# "COMBINATION OF EPIGENETIC REGULATORS WITH CHEMOTHERAPEUTIC AGENTS: A NOVEL APPROACH FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA"

**A Thesis Submitted to**

**NIRMA UNIVERSITY**

**In Partial Fulfillment for the Award of the Degree of**

## MASTER OF PHARMACY

## IN

## PHARMACOLOGY

**BY**

**AYUSH SHARMA (21MPH203), B. PHARM.**

**Under the guidance of**

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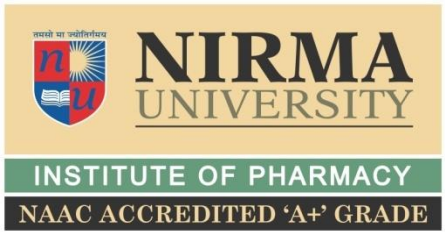


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# CERTIFICATE

*This is to certify that the dissertation work entitled "Combination of Epigenetic Regulators with Chemotherapeutic Agents: A Novel Approach for the Treatment of Hepatocellular Carcinoma" submitted by Mr. Ayush Sharma with Regn. No. (21MPH203) in partial fulfillment for the award of Master of Pharmacy in "Pharmacology" is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under my/our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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# CERTIFICATE OF ORIGINALITY OF WORK

*This is to undertake that the dissertation work entitled "Combination of Epigenetic Regulators with Chemotherapeutic Agents: A Novel Approach for the Treatment of Hepatocellular Carcinoma" Submitted by Mr. Ayush Sharma (21MPH203) in partial fulfillment for the award of Master of Pharmacy in "Pharmacology" is a bonafide research work carried out by me at the "Department of Pharmacology", Institute of Pharmacy, Nirma University under the guidance of "Dr. Snehal S. Patel". I am aware about the rules and regulations of Plagiarism policy of Nirma University, Ahmedabad. According to that, this work is original and not reported anywhere as per best of my Knowledge.*

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# *DECLARATION*

*I hereby declare that the dissertation entitled "Combination of Epigenetic Regulators with Chemotherapeutic Agents: A Novel Approach for the Treatment of Hepatocellular Carcinoma", is based on the original work carried out by me under the guidance of Dr. Snehal S. Patel, Associate Professor under the Department of Pharmacology, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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***बस कर्म तुम्हारा कल होगा, और कर्म में अगर सच्चाई है तो, कर्म कहाँ निष्फल होगा ।***
***हर एक संकट का हल होगा, वो आज नहीं तो कल होगा ।***
***ज़्यादा वक्त लगेगा, मगर संघर्ष ज़रूर सफल होगा ।***
***हर एक संकट का हल होगा, वो आज नहीं तो कल होगा ।***

***~ श्रीकृष्ण***

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***~ AYUSH SHARMA***

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# *LIST OF ABBREVIATION*

| | | |
|---|---|---|
| 5-ASA | : | 5-Aminosalicylic Acid |
| AFB1 | : | α-fetoprotein levels |
| BCLC | : | Barcelona Clinic Liver Cancer |
| $CCL_4$ | : | Carbon Tetrachloride |
| DNA | : | Deoxy-ribonucleic Acid |
| DNMT | : | DNA Methyl Transferase |
| DNMTi | : | DNA Methyl Transferase Inhibition |
| GSH | : | Reduced Glutathione |
| HBV | : | Hepatitis B Virus |
| HCC | : | Hepatocellular Carcinoma |
| HCV | : | Hepatitis C Virus |
| HIV | : | Human Influenza Virus |
| LPO | : | Lipid Peroxidation |
| NAD | : | Nicotinamide Adenine Dinucleotide |
| NADH | : | Nicotinamide Adenine Dinucleotide Hydrogen |
| NAFLD | : | Nonalcoholic Fatty Liver Disease |
| NASH | : | Non-alcoholic Steatohepatitis |
| NDEA | : | N-Nitrosodiethyl Amine |
| SOD | : | Superoxide Dismutase |

# CHAPTER 1

# ABSTRACT

**Background:** Epigenetic modification is a common feature of hepatocellular carcinoma. DNA Methyl Transferases (DNMT) inhibitors in combination with chemotherapeutic agents show their inhibitory effects on the DNMT while increasing the sensitivity of chemotherapeutic agents. In the present study, we aimed to evaluate the effect of Olsalazine (a DNMT inhibitor) in combination with gemcitabine (a chemotherapeutic agent) on the development of NDEA induced hepatic cancer in Swiss albino mice.

**Method:** We tested our hypothesis using *in silico, in-vitro* and *in-vivo* models. For the *in silico* analysis, molecular docking was performed for the initial screening of the selected ligands on DNMT1 protein (PDB ID: 4WXX) using AutoDockTools-1.5.6. software. Ligand preparation and energy minimization step was performed using Chemdraw Professional 15.0 and Chem3D softwares, respectively. *In-vitro* experiments were performed on human hepatocellular carcinoma HepG2 cells. MTT assay was performed to evaluate the effects of gemcitabine alone or in combination with Olsalazine pretreatment on the viability of selected cell line. For animal experiments, thirty animals were allocated to five different groups (n=5 per group): Normal Control (NC), Diseased control (DC) treated with N-Nitrosodiethylamine (NDEA), Disease control treated with standard drug (DC-SD) gemcitabine (50mg/kg) once a week at a dose of intraperitoneally, Disease control treated with standard drug (Gemcitabine) and a low pretreatment dose (50mg/kg) of Olsalazine (DC-SD-LD) and Disease control treated with standard drug (Gemcitabine) and a higher pretreatment dose (100mg/kg)of Olsalazine (DC-SD-HD). For induction of Hepatocellular carcinoma, Carbon tetrachloride (CCL4) at a dose of 50 µl/kg body weights in liquid paraffin was administered orally for 4 consecutive days followed by NDEA administration at a cumulative dose of 350mg/kg twice a week intraperitoneally for six weeks. NDEA was administered for two weeks at 25mg/kg followed by increasing doses at 50mg/kg and 100mg/kg, respectively for next two weeks. Following induction, treatment with Gemcitabine alone or in combination with Olsalazine pretreatment was continued for 3 weeks. Bodyweight, food and water intake were measured for 3 weeks. At the end of third week, blood samples were collected and serum biochemistry and ELISA were performed. Isolated livers at the end of third week of treatment were weighed and subjected to histopathological analysis and immunohistochemistry (IHC). Oxidative stress parameters were performed on liver tissue homogenates.

**Results:** Among the selected ligands Olsalazine, showed the highest binding affinity (-5.54) among all selected ligands. Therefore, Olsalazine was selected for further *in-vitro* and *in-vivo* evaluation. Cytotoxicity of gemcitabine against the HepG2 cells was improved upon pretreatment with Olsalazine (at 10um concentration). In *in-vivo* study, reduced tumor lesions were seen in animals pretreated with Olsalazine (20mg/kg and 40mg/kg) followed by treatment with gemcitabine (50mg/kg). Pretreatment with Olsalazine improved the treatment response to gemcitabine in comparison to gemcitabine alone treated group. Cancer progression was reduced, which was evident from improved body weight, food and water intake. Serum biochemistry showed reduced levels of ALP, LDH, CRP, SGPT, SGOT and total bilirubin levels in Olsalazine pretreated groups in comparison to diseased animals. Reduced levels of IL-6 and TNF-alpha were seen in Olsalazine pretreated animals, with a significant increase in P53 levels, suggesting improved treatment response. Furthermore, treatment showed reduced levels of LPO and increased activity of GSH and SOD in liver tissues. Results from histopathological examination showed increased number of double nucleus, congestion in central and portal veins, decreased sinusoidal space in disease control group. In contrast, the sinusoidal space was increased with decreased number of double nucleus in treated group compared to the diseased control group. Immunohistochemistry results showed increase in apoptosis evident with increased levels of p53 in treated group as compared to the disease control group.

**Conclusion:** Our findings from the current study indicate that combining the chemotherapeutic drug gemcitabine with the epigenetic regulator Olsalazine enhances the antitumor response and also increases the sensitivity of gemcitabine for antitumor responses.

# CHAPTER 2

# INTRODUCTION

Cancer is a condition of abnormal growth of a specific population of cells in the body that continue to grow in an uncontrolled manner and spread to other parts of the body. Cancer is the second-leading cause of death worldwide and a significant issue for public health (Hassanpour & Dehghani, 2017). Uncontrolled cell proliferation and the development of metastatic features are the main characteristics of cancer (Sarkar et al., 2013).The six compatibilities that contribute to the hallmarks of cancer include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011). Since the human body is composed of an infinite number of cells, almost system of human body is prone to develop cancer. Human cells may typically expand and divide to create new cells as needed by the body. Cells die when they aged or harmed, and new cells replace them. Occasionally, this systematic process fails, causing damaged or aberrant cells to proliferate when they shouldn't. These cells have the ability to grow into tumors. Tumors can be cancerous (Malignant) or non-cancerous (Benign). Cancerous tumors have the ability to spread to remote regions of the body, infiltrate nearby tissues, or both. A number of environmental (external) factors (tobacco, chemicals, radiation, and infectious organisms), as well as internal factors (inherited mutations, hormones, immune conditions and random mutations), are involved in the development of cancer. These components may interact to initiate or promote human carcinogenesis (Tyagi et al., 2017).

In general, cancer changes how cells communicate with one another and make important genes fail. This disruption affects the cell cycle, leading to aberrant proliferation (Cigudosa et al., 1999; Matlashewski et al., 1984; Seto et al., 2010). During the process of carcinogenesis, Proto-oncogenes, which are typically in-charge of cell growth and division, are transformed into oncogenes following gain of function mutations, leading to an uncontrolled growth signalling and proliferation of transformed cell. Additionally, loss of function mutations in tumor suppressor genes causes uncontrolled cell division.

Liver cancer is one of the most common cancers reported worldwide. By 2025, it is anticipated that liver cancer would affect more than 2 million individuals annually. Hepatocellular carcinoma (HCC), the most common kind of liver cancer, accounts for around 90% of cases (Llovet et al., 2021). Hepatocellular carcinoma is currently the fifth leading cause of cancer

related deaths for men and the seventh leading cause of cancer related deaths in women globally (Patel et al., 2021). HCC is a hyper-vascular, rapidly expanding tumor that is fuelled by many key processes, including the formation of tumor at metastatic sites, the breakdown of extracellular matrix components, the migration of the tumor cells through the basement membrane, and cell proliferation (Wong et al., 2014).

Several oncogenes and tumor suppressor genes are activated and inactivated at the start of carcinogenesis, which encourages molecular processes leading to the development of HCC (Bhatia et al., 2015). The greatest risk factor for developing HCC is infection with the hepatitis B virus (HBV), which accounts for around 50% of liver cancer cases (Llovet et al., 2021). Infection or injury to the liver can cause inflammation. If the problem persists, fibrogenesis develops, eventually leading to hepatocellular carcinoma (Yang et al., 2019b). Additionally, the poor prognosis of those with HCC is linked to the increased resistance of this cancer to radiation and chemotherapy (Patel et al., 2021).Local ablation, surgical resection, or liver transplantation are curative options for early-stage HCC. The characteristics of the tumor, severity of the underlying liver dysfunction, age, or other medical comorbidities, availability of local medical resources, and other factors affect the choice of the course of treatment. Kinase and immune checkpoint inhibitors have been used to successfully treat patients with advanced-stage HCC (Yang & Heimbach, 2020; Zeng et al., 2017).

The study of heritable variations in gene expression that take place without a change in DNA sequence and are potent enough to control the dynamics of gene expression is known as epigenetics (Rodenhiser & Mann, 2006). Epigenetic modification are widely known to occur in cancer and in a number of other illnesses, such as diabetes, lupus, asthma, and a number of neurological conditions (Kelly et al., 2010). The majority of epigenetic alterations are enzymatic and potentially reversible, as opposed to genetic changes involving mutations. Because epigenetic changes can be undone, they appear to be the best targets for cancer therapy (Bianco & Gévry, 2012).Three groups of epigenetic proteins—writers, readers, and erasers—control the intricate interplay between epigenetic events (Ahuja et al., 2016; Simó-Riudalbas & Esteller, 2015). Writer introduces and Readers recognise and understand the numerous chemical changes that makes modification on DNA and histones, while erasers delete the alterations. With the

growing development of technologies to investigate the epigenome, pharmaco-epigenomics, a novel field where epigenetic profiles may be utilised to find molecular pathways for cancer drug sensitivity and be used in determining the best treatment approach, is emerging (Kelly et al., 2010). Numerous epigenetic drugs have been made accessible for the treatment of cancer, and others are presently undergoing clinical testing. The medications 5-azacytidine and 5-aza-20-deoxycytidine, which are used as first-line therapies for patients with, for example, myelodysplastic syndrome, inhibit the DNA methyltransferases DNMT1 and DNMT3B, which are regarded as writers. Drugs like romidepsin and vorinostat target histone deacetylases (HDACs), which serve as erasers (SAHA). Clinical trials for medications that target epigenetic readers, such as bromodomain (BET) inhibitors, are currently ongoing (Majchrzak-Celinska et al., 2021). DNA and histone modifications are tightly regulated in normal tissues, and when they are dysregulated, nucleosome organization, which affects gene expression, DNA replication, and repair, is significantly impacted (Wang et al., 2021). Epigenetics importance in preserving normal development and biology is demonstrated by the fact that many diseases develop when the wrong epigenetic marks are added, removed, or introduced at the wrong time or location (Wu & Zhang, 2010).

The three primary kinds of epigenetic alterations are DNA methylation, histone modifications, and nucleosome positioning (Wang et al., 2021).Maintaining genome integrity, genomic imprinting, transcriptional regulation, and developmental processes depend on the epigenetic DNA modifications brought on by methylation (Wu & Zhang, 2010). DNA methylation, which normally takes place at the 5-position of the cytosine ring inside CpG dinucleotides, silences genes and non-coding genomic regions. DNMTs are the enzymes that catalyse the alterations at 5-methylcytosine in the DNA (Wu & Zhang, 2010).To date, methylation, acetylation, and phosphorylation of histones have all been discovered. Together, these modifications control the chromatin state, gene expression, and other nuclear processes (Wang et al., 2021). Since aberrant histone modification levels in sick tissue are the consequence of an imbalance in these modifying enzymes, restoring the elevated or reduced quantity of a particular enzyme would restore the injured cells' natural equilibrium. Histone deacetylases (HDAC) are extensively expressed resulting in a general reduction in histone acetylation levels. Histone methyltransferase and histone demethylase enzymes are both dysregulated in cancer (Kelly et al., 2010). Dynamic

chromatin structural changes that have an impact on transcription, replication, and DNA repair processes have been linked to these abnormalities and could possibly be used as cancer therapeutic targets (Majchrzak-Celinska et al., 2021).

DNA Methylation is one of the epigenetic processes of methylation of cytosine bases at the C5 site in the CpG islands of chromosomal DNA. The majority of the active enzymes in the methylation pathway, the DNMT family of enzymes, catalyse the transfer of a methyl group from s-adenosyl-l-methionine (SAM) to DNA (Wang et al., 2021).A subset of the gene promoters located within CpG islands are hypermethylated in cancer, which silences the genes as a result of global hypomethylation and hypermethylation (Kelly et al., 2010).The *de novo* enzymes DNMT3A and DNMT3B, which target unmethylated CpGs to begin methylation and are extensively expressed during embryogenesis and hardly expressed in adult tissues, target unmethylated CpGs to preserve the current methylation patterns during DNA replication. Another member of the family is DNMT-3L, which interacts with DNMT3a and 3b to promote the methylation of retro transposons despite lacking intrinsic methyltransferase activity (Deplus et al., 2002; Friedman et al., 2009; R. Kanwal & Gupta, 2012).Currently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved two DNMT inhibitors (DNMTi), the nucleoside analogues 5-azacitydine (azacitidine) and 5-aza-20-deoxycytidine (decitabine), to treat myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myelomonocytic leukemia(Majchrzak-Celinska et al., 2021).

Hypomethylating drugs that target the isoforms of DNA methyltransferase (DNMT) are promising anticancer treatments. It has been previously shown that the FDA-approved anti-inflammatory drug Olsalazine also functions as a DNA hypomethylating agent. Olsalazine almost exactly mimics the effects of the well-known hypomethylating drug 5-aza-2'-deoxycytidine while exhibiting no cytotoxicity at the studied levels (Méndez-Lucio et al., 2014). In the treatment of inflammatory bowel illness, olsalazine and 5-Aminosalicylic Acid (5-ASA) are two anti-inflammatory drugs that are often used (Staerk Laursen et al., 1990). Olsalazine is a prodrug made of two 5-ASA moieties joined together by an azo bond. Microorganisms in the colon break down the azo bond to release 5-ASA. Because it is active in the colon's lumen, olsalazine is appropriate for colonic inflammatory illnesses including ulcerative colitis (Ryde et al., 1991). Scavenging oxygen-derived free radicals, inhibiting lipoxygenase, preventing

leukotriene synthesis from arachidonic acid, altering the prostaglandin profile by affecting prostaglandin 15-hydroxydehydrogenase, and interfering with leukocyte function are a few potential mechanisms for the anti-inflammatory effects of olsalazine (Brown et al., 2000; Enna & Bylund, 2007).

A strong and focused deoxycytidine analogue is gemcitabine. It is a well-known anticancer drug that has received FDA approval to be used either alone or in combination with other medications (paclitaxel, carboplatin, or cisplatin) to treat locally advanced or metastatic cancer(Gray et al., 2012). Failure of apoptosis caused by gemcitabine to have cytotoxic effects is one of the main mechanisms of gemcitabine resistance (S. Xu et al., 2021). The medication is either activated by deoxycytidine kinase to produce dFdC-5'-monophosphate (dFdCMP) or deaminated by deoxycytidine deaminase to produce 2',2'-difluorodeoxyuridine (dFdU). While dFdCMP is further metabolized to dFdC 5'diphosphate and dFdC 5'triphosphate, which when integrated into DNA, cause chain termination (Mini et al., 2006). Numerous studies have been carried out to assess the effectiveness and tolerance of gemcitabine in combination with other cytotoxic drugs due to the synergistic action with other chemotherapeutic substances and epigenetic regulators. The effect of gemcitabine in conjunction with novel targeted medicines is being studied in ongoing clinical studies (Toschi et al., 2005).

Utilizing the Synergy of Epigenetic Regulators and Chemotherapeutic Agents: A Promising Approach for Hepatocellular Carcinoma Treatment

The aim of this study was to explore the potential of combining epigenetic regulators with chemotherapeutic agents as a novel therapeutic approach for treating hepatocellular carcinoma. Specifically, we investigated the binding effects between a selected hypomethylating agent and a DNMT1 inhibitor to understand their interactions. Furthermore, we assessed the *in vitro* effects of the chosen hypomethylating agent in combination with a chemotherapeutic agent. Additionally, we sought to evaluate the anti-tumor efficacy of this selected combination through experiments conducted on animal models. By examining the synergistic effects of epigenetic regulators and chemotherapeutic agents, we aimed to provide valuable insights into a promising strategy for enhancing the treatment of hepatocellular carcinoma.

# CHAPTER 3

# REVIEW OF LITERATURE

### 3.1 Hepatocellular Carcinoma

The majority of liver cancer cases are discovered at advanced stages (Singal et al., 2020).HCC is a hypervascular, quickly growing tumor that is driven by a number of important mechanisms, including tumor development at metastatic locations, extracellular matrix component breakdown, tumor cell migration through the basement membrane, and cell proliferation (Yang & Heimbach, 2020). At the beginning of carcinogenesis, several oncogenes and tumor suppressor genes are activated and inactivated, which stimulates the molecular processes that result in the development of HCC (Bhatia et al., 2015).Early-stage HCC is asymptomatic, which causes a considerable delay in prompt detection. Advanced HCC patients have few effective and readily available therapy choices, and those with advanced illness are not candidates for curative surgery (Yang & Heimbach, 2020).The most popular monitoring diagnostics are liver ultra sonography (Yang & Heimbach, 2020). By eliminating the requirement for a liver sample for histologic confirmation, improved imaging methods have made non-invasive diagnosis of hepatocellular carcinoma exceedingly accurate and trustworthy. Biopsies can still be performed on rare occasions to confirm hepatocellular carcinoma in tumors that lack any distinctive radiologic characteristics (Yang & Heimbach, 2020).

Numerous risk factors for HCC have been identified, the most prevalent of which include chronic infections with the hepatitis B and C viruses, non-alcoholic steatohepatitis, aflatoxin-contaminated food intake, and chemical carcinogens (Saber et al., 2018).The majority of instances of HCC are caused by hepatitis B virus (HBV) infection, which accounts for around 50% of cases. Even after HCV eradication, individuals with cirrhosis are still thought to be at a significant risk for developing HCC. Estrogen-mediated suppression of IL-6 has long been proposed as a mechanism by which oestrogen exerts its protective effect against HCC. Studies indicate that oestrogen protects and androgen promotes HCC. Systemic treatments for HCC, including as immune checkpoint inhibitors (ICIs), tyrosine kinase inhibitors (TKIs), and monoclonal antibodies, are currently posing a threat to their usage. On average, systemic medications are thought to be used throughout the lives of people with HCC, especially when the illness is advanced (Llovet et al., 2018, 2021).

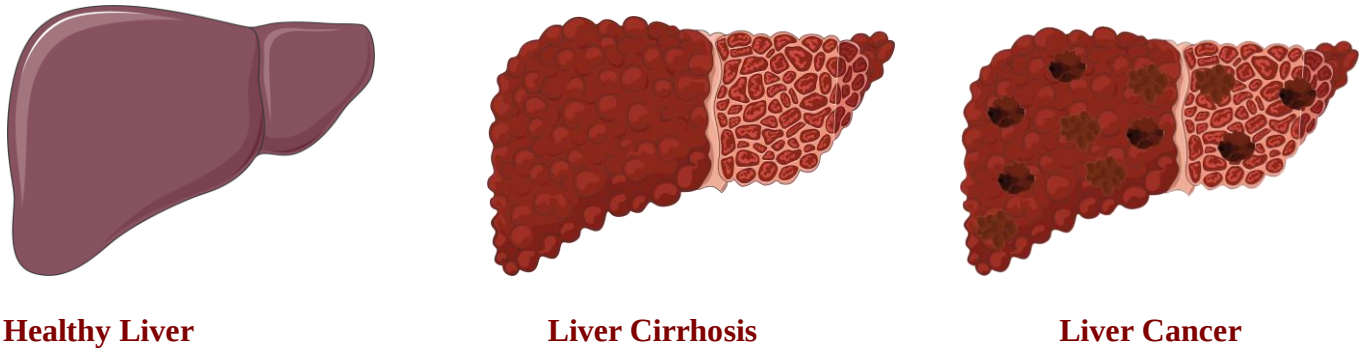



**Figure 1: Schematic representation of hepatocellular carcinoma progression**

### 3.2 Epidemiology

Liver cancer is the sixth leading cancer globally with 905,677 (4.7%) new liver cancer cases reported alone in 2020, making it one of the most common cancers in the world. The highest incidence and mortality of HCC are observed in East Asia and Africa, although HCC incidence and mortality are increasing in different parts of Europe and in the USA. HCC is projected to become the second leading cause of cancer-related death by 2030 if these trends continue (Llovet et al., 2021). HCC incidence decreased between 1978 and 2012 in several Asian nations and Italy, but rose in India, the Americas, Oceania, and the majority of European nations (Yang & Heimbach, 2020). However, in more recent years, the growth has decreased in certain nations, including the US, as rates in various subgroups have plateaued or decreased (McGlynn et al., 2021). In different places of the world, HCC develops at different ages. In nations like Japan, North America, and Europe, where the median age of start is above 60 years old, HCC tends to manifest itself later in life. HCC is often detected in patients between the ages of 30 and 60 in much of Africa and certain areas of Asia (Yang et al., 2019b).

Figure [1] show epidemiology of hepatocellular carcinoma.

#### 3.2.1 Epidemiology of hepatocellular carcinoma in Asia

Liver cancer is the second most common cancer mortality cause in Asia, accounting for 72.5% of all cases worldwide in 2020. Hepatocellular carcinoma (HCC), the most prevalent histological form, is responsible for the majority of liver cancer cases in terms of incidence and death. In most Asian countries, hepatitis B and C virus (HBV and HCV) infection rates have slightly decreased, which is primarily attributable to newborn HBV vaccination, HCV horizontal

transmission control, and chronic hepatitis treatment. However, the frequency of HCC brought on by metabolic variables, such as the metabolic syndrome, obesity, and non-alcoholic fatty liver disorders, is rising quickly in Asian nations and may soon overtake other causes as the main factor in HCC. Given that the average alcohol intake is still rising, excessive alcohol use remains a significant risk factor (C. hao Zhang et al., 2022).

### 3.2.2 Epidemiology of hepatocellular carcinoma in India

One of the most prevalent malignancies in both men and women in India is liver cancer. According to the Globocon report 2020, there were 33,793 (0.4%) fatalities from liver cancer in 2020, and 34,743 (0.5%) new instances of liver cancer were recorded in India. Liver cancer is the ninth leading cause of cancer related deaths in India. India has a higher frequency of liver cancer than other continents, according to the World Health Organization. Male patients have a larger risk than female patients, and the risk rises with advancing age. According to recent statistics, the overall male-to-female ratio for age-standardized liver cancer incidence and death is 2.4 (E. Kim & Viatour, 2020, McGlynn et al., 2021). Up to 2040, there is a possibility that the number of liver cancer cases may double compared to lung cancer instances.

Figure [2] show epidemiology of hepatocellular carcinoma in India.

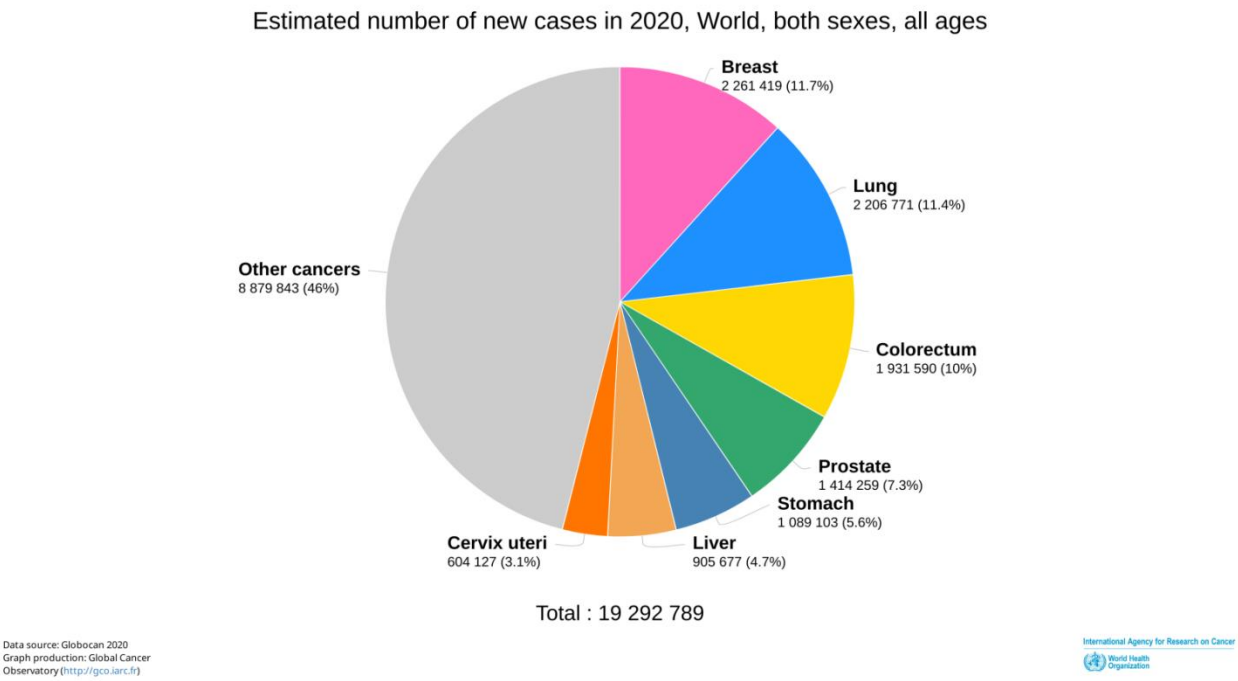



**Figure 2: Epidemiology of Hepatocellular Carcinoma**

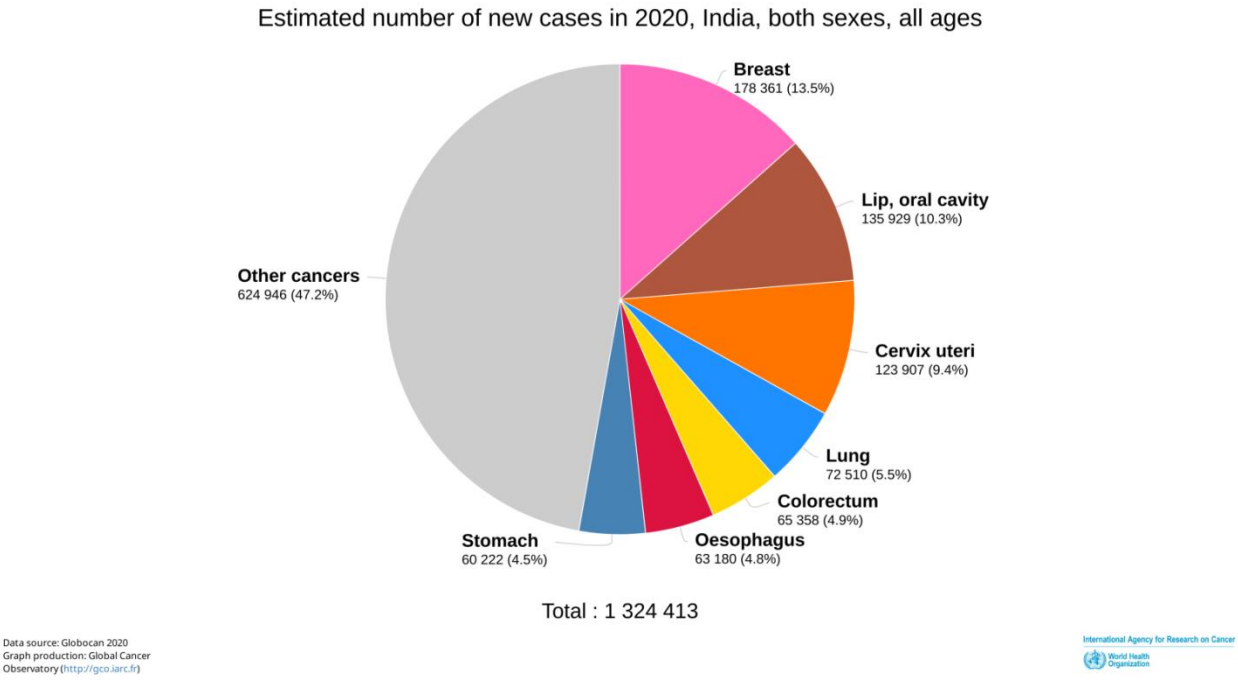



**Figure 3: Epidemiology of Hepatocellular Carcinoma in India**

**Table 1: The list of the most prevalent malignancies according to the WHO Global Cancer Observatory (GLOBOCAN) 2020**

| S. No. | All Cancer | Number of Cases | | |
|---|---|---|---|---|
| | | **Both Sex** | **Male** | **Female** |
| **1** | All Cancer | 19292789 | 10065305 | 9227484 |
| **2** | Breast | 2261419 | - | 2261419 |
| **3** | Lung | 2206771 | 1435943 | 770828 |
| **4** | Colorectal | 1931590 | 1065960 | 865630 |
| **5** | Prostate | 1414259 | 1414259 | - |
| **6** | Stomach | 1089103 | 719523 | 369580 |
| **7** | Liver | 905677 | 632320 | 273357 |
| **8** | Cervix Uteri | 604127 | - | 604127 |
| **9** | Oesophagus | 604100 | 418350 | 185750 |
| **10** | Thyroid | 586202 | 137287 | 448915 |
| **11** | Bladder | 573278 | 440864 | 132414 |
| **12** | Non-Hodgkin Lymphoma | 544352 | 304151 | 240201 |
| **13** | Pancreas | 495773 | 262865 | 232908 |

**Table 2: The list ofhepatocellular carcinoma according to the WHO Global Cancer observatory (GLOBOCAN) 2020.**

| S. No. | Country | Total Cases | Male | Female | Death |
|---|---|---|---|---|---|
| **1** | All Cases | 9,05,677 | 6,32,320 | 2,73,357 | 8,30,180 |
| **2** | Africa | 70,542 | 45,142 | 25,400 | 65,944 |
| **3** | America | 82,711 | 52,062 | 30,694 | 69,202 |
| **4** | Asia | 6,56,992 | 4,71,999 | 1,84,993 | 6,08,898 |
| **5** | Europe | 87,630 | 58,089 | 29,551 | 78,415 |
| **6** | Australia &NewZealand | 3,344 | 2,429 | 915 | 2,503 |

### 3.3 Risk Factors:

Chronic infections with the hepatitis B or C viruses, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) are major risk factors for HCC (El-Serag, 2011). While HBV has been identified as the most frequent etiological agent globally, HCV is the most prevalent cause of HCC in the USA. Many experts believe that NAFLD will soon overtake obesity as the primary cause of HCC, particularly in the USA and other Western nations (Z. M. Younossi et al., 2015), given the prevalence of obesity-related NAFLD. It's well accepted that any long-term liver condition that results in cirrhosis can put a patient at risk for HCC. HCC, however, can sporadically develop in the absence of cirrhosis (Sayiner et al., 2019; Z. Younossi et al., 2019).

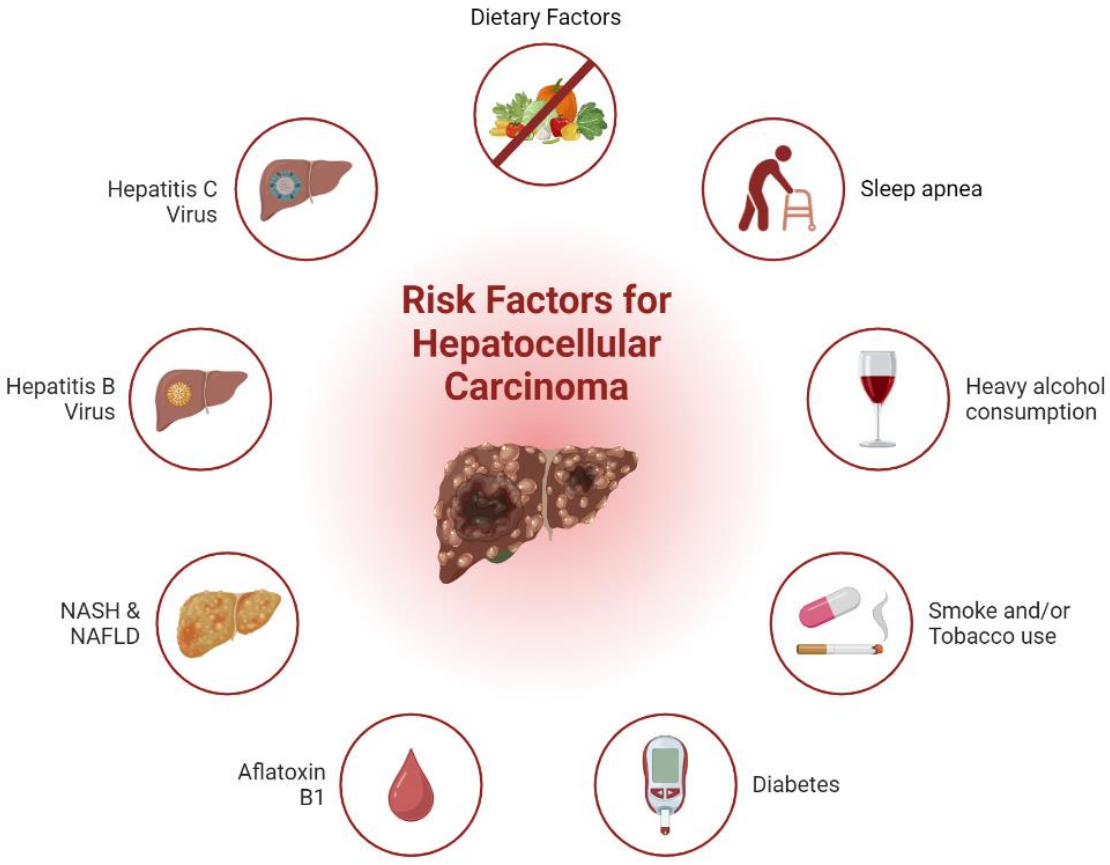



**Figure 4: Risk Factors for Hepatocellular Carcinoma**

### 3.3.1 Hepatitis B Virus:

In nations with few medical resources, chronic hepatitis B is the most common cause of HCC. In fact, chronic hepatitis B is responsible for more than half of all HCCs worldwide (Yang et al., 2019b). HBV is a DNA virus that causes a persistent necro inflammatory condition that encourages liver cell alterations and results in HCC (Bouvard et al., 2009). HBV DNA is frequently incorporated into the genome when analyzing tumor tissue from HBV carriers (McGlynn et al., 2021). Demographic factors (such as male sex, older age, Asian or African ancestry, and family history of HCC), viral factors (such as high HBV replication levels, HBV genotype, infection duration, and co-infection with HCV or HIV), and environmental exposures (such as aflatoxin, alcohol, tobacco, obesity, and diabetes) are cofactors that also increase risk among HBV carriers (McGlynn et al., 2021).As a result of the high prevalence of endemic HBV infection in East Asia, the risk of HCC in males (40 years of age) and women (50 years of age) is more than the cost-effectiveness thresholds, which justifies the use of monitoring programmes. In several Asian regions, HBV vaccination programmes have reduced the prevalence of HCC, while many governments have not yet implemented universal immunization programmes (Llovet et al., 2021).

### 3.3.2 Hepatitis C Virus:

In North America, Europe, Japan, several regions of central Asia, including Mongolia, and northern Africa and the Middle East, notably Egypt, HCV is the most common virus-related cause of HCC (Yang et al., 2019b). The most prevalent underlying liver condition in people with HCC in North America, Europe, and Japan is chronic HCV infection (Llovet et al., 2021). Since HCV is an RNA virus and does not integrate into the host genome like HBV does, only people who develop cirrhosis or chronic liver injury with bridging fibrosis are at an increased risk of developing HCC. An growing number of HCV-infected individuals are being effectively treated with direct-acting antiviral (DAA) medication to obtain an SVR, which lowers the risk of HCC by 50–80% (F. Kanwal et al., 2017). However, the frequency of HCC brought on by metabolic variables, such as the metabolic syndrome, obesity, and non-alcoholic fatty liver disorders, is rising quickly in Asian nations and may soon overtake other causes as the main factor in HCC.

Given that the average alcohol intake is still rising, excessive alcohol use remains a significant risk factor (Llovet et al., 2021). HCV is often spread through many sexual encounters or high-

risk sexual conduct that involves direct blood contact, such as intravenous drug use (Yang et al., 2019b).

### 3.3.3 Tobacco:

According to the 2014 US Surgeon General's study, current smoking raised the risk of liver cancer by 70%, but prior smoking increased the risk by 40% (Alberg et al., 2014). However, a recent study revealed that the number of years after smoking cessation was inversely related to HCC risk, with people who stopped smoking more than 30 years ago having an HCC risk that was comparable to never-smokers (McGlynn et al., 2021; Petrick et al., 2018).

### 3.3.4 Alcohol:

The second most frequent risk factor for HCC in the USA and Europe is alcoholic cirrhosis (Yang et al., 2019b). Due to sex variations in alcohol metabolism, women are more vulnerable than males to liver damage from alcohol consumption, and are also more likely to develop cirrhosis (Frezza et al., 1990). Drinking too much alcohol raises the likelihood of having hepatocellular carcinoma (HCC) when viral hepatitis is present (Yang et al., 2019b). Currently, cirrhosis from persistent alcohol use or NASH affects a growing number of people. Depending on the location, alcohol-related cirrhosis contributes for between 15% and 30% of HCC cases each year, with yearly incidence ranging from 1% in population-based studies to 2-3% in tertiary care referral centres (Llovet et al., 2021).

### 3.3.5 Dietary Factors:

The use of coffee has repeatedly been linked to a lower incidence of liver cancer (Clinton et al., 2020). According to a 2017 meta-analysis of cohort and case-control studies, drinking an additional two cups of coffee per day was linked to a 35% lower risk (Kennedy et al., 2016). Additionally, coffee has been linked to lower liver enzyme levels, slower fibrosis progression, and a decreased chance of developing diabetes. However, it is unclear what mechanisms underlie a potential protective benefit of coffee. Caffeine and many other coffee ingredients, such as diterpenes, may have positive effects on inflammation, fibrosis, insulin resistance, and oncogenesis, according to experimental studies (Torres & Harrison, 2013).

### 3.3.6 Age, Sex and other factor:

HCC has been linked to a number of socio-demographic factors, particularly in cirrhotic individuals. Age is a significant risk factor, with persons >70 years of age reporting the greatest age-specific incidence. Furthermore, there is a high male preponderance in HCC (male to female ratio of 2-3:1), which is probably due to a concentration of risk factors in males and variations in sex hormones (Sung et al., 2021). Studies on the epidemiology of disease have also shown that smoking increases the risk of HCC. With the exception of research demonstrating the preventative effects of coffee and aspirin, the impact of food in reducing the risk of HCC is still unknown (Bravi et al., 2013; Y.-C. A. Lee et al., 2009; Llovet et al., 2021).

### 3.3.7 Diabetes Mellitus:

A twice to threefold greater risk of HCC is linked to diabetes mellitus. Hepatocarcinogenesis is hypothesized to be influenced by insulin resistance and the subsequent formation of reactive oxygen species that cause hepatic inflammation. According to one study, hyperglycemia raises the risk of HCC in those with liver cirrhosis as well. It is still debatable whether interactions between diabetes and the aetiology of liver cirrhosis affect the risk of HCC (El-Serag et al., 2006; Huang et al., 2018; Hui et al., 2008; Yang et al., 2016, 2019b).

### 3.3.8 Aflatoxin B1:

A range of foods, most notably maize, ground nuts, and tree nuts, are contaminated by aflatoxins, mycotoxins made by fungus of the Aspergillus species. Aflatoxin B1 (AFB1) is the most powerful of the four main aflatoxins, along with aflatoxins B2, G1, and G2. Many places on earth have AFB1, particularly in those with warm, muggy climates. AFB1 is particularly cancerous when it co-occurs with persistent HBV infection since the two conditions together increase the risk of HCC (McGlynn et al., 2021).HBV and aflatoxin exposure have a significant impact on the chance of developing liver cancer (Ross et al., 1992). The cytochrome P450s that convert inactive AFB1 to the mutagenic AFB1-8,9-epoxide may be activated by chronic HBV infection. The likelihood of TP53 mutations brought on by AFB1 is likewise increased by hepatocyte necrosis and regeneration from persistent HBV infection. Additionally, HBV oncogenic protein inhibits nuclear excision repair, which typically removes AFB1-DNA adducts (Kew, 2003; Yang et al., 2019b).

### 3.3.9 NASH:

NASH, which serves as the precursor stage in the development of HCC in individuals with diabetes mellitus or obesity, is another prevalent aetiological reason for cirrhosis in humans. NASH is now the most frequent cause of cirrhosis in most parts of the world due to the rising incidence of obesity. Since 2010, the percentage of cases of HCC in the West that may be ascribed to NASH has quickly climbed to 15-20%. (Estes et al., 2018; Llovet et al., 2021).

### 3.3.10 NAFLD:

In the majority of developed nations, nonalcoholic fatty liver disease (NAFLD) is currently the most prevalent liver disease and a significant risk factor for HCC. NAFLD is currently responsible for 10% to 20% of HCC cases in the United States (Yang et al., 2019b). Clinical factors (cirrhosis, diabetes, obesity, and hypertension), demographic traits (age, race/ethnicity), and genetic susceptibility (e.g., genetic variation in PNPLA3) are all associated with an increased risk of developing HCC in people with NAFLD (McGlynn et al., 2021).

### 3.3.11 Other factors for cirrhosis:

The prevalence of HCCs due to these additional aetiologies is between 5% and 10% worldwide. Other chronic liver illnesses, such as chronic biliary disease, hereditary liver diseases, and metabolic liver diseases, can produce cirrhosis and encourage the development of HCC (Yang et al., 2019b).

### 3.4 Pathogenesis

Due to the fact that advanced liver disease rather than HCC determines a patient's risk for morbidity and mortality, patients with advanced liver disease are typically ineligible for sorafenib therapy. Due to HCC's high level of drug resistance and drug toxicity when combined with underlying liver disease, there is no established systemic chemotherapy regimen. A medical oncologist should think about systemic chemotherapy in light of hepatic reserve, treatment objectives, and the availability of and eligibility for clinical trials (Dhanasekaran et al., 2016). There are multiple factors showed in a table 3 and figure no. 4.

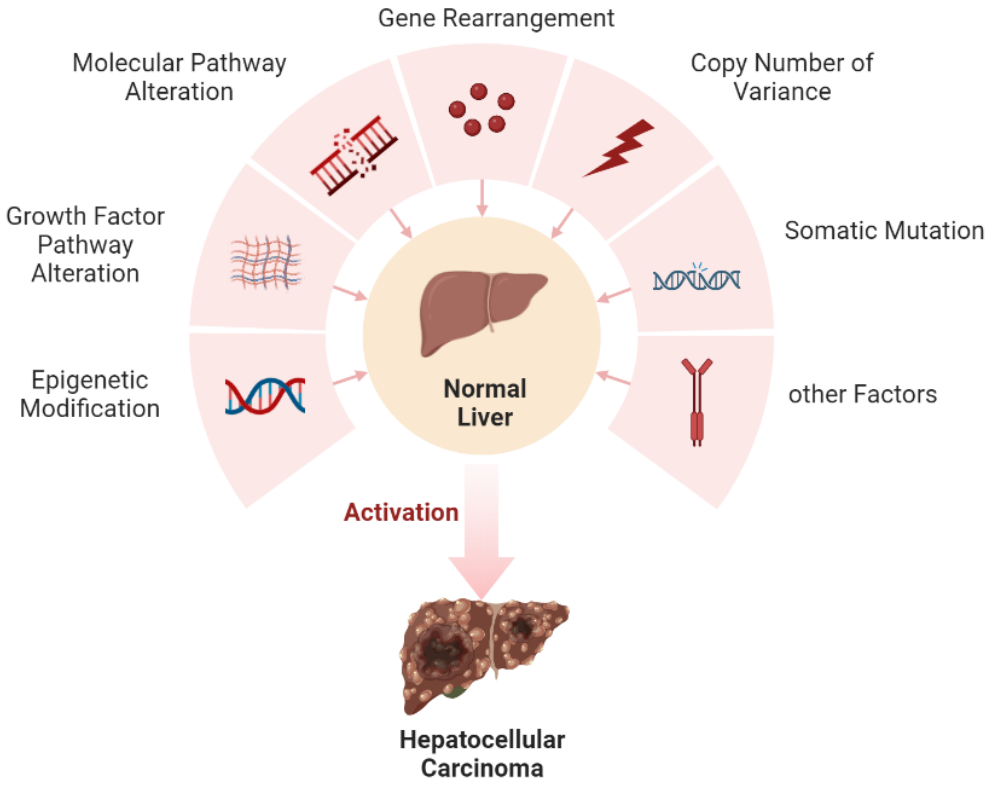



**Figure 5: Pathogenesis of Hepatocellular Carcinoma**

**Table 3: Major events in pathogenesis of Hepatocellular Carcinoma**

| S. No. | Factors | |
|---|---|---|
| 1 | Epigenetic Modification | **DNA Methylation** |
| | | MicroRNA |
| | | Lnc RNA |
| 2 | Growth Factor Pathway Alteration | Wnt/β-catenin |
| | | Tyrosine kinase pathways |
| | | Hedgehog |
| 3 | Molecular Pathway Alteration | Wnt-β Catenin |
| | | Tyrosin Kinase |
| | | JAK/STAT |
| | | Ubiquitin-proteosome |
| 4 | Gene Rearrangements | DNAJB1-PRKACA fusion in fibrolamellar HCC |
| 5 | Copy Number of Variation | Myc |
| | | VEGF-A |
| | | MET |
| | | CCDN1 |
| | | CDKNA2 |
| 6 | Somatic Mutation | TERT Promoter |
| | | TP53 |
| | | CTNNB1 |
| | | AXIN1/2 |
| | | ARID1A/ARID2 |
| | | RB1 |
| 7 | Other Factors | Gene mutations |
| | | Gene amplification/Deletions |

### 3.5 Stages:

Tumor load, liver function, cancer-related symptoms (such as performance status (PS), Karnofsky Index), and the effects of treatment are taken into account when predicting survival (Díaz-gonzález, 2016). The Barcelona Clinic Liver Cancer (BCLC) classification is currently the most popular standard staging system for HCC and is the only staging system that has been prospectively validated (Yang et al., 2019b). It aims to combine prognosis estimation and potential treatment advances in a single unified proposal (L, 2020). The staging of HCC is essential for prognostic assessment and selection of appropriate treatment strategy (Bruix et al., 2014). According to BLCL classification there are 5 stages very early stage (Stage 0), early stage (Stage A), intermediate stage (Stage B), advanced stage (Stage C), end stage (Stage D)

#### 3.5.1 Stage 0:

Patients included in the very early stage (BCLC 0) are those who are diagnosed with a solitary tumor, which measures less than 2 cm. They would respond well to a drastic strategy and resection (L, 2020, Díaz-gonzález, 2016).

#### 3.5.2 Stage A:

Early stage, BCLC A, involves individuals identified with one single nodule irrespective of its size or with 3 tumors measuring up to 3 cm each. Besides these qualities, individuals included in this stage must have an intact liver function, being Child-Pugh A and no cancer-related symptoms with ECOG-Performance Status (PS) 0. Patients may be candidates for liver resection, local ablation, or transplantation depending on their physical state and coexisting diseases (Díaz-gonzález, 2016). Transplantation is an option for patients with three nodules or a single tumor under 5 cm that have liver function impairment. Adjuvant resection or percutaneous therapies are suggested when there is lengthy waiting periods. Living donor liver transplantation can also be considered. In minor nonsurgical HCC, percutaneous therapies, are used (L, 2020).Surgery is not limited by the patient's size because resection may still be beneficial if the HCC has reached a large size without spreading or producing symptoms (Bruix et al., 2014; de Lope et al., 2012; Llovet et al., 2003).

### 3.5.3 Stage B:

The best candidates for chemoembolization are asymptomatic individuals with multinodular noninvasive tumors (stage B), especially those with Child-Pugh A compensated cirrhosis (L, 2020). Liver function has to be maintained and first-line therapy is trans-arterial chemoembolization (TACE) (Bruix et al., 2014). Patients who have a multinodular disease that is restricted to the liver alone, without extrahepatic spread, without tumoral portal venous invasion, and who have preserved - or almost preserved - liver function (Child-Pugh A or B without ascites) without cancer-related symptoms are considered to be in the BCLC B stage (Díaz-gonzález, 2016).

### 3.5.4 Stage C:

Patients with advanced malignancies (stage C) indicating vascular involvement/extrahepatic dissemination or physical disability (PST1–2) are examined for novel antitumoral agents(L, 2020). Same heterogeneity impacts the amount of tumor burden, but rigorous clinical examination in multifocal HCC covering both lobes frequently exposes cancer-related symptoms and therefore patients should be classed as BCLC C. This stage also includes individuals with extrahepatic dissemination and/or vascular invasion. They benefit from sorafenib therapy (Bruix et al., 2014).

### 3.5.5 Stage D:

Patients who have considerable cancer-related symptoms (ECOG-PS >2) and a severe impairment of liver function (Díaz-gonzález, 2016), physical status (PST 2), or tumor load (Okuda Stage 3) are defined as end stage, BCLC D patients, and they have symptomatic treatment that is classed as Child-Pugh C(L, 2020). The best line of action at this stage is to give the maximum supportive care available. HCC diagnosis may simply become a transplant contraindication in these people (Bruix et al., 2014).

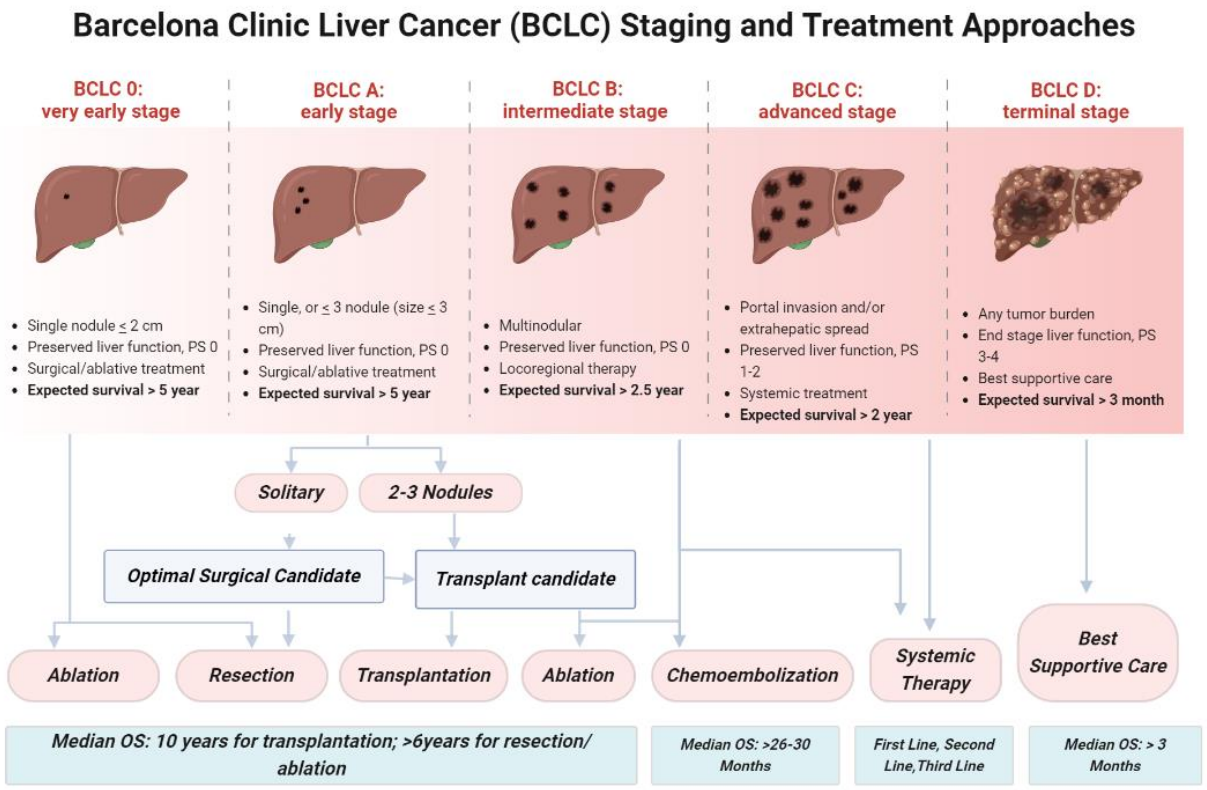



**Figure 6: Stages and Treatment Approaches of Hepatocellular Carcinoma**

### 3.6 Treatment Approaches:

Since most therapies for HCC might aggravate existing liver disease, the management of HCC includes a complicated decision-making process that takes into account not only the amount of the tumor and the patient's comorbidities and also the severity of liver dysfunction. Medical facilities in diverse nations with varying degrees of experience and resources provide a wide range of treatment alternatives. In order to obtain the better results, HCC management demands a multidisciplinary team approach (Serper et al., 2017; Yang et al., 2019b).

HCC can be treated with a variety of treatment techniques, including ablative electrochemical therapies, surgery (liver resection or transplantation (LT)), and radiation therapy (e.g. radiofrequency ablation or ethanol injection). Non-ablative treatments include stereotactic body radiation and catheter-based embolic therapies like chemoembolization and radioembolization (SBRT). Finally, systemic treatment today is non-curative and only consists of the multikinase

inhibitor sorafenib. These treatment approaches can be generally categorised according to whether they aim to treat or control the tumor. While the majority of loco regional therapy (catheter-based therapies, SBRT, and sorafenib) are not thought to be curative, surgical and ablative treatments are administered with the goal of curing the disease. These treatments must be taken into account as a way to control HCC and, in some cases, as a way to downsize or bridge the patient to more conclusive options like LT, even though they are frequently linked to durable responses (Hartke et al., 2017).

### 3.6.1 Surgical Resection:

As long as the vigorous treatment is well tolerated and the risk of liver function impairment is modest, it is the treatment of choice for patients with HCC who do not have cirrhosis (Díaz-gonzález, 2016). In the absence of clinically significant portal hypertension, which is indicated by a hepatic venous pressure gradient of less than 10 mmHg and is clinically determined by the presence of ascites, oesophageal varices, or a platelet count of less than 100,000/mm3 in conjunction with clinically significant splenomegaly, it is a recommended course of treatment for patients with resectable disease (Berzigotti et al., 2015; Boleslawski et al., 2012; Teh et al., 2005; Yang et al., 2019b). On the other hand, people with cirrhosis run the risk of experiencing complications from surgery connected to the liver. A thorough preoperative examination is necessary as a result. Complication rates have decreased thanks to advancements in candidate selection, surgical technique, and postoperative care.

The risk of vascular invasion and extra hepatic dissemination increases along with tumor growth, although it is crucial to note that tumor size is not a limiting factor for surgery. Surgical resection may be possible when multinodular disease is present, but this does not always guarantee that it will be advantageous. Survival may not be improved compared to alternative therapies or even no therapy at all. Additionally, there is a considerable rise in the probability of recurrence, which might potentially reach 100% (Ishizawa et al., 2008). This reveals that despite an active attempt, a remedy cannot be found. In order to provide the optimal treatment choice based on tumor burden, physical state, and comorbidities, doctors should consider all available options (Díaz-gonzález, 2016).

Non-anatomic resection, microvascular invasion, a moderately to poorly differentiated tumor, the quantity of tumor nodules, satellite lesions, and a high AFP level are factors that are now well known to be linked to early recurrence. However, the quantity of tumor nodules, the degree of liver fibrosis, and the severity of hepatitis are all linked to late phase recurrence. Despite this possibility of recurrence, surgical resection is still the treatment of choice, particularly for HCC patients who are not cirrhotic (Grandhi et al., 2016).

### 3.6.2 Liver transplant:

A possibly curative HCC therapy is surgical resection. The patient's leftover liver tissue is still at risk of cancer development, and the 5-year chance of recurrence is greater than 70%. Candidate selection for surgical excision should involve a comprehensive assessment of the size of the tumor as well as the underlying liver function. In Asian nations, the indocyanin green retention test is used to measure hepatic reserve, while in western nations, increased bilirubin and clinical symptoms of portal hypertension (such as thrombocytopenia, splenomegaly, and varices) are used (Imamura et al., 2003; Yang & Roberts, 2010).

The removal of the neoplasm, any potential microscopical foci, and the underlying oncogenic liver disease are two ways in which this treatment strategy may be helpful. Liver transplantation yields a 5-year overall survival rate of 70% when using the Milan criteria (one HCC 5 cm or up to three nodules up to 3 cm). Recurrence rates are around 15% (Clavien et al., 2012; Díaz-gonzález, 2016).

### 3.6.3 Ablation:

Patients with early-stage HCC may benefit from the possibly curative procedure of percutaneous local ablation. The two techniques that are most often employed are microwave and radiofrequency ablation. A more popular local ablation technique is microwave ablation (Shiina et al., 2018). Both methods transmit heat directly into tumors to cause necrosis, however microwave ablation has some advantages over radiofrequency ablation due to its better thermal efficiency and unique method of heat production (Yang et al., 2019b).

This therapy's primary goal is to cause the necrosis of cancerous cells by injecting chemical substances, such as ethanol, or by changing the local temperature with radiofrequency (RFA),

microwave, or cryotherapy (percutaneous ethanol injection, PEI). When the tumor is less than 2 cm in size, these 2 approaches cause necrosis that spreads to approximately 100% of the tumor (Germani et al., 2010). RFA may still be quite effective even for bigger nodules, when PEI efficacy is reduced. 70% of patients survive after receiving these therapies after five years. With good response rates, microwave ablation has emerged as a viable technique (Breen & Lencioni, 2015; Bruix et al., 2015; Díaz-gonzález, 2016).The most recent AASLD recommendations suggest that persons with Child-Pugh score class A cirrhosis and resectable small tumors receive resection rather than ablation, which is based on a meta-analysis of three RCTs (Chen et al., 2006; Heimbach et al., 2018; Yang et al., 2019b).

### 3.6.4 Transarterial Chemoembolization:

The most widely used therapy for hepatocellular cancer is TACE. It works by injecting cytotoxic chemotherapy right into the tumor and, by embolizing particles causing intratumoral ischemia, by restricting tumor feeding arteries. Hepatocellular carcinoma in the intermediate stage is typically treated with TACE. It is unknown at this time whether the survival advantage of TACE is caused by embolic or chemotherapeutic effects (Llovet et al., 2002; Lo et al., 2002; Yang & Heimbach, 2020)**.**TACE contains two basic steps: delivering embolization particles into the tumor-feeding artery, which results in ischemic necrosis of the tumor, and intra-arterial infusion of cytotoxic chemotherapy drugs. Doxorubicin, epirubicin, or cisplatin are the most frequently utilised medications during traditional TACE (Lencioni et al., 2016; Yang et al., 2019b).TACE is the first-line treatment for intermediate-stage hepatocellular carcinoma, but it is also a successful option for patients with early-stage disease who are ineligible for surgical treatment or ablation because of the location of the tumor, or as a temporary solution for those awaiting liver transplantation. Patients, however, may have TACE refractory tumor growth, which is indicated by either up trending tumor markers after TACE treatment or a failure to control the local tumor despite two TACE treatments in quick succession(Cheng et al., 2014). Repeated TACE treatments may be a viable choice for local tumor development or recurrence. In these cases, systemic treatment should be considered (Kudo et al., 2014; Yang & Heimbach, 2020).

### 3.6.5 Transarterial radioembolization:

For the treatment of patients with unresectable, multifocal HCC, including tumors with portal-vein invasion, Transarterial radioembolization (TARe) using Y-impregnated glass microspheres (Therasphere®; mDsnordion, Ottawa, Canada) or resin beads (Sirsphere®; sirtex, Sydney, Australia) is becoming more popular. With acceptable safety and enhanced tolerability, TARe appears to provide clinical results that are comparable to those of TACe (Geschwind et al., 2004; Sangro et al., 2006). Further randomized trials are needed to assess TARe's long-term effectiveness and adverse-effect profile(Yang & Roberts, 2010).

### 3.6.6 Systemic therapy:

The majority of HCCs are resistant to common chemotherapy drugs. Additionally, due to underlying liver disease, individuals with HCC typically have low tolerance for systemic treatment. However, a number of significant molecules and pathways, such as receptor tyrosine kinases, wnt—catenin signalling, the ubiquitin- proteasome system, epigenetic DnA modification (promoter methylation and histone acetylation), the PI3K-AKT-mTOR pathway, proangiogenic molecules, and telomerase, have been linked to liver carcinogenesis (Roberts & Gores, 2005). The development of drugs that target these pathways is ongoing. For the treatment of patients with advanced HCC, sorafenib, an oral multikinase inhibitor that targets the Raf kinase, veGFR, and PDGFR signalling pathways, has been approved (Yang & Roberts, 2010).

Sorafenib is the first systemic therapy that has been demonstrated to extend the survival of patients with advanced-stage HCC in phase III RCTs, with an improvement in median overall survival of 2-3 months. It was the first medication approved for use as the first-line systemic therapy for patients with advanced-stage HCC (Yang et al., 2019b).Anorexia, nausea, vomiting, weight loss, hoarseness of voice, esthesia, and hypertension are the main side effects. Because of the side effect profile, sorafenib can be difficult to tolerate; as a result, dose reduction (54%) or treatment interruption (40%) are frequently required. It is advised to continue therapy despite the negative effects until tumor development is seen (Balogh et al., 2016).

For patients whose disease worsened or who were intolerant to sorafenib, a number of medications have recently received approval as second-line treatments Lenvatinib, Atezolizumab, bevacizumab, Regorafenib, Cabozantinib, Ramucirumab, Nivolumab, Pembrolizumab (Yang & Heimbach, 2020).Because their underlying liver illness rather than

HCC determines their risk for morbidity and death, individuals with advanced liver disease are often ineligible for sorafenib treatment. Due to the very drug-resistant character of HCC as well as drug toxicity in the context of underlying liver disease, there is no established systemic chemotherapy treatment. A medical oncologist should think about systemic chemotherapy in the context of hepatic reserve, therapeutic objectives, and availability and eligibility for clinical trial (Hartke et al., 2017).

### 3.7 Epigenetic Modification

Epigenetics is the study of heritable variations in gene expression unrelated to changes in DNA sequence. Covalent modifications to DNA or histones known as epigenetic changes impact the genome's interpretation rather than its sequence (Mohammad et al., 2019). While somatically heritable, epigenetic alterations also have the advantage of being reversible, this makes them promising therapeutic targets. They function in conjunction with genetic pathways to control transcriptional activity. Epigenetic alterations can occur before disease pathology manifests, serving as diagnostic risk markers as well as prognostic markers for disease development. Normal tissues have highly regulated DNA and histone modifications, and their dysregulation has a significant influence on how nucleosomes are organized, which in turn impacts gene expression, DNA replication, and DNA repair(Wang et al., 2021). (1)Dysregulation of transcription and chromatin tissue results in alterations in gene expression profiles, which promote the growth and spread of tumors. These changes are caused by variations in normal DNA methylation and histone acetylation/ deacetylation patterns. Early and late phases of cancer frequently see an accumulation of epigenetic alterations (López-Camarillo et al., 2019).

#### 3.7.1 Histone Modification

Comparatively speaking, histone modifications are more labile, and a delicate balance between histone modifying enzymes, which add or remove certain modifications, keeps their levels stable. Correcting the increased or reduced amount of a specific enzyme will restore the natural equilibrium in the afflicted cells (Kelly et al., 2010). In sick tissue, abnormal histone modification levels are the consequence of an imbalance in these modifying enzymes. The "histone code" concept, which postulates that epigenetic alterations are linked to the onset and progression of a variety of cancers, was made possible by histone modifications (Wang et al.,

2021).The equilibrium between the activity of histone-modifying enzymes that add or remove certain modifications keeps levels of histone modifications constant. Correcting the increased or reduced amount of a certain enzyme should restore the natural equilibrium in the damaged cells, since abnormal histone modification levels originate from an imbalance in these modifying enzymes in sick tissue (Kelly et al., 2010).Acetylation, methylation, phosphorylation, ADP-ribosylation, phosphodi-esterylation, deimination, isomerization, ubiquitination, parylation, citrullination, and sumoylation are some of the changes that may happen to histone tails (Akone et al., 2020). These alterations have been connected to dynamic chromatin structure changes that have an effect on processes including transcription, replication, and DNA repair. They might potentially be the target of anticancer medications (Majchrzak-Celinska et al., 2021).The acetylation and methylation of lysine residues on H3 and H4 have been the subjects of the most extensive research among the aforementioned post-translational histone changes. The supposed "charge neutralisation model" describes how histone acetylation works. This concept states that lysine residues on histone H3/H4 with positive charges make it easier to tightly package negatively charged DNA. The tight chromatin compaction can be loosened by the addition of an acetyl group, giving transcription factors access and permitting DNA transcription (Y. Lu et al., 2020; Majchrzak-Celinska et al., 2021).Histone deacetylase inhibitors (HDACi) can alter both the acetylation of histones and non-histone proteins, possibly having more extensive effects. Furthermore, only around 10% of all acetylation sites are targeted by non-isoform selective HDACi. Therefore, additional research is required to comprehend the underlying rationale for the target definition of both generic and isoform-specific HDACi. There is now a lot of work being done to discover novel compounds that can specifically block particular HDACs and prevent the negative effects associated with a global HDACi (Kelly et al., 2010; Wang et al., 2021).One to three methyl groups are post-translationally added to lysine residues in proteins by lysine histone methyltransferases (KMTs). Depending on the particular lysine residue involved, lysine methylation has the ability to either stimulate or quiet gene transcription. Targeted treatment is thought to include inhibiting the histone methyltransferase enhancer of zeste homologue 2 (EZH2), which targets Lys27 of histone H3. Clinical studies are now testing inhibitors of EZH2, a transcriptional repressor that belongs to the Polycomb family. EZH2 gain of function mutations in lymphomas can be treated with EZH2 inhibitors (Majchrzak-Celinska et al., 2021).

### 3.7.2 DNA Methylation

Epigenetic DNA alterations known as methylation in eukaryotes are crucial for maintaining genome integrity, genomic imprinting, transcriptional control, and developmental processes (Wu & Zhang, 2010). Massive worldwide DNA methylation loss is a hallmark of cancer cells. Global hypomethylation mostly affects repetitive sequences, which encourages translocations, gene disruption, chromosomal instability, and the reactivation of endoparasitic sequences (Gaudet et al., 2003; Goelz et al., 1985).Genes and non-coding genomic areas are silenced as a result of DNA methylation, which often occurs at the 5 position of the cytosine ring inside CpG dinucleotides. The enzyme DNA methyltransferases (DNMTs) catalyses the alteration at 5-methyl cytosine (Rodríguez-Paredes& Esteller, 2011). The human genome contains around 29,000 CpG islands, most of which are situated in the promoter regions, adjacent to the transcription start site, or first exons. Other locations that have been shown to have CpG methylation include gene bodies, which can silence alternative transcription start sites, repetitive sequences like centromeres and transposon elements, CpG island coastlines, and noncoding regions like enhancer regions and miRNAs. Additionally, the X chromosome exhibits methylation, which results in its inactivation, and is the cause of genomic imprinting (Majchrzak-Celinska et al., 2021). There are three primary DNMTs: DNMT1 (maintains the current methylation patterns after DNA replication), DNMT3A (de novo enzymes that target unmethylated CpGs to induce methylation), and DNMT3B (minimally expressed in adult tissues). DNMT1 maintains the current methylation patterns following DNA replication).DNMT-3L, a member of the same family but lacking intrinsic methyltransferases activity, collaborates with DNMT3a and 3b to promote retro transposon methylation (Deplus et al., 2002; Friedman et al., 2009; R. Kanwal & Gupta, 2012).Genes involved in the primary biological processes are impacted by promoter hypermethylation's transcriptional inactivation: DNA repair (hMLH1, MGMT, WRN, BRCA1), vitamin response (RARB2, CRBP1), Ras signaling (RASSFIA, NOREIA), cell cycle control (p16INK4a, p15INK4b, RB), p53 network (p14ARF, p73 (also known as TP73), HIC-1) and apoptosis (TMS1, DAPK1, WIF-1, SFRP1), among others (Kelly et al., 2010). Clinical experiments have thoroughly characterised and assessed DNA methylation inhibitors35. 5-Azacytidine, a nucleoside analogue that is integrated into RNA and DNA, has recently been demonstrated to be effective in treating individuals with high-risk myelodysplastic syndromes (MDS). The deoxy derivative of 5-Aza-CR, 5-Aza-2-

deoxycytidine, is solely integrated into DNA. Both azanucleosides function at low concentrations by securing DNMT enzymes after incorporation into DNA, causing widespread demethylation when cells divide (Kelly et al., 2010). More crucially, unlike the DNMTis listed above, procaine was discovered to be a protein-protein interactions (PPI) disrupter. By interfering with DNMTs' ability to attach to DNA, procaine lowers the degree of DNA methylation in the promoter regions of CDKN2A and RAR. Gastric cancer (GC) cells (SGC-7901, BGC-823, and MKN-45) showed increased apoptosis and reduced proliferation, suggesting that procaine, a non-nucleoside DNMT, may have therapeutic effects on cancer (Y. C. Li et al., 2018; Wang et al., 2021).

**3.8 Olsalazine*:***

The anti-inflammatory drug olsalazine with IUPAC name 5-[(3-carboxy-4-hydroxyphenyl)diazenyl]-2-hydroxybenzoic acid and chemical formula $C_{14}H_{10}N_2O_6$ and 302.24 molecular weight (sodium azodisalicylate; azodisal sodium) is made to deliver its active moiety. mesalazine (5-aminosalicylic acid; mesalamine). Reaching the colon without suffering from the negative consequences brought on by using a sulfapyridine carrier. as an additive. Patients with Crohn's colitis may find salazine to be helpful since it is an excellent oral therapy for both active ulcerative colitis and maintaining disease remission (Enna & Bylund, 2007).

Amesalazine is efficiently delivered to the colon via olsalazine. The prodrug itself speeds up gastrointestinal transit of food and raises net luminal water secretion. The high water content and lack of blood make the subsequent diarrhoea different from that linked to inflammatory bowel disease (occurring in around 17% of patients and resulting in withdrawal from medication in 6% of patients) (Brown et al., 2000).

Olsalazine-induced diarrhoea often started soon after olsalazine medication was started or the dosage was increased. Was often brief and more frequent with greater dosages. Decrease in dosage. Increases in dosage frequency and simultaneous administration with meals reduced the severity of persistent olsalazine-induced diarrhoea in many individuals. the exception being diarrhoea. In general, olsalazine was well tolerated. Less than 14% of individuals who were intolerant or allergic to sulfasalazine responded similarly to olsalazine. For the treatment of initial episodes as well as acute exacerbations of mild to severe acute ulcerative colitis,

olsalazine appears to be an effective medication. and for people with persistent ulcerative colitis to keep their remission (Enna & Bylund, 2007; Staerk Laursen et al., 1990).

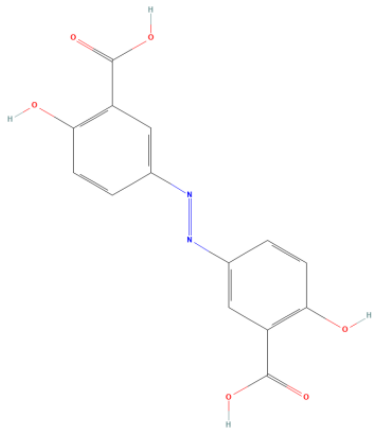


**Figure 7: Structure of Olsalazine**

As a brand-new DNA hypomethylating agent, olsalazine, an authorised anti-inflammatory medication. The cell-based screen employed in this study is extremely tractable, internally controlled, and suitable for an epigenetics medication repurposing technique. Olsalazine exhibits low cytotoxicity at the tested doses while acting in a manner that is quite similar to that of the well-known hypomethylating medication 5-aza-2'-deoxycytidine (Méndez-Lucio et al., 2014).Olsalazine demonstrated a similarity value of 1.032 with NSC14778 in spite of the generally low similarity between the reference molecule and licenced medications in Drug Bank. Due to its strong structural resemblance to the query molecule, olsalazine was selected as a promising candidate for DNMT1 inhibition. Additionally, 5-azacytidine was used to calibrate an in vivo investigation using HeLa cells modified to report gene expression visually using Green fluorescent protein in order to detect hypomethylation. It has been explained how to appropriately report DNMT1 activity, which is in charge of preserving methylation patterns. This approach was used to evaluate olsalazine, and the findings suggested a hypomethylation pattern. Olsalazine was interestingly well tolerated by cells, which was not the case with 5-azacytidine treatments (Brown et al., 2000; Jesús Naveja et al., 2016).

### 3.9 Gemcitabine:

Gemcitabine is the most significant cytidine analogue created since cytosine arabinoside (29,29-difluoro 29-deoxycytidine, dFdC) (Ara-C). Its powerful anticancer activity has been effectively demonstrated in the clinical context using data from a variety of in vitro and in vivo tumor models (Mini et al., 2006).

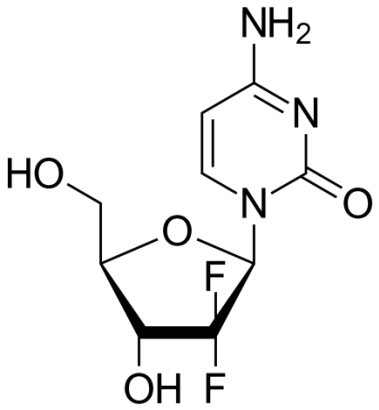



**Figure 8: Structure of Gemcitabine**

Gemcitabine undergoes a complicated intracellular conversion to the nucleotides gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP), which are responsible for its cytotoxic effects, following influx over the cell membrane via nucleoside transporters. Numerous effects on DNA synthesis may be the cause of gemcitabine's cytotoxic activity. As a DNA polymerase inhibitor, dFdCTP competes with deoxycytidine triphosphate (dCTP). Due to the depletion of the deoxyribonucleotide pools required for DNA synthesis caused by dFdCDP's powerful inhibition of ribonucleosidereductase, dFdCTP's effects are amplified. After one additional nucleotide is integrated into DNA, dFdCTP causes the termination of the DNA strand. Given that integration of dFdCTP into DNA appears to be resistant to the standard methods of DNA repair, this additional nucleotide may be crucial in protecting the dFdCTP from DNA repair enzymes. Deoxycytidine deaminase converts 2,29-difluorodeoxyuridine into 2,29-difluorodeoxyuridine, which efficiently inactivates gemcitabine (Mini et al., 2006). HCC cell proliferation was greatly slowed down by gemcitabine in a dose- and time-dependent manner. Gemcitabine caused cell cycle arrest at the G1 phase, but neither the sub-G1 fraction nor nuclear morphology suggested that apoptosis had been triggered. In HCC cells, respectively, gemcitabine elicited differential activation of the checkpoint kinases Chk2 and Chk1, and it also activated extracellular signal-regulated kinase (Routhier A et al., 2010).

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 Materials and Chemicals

### 4.1.1 Cell Line:

HepG2 is an immortal human liver cancer cell line that was obtained from NCCS.10% Foetal Bovine Serum (FBS), 1-2% antibiotics, and Dulbecco's Modified Eagle Medium (DMEM) were used to support the growth of HepG2 cells. Cells were grown at 37°C in a humidified, 5% CO2 incubator.

### 4.1.2 Chemicals and Reagents

The HepG2 Cell line's growth medium, Dulbecco's Modified Eagle Medium (DMEM), was purchased from Himedia. HepG2 cells received growth support from foetal bovine serum (FBS).N-Nitrosodiethylamine (NDEA), was purchased from Chemscene, China.Olsalazine and Gemcitabine were purchased from Emcure Pharmaceuticals, India. Alkaline Phosphatase (ALP), Asparatate Aminotransaminase (AST), Alanine Aminotransaminase (ALT), Lactate Dehydrogenase (LDH), Total Bilirubin (TBIL), Total Protein , C-Reactive Protein kits were obtained from Labcare diagnostics. TNF-α, IL-6 and p53 Elisa kits were obtained from Krishgen Biosystems, India. All other chemicals used were of analytical grades.

### 4.1.3 Animal Care

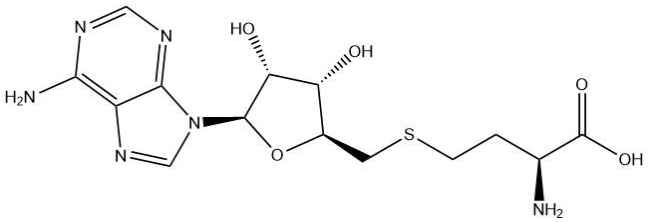
Male Swiss albino mice, weighing 25-30gms were procured from the animal house of the Institute of Pharmacy, Nirma University, Ahmedabad, India. Mice were acclimatized for 7 days for adapting to the surrounding environment. Animals were maintained at 25°C ± 5°C, 55 ± 5 % humidity, and 12 hours of light/dark were also maintained. Free access to food and water was given to mice. The experimental design of this study was approved by the Institutional Animal Ethics Committee (IAEC) of Nirma University, Institute of Pharmacy (IPNU), Ahmedabad as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi with protocol no. IP/PCOL/MPH/34/2023/001. Mice were kept under regular observation.

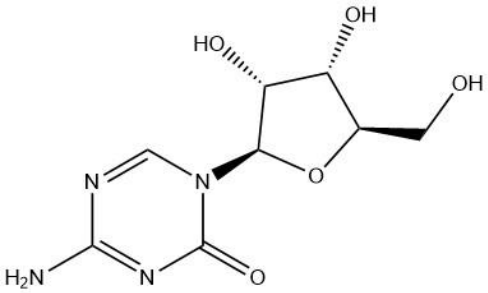
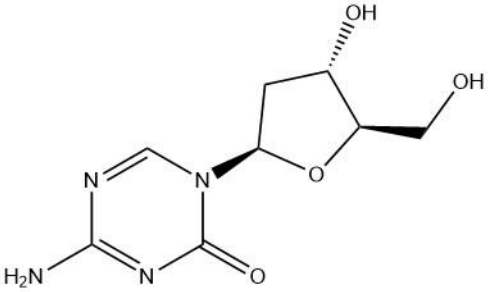
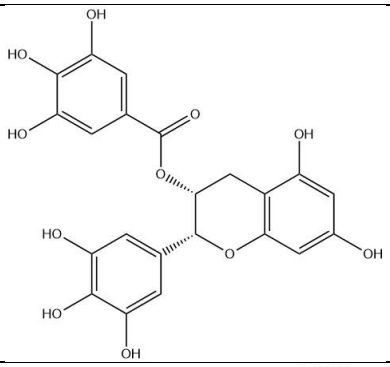
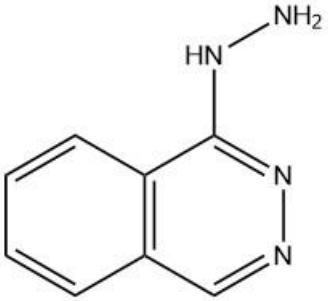
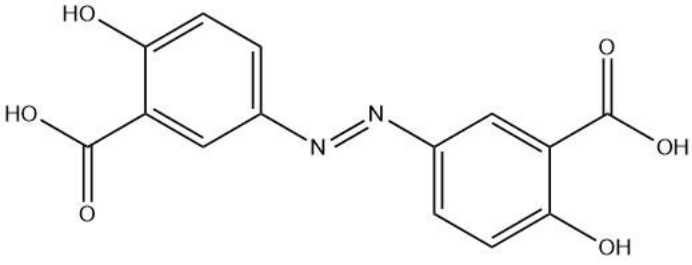
## 4.2 Methods

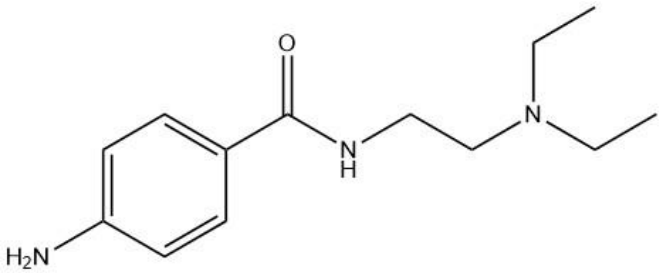
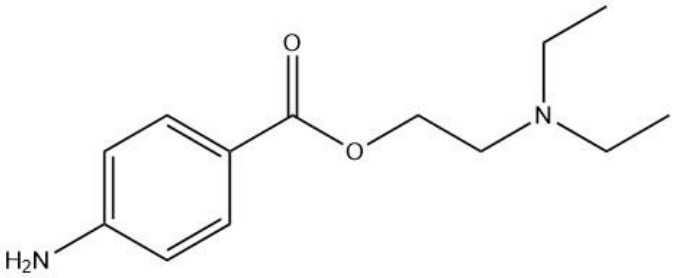
### 4.2.1 *In-Silico* Studies

Molecular docking is one of the most useful tools for predicting the interactions between the target protein and small molecule through computational methods (Astalakshmi et al., 2022). During the literature survey, we selected various small molecules showing hypomethylating activity namely Azacitidine, Decitabine, EGCG, hydralazine, Olsalazine, Procainamide & Procaine, and were initially screened using molecular docking on human DNMT1 (PDB ID: 4WXX) protein. AutoDockTools-1.5.6 software was used for performing protein preparation and molecular docking. Ligands were prepared using ChemDraw Professional 15.0 and ChemDraw 3D. Briefly, ligands 2D structures were prepared in ChemDraw Professional 15.0 software which was then converted to a 3D structure and then energy was minimized by using ChemDraw 3D software, file was then saved in PDB format. The ligand PDB file was then converted and saved in PDBQT format by the software AutoDockTools-1.5.6. Protein was prepared by deleting extra chains, extracting co-crystallized ligands, deleting water molecules, the addition of polar hydrogens, the addition of Kollman charges, assigning AD4-type atoms, and finally saving a file in a PDBQT format. Grid was generated by focusing it on the Centre of the ligand and docking was performed, files were saved in GPF and DPF format respectively. Grid and dock were run by choosing the proper pathname and files were saved in GLG and DLG format. Binding energy was obtained by analysing the DLG file in AutoDockTools 1.5.6 software. Interactions of the ligands with the protein are visualized by using discovery studio 2021 client software.

**Table 4: 2D structure of the Ligands**

| Sr.no. | Ligand | 2D |
|---|---|---|
| 1 | Co-crystal |  |

| | | |
|---|---|---|
| 2 | Azacitidine |  |
| 3 | Decitabine |  |
| 4 | EGCG |  |
| 5 | Hydralazine |  |
| 6 | Olsalazine |  |

| | | |
|---|---|---|
| 7 | Procainamide |  |
| 8 | Procaine |  |

## 4.2.2 *In-Vitro* Studies

### 4.2.2.1 Media Preparation

Media preparation is done in a biosafety cabinet with all the necessary precautions to keep the environment sterile. All of the methods listed below included the usage of the media created here. Each time, hands are cleaned with 70% isopropyl alcohol before being kept in the biosafety cabinet at regular intervals. When preparing media, the following procedures are followed:

- 40,000 µl of DMEM was transferred to the sterile centrifuge tube of 50 ml with the help of a micropipette.
- Then, 4000 µl of FBS (10%) was transferred to the same centrifuge tube in which media was transferred.
- Antibiotic solution, 1 % of the total volume of DMEM is transferred to the same.
- Sterilization of prepared media is done immediately after preparing the media by filtering the same through a 0.2 µm filter.
- Media prepared is then stored at 4°C.

### 4.2.2.2 Revival of the cell line

The revival procedure of the HepG2 cell line followed was:

- 2ml of sterile media was transferred into a T-25 flask and was kept in an incubator to maintain a temperature of 37°C of media.
- The cryovial was removed from the Liq. Nitrogen and wiped with 70% Isopropyl Alcohol.
- Then the cryovial was thawed at 37°C.
- Cryovial was centrifuged at 5000 rpm for 10 minutes.
- The supernatant was discarded and the 1-2 ml media is added to the vial containing cells and centrifuged again at 5000 rpm for 10 minutes.

- Supernatant was discarded without disturbing the pallet and then the cells are resuspended in the 1 ml of media and shaken vigorously.
- Cells are then transferred to the same T-25 flask.
- Incubate it for 24 hrs and after 24 hrs check the morphology of the cells for the confirmation of successful revival of the cells.

#### 4.2.2.3 Cell Culture:

In the media, the resurrected cells persisted in expanding. The flask was examined daily under an inverted microscope to look for contamination and morphology. The media in the flask is changed out every other day for new media. When the medium turns a pale yellow, pipette it out of the flask. After that, give the cells a wash with 1-2 ml of PBS. Transfer the new media into the flask after pipetting out the PBS. Finally, maintain the flask in the CO2 incubator at 37°C in a humid environment.

#### 4.2.2.4 Subculturing of cells:

Maintaining cells in an exponential growth phase requires subculturing. The HepG2 cell line has a 38-hour doubling time. When cells achieve 70–90% of confluence, they are prepared for subculturing. The cells' subculture was carried out by:

- Discard the growth media from the flask.
- Give a wash to a cell layer from PBS for the removal of residual growth media which can act as a trypsin inhibitor.
- 1ml of Trypsin EDTA solution was added and incubated for 2-3 minutes at the proper temperature.
- Observe cells under a microscope at regular intervals till they get detached from the substrate.
- Add 4-5ml of growth media to the flask.
- Centrifuge the cell suspension at 5000 rpm for 10 minutes.

- Remove the supernatant and add 1 ml growth media to the pallet and mix it properly.
- Count the cells using a hemacytometer, by taking 10µl of cell suspension.
- Calculate the cell count by multiplying 104 from the cells counted.
- Add appropriate cell suspension volume to the new flask.
- Incubate it in a CO2 incubator at 37°C under humified conditions

#### 4.2.2.5 Cell Proliferation Assay (MTT Assay):

Cell viability is assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), a tetrazolium salt. The NAD(P)H-dependent oxidoreductase enzymes of live cells are used to reduce yellow MTT to purple formazan crystals in the MTT test, a colorimetric assay. The following stages are taken during the MTT test procedure:

- HepG2 (10,000 cells) were seeded in a 96-well plate.
- Incubate it for 24 hrs.
- After 48 hrs. cells were treated with the different concentrations of the olsalazine and gemcitabine (0.1µM, 1µM, 10µM,) and (25µM,50µM, 100µM, 200µM, 400µM) in a triplicate and incubate it for 48hrs respectively.
- After completion of the incubation period, MTT 5mg/ml is added to each well and incubated for another 4 hrs.
- After the incubation for 4 hrs., MTT was discarded and 100µl of DMSO would be added per well.
- By microplate plate reader absorbance was measured at 570nm.
- Cell viability was calculated using the following formula:

  % Cell viability = (Absorbance of test / Absorbance of control) * 10

## 4.2.3 *In-Vivo* Studies:

### 4.2.3.1 Experimental Design:

Male Swiss albino mice were obtained and acclimatized for one week. After the completion of the acclimatization period, mice were randomized into five groups each group contain 5 animals:

- ***Group A:*** Normal Control, mice that are given no treatment.
- ***Group B****:* Disease Control: Animals were fed to normal diet with intraperitoneal (i.p.) administered of $CCl_4$ (50 µl/kg body weight in liquid paraffin) for four consecutive days followed by 25 mg/kg NDEA was administered through i.p. route for 2 weeks and the dose was increased to 50mg/kg & 100mg/kg for another 2-2 successive weeks.
- ***Group C****:* Positive Control: Animals were fed to normal diet with i.p. administration of $CCl_4$ (50 µl/kg body weight in liquid paraffin) for four consecutive days followed by 25 mg/kg NDEA was administered through i.p. route for 2 weeks and the dose was increased to 50mg/kg & 100mg/kg for another 2-2 successive weeks. A single weekly 50mg/kg ip dosage of gemcitabine acetate was given to the animals in this group.
- ***Group D****:* Treatment Group 1:, Animals were fed to normal diet with i.p. administration of $CCl_4$ (50 µl/kg body weight in liquid paraffin) for four consecutive days followed by 25 mg/kg NDEA was administered through i.p. route for 2 weeks and the dose was increased to 50mg/kg & 100mg/kg for another 2-2 successive weeks. The animals in this group received a single 50mg/kg ip dose of gemcitabine acetate per week. Three times per week, 50 mg/kg of olsalazine will be administered to the animals of this group.
- ***Group E****:* Treatment Group 2: Animals were fed to normal diet with i.p. administration of $CCl_4$ (50 µl/kg body weight in liquid paraffin) for four consecutive days followed by 25 mg/kg NDEA was administered through i.p. route for 2 weeks and the dose was increased to 50mg/kg & 100mg/kg for another 2-2 successive weeks. The animals in this group received a single 50mg/kg i.p. dose of gemcitabine acetate per week. Three times per week, 100 mg/kg of olsalazine will be administered to the animals of this group.

**Table 5: Group of Animals and drug administered during the study**

| S. No. | Group | Animal | Dose |
|---|---|---|---|
| 1. | I | 5+1* | Normal Control |
| 2. | II | 5+1* | Disease Control administered with CCL4 ($CCl_4$(50 µl/kg), NDEA (25 mg/kg, 50 mg/kg, 100 mg/kg) and 2-Acetylaminofluorene (0.02% ad-libitum) |
| 3. | III | 5+1* | Disease control group treated with Gemcitabine Acetate (50mg/kg) alone. |
| 4. | IV | 5+1* | Disease control group treated with Gemcitabine Acetate (50mg/kg) and Olsalazine (50mg/kg). |
| 5. | V | 5+1* | Disease control group treated with Gemcitabine Acetate (50mg/kg) and Olsalazine (100mg/kg) |

At the end of the study, the animals were slaughtered, blood samples were taken by a retro-orbital puncture, and the serum was separated at 4300 rpm for 15 minutes to perform biochemical tests for C-reactive protein, total protein, total bilirubin, AST, ALT and ALP. Using liver tissue homogenate, several oxidative parameters including GSH, lipid peroxidation, SOD, and CAT test were carried out. For the purposes of immunohistochemistry and histological analysis, liver tissue was fixed in 10% formalin.

#### 4.2.3.2 Induction of Hepatocellular Carcinoma:

For inducing Hepatocellular Carcinoma, $CCl_4$-NDEA-induced HCC model was used. Firstly, after procuring mice, weighing 25-30 gm, were acclimatized for a week. Then, $CCl_4$ (50 µl/kg body weight in liquid paraffin) was administered for consecutive 4 days through the intraperitoneal route. After the administration of $CCl_4$for 4 consecutive days, NDEA was administered at a dose of 25 mg/kg from the $5^{th}$ day for 2 successive weeks and then dose will be increased to 50 mg/kg for next 2 successive weeks and 100 mg/kg for another 2 successive weeks. Treatment will be started after the induction with Olsalazine for 3 consecutive days and after the break of 72hours there will be administration of gemcitabine acetate in a week till 6 successive weeks (Bhatia et al., 2015; Pal et al., 2021; Sur, Pal, Mandal, et al., 2016; Sur, Pal, Roy, et al., 2016; Zeng et al., 2017).

#### 4.2.3.3 Biochemical Estimation:

At the end of the study, the animals were slaughtered, blood samples were taken by a retro-orbital puncture, and the serum was separated at 4300 rpm for 15 minutes to perform biochemical tests for ALP, AST, ALT, C-reactive protein, LDH, Total Bilirubin, Total Protein levels in the serum will be measured using their respective colorimetric kits (kits from Lab-Care Diagnostics (India) Pvt. Ltd.) and an UV-visible spectrophotometer (Shimadzu UV1601, Japan) (Patel et al., 2021; Sur, Pal, Roy, et al., 2016).

##### 4.2.3.3.1 Alkaline Phosphatase Estimation

Principle:

Alkaline phosphatase breaks down p-nitrophenyl phosphate into p-nitrophenol and phosphate. The rise in absorption at 405 nm increases in direct proportion to the sample's alkaline phosphatase content.

Reagent**:** Ready to use

Procedure**:**

| | |
|---|---|
| Aspirate the required volume of serum and working reagent as given in the table below | |
| Sample | 20µl |
| Reagent | 1000µl |
| Mix well and incubate it for 60 sec. at 37°C | |
| For two minutes, record the absorbance rise at intervals of 30 seconds to calculate the Δ A/min | |

Calculation*:*

A/min. x 2720 = U/l Alkaline Phosphatase

#### 4.2.3.3.2 Aspartate Transaminase Estimation

Principle:

L-aspartate and -ketoglutarate are reversibly transaminated by the AST into oxaloacetate and L-glutamate. Malate dehydrogenase is then used to convert the oxaloacetate to malate while simultaneously oxidising NADH to NAD.

The rate of decreased level of NADH is determined photo metrically and is directly proportional to the activity of GOT in our used sample

Reagent**:**

Reagent I: Buffer reagent

Reagent II: Enzyme reagent

Preparation of Reagents

| | |
|---|---|
| Working Reagent | 4 Parts of Buffer Reagent |
| | 1 Part of Enzyme Reagent |

Procedure**:**

| | |
|---|---|
| Aspirate the required volume of serum and working reagent as given in the table below | |
| Sample | 100µl |
| Reagent | 1000µl |
| Mix well and incubate it for 1Min. at 37°C at 340 nm read absorbance values | |
| For two minutes, record the absorbance decrease every 30 seconds during 2 mins & calculate the ΔA/min | |

Calculation**:**

AST (SGOT) (IU/L) = A/min X 1746

#### 4.2.3.3.3 Alanine Transaminase Estimation

Principle**:**

L-alanine and -ketoglutarate are reversibly transaminated by ALT into pyruvate and L-glutamate. In the presence of lactate dehydrogenase (LDH), the pyruvate is subsequently converted to lactate while NADH is simultaneously oxidised to NAD.

The rate of consumed NADH is determined photo metrically and it is directly proportional to the activity of GPT in used sample.

Reagent**:**

Reagent I: Buffer reagent

Reagent II: Enzyme reagent

Preparation of Reagents

| | |
|---|---|
| Working Reagent | 4 Parts of Buffer Reagent |
| | 1 Part of Enzyme Reagent |

Procedure**:**

| | |
|---|---|
| Aspirate the required volume of serum and working reagent as given in the table below | |
| Sample | 100µl |
| Reagent | 1000µl |
| Mix well and incubate it for 1Min. at 37°C at 340 nm read absorbance values | |
| For two minutes, record the absorbance decrease every 30 seconds during 2 mins & calculate the ΔA/min | |

Calculation**:**

ALT (SGPT) (IU/L) = A/min X 1746

#### 4.2.3.3.4 C-reactive Protein Estimation

Principle**:**

C-reactive protein (CRP) in human blood or plasma may be measured quantitatively using the CRP-Turbilatex. When combined with samples containing CRP, latex particles coated with a particular anti-human CRP agglutinate. According to the amount of CRP present in the patient sample, the agglutination alters the absorbance, which may be measured by comparing it to a calibrator with a known amount of CRP.

Reagent:

Reagent I: Tris buffer 20mmol/l, pH 8.2 Sodium Azide 0.95 g/l

Reagent II: Latex particles coated with goat IgG anti-human CRP, pH 8.2

Preparation of Reagents

| | |
|---|---|
| Working Reagent | 9 Parts of Reagent 1 |
| | 1 Part of Reagent 2 |

Procedure:

| Aspirate the required volume of serum and working reagent as given in the table below | | |
|---|---|---|
| | Cal | Sample |
| Reagent | 1000 µl | 1000 µl |
| Standard | 10 µl | - |
| Sample | - | 10 µl |
| Mix well and read absorbance immediately A1 and after 2 minutes A2 of the sample addition. | | |

Calculation:

CRP (mg/L) = (A2-A1) sample/ (A2-A1) Calibrator X Calibrator Concentration

#### 4.2.3.3.5 Lactate Dehydrogenase Estimation

Principle:

Lactate dehydrogenase causes lactate to be converted to pyruvate when NAD is present. LDH concentration directly relates to the rate of NADH synthesis

Preparation of Working Reagent:

| | |
|---|---|
| Working Reagent | 9ml of Buffer |
| | 1ml of Substrate reagent |

Procedure:

| | |
|---|---|
| Aspirate the required volume of serum and working reagent as given in the table below | |
| Sample | 25µl |
| Reagent | 1000µl |
| Mix well and incubate it for 1 minute | |
| Change in absorbance was recorded for the next 2 minutes (ΔA/min.) | |

Calculation:

340: Activity (U/L) = Δ A/min*6592

#### 4.2.3.3.6 Total Bilirubin Estimation

Principle:

This procedure couples 3, 5- dichlorophenyldiazonium with total bilirubin in the presence of a solubilizing agent in a highly acidic solution. A photometric assay (546 nm) may be used to measure the intensity of the red azo dye produced, which is directly proportional to the total bilirubin.

Reagent**:**

Reagent I: Bilirubin Total Reagent

Reagent II: Total Nitrite Reagent

Procedure**:**

| | |
|---|---|
| Aspirate the required volume of serum and working reagent as given in the table below | |
| Sample | 50µl |
| Reagent | 1000µl |
| Mix well and incubate it for 5 minute at 37ºC or 10min | |
| Change in absorbance was measure at 546/ 630nm | |

Calculation**:**

Total Bilirubin (mg/dl) = Abs of test X 24

#### 4.2.3.4 Oxidative Parameters

##### 4.2.3.4.1 Tissue Preparation and Storage

The removed liver was cleaned of tissue debris using phosphate buffer saline (50 mM, pH 7.0), and each piece was then blotted on ash-free filter paper. Unless otherwise specified, the liver tissues were homogenized in a 1:3 w/v solution of Tris-HCl buffer (50 mM, pH 7.5). The crude homogenates were centrifuged at 8000 rpm at 4 °C for 15 minutes to get clear supernatants, and then aliquots were kept at 20 °C until further analysis (Husain et al., 2018).

##### 4.2.3.4.2 Total Protein Estimation

Principle**:**

In an alkaline media, protein produces an intense violet-blue complex with copper salts. As an antioxidant, iodide is used. The ratio of the total protein contents in the sample to the color intensity that result.

Reagent:

Reagent I: Biuret reagent

Protein Standard: 6g/dl

Procedure:

| Aspirate the required volume of serum and working reagent as given in the table below | | | |
|---|---|---|---|
| | **Blank** | **Standard** | **Sample** |
| **Reagent 1** | **1000** µl | **1000** µl | **1000** µl |
| **Standard** | **-** | **20** µl | **-** |
| **Sample** | **-** | **-** | **20** µl |
| Mix well and incubate it for 10 minute at 25-27ºC. | | | |

| Measure absorbance of the sample (Ac) and standard (As) against reagent blank. |
|---|

Calculation**:**

Total protein g/dl = Absorbance of sample / Absorbance of standard X standard value (6g/dl)

#### 4.2.3.4.3 Assessment of Lipid Peroxidation

Lipid peroxidation (LPO) in liver tissue was estimated in terms of malondialdehyde (MDA) by following the method of Ohkawa (Husain et al., 2018; Ohkawa et al., 1979).

Principle:

Multiple acute and chronic brain illnesses are known to have a harmful mechanism involving lipid peroxidation by reactive oxygen species (ROS). Thiobarbituric acid assay (TBA test) is the most well-known and currently utilised assay as an indicator for lipid peroxidation products. It is based on the reaction of malondialdehyde (MDA), a byproduct of lipid peroxidation, with TBA to create a red adduct(Garcia et al., 2005). This test is likewise based on a condensation reaction between two TBA molecules and one MDA molecule, where the pace of the reaction is affected by temperature, pH, and TBA concentration (Khoubnasabjafari et al., 2015).

Chemicals and Reagent:

| S. No. | Name | Concentration | Preparation |
|---|---|---|---|
| ***1.*** | ***Sodium Dodecyl Sulphate*** | ***8.1%*** | ***8.1gm SDS in 100ml water*** |
| ***2.*** | ***Acetic Acid (3.5pH)*** | ***20%*** | ***20ml Acetic acid in 100ml water*** |
| ***3.*** | ***Aqueous solution of thiobarbituric acid*** | ***0.8 %*** | ***0.8gmthiobarbituric acid in 100ml water*** |
| ***4.*** | ***n-Butano and Pyridine mixture*** | ***15:1 v/v*** | ***90ml n-butanol + 6ml pyridine*** |

Procedure

| ***Blank*** | ***Test*** |
|---|---|
| 0.2ml of 8.1% SDS | 0.2ml of 8.1% SDS |
| 1.5ml of 20% Acetic acid (pH 3.5) | 1.5ml of 20% Acetic acid (pH 3.5) |
| 1.5ml of 0.8% Thiobarbituric acid | 1.5ml of 0.8% Thiobarbituric acid |
| 0.2ml Distilled water | 0.2ml Crude liver homogenate |
| Raised volume upto 4ml | Raised volume upto 4ml |
| Heating for 1hr at 95 ℃, the mixture takes a pink color that was cooled until it attained room temperature | |
| 5ml of n-butanol and pyridine mixture (15:1v/v) was added to the solution and vortexed. | |
| Centrifuged at 8000 rpm for 10min | |
| The absorbance of organic layer was read at 532nm | |

#### 4.2.3.4.4 Assessment of Reduced Glutathione

GSH level was determined according to the method of Ellman (ELLMAN, 1959; Tsai et al., 2010).

Principle

The body's natural antioxidant glutathione is found in virtually all of its cells and aids in the detoxification of xenobiotics and medicines. It is also used as a supplement to alleviate cachexia brought on by cancer (Rahman, Kode, et al., 2006). Cysteine, glutamic acid, and glycine make up the tripeptide known as reduced glutathione (GSH), which is essential for the regulation of detoxification, signalling, and other cellular functions. A glutathione oxidised form is glutathione disulfide (GSSG). When NADPH is present, glutathione reductase (GR) converts it to GSH. Hydrogen peroxide is changed into water by the enzyme glutathione peroxidase (GP) (Salbitani et al., 2017).

Chemicals and Reagent

| S. No. | Name | Concentration | Preparation |
|---|---|---|---|
| *1.* | ***Phosphate buffer*** | ***pH 8*** | ***Ice-cold phosphate buffer*** |
| *2.* | ***Trichloroacetic acid (TCA)*** | ***10%*** | ***10gm TCA in 100ml water*** |
| *3.* | ***Sodium Citrate*** | ***1 %*** | ***1gm sodium citrate in 100ml water*** |
| *4.* | ***Dithiobis nitro benzoic acid (DTNB)*** | ***-*** | ***40 mg in 100 ml of 1% Sodium citrate and cover with aluminum foil.*** |

Procedure:

| ***Blank*** | ***Test*** |
|---|---|
| | Take homogenate |
| | Add 0.1 ml of 10% TCA |
| Centrifuge at 3000 rpm at 25℃ for 10 min | |
| | 1ml of supernatant |
| 2ml of Phosphate buffer | 1ml of Phosphate buffer |
| 1ml of DTNB reagent | |
| Mix it well and vortexed it thoroughly for 1 minute | |
| Incubate it for 5 minutes at room temperature | |
| Absorbance was recorded at 412nm | |

#### 4.2.3.4.5 Assessment of Superoxide Dismustase

SOD enzymatic activity was determined according to the method of Misra and Fridovich (Misra & Fridovich, 1972; Tsai et al., 2010).

Principle

The idea behind this test is that when $O_2$ interacts with NBT, it can turn the yellow tetrazolium in the gel into a blue precipitate. A clear area (achromatic bands) forms in SOD-active regions, fighting with NBT for $O_2$ (Weydert & Cullen, 2010).it is also based on Superoxide anion radicals were produced using xanthine and xanthine oxidase, and these radicals then quantitatively react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to give a red formazan dye. By converting the superoxide radical to oxygen, SOD prevents the process from happening (Assady et al., 2011).

Chemicals and Reagent

| S. No. | Name | Concentration | Preparation |
|---|---|---|---|
| *1.* | ***EDTA*** | ***0.0001M*** | ***9.3gm in 250ml water*** |
| *2.* | ***Carbonate buffer*** | ***pH 9.7*** | ***8.4gm $NaHCO_3$+10.6gm $Na_2CO_3$ in water*** |
| *3.* | ***Epinephrine*** | ***0.003M*** | ***50mg/100ml in 2pH HCL Cover it with aluminum foil*** |

Procedure:

| ***Blank*** | ***Test*** |
|---|---|
| 100 µl D.W. | 100 µl homogenate |
| 0.75 ml ethanol and 0.15 ml ice-cold chloroform | |
| Centrifuge at 3000 rpm at 25℃ for 10 min | |
| 0.5 ml supernatant + 0.5 ml EDTA + 1 ml carbonate buffer + 0.5 ml epinephrine | |
| Absorbance was read against blank at an interval of 30 sec for 3 min. at 480 nm | |

#### 4.2.3.5 Effect of treatments on inflammatory markers: TNF-α, IL-6 Levels:

Following the recommendations of the manufacturer, TNF-α and IL-6 level will be determined using a highly sensitive Elisa kit (Krishgen Biosystems, India) according to the manufacturer's recommendations using ELISA reader (Matrix BioMedical) (Patel et al., 2021; Sur, Pal, Mandal, et al., 2016).

##### 4.2.3.5.1 TNF-α Estimation

Principle**:**

The homotrimeric 17 kDa protein tumor necrosis factor-alpha (TNF-alpha) is a powerful modulator of inflammatory and metabolic processes. TNF- was first identified as a cytokine with a strong cytotoxic potential for tumor cells; in vivo, it induces tumor necrosis, and in vitro, it has cytolytic action against tumor cells. Additionally, it has been shown that TNF- is a key mediator in the shock brought on by gram-negative bacteria. TNF serves as a crucial mediator in a variety of inflammatory and immunological processes. It can be caused by a variety of microorganism products and cytokines, but it can also stimulate the creation of a large number of cytokines. The p55 and p75 TNF-receptors are two different kinds of TNF-receptors that carry out signal transduction. The intracellular signalling routes used by the receptors vary significantly.

Materials**:**

1. Microtiter Coated Plate (12X8 wells) – 1 no.
2. Recombinant Rat TNF-α Standard Lyophilized (2000pg/ml) –1 vial
3. Rat TNF-α Biotin Conjugated Detection Antibody – 1 vial
4. Concentrated Streptavidin Horseradish Peroxidase - 1 vial
5. (20X) Wash Buffer – 25ml
6. (5X) Assay Diluent – 10ml
7. TMB Substrate – 12ml

8. Stop Solution – 12ml
9. Instruction Manual

Procedure**:**

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicates. A standard curve is required for each assay.
2. **Standards Preparation:** Reconstitute the lyophilized vial with 730 ul of Distilled water to generate a 2000 pg/ml top standard. Keep the standard for 15 mins with gentle agitation before making further dilutions. Perform serial dilutions by using main stock solution as per the below table. Thus the Rat TNF-α Standards concentration are 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml and 31.25 pg/ml. Assay Diluent (1x) serves as the zero standard (0 pg/ml).
3. Add 100µl/well of **Standards** and **Samples** to the plate, Seal plate and incubate for 2 hours at Room Temperature (18-25°C).
4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
5. Add 100µl of diluted **Detection Antibody** solution to each well, seal plate and incubate for 1 hour at Room Temperature (18-25°C).
6. Wash plate 4 times with **Wash Buffer (1X)** as in step 4.
7. Add 100µl of diluted **Streptavidin-HRP** solution to each well, seal plate and incubate for 30 minutes at Room Temperature (18-25°C).
8. Wash plate 4 times with **Wash Buffer (1X)** as in step 4.
9. Add 100µl of **TMB Substrate** solution and incubate in the dark for 30 minutes at Room Temperature. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.

**10.** Stop reaction by adding 100µl of **Stop Solution** to each well. Positive wells should turn from blue to yellow.

**11.** Read absorbance at 450 nm within 30 minutes of stopping reaction.

Calculation**:**

Calculate the average absorbance for each duplicate set of standards and samples. Deduct the average absorbance of the blank standards (representing the background) from each well's absorbance reading. Construct a standard curve using standard graph paper, plotting cytokine concentration on the x-axis and absorbance on the y-axis. Draw a straight line that best fits the standard data points. To determine the concentrations of unknown cytokine samples, locate the mean absorbance value of the unknowns on the y-axis and draw a horizontal line to intersect the standard curve. Then, draw a vertical line from the intersection point to the x-axis to read the corresponding cytokine concentration. If the samples were diluted, multiply the obtained concentration by the appropriate dilution factor.

#### 4.2.3.5.2 IL-6 Estimation

Principle**:**

Interleukin-6 (IL-6) is a multifunctional cytokine that controls hematopoiesis, acute phase reactions, immunological responses, and other processes. It may be a key component of the host defence systems. In patients with cardiac myxoma, polyclonal B-cell activation with autoantibody synthesis was originally proposed to be connected to aberrant IL-6 production. Since then, it has been proposed that IL-6 has a role in the patophysiology of several disorders. Thus, more thorough understanding of diverse medical circumstances is provided by measurement of IL-6 levels in serum and other bodily fluids.

Materials**:**

1. Microtiter Coated Plate (12 x 8 wells) – 1 no
2. Recombinant Rat IL-6 Standard (230 ng/ml; lyophilized) – 1 vial
3. Rat IL-6 Biotin Conjugated Detection Antibody (lyophilized)– 1 vial
4. Concentrated Streptavidin Horseradish Peroxidase – 1 vial
5. (20X) Wash Buffer – 25 ml
6. (1X) Assay Diluent – 50 ml
7. Rat IL-6 Biotin Conjugated Detection Diluent – 10 ml
8. TMB Substrate – 12 ml
9. Stop Solution – 12 ml
10. Instruction Manual

Procedure**:**

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicates. A standard curve is required for each assay.
2. **Standards Preparation:** Reconstitute the lyophilized vial with 40 ul of Assay Diluent (1X) to generate a 230 ng/ml. Dilute 34.78 ul of original Standard (230 ng/ml) with 965.22 ul of Assay Diluent (1X) to generate a 8000 pg/ml top standard. Perform serial dilutions by using top 8000 pg/ml top standard as per the below table. Thus, the Rat IL-6 standard concentrations are 8000 pg/ml, 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml and 125 pg/ml. Assay Diluent (1X) serves as the zero standard (0 pg/ml).
3. Add **50 ul** of diluted Biotin conjugated **Detection Antibody** to all wells.
4. Add **100 ul** of **Standards** and **Samples** to respective wells.
5. Seal plate and incubate at 37°C for 2 hours.
6. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
7. Add **100 ul** of diluted **Streptavidin-HRP** solution to each well, seal plate and incubate for 30 minutes at 37°C.
8. Wash plate 4 times with **Wash Buffer (1X)** as in step 6.
9. Add **100 ul** of **TMB Substrate** solution and incubate in the dark for 30 minutes at 37°C. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.
10. Stop reaction by adding **100 ul** of **Stop Solution** to each well. Positive wells should turn from blue to yellow.
11. Read Absorbance at 450 nm within 30 minutes of stopping reaction

Calculation**:**

Calculate the average absorbance for each set of duplicate or triplicate standards and samples. Subtract the average absorbance of the blank standards (representing the background) from the absorbance values of each well. Create a standard curve using semi-log graph paper, where the cytokine concentration is plotted on the x-axis and absorbance is plotted on the y-axis. Draw a straight line that best fits the data points of the standards. To determine the concentrations of unknown cytokine samples, locate the mean absorbance value of the unknowns on the y-axis and draw a horizontal line to intersect the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding cytokine concentration. If the samples were diluted, multiply the obtained concentration by the appropriate dilution factor. It is recommended to utilize computer-based curve-fitting software for accurate analysis and curve fitting.

#### 4.2.3.6 Effect of treatments on apoptotic markers:

##### 4.2.3.6.1 p53/Tumor Protein p53/Tp53 :

Following the recommendations of the manufacturer, p53 level will be determined using a highly sensitive Elisa kit (Krishgen Biosystems, India) according to the manufacturer's recommendations using ELISA reader (Matrix BioMedical) (Patel et al., 2021; Sur, Pal, Mandal, et al., 2016).

Principle:

The method employs sandwich ELISA technique Rat p53/Tumor Protein p53/Tp53 monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Rat p53/Tumor Protein p53/Tp53 present in the sample are bound by the antibodies. Biotin labeled p53/TP53 antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Rat p53/Tumor Protein p53/Tp53 in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Material**:**

1. Rat p53/TP53 Antibody Coated Microtiter Plate (8 x 12 wells) – 1 no
2. Standard, Rat p53/TP53 (concentrated, 800 pg/ml) – 0.5 ml
3. Biotinylated p53/TP53 Antibody – 1 ml
4. Streptavidin: HRP Conjugate – 6 ml
5. Standard Diluent – 3 ml
6. (20X) Wash Buffer – 25 ml
7. TMB Substrate – 12 ml
8. Stop Solution – 12 ml
9. Instruction Manual

Procedure**:**

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.
2. Add 50 ul prepared Standards to respective standard wells and add 40 ul Samples to respective sample wells.
3. Pipette 10 ul Biotinylated p53/Tp53 Antibody to respective sample wells.
4. Pipette 50 ul Streptavidin: HRP Conjugate to all wells. Mix well.
5. Cover the plate with a sealer and incubate for 60 minutes at 37°C.
6. Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
7. Pipette 100 ul TMB Substrate to all wells.
8. Incubate the plate at 37°C for 10 minutes. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
9. Pipette 100 ul of Stop Solution to all wells. The wells should turn from blue to yellow in color.
10. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation**:**

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph Paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Rat p53/Tumor Protein p53/Tp53 concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Rat p53/Tumor Protein p53/Tp53 Concentration.

#### 4.2.3.7 Histopathological Analysis

Following the completion of the 7-week induction and treatment period, the mice will be humanely euthanized using an overdose of ether. The liver will be carefully extracted and placed in a 10% formalin solution for preservation. Decalcification of the liver tissue will be conducted using a 5% formic acid solution. Subsequently, the liver samples will undergo paraffin embedding to facilitate sectioning. Sections of approximately 5 μm thickness will be prepared and stained with hematoxylin and eosin. These stained sections will be utilized to examine and assess liver fibrosis and hyperplasia, providing valuable insights into the tissue's condition and any potential pathological changes (Pal et al., 2021; Patel et al., 2021; Sur, Pal, Mandal, et al., 2016).

#### 4.2.3.8 Immunohistochemistry

The tissue sections will undergo a series of steps for immunohistochemical analysis. First, the sections will be deparaffinized and washed with tap water. To block endogenous peroxidase activity, the sections will be treated with a 3% hydrogen peroxide solution. Then, the primary antibody specific to p53 will be applied to the sections and allowed to bind to the target antigens for 35 minutes at room temperature. Afterward, the unbound primary antibodies will be washed away using phosphate buffer saline. Horseradish peroxidase (HRP) labeled secondary antibodies, which are specific to the primary antibody, will be added and allowed to react for 30 minutes at room temperature. Any unreacted secondary antibodies will be removed by treating the sections with PBS. To visualize the binding sites, 3,3'-diaminobenzidine (DAB), a substrate agent for HRP, will be added, resulting in the development of a color reaction. Excess DAB will be washed away with running water. To stain the nuclei, the sections will be treated with hematoxylin. Following this, the sections will be washed, dehydrated, and mounted on slides. The slides will be examined under a phase contrast microscope at a magnification of 40x. Immunoreactivity against the respective markers will be analyzed in similar fields within the liver sections from all experimental groups. The images obtained will be analyzed using specialized software to quantify the total field and the area stained by immunohistochemistry (IHC). The percentage of the IHC stained area will be calculated based on these measurements (Patel et al., 2021).

**Statistical analysis:**

All statistical analysis performed in this study was done by GraphPad Prism 9.5.1. The results were analyzed by using the one-way ANOVA and the method used was Bonferroni Multiple Comparison Test. The results were expressed as mean ± SD. Statistical significance was obtained when p value was less than 0.05.

# CHAPTER 5

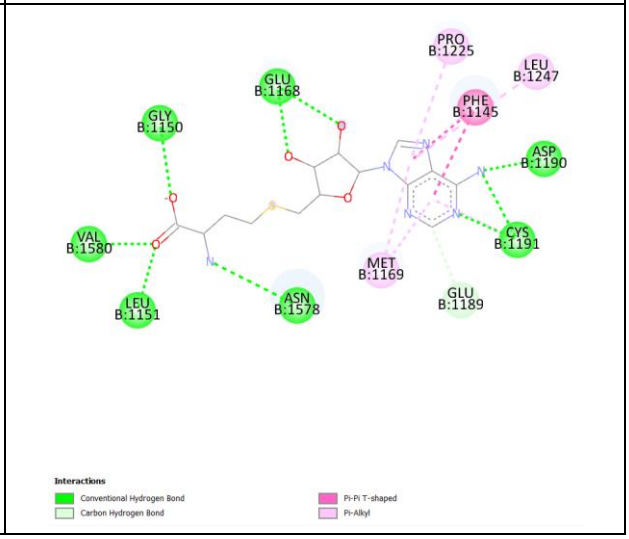
# RESULTS

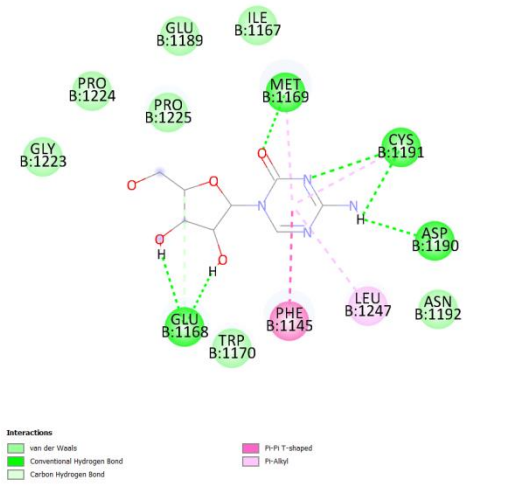
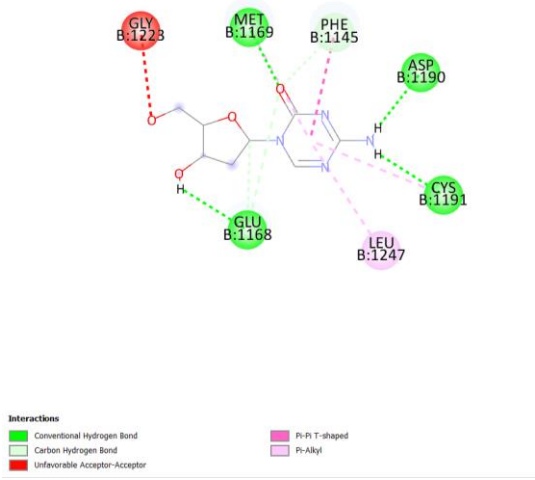
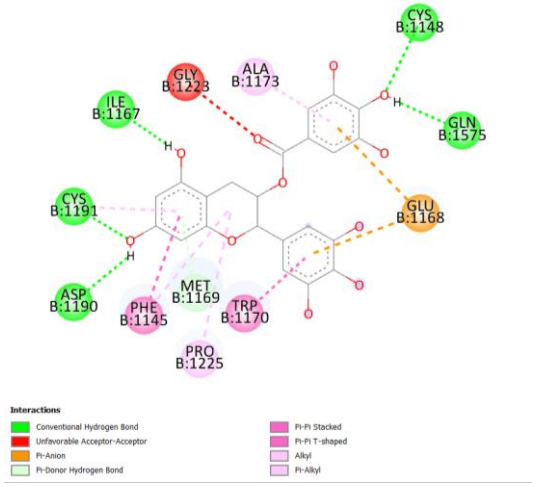
## 5.1 In-Silico Study

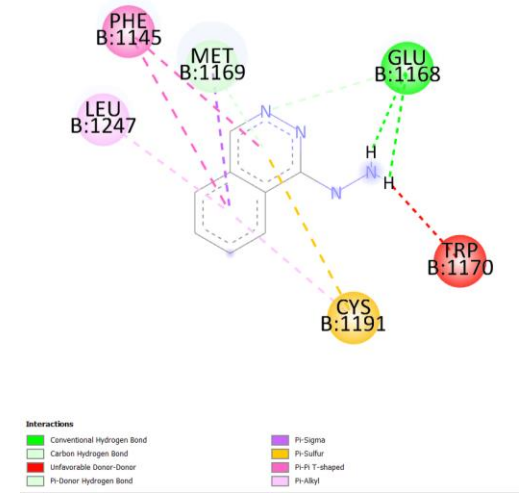
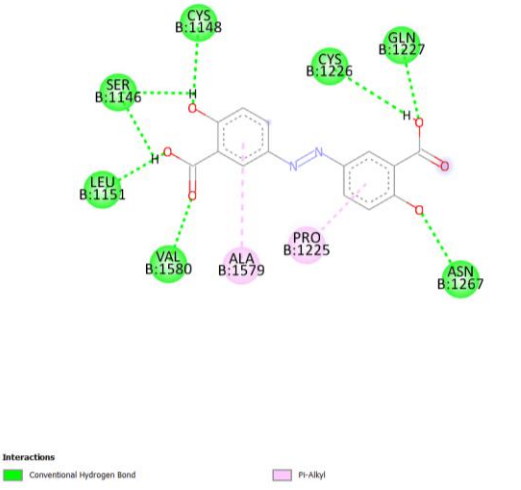
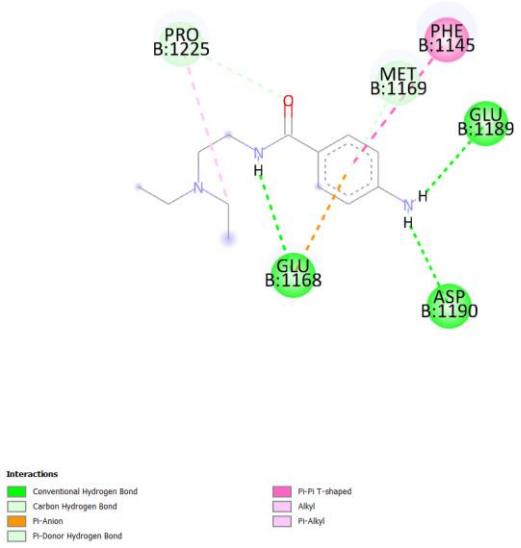
### 5.1.1 Olsalazine, as an epigenetic regulator, exhibits strong binding affinity as a DNMT1 inhibitor.

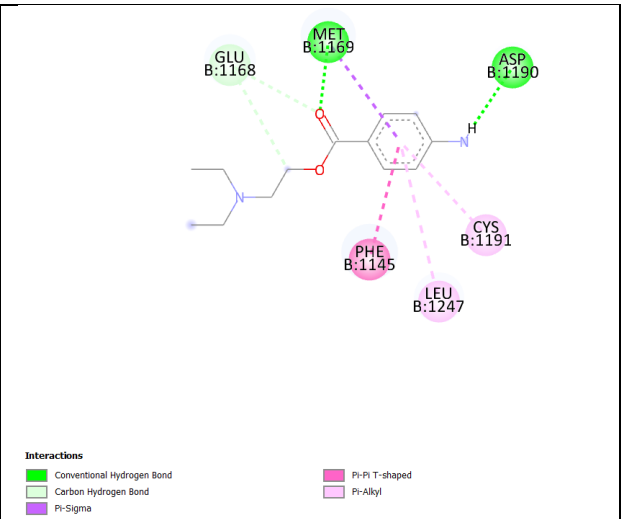
All seven selected ligands i.e., Azacitidine, Decitabine, EGCG, Hydralazine, Olsalazine, Procainamide, and Procaine were docked against human DNMT1 protein (PDB ID: 4WXX). Binding energies are shown to be negative, ranging from -3.41 to -5.54 kcal/mol. More negative binding energy stronger the interaction. Among all the ligands Olsalazine showed the highest binding affinity. Hence, Olsalazine was selected for further evaluation.

**Table 6: Binding affinity and interactions of ligand**

| Sr. no. | Ligand | Reference RMSD | Binding Affinity | Diagram |
|---|---|---|---|---|
| 1 | Co-crystal | 1.10 | -5.64 kcal/mol |  |

| | | | | |
|---|---|---|---|---|
| 2 | Azacitidine (L1) | 61.05 | -3.43 kcal/mol |  |
| 3 | Decitabine (L2) | 61.48 | -3.38 kcal/mol |  |
| 4 | EGCG (L3) | 61.59 | -5.48 kcal/mol |  |

| | | | | |
|---|---|---|---|---|
| 5 | Hydralazine (L4) | 62.38 | -5.16 kcal/mol |   |
| 6 | Olsalazine (L5) | 62.36 | -5.54 kcal/mol |   |
| 7 | Procainamide (L6) | 62.15 | -3.64 kcal/mol |   |

| | | | | |
|---|---|---|---|---|
| 8 | Procaine (L7) | 61.96 | -3.20 kcal/mol |   |

The ligand-protein complex interactions in 2D figures were shown in table 2. Except hydralazine, every selected ligand was able to make hydrogen bonds some of the amino acids were in common like MET A:1169, PHE A:1145, GLU A:1168, SER A:1146, PRO A:1225 and GLU A:1189. Mean RMSD of all tested ligands was 0.00 Å. Less RMSD value indicates that the docked complex is stable, and the docking procedure is validated.

**Table 7: The obtained molecular docking analysis of several compounds against DNMT1 protein using AutoDockTools 1.5.6**

| | L1 | L2 | L3 | L4 | L5 | L6 | L7 |
|---|---|---|---|---|---|---|---|
| **Run** | 6 | 3 | 6 | 4 | 7 | 7 | 10 |
| **Cluster Rank** | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Cluster RMSD(Å)** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Reference RMSD (Å)** | 61.05 | 61.48 | 61.59 | 62.38 | 62.36 | 62.15 | 61.96 |
| **Estimated Inhibition Constant, Ki** | 1.07 mM | 746.24 µM | 44.94 µM | 115.89 µM | 29.40 µM | 1.23 mM | 3.18 mM |

## 5.2 In-Vitro Study

### 5.2.1 Olsalazine Pre-treatment Improves Cytotoxicity of Gemcitabine in HepG2 Cell Line

#### 5.2.1.1 MTT Assay

##### 5.2.1.1.1 Effect of Gemcitabine on HepG2 Cell Line to Find $IC_{50}$

The MTT assay was utilized to determine the $IC_{50}$ of Gemcitabine as a monotherapy. This assay, commonly employed to assess cell viability and growth inhibition, plays a crucial role in evaluating the effectiveness of anticancer drugs. By subjecting HepG2 cells, a human liver cancer cell line, to varying concentrations of Gemcitabine, researchers were able to determine the $IC_{50}$ value. This information is essential for understanding the potency of Gemcitabine as a standalone treatment for liver cancer.

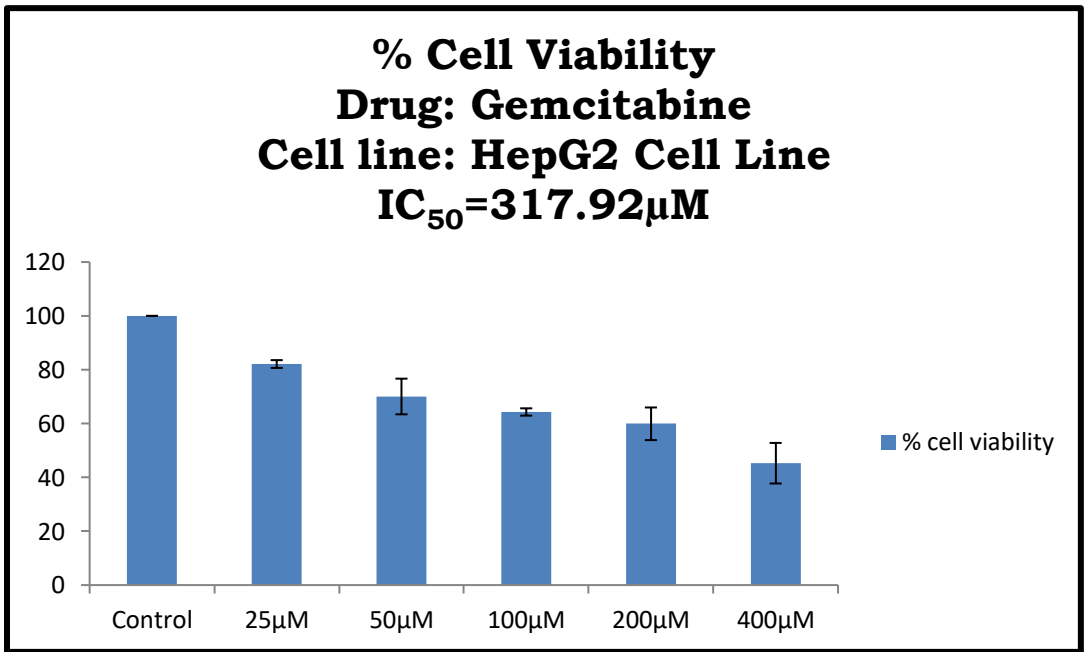



**Figure 9: Effect of Gemcitabine on HepG2 Cell Line to Find IC50**

#### 5.2.1.1.2 Effect of Treatment on HepG2 Cell Line

Additionally, the study aimed to investigate the direct impact of sequential treatment involving olsalazine and gemcitabine at different concentrations on HepG2 cells. Sequential treatment involves administering olsalazine, followed by gemcitabine, to assess any potential synergistic or additive effects. By utilizing the MTT assay, researchers were able to evaluate the efficacy of this combination therapy. This investigation provides valuable insights into the effectiveness of sequential treatment with olsalazine and gemcitabine, offering potential therapeutic strategies for liver cancer management.

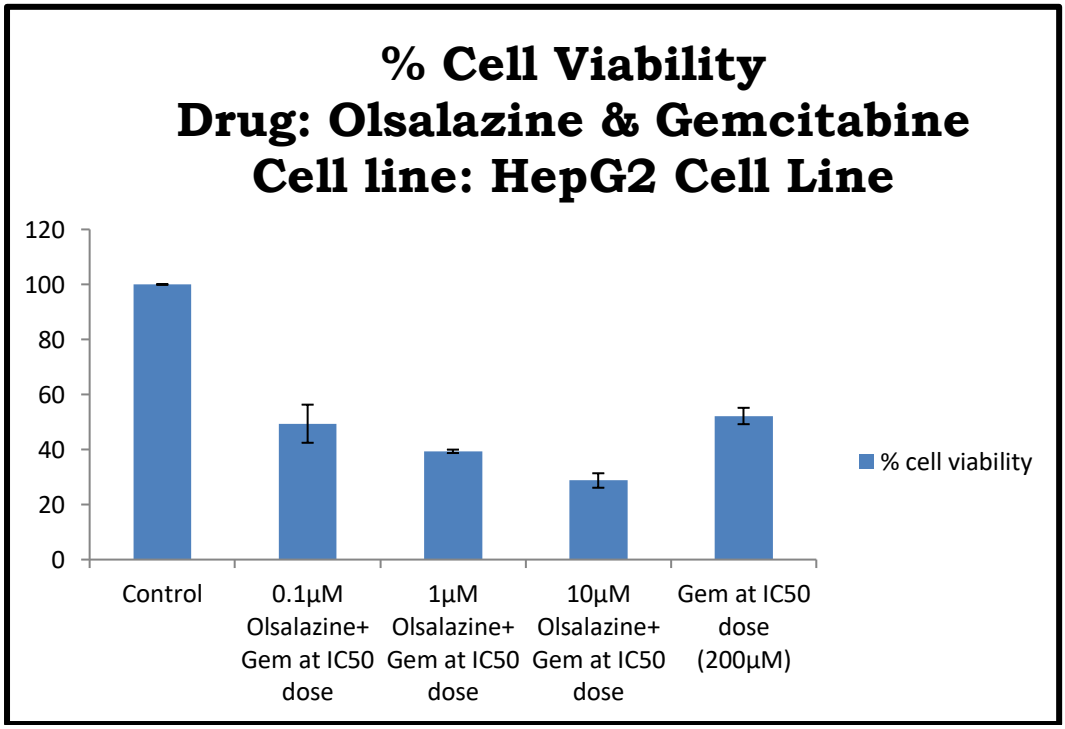



**Figure 10: Effect of Treatment on HepG2 Cell Line**

## 5.3 In-Vivo Study

### 5.3.1 Olsalazine Pretreatment Slows the Carcinogenesis in NDEA Induced Hepatocellular Carcinoma in Swiss Albino Mice

#### 5.3.1.1 Effect of treatment on the body weight

In comparison to the Normal Control group, the Disease Control (DC) group experienced a significant decrease in body weight ($p < 0.0001$). However, when comparing the Standard Dose (DC-SD) group, and the treatment - low dosage (DC-SD-LD) and treatment high dose(DC-SD-HD) groups with the DC group, there was a non-significant increase in body weight observed. These findings suggest that the administered doses had no significant impact on body weight compared to the disease control group.

**Table 8: Effect of treatments on body weight in CCL4-NDEA induced Hepatocellular carcinoma in mice.**

| Groups | Body Weight (gm) |
|---|---|
| NC | 37.23 ±1.349 |
| DC | 30.20 ± 3.557**** |
| DC-SD | 30.57 ± 2.712 |
| DC-SD-LD | 31.29 ± 2.981 |
| DC-SD-HD | 30.29 ± 2.811 |

All Values are expressed as Mean ± SD [NC = Normal Control; DC = Disease Control; DC-SD = Disease control group treated with 50mg/kg gemcitabine (Standard); DC-SD-LD = Disease control group treated with 50mg/kg gemcitabine (Standard) and 50mg/kg Olsalazine; DC-SD-HD = Disease control group treated with 50mg/kg gemcitabine (Standard) with 100mg/kg Olsalazine. * *Significantly different from normal control group* (****p<0.0001).One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

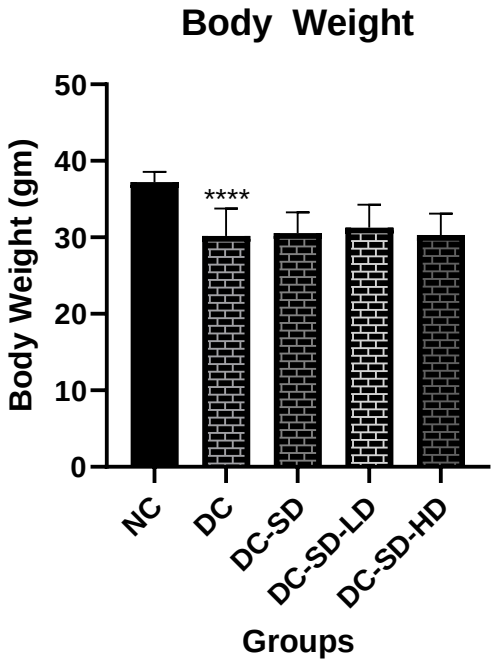



**Figure 11: Effect of treatments on the body weight**

All Values are expressed as Mean ± SD [NC = Normal Control; DC = Disease Control; DC-SD = Disease control group treated with 50mg/kg gemcitabine (Standard); DC-SD-LD = Disease control group treated with 50mg/kg gemcitabine (Standard) and 50mg/kg Olsalazine; DC-SD-HD = Disease control group treated with 50mg/kg gemcitabine (Standard) with 100mg/kg Olsalazine. * *Significantly different from normal control group* (****$p<0.0001$).One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.1.2 Effect of treatments on food intake

The DC group exhibited a notable significant ($p < 0.0001$) reduction in their food consumption compared to the normal control group. During the induction period, there was no discernible difference in food intake between the all treatment groups and the disease control group. However, following the initiation of treatment with olsalazine and gemcitabine, the treatment group experienced a slight non significant improvement in food intake in comparison to the disease control group.

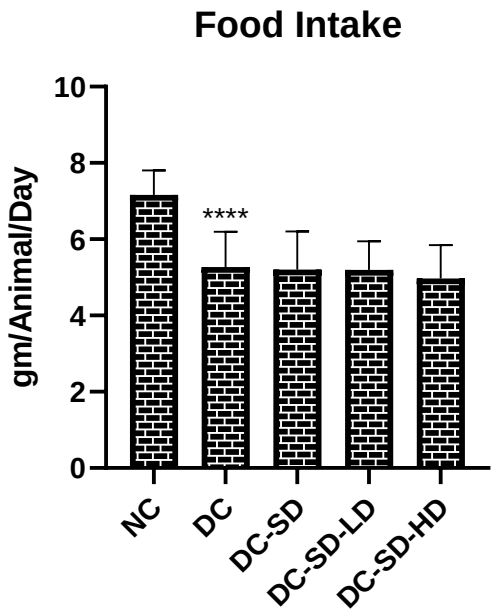



**Figure 12: Effect of treatments on food intake**

All Values are expressed as Mean ± SD [NC = Normal Control; DC = Disease Control; DC-SD = Disease control group treated with 50mg/kg gemcitabine (Standard); DC-SD-LD = Disease control group treated with 50mg/kg gemcitabine (Standard) and 50mg/kg Olsalazine; DC-SD-HD = Disease control group treated with 50mg/kg gemcitabine (Standard) with 100mg/kg Olsalazine. * *Significantly different from normal control group* (****$p<0.0001$).One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.1.3 Effect of treatments on water intake

The DC group demonstrated a note worthy but significant ($p < 0.0001$) decrease in their water consumption compared to the normal control group. Throughout the induction period, there were no notable distinctions in water intake between the various treatment groups and the disease control group. Nevertheless, after the introduction of olsalazine and gemcitabine treatment, the treatment group displayed a slight, albeit statistically insignificant, enhancement in water intake when compared to the disease control group.

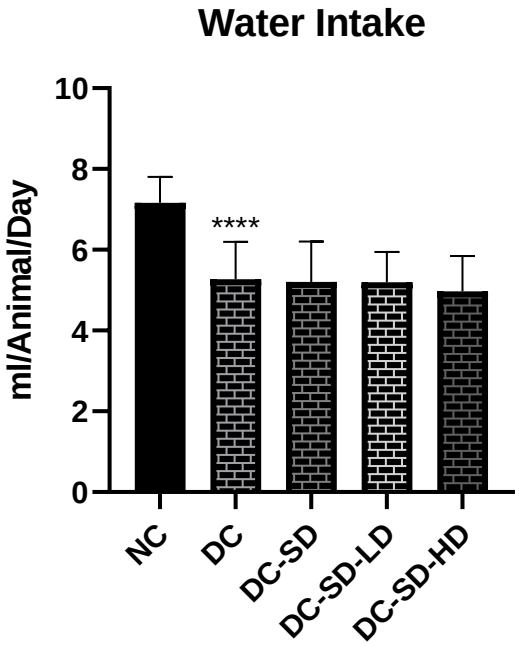



**Figure 13: Effect of treatments on water intake**

All Values are expressed as Mean ± SD [NC = Normal Control; DC = Disease Control; DC-SD = Disease control group treated with 50mg/kg gemcitabine (Standard); DC-SD-LD = Disease control group treated with 50mg/kg gemcitabine (Standard) and 50mg/kg Olsalazine; DC-SD-HD = Disease control group treated with 50mg/kg gemcitabine (Standard) with 100mg/kg Olsalazine. * *Significantly different from normal control group* (****$p<0.0001$).One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.1.4 Effect of treatments on absolute liver weight

When compared to the NC group, the Disease Control (DC) group significantly ($P < 0.05$) increased the absolute weight of the liver. When compared to DC group, the standard dose group is administered with Gemcitabine to and shows significantly ($p < 0.05$) decreased in the absolute liver weight. When compared to the DC group, the animals treated with the treatment - low dosage (DC-SD-LD)and treatment high dose(DC-SD-HD) groups of olsalazine and gemcitabine combination both demonstrated significant reduction of absolute liver weight ($p < 0.01$) and ($p < 0.01$), respectively.

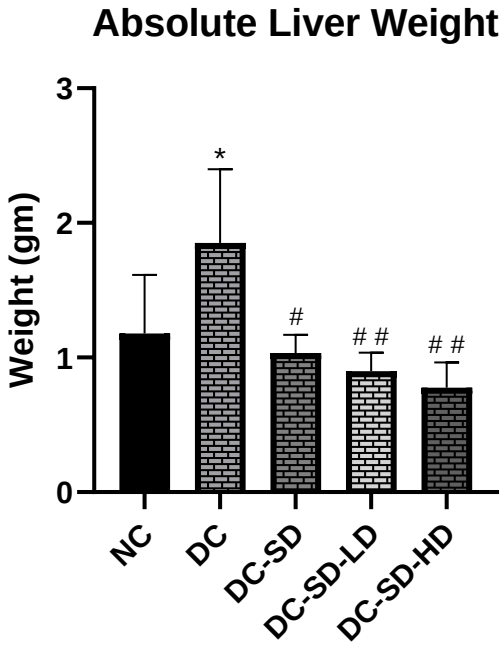



**Figure 14: Effect of treatments on liver weight**

All Values are expressed as Mean ± SD [NC = Normal Control; DC = Disease Control; DC-SD = Disease control group treated with 50mg/kg gemcitabine (Standard); DC-SD-LD = Disease control group treated with 50mg/kg gemcitabine (Standard) and 50mg/kg Olsalazine; DC-SD-HD = Disease control group treated with 50mg/kg gemcitabine (Standard) with 100mg/kg Olsalazine. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC),* ($^{*}p<0.01$, $^{\#}p<0.05$, $^{\#\#}p<0.01$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.1.5 Effect of treatment on relative liver weight

Compared to the NC group at the 10-week mark, the Disease Control (DC) group exhibited a notable increase in the relative weight of the liver, which was statistically significant ($P < 0.05$). However, in comparison to the disease control alone therapy, in standard dose group the administration of Gemcitabine resulted in a significant ($p < 0.05$) decrease in the relative liver weight. Additionally, when compared to the DC group, both the treatment - low dosage (DC-SD-LD)and treatment high dose(DC-SD-HD) groups, combinations of olsalazine and gemcitabine in the treated animals demonstrated a significant reduction in relative liver weight, with the high dosage showing ($p < 0.01$) reduction and the low dosage exhibiting ($p < 0.01$) reduction.

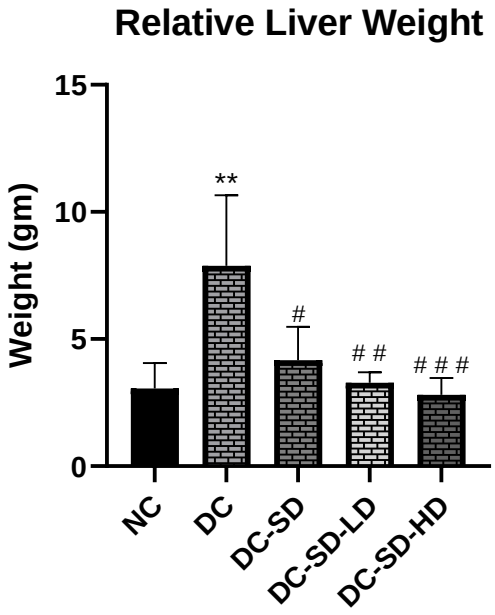



**Figure 15: Effect of treatments on relative liver weight**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), ($^{**}p<0.01$, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.1.6 Effect of treatments on survival rate

According to the Kaplan-Meier analysis, the disease control group which isCCL4/NDEA-treated model mice exhibited higher mortality rates compared to the standard dose (DC-SD) group, the treatment - low dosage (DC-SD-LD) and treatment high dose (DC-SD-HD) groups group. Additionally, the survival proportion of mice decreased following the induction of hepatocellular carcinoma when compared to the normal control group. However, after the treatment of hepatocellular carcinoma in the DC-SD, DC-SD-LD, and DC-SD-HD groups, the survival proportion of mice significantly increased in comparison to the disease control group.

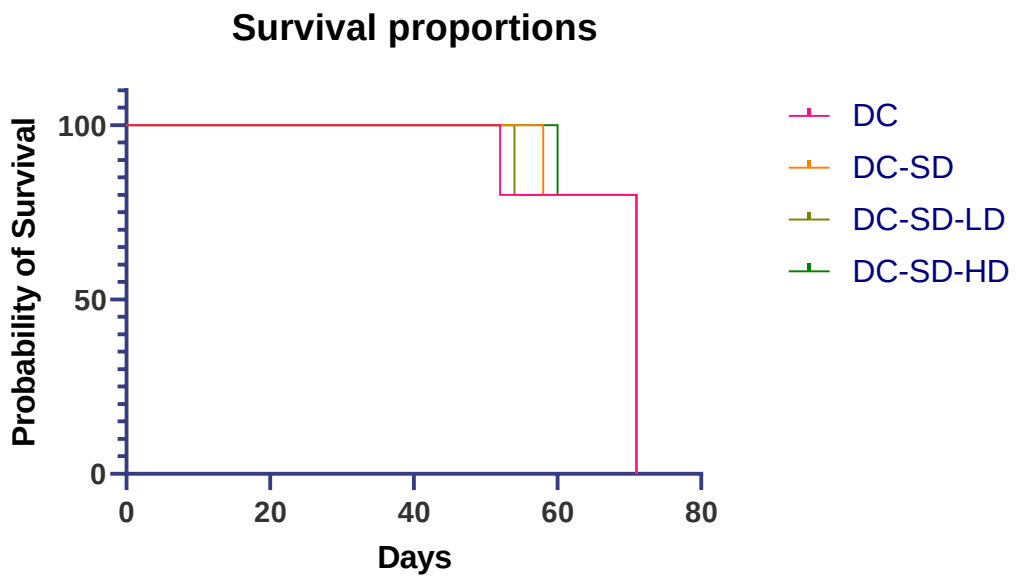



**Figure 16: Effect of treatments on survival rate**

All values are expressed in Mean ± SD [DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. Shown are the Kaplan-Meier survival curves for a DC vs. DC-SD vs. DC-SD-LD vs. DC-SD-HD . Statistical analysis was performed using the log-rank test (Mantel-Cox method). Log Rank (Montex-Cox Test P = 0.9995).

### 5.3.2 Olsalazine Pre-treatment Reduces Levels of Liver Injury markers in NDEA Induced Hepatocellular Carcinoma In Swiss Albino Mice

#### 5.3.2.1 Alkaline Phosphatase (ALP) levels:

The disease control (DC) group exhibited significantly higher levels of serum ALP compared to the healthy normal control group, with a p-value ($p < 0.0001$). In contrast, the standard group (DC-SD) group demonstrated significantly lower serum ALP levels than the DC group, also with a p-value ($p < 0.0001$). Similarly, both the treatment - low dosage (DC-SD-LD) and treatment high dose(DC-SD-HD) groups showed a substantial reduction in blood ALP levels, with p-values ($p < 0.0001$) for both groups. These findings indicate that the treatment groups, regardless of dosage, were able to effectively lower serum ALP levels similar to that observed in the disease control group. These results suggest the potential of the standard and varying dosages of the treatment regimen in mitigating the elevation of ALP levels associated with the disease.

#### 5.3.2.2 Alanine Transaminase (ALT) levels:

Comparatively, the disease control (DC) group displayed significantly higher levels of serum ALT than the normal control group, with a p-value ($p < 0.0001$). However, in the standard dose (DC-SD) group, it was observed that serum ALT levels were significantly lower than those in the DC group, with a p-value ($p < 0.01$). Similar to the disease control group, both the treatment - low dosage (DC-SD-LD)and treatment high dose(DC-SD-HD) groups exhibited a substantial decrease in serum ALT levels, with p-values ($p < 0.001$) for both groups. These findings indicate that the standard dosage as well as the low and high dosages of the treatment regimen effectively reduced serum ALT levels comparable to that observed in the disease control group. These results highlight the potential of the treatment in mitigating the elevation of ALT levels associated with the disease.

#### 5.3.2.3 Aspartate Transaminase (AST) levels:

The disease control (DC) group exhibited significantly higher levels of serum AST compared to the normal control group, with a p-value ($p < 0.0001$). In contrast, the standard dose (DC-SD) group displayed significantly lower serum AST levels than the DC group, with a p-value (p

<0.001). Similarly, both the treatment - low dosage (DC-SD-LD)and treatment high dose(DC-SD-HD) groups showed a substantial reduction in blood AST levels, with p-values ($p < 0.0001$) and ($p < 0.001$), respectively. These findings demonstrate that the treatment groups, including the standard and varying dosages, were able to effectively lower serum AST levels similar to those observed in the disease control group. These results indicate the potential therapeutic benefit of the treatment regimen in reducing elevated AST levels associated with the disease.

#### 5.3.2.4 C- Reactive Proteins (CRP) levels:

The disease control (DC) group exhibited significantly higher levels of serum CRP compared to the normal control group, with a p-value ($p < 0.001$). In contrast, the standard dose (DC-SD) group displayed significantly lower serum CRP levels than the DC group, with a p-value ($p < 0.01$). Similarly, both the treatment - low dosage (DC-SD-LD) and treatment - high dosage (DC-SD-HD) groups demonstrated a substantial reduction in blood CRP levels, with p-values ($p < 0.01$) for both groups. These findings highlight that the treatment groups, including the standard and varying dosages, were effective in lowering serum CRP levels akin to those observed in the disease control group. These results emphasize the potential therapeutic value of the treatment regimen in reducing elevated CRP levels associated with the disease.

#### 5.3.2.5 Lactate Dehydrogenase (LDH) levels:

The disease control (DC) group exhibited significantly higher levels of serum LDH compared to the normal control group, with a p-value ($p < 0.001$). Conversely, the standard dose (DC-SD) group displayed significantly lower serum LDH levels than the DC group, with a p-value ($p < 0.01$. Similarly, both the treatment - low dosage (DC-SD-LD) and treatment - high dosage (DC-SD-HD) groups demonstrated a substantial reduction in serum LDH levels, with p-values ($p < 0.001$) and ($p < 0.01$), respectively. These findings indicate that the treatment groups, including the standard and varying dosages, effectively decreased serum LDH levels similar to those observed in the disease control group. This suggests the potential therapeutic benefit of the treatment regimen in reducing elevated LDH levels associated with the disease.

### 5.3.2.6 Total Bilirubin (TBIL) levels:

The disease control (DC) group exhibited significantly higher levels of serum TBIL compared to the normal control group, with a p-value less than 0.0001. In contrast, the standard dose (DC-SD) group displayed significantly lower serum TBIL levels than the DC group, also with a p-value less than 0.0001. Similarly, both the treatment - low dosage (DC-SD-LD) and treatment - high dosage (DC-SD-HD) groups demonstrated a substantial decrease in serum TBIL levels, with p-values less than 0.0001 for both groups. These findings indicate that the treatment groups, including the standard and varying dosages, effectively reduced serum TBIL levels comparable to those observed in the disease control group. This suggests the potential of the treatment regimen in mitigating the elevation of TBIL levels associated with the disease.

**Table 9: Effect of treatments on serum biochemical parameters**

| | NC | DC | DC-SD | DC-SD-LD | DC-SD-HD |
|---|---|---|---|---|---|
| **ALP (IU/L)** | 109.5 ± 12.78 | 489.4 ± 69.87$^{****}$ | 196.7 ± 52.09$^{\#\#\#\#}$ | 148.8 ± 40.34$^{\#\#\#\#}$ | 199.5 ± 17.20$^{\#\#\#\#}$ |
| **ALT (IU/L)** | 35.79 ± 10.13 | 186.6 ± 22.82$^{****}$ | 112.6 ± 37.45$^{\#\#}$ | 84.96 ± 27.15$^{\#\#\#}$ | 86.27 ± 20.65$^{\#\#\#}$ |
| **AST (IU/L)** | 80.75 ± 19.36 | 279.8 ± 51.02$^{****}$ | 146.8 ± 40.47$^{\#\#\#}$ | 131.3 ± 7.434$^{\#\#\#\#}$ | 146 ± 18.63$^{\#\#\#}$ |
| **CRP (MG/L)** | 0.71 ± 0.333 | 2.54 ± 0.918$^{***}$ | 1.208 ± 0.299$^{\#\#}$ | 1.048 ± 0.337$^{\#\#}$ | 1.055 ± 0.134$^{\#\#}$ |
| **LDH ((IU/L))** | 277.7 ± 57.23 | 1153 ± 526.3$^{***}$ | 422.1 ± 65.84$^{\#\#}$ | 269.7 ± 95.22$^{\#\#\#\#}$ | 397.2 ± 19.15$^{\#\#}$ |
| **TBIL (MG/DL)** | 0.055 ± 0.013 | 1.373 ± 0.442$^{****}$ | 0.2225 ± 0.123$^{\#\#\#\#}$ | 0.1575 ± 0.049$^{\#\#\#\#}$ | 0.2075 ± 0.22$^{\#\#\#\#}$ |

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001^{****}p<0.0001$, $^{\#}p<0.\ 05$, $^{\#\#}p<0.\ 01$, $^{\#\#\#}p<0.\ 001$, $^{\#\#\#\#}p<0.\ 0001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.2.1 Alkaline Phosphatase (ALP) levels:

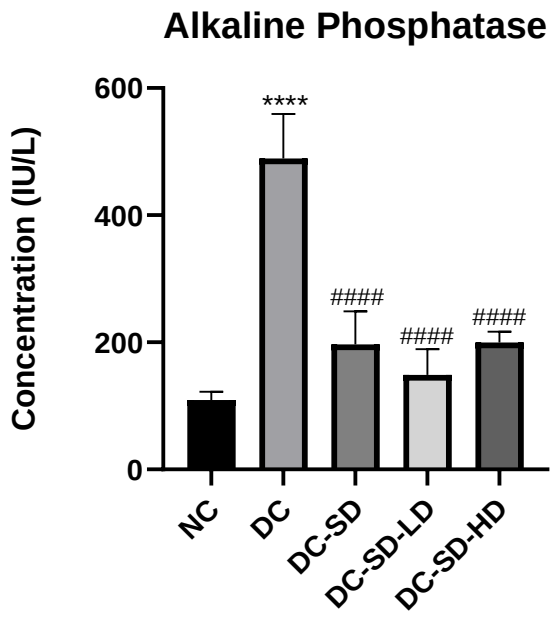



**Figure 17: Effect of treatments on alkaline phosphatase**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), ($^{****}p<0.0001$, $^{####}p<0.0001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.2.2 Alanine Transaminase (ALT) levels:**

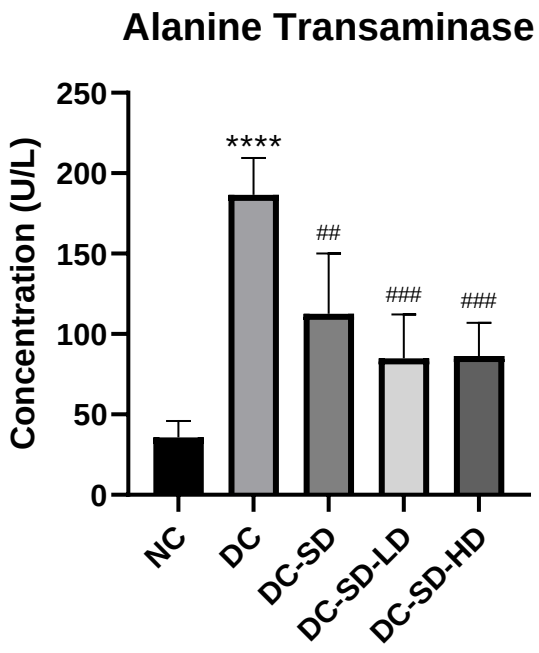



**Figure 18: Effect of Treatments on alanine transaminase**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC),* ($^{****}p<0.0001, ^{##}p<0.01, ^{###}p<0.001$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.2.3 Aspartate Transaminase (AST) levels:**

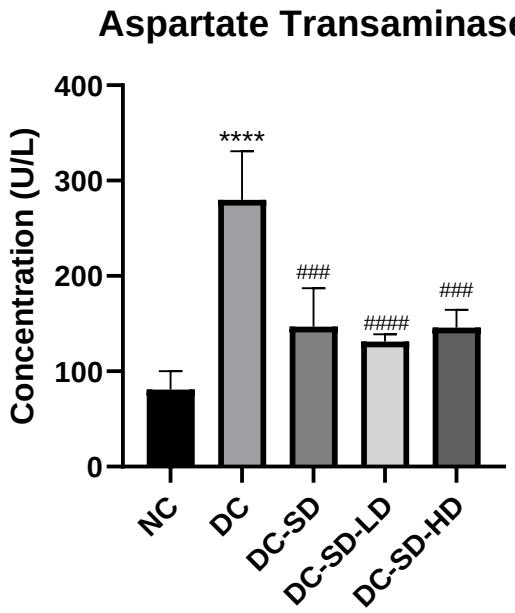



**Figure 19: Effect of treatments on aspartate transaminase**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC),* ($^{****}p<0.0001, ^{###}p<0.\ 001, ^{####}p<0.\ 0001$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.2.4 C-Reactive Protein (CRP) levels:

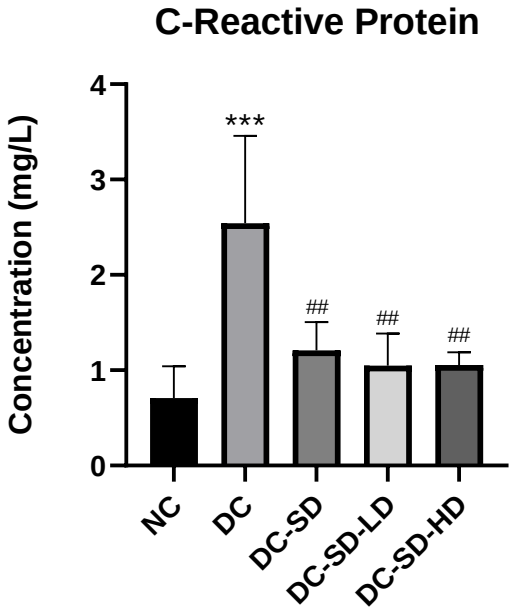



**Figure 20: Effect of Treatments on c-reactive protein**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC),* ($^{***}p<0.001, ^{\#\#}p<0.01$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.2.5 Lactate Dehydrogenase (LDH) levels:**

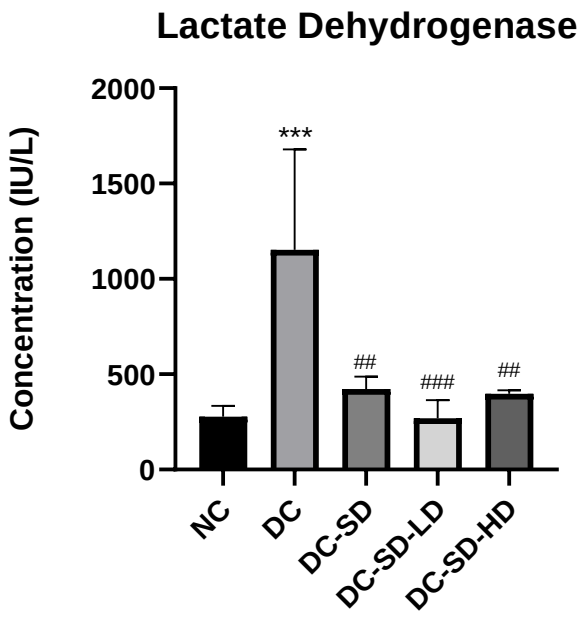



**Figure 21: Effect of treatments on lactate dehydrogenase**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), (*** $p<0.001$, ## $p<0.01$, ### $p<0.001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.2.6 Total Bilirubin (TBIL) levels:**

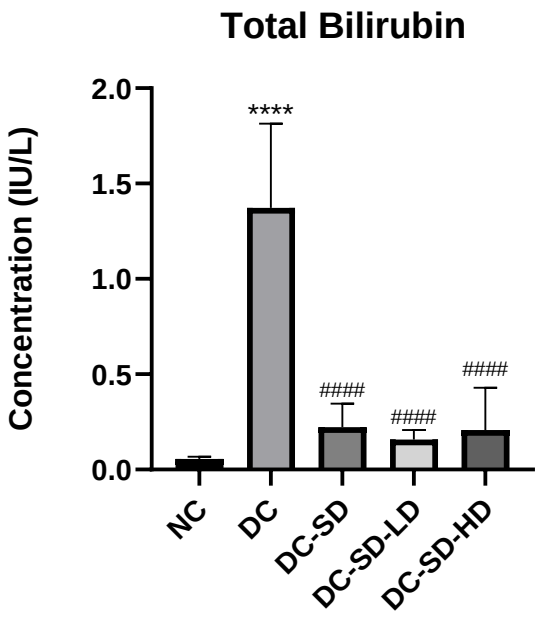



**Figure 22: Effect of treatments on total bilirubin**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), ($^{****}p<0.0001, ^{####}p<0.0001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.3 Olsalazine Pre-treatment Reduces Levels of Tissue Oxidative Parameters in NDEA Induced Hepatocellular Carcinoma In Swiss Albino Mice

#### 5.3.3.1 Total Protein levels:

The disease control (DC) group demonstrated significantly higher levels of tissue total protein compared to the normal control group, with a p-value ($p < 0.0001$). Conversely, the standard dose (DC-SD) group exhibited considerably lower tissue total protein levels than the DC group, also with a p-value ($p < 0.0001$). Similarly, both the treatment - low dosage (DC-SD-LD) and treatment - high dosage (DC-SD-HD) groups displayed significant decreases in blood total protein levels, with p-values ($p < 0.0001$) for both groups, in comparison to the DC group. These findings suggest that the treatment groups, including the standard and varying dosages, effectively reduced tissue total protein levels similar to those observed in the disease control group. This highlights the potential of the treatment regimen in normalizing protein levels associated with the disease.

#### 5.3.3.2 Reduced Glutathione levels:

The disease control (DC) group exhibited significantly lower levels of glutathione in liver tissue compared to the normal control group, with a p-value ($p < 0.01$). However, it was found that the standard dose (DC-SD) group had slightly higher glutathione levels in liver tissue compared to the DC group, although this difference was not statistically significant. Additionally, the treatment - high dosage (DC-SD-HD) group showed a statistically significant reduction in glutathione levels in liver tissue, with a p-value ($p < 0.05$). On the other hand, the treatment - low dosage (DC-SD-LD) group displayed a non-significant decrease in glutathione levels in liver tissue compared to the DC group. These findings suggest that the treatment groups, especially the high dosage group, had varying effects on glutathione levels in liver tissue, with the high dosage group showing a significant reduction.

#### 5.3.3.3 Lipid Peroxidation levels:

The disease control (DC) group exhibited significantly higher levels of lipid peroxidation in liver tissue compared to the normal control group. However, it was observed that the standard dose (DC-SD) group had slightly lower lipid peroxidation levels in tissue compared to the DC group,

although this difference was not statistically significant. Furthermore, the treatment - low dosage (DC-SD-LD) group showed a statistically significant reduction in lipid peroxidation levels in liver tissue, with a p-value ($p < 0.05$). Conversely, the treatment - high dosage (DC-SD-HD) group exhibited a non-significant decrease in lipid peroxidation levels in liver tissue compared to the DC group. These findings suggest that the treatment groups, particularly the low dosage group, had varying effects on lipid peroxidation levels in liver tissue, with the low dosage group showing a significant reduction. These results highlight the potential of the treatment regimen in mitigating lipid peroxidation, which is associated with oxidative stress.

#### 5.3.3.4 Superoxide Dismutase levels:

The levels of superoxide dismutase (SOD) in liver tissue of the disease control (DC) group were found to be slightly lower than those of the normal control group, although this difference was not statistically significant. Similarly, it was observed that the standard dose (DC-SD) group had slightly higher SOD levels in liver tissue compared to the DC group, but again, this difference was not statistically significant. Additionally, both the treatment - low dosage (DC-SD-LD) and treatment - high dose (DC-SD-HD) groups exhibited non-significant increases in SOD levels in liver tissue compared to the DC group. These findings indicate that the treatment regimen did not significantly impact SOD levels in liver tissue.

**Table 10: Effect of treatments on tissue oxidative parameters**

| | NC | DC | DC-SD | DC-SD-LD | DC-SD-HD |
|---|---|---|---|---|---|
| **Total Protein (IU/L)** | 2472 ± 138.5 | 4816 ± 166.9**** | 1977 ± 611.2#### | 2110 ± 121.2#### | 2101 ± 255.6#### |
| **Reduced Glutathione (µg/mg of protein)** | 0.0204 ± 0.003 | 0.0054 ± 0.003** | 0.0146 ± 0.007 | 0.0159 ± 0.002# | 0.0187 ± 0.002 |
| **Lipid Peroxidation (nmol/mg of protein)** | 4.862 ± 0.939 | 7.09 ± 1.303 | 4.716 ± 1.176 | 2.875 ± 2.145# | 4.405 ± 0.906 |
| **Superoxide Dismutase (SOD/mg of protein)** | 0.6571 ± 0.252 | 0.2391 ± 0.078 | 0.3923 ± 0.183 | 0.6285 ± 0.135 | 0.6596 ± 0.135 |

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), (*$^{****}$*p<0.0001,*$^{**}$*p<0.01,* $^{\#}$*p<0. 05,* $^{\#\#\#\#}$*p<0. 0001).* One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.3.1 Total Protein levels

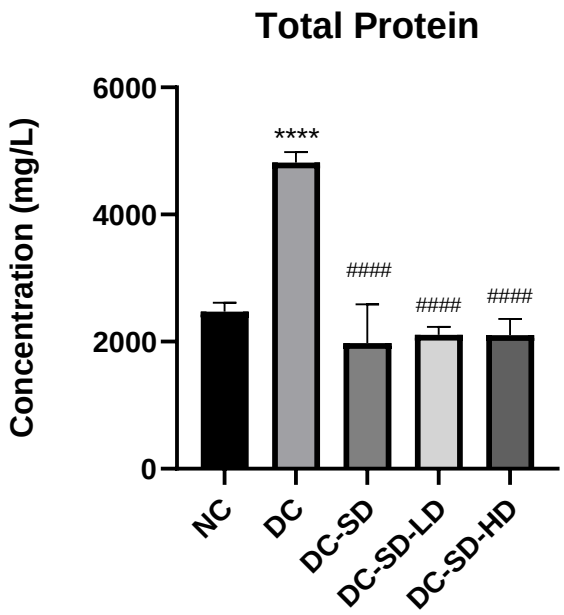



**Figure 23: Effect of treatments on total protein**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC),* ($^{****}p<0.01, ^{####}p<0.\ 01$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.3.2 Reduced Glutathione levels:**

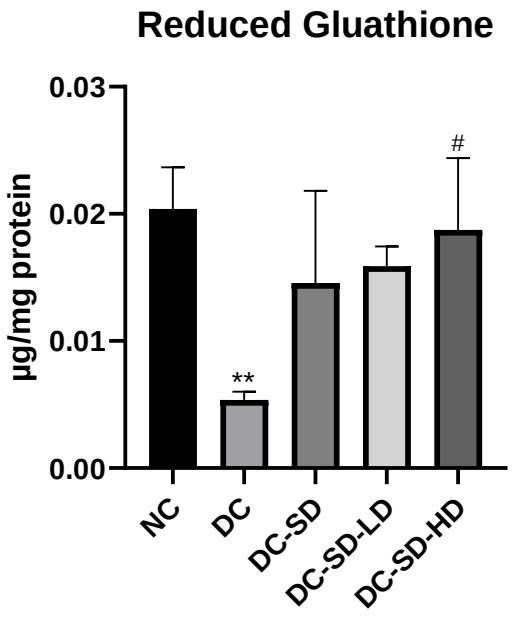



**Figure 24: Effect of treatments on reduced glutathione**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC),# indicates significantly different from disease control group (DC),*($^{**}p<0.01$, $^{\#}p<0.05$). One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.3.3 Lipid Peroxidation levels:

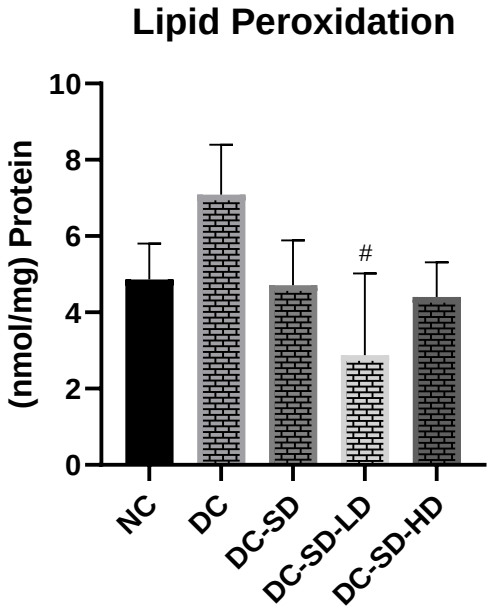



**Figure 25: Effect of treatments on lipid peroxidation**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose].*# indicates significantly different from disease control group (DC),($^{\#}p<0.05$).* One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

**5.3.3.4 Superoxide Dismutase level:**

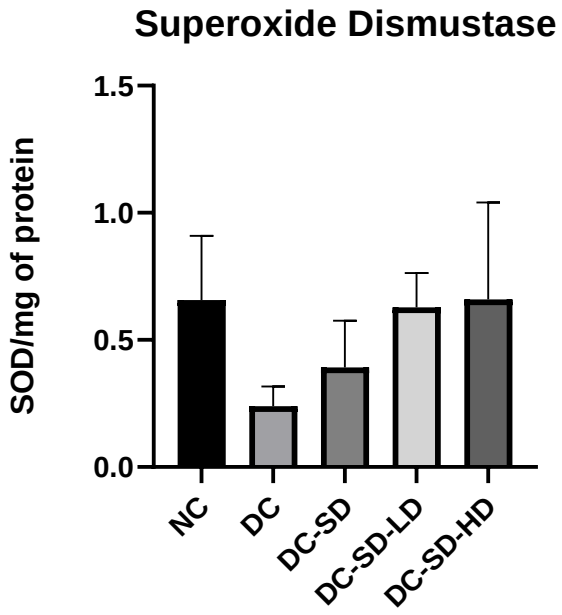



**Figure 26: Effect of treatments on superoxide dismutase**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.4 Olsalazine Pre-treatment Reduces Levels of Tumor Inflammatory Markers in NDEA Induced Hepatocellular Carcinoma In Swiss Albino Mice

#### 5.3.4.1 Effect of treatments on TNF-α

The disease control (DC) group exhibited significantly elevated levels of serum TNF-α compared to the normal control group, with a statistically significant p-value ($p < 0.01$). In contrast, the standard dose (DC-SD) group displayed lower serum TNF-α levels compared to the DC group. Likewise, both the treatment - low dosage (DC-SD-LD) and treatment - high dose (DC-SD-HD) groups demonstrated substantial reductions in serum TNF-α level. These findings indicate that the treatment regimens effectively lowered TNF-α level in the serum, suggesting a potential modulation of inflammatory processes. The significant decrease in TNF-α levels observed in the treatment groups suggests the therapeutic potential of the interventions in regulating inflammatory responses. Further investigations are required to elucidate the precise mechanisms by which these treatments influence TNF-α level and to explore the clinical implications of reducing TNF-α level in the specific context of the disease under investigation. Overall, these results underscore the effectiveness of the treatment in attenuating TNF-α level and highlight its potential role in managing the associated inflammation.

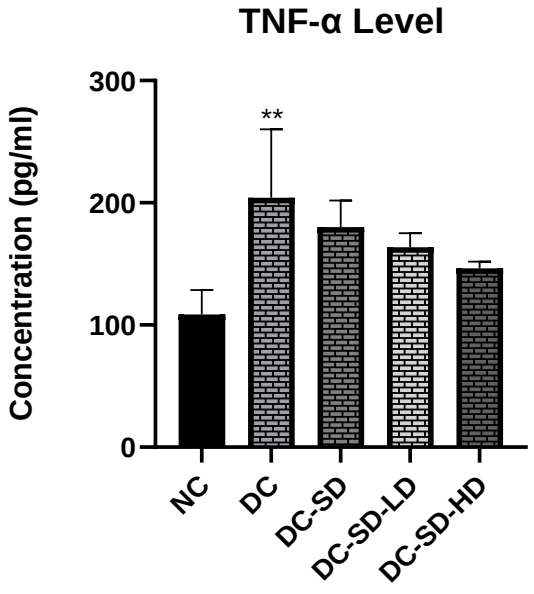



**Figure 27: Effect of treatments on TNF-alpha**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.4.2 Effect of treatment on IL-6

In the study, it was observed that the disease control (DC) group had significantly higher levels of serum IL-6 compared to the normal control group, with a statistically significant p-value of ($p < 0.05$). Conversely, the standard dose (DC-SD) group exhibited considerably lower serum IL-6 levels in comparison to the DC group. Additionally, the treatment - low dosage (DC-SD-LD) groups demonstrated significantly lower serum IL-6 concentrations compared to the DC (disease control) group. Moreover, there was a decrease in the level of IL-6 concentration in the treatment – high dose (DC-SD-HD) group when compared to the DC group. These findings indicate the potential of the standard and low dosage treatments to mitigate the elevated levels of IL-6, a pro-inflammatory cytokine associated with various pathological conditions. The significant ($p < 0.05$) reduction in serum IL-6 levels observed in the DC-SD-LD and non-significant decrease is observed in DC-SD-HD groups suggests the efficacy of the treatments in modulating the inflammatory response. Further investigations are warranted to elucidate the underlying mechanisms and clinical implications of these treatment-induced changes in IL-6 levels. Overall, these results highlight the therapeutic potential of the interventions in attenuating IL-6 levels and suggest their relevance in managing inflammation-associated disorders.

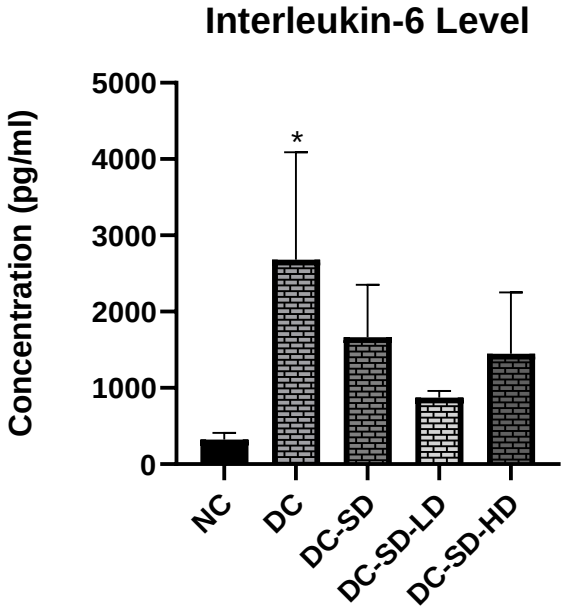



**Figure 28: Effect of treatments on IL-6**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. * *indicates significantly different from normal control group (NC), # indicates significantly different from disease control group (DC), ($^{****}p<0.0001, ^{\#\#}p<0.001, ^{\#\#\#\#}p<0.0001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

### 5.3.5 Olsalazine Pretreatment Improve Gemcitabine sensitivity by Increasing p53 Induction

#### 5.3.5.1 Effect of treatment on p53

The comparison of serum p53 levels between the disease control (DC) group and the normal control group revealed no statistically significant difference. Similarly, there was no significant variation in serum p53 levels between the DC-standard dose (SD) group and the DC (disease control) group. However, interestingly, both the treatment - low dose (DC-SD-LD) and treatment - high dose (DC-SD-HD) groups demonstrated significantly higher serum p53 levels when compared to the DC group. The p-values for these comparisons were found to be 0.01 for both the low dose and high dose treatment groups. These findings suggest that the administered treatments at both low and high doses have the potential to positively impact serum p53 levels, potentially indicating a restoration or enhancement of p53-mediated cellular functions. Further investigation is warranted to explore the underlying mechanisms and evaluate the clinical implications of these observed changes in p53 levels. Overall, these results highlight the potential therapeutic significance of the treatments in modulating p53 levels, a crucial protein involved in cellular processes such as DNA repair, cell cycle regulation, and apoptosis.

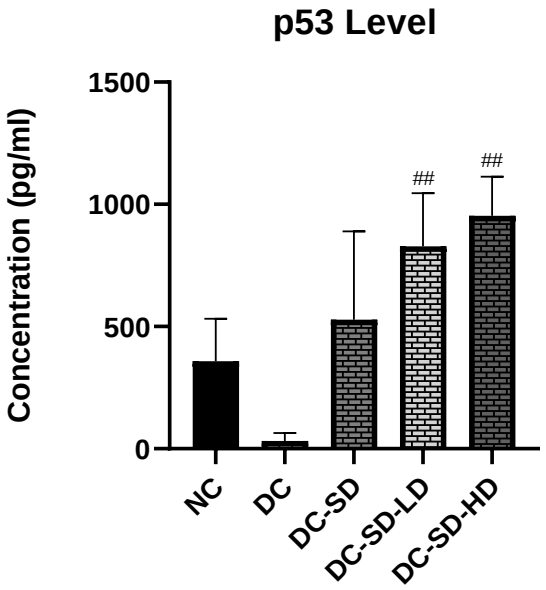


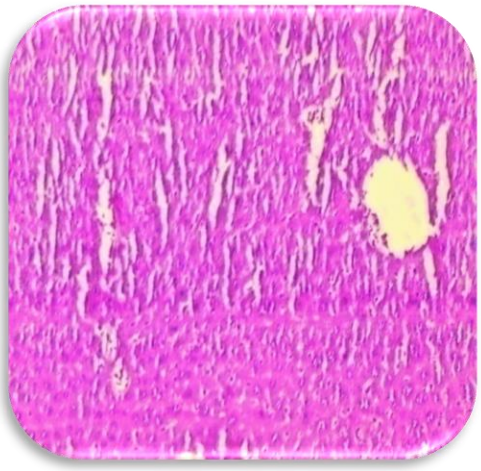

**Figure 29: Effect of treatments on p53**

All values are expressed in Mean ± SD [NC: Normal Control, DC: Disease Control, DC-SD: Disease Control + Standard Dose, DC-SD-LD: Disease Control + Standard Dose + Low Dose, DC-SD-HD: Disease Control + Standard Dose + High Dose]. *# indicates significantly different from disease control group (DC)*, (*$^{\#\#}p<0.001$, $^{\#\#\#\#}p<0.001$)*. One way ANOVA with Bonferroni Multiple Comparison Test was applied for statistical analysis.

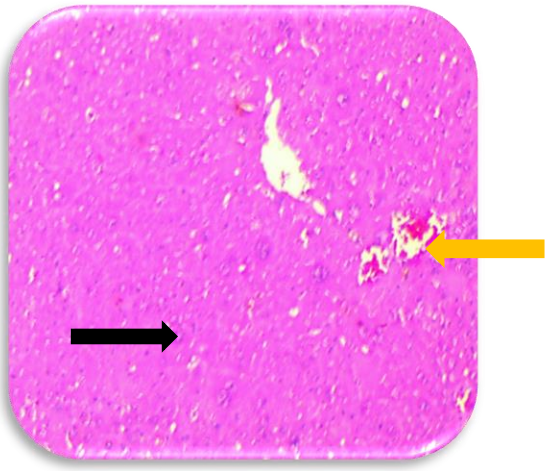
### 5.3.6 Histopathology and Immunohistochemistry

The tissue of mice in the normal control group is shown to have a lower ratio of double nuclei to cytoplasm and to have no congestion in the central and portal veins. However, in the disease-control group, the mice's liver tissue is invaded by the cells, increasing the double nucleus to cytoplasm ratio (black arrow) and clogging the sinusoidal space, portal vein, and central vein (yellow arrow). With a reduction in the number of central veins and portal veins as well as a decrease in the double nucleus in the cytoplasm, cell invasion in DC-SD-LD and DC-SD-HD was inhibited.

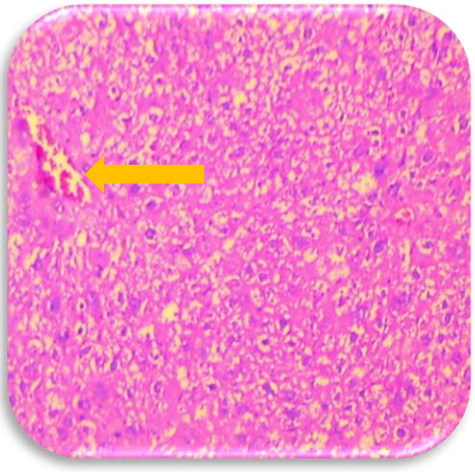
The disease control group did not exhibit any visible brown spots, while the normal control group showed clear expression of p53, as indicated by the arrow. Interestingly, in the treatment group, an increase in p53 expression was observed, which may be associated with the stimulation of apoptosis. This finding suggests that the administered treatment may have a role in enhancing p53-mediated apoptosis, leading to the observed increase in p53 expression. The presence of brown spots and the corresponding p53 expression in the normal control group further supports the notion that p53 plays a crucial role in cellular processes related to apoptosis. Further investigation is required to better understand the specific mechanisms underlying the relationship between p53 expression and the development of brown spots, as well as the potential therapeutic implications of modulating p53 activity in this context. Overall, these findings shed light on the association between p53 expression, apoptosis, and the observed phenotypic changes in the treatment group.



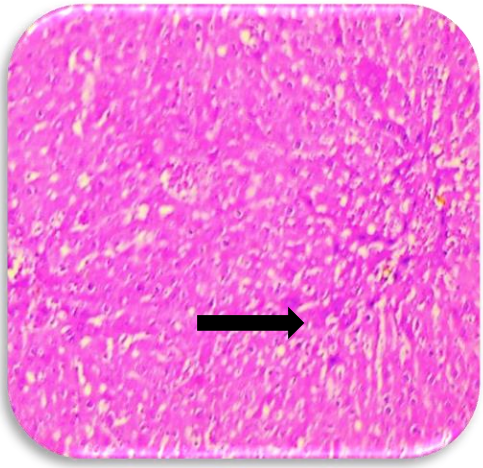
Normal Control



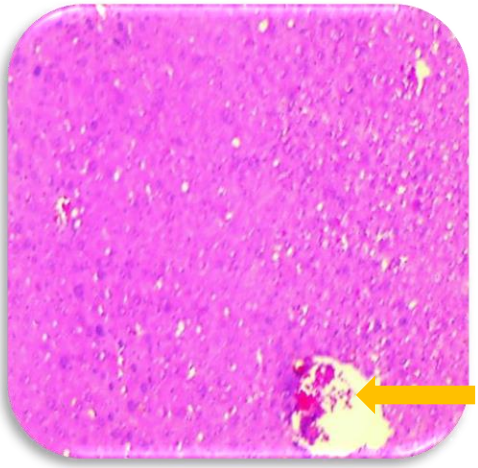
Disease Control



Disease Control-Standard Dose

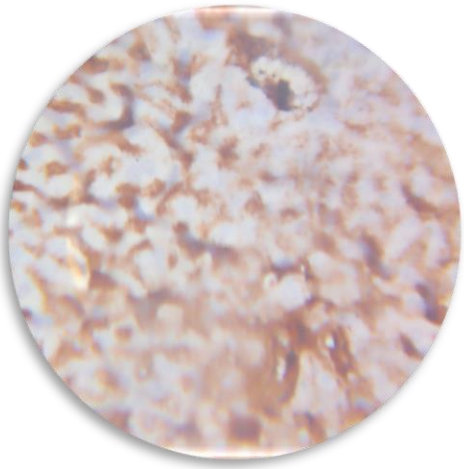


Disease Control-Standard Dose-Low Dose

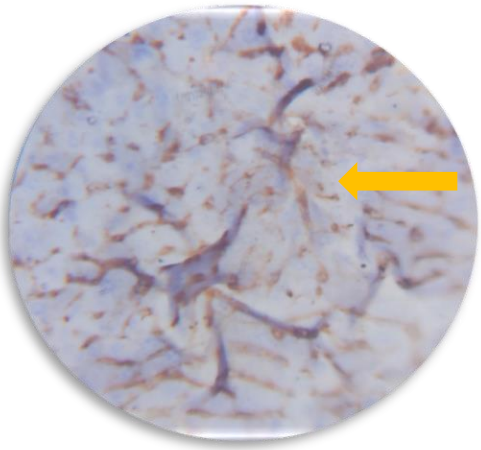


Disease Control-Standard Dose-High Dose

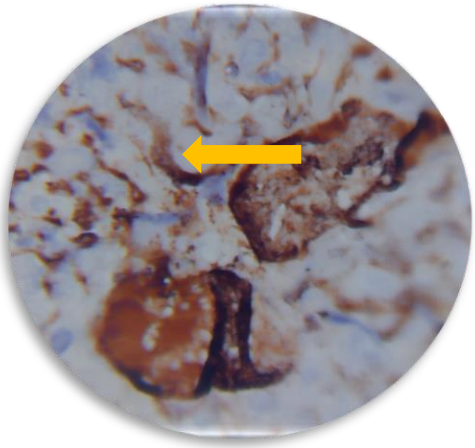
**Figure 30: Effect of treatments on histology of NDEA induced hepatocellular carcinoma**



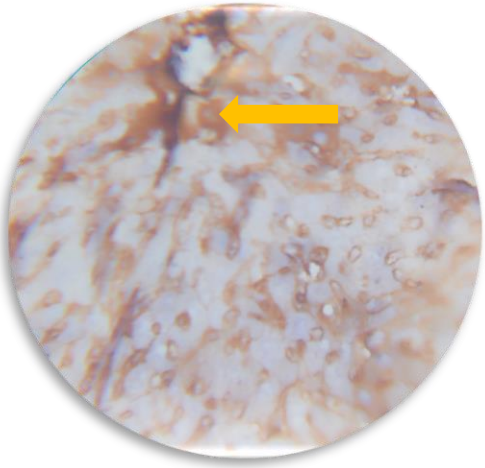
Normal Control



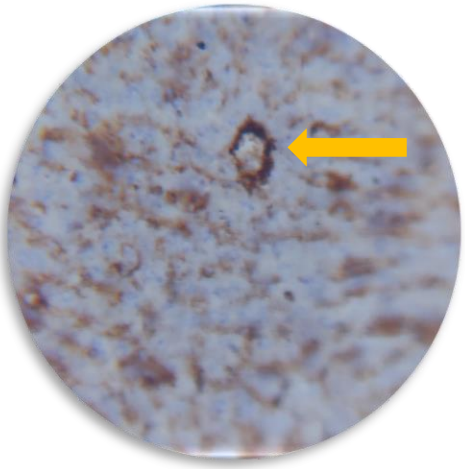
Disease Control



Disease Control-Standard Dose



Disease Control-Standard Dose-Low Dose



Disease Control-Standard Dose-High Dose

**Figure 31: Effect of treatments on p53 expression of NDEA induced hepatocellular carcinoma**

# CHAPTER 6

# DISCUSSION

The term "cancer" refers to a collection of more than 100 disorders that progressively manifest over time and include unchecked cell proliferation. It is a disorder of abnormal metabolism and signalling that results in unchecked cell division and survival. Although practically every tissue in the body can acquire cancer, and each kind of cancer has its own distinctive characteristics, the fundamental mechanisms that cause cancer are relatively similar in all cancer types (Upadhyay, 2021). We present an overview of the most prevalent cancers today using data from the official World Health Organisation and American Cancer Society databases, as well as the frequency, mortality, and life expectancy of the world's top 15 malignancies. In terms of cause-specific Disability-Adjusted Life Years (DALYs), cancer is the human disease with the greatest clinical, social, and economic costs. The risk of cancer is 20.2% for those aged 0-74 (22.4% for males and 18.2% for women, respectively (Akram et al., 2017; Mattiuzzi & Lippi, 2019). There were 18 million new instances of cancer diagnosed in 2018, with lung, breast, and prostate cancers accounting for the majority of those cases (2,09 million, 2,09 million, and 1,28 million, respectively).Other than sex-specific cancers, the frequency ratio of men to women is more than one for all cancers, with the exception of thyroid (i.e., 0.30). Cancer is now the second leading cause of death in the world (8.97 million deaths) behind ischemic heart disease, but it is predicted that it will overtake it in 2060 (18.63 million fatalities). The three most lethal malignancies in the general population are lung, liver, and stomach cancers, whereas lung and breast cancers are the leading causes of cancer-related death in men and women, respectively(Siegel et al., 2016).The procedure of treating cancer has been quite difficult. Single molecular aberrations or cancer pathways have been the focus of successful treatment interventions that have marginally improved survival in several malignancies (Zugazagoitia et al., 2016). Aside from recent significant advancements in stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, chemo dynamic therapy, sonodynamic therapy, and ferroptosis-based therapy, traditional treatment modalities like surgery, chemotherapy, and radiotherapy are still in use. Oncology practises today concentrate on creating effective and secure cancer nanomedicines. The development and spread of particular cancer cells can be prevented by targeted treatment, which also protects good cells from harm (Debela et al., 2021).

With over 90% of cases, hepatocellular carcinoma (HCC) is the most prevalent kind of liver cancer. A quarter of HCC cancers have mutations that can be treated; however, because most mutations only occur in around 10% of cases, proof-of-concept studies are made more difficult. The primary risk factors for HCC development are hepatitis B and C virus infection, while non-alcoholic steatohepatitis linked to metabolic syndrome or diabetes mellitus is increasingly common in the West. With 905 677 new cases expected to be diagnosed by 2020, liver cancer is the sixth most prevalent cancer worldwide and the fourth main cause of cancer-related mortality worldwide (Llovet et al., 2021; Schulze et al., 2015). Early identification and HCC surveillance improve the likelihood of possibly curative therapy. Curative treatments for early-stage HCC include local ablation, surgical resection, or liver transplantation. The choice of a course of treatment is influenced by the features of the tumor, the degree of the underlying liver dysfunction, age, other medical comorbidities, the accessibility of local medical resources, and other factors. Patients with cancer that is in an intermediate stage receive loco regional therapy using catheters. Patients with advanced-stage HCC have been successfully treated with kinase and immune checkpoint inhibitors(Yang et al., 2019a). Six systemic medications (atezolizumab with bevacizumab, sorafenib, lenvatinib, regorafenib, cabozantinib, and ramucirumab) have been licenced based on phase III studies, while three other medications have received accelerated FDA approval because of evidence of effectiveness (Llovet et al., 2021).

The study of methods that change gene expression without changing the primary DNA sequence is known as epigenetics. Changes in DNA methylation, histone modifications, and tiny non-coding microRNAs (miRNA) are examples of reversible and heritable epigenetic mechanisms(R. Kanwal & Gupta, 2012).Since epigenetic alterations frequently occur before disease pathology, they can serve as helpful diagnostic or prognostic markers for disease progression(Kelly et al., 2010). Both genetic and epigenetic processes regulate the onset and spread of cancer. Aberrant epigenetic alterations are frequently referred to as key actors in the course of cancer and are thought to begin at a very early stage in the creation of neoplastic cells (Kanwal& Gupta, 2012). Multiple epigenetic abnormalities can be seen in malignant tissue, and cells have the capacity to become resistant to therapy, therefore it is likely that epigenetic therapies work best when paired with other anticancer tactics like cytotoxic or signal transduction inhibitors (López-Camarillo et al., 2019). Prior to chemotherapy, or in combination with chemotherapy to produce synergistic

effects and maximise therapeutic efficacy, epigenetic medicines are given to cancer cells to make them more sensitive to the drug (Majchrzak-Celinska et al., 2021).

Hepatocellular cancer was produced in experimental male mice using the CCL4/NDEA-induced hepatocellular carcinoma paradigm in the current investigation. The inflammation of secondary lymphoid organs and a prolonged oxidative stress situation were brought on by CCl4's induction of liver fibrosis. Hepatotoxins like CCl4 are often utilised in animal models of liver fibrosis (Sayed et al., 2021).NDEA is a strong, pervasive environmental carcinogen that is also an extensively used experimental model for examining the disease's underlying pathophysiology (Bhatia et al., 2015). It has been shown in various experimental animal models that administering NDEA intraperitoneally, intradermally, intratracheally, or intragastrially, either alone as a carcinogen or in combination with other carcinogens, can cause carcinogenesis in the lungs and nasal cavity, followed by the liver, stomach, and kidney (Sur, Pal, Roy, et al., 2016).

In-silico methods are getting very popular nowadays, because they predict the how a selected ligand will interact with the target providing the method for initial screening. During the literature survey, seven hypomethylating drugs were selected. Molecular docking was performed of the seven drugs namely Azacitidine, Decitabine, EGCG, Hydralazine, Olsalazine, Procainamide, and Procaine. Olsalazine showed the highest binding affinity with the binding energy of -5.54 kcal/mol. Therefore, Olsalazine was opted for the in-vitro and in-vivo studies.

In order to assess the direct effects of olsalazine and gemcitabine on the HepG2 cell line, an MTT experiment was conducted. The MTT experiment was initially performed to determine the $IC_{50}$ of gemcitabine when administered alone. Subsequently, the combination of gemcitabine and olsalazine was evaluated using the same assay to further investigate their potential synergistic effects on the HepG2 cell line. The results indicated that the combination of both medications had positive outcome impact on the HepG2 cell line. From the MTT data, it was inferred that the combined use of an epigenetic regulator and a chemotherapeutic drug may be exerting a preventive effect on the development of hepatocellular carcinoma. This effect is likely achieved through modifications in the epigenetic pathway and by increasing the sensitivity of the chemotherapeutic agents activity.

Throughout the experiment, a sudden drop in the animals' body weight following the production of hepatocellular carcinoma in the NDEA model was seen. However, there was a significant change in body weight in the disease control group compared to the normal control group, and there was a non-significant change in the standard and drug treated group compared to the disease control group, suggesting that the hepatocellular carcinoma induction was the cause of the weight loss in all other groups. Furthermore, mice with the disease are significantly less likely to drink water than mice without the disease, but there was significant difference in water intake between the disease control group and the normal control group. However, there is no significant change is observed between the standard and drug-treated groups as compared to disease control group. After the hepatocellular carcinoma was induced, food intake gradually decreased, but there was a significant difference between the disease control and normal control groups when it came to food intake, while there was no significant difference is observed between the standard and drug-treated groups.

After the animal was slaughtered, the liver's absolute and relative weights were continually recorded. Over the course of the investigation, it was discovered that both absolute and relative weight were rising. When compared to the disease control group at the conclusion of the research, a substantial reduction in absolute and relative liver weight was seen in the groups treated with gemcitabine alone as well as in combination with olsalazine and gemcitabine. In contrast, the development of nodules and tumors in the liver as well as unchecked cell proliferation after exposure to carcinogens may be to blame for the rise in liver weight in the disease control group. Gradual weight gain and decreased liver weight following olsalazine and gemcitabine treatment may indicate the combination's ability to protect against proliferative effects on NDEA-induced HCC as well as its ability to improve its capacity for doing so (Panchal et al., 2017; Uemitsu et al., 1986).

The survival rate in HCC is an important topic of discussion, as it directly reflects the effectiveness of diagnostic methods, treatment options, and patient outcomes. After the induction with CCL4/NDEA there is reduction in body weight, food intake and water intake and also it is affected in a number of variables, including as the stage of the tumor, the health of the liver, any

underlying hepatic disease. The survival rates in HCC treated animals have increased, with non significantly increased in their body weight, food intake and water intake and low mortality rate in the treatment period or after the treatment period (Saber et al., 2018, 2019).

An important enzyme, alkaline phosphatase (ALP), is involved in a number of physiological processes, such as bone mineralization, liver function, and bile acid metabolism. It is present in large amounts in all bodily tissues, with notable concentrations in the liver, bones, kidneys, and intestines. The dephosphorylation of several compounds, including nucleotides, proteins, and alkaloids, is facilitated by ALP (Saif et al., 2005).As a diagnostic and prognostic tool for several illnesses, such as liver problems, bone diseases, and some cancers, the measurement of ALP levels has important therapeutic value. Increased blood levels of ALP frequently signify biliary blockage, bone diseases, or liver malfunction. On the other hand, decreased ALP levels may be seen in situations of starvation or some hereditary diseases. An enzyme called ALP is mostly produced by the liver. ALP levels in HCC patients can indicate both healthy liver function and hepatic impairment. In order to evaluate the overall liver health and function in HCC patients, monitoring ALP levels in conjunction with other liver function indicators is recommended (Crum-Cianflone et al., 2010; X.-S. Xu et al., 2014). The serum ALP level in the disease control group was found to be significantly greater than that of the normal control group. However, both the low and high dose combinations of olsalazine and gemcitabine markedly reduced the blood ALP level as compared to the disease control group.

Serum glutamic-pyruvic transaminase (SGPT), also known as alanine transaminase (ALT), is an enzyme that is largely present in the liver and to a lesser extent in the kidneys, heart, and skeletal muscles. By aiding the reversible transfer of an amino group from alanine to alpha-ketoglutarate, which results in the generation of pyruvate and glutamate, ALT plays a critical function in the metabolism of amino acids (Kawamoto et al., 2012). As a biomarker for both healthy liver function and liver disorders, ALT is frequently tested in clinical settings. An essential diagnostic tool for liver illnesses such viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), and drug-induced liver injury, elevated ALT levels in the blood suggest hepatocellular injury or liver malfunction (W. R. Kim et al., 2008; J. Li et al., 2019; Lok & McMahon, 2009). During HCC treatment, keeping an eye on changes in SGPT levels can assist

determine how well the medication is working. While chronically increased or rising SGPT levels may indicate treatment resistance or disease progression, decreasing SGPT levels over time may indicate therapy efficacy and better liver function. The serum ALT level in the disease control group was found to be significantly increased than that of the normal control group. However, both the low and high dose combinations of olsalazine and gemcitabine helped to reduce the serum ALT level significantly as compared to the disease control group.

The greatest concentrations of the enzyme aspartate transaminase (AST), also known as serum glutamic-oxaloacetic transaminase (SGOT), are found in the liver, heart, skeletal muscles, kidneys, and brain. AST plays a crucial function in the metabolism of amino acids by catalysing the reversible transfer of an amino group from aspartate to alpha-ketoglutarate, which results in the creation of oxaloacetate and glutamate (Chiopris et al., 2021; Giannini et al., 2005; Maurice & Manousou, 2018). The biomarker AST is frequently used to assess liver health and disorders. A important diagnostic tool for liver illnesses such viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), and drug-induced liver injury, elevated AST levels in the blood suggest hepatocellular injury or liver malfunction (J. K. Lee et al., 2010; Lok & McMahon, 2009; Testino et al., 2014). SGOT levels can be measured repeatedly to track the development of HCC. The advancement of the illness, a deterioration of liver function, or resistance to therapy may all be indicated by rising SGOT levels over time. Assessing SGOT levels can be useful in determining therapy efficacy and spotting illness recurrence. The serum AST level in the disease control group was found to be significantly increased than that of the normal control group. However, both the low and high dose combinations of olsalazine and gemcitabine helped to reduce the serum AST level significantly as compared to the disease control group.

C-reactive protein (CRP) is a well-known acute-phase reactant and an important marker of inflammation in the body. The liver produces it as a reaction to interleukin-6 (IL-6) and other pro-inflammatory cytokines. It is a pentameric, annular protein. Since CRP increases significantly in inflammatory conditions, it is a helpful biomarker for monitoring systemic inflammation and several illnesses (Black et al., 2004; Pepys & Hirschfield, 2003; Ridker et al., 2002). CRP is frequently used in clinical practises as a diagnostic and prognostic tool for a

variety of disorders. Increased blood levels of CRP are associated with infections, autoimmune diseases, cardiovascular diseases, and various forms of cancer. Additionally, myocardial infarction and stroke have been linked to CRP as an independent predictor of future cardiovascular events (Danesh et al., 2004; Pepys & Hirschfield, 2003; Ridker et al., 2002). Changes in CRP levels throughout HCC therapy can help with disease surveillance and treatment response. While consistently increased or rising CRP levels may indicate treatment resistance or disease progression, a decrease in CRP levels after therapy may indicate therapeutic efficacy and a better prognosis. The serum CRP level in the disease control group was found to be significantly increased than that of the normal control group. However, both the low and high dose combinations of olsalazine and gemcitabine helped to reduce the serum CRP level significantly as compared to the disease control group.

A key enzyme in cellular energy metabolism is lactate dehydrogenase (LDH). It catalyse the simultaneous interconversion of NADH to NAD+ and the reversible conversion of lactate to pyruvate. The glycolytic pathway, which is in charge of breaking down glucose and producing energy in the form of ATP, depends heavily on LDH (Brooks, 2018; Draoui et al., 2014; Fantin et al., 2006).Many tissues and organs, including the heart, liver, kidneys, skeletal muscles, and red blood cells, have large quantities of LDH. There are several LDH isoforms, and each one is found in different organs. LDH is made up of four subunits, and the combination of these subunits determines the composition of each isoforms (Faloppi et al., 2016; M. Lu et al., 2008; Ponc et al., 2001). LDH level variations during HCC therapy can help with disease surveillance and treatment response. While consistently increased or rising LDH levels may indicate treatment resistance or illness progression, decreasing LDH levels after therapy may indicate therapeutic efficacy and an improved prognosis. LDH is a biomarker that may be used to evaluate therapy effectiveness and direct therapeutic choices. It was discovered that the serum LDH level in the disease control group was noticeably higher than that of the normal control group. However, compared to the disease control group, both the low and high dosage combinations of olsalazine and gemcitabine significantly reduced the blood LDH level.

A vital biomarker for diagnosing many liver illnesses and evaluating liver function is total bilirubin. It is a yellow pigment made from heme that is largely produced during the breakdown

of red blood cells. Bilirubin is carried to the liver, where it combines with glucuronic acid to generate bilirubin glucuronides, which may be expelled in bile and are water-soluble (Choi et al., 2013; Vitek, 2012; Zelenka et al., 2012). Insights regarding treatment response and disease progression can be gained from changes in total bilirubin levels after HCC therapy. Total bilirubin levels that gradually decrease may signify better liver function and a positive response to treatment, whereas levels that remain consistently high or increase can be an indication of disease progression or treatment resistance. It's critical to track total bilirubin levels in order to evaluate therapy success and modify therapeutic approaches as necessary. Serum TBIL levels were observed to be considerably higher in the disease control group than in the normal control group. However, compared to the disease control group, both the low and high dosage combinations of olsalazine and gemcitabine significantly reduced the blood TBIL level.

Total protein is a crucial component of regular blood tests and is used as a major measure to evaluate a person's overall health, liver function, and nutritional condition. It indicates the total amount of blood proteins, which are important for a number of biological activities. The measurement of all blood proteins, including albumin and globulins, is known as total protein. It is a commonly used laboratory test that offers insightful data on the general protein status and aids in the evaluation of many medical diseases. Total protein levels that are abnormal can offer valuable diagnostic and prognostic data. While increased amounts can be detected in dehydration, inflammation, certain infections, or plasma cell abnormalities, low total protein levels can signify malnutrition, liver or kidney illness, or protein-losing conditions (dos Santos et al., 2015). In HCC patients, total protein levels are frequently utilised as a measure to assess liver function. Reduced total protein concentrations may be a sign of poor hepatic synthetic function, such as decreased liver albumin synthesis, which can happen in advanced HCC (J.-B. Zhang et al., 2011). Total protein levels in liver tissue were found to be significantly greater in the disease control group than in the normal control group. However, both the low and high dose combination of olsalazine and gemcitabine significantly decreased the level of total protein in the liver tissue as compared to the disease control group.

Glutamate, cysteine, and glycine are the three amino acids that make up the tripeptide known as reduced glutathione (GSH). It is produced intracellularly and is found in significant quantities

throughout cells, particularly in the cytoplasm. Together with other antioxidants, GSH functions as an essential part of the cellular antioxidant defence system, neutralising damaging ROS and preserving redox equilibrium. Reactive oxygen species (ROS) may be effectively scavenged by GSH, which also takes part in a number of enzymatic processes that aid in detoxification and oxidative damage prevention (Hayes & McLellan, 1999; Meister & Anderson, 1983; Rahman, Biswas, et al., 2006). Changes in GSH levels and the GSH/GSSG (glutathione disulfide) ratio can signify oxidative stress and are linked to a number of illnesses, such as cancer, liver disease, and neurological disorders (Jones, 2006). The function of reduced glutathione (GSH) and its levels in hepatocellular carcinoma (HCC) can be complicated and context-dependent. In HCC, abnormalities in redox homeostasis and GSH levels can affect the growth and spread of the tumor. Increased GSH levels may encourage cancer cell survival, growth, and resistance to apoptosis, which would increase the size and invasiveness of tumors (Reuter et al., 2010; Traverso et al., 2013). GSH levels in liver tissue were found to be significantly decreased in the disease control group than in the normal control group. However, the combination of olsalazine and gemcitabine with low dose increased significantly and non significantly in high dose in the level of LPO in the liver tissue as compared to the disease control group.

One of the primary steps in cellular oxidative damage is lipid peroxidation. It describes the reactive lipid peroxidation products that are produced as a result of the oxidative destruction of lipids, especially polyunsaturated fatty acids (PUFAs). The integrity of the cell membrane may be compromised as a result of this process and dangerous reactive oxygen species (ROS) may be produced. Lipid hydro-peroxides are created when ROS attack PUFAs, which starts the process of lipid peroxidation. The degradation of these hydroperoxides can lead to the production of a number of reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are frequently employed as indicators of lipid peroxidation (Ayala et al., 2014; Esterbauer et al., 1991; Turrens, 2003). Numerous clinical problems, such as cancer, liver ailments, neurological disorders, and cardiovascular diseases are linked to excessive lipid peroxidation. Cellular malfunction, inflammation, and tissue damage can result from the buildup of lipid peroxidation products in the body (Halliwell & Gutteridge, 2015; Stadtman & Oliver, 1991). Hepatocellular carcinoma (HCC) has been shown to be significantly influenced by lipid peroxidation (LPO), both during its early stages and as it progresses. Increased oxidative stress,

DNA damage, and cellular dysfunction can result from LPO dysregulation, which can aid in the aetiology of HCC (Gallelli et al., 2018; Pompella et al., 2003). LPO levels in liver tissue were found to benon significantly increased in the disease control group than in the normal control group. However, the combination of olsalazine and gemcitabine with low dose decreased significantly and non significantly in high dose in the level of LPO in the liver tissue as compared to the disease control group.

The antioxidant enzyme superoxide dismutase (SOD) is vital for cellular protection against oxidative stress. Superoxide radicals (O2•-) are catalysed into less reactive molecules such molecular oxygen (O2) and hydrogen peroxide (H2O2). SOD works to shield cells from oxidative damage and to stop the build-up of dangerous superoxide radicals (Fridovich, 1995; McCord, 2000; McCord & Fridovich, 1969; Valko et al., 2007). In cancer, oxidative stress has a dual function in tumor genesis and progression. The redox status of cancer cells is modulated by SOD enzymes, which has an impact on cancer cell survival, proliferation, and metastasis. Changes in SOD activity have been seen in a number of cancer forms, underlining the possible contribution of this enzyme to tumor growth (Heng et al., 2021; Zou et al., 2016). Hepatocellular carcinoma (HCC) is a condition in which superoxide dismutase (SOD) is significant. SOD activity and levels have been found to be dysregulated in HCC, which may have an effect on oxidative stress levels and aid in the growth and development of tumors (Hayes et al., 2020; X. Zhang et al., 2016). SOD levels in liver tissue were found to be non-significantly decreased in the disease control group than in the normal control group. However, both the low and high dose combination of olsalazine and gemcitabine non-significantly increased the level of SOD in the liver tissue as compared to the disease control group.

The main producers of tumor necrosis factor-alpha (TNF-alpha) are activated immune cells such macrophages, monocytes, and T cells. TNF-alpha is a cytokine, a tiny protein involved in cell signalling. It is essential for controlling cell death, inflammation, and the immunological response. TNF-alpha is a pleiotropic cytokine, which means that it may affect many cell types and biological functions in various ways (Balkwill, 2009; Tracey et al., 1986). However, TNF-alpha production that is excessive or dysregulated can cause chronic inflammation and aid in the pathogenesis of a number of illnesses, including rheumatoid arthritis, inflammatory bowel

disease, psoriasis, sepsis, and several cancers. As a result, TNF-alpha has emerged as a key therapeutic target for the creation of medications that can modify its activity and treat disorders linked to inflammation (Dinarello, 2004). The most prevalent form of primary liver cancer, hepatocellular carcinoma (HCC), is complicatedly impacted by TNF-alpha, a pleiotropic cytokine. Depending on the stage of the illness, the tumor microenvironment, and interactions with other molecules, the levels of TNF-alpha in HCC can change. In HCC, TNF-alpha has been linked to accelerating tumor development and growth. Through the activation of several signalling pathways, such as nuclear factor-kappa B (NF-B) and mitogen-activated protein kinases (MAPK), it can promote the growth and survival of cancer cells. TNF-alpha further encourages angiogenesis, the creation of new blood vessels that feed the tumor with nutrition and oxygen, promoting tumor growth and metastasis (Cha et al., 2007; Liu et al., 2020; Tan et al., 2019). TNF-alpha serum levels were found to be significantly increased in the disease control group than in the normal control group. However, the combination of olsalazine and gemcitabine with low dose decreased significantly and non significantly with high dose in the level of serum TNF-alpha as compared to the disease control group.

The multifunctional cytokine interleukin-6 (IL-6) is a member of the interleukin family. It is generated by a variety of cell types, including fibroblasts, endothelial cells, and immune cells (including T cells and macrophages). IL-6 is essential for controlling inflammatory processes, immunological responses, and tissue homeostasis. It works by way of a particular receptor complex made up of glycoprotein 130 (gp130) and the IL-6 receptor (IL-6R) (Kishimoto, 2010; Naugler et al., 2007). Involved in both acute and chronic inflammatory processes, IL-6 affects several cell types pleiotropically. It can enhance the activation of immune cells, increase the synthesis of acute-phase proteins, control hematopoiesis, and affect how different organs and tissues work. Additionally, IL-6 has been linked to the development of a number of illnesses, including cancer, autoimmune diseases, and chronic inflammation (Schaper& Rose-John, 2015; Tanaka & Kishimoto, 2012). The most frequent kind of primary liver cancer, hepatocellular carcinoma (HCC), is significantly influenced by IL-6, a multifunctional cytokine. In HCC, IL-6 levels are frequently increased, and this cytokine has a role in several aspects of the growth and development of HCC. IL-6 promotes the formation of tumors by acting as a growth factor for HCC cells. Through the activation of several signalling pathways, including the signal transducer and activator of transcription 3 (STAT3) pathways, it promotes the proliferation and survival of

cancer cells. Additionally, IL-6 promotes angiogenesis, which aids in the creation of new blood vessels necessary for tumor support (Kishimoto, 2010; Naugler et al., 2007). IL-6 serum levels were found to be significantly increased in the disease control group than in the normal control group. However, the combination of olsalazine and gemcitabine with low dose decreased significantly and non-significantly with high dose in the level of serum TNF-alpha as compared to the disease control group.

A tumor suppressor protein called p53 is essential for preserving genomic integrity and thwarting the growth of cancer. The TP53 gene encodes it, and it is frequently referred to as the "guardian of the genome." As a transcription factor, p53 controls the expression of a wide range of target genes involved in DNA repair, cell cycle regulation, apoptosis, and senescence(Lane & Crawford, 1979).Cellular responses to stress, such as DNA damage, oncogene activation, hypoxia, and telomere erosion, are significantly regulated by the protein p53. When cells are exposed to stress signals, p53 is activated, which sets off the proper cellular reactions to protect genomic integrity and prevent tumor growth. P53 encourages cell cycle arrest to allow DNA repair or initiates death if damage is irreversible through its several downstream targets(Lane, 1992; Muller & Vousden, 2013). One of the most frequent genetic changes in human malignancies is TP53 gene mutations. Loss or inactivation of p53 activity increases the likelihood of uncontrolled cell proliferation and the accumulation of genetic defects, which aid in the initiation and development of many cancers (Vousden & Lane, 2007). Hepatocellular carcinoma (HCC), the most prevalent form of primary liver cancer, is significantly influenced by p53, a critical tumor suppressor protein. The genetic changes, underlying liver disease and interactions with other molecules can all affect the levels and functioning of p53 in HCC. By controlling cell cycle progression, DNA repair, apoptosis, and senescence, wild-type p53 functions as a powerful tumor suppressor. It contributes to the preservation of genomic integrity and stops the accumulation of genetic defects that might result in the development of HCC. HCC generally displays loss or inactivation of p53 function, which usually results from TP53 mutations and aids in the development of the tumor (El-Deiry, 2003; Hsu et al., 1991; Hussain et al., 2007). Serump53 levels were found to be non-significantly decreased in the disease control group than in the normal control group. However, both the low and high dose combination of olsalazine and gemcitabine non-significantly increased the serum level of p53 as compared to the disease control group.

# CHAPTER 7

# CONCLUSION

In summary, our study provides strong evidence supporting the significant improvement in antitumor response achieved by combining Olsalazine and Gemcitabine. The synergistic effect observed between the epigenetic regulator and the chemotherapeutic drug suggests a promising strategy for enhancing therapeutic outcomes in cancer treatment. By incorporating Olsalazine, we not only witnessed enhanced antitumor effects but also observed increased sensitivity of Gemcitabine, which may help overcome drug resistance mechanisms. These findings highlight the potential of this combination therapy in improving treatment outcomes for cancer patients.

Furthermore, our investigation elucidated two distinct mechanisms by which Olsalazine and Gemcitabine contribute to the treatment of hepatocellular cancer. Olsalazine exerts its effect through epigenetic modification by inhibiting DNMT1, while also enhancing the sensitivity of Gemcitabine. The second pathway involves the activation of cell apoptosis, demonstrated by an increase in the p53 apoptotic factor and a decrease in inflammatory markers such as TNF-alpha and IL-6. Further gene expression studies are warranted to provide additional evidence for the role of epigenetic alterations in this therapeutic approach.

Overall, our findings underscore the importance of exploring innovative combination treatments that leverage synergistic effects to enhance antitumor responses and improve cancer treatment success. While more research is needed to fully understand the underlying processes and confirm the therapeutic value of this strategy, our study highlights the potential of combining Olsalazine and Gemcitabine as an effective therapeutic approach for hepatocellular cancer, opening avenues for further investigation in the field of epigenetic modifications and their impact on cancer treatment.

# CHAPTER 8

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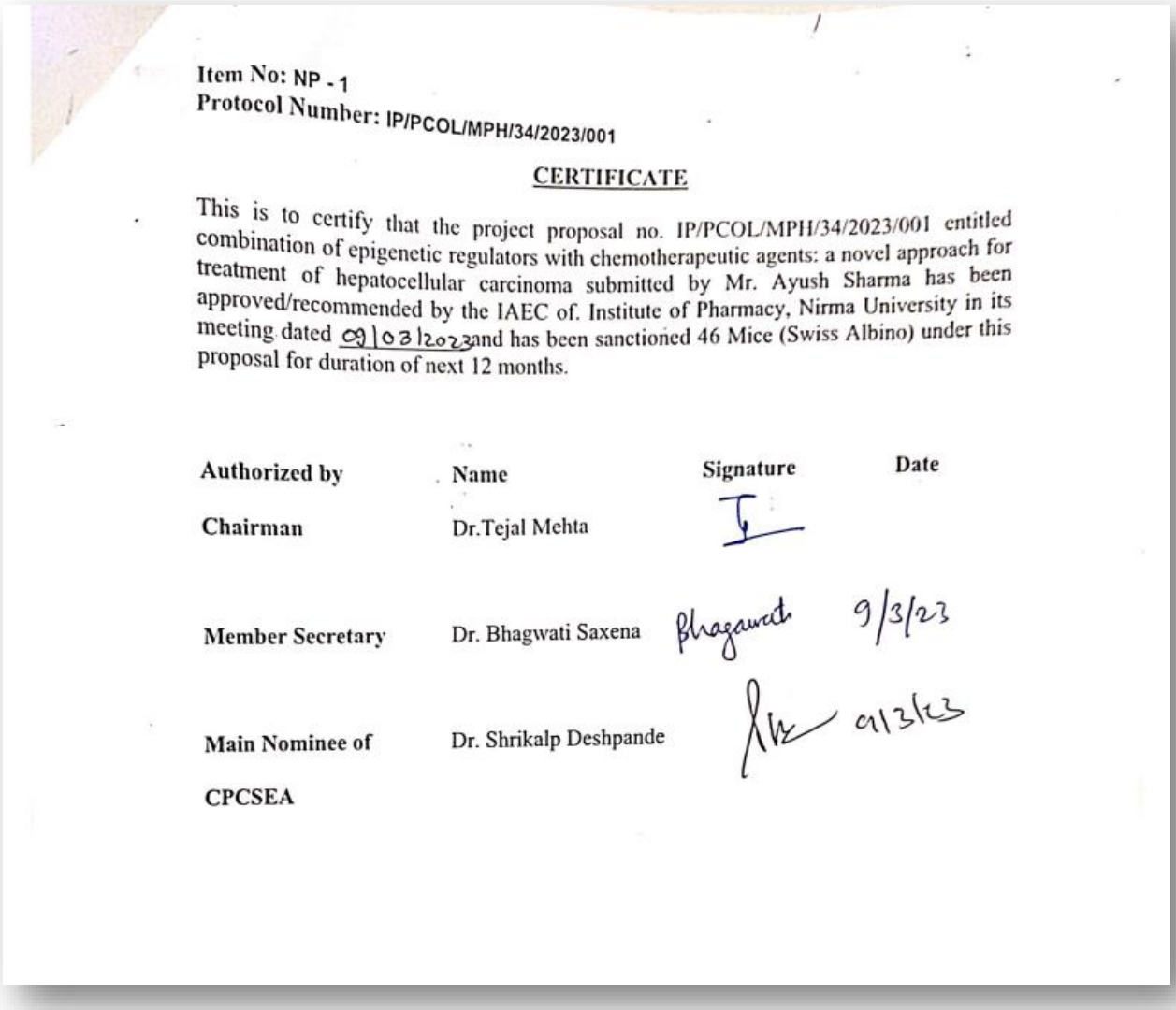
# CHAPTER 9

# ANNEXURE



Item No: NP - 1
Protocol Number: IP/PCOL/MPH/34/2023/001

**CERTIFICATE**

This is to certify that the project proposal no. IP/PCOL/MPH/34/2023/001 entitled combination of epigenetic regulators with chemotherapeutic agents: a novel approach for treatment of hepatocellular carcinoma submitted by Mr. Ayush Sharma has been approved/recommended by the IAEC of. Institute of Pharmacy, Nirma University in its meeting dated 09/03/2023 and has been sanctioned 46 Mice (Swiss Albino) under this proposal for duration of next 12 months.

| Authorized by | Name | Signature | Date |
|---|---|---|---|
| Chairman | Dr.Tejal Mehta | | |
| Member Secretary | Dr. Bhagwati Saxena | Bhagwati | 9/3/23 |
| Main Nominee of CPCSEA | Dr. Shrikalp Deshpande | | 9/3/23 |

**Figure 32: Certificate of IAEC Approval**

## Research & Review Publications

- Completed project with research paper with topic stating “Efficacy pharmacological study of Bacillus coagualans BCP92 (MTCC 25460) in animal model of castor oil induced diarrhea”. It will be submitted for publication in the month of May 2023.

- Review article with topic stating “Transient Receptor Potential (TRP): A Friend or Foe?” It will be submitted for publication in the month of May 2023.

- Currently working on research project with topic stating “combination of epigenetic regulators with chemotherapeutic agents: a novel approach for treatment of Hepatocellular carcinoma”. It will be submitted for publication in the month of June 2023.

- Currently working on IDEA Lab project research paper with topic stating “Pharmacological evaluation of nicotine for COVID 19 treatment”. It will be submitted for publication in the month of May 2023.

NIRMA UNIVERSITY
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**2nd NIRMA e-CONFERENCE FOR INTERNATIONAL CONNECT (NCIC) – 2023**

"Recent Advances in Nanotechnology: Drug Discovery & Therapeutics"
January 24 - 25, 2023

**CERTIFICATE**

***Prof./Dr./Mr./Ms. Ayush Sharma*** *has participated as a* ***Delegate*** *and Presented a* ***Paper (Oral)*** *in the* ***2nd Nirma e-Conference for International Connect (NCIC) – 2023*** *on* ***"Recent Advances in Nanotechnology: Drug Discovery & Therapeutics"*** *organized by* ***Institute of Pharmacy, Nirma University*** *during January 24-25, 2023.*

| **Dr. Mayur Patel** | **Prof. Tejal Mehta** | **Dr. Shital Butani** |
|---|---|---|
| Organizing Secretary, NCIC 2023 | Convenor, NCIC 2023 | Scientific Committee, NCIC 2023 |

NIPiCON-IPS 2022

6th Nirma Institute of Pharmacy International Conference
Jointly organized with Indian Pharmacological Society

NIRMA UNIVERSITY
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NAAC ACCREDITED 'A' GRADE

*"Emerging Opportunities and Challenges in Pharmacology and Pharmaceutical Sciences for Drug Discovery and Healthcare Innovations"*

February 17-19, 2022

Certificate

This is to certify that Dr. / Mr. / Ms. ***Sharma Ayush*** attended 6th Nirma Institute of Pharmacy International Conference jointly organized with Indian Pharmacological Society held during February 17-19, 2022 as ***Delegate*** and ***Oral paper presenter*** and secured ------ prize.

| Prof. Tejal Mehta | Prof. Jigna Shah | Dr. BDP. Kala Kumar | Prof. Manjunath Ghate | Dr Shivprakash Rathnam |
|---|---|---|---|---|
| Coordinator, Scientific Committee | Organizing Secretary | Secretary, IPS | Convener | President, IPS |

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# Olsalazine in HCC

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