Studies On The Protective Role Of Bioactive Compound Against Hepatocellular Carcinoma

Thesis Submitted for The Degree of

Doctor of Philosophy

in

Science

By

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Registration No. SLSBT1123618

Index No. 236/18/Life Sc./26

DEPARTMENT OF LIFE SCIENCE & BIOTECHNOLOGY

JADAVPUR UNIVERSITY

2023

Dedicated

To

Liver Cancer Patients

CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled **"Studies on the Protective Role of Bioactive Compound Against Hepatocellular Carcinoma"**, submitted by **Sayanta Sarkar** who got his name registered on **25/09/2018** for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Ratan Gachhui** and co-supervision of **Dr. Parames Chandra Sil** and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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ACKNOWLEDGEMENTS

It's been years of toil and relentless perseverance every day to complete this journey. I have done my work with absolute honesty. There have been many ups and downs along the journey, but never has there been a lack of effort. I have always believed in giving my best in everything I do.

*First and foremost, I would like to acknowledge my gratitude to God for providing me with this opportunity. Many people have contributed to the successful accomplishment of this thesis. I feel my heartful gratitude for all the people who have been directly or indirectly helped in this work. I express profound respect to my supervisor "***Professor Ratan Gachhui***, Jadavpur University" and co-supervisor "***Professor Parames Chandra Sil***, Bose Institute". Their supervision helped me to overcome various critical situations when my experiments were not working or during experiment standardization periods. Their passion for knowledge, scientific intuition, and scientific thinking helped me in molding myself into a better researcher. It was a great privilege and honor to work under their guidance.*

I am extremely thankful to ***Dr. Joydeep Mukherjee***, ***Dr. Parimal Karmakar*** *for being my all-time cooperative RAC members.*

I am grateful to the ***University Grant Commission (UGC), Jadavpur University and Bose Institute*** *for providing the working space and material for the experiments.*

I express my sincere regards to all the faculty members and colleagues of the Department of Life Science & Biotechnology and the Division of Molecular Medicine for their constant support and guidance.

I am also blessed with a few wonderful seniors and colleagues Dr. Sumit Ghosh and Mr. Abhishek Kumar Das, Ms. Noyel Ghosh, Mrs. Ankita Mondal, Ms. Sharmistha Chatterjee, Mrs. Jhilam Majumdar, Ms. Rubia Parvin who always showed their trust in me during my research.

I am grateful to the Animal facility of Bose Institute for providing the experimental mice and to all the technical officers for providing their support.

ABSTRACT

INDEX No.: 236/18/Life Sc./26

Title: Studies on the protective role of bioactive compound against hepatocellular carcinoma

Submitted by: Sayanta Sarkar

Hepatocellular carcinoma (HCC) refers to a major class of liver cancer. Bioactive compounds exhibit protective effects against DEN+CCl_4-induced hepatocellular carcinoma model in mice. Owing to the many anti-cancer effects of isorhamnetin, we investigated its protective effects in the DEN+CCl_4-induced HCC mice model. Isorhamnetin was found to have potential anti-tumor properties against HCC. Its administration inhibits TNF-α and some other pro-inflammatory cytokines to suppress cancer-inducing inflammation. Additionally, it regulates Akt and MAP kinase signaling molecules to suppress Nrf2 signaling. It also suppresses cell cycle progression and induces cancer death in DEN+CCl_4 administered mice. Isorhamnetin administration negatively regulates cancer cell metabolism and cell cycle progression. These findings in the DEN+CCl_4-induced mice HCC model indicate anti-TNF-α properties of isorhamnetin and strongly suggest that it could be used in inhibiting TNF-α mediated sorafenib resistance in liver cancer patients. Isorhamnetin also functions as a PPAR-γ activator and Nrf2-inhibitor to suppress cancer progression. Regulation of diverse cellular signaling pathways makes isorhamnetin a better anti-cancer chemotherapeutic candidate. It can be further studied specifically as an inhibitor of the above-mentioned cancer-promoting proteins or an agonist of anti-cancer proteins to develop a more promising treatment for hepatocellular carcinoma.

Submitted by: Sayanta Sarkar

The thesis has been divided into 5 chapters:

Chapter 1 gives a brief outline and general introduction the entire study.
Chapter 2 encloses a detailed review of the literature on hepatocellular carcinoma.
Chapter 3 details the introduction and literature review on isorhamnetin.
Chapter 4 gives information related to the materials and methods used in the study.
Chapter 5 describes *in vivo* the antineoplastic properties of isorhamnetin against HCC.
Chapter 6 describes the detailed molecular mechanism of anti-HCC properties of isorhamnetin.

Here, my research work showed the hepatocellular carcinoma ameliorative properties of isorhamnetin with a detailed mechanism.

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CONTENTS

Chapter 1

General Introduction

There is an increasing incidence of liver cancer around the world, which is a significant health concern nowadays. The number of people who will suffer from liver cancer is expected to exceed one million by 2025 (Llovet et al. 2021). Approximately 90% of cases of liver cancer are caused due to hepatocellular carcinoma (HCC). In around 50% of cases, the most important reason for HCC development is hepatitis B virus (HBV) infection (Global Burden of Disease Liver Cancer et al. 2017). Due to successful antiviral treatment leading to sustained virological response (SVR), hepatitis C virus (HCV) infection risk has decreased significantly. The incidence of HCC remains high in patients with cirrhosis despite of clearance from HCV (Kanwal et al. 2017). Western countries are experiencing an increase in non-alcoholic steatohepatitis (NASH) as a result of metabolic syndrome and diabetes. Moreover, mutational signatures have been found to indicate that aristolochic acid and tobacco may contribute to HCC pathogenesis (Estes et al. 2018).

In the year 2018, 841,080 new liver cancer cases were documented in the world. Currently, it is the sixth most common cancer globally and the fourth leading cause of death due to cancer. HCC incidence and mortality are highest in East Asia and Africa, but mortality rates have increased in parts of the USA and Europe as well (McGlynn, Petrick, and London 2015). Since the early 2000s, HCC has been the fastest-growing reason for deaths related to cancer in the USA, as per reports by Surveillance Epidemiology End Results (SEER), and by 2030, it will move from fourth to the third leading cause of death due to cancer if these statistics persist (Rahib et al. 2014). Various risk factors are responsible for the varying incidence of HCC worldwide. Over half of the cases occur in China, whereas 10%, 7.8%, 5.1%, 4.6%, and 0.5% occur in Europe, Africa, North America, Latin America, and Oceania, respectively. ASIR is the highest in eastern Asia at 17.7 per 100,000, with Mongolia having the highest ASIR at 93.4. South-East Asia has the highest ASIR at 13.3, while Africa has the highest ASIR at 8.4. For example, Central and Eastern Europe has the lowest ASIR at 2.5, followed by Western Asia with an equal rate of around 4.0 (Singal, Lampertico, and Nahon 2020). In 2018, Eastern Asia and Northern Africa had the highest mortality rates for HCC, followed by South East Asia (13.2). As a result, South Central Asia has a low mortality rate of around 2.3 (in Central, Northern, and Eastern Asia), followed by Central, North, and East Europe, and West Asia. The ASMRs of Egypt and Mongolia are the highest, while Morocco and Nepal have the lowest ASMRs. HCC is a highly lethal disease based on ASMR and ASIR that are similar throughout the world (Singal, Lampertico, and Nahon 2020).

CC development is caused and mediated by a variety of factors. Even though we know more about the mechanisms underlying disease, we have not yet applied this knowledge in clinical practice. It is difficult to study HCC tumours since only about 25% of them have actionable mutations, and most mutations are rarer than 10% (Schulze, Nault, and Villanueva 2016). A number of HCC mutations, including TERT, TP53, and CTNNB1, are currently incurable. The use of molecular and immune classifications as biomarkers for guiding treatment is also under investigation. New research has shed light on the importance of the tumour microenvironment for the development of NASH-associated HCC, including the immune system and platelet activation (Zucman-Rossi et al. 2015).

In clinical practice, tumour tissue from biopsies is increasingly used for molecular analysis to diagnose HCC. HCC can be prevented by HBV vaccines and antiviral therapies, as well as by drinking coffee and taking aspirin. Histologically proven treatment for HCC, including hepatic resection and liver transplantation, has greatly improved since the early 2010s (Marrero et al. 2018). A reduction in tumour stage beyond Milan criteria has resulted in improved surgical resection outcomes and remarkable survival rates for liver transplants. Radiofrequency ablation remains the most common procedure for non-surgical ablation of early-stage HCC. Although randomized controlled trials have shown negative results, adjuvant therapies are still needed in order to prevent relapse after these curative approaches. HCC that has reached the intermediate stage has been treated with transarterial chemoembolization (TACE). A phase II trial of transarterial radioembolization (TARE) showed promise, but guidelines haven't yet recommended it as the standard of care. Intermediate treatment options will not become better in the near future by using radiation oncology or locoregional devices (Salem et al. 2016).

Currently, there are a number of systemic therapies being used for HCC to overcome the difficulties associated with conventional therapies. These therapies include monoclonal antibodies, tyrosine kinase inhibitors (TKIs), and immune checkpoint inhibitors (ICIs) (Finn et al. 2020). A majority of HCC patients undergo systemic therapies, especially when the disease is in its advanced stages. During the past five years, systemic therapies have made significant progress, increased patients' survival rates and improving their quality of life (Kudo 2012). Patients' reported outcomes improved after the combination of atezolizumab and bevacizumab (anti-PDL1 antibodies) was combined with advanced-stage HCC cases. In cases where single-agent regimens failed, regorafenib, cabozantinib, and ramucirumab

showed improved survival benefits compared to sorafenib and lenvatinib (Bruix, Gores, and Mazzaferro 2014). Some 15-20% of responders have found benefits from single-agent ICIs, but biomarkers have not been successful in identifying them. A phase III trial examining the effectiveness of combining TKIs and ICIs or CTLA4 inhibitors and PD1/PDL1 axis inhibitors is currently underway, and its outcomes are expected to influence treatment options for HCC.

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Chapter 2

Review of Literature:

Hepatocellular Carcinoma

1. Etiology

HCC is a multi-factorial condition and a lot of different factors contribute to its etiology. Each of the etiological factors is described below:

1.1. Cirrhosis

Liver cirrhosis is the primary risk factor for HCC, which is mostly caused by infection with HBV and HCV. There is an increase of fibrous tissue in the liver along with the destruction of hepatocytes which paves the way for the growth of a tumour. Cirrhosis also leads to the activation of stellate cells which leads to an influx of cytokines, oxidative stress products, and growth factors, most of which have been shown to contribute to tumorigenesis.

1.2 HBV and HCV

Chronic liver infection caused by HBV and HCV is one of the primary causes of HCC development. Mechanisms that lead to hepatocarcinogenesis due to viruses are complicated and lead to liver cirrhosis which eventually develops into HCC in almost 90% of individuals (Simmonds, 2001). The mechanism of HBV infection involves incorporating the genetic material of the virus into the human genome leading to the inactivation of p53, oxidative stress, and inflammation, which eventually lead to hepatocarcinogenesis. Among virus-related causes, human immunodeficiency virus (HIV) and hepatitis D virus (HDV) can also lead to the development of HCC although to a lesser extent.

1.3 Autoimmune Hepatitis (AIH)

AIH is a condition whose cause is yet uncertain, and it is characterized by the destruction of the liver parenchymal cells which leads to fibrosis and cirrhosis. AIH contributes to very few (less than 1%) cases of HCC worldwide and it is usually seen in chronic liver cirrhosis patients.

1.4 Carcinogens

Apart from viruses, chemical carcinogens are also involved in the development of HCC. Examples include tobacco smoke, aflatoxins, arsenic, and vinyl chloride among others that may either function independently or along with viruses to result in cirrhosis contributing to HCC development (Zhang, 2010).

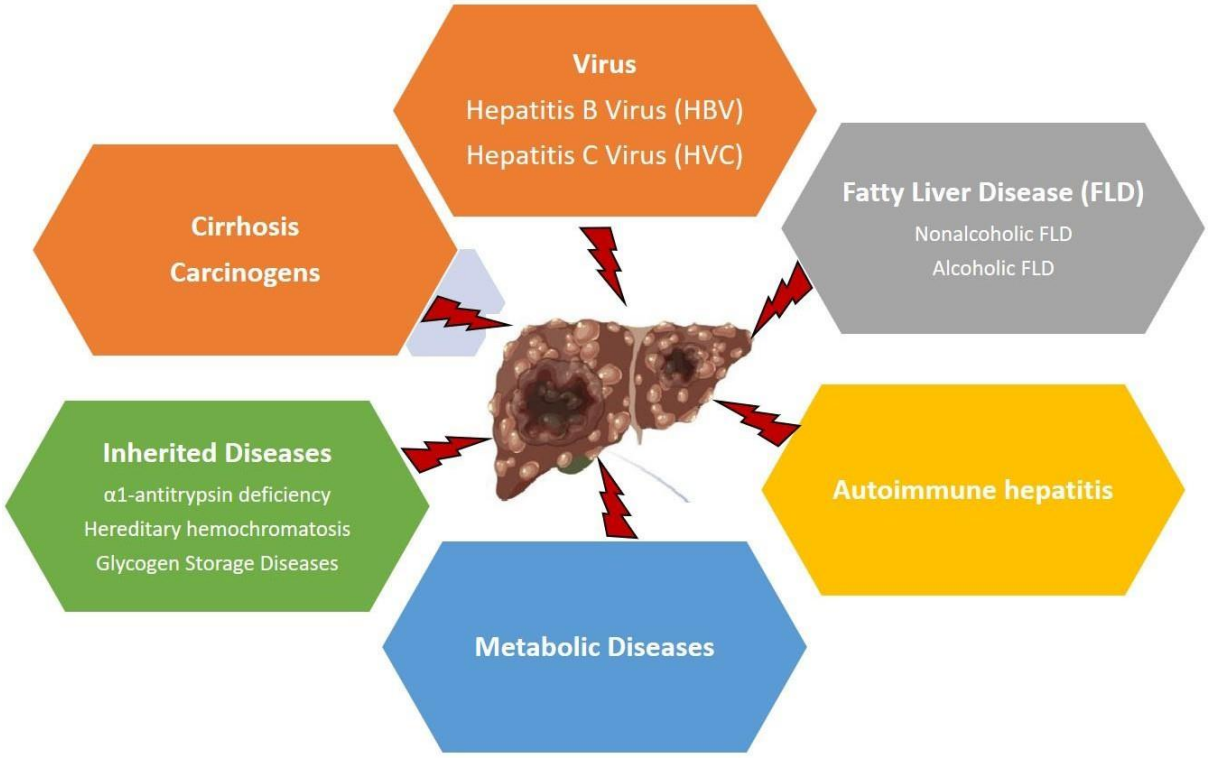

Figure: HCC inducing factors

1.5 Inherited Diseases

Some hereditary metabolic syndromes such as α1-antitrypsin deficiency, Wilson's disease, hemochromatosis, and hepatic porphyria are known to promote hepatocarcinogenesis as a consequence of liver damage and inflammation (Okuda, 2000).

1.5.1 α1-antitrypsin deficiency (A1ATD)

α1-antitrypsin (A1AT) is a part of the protein superfamily, Serine Proteinase Inhibitor (SERPIN), which is characterized by the presence of three beta sheets and one reactive mobile loop. In A1ATD, the serum levels of A1AT decrease leading to liver disease and enhancing the risk of liver cirrhosis and, consequently, HCC (Okuda, 2000).

1.5.2 Hereditary hemochromatosis (HH)

Hemochromatosis is characterized by excessive iron absorption from food which eventually accumulates in organs such as the heart, joints, pancreas, and liver. An overload of iron in the organs, especially the liver, promotes tumourigenesis by increasing the proliferation of

hepatocytes, increases levels of ROS, leads to DNA damage, leads to membrane damage of cells and organelles, and causes peroxidative damage (Okuda, 2000).

1.5.3 Glycogen Storage Diseases (GSD)

Glycogen is a complex molecule that serves as a reserve energy source for the body. Genetic disorders known as Glycogen Storage Diseases (GSDs) occur due to problems in the synthesis or breakdown of glycogen. According to a recent study, 57% of patients with glycogen storage disease Type 1 are diagnosed with hepatocellular adenoma, while 8% develop HCC (Okuda, 2000). The incidence of hepatocellular carcinoma in patients with type III GSD is lower than 10 per 100,000, despite rare reports of patients with liver cirrhosis (Okuda, 2000). Hepatocellular carcinoma can develop in patients with type VI GSD, although there are limited reports of it in these patients (Okuda, 2000).

1.6 Metabolic Diseases

An important metabolic disease is diabetic mellitus that contributes to around 7% of all HCC cases around the world. The pathophysiology associated with diabetes involving hyperinsulinemia, hyperglycemia, insulin resistance, and activation of signalling pathways are predisposing factors for HCC.

1.7 Fatty Liver Disease

Fatty liver disease has emerged as an important risk factor for chronic liver disease which may lead to the development of HCC.

1.7.1 Non-Alcoholic Fatty Liver Disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is characterized by an excessive accumulation of lipids in the liver known as steatosis, which subsequently leads to inflammation and develops into steatohepatitis, followed by cirrhosis and HCC (Okuda, 2000).

1.7.2 Alcoholic Fatty Liver Disease (AFLD)

AFLD is a type of fatty liver disease which, as its name indicates, occurs due to excessive consumption of alcohol which eventually leads to the accumulation of lipids, inflammation, and scarring of the liver characteristic of hepatic injury leading to the development of HCC. It has been shown to contribute to around 30% of all HCC cases worldwide (Okuda, 2000).

2. Markers

2.1. Oncofoetal and glycoprotein antigens

2.1.1. α-fetoprotein and α-fetoprotein-L3

A glycoprotein which is primarily produced in the liver of a foetus is known as alpha fetoprotein (AFP). The tumour excretion of AFP is responsible for a high serum AFP concentration in over 70% of HCC patients. A common cut-off value of 20ng/mL has been used in screening HCC patients for over 40 years (Agarwal, 2017).

2.1.2. Glypican-3

The plasma membrane contains glycopican-3 (GPC3), a type of heparan sulphate proteoglycan. Growth factors can be altered by its interaction with them. As opposed to healthy individuals and patients diagnosed with non-malignant hepatopathy, HCC patients have significantly higher serum levels of GPC3 in both protein and mRNA (Agarwal, 2017).

2.1.3. Zinc α-2 glycoprotein (ZAG)

A soluble glycoprotein of major histocompatibility complex class I, ZAG belongs to the ZAG family. The overexpression of the ZAG protein was confirmed in the HCC group from the serum proteomes of healthy adults, liver cirrhosis groups, and HCC groups (Agarwal, 2017).

2.1.4. Tumour-associated glycoprotein 72 (TAG-72)

There is a glycoprotein complex called TAG-72 that is similar to mucin-1 and this complex is commonly found in human adenocarcinoma tissues such as gastric, colon, and pancreatic cancers, but it does not appear in normal tissues. TAG-72 expression in HCC tissues has been shown to be significantly increased, and this overexpression may contribute to tumour invasion and metastasis (Agarwal, 2017).

2.1.5. Golgi protein 73 (GP73)

The GP73 protein is considerably increased among different cancer types such as lung, seminoma, and renal cell cancers, but it is becoming increasingly recognized for its close association with liver diseases, particularly HCC.

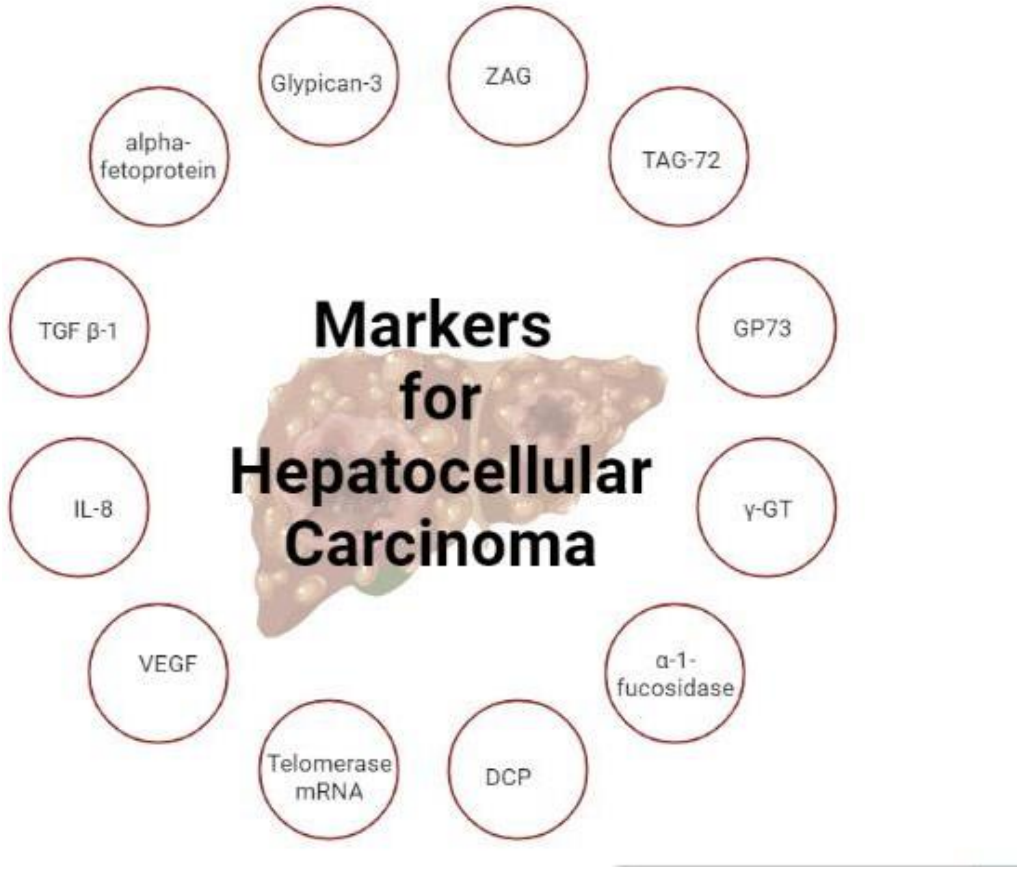

Figure: Depiction of markers used for HCC detection

2.2. Cytokines

2.2.1. Vascular endothelial growth factor (VEGF)

HCC's neovascularization is regulated by VEGF, a cytokine involved in regulating tumour neovascularization. Recent studies have shown that HCC tissues and HCC with microscopic venous invasion tissues express considerably increased levels of VEGF compared to normal tissues and HCC tissues without microscopic venous invasion, as evidenced by high levels of vascularization observed in HCC tumours (Bou-Nader, 2020).

2.2.2. Interleukin-8

In recent studies, IL-8 levels in serum of HCC patients are considerably higher than those in normal individuals (17.6 pg/mL versus 1.0 pg/mL, P = 0.046). Therefore, IL-8 may be useful for detecting HCC or predicting its prognosis (Bou-Nader, 2020).

2.2.3. Transforming growth factor β-1 (TGF β-1)

HCC patients have significantly higher serum levels of TGF-1 than healthy individuals and those with non-malignant hepatitis (P = 0.0001). Its sensitivity is higher than that of AFP (24%), but its specificity at 200 ng/mL is similar to that of AFP at over 95% (Bou-Nader, 2020).

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Chapter 3

Review of Literature:

Isorhamnetin

1. Introduction

Various parts of plants are being used for medicinal purposes since ancient times. Flavonoids are majorly used plant-derived compounds since they possess a vast variety of medicinal properties. Isorhamnetin is one of the 4000 flavonoids that have a cure for multiple disorders ranging from fever to cancer. It is present in the leaves of *Ginkgo biloba L.*, fruits of *Hippophe rhamnoids L.*, Kombucha tea, *Brickellia cavanillesii, Strychnos pseudoquina, Oenanthe javanica,* and many other plants. Isorhamnetin is isolated chromatographically from medicinal plant parts. Moreover, pears and tomatoes are a rich dietary and direct source of it (Lassus et al. 1989).

Fig. Ginkgo biloba

Fig. Hippophae rhamnoides

(Image source: Lassus, A., et al., *Ciprofloxacin versus amoxycillin and probenecid in the treatment of uncomplicated gonorrhoea.* Scand J Infect Dis Suppl, 1989. **60**: p. 58-61.**)**

2. Chemical structure

The chemical structure of isorhamnetin contains four hydroxy groups and therefore, it comes in the class of flavanols of flavonoids. It has an IUPAC name 3,4',5,7-Tetrahydroxy-3'-methoxyflavone. It is synthesized by the 3' methylation of quercetin by the enzyme quercetin 3-O-methyltransferase. The hydroxyl groups present in isorhamnetin assist in its isolation by chromatography followed by mass spectrometry (Lassus et al. 1989).

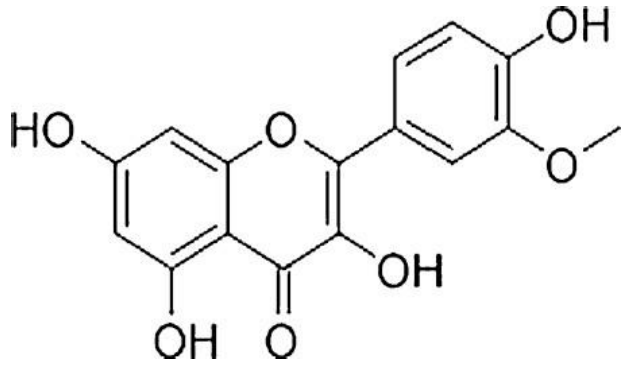

Fig. Chemical structure of isorhamnetin

(Image source: Lassus, A., et al., *Ciprofloxacin versus amoxycillin and probenecid in the treatment of uncomplicated gonorrhoea.* Scand J Infect Dis Suppl, 1989. **60**: p. 58-61.**)**

3. Properties

In HepG2 liver cancer cells, isorhamnetin exhibits hepatoprotective effects against AA+ iron-induced hepatotoxicity. It reduces the AA+ iron-induced reactive oxygen species production and apoptosis in hepatocytes (Dong et al. 2014).

4. Usage in liver diseases

It is used in various diseases including cardiovascular diseases, diabetes, neurodegenerative disorders and several liver disorders like non-alcoholic steatohepatitis, hepatic fibrosis. In-vitro studies also reported the protective effects of isorhamnetin in cardiomyocytes. It protects the cells from doxorubicin-induced cytotoxicity by preventing the LDH release and scavenging ROS produced by doxorubicin treatment. It also protects cardiomyocytes from doxorubicin-induced DNA fragmentation. Isorhamnetin shows *in-vitro* and *in-vivo* anti-diabetic properties at different doses. Isorhamnetin cures diabetes by regulating the PPARα, PPARγ-mediated signalling. It acts as a PPAR agonist to reduce blood sugar levels. It inhibits adipocyte differentiation in 3T3-L1 cells by reducing TG storage and GAPDH activity (Lee et al. 2009). Isorhamnetin regulates the transcriptional program of adipocytes by regulating the levels of PPARγ and C/EBPα. Isorhamnetin increases the transport of GLUT4 transporters to the plasma membrane to enhance the glucose uptake by the cells. Several neuroprotective properties of isorhamnetin have been reported to date. One of the mechanisms is the reduction of endoplasmic reticulum-induced neurotoxicity. Endoplasmic reticulum stress (ERS) induces apoptosis in neuronal cells that results in the development of

Alzheimer's disease. Isorhamnetin suppresses the activation of ASK and p38 kinase to eliminate the ERS-induced neuronal cell apoptosis. It also reduces ROS levels to mitigate the apoptotic effects of ERS in N2a cells (Qiu et al. 2017).

4.1. Liver Diseases

4.1.1 Non-Alcoholic Steatohepatitis

Non-alcoholic steatohepatitis (NASH) arises from the accumulation of toxic fatty acids inside the liver cells. It is also called as fatty liver disease and can be stated as non-alcoholic fatty liver disease (NAFLD) in its initial stages. Isorhamnetin administration shows a significant reduction in lipogenesis in the liver cells. Isorhamnetin reduces liver weight with a reduction of 15 % of oil red staining in comparison to NASH-induced mice. Additionally, its administration reduces the increased serum levels of AST, and ALT up to control mice groups. Its treatment reverses the gene expression profile in NASH mice models.

4.1.2 Hepatic fibrosis

Excessive formation of connective tissue in the liver results in fibrosis. NASH is one of the major causes of hepatic fibrosis which elevates the cytokine-induced hepatic stellate cell-mediated collagen production. In addition to inhibiting HSCs, isorhamnetin reduces excessive extracellular matrix formation by downregulating TGF-b signalling-dependent fibrogenic gene expression (Yang et al. 2016). Isorhamnetin inhibits macrophage recruitment and collagen type I and II expressions to reduce liver fibrosis. It elevates MMP-2 by downregulating TIMP-2 to inhibit ECM production in liver fibrosis. Isorhamnetin inhibits liver fibrosis by modulating the levels of autophagy also. It decreases LC3 and beclin-1 to inhibit autophagy in liver fibrosis mice models. Isorhamnetin also reduces p-Smad3 and p-p38, which are downstream proteins in TGF-β signalling to inhibit the fibrogenic effects of TGF (Liu et al. 2019)-b. In-vitro studies show that it also inhibits other TGF-β regulated proteins such as PAI-1 and a-SMA. There, isorhamnetin inhibits TGF-β dependent phosphorylation of Smad protein and subsequent transcription of its target genes. In LX-2 cells, isorhamnetin increases the nuclear localization of Nrf2 for up to 3 hrs thereby increasing the expression of Nrf2-dependent genes involved in oxidative stress response. Similar isorhamnetin activities were reported in *in-vivo* studies as well.

4.2 Inflammatory disorders

Isorhamnetin is used to cure many medical disorders due to its anti-inflammatory properties. Isorhamnetin reduces the serum levels of pro-inflammatory cytokines such as TNF-α, IL- β, and IL-6 in LPS-treated immune cells. It also minimizes the increased weight of the lung due to LPS-induced water accumulation, thereby reducing the wet/dry weight ratio of the lung in the mice model. Additionally, isorhamnetin reduces the activation of NF-kB to minimize the adverse effects of LPS-mediated cytokine production in mice lungs (Li et al. 2016). According to another report, isorhamnetin reduces the levels of IL-6, and IL-8 in LPS-induced human gingival fibroblasts. Isorhamnetin reduces the NF-kB activation in these cells also to exert its anti-inflammatory properties. The anti-inflammatory mechanism involves the activation of Nrf-2 and its target genes by isorhamnetin (Qi et al. 2018).

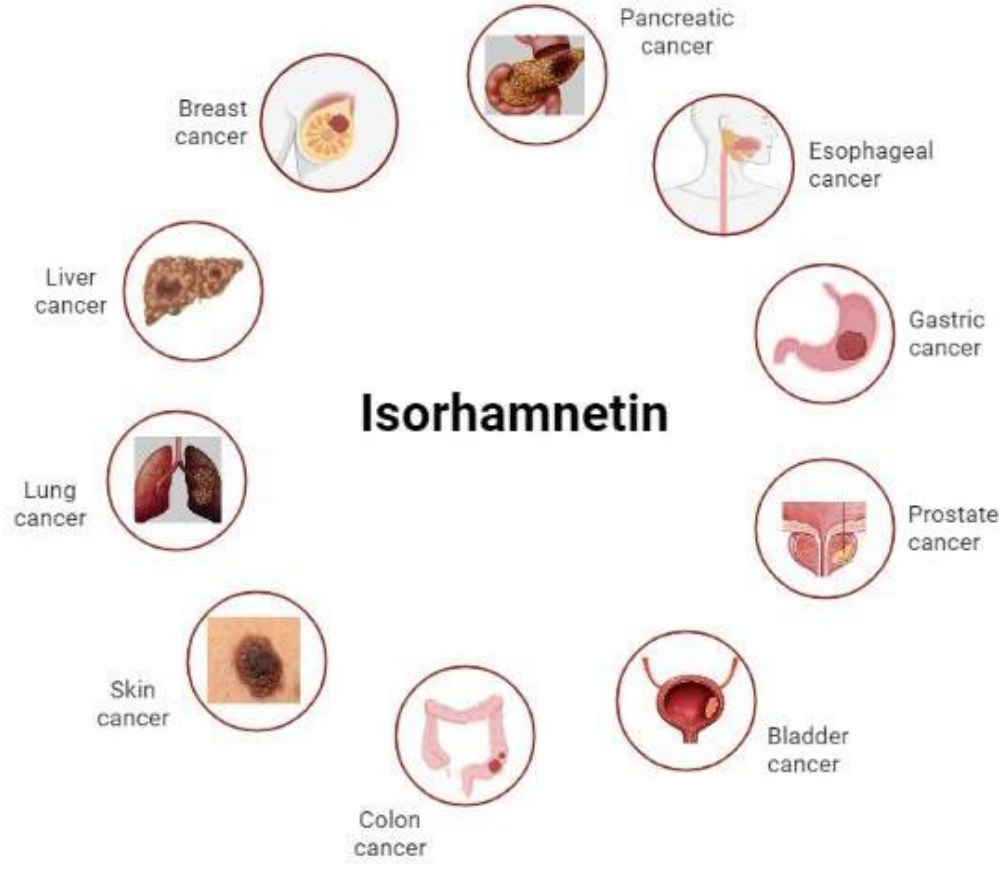

Figure: Depicting types of cancers affected by isorhamnetin

(Image source: Created with BioRender.com)

4.3 Cancers

Anti-cancer effects of isorhamnetin have been studied in various cancers including lung skin breast prostate as well as several other types of cancer related to alimentary canal. Roles of isorhamnetin in gut-cancer have been discussed in the following portion. Some studies are described below pictorially for the anti-cancerous properties of isorhamnetin.

4.3.1 Oesophageal cancer

Oesophageal cancer has a very high malignancy rate and stands at sixth position in mortality rate. While new treatment regimens to treat oesophageal cancer are under investigation, isorhamnetin administration showed promising results in *in-vivo* and *in-vitro* studies. Isorhamnetin kills the oesophageal cancer cells, Eca-109, and inhibits their growth. It upregulates the protein levels of apoptotic proteins such as Bax and Mcl-1 to induce cell death. Isorhamnetin also activates NF-κB and induces the expression of COX-2 to execute its anti-tumorigenic effects against oesophageal cancer (Shi et al. 2012).

4.3.2 Gastric cancer

Isorhamnetin administration also suppresses gastric cancer which is a very lethal and 5^{th} most common cancer worldwide. Chemoresistance of the gastric cancer patients makes this disease even more complex and increases the recurrence rate as well. Hence, the search for candidate drugs is still an ongoing process to overcome the chemoresistance as well as to increase the prognosis of this disease. It reduces the levels of cyclin D1 to inhibit cell proliferation and suppresses VEGFA and CXCR4 to inhibit angiogenesis and metastasis in gastric cancer cells. Isorhamnetin induces cell apoptosis by upregulating the pro-apoptotic proteins like cleaved PARP and Bak. Isorhamnetin uses its PPARγ agonist properties to perform its anti-tumoural activity in gastric cancer cells. PPARγ levels get upregulated upon isorhamnetin administration in mice tumour tissues (Ramachandran et al. 2012).

Isorhamnetin synergizes the anti-tumour effects of capecitabine in human gastric cancer cells which are chemoresistant. Isorhamnetin also downregulates NF-kB which gets abruptly upregulated by capecitabine treatment. These additive effects of isorhamnetin with capecitabine are also observed in the xenograft gastric cancer mouse model. A significant change can be observed in the levels of biomarkers of proliferation and angiogenesis in

mouse models administered with a combination of isorhamnetin and capecitabine (Manu et al. 2015).

4.3.3 Colon cancer

Colon cancer is one of the most prevalent cancers in men and accounts for almost 10% of all tumours. It majorly arises from bad lifestyle habits and therefore can be avoided by altering the food habits and lifestyle. Foods containing flavonoids are reported to reduce the risks of colon cancer. In-vitro study suggests that isorhamnetin has the ability to reduce the proliferation of HCT-116, a colon cancer cell line in a dose and time-dependent manner. Use of 100 uM isorhamnetin for 48 hrs induces cell death in colon cancer cells. This cell death is due to the arrest of the cell cycle in the G0/G1 phase and G2/M phase as well (Li et al. 2014).

Isorhamnetin also shows chemoprotective effects in colorectal cancer mice models and cell lines. In the colorectal cancer-induced mice model, isorhamnetin inhibits c-Src activation by upregulating the expression of its negative regulator, CSK, to inhibit tumour development. It also suppresses the activation of Akt, and ERK to control Src expression. By activating Src, isorhamnetin blocks the translocation of b-catenin into the nucleus. These effects were also observed in colon cancer cell line, HT29 (Saud et al. 2013).

4.3.4 Pancreatic cancer

Pancreatic cancer is a very lethal disease with a survival rate of 5% only. Chemoprevention is a reliable method for pancreatic cancer reversal; however, the finding of the more specific chemo-based compounds is still an ongoing process. Isorhamnetin shows promising inhibitory properties in pancreatic cancer cells. It suppresses the proliferation of PANC-1 cells by inducing arrest in the G2/M phase and S-phase of the cell cycle (Wang et al. 2018). Cell cycle arrest is induced by the downregulation of Cyclin A and upregulation of Cdk2 at the mRNA level. Additionally, apoptosis is induced by isorhamnetin in the same pancreatic cancer cells. Isorhamnetin blocks the activation of MEK protein and Stat3 to reduce the migration and invasiveness of pancreatic cancer cells (Wang et al. 2018).

4.3.5 Liver cancer

Liver cancer arises mainly with the occurrence of hepatocellular carcinoma which accounts for more than 90% of cases of liver cancer. It is one of the leading causes of cell death worldwide.

The use of many flavonoids including isorhamnetin has been reported in *in vitro* studies as having anti-tumorigenic properties. Work on the anti-cancerous properties of isorhamnetin in an *in-vivo* system is still lacking. Various studies have been done in liver cancer cell lines, HepG2 cells, to investigate the possibility of anti-cancer effects of isorhamnetin in liver cancer. It activates Nrf2 signalling in a dose-dependent manner and induces Nrf2 translocation into the nucleus. Nrf2 accumulation in the nucleus increases the expression of the anti-oxidant enzyme, HO-1, and GCL, which is involved in glutathione biosynthesis. Isorhamnetin-mediated Nrf2-induction involves Erk1/2 activation that reduces t-BHP toxicity-induced oxidative stress and apoptosis in liver cancer cells (Yang et al. 2014). Isorhamnetin isolated from *Hippophae rhamnoids L*. shows anti-tumour activity in the BEL-7402 liver cancer cell line. It induces dose and time-dependent cytotoxicity in BEL-7402 cells. Isorhamnetin disrupts chromatin architecture that results in cancer cell death (Teng et al. 2006).

Anti-tumour properties of isorhamnetin isolated from *Cedrus deodara* were demonstrated in liver cancer HepG2 cells. At a dose of 1.22 mg/g, isorhamnetin shows cytotoxic activity with an IC50 value of 114.12 ug/ml. It kills liver cancer cells by arresting them in the G0/G1 stage of the cell cycle (Shi et al. 2016).

To evaluate the anti-tumour properties of isorhamnetin in against liver cancer cells, mass spectrometry was performed. This study sheds light upon all the molecular targets of isorhamnetin. Among all isorhamnetin-targeted upregulated and downregulated proteins, many were related to cell cycle regulators (Cdk2), cell proliferation, replication (PCNA), redox-homeostasis (PRDX1, Glutaredoxin-3, PRDX5), cytoskeletal proteins (tubulin, cofilin-1), ribosomal proteins (RPS17, RPS10, NPM). Additionally, proteins involved in autophagy (Hsp90), transcription (RBMX, hnRNPK, hnRNPH1, hnRNPA1, hnRNPD, SNRNPD2), chromatin organization (H1b, NAP1L1) are also regulated by isorhamnetin. Some mitochondrial proteins, mRNA transport proteins are also regulated by isorhamnetin hepatocytes (2018).

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Chapter 4

Materials and Methods

1. Chemicals

DEN (#N0756), CCl_4 (#289116), and Isorhamnetin (#17794) were purchased from Sigma Aldrich India. The bicinchoninic acid (BCA) assay kit was procured from Thermo Fisher Scientific, USA. ELISA kits were purchased from Abcam (Cambridge, UK), and antibodies, required for detection of specific proteins, were purchased from Abcam (Cambridge, UK) Cell Signaling Technology, USA. Other required chemicals were bought from Sisco Research Laboratory (SRL), India.

2. Animal

Nearly 4 wks old male Swiss albino mice were procured from Animal House Facility of Bose Institute, Kolkata. All mice were allowed to nurture with proper food pellets and drinking water along with other factors including stable temperature of 25 °C temperature, alternative cycle of 12 h light/ 12 h dark and 45 % - 55 % humidity. After being acclimatized for two wks according to aforementioned laboratory factors, mice were subjected to chemical treatment for development of HCC. All animal experiments were accomplished following the rules and regulation of Institutional Animal ethics committee (IAEC) and CPCSEA, the Ministry of Environment and Forest (1796/GO/EReBiBt/S/14/CPCSEA).

3. Development of *in vivo* HCC model

Following the protocol, described elsewhere (Sur et al. 2016) doses of DEN were selected for induction of HCC in male Swiss albino mice. in short mice were initially injected intraperitoneally with 15 µl/kg body weight of CCl_4 on every day for a continuous stretch of 4 days. After completion of CCl_4 injection DEN at a concentration of 75 mg/kg body weight was injected intraperitoneally once in a week for 3 successive wks. After the end of 3 wks mice were again treated with DEN at a higher dose of 100 mg/kg body wight once in a week for three more successive wks. Meanwhile, it is important to say that prior and after DEN injection not given access to drinking water. After completion of entire HCC induction procedure (after eleven wks) HCC bearing mice were sacrifices though cervical dislocation.

4. Dose determination and administration of isorhamnetin

As there is no definite information regarding dosage of isorhamnetin in Swiss albino mice for curing HCC, we treated HCC bearing Swiss albino mice with several different doses of isorhamnetin corresponding to the concentrations mentioned elsewhere for *in vitro* (Sur et al. 2016) and *in vivo* evaluation (Park et al. 2016, Ramachandran et al. 2012). In brief, three

different doses of isorhamnetin, i.e., 25 mg/kg,50 mg/kg, and 100 mg/kg body weight were prepared in phosphate buffered saline (pH 7.4) (Ye et al. 2008). Post-carcinogen administration, different doses of isorhamnetin were injected into intraperitoneal cavities of 30 mice (12 wks old) bunched randomly in 5 groups (Yang et al. 2014). Mice werxxxxe treated with different dose of isorhamnetin once in a week till the end of 11th week (Fei and Wei et al. 2018, Teng et al, 2006).

Group 1 (control): Wild type receiving no treatment

Group 2 (negative control group): Mice receiving isorhamnetin intraperitoneally from 7th to 17th week.

Group 3 (carcinogen only group): Mice receiving intraperitoneal injection of CCl_4+DEN.

Group 4 (positive control): HCC bearing mice receiving doxorubicin injection (1 mg/kg body weight) intraperitoneally from 7th to 17th week.

Group 5 (isorhamnetin treatment group): HCC bearing mice receiving dose dependent isorhamnetin treatment intraperitoneally from 7th to 17th week.

5. Tissue and blood collection

After scarifying mice on 18th week bold was collected by arterial puncture and was processed from serum isolation (Manna, Sinha and Sil 2007). briefly, serum was isolated by collecting blood were kept K_2-EDTA cuvette for 30 min at 37 ℃ followed by a 30 min centrifugation at 3000 x g. collected serum in the supernatant was then stored at -80 ℃ temperature for further biochemical studies. The hepatic tissues were aseptically resected and weighed. Resected tissues were either stored at – 80 ℃ for transcriptional as well as translational studies or fixed in formalin for histopathological as well as immunohistochemical evaluation.

6. Evaluation of hepatic histopathological morphology

Formalin fixed liver tissues were processed for paraffin blocking followed by microtome sectioning into 5 µm thick section. Slides containing liver tissue sections were dehydrated and rehydrated in graded ethanol for the purpose of H & E staining. Later on, stained sections were viewed under bright field microscope at 40 X magnification (Chowdhury et al. 2016).

7. Assessment of liver function by biochemical assays

Isolated serum was used for evaluation for liver function to assessing level of serum alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST). Also, mice bilirubin content was examined parallelly (Chowdhury et al. 2016).

7.1. Determination of ALP, ALT & AST

These biochemical processes were performed spectrophotometrically using standard estimation kits (Span Diagnostic Ltd, India). Serum ALP level was measured kinetically and calorimetrically at 405 nm wavelength following manufactures# standards. Also, serum ALT level was evaluated in order to check extent of hepatic necrosis associated with HCC. It was measured spectrophotometrically using the kit at 340 nm wavelength. Another enzyme associated with liver necrosis; AST was also calculated colorimetrically at 340 nm.

7.2. Evaluation of serum bilirubin content

Measurement of azobilirubin concentration is directly proportional to the bilirubin. It was measured by a spectrophotometer by red-purple colour compound wavelength.

8. Tissue homogenization and total protein isolation

Dissected hepatic tissue samples from wild type and HCC bearing mice were washed in 1 X PBS (pH 7.4) prior any processing. Sections of hepatic tissue samples each weighing about 200 mg were then homogenized using ice-cold RIPA lysis buffer of pH 8.0 containing 0.15 M NaCl, 0.05 M Tris, 0.5 % Sodium deoxycholate, 0.1 % SDS, and Triton X supplemented with protease and phosphatase inhibitor cocktail (Thermo Fischer Scientific, US). Next, the tissue homogenates were cleared up to collect protein containing supernatants by centrifugation at 12000 rpm for 20 min at 4 °C temperature. Collected supernatants were evaluated for protein concentration spectrophotometrically at 562 nm by using BCA assay kit (Ghosh et al. 2022). Thereafter, protein rich supernatants were aliquoted and reserved at -80 °C for future use.

9. Immunoblotting

Protein samples isolated from tissue homogenates were analyzed for changes in translational expression through western blotting (Ghosh et al. 2022). Equal amounts (40 µg) of protein s of interest from each experimental group were separated across 10 – 12 % SDS PAGE electrophoretically resolved proteins were then transferred onto a PVDF membranes

according to previously described method. Later on, nonspecific protein interactions were avoided by using 5 % BSA blocking solution for 45 min. Later on, primary antibodies against TNF-α, phosphor-JNK, phosphor-p38, phosphor-c-Jun, PPAR-γ, phospho-ERK, C/EBP-δ, TGF-β, Nrf2, Keap1, SOD-2, HO-1, phosphor-GSK3β, phospho-mTOR, phosphoMcl-1, phosphor-Akt (T308) & (S473), p27, phosphor-Bad were used in 1:1000 dilution for incubating protein loaded membranes at 4 °C temperature. Next day, HRP-flagged secondary antibody in 1:10000 dilution was used for 2 hrs for cross reaction finally translational expression of proteins were visualized using enhanced chemiluminescence. β- actin was used as universal protein loading control.

10. Immunohistochemistry

Paraffinized liver tissue sections were affixed upon poly-L lysin coated glass slides and were deparaffinized by dipping into xylene for immunohistochemical processing. Later on, tissues were rehydrated, internal peroxidases were quenched, antigens were demasked, and nonspecific proteins were blocked (Ghosh et al. 2023). Thereafter liver tissue sections were checked for immunological expressions of p21 and MMP-9 using anti-p21 and anti-MMP-9 primary antibodies in 1:300 dilution along with Alexa-fluor 488 bound secondary antibody cross reaction. Afterwards, slides were mounted using Vectashield containing in built DAPI and visualized employing fluorescence microscope.

11. RNA isolation and quantitative real time PCR (RT PCR)

Extraction of total RNA from dissected liver tissues were made by homogenizing tissue sections employing TRIzol reagents (Banerjee et al. 2018). Extracted RNA concentrations were calculated spectrophotometrically with the help of nanodrop. Following the concentrations, optimal amount of RNA was converted into cDNA using Verso cDNA synthesis kit. Next, synthesized cDNAs were amplified by real time PCR (ABI Prism 7500, USA) using cognate primers, and SYBR Green Master Mix. Experiments was repeated in triplicate and gene expression was normalized against expression of GAPDH.

12. Statistical analysis

Results have been represented as mean ± standard error/ standard deviation. All experiments were run in triplicate and statical analysis were made through one-way analysis of variance (ANOVA). Post-ANOVA Tukey test was employed to find out any statistically significant

difference of variance. P-value < 0.05 has been considered to be statistically significant throughout the study. All statistical analysis were made using GraphPad Prism (version 6).

Table: The sequence of primers used for the qRT-PCR analysis of respective genes.

Gene name	Forward/Reverse	Primer sequence
GAPDH	Forward	5′-ACCACAGTCCATGCCATCAC-3′
	Reverse	5′-TCCACCACC CTGTTGCTGTA-3′
c-Jun	Forward	5′-AAAACCTTGAAAGCGCAAAA-3′
	Reverse	5′-CGCAACCAGTCAAGTTCTCA-3′
Nrf2	Forward	5′-ACACGGTCCACAGCTCATC-3′
	Reverse	5′-TGCCTCCAAGTATGTCAATA-3′
TNF-α	Forward	5′-AGAAACACAAGATGCTGGGACAGT-3′
	Reverse	5′-CCTTTGCAGAACTCAGGAATGG-3′
Arg1	Forward	5′-CATTGGCTTGCGAGACGTAGAC-3′
	Reverse	5′-GCTGAAGGTCTCTTCCATCACC-3′
PCNA	Forward	5′-CAAGTGGAGAGCTTGGCAATGG-3′
	Reverse	5′-GCAAACGTTAGGTGAACAGGCTC-3′
PPAR-γ	Forward	5′-GTACTGTCGGTTTCAGAAGTGCC-3′
	Reverse	5′-ATCTCCGCCAACAGCTTCTCCT-3′

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Chapter 5

Investigating the anti-tumour effects of isorhamnetin against DEN+CCl_4 mediated HCC in Swiss albino mice

1. Introduction

HCC, also known as primary liver cancer, is the most common form of liver cancer and one of the leading causes of cancer-related deaths worldwide. It originates in the main type of liver cells called hepatocytes. HCC usually develops in the setting of chronic liver diseases, such as cirrhosis, hepatitis B or C infection, or non-alcoholic fatty liver disease. HCC is a complex and aggressive disease that often presents challenges in diagnosis, treatment, and management. It typically exhibits a high potential for invasiveness, metastasis, and recurrence, making early detection and intervention crucial for improving patient outcomes (Sung et al. 2021, Chavda 2021).

The risk factors for developing hepatocellular carcinoma include chronic viral hepatitis, alcohol abuse, exposure to certain chemicals and toxins, metabolic disorders, obesity, and certain genetic conditions (Llovet et al. 2018). In regions where hepatitis B is endemic, such as parts of Asia and Africa, chronic hepatitis B infection is a major risk factor for HCC (Villanueva 2019). In other parts of the world, hepatitis C infection and excessive alcohol consumption are more commonly associated with HCC (Villanueva 2019). Symptoms of HCC may vary depending on the stage and extent of the disease, but they can include abdominal pain or discomfort, weight loss, loss of appetite, fatigue, jaundice (yellowing of the skin and eyes), and swelling in the abdomen. However, in the early stages, HCC may not cause noticeable symptoms, which further emphasizes the importance of regular screening for high-risk individuals (Villanueva 2019). Diagnosis of HCC involves a combination of imaging tests, such as ultrasound, computed tomography (CT) scan, or magnetic resonance imaging (MRI), along with blood tests to assess liver function and detect tumour markers. In some cases, a liver biopsy may be necessary to confirm the diagnosis (Llovet et al. 2018, (Villanueva 2019). Treatment options for HCC depend on several factors, including the stage of cancer, patients' overall health, and the availability of liver transplantation. Treatment modalities can include surgical resection, liver transplantation, locoregional therapies (such as radiofrequency ablation or transarterial chemoembolization), systemic therapies (such as targeted therapies or immunotherapy), and supportive care to manage symptoms and improve quality of life (Llovet et al. 2018). Prevention of HCC involves reducing the risk factors associated with chronic liver diseases. This includes practicing safe behaviour to prevent hepatitis B and C infections, receiving appropriate vaccinations, maintaining a healthy

weight, limiting alcohol consumption, and managing underlying liver diseases (Llovet et al. 2018, (Villanueva 2019).

DEN, a potent carcinogen, is usually administered to mice through a single intraperitoneal injection during the neonatal or early postnatal period. DEN induces genetic mutations and DNA damage in hepatocytes, initiating the carcinogenic process (Uehara, Pogribny, and Rusyn 2014, Tolba et al. 2015)

CCl_4, a hepatotoxin, is commonly used to promote liver injury and fibrosis in this model (Uehara, Pogribny, and Rusyn 2014). It is typically administered to mice through repeated injections or by gavage over an extended period. CCl_4 causes hepatocyte damage, inflammation, oxidative stress, and the activation of hepatic stellate cells, leading to liver fibrosis and subsequent progression to hepatocellular carcinoma. The chronic liver injury caused by CCl_4 administration leads to the activation of hepatic stellate cells and the deposition of extracellular matrix proteins, resulting in liver fibrosis. Liver fibrosis creates a pro-tumorigenic microenvironment that promotes the growth and progression of hepatocellular carcinoma (Uehara, Pogribny, and Rusyn 2014).

Isorhamnetin is a naturally occurring flavonoid compound that belongs to the flavanol subgroup (Bhattacharya et al. 2020). It is found in various plant sources, including fruits, vegetables, herbs, and grains. Isorhamnetin exhibits a range of biological activities and has gained attention for its potential health benefits. Structurally, isorhamnetin is closely related to another well-known flavanol called quercetin. It differs from quercetin by having a methyl group attached to its B-ring. This subtle difference in structure can influence its bioavailability, metabolism, and specific biological effects (Bhattacharya et al. 2020). Isorhamnetin is known for its antioxidant properties, which contribute to its potential health benefits. As an antioxidant, it helps protect cells and tissues from oxidative damage caused by free radicals, unstable molecules that can lead to cellular dysfunction and contribute to the development of various diseases, including cardiovascular disease, cancer, and neurodegenerative disorders. Additionally, isorhamnetin has demonstrated anti-cancer properties in preclinical studies (Gong et al. 2020). It has been found to inhibit the growth of cancer cells and induce apoptosis (programmed cell death) in various types of cancer, including breast, colon, prostate, and liver cancer (Li et al. 2022, Zhang et al. 2015, Hu et al. 2015, Antunes-Ricardo et al. 2014). These anticancer effects are attributed to its ability to modulate various signalling pathways involved in cell growth, differentiation, and survival.

Studies have suggested that isorhamnetin may exert anticancer effects against liver cancer cells. It has demonstrated the ability to inhibit cell proliferation, induce cell cycle arrest, and promote apoptosis (programmed cell death) in HCC cells in laboratory experiments (Hu et al. 2015, Teng et al. 2006). These actions are attributed to its ability to modulate various signalling pathways involved in cell growth, survival, and invasion. Furthermore, isorhamnetin has been shown to have antioxidant and anti-inflammatory properties, which can help reduce oxidative stress and inflammation in the liver (Yang et al. 2014). Chronic inflammation plays a significant role in the development and progression of liver cancer, and targeting this inflammatory process can be beneficial in managing the disease. Additionally, isorhamnetin may enhance the effectiveness of other chemotherapeutic agents used in liver cancer treatment (Zhang et al. 2015). Studies have demonstrated its ability to sensitize liver cancer cells to the cytotoxic effects of certain anticancer drugs, thereby potentially improving the efficacy of combination therapies (Zhang et al. 2015, Hu et al. 2015, Teng et al. 2006).

While the preclinical evidence is promising, it is important to note that the use of isorhamnetin as a treatment for liver cancer is still in the early stages of research. Further studies are needed to determine its optimal dosage, safety profile, and efficacy in human clinical trials. Additionally, understanding its mechanism of action and potential interactions with other treatment modalities will be crucial for its successful integration into liver cancer treatment strategies.

2. Material and Methods

Refer to the detailed description in Chapter 4.

3. Results

3.1. Effect of isorhamnetin and DEN on body weight, liver-function

To investigate the protective effects of isorhamnetin on DEN+CCl_4-induced HCC, four-week-old male Swiss albino mice were subjected to DEN+CCl_4 treatment for seven weeks (Figure 1). Since the dose of DEN and CCl_4 to induce HCC is well established (Sur et al. 2016), we used the same dose to induce tumours in mice livers.

Mice developed tumour nodules 126 days as a result of carcinogen exposure and HCC induction was measured by one of the authentic serum protein markers that is AFP (Figure 2).

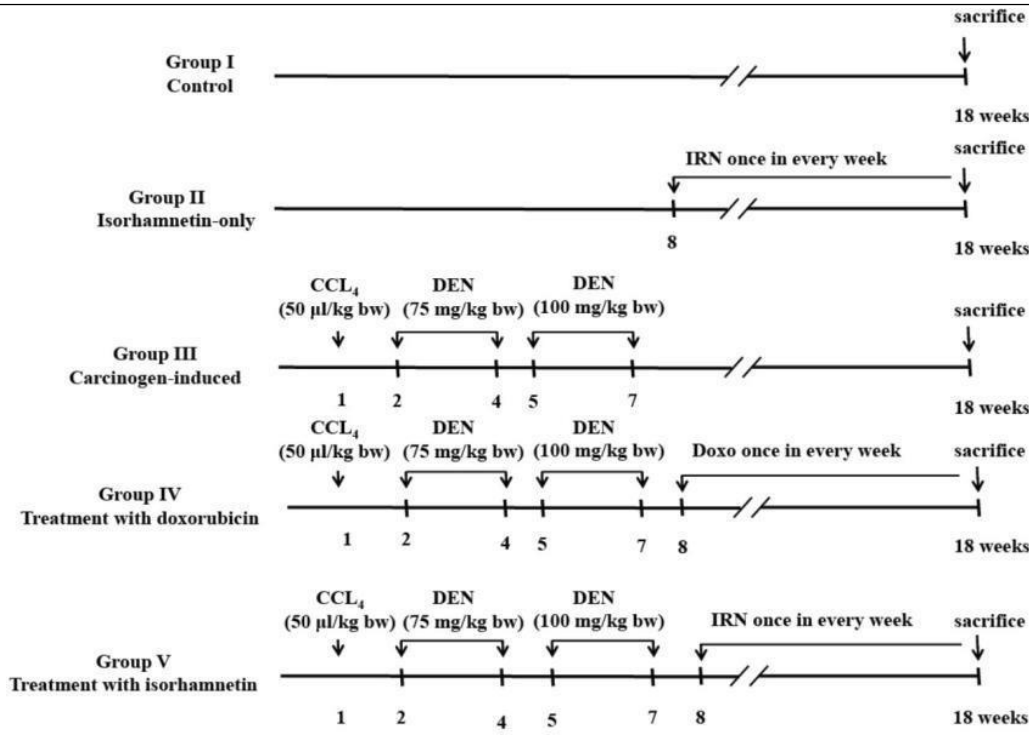

Figure 1 ***In vivo*** **experimental protocol** A scheme describing the experiments for HCC induction and cure was performed in animals. Swiss albino mice were treated with CCl_4 in the first week followed by DEN treatment in different doses for the next six weeks (Group III). One group was further administered isorhamnetin (IRN) till the end of the experiment (Group V). Isorhamnetin was also administered without carcinogen treatment (Group II). Doxorubicin (Doxo) was taken as a positive control in a parallel group (Group IV)

Although the dose of the toxin was predetermined to induce a tumour, there are no reports of a definite dose of isorhamnetin in curing HCC in mice. Therefore, to investigate the *in-vivo* anti-tumour dose of isorhamnetin, we treated the mice groups with various isorhamnetin doses considering those reported in cellular studies in different liver cancer cell lines (Dong et al. 2014, Teng et al. 2006, Runhuan and Haiyan 2018) and in treating other cancers *in-vivo* (Wu et al. 2018, Zhang et al. 2015, Park et al. 2019, Cai et al. 2020, Ramachandran et al. 2012, Sun et al. 2013, Wang et al. 2018). To get the optimum concentration of isorhamnetin at which it shows maximum protection of liver physiology and ideal enzymatic activity we went for a dose-dependent trial and found out that a dose of 100 mg/kg of isorhamnetin can demonstrate a considerable mode of protection of the liver from carcinogen exposure, hence, this particular dose was chosen for following experimental works (Figure 3).

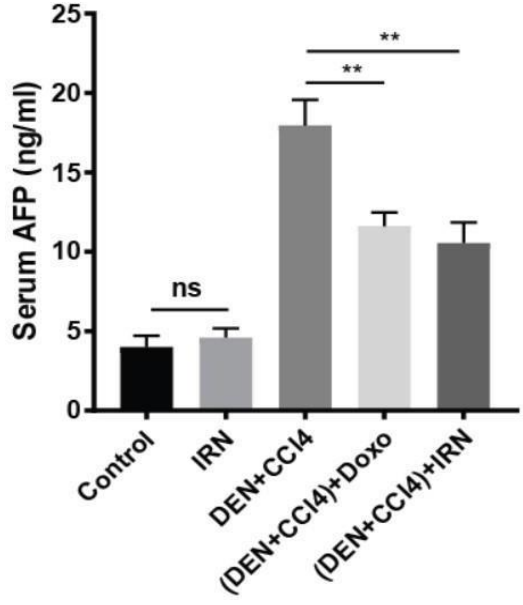

Figure 2. Serum ALP levels of experimental mice groups.

The exposure to DEN+CCl_4 drastically altered the physiological level of liver enzymes like ALP, ALT, AST, and bilirubin, which was normalized after isorhamnetin administration. To check whether isorhamnetin can alter the physiological level of liver enzymes a group of mice were treated with only isorhamnetin, and the results showed isorhamnetin helped to maintain normal liver function by keeping those enzyme levels at normal condition.

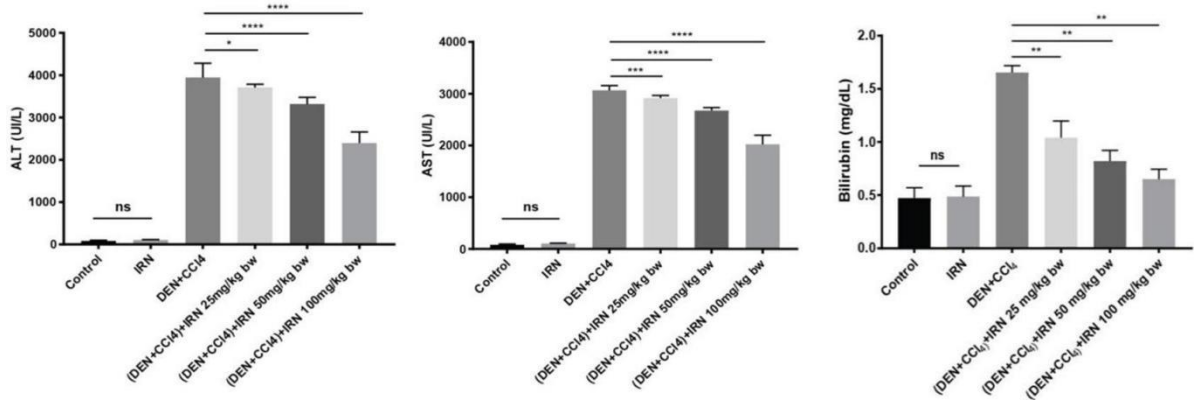

Figure 3. Serum ALT, AST, and bilirubin levels of experimental mice groups.

We harvested male Swiss albino mice of body weight approximately 25 to 30 gm and proceed with the experiment, we measured that the body weight of each group of mice thoroughly. We observed the DEN+CCl_4 administration reduced body weight in a drastic manner which finally goes down to half of the body weight measured initially on the other hand neither control nor isorhamnetin-only groups have any issues of body weight alterations.

DEN+CCl_4 exposure intended to disrupt the liver architecture and to heal that damage, liver cells started proliferation which caused the weight gain of that particular organ compared to the control mice group. Hence when we measured the liver/body weight ratios of all mice groups DEN+CCl_4-treated group showed a higher value, due to an increase in liver weight and a decrease in body weight. On the other hand, isorhamnetin brought back the liver/body weight ratios near normal by easing the tumour burden (Table 1).

Table 1. Body weights, liver weights (mean ± SD) (n=6), and their ratios in Swiss albino mice

Group no.	Treatment	Weight (gm)		Weight ratio
		Liver	Body	Liver/Body
I	Control	1.55 ± 0.15	25.4 ± 2.0	0.061
II	IRN	1.55 ± 0.12	25.0 ± 2.4	0.062
III	DEN+CCl_4	2.05 ± 0.90	12.6 ± 1.6	0.162
IV	(DEN+CCl4) + Doxo	1.55 ± 0.30	24.6 ± 1.7	0.063
V	(DEN+CCl4) + IRN	1.46 ± 0.23	24.8 ± 1.8	0.058

High expression of AFP is associated with hepatocyte proliferation and HCC progression (Ambade et al. 2016, Attallah et al. 2015, Sur et al. 2016). Therefore, we investigated the expression of AFP which is a characteristic marker in HCC diagnosis (Ambade et al. 2016). DEN+CCl_4 treatment increased serum AFP indicating HCC development while subsequent treatment of isorhamnetin lowered AFP expression to the levels of the control and doxorubicin-administered group (Figure 2). Additionally, isorhamnetin did not induce any changes in AFP levels when administrated alone (Figure 2).

The induction of cancer starts with the initiation of cellular proliferation which must require proper validation through assessing some well-known markers present in the blood serum. PCNA, a core member of the replication machinery and repair complex, also is a well-validated marker for checking proliferation. We assessed PCNA mRNA expression in real time and among all mice groups the DEN+CCl_4 mice group showed the maximum expression of that particular gene expression. Arginase-1 a protein whose expression is being restricted only in hepatocytes makes it a well-desired protein marker to check any physiological change

specific to hepatocytes. Our results showed that the alleviation of arginase-1 after DEN+CCl_4 treatment was mitigated by isorhamnetin administration in HCC mice.

Expression of PCNA and arginase-1 was not affected by isorhamnetin when mice were not treated with a toxin (Figure 4).

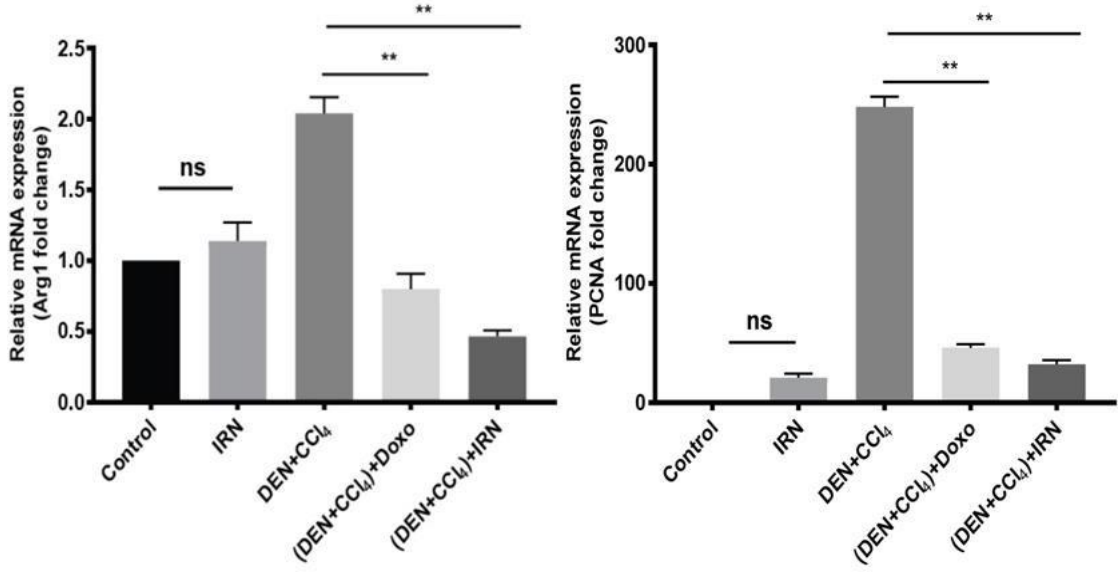

Figure 4. qPCR analysis of cell proliferation marker PCNA and HCC marker Arginase-1 in all experimental groups.

Overall, these results indicate that isorhamnetin shows hepatoprotective effects in the DEN+CCl_4-induced HCC mice model. Serum AFP, ALT, ALP, AST, bilirubin, and arginase-1 levels become near normal in isorhamnetin-administered HCC mice. Additionally, isorhamnetin administration regresses the tumour size, amount, and incidence after carcinogen treatment.

3.2. Isorhamnetin restores liver internal structure in carcinogen-exposed mice and reduces tumour burden

Tumour nodules were also counted in each group. Carcinogen-treated and isorhamnetin-administered groups harboured much fewer tumours similar to the doxorubicin group in comparison to the carcinogen-treated group (Figure 5). After 18 weeks of first carcinogen administration, all mice of this group developed visible tumours whereas no tumours were detected in control or isorhamnetin-only groups. However, tumours were rarely detected on the liver surface in DEN+IRN groups suggesting that isorhamnetin administration somehow

lowers the tumour incidence in mice (Figure 5). When the size of these tumours was measured, post-isorhamnetin treated mice showed strikingly smaller tumours than DEN+CCl_4 treated mice (Figure 5).

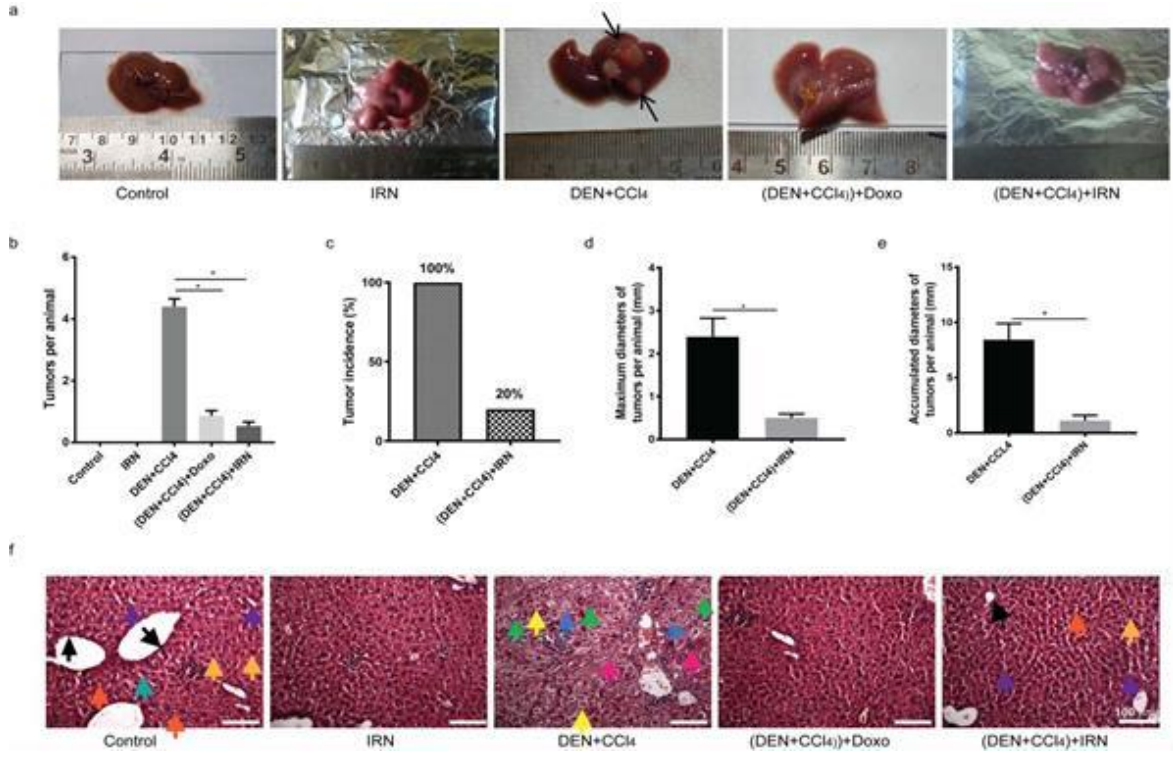

Figure 5. Isorhamnetin restores liver morphology in carcinogen-exposed mice (a) Representative macroscopic images, black arrow indicates tumour nodule, (b) Numbers of visible nodules on the liver surface of mice in each group (n=6), (c) Tumour incidence in group III and group V, (n=6), (d) The maximum diameters and (e) accumulated diameters of tumours per mouse are depicted (n=6). (f) Representative images of HE-stained liver sections of indicated mice groups; arrowheads; Black (endothelial cells), Purple (sinusoids), Mustard-yellow (regular nuclei), Teal (nucleoli), Orange (Kupffer cells), Yellow (enlarged hepatocytes), Green (multinucleation, nuclear atypia), Pink (hyperchromasia), Blue (ballooned hepatocytes); Magnification 40X; Scale bar, 100µm

In macroscopic aspects the hepatoprotective ability of isorhamnetin was apparent. Those isorhamnetin-given groups previously exposed to carcinogen harboured much fewer tumour nodules as same as the doxorubicin group in comparison to the DEN+CCl_4-treated group. To demonstrate isorhamnetin does not get involved in tumour formation only isorhamnetin was administrated to a group of mice who did not get any dose of DEN+CCl_4. After 18 weeks of the first isorhamnetin introduction, macroscopic studies showed similar results to the control

group which is no perceptible tumour establishment. On the other hand, isorhamnetin treatment after carcinogen administration indeed brings down the tumour incidence and size in mice liver.

Standard diagnosis helps to screen tumours caused by HCC from other tumours that may be seen in the liver or its periphery and to examine problematic tumours that require contextual assessment.

Analysis of H & E-stained liver tissue morphology revealed that isorhamnetin secured hepatic tissues from DEN+CCl_4-induced HCC. Hepatocellular carcinomas comprehend the multifaceted prospect of typical hepatocyte microscopic anatomy and architecture with heterogeneous microscopic appearances. A characteristic cytological arrangement that signifies HCC is the hepatic trabeculae architectural disruption which was found in DEN+CCl_4 treated groups. In contrast, control groups displayed neatly ordered trabeculae which were lined by the reticulin system, and spaced by sinusoids. In DEN+CCl_4 treated groups multiple inappropriately patterned cells aggregated to form neoplastic pseudo trabeculae, with disoriented to completely missing reticulin framework. A vascular network comprising endothelial cell lining forms a signature microscopic appearance around these neoplastic pseudo trabeculae which is a prognostic sign of HCC. In DEN+CCl_4 treated groups some trabeculae degenerated to form a pseudo glandular pattern, where cellular debris and Kupffer cells aggregated. These pseudo-glandular pattern act as a framework where proliferative neoplastic cells can propagate and get nourishment from the lining of endothelial cells surrounding the mass of cells. Neoplastic cells happened to be sat on one another maintaining compact or solid patterns marking sinusoidal networks inapparent in DEN+CCl_4 treated groups. In DEN+CCl4 treated groups within compact growth patterns some cells were found to have disoriented nuclear membranes and unevenly diffused heterochromatin with higher nuclear to cytoplasmic ratios where mitosis and necrosis were not in order. DEN+CCl_4 group mice after isorhamnetin treatment as well as doxorubicin treatment revived liver anatomical structure. An evidential decrement in the swelling of liver plates, the display of portal tracts, and regular cellular and nuclear configuration were ascertained.

Results from the liver histopathological assessment suggest that isorhamnetin improves the internal liver structure in carcinogen-exposed mice. These effects are certainly due to the anti-tumour properties of isorhamnetin.

4. Discussion

Chemotherapeutics are widely used and the most accepted treatment to cure almost all cancers. Anti-cancer potential of isorhamnetin, a plant-derived flavonoid, was examined in this study. In vivo study was performed in the DEN+CCl_4-induced Swiss albino mice HCC model. The aim of this study is to understand the molecular mechanism of DEN+CCl_4-induced liver carcinogenesis regression by isorhamnetin.

Isorhamnetin regresses HCC as evidenced by a decrease in serum AFP levels. AFP is a diagnostic marker of hepatocarcinogenesis (Sur et al. 2016). It has a functional role in tumour growth by regulating hepatic cell proliferation and its high expression is correlated with poor prognosis in HCC (Ambade et al. 2016). High serum AFP is a commonly used marker in HCC screening (Wang et al. 2018). We also observed a high level of AFP in the serum of DEN+CCl_4 treated mice which was decreased by subsequent administration of isorhamnetin. The near-normal level of AFP in the isorhamnetin-treated group indicates the chemoprotective potential of isorhamnetin against HCC.

Isorhamnetin maintains the normal function of liver enzymes by suppressing DEN+CCl4-induced liver damage. Liver function is impaired in carcinogen-induced liver damage that can be detected by increased serum levels of liver functional markers. In the current research, it has been shown that the serum levels of liver function marker enzymes such as ALT, ALP, and AST were reverted to normal after treatment with isorhamnetin in the DEN+CCl_4-induced HCC mice model. Reduction in DEN+CCl_4-mediated liver damage by isorhamnetin is seen in liver histology also.

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Chapter 6

Investigating the detailed mechanism of anti-HCC properties of isorhamnetin against DEN+CCl4 mediated HCC in Swiss albino mice

1. Introduction

In mice, the suppression of liver cancer involves the modulation of several signalling pathways that play critical roles in tumour development, progression, and metastasis. The PI3K/Akt/mTOR pathway plays a crucial role in regulating cell growth, survival, and metabolism (Whittaker, Marais, and Zhu 2010). Dysregulation of this pathway is commonly observed in liver cancer and contributes to tumour development and progression. Inhibiting this pathway can induce cell cycle arrest, inhibit cell proliferation, and promote apoptosis in liver cancer cells. The MAPK/ERK pathway is involved in cell proliferation, differentiation, and survival (Schmitz et al. 2008). Aberrant activation of this pathway is frequently observed in liver cancer and contributes to tumour growth and metastasis. Targeting this pathway through inhibitors or modulators can suppress tumour growth and invasion in liver cancer. The TGF-β/Smad pathway has dual roles in liver cancer, acting as both a tumour suppressor and tumour promoter depending on the stage of the disease (Philip et al. 2005, Roberts and Wakefield 2003). In the early stages, TGF-β signalling suppresses tumour growth by inhibiting cell proliferation and inducing apoptosis. However, in advanced stages, the TGF-β pathway promotes tumour progression and metastasis (Roberts and Wakefield 2003). Modulating this pathway to restore its tumour-suppressive functions or inhibit its tumour-promoting effects holds therapeutic potential in liver cancer. Isorhamnetin shows significant improvement in these signalling thereby protecting hepatic tissues from damages caused by DEN+CCl4.

The DEN+CCl4 induced HCC (hepatocellular carcinoma) mouse model is a widely used experimental model to study the development and progression of liver cancer in preclinical research (Uehara, Pogribny, and Rusyn 2014). The combination of DEN and CCl4 administration induces chronic liver injury, inflammation, and continuous cycles of liver regeneration. This chronic liver injury promotes the development of hepatocyte abnormalities and sets the stage for the subsequent development of liver tumours. This model mimics key aspects of human hepatocellular carcinoma, including chronic liver injury, inflammation, fibrosis, and the subsequent development of liver tumours. Over time, the repeated cycles of liver injury, inflammation, and fibrosis in the DEN+CCl4 model contribute to the development of hepatocellular carcinoma. The process involves the accumulation of genetic alterations, dysregulation of signalling pathways, and clonal expansion of transformed hepatocytes, eventually leading to the formation of liver tumours.

Isorhamnetin, a significant monomethoxyflavonol, has a wide range of pharmacological effects, including anti-inflammation, anti-tumour and anti-oxidation and hence can be used as a potential medicine for the prevention and treatment of various diseases (Bhattacharya et al. 2020). It inhibits tumour cell proliferation and promotes apoptosis by regulating the expression of tumour-related genes. Isorhamnetin has extensive pharmacological effects. The specific mechanism of action is still unclear and further experimental verification is needed. Therefore, the pharmacological action and mechanism of isorhamnetin is currently a major research area. The hepatoprotective role of isorhamnetin has been described in the previous chapter of this thesis. However, the specific mechanism of action is unclear and needs further research. The current chapter describes the mechanisms of action which involve anti-inflammation, antioxidation, and regulation of apoptosis to overcome dysregulation of signalling pathways induced by DEN+CCl_4. These pharmacological activities often play an important role in the treatment of HCC.

HCC development involves the induction of inflammation which in physiological conditions is aroused by hepatitis B or hepatitis C virus infection (Llovet et al. 2021). Inflammation leads to cirrhosis which is another major risk factor in HCC development and helps in its initiation and progression. The anti-inflammatory activity of isorhamnetin reduced the damages caused by DEN+CCl_4 induced inflammation. PPAR-γ reduces HCC progression by inducing the expression of cell adhesion genes, extracellular matrix regulator genes, and tumour suppressor genes (Hsu and Chi 2014). Isorhamnetin acted as a TNF-α antagonist and helps to activate PPAR-γ for the reduction of HCC advancement. Isorhamnetin increased ROS in tumour tissues by regulating the Nrf2 pathway. YAP overexpression is associated with HCC severeness with elevated serum α-fetoprotein levels (Yu et al. 2014). Isorhamnetin balanced DEN+CCl_4 induced upregulated YAP expression.

2. Material and Methods

The details have been described in Chapter 4.

3. Results

3.1 Isorhamnetin shows anti-TNF-α properties and inhibits pro-inflammatory cytokines to regress HCC

HCC development initiates with inflammation and tumour necrosis factor (TNF)-α plays a

crucial role in triggering the immune reaction so we surveyed the status of the particular proinflammatory cytokine (Figure 1).

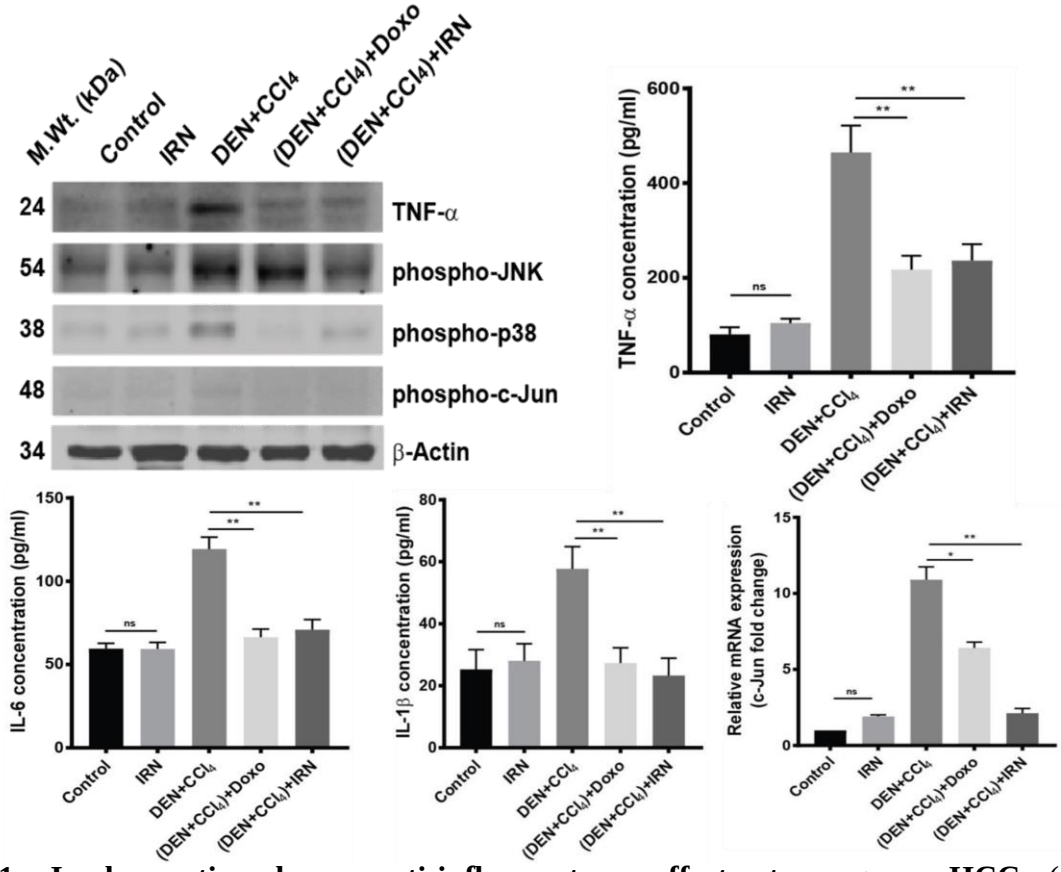

Figure 1. Isorhamnetin shows anti-inflammatory effects to regress HCC (a) Representative immunoblot images of indicated proteins. (b, c, d, e) Graphs represent the serum levels of inflammatory cytokines TNF-α (b) IL-1β (c) IL-6 (d) and IL-17 (e) of indicated groups. (f) qPCR analysis of c-Jun in all experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, non-significant variance existed between the control and IRN group, *P< 0.05, **P< 0.001

The stimulation in the magnitude of TNF-α in DEN+CCl_4 treated mice liver in western blot, and the subsequent downregulation of expression of the particular proinflammatory cytokine after isorhamnetin administration near normal level was ascertained (Figure 1). After that, we ran a qPCR for the survey of TNF-α at the mRNA level to justify our former western blot result. Both results conveyed that isorhamnetin diminished the overexpression of TNF-α in DEN+CCl_4 exposed mice (Figure 1).

To establish the stimulation of downstream signalling pathways of TNF-α engaged in activating interleukins (IL-1β, IL-6, IL-17) we performed ELISA experiments. ELISA results showed in DEN+CCl_4-induced mice, a raise in TNF-α levels with a succeeding increase in the magnitudes of IL-1β, IL-6, and IL-17.

MAPK activation leads to stimulating hepatitis which later has a chance to get mature to develop HCC through TNF-α activation. Thus, we checked JNK and p38 stimulation in DEN+CCl_4 treated groups. The heightened level of phospho-JNK and phospho-p38 in carcinogen-exposed mice were attenuated by consequent isorhamnetin treatment. The same experiment was performed with doxorubicin and interestingly, isorhamnetin performed better than doxorubicin in suppressing JNK (Figure 1).

At the early stage of HCC, the newly generated tumour cell needs the environment to survive, among many factors c-Jun controls apoptosis by blocking p53 tumour suppressor activation, thereby boosting tumour cell viability. Our western blot and qPCR experiments conveyed that c-Jun stimulation was diminished by isorhamnetin treatment to control HCC early induction and to stimulate hepatocyte apoptosis via p53 induction better than doxorubicin.

Finally, these data indicate that isorhamnetin exhibits anti-TNF-α properties and might regress tumours by modulating tumour-promoting inflammation in HCC. We also conclude that this anti-TNF-α activity is mediated by the suppression of MAPK signalling by isorhamnetin. Results also suggest that isorhamnetin reduces the activation of c-Jun to induce cancer cell apoptosis.

3.2. Isorhamnetin upregulates PPAR-γ by reversing the elevated levels of MAPK & TGF-β signalling proteins

Peroxisome proliferator-activated receptor (PPAR)-γ is a major transcription regulator of lipid and glucose metabolism (Hernandez-Quiles, Broekema, and Kalkhoven 2021). Inhibition of PPAR-γ by TNF-α and other inflammatory cytokines aids in inflammation and cancer progression (Ye 2008). Hence, we analyzed the mRNA expression of PPAR-γ and found a decrease in mRNA expression in DEN+CCl_4 treated mice groups which was increased by isorhamnetin treatment. These results were validated at protein levels by western blot analysis. Results demonstrated a significant downregulation of PPAR-γ in HCC mice livers, however, levels were rescued by isorhamnetin administration.

Since TNF-α mediated regulation of PPAR-γ depends on the inhibition of C/EBP-δ (Kudo et al. 2004), we checked its protein levels by western blotting. As predicted, C/EBP-δ was downregulated in HCC mice liver samples which were upregulated in isorhamnetin-treated mice (Figure 2). Besides TNF-α mitigates PPAR-γ levels via stimulating MAPK signalling (Adams et al. 1997, Hu et al. 1996). When we enquired about the expression of different MAP kinases in immune blot experiments, the results pointed to a significantly elevated level of ERK, JNK, and p38 kinase (Figure 2).

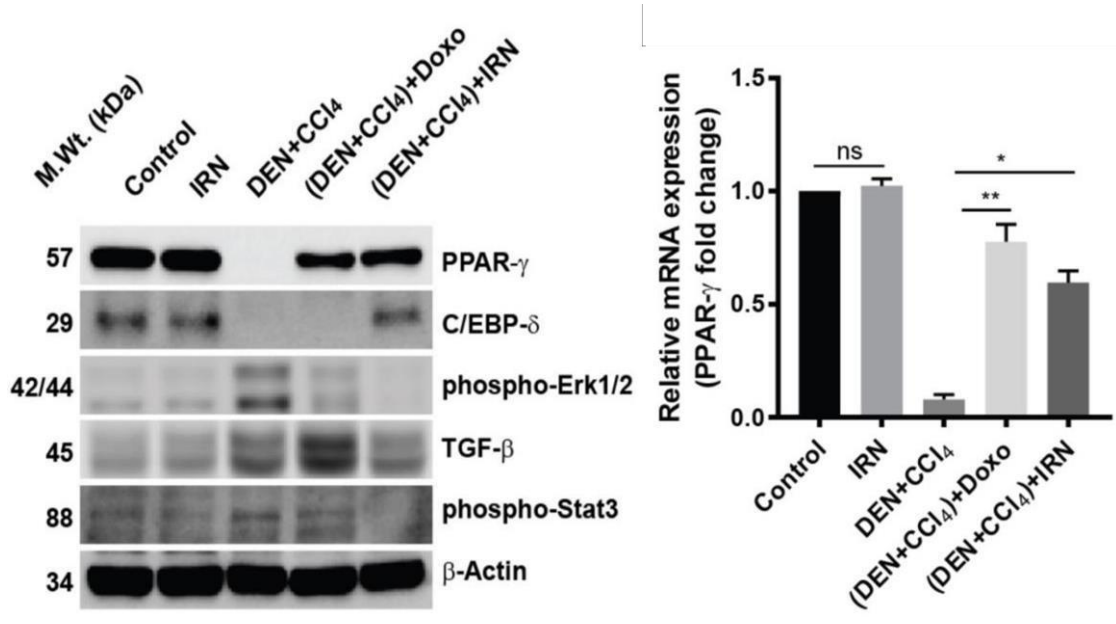

Figure 2. Isorhamnetin regulates PPAR-γ through MAPK and TGF-β (a) Representative immunoblot images in liver lysates of different groups. (b) qPCR analysis of PPAR-γ in all experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. The graph represents mean fold change ± SD for three different experimental sets, non-significant variance existed between the control and IRN group, *P < 0.05, **P < 0.001, ***P<0.0002

Since we found a reduced level of PPAR-γ, we predicted that it would also be affecting TGF-β levels and might be upregulated in HCC mice liver lysates. Western blot results confirmed the stimulation in TGF-β expression in DEN+CCl_4 treated mice groups (Figure 2). The levels of TGF-β became low as PPAR-γ was upregulated by isorhamnetin administration (Figure 2).

TGF-β increases the levels of MMP-9, a key factor to heighten the severeness of HCC. Therefore, we checked the expression of MMP-9 by IHC and found it to be upregulated in HCC tissue, whereas, isorhamnetin suppressed the levels of MMP-9 in carcinogen-treated mice (Figure 3).

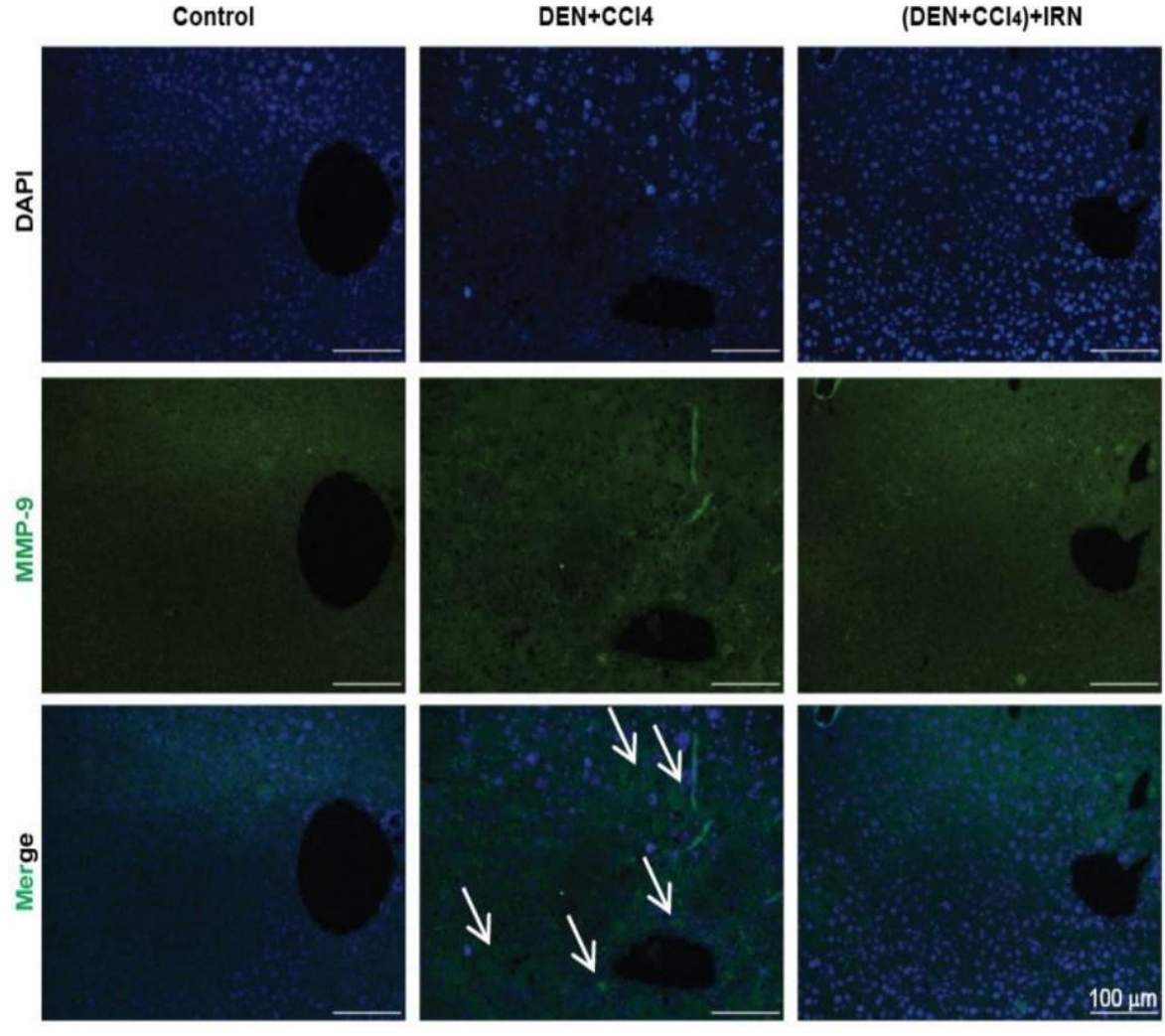

Figure 3. Isorhamnetin regulates MMP9 expression. Representative images of MMP-9 immunostaining of liver sections from different groups (arrows indicate high MMP-9 in DEN+CCl_4 group). Magnification 40X; Scale bar, 100µm

These results suggest that DEN+CCl_4 treatment induces hepatocellular carcinoma in mice by upregulating the levels of ERK, JNK, and p38 MAP kinases and by downregulating the PPAR-γ levels. TGF-β is also upregulated due to decreased inhibitory effects of PPAR-γ to promote metastasis by the carcinogen. Activation of TGF-β stimulates MMP-9 levels to enhance the aggressiveness of HCC in carcinogen-treated mice. These results also suggest

that isorhamnetin treatment of carcinogen-exposed mice reduces the MAP kinase and TGF-β signalling and induces PPAR-γ signalling to inhibit tumour growth and metastasis.

3.3. Nrf2 signalling is negatively regulated by isorhamnetin to increase ROS load in tumour cells

Cancer cells need to overcome the oxidative stress conditions generated due to the high rate of cellular proliferation and exposure to chemical carcinogens for their sustenance as well as progression (Sarkar et al. 2020). Nrf2 is a master regulator of cellular antioxidant response and its activation is reported in HCC development and progression. It suppresses ROS-induced cancer cell death, thereby, promoting proliferation and metastasis in HCC (Sarkar et al. 2020).

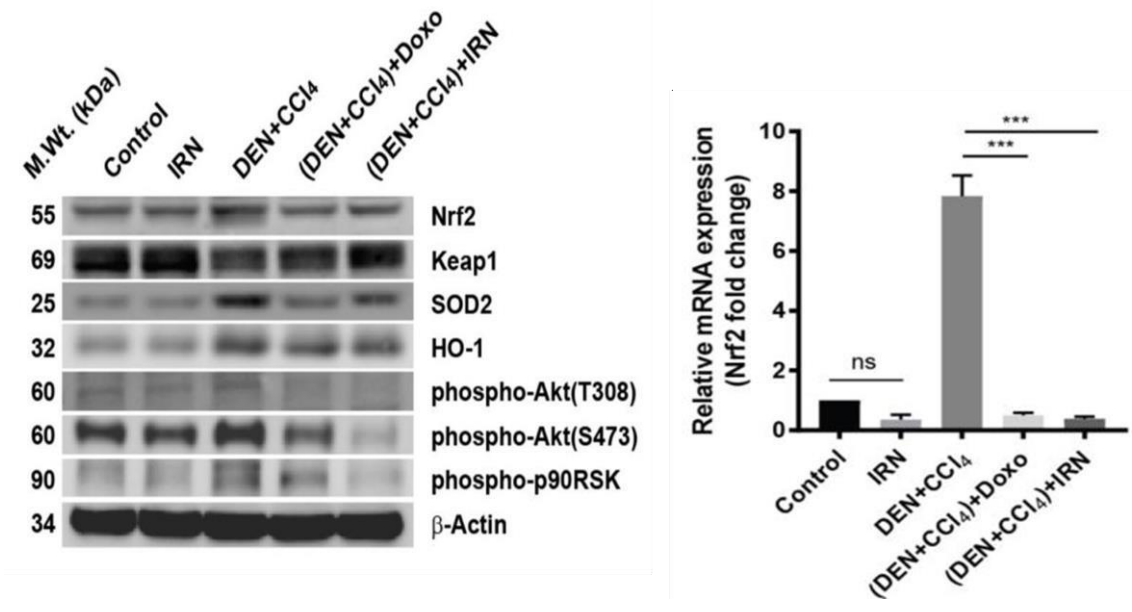

Figure 4. Isorhamnetin suppresses Nrf2 signalling (a) Representative immunoblot images of indicated proteins in liver lysates of different groups. (b) qPCR analysis of Nrf2 in experimental groups. Expression was normalized against β-actin and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, non-significant variance existed between control and IRN group, ***P< 0.0002

Western blot results showed an upregulated level of Nrf2 in DEN+CCl_4 induced liver cancer tissues which were reverted to near control levels by isorhamnetin treatment (Figure 4). Nrf2 mRNA expression analysis by qPCR validated the previous western blot results. To validate

the effect of the carcinogen on Nrf2 signalling and how isorhamnetin treatment modulates the outcome we checked Keap1 levels through western blot, an upstream controller of Nrf2 signalling. In the presence of isorhamnetin, the downregulation of Keap1 was reverted.

Nrf2 supports cancer cell proliferation by changing the transcription of several cancer-promoting and ROS-suppressing genes (Sarkar et al. 2020). In DEN+CCl_4 treated mice where Nrf2 levels were alleviated, HO-1 and SOD2 levels were also upregulated. These results are expected because HO-1 and SOD2 are controlled by Nrf2. These results suggest that DEN+CCl_4 upregulates Nrf2 signalling in mice liver which is downregulated by isorhamnetin to suppress tumour growth. We next tried to find out the upstream regulators of Nrf2 changes by isorhamnetin. Certain phytochemicals elicit improved sensitization of chemo resistant HCC cells by inhibiting Nrf2 signalling via downregulation of the Akt and ERK pathways (Sarkar et al. 2020). Therefore, we looked into whether isorhamnetin modulates Nrf2 via Akt and ERK modulation. Both the proteins, Akt and ERK were stimulated in HCC mice liver and their expression was weakened in the isorhamnetin-treated liver. Since ERK is activated by DEN to induce HCC and inhibited by isorhamnetin to regress the same, we checked some of its upstream proteins and downstream targets of ERK to investigate its additional purpose other than regulating Nrf2. Western blot indicated that isorhamnetin affected the canonical ERK signalling molecules also. Levels of Raf, which is an upstream protein in the ERK pathway, and of Jun, which is a target gene of the ERK pathway, were found to be upregulated by DEN, whereas, levels of both were reverted to normal by isorhamnetin.

To start cell migration for invasion into other neighbouring noncancerous organs cancer cells switch on the RAS-ERK signalling pathway hence we checked the condition of RSK. RSK was phosphorylated in HCC mice groups showing its high activity in HCC livers, on the other hand, isorhamnetin suppressed the phosphorylation state of RSK.

These results suggest that isorhamnetin reduces the Nrf2 signalling to induce ROS accumulation in the tumour microenvironment. These changes in Nrf2 levels are mediated by Akt and ERK kinases. In addition to regulating Nrf2 signalling, decreased ERK signalling also hampers RSK activation which potentiates the anti-tumour properties of isorhamnetin.

3.4. Isorhamnetin decreases cancer cell metabolism and cell cycle progression to regress HCC

Akt regulates several proteins of the cell cycle and glycogen metabolism to maintain a high rate of cancer cell proliferation and metabolism (Luo, Manning, and Cantley 2003). Among its several targets, Akt inhibits Bad to suppress apoptosis in cancer cells (Luo, Manning, and Cantley 2003), activates mTOR to enhance translation, and inhibits GSK-3β to elevate cancer cell metabolism (Luo, Manning, and Cantley 2003). From earlier results, we found an increment in Akt expression which made us think about the expression levels of GSK-3β, cyclins, Bad, and mTOR which are regulated by Akt. A considerable increment in the phosphorylation of GSK-3β thereby inactivation in DEN+CCl_4 induced HCC liver tissue which became reverted back into functional form in the presence of isorhamnetin (Figure 5). Similarly, DEN+CCl_4 induced the phosphorylation of Bad and mTOR in HCC livers tissues which was suppressed by isorhamnetin.

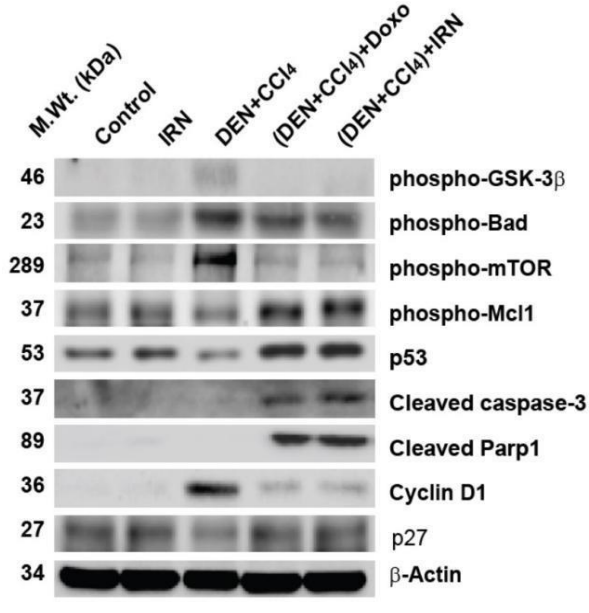

Figure 5. Isorhamnetin disrupts cancer cell homeostasis. Representative immunoblot images of indicated proteins in liver lysates of different groups. Expression was normalized against β-actin and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, non-significant variance existed between the control and IRN group, ***P< 0.0002

Mcl-1 is an anti-apoptotic protein that is targeted for degradation upon phosphorylation (Tong et al. 2017). To activate cell death in tumour cells, many chemotherapeutic treatments are

based on the degradation of Mcl-1. It is phosphorylated by GSK-3β (Wakatsuki et al. 2017) and similar to GSK-3β, we found reduced phosphorylation of Mcl-1 to inhibit its anti-apoptotic properties on cancer cells. In contrast, isorhamnetin increases its phosphorylation via GSK-3β, thereby, its degradation, hence cancer cells undergo apoptosis (Figure 5). In our western blot results, we observed that isorhamnetin adversely affects cancer cell metabolism by suppressing GSK-3β (Figure 5).

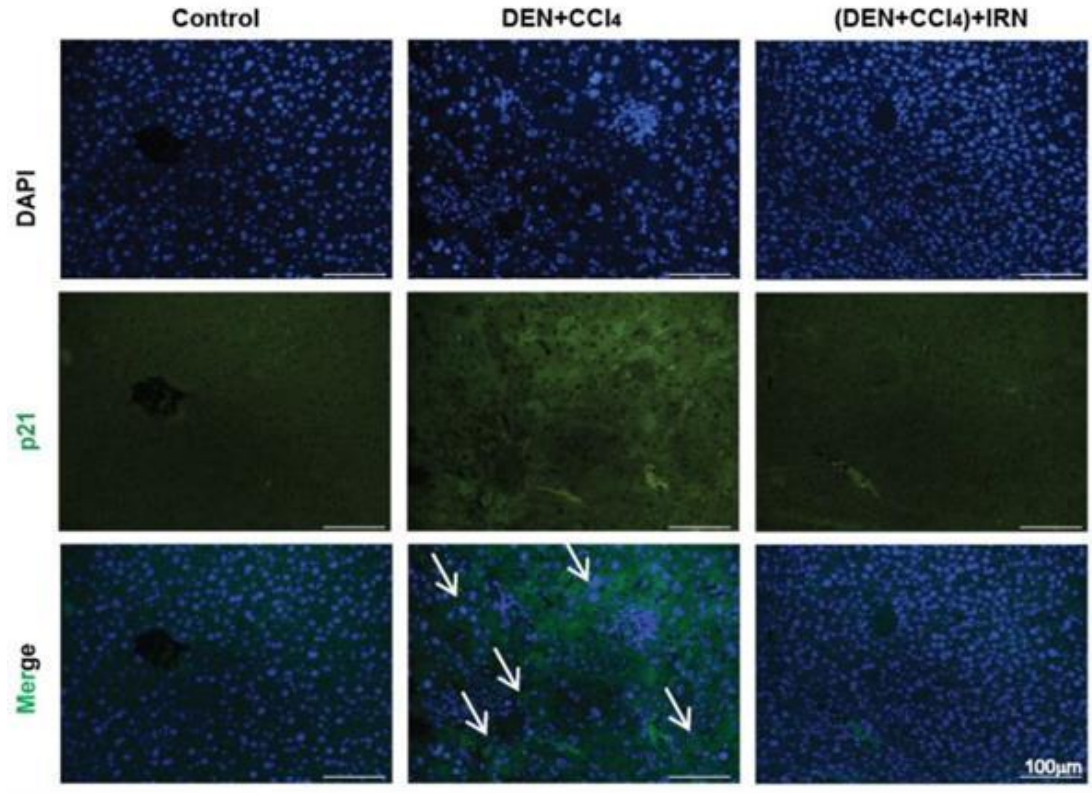

Figure 6. Isorhamnetin regulates p21 expression. p21 IHC, Representative images of p21 immunostaining of liver sections from different groups (arrows indicate cytoplasmic p21 in toxin group). Magnification 40X; Scale bar, 100µm.

We wanted to enquire whether cyclin D1 expression, which can be controlled by ERK, was regulated in the presence of isorhamnetin. In DEN+CCl_4-exposed mice Cyclin D1 was overexpressed and induced cell cycle progression presumably by activated ERK which was reverted back by isorhamnetin treatment.

With these results, we investigated the PCNA status, which should show a similar pattern as cyclin D1. When checked in the qPCR experiment, we observed that PCNA expression was significantly high in HCC liver tissue whereas isorhamnetin reduced the levels to that of normal liver samples (Figure 5).

p27, a protein dedicated to suppressing cell proliferation, was downregulated in DEN+CCl_4-exposed mice and the expression of p27 was reverted when isorhamnetin was given. The increase in cytoplasmic localization of the p21 in DEN+CCl_4-exposed mice liver which is generally seen in many HCC cases were revealed to normal condition by isorhamnetin.

These results indicate that isorhamnetin adversely affects tumour cell metabolism by activating GSK-3β and suppressing mTOR. It also induces cell death by upregulating Bad and downregulating Mcl-1. Isorhamnetin reduces cyclin D1 and PCNA levels and induces p27 expression to inhibit carcinogen-induced hepatocyte cell cycle progression. Additionally, isorhamnetin reduces the expression of p21 in the cytoplasm to reduce cancer cell proliferation and motility (Figure 6).

3.5. Autophagy mediated YAP1 degradation is restored by isorhamnetin in DEN+CCl4-treated mice to reduce HCC progression

Hippo signalling has a pivotal role in organ size and tissue growth regulation, in which, YAP acts as a transcriptional co-activator (Valero, Pawlik, and Anders 2015). YAP is the major protein in Hippo signalling and its overexpression or deregulation has been observed in hepatocellular carcinoma (Valero, Pawlik, and Anders 2015). YAP interacts with DNA binding proteins such as PPAR-γ, and RUNXs to regulate transcription (Yu et al. 2014). YAP-induced chemoresistance is also reported in MAPK-mediated signalling (Zhou et al. 2019). An efficient drug is needed to overcome the YAP-induced HCC progression and chemoresistance. Therefore, we checked whether our HCC mice model has high YAP levels and if isorhamnetin could also influence the YAP signalling. YAP level was upregulated in DEN+CCl_4-exposed mice liver and the protein expression was reduced in the presence of isorhamnetin. Similarly, the administration of doxorubicin also decreased the levels of YAP (Figure 7). RUNX2 also showed similar western blot results like YAP in the DEN+CCl_4-treated mice group and its levels were downregulated in the presence of isorhamnetin (Figure 7).

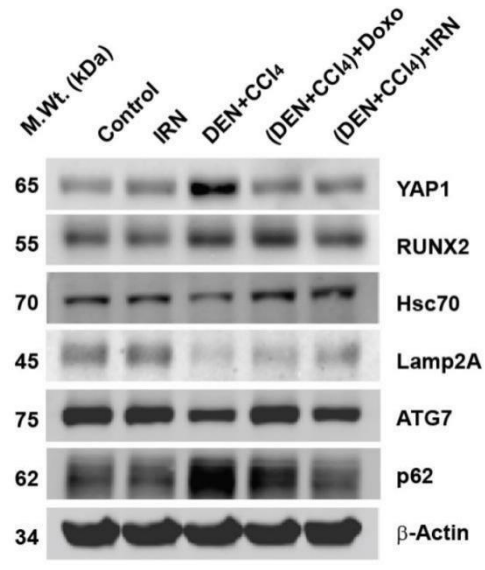

Figure 7. Isorhamnetin regulates Hippo signalling through Autophagy. Representative immunoblot images of indicated proteins in liver lysates of different groups. Expression was normalized against β-actin and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, non-significant variance existed between control and IRN group, ***P< 0.0002

Next, we tried to find the YAP regulatory proteins. Suppression of chaperon-mediated autophagy has a connection with impaired degradation of YAP (Lee et al. 2018, Zhou et al. 2019). ATG7, an autophagy-related protein was downregulated in DEN+CCl_4-treated mice and isorhamnetin reversed this process (Figure 7). We validated the results by investigating the levels of p62, that accumulate upon autophagy downregulation. As expected, p62 was considerably upregulated in the DEN+CCl_4-exposed mice group where autophagy was downregulated with an associated increase in YAP1 and RUNX2 to induce tumour growth (Figure), whereas isorhamnetin and doxorubicin reverse the process similar to normal condition.

These findings suggest that isorhamnetin upregulates autophagy to reduce the YAP1-mediated HCC inducing-effects of DEN+CCl_4. Isorhamnetin treatment could improve the clinical outcomes in HCC patients with either YAP overexpression or YAP-induced chemoresistance.

4. Discussion

In this study, we try to find out which signalling pathways are used by isorhamnetin to protect the liver from carcinogen exposure. Isorhamnetin utilizes its anti-inflammatory property to inhibit the production of TNF-α, IL-1β, IL-6, and IL-17. Many HCC patients with severe conditions have been recommended for sorafenib medication, but in some patients due to increased expression of TNF-α, sorafenib failed to perform at its best because TNF-α tends to resist sorafenib performance.

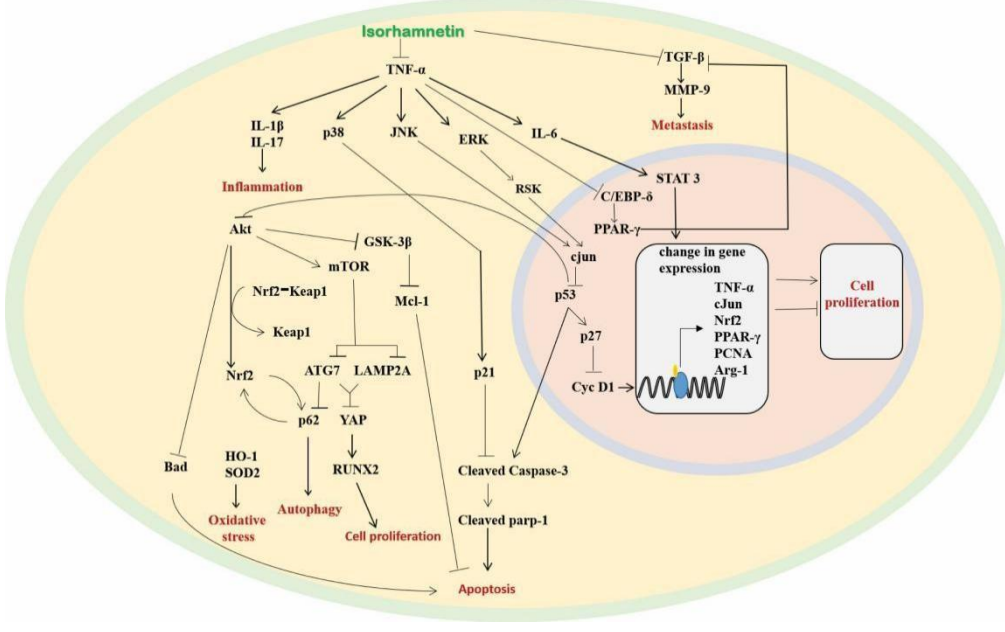

Figure 8. Model representing possible signalling pathways and their cross-talks regulated by isorhamnetin to inhibit DEN+CCl_4-induced HCC in Swiss albino mice.

TNF-α regulates the PPAR-γ signalling pathway, which checks tumour advancement by downregulating MAPK pathways and TGF-β signalling. Our results suggest that DEN+CCl_4 exposure increases the production of many members of proteins involved in ERK, JNK, and p38 MAPK pathways presumably regulating PPAR-γ signalling. Despite its widespread application as a chemotherapeutic agent doxorubicin has some side effects in human and murine breast cancer cells regarding metastasis due to doxorubicin-mediated activation of TGF-β signalling. Our results indicate that isorhamnetin has regulatory action on TGF-β signalling suggesting that isorhamnetin can be considered to use along with doxorubicin to get the better effects of doxorubicin mediated metastasis and suppress EMT.

DEN protects tumour cells from oxidative stress by maintaining a high level of Nrf2 and thereby its target proteins, involved in maintaining the production of reactive oxygen species, namely HO-1, and SOD2. DEN regulates Keap1 to assure an uninterrupted amount of Nrf2. Isorhamnetin acts as an Nrf2 inhibitor and reverts this process to induce apoptosis in liver tumour cells hence can be considered as a therapeutic treatment for HCC. The overexpressed Bad and inhibition of Mcl-1 protein cause apoptosis in liver tumours which is governed by isorhamnetin. In addition to inducing tumour cell death, isorhamnetin downregulates some proteins that help in tumour cell metabolisms such as GSK-3β and mTOR. Earlier, LY2090314 was used in clinical trials to inhibit GSK-3β as cancer co-therapy, however, it did not show efficacy (Mancinelli et al. 2017). Isorhamnetin can be used in the clinical trial of HCC for having GSK-3β inhibitory properties. In the isorhamnetin-treated group, overexpressed p27, inhibited PCNA and cyclin D1 cause inhibition of the cell cycle which suggests that isorhamnetin is an effective chemotherapeutic agent to be considered.

Overall, data presented in this report indicate that DEN+CCl_4 exposure induces hepatocellular carcinoma in mice and intraperitoneal administration of isorhamnetin can restrict this carcinogenesis in the same mouse model. Isorhamnetin shows better chemo-preventive effects than doxorubicin in regressing mice HCC.

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Summary

SUMMARY

Studies on the protective role of bioactive compound against hepatocellular carcinoma

HCC mortality and incidence are on the rise worldwide, particularly in the United States and Europe. The medical community should prioritize and improve HCC surveillance since early detection leads to better survival rates. We need to develop more sensitive and specific surveillance tools (such as blood-based biomarkers and new imaging techniques) in order to improve the risk stratification of high-risk patients, and we need to create interventions that encourage physicians and patients to use surveillance more effectively. In order to increase the number of HCC patients eligible for curative procedure, healthcare systems need to adapt to changing epidemiology and economic burdens of chronic liver disease. By taking this action, we may be able to improve the prognosis for this challenging disease. In addition to surgical resection, liver transplantation is limited by the availability of donor organs. While interventional oncology has made significant progress in treating small cancers with minimally invasive techniques such as RFA, MWA, TACE, TARE, and IRE, transplantation has not been a viable option for small cancers. Side effects limit the effectiveness of sorafenib, which prolongs survival. Treatment options for HCC are expected to improve with immunotherapy and novel chemotherapeutic agents like regorafenib. Over the next decade, HCC patients will benefit from a personalized multidisciplinary approach. Identifying tumours earlier and increasing cure chances are possible in the short term by increasing surveillance of at-risk patients.

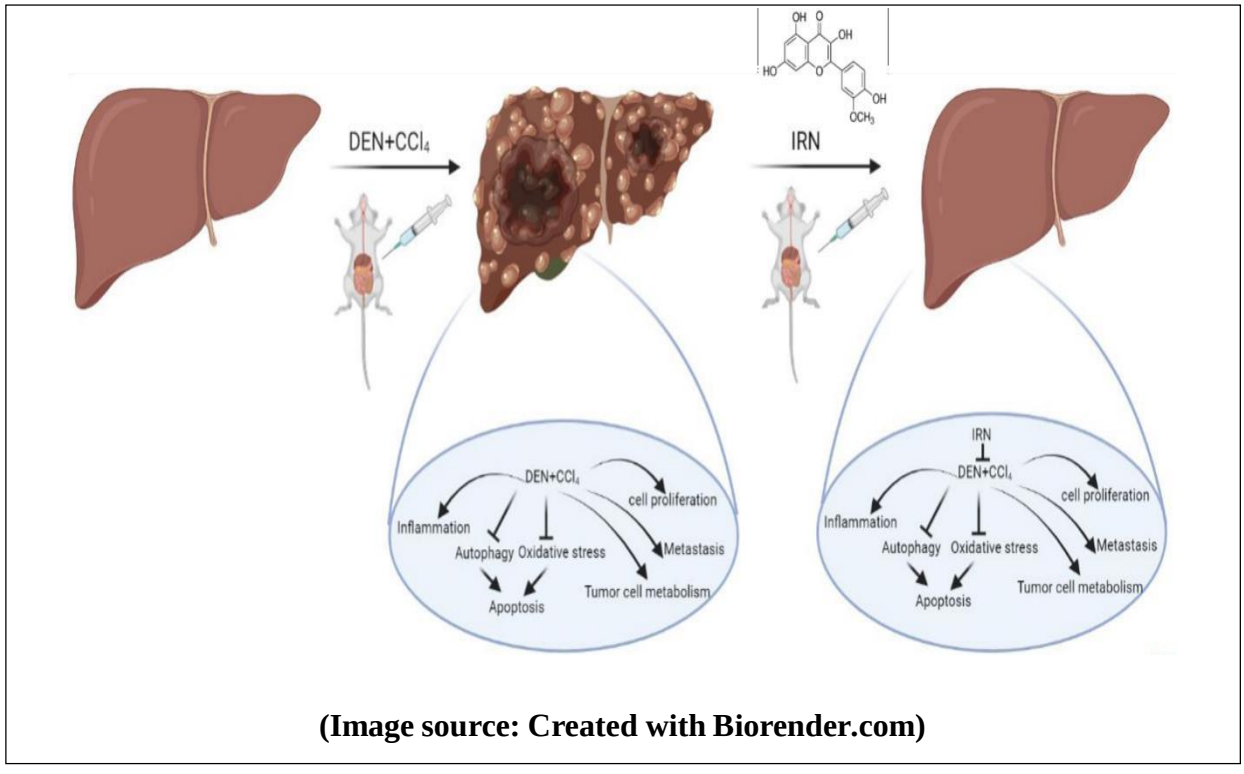
It is a growing concern that hepatocellular carcinoma is responsible for an increasing number of deaths. By vaccinating everyone against HBV, increasing cure rates for HCV, and improving surveillance, the burden can be reduced. A multidisciplinary approach is required for the management of hepatocellular carcinoma, especially for patients with cirrhosis. Despite improvements in clinical management for patients with advanced cancer, other areas of management, such as chemotherapy and adjuvant therapy after surgery or ablation, still need improvement. There is a challenge in determining the best order of sequential systemic therapy that maximizes clinical benefits while minimizing side effects and cost as more systemic agents are found to be effective in phase 3 trials. In the near future, research initiatives for hepatocellular carcinoma will be shaped by combination therapies and the use of systemic drugs sooner rather than later.

Here, we studied the organ pathophysiology of the liver with malignant conditions. We chose isorhamnetin as a bioactive molecule to study the protective strategies. The ameliorative effects against inflammation, metastasis, cell cycle progression, proliferation, and promoting oxidative stress, and apoptosis in cancer tissues were extensively studied. Our work mainly covered the hepatoprotective effects of the concerned bioactive molecule against hepatocellular carcinoma.

Being an acute hepatotoxin DEN specifically operates in the liver to be specific in the centrilobular hepatocytes where the liver specific isoform of cytochrome P450 expresses. A methylated intermediate is generated after the reaction with cytochrome P450, results in release of electrophilic methyl ions, which form DNA adduct, causing various mutations. CCl_4, on the other hand, accelerates the toxin micro-environment within liver to create near to physiological condition usually observed in HCC.

Hepatocellular carcinoma caused by multiple sequential events. Inflammation initiates the beginning of disease progression, which is supplemented by oxidative stress, autophagy, apoptosis suppression for long survival of cancerous cells and finally metastasis by breaking extracellular matrix barrier. TNF-α along with other interleukins induce hyperimmune response that promote favourable tumour microenvironment due to localized invasion of lymphocytes and Kupffer cells and produce reactive oxygen species. TNF-α also activates IL-6 which promotes STAT3 activation generating liver cancer stem cells very commonly seen in HCC. To initiate metastasis membrane metalloproteases are in general activated by TGF-β. Isorhamnetin blocks the expression of TGF-β most probably through activation of PPAR-γ. The increment in cytosolic concentration of p21 causes cellular transformation. In HCC PI3K-Akt-mTOR pathway stays operational and many chemotherapeutic agents shows anti-proliferative properties by suppressing mTOR molecule. PI3K mediated activated Akt responses to external stimuli via mTORC1 stimulation, downregulating autophagy. When there are scarce in nutrients and oxygen activated autophagy blocks further proliferation. In HCC when Nrf2 phosphorylates p62 cancer cells tend to acquire a tolerance for chemotherapy. When Hippo signalling is off the YAP1 responses to external stimuli via MAPK pathway and its activation bypass its degradation and allow translocation into nucleus and allow many activities to transform cells into malignant ones which lead to increment in liver mass with tumour tissues.

Thus, the therapeutic strategies of isorhamnetin involved anti-inflammatory, cytotoxic effects against hepatocellular carcinoma.



(Image source: Created with Biorender.com)

Abbreviations

Abbreviations

AST	Aspartate aminotransferase
ATG7	Autophagy related 7
ALT	Alanine aminotransferase
Bad	Bcl-2 associated agonist of cell death
Bcl-2	B-cell lymphoma 2 protein
CCl_4	Carbon tetrachloride
C/EBP-δ	CCAAT/enhancer-binding protein delta
DEN	N-diethylnitrosamine
Doxo	Doxorubicin
ERK	Extracellular signal-regulated kinase
EMT	Epithelial-mesenchymal transition
GSK-3β	Glycogen synthase kinase-3 beta
HCC	Hepatocellular carcinoma
HO-1	Heme Oxygenase-1
HRP	Horseradish peroxidase
IRN	Isorhamnetin
JNK	c-Jun N-terminal kinase
Keap-1	Kelch-like ECH-associated protein 1
Lamp2A	Lysosomal-associated membrane protein-2
MMP-9	Matrix metalloproteinases 9
Mcl-1	Myeloid cell leukemia-1
mTOR	Mammalian target of rapamycin
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung carcinoma
PPAR-γ	Peroxisome proliferator-activated receptor-gamma

PBS	Phosphate buffered saline
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
RIPA	Radioimmune precipitation assay
RUNX2	Runt-related transcription factor 2
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate
SVR	Sustained virological response
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
YAP1	Yes-associated protein 1

Publications

List of Publications

Original Article

- Sarkar S, Das AK, Bhattacharya S, Gachhui R, Sil PC. Isorhamnetin exerts anti-tumour activity in DEN+CCl_4-induced HCC mice. Medical Oncology. 2023 May 24;40(7):188.

Book Chapter

- Sarkar, S., Sil, P.C. (2023). Curcumin and Its Role in Cancer Prevention. In: Kathryn Woltjer. (eds) Curcumin and Its Role in Health and Disease ISBN: 979-8-88697-491-1. © 2023 Nova Science Publishers, Inc.

- Sarkar, S., Ghosh, N., Kundu, M., Sil, P.C. (2020). Nrf2 and Inflammation-Triggered Carcinogenesis. In: Deng, H. (eds) Nrf2 and its Modulation in Inflammation. Progress in Inflammation Research, vol 85. Springer, Cham. https://doi.org/10.1007/978-3-030-44599-7_6

Original Reprint

Medical Oncology (2023) 40:188
https://doi.org/10.1007/s12032-023-02050-5

ORIGINAL PAPER

Isorhamnetin exerts anti-tumor activity in DEN + CCl_4-induced HCCmice

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Received: 10 April 2023 / Accepted: 6 May 2023

Abstract

Background Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer and the main cause of cancer death globally. The use of medicinal herbs as chemotherapeutic agents in cancer treatment is receiving attention as they possess no or minimum side effects. Isorhamnetin (IRN), a flavonoid, has been under attention for its anti-inflammatory and anti-proliferative properties in a number of cancers, including colorectal, skin, and lung cancers. However, the *in vivo* mechanism of isorhamnetin to suppress liver cancer has yet to be explored.

Methods and Result HCC was induced by *N*-diethylnitrosamine (DEN) and carbon tetrachloride (CCL_4) in Swiss albino mice. Isorhamnetin (100 mg/kg body weight) was given to examine its anti-tumor properties in HCC mice model. Histological analysis and liver function assays were performed to assess changes in liver anatomy. Probable molecular pathways were explored using immunoblot, qPCR, ELISA, and immunohistochemistry techniques. Isorhamnetin inhibited various pro-inflammatory cytokines to suppress cancer-inducing inflammation. Additionally, it regulated Akt and MAPKs to suppress Nrf2 signaling. Isorhamnetin activated PPAR-γ and autophagy while suppressing cell cycle progression in DEN +CCl_4-administered mice. Additionally, isorhamnetin regulated various signaling pathways to suppress cell proliferation, metabolism, and epithelial–mesenchymal transition in HCC.

Conclusion Regulating diverse cellular signaling pathways makes isorhamnetin a better anti-cancer chemotherapeutic candidate in HCC. Importantly, the anti-TNF-α properties of isorhamnetin could prove it a valuable therapeutic agent in sorafenib-resistant HCC patients. Additionally, anti-TGF-β properties of isorhamnetin could be utilized to reduce the EMT-inducing side effects of doxorubicin.

Keywords Isorhamnetin · Hepatocellular carcinoma · Inflammation · MAPKs · Nrf2 signaling

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATG7	Autophagy-related 7
Bad	BCL2-associated agonist of cell death
CCl_4	Carbon tetrachloride
C/EBP-δ	CCAAT/enhancer-binding protein delta
DEN	*N*-Diethylnitrosamine
Doxo	Doxorubicin
ERK	Extracellular signal-regulated kinase
EMT	Epithelial–mesenchymal transition
GSK-3β	Glycogen synthase kinase-3 beta:
HCC	Hepatocellular carcinoma
HO-1	Heme Oxygenase-1
HRP	Horseradish peroxidise
IRN	Isorhamnetin
JNK	C-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
Lamp2A	Lysosomal associated membrane protein-2
MMP-9	Matrix metalloproteinases 9
Mcl-1	Myeloid cell leukemia-1
mTOR	Mammalian target of rapamycin
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung carcinoma

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Published online: 24 May 2023

PPAR-γ	Peroxisome proliferator-activated receptor-gamma
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
RUNX2	Runt-related transcription factor 2
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulfate
SVR	Sustained virological response
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
YAP1	Yes-associated protein 1

Introduction

Liver carcinoma is the sixth most prevalent cancer worldwide accounting for almost a million new cases and causing 8,30,180 deaths globally in 2020 [1]. In India, 37,410 cases of liver cancer were reported in the year 2020, with a ratio of 4:1 for males to females [2]. Additionally, the World Health Organization projects approximately 1 million deaths from liver cancer in 2030 globally [3]. Rapidly rising occurrences of liver cancer, along with a low survival rate despite treatments, have emerged as a global health problem. Hepatocellular carcinoma (HCC) accounts for approximately 90% of all liver cancer cases [4]. Several risk factors like hepatitis virus infection, cirrhosis, non-alcoholic steatohepatitis, alcohol consumption, and obesity are accountable for the induction of HCC [4]. Additionally, cancer-driven genomic mutations induced by potentially harmful food products can also increase the risk of liver cancer [4]. Although liver cancer-targeted therapy, immunotherapy, radiation therapy, transarterial therapy, and precision medicine are available to treat patients, the low survival rate following these therapies continues to be a big issue [4]. Parallel research to develop promising compounds from natural products is also in progress for various pathological conditions, including cancer treatment. The use of plant-derived drugs has increased significantly in the past few years [5, 6]. Isorhamnetin, a 3'-O-methylated metabolite of quercetin, is a flavonoid compound present in the leaves of *Ginkgo biloba* L. and flowers and fruits of *Hippophae rhamnoides* L. (sea buckthorn), *Oenanthe javanica*, kombucha tea, and in many other plants [7]. The total flavonoids of *Hippophae rhamnoides* have been frequently employed in traditional Chinese medicine for their anti-cancer properties. Pharmacological effects of isorhamnetin have been shown to cure cardiovascular diseases, neurodegenerative diseases, and kidney diseases through its anti-inflammatory and antioxidant activity [8]. It shows anti-tumor efficacy both in vitro and in vivo against melanoma, stomach cancer, breast cancer, oesophageal cancer, colon cancer, and gastric cancer [9–12]. Isorhamnetin shows hepatoprotective effects in carbon tetrachloride (CCL_4)-induced and bile duct ligation (BDL)-induced fibrosis in mice [13]. Isorhamnetin exhibits its hepatoprotective effect by modulating TGF-β1/p38 and TGF-β1/Smad3 pathways [14]. It reduces the levels of pro-inflammatory cytokines by modulating p38/PPAR-α pathway in concanavalin A-induced acute hepatitis in mice. In another study, isorhamnetin was found to activate Nrf2 signaling in response to oxidative stress in HepG2 cells [15].

N-diethylnitrosamine (DEN), a well-known chemical carcinogen, is the most commonly recommended in animal models for liver cancer studies [16]. DEN is a genotoxic carcinogen found in a variety of foods (soft drinks, milk products, smoked meat, fish, alcohol, and preservatives), tobacco products, cosmetics, and cigarette smoke [16]. DEN-induced carcinogenesis generates reactive oxygen species (ROS) leading to cellular damage. The DEN-induced HCC rodent model is the most recognized since it similarly reproduces gene expression profiles to that of a human. It takes longer exposure to treat DEN alone to cause HCC. So, in order to accelerate the development of DEN-induced HCC, we employed CCl_4 as a tumor promoter. CCl_4 induces hepatic fibrosis and compensatory cell proliferation [17].

In the present study, we investigated the anti-tumor potential of isorhamnetin toward DEN + CCl_4-induced HCC in Swiss albino mice. We found that isorhamnetin modulates a wide range of cellular signaling pathways in carcinogen-exposed Swiss albino mice to prevent hepato-carcinogenesis. The effects of isorhamnetin were compared to that of doxorubicin which is currently being used as a chemotherapeutic drug in liver cancer.

Materials and methods

Chemical compounds

DEN (#N0756), CCl_4 (#289116), and Isorhamnetin (#17794) were purchased from Sigma-Aldrich, India. ELISA kits were purchased from Abcam, UK and Span Diagnostic Ltd., India. Antibodies required for the detection of specific proteins were purchased from Cell Signaling Technology. Other required chemicals were bought from SRL, India.

Animals

Four week-aged male Swiss albino mice were obtained from the Animal House Facility of Bose Institute, Kolkata, India. Animals were maintained at 25 ± 4 °C temperature, with an alternating 12-h light/dark cycle and 45–55% humid conditions. Food pellets (Agro Corporation Private Ltd., Bangalore, India) and drinking water were provided routinely.

Mice were under regular observation for their well-being, body weight, toxicity, and survival. Animals were acclimatized to standard laboratory state for 2 weeks before the start of any dosing. All the animal experiments were prosecuted according to the institutional ethical committee and with the permission of IAEC, CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals), and the Ministry of Environment and Forests, New Delhi, India [1796/GO/EReBiBt/S/14/CPCSEA].

Development of the mouse model

The dose selection of the DEN-induced HCC model was based on a previous study [17]. Briefly, mice were intraperitoneally treated with CCl_4 (50 µL/kg body weight in liquid paraffin) for 7 days successively followed by intraperitoneal injection of DEN at 75 mg/kg body weight weekly for three successive weeks and 100 mg/kg body weight for another 3 successive weeks. Mice were kept from consuming any food or water for 1 h earlier and later the DEN application and were sacrificed after 11 weeks of carcinogen dose completion.

Isorhamnetin and doxorubicin administration

There are no reports of a definite dose of isorhamnetin in curing HCC in mice. Therefore, to find out the optimum *in vivo* activity of isorhamnetin against HCC, we treated mice with various isorhamnetin doses considering those reported in cellular studies of various liver cancer cell lines [18, 19] and *in vivo* [20–23] studies of other cancers. Isorhamnetin (25 mg/kg, 50 mg/kg, and 100 mg/kg body weight) solution was made in phosphate-buffered saline (PBS) before treatment. It was given post-carcinogen administration by intraperitoneal route weekly till the end of the experiment for 11 weeks. Doxorubicin (1 mg/kg body weight) was administered in PBS, intraperitoneally post-carcinogen administration weekly till the end of the experiment for 11 weeks [24].

In vivo experimental design

The experimental design was adapted from previous study [15]. Animal experiments were performed in three independent sets. Male Swiss albino mice (5–6 weeks) with an average body weight of 20–25 g were divided into the following experimental groups, having 6 mice in each group (Fig. 1a):

Group I (normal control group): Mice without any treatment.

Group II (isorhamnetin-only group): Mice of this group received only isorhamnetin via intraperitoneal injection from the start of the 8th week and continued till the end of the experiment (18th week).

Group III (carcinogen group): Mice in this group received CCl4 (1st week) followed by DEN administration (2nd–7th week).

Group IV (positive control group): Mice in this group received doxorubicin after 7 weeks of first carcinogen administration and continued till the end of the experiment (18th week).

Group V (post-treatment group): Mice in this group received isorhamnetin after 7 weeks of first carcinogen administration and continued till the end of the experiment (18th week).

Mice from different experimental groups were sacrificed at the end of 18th week.

Collection of blood, liver, and extraction of serum

Mice were sacrificed upon completion of the treatment period. Blood samples were collected followed by serum isolation through incubation of the collected blood at 37 °C for 30 min and centrifugation at 3000 xg for half an hour. The isolated serum samples were acquired and stored at – 80 °C for further use. The livers were aseptically dissected and weight was recorded. After weighing, liver tissues were dissected and divided into different parts. One part was fixed in formalin and paraffin sections were prepared for histopathological analysis and immunohistochemistry analysis. The other fresh tissue parts were used for RNA/protein isolation and further experiments [25].

Gross morphological assessment of liver

All mice were weighed periodically and the data were recorded. After the sacrifices, livers were collected, weighed, and examined for visibly present tumors and other morphological alterations.

Histological studies

For histological studies, a small portion of livers from the normal and experimental mice were fixed in 10% buffered formalin and was processed for paraffin sectioning. Sections of about 5 mm thickness were stained with hematoxylin and eosin to evaluate the pathophysiological changes under the light microscope at a magnification of 40X [26].

Assessment of liver function by enzyme assay

Blood from five different groups of mice was collected from the tail vein after a specific time interval. Serum was obtained by centrifugation at 3000xg for 10 min at 4 °C. Biochemical parameters, i.e., alkaline phosphatase (ALP),

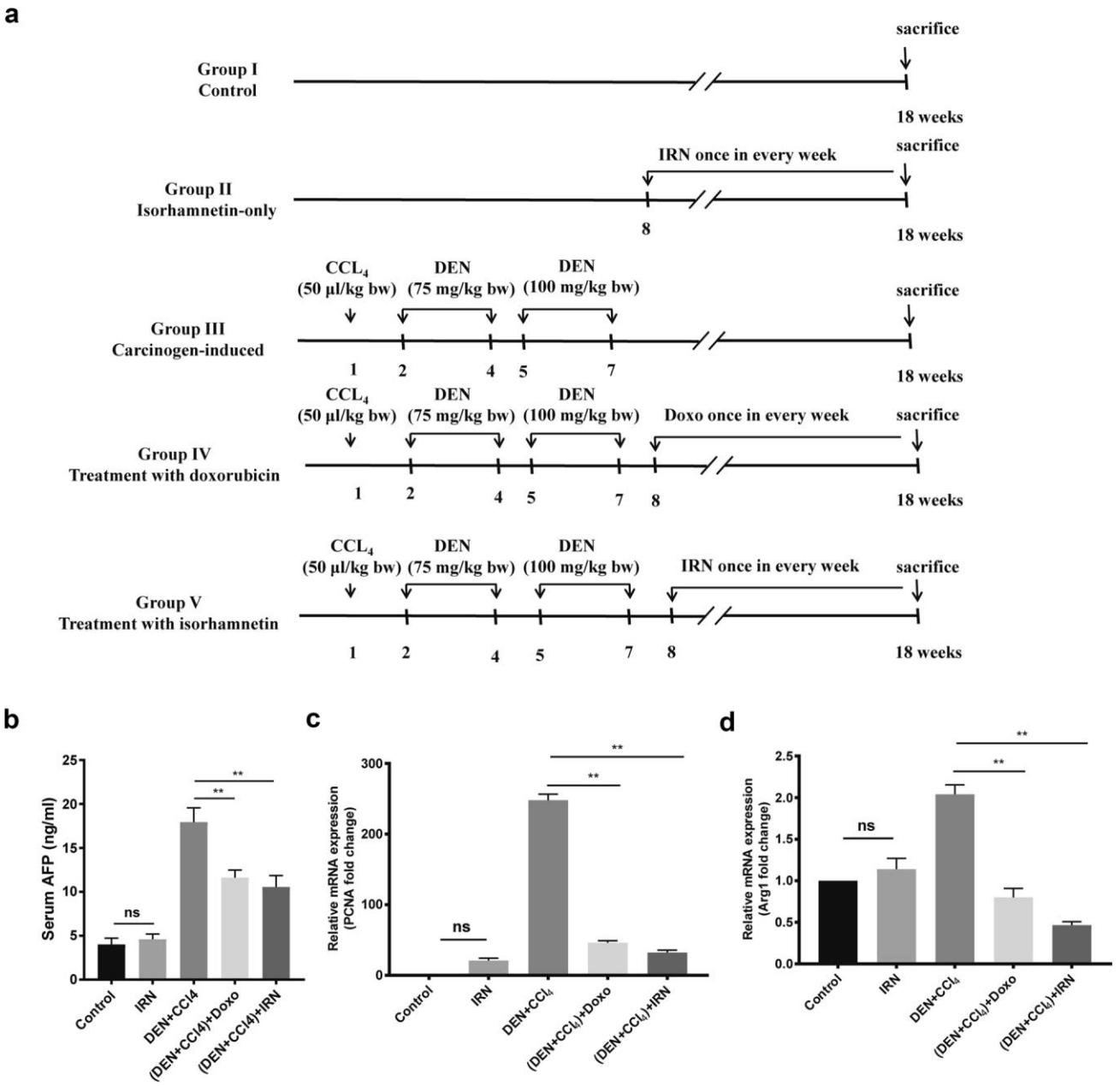

Fig. 1 Isorhamnetin maintains body weight and liver function in HCC mice model **a** Scheme describing the experiments for HCC induction and cure were performed in animals. Swiss albino mice were treated with CCl_4 in the first week followed by DEN treatment in different doses for the next 6 weeks (Group III). One group was further administered isorhamnetin (IRN) till the end of the experiment (Group V). Isorhamnetin was also administered without carcinogen treatment (Group II). Doxorubicin was taken as a positive control in a parallel group (Group IV). **b** Serum AFP levels in indicated mice groups. qPCR analysis of **c** PCNA and **d** Arginase-1 in all experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. Graphs represent mean fold change ± SD, $^{**}P < 0.001$, ns represents non-significant different between Control and IRN group

aspartate aminotransferase (AST), alanine transaminase (ALT), and total bilirubin, were analyzed spectrophotometrically using kit's protocol (Span diagnostic Ltd., India).

Preparation of tissue homogenate

Livers were collected post-sacrifice of the experimental mice and washed in PBS. The liver sections of approximately 1 mm diameter were then placed in a Dounce glass homogenizer and subjected to homogenization by 1/5 (w/v)

ice-cold radioimmune precipitation assay (RIPA) lysis buffer [150-mM NaCl, 50-mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, Triton X-100] and then added with phosphatase and protease inhibitor cocktails (Inhibitor Cocktails, Thermo Fisher Scientific). The homogenized tissues were then centrifuged at 16,000 xg at 4 °C for 20 min. The supernatants were collected and stored at – 80 °C until the further experiment.

Total protein estimation

After the collection of supernatants, the protein content of the supernatant obtained after homogenization was estimated with the Bicinchoninic Acid (BCA) assay kit. Afterward, the remaining supernatants were aliquoted based on protein content in samples of each group and stored at − 80 °C for subsequent experiments.

Immunoblot analysis

For the immunoblot study, 40 µg of protein from each sample of liver tissue was resolved by 10% SDS-PAGE following the standard protocol. Primary antibodies used for analysis were purchased from Cell Signaling Technologies, Santa Cruz Biotechnology or Abcam. The dilution was set at 1:1000 for primary antibodies. Respective HRP-conjugated secondary antibody was needed to identify primary antibodies (1:10,000) using the enhanced chemiluminescence method. The band intensities were quantified by ImageJ and peak density normalization was achieved with β-actin [25–31].

Immunohistochemistry

Paraffinized tissue microsections were placed on poly-L-lysine-coated slides. Tissue staining was done as described in our previous study. Briefly, tissue sections were first deparaffined followed by rehydration and then incubation in a blocking solution of bovine serum albumin for 1 h at room temperature. Tissues were then incubated with primary antibody diluted in blocking solution (1:100) overnight at 4 °C. Slides were washed thrice with 1X PBS followed by Alexa fluor 488-tagged secondary antibody (1:300) incubation for 1 h at room temperature. After washing with PBS, slides were mounted with DAPI containing Vectashield and stored at 4 °C till microscopic analysis.

RNA extraction and real-time PCR (qPCR)

Extraction of the liver RNA of each group of mice was done by the TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA). RNA concentrations were estimated spectrophotometrically by a nanodrop (Hellma TrayCell Type 105.810). Verso cDNA synthesis kit (Thermo Scientific, USA) was used for the conversion of 2 µg of RNA into cDNA. cDNA was amplified by real-time PCR (ABI Prism7500; Life Technology, MA, USA) using specific forward primer (FP) and reverse primer (RP) given in supplementary table S2 and Power SYBR Green Master Mix (Applied Biosystems, Life Technology, USA). Relative gene expression was normalized by GAPDH expression and represented by the graph.

Statistical analysis

All results in this study have been represented as mean ± standard deviation (SD) after performing at least three independent set of experiments. All statistical analyses of data were done by the one-way analysis of variance (ANOVA) method. A *P*-value less than 0.05 have been regarded as statistically significant.

Results

Effect of isorhamnetin and DEN on body weight and liver function

Mice treatments were performed as given in the schematic (Fig. 1a). Mice developed HCC 18 weeks after carcinogen treatment as observed by significantly increased serum AFP levels (Fig. 1b), which is a characteristic marker in HCC diagnosis [17, 32]. Isorhamnetin administration post-DEN + CCl_4 treatment kept the ALT, AST, ALP, and bilirubin values near normal, suggesting that isorhamnetin might prevent DEN-CCl_4-induced tumors in the mice model (Fig. S1). In our dose-dependent study, we observed optimum protection in liver enzyme function when isorhamnetin was administered at a dose of 100 mg/kg (Fig. S1), and this dose was selected for subsequent studies. Isorhamnetin alone did not affect the activity of these enzymes in mice suggesting that it acts to maintain normal liver functions in hepatotoxic conditions.

Compared with control and isorhamnetin-only groups, DEN + CCl_4 treatment significantly reduced the body weight, with a final reduction of 50% before the end of the experiment (Supplementary Table S1). Correspondingly, the DEN +CCl_4-treated group exhibited significantly higher liver/body weight ratios compared with the control group which might be due to the DEN +CCl_4-induced liver damage (Supplementary Table S1). In contrast, the liver/body weight ratios of isorhamnetin-administered mice were decreased compared to DEN + CCl_4-treated mice, suggesting that isorhamnetin alleviated the tumor burden (Supplementary Table S1).

To further validate the carcinogen-induced hepatic cell proliferation in the DEN +CCl_4 mice group, we performed qPCR analysis for PCNA which is a marker for cell proliferation. PCNA expression was highly upregulated in the DEN + CCl_4 mice group contrary to all other groups (Fig. 1c). We also checked arginase-1 levels which is another potential marker of HCC [33, 34]. Arginase-1 was highly upregulated after DEN + CCl_4 treatment; however, its levels were suppressed by isorhamnetin administration in the HCC mice model (Fig. 1d) suggesting that isorhamnetin prevents hepato-carcinogenesis in Swiss albino mice.

Isorhamnetin restores liver internal structure in carcinogen-exposed mice

Anti-tumor activity of isorhamnetin was evident in macroscopic studies (Fig. 2a). Carcinogen-exposed isorhamnetin-administered groups harbored much fewer tumors similar to the doxorubicin group in comparison to the carcinogen-treated group (Fig. 2b). After 18 weeks of the first carcinogen administration, all mice in this group developed visible tumors, whereas no tumors were detected in control or isorhamnetin-only groups. However, tumors were rarely detected on the liver surface in DEN + IRN groups, suggesting that isorhamnetin administration lowers the tumor incidence in mice (Fig. 2c). When the size of these tumors was measured, post-isorhamnetin-treated mice showed strikingly smaller tumors than DEN +CCl_4-treated mice (Fig. 2d, e).

To ascertain the anti-tumor activity of isorhamnetin against DEN + CCl_4-induced HCC, we checked the morphology of H&E-stained liver sections by histological assessment. The results showed that isorhamnetin protected the liver from DEN +CCl_4-induced HCC (Fig. 2f). In control and isorhamnetin-treated groups, hepatocytes were having a polygonal shape, separated by sinusoids having well-defined round nuclei with 2–3 prominent nucleoli.

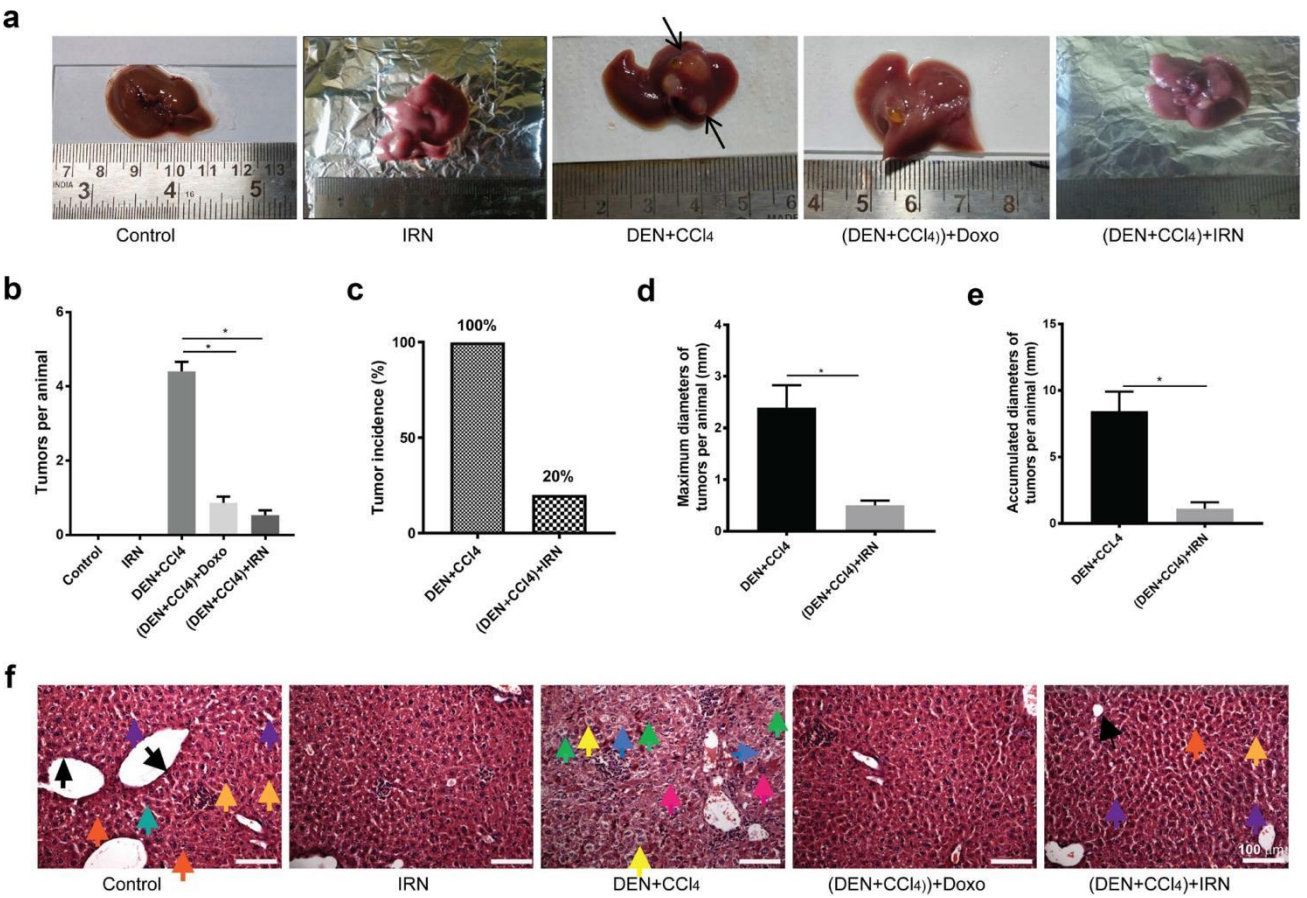

Fig. 2 Isorhamnetin restores liver morphology in carcinogen-exposed mice. **a** Representative macroscopic images; black arrow indicates tumor nodule. **b** Numbers of visible nodules on the liver surface of mice in each group ($n = 6$). **c** Tumor incidence in group III and group V ($n = 6$). **d** The maximum diameters and **e** accumulated diameters of tumors per mouse are depicted ($n = 6$). **f** Representative images of HE-stained liver sections of indicated mice groups; arrowheads: Black (endothelial cells), Purple (sinusoids), Mustard yellow (regular nuclei), Teal (nucleoli), Orange (Kupffer cells), Yellow (enlarged hepatocytes), Green (multinucleation, nuclear atypia), Pink (hyperchromasia), and Blue (ballooned hepatocytes); Magnification 40X; Scale bar, 100 μm

Sinusoids were lined with endothelial cells and had Kupffer cells (Fig. 2f, control). However, in DEN + CCl_4-treated groups, progressed HCC showed an expansive and infiltrative histologic growth pattern with cytological atypia and architectural deformities. There were no definite portal tracts seen within the tumor along with the thickening of hepatic plates. DEN + CCl_4-induced liver section showed steatohepatitis HCC-like appearance or nuclear inclusions (Fig. 2f; DEN + CCl_4). We observed ballooning of the hepatocytes along with infiltration in inflamed areas. Additionally, multinucleation with enlarged nuclei (nuclear atypia) containing prominent nucleoli (hyperchromasia) and dispersed chromatin was similar to benign tumor cells (Fig. 2f; DEN + CCl_4). DEN + CCl_4 group mice with subsequent isorhamnetin administration rescued liver morphology. A significant decrease in the thickening of liver plates, the appearance of portal tracts, and normal cellular and nuclear shapes were observed upon isorhamnetin treatment (Fig. 2f; (DEN + CCl_4) + IRN). We found a similar effect of isorhamnetin when compared to doxorubicin in protecting the liver against carcinogen-induced tumors (Fig. 2f; (DEN + CCl_4) + Doxo).

Isorhamnetin shows anti-TNF-α properties and inhibits pro-inflammatory cytokines to regress HCC

Since inflammation is among the initial steps of HCC development [35], we analyzed the status of tumor necrosis factor (TNF-α) which is a key mediator of tumor-promoting inflammation [36]. Western blot results showed a significant upregulation in the levels of TNF-α in DEN +CCl_4-treated mice liver and isorhamnetin reduced the levels of TNF-α comparable to the control and doxorubicin groups (Fig. 3a, S3a). We then validated the western blot result by checking the TNF-α at the mRNA level through a qPCR experiment. Results presented that isorhamnetin reduced the elevated mRNA levels of TNF-α in DEN +CCl_4-exposed mice near

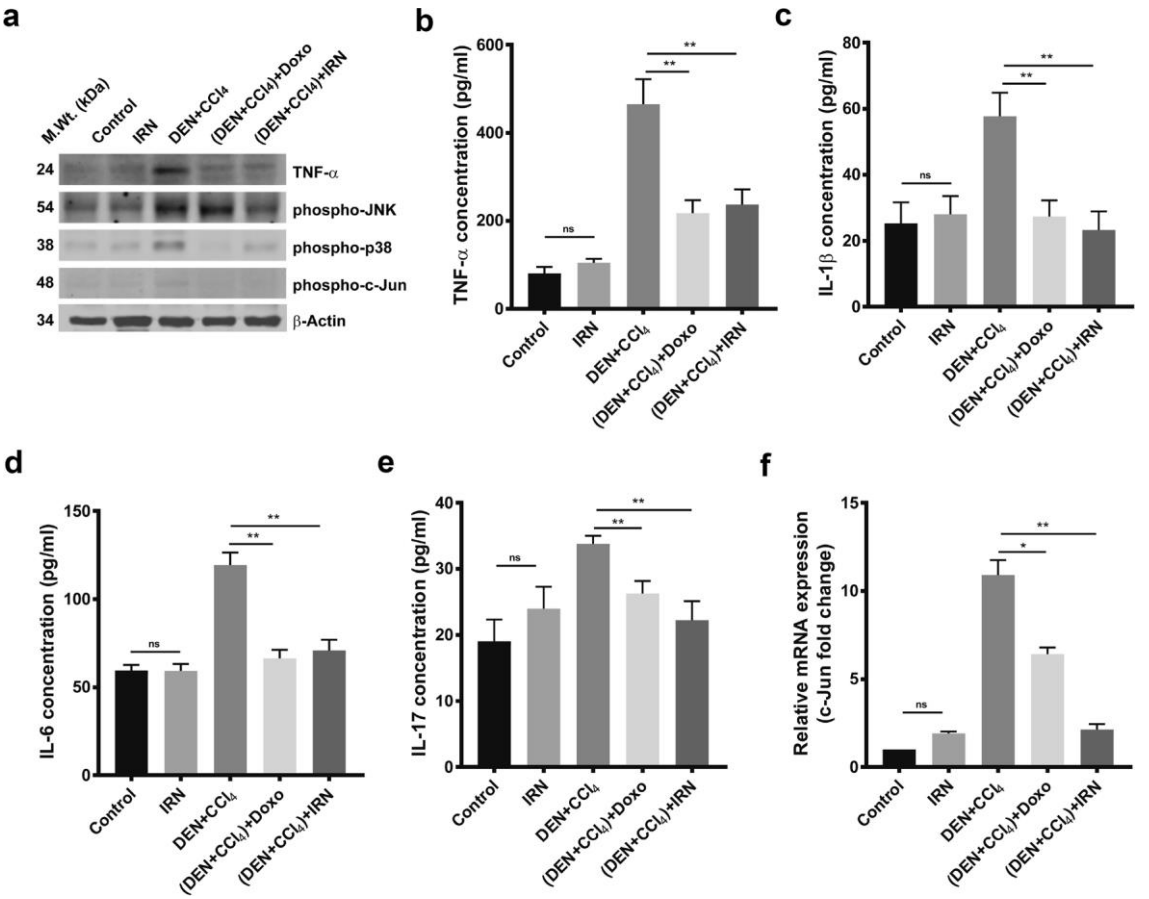

Fig. 3 Isorhamnetin shows anti-inflammatory effects to regress HCC. **a** Representative immunoblot images of indicated proteins. **b**–**d** Graphs represent the serum levels of inflammatory cytokines TNF-α **b**, IL-1β **c**, IL-6 **d**, and IL-17 **e** of indicated groups. **f** qPCR analysis of c-Jun in all experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, non-significant variance existed between the control and IRN group, $*P<0.05$ and $**P<0.001$

the levels of control and doxorubicin groups (Fig. S3.b). We validated the findings further by looking at TNF-α-induced interleukins using an ELISA. In the carcinogen-exposed mice group, we found higher TNF-α levels (Fig. 3b), as well as an increase in IL-1β, IL-6, and IL-17 levels. However, the expression of these pro-inflammatory cytokines was reduced by isorhamnetin administration (Fig. 3c–e).

Next, we tried to find the regulatory molecule for DEN + CCl_4-induced TNF-α expression. Several findings pointed toward MAP kinase activation since it induces TNF-α-mediated hepatitis, the most common risk factor in HCC induction [37]. Therefore, we analyzed JNK and p38 activation in DEN and isorhamnetin-administered mice and the results showed elevated levels of phospho-JNK and phospho-p38 in DEN + CCl_4-treated groups (Fig. 3a, S3a). Their levels were reduced by subsequent isorhamnetin treatment in the carcinogen-exposed mice group. Interestingly, isorhamnetin showed better results than doxorubicin in suppressing JNK.

During HCC initiation, c-Jun activation blocks p53-induced apoptosis and helps in tumor cell survival [38]. Additionally, c-Jun protects tumor cells from TNF-α-induced cell death by hindering p53 activation [38]. Therefore, we analyzed the phospho-c-Jun levels by western blotting which indicated elevated activation of c-Jun in DEN + CCl_4-exposed mice (Fig. 3a, S3.a). This c-Jun activation was reduced by isorhamnetin treatment, most probably to inhibit HCC induction and induce p53-mediated hepatocyte apoptosis. The mRNA levels of c-Jun were also analyzed by qPCR. We observed that carcinogen-induced c-Jun expression was decreased by isorhamnetin administration and showed better results than doxorubicin (Fig. 3f).

Isorhamnetin upregulates PPAR-γ by inhibitingMAPK & TGF-β signaling

To check the possible role of isorhamnetin in HCC regression by modulating PPAR-γ, we analyzed its mRNA expression and found a decrease in mRNA expression in DEN + CCl_4-treated mice group which was increased by isorhamnetin treatment (Fig. 4b). These findings were confirmed at the protein level, revealing a significant downregulation of PPAR-γ in HCC mouse livers, which was reversed by isorhamnetin administration (Fig. 4a, S4).

Because C/EBP-δ inhibition is required for TNF-α-mediated PPAR-γ regulation [39], we used western blotting to examine its protein level and discovered that C/EBP-δ was downregulated in HCC mouse liver samples that were upregulated by isorhamnetin treatment (Fig. 4a, S4). Incidentally, Isorhamnetin outperformed doxorubicin in terms of upregulating C/EBP-δ. TNF-α also reduces PPAR-γ expression by increasing MAPK signaling [40]. Therefore, we checked different MAPK in western blot experiments. In addition to our previous results (Fig. 4a), isorhamnetin downregulates ERK levels suggesting possible crosstalk between TNF-α, PPAR-γ, and MAPK signaling (Fig. 4a, S4).

PPAR-γ plays an inhibitory role for TGF-β which is a crucial factor in the maintenance of cancer cell growth, migration, and EMT during HCC progression [41]. Immunoblot results confirmed the upregulation in TGF-β levels in DEN + CCl_4-treated mice groups (Fig. 4a, S4). The levels of TGF-β became low as PPAR-γ was upregulated by isorhamnetin administration. We found that isorhamnetin is more effective than doxorubicin in suppressing TGF-β. TGF-β can induce phosphorylation of Stat3 which has a pivotal role in the early stage of HCC and protect cancerous cells from immune cells by suppressing the immune system of our body. We, therefore, found an increase in the phosphorylation status of Stat3 in our western blot results (Fig. 4a). TGF-β increases the levels of MMP-9, a key protein to enhance the aggressiveness of HCC [42]. Additionally, PPAR-γ plays an inhibitory role toward MMP-9 [43]. Therefore, we checked MMP-9 by IHC and found it to be upregulated in HCC liver sections, whereas isorhamnetin suppressed the levels of MMP-9 in carcinogen-treated mice comparable to the control group (Fig. 4c).

Nrf2 signaling is negatively regulated by isorhamnetin to increase ROS load in tumor cells

We checked whether isorhamnetin regulates Nrf2-Keap1 signaling to regress HCC development and progression. We observed an upregulated protein level of Nrf2 in DEN + CCl_4-induced liver cancer tissues which was reverted to near control levels by isorhamnetin treatment (Fig. 5a, S5). We also evaluated the changes in Nrf2 expression at the mRNA level by qPCR, which validated the western blot results (Fig. 5b). To further confirm the Nrf2 changes in HCC versus isorhamnetin-treated liver samples, we tested the Keap1 protein level, which is the upstream regulator of Nrf2 [44]. The results showed a downregulation of Keap1 in HCC tissue, whereas it was upregulated by isorhamnetin treatment (Fig. 5a, S5). The outcomes of the isorhamnetin treatment were comparable to those of the control and doxorubicin groups. We checked the levels of some proteins regulated by Nrf2, such as HO-1 and SOD2. Upregulation of SOD2 and HO-1 levels was observed in DEN + CCl_4-treated livers where Nrf2 levels were high (Fig. 5a, S5). In contrast to HCC samples, SOD2 and HO-1 levels were downregulated by isorhamnetin treatment in the carcinogen-exposed group.

We investigated whether isorhamnetin regulates Nrf2 via Akt and ERK modulation. Both the proteins, Akt (Fig. 5a, S5) and ERK (Fig. 4a) were found to be upregulated in HCC liver samples and their downregulation was observed in the isorhamnetin-treated liver. We checked some other

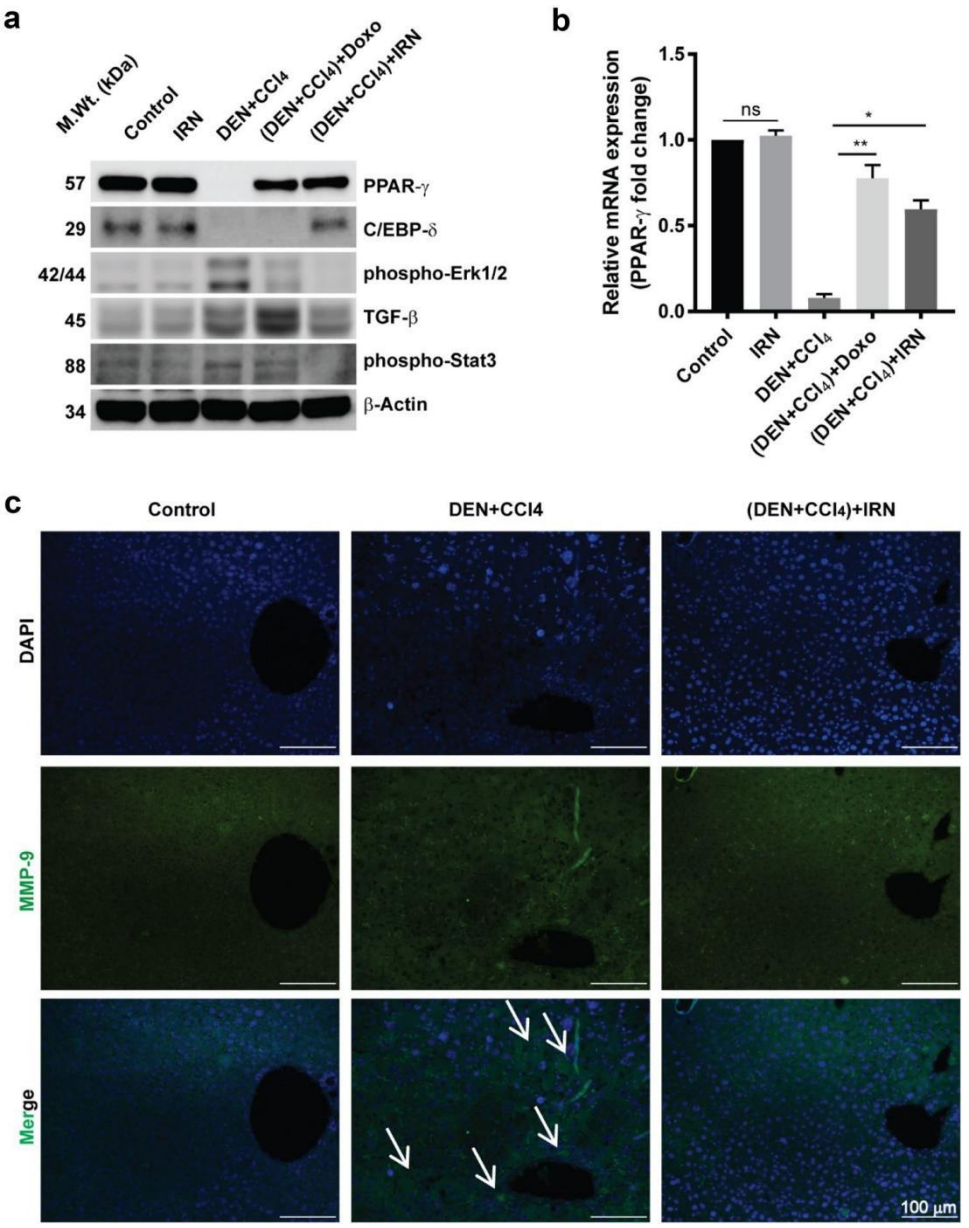

Fig. 4 Isorhamnetin regulates PPAR-γ through MAPK and TGF-β. **a** Representative immunoblot images in liver lysates of different groups. **b** qPCR analysis of PPAR-γ in all experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. Graph represents mean fold change ± SD for three different experimental sets and non-significant variance existed between control and IRN group, $*P < 0.05$, $**P < 0.001$, and $***P < 0.0002$. **c** Representative images of MMP-9 immunostaining of liver sections from different groups (arrows indicate high MMP-9 in DEN + CCl_4 group). Magnification 40X; Scale bar, 100 µm

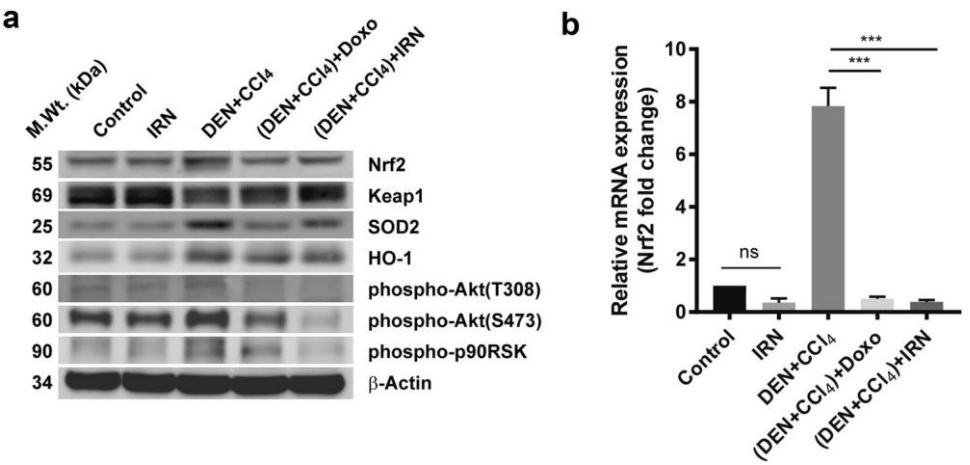

Fig. 5 Isorhamnetin suppresses Nrf2 signaling. **a** Representative immunoblot images of indicated proteins in liver lysates of different groups. **b** qPCR analysis of Nrf2 in experimental groups. Expression was normalized against GAPDH and fold change was calculated with respect to control. Graphs represent mean fold change ± SD for three different experimental sets, and non-significant variance existed between control and IRN group, $***P < 0.0002$

proteins of ERK signaling to investigate additional functions of it besides regulating Nrf2. We checked the status of RSK, which is the principal effector of ERK signaling and regulates cancer cell migration and invasiveness [45]. Phosphorylation of RSK was significantly upregulated in HCC liver indicating its high activity in HCC livers, whereas this activity was suppressed by isorhamnetin by reducing the phosphorylation status of RSK (Figs. 5a, S5).

Isorhamnetin decreases cancer cell metabolism and cell cycle progression and induces cell death to regress HCC

Since Akt was activated in DEN-induced HCC liver tissues and its levels were reduced by the use of isorhamnetin (Fig. 5a), we checked the phosphorylation status of some of its target proteins such as GSK-3β, cyclins, Bad, and mTOR by western blot. Results indicated a significant increase in the inhibitory phosphorylation of GSK-3β in DEN + CCl_4-induced HCC liver tissue which became significantly low by isorhamnetin administration (Figs. 6a, S6). Similarly, Akt induced the phosphorylation of Bad in DEN+CCl_4-treated HCC liver tissues which was suppressed

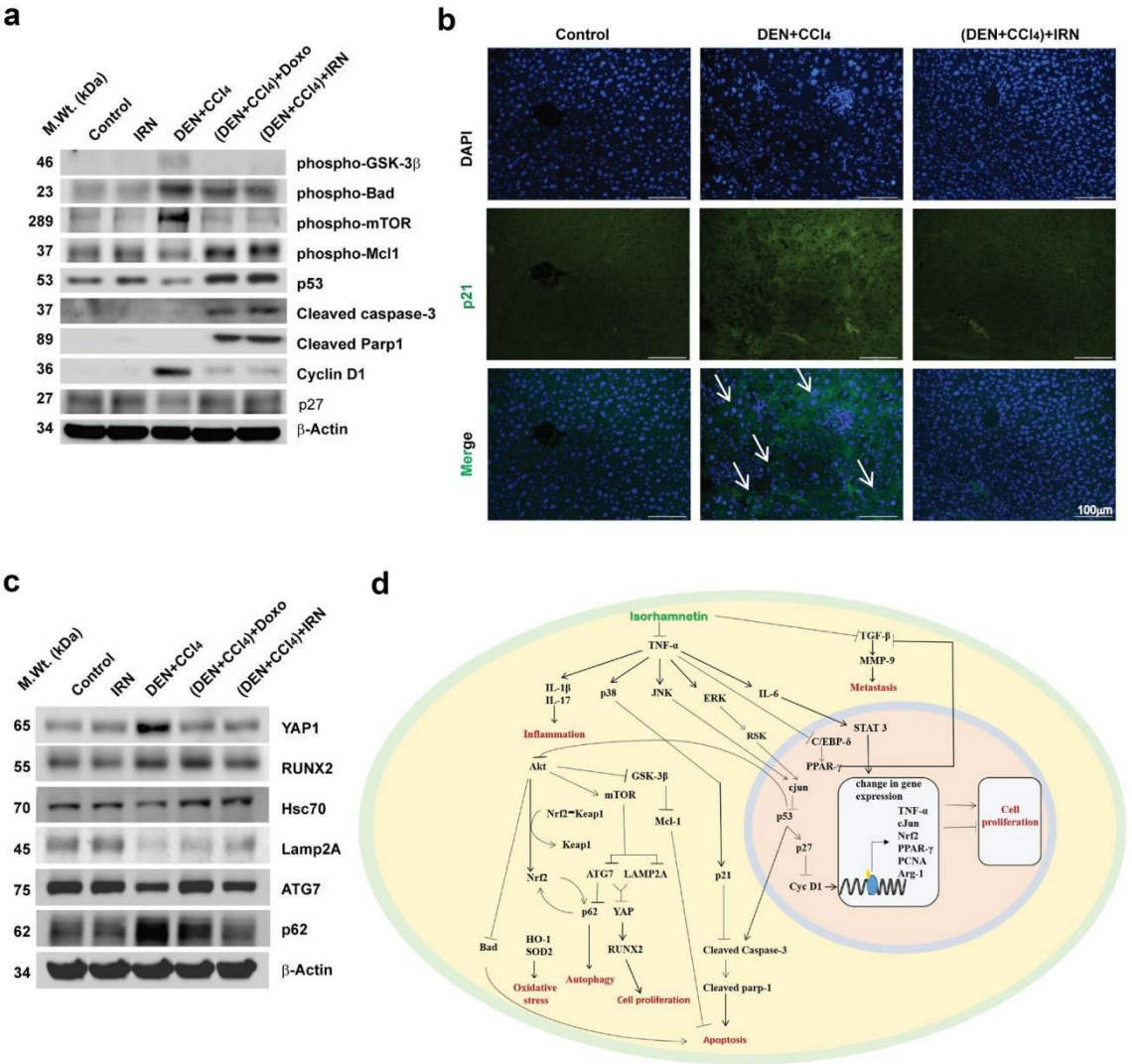

Fig. 6 Isorhamnetin disrupts cancer cell homeostasis. **a** Representative immunoblot images of indicated proteins in liver lysates of different groups. **b** Representative images of p21 immunostaining of liver sections from different groups (arrows indicate cytoplasmic p21 in DEN+CCl_4 group). Magnification 40X; Scale bar, 100 μm. **c** Representative immunoblot images of indicated proteins in liver lysates of different groups. **d** Model representing possible signaling pathways and their crosstalks regulated by isorhamnetin to inhibit DEN+CCl_4-induced HCC in Swiss albino mice

by isorhamnetin, most probably to induce apoptosis in cancer cells (Figs. 6a, S6). In contrast, Akt phosphorylated mTOR to induce its activation which was reduced to normal levels by isorhamnetin administration (Figs. 6a, S6).

Many cancer therapies include Mcl-1 degradation to induce apoptosis in cancer cells [46]. It is phosphorylated by GSK-3β. Isorhamnetin increases its phosphorylation via GSK-3β, therefore, degradation of Mcl-1, so that cancer cells undergo apoptosis (Figs. 6a, S6). To further confirm the apoptosis-inducing effects of isorhamnetin in the carcinogen-exposed liver, we checked the activation of caspase-3 and PARP1. Cleavage and therefore activation of both the proteins were observed in isorhamnetin-administered carcinogen-treated mice liver (Fig. 6a) suggesting apoptosis-inducing effects of isorhamnetin in cancer cells. These effects were similar to those observed in doxorubicin treatment. We also checked the levels of p53 which is the regulator of caspase-mediated apoptosis. Levels of p53 were found to be upregulated in carcinogen-exposed doxorubicin and isorhamnetin-treated groups (Figs. 6a, S6), suggesting that isorhamnetin treatment activates p53 to induce caspase and PARP-mediated cancer cell death.

To further evaluate the effect of isorhamnetin on cell cycle regulation, we checked the levels of different cell cycle regulatory proteins. Cyclin D1 overexpression is associated with HCC and is sufficient to promote HCC cell cycle progression even in the absence of mitogens [47]. Activation of ERK increased cyclin D1 levels in HCC livers and these levels were restored by isorhamnetin treatment (Figs. 6a, S6). We also examined other cell cycle regulators like p27, a tumor suppressor protein [48]. Isorhamnetin induces the expression of p27 which is otherwise suppressed in HCC liver tissues to promote tumor cell growth (Fig. 6a, S6). Next, levels of p21 protein were investigated, the upregulation of which has already been reported in HCC [49]. p21 is majorly expressed in the cytoplasm and its expression can be found in the nucleus to a lesser extent [50]. Cytoplasmic p21 inhibits caspase-3 to inhibit apoptosis [51]. In our HCC liver tissues, we found a significant increase in p21 cytoplasmic staining intensity, while isorhamnetin treatment downgrades its level (Fig. 6b).

Autophagy-mediated YAP1 degradation is restored by isorhamnetin to inhibit HCC progression

The western blot results showed an upregulation of YAP protein levels in the DEN + CCl_4-exposed mice group (Figs. 6a, S7). On the contrary, YAP levels were decreased by the administration of isorhamnetin after carcinogen treatment which was similar to the effects of doxorubicin. Next, we checked the levels of RUNX2, which is a YAP-interacting DNA-binding protein. RUNX2 was also upregulated similarly to YAP in the DEN+CCl_4-treated mice group and its levels were downregulated by isorhamnetin (Fig. 6a, S7). We also sought to identify the YAP-regulating proteins and a literature survey suggested that inhibition of chaperon-mediated autophagy (CMA) is associated with impaired degradation of YAP [52]. Lamp2A was downregulated in the DEN + CCl_4-treated mice group which suggests a possible reason for YAP1 upregulation due to its reduced degradation by autophagy (Figs. 6c, S7). Similar to Lamp2A levels, Hsc70 was also downregulated by carcinogen exposure (Fig. 6c). Isorhamnetin administration in the carcinogen-exposed group upregulated the levels of Lamp2A and Hsc70 near those of control and doxorubicin groups (Fig. 6c), suggesting that isorhamnetin could induce CMA to inhibit YAP1-mediated tumor growth.

The influence of isorhamnetin on macroautophagy was also checked in HCC mice models. ATG7 levels were found to be downregulated by the HCC-inducing carcinogen group, whereas isorhamnetin reversed this phenomenon (Fig. 6c, S7). The level of p62 was significantly upregulated in the mice group where autophagy was downregulated with an associated increase in YAP1 and RUNX2 in the carcinogen-treated group (Fig. 6c, S7). Isorhamnetin administration in the carcinogen-treated group decreased p62 accumulation up to the levels of the control and doxorubicin group indicating that isorhamnetin could upregulate autophagy to regress tumor growth.

Discussion

The study dissects the mechanism of anti-cancer and hepatoprotective properties of isorhamnetin. Our results suggest that isorhamnetin shows anti-inflammatory properties by decreasing TNF-α, IL-1β, IL-6, and IL-17 levels (Fig. 6d). High TNF-α expression causes sorafenib resistance in HCC patients, despite the fact that sorafenib was the first drug approved for the treatment of advanced stage HCC [53, 54]. TNF-α inhibition has been shown to reduce epithelial–mesenchymal transition (EMT) and thus overcome sorafenib resistance [54]. Our findings suggest that isorhamnetin's TNF-α inhibitory property could be used to improve sorafenib resistance in HCC patients.

Several inflammatory cytokines including TNF-α inhibit PPAR-γ, which aid in inflammation and cancer progression [55]. Our results suggest that DEN + CCl_4 treatment induces hepatocellular carcinoma in mice by upregulating the levels of ERK, JNK, and p38 MAP kinases and by downregulating the PPAR-γ levels. TGF-β is also upregulated due to decreased inhibitory effects of PPAR-γ to promote metastasis. Our results also suggest that isorhamnetin treatment in carcinogen-exposed mice reduces the MAP kinase and TGF-β signaling and induces PPAR-γ signaling to inhibit tumor growth and metastasis (Fig. 6d). Although

doxorubicin use is a safety measure in many cancers' chemotherapeutic regimes, it activates a number of proteins that are supportive of tumor growth. Doxorubicin activates TGF-β signaling in human and murine breast cancer cells resulting in the induction of metastasis which is one of the side effects of doxorubicin administration. Therefore, there is a need for some other drug in combination with doxorubicin that can suppress the adverse effects caused by it. Our results indicate that isorhamnetin has inhibitory effects on TGF-β signaling suggesting that the use of isorhamnetin along with doxorubicin could overcome the side effects of doxorubicin and suppress EMT. This TGF-β inhibitory property of isorhamnetin is mediated by PPAR-γ and ERK signaling (Fig. 6d).

Isorhamnetin also induces oxidative stress to induce tumor cell death. Elevation of oxidative stress is mediated by downregulating Nrf2 and its targets such as HO-1 and SOD2 and upregulating its inhibitor Keap1 (Fig. 6d). Identification of Nrf2 inhibitors is ongoing research in the treatment of liver cancer as Nrf2 is a key player in HCC progression [44]. Hence, inhibition of Nrf2 signaling by isorhamnetin could be used as a treatment for HCC.

Isorhamnetin-induced tumor cell death involves the upregulation of Bad, cleaved caspase-3, and cleaved-PARP and degradation of Mcl-1 proteins (Fig. 6d). Isorhamnetin alleviates the inhibitory role of Akt on Bad by inactivating Akt through p53 (Fig. 6d). Additionally, isorhamnetin-mediated p53 upregulation caused activation of caspase-3 and PARP1 signaling to induce cancer cell death in the carcinogen-exposed mice group (Fig. 6d). Since activation of caspase-3 is also mediated by cytoplasmic p21 to inhibit tumor cell apoptosis, cytoplasmic p21 in post-isorhamnetin-treated mice would no longer be available to inhibit caspase-3 (Fig. 6d). Upregulation of autophagy by isorhamnetin could be an additional pathway to induce cell death in our HCC mice model. Hippo signaling regulates organ size and tissue growth, in which YAP acts as a transcriptional co-activator. YAP deregulation has been observed in hepatocellular carcinoma. An efficient drug is needed to overcome the YAP-induced HCC progression and chemoresistance. Our results suggest that elevated autophagy by isorhamnetin regulates YAP1 degradation to inhibit HCC development and progression (Fig. 6d). Upregulation of p27 and downregulation of PCNA and cyclin D1 in the isorhamnetin group suggest that it inhibits cell cycle progression of liver cancer cells (Fig. 6d). These changes exhibit the chemopreventive effects of isorhamnetin in mice with hepatocellular carcinoma.

Conclusion

In this study, isorhamnetin administration displayed chemopreventive properties in DEN+CCl_4-induced HCC mice. Isorhamnetin treatment reduces the serum AFP, arginase-1, ALT, AST, ALP, and bilirubin indicating a potential hepatoprotective effect upon carcinogen exposure. The molecular mechanism of isorhamnetin-mediated HCC regression involves the suppression of diverse cellular pathways that are known to support tumor initiation and progression. We observed downregulation of MAP kinases, mTOR, GSK-3β, YAP1, and Nrf2 signaling while elevated autophagy proteins, apoptotic signaling proteins, and tumor suppressor proteins. Importantly, the anti-TNF-α properties of isorhamnetin could prove it a valuable therapeutic agent in sorafenib-resistant HCC patients. Alternatively, anti-TGF-β properties of isorhamnetin could be utilized to reduce the EMT-inducing side effects of doxorubicin. Our studies suggest that the administration of isorhamnetin might be a promising treatment for liver cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12032-023-02050-5.

Acknowledgements The authors are deeply grateful to Ms. Noyel Ghosh and Ms. Ankita Mandal for their valuable inputs during the preparation of the manuscript.

Author contributions SS contributed to conceptualization, methodology, software, validation, data curation, formal analysis, investigation, writing of the original draft, visualization, and writing, reviewing, & editing of the manuscript. AKD contributed to methodology, validation, and writing, reviewing, & editing of the manuscript. SB contributed to conceptualization. RG contributed to conceptualization, validation, data curation, formal analysis, visualization, and writing, reviewing, & editing of the manuscript. PCS contributed to conceptualization, validation, data curation, formal analysis, visualization, and writing, reviewing, & editing of the manuscript. All authors read and approved the final manuscript.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration

Competing interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All the animal experiments were prosecuted according to the institutional ethical committee and with the permission of IAEC, CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals) and the Ministry of Environment and Forests, New Delhi, India [1796/GO/EReBiBt/S/14/CPCSEA].

Consent to participate Not applicable.

Consent to publish Not applicable.

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