Nitric oxide synthesis inhibition and cytotoxicity of Korean horse mussel *Modiolus modiolus* extracts on cancer cells in culture

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Abstract The Korean horse mussel extract was purified and fractionated by a bioassay-guided purification step. The final fraction contained seven steroid and one polycyclic aromatic compounds, in which cholest-7-en-3-ol, (3 β ,5 α)- (58.7 %) was a main component followed by ergosta-7,22dien-3-ol (3 β ,5 α ,22E) (13.0 %). This extract exhibited strong anti-inflammatory activity determined solely through the nitric oxide inhibition assay in a dose-dependant manner with the IC₅₀ value of 9.6 μ g/mL and no cytotoxic effect on the macrophages. Moreover, it also exhibited strong cytotoxicity with the IC₅₀ values of 21.4, 36.4, and 37.1 μ g/mL against AGS, DLD-1, and HeLa cells, respectively. These results indicated that the horse mussel extract might be a functional ingredient in the prevention of inflammation and human cancers.

Keywords Anti-inflammatory · Cytotoxic · Steroid · Horse mussel

Introduction

The use of natural products as anti-inflammatory and anticancer agents has a long history that began with folk medicine and has been incorporated into traditional and allopathic medicine through the years. Several drugs currently used in chemotherapy were isolated from plant/animal species or derived from a natural prototype.

NO is a reactive radical molecule produced from the guanidino nitrogen of L-arginine, which is oxidized by NO synthase (NOS). NO is essential for host innate immune responses to pathogens such as bacteria, viruses, fungi, and parasites (Bogdan et al. 2000). However, the excessive NO production can result in the development of inflammatory diseases such as rheumatoid arthritis and autoimmune disorders (Yoon et al. 2009). Thus, the inhibition of NO production is a major target for the development of anti-inflammatory agent.

DNA, cell membranes, proteins, and other cellular constituents are target sites of the degradation processes, and consequently induce different kinds of serious human diseases including neurological disorders and some types of cancer (Sahreen et al. 2010). Cancer is a major cause of mortality worldwide. Therefore, chemoprevention has been developed as a promising cytotoxic approach for reducing the morbidity and mortality of cancer.

Marine organisms are attractive sources of novel and biologically-active compounds due to their tremendous biodiversity. Over the past two decades, about 3,000 new compounds have been discovered

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from various marine sources, and some of these compounds have been employed in clinical therapies (Kwon et al. 2007). The solvent extracts from fisheries such as seaweeds (Yuan and Walsh 2006), ascidian (Schupp et al. 2001) and mollusc (Zhang et al. 2008) were reported to have inhibitory effects against inflammation and cancer. The molluscs have become one of the most important sources of marine natural products in the last decades, and a significant number of compounds with unusual structures and bioactivities have been isolated from various molluscs. There are a few reports on the biological activities of shellfishes origin steroid compounds including cholesterol: a mediator of inflammation (Antonio et al. 2011), proinflammatory cytokine and TNF- α (Muthusamy et al. 2011), anti-inflammatory activity of New Zealand green-lipped mussel (Halpern 2000), antibacterial activity of horse mussel *Modiolus modiolus* (Haug et al. 2004), and antioxidant activities of black mussel *Mytilus galloprovincialis* (Gorinstein et al. 2003). In particular, molluscs are considered to be one of the most promising sources due to their variety of species and applications.

The mussel is a benthic, filter-feeding organism which can thrive on hard surfaces, and has been used as food for thousands of years. *M. modiolus* belongs to Bivalvia class, Mollusca Phylum, Animalia Kingdom. The pharmacological functions of mussel include antibacterial and antiviral (Defer et al. 2009), anti-cyclooxygenase (McPhee et al. 2007), antioxidant (Kaloyianni et al. 2009), agglutinating (Jayaraj et al. 2008), and anti-inflammatory activity (Singh et al. 2008). Even though horse mussel is abundant and ubiquitous in the oceans of Korea and Japan, it has not been utilized as food or other ingredients because of no deliciousness. The objectives of this study were to investigate the anti-inflammatory and cytotoxic activities of horse mussel against the macrophage cell line RAW 264.7 and three typical human cancer cells, and then to isolate and characterize the anti-inflammatory and anticancer compounds.

Materials and methods

Materials

Live specimens of horse mussel *M. modiolus* with about 10 cm length were collected in August 2012 at

Jollanam-do, Korea. Samples were brought to the laboratory in fresh condition, immediately dissected, rinsed with tap water to eliminate contaminant, and then stored at -40°C until used. The horse mussel was thawed at 4°C overnight and then sliced with knife. The horse mussel was placed in a stainless steel container, frozen in liquid nitrogen, and then ground in a blender.

Reagents

Dimethyl sulphoxide (DMSO), lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8, N $^{\Psi}$ -Nitro-L-Arginine methyl ester hydrochloride (L-NAME), ascorbic acid, α -tocopherol, Griess reagent, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Lonza (Walkersville, MD, USA). All other chemicals and reagents were of analytical grade commercially available.

Cell lines and culture

The macrophage cell line RAW 264.7 (Korean Cell Line Bank, Seoul, Korea) was grown in a plastic culture flask in RPMI-1640 with L-glutamine supplemented with 10 % FBS and 1 % antibiotic/antimycotic solution (penicillin/streptomycin) under 5 % CO_2 at 37°C . After 3 days, cells were removed from the culture flask by scraping and centrifuging at $420\times g$ for 3 min (Eppendorf, Hamburg, Germany). The medium was then removed and the cells were resuspended with 4 mL of fresh RPMI-1640. Three cancer cell lines, namely human gastric carcinoma cell (AGS, ATCC $^{\text{®}}$ CRL-1739 $^{\text{TM}}$), human colon cancer cell (DLD-1, ATCC $^{\text{®}}$ CCL-221 $^{\text{TM}}$), and human cervical cancer cell (HeLa, ATCC $^{\text{®}}$ CRM-CCL-2 $^{\text{TM}}$) (ATCC, Rockville, MD, USA) were grown in RPMI-1640 supplemented with 10 % FBS, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a humidified atmosphere of 5 % CO_2 .

Preparation and purification of the horse mussel extract

The body fluid of fresh horse mussel was removed and then the body wall was washed with tap water. The

body wall (1 kg) was cut into small pieces and then extracted overnight with 2 L of 95 % EtOH at 4 °C, which was repeated three times. The combined extracts were filtered and then evaporated in a vacuum at 40 °C to dryness yielding the crude extract (52.3 g), and then suspended in water (500 mL) in a separatory funnel. The solution was partitioned with hexane to give the hexane fraction (11.8 g). Next, the aqueous layer was successively partitioned with each of CHCl₃ and EtOAc to give the CHCl₃ (2.5 g) and EtOAc (3.4 g) fraction, and aqueous residue (26.7 g), respectively.

Since the hexane fraction resulted in high anti-inflammatory and cytotoxic activities as well as high extraction yield, this was coarsely fractionated on a silica gel column (3.0 × 45.0 cm) chromatography by step-gradient elution with hexane:EtOH solvent system in increasing polarity (100:0 to 0:100, v/v, 400 mL) to give nine fractions (HM₁–HM₉). The highest yield fraction HM₄ was further fractionated on LH-20 gel filtration column (3.5 × 36.0 cm) using MeOH as an eluent to give two fractions, HM_{4,1} and HM_{4,2}. The fraction HM_{4,2} was further purified with a recycling preparative HPLC LC-9104 (Japan Analytical Industry Co. Ltd., Tokyo, Japan) equipped with a JAIGEL-GS310 column (21.5 × 500 mm) by eluting with CHCl₃:MeOH:Water (5:1:0.1) (v/v/v). The elution profile was monitored at 254 nm. Each elution gave 5 fractions (HM_{4,2,1}–HM_{4,2,5}). The solvent in each fraction was removed using a vacuum evaporator and finally vacuum-dried in a vacuum drying oven. The fraction HM_{4,2,4} was then subjected to anti-inflammatory and cytotoxic assays.

For GC/MS analysis, a system combining a GC 7890A with a quadrupole MS 5975C (Agilent Technologies, Waldbronn, Germany) and A HP-35 column (30 m × 0.25 mm i.d., 0.25 µm film thickness) was used. The injection volume was 2 µL and the injection port temperature was 250 °C. Temperature of the column was started at 100 °C for 2 min, raised to 300 °C at 20 °C/min, and held for 6 min. Helium was used as a carrier gas at linear flow rate of 1 mL/min. All spectra were scanned within the range m/z 33–600.

Anti-inflammatory activity

Anti-inflammatory activity was assayed according to the modified method of Abas et al. (2006), which was determined solely through the nitric oxide inhibition

assay. Briefly, RAW 264.7 cells were seeded in 96-well flat-bottom tissue culture plates (1 × 10⁵ cells/100 µL) and incubated at 37 °C in 5 % CO₂ for 24 h. Cells were then stimulated with 200 µL solution containing the extract, LPS, and RPMI 1640 without phenol red for 24 h. One hundred µL of cell culture supernatant was combined with 100 µL of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 540 nm using a microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) after 10 min. The hexane fraction was prepared from a stock (1,000 µg/mL) in 1 % DMSO and then added to the RPMI 1640 medium at concentrations range of 5–50 µg/mL.

The percentage of nitric oxide inhibition was calculated based on the ability of extract to inhibit nitric oxide formation by cells compared with the control. The volume of extracts added to the cell culture medium was accurately performed as an equivalent amount in the control group. A commercial drug, N^ω-Nitro-L-Arginine methyl ester hydrochloride (L-NAME), was used as a positive control. The result was expressed as percentage of NO production compared to the control as the follows:

$$\text{NO inhibition (\%)} = 100 \times \frac{[(\text{NO}_2^-)_c - (\text{NO}_2^-)_s]}{(\text{NO}_2^-)_c}$$

where (NO₂[−])_c was the concentration of nitrite released by cells without the addition of the extract, and (NO₂[−])_s was the concentration of nitrite released by the cells in the presence of the extract.

Cytotoxic activity on RAW 264.7 cells

To determine that the observed nitric oxide inhibition was not false positive due to cytotoxic effects, a cytotoxicity assay on RAW 264.7 cells was also performed according to the method of Abas et al. (2006). RAW 264.7 cells at a density of 1 × 10⁵ cell/well were plated in a 96-well flat-bottom tissue culture plate (SPL Life Sciences Inc., Gyeonggi-do, Korea), and then incubated at 37 °C for 3–4 h. Plated cells were then treated at four different concentrations (100, 250, 500, 1,000 µg/mL). After incubated for 24 h, 100 µL of MTT (2 mg/mL) was added to each well and then incubated at 37 °C for 4 h in 5 % CO₂. The medium was then carefully discarded and the formed formazan salt was dissolved in 120 µL DMSO. The

plates were placed at room temperature for 5 min with a mild shaking. One hundred μL of the MTT formazan was transferred to a new 96-well flat-bottom tissue culture plate. The absorbance was determined at 540 nm by UV-spectrophotometric plate reader (Molecular Devices Inc.). Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cell to untreated cell.

Cytotoxicity on AGS, DLD-1, and HeLa cancer cells

The cytotoxicity of the horse mussel solvent extract against AGS, DLD-1, and HeLa cancer cells were assayed according to the modified method of Kim et al. (2010a, b). Cells were cultured in RPMI-1640 medium with L-glutamine supplemented with heat inactivated 10 % fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ streptomycin and 100 U/mL penicillin at 37 °C in 5 % CO_2 . For experiments, cells were plated in a 96-well flat-bottom tissue culture plate (10^4 cell/well in 100 μL of medium). After 4 h, the extracts dissolved in PBS were added to each well and incubated for 72 h. At the end of 72 h incubation, PBS (100 μL) containing 2 mg/mL of MTT was added in each well. Three hours later, the formazan product of MTT reduction was dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured with a microplate reader at 540 nm. The inhibition of cellular growth by the tested sample was calculated as the percent inhibitory activity and expressed as the IC_{50} value (the concentration of the tested sample to inhibit 50 % growth of the cell).

Statistical analysis

All assays were carried out in at least triplicates and results are expressed as mean $\pm$ SD. ANOVA test was used to analyze the differences among fractions for different bioactivity assays with least significance difference (LSD) at $p < 0.05$ as a level of significance.

Results and discussion

Extraction yield

Fresh horse mussel extracted with 95 % EtOH resulted in 5.2 % of yield (w/w) (Table 1). It was found that the

partition of the EtOH extract with water produced the highest yield (51.0 %, w/w), whereas the yield with CHCl_3 was lowest (4.3 %, w/w). This result suggested that most compound of the horse mussel extract consisted of polar compounds and more water-soluble substances, which was proven by the lower yield in hexane (22.6 %, w/w) and EtOAc fraction (6.5 %, w/w). The highest yield of water fraction in this partition was in line with other studies; green seaweed *C. fulvescens*, *C. monoiliger*, and *U. pertusa* (Cho et al. 2010), and medicinal herb *Smilax excelsa* L. leaf extract (Ozsoy et al. 2008), where the water fraction resulted in the highest extraction yield. These results indicated that each solvent fraction could be different in both the composition and the ratio of their constituents.

Anti-inflammatory activity

The horse mussel solvent extract attenuated NO production in a dose-dependent manner (Fig. 1a). At 100 $\mu\text{g}/\text{mL}$, the EtOAc fraction inhibited 93.2 % of NO production (IC_{50} value, 29.3 $\mu\text{g}/\text{mL}$), which was much higher than those of the other fractions (Table 1). The beneficial effect of the horse mussel extract and its solvent fractions on the inhibition of inflammatory mediators production in macrophages may be mediated by oxidative degradation of phagocytes products such as O_2^- and HOCl (Loizzo et al. 2009). The decline in NO production may be attributed to the decrease of inducible NO synthase (iNOS) at mRNA or protein level and the respective alterations of iNOS gene transcription (Wu et al. 2008). New Zealand green-lipped mussel powder (lyprinol) had significant anti-inflammatory activity when given to animals and humans (Halpern 2000), where much of this activity was associated with lipid (omega-3 PUTAs) and carotenoids. 5 β -Scymnol from mussel oil *Perna canaliculus* prevented the UV damage to melanocytes and regulated the proinflammatory cytokine and $\text{TNF-}\alpha$ (Muthusamy et al. 2011). However, there is no detailed information on the anti-inflammatory compounds of horse mussel.

Cytotoxic activity on RAW 264.7 cell

To ensure that the horse mussel extract did not interfere with the survival of the macrophages, the cytotoxic effects of these extracts on macrophages RAW 264.7 was determined by MTT assay. After 24 h

Table 1 Anti-inflammatory activity and cytotoxicity against cancer cells of the horse mussel solvent extract

Fraction	Extraction yield (% w/w)	Anti-inflammatory activity (IC ₅₀ , µg/mL)	Cytotoxicity (IC ₅₀ , µg/mL)		
			AGS	DLD	HeLa
EtOH	5.2 ^a	1,910.2	>1,000	>1,000	>1,000
Hexane	22.6 ^b	290.2	382.4	844.1	561.4
CHCl ₃	4.3 ^b	1,009.6	184.0	187.6	365.5
EtOAc	6.5 ^b	29.3	>1,000	>1,000	>1,000
Water	51.0 ^b	1,821.0	>1,000	>1,000	>1,000
L-NAME ^c		50.0	–	–	–
5-Fluorouracil ^d		–	5.5	34.5	3.4
Paclitaxel ^d		–	3.2	24.6	2.2

^a Percentage of the fresh horse mussel^b Percentage of the dried EtOH extract^c Anti-inflammatory positive control^d Cytotoxic positive control **Fig. 1** Biofunctional activities of the horse mussel solvent extract in RAW 264.7 macrophages. **a** anti-inflammatory activity, **b** cell viability. Means in the same group of bar graph with different superscript letters are significantly different ($p < 0.05$)

incubation, the EtOH extract of horse mussel and its fractions (hexane, CHCl₃, EtOAc, and water) at 100–1,000 µg/mL did not significantly alter cell viability (Fig. 1B). The cell viability in all fractions was >90 %, which was similar to that of L-NAME as a positive control. Therefore, the horse mussel extract exhibited no cytotoxic effects at the concentration tested in this study.

Cytotoxicity on human cancer cells

The EtOH extract of horse mussel inhibited the growth of AGS, DLD-1, and HeLa cancer cells in a concentration-

dependent manner (Fig. 2). There was a considerable difference in the sensitivity of these cells to the EtOH and EtOAc extracts. The DLD-1 cell was more resistant to the horse mussel extract than were AGS and HeLa cells. At 1,000 µg/mL, the EtOH extract inhibited the growth of AGS, DLD-1, and HeLa cells line by 22.1, 17.0, and 28.1 %, respectively. The cytotoxicity of the EtOH extract against HeLa was relatively higher than 0.7–7.8 % of the EtOH extract of various edible seaweeds including *L. setchellii*, *M. integrifolia*, and *N. Leutkeana* against the same cell at the same concentration (Yuan and Walsh 2006), but lower than 78.8 and 95.5 % of the EtOH extract of *C. pilulifera* against HeLa cell at

Fig. 2 Cytotoxicity of the horse mussel solvent extract against **a** AGS, **b** DLD-1, and **c** HeLa cancer cell. Means in the same group of bar graph with different superscript letters are significantly different ($p < 0.05$)

200 µg/mL after treatment for 3 and 7 days, respectively (Kwon et al. 2007). In order to isolate and identify the bioactive compound responsible for the cytotoxicity, the EtOH extract was partitioned successively with hexane, CHCl₃, and EtOAc. The CHCl₃ fraction inhibited more significantly the growth of cancer cells than the EtOH extract/other fractions. The IC₅₀ values of the CHCl₃ fraction against AGS, DLD-1, and HeLa cell were 184.0, 187.6, and 365.5 µg/mL, respectively. This was in line with the finding of Kim et al. (2010a, b) where the CHCl₃ fraction of herb *C. grandis* Osbeck (Dangyuja) had the highest cytotoxic activity. This fraction may alter the regulation of the cell cycle machinery, resulting in cellular arrest at different phases of the cell cycle and, thereby, reducing the growth and proliferation of cancerous cell (Hseu et al. 2008). Wang et al. (2006) reported that the cytotoxic activity might result, at least in part, from the inhibition of DNA synthesis, proliferation, as well as apoptosis induction of cancer cell. Moreover,

the hexane fraction inhibited the growth of cancer cells moderately with the IC₅₀ values of 382.4, 844.1, and 561.4 µg/mL against AGS, DLD-1, and HeLa cell, respectively (Table 1). The water fraction exhibited a very low cytotoxic activity which might be due to the elimination of cytotoxic active lipid-soluble compounds. These considerable variations in the cytotoxic activity between the extract and samples are probably due to the various chemical compositions of different species, anatomical regions, and extraction and purification procedures as well as the use of different cancer cells. Since the EtOAc fraction had the highest anti-inflammatory activity, whereas the CHCl₃ fraction had the highest cytotoxic activity against three different cancer cells, it was indicated that anti-inflammatory compounds in the EtOAc fraction might be different from the cytotoxic compound in CHCl₃ fraction. However, the hexane fraction exhibited potent both anti-inflammatory and cytotoxicity activities against three different cancer cells

as well as higher yield than the other fractions. Therefore, the hexane fraction was used for further purification using a thin layer, silica gel, LH-20 gel filtration chromatography, and recycling preparative HPLC in order.

Purification of anti-inflammatory and anticancer compounds

The hexane fraction of horse mussel was further purified by a thin layer and silica gel chromatography, respectively, to give nine fractions (HM₁–HM₉). The fraction HM₁–HM₉ were then tested for their anti-inflammatory activity determined solely through the nitric oxide inhibition assay. The fraction HM₄ with the highest yield exhibited moderate anti-inflammatory activity, in which NO release was 56.9 % at 250 µg/mL (IC₅₀ value, 211.5 µg/mL). The cytotoxicity of the fraction HM₄–HM₉ against AGS, DLD-1, and HeLa cancer cells were also determined. The fraction HM₄ inhibited the growth of AGS, DLD-1, and HeLa cells at 100 µg/mL by 27.4, 14.5, and 19.9 %, respectively. The inhibitory activity of the fraction HM₄ was stronger against AGS cell proliferation, while relatively lower against HeLa and DLD-1 cells at the concentration range of 100–1,000 µg/mL.

Considering the extraction yield and activity, only the fraction HM₄ was chosen for further purification by Sephadex LH-20 gel filtration chromatography and eluted with MeOH. The resulting fraction, HM_{4,1} and HM_{4,2}, were then collected, evaporated and assayed for their activity. As shown in Fig. 3, HM_{4,1} and HM_{4,2} at 50 µg/mL significantly inhibited NO release by 88.2 and 96.9 %, respectively, which suggested that the NO inhibitory compounds would be contained in these fractions.

The fractions HM_{4,1} and HM_{4,2} were also tested for their inhibitory activities against cancer cells. At 100 µg/mL, HM_{4,2} significantly inhibited the growth of AGS and HeLa cell by 93.7 and 87.7 %, respectively. However, the 51.5 % inhibition on DLD-1 was relatively lower at the same concentration (Fig. 4). Moreover, at 100 µg/mL, HM_{4,1} moderately inhibited the proliferation of AGS, DLD-1 and HeLa cancer cells by 47.6, 28.2, and 56.5 %, respectively. It was found that the fraction HM_{4,2} with lower molecular weight had more potent inhibitory activity against the growth of AGS, DLD-1, and HeLa cells than HM_{4,1}. This might be due to the differences in the molecular weight and the efficacy of the extracted

Fig. 3 Effect of the horse mussel HM_{4,1} and HM_{4,2} fractions on LPS-induced nitrite production in RAW 264.7 macrophages. Means in the same group of bar graph with different superscript letters are significantly different ($p < 0.05$)

fractions. It is also assumed that the HM_{4,2} fraction with low molecular weight may have greater molecular mobility and diffusivity than the high molecular weight HM_{4,1} fraction, which appears to improve the interaction with cancer cell components, and thus enhanced cytotoxic activity. This finding was in line with the results of Chen et al. (2010) where low molecular weight OCAP-3-1 (22.8 kDa) from Chinese folk medicine *Ornithogalum caudatum* Ait exhibited stronger cytotoxic activities against human leukemic K562 cells than high molecular weight OCAP-3-2 (46.4 kDa).

The fraction HM_{4,2} with higher anti-inflammatory and cytotoxicity against three different cancer cells was further purified on recycling preparative HPLC to give 5 subfractions, where the subfraction HM_{4,2,4} had the highest yield (0.05 % of the dried of EtOH extract). Hence, HM_{4,2,4} was further analyzed using gas chromatography/mass spectrometer to give 8 compounds (Fig. 5). The spectra in Fig. 5 were compared with Wiley 7N library, resulting in seven steroid compounds and one polycyclic aromatic compound (Table 2). The steroid compound based on area containing cholest-7-en-3-ol, (3 β ,5 α)-(58.7 %), ergosta-7,22dien-3-ol (3 β ,5 α ,22E) (13.0 %), stigmast-7-en-3-ol, (3 β ,5 α ,24S) (6.9 %), two desmosterols (4.8 and 3.5 %), androstan-11-one,3-(acetyloxy)-17-iodo-, (17 α)- (4.2 %), and cholest-5-en-3-ol (3 β) (1.8 %), while polycyclic aromatic compound was 9,10,12-trimethyl-benz(A)acridine (1.8 %).

Fig. 4 Cytotoxicity of the horse mussel HM_{4.1} and HM_{4.2} fractions against **a** AGS, **b** DLD-1, and **c** HeLa cancer cell. Means in the same group of bar graph with different superscript letters are significantly different ($p < 0.05$)

The subfraction HM_{4.2.4} exhibited the strong anti-inflammatory activity in a dose-dependent manner. At 50 µg/mL, it inhibited NO production as high as 100 % with a IC₅₀ value of 9.6 µg/mL (Fig. 6a). Additionally, there was no cytotoxic effect at the concentration tested. Moreover, The subfraction HM_{4.2.4} also exhibited excellent cytotoxicity against AGS, DLD-1, and HeLa cells, in which its IC₅₀ values were 21.4, 36.4, and 37.1 µg/mL, respectively (Fig. 6b). The cytotoxicity of this fraction against cancer cells was comparable to those of the positive controls, Paclitaxel and 5-Fluorouracil, in which the IC₅₀ values of 5-Fluorouracil were 5.5, 34.5, and 3.4 µg/mL against AGS, DLD-1, and HeLa cancer cells, respectively (Table 1). On the other hand, Paclitaxel had the strongest cytotoxicity against cancer cells with the IC₅₀ values of 2.2, 24.6, and 3.2 µg/mL against AGS, DLD-1, and HeLa cancer cells,

respectively (Table 1). The steroids may exert as cytotoxic agent mediated by multiple signalling pathways. The growth of cancer cells might be inhibited through the interaction with tubulin in which it promotes the polymerisation of tubulin, thereby causing cell death by disrupting the normal microtubule dynamics required for cell division and vital interphase processes (Cragg and Newmon 2005). Furthermore, the steroids also inhibit STAT3 proteins (signal transducers and activators of transcription), which are important in cancer cell survival and proliferation (Aggarwal and Shishodia 2006). In addition, the mussel oil extract did not affect the release of TNF-α, an inflammatory and cancer mediator, by suppressing p38 MAPK pathway in human melanocyte cell (Muthusamy et al. 2011). On the contrary, cholesterol treatment increased endothelial nitric oxide synthase (eNOS) expression (synthesis of

Fig. 5 Ion chromatogram of HM_{4.2.4} analyzed with gas chromatography/mass spectrometer. The spectra were compared with the Wiley 7 N library

Table 2 Chemical composition of the fraction HM_{4.2.4} from the horse mussel *M. modiolus* extract analyzed with GC/MS

Peak	RT (min)	Library	Mol. formula	Mol. weight	Match (%)	Area (%)
1	11.467	Cholest-5-en-3-ol (3β)	C ₂₇ H ₄₆ O	386.36	99	1.8
2	11.867	Desmosterol	C ₂₇ H ₄₄ O	384.34	94	3.5
3	12.141	Desmosterol	C ₂₇ H ₄₄ O	384.34	90	4.8
4	12.822	Cholest-7-en-3-ol, (3β,5α)-	C ₂₇ H ₄₆ O	386.36	94	58.7
5	13.652	Ergosta-7,22dien-3-ol (3β,5α,22E)	C ₂₈ H ₄₆ O	398.36	62	13.0
6	15.069	Androstan-11-one,3-(acetyloxy)-17-iodo-, (17α)-	C ₂₁ H ₃₁ IO ₃	458.13	58	4.2
7	16.848	Stigmast-7-en-3-ol, (3β,5α,24S)	C ₂₉ H ₅₀ O	414.39	94	6.9
8	17.803	9,10,12-trimethyl-benz(A)acridine	C ₂₀ H ₁₇ N	271.14	64	1.8

nitric oxide) (Peterson et al., 1999), which was similar to the results of this study that cholest-7-en-3-ol did not show both cell toxicity and anti-inflammatory activity determined through the NO inhibition and cytokine assays on COX-2 and TNF- α (data not shown). Hexane fraction of *Bursera simaruba* (Linneo) Sarg. leaves showed anti-inflammatory activity, in which 24S-stigmast-5,22E-dien-3 β -ol and 24S-stigmast-5-en-3 β -ol were main compounds (Carretero et al. 2008). In addition, Guevara et al. (1996) reported that stigmast-7-enol from the green leaves of *Plumeria acuminata* Ait reduced the number of micronucleated polychromatic erythrocytes induced by the mugaen by 80 %. Ergost-5-en-3-ol (3 β), stigmasta-5,22E-dien-3 β -ol, and stigmast-

5-en-3-ol (3 β) purified from leaves of *Aegle marmelos* showed anti-inflammatory activity, while cholest-5-en-3-ol (3 β) did not (Mujeeb et al. 2014). In addition, anti-inflammatory activity of ergosta-7,22-dien-3-ol of spiny sea-star was stronger than that of two fatty acids, *cis* 11-eicosenoic and *cis* 11,14-eicosadienoic acids. However, maximum activity was obtained when both compounds were tested in combination, thus suggesting a potentially synergistic activity of both classes of metabolites (Pereira et al. 2014). In addition, macrophage activation in atherosclerotic lesions resulted from extrinsic, proinflammatory signals generated within the artery wall that suppressed homeostatic and anti-inflammatory functions of desmosterol by the

Fig. 6 Biofunctional activities of the fraction HM_{4.2.4} from the horse mussel *M. modiolus*. **a** anti-inflammatory activity and cell viability, **b** cytotoxicity against three cancer cells. Means in the

same group of bar graph with different superscript letters are significantly different ($p < 0.05$)

suppression of inflammatory-response genes in macrophage foam cells (Spann et al. 2012).

Based on our best knowledge, a publication on anti-inflammatory and cytotoxic compounds from horse mussel *M. modiolus* is not available so far. McPhee et al. (2007) reported that sterol isolated from the New Zealand green-lipped mussel *Perna canaliculus* exhibited anti-cyclooxygenase (COX-2), a lipid metabolising enzyme that catalyses the oxygenation of polyunsaturated fatty acids (PUFA), preferably arachidonic acid, to form the prostanoids, potent cell-signalling molecules associated with the inflammatory processes. Tenikoff et al. (2005) reported that desmosterol-containing lyprinol isolated from the New Zealand green-lipped mussel had potential effect in the treatment of inflammatory bowel disease in mice. Cholesterol was the most predominant sterol (31 % of total sterols). Other major sterols were desmosterol/brassicasterol (co-eluting), 24-methylenecholesterol, trans-22-dehydrocholesterol, 24-nordehydrocholesterol and ocellasterol (Murphy et al. 2003). Even though there are many bioactive compounds presented in horse mussel, steroids might be a strong anti-inflammatory and cytotoxic compound.

Taken together, these data suggest the efficiency of the horse mussel extract to prevent inflammation and inhibit cancer cell proliferation. These data can be considered as an important step before going to patients or in food and drug products. However, further in vivo studies using animal models and human patients are necessary to elaborate and exploit this nascent promise.

Conclusions

The anti-inflammatory and cytotoxic activities of horse mussel were evaluated to obtain an insight of its beneficial effects to prevent inflammation and cancer. The horse mussel extract containing seven steroids and one polycyclic aromatic compounds exerted strong anti-inflammatory activity and cytotoxicity against the three human cancer cells. Therefore, the horse mussel could be utilized as a raw material for the production of anti-inflammatory and anticancer compounds.

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