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A Comparative Investigation of Antimicrobial Activities of Proteins Derived From *Anodonta Rubens*

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ABSTRACT

Protein extracted from *Anodonta rubens* (Mollusca) was evaluated to investigate in vitro the anti-microbial and the antiviral activities. The crude protein was fractionated by precipitation by different methods. Different measures were determined such as inhibition zone (mm), MIC (minimum inhibitory concentration), EC50. The toxicity of the most active fractions was measured by the XTT assay then CC50 and selective index value were calculated. Amino acid analysis was performed for the purified fraction. Some of the tested protein showed activities with various degrees. The most potential antiviral activity, about 96%, was achieved in case of 75-90% saturation of Polyethylene glycol of molecular weight 4000 (PEG 4000). The protein fractions showed low toxicity. The fraction is rich in essential amino acids, alanine, arginine, glutamine, glycine and phenylalanine. To the best of our knowledge, no similar works were reported with this organism and these compounds..

Keyword: *Anodonta rubens*, antiviral and antimicrobial. Protein fractions

INTRODUCTION

In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. In addition, Viruses cause many important diseases in humans, with viral-induced emerging and re-emerging infectious diseases representing a major health threat to the public. Viruses can also infect livestock and marine species, causing huge losses of many vertebrate food species. This situation, the undesirable side effect of certain antibiotics, and the emergence of previously uncommon infections, has forced scientists to look for new antimicrobial substitutions from various sources such as from plant origin and animal origin most of the publications concerning antimicrobial activity. There is a vital interest in discovering new antimicrobial compounds with fewer environmental and toxicological risks and no resistance developed by the pathogens. The study of natural products that exhibit biological activity, derived from plants and animals has long been showing significant biomedical value and crude products isolated from marine organisms have served as the source of many drugs [1,2,3].

Mollusks are widely distributed throughout the world and have many representatives. Mollusca is the second largest animal phylum, with an estimated diversity of up to 200000 extant species [4]. In Mollusca, either single body compartment alone, like haemolymph and egg masses, or extracts of whole bodies have been tested for activity and many active compounds have been reported worldwide [5,67]. Antimicrobial peptides are important in the first line of the host defense system of many animal species [8.9]. Their value in innate immunity lies in their ability to function without either high specificity or memory. Moreover, they are synthesized without dedicated cells or tissues and they can rapidly diffuse to the point of infection. The swan mussel (*Anodonta cygnea*) is one of the largest freshwater mussels (it tolerates salinity of 0.1-0.2 PSU). The swan mussel prefers fertile bottom sediments, and while it occurs both at greater distances from the shore and at greater depths, it also is noted in the shallow littoral zone devoid of macrophytes, which differentiates this species from other representatives of the Uniondale. It inhabits the zone from approximately 0.2 m to several meters in depth. The purpose of this study was to establish an in vitro evidence for that the protein fractions from *Anodonta rubens* have antimicrobial activities. The crude and the partially purified protein fractions were screened against some pathogenic bacteria and fungi while the antiviral activity was tested against Herpes simplex, mumps virus and hepatitis B virus. To the best of our knowledge, there were no previous works concerned with biological activities of the protein extracted from this organism.

MATERIALS AND METHODS

Collection and identification of the organism.

The organism was collected from Nile River. This organism was identified as *Anodonta rubens* according to its morphological, anatomical and histological characteristics cited by Luiz [10] and Burch, [11]. To avoid taxonomic

uncertainties due to the usual misidentification, identities were confirmed by genetic analysis. For this, tissue samples were collected and placed directly into 96% ethanol. The whole genomic DNA was extracted from small tissue samples pieces (2 mm3) using a standard high-salt protocol [12]. A fragment of approximately 700 bp of the mtDNA cox1 gene was amplified by polymerase chain reaction (PCR) using the primers (5-GGTCAACAAAYCATAARGATATTGG-3) and (50-TCAGGGTGACCAAAAAAYCA-30) [13,14]. The cycle parameters were: initial denaturation at 94°C for 3 min, denaturation at 94°C (30 sec), annealing at 48°C (45 sec), and extension at 72°C (45 sec) repeated for 35 cycles and a final extension at 72°C for 5 min. Amplified DNA templates were sequenced and the sequences obtained in this study were submitted to Gene Bank (Nucleotide BLAST). The evolutionary relationship, the parameters of genetic similarity and distances of the compared sequences were estimated using MEGA 5 software [15].

Test microorganisms.

Seven different bacterial strains were used, *Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, representative for G+ve bacteria and *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella typhi*, representative for G-ve bacteria to investigate the antibacterial activities. Four fungal strains, *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Trichophyton mentagrophytes* were used to test the antifungal activities. The bacterial strains were cultured and maintained on Nutrient agar media except *S. pneumonia* was cultivated on blood agar media using anaerobic jar ate 37ºC. The bacterial strain are isolates from clinical specimens The fungal strains were cultured and maintained on Sabouroud's dextrose agar media and kept refrigerated at 4º C.

Extraction of protein.

Two hundred grams of the animal tissues (whole body) were homogenized in electric blender with 500 ml of phosphate buffer, and the homogenate was put on magnetic stirring for 24 hours. The pH was adjusted to 7.5 using pH meter, Centrifuge at 4000 rpm for 20 min to remove sediment. Dialyze against deionized water for 48 hour was carried out (in refrigerator) and the water was replaced each that 6 hour and lyophilize by Freeze drier [16].

Protein determination.

Determination of total protein in the crude extract and the protein fractions was by the Bradford method [17].

Protein Fractionation.

Precipitation of protein using solvents:

Different quantities of Acetone, Ethanol or Tricholoroacetic acid (TCA) (4ºC) were added sequentially to the crude extracted (cooled 500 ml). The final saturation of the solvent was adjusted at 15, 30, 45, 60, 75 and 90 %. The protein content of each fraction was assayed after its separation using centrifugation at 10,000 rpm for 30 min at 4ºC.

Precipitation with polyethylene glycol.

The precipitation of the protein using PEG (polyethylene glycol) was carried out according to the method described by Walker [18]. PEG 400, 1000 and 4000 each with several conc. 15, 30, ……to 90% (W/V) were added slowly to cold crude extract in a jacked beaker at 4˚C and homogenized to 20 min. The resultant solution were centrifuged at 10000 rpm for 30 min at 4ºC. The resulting pellet was collected and dialyzed against deionized water.

Precipitation of the protein by salting off with ammonium sulfate

The powder of amm. Sulfate was added slowly but steadily with thorough mixing. The precipitate was allowed to form for 30 minutes at 4°C with stirring. The precipitate was recovered by centrifugation at 10000 rpm for 30 min. The pellets were resuspend in a volume of buffer equal to the volume of the extract.

Antibacterial and antifungal assay

In vitro antibacterial activity was determined by agar well diffusion technique [19], 20 mg of protein was dissolved in 1ml of water. The prepared nutrient agar plates were seeded with 0.1 ml of 3x106 cells /ml from each bacterial strain (*S. pneumoniae* was cultivated on blood agar media using anaerobic jar ate 37ºC) and the sabouroud's dextrose agar plates were seeded with 0.1ml of 4x105 sofa from each fungal strain. The agar plates were incubated at 37˚C for 24 h for bacteria and at 25º C for two days to on week for fungi (according to the type of fungi). Three replicates were formed for every sample [20], Inhibition zone was measured (mm). Tetracycline (30µg/ml) and Amoxicillin (30µg/ml) were used as controls for *S. aureus* and E. coli for bacteria and Itraconazol 0.8 mg/ml was used as control for fungi.

Determination of Minimal inhibitory concentration (MIC):

All bacterial strains were grown on Mullar Hington media and (*S. pneumonia* Mullar-Hington medium was supplemented with lysed blood cells) the concentration of the bacterial suspension was standardized to be at 0.5 McFarland. Different concentrations of the most active protein fractions (as specified) was adjusted at different values (25, 50, 100, 150, 200, 250, 300, 350, 400, 500, 1000 µg/ml).The cultures were incubated at 37ºC for 24h. The resulting turbidity was observed, MIC was determined to be where growth was no longer visible by assessment of turbidity by optical density readings at 600 nm using Cecil UV-Vis single beam Spectrophotometer. Tetracycline (100 µg/ml) and Amoxicillin (100 µg/ml) were used as controls for the *S. aureus* and *E. coli*. For fungi Sabouraud's glucos medium was used as growth medium where the spore/cell suspension was adjusted at 1x 104 cfu/ml through plat count method. Different concentrations of the most active protein fraction (as specified) were adjusted at 25, 50, 100, 150, 200, 250, 300, 350, 400, 500, 1000 µg/ml separately. After incubation at 27ºC for 2days to one week. The culturs were plated on PDA media. After incubation at 27 C for different periods of time according to the type of the fungus (2days to 10 days). MIC was determined to be where growth was no longer visible. Itraconazol 0.8 mg/ml was used as control.

Antiviral activity.

The assay was performed for herpes simplex (HSV) and mumps virus by using monolayers of Vero cells in 24-well plates seeded with 1 mL of 3 × 104 Vero cell suspension per well in minimum essential media (MEM) with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 100 units of penicillin and streptomycin. The cell viability was determined using viable cell count technique with tryban blue

stain [21]. Cells were incubated for 3 to 4 days at 35°C. The virus-extract mixture was inculcated and after contact for 2h 3 ml of MEM with 1% agarose was, overlay the cell monolayer. After solidification of the agarose the plate incubated then the cells were fixed with 10% formalin solution for 2 hr, and stained with crystal violet stain. Finely, Virus plaques were counted and the percentage of reduction was calculated. For hepatitis B (HBV) the Hepatitis B surface antigen (HBsAg) binding activity inhibition was performed according to the method described by Venkateswaran et al., [22]. EC50 is the concentration of the extract which cause 50% inhibition of virus effect.

Cytotoxicity test:

HepG2 cells were cultured in DMEM media supplemented with 10% (v/v) fetal bovine serum and 0.2% (v/v) of a penicillin/streptomycin solution. Antibiotics were added to the medium to eliminate potential interference from microbial contamination that may be present on the test material and control samples. Cells were cultured at 37 °C and 5% CO2; complete medium was changed every 2 to 3 days. For experimental procedures, cells were seeded in 96-well plates at a concentration of 1x 105 cells / well. The toxicity was measured by the XTT assay [23] (Roche 2005). Different concentrations of the protein extracts was incubated with the cells in 96 well plate at 37ºC in humated incubator (5% CO2). After 24 h of the incubation 50 µl XTT reagent was added for 1.4 h. Control was the cell without the addition of the protein extract. Blank was the medium without cells. The color was measured at 450 nm cell viability was determined by comparing the sample with control according this the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where, At = Absorbance of Test, Ab = Absorbance of Blank (Media), Ac = Absorbance of control (cells)

% cell inhibition = 100 − % cell survival. CC50 is the concentration at which the extracts causing 50% toxicity to the cells. SI (selective index) = CC50 / EC50, SI express the affinity of the extract.

Purification of the fractions.

Gel filtration.

Gel filtration on Sephadex G-75. Sephadex G-75 slurry was packed into a column (0.7×50 cm) and equilibrated with phosphate buffer (pH 7). The fractions with the highest antiviral activity from PEG4000 precipitation were placed on column. Fractions (2 ml) were collected at a flow rate of 0.25 ml/min. The elute was monitored for protein concentration at 280 nm and was also assayed for antiviral activity. Fractions with high activity were pooled and precipitated with PEG4000. Again, the precipitate was collected by centrifugation at 10, 000 rpm at 4°C for 20 min, redissolved in a minimal amount of buffer and then dialysed against the distilled water overnight.

Ion exchange.

DEAE cellulose was packed into a column (1.5×17 cm). The column was equilibrated with 4 bed volumes of phosphate buffer. The active fractions obtained by gel filtration on Sephadex G-100 were dialyzed overnight against the distilled water and applied to the column. The column was washed to remove all unbound proteins and the bound protein was eluted stepwise by 0 to 1 M NaCl. Fractions (2 ml) were collected at a flow rate of 0.5 ml/min. Fractions exhibiting antiviral activity were pooled and concentrated with PEG4000. The precipitate was collected by centrifugation at 10000 rpm at 4°C for 20 min, redissolved in buffer and dialyzed against distilled water overnight.

Electrophoresis.

After pooling of the active fraction that were obtained from the ion exchange column. The sample obtained from the column was loaded into polyacrylamide gel along with molecular weight markers [24].

Amino acid analysis.

The purified protein was subjected to amino acid analysis, samples were hydrolyzed in 6 N HCl at 110°C for 24 hrs. Amino acid analysis was carried out using LC3000 Amino acid analyzer (Eppendorf, Biotronic, Maintal, Germany), (75•6.0 mm) BTC guard column and BTC 2140 main column (145•3.2 mm) under the following conditions: Flow rate: 0.2 ml min , pressure of buffer from 0.0 to 50 bar, pressure of reagent from 0.0 to 150 bar and reaction temperature 123°C.

Statistical analysis.

All data are presented as a means from at least two repeat experiments.

RESULTS

Identification of the organism.

According to the obtained sequence the organism was identified as *Anodonta* to the genus level. The phylogenic tree was constructed as in Fig.1 (gene accession no. lcl62359). Further identification on the species level was conducted depending on the morphological anatomical and histological characteristics.

Antibacterial and Antifungal activities.

Preliminarily investigation of the growth inhibition by well diffusion.

Forty-two protein fractions from one species of mollusca, *Anodoonta rubens* were screened against seven human pathogenic bacteria and four fungal pathogens for testing their antimicrobial activities. Preliminarily, the inhibition which caused by the protein fractions was expressed as inhibition zones (mm). The inhibition zones for the protein fraction precipitated with ethanol, acetone, tricholoroacetic acid (TCA), ammonium sulphate (amm sulf) and polyethylene glycol (PEG, 400, 1000 and 4000) were given in Fig. 2 (a),(b). The maximum inhibition zone, (19-22mm), was observed in case of fractions precipitate with PEG 4000 (45-60%). This inhibition was against *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia*e, *Proteus mirabilis*, *Staphylococcus aureus, Streptococcus pneumoniae, Candida albicans and Trichophyton mentagrophytes*. Furthermore, fraction precipitated by PEG400 (45-60%) caused growth inhibition of *Aspergillus niger* and *Aspergillus flavus*. Upon using TCA (15-30%) for protein precipitation the obtained fractions showed activities against *E. coli*, *K. pneumonia*, *S. aureus* and *Proteous mirabilis* , *Salmonella. typhi* and *Streptococcus pneumoniae*. The inhibition zones ranged from 15 to 17 mm with bacterial strains while it reached 12-18 mm with the fungal strains. Upon use of the amm. Sulfate (75-90%) saturation for

protein precipitation inhibition zones of 19 mm, was recorded in case of *Candida albicans* and 14-15 mm in case of *K. pneumoniae*, *S. aureus* and *Proteous mirabilis, S. typhi* and *Streptococcus pneumonia.* While inhibition zone, 11mm was resulted in case *T. mentagrophytes* and *A. niger*. It is noticeable that the lowest antibacterial and antifungal activities against the tested microorganisms were observed after precipitation with ethanol and acetone. Tetracycline (30 µg/ml) and Amoxicillin (30µg /ml) were used as controls for the *S. aureus* and *E. coli*. Inhibition zone of 22, 23 mm was recorded for tetracyclin and amoxillin with *S.aureus*, respectively. Inhibition zones of 19 mm were recorded for both antibiotics with *E. coli*.

Minimum inhibitory concentration.

Further investigation to assess the antimicrobial activities for the protein extract included determination minimum inhibition concentration (MIC) for the protein fraction after partial purification by precipitation. The results in fig. 3(a), (b) indicated that some protein fractions have antimicrobial activities at concentrations below 250µg/ml while the protein fractions having MIC greater than 250 µg/ml were excluded. MIC (230 µg/ml) was recorded in case of protein fraction precipitated with PEG 1000 (45-60%) against *B. cereus* and this was the highest one among all the fractions tested. In case of *S. pneumonae* the MIC was 120 µg/ml for protein precipitated by PEG4000 (45-60%) and this was the lowest MIC against the bacterial strains. The lower the MIC the better activity. The obtained results indicated that the protein fractions that were precipitated by PEG of high molecular weight 1000 and 4000 have better antibacterial activities after comparison with the other fractions. Besides the determination of the MIC against the bacterial strains it was found that some protein factions having antifungal activity at MIC below 250 µg/ml as shown in Fig.3 (b). The lowest MIC (210 µg/ml) was recorded against *C. albicans* by protein precipitate by PEG1000 (45-60%).

Antiviral activates:

The antiviral activities of the precipitated protein fraction were investigated using three viruses, herpes simplex virus (V1), mumps virus (V2) and hepatitis B virus (V3). For each fraction the antiviral activities were measured at two different concentration 10 and 20 µ. As indicated in Table S1 the protein fraction, which was precipitated using 75-90%, acetone inhibit 78%, 80.55 and 82.11 % of $4.5x10^7$ /ml for V1, V2 and V3. Near to this value, the inhibition by protein precipitate using 45-60% of ethanol. (Table 2). Trichloro acetic acid at concentration 75-90% precipitated protein fraction able to inhibit 85.55, 75 and 88% of V1, V2 and V3, respectively when the dose 20ug (Table 3). In case of precipitation by salting with Amm. Sulfate at 75-90% saturation the protein fractions achieved 88.89, 85.67 and 88.89% inhibition for V1, V2 and V3, respectively (Table 4).In spite of the noticeable antiviral activities of the protein fractions precipitated with the previous materials however, the proteins that precipitated by polyethylene glycol showed a significant antiviral activity if it compared with Amm. Sulfate and the other solvents (Table 5). The inhibition was 96% by protein fraction precipitated with 75-90% of PEG-4000. In general extracts with a good antimicrobial activity of low EC50 (Efficient concentration) and high selective index (SI). As shown in table (6) the protein fraction which precipitated with PEG400 (75-90%) has SI value of 11.26 against HBV. This probably indicates its high selectivity when compared with the other extracts. EC50 was least (3.42 µg) with extract precipitated with ethanol (30-45%) against mumps virus.

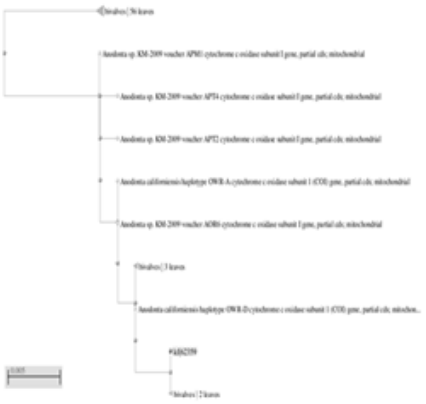

Fig.1. Phylogenetic relationships as shown by the neighbor joining

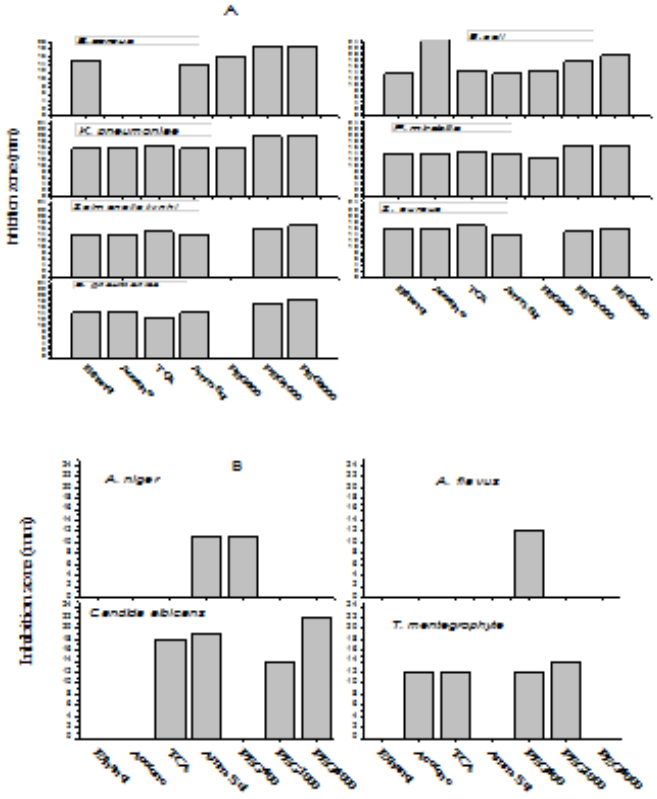

Fig. 2 The antimicrobial activities.

Antibacterial activities (a) of protein fractions after partial purification. Inhibition zone by mm. the active fractions that precipitated with ethanol conc. (0-15%), acetone, TCA (15-30%), Amm. Sulfate (75-90%) and PEG 400, 1000 and 4000 at concentration (45-60%). Antifungal activities (b) Inhibition zone by mm. the active fractions that precipitated with TCA (15-30%), Amm. Sulfate (75-90%) and PEG 400, 1000 and 4000 at concentration (45-60%).

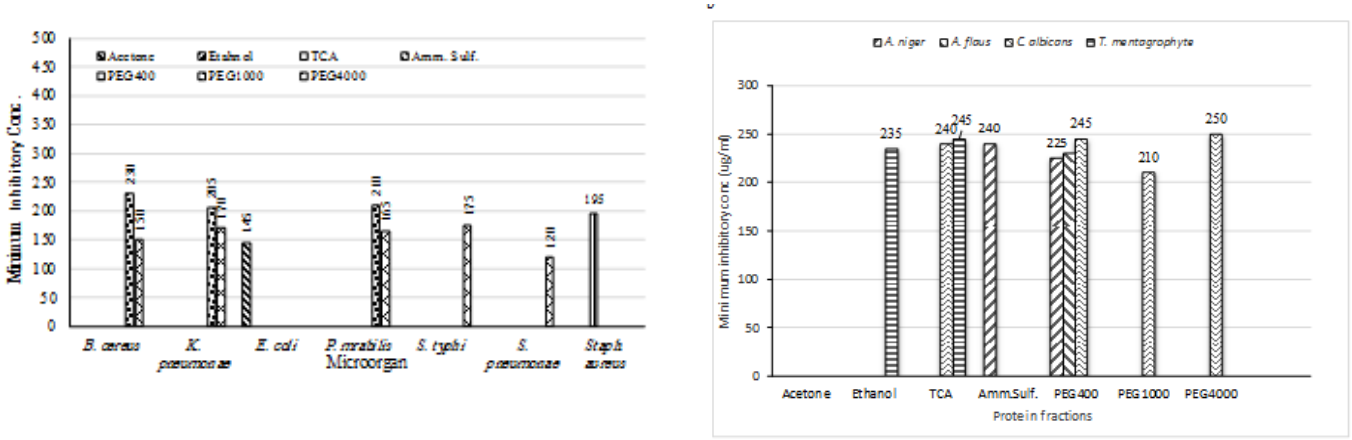

Fig. 3.Minimum inhibitory concentration (MIC): Antibacterial activities (a) of protein fractions after partial purification the active fractions that precipitated with ethanol conc. (0-15%), acetone, TCA (15-30%), Amm. Sulfate (75-90%) and PEG 400, 1000 and 4000 at concentration (45-60%). Antifungal activities (b) the active fractions that precipitated with TCA (15-30%), Amm. Sulfate (75-90%) and PEG 400, 1000 and 4000 at concentration (45-60%).

Table 1- Viral inhibition by different protein fractions obtained after precipitation with acetone using antiviral assay strategies.

Fraction	Protein content mg/ml	Recov protein	Dose conc.	Initial viral conc. $X10^7$	Virus conc Pfu/ml$X10^7$			Viral inhibition (%)		
					V1	V2	V3	V1	V2	V3
Crude	38	100	10	4.5	1.1	3.80	4.50	75.55	15.65	0.00
			20	4.5	1.5	3.76	4.50	66.66	16.36	0.00
0-15	1.9	5.00	10	4.5	4.80	4.50	4.50	0.00	0.00	0.00
			20	4.5	4.00	4.50	4.50	11.11	0.00	0.00
15-30	3.20	8.42	10	4.5	5.10	4.50	4.50	0.00	0.00	0.00
			20	4.5	6.00	4.50	4.50	0.00	0.00	0.00
30-45	4.21	11.07	10	4.5	4.00	4.10	4.02	11.11	9.00	10.75
			20	4.5	3.34	4.10	4.01	25.77	9.00	10.85
45-60	5.30	13.94	10	4.5	3.10	3.94	3.44	31.11	12.45	23.61
			20	4.5	3.98	3.84	3.08	11.55	14.76	31.65
60-75	18.94	49.84	10	4.5	1.11	2.02	1.56	75.33	55.13	65.30
			20	4.5	0.95	1.88	1.45	78.88	58.18	67.68
75-90	4.10	10.78	10	4.5	0.92	1.39	1.11	79.55	69.17	75.35
			20	4.5	0.65	1.10	0.52	85.55	75.65	88.34

Table 2- Viral inhibition by different protein fractions obtained after precipitation with ethanol using antiviral assay strategies.

Fraction	Protein content mg/ml	Recov. protein	Dose conc.	Initial viral conc. $X10^7$	Virus conc. pfu/mlx10^7			Viral inhibition (%)		
					V1	V2	V3	V1	V2	V3
Crude	36	100	10	4.5	3.46	3.46	3.36	23.10	22.3	21.45
			20	4.5	3.36	3.36	3.28	25.22	24.65	25.34
0-15	2.7	10.27	10	4.5	3.33	3.24	3.19	26.00	28.11	27.13
			20	4.5	3.21	3.03	2.86	28.67	32.67	29.12
15-30	3.4	11.66	10	4.5	2.97	2.86	2.75	34.00	36.34	36.34
			20	4.5	2.99	2.82	2.64	33.56	37.34	39.00
30-45	10.25	34.86	10	4.5	2.87	2.78	2.19	36.22	38.23	41.34
			20	4.5	2.90	2.47	2.01	35.56	45.15	51.34
45-60	18	50.00	10	4.5	1.99	1.96	1.53	55.78	56.34	55.23
			20	4.5	1.183	1.02	1.40	73.71	77.23	66.11
60-75	1.20	7.00	10	4.5	1.33	1.06	1.11	70.44	76.44	69.00
			20	4.5	1.11	1.05	1.02	75.33	76.63	75.34
75-90	0.5	4.10	10	4.5	1.00	1.33	0.81	77.78	70.34	77.33
			20	4.5	0.99	0.88	3.36	78.00	80.55	82.11

Dose conc. is the dose of the protein fraction pfu plaque forming unit, V1: Herpes simplex virus, V2 mumps virus and V3 HBV

Table 3- Viral inhibition by different protein fractions obtained after precipitation with TCA using antiviral assay strategies

Fraction	Protein content mg/ml	Recov. protein	Dose conc.	Initial viral conc. $X10^7$	Virus conc. Pfu/mlx10^7			Viral inhibition (%)		
					V1	V2	V3	V1	V2	V3
Crude	36	100	10	4.5	3.46	3.82	4.18	23.10	15.10	07.00
			20	4.5	3.36	3.64	4.13	25.22	19.22	08.22
0-15	2.7	10.27	10	4.5	3.68	3.91	3.95	18.11	13.10	12.10
			20	4.5	3.62	3.85	3.85	19.50	14.34	14.34
15-30	3.40	11.66	10	4.5	3.89	3.99	3.77	13.55	11.35	16.11
			20	4.5	3.84	3.90	3.72	14.65	13.23	17.23
30-45	10.25	34.86	10	4.5	1.57	1.26	1.29	65.10	72.10	71.12
			20	4.5	1.46	1.20	1.02	67.34	73.33	77.32
45-60	18.00	50.00	10	4.5	1.29	1.57	1.99	71.30	65.11	55.60
			20	4.5	1.14	1.49	1.97	74.65	66.96	56.15
60-75	1.2	7.00	10	4.5	3.82	4.00	4.04	15.10	11.11	10.13
			20	4.5	3.77	3.77	3.95	16.11	16.21	12.01
75-90	0.5	4.16	10	4.5	3.95	4.09	3.85	12.15	9.17	14.25
			20	4.5	3.90	4.08	3.80	13.14	9.23	15.41

Initial Dose conc. is the dose of the protein fraction pfu plaque, V1: Herpes simplex virus, V2 mumps virus and V3 HBV

Table 4- Inhibition of viral replication by different protein fractions obtained after precipitation with Amm. sulfate using antiviral assay strategies.

Fraction	Protein content mg/ml	Recov. protein	Dose conc (µg)	Initial viral conc. $X10^7$	Virus conc Pfu/ml			Viral inhibition (%)		
					V1	V2	V3	V1	V2	V3
Crude	36	100	10	4.5	3.46	4.50	0.89	23.11	0.00	13.45
			20	4.5	3.36	4.50	3.75	25.33	0.00	16.67
0-15	5.2	14.44	10	4.5	2.7	3.94	2.90	40.00	12.34	35.56
			20	4.5	2.8	3.43	2.44	37.78	23.67	45.76
15-30	3.6	10	10	4.5	2.5	2.93	4.50	44.44	34.78	0.00
			20	4.5	2.8	2.45	4.50	37.78	45.66	0.00
30-45	3.4	9.4	10	4.5	2.4	3.44	2.44	46.67	23.56	45.78
			20	4.5	2.30	3.21	2.08	48.89	28.67	53.78
45-60	2.70	7.50	10	4.5	2.61	2.94	1.45	42.00	34.67	67.78
			20	4.5	2.66	2.63	1.03	40.89	41.45	77.10
60-75	3.12	8.66	10	4.5	1.40	1.95	1.26	68.89	56.56	71.98
			20	4.5	1.22	1.68	1.01	72.89	62.76	77.45
75-90	16.98	47.16	10	4.5	0.7	0.96	0.95	84.44	78.65	79.00
			20	4.5	0.5	0.64	0 .50	88.89	85.67	88.89

Dose conc. is the dose of the protein fraction, pfu plaque forming unit, V1: Herpes simplex virus, V2 mumps virus and V3 HBV

Table 5- Inhibition of viral replication by protein fractions obtained after precipitation with PEG using antiviral assay strategies against Herpes simplex virus.

Fraction	Protein content mg/ml			Recovered protein (%)			Dose conc µg	Initial viral conc. $X10^7$	Virus conc. pfu/ml			Viral inhibition (%)		
	a	b	c	a	b	c			a	b	c	a	b	c
Crude	38	38	38	100	100	100	10	4.5	1.7	1.7	1.7	62.2	62.2	62.2
							20	4.5	1.5	1.5	1.5	66.7	66.7	66.7
0-15	12.5	5.1	6.6	32.8	13.4	17.2	10	4.5	2.2	1.98	1.75	51.1	56.0	61.1
							20	4.5	2.0	1.60	1.48	55.6	64.4	67.1
15-30	3.1	4.1	5.1	8.2	10.2	13.4	10	4.5	2.0	1.58	1.40	55.6	64.9	68.9
							20	4.5	1.8	1.45	1.31	60.0	67.8	70.9
30-45	4.5	3.5	5.8	11.2	9.21	15.3	10	4.5	3.6	1.34	1.24	20.0	70.2	72.4
							20	4.5	3.4	1.30	1.19	24.4	71.1	73.6
45-60	5.2	7.1	6.1	13.6	18.7	16.0	10	4.5	2.71	125	1.11	39.8	72.2	75.3
							20	4.5	2.30	1.22	1.00	48.9	72.9	77.8
60-75	5.2	13.6	8.2	13.4	35.7	21.5	10	4.5	1.81	1.20	0.92	59.8	73.3	79.6
							20	4.5	1.44	1.15	0.87	68.0	74.4	80.7
75-90	6.6	5.0	6.0	18.0	13.1	15.7	10	4.5	1.98	1.00	o.75	70.4	77.8	91.3
							20	4.5	1.85	0.90	0.69	72.2	80.0	95.6

a =PEG 400*, b =PEG 1000* and c = PEG4000* are the different molecular weights of the polyethylene, Dose conc.: the dose of the protein fraction, pfu: plaque-forming unit.

Table 6- The values of CC50, EC50 and selective index (SI) for the protein fraction of the best antiviral activities. Fraction of proteins precipitated with: I: acetone (75-90%), II: ethanol (30-45%), III: ethanol (45-60%), IV: TCA

Fractions	CC_{50} (µg)	EC_{50} (µg)			SI		
		V1	V2	V3	V1	V2	V3
I	44.26	8.78	7.78	6.56	5.04	5.69	6.75
II	23.56	3.44	3.42	3.35	6.85	6.89	7.03
III	31.35	3.55	3.62	3.66	8.83	8.66	8.57
IV	27.48	5.42	4.77	5.31	5.07	5.76	5.18
V	77.86	9.50	9.31	9.31	8.20	8.36	8.36
VI	66.21	6.90	6.96	5.88	9.60	9.51	11.26
VII	44.25	6.52	5.98	5.74	6.79	7.40	7.71
VIII	48.54	7.11	7.97	6.96	6.83	6.09	6.97

Fraction of proteins precipitated with: I: acetone (75-90%), II: ethanol (30-45%), III: ethanol (45-60%), IV: TCA (75-90%), V: Amm. Sulfate (75-90%), VI: PEG400 (75-90%), VII: PEG 1000 (75-90%), VIII: PEG4000 (75-90%). V1: Herpes simplex virus, V2 mumps virus and V3 HBV.

Cytotoxicity test:

The Cytotoxicity for the protein fractions of the most antiviral ativities was performed as described above Figure S4. The CC50 for protein fraction precipitated with ethanol at (30-45%) was (23.56 µg) and this was the lowest values if it compared with other tested fractions. The protein fraction precipitated with Amm. Sulfate at (75-90%) had CC50 value of 77.86 µg which is the highest one among the tested fractions.

Protein purification.

The most active fraction, which precipitated with PEG 4000, was purified by chromatographic methods. The purification started with gel filtration and the results are indicated in Fig. 5(a) where one peak of high antiviral activity. The active fractions were pooled (fractions 20-31) and applied to ion exchange column (DEAE cellulose) where the protein was eluted stepwise with NaCl 0.2 -1M. As indicated in Fig.5 (b) the fractions 18-30 were pooled. The antiviral activities for the purified fraction were found to be 91.16, 89.18, 88.98 % against HSV, Mumps and HBV, respectively Table 7. Gel electrophoresis revealed a single band for the antiviral active fraction (Fig.6) of Molecular weight of the purified protein was found to be about 40 KDa.

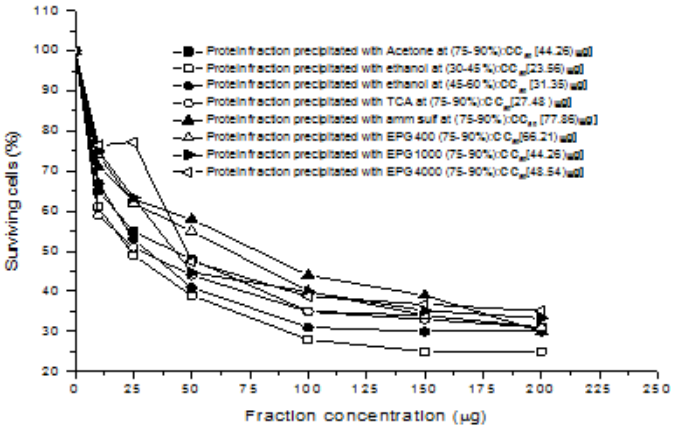

Fig. 4. Cytotoxicity for the protein fraction of the most antiviral activities

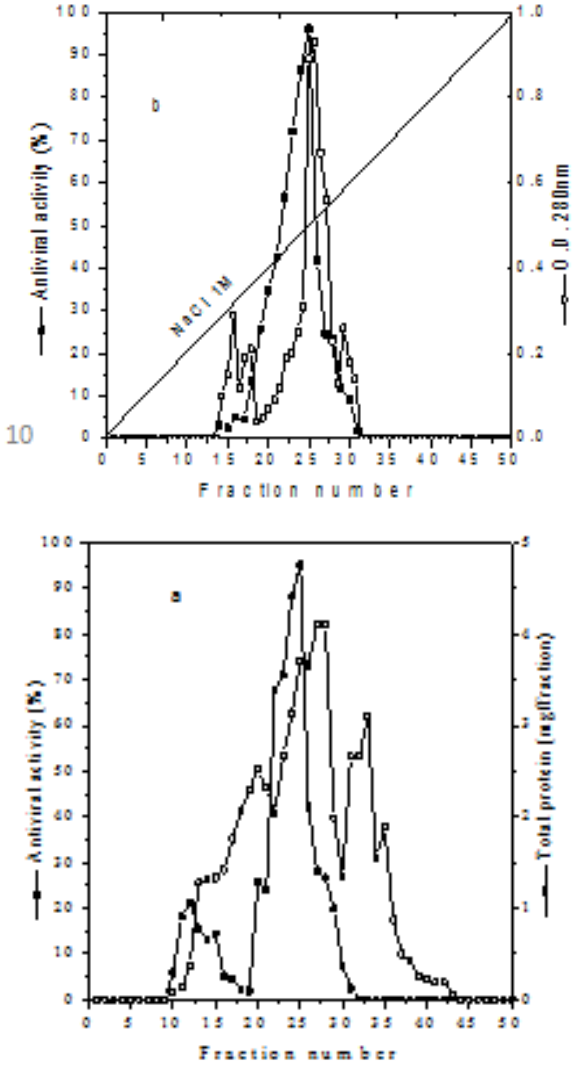

Fig.5. Gel filtration (a) and ion exchange (b) of the most active antiviral protein fraction

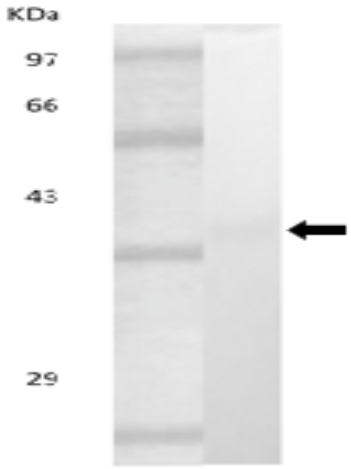

Fig.6. Electrophoresis gel showing the single band (the arrow) of the purified protein

Table 7 Antiviral activities of the purified fractions

Fraction	Protein content (mg/ml)	Recovered content (%)	Dose (µg)	Antiviral Inhibition (%) V1	V2	V3
Crude	38	100%	20	62.2	59.11	58.19
F1	6.0	15.70	10 20	95 90.9	93.14 89.11	89.22 85.11
F2	4.5	11.8	10 20	93.40 89.14	90.11 83.44	88.10 79.40
F3	4.2	11.05	10 20	91.6 89.55	89.18 79.70	88.89 80.51

Amino acid analysis

After amino acid analysis a group of essential amino acids was detected. The fraction is rich in essential amino acids like alanine, arginine, glutamine, and glycine and phenylalanine (Fig. 7). .

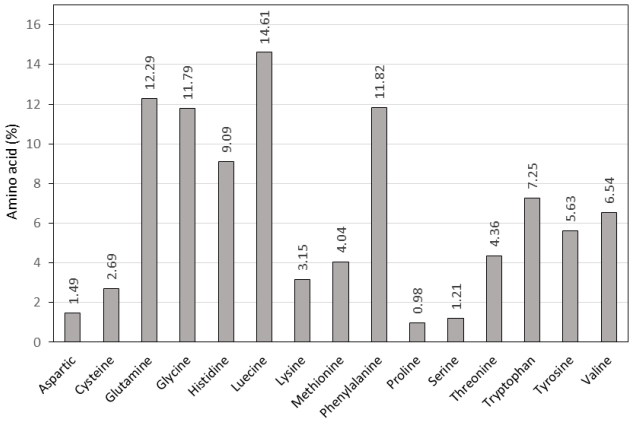

Fig. (7)- Amino acid content of the active fraction protein.

DISCUSSION

Viral infections are the cause of many human and animal diseases that have tremendous economic impacts. The limited availability of antiviral measures, along with the appearance of new virus types and drug-resistant viral strains, have led scientists to expand their search for novel drug candidates, recently turning back to nature. In the present study antimicrobial activity has been observed against bacterial and fungal strains. Antibacterial activity has previously been described in a wide range of mollusk species [25, 26, 27, 28]. The results are in agreement with that recorded by Pakrashi, et.al. [29](2001) who found that ammonium sulfate precipitated protein (SF-50) isolated from the sperm theca gland of *Telescopium sp*., showed antimicrobial effect on *Escherichia coli*. The antimicrobial activity of oyster *Crassostrea madrasensis* and mussel *Perna viridis* was cited by Annamalai et al., [30]. While nearly all the obtained protein fractions showed the antibacterial, antifungal and antiviral activities, but the protein fractions precipitated by PEG of high molecular weight was the most potent antiviral one. It was noted that with increase of molecular weight of PEG the antiviral activities increased. The molecular basis of the protein-precipitating action of PEG and other polymers is poorly understood. The original work of Polson et al. [31] documented the increasing effectiveness of PEG as the size of the polymer is increased. Juckes [32] subsequently called attention to the tendency of larger proteins to precipitate at lower concentrations of PEG and noted a correlation between the slope of the precipitation curve and the size of the protein. It is worthy to note that low toxicity for these protein fractions and could be no side effect. The potent protein fraction was found to be rich in essential amino acids like arginine, glutamine, glycine and phenylalanine. Wang et al. [33] suggested that amino acids can design a peptide of a desired activities.

CONCLUSION

Some protein fractions from *Anodonta rubens* which was collected from the fresh water of the Nile river was found to possess pharmacological action. A set of experimental tests in vitro determined the safety and effectiveness of these extracts. Experimental results revealed that the protein extracts have a varied and significant levels of antimicrobial activities with no toxic effect. This was the first citation for such compounds from *Anodonta rubenes* to be studied for its antimicrobial activity. The actions of these proteins may guide further research.

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