

Selective Toxicity of Non-polar Bioactive Compounds of Sea Cucumber (*Holothuria sabra*) Extracts on Isolated Mitochondria and Hepatocytes of Induced Hepatocellular Carcinoma Rat Model

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Abstract

objective: Hepatocellular carcinoma (HCC) is the fifth most malignant of liver cancer globally. Anti-inflammatory and anti-cancer properties, scientists have begun to further investigate the natural bioactive compounds found in marine animals. *Holothuria sabra* (*H. sabra*), a sea cucumber is known to show the mentioned properties. **Method:** This study examined the selective toxicity of different dilutions of polar and non-polar extracts (n-hexane, diethyl ether, methanolic and aqueous) obtained from *H. sabra*, on hepatocytes and isolated mitochondria obtained from hepatocellular carcinoma induced rats. In order to induce HCC in rats, diethylnitrosamine (DEN) was injected followed by 2-acetylaminofluorene (2-AAF). Finally, hepatocytes and mitochondria isolated from cancerous and normal hepatocytes were applied for subsequent investigations. **Results:** Our results show that different concentrations (250, 500 and 1000µg/ml) of the n-hexane, diethyl ether and methanolic extracts significantly ($p < 0.05$) induces reactive oxygen species (ROS) formation and mitochondrial swelling, decreased mitochondrial membrane potential (MMP) disruption, increased cytochrome c release and induced the cell apoptosis phenotypes only in HCC hepatocytes and mitochondria in a time and concentration-dependent manner. **Conclusion:** Our results suggest that bioactive compounds found in *H. sabra* can potentially serve as anti-HCC molecules if further studies such as molecular identification, confirmatory in vivo experiments and clinical trials receive satisfactory results.

Keywords: HCC- *Holothuria sabra*- non-polar bioactive compounds- mitochondrial and cellular targeting- selective toxicity

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Introduction

Hepatocellular Carcinoma (HCC) is categorized among the deadliest cancers and the fifth principal cause of cancer across the world [1, 2]. Causative factors of HCC include Hepatitis C, which has annually increased the rate of HCC up to 3.7 per 100 patients. Hepatitis B has also increased the rate to 2.2 per 100 patients per year [3]. Additional causative factors include food additives, non-alcoholic fatty liver disease, obesity, and various forms of pollutions and industrial chemical toxins found in the environment [4, 5].

Current therapies such as chemotherapy, surgery, and

liver transplantation have not been successful treatments given their low efficiency, high recurrence rate, and metastasis [1]. Therefore, an urgent need exists to identify alternative methods for treating various cancers. More than 300 patents have been published within the decade exploring the effects of sea products on cancer therapy [6]. According to the research, approximately 60% of drugs used for cancer treatments have been obtained from natural products [7-9]. Among them, immense diversity of active compounds have been isolated from marine animals and plants [10]. In the past decade, natural marine products have been considered as one of the most promising anti-cancer, anti-tumoral, and

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anti-inflammatory sources.

The sea cucumber is a popular natural marine animal that resides within deep hot waters. It is an invertebrate marine animal of Holothuroidea (Holothuroidea). Prominent characteristics of the marine animal include anti-tumoral and anti-inflammatory properties. Sea cucumbers have been used as a source of food among Asian countries (China, Korea, and Taiwan) because of its nutritious and medicinal properties [11, 12]. In developed countries (the US and Canada), the marine animal has been dried and encapsulated as nutraceuticals for inflammatory conditions in humans and animals [13].

Although the identification and isolation of bioactive compound extracts of marine animals are within the preliminary stages in comparison to terrestrial dwellers, several forms of the mentioned extracts are undergoing more investigations in clinical trials and are being used as anti-disease drugs. The aim of this is to identify the toxicity effect of polar and non-polar extracts

(n-hexane, diethyl ether, methanolic and aqueous extracts) of *Holothuria sabra* on the cancerous hepatocyte mitochondria, and also on the cancerous hepatocytes of induced HCC rat models, in order to promote further research.

Materials and Methods

Preparation of *Holothuria sabra* Extraction

Holothuria sabra samples were gathered from deep hot water regions of the Persian Gulf in the province of Hormozgan, south of Iran. Samples were prepared at the Persian Gulf and Oman Sea Ecological Research Institute, Bandar Abbas, Iran. Different forms of extractions: methanolic, n-hexanoic, diethyl ether, and aqueous extracts, were prepared according to the methods described by Sarhadizadeh et al., (2014) [14]. Bioactive compounds were extracted based on their polarity by using water and different organic solvents. For this reason, sea cucumber samples were washed with distilled water then cut into small pieces, homogenized by blender, and finally, suspended in mentioned extracts for 72 h at room temperature. Subsequently, the suspension was centrifuged for 15 minutes (30,000×g, 4°C) and evaporated by a rotary evaporator at 45°C under vacuum. The obtained powder was stored at -20 °C.

Standardization of extracts by GC-MS analysis

The different extracts of *H. sabra* were analyzed by GC-MS (Agilent7000 series Triple Quad GC/MS MainFrame). The 30 mm, 0.25 mm, 0.5 mm AB-35MS fused silica capillary column was used as the GC column dimension. This experience was analyzed under the following conditions: injector temperature was 250°C, column temp isothermal was 100°C increased to 250°C at 6°C/min and held at this given temperature for 10 minutes, the ion source temperature was 200°C and the interface temperature was 250°C. In addition, helium gas was engaged as the carrier gas at the rate of 1ml/min. Spectra were obtained in the EI mode with 70eV ionization energy. Finally, the compounds were

identified by comparison with the standards.

Animals

Male Sprague-Dawley rats (120 – 130g) purchased from the Institute Pasteur (Tehran, Iran) were kept in an air-controlled room at 20 - 25°C; in a humidity of 50% - 60%, and for 12 hours daylight. All experiments were accomplished according to the ethical standards of the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences in Tehran, Iran. The rats were divided into two groups, healthy rats, and induced rats, (A) healthy rats as a control group and (B) rats induced with Hepatocarcinogenesis by a single intraperitoneal (i.p.) injection of DEN (a single dose of 200mg/kg body wt.). After two weeks 2-acetylaminofluorene (2-AAF 0.02%, w/w) was injected every 2 weeks to group B, in order to promote cancer [1, 15].

Cancer Diagnosis Tests

In order to determine the induction of cancer, several experiments were executed. Serum alpha-fetoprotein (AFP) as blood levels of liver cancer-specific marker, liver function tests and liver histopathology of treated rats were examined. The ADVIA Centaur AFP bioassay (Siemens, Germany) was used to determine the concentration of Serum alpha-fetoprotein (AFP). Moreover, alkaline phosphatase (ALP), Serum alanine transaminase (ALT), and aspartate transaminase (AST) as liver enzymes were tested by Hitachi-912 chemistry analyzer (Mannheim, Germany) and by standard diagnostic kits (Roche Diagnostics). In addition, a histopathological evaluation as a complementary test was assayed.

Isolation of the Mitochondria from Hepatocytes

Hepatocytes were isolated by using the four-step collagenase liver perfusion method. After that, to examine the cell viability, trypan blue exclusion test was used. For mitochondrial parameters assay, the mitochondria was prepared and extracted from hepatocytes. Briefly, the hepatocytes were suspended in 10mL of solution A (0.25M of sucrose, 0.01M of tricine, 1mM of EDTA, 10mM of NaH₂PO₄, and 2mM of MgCl₂; pH=8) and subsequently frozen at -80°C for 10 min and centrifuged at 760 g for 5 min in order to break the plasma membrane. The supernatant was kept while the pellet was homogenized for 10 min, followed by centrifugation at 760 g for 5 min. The supernatants from the two previous steps were combined and centrifuged for 20 min at 8,000 g. The final mitochondria containing pellet was suspended in Tris buffer (0.05M of Tris-HCl, 0.25M of sucrose, 20 mM of KCl, 2.0mM of MgCl₂, and 1.0mM of Na₂HPO₄; pH=7.4) at 4°C for further tests [1, 16-18].

Succinate Dehydrogenase (SDH) Activity Assay

The activity of mitochondrial complex II, SDH activity, was determined by using a MTT test. In order to do this assessment, 100µl of mitochondrial suspension was incubated with different concentrations of the mentioned polar and non-polar extracts of *H. sabra* (10,

25, 50, 100, 250, 500 and 1000 µg/ml) at 37°C for 1 hour. Then, 0.4% of MTT was added to the above suspensions and incubated for 30 min at the previous situation. Finally, 100 µl DMSO (5%) was added and the absorbance at 580 nm was measured by an ELISA reader (Tecan, Rainbow Thermo, Austria) [19, 20].

Determination of Reactive Oxygen Species (ROS)

At first, the mitochondrial suspension from both groups of animals (HCC induced and normal) was suspended in respiration buffer (20 mM of Mops, 10 mM of Tris, 0.32 mM of sucrose, 0.5 mM of MgCl₂, 0.1 mM of KH₂PO₄, 5 mM of sodium succinate, and 50 µM of EGTA). After that, Dichlorofluorescein Diacetate (DCFH-DA, at final concentration=10 µM) was added then, incubated for 10 minutes at 37°C to measure the ROS level induced by *H. sabra* extracts. Finally, to determine the intensity of fluorescence induced by dichlorofluorescein (DCF), the Shimadzu RF-5000 U fluorescence spectrophotometer (EX=488 nm and EM=527 nm) was used [21, 22].

Mitochondrial Membrane Potential Assay (MMP)

The cationic fluorescent dye (Rhodamine123, Rh 123) was used to determine the amount of uptake of mitochondrial membrane potential (MMP). Rh 123 (at final concentration=10 µM) was added to the mitochondrial suspensions obtained from HCC and normal groups (1000 µg mitochondrial protein/ml) in the MMP assay buffer including 2 mM of MgCl₂, 10 mM of KCl, 5 mM of KH₂PO₄, 68 mM of D-mannitol, 50 µM of EGTA, 5 mM of sodium succinate, 10 mM of HEPES, 220 mM of sucrose and 2 µM of rotenone. Then, the amount of uptake was determined by a Shimadzu RF5000U fluorescence spectrophotometer which was set at EX=490 nm and EM=535 nm [20, 21, 23].

Determination of Mitochondrial Swelling

The isolated mitochondria were suspended in swelling buffer (70 mM of sucrose, 230 mM of mannitol, 3 mM of HEPES, 2 mM of Tris-phosphate, 5 mM of succinate, and 1 µM of rotenone) and incubated at 30°C with different concentrations of various forms of non-polar (n-hexane (250, 500 and 1000 µg/ml), diethyl ether (250, 500 and 1000 µg/ml)) and both polar and non-polar (methanolic (250, 500 and 1000 µg/ml)) extracts. The absorbance was determined at 15 min interval, by an ELISA reader (Tecan, Rainbow Thermo, Austria) at 540 nm [(22, 24, 25)].

Effect of H. sabra on the Cytochrome C Release

Different extracts of *H. sabra* at IC₅₀ µg/ml (half-maximal inhibitory concentration) induced a significant (P<0.05) release of cytochrome c of the mitochondria isolated from the HCC group. The release of cytochrome c acquired by the mentioned extracts was assayed by the Quantikine Rat/Mouse Cytochrome c Immunoassay kit provided by R&D Systems, Inc. (Minneapolis, MN, USA).

Assessment of Holothuria sabra toxicity on hepatocytes

To evaluate the selective cytotoxicity effect of different

extracts of *H. sabra* on the viability of HCC and normal hepatocytes, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used. Hepatocytes obtained from normal and HCC cells (1×10⁴ cells/well) were treated with different concentrations (10-1000 µM) of non-polar extracts of *H. sabra* (n-hexane and diethyl ether) and also with polar and non-polar extract (methanolic extract) and incubated in 96-well plates for 90 min, at 37°C in a humidified incubator. Subsequently, MTT (5 mg/ml in RPMI 1640 to each well) was added to the above-obtained hepatocytes which were maintained in RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 µg/ml streptomycin) and then incubated. In the next step, centrifugation began at 1800 xg for 5 min at 4°C. The buffer solution (containing different extracts) was inserted into each well and 100 µl of DMSO was then added to dissolve the formazan crystals. Finally, the absorbance was measured at 570 nm by an ELISA reader. Each extract concentration was assessed in three separate experiments (n=3).

Quantification of apoptosis

The percentage of apoptosis versus necrosis induced on hepatocytes obtained from both normal and HCC groups following IC₅₀ µg/ml treatment of n-hexane and diethyl ether (contain non-polar compounds) and methanolic (contains both polar and non-polar compounds) extracts of *H. sabra* was determined by flow cytometry (equipped with a 605 nm argon ion laser, the flowing software 1.2.5. and using a 530nm bandpass filter (FL-2 channel). Beforehand, the given cells were seeded overnight in 12-well culture plate (75000 cells/well) and treated with the mentioned extracts for 90 min. Then, floating and adherent cells were incubated with 750 µL of a hypotonic buffer (50 µg/mL propidium iodide (PI) in 0.1% sodium citrate containing 0.1% Triton X-100) at 4°C and under dark conditions. Each determination is based on the mean fluorescence intensity of 10,000 counts.

Statistical Analysis

Graph Pad Prism software (version 5) was utilized for all statistical analyses. One-way ANOVA test with post hoc Tukey test was used. Two-way ANOVA test, followed by post hoc Bonferroni test was performed in certain experiments as well. The assays were performed five times (n=5), and results were presented as mean ± SD. Statistical significance was set at P<0.05.

Results

Effect of DEN/2-AAF on liver function markers and AFP

A significant (P<0.05) increase in serum ALT, AST, and ALP (liver function markers), as well as AFP (The liver marker for HCC exists in the bloodstream) concentrations in HCC group, were shown in Table 1.

GC-MS Analysis

The GC-MS analysis has shown that the non-polar extracts of mentioned marine animal reveal the presence of

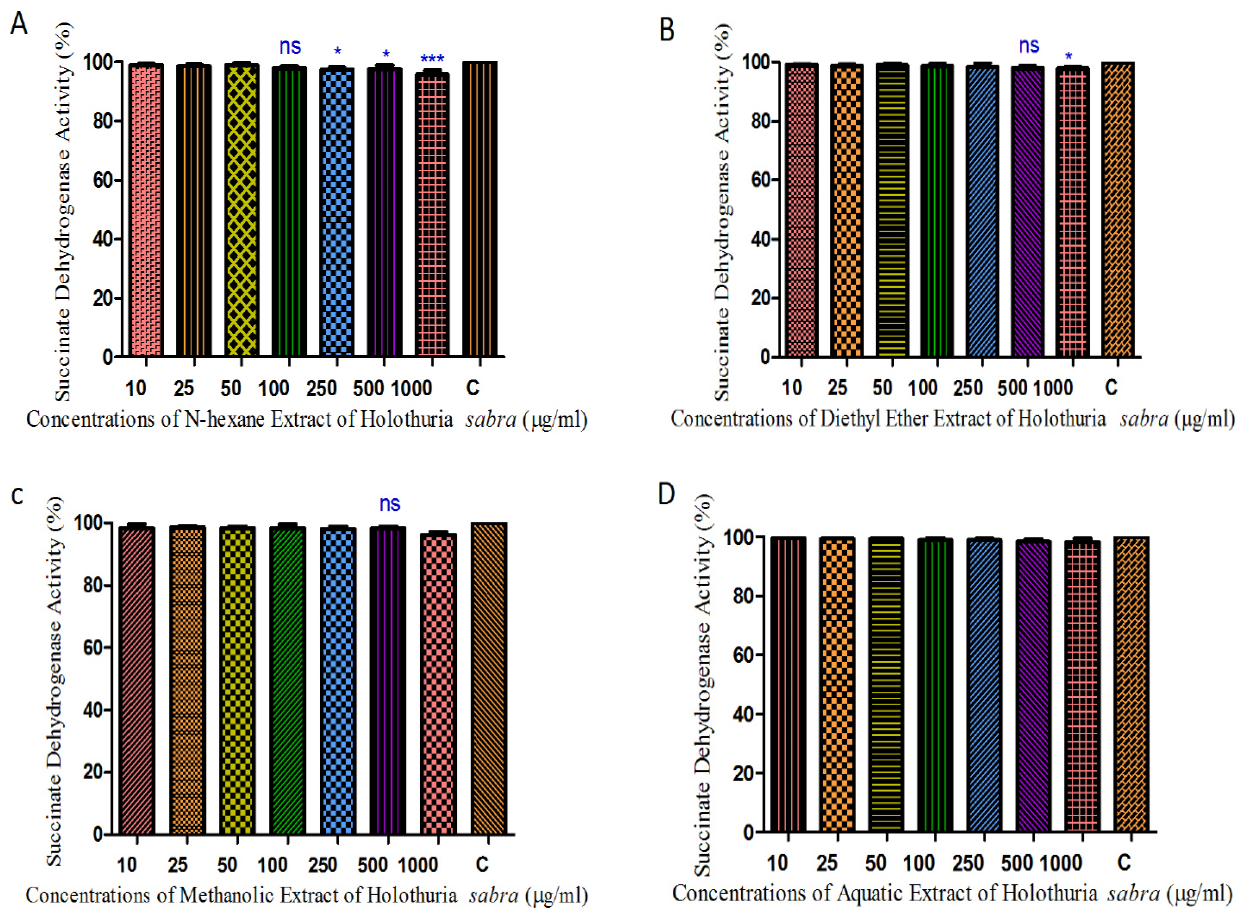


Figure 1. The Effect of Different Concentrations of *H. sabra* on the SDH Activity of Normal Hepatocytes. (A) n-hexane extract, (B) diethyl ether extract, (C) methanolic extract of the *H. sabra* concentrations and (D) aqueous extract on SDH activity in the mitochondria obtained from hepatocytes. Values are presented as mean \pm SD (n = 3). *, ** and *** p<0.05, p<0.01 and p<0.001, respectively, compared to the corresponding control (*H. sabra* concentration="0 μ g/ml").

triterpenoids, steroids, and dihydrotestosterone according to the Table 2. According to this table, the n-hexane extract including, Squalene (12%), Ambrein (15%), β -amyirin (9%), diethyl ether extract including, Renanolone (28%), Lupeol (8%), Drostanolone (15%), and methanolic extract including Lanosterol (14%).

Effect of *Holothuria sabra* extracts on SDH (Succinate Dehydrogenase) Activity

The effects of different concentrations (10, 25, 50, 100, 250, 500 and 1000 μ g/ml) of different extracts of *H. sabra* on SDH activity of mitochondria obtained from normal and HCC groups were examined by the MTT assay in order to identify the amount of IC₅₀ μ g/ml.

Normal and HCC mitochondria were incubated for 1 h in the presence of different concentrations of different extracts of *H. sabra* (10, 25, 50, 100, 250, 500 and 1000 μ g/ml). All applied concentrations

of non-polar extracts of *H. sabra* (n-hexane and diethyl ether) polar and non-polar extract (methanolic extract) indicated a significant (P<0.05) decrease only in the SDH activity of the HCC mitochondria group but did not show any significant effect on normal healthy mitochondria Figures (1, 2). More importantly, the above result did not occur in the polar extract containing aqueous extract.

In addition, as shown in Figure 2 (A, B), the mentioned non-polar extracts of *H. sabra* in the highest concentrations (1000 μ g/ml) significantly (P<0.05) reduced the activity of complex II in the HCC mitochondria compare to the normal group. Also, the methanolic extract revealed the same trend concentration-dependent result but at a lower rate Figure 2 C. But the aqueous extract of *H. sabra* at all applied concentration (10, 25, 50, 100, 250, 500 and 1000 μ g/ml) did not significantly affect SDH activity Figure 2 D.

Table 1. Effect of DEN/2-AAF on the ALT, AST, ALP, and AFP Markers

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	AFP (IU/L)
Control group (A)	93 \pm 8.5	67 \pm 4.8	618 \pm 8.5	0.463 \pm 0.02
HCC group (B)	342 \pm 17***	627 \pm 54***	778 \pm 10***	2.316 \pm 0.09*

*** Values are presented as mean \pm SD (n=9). P < 0.001 compared with group B.

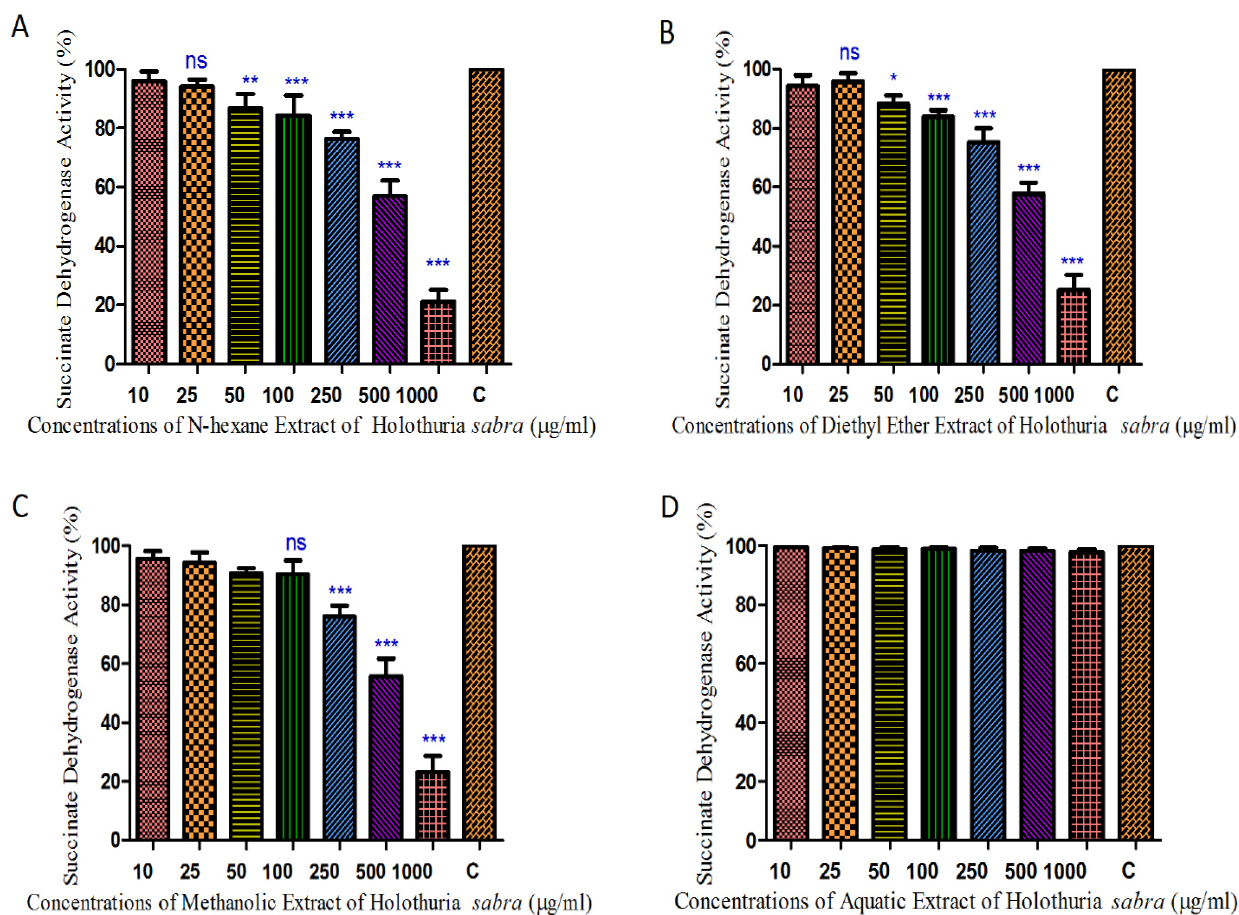


Figure 2. The Effect of Different Concentrations of *H. sabra* on the SDH Activity of HCC Hepatocytes. (A) n-hexane extract, (B) diethyl ether extract, (C) methanolic extract of the *H. sabra* concentrations and (D) aqueous extract on SDH activity in the mitochondria obtained from HCC hepatocytes. Values are presented as mean \pm SD (n = 3). *, ** and *** p<0.05, p<0.01 and p<0.001, respectively, compared to the corresponding control (*H. sabra*

Effect of *Holothuria sabra* on ROS Production

As shown in Figure 3 (A-C), different concentrations of non-polar extracts (n-hexane and diethyl ether) and both polar and non-polar extract (methanolic extract) of *H. sabra* significantly (P<0.05) increased ROS generation (demonstrated as fluorescence intensity units emitted from highly fluorescent DCF) in the HCC group. The same result did not occur in the control group. The results of our study show that the n-hexane extract (250, 500 and 1000µg/ml) obtained from the *H. sabra* on ROS formation is stronger than diethyl ether extract (250, 500 and 1000µg/ml) and methanolic extract (250, 500 and 1000µg/ml) Figure 3. This activity occurred in a time and concentration-dependent manner.

Effect of *Holothuria sabra* on Mitochondrial Membrane Potential (MMP)

To measure the effect of non-polar and polar extracts of *H. sabra* on MMP, Rh123 staining test was applied. As shown in Figure 4 (A-C), all non-polar (n-hexane and diethyl ether) and both polar and non-polar (methanolic) extracts of *H. sabra* at different concentrations (250, 500, 1000µg/ml) significantly (P<0.05) raised the absorbance of the fluorescence intensity which determined the collapse of MMP. Actually, the declined MMP demonstrates the distributed damaged mitochondria into the cytosol. According to our results, MMP was significantly (P<0.05) decreased after the addition of the above extracts especially, n-hexane,

Table 2. The GC-MS Analysis of Different *H. sabra* Extracts

Extracts	Components	percentages	Structure chemical	Molecular formula	MW
N-hexane	Squalene	25%	Triterpenoids	C ₃₀ H ₅₀	410.718
	Ambrein	18%	Triterpenoids	C ₃₀ H ₅₂ O	428.75
	β-amyrin	19%	Triterpenoids	C ₃₀ H ₅₀ O	426.72
Diethyl ether	Renanolone	28%	Steroids	C ₂₀ H ₃₀ O ₄	334.450
	Lupeol	18%	Triterpenoids	C ₃₀ H ₅₀ O	426.72
	Drostanolone	10%	Dihydrotestosterone	C ₂₀ H ₃₂ O ₂	304.46
Methanol	Lanosterol	24%	Triterpenoids	C ₃₀ H ₅₀ O	426.71

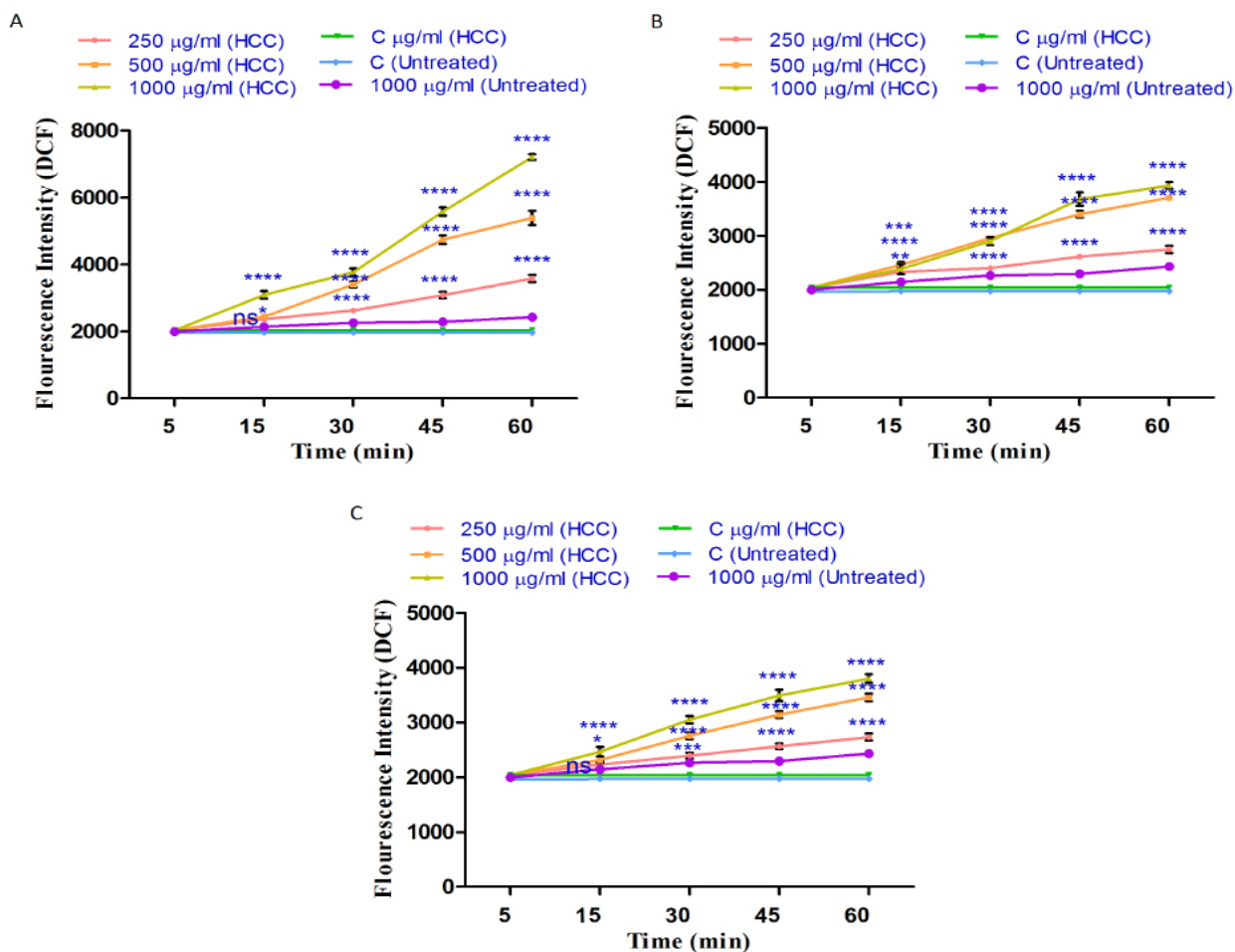


Figure 3. The Effect of Different Concentrations of *H. sabra* on the ROS Generation. (A) n-hexane extract (250, 500 and 1000 µg/ml), (B) diethyl ether extract (250, 500 and 1000 µg/ml), (C) methanolic extract (250, 500 and 1000 µg/ml) of the *H. sabra* concentrations induced a significant increase of mitochondrial ROS formation in HCC but not control mitochondria. Values are presented as mean \pm SD (n = 3). *, **, ***and ****p<0.05, p<0.01, p<0.001 and p<0.0001, respectively, compared to the corresponding control (*H. sabra* concentration="0 µg/ml").

in a time and concentration-dependent manner by passing 60 min and in a higher concentration. The above results were seen only in the mitochondria obtained from HCC hepatocytes.

Effect of *Holothuria sabra* on Mitochondrial Swelling

The mitochondrial swelling, as another indicator of the mitochondrial permeability transition (MPT), was assayed. The decrease of absorbance of the mitochondria measured at 540 nm was shown to induce mitochondrial swelling. Increasing rate of mitochondrial swelling of HCC group by the addition of different concentrations of non-polar (n-hexane and diethyl ether (250, 500 and 1000µg/ml)) and both polar and non-polar (methanolic (250, 500 and 1000µg/ml)) extracts of *H. sabra* is significant (P<0.05), shown in Figure 5. This trend was seen in higher concentration of n-hexane (1000µg/ml), diethyl ether (1000µg/ml) and methanolic (1000µg/ml) extracts of *H. sabra* in a concentration and time-dependent manner. In addition, the n-hexane extract obtained from *H. sabra* on mitochondrial swelling is stronger than other extractsFigure 5A.As shown in Figure 5 (A-C) only the highest applied concentration

(1000µg/ml) of all n-hexane, diethyl ether and methanolic extracts significantly influenced mitochondrial swelling of the mitochondria obtained from the normal group.

Effect of *Holothuria sabra* on the Cytochrome C Release

As shown in Figure 6 (A-C), the non-polar (n-hexane and diethyl ether) and both polar and non-polar (methanolic) extracts of *H. sabra* (at IC50 µg/ml) induced a significant (P<0.05) release of cytochrome c only in the mitochondria isolated from the HCC group. Among them, the n-hexane extract showed the highest effect on the HCC group. More importantly, our results show that pretreatment of n-hexane (500 µg/ml), diethyl ether (500 µg/ml) and methanolic extracts (500 µg/ml)-treated mitochondria from the HCC group by cyclosporine A (Cs.A) as a MPT inhibitor, and butylated hydroxyl toluene (BHT) as an antioxidant, inhibited the release of the cytochrome c compared to sole extract of *H. sabra* treated HCC group without any pretreatments.

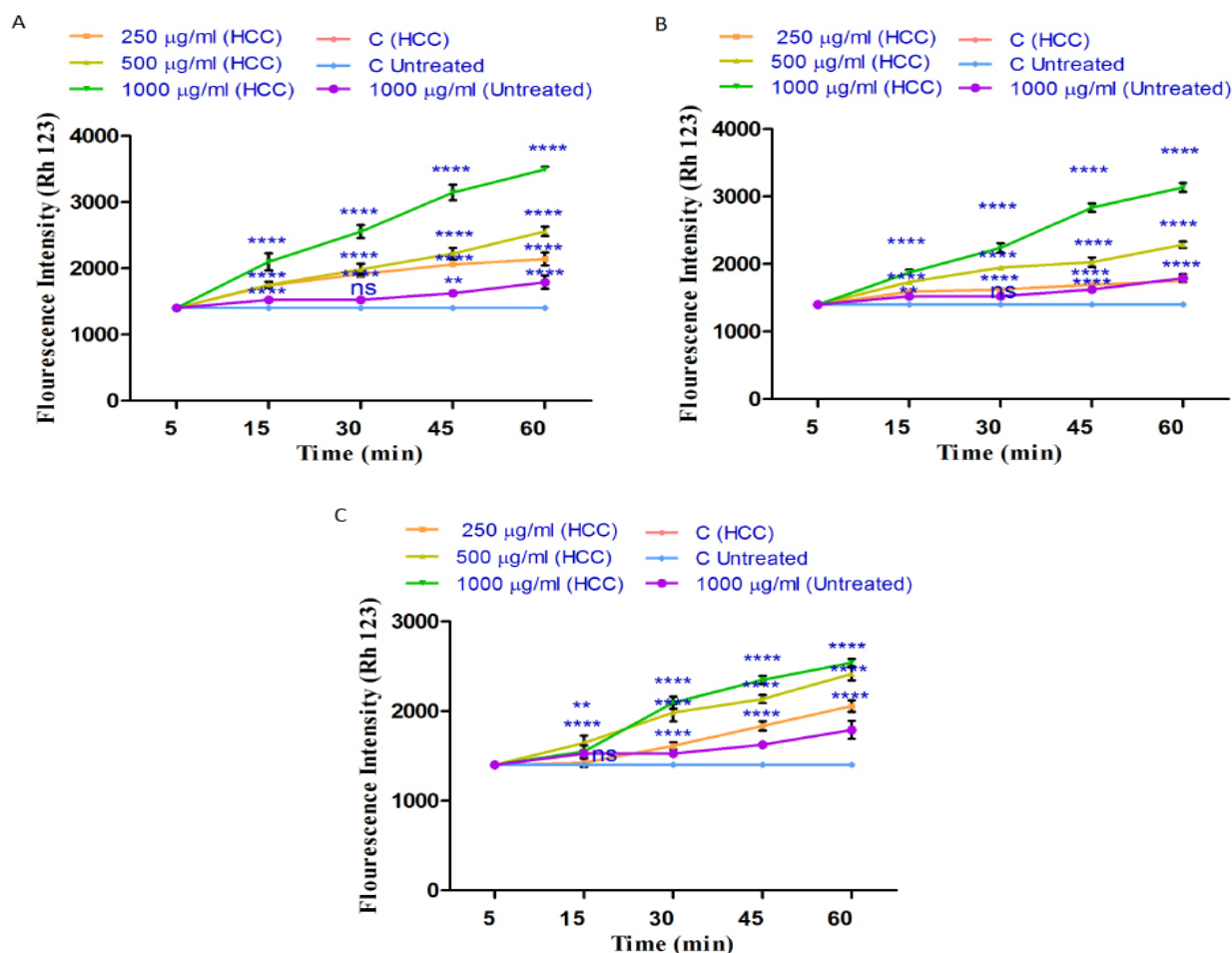


Figure 4. The Effect of Different Concentrations of *H. sabra* Extracts on the MMP Collapse. (A) n-hexane extract (250, 500 and 1000 µg/ml), (B) diethyl ether extract (250, 500 and 1000 µg/ml), (C) methanolic extract (250, 500 and 1000 µg/ml) of the *H. sabra* concentrations induced a significant decrease at MMP in HCC but not control mitochondria. Values are presented as mean \pm SD (n = 3). *, **, *** and **** p<0.05, p<0.01, p<0.001 and p<0.0001, respectively, compared to the corresponding control (*H. sabra* concentration="0 µg/ml").

Effect of *Holothuria sabra* on cell apoptosis

The percentage of apoptosis/necrosis caused by non-polar (n-hexane and diethyl ether) and both polar and non-polar (methanolic) extracts of *H. sabra* was assessed by annexin V-PI double staining at 90 min by flow cytometry. According to the related plots Figure 7, the percentage of annexin V+/PI- cells (CD19-gated) significantly (P<0.05) increased following the addition of n-hexane > diethyl ether > methanolic extracts of *H. sabra* on the HCC hepatocytes at IC₅₀µg/ml. This complementary test confirmed the previous results which were induced by mentioned extracts.

Discussion

Given the rise of HCC rates globally coupled with inefficient methods for HCC treatment [23], the authors of this paper sought to investigate novel treatment methods and materials to solve this mentioned problem. Marine animals containing various bioactive compounds present strong anticancer and anti-tumor properties [20, 21, 26]. In addition, beyond 70% of anti-cancer agents used, are originally sourced with varieties of lead compounds for different cancer activities [25, 27].

Previous studies have shown that marine animals containing several numbers of functional bioactive compounds significantly induce inhibition of tumor growth rate in a dose-dependent manner [28] and suppress tumor viability [21, 26, 29]. Such properties of sea cucumbers are attributed to their bioactive compounds such as triterpenoid glycosides, sulfated polysaccharides, and sterols. These bioactive compounds are also popular given their natural origin, long nutritional history, and negligible toxic effects. In addition, induction of apoptosis is a prominent anti-cancer mechanism deployed by these compounds [30, 31]. More importantly, this research has shown that a GC-MS of the n-hexane extract of mentioned marine animals reveals the presence of Squalene, Ambrein, β -amyryn, diethyl ether extract reveals Renanolone, Lupeol, Drostanolone and methanolic extract reveals Lanosterol.

This study was designed to discern the screening toxicity of diethyl ether and n-hexane (non-polar), and methanolic extract of both polar and non-polar extracts of *H. sabra* on HCC isolates from rats.

According to the results of this study, the significant increase in serum markers such as ALT, AST, and ALP (indicators of hepatic dysfunction in the HCC group),

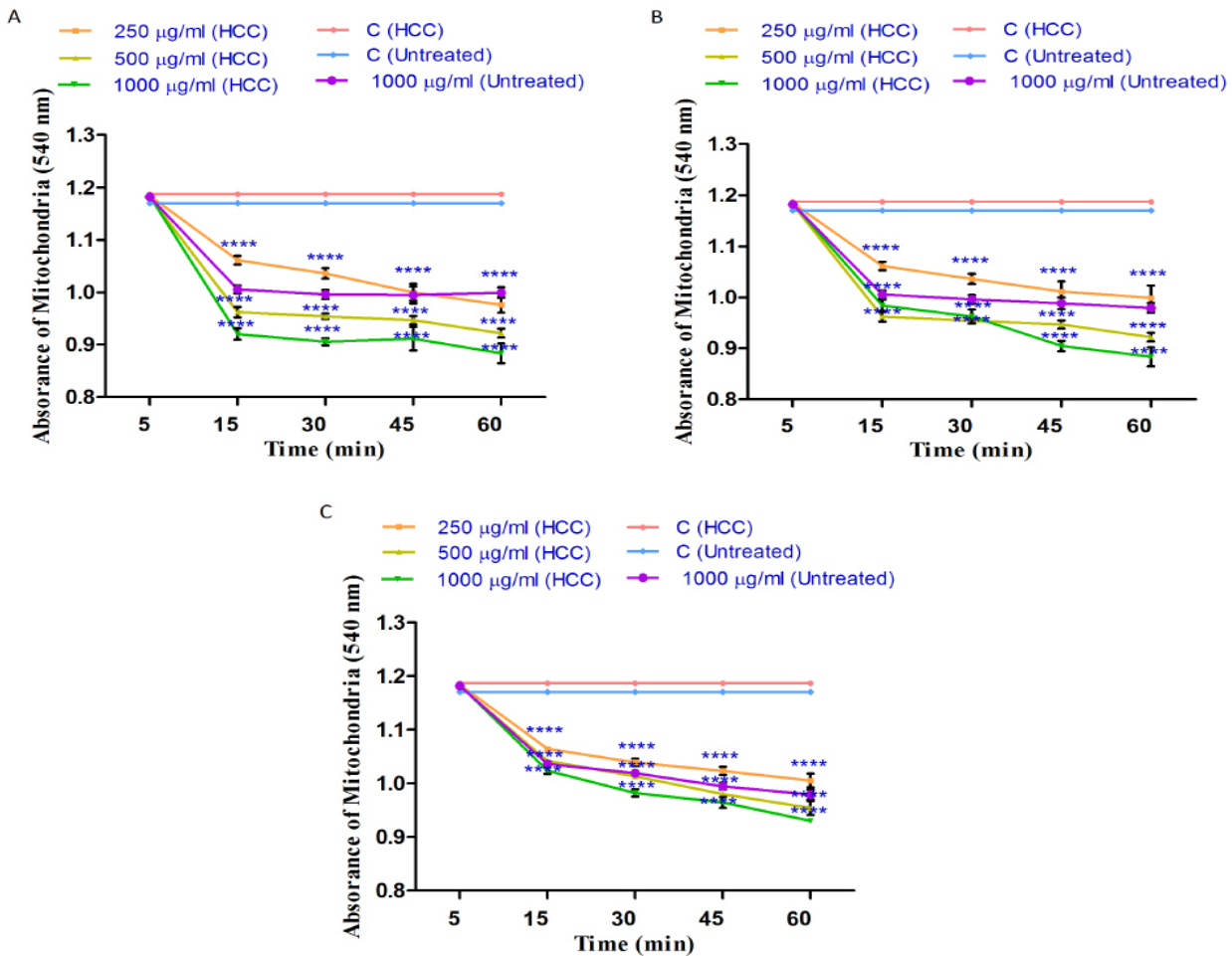


Figure 5. The Effect of Different Concentrations of *H. sabra* Extracts on the Mitochondrial Permeability. (A) n-hexane extract (250, 500 and 1000 µg/ml), (B) diethyl ether extract (250, 500 and 1000 µg/ml), (C) methanolic extract (250, 500 and 1000 µg/ml) of the *H. sabra* concentrations induced a significant increase at mitochondrial swelling in HCC but not control mitochondria. Values are presented as mean \pm SD (n = 3). *, **, ***and****p<0.05, p<0.01, p<0.001 and p<0.0001, respectively, compared to the corresponding control (*H. sabra* concentration="0 µg/ml").

illustrate that the liver cancer in rats was induced by DEN/2-AAF. It has been well documented, that increasing levels of the given enzymes in the blood represent liver damage. AFP as another complementary HCC marker increased in HCC-induced rats supporting our hypothesis. Hepatic dysfunction appears to be due to DEN/AAF regimen generating genetic alterations in liver hepatocytes [16, 17, 20, 22, 32-34]. In addition, histopathological tests reveal HCC hepatocytes with disorganized hepatic lobular architecture and cellular damage to the liver treated by DEN/2-AAF regimen. All of the above findings significantly differ between the HCC group and the control group [23].

Our results show that three extracts of *H. sabra* at all applied concentrations (250, 500 and 1000 µg/ml) significantly (p<0.05) decrease SDH activity or complex II in the HCC mitochondria in comparison to control rat hepatocytes. On the other hand, the aqueous extract of *H. sabra* did not have any significant effect on SDH activity.

It has been documented that the mitochondria are the specific source of ROS. Oxygen consumed by the respiratory

chain of HCC mitochondria is converted to O_2^- . Subsequently, superoxide anions generated in the mitochondria are rapidly converted to H_2O_2 ; up to 60 to 80% of the cellular generation rate is by the mitochondrial superoxide dismutase (MnSOD). The induced HCC mitochondria (by three extracts of *H. sabra*), increased the generation of H_2O_2 formation, and the subsequent and numerous irreversible cellular damages [35].

So, the level of ROS formation as mitochondria parameter toxicity was evaluated. In this study, three extracts of *H. sabra* at the different time and concentration significantly (p<0.05) promoted ROS formation in the mitochondria of the HCC group compared to the control group. Among them, n-hexane extract was shown significantly (P<0.05) increase the rate of ROS formation in a time and concentration-dependent manner more than the other extracts.

Our findings indicate that the above concentrations of the three extracts of *H. sabra* significantly induced the collapse of MMP ($\Delta\Psi_m$) in the mitochondria isolated from the HCC group in comparison to the control group. The n-hexane extract of *H. sabra* significantly reduced

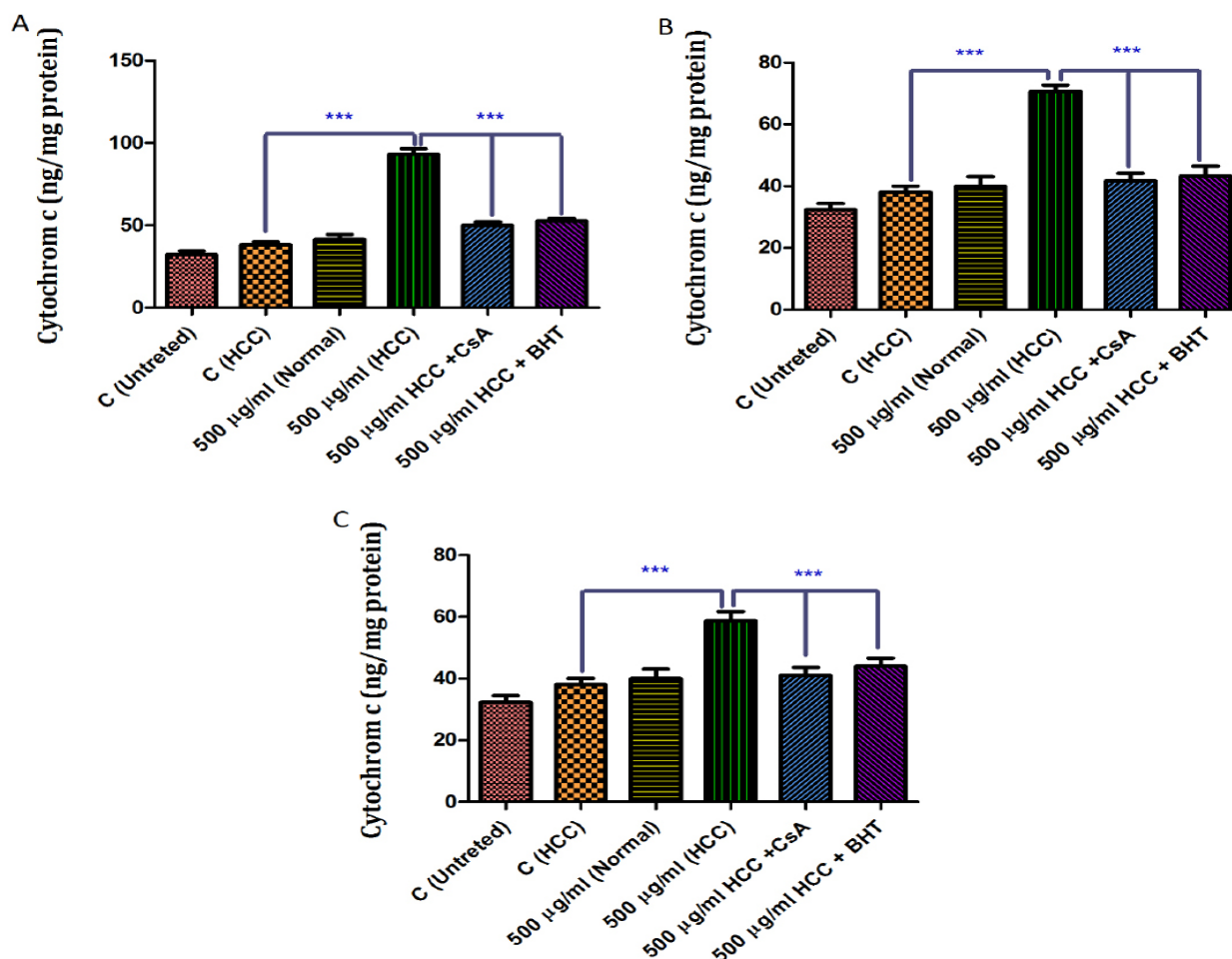


Figure 6. The Effect of Different Concentrations of *H. sabra* on the Cytochrome c Release. (A) N-hexane extract (250, 500 and 1000 µg/ml), (B) diethyl ether extract (250, 500 and 1000 µg/ml), (C) methanolic extract (250, 500 and 1000 µg/ml) of the *H. sabra* significantly increased the cytochrome c release only in HCC but not control mitochondria ($P < 0.05$). Values are presented as mean \pm SD ($n = 3$). *** $P < 0.001$ compared to corresponding control (extract concentration = "0 µg/ml").

the MMP more potently than the other extractions of *H. sabra*. The collapse of MMP plays a vital role in subsequent processes and apoptosis. Mitochondrial membrane damage can cause MPT pore-opening, the release of cytochrome c into the cytosol, which has been resulted in apoptosis and necrosis [36].

Mitochondrial swelling as another indicator of MPT is significantly elevated by the given concentrations of three extracts of *H. sabra* in the mitochondria obtained from HCC in comparison to the control group (5 A-B). As hypothesized, apoptosis induction is due to oxidative stress, the opening of the MPT pore; the subsequent release of cytochrome c from mitochondria to cytosol after mitochondrial swelling and the collapse of MMP are another disorder pathway.

The most important results were that the mentioned extracts (500 µg/ml) of *H. sabra* significantly induced the release of cytochrome c only from the HCC but not from the control mitochondria.

A defected apoptosis pathway plays a critical role in most malignant tumors and can be effective for cancer therapies. Research has shown that apoptosis induced by marine species are due to the presence of bioactive

compounds, which are considered as a new anti-cancer drug [37].

The distinctive function and characteristic of mitochondria between cancer and normal cells such as the different size, number, and shape of the mitochondria, different structure, and genomic mitochondrial alterations have also been shown in cancer cells in comparison to normal cells [20, 38, 39]. Therefore, the mitochondria were chosen as the main gateway for design and development of anti-cancer drugs [40]. Our results show that non-polar extracts (n-hexane and diethyl ether) of *H. sabra* (500 µg/ml of n-hexane and 500 µg/ml of diethyl ether) induce apoptosis in the HCC cell group. The efficacy of sea cucumber extracts at inducing apoptosis of n-hexane was obviously shown in figure 7 A-C. The methanolic extract by both polar and non-polar can induce apoptosis only in the highest concentration (1000 µg/ml).

In conclusion, we suggest that the effects of all extracts of *H. sabra* on mitochondria and hepatocyte cells may be attributed to the presence of numerous arrays of bioactive compounds such as phenolic, saponins, sterols, cerebrosides and sulfated polysaccharides. As the results show, only non-polar compounds containing extracts

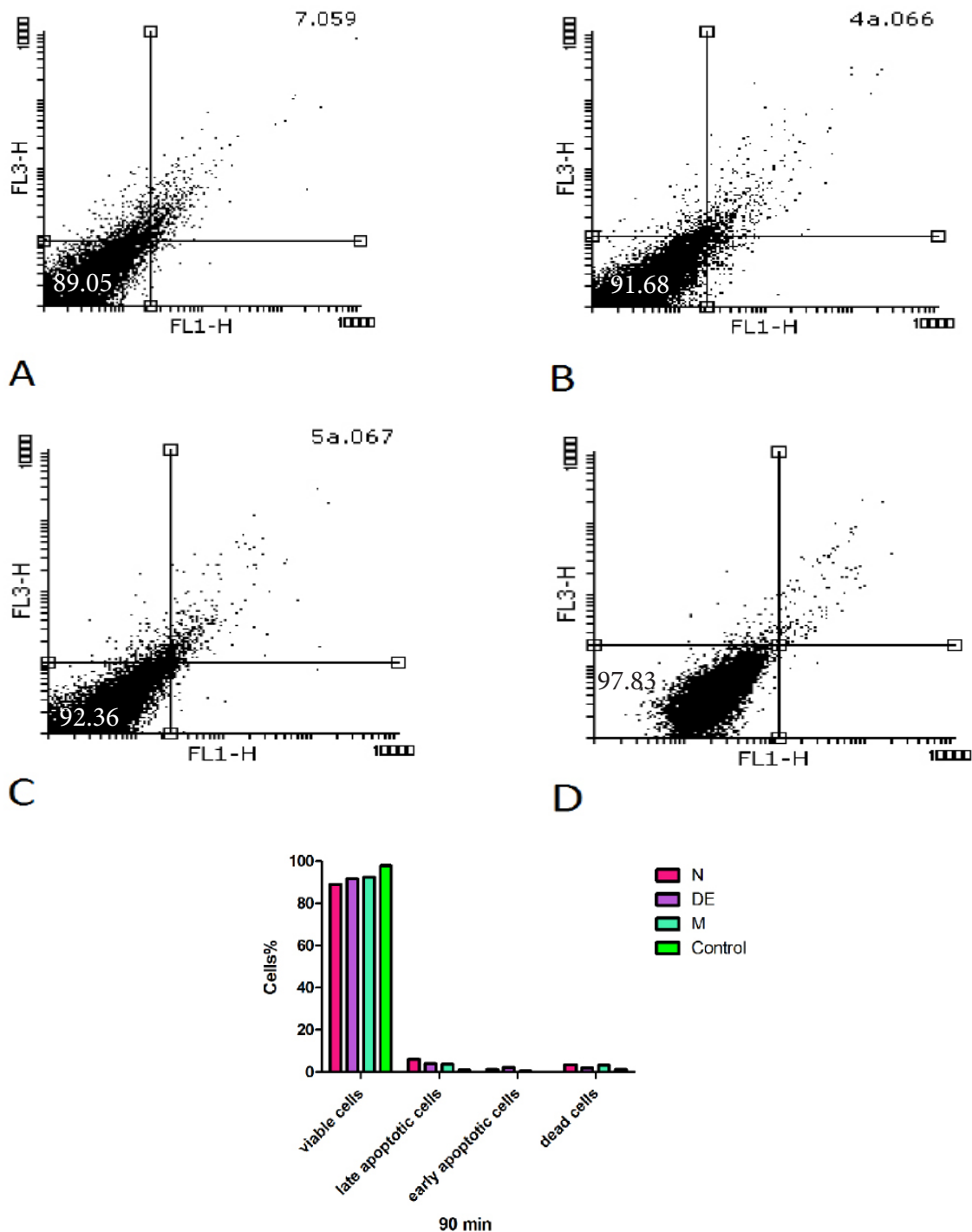


Figure 7. The Non-polar (N-hexane and Diethyl ether) and both Polar and Non-polar (methanolic) Extracts of *H. sabra* Extracts at 500 $\mu\text{g/ml}$ Concentration ($\text{IC}_{50}\mu\text{g/ml}$) Induced Apoptosis in Freshly Isolated from HCC and Normal Group. Sum of both early and late apoptotic features were measured by the annexin V assay using flow cytometry at 90 min after incubation in both groups. Results are expressed as means \pm SD (n=3), $P < 0.05$.

can cause selective toxicity alterations in mitochondrial parameters. Hence, we posit non-polar extracts of *H. sabra* as new anti-HCC drug candidates due to their ability to induce changes in mitochondrial and cellular parameters. In addition, the lower effects of methanolic extract of *H. sabra* on mitochondria isolated from HCC hepatocytes may be due to the presence of lower non-polar bioactive compounds comparing to n-hexane and diethyl ether extracts. This study provides evidence that *H. sabra* should be strongly considered as a potential new anti-HCC drug

candidate, as we demonstrate that mitochondrial targeting is a vital mechanism for the Persian Gulf sea cucumber extracts.

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Conflict of Interest

The authors declare no conflict of interest.

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