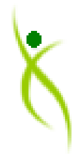

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Research Article

Zoology for Novel drug discovery

Evaluation of Anticancer, Antibacterial and Haemolytic Activities of Crude Mucus from Marine Catfish *Tachysurus Dussumieri*

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Abstract: Marine organisms are one of the diverse groups of animals that are highly specialized for their aquatic existence. The skin mucus share protective activities against environment that are rich in infectious agents. The current study aims to analyses the anticancer, antibacterial and haemolytic properties of crude epidermal mucus from marine catfish *Tachysurus dussumieri.* Cell viability and Cytotoxcity was assessed by MTT method on HT 29 (human colon carcinoma) and Vero (kidney epithelial cells of African Green Monkey) cell line. In Vero up to 1000 µg/ml of crude mucus shows cell viability of 80% and hence mucus concentration for treatment of HT 29 cells was fixed between100 to 1000 µg/ml. The proliferation inhibition on HT 29 at a concentration of 400µg/ml mucus from *T. dussumieri* at 24 h. Cellular and nuclear morphological variations was observed in different concentration of mucus, followed by DNA fragmentation to confirm the induction of apoptosis. Moreover, mucus sample was tested against Gram positive and negative bacteria, The maximum zone of inhibition was observed in *S. aureus* (15.61 ± 0.7 mm), followed by *B. subtilis* (8.32 ± 0.32 mm) and *E. coli* (8.14 ± 0.08 mm). Interestingly mucus from *T. dussumieri* showed haemolytic activity on different blood samples of goat, rat, sheep, buffalo, rabbit , cow, hen and human. Mucus showed maximum haemolytic activity against goat, rat, sheep and buffalo when compared to other RBCs. All these results showed that the epidermal mucus has more efficacy on therapeutic application in near future..

Keywords: Catfish, Mucus, Antimicrobial property, Cytotoxicity, DNA damage

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1. INTRODUCTION

Global burden of cancer in economically developing countries continues due to aging and growth of the world population alongside cancer-causing behaviors, such as smoking, consumption of alcohol etc. In normal cells, the function of tissue homeostasis, proliferation and apoptosis are balanced, but in cancer cells it is characterized by the uncontrolled growth and spread of abnormal cells [1]. In India 30 percent of the population belonging to the age group of 45 to 64 years die due to colorectal cancer, hence it has become important to analyze, the cause and to take measures for prevention [2]. The Annual incidence rates (AAR) for colon cancer in men are 4.4 per 10, 0000 and women is 3.9 per 10, 0000. It ranks 8th among men and 9th among women. In the 2013 report of Indian council of medical research 1996, the highest AAR in men for colon rectal cancer (CRCs) was recorded in Thiruvananthapuram. As per the international correlation studies, there is a positive association between dietary fat, red meat consumption and colorectal incidence. The richness of diversity offers a great prospect for the discovery of new bioactive compounds. Every year, hundreds of new compounds are being discovered. Nowadays, an increasing number of natural products isolated from marine animals is rapidly increasing [3]. From the world fisheries data, almost 20 million metric tons of fish by-products are castoff annually whereby their by-products are rich in proteins, minerals, enzymes, pigments and flavors[4]. Recently, an increasing diverse number of bioactive peptides from fish processing materials are shown to possess good antioxidant [5], anticoagulant [6], antihypertensive [7], anticancer [8] and antimicrobial properties [9]. Skin of teleost is distinctive when compared to other mammals, because it secretes mucus which is involved in immune functions. The aquatic environment is rich in pathogenic organisms; it has various adaptations like physical, chemical, and biological properties [10]. The epidermal mucus is secreted by the goblet cells which are present in the epidermis. Mucus has mucin, glycoproteins and gel forming macromolecules, along with innate immunity which is a key component in the epidermal mucus that protects it from foreign substances [11].The osmolarity and pH of the mucus change accordingly in response to microbial exposure or environmental fluctuations where the rate of mucus secretion increases [12]. Mucus secretion perform numeral functions most importantly lubrication [13], mechanical protection, osmoregulation, locomotion, immunological role and intraspecific chemical communication [14]. Marketed antimicrobial drugs are well developed for treatments, where most of the bacteria have already developed resistance against existing drugs [15]. Hence the current attention of investigators is to look for other substitute antibiotics and antibacterial drugs [16]. In twentieth century one of the greatest medical achievements is antibiotic drugs; these drugs either kill the bacteria or prevent them from replicating. Penicillin, the first antibiotic, is still used to treat some infections and other widely used antibiotics are amoxicillin, bacitracin, cephalosporins, fluoroquinolones erythromycin, and tetracycline. Identifying a novel antimicrobial compound is essential, when put together to fight the drug resistant bacterial pathogens. In this regard several studies have been made to discover new antimicrobial drugs from natural resources including animal and plant origins. A key factor of designing a drug for active molecules by a way of haemolytic activity and the interaction between molecules and biological entities at cellular level provides the leading information for pharmacological studies. Haemolytic assay is an indicator of general cytotoxicity to normal healthy cells [17]. The present study investigates antibacterial, haemolytic and anticancer properties of crude epidermal mucus from marine catfish *T. dussumieri.*

2. MATERIALS AND METHODS

2.1 *Collection of marine catfish*

The marine catfish *T. dussumieri* were collected from South East coast of India. Collected catfish were transported to the laboratory in the sea water and dissolved oxygen was maintained by the aerator. The fish were acclimatized to the laboratory conditions and were maintained with filtered seawater at 35 ppt, pH 8, 1000 mOsm/l.. It was identified and authenticated by zoologist Dr. C. Arulvasu Assistant Professor, Department of Zoology, University of Madras based on examination of the morphological characteristics and palatine teeth patterns referred from Marine Catfish Resources of India (Exploitation and Prospects) CMFRI Bulletin 40, Chapter Two and also referred Records of Indian museum, Vol no. 51, Pg no. 6-7

2.2 *Preparation of mucus from T. dussumieri*

Mucus was collected from healthy live fish, which were placed on a surgery bed. Epidermal mucus from the dorsal side of the fish was collected by a cell scraper and transferred into a tube with buffer solution containing 0.013 M Tris, 0.12 M NaCl, and 0.003 M KCl (pH-7.4) [8]. In order to avoid any blood, intestinal and sperm contamination the mucus is not collected from the ventral side. Then samples were stored at -80°C until use. Stored mucus samples were allowed to thaw at room temperature and homogenized with pestle and mortar. The insoluble particles were eliminated by centrifugation at 1000 *x g* for 10 min at 4°C and the supernatant was collected, lyophilized and stored at -60°C for further examination.

2.3 *Cancer cell lines and chemicals*

Human Colon adenocarcinoma cell line (HT 29) was obtained from National Centre for Cell Science, Pune, India. DMEM, Trypsin-EDTA, Fetal Bovine Serum (FBS), 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT), sodium bicarbonate, Dimethyl sulphoxide (DMSO) and antibiotic solution were purchased from Hi Media Laboratories, Mumbai, India. 96 well plates, 6 well plates, Tissue culture flasks (25 and 75 mm^2), (15 and 50 ml) Centrifuge tubes were bought from Tarsons Products Pvt, Kolkata, India.

2.4 *Cytotoxicity on Vero cell line*

Vero cells were maintained in DMEM medium, supplemented with 10% of FBS, at 5% CO2 and 37°C. For the experiments, cells were detached and centrifuged pellet was suspended and seeded in 96 well plates. The concentration of epidermal mucus was fixed between 200 to 1000 µg/ml after 24 h incubation. Cells received 10µl of 5 mg/mL MTT solution, after incubation time formazan crystals were formed and dissolved with organic solvent.

The absorbance was taken at 570 nm. Viable cells showed dark purple color.

2.5 MTT Assay

The Cytotoxicity of samples on Colon cancer cell lines were determined by MTT assay[18]. Human cancer cells (1 x 10^6/well) were plated with Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS in 96 well plates. The cells were incubated for 24 h under 5% CO_2, 95% O_2, and 37°C, allowing the cells to attach to the bottom of the well. The medium was removed from the cell culture plates and different concentration of crude mucus was added to each well for 24 h incubation. Control cells were maintained absence of crude mucus samples. After 24 h, 20μl of 5 mg/mL MTT dye was added to each well and the plates were further incubated for 3 h at 37°C and then 100 μl of DMSO was added and the formed crystals were dissolved gently by pipetting 2 to 3 times and measuring absorbance at 570 nm.

2.6 Morphological observation of HT 29 cells

Human cancer cells were trypsinized and plated in 6 well plate, the cells were incubated for 24 h for attachment with DMEM medium containing 10% FBS. Subsequently, various concentrations of crude mucus sample was added to the cell lines. Control cells were maintained with serum less medium, and on completion of 24 h incubation, the cells were observed under Inverted microscope [8].

2.7 Propidium iodide staining for nuclear damage

Fluorescent staining of apoptotic nuclei were performed[19]. Cancer cells (5 x 10^4 cells/ml) were seeded in 6 well plates with medium containing 10% FBS. Cells were treated with the crude mucus ~~protein~~, control cells are maintained with the serum less media. After completion of incubation the media is removed from the cells , washed with PBS and fixed with methanol: acetic acid (3:1 v/v) for 10 minutes and stained with 50 μg/ml of propidium iodide for 20 minutes. Then the stained cells were observed under confocal microscope (LSM 710, Carl Zeiss) at 20 X magnification.

2.8 DNA-fragmentation analysis

DNA extraction and agarose gel electrophoresis done with horizontal gel apparatus[20]. Cancer cells (3×10^6 cells/ml) were plated and maintained in controlled condition. The different concentration of sample was added. After 24h incubation, the cells were scraped and taken in an eppendorf for DNA extraction. of Agarose gel (0.8%) was electrophoresed for DNA containing ethidium bromide in a mini gel tank containing TAE buffer for 1 hour under 90 V. Then the gel was examined under UV transilluminator (Biorad) and photographed.

2.9 Tested strains

Pure cultures of microbes were obtained from Center for Advanced studies in Botany, University of Madras, and Chennai. Two gram negative organisms suspension (*Escherichia coli* and *Klebsiella pneumonia*) and two gram positive organisms suspension (*Staphylococcus aureus* and *Bacillus subtilis*) were taken for the antibacterial activity using well diffusion method. The optical density of each bacterial suspension was adjusted to 0.5 OD using saline (0.9%) at 570nm. (UV-VIS Shimadzu160)

2.10 Antibacterial activity

The nutrient agar medium was prepared as per required volume. Then the medium is transferred into the petri plates and allowed to solidify. Then the bacterial suspension of 0.5 McFarland (1.5×10^8 CFU/ml) was prepared and bacterial strains from this prepared suspension was used to streak for confluent growth on the surface of agar plate with sterile swab. Then four well were cut using well-cutter of diameter 6 mm. The concentration of 20 μl from stock solution of mucus 10 mg/ml was added into the first well and the second well, the positive control of gentamycin was added to the third well and the negative control of sample buffer was added into the fourth well. The Petri plates were then incubated in incubator at 37ºC for 12 h. The results were noted by measuring the zone of inhibition surrounding the well by well diffusion method [21].

2.11 Haemolytic activity

The haemolytic activity of epidermal mucus on various blood samples such as chicken, goat, cow, rat, rabbit and human RBCs [22] were determined. The sample was prepared in different concentrations. The 1% RBC suspension of 10 μL was added to each tube. The eppendorf was gently shaken and incubated for two hours at room temperature. The results were indicated as Uniform red color suspension, which was considered as positive haemolysis and a button formation in the bottom of the tube was considered as lack of haemolysis. The absorbance was measured at 540 nm.

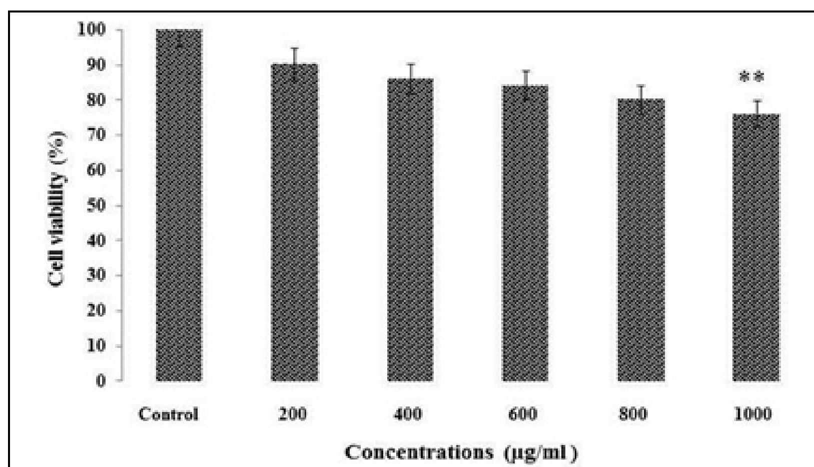
3 STATISTICAL ANALYSIS

The data generated in this study was analyzed using one-way ANOVA (SPSS) and p value is less than 0.01 is considered to be significant.

4. RESULTS

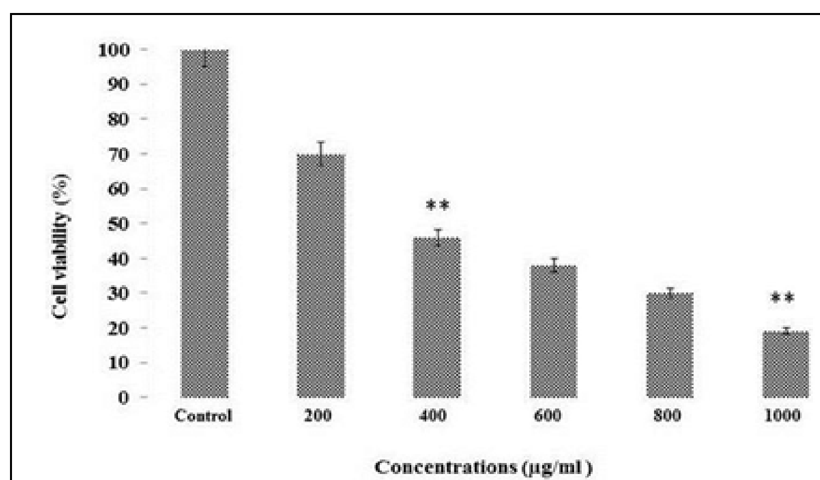
4.1 Cytotoxicity and Cell viability

Cytotoxicity and cell viability was analyzed by using Vero and HT 29 cell line followed by MTT method. Study showed the cell sustainability of Vero cells after treatment with crude mucus. It clearly demonstrated up to 80% of cells are viable at the concentration of 800 μg/ml incubation of 24 h (Graph 1). Hence the crude epidermal mucus is safe for normal cell line. The cytotoxic effect of mucus of *T. dussumieri* was tested upon colon adenocarcinoma cancer cell line HT 29. Graph indicated that decrease in the cell viability at concentration ranging from 200-1000 μg/ml of treated cells have very minimum cell death was observed. In the present study the inhibition concentration (IC_{50}) value is found to be 400 μg/ml of crude mucus at 24 h incubation (Graph 2).



The values are Mean ± S.D. of triplicates **(P<0.01)..

Graph 1. ***Cytotoxic effect on vero cells treated with crude mucus extract of catfish T. dussumieri***

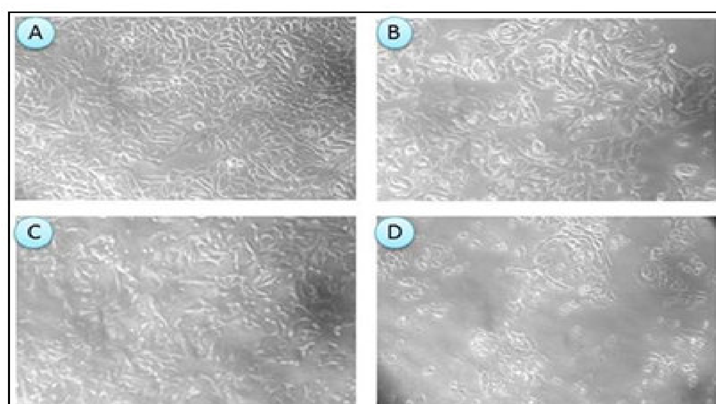


The values are Mean ± S.D. of triplicates**(P<0.01) …

Graph 2. ***Cell viability assessment of crude mucus extract from catfish T. dussumieri on the HT 29 cells***

4.2 Morphological observation

Morphological variations were observed in HT 29 cells both (control and treated) cells with crude mucus (Figure 1). Control cells showed irregular confluent aggregates, with rounded and cell morphology. The treated cells at 24 h incubation, the polygonal cells begin to shrivel and appear spherical in shape.

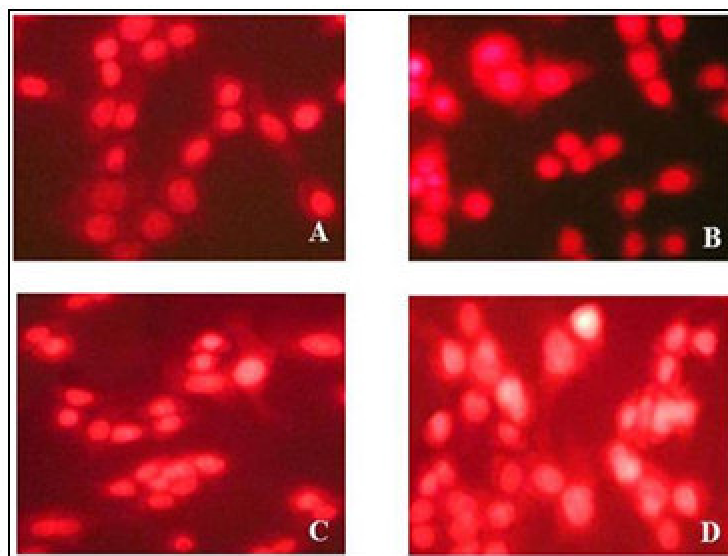


A- Control cells, B- Minimum concentration (200 µg/ml), C- IC_{50} concentration (400 µg/ml), D- Maximum concentration (1000 µg/ml)

Fig 1. ***Morphological observation of HT 29 cells treated with crude mucus extract of T. dussumieri***

4.2 PI staining

The method used to study the nuclear damage was done by propidium iodide staining. In the event of control cells, a very small number of cells were stained with propidium iodide. where cells treated with 400 µg/ml of crude mucus observed very least number of stained cells along fluorescence in the nucleus with incubation time of 24h and a progressive increase in the number of propidium iodide stained bright fluorescence occurred in nuclear condensation of cells were observed for maximum concentration (1000 µg/ml) (Figure 2).

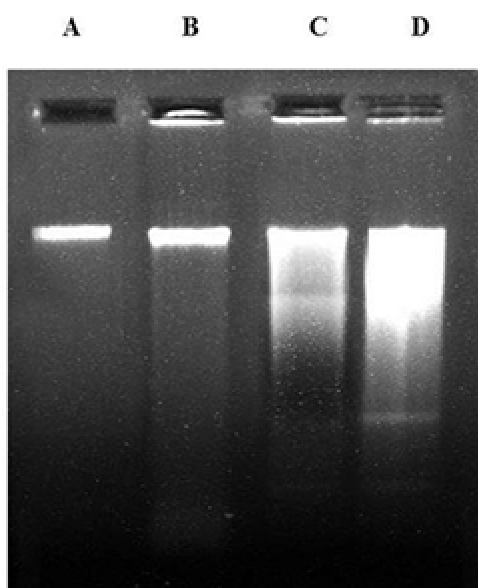


A- Control cells, B -Minimum concentration (200 µg/ml), C - IC_{50} concentration (400 µg/ml), D - Maximum concentration (1000 µg/ml)

Fig 2. ***Nuclear Morphological observation of HT 29 cells treated with crude mucus extract from T. dussumieri by propidium iodide staining***

4.3 DNA fragmentation assay

DNA fragmentation assay was done to confirm whether the cytotoxic induced by extracted crude mucus of *T. dussumieri* involves nuclear damage which leads to be an apoptotic changes and this can be detected by this DNA fragmentation assay. Treatment with the crude mucus ~~protein~~ (Figure 3) displayed DNA bands from the control group of HT29 cells remained intact where as the treated cells appeared as fragments and streaks were observed. These results indicated that crude mucus could induce apoptosis in HT29 cells and the number of fragments increased as the concentration of mucus increased. Higher concentration has more fragmented streaking compared to IC_{50} value.



A- Control cells, B -Minimum concentration (200 µg/ml), C - IC_{50} concentration (400 µg/ml), D - Maximum concentration (1000 µg/ml)

Fig 3. ***DNA Fragmentation of HT 29 cell line treated with crude mucus extract from T. dussumieri***

4.4 Antibacterial activity

The antibacterial activity was confirmed by zone of inhibition formed on the bacterial culture lawn. Zone of inhibition was observed maximum against *S. aureus* (15.61 ± 0.7 mm), followed by *B. subtilis* (8.32 ± 0.32 mm) and *E. coli* (8.14 ± 0.08 mm). The minimum zone of inhibition was observed in *K. pneumonia* (5.18 ± 0.16 mm). The inhibition zone indicating antibacterial activity of epidermal mucus of *T. dussumieri* (Table. 1) showed antibacterial activity of epidermal mucus on gram negative and gram positive bacteria respectively.

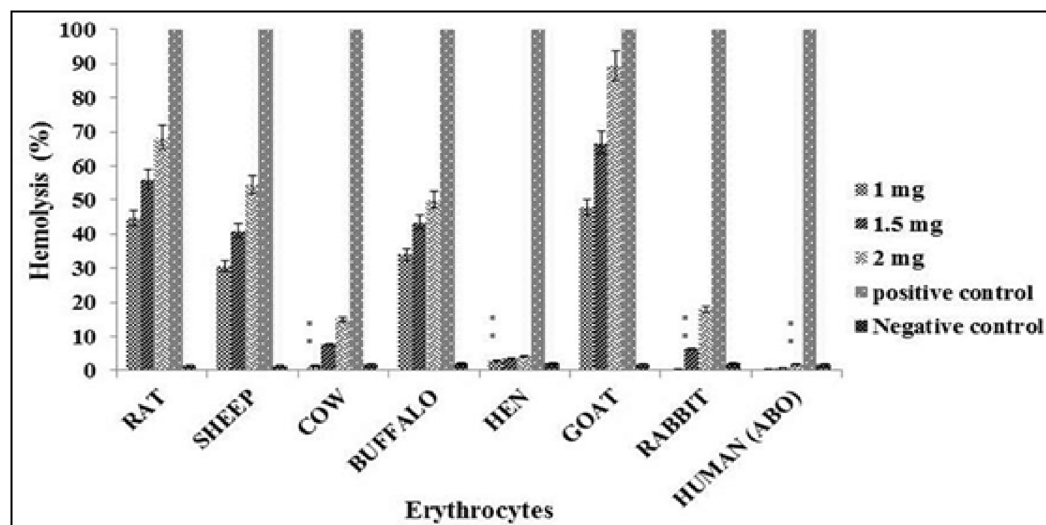
Table 1.*Zone of Inhibition (mm) indicating antibacterial activity of epidermal mucus from catfish T. dussumieri*

Bacterial pathogens	Zone of inhibition (mm)
(Gram-negative bacteria)	
E. coli	8.14 ± 0.08
K. pneumonia	5.18 ± 0.16
(Gram-positive bacteria)	
S. aureus	15.61 ± 0.7
B. subtilis	8.32 ± 0.32

This data expressed as mean ± standard deviation for the three discrete experiments (n=3)

4.5 Haemolytic activity

The epidermal mucus of catfish *T. dussumieri* was subjected to haemolytic activity against Rat, Sheep, Cow, Buffalo, Hen, Goat, Rabbit and Human blood. Maximum haemolysis observed against Goat (89.4%), Rat (68.4%), Sheep (54.6%) and buffalo (50%) blood. Minimum haemolysis was seen in Rabbit (18%), Cow (15%), Hen (4.3%) and Human blood A, B and O (1.8, 1.6 and 1.9%). The percentage of haemolysis increases on increase in the concentration of sample. The comparison of haemolytic activity on different blood samples were shown in the (Figure. 4).



***** Denotes degree of haemolysis of RBCs when compare to positive control Data are presented as mean ± SD from three different experiments. Significantly different from control **p< 0.01***

Fig 4. *Haemolytic activity of different concentration of epidermal mucus extract from T. dussumieri on various blood samples*

5. DISCUSSION

Mucus has potential defense on microbes and cancer cells. In present study the fact that crude mucus of *T. dussumieri* has good content of protein, the quantitative and qualitative analysis of mucus has been already published in our laborator. The results on antibacterial activity suggest that the epidermal mucus was capable to inhibit microbial growth against human pathogens. Most important requirements of killing pathogens is to first allow the attraction of bacterial membranes having negative charge, and the second property allows the interaction and absorption inside bacteria antimicrobial peptides and should have the net cationic charge and the ability to take amphipathic structures [23]. The skin mucus extract of two freshwater fishes *Tilapia mossambicus* and *Clarias batrachus* exhibited antibacterial activity. The zone of inhibition for the mucus extract of *Channa batrachus* maximum was observed *against A. hydrophila* (17.52 ± 1.02 mm), *E. coli* (15.48 ± 0.56 mm), *P. aeruginosa* (14.45 ± 1.52 mm), then *P. vulgaris* (12.80 ± 0.60 mm), *K. pneumonia* (8.51 ± 0.92 mm), then *S. aureus* (10.76 ± 0.94 mm). The maximum zone of inhibition for the mucus extract of *T. mossambicus* was observed against *A. hydrophila* (12.76 ± 1.68 mm), *E. coli* (10.83 ± 1.21 mm), *P. vulgaris* (10.49 ± 0.82 mm), and *Staphylococcus aureus* (8.55 ± 0.45 mm) [24]. In our studies, mucus of the catfish *T. dussumieri* having remarkable antimicrobial activity against *S. aureus* (15.61 ± 0.7 mm), *B. subtilis* (8.32 ± 0.32 mm), *E. coli* (8.14 ± 0.08 mm) and *K. pneumonia* (5.18 ± 0.16 mm). Haemolytic activity has been shown to be present in the mucus of various catfishes such as *A. thalassinus* [25], *A. bilineatus* [26], *P. lineatus* [27-28], *P. canius* [29], Eels such as *Anguilla japonica* [30],

Lycodontis nudivomer [31], Soap fishes *Pogonoperca punctata* [32]. Our results of haemolytic assay showed good activity in blood samples. The crude extracts of epidermal mucus showed maximum activity in goat, rat, sheep, and buffalo, whereas minimum haemolysis seen in rabbit, cow, hen and human. During drug treatment the toxicity connected with cancer specificity is lacking between the tumor and cancer cells in chemotherapy. Hence, recently many studies about natural compounds obtained from marine organisms obstruct more selectively the cancer cells than the normal cells. The different concentration of crude mucus on Vero cells showed maximum cell viability upto 800µg/ml. Hence the concentration was fixed between 100 to 1000 µg/ml concentrations for HT 29 cells. Here, they determined the cell viability concentration in which the catfish crude mucus significantly inhibited growth of colon adenocarcinoma cells [33]. The present study shows that catfish mucus is active against colon cancer cells. The MTT assay indicated that cytotoxin strongly inhibits HT 29 cell proliferation with an IC_{50} . The role of apoptosis study was observed with propidium iodide staining of treated and untreated cells it make to permeable in dead cells and exhibit the dead cell count of the HT 29. There was an optimum dose and time for induction of apoptosis. In our study, fluorescence red color shows the stain bind to nuclei acid of dead cells HT 29. Apoptotic cell activates the proteolytic enzymes indicating the morphological changes in cells which eventually mediate the DNA cleavage. This nuclear damage was observed by fluorescent microscopy with propidium iodide staining method. An association between apoptosis and DNA fragmentation was confirmed with several studies [34]. A significant manner of cell death after cytotoxic treatment of drug has shown Apoptosis [35]. In programmed cell death, cells undergo membrane blebbing, of cytoplasmic condensation through this activation of specific proteases alter the genome which turned into smaller apoptotic bodies [36]. In this process of apoptosis, the unwanted or dysfunctional cells had been expelled [37].

6. CONCLUSION

The present investigation revealed that the epidermal mucus possesses anticancer (Human colon adenocarcinoma cells) and antibacterial properties (gram positive and negative bacteria). The haemolytic activity results showed that the epidermal mucus does not explore significant haemolysis on all human RBCs. Thus crude mucus can be considered for further investigation on effective and safe discovery of novel drug through purification and structural identification.

7. ACKNOWLEDGEMENT

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8. AUTHOR CONTRIBUTION STATEMENT

The framework for this study and subsequent data collection and processing was done by Ms.R. Kavitha. Mr. Tharun Kumar aided in the collection of animal and performed the antibacterial study. The statistical analysis was done by Ms. Gracy Jenifer, Mr.Deepakrajasekar and Mr. Kathirvel Neelan. Valuable input for the compilation of this work was provided by Dr.C.Arulvasu.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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