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Mulberry leaf extract inhibits obesity and protects against diethylnitrosamine-induced hepatocellular carcinoma in rats

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ABSTRACT

Mulberry leaf has been recognized as a traditional Chinese medicinal plant, which was distributed throughout the Asia. The aqueous extract of mulberry leaf extract (MLE) has various biologically active components such as polyphenols and flavonoids. However, the inhibitory effect of MLE in hepatocarcinogenesis is poorly understood. In this study, we determined the role of MLE supplementation in preventing hepatocarcinogenesis in a carcinogen-initiated high-fat diet (HFD)-promoted Sprague-Dawley (SD) rat model. The rats were fed an HFD to induce obesity and spontaneous hepatomas by administering 0.01% diethylnitrosamine (DEN) in their drinking water for 12 weeks (HD group), and also to fed MLE through oral ingestion at daily doses of 0.5%, 1%, or 2%. At the end of the 12-week experimental period, the liver tumors were analyzed to identify markers of oxidative stress and antioxidant enzyme activities, and their serum was analyzed to determine their nutritional status and liver function. Histopathological analysis revealed that MLE supplementation significantly suppressed the severity and incidence of hepatic tumors. Furthermore, compared with the HFD + DEN groups, the expression of protein kinase C (PKC)-α and Rac family small GTPase 1 (Rac1) was lower in the MLE groups. These findings suggest that MLE prevents obesity-enhanced, carcinogen-induced hepatocellular carcinoma development, potentially through the protein kinase C (PKC)α/Rac1 signaling pathway. MLE might be an effective chemoprevention modality for nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH)-related hepatocarcinogenesis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is linked to metabolic disorders, and in adults with obesity, it carries a high risk of progression to chronic liver disease. NAFLD incorporates a wide range of conditions ranging from steatosis to nonalcoholic steatohepatitis (NASH). NASH is an aggressive form of inflamed fatty liver that can lead to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) and increase mortality.[1] According to the International Agency for Research on Cancer, HCC is the third most common cause of cancer deaths globally. The most accepted theory for NAFLD pathogenesis is the "two-hit theory": The initial hit is obesity-associated or abnormal glucose tolerance–associated insulin resistance caused by excessive free fatty acid flux into the liver, and the second hit is inflammation due to oxidative stress, inflammatory cytokines, iron, and endotoxins.[2] Proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 play pivotal roles in obesity-related steatohepatitis and subsequent hepatocarcinogenesis.[3]

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NAFLD/NASH is a common liver disorder that can cause HCC.[1–3] The prevalence of NASH and NASH-related HCC has been increasing annually.[4,5] Chronic liver diseases cause the development of various pathological events, including metabolic syndrome (e.g., obesity), insulin resistance, inflammation, and oxidative stress. Increased reactive oxygen species (ROS) generation contributes to nonalcoholic steatohepatitis in HFD-fed rats. Additionally, DEN is regarded as carcinogenic because of its role in ROS generation, which results in cellular damage, oxidative stress, and inflammatory responses.[6] HFD promotes the progression of DEN-induced liver cancer.[3,7,8] In our study, in using HFD + DEN-treated SD rats as a model of HCC, we observed that the HD group exhibited increased oxidative damage, 8-OHdG accumulation, TBARS (a reliable indicator of lipid peroxidation) concentrations, and proinflammatory cytokines (IL-6 and TNF-α) levels in steatotic hepatocytes. However, oxidative stress can increase because of ROS generation and defects in redox defense mechanisms involving the representative antioxidants. Antioxidative modes of action can be classified as enzymatic and nonenzymatic. These actions may include (1) scavenging and quenching ROS (e.g., with vitamin C, vitamin E, and GSH) and (2) upregulating antioxidant enzymes (e.g., SOD, glutathione peroxidase (GSH-P), and catalase).[9] In the current study, the HD-induced HCC rats demonstrated high hepatic oxidative stress and altered levels of endogenous antioxidant enzymes, which were reversed by adding MLE. Our results on the potential mechanisms by which MLE may protect against HCC are similar to those regarding other extracts mentioned in previous studies. For example, tomato extract supplementation exerted hepatoprotective effects by decreasing hepatic inflammation and hepatic lipid accumulation in HFD + DEN rats.[10] Curcumae reduced the incidence of HFD + DEN-induced HCC by suppressing oxidative stress, inflammatory reactions, and increasing the gut microbial diversity.[11] To our knowledge, this is the first study to demonstrate that MLE treatment attenuated HFD + DEN induced by enhancing enzymatic antioxidant defense.

Mechanistic studies have revealed that obesity is an independent risk factor for HCC.[12] Diethylnitrosamine (DEN) is a highly toxic organic compound that is frequently used in cancer research to induce liver tumors in experimental animals.[13] Wang et al. reported that NASH induced by a high-fat diet (HFD) accelerated DEN-initiated hepatocarcinogenesis in Sprague Dawley (SD) rats through increased oxidative stress and inflammation.[14] This animal model provides a sound basis for analyzing the efficacy of synthetic compounds or phytochemicals in preventing NASH-related hepatocarcinogenesis.[15] On the other hand, the protein kinase C (PKC) signaling pathways play a vital role in the malignant proliferation, migration and invasion of human liver cancer, suggesting a role for the PKCα in the malignant progression of human HCC.[16] Moreover, it has been observed that PKCα isoform is required for the clonal expansion of DEN-initiated hepatocytes.[17,18] PKCα is overexpressed and directly associates with Rac1, a small GTPase protein, resulting in enhanced cell motility,[19] and that contribute to the migration and metastatic potential of breast cancer cells.[20] Recently an interesting paper demonstrated that PKCα positively regulates the activation of Rac1 during structural plasticity.[21,22] This link between PKCα and Rac1 supports a growing understanding of the molecular mechanisms underlying the biological effects. Here, we investigated the incorporation of an atherogenic HFD into a DEN model of HCC and hypothesized that MLE might inhibits obesity promoting diethylnitrosamine-induced hepatocellular carcinoma at least partly by the PKCα and Rac1 pathways.

Because the available treatment options for HCC have limited efficacy, chemoprevention strategies were proposed as potential options for reducing HCC occurrence and associated mortality.[23] Phytochemicals can serve as chemopreventive and therapeutic agents that may also prevent the development of metabolic syndrome risk factors, such as obesity, diabetes mellitus, cardiovascular diseases, and NAFLD.[5] Mulberry, *Morus alba* L., is a member of the Moraceae family and is extensively planted in various Asian countries, including Taiwan. The leaves, fruits, roots, branches, and bark of the *Morus* species provide many health benefits. Mulberry leaves have long been used as a traditional treatment in Chinese medicine for various diseases; they have been reported to have antiobesity, antitumor, anti-inflammatory, antioxidant, antidiabetic, hypotensive, and hepatoprotective properties[4,24] because of their high concentrations of compounds such as flavonoids, alkaloids, and phenolic acids.[25] In one study, only 33% (2/6) of the liver samples obtained from NASH mice (a stelic animal model) treated with mulberry leaves developed adenoma, suggesting they have potential protective effects against HCC.[19] Mulberry leaf extract (MLE) supplementation not only attenuates dyslipidemia and lipid accumulation in HFD-fed mice but also prevents diethylnitrosamine (DEN)-induced hepatocarcinogenesis.[26–28] Because no effective treatment or chemoprevention strategies are available for HCC related to NASH, an animal model with the same clinical features as human NASH is required. In addition, no study has evaluated whether MLE can inhibit NASH-related hepatocarcinogenesis. In the present study, we explored the mechanisms underlying the antitumorigenesis effect of MLE-specifically, we explored its effect against HFD + DEN-induced hepatic inflammation and tumorigenesis.

2. Materials and methods

2.1. Reagents and chemicals

Diethylnitrosamine (N0258) and beef tallow were obtained from Sigma-Aldrich (St. Louis, MO, USA). The diagnostic kits for triglyceride, total cholesterol, leptin, adiponectin, aspartate aminotransferase (AST) (AS8306), alanine aminotransferase (ALT) (AL8006), gamma glutamyltranspeptidase (γGT) (GT8146), alpha-fetoprotein (AFP)/TNF-α/IL-6 (EV3561), glutathione (GSH) (GR2609), superoxide dismutase (SOD) (SD125), glutathione peroxidase (GPx) (SC10154), catalase/GST-α/total GST (NX2332) were purchased from Randox Laboratories (London, UK). In addition, 8-Hydroxy-2′-deoxyguanosine (8-OHdG) (sc-393871), protein kinase C (PKC)α (sc-8393), and Rac1 (sc-514583) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-mouse (code 115-075-020), anti-rabbit (code 111-155-144), and anti-goat (code 101-005-165) antibodies conjugated with horseradish peroxidase were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

2.2. Preparation of plant extract

Mulberry leaves were harvested from a mulberry garden in Dadu Township in central Taiwan. MLE was prepared as previously described.[29] Fresh mulberry leaves (200 g) were dried at 50 °C and then heated at 90 °C in 3 L of deionized water for 1 h. After filtration, the residue was removed, and the supernatant was lyophilized in a freeze dryer (VirTis; SP Scientific, Warminster, PA, USA) to prepare MLE. The dried powder was stored at −80 °C for further use. The powder was weighed, and the yield was calculated (yield: 38.63%). According to literature previously published by our laboratory, the HPLC analysis and LC-MS identification results have revealed the presence of the following phenolic compounds: 0.238% chlorogenic acid, 0.317% cryptochlorogenic acid, 0.355% neochlorogenic acid, 0.013% protocatechuic acid, 0.035% nicotiflorin, 0.092% rutin, 0.056% isoquercitrin, and 0.053% astragalin.[29,30]

2.3. Animals and experimental design

Male SD rats (170–200 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All animals in this study were maintained according to the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University for the care and use of laboratory animals (approval number: 1211). The rats were housed at 22 ± 2 °C, with a relative humidity of 55 ± 5% and 12-h light–dark cycles. All rats were subjected to experimental procedures

after acclimatization for 1 week. The technology roadmap used in this study is provided in Supplementary Fig. 1.

The experimental SD rats were distributed into seven groups (10 rats per group):

Group 1: Rats fed a standard pellet diet and water ad libitum (control group).

Group 2: Rats fed an HFD (40% beef tallow) instead of regular chow (HFD group).

Group 3: Rats with HCC induced by adding 0.01% DEN to their drinking water throughout the experimental period (DEN group).

Group 4: Rats receiving 0.01% DEN, as in the DEN group, and fed an HFD (HD group).

Groups 5–7: Rats receiving HFD and 0.01% DEN, as in the DEN group, and a daily dose of 0.5%, 1%, or 2% MLE through oral ingestion (HD + MLE groups; HD + MLE 0.5%–2%).

The HFD diet compositions have been described previously.[31] The DEN-induced model based on previous studies were employed.[32] The total experimental period was 12 weeks. Their body weights were monitored weekly. At the end of 12 weeks, all animals were euthanized using CO_2 inhalation. Before dissecting the rats, we measured the rats' weights. The liver was washed with phosphate-buffered saline and weighed. We collected liver samples to determine the liver-to-body weight ratio (organ weight/body weight), controlling for individual variations. Their blood and liver tissue samples were collected and maintained at −80 °C until analysis.

2.4. Histopathological evaluation

Hematoxylin and eosin (H&E) and immunohistochemical staining were performed as previously described.[33] The livers were immersion-fixed overnight in 10% buffered neutral formalin. Next, the tissues were routinely processed for paraffin embedding; 3-5-μm-thick sections were cut, mounted on glass slides, and processed for H&E staining. The histopathological changes, such as those in the cellular lipid vesicles and cell morphology, were observed under a light microscope. The sections were subjected to immunohistochemical staining for 8-OHdG, PKCα, and Rac1 according to the manufacturer's instructions. The sections were initially blocked with a solution containing 10% FBS, 0.03% Triton X-100, and TBS for 1 h at room temperature. Subsequently, they were incubated overnight at 4 °C with primary antibodies. The primary antibodies used were mouse anti-8-OHdG (1:1000, Santa Cruz, California, USA), mouse anti-PKCα (1:200, Santa Cruz, California, USA), and rabbit anti-Rac1 (1:250, Santa Cruz, California, USA). The positive staining was visualized using a DAB peroxidase substrate kit, and these sections were counterstained with hematoxylin. The positive for expression of 8-OHdG, PKCα and Rac1 product exhibited brown yellow granules in the hepatocytes. The Image-Pro Plus 6.0 software (Media Cybernetics, Washington, USA) was used to quantify and analyze the average number of cells exhibiting IHC-positive staining. For this quantification, five random areas were selected from each slide of rat. The number of cells showing positive labeling and the total number of cells counted were recorded. Subsequently, an average percentage was calculated based on the total number of positive staining cells across the five selected fields. Results are expressed as mean ± SD.

2.5. Lipid peroxidation assays

Tissue homogenate was used for the determination of lipid peroxidation and antioxidant enzymes. A thiobarbituric acid reactive substances (TBARS) assay was used to measure the tissue concentration of malondialdehyde, a product of lipid peroxidation, as an indicator of oxidative stress. The results are expressed as malondialdehyde equivalents (mmol/mg of protein) with 1,1,3,3-tetramethoxypropane used as a standard.[34] Absorbance of the supernatant was detected at 532 nm.

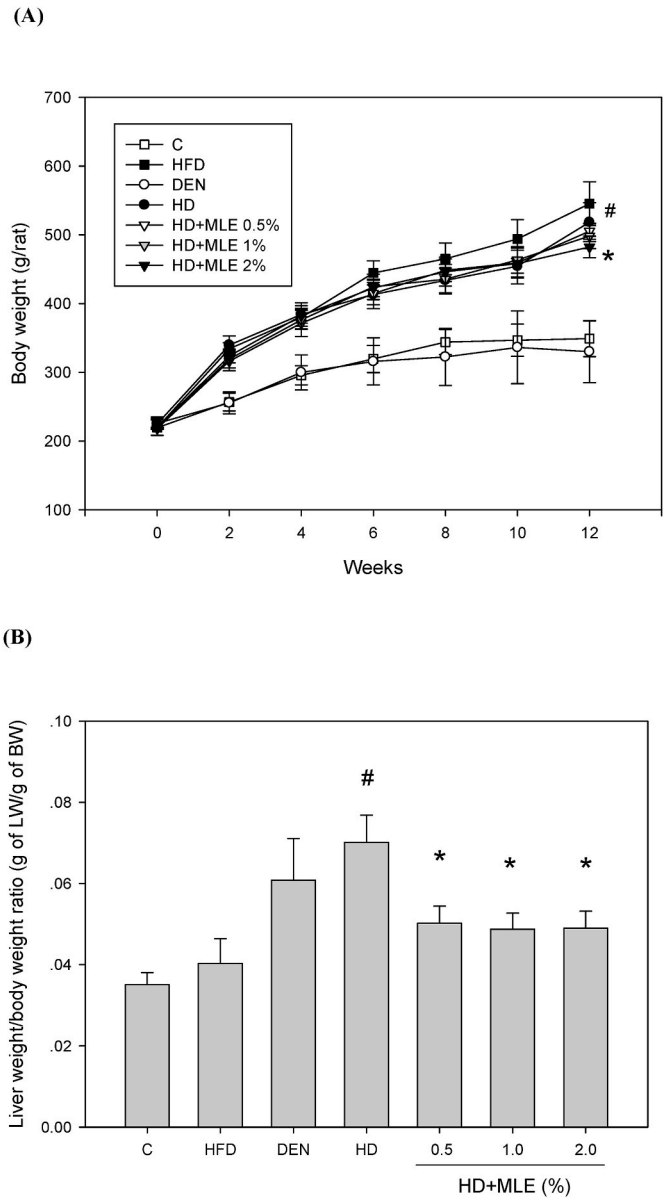

Fig. 1. Effect of MLE on body weight changes in HFD plus DEN-induced rat. Changes in (A) body weight and (B) ratio of the liver weight to body weight in the DEN-treated rat on a HFD. SD rats fed on normal diet without DEN water (Control). HFD, SD rats fed on HFD without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. HD + MLE 0.5%, HFD containing 0.5% MLE with DEN water. HD + MLE 1%, HFD containing 1% MLE with DEN water. HD + MLE 2%, HFD containing 2% MLE with DEN water. Values are mean ± standard deviation of 10 rats. #, $p < 0.05$ as compared with the C group; *, $p < 0.05$ as compared with the HD group. C, control; DEN, diethylnitrosamine; HFD, high-fat diet; HD, HFD + DEN; MLE, mulberry leaf extract.

2.6. Measurement of biochemical parameters

Hepatic function tests: A portion of the liver (500 mg) was used for lipid extraction. The tissues were homogenized in phosphate-buffered saline, and the lipids were extracted using *n*-hexane-isopropanol (3:2, v/v). Once dry, the lipid extracts were redissolved in 200 μL of isopropanol, and a small volume was used to measure the cholesterol and triglyceride concentrations through enzymatic assays. The activity of the antioxidant enzymes (GSH, GPx, SOD, catalase, GST-α, and total GST assay) was assayed using commercial kits (Randox Laboratories, Antrim, UK). The enzymes were measured using enzymatic colorimetric methods, according to the manufacturer's protocols.

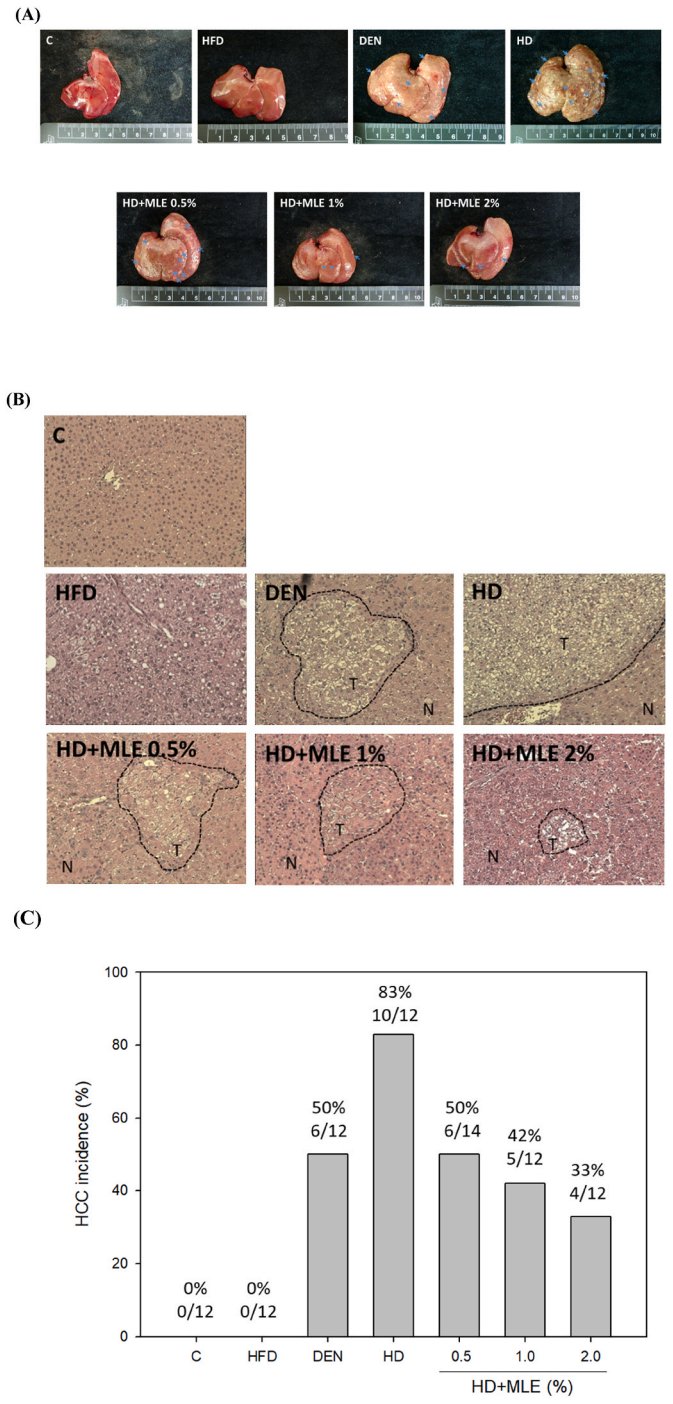

Fig. 2. Effect of MLE on liver histology in HFD plus DEN-induced rat. (A) Macroscopic observation in the DEN-treated rat on a HFD. In macroscopic photomicrographs, tumors are indicated by arrows. (B) Histopathological findings of the livers from the rat on hematoxylin and eosin (H&E) stained sections. H&E staining results of the tumor areas are presented (original magnification, ×100). The area in the dotted line was shown as tumor area. Representative of three independent experiments. (C) Liver tumor incidence at sacrifice in the different treatment groups. N, normal hepatocyte; T, tumor; HCC, hepatocellular carcinoma.

Serum analysis: The concentrations of the total triglyceride (TG), total cholesterol (TC), leptin, adiponectin, AST, ALT, γGT, AFP, TNF-α, and IL-6 were measured using enzymatic colorimetric methods with commercial kits (Randox Laboratories) according to the manufacturer's protocols. The serum analyses were conducted using an automatic analyzer (Olympus AU2700, Olympus, Tokyo, Japan).

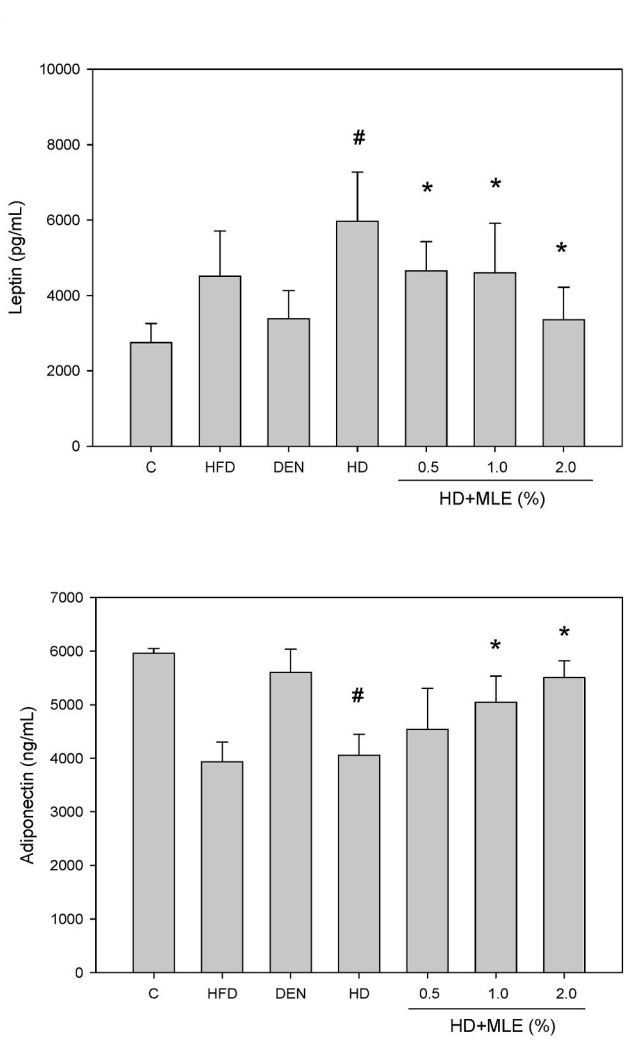

Fig. 3. Effect of MLE on levels of plasma leptin and adiponectin in HFD plus DEN-induced rat. The levels of (A) leptin and (B) adiponectin were determined by ELISA. Data are shown as the means ± SD. #, $p < 0.05$ as compared with the C group. *, $p < 0.05$ as compared with the HD group.

2.7. Statistical analysis

The significance of the differences in the results for all parameters studied was evaluated using analysis of variance, followed by Duncan's multiple-range test. $P < 0.05$ was set as significant (Sigma Stat, Jandel Scientific, San Rafael, CA).

3. Results

3.1. General observations

To investigate whether MLE contributes to tumorigenesis, we used an HCC animal model to monitor progression from obesity to HCC according to the method of Chen and colleagues.[32] At the end of the 12-week experimental period, the body weights of all HFD-fed groups were significantly higher than those of the control group; however, the body weights were lower in the three HD + MLE groups than they were in the HFD + DEN (HD) group (Fig. 1A). In addition, the liver-to-body weight ratio was significantly lower in the HD + MLE groups (Fig. 1B).

Table 1
MLE supplement ameliorated the biomarkers expression of hepatic injury and inflammation in HFD + DEN-induced rat.

	C	HFD	DEN	HD	HD + MLE 0.5%	HD + MLE 1%	HD + MLE 2%
AST (U/L)	38.00 ± 4.97	80.50 ± 5.13	147.00 ± 17.31	207.10 ± 37.48$^{\#}$	162.30 ± 46.65*	143.90 ± 30.59*	126.80 ± 41.91*
ALT (U/L)	78.70 ± 10.92	106.40 ± 33.15	117.20 ± 32.85	211.70 ± 60.06$^{\#}$	171.20 ± 27.83*	163.20 ± 38.49*	148.90 ± 33.63*
γGT (U/L)	3.80 ± 1.62	4.00 ± 1.63	54.80 ± 17.61	84.80 ± 17.61$^{\#}$	58.20 ± 16.27*	48.40 ± 11.55*	46.90 ± 11.62*
AFP (ng/mL)	4.60 ± 2.27	4.30 ± 1.77	57.40 ± 16.93	103.70 ± 26.42$^{\#}$	57.50 ± 15.41*	48.60 ± 11.23*	47.10 ± 10.16
TNFα (pg/mL)	79.29 ± 6.81	109.87 ± 22.23	249.04 ± 72.81	276.04 ± 55.31	241.60 ± 65.28	155.83 ± 59.88*	151.58 ± 42.02*
IL-6 (pg/mL)	37.30 ± 2.79	39.64 ± 2.42	44.00 ± 2.51	44.85 ± 1.92$^{\#}$	41.55 ± 3.68	40.96 ± 3.35*	40.31 ± 3.71*

C: rats fed with normal diet as a normal control group; **HFD:** rats fed with high fat diet as a HFD control group; **DEN:** rats fed with normal diet and 0.01% diethylnitrosamine (DEN) via drinking water; **HD:** rats fed with HFD and DEN via drinking water; **HD + MLE 0.5%:** rats fed with HFD, 0.5% MLE, and DEN via drinking water; **HD + MLE 1%:** rats fed with HFD, 1% MLE, and DEN via drinking water; **HD + MLE 2%:** rats fed with HFD, 2% MLE, and DEN via drinking water. Each value is expressed as the mean ± SD ($n = 10$/group). Results were statistically analyzed with ANOVA and significantly different at $p < 0.05$ according to Duncan's multiple range test. #, $p < 0.05$ compared with the C group. *, $p < 0.05$ compared with the HD group.

Table 2
MLE supplement ameliorated the serum and hepatic lipid in HFD + DEN-induced rat.

	C	HFD	DEN	HD	HD + MLE 0.5%	HD + MLE 1%	HD + MLE 2%
Total cholesterol (mg/dL)	54.00 ± 11.43	137.50 ± 11.87	117.20 ± 21.75	162.50 ± 31.00	136.60 ± 8.98*	127.90 ± 13.94*	106.50 ± 19.00*
Total triglyceride (mg/dL)	24.40 ± 3.86	65.60 ± 10.96	43.50 ± 6.11	77.40 ± 12.57$^{\#}$	60.10 ± 9.48*	47.50 ± 8.55*	44.30 ± 7.54*
Liver cholesterol (mg/g liver weight)	176.63 ± 23.17	312.10 ± 47.28	211.96 ± 27.80	343.31 ± 52.01$^{\#}$	250.45 ± 46.91*	243.43 ± 48.78*	236.94 ± 47.60*
Liver triglyceride (mg/g liver weight)	293.49 ± 31.6	725.62 ± 123.52	381.54 ± 40.38	739.85 ± 105.10$^{\#}$	626.37 ± 95.99*	589.17 ± 114.56*	596.17 ± 124.94*

C: rats fed with normal diet as a normal control group; **HFD:** rats fed with high fat diet as a HFD control group; **DEN:** rats fed with normal diet and 0.01% diethylnitrosamine (DEN) via drinking water; **HD:** rats fed with HFD and DEN via drinking water; **HD + MLE 0.5%:** rats fed with HFD, 0.5% MLE, and DEN via drinking water; **HD + MLE 1%:** rats fed with HFD, 1% MLE, and DEN via drinking water; **HD + MLE 2%:** rats fed with HFD, 2% MLE, and DEN via drinking water. Each value is expressed as the mean ± SD ($n = 10$/group). Results were statistically analyzed with ANOVA and significantly different at $p < 0.05$ according to Duncan's multiple range test. #, $p < 0.05$ compared with the C group. *, $p < 0.05$ compared with the HD group.

3.2. Effect of MLE on DEN + HFD-induced liver tumors

Images of the livers with the median tumor burden for each group are presented in Fig. 2A, and the H&E-stained liver sections are presented in Fig. 2B. In the control and HFD groups, no tumors were observed, with the liver histology exhibiting a normal appearance. In the DEN-treated groups, small white nodules were macroscopically observed (indicated by arrows) and a tumor burden (dotted line) was developed. In the HD groups, the numbers and sizes of tumors were higher than those in the control group. However, the numbers and sizes of tumors in the three HD + MLE groups were significantly lower than those in the HD group. The incidence of HFD + DEN-induced hepatic tumors was 80% (Fig. 2C). By contrast, the incidence of hepatic tumors in the three HD + MLE groups was 30%–50%. Leptin and adiponectin are adipokines that are altered with obesity, and both hormones are linked to cancer. The adiponectin adipokine appears to play an opposing role to leptin in cancer development.[35] Compared with the control group, leptin levels were significantly higher ($p < 0.05$, Fig. 3A) and plasma adiponectin levels were lower in the HD-treated rats ($p < 0.05$, Fig. 3B). MLE exerted a substantial protective effect, with decreased plasma leptin and increased plasma adiponectin compared with the HD group. These results suggest that MLE may protect against HFD + DEN-induced hepatocarcinogenesis.

3.3. Effect of MLE on serum markers of liver injury

Compared with the control rats, the serum ALT, AST, γGT, AFP, TNF-α, and IL-6 levels were significantly higher in the HD-treated rats (Table 1). Compared with the HD group, the HD + MLE groups exhibited significantly lower serum ALT, AST, γGT, AFP, TNF-α, and IL-6 levels (Table 1).

The liver and plasma TG and TC concentrations were significantly higher in the HD group than they were in the control group (Table 2). Compared with the HD group, the HD + MLE group exhibited significantly reduced liver and plasma TG and TC concentrations. Overall, these observations demonstrate that MLE treatment has a hepatoprotective effect against HFD + DEN-induced HCC.

3.4. Effects of MLE on oxidative stress in rats

Oxidative stress plays a critical role in NAFLD-to-NASH progression and HCC development.[36] Therefore, we analyzed the levels of oxidative stress and antioxidant biomarkers in the rats. The amount of TBARS was significantly higher in the HD-only group than it was in the control rats (Fig. 4A) but significantly lower in the HD + MLE groups than it was in the HD-treated animals. To confirm malignancy, we immunostained the tumors for 8-OHdG. We discovered 8-OHdG-positive HCC in the DEN and HD specimens (Fig. 4B) although not all tumors exhibited positive staining. MLE administration in the HD group reduced the intensity of 8-OHdG staining in the liver compared with the HD-only group.

The activity levels of the antioxidant enzymes (GSH, GPx, SOD, catalase, GST-α, and total GST) were significantly increased in the livers of the HD + MLE rats compared with the HD-only group (Fig. 5). These results indicate that MLE attenuated hepatic oxidative stress in our rat model of NAFLD/NASH-related hepatocarcinogenesis.

3.5. Effect of MLE on PKCα and Rac1 expression in rats

The overexpression of PKCα regulates Rac1 (a small GTPase protein) and enhances cell motility.[37] Furthermore, PKCα may regulate several downstream processes, such as Rac1, that promote the migration and metastatic potential of breast cancer cells.[38] In our study, immunostaining analyses revealed markedly lower numbers of PKCα-positive and Rac1-positive hepatocytes in the HD + MLE group than were in the HD group (Fig. 6A and B), indicating MLE treatment-induced downregulation of PKCα and Rac1, which was beneficial for hepatocarcinogenesis in our rat model.

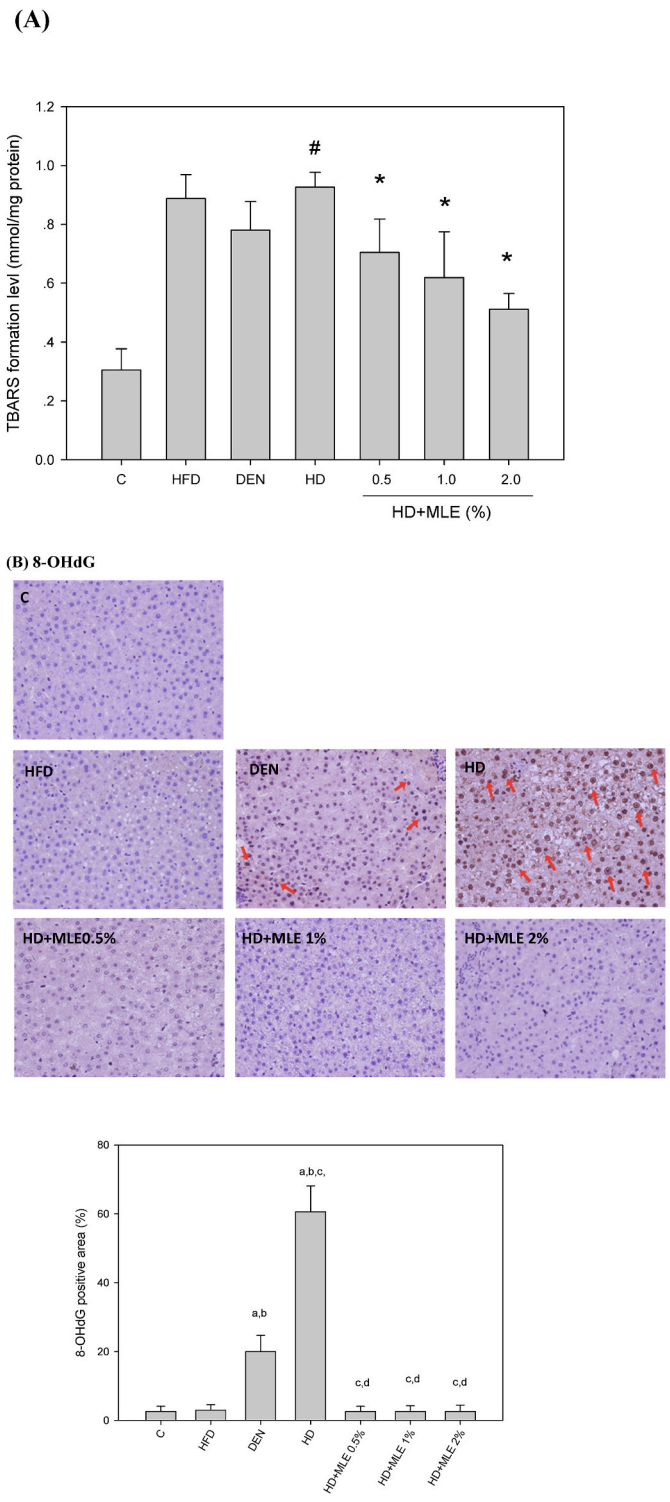

Fig. 4. Effect of MLE on oxidative stress in HFD plus DEN-induced rat. (A) The levels of TBARS formation in liver was quantitated by spectrophotomic analysis. Data are shown as the means ± SD. #, $p < 0.05$ as compared with the C group; *, $p < 0.05$ as compared with the HD group. (B) The liver 8-OHdG protein expression shown by immunohistochemistry in the different study groups (original magnification, ×200). Arrows highlight 8-OHdG positive cells. Results were statistically analyzed with ANOVA and significantly different at $p < 0.05$ according to Duncan's multiple range test. a, $p < 0.05$ compared with the C group. b, $p < 0.05$ compared with the HFD group. c, $p < 0.05$ compared with the DEN group. d, $p < 0.05$ compared with the HD group.

4. Discussion

In the present study, the addition of HFD to DEN led to an increased number of confirmed HCC lesions at 12 weeks of age. Compared with the HD group, rats receiving HD + MLE exhibited stronger hepatoprotective (measured using liver and body weight, histopathology, and AST and ALT levels), lipid-lowering (measured as serum TC, triglyceride, leptin, and adiponectin levels), and antioxidant (measured as GSH, GPx, SOD, catalase, GST-α, and total GST activity) effects. The MLE-treated rats also exhibited lower PKCα and Rac1 expression than the HD group did. Our previous study indicated an inhibitory effect of MLE and its major components, chlorogenic acid and neochlorogenic acid, which can ameliorate hepatic lipid accumulation and peroxidation in patients with alcoholic steatohepatitis.[30] Therefore, chlorogenic and neochlorogenic acid may contribute to the cancer chemopreventive properties of DEN plus HFD-induced hepatocarcinogenesis.

Mulberry is distributed throughout Taiwan. MLE contains various phytoconstituents, including phenolic acids, flavonoids, and alkaloids (e.g., 1-deoxynojirimycin), and γ-aminobutyric acid, tannins, and terpenes.[4] The widespread use of MLE in traditional Chinese medicine and the presence of phytoconstituents encouraged us to explore the hepatoprotective and hypoglycemic effects of MLE against streptozotocin (STZ)-induced diabetic animals. Serum glucose levels, insulin levels, antioxidant enzyme activities, and histopathological changes were observed during the experimental period in STZ-treated and STZ + MLE-treated animals. Clinical studies have demonstrated that oral administration of MLE improves glucose metabolism and plasma cholesterol levels.[39–41] MLE has also been reported to have anti-melanogenesis, antigout, anti-ischemic antidepressant-like, and neuroprotective effects. Li et al. reported that a 70% ethanol extract of MLE significantly inhibited melanin production by regulating melanogenesis-related protein expression (cAMP-responsive element-binding protein and p38) in B16–F10 mouse melanoma cells.[42] Wan et al. discovered MLE to reduce xanthine oxidase activity and hydrogen peroxide production, thus demonstrating its potential as a new antigout drug.[43] Ma et al. noted that oral MLE enriched with 1-deoxynojirimycin can reduce C-reactive protein, IL-6, TNF-α, SOD, and malondialdehyde levels in patients with stable angina pectoris, thereby increasing their antioxidant and anti-inflammatory capacities.[44] MLE also effectively inhibits glutamate-induced cell death in the hippocampal and cortical slices, implicating neuroprotection activity in the antidepressant-like effect.[9] It may also exert a neuroprotective effect by blocking aldose reductase activity and alleviating neuroinflammation.[45]

Adipokines, such as leptin and adiponectin, are bioactive proteins synthesized and secreted by adipocytes, which play an essential role and are important mediators of various metabolic processes, including energy homeostasis.[46] The association between adipokines (leptin and adiponectin) and HCC in NAFLD/NASH is well documented and widely recognized.[47] Epidemiologic evidence suggests that in people with obesity, hepatocyte-specific leptin signaling can promote HCC development.[48] Shimizu et al. demonstrated the preventive effect of pitavastatin against obesity-related hepatocarcinogenesis in DEN-treated db/db mice.[49] In DEN-treated obese mice models, Tajima et al. observed metformin to markedly increased serum adiponectin levels and decrease serum leptin levels.[50] Metformin has direct effects on adipose tissue: it induces adiponectin secretion and reduces leptin secretion.[51] MLE decreased plasma leptin levels and increased adiponectin levels in HFD-fed rats (Peng et al., 2018) and increased adiponectin levels in murine 3T3-L1 adipocytes.[52] Consistent with these findings, our results indicate that MLE treatments prevent HFD + DEN-induced hepatocarcinogenesis by improving adipokine imbalance.

Several signaling cascade are required for the progression of chronic liver disease to HCC. Fig. 6 shows that MLE treatment downregulates PKCα and Rac1. PKC has also been demonstrated to increases Rac1 activity and regulate hyaluronic acid-promoted melanoma cell motility.[53] Furthermore, phorbol ester treatment leads to PKC-dependent activation

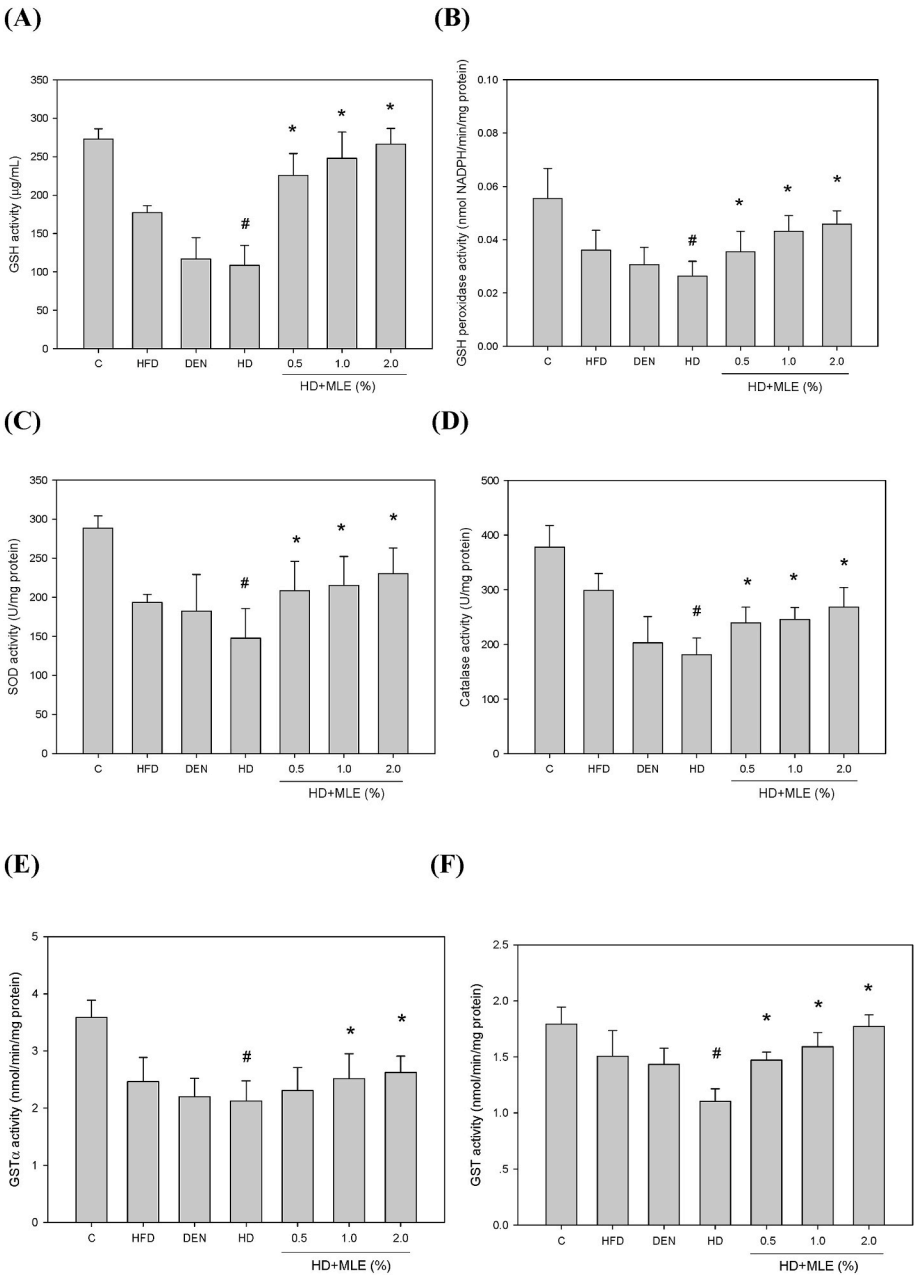

Fig. 5. Effect of MLE on liver antioxidant status in experimental rats. The levels of (A) GSH, (B) GPx, (C) SOD, (D) Catalase activity, (E) GST-α and (F) total GST enzymatic activity of liver from experimental rat. Enzyme activity was quantitated by spectrophotometer analysis. The crude extracts of liver obtained from experimental rats. SD rats fed on normal diet without DEN water (control). Data are shown as the means ± SD. #, $p < 0.05$ as compared with the C group; *, $p < 0.05$ as compared with the HD group.

of Cdc42 and Rac1.[54] However, Turkson et al. indicated that Rac1-mediated C-Jun N-terminal kinase (JNK) signals potentially play critical roles in cell transformation and human cancer development.[55] Because increased and sustained JNK activation in the liver is associated with inflammation, hepatocyte death, and hepatocarcinogenesis, which is often found to contribute to the development of HCC.[56] Oral administration of an antioxidant around the time of DEN exposure blocked prolonged JNK activation and prevented excessive DEN-induced carcinogenesis in $Ikk\beta^{\Delta hep}$ mice.[57] PKCα-mediated c-JNK activation regulates multiple biological functions, such as cell growth, survival, migration, and adhesion.[38] In the present study, western blotting revealed that phosphorylated JNK1/2 expression was significantly lower in the HD + MLE groups than it was in the HD group (data not shown). These data indicate that the coordinated activation of Rac1-mediated JNK pathways, which may play distinct roles in attenuating tumor development and progression, is controlled by activated PKCα.

PKCα and Rac1 play a critical role in regulating various cellular processes. Previous studies have documented the nuclear translocation of PKCα or Rac1 following DEN-induced exposure.[58,59] Specifically, administering a high dose of DEN (90–200 mg/kg) intraperitoneal (IP) injection has been shown to induce the nuclear translocation of PKCα and Rac1. In contrast, our data analysis reveals that exposure to 0.01% DEN through drinking water confines the expression of PKCα and Rac1 to intracellular and pericellular compartments. Administration protocol discrepancies could influence DEN absorption, metabolism, and consequent biological effects, potentially yielding distinct physiological or molecular responses that could explain the observed absence of PKCα and Rac1 translocation to the nucleus. However, the exact molecular

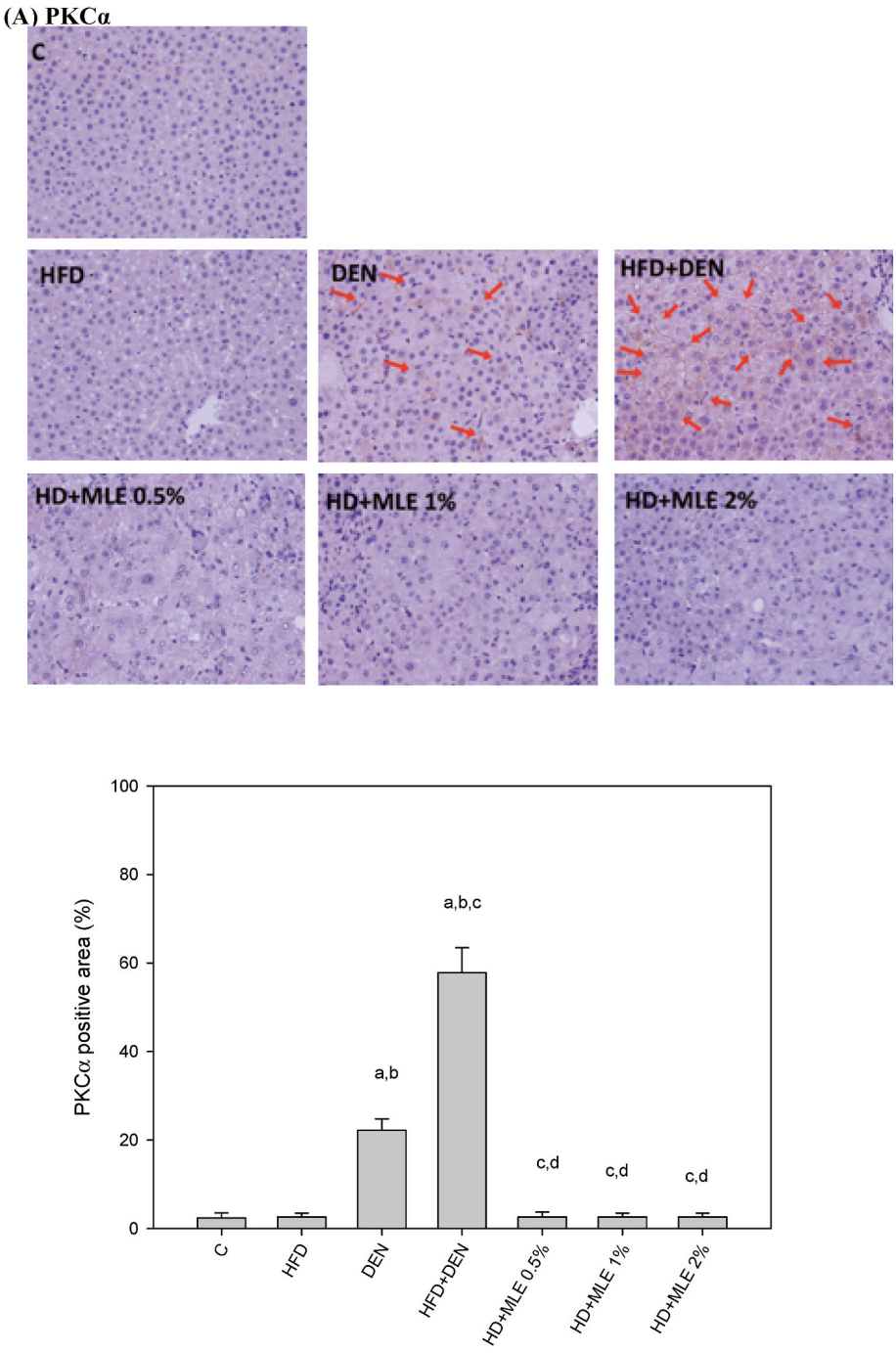

Fig. 6. Effect of MLE treatment on protein expression PKCα and Rac1. Immunohistochemistry labeling for (A) PKCα, (B) Rac1 expression in the livers of the experimental rats (original magnification, ×200). Arrows highlight PKCα or Rac1 positive cells. Results were statistically analyzed with ANOVA and significantly different at $p < 0.05$ according to Duncan's multiple range test. a, $p < 0.05$ compared with the C group. b, $p < 0.05$ compared with the HFD group. c, $p < 0.05$ compared with the DEN group. d, $p < 0.05$ compared with the HD group.

mechanisms by which MLE exerted anticancer effects in the HFD + DEN-induced HCC model remain unclear. Our previous in vitro study clarified that mulberry leaf polyphenol extract activated the p53 pathway, which resulted in the downstream upregulation of phosphatidylinositol-3 kinase (PI3K), *p*-AKT, and fatty acid synthase in human hepatoma HepG2 cells.[60] Future in vivo studies should explore the signaling molecules involved in MLE's anticancer effects.

5. Conclusions

We investigated the effects of MLE supplementation in an HFD + DEN-induced HCC rat model. This is the first study to report that MLE suppressed oxidative stress, decreased liver injury, and protected against the initiation and progression of hepatocarcinogenesis. MLE might be one of the most promising candidates for preventing NAFLD/NASH-related hepatocarcinogenesis. Further research is warranted to better delineate the mechanisms underlying MLE-induced inhibition of hepatocarcinogenesis.

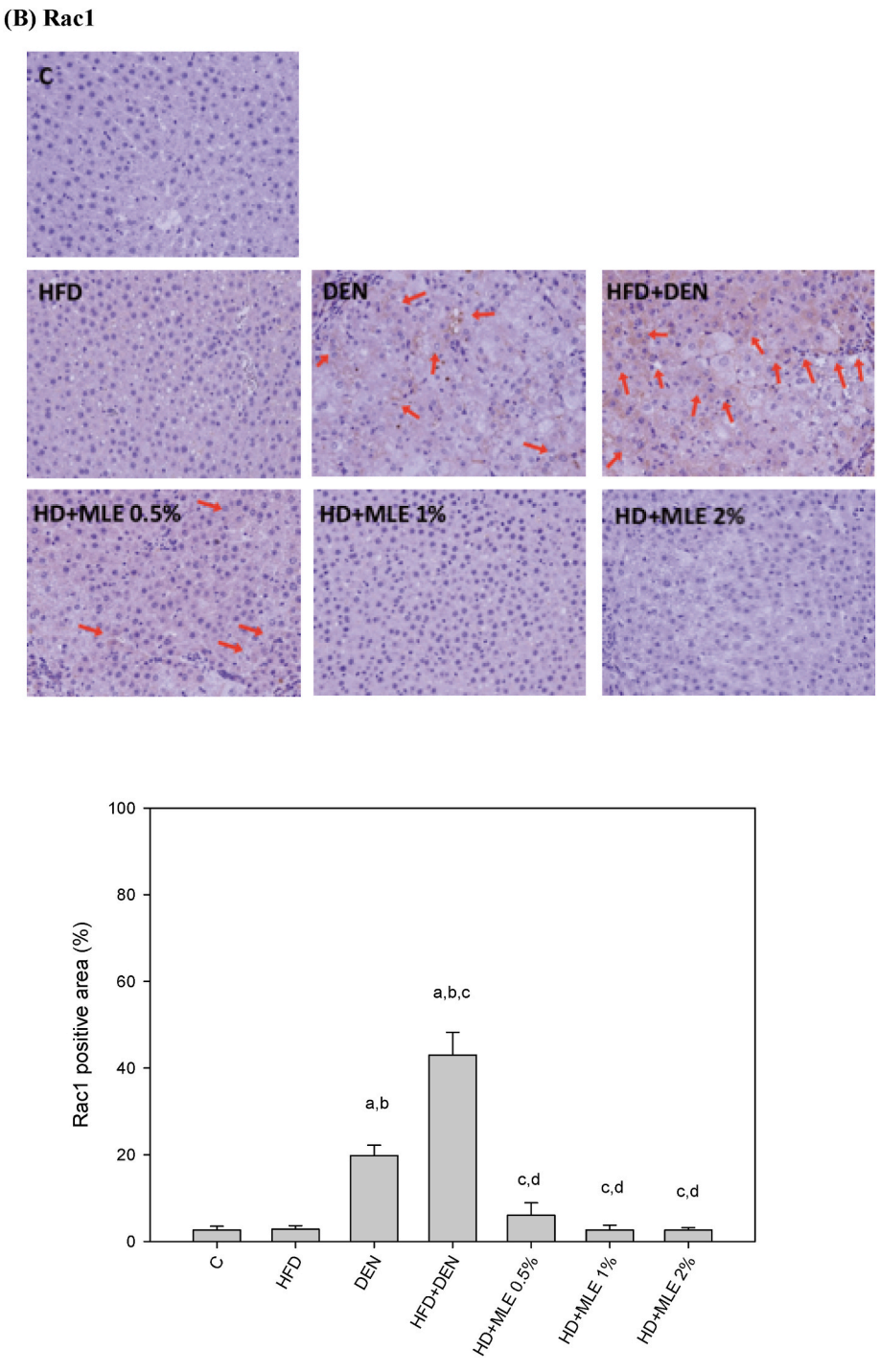

Fig. 6. (*continued*).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.01.007.

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