



## A Search for Anti-Carcinogenic and Cytotoxic Effects of Persian Gulf Sea Snake (*Enhydrina schistosa*) Venom on Hepatocellular Carcinoma Using Mitochondria Isolated from Liver

Enayatollah Seydi<sup>a,b</sup>, Pardis Tajarri<sup>c</sup>, Parvaneh Naserzadeh<sup>c</sup>, Jalal Pourahmad<sup>c\*</sup>

<sup>a</sup>Department of Occupational Health and Safety Engineering, School of Health, Alborz University of Medical Sciences, Karaj, Iran, <sup>b</sup>Research Center for Health, Safety and Environment, Alborz University of Medical Sciences, Karaj, Iran, <sup>c</sup>Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

### Abstract

Common techniques for the treatment of Hepatocellular carcinoma (HCC) have not been successful, and thus the design and discovery of new compounds with better anti-cancer function are needed. Snake venom is among the most important compounds used by researchers to the treatment of various cancers. This study was designed to evaluate the toxicity effect of Persian Gulf snake venom (*Enhydrina schistosa*) on hepatocytes and mitochondria isolated from HCC rats model. HCC has been induced in rats with diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF). Then rat hepatocytes were isolated with collagen perfusion technique. The results showed that *E. schistosa* (5, 10, 20 and 40 µg/ml) increases the level of reactive oxygen species (ROS) generation, collapse in mitochondrial membrane potential (MMP), swelling in mitochondria, and cytochrome c release only in hepatocytes and mitochondria isolated from the HCC group. These results proposed that *E. schistosa* could be considered as a promising complementary therapeutic agent for the treatment of HCC.

**Keywords:** *Enhydrina schistosa*; Apoptosis; Hepatocellular carcinoma; Hepatocytes; Mitochondria, Reactive Oxygen Species.

### 1. Introduction

Cancer is one of the diseases that is considered one of the most important public concerns and problems. Also, statistics show that cancer causes a high mortality rate in people around the world [1, 2]. Hepatocellular

carcinoma (HCC) is one of the most important forms of liver cancer that occurs in adults. Unfortunately, the HCC prognosis is very poor and the common techniques and agents used to treatment of HCC have not been successful [3, 4]. Therefore, the use of new compounds for

Corresponding Authors: Jalal Pourahmad, Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences Tehran, Iran

Tel: +98-2122558786

E-Mail: j.pourahmadjaktaji@utoronto.ca

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the treatment of HCC is very important. A new and ideal anti-cancer compound should have selective toxicity on cancer cells and has no effect on healthy cells [5]. In recent years, compounds of natural origin have been considered for the treatment of cancer, and one of these compounds is snake venom. Snake venom contains a number of different compounds, and each of them has unique pharmacological properties [1, 6, 7, 8, 9]. The anti-cancer effects of snake venom were investigated on various cell line including colon, breast, and lung [10].

Studies have shown that snake venom can kill cancer cells through reactive oxygen species (ROS) generation. Also, it was shown that ROS can cause cell death through changes in mitochondrial membrane potential (MMP) and cytochrome release [11-13]. It has also been shown that excessive generation of ROS leads to inhibition of tumor growth, and subsequent induction of apoptosis. In addition, the collapse of the MMP leads to induction of apoptosis through the mitochondria-dependent pathway [12]. Mitochondrial is one of the most vital organelles that is the source of ROS generation and induction of apoptosis. Furthermore, compounds that can directly

target mitochondria are considered by researchers [14, 15]. The results of previous studies indicate that in the HCC the level of anti-apoptotic proteins expression is higher than that of pro-apoptotic proteins. In fact, in the HCC there is no equilibrium between the levels of anti-apoptotic and pro-apoptotic proteins and the apoptotic process is suppressed [16, 17].

Thus, the discovery of new compounds that induce ROS mediated apoptosis in cancer cells is essential for the treatment of HCC. The cytotoxicity mechanisms of Persian Gulf sea snake (*Enhydrina schistosa*) venom on hepatocytes and mitochondria isolated from the HCC rat model were not completely reported till now. This study aimed to investigate the selective apoptotic effect and oxidative stress caused by crude venom of the *Enhydrina schistosa* (*E. schistosa*) on hepatocytes and mitochondria obtained from the liver of HCC rats and the detailed mechanisms.

## 2. Materials and Methods

### 2.1. Crude Venom

Crude venom of the *E. schistosa* was supplied from the Hara (Qeshm, Iran). The Hara forest is located between the Qeshm Island and the southern coast of Iran in the north-eastern part of the Persian Gulf. Venom is aspirated from each fang using a pipette tip. Snakes are released after milking. Pooled venom was stored in liquid nitrogen, lyophilized, and kept at  $-20^{\circ}\text{C}$  until analysis.

## 2.2. Animals

The male rats (Sprague-Dawley) were purchased from the Institute Pasteur (Tehran, Iran). Rats were kept in a temperature-controlled environment on a 12:12 h light/dark cycle. All investigations were performed according to the guidelines of ethical standards and Institutional Animal Care and Use Committee (IACUC) of Shahid Beheshti University of Medical Sciences in Tehran, Iran.

## 2.3. HCC Induction Protocol

HCC was induced by intraperitoneal injection of DEN (200 mg/kg body wt, single dose) and oral intake of 2-AAF (0.02%, w/w) for two weeks. At the beginning of the study, DEN was injected into rats. After two weeks, the rats received 2-AAF for two weeks from food. The time required for the induction of HCC was 15 weeks. All tests were done after 15 weeks.

## 2.4. Mitochondria Isolation

At the end of the week 15, hepatocytes from rat liver were isolated using collagenase liver perfusion technique. The viability of hepatocytes was evaluated using the trypan blue exclusion assay. To isolate mitochondria, hepatocytes ( $30 \times 10^6$  cells) were centrifuged at 300g for 3 minutes and 760g for 5 minutes. In the following, the supernatant was kept and the pellet was centrifuged at 760 g for 5 minutes. Finally, the supernatants from the previous two steps were combined, and centrifuged at 8000 g for 20 minutes. To conduct the desired tests, the final

mitochondrial pellets were suspended in corresponding buffer [14, 15].

## 2.5. Succinate Dehydrogenase (SDH) Activity Assay

MTT probe was used to evaluate the effects of crude venom of the *E. schistosa* (0, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) on SDH activity. At first, mitochondria isolated from both groups (normal and cancer) were exposed to all applied concentrations of crude venom of the *E. schistosa*. Then, MTT probe at concentration of 0.4% was added to the medium and incubated at 37 °C for 30 minutes. Finally, DMSO is been used to dissolve formazan crystals, and the absorbance at 570 nm was measured using an ELISA reader (Tecan, Rainbow Thermo, Austria) [14, 15].

## 2.6. ROS Level Assay

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe was used to evaluate the effects of crude venom of the *E. schistosa* (0, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) for 5, 15, 30, 45 and 60 minutes on ROS level assay. Briefly, mitochondria isolated from both groups (normal and cancer) were exposed to all applied concentrations of crude venom of the *E. schistosa*. Then, DCFH-DA (10  $\mu\text{M}$ ) probe was added to the medium and incubated at 37 °C for 5, 15, 30, 45 and 60 minutes. Finally, the fluorescence intensity at  $\text{EX}\lambda=488$  nm and  $\text{EM}\lambda=527$  nm was measured with a fluorescence spectrophotometer (Shimadzu RF5000U) [14, 15].

### 2.7. MMP Assay

Rhodamine 123 (Rh 123) probe was used to evaluate the effects of crude venom of the *E. schistosa* (0, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) for 5, 15, 30, 45 and 60 minutes on MMP collapse assay. Briefly, mitochondria isolated from both groups (normal and cancer) were exposed to all applied concentrations of crude venom of the *E. schistosa*. Then, Rh 123 (10  $\mu\text{M}$ ) probe was added to the medium and incubated at 37 °C for 5, 15, 30, 45 and 60 minutes. Finally, the fluorescence intensity at  $\text{EX}\lambda=490\text{ nm}$  and  $\text{EM}\lambda=535\text{ nm}$  was measured with a fluorescence spectrophotometer (Shimadzu RF5000U) [14, 15].

### 2.8. Mitochondrial Swelling Assay

Briefly, isolated mitochondria from both groups were suspended in swelling assay buffer. In the next step, mitochondrial suspension was incubated with 5, 10, 20 and 40  $\mu\text{g/ml}$  of crude venom of the *E. schistosa*. Eventually, absorbance was measured at 540 nm was measured using an ELISA reader (Tecan, Rainbow Thermo, Austria).

### 2.9. Release of cytochrome c

Briefly, the Quantikine Rat/Mouse Cytochrome c Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) was used for determination of cytochrome c release.

### 2.10. Statistical Analysis

The data were shown as the means  $\pm$  standard deviation (SD). The one and two - way ANOVA analysis (GraphPad Prism software, version 5) were used for the

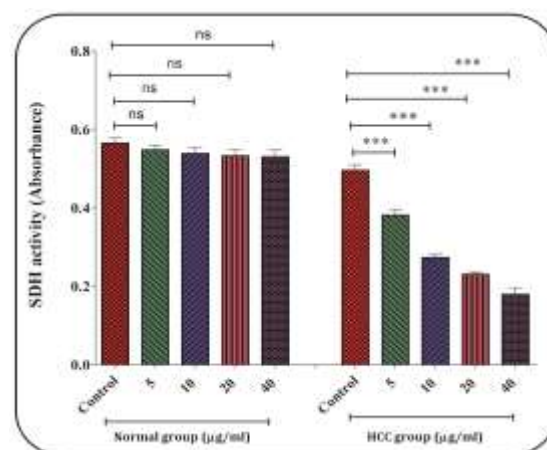
determination of differences between the mean values.  $P < 0.05$  was considered to display a statistically significant difference.

## 3. Results and Discussion

### 3.1. Results

#### 3.1.1. *E. Schistosa* Decreased SDH Activity

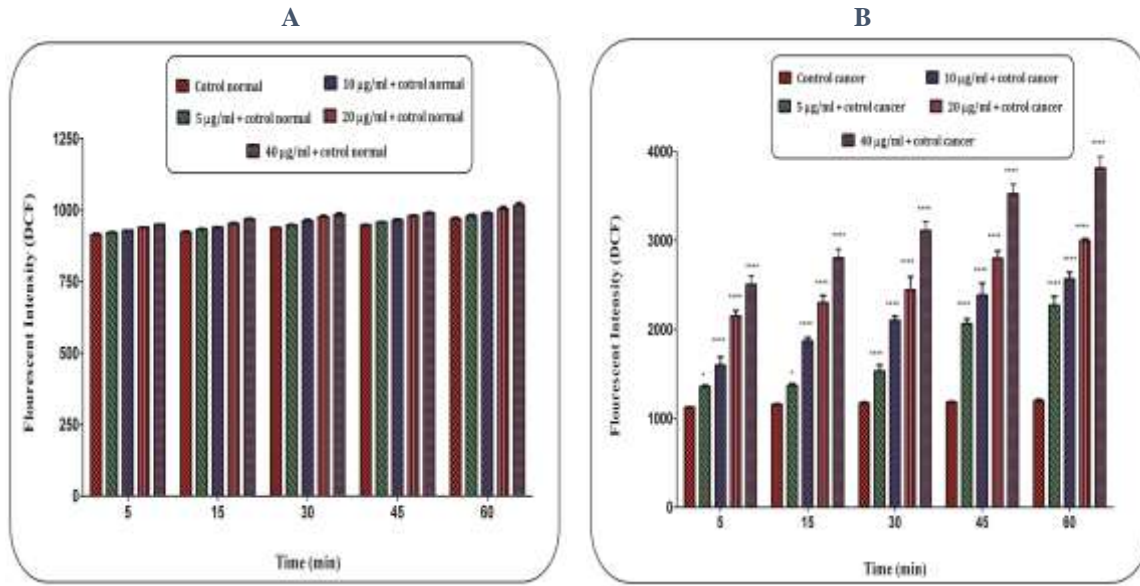
As is shown in figure 1, the crude venom of *E. schistosa* at all concentrations (0, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) has been able to significantly decrease the mitochondrial activity, while this change has not been reported in the normal group (Figure 1).



**Figure 1.** SDH activity assay. The effect of *E. schistosa* (0, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) venom on SDH activity in the liver mitochondria isolated from the normal (a) and cancer (b) groups. Data are showed as mean  $\pm$  SD (n=3). \*\*\*  $P < 0.001$  shows a significant difference in comparison with the corresponding control.

#### 3.1.2. *E. Schistosa* Increased ROS Level

In cancer group, ROS level significantly increase compared with the corresponding control after 5, 15, 30, 45 and 60 minutes of incubation with *E. schistosa* at concentration of 5, 10, 20 and 40  $\mu\text{g/ml}$  (Figure 2B). In mitochondria isolated from the normal group, a change in the level of ROS has not been reported (Figure 2A).

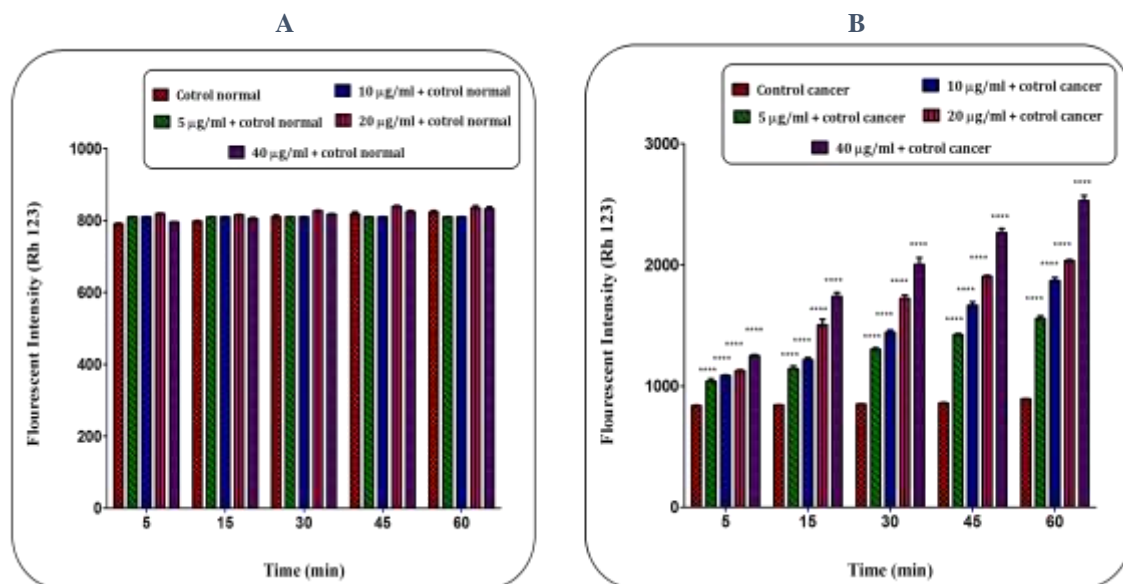


**Figure 2.** ROS level assay. The effect of *E.schistosa* (5, 10, 20 and 40  $\mu\text{g/ml}$ ) venom on ROS in the liver mitochondria isolated from the normal (a) and cancer (b) groups. Data are showed as mean  $\pm$  SD (n=3). \* and \*\*\*\* show a significant difference in comparison with the corresponding control ( $P<0.05$  and  $P<0.0001$ , respectively).

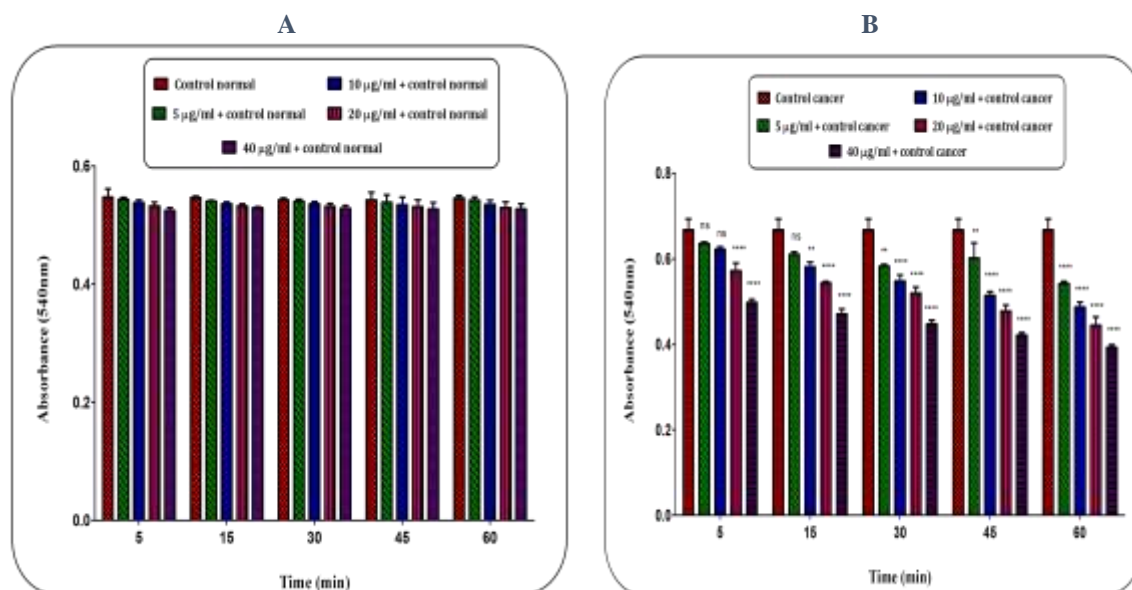
### 3.1.3. *E.Schistosa* Induced MMP Collapse

The collapse at the mitochondrial membrane potential (MMP) is observed only in the mitochondria isolated from the cancerous group (Figure 3B). The results of

Figure 3A show that *E.schistosa* at different concentrations (5, 10, 20 and 40  $\mu\text{g/ml}$ ) not affect the MMP in the normal group (Figure 3A).



**Figure 3.** Mitochondrial membrane potential (MMP) assay. The effect of *E.schistosa* (5, 10, 20 and 40  $\mu\text{g/ml}$ ) venom on MMP collapse in the liver mitochondria isolated from the normal (a) and cancer (b) groups. Data are showed as mean  $\pm$  SD (n=3). \*\*\*\*  $P<0.0001$  show a significant difference in comparison with the corresponding control.



**Figure 4.** Mitochondrial swelling assay. The effect of *E.schistosa* (5, 10, 20 and 40 µg/ml) venom on mitochondrial swelling in the liver mitochondria isolated from the normal (a) and cancer (b) groups. Data are showed as mean ± SD (n=3). \*\* and \*\*\*\* show a significant difference in comparison with the corresponding control (P<0.01 and P<0.0001, respectively).

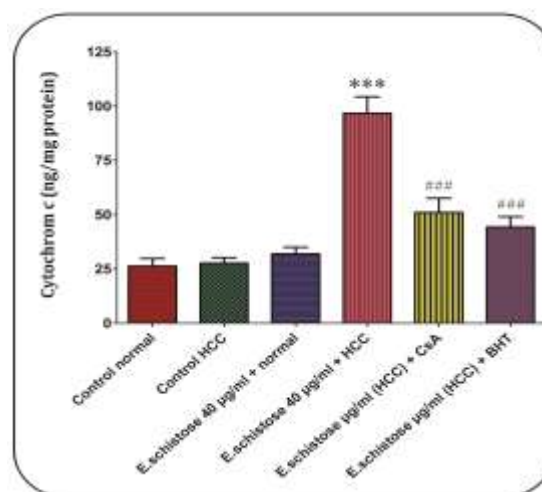
### 3.1.4. *E.Schistosa* Increased Mitochondrial Swelling

The results showed that *E.schistosa* at concentration of 5, 10, 20 and 40 µg/ml and 5-60 minutes after incubation increased the swelling in isolated mitochondria from the cancerous group (Figure 4B). Furthermore, *E.schistosa* venom at all applied concentrations did not induce swelling in the isolated mitochondria from the normal group (Figure 4A).

### 3.1.5. *E.Schistosa* Increased Cytochrome C Release

Results showed that *E.schistosa* venom induced a significant release of cytochrome c only in the mitochondria isolated from the HCC hepatocytes group (Figure 5). As shown in Figure 5, pre-exposure to cyclosporine A (Cs.A) and butylated hydroxyl toluene (BHT) has reduced the release of cytochrome c

by *E. schistosa* venom at concentration of 40 µg/ml. Cs.A is a MPT inhibitors like, and BHT is a antioxidants.



**Figure 5.** Cytochrome c release assay. The amount of expelled cytochrome c from the mitochondrial fraction into the suspension buffer was determined using a rat/mouse cytochrome c ELISA kit. Data are presented as mean ± SD (n=3). \*\*\* show significant difference in comparison with the corresponding control (p<0.001). ### Show significant difference in comparison with *E.schistosa* venom (40 µg/ml) - treated HCC group (P<0.001).

### 3.2. Discussion

In recent years, many methods have been used for the treatment of liver cancer, but these methods have not been successful in perfectly curing liver cancer. Therefore, designing a proper therapeutic option is essential [15]. HCC is known as a very deadly malignancy with poor prognosis [18]. Furthermore, research suggests that apoptosis signaling in the disease is disturbed and suppressed [16]. Therefore, the design and recognition of compounds that induce apoptosis can be useful in the HCC treatment [18]. Today, animal venoms including snake venoms have been considered by researchers due to active biological compounds and functional mechanisms. Also, snake venoms have been used as alternative strategy for cancer treatments [19, 20]. Accordingly, we determine the selective toxicity effects of *E. schistosa* crude venom on normal and HCC mitochondria and evaluate the effect of *E. schistosa* crude venom on oxidative stress (through ROS assay) and mitochondrial damage (MMP collapse assay and cytochrome c release) after *E. schistosa* crude venom exposure. The first step was to measure the SDH activity using MTT probe. The results showed that crude venom has selective toxicity effect on mitochondria isolated from HCC group and found that *E. schistosa* promoted decrease of the SDH activity. Our results are in agreement with previous studies that have shown that snake venoms cause the alteration in mitochondria [5, 11].

It has been shown that cell death (especially apoptosis) occurs due to mitochondria. Mitochondria as an essential organelle play an important role in the regulation of the apoptotic process. In this research, to better understand the mechanism of action of the *E. schistosa* venom, we determined the MMP using Rh 123 in mitochondria isolated from normal and HCC groups. Results showed that *E. schistosa* crude venom declined the MMP only in the mitochondria isolated from HCC but not the normal group. The results are in agreement with previous studies [11, 14]. Research has shown that MMP plays an important role in mitochondrial function. The collapse at the MMP is one of the critical points in apoptosis [14, 15]. Studies have shown that snake venoms have inhibited the growth of cancerous cells via inducing apoptosis. Also, the results of studies indicate that the level of ROS increases during the process of apoptosis. Furthermore, the generation of ROS and oxidative stress has been suggested as common mediators of apoptosis process [21, 22]. The results showed that exposure to *E. schistosa* crude venom increases the generation of ROS only in isolated mitochondria from HCC but not normal hepatocytes. The regulation of cellular redox has been considered as one of the most important target of anticancer compounds. In recent years, the “oxidation therapy” strategy has been considered by researchers; the use of ROS to kill cancer cells [21]. ROS have been used at high levels to induce an apoptotic process through mitochondrial pathways [18].

principal mechanisms of *E. schistosa* in venom ROS generation and induction of apoptosis has not been so far investigated.

In this study, mitochondrial swelling has been evaluated as one of the most important MPT pore opening indicators. The results showed that *E. schistosa* crude venom increases the mitochondrial swelling only in mitochondria isolated from HCC hepatocytes. Finally, cytochrome c release was evaluated as one of the apoptotic indexes. Furthermore, the results showed that *E. schistosa* crude venom released cytochrome c from HCC mitochondria.

#### 4. Conclusion

In conclusion, the results indicated that crude venom of *E. schistosa* increases the ROS generation only in the mitochondrial isolated from the HCC rats. This process resulted in a decline of MMP, alteration of mitochondrial swelling and release of cytochrome c, which can induce starting apoptosis signaling in liver hepatocytes of HCC rats. Furthermore, the results proposed that increase of ROS generation is the main regulator of mitochondria-mediated apoptosis.

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