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Original Article

Therapeutic efficacy of a *Dioscorea membranacea* extract in a rat model of hepatocellular carcinoma: Histopathological aspects

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ABSTRACT

Hepatocellular carcinoma (HCC) is most common in adults and has a high mortality rate because of a lack of effective treatment options. We investigated the effect of a medicinal plant as a potential source of drugs against HCC. The rhizomes of *Dioscorea membranacea* Pierre (DM), Hua-Khao-Yen in Thai, are commonly used as ingredients for alternative treatment of cancer in Thailand. In this study, the anticancer effects of DM extract in HCC-bearing rats were evaluated with respect to gross morphology, histopathology, and leakage of liver enzymes. In untreated HCCs, typical features of liver cancer, including hepatic nodules, thick-cell cords, and pseudoglandular cell arrangements, were observed. In addition, the HCCs showed abnormal reticulin patterns and a high glypican3 expression. In HCC-bearing rats treated with DM the cancer areas and reticulin expression were significantly reduced compared to the untreated group ($p < 0.01$). Sorafenib, the standard drug to treat HCC, reduced the cancer area further, but increased leakage of liver enzymes and decreased serum albumin concentration, indicating liver toxicity. These findings suggest that DM has an anticancer effect on HCCs in an animal model *in vivo* with potentially less severe side effects than sorafenib. Therefore, further studies of DM's mechanism of action in HCC should be carried out.

1. Introduction

Hepatocellular carcinoma (HCC) is a liver cancer that is most common in adults. Among all cancers, HCC was the fourth most frequent cause of cancer-related deaths worldwide in 2018, with almost 800,000 deaths.[1] HCC is, therefore, a major health problem with a high mortality rate. Its high incidence in Asia and Africa is ascribed to the prevalence of hepatitis B or C virus infections.[2,3] In histological sections, cancer cells are arranged in hepatic cell plates of more than three layers thickness (thick-cell cord).[4] This feature is best visible if sections are stained for reticulin. Another feature of HCC is its pseudoglandular architecture, which shows gland-like dilatations of bile canaliculi between the tumor cells.[5,6] Furthermore, immunohistochemical stains can be used to diagnose HCC. Glypican3 (GPC3) is, for example, expressed in approximately 80% of HCCs.[7] GPC3 is a heparan sulfate proteoglycan family member that becomes more highly expressed in HCC than in normal livers or livers with benign hepatic lesions.[8,9]

Sorafenib (SF) administration is considered the standard of care for patients with advanced HCC.[10] SF is a small-molecule multikinase inhibitor that inhibits tumor cell proliferation and tumor angiogenesis in a wide range of tumor models.[11] However, apart from toxic side effects, tumors quickly develop resistance against

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; DEN, diethylnitrosamine; DM, *Dioscorea membranacea*; GPC3, glypican3; HCC, hepatocellular carcinoma; PBS, phosphate-buffered saline; SF, sorafenib; TAA, thioacetamide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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the drug.[12] Traditional herbal medicines are commonly used to treat cancer in Thailand.[13] The rhizomes of *Dioscorea membranacea* Pierre (DM) are an example of a commonly used ingredient in Thai traditional medicine drug formulations for alternative treatment of cancer.[14] DM extract exhibits high cytotoxic activity against several cancer cell lines, including breast, lung, colon, prostate, and liver cancer, but is less toxic to normal cells.[14–16] The extract has also anti-inflammatory, antioxidant, and anti-allergic properties.[17–20] Therefore, this study aimed to determine whether DM extract has any anti-cancer effect *in vivo*, in HCC-bearing rats, using gross morphology, histopathological analysis, and liver function tests as parameters.

2. Materials and methods

2.1. Plant extract

Dioscorea membranacea (DM) extract was prepared at the Center of Excellence of Applied Thai Traditional Medicine (Thammasat University, Thailand). Dried ground plant material (100 g) was macerated with 95% ethanol and concentrated to dryness under reduced pressure to prepare a stock of the plant extract.[15,21] The extract quality and compound fingerprints were comparable to those established in previous studies.[22,23] Briefly, 13 compounds are known to be present in ethanolic DM extract including I) dioscorealides A, II) dioscorealides B, III) dioscoreanone,[19,24] IV) β-sitosterol, V) stigmasterol, VI) β-D-sitosterol glucoside, VII) diosgenin-(3-O-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside, VIII) diosgenin 3-O-β-D-glucopyranosyl (1 → 3)-β-D-glucopyranoside,[19,20] IX) 2,4 dimethoxy-5,6-dihydroxy-9,10-dihydrophenanthrene, X) 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene, and XI) 5,6,2 -trihydroxy 3,4-methoxy, 9,10-dihydrophenanthrene,[14] XII) epipanthogenin B, and XIII) panthogenin B.[25] The working solution of DM was prepared in propylene glycol:tween80:water (4:1:4 v/v).

2.2. Animals

Male 6-7 week-old Wistar rats (*Rattus norvegicus*) weighing 200–250 g were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were allowed free access to food and water in a temperature- and humidity-controlled environment and were maintained on a 12 h light/dark cycle. All procedures involving animals were followed and approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (Bangkok, Thailand; approval no. 2/2559).

2.3. Experimental design

After acclimatization, the rats were weighed and randomly divided into six groups of six rats each. Group 1 was the standard control group and group 2 the control group treated with *D. membranacea* extract only. Groups 3–6 were subjected to HCC induction, which was started with a single intraperitoneal dose of 200 mg/kg diethylnitrosamine (DEN; Sigma-Aldrich, St. Louis, MO, USA). Starting 2 weeks later, the rats were intraperitoneally administered 300 mg/kg of thioacetamide (TAA; Sigma-Aldrich, St. Louis, MO, USA) three times per week for 4 consecutive weeks. The rats were then left for a further 2 weeks without any treatment to allow the cancer to develop. After 8 weeks, all groups were subjected to daily oral administration of different compounds for another period of 8 weeks. HCC-bearing rats were now randomly assigned to groups 3–6. Groups 1 and 3 were administered the vehicle (propylene glycol:tween80:water (4:1:4 v/v), group 4 was administered 4 mg/kg of *D. membranacea* extract, groups 2 and 5 40 mg/kg of *D. membranacea* extract, and group 6 30 mg/kg sorafenib.[26,27] Humane endpoint criteria were appearance of a visible lesion of the skin or weight loss exceeding 20% of the body weight of rats in the control group. At the end of the experiment (16 weeks), rats were anesthetized with 45 mg/kg Nembutal before collecting blood by cardiac puncture and decapitation with a rodent guillotine.

2.4. Liver function tests

Blood serum collected after a heart puncture was used to assess liver function. The serum markers were measured in a standard clinical lab (Bangkok R.I.A. Laboratory, Bangkok, Thailand) using commercially available standard enzymatic reagents (Abbott Pharmaceutical Co. Ltd.) in a biochemical autoanalyzer (ARCHITEC model Ci16200, Abbott Pharmaceutical Co. Ltd.). The assays included aspartate aminotransferase (AST; EC 2.6.1.1, cat. no. 7D81-21), alanine aminotransferase (ALT; EC 2.6.1.2, cat. no. 7D56-21), alkaline phosphatase (ALP; EC 3.1.3.1, cat. no. 7D55), and γ-glutamyltransferase (GGT; EC2.3.2.2 cat. no. 7D65) activity, and albumin (cat. no. 7D53) and total protein concentration (cat. no. 7D63).

2.5. Gross and histopathological examination of the liver

The rat livers were excised, dissected, and rinsed in phosphate-buffered saline (PBS). Examination of the abdominal cavity was carried out to identify gross abnormalities of the organs and photographs were taken. The relative liver weight was calculated as the percentage of liver weight/final body weight. For histopathological examination, the liver tissues were fixed in 4% formaldehyde and then dehydrated in a graded alcohol series (100, 95, 90, 80, 70, and 50%). Liver tissues were embedded in paraffin blocks and sectioned at 5 μm thickness. The sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin and for reticulin (Gordon-Sweet, Bio-Optica, Milano, Italy). The sections were mounted with Permount and analyzed for histopathological features of HCC with a panoramic digital slide scanner program (3Dhistech, Hungary). Randomly selected areas of 10 mm^2 were observed by three specialists in liver histopathology for the presence of HCC. The specialists were not informed about the treatment corresponding to the sections. The criteria for cancer detection were thick cell cords and a pseudoglandular cell arrangement in hepatic nodules. The percentage of HCC-containing and reticulin-positive areas (30 samples/groups) were calculated by using the case viewer program (v1.3.0.41885; https://www.3dhistech.com/caseviewer) and Cell Sense Dimensions software (Olympus, Hamburg, Germany), respectively.[28]

2.6. Immunohistochemistry

Liver sections were deparaffinized and rehydrated in a descending graded series of ethanols. The immunostaining was performed using ImmunoCruz® rabbit ABC Staining System (sc-2018, Santa Cruz, CA, U.S.). The liver samples were incubated with ethylenediaminetetraacetic acid (EDTA) (pH 8.0) for 15 min at 70 °C to perform antigen retrieval. After cooling to room temperature and washing in distilled water, the sections were incubated in 3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity and washed with PBS twice. The sections were incubated with 1.5% blocking serum in PBS for 1.5 h in a humidified chamber. The sections were then incubated with rabbit polyclonal anti-glypican 3 antibody (1:500, cat. no. ab186872; Abcam, Cambridge, UK) overnight at 4 °C in a humidified chamber. The negative control was incubated and treated similarly except that the primary antibody was omitted. After incubation with primary antibody, the

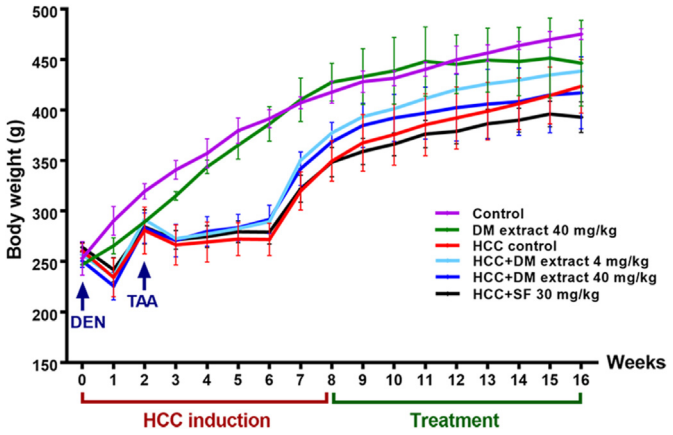

Fig. 1. Changes in body weight of rats during the 16 weeks of the experiment. Results were presented as the mean ± standard deviation. DEN: diethylnitrosamine; TAA: thioacetamide.

sections were washed with PBS and incubated for 1.5 h with biotinylated secondary antibody (1:100, sc-2018, Santa Cruz, CA, U.S.). After washing with PBS, the sections were incubated with avidin and biotinylated horseradish peroxidase (AB reagents) for 30 min, washed with PBS, further incubated with peroxidase substrate, and finally washed with PBS. Counterstaining was performed by incubating the sections in Mayer's hematoxylin (Bio-Optica, Milano, Italy) for 1 min. After washing in distilled water, the sections were dehydrated, cleared in xylene, and mounted with Permount. The sections were scanned with a panoramic digital slide scanners program (3Dhistech, Hungary).

2.7. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using TUNEL assay kit - horseradish peroxidase (HRP)- 3,3′-diaminobenzidine (DAB) (cat. no. ab206386; Abcam, Cambridge, UK). Briefly, the tissue sections were rehydrated and permeabilized before inactivation of endogenous peroxidase. After labeling the 3′-hydroxyl ends of fragmented DNA with terminal deoxynucleotidyl transferase (TdT) enzyme, the sections were incubated in blocking buffer and then incubated with streptavidin-HRP conjugated solution. The positive cells were developed with DAB substrate. All sections were counter-stained with methyl green solution. Finally, the sections were mounted and photographed under a light microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

All data are presented as means ± SEM. The data was analyzed using GraphPad Prism 7.0, one-way analysis of variance (ANOVA) with a Bonferroni test to compare between groups.

3. Results

3.1. Liver weight as a fraction of body weight

The body weight of the rats decreased upon injection with initiator (DEN) and promoter (TAA), but gradually increased after discontinuing TAA administration (week 6 till week 16) (Fig. 1).[28] At the end of the experiments, the final body weight of sorafenib-treated group was significantly decreased compared to control group ($p < 0.05$). The final liver weight was significantly decreased

Table 1
Body weight, liver weight, and the liver-body weight ratio (mean ± SEM.).

Groups	Body weight (g)	Liver weight (g)	Liver weight/body weight (%)
Control	475 ± 3	12.9 ± 0.5	2.7 ± 0.1
DM extract 40 mg/kg	446 ± 24	11.9 ± 0.4	2.7 ± 0.1
HCC control	423 ± 10	14.1 ± 0.6	3.3 ± 0.1 *
HCC + DM extract 4 mg/kg	438 ± 18	14.0 ± 0.7	3.2 ± 0.1
HCC + DM extract 40 mg/kg	417 ± 13	13.6 ± 0.7	3.3 ± 0.1 *
HCC + SF 30 mg/kg	390 ± 5 *	10.3 ± 0.3#	2.6 ± 0.1#

*$p < 0.05$ compared to control group, #$p < 0.01$ compared to all HCC group.

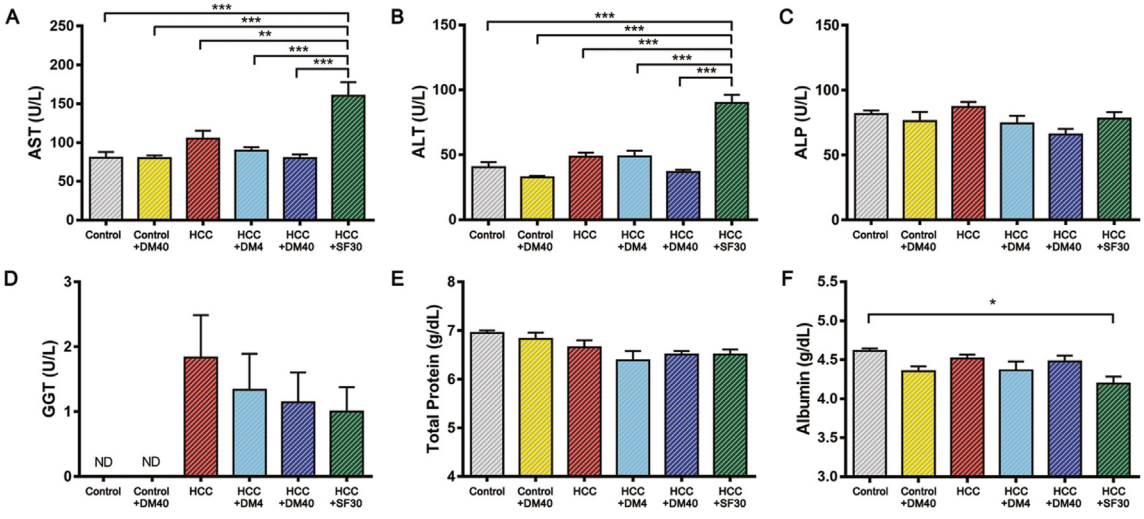

Fig. 2. Serum levels of A) aspartate aminotransferase (AST), B) alanine aminotransferase (ALT), C) alkaline phosphatase (ALP), and D) gamma-glutamyl transferase (GGT) activities, and E) total protein and F) albumin content (*$p < 0.05$, ***$p < 0.001$, ND: non-detectable).

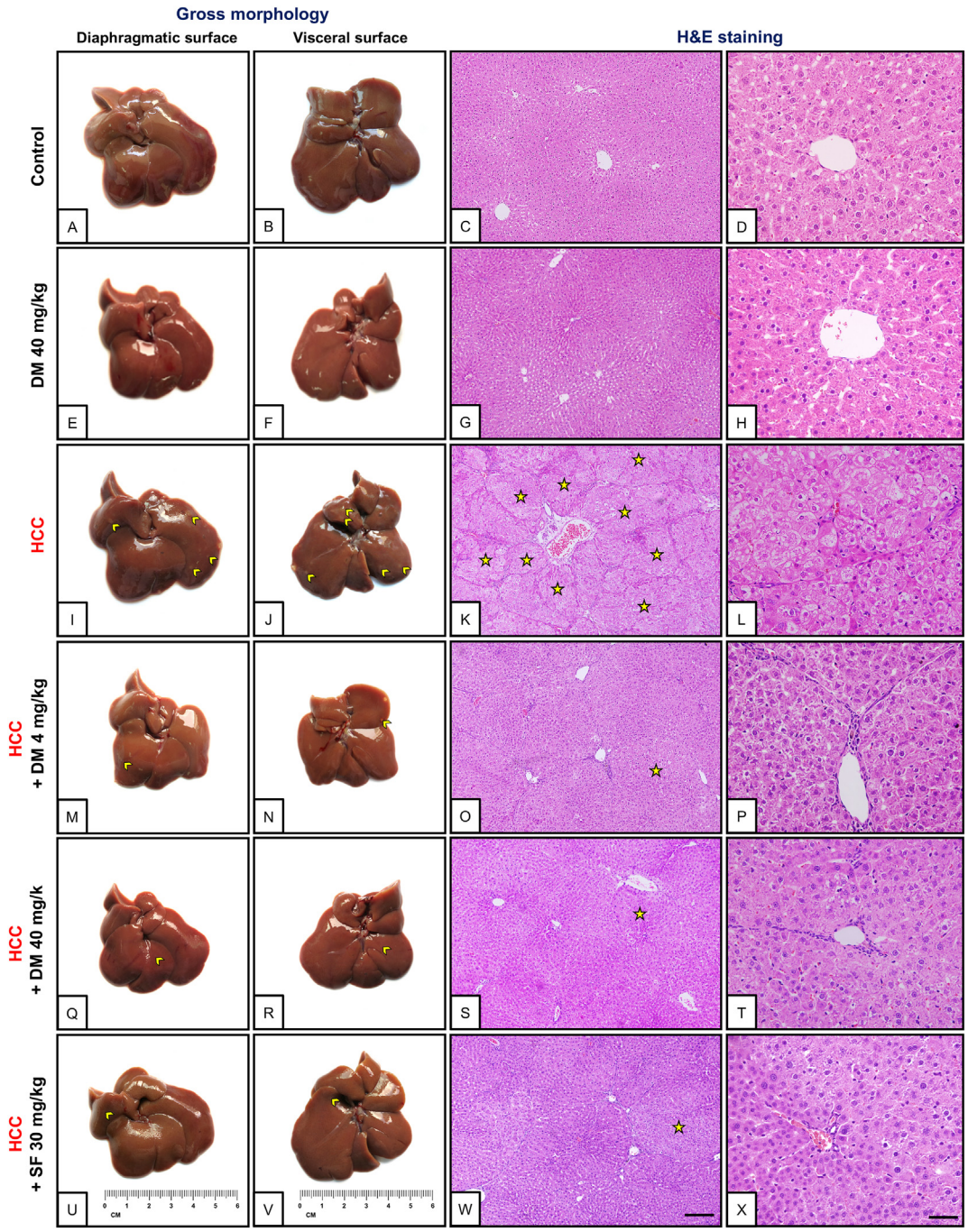

Fig. 3. Gross morphology of the diaphragmatic and visceral surfaces of the livers of the six groups in the left two columns (scale bar: ruler in cm). The right two columns show histological liver sections stained with H&E (scale bar: 200 and 50 μm, respectively). Yellow arrowheads; hepatic nodules, Stars; centers of tumor cell masses.

in sorafenib-treated group compared to all HCC groups (Table 1; $p < 0.01$). Furthermore, the HCC control and the 40 mg/kg *D. membranacea* extract-treated HCC group had a significantly increased liver-to-body weight ratio compared to the control group ($p < 0.05$). However, there was no significant difference in the liver-to-body weight ratio between the control and *D. membranacea* extract-treated control groups. The liver-to-body weight ratio in the sorafenib-treated HCC-bearing group was significantly lower than that in the other HCC groups (Table 1).

3.2. Liver enzyme and function tests

The serum concentrations of all "liver" enzymes and albumin in the control and *D. membranacea* groups were similar, but serum AST and ALT concentrations were significantly increased in the sorafenib-treated group compared to all other groups (Fig. 2A and B; $p < 0.001$). In addition, the serum concentration of albumin in the sorafenib-treated group was significantly decreased compared to the control group (Fig. 2F; $p < 0.05$). GGT activity was

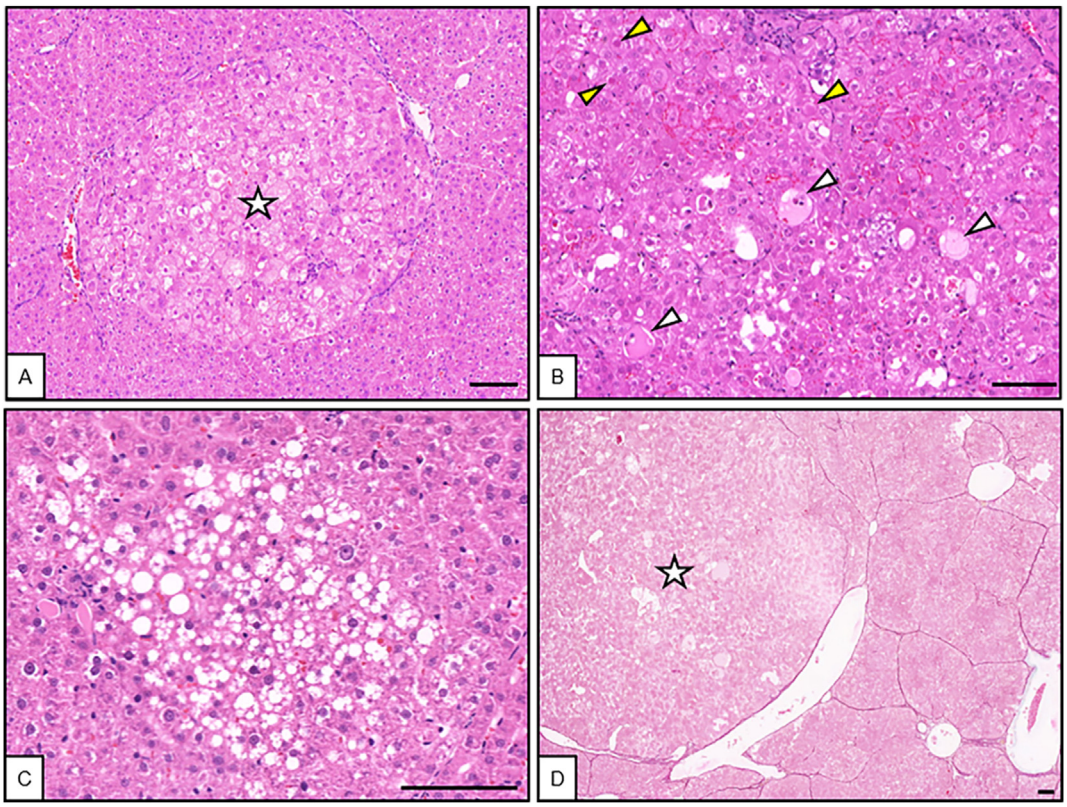

Fig. 4. Histopathology of liver nodules of HCC-bearing rats. The solid tumor nest (white star; A), its pseudoglandular architecture (white arrowheads) and intracellular hyaline globules (yellow arrowheads; B), micro-steatosis (C), and absence of internal reticulin staining show the complex make-up of each hepatic nodule (D) (scale bars: 100 μm).

undetectable in rats not carrying an HCC, but rose above the level of detection in all HCC-induced groups (Fig. 2D). Serum ALP activity and total protein concentration did not change significantly as a result of the interventions (Fig. 2C and E).

3.3. Gross morphology and histopathological analysis of the livers

The gross morphology of livers in the *D. membranacea*-only group was similar to that in the control group. Liver in the HCC groups had clearly visible hepatic nodules (Fig. 3), but the *D. membranacea*-treated and sorafenib-treated HCC groups had fewer hepatic nodules than the untreated HCC group.

Photomicrographs of liver sections of the control and *D. membranacea*-only groups showed a normal architecture of the hepatocytes. Hepatocytes had an eosinophilic cytoplasm with numerous mitochondria and rough endoplasmic reticulum stacks. Hepatocytes were organized into cell plates separated by sinusoids that radiated from the central vein (Fig. 3C,D,G and H). These findings imply that vehicle and *D. membranacea* extract did not affect liver architecture in rats. The HCC groups did show numerous hepatic nodules (Fig. 3K and L). In contrast, the number of hepatic nodules and abnormal hepatocytes were reduced in both doses of *D. membranacea*-treated and sorafenib-treated HCC rats (Fig. 3M–X). Moreover, liver cancer histopathological features, including pseudoglandular structures, hepatic cell cords greater than three cell layers in thickness, intracellular hyaline globules, and micro-steatosis (Fig. 4A–C) were not markedly visible in *D. membranacea*- and sorafenib-treated HCC rats.

3.4. Reticulin staining, cancer area, and GPC3 immunoreactivity

Staining liver sections with reticulin can be used to visualize the presence of HCC. In all HCC groups the reticulin staining pattern was abnormal. The near absence of reticulin fibers between the hepatoma cells within tumor nodules indicated the loss of normal architecture of hepatic cell plates (Figs. 4D and 5). In addition, a thick reticulin-positive fiber outlining each tumor nodule was markedly present. Due to fewer, but much thicker reticulin-positive fibers surrounding the tumor nodules, the reticulin-positive area of HCC groups was significantly increased compared to the control group ($p < 0.001$). In contrast, the reticulin-positive areas in livers of *D. membranacea*- (both concentrations) and sorafenib-treated HCC rats were significantly lower than the untreated HCC groups (Fig. 6A). Likewise, histopathologically the percentage of cancer areas in *D. membranacea*-treated (~5%), and sorafenib-treated HCC-bearing rats (~2%) were highly significantly reduced compared to the untreated HCC rats (~20%) (Fig. 6B; $p < 0.001$).

In order to confirm HCC, we immunostained sections for the presence of GPC3. The control and *D. membranacea*-only groups showed a weak GPC3 staining in a wide area around the central vein (Fig. 7A and B), whereas HCC nodules showed a diffuse, more intense staining pattern (Fig. 7C). The staining intensity of GPC3 in untreated HCCs was slightly greater than that in the *D. membranacea*- or sorafenib-treated HCC groups (Fig. 7D–F).

3.5. TUNEL assay

In order to investigate apoptosis as a potential mechanism of the beneficial effect of *D. membranacea* extract, we performed TUNEL assay to demonstrate DNA fragmentation. After TUNEL staining, most of the area in the tissue sections did not develop a positive signal in untreated HCC group (Fig. 8A). In contrast, liver sections of rats treated with either dose of *D. membranacea* extract showed TUNEL-positive nuclei in the liver cancer area (Fig. 8B and C).

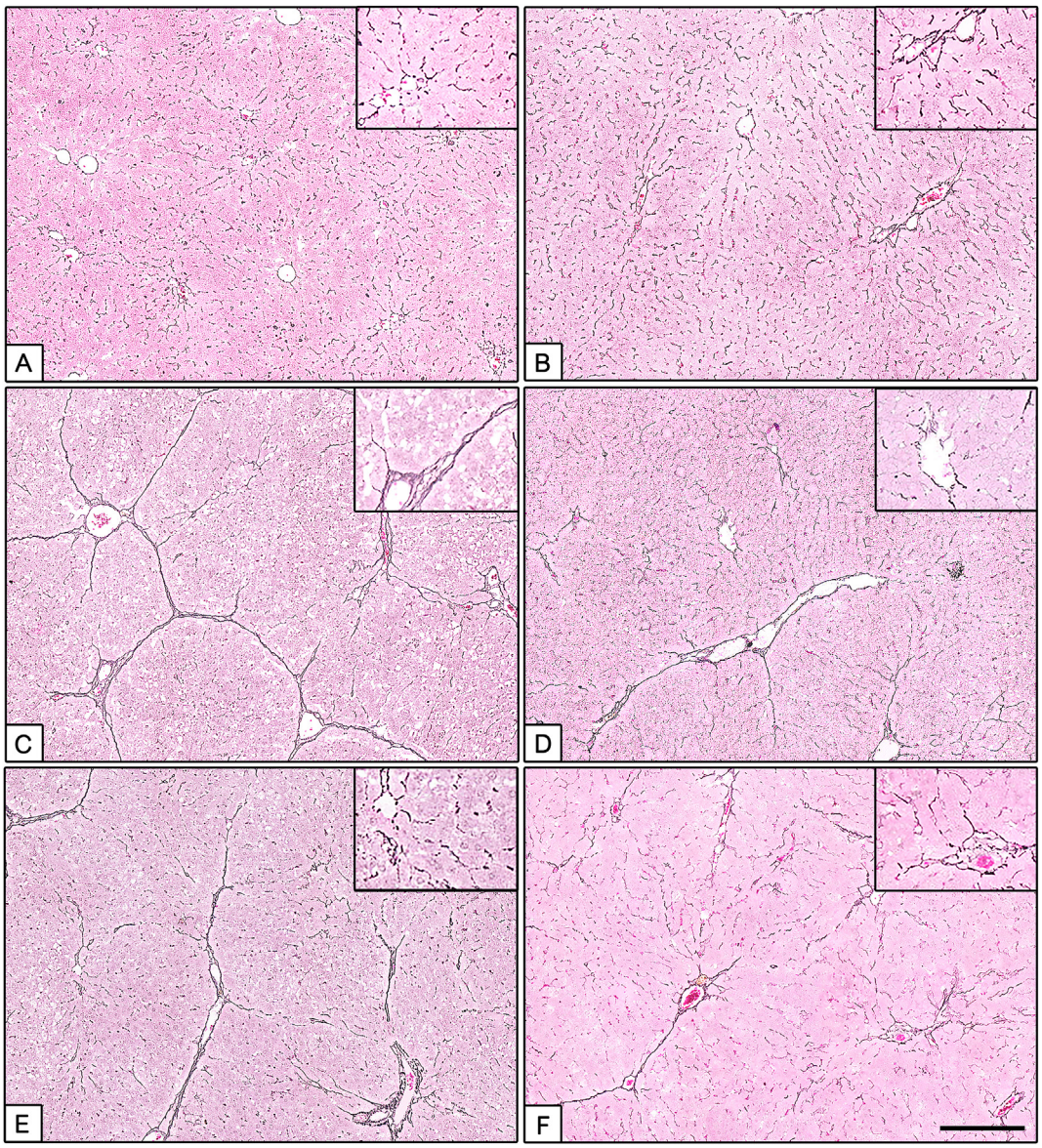

Fig. 5. Liver sections after staining for the presence of reticulin, with magnifications in the insets. Control group (A), control + *D. membranacea* 40 mg/kg group (B), untreated HCC group with abnormal reticulin pattern in most areas (C), HCC + *D. membranacea* 4 mg/kg (D), HCC + *D. membranacea* 40 mg/kg (E), and HCC + sorafenib 30 mg/kg group (F) (scale bar: 200 μm).

4. Discussion

The histopathological analysis of the effects of *D. membranacea* extract in HCC-bearing rats revealed its potential to reduce the cancer volume and ameliorate its presentation. The hepatoma cells in the nodules of HCC-bearing rats lost their trabecular arrangement with reticulin staining confined to the nodular periphery. Furthermore, the hepatoma cells expressed more GPC3. Sorafenib treatment reduced the cancer volume most, but inflicted more liver injury, as shown by the increased concentration of serum liver enzymes and the lower serum albumin concentration. *D. membranacea* treatment was twofold less effective in reducing the cancer volume, but did not cause liver injury.

Hepatocarcinogenesis is the development of liver cancer due to exposure to a carcinogen that promotes gene damage that, in turn, causes hepatocyte transformation. It is a multistep process that includes initiation, promotion, and progression.[29] In this study, the initiation process, the exposure to the initiator DEN, probably causes DNA mutations in hepatocytes that transform normal cells into cancer cells.[30] DEN is a widely accepted and commonly used initiator of liver carcinogenesis in rats.[31] We used the toxic agent TAA as promoter. This compound causes centrilobular liver damage and inflammation, and sustains proliferation of cancer cells.[32] At low dose, TAA induces liver fibrosis and cirrhosis in the rat.[33] Its hepatotoxic response depends on the dose and duration of administration.[34] Our protocol was adapted from mice[35] to rats by increasing dose and duration of TAA treatment, because a lower dose and shorter treatment from our preliminary study only caused regenerative nodules instead of cancer in some of the experimental rats. The presently used protocol results in the development of tumors in all rats. Although TAA treatment caused weight loss, all animals survived after the period of cancer progression. HCC-bearing rats treated with either 4 or 40 mg/kg *D. membranacea* gained weight.

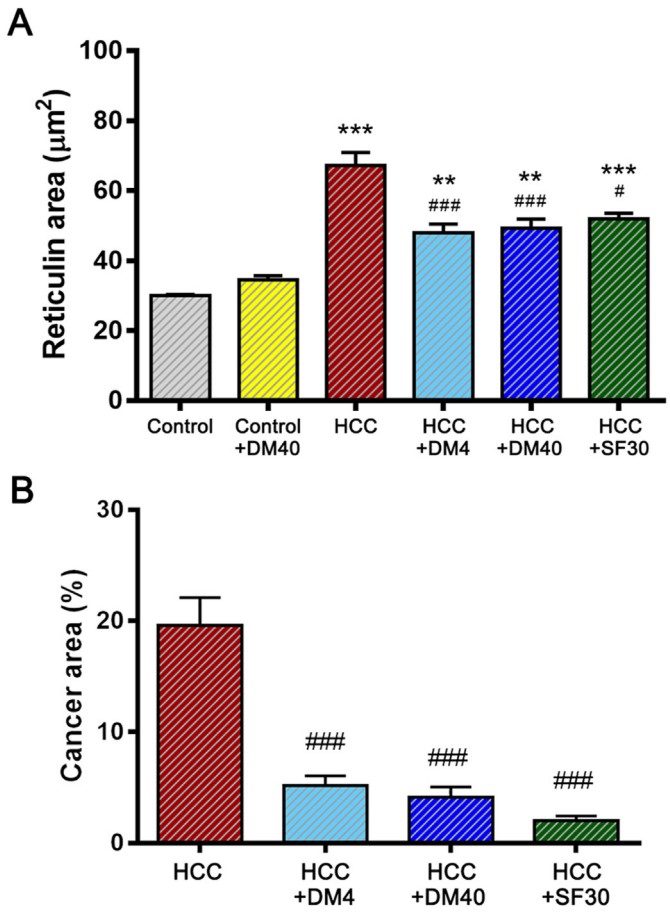

Fig. 6. Surface of the reticulin areas per section (A) and relative cancer area (B) in rat livers (**$p < 0.01$ vs. control group, ***$p < 0.01$ vs. control group, $^{\#}p < 0.01$ vs. HCC group, $^{\#\#\#}p < 0.001$ vs. HCC group).

Since the HCCs had formed a thick sheath of reticular fibers or fibrous tissue around the tumor cell nest,[36] the reticulin stain, therefore, seems to be a promising technique to grade malignancy in HCC. From our present study, a high GPC3 expression was present inside each of the hepatic nodule in which the reticular framework along the hepatic cell cord has lost. Several studies reported that GPC3 is a good marker to detect HCC in sections.[7,37] GPC3 can, via its heparan sulfate side chain, interact with Wnt and Frizzled, which are both markers of tumor proliferation.[38] This protein is not or only weakly expressed in healthy livers.[39] Our observations reveal that GPC3 is expressed in the pericentral zone of normal (rat) livers. In agreement, Wnt signaling is exclusively active in pericentral hepatocytes.[40,41]

Liver function tests are useful for the evaluation of liver diseases. Elevated concentrations of liver enzymes, such as AST and ALT, in serum are a response to liver injury.[42,43] Previously, we showed that administration of solely TAA three times weekly for eight weeks also increased serum ALT, AST, and ALP levels and induced severe fibrosis in rat.[44] Many studies report a significant increase in circulating liver enzyme levels in rats with DEN-induced tumors.[45,46] Both compounds are, therefore, hepatotoxic, but from our present study administration of DEN followed by TAA was no longer associated with elevated circulating liver enzymes 8 weeks after discontinuing TAA administration, even though many HCC nodules had developed. These findings, therefore, indicate that elevation of circulating liver enzymes is not a reliable HCC marker.

The liver-to-body weight ratio in both normal control and *D. membranacea* 40 mg/kg only groups were not different, implying that *D. membranacea* does not affect animal health. However, this ratio was increased in the HCC-bearing rat probably due to the presence of numerous liver tumor mass together with the lower body weight. This trend is similar to what was reported for the HCC condition in animal study.[28,47] Even though treatment of HCC-bearing rat with *D. membranacea* (both concentrations) did not change this ratio compared with the untreated HCC group, the cancer area was histopathologically about four-fold decreased. Therefore, the liver-to-body weight ratio alone is not a reliable parameter to gauge the efficacy of a treatment. Similarly, the ratio in the sorafenib-treated HCC rats was markedly decreased compared to the untreated HCC group. This is probably due to the fact that sorafenib has anti-angiogenic, anti-proliferative, and anti-metastasizing activity,[10–12,27] which results in a decrease of liver tumor mass. This is confirmed by our histopathological study that showed a smaller cancer area. Nevertheless, our study found that sorafenib treatment caused lower liver and body weights resulting in the lowest value of liver-to-body weight ratio. Furthermore,

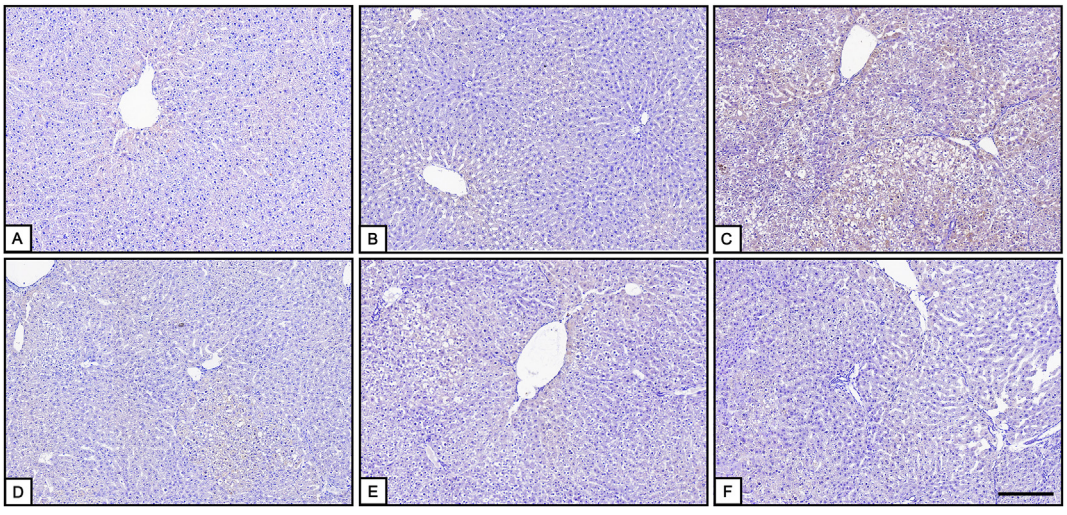

Fig. 7. Liver sections stained for the presence of GPC3. Control group (A), control + *D. membranacea* 40 mg/kg group (B), untreated HCC group (C), HCC + D. *membranacea* 4 mg/kg (D), HCC + *D. membranacea* 40 mg/kg (E), and HCC + sorafenib 30 mg/kg group (F) (scale bar: 200 μm).

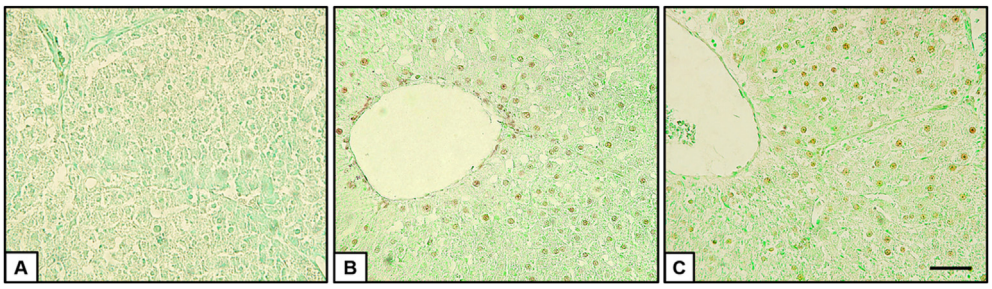

Fig. 8. TUNEL assay of liver tissues in the untreated HCC group (A), the HCC + *D. membranacea* 4 mg/kg (B), and the HCC + *D. membranacea* 40 mg/kg (C). Nuclei stained with the TUNEL assay are brown. Sections were counter-stained with methyl green (scale bar: 50 μm).

sorafenib also caused an elevation of serum ALT and AST liver enzymes and a decreased serum albumin. These observations, like our earlier study,[43] do suggest that sorafenib is hepatotoxic. We selected the dosage of sorafenib based on the effective doses commonly used in HCC rat models.[26,27] Sorafenib-induced hepatotoxicity and severe liver dysfunction do, however, only rarely occur in HCC patients,[48,49] even though liver necrosis with inflammatory cell infiltration have been reported in HCC patients.[50]

We selected *D. membranacea* extract from five species of Thai herbal plants called Hua-Khao-Yen. From all five species, *D. membranacea* extract showed the highest cytotoxicity *in vitro* against many cancer cell lines, including the human liver cancer cell line HepG2.[14] Thus far, there was no earlier investigation of the effects of *D. membranacea* extract in an animal HCC model *in vivo*. This study is, therefore, the first to show the beneficial effects of this plant in HCC-bearing rats. Doses of the extract used in the present study were based on those used *in vitro*[14] and were demonstrated to be safe in an acute and chronic toxicity test in rats.[51] In agreement, liver sections of rats only treated with a high dose of *D. membranacea* extract showed the same, unaltered architecture of hepatocytes as found in control livers, which underscores that *D. membranacea* extracts are not toxic. The present study shows an antiproliferative effect of *D. membranacea* extract in DEN-and TAA-induced HCC *in vivo*, but the effect was not dose-dependent, which needs further study.

In a previous study, we showed that another herbal extract, Benja-ummarit,[28] also effectively reduced the size of hepatic nodules in the same model of HCC-bearing rats. Benja-ummarit remedy appeared to have a similar effect on reducing the cancer area (~2%), but this effect was based on the scoring of H&E-stained sections only. Based on our present findings, the reticulin staining assay is more reliable. Benja-ummarit also reduced VEGF expression and content in the liver, which is also one of the mechanisms of action of sorafenib. The putative active compound of Benja-ummarit is gambogic acid, whereas dioscorealide B is candidate for active principle in *D. membranacea* extracts. Dioscorealide B reportedly has a high cytotoxic effect for human liver cancer cells[25] and a pro-apoptotic activity in breast cancer cells.[28] Moreover, our research group found that HCC-bearing rats treated with a crude extract of *D. membranacea* showed DNA fragmentation by TUNEL assay, indicating apoptosis of cancer cells. In addition, a related compound, dioscoreanone may have an additive effect for HCC treatment due to its anti-inflammatory and antioxidant properties.[18,19] Future studies should identify the active components in Benja-ummarit and *D. membranacea* extracts and establish whether both compounds do exert their effects independently or not.

5. Conclusion

This study suggests that *D. membranacea* treatment has anti-cancer effects in HCC-bearing rats and causes much less liver injury than the standard treatment with sorafenib.

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Author contributions

VK performed the experiment, immunohistochemistry studies and interpreted the data, NK collected the liver and performed histological study, SP carried out the gross morphology and pathological studies and reticulin staining, AI carried out the DM extraction, and WP designed the study, supervised the research project, interpreted the data and edited the manuscript. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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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.2021.02.001.

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