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RESEARCH ARTICLE

Effect of crude and partially purified epidermal mucus proteins of marine catfish *Tachysurus dussumieri* on human cancer cell line

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Abstract

Epidermal mucus protein of marine catfish *Tachysurus dussumier* was screened for its anticancer properties. The mucus sample was collected and categorized as crude and partially purified. Crude and partially purified samples consist of 0.48 ± 0.02 mg/mL and 0.82 ± 0.05 mg/mL proteins, 1.15 ± 0.06 mg/mL and 0.54 ± 0.02 mg/mL carbohydrates and 0.78 ± 0.04 mg/mL and 0.32 ± 0.03 mg/mL of lipids respectively. Cytotoxicity was expressed in the inhibition concentration (IC_{50}) value 5 mg/mL at 72 h for crude sample and 3 mg/mL at 48 h for partially purified protein against human lung adenocarcinoma epithelial cell line (A549). DNA fragmentation revealed that the control cells has intact DNA, whereas cells treated with both the mucus proteins confirmed DNA fragments of different molecular range due to the induction of apoptosis.

Keywords: *Tachysurus dussumier*, mucus protein, cytotoxicity, adenocarcinoma, DNA fragmentation.

Introduction

Improvements in living standards and emergence of communicable diseases have led to cancer and other chronic diseases emerging as major public health problems in certain quarters of the Indian sub-continent. These diseases are lifestyle related, have a long latent period and needs specialized infrastructure and human resource for treatment (Nair and Sankaranarayanan, 1991). Animals have been used as medicinal resources for the treatment and relief of a myriad of illnesses and diseases in practically every human culture (Costa Neto, 2005). The innate antimicrobial compounds with a broad antimicrobial effect have been identified in a variety of multicellular organisms (Zasloff, 2002), ranging from insects to several groups of vertebrates such as fishes, amphibians (Zasloff *et al*., 1987) and mammals (Harder *et al*., 1997).

In fish the epidermal mucus is considered as key component of innate immunity (Ellis, 1974; Ingram, 1980). Antimicrobial compounds have been found associated with the epithelial mucus-secreting cells of fishes (Diomand *et al*., 1997). The epidermal mucus is produced primarily by epidermal goblet or mucus cells and is composed mainly of water and gel-forming macromolecules including mucins and other glycoproteins (Negus, 1963; Shephard, 1993). The composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental perturbations such as hyperosmolarity and acidity (Agarwal *et al*., 1979; Zuchelkowski *et al*., 1981). The mucus layer on the fish surface performs a number of functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Negus, 1963; Ingram, 1980; Shephard, 1994).

Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavours (Hellio *et al*., 2002). Some antimicrobial agents are present in the mucus of bony fishes which bind to the microbes and destroy it (Ebran *et al*., 1999). The lysozyme is isolated from fish was an enzyme with bacteriostatic properties and ubiquitous in its distribution among living organisms. Sloughing of microbes in the mucus contains many antibacterial substances including antibacterial peptides, lysozyme, lectins and proteases (Barnes *et al*., 2003). The biological active components present in mucus of fish have found to be useful for pharmacological reagents including antibacterial, antifungal, antiparasites, antioxidant and disease resistant. To date a large number of antifungal proteins have been reported. The antifungal and anti-insect activities of lectins can be made use of in the control of pathogens. The production of anti-tumor and anti-viral drugs based on lectins may also be feasible (Sze Kwan Lam and Tzi Bun Ng, 2011). Although, nowadays researchers have found alternative chemotherapic method for the treatment of cancer in several ways, one such method is innate antimicrobial protein or peptide. Hence, this study has been attempted for screening the potent anticancer compound and evaluates the anticancer properties of marine cat fish *Tachysurus dussumieri* epidermal mucus.

Materials and methods

Collection of marine catfish: The marine catfishes, *Tachysurus dussumieri* were collected from East Coast Bay of Bengal, Kasimedu, Chennai, Tamil Nadu, India. The catfishes weighing approximately 250 g were collected and transported to laboratory in their native water (tap water containing 0.2 mM Ca^{2+}). During transportation, the water was kept at 4°C with dissolved oxygen always exceeding 90% of saturation values.

The collected fishes were acclimatized to laboratory condition and they were maintained for 4 d. After 4 d these fishes were used for mucus collection.

Isolation of mucus from marine catfish: Mucus was collected as described by Ross *et al*. (2000) with slight modifications. The mucus was obtained only from healthy fishes. Fishes were anesthetized in Tris-buffer (pH 7.4) and placed on the ventral side of the body facing downward on a "surgery-bed", cutaneous mucus from the dorsal side of the fish was collected by a cell-scraper and transferred to a tube with 10 mL of buffer solution containing 0.013 M Tris, 0.12 M NaCl and 0.003 M KCl (pH 7.4). Mucus was not collected from the ventral side in order to avoid any blood, intestinal and sperm contamination. The mucus samples were stored at –80°C until use.

Preparation of crude mucus from experimental animal: Stored mucus samples were allowed to thaw at room temperature and the insoluble particles were removed by centrifugation at 1000xg for 10 min at 4°C. The supernatant was centrifuged at 20,000xg for 10 min at 4°C (Beckman E 80) and the pellet was discarded and supernatant was lyophilized. The lyophilized sample was suspended in a small volume of distilled water and heated for 5 min at 100°C (Flik *et al.,* 1984). After centrifugation at 10,000xg for 5 min at 4°C, the supernatant was used for further studies.

Partial purification of catfish mucus: Partial purification of catfish mucus was performed by ammonium sulphate precipitation and dialysis. Saturated ammonium sulphate solution (pH 7.4) was added to the supernatant to obtain final saturation around 55%. The solution was left on ice for 2 h and centrifuged at 25,000xg for 30 min at 4°C (Beckman E80). The supernatant was adjusted to pH 4 with H_2SO_4 and centrifuged at 1,00,000×g for 30 min at 4°C. The pellet was resuspended in distilled water and desalted by dialysis against distilled water, samples were placed in dialysis tube 6 cm x 15 mm (molecular cut off 10-12 KDa) and the tube were placed on an orbital shaker and kept at room temperature for overnight (37 ± 2°C). After dialysis the sample was recovered and used to further assays.

Biochemical parameters:

Estimation of protein in mucus: Both the crude and partially purified samples were quantified using the method described by Bradford (1976). Briefly, 20 mg of the mucus sample was homogenized separately in 1 mL of 10% trichloroacetic acid (TCA) to precipitate the protein. Then the sample was centrifuged at 4000×g at 4°C for 10 min. The sample was mixed with 5 mL of Bradford reagent. The sample were incubated for 10 min and the developed color was read at 595 nm using Shimadzu160 UV-VIS double beam spectrophotometer and the amount of protein was calculated.

Estimation of carbohydrate in mucus: The total carbohydrate was quantified using the method described by Carroll, *et al.* (1956). Twenty milligram of the mucus sample was homogenized in 80% ethanol and centrifuged at 4000xg for 10 min and the supernatant was collected. Supernatant (0.5 mL) was taken and made up to 1 mL with distilled water to which 4 mL of anthrone reagent was added and heated for 10 min in a boiling water bath. After 10 min the solution was cooled at room temperature (dark) for 30 min and the resultant blue color complex was measured at 620 nm using Shimadzu 160 UV-VIS double beam Spectrophotometer. A standard was prepared by using saturated benzoic acid and the amount of carbohydrate present in the sample was calculated using standard value.

Estimation of lipid in mucus: The extraction of lipid from the sample was done according to Folch *et al.* (1957) and the estimation was done according to Barnes and Blackstock (1973). The known volume (20 mg) of sample was homogenized in 5 mL of chloroform-methanol (2:1) mixture to which 0.5 mL of 0.9% NaCl solution was added and shaken well. This mixture was then transferred to separating funnel and allowed to stand for 12-14 h. The lower phase containing lipid was collected in test tube and the volume was made up to 5 mL with chloroform. Aliquot of 0.5 mL lipid samples was digested with 0.5 mL of concentrated sulphuric acid in a boiling water bath for 15 min. A volume of 0.2 mL of acid digest was taken in 4 different test tubes to which 5 mL of phosphovanillin reagent was added to each. The mixture was allowed to stand for 30 min and the color developed was read at 520 nm using 160 UV-VIS double beam spectrophotometer. Lipid standard and blank was prepared simultaneously. The amount of total lipid was calculated using the lipid standard value.

Protein profile analysis by SDS-PAGE: The protein profile of crude and partially purified proteins were qualitatively analyzed by SDS-PAGE (sodium dodecyl sulphate-poly acrylamide gel eletrophoresis) as described by Laemmli, (1970). Briefly, SDS-PAGE is carried out by 12% resolving gel and 5% stocking gel.

Cancer cell line and chemicals: Human Lung Adenocarcinoma epithelial cell line (A549) was obtained from National Center for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle Medium (DMEM), Trypan blue(TB), Trypsin-EDTA, Fetal Bovine Serum (FBS), 3-(4, 5-Dimethyl thiazol-2yl)-2, 5-dimethyltetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), Sodium bicarbonate and antibiotic solution were purchased from Hi-Media laboratories Ltd, Mumbai, India. 96 well plates, 6 well plates, tissue culture flasks (25 mm^2 and 75 mm^2), 15 mL and 50 mL centrifuge tubes were purchased from Tarson products. Pvt., Kolkata, India.

Cytotoxicity assay: The cytotoxic effect of mucus protein was assessed by MTT method (Mossman, 1983). Briefly, Human cancer cells (1 x 10^5 /well) were plated in 96 well plates with DMEM medium containing 10% FBS. 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 DMEM medium incubated was removed from the cell culture plates, the cells were washed with PBS and the cells were treated with medium containing different concentration (1-10 mg/mL) of crude and partially purified mucus extracts for 24, 48 and 72 h incubation. Control cells were maintained without/absence of mucus protein samples. After 24, 48 and 72 h 20 µL of 5 mg/mL MTT solution was added to each well and the cultures were further incubated for 4 h at 37°C and then 100 µL of DMSO was added and the formed crystals were dissolved gently be pipetting 2 to 3 times and measured absorbance at 570 nm. Growth inhibition rate was calculated as followed formula:

$$\text{Percentage of growth inhibition} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

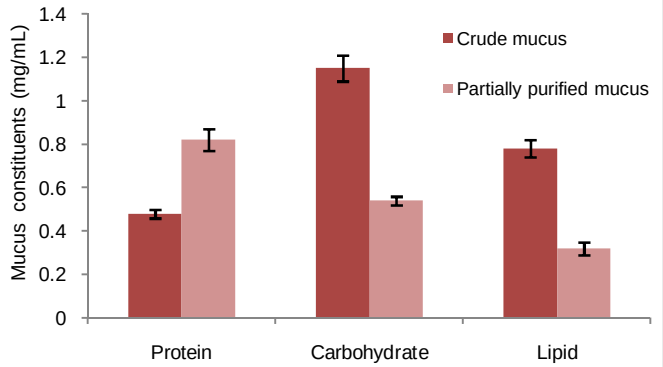
Morphological examination: Human cancer cell line (A549) was trypsinized and 5 mL of growth medium was added to trypsinized cells and cell suspension was mixed well. Then 2 mL of cell suspension was added to the sterilized test tubes containing cover slip. The test tubes were placed in a slanting position and kept in CO_2 incubator for 2 d. The monolayer of cells formed in the cover slip was observed under Inverted microscope and photographed.

DNA-fragmentation analysis: DNA extraction and agarose gel electrophoresis were performed by the method of Luisa *et al*. (2006). Cancer cells (3×10^5 cells/mL) were plated per well in 6 well plates with DMEM medium containing 10% FBS. The control plates received 0.01% DMSO containing medium and treatment plates received 1–10 mg/mL of fish mucus protein samples containing medium. After 48 and 72 h of treatment, the DNA was extracted from the cell lysate. The cells were washed with PBS and then added 0.5 mL of lysis buffer, transferred to a microfuge tube and incubated for 1 h at 37°C. To this, 4 µL of proteinase K was added and the tubes were incubated at 50°C for 3 h. To each tube, 0.5 mL of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed and centrifuged at 10,000 rpm for 10 min to separate the DNA containing upper aqueous phase. To the resulting aqueous phase, 2 vol of ice-cold absolute ethanol and 1/10^{th} volume of 3 M sodium acetate were added and incubated for 30 min on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 rpm for 10 min at 4°C and the pellet was washed with 1 mL of 70% ethanol. The pellet was allowed to dry at room temperature for approximately 30 min and resuspended in 50 µL of TE buffer. The DNA was quantified by UV-visible spectroscopy and 10 µg of DNA was electrophoresed in 1.2% agarose gel containing ethidium bromide in a mini gel tank containing TBE buffer for 1 h under 90 V. Then, the gel was examined under UV trans-illuminator (Bio-Rad) and photographed.

Results and discussion

The epidermal mucus obtained from marine catfish *T. dussumieri* was pooled to yield mucus sample. The pooled mucus was then separated into 2 parts as crude mucus and partially purified mucus samples. Lung Adenocarcinoma cell line (A549) were subjected to experiments when reach the confluence after 24 h. The crude mucus that was obtained from epidermal layer of marine catfish was found to contain most of the basic biochemical components like proteins, carbohydrates and lipids etc., The partial purification was performed to separate out or to isolate the higher molecular weight proteins (above 10 kDa). In this process certain other carbohydrates and lipid molecules get separated out. The protein content was estimated by using Bradford method. From the standard graph obtained using optical density value, the total protein content in crude mucus was estimated to be 0.48±0.02 mg/mL and in partial purification the total protein was found to be 0.82±0.05 mg/mL (Fig. 1). Hence it can be inferred that the partially purified mucus samples has almost double the amount of protein when comparing with that of the crude mucus sample.

Fig. 1. The biochemical constituents of crude and partially purified mucus of *T. dussumieri.*

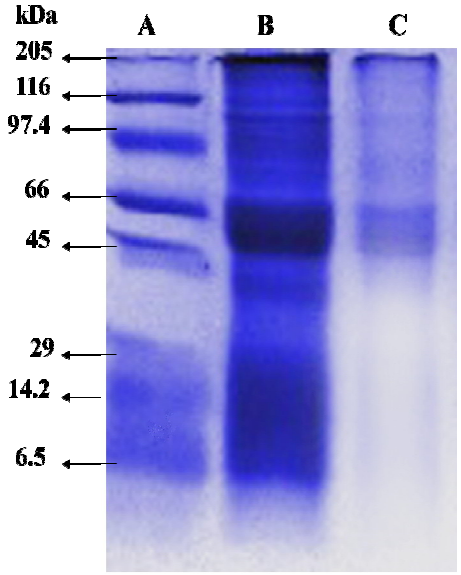


The carbohydrate content of both crude and partially purified mucus samples were quantified using standard method. From the standard graph the total amount of carbohydrate was observed to be 1.15±0.06 mg/mL in crude and 0.54±0.02 mg/ml in partially purified mucus sample (Fig. 1). Hence the result shows that the partially purified mucus has lower amount of carbohydrate content when comparing with that of the crude sample. The lipid present in the fish epidermal mucus samples was extracted and estimated. From the standard graph obtained using optical density values the lipid level in crude sample was estimated to be 0.78±0.04 mg/mL and 0.32±0.03 mg/mL in the partially purified mucus sample.

From the result it was clearly understood that the crude mucus sample has comparatively higher amount of lipid content than in partially purified mucus sample. The results obtained from basic biochemical analysis clearly listed out the total amount of proteins, carbohydrates and lipids present in both crude and partially purified epidermal mucus samples of marine catfish *T. dussumieri*. Our results were in accordance with the earlier results of Shephard (1994) and Chong *et al*. (2005).

The protein profiles of the crude and partially purified mucus protein samples of marine catfish on SDS-PAGE gel showed proteins ranging from 205 kDa to less than 6.5 kDa (Fig. 2). The crude protein sample showed both low and high molecular weight protein bands, and the low molecular mass proteins below 29.0 kDa were more prominent. The protein profile of the partially purified mucus extracts showed mainly high molecular weight proteins ranging from 45-205 kDa. These results were similar to the reports of Chong *et al*. (2005).

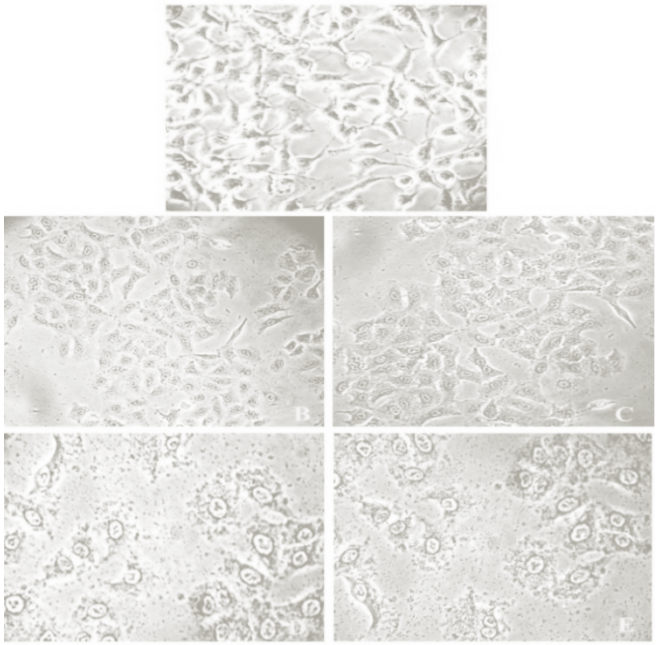
Fig. 2. SDS-PAGE showing the protein profiles of crude and partially purified mucus protein samples of *T. dussumieri.*



A-Protein markers (Molecular weight range for 6.5 kDa-205 kDa);
B-Crude mucus protein sample;
C-Partially purified mucus protein sample.

Cell death in tumors, whether spontaneous or treatment-induced, occurs predominantly via apoptosis rather than necrosis (Wyllie *et al.,* 1980; Arend *et al.,* 1990). Morphological evaluation of cancer cells after treatment with mucus samples at different concentration revealed the characteristic apoptotic morphology such as loss of membrane integrity, rupture of cells etc. In this connection cells (A549) were seeded at a density of 1×10^4 cells per well onto 24-well plate after 24 h. The control plate did not show any morphological changes and were irregular confluent aggregates with rounded and polygonal cell morphology.

Fig. 3. Morphological changes of A549 cell line due to the effect of crude and partially purified mucus protein samples of *T. dussumieri.*



A-Control A549 cells; B&C-A549 cells treated with 5 mg/mL of crude mucus at 72 h and 3 mg/mL of partially purified mucus at 48 h; D&E-A549 cells treated with 10 mg/mL of crude and partially purified mucus at 72 h.

The cells incubated for 72 h with concentration (5 mg/mL) of crude mucus protein and for 48 h with partially purified mucus protein (3 mg/ mL) have shown the characteristic morphological changes of apoptosis including membrane blabbing, chromatin condensation and the formation of apoptotic bodies. And also the cells were incubated for 72 h with maximum concentration (10 mg/ mL) of both the mucus samples. The cell shrinkage increased progressively in dose and time dependent manner. This shrinkage may be due to the growth inhibitory effect of mucus samples of *T. dussumieri* (Fig. 3).

Apoptosis, which is caused by a variety of instances, is thought to have a key role in killing cancer cells. A large number of studies confirm that almost all apoptosis stimulating factors can cause structural damage and mitochondrial dysfunction (Marchetti *et al.,* 1996). Mitochondrial trans-membrane potential and mitochondrial permeability changes during apoptosis play an important role in the process (Newmeyer and Ferguson-Miller, 2003). Interestingly in this study, treatment of cancer cells (A549) with the mucus protein samples isolated from marine catfish *T. dussumieri* produced a significant dose-dependent decrease in cell viability as measured by the MTT assay. Cell viability of A549 cells was assessed by treating the mucus protein samples with different concentrations (1-10 mg/mL) for different hours (24, 48 and 72) of incubation. A significant decrease was seen in the cell viability at different concentration of crude and partially purified mucus protein.

The IC_{50} value of the cells treated with crude mucus protein (50.11%) was 5 mg/mL at 72 h and for the partially purified mucus protein (50.83%) was 3 mg/mL at 48 h. There was no significant change in IC_{50} after 24 h incubation in both the samples (Fig. 4a and b). In addition to the morphological evaluation, apoptosis induction by mucus samples was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptosis.

Fig. 4a. Effect of crude mucus protein of *T. dussumieri* on the growth of A549 cells.

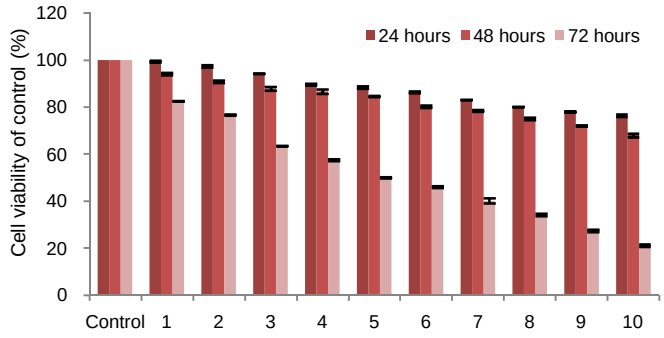


Fig. 4b. Effect of partially purified crude mucus protein of *T. dussumieri* on the growth of A549 cells.

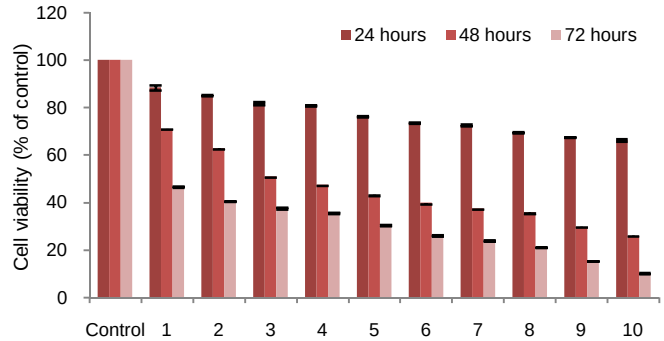
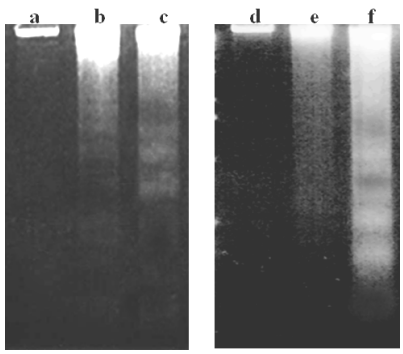


Fig. 5. Agarose gel showing the DNA fragmentation of A549 cell line due to the effect of crude and partially purified mucus protein samples of *T. dussumieri.*



a&d-Control A549 cells; b&e-A549 cells treated with 5 mg/mL of crude mucus at 72 h and 3 mg/mL of partially purified mucus at 48 h; c&f-A549 cells treated with 10 mg/mL of crude and partially purified mucus at 72 h.

As illustrated in figure 5, 1.2% agarose gel electrophoresis of DNA extracted from A549 cells treated with crude mucus proteins (5 mg/mL) for 72 h incubation and partially purified mucus proteins (3 mg/ mL) at 48 h incubation. It was found that the DNA obtained from control samples were intact and represented as prominent single band on the gel. On the contrary the electrophoretic DNA obtained from cell lines treated with both the mucus protein samples were released DNA fragments of different molecular range. Comparatively cell lines treated with 10 mg/mL of both mucus protein samples showed higher fragmentation compared to control. DNA damage in responsive mammalian cells is implicated as a critical event in the induction of apoptosis (Venkatachalam *et al.,* 1993; Walker *et al.,* 1997). Data from morphological change, DNA laddering and nucleosomal DNA fragmentation showed that MAP inhibits HL-60 cells proliferation by apoptosis. The mechanisms underlying these effects vary depending on the initiating stimulus, but a common feature is the activation of certain endonucleotidases leading to DNA fragmentation (Wyllie *et al.,* 1984; Barry and Eastman, 1992). Endonucleotidase activation may result from disruption of DNA supercoil structure, interference with DNA repair mechanisms or interference with normal cellular signaling pathways (Bergamaschi *et al.,* 1993; Yousefi *et al.,* 1994).

Conclusion

The mucus sample collected from marine catfish *Tachysurus dussumieri* was categorized as crude and partially purified samples consist of 0.48 ± 0.02 mg/mL and 0.82 ± 0.05 mg/mL proteins, 1.15 ± 0.06 mg/mL and 0.54 ± 0.02 mg/mL carbohydrates and 0.78 ± 0.04 mg/mL and 0.32 ± 0.03 mg/mL of lipids. Cytotoxicity was expressed in the inhibition concentration (IC_{50}) value 5 mg/mL at 72 h for crude protein sample and 3 mg/mL at 48 h for partially purified protein against human lung adenocarcinoma epithelial cell line (A549). DNA fragmentation revealed that the control cell has intact DNA, whereas cells treated with both the mucus proteins confirmed DNA fragments of different molecular range due to the induction of apoptosis. To conclude, screening of mucus protein from *T. dussumieri* has strong anticancer properties against human lung adenocarcinoma epithelial cell line A549.

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