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## Bioactive potency of epidermal mucus extracts from greasy grouper, *Epinephelus tauvina* (Forsskal, 1775)

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### ABSTRACT

**Objective:** To study the bio-potency of epidermal mucus from *Epinephelus tauvina*.

**Methods:** Mucus was extracted with acidic, organic and aqueous solvents. Protein, carbohydrate, lipid, amino acid and fatty acid content of mucus extracts were quantified by UV-spectrophotometer, high performance liquid chromatography and gas chromatography-mass spectrometer, respectively. Antimicrobial activity was tested against five human and fish pathogens by using agar well diffusion method. The molecular weight of peptides was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The haemolytic activity of extracts was tested against chick, goat, cow and human red blood cell.

**Results:** Protein contributed with maximum of 26.25% in crude mucus. Arginine was recorded maximum of (133.9 nmol/mL) in crude mucus. 2,4,6-Decatrienoic acid and bis (a-chloroethyl) sulfone were confirmed in organic extract. The antimicrobial activity of acidic extract was significant. Among the human pathogens, maximum zone of inhibition [(26.0 ± 0.3) mm] was observed against *Proteus mirabilis*. Whereas, among fish pathogens maximum zone of inhibition [(25.0 ± 0.1) mm] was observed against *Vibrio parahaemolyticus*. The activity of other two extracts was not remarkable. The molecular weight of peptides ranged from 115.5–37.1 kDa in acidic extract was determined. Chicken and goat blood were found to be highly vulnerable to the lysis.

**Conclusions:** The whole mucus could be a promising source with numerous bioactive-potency. Consequently, this preliminary information suggested that mucus is a source of novel antimicrobial agents for fish and human health related applications.

## 1. Introduction

The broad usage of antibiotics has led to resistance and emergence of various potent pathogens, nullifying the effect of active drug components. Hence, the world is in need of novel active bio-molecules to combat the emerging and re-emerging pathogens hindering aquatic and human health status. Fishes are highly diverse group of animals and are highly specialized for their aquatic existence. They comprised today almost half the number of existing vertebrate species. They are in intimate contact with their environment, which can contain very high load

of bacteria and viruses. Many of these pathogens are saprophytic, while some are pathogenic and both are capable of digesting and degrading the fish tissues. However, under normal conditions the fish maintains a healthy state by defending itself against these potential invaders by a complex system of innate and adaptive defence mechanisms[1].

Gut, skin and gills are the major mucosal surfaces and immune barriers. The epidermal layer contains specialized glandular cells that generate mucins and alarm substances. These substances having potential antimicrobial and noxious properties[2]. Mucus gives physical defence to epidermis and also serves as an anti-predatory activity in fishes[3]. It is advantageous not only to fish but also to mankind for various purposes like immunological defence and also in aquaculture industry. Mucus is one of the most vital components for fish mucosal immunity. The innate immune components present in the epidermal mucus are glycoproteins, lysozyme, complement proteins, lectins, C-reactive proteins, flavoenzymes, proteolytic enzymes, and antimicrobial

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peptides as well as immunoglobulins[4,5]. On the other hand, there are many important adaptive immunity components such as immunoglobulins, B and T lymphocytes, *etc.*[6]. The specific immune mechanisms of fishes are slow due to their poikilothermic nature. Therefore, fishes are relying highly on their innate immunity, which is epidermal mucus. The vertebrate immune system includes lymphoid organs that, according to their ontogeny and functional characteristics, are considered to be either primary or secondary and one of the secondary organs working on it is the mucosa associated lymphoid tissue[7]. Gut-associated lymphoid tissue has been the extension of mucosa associated lymphoid tissue[8]. Mucosal immunity in fish is very rarely studied research field, although there is currently great interest in this knowledge and the study of the gut-associated lymphoid tissue has intensified in recent years[9,10]. Fish mucosa associated lymphoid tissue has defence mechanisms (both innate and adaptive) that constitute the first line of defence against the infectious agents[10-12].

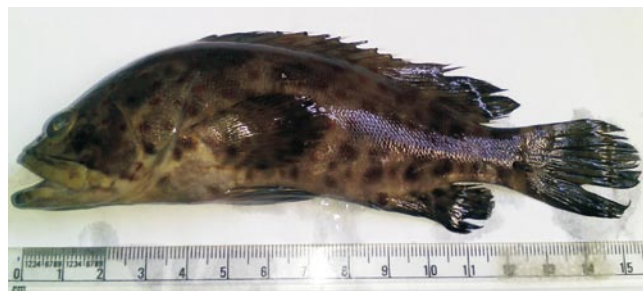
Recent studies on mucus of other fishes like catfishes, eels, rainbow trouts *etc.*, stated that the epithelial surfaces of skin, gills, are inhabited with potential pathogens. Fish live in a microbe-rich environment and is vulnerable to infestation by pathogenic or opportunistic micro-organisms. Over the past years, it has also been shown that mucus plays a vital role in the prevention of colonization of parasites, bacteria and fungi[13,14].

Albeit, more frequently mucus prevents the pathogen adherence to the underlying tissues being an indispensable barrier in the self-defence system of fishes. Fish epidermal mucus has antimicrobial peptides which are known as host defence peptides in fact they are evolutionary conserved components of innate immune system found among all cases of life[15]. Antimicrobial peptides showed a broad spectrum of activity that is 12–100 times more potent than that of amphibian antimicrobial peptides against various fish and human pathogens[16]. Based on the above information, the present study was carried with a goal in order to prove that, epidermal mucus are immune potent source against invading pathogens and acting as an immunological barriers of those aquatic edible fishes.

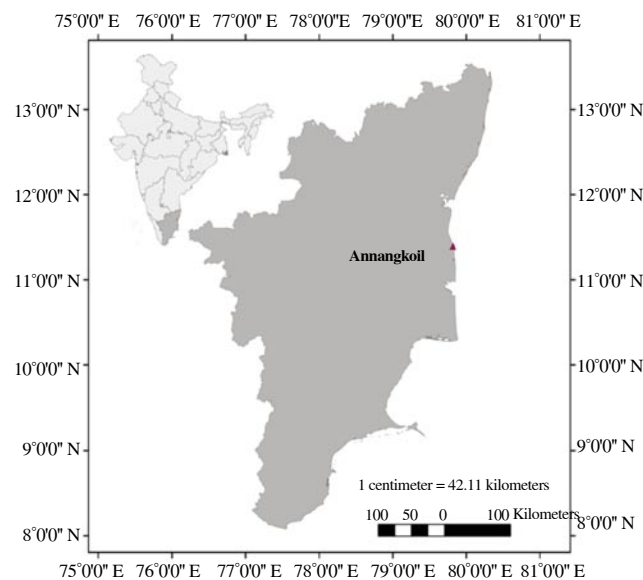
## 2. Materials and methods

### 2.1. Sample collection and preservation

The epidermal mucus from common edible fish *Epinephelus tauvina* (*E. tauvina*) (Figure 1) were obtained as described by Ross *et al.*[17]. Fish were collected from Annangkovil landing centre of Parangipettai (Figure 2). The mucus was suspiciously scraped from the dorsal body surface using a sterile spatula. Mucus from the ventral skin was not collected in order to avoid urinal and spermal contaminations. It was then transported immediately to the laboratory and was centrifuged at 8000 r/min for 10 min. The supernatant was collected and lyophilized properly. The lyophilized samples were stored at  $-20^{\circ}\text{C}$  for further analysis.



**Figure 1.** The study animal, *E. tauvina* (greasy grouper).



**Figure 2.** Map showing the study area, Annangkovil, Parangipettai coastal waters.

### 2.2. Biochemical composition

#### 2.2.1. Protein estimation

Total protein was estimated by following the method of Lowry *et al.*[18]. Known quantity of sample was taken and ground it well with 80% ethanol. Then it was centrifuged at 5000 r/min for 15 min. The precipitate was taken and dissolved in 1 mol/L NaOH and made up to 5 mL. From this, 0.5 mL was taken, followed by 5 mL of solution C (50 mL of solution A with 1 mL of solution B) was added and kept it for 10 min. Finally, 0.5 mL of folin phenol reagent was added and the intensity of colour developed was read at 640 nm in a spectrophotometer. The concentration of the protein content present in the samples were calculated by using the formula:

Protein present in the test sample (mg/g) = optical density of the sample  $\times$  concentration of the standard optical density/weight of the sample.

#### 2.2.2. Carbohydrate estimation

Total carbohydrate was estimated by following the method of Seifter *et al.*[19]. To an aliquot of sample homogenate, 4 mL of anthrone reagent was added and incubated in a boiling water bath for 15 min. Tubes were then cooled at room temperature at dark condition. Then the optical density was measured at 750 nm by using spectrophotometer. Here glucose (100 mg/100 mL distilled water) was used as the standard. The concentration of the carbohydrate

content present in the samples were calculated by using the formula:

Carbohydrate present in the test sample (mg/g) = optical density of the sample  $\times$  concentration of the standard optical density/weight of the sample.

### 2.2.3. Lipid estimation

Total lipid was estimated by following the method of Folch *et al.*[20]. Known weight of test sample was taken and homogenized well with 4 mL of chloroform methanol (2:1) mixture. After mixing well, 0.2 mL of 0.9% sodium chloride was added and kept the mixture for overnight at room temperature. The lower layer of lipid was collected carefully and dried in vacuum desiccators. The dried total lipid was dissolved by using concentrated sulphuric acid by keeping in boiling water bath for 10 min. From the above prepared total lipid sample, 0.2 mL was taken in test tube and 5 mL of sulfo-phospho-vanillin reagent was added, shaken well and kept for 30 min. The intensity of red colour was measured at 520 nm by using spectrophotometer. The concentration of the lipid content present in the samples were calculated by using the formula:

Lipid present in the test sample (mg/g) = optical density of the sample  $\times$  concentration of the standard optical density/weight of the sample.

### 2.3. Analysis of amino acid composition using o-phthalaldehyde derivatization for high performance liquid chromatography (HPLC)

Amino acid composition was analysed by following the method of Bruckner *et al.*[21] and pre-column o-phthalaldehyde derivatization for HPLC analysis of amino acids was done in Agilent 1100 HP-HPLC using C18, 150 mm length, 5.0  $\mu$ m particle sizes, 4.6 diameter column and the results were analysed using Chemstation software (Agilent Technologies Inc, Alpharetta, US). Standards, buffers and o-phthalaldehyde used in this analysis were procured commercially from Hewlett Packard (Agilent Technologies Inc, Alpharetta, US).

The instrument was calibrated using 1.0 nm, 500 pm, 250 pm standards individually. Mix 10  $\mu$ L of the standard in 60  $\mu$ L borate buffer and 10  $\mu$ L of o-phthalaldehyde reagent in dilution vial then cyclomixed it. From this mixture 50  $\mu$ L was injected in the HPLC using Hamilton syringes. Each standard was individually run in the gradient program mentioned below. The chromatogram for them was obtained. The two consecutive runs that have the same retention time were taken and the average of them was used for plotting the graphs in the calibration table. The procedure was termed as calibration and the curve obtained for the same was calibration curve. Mixed 10  $\mu$ L of the sample in 60  $\mu$ L borate buffer and 10  $\mu$ L of o-phthalaldehyde reagent in dilution vial then cyclomixed it.

From this mixture 50  $\mu$ L was injected in the HPLC using Hamilton syringes. After running the sample the chromatogram was obtained for the same. The area of peaks in the chromatogram were recorded and calibrated along with the standards.

### 2.4. Functional group analysis in crude mucus using fourier transform infrared spectroscopy (FTIR)

The powdered crude mucus sample (5 mg) was mixed with KBr 100 mg and subjected to a pressure of about  $5.10^6$  Pa in an emigrant dye to produce a clear transparent disc of 13 mm diameter and 1 mm thickness. FTIR spectra in the region from 4000 to 500  $\text{cm}^{-1}$  were recorded at room temperature [ $(25 \pm 1)^\circ\text{C}$ ] on a Nicolet Avater 360. FTIR spectrometer were equipped with an air-cooled deuterated triglycine sulfate and purged with nitrogen. For each spectrum 100 interferograms providing a spectral resolution of 4  $\text{cm}^{-1}$  were co-added. Each sample was scanned with three different pellets under identical conditions. The spectra were analyzed using Origin 8.0 software (Origin Lab Corporation, Massachusetts, US).

### 2.5. Preparation of extracts from mucus

#### 2.5.1. Aqueous extract

The lyophilized mucus sample was re-suspended in 100 mmol/L (w/v) of ammonium bicarbonate at 1 mg/mL. The mixture was centrifuged at 9000 r/min for 10 min at  $4^\circ\text{C}$ . Then the supernatant was collected and checked for antimicrobial activity.

#### 2.5.2. Organic extract

The organic extract of mucus was prepared as described by Hellio *et al.*[22] with minor modifications. The lyophilized powder was suspended in 95% ethanol at 1 mg/mL and centrifuged at 11000 r/min for 30 min at  $4^\circ\text{C}$ . The supernatant was discarded and pellet was collected. The ethanol extract pellet was evaporated overnight and the extract was suspended in 50 mL of distilled water, and then partitioned 4 times with 200 mL of dichloromethane. The remaining was re-dissolved in water and 5% dimethylsulfoxide, which was then checked for antimicrobial activity.

#### 2.5.3. Acidic extract

The acidic extracts of mucus were prepared using the method described by Diamond *et al.*[23] with minor modifications. The lyophilized mucus sample was mixed with 150 mL of 10% (w/v) acetic acid and placed in boiling water bath for 5 min. The acid mucus mixture was then centrifuged at 10000 r/min for 35 min at  $4^\circ\text{C}$ . Supernatant was collected and checked for the antimicrobial activity.

### 2.6. Analysis of spectral property of the extracts using UV-visible spectroscopy

UV spectral absorbance curve of each dilution extract was determined by JASCO UV 2D Spectrophotometer, Japan. The UV absorbance curve was obtained by scanning wavelength between 200 and 800 nm. Maximum absorbance and peak reading were plotted using Origin 8.0 software.

### 2.7. Functional group analysis in organic extract using FTIR

The powdered organic extract of mucus sample (5 mg) was mixed with KBr (100 mg) and subjected to a pressure of about  $5.10^6$  Pa in an emigrant dye to produce a clear transparent disc of 13 mm diameter and 1 mm thickness. FTIR spectra in the region  $4000\text{--}500\text{ cm}^{-1}$  were recorded at room temperature as ( $25 \pm 1^\circ\text{C}$ ) on a Nicolet Avater 360. FTIR spectrometer equipped with an air-cooled deuterated triglycine sulfate and purged with nitrogen. For each spectrum 100 interferograms providing a spectral resolution of  $4\text{ cm}^{-1}$  were co-added. Each sample was scanned with three different pellets under identical conditions. The spectra were analysed using Origin 8.0 software.

### 2.8. Fatty acid composition analysis in organic extract using gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the organic extract of mucus sample was performed by using JEOL, GC-MS, Japan. The mass spectrometer was operated in the EI mode at 70 eV. Ion source and transfer line temperature were kept at  $300^\circ\text{C}$ . The mass spectra were obtained by centroid scan of the mass range from 50 to 800 amu. Identification of organic compounds was done by matching their recorded spectra with the data bank mass spectra of National Institute of Standards and Technology library V11 provided by the instruments software.

### 2.9. Antimicrobial activity

Nutrient medium was used for the growth of marine bacteria. Nutrient broth powder was dissolved in appropriate amount of distilled water. The pH was adjusted to 7.0 and autoclaved at  $121^\circ\text{C}$  for 20 min. The media were cooled at room temperature and kept for further use. For nutrient agar, the medium was poured into Petri plate and left at room temperature for solidification.

### 2.10. Antimicrobial activity of extracts

The aqueous, organic and acidic extracts were checked for their antimicrobial activity using agar well diffusion method against selected clinical human and fish pathogens with ampicillin as the positive control and sterile phosphate buffer solution as the negative control. Each nutrient agar Petri dish plates were bored with four different wells and named as 1) aqueous, 2) organic, 3) acidic, and 4) control. These bored Petri dishes were inoculated (swabbed) with overnight grown fresh human pathogens such as *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Proteus mirabilis* (*P. mirabilis*), *Staphylococcus aureus* besides fish pathogens like *Vibrio alginolyticus*, *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Vibrio harveyi*. The extracts were added to the wells and incubated overnight at  $37^\circ\text{C}$  for human pathogens and at  $28^\circ\text{C}$  for fish pathogens. The plates were observed next day for the formation of inhibitory zone and the zones were measured.

### 2.11. Active antimicrobial extract purification using diethylaminoethyl cellulose (DEAE-C) column chromatography

Partial purification of the crude extract was carried out using DEAE-C anion exchange chromatography according to the procedure of Stempein *et al.*[24]. A total of 26 g of DEAE-C was taken and was swelled in 650 mL of distilled water and incubated for 3 h. The supernatant was then discarded. The sediment was mixed with half a litre of 1 mol/L NaOH. This mixture was incubated for 30 min and again the supernatant was discarded. The collected sediment was mixed with half a litre of 1 mol/L HCl and was incubated for 30 min and the supernatant was discarded. This was then washed with distilled water and again with phosphate buffer solution until the pH reached 7.4.

### 2.12. Separation of peptide mixtures in the active antimicrobial fraction using thin layer chromatography (TLC)

The crude and acidic extracts of the mucus samples exhibiting intense antimicrobial activity were run on a TLC plate with trypsin as standard. The sample was loaded onto the TLC plate (stationary phase) with 1.5 cm spacing between the sample points, in order to avoid the collision of bands on run. The sample loaded onto the stationary phase was then placed in the scintillation chamber/clean glass beaker containing the solvent system (mobile phase) comprising butanol, acetic acid and water in the ratio of 4:1:5. The solvent system was then allowed to develop until it reached approximately 1 cm below the top of the stationary phase. Once the solvent system reached near the top of the stationary phase, the TLC plate was taken out from the chamber and then kept in hot air oven at  $50^\circ\text{C}$ , which favoured the evaporation of the solvent system. A 0.2% ninhydrin reagent was then sprayed onto the solvent evaporated TLC plate and was then again kept in a hot air oven for 5–10 min in order to develop colour, which indicated the presence of the peptides. The colour developed was then observed visually and could also be observed under UV-transilluminator[25].

### 2.13. Separation and identification of bio-active peptides using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

One dimension SDS-PAGE was done following the modified method[26]. SDS-PAGE was run on vertical slab gel system. Proteins were electrophoresed on 12% separating gel (0.7 mm thickness) overlaid with 5% stacking gel. A 10% (w/v) stock solution was prepared in de-ionized water and stored in room temperature. The samples were solubilized in reducing sample buffer and equal amount of protein was loaded into 12% SDS-PAGE and was carried out at constant current (30 mA). The gel was stained briefly in staining solution and was destained in destaining solution. The proteins profile was visualized, documented and preserved.

### 2.14. Haemolytic activity of the extracts using chick, goat, cow and human red blood cell (RBC)

The haemolytic activity of mucus extracts on chick, goat, cow and human RBC was tested following the haemolytic method of Venkateshvaran[27]. The crude (3), aqueous (4), organic (5) and acidic (6) extracts of the epidermal mucus of *E. tauvina* were subjected to haemolytic activity against chicken (A), goat (B), cow (C) and human blood (D). The absorbance was calculated at 542 nm and compared with the positive (1) and negative (2) control.

#### 2.14.1. Preparation of erythrocyte suspension

Blood was obtained from a nearby slaughter house at Parangipettai using 5% ethylene diamine tetraacetic acid solution (2.7 g in 100 mL of distilled water) as an anticoagulant and was transported to the laboratory. The anticoagulated blood was centrifuged at 5000 r/min for 5 min. The supernatant was discarded and the pellet was resuspended in phosphate buffer saline (pH 7.4). This process was repeated thrice. A 1% erythrocyte suspension was prepared by adding 99 mL phosphate buffer saline to 1 mL of packed RBC.

#### 2.14.2. Haemolytic assay

The micro haemolytic test was performed in 96 well micro titre plates. Different rows were selected for chick, goat, cow and human blood. The crude, aqueous, acidic and organic extracts were checked for their haemolytic efficacy at 10 µL/mL diluted using sterile phosphate buffer saline. Its efficacy was tested using 100 µL of 1% RBC. Appropriate controls were included in the test such as 0.1% SDS as the positive control and 0.5% dimethylsulfoxide as the negative control as per Hoffman standard. The plate was gently shaken and then allowed to stand for 2 h at room temperature and the results were

recorded. Uniform red colour suspension in the wells was considered as positive haemolysis and a button like formation in the bottom of these wells was considered as lack of haemolysis. The absorbance was then read at 542 nm using an ELISA plate reader.

## 3. Results

### 3.1. Biochemical composition

The protein, lipid and carbohydrate percentages in the crude mucus sample of *E. tauvina* were calculated to be 26.25%, 24.99% and 2.03% respectively.

### 3.2. Amino acid composition

Arginine (133.9 nmol/mL) and glutamic acid (73.5 nmol/mL) were recorded at maximum level whereas glycine and lysine (5.3 and 6.5 nmol/mL) were recorded at minimum level in the crude mucus. Higher level of glutamic acid represented antimicrobial activity and thus exhibited physiological barrier for these microbes. Other amino acids like aspartic acid, serine, histidine, threonine, alanine, tyrosine and valine constituted minimum percentages (Figure 3).

### 3.3. Analysis of functional groups in crude mucus

The FTIR spectrum of crude mucus of *E. tauvina* revealed the characteristic functional groups based on the peaks obtained (Figure 4). A stretching of C-O-C, C-O at 1000–1200 cm<sup>-1</sup> corresponded to the presence of carbohydrates. Absorption peaks between 910–665 and 690–515 cm<sup>-1</sup> corresponded to N-H of primary amine and C-X stretch of alkyl halides, respectively. IR peak observed in the range of

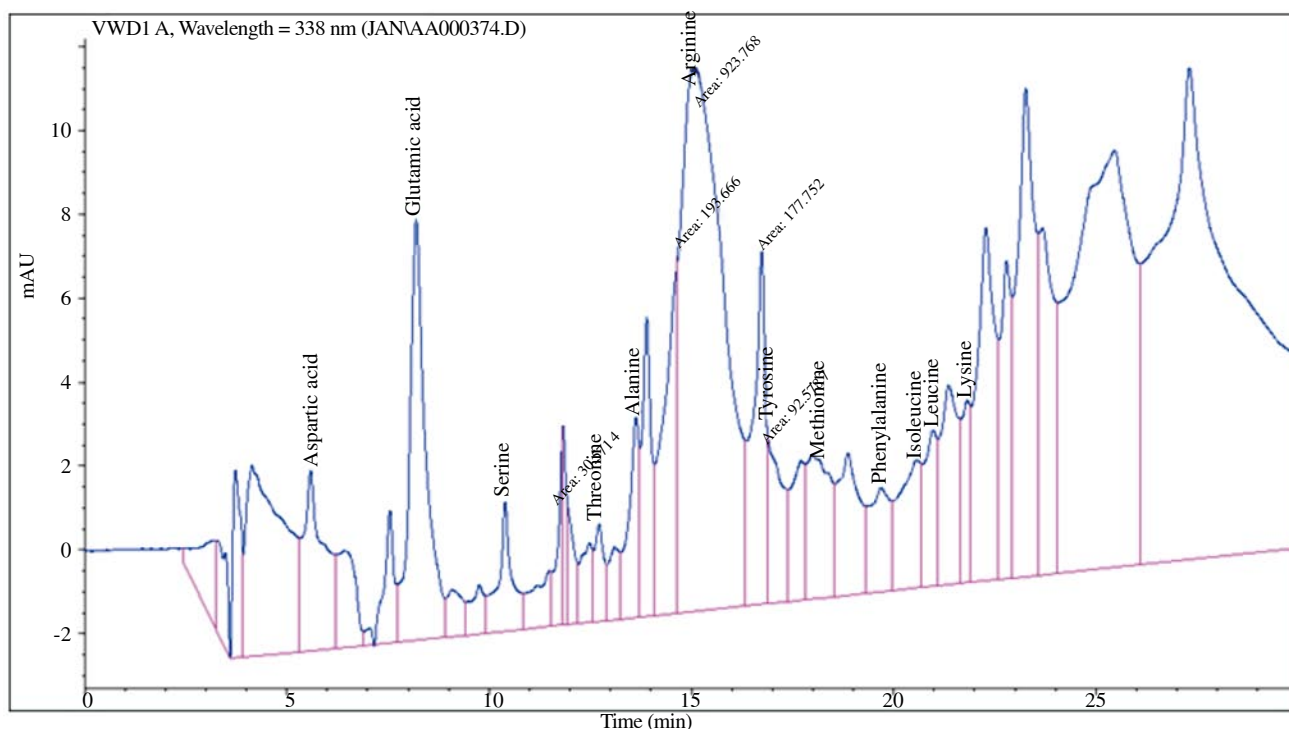
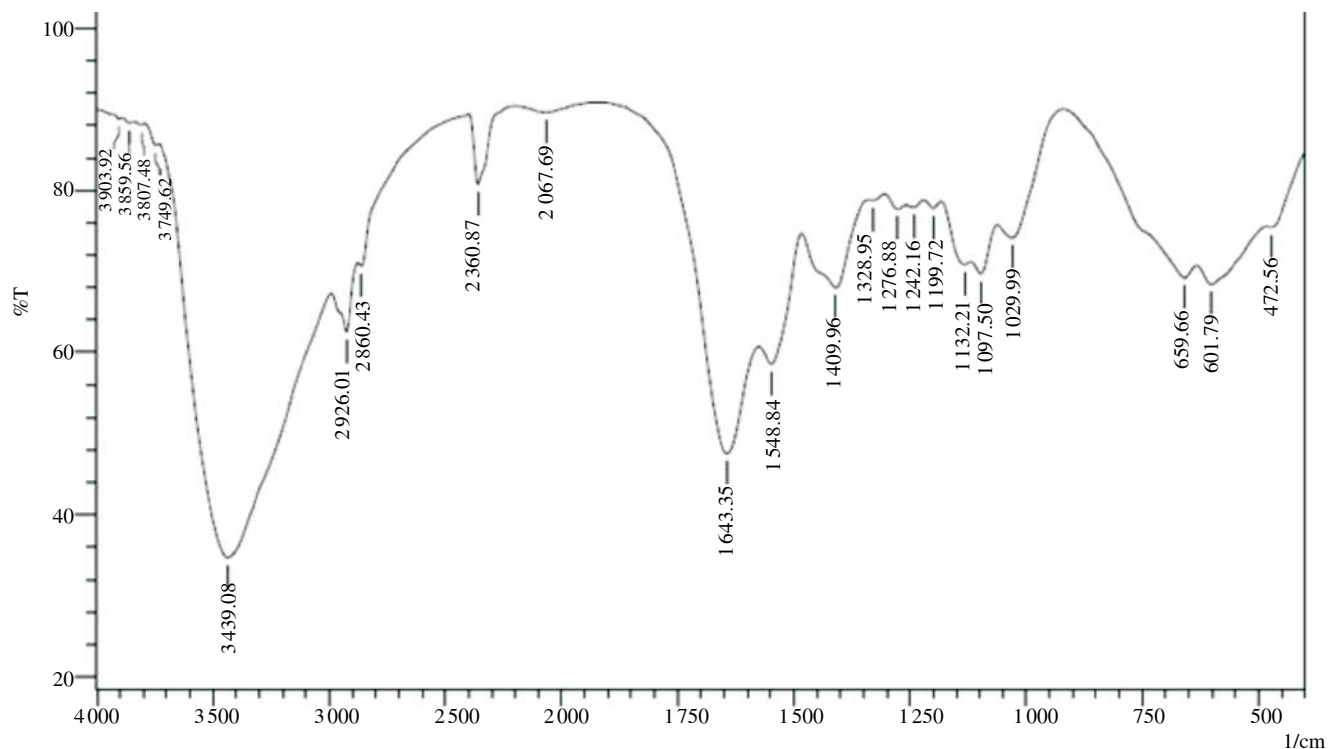


Figure 3. Chromatogram showing the area of peaks of the amino acids for crude epidermal mucus.

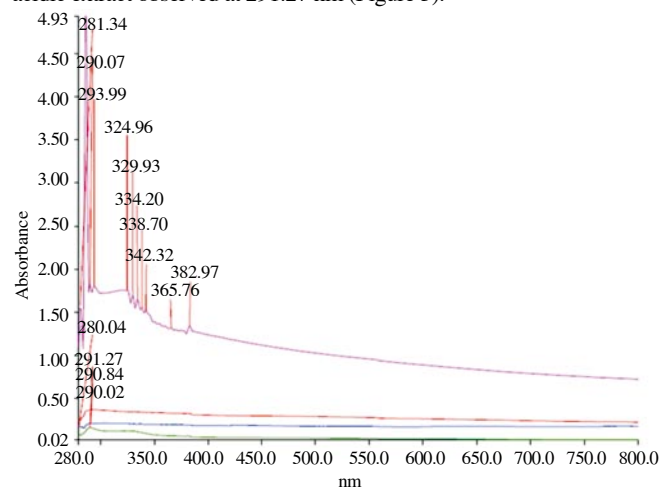


**Figure 4.** FTIR analysis of *E. tauvina* crude epidermal mucus.

2350–2360  $\text{cm}^{-1}$  may be of  $\text{CO}_2$  adsorption or asymmetric stretching of group N-C-O. The FTIR spectrum of mucus from fish confirmed the presence of primary amine group, aromatic compounds, halide group, aliphatic alkyl group and polysaccharides (carbohydrates). Consequently, IR spectra may be attributed to the presence of alkyl amine or cyclic amine with polysaccharides in the epidermal mucus of *E. tauvina*.

### 3.4. UV spectral analysis of the extracts

The mucus extracts of *E. tauvina* were subjected to UV spectral analysis (280 to 800 nm). In all the extracts majority of the peaks were observed between 280 nm to 400 nm. The sharp peaks of crude mucus extract were observed between 280 to 382 nm, aqueous extract observed at 290.02 nm, organic extract observed at 290.24 nm and acidic extract observed at 291.27 nm (Figure 5).



**Figure 5.** UV spectral analysis of various extracts of *E. tauvina* epidermal mucus.

### 3.5. Analysis of functional groups in organic extract

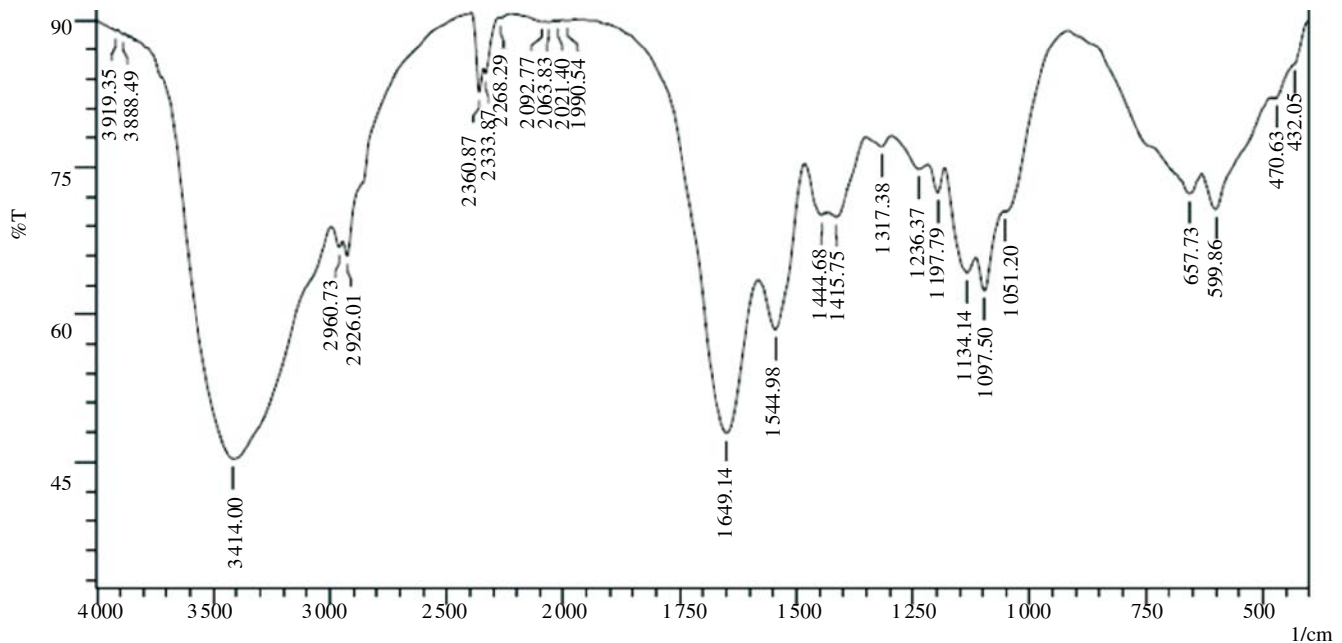
The FTIR spectrum of organic extract of mucus of *E. tauvina* showed the characteristic functional groups based on the peaks obtained (Figure 6). The absorption peak at 2100–2350  $\text{cm}^{-1}$  showed the presence of thiocyanates, nitrile, isonitriles, isocyanides and diazonium salts. The absorption peak at 1120–1170  $\text{cm}^{-1}$  revealed the presence of sulfones group in the organic extract of the epidermal mucus of *E. tauvina*.

### 3.6. Fatty acid composition

The fatty acid profile of the organic extract showed the presence of 2,4,6- decatrienoic acid (Figure 7) and bis (a-chloroethyl) sulfone (Figure 8) and the peaks were compared by making use of National Institute of Standards and Technology library.

### 3.7. Antimicrobial activity

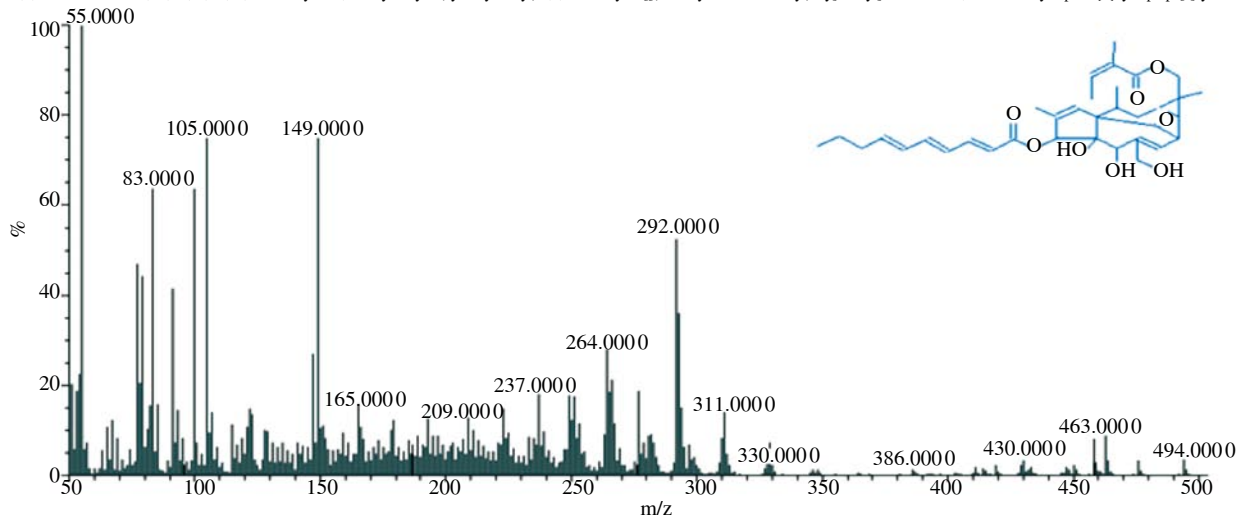
The acidic extracts of the mucus showed intense antimicrobial activity exhibiting higher zone of inhibition against both human and fish pathogens. The activity of aqueous and organic extract was not remarkable and hence only the activity of acidic extract was recorded (Table 1). In human pathogens, the activity was very high against *P. mirabilis* (Figure 9). In fish pathogen, *V. parahemolyticus* (Figure 10) exhibited high level of sensitivity against the acidic extract of mucus of *E. tauvina*. The acidic extract showed high antimicrobial activity than the antibiotic ampicillin.



**Figure 6.** FTIR analysis of *E. tauvina* epidermal mucus organic extract.

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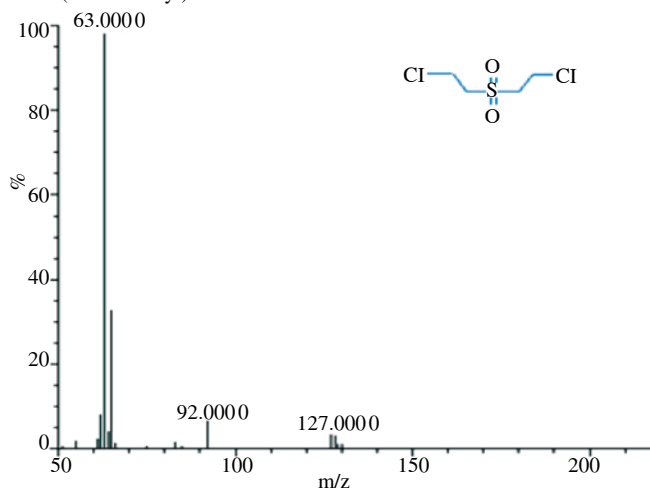
2,4,6-Decatrienoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,7,9-trimethyl-1-[(2-methyl-1-oxo-2-butenyl)oxy]methyl]-11-oxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl ester



**Figure 7.** The gas chromatogram showing the compound, 2,4,6-decatrienoic acid of *E. tauvina* epidermal mucus of organic extract which was compared with the National Institute of Standards and Technology library.

NIST MS 7 of 100(471-03-4) #ions=27

Bis (a-chloroethyl) sulfone



**Figure 8.** The gas chromatogram showing the compound, bis (a-chloroethyl) sulfone of *E. tauvina* epidermal mucus organic extract.

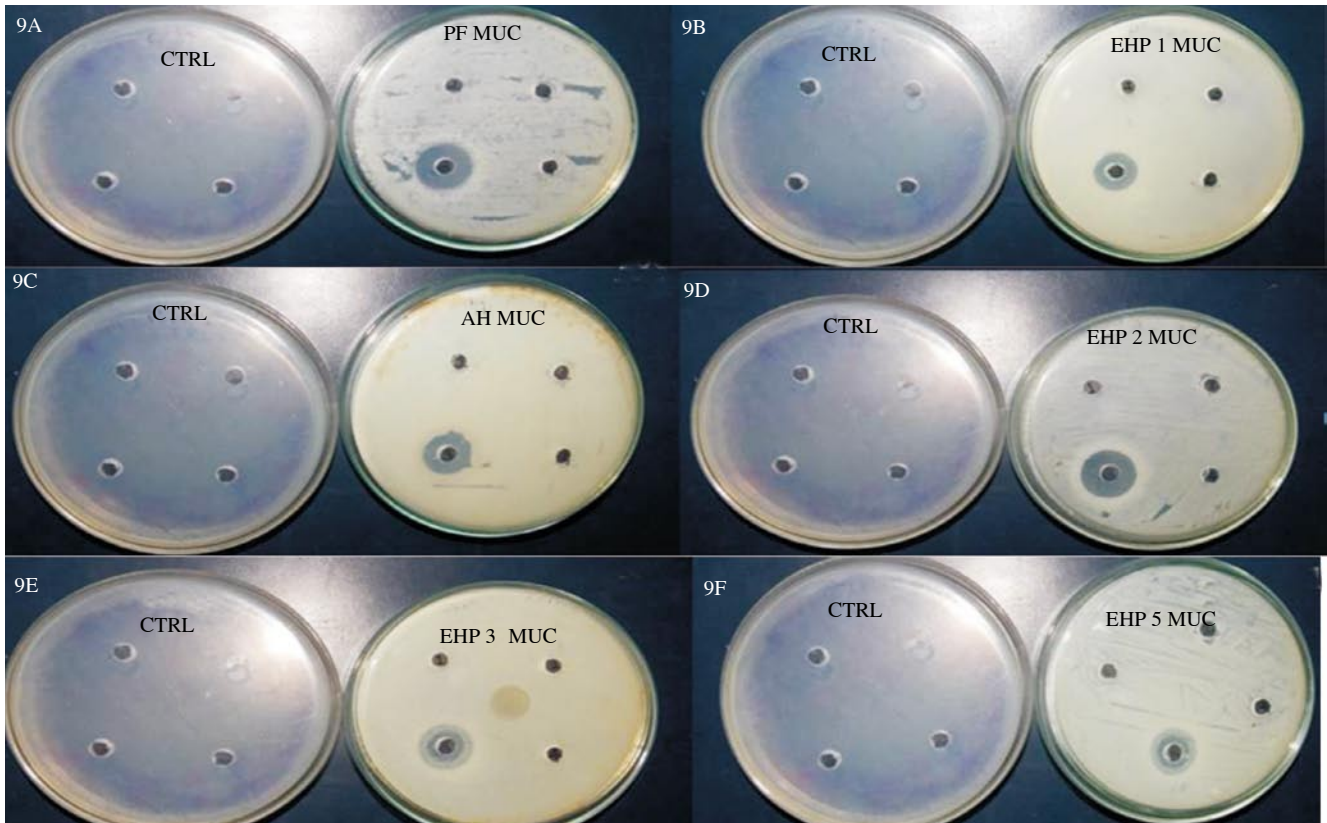
**Table 1**

Zone of inhibition exhibited by the acidic extract against various human and fish pathogens.

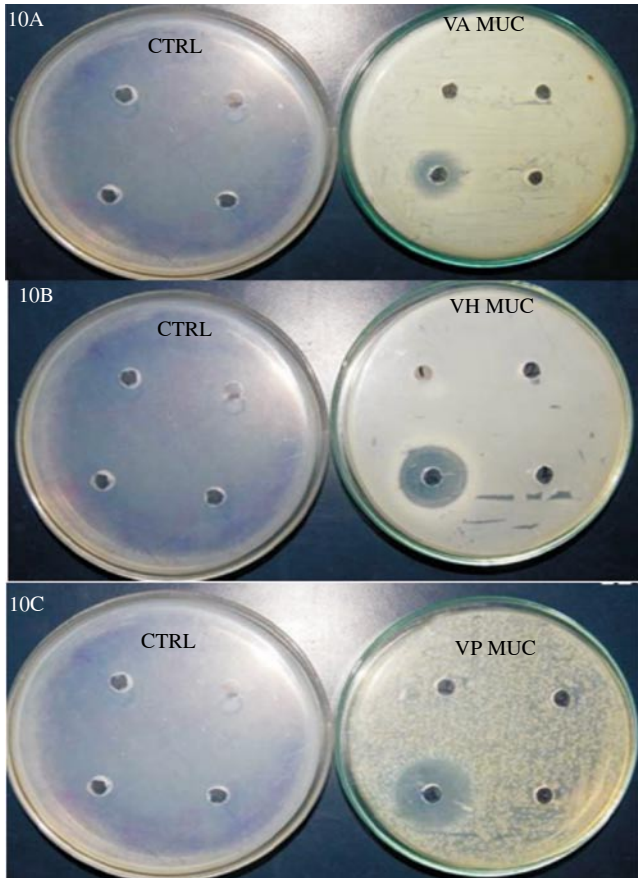
Pathogens	Code	Zone of inhibition (mm)		
		Extract	Positive control	Negative control
Human pathogens	<i>Escherichia coli</i> EHP 1	14.0 ± 0.3	9.0 ± 0.2	0.0 ± 0.0
	<i>Salmonella typhi</i> EHP 2	18.0 ± 0.2	11.0 ± 0.2	0.0 ± 0.0
	<i>Klebsiella pneumonia</i> EHP 3	17.0 ± 0.3	10.0 ± 0.3	0.0 ± 0.0
	<i>P. mirabilis</i> EHP 4	26.0 ± 0.3	14.0 ± 0.1	0.0 ± 0.0
	<i>Streptococcus aureus</i> EHP 5	16.0 ± 0.1	11.0 ± 0.3	0.0 ± 0.0
Fish pathogens	<i>Vibrio alginolyticus</i> VA 1	15.0 ± 0.1	9.0 ± 0.1	0.0 ± 0.0
	<i>Vibrio parahaemolyticus</i> VP 2	25.0 ± 0.1	13.0 ± 0.3	0.0 ± 0.0
	<i>Aeromonas hydrophila</i> AH 3	16.0 ± 0.3	11.0 ± 0.2	0.0 ± 0.0
	<i>Pseudomonas fluorescens</i> PF 4	18.0 ± 0.1	10.0 ± 0.3	0.0 ± 0.0
	<i>Vibrio harveyi</i> VH 5	21.0 ± 0.2	15.0 ± 0.2	0.0 ± 0.0

### 3.8. DEAE-C column chromatography

The antimicrobial active acidic fraction was purified using DEAE-C column chromatography in order to obtain crude fraction of the extract to reconfirm its antimicrobial activity and the activity was successfully duplicated.



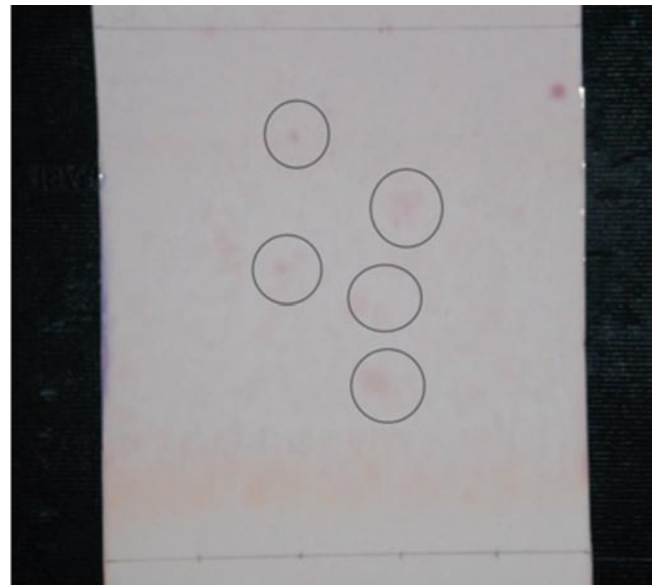
**Figure 9.** The extracts namely acidic, aqueous and organic extracts were checked for its antimicrobial efficacy against selected human pathogens. In human pathogens, the activity was very high against *P. mirabilis* (Figure 9A).



**Figure 10.** The extracts, namely, acidic, aqueous and organic extracts were checked for its antimicrobial efficacy against selected fish pathogens. In fish pathogens, *V. parahemolyticus* (Figure 10C) exhibited high level of sensitivity against the acidic extract of mucus of *E. tauvina*.

### 3.9. TLC

The peptides were visualized as pink spots in the silica gel plate (Figure 11). The  $R_f$  values were calculated to be 0.58 and 0.72 in crude extract and 0.31, 0.52 and 0.65 for acidic extract.



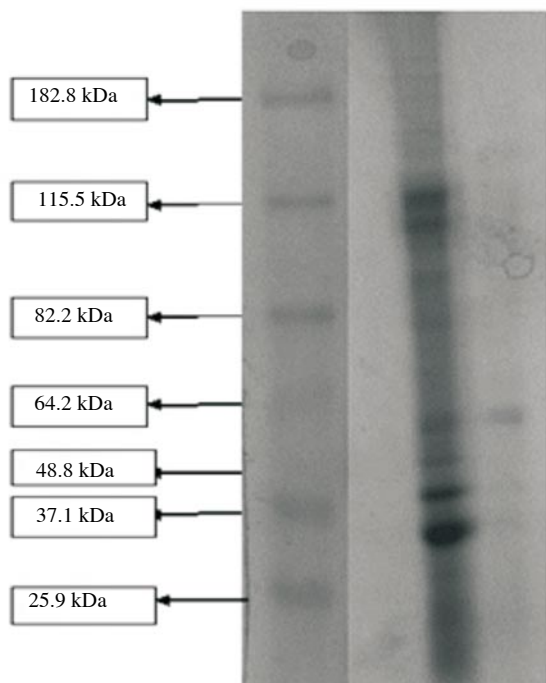
**Figure 11.** TLC plate showing the pink spots after spraying with 0.2% ninhydrin.

### 3.10. SDS-PAGE

Twelve bands ranging from 115.5 to 13.0 kDa in the crude



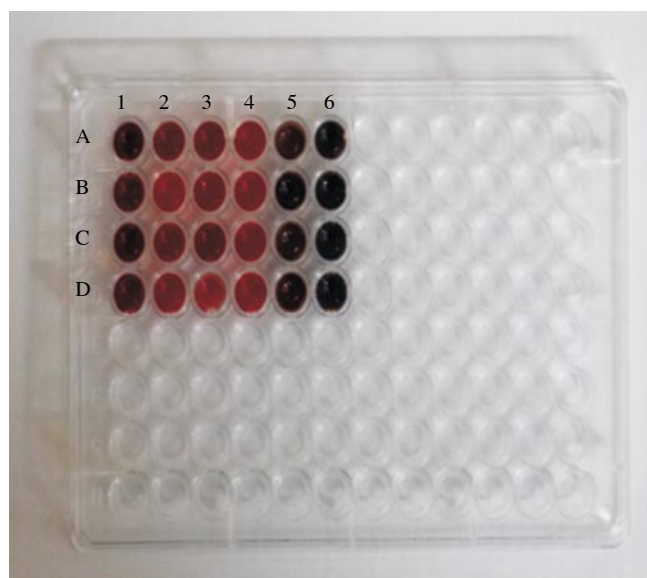
extract were observed (Figure 12). The molecular weight of seven peptides present in the acidic extract was found to be ranging from 115.5 to 37.1 kDa. Certain bands were not clearly visible.



**Figure 12.** SDS-PAGE gel showing the bands and molecular weight markers.

### 3.11. Haemolytic activity

The crude and organic extract exhibited good haemolytic activity and partial haemolysis was observed in the aqueous and acidic extracts. However, considerable variation of the haemolysis was observed depending on the type of blood used. Chicken and goat blood was found to be highly vulnerable to the lysis (Figure 13).



**Figure 13.** Hemolytic activity of the various extracts of *E. tauvina* against chick, goat, cow and human blood.

## 4. Discussion

Exploit of mucus for bio-active compounds were in research at present, the majority of antimicrobial macromolecules have been isolated from the fish skin that constitutes a first line of barrier against microbial infestation[28]. Many organisms possess antimicrobial properties, although most of the antimicrobial agents that have been isolated from marine sources are not active enough to compete with conventional antimicrobials obtained from microorganisms[29,30]. The ability of bacteria to adhere to host surfaces is necessary for colonization and then for eliciting the infection. As bacterial colonization is required for pathogenicity, genes involved in bacterial colonization have been regarded as virulence genes. Secretory mucins are the major constituents of the mucus layer in which several biochemical compounds have been identified such as antimicrobial peptides and antibodies[31].

Fish mucus is multi-functional, playing a major role in communication, resistance to diseases, respiration, ionic and osmotic regulation, feeding, nest building, reproduction and excretion[32]. In the present study, estimation of bio-molecules confirmed the presence of proteins, carbohydrates and lipids. The spectral analysis of *E. tauvina* showed sharp peak values between 200 and 400 nm in all the mucus extracts. Most of the fish mucus had more than one peak in spectral analysis reported[33]. The FTIR analysis showed distinct spectral profile confirming the presence of primary amine group, aromatic compound, halide group, aliphatic alkyl group and polysaccharides.

The acidic extract of *E. tauvina* mucus showed high level of inhibition zone against both human and fish pathogens. Similar results were observed in *Channa punctatus* and *Cirrhinus mrigala*[29]. Many workers demonstrated the antimicrobial property of epidermal mucus in fishes: hagfish-*Myxine glutinosa*[34], cat fish-*Arius maculatus*[35], eel fish-*Anguilla anguilla*[30]. Videler *et al.*[36] stated that mucus secreted by external epithelial globlet cells possess antibiotic compounds. In gold fish (*Carassius auratus*) the primary activating antimicrobial compound was a serum protein-transferring[37]. Cole *et al.*[31] and Ellis[32] reported that epidermal mucus contains variety of antimicrobial components such as antimicrobial peptides, lysosomes, proteases and lecithin. The innate immunity of mucus has broad antimicrobial effect, and has been identified in a variety of multi-cellular organisms[38].

Inhibitory effect of the mucus extract may be due to the pore forming properties against several bacterial strains and this suggested that fish secrete antibacterial proteins in epidermal mucus which acts as a defence barrier. The antibacterial activity may be due to the glycol-proteins present in the mucus that are able to kill bacteria by forming large pores in the target membrane[39-42]. Results of the present study showed the folkloric usage of fishes and suggested that

the mucus of fishes possess certain constituents with antimicrobial agents which could be used as new drugs for the therapy of infectious diseases caused by pathogens. Once running the crude and acidic extracts of the epidermal mucus in SDS-PAGE, the bands were found to be 25.9–115.5 kDa. In the silver staining method, many researchers isolated proteins from different tissue and mucus from various fishes: Atlantic hagfish[43], winter flounder[31], Atlantic halibut[44].

The mucus secretion of *E. tauvina* has proteinaceous substances which showed potent bioactivity (haemolytic) when mixed with blood cells of chicken, goat, cow and human. Mucus extracts such as acidic and crude exhibited high level of haemolytic activity. Some antimicrobial agents present in the mucus of bony fishes bind with microbes and destroy the blood cells exhibiting haemolysis[45]. Shinmar *et al.*[46] reported that lysozyme in the mucus has bacteriostatic properties and was ubiquitous in its distribution among living organisms. Disease control is an important aspect in aquaculture industries. Outbreak of diseases can seldom be recognized as a single causative factor. Establishment of hatcheries for breeding fishes particularly on a commercial basis, the prospect of frequent disease outbreak becomes a threat. Generally the bacterial, fungal and protozoan parasite diseases of fish are quite natural in aquaculture industries. Once these diseases outbreaks, the survival rate will be reduced drastically[22].

The present study emphasized that the acidic mucus extract of *E. tauvina* have potential antimicrobial activity against fish and human pathogens. The acidic soluble proteins are responsible for the defensive role and thus the epidermal mucus could be a potential source of novel antimicrobial components for human health related applications. Further studies on the characterization of the antimicrobial substances in these acidic mucus extract will be helpful in better understanding the composition and function of antimicrobial components responsible for mucosal defence mechanism of candidate species in aquaculture industry.

### Conflict of interest statement

We declare that we have no conflict of interest.

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