2.1. INTRODUCTION

2.1.1. HEPATOCELLULAR CARCINOMA (HCC)

Globally, liver cancer stands fifth among cancer types and is the second most common cause of cancer mortality. HCC, the major histological subtype and one among the most aggressive human cancers, is associated with multiple risk factors, including hepatitis B and C virus infection, alcohol consumption, obesity and diet contamination (Jemal et al., 2011). In India, the incidence of HCC is seen rising 1.6% per year with mortality rate of 6.8/100,000 for men and 5.1/100,000 for women (Kumar et al., 2014). More than 80% of these HCC cases were associated with chronic hepatitis C (Sievert et al., 2011) and active hepatitis B viral infections (Batham et al., 2007) which are sufficient for the tumour initiation and development of HCC (Kumar et al., 2014). All these significant epidemiological factors ensure further worldwide rise in HCC incidence over the next decade (Kumar et al., 2014), eventually leading to urgency in understanding HCC development in diseased liver. Hence, an effective approach for the prevention of HCC is highly needed with the focus on the molecular regulators of the liver disease through the inflammation-fibrosis-cancer axis.

2.1.1.1. INFLAMMATION-FIBROSIS-CANCER (IFC) AXIS.

Chronic liver injury is associated with the continuous expression of cytokines and recruitment of immune cells, eventually end up releasing free radicals causing DNA damage, thus fostering the neoplasic transformation. This neoplasic transformation includes structural and/or functional modifications of proteins involved in cell-cycle control, apoptosis, oxidative stress, lipid peroxidation and DNA repair damage (Budhu et al., 2006 and Leonardi et al., 2012), followed by hepatocyte proliferation and wound healing response mediated by stellate cells leading to liver fibrosis and eventually result in HCC (Farazi et al., 2006 and Stauffer et al., 2012). Hence, determining the molecular
drug target for an effective HCC treatment should be carried out in preneoplastic lesion, fibrosis and HCC axis.

2.1.1.1. PpReNEOPLASTIC LESION

Any injury to liver in due course leads to tissue repair and regeneration involving deregulated growth and death of damaged hepatocytes. The high cell turnover influences severe changes in the liver mainly characterized by the development of preneoplastic lesions which will eventually develop into HCC (Borzio et al., 1998). In animal experiments of early stages of hepatocarcinogenesis, the oxidative stress associated with inflammation caused by the hepatocarcinogen induces genomic instability, rearrangements and mutations (Hussain et al., 2007) leading to uncontrolled transactivation of major cellular pathways such as NFkB, β-catenin, p53, EGF, HGF and TGFβ (Arsura et al., 2005, Branda and Wands, 2006 and Villanueva et al., 2007). Likewise, numerous studies in recent times have shown the animal models recapitulating early preneoplastic alterations like that of human liver carcinogenesis showcasing the evolution of preneoplastic lesions to malignancy and molecular alterations of signal transduction pathways (Farazi and De Pinho, 2006, Feo et al., 2006, Lee and Thorgeirsson, 2006 and Feo et al., 2008). Therefore, studies on the role of predisposing genes in the deregulation of key pathways in the early stages of hepatocarcinogenesis will impact detailed knowledge leading to development of novel therapies for HCC.

2.1.1.1.2. FIBROSIS

Liver fibrosis is the result of the wound-healing response of the liver to repeated injury, where the liver parenchymal cells regenerate and replace the necrotic or apoptotic cells. Upon chronic liver insults, the liver regeneration fails and the hepatocytes are replaced with abundant extracellular matrix (ECM) proteins including fibrillar collagens (I & III) (Bataller et al., 2005). Accumulation of these ECM proteins distort the liver architecture by forming fibrous scar, eventually leading to cirrhosis, associated with the development of nodules of
regenerating hepatocytes and organ contraction (Friedman, 2003 and Lotersztajn et al., 2005).

Hepatic stellate cells (HSCs) are the principal fibrogenic cell types of the liver. Upon injury, HSCs undergo a well-characterized activation process during which they lose their characteristic vitamin A and lipid stores and obtain a myofibroblastic phenotype (Bataller et al., 2005 and Friedman et al., 2008). In addition, some transcription factors such as NF-κB, c-myb, Sp-1, STAT-1 also favour HSC activation (Kawada et al., 1997). On activation, HSCs generate α-smooth muscle actin (α-SMA)-positive myofibroblast like cells that are responsible for the deposition of majority of excess ECM in the fibrotic liver forming fibrous scar (Friedman, 1993 and Eng et al., 2000). Based on current experimental evidences, the targets of antifibrotic therapy include reducing the inflammation and immune responses that trigger fibrosis, inducing apoptosis of activated HSC thereby downregulating HSC activation and inhibiting stellate cell proliferation, that eventually increase the degradation of ECM, thus resulting in regression of liver fibrosis (Issa et al., 2004).

2.1.2. Experimental model for liver preneoplastic lesion, liver fibrosis, liver cancer

The use of experimental animal models has made significant contributions towards understanding the cellular and molecular mechanisms underlying liver preneoplastic lesions, fibrosis and HCC. Numerous hepatotoxins were used to experimentally induce preneoplastic lesions, liver fibrosis and HCC in animal models. Among them, diethylnitrosamine (DEN) is hailed as an ideal hepatocarcinogen to induce HCC in rodents, which showed similar biochemical, histological, molecular expression profiles (Feo et al., 2000) and proceeds in similar stages to that of human liver cancer i.e., formation of preneoplastic foci, neoplastic nodules and HCC nodules (Peto et al., 1991).
2.1.2.1. DEN

DEN, an important environmental carcinogen of the N-nitrosamine class is a light-sensitive, volatile, clear yellow oil that is soluble in water, lipids and other organic solvents.

**Physical properties**

Molecular Weight - 102.135 g/mol  
Molecular Formula - C₄H₁₀N₂O  
Boiling Point - 175-177°C  
Density - 0.9422 at 20 °C

![Chemical structure of DEN](image)

**Chemical structure of DEN**

DEN, an established liver carcinogen is majorly seen in tobacco smoke, ground water, cured and fried meats, cheddar cheese, alcoholic beverages, occupational settings, cosmetics, agriculture chemicals and pharmaceutical products (Sivaramakrishnan et al., 2008 and Gupta et al., 2010). DEN induced progression of HCC in Wistar rats is considered the most accepted and widely used experimental model to study liver carcinogenesis (Ha et al., 2001). Human livers metabolize nitrosamines similar to that of rat liver and also exhibit considerable similarities with regard to morphology, genomic alterations and gene expression, despite their different disease etiologies (Feo et al., 2000). DEN metabolism in the liver by cytochrome 2E1 (CYP 2E1) generates reactive oxygen species (ROS) causing oxidative stress by the formation of hydrogen peroxide and superoxide anions (Verna et
DEN, being a genotoxic carcinogen, forms alkyl DNA adducts, induces chromosomal aberrations, micronuclei and sister chromatid exchanges in the rat liver (Jagadeesh et al., 2009). These mutations induced by DEN are responsible for the development of hepatocarcinogenesis (Mandal et al., 2008). Also, DEN causes perturbations in the nuclear enzymes involved in DNA repair/replication (Sivaramakrishnan et al., 2008). The application of DEN as the primary liver carcinogen has depicted numerous key cellular pathways involved in HCC development and also an asset in identifying new molecular targets for HCC treatment (Naugler et al., 2007 and Grivennikov et al., 2010).

2.1.3. Novel Therapeutic Targets for HCC

Studies with experimental animals have largely contributed to the better understanding of the pathogenesis of liver diseases by enlightening the role of various signaling pathways that link chronic liver inflammation to HCC. Among them, the most altered pathways include Wnt/β-catenin, NF-κB, p53, EGF, TGFβ/Smad and Hippo/YAP pathways where they play a crucial role in liver homeostasis and progression of liver cancer (Villanueva et al., 2007, Han et al., 2012 and Yu et al., 2015).

2.1.3.1. NF-κB Signaling

The NF-κB pathway is a highly conserved evolutionary pathway, with key functions in the regulation of immune and inflammatory responses in mammals (Xiao et al., 2005). NF-κB, a hetero or homodimer of various members of the Rel family of DNA-binding proteins, regulates the transcription of genes containing κB binding sites. The human NF-κB family consists of five cellular DNA-binding subunits: p50, p52, cRel, p65 (Rel A) and Rel B, all share an N-terminal Rel homology domain (RHD), mediating dimerization, interaction with the inhibitor of κB (IκB) proteins, nuclear translocation and DNA binding (Schmitz et al., 2004). In addition, p65, RelB and cRel contain C-terminal transactivation domains that trigger gene transcription. NF-κB p65 contains two
potent transactivation domains within its C-terminus and is probably the strongest activator of most genes with κB sites (Schmitz et al., 1991). Two other members of the human NF-κB family, p52 and p50, become active DNA-binding proteins after cleavage of their larger precursors p100 and p105, respectively, which activate transcription when they form heterodimers with subunits that contain transactivation domains (p65, Rel B or c Rel), but may also repress gene transcription when forming dimers without a transactivation domain (p50-p50 homodimers) (Haecker et al., 2004 and Saha et al., 2008).

NF-κB is activated by various stimuli such as pathogen-derived molecules (such as lipopolysaccharide (LPS), viral and bacterial DNA and RNA that stimulate Toll-like receptors (TLRs), as well as inflammatory cytokines such as tumour necrosis factor-α, interleukin-1 (Karin et al., 2005 and West et al., 2006) resulting in the initiation of inflammatory, immune and wound-healing responses and the clearance of pathogens. The activation of NF-κB leads to the transcription of genes with κB binding sites, which are involved in the regulation of inflammation, immune responses and cell survival (Pahl et al., 1999).

2.1.3.1.1. NF-κB LINKS LIVER INJURY, FIBROSIS AND HCC

Several studies have validated the hypothesis of inflammation associated with chronic liver disease promoting HCC progression (Karin et al., 2009, Mencin et al., 2009 and Wasmuth et al., 2010). NF-κB modulates liver fibrogenesis predominantly in three cellular compartments; firstly by regulating hepatocyte injury, the primary trigger of fibrogenic responses, secondly by regulating inflammatory signals elicited in macrophages and other inflammatory cells in the liver and finally by regulating fibrogenic responses in HSCs (Luedde et al., 2011). NF-κB also regulates three key aspects of HSC biology such as activation, survival and inflammatory responses, which eventually drive fibrosis to HCC. Reports showed NF-κB as a key mediator of HSC survival during fibrogenic responses, as demonstrated by the ability of NF-κB inhibitors (sulfasalazine) or NF-κB inhibitory NEMO-binding peptide to promote HSC apoptosis (Oakley et al., 2005 and Oakley et al., 2009).
Genetic ablation of NF-κB regulators, by itself, is sufficient to initiate and promote liver carcinogenesis in experimental animals and does not require any mutations in the oncogenes or tumour suppressor genes, emphasizing the critical role of NF-κB in the progression of liver diseases (Schmitz et al., 2004, Luedde et al., 2007 and Inokuchi et al., 2010). In liver, NF-κB mediates key functions like influencing the survival of hepatocytes, inflammation in Kupffer cells, inflammation and activation of HSCs and cell survival. Overall, NF-κB plays a key role in the inflammatory and anti-apoptotic pathways during HCC progression and the animal models with chronic injury, inflammation and fibrosis will ensure better understanding.

2.1.3.2. Wnt/β-CATENIN SIGNALING

The Wnt pathway plays an important role in liver development and regeneration (Nejak-Bowen et al., 2011). β-catenin, the effector molecule of wnt pathway is inhibited by a protein complex that includes axin, GSK-3β and APC (Dajani et al., 2003 and Ha et al., 2004). The binding of Wnt protein to the Frizzled receptor activates the Dishevelled (DSH) protein which subsequently inhibits the destruction complex (axin/GSK-3/APC) thereby stabilizing β-catenin in the cytoplasm. The stabilized β-catenin enters the nucleus where it binds to TCF/LEF transcription factor promoting the expression of specific genes such as cyclin D1, Myc and TCF-1 (Tetsu et al., 1999, Staal et al., 2001, Yochum et al., 2010 and Tauriello et al., 2012).

In liver, the activation of the canonical Wnt signaling causes tumour formation driven by liver stem cells (Takigawa et al., 2008 and Taniguchi et al., 2008). Aberrant nuclear accumulation of β-catenin has been observed in 17-40% of HCC cases, mainly due to the methylation of Wnt inhibitors, inactivation of GSK3β, Wnt3/Fz7 upregulation, mutations in β-catenin gene (19-44%), axin-1 (5-14%) and axin–2 (3-10%) (Thompson et al., 2007, Zucman-Rossi et al., 2007, Wang et al., 2008 and Breuhahn et al., 2009).
In rodents, the aberrant activation of Wnt/β-catenin signaling in hepatocyte leads to the development of HCC (Colnot et al., 2004). The activation of Wnt/β-catenin pathway was more strongly associated with progression than with tumour initiation (Osada et al., 1999) and appears to be a major oncogenic event in liver cancer. Hence, studies on possible roles of the Wnt/β-catenin pathway in tumour initiation/early HCC would further make it a strong candidate for molecular therapeutic interventions in liver diseases.

2.1.3.3. HIPPO SIGNALING

The hippo signaling cascade, composed of a highly conserved kinase cassette regulating Yap and TAZ, plays an important role in the organ-size control during embryonic development and conserved as a tumour suppressor pathway in mammals (Yin et al., 2011). The pathway is activated when the kinases Mst1/2 phosphorylate and activate the Lats1/2 kinases that in turn phosphorylate and inhibit Yap and TAZ (Pan et al., 2010 and Yu et al., 2013). Two cofactors, Salvador (SAV or WW45) and MOB1 are also involved as Mst and/or Lats partners. The mechanism of Yap/TAZ inhibition by phosphorylation is dual: degradation by the proteasome and/or sequestration in the cytoplasm through anchoring proteins. In the nucleus, Yap and TAZ do not bind DNA directly but are co-activators that regulate gene expression resulting in proliferation and organ growth, thereby associating with specific transcription factors, and in particular with TEAD factors. The inactivation of Hippo pathway could lead to excessive cell proliferation, inhibition of apoptosis resulting in carcinogenesis (Pan et al., 2010).

Deregulation of Hippo pathway is reported with high frequency in human carcinomas, including lung, colorectal, ovarian, liver and prostate cancers (Dong et al., 2007, Zhao et al., 2007 and Steinhardt et al., 2008). Studies in D. melanogaster showed, YKI (Yap) activity is hyperactivated in cells immediately adjacent to epithelial tissue wounds, suggesting that the hippo pathway is locally repressed to allow the proliferation and repair (Grusche et al., 2011). Therefore, the Yap and TAZ hyperactivation could be a driver of liver carcinogenesis.
Hippo pathway activity is tightly coupled to tissue and cellular architecture and function. Derangement of tissue architecture is a fundamental feature of solid human tumours, and thus might underlie the deregulated Hippo pathway activity that has been reported in different human cancers. The mechanical properties of tumours have been linked to their growth and progression (Levental et al., 2009). This implies that signaling pathways that are sensitive to the mechanical properties of their surrounding environment modulate tumour cell behaviour. Recent studies showed that the Hippo pathway controls organ size partly by responding to mechanical cues such as stretch and compression. In cultured mammalian cells, the Hippo pathway responds to the tensile state of the actin cytoskeleton; Yap and TAZ activity is promoted when cells are stretched, and repressed when cells are compressed (Dupont et al., 2011 and Wada et al., 2011). Also, studies in D. melanogaster epithelial tissues showed that YKI (Yap) drives tissue overgrowth in response to mutations that caused increased actin polymerization (Fernández et al., 2011 and Sansores-Garcia et al., 2011). This ability of the Hippo pathway to respond to mechanical cues might partially underlie the frequent hyperactivation of Yap and TAZ that has been observed in human tumours, as tumour tissue has altered mechanical properties owing to extracellular matrix modifications and the infiltration of stromal and immune cells (Butcher et al., 2009).

In fibrosis, epithelial to mesenchymal transition (EMT) describes the process of epithelial cells that undergo transdifferentiation toward a myofibroblast-like phenotype, a phenomenon observed in both cancer metastasis and fibrosis (Chapman et al., 2011 and De Craene et al., 2013). Upon injury, epithelial cells lose their characteristic cellular junctions and acquire a spindle-like morphology. Cells undergoing EMT often show increased motility, de novo expression of α-SMA, and elevated expression of ECM components, such as collagens and fibronectin. Also during EMT, evidences propose that TGF-β, Wnt and Yap/TAZ interact with each other to drive the transformation toward a
mesenchymal-like cell type (Varelas et al., 2008, Fujii et al., 2012 and Zhou et al., 2012).

Studies on Hippo pathway deregulation mostly showed immunohistochemical detection of Yap in the nucleus of tumour tissue (Steinhardt et al., 2008). Further studies also confirmed Yap localizing in nucleus in approximately 60% of HCCs (Xu et al., 2009), 15% of ovarian cancers (Zhang et al., 2011) and 65% of non-small-cell lung cancers (Wang et al., 2010), deliberating its oncogenic function. Somatic mutations, the amplification and overexpression of Yap have been reported in various human and murine tumours (Overholtzer et al., 2006 and Zender et al., 2006) and interestingly no mutations were found in the other key hippo kinases such as Mst1, Mst2, Lats1 and Lats2 (St et al., 1999, Lu et al., 2010 and Zhou et al., 2011).

Although direct mutations affecting the Hippo pathway seem to be uncommon, Hippo pathway activity is clearly perturbed through crosstalk with signaling pathways that frequently harbour oncogenic mutations. Multiple cancer associated signaling networks engage in regulatory crosstalk with the Hippo pathway, often at the level of the Yap and TAZ oncoproteins. The Wnt, TGF-β, bone morphogenetic protein (BMP), Hedgehog (HH), Notch and mTOR pathways are reported to functionally interact with the Hippo pathway. In contrast to the Hippo pathway, these pathways are frequently mutated in different human cancers (Irvine et al., 2012).

Evidences propose the deregulated transcriptional modulators of the TGF-β, Wnt and Yap/TAZ pathways are integral factors in the cross regulation between these pathways. Cytoplasmic retention of transcription factors and transcriptional activators proves to be an ingenious system through which the three different pathways tightly regulate their own and each other’s activity and drive the progression of liver diseases.
AIM OF THE STUDY

Recent studies from this laboratory have shown the hepatoprotective effects of morin in experimental liver diseases, where morin exhibited anti-cancer effects by regulating the expression of key inflammatory proteins, and, by fostering apoptosis, attenuated DEN induced HCC in Wistar rats (Sivaramakrishnan et al., 2009 and Sivaramakrishnan et al., 2010). Also, morin demonstrated anti-fibrotic effects by inhibiting HSC proliferation and inducing apoptosis in activated HSCs by suppressing Wnt/β-catenin and NFκB signaling (Madankumar et al., 2014 and Madankumar et al., 2015). However, the mechanistic actions of morin on the progressive stages of HCC still remain unclear. Hence, the main objective of this study was to investigate the molecular action of morin on dysregulated signaling pathways in all the three progressive stages in experimental HCC viz., preneoplastic lesion, fibrosis and HCC.

Liver preneoplastic lesion, fibrosis and HCC were induced in Wistar rats using DEN as described earlier (Sivaramakrishnan et al., 2009, Khan et al., 2011 and Madankumar et al., 2014). The activities of liver marker enzymes, lipid peroxide levels, protein carbonyl levels, antioxidant enzymes and liver architecture were monitored to cast light on the antioxidant and hepatoprotective nature of morin. To establish the anti-inflammatory, anti-fibrotic, anti-carcinogenic and apoptosis inducing effect of morin, the mRNA and protein levels of key signalling molecules and key apoptotic molecules which are strongly associated with the Hippo signaling pathway, NFκB and Wnt/β-catenin, were determined by qPCR, western blotting and immunofluorescence analysis.
2.2. MATERIALS AND METHODS

2.2.1. ANIMALS

Male Wistar rats of albino strain, each weighing about 200g, obtained from Kings Institute of Preventive Medicine (Guindy, Chennai), were used in this study. All rats were housed in polypropylene cages at controlled temperature (24 ± 4°C) with relative humidity (60 ± 5%) and proper lighting and fed with standard rat pellet diet (Hindustan Lever Ltd., Bangalore, India) and water, *ad libitum*. The rats received humane care and all experimental procedures were conducted in accordance with the guidelines set by the institutional ethical committee for the use of small animals in biomedical research at University of Madras, Chennai, India (IAEC No.03/02/2012 and IAEC No.01/01/2014).

2.2.1.1. DEN INDUCED PRENEOPLASTIC LESION, FIBROSIS AND HCC

DEN administration to rats resulted in acute liver injury with significant DNA damage to the liver cells, subsequently leading to inflammation and development of neoplastic liver lesions. Earlier reports from this laboratory have shown DEN administration to Wistar rats successfully induced the progressive stages of HCC viz., preneoplastic lesions, Fibrosis and HCC (Sivaramakrishnan *et al.*, 2009, Khan *et al.*, 2011 and MadanKumar *et al.*, 2014).

*Preneoplastic lesion model* - To induce preneoplastic lesion, male Wistar rats, weighing 200g were given intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight (BW)), in saline, once a week, for 3 alternate weeks, as previously described (Khan *et al.*, 2011).

*Fibrotic model* - To induce fibrosis, male Wistar rats, weighing 200g were given i.p. injection of DEN (100 mg/kg BW), in saline, once a week, for 6 weeks, as previously described (MadanKumar *et al.*, 2014).

*HCC model* - To induce HCC, male Wistar rats, weighing 200g were administered DEN (200 mg/kg BW) in drinking water, orally for 16 weeks, as previously described (Sivaramakrishnan *et al.*, 2009).
2.2.1.2. MORIN TREATMENT

The ideal dose for morin was determined earlier in a study where DEN induced Wistar rats, when treated with morin (50 mg/kg BW), showed maximum hepatoprotective efficacy (MadanKumar et al., 2014). For this study, morin (50 mg/kg BW) was administered to Wistar rats by oral gavage. Morin solution was prepared initially by dissolving morin in few drops of propylene glycol and finally suspended in water.

2.2.1.3. EXPERIMENTAL GROUPING OF RATS

Rats were randomly divided into ten groups, each group consisting of six rats as follows (Scheme 2.1).

**Group I (Control):** Rats received standard rat pellet diet and water, *ad libitum.*

**Group II (Preneoplastic lesion - morin control):** Rats received morin (50 mg/kg BW) orally, thrice a week, from third week till the end of the experimental period.

**Groups III (Preneoplastic lesion - induced):** Rats received i.p. injection of DEN (200 mg/kg BW, in saline), once a week, for three alternate weeks.

**Group IV (Preneoplastic lesion - morin treated):** Rats received DEN as in group III and morin as in group II, till the end of the experimental period.

**Group V (Fibrosis - morin control):** Rats received morin (50 mg/kg BW) orally, thrice a week, from fifth week till the end of the experimental period.

**Groups VI (Fibrosis - induced):** Rats received i.p. injection of DEN (100 mg/kg BW, in saline), once a week, for 6 weeks.

**Group VII (Fibrosis - morin treated):** Rats received DEN as in group VI and morin as in group V, till the end of the experimental period.
**Group VIII (HCC - morin control):** Rats received morin (50 mg/kg BW) orally, thrice a week, from tenth week till the end of the experimental period.

**Group IX (HCC - induced):** Rats received DEN (200 mg/kg BW, in drinking water), orally, for sixteen weeks.

**Group X (HCC - morin treated):** Rats received DEN as in group IX and morin as in group VIII, till the end of the experimental period.

**Scheme 2.1. Experimental regime for DEN induced progressive stages of HCC and morin treatment.**

**2.2.2. BIOCHEMICAL ANALYSIS**

**2.2.2.1. SERUM PREPARATION**

After the experimental period, the control and experimental rats were anesthetized with ketamine hydrochloride intravenously (30 mg/kg BW). Blood samples were collected and allowed for complete clotting of blood, then
centrifuged at 2500 rpm for 10 min at 4°C. The resultant clear supernatant (serum) was used for various biochemical analyses.

2.2.2.2. **10% LIVER HOMOGENATE PREPARATION**

1g of liver tissue from the control and experimental rats were cut into small pieces and homogenized in 10 ml of ice cold 0.1 M Tris - HCl buffer (pH 7.4). Protein content was estimated in this liver homogenate and biochemical assays were carried out.

2.2.2.3. **ESTIMATION OF TOTAL PROTEIN**

The protein content was estimated by the method of Lowry *et al.*, (1951).

**Reagents**

1. Alkaline copper reagent
   
   Solution A: 2% sodium carbonate in 0.1 N NaOH
   
   Solution B: 0.5% copper sulphate in water
   
   Solution C: 1% sodium potassium tartarate in water
   
   50 ml of solution A was mixed with 0.5 ml of solution B and 1.0 ml of solution C just before use.

2. Folin’s phenol reagent: Diluted 1:2 with distilled water just before use.

3. Standard BSA: 10 mg of BSA was dissolved in 100 ml of distilled water.

**Procedure**

0.1 ml of the serum/liver homogenate was taken in clean, dry test tubes containing 0.9 ml of distilled water. To this 4.5 ml of alkaline copper reagent was added and allowed to stand for 10 minutes. Finally 0.5 ml of Folin's phenol reagent was added to the above contents and incubated at room temperature for 20 minutes. Standard BSA solution with concentrations (20 - 100 µg), water
blank and tris blank were also treated in similar manner. The blue colour developed was read at 620 nm in a Shimadzu UV-Visible double beam spectrophotometer. The total protein content was expressed as mg/dl in serum and mg/g tissue in liver.

2.2.2.4. **ESTIMATION OF LIVER MARKER ENZYMES**

2.2.2.4.1. **ASSAY OF ASPARTATE AMINOTRANSFERASE**

Aspartate aminotransferase (AST) was assayed by the method of King *et al.*, (1965a).

**Reagents**

1. 0.1 M Phosphate buffer, pH 7.4
2. 1 mM DNPH in 2N HCl
3. 0.4 N NaOH
4. Substrate solution: 1.33 g of aspartic acid and 15 mg of α-oxoglutarate were dissolved in 100 ml of phosphate buffer containing 0.5 mM NaOH.
5. Standard Pyruvate

**Procedure**

To preincubated buffered substrate at 37°C for 10 minutes, 0.1 ml of serum was added and the mixture was incubated at 37°C for 1 hour. The reaction was stopped by the addition of 1.0 ml of DNPH reagent and incubated for 20 minutes. In blank tubes, 0.1 ml of serum was added after the addition of DNPH reagent. The tubes were kept at room temperature for 20 minutes. Finally, 10 ml of sodium hydroxide was added to all the test tubes. The colour developed was read at 540 nm using a Shimadzu UV-Visible double beam spectrophotometer. The pyruvate serves as standard for estimation. The enzyme activity was expressed as IU/L in serum.
2.2.2.4.2. ASSAY OF ALANINE AMINOTRANSFERASE

Alanine aminotransferase (ALT) was assayed by the method of King et al., (1965a).

**Reagents**

1. 0.1 M Phosphate buffer, pH 7.4
2. 1 mM DNPH in 2N HCl
3. 0.4 N NaOH
4. Substrate solution: 1.78g of D-L-Alanine and 38mg of 2-ketoglutaric acid were dissolved in 0.5 ml of 0.4 N NaOH and made upto 100 ml with phosphate buffer.
5. Standard Pyruvate

**Procedure**

To preincubated 1.0 ml of buffered substrate at 37°C for 10 minutes, 0.1 ml of serum was added and the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1.0 ml of DNPH reagent and allowed the tubes to stand for 20 minutes. In blank tubes, the 0.1 ml of serum was added after the addition of DNPH reagent. The tubes were kept at room temperature for 20 minutes. Finally, 10 ml of sodium hydroxide was added to all the test tubes. The colour developed was read at 540 nm using a Shimadzu UV-Visible double beam spectrophotometer. The pyruvate serves as standard for estimation. The enzyme activity was expressed as IU/L in serum.

2.2.2.4.3. ASSAY OF ALKALINE PHOSPHATASE

Alkaline phosphatase (ALP) was assayed by the method described by King et al., (1965b).
Reagents

1. 0.1 M Carbonate bicarbonate buffer, pH 10.0
2. 0.01 M Disodium phenyl phosphate
3. 0.1 M Magnesium chloride
4. Folin’s reagent
5. 15% Sodium carbonate
6. Standard Phenol: 22.6 mg of pure and crystalline phenol was dissolved in 0.1 N HCl and made upto 100 ml with the same acid.

Procedure

To 1.5 ml of buffer, 1 ml of substrate and 0.1 ml of magnesium chloride was added followed by the addition of 0.1 ml serum only to the ‘test’ test tubes. The tubes were incubated at 37°C for 15 minutes, and 1.0 ml of Folin’s reagent was added to all the tubes. Finally, 0.1 ml of serum was added to the ‘blank’ test tubes. To aliquots of clear supernatants 15% sodium carbonate was added, incubated at 37°C for 15 minutes and the blue colour developed was read at 640 nm in a Shimadzu UV-Visible double beam spectrophotometer. Standard phenol and a reagent blank were treated similarly. The enzyme activity was expressed as IU/L in serum.

2.2.2.4.4. Assay of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) was assayed by the method of King et al., (1965c).

Reagents

1. 0.1 M Glycine buffer, pH 10.0
2. 0.4 N NaOH
3. NAD⁺ solution: 10 mg of NAD⁺ was dissolved in 2 ml of distilled water.
4. DNPH reagent: 20 mg of DNPH in 100 ml of 1N HCl.
5. Lithium lactate: 1.28 g of lithium lactate was dissolved in 50 ml of 31.25ml glycine buffer and 18.75ml of 0.1N NaOH.
6. Standard pyruvate

**Procedure**

To 1.0 ml of the buffered substrate, 0.1 ml of serum and 0.2 ml of NAD$^+$ was added to ‘test’ test tubes and incubated at 37°C for 15 minutes. To the ‘blank’ test tubes 0.2 ml of NAD$^+$ and 1.0 ml of buffered substrate solution was added. Then 1.0 ml of DNPH reagent was added to all the tubes. 0.1 ml of serum was then added to ‘blank’ test tubes only and all test tubes were incubated at 37°C for 15 minutes. Finally, 7.0 ml of 0.4 N sodium hydroxide was added to all the tubes and the colour developed was read at 420 nm within 5 minutes in a Shimadzu UV-Visible double beam spectrophotometer. A set of standard pyruvate was also treated in the similar manner. The enzyme activity was expressed as IU/L in serum.

**2.2.2.4.5. ASSAY OF γ-GLUTAMYLTRANSFERASE**

γ-glutamyl transferase (γ-GT) was assayed by the method of Rosali *et al.*, (1972).

**Reagents**

1. 0.18 M Tris-HCl, pH 8.5
2. Substrate: 0.00625 M L-γ-glutamyl-p-nitroanilide in Tris-HCl buffer; dissolved by heating at 50-60°C.
3. 0.1 M glycylglycine
4. Standard: 13.8 mg of p-nitroaniline was dissolved in 1 liter of ddH$_2$O.
Procedure

To 0.2 ml of serum, 0.5 ml of substrate, 1 ml of Tris-HCl and 2.2 ml of glycyglycine was added to clean test tubes. The total volume was made up to 4 ml with distilled water. After incubating the mixture for 30 minutes at 37°C, the samples were heated at 100°C for 5 minutes and centrifuged. The amount of p-nitroaniline in the supernatant was measured at 410 nm using a Shimadzu UV-Visible double beam spectrophotometer. The activity of γ-GT was expressed as nM of p-nitroaniline formed/min/mg protein.

2.2.2.4.6. ESTIMATION OF LIPID PEROXIDATION PRODUCTS

TBARS level was determined by the method described by Ohkawa et al., (1979).

Reagents

1. 20% TCA
2. 0.12 M TBA
3. Tetramethoxy propane (Standard)

Procedure

To 0.5 ml of liver homogenate, 2.0 ml of 20% TCA was added and the contents were mixed and centrifuged at 4000 rpm for 20 minutes. To 2.0 ml of the supernatant 2.0 ml of TBA reagent was added and mixed. Reagent blank and standards were also treated similarly. The contents in the tubes were heated for 20 minutes in a boiling water bath. The tubes were cooled to room temperature and the absorbance was read at 532 nm in a Shimadzu UV-Visible double beam spectrophotometer. The lipid peroxide content was expressed as nmole of TBARS formed/mg protein.

2.2.2.4.7. ESTIMATION OF PROTEIN CARBONYLS

Protein carbonyl content was measured as an index of protein oxidation as described by Levine et al. (1990).
Reagents

1. 0.1% (w/v) 2, 4-Dinitrophenylhydrazine (2, 4-DNPH) in 2 N HCl
2. 2N HCl
3. 20% Trichloroacetic Acid (TCA)
4. (1:1 V/V) Ethanol/Ethyl Acetate
5. 8 M Guanidine hydrochloride
6. 133 mM Tris-HCl (pH 7.2)
7. 13 mM EDTA

Procedure

To 0.8 ml of the liver homogenate, 0.8 ml of 0.1% (w/v) DNPH was added and incubated for 1 hour. The control tubes include 0.8 ml of liver homogenate and 0.8 ml of 2 N HCl. After incubation, 0.8 ml of 20% TCA was added to all the test tubes and the contents were centrifuged at 1900 rpm for 10 minutes. After washing with ethanol/ethyl acetate (1:1 v/v), residues were dissolved in 2.0 ml of 8 M guanidine hydrochloride prepared in 133 mM Tris-HCl (pH 7.2) containing 13 mM EDTA and centrifuged at 1900 rpm for 10 minutes. The absorbance of each sample was read at 365 nm against the control. The results were expressed as mol of 2,4-DNPH incorporated/mg protein based on a molar extinction coefficient of 2.1 x104 M cm⁻¹ for aliphatic hydrazones. The level of protein carbonyl is expressed as nmoles/mg protein.

2.2.2.5. Estimation of Antioxidant Enzymes

2.2.2.5.1. Assay of Superoxide Dismutase

The activity of superoxide dismutase (SOD) was measured by the method of Misra and Fridovich (1972).

Reagents

1. 0.1 M Carbonate-bicarbonate buffer, pH 10.2
2. EDTA
3. 3 mM Epinephrine
4. Chloroform
5. Ethanol

**Procedure**

To 0.1 ml of serum/liver homogenate, 0.75 ml ethanol and 0.15 ml ice cold chloroform was added and the contents were centrifuged. To 0.5 ml of the supernatant, 0.5 ml of EDTA solution and 1.0 ml of carbonate-bicarbonate buffer was added. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance at 480 nm was measured in a Shimadzu UV-Visible double beam spectrophotometer. The activity of SOD is expressed as 50% inhibition of epinephrine auto oxidation/min/mg protein.

**2.2.2.5.2. ASSAY OF CATALASE**

The activity of Catalase was measured by the method of sinha et al (1972).

**Reagents**

1. 50 mM Phosphate buffer, pH 7.0
2. 30 mM Hydrogen peroxide
3. Dichromate acetic acid reagent: 5% potassium dichromate was prepared with dilute acetic acid (1:5 v/v in distilled water).

**Procedure**

To 0.1 ml of serum/liver homogenate, 1.0 ml of phosphate buffer and 0.4 ml of \( \text{H}_2\text{O}_2 \) was added. The reaction was arrested at different time intervals by the addition of 2.0 ml of dichromate acetic acid reagent. Standard \( \text{H}_2\text{O}_2 \) in the range of 4-20 \( \mu \text{m} \) were taken in clean, dry tubes and treated similarly. The tubes are heated in a boiling water bath for 10 minutes. The green colour developed was
read at 570 nm. The activity of catalase was expressed as μm of H₂O₂ consumed/min/mg protein.

### 2.2.2.5.3. ASSAY OF GLUTATHIONE PEROXIDASE

The activity of glutathione peroxidase (GPx) was measured by the method of Rotruck et al., (1973) with modifications.

**Reagents**

1. 0.4 M Phosphate buffer, pH 7.0
2. 4 mM EDTA
3. 10 mM Sodium azide
4. 2 mM Glutathione
5. 2.5 mM Hydrogen peroxide
6. 10% Trichloro acetic acid
7. DTNB reagent
8. Standard GSH: 20 mg of reduced glutathione dissolved in 100 ml of distilled water.

**Procedure**

To clean, dry tubes 0.4 ml phosphate buffer, 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml of serum/liver homogenate, 0.1 ml H₂O₂ was added and all tubes were made up to final incubation volume of 2.0 ml using distilled water. All the tubes were incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 0.5 ml of TCA. To determine the residual glutathione content, the supernatant was removed by centrifugation and to this 3.0 ml of disodium hydrogen phosphate and 1.0 ml of DTNB reagent were added. The colour developed was read at 412 nm immediately using a Shimadzu UV-Visible double beam spectrophotometer. A blank was treated with only disodium hydrogen phosphate and 1 ml of DTNB reagent. Suitable aliquots of standards were taken and treated in the same way. Glutathione peroxidase activity was expressed as μg of glutathione consumed/min/mg protein.
2.2.2.5.4. Assay of Reduced Glutathione

Reduced glutathione was determined by the method of Maron et al., (1979).

Reagents

1. 0.2 M Phosphate buffer, pH 8.0
2. DTNB reagent
3. 5% TCA
4. Standard GSH: 10 mg GSH in 100ml of distilled water.

Procedure

To 0.5 ml of serum/liver homogenate, 1.0 ml of 5% TCA was added and the precipitate thus formed was removed by centrifugation. To 1.0 ml of the supernatant, 2.0 ml of DTNB reagent was added and the final volume was adjusted to 4.0 ml with phosphate buffer, pH 8.0. The standards and a reagent blank were also treated in a similar manner. The colour developed was read at 412 nm in a Shimadzu UV-Visible double beam spectrophotometer. The amount of glutathione in tissues was expressed as nm/mg protein.

2.2.3. Enzyme Linked Immunosorbent Assay (ELISA) of Alpha Fetoprotein

The serum levels of Alpha Fetoprotein (AFP) were determined using ELISA (Enzyme-Linked Immunosorbent Assay) kit (Abcam (ab108838)). ELISA is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human AFP in serum, plasma and cell culture supernatants.

Reagents

1. 1X Sample Diluent N
2. 1X Wash Buffer
3. 1X Biotinylated alpha Fetoprotein Antibody
4. 1X Streptavidin-Peroxidase Conjugate
**Procedure**

Before the start of the experiment, all reagents prepared are maintained to room temperature (18 - 25°C). 100 µL of standard and serum samples are added to the 96 well plate and incubated for 2.5 hours at room temperature or overnight at 4°C with gentle shaking. After incubation, solution was discarded and washed 4 times with 1X Wash Solution provided with the kit. Invert the plate and blot it against clean paper towels to remove the residual wash buffer and add 100 µL of 1X Biotinylated AFP Detection antibody and incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and washed 4 times with 1X wash buffer. 100 µL of 1X HRP-Streptavidin solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking. The plate was washed 4 times with 1X wash buffer. 100 µL of TMB One-Step Substrate Reagent was added to the wells and incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µL of Stop Solution was added to each well and the colour developed was read at 450 nm, immediately. AFP levels are expressed as ng/ml.

**2.2.4. GELATIN ZYMOGRAPHY**

Gelatin zymography is employed to detect the activity of both latent and active forms of the gelatinases (MMP-2 and MMP-9) as described earlier (Liota *et al.*, 1990). The resolving gel contains gelatin, a substrate for the gelatinases. MMP proteins are visualised at the site of enzymatic activity, identified as a clear band in a dark-stained gel. The latent pro-forms of the gelatinases are detected by this method due to SDS in the gel exposing the active site via a conformational change, as well as dissociating active forms from their inhibitors.

**Reagents**

1. Gelatin substrate: Dissolve 25 mg of gelatin in 10 ml of distilled water. Add this substrate solution in resolving buffer instead of distilled water.
2. Zymography digestion buffer: 50 mM Tris-Hcl, pH 7.5, 5 mM CaCl$_2$, 0.2M NaCl, 1% Triton-X-100
3. 2.5% Triton-X-100

Procedure

After preparing 8% resolving gels with gelatin, the samples were mixed with sample application buffer (SSB) (1:1, ratio) were loaded into the wells. After, electrophoresis at 100 V for 90 minutes, gels was washed twice (for 30 minutes) with 2.5% Triton-X-100 and incubated in zymography digestion buffer for 18 hours at 37°C. The gel was stained with CBB R250 staining solution for one hour at room temperature. Enzyme activities were revealed as clear bands against the dark blue background of the substrate gel and were quantified by scanning densitometry.

2.2.5. Western Blotting

For western blotting, protein lysates from liver tissue of control and experimental groups were prepared and protein concentrations were determined by Bradford’s method as described in section (1.2.5.). The samples were resolved by 10% SDS-PAGE and transferred on to PVDF membrane using standard procedures. Membranes were blocked with 5% milk powder for one hour and incubated with anti-α-SMA, anti-GSTpi, anti-PCNA, anti-Mst1, anti-Lats1, anti-Yap, anti-NFκB p65, anti-IκBα, anti-β catenin, anti-Gsk-3β, anti-MMP-2, anti-MMP-9, anti-Bcl-2, anti-Bax and anti-cleaved caspase 9 respectively, overnight at 4°C. Binding of primary antibody was detected with HRP-conjugated host specific secondary antibodies for one hour, washed thoroughly and developed with ECL kit according to the manufacturer’s protocol. GAPDH served as loading control.
2.2.6. **GENE EXPRESSION ANALYSIS**

2.2.6.1. **RNA EXTRACTION**

Total RNA was extracted according to Chomczynski and Sacchi (1987) method as described in section 1.2.8.1.

2.2.6.2. **cDNA SYNTHESIS**

cDNA from each sample was synthesised as described in section 1.2.8.2.

2.2.6.3. **QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qPCR)**

Quantitative Real Time Polymerase Chain Reaction (qPCR) was performed as described in section 1.2.8.3.

2.2.7. **LIVER HISTOLOGY**

A portion of the liver tissue was fixed in 10% neutral buffered formalin for 24 h and dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin and stored indefinitely.

2.2.7.1. **HAEMATOXYLIN AND EOSIN STAINING**

Formalin fixed and paraffin embedded liver sections were dewaxed by incubating in xylene at 60°C for 30 min and cleared in xylene for 5 min. Descending grades of ethanol from 100% to 30% were used to rehydrate the slides and finally dipped in distilled water for 5 min, followed by rinsing in TBS. Sections were stained with Mayer’s haematoxylin for 30 min and washed with water until the colour changed to blue. Then the sections were differentiated in 0.1 % HCl for 45 sec, washed with water and stained with 1% eosin for 1 min. After washing, the sections were dehydrated in ascending grades of ethanol (30%, 50%, 70%, 90% and 100%), cleared in xylene and sections were mounted using DPX. Finally, the sections were viewed under light microscope for histopathological changes.
2.2.7.2. AgNOR STAINING

AgNOR staining is a simple, quick and reliable method for evaluating cell proliferation by detecting AgNORs size and dispersion. The staining was performed according to the method described by Bukhari et al., 2007.

Procedure

4µm thick liver sections were deparaffinized by incubating in xylene at 60°C for 30 min and cleared in xylene for 5 min. Rehydration was then performed using descending ethanol concentrations and finally in several changes of ultrapure distilled water. The sections were incubated in acid alcohol (3 parts ethanol: 2 parts acetic acid) for 5 min and then rinsed in distilled water. Then the sections were incubated with silver nitrate solution in a humidified chamber for 45 min at RT. The silver staining solution consisted of 2 parts of 50% solution of silver nitrate and 1 part of 2% gelatin in 1% formic acid solution. Sections were then incubated with a 10% solution of sodium thiosulphate for 5 min, washed in distilled water, dehydrated in graded ethanol, cleared in xylene and mounted using DPX. AgNOR Stained sections were examined under the light microscope (Carl Zeiss Microscopy, Germany).

2.2.7.3. Picro-sirius red staining

Picrosirius red staining is commonly employed to evaluate degrees of liver fibrosis on paraffin-embedded sections. The staining was performed according to the method described by Junqueira (1979). The strong anionic dye Sirius Red F3B (Direct Red 80) stains collagen by reacting with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fibre in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in an enhanced birefringency. When observed by bright field microscopy, collagen appears red.
Procedure

Paraffin embedded liver sections were dewaxed and cleared in xylene, rehydrated in descending grades of ethanol (100 to 30%) and washed in distilled water. Sections were stained with 0.1% Sirius-red (Direct Red 80, Sigma) in saturated picric acid solution for 1 h, followed by washes with 0.5% acetic acidified water. After washing, the sections were dehydrated in ascending grades of ethanol (30 to 100%), cleared in xylene and sections were mounted using DPX. The morphology of collagen fibers was captured with a light microscope (Carl Zeiss Microscopy, Germany).

2.2.7.4. Masson’s trichrome staining

The trichrome stain uses three dyes to impart three different colours, on collagen, cytoplasm and nuclei respectively. The Masson’s trichrome stain consists of sequential staining with iron hematoxylin which stains nuclei black, Biebrich scarlet which stain cytoplasm red and aniline blue which stain collagen blue, respectively.

Procedure

Bouin’s fixed and paraffin embedded liver sections were first dewaxed, cleared with xylene and hydrated in a series of graded ethanol solution. The hydrated liver sections were incubated in Weigert’s Iron hematoxylin solution, stained with Biebrich scarlet-acid fuchsin and aniline blue. The sections were then dehydrated in increasing ethanol series, cleared in xylene and mounted using DPX. The collagen fibers were stained blue, the nuclei were stained black and hepatocytes were stained red.

2.2.8. Immunofluorescence analysis

Paraffin embedded liver sections were dewaxed and cleared in xylene, hydrated with descending grades of ethanol from 100% to 30%, washed with distilled water. The heat-induced epitope retrieval was performed using citrate based
solution, thus enhancing staining intensity of antibodies. After washing in TBS, the sections were blocked with 3% BSA in TBST for 1 h. The sections were incubated with the anti-PCNA, anti-Mst1, anti-Lats1, anti-Yap, anti-NFκB p65, anti-β-catenin antibody at 4°C overnight. The corresponding FITC conjugated secondary antibodies were added and incubated for 1 h at RT. The liver cells were counterstained using PI (1 mg/ml) diluted in TBS (1:1000) and visualized using a fluorescence microscope (Carl Zeiss Microscopy, Germany).

2.2.9. Statistical Analysis

All the grouped data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. * p <0.05, ** p <0.01, *** p <0.001 were considered as statistically significant. All results were expressed as mean ± SD for 6 animals in each group.
2.3. RESULTS AND DISCUSSION

2.3.1. EFFECT OF MORIN ON DEN INDUCED RATS

The differences in the body weight, liver weight and relative liver weight between the control and experimental rats are presented in Table-2. The rats in preneoplastic lesion-induced group and HCC-induced group showed significant (p<0.05) decrease in the final body weight with relatively increased liver weight, whereas in fibrosis-induced rats (Group VI) both the body weight and liver weight were significantly decreased compared to control rats (Group I). However, oral treatment of morin (50 mg/Kg BW) markedly reversed the above changes to near normal range as observed in treated groups (IV, VII and X).

The activities of serum marker enzymes (AST, ALT, ALP, LDH and γ-GT) were significantly (p <0.05) elevated in all the three progressive stages of DEN induced rats (Group III, VI and IX) compared to control rats (Group I) as shown in Figure-2.1. In contrast, the activities of these markers were markedly reverted to near normal levels in morin treated groups (Group IV, VII and X). The increased levels of serum transaminases (AST and ALT), ALP, LDH and γ-GT are mainly due to the effect of DEN causing significant liver damage leading to distinctive changes in the serum activities (Plaa et al., 1989). Several studies reported that the elevation of ALT activity was repeatedly credited to hepatocellular damage and was accompanied by a rise in AST, after DEN administration (Bansal et al., 1996). The increase in LDH reflects a non-specific alteration in the plasma membrane integrity and/or permeability. γ-GT plays a key role in the production and degradation of glutathione and located in cellular membrane of many tissues with its active site present on the outer surface of the membrane. Chemical carcinogens that enter the liver initiate systematic induction of γ-glutamyl transferase synthesis, which was found to be elevated in cancerous condition (Ngo et al., 1994). This elevation of γ-glutamyl transferase activity shows the stages of carcinogenic process, since its levels are correlated with growth rate, histological differentiation and survival time of the host (Koss et al., 1982).
<table>
<thead>
<tr>
<th>Group</th>
<th>Final Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Relative Liver Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Group I)</td>
<td>246.00 ± 7.07</td>
<td>7.10 ± 0.54</td>
<td>2.89 ± 0.25</td>
</tr>
<tr>
<td>Morin Control (Group II)</td>
<td>239.16 ± 8.01</td>
<td>7.00 ± 0.60</td>
<td>2.93 ± 0.31</td>
</tr>
<tr>
<td>DEN Induced (Group III)</td>
<td>173.66 ± 7.11</td>
<td>8.77 ± 0.75</td>
<td>5.05 ± 0.43</td>
</tr>
<tr>
<td>Morin Treated (Group IV)</td>
<td>229.16 ± 10.68</td>
<td>7.47 ± 0.49</td>
<td>3.26 ± 0.28</td>
</tr>
<tr>
<td>Morin Control (Group V)</td>
<td>241.36 ± 6.01</td>
<td>7.35 ± 0.80</td>
<td>3.03 ± 0.51</td>
</tr>
<tr>
<td>DEN Induced (Group VI)</td>
<td>175.33 ± 6.53</td>
<td>4.08 ± 0.86</td>
<td>2.31 ± 0.43</td>
</tr>
<tr>
<td>Morin Treated (Group VII)</td>
<td>203.83 ± 5.30</td>
<td>5.97 ± 0.72</td>
<td>2.93 ± 0.39</td>
</tr>
<tr>
<td>Morin Control (Group VIII)</td>
<td>240.56 ± 7.31</td>
<td>7.20 ± 0.45</td>
<td>2.99 ± 0.39</td>
</tr>
<tr>
<td>DEN Induced (Group IX)</td>
<td>165.66 ± 7.25</td>
<td>21.66 ± 3.26</td>
<td>13.05 ± 1.62</td>
</tr>
<tr>
<td>Morin Treated (Group X)</td>
<td>195.33 ± 8.75</td>
<td>10.09 ± 1.14</td>
<td>5.18 ± 0.73</td>
</tr>
</tbody>
</table>

**Table-2.1: Effect of DEN and morin on body weight, liver weight and relative liver weight of control and experimental rats.** Values are expressed as mean ± SD (n = 6). Statistical significance at p<0.05. Comparisons are made with ‘a’ Control (Group I), ‘b’ DEN induced preneoplastic rats (Group III), ‘c’ DEN induced fibrotic rats (Group VI) and ‘d’ DEN induced HCC rats (Group IX). Body weight expressed in grams, liver weight expressed in grams and relative liver weight expressed as the average of liver weight at final body weight multiplied by 100.
Figure 2.1. Effect of morin on serum marker enzymes AST, ALT, ALP, LDH and γ-GT in progressive stages of HCC in control and experimental animals. Values are expressed as mean ± S.D (n = 6). Statistical significance at * p<0.05. Comparisons are made with 'a' control (Group I), 'b' DEN induced preneoplastic rats (Group III), 'c' DEN induced fibrotic rats (Group VI) and 'd' DEN induced HCC rats (Group IX). AST, ALT, ALP, LDH and γ-GT activities are expressed as IU/L.
The above results showed that morin treated rats (Group IV, VII and X) reverted
the elevated levels of serum marker enzymes markedly which augments the
previous reports from this laboratory (Sivaramakrishnan et al., 2009 and
Madankumar et al., 2014). This shows that morin prevents liver damage by
maintaining the membrane integrity, thereby suppressing the leakage of enzymes
through membranes, thus exhibiting its hepatoprotective potential.

2.3.2. EFFECT OF MORIN ON LIPID PEROXIDES, PROTEIN CARBONYL AND
ANTIOXIDANT ENZYME ACTIVITIES

**Figure-2.2 and Table-2.2** illustrate the levels of MDA and TBARS (liver lipid
peroxidation (LPO) end-products), protein carbonyl (PCO) and serum
antioxidant enzymes in the control and experimental groups of rats. Rats in
DEN-induced groups (III, VI and IX) showed significant increase in lipid
peroxidation levels with concomitant decrease in antioxidant enzymes
(superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)
and reduced glutathione (GR)) levels compared to control rats (Group I).
However, morin treated rats in experimental groups (IV, VII and X) showed
markedly reduced levels of LPO and PCO with significant rise in antioxidant
levels compared to DEN induced groups.

Free radicals are often produced *in vivo* as a result of carcinogen exposure
causing oxidative stress that in turn leads to damage of nucleic acids, proteins
and lipids resulting in chromosomal instability, mutations, loss of organelle
function and membrane damage, which play an important role in the
development of cancer (Waris et al., 2006). As reported earlier, it is obvious that
DEN administration causes significant liver damage resulting in lipid
peroxidation and enhanced generation of the reactive oxygen species (ROS) in
the preneoplastic lesion, fibrosis and HCC rats (Sivaramakrishnan et al., 2009,
Khan et al., 2011b and Madankumar et al., 2014). LPO result because of the
peroxidative loss of unsaturated lipids, thus bringing about cellular lipid
degradation and membrane disordereding. Numerous studies have shown that
metabolism of DEN in liver generates ROS, hence resulting in oxidative stress
Figure-2.2. Effect of morin on lipid peroxidation levels MDA, TBARS and protein carbonyls in liver tissues in progressive stages of HCC in control and experimental animals. Values are expressed as mean ± S.D (n = 6). Statistical significance at *p<0.05. Comparisons are made with ‘a’ control (Group I), ‘b’ DEN induced preneoplastic rats (Group III), ‘c’ DEN induced fibrotic rats (Group VI) and ‘d’ DEN induced HCC rats (Group IX). LPO-MDA levels are expressed as nmole of MDA formed/min/mg protein, LPO-TBARS levels are expressed as nmole of TBARS formed/mg protein, PCO levels are expressed as nmole/mg protein.
and cellular damage (Bartsch et al., 1989 and Sivaramakrishnan et al., 2009). The universal marker of protein oxidation - PCO, a product of irreversible non-enzymatic oxidation or carbonylation of protein, is an indicator of free radical generation in cells (Dalle-donne et al., 2006). PCOs are reflective of more severe cases of oxidative stress which are mainly due to the presence of reactive oxygen mediated protein oxidation (Berlett et al., 1997 and Shacter et al., 2000).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>SOD</th>
<th>CAT</th>
<th>GPX</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Group I)</td>
<td>2.49 ± 0.22</td>
<td>1.24 ± 0.21</td>
<td>11.70 ± 0.51</td>
<td>13.93 ± 0.37</td>
</tr>
<tr>
<td><strong>Proneoplastic Lesion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morin Control (Group II)</td>
<td>2.66 ± 0.08</td>
<td>1.15 ± 0.11</td>
<td>12.21 ± 0.70</td>
<td>13.68 ± 0.78</td>
</tr>
<tr>
<td>DEN Induced (Group III)</td>
<td>1.30 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.03 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.22 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morin Treated (Group IV)</td>
<td>1.84 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.98 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.37 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.96 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fibrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morin Control (Group V)</td>
<td>2.58 ± 0.18</td>
<td>1.38 ± 0.41</td>
<td>11.91 ± 0.75</td>
<td>12.98 ± 0.88</td>
</tr>
<tr>
<td>DEN Induced (Group VI)</td>
<td>1.42 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.61 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.76 ± 0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.94 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morin Treated (Group VII)</td>
<td>1.91 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.04 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.49 ± 0.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.2 ± 0.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>HCC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morin Control (Group VIII)</td>
<td>2.38 ± 0.28</td>
<td>1.19 ± 0.68</td>
<td>12.41 ± 0.80</td>
<td>13.18 ± 0.34</td>
</tr>
<tr>
<td>DEN Induced (Group IX)</td>
<td>1.05 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.54 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.83 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morin Treated (Group X)</td>
<td>1.75 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.92 ± 0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.73 ± 0.70&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.29 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table-2.2: Effect of DEN and morin on the activities of serum antioxidant enzymes in control and experimental rats.** Values are expressed as mean ± SD (n = 6). Statistical significance at p<0.05. Comparisons are made with 'a' control (Group I), 'b' DEN induced proneoplastic rats (Group III), 'c' DEN induced
fibrotic rats (Group VI) and DEN induced HCC rats (Group IX). SOD activity is expressed as 50% inhibition of epinephrine auto oxidation, CAT activity is expressed as μmole of H₂O₂ decomposed/min/mg protein, GPx activity is expressed as μmole of GSH oxidized/min/mg protein and GR activity is expressed as μmole of NADPH oxidized/min/mg protein.

DEN administration induces major hepatocellular damage in group III, VII and IX rats which are evidenced by significant decrease in the activities of serum antioxidant enzymes. In response to oxidant injury, the endogenous antioxidant system reacts by counteracting the ROS with the help of enzymatic antioxidants such as SOD, CAT, GPx and GR. SOD dismutates superoxide radicals (O₂⁻) into hydrogen peroxide (H₂O₂) and O₂. CAT and GPx further detoxify H₂O₂ to H₂O. Thus SOD, CAT and GPx act mutually and constitute the enzymatic antioxidant defense mechanism against ROS (Bhattacharjee et al., 2006). GR plays an essential role in regenerating GSH from GSSG (Oxidised glutathione), thus maintaining the balance between the redox couple. Thus, decrease in the activities of these enzymes in the current study could be attributed to the excessive utilization of the enzymes in deactivating the free radicals generated during DEN metabolism. Morin, as reported earlier (Sivaramakrishnan et al., 2009 and Madankumar et al., 2014) reduced the oxidative stress and decreased the burden of antioxidant enzymes (Group IV, VII and X) suggesting the antioxidant efficacy of morin treatment against DEN induced liver injury in progressive stages of HCC.

2.3.3. EFFECT OF MORIN ON LIVER HISTOLOGY

Figure-2.3 depicts the macroscopic and microscopic analysis of the liver of control and experimental rats. The liver of control (group I) and morin control rats (group II, V and VIII) appeared normal showing normal morphology while the livers of DEN induced rats in groups III, VI and IX showed significant damage with distinct numerous nodules on their surfaces. Liver of morin treated rats in groups IV, VII and X showed markedly decreased damage with lesser nodules compared to DEN induced rats.
Figure-2.3. Effect of morin on liver morphology and histology. Macroscopic examination and Microscopic examination of liver by haematoxylin and eosin staining in control, morin control, DEN induced preneoplastic lesion, fibrosis and HCC and morin treated preneoplastic lesion, fibrosis and HCC rats. Scale bar: 50μm.
Also, the liver sections from control and experimental groups were stained with hematoxylin and eosin (HE) to check for microscopic changes. When viewed under light microscope, the liver sections of control and morin control groups showed normal liver cell architecture with granulated cytoplasm, small uniform nuclei, normal lobular architecture with central veins and radiating hepatic cords. The liver sections of DEN-induced preneoplastic group showed binucleated large irregular cells that were pleiomorphic and hyperchromatic, characterized by a nucleolus at the centre with enlarged cytoplasm consisting extensive vacuolation with masses of acidophilic material. The liver sections of DEN-induced fibrotic group showed more inflammatory infiltration, steatosis and numerous thick fibrous septa, while the DEN-induced HCC group showed loss of architecture and presence of abundant tumour cells with more inflammatory infiltration, eosinophilic granular cytoplasm, rounded nuclei and prominent nucleoli. However, the liver sections of morin treated groups showed very fewer neoplastic cells, lesser infiltration with near-normal architecture compared to DEN-induced groups.

2.3.4. Effect of Morin on the markers on progressive stages of HCC

Figure-2.4 presents the evaluation of different stage-specific markers such as Glutathione S-transferase -pi (preneoplastic lesion), Alpha-smooth muscle actin (fibrosis), Proliferating cell nuclear antigen and Alpha-Feto protein (HCC) as determined by western blot analysis and ELISA.

Glutathione S-transferase (GST), a phase II detoxification enzyme, protects cells from the toxic and neoplastic effects of the carcinogens (Talalay et al., 1988). Of the three classes of GSTs (alpha, mu and pi), GST-pi is the predominant form seen in cancer cells. Placental GST, the only GST of the pi class in rats, is absent in the normal rat hepatocytes and is specifically induced at an early stage of chemical hepatocarcinogenesis (Power et al., 1987). In the hepatocytes of preneoplastic lesions and HCCs, GST-pi was constitutively expressed at high levels thus serving as a marker for early hepatocellular carcinogenesis. Numerous studies reported that lead nitrate, aflatoxin metabolites and DEN
Figure-2.4. Evaluation of stage-specific markers in progressive stages of HCC. (a). Western blot analyses of GST-pi, α-SMA, PCNA and densitometric analysis in preneoplastic lesion, fibrosis and HCC progressive stages of DEN induced and morin treated rats, respectively. (b). ELISA of Serum AFP in DEN induced HCC and morin treated rats. Values are expressed as mean ± S.D (n = 6). Statistical significance at * p<0.05. Comparisons are made with ‘a’ control (Group I), ‘b’ DEN induced preneoplastic rats (Group III), ‘c’ DEN induced fibrotic rats (Group VI) and ‘d’ DEN induced HCC rats (Group IX). Serum AFP levels are expressed in ng/ml. Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated.
induce significant increase in GST-pi mRNA (Vandenberghe et al., 1989, Hatayama et al., 1991, Muramatsu et al., 1991 and Khan et al., 2011b). Alpha smooth muscle actin (α-SMA) is an actin isoform often used as a reliable marker for smooth muscle cell differentiation (Skalli et al., 1986). HSC proliferation and differentiation are closely associated with the development of liver fibrosis. In response to stimuli such as inflammation, HSCs exhibit intense cytoplasmic α-SMA immunoreactivity and hence, regarded as a reliable marker of the activated HSCs (Nouchi et al., 1991, Yamaoka et al., 1993, Guid et al., 1996 and Dooley et al., 2001). Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in DNA synthesis and repair. A frequently used marker of cellular proliferation, PCNA identifies cells in the G1/S phase of the cell cycle. PCNA expression is reported to predict several tumour recurrences, including HCC (Na et al., 1994; Suehiro et al., 1995). Alpha-Feto protein (AFP) is the most widely used tumour biomarker for the early detection of HCC. Numerous clinical findings demonstrated that serum AFP had a sensitivity of 41-65% and specificity of 80-94% when the cut-off value is 20 ng/ml (Debruyne et al., 2008).

In the current study, DEN-induced progressive stages of HCC was successfully established which is evident from the elevated expression of GST-pi, α-SMA, PCNA and AFP in DEN-induced group and morin treatment to DEN induced rats showed marked reduction in all these markers which supplement the previous reports from this laboratory (Sivaramakrishnan et al., 2009, Khan et al., 2011b and Madankumar et al., 2014).

2.3.5. EFFECT OF DEN ON KEY SIGNALING PATHWAYS INVOLVED IN PROGRESSIVE STAGES OF HCC

Among various important signaling pathways, the most altered ones includes NF-κB, β-catenin and Hippo, all of which are expected to play a crucial role in liver homeostasis and HCC progression.
**Figure-2.5** depicts the expression profile of key molecules of NF-κB and Wnt/β-catenin pathways determined by Western blot analysis. The protein levels of NF-κBp65 and β-catenin in DEN-induced preneoplastic lesion, fibrosis and HCC groups were significantly elevated (p<0.001) compared to control group. However, when analysed between the three progressive stages of HCC, the expression of both NF-κBp65 and β-catenin in fibrotic group was found to be significantly (p<0.05) increased compared to preneoplastic lesion group. Also, the HCC induced group showed significantly (p<0.05) increased expression compared to both preneoplastic lesion and fibrotic rats.

Numerous studies showed that elevated NF-κB activity was associated with HSC activation in fibrosis (Hellerbrand *et al*., 1998, Lang *et al*., 2000 and Wright *et al*., 2001). An IKK knockout study in mouse model showed that proinflammatory milieu of the injured liver generates a microenvironment favouring the development of HCC (Maeda *et al*., 2005). Likewise, the activation of the Wnt/β-catenin pathway was implicated in various fibrotic diseases such as systemic sclerosis, pulmonary, cardiac, renal, skin and liver fibrosis (Surendran *et al*., 2002, He *et al*., 2009, Liu *et al*., 2009, He *et al*., 2010, Henderson *et al*., 2010 and Wei *et al*., 2011) and were also found to be upregulated in HSC-derived myofibroblast like cells (Jiang *et al*., 2006). Also, in HCC, β-catenin was reported to play a role in promoting tumour progression by stimulating tumour cell proliferation and reducing the activity of cell adhesion systems (Inagawa *et al*., 2002). The accumulation of nuclear β-catenin in early liver progenitor phenotype induces HCC development and promotes tumour recurrence (Zulehner *et al*., 2010). Moreover, this result was in line with the earlier reports from this laboratory confirming that DEN administration to rats deregulated the NF-κB and Wnt/β-catenin pathways (Sivaramakrishnan *et al*., 2009, Khan *et al*., 2011, Madankumar *et al*., 2014 and Madankumar *et al*., 2015). All these reports confirmed that both NF-κB and β-catenin actively participated in all the three progressive stages of HCC.
Figure 2.5. DEN altered the expressions of key molecules of Hippo, NFκB and Wnt/β-catenin signaling pathways in progressive stages of HCC. (a). Western blot analyses of expression of NFκBp65, β-catenin and densitometric
analysis are expressed as “fold change” and (b). western blot analyses of expression of key Hippo signaling molecules Mst1, Lats1, Yap and densitometric analysis are expressed as “fold change”. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3). Statistical significance at * (p<0.001) compared to control rats (Group I); # (p<0.05) compared to DEN induced preneoplastic lesion rats (Group III); $ (p<0.05) compared to DEN induced fibrotic rats (Group VI). Lane 1 - Control (Group I), Lane 2 - DEN induced preneoplastic lesion (Group III), Lane 3 - DEN induced fibrosis (Group VI) and Lane 4 - DEN induced HCC (Group IX).

To elucidate whether DEN induction affected Hippo pathway, the protein levels of Mst1, Lats1 and Yap were determined by Western blotting. DEN administration resulted in significant (p<0.001) reduction in the protein levels of Mst1 and Lats1 in preneoplastic lesion and fibrotic groups with concomitant increase in the Yap protein as compared to control group. When the levels of Mst1 and Lats1 were compared between fibrotic and preneoplastic lesion groups, no significant changes were observed, whereas Yap showed significant (p<0.05) changes. In HCC group both Mst1 and Yap showed significant (p<0.05) changes with decreased expression of Lats1 compared to preneoplastic lesion and fibrotic groups.

In mammals, Hippo signaling plays an important role in organ-size control during embryonic development and is conserved as a major tumour suppressor pathway (Yin et al., 2011). Hippo signaling was deregulated in most of the human cancers including lung, colorectal, ovarian, liver and prostate cancers (Dong et al., 2007, Zhao et al., 2007 and Steinhardt et al., 2008). Increased nuclear staining (about 60%) for Yap was reported in human HCC samples which further revealed its oncogenic role and as a potential marker for HCC (Steinhardt et al., 2008). Yap overexpression promoted epithelial-mesenchymal transition (EMT) of cultured cells and suppressed anoikis (Overholtzer et al., 2006). Likewise, DEN-induced preneoplastic lesion in Fischer F-344 rats showed increased accumulation of Yap, confirming Yap activation as an early
event in HCC development (Perra et al., 2014). Indeed, studies employing genetically modified animals showed that the overexpression of Yap and the combined Mst1/2 deficiency led to massive liver overgrowth and development of HCC (Camargo et al., 2007 and Zhou et al., 2009). Mst1/2 double knockout mice showed decreased Yap phosphorylation with Yap genomic amplification (Overholtzer et al., 2006 and Zender et al., 2006) and elevated nuclear localization in human HCC (Dong et al., 2007 and Zhao et al., 2007). Recently, results from an in vivo study with CCl4 administered mice and in vitro studies with Yap knockdown cells confirmed Yap activation as a critical driver for HSC activation promoting liver fibrosis (Mannaerts et al., 2015). Hence, Yap activation seemed to play an important role in human HCC and an impaired Hippo pathway might be a common mechanism for Yap activation during HCC progression (Zhao, et al., 2009).

Altogether, the present findings (Figure-2.5) showed significantly increased levels of NF-κBp65, β-catenin and Yap1, the key molecules of the inflammatory, cell proliferation and Hippo signaling in DEN-induced rats which appeared to be the successful animal model for further molecular target studies in the progression of HCC.

2.3.6. Effect of Morin on Hippo Signaling

To examine the effect of morin on Hippo pathway, the levels of key Hippo components such as Mst1 (Figure-2.6), Lats1 (Figure-2.7), TAZ (Figure-2.7) and Yap1 (Figure-2.8) were determined by qPCR, Western blotting and immunofluorescence analysis.

In DEN-induced preneoplastic lesion and fibrotic groups, the expression levels of Mst1 and Lats1 were significantly decreased with marked increase in Yap and TAZ expression compared to the control group. Morin treatment to DEN-induced rats showed marked increase in the expression levels of Mst1 and Lats1 with significant decrease in Yap and TAZ levels compared with DEN induced rats. With respect to HCC group, Yap and TAZ were significantly increased with
(c). Immunofluorescence analysis of Mst1

Figure-2.6. Morin activates Hippo signaling by altering the expressions of the key molecule Mst1 in progressive stages of HCC.(a). Quantitative PCR analyses of expression of Mst1, (b). western blot analyses of expression of Mst1 and densitometric analysis are expressed as “fold change” and (c). immunofluorescence analyses of Mst1 (Mst1 (FITC- Green) and propidium iodide (PI- Red) nuclear counterstain) in control and experimental groups of rats. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3) relative to control. Statistical significance at * p<0.05; ** p<0.01; *** p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III); ‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated. Scale bar: 50μm.
(a).

![Graph showing relative mRNA expression of TAZ](image)

(b).

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![Western blot images](image)

**Relative mRNA Expression**

- **TAZ**
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  - Morin Control
  - Induced
  - Treated

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**FOLD CHANGE**

- **Lats1**
  - Control
  - Morin Control
  - Induced
  - Treated

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Morin activates Hippo signaling by altering the expression of the key molecules Lats1 and Transcription co-activator TAZ in progressive stages of HCC. (a). Quantitative PCR analyses of expression of TAZ, (b). western blot analyses of expression of Lats1 and densitometric analysis are expressed as “fold change” and (c). immunofluorescence analyses of Lats1 (Lats1 (FITC-Green) and propidium iodide (PI- Red) nuclear counterstain) in control and experimental groups of rats. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3) relative to control. Statistical significance at * p<0.05; ** p<0.01; *** p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III); ‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated. Scale bar: 50μm.
Preneoplastic Lesion
Fibrosis
HCC

Relative mRNA Expression

Control Morin Control Induced Treated

Yap (75 kDa)

GAPDH (37 kDa)
(c) Immunofluorescence analysis of Yap

**Figure-2.8.** Morin activates Hippo signaling by regulating the expression of Transcription co-activator Yap in progressive stages of HCC. (a). Quantitative PCR analyses of expression of Yap, (b). western blot analyses of expression of Yap and densitometric analysis are expressed as “fold change” and (c). immunofluorescence analyses of Yap (Yap (FITC- Green) and propidium iodide (PI- Red) nuclear counterstain) in control and experimental groups of rats. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3) relative to control. Statistical significance at *p<0.05; ** p<0.01; *** p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III); ‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated. Scale bar: 50μm.
decreased levels of Lats1 compared to control group, whereas morin treated group showed marked increase of Mst1 and Lats1 levels with significant reduction in Yap expression compared to DEN induced group. Immunofluorescence results further complemented the qPCR and Western blot results. In brief, morin treated groups showed prominent nuclear signals for Mst1 and Lats1 with substantial reduction in nuclear Yap localization which confirmed the activation of Hippo signaling.

During the development process and in cancer, tissue and cell growth control require coordinated regulation of cell proliferation and apoptosis. Hippo pathway is one such tumour suppressive pathway which plays a key role in regulating organ size, inhibiting cell proliferation, promoting apoptosis by directly phosphorylating and inhibiting Yap and TAZ (Zhao et al., 2007). Studies using Mst1/2 double knockout mice showed significant abolition of Yap phosphorylation with increased Yap nuclear localization causing liver tumourigenesis. The knockdown of Yap reversed the transformed phenotype of HCC derived cells in these experimental mice (Zhou et al., 2009). Over 70% of HCC cases showed significantly reduced active Mst1/2, decreased Yap phosphorylation with increased Yap accumulation in the nucleus eventually leading to increased transcription of c-myc, cyclin D1, inhibitors of apoptosis cIAP1 (Birc2) and survivin (Birc5) (Overholtzer et al., 2006, Zender et al., 2006, Dong et al., 2007 and Zhao et al., 2007).

In humans, the tumour suppressor kinase, Lats (Lats1/2), phosphorylates Yap and TAZ on serine residues and in the conserved HXRXXS motif (S127 and S381) and (S381), respectively creating 14-3-3 binding sites leading to cytoplasmic retention and functional inactivation of Yap and TAZ. The ectopic expression of Yap and TAZ stimulated cellular proliferation, reduced cell contact inhibition, and promoted EMT (Lei et al., 2008 and Sudol et al., 2013). Overexpression of Lats1/2 in experimental animals suppressed EMT and tumour growth of cancer cells (Xia et al., 2002, Hao et al., 2008 and Zhang et al., 2008). Several animal studies highlighted the tumour suppressor function of Mst1/2 and
Lats1/2 (St et al., 1999 and Zhou et al., 2009) and epigenetic silencing of these genes resulted in cancer progression (Takahashi et al., 2005, Seidel et al., 2007 and Harvey et al., 2013). Recent reports claimed the applicability of TAZ mRNA as a prognostic factor in preneoplastic lesions of HCC where increased levels of TAZ mRNA were observed in human HCC cases. Of these, 26% corresponded to peritumoural tissues and 65% to tumour tissues which relatively showed significant association with tumour size and reduced survival indicating TAZ as a pro-growth factor in the progression of HCC (Hayashi et al., 2015 and Xiao et al., 2015). Also, studies on silencing TAZ in HCC cells showed increased Yap expression, which compensated the TAZ expression conferring chemo resistance during cancer progression. Likewise, knocking down TAZ in nude mice resulted in increased ability of tumour formation compared to control and double knockdown of TAZ and Yap. All these findings suggested that Yap stood on an upper level of hierarchy than TAZ during HCC progression (Hayashi et al., 2015).

An in vitro study showed that curcumin effectively exhibited anticancer effects by suppressing TAZ/Yap expressions (Hayashi et al., 2015). Likewise, in this study, it was observed that morin treatment to DEN-induced rats resulted in significant elevation in the levels of Mst1 and Lats1 with notable reduction in Yap and TAZ levels, which confirmed the activation of Hippo signaling and inhibition of HCC progression.

2.3.7. Effect of Morin on Wnt/β-catenin Signaling

To explore the anti-proliferative effects of morin in vivo, the levels of key Wnt/β-catenin molecules such as β-catenin and GSK-3β were determined (Figure-2.9). Both, β-catenin and GSK-3β were significantly increased in DEN-induced groups (III, VI and IX) compared to control group. Morin treated groups (IV, VII and X) showed marked reduction in the levels of β-catenin and GSK-3β compared to DEN-induced groups. Immunofluorescence staining for β-catenin showed intense nuclear expression in DEN-induced group (III, VI and IX),
(a).

![Graph showing relative mRNA expression of β-catenin, Fibrosis, and HCC stages across different conditions.]

(b).

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Figure 2.9. Activation of Hippo signaling by morin regulates Wnt/β-catenin signaling in progressive stages of HCC. (a). Quantitative PCR analyses of expression of β-catenin, (b). western blot analyses of expression of β-catenin, Gsk-3β and densitometric analysis are expressed as “fold change” and (c). immunofluorescence analyses of β-catenin (β-catenin (FITC- Green) and propidium iodide (PI- Red) nuclear counterstain) in control and experimental groups of rats. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3) relative to control. Statistical significance at * p<0.05; ** p<0.01; *** p<0.001, 'a' compared to control rats (Group I); 'b' compared to DEN induced preneoplastic lesion rats (Group III); 'c' compared to DEN induced fibrotic rats (Group VI); 'd' compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated. Scale bar: 50µm.
Figure-2.10. Morin restrains cell proliferation markers in progressive stages of HCC. Histological silver stained nucleolar organiser region (AgNOR staining) and PCNA immunofluorescence staining (PCNA (FITC- Green) and propidium iodide (PI- Red) nuclear counterstain) of liver sections in control, morin control, DEN induced preneoplastic lesion, fibrosis and HCC, and morin treated Preneoplastic lesion, fibrosis and HCC rats. Scale bar: 50μm.
whereas morin treated groups exhibited prominent cytoplasmic staining with occasional nuclear staining. Also, the histological staining for silver stained nucleolar organiser region (AgNOR) and immunostaining for PCNA were carried out in the liver sections of control and experimental groups to validate the anti-proliferative effects of morin (Figure-2.10). Liver sections of DEN-induced groups (III, VI and IX) showed hypertrophic hepatocyte nucleus with greater amount of AgNOR proteins and increased PCNA levels as evident from the strong fluorescence signals compared to control groups. On the other hand, liver sections from morin treated groups (IV, VII and X) showed markedly reduced AgNOR and PCNA staining compared to DEN-induced groups which confirmed the anti-proliferative effects of morin during HCC progression.

During HCC, frequent mutations in the phosphorylation domain of glycogen synthase kinase 3β (Gsk3β) result in the inhibition of the ubiquitin proteasome degradation pathway leading to β-catenin accumulation in the nucleus (Aberle et al., 1997). Mutation in the β-catenin gene at exon 3 has been reported in 30% of HCC cases where this active phenotype forms a complex with T cell-factor (TCF)/lymphoid enhancer-factor (LEF), transiting into the nucleus and activating the target genes such as c-myc, c-jun and cyclin D1 (Miyoshi et al., 1998 and Prieve et al., 1999). Recently, a study showed that TAZ cross talks with the Wnt/β-catenin signaling by forming β-catenin/TAZ/β-TrCP in the cytoplasm. In presence of a Wnt ligand, GSK3β dissociates from the destruction complex β-catenin/TAZ/β-TrCP, β-catenin is dephosphorylated, translocated into the nucleus, with TAZ (Azzolin et al., 2012). Upon Hippo signaling activation, phosphorylated Yap binds with β-catenin in the cytoplasm thereby inhibiting its translocation to the nucleus, and, subsequently the transcription of LEF/TCF target genes (Imajo et al., 2012).

A previous report from this laboratory showed that morin treatment to fibrotic rats resulted in significantly decreased levels of β-catenin and its target genes and exhibited anti-fibrotic effects by suppressing Wnt/β-catenin pathway (Madankumar et al., 2014). Results from this study further confirmed the
antiproliferative action of morin against DEN-induced HCC progression, mainly by activating the hippo signaling which inactivated Yap and TAZ, this inactivated Yap sequestering in the cytoplasm forming a destruction complex with β-catenin leading to its ubiquitination and proteosomomal degradation, thereby inhibiting cell proliferation and cell survival.

2.3.8. EFFECT OF MORIN ON MATRIX METALLOPROTEINASES AND COLLAGEN

Figure-2.11 presents the expression pattern of MMP-2 and MMP-9 in the control and experimental groups determined by gelatin zymography and western blotting. DEN administered groups (III, VI and IX) showed significant increase in MMP-2 and MMP-9 levels compared to control group, while morin treatment in groups (IV, VII and X) markedly reverted the expression to near normal levels. The elevated levels of MMPs in DEN-induced rats revealed the occurrence of cancer cell invasion, inflammation and metastasis; however morin treatment to DEN-induced rats attenuated these changes confirming its inhibitory effect on MMPs.

Further, to examine the effect of morin on collagen levels in DEN-induced groups, the liver sections from control and experimental groups were stained by picrosirius red and masson’s trichrome methods (Figure-2.12.). Liver sections stained by picrosirius red and trichrome method, showed prominent red and blue staining pattern for collagen, respectively, in and around the fibrotic septa and between the nodules in fibrotic and HCC groups whereas the preneoplastic lesion group showed lower level deposition of collagen, as evidenced by decreased red and blue stain intensities. Liver sections from morin treated group (IV, VII and X) exhibited remarkably reduced collagen accumulation when compared to DEN-induced group (III, VI and IX). Control and morin control liver sections showed normal collagen deposition surrounding the portal area and lower percentage of sirius red-positive area.

MMPs play a significant role in tissue remodeling, inflammation, tumour cell growth, migration, invasion and metastasis in many cancers, and they are the
Figure 2.11. Activation of Hippo signaling by morin inhibits tumor invasion through altered levels of MMPs in progressive stages of HCC. (a). Gelatin zymography analyses and (b). Western blot analyses of expression of MMP-2, MMP-9 and densitometric analysis are expressed as “fold change”. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3). Statistical significance at * p<0.05; ** p<0.01; *** p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III); ‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group XI). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated.
Figure 2.12. Activation of Hippo signaling by morin regulates tumour microenvironment through altered levels of collagens in progressive stages of HCC. Psirius red and Masson tri-chrome staining of liver sections in control, morin control, DEN induced preneoplastic lesion, fibrosis and HCC, and morin treated Preneoplastic lesion, fibrosis and HCC rats. Scale bar: 50 μm.
major modulators of the tumour microenvironment, which plays key roles in HCC tumourigenesis (Kessenbrock et al., 2010). The increased proteolytic activities of MMPs degrade the surrounding stroma and allow the tumour cells to spread, thus coordinating tumour invasion. Also, studies have shown that MMPs not only degrade ECM but also modulate and activate cancer signaling pathways such as TGF-β1, which is a key modulator of EMT in HCC (Yu et al., 1999). *In vitro* studies showed that increased availability of ECM components, particularly collagens can promote tumour cell viability, increase motility and induce expression of MMP by invading tumour cells (Ogata et al., 1998 and Enjoji et al., 2000). In a recent study using TAZ knockdown mice, researchers described that TAZ increased the levels of MMP-2 and MMP-9 favouring cell proliferation and EMT in HCC (Xiao et al., 2015).

This study confirmed the previous findings from this laboratory where morin inhibited the activation of HSCs by suppressing TGF-β/Smad signaling, hence prevented the accumulation of excess collagen and reduced the levels of MMP-2, MMP-9 and TIMP-1 (data not published). Also, morin treatment to DEN-induced HCC rats regulated the expressions of MMP-2 and MMP-9 (Sivaramakrishnan et al., 2009). Altogether, morin as described earlier, activated the hippo pathway thereby attenuating TAZ levels, which, in turn, decreased MMP-2 and MMP-9 levels.

**2.3.9. Effect of Morin on NF-κB Signaling**

To determine the effect of morin on NF-κB signaling, the expression levels of key NF-κB signaling molecules such as NF-κBp65 and IkBα were determined (*Figure-2.13*). The qPCR and Western blot results showed significant increase in NF-κBp65 levels with marked decrease in IkBα levels in DEN-induced groups (III, VI and IX) compared to control group. Morin treated groups (IV, VII and X) showed reduced NF-κBp65 levels and markedly increased IkBα level. Immunofluorescence staining of NF-κBp65 (*Figure-2.13(c)*) also confirmed the above western blot results, where prominent nuclear signals for NF-κBp65 were observed in DEN induced groups compared to control group,
(a).

![Graph showing relative mRNA expression of NFκB-p65, IκB-α, and GAPDH in control, morin control, induced, and treated conditions across preneoplastic lesion, fibrosis, and HCC stages.](image1)

(b).

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<td>IκB-α (39 kDa)</td>
<td><img src="image5" alt="Image of IκB-α" /></td>
<td><img src="image6" alt="Image of IκB-α" /></td>
<td><img src="image7" alt="Image of IκB-α" /></td>
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<tr>
<td>GAPDH (37 kDa)</td>
<td><img src="image8" alt="Image of GAPDH" /></td>
<td><img src="image9" alt="Image of GAPDH" /></td>
<td><img src="image10" alt="Image of GAPDH" /></td>
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![Graph showing fold change in NFκB-p65 and IκB-α expression across preneoplastic lesion, fibrosis, and HCC stages.](image11)

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(c). Immunofluorescence analysis of NFκB p65

**Figure-2.13. Morin attenuates the expression levels of NFκB p65 and IκB-α in progressive stages of HCC.** (a). Quantitative PCR analyses of expression of NFκB p65, (b). western blot analyses of expression of NFκB p65, IκB-α and densitometric analysis are expressed as “fold change” and (c). immunofluorescence analyses of NFκB p65 (NFκB p65 (FITC- Green) and propidium iodide (PI- Red) nuclear counterstain) in control and experimental groups of rats. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3) relative to control. Statistical significance at * p<0.05; ** p<0.01; *** p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III); ‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated. Scale bar: 50μm.
which in turn showed basal cytoplasmic staining. Morin treated groups (IV, VII and X) exhibited remarkably strong cytoplasmic signals with weak nuclear staining compared to DEN-induced groups (III, VI and IX).

Canonical NF-κB signaling actively participates and plays a major role in numerous chronic liver diseases. Studies with genetic Rel A−/− knockout mice evidently showed NF-κB as the main transcriptional factor protecting hepatocytes against TNF and LPS induced cell death (Beg et al., 1996). NF-κB is activated in hepatocytes in response to stimuli such as alcohol, endotoxin, TNF-α and cholestasis (bile duct ligation) showing the protective response that limits apoptotic loss of the parenchyma and further promote regeneration of hepatocyte mass by stimulating hepatocyte proliferation. However, such insistently elevated NF-κB levels in hepatocytes lead to chronic inflammatory state driving the development of liver fibrosis and HCC (Chaissson et al., 2002 and Spitzer et al., 2002). A wide range of proinflammatory mediators including LPS, TNF, IL-1β, angiotensin II and CD40L activate NF-κB signaling in liver leading to the activation of HSCs (Schwabe et al., 2001, Paik et al., 2003 and Oakley et al., 2009). Moreover, activated NF-κB signaling is frequently seen in culture activated HSCs (Elsharkawy et al., 1999 and Oakley et al., 2009). Also, CCl4-induced fibrosis rats showed increased NF-κB activation mediating the activation of HSCs, thus stimulating the expression of proinflammatory molecules and anti-apoptotic factors required for activated HSCs survival (Elsharkawy et al., 1999 and Lv et al., 2007). The increased activation of NF-κB in HSCs also contributes to a tumour-friendly microenvironment providing inflammatory cytokines and chemokines as well as accumulation of the ECM proteins ultimately leading to HCC progression.

Previously, in this laboratory, the anti-cancer effects of morin were elucidated, where oral treatment of morin to DEN-induced and DMBA-induced cancer rats resulted in the regulation of exacerbated NF-κB signaling (Sivaramakrishnan et al., 2009 and Nandhakumar et al., 2012). Also, morin exhibited anti-fibrotic effects, where it suppressed NF-κB signaling in activated HSCs and DEN-
induced fibrotic rats (Madankumar et al., 2015). The current result showed that DEN administration to experimental rats in DEN-induced groups led to the activation of NF-κB signaling as evident from the elevated nuclear NF-κBp65 levels. On the other hand, morin treatment to DEN-induced rats exhibited marked reduction in NF-κBp65 and significant increase in IκBα levels thus attenuating the activation of NF-κB signaling.

2.3.10. EFFECT OF MORIN ON APOPTOTIC PATHWAYS

To investigate the pro-apoptotic effects of morin, the expression levels of Bcl-2, Bax and cleaved caspase-9 were determined in the control and experimental groups (Figure-2.14). DEN-induced rats (Groups III, VI and IX) showed significant increase in the levels of Bcl-2 (anti-apoptotic protein) with marked decrease in levels of Bax and cleaved caspase-9 (pro-apoptotic proteins) compared to control. However, morin treatment to rats in groups IV, VII and X resulted in significant increase in Bax and cleaved caspase-9 levels with a concomitant decrease in Bcl-2 levels.

Members of the Bcl-2 family maintain the balance of cell death and survival due to the involvement of pro-apoptotic and anti-apoptotic proteins (Degli et al., 2003 and Letai et al., 2005). Bax has been shown to localize to the mitochondrial membrane and is engaged in the release of cytochrome c during apoptosis (Krajewski et al., 1993). Caspases play a central role in both induction as well as execution of apoptosis. Caspase-9 and caspase-3 are the main effectors and executor caspases involved in intrinsic pathway of apoptosis and play a critical role in the disintegration of the cells undergoing apoptosis. In HCC, the balance between cell death and cell survival is disrupted because of hyperactivation of anti-apoptotic proteins mainly due to the involvement of dysregulated signaling pathways such as NFκB and hippo signaling (Dong et al., 2007 and Lv et al., 2007).
Figure 2.14. Morin induces apoptosis by altering the expression of key apoptotic molecules in progressive stages of HCC. (a), western blot analyses of expression of Bcl-2, Bax, Cleaved Caspase -9 and densitometric analysis are expressed as “fold change”. Data were normalised to GAPDH expression, and shown as mean ± standard deviation (SD) (n=3). Statistical significance at *p<0.05; **p<0.01; ***p<0.001, ‘a’ compared to control rats (Group I); ‘b’ compared to DEN induced preneoplastic lesion rats (Group III);
‘c’ compared to DEN induced fibrotic rats (Group VI); ‘d’ compared to DEN induced HCC rats (Group IX). Lane 1- Control, Lane 2- Morin Control, Lane 3- DEN Induced and Lane 4- Morin Treated.

Several in vivo and in vitro reports emphasise the importance of apoptosis. Liver-specific transgenic Yap mice displayed enlarged liver phenotype and when the Yap overexpression was turned off the liver returned to its normal size via apoptosis (Camargo et al., 2007 and Dong et al., 2007). Similar results were obtained in liver-specific Mst1/2 and Sav1 knockout models (Zhou et al., 2009). Also, overexpression of Mst1 in HepG2 cells showed enhanced sensitivity to cisplatin and thereby inhibited the growth of HepG2 cells through induction of apoptosis (Xu et al., 2013).

Earlier reports in this laboratory confirmed the pro-apoptotic effects of morin in DEN-induced and DMBA-induced cancer rats (Sivaramakrishnan et al., 2009 and Nandhakumar et al., 2012). Also, morin was shown to potentiate apoptosis in activated HSCs and DEN-induced fibrotic rats (Madankumar et al., 2015). Likewise in this study, morin exhibited its pro-apoptotic effects mainly by suppressing NF-κB activation and activating the hippo signaling in DEN-induced rats.

2.4. CONCLUSION

In the in vivo study, DEN-induced preneoplastic lesion, fibrosis and HCC rat models were successfully established. The study, for the first time, determined possible cross talk between the Hippo, Wnt/β-catenin and NF-κB signaling pathways in DEN induced rats. Overall, this study is the first to demonstrate the mechanistic action of morin on hippo signaling in DEN-induced progressive stages of HCC. The positive outcome from the animal study therefore encouraged to perform an in vitro study using HepG2 cells which may provide novel insights about the molecular mechanisms behind the hepatoprotective effects of morin.