Summary

The objective of this study was to investigate the molecular mechanism behind the activation of Hippo pathway by morin and to determine its co-ordinated regulation of β-catenin and NF-κB signaling in experimental liver cancer. To examine the hypothesis, human liver carcinoma (HepG2) cells and diethylnitrosamine (DEN) induced progressive stages of experimental liver cancer models were used. The key findings are summarized below:

Objective 1: To elucidate the anti-proliferative and pro-apoptotic effects of morin in HepG2 cells.

- Morin treated HepG2 cells showed concentration and time dependent cytotoxicity with an estimated IC₅₀ value of 50 µM at 24 and 48 h, as determined by MTT and LDH assays.

- Expression profile of key hippo signaling molecules such as Mst1, p-Mst1, Lats1, p-Lats1 and p-Yap were significantly increased with marked decrease in Yap expression upon morin treatment, indicating the activation of hippo pathway. Immunofluorescence analysis revealed prominent nuclear staining for Mst1 and Lats1 in morin treated cells with marked increased cytosolic staining for Yap which further complemented the above said phenomena.

- Morin treated cells showed significantly decreased levels of β-catenin, GSK-3β and c-Myc compared to the untreated cells, emphasizing the anti-proliferative role of morin, mainly by inhibiting β-catenin activation. The immunofluorescence staining of β-catenin confirmed the anti-proliferative action of morin with marked cytosolic expression compared to intense nuclear staining in untreated HepG2 cells.

- Morin treatment for 24 and 48 h induced G0/G1 cell cycle arrest in HepG2 cells with significantly decreased cyclin D1 protein levels, an
active regulatory switch in cell cycle progression from G1 to S phase augmenting the anti-proliferative potential of morin.

- Morin treatment for 24 and 48 h resulted in significant increase in the levels of Bax and cytochrome c with marked decrease in Bcl-2 levels thereby promoting the activation of caspase-9 and caspase-3 favoring apoptosis in HepG2 cells. Also, annexin-V/PI staining in morin treated HepG2 cells further validated the induction of apoptosis determined by flow cytometry showing the translocation of phosphatidylserine from the inner leaflet of the phospholipid bilayer to its cell surface. DNA fragmentation analysis demonstrated that morin induced apoptosis in HepG2 cells. Nuclear condensation and fragmentation, the characteristic features of apoptotic cells, were observed in morin treated HepG2 cells which were evident from AO/EB, PI and DAPI staining. The number of apoptotic cells was significantly increased in cells exposed to morin in a time dependent manner.

**Objective 2: To understand the molecular regulatory mechanism of morin behind the activation/inhibition of key molecular pathways in the progressive stages of experimental liver cancer.**

- Increased levels of the serum marker enzymes (AST, ALT, ALP, LDH and γ-GT) in DEN induced rats (preneoplastic lesion, fibrosis and HCC) were significantly brought back to near normal levels upon morin treatment in all the three progressive stages of liver cancer (preneoplastic lesion, fibrosis and HCC), exhibiting its hepatoprotective potential.

- Oxidative stress induced by DEN administration, as evidenced from elevated lipid peroxides and protein carbonyl levels with reduced antioxidant defense mechanism, played a major role in liver damage. Morin treatment significantly decreased the oxidative stress and increased the antioxidant enzymes due to its antioxidant potential in DEN induced progressive stages of liver cancer.
The levels of tumour stage specific markers GST-pi, α-SMA and PCNA were significantly increased in DEN induced preneoplastic lesion, fibrotic and HCC rats, respectively. Their expression levels were significantly reverted to near normal upon morin treatment, indicating the anti-cancer action of morin against progressive stages of liver cancer.

The levels of Mst1 and Lats1 were significantly increased with marked decrease in Yap and TAZ levels in all the morin treated groups suggesting the activation of hippo signaling compared to DEN induced experimental groups (preneoplastic lesion, fibrosis and HCC).

Morin suppressed NF-κB signaling by decreasing the levels of active NF-κBp65 with increased IκBα levels compared to DEN induced rats (preneoplastic lesion, fibrosis and HCC), thereby inhibited IκB-α degradation and NF-κBp65 translocation as evident from western blot and immunofluorescence analysis.

Histopathological analysis of collagen deposition in liver (picro-sirius red and masson’s trichrome staining) confirmed significant reduction in the liver collagen content in morin treated groups compared to DEN induced experimental groups.

Morin treatment significantly decreased MMP-2 and MMP-9 levels as evident from gelatin zymography and western blot analysis compared to DEN induced experimental groups.

In DEN induced group, the elevation of β-catenin and its target gene cyclin D1 along with prominent nuclear β-catenin expression warrants the active participation of Wnt/β-catenin pathway. Also, significant downregulation of GSK 3β, β-catenin and its associated proteins by morin demonstrated the anti-proliferative potential of morin in DEN induced progressive stages of liver cancer.
- Morin potentiated apoptosis through activation of hippo signaling by altering the apoptotic proteins with significant increase in Bax protein along with decreased expression of Bcl-2, promoting the activation of caspases-9 leading to apoptosis compared to DEN induced experimental groups.

**Objective 3:** To elucidate the molecular mechanisms behind the activation of Hippo pathway by morin in Mst1 transfected HepG2 cells.

- Morin treated F-Mst1 transfected cells showed existence of a prominent 35kDa kinase active cleaved Mst1 protein which was absent in F-Mst1 overexpressed cells and untreated HepG2 cells. This cleaved form resembled the function of kinase active N-Mst1 overexpressed HepG2 cells, as evident from immunofluorescence staining showing increased nuclear staining for Mst1, indicating activation of Mst1.

- Morin treated F-Mst1 transfected cells showed significantly increased levels of p-Mst1, Lats1, p-Lats1 and p-Yap with marked decrease in Yap expression resembling similar expression profile of kinase active N-Mst1 overexpressed cells that in-turn indicated the activation of hippo signaling in morin treated HepG2 cells. The immunofluorescence results substantiated the activation of hippo signaling by morin in F-Mst1 transfected cells which showed increased nuclear staining for Mst1 and Lats1 with decreased Yap nuclear expression, as similar to N-Mst1 expressing HepG2 cells.

- F-Mst1 overexpressed cells upon morin treatment showed significantly decreased levels of β-catenin, Gsk-3β and cyclin D1 compared to the untreated and F-Mst1 overexpressed HepG2 cells highlighting the anti-proliferative action of morin, which activated hippo signaling and inhibited the β-catenin through Mst1 mediated mechanism.

- This study is the first to report the molecular link between hippo signaling and NF-κB signaling where the cleaved active Mst1 fragment in morin
Schematic representation of the molecular mechanistic action of morin on attenuation of progression of Hepatocellular carcinoma (HCC) through Mst1/Hippo mediated regulation.
treated F-Mst1 transfected cells suppressed the activation of NF-κBp65 by downregulating the IκB-α degradation. Decreased NF-κBp65 levels with increased IκB-α levels compared to the untreated and F-Mst1 overexpressed HepG2 cells, thus prevented nuclear translocation of NF-κBp65 as evident from NF-κB p65 GFP reporter assay and NF-κB ELISA assay.

- Induction of apoptosis through the activation of hippo signaling via Mst1-JNK mediated mechanism was shown in morin treated F-Mst1 transfected cells with significantly increased levels of JNK, p-JNK, c-JUN and Bax and concomitant decrease in Bcl-2 levels, promoting the activation of caspase-9 and caspase-3 and favoring apoptosis. Annexin V-FITC/PI staining further validated the induction of apoptosis in morin treated F-Mst1 transfected cells and N-Mst1 transfected cells with increased nuclear condensation and fragmentation, the characteristic features of apoptotic cells as evident from AO/EB and PI staining.

**Conclusion**

Altogether, both in vitro and in vivo studies clearly showed the anti-cancer effect of morin through the activation of hippo pathway, mainly regulating Wnt/β-catenin and NF-κB signaling and favouring apoptosis via Mst1 mediated mechanism. Thus, this study holds a significant attempt for an effective therapeutic approach with the use of a dietary flavonoid targeting multiple molecular signaling events in the progression of liver diseases.