RESEARCH ARTICLE

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Toxicity Effect of *Hypnea flagelliformis* Algae on Cancerous Mitochondria Obtained from Rat Model of Hepatocellular Carcinoma

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Abstract

Background and objective: Hepatocellular carcinoma (HCC) is recognized as one of the major public health problems and deadly malignancies worldwide. Today, the use of compounds of natural origin in the treatment of cancer and other diseases has been of interest to researchers. Marine compounds such as algae have anti-cancer effects. In addition, Sea algae have nutritional value. This research is designed to investigate the cytotoxic effects of *Hypnea flagelliformis* (*H. flagelliformis*) extracts (methanolic, diethyl ether and n-hexane) on HCC mitochondria. **Material and Method:** HCC was induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) in rats. Then, toxicity parameters were evaluated. **Results:** The results showed that all *H. flagelliformis* extracts (250, 500 and 1000 µg/ml) significantly caused toxicity in HCC mitochondria, and no effect on healthy mitochondria was reported. **Conclusion:** The results indicate that the use of *H. flagelliformis* along with selected drugs in the treatment of HCC can help in the treatment of this cancer.

Keywords: Carcinoma- hepatocellular- marine algae- hypnea- mitochondria- models- animal

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Introduction

Cancer is known as one of the most important public health and mortality problems in the world. Cancer has imposed a heavy burden on public healthcare systems, especially in developed and developing countries [1, 2]. Statistics show that the incidence and mortality due to it are increasing worldwide. There are many approaches to treatment of cancer, but these methods have the ability to cause irreversible damage to healthy tissues around the tumor [3, 4]. Hepatocellular carcinoma (HCC) is the most important type of liver cancer and its incidence has increased in recent years [5, 6]. The incidence of HCC is higher in developing countries and hepatitis B (HBV) and C (HCV) are the most common risk factors. There are different approaches to treat HCC, but these approaches have not been successful and are associated with damage to normal tissue and cells. Therefore, there is a need for new treatment approaches that cause minimal damage to normal cells and tissues [5, 7, 8].

In recent years, products of natural origin have become an important source for the discovery of compounds with anti-cancer activity. Due to the fewer side effects of compounds of natural origin than compounds of chemical origin, the attention of cancer researchers has increased to natural compounds [4, 9-11]. Marine algae have been considered as natural marine products due to their many bioactive compounds. Marine algae have many pharmacological effects, including anti-cancer. Also, these compounds can increase the efficiency and improve the effects of anti-cancer drugs [1, 2, 12, 13]. The genus Hypnea is one of the most important algae that is economically important. Hypnea flagelliformis (*H. flagelliformis*) is one of the red algae that is widely found on the shores of Bandar Abbas (the south of Iran, Persian Gulf). *H. flagelliformis* has many bioactive compounds that are involved in biological activities. Previous research has shown that *H. flagelliformis* can

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cause toxicity in cancer cells, which could indicate its anti-cancer effects [10, 14, 15].

Our previous studies showed that compounds of natural origin have anti-cancer effects by targeting mitochondria [16, 17]. Mitochondria is one of the most important organelles that plays important roles in eukaryotic cells and is involved in many physiological processes [18, 19]. In recent years, mitochondria have been used as a therapeutic target for cancer [20-22]. The use of compounds that can target cancerous mitochondria and have less side effects on normal cells and organs is important in cancer treatment. Therefore, we investigated the effects of *H. flagelliformis* algal extracts (methanolic, diethyl ether and n-hexane) on mitochondria isolated from a rat model of HCC. In this study, diethylnitrosamine (DEN) was used as a cancer-initiating agent and 2-acetylaminofluorene (2-AAF) as a cancer-promoting agent to induce HCC in rats. In the following, mitochondrial toxicity parameters were investigated by measuring mitochondrial function, mitochondrial ROS level, mitochondrial membrane potential (MMP) collapse, mitochondrial swelling and cytochrome c release.

Materials and Methods

Animals

After purchase, the animals (Male Wistar rats) were kept under standard conditions of temperature, humidity, and lighting cycle. All the experiments were performed according to the guidelines of the ethics and animal supervision committee (ID: IR.MUI.AEC.1402.079). In this study, an effort was made to cause minimal suffering to the animals.

Chemicals

2,7-dicloroflurescein diacetate (DCHF-DA) and rhodamine 123 (Rh 123) were purchased from Sigma Aldrich Company (St. Louis, MO, USA). Cytochrome-c release was evaluated using a Quantikine Rat/Mouse Cytochrome c Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). Furthermore, all chemicals were of the highest commercial grade available.

Experimental design

6 rat were placed in the HCC group and 6 rat were placed in the control group for investigation. After 1 week of habituation, the animals were separated into 2 groups for HCC induction. 1 group was placed as control and the other group was exposed to DEN (200 mg/kg, ip) and 2-AAF (dietary, 0.02% w/w) to induce HCC cancer. The duration of the study was 15 weeks. After the 15th week, biochemical and pathological tests were performed to prove HCC (data not reported) [23]. In the next step, mitochondria were incubated with different concentrations of *H. flagelliformis* algae extracts (methanolic, diethyl ether and n-hexane) and toxicity tests were evaluated.

Collection of H. flagelliformis

H. flagelliformis samples were collected from the Qeshm Island Persian Gulf coastal waters (from 1-3 meters depth). The collection and identification of *H. flagelliformis* algae samples was done by Dr. Melika Nazemi in Bandar Abbas, Iran. To remove microscopic organisms, the samples were washed with distilled water and freshwater. The *H. flagelliformis* were washed twice with distillated water and stored at −20°C until use.

Preparation of H. flagelliformis extracts

H. flagelliformis samples (200 g dry powder weight) combined with three different solvents: n- hexane, diethyl ether and methanol. In order to collect n-hexane (non-polar extract), diethyl ether (semi-polar extract) and methanol (polar extract) extracts, *H. flagelliformis* algae samples were placed in n-hexane, diethyl ether and methanol for 24 hours, 48 hours and 72 hours, respectively. Then, each of the extracts was filtered and evaporated using Rota vapor under low pressure for n-hexane, diethyl ether and methanolic extracts at 35-40 °C, 38-40 °C and 40-45 °C, respectively.

Isolation of Mitochondria from Rat Hepatocytes

At the end of week 15, the animals were anesthetized with ketamine (intraperitoneal) and xylazine (intraperitoneal) in doses of 80 mg/kg, and 5 mg/kg, respectively. At first, hepatocytes were isolated according to standard protocol [24, 25]. Then, the hepatocytes were centrifuged in two steps to isolate the mitochondria. First step: 760 × g for 5 min, and two step: 8000 × g for 20 min [26, 27]. In order to evaluate toxicity parameters, mitochondria were exposed to different concentrations of *H. flagelliformis* algae extracts.

Evaluation of succinate dehydrogenase (SDH) activity

MTT reagent/test was used to measure mitochondrial SDH activity. 1 mg protein/ml of mitochondria was used for each test. To measure the activity of this parameter, mitochondria were suspended in corresponding assay buffer and incubated to 0-1,000 µg/ml of *H. flagelliformis* algae extracts (methanolic, diethyl ether and n-hexane) for 1hour. Finally, absorbance at a wavelength of 570 nm was evaluated to measure the activity of mitochondria SDH in both groups [28].

Evaluation of ROS formation

In order to measure the level of ROS using DCFH-DA probe, mitochondria were suspended in respiration buffer assay. Then, mitochondria incubated with *H. flagelliformis* algae extracts (250, 500 and 1000 µg/ml). Finally, the measured fluorescence intensity level (EXλ = 488 nm / EMλ = 527 nm) indicates the generation level of mitochondria ROS. The ROS level in both groups was measured at 15, 30 and 60 min after incubated to different concentrations of *H. flagelliformis* extracts [23].

Evaluation of mitochondrial membrane potential (MMP) collapse

Rh123 probe was used to measure MMP collapse. In order to measure this parameter, mitochondria were suspended in corresponding assay buffer, and then incubated with different concentrations of *H. flagelliformis* algae extracts (250, 500 and 1000 µg/ml). Finally, fluorescence intensity (EXλ = 490 nm/EMλ = 535 nm)

was assayed to measure the MMP collapse in both groups. The MMP collapse in both groups was measured at 15, 30 and 60 min after incubated to different concentrations of *H. flagelliformis* extracts [24].

Evaluation of mitochondrial swelling

First, mitochondria were suspended in corresponding assay buffer. In the next step, mitochondria were incubated with different concentrations of *H. flagelliformis* algae extracts (250, 500 and 1000 µg/ml). The absorbance of the samples was recorded at a wavelength of 540 nm. This test was measured at 15, 30 and 60 min after incubated to different concentrations of *H. flagelliformis* extracts.

Measurement of cytochrome c release

Cytochrome c release was measured step by step after incubating mitochondria with *H. flagelliformis* extracts, and also evaluation the effects of inhibitory compounds according to the manufacturer's kit instructions.

Statistical analysis

Results are reported as mean ± S.D. GraphPad Prism software (version 8) was used for data analysis. The significance level was $p < 0.05$. Statistical significance was assessed using the one-way ANOVA test and post hoc Tukey (SDH activity, and cytochrome c release), and the two-way ANOVA test and post hoc Bonferroni (mitochondrial ROS level, MMP collapse, and mitochondrial swelling).

Results

Effects of algal H.flagelliforms on mitochondrial SDH activity

In HCC group, the results showed that all extracts of H.flagelliformis (250, 500, and 1,000 µg/ml) have significantly decreased the SDH activity (Figure 1 A-C). The effects of extracts on SDH activity in HCC mitochondria were as follows: n-hexane> diethyl ether> methanolic. In the normal group, this effect of *H. flagelliformis* extracts was not reported. These results can indicate the selective effects of *H. flagelliformis* extracts only in HC mitochondria.

Effects of algal H.flagelliforms on mitochondrial ROS

As presented in Figure 2 A-C, all *H. flagelliformis* extracts at all concentrations, and all time incubation (15, 30, and 60 min) significantly increased ROS levels in the HCC group. In the n-hexane extract group, the level of ROS was higher compared to the other two extracts. A raise in the level of ROS was not reported in the normal group.

Effects of algal H.flagelliforms on MMP collapse

A collapse in the MMP has been reported in the HCC group exposed to all concentrations of *H. flagelliformis* extracts at 15, 30, and 60 min (Figure 3 A-C). The level of collapse in the MMP was higher in diethyl ether and n-hexane groups compared to methanolic group (Figure 3 A-C). *H. flagelliformis* extracts had no effect on MMP collapse in the normal group.

Effects of algal H.flagelliforms on mitochondrial swelling

As presented in Figure 4 A-C, all *H. flagelliformis* extracts at all concentrations, and all time incubation (15, 30, and 60 min) significantly raised mitochondrial swelling in the HCC rat group (Figure 4 A-C). In the normal group, this effect of *H. flagelliformis* extracts was not reported. These results can indicate the selective effects of *H. flagelliformis* extracts only in HC mitochondria.

Effects of algal H.flagelliforms on cytochrome release

A release of cytochrome c has been reported in the HCC group exposed to *H. flagelliformis* extracts (500 µg/ml). Compared to the other two extracts, the amount of cytochrome c released by incubation of HCC mitochondria with n-hexane extract was higher (Figure 5 A-C). In the normal group, this effect of *H. flagelliformis* (1,000 µg/ml) in the highest concentration was not reported (Figure 5 A-C). Furthermore, the results revealed that CsA and BHT reduce the effect of *H. flagelliformis* (500 µg/ml) extracts on the cytochrome c release from HCC mitochondria

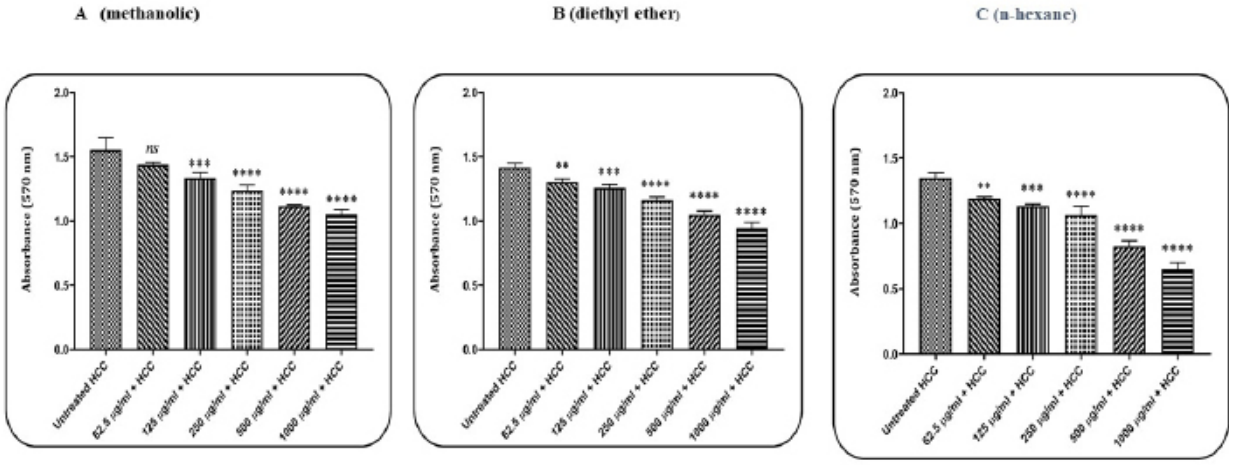

Figure 1. SDH Activity. The effect of *H. flagelliformis* (250, 500, and 1000 µg/ml) on mitochondrial SDH activity. Data were represented as the mean ± SD (n = 3). ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$) significant difference with untreated HCC group.

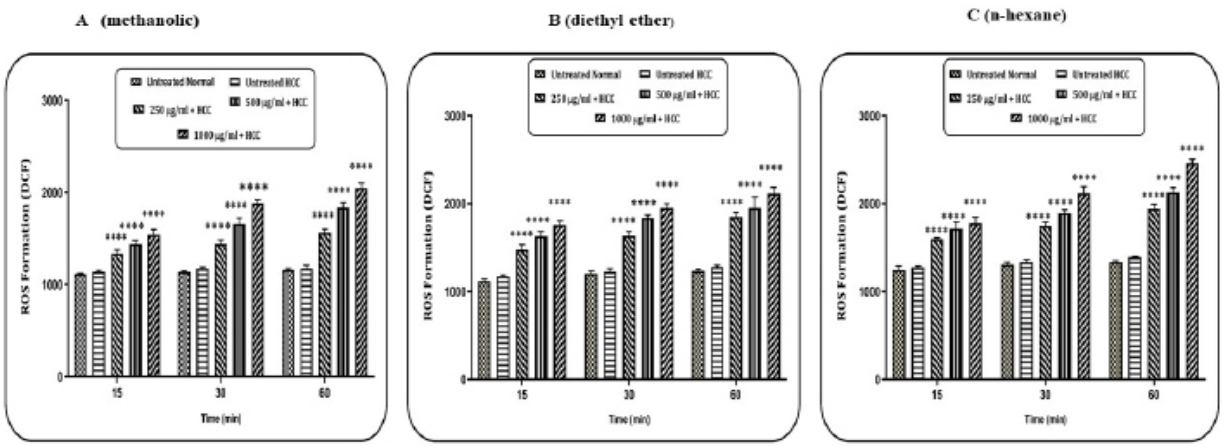

Figure 2. ROS شssay. The effect of *H. flagelliformis* (250, 500, and 1000 µg/ml) on mitochondrial ROS. Data were represented as the mean ± SD (n = 3). **** ($p<0.0001$) significant difference with untreated HCC group.

(Figure 5A-C).

Discussion

This study was conducted to investigate the effects of *H. flagelliformis* algae extracts on cancerous mitochondria of rats with HCC. To achieve this goal, factors such as mitochondrial SDH activity, ROS level, mitochondrial swelling, collapse in MMP, and cytochrome c release were evaluated. Cancer is known as one of the most

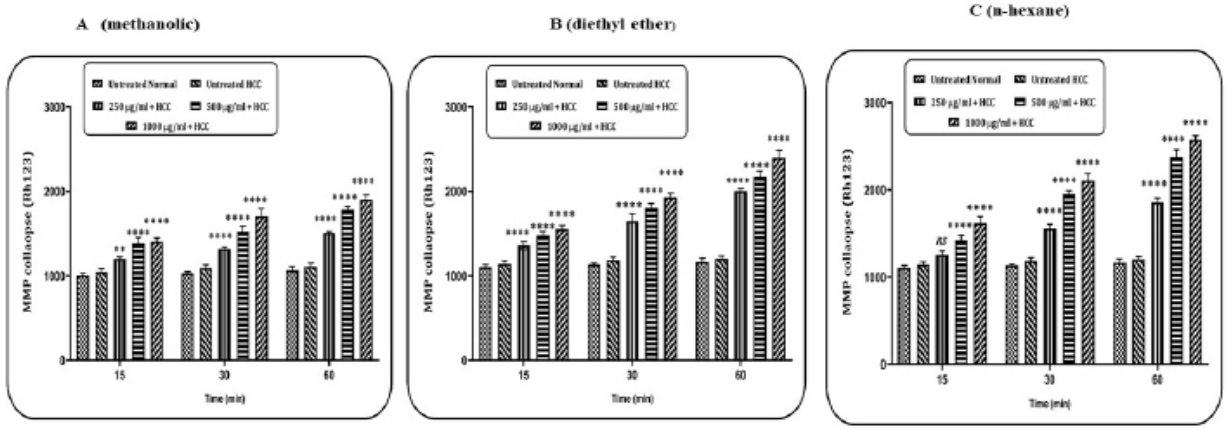

Figure 3. MMP Collapse Assay. The effect of *H. flagelliformis* (250, 500, and 1000 µg/ml) on MMP collapse. Data were represented as the mean ± SD (n = 3). ** ($p<0.01$), and **** ($p<0.0001$) significant difference with untreated HCC group.

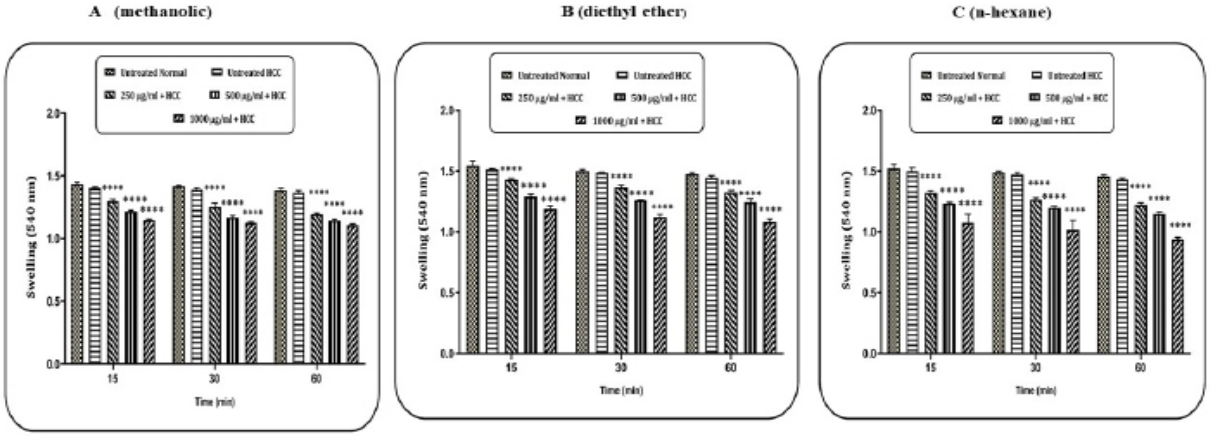

Figure 4. Mitochondrial Swelling Assay. The effect of *H. flagelliformis* (250, 500, and 1000 µg/ml) on mitochondrial swelling. Data were represented as the mean ± SD (n = 3). **** ($p<0.0001$) significant difference with untreated HCC group.

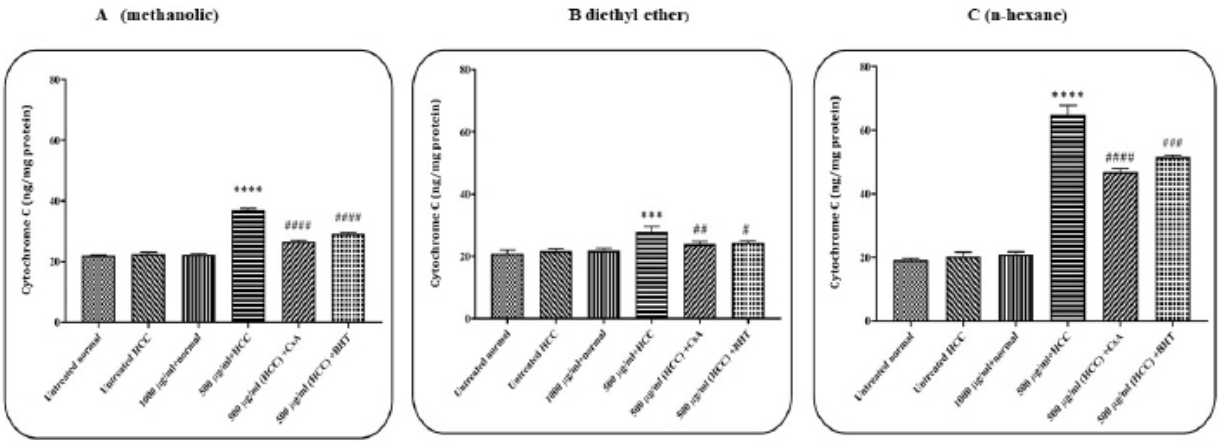

Figure 5. Cytochrome c Release assay. The effect of *H. flagelliformis* (250, 500, and 1000 µg/ml) on cytochrome c release. Data were represented as the mean ± SD (n = 3). *** ($p<0.001$), and **** ($p<0.0001$) significant difference with untreated HCC group. # ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$) and #### ($p<0.0001$) significant difference with 500 µg/ml + HCC group.

important diseases in the field of health, which is deadly and its global statistics are increasing. This disease affects the economic status of society and the quality of life of patients [29-31]. HCC is one of the most dangerous and deadly cancers in the world and its incidence is increasing. Therefore, it is necessary to use new approaches in its treatment [32, 33]. There are many approaches to treat different cancers, but they have not been successful and are associated with side effects on normal tissues/cells. Accordingly, a targeted therapeutic approach is needed [16]. The targeted therapy is one of the important approaches that can help cancer treatment and increase the survival rate of cancer patients [34].

Compounds of marine origin have many known pharmacological effects, including anticancer, through bioactive metabolites. Marine algae are one of the most important marine compounds that have nutritional importance and do not have cytotoxic effects. In addition, anticancer effects of these compounds have been reported [35, 36]. The results of our studies showed that compounds of natural origin cause toxicity in cancer cells by targeting mitochondria [16, 17]. Mitochondria are recognized as a vital organelle that is of interest in cancer research. In recent years, various compounds have been used to target mitochondria in cancer treatment. These compounds are called 'mitocans', which can target the mitochondrial transport chain (MTC), apoptosis pathways, ROS level and mitochondrial membrane permeability. These approaches can be used to kill cancer cells [37-39]. It has been shown that there are many differences between mitochondria in normal and cancer cells. Mitochondria in cancer cells (such as liver tumors) are more vulnerable compared to normal cells [23, 40].

Our results showed that *H. flagelliformis* extracts cause a decrease in mitochondrial SDH activity, an increase in the level of mitochondrial ROS, a collapse in MMP, mitochondrial swelling and the release of cytochrome c from mitochondria. These results are in agreement with the results of previous studies [16, 17, 41]. SDH is part of the mitochondrial respiratory chain (MRC) and plays a role in the connection between this chain and the TCA cycle. In addition, this complex can play a role in the generation of ROS. Therefore, disruption of SDH function by *H. flagelliformis* extract can be associated with disruption of mitochondrial function and generation of ROS [42]. An increase in the level of ROS is associated with the induction of apoptosis signaling in cancer cells. Therefore, the compounds that cause an increase in the level of ROS in cancer cells can be used as anti-cancer agents [39]. The level of ROS in tumor cells is higher compared to normal cells. In addition, approaches that are used to generate ROS can help in cancer treatment [43, 44]. It has been shown that cancer cells are vulnerable to the oxidative stress of ROS. Therefore, increased ROS can kill cancer cells [23, 45]. *H. flagelliformis* extract caused an increase in the level of ROS only in cancer mitochondria, which can indicate the targeting of cancer mitochondria. This result is in agreement with our previous studies, which have shown that compounds of natural origin increase the level of ROS in cancer mitochondria [16, 17, 41]. ROS can disrupt the permeability of the mitochondrial membrane. This event can be accompanied by mitochondrial swelling, collapse in MMP and release of cytochrome c [16, 17, 23, 41]. *H. flagelliformis* may have caused a disruption in the permeability of the mitochondrial membrane and its consequences through an increase in the level of ROS.

In conclusion, we concluded that *H. flagelliformis* algae extracts can be helpful in the treatment of HCC along with selected drugs due to their biologically active compounds. *H. flagelliformis* extracts by targeting only cancerous mitochondria could be promising in HCC treatment. In spite of these effects, the use of *H. flagelliformis* needs further studies in animal and clinical trials. These compounds can be a source of anti-cancer drugs. This research is an animal study and requires further investigations of these compounds on cell lines.

Author Contribution Statement

Mahsa Barzegar and Nazanin Shahbazi contributed to this research in carrying out the experiments. Melika

Nazemi contributed to this research in sample collection and Identification. Zhaleh Mohsenifar contributed to this research in the investigation of biochemical and histological parameters. Jalal Pourahmad, Abas Jafarian and Enayatollah Seydi contributed to this research in formulating the research question (s), designing the study, carrying it out as thesis supervisor, analyzing the data, and writing paper.

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Ethics Committee Approval

All the experiments were performed according to the guidelines of the ethics and animal supervision committee (ID: IR.MUI.AEC.1402.079).

Availability of data

Data will be made available on request.

Declaration of Conflict of interest

The authors declare that they have no conflict of interest.

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