

## Original Research Article

# Effect of ganoderic acid on diethylnitrosamine-induced liver cancer in mice

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### Abstract

**Purpose:** To investigate the hepatoprotective role of ganoderic acid A (GAA) on liver cancer induced by diethylnitrosamine (DEN) via Nrf-2/HO-1/NF-κB signal pathway in mice.

**Methods:** Sixty male C57BL/6J mice were randomly divided into 4 groups: (1) control group, (2) DEN (25 mg/kg) group, (3) GAA (20 mg/kg) + DEN group, (4) GAA (40 mg/kg) + DEN group. The protective effect of GAA on liver was evaluated by determining malondialdehyde (MDA), superoxide dismutase (SOD), inflammatory cytokines including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and the expression of heme oxygenase-1 (HO-1), nuclear factor erythroid-2-related factor-2 (Nrf-2), IκBα, p-IκBα, p65, p-p65, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in serum.

**Results:** The results demonstrate that GAA treatment significantly suppressed the generation of MDA, proinflammatory cytokines, and restored the activity of SOD in the serum of DEN-induced liver cancer in mice. Western blots analysis revealed that GAA significantly restored Nrf-2/HO-1/NF-κB signal pathway-related protein levels in DEN-induced mice liver cancer model.

**Conclusion:** This research reveals the anticancer activity of GAA in liver tissue, and suggests that GAA counters DEN-induced liver cancer through Nrf-2/HO-1/NF-κB signal pathway.

**Keywords:** Ganoderic acid A, Nrf-2/HO-1/NF-κB pathway, Liver cancer, MDA, GAPDH, SOD

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## INTRODUCTION

In recent years, hepatocellular carcinoma (HCC) has been the sixth most common form of hepatic malignancy and the third leading cause of cancer-related mortality around the world [1]. Although surgical resection and traditional chemotherapy are effective treatment strategies for HCC patients, the poor prognosis of patients

after the surgery remains worthy of concern [2]. In the development and progression of HCC, there are many pathological changes in the oxidative stress and inflammatory response, which triggers cirrhosis and hepatic dysfunction in HCC patients [3,4]. Diethylnitrosamine (DEN)-caused hepatocellular carcinoma (HCC) in mice is one of most widely used animal models to simulate human liver cancer. Although documents have pointed that DEN induced the

excessive inflammatory and oxidative modulator, its underlying molecular mechanisms during tumor progression remain not fully understood.

In the process of carcinogenesis, the promotion of reactive oxygen species (ROS) and oxidative stress disturbs redox balance, which leads to activation of anti-oxidative repair mechanisms [5]. Nuclear factor erythroid-2-related factor-2 (Nrf-2) as one of the classical transcription factors, is activated by the oxidative stress injury implicated in the initiation of cancer [6]. Nuclear erythroid 2-related factor 2(Nrf-2) translocates from the cytoplasm to cell nucleus and combines antioxidant response element (ARE) to maintain cellular redox balance [7]. Therefore, the ARE-controlled genes are activated. Nrf-2 activation initiates NF- $\kappa$ B mediated tumorigenesis progression in liver cancer and upregulated the expression of several multi-functional anti-oxidative proteins such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and heme oxygenase-1 (HO-1) [8]. As a downstream molecule of Nrf-2, over-expression of HO-1 attenuated ROS generation, thus modulating oxidative stress and anti-cancer effects [9]. Taken together, inhibition of Keap-1, NF- $\kappa$ B and induction of Nrf-2, HO-1 appear to be a potential therapeutic approach in mice liver cancer induced by DEN.

*Ganoderma lucidum* (Basidiomycete), is a clinically effective herb in Traditional Chinese Medicine (TCM) for the prevention and treatment of a variety of human physical disorders, including hypertension, coronary heart disease, chronic hepatitis and immunological disorders. Ganoderic acid A (GAA) is a natural compound extracted from *G. lucidum*, which are potential low-cost therapeutic candidates with multiple pharmacological activities. At present, more than 130 ganoderic acids and associated derivatives have been isolated and identified from *G. lucidum* [10]. Ganoderic acid A had been shown to suppress tumorigenesis in several types of human cancers including lung, colon, prostate, pancreatic and ovary tumor [11].

In addition, GAA was reported to promote apoptosis and anti-angiogenic activity with low side-effect and cytotoxicity in cancer cells [12]. Ganoderic acid A inhibits tumor growth by inducing apoptosis, this study is to explore whether or how the Nrf-2/HO-1/NF- $\kappa$ B signal pathway participant in anti-oxidative and anti-inflammatory effects of GAA. In summary, GAA might play a key role in attenuating oxidative stress and inflammation through Nrf-2/HO-1/NF- $\kappa$ B signaling pathway in mice liver cancer.

In this study, the role of GAA on DEN-induced mice liver cancer and the potential mechanism were explored.

## EXPERIMENTAL

### Reagents

Ganoderic acid A (CAS no. 81907-62-2) and DEN (#N0756), bought from Sigma-Aldrich (St. Louis, MO, USA), were dissolved in Dimethyl sulfoxide (DMSO) and saline (the concentration of Dimethyl sulfoxide (DMSO) were less than 0.1 % [v/v]). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (#E-EL-M0049c), IL-6 (#E-EL-M0044c) and IL-1 $\beta$  (E-EL-M0037c) enzyme-linked immuno-sorbent assay (ELISA) kits were supplied by Elabscience Biotech. Co. Ltd (Wuhan, China). Malondialdehyde (MDA, #A003-1) and superoxide dismutase (SOD, #A001-3) commercial kits were produced by Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against I $\kappa$ B $\alpha$  (#4814), p-I $\kappa$ B $\alpha$  (#2859), p65 (#8242), p-p65 (#3033), Nrf2 (#12721), HO-1 (#82206) and GAPDH (#5174) were acquired from Cell Signaling Technology (Danvers, USA).

### Animals and experimental design

Sixty male C57BL/6J mice (2 weeks old) were purchased from Jiangning Qinglongshan Animal Cultivation Farm (Nanjing, China). All mice were given free water and chow, maintained under a 12-h light/dark cycle with a constant temperature of  $25 \pm 2$  °C in the Animal Center of China Pharmaceutical University. The animals study got approval from Ethic Committee of Qingdao Commercial Staff Hospital, the approval no. 201706325. All the experimental procedures were performed in line with WHO guidelines [13].

Murine model hepatocellular carcinoma was induced according to the previous literature with minor modification [14]. Experimental mice were randomly divided into 4 groups (n = 15) as follows: control group, DEN (25 mg/kg) group, DEN + GAA (20 mg/kg) group and DEN + GAA (40 mg/kg) group. Mice in all groups except control group were injected intraperitoneally with DEN (25 mg/kg). At the same time, control mice were intraperitoneally treated with saline. One week later, the animals intragastrically received low doses(20mg/Kg) and high doses(40mg/Kg) of Ganoderic acid A 3 times a week for 32 weeks.

Simultaneously, mice in control group and DEN group were given equal volumes of saline. At the end of the experiment, the animals were sacrificed and the blood samples were prepared

for ELISA and commercial kits detection. Mice liver tissues were used for western blot, biochemical and pathology analyses.

#### Determination of MDA and SOD in serum

The blood samples were centrifuged at 3000 rpm for 15 min and the serum were frozen at  $-80^{\circ}\text{C}$ . The levels of oxidative stress biomarkers MDA and SOD in serum were assessed using spectrophotometry using commercially available kits. MDA can interact with thiobarbituric acid (TBA) under acidic and heating condition, and generate chromogenic reaction under 539nm; SOD activity was determined by nitro-blue tetrazolium (NBT).

#### Histopathological analysis of liver tissue

Liver tissues were fixed in 10 % formalin solution and embedded in paraffin for histopathological analysis. Then, the liver sections were stained with hematoxylin and eosin (H & E). Histopathological changes were observed under an optical microscope.

#### Determination of cytokines in serum

The levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in serum of DEN mice were determined using ELISA kits. All experimental procedures were performed according to the kit manufacturer's instructions.

#### Western blot analysis

Liver tissues were homogenized in lysis buffer containing 1 ml RIPA and 10  $\mu\text{L}$  PMSF. I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , p-65, p-p65 were quantified using BCA kit. Protein samples were loaded on 12 % SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked with 5 % milk for 2 h and washed with TBST three times.

The membranes were incubated with primary antibodies against p-I $\kappa$ B $\alpha$  (1:1000), I $\kappa$ B $\alpha$  (1:1000), p-p65 (1:1000), p65 (1:1000), GAPDH (1:1000) overnight at  $4^{\circ}\text{C}$ . HRP-labeled secondary antibodies were added, and incubated with membranes for 2h. Immunoblots were visualized using the ECL detection system and a gel imaging system.

#### Statistical analysis

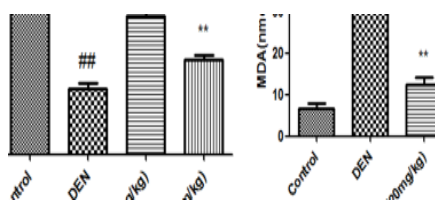
The data was analyzed with spss 19.0, and expressed as means  $\pm$  SDs. Significant differences were checked with one-way ANOVA followed by Tukey multiple comparison tests.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of GAA on oxidative stress in serum of GEN-induced mice

The serum content of MDA and SOD in each group were detected and presented in Figure 1. The level of MDA in serum was significantly increased in DEN-induced mice compared with control group. Simultaneously, the level of SOD was significantly decreased in DEN-induced mice compared with control group. Ganoderic acid A administration significantly reverses the level of SOD and MDA of DEN-induced mice. The high dose of GAA (40 mg/kg) was more effective compared to GAA (20 mg/kg).

The data confirmed that GAA administration could attenuated the oxidative stress in DEN mice.



**Figure 1:** Serum content of MDA and SOD. Different superscript letters meant significant differences between groups; # $p < 0.01$  vs. control group; \* $p < 0.05$ , \*\* $p < 0.01$  vs. DEN group

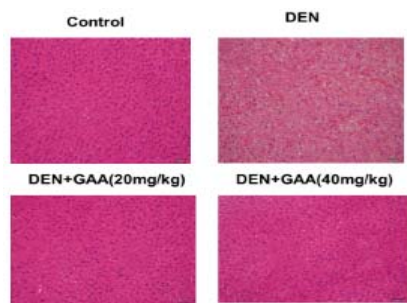
### Effect of GAA on histopathological examination

The H&E staining results are presented in Figure 2. There were no abnormal findings in the liver tissue of the control group. However, in the DEN-induced group, the study indicated evident disruption of hepatic lobule structure and hepatoma nodules. In different GAA treatment groups, antitumor effects were observed. The H & E staining of GAA-treated groups indicated GAA (40mg/kg) is more effective than GAA (20mg/Kg) on HCC, suggesting that GAA could ameliorate the DEN-induced liver tumor at the dose of 40 mg/kg.

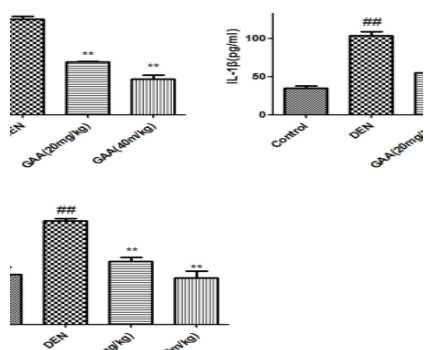
### Effect of GAA on serum TNF- $\alpha$ , IL-1 $\beta$ and IL-6 of GEN-induced liver tumor mice

The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in serum were measured to evaluate the hepatoprotective effect of GAA. As indicated in Figure 3, significant increases were observed in the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 contents in the DEN group when compared with those in control group. GAA (40 mg/kg) group is more efficient than GAA (20

mg/kg) in suppressing the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6.



**Figure 2:** The H & E staining results (x200) indicate that GAA treatment could ameliorate the DEN-induced liver tumor



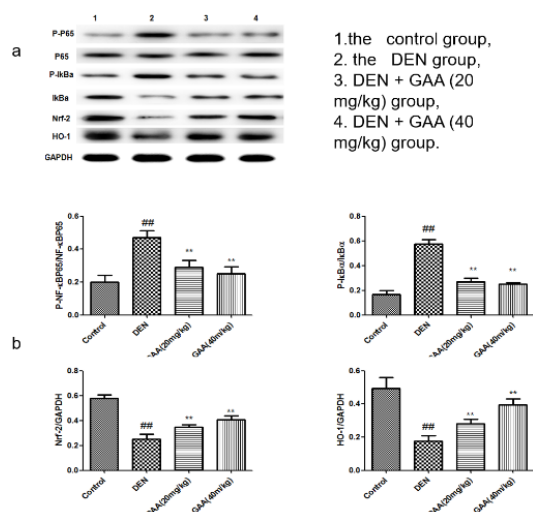
**Figure 3:** Effect of GAA on TNF- $\alpha$  (A), IL-1 $\beta$  (B), IL-6 (C) contents in serum of mice. Results are expressed as means  $\pm$  SDs (n = 8); #*p* < 0.01 vs. control group; \**p* < 0.05, \*\**p* < 0.01 vs. DEN group

### Effect of GAA on Nrf-2/HO-1/NF- $\kappa$ B pathway-related proteins in DEN-induced mice

The results in Figure 4 show the expressions of relative proteins. According to the results obtained, the expression levels of I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , p-65, p-p65 proteins were increased in the DEN-induced group when compared with the control group. However, all the proteins were down-regulated by GAA treatment at 20, 40 mg/kg, especially the group of DEN + GAA (40 mg/kg) group. The findings indicate the anti-tumor and anti-inflammatory effects of GAA.

## DISCUSSION

The present study revealed that GAA administration significantly inhibited the development of liver cancer in DEN mice. To the best of our knowledge, the present study evaluated the protective effect of GAA on DEN-induced hepatocellular carcinoma by suppressing inflammation and oxidative response for the first time.



**Figure 4:** Effect of GAA on Nrf-2/HO-1/NF- $\kappa$ B pathway-related proteins in DEN-induced mice. Expressions of I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , p65, p-p65, Nrf-2, HO-1 and GAPDH were quantified using western blot analysis. Analysis was confirmed with three different sets of experiments. GAPDH served as a loading control. The data were given as mean  $\pm$  SD (n = 3 - 4). \*\*\**p* < 0.001 Vs respective untreated control groups

As an inflammation-associated cancer, hepatocellular carcinoma is linked to angiogenesis, immune suppression and tumor progression. The DEN-induced murine model is a well-established model, and widely used in liver cancer study [15]. The pathological process in the DEN model is similar to the pattern in human liver cancer. These findings suggest that GAA alleviated DEN-induced liver cancer through the Nrf-2/HO-1/NF- $\kappa$ B signaling pathways.

The significant reduction in SOD level and significant increase in MDA level were observed in the DEN-induced mice compared with those in control group. As a product of lipid peroxidation, MDA is mutagenic and carcinogenic through reacting with DNA to induce live tissue damages. SOD plays an important role in eliminating harmful metabolism produced by human [16,17]. GAA treatment could significantly suppress the content of MDA and elevate the level of SOD, suggesting that GAA administration might play a key role in attenuating the DEN-induced hepatic carcinogenesis and tumorigenesis by inhibiting oxidative stress. Recent evidence has revealed that GAA exerts a crucial role in enhancing the anti-inflammatory and anti-oxidative properties in DEN-induced mice [18]. Of note, in the progress of liver cancer, there were overproduction of proinflammatory cytokines including TNF- $\alpha$ , IL-6 and IL-1 $\beta$  [19]. This research detected significant differences of these cytokines in the comparison between the DEN-induced mice and normal

mice. TNF- $\alpha$  is considered as a NF- $\kappa$ B activator, could promote the inflammatory cells translocating from cytosol into nucleus and accelerate cell proliferation in an inflammatory reaction [20]. The transcription factor, NF- $\kappa$ B, was combined with its inhibitor I $\kappa$ B $\alpha$  in cytosol. NF- $\kappa$ B was activated via the overproduction of TNF- $\alpha$  and the phosphorylation of I $\kappa$ B $\alpha$ , then dissociated from I $\kappa$ B $\alpha$  in cytosol [21]. Consequently, NF- $\kappa$ B trigger an increase in expression of certain pro-inflammatory cytokines, such as IL-6 and IL-1 $\beta$ . IL-1 $\beta$  is an important pro-inflammatory cytokine in the progression of liver tumorigenesis. IL-6 played a pivotal role in inhibiting the degradation of extracellular matrix [22]. The results showed that GAA treatment significantly exert inhibitory activities against liver tumor growth and inflammatory response by suppressing the release of cytokines.

The study investigated the underlying mechanism of GAA on the role of anti-inflammation and anti-oxidative, which might relate to Nrf-2/HO-1/NF- $\kappa$ B signaling pathway. Nrf-2 is a redox-sensitive transcription factor, which plays a crucial role in the process of inflammation. Nrf-2 is known to bind to anti-oxidant response elements (AREs) in promoter regions and then regulate the expression of rate-limiting enzyme HO-1 [23]. Under physiological conditions, Nrf-2 binds to Keap1 in the cytoplasm to maintain inactivated. Nrf-2 might be activated by the increased level of ROS. When oxidative stress occurs, the hepatic Nrf-2 dissociate from Keap1, then translocate from the cytoplasm to nucleus [24]. HO-1 exerts anti-inflammatory and anti-oxidative functions, the expression of HO-1 is regulated by Nrf-2 [25]. This study showed down-regulated expression of Nrf-2 and HO-1 in DEN-induced group. GAA administration significantly upregulated the expressions of Nrf2 and HO-1 in liver tumor, suggesting that GAA might exert anti-inflammatory and anti-oxidant via the Nrf-2/HO-1 signaling pathway.

## CONCLUSION

The findings of this study demonstrate that GAA ameliorates liver cancer by inhibiting oxidative stress and inflammation responses in DEN-induced mice liver cancer model via Nrf-2/HO-1/NF- $\kappa$ B signal pathway. Thus, it has potentials for the clinical management of liver cancer.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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