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Anticancer Potential of Intestinal Mucus from Marine Catfish Tachysurus dussumieri on MDA-MB-231-Bio-Processing of Intestinal Mucus from Marine Catfish Tachysurus dussumieri on Human Breast Cancer Cell line

Kavitha Raja, Lekshmi Bose, Livya Catherene Martin, Gracy Jenifer Sahayanathan, Deepakrajasekar Padmanaban and Arulvasu Chinnasamy*

Department of Zoology, University of Madras, Guindy Campus, Chennai, India

Research Article

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***For Correspondence**

Arulvasu Chinnasamy, Department of Zoology, University of Madras, Guindy Campus, Chennai, India

E-mail: carulvasu@gmail.com

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ABSTRACT

Fish mucus is rich in several bioactive substances and an exciting source of natural compounds with multidimensional properties. The present study investigates the anticancer effect of intestinal mucus of marine catfish Tachysurus dussumieri against the human breast cancer cell line (MDA-MB-231). The biochemical analysis of crude intestinal mucus revealed that Protein (1.35 mg/ml), Lipid (1.086 mg/ml), and Carbohydrate (2.095 mg/ml). The protein profiling via Native and SDS-PAGE have bands of both low and high molecular weight. The cell viability of MDA-MB-231 cells on treatment with crude intestinal mucus expressed an IC50 value at 800 µg/ml. Nuclear damage was observed by propidium iodide staining and the intensity of the membrane potential of mitochondria was investigated through Rh-123 staining. The incidence of DNA damage was further established by Agarose gel electrophoresis and the cell cycle arrest at the S phase was confirmed by way of Flow cytometry.

INTRODUCTION

Cancer is a paramount problem in developing as well as developed nations. It is distinguished by the unusual growth and differentiation of cells in the body[1]. Breast cancer is the most frequently diagnosed cancer in women (24.2%) and the second most prevalent cancer on the whole[2] .It is a multi-stage disease and thus early detection makes it possible to increase the chance of a patient's survival as well as enhancing their response to treatments[3]. Chemotherapy is one of the most extensively followed procedures for treating cancer in various stages. But it has certain downsides such as resistance to the drug, probability of recurrence, and supplementary side effects that affect the standard of patient's life[4]. There is a need for novel remedies having subsidiary side effects and more constructive retaliation towards prognosis[5] .The antitumor drugs built from natural constituents are said to modulate and enrich the immune system, impede cellular multiplication, and boost apoptosis. The microbes, plants, and marine animals are a significant source of anticancer drugs[4]. About 25,000 active compounds have been extracted from marine animals[6]. The active peptides and proteins reaped from them have displayed an enormous number of activities like anticoagulant, antioxidant, anti-diabetic, antimicrobial, antitumor, immune-modulatory, and antihypertensive. Marine fishes are more than 13,000 species from which more than 100 species have been used for various pharmaceutical treatments[7]. Fishes live in a very challenging environment as a result of which they produce a large variety of novel compounds[8]. Several bioactive compounds have been obtained from various fishes such as pufferfish Arothron immaculatus[9], Mugil cephalus[10], Rita rita, Channa punctatus[11], orange banded sting fish, Choridactylus multibarbus[12], marine catfish, Plotosus lineatus[13]. Marine fishes are a dominant part of the human diet. The capture fisheries make up half of the gross global fish production and about 70% of this goes for processing. The industries processing fish throw away immense quantities of head, viscera, skin, and fins. These byproducts can be used to isolate bioactive compounds[14]. A large number of peptides acquired from fishes have demonstrated potential curative properties depending upon the constituents and framework of the amino acids[15]. Hence this study attempts to investigate the anticancer activities of crude intestinal mucus of Tachysurus dussumieri against the human breast cancer cell line (MDA-MB-231).

MATERIALS AND METHODS

Preparation of intestinal mucus

Ailment free fishes were selected and sacrificed. The fishes were cut open with the help of sterile scissors. The intestine

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was then set apart from the internal organs[16] (Figure 1). The intestinal wall was scrapped gently with the help of a spatula. The entire process was carried out on the ice[17]. To the intestinal mucus which was collected with equivalent amounts of sterile physiological saline (0.85%) was added, homogenized with a motor and pestle, and centrifuged at 5000 rpm for about 15 minutes. The supernatant was collected and was stored at -20°C.

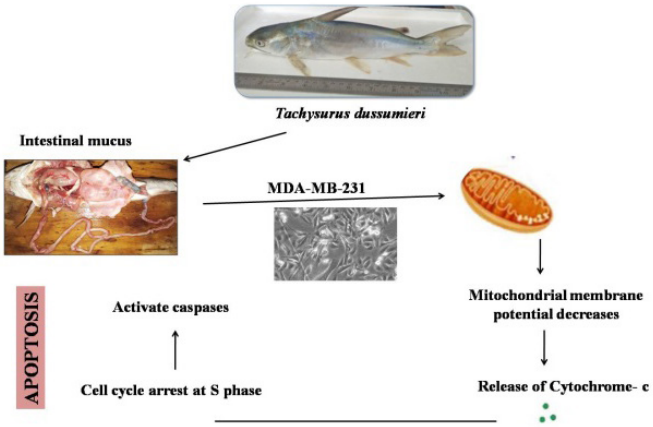

Figure 1: Schematic representation of steps involved in extraction of intestinal mucus.

Quantitative and qualitative analysis

The total protein content was estimated through the standard method of Lowry[18]. The gross carbohydrate and lipid contents were assessed by the method of Barnes and Blackstoc[19] and Roe[20] respectively. The protein profile of the crude intestinal mucus was appraised by SDS and NATIVE- PAGE following the standardized procedures of Maurer[21], and Laemmli[22] respectively.

Cancer cell line and chemicals

The breast cancer (MDA-MB-231) and African green monkey kidney cells were obtained from National Center for Cell Science (NCCS), Pune, India. DMEM, Trypsin-EDTA, Fetal Bovine Serum (FBS), 3-(4,5-dimethylthiazol-2yl)- 2, 5-diphenyltetrazolium bromide (MTT), sodium bicarbonate, Dimethyl sulphoxide (DMSO), anti-infection solutions were bought from Hi-Media Laboratories, Mumbai, India. 6 well plates, 96 well plates, Centrifuge tubes (15 and 50 ml) and Tissue culture flagons (25 and 75 mm) were bought from Tarsons Products Pvt, Kolkata, India. Synthetic compounds utilized in the current studies were none accessible locally.

MTT assay

The cytotoxicity and cell viability of the cells were tested analogously with the method of Mosmann[23]. The Vero and Breast cancer cells (5 × 103 cells/ml) were placed in 96 well plates along with a DMEM medium containing 10% FBS. The cells were incubated for 24 hours under the standard conditions of 95% O2, 5% CO2 at 37ºC. The medium was cleaned off, the control wells were provided with the medium again and treatment cells (Vero and breast cancer) were received 200 to 1000 µg/ml of medium containing intestinal mucus. The culture plates were incubated again as above 24 hours after 10 µl of MTT solution was added to all the wells and the cultures were incubated again for 4 hours and then 100 µl of DMSO was added and the crystals formed were dissolved gently by pipetting 2 to 3 times. A microplate reader was used to determine the absorbance at 570 nm.

General morphological observation

The changes in the morphology of the MDA-MB-231 cancer cell line treated with the crude intestinal mucus were assessed by using light microscopy (Nikon, Sclipse TS 100). The breast cancer cells (5 ×104 cells/ml) were plated in 40 × 10 mm dishes with DMEM medium constituting 10% FBS. The cells were incubated at 37˚C under 5% CO2, 95% O2 for 24 hours. The medium was then removed and the control dishes were provided with the fresh medium again and the treatment dishes were given IC50 and maximum concentrations of (800 and 1000 µg/ml) crude intestinal mucus then the culture plates were incubated. After the incubation time (24hr), the cells were visualized and photographed under an inverted light microscope at 20X magnification[24].

Propidium iodide staining

Fluorescent staining of apoptotic nuclei was performed by the method of Keum[25].MDA-MB-231cells (5 × 104 cells/ml) were introduced to 6 well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 hours with 5% CO2, 95% O2 at 37ºC. Then the medium was removed and the control well-received fresh medium and the treatment plates received 800 and 1000 µg/ml concentrations of crude intestinal mucus. Then the culture plates incubated as above. After completion of incubation time the cells were washed with PBS and fixed in methanol: acetic acid (3:1 v/v) for 10 minutes and stained with 50

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µg/ml of propidium iodide for 20 minutes. After staining the cells were visualized immediately under a confocal microscope (LSM 710, Carl Zeiss) at 20X magnification.

Rhodamine 123 staining

Rhodamine 123 is a green fluorescent dye that binds and enhances the membrane depolarization in the mitochondria followed by the method of Jhonson[26]. MDA-MB-231 cells were treated with the Intestinal mucus of T. dussumieri for 24 h. The cells were washed with PBS (pH 7.4) and stained with 5 g/ml rhodamine 123 at 37°C for 30 min. The cells were then washed with PBS to remove the excess staining and visualized at 20X magnification under a fluorescent microscope (LSM 710, Carl Zeiss)

DNA-fragmentation analysis

MDA-MB-231 cells were maintained at a density of 1 × 105 cells/well in six-well plates for 24 h and treated with intestinal mucus at concentrations of 800 and 1000 µg/ml for 24 h. The trypsinized cells were by centrifugation at 9000 × g for 5 min at 4°C, washed twice with cold PBS (pH 7.2), and treated with 500 µl of lysis buffer at 50°C. After 12 h, an equivalent volume of phenol-chloroform-isoamyl was added. Then the supernatant was indeed moved to new Eppendorf tubes, treated with 60 µL of ammonium acetate (10 M) and 600 µL of supreme ethanol was put away at −20◦C. After 12 hr, the centrifuged at 12,000 × g for 10 min, the accelerate was collected and air-dried for 30 min. The DNA was resuspended in 40 µL of Tris- (EDTA) (TE buffer pH 7.4) and electrophoresed on a 0.8% agarose gel. The gel was analyzed and captured with gel documentation (Med Care) and the experiment was followed by Bortner[27].

Cell cycle analysis

Progress through the cell cycle was examined by Zhi[28]. MDA-MB-231cells (1 × 105 cells) were treated with IC50 and maximum concentrations (800 and 1000 µg/ml) of intestinal mucus for 24 hr. Then the cells were collected, washed by super cold PBS, fixed with 70% ethanol at 4˚C for 12 hr, and stained with propidium iodide within the sight of 1% RNAase at 37˚C for 30 min before examination utilizing stream cytometry (BD FACS Calibur Flow Cytometer).

Statistical analysis

In all experiments was performed in triplicate and the final values were represented as mean ± Standard Deviation (SD). Differences with *p value<0.05, **p<0.01, ***p<0.001 were considered to be statistically significant.

RESULTS

Biochemical and protein profiles of crude intestinal mucus from T.dussumieri

The crude intestinal of mucus T. dussumieri was collected to examine the biochemical components present in it. The amount of proteins, lipids, and carbohydrates was 1.35, 1.086, and 2.095 mg/ml respectively. The protein profiles were analyzed with the help of NATIVE and SDS-PAGE. The native form of proteins shows seven prominent and clear bands with CBB Staining and SDS-PAGE showed proteins band ranging from 245 to 25 kDa. Thus the crude sample showed both low and high molecular weight protein bands (Figure 2).

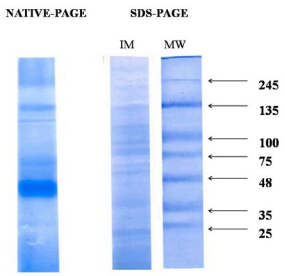

Figure 2: Qualitative analysis of intestinal mucus from Tachysurus dussumieri by Gel electrophoresis.

IM=Intestinal Mucus, MW=Molecular Weight Marker.

Cytotoxicity and cell viability

The cytotoxic and cell viability of crude intestinal mucus of T. dussumieri was analyzed using the MTT method on the Vero (normal) and human breast cancer cell lines. The cytotoxicity of normal cells results revealed that up to 75% of cells are viable in the concentration of 1000 µg/ml. Hence the dosage of crude intestinal mucus was fixed to be between 200 to 1000 µg/ml on breast cancer cell line. The cell viability of MDA-MB-231 cells results showed, 50% viability was achieved at the concentration of 800 µg/ml and this concentration was taken as Inhibitory Concentration (IC50) of intestinal mucus on the MDA-MB-231 cell line (Figure 3).

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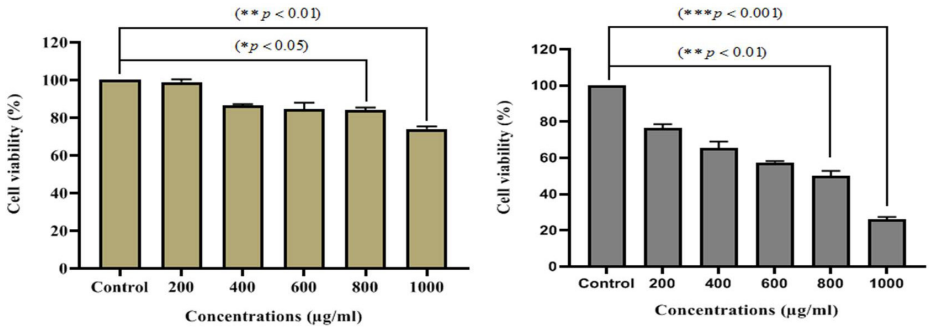

Figure 3: Cytotoxicity and cell viability of crude intestinal mucus on Vero and MDA-MB-231 cells.

General morphological observation

Morphological variations were observed in the control and treated MDA-MB-231 cells by using a light microscope. The control cells show the complete confluence of spindle-shaped cells with the uniform monolayer. While the treated cells at the concentration of IC50 (800 µg/ml) showed minimum confluence of cells; cell to cell interactions are disrupted. The maximum concentration (1000 µg/ml) of the intestinal mucus treated cells was disturbed in the monolayer and cells were found to be aggregated (Figure 4a). This shrinkage is due to the growth inhibitory effect of T. dussumieri crude intestinal mucus.

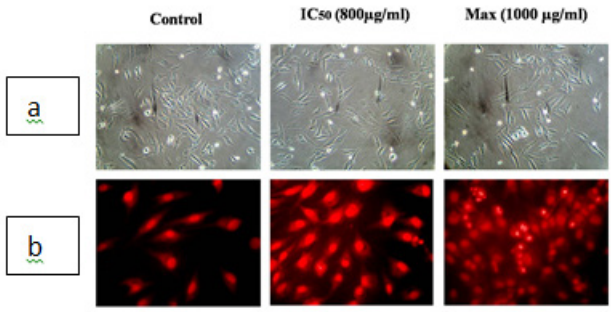

Figure 4: General (a) and nuclear morphological (b) observation of MDA-MB-231 cells treated with crude intestinal mucus.

Nuclear morphological observation

Nuclear damage of MDA-MB-231 cells on treatment with crude intestinal mucus was observed by Propidium iodide staining. The control cells were examined a very negligible amount of nuclear-damage. When the treated cells at the IC50 (800 µg/ml) concentration show a moderated number of cells with nuclear shrinkage whereas the cells treated with maximum dosage (1000 µg/ml) of intestinal mucus showed higher nuclear-damaged cells visualized fluorescence in the nucleus with fragmented DNA (Figure 4b). Hence there is an increase in the degree of apoptosis in cells upon an increase in the concentration of a drug.

Mitochondrial membrane potential

A significant and demonstrative stage in the characteristic apoptotic pathway is the depolarization of the mitochondrial layer and the ensuing increases in permeability of the external membrane. This is joined by the outcoming of proapoptotic molecules and cytochrome-c. The rhodamine 123 is a particular stain for the recognition of changes in Δψm in living cells. The study exhibited that crude intestinal mucus prompted a critical decrease in the number of cells with flawless membrane potential and expanded the number of cells with low Δψm after 24 hr. The adversity of Δψm was decreased where the concentration of intestinal mucus increased vice versa in MDA-MB-231 cells. The intensity of membrane potential was calculated and shown in the graph (Figure 5).

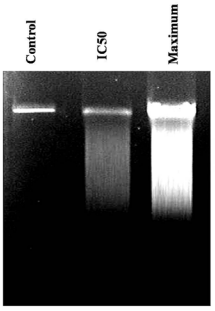

Figure 5: DNA fragmentation analysis of MDA-MB-231 cells treated with crude intestinal mucus at 24 hr.

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DNA fragmentation

DNA-fragmentation analysis confirms the incidence of damage in the DNA of apoptotic cells. MDA-MB-231 cells are treated with crude intestinal mucus for incubation of 24 hr. The control cells show a single band of intact DNA in the agarose electrograph. Cells treated with IC50 concentration of 800 µg/ml showed DNA fragmentation in the form of streaks. This streaking indicates the presence of DNA damage. In maximum (1000 µg/ml) concentration, DNA damage is higher hence the intensity level of the streaking pattern is also visualized with higher fluorescence (Figure 6).

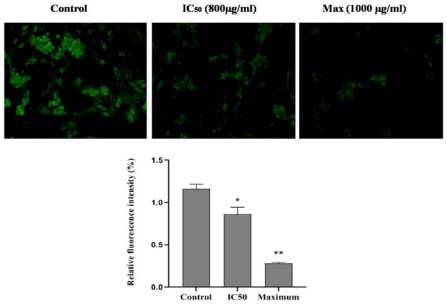
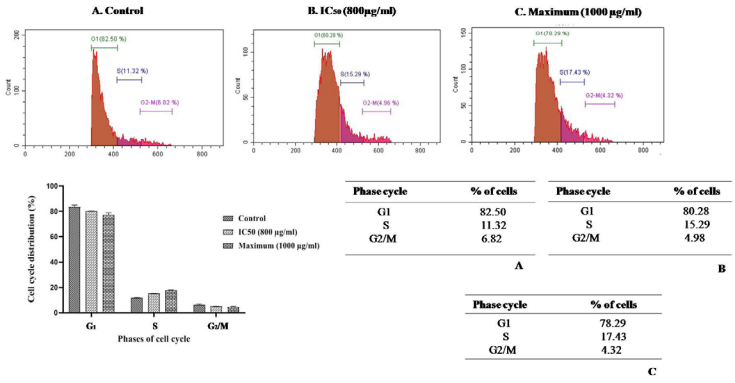


Figure 6: Observation of mitochondrial membrane potential depolarization of MDA-MB-231 cancer cells treated with intestinal mucus by using Rhodamine 123 staining.

Cell cycle

MDA-MB-231 cells were treated with various concentrations of intestinal mucus for 24 hr, subsequently, the cell cycle examinations and sample treated cells arrested at S phase compared with the control group of cell cycle phases (Figure 7). In IC50 concentration of treated cells revealed the quantities of cells arrested in S phase was confirmed with the percentage of cells occurrence in G1(80.28%), S(15.29%), G2-M(4.96%) with following maximum dosage (1000 µg/ml) showed a sequential increase of cells percentage in synthesis phase of DNA. These result outcomes demonstrated the progression of hindrances and induction of apoptosis through intestinal mucus in breast cancer cell line.



Phase cycle	% of cells
G1	82.50
S	11.32
G2/M	6.82

A

Phase cycle	% of cells
G1	80.28
S	15.29
G2/M	4.98

B

Phase cycle	% of cells
G1	78.29
S	17.43
G2/M	4.32

C

Figure 7: Cell cycle analysis of MDA-MB-231 cell line treated with crude intestinal mucus by flow cytometry.

DISCUSSION

In the present study, to explore the antiproliferative effect of intestinal mucus from marine catfish Tachysurus dussumieri on human breast cancer cell line (MDA-MB-231). The biochemical analysis of intestinal mucus showed the presence of protein, lipid, and carbohydrate in substantial amounts. The fish proteins are used in many fields related to inflammation, metabolic syndrome, osteoporosis, insulin resistance, and cancer treatment[29]. The total protein, lipid and carbohydrate contents of intestinal mucus were found to be 1.35, 1.086, 2.095 mg/ml respectively. The previous report result showed lesser amount of biochemical constituents in epidermal mucus of various species of catfishes such as, Clarias species, Clarias gariepinus and Tachysurus dussumeiri[23,30,31]. respectively when compare to intestinal mucus of T. dussumieri.

The qualitative analysis of crude intestinal mucus was determined by Native and SDS Phage using CBB staining. The molecular weight of the crude intestinal protein was determined by SDS PAGE on 12% polyacrylamide gel using a standard protein marker 25 to 245 kDa with a lower range of peptides. According to Stabili[32], there was fourteen major protein bands were observed ranging from 12 to 200 kDa on the mucus of Actinia equine by SDS-PAGE. Similarly, various studies done on the mucus of fish species such as Eel, Carp, Tench, and Trout had protein bands ranging from 94 kDa to 20.1 kDa examined by Ebran[33].

The prevalent motive of the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay is to calculate the viable cells. This assay is useful for computing the sensitivity of drug in cell lines as well as cells undergoing division. The reduction in the number of cells shows inhibition in cell growth and the drug sensitivity is then stated as the concentration of the drug required to

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attain 50% growth inhibition (IC50) when compared to the growth of the cells in the untreated control[34].Cytotoxicity of intestinal mucus was checked with a normal cell line by MTT assay, which exhibits 75% of cells are viable at a higher concentration of 1000 µg/ml. Hence the drug concentration of MDA-MB-231 cells was fixed to be 200 to 1000 µg/ml. The crude intestinal mucus showed a dose and time-dependent decrease in cell viability. Increasing the time of incubation showed a further decrease in cell viability. Approximately 50% inhibition of cell viability was seen at 800 µg/ml upon 24 hr exposure.

There are two basic principles of apoptotic pathways. One includes the ligation of death ligands, for example, tumor necrosis factor and Fas to their cytoplasmic layer receptors and is alluded to as the extrinsic pathway[35].The other pathway includes mitochondria and is known as the intrinsic pathway. The intrinsic pathway is started by the loss of MMP which prompts the opening of the mitochondrial porousness and the arrival of effectors including cytochrome c and apoptosis instigating factor from the mitochondria into the cytosol. The cytochrome c triggers the proteolytic movement of caspase-3 and caspase-9 in the cytosol, and these enacted caspases corrupt (poly [ADP-ribose] polymerase) and caspase-actuated DNase, which starts DNA debasement[36]. MDA-MB-231 cells treated with intestinal mucus exhibit the MMP exhaustion along with that the fluorescence of IC50 concentration determines the loss of MMP uprightness. Rhodamine-123 can bind to the live mitochondria and discharges the fluorescence because of high extremity, however, dead cells neglect to hold this fluorescence color since the loss of mitochondrial membrane potential[37] whereas due to the treatment of intestinal mucus, cells achieve the higher degree of ROS with loss of MMP and the degradation of DNA was visualized with the help of agarose gel electrophoresis, the streaking pattern of fluorescence indicates the fragmentation of DNA.

The cell cycle progression was directed towards many control focuses at various points in the cell cycle; the three ways of being a progression of phases G1,S,G2/M, at metaphase/anaphase transmission during mitosis. The failure of these checkpoints can prompt uncontrolled cell growth. The G1 and S checkpoints are generally basic for control of cell expansion through intracellular and extracellular signs identified with transportation and molecule of atoms into the core[38] whereas the drug boldine induces cell cycle arrest dose-dependently at the G2/M phase of MDA-MB-231 cells[39]. A report from Yang[40] the peptide SA12 which inhibits the cell cycle at the G1 phase of MCF-7 and MDA-MB-231. In our study, the treatment of intestinal mucus induces the cell cycle arrest at the synthesis phase which may be inhibiting the Cyclin-B-CDK1 complex, which slowly accumulates throughout the cell cycle to promote mitotic entry. These S intraphase checkpoints identify the damages of double bonds through that it activates the kinases of ATM and ATR[41].

CONCLUSION AND RECOMMENDATION

In conclusion, our study has shown that the intestinal mucus exerted an inhibitory effect on the human breast cancer cells (MDA-MB-231) and this effect was related to the induction of apoptosis through depolarization of the mitochondrial membrane. The crude intestinal mucus of the catfish Tachysurus dussumieri might represent a novel source of bioactive molecules with potential applicative purposes in drug discovery. Further investigation will be required in order to purify and characterize the molecules responsible for the cellular apoptosis.

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