the population than SCC with a transformation rate of 6-10% over 10 years and provide an excellent target for the development of skin cancer chemoprevention strategies.

Although only 6% of skin cancers are melanomas, melanoma is the most deadly form of skin cancer. Usually 132,000 of skin melanomas are diagnosed every year. Dysplastic nevi, a likely precursor of melanoma, are also potential targets for chemoprevention as they are also vitally important in the reduction of skin cancer mortality. However, chemoprevention studies of melanoma have been limited (Figure 5).

Skin serves as a protective barrier against the deleterious effects of environmental factors. For more than 50 years, the multistage model of mouse skin carcinogenesis has provided a conceptual framework to study the carcinogenic process in tissues of epithelial origin. The DMBA-induced skin is the prototypical and best-characterized member of the polycyclic aromatic hydrocarbon (PAH) family of chemical carcinogens, which are widespread in the environment. PAHs are suspected human carcinogens and could serve as the best model to study chemoprevention by various pharmacological agents.

The pharmacological intervention can prevent or delay the occurrence of cancer in high-risk populations, such as those with premalignant lesions or previously resected cancer. Exposure to UV light induces a number of molecular pathways and results in specific genetic alterations (i.e. ~53 mutations) that are critical to progression from normal skin to precancerous lesions and ultimately the cancer. These UVB-induced changes serve as a basis for the development of chemopreventive agents. Targets may include inhibition of polyamine synthesis, inhibition of prostaglandin synthesis, specific retinoid receptors, as well as inhibition of specific components of the Ras and MAP kinase signal transduction pathways.

Considerable benefits for developing countries are possible when the local medicinal plants of traditional sources used, are subjected to scientific methods of validation and quality control. Plants sources used in traditional medicine therefore

have an important role to play in the maintenance of health in all parts of the world and in the introduction of new treatment strategies. The dietary ingredients could also be potential source of a strategy to suppress the occurrence of cancer. The benefit of this strategy is that dietary sources are used regularly and they are non-toxic, safe, acceptable and can be manipulated easily.

HESPERIDIN

Hesperidin is a solid substance with low solubility in water. It is, however, much more soluble in water than its aglycone hesperetin. Hesperidin has a molecular formula of $C_{28}H_{34}O_{15}$, with a molecular weight of 610.57 Daltons. The disaccharide of hesperidin, rutinose, is comprised of the sugars rhamnose (6-deoxy-L-mannose) and glucose. The other names of hesperidin are hesperetin 7-rhamnoglucoside, hesperetin-7-rutinoside and (S)-7-[[6-0-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl] oxy]-2, 3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one. The structure of hesperidin is shown in Figure 6.

Hesperidin was first discovered by Lebreton in the year 1827 in an impure form and has been investigated for its various properties since then (Fluckiger and Hanbury, 1986). Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), is a predominant bioflavonoid, present in large amounts in the discarded rinds 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 Citrus genus. It is also found in many plants other than citrus species, such as Fabaceae (Bhalla and Dakwake, 1978), Betulaceae (Pawlowska, 1980), Lamiaceae (Kokkalou and Kapetanidis, 1988) and Papilionaceae. Hesperidin is also present in the bark of *Zanthoxylum avicennae* and *Z. cuspidatum* belonging to family Rutaceae (Arthur *et al.,* 1956). It has been also isolated from the roots of *Acanthopanax setchuenensis i*n China (Zhao *et al.,* 1999). The highest concentration of hesperidin has been found in the green fruits, which increases during storage (Higby, 1941). It is reported to be present in the epicarp, mesocarp, endocarp and juice of Citrus fruits (Kawaguchi *et al.,* 1997). The hesperidin contents have been found to increase after germination of seeds (Barthe *et al.,* 1988).

Both hesperidin and its aglycone hesperitin have been reported to possess a wide range of pharmacological properties. Hesperidin has been reported to possess significant anti-inflammatory, analgesic, antihypertensive, diuretic antibacterial and

antiviral effect (Galati *et al.,* 1994; Emim *et al.,* 1994; Bae *et al.,* 2000; Kim *et al* 2001; Ohtsuki *et al.,* 2003). Hesperidin has been reported to inhibit tumor initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts *in vitro* (Berkarda *et al.,* 1998; Tanaka *et al.,* 1997; Franke *et al.,* 1998). It has been found to reduce cholesterol levels in humans (Kurowska *et al.,* 2000) and retard the bone loss (Chiba *et al.,* 2003; Hasanoglu *et al.,* 2001). Its deficiency has been indicated in abnormal capillary leakage. Hesperidin has been found to possess beneficial effects on the abnormal capillary permeability, fragility and protection against various traumas and stresses (Felicia *et al.,* 1996). It has been found to be non-toxic in animals and humans (Sieve, 1952; Kawabe *et al.,* 1993; Kawaguchi *et al.,* 1997). The chemoprotective effect of hesperidin has not been evaluated therefore the present study envisages to investigate the chemoprotective activity of hesperidin in skin carcinogenesis mouse model and related molecular mechanisms. Hesperidin is represented by the following chemical structure:

AIM AND OBJECTIVE OF THE STUDY:

Increasing environmental pollution, altered life style and various other factors have increased the frequency of cancer in human population. The cancer is the second largest killer disease in the modern world. In spite of the availability of large paraphernalia of treatment strategies, complete cure of cancer still remains elusive. The present modalities of cancer treatment are non-specific and also change the fidelity of genome of normal cells as a result secondary tumors have been reported in the survivors (Pendelton *et al*., 2014). Further, treatment of cancer is highly expensive and beyond the reach of common man. An age old saying that **"Prevention is better than cure"** could be a prudent strategy to reduce the occurrence of cancer worldwide. The most important imaginative approach to reduce the cancer cases worldwide could be inhibition of the induction of carcinogenesis or cancer by pharmacological intervention. These pharmacological agents are expected to spare the cellular DNA from the mutagenic changes induced by various factors and preserve genome in its native form despite the onslaught from the various physical and chemical challenges. Common dietary agents may play an important role in the inhibition of carcinogenesis. Therefore, present study aims to investigate the chemopreventive effects of Hesperidin in mice by studying the;

1. Chemopreventive activity of Hesperidin in DMBA—TPA at initiation and proliferation stage in chemical carcinogenesis.
2. Alteration in the Biochemical responsible for the prevention of chemical carcinogenesis by Hesperidin.

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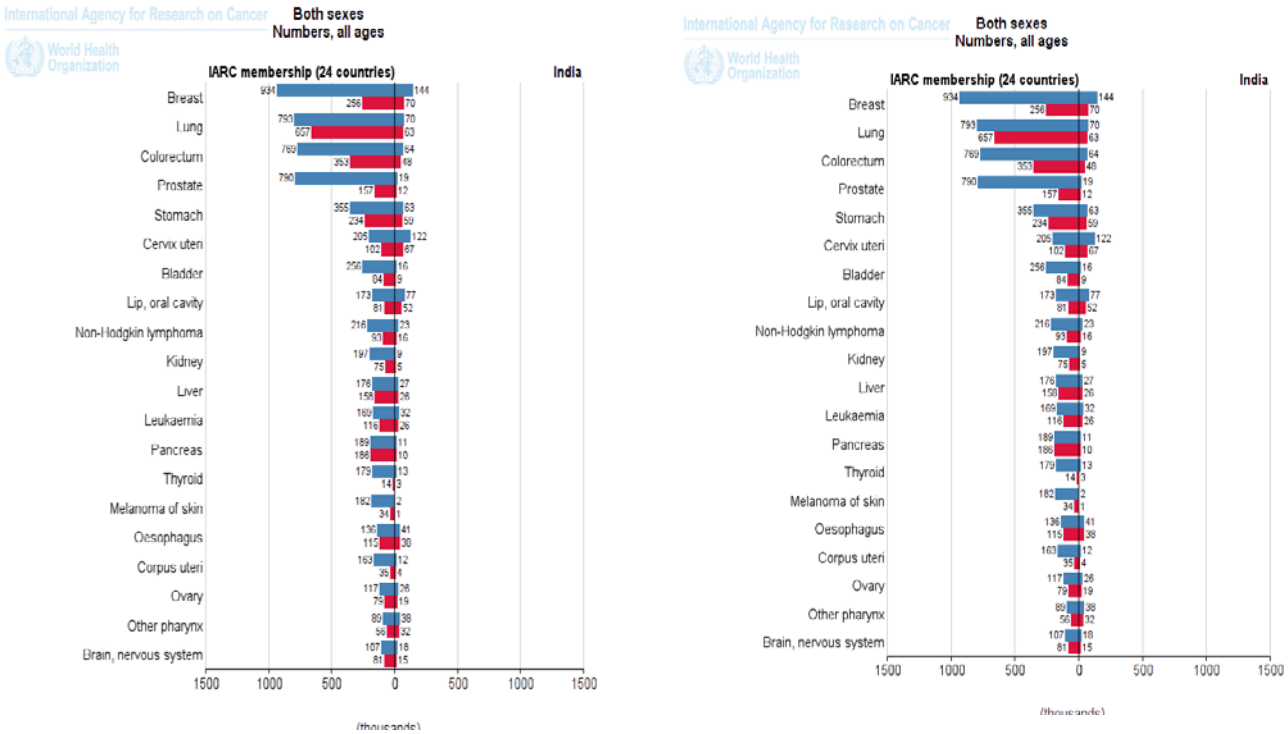

Figure 1. Occurrence of cancer in 2012. (a) IARC nations (b) India
(World Cancer Report 2014)

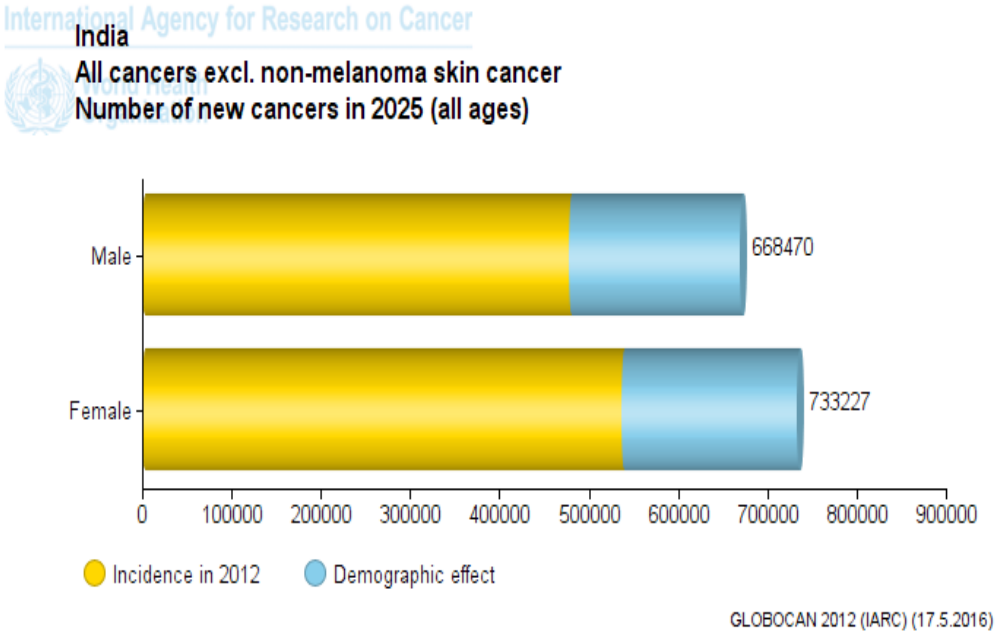

Figure. 2. Projection of new cancer cases in India (WHO/ IARC, 2012).

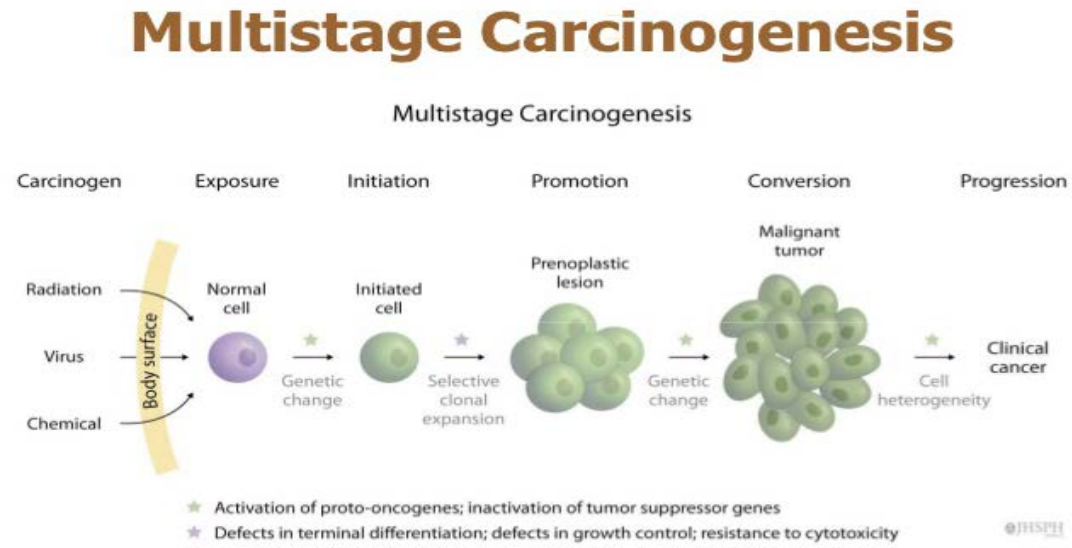

Figure 3: Diagrammatic representation of multi-stage carcinogenesis in humans.

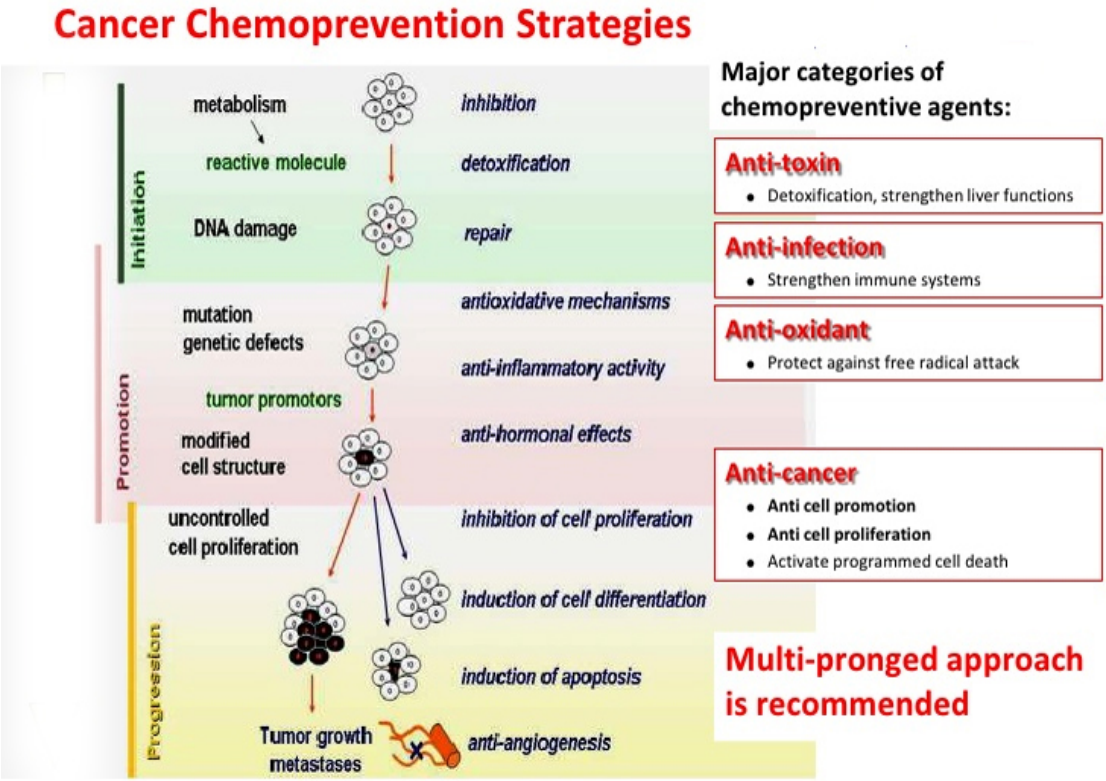

Figure 4: Chemoprevention and strategies to inhibit carcinogenesis

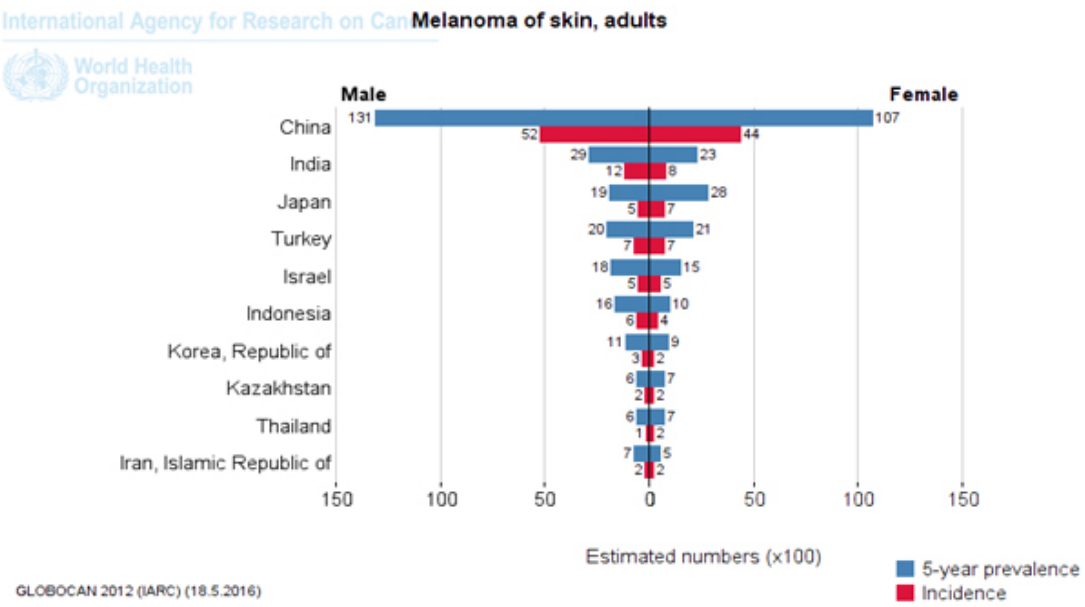

Figure 5. The report of 5 years prevalence of skin cancer in top ten Asian nations (IARC, 2012).

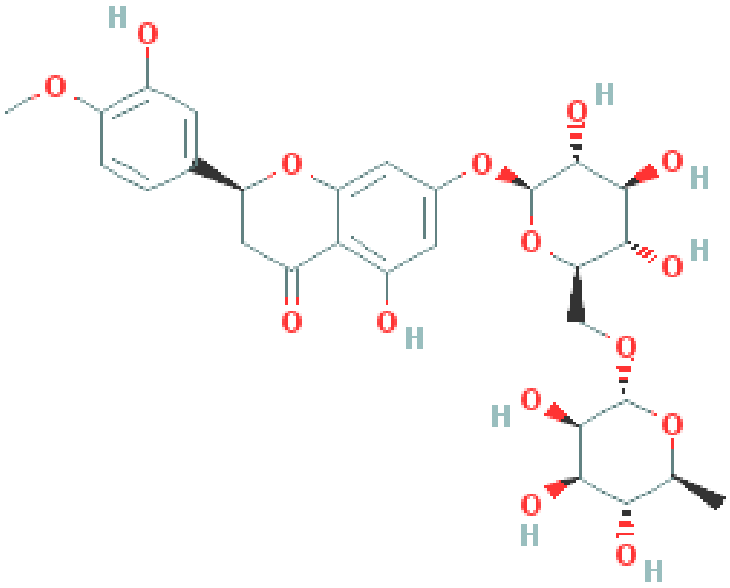

Figure 6. Chemical structure of hesperidin

Table 1: **Body weights changes in mice orally administered chronically with various doses of hesperidin. The data were expressed as Mean ± SEM, n=10.**

Treatment (90 days)	Body Weight (g) (Mean ± SEM)			
	Day 0	***Day 30***	***Day 60***	***Day 90***
Control (SPS)	23.4 ± 1.3	25.3 ± 1.2	28.6 ± 1.6	31.8 ± 1.1
HPD 100mg	22.9 ± 1.2	25.9 ± 1.2	30.5 ± 1.7	33.0 ± 1.2
HPD 200mg	24.2 ± 1.1	27.2 ± 1.2	30.2 ± 1.2	34.6 ± 1.3
HPD 300mg	23.3 ± 0.9	26.3 ± 1.2	29.3 ± 0.7	32.7 ± 1.0
HPD 400mg	24.3 ± 0.6	28.3 ± 1.2	30.2 ± 0.7	35.6 ± 1.20

There was no statistically significant difference between groups.

Table 2: **Effect of chronic administration of various doses of hesperidin on sperms of Swiss albino mice. The data were expressed as Mean ± SEM, n=10.**

Group	% of Sperm Viability	% of Sperm Motility
Control (SPS)	78.0 ± 0.42	52.1 ± 0.38
HPD 100mg	75.1 ± 0.50	47.9 ± 0.26
HPD 200mg	76.5 ± 0.41	50.2 ± 0.29
HPD 300 mg	78.1 ±0.44	51.15 ± 0.51
HPD 400 mg	79.2 ±0.53	51.25 ± 0.39

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Table 3: Hematological studies on Swiss albino mice after chronic treatment with different doses of hesperidin. The data were expressed as Mean ± SEM, n=10.

Treatment Group	Hematological studies on mice after chronic treatment with hesperidin		
	WBC ($\times 10^3$)	**RBC ($\times 10^6$)**	**Haematocrit (vol. %)**
Control(SPS)	5.4 ± 0.7	6.9 ± 0.2	32.01 ± 0.25
HPD 100mg	5.6 ± 0.9	7.8 ± 0.2	30.08 ± 0.59
HPD 200mg	5.7 ± 0.7	8.2 ± 0.3	30.98 ± 0.36
HPD 300mg	5.8 ± 0.8	8.6 ± 0.3	29.59 ± 0.72
HPD 400mg	5.9 ± 0.6	8.7 ± 0.2	29.87 ± 0.84

Table 4: Effect of chronically administered hesperidin on the micronuclei induction in the splenocytes of mice. The data were expressed as Mean ± SEM, n=10.

Treatment	MNBNC per 1000±SEM			
	One MN	Two MN	Multi MN	Total MN
Control (SPS)	20.3 ± 1.23	0.29 ± 0.03	0.43 ± 0.16	21.02 ± 1.55
HPD 100mg	20.65 ± 1.45	1.46 ± 0.33	0.56 ± 0.15	22.67 ± 1.58
HPD 200mg	25.97 ± 1.76*	2.25 ± 0.37*	0.89 ± 0.12	29.11 ± 2.11*
HPD 300mg	28.78 ± 1.88*♣	2.88 ± 0.42*♣	1.19 ± 0.25*♣	32.85 ± 2.34*♣
HPD 400mg	26.87 ± 0.76*♣	2.55 ± 0.87*♣	1.00 ± 0.22*♣	30.42 ± 2.11*♣

** p<0.01 when compared with control*

♣p<0.01 when compared to 100 mg/kg hesperidin.

Table 5: Biochemical estimations

Dose (mg/ kg b.wt.)	AST (Units/ml)	ALT (Units/ml)	Creatinine (mg%)	Uric acid (mg/dl)
Control (SPS)	20.23±2.21	18.56±2.32	1.15±0.68	2.25±0.05
HPD 100mg	24.20±1.35	23.24±1.12	1.13±0.72	3.20±0.45
HPD 200mg	34.85±2.23*	24.92±1.21	1.13±0.25	2.54±0.42
HPD 300mg	33.59±1.29*	28.84±1.31*	0.88±0.02	4.32±0.78*
HPD 400mg	31.92±2.52*	28.28±2.41*	1.15±0.27	3.06±0.88*
Normal ref value	8.00-40.00	5.00-35.00	0.60 - 1.20	3.40-7.00

The data are expressed as Mean ± SEM, n=10.

**p<0.05 when compared to control group.*

CHAPTER
2

Evaluation of acute and chronic toxicities of hesperidin in Swiss albino mice

ABSTRACT

The acute toxicity evaluation is necessary to find out the maximum tolerated dose as well as its toxic side effects. The acute and chronic toxicity of hesperidin was studied in male Swiss albino mice orally administered with 0.5, 1, 2, 2.5 and 3 g/kg body weight. The acute toxicity studies showed that hesperidin is non-toxic up to a dose of 3 g/kg body weight. The chronic administration of 100, 200 300 and 400 mg/kg body weight hesperidin for 90 days did not show any signs of toxicity, which indicate that 400 mg/kg/day is safe in mice. The DNA damage study by micronucleus assay revealed a significant alteration in the frequency of micronuclei in the splenocytes after chronic administration of hesperidin except 100 mg/kg, where this increase was within control range. The sperm dysfunction test showed no distinctive alteration in the motility and viability of sperms after chronic administration of hesperidin. Similarly, the analysis of blood RBC showed an increase in their numbers whereas WBC remained unaltered. The biochemical profiling showed that chronic administration of various doses of hesperidin did not alter the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and uric acid in comparison to baseline levels. From the present study it is clear that hesperidin is safe up to 3 g/kg body weight and does not have any undesirable side effects after acute and chronic administrations.

1. INTRODUCTION

The use of herbal and natural products in healthcare is as old as the advent of human history. Traditional practices of folk medicines in different cultures have a long history of ancestors creating primitive medicines during their struggle against natural calamity and disease. The beginning of herbal medicine dates back to early humans, who found that some food/food ingredients had specific properties of relieving or eliminating certain diseases, and maintaining good health. Since then herbal medicine has been playing an important role in the human healthcare. The fact that oldest system of medicine, the Ayurveda uses medicines based on herbs and natural products reinforces their value in human healthcare as these formulations have been usually found non-toxic or with negligible toxic side effects. Despite the long history of their use, the natural products did not draw the attention they deserve by the practitioners of modern medicine for healthcare, which give too much emphasis on the isolated chemicals. The interest in herbs and natural products got rekindled to the sheer fact that 75% of the modern drugs are either directly or indirectly derived from natural products (Cragg and Newman, 2013). This indicates the importance and usefulness of herbal medicine in human healthcare. Many scientists and medical professionals thought it sensible to establish their usefulness in human healthcare by scientifically

evaluating the various properties of natural products or sources of treatment in the experimental systems. Since the scientific evaluation of herbal and natural products could provide new insight for development of novel therapies for human healthcare.

Recent years have witnessed a renewed interest in plants as pharmaceuticals worldwide. This interest is channeled into the discovery of new biologically-active molecules by the pharmaceutical industry and into the adoption of plants source for self-medication by the general public. In both of these areas some attention is being paid to the investigation and use of ethnomedicine, the traditional use of plants for medicinal purposes by particular cultural groups (Cordell, 2011). Considerable benefits for developing countries are possible when the local medicinal plants are subjected to scientific methods of validation of traditional use and quality control. This approach has met with success in some parts of the world but is not always appreciated by national governments and international agencies. Related areas of concern such as conservation of ecology and culture must be integrated with any such program.

The currently observed rapid increase in consumption of herbal remedies worldwide has been stimulated by several factors, including the notion that all herbal products are safe and effective (Farnsworth *et. al.,* 1985; Said O *et. al.,* 2002). However, over the past decade, several news-catching episodes in developed communities indicated adverse effects, sometimes life-threatening, allegedly arising consequential to taking herbal products or traditional medicines from various ethnic groups (Elvin-Lewis, 2001; Chan, 2003). In some cases, adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions or uses has led to adverse reactions that are sometimes life-threatening or lethal to patients (Ernst, 1998; Ernst, 1999; Abu-Irmaileh and Afifi, 2003). Most reports of toxic effects due to the use of herbal medicines and dietary supplements are associated with hepatotoxicity although reports of other toxic effects including kidney, nervous system, blood, cardiovascular and dermatologic effects, mutagenicity, and carcinogenicity have also been published in medical literature. On the basis of various case reports, the toxicity from herbal remedies has ranged from mild elevations of liver enzymes to fulminated liver failure requiring liver transplantation. The reported toxicity of herbal formulations may be the result of several factors, including contamination with pesticides, microbes, heavy metals, toxins or adulteration with orthodox drugs (El Nahhal, 2004). Therefore, for safety and quality assurances, chemical analytical techniques should be applied at different stages for good practices in quality

assurances of natural or herbal products, including good agricultural practice by farmers, good sourcing, and laboratory practices by pharmaceutical companies, good manufacturing practices and innovative clinical trial by researchers and physicians (Chan, 2003). It also indicates that safety evaluation is of paramount importance to avoid any toxic effect of any pharmacological agents including dietary ingredients.

Hesperidin is a bioflavanoid present in citurs fruits and several other plants and it is consumed in the form of citrus fuit huices by the humans (Lia and Schluesener, 2015). It has been reported to reduce the generation of ROS and caspase-dependent apoptosis in human polymorphonuclear neutrophils in vitro (Ross and Kasum, 2002). It is found to have an inhibitory effect on lipopolysaccharide (LPS)-induced over expression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), over-production of prostaglandin E2 and nitric oxide (NO) (Sakata *et al*., 2003). Hesperidin has been found to reduce the severity of bleomycin induced lung injury (Görmeli, 2016) and it has also shown to possess renoprotective effect against gentamicin-induced nephrotoxicity (Jain and Somani, 2015). It prevents the emergence of GC-induced, epidermal functional abnormalities (Man *et al*., 2014). Diosmin is a compound converted from hesperidin has been shown to improve factors associated with diabetic complications. The intervention with a diosmin-containing flavonoid mixture showed a decrease in hemoglobin A1c accompanied by an increase in glutathione peroxidase, demonstrating long-term decreased blood glucose levels and increased antioxidant activity (Manuel, 1999). Diosmin can normalize capillary filtration rate and prevent ischemia in diabetics. It can facilitate hemorheological improvements due to decreased RBC aggregation, which decreases blood flow resistance, resulting in reduction of both stasis and ischemia (Lacombe *et al.,* 1988, 1989). Hesperidin is used in cosmetic industry and also medication to stop vessels capillary bleeding (Cazarolli *et al.,* 2008). Consumption of hesperidin by humans may be high due to eating of citrus fruits and drinking of citrus juices. Therefore, it was desired to investigate the acute and chronic toxic effect of hesperidin in Swiss albino mice.

2. MATERIALS AND METHODS

2.1. Chemicals

Hesperidin (HPLC grade >98%) was purchased from HiMedia Ltd. Mumbai, India. Phosphate buffered saline (PBS) sodium carboxy methyl cellulose (CMC), NaOH (sodium hydroxide), NaCl (Sodium chloride), boric acid, ammonium oxalate, glacial

acetic acid, methanol, absolute alcohol and DMSO (dimethylsulphoxide) were procured from Ranbaxy (Mumbai - India), agarose (Cat No. A-4718), Trizma base, Na_2EDTA, concanavalin-A, Fetal calf serum (FCS), cytochalasin-B, RPMI-1640 medium, was supplied by Sigma-Aldrich Co. Ltd. (Bangalore, India). Acridine orange (Cat. No. 34001 9704640E) and Eosin Y, Ethidium bromide were requisitioned from BDH, England.

2.2. Animal care and handling

The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, six to eight week old male and female Swiss albino mice weighing 22 to 25 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and 12 h of light and dark cycle, respectively. The animals had free access to the sterile food and water, five animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was undertaken after an approval by the Institutional Animal Ethics Committee of Mizoram University, Aizawl vide letter no. IAEC/4503.

2.3. Preparation of drug and mode of administration

The hesperidin was dissolved freshly in sterile physiological saline (SPS) containing 0.5% carboxymethyl cellulose (CMC) as required. The animals were orally administered with hesperidin according to the experimental protocol. Henceforth the hesperidin will be called as HPD.

2.4. Acute toxicity

The acute toxicity of hesperidin was determined according to Prieur *et. al.*, (1973) and Ghosh (1984) adhering strictly to OECD guidelines (OECD, 2001). Briefly, the animals were allowed to fast by withdrawing the food and water for 18 h. The fasted animals were divided into six groups of 10 each including control group. The animals were orally administered with a single dose of 0, 0.5, 1.0, 2, 2.5 or 3 g/kg body weight hesperidin. The animals were continuously monitored for first 24 h and thereafter twice daily up to 14 days post-hesperidin treatment for signs of toxicity and mortality (Shah *et. al.*, 1989).

2.5. Chronic toxicity

The chronic toxicity of hesperidin was evaluated by randomizing the equal number of male and female mice in each group. A minimum of ten animals were used in each

group and the animals were orally administered with a single dose of 100, 200, 300 or 400 mg/kg body weight of hesperidin once daily consecutively for 90 days (WHO Scientific Group, 1967), whereas the control group received 0.5 % CMC in SPS in a similar fashion. The animals were monitored for all external general signs and symptoms of toxicity, body weight changes, and mortality until the end of 90^{th} day. The body weights of the animals were recorded before and after hesperidin treatment once in a week until the termination of the study.

2.6. Hematological assessment

The blood was collected from each mouse trough eye orbit under anesthesia. The WBC, RBC and haematocrit were counted using a hemocytometer.

2.7. Spermatogenic dysfunction

The spermatogenic dysfunction in chronically treated males was determined using the sperm abnormality test, which is considered to be a reliable parameter for assessing germ cell mutagenicity and carcinogenicity (Wyrobek *et. al.,* 1983). The cauda epididymides and vas deferens from the each animal were dissected out and transferred to individual centrifuge tubes containing 3 ml Krebs - Ringer's bicarbonate buffer (Qureshi *et al*., 1990). The sperm suspension was filtered and subsequently stained in 0.5 ml of 1% eosin-Y in a test tube. The contents were thoroughly mixed and one drop of the suspension was placed on to a slide before spreading. The slides were screened and observed for sperm abnormalities under microscope (Leica DM2500, Wetzlar, Germany).

2.8. Splenocytes Micronuclei Assay

The chronically treated animals were euthanized by cervical dislocation. The animals were thoroughly wiped with 70% alcohol and their abdominal cavities were opened with the sterile scissors and forceps under aseptic conditions. The spleens of the animals were removed aseptically and washed twice in the sterile phosphate buffered saline (PBS). The splenocytes were extracted as described earlier (Jagetia *et. al.,* 2001) and cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and concanavalin-A as the mitogen. The cultures were incubated at 37^{o}C for 72 h. Duplicate cultures were set from each animal for each drug dose.

Micronuclei were prepared according to the method of Fenech and Morley (1985) with minor modifications. Briefly, 40 h after the splenocyte culture, 5 µg/ml of cytochalasin-B was added to each culture and allowed to grow for next 32 h. The splenocytes were harvested after 72 h of initiation of the cultures, subjected to mild

hypotonic treatment (0.7% potassium chloride) so as to retain the cytoplasm and fixed in Carnoy's fixative (3:1 methanol: acetic acid). The cells were centrifuged again and resuspended in a small volume of fixative and dropped on to precleaned coded slides to avoid observer's bias.

Cells were stained with acridine orange (2.5 mg/ ml) in Sorensen's buffer (pH 6.8). The slides were washed twice in Sorensen's buffer, mounted and observed under a fluorescent microscope, equipped with 450-490 nm BP filter set with excitation at 453 nm (Leica DM2500, Wetzlar, Germany), using a 40X N Plan objective lens. A minimum of one thousand binucleate splenocytes (BNC) with well-preserved cytoplasm was scored from each culture and the frequency of micronucleated binucleate cells (MNBNC) was determined. The micronuclei identification was done as described earlier (Kirsch-Volders *et. al*., 2000; Fenech, 2003).

2.9. Biochemical studies

The blood was collected aseptically from the retro-orbital sinuses using capillary tube of each animal receiving acute or chronic hesperidin treatment on the day of termination under ketamine anesthesia. The blood was allowed to stand at 4ºC for 30 min. It was then separated by centrifugation at 1000 rpm for 5 min. The serum was separated and analyzed for aspartate amino transferase (AST/SGOT) (Reitman & Frankel, 1957), alanine amino transferase (ALT/SGPT) (Reitman & Frankel, 1957), creatinine (Bones *et al.,* 1945) and uric acid (Trinder P, 1969; Fossati & Prencipe, 1980) spectrophotometric method using Eppendorf Biospectrometer, Germany.

3. Statistical Analyses

The statistical analyses were performed using Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. The significance between the treatments was determined by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. A *p-value* of < 0.05 was considered to be statistically significant.

4. RESULTS

The results of acute and chronic toxicities after hesperidin treatment are summarized in the Tables 1–5 and Figures 1-5.

4.1. Acute toxicity

Single oral administration of different doses of hesperidin did not induce any visible signs of toxicity and drug related mortality up to a dose of 3 g/kg body weight and therefore it was considered as no-observed-adverse-effect level (NOAEL) dose.

The higher doses could not be assessed for acute toxicity owing to the problems faced in dissolving higher amounts of hesperidin.

4.2. Chronic toxicity

Oral administration of 100, 200 300 or 400 mg/kg b. wt. hesperidin once daily consecutively for 90 days did not elicit any visible signs of toxicity and mortality up to a dose of 400 mg/kg body weight until the end of the study and therefore this dose was considered as the safe dose.

4.3. Body weight changes

The body weights of the animals were recorded every week before and after hesperidin treatment until the end of 90 days. Both hesperidin treatment and non-drug treated control group did not showed any significant difference in body weights between these two groups (Table 1, Figure 1).

4.4. Sperm dysfunction

The spermatogenic dysfunction in chronically treated males receiving various doses of hesperidin for 90 days was determined using the sperm abnormality test. The analysis of data revealed that hesperidin treatment up to 400 mg/kg b. wt. did not alter the sperm viability and motility indicating that it does not have any adverse toxic effect. Hesperidin also did to cause any significant change in the sperm morphology when compared with non-drug treated control group (Table 2).

4.5. Blood Analysis

Chronic administration of mice with various doses of hesperidin revealed an insignificant rise in the number of RBCs *($p < 0.05$),* whereas the WBC counts remained unchanged. Chronic hesperidin treatment also did not induce hemolysis (Table 3).

4.6. Splenocytes micronuclei assessment

The chronic administration of different doses of hesperidin resulted in a dose dependent increase in the frequency of micronucleated binucleate cells (MNBNC) however; this rise in MNBNC was statistically non-significant, except 300 and 400 mg/kg body weight of hesperidin, where a maximum rise in MNBNCs was observed however, 300 mg/ kg b. wt. hesperidin showed a marginally higher number of MNBNC than 400 mg/ kg body weight, however the difference between 300 and 400 mg was statistically non-significant (Table 4).

4.7. Biochemical estimations

The oral administration of hesperidin for consecutive 90 days did not significantly alter aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and uric acid (Table 5, Figures 2-5).

DISCUSSION

The application of plant based products as a source of alternative medicine and therapies has increased in many developed countries (Eisenberg *et. al.,* 1998; Ernst 2003). The world health organization reported that more than 75- 80% of the world population use herbal medicines and natural products for their healthcare especially developing nations (Drew, 1997; WHO, 2003). In spite of their well-known use, scientific evaluation of most of these medicines is deficient and plant as medicines though effective are unable to establish their utility as medicine in the modern context (Roberts & Becker, 1993; Brevoort, 1998). In several cases of chronic diseases, where modern medicinal system has either failed or does not provide hope of cure, the traditional herbal medicines have been highly successful (Rivera *et al.,* 2007). This reposes faith in the traditional herbal medicine and more and more people are inclined to use them. The major constrain about the herbal drugs have been their quality control and absence of scientific data on the safety and toxicity profiles (Raynor *et al*., 2011, Martins Ekor, 2013). Therefore the present study was undertaken to evaluate the toxicity profile of different doses of hesperidin, a natural bioflavanoid present in citrus juices in mice.

The systematic evaluation of acute toxicity of hesperidin has shown that it is nontoxic up to a dose of 3 g/kg b.wt. Administration of 5% methyl hesperidin has been found to be nontoxic in mice earlier (Kawabe *et al*., 1993). There are no systematic reports of hesperidin toxicity in humans however a mixture of diosmin (450 mg) and hesperidin (50 mg) known as daflon administered as two tablets per day in human for six weeks to 1 year have shown some minor adverse effect in 10% of the subject when compared 13.9% in placebo treated group (Meyer, 1994). The chronic admiration of hesperidin up to 400 mg/kg for 90 days did not induce toxic side effect in the form of DNA damage, sperms, body weight, blood profile, and biochemical parameters. It did not show heptotoxicity as the activity of AST and AST remained unaltered. Likewise, no nephrotoxicity was observed as creatinine and uric acid levels were within normal range. The results of our study clearly indicate that that hesperidin

is nontoxic up to 3 g/kg in acute toxicity tests whereas 400 mg/kg b. wt. administration for 90 days for chronic toxicity assay. Another flavonoid naringin has been found to be nontoxic when rats were administered at a dose of 1250 mg/kg/day for 13 weeks and six months as the animals did not show any adverse effect on food consumption, opthalmoscopic examination, hematology, clinical biochemistry, serum sex hormone, macroscopic findings, organ weights and histopathological examination similar to the present study. The acute toxicity study showed tolerance of 16 g/kg b.wt. naringin without any side effects (Li *et al.,* 2013; 2014).

The present study demonstrates that hesperidin is very safe up to 3 g/kg b. wt. and 40 mg/kg b. wt. in acute and chronic toxicity studies when administered orally as it did not shown any toxicity on blood cells, liver and kidney function tests. It also did not alter sperm motility and morphology and also did not increase sperm mortality. The DNA damage-induced by chronic administration was also within normal range.

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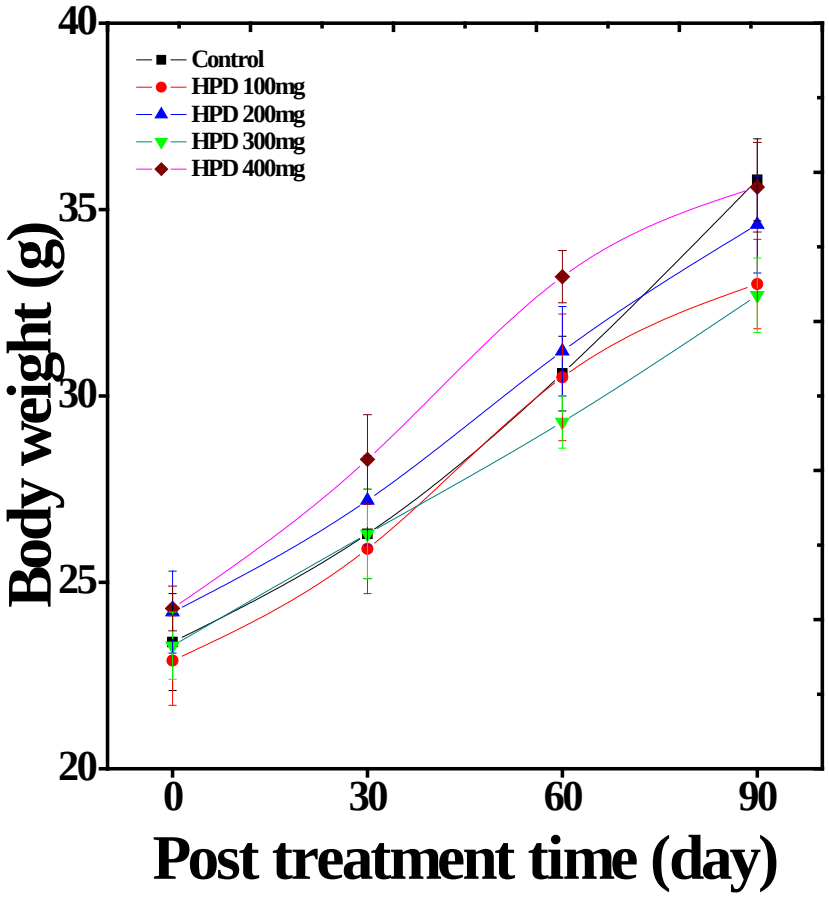
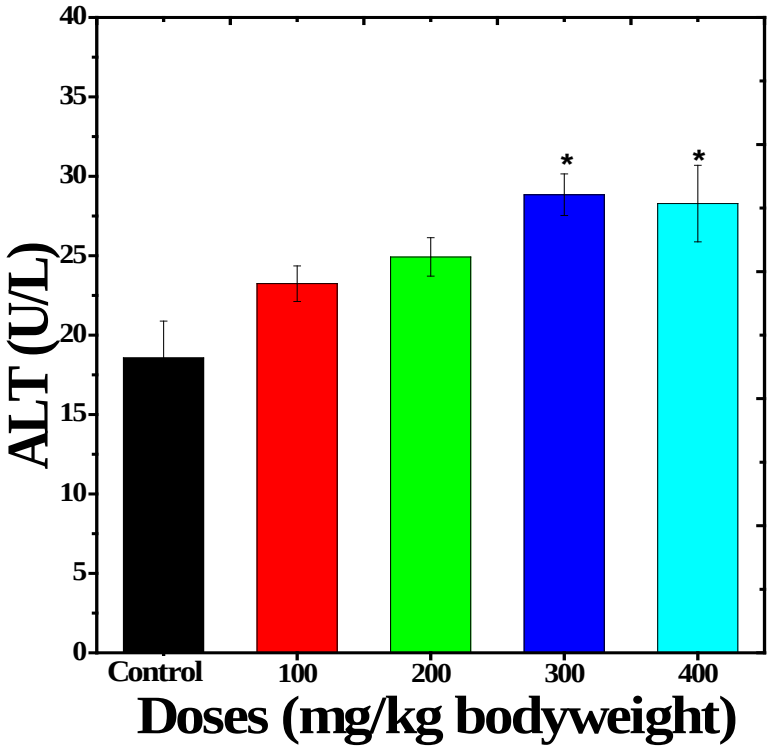
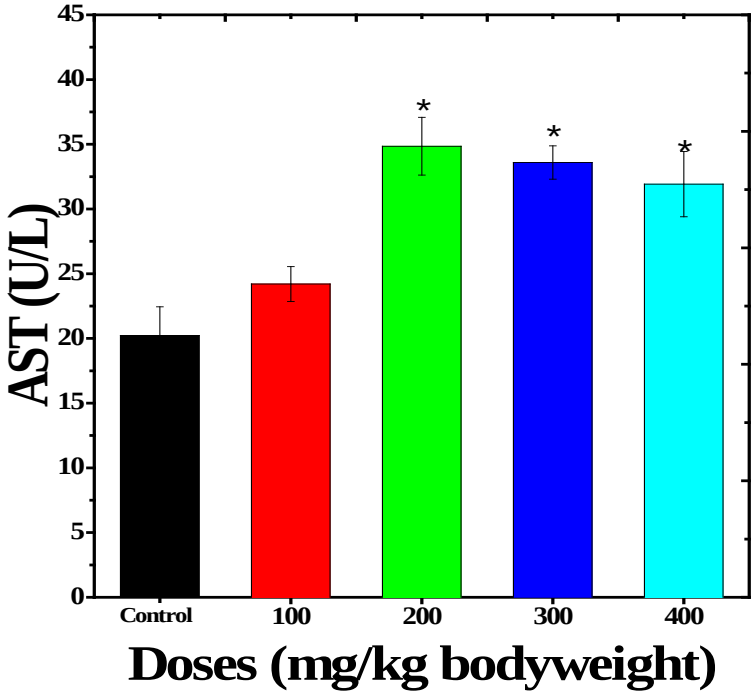


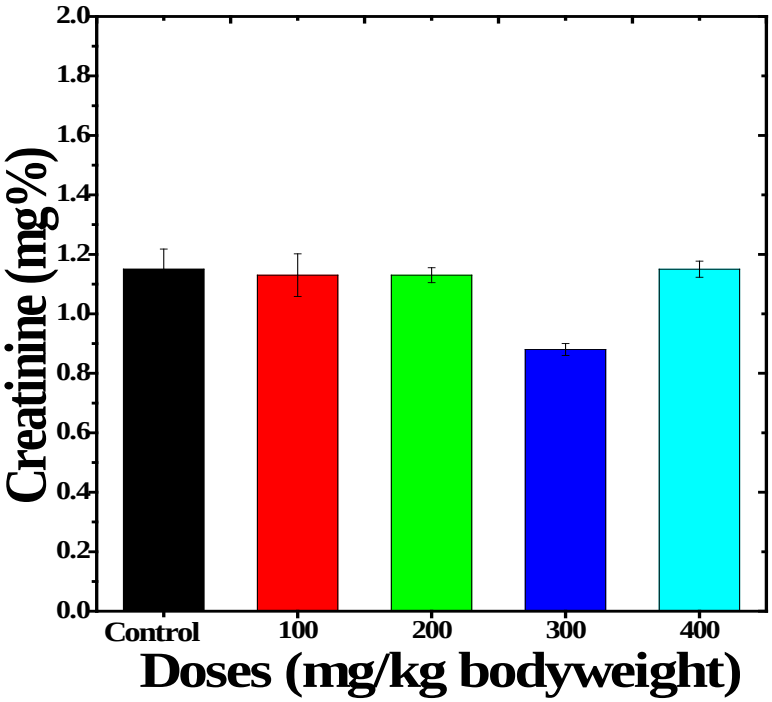
Figure 1: Body weights changes in mice orally administered chronically with different doses of hesperidin. Closed squares (black): Control; Closed circles (red): 100 mg/kg. body weight, Up triangles (blue): 200 mg/kg. body weight, Down triangles (green): 300 mg/kg. body weight and Diamond (wine red): 400 mg/kg. body weight. *The data expressed as Mean ± SEM, n=10, p<0.05.*



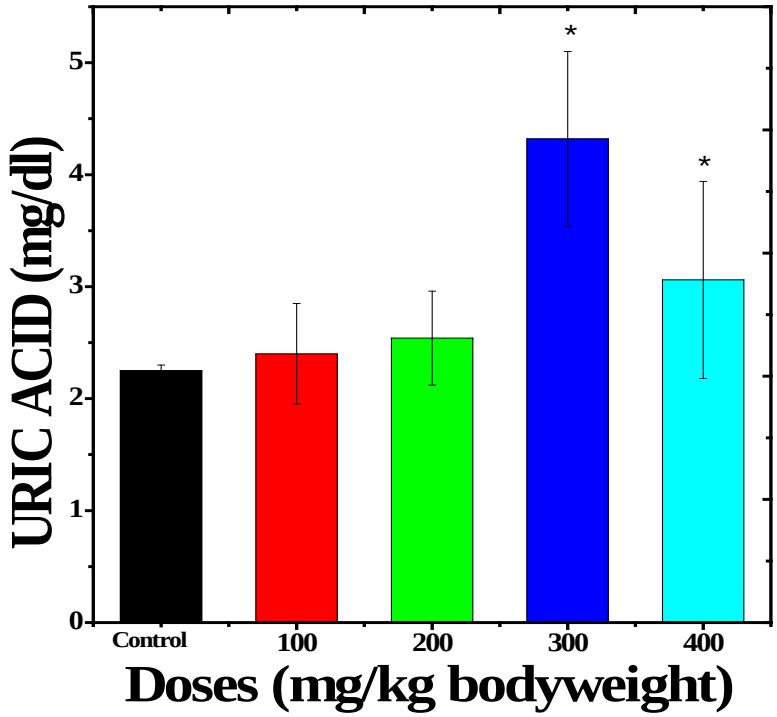
Figures 2: Effect of chronic administration of different doses of hesperidin on alanine transaminase (ALT) in mice. ***The data are expressed as Mean ± SEM, n=10.* p<0.01 when treatment are compared with control. No symbol = no significance.***



Figures 3: Effect of chronic administration of different doses of hesperidin on aspartate transaminase (AST) in mice. ***The data are expressed as Mean ± SEM, n=10.* p<0.01 when treatment are compared with control. No symbol = no significance.***



Figures 4: Alteration in the serum creatinine contents in mice chronically administered with different doses of hesperidin. ***The data are expressed as Mean ± SEM, n=10.* p<0.01 when treatment are compared with control. No symbol = no significance.***



Figures 5: Alteration in the serum uric acid contents in mice chronically administered with different doses of hesperidin. ***The data are expressed as Mean ± SEM, n=10. * p<0.01 when treatment are compared with control. No symbol = no significance.***

CHAPTER 3

Determination of anti-inflammatory and analgesic activities of a citrus bioflavanoid, hesperidin in mice.

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Chapter 3

ABSTRACT

Inflammation is one of the important responses elicited by organisms to counteract obnoxious stimuli. However, continuous inflammation has been responsible for the induction of several diseases. Therefore, it is essential to combat excess inflammation by devising countermeasures to neutralize excess inflammation. The present study was undertaken to investigate the analgesic and anti-inflammatory activities of hesperidin, a citrus flavonoid in mice using standard procedures employed for these activities including hotplate, acetic acid, tail immersion, xylene and formalin-induced edema tests. Treatment of mice with different doses of hesperidin revealed that hesperidin induced analgesic and anti-inflammatory activities in a dose dependent manner as indicated by pain inhibition and reduced inflammation. The maximum effect was observed for 300 mg/kg b. wt. hesperidin. Our study demonstrates that hesperidin has analgesic as well as anti-inflammatory action.

1. INTRODUCTION

Inflammation is a complex biological response of body tissues, which is elicited against the harmful stimuli, pathogenic attacks, and irritants. The inflammation is characterized by redness, warmth, swelling and pain (Sherwood and Toliver-Kinsky, 2004; Ferrero-Miliani *et al*., 2007). The sustained inflammation causes rheumatoid arthritis, atherosclerosis, hay fever, ischemic heart diseases, and other disorders (Black and Garbutt, 2002; Stevens *et al*., 2005; Libby, 2008). Inflammation is a common manifestation of infectious diseases including leprosy, tuberculosis, syphilis, asthma, inflammatory bowel disease, nephritis, vascularitis, celiac diseases, and numerous auto-immune diseases (Robbins and Cotran, 1979). Inflammation is known for a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Lichtman, 2009). The non-steroidal anti-inflammatory drugs (NSAIDs) employed in the treatment of inflammation are one of the most widely used drugs throughout the globe. However, the use of NSAIDs as anti-inflammatory agents is limited due to induction of undesirable side effects on gastric mucosa, kidney, bronchus and cardiovascular system (Burke *et al.*, 2006; Wallace and Vong, 2008). The NSAIDs are mainly used to alleviate the inflammation related swelling and pain and their persistent use is accompanied by the risk of gastrointestinal, cardiovascular and other toxicities.

Hesperidin is a naturally occurring flavonoid present in citrus fruits and was first discovered by Lebreton in 1827 (Flückiger and Hanbury, 1986). Hesperidin occurs in all parts of plants including fruit, vegetables, nuts, seeds, leaves, flowers and bark. It is an abundant and inexpensive byproduct of citrus family (Middleton, 1984; Barthe *et al*., 1988)

Hesperidin is also present in plants belonging to family Fabaceae apart from the plants belonging to citrus family including Betulaceae and Lamiaceae (Bhalla and Dakwake, 1978; Pawlowska,1980; Kokkalou and Kapetanidis, 1988). The citrus peel flavonoids were found effective in preventing capillary bleeding associated with scurvy as early as 1938 (Szent-Győrgi, 1938), since then hesperidin has undergone several investigations. Hesperidin has been found to protect against inflammation, oxidative stress, hypotension, nitric oxide synthase inhibition, apoptosis and infection (Galati *et al.,* 1994; Kawaguchi *et al*., 1999; Boisseau, 2002; Olszanecki *et al*., 2002; Chen *et al*., 2010). Hesperidin has been reported to protect against neurotoxicity by normalizing oxidative stress and inflammation (Cho, 2006; Tamilselvam *et al*., 2013). It also acts as an anti-hypercholesterolemic and anticarcinogenic agent (Tanaka *et al*., 1997a; Kawaguchi *et al*., 1997; Son *et al*., 1991; Lee *et al*., 1999). Hesperidin has been found to be useful in inflammatory bowel disease and it has been reported to be antiarthritic, antiatherogenic and also to protect against platelet and erythrocyte aggregation (Kim and Chung, 1990; Loguercio *et al*., 1996; Robbins, 1967; Zaragoza et al., 1986; Zaragoza *et al*., 1985). Subchronic admistration of Hesperidin for 13 weeks has been reported to be non-toxic up to 5% in mice receiving it (Kawabe *et al*., 1993). The humans can orally tolerate as high as less than 150 g of hesperidin. However, reports regarding the anti-inflammatory activity of hesperidin in animal model are scanty**.** Therefore, the present study was carried out to obtain an insight into the anti-inflammatory and analgesic efficacy of the hesperidin in mice treated with different doses of hesperidin.

2. MATERIALS AND METHODS

2.1. Chemicals

Hesperidin PG 95% was purchased from Himedia Laboratories, Mumbai, India, whereas diclofenac sodium was procured from NEON Laboratory Ltd, Mumbai, India**.** The acetic

acid, formaldehyde, Xylene and other routine chemicals were supplied by Merck India Ltd., Mumbai, India.

2.2. Animal care and handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were culled from an inbred colony maintained under the controlled conditions of temperature (25 ± 2ºC) and humidity (55–60%) and 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing wood powder (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH, USA and Indian National Science Academy, New Delhi, India guidelines, after getting the approval of the Institutional Ethics Committee of the Mizoram University, Aizawl, Mizoram, India.

2.3. Preparation of drug and mode of administration.

Hesperidin was weighed and dissolved in distilled water, henceforth it will be called as HPD. HPD was administered orally using an oral gavage (Popper and Sons, New Hyde Park**,** USA).

2.4. Experimental

The anti-inflammatory and analgesic activities were determined by dividing the animals into the following groups:

2.4.1. Saline group: The animals of this group did not receive any treatment except sterile physiological saline (SPS).

***2.4.2. Diclofenac group*:** The animals of this group were injected with 20 mg/kg b. wt. of diclofenac sodium (DIF) intraperitoneally.

***2.4.3. Hesperidin group*:** The animals of this group were administered with 100, 200, 300 and 400 mg/kg b. wt. hesperidin.

The analgesic and anti-inflammatory activities were determined 30 minutes after the administration of SPS or diclofenac (DIF) or hesperidin *(*HPD) as the case may be.

2.5. Analgesic activity

The analgesic activity of hesperidin was determined by carrying out the following tests:

2.5.1. Hot-plate test

The hot plate test was carried out as described earlier (Asongalem *et al.*, 2004) where the grouping and other conditions were essential similar to that described above. The hotplate contained metallic surface (diameter 20 and 10 cm high) and its temperature was set at 55ºC. Briefly, each mouse was placed onto the hotplate and covered with a glass beaker to avoid heat loss. Each mouse also acted as its own control. The time taken to lick the fore paws or jump was recorded. The latency is defined as the reaction time taken by each mouse to respond to licking of the fore paws or jumping. Untreated animals exhibited a latency of 5–20 seconds. Thirty minutes after administration, the latency period/reaction time for all groups was recorded. Usually10 mice were used for each group.

The pain inhibition (%) was calculated as follows:

$$\frac{\text{Post-treatment latency (s) - Pre-treatment latency (s)}}{\text{Pre-treatment latency (s)}} \times 100.$$

2.5.2. Acetic acid induced writhing test

A separate experiment was conducted to evaluate the analgesic activity by the acetic acid-induced writhing test as described earlier (Hosseinzadeh and Younesi, 2002). The grouping and other conditions were essentially similar to that described in the experimental section. The mice of all groups were administered intraperitoneally with 0.7% v/v acetic acid (volume of acetic acid did not exceed 10 µl/g b. wt.). Immediately after acetic acid administration, the mice were individually placed into glass beakers and the number of writhes induced in these animals was counted up to 30 min after five minutes of acetic acid administration. The stretching of the abdomen with simultaneous stretching of at least one hind limb was scored as a writhe. Usually10 mice were used for each group.

Inhibition of writhing (%) was calculated as:

$$\frac{\text{Control - Treated}}{\text{Control}} \times 100.$$

2.5.3. Tail-immersion test

A separate experiment was performed to evaluate the analgesic activity of HPD by the tail immersion test. The grouping and other conditions were similar to that described above in experimental section. The tail-immersion test was carried out in a hot water bath set at a temperature of 55 ± 0.5ºC, where 3 cm of animal tail was immersed into the hot water and tail withdrawal reaction was recorded as time in seconds in all groups using a digital stopwatch at 0, 0.5, 1, 2, 3, 4 and 6 hours after administration of HPD or DIF. A minimum of three observations were made for each animal in control group, immediately and 10 min after the initial reading. Usually10 mice were used for each group.

2.6. Anti-inflammatory activity

The anti-inflammatory activity of hesperidin was investigated by undertaking the following tests:

2.6.1. Xylene-induced ear edema

A separate experiment was conducted to evaluate the anti-inflammatory activity of hesperidin by xylene-induced ear edema, where the grouping and other conditions were essentially similar to that described above in the experimental section. Mice were divided into three groups of 10 each. Thirty minutes after administration of SPS, HPD or DIF, the ear edema was produced by applying 0.03 ml of xylene on the inner surface of the right ear, whereas untreated left ear served as control. Fifteen minutes after the application of xylene, the mice were killed under ketamine anesthesia. Circular sections of both the ears were taken, using a cork borer with a diameter of 6 mm and weighed.

$$\text{Inhibition (\%)} = \frac{\text{Difference in ear weight (control) - Difference in ear weight (test)}}{\text{Difference in ear weight (control)}} \times 100.$$

2.6.2. Formalin induced inflammation

The anti-inflammatory activity of hesperidin was investigated by formalin induced inflammation in a separate experiment, where the grouping and other conditions were similar to that described above in experimental section. The assessment of anti-inflammatory activity using the formalin induced inflammation was carried out as described earlier (Saxena *et al.*, 1984). The inflammation was produced by subaponeurotic administration of 0.1 ml 2% formaldehyde in the right hind paw of the

mice on the first and third day. The animals were intraperitoneally administered daily with the HPD or DIF for 10 days. Alteration in paw size was estimated daily by wrapping a piece of cotton thread around the paw and measuring the circumference with a meter scale. Ten mice were utilized for each group.

3. Statistical Analysis

The statistical significance between the treatments were determined using students't' test and one way ANOVA with the application of Tukey's test for multiple comparison for different parameters between the groups, using Origin Pro 8 (Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. statistical software was used for all analyses. A P value of $\leq$ 0.05 was considered as statistically significant.

4. RESULTS

The results of analgesic and anti-inflammatory activities of hesperidin have been expressed as mean ± SEM (Table 1-5 and Figures 1-4).

4.1. Analgesic Activity

4.1.1. Hot-plate test

The results of analgesic activity, which was assessed using the hot plate method, are presented in table 1. The mice treated with different doses of hesperidin showed a significant analgesic activity at all doses (Figure 1). However, the maximum analgesic effect was observed at a dose of 300 mg/kg b. wt. hesperidin, which showed a maximum pain inhibition of 62.22% as compared to other doses i.e. 100 (25.54%), 200 (34.2%), and 400 (61.85%) mg/kg b. wt. The diclofenac (positive control) treated animals exhibited a pain inhibition of 76.31% at a dose of 20 mg/kg b. wt. (Figure 1).

4.1.2. Acetic acid induce writhing test

The analgesic effect of hesperidin was further studied by acetic acid induced writhing test and the data are shown in (Figure 2). The administration of acetic acid in control mice produced 66.2±1.16 writhes, whereas pretreatment of mice with 100, 200, 300 and 400 mg/kg b. wt. of hesperidin reduced the number of writhes in a dose dependent manner *i.e.* 50.6±1.45 (23.56%), 45.3±1.87 (31.62%), 24.52±0.16 (62.86 %), and 26±1.38 (60.67%) for 100, 200, 300 and 400 mg/kg HPD, respectively (Figure 2) when compared with the saline

treated controls. The standard anti-inflammatory drug, 20 mg/kg b. wt. diclofenac reduced the number of writhes to 10.8±0.74 (83.68 %) (Table 2).

4.1.3. Tail immersion test

The analgesic activity was also estimated by tail immersion test and the results are depicted in table 3. This test revealed that hesperidin as well as the positive controls exhibited a significant analgesic activity as compared to the negative saline control. However, diclofenac treatment was superior to the hesperidin treatment. The 300 mg/ kg. b. wt. hesperidin post treatment and diclofenac showed 56.98% and 72.89 % inhibition, respectively.

4.2. Anti-Inflammatory Activity

4.2.1. Xylene induced ear edema.

The saline treated control mice showed 13.98±0.60 mg increase in ear weight when compared to untreated ear indicating that xylene induced inflammatory changes (Figure 3). Treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. HPD inhibited the induction of ear edema by 26.03% (10.34±1.05 mg), 34.54 % (9.15±1.09 mg), 47.21 % (7.38±1.2 mg) and 43.63 % (7.88±0.63 mg), respectively, whereas the positive control diclofenac treatment at a dose of 20 mg/kg b. wt. inhibited the development of edema by 52.7182 % (6.61±0.49 mg) which was greater when compared to all the doses of the hesperidin tested (Table 4).

4.2.2. Formalin induced inflammation

The anti-inflammatory activity of hesperidin was further confirmed by formalin induced inflammation in the mouse paw. The treatment of mice with formalin induced inflammation in the mouse paw as evidenced by increased paw diameter (Figure 4). However, treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. hesperidin significantly reduced the paw diameters (Table 5). Diclofenac treatment also reduced the formalin-induced paw diameter significantly (Table 5).

DISCUSSION

Inflammation is a natural defense process, which is evoked by a cascade of events in response to disturbances caused by agents that are unwelcome by the body and its principal role is to neutralize the cause of disturbance, remove damaged cells/tissues and

restore normal state (Medzhitov, 2008; Soehnlein and Lindbon, 2010). Despite this fact, persistence of inflammation is a great cause of concern as it leads into the development of numerous human diseases including asthma, cardiovascular diseases, allergy, type 2 diabetes, autoimmunity, atherosclerosis, Alzheimer disease, obesity, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematous, cancer, certain psychiatric disorders and many more (Dos *et al*., 2012; Elinav *et al*., 2013; Alexander and O'Connell, 2015; Kotas and Medzhitov, 2015). Although many anti-inflammatory agents are in vogue, their constant use is not without harmful side effects implying that a continuous search is needed to screen newer and safer agents, which can reduce inflammation without any side effect or minimum side effects. Therefore, the present study was designed to evaluate the anti-inflammatory and analgesic activities of a citrus bioflavonoid, hesperidin in mice.

The classical methods of hot plate and tail immersion techniques are suitable to evaluate the analgesic activity of any substance that acts on the central nervous system (Woolfe and MacDonald, 1994). The pain is regarded as unpleasant sensory, emotional and cognitive experience elicited by nociceptors against pain-inducing physical or chemical stimuli (Dubin and Patapoutian, 2010). The management of chronic pain has been a constant problem in humans as it has a deleterious impact on the sufferers (Olesen *et al*., 2012). Pain can be alleviated by administering analgesic drugs, which will interrupt nociceptor pathways. The opioid and non-steroidal anti-inflammatory drugs have been used for the clinical management of pain in humans since a long time, however, their constant use make them ineffective and moreover, they have been reported to induce adverse effects (Kissin, 2010). The evaluation of analgesic effect of hesperidin by different methods revealed that it possessed analgesic activity at a dose of 300 and 400 mg/kg b. wt. in the analgesic animal model used to evaluate centrally acting analgesic drugs. The hot plate test allows precise determination of the analgesic activity of drugs that act on the central nervous system. The analgesic activity of hesperidin was also tested by the acetic acid-induced abdominal constriction method, where the pain is indirectly initiated via endogenous mediators like prostaglandins, which stimulates peripheral nociceptive neurons. The hesperidin inhibited both the heat and acetic acid-induced pain in the animals indicating that it possessed analgesic activity. A similar effect

has been observed for quercetin earlier (Filho *et al.*, 2008). The neuronal fibers respond equally to both narcotics and non-steroidal anti-inflammatory drugs (Collier *et al*., 1968). Inflammation is all pervasive phenomenon and its main function is to neutralize the cause, destroy the source, repair the damaged tissue and regain the homeostatic state of the tissues (Medzhitov, 2008; Soehnlein and Lindbon, 2010). It may or may not be associated with pain depending on the stimuli of inflammation. A neurogenic and inflammatory pain model, the formalin-induced paw edema test, was used to assess further the antinociceptive properties of hesperidin. Formalin administration evokes behavioral effects, which are related to the direct chemical stimulation of nociceptors. The pain induced by inflammatory phase involves a combination of stimuli, including inflammation of peripheral tissues and mechanisms of central sensitization (Shibata *et al.*, 1989; Le Bars *et al*., 2001). The drugs that act on central nervous system including opioids, inhibit both phases equally, however drugs that interact with peripheral nervous system such as NSAIDs and corticosteroids, only inhibit the second phase (Shibata *et al*., 1989). Hesperidin seems to be effective on both the central and peripheral nervous systems as it is able to desensitize neurons of both central and peripheral nervous systems equally as indicated by the attenuation of pain and inflammation. Hesperidin has been reported to exert anti-inflammatory effect in rat and *Aeromonas hydrophila* earlier (Emim *et al.*, 1994; Abuelsaad *et al*., 2014). The anti-inflammatory effect of hesperidin was further confirmed by Xylene, induced mouse ear edema, which causes serious edematous changes in the skin when applied to the ear surface (Sowemimo *et al*., 2013). The ear edema model induced by xylene has certain advantages in the evaluation of anti-inflammatory activity of steroids as well as non-steroidal anti-inflammatory agents (Kumawat *et al.*, 2012; Zhang and An, 2007). The reduction in ear edema by hesperidin indicates that it has anti-inflammatory potential.

The direct comparison of the results obtained for analgesic and anti-inflammatory activities of diclofenac in the present study with that of other studies may not be feasible due to differences in experimental protocol, dosage and animal species used. However, we have observed analgesic and anti-inflammatory activities in the present study. Similarly, diclofenac has been reported to possess analgesic and anti-inflammatory activities earlier and it has been used as a standard drug while evaluating the analgesic

and anti-inflammatory activities of new natural products/drugs (Hosseinzadeh and Younesi, 2002; Malhotra *et al*., 2013; Rambabu *et al*., 2014; Sheorey *et al*., 2013).

The exact mechanism of analgesic and anti-inflammatory action of hesperidin is not known. However, inflammation has been reported to be induced by secretion of proinflammatory cytokines like TNF-α, IL1β (Bentli *et al*., 2013). It seems that hesperidin has been able to inhibit the secretion of proinflammatory cytokines including TNF-α, IL-6 and IL1β leading to alleviation in the inflammatory response. In fact hesperidin treatment has been reported to bring the 2,3,7,8-tetrachlorodibenzo-ρ-dioxin-induced TNF-α, and IL1β levels to normal in rats (Bentli *et al*., 2013). Cyclooxygenases (COX-1 and COX-2) are involved in inflammation and hesperidin has been reported to suppress the expression of COX-II gene earlier (Hirata *et al*., 2005). The inhibition of COX-II gene by hesperidin may have blocked the production of prostaglandins leading to the suppression of inflammatory response in the present study. The NF-κB arouses proinflammatory pathway by stimulating the expression of various inflammatory cytokines, chemokines and adhesion molecules (Lawrence, 2009). Therefore, inhibition of NF-κB by hesperidin may have blocked the NF-κB-induced inflammatory pathway and subsequently invoked the analgesic and anti-inflammatory action. Hesperidin has been reported to suppress the transcription of NF-κB earlier (Ghorbani *et al.*, 2012).

CONCLUSIONS

Our study demonstrates that hesperidin has been able to exert analgesic and anti-inflammatory actions in mice by reducing pain and inflammatory changes, which may be due the inhibition of inflammatory cytokines like TNF-α, IL1β and IL-6 and also the suppression of transcription of NF-κB and COX-II genes that eventually blocked the production of prostaglandins and inflammatory pathway.

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CHAPTER 3

Determination of anti-inflammatory and analgesic activities of a citrus bioflavanoid, hesperidin in mice.

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Chapter 3

ABSTRACT

Inflammation is one of the important responses elicited by organisms to counteract obnoxious stimuli. However, continuous inflammation has been responsible for the induction of several diseases. Therefore, it is essential to combat excess inflammation by devising countermeasures to neutralize excess inflammation. The present study was undertaken to investigate the analgesic and anti-inflammatory activities of hesperidin, a citrus flavonoid in mice using standard procedures employed for these activities including hotplate, acetic acid, tail immersion, xylene and formalin-induced edema tests. Treatment of mice with different doses of hesperidin revealed that hesperidin induced analgesic and anti-inflammatory activities in a dose dependent manner as indicated by pain inhibition and reduced inflammation. The maximum effect was observed for 300 mg/kg b. wt. hesperidin. Our study demonstrates that hesperidin has analgesic as well as anti-inflammatory action.

1. INTRODUCTION

Inflammation is a complex biological response of body tissues, which is elicited against the harmful stimuli, pathogenic attacks, and irritants. The inflammation is characterized by redness, warmth, swelling and pain (Sherwood and Toliver-Kinsky, 2004; Ferrero-Miliani *et al*., 2007). The sustained inflammation causes rheumatoid arthritis, atherosclerosis, hay fever, ischemic heart diseases, and other disorders (Black and Garbutt, 2002; Stevens *et al*., 2005; Libby, 2008). Inflammation is a common manifestation of infectious diseases including leprosy, tuberculosis, syphilis, asthma, inflammatory bowel disease, nephritis, vascularitis, celiac diseases, and numerous auto-immune diseases (Robbins and Cotran, 1979). Inflammation is known for a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Lichtman, 2009). The non-steroidal anti-inflammatory drugs (NSAIDs) employed in the treatment of inflammation are one of the most widely used drugs throughout the globe. However, the use of NSAIDs as anti-inflammatory agents is limited due to induction of undesirable

side effects on gastric mucosa, kidney, bronchus and cardiovascular system (Burke *et al.*, 2006; Wallace and Vong, 2008). The NSAIDs are mainly used to alleviate the inflammation related swelling and pain and their persistent use is accompanied by the risk of gastrointestinal, cardiovascular and other toxicities.

Hesperidin is a naturally occurring flavonoid present in citrus fruits and was first discovered by Lebreton in 1827 (Flückiger and Hanbury, 1986). Hesperidin occurs in all parts of plants including fruit, vegetables, nuts, seeds, leaves, flowers and bark. It is an abundant and inexpensive byproduct of citrus family (Middleton, 1984; Barthe *et al*., 1988)

Hesperidin is also present in plants belonging to family Fabaceae apart from the plants belonging to citrus family including Betulaceae and Lamiaceae (Bhalla and Dakwake, 1978; Pawlowska,1980; Kokkalou and Kapetanidis, 1988). The citrus peel flavonoids were found effective in preventing capillary bleeding associated with scurvy as early as 1938 (Szent-Győrgi, 1938), since then hesperidin has undergone several investigations. Hesperidin has been found to protect against inflammation, oxidative stress, hypotension, nitric oxide synthase inhibition, apoptosis and infection (Galati *et al.,* 1994; Kawaguchi *et al*., 1999; Boisseau, 2002; Olszanecki *et al*., 2002; Chen *et al*., 2010). Hesperidin has been reported to protect against neurotoxicity by normalizing oxidative stress and inflammation (Cho, 2006; Tamilselvam *et al*., 2013). It also acts as an anti-hypercholesterolemic and anticarcinogenic agent (Tanaka *et al*., 1997a; Kawaguchi *et al*., 1997; Son *et al*., 1991; Lee *et al*., 1999). Hesperidin has been found to be useful in inflammatory bowel disease and it has been reported to be antiarthritic, antiatherogenic and also to protect against platelet and erythrocyte aggregation (Kim and Chung, 1990;

Loguercio *et al*., 1996; Robbins, 1967; Zaragoza et al., 1986; Zaragoza *et al*., 1985). Subchronic admistration of Hesperidin for 13 weeks has been reported to be non-toxic up to 5% in mice receiving it (Kawabe *et al*., 1993). The humans can orally tolerate as high as less than 150 g of hesperidin. However, reports regarding the anti-inflammatory activity of hesperidin in animal model are scanty**.** Therefore, the present study was carried out to obtain an insight into the anti-inflammatory and analgesic efficacy of the hesperidin in mice treated with different doses of hesperidin.

2. MATERIALS AND METHODS

2.1. Chemicals

Hesperidin PG 95% was purchased from Himedia Laboratories, Mumbai, India, whereas diclofenac sodium was procured from NEON Laboratory Ltd, Mumbai, India**.** The acetic acid, formaldehyde, Xylene and other routine chemicals were supplied by Merck India Ltd., Mumbai, India.

2.2. Animal care and handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were culled from an inbred colony maintained under the controlled conditions of temperature (25 ± 2ºC) and humidity (55–60%) and 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing wood powder (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH, USA and Indian National Science Academy, New Delhi, India

guidelines, after getting the approval of the Institutional Ethics Committee of the Mizoram University, Aizawl, Mizoram, India.

2.3. Preparation of drug and mode of administration.

Hesperidin was weighed and dissolved in distilled water, henceforth it will be called as HPD. HPD was administered orally using an oral gavage (Popper and Sons, New Hyde Park**,** USA).

2.4. Experimental

The anti-inflammatory and analgesic activities were determined by dividing the animals into the following groups:

2.4.1. Saline group: The animals of this group did not receive any treatment except sterile physiological saline (SPS).

***2.4.2. Diclofenac group*:** The animals of this group were injected with 20 mg/kg b. wt. of diclofenac sodium (DIF) intraperitoneally.

***2.4.3. Hesperidin group*:** The animals of this group were administered with 100, 200, 300 and 400 mg/kg b. wt. hesperidin.

The analgesic and anti-inflammatory activities were determined 30 minutes after the administration of SPS or diclofenac (DIF) or hesperidin *(*HPD) as the case may be.

2.5. Analgesic activity

The analgesic activity of hesperidin was determined by carrying out the following tests:

2.5.1. Hot-plate test

The hot plate test was carried out as described earlier (Asongalem *et al.,* 2004) where the grouping and other conditions were essential similar to that described above. The hotplate contained metallic surface (diameter 20 and 10 cm high) and its temperature was set at

55ºC. Briefly, each mouse was placed onto the hotplate and covered with a glass beaker to avoid heat loss. Each mouse also acted as its own control. The time taken to lick the fore paws or jump was recorded. The latency is defined as the reaction time taken by each mouse to respond to licking of the fore paws or jumping. Untreated animals exhibited a latency of 5–20 seconds. Thirty minutes after administration, the latency period/reaction time for all groups was recorded. Usually10 mice were used for each group.

The pain inhibition (%) was calculated as follows:

$$\frac{\text{Post-treatment latency (s) - Pre-treatment latency (s)}}{\text{Pre-treatment latency (s).}} \times 100.$$

2.5.2. Acetic acid induced writhing test

A separate experiment was conducted to evaluate the analgesic activity by the acetic acid-induced writhing test as described earlier (Hosseinzadeh and Younesi, 2002). The grouping and other conditions were essentially similar to that described in the experimental section. The mice of all groups were administered intraperitoneally with 0.7% v/v acetic acid (volume of acetic acid did not exceed 10 µl/g b. wt.). Immediately after acetic acid administration, the mice were individually placed into glass beakers and the number of writhes induced in these animals was counted up to 30 min after five minutes of acetic acid administration. The stretching of the abdomen with simultaneous stretching of at least one hind limb was scored as a writhe. Usually10 mice were used for each group.

Inhibition of writhing (%) was calculated as:

$$\frac{\text{Control - Treated}}{\text{Control}} \times 100.$$

2.5.3. Tail-immersion test

A separate experiment was performed to evaluate the analgesic activity of HPD by the tail immersion test. The grouping and other conditions were similar to that described above in experimental section. The tail-immersion test was carried out in a hot water bath set at a temperature of 55 ± 0.5ºC, where 3 cm of animal tail was immersed into the hot water and tail withdrawal reaction was recorded as time in seconds in all groups using a digital stopwatch at 0, 0.5, 1, 2, 3, 4 and 6 hours after administration of HPD or DIF. A minimum of three observations were made for each animal in control group, immediately and 10 min after the initial reading. Usually10 mice were used for each group.

2.6. Anti-inflammatory activity

The anti-inflammatory activity of hesperidin was investigated by undertaking the following tests:

2.6.1. Xylene-induced ear edema

A separate experiment was conducted to evaluate the anti-inflammatory activity of hesperidin by xylene-induced ear edema, where the grouping and other conditions were essentially similar to that described above in the experimental section. Mice were divided into three groups of 10 each. Thirty minutes after administration of SPS, HPD or DIF, the ear edema was produced by applying 0.03 ml of xylene on the inner surface of the right ear, whereas untreated left ear served as control. Fifteen minutes after the application of xylene, the mice were killed under ketamine anesthesia. Circular sections of both the ears were taken, using a cork borer with a diameter of 6 mm and weighed.

$$\text{Inhibition (\%)} = \frac{\text{Difference in ear weight (control) - Difference in ear weight (test)}}{\text{Difference in ear weight (control)}} \times 100.$$

2.6.2. Formalin induced inflammation

The anti-inflammatory activity of hesperidin was investigated by formalin induced inflammation in a separate experiment, where the grouping and other conditions were similar to that described above in experimental section. The assessment of anti-inflammatory activity using the formalin induced inflammation was carried out as described earlier (Saxena *et al.,* 1984). The inflammation was produced by subaponeurotic administration of 0.1 ml 2% formaldehyde in the right hind paw of the mice on the first and third day. The animals were intraperitoneally administered daily with the HPD or DIF for 10 days. Alteration in paw size was estimated daily by wrapping a piece of cotton thread around the paw and measuring the circumference with a meter scale. Ten mice were utilized for each group.

3. Statistical Analysis

The statistical significance between the treatments were determined using students‘t’ test and one way ANOVA with the application of Tukey’s test for multiple comparison for different parameters between the groups, using Origin Pro 8 (Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. statistical software was used for all analyses. A P value of $\leq$ 0.05 was considered as statistically significant.

Chapter 3

4. RESULTS

The results of analgesic and anti-inflammatory activities of hesperidin have been expressed as mean ± SEM (Table 1-5 and Figures 1-4).

4.1. Analgesic Activity

4.1.1. Hot-plate test

The results of analgesic activity, which was assessed using the hot plate method, are presented in table 1. The mice treated with different doses of hesperidin showed a significant analgesic activity at all doses (Figure 1). However, the maximum analgesic effect was observed at a dose of 300 mg/kg b. wt. hesperidin, which showed a maximum pain inhibition of 62.22% as compared to other doses i.e. 100 (25.54%), 200 (34.2%), and 400 (61.85%) mg/kg b. wt. The diclofenac (positive control) treated animals exhibited a pain inhibition of 76.31% at a dose of 20 mg/kg b. wt. (Figure 1).

4.1.2. Acetic acid induce writhing test

The analgesic effect of hesperidin was further studied by acetic acid induced writhing test and the data are shown in (Figure 2). The administration of acetic acid in control mice produced 66.2±1.16 writhes, whereas pretreatment of mice with 100, 200, 300 and 400 mg/kg b. wt. of hesperidin reduced the number of writhes in a dose dependent manner *i.e.* 50.6±1.45 (23.56%), 45.3±1.87 (31.62%), 24.52±0.16 (62.86 %), and 26±1.38 (60.67%) for 100, 200, 300 and 400 mg/kg HPD, respectively (Figure 2) when compared with the saline treated controls. The standard anti-inflammatory drug, 20 mg/kg b. wt. diclofenac reduced the number of writhes to 10.8±0.74 (83.68 %) (Table 2).

4.1.3. Tail immersion test

The analgesic activity was also estimated by tail immersion test and the results are depicted in table 3. This test revealed that hesperidin as well as the positive controls exhibited a significant analgesic activity as compared to the negative saline control. However, diclofenac treatment was superior to the hesperidin treatment. The 300 mg/ kg. b. wt. hesperidin post treatment and diclofenac showed 56.98% and 72.89 % inhibition, respectively.

4.2. Anti-Inflammatory Activity

4.2.1. Xylene induced ear edema.

The saline treated control mice showed 13.98±0.60 mg increase in ear weight when compared to untreated ear indicating that xylene induced inflammatory changes (Figure 3). Treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. HPD inhibited the induction of ear edema by 26.03% (10.34±1.05 mg), 34.54 % (9.15±1.09 mg), 47.21 % (7.38±1.2 mg) and 43.63 % (7.88±0.63 mg), respectively, whereas the positive control diclofenac treatment at a dose of 20 mg/kg b. wt. inhibited the development of edema by 52.7182 % (6.61±0.49 mg) which was greater when compared to all the doses of the hesperidin tested (Table 4).

4.2.2. Formalin induced inflammation

The anti-inflammatory activity of hesperidin was further confirmed by formalin induced inflammation in the mouse paw. The treatment of mice with formalin induced inflammation in the mouse paw as evidenced by increased paw diameter (Figure 4). However, treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. hesperidin

significantly reduced the paw diameters (Table 5). Diclofenac treatment also reduced the formalin-induced paw diameter significantly (Table 5).

DISCUSSION

Inflammation is a natural defense process, which is evoked by a cascade of events in response to disturbances caused by agents that are unwelcome by the body and its principal role is to neutralize the cause of disturbance, remove damaged cells/tissues and restore normal state (Medzhitov, 2008; Soehnlein and Lindbon, 2010). Despite this fact, persistence of inflammation is a great cause of concern as it leads into the development of numerous human diseases including asthma, cardiovascular diseases, allergy, type 2 diabetes, autoimmunity, atherosclerosis, Alzheimer disease, obesity, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematous, cancer, certain psychiatric disorders and many more (Dos *et al*., 2012; Elinav *et al*., 2013; Alexander and O'Connell, 2015; Kotas and Medzhitov, 2015). Although many anti-inflammatory agents are in vogue, their constant use is not without harmful side effects implying that a continuous search is needed to screen newer and safer agents, which can reduce inflammation without any side effect or minimum side effects. Therefore, the present study was designed to evaluate the anti-inflammatory and analgesic activities of a citrus bioflavonoid, hesperidin in mice.

The classical methods of hot plate and tail immersion techniques are suitable to evaluate the analgesic activity of any substance that acts on the central nervous system (Woolfe and MacDonald, 1994). The pain is regarded as unpleasant sensory, emotional and cognitive experience elicited by nociceptors against pain-inducing physical or chemical

stimuli (Dubin and Patapoutian, 2010). The management of chronic pain has been a constant problem in humans as it has a deleterious impact on the sufferers (Olesen *et al.*, 2012). Pain can be alleviated by administering analgesic drugs, which will interrupt nociceptor pathways. The opioid and non-steroidal anti-inflammatory drugs have been used for the clinical management of pain in humans since a long time, however, their constant use make them ineffective and moreover, they have been reported to induce adverse effects (Kissin, 2010). The evaluation of analgesic effect of hesperidin by different methods revealed that it possessed analgesic activity at a dose of 300 and 400 mg/kg b. wt. in the analgesic animal model used to evaluate centrally acting analgesic drugs. The hot plate test allows precise determination of the analgesic activity of drugs that act on the central nervous system. The analgesic activity of hesperidin was also tested by the acetic acid-induced abdominal constriction method, where the pain is indirectly initiated via endogenous mediators like prostaglandins, which stimulates peripheral nociceptive neurons. The hesperidin inhibited both the heat and acetic acid-induced pain in the animals indicating that it possessed analgesic activity. A similar effect has been observed for quercetin earlier (Filho *et al.*, 2008). The neuronal fibers respond equally to both narcotics and non-steroidal anti-inflammatory drugs (Collier *et al.*, 1968). Inflammation is all pervasive phenomenon and its main function is to neutralize the cause, destroy the source, repair the damaged tissue and regain the homeostatic state of the tissues (Medzhitov, 2008; Soehnlein and Lindbon, 2010). It may or may not be associated with pain depending on the stimuli of inflammation. A neurogenic and inflammatory pain model, the formalin-induced paw edema test, was used to assess further the antinociceptive properties of hesperidin. Formalin administration evokes

behavioral effects, which are related to the direct chemical stimulation of nociceptors. The pain induced by inflammatory phase involves a combination of stimuli, including inflammation of peripheral tissues and mechanisms of central sensitization (Shibata *et al.*, 1989; Le Bars *et al*., 2001). The drugs that act on central nervous system including opioids, inhibit both phases equally, however drugs that interact with peripheral nervous system such as NSAIDs and corticosteroids, only inhibit the second phase (Shibata *et al*., 1989). Hesperidin seems to be effective on both the central and peripheral nervous systems as it is able to desensitize neurons of both central and peripheral nervous systems equally as indicated by the attenuation of pain and inflammation. Hesperidin has been reported to exert anti-inflammatory effect in rat and *Aeromonas hydrophila* earlier (Emim *et al.*, 1994; Abuelsaad *et al*., 2014). The anti-inflammatory effect of hesperidin was further confirmed by Xylene, induced mouse ear edema, which causes serious edematous changes in the skin when applied to the ear surface (Sowemimo *et al*., 2013). The ear edema model induced by xylene has certain advantages in the evaluation of anti-inflammatory activity of steroids as well as non-steroidal anti-inflammatory agents (Kumawat *et al.*, 2012; Zhang and An, 2007). The reduction in ear edema by hesperidin indicates that it has anti-inflammatory potential.

The direct comparison of the results obtained for analgesic and anti-inflammatory activities of diclofenac in the present study with that of other studies may not be feasible due to differences in experimental protocol, dosage and animal species used. However, we have observed analgesic and anti-inflammatory activities in the present study. Similarly, diclofenac has been reported to possess analgesic and anti-inflammatory activities earlier and it has been used as a standard drug while evaluating the analgesic

and anti-inflammatory activities of new natural products/drugs (Hosseinzadeh and Younesi, 2002; Malhotra *et al*., 2013; Rambabu *et al*., 2014; Sheorey *et al*., 2013).

The exact mechanism of analgesic and anti-inflammatory action of hesperidin is not known. However, inflammation has been reported to be induced by secretion of proinflammatory cytokines like TNF-α, IL1β (Bentli *et al*., 2013). It seems that hesperidin has been able to inhibit the secretion of proinflammatory cytokines including TNF-α, IL-6 and IL1β leading to alleviation in the inflammatory response. In fact hesperidin treatment has been reported to bring the 2,3,7,8-tetrachlorodibenzo-ρ-dioxin-induced TNF-α, and IL1β levels to normal in rats (Bentli *et al*., 2013). Cyclooxygenases (COX-1 and COX-2) are involved in inflammation and hesperidin has been reported to suppress the expression of COX-II gene earlier (Hirata *et al*., 2005). The inhibition of COX-II gene by hesperidin may have blocked the production of prostaglandins leading to the suppression of inflammatory response in the present study. The NF-κB arouses proinflammatory pathway by stimulating the expression of various inflammatory cytokines, chemokines and adhesion molecules (Lawrence, 2009). Therefore, inhibition of NF-κB by hesperidin may have blocked the NF-κB-induced inflammatory pathway and subsequently invoked the analgesic and anti-inflammatory action. Hesperidin has been reported to suppress the transcription of NF-κB earlier (Ghorbani *et al.,* 2012).

CONCLUSIONS

Our study demonstrates that hesperidin has been able to exert analgesic and anti-inflammatory actions in mice by reducing pain and inflammatory changes, which may be due the inhibition of inflammatory cytokines like TNF-α, IL1β and IL-6 and also the

suppression of transcription of NF-κB and COX-II genes that eventually blocked the production of prostaglandins and inflammatory pathway.

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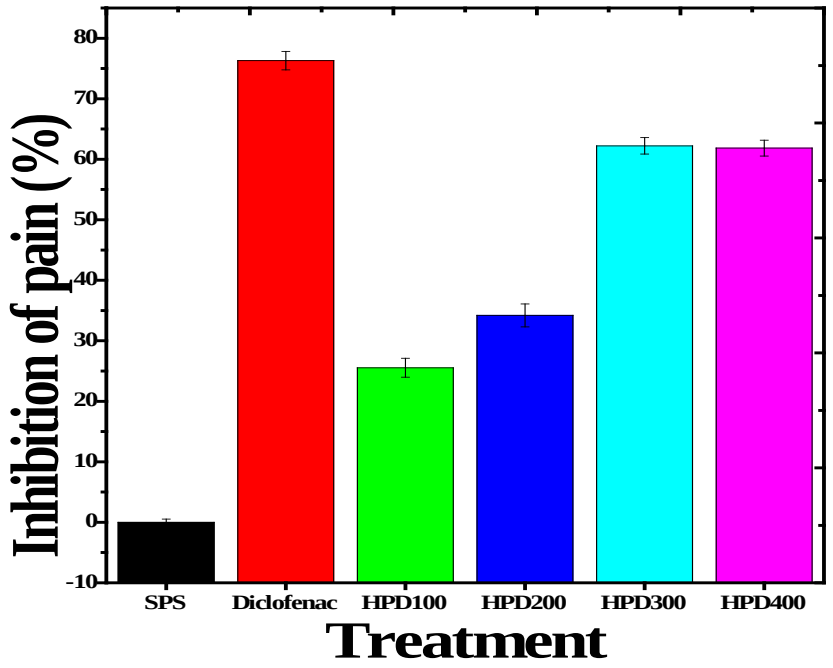

Figure 1: Effect of different doses of hesperidin on the analgesic activity in Swiss albino mice by hot plate test. The results are expressed as Mean ± SEM, n=10.
No symbol= no significance.

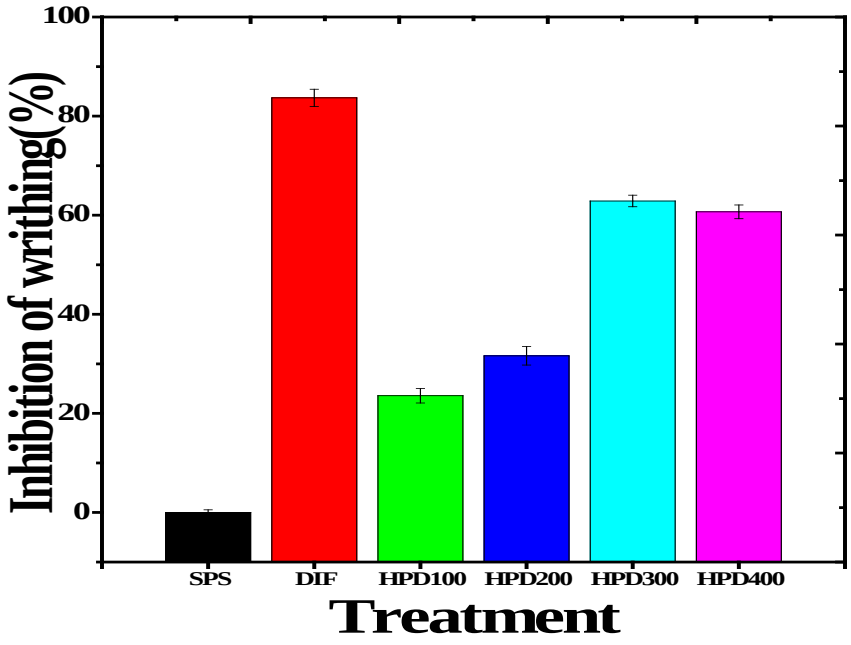

Figure 2: Alteration in the analgesic activity by acetic acid induced writhing in Swiss albino mice treated with different doses of hesperidin. The results are determined as percent and expressed as Mean ± SEM, n=10.
No symbol= no significance.

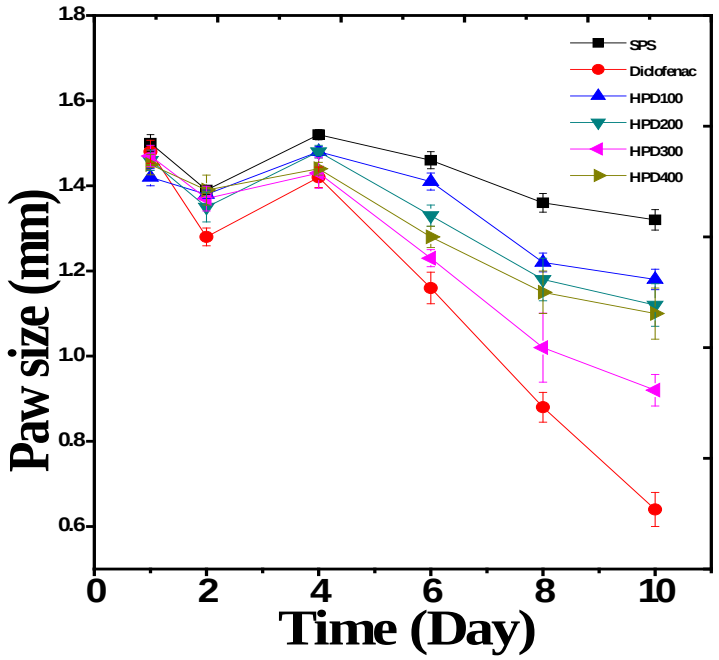

Figure 3: Effect of various doses of hesperidin on formalin induced paw edema in Swiss albino mice. The results are expressed as Mean ± SEM, n=10.
No symbol= no significance.

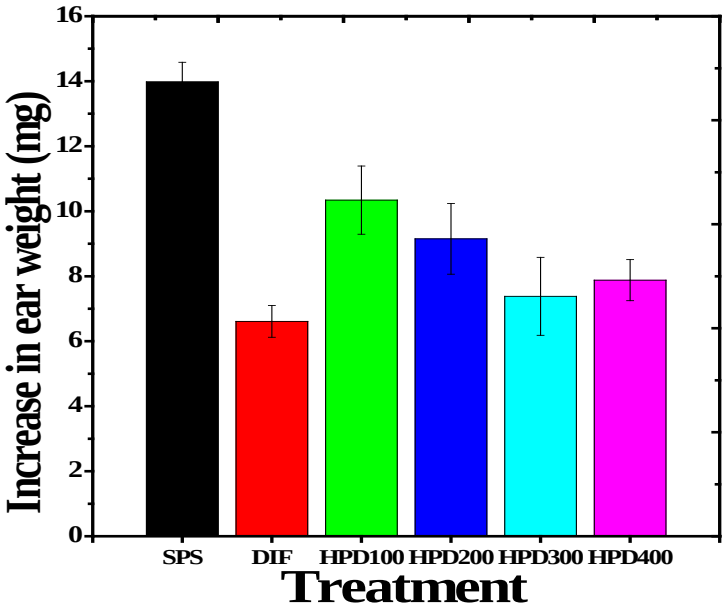

Figure 4: Effect of various doses of hesperidin on xylene induced ear edema in Swiss albino mice edema in mice. The results are expressed as Mean ± SEM, n=10.
No symbol= no significance.

Table 1: Effect of various doses hesperidin administered orally on the analgesic activity in mice by hot plate test. The results were expressed as Mean ± SEM, n=10.

Treatment	Dose (mg/kg b.wt)	Mean ±SEM		Inhibition (%)
		Pre-treatment Reaction-latency (s)	Post-treatment reaction-latency (s)	
SPS	10ml	7.60±0.58	7.60±0.45	0.000
DIF	20	7.60±0.55	13.40±0.84	76.31
HPD	100	8.00±0.32	10.05±1.57	25.54
	200	8.52±0.65	11.45±1.89	34.2
	300	6.50±0.55	14.60±1.35	62.22
	400	9.00±0.32	14.55±1.31	61.85

Table 2: Alteration in the analgesic activity by acetic acid induced writhing in mice treated with different doses of hesperidin. The results were expressed as Mean ± SEM, n=10.

Treatment	Dose (mg/kg b.wt)	No. of writhings (Mean ±SEM)	Inhibition of writhing (%)
SPS	10ml	66.2±1.16	0.000
DIF	20	10.8±0.74	83.68
HPD	100	50.6±1.45	23.56
	200	45.3±1.87	31.62
	300	24.52±0.16	62.86
	400	26±1.38	60.67

Table 3: Alteration in the response time in mice treated with hesperidin before subjecting them to tail immersion test. The results were represented as Mean ± SEM, n=10.

Treatment	Dose (mg/kg b. wt)	Response time in seconds ±SEM Assessment time (h)					
		0	**0.5**	**1**	**2**	**4**	**6**
SPS	10ml	4.84±0.19	4.86±0.22 (0.5)	4.89±0.18 (1.07)	4.96±0.18 (2.44)	4.91±0.19 (1.32)	4.82±0.20 (-0.54)
DIF	20	4.28±0.04	5.38±0.07 (25.7)	6.69±0.67 (56.3)	7.40±0.24 (72.89)	6.82±0.19 (59.34)	5.50±0.31 (28.5)
HPD	100	4.85±0.29	4.88±0.22 (0.62)	4.94±0.18 (1.85)	4.98±0.18 (2.68)	4.99±0.19 (3.92)	5.05±0.20 (3.54)
	200	5.01±0.15	5.18±0.30 (3.39)	5.40±0.33 (7.78)	5.68±0.37 (13.37)	5.75±0.29 (14.77)	5.38±0.39 (7.38)
	300	5.30±0.29	6.01±0.33 (13.39)	6.25±0.82 (17.92)	8.32±0.48 (56.98)	6.58±0.08 (24.15)	6.26±0.35 (18.11)
	400	5.32±0.29	6.00±0.33 (12.78)	6.24±0.82 (17.29)	7.25±0.48 (36.27)	6.37±0.08 (19.73)	6.19±0.35 (16.35)

Inhibition (%) is shown in brackets

Table 4: Effect of various doses of hesperidin on xylene induced ear edema in Swiss albino mice. The results were expressed as Mean ± SEM, n=10.

Treatment	Dose (mg/kg b.wt).	Increase in ear weight (mg)	Inhibition (%)
		Mean ±SEM	
SPS	10ml	13.98±0.60	0.000
DIF	20	6.61±0.49	52.71
HPD	100	10.34±1.05	26.03
	200	9.15±1.09	34.54
	300	7.38±1.2	47.21
	400	7.88±0.63	43.63

Table 5: Effect of various doses of hesperidin on xylene induced paw edema in Swiss albino mice. The results were expressed as Mean SEM, n=10.

Treatment	Dose (mg/kg b.wt)	Diameter of paw in mm (Mean ±SEM) Assessment time (day)					
		1	2	4	6	8	10
SPS	10ml	1.50±0.02	1.39±0.02	1.52±0.033	1.46±0.02	1.36±0.02	1.32±0.02
DIF	20	1.48±0.02	1.28±0.02	1.42±0.024	1.16±0.04	0.88±0.03	0.64±0.04
HPD	100	1.42±0.02	1.38±0.01	1.48±0.012	1.41±0.02	1.22±0.02	1.18±0.02
	200	1.46±0.03	1.35±0.03	1.48±0.015	1.33±0.03	1.18±0.05	1.12±0.05
	300	1.47±0.03	1.37±0.03	1.43±0.036	1.23±0.02	1.02±0.08	0.92±0.03
	400	1.45±0.03	1.39±0.04	1.44±0.015	1.28±0.03	1.15±0.05	1.10±0.06

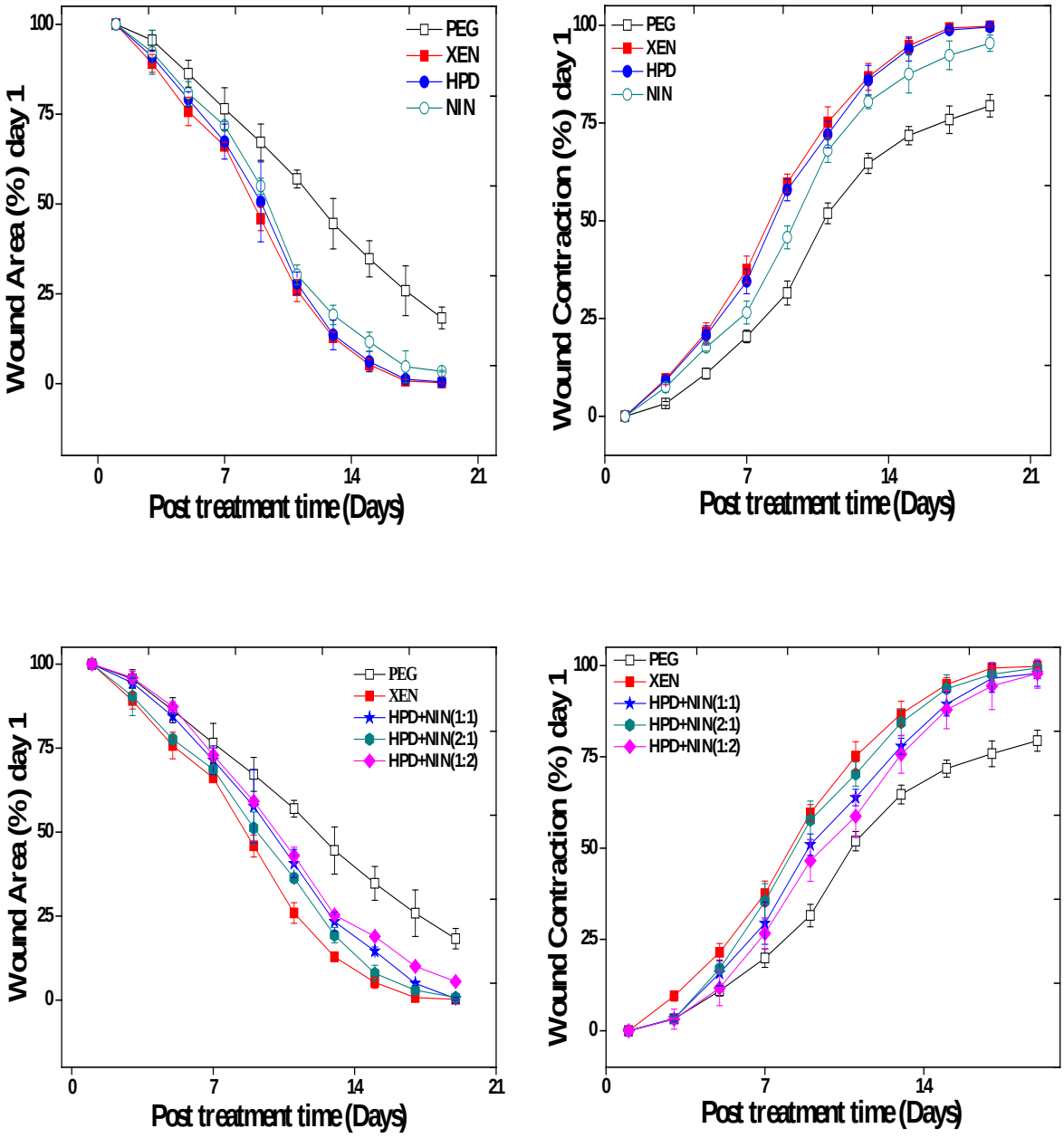

Figure 1: Effect of various concentrations of topical application of hesperidin and naringin and their different combinations on the contraction of regenerating excision wound in mice. The data are expressed as Mean± SEM, n=10, *p<0.05*.

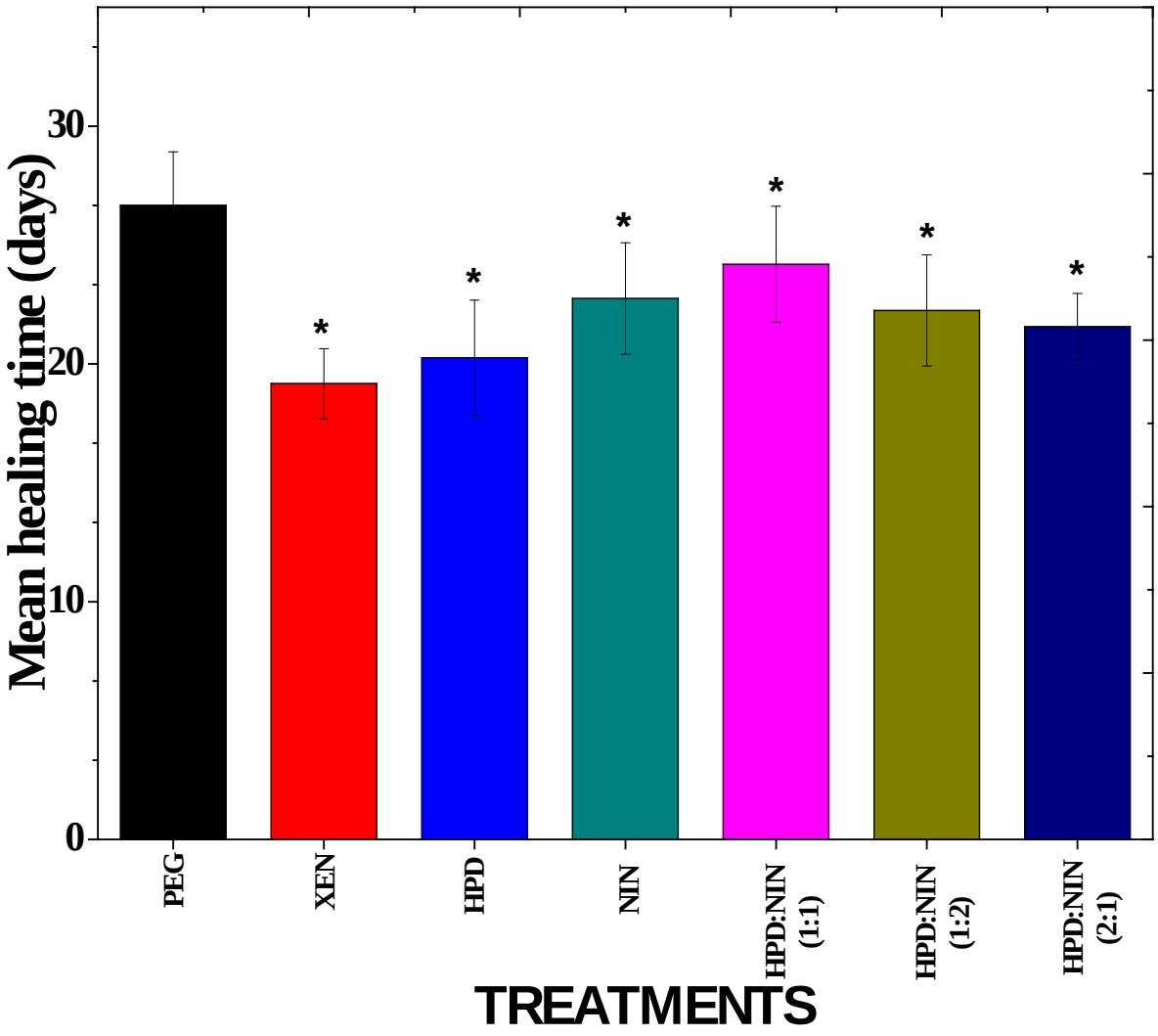

Figure 2: Effect of topical application of hesperidin and naringin and their different combinations on the mean wound healing time in mice. The results are expressed as Mean± SEM, n=10. *$p < 0.01$ when treatment groups are compared with polyethylene group.

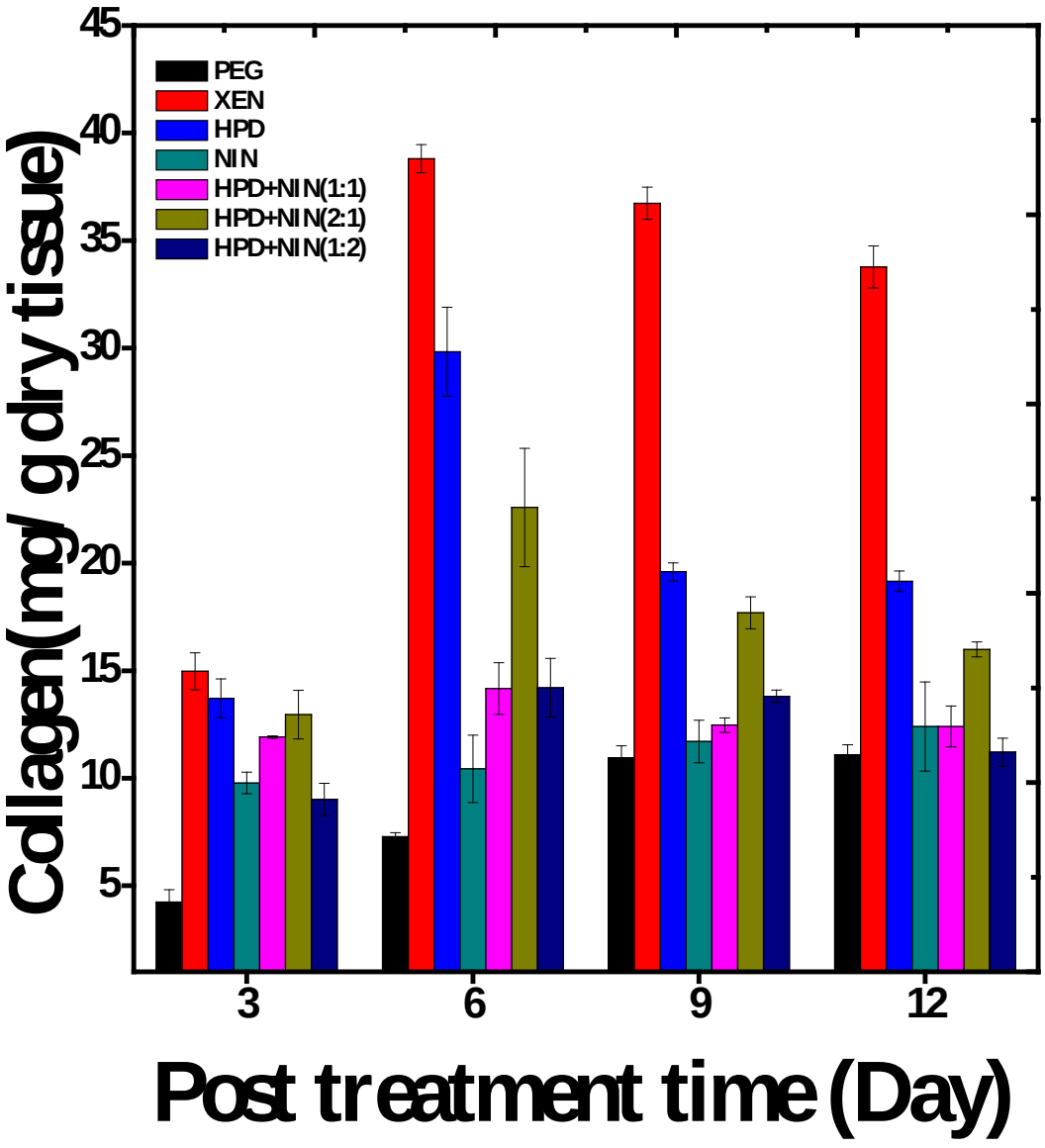

Figure 3. Effect of hesperidin and naringin and their different combinations on the biosynthesis of collagen in the regenerating excision wounds of mice at different post wounding days. The results indicate Mean± SEM, n=10, *$p < 0.01$ when treatment groups are compared with polyethylene group.

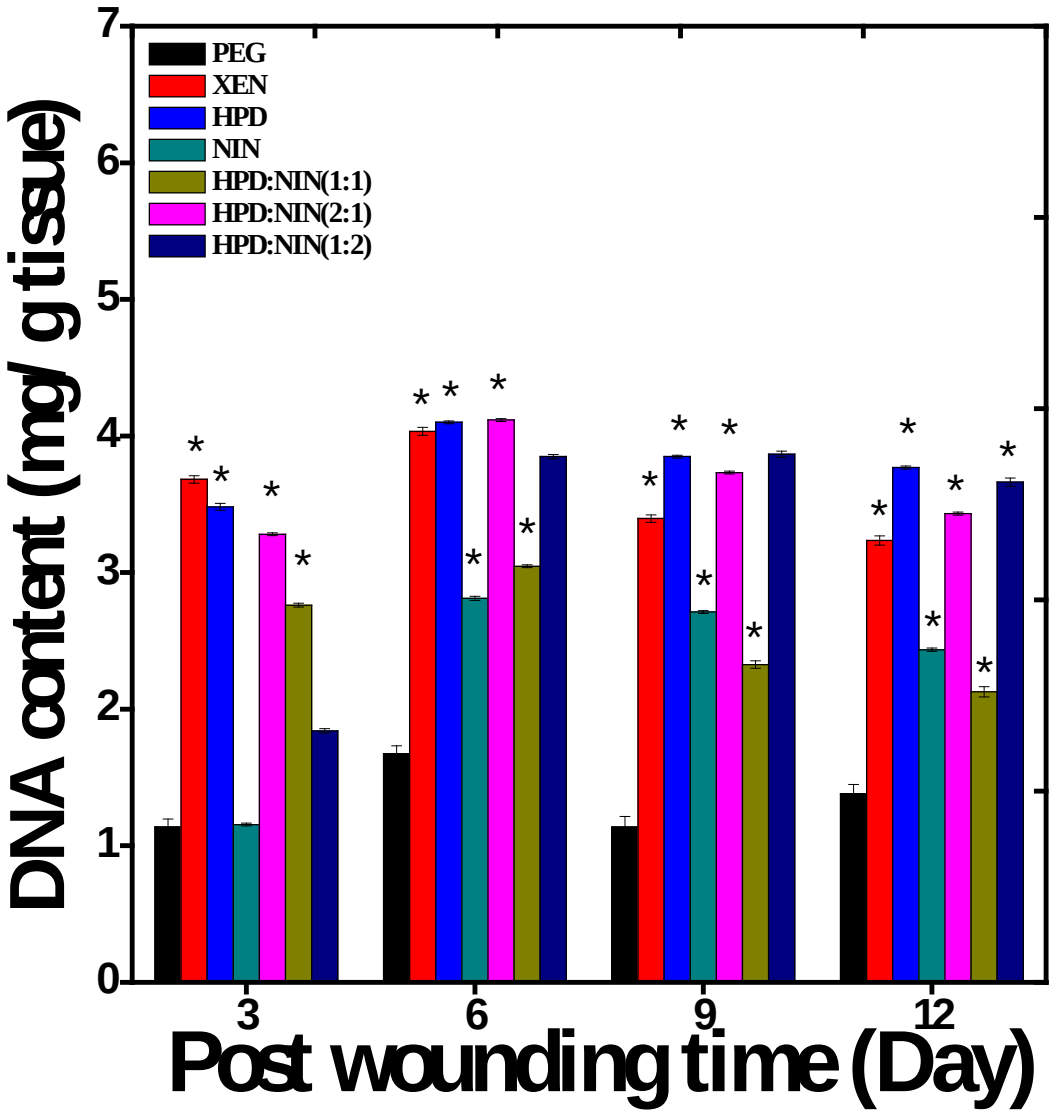

Figure 4: Effect of hesperidin and naringin and their different combinations on the biosynthesis of deoxyribonucleic acid in the regenerating excision wound of mice at different post wounding days. The results are Mean± SEM, n=10, *$p < 0.001$ when treatment groups are compared with polyethylene group.

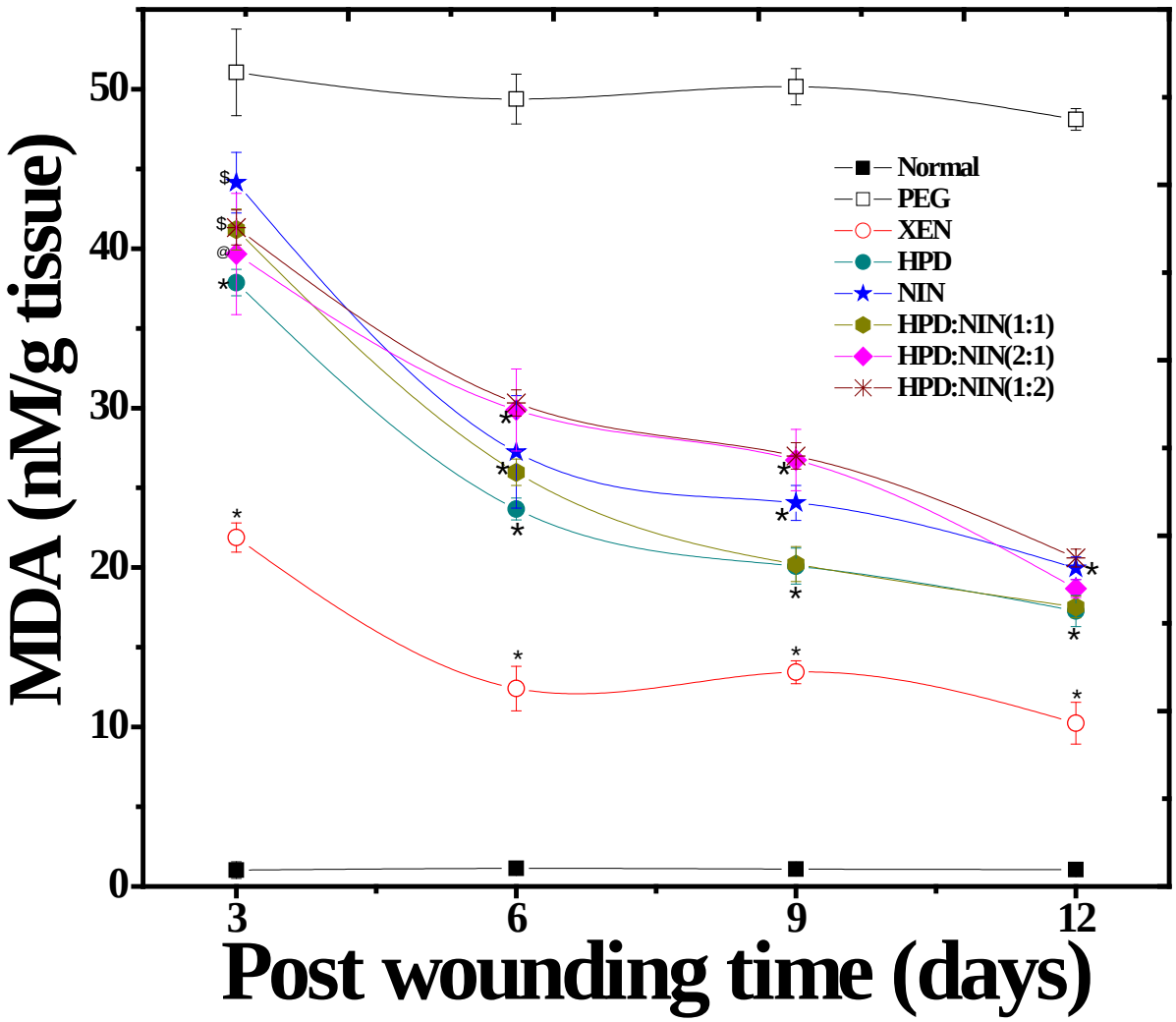

Figure 5: Effect of hesperidin and naringin and their different combinations on the lipid peroxidation in the regenerating wounds of mice excised at different post wounding days. The results are Mean± SEM, n=10, *$p<0.001$; $^{@}p< 0.02$, $^{\$}p< 0.05$ when treatment groups are compared to control (PEG) group. No symbol= no significance.

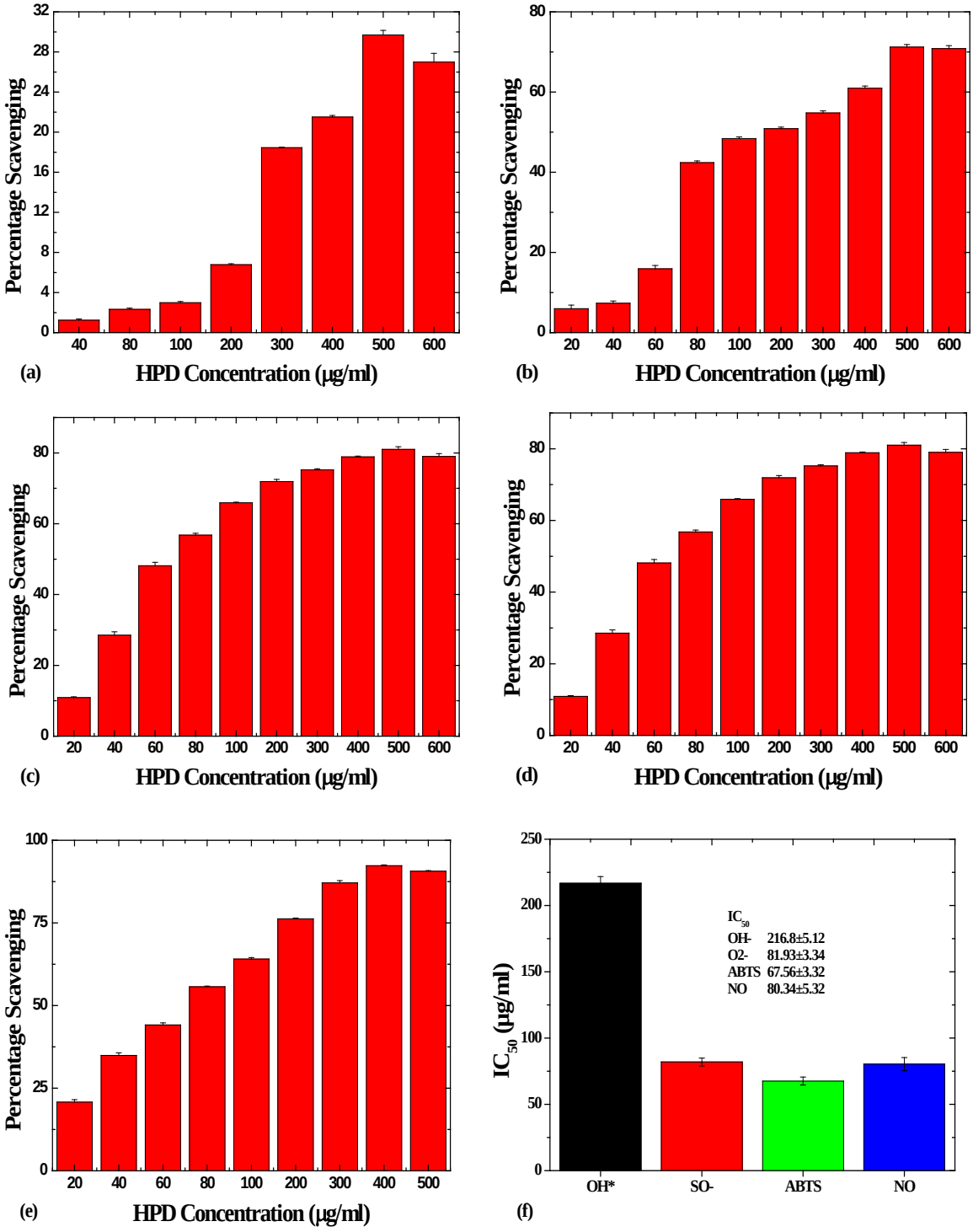

Figure 5. Effect of various concentrations of HPD on the free radical scavenging activity. a: DPPH, (b) Hydroxyl, (c) Nitric oxide, (d) Superoxide anion, (e) ABTS (f) IC_{50}. The results are expressed as Mean ±SEM, n=3.

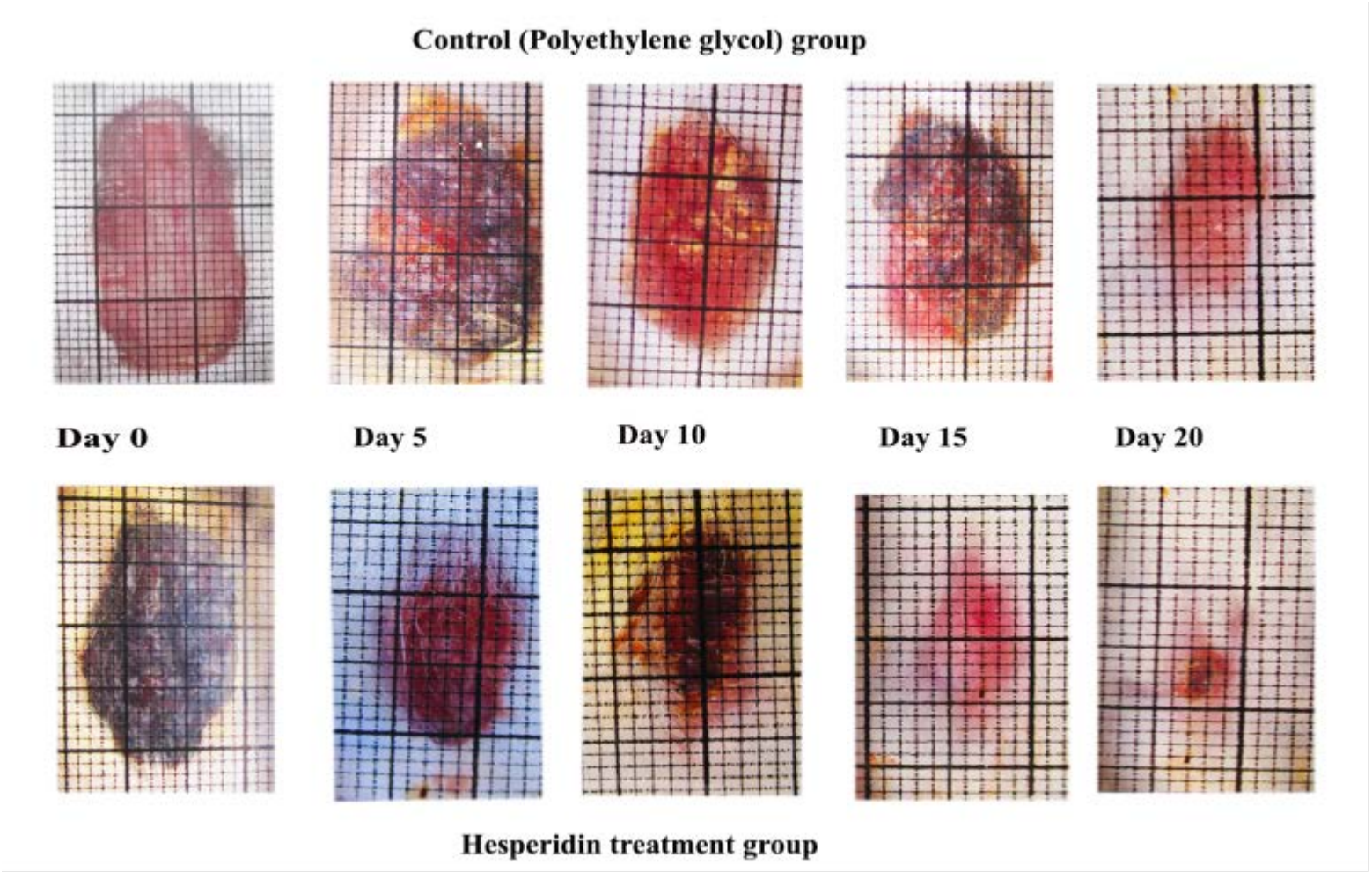

Plate 1: Effect of topical application of hesperidin on healing of deep dermal excision wounds in Swiss albino mice. The next wounding day considered as Day 1. The excised wound was observed until complete closure.

Table 1: The effect of 5 % hesperidin and naringin or their combination on the regeneration of dermal excision wound in mice. The result are expressed as Mean ± SEM, n=10.

Treatment	Mean wound healing time (days) (Mean ± SEM)
PEG	26.38±0.12
XEN	19.54±0.18*
HPD	20.68±0.42*
NIN	22.97±0.27*
HPD+ NIN (1:1)	22.38±0.09*
HPD+ NIN (2:1)	21.49±0.12*
HPD+ NIN (1:2)	24.43±0.17

***p<0.01 when treatment groups are compared to control group (PEG).*

No symbol=no significant different. Standard error of the mean (SEM).

Table 2: Effect of hesperidin and naringin or their combination on the biosynthesis of collagen in the regenerating excision wounds of mice

Treatment time (day)	Collagen (mg/g tissue±SEM)						
	PEG	**XEN**	**HPD**	**NIN**	**HPD+ NIN (1:1)**	**HPD+ NIN (2:1)**	**HPD+ NIN (1:2)**
3	4.24±0.57	14.97±0.85*	13.71±0.91*	9.77±0.51*	11.91±0.05*	12.95±1.13*	9.01±0.75*
6	7.27±0.19	38.80±0.66*	29.83±2.06*	10.43±1.56$^{\alpha}$	14.17±1.19*	22.59±2.76*	14.21±1.35*
9	10.94±0.56	36.73±0.75*	19.59±0.41*	11.70±0.98	12.46±0.33♠	17.68±0.74*	13.81±0.24♠
12	11.07±0.48	33.77±0.99*	19.15±0.47*	12.40±2.07	12.40±0.94	15.98±0.35*	11.21±0.64

**p < 0.01, ♠p< 0.02, $^{\alpha}$p< 0.05 when treatment groups are compared to polyethylene group.*

No symbol= no significance difference. N=10.

Table 3: Effect of hesperidin and naringin or their combination treatment on the biosynthesis of deoxyribose nucleic acid in the regenerating excision wounds of mice.

Treatment time (day)	DNA (mg/g tissue± SEM)						
	PEG	XEN	HPD	NIN	HPD+ NIN (1:1)	HPD+ NIN (2:1)	HPD+ NIN (1:2)
3	1.13±0.05	3.68±0.02*	3.48±0.03*	1.15±0.01	3.28±0.02*	1.84±0.02	2.76±0.02*
6	1.67±0.06	4.03±0.02*	4.10±0.01*	2.81±0.01*	4.11±0.01*	3.84±0.01*	3.04±0.07*
9	1.13±0.07	3.39±0.03*	3.84±0.01*	2.71±0.01*	3.73±0.01*	3.86±0.02*	2.32±0.02*
12	1.14±0.08	3.29±0.02*	3.76±0.01*	2.65±0.02*	3.64±0.01*	3.84±0.02*	2.28±0.02*

**p<0.001 when treatment groups are compared to concurrent control group.*
No symbol= no significance. N=10.

Table 4: Effect of hesperidin on lipid peroxidation level in the regenerated skin of mice excised wound.

Treatment time (day)	MDA(nmol/g tissue± SEM)							
	Normal	PEG	XEN	HPD	NIN	HPD+ NIN (1:1)	HPD+ NIN (2:1)	HPD+ NIN (1:2)
3	1.03±0.51	51.05±2.71	21.87±0.91*	37.87±0.82*	44.14±1.89$^{\alpha}$	41.19±1.28$^{\alpha}$	39.66±3.94♠	41.32±1.09$^{\alpha}$
6	1.13±0.12	49.38±1.58	12.41±1.39*	23.67±0.68*	27.25±3.52*	25.97±0.82*	32.88±2.56*	30.32±0.81*
9	1.10±0.11	50.15±1.13	13.43±0.71*	20.08±1.13*	24.05±1.09*	20.21±1.09*	26.74±1.92*	26.99±0.87*
12	1.06±0.21	48.10±0.68	10.235±1.31*	17.27±0.97*	19.95±0.72*	17.52±0.69*	18.68±0.56*	20.59±0.66*

**p<0.001; ♠p< 0.02, $^{\alpha}$p< 0.05 when treatment groups are compared to control group.*
No symbol= no significance. N=10.

Table 5: Effect of different concentrations of hesperidin on the scavenging of various free radicals in cell free system.

Concentration (µg/ml)	Free radical inhibition (Mean ± SEM)				
	DPPH	**$^{\cdot}OH$**	**$NO^{\cdot}$**	**$O_2^{\cdot-}$**	**$ABTS^{\cdot+}$**
20	1.77±0.42	6.25±0.15	11.28±0.37	11.14±0.63	20.40±0.61
40	2.19±0.62	6.90±0.19	26.78±0.73	27.91±0.41	34.51±0.88
60	4.48±0.58	14.54±0.72	46.64±0.55	45.96±0.43	43.99±0.45
80	9.318±0.76	32.26±0.58	57.54±0.42	57.00±0.33	54.88±0.36
100	18.09±0.32	41.26±0.45	65.34±0.43	64.54±0.51	64.66±0.23
200	20.89±0.34	51.23±0.45	71.49±0.33	70.59±0.74	75.81±0.75
300	25.98±0.43	55.69±0.75	74.46±0.93	74.92±0.36	85.88±0.67
400	26.99±0.38	61.22±0.48	79.62±0.13	77.45±0.62	92.62±0.26
500	29.08±0.13	71.35±0.46	81.25±0.27	80.77±0.28	90.80±0.34
600	26.99±0.38	71.27±0.16	79.42±0.26	78.51±0.65	90.77±0.31
IC_{50}	---	216.8±5.21	80.34±5.12	81.93±3.34	67.56±3.43

N=5.

CHAPTER
4

Topical application of hesperidin, citrus bioflavanone accelerates healing of deep dermal excision wounds in mice

ABSTRACT

The wound healing activity of different concentrations of hesperidin and naringin ointments was investigated in mice after infliction with a full thickness rectangular excision wound of 2.5 x 1.5 cm^2 area by measuring wound contraction using superimposed transparent graph sheet containing 1 mm^2 grid squares and mean wound healing time. Topical application of hesperidin and naringin ointment once a day accelerated the healing of excision wounds when compared to placebo treatment. A maximum wound contraction was observed for 5% hesperidin application when compared to naringin or application of combination ofhesperidin and naringin. Application of 5% hesperidin reduced the mean wound healing time (MHT) significantly, where it was shorter by 5.7 days when compared to the placebo controls. Naringin or combination of hesperidin and naringin also enhanced the wound contraction; however, it was lesser than hesperidin treatment alone. The application of naringin and its combination with hesperidin also led to a reduction in the MHT but this alleviation was lesser than hesperidin alone. The collagen and DNA synthesis studies revealed a similar correlation where maximum syntheses of collagen and DNA were observed for hesperidin treatment alone, when compared to all other groups at all post wounding days. In vitro studies have indicated that hesperidin scavenged DPPH, OH, O_2^-, ABTS and NO radicals in a concentration dependent manner up to 500 µg ml except ABTS radicals, where a maximum scavenging activity was observed at 400 µg/ml. The present study clearly demonstrates that 5% hesperidin accelerated the healing of regenerating wounds by increasing synthesis of collagen and DNA, which may be due to its antioxidant effect.

1. INTRODUCTION

The concept of developing drugs from plants used in indigenous medical system is much older, while in some cases direct links between a local and biomedical use exists, in other cases the relationship is much more complex (Heinrich and Gibbons, 2001). Wound healing is a complex and dynamic process in which cellular structure and tissue layer of the damaged tissue are restored to its normal state as closely as possible during wound repair and regeneration (Diegelmann and Evans 2004). Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin (Shaw and Paul, 2009). Wound healing involves continuous cell–cell and cell–matrix interactions that allow the process to proceed in three overlapping phases: inflammation (0–3 days), cellular proliferation (3–12 days) and remodeling (3–6 months) (Shaw and Paul, 2009; Velnar *et al*., 2009; Eckes *et al*., 2010). Wounds and

particularly chronic wounds are major concerns for the patient and clinicians alike as chronic wounds affect a large number of patients and seriously reduce their quality of life (Diegelmann and Evans 2004). It has been reported that only 1–3% of drugs listed in Western pharmacopoeia are intended for use in the skin and for wound repair (Balick and Cox, 1996). Both traditional and Western systems of medicine for wound healing suffer from the lack of resources and awareness. Wound healing constitutes a major problem due to the high cost of therapy and the presence of unwanted side effects (Porras-Reyes *et al*., 1993; Suh *et al*., 1998). Research on wound healing agents is one of the developing areas in modern biomedical sciences.

Despite the fact the reactive oxygen species (ROS) are deleterious to wound healing process due to their harmful effects on cells and tissues, a, certain amount of ROS is required to stimulate wound healing during inflammatory phase as they act as signaling molecules to initiate wound healing. Superoxide and H_2O_2 NAD(P)H oxidase are essential for recruitment of platelets for blood clot formation an indispensible step in wound repair (Suzuki and Mittler, 2012). Recruitment of neutrophils is one of the essential steps in repair of the tissue injury as these cells are required to produce a burst of free radicals including nitric oxide, which act as a signaling molecule for endothelial cells to form new blood vessels (Traci *et al*., 2013). However, excess production of free radicals is detrimental to wound repair as they will delay the tissue regeneration and could even lead to fibrotic changes.

Hesperidin, a naturally occurring flavonoid present abundantly in vegetables and fruits (Kanes *et al.*, 1993; Emim *et al*., 1994, Justesen *et al*., 1998). Hesperidin is an inexpensive byproduct of citrus family and is the major bioflavonoid in the sweet oranges and lemons (Garg *et al*., 2001). Hesperidin exerts many beneficial effects such as antioxidant, anti-allergic and anti-inflammatory (Galati *et al.,* 1994; Garg *et al.,* 2001, Chen *et al*., 2010; Vaberyureilai *et al*., 2015). It has been reported to possess anticarcinogenic effects in tongue, esophagus, colon, and urinary bladder carcinogenesis models in rat (Tanaka *et al*., 2000). Hesperidin has been reported to inhibit tumor initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts (Berkarda *et al.,* 1998; Tanaka *et al*., 1997; Franke *et al*., 1998). Hesperidin is antihypertensive, diuretic, hypocholesterolemic, hypoglycemic and found to retard bone

loss in humans (Galati *et al*., 1996; Kurowska *et al.*, 2000; Chiba *et al*., 2003; Bensaoula *et al*., 2015). Hesperdin has been reported to protect heart against the ischemic heart disease in diabetic rats (Agrawal *et al*., 2014). Purified micronized flavanoid fraction, containing 90% diosmin and 10% hesperidin has been reported to offer protection against reactive oxygen radicals both *in vivo* and *in vitro*. It is also effective in the healing of clean and infected wounds, both orally and topically (Lonchampt *et al*., 1989; Cypriani *et al*., 1993; Hasanoglu *et al*., 2001). Deficiency of hesperidin has been indicated in abnormal capillary leakage. Hesperidin has been found to possess beneficial effects on the abnormal capillary permeability, fragility, and protection against various traumas and stresses (Felicia *et al*., 1996). Normal intake of hesperidin or related compounds did not induce signs of toxicity in humans. Both hesperidin and its aglycone hesperitin have been reported to possess a wide range of pharmacological properties. Therefore the aim of this study was to assess the wound healing ability of hesperidin in mice inflicted with deep dermal excision wound.

2. MATERIALS AND METHODS

2.1. Chemicals

Hesperidin (98%) was procured from Himedia Ltd, Mumbai, India. Xenaderm (XEN) was procured from Health Point Ltd., Fort Worth, Texas, USA. Polyethylene glycol (PEG), hydroxyproline (catalog No: H5534), chloramine-T (catalog No: C9887), deoxyribonucleic acid (catalog No: D4522), diphenylamine (catalog No: D2385), p-dimethylamino-benzaldehyde (catalog No: 42363–0250), thiobarbituric acid (TBA), 1-Diphenyl-2-picryl hydrazyl (DPPH), naphthyl ethylene diamine dihydrochloride (NED) and sodium nitroprusside were procured from Sigma Aldrich Chemical Co., St. Louis, MO, USA, while methanol, ethanol, trichloroacetic acid, perchloric acid, sodium hydroxide, hydrochloric acid, sodium chloride , dimethyl sulphoxide (DMSO), orthophosphoric acid, acetic acid, n-butanol, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium carbonate, sodium bicarbonate, sodium carboxy methylcellulose, vitamin E, ethylene diamine tetra acetic acid (EDTA), and potassium chloride were supplied by SD fine-chemicals Ltd., Mumbai, India.

2.2. Animal care and handling:

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were selected from an inbred colony maintained under the controlled conditions of temperature (25±2ºC), humidity (55–60%) and 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH and Indian National Science Academy, New Delhi, India guidelines. The study was approved by the Institutional Animal Ethics Committee of the Mizoram University, Aizawl, India vide letter no. IAEC/4503.

2.3. Experimental protocol

The wound healing activity of hesperidin was evaluated in the deep dermal excision wound created in mice according to the details given below and a total of 150 animals were used to complete all experiments.

2.3.1. Preparation of drug and mode of administration

Different concentrations of hesperidin and naringin ointments were prepared using standard procedures. Henceforth, the hesperidin ointment will be referred to as HPD whereas as naringin will be NIN. Different concentrations: hesperidin alone (5%), naringin alone (5%), hesperidin: narinign (1:1, 2:1, 1:2) were prepared in polyethylene glycol (PEG). The ointment was topically applied on the excision wound/s one day after wound creation until completing healing of the wounds.

2.3.2. Production of full-thickness skin wound.

The full thickness dermal wound on the dorsum of mice was produced as described earlier (Jagetia *et al*., 2003). Briefly, the fur of the dorsum of each animal was removed with a cordless electric mouse clipper (Wahl Clipper Corporation, Illinois, USA). The animals were anaesthetized using ketamine and the entire body was cleaned and decontaminated by wiping with 70 % ethanol. The cleared dorsal surface of the skin was marked with a sterile rectangular (2.5 x 1.5 cm) acrylic stencil. A full thickness dermal wound was created by excising the full thickness skin flap in an aseptic environment

under a vertical laminar flow apparatus using sterile forceps and scissors. Each wounded animal was housed in a separate sterile polypropylene cage until the termination of experiments.

2.3.3. Study of Wound Contraction

The wound healing activity was determined by dividing the animals into the following groups:

2.3.3.1. PEG group: The wounds of animals of this group were topically covered with PEG once daily.

2.3.3.2. XEN group: The animals of this group were topically applied with xenaderm, a standard wound healing ointment, which was used as a positive control.

2.3.3.3. HPD group: The animals of this group received topical application of 5% of HPD ointment on their wounds.

2.3.3.4. NIN group: The animals of this group were topical applied 5% NIN ointment on their wounds.

2.3.3.5. HPD + NIN group: The animals of this group received topical application of 1:1, 1:2, 2:1 HPD and NIN ointments on their wounds.

The PEG, HPD or NIN or XEN was applied usually at 11 AM to each deep dermal excision wound of each animal of respective groups once every day from day 1 post wounding so as to cover the whole area of the wound until the complete healing of the wounds. The animals from the each group were monitored daily until complete wound healing.

2.4. Measurement of the wound contraction

The contraction of the wound was measured every alternate days starting from day 1 post wounding. The whole wound was covered by superimposing with a transparent grid of 1 cm^2 consisting of 100 squares each of 1 mm^2. The wound area was determined by counting the number of squares, which exactly covered the whole wound area.

2.4.1. Mean wound healing time

A separate experiment was conducted to determine the mean wound healing time (MHT), where grouping and other conditions remained essentially similar to that described above, except that the area of the wounds was not measured and the animals were left undisturbed after the various treatments. The animals of each group were monitored until

complete healing of wounds and the day at which each wound healed completely in each group was recorded. Mean of all healed wounds was determined and has been expressed as MHT in days.

2.5. Biochemical Analysis

A separate experiment was conducted to study the effect of topical application of PEG, HPD or NIR or XEN, where grouping and other conditions were essentially similar to that described for wound contraction except that the granulation tissue from each regenerating wound from each group was collected on the 3, 6, 9 and 12th day post wounding. The granulation tissues were homogenized and collagen, DNA contents, and lipid peroxidation were estimated as described below:

2.5.1. Collagen

Hydroxyproline (an indication of collagen formation) concentration was determined as described by Woessner (1961) with minor modifications. The weighed granulation tissues were hydrolysed in 6 N HCl for 3 hours at 130ºC, neutralised with 2.5 N NaOH to pH 7 and diluted with Milli-Q water. The diluted solution was mixed with chloramine-T reagent and kept at room temperature for 20 minutes, followed by the addition of freshly prepared ρ-dimethylaminobenzaldehyde (Ehrlich's reagent). The whole mixture was incubated for 15 minutes at 60ºC. The absorbance of each sample was measured at 550 nm using a double beam ultraviolet (UV)-visible spectrophotometer (Eppendorf Biospectrometer SW 3.5.1.0., Germany). The amount of hydroxyproline was determined by comparing with the standard curve. Total collagen from hydroxyproline analysis was determined by multiplying with a factor of 6.94 (Gallop and Paz, 1975). Collagen contents of granulation tissues have been expressed as milligram per gram dry tissue weight.

2.5.2. Deoxyribonucleic acid

The DNA estimation in regenerating wound gives an indication of cell proliferation. The DNA contents were measured by homogenizing the dry granulation tissue from each regenerating wound from each group in 5% TCA followed by centrifugation. The pellets were washed with 10% TCA, resuspended in 5% TCA and incubated at 90ºC for 15 minutes. The contents were centrifuged again and the resultant supernatant was used for the estimation of DNA by the method of Burton (1956). The DNA was hydrolysed with

60% perchloric acid at 80ºC for 20 minutes followed by the addition of Burton's diphenylamine reagent and overnight incubation at room temperature. Thereafter, 95% ethanol was added and absorbance was read at 600 nm using a UV-VIS spectrophotometer. The amount of DNA was determined by comparing with the standard curve and has been expressed as mg/g dry tissue weight.

2.5.3. Lipid Peroxidation

Lipid peroxidation (LOO) assay was carried out following the method of Buege and Aust (1978). One ml of granulation tissue homogenate was mixed with 2 ml of TCA-TBA-HCl reagent thoroughly. The mixture was heated in a boiling water bath for 15 minutes and cooled immediately at room temperature. After cooling, it was centrifuged at 1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS spectrophotometer. The blank contained all the reagents, except the cell homogenate that was substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^6$ $M^{-1}cm^{-1}$.

2.6. Free radical scavenging activity

2.6.1. Preparation of different concentration

For *in vitro* experiments usually 1 mg/ ml of hesperidin stock concentration was prepared in DMSO and was serially diluted with solvents to lower dilutions *i.e*. 20, 40, 60, 80, 100, 200, 300, 400, 500, 600 µg/ ml from stock solution for different analyses.

2.6.2. DPPH radical scavenging activity

Radical scavenging activity of hesperidin against stable DPPH˙ (2, 2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically. Hydrogen donation by an antioxidant compound during its interaction reduces DPPH˙ and the change in colour (from deep—violet to light - yellow) can be measured at 515 nm spectrophotometrically. Radical scavenging activity of hesperidin was measured by the slightly modified method of Leong and Shui, (2002). Briefly, each sample stock solution (1.0 mg/ ml) was diluted by DMSO so as to obtain different concentrations of 20 to 600 µg /ml. The reaction mixture of 1 ml 0.3 mM DPPH in methanol and 2.5 ml sample solution containing different concentrations of hesperidin were allowed to react at room temperature for 30 min and the absorbance was recorded at 515 nm using a UV/VIS spectrophotometer. The data obtained were converted into the percent antioxidant activity as described by Mensor

et. al., (2001). DMSO (1 ml) was used as a blank, whereas DPPH solution with DMSO was used as a negative control. The IC_{50} values were calculated by linear regression, against the concentration of hesperidin. Usually scavenging activity is an average of three replicates for each concentration. The results were confirmed by repetition of the experiment twice. The percentage of DPPH radical scavenging activity was calculated as follow:

$$\text{DPPH inhibition (\%)} = 100 - \frac{(\text{Abs of control} - \text{Abs of sample}) \times 100}{\text{Abs of control}}$$

2.6.3. Hydroxyl radical scavenging activity

Various concentrations of hesperidin in DMSO (0.2 ml) were added to the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 mL), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 mL) in phosphate buffer (pH, 7.4, 20 mM) to make up the final volume up to 1.2 ml (Halliwell *et. al.,* 1987). The reaction mixture was incubated for 30 min at 37ºC. This was followed by the addition of ice-cold trichloro acetic acid (0.2 ml, 15%, w/v) and thiobarbituric acid (0.2 ml, 1%, w/v) in 0.25 N HCl . The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was read at 532 nm against the corresponding blank in a UV/ VIS spectrophotometer. The results were confirmed by repetition of the experiment twice.

2.6.4. Superoxide anion scavenging activity

Superoxide scavenging activity was estimated as described earlier (Hyland *et al*., 1983). To the reaction mixture containing 0.2 ml of NBT (1 mg/ml of solution in DMSO), 0.6 ml of different concentrations of hesperidin, 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml H_2O) was added to give a final volume of 2.8 ml. The absorbance was read at 560 nm using a UV-VIS double beam spectrophotometer. The blank consisted of pure DMSO instead of alkaline DMSO. The results were confirmed by repetition of the experiment twice.

2.6.5.

2.6.6. ABTS radical cation radical scavenging activity

Total antioxidant potential of hesperidin was determined by ABTS (2, 2- azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt) assay spectrophotometrically as described earlier (Miller *et. al.*, 1996). The reaction between ABTS and potassium persulphate produces the ABTS radical cation, blue green in colour, in the presence of the antioxidant reductant; the coloured radical cation is converted back to colourless ABTS. This technique measures the relative ability of antioxidant substances to scavenge the $ABTS^{\bullet+}$ cation radical generated in the aqueous phase. The reaction mixture contained ABTS (0.00017 M), hesperidin (20-600 μg /ml) and buffer in a total volume of 3.5 ml. The absorbance was measured at 734 nm in UV-Visible double beam spectrophotometer. The results were confirmed by repetition of the experiment twice.

2.6.7. Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Ilosvoy reaction (Marcocci *et. al.*, 1994). The Griess Ilosvoy reaction was modified by using naphthyl ethylene diamine dihydrochloride (0.1%, w/v) instead of 1-napthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to a reduced production of nitric oxide. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffered saline (0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Thereafter, 1 ml of naphthylethylenediamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance was recorded at 540 nm against the corresponding blank in a UV/VIS spectrophotometer. The results were confirmed by repetition of the experiment twice.

3. Statistical analyses

The statistical significance between the treatments were determined using students‘t’ test and one way ANOVA with the application of Tukey’s test for multiple comparison for different parameters between the groups, using Origin Pro 8 (Pro 8 SRO v8.0724 (B724),

Northampton, MA, USA. statistical software was used for all analyses. A P value of ≤ 0.05 was considered as statistically significant.

4. RESULTS

The results are expressed as the mean ±standard error of the mean (SEM), and presented as Tables 1-5 and Figures 1-5.

The topical application of hesperidin ointment enhanced the wound contraction and accelerated healing process at various post treatment times (Figure 1). Treatment of mice with different concentrations of hesperidin ointment resulted in a steady contraction of excision wounds with time and a significant enhancement of wound contraction was observed in regenerating wound receiving the application of hesperidin alone ointment when compared with polyethylene group at post wounding days (Figure 1). The application of naringin ointment (5%) also steadily contracted the wound as indicated by shrinking wound area with time however its effect was slightly lesser than the hesperidin treatment alone (Figure 1). The combination of hesperidin with naringin in the ratios of 1:1 or 1:2 or 2:1 resulted in an increase in wound contraction when compared to PEG treatment alone. However, maximum contraction was observed for the group receiving topical application of a mixture of 2 parts hesperidin and 1 part of naringin ointment (Figure 1). The wound contraction for this mixture was almost at par with the positive control xenaderm (Figure 1).

The analysis of mean wound healing time showed the complete closure of wounds receiving topical application of PEG alone was achieved by 26.38 day post wounding whereas topical application of hesperidin alone advanced the wound healing time by 5.7 days, where complete healing of wound was attained by day 20.68. The topical application of naringin ointment brought down the mean wound healing time by 3.4 days and complete closure of wounds was observed at 22.97 day. When both hesperidin and naringin were combined in different ratios the mean wound healing time reduced for all three combinations, when compared with the PEG alone (Figure 2). The maximum decline in wound healing time was observed for HPD+NIN (2:1) with a MHT of 21.49 days followed by 22.38 days for HPD+ NAR (1:2) group, and 24.43 days in case of HPD+ NAR (1:1) group when compared to PEG treatment alone. This has led to the

reduction in MHT of 4.89, 4 and 2 day for HPD+ NIN groups receiving topical application in the ratio of 2:1, 1:2 and 1:1 of hesperidin and naringin, respectively (Table 1 and Figure 2).

The amount of neocollagen synthesized in the regenerating wounds was measured as hydroxyproline contents. The collagen synthesis elevated on day 3 and continued to rise up to day 12 post wounding. However a maximum synthesis of collagen was observed at day 6 post wounding in all the groups, when compared to PEG treatment alone (Figure 3). The collagen synthesis was almost 4 folds higher in the HPD group when compared to PEG treatment alone. In other groups the quantum of collagen synthesis was highest on day 3 post wounding, where this was 2 to 3 fold greater than PEG treatment alone depending on the type of treatment (Table 2). The collagen synthesis declined with the progression in wound healing and reduced on day 12 post wounding in all groups. Despite this alleviation the collagen synthesis was higher than day three post wounding (Table 2 and Figure 3). The collagen synthesis was always higher in the HPD group at all post wounding days (Figure 3).

The increase in DNA contents in regenerating wounds indicates proliferation of cells. The topical application of HPD alone on regenerating wound increased the DNA contents significantly on all post wounding days and the DNA synthesis was 3.5 to 4 folds more on all estimation days when compared to PEG application alone (Table 3 and Figure 4). Naringin application marginally increased the DNA synthesis as there was no significant elevation in DNA synthesis in the granulation tissue at 3rd day post wounding. However, there has been a significant rise in DNA synthesis on 6, 9 and 12 days post wounding after naringin application alone. The application of different combinations of HPD and NIN resulted in a significant rise in the DNA synthesis at all post wounding times except day 3 for HPD and NIN in the ration of 2:1 (Table 3).

The deep dermal wound increased lipid peroxidation and this remained almost similar on all post wounding days in PEG group as there was no significant alteration when compared to day 3 (Table 4). Application of HPD alone reduced induction of LOO and this attrition in LOO was between 3.5 to 4 fold lower, depending on the post wounding time of assessment (Table 4). Application of NIN ointment also reduced the LOO significantly when compared to PEG treatment alone; however, this reduction was

higher in the HPD group (Table 4 and Figure 5). The topical application of different combinations of HPD and NIN also reduced the induction of LOO significantly; however, this reduction was always lower than HPD application alone (Figure 5).

Free radical Scavenging

The effect of different concentration of HPD on the inhibition of different free radicals is shown in table 5 and figure 5. The hesperidin inhibited the generation of DPPH, free radicals in a concentration dependent manner however, it was not very effective as it inhibited the generation of DPPH only by 29% (Table 5 and Figure 6) and this has been the reason that it was not possible to determine IC_{50} for DPPH scavenging. However, hesperidin showed a concentration dependent inhibition on the generation of $^{\bullet}OH$, $NO^{\bullet}$, and $O_2^{\bullet-}$ radicals and a maximum inhibition was observed at 500 μg/ml for $^{\bullet}OH$, $NO^{\bullet}$ and $O_2^{\bullet-}$ that declined thereafter (Table 5 and Figure 6). The total antioxidant activity was measured using ABTS assay and the inhibition of $ABTS^{\bullet+}$ radicals showed a dose dependent scavenging up to 400 μg/ml and plateaued thereafter (Figure 5). The IC_{50} concentration for $^{\bullet}OH$, $NO^{\bullet}$, $O_2^{\bullet-}$ and $ABTS^{+\bullet}$ scavenging were 216.8±5.12, 80.34±5.32, 81.93±3.3 and 67.56±3.32 μg/ml, respectively.

DISCUSSION

Wound healing process begins with the restoration of a damaged tissue. The healing primarily depends on the degree of damage, general health status, and repair and regeneration ability of the tissue (Pesin *et al.,* 2009). Wound healing process is conveniently divided into four major overlapping phases including hemeostasis; inflammation, proliferation, maturation and remodeling (Enoch *et al.,* 2006; Janis and Harrison, 2014). Immediately after wounding the hemostasis begins which activates arrest of bleeding and formation of fibrin clot leading to formation of a plug (Monroe *et al.,* 2010). This is followed by inflammatory phase where, platelets get activated, and neutrophils, macrophases and leucocytes migrate to wound bed and become active to clear the dead and damaged cells (Enoch and Leaper 2008; Janis and Harrison, 2014). The growth factors secreted by these cells attract fibroblast and endothelial cells which aid in the formation of fibrin clot (Janis and Harrison, 2014). The proliferative stage begins by 4 day with the recruitment of fibroblasts from the undamaged tissue at the

wound site and keratinocytes, which initiate epithelialization of the regenerating wound, where the cells undergo division stimulated by various growth factors (Janis and Harrison, 2014). The final stage involves deposition of collagen by fibroblasts and regaining of strength (Janis and Harrison, 2014). The failure of this stage or deviation from the normal regenerative process may lead to non-healing wounds or improper healing. This indicates that pharmacological intervention may properly guide the wound regeneration and improve healing of wound/s. Therefore the present study was under taken to evaluate the effect of topical application of hesperidin or naringin or their different combinations on healing of deep dermal excision wound in mice.

Wound healing is dynamic process and wound contraction can be defined as the centripetal movement of the edges of the regenerating wound/s that eventually leads into the complete closure of the wound (Tejero-Trujeque, 2001). The progress of wound healing of full thickness dermal wounds can be easily assessed by measuring wound contraction regularly (Jagetia *et al*., 2003; 2007; Jagetia and Rajankikant, 2005, 2012; Jagetia and Ravikiran, 2015). Fibronectin an extracellular cell matrix (ECM) component is crucial in healing of regenerating wounds as it forms fibronectin clot after binding with platelets and fibrin immediately after wound healing and forms matrix with other ECM components that determines the course of wound healing (Lenselink, 2013; Janis and Harrison, 2014). The wound contraction is initiated by the migration of dermal fibroblasts, their conversion into myofibroblasts and formation of the granulation bed in the regenerating wound. The fibroblasts are not only responsible for collagen synthesis but also for the formation of other ECM components that are very essential for wound closure (Hinz, 2007; Driskell *et al.,* 2013). Apart from this fibroblast also secrete different factors like vascular endothelial growth factor (VEGP), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and basic fibroblast growth factor (bFGF), which are essential for cell proliferation and formation of new blood vessel for the repair of wounds (Barrientos *et al*., 2008; Bao *et al*., 2009). Keratinocytes have been found to mediate wound contraction in dermal wounds in concert with fibroblasts (Issac *et al*., 2011). The integrins have been reported to play a crucial role in wound contraction apart from collagen (Racine-Samson *et al*., 1997). The observation that hesperidin and its combination of naringin enhanced the contraction of wound when compared to PEG

application alone may be due to the enhanced proliferation of fibroblasts and keratinocytes that may have increased the synthesis of collagen and other ECM components. The study regarding the healing of dermal excision wound after topical application of hesperidin or its combination with naringin are unavailable however, hesperidin has been found to enhance proliferation of keratinocytes and aid in epidermal cell proliferation and differentiation (Hou *et al*., 2012) that are necessary for healing of wounds. Earlier ascorbic acid, curcumin and extract of nigella sativa have been found to enhance wound contraction in deep dermal wound in mice (Jagetia *et al*., 2003; Jagetia and Rajanikant, 2005, Jagetia and Ravikiran, 2015).

The exact mechanism of action by which hesperidin and its combination with naringin accelerated the healing of deep dermal excision wounds of mice is not known. It is speculated that hesperidin and its combination with naringin may have employed multiple putative mechanisms to enhance the wound healing in the present study. Hesperidin and naringin may have reduced the free radical generation and kept it to the optimum level to generate inflammatory responses necessary for healing of the excision wounds. Naringin has been reported to scavenge free radicals in vitro (Jagetia *et al*., 2003). Hesperidin has also been found to reduce the free radical generation in the present study. Collagen is an important protein which is necessary for healing of the wounds and provides strength to the regenerating wound during the late phase of wound healing. Hesperidin and its combinations have increased the synthesis of collagen leading to accelerated wound repair and regeneration. Increased DNA synthesis is an index of cell proliferation and hesperidin and its combination with naringin elevated the DNA synthesis, which may be due to increased fibroblasts, keratinocytes and other cell proliferation in the granulation tissue of regenerating wound. This increased cell proliferation of cells may have led to accelerated repair of the wound in our study. Hesperidin and its combination with naringin may have elevated the VEGF, TNF α, and TGF β, which would have helped the fibroblasts, keratinocytes and endothelial cells to proliferate and laid the foundation of granulation tissue for wound repair (Sinno and Prakash, 2013). Hesperidin has been reported to elevate the VEGF, TNF α, and IL-1β (Shi *et al*., 2012). The hesperidin has been found to increase GSH contents, GSHpx, SOD in the regenerating wound and decrease lipid peroxidation (Jagetia and Rao, 2015), which

may have helped in earlier repair in the hesperidin and combination groups. The wounding has been reported to increase the expression of NF-κB, COX-II and LOX (Zhou *et al*., 1999; Gaddipati *et al*., 2003) and hesperidin and naringin have been found to inhibit the NF-κB, and COX-II activation that would have accelerated the healing of regenerating wounds (Hirata *et al.*,2005).

Conclusions: Topical application of hesperidin and its combination with naringin increased the regenerating capacity of wounds if applied once daily and it also helped to accelerated wound repair and regeneration in wounds as evidenced by increased wound contraction and reduced mean wound healing time. The observed effect may be due to scavenging activity of free radicals, and their ability to modulate various growth factors including VEGF, TNF α, and TGF β. Hesperidin and naringin may have also suppressed the activation of NF-κB, and COX-II accelerating the wound repair processes. Hesperidin and naringin, the natural non-toxic compounds found in citrus fruits, are consumed daily by humans and their topical application may accelerate cutaneous healing of wounds in clinical setup.

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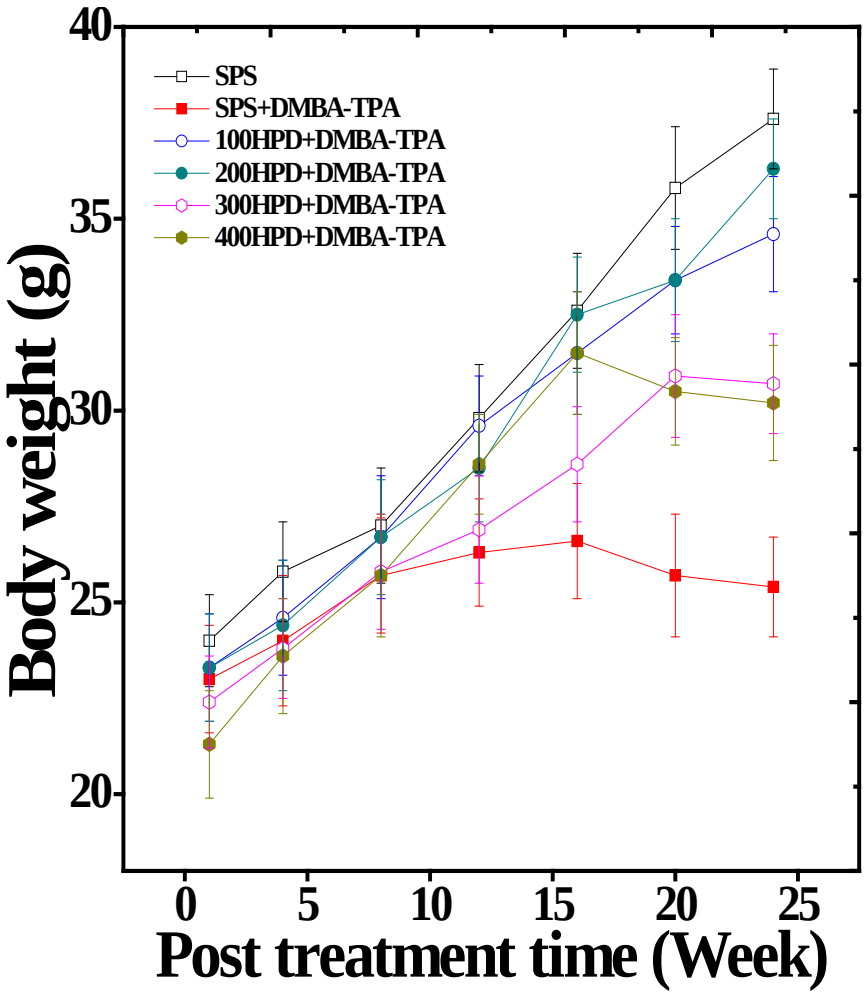

Figure 1: Effect of pretreatment of various doses of hesperidin on body weight changes of albino mice receiving DMBA-TPA application for the induction of skin papilloma. The data are expressed as Mean ±SEM, n=10, $p<0.05$.

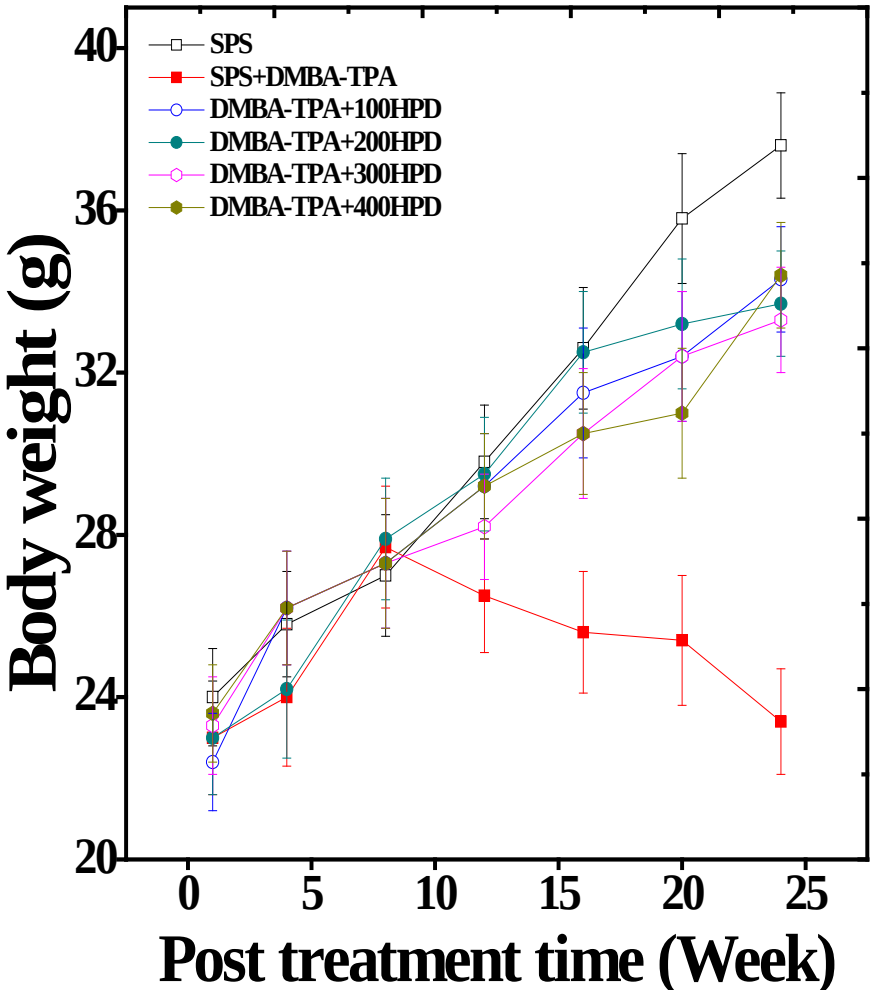

Figure 2: Effect of posttreatment of various doses of hesperidin on body weight changes of albino mice receiving DMBA-TPA application for the induction of skin papilloma. The data are expressed as Mean ±SEM, n=10, p<0.05.

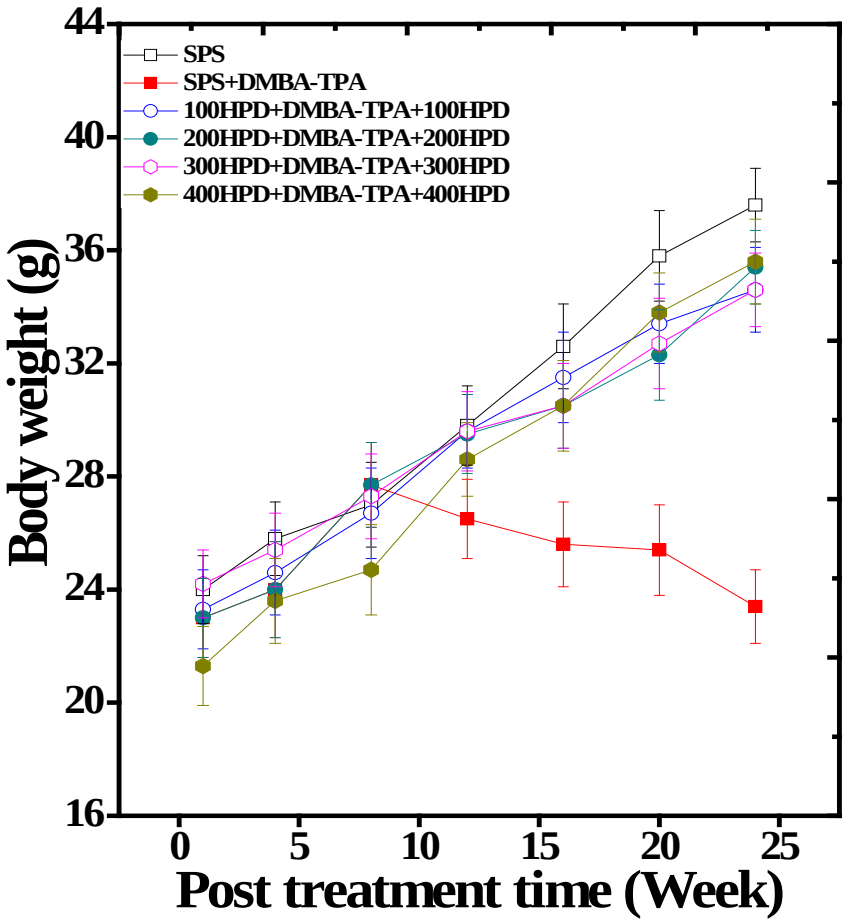

Figure 3: Effect of pre-post treatment of various doses of hesperidin on body weight changes of albino mice receiving DMBA-TPA application for the induction of skin papilloma. The data are expressed as Mean ±SEM, n=10, p<0.05.

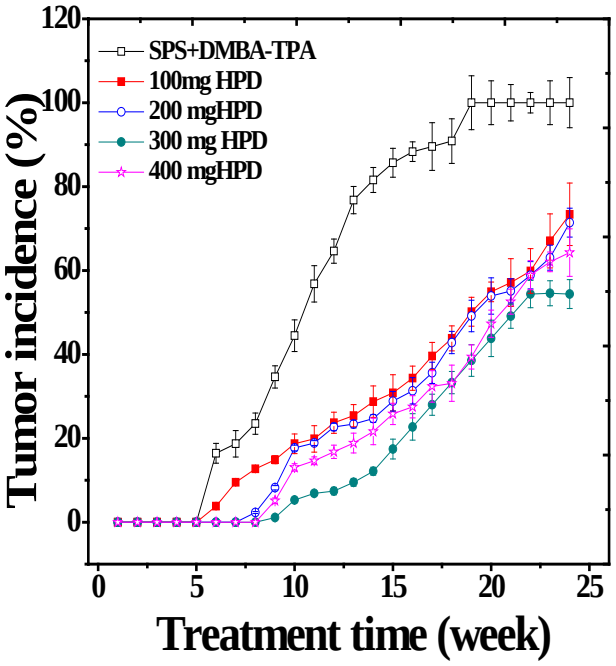

Figure 4: Incidence of skin papilloma after application of DMBA-TPA or oral administration of various doses of hesperidin treatment in Swiss albino. The data are expressed as Mean ±SEM, n=10, p<0.01.

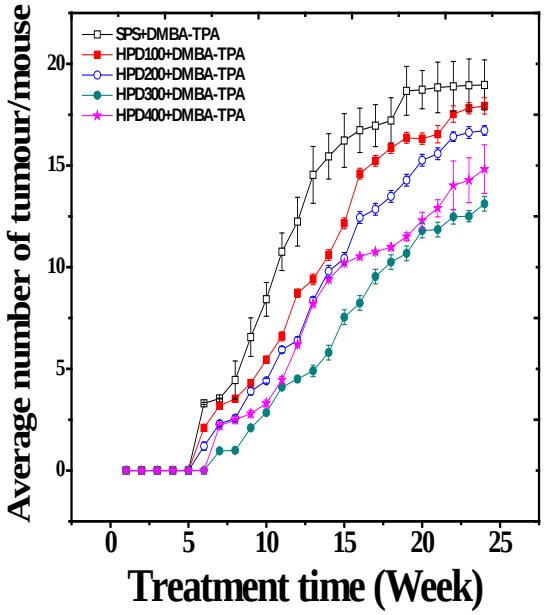

Figure 5: Alteration in the DMBA-TPA induced skin papilloma in mouse administered with various doses of hesperidin for two weeks before carcinogen application. The data are expressed as Mean ±SEM, n=10, p<0.01.

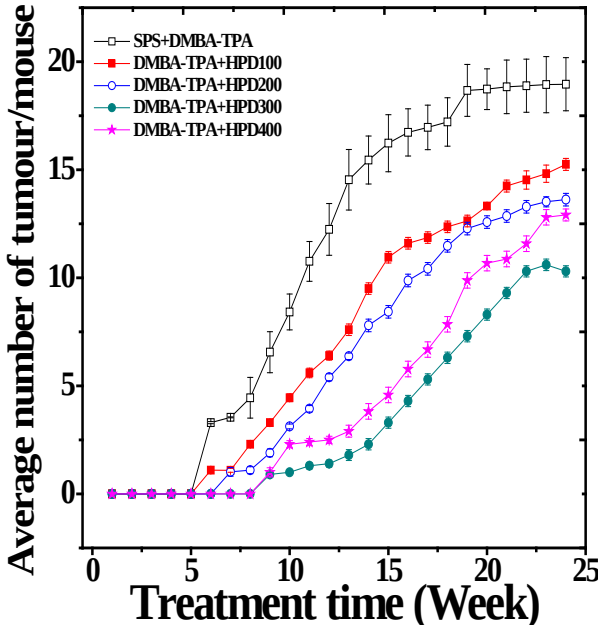

Figure 6: Alteration in the DMBA-TPA induced skin papilloma in mouse administered with various doses of hesperidin after carcinogen application.. The data expressed as Mean ±SEM, n=10, p<0.01.

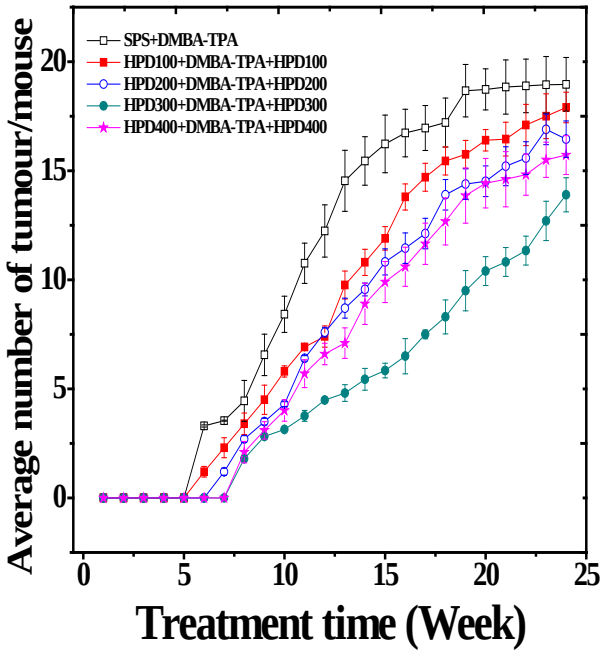

Figure 7: Alteration in the DMBA-TPA induced skin papilloma in mouse administered with various doses of hesperidin for two weeks before and after (pre-post) carcinogen application. The data expressed as Mean ±SEM, n=10, p<0.01.

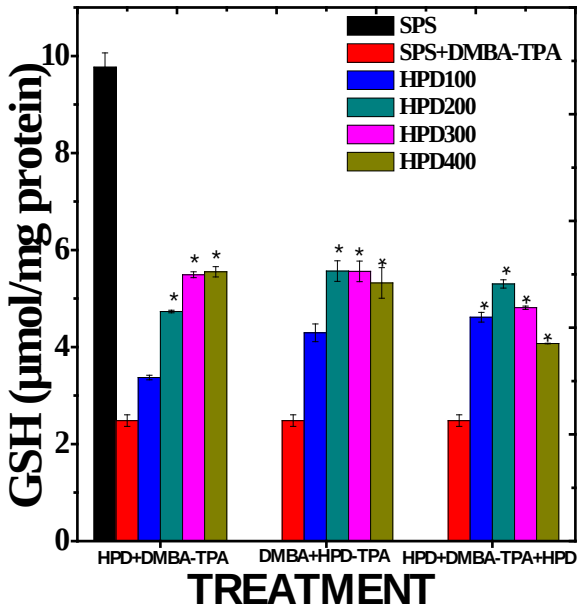

Figure 8: Alteration in the glutathione (GSH) concentration in the skin papilloma of mice treated with different doses of hesperidin. The data represent Mean ± SEM, n=10. * *p<0.01 when treatment groups are compared to DMB-TPA group.***

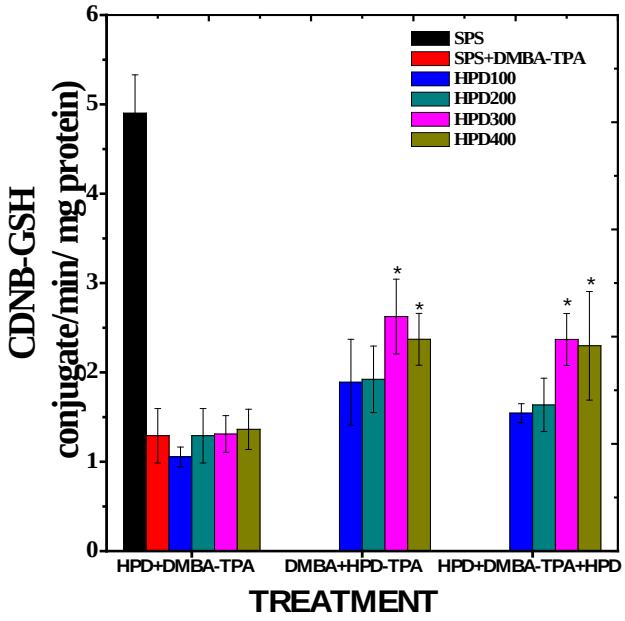

Figure 9: Alteration in the glutathione glutathione -S -transferase (GST) activity in the skin papilloma of mice treated with different doses of hesperidin. The data represent Mean ± SEM, n=10. * p<0.01 when treatment groups are compared to DMB-TPA group.

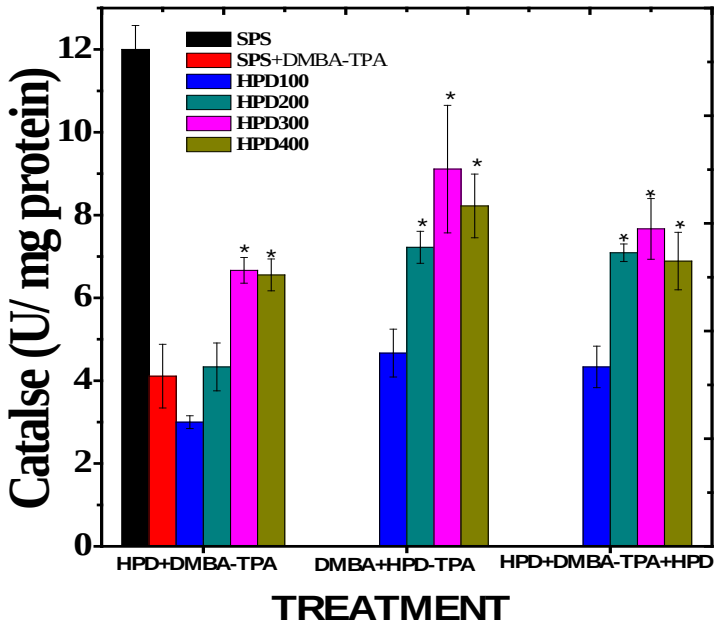

Figure 10: Alteration in the catalase activity in the skin papilloma of mice treated with different doses of hesperidin. The data represent Mean ± SEM, n=10.

**** p<0.01 when treatment groups are compared to DMB-TPA group.***

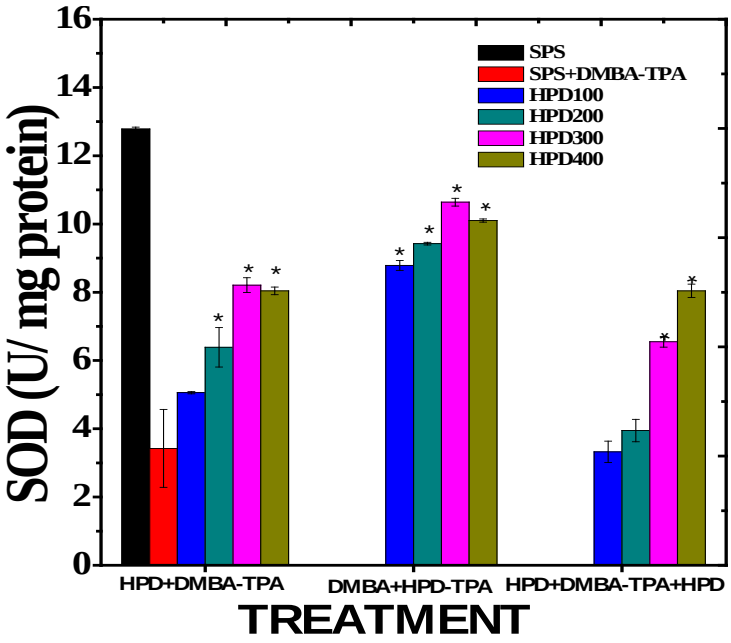

Figure 11: Alteration in the superoxide dismutase (SOD) activity in the skin papilloma of mice treated with different doses of hesperidin. The data represent Mean ± SEM, n=10.

****p<0.01when treatment groups are compared to DMB-TPA group.***

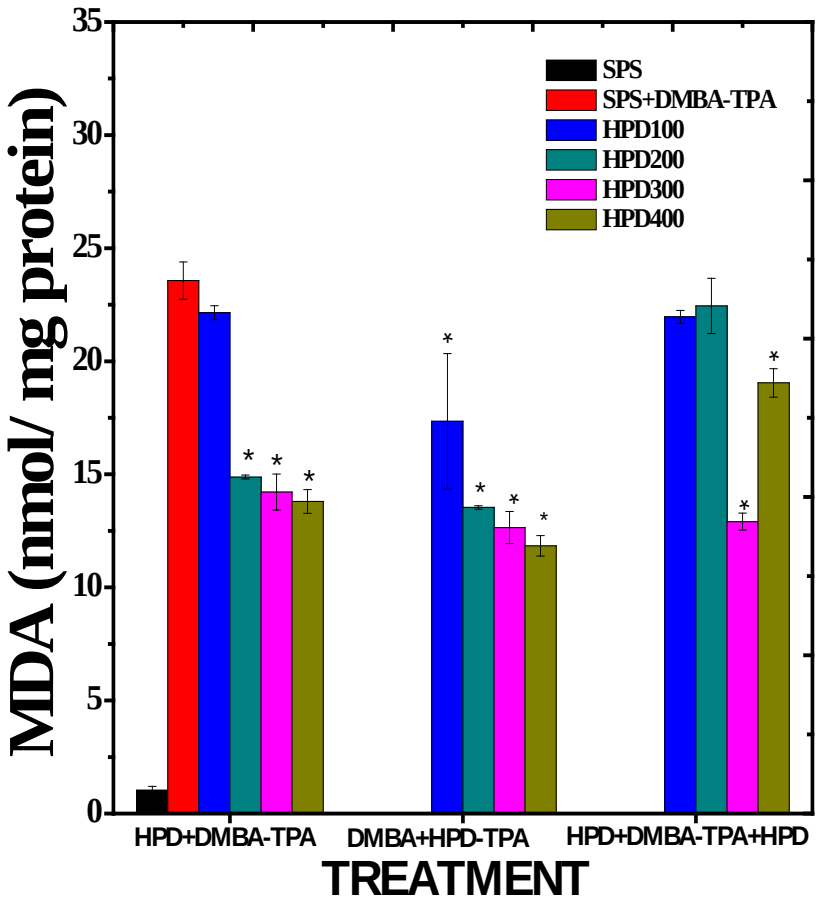

Figure 12: Alteration in the lipid peroxidation (LOO) in the skin papilloma of mice treated with different doses of hesperidin. The data represent Mean ± SEM, n=10.

**** p<0.01 when treatment groups are compared to DMB-TPA group*.**

CHAPTER
5

Investigation of the chemopreventive potential of hesperidin, bioflavonoid in multi-stage cutaneous carcinogenesis

ABSTRACT

The chemopreventive potential of hesperidin was investigated on the 7, 12-dimethylbenz[a]anthracene (DMBA)-TPA-induced skin carcinogenesis in Swiss albino mice. The skin carcinogenesis was initiated by the topical application of DMBA followed by the application of 12-O-tetradecanoyl phorbol-13-acetate (TPA) as a promoting agent on the shaved dorsum of mice treated or not with various doses of hesperidin. The application of DMBA followed by subsequent application of TPA led to 100% tumor incidence and increased average number of tumors in mice, whereas the administration of hesperidin before or after and continuous (pre and post) carcinogen application significantly reduced the tumor incidence and average number of tumors when compared to DMBA-TPA alone. The tumor formation was also delayed by hesperidin treatment. Topical application of DMBA-TPA increased the oxidative stress as evident by a significant rise in TBARS and decline in various antioxidants when compared to the untreated control group. The hesperidin treatment significantly reduced TBARS in the skin of mice treated with DMBA-TPA and significantly elevated the glutathione concentration and glutathione-S-transferase, superoxide dismutase and catalase activities when compared with the DMBA-TPA application alone. Our study demonstrates that hesperidin protected mice against chemical carcinogenesis and the chemopreventive effect of hesperidin may be due to the protection of DMBA-induced DNA damage, inhibition of TPA induced-inflammatory response and increased antioxidant status.

1. INTRODUCTION

Cancer is a multifaceted disease which is variable in its presentation, development and outcome from one patient to the other, which is due the existence of heterogeneity and variability at the cellular and molecular level. Carcinogenesis is a multistep, multipath and multifocal process encompassing a series of genetic and epigenetic changes in the cell leading to genomic instability, which finally ends up in the development of the disease cancer (O'Shaughnessy *et al*., 2002; Hanahan and Weinberg *et al*., 2011). During carcinogenesis the cells undergo profound, genetic, metabolic and behavioural changes, which cause them to proliferate excessively and untimely, they also escape surveillance by the immune system, and finally acquire invasive characteristics that results in distant metastases (van Gent *et. al.,* 2001; Lea˜o *et al*., 2005; Hanahan and Weinberg *et al.,* 2011).

The systematic approach to carcinogenesis has made immense progress in acquiring the knowledge required to prevent cancer during past few decades. Many scientists deliberately investigated the potential causes of cancer induction including the role of environmental factors, diets and lifestyle in carcinogenesis (Garcia-Closas *et al*., 2005; Huxley, 2009; Smith *et al.,* 2014). These studies did provide an insight

into the mechanisms of cancer occurrence. It has also been revealed that carcinogenesis results from the accumulation of multiple sequential mutations and alterations in nuclear and cytoplasmic molecules, which finally culminates into invasive neoplasms (Loeb and. Harris, 2008; Ward and Thompson, 2012; Nahata *et al*., 2015; Alameda *et al*., 2016; Poirier, 2016). Numerous pharmacological agents have been tested for their safety and effectiveness in preclinical trials and still continue to be tested in Phase I, II and III clinical interventions for the treatment of cancers or as cancer chemopreventive agents at various sites including skin, breast, colon, prostate, esophagus, mouth, lung, cervix, endometrium, ovary, liver and bladder (Li *et al.,* 2002; Szumiło, 2008; Landis-Piwowar and Iyer, 2014).

The term chemoprevention was introduced by Michael Sporn in the year 1976, which is reversal of the process of carcinogenesis. The main aim of chemoprevention is to prevent or arrest or reverse either the initiation phase of carcinogenesis or the progression of neoplastic cells to cancer (Sporn and Suh, 2000). Chemoprevention is by now an emerging area of clinical oncology addressed to healthy individuals at higher risk for cancer, subjects with precancerous conditions, and patients who are at risk for a secondary cancer (Veronesi and Bonanni, 2005). Chemoprevention deals with the pharmacological intervention of the process of any disease so that the manifestation of the disease can be delayed or inhibited (Boone *et. al.,* 1990; Lippman and Levin, 2005; Lippman and Hawk, 2009). Chemoprevention is widely used and readily accepted by doctors and patients in the form of drugs that lower cholesterol concentrations and blood pressure to reduce the risk of cardiovascular disease. It can also be used in some apparently healthy people to prevent or reduce the risk of cancer development using various pharmacological agents. The biomedical community needs to recognize and advocate approaches to prevent cancer with the same enthusiasm that it currently directs towards treating it (Greenwald, 2002).

The inhibitors of cancer that are able to either arrest or reverse cancer development by interfering with one or more steps of carcinogenesis have been identified and systematically evaluated for their potential as chemopreventive agents (Huang *et al.,* 1997). Various environmental and genetic factors are involved in the induction of skin cancer, but exposure to chemical carcinogens and solar ultraviolet (UV) radiations seems to be primarily responsible for several skin diseases including skin cancer (Gupta and Mukhtar, 2002). Chronic exposure of skin to harmful chemicals, like synthetic cosmetics leads to molecular alteration in the skin epithelium

causing basal cell and squamous cell carcinoma and melanoma (Gupta and Mukhtar, 2002; Katiyar, 2005). The skin cancers are more prevalent in Western world and Australian subcontinent and contribute to significant mortality. The cancer related mortality rate may be brought down by pharmacological intervention before the onset of carcinogenesis, and the use of dietary or herbal products looks an attractive proposition because of their daily use and non-toxicity (Dragsted *et al.,* 1993; Mukhtar and Agarwal, 1996; Kris-Etherton *et al.,* 2002; Rabi and Gupta, 2008; Lippman and Hawk, 2009). The dietary factors play an important role in human health and in certain chronic diseases including cancer (Aggarwal and Shishodia, 2006). The role of dietary modification in the reduction of cancer risk has recently drawn widespread attention, because the differences in worldwide human cancer mortality often depend on lifestyle and dietary habits (De flora *et al.,* 1993; García-Closas *et al*., 2005).

The mouse skin carcinogenesis model has become very useful in studying the genetic and biological changes involved in tumour promotion (Holden *et al.,* 1997). For more than 50 years, mouse skin has been used as a conventional model for studying the mechanisms of carcinogenesis and the modulation of sequential steps involved in this process (DiGiovanni, 1992; Yuspa, 1994). The two stage model of mouse skin carcinogenesis involves initiation step stimulated by topical application of a suboptimal dose of a carcinogen such as DMBA and promotion step requiring multiple treatments of a tumor promoting agent like TPA (Boutwell, 1964). Initiation process is irreversible and probably involves somatic mutation whereas promotion phase is reversible at least in early stages and involves induction of altered gene expression (Boutwell, 1974). There are various factors that accelerate the formation of skin cancer, one of which includes accumulation of unsaturated lipids. It has been suggested that accumulation of unsaturated lipids plays an important role in carcinogenesis just as they are required in certain cancers (Black, 1983). The mouse skin carcinogenesis model has become very useful in studying the genetic and biological changes involved in tumor promotion (Vogelstein *et al.,* 1988; O'Shaughnessy *et al*., 2002). Some of the genetic changes associated with the chemical initiation of benign papillomas and their transition into squamous cell carcinoma have been well characterized in this system (Hong and Sporn, 1997).

A large number of naturally occurring as well as synthetic chemopreventive agents have properties to control carcinogen-induced hyper-proliferation of cells in

the target organ/s during the initiation as well as post-initiation phases of carcinogenesis (Mori *et al*., 1997; Mori *et al*., 1999). Several agents including fruits, vegetables, beverages, isolated single compounds, spices and medicinal plants have been screened for their chemopreventive activity during the past few decades in various models including humans (Surh, 1999; Gupta and Mukhtar, 2002). The epidemiological survey also indicates that intake of citrus fruits had beneficial effect on the prevention of cancer and the polyphenolic fraction isolated from grape seeds has been reported to provide a significant protection against tumor promotion in the mouse skin tumorigenesis model (Zhao *et al.*, 1999).

Hesperidin (3,5,7 – trihydroxyflavanone 7- rhamnoglucoside) a bio flavonoid, is the food-bound form of hesperitin. It was first isolated in 1828 by French chemist Lebreton from the albedo (the spongy inner portion of the peel) of oranges, and has since been found in lemons and other citrus fruits (Dakshini, 1991, Manthey and Grohmann, 1998, Garg *et al*., 2001). Hesperidin is mainly used as antioxidant, as it remarkably prevented indicators of oxidative stress, such as the ROS and lipid peroxidation levels in a dose-dependent manner (das Neves *et al.*, 2004). It has also been reported to reduce the generation of ROS and caspase-dependent apoptosis in human polymorphonuclear neutrophils in vitro (Ross and Kasum 2002; Zielinska-Przyjemska and Ignatowicz 2008). Hesperidinhas been found to be anti-allergic, antihypotensive, antimicrobial, vasodilator, anti-inflammatory antihyperlipidemic antihypertensive, and cardioprotective in ischemic heart disease in diabetic rats (Galati *et al.*, 1994; Emim *et al*., 1994; Monforte *et al.*,1995; Garg *et al.*, 2001; Ohtsuki *et al.*, 2003; Chen *et al*., 2010; Agrawal *et al.*, 2014; Vaberyureilai *et al*., 2015). Hesperidin has been reported to inhibit tumour initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts (Berkarda *et al.*, 1998; Tanaka *et al*., 1997; Franke *et al*., 1998). It is also effective in the healing of clean and infected wounds, both orally and topically (Lonchampt *et al*., 1989; Cypriani *et al*., 1993; Hasanoglu *et al*., 2001). Deficiency of hesperidin has been indicated in abnormal capillary leakage. Hesperidin has been found to possess beneficial effects on the abnormal capillary permeability, fragility and protection against various traumas and stresses (Felicia *et al*., 1996). It has got hepatoprotective effect against DMN-induced fibrosis in rats, which is attributed to its antioxidant effect (El-Samaligy *et al*., 2006). Hesperidin has been reported to provide strong cellular antioxidant protection against the damaging effects of paraquat and hydrogen

peroxide (Wilmsen *et al.,* 2005). Hesperidin treatment effectively protected aged rat heart by increasing the activity of enzymic antioxidants and upregulated the protein levels of nuclear factor erythroid 2-related factor 2, which is responsible for maintaining the antioxidant status of the cell (Elavarasan *et al.,* 2012). It has been found to protect against renal dysfunction through inhibiting free-radical formation and restoration of the antioxidant defense systems (Jain and Somani, 2015). However, the chemo-preventive potential of hesperidin has not been studied therefore; the aim of the present study was to investigation the chemopreventive potential of hesperidin in the two stages DMBA-TPA induced skin carcinogenesis in Swiss albino mice.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Hesperidin (98%) was procured from Himedia Ltd, Mumbai, India. 7,12-dimethylbenzanthracene (DMBA), 12-O-tetradecanoyl-13-phorbol acetate (TPA), 1-Chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), triton X-100, ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA), pyruvic acid, reduced, and nicotinamide adenine dinucleotide (NADH) were obtained from Sigma Chemical Co. (Bangalore, India). Sodium carbonate (Na_2CO_3), Trichloroacetic Acid (CCl_3.COOH), Potassium Chloride (KCl), Potassium Sodium-Tartrate were procured from SD Fine Chemicals, Mumbai, India, whereas Sodium Hydroxide (NaOH), Sodium Chloride (NaCl), Sulphuric Acid (H_2SO_4), Hydrochloric Acid (HCl), Tris Buffer GR (Tris (hydroxymethyl) aminomethane (Merck India Limited, Mumbai, India).

2.2. *Animal Care and Handling*

Six to eight weeks old adult male Swiss albino mice weighing (22 ± 5) g were selected from a pathogen free inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and light (12 h of light and dark, respectively). The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). The animals had free access to sterile food and water during the experiments. Usually 5 animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. All experiments were carried out in compliance

with the regulations of Institutional Animal Ethics Committee of the Mizoram University, Aizawl vide letter no. IAEC/4503.

2.3. *Preparation of Drug (hesperidin)*

The hesperidin was weighed and dissolved in normal saline containing 0.5 % Carboxyl methyl cellulose (CMC). The animals were given with different doses of freshly prepared hesperidin in drinking water daily until termination of the experiment.

2.4. Experimental Protocol

2.4.1. **Induction of Skin papillomas.**

Skin papillomas in male Swiss albino mice were induced using DMBA-TPA protocol (Dhawan *et al.,* 1999). Briefly, the hairs of mice dorsum were depilated with an electric clipper (Wahl clipper) followed by the application of depilatory cream (Jolene India Ltd - Mumbai) at least 48 h before the application of the DMBA to avoid hair regrowth. The mice that did not show signs of hair regrowth were used for the experiment. The shaved dorsum of animals was topically applied with 20 µg DMBA in 200 µl acetone/animal twice a week with a gap of 72 hours between the two applications for two weeks. This was followed with the application of 5.0 µg TPA in 200 µl acetone/animal twice a week until 24 weeks when the study was terminated.

2.4.2. **Experimental**

The chemopreventive activity of hesperidin was determined by dividing the animals into the following treatment groups:

2.4.2.1.1 **NORMAL (untreated) Group:**

Animals received topical application of acetone (0.2 ml/mouse) on the skin of shaved dorsum and were given 0.5 % CMC in 0.9 % saline in drinking water daily during the experimental period and served as control group.

2.4.2.2. **DMBA-TPA (Control) Group:**

The animals of this group received DMBA and TPA as described above for induction of skin papillomas and received no other treatment.

2.4.2.3. **HDP+DMBA-TPA (Pre treatment) Group:**

The animals of this group were given 100, 200, 300 and 400 mg/ml hesperidin two weeks before the 1st application of DMBA-TPA in acetone (0.2 ml/mouse) and daily thereafter, until the termination of experiment.

2.4.2.4. **DMBA-TPA+ HDP (Post treatment) Group:**

Animals of this group received 100, 200, 300 and 400 mg/ml hesperidin orally in drinking water after 1st application of DMBA-TPA application and until the termination of the experiment.

2.4.2.5. **HDP+DMBA-TPA+HPD (Continuous treatment) Group:**

Animals of this group received 100, 200, 300 and 400mg/ml hesperidin orally in drinking water 15 days before and after DMBA-TPA application and until the termination of the experiment.

Animals were weighed initially, then weekly and finally before autopsy. Papillomas appearing in the shaved area were recorded at weekly intervals and papillomas >1 mm in diameter were included in data analysis only, if they persisted for 2 weeks or more. Animals were sacrificed 24 weeks after the commencement of the treatments. The mice were euthanized under ketamine anesthesia, their dorsal skin/skin containing papillomas were removed surgically and washed in cold physiological saline. One part of skin/skin tumor was stored at -80°C for biochemical estimations, whereas the other part was stored in 10% buffered formalin for histopathological examination.

2.5 Histopathological examination

The fixed tumors were dehydrated sequentially in 50%, 70%, 80%, 90% alcohol and finally absolute alcohol and xylene. The tissue were transferred to molten paraffin wax for impregnation and then allowed to harden. The embedded tissues were cut into 5 µm thick sections using a rotary microtome (Leica RM 2125 RTS, Germany) and mounted on to the glass microslides (Axiva, New Delhi). The slides containing sections were processed and stained with hematoxylin and eosin for the histopathological examination. The slides were scored blindly by a pathologist under a transmitted light microscope (Leica DM 2500, Germany) and the tumors were classified according to well-established criteria.

2.6 ***Biochemical Assays***

For biochemical assays the tissues were homogenized.

2.6.1. Preparation tissue homogenate

The treated animals were sacrificed by euthanasia after 24 week of 1st application of DMBA. For biochemical studies, a known amount of skin tumors were washed in ice cold 0.9% saline and the tissue was minced into small pieces with the help of a scissors and forceps and 10% homogenate (W/V) was prepared in homogenizing

buffer (50mM phosphate buffer, pH 7.4) using a digital sonicator (PCI 500F analytics) as described earlier (Alam *et. al.*, 2000). The homogenates were centrifuged at 10,000 rpm at 4ºC for 20 min and stored at -20º C until further use for the biochemical estimations.

2.6.2. Estimation of reduced glutathione (GSH)

The GSH in skin papillomas was determined by the method of Moron *et. al.*, (1984). Briefly, 100 µl of sample homogenate (10% w/v) was mixed with 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (4 mg/1 ml) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm using a UV-VIS spectrophotometer ((Eppendorf Biospectrometer SW 3.5.1.0., Germany). The GSH concentration was calculated as µmol GSH/g tissue from a standard curve.

2.6.3. Glutathione S-transferase (GST) activity

The glutathione-S-transferase activity was measured by the method of Habig *et. al.*, (1974). Briefly, 100 µl sample homogenate, 2.4 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1.0 mM), 0.2 ml CDNB (1.0 mM) and 0.2 ml of cytosolic fraction was added in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and the enzymatic activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

2.6.4. Catalase (CAT) activity

The catalase activity was measured by the method described previously (Abei *et al.*, 1984). Briefly, the tissues were homogenized (10% w/v) in 50 mM phosphate buffer, pH 7.4, containing 1mM EDTA and 1mM PMSF. Catalase was estimated at 240 nm by monitoring the decrease of H_2O_2. In brief, the reaction mixture (1 ml) contained 0.02 ml of suitably diluted cytosolic sample in phosphate buffer (50 mM, pH 7.4) and 0.1ml of 30 mM H_2O_2 in phosphate buffer. Changes in absorbance were recorded at 240 nm using a UV-VIS double beam spectrophotometer every 30 seconds. The catalase activity was calculated in terms international unit (UI)/mg protein.

2.6.5. Superoxide dismutase

Total SOD activity was measured by the method of Fried (1975). Briefly, 900 µl buffer was mixed with 100 µl each of tissue homogenate, nitroblue tetrazolium (NBT), phenazine methosulphate and NADH. The control consisted of all the reagents except the homogenate, while the blank consisted of buffer and the homogenate without any reagents. The absorbance of sample, control and blank was

read at 560 nm using a UV-Visible Spectrophotometer and the enzyme activity has been expressed in units (1 U = 50% inhibition of NBT reduction).

2.6.6. Estimation of lipid peroxidation (TBARS)

The assay for lipid peroxidation was done according to the method of Wright *et. al.*, (1981). Lipid peroxidation in the microsomes was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, and is expressed in terms of malondialdehyde (MDA) formed per mg protein. In brief, 0.4 ml of homogenate was mixed with 1.6 ml of 0.15 M Tris KCl buffer, 0.5 ml of 30% TCA and 0.5 ml of 52 mM TBA and placed in a water bath for 45 min at 80°C. The tubes were removed, cooled in ice and centrifuged at room temperature for 10 min at 3,000 rpm. The absorbance of the clear supernatant was measured against the blank of distilled water at 538 nm in spectrophotometer. The content of MDA has been expressed as nmol/mg protein.

2.6.6. Protein estimation

The protein concentration in all samples was determined by the standard method (Lowry *et. al.,* 1951) using bovine serum albumin (BSA) as standard at 650 nm.

2.7. Statistical analyses

The level of significance in the alteration in the body weights after various treatments was determined using Student's t-test. The statistical significance for biochemical test was carried out using one-way analysis of variance (ANOVA) with the application of Tukey's Post-hoc test for multiple mean comparison wherever necessary.

3. RESULTS

The results are expressed as the mean ± standard error of the mean (SEM), wherever required and are presented as table 1-4 and Fig 1-7.

3.1. Effect of various doses of HPD treatment on body weight.

The body weights of mice ranged between 21.3 to 24.2 g at the beginning of the experiment. The average body weight increased in all groups with time and a highest increase was observed at 24th weeks except in SPS + DMBA + TPA group, where a sharp statistically significant decline was observed ($p<0.01$**)**. The HPD treatment had conducive effect as indicated by an increase in body weights in animals applied with carcinogen when compared with the DMBA-TPA alone group (Figure 2).

3.2. Effect of various doses of HPD treatment on tumor incidence

The chemo-preventive effect of HPD on DMBA-TPA induced tumors in mice is depicted in Figure 1 and Table 1. Application of DMBA-TPA caused appearance of skin papilloma after 6 weeks of first DMBA application. The evaluation of tumor incidence at the end of the experiment (24 week) showed 100% tumor incidence in DMBA-TPA controls, whereas HPD treatment resulted in a steady decline in the tumor incidence with increasing dose of HPD administration. Treatment of mice with HPD before, after and continuous prior to carcinogen application and thereafter up to 24 weeks resulted in a HPD dose dependent inhibition of skin papilloma (Figure1 and 2). HPD treatment reduced the average number of tumors in mice in a dose dependent manner and a maximum reduction of tumors ($p<0.01$) was observed for 300 mg/ kg body weight of HPD (Figure 1). Likewise, HPD treatment also reduced the tumor incidence in a dose dependent manner in comparison with DMBA-TPA control group and the lowest incidence was recorded for 300 mg/ kg body weight of HPD. The tumor incidence reduced to 26.61, 28.61, 45.61 and 35.72 % in the animals receiving 100, 200, 300 and 400 mg/kg body weight (HPD), respectively. The frequency of tumors reduced by 5.33, 6.74, 21.53 and 12.95 % in the animals administered with 100, 200, 300 and 400 mg/ml HPD in drinking water before and 19.48, 10.68, 24.37 and 25.33 % after the DMBA-TPA application. The frequency of tumors reduced by 5.49, 8.11, 15.44 and 13.01% in the animals receiving continuously 100, 200, 300 and 400 mg/ kg b. wt. HPD in drinking water (Table 1 and Figure 1).

3.3. Determination of antioxidant status

The effect of HPD treatment on DMBA-TPA induced oxidative stress was examined by determining various antioxidants in tumor tissue including glutathione, glutathione-S-transferase, catalase, superoxide dismutase and lipid peroxidation (Table 3 Figure 3 to 7).

3.3.1. Glutathione estimation

Induction of chemical carcinogenesis resulted in an approximate 3.2 folds reduction in the GSH concentration in the skin/skin tumors (Figure 3). This decline in GSH concentration was significantly higher ($p<0.01$) when compared with non-drug-non carcinogen treated control group. Administration of various doses of HPD to the animals receiving topical application of chemical carcinogens significantly increased the GSH levels in a dose dependent manner in all groups when compared to carcinogen treatment alone. The increased in GSH was approximately 1.5 fold

($p<0.01$) for 100, 200, 300 and 400 mg/kg body weight of HPD in drinking water before DMBA-TPA application. The post treatment of various doses of HPD enhanced the GSH approximately by 2.2 fold ($p<0.01$) for 100, 200, 300 and 400 mg/kg body weight, whereas the continuous treatment with 100, 200, 300 and 400 mg/kg body weight HPD in drinking water resulted an increased in GSH concentration by approximately 1.7 fold ($p<0.01$).

3.3.2. Glutathione S-transferase (GST) activity

Application of chemical carcinogens to mouse skin resulted in an approximate 3.8 folds ($p<0.01$) reduction in the GST activity in mouse skin/skin tumors at 24 weeks post-carcinogen treatment (Figure 4). HPD treatment resulted in an elevation in the GST activity when compared to the spontaneous level. The pretreatment of HPD in drinking water showed a significant increase in GST activity ($p<0.01$) at 24 weeks post-carcinogen treatment. The post treatment of HPD at a dose of 100, 200, 300 and 400 mg/kg b. wt. in drinking water revealed significant rise in the GST activity when compared to DMBA-TPA treatment alone and it was approximately 1.7 folds higher for this group, whereas the continuous treatment also enhanced GST activity ($p<0.01$) approximately 1.5 fold (Figure 4).

3.3.3. Catalase (CAT) activity

Application of DMBA-TPA on mouse skin resulted in a decline in the catalase activity approximately by 2.9 folds when compared to untreated group. However, administration of different doses of HPD to carcinogen treated mice led to a significant ($p<0.01$) elevation in the catalase activity in skin tumors at 24 weeks post-carcinogen treatment (Figure 5). The maximum elevation in the catalase activity was observed for 300 mg/kg b. wt. HPD in all the groups and this rise at this dose was approximately 1.6, 2.2 and 1.9 folds for pre, post and pre-post HPD treatment when compared to carcinogen treatment (Table 6).

3.3.4. Superoxide dismutase (SOD) activity

Chemical carcinogenesis significantly ($p<0.01$) reduced the activity of SOD in the skin tumors of mice at 24 weeks post-carcinogen treatment (Figure 6). Treatment of mice with different doses of HPD during carcinogenesis elevated SOD activity significantly ($p<0.01$) in the skin.skin tumors at 24 weeks post-carcinogen treatment in all the groups**.** Adminstration of different doses of HPD caused a dose dependent elevation in the SOD activity in all the groups up to 300 mg/kg b. wt. where the SOD activity was greatest. This increase in SOD activity was approximately 2.4, 3 and 1.9

folds higher at 300 mg/kg in pre, post and pre-post HPD treated groups when compared to DMBA-TPA treatment alone (Table 7).

3.3.5. Lipid peroxidation (TBARS)

Application of DMBA followed by TPA on mouse skin for the induction of chemical carcinogenesis caused a time dependent elevation in TBARS that were approximately 23 folds ($p<0.01$) higher in the skin/skin tumors after 24 weeks post-carcinogen treatment (Figure 7). Administration of 100, 200, 300 and 400 HPD mg/kg body weight in drinking water before, during and after carcinogen treatment significantly inhibited the induction of TBARS in the skin/skin tumors of mice in a dose dependent manner and the lowest lipid peroxidation was observed at 400 mg/kg in all the groups, except pre-post HPD group where it was greater than 300 mg/kg HPD (Table 8). The decline in lipid peroxidation was 1.7, 2 and 1.24 fold lowere than DMBA-TPA treatment in pre, post and pre-post HPD treatment at 400 mg/kg HPD (Table 8).

4. DISCUSSION

The increasing incidence of cancer and cancer induced mortality indicates that strategies are required to reduce the occurrence of cancer. Skin cancer is emerging as one of the common malignancies throughout the world as its incidence is increasing and the experimental models of skin carcinogenesis model provide a tool to understand the process of cancer development and devise strategies to control it in a preclinical setting (Abel *et al*., 2009; Neagu *et al*., 2016). If the cancer induction is suppressed by dietary or pharmacological intervention, it will be able to reduce the cancer occurrence worldwide. In fact vegetarian diet and several phytoceuticals have been found to reduce the risk of cancer development (Lampe, 2003; Pan and Ho, 2008). It may be worthwhile to explore the possible chemopreventive action of dietary ingredients in preclinical experimental models. Therefor,e the present study was undertaken to investigate the chemopreventive effect of hesperidin, a bioflavanoid present in various citrus fruits in two stage skin carcinogenesis in the Swiss albino mice.

The two stage cutaneous mouse model of skin carcinogenesis is an appropriate preclinical model to study the chemopreventive effect of any pharmacological agent (Abel *et al.*, 2009; Neagu *et al.*, 2016). The topical application of DMBA–TPA on mouse skin induced skin papillomas efficiently in all the mice who presented with tumors in the present study. DMBA-TPA application has been reported to induce skin

papillomas in various strains of mouse earlier (Frei and Stephens, 1968; Hennings *et. al.*, 1983; DiGiovani, 1992; Jifu *et. al.*, 1999; Coghlan *et. al.*, 2000; Singh *et. al.*, 2002; Dasgupta *et. al.*, 2003; Jagdeep *et. al.*, 2008; Abel *et al*., 2009; Neagu *et al*., 2016). Administration of different doses of HPD in drinking water to carcinogen treated mice significantly reduced the tumor incidence and average number of tumors. A recent study has reported suppression in benzo-a-pyrene induced lung carcinoma in mice **(**Bodduluru *et al*., 2015). Hesperidin has been reported to inhibit tumour initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts (Berkarda *et al.*, 1998; Tanaka *et al.*, 1997; Franke *et al*., 1998). Studies of rats treated with orange juice have shown a 22% reduction in colon cancer (Miyagi *et al*., 2000). Similarly, (−)-epi-gallocatechin-3-gallate, baicalein, genistein, oroxylin A, galangin and quercetin have been reported to be active against hepatocarcinogenesis (Liao *et al*., 2013). Another flavonoid, isorhamnetin has been found to inhibit colorectal cancer in mouse (Saud *et al*., 2013). Many other fruit, plant extracts and phytochemicals have been reported to exert chemopreventive action in vitro and in vivo (Birt, 2001; Jagetia *et. al.*, 2003; Jagetia and Reddy, 2005; Katiyar, 2005; Aggarwal *et. al.*, 2006; Xiao *et al*., 2013).

The chemopreventive effect of HPD may be due to the inhibition of initiation and promotion phases of carcinogenesis. Most in vivo studies using chemopreventive agents, reporting significant reduction in the tumor formation, have been followed only until week 20 (Lu *et. al.*, 1997), whereas we have followed it until 24 weeks. However, the percentage of inhibition was statistically significant until week 21 (47.4%; $P< 0.05$) when analysis were carried out for 21 and 24 week the difference between these two time points was statistically non-significant indicating that a maximum effect was reached by 21 weeks.

In multistep carcinogenesis, reactive oxygen species have been shown to play a role mostly in the promotion phase by sustained inflammation (Cerutti, 1985; Machlemater *et. al.*, 1988; Oberley and Oberley 1988; Perchellet and Perchellet, 1989; Sun, 1990; Oberley and Oberley, 1993; Closa and Folch-Puy, 2004), which is essential for cell proliferation and tumor progression. The inflammatory response induced by TPA changes the fidelity of genome by inducing additional mutations in the cell and offers advantage to the initiated cell to proliferate and form tumors (Rundhaug and Fischer, 2010). The development of tumors after DMBA-TPA application in the present study may be due to increased oxidative stress and sustained

inflammatory response as indicated by a sharp reduction in GSH, GST, CAT and SOD accompanied by increased lipid peroxidation. HPD treatment inhibited the DMBA-TPA-induced reduction in GSH, GST, CAT and SOD which may be directly related to reduction in tumor incidence and tumor multiplicity indicating that HPD has been instrumental in intervening oxidative stress-mediated carcinogenesis in this study. The GSH is a biological antioxidant present in high concentrations, and imparts protection against oxidative damage (Dhawan *et. al.,* 1999; Jagetia and Reddy, 2005; 2014; Jagetia and Rao., 2015). The reduced levels of GST have been correlated with tumor-induction provoked by DMBA/TPA treatment (Huang *et. al.,* 1997; Dhawan *et. al.,* 1999; Dasgupta *et. al.,* 2003). DMBA/TPA treatment has been reported to attenuate antioxidant enzymes including SOD and CAT in mice as well as squamous cell carcinomas (Oberly *et. al.,* 1993). Several reports suggest that GSH is a more efficient antioxidant agent than SOD or CAT (Artali *et. al.,* 2009). The GSH has been also reported to alter the profiles of lipoxygenase and cyclooxygenase (Bryant *et. al.,* 1982; Capdevila *et. al.,* 1995), which are involved in tumorigenesis. However, GSH has been found to be highly variable and contradictory, depending on the cell type, nature of the carcinogen and its modulatory pathways (Oberley and Oberley, 1988; Machlemater *et. al.,* 1988; Oberley and Oberley, 1993). Increase in the level of GSH by the chemopreventive action of flavonoids and other phytochemicals in mouse skin has also been reported earlier (Elangovan *et. al.,* 1994; Surh, 1999; Dasgupta *et. al.,* 2003; Kumar *et. al.,* 2006).

It is the combined effort of modulating antioxidant enzyme(s) that leads to a shift in the intracellular oxidation/reduction balance. This could lead to a changed cell and organ sensitivity to tumorigenesis induced by physical or chemical agents. Oxidative stress stimulates the production of MDA and/or other aldehydes in the biological systems. These oxidative products can react with amino acids and/or DNA introducing cross linkages between proteins and nucleic acids, and may also induce alterations in replication and transcription (Perchellet and Perchellet, 1984; Zhong and Yin, 2015) leading to tumor formation. Lipid peroxidation induced by reactive oxygen species (ROS) might be involved in tumor progression and promotion of carcinogenesis (Guyton and Kesler, 1993; Sánchez-Pérez *et al*., 2005; Zhong and Yin, 2015). Elevated levels of MDA were observed in skin tumors of animals treated with DMBA-TPA indicating the pivotal role played by oxidative stress in DMBA-TPA-induced mouse skin carcinogenesis. Earlier an identical effect has been observed in

the mouse skin model (Dhawan *et. al.,* 1999; Lee *et al*., 2012). Treatment of HPD in DMBA-TPA applied mice significantly lowered the oxidative stress as evidenced by reduced MDA levels and causing reduction in tumor production. Certain flavonoids and *Opuntia humifusa* have been reported to lower MDA levels in mouse skin/skin tumors earlier (Elangovan *et. al.,* 1994; Lee *et al*., 2012). This reduction in MDA level may be due to the increase in the antioxidant enzymes as indicated above. The use of antioxidants has been reported to act as protective agents against cancer (Kozoumbo *et al.,* 1983; Huang *et. al.,* 1997; Aggarwal and Shishodia, 2004; Katiyar, 2005; Jagetia, 2007).

The exact mechanism of action of hesperidin to suppress DMBA-TPA induced carcinogenesis is not well understood. It may have acted through multiple putative mechanisms to inhibit DMBA-TPA induced carcinogenesis. Hesperidin is known to inhibit free radical formation and this action of hesperidin may have inhibited the DMBA/TPA induced free radicals that may have subsequently reduced the mutagenic effect of latter reducing the tumnor incidence. The attrition of lipid peroxidation and increase in the GSH contents, GST, catalase and superoxide dismutase activity may have reduced the DMBA/TPA induced oxidative stress and inflammation, which are the main culprit during carcinogenesis and thereby reducing the tumor incidence. Although we have not investigated the molecular mechanisms, we believe hesperidin may have inhibited the transcriptional activation of NF-κB and COX-II, which play a major role in inflammation and cell proliferation, alleviating tumor incidence. Hesperidin and naringin have been found to inhibit the NF-κB, and COX-II activation (Hirata *et al*., 2005; Ghorbani *et al*., 2012; Parhiz *et al.,* 2015). The hesperidin may have upregulated the transcriptional activation of Nrf2 leading to increased activities of GST, catalase and SOD and GSH contents reducing the tumor incidence. Hesperidin has been reported to increase ERK/Nrf2 earlier (Chen *et al.,* 2010; Elavarasan *et al.,* 2012; Parhiz *et al*., 2015).

CONCLUSIONS

The chemopreventive action of HPD may be due suppression of free radical formation by DMBA/TPA increase in antioxidant enzymes and reduction in the oxidative stress as revealed by reduced lipid peroxidation in the HPD treated animals. This may have reduced neoplastic transformation and reduced the tumor incidence in the HPT treated groups. Hesperidin may have inhibited the transcriptional activation of NF-κb and COX-II involved in cell transformation by sustained inflammation reducing the tumor

incidence. It may have accelerated the transcriptional activation of ERK/Nrf2 signalling pathway and increased the antioxidant status as observed in the present study causing reduced tumor formation.

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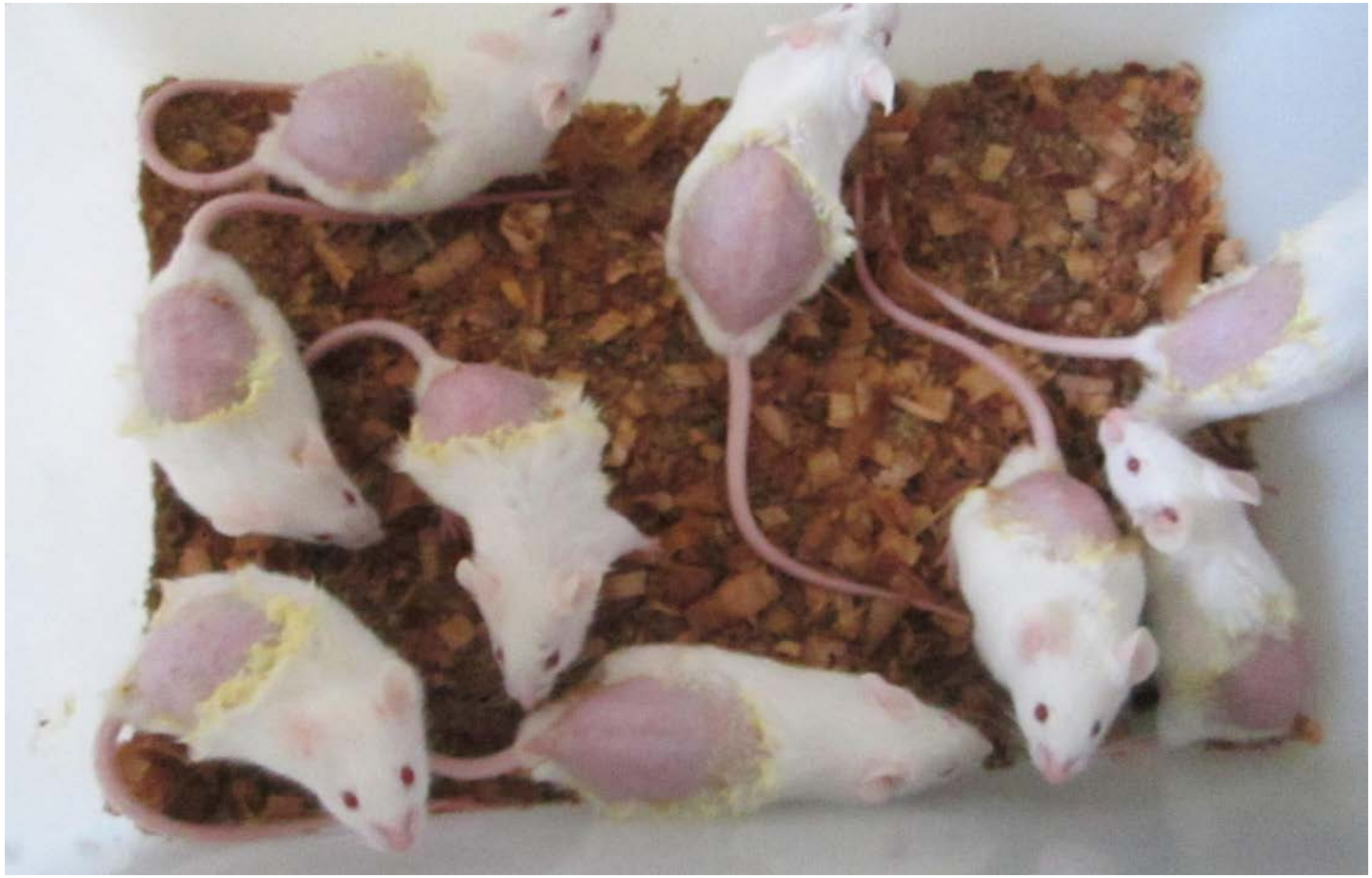


Plate 1: Shaved dorsum of Swiss albino mice prior to DMBA-TPA application.
The animal did not show any sign of regrowth of hairs before the oo set of experiment.

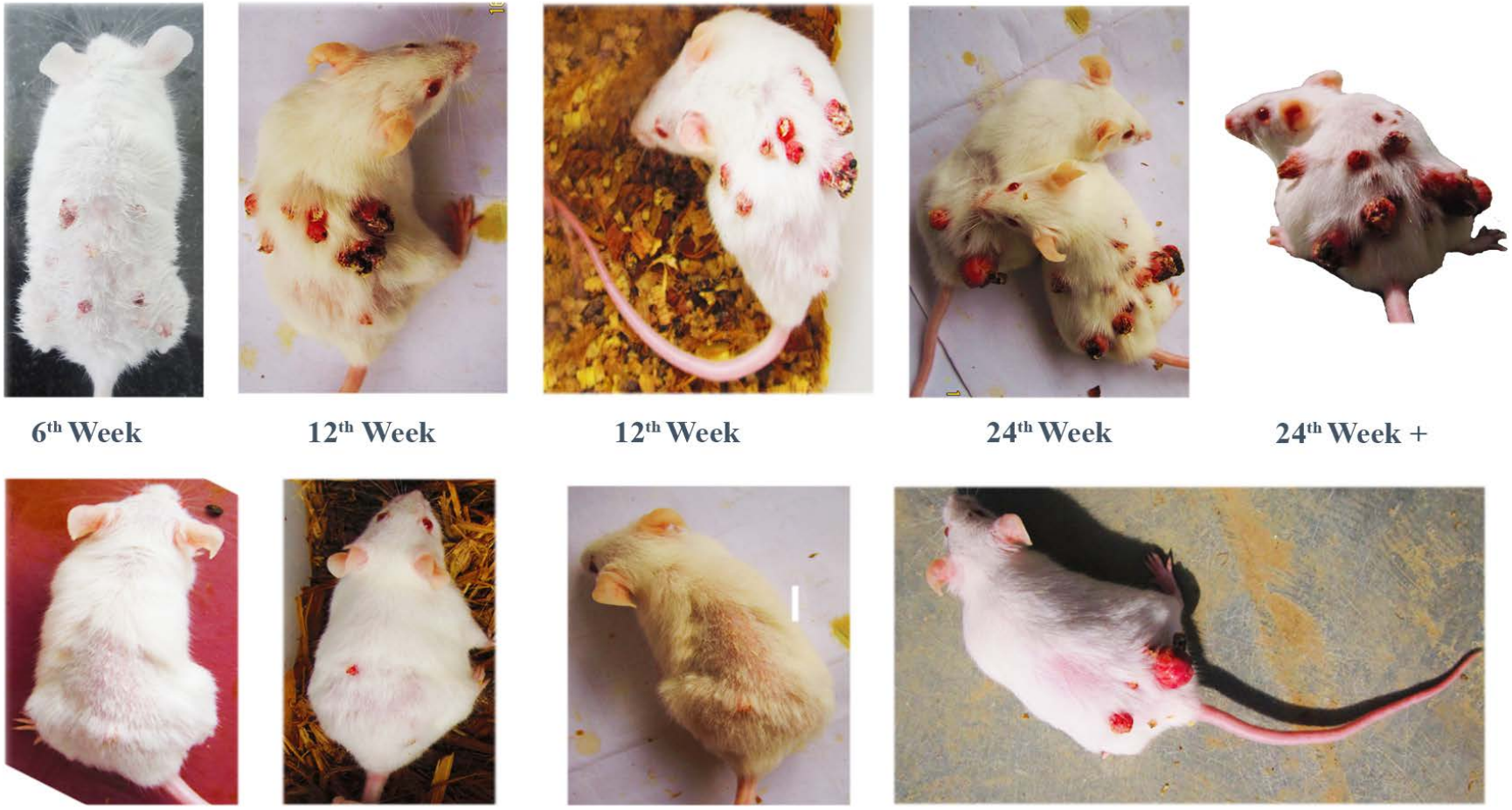
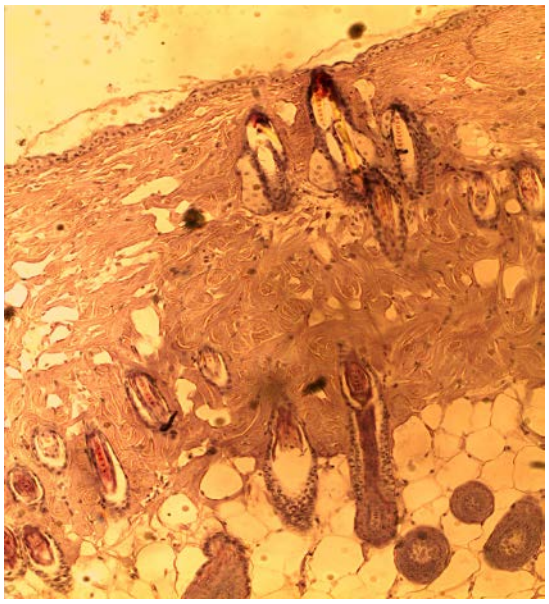
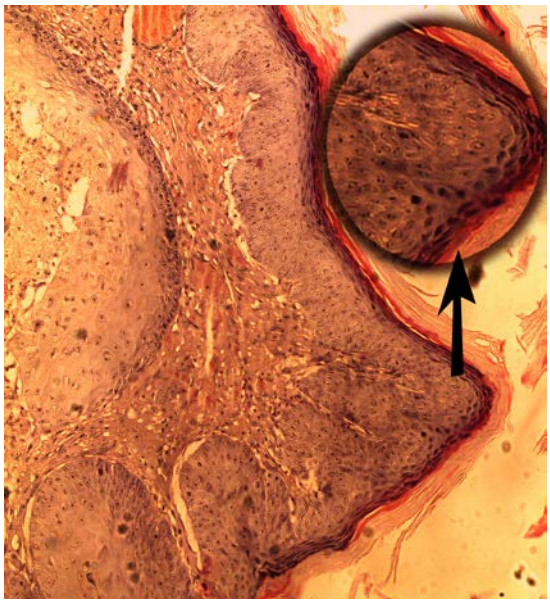


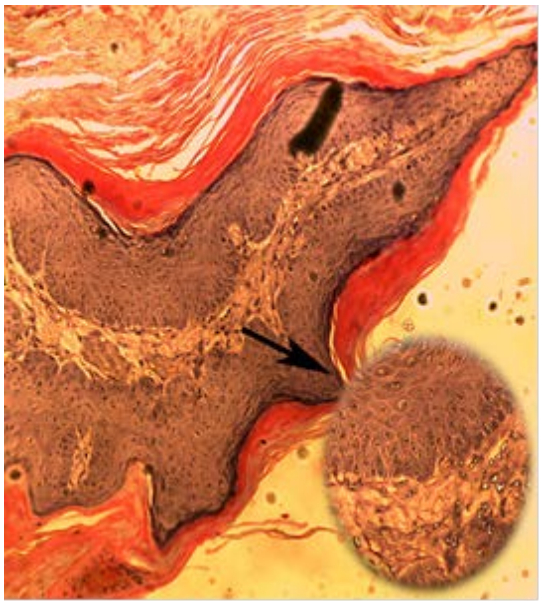
Plate 2: The Swiss albino mice showing papilloma induced by DMAB-TPA with or without hesperidin treatment .The mice developed papilloma after 6th Week of DMBA-TPA application. Upper panel: DMBA-TPA alone and Lower panel: Hesperidin treatment.



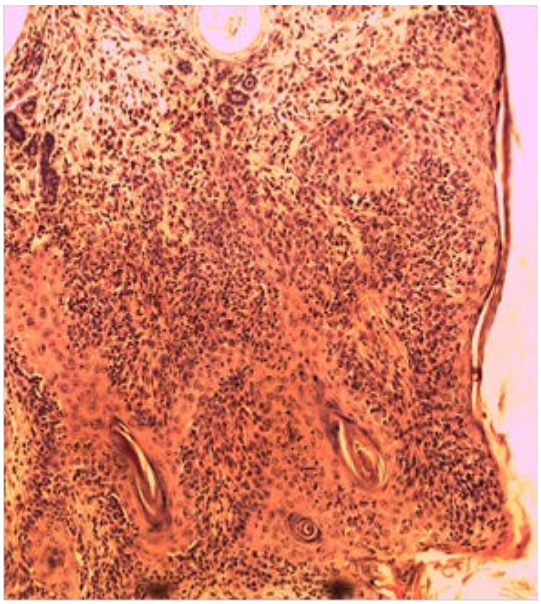
Normal skin



Papilloma



Hyperplastic epidermis with papilloma



Squamous cell carcinomas (SCC)

Plate 3: Cross section of skin and skin papilloma on 24th week post DMBA-TPA application representing malignant transformation in skin of Swiss albino mice.

Table 1: Alteration in the body weight of mice receiving topical application of DMBA-TPA with or without various doses of hesperidin orally

Body weight in grams (Mean ±SEM)														
Treatment type														
Weeks	SPS	SPS+ DMBA-TPA	HPD+DMBA-TPA				DMBA-TPA+HPD				HPD+DMBA-TPA+HPD			
			100	200	300	400	100	200	300	400	100	200	300	400
1	24.0±1.2	23.0±1.4	23.3±1.4	23.3±1.4	22.4±1.2	21.3±1.4	22.4±1.2	23.0±1.4	23.3±1.2	23.6±1.2	23.3±1.3	23.0±1.3	24.2±1.2	21.3±1.4
4	25.8±1.3	24.0±1.7	24.6±1.5	24.4±1.7	23.8±1.3	23.6±1.5	26.2±1.4	24.2±1.7	26.2±1.4	26.2±1.5	24.6±1.4	24.0±1.7	25.4+1.3	23.6±1.5
8	27.0±1.5	25.7±1.5	26.7±1.6	26.7±1.5	25.8±1.5	25.7±1.6	27.3±1.6	27.9±1.5	27.3±1.6	27.3±1.7	26.7±1.2	27.7±1.8	27.3±1.5	24.7±1.6
12	29.8±1.4	26.3±1.4*	29.6±1.3	28.5±1.4	26.9±1.4	28.6±1.3	29.2±1.3	29.5±1.4	28.2±1.3	29.2±1.3	29.6±1.3	29.5±1.4	29.6±1.4	28.6±1.3
16	32.6±1.5	26.6±1.5^{a}	31.5±1.6	32.5±1.5^{c}	28.6±1.5	31.5±1.6	31.5±1.6	32.5±1.5^{c}	32.6±1.6^{c}	32.6±1.4^{c}	31.5±1.5	30.5±1.5	30.5±1.5	30.5±1.6
20	35.8±1.6	25.7±1.6$^{\clubsuit}$	33.4±1.4*	33.4±1.6^{c}	30.9±1.6*	30.5±1.4*	32.4±1.6*	33.2±1.6*	33.4±1.6*	33.0±1.2*	33.4±1.4*	32.3±1.2*	32.7±1.3*	33.8±1.4
24	37.6±1.3	25.4±1.3$^{\clubsuit}$	34.6±1.5^{*c}	36.3±1.3^{c}	30.7±1.3^{*c}	30.2±1.5*	34.3±1.3^{*c}	33.7±1.3^{*c}	33.6±1.2^{*c}	34.4±1.3^{*c}	34.6±1.3^{*c}	35.4±1.3^{c}	34.6±1.9	35.6±1.8

**P<0.05, ^{a}p<0.01, $^{\clubsuit}$p<0.001 when compared to SPS group.*

^{c}p<0.05 when treatment are compared to DMBA-TPA group.

No symbol=no significant difference.

Standard error of the mean (SEM).

N=10.

Table 2: Alteration in the DMBA-TPA induced tumor induction in Swiss Albino mice administered with various doses of hesperidin (mg/kg. body weight).

Time in weeks	SPS+ DMBA-TPA	Number of papilloma (Mean ± SEM)											
		HPD+DMBA-TPA				DMBA-TPA+HPD				HPD+DMBA-TPA+HPD			
		100	200	300	400	100	200	300	400	100	200	300	400
6	3.3±0.05	2.1±0.1*	1.2±0.2*	0	0	0	0	0	0	0	0	0	0
7	3.54±0.02	3.19±0.09*	2.3±0.15*	0.97±.01$^{\alpha}$	2.2±0.15*	1.09±0.09*	1.02±0.2*	0	0	1.2±0.24*	1.2±0.16*	1.8±0.15*	2.1±0.48*
8	4.45±0.94	3.53±0.15	2.57±0.16*	0.99±0.01$^{\alpha}$	2.5±0.16	2.3±0.15*	1.1±0.16	0	0	3.4±0.49*	2.7±0.45*	2.41±0.13*	2.6±0.49*
9	6.56±0.95	4.3±0.15*	3.9±0.17 $^{\alpha}$	2.1±0.01$^{\alpha}$	2.8±0.16*	3.3±0.15*	1.9±0.17 $^{\alpha}$	0.9±0.10 $^{\alpha}$	1.6±0.16$^{\clubsuit}$	4.5±0.67*	3.5±0.44*	2.82±0.14 $^{\alpha}$	3.1±0.40*
10	8.42±0.83	5.45±0.17*	4.41±0.11 $^{\alpha}$	2.85±0.01$^{\alpha}$	3.3±0.16^{a}	4.45±0.17*	3.12±0.11 $^{\alpha}$	1±0.00 $^{\alpha}$	2.3±0.16 $^{\alpha}$	5.8±0.26*	4.3±0.11*	3.54±0.02 $^{\alpha}$	4.01±0.48*
11	10.76±0.92	6.6±0.22*	5.94±0.15*	4.1±0.02$^{\alpha}$	4.46±0.16^{a}	5.6±0.22*	3.94±0.15 $^{\alpha}$	1.3±0.15$^{\clubsuit}$	2.4±0.16 $^{\alpha}$	6.92±0.14*	6.4±0.13*	3.76±0.24 $^{\alpha}$	5.7±0.64*
12	12.24±1.2	8.72±0.21*	6.4±0.16 $^{\alpha}$	4.5±0.16 $^{\alpha}$	6.2±0.03^{a}	6.4±0.21*	5.4±0.16 $^{\alpha}$	1.4±0.16$^{\clubsuit}$	2.5±0.17 $^{\alpha}$	7.4±0.48*	7.6±0.22*	4.49±0.13 $^{\alpha}$	6.6±0.48*
13	14.54±1.4	9.4±0.26*	8.37±0.15 $^{\alpha}$	4.9±0.27 $^{\alpha}$	8.2±0.01*	7.6±0.26*	6.37±0.15 $^{\alpha}$	1.8±0.24$^{\clubsuit}$	2.9±0.27 $^{\alpha}$	9.76±0.64*	8.7±0.45*	4.81±0.38 $^{\alpha}$	7.1±0.70*
14	15.45±1.11	10.6±0.26*	9.8±0.29 $^{\alpha}$	5.8±0.35$^{\alpha}$	9.4±0.02*	9.5±0.26 $^{\alpha}$	7.8±0.29 *	2.3±0.24$^{\clubsuit}$	3.82±0.35 $^{\alpha}$	10.8±0.60*	9.56±0.89*	5.44±0.49$^{\clubsuit}$	8.9±0.94*
15	16.23±1.32	12.16±0.26*	10.43±0.29 $^{\alpha}$	7.54±0.35$^{\alpha}$	10.2±0.04*	10.95±0.27*	8.43±0.29*	3.3±0.25$^{\clubsuit}$	4.58±0.35 $^{\alpha}$	11.9±0.53*	10.82±0.60*	5.84±0.33$^{\clubsuit}$	9.9±0.94*
16	16.73±1.09	14.6±0.26*	12.44±0.28 $^{\alpha}$	8.24±0.35$^{\alpha}$	10.53±0.03*	11.6±0.27 $^{\alpha}$	9.874±0.29*	4.3±0.24$^{\clubsuit}$	5.78±0.36 $^{\alpha}$	13.8±0.60*	11.45±0.70$^{\alpha}$	6.5±0.81$^{\clubsuit}$	10.6±0.89*
17	16.96±1.03	15.23±0.27*	12.85±0.29 $^{\alpha}$	9.54±0.36$^{\alpha}$	10.76±0.03*	11.86±0.27$^{\alpha}$	10.41±0.29*	5.3±0.24$^{\clubsuit}$	6.68±0.35 $^{\alpha}$	14.7±0.64*	12.12±0.70	7.5±0.20$^{\clubsuit}$	11.65±0.94*
18	17.21±1.12	15.86±0.26	13.48±0.29*	10.25±0.36$^{\alpha}$	10.98±0.10*	12.36±0.27*	11.48±0.28*	6.3±0.23$^{\clubsuit}$	7.85±0.35 $^{\alpha}$	15.45±0.64*	13.9±0.70*	8.3±0.78$^{\clubsuit}$	12.68±1.07*
19	17.42±1.2	16.36±0.26	14.28±0.29*	10.68±0.36$^{\alpha}$	11.5±0.20*	12.63±0.26$^{\alpha}$	12.28±0.29*	7.3±0.24$^{\clubsuit}$	9.88±0.35 $^{\alpha}$	15.75±0.64*	14.39±0.70*	9.5±0.92 $^{\alpha}$	13.86±1.26*
20	18.67±0.94	16.32±0.27	15.26±0.29*	11.8±0.35$^{\alpha}$	12.3±0.40 *	13.32±0.15$^{\alpha}$	12.58±0.29$^{\alpha}$	8.3±0.24$^{\clubsuit}$	10.68±0.36*	16.4±0.48	14.52±0.71*	10.4±0.66 $^{\alpha}$	14.43±1.13*
21	18.73±1.24	16.54±0.42	15.58±0.24*	11.85±0.35*	12.9±0.42 *	14.25±0.27$^{\alpha}$	12.86±0.27$^{\alpha}$	9.3±0.24$^{\clubsuit}$	10.87±0.35*	16.45±0.77	15.21±0.89*	10.82±0.66$^{\alpha}$	14.61±1.26*
22	18.84±1.23	17.53±0.4	16.42±0.23*	12.48±0.35*	14.02±1.2*	14.53±0.42$^{\alpha}$	13.29±0.29 *	10.3±0.22$^{\clubsuit}$	11.58±0.35*	17.1±0.94	15.58±0.74*	11.34±0.66$^{\alpha}$	14.82±0.94*
23	18.89±1.3	17.82±0.28	16.62±0.29	12.51±0.28*	14.28±1.1*	14.82±0.40$^{\alpha}$	13.52±0.29*	10.6±0.24$^{\clubsuit}$	12.80±0.35*	17.5±1.02	16.9±0.70*	12.7±0.90 $^{\alpha}$	15.50±0.81*
24	18.94±1.23	17.93±0.4	16.72±0.23	13.12±0.36*	14.82±1.2*	15.25±0.28*	13.62±0.24*	10.3±0.22$^{\clubsuit}$	12.91±0.35*	17.9±0.71	16.45±0.83*	13.90±0.78$^{\alpha}$	15.72±0.89*

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001 when treatment group are compared to DMBA-TPA groups.*

No symbol=no significant difference. Standard error of the mean (SEM). N=10.

Table 3: Chemopreventive effect of various doses of hesperidin on DMBA-TPA -induced skin tumor in Swiss Albino mice.

Assay Time (Weeks)	Tumor inhibition (percent ±SEM)											
	SPS+DMBA-TPA	Hesperidin mg/kg body weight											
		HPD+DMBA-TPA				DMBA-TPA+HPD				HPD+DMBA-TPA+HPD			
		100	200	300	400	100	200	300	400	100	200	300	400
6	0	36.36*	63.64^{a}	100^{a}	100^{a}	100	100^{a}	100^{a}	100	100	100	100	100
9	0	34.45*	40.54*	67.84^{a}	57.32^{a}	48.93*	70.78$^{\clubsuit}$	86.12$^{\clubsuit}$	74.69$^{\clubsuit}$	30.38*	45.97	56.95$^{\clubsuit}$	52.13$^{\clubsuit}$
15	0	25.07*	35.73*	53.51^{a}	37.15*	32.53*	48.04*	79.51$^{\clubsuit}$	71.75$^{\clubsuit}$	26.35*	33.33	63.99$^{\clubsuit}$	38.42*
19	0	12.84	23.92*	43.06*	38.70*	32.69*	34.53	60.89$^{\clubsuit}$	47.34^{a}	16.06*	23.29	48.89	26.12*
24	0	04.42	10.87	30.06*	21.00*	18.67	27.37	44.98*	31.16*	4.205	12.27	25.86*	16.15*

**P<0.05, ^{a}p<0.01, $^{\clubsuit}$p<0.001 when treatment groups are compared to DMBA-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).* N=10.

Table 4: Alteration in the glutathione concentration in the skin papilloma of mice topically applied with DMBA-TPA with or without different doses of hesperidin administration.

Hesperidin (mg/kg)	GSH (µmol) Mean ±SEM		
	HPD+DMBA-TPA	DMBA-TPA +HPD	HPD+DMBA-TPA+HPD
SPS	**9.76±0.29**	**9.76±0.29**	**9.76±0.29**
DMBA-TPA	2.48±0.11*	2.48±0.11*	2.48±0.11*
100	3.37±0.05$^{\#}$	4.29±0.18$^{\#}$	4.61±0.09^{a}
200	4.73±0.02^{a}	5.26±0.21^{a}	5.30±0.08^{a}
300	5.49±0.05^{a}	5.56±0.09^{a}	8.81±0.03^{a}
400	5.55±0.11^{a}	5.32±0.31^{a}	8.07±0.01^{a}

***p<0.001, when untreated (SPS) are compared to DMBA-TPA. $^{\#}$p<0.05, ^{a}p<0.01, $^{\clubsuit}$p<0.001when treatment groups are compared to DMB-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).* N=10

Table 5: Alteration in the glutathione-s-tranferase activity in the skin papilloma of mice topically applied with DMBA-TPA with or without different doses of hesperidin administration.

Hesperidin (mg/kg)	GST (U/mg protein) Mean ±SEM		
	HPD+DMBA-TPA	DMBA-TPA +HPD	HPD+DMBA-TPA+HPD
SPS	**4.90±0.08**	**4.90±0.08**	**4.90±0.08**
DMBA-TPA	1.29±0.03*	1.29±0.03*	1.29±0.03*
100	1.05±0.01	1.88±0.04a	1.54±0.01a
200	1.29±0.03	1.92±0.06a	1.63±0.05a
300	1.31±0.02#	2.62±0.04♣	2.36±0.09♣
400	1.36±0.02#	2.37±0.02♣	2.29±1.04♣

**p<0.001, when untreated (SPS) are compared to DMBA-TPA.*
#p<0.05, ap<0.01, ♣p<0.001when treatment groups are compared to DMB-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).
N=10.

Table 6: Alteration in the catalase activity in the skin papilloma of mice topically applied with DMBA-TPA with or without different doses of hesperidin administration.

Hesperidin (mg/kg)	CAT (U/mg protein) Mean ±SEM		
	HPD+DMBA-TPA	DMBA-TPA +HPD	HPD+DMBA-TPA+HPD
SPS	**12.0±0.157**	**12.0±0.157**	**12.0±0.157**
DMBA-TPA	4.11± 0.17*	4.11±0.176*	4.11±0.176*
100	3.00±0.15	4.66±0.51	4.33±0.50a
200	4.32±0.20a	7.22±0.38♣	7.08±0.21♣
300	6.66±0.30a	9.11±1.15♣	7.66±1.71♣
400	6.55±0.38a	8.22±0.76♣	6.88±1.29♣

**p<0.001, when untreated (SPS) groups are compared to DMBA-TPA.*
#p<0.05, ap<0.01, ♣p<0.001when treatment groups are compared to DMB-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).
N=10

Table 7: Alteration in the superoxide dismutase activity in the skin papilloma of mice topically applied with DMBA-TPA with or without different doses of hesperidin administration.

Hesperidin (mg/kg)	SOD (U/mg protein) Mean ±SEM		
	HPD+DMBA-TPA	**DMBA-TPA +HPD**	**HPD+DMBA-TPA+HPD**
SPS	**12.78±0.05**	**12.78±0.05**	**12.78±0.05**
DMBA-TPA	3.42±0.14*	3.42±0.14*	3.42±0.14*
100	5.05±0.03^{a}	8.78±1.14♣	3.32±0.31
200	6.38±0.57^{a}	9.42±0.04♣	3.94±0.32
300	8.20±0.21♣	10.63±0.11♣	6.54±0.15^{a}
400	8.04±0.12♣	10.10±0.04♣	8.06±0.19♣

**p<0.001, when untreated (SPS) group are compared to DMBA-TPA. $^{\#}$p<0.05, ^{a}p<0.01, ♣p<0.001when treatment groups are compared to DMB-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).* N=10.

Table 8: Alteration in the lipid peroxidation in the skin papilloma of mice topically applied with DMBA-TPA with or without different doses of hesperidin administration.

Hesperidin (mg/kg)	LOO (nmol/mg protein) Mean ±SEM		
	HPD+DMBA-TPA	**DMBA-TPA +HPD**	**HPD+DMBA-TPA+HPD**
SPS	**1.02±0.17**	**1.02±0.17**	**1.02±0.17**
DMBA-TPA	23.56±0.82*	23.56±0.82*	23.56±0.82*
100	22.14±0.30	17.34±1.99$^{\#}$	21.96±0.28
200	14.80±0.08^{a}	13.53±0.07♣	22.44±0.73
300	14.21±0.79♣	12.64±0.70♣	12.91±0.37♣
400	13.79±0.52♣	11.83±0.46♣	19.04±0.62$^{\#}$

**p<0.001, when untreated (SPS) groups are compared to DMBA-TPA. $^{\#}$p<0.05, ^{a}p<0.01, ♣p<0.001when treatment groups are compared to DMB-TPA group. No symbol=no significant difference. Standard error of the mean (SEM).* N=10.

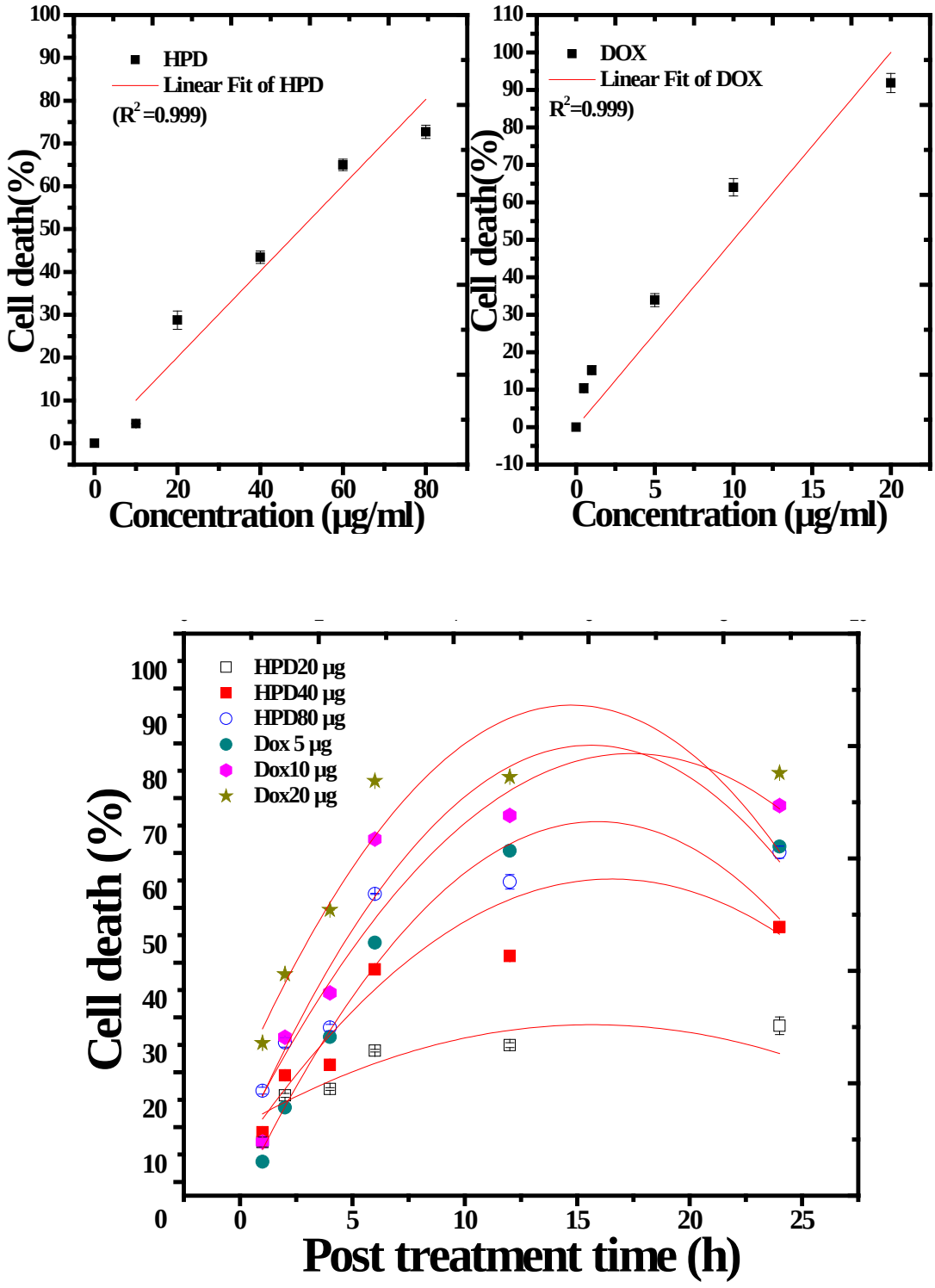


Figure 1: Cytotoxic effect of different concentrations of HPD and DOX in cultured human epidermoid carcinoma A431 cells by MTT assay. Upper panel: effect of different concentrations of HPD (left)or DOX (right) and lower panel: Effect of different exposure times. N=3.

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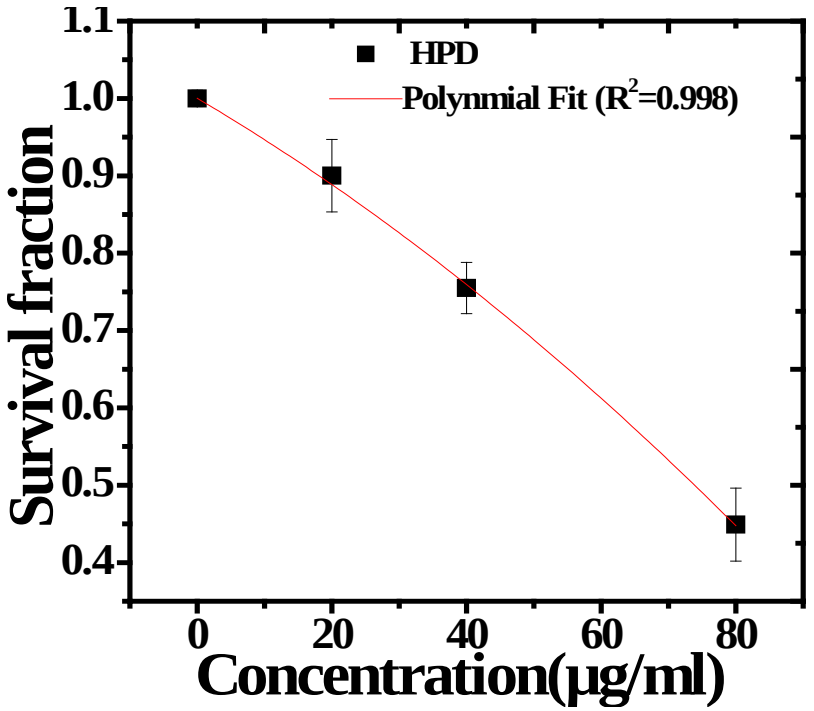

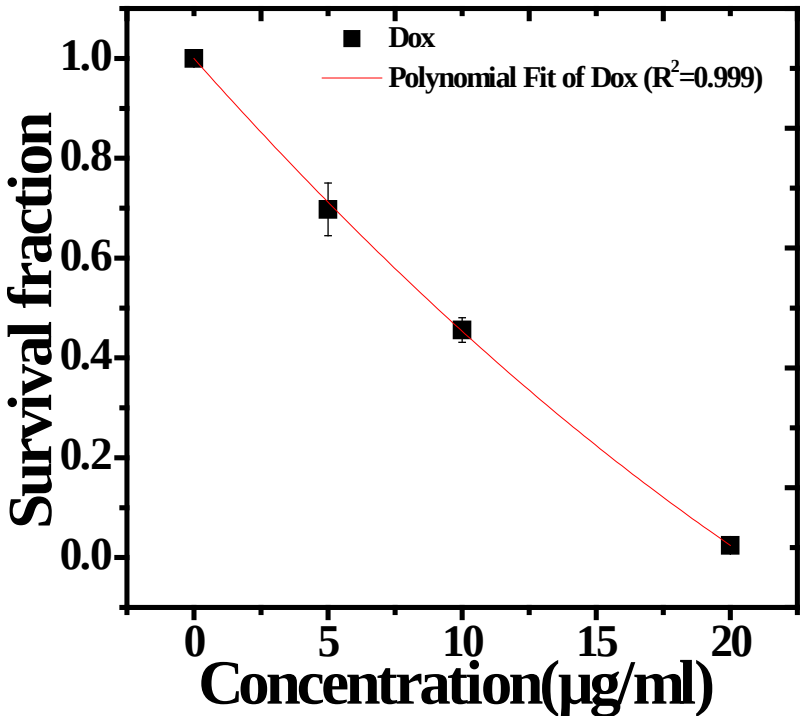

Figure 2: Effect of different concentrations of HPD and DOX on the clonogenicity of cultured human epidermoid carcinoma A431 cells. N=3, R^2=0.99.

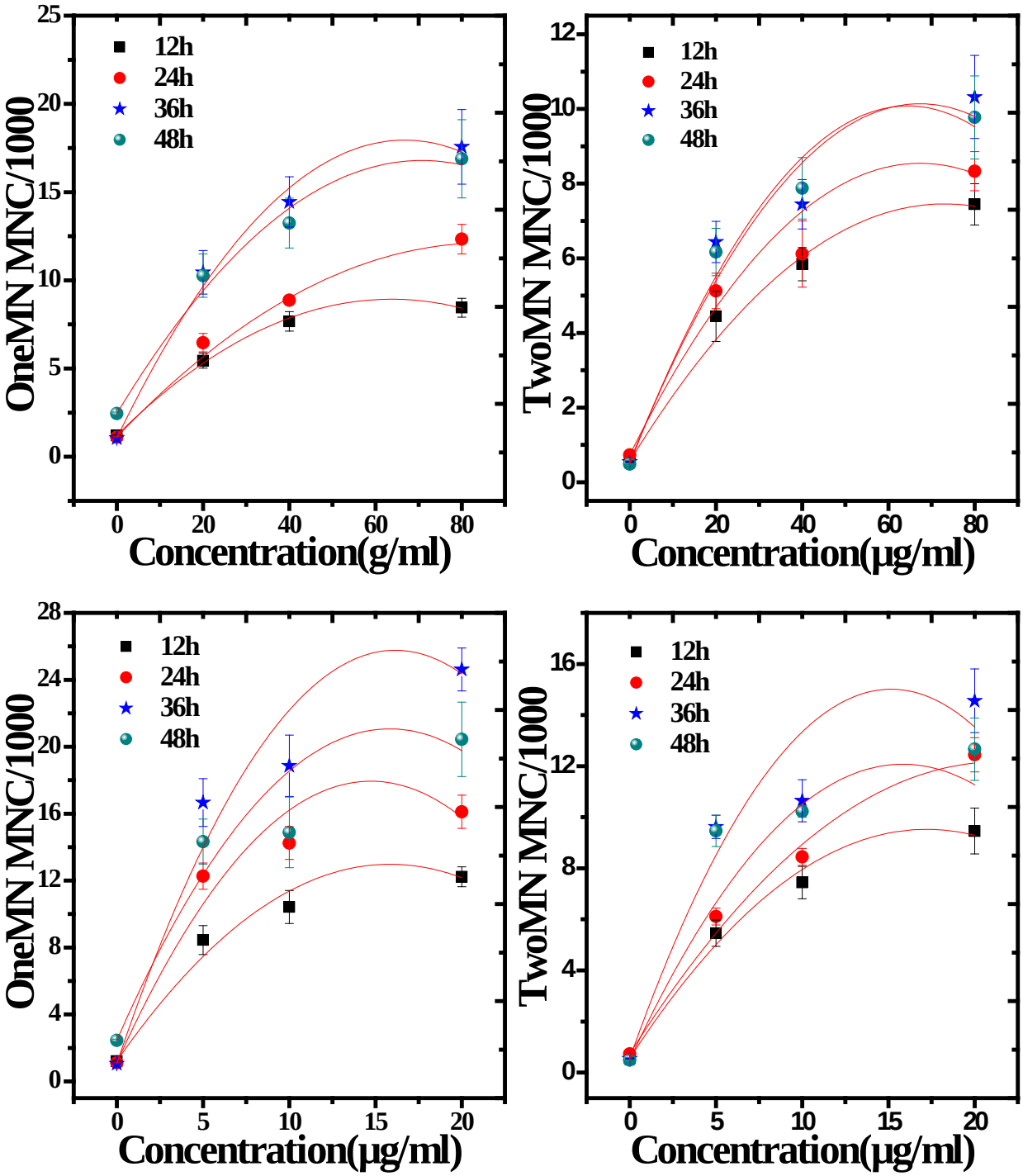

Figure 3: Effect of different concentrations of HPD or DOX on the induction of micronuclei in the mononucleated cell (MNMNC) at different treatment post treatment times in the cultured human epidermoid carcinoma A431 cells. The data represent Mean±SEM, n=3, $p<0.05$.

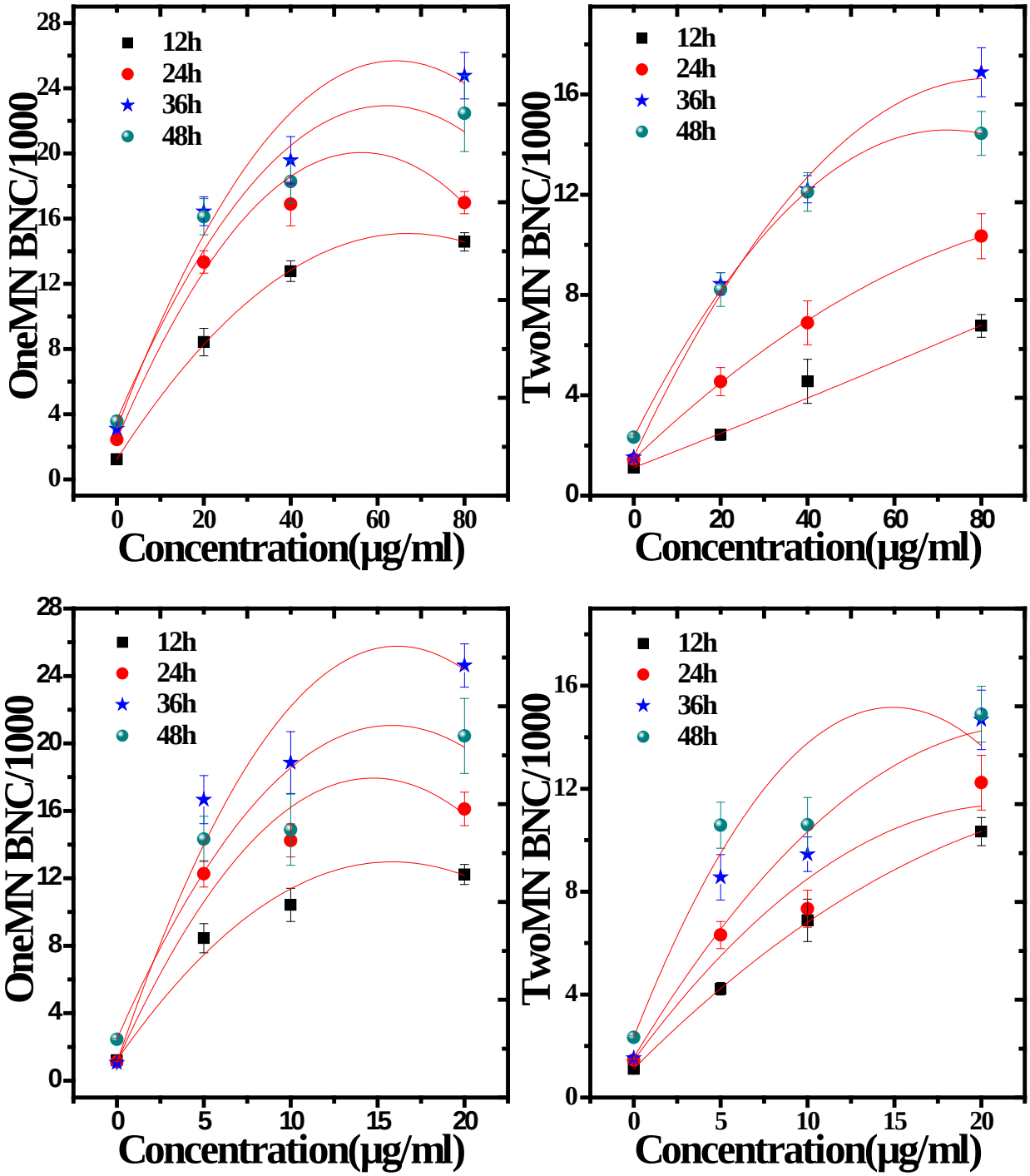

Figure 4: Effect of different concentrations of HPD or DOX on the micronuclei induction in the binucleated cells (MNBNC) at different treatment post treatment times in the cultured human epidermoid carcinoma A431 cells. The data represent Mean ±SEM, n=3, $p<0.05$.

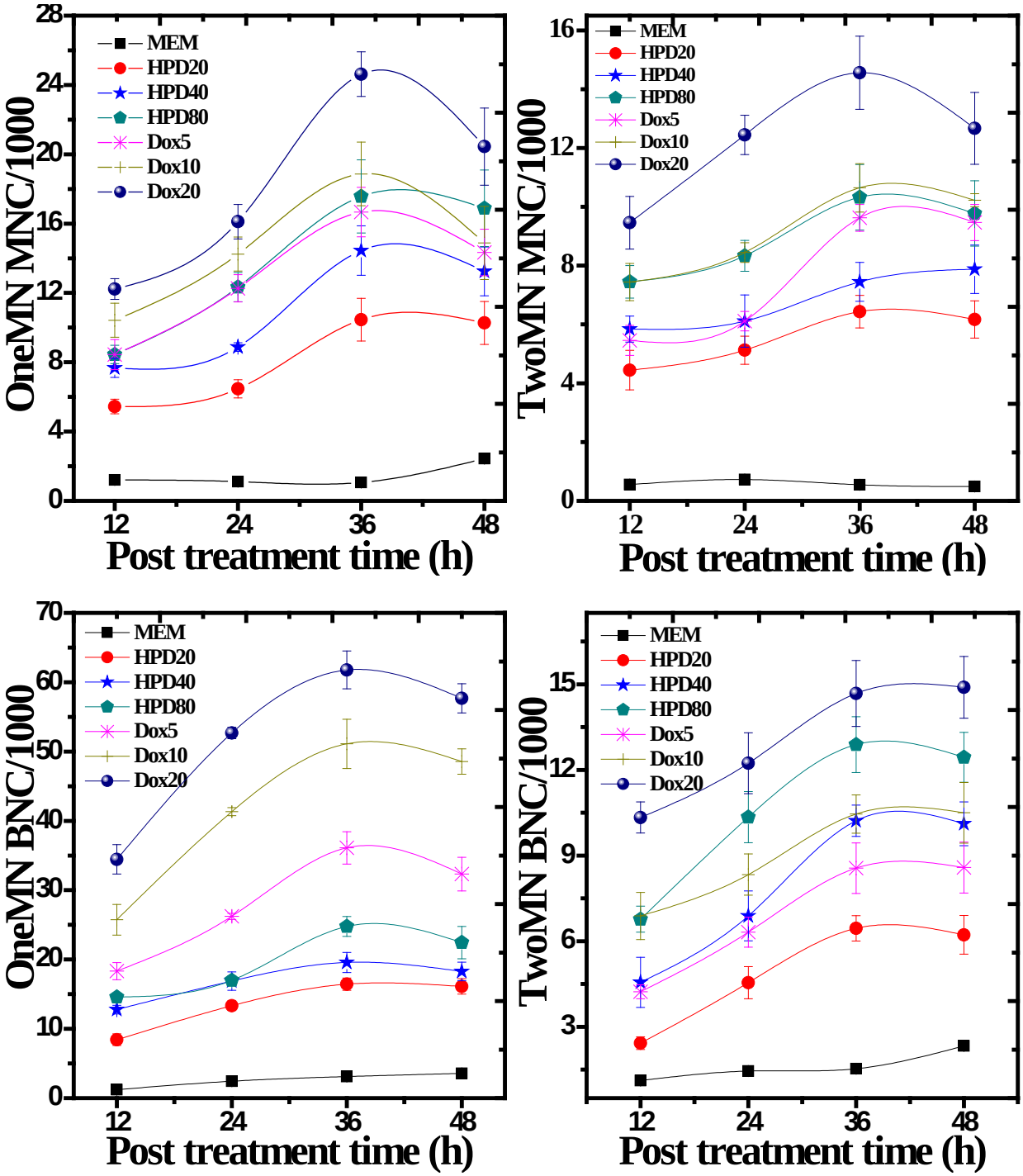

Figure 5: Alteration in the frequency of on micronuclei with time in mononucleate (upper panel) and binucleate cells (lower panel) at different post treatment times in the cultured human epidermoid carcinoma A431 cells. The data represent Mean ±SEM, N=3, $p<0.05$.

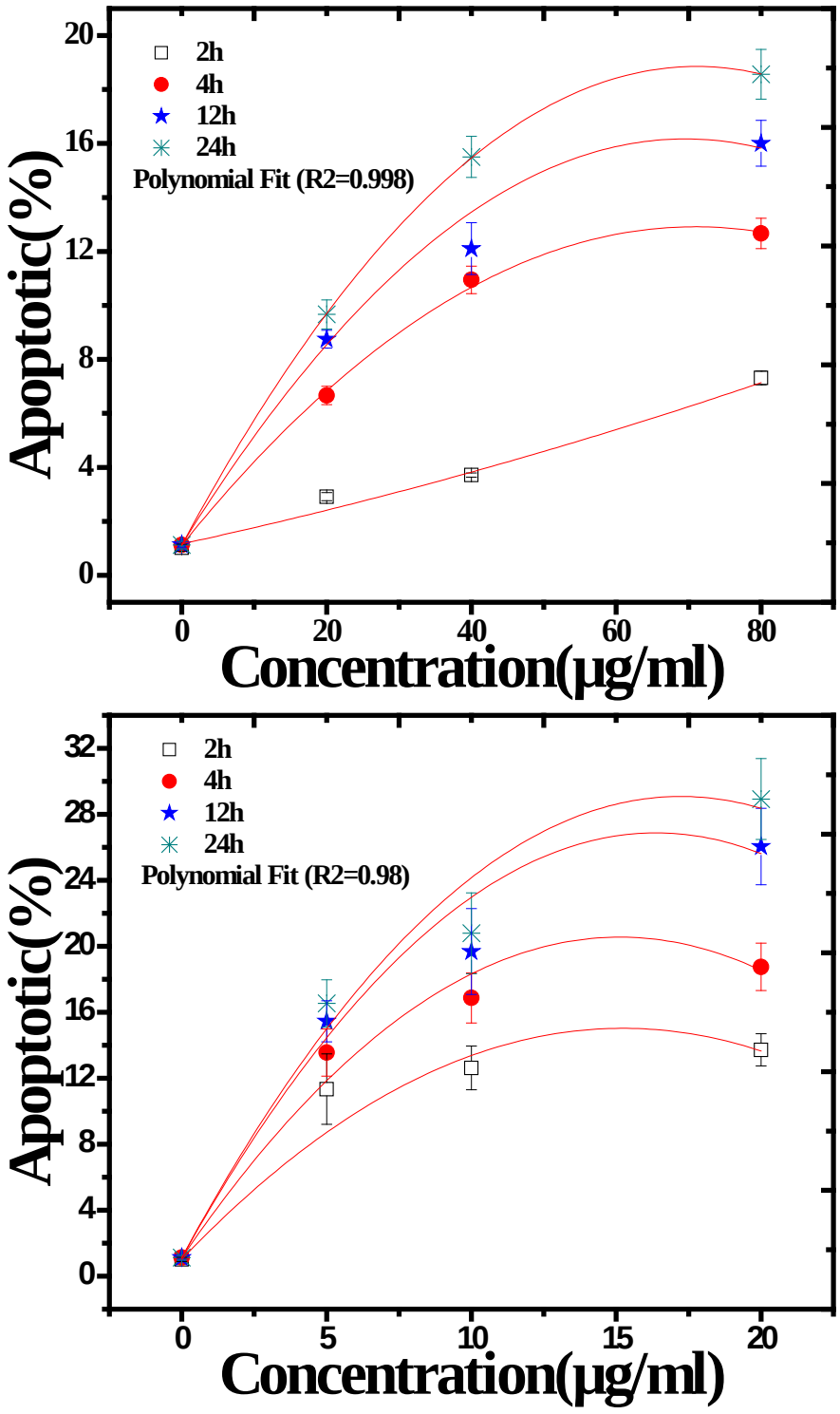

Figure 6: Effect of different concentrations of HPD or DOX on apoptosis induction at different post treatment times in the cultured human epidermoid carcinoma A431 cells. The data represent Mean ±SEM, n=3, R^2=0.98.

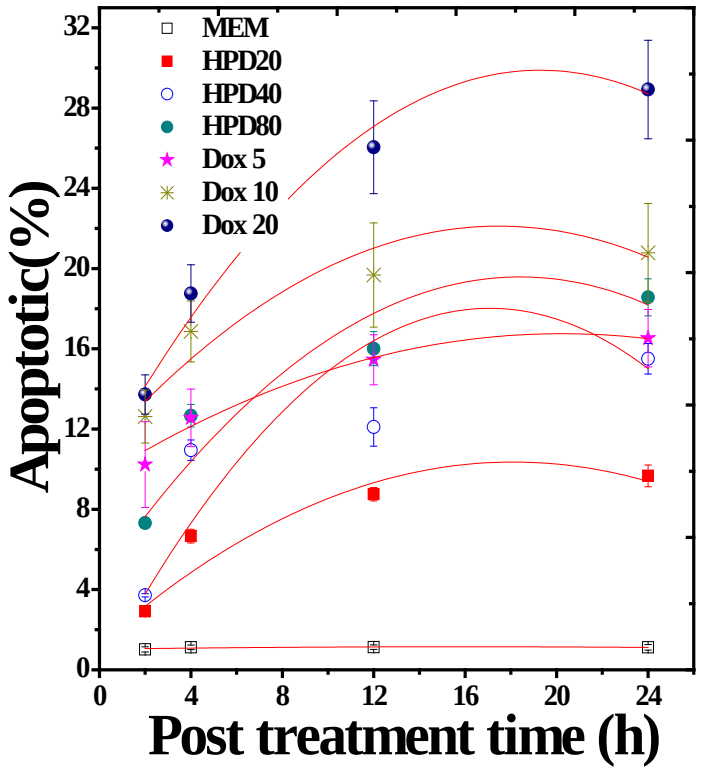

Figure 7: Alteration in the apoptosis index at different post-treatment times in the cultured human epidermoid carcinoma A431 cells treated with HPD or DOX. N=3, R^2=0.98.
The data represent Mean ±SEM, n=3, p<0.05.

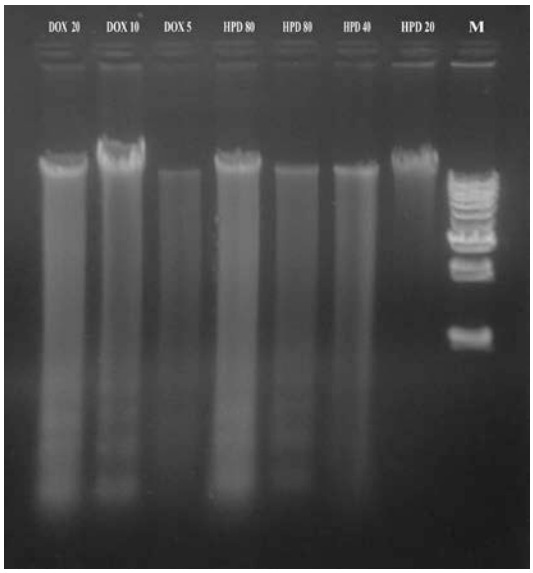

Figure 8: DNA fragmentation in A431 cells after treatment with different concentrations of HPD or DOX formed the ladder like patterns at 6 h post drug treatment times.

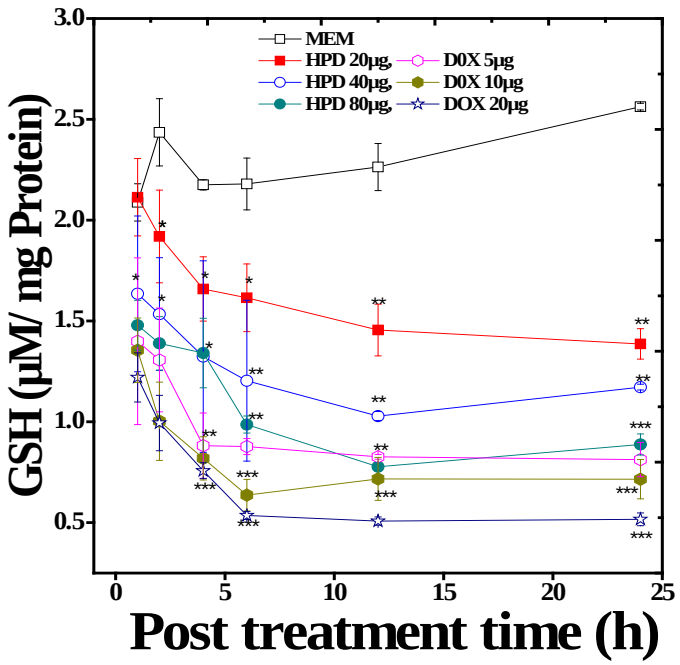

Figure 9: Alteration in the glutathione concentration in cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) ordoxorubicin (DOX). The data represent Mean ±SEM, N=5. ***p<0.01, **p<0.001 when treatment groups are compared to MEM group. No symbol=no significant difference. Standard error of the mean (SEM).***

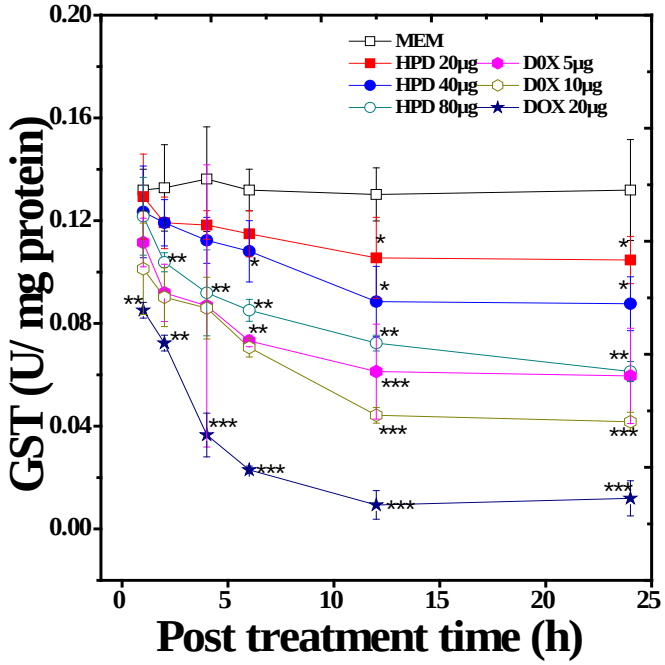

Table 10: Alteration in glutathione-s-tranferase activity in cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) ordoxorubicin (DOX). The data represent Mean ±SEM, N=5. ***p<0.01, **p<0.001 when treatment groups are compared to MEM group. No symbol=no significant difference. Standard error of the mean (SEM).***

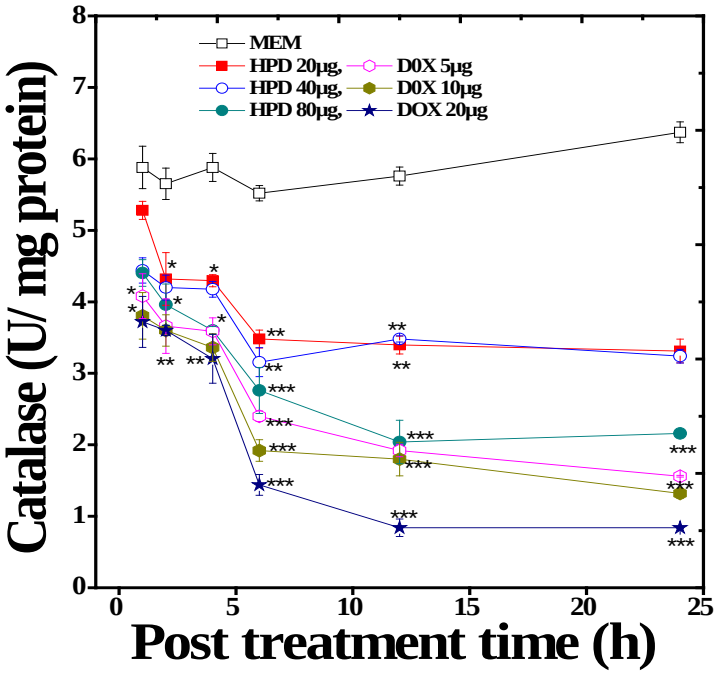

Figure 11: Alteration of catalase activity in cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) ordoxorubicin (DOX). The data represent Mean ±SEM, N=5. **p<0.01, **p<0.001 when treatment groups are compared to MEM group. No symbol=no significant difference. Standard error of the mean (SEM).*

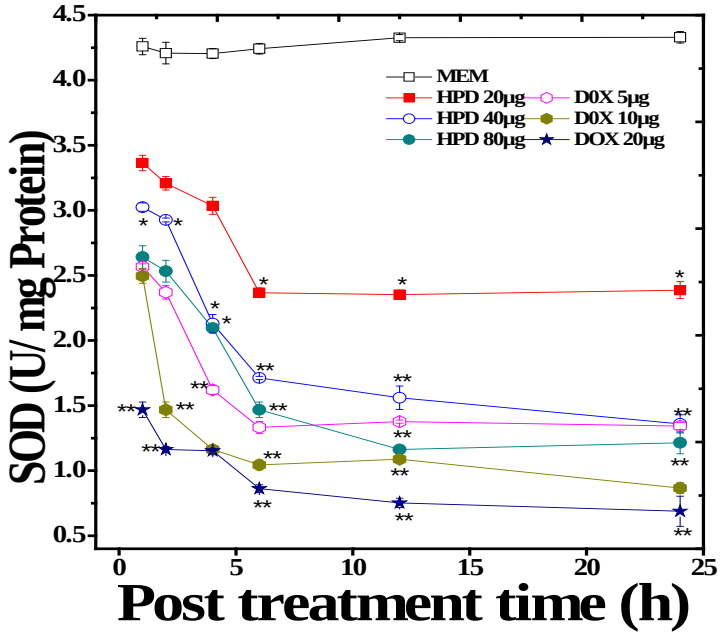

Figure 12: Alteration of superoxide dismutase activity in cultured human epidermoid carcinoma A431 cells reated with different concentrations of hesperidin (HPD) or doxorubicin (DOX). The data represent Mean ±SEM, N=5. **p<0.01, **p<0.001 when treatment groups are compared to MEM group. No symbol=no significant difference. Standard error of the mean (SEM).*

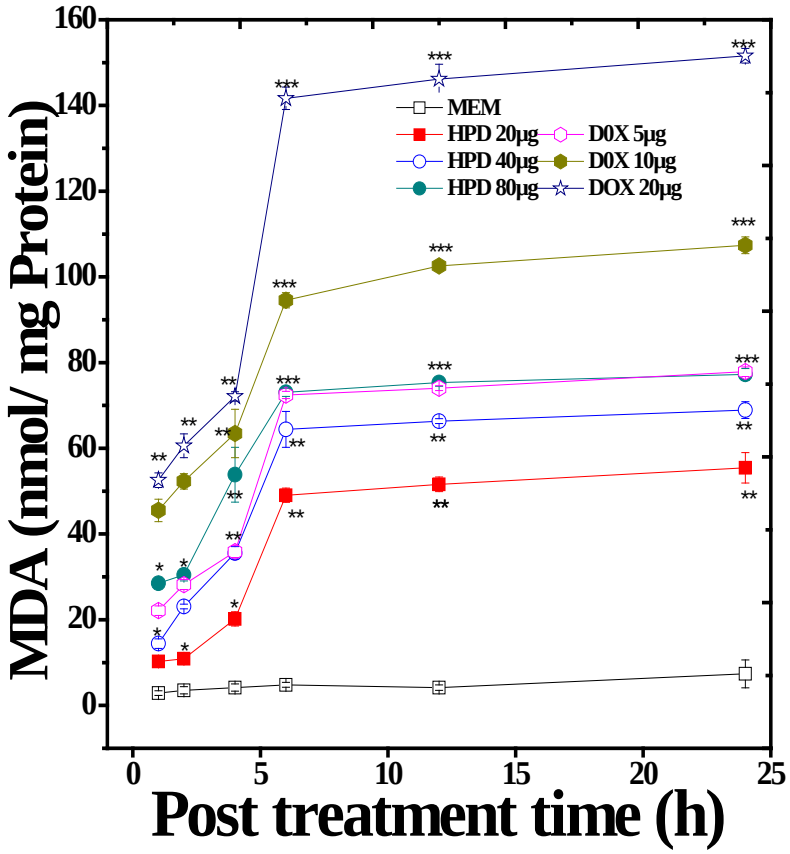

Figure 13: Alteration of lipid peroxide level in cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX). The data represent Mean ±SEM, N=5. *p<0.05, **p<0.01, ***p<0.001 when treatment groups are compared to MEM group. No symbol=no significant difference. Standard error of the mean (SEM).

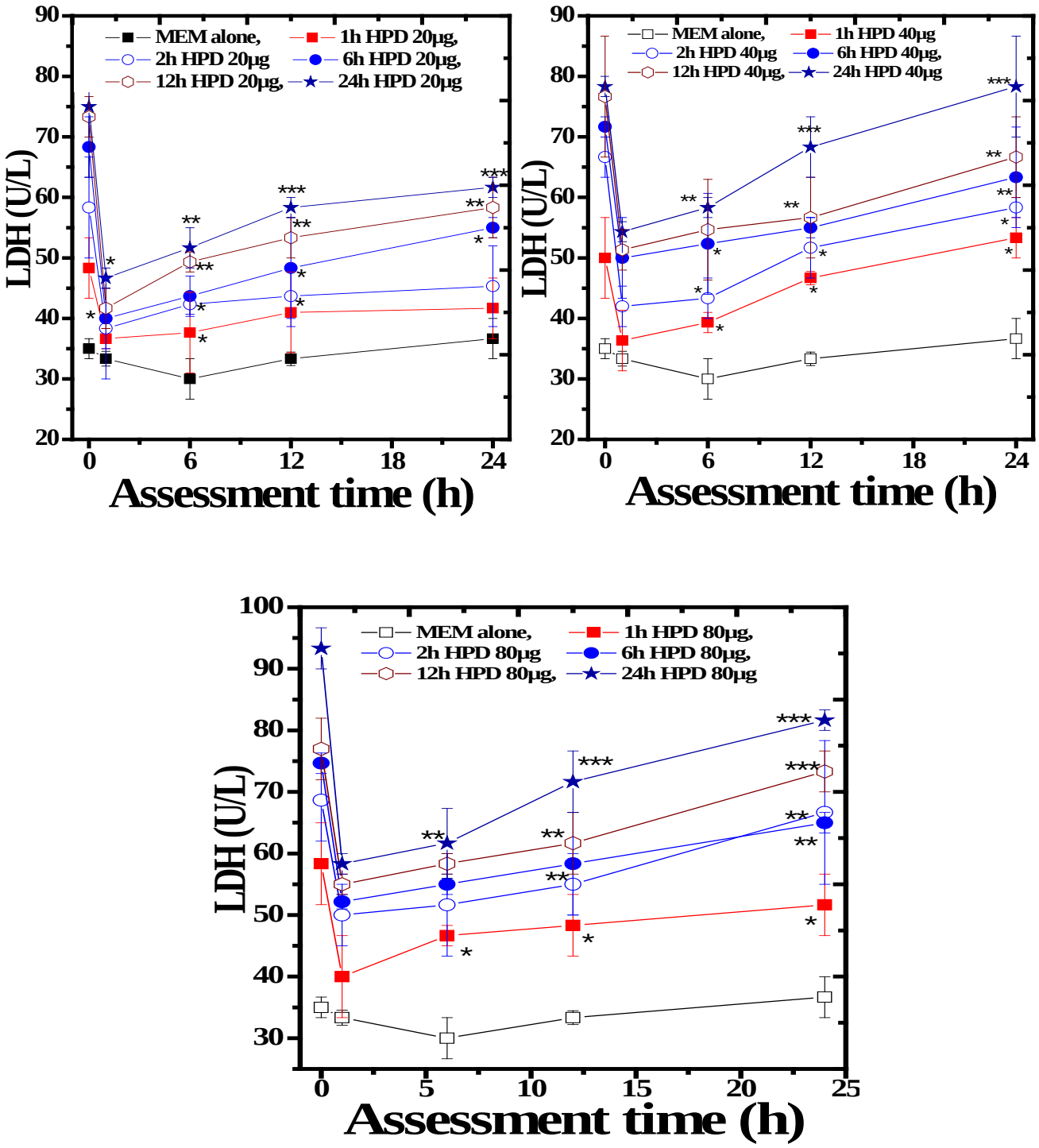

Figure 14: Alteration in LDH released by human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) ordoxorubicin (DOX).

The data represent Mean ±SEM, N=5.

***p<0.05, **p<0.01, ***p<0.001when treatment groups are compared to MEM group.*

No symbol=no significant difference. Standard error of the mean (SEM), n=5.

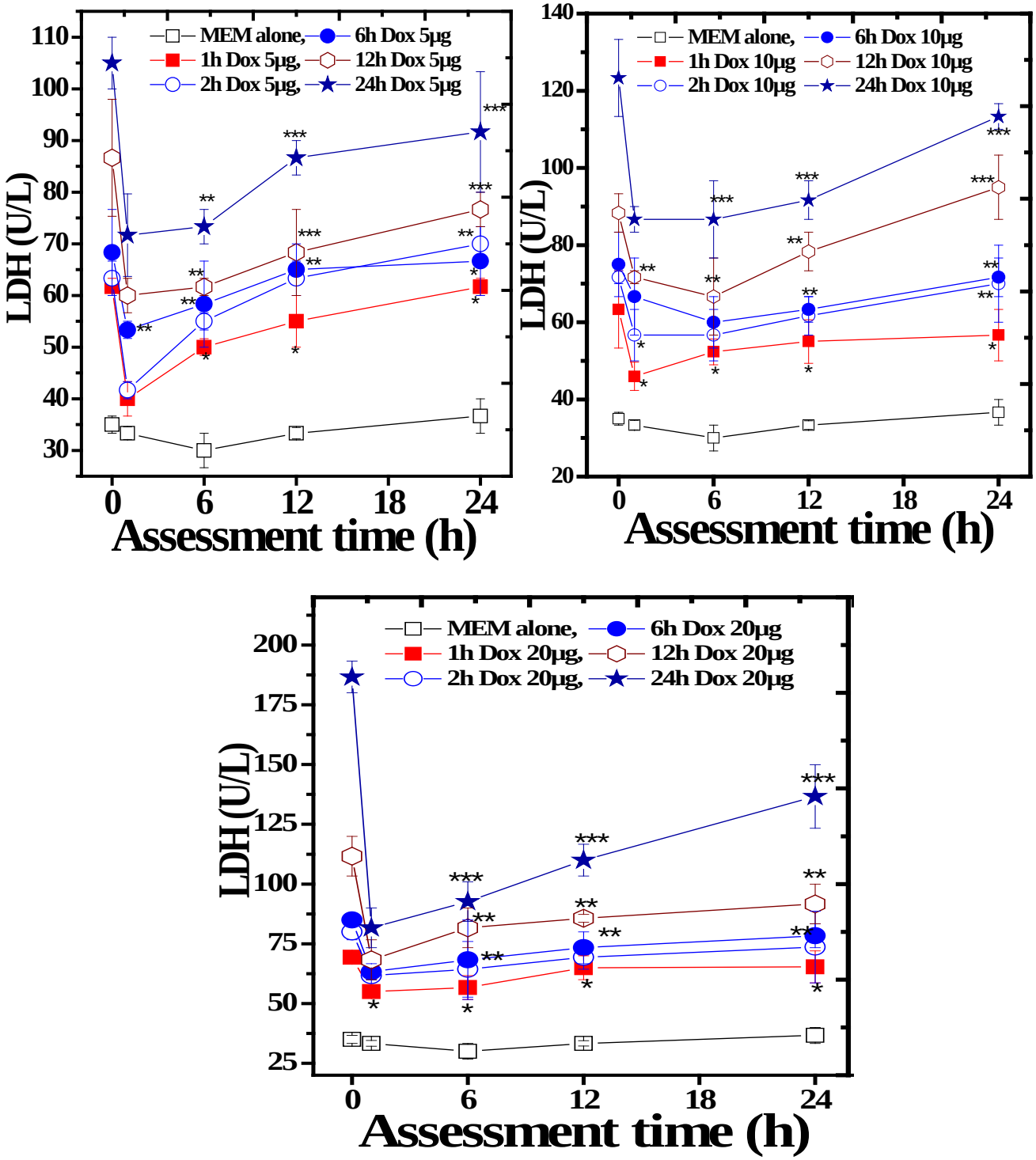

Figure 15: Alteration of LDH released by human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX). The data represent Mean ±SEM, *N=5.*p<0.05, **p<0.01, ***p<0.001when treatment groups are compared to MEM group. No symbol=no significant difference.*

CHAPTER
6

Evaluation of anticancer activity of hesperidin on cultured human epidermoid carcinoma A431 cells

ABSTRACT

The effect of hesperidin on cell growth DNA damage, apoptosis and antioxidant status was studied in cultured human epidermoid carcinoma A431 cells. Exposure of A431 cells to various concentrations of hesperidin resulted in a concentration dependent decline in the cell survival, which reflected as a reduction in the clonogenicity of these cells. The DNA damage assay revealed that hesperidin increased the frequency of micronuclei in both mononucleate cells and binucleate cells in a concentration dependent fashion with assay time up to 36 h post treatment. Similarly, hesperidin induced apoptosis in A431 cells in a concentration dependent manner and with increasing assay time. Exposure of A431 cells to hesperidin resulted in a decline in glutathione concentration, and glutathione-s-transferase, catalase, and superoxide dismutase activities followed by a rise in the lipid peroxidation and lactate dehydrogenase release. The hesperidin has been able to suppress cell proliferation and this effect of hesperidin seems to be due induction of micronuclei formation, apoptosis and reduction in glutathione concentration, and glutathione-s-transferase, catalase, and superoxide dismutase activities accompanied by an increased lipid peroxidation and lactate dehydrogenease release.

1. INTRODUCTION

Cancer is a leading single cause of mortality worldwide and it is responsible for one in eight deaths worldwide (Garcia *et al*., 2007; Center *et al*., 2011). The burden is expected to grow worldwide due to the growth and aging of the population, particularly in less developed countries, in which about 82% of the world's population resides. The disease cancer is lifestyle related, have a long latent period and need specialized infrastructure and human resource for treatment. The adoption of lifestyle behaviors that are known to increase cancer risk, such as smoking, poor diet, physical inactivity, and reproductive changes (including lower parity and later age at first birth), have further increased the cancer burden in less economically developed countries (Lindsey *et al*., 2015). It has been estimated that of all new cancers diagnosed annually in the world almost one-third originates in the skin (Parker *et al*., 1998). The prevention of skin cancer is a desirable goal due to increasing rise in incidence of skin cancer patients world-wide (Greenlee, 2001; Gupta, 2001). In the United States alone 1.2 million new cases with different forms of skin cancer are identified each year (Parker *et al*., 1998; Greenlee, 2001). The two more common types and high occurrence of human skin cancers are basal cell carcinomas (BCC) and squamous cell carcinomas (SCC), grouped together as non-

melanoma skin cancer (Limmer, 2001; Alam, 2001). Solar keratosis is common dysplastic epidermal lesions, which are generally regarded as precursor lesions for SCC (Heaphy and Ackerman, 2000).

Exposure to various xenobiotics, such as industrial chemicals, arsenic, pesticides, cigarette smoke or other pollutants have resulted in increasing episode of skin related occupational health problems including skin cancer (Rockley *et al.,* 1994). There is convincing evidence that solar ultraviolet (UV) radiation is the major cause of skin cancer in the human population (Yamaguchi, 1999; de Gruijl, 2000). Excessive UV exposure, particularly the ultraviolet-B light (UVB) (290–320 nm), a component of the solar spectrum is the principal cause of DNA damage (de Gruijl, 2000). Acute UVB exposure can cause a marked sunburn reaction that leads to severe edema and blistering of the skin (Soter, 1990) while the chronic exposure could result in premature skin aging, wrinkling, alterations in immune response and cancer (Wenk, 2001; Iwai, 1999; Krutmann, 1995; Drouin, 1997; de Gruijl, 1999). Exposure of skin to various chemical and physical agents results in (i) stimulation of DNA synthesis and proliferation, (ii) epidermal hyperplasia, (iii) depletion of antioxidant defense system, (iv) induction of ornithine decarboxylase, (v) increase in prostaglandin production and (vi) impairment of signal transduction pathway and faulty repair. The natural products may play a significant role in reducing the incidence of cancer worldwide.

Hesperidin, a bioflavonoid was first isolated in 19th century by French chemist Lebreton (1828) from the spongy inner portion of the peel of oranges. Hesperidinis found in lemons and other citrus fruits (Dakshini, 1991; Manthey and Grohmann, 1998, Garg *et al*., 2001). It is useful in many illnesses including genetic disorders. Hesperidin has been found to suppress has b ROS and lipid peroxidation (das Neves *et al.,* 2004) and reported to alleviate the generation of ROS and caspase-dependent apoptosis in human polymorphonuclear neutrophils in vitro (Ross and Kasum 2002; Zielinska-Przyjemska and Ignatowicz 2008). Hesperidin is an anti-allergic, antihypotensive, antimicrobial, vasodilator anti-inflammatory, antihyperlipidemic, antihypertensive, hepatoprotective and cardioprotective (Galati *et al.,* 1994; Emim et al., 1994; Monforte *et al.*,1995; Garg *et al.,* 2001; Ohtsuki *et al.,* 2003; El-Samaligy *et al*., 2006; Chen *et al*., 2010; Agrawal *et al.,* 2014; Vaberyureilai *et al*., 2015). Hesperdin has been reported to heal clean and infected

wounds, both orally and topically (Lonchampt *et al*., 1989; Cypriani *et al*., 1993; Hasanoglu *et al*., 2001). Deficiency of hesperidin results in abnormal capillary leakage and its intake has been found to produce beneficial effects on the abnormal capillary permeability, fragility and various traumas and stresses (Felicia *et al*., 1996)., Hesperidin has been reported to protect against paraquat and hydrogen peroxide induced damage by increasing cellular antioxidants (Wilmsen *et al.,* 2005). Prostaglandin E2 (PGE2) significantly levels were found to be reduced by hesperidin (Sakata *et al.,* 2003). Hesperidin and hesperetin have been shown to exert a mild inhibitory effect on NO production, mild suppression on COX-1 activity but a reasonably good inhibitory effect on COX-2 activity (Lee and Kim, 2010). Hesperidin significantly suppressed TNF-α-induced VCAM-1 protein expression, but did not have any impact on ICAM-1. It was hypothesized that this inhibition might perform via NF-κB-independent mechanism (Nizamutdinova *et al.,* 2008). Hesperidin is bioflavanoid with pleiotropic activities, therefore it was desired to obtain an insight into the effect of hesperidin in the cultured human epidermoid carcinoma A431cells.

2. MATERIALS AND METHODS

2.1 Chemicals

MEM medium, fetal bovine serum (FBS) Nitroblue tetrazolium lt (NBT), phenazine methosulphate (PMS), 5,5'dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitronbezene (CDNB), DMSO (Dimethyl sulphoxide). 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), triton X-100, ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA), agarose, , ethidium bromide, acridine orange, crystal violet, chrysin and cytochalasin B were obtained from Sigma Aldrich Chemicals Pvt. Ltd. (Bangalore, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, hydrogen peroxide (H_2O_2), and Folin-Ciocalteu reagent were supplied by the SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium Chloride (NaCl), disodium hydrogenphosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol, Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were procured from Merck India Limited, Mumbai, India. Hesperidin (98% HPLC grade), trypsin EDTA 1X, MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-

diphenyl tetrazolium bromide), MEM medium, Phenol-Chloroform-isoamyl alcohol (PCI), fetal bovine serum (FBS), reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin, was supplied by Biochem Pharmaceutical Industries, Mumbai, India. LDH kit was procured from Coral Clinical Systems, Verna Industrial Estate, Verna, Goa, India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

2.2 Preparation of Drugs

The hesperidin (HPD) was dissolved in suitable volume of DMSO and the final concentration of the DMSO never exceeded 0.02%.

2.3 Cell culture and treatment

The human epidermoid carcinoma A431 cells were procured from the National Centre for Cell Science, Pune, India. The cells were routinely grown in 25 cm^2 culture flasks (HiMedia, Mumbai, India) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate with loosened caps at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

2.4. Experimental Design

Usually 10^4 exponentially growing A431 cells were seeded into several 96 well microplated containing MEM supplemented with 10% FBS and the flasks were divided into the different groups depending on the experimental protocol:

2.4.1. **MEM group**: The cells of this group were treated with 2 µl/ml of DMSO.

2.4.2. **HPD group**: This group of cells was treated with 10, 20, 40, 60, 80 and 100 µg/ml of HPD.

2.4.3. **DOX group:** The cell cultures of this group were treated with 5, 10 or 20 µg/ml of doxorubicin (DOX) that served as positive control.

2.4.4. Determination of cytotoxicity

The cytotoxic effect of different concentrations of HPD or DOX was studied by MTT assay in A431 cells as described by Mosmann (1983). Briefly, 10^4 cells were seeded in 96 well plates containing 100 µl minimum essential medium (MEM) in each microwell. The cells were incubated at 37ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air and were allowed to attach for 24 hours. Different concentrations of HPD

or doxorubicin were added into each well of the microplates and incubated in the CO_2 incubator. After 72 hours of cell plating, 20 µl of MTT was added into each well and the microplates were incubated for another 2 hours. The drug containing media were removed and the insoluble purple formazan formed was dissolved with DMSO and incubated once again for 4 hours after which the absorbance was measured at 560 nm. The cytotoxicity was calculated by using the following formula:

Cytotoxicity= Control-Treatment/Control X 100.

The IC_{50} was also calculated using GraphPad Prism 6 software.

2.5. Determination of optimum exposure time for cytotoxicity

A separate experiment was conducted to study the effect of treatment time of HPD on the cytotoxicity, where grouping and other conditions were essentially similar to that described above except that the cells were exposed to HPD for different times and the cytotoxicity was determined by MTT assay as described above.

2.6. The Determination of anticancer activity

The anticancer activity of HPD was determined by inoculating $1x10^6$ exponentially growing A431 cells into several culture flasks. The cells were allowed to attach for 24 h and were divided into the following groups:

2.6.1.1. **MEM group**: The cells of this group were treated with 2 µl/ml of DMSO.

2.6.1.2. **HPD group**: This group of cells was treated with 20, 40, or 80 µg /ml HPD.

2.6.1.3. **DOX group:** The cell cultures of this were treated with 5, 10 or 20 µg/ml DOX and served as a positive control.

After 6 hours of drug/s treatment the media were removed and the flasks were washed twice with sterile PBS, and cells were dislodged from the culture flasks by trypsin EDTA treatment and the following studies were conducted.

2.7. Clonogenic Assay

Usually 200 A431 cells were seeded into several individual petridishes containing 5 ml MEM and allowed to grow for another 12 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. Plating efficiency (PE) of the cells was determined and surviving fraction (SF) was calculated using the formulae given below:

PE = (Number of colonies counted x 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

2.8. Micronucleus Assay

Usually $5X10^5$ cells left after clonogenic assay were seeded in triplicate for each concentration and allowed to grow for 12, 24, 36 and 48 h. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). Briefly, the cells were allowed to attach for 6 h, after which 5 µg/ml of cytochalasin-B was added to inhibit cytokinesis. The cells were left undisturbed and the cultures were terminated at 12, 24, 36 and 48 h post-drug-treatment. The media containing cytochalasin-B were removed and the cells were washed with PBS, dislodged by trypsin-EDTA treatment and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and subjected to mild hypotonic treatment (0.75% ammonium oxalate) at 37°C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative 3:1 (Methanol: Acetic acid). After centrifugation, the cells were resuspended in a small volume of fixative and spread on to pre cleaned coded slides to avoid observer's bias. The slides containing cells were stained with 0.025% acridine orange (BDH, England, Gurr Cat. no. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed twice in the buffer to remove excess stain. The slides mounted in Sorensen's buffer were observed under a fluorescence microscope (DM-2500, Leica Microsystems, Wetzlar, Germany) equipped with 450–490 nm BP filter set with an excitation at 453 nm using a 40 X N Plan objective. A minimum of one thousand binucleate cells with well-preserved cytoplasm was scored for each concentration and each post-treatment time. The frequency of mononucleated cells bearing micronuclei as well as binucleated cell bearing micronuclei was determined. The micronucleated cells were scored according to the criteria of Kirsch-Volders *et al.*, (2003) and Fenech *et al*., (2003).

2.9. Apoptosis Assay

Approximately $4x10^5$ A431 cells remaining after clonogenic and micronucleus assay were inoculated in several six well culture plates (Himedia, Mumbai, India) and the cultures from all the groups were terminated at 2, 4, 12, and 24 h post-drug treatment. The ability of the drugs to induce apoptosis was studied using standard protocol. Briefly, the cells were labeled with nucleic acid-binding dye mix of 25 µg/ml acridine orange and

25 µg/ml ethidium bromide in the ratio of (1:1) in PBS (Cohen, 1993). The cells were examined under a fluorescence microscope (DM-2500, Leica Microsystems, Wetzlar, Germany). For each sample, at least 1000 cells were scored, and apoptotic cell index was determined as follows:

Apoptotic index (%) = Number of apoptotic cells scored X 100/Total number of cells counted.

2.10. DNA fragmentation assay

Since DNA fragmentation is biochemical marker of apoptosis, the ability of HPD to induce apoptosis in A431 cell was studied by conducting a separate experiment, where grouping and other conditions were essentially to that described above except that 1×10^6 exponentially growing A431 cells were inoculated into individual wells of six well plates and treated with HPD or DOX as described in section 2.4.3.1. The cells were harvested at 2, 4, 12 and 24 h post-drug treatment and the DNA ladder formation was detected by SDS/proteinase-K/RNase method that allowed the isolation of only fragmented DNA without contamination with RNA (Herrmann *et. al.*, 1994). Briefly, the cells were removed from the culture plates by trypsin EDTA treatment, pelleted, washed in cold PBS, lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2 % Triton X-100), kept at 4°C for 20 min, and the contents were centrifuged at 14,000 g for 15 min. The supernatant was collected and mixed with proteinase K (0.5 mg/ml) and incubated for 1 h at 37°C followed by the RNase-A (0.5 mg/ml) treatment for 1 h at 50°C. The DNA was extracted with phenol, chloroform and isoamyl alcohol (1:1:1) and precipitated with ammonium acetate (3M) and chilled isopropanol. Various samples were loaded on to 1 % agarose gel placed in a horizontal electrophoresis tank containing TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) buffer. The DNA was resolved at 100 Volts and 40 mA and the resultant DNA fragmentation ladder was visualized under 265 nM UV light by staining the gel with ethidium bromide (0.5 µg/ml).

2.11. Biochemical Assay

A separate experiment was carried out to estimate the effect of HPD on the activities of various antioxidant enzymes, lactate dehydrogenase and lipid peroxidation in A431 cells, where grouping and other conditions were essential similar to that described for anticancer activity except that the cell cultures were terminated at 1, 2, 6, 12 and 24 h

post drug treatment. The drug containing media were removed**;** the cells were washed with sterile PBS and the dislodged using trypsin EDTA treatment. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

2.11.1. Estimation of reduced glutathione (GSH)

Glutathione was estimated as described earlier (Moron *et al*., 1929). Glutathione concentration was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. Briefly, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The blank consisted of distilled water instead of cell homogenate. The mixture was allowed to stand for 2 minutes and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata).

2.11.2. **Glutathione - S – transferase (GST) estimation**

Glutathione-s-transferase activity was estimated by the method of Habig *et al*., (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20 mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min. After incubation, 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate were added. The absorbance was read against the blank (distilled water) at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer.

2.11.3. **Catalase (CAT) estimation**

Catalase was assayed according to technique described by Aebi (1984). Briefly, in a 3 ml cuvette, 20 µl of sample was diluted with 1.98 ml of 50 mM phosphate buffer (pH 7.0). The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2 and the decrease in absorbance was monitored 15 every seconds at 240 nm for 60 seconds in a UV-VIS Biospectrophotometer.

2.11.4. **Superoxide dismutase (SOD) estimation**

SOD is an enzyme that catalyzes dismutation of two superoxide anions (O_2^-) into hydrogen peroxide and molecular oxygen and its activity was estimated as described by Fried (1975). Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, 200 µl of 780 µM NADH were incubated for 90 seconds at 30° C. The reaction was terminated by adding 1000 µl of

acetic acid and followed by the addition of 4 ml n-butanol. The absorbance was recorded at 560 nm using a UV-VIS Biospectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme and the test with SOD enzyme samples using the formula (Blank-Sample)/Blank X 100. The enzyme activity has been expressed in units (1 U = 50% inhibition of NBT reduction).

2.11.5. Lipid peroxidation (LOO) estimation

Lipid peroxidation (LOO) assay was carried out following the method of Buege and Aust (1978). Briefly, 1 ml of tissue homogenate was mixed with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The reaction mixture was heated in a boiling water bath for 15 minutes, cooled immediately to room temperature, centrifuged at 1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against the blank in a UV-VIS Biospectrophotometer. The blank contained all the reagents minus the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

2.11.6. Protein estimation

Protein concentration in all samples was determined by the method of (Lowry *et. al.,* 1951) using bovine serum albumin (BSA) as standard at 650 nm.

2.12. Lactate dehydrogenase estimation

A separate experiment was conducted to study the activity of the enzyme lactate dehydrogenase as described earlier (Decker and Lohmann-Matthes, 1988) using LDH kit. Briefly, $1x10^{5}$ cells were seeded into several twelve well culture plates and treated with different concentrations (20, 40 and 80 µg/ml) of HPD for 1, 2, 6, 12 and 24 h. After the drug treatment the whole media was removed and the cells were washed with PBS and fed with a fresh media of same volume and the LDH activity was estimated. This was considered as 0 hour for each exposure time. The LDH activity was estimated at 1, 6, 12 and 24 h post drug treatment where 50 µl of the medium was removed at each assay time and replaced with an equal volume of fresh medium at different time intervals (h). It was mixed with 1 ml of working reagent prepared by mixing buffer reagent and starter reagent in the ratio of 8:2 according to the manufacturer's protocol. The activity of LDH was estimated by recording the absorbance of the medium every minute for four minutes at 25°C in a UV-VIS Biospectrophotometer at 339 nm and the mean absorbance change

per minute (ΔA/ min) was calculated. The activity of LDH has been expressed as unit per liter using the formula LDH activity in U/L = ΔA/ min. x 3333.

3. Statistical Analyses

The statistical significance between the treatments were determined using students 't' test, Wilcoxon's signed rank test and one way ANOVA with the application of Tukey's test for multiple comparison for different parameters between the groups, using Origin Pro 8 (SRO v8.0724 (B724), Northampton, MA, USA.) statistical software was used for all analyses. A P value of ≤ 0.05 was considered as statistically significant.

4. RESULTS

The results have been expressed as the mean ± standard error of the mean (SEM), wherever required and are presented as Tables 1-10 and Figures 1-15.

4.1. Determination of cytotoxicity

Treatment of human epidermoid carcinoma A431 cells with different concentrations of HPD caused a concentration dependent increase in the cell cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of HPD used. The cytotoxicity was 4.56% 28.75% ,43.45%, 64.98% and 72.70% for 10, 20, 40, 60 and 80 µg/ml HPD, respectively(). The IC_{50} was determined as 42.70 µg/ml HPD. Treatment of A431 cells with different concentrations of HPD induced highest toxic effect at a concentration of 80 µg/ml. However, there was no statistically significant difference between 60 and 80µg/ml HPD (Table 1and Figure 1).

4.2. Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxic effect of HPD was also evaluated by MTT assay. The HPD treatment resulted in a time dependent increase in the cytotoxicity in A431 cells. The optimum cytotoxic effect was observed in the A431 cells treated with HPD for 6 h (52.54%) at a concentration of 80 µg/ml (Table 2 and Figure 1). Thereafter, the cytotoxicity HPD increased gradually. However, this increase was statistically insignificant when compared to 6 h treatment.

4.3. Clonogenic Assay

Clonogenic assay is a gold standard to assay the cell survival and treatment of A431 cells with different concentrations of HPD caused a concentration dependent decline in the

clonogenicity of A431 cells, which was indicated by a constant decline in the colony formation with increasing concentration of HPD as compared to the MEM group. The 80 µg/ml concentration showed the greatest reduction in the cell survival. Treatment of A431 cells with different concentrations of doxorubicin exhibited the pattern similar to that of HPD (Table 2 and Figure 3). The cell survival of 80 µg/ml HPD was equal to 10 µg/ml DOX the positive control used in this study (Table 2 and Figure 2).

4.4. Micronucleus assay

The frequency of micronuclei bearing mononucleate (MNMNC) and bi nucleate cells (MNBNC) has been represented separately (Table 4, Figure 3 - 5). Treatment of A431 cells with different concentrations of HPD resulted in the concentration dependent rise in the frequency of mononucleate cells bearing one and two micronuclei increased in a concentration dependent manner and the maximum number of both micronuclei was scored for a concentration of 80 µg/ml HPD (Table 4, Figure 3-5). The analysis of MNMNC kinetics with scoring time revealed that their frequency elevated with scoring time with a maximum rise at 36 h post HPD or DOX treatment and decline thereafter. However, the difference between 36 and 48 h was not statistically significant. The highest frequency of micronuclei in mononucleated cell was observed at the concentration of 80 µg/ml for approximately 16 fold increase when compared with concurrent control group ($p<0.001$) at 36 h post treatment (Table 4, Figure 3). The frequency of micronuclei in the binucleated cells also increased in a concentration dependent manner and a peak rise in the MNBNC was recorded at 36 h post-drug treatment. The frequency of BNC with one and two micronuclei also followed a similar pattern and a maximum rise ($p<0.001$) was observed for a concentration of 80 µg/ml HPD, where approximately 9 fold rise was observed in the frequency of MNBNCs at 36 h post drug treatment in the HPD treated group (Table 4, Figure 4 and 5). The frequency of MNBNC increased with time starting from 12 h post drug treatment and continued to increase up to 36 h post drug treatment where this increase was maximum and declined thereafter at 48 h post drug treatment.

4.5. Apoptosis

The treatment of A431 cells with different concentration of HPD revealed that HPD induced apoptosis in A431 cells as early as 2 h post drug treatment. The number of

apoptotic cells in HPD treated A431 cells significantly ($p<0.001$) increased in a concentration dependent manner at all the post drug treatment evaluation times with a maximum rise for 80 μg/ml HPD (Table 5 and Figure 6). The analysis of apoptotic cells at different time revealed that HPD treatment increased their frequency with increasing assay time and maximum numbers of apoptotic cells were scored at 25 h post drug treatment (Table 5 and Figure 6). The pattern of apoptosis was exactly similar in the DOX treated A431 cells (Table 5 and Figure 6, 7).

4.6. DNA Fragmentation

The induction of apoptosis was further confirmed by DNA fragmentation assay in the form of DNA ladder formation. Treatment of A431 cells with different concentration of HPD or DOX formed the ladder like patterns at different post drug treatment times (Figure 8), which confirmed the ability of HPD to produce DNA fragments in A431 cells.

4.7. Biochemical assay

4.7.1. Estimation of Glutathione (GSH)

Exposure of A431 cells to different concentrations of HPD significantly reduced the GSH concentration in a dose dependent manner when compared to untreated MEM group and a maximum attrition in the GSH contents was observed at 80 μg/ml HPD (Table 6, Figure 8). The GSH concentration continuous declined with time starting from 1 h post drug treatment and continued to decrease up to 24 h where a 1.8, 2.2 and 2.9 fold decline was observed for 20, 40 and 80 μg/ml HPD, respectively. However, the difference between 12 and 24 was negligible (Table 6, Figure 9).

4.7.2. Glutathione-S-transferases (GST)

The activity of GST enzyme reduced in a concentration dependent manner in A431 cell treated with different concentration of HPD and a great decline was observed in cells treated with 80 μg/ml HPD (Table 7 and Figure 10). The GST activity reduced with assay time and a maximum alleviation in the GST activity was observed at 24 h drug treatment where it was 1.3, 1.6 and 2.2 fold lower than the MEM group at 20, 40 and 80 μg/ml HOD, respectively (Table 7, Figure 10).

4.7.3. Catalase (CAT)

The catalase activity declined in a concentration dependent fashion in the A431 cells treated with different concentrations of HPD at all the assay times (Table 8 and Figure

11). The catalase activity declined significantly at 1 h post drug treatment and continued to decline up to 24 h post drug treatment where it was 1.9 to 2.9 fold lower for 20, 40 and 80 μg/ml HPD respectively in comparison with MEM treatment alone (Table 8, Figure 11).

4.7.4. Superoxide dismutase (SOD)

The treatment of A431 cells with various concentrations of HPD resulted in a gradual decrease in the activity of SOD at all post drug treatment assay times (Table 9 and Figure 12). The SOD activity reduced with time and a maximum attrition in SOD activity was observedat 12 h post drug treatment, where it was 1.8, 2.8 and 3.7 folds lesser at 20, 40 and 80 μg/ml HPD, respectively when compared to MEM treatment (Table 9).

4.7.5. Lipid Peroxidation (LOO)

The treatment of A431 cells with different concentrations of HPD resulted in significant increase in the oxidation of lipids in all treatment groups and this increase was concentration dependent (Table 10, Figure 13). Treatment of A431 cells with different concentration of HPD led to a drastic reside in LOO at all post treatment times and a maximum rise was seen at 24 h post treatment (Table 10 and Figure 13).

4.7.6. Lactate dehydrogenase activity (LDH)

The LDH release is a signature of toxicity and exposure of A431 cells to different concentrations of HPD for different times significantly elevated the LDH activity in a time and concentration dependent manner (Table 14-15). The LDH release from A431 cells exposed to HPD for different times showed an assay time dependent increase and a maximum increase in the LDH activity was observed at 24 h post-treatment in all the groups and at a concentration of 80 μg/ml HPD (Tables 12-14 and Figure 12). The maximum released in LDH was observed at 0 h post-treatment because h release corresponds to the amount of LD accumulated in the medium after 1, 2, 6, 12 and 24 of drug treatment.

DISCUSSION

The incidence of cancer has been increasing despite the availability of various modern treatment modalities. The incidence of cancer may be reduced by evolving strategies/modalities that do not allow the cells to get transformed in to the malignant

phenotype. The pharmacological intervention that reduces the development of cancer is known as chemoprevention. The use of chemopreventive agents will certainly reduce the risk of cancer and several epidemiological studies supports this strategy. The natural products have been used by humans as diet and even medicine and their judicious use may actually inhibit the occurrence of cancer in humans. The advantage of natural products is that they are natural in origin and hence most biocompatible with minimum side effects in comparison to chemical synthetic products. Therefore, the present study was undertaken to evaluate systematically the anticancer activity of hesperidin in cultured epidermoid carcinoma A431 cells.

Hesperidin, a bioflavonoid was able to damage human epidermoid carcinoma A431 cells in a concentration dependent fashion and maximum cytotoxicity was attained at a concentration of 80 µg/ml by exposing the cells to hesperidin for 6 h in all groups. Cytotoxicity determination by MTT assay is an indicator of the cell killing effect of any pharmacological agent and it has been used to test anticancer activity (McCauley et al., 2013 Chemotherapy as a treatment of cancer often relies on the ability of cytotoxic agents to kill or damage rapidly proliferating cells (Gerber, 2008; Anonymous, 2014*).* The cytotoxic effect of bioflanovoid was further confirmed by clonogenic assay where hesperidin arrested the reproductive capacity of A431 cells. The maximum reduction in the cell survival was found at highest concentration used (80 µg/ml), where the surviving fraction declined to 0.45 (55% reduction in survival). This indicates that use of hesperidin is able to reduce the reproductive integrity of cancer cells and subsequently alleviate the clonogenic potential. The clonogenic assay has been used frequently to determine the efficacy of chemotherapy drugs and ionizing radiation to kill cancer cells (Buch *et al*., 2012; Jagetia and Venkatesha, 2016; Patties *et al.,* 2016). Chemosensitivity in vitro has been found to be well correlated in vivo earlier (Su, 2014**).** Cytotoxic agent cause damage to cells and prevent mitosis by various mechanisms including damaging DNA and inhibition of the cellular machinery involved in cell division *(*Malhotra and Perry, 2003).

The hesperidin has been able to induce DNA damage in the A431 cells as indicated by the formation of micronuclei in mononucleate as well as binucleate cells. The micronuclei are considered as surrogate marker of the DNA damage and their assessment certainly provide a definite indication of DNA damage mediated cell death (Jagetia and

Rao, 2011; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016). The assessment of micronuclei also provide insight into the cell death as production of multiple micronuclei indicates production of complex multiply sites of damaged DNA and DNA exchanges that leads to cell death (Sage and Harrison, 2011) . Hesperidin produced multiple micronuclei in A431 cells indicating that it has induced complex multiply sites of DNA damage that may have reduced the clonogenicity of A431 cells in the present study. The formation of DNA DSBs and MN is often the result of simultaneous excision repair of damages and wrong base incorporation. A failure of the appropriate gap-filling event leads to DSB (Dianov *et al*., 1991) and influence on the development of genomic instability and cancer (Aypar *et a*l., 2011). This happens only if the level of DSBs exceeds the repair capacity of dividing cells, which is mainly due to either the misrepair of DSBs by the dysfunctional homologous recombination (O'Donovan and Livingston, 2010). The micronuclei bearing cells are dying cells and this may be the reason for cytotoxic effect hesperidin.

The other mechanism of cell death is induction of apoptosis and our results indicates that hesperidin induced apoptosis in the A431 cells in a dose and time dependent manner. Hesperidin has been reported to induce aopotosis in HeLa cells (Wang *et al.,* 2015). The apoptosis is characterized by DNA condensation, fragmentation and subsequent molecular and biochemical events that finally lead to cell death (Cotter, 2009; Nikoletopoulou *et al.,* 2013). We have not studied how hesperidin induced apoptosis in A431 cells but assume that it may have either used non-mitrochondrial pathways or mitochondrial pathway or both pathways to induce apoptosis. Hesperidin has certainly induced DNA fragmentation in A431 cells as indicated by the formation of DNA ladder, which may have been due the activation of endonucleases that degrades DNA into multiple fragments of 185 base pairs that can be easily visualized in agarose gel (Wyllie, 1980; Bortner *et al.,* 1995) and also micronuclei, which are large DNA fragment of the genome.

The GSH plays a crucial role in the elimination, and neutralizing toxins and reduced GSH levels cause oxidative stress (Ganesaratnam *et al.,* 2004). It is also known to play crucial role in the cell differentiation, proliferation and apoptosis (Traverso *et al*., 2013). However, a decrease in GSH levels is closely associated with certain pathologies in

humans (Colell *et al.,* 1998; Lluis *et al*., 2005). The reduced level of GSH is implicated in raised oxidative stress and also sensitizing cells against chemotherapy and radiotherapy (Ortega *et al.,* 2012). Hesperidin reduced GSH and several other antioxidant enzymes including catalase, GST and SOD that may have increased lipid peroxidation and LDH activity, which indicate that the concentrations of hesperidin used in the present study unequivocally induced oxidative stress leading to damaged cell DNA and subsequently causing death of A431 cells. Our results show that hesperidin induced lipid peroxidation very efficiently and these peroxides would have reacted with the DNA inducing a cascade of events that led to the death of A431 cells. Reduced glutathione has been reported to sensitize cancer cells earlier (Rocha *et al.,* 2014). The exact mechanism of action of hesperidin in bringing the death of A431 cells is not known however, it is assumed that hesperidin may have interacted with these cells by multiple ways to induce cell death. The most important mechanism may be the depletion of antioxidant status due to the attrition of GSH and antioxidant enzymes like GST, catalase and SOD, which might have increased the oxidative stress and lipid peroxidation. This is substantiated by the decline in all antioxidants and rise in LOO. The increased LOO may have interacted with cell DNA leading to damaged DNA as indicated by the increased micronuclei formation and induction of apoptosis in the A431 cells by HPD. Hesperidin may have increased free radical formation, release of cytochrome C and apoptosis inducing factor and activated caspase-3 to bring cell death in the present study. Hesperidin has been reported to elevate ROS formation, mobilize Ca^{2+}, release cytochrome C and apoptosis inducing factor from cell mitochondria and activate caspase-3 in HeLa cells (Wang *et al*., 2015). Hesperidin is may have stimulated preapoptotic pathway including Bax and p21 leading to cell death (Ismail *et al*., 2012). HPD is also known to trigger death receptor pathway to induce apoptosis (Banjerdpongchai *et al.,* 2016). Hesperidin has been reported to down regulate the transcription activation of NF-κB and COX-II which elevated in cancer cells activation (Hirata *et al.,* 2005; Ghorbani *et al*., 2012; Parhiz *et al*., 2015).

The hesperidin produced its anticancer activity by inducing micronuclei and apoptosis in A431 cells characterized by DNA fragmentation. The hesperidin action seems to be mediated by its ability to induce ROS in A431 cells that may have led to decreased

synthesis of GSH, GST, catalase and SOD and increased production of lipid peroxide and LDH. The hesperidin may have induced apoptosis by suppressing the transcriptional activation of NF-κB and COX-II and activation of Bax and P21. It may have also released cytochrome C and apoptosis inducing factor from mictcohondria and activated executioner caspase-3 causing apoptosis of A431 cells.

CONCLUSION

Our study revealed that decreased GSH has been. It is fair to know that hesperidin induce lipid peroxidation that results in the membrane damage and functional loss and protein modification, DNA damage, cell death and pathogenesis of various diseases (Jagetia and Rajanikant, 2014). The increased lipid peroxidation by hesperidin may have led to the increase in DNA damage leading to increased micronuclei formation and apoptosis. The increased LOO may have also increased membrane damage and the DNA damage leading to increased micronuclei formation and apoptosis and cell death in the present study. LOO has been reported to induced DNA damage earlier (Łuczaj and Skrzydlewska, 2003).

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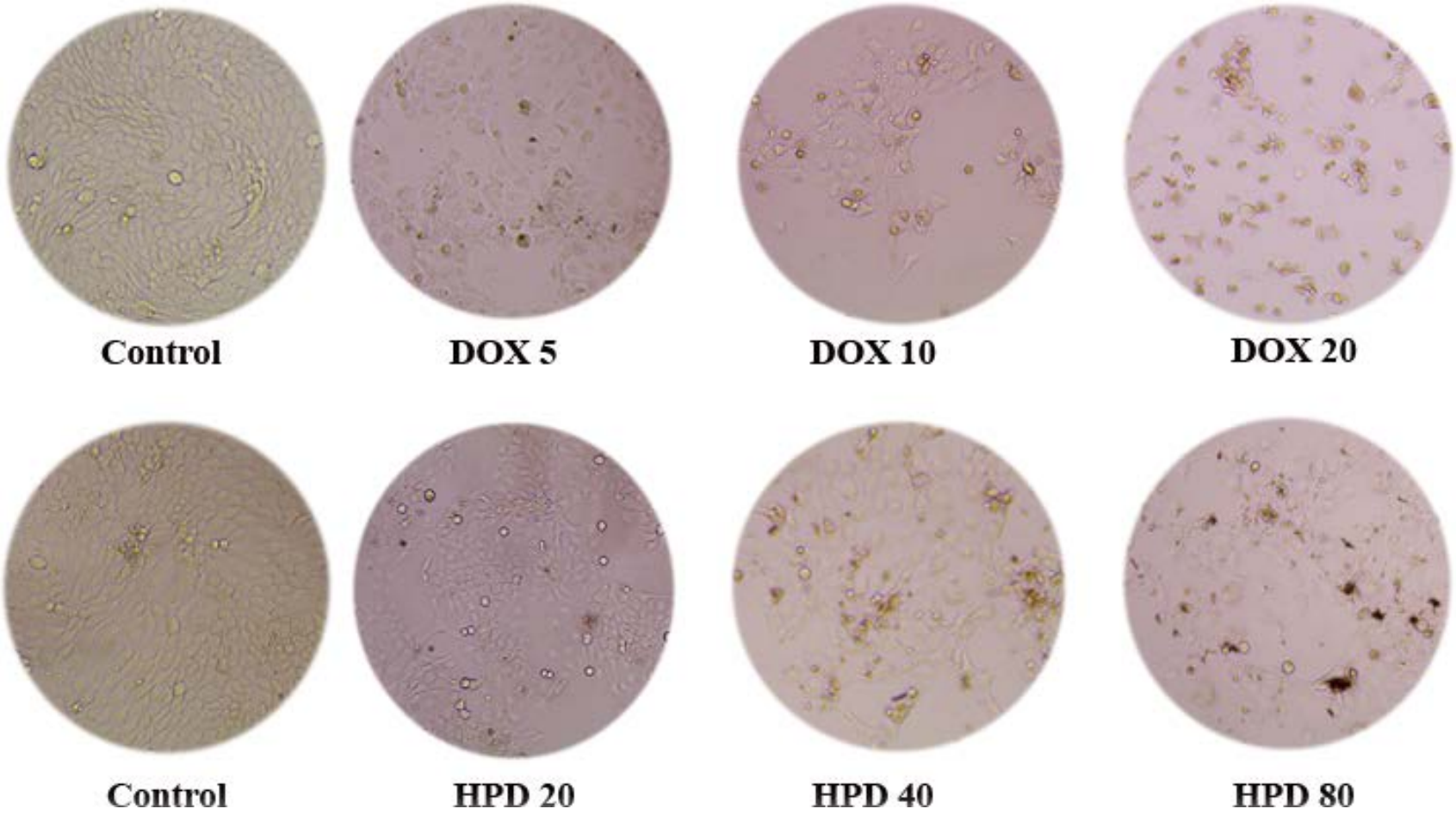

Plate 1: The effect of hesperidin (HPD) or doxorubicin (DOX) treatment on the cultured human epidermoid carcinoma A431 cells after 12 h. The HPD or DOX treatment showing cell death when compared to the control (untreated) group.

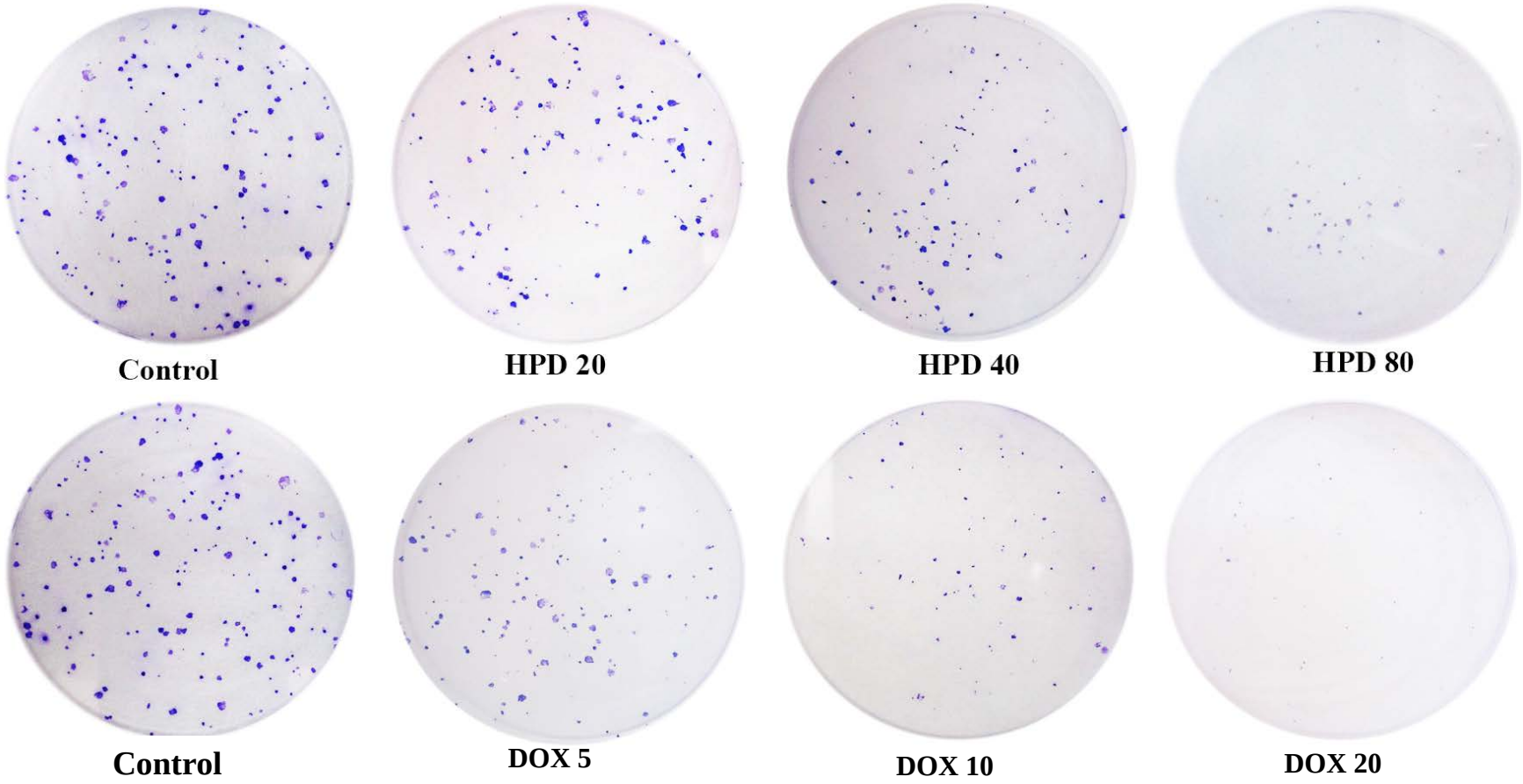

Plate 2: The effect of various concentrations of hesperidin (HPD) ordoxorubicin (DOX) on the clonogenicity of cultured human epidermoid carcinoma A431 cells. 200 cells were seeded in each plate, allowed cells to attach for 16 h, treated with HPD or DOX, whole drug containing media were removed and replaced with fresh media and the cells were allowed to grow for another 12 days.

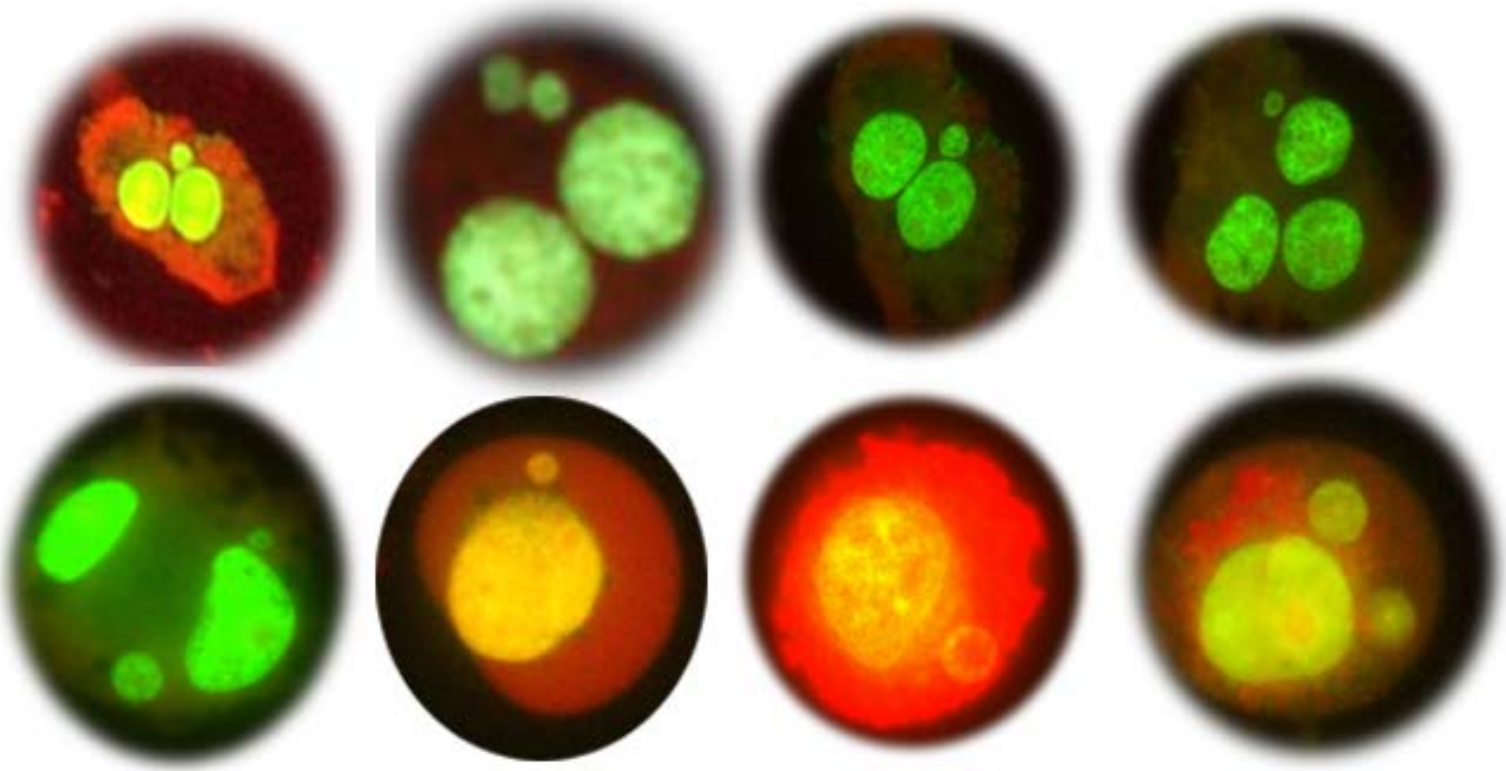

Plate 3: Micronuclei images of A431 cells treated with HPD or DOX: Upper lane – Micronuclei binucleated cells lower lane- Micronuclei in mononucleated cell

Table 1: Cytotoxic effects of different concentrations of hesperidin or doxorubicin on human epidermoid carcinoma A431 cells by conventional MTT assay. () indicates the dose for doxorubicin.

Concentration (µg/ml)	Treatment (Percent ± SEM)	
	HPD	DOX
10(0.5)	4.56±0.04	10.34±1.22
20(1)	28.75±2.12	15.21±1.21
40(5)	43.45±1.44	33.92±1.73
60(10)	64.98±1.34	64.02±2.31
80(20)	72.70±1.54	91.86±2.57

N=5. p<0.05

Table 2: Effect of different exposure times on the cytotoxic effects of hesperidin (HPD) or doxorubicin (DOX) on human epidermoid carcinoma A431 cell by MTT assay

Treatment (µg/ml)	Cytotoxicity (Percent ± SEM)					
	Assessment time (h)					
	1	2	4	6	12	24
Hesperidin (HPD)						
20	7.28±0.91	15.78±0.36	16.96±0.23	23.95±0.25	24.94±0.44	28.49±1.61
40	9.07±0.25	19.46±0.36	21.37±0.72	38.77±0.23	41.18±0.48	46.51±0.45
80	16.65±0.62	25.46±0.87	28.16±0.59	52.54±0.12	54.74±1.34	60.14±1.11
Doxorubicin (DOX)						
5	3.71±0.79	13.56±0.05	26.42±0.78	43.66±0.11	60.39±0.09	61.17±0.71
10	7.32±0.08	26.42±0.08	34.48±0.09	62.52±0.08	66.83±0.05	68.67±0.05
20	25.35±0.01	37.91±0.15	49.64±0.07	73.13±0.01	73.87±0.02	74.59±0.05

N=5. p<0.05

Table 3: Effect of different concentrations of HPD and DOX on the clonogenic potential of the cultured human epidermoid carcinoma A431 cells. *N=3 p<0.05*

Concentration (µg/ml)	Survival fraction(Mean± SEM)	
	Treatment	
	HPD	DOX
0	1.00±0.01	1.00±0.01
20(1)	0.90±0.04	0.68±0.05
40(5)	0.75±0.03	0.45±0.05
80(20)	0.45±0.05	0.06±0.00

Table 4: Effect of different concentrations of HPD or DOX on micronuclei induction at different post treatment times in the cultured human epidermoid carcinoma A431 cells.

Cell type	Treatment (µg/ml)	Cells bearing micronuclei (Mean ± SEM)											
		Post treatment time (h)											
		12 h			24 h			36 h			48 h		
		One	Two	Total	One	Two	Total	One	Two	Total	One	Two	Total
Mononucleate cells	**HPD**												
	0	1.21±0.05	0.55±0.01	**1.76±0.02**	1.11±0.03	0.72±0.03	**1.83±0.01**	1.06±0.02	0.54±0.02	**1.60±0.02**	2.44±0.08	0.49±0.01	**2.93±0.11**
	20	5.45±0.42*	4.44±0.67$^{\alpha}$	**9.89±0.05**	6.47±0.52$^{\alpha}$	5.12±0.48$^{\alpha}$	**11.59±0.05**	10.45±1.23$^{\alpha}$	6.44±0.55$^{\alpha}$	**16.89±1.10**	10.26±1.23$^{\alpha}$	6.16±0.63$^{\alpha}$	**16.42±1.02**
	40	7.67±0.55*	5.84±0.44$^{\alpha}$	**13.51±0.14**	8.88±0.24$^{\alpha}$	6.11±0.89$^{\alpha}$	**14.99±0.08**	14.44±1.42$^{\alpha}$	7.44±0.64$^{\alpha}$	**21.88±1.23**	13.24±1.41$^{\alpha}$	7.88±0.82$^{\alpha}$	**21.08±1.27**
	80	8.45±0.53*	7.44±0.55$^{\alpha}$	**15.89±1.05**	12.33±0.84$^{\alpha}$	8.33±0.52$^{\alpha}$	**20.66±0.23**	17.56±2.11$^{\alpha}$	10.32±1.11$^{\alpha}$	**27.87±1.52**	16.89±2.21$^{\alpha}$	9.77±1.13$^{\alpha}$	**26.59±1.09**
	DOX												
	5	6.45±0.86$^{\alpha}$	5.45±0.51$^{\alpha}$	**11.90±1.02**	11.27±0.78	6.11±0.33$^{\alpha}$	**11.38±0.06**	16.67±1.42$^{\alpha}$	9.62±0.45$^{\alpha}$	**26.29±1.22**	14.33±1.23$^{\alpha}$	9.46±0.61$^{\alpha}$	**23.79±1.55**
	10	10.42±0.98$^{\alpha}$	7.44±0.63$^{\alpha}$	**17.86±1.12**	14.24±0.98	8.45±0.32$^{\alpha}$	**22.68±0.14**	18.86±1.83$^{\alpha}$	10.64±0.82$^{\alpha}$	**29.50±1.48**	14.88±0.73$^{\alpha}$	10.22±0.22$^{\alpha}$	**25.10±1.89**
	20	12.22±0.59$^{\alpha}$	9.45±0.88$^{\alpha}$	**21.67±0.75**	16.11±0.99	12.44±0.67$^{\alpha}$	**28.55±1.20**	24.62±1.28$^{\alpha}$	14.56±1.24$^{\alpha}$	**39.18±2.58**	20.44±1.20$^{\alpha}$	12.66±1.22$^{\alpha}$	**33.10±1.13**
Binucleate cells	**HPD**												
	0	1.23±0.11	1.11±0.06	**2.34±0.04**	2.44±0.12	1.45±0.05	**3.84±0.07**	3.11±0.11	1.53±0.11	**4.64±0.82**	3.56±0.33	2.33±0.07	**5.88±0.83.**
	20	8.42±0.84$^{\alpha}$	2.43±0.22*	**10.85±0.27**	13.33±0.68$^{\alpha}$	4.55±0.56*	**17.83±1.31**	16.44±0.88$^{\alpha}$	8.45±0.44$^{\alpha}$	**24.89±1.11**	16.11±1.11$^{\alpha}$	8.22±0.67$^{\alpha}$	**24.32±1.56**
	40	12.76±0.63$^{\alpha}$	4.56±0.88	**17.32±0.55**	16.89±1.33$^{\alpha}$	6.89±0.87*	**27.77±0.68**	19.58±1.44$^{\alpha}$	12.22±0.54$^{\alpha}$	**31.78±1.17**	18.26±1.32$^{\alpha}$	12.11±0.76$^{\alpha}$	**30.36±2.05**
	80	14.56±0.56$^{\alpha}$	6.77±0.45$^{\alpha}$	**21.33±1.25**	16.98±0.67$^{\alpha}$	10.35±0.89$^{\alpha}$	**27.33±1.54**	24.77±1.42$^{\alpha}$	16.88±0.97$^{\alpha}$	**41.65±2.05**	22.45±2.33$^{\alpha}$	14.44±0.87$^{\alpha}$	**36.87±1.88**
	DOX												
	5	18.33±1.23$^{\alpha}$	4.22±0.24$^{\alpha}$	**22.55±1.19**	26.22±0.44$^{\alpha}$	6.31±0.52$^{\alpha}$	**32.53±1.25**	36.11±2.32$^{\alpha}$	8.56±0.88$^{\alpha}$	**44.67±1.88**	32.32±1.42$^{\alpha}$	10.57±0.89$^{\alpha}$	**42.86±1.84**
	10	25.73±2.22$^{\alpha}$	6.88±0.82$^{\alpha}$	**32.61±1.45**	41.33±1.57$^{\alpha}$	7.33±0.72$^{\alpha}$	**48.63±2.43**	51.11±1.22$^{\alpha}$	9.45±0.67$^{\alpha}$	**60.56±1.52**	48.56±1.83$^{\alpha}$	10.59±1.06$^{\alpha}$	**59.24±1.05**
	20	34.44±1.21$^{\alpha}$	10.33±0.54$^{\alpha}$	**44.74±1.87**	52.66±0.82$^{\alpha}$	12.23±1.05$^{\alpha}$	**64.89±2.81**	61.77±1.34$^{\alpha}$	14.67±1.15$^{\alpha}$	**76.42±1.67**	57.68±2.11$^{\alpha}$	14.89±1.08$^{\alpha}$	**72.44±1.85**

**P<0.01, $^{\alpha}$P<0.001 when different treatment time are compared to 0h with respective post treatment time.*

Standard error of the mean (SEM); No symbol=no significance; N=5.

Table 5: Effect of different concentration of HPD and DOX on apoptosis induction at different post treatment time in cultured human epidermoid carcinoma A431 cells.

Treatment (µg/ml)	Apoptotic index (mean±SEM)			
	Post Treatment Time (Hours)			
	2	4	12	24
MEM	1.02±0.13	1.12±0.11	1.13±0.11	1.12±0.14
HPD 20	2.91±0.15*	6.67±0.34*	8.76±0.33*	9.67±0.15*
HPD 40	3.72±0.08*	10.95±0.51*	12.11±0.96*	15.50±0.76*
HPD 80	7.31±0.25*	12.67±0.56*	16.01±0.85*	18.56±0.92*
DOX 5	11.23±0.14*	13.56±1.43*	15.45±1.21*	16.53±1.43*
DOX 10	12.63±1.23*	16.86±1.52*	19.68±2.51*	20.79±2.21*
DOX 20	13.72±0.98*	18.75±1.43*	26.05±2.31*	28.93±2.45*

**P<0.001 when treatment groups are compared to MEM group with respective post treatment time. Standard error of the mean (SEM).*
No symbol=no significance. N=5.

Table 6: Alteration in the glutathione concentration in the cultured human epidermoid carcinoma A431 cells treated with different concentration of hesperidin (HPD) or doxorubicin (DOX).

Treatment (µg/ml)	GSH (µmol/mg protein) Mean ±SEM					
	Assessment time (h)					
	1	2	4	6	12	24
MEM	2.08±0.09	2.44±0.18	2.18±0.02	2.17±0.12	2.26±0.11	2.56±0.01
Hesperidin (HPD)						
20	2.11±0.19	1.91±0.23	1.65±0.15$^{\alpha}$	1.62±0.12$^{\alpha}$	1.45±0.12$^{\alpha}$	1.38±0.07$^{\clubsuit}$
40	1.63±0.38	1.53±0.27*	1.32±0.147$^{\clubsuit}$	1.20±0.38*	1.02±0.02$^{\clubsuit}$	1.17±0.01$^{\clubsuit}$
80	1.47±0.12$^{\alpha}$	1.38±0.13$^{\alpha}$	1.34±0.17$^{\alpha}$	0.98±0.04$^{\clubsuit}$	0.77±0.02$^{\clubsuit}$	0.88±0.05$^{\clubsuit}$
Doxorubicin(DOX)						
5	1.39±0.41	1.31±0.25$^{\clubsuit}$	0.88±0.16$^{\clubsuit}$	0.87±0.03$^{\clubsuit}$	0.82±0.01$^{\clubsuit}$	0.81±0.08$^{\clubsuit}$
10	1.35±0.12$^{\alpha}$	1.00±0.02$^{\clubsuit}$	0.81±0.10$^{\clubsuit}$	0.64±0.07$^{\clubsuit}$	0.72±0.10$^{\clubsuit}$	0.71±0.09$^{\clubsuit}$
20	1.22±0.12$^{\clubsuit}$	0.99±0.13$^{\clubsuit}$	0.76±0.03$^{\clubsuit}$	0.54±0.01$^{\clubsuit}$	0.51±0.02$^{\clubsuit}$	0.52±0.03$^{\clubsuit}$

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment groups are compared spontaneous control group. Standard error of the mean (SEM)*
No symbol=no significant difference. N=5.

Table 7: Alteration in the glutathione-s-transferase activity in the cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX).

Treatment (µg/ml)	GST (U/mg protein) Mean ±SEM					
	Assessment time (h)					
	1	2	4	6	12	24
MEM	0.13±0.01	0.13±0.02	0.14±0.02	0.13±0.00	0.13±0.01	0.13±0.02
Hesperidin (HDP)						
20	0.13±0.01	0.12±0.01	0.12±0.01	0.11±0.01	0.10±0.02	0.10±0.01
40	0.12±0.01	0.12±0.01	0.11±0.01	0.10±0.01*	0.09±0.01*	0.08±0.01$^{\alpha}$
80	0.12±0.01	0.10±0.01	0.09±0.01	0.08±0.01$^{\alpha}$	0.07±0.01$^{\clubsuit}$	0.06±0.01$^{\clubsuit}$
Doxorubicin (DOX)						
5	0.11±0.01	0.09±0.01*	0.07±0.01*	0.07±0.01$^{\alpha}$	0.06±0.00$^{\clubsuit}$	0.05±0.00$^{\clubsuit}$
10	0.10±0.01	0.09±0.01*	0.08±0.01*	0.07±0.00$^{\alpha}$	0.04±0.00$^{\clubsuit}$	0.04±0.00$^{\clubsuit}$
20	0.08±0.00*	0.072±0.00*	0.04±0.00*	0.023±0.00$^{\clubsuit}$	0.01±0.00$^{\clubsuit}$	0.01±0.00$^{\clubsuit}$

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment groups are compared to spontaneous control group. Standard error of the mean (SEM).*
No symbol=no significant difference. N=5.

Table 8: Alteration in the atalase activity in cultured human epidermoid carcinoma A431 cells treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX).

Treatment (µg/ml)	CAT(U/mg protein) Mean ±SEM					
	Assessment time (h)					
	1	2	4	6	12	24
MEM	5.88±0.29	5.65±0.21	5.88±0.19	5.52±0.11	5.76±0.12	6.372±0.14
Hesperidin(HPD)						
20	5.28±0.12	4.32±0.36*	4.29±0.08$^{\alpha}$	3.48±0.13$^{\clubsuit}$	3.39±0.12$^{\clubsuit}$	3.31±0.17$^{\clubsuit}$
40	4.44±0.17$^{\alpha}$	4.20±0.17$^{\alpha}$	4.18±0.11$^{\alpha}$	3.16±0.20$^{\clubsuit}$	3.48±0.04$^{\clubsuit}$	3.24±0.09$^{\clubsuit}$
80	4.40±0.18$^{\alpha}$	3.96±0.29$^{\alpha}$	3.60±0.05$^{\alpha}$	2.76±0.32$^{\clubsuit}$	2.04±0.30$^{\clubsuit}$	2.16±0.04$^{\clubsuit}$
Doxorubicin (DOX)						
5	4.08±0.31$^{\alpha}$	3.66±0.38$^{\alpha}$	3.58±0.18$^{\alpha}$	2.40±0.07$^{\clubsuit}$	1.92±0.08$^{\clubsuit}$	1.56±0.01$^{\clubsuit}$
10	3.80±0.32$^{\alpha}$	3.60±0.21$^{\alpha}$	3.36±0.19$^{\alpha}$	1.92±0.15$^{\clubsuit}$	1.80±0.23$^{\clubsuit}$	1.32±0.06$^{\clubsuit}$
20	3.72±0.35$^{\alpha}$	3.60±0.07$^{\alpha}$	3.20±0.34$^{\alpha}$	1.44±0.15$^{\clubsuit}$	0.84±0.12$^{\clubsuit}$	0.84±0.01$^{\clubsuit}$

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment groups are compared to MEM group. Standard error of the mean (SEM). No symbol=no significant difference.* N=5.

Table 9: Alteration in the superoxide dismutase activity in cultured human epidermoid carcinoma A431 cells y treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX).

Treatment (µg/ml)	SDO (U/mg protein) Mean ±SEM					
	Assessment time (h)					
	1	2	4	6	12	24
MEM	4.25±0.06	4.21±0.08	4.20±0.03	4.24±0.04	4.33±0.02	4.33±0.04
Hesperidin						
20	3.36±0.05$^{\alpha}$	3.21±0.05$^{\alpha}$	3.04±0.06$^{\alpha}$	2.37±0.03$^{\clubsuit}$	2.35±0.02$^{\clubsuit}$	2.38±0.06$^{\clubsuit}$
40	3.02±0.03$^{\alpha}$	2.93±0.01$^{\alpha}$	2.13±0.07$^{\clubsuit}$	1.71±0.01$^{\clubsuit}$	1.56±0.08$^{\clubsuit}$	1.36±0.06$^{\clubsuit}$
80	2.64±0.08$^{\alpha}$	2.53±0.08$^{\alpha}$	2.09±0.01$^{\clubsuit}$	1.47±0.06$^{\clubsuit}$	1.16±0.02$^{\clubsuit}$	1.21±0.08$^{\clubsuit}$
Doxorubicin						
5	2.56±0.02$^{\alpha}$	2.37±0.05$^{\alpha}$	1.62±0.03$^{\clubsuit}$	1.33±0.04$^{\clubsuit}$	1.38±0.01$^{\clubsuit}$	1.34±0.02$^{\clubsuit}$
10	2.49±0.05$^{\alpha}$	1.46±0.06$^{\alpha}$	1.16±0.02$^{\clubsuit}$	1.04±0.03$^{\clubsuit}$	1.09±0.02$^{\clubsuit}$	0.87±0.04$^{\clubsuit}$
20	1.47±0.06$^{\alpha}$	1.16±0.02$^{\alpha}$	1.15±0.01$^{\clubsuit}$	0.86±0.03$^{\clubsuit}$	0.75±0.03$^{\clubsuit}$	0.67±0.11$^{\clubsuit}$

$^{\alpha}p<0.01$, $^{\clubsuit}p<0.001$ when treatment groups are compared to spontaneous control group. Standard error of the mean (SEM). No symbol=no significant difference. N=5.

Table 10: Alteration in the lipid peroxidation in cultured human epidermoid carcinoma A431 cells by treated with different concentrations of hesperidin (HPD) or doxorubicin (DOX).

Treatment (µg/ml)	LOO (nmol/mg protein) Mean ±SEM					
	Assessment time (h)					
	1	2	4	6	12	24
MEM	2.88±0.55	3.53±0.85	4.17±0.84	4.81±0.56	4.17±0.64	4.27±0.25
Hesperidin (HPD)						
20	10.26±0.85$^{\alpha}$	10.89±1.15$^{\alpha}$	20.19±1.67$^{\clubsuit}$	49.04±1.65$^{\clubsuit}$	51.60±1.68$^{\clubsuit}$	55.44±3.56$^{\clubsuit}$
40	14.42±1.11$^{\clubsuit}$	23.08±0.56$^{\alpha}$	35.57±1.46$^{\clubsuit}$	64.42±4.19$^{\clubsuit}$	66.35±0.56$^{\clubsuit}$	68.91±1.94$^{\clubsuit}$
80	28.53±0.85$^{\clubsuit}$	30.45±1.39$^{\clubsuit}$	53.85±6.40$^{\clubsuit}$	73.08±0.96$^{\clubsuit}$	75.32±0.84$^{\clubsuit}$	77.24±1.38$^{\clubsuit}$
Doxorubicin (DOX)						
5	22.12±1.11$^{\clubsuit}$	28.20±1.15$^{\clubsuit}$	35.89±1.38$^{\clubsuit}$	72.44±0.84$^{\clubsuit}$	74.04±1.55$^{\clubsuit}$	77.88±1.12$^{\clubsuit}$
10	45.51±2.62$^{\clubsuit}$	52.29±1.78$^{\clubsuit}$	63.46±5.63$^{\clubsuit}$	94.55±1.78$^{\clubsuit}$	102.56±1.85$^{\clubsuit}$	107.37±1.96$^{\clubsuit}$
20	52.56±1.69$^{\clubsuit}$	60.57±2.77$^{\clubsuit}$	72.11±1.11$^{\clubsuit}$	141.67±2.62$^{\clubsuit}$	146.15±3.46$^{\clubsuit}$	151.60±1.69$^{\clubsuit}$

$^{\alpha}p<0.01$, $^{\clubsuit}p<0.001$when treatment groups are compared to MEM group with respective treatment time. Standard error of the mean (SEM). No symbol=no significant difference. N=5.

Table 11: Alteration in the LDH release by A431 cells treated with various concentrations of HPD and DOX at different post exposure times.

Treatment	Concentration (µg/ml)	Lactate dehydrogenase (U/L) Mean± SEM				
		Post treatment time (h)				
		1	2	6	12	24
Control	MEM	34.99±1.67	33.33±1.21	29.99±3.33	33.33±1.11	36.66±3.33
HPD	20	48.32±4.98	58.32±8.33*	68.32±4.99$^{\alpha}$	73.32±3.32$^{\alpha}$	74.99±11.6$^{\alpha}$
	40	49.99±6.66	66.66±3.33$^{\alpha}$	71.65±1.66$^{\alpha}$	76.65±9.99$^{\alpha}$	78.32±1.66$^{\alpha}$
	80	58.32±2.65*	68.66±3.65$^{\alpha}$	74.65±1.65$^{\alpha}$	76.99±4.99$^{\alpha}$	93.32±3.45$^{\alpha}$
DOX	5	61.66±1.66α	63.32±3.32$^{\alpha}$	68.32±8.32$^{\alpha}$	86.65±11.33$^{\alpha}$	104.98±4.98$^{\alpha}$
	10	63.32±3.99*	71.65±1.78$^{\alpha}$	74.99±8.33$^{\alpha}$	88.32±4.99$^{\alpha}$	123.32±9.94$^{\alpha}$
	20	69.32±1.62α	79.99±3.33$^{\alpha}$	84.99±1.66$^{\alpha}$	111.65±8.83$^{\alpha}$	186.64±6.66$^{\alpha}$

**p<0.05, $^{\alpha}$p<001 when treatment groups are compared with spontaneous control group, Standard error of the mean (SEM). No symbol=no significant difference.* N=5.

Table 12: Alteration in the LDH release by A431 cells treated with 20 µg/ml of HPD or 5 µg/ml of DOX for different times at different post treatment times.

Exposure time (h)	Lactate dehydrogenase (U/L) Mean± SEM									
	Assessment time (h)									
	0		1		6		12		24	
	HPD 20	DOX 5	HPD 20	DOX 5	HPD 20	DOX 5	HPD 20	DOX 5	HPD 20	DOX 5
MEM	34.99±1.66		33.33±1.21		29.997		33.33±1.11		36.66±3.33	
1	48.32±4.98	61.66±1.66$^{\alpha}$	36.66±1.66	39.99±3.32	37.66±6.67	49.99±1.62*	40.99±6.66	54.99±4.89*	41.66±4.95	61.66±1.66$^{\alpha}$
2	58.32±8.33*	63.32±3.32$^{\alpha}$	38.33±8.32	41.66±1.66*	42.32±1.69	54.99±1.65*	43.66±4.94	63.32±3.33*	45.32±6.66	62.99±3.31$^{\alpha}$
6	68.32±4.99$^{\alpha}$	68.32±8.32$^{\alpha}$	39.99±4.99	53.32±1.67*	43.66±3.32	58.32±8.31$^{\alpha}$	48.32±8.31	64.99±4.98$^{\alpha}$	54.99±1.66$^{\alpha}$	66.66±6.67$^{\clubsuit}$
12	73.32±3.32$^{\alpha}$	86.65±11.33$^{\alpha}$	41.66±3.33	59.99±3.43*	49.32±1.65*	61.66±1.68$^{\alpha}$	53.32±3.33$^{\alpha}$	68.32±8.33$^{\clubsuit}$	58.32±4.98*	76.65±3.31$^{\clubsuit}$
24	74.99±11.6$^{\alpha}$	104.98±4.98$^{\alpha}$	46.66±1.66*	71.65±7.99$^{\alpha}$	51.66±3.32*	73.32±3.33$^{\clubsuit}$	58.32±1.68$^{\alpha}$	86.65±3.33$^{\clubsuit}$	61.66±1.66$^{\alpha}$	91.65±11.65$^{\clubsuit}$

p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment are compared to spontaneous control with respective assessment time. No symbol= no significant. Standard error of the mean (SEM).* N=5.**

Table 13: Alteration in the LDH release by A431 cells treated with 40 µg/ml of HPD or 10 µg/ml of DOX for different times at different post treatment times.

Exposure time (h)	Lactate dehydrogenase (U/L) Mean± SEM									
	Assessment time (h)									
	0		1		6		12		24	
	HPD 40	DOX 10	HPD 40	DOX 10	HPD 40	DOX 10	HPD 40	DOX 10	HPD 40	DOX 10
MEM	34.99±1.66		33.33±1.21		29.99±2.17		33.33±1.11		36.66±3.33	
1	49.99±6.66	63.32±9.99*	36.32±4.97	45.99±3.66	39.32±1.63*	52.32±3.33*	46.66±1.12	54.99±5.66	53.32±3.32	56.66±6.65
2	66.66±3.33$^{\alpha}$	71.25±1.78$^{\clubsuit}$	41.99±3.31	56.66±6.68$^{\alpha}$	43.32±3.33*	56.66±6.67*	51.66±4.99	61.66±4.99	58.32±1.66	69.99±9.98
6	71.65±1.66$^{\alpha}$	74.99±8.33$^{\alpha}$	49.99±6.66	66.66±9.92$^{\alpha}$	52.32±2.95	59.99±6.66	54.99±1.66	63.32±3.34	63.32±1.65	71.65±4.98
12	76.65±9.99$^{\clubsuit}$	88.32±4.99$^{\clubsuit}$	51.32±3.33$^{\alpha}$	71.65±1.68$^{\alpha}$	54.66±8.42	66.66±9.98	56.66±6.68	78.32±4.97	66.66±6.67	94.99±8.32
24	78.32±1.66$^{\clubsuit}$	123.32±9.94$^{\clubsuit}$	54.32±1.68$^{\alpha}$	86.65±3.43$^{\clubsuit}$	58.32±1.65	86.65±9.98	68.32±4.89	91.65±4.93	78.32±8.32	113.32±3.33

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment are compared to spontaneous control with respective assessment time.*
No symbol= no significant. Standard error of the mean (SEM), N=5.

Table 14: Alteration in the LDH release by A431 cells treated with 80 µg/ml of HPD or 20 µg/ml of DOX for different times at different post treatment times.

Exposure time (h)	Lactate dehydrogenase (U/L) Mean± SEM									
	Assessment time (h)									
	0		1		6		12		24	
	HPD 80	DOX 20	HPD 80	DOX 20	HPD 80	DOX 20	HPD 80	DOX 20	HPD 80	DOX 20
MEM	34.99±1.66		33.33±1.21		29.997		33.33±1.11		36.66±3.33	
1	58.32±6.65	69.32±1.62	39.99±6.66	54.99±1.65	46.66±4.56	56.66±4.93	48.32±3.34	64.99±4.89	51.66±1.98	65.32±6.48
2	68.66±6.65	79.99±3.33	49.99±4.99	61.66±1.66	51.66±8.32	64.32±11.65	54.99**±6.65**	69.32±4.98	66.66±6.65	73.65±14.91
6	74.65±1.65	84.99±1.66	52.16±1.15	63.32±3.31	54.99±1.66	68.32±16.65	58.32±8.32	73.32±6.67	64.99±1.67	78.32±4.99
12	76.99±4.99	111.65±8.83	54.99±1.66	68.32±8.32	58.32±1.67	81.65±8.33	61.66±4.56	85.65±1.63	73.32±3.33	91.65±8.31
24	93.32±3.45	186.64±6.66	58.32±1.76	81.65±8.33	61.66±5.66	92.65±8.33	71.65±4.99	109.98±6.68	81.65±1.66	136.65±13.21

**p<0.05, $^{\alpha}$p<0.01, $^{\clubsuit}$p<0.001when treatment are compared to spontaneous control with respective assessment time.*
No symbol= no significance. Standard error of the mean (SEM), N=5.

Summary

&

Conclusions

Summary and Conclusions

Cancer is usually believed to be disease of modern world however, it is not the case as humans and other animals have shown the presence of cancer throughout recorded history. The earliest evidence of cancer is found among fossilized bones and human mummies preserved in ancient Egypt, and also the ancient manuscripts, which has recorded the occurrence of cancer (Grmek, 1975-76; Cassileth, 1983). The earliest known descriptions of cancer appeared in several Papyri from ancient Egypt (American Cancer Society, 2009*).* Hippocrates (460-370 BC), is considered as the "Father of Medicine and he used the terms *karkinos* and carcinoma to describe non-ulcer forming and ulcer-forming tumors for the first time. In Greek, these words refer to a crab, most likely applied to the disease because like crab the cancer has a central core and the finger-like spreading projections from a cancer resembling the legs of a crab. The Roman physician, Celsus (28-50 BC), later translated the Greek term into cancer, the Latin word for crab. Galen (130-200 AD), another Greek physician, used the word *oncos* (Greek for swelling) to describe tumors. Although the crab analogy of Hippocrates and Celsus is still used to describe malignant tumors, Galen's term is now used as a part of the name for cancer specialists – oncologists (Woelfer, 1881; Breasted, 1930; Ebbell, 1937).

The carcinogenesis is a multistep process that begins with normal cell populations, which are transformed into a pre-neoplastic cell population and subsequently form a highly malignant tumor (Sugimura, 1992). Each step during carcinogenesis involves a varying degree of stability and reversibility. This multistep process of experimental carcinogenesis can be divided into initiation, promotion and progression (Farber, 1984; Pitot and Riegel, 1987; Shields and Harris, 1991). The earliest studies of multistage carcinogenesis in experimental animals were based on studies by Rous and Friedewald (1941) on mouse skin indicating the presence of two stages during carcinogenesis namely: initiation and promotion. Foulds (1954) proposed the concept of tumor progression describing the characteristics of malignant neoplasia and its evolution to higher degrees of autonomy and malignancy, which is now regarded as the third stage of tumor development. Thus it is widely accepted that cancer development is a multistep event proceeding through discrete morphological and biochemically altered stages from normal to pre-neoplastic lesions to highly malignant tumors (Sugimura *et. al.*, 1991; Rundhaug and Fischer, 2010) involving dysfunction of genes involved in cell growth, differentiation and cell cycle control, which includes proto-oncogenes and tumor suppressor genes (Harris, 1991; Coleman and Tsongalis,

2006). The modern view of carcinogenesis is the multistep process of experimental carcinogenesis can be divided mainly into three stages namely, *"initiation", "promotion"* and *"progression"* (Pitot *et al*., 1987; Shields and Harris, 1991; Farber, 1984; Rundhaug and Fischer, 2010).

Majority of the chemical carcinogens are not capable of causing hazardous effects but the metabolism of these compounds play a crucial role in the initial host response to the environmental exposure. Disturbance in the balance between capacity of activation and detoxification may thus explain the individual variations in response to exposures to carcinogens. The amount of ultimate carcinogen produced depends on the action of competing activation and detoxifying pathways involving phase I and phase II enzymes (Kensler and Cooney, 1981; Cooney, 1982; Guengrich, 1988). There are two theories of carcinogenesis: somatic mutation theory, where a carcinogen causes mutation and confers selective advantage of cell proliferation leading to neoplastic transformation and thus the neoplasia is monoclonal in origin and that the default state of a metazoan cells is quiescence. This theory was proposed by Boveri in the year 1914. According to this theory cancer is irreversible. The other theory of carcinogenesis is the tissue organization field theory, which states that carcinogenesis is primarily a problem of tissue organization, where a carcinogen destroys the normal tissue architecture and disrupts cell-to-cell signaling, and compromising genomic integrity (Sonnenschein and Soto, 2000). Hence, in this theory the DNA mutations are the effect, and not the cause, of the tissue-level events (Rosenfeld *et al*., 2013). According to this theory cancer is reversible and curable. It is well known that carcinogens cause a point mutation leading to a change in the single base pair or change several base pairs encoding an abnormal protein. These genetic changes may lead in the breakage of chromosomes or duplication or loss of chromosomes during or after DNA replication. This results in genomic instability, where the cancer cells have unstable genome including aneuploidy. The other way is that carcinogen may change the way of DNA packaging, the epigenetic change. Several forms of genetic changes have been reported including gene amplifications, deletions, insertions, rearrangements, and point mutations in the neoplastic cells indicating that these process are fundamental to carcinogenesis (Lengauer *et al.,* 1998)

The major mechanisms of chemical protection against mutagenesis, carcinogenesis and other forms of toxicity is the induction of phase II metabolizing enzymes. The phase II metabolizing enzymes include various transferases such as UDP-

glucuronosyl transferase, glutathione S-transferase and NADPH quinone reductase. The phase II enzymes act on the electrophilic products generated in the phase I reaction by incorporating them into endogenous moieties (glucuronide, glutathione, sulphate) to produce extremely electrophilic products that are excreted from the cell (Cooney, 1982; Guengrich, 1988).

There is a substantial evidence of involvement of free radicals during the enzymatic activation of various carcinogens such as benzo(a)pyrene (BaP), dimethylbenz(a)anthracene (DMBA), aromatic amines and N-nitrasocompounds (Clemens, 1991). All these compounds induce free radicals through normal metabolic pathways, which interact with DNA to form DNA adducts (Klaunig *et. al.*, 1998). The reactive oxygen species generated during tumor promotion interact with a wide variety of cellular biomolecules resulting in altered phenotypic expression, which may be mediated through direct modification of the genome or epigenetic pathways. The pharmacological intervention may inhibit the generation of free radicals by various sources and offer protection to the cellular genome.

Chemoprevention is a strategy to prevent the development of cancer by various means. Despite of the immense efforts to improve treatment of cancer and find its cure, the overall mortality rates for most form of cancer have not significantly declined in the past 50 years (Jemal *et. al.,* 2008). Conventional therapeutic (chemotherapy and radiation) and surgical approaches have not been able to control the incidence of most of cancer types. The major treatment strategies of cancer cause damage to the cellular genome of not only the neoplastic cells but also the normal cells, which in turn become neoplastic in due course of time. Therefore, strategies that could inhibit the occurrence of cancer could be most desirable than those that allows the development of cancer and treat it thereafter. Chemoprevention is a term used frequently to describe the paradigm that can block the occurrence of cancer.

The old age saying that *"prevention is better than cure"* could be an important strategy to reduce the risk of cancer in human population and the most important imaginative approach to reduce the cancer cases worldwide, could be to inhibit the induction of carcinogenesis or cancer by pharmacological intervention, which will not allow the cellular DNA to undergo mutagenic changes, in other words the cellular DNA will be preserved in its native form despite the onslaught from various physical and chemical sources (Liu, 2004; Liu *et. al.*, 2007). This new pharmacological approach to arrest or reverse the process of carcinogenesis, and thus prevent cancer, is called

"*chemoprevention*". For the first time, it has been shown convincingly that the use of chemopreventive agents in men and women with premalignant lesions can substantially reduce the subsequent development of truly invasive cancer.

Skin cancer chemoprevention is theoretically similar to chemotherapeutics, but focuses on pre-invasive rather than invasive lesions. Accessibility of the skin allows for easier detection and removal of neoplastic or preneoplastic lesions, using noninvasive or minimally invasive techniques. The skin, therefore, is a model organ for investigating cancer prevention processes that may be relevant to other organs as well. The incidence of skin cancer has been increasing at an alarming rate with an estimated 3.6 million cases in 2005; accounting for 40% of all cancer diagnoses in Western world. The majority of skin cancers are nonmelanomas (NMSCs) and include epidermal keratinocyte derived squamous cell (SCC) and basal cell carcinomas (BCC) both of which are closely associated with chronic exposure to ultraviolet light (UV). A pre-malignant lesion or actinic keratosis (AK) has been identified for SCC, but not for BCC.

Although only 6% of skin cancers are melanomas, melanoma is the most deadly form of skin cancer. Usually 132,000 of skin melanomas are diagnosed every year. Dysplastic nevi, a likely precursor of melanoma, are also potential targets for chemoprevention as they are also vitally important in the reduction of skin cancer mortality. However, chemoprevention studies of melanoma have been limited.

It is believed that dietary factors may contribute to the reduction of as much as one-third of potentially preventable cancers. The long-term preventive effect of plant-based agents for chemoprevention of cancer and several other chronic diseases is well documented (Jang and Pezzuto, 1999; Link *et al.*, 2010). The development of a malignant tumor involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, and immunological). In addition, carcinogenesis often proceeds through multiple discernible but often overlapping stages. The transitions between successive stages can be enhanced or inhibited by various agents. Several types of evidences indicate that 50–80% of human cancers are potentially preventable, because its causation, i.e., the factors that determine the incidence, is largely exogenous. Exogenous causative agents or factors that have been identified in humans include cigarette smoking, exposure to occupational and environmental chemicals, radiation, carcinogenic factors present in the diets, lifestyle, socioeconomic factors, specific viruses, bacteria, and/or parasites. Apart from these

factors hereditary factors play a critical role in influencing individuals' susceptibility to cancer and that in certain rare forms of human cancer; it is the hereditary factors that determine the development of cancer in an individual. However, in the majority of human cancers, the exogenous factors present the most likely opportunities for interventions targeted to primary prevention of cancer.

Chemopreventive compounds have been classified into blocking agents or suppressing agents according to the carcinogenic stages they interrupt (Wattenberg, 1997). The blocking agents are compounds, which discourage the metabolic activation of procarcinogens and subsequent formation of reactive carcinogens or prevent active carcinogens from reaching or reacting with critical cellular targets such as DNA, RNA and proteins, whereas the suppressing agents are those pharmacological agents that deter malignant transformation of initiated cells after reaction of carcinogens with important cellular targets during promotion or progression.

Dietary polyphenols having antioxidant, antimutagenic activities and modulating effects on certain cytochrome P-450 enzymes and may play an important role in chemoprevention strategies. Because of the expected safety following long-term administration to human, the diet has been considered as a rich source of potential chemopreventive agents. In fact, a number of natural compounds with inhibitory effects on tumorogenesis have been identified from human diet or sources of diet. These compounds include isothiocyanates from cruciferous vegetables, catechins from green tea, resveratrol from grape seeds, red wine, curcuminoids from turmeric, procyanidins from various fruits and nuts, isoflavones from soybean, and antioxidant vitamins in various foods. With a significant advancement in our understanding of the cellular events leading to cancer, synthetic chemopreventive agents have been also developed, which include selective inhibitors of ornithine decarboxylase (ODC), selective estrogen receptor modulators (SERM), selective inhibitors of retinoid X receptors (rexinoids), and inhibitors of inducible cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS).

Hesperidin was first discovered by Lebreton in the year 1827 in an impure form and has been investigated for its various properties since then (Fluckiger and Hanbury, 1986). Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), is a predominant bioflavonoid, present in large amounts in the discarded rinds 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 Citrus genus. It is also found in many plants other than citrus species, such as Fabaceae (Bhalla and Dakwake, 1978), Betulaceae (Pawlowska, 1980), Lamiaceae (Kokkalou and Kapetanidis, 1988) and Papilionaceae. Hesperidin is also present in the bark of *Zanthoxylum avicennae* and *Z. cuspidatum* belonging to family Rutaceae (Arthur *et al.,* 1956). It has been also isolated from the roots of *Acanthopanax setchuenensis i*n China (Zhao *et al.,* 1999). The highest concentration of hesperidin has been found in the green fruits, which increases during storage (Higby, 1941). It is reported to be present in the epicarp, mesocarp, endocarp and juice of Citrus fruits (Kawaguchi *et al.,* 1997).

Both hesperidin and its aglycone hesperitin have been reported to possess a wide range of pharmacological properties. Hesperidin has been reported to possess significant anti-inflammatory, analgesic, antihypertensive, diuretic antibacterial and antiviral effect (Galati *et al.,* 1994; Emim *et al.,* 1994; Bae *et al.,* 2000; Kim *et al* 2001; Ohtsuki *et al.,* 2003). Hesperidin has been reported to inhibit tumor initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts *in vitro* (Berkarda *et al.,* 1998; Tanaka *et al.,* 1997; Franke *et al.,* 1998). It has been found to reduce cholesterol levels in humans (Kurowska *et al.,* 2000) and retard the bone loss (Chiba *et al.,* 2003; Hasanoglu *et al.,* 2001). Its deficiency has been indicated in abnormal capillary leakage. Hesperidin has been found to possess beneficial effects on the abnormal capillary permeability, fragility and protection against various traumas and stresses (Felicia *et al.,* 1996). It has been found to be non-toxic in animals and humans (Sieve, 1952; Kawabe *et al.,* 1993; Kawaguchi *et al.,* 1997). The chemoprotective effect of hesperidin has not been evaluated therefore the present study envisages to investigate the chemoprotective activity of hesperidin in skin carcinogenesis mouse model.

AIM AND OBJECTIVE OF THE STUDY:

Increasing environmental pollution, altered life style and various other factors have increased the frequency of cancer in human population. The cancer is the second largest killer disease in the modern world. In spite of the availability of large paraphernalia of treatment strategies, complete cure of cancer still remains elusive. The present modalities of cancer treatment are non-specific and also change the fidelity of genome of normal cells as a result secondary tumors have been reported in the survivors (Pendelton *et al*., 2014). Further, treatment of cancer is highly expensive

and beyond the reach of common man and chemoprevention could be a prudent strategy to reduce the occurrence of cancer worldwide by use of different pharmacological agents that are expected to spare the cellular DNA from the mutagenic changes induced by various factors and preserve it in native form. Common dietary agents may play an important role in the inhibition of carcinogenesis. Therefore, present study aims to investigate the chemopreventive effects of hesperidin in mice.

CHAPTER 1

This chapter gives the linkage of contemporary context on chemoprevention and scientific knowledge. A brief description on the aim and scope of the thesis is enlisted at the end of this chapter.

CHAPTER 2

In this chapter**,** the acute toxicity evaluation, necessary to find out the maximum tolerated dose as well as its toxic side effects has been carried out, where the male Swiss albino mice were orally administered with 0.5, 1, 2, 2.5 and 3 g/kg body weight of hesperdin. The acute toxicity studies showed that hesperidin is non-toxic up to a dose of 3 g/kg body weight. The chronic administration of 100, 200 300 and 400 mg/kg body weight hesperidin for 90 days did not show any signs of toxicity, which indicate that 400 mg/kg/day is safe in mice. The DNA damage study by micronucleus assay revealed a significant alteration in the frequency of micronuclei in the splenocytes after chronic administration of hesperidin except 100 mg/kg, where this increase was within control range. The sperm dysfunction test showed no distinctive alteration in the motility and viability of sperms after chronic administration of hesperidin. Similarly, the analysis of blood RBC showed an increase in their numbers whereas WBC remained unaltered. The biochemical profiling showed that chronic administration of various doses of hesperidin did not alter the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and uric acid in comparison to baseline levels. From the present study it is clear that hesperidin is safe up to 3 g/kg body weight and does not have any undesirable side effects after acute and chronic administrations.

CHAPTER 3

The Inflammation is one of the important responses elicited by organisms to counteract obnoxious stimuli. However, continuous inflammation has been responsible for the induction of several diseases. Therefore, it is essential to combat

excess inflammation by devising countermeasures to neutralize excess inflammation. The chapter 3 provides an inkling of the analgesic and anti-inflammatory activities of hesperidin, which were studied in mice using standard procedures including hotplate, acetic acid, tail immersion, xylene and formalin-induced edema tests. Treatment of mice with different doses of hesperidin revealed that hesperidin induced analgesic and anti-inflammatory activities in a dose dependent manner as indicated by pain inhibition and reduced inflammation. The maximum effect was observed for 300 mg/kg b. wt. hesperidin.

CHAPTER 4

The chapter 4 gives an account of the wound healing ability of different concentrations of hesperidin and naringin ointments in mice after infliction with a full thickness rectangular excision wound of 2.5 x 1.5 cm^2 area and by measuring wound contraction using superimposed transparent graph sheet containing 1 mm^2 grid squares and mean wound healing time. Topical application of hesperidin and naringin ointment once a day accelerated the healing of excision wounds when compared to placebo treatment. A maximum wound contraction was observed for 5% hesperidin application when compared to naringin or application of combination of both the hesperidin and naringin. Application of 5% hesperidin reduced the mean wound healing time (MHT) significantly by 5.7 days when compared to the placebo controls. Naringin or combination of hesperidin and naringin also enhanced the wound contraction; however, it was lesser than hesperidin treatment alone. The application of naringin and its combination with hesperidin also led to a reduction in the MHT but this alleviation was lesser than hesperidin alone. The collagen and DNA synthesis studies revealed a similar correlation where maximum syntheses of collagen and DNA were observed for hesperidin treatment alone, when compared to all other groups at all post wounding days. In vitro studies have indicated that hesperidin scavenged DPPH, OH, $O_2^{-\bullet}$, ABTS and NO radicals in a concentration dependent manner up to 500 µg ml except ABTS radicals, where a maximum scavenging activity was observed at 400 µg/ml. The present study clearly demonstrates that 5% hesperidin accelerated the healing of regenerating wounds by increasing synthesis of collagen and DNA, which may be due to its antioxidant effect.

CHAPTER 5

In this chapter, the chemopreventive potential of hesperidin was investigated on the 7, 12-dimethylbenz[a]anthracene (DMBA)-TPA-induced skin carcinogenesis in Swiss

albino mice. The skin carcinogenesis was initiated by the topical application of DMBA followed by the application of 12-O-tetradecanoyl phorbol-13-acetate (TPA) as a promoting agent on the shaved dorsum of mice treated or not with various doses of hesperidin. The application of DMBA followed by subsequent application of TPA led to 100% tumor incidence and increased average number of tumors in mice, whereas the administration of hesperidin before or after and continuous (pre and post) carcinogen application significantly reduced the tumor incidence and average number of tumors when compared to DMBA-TPA alone. The tumor formation was also delayed by hesperidin treatment. Topical application of DMBA-TPA increased the oxidative stress as evident by a significant rise in TBARS and decline in various antioxidants when compared to the untreated control group. The hesperidin treatment significantly reduced TBARS in the skin of mice treated with DMBA-TPA and significantly elevated the glutathione concentration and glutathione-S-transferase, superoxide dismutase and catalase activities when compared with the DMBA-TPA application alone. Our study demonstrates that hesperidin protected mice against chemical carcinogenesis and the chemopreventive effect of hesperidin may be due to the protection of DMBA-induced DNA damage, inhibition of TPA induced-inflammatory response and increased antioxidant status.

CHAPTER 6

This chapter explains the effect of hesperidin on cell growth, DNA damage, apoptosis and antioxidant status in cultured human epidermoid carcinoma A431 cells. Exposure of A431 cells to various concentrations of hesperidin resulted in a concentration dependent decline in the cell survival, which reflected as a reduction in the clonogenicity of these cells. The DNA damage assay revealed that hesperidin increased the frequency of micronuclei in both mononucleate cells and binucleate cells in a concentration dependent fashion with assay time up to 36 h post treatment. Similarly, hesperidin induced apoptosis in A431 cells in a concentration dependent manner and with increasing assay time. Hesperidin treatment of A431 cells resulted in a decline in glutathione concentration, and glutathione-s-transferase, catalase, and superoxide dismutase activities followed by a rise in the lipid peroxidation and lactate dehydrogenase release. The hesperidin has been able to suppress cell proliferation and this effect of hesperidin seems to be due induction of micronuclei formation, apoptosis and reduction in glutathione, and glutathione-s-transferase, catalase, and

superoxide dismutase activities accompanied by increased lipid peroxidation and lactate dehydrogenease release.

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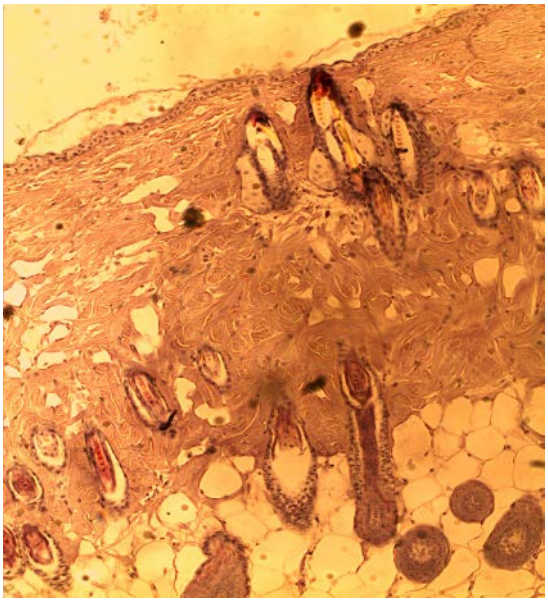
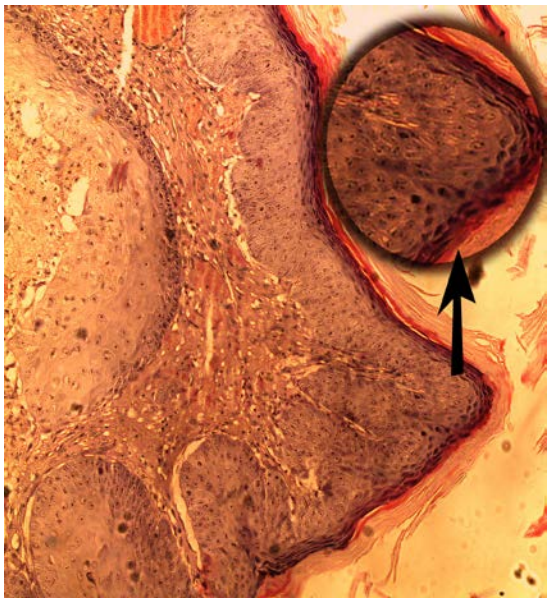
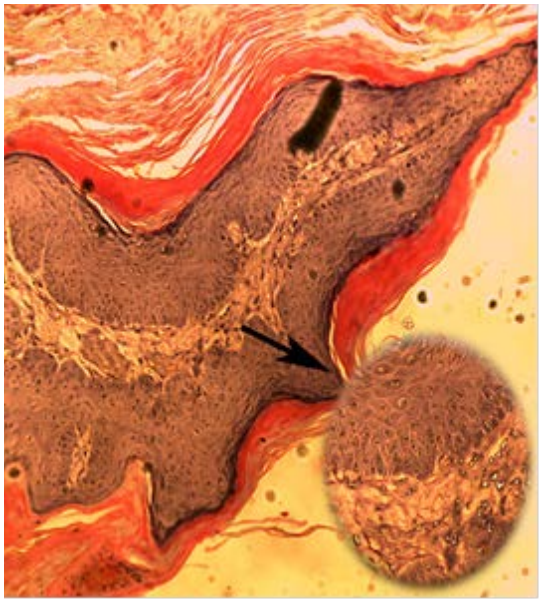


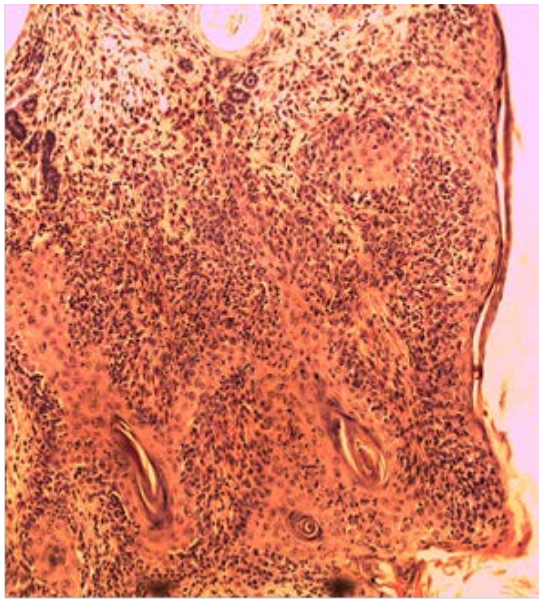
Normal skin



Papilloma



Hyperplastic epidermis with papilloma



Squamous cell carcinomas (SCC)

Plate 1: Cross section of skin and skin papilloma on 24th week post DMBA-TPA application representing malignant transformation in skin of Swiss albino mice.