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Potential Therapeutic Effect of Sinigrin on Diethylnitrosamine-Induced Liver Cancer in Mice: Exploring the Involvement of Nrf-2/HO-1, PI3K−Akt−mTOR Signaling Pathways, and Apoptosis

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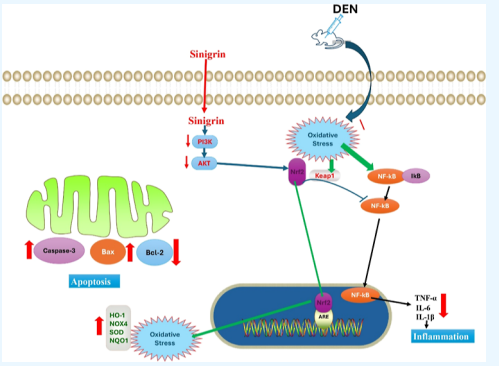
ABSTRACT: Sinigrin is a glucosinolate present in plants of the family Brassicaceae and has been considered for its anticancer potential. This study examines the efficacy of sinigrin on the liver cancer caused by diethylnitrosamine (DEN) in mice through the analysis of its impact on the Nrf-2/HO-1, PI3K−Akt−mTOR, and apoptotic pathways. Development of liver cancer was induced by intraperitoneal injection at the age of 14 days with DEN (25 mg/kg) in mice. Thereafter, sinigrin was orally administered at doses of 10 and 20 mg/kg body weight per day the last 28 days. At the end of 10 weeks, mice were sacrificed and then we conducted hepatic biochemical and molecular assessments. Sinigrin reduced the serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), alpha-fetoprotein (AFP), and bilirubin but increased total protein, and albumin, levels. Sinigrin increased the antioxidant enzymes (SOD, CAT, GPx, and GST) as indicated by reduced 8-OHdG, TBARS and increased glutathione. Sinigrin reduced the levels of inflammatory cytokines (IL-6, IL-1β, TNF-α, and NF-κB p65) and PI3K/AKT/mTOR signaling pathway. Sinigrin also activated the intrinsic mitochondrial apoptosis pathway mediated by p53, downregulated antiapoptotic proteins (Bcl-2), upregulated pro-apoptosis regulatory proteins like Bax and caspase-3. All these results indicate that the protective effects of sinigrin against liver cancer are likely to be applied as an effective therapeutic agent through its antioxidant and pro-apoptotic activities.



1. INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most frequent liver cancers with elevated incidence and increased morbidity which becoming a serious public health problem worldwide. HCC ranks the third leading cause of cancer death and more than 900,000 new cases in the year 2020.[1] Therefore, conditions like nonalcoholic fatty liver disease (NAFLD) and toxins like DEN are considered to increase the risk of HCC.[2] Therefore, the processes that contribute to hepatocarcinogenesis mediated by DEN must be understood to prevent and treat the disease effectively. HCC is mainly induced by genetic and epigenetic changes, chronic inflammation, and environmental factors.[3] Dysmetabolic scenario and increased oxidative stress in nonalcoholic steatohepatitis raise the chances of HCC. For instance, the global mortality rate of liver cancer in 2020 was estimated to be 830,180, which proves that there is a need to address the issue of HCC treatment and prevention.[4]

DEN, which is a potent hepatocarcinogen used in research, causes DNA damage and oxidative stress followed by inflammation to develop liver tumors.[5] Initiation was caused by the formation of DNA adducts in a process of activation of DEN through cytochrome P450 enzymes.[5] It is for this reason that antioxidants play a significant role in the prevention of liver cancer given the fact that DEN-induced oxidative stress was evident. Previous Mahmoud et al.[6] has been reported that several signaling pathways such as Nrf-2/HO-1 is activated during hepatocarcinogenesis induced by DEN. These pathways regulate the cellular defense mechanism of antioxidants that fight free radicals induced oxidation. Stimulating the Nrf-2/HO-1 pathway reduces the extent of hepatotoxicity and prevents DEN-induced carcinogenesis.[7] Also, the derangement of the PI3K/AKT/mTOR pathway is involved in HCC development. Cell survival/death and growth of cells is one of the processes controlled by signaling in the PI3K/AKT/mTOR pathway that includes cell proliferation/apoptosis.[8] Any alteration to this pathway can lead to abnormal cell growth and metastasis as well as the development of tumors. The PI3K/AKT/mTOR pathway was reported to be hyperactivated in the HCC context which is one of the known

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oncogenic events that drive the malignant change of hepatocytes.

Sinigrin, a glucosinolate present in plants belonging to the Brassicaceae family, is a compound being explored for its anticancer potential. Sinigrin is isolated from vegetables like black mustard, *Brussels sprouts*, and broccoli seeds and exhibits various biological properties, including anticancer, anti-inflammatory, and antioxidant activities.[9−11] The hydrolysis of sinigrin produces isothiocyanates, such as allyl isothiocyanate, which exhibit anticancer effects by inhibiting cancer cell proliferation, inducing apoptosis, and modulating signaling pathways associated with tumor progression.[12] Previous results shown that sinigrin had potential to be an anticancerous compound due its effects on cancer cell proliferation, apoptosis induction and signaling pathways related to poor outcome.[13] It regulates several molecules including p53, Bcl-2, caspases and cell cycle arrest.[14] Therefore, this leads to the formation of hindrance to tumor growth and increase apoptosis and improvement of the liver in DEN-induced liver cirrhosis. In the present research, we aimed to examine the impact of sinigrin on the Nrf-2/HO-1 and PI3K/AKT/mTOR pathways.

Sinigrin has anticancer effect and offers cardiovascular benefits through the prevention of atherosclerosis through the inhibition of inflammatory mediators.[9,15] Besides, this substance possesses antibacterial and antifungal activity, it may be used in combating microbial infections. Also, the antioxidant activity reduces oxidative stress and DNA damage which are critical in the development of cancer. Besides, studies have demonstrated that sinigrin exhibits minimal toxicity toward normal cells, particularly human keratinocyte cells (HaCaT), while selectively targeting cancer cells.[16] Based on these assumptions, it is believed that sinigrin can prevent the negative impacts of DEN on liver cells by increasing apoptosis and blocking cell proliferation pathways, which may provide a new strategy for the treatment of liver cancer. The novelty of this study lies in investigating the exact molecular pathways via which sinigrin may have anticancer benefits in HCC. More precisely, the study examines the impact of sinigrin on the Nrf-2/HO-1 and PI3K/AKT/mTOR signaling pathways. This research aims to further our understanding of the pathways involved in order to investigate the possibility of sinigrin as a therapeutic agent for liver cancer treatment.

2. MATERIALS AND METHODS

2.1. Animals. An animal model including 24 male 14 days old ICR mice was used for the experimental research. All mice were housed under specific pathogen-free conditions in facilities with a standard temperature, light/dark cycle and humidity with free access to food and water throughout the study periods. This study was approved by the Ethics Committee of Shanxi Province Cancer Hospital/Shanxi Hospital Affiliated to Cancer Hospital (approval number: SPCHC-2023-136).

2.2. Experimental Design. Thirty male ICR mice (n = 6 per group) were randomly assigned to one of four groups.

Group I (control): Mice were given normal saline (0.1% NaCl) throughout the experiment.

Group II (DEN): DEN dissolved in sterile saline was intraperitoneally administered to mice at 25 mg/kg of body weight when the animals reached 14 days old, and they were euthanized after reaching ten months following DEN injection.[17]

Group III (DEN + 10sinigrin): The mice were injected with a dose of about 25 mg/kg body weight intraperitoneally at 14 days of age. 10 mg/kg sinigrin administered orally for 28 days before the termination of the experiment. These animals were euthanized by cervical dislocation 10 months after the DEN injection.

Group IV (DEN + 20sinigrin): The mice were injected with a dose of about 25 mg/kg body weight intraperitoneally at 14 days of age. 20 mg/kg sinigrin administered orally for 28 days before the termination of the experiment. These animals were euthanized by cervical dislocation 10 months after the DEN injection as indicated in Figure 1A.

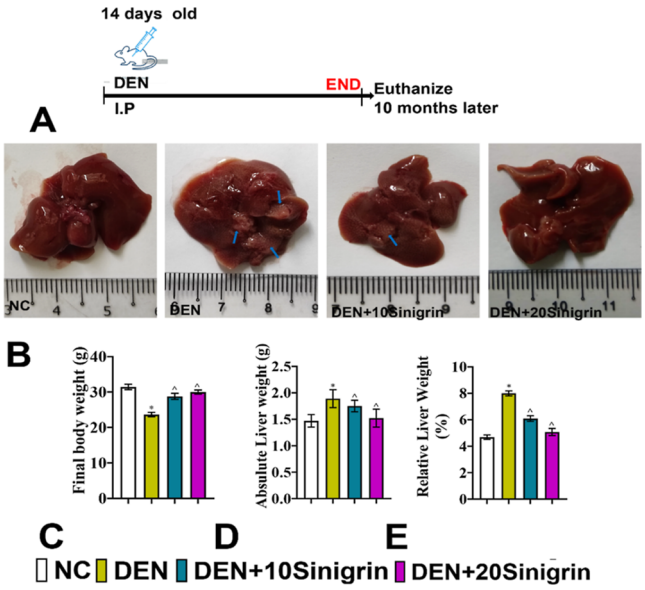

Figure 1. Effects of sinigrin on liver morphology and weight in DEN-treated mice. (A) Shows experimental design, (B) representative images of liver from each treatment group, (C) final body weight of mice, (D) absolute liver weight, (E) relative liver weight (liver weight/body weight ratio) of mice from each treatment group. Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; $\hat{p}$ < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

We selected a dose of 25 mg/kg of DEN based on its effectiveness in generating hepatic carcinoma in mouse models. We delivered DEN to the mice at 14 days of age because of the critical nature of this developmental period for liver growth, which renders the liver more susceptible to carcinogenic chemicals.[17] The period chosen for administering DEN guarantees that it reliably and consistently causes the formation of tumors. This is crucial for accurately assessing the therapeutic capabilities of sinigrin. The dosages of 10 and 20 mg/kg sinigrin in the present study were selected based on the findings of previous studies on the therapeutic value of this compound. For Group III, the dose of 10 mg/kg was chosen, as Jie et al.[14] used the lower dose of 10 mg/kg, which demonstrated considerable anticancer properties with mild side effects. For Group IV, a dose of 20 mg/kg was selected as it was in the middle between the 15 and 25 mg/kg doses used in other studies, to maximize therapeutic efficacy while minimizing the risk of toxicity.

2.3. Body Weight and Liver Weight Assessment. At the end of study, body weight was monitored in mice. At the

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end of the experiment, all animals were first sedated using ketamine /xylazine at a dose of 90/10 mg/kg and then killed by cervical displacement. The liver tissues were also removed, weighed and prepared for other tests. At the same time, blood samples were taken for biochemical examination.

2.4. Organ Indices Assessment. After the excision, the liver weight of each mouse was taken and the tissue washed with buffered saline. The liver was then dried in an oven until it was totally dry and the dry weight was recorded. To compare the liver size with the body mass, the liver weight was presented as a percentage of the body weight of the animal.

2.5. Assessment of Liver Tissue Injury Markers. The markers of liver injury, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-GGT in serum were assayed by ELISA kits according to the manufacturer's instructions and are expressed as described figures per IU/L.

2.6. Biochemical Analyses for Liver Function Test. The serum total protein, albumin (ALB), globulin (GLB), and total bilirubin (TBIL) concentrations were measured by the automatic biochemical analyzer (Roche Cobas c 311) and the corresponding kits (Elabscience, Wuhan, China).

2.7. Assessment Marks of the 8-Hydroxy-2′-deoxyguanosine (8-OHdG). Serum 8-OHdG was calculated by ELISA kit (Elabscience, Wuhan, China), as a typical biomarker of oxidative DNA impairment. The 8-OHdG levels were determined by means of an ELISA method according to the manufacturer's instructions with slight modifications and presented results as ng/dl.

2.8. Measurement of Oxidative Stress in Liver Tissue. Lipid peroxidation was indicated by hepatic MDA, and only glutathione (GSH) measurements were performed as a parameter for the cellular antioxidant status. Absorbance was read at 532 nm and MDA is recorded in terms of nmol MDA/mg protein. The content of reduced GSH was determined by its reaction with 1,2-dithio-bis nitrobenzoic acid (DTNB) and the absorbance at 412 nm is read on plate reader and expressed as nmol/mg protein.

2.9. Antioxidant Enzymatic Assays of Liver. Antioxidant enzyme activities in the liver supernatants were assayed using specific colorimetric assay kits for several antioxidant enzymes. SOD, CAT. GPx, and GST were used as antioxidant

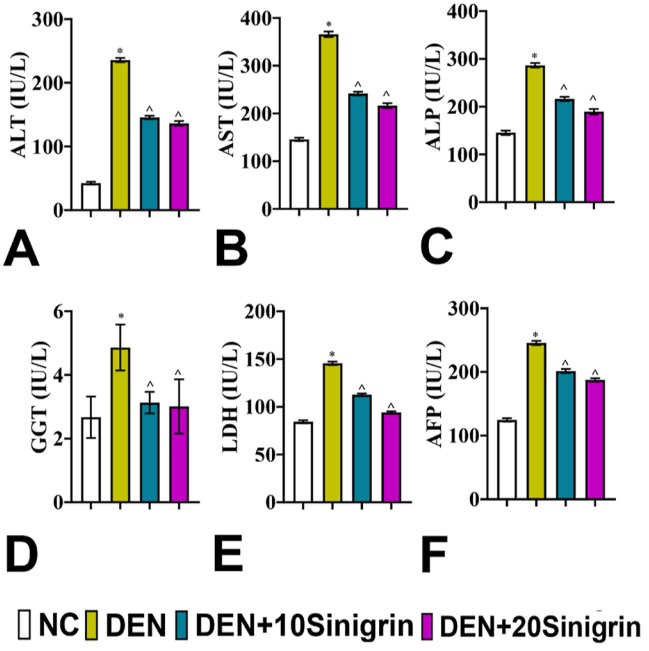

Figure 2. Effects of sinigrin on liver enzyme levels in DEN-treated mice. Serum levels of (A) ALT, (B) AST, (C) ALP, (D) GGT, (E) LDH, (F) AFP. Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; ^p < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

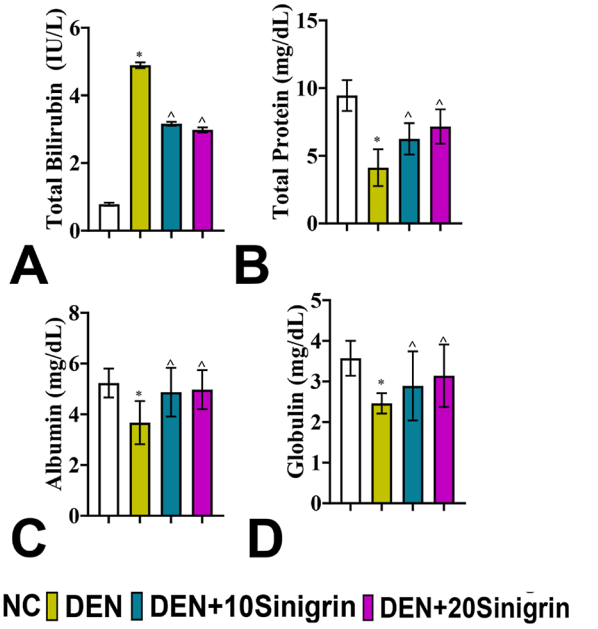

Figure 3. Effect of sinigrin on serum protein levels in DEN-induced HCC in mice. Serum levels of (A) Total Bilirubin (TBIL), (B) total protein, (C) Albumin (ALB), (D) Globulin (GLB). Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; ^p < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

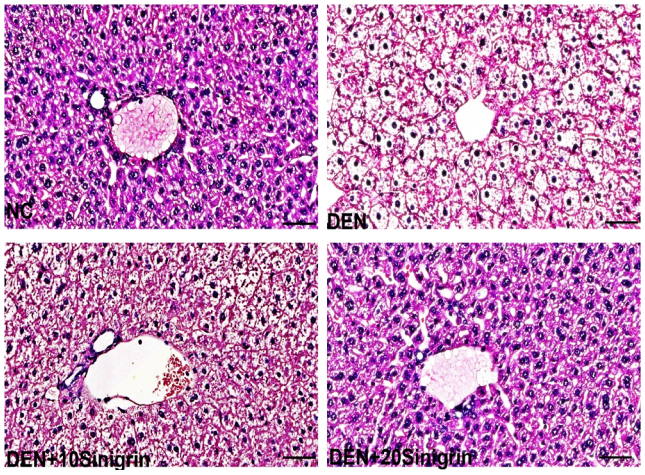

Figure 4. Histopathological changes of liver tissue in DEN-induced hepatocarcinogenesis. Representative images of liver tissue sections stained with H&E. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin. Scale: 50 μm, magnification: 40×.

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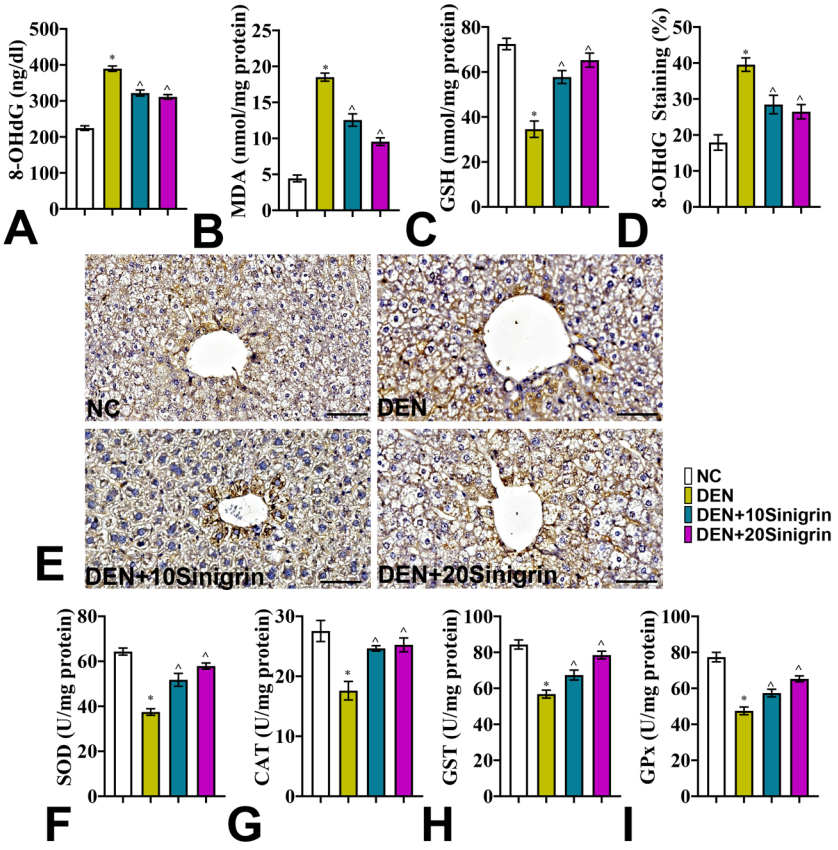

Figure 5. Protective effects of sinigrin on oxidative stress markers and antioxidant enzyme activities in DEN-induced hepatotoxicity in mice. (A) 8-OHdG levels in serum (ng/dL), (B) MDA levels (nmol/mg protein), (C) GSH levels (nmol/mg protein) in liver tissue, (D) 8-OHdG staining (%) in liver tissue, (E) Representative photomicrographs of liver tissue stained for 8-OHdG (magnification: 40×; scale bar = 50 μm), (F) SOD activity (U/mg protein), (G) CAT activity (U/mg protein), (H) GST activity (U/mg protein), (I) GPx activity (U/mg protein) in liver tissue. Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; ^p < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

defense means to combat reduction of oxidants and free radicals. According to the manufacturer's instruction, assays were determined as U/mg of protein.

2.10. Cytokines/Inflammatory Mediators Evaluation. Measurement of IL-6, IL-1β, and TNF-α in liver tissue using ELISA kits (Elabscience, Wuhan, China).

2.11. Reverse Transcription Polymerase Chain Reaction. Total RNA was extracted from liver tissues with the use of RNAiso Plus reagent (Takara, China). Then RNA was reverse transcribed into cDNA (1 μg), using PrimeScript RT reagent Kit (Takara, China). An ABI stepone real-time qPCR system using TB green premix Ex *Taq*II (Takara, China) was used to perform the Real time PCR. All reactions were conducted three times. The genes of interest were quantified relative to the housekeeping reference *Gapdh* mRNA expression levels by $2^{-\Delta\Delta Ct}$ method. Primers used in the present study includes:

PIK3CA (5′CTCAGCTCTCACCCTCCTCT3′; 5′GGTCTCTCTTTCCGCTCACA3′), Akt1 (5′TAGGCCCAGTCGCCCG3′; 5′GGGTAACCCAGGGATGATGC3′), Mtor (5′ACAAGCTCTGTTTGTGGCTCT3′; 5′GGCTGGGTTCATGCTGCTTA3′), Tp53i11 (5′GCCACATCCTTCTCACCCTAC3′; 5′CTGGCCTCAGTCATCGTTCC3′), Bcl2 (5′AGCATGCGACCTCTGTTTGA3′; 5′ATGCACCCAGAGTGATGCAG3′), Bax (5′CTGGATCCAAGACCAGGGTG3′; 5′GTGAGGACTCCAGCCACAAA3′), Casp3 (5′GGGAGCTTGGAACGGTACG3′; 5′GGATTTTGAATCCACTGAGGTTTTG3′).

2.12. Histopathological Evaluation. Mice livers were harvested and immediately fixed in 10% buffered neutral formalin, followed by paraffin embedding sectioning for hematoxylin−eosin (H&E) staining. The thin sections with a thickness of 5 μm were stained using H&E reagent (E-IR-R117A; Elabscience, Wuhan, China) for microscopic examination.

2.13. Immunohistochemistry. The liver sections were then dewaxed and subjected to antigen retrieval before being blocked in a blocking solution to reduce background staining. The sections were then exposed to primary antibodies selective for 8-OHdG (BS-1278R; Thermo Fisher Scientific, California, USA; 1:200 dilution), NF-κB p65 (ab16502; Abcam, Cambridge, MA, USA; 1:500 dilution), PI3K (MA5−32917; Thermo Fisher Scientific, California, USA; 1:200 dilution), Akt1 (sc-5298 Santa Cruz Biotechnology, Santa Cruz, CA, 1:100) overnight at 4 °C. The next day, after washing in PBS they were processed using HRP-conjugate secondary antibody (Vector, MP-7714-15, Burlingame, CA, USA). Visualization was done on DAB/magenta substrate which gives color in the presence of peroxidase. The sections were then counterstained with hematoxylin for staining of cell nuclei. Finally, the number of IHC-positive cells was quantified using ImageJ was used.

2.14. Immunofluorescence. The liver sections were dewaxed and then subjected to antigen retrieval. Sections

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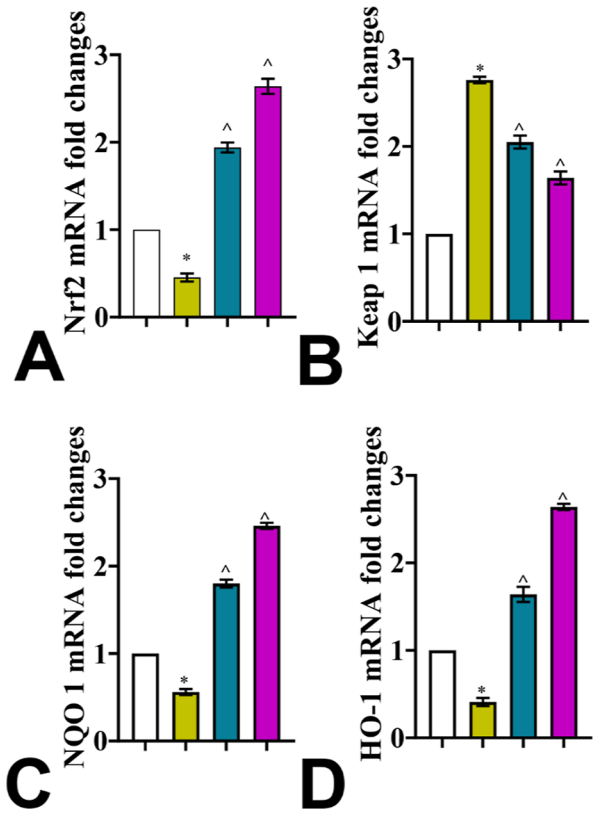

Figure 6. Gene expression changes in response to sinigrin treatment in DEN-induced hepatocarcinogenesis. mRNA levels of (A) Nrf2, (B) Keap1, (C) NQO1, (D) HO-1 fold changes in liver tissue. Data are presented as mean ± SD ($n = 6$). *$p < 0.05$ vs NC group; ^$p < 0.05$ vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

were blocked in 5% BSA in PBS at room temperature for adsorption of background staining. The sections were then incubated with primary antibody (Caspase-3, ab179517, Abcam, Cambridge, MA, USA, 1:200 dilution) in blocking buffer at 4 °C for overnight. The next day, the sections were washed three times in PBS then incubated with FITC-conjugated secondary antibodies for another hour at room temperature. After 3 times with PBS, incubation in DAPI solution with mounting medium. The sections were observed on a confocal microscope in order to the fluorescent signals throughout the sections of samples.

2.15. Statistical Analysis. Statistical data were represented in GraphPad Prism version 9.0.2 and was analyzed for its significance. Quantitative data are shown as the mean ± standard deviation (SD). One way Analysis of variance (ANOVA) test was performed for the comparison of groups, and a p value less than 0.05 it would be considered significant.

3. RESULTS

3.1. Effects of Sinigrin on Body and Liver Weight. The final body weight of the mice ($n = 6$ per group) that received DEN was significantly ($p < 0.001$) lower than the final body weight of the control group, which depicts the effect of DEN. But the mice that were treated with sinigrin by the administration lost less weight as seen from the Figure 1C. Furthermore, absolute and relative liver weight was also significantly ($p < 0.001$) increased in the DEN group showing that hepatomegaly was due to carcinogenesis (Figure 1B,D,E). The administration of sinigrin also produced a noticeable ($p < 0.01$) decrease in the liver weight which may be of therapeutic value in the treatment of hepatic hypertrophy induced by DEN.

3.2. Effect of Sinigrin on Serum Hepatic Parameters. The results depicted in Figure 2 revealed that there was a highly significant ($p < 0.01$) increase in the serum concentrations of liver damage markers after exposure to DEN these include; ALT (Figure 2A), AST (Figure 2B), alkaline phosphatase (ALP) (Figure 2C), gamma-glutamyl transferase (GGT) (Figure 2D), lactate dehydrogenase (LDH) (Figure 2E), and alpha-fetoprotein (AFP) (Figure 2F). The observed decrease in these levels after treatment with sinigrin indicates its potential to improve liver health and reduce liver damage.

3.3. Effects of Sinigrin on Liver Serum Marker Enzymes. DEN led to significantly increased levels of TBIL (Figure 3A), with decreased total protein (Figure 3B), ALB (Figure 3C), and GLB (Figure 3D) that are vital for liver function. Supplementation of sinigrin to DEN treated mice led to significant increase in the total protein, ALB, GLB, but decreased TBIL content in their serum. The studies show that sinigrin enhances the liver's biosynthetic and detoxifying capacity to help it heal from carcinogenic injury.

3.4. Histology of Hepatic Tissue. Exposure to DEN caused changes in the liver tissue and the shape of the cells in mice. There were more inflammatory cells infiltrating in the liver tissue of DEN-treated mice. Moreover, the cytoplasm of the hepatocytes in the sinigrin treated animals was more ordered and the general organization of the liver was more preserved. But decrease in the infiltration of inflammatory cells, which is indicative of reduced inflammation following the treatment of sinigrin. These data suggest that sinigrin may have the ability to regain the normal histology of the liver after being damaged by DEN and thus strengthen the possibility of using it as a therapeutic agent in liver cancer (Figure 4).

3.5. Effects of Sinigrin on Oxidative Stress Markers and 8-OHdG Markers in Liver Tissue. The observed significant ($p < 0.01$) increase in serum 8-OHdG levels after DEN treatment indicates that hepatic protein synthesis is affected. DEN-treated mice that received sinigrin had significantly lower ($p < 0.01$) levels of 8-OHdG compared to mice only treated with DEN (Figure 5A). Likewise, the immunohistochemistry (IHC) results also indicated that there was increased distribution of 8-OHdG protein in the liver tissue of DEN-treated mice than the normal control mice. The 8-OHdG protein distribution in the liver tissue of mice administered with 10 and 20 mg/kg sinigrin was significantly reduced compared to the DEN-treated mice (Figure 5E,D). The liver cancer model established by DEN showed a remarkable elevation ($p < 0.01$) in MDA indicating that the process of lipid peroxidation and oxidative stress was enhanced (Figure 5B). On the other hand, GSH is a potent antioxidant in liver tissues and plays a role in combating oxidative stress. The levels of GSH reduced ($p < 0.01$) which suggests the defensive antioxidant response to the oxidative stress (Figure 5C). The treatment of DEN to mice significantly elevated the levels of MDA and GSH in the liver tissue of mice compared with the mice treated with sinigrin alone at $p < 0.01$.

3.6. Effect of Sinigrin on Antioxidant Enzymes in Liver Tissue. The present investigation revealed that the mice bearing DEN induced liver cancer exhibited significantly ($p <$

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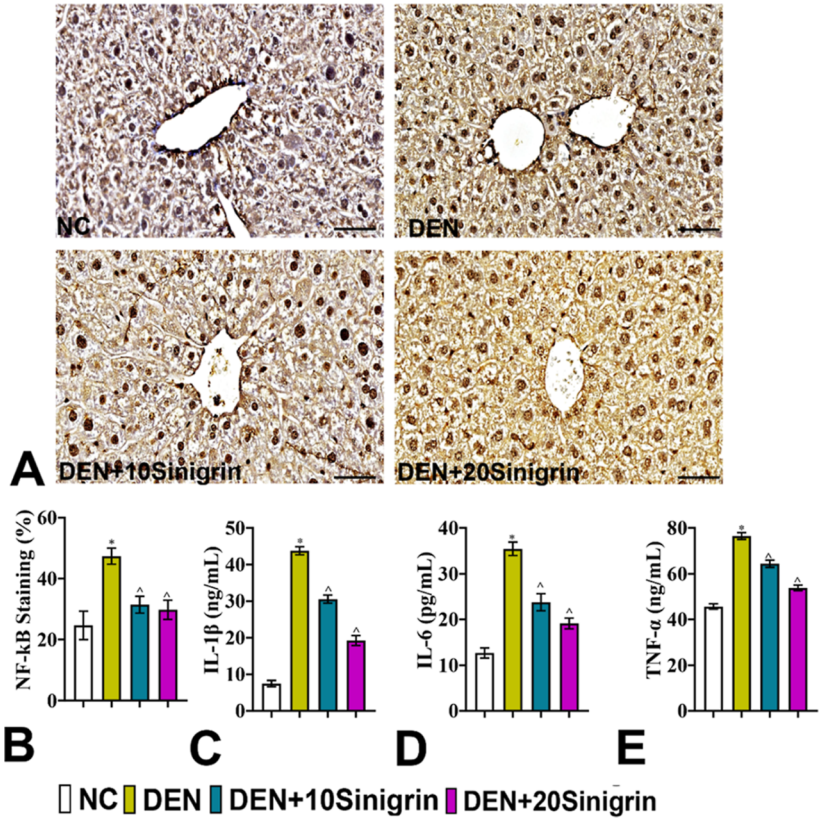

Figure 7. Effects of sinigrin on inflammatory markers in DEN-induced mice. (A) representative photomicrographs of liver tissues stained with IHC showing the NF-κB p65 brown staining (B) NF-κB staining (%), (C) IL-1β levels (ng/mL), (D) IL-6 levels (pg/mL), (E) TNF-α levels (ng/mL). Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; ^p < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

0.01) decreased levels of vital antioxidant enzymes including SOD (Figure 5F), CAT (Figure 5G), GST (Figure 5H), and GPx (Figure 5I) in their liver. This decline indicates that the antioxidant defense system has been affected. But the present data revealed that sinigrin administration caused a significant (p < 0.01) enhancement in these important antioxidant enzymes (SOD, CAT, GPx, and GST) in the liver of DEN-induced mice. Therefore, sinigrin administration enhanced the enzymatic antioxidant levels and proved to have the potential to enhance the antioxidant defense system of the liver and inhibit the development of liver cancer. The q-PCR analysis of mRNA level of Nrf2 (Figure 6A), Nqo-1 (Figure 6C), and Ho-1 (Figure 6D) were significantly decreased whereas the Keap1 gene expression (Figure 6B) was upregulated in the liver tissue of DEN treated mice as compared to normal control mice. However, DEN-induced mice that received sinigrin showed a marked upregulation of Nrf2, Ho-1, and Nqo-1 gene expressions and a downregulation of Keap1 gene expression than the DEN alone group.

3.7. Impact of Sinigrin on Liver Inflammatory Markers. NF-κB was determined by immunohistochemical staining in mouse liver tissue of the present study. Therefore, this research revealed that the expression of NF-κB protein levels was significantly higher in liver tissue from mice with DEN than control. However, a lower expression of NF-κB protein in the liver tissue was observed in sinigrin-treated mice than that induced-DEN untreated mice (Figure 7A,B). DEN induced hepatocarcinogenesis resulted in enhanced (p < 0.001) levels of pro-inflammatory cytokines IL-1β (Figure 7C), IL-6 (Figure 7D), and TNF-α (Figure 7E). These cytokines are associated with inflammation, and this is an important process in the development of liver cancer. But the findings of the present study showed that sinigrin treatment significantly decreased (p < 0.01) the level of IL-1β, IL-6, and TNF-α inflammatory markers.

3.8. Effects of Sinigrin on PI3K/AKT/mTOR Signaling Pathways. Under DEN induced model of hepatocarcinogenesis in mice, proteins belonging to PI3K/AKT/mTOR pathway which are known for cell proliferation and survival were activated. In the present investigation, we found the mRNA transcript levels upregulation of PI3K (Figure 8A), AKT (Figure 8B), and mTOR (Figure 8C) which are involved in cell survival and growth. The treatment of sinigrin caused a significant (p < 0.001) decrease in the activation of the PI3K/AKT/mTOR pathway. In the present study, the liver tissue of mice was observed to analyze the double immunohistochemical staining of PI3K (Figure 8D,F) and AKT (Figure 8E,F). In the present study, the immunohistochemical analysis showed that expression of PI3K (Figure 8D,F) and AKT (Figure 8E,F) proteins was increased in the liver tissue of DEN-induced mice compared to control group. However, liver tissue of DEN-induced mice treated with sinigrin expressed less PI3K (Figure 8D,F) and AKT (Figure 8E,F) proteins as compared to untreated DEN-induced mice.

3.9. Modulation of Hepatic Apoptosis Markers by Sinigrin. In the model of DEN induced hepatocarcinogenesis, there were changes in protein levels that affected apoptosis regulation in mice. The downregulation of p53 (Figure 9A),

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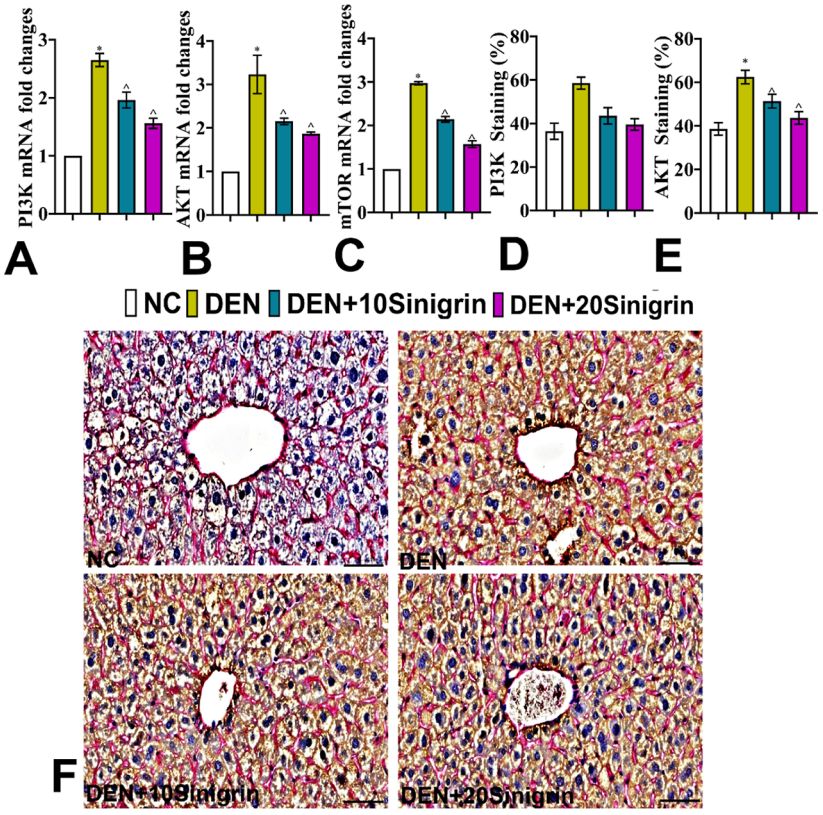

Figure 8. Effects of sinigrin on PI3K/AKT/mTOR pathway in DEN-induced mice. (A–C) Bar graphs showing the mRNA levels of PI3K/AKT/mTOR pathway-related genes in liver tissues: mRNA levels of (A) PI3K, (B) AKT, (C) mTOR, (D) PI3K staining (%), (E) AKT staining (%). (F) Representative photomicrographs of liver tissues stained with double IHC showing the PI3K (red) and AKT (brown) staining (magnification: 40×; scale bar = 50 μm). Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; $\hat{p}$ < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

Bax (Figure 9B), and Caspase-3 (Figure 9D) mRNA transcript levels enabled the process of apoptosis which is a form of programmed cell death. On the other hand, there was increased in Bcl-2, which is known to enhance cell death in hepatocarcinogenesis caused by DEN (Figure 9C). These changes further highlighted the interference of programmed cell death pathways that result in survival and growth of abnormal cells during the hepatocarcinogenesis induced by DEN. In the present study, the liver tissue of mice was observed to analyze the immunofluorescence (IF) staining of Caspase-3 (Figure 9E,F). The results showed that the level of Caspase-3 proteins was down-regulated in the liver tissue of DEN-induced mice as compared to the control group. But, in the case of mice that were treated with sinigrin, the level of Caspase-3 proteins in the liver tissue was comparatively higher than the DEN-induced mice without sinigrin treatment.

4. DISCUSSION

Diethylnitrosamine (DEN) is a potent hepatotoxin and carcinogen which has been used extensively to induce HCC in animal models.[5] DEN is injected and is metabolized by the hepatic cytochrome P450 enzymes to form DNA adducts and thus triggers the process of carcinogenesis. In this study, we used 25 mg/kg DEN injected intraperitoneally at 14 days of age to develop HCC model in mice. The mice were sacrificed 10 months after the DEN injection which acted as a base for assessing the hepatoprotective property of sinigrin.[17]

The outcomes of our study revealed that all the mice treated with DEN experienced a reduction in body weight and an augmentation in liver weight. These findings are consistent with previous studies, such as those by Nakatani et al.[18] and Assar et al.,[19] which also reported similar changes in body and liver weights in DEN-treated mice. These data indicate that there is chronic liver injury and that hepatomegaly might be present. These changes are in line with the generally accepted effects of chronic liver disease, which lead to reduced appetite and limitation of food intake.[20] The administration of sinigrin reduced the negative impact of DEN to a significant level as it reversed the loss of body weight and brought the liver weight to normalcy. This suggests potential clinical implications for sinigrin as a therapeutic agent in the management of liver damage and improving overall health in individuals with chronic liver disease. This reversal underscores the possibility of sinigrin in boosting general health and treating liver damage caused by DEN.[14]

The current study also underlines the importance of evaluating the total protein, ALB and GLB levels, as well as 8-OHdG for the assessment of liver function and protein synthesis potential.[21] Chronic impairment in the synthesis of liver proteins was observed after administration of DEN as evidenced by the reduction in serum 8-OHdG levels.[22] The exposure to DEN causes the detachment of polyribosomes from the surface of the endoplasmic reticulum which interrupts protein synthesis.[23] Serum enzymes like AST, ALT, ALP, GGT, and LDH are used in the assessment of the liver injury especially in HCC investigations arising from DEN.[24] The decrease in serum 8-OHdG levels after sinigrin therapy correspond that sinigrin can prevent oxidative damage from

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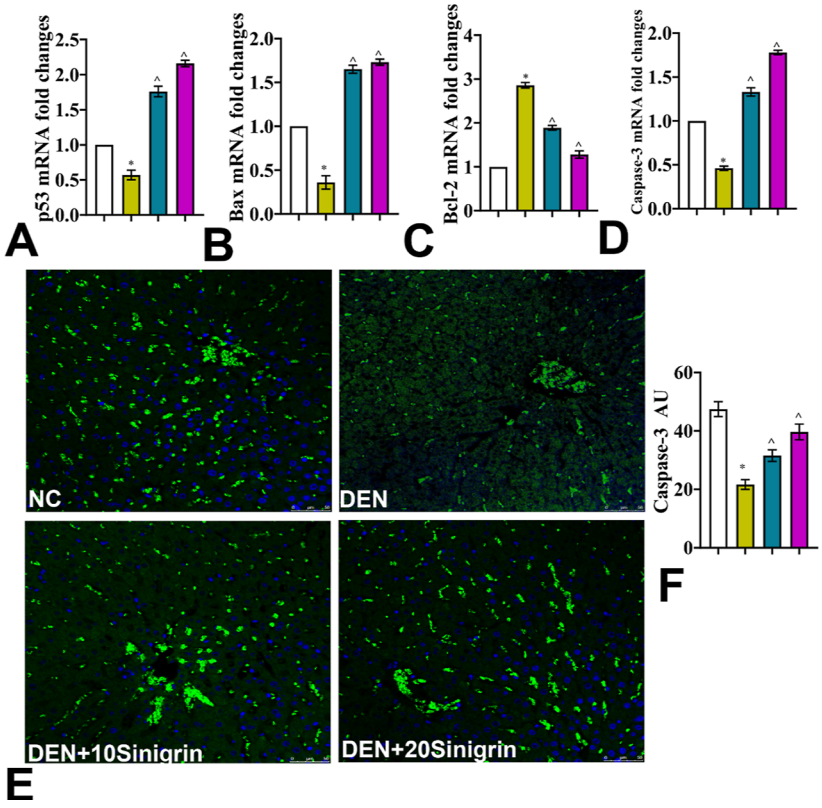

Figure 9. Effects of sinigrin on apoptosis markers in DEN-induced hepatocarcinogenesis. (A−D) Bar graphs showing the mRNA levels of apoptosis-related proteins in liver tissues: (A) p53 (B) Bax, (C) Bcl-2, (D) Caspase-3. (E) IF staining of Caspase-3 in liver tissues (magnification: 40×; scale bar = 100 μm), (F) IF intensity (AU). Data are presented as mean ± SD (n = 6). *p < 0.05 vs NC group; ^p < 0.05 vs DEN group. Treatment groups: NC—normal control, DEN—diethylnitrosamine, DEN + 10sinigrin—DEN with 10 mg/kg sinigrin, DEN + 20sinigrin—DEN with 20 mg/kg sinigrin.

DEN. This would imply that sinigrin has potential in the treatment of liver cancer. These studies demonstrate the relevance of these biochemical markers and possible therapies in the understanding and management of liver pathologies.

Antioxidants are needed to minimize the effects of cancer promoting chemicals like DEN since they cause oxidative stress. DEN activates the formation of free radicals which are capable of generating LPO and can potentially contribute to the development of cancer through interaction with DNA.[25] ROS are involved in oxidative stress and particularly in cancer cells which results in cell damage and death.[26] In this study, there was a higher level of TBARS in the mice treated with DEN. This implies the presence of LPO and shows that there is oxidative stress present in the sample. However, the administration of sinigrin significantly increased the antioxidant reaction, reducing LPO levels and increasing the activity of antioxidants.[11] The ability of sinigrin to scavenge free radicals makes it a promising option for an antioxidant therapy of liver cancer. Sinigrin improves the antioxidative protection by increasing the level of antioxidant enzymes including SOD, catalase, and GPx. Furthermore, sinigrin activates the Nrf2 signaling pathway to enhance the antioxidant and cytoprotective genes such as keap1 Nqo1 and heme oxygenase-1 (HO-1).[27] This improve enhances the tolerance capacity of liver cells to toxicants; therefore, sinigrin may use for the treatment of liver cancer.[28]

Inflammation in the liver, which is caused by many insults, plays a crucial role in the advancement of liver illnesses such as hepatitis, fibrosis, and HCC.[29] The above-mentioned response mirrors that generated by pro-inflammatory cytokines (such as IL-6, IL-1β, and TNF-α) acting through canonical pathway of NF-κB activation.[30] Sinigrin, which has been researched for its effects on liver injury, can potentially lower pro-inflammatory cytokines and NF-κB levels, indicating its potential as an anti-inflammatory medication. Sinigrin can regulate the inflammatory response,[10] which can help reduce chronic inflammation associated with the advancement of liver disease. The results emphasize the function of sinigrin as a regulator of inflammatory responses, providing valuable information on its potential use in treating liver disorders and other conditions associated with chronic inflammation.[31] The PI3K/AKT/mTOR pathway regulates cell growth, survival, proliferation, and metabolism.[32] Deregulation of this pathway is a commonality in many cancers which includes HCC. This cascade of events starts with the activation of PI3K and production of PIP3, which bring AKT to the cell membrane. AKT, a protein kinase that targets serine and threonine residues, is crucial in various physiological processes, especially in promoting cell survival by inhibiting apoptosis.[33] Downstream, mTOR is essential in regulating cell growth and development by sensing external signals and orchestrating cellular metabolism. S6K, a downstream target of mTOR, regulates protein synthesis and cell growth. Our study discovered that the administration of sinigrin led to a significant reduction in the expression of PI3K, AKT, and mTOR in mice with HCC induced by DEN. The confirmation was conducted using qPCR analysis. The results suggest that sinigrin has the capacity to impede the growth of cancer cells

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by disrupting the PI3K/AKT/mTOR pathway, resulting in reduced cell proliferation and enhanced apoptosis. Sinigrin may inhibit cancer cell growth by disrupting the PI3K/AKT/mTOR pathway.[34]

Apoptosis can be triggered by specific signaling pathways associated with different regulatory molecules. The p53 gene, which functions as a tumor suppressor, triggers both extrinsic and intrinsic apoptotic pathways.[35] Intrinsic apoptosis is induced by various conditions such as DNA damage, increased ROS levels, lack of growth factor, endoplasmic reticular stress.[36] Different types of cancers have been observed to have increased levels of Bcl-2 and decreased levels of Bax, ultimately leading to the suppression of apoptosis. The caspase family proteins are crucial in the molecular processes during apoptosis. Increased production of pro-apoptotic proteins could enhance the activation of apoptosis by increasing caspase cleavage, a well-established mechanism for triggering apoptosis in cancer cells.[37]

The present study showed that in mice with HCC generated by DEN lower Bax and caspase-9 mRNA accompanied by upregulation of the levels of Bcl-2 but sinigrin supplementation reversed the DEN induced effects.[14] Our findings showed that administering sinigrin to mice with DEN-induced HCC resulted in a considerable rise in Bax and caspase-9 mRNA levels, and a decrease in Bcl-2 levels. These findings indicate that sinigrin induces apoptosis via the intrinsic route by regulating these crucial apoptotic markers. The main process of apoptosis is the mTOR/PI3K/AKT-mediated signaling pathway, which represents a vital oncogenic kinase for cancer progression.[38] Sinigrin decreased mTOR/PI3K/AKT mRNA expression in mice stimulated by DEN. Therefore, suppression of the mTOR/PI3K/AKT signaling pathway hinders the proliferation and infiltration of HCC cells by inducing apoptosis. This suggests that a combination of different chemicals work together to elucidate the mechanisms by which sinigrin inhibits HCC.[32]

In conclusion, the effective regulation of key signaling pathways and mitigation of oxidative stress by sinigrin along with simultaneous inhibition on inflammation demonstrates that sinigrin is a potential agent for hepatic cancer therapy. This makes it a highly potential drug for liver cancer by virtue of its diverse actions with antioxidative, anti-inflammatory and apoptotic function besides modulating various signaling pathways. Gaining a comprehensive comprehension of sinigrin's interactions with these pathways can enhance the efficacy and precision of cancer treatments, hence enhancing outcomes for patients with liver cancer.

■ ASSOCIATED CONTENT

Data Availability Statement

All the data generated or analyzed during this study are included in this published article.

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Author Contributions

Z.B. and B.J. conceived the idea and designed the study. Z.B. and H.L. performed the experiments. Z.B. and H.L. helped in statistical analysis. Z.B. and H.L. wrote the manuscript. B.J., edited manuscript. All authors have read and approved the final version of the manuscript. The conception process, methodology, and funding acquisition.

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Notes

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[§]Z.B. and H.L. are the co-first authors.

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