Antimicrobial, Antioxidant And Cytotoxicity Activities Of Hemolymph Peptide Fraction Of The Algerian Mussel *Mytilus Galloprovincialis* (Lamarck)

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Abstract
In addition to their nutritional value and their wellness effects, Molluscs in general and specially Mussels are a potential source of several bioactive compounds that have gained much more importance over the last decade due to their protein richness and their various uses as nutraceutical, pharmaceutical, therapeutic and functional food agents. This study aims to assess the antimicrobial, antioxidant and cytotoxicity activities of peptide fraction extracted from hemolymph of Algerian blue mussel, *Mytilus galloprovincialis*. Antimicrobial activity of hemolymph peptide fraction (HPF) against a panel of Gram-positive and Gram negative pathogenic bacteria, lactic acid bacteria (LAB) and fungi strains was determined with well diffusion method. Antioxidant activity of HPF was determined using DPPH and FRAP tests, as well as its ability to scavenge hydroxyl radicals (OH). HPF toxicity towards several tumor cells was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test. The growth of most pathogenic bacteria and *Candida albicans* tested were inhibited by HPF of *M. galloprovincialis* (inhibition zone diameters ranging from 10 to more than 24 mm); whereas that of LAB was not affected. Antioxidant activity of 3 mg HPF/mL was about 78.14±2.59, 53.86±1.25 and 84.86±5.68 %, respectively, when determined with DPPH, FRAP, and OH tests. HPF of *M. galloprovincialis* did not exert any toxicity against any normal cell line, human skin keratinocyte, cancer cell lines, human lung carcinoma, human liver hepatoblastoma, human fibrosarcoma, HPV-16 E7-expressing mouse lung epithelial cell (- MHC class I). According to the results of mouse macrophage cell line (preosteoclast) (RAW264.7), HPF had a slight immunosuppressant activity; while results on human stomach adenocarcinoma (AGS) have shown a marginal cancer cytotoxicity of HPF.

Keywords: *Mytilus Galloprovincialis*, hemolymph peptide fraction, antioxidant, antimicrobial, cytotoxicity.

1. Introduction

The immensity of marine biodiversity is an asset of richness in various animal and plant species which are a potential source of active biomolecules that can contribute to human well-being (Destoumieux-Garzón et al., 2016).
Bivalves are one of the most successful groups of animals on Earth that play a key role in marine ecosystems and have colonized the interface between land and sea over the last 500 million years, providing ecosystem benefits such as habitat for other organisms and clarification of water (Guo et al., 1999). Mussels, comprising of a variety of *Mytilidea* bivalve mollusks species, are the largest phylum after arthropods (Langdon and Newell, 1990), and represent 13% of the world aquaculture production (FAO, 2014).
Like all invertebrates, mollusks lack adaptive immune system; however, they are extraordinarily well adapted to adverse environmental conditions. Most marine bivalve mollusks are filter-feeders and uptake microalgae, bacteria, and detritus from surrounding water or sediment. The bacterial density of bivalve tissues is higher than that of seawater, and can be lethal if the accumulated bacteria are pathogenic (Cavallo et al., 2009). Bivalves protect themselves against pathogenic microorganisms through cellular defense mechanisms (phagocytosis, encapsulation) and humoral defense factors (lectins, agglutinins, lysosomal enzymes, antimicrobial peptides) (Tsankova et al., 2012).
Antimicrobial peptides (AMP) are small peptides existing widely in mammals, amphibians, marine invertebrates and insects (Qin et al., 2014), and they appear to be one of the actors in innate immunity defense system (humoral immunity). These peptides are mainly located in circulating cells (Bulet et al., 2004). Marine bioactive peptides, as a source of unique bioactive compounds, are the focus of current research (Chakraborty and Joy, 2020; Panayotova et al., 2020). They exert various biological activities such as antimicrobial (Tsankova et al., 2012), antioxidant (Jung et al., 2007) anticancer (Kim et al., 2013), anti-hypertensive (Je et al., 2005), anti-inflammatory (Kim et al., 2016),

anticoagulant (Jung and Kim, 2009), and antiviral (Balseiro et al., 2011). AMP_s have been shown to exert little or no toxic effect on healthy eukaryote cells; their real target being prokaryotes and abnormal eukaryote cells (Venier et al., 2011). Because marine invertebrates rely solely on innate immune mechanisms for defense, they represent a potentially rich source for pharmacologically useful antimicrobial peptides (Otero-Gonzáiez et al., 2010).
This study fills the lack of information on the biological properties of the hemolymph proteins of the mussel, *Mytilus galloprovincialis*, caught in Algerian West cost by highlighting their activities as antimicrobial against 15 pathogenic strains, antioxidant, and cytotoxic against several tumor cell lines.

2. Materials and methods

2.1. Site and hemolymph sampling, and mussel farming

The 4-6 cm sized mussels, *M. galloprovincialis*, were collected in an ice container as needed for the experiment from the seawater of the port of Mostaganem in Algeria (35°55'52"N and 0°05'21''E) where they were raised to be available all year long.
The shells of the mussels were notched at the posterior adductor muscle level to facilitate hemolymph collection. Hemolymph (0.1mL/animal) was immediately taken from the posterior adductor muscle using a sterile 1mL syringe with a 30 G×1/2 (0.30×12.7mm) needle directly into an equal volume of Modified Alsever Solution (MAS) as anti-aggregation buffer (Gonzalez et al., 2007). Hemolymph samples were held in ice to reduce hemocyte clumping, then immediately centrifuged at 800 x g for 15 min at 4 °C (Tasiemski et al., 2007; Mitta et al., 2000). Plasma (cell-free hemolymph) was kept at -20°C until use.

2.2. Peptide extraction

The plasma was first diluted in water (1v/1v) containing 0.1% (v/v) trichloroacetic acid. The pH was brought to 3.9 with 1 M HCl in an ice-cold water bath under gentle stirring for 30 min, then the mixture was centrifuged at 4°C during 20 min at 10 000 x g (Mitta et al., 2000).

2.3. Solid Phase Extraction and Pre-purification

Plasma extracts were loaded onto SepPak C18 Vac cartridges (Waters Associates, Switzerland) previously washed and equilibrated with acidified water (0.05% trifluoroacetic acid in Ultra-Pure Water (UPW). After what, two successive elutions were performed with 5 and 40 % (v/v) acetonitrile in acidified water, successively. Then, the 5% (v/v) eluted fraction was discarded; while that of 40% (v/v) was lyophilized and reconstituted with UPW (Mitta et al., 2000).

2.4. Microbial strains

Growth of several pathogenic microbial strains was tested in presence of hemolymph peptide fraction (HPF) of *M. galloprovincialis*. The Gram negative bacteria tested are *Escherichia coli* ATCC 10536, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 2785, *Shigella dysenteriae* CECT 457 and *Serratia marcescens* DSM 30121; while the Gram positive bacteria are *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Proteus mirabilis* ATCC 13315, *Enterococcus hirae* ATCC 10541, *Micrococcus luteus* ATCC 10420, and *Bacillus cereus* ATCC 9884. Two Lactic bacteria, *Bb V2* (*Bifidobacterium* sp, an experimental strain) and *Lactobacillus plantarum* ATCC 748; as well as two fungal strains, *Candida albicans* ATCC 10231 and *Aspergillus fumigates* ATCC 106404, were also tested. Pathogenic strains and lactic acid bacteria were individually incubated 24h at 37 °C in Muller Hinton and in MRS broth media, respectively; whereas *Candida albicans* and *Aspergillus niger* were grown 48-72h in Sabouraud media and 7 days in PDA (potatoe Dextrose Agar) media respectively at 30°C (NCCLS, 2001; Pfaller et al., 1998).

2.5. Antimicrobial assay

The HPF antimicrobial activity was evaluated by agar well diffusion method. The bacterial strains were cultured on Muller Hinton semi-agar, and potatoe Dextrose semi-agar for antifungal activity. Bacterial cultures were adjusted to a concentration of 10^8 UFC/mL, 10^6 UFC/mL for the yeasts (Haddouchi et al., 2009) and 10^6 spores/mL for fungi (Braga et al., 2007). 100 µL portions of HPF (3 mg/mL) were pipetted into each well (6 mm in diameter). Sterile water was used as negative control. The plates were then incubated for 2 h at 4 °C, and incubated at 37 °C for 18-24 h for bacterial strains, at 30°C for 24-48 h for yeast strains and 7 days for fungal strains. The zone of inhibition was measured in mm. The assay was repeated three times and the averages of the three experiments taken.

2.6. Antioxidant activity

2.6.1. DPPH radical scavenging activity

The DPPH free radical scavenging activity of hemolymph peptide fraction (HPF) was determined using the original method of Blois (Blois, 1958). The mixture of 1 mL of different concentrations of HFP (3, 1.5, 0.75 and 0.37 mg/mL) and 0.5 mL of 0.16 mM of freshly prepared methanolic DPPH solution was incubated at 27°C in a dark for 30 min. Sample absorbance was measured at 517 nm against a negative control (methanolic solution) and compared to a positive control run with the same concentrations of ascorbic acid in the same operating conditions. The percentage of scavenging activity was calculated using the following formula:

(Abs Control –Abs Sample) x 100/ Abs control (1)
Where Abs control: absorbance of the control; Abs sample: absorbance of the sample
The concentration of HPF or ascorbic acid giving 50% of DPPH scavenging activity, named IC50, was determined graphically from the relation:
% DPPH scavenging activity = f (concentration of HPF)

2.6.2. Ferric Reducing Antioxidant Power (FRAP) activity
The ferric reducing antioxidant power (FRAP) was determined according to the method described by Benzie and Strain (Benzie and Strain, 1996). To do so, 2.5 mL of different HPF concentrations (3, 1.5, 0.75 and 0.37 mg/mL) were mixed with 2.5 mL of 0.2 M/pH 7.4 phosphate buffer and 2.5 mL of 1% (p/v) potassium ferricyanide ($K_3Fe\ C_6\ N_6$). This mixture was incubated 30 min at 50 °C, then a solution of 2.5 mL of 10 % (p/v) trichloroacetic acid (TCA), 2.5 mL of distilled water, and 0.5 mL of 0.1 % (p/v) $FeCl_3$ was added. The absorbance was measured at 700 nm against a buffer phosphate blank, and compared to the same concentrations of ascorbic acid as a positive control run in the same operating conditions. The percentage of scavenging activity was calculated using the previous formula (1).

2.6.3. Hydroxyl radical-scavenging activity
Hemolymph peptide fraction (HPF) ability to scavenge hydroxyl radical was evaluated with the deoxyribose method as described by Chung et al., 1997. Different HPF concentrations (3, 1.5, 0.75, and 0.37 mg/ml) were prepared, and 1 mL of each was mixed with 0.8 mL of 10 mM FeSO4-7 H2O, 1 mL of 10 mM EDTA and 2 mL of 0.2 M, pH 7.4 phosphate buffer. Then, 0.2 mL of 10 mM H_2O_2 was added to the reaction mixture and incubated for 4 h at 37ºC. After what, 1 mL of 2.5% (p/v) TCA was added, vigorously mixed and heated in a water bath at 100 ºC for 15 min. After cooling, the absorbance was measured at 532 nm. The percentage of inhibition of hydroxyl radical was calculated using the previous formula (1).

2.7. Cytotoxicity
2.7.1. Cells
The following 7 different cell types were used in the cytotoxicity test: 2 normals cells, Human epidermal keratinocyte (HaCaT) and Mouse macrophage cell line (preosteoclast: RAW264.7); and 5 cancer cells, human stomach adenocarcinoma (AGS), Human lung carcinoma (A549), Human liver hepatoblastoma (HepG2), Human fibrosarcoma (HT1080), TC-1 P3 HPV-16 E7-expressing mouse lung epithelial cell (- MHC class I). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum in humidified incubator containing 5% CO_2 at 37°C. The cells were obtained from the Department of biochemistry and molecular biology, college of medicine, Korea University, Seoul, South Korea.

2.7.2. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay
The MTT assay was used to determine the in vitro cytotoxicity of hemolymph peptide fraction (HPF) towards the cells cited above. The cells were grown in 96-well plate at a density of 5.10^4 cells/100 µL /well. After 4h of incubation, the cells were treated with 100 µL of fresh RPMI medium (Roswell Park Memorial Institute medium) containing three different HPF concentrations (10 µM, 100 µM, and 1000 µM) and incubation for 72 h. Then, 25 µL of MTT solution (5 mg/ mL) was added to each well, and the plate was re-incubated for 4 h. Finally, the medium was removed and 100 µL of DMSO was added to dissolve the formazan crystal. Cell survival rate was calculated by measuring the absorbance at 492 nm using micro plate spectrophotometer (Meerloo et al., 2011). The relative cell viability in percentage was calculated as follow (Zheng et al., 2023):
% cell viability = (Abs_s of treated samples /Abs_c of untreated sample) X 100
ABSc: absorbance of the control (culture medium without HPF)
ABSs: absorbance of the sample (culture medium containing different HPF concentrations).

2.8. Statistical analysis
Statistical analysis was conducted using ANOVA analysis (StatBox logiciel, GrimmerSoft; version 6.4, France). Comparisons were made using Student–Newman–Keuls test at threshold 5%. All data presented are mean values of triplicates obtained from three separate runs.

3. Results and discussion
3.1. Antimicrobial activity
The antimicrobial activity tests have shown that HPF of *M. galloprovincialis* inhibited the growth of the pathogenic strains used in this experiment (Table 1). The highest inhibition zone diameters recorded in a decreasing order were about 24.33 ±1.15 mm for *Staphylococcus aureus* ATCC 25923, 15 ± 0.00 mm for *Pseudomonas aeruginosa* ATCC 27853, 14.33 ± 1.15 mm for *Escherichia coli* ATCC 10536 and *Enterococcus hirae* ATCC 1059, 11.33 mm for *Listeria monocytogenes* ATCC 7644 and *Shigelladys enteriae* CECT 457, and 10 ± 0.00 mm for *Bacillus cereus* ATCC 10420. However, the HPF failed to inhibit the growth of *Micrococcus luteus* ATCC 10420, *Proteus mirabilis* ATCC 13315, *Serratia marcescens* DSM 3012, *Salmonella typhimurium* ATCC 1331, and the two beneficial bacteria, Bb v2 and *Lactobacillus plantarum*. On the other hand, HPF extract was very effective in inhibiting the growth of the fungi strain, *Candida albicans* ATCC 10231, with an inhibition zone of 20 mm; while failed to inhibit the growth of *Aspergillus*

fumigates ATCC 106404. The obtained results evidenced that crude peptide extract of *Mytilus galloprovincialis* hemolymph has a potential antimicrobial property.

Antimicrobial peptides (AMP_s) are a key component of the innate immune system of marine invertebrates (Pereiro et al., 2021; Moreira et al., 2020; Kyoung Kang et al., 2019). The first study on bivalve AMPs using reverse genomics was conducted in the late 1990 (Hubert et al., 1996). Clearly, the field of marine invertebrate antimicrobial peptides is underexplored and offers an opportunity for extensive antimicrobial peptide research. As pathogenic microorganisms continuously develop resistance to conventional antibiotics, the development of new antimicrobial agents becomes an urgent problem (Hooper et al., 2007). The activity of some marine invertebrate AMPs was previously reported (Jeyasanta et al., 2020; Queensley et al., 2019). Work in the molluscs *Mytilus edulis* and *Mytilus galloprovincialis* led to the isolation of several AMPs (defensin, mytilin, mitomycin and mytisin) (Chisholm June and Smith, 2009). Furthermore, Sathyan et al. (2014) reported the antimicrobial activities of crude extracts of 24 different mollusc peptides.

Table 1. Antimicrobial activity (diameter of inhibition zone in mm) of Hemolymph Peptide Fraction (HPF) of *Mytilus galloprovincialis* against thirteen pathogenic and two beneficial microorganisms.

Bacterial strains	**HPF extract (mm)**
Staphylococcus aureus ATCC 25923	24.33 ± 1.15
Pseudomonas aeruginosa ATCC 27853	15 ± 0.00
Escherichia coli ATCC 10536	14.33 ± 1.15
Enterococcus hirae ATCC 10591	14.33 ± 1.15
Listeria monocytogenes ATCC 7644	11.33 ± 1.15
*Shigelladysenteriae*CECT 457	11.33 ± 1.15
*Proteus mirabilis*ATCC 13315	-
*Serratiamarcescens*DSM 30121	-
*Salmonella typhimurium*ATCC 13311	-
*Bacillus cereus*ATCC 9884	10 ± 0.00
*Micrococcus luteus*ATCC10420	1.00 ± 0.00
*Bifidobacterium*spBb v2	-
Lactobacillus plantarum	-
*Candida albicans*ATCC 10231	20 ± 0.00
Aspergillus fumigates ATCC 106404	2 ± 0.00

Values reported as mean ± standard deviation. Mean of three experiments. Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.
(-) indicates non inhibition of microbial growth.

AMPs are distinguished by their net positive charge and amino acidic residue amphipathic distribution; these key features explain their mode of action with the membrane of target microorganisms (Marshall and Arenas, 2003). In order to maintain their antimicrobial activity, the synthetized AMP_s must have an amphipathic structure, and should be organized in hydrophobic and hydrophilic amino acid zones (Arenas et al., 2009), since they use different modes to inhibit Gram-positive and Gram-negative bacteria, and fungi (Sathyan et al., 2014).

Sheringham et al. (2015) have shown that the crude acetone peptide extracts of two aquatic invertebrates *Galatea paradoxa* (G. *paradoxa*) and *Patella rustica* (*P. rustica*) were a potential sources of antimicrobial peptides. Although extract from *G. paradoxa* has a high antibacterial activity and the highest zone of inhibition against both Gram-positive and Gram-negative bacteria such as *Escherichia coli* (19.7 ± 0.6 mm), *Staphylococcus aureus* **(**20.0 ± 0.0 mm), *Bacillus subtilis* (20.3 ± 0.6 mm), *Pseudomonas aeruginosa* (20.3 ± 1.5 mm), *Salmonella typhimurium* (15.3 ± 0.6 mm), *P. rustica* extracts were much more efficient towards fungi like *Candida albicans*, with an inhibition zone of 37.0 ± 1.0 mm. The inhibition zones of 16.7 ± 1.15 mm and 15.0 ± 1.0 mm were recorded for *Klebseilla pneumoniae* and *Enterococcus feacalis*, respectively.

The aqueous fractions extracts of two Marine Molluscs, *Pachymelania aurita* (*P. aurita*) and *Tympanatonus fuscatus Var Radula* (*T. fuscatus*) collected from the Niger-Delta region of Nigeria do not exhibit any antimicrobial activity against the organisms tested, even at the highest concentration tested (100 mg/mL); whereas the acetone-methanol fraction extracts of *P. aurita* and *T. fuscatus* showed broad-spectrum antibacterial activity against five bacterial isolates at the highest concentration (100 mg/mL): *Staphylococcus aureus* ATCC 43300 (12 ± 1.4 mm with *P. aurita* and 11.5 ± 0.7mm with *T. fuscatus*), *Micrococcus luteus* NCIB 196 (12 ± 1.4 mm with *P. aurita* and 12.0 ± 1.4 mm with *T. fuscatus*), *Klebsiella pneumoniae* NCIB 418 (11.5 ± 0.7 mm with both *P. aurita* and *T. fuscatus*), *Clostridium sporogenes* NCIB 532 (11.5 ± 0.7 mm with *P. aurita* and 11 mm with *T. fuscatus*), *Bacillus stearothermophilus* NCIB

8222 (10.55 ± 0.1 mm with both *P. aurita* and *T. fuscatus*), *Candida albicans* (12 mm with both *P. aurita* and *T. fuscaus*). Alcohol extracts of *P. aurita* and *T. fuscatus* do not exhibit any antimicrobial activity against *Proteus vulgaris* NCIB 67, *Pseudomonas aeruginosa* NCIB 950, *Bacillus subtilis* NCIB 3610; *Escherichia coli* NCIB 86, and *Serratia marcescens* NCIB 1377 at a concentration of 100 mg/mL of extract (Queensley et al., 2019)

The study of *Jeyasanta* et al. (2020) confirm that the antimicrobial activity of proteins extracted from *Perna Perna* (brown mussel), *Perna Viridi* (green mussel) and parrot mussel–depends on the extraction solvent used, given that methanolic, ethanolic, and acetonic extracts markedly inhibited several bacteria (inhibition zone diameters ranging from 7 to 20 mm); while ethyl acetate, hexane, or butanol extracts were less efficient (inhibition zone diameters ranging from 1 to 7 mm).

3.2. Antioxidant activity

3.2.1. DPPH Radical Scavenging Activity of Hemolymph Peptide Fraction (HPF)

The results of the DPPH radical scavenging ability of different concentrations of the hemolymph peptide fraction (HPF) compared to ascorbic acid as a standard (positive control) are reported in Figure 1. The HPF and ascorbic acid DPPH scavenging activity was dose-dependent. The highest trapped DPPH radical rate with 3 mg/mL of HPF was 78.14 ± 2.59 %; while that of the same concentration of ascorbic acid was about 93.43 ± 1.32 %. This HPF scavenging activity represents almost 84 % that of the ascorbic acid.

3.2.2. Ferric Reducing Antioxidant Power (FRAP) of Hemolymph Peptide Fraction (HPF)

In the present study, the trend for ferric ion reducing activities of peptide fraction and ascorbic acid are shown in Figure 2. The absorbance of HPF clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentrations. FRAP essay was used as quick and simple method to perform; as well as for the good reproducibility of its reaction (Müller et al., 2010). Highest antioxidant activity (53.86 ± 1.246 %) was observed with 3 mg/mL HPF extract. The HPF ferric reducing power increased with the increasing concentration of HPF; suggesting a dose dependent activity, since it raised from 45.132 ± 0.141 % at 0.37 mg/mL to 53.86 ± 1.246 at 3mg/mL.

3.2.3. Hydroxyl Radical scavenging activity of Hemolymph Peptide Fraction (HPF)

Hydroxyl radical is strongly oxidizing, mainly towards lipids. The ability of scavenging hydroxyl radicals by an antioxidant is directly related to the prevention of propagation of lipid peroxidation process (Batista et al., 2010). Antioxidant scavenges the hydroxyl radical and inhibits the degradation of deoxyribose, and subsequent color formation (Bektaşoglu et al., 2006). Scavenging these radicals is of great importance in biological systems to keep cell membrane intact. In the present experiment, HPF of *M. galloprovincialis* has shown a highly significant hydroxyl radical scavenging activity, reaching a maximum of 84.86 ± 5.685 % when used at 3mg HFP/mL extract; while that of ascorbic acid was about 95.71 ± 0.023% (Fig. 3).

In the study of Sheringham et al. (2015), a maximum DPPH scavenging activity of 56.77 and 79.77% was obtained with 0.39 mg HPF/mL extract of *Galatea paradoxa* and *Patella rustica*, respectively; while a minimal activity of 24.27 and 21.40 %, respectively, was recorded with 0.003 mg HPF/mL extract for the same molluscs. The maximum and the minimal DPPH scavenging activity registered with 0.39 and 0.003 mg/mL ascorbic acid as a standard were 86.77 and 42.27 %, respectively.

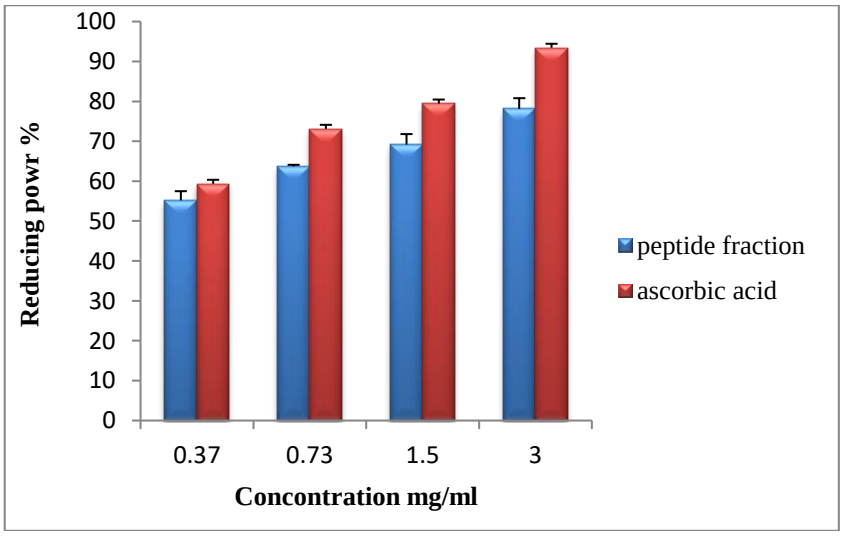

Figure 1: DPPH free radical scavenging activity of different concentrations of *Mytilus galloprovincialis* hemolymph peptide fraction (HPF) extract and ascorbic acid as a standard. Values represent the mean ± SEM of three determinations. ($p<0.001$).

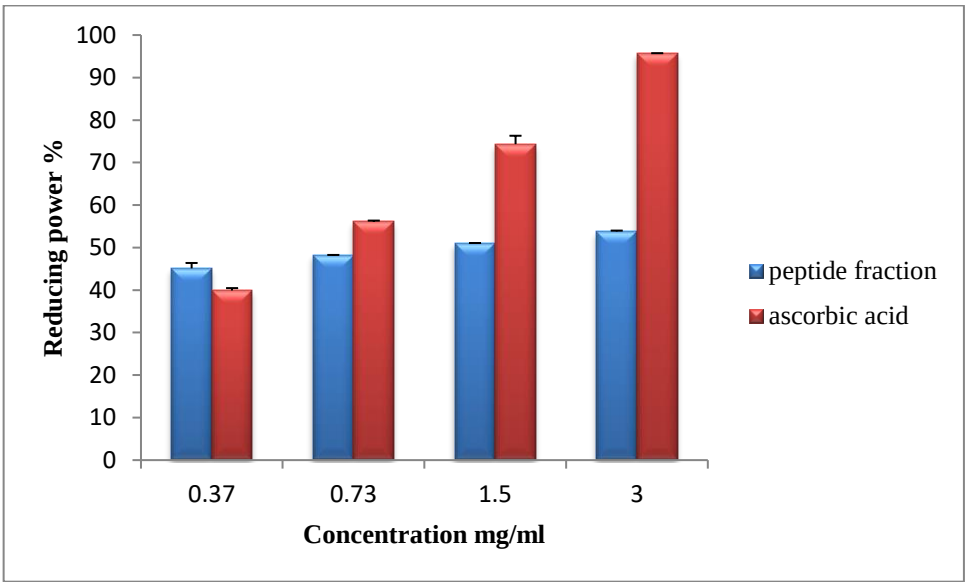

Figure 2: Ferric Reducing Antioxidant Power (FRAP) of *Mytilus galloprovinciallis* hemolymph peptide fraction. Values represent the mean ± SEM of three determinations). ($p<0.001$).

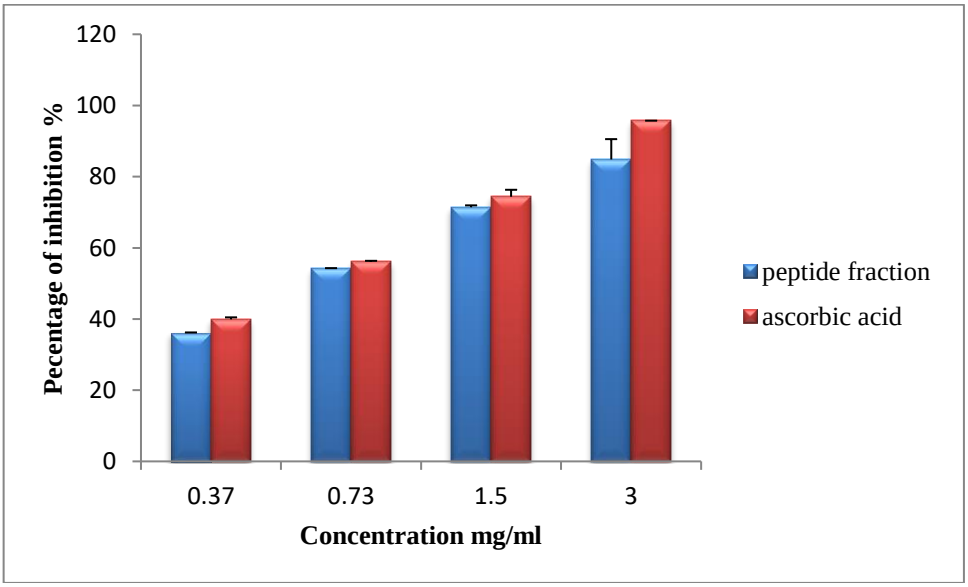

Figure 3: hydroxyl radical scavenging activity of *Mytilus galloprovinciallis* hemolymph peptide fraction. Values represent the mean ± SEM of three determinations. ($p<0.001$).

According to Zamorano-Apodaca et al. (2020), the recorded DPPH free radical scavenging activity of the peptide fraction with a molecular weight ranging from 1 to 5 kDa was 66 %. Peptides with low molecular weight (MW) could efficiently react with free radicals and inhibit the lipid peroxidation cycle spread; therefore, they exhibit higher antioxidant activity (Ranathunga et al., 2006). Besides, the high DPPH radical scavenging activity of the peptide fractions has been reported to be usually associated with a high level of hydrophobic amino acid content (Karoud et al., 2017). Bioactive peptides derived from marine organisms are the focus of current research due to their many health benefits. Antioxidant activity is one of the most important biological functions of the bioactive peptides. Dai-Hung and Se-Kwon, (2013) reported an inverse relationship between antioxidant intake and disease. Niranjan et al. (2005) have shown that the radical scavenging activity of fermented mussel sauce used at 0.2mg/mL towards various free radicals was about 41.3 ± 0.54 %, 40.1 ± 0.76 %, and 35.4 ± 0.87 % against superoxide, hydroxyl and carbon-centered, respectively.

Ability of purified peptides to reduce Fe3+ to Fe2+ was used to evaluate their reducing power, and the absorbance values obtained herein (0.394 with 3mg HFP/mL and 0.297 with 0.37 mg HFP /mL) are slightly lower than that reported by Zhouyong et al. (2017) (0.441 with 2 mg/mL of peptides of MW lower than 3 kDa). This suggests that chromatography purification could improve the reducing power of peptide fractions.

The exact mechanism underlying the antioxidant activity of peptides has not been fully elucidated. However, the antioxidant activity of peptides is more related to their amino acid composition, structure and hydrophobicity (Chen et al., 1998). Depending on the formation process and chemical characteristics of free radicals, antioxidant peptides can be

lipid peroxidation inhibitors, free radical scavengers, or transition metal ion chelators in antioxidant reactions (Niranjan et al., 2005). Peptides with hydrogen donor activity can scavenge free radicals or effectively inhibit lipid oxidation, while those with metal ion chelating activity can decrease free radicals generated by metal ion catalysis (RiBang et al., 2015).

3.3. Cytotoxicity

The active HPF of *Mytilus galloprovincialis*, used at a concentration of 1000 µM, had no cytotoxicity against a normal cell line, human skin keratinocyte (HaCaT) and cancer cell lines such as human lung carcinoma (A549), human liver hepatoblastoma (HepG2), human fibrosarcoma (HT1080) and HPV-16 E7-expressing mouse lung epithelial cell (- MHC class I), with a respective cell viability of 88.055%, 100.561 %, 91.327%, 127.25%, and 81.64±2%.

Cell viability is commonly used in oncology and pharmacology to determine the Half-maximal inhibitory concentration (IC50) value of a cytotoxic compound in cell culture. So, IC50 was measured as the concentration of peptides able to reduce by 50 % the cell viability in comparison with the untreated cells. At the concentration of 1000µM/mL, an average HPF immunosuppressive activity towards mouse macrophage cell line (preosteoclast) (RAW264.7) with a cell viability of 65.53 %, and a marginal cancer cytotoxicity against human stomach adenocarcinoma (AGS) with a cell viability of 56.08 % were recorded (Table 2).

Table 2. Cytotoxic activity of hemolymph peptide fraction of *Mytilus galloprovincialis* against Human epidermal keratinocyte (HaCaT), Mouse macrophage cell line (RAW264.7, preosteoclast), Human stomach adenocarcinoma (AGS), Human lung carcinoma (A549), Human liver hepatoblastoma (HepG2), Human fibrosarcoma (HT1080), HPV-16 E7-expressing mouse lung epithelial cell (TC-1 P3, MHC class). Cells were treated with different concentrations of peptides fraction. The effect was measured by MTT cell viability assay (Survival %). Values represent the mean ± SEM of three determinations.

Cells lines	10µM	100µM	1000µM
HT1080	130.83±0.98	121.27±1.14	127.25±0.82
AGS	99.24±5.70	85.067±2.32	56.085±1.48
HaCaT	99.705±5.48	96.19±2.73	88.055±5.31
HepG2	95.865±5.15	93.47±3.29	91.327±0.00
A549	*109.47±0.5*	106.203±3.84	100.5615±3.27
TC-1 P3	100.35±1.38	97.62±1.21	81.64±2.34
Raw 264.7	105.47±8.76	102.11±0.42	65.53±2.39

The IC50 values of *M. galloprovincialis* HPF, determined on the basis of MTT test, towards cancer cell lines in comparison with normal cells are reported in Table 3.

IC50 greater than 1000 µM has been observed for cells like human cutaneous keratinocytes (HaCaT), and cancer cell lines such as human lung carcinoma (A549), human hepatic hepatoblastoma (HepG2), human fibrosarcoma (HT1080), mouse expressing HPV-16 E7 lung epithelial cell (- MHC class I) (TC-1 P3).

Table 3. IC50 (µM) determined on the basis of MTT Assay

Compounds	IC50 (µM) Based on MTT Assay						
	Normal cell		Cancer cell				
	HaCaT	RAW264.7	AGS	A549	HepG2	HT1080	TC-1 P3
HPF	>1000	158±8.6	105±4.1	>1000	>1000	>1000	>1000

According to the obtained findings, the hemolymph peptide fraction (HPF) could be generally regarded as non-toxic to human except a marginal cytotoxicity against mouse macrophage cell line (RAW264.7) (IC50 158 ± 8.6 µM) and no cancer-specific cytotoxicity except a marginal anti-cancer activity against human stomach adenocarcinoma cancer (AGS) (IC50 105 ± 4.1 µM).

According to the results of Gerdol et al. (2015), the MTT assays indicated that Cysteine-Rich Peptides (MgCRP-I) from *Mytilus galloprovincialis* were not cytotoxic on the Human colorectal carcinoma (HT-29), human neuroblastoma (SHSY5Y), and breast cancer (MDAMB231) cell lines up to a concentration of 10 mM.

Given the antimicrobial and antioxidant activities of the hemolymph peptide fraction observed in this experiment, it can be considered that *M. galloprovincialis* hemolymph is a potential source of bioactive peptides which are generally regarded as non-toxic to human except a marginal cytotoxicity against macrophage/monocyte and no cancer-specific cytotoxicity except a marginal anticancer activity against human stomach cancer.

In conclusion, the obtained results of antimicrobial activity of HPF extracted with 0.1% trichloroacetic acid and acetonitrile from an aquatic invertebrate, *M. galloprovincialis*, have shown that it is a good source of antimicrobial peptides; whereas those regarding the antioxidant activity, argue for their use as a natural functional food preservative. Further work could focus on the isolation, purification and characterization of these bioactive peptides. Therefore, molluc peptide fractions with antimicrobial, antioxidant, and anti-proliferative effects are worthy for further investigation as health-promoting functional food ingredients.

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Author contribution
Conceptualization: SOEH and AR; experimental design and investigation: SOEH, AR and GHP; writing and original draft preparation: SOEH; data curation: SOEH and AR; supervision: AR and GHP; project administration: AR. All authors have read and approved the final manuscript. Funding Algerian Ministry of Higher Education and Scientific Research, grant number DOOL01UN27012019.

Conflict of interest
Authors declare no conflict of interest

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