

SUPPLEMENTARY TEXT

Nimbolide inhibits tumor growth by restoring hepatic tight junction protein expression and reduced inflammation in an experimental hepatocarcinogenesis

Ram AK *et al.* Nimbolide inhibits tumor growth in hepatocellular carcinoma

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MATERIALS AND METHODS-MOLECULAR DOCKING STUDIES

Protein preparation

The 3D X-ray crystal structure of ZO-1 (PDB ID: 2RRM), NF- κ B (PDB ID: 1VKX) and TNF- α (PDB ID: 2TNF) were obtained from RCSB protein data bank (<http://www.rcsb.org>). The retrieved proteins were prepared before molecular docking using PyMol software (Schrodinger LLC, Cambridge, USA). The water molecules were removed from the protein crystallographic structure and polar hydrogens, koliman charge were added using AutoDock 4.0 and saved in .pdb script for further molecular docking analysis.

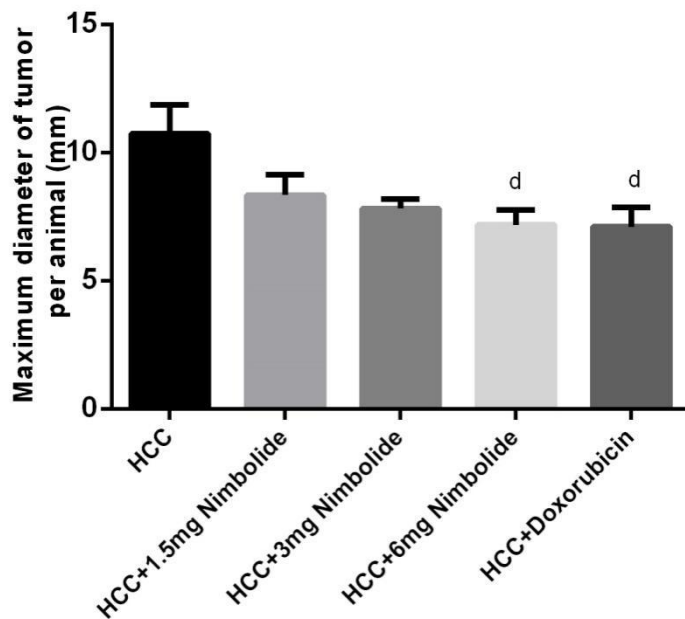
Ligand preparation

Nimbolide (CID: 100017) was retrieved from Pubchem database (<https://pubchem.ncbi.nlm.nih.gov/compound>) in.SDF file format and then converted to .mol2 format using Open Babel software version 2.4.1. The ligand nimbolide was prepared and optimized by means of ligand preparation script in AutoDock 4.0 program. The ligand was prepared for docking as detecting root, torsion tree were set and saved in .pdbqt file format.

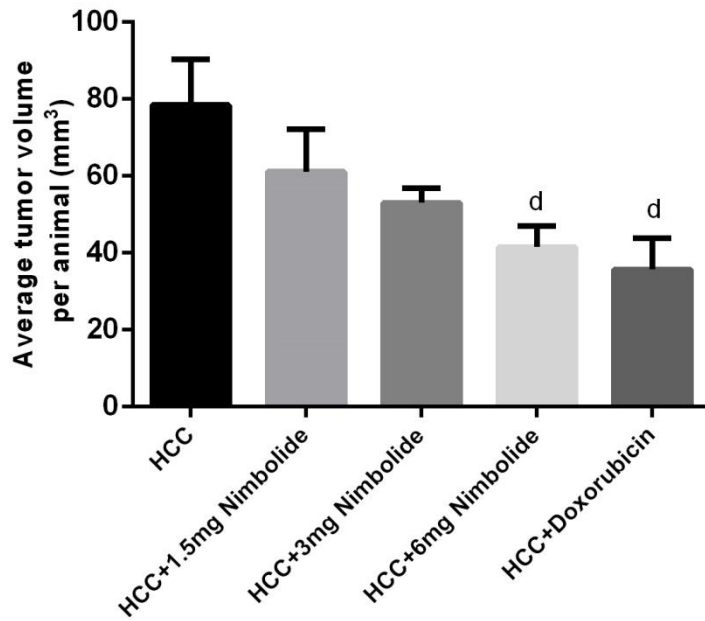
Grid generation

Grid generation was carried out using the prepared crystallographic structures of ZO-1, NF- κ B and TNF- α protein. The ligand molecules nimbolide in the complexes of protein structure were picked to form grid. Thereafter, the centroid of ligand molecules in complex structures were chosen to generate grid points X = 60, Y = 60, and Z = 60 axis set for docking analysis. The grid file was generated by means of “grid generation panel” in AutoDock software version 4.0.

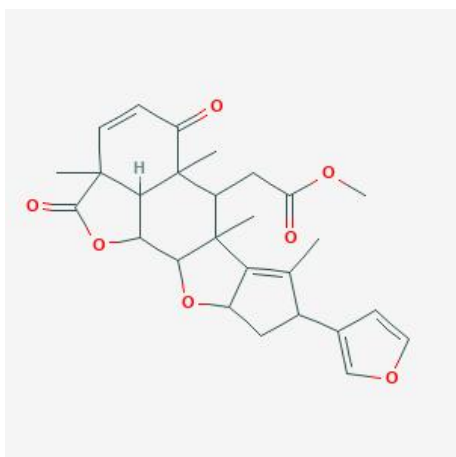
Figure Legends



Supplementary Figure 1 Dose response effect of nimbolide on maximum tumor diameter in experimental mice groups ($n = 6-7$). Values were expressed as mean \pm standard error of mean. Comparison between the groups were analysed by one way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. ^d $P < 0.05$ compared to hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

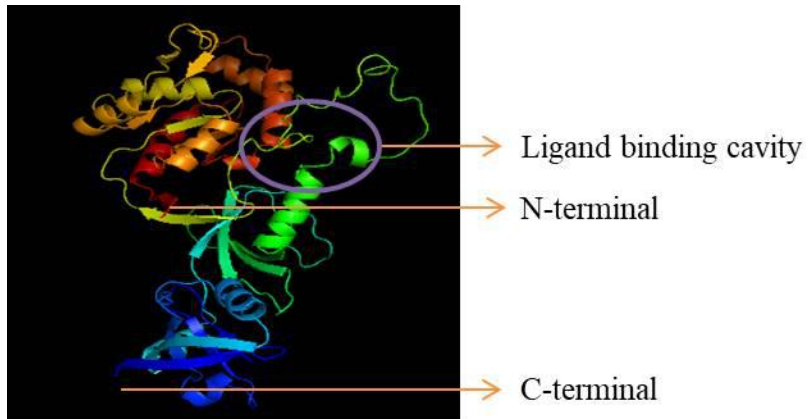


Supplementary Figure 2 Dose response effect of nimbolide on tumor volume in experimental mice groups ($n = 6-7$). Values were expressed as mean \pm standard error of mean. Comparison between the groups were analysed by one way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. ^d $P < 0.05$ compared to hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

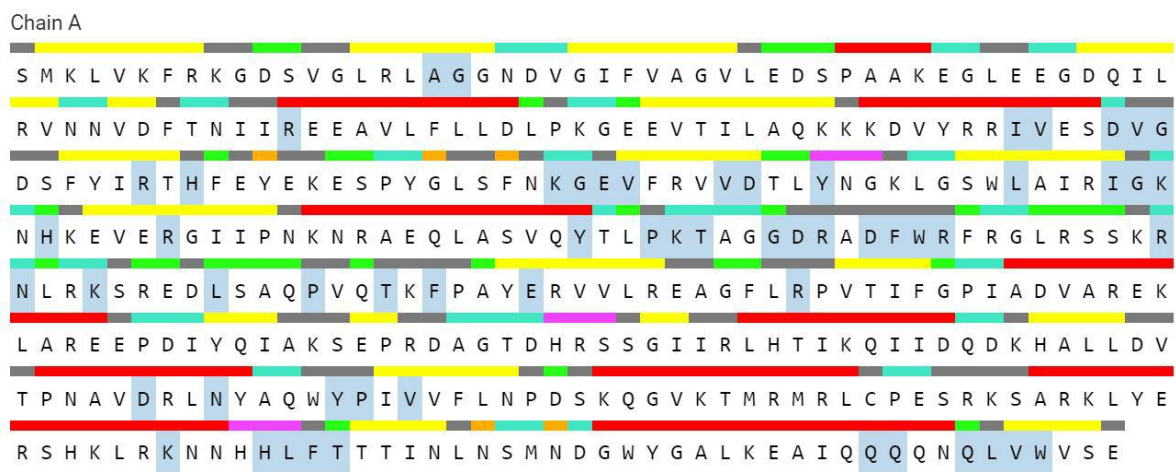


Supplementary Figure 3 Representation of 2D structure of nimbolide.

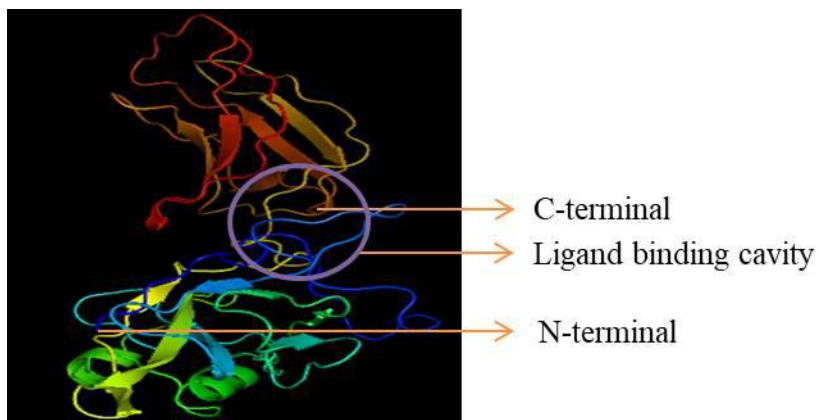
A

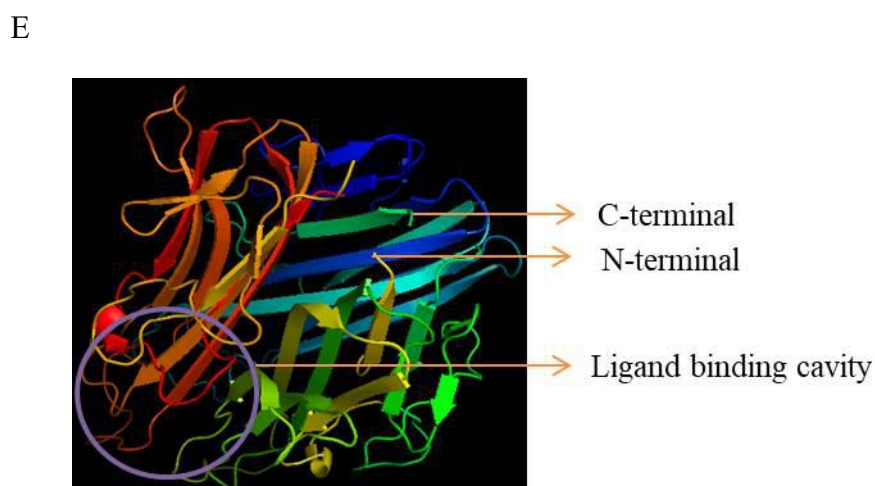
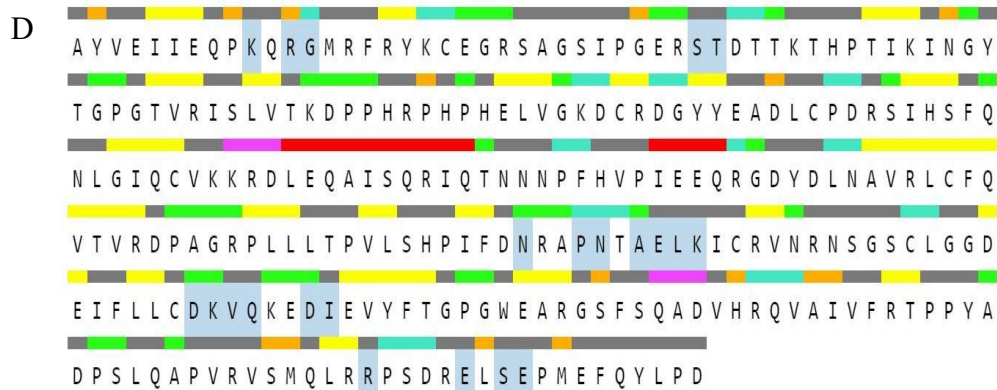


B

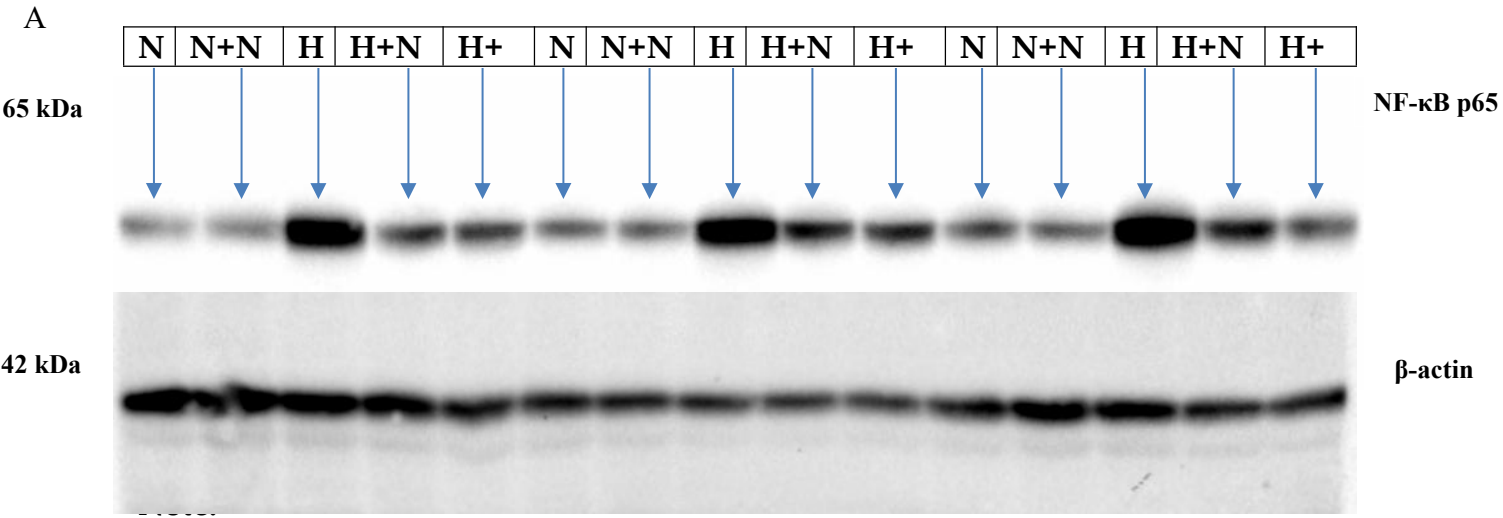


C



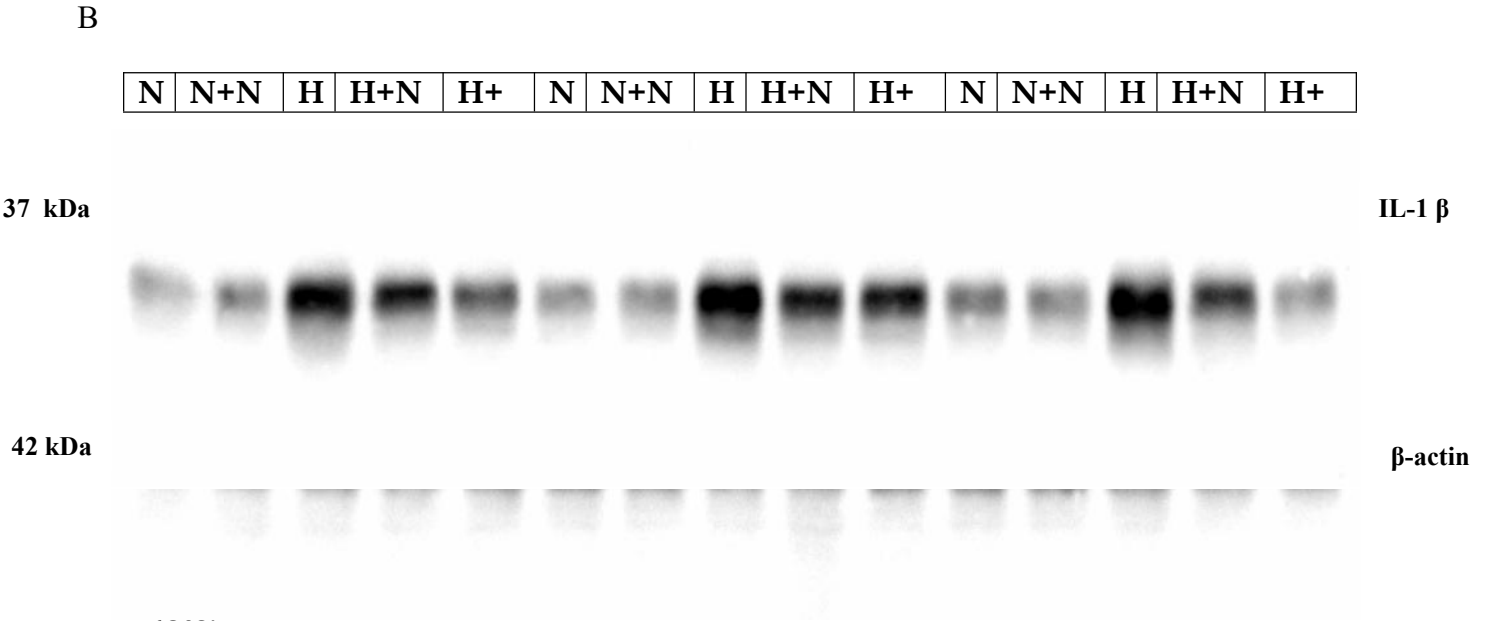


Supplementary Figure 4 3D and 2D structures. A and B: Ligand binding cavity of ZO-1; C and D: Ligand binding cavity of NF-Kb; E and F: Ligand binding cavity of TNF- α .



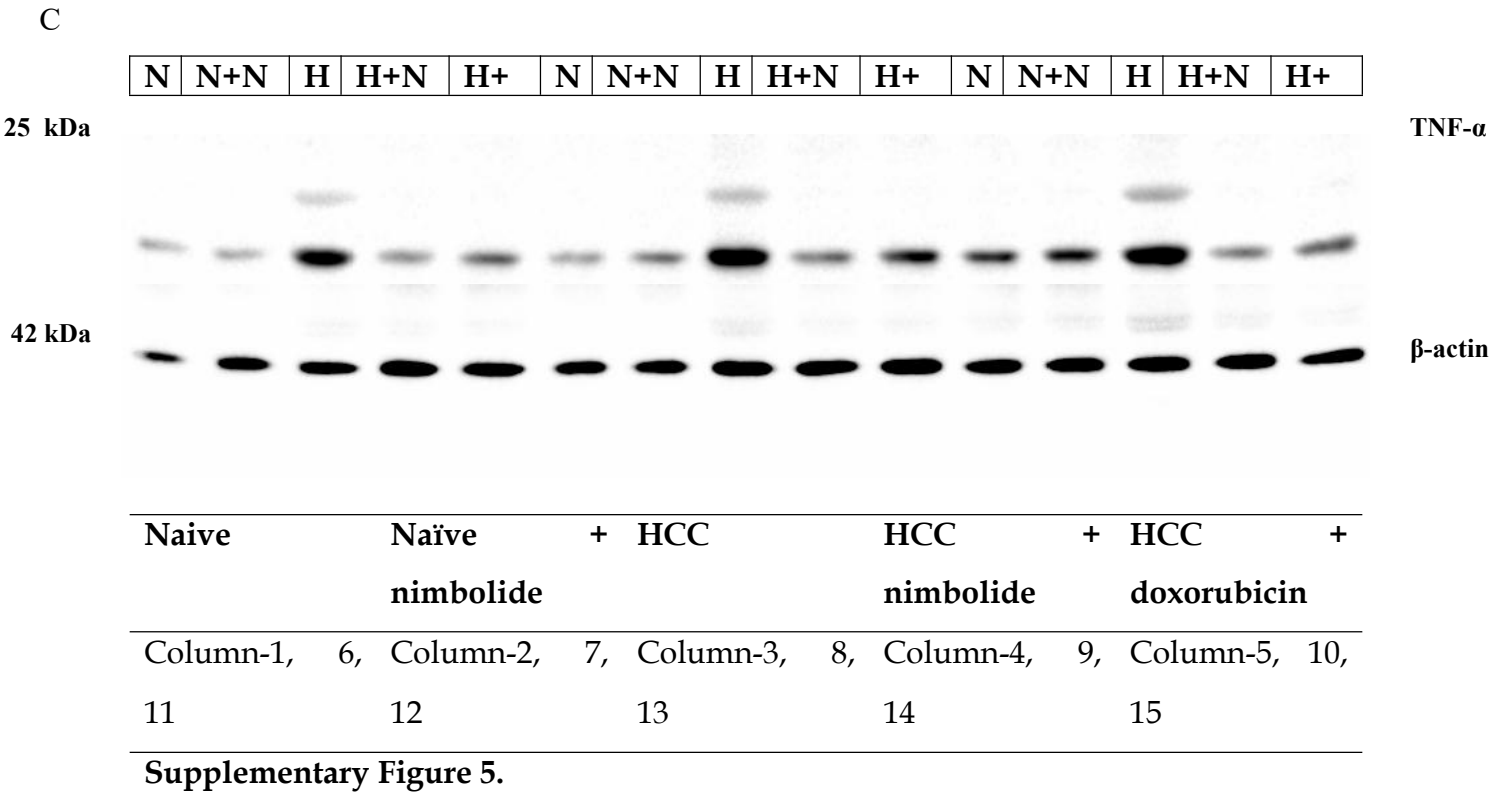
N: Naïve; H: HCC; NL: Nimbolide; D: Doxorubicin.

Naïve		Naïve + HCC		HCC + HCC		HCC + HCC		HCC + HCC		HCC + HCC	
		nimbolide				nimbolide		doxorubicin			
Column-1, 6, 11	Column-2, 7, 12	Column-3, 8, 13	Column-4, 9, 14	Column-5, 10, 15							



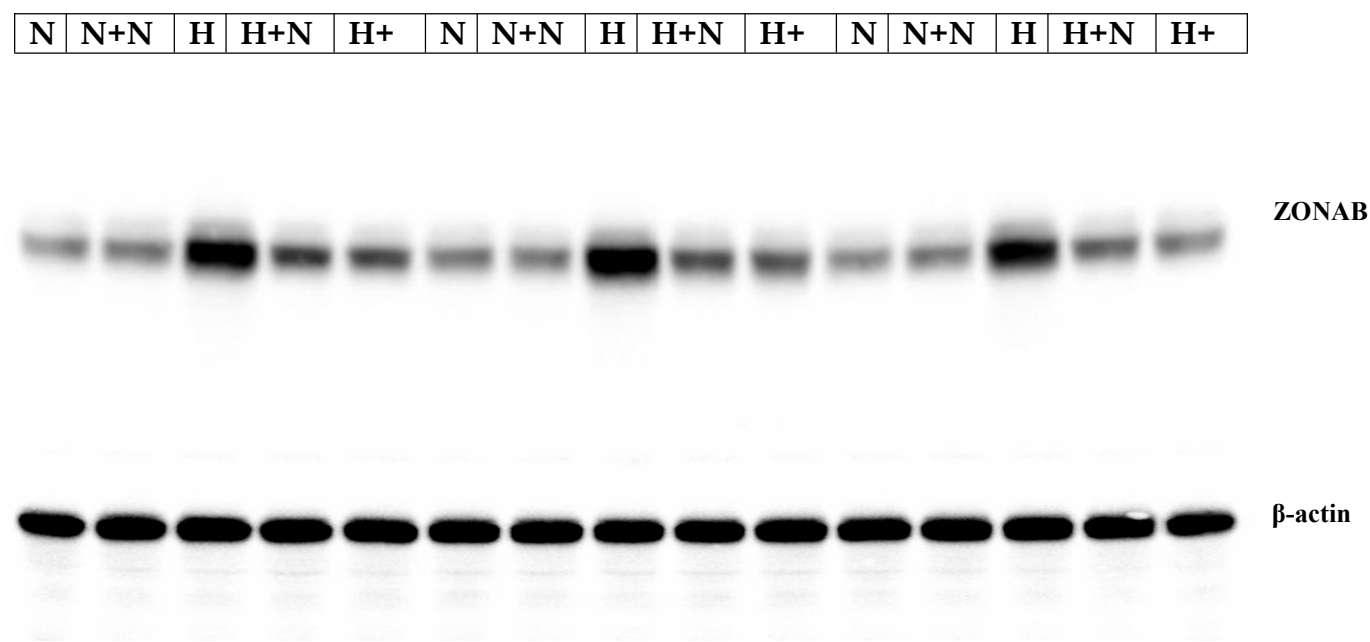
N: Naïve; H = HCC; NL: Nimbolide; D: Doxorubicin.

Naive		Naïve		+ HCC		HCC		+ HCC		+
		nimbolide				nimbolide		doxorubicin		
Column-1, 6, 11		Column-2, 7, 12		Column-3, 8, 13		Column-4, 9, 14		Column-5, 10, 15		



Supplementary Figure 5.

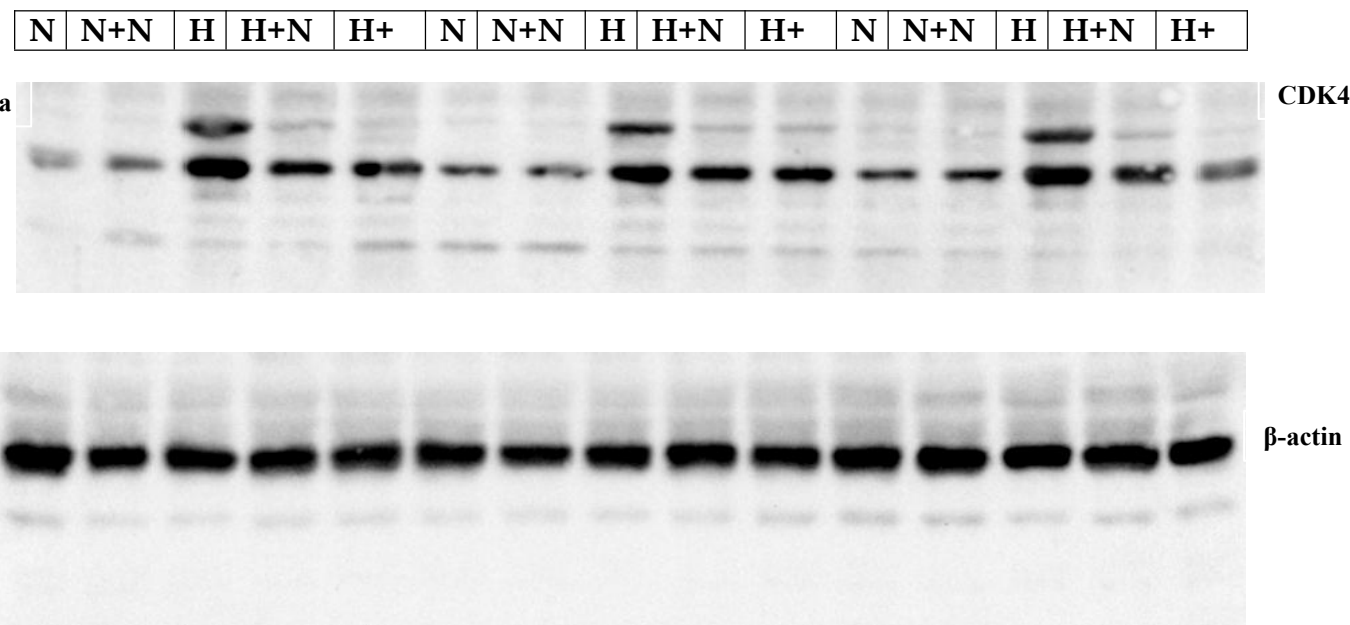
A



N: Naïve; H: HCC; NL: Nimbolide; D: Doxorubicin.

Naïve		Naïve + nimbolide		HCC		HCC + nimbolide		HCC + doxorubicin	
Column-1, 11	6, 12	Column-2, 12	7, 13	Column-3, 13	8, 14	Column-4, 14	9, 15	Column-5, 10, 15	

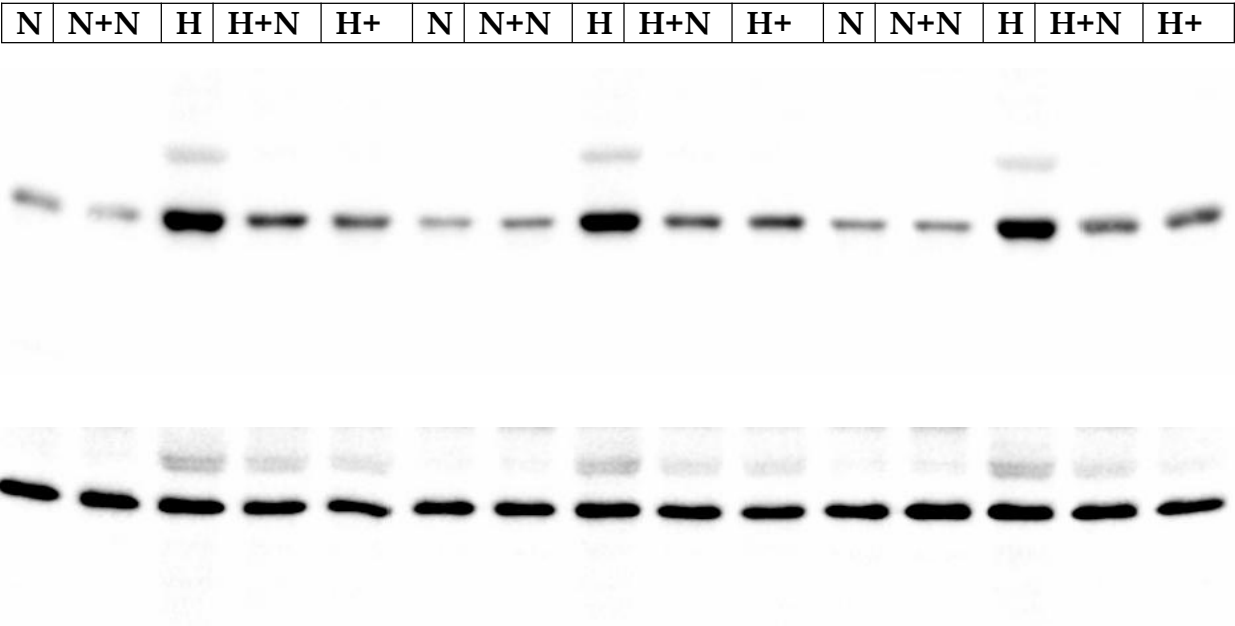
B



N: Naïve; H: HCC; NL: Nimbolide; D: Doxorubicin.

Naïve		Naïve + HCC		HCC + HCC		HCC + HCC		HCC + HCC	
		nimbolide				nimbolide		doxorubicin	
Column-1, 6, 11	Column-2, 7, 12	Column-3, 8, 13	Column-4, 9, 14	Column-5, 10, 15					

C

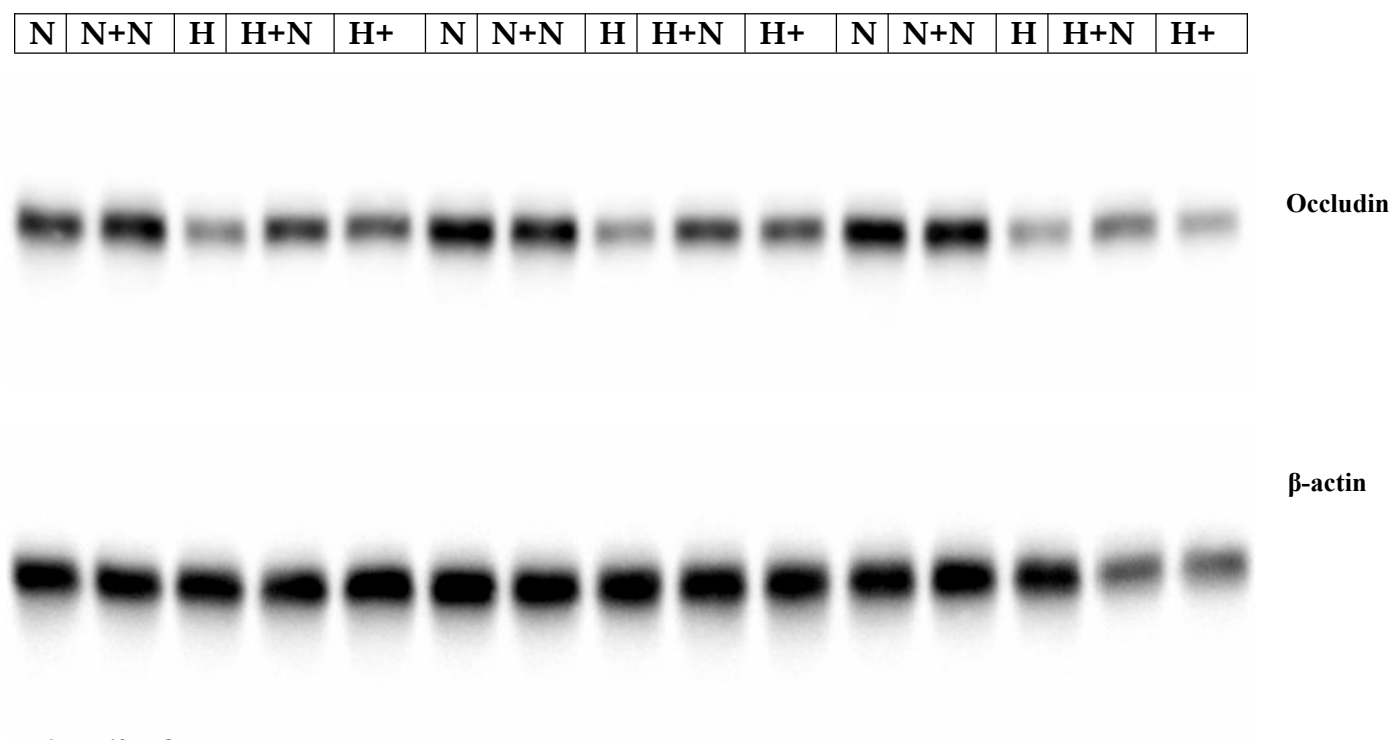


N: Naïve; H: HCC; NL: Nimbolide; D: Doxorubicin.

Naïve		Naïve + HCC		HCC + HCC		HCC + HCC		HCC + HCC	
		nimbolide				nimbolide		doxorubicin	
Column-1, 6, 11	Column-2, 7, 12	Column-3, 8, 13	Column-4, 9, 14	Column-5, 10, 15					

Supplementary Figure 6.

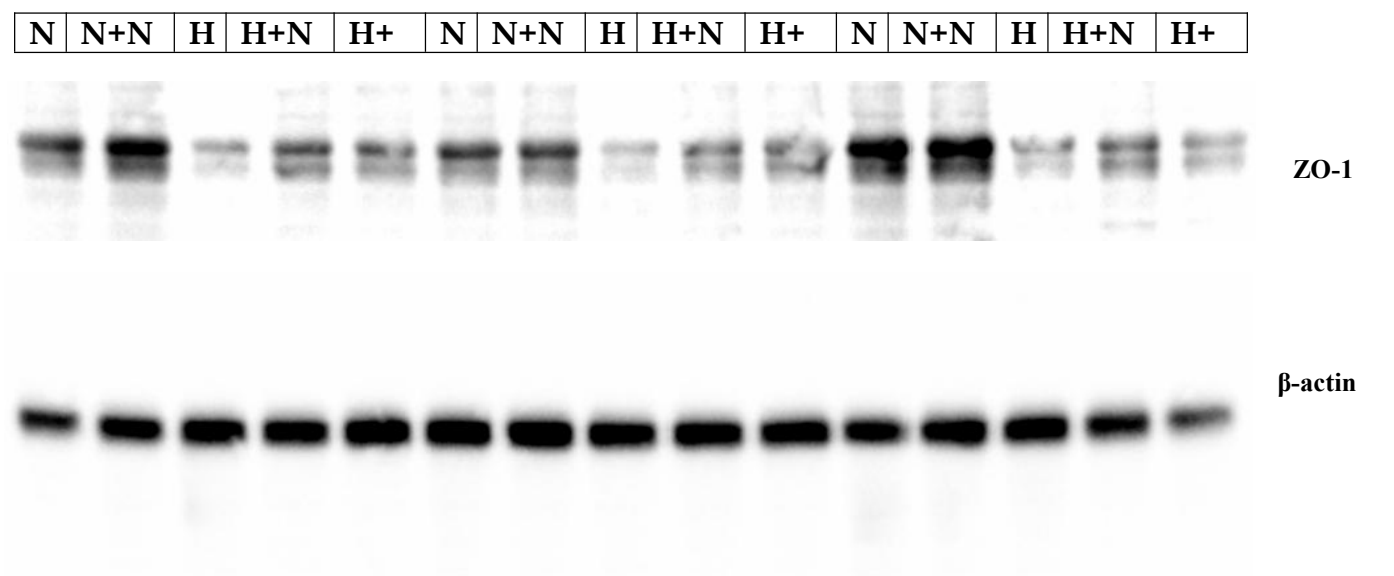
A



H: HCC; NL: Nimbolide; D: Doxorubicin.

Naive		Naïve		+ HCC		HCC		+ HCC		+	
		nimbolide				nimbolide		doxorubicin			
Column-1, 6, 11	Column-2, 7, 12	Column-3, 8, 13	Column-4, 9, 14	Column-5, 10, 15							

B



Naive		Naïve	+	HCC		HCC	+	HCC	+
		nimbolide				nimbolide		doxorubicin	
Column-1, 6, 11		Column-2, 7, 12		Column-3, 8, 13		Column-4, 9, 14		Column-5, 10, 15	

HCC: Hepatocellular carcinoma.

Supplementary Figure 7.

Supplementary table 1 Dose response effect of nimbolide on plasma biochemical parameters and alpha-fetoprotein levels in naïve and experimental mice

Parameter s	Naïve	HCC	HCC + 1.5 mg/kg nimbolide	HCC + 3 mg/kg nimbolide	HCC + 6 mg/kg nimbolide	HCC + doxorubicin
AST (IU/L)	58.67 ± 6.11	236.7 ± 12.99 ^c	217.0 ± 15.63	188.3 ± 17.47	133.3 ± 21.15 ^e	138.7 ± 16.95 ^e
ALT (IU/L)	38.33 ± 3.48	153.0 ± 13.89 ^c	135.7 ± 9.93	117.0 ± 6.42	90.67 ± 7.83 ^e	92.0 ± 10.12 ^e
ALP (IU/L)	66.33 ± 8.09	296.7 ± 30.6 ^c	257.0 ± 15.5	187.7 ± 16.22 ^d	150.3 ± 16.38 ^f	142.3 ± 26.27 ^f
AFP (ng/mL)	1.74 ± 0.10	8.15 ± 0.85 ^c	7.61 ± 0.60	6.03 ± 0.78	5.22 ± 0.53 ^d	4.93 ± 0.59 ^d

Values were expressed as mean ± standard error of mean ($N = 3-6$). Comparison between the groups were analysed by one way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. ^c $P < 0.0001$ compared to naïve; ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ compared to HCC. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.