Iranian Journal of Fisheries Sciences 21(1) 157-173 2022
DOI: 10.22092/ijfs.2022.350884.0

Research Article

Antiproliferative activity of *Portunus segnis* muscle extract on apoptosis of colon cancer cell line (HT-29)

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Received: January 2020 Accepted: December 2021

Abstract

Recently, marine natural products have had a critical role in the development of medicinal goods. The present study aimed to determine and compare *in vitro* effects of hexane, butanol, ethyl acetate, and water extracts of the muscle of *Portunus segnis* on a colon cancer cell line. In this experimental study, the HT29 colon cancer cell line was treated with various concentrations of crude extracts in three periods of 24, 48, and 72 h. HT29 viability was evaluated by Trypan Blue staining and MTT assay. Cell apoptosis was determined by flow cytometry assays. Also, reactive oxygen species (ROS), lactate dehydrogenase, and caspase 3/7-9 activities were tested in butanol extract-treated HT29 cells. Bioactive compounds of butanol extract were analyzed by gas chromatography-mas spectrometry (GC-MS). Palmitoleic acid (4.83%), 9-octadecenoic acid (4.82%), docosane (4.66%) and eicosane (4.34%) were found in muscle butanol extract of *P. segnis*. The muscle butanol extract demonstrated an IC50 of 10.12±0.35 µg/mL, towards the cell line. The results also indicate that decreasing cell viability depends on both dose and time. The bioactive compounds led to a significant elevation in ROS production, as assessed by the measurement of fluorescence intensity in stained cells. Furthermore, activation of caspases-3/7 and -9 induced apoptosis. After treatment with the butanol extract compound, activation of caspases-3/7-9 was illustrated and confirmed the involvement of mitochondrial-mediated apoptosis. Butanol extract compounds of the muscle of *P. segnis* can be introduced as a potential candidate for the development of anticancer chemotherapeutic agents.

Keywords: *Portunus segnis*, Colon cancer, LDH, ROS, Caspases 3/7, 8, and 9.

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[DOR: 20.1001.1.15622916.2022.21.1.7.3]

Introduction

Cancer is a group of diseases described as uncontrolled and abnormal growth of cells (Wallace, 2000; Zhang *et al.*, 2015). In 2018, an estimated 18.1 million new cancer patients were diagnosed and 9.6 million cancer deaths occurred worldwide (IARC, 2018). Cancer occurrence is still increasing quickly, especially for gastrointestinal cancers. Colon cancer, which is one of the most common types of cancer, is the third leading cause of cancer death worldwide (Yang *et al.*, 2009; Araújo *et al.*, 2011; Bray *et al.*, 2018). One of the most important contributors to main risk of colon cancer is dietary behavior, such as high-fat, low-fiber diet (Saetang and Sangkhathat, 2017; Siegel *et al.*, 2017).

In cancer cases surgery, radiotherapy and chemotherapy are still first chosen treatments. However, for over 40 years, humankind have benefited greatly from natural products in its struggles with cancer. The role of natural products is becoming an important research subject for drug discovery and development. Main sources of extracted natural products are microbes, plants and animals from terrestrial and marine environments (Cragg and Newman, 1999; Sarker *et al.*, 2005; Colegate and Molyneux, 2007). Although over 70% of the earth's surface is covered with aquatic ecosystems, in past decades, fewer studies are done in marine than terrestrial ecosystems. Crude substances extracted from marine creatures, especially invertebrate, such as crabs, shrimps, algae and fungi have unique chemical components, which are structurally diverse. Containing important bioactive compounds, they play a key role in inhibition of various stages of tumorigenesis associated with inflammatory processes. Natural marine based products from crabs (Mirzapur *et al.*, 2015), molluscs (Patra *et al.*, 2020; Summer *et al.*, 2020) and sea cucumbers (Cui *et al.*, 2020), and sessile organisms, such as corals, seaweeds and sponges (Conte *et al.*, 2021) have recently been considered as new therapeutic agents for cancer.

Crabs are an extraordinarily successful group, found all over the world. *Portunus segnis* is a marine nocturnal crab, native to northern and western Indian Ocean, from Pakistan westwards to Persian Gulf and Red Sea, and along eastern coasts of South Africa (Deidun and Sciberras, 2016; Fujaya *et al.*, 2016). Blue swimming crab species, *P. segnis*, belongs to Portunidae family. Among Portunidae crabs, *P. segnis* is one of the most commercially important true crabs in coastal waters of Persian Gulf and other areas worldwide (Hosseini *et al.*, 2014).

This species can inhabit a wide range of coastal and continental shelf ecosystems, including muddy, sandy and seagrass environments, from intertidal zone to at least 50 m depth (Rabaoui *et al.*, 2015). However, few studies have been done on advantages of natural products extracted from this crab as drug's raw material (Anjugam *et al.*, 2016; Bejaoui *et al.*, 2017; Hamdi *et al.*, 2018, 2020).

Many marine bioactive compounds from crabs, sponges, soft corals, bryozoans,

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cephalopods and echinoderms are identified and extracted, and used as new drugs to treat diseases such as cancer (James, 1994; Ibrahim *et al.*, 2017). Studies demonstrated that bioactive compounds, including fatty acids, oleic acid, linoleic acid, carotenoids, alanine-betaxanthin, peptides are extracted from various crab species (Tincu and Taylor, 2004; Hamdi *et al.*, 2020; Rehman *et al.*, 2020).

The main purpose of the present study was to investigate cytotoxic activity of hexane, butanol, ethyl acetate and water extracts from muscle of *P. segnis* and compare its treated HT-29 cells with different concentrations of crude extracts.

Materials and methods

Sampling and sample preparation

75 samples of the blue swimming crab, *P. segnis*, were collected from Bushehr, Iran (28° 54′ 83″ N and 50° 46′ 26″ E) in summer 2017. Sampling was done using gillnets and trawls. Afterward, all samples were frozen and placed in ice box and moved to Pasteur Institute of Iran laboratory for analysis (Sahebi *et al.*, 2018). Crabs were carefully washed with sterile distilled water and muscle tissues were separated and dried using freeze-drying method at -70°C.

For extraction of bioactive compounds from the crab's muscle, a volume of 600 mL of methanol/ ethyl acetate (1:1, v/v) mixture solution was added to 100 g of sample and let the prepared mixture to be incubated for 48 h. Then extracts were filtered, concentrated and kept in dark condition. Following the methodology, a volume of 250 mL distilled water was used for dilution and extraction of muscle extract, then using 250 mL of n-Hexane, ethyl acetate, and butanol the useful compounds were extracted in a separator funnel based on their polarity. Finally, the extract solvents were dried with freeze drier at -70°C (Sahebi *et al.*, 2020).

GC-MS analysis

Determination of extract contents was performed using Agilent 6890 gas chromatography-mass spectrometer (GC-MS) (Agilent Technologies, Santa Clara, CA) in Danesh Pazhoohan Payesh Amin laboratory. For detection of compounds, an electron ionization system with ionization energy of 70 eV was used. Inert helium gas was used as carrier at a flow rate of 1 mL/min. Temperature of injector and mass transfer line was set at 250 and 285°C, respectively. The oven temperature plan consisted of initial temperature of 70°C for 1 min, and then gradually increased to 280°C at a rate of 10°C/min. Total time of analysis was 35 minutes.

Cell culture and MTT assay

HT-29 human colon cancer cells and CCD 841 normal human colon epithelial cells were obtained from Pasteur Institute of Iran. To evaluate safety of the extracted crude, CCD 841 cell line was used as normal human cells. RPMI medium was employed to cultivate HT-29 cell line. The effect of different sample concentrations, including 0.001, 0.01, 0.02, 0.1 and 0.5 µg/mL on the cells was examined. Cytotoxicity of the

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extracts was evaluated using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

LDH release assay

Lactate dehydrogenase (LDH) release served as biomarker to determine cytotoxicity of compounds. LDH release assay was performed using Thermo Company protocol (cat. no. 88953) (Hajrezaie *et al.*, 2015a).

Caspase 3/7, 8 and 9 activities

Caspase 3/7, 8 and 9 activities were determined using the commercial kit Caspase-Glo® 3/7, 8 and 9 assays (Promega, Madison, WI). In brief, HT-29 cells (1.0×10^4 cells/well) were treated with different concentrations of butanol extract for 24 h. Pursuant to the manufacturer's protocol, after 30 min incubation at temperature room with the Caspase-Glo reagent (100 µL) the active caspases from apoptotic cells affected the cleavage of aminoluciferin-labeled synthetic tetrapeptide, causing release of the substrate for the luciferase enzyme. Caspase activities were determined using Tecan Infinite® 200 Pro (Tecan, Männedorf, Switzerland) microplate reader.

Reactive oxygen species (ROS) assay

HT-29 cells (1×10^4 cells/mL) were treated with different concentrations (1.25, 3.12, 6.25, 12.5, 25 µg/mL) of butanol extract and dimethylsulfoxide (DMSO) as negative control for 24 h. Then, cell lines were treated with dihydroethidium (DHE) dye for 30 min. Afterwards, cells were fixed and washed with wash buffer per manufacturer's instructions. DHE dye was oxidized to ethidium in attendance of superoxides. The fluorescence intensity was determined by fluorescent plate reader at extension wavelength of 520 nm and emission wavelength of 620 nm (Rouhollahi *et al.*, 2015).

Flow cytometry

To determine and confirm growth inhibitory activity of butanol extract of *P. segnis* muscle, this study evaluated induction of apoptosis by flow cytometry. This evaluation was based on measurement of DNA content of nuclei labeled with propidium iodide and annexin (Sigma-Aldrich) similar to the method of Gamet-Payrastre *et al.* (2000) with slight rectifications.

Statistical analyses

Predictive Analysis Software (PASW) v. 18 was used to analyze data in the present study. Experimental data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are triplicated and displayed as mean±standard deviation (SD). Level of significance was set at $p<0.05$.

Results

Cytotoxic effect of Portunus segnis muscle on HT-29 cells

To determine cytotoxicity of various fractions of *P. segnis* muscle on human cancer cells, MTT assay was used, and its results are shown in Table 1. The strongest and weakest inhibitory effect on HT-29 colon cancer cells were

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observed in butanol and ethyl acetate extracts with IC50 value of 10.12±0.35 and 48.14±0.62 µg/mL after 72 and 24h of treatment cells, respectively. According to the obtained results, the strongest suppressive effect on HT-29 cells observed in treatment with muscle butanol extract of *P. segnis*. It was also the most effective in subsequent evaluations (Table 1).

Table 1: Inhibitory effects of different *P. segnis* muscle extracts on proliferation of normal human (CCD841) and HT29 cell line.

Cell line		IC50 (µg/mL)		
		24 h	48 h	72 h
HT-29	hexane extract	35.27±0.71	25.07±0.68	19.25±0.22
CCD 841		50<	50<	50<
HT-29	butanol extract	26.63±0.20	15.13±0.21	10.12±0.35
CCD 841		50<	50<	50<
HT-29	ethyl acetate extract	48.14±0.32	34.63±0.38	22.86±0.51
CCD 841		50<	50<	50<
HT-29	H_2O extract	44.33±0.33	31.97±0.62	19.38±0.23
CCD 841		50<	50<	50<

Cells were treated with various concentrations for 24, 48 and 72 h. IC_{50} values were analyzed by nonlinear regression analysis.

LDH release assay

Results of LDH release assay illustrated that the cell line treated with butanol extracts of *P. segnis* induced significant elevation in LDH release (p<0.05), indicating cytotoxicity at 12 and 25 µg/mL concentrations in comparison with the control cells (Fig. 1).

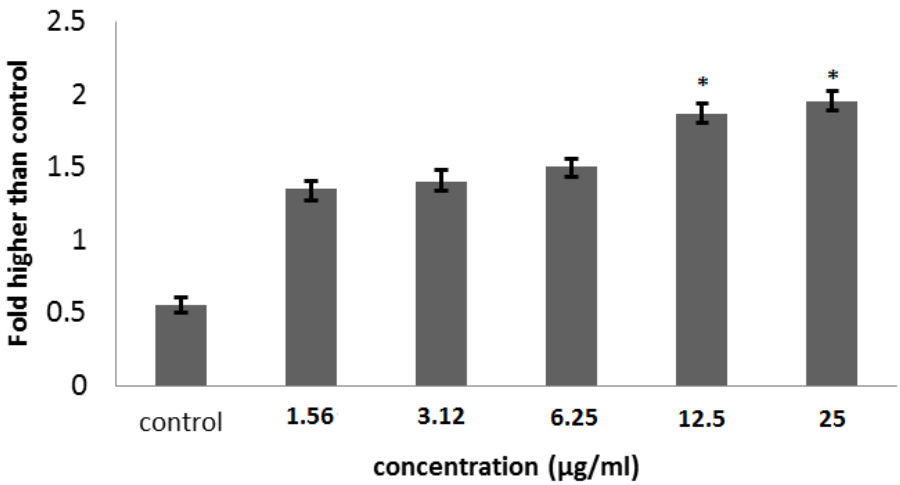

Figure 1: Mean lactate dehydrogenase (LDH) assay values demonstrating cytotoxicity of butanol extract of *P. segnis* against HT-29 cells. Results showed significant cytotoxicity at concentrations of 12.5 and 25 µg/mL. Error bars represent standard deviation. *Significantly different from control treatment cells (p<0.05).

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ROS analysis

ROS production plays a vital role in activation of mitochondrial-initiated events causing apoptosis. Accordingly, the apoptotic death caused by ROS-induced oxidation damage is a critical agent in cytotoxic effects of different natural compounds (Zorofchian Moghadamtousi *et al.*, 2014). As shown in Figure 2, exposure of HT-29 cells to concentrations of 12.5 and 25 µg/mL of butanol muscle extract led to significant increase in their generated ROS levels. ROS assay found oxidation of DHE to ethidium in presence of butanol muscle extract of *P. segnis* after 24 h of treatment.

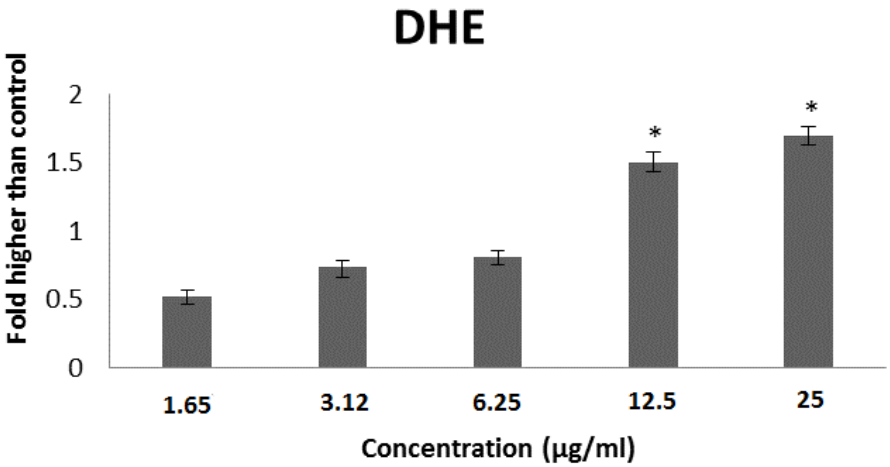

Figure 2: Mean ROS generation in presence of butanol extract at concentrations of 12.5 and 25 µg/mL, the butanol extract caused significant ROS formation in HT-29 cells. Error bars represent standard deviation. *Significantly different from control treatment cells ($p<0.05$).

Effect of butanol extract of P. segnis muscle on caspases 3/7, 8 and 9 activities

To assess the effect of butanol extract of *P. segnis* on activities of caspases 3/7, 8 and 9 in HT-29 cell line, the line was treated with different concentrations of butanol extract of muscle tissue of *P. segnis* for 24 h. The results showed significant increase of caspases activity 3/7 and 9 in 12.5 and 25 µg/mL doses compared with the control group ($p<0.05$), demonstrating activity of caspases 3/7 and 9 in dose-dependent manner. However, there was no significant difference in caspase 8 activity compared to control group ($p>0.05$, Fig. 3).

Flow cytometry analysis of cell apoptotic in HT-29 cells

The obtained results found significant increase in percentage of apoptotic cells in treated cells with 9 mg/mL of butanol extract of *P. segnis* compared with cells in the control groups for 24, 48 and 72 h (Fig. 4). Percentage of apoptotic cells were obtained in treated and control cells groups as 15.57 and 13.23% in 24 h, 27.39 and 12.4% in 48 h, and 30.43 and 7.95% in 72 h, respectively. Hence, in 72 h, the highest numbers of apoptotic cells were illustrated.

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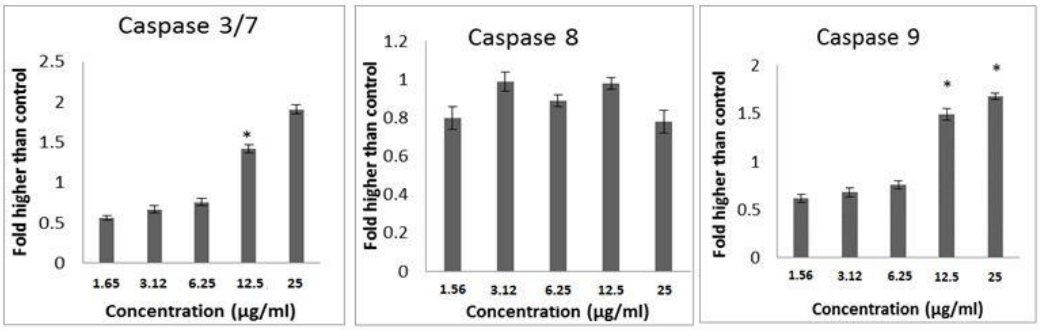

Figure 3: Mean effect of various concentrations of butanol extract on caspase 3/7, 8 and 9 activities on HT-29 cells: results revealed significant activation of caspases-3/7and -9. Error bars represent standard deviation. *Significantly different from control treatment cells (p<0.05).

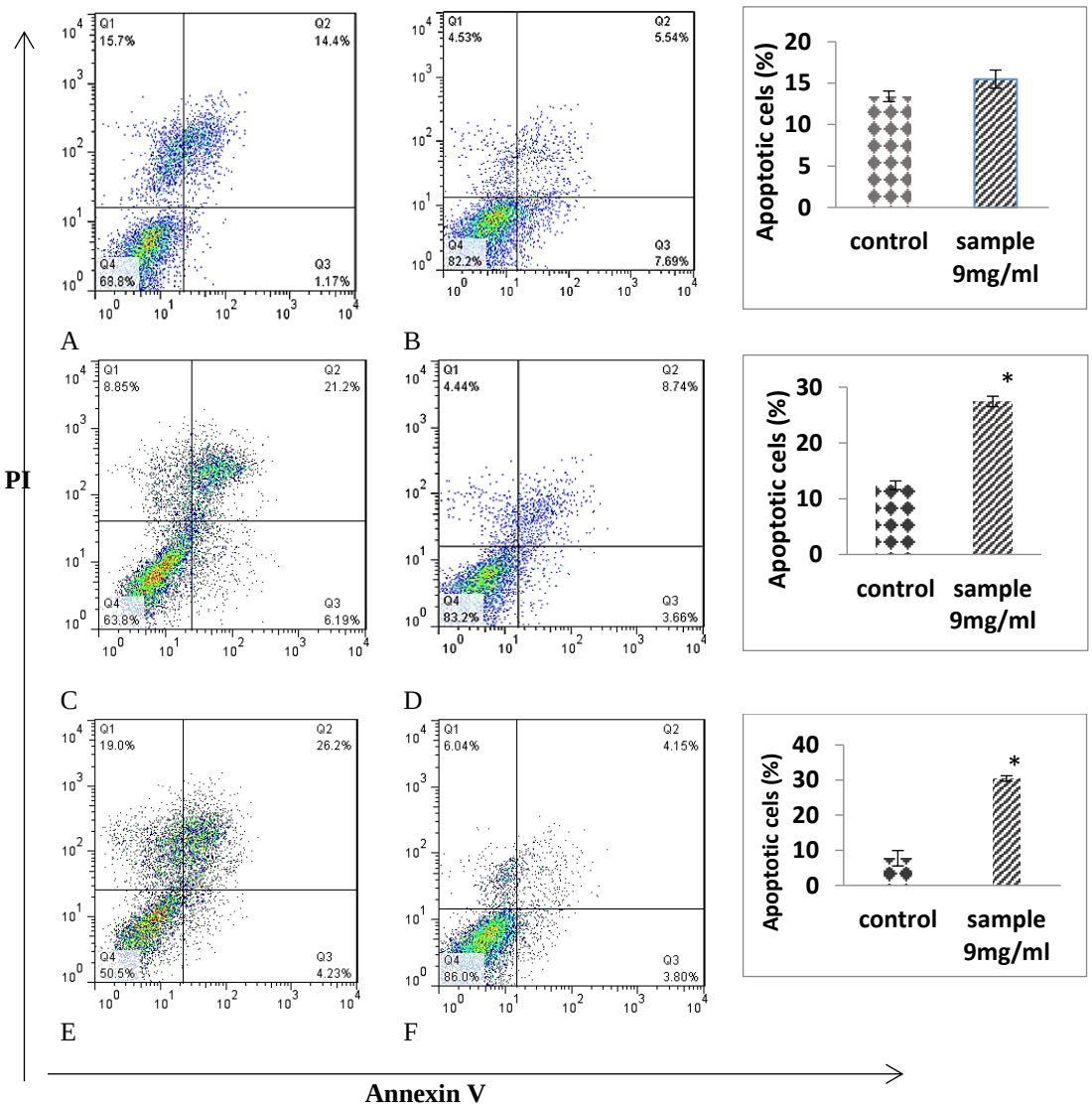

Figure 4: Apoptosis induced by butanol extract in HT-29 cells after treatment for (A) sample 24 h; (B) control 24 h; (C) sample 48 h; (D) control 48 h; (E) sample 72 h; (F) control 72 h. Error bars represent standard deviation. *Significantly different from control treatment cells (p<0.05).

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Extract composition of muscle

Butanol crude extract of muscle was analyzed using GC-MS, to identify different groups of organic compounds. Figure 5 displays GC MASS chromatogram of identified compounds of butanol crude extract from muscle of *Portunus segnis*.

The chemical compounds were identified by mass spectra of GC-MS according to their retention time on a fused-silica capillary column. A total of 15 compounds were identified, accounting for 68.47% of butanol crude extract.

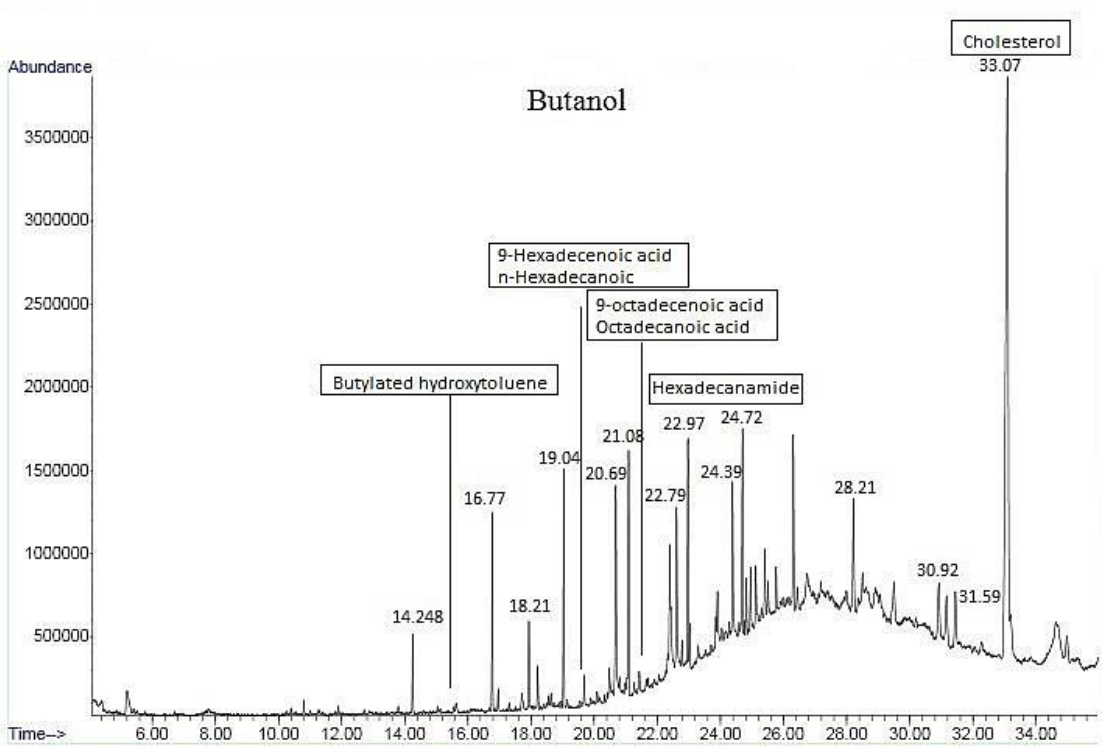

Figure 5: GC MASS chromatogram of identified compounds of butanol crude extract from muscle of *Portunus segnis*.

Discussion

It has been proven that natural products and their active constituents exert their chemopreventive effects in a wide range of colon cancer cell lines, such as HT-29, CaCo2 and HCT-116 (Rajamanickam and Agarwal, 2008). In the present study, evaluation of cytotoxicity of natural products of different doses of butanol, hexane, ethyl acetate and water extracts from muscle tissue of *P. segnis* was investigated on HT-29 colon cancer cell line. The results certified safety of extracted natural compound, while no effect was observed in normal cell line (CCD841). In addition, the results showed that inhibitory properties of muscle extracts on cancer cells were dose- and time-dependent. Some believe that crabs contain little fat, but generally crabs are very good source of unsaturated fatty

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acids (PUFA n-3), especially EPA and DHA (Ayas, 2016). Also high protein levels in *Portunus* sp. were reported (Gökoðlu and Yerlikaya, 2003; Kuley *et al.*, 2008).

Over the past few decades, many studies are conducted to evaluate the effects of unsaturated fatty acids on colon cancer (Ayas, 2016; Eltweri *et al.*, 2017). Research has illustrated a clear role for omega-3 PUFAs in preventing cancer extension at various stages including cancer cell proliferation, survival, inflammation, angiogenesis and metastasis (Eltweri *et al*., 2017). Therefore, these attributes of omega-3 PUFAs suggest that they have inhibitory properties in cancer handling (Anti *et al.*, 1992; Jordan and Stein, 2003; Berquin *et al.*, 2008; Volpato and Hull, 2018). Accordingly, one of the reasons behind anticancer properties of *P. segnis* extracts is anticancer effects of unsaturated fatty acids.

In the present study, butanol extract of muscle of *P. segnis* could be a promising source of drug development process owing to the fact that it demonstrates a high antiproliferative effects of butanol extract on HT29 cell lines. According to results of GC–MS, bioactive compounds including palmitoleic acid, 9-octadecenoic acid, eicosane, hexacosane, (Z)-9-octadecenamide and n-hexadecanoic acid, oleic acid and 9-octadecanoic acid were identified in butanol extract of muscle of *P. segnis*. For example, hexadecanoic acid has significant biological functions, such as antioxidant, anti-inflammatory and antimicrobial activities (Yu *et al.*, 2005; Ukwubile *et al.*, 2019). Relatively polar solvents such as butanol are ideal solvents for releasing all lipids from cell membranes or lipoproteins. Accordingly, completely polar solvents such as water and methanol are not suitable for lipid extraction due to their chemical reaction with lipids. As results of qualitative analysis of GC-MS showed, chemical compositions that were characterized in butanol crude extract of muscle were cholesterol (30.96%), palmitoleic acid (4.83%), 9-octadecenoic acid (4.82%), docosane (4.66%), eicosane (4.34%), hexadecane (3.21%), heptadecane (3.72%), hexacosane (3.21%), tricosane (2.36%), (Z)-9-octadecenamide (2.73%), 1-phenanthrenecarboxylic acid (1.55%), tetradecane (0.31%), palmitamide (0.84%), phthalic acid (0.51%) and butylated hydroxytoluene (BHT) (0.42%). Cancer inhibitory properties of butanol extract can be considered due to the presence of these compounds as well as the synergistic and co-existing effects of such materials (Fini *et al.*, 2008).

Oleic acid has a significant effect in activation of various intracellular pathways, including carcinoma cell development (Karan and Erenler, 2018). Anticancer effects of 9-octadecenoic acid and (Z)-9-octadecenamide compounds have been reported on prostate cancer cells (Yu *et al.*, 2005; Ukwubile *et al.*, 2019). Likewise, eicosane is reported to exhibit anticancer activities; eicosane produces cytotoxicity both in vitro and in nude

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mice bearing human ovarian carcinoma cells (Mishra *et al.*, 2019).

The results of the MTT and LDH assays clearly displayed that butanol extract of muscle tissue of *P. segnis* caused inhibition of cell population growth of HT29 cells. It is reported that different cytotoxicity assays can have different results, depending on employed cytotoxicity assay and used agent (Mehdi *et al.*, 2011). However, LDH assay is based on release of the enzyme into the culture medium after cell membrane damage, whereas MTT assay is usually based on enzymatic conversion of MTT in the mitochondria. Note, however, that both cytotoxicity assays, applied in vitro to assessable butanol extract toxicity showed similar results (Mehdi *et al.*, 2011).

Cytotoxic effect of butanol extract was also confirmed by measuring release of lactate dehydrogenase from the exposed cells. According to the results obtained in the present study, significant toxicity was observed at different concentrations, 12.5 and 25.5 µg/mL, of butanol extract. Therefore, significant release of LDH showed that toxicity of butanol extract was potentially lost through membrane integration, which occurs through activation of apoptosis or necrosis pathway (Choi *et al.*, 2012; Hajrezaie *et al.*, 2014). Increased release of lactate dehydrogenase as a result of cell apoptosis, colon cells of CaCO2 cell line confirm the results of the present study regarding release of LDH during apoptosis (Manerba *et al.*, 2017). Inhibitory properties of Lnula graveolens on cell lines of HT-29, A549, MCF7 and changes in LDH enzyme levels is investigated and results showed the highest cytotoxic effect related to the cell line HT-29, and as a result of cell death, LDH levels increased (Karan and Erenler, 2018).

Apoptosis, a type of programmed cell death, operates by various extracellular and intracellular signals (Xu *et al.*, 2017). Apoptosis plays an important role in health of the body. Impaired apoptosis is involved in a range of diseases such as cancers, due to some extent to the decrease in cell death.

In the current study, flow cytometry analysis of HT29 cell line exposed to butanol extract of *P. segnis* demonstrated that apoptotic cells increased in a time-dependent manner. Another study showed that apoptosis proportion of cells was increased by treatment by natural compounds on various cancerous cells, such as cervical cancer cell lines (Mehdi *et al.*, 2011), colon cancer HT29 and CaCO2 (Esmaeelian *et al.*, 2013, 2014), breast cancer (Zhong *et al.*, 2012) and primary gastric and HepG2 cells (Specian *et al.*, 2016). Disorder of mitochondrial membrane potential is one of the first intracellular events occurring after induction of apoptosis. According to the research, balance between ROS levels in the body is key to cellular function and to apoptotic pathway in precancerous cells (Hajrezaie *et al.*, 2015b).

The result of ROS assay illustrates that generation of ROS levels increased in a dose-dependent manner. Agarwal *et al.* (2018) showed that the curcumin-

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induced ROS generation in HT-29 cells led to DNA fragmentation, and significantly increased apoptotic cells in a dose-dependent manner. Moreover, Kee *et al.* (2016) examined properties of quercetin natural compounds on the colon 38 (MC38) and CT26 cell lines, and their results were similar to those of the present study, and properties of inhibitory compound and cell apoptosis are proven. According to the results of Chen *et al.* (2011), inhibitory properties of natural compounds of *Ginkgo biloba* extract on colon cancer of the HT-29 cell line were confirmed, showing that, the rate of caspase 3 has significant increased. In the present study, butanol extract caused a significant increase in caspase activities of 7/3 and 9. While caspase 8 did not show any significant difference compared with the control group, suggesting that the apoptosis induced in HT-29 cells by the intrinsic mitochondrial pathway, whereas the caspase 8 pathway is dependent on the external death receptor.

The results showed that butanol extracts of muscle of *Portunus segnis* had anti-cancer properties on HT-29 cell line. In addition, treatment of HT-29 cells with butanol extract significantly produced reactive oxygen species, which led to mitochondrial events and eventually cell apoptosis. Role of the mitochondrial-dependent apoptotic pathway was also demonstrated by significant activation of caspase 9 and caspase 3 and 7. This study can be beneficial as a potential candidate for development of anticancer agents.

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