SCREENING OF ANTIBACTERIAL AND CYTOTOXIC ACTIVITY OF EXTRACTS FROM EPIDERMIS AND EPIDERMAL MUCUS OF BARBONYMUS SCHWANENFELDII (TINFOIL BARB FISH)

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Abstract

Aim: *The main objective of this work is to increase the utilization of by-products like mucus and skin from Tinfoil barb fish in order to isolate antibacterial protein against gram positive and gram negative microorganism.*

Materials and Methods: *The fish epidermis and epidermal mucus samples were extracted with crude, ethanol and dichloromethane to identify potential antimicrobial agents including the ethanol and dichloromethane soluble compounds. Preliminary screening for antimicrobial activity of the extracts were tested against two Gram-positive bacteria Bacillus cereus (MTCC 1306)and Staphylococcus aureus(MTCC 3160) and two Gram-negative bacteria Shigellaboydii and E.coli(MTCC 1303) using standardized disc susceptibility test method recommended by the British Society of Antimicrobial Chemotherapy(BSAC). The activity was measured in terms of zone of inhibition in mm. Also Mouse fibroblast 3T3 L1 cell lines were used to measure cytotoxic activity. Minimum cell viability was shown by sample of 10 mg/ml concentration of the epidermal mucus.*

Result: *The ethanol extract exhibited a bactericidal activity and inhibited the growth of Bacillus cereus, Staphylococcus aureus, Shigellaboydii and E.coli. The dichloromethane exhibited activity against Bacillus cereus, Staphylococcus aureus, E.coli and no activity against Shigellaboydii. The aqueous showed no bactericidal activity for any of the human pathogen tested. These results show the presence of antimicrobial agent and the role of fish byproducts like mucus and epidermis in antimicrobial protection.*
Conclusion: *the present result suggested that the mucus and epidermal extract of Barbonymus schwanenfeldii (Tinfoil barb fish) may be a potent source of antimicrobial agent for human pathogen.*

Key words: *Barbonymus schwanenfeldii, Tinfoil barb fish, antibacterial activity, epidermal mucus, epidermis.*

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1. INTRODUCTION

The recent development in the field of chemotherapeutic technique plays a vital importance. The need of new therapeutic agents are increasingly important for the public health issues in the world[1].The compounds isolated from the natural source are highly used since the ancient times for the treatment of various disease and for the improvement of live[2]. Antibacterial proteins play an important role in the innate immunity of animals which act as the first line of the host defense mechanism. The use of antibiotics repeatedly has given multiresistance to the bacterial strains throughout the world. The increase in the resistance to antibiotics is a major problem for the human society at present. This leads to the search of new therapeutic compounds from natural sources by which the organism will not obtain resistance[3]. Among the total 252 traditional medicines selected by WHO, 8.7% are from animals[4]. Fishes contribute a major resource for variety of bioactive compounds. Apart form the resources for variety of bioactive compounds. Apart from the muscles that is consumed by human, the external and the waste parts like mucus, epidermis and gills may also act as the good source of antimicrobial components since these layers act as a lubricant which act as a mechanical protective barrier[5-6]. Many studies have been reported on the antimicrobial activity of mucus, epithelial tissues and other mucus secretion against marine microbial strain[7]. It is also proved that mucus plays a role in the prevention of colonization of parasites, bacteria and fungi[8-10]. Application of such natural defense system is one of the developing methods for producing alternative antimicrobial drugs[11]. The epidermal mucus is composed of gel forming macromolecules and glycoproteins; the mucus is secreted by epidermal goblet or mucus cells[12-13]. The mucus layer on the fish surface performs a number of inevitable functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building[14-16].It has been known that fish mucus contains a variety of biologically active compounds such as lysozyme, lectins, proteolytic enzymes, Flavoenzymes, immunoglobulin, C-reactive protein, Apo lipoprotein A-I and

antimicrobial peptides that are constitutively expressed to provide immediate protection to fish from potential pathogenic microbes and parasites17. Antibacterial activity in fish mucus has been demonstrated in several fish species20; yet this activity seems to vary from species to species such as rockfish (Sebastes schlegelii)18, rainbow trout (Oncorhynchus mykiss)19 , tilapia (Tilapia hornorum)20 and Bloch (Channa striatus)21 and can be specific towards certain bacteria.

Cytotoxicity is the quality of being toxic to cells. Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, if they are interested in developing a therapeutic that targets rapidly dividing cancer cells, for instance; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical22-23. Cytotoxicity can also be monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product24. The MTT Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. 3T3 cells come from a cell line established in 1962 by two scientists then at the Department of Pathology in the University School of Medicine, George Todaro and Howard Green. The 3T3 cell line has become the standard fibroblast cell line. The '3T3' designation refers to the abbreviation of "3-day transfer, inoculum 3 x 105 cells." 3T3 cells are often used in the cultivation of keratinocytes, with the 3T3 cells secreting growth factors favourable to these kinds of cells.

To date no studies have been made on the antibacterial and cytotoxic property of Barbonymus schwanenfeldii (Tinfoil barb fish). Hence an attempt was made to find out the antibacterial activity and cytotoxicity in the epidermis and the mucus of Barbonymus schwanenfeldii (Tinfoil barb fish) against selected gram positive and gram negative bacteria.

2. MATERIAL AND METHODS:

Collection of Sample: The fish of approximately 3 months old was collected from the nearby culture pond with the body weight of 150g ± 5g.This was maintained in lab condition for 2 days, the mucus and epidermis collected from them.

Collection of mucus: The mucus sample was collected from the dorsal side of the body using a spatula the ventral side is usually avoided to prevent the intestinal and sperm contamination. The mucus is immediately frozen to prevent bacterial contamination then lyophilized and stored at -400C.

Collection of epidermal layer and muscle: The fish epidermis was dissected using the scalpel and washed with 5% ethanol to remove the associated micro flora. The epidermis was frozen, lyophilized and stored at -400C.

Extraction procedure and protein estimation: Both the epidermis and mucus sample were used for the preparation of the aqueous, ethanol and dichloromethane extracts. Both the epidermis and mucus sample were suspended in distilled water using magnetic stirrer for 3hrs. It was then centrifuged at 30,000xg for 30mins at 40C followed by filtration with whatmann filter paper. The supernatant was lyophilized to the give the aqueous extract. When this was tested for antibacterial activity it showed negative against microorganism so the results are no shown. One part of the aqueous extract was used for ethanol and dichloromethane extraction.

For organic extracts, the biological material was suspended with stirring in 95% ethanol. It was then centrifuged at 30,000 xg for 30 mins at 4ºC.The resultant pellet was re-extracted five times in the same way. The alcoholic extracts were combined and evaporated under vacuum at temperature below to 40 ºC. Distilled water (50 ml) was then added and partitioned with dichloromethane (CH2Cl2) (200 ml).The aqueous phases were collected, lyophilized, re-suspended in absolute ethanol (100 ml). It was then filtered and concentrated under vacuum at temperature below to 40ºC. This was collected as ethanol extract. The organic phases were collected, dried for 24 h under Na2SO4, filtered and concentrated under vacuum at temperature below 40ºC. This was collected as dichloromethane extract. These three phases were stored at -40ºC. Protein quantification was determined based on Bradford protein assay 24 by using bovine serum albumin as standard.

Bacterial Culture Condition: Antimicrobial activity of the mucus and epidermal extracts were tested against two Gram-positive bacteria Bacillus cereus (MTCC 1306) and Staphylococcus aureus(MTCC 3160) and two Gram-negative bacteria Shigellaboydii and E.coli(MTCC 1303) all the microorganism were grown and maintained in nutrient broth at 37oC.

Screening of antimicrobial activity: The screening for antimicrobial activity of the aqueous, ethanol and dichloromethane extract from Barbonymus schwanenfeldii (Tinfoil barb fish) was carried out against Gram-positive bacteria Bacillus cereus (MTCC 1306) and Staphylococcus aureus (MTCC 3160) and two Gram-negative bacteria Shigellaboydii and E.coli (MTCC 1303) by following the protocol of British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility testing Method25. The bacterial suspension was diluted up to 10^3 CFU units before plating. 20 µl of mucus and epidermal extracts were impregnated onto a disc. The disc with extracts were then

transferred into the Mueller Hinton agar plate with bacterial culture.5% DMSO which was used for the dilution of the extracts , the ethanol and dichloromethane used for the extraction was also loaded on the disc and plated for comparison. The plates were left at ambient temperature for 15 minutes to allow excess pre diffusion of extracts prior to incubation at 37 °C for 24 h. Diameters of inhibition zones were measured. This method was employed for both the epidermis and mucus sample extracts.

3. DETERMINATION OF CYTOTOXICITY

Cell Lines: Mouse fibroblasts (3T3 cell line) were used to determine cytotoxicity. The '3T3' designation refers to the abbreviation of "3-day transfer, inoculums 3 x 105 cells."

Sub culturing and maintenance of cell line: Bring the medium and TPVG to room temperature for thawing. Observe the tissue culture bottles for growth, cell degeneration, pH and turbidity by seeing in inverted microscope. If the cells become 80% confluent it goes for sub culturing process. The mouth of the bottle was wiped with cotton soaked in spirit to remove the adhering particles. The growth medium was discarded in a discarding jar.4-5 ml of MEM without FCS was added and gently rinsed with tilting. The dead cells and excess FCS were washed out and then the medium was discarded. TPVG was added over the cells. It was incubated at 37ºC for 5 mins for disaggregation. The cells become individual and it's present as suspension.5 ml of 10% MEM with FCS was added by using a serological pipette. It was passage gently using a pipette. After passaging the cells were split into 1:2 ratios for cytotoxicity studies for plating method.

Seeding of cells: After homogenization 1 ml of the suspension was taken and poured into 24 well plates. In each well 1 ml of the suspension was added and kept in desiccators in 5% CO2 atmosphere. After 2 days of incubation the cells were observed in inverted microscope. Assay is carried out when the cells become 80% confluent.

Cytotoxicity assay: In order to study the antitumor activity of a new drug, it is important to determine the cytotoxicity concentration of the drug. Cytotoxicity tests define the upper limit of the extract concentration which is non toxic to the cell line. The concentration non toxic to the cells is chosen for antiviral assay.

After the addition of the drug, cell death and cell viability was estimated. The result was confirmed by additional metabolic intervention experiment such as MTT assay.

Stock concentration: The working concentration was prepared fresh and filtered through 0.45 μfilter before each assay.10 mg/ml of the sample was taken in an eppendroff tube.500 µl of MEM without FCS was taken in eppendroff tubes. Then 500 µl of the working standard was added to the first eppendroff tube and mixed well then 500 µl of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the drug. As a result, the volume remains constant but there is a change in concentration.

Sampling: The 48 hr monolayer culture of mouse fibroblast cells (80%CONFLUENT) was seeded in 24 well titer plates. The plates were microscopically examined for confluent monolayer, turbidity and toxicity if the cells become confluent. The growth medium (MEM) was removed using a micropipette. The monolayer was washed twice with MEM without FCS to remove the dead cells and excess F-Stop the washed cell sheet, add 1 ml of medium (without FCS) containing defined concentration of the drug in respective wells. Each dilution of the drug ranges from 1:1 to 1:16 and they were added to the respective wells. To the cell control wells 1ml of MEM without FCS was added. The plates were incubated at 37ºC in 5% CO2 environment and observed for cytotoxicity using a compound microscope.

4. MTT ASSAY

1 ml of medium without FCS containing each dilution of sample ranging from 1:1 to 1:64 were added to the respective wells. To the cell control well1 ml MEM without FCS was added. The plates were incubated at 37ºC for 24 hrs. After incubation the medium was removed from the wells and 200µl MTT dye was added to it and incubated for 4 hrs. After incubation 1ml of DMSO was added in each well and left for a minute. This suspension was transferred to the curette and O.D. values were read at 595nm by taking DMSO as a blank. Cell viability % = Mean O.D. / Control O.D. * 100

5. RESULTS

In these study three different extraction methods was used to screen epidermal mucus and epidermis for antimicrobial activities. Aqueous, ethanol and dichloromethane extracts were prepared from the epidermal mucus and epidermis of Barbonymus schwanenfeldii (Tinfoil barb fish). An aqueous extraction protocol was used to prepare an extract containing all the aqueous soluble components in the mucus, such as proteases, lysozyme and glycoproteins26. The results of the antibacterial activity of mucus extracts of Barbonymus schwanenfeldii (Tinfoil barb fish) are presented in Table I. These extracts were then screened against Gram-positive bacteria Bacillus cereus (MTCC 1306) and Staphylococcus aureus (MTCC 3160) and two Gram-negative bacteria Shigellaboydii and E.coli (MTCC 1303) by using disc diffusion method. Antimicrobial activity was confirmed by the zone of inhibition. Among the three extracts, aqueous extracts showed no activity against human pathogens. The ethanol extract exhibited a bactericidal activity and inhibited the growth of Bacillus cereus, Staphylococcus aureus, Shigellaboydii and E.coli. The dichloromethane exhibited activity against Bacillus cereus, Staphylococcus aureus, E.coli and no activity against Shigellaboydii... The controls

incubated with solvents and bacterial culture showed negative results, demonstrating that the solvents themselves did not account for the antimicrobial activity observed in fish mucus and epidermal extracts. The maximum inhibitory zone was seen in ethanol extract of mucus against E.Coli, Bacillus cereus and in dichloromethane extract of mucus against Staphylococcus aureus and Bacillus cereus respectively. The zone of inhibition was maximum against E.Coli in both ethanol and dichloromethane extracts.

The protein content of the 3 extracts are tabulated in table II. The highest concentration was obtained in aqueous extraction (0.439mg/ml) of mucus followed by ethanol extract (0.321mg/ml) and dichloromethane extract (0.294mg/ml).The concentration of the protein in epidermis was aqueous extract (0.311mg/ml), ethanol extraction (0.265mg/ml) and dichloromethane extraction (0.255mg/ml). The fish mucus ethanol extract was found to be toxic against Mouse fibroblast 3T3 cell lines. The readings are tabulated in table III and graphically represented in graph I.

Table I. Screening of antimicrobial activity of the mucus extracts and epidermis of Barbonymus schwanenfeldii (Tinfoil barb fish).

Organism	**Aqueous extract**		**Ethanol extract**		**Dichloromethane extract**	
	Mucus	**Epidermis**	**Mucus**	**Epidermis**	**Mucus**	**Epidermis**
E.coli	-	-	9mm	12 mm	8 mm	12 mm
Staphylococcus aureus	-	-	8 mm	10 mm	9 mm	11 mm
Shigellaboydii	-	-	7 mm	9 mm	8 mm	-
Bacillus cereus	-	-	9 mm	7 mm	9 mm	10 mm

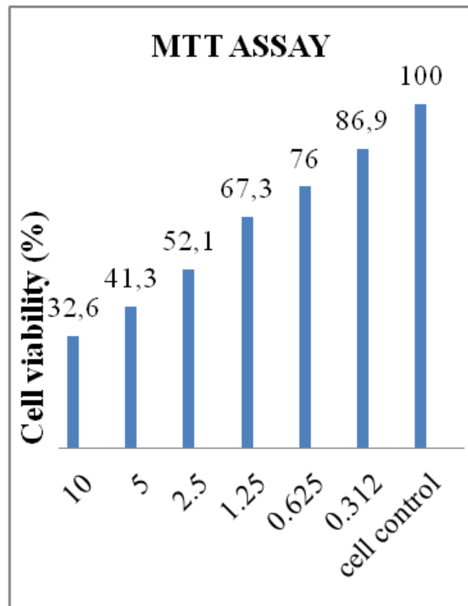
– indicates no antimicrobial activity, values indicates the zone of inhibition. The values are the mean of triplicates.

Table II. Protein content of different mucus extracts of Barbonymus schwanenfeldii (Tinfoil barb fish).

Sample extract	Epidermis	mucus
Aqueous	0.311mg/ml	0.439mg/ml
Ethanol	0.265mg/ml	0.321mg/ml
Dichloromethane	0.255mg/ml	0.294mg/ml

Table III
MTT ASSAY RESULT
MUCUS SAMPLE- ETHANOLIC EXTRACT

S.No	Concentration (mg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	10	Neat	0.15	32.6
2	5	1:1	0.19	41.3
3	2.5	1:2	0.24	52.1
4	1.25	1:4	0.31	67.3
5	0.625	1:8	0.35	76.0
6	0.312	1:16	0.40	86.9
7	Cell control		0.46	100



GRAPH 1 Cell viability Vs Concentration

6. DISCUSSION

The artificial source of medicine, its side effects and resistance have impacted a high persistency in the recent biological environment; to overcome this many studies are being carried out to use the natural sources and safer source on the other hand. Animals have been a medicinal source for various diseases of humans24. Multi-cellular organisms are very good source of innate antimicrobial componds26. The antimicrobial peptides have activity against gram positive bacteria, gram negative bacteria, fungi, protozoa and viruses27. Fishes are habitually surrounded by water, which contains a wide variety of pathogenic and non-pathogenic microorganisms. The role of mucus and its components in various fish species suggest that the epidermal mucus acts as a first line of defense against the pathogens. In our report, the mucus and epidermis extracted with ethanol and dichloromethane show potent antimicrobial activity28-29. Several studies have been carried out to explore the properties of fish mucus, whereas no information is available for the antimicrobial properties of Barbonymus schwanenfeldii (Tinfoil barb fish). In the present study, epidermis and mucus of Barbonymus schwanenfeldii was collected and extracted in three different ways to obtain different components. Protein quantification results also that available of high amount of proteins may be a potential antimicrobial source. The physiological conditions like salinity, pH, stress and stage of growth and maturity alter the biological substance of mucus30-31. The variations in amount of mucus secretion between fish species had been observed to play a role in the susceptibility of the fish to infection. Studies have shown that fish mucus contains variety of enzymes including lysozymes, proteases, alka- line phosphatase and cathepsin B that play a significant role in innate immune system of fish32. Antimicrobial screening results showed that no detectable levels of antimicrobial activity was observed in aqueous mucus extract against human pathogens tested. Earlier studies also have reported that, no microbial growth inhibition observed in aqueous fish mucus extracts of a wider range of fish species including Arctic char (Salvelinus alpinus), brook trout (Salvelinus fontinalis), koi carp (Cyprinus carpio), striped bass (Morone saxatilis), haddock (Melanogrammus aeglefinus) and hagfish (Myxine glutinosa)33-34.

A wide array of bio-indicators is available and applicable for the detection of a chemical-induced cytotoxicity resulting in necrosis. In this study, we use MTT bio-transformation to detect mitochondrial functionality. No cytotoxic on the 3T3-cell line was observed using epidermis extract. But cytotoxic activity on 3T3 cell lines was observed by using Mucus ethanol extract.

In the present study the mucus and epidermal extracts isolated from Barbonymus schwanenfeldii shows an inhibiting effect on the microorganisms. The glycoprotein present in the mucus may be one of the important components for the antibacterial activity. The glycoprotein have the capacity to form holes on the membrane of bacteria thereby leading to its death35. Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus acts as a chemical defense barrier.

In conclusion, this study investigated the antimicrobial activities for Barbonymus schwanenfeldii (Tinfoil barb fish) mucus and epidermal extract (aqueous, ethanol and dichloromethane) against 2 gram positive and 2 gram positive bacteria. From the results, the ethanol extract exhibited a bactericidal activity and inhibited the growth of Bacillus cereus, Staphylococcus aureus, Shigellaboydii and E.coli. The dichloromethane exhibited activity against Bacillus cereus, Staphylococcus aureus, E.coli and no activity against Shigellaboydii. In the present study it is proved that Barbonymus schwanenfeldii (Tinfoil barb fish) can be a good source of an antibacterial activity against human pathogens. In future, further investigations can be focused on other human bacterial pathogens as well as fungal pathogens.

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