Therapeutic effects of the Egyptian horned viper LAAO against hepatocellular carcinoma induced in rats

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Background
The most common kind of liver cancer, hepatocellular carcinoma (HCC), is the fourth leading cause of cancer-related mortality worldwide and has poor prognosis. Strong hepatocarcinogen diethyl nitrosamine (DENA) is a well-known substance. It is well known that DENA damages DNA repair enzymes and is typically used to cause liver cancer in experimental animal models, such as rats. *Cerastes cerastes* L-amino acid oxidase (Cc-LAAO) has hepatoprotective, antioxidant, and anticancer effects.

Objective
To assess the effectiveness of L-amino acid oxidase (LAAO) as a hepatoprotective agent in comparison to paclitaxel (PAC) as a conventional anticancer medicine in the early identification of HCC using biomarkers [alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA)], various liver function tests, and oxidant and antioxidant tests.

Materials and methods
CCl4 (200 mg/kg b.wt.) was injected subcutaneously once a week for 3 weeks after a single IP dose of DENA (200 mg/kg b.wt.) to develop hepatocellular cancer in rats. Twenty-five adult, mature, healthy rats were used in this investigation; their average weight was 100±10 g, and they were divided into five groups, each with five rats. After the experiment, some hepatic tests, histology of the liver, a tumor biomarker, and some kidney functions were assessed for all groups.

Results and conclusion
ASAT, ALAT, ALP, total bilirubin, tumor markers AFP, CEA, and lipid peroxides malondialdehyde (MDA) significantly rose in serum after DENA administration in rats, whereas activating antioxidants like SOD, CAT, GPx, and GSH decreased. LAAO and paclitaxel significantly ameliorated biomarkers for liver damage, lipid peroxides (MDA), antioxidants such as (SOD), (CAT), (GSH), (GPx), tumor marker (AFP), and (CEA) compared with the HCC group. Histopathology showed vacuolar hepatocytes with dispersed hepatocyte necrosis and infiltration of mononuclear cells. When used with DENA, the LAAO administration reduced negative effects and produced positive effects. These findings demonstrate that LAAO prevents liver HCC caused by DEN by preventing lipid peroxidation, hepatic cell oxidative stress, and boosting the antioxidant system.

Keywords:
cerastes cerastes, diethylnitrosamine, hepatocellular carcinoma, l-amino acid oxidase, liver, paclitaxel

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Introduction

Hepatocellular carcinoma (HCC), the most prevalent form of liver cancer, is the fourth biggest cause of cancer-related death globally and has a dismal prognosis [1]. The World Health Organization

(WHO) estimates that there will be 10.3 million cancer deaths and 19.3 million new cases worldwide in 2020. The second most prevalent cause of cancer death (830180 fatalities, 8.3%) and the seventh most common cancer worldwide, respectively, is liver cancer (905677 cases, or 4.7% of the total). By 2040, liver cancer cases and fatalities are expected to increase by more than 55% [2]. Various pathological changes caused by the liver disease include fatty liver, increased ROS or oxidative stress, hepatocyte necrosis, hepatitis, steatosis, cholestasis, vascular lesions, granulomatous and veno-occlusive disease, inflammatory markers, and increased levels of hepatocellular carcinoma. Portal hypertension and liver failure continue to increase [3]. Combining surgery, radiation therapy, and chemotherapy is the current gold standard of cancer treatment [4]. Traditional approaches have limitations, though, including a lack of early detection screening tools and tumor-specific drug delivery systems. In addition, because most traditional anticancer medications are unable to distinguish between malignant and healthy cells, they can have hazardous systemic consequences. To solve this issue, new medications that are more efficient and selective are urgently needed. Bioactive peptides are being viewed more favorably as potential pharmacological targets for cancer treatment. There is growing evidence that peptides derived from natural animal sources have physiological properties like immunomodulatory, antibacterial, anticancer, and antioxidative effects [5–8]. The most effective cancer treatment now on the market, paclitaxel (PAC), has negative effects. Nail color changes, tingling in the hands or toes, loss of appetite, taste changes, thinning or brittle hair, and soreness in the joints of the arms or legs lasting 2–3 days are some of the most frequent adverse effects. More severe side effects can also occur, including unusual bleeding or bruising, pain, redness, or swelling at the injection site, hand–foot syndrome, change in regular bowel habits for longer than 2 days, fever, chills, cough, sore throat, difficulty swallowing, dizziness, shortness of breath, extreme exhaustion, skin rash, and facial flushing. Moreover, female infertility due to ovarian damage and chest pain can also happen. There may also be neuropathy [9]. Common names for *Cerastes cerastes* include Egyptian sand viper and desert horned viper. It is one of the snakes that people in the vast deserts of North Africa and the Middle East are most familiar with. It is a common, venomous snake in Africa that lives in Egypt's sandy deserts [10–12]. LAAO is one of the enzymes found in the venom of *C. cerastes*. When used against different cancer cell lines, LAAO demonstrates anticancer therapeutic efficacy [13]. Several studies on snake venoms demonstrate that SVLAAOs can promote cytotoxicity in a variety of cell lines, including EAT (Ehrlich ascites tumor), S180 (murine sarcoma 180 tumor), SKBR-3 (breast adenocarcinoma), Jurkat (human acute T-cell leukemia), B16F10 (murine melanoma), and PC12 (rat adrenal gland pheochromocytoma) [14]. It is noteworthy that the damage in normal cells is usually negligible when compared with the damage caused in tumor cells [15]. Since then, a variety of studies have characterized the apoptotic effect of LAAOs in various cell lines (in vitro), indicating that this enzyme class is connected to the cytotoxic activity of venoms, although no study on animal model cancer has been done (in vivo). Therefore, i the current study was evaluated for its anticancerous activity against the proliferation of in vivo model of liver cancer compared with a conventional anticancer drug (PAC) to explore the apoptotic mechanism and provide new anticancer agents in the future after further studies.

Materials and methods

Materials

Egyptian Cerastes cerastes (Cc) venom was purchased from the Medical Research Center, The Holding Corporation for Biological Products and Vaccines and Pharmaceuticals (VACSERA), Egypt's Laboratory Animal Division. The venom was combined, centrifuged at a low speed of 10,000 rpm for 10 min, and the supernatant was collected and kept at −80 °C until needed. Using size-exclusion and anion-exchange chromatography, CC-LAAO from Cerastes cerastes venom was purified [16]. Diethylnitrosamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Taxol (PAC) was purchased from (Hikma Co, Egypt). The high analytical grade chemicals and reagents used in the rest of the study were acquired from regular commercial providers.

Experimental animals and *in vivo* study

Adult male albino rats (100±10 g) were carried out in accordance with the institutional policies on ethical behavior for the use and treatment of research animals. The Institutional Animal Ethics Committee (URAF) approved the experiment's protocol, which was given the approval code URAF/3/23. For the current in vivo study, 25 healthy albino rats were used. All of the experimental rats were exposed to the standard conditions (23±2oC, humidity 55±5, and a 12 h cycle of light and darkness throughout the experimental period). Throughout the trial, all of the

rats were housed in polypropylene cages with free access to water and a normal meal.

Induction of hepatocellular carcinoma

Rats received a single intraperitoneal injection of diethyl nitrosamine (DENA) (200 mg/kg body weight), followed by weekly for three weeks subcutaneous injections of (CCl_4) (200 mg/kg body weight), according to Sundaresan and Subramanian [17].

Experimental design

Rats with and without HCC were placed into five groups of five rats each after HCC induction as follows:

Healthy animals made up group one and acted as the control. Healthy mice in group 2 received an intraperitoneal injection of LAAO diluted in PBS (2.5 μg/ml/48 hours) for 1 week [18]. In group 3, untreated HCC animals were used. HCC animals in group (4) received intraperitoneal injections of LAAO diluted in PBS (2.5 g/ml/48 hours) for 1 week. Animals in group 5 (HCC) received an intraperitoneal injection of Taxol (PAC) at a dose of 1/10 LD50 (3.13 μg/kg/48 hr.) for 1week [19].

Blood and tissue sampling

Following anesthesia, blood samples were taken from the rats on completion of the treatment period. The blood samples were then centrifuged at 3000 rpm for 15 min to separate the sera, which were then divided into aliquots and stored at −80 °C till the completion of biochemical tests. Following blood collection, the animals were quickly decapitated and slaughtered. The livers of each animal were then removed, separated, and preserved at −80 °C for the identification of oxidative stress indicators.

Hepatic tissue homogenization

An ultrasonic homogenizer (Sonics VCX-750 Vibra-Cell, USA) was used to homogenize a sample of liver tissue in ice-cold phosphate buffer (50 mM, pH 7.4) to produce 10% homogenate (w/v). The homogenate was then centrifuged in a cool centrifuge (ROTANTA 460 R, Germany) at 3000 rpm for 20 min to remove nuclear and mitochondrial fractions. The supernatant was collected, divided into aliquots, and kept at −80 °C until the oxidative stress markers were determined [20].

Biochemical determinations

Liver function

The Breuer technique was used to measure the blood levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) using a spectrophotometer (Cary 100 UV-Vis, USA) [21]. Total bilirubin (TBIL) and alkaline phosphatase (ALP) (the German Society for Clinical Chemistry 1972) were measured using reagent kits purchased from the Egyptian Corporation for Biotechnology Spectrum, Egypt [22].

Kidney function

Using reagent kits purchased from the Egyptian Corporation for Biotechnology Spectrum, Egypt, the serum's urea and creatinine concentrations were measured following the procedures outlined by Tiffny and colleagues [23] and Bowers and Wong [24].

Hepatic oxidative stress markers

Glutathione peroxidase (GPx) activity was measured using the Paglia technique [25] using kits purchased from Bio-diagnostic Co., Dokki, Giza, Egypt. Reduced glutathione (GSH) level was assessed using the technique described by Beutler and colleagues [26], and superoxide dismutase (SOD) activity was assessed using the technique reported by Nishikimi and colleagues [27]. Malondialdehyde (MDA) level, an indirect indicator of lipid peroxidation, was measured chemically following Ruiz-Larrea and colleagues [28].

Serum tumor markers

Alpha-fetoprotein (AFP) (LifeSpan BioSciences, Inc. Seattle, WA, USA) and carcinoembryonic antigen (CEA) in which CEA and sample concentration is determined on Cobas e411 according to Guder and colleagues [29].

Histological examination

The placed organs were washed in formalin and then dried with alcohol. For microscopic examination, paraffin-embedded sections of 5–6 μm thickness were cut and stained with hematoxylin (H) and eosin (E).

Statistical analysis

Using the Statistical Package for the Social Sciences (SPSS/PC) computer application (version 26), all numerical data were statistically evaluated using one-way analysis of variance (ANOVA), followed by the post hoc (LSD) test at $P \leq 0.05$.

Result

Liver function tests

Our results have shown that when compared with their comparable values in the control group, the DENA (HCC) groups recorded a significant rise in ALAT, ASAT, ALP, and total bilirubin, whereas the LAAO-

administered groups showed minor changes. Besides that, as compared with the group treated with DENA alone, the groups treated with LAAO or PAC with DENA demonstrated a significant decrease in ALAT, ASAT, ALP, and total bilirubin enzyme activity values (Fig. 1).

Kidney function tests

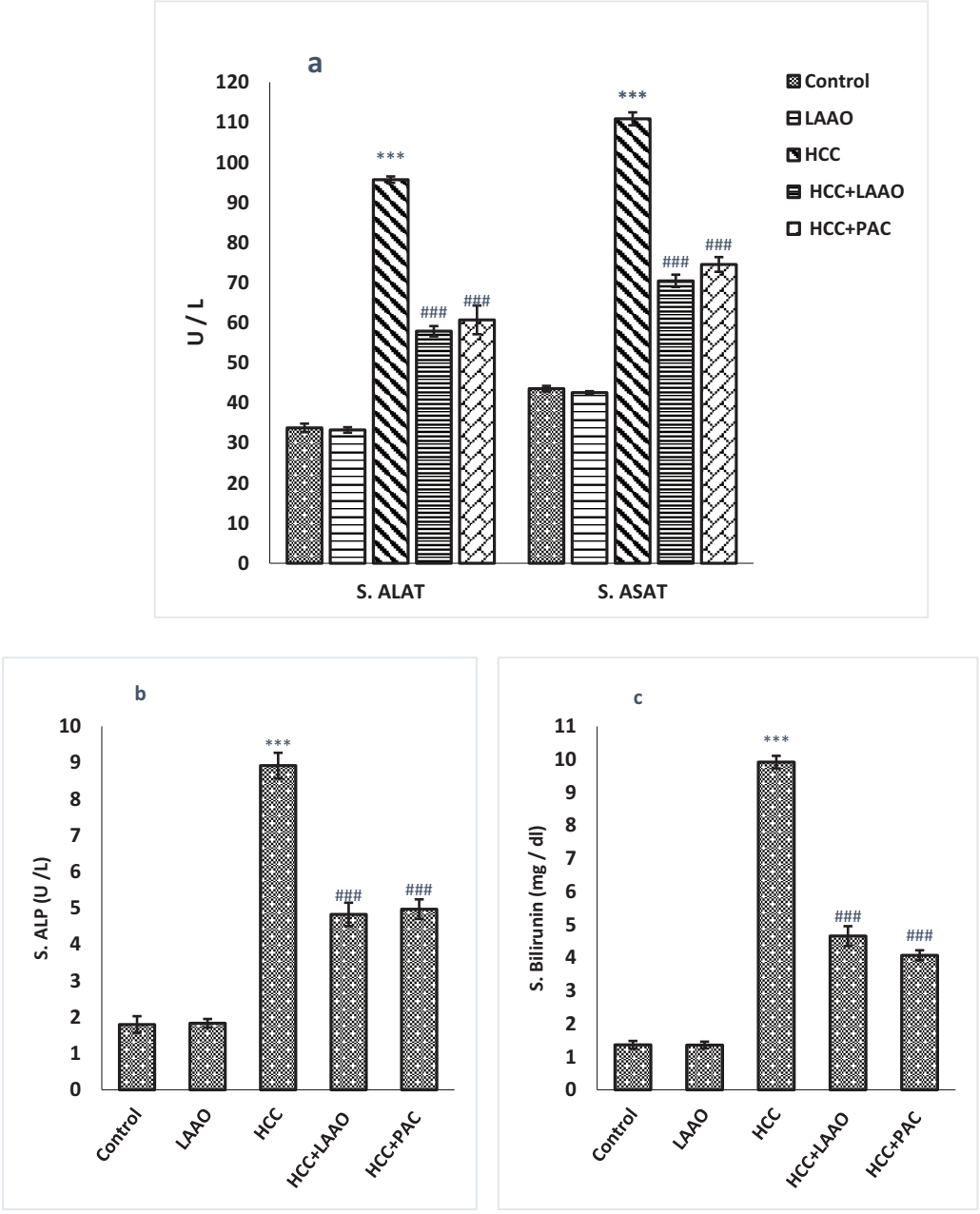
Figure 2 shows a substantial rise in blood levels of urea and creatinine in the HCC group compared with the control group. When compared with the HCC animal group, the LAAO- and PAC-treated HCC animal group showed a substantial decrease in blood urea and creatinine levels.

Oxidative and antioxidant markers

The results of the current study showed that when compared with the control group, the induced HCC animals' group showed a significant increase in the hepatic MDA level along with a significant decrease in the hepatic CAT, SOD, GPx, and GSH, while the LAAO-administered group showed insignificant changes.

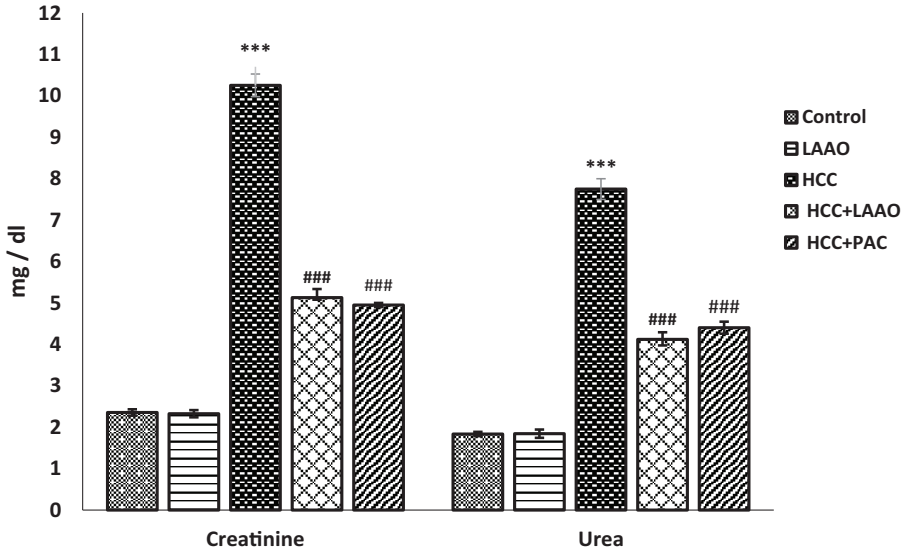
Compared favorably with the untreated HCC group, LAAO and PAC therapy of HCC mice produced a significant decline in both MDA levels and a marked increase in CAT, SOD, GPx, and GSH values. (Fig. 3).

Figure 1



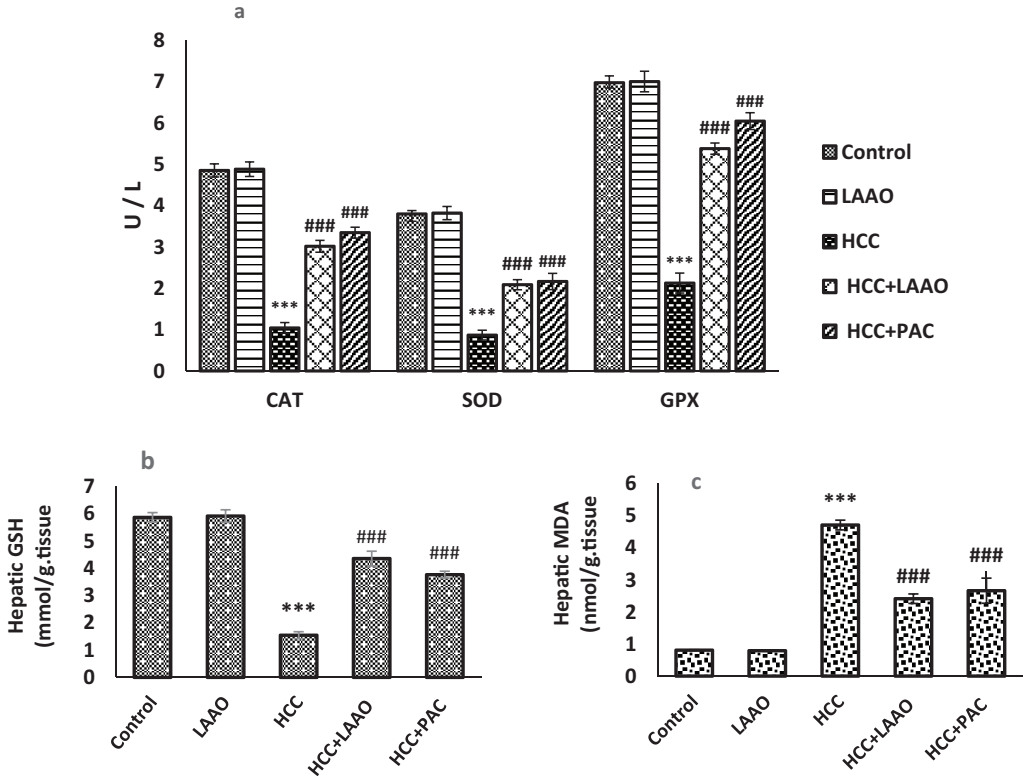
Compared with the control group, the treated HCC-animal groups' liver function was: (a): serum ALAT and ASAT activities, (b) serum ALP activity, and (C) serum bilirubin level. * Differs from the control group, while # differs significantly from the HCC group.

Figure 2



In comparison to the control group, the treated HCC-animal groups' serum creatinine and urea levels show that: *** is significantly different ($P \leq 0.001$) from the control group and ### is significantly different ($P \leq 0.001$) from HCC group.

Figure 3

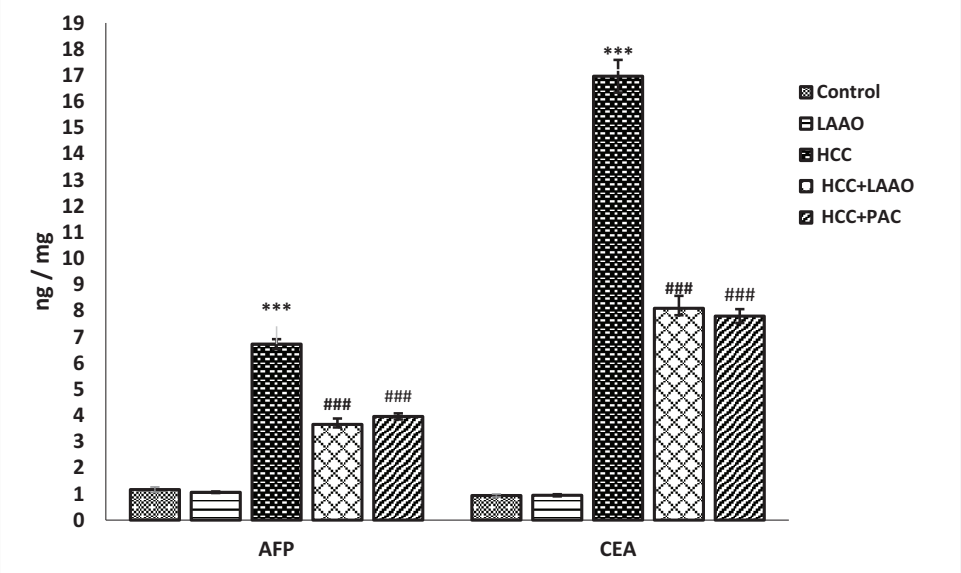


Serum hepatic level of antioxidants (CAT, SOD, and GPx activities and GSH level) and the level of (MDA) markers of the treated HCC-animals' groups in comparison with the control one. (a): CAT, SOD, and GPx activities in hepatic tissues; (b) GSH level in hepatic tissues; (c) MDA level in hepatic tissues. ### is significantly different ($P \leq 0.001$) from the HCC group and *** is significantly different ($P \leq 0.001$) from the control group.

Hepatocellular carcinoma tumor biomarkers

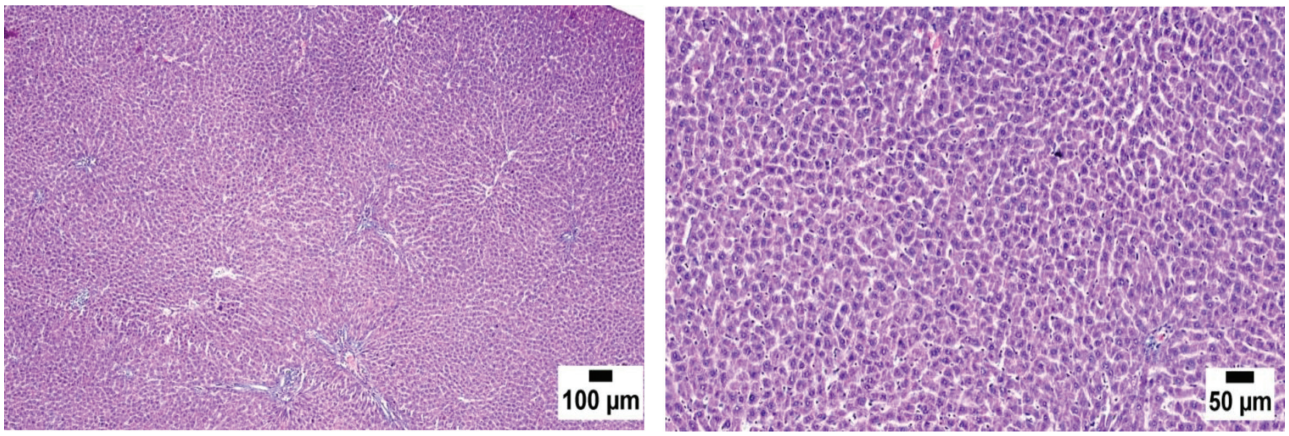
The levels of CEA and AFP at the end of the experiment were significantly higher in the DENA-administered group when compared with their respective values in the control group, according to the results of the administration of LAAO and DENA, respectively. In addition to CEA and AFP levels, treated LAAO (HCC + LAAO group) and

Figure 4



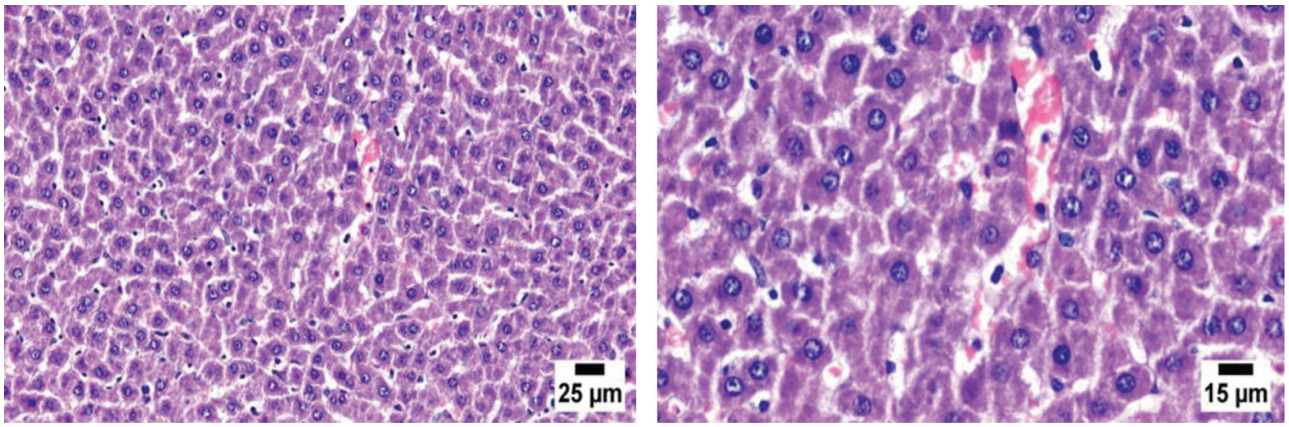
AFP alpha-fetoprotein and CEA carcinoembryonic antigen tumor markers in adult male albino rats treated under various settings. * is significantly different from the control group and # is significantly different from the HCC group.

Figure 5



Liver tissue from the control group showing a normal histological structure (H and E). 400x magnification.

Figure 6



Liver tissue from the LAAO-administrated group showing normal histological structure (H and E). 400 x magnification.

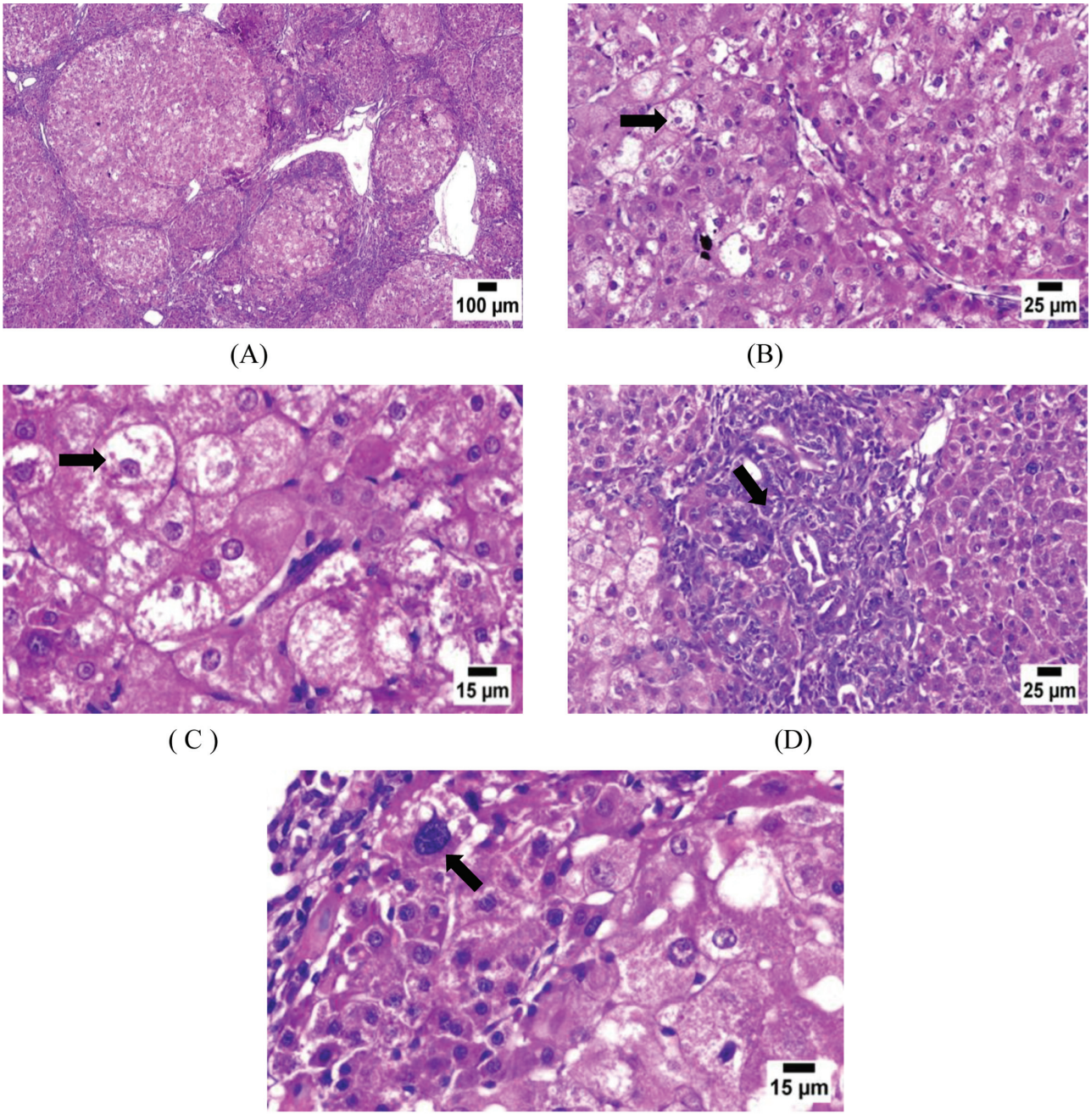
PAC (HCC+PAC group) showed a substantial decline when compared with the group treated with DEN (HCC) only. (Fig. 4).

Histological examination

The livers from the control group and the LAAO-treated group underwent microscopic analysis, which revealed normal hepatocytes that were arranged in a typical lobular architecture with central veins and radiating hepatic cords. The hepatic artery, portal vein, and bile duct branches in the portal triads had normal histology. (Figs. 5 and 6).

Liver of the HCC group (Fig. 7) showed marked histopathological changes. The hepatic parenchyma was replaced by a dense cellular neoplasm that appeared unencapsulated, multilobulated, and composed of polygonal cells. Neoplastic cells showed

Figure 7



Liver tissue from the HCC group. (A) multilobulated neoplastic cells separated by fibrous connective tissue with hyperplasic biliary epithelium and inflammatory cell infiltration (H and E); (B) vacuolar degeneration of the hepatic parenchyma (arrow) (H and E); (C) excessive vacuolation of the hepatocytes (arrow) (H and E); (D) hyperplasia of the biliary epithelium (arrow) (H and E); (E) marked karyomegaly and hyperchromasia (arrow). 400 x magnification.

defined cell boundaries, an abundance of eosinophilic granular to vacuolated cytoplasm, spherical nuclei with one to three distinct magenta nucleoli, and finely stippled chromatin. There were moderate amounts of cytomegalic, multinucleated neoplastic cells as well as moderate levels of anisocytosis, anisokaryosis, and anisocytosis. The portal areas revealed variable numbers of inflammatory cells infiltration admixed with hyperplastic biliary epithelial cells that form small irregular bile ducts with cystic dilation in some instances.

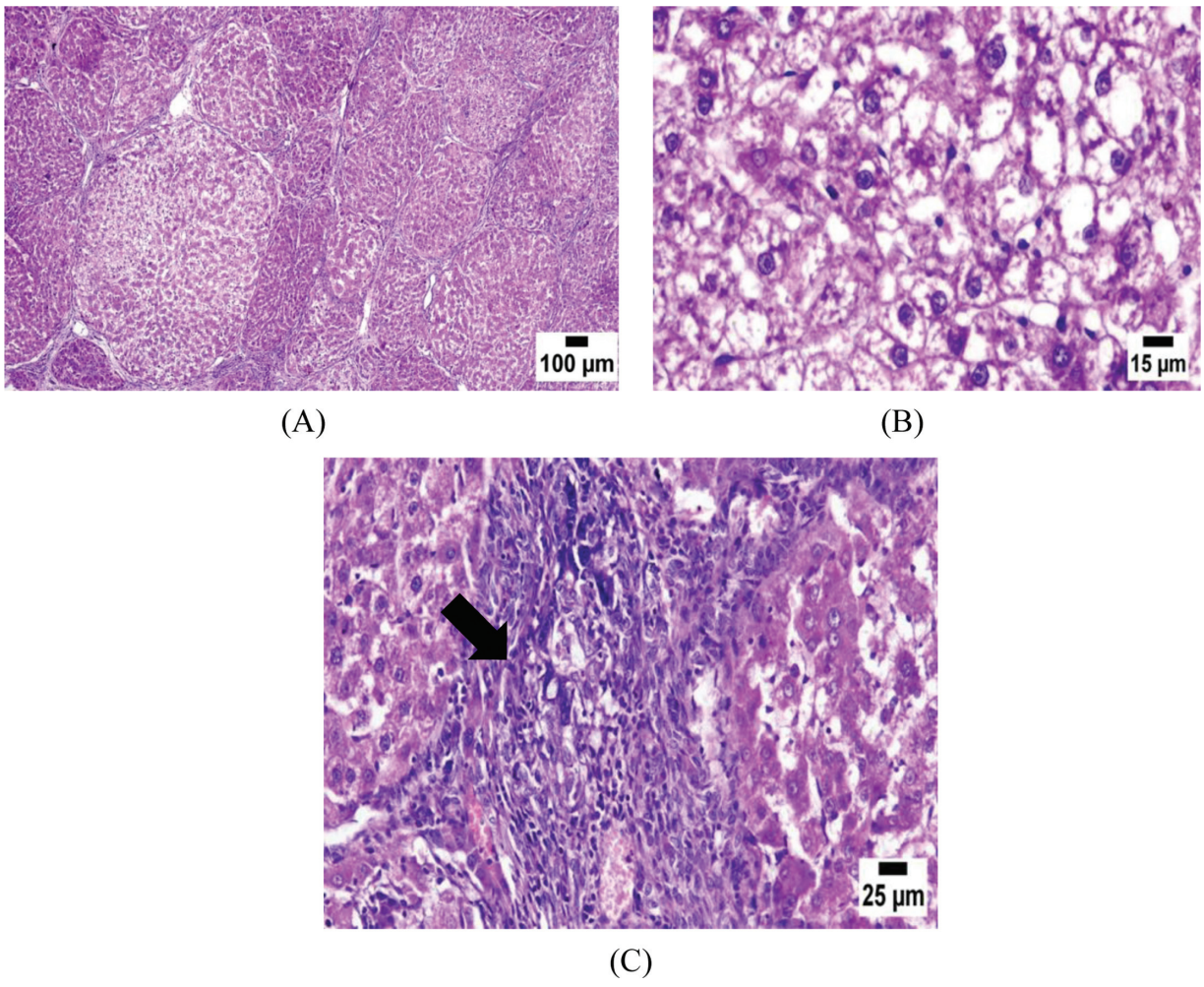
Moderate improvement was detected in the LAAO group (Fig. 8), which characterized mild to moderate vacuolation of hepatocytes with fewer altered foci. Meanwhile, some examined sections showed severe hyperplasia of the biliary epithelium with fibroplasia and newly formed bile ductules.

Meanwhile, a less protective effect was observed in the PAC group (Fig. 9). Numerous regenerative nodules were accompanied by vacuolation of the hepatic parenchyma. Abundant portal fibrosis with bile duct hyperplasia and several newly formed bile ductules lined with the cuboidal epithelium. Cystically, a dilated bile duct was less frequently observed.

Discussion

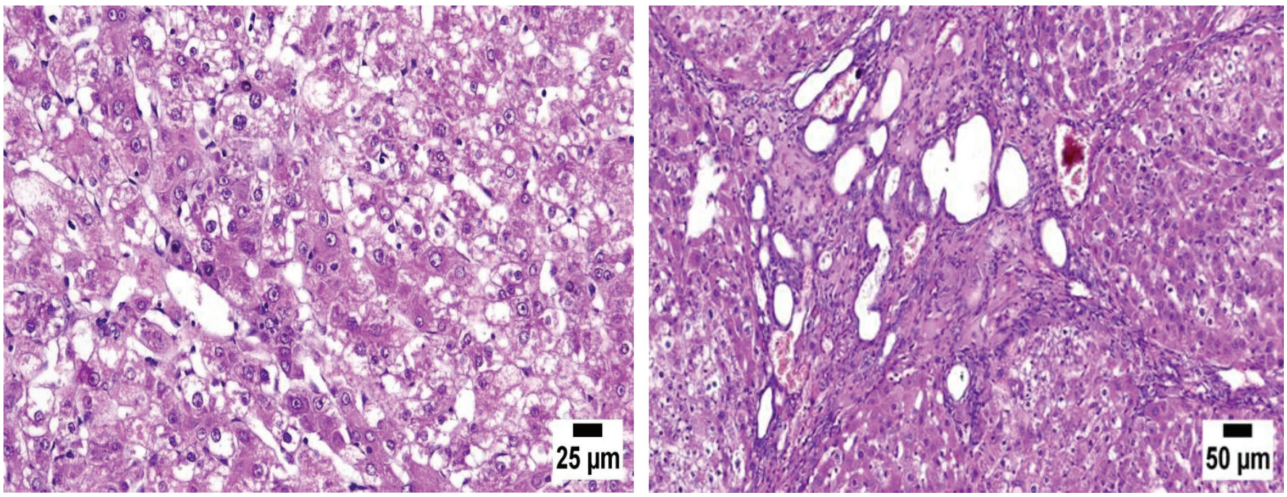
HCC is a prominent cause of death and affects a huge portion of the global population. As HCC is the most common type of liver cancer, it is extensively researched to determine its potential origins and mechanisms [30]. Hepatitis, heavy alcohol consumption, DENA, hormone exposure, and hemochromatosis were found to be the main causes of hepatocellular cancer. Being a hepatocarcinogen and a substrate of the hepatic cytochrome CYP2E1 that results in the production of intracellular reactive oxygen species, DEN is a substance that causes cancer and causes the formation of carcinogenic activity in organs (ROS) [31].

Figure 8



Liver tissue from the LAAO group. (A) limited dysplasia of the hepatic lobules that are separated by fewer fibroplasia (H and E), (B) limited vacuolation of the hepatic parenchyma (H and E); (C) oval cell hyperplasia with moderate portal fibrosis and inflammatory cells infiltration (arrow) (H and E). 400 x magnification.

Figure 9



Liver tissue from the paclitaxel group. (A) moderate vacuolation of the hepatic tissue (H and E); (B) abundant portal fibrosis with cystically dilated bile ducts (H and E). 400x magnification.

In all our results, we found that the LAAO did not affect the animals, and its results were insignificant when compared with the control group. This means that it has no side effects on experimental animals when they are injected with 2.5 µg/ml in agreement with previous results [18], unlike PAC, which has many serious side effects [9].

When compared with the same results in the control group, the liver function tests of the DENA (HCC) group in the current study revealed a significant increase in the liver function parameters ASAT, ALAT, ALP, and T.Bil. Similar findings were published by Tiffney and colleagues [23], Ahmed and colleagues and Fathy and colleagues [32–34], showing that the liver damage brought on by DENA typically reflected alterations in the serum enzyme activity and the instability of the liver metabolism; and an increase in the level of ASAT, ALAT, ALP, and T.Bil in the serum indicated the extent of hepatocellular damage and liver injury. In addition, the elevated liver enzymes in the serum are a result of lipid peroxidation of the liver cell membranes, which was triggered by free radicals. This process resulted in protein carbonylation and abnormal structural changes of the biomembranes, as well as a loss of liver integrity and reduced metabolic activity [34,35]

Our results showed that ASAT, ALAT, ALP, and T. Bil were significantly lower in rats treated with LAAO and PAC than in the DENA-treated rats, indicating that LAAO may have a protective effect against liver damage. The reduction in cellular damage may be the cause of the treated groups' lower serum levels of ALAT, ASAT, ALP, and T. BIL [36,37].

This has been strengthened by the findings of Costa and colleagues [38], who found that LAAO treatment has potent chemopreventative activity against a wide range of tumors, has exciting potential in the prevention and treatment of hepatocarcinogenesis, and may be a promising candidate to inhibit inflammation and apoptosis signaling.

The current investigation found that as compared with the comparable values in the control group, the evaluated kidney function parameters significantly changed when diethylnitrosamine was supplemented in agreement with a previous study [39,40]. A significant sign of renal failure is an increase in serum creatinine levels, and this range indicates glomerular function [41,42]. LAAO and PAC significantly decreased the elevated levels of serum urea and creatinine when compared with the DEN control group suggesting the protective effects of LAAO and PAC.

Hepatic oxidative voltage (MDA levels) significantly increased in HCC animals (DENA) who had not received any treatment, whereas the hepatic antioxidant batteries significantly decreased (SOD, GSH, GPx, and CAT activities) in agreement with Zhang [43].

The antioxidant enzymes act as antioxidant regulators and oversee removing ROS and other free radicals from tissues and cells [44].

Hepatic MDA levels significantly decreased after LAAO and PAC treatment for HCC mice, and hepatic SOD, GSH, GPx, and CAT activities significantly increased. The increase in SOD activity observed is thought to be a sign of increased H_2O_2 generation in a liver cancer cell line (HepG2) treated with LAAO [45]. According to reports, numerous cell lines' overexpression of SOD increased the flow and level of H_2O_2 generation [46,47]. It is well known in the literature that LAAO causes apoptosis as a byproduct of the release of intracellular H_2O_2 to cause its cytotoxic effects. [48].

By interrupting ROS attack, preventing ROS synthesis, promoting ROS-caused damage repair, and supplying cofactors for other antioxidants to work properly, antioxidants can shield membranes against ROS toxicity [49]. The availability of these antioxidants is related to the development of fatal diseases like cancer [50]. Natural antioxidants can reduce the related intracellular oxidative stress by blocking the generation of ROS [51]. The antioxidant system's first line of defense against oxidative damage caused by superoxide radicals is SOD [52]. Superoxide dismutase catalyzes the conversion of superoxide radicals to hydrogen peroxide and water [53].

In the current study, as compared with the similar values in the control groups, the HCC group showed substantial changes in all examined tumor markers. When compared with the corresponding values in the control group, the percentages of CEA and alpha-fetoprotein (AFP) in the DEN-induced group demonstrated a significant rise. Equivalent results were reported by Zhang *et al*. [43] and Gokuladhas and colleagues [54].Moreover, when compared with the HCC group, LAAO and PAC showed a significantly lower level of CEA and AFP. This demonstrates that LAAO and PAC were able to stop the growth of the tumor because CEA levels are unique to liver metastasis. Two significant biomarkers for early-stage tumor development and metastasis of cancer in the organ are CEA and AFP. The most popular tumor biomarker for the early identification of HCC was AFP [43,55,56]. It is possible that the necrosis of hepatocytes, which control AFP synthesis at the cellular level, is the cause of the elevation of the AFP gene expression in DEN-administered rats [34,57].

To further substantiate the occurrence of apoptotic morphological changes at the cellular level, histological abnormalities of the rat liver were carried out. The nucleus and the cytoplasm of the control animals were normal. The DENA-induced animals displayed many irregularly shaped nuclei adjacent to one another and uneven cytoplasm. The results are consistent with earlier reports, which may be caused by an excessive amount of free radicals being produced after DEN administration [39,58]. Animals treated with LAAO alone displayed normal architecture, demonstrating that the substance did not alter the intracellular morphology of liver cells and, as a result, was harmless at the prescribed dosage in agreement with a previous result [18]. Moderate improvement was detected in HCC treated by the LAAO group which characterized mild to moderate vacuolation of hepatocytes with fewer altered foci, which reveal that the LAAO may induce apoptosis. Meanwhile, a less protective effect was observed in the PAC group. Numerous regenerative nodules accompanied vacuolation of the hepatic parenchyma.

Conclusion

LAAO isolated from *Cerastes cerastes* demonstrated anticancer activity against HCC in the rat liver compared with PAC, which is used as a conventional therapy for HCC with a minimal effect on normal cells encouraging its application in cancer therapy in the future. Regarding DEN-induced hepatocellular carcinoma, LAAO significantly reduced oxidative stress, avoided hepatic damage supported by histological changes, and advanced tumor occurrences. Thus, continued research into LAAO as a potential therapeutic option for the treatment of liver cancer is advised. This is the first report demonstrating the effect of Cc-LAAO on rat model cancer, which may lead to the development of cancer treatment.

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Conflicts of interest

The authors have no relevant financial or nonfinancial interests to disclose.

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