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# Antimicrobial And Anticancer Activity Of Protein Hydrolysate And Crude Extract Of Snail Clithon Oualaniense (Lesson 1831)

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***Abstract: Clithon oualaniense is a brackish water snail with an operculum. It is an aquatic gastropod Mollusca with variable colouration pattern and small in size. Many bioactive compounds have been investigated for their antimicrobial, cytotoxic, anti-tumor and anti-inflammatory, and antiviral properties of mollusks. The present study is to investigate the antimicrobial activity and anticancer activity of protein hydrolysate and crude extract of snail Clithon oualaniense (Lesson 1831). Protein hydrolysate was prepared from tissue of Clithon oualaniense by enzymatic hydrolysis. 2.9 mg/ml protein concentration was estimated by Bradford's method and 20 to 235 kDa protein bands were found in SDS PAGE analysis. Antibacterial assay was carried out against six bacterial pathogens by agar disc diffusion method. In antibacterial activity, the maximum zone of inhibition was observed against Staphylococcus aureus (23 mm) at 100µg/ml concentration of crude extract and 20mm at 100µg/ml concentration of protein hydrolysate. This study showed that the snail Clithon oualaniense crude extract and protein hydrolysate could be used as an antibiotic in biomedical research.***

***Keywords: Antimicrobial, Clithon oualaniense, Protein hydrolysate, Staphylococcus aureus***

## 1. INTRODUCTION

Molluscs are the species with a wide range of uses in pharmacology and are considered as an important natural source to derive many novel bioactive compounds [1]. They are rich sources of biologically active secondary metabolites. Today, more than 60% of the anticancer drugs commercially available are of natural origin [2]. The major sources of biomedical compounds are sponges (37%), coelenterates (21%) and microorganisms (18%) followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%), bryozoans (1%) [3]. Several molluscan derived therapies are listed on the homoeopathic Materia Medica [4]. The majority of research on natural products from the phylum Mollusca has been focused on primarily soft-bodies or shell-less molluscs, particularly nudibranches and opisthobranches [5]. Some studies have also been reported by biological activity from shelled molluscs [6, 7]. Snail mucus secretions could be a source for antibacterial agents that can serve as a drug for wound treatment [8].

Many bioactive compounds of mollusc have been reported predominantly for their antimicrobial, anti- leukemic, antineoplastic and antiviral properties [9]. Anti- inflammatory and antitumor activities [10]and they have the potential to prevent free radical oxidation process that causes cell damage, cancer and degenerative diseases [11]. The hemolymph of *Galleria mellonella* showed antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* [12].

Enzymatic proteolysis from animal and plant sources has been studied extensively and described by several different authors over the last 60 years [13]. Hydrolysis of proteins leads to the cleavage of peptide bonds which makes proteins to peptides and amino acids. These mixtures of peptides and amino acids are called protein hydrolysates. Many protein

hydrolysates possess antioxidant activity [14]. They are important ingredients for food and industrial applications as it contains more bioactive compounds [15].

The peptides remain inactive some times when it presents inside the protein sequencing, enzymatic hydrolysis process releases the peptides from protein and these protein hydrolysate possess many activities like antimicrobial, antioxidant, antidiabetic, anti-inflammatory, and anti- hypertensive [16, 17]. It is also reported that the peptides recovered after cleavage are more bioactive and antioxidant in nature [18]. The protein hydrolysate of *Cryptozona bistrialis* showed antibacterial activity against pathogenic bacteria and a natural source of antioxidant agents [19].

Molluscs bioactive compounds are currently used for a range of therapeutic applications in pharmaceuticals as crude or semipurified or purified extracts as nutraceuticals [20]. Therefore, the aim of the present study was to evaluate the antimicrobial activity and anticancer activity of the crude extract and protein hydrolysates of snail *Clithon oualaniense* against different pathogenic bacteria and anticancer activity with lung cancer cell (A549) and also to analyze the cytotoxicity of hydrolysates using normal cell (L929) to identify its biomedical potential.

## 2. MATERIALS AND METHODS

### Collection and identification

*Clithon oualaniense* (Lesson 1831), brackish water snails were collected from, Kappil Beach, Varkala, Kerala, India. They were identified by Dr. R. Venkitesan, Scientist - C, Zoological Survey of India, 130, Santhome High Road, Chennai-600028.

### Sample preparation

The collected snails were brought to the laboratory, broken the shells and the soft body were separated and stored at -20° C until used. The crude sample was prepared by blending the tissues with 0.7% cold saline, then the mixture was centrifuged for 10 minutes at 7000 rpm and supernatant obtained was used as crude extract and stored at 4°C.

### Protein hydrolysate preparation

The proteolytic digestion of *Clithon oualaniense* was performed according to the method described by Je, 2007. To produce peptides from tissue of *Clithon oualaniense*, enzymatic hydrolysis was carried out with the enzyme Trypsin. The enzyme trypsin was taken with 0.1 M phosphate buffer under optimal condition with pH 8, temperature at 37°C at enzyme/substrate ratio of 1:250 (w/w). Tissue of *Clithon oualaniense* was homogenized with blender and then thoroughly mixed with enzyme. The enzyme substrate mixture was incubated for a period of 6 hours with constant stirring. At the end of the incubation period,

the content was heated in a water bath for 10 minutes at 100°C. This heating inactivates and stops the enzyme activity. Then the mixture was centrifuged for 15 minutes at the speed of 10000 rpm. The supernatant obtained was the protein hydrolysate. The hydrolysates were lyophilized to get a powdered sample and were stored at -20° C [21].

**Determination of protein concentration**

The concentrations of protein hydrolysate were estimated using Bovine Serum albumin as a standard [22].

**SDS PAGE analysis**

The molecular weight of the protein hydrolysate was confirmed by the SDS PAGE analysis with the molecular weight marker ranging from 11-250 kDa [23].

**Antibacterial assay**

Antimicrobial activity of the crude and protein hydrolysate from the snail, *Clithon oualaniense* was assessed against 6 human pathogenic bacterial strains. Among the pathogenic bacteria, 3 were gram+ve (*Staphylococcus aureus*, *Streptococcus mutans* and *Bacillus subtilis*) and 3 were gram -ve (*Klebsilla pneumonia*, *Proteus vulgaris* and *Escherichia coli*). The strains were obtained from Inbiotics Research lab, Nagercoil, Tamil Nadu, India and were periodically sub cultured and maintained in a respective medium at 4°C. Antibacterial activity of crude and protein hydrolysate of the snail was determined by disc diffusion method [24]. The crude and protein hydrolysate were prepared with various concentration of 50 and 100 µg/ml, and 25µl of respective concentration were added to each disc. Sterile disc were used as negative control and standard streptomycin (25mg) was used as positive control. The diameters of zone of inhibition were measured in millimeter by using the antibiotic zone measuring scale.

**Cytotoxicity assay**

The Mouse Fibroblast Cell lines procured from NCCS, Pune were cultured and maintained until they reach 70% Confluence in T25 Flask at 37ºC in a 5% $CO_2$ Incubator. Afterwards, 200µl cell suspension was seeded in a 96-well plate at the required cell density (20,000 cells per well), without the test agent and allowed the cells to grow for about 24 hours. Appropriate concentrations of crude extract and protein hydrolysate of *Clithon oualaniense* (6.25, 25, 50, 100, 200 and 400µg/mL) dissolved in DMEM Media high glucose (Cat No. AL111, Himedia) were added and incubated for 24 hrs at 37°C in a 5% $CO_2$ atmosphere. Camptothecin with the concentration of 25µM is used as a positive control. After the incubation period, the Spent Media was removed and 100uL of MTT reagent (Cat No:4060, Himedia) was added and incubated for 3 hrs at 37°C. After incubation period, the formed formazan crystals were dissolved with 100 µl of DMSO (Cat No.1309, Sigma) and the absorbance readings were taken by ELISA Reader (ELX 800, Biotek) at 570 nm and the $IC_{50}$ value is calculated using linear regression equation i.e. Y=Mx+C derived from the cell viability graph.

## 3. RESULTS

### Antibacterial activity

The crude extract and protein hydrolysate of *Clithon oualaniense* exhibited variable inhibitory response against pathogens. The 100 µg concentration of crude extract of *Clithon oualaniense* showed the maximum inhibitory activity (23 mm) against S*taphylococcus aureus* and protein hydrolysate with minimum zone of inhibition (20 mm). Both 100 µg concentration crude and protein hydrolysate showed same activity against *Klebsiella pneumonia* with 18 mm. The protein hydrolysate showed activity against most of the gram positive and gram negative organism with maximum inhibition zone except *Staphylococcus aureus* (Table 1, Figure 1).

Table 1: Antibacterial activity of crude extract of protein hydrolysate from *Clithon oualaniense*

| **Sample Code** | **Bacteria Strains Name** | | | | | |
|---|---|---|---|---|---|---|
| | ***Staphylococcus aureus* (G+)** | ***Streptococcus mutans* (G+)** | ***Bacillus subtilis* (G+)** | ***Klebsiella pneumonia* (G-)** | ***Proteus vulgaris* (G-)** | ***Escherichia coli* (G-)** |
| CSC 50 | 18 | 14 | 12 | 11 | 9 | 10 |
| CSC 100 | 23 | 17 | 14 | 18 | 13 | 14 |
| CSP 50 | 14 | 13 | 12 | - | 14 | 13 |
| CSP 100 | 20 | 19 | 17 | 18 | 15 | 16 |
| PC | 19 | 21 | 21 | 16 | 19 | 18 |
| NC | - | - | - | - | - | - |

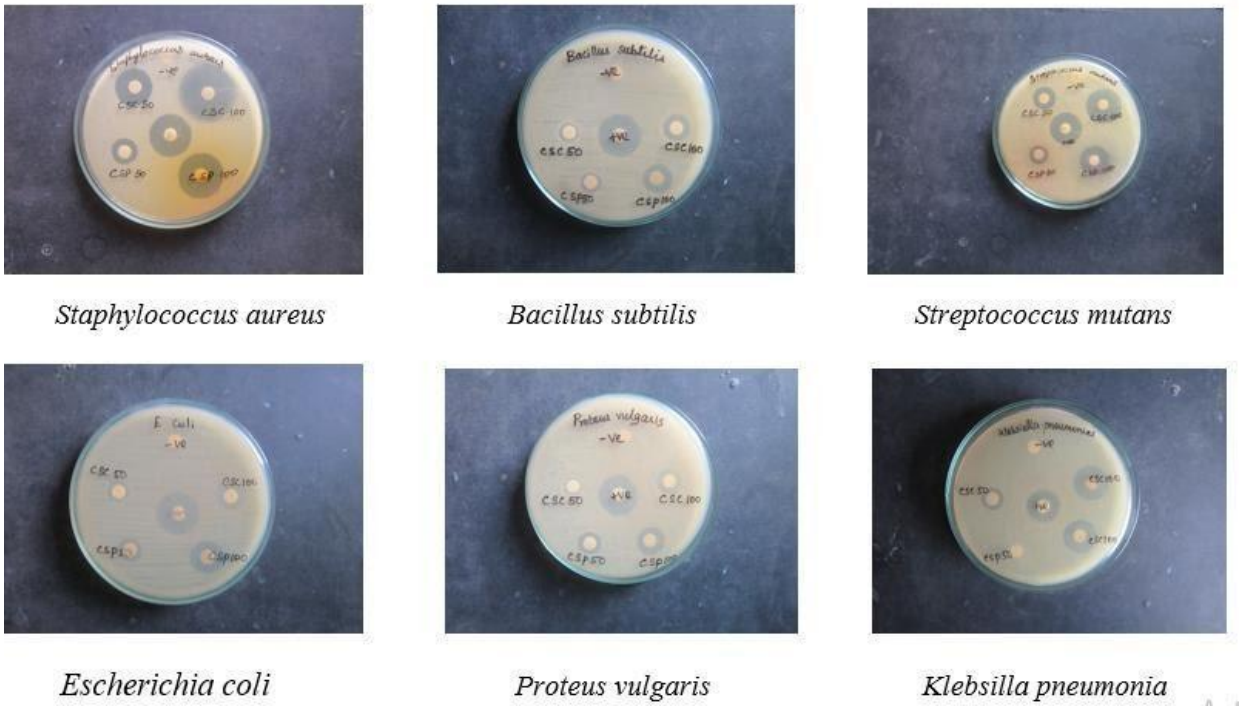



Figure 1: Antibacterial activity of crude extract of protein hydrolysate from *Clithon oualaniense*

**Anticancer activity and cytotoxicity assay**

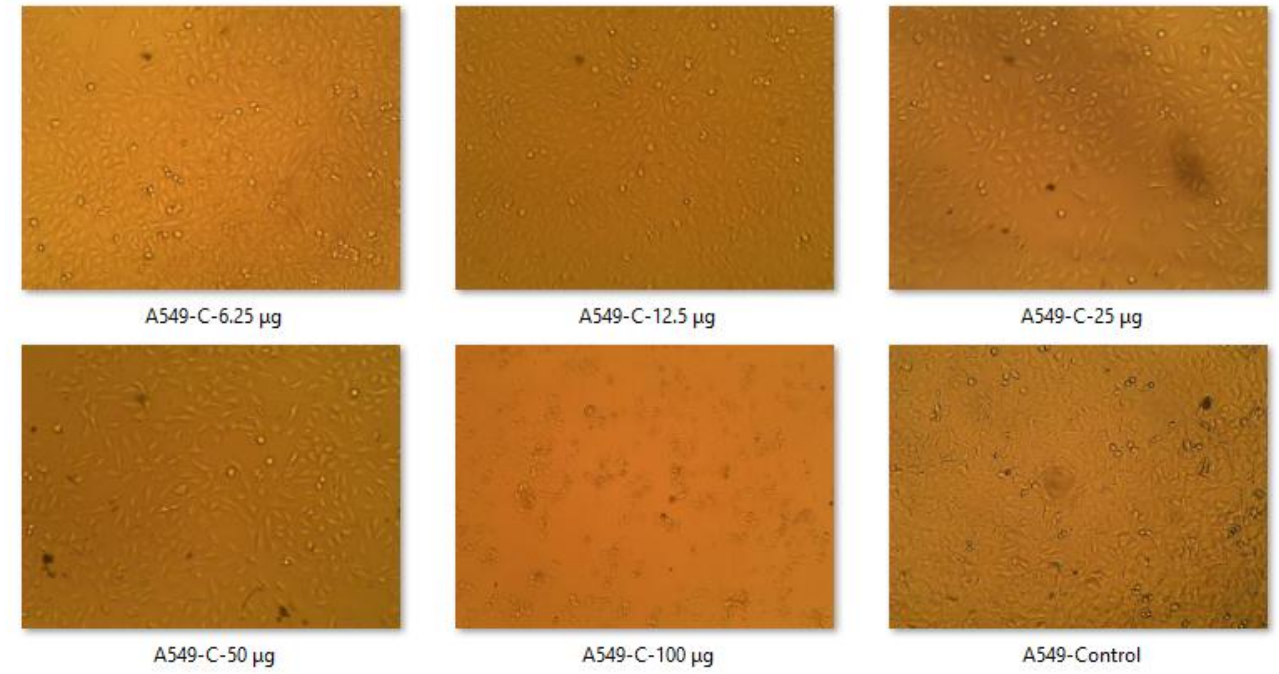
The protein hydrolysate and crude extract of *Clithon oualaniense* were tested against A549 cells (Lung cancer cells) to test the anticancer property of the compounds. Here, the protein hydrolysate showed $IC_{50}$ value of 88.84 µg/ml and crude extract with $IC_{50}$ 103.88 µg/ml. Hence the protein hydrolysate showed more activity against A549 cells, it is further analyzed to observe the cytotoxicity. The protein hydrolysate is tested with normal fibroblast cells (L929) and it showed less toxicity with143.12 µg/ml (Table 2, Figure 2). The microscopic images are described in figure 3 and 4.

Table 2: The Anticancer and Cytotoxicity effect of crude extract and protein hydrolysate of Clithon oualaniense against the A549 and L929 Cell lines

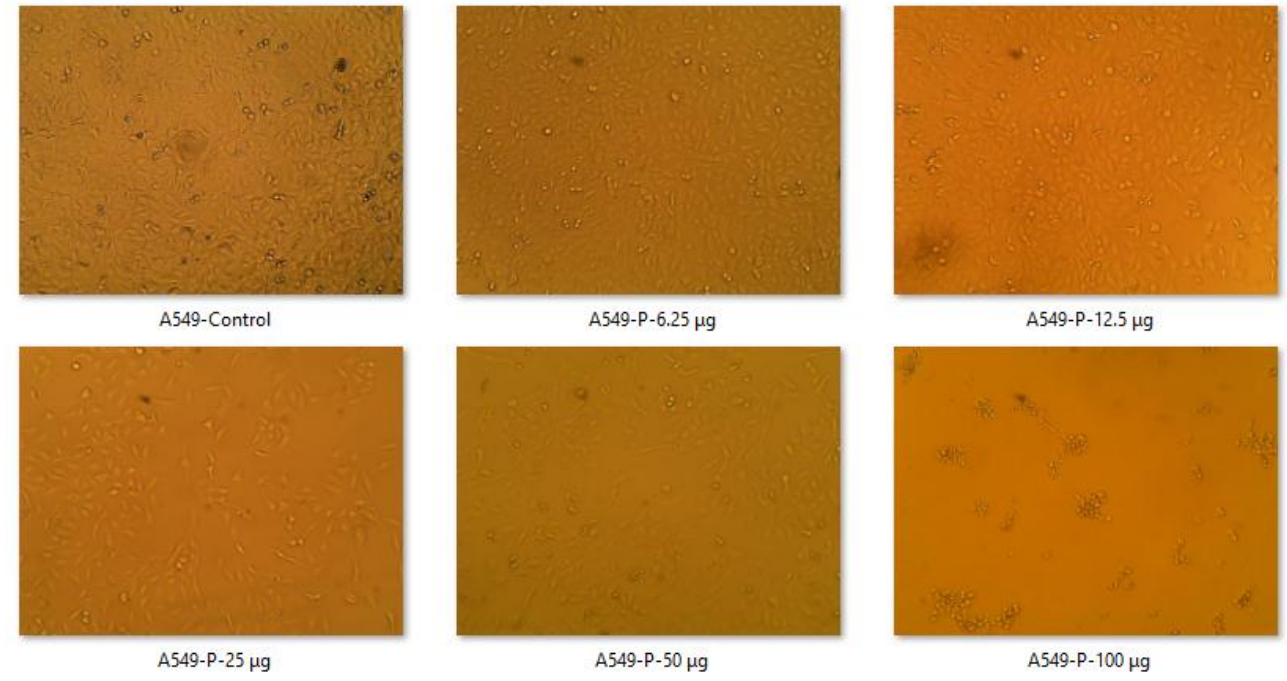
| **Cell lines** | **Percentage (%) of viability** | | | | | **$IC_{50}$ value µg/ml** |
|---|---|---|---|---|---|---|
| | **6.25** | **12.5** | **25** | **50** | **100** | |
| **L929(P)** | 98.97 | 90.88 | 81.14 | 73.57 | 66.82 | **143.12** |
| **A549(P)** | 91.51 | 87.95 | 72.82 | 67.38 | 46.29 | **88.84** |
| **A549(C)** | 93.73 | 89.12 | 71.10 | 64.20 | 55.94 | **103.88** |

P – Protein Hydrolysate, C – crude extract

Figure 2: The Anticancer and Cytotoxicity effect of crude extract and protein hydrolysate of *Clithon oualaniense* against the A549 and L929 Cell lines.




**(a)**




**(b)**

Figure 3: The Anticancer effect of (a) crude extract and (b) protein hydrolysate of *Clithon oualaniense* against the A549 Cell line

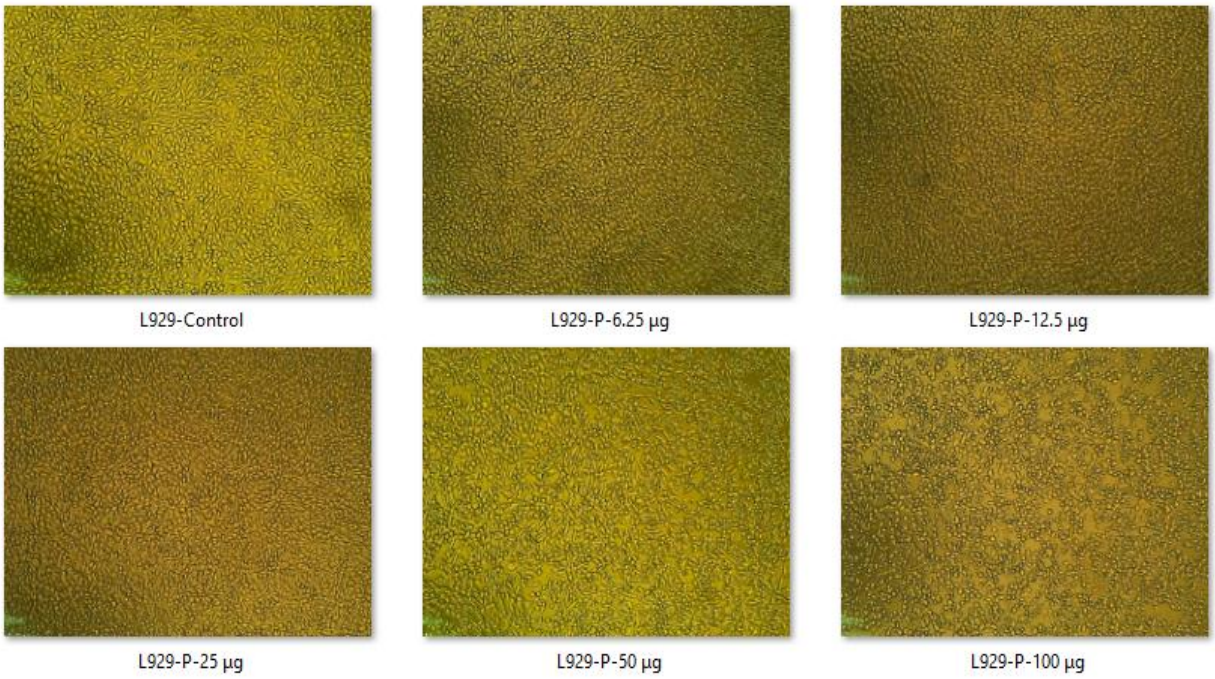



Figure 4: The Cytotoxicity effect of protein hydrolysate of *Clithon oualaniense* against the L929 Cell line.

**Determination of protein concentration**

The protein concentration was quantified as 2.2 mg/ml and molecular weight of protein hydrolysate is around 20 – 135 kDa. It was identified using SDS-PAGE (Figure 5).

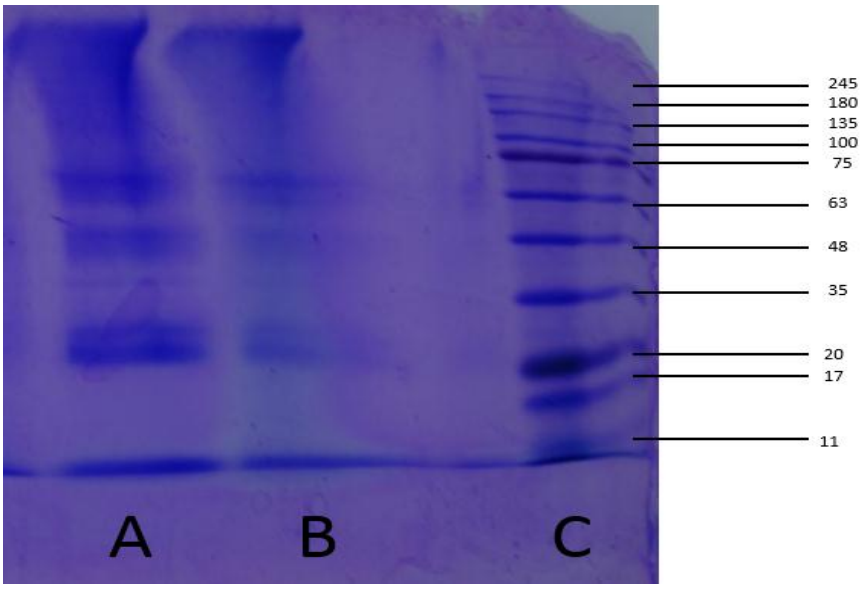



Fig 5 SDS Analysis A and B- protein Hydrolysate, M-molecular weight marker

## 4. DISCUSSION

Mollusks have great diversity of novel bioactive compounds with potential source of drug. The protein extract from mollusca has several advantages as a very promising material for antimicrobial and antitumor drugs without any side effects. The crude protein from green mussel, *Perna viridis* had more anticancer effects with less cytotoxicity towards Vero cells than *Meretix meretix* [25]. In our investigation, the protein concentration of *Clithon oualaniense*

showed 2.2 mg/ml with molecular weight 11 – 135 kDa. Previous report showed that the protein concentration of *Babylonia spirata* was 2.6 mg/ml with molecular weight 40 – 200 kDa and its protein hydrolysate has many potential antibiotics.

Biological activities of mollusca have been previously reported that Hemocyanins possess an antimicrobial, an antifungal, an antiviral and antitumor activity [26], ethanol extracts of gastropod *B. spirata* and *Turbo brunneus* showed maximum antibacterial activities against *E. coli, K. pneumoniae, P. vulgaris* and *S. typhi* respectively [27] and *Pseudomonas aeruginosa* [28]. The mucin found in the mucous secretion of *Achatina fulica* is related to antibacterial factors found in its protein with antibacterial activity [29]. The antibacterial activities of ethanol extracts of gastropod, *Babylonia spirata* and *Turbo brunneus* observed highest activity against *E. coli, K. pneumoniae, P. vulgaris* and *S. typhi* [9]. The bactericidal proteins from the Littoral Mollusk *Cenchritis muricatus* have antimicrobial activity [30].

Biochemically and pharmacologically active peptides in the hemolymph of garden snail *Helix lucorum* and marine snails *R. venosa* were analysed [31], which are rich in Cys, Pro, Ser or Gly residues showed high antimicrobial activity against *S. aureus*. [32]. Antibacterial and antiviral activities also have been previously described in the hemolymph of several molluscan species such as, sea hares, sea slung, oysters, and mussels [33]. Acetone extract of various mollusks also displayed broad spectrum of antibacterial activity against pathogens. The tissue extracts are rich in antibacterial lipophilic constituents such as sterol esters, glycerides, free fatty acids, sterol and polar lipids [34]. Acetone and methanol tissue extracts of *H. pugilinus* exerted promising in vitro antibacterial, antioxidant activity and cytotoxicity [35]. In our study it was clearly observed that the crude extract and protein hydrolysate of *Clithon oualaniense* had many potential antibacterial compounds which was proved by showing activity against all tested pathogens.

Similar to antimicrobial activity previous study reports were described about the anticancer activity of low toxic compounds from mollusks. The antitumor and immunostimulating effects of oyster hydrolysates prepared in this study revealed its potential for tumor therapy and as a dietary supplement with immunostimulatory activity [36]. The purified achacin from *A. fulica* mucus could induce death of HeLa cells at the $IC_{50}$ of 10 µg/ml [37] and the antiproliferative effect of isolates from hemolymph of *Helix lucorum* and *R. venosa* against bladder cancer cell line T-24. The effect was found to be dose- and time-dependent and similar to the effects of doxirubicin [31]. *P.virens* methanolic extract possessed the potential antioxidant and anticancer activity [1]. Our study also revealed that the protein hydrolysate showed activity against A549 cancer cell with Ic50 value 88.84 µg/ml and Ic50 value 143.12 µg/ml against normal cell L929. It was clear that snails have anticancer property with low cytotoxicity and it could be used as natural source for treating human diseases.

## 5. CONCLUSION

The present study revealed that the crude extract and protein hydrolysate of *Clithon oualaniense* showed a potent antibacterial against pathogenic microorganisms and anticancer activity against cancer cells with low cytotoxicity in normal cells. Further intention was to purify these potent bioactive compounds.

## 6. ACKNOWLEDGEMENT

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