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Epidermal Mucus of *Anabas testudineus* as a Promising Source of Antibacterial and Anticancer Agents

(Mukus Epidermis *Anabas testudineus* sebagai Punca Agen Antibakteria dan Antikanser yang Menggalakkan)

AHMED ABDULKAREEM NAJM[1], HERRYAWAN RYADI EZIWAR DYARI[3], BABUL AIRIANAH OTHMAN[2,4], SHARIFAH SAKINAH SYED ALWI[5], AHMAD AZFARALARRIFF[2], MUHAMMAD SHAHID[1], SITI AISYAH SANUSI[2], DOUGLAS LAW[2,6] & SHAZRUL FAZRY[2,4,7,*]

[1]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia
[2]Department of Food Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia
[3]Department of Earth Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia
[4]Innovative Center for Confectionery Technology (MANIS), Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia
[5]Department of Biomedical Science, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
[6]Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
[7]Chini Lake Research Centre, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia

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ABSTRACT

Anabas testudineus is a sturdy freshwater fish that can live in a polluted environment due to the epidermal mucus (EM) that protects the fish from pathogens or germs. This study explored the functional properties of the EM as a potential antimicrobial and anticancer agent. Inactive *Pseudomonas aeruginosa* was introduced into fish tanks to stimulate the production of EM. This stimulus significantly increased EM production by more than 100% after 10 days of stimulation, indicating that EM production was influenced by environmental biotic stress. *In vitro* antibacterial activity tests showed that EM has significant antibacterial activity against *Escherichia coli* (12 ± 0.23 mm) and *P. aeruginosa* (10 ± 0.13 mm) at the tested concentration of 1000 µg/mL. Further characterisation against cells showed that EM has a cytotoxic effect against human breast cancer (MCF7) and human melanoma (A375.S2) producing an IC_{50} value of 4.97 ± 0.25 and 6.27 ± 0.17 mg/mL, respectively. In contrast, no cytotoxicity against normal fibroblast skin cells (HS27) was observed. In addition, apoptosis analysis showed that EM could cause DNA fragmentation of cancer cells, while no effect on normal cells was observed. These findings indicated that EM from *A. testudineus* could be further studied and explored as an anticancer agent.

Keywords: *Anabas testudineus*; anticancer; antimicrobial peptide; epidermal mucus; mucus secretion

ABSTRAK

Anabas testudineus adalah ikan air tawar yang lasak dan dapat hidup dalam persekitaran yang tercemar kerana mempunyai mukus epidermis (EM) yang melindungi ikan ini daripada patogen atau kuman. Penyelidikan ini mengkaji tentang sifat berfungsi EM sebagai agen antimikrob dan antikanser yang berpotensi. *Pseudomonas aeruginosa* yang tidak aktif dimasukkan ke dalam tangki ikan untuk merangsang pengeluaran EM. Rangsangan ini meningkatkan pengeluaran EM dengan ketara lebih daripada 100% selepas 10 hari rangsangan yang menunjukkan bahawa

pengeluaran EM dipengaruhi oleh tekanan biotik persekitaran. Ujian aktiviti antibakteria secara *in vitro* menunjukkan bahawa EM mempunyai aktiviti antibakteria terhadap *Escherichia coli* (12 ± 0.23 mm) dan *P. aeruginosa* (10 ± 0.13 mm) pada kepekatan 1000 µg/mL. Pencirian lebih lanjut terhadap sel menunjukkan bahawa EM mempunyai kesan sitotoksik terhadap sel barah payudara manusia (MCF7) dan sel melanoma manusia (A375.S2), masing-masing menunjukkan nilai IC_{50} 4.97 ± 0.25 dan 6.27 ± 0.17 mg/mL. Sebaliknya, tiada kesan toksik terhadap sel kulit fibroblas normal (HS27) yang diperhatikan. Di samping itu, analisis apoptosis menunjukkan bahawa EM dapat menyebabkan fragmentasi DNA bagi sel kanser, tetapi tiada kesan ke atas sel normal yang diperhatikan. Hasil kajian ini menunjukkan bahawa EM daripada *A. testudineus* boleh dilakukan kajian selanjutnya dan diterokai dengan lebih mendalam sebagai agen antikanser.

Kata kunci: *Anabas testudineus*; antikanser; mukus epidermis; peptida antimikrob; rembesan mukus

Introduction

Anabas testudineus, the climbing perch, is a native species found in Asia belonging to the Anabantide family. It inhabits brackish water and freshwaters of Asia, including Malaysia, Pakistan, Bangladesh, the Philippines, Myanmar, Thailand, Pakistan, India, Sri Lanka and China (Sarkar et al. 2005). It is highly adaptive as it can live in various environments and conditions (temperatures, reduced dissolved oxygen, and pH) and live without water for 6 to 10 h (Rahman 2005).

One of the main contributing factors for fish well-being and survivability is the epidermal mucus (EM), which functions as a protective layer from both environmental biotic and abiotic factors (Al-Rasheed et al. 2018). It can also be characterised as a slimy, oily viscous colloid known as mucins composed of mucopolysaccharides, antibacterial enzymes, proteins and water (Dash et al. 2018) that coat the epithelial surfaces and act as a stable physical and chemical shield against invading pathogens (Arockiaraj et al. 2014). In addition, this mucus also contains various essential proteins such as proteases, antimicrobial peptides (AMPs), lectins, lysozymes, immunoglobulins, c-reactive protein (CRPs), transferrin, amphipathic lipid packing sensor (ALPs), and numerous other antibacterial proteins that play a significant role in fish immunity (Arockiaraj et al. 2013) the recombinant MrCrs gene was constructed and expressed in Escherichia coli BL21 (DE3. For example, Al-Rasheed et al. (2018) reported that the skin mucus of *A. testudineus* has the ability to inhibit the growth of several bacteria.

Various studies have reported the antibacterial activity of EM against various bacterial species. For example, epidermal mucus from Atlantic flatfish has demonstrated antibacterial activities against several bacterial strains (Patrzykat et al. 2003). Moreover, antibacterial activities against *Bacillus megaterium*, *Escherichia coli,* and *Candida albicans* have also been reported for Atlantic cod (*Gadus morhua*) (Bergsson et al. 2005). Furthermore, antibacterial activity against the Gram-negative pathogenic bacteria, *Pseudomonas aeruginosa*, has been reported for snakehead fish (*Channa striata*) epidermal mucus (Wei et al. 2010). On top of this, Kumari et al. (2019) found that the EM of freshwater fish such as *Hippelates nobilis*, *Ctenopharyngodon idella,* and *Cyprinus carpio* showed antibacterial activity against various types of pathogens compared to snakehead fish (*C. striata*).

In addition to antimicrobial activity, there is some scientific evidence showing that the epidermal mucus (EM) of freshwater fish and marine fish is cytotoxic against some tumour cells such as human lung cancer cells (Arulvasu et al. 2012), human breast cancer cells (Alijani Ardeshir et al. 2020; Teerasak et al. 2016) and colon cancer cells (Buhari et al. 2015). The cytotoxicity effect against human breast cancer cells by EM obtained from goby (*Neogobius melanostomus*) and common carp (*C. carpio*) fish have also been reported (Alijani Ardeshir et al. 2020). Not long ago, Fuochi et al. (2017) has found that the *Dasyatis pastinaca* (Linnaeus 1758) skin mucus blocked the proliferation of toxic leukaemia cells (HL60) while not affecting healthy neuroblastoma cells (SH-SY5Y) and multiple myeloma cell lines (MM1, U266).

As part of EM, in recent years, AMPs have gained the attention of researchers due to their ability to inhibit both microbes and neoplasm (Jin & Weinberg 2019). The number of AMPs shown to have anticancer properties has steadily increased. An example of such AMP is the Aurein 1.2 (GLFDIKKIAESF), a peptide extracted from frog (*Litoria aurea*). The Aurein 1.2 was reportedly a potential toxin against different cancer cell lines in *in vitro* experiments. Conversely, Aurein 1.2 did not show any adverse effect against normal cell lines, but remarkably

suppressed drug-resistant breast cancer cells both in *in vitro* and *in vivo* (Dennison et al. 2007; Rozek et al. 2000). More interestingly, these AMPs are not toxic to fibroblasts or erythrocytes (Hilchie et al. 2011). The physicochemical parameters that contribute to the AMP activity against cancers are mostly unknown, although the properties of AMP and anticancer peptides are very similar. Despite decades of medical and scientific research on most aquatic animals, *A. testudineus* is still under studied. Currently, *A. testudineus* could be one of the promising elected species for aquaculture activities and scientific research due to its robust characteristics mentioned previously (Al-Rasheed et al. 2018; Ndobe et al. 2019). Therefore, this study aimed to characterise the functional properties of pseudo-pathogenically induced *A. testudineus* EM extract and observe their antibacterial and anticancer activities.

Materials and Methods

SAMPLE PREPARATION

A total of 30 climbing perch with an average weight of 300 g was obtained from a local fish farm in Jelebu, Negeri Sembilan, Malaysia. The fish were acclimatised for 7 days (in groups of threes) in a 26×17×19 cm^3 plastic aquarium at 28 °C, at a pH range between 6 and 6.5. The water used in this experiment was dechlorinated tap water. The fish were fed a commercial pelleted diet (*ad libitum*) composed of protein (18 to 50%), lipids (10 to 25%), carbohydrate (15 to 20%), ash (<8.5%), phosphorus and trace amounts of vitamins, minerals, and water for the entire period of the experiment. All experiments were approved by Universiti Kebangsaan Malaysia (UKM) animal ethics committee (Ethics code: FST/2019/MOHD SHAZRUL FAZRY/25 SEPT./1034-SEPT.-2019-FEB-2020).

PSEUDO-PATHOGENICALLY FISH CHALLENGE

To stimulate the innate immune response of the *A. testudineus*, the fish were challenged with the inactive *P. aeruginosa* to stimulate the fish innate immunity and secrete the EM layer as reported by Thomas et al. (2014) with slight modification. The culture of *P. aeruginosa* (ATCC® 27853) was prepared in nutrient broth (HI media) and incubated at 37 ºC for 48 h. The concentration of the microbial was monitored by counting the number of colonies using a hemocytometer. When the concentration of *P. aeruginosa* reached 10^7 CFU/mL, the culture was deactivated using autoclave at 121 °C for 20 min at high pressure steam of 15 psi. The inactivated *P. aeruginosa* was added into three tanks having 3 fish, respectively. One tank was prepared as negative control. The mucus was collected on days 1, 7, 10 and 14. The experiments were done at 28 °C, pH range between 6 and 6.5, ammonia level of 0.01 ppm, nitrite level of 0.72 ppm, and nitrate level of 25.0 ppm.

MUCUS COLLECTION

The EM of the fish was collected as described by Ross et al. (2000) and Topic et al. (2012), with slight modifications. The selected fish were anesthetised using Tricaine methane-sulfonate (MS-222). The mucus was collected from the dorsal side using a cell scraper, then transferred to a 15 mL falcon tube containing 3 mL buffer solution containing 0.013 M Tris, 0.12 M NaCl, and 0.003 M KCl, pH 7.4. The collected mucus was centrifuged at 1,100 × g for 10 min at 4 °C. The mucus samples were stored at 80 °C until they were ready for use.

MUCUS EXTRACTION

The EM was centrifuged at 1,000 × g for 10 min at 4 °C to remove insoluble particles. Then, the collected supernatant was centrifuged at 2,000 × g for 10 min at 4 °C. The supernatant from this second centrifugatition was then collected and lypholised using freeze dryer (Labconco 74200-30). The lyophilised sample was suspended in 5 mL of distilled water, as reported by Ross et al. (2000). Then, the mucus samples were centrifuged at 2,500 × g for 5 min at 4 °C, and the supernatant was used for further studies.

MUCUS COMPONENT ANALYSIS

This study employed the Nutritional Labelling Analysis (Energy, Fat, Protein, Carbohydrate, Ash, and Moisture) AOAC Analytical Methods (Lee 1995) to analyse the components found on day 14. Untreated fish skin mucus was used to measure their compositions (carbohydrate, protein, fat, ash and energy). The experiment was done in Unipeq laboratory, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia.

ESTIMATION OF PROTEIN IN MUCUS

The total protein of EM was quantified using the Bradford (1976) technique and recommendations. Approximately 1.5 mL of Bradford reagent was added to the samples and kept at room temperature for 10 min. The absorbance was read at 595 nm using a UV-Vis

double beam Spectrophotometer model (Shimadzu 160 UV-Vis Inc. USA).

PROTEIN PROFILE ANALYSIS BY SDS-PAGE

The crude mucus protein profile was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Briefly, SDS-PAGE was employed by 12% resolving gel and 5% stacking gel.

ANTIMICROBIAL ACTIVITY

The antimicrobial activity of EM mucus extract obtained from *A. testudineus* fish was tested against human pathogens namely *P. aeruginosa*, *E. coli*, *B. subtilis,* and *B. cereus*. The selected microbes were spread on nutrient agar plates using cotton bud and incubated for 24 h at 37 °C. The antimicrobial activity test was adapted from Kourmouli et al. (2018) by adding 20 µL (1000 µg/mL) of mucus extract diluted in 10% methanol on a 6 mm blank antibiotic disc. Then, the disc was placed onto the nutrient agar plate of the bacterial culture and incubated at 37 °C for 24 h. A standard antibiotics disk (10 µg/mL streptomycin) was used as the positive control while a blank was repeated with the non-activated disc as negative controls. After the incubation, the inhibition zones that appeared around the discs were recorded. The inhibition zone was measured to the nearest millimetre (mm), and expressed as the means of three separate experiments.

The extracted mucus that showed antimicrobial activity was used to measure the minimum inhibitory concentration (MIC) needed to inhibit the growth of pathogens. The microdilution method was applied using nutrient broth on the MIC test, as described by Wei et al. (2010) with slight modification. To determine the MIC for inhibiting pathogen growth, EM was twice-diluted sequentially with 100 µL of the nutrient broth. The 50 µL of overnight growth pathogens were applied to each tube containing different concentrations of EM (1000, 500, 250, 125, 62.5 µg/mL) and incubated at room temperature for 18 to 24 h.

ANTICANCER ACTIVITY TEST CELL LINES

Cancer and normal cell lines were used in this study and purchased from the American Type Culture Collection Organization (ATCC). A human breast cancer cell (MCF7), human malignant melanoma (A375.S2), and normal human cell of skin from a newborn fibroblast (HS27) were used in this study to determine cytotoxicity. Dulbecco's Modified Eagle Medium (DMEM) was filled up with 10% FBS and 1% antibiotic mix (penicillin-streptomycin). The cells were kept at 37 °C in 5% CO_2 and 85 to 95% humidity.

CELL CYTOTOXICITY ASSAY

Cytotoxicity of EM was investigated using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described by Mohammed et al. (2018). When the confluence of cells reached 80%, the medium was discarded from the flask, and cells were washed with 1X Phosphate-buffered saline (PBS) three times. One millilitre of trypsin was used to detach the cells from the flask, and 5 mL of the medium was added into the flask and mixed well. Then, it was transferred into a new tube and centrifuged for 5 min at 2,500 × g. A hemocytometer was used to count the cells (1×10^5), and 100 µL concentration of cell suspension was seeded into each well of 96 well plates. After 24 h, 100 µL of EM was added into each well of both plates at concentrations 1 to 10 mg/mL and incubated for 24 and 48 h, respectively. The cell cytotoxicity was measured by adding 10 µL of MTT reagent into the cell suspension and left for 4 h. Finally, the content of the wells was discarded, and 200 µL DMSO (100%) was added to each well and left for 15 min. The absorbance was measured at 570 nm.

MORPHOLOGICAL EXAMINATION

The morphological examination was performed according to Mohammed et al. (2018), the human cancer cell line (MCF7 and A375.S2) and normal fibroblast were trypsinised, and the trypsinised cells were mixed with 5 mL of growth medium. Then 2 mL of the mixture was added to the sterilised tubes containing the coverslip. The tubes were kept in a slanting position and kept in a CO_2 incubator for 48 h. Then they were stained with Giemsa-stain and observed under a microscope and recorded.

DNA FRAGMENTATION ANALYSIS

DNA extraction and agarose gel electrophoresis were performed by the method described by Panno et al. (2006). Cancer and normal cells (3×10^5 cells/mL) were plated per well in 6 well plates with DMEM medium containing 10% FBS. 4.97 mg/mL of fish mucus protein samples were added to cancer cell plates. After 48 h of treatment, the DNA was extracted from the cell lysate. Then the cell lysate was washed with PBS followed by

the addition of 0.5 mL of lysis buffer. Next, the solution was transferred to a micro tube and stored for 1 h at 37 °C. Next, 4 µL of proteinase K was added and kept at 50 °C for 3 h. Finally, 0.5 mL of phenol: chloroform: isoamyl alcohol (25:24:1) was mixed and centrifuged at 15,000 × g for 10 min to separate the upper aqueous phase containing DNA. To precipitate the DNA, two volumes of absolute ice-cold ethanol and 1/10 volume of 3 M sodium acetate was incubated for 30 min on ice. DNA was pelleted by centrifuging at 16,000 × g for 10 min at 4 °C, and the pellet was washed with 1 mL of 70% ethanol. The pellet was left to dry at room temperature for approximately 30 min and suspended in 50 µL of TE buffer.

STATISTICAL ANALYSIS

This research utilised descriptive statistics, including standard deviation (SD), average and percentage to analyse mucus components of the extract and the analysis of the IC_{50} based on Swinney (2011). The bivariate analysis was used to find the correlation between control and mucus using a p-value <0.05. A Statistical Package for the Social Sciences programme (SPSS) version 23 and Microsoft Excel 2016 were the software utilised to analyse the data.

RESULTS AND DISCUSSION

The protein content of the epidermal mucus (EM) was estimated using the Bradford test. The results showed that the protein content of EM gradually increased during the treatment (Table 1). Interestingly, the protein content in EM produced by fish treated for 14 days increased by up to 33 times compared to control. There was no change in the protein content in EM for all fish treated as controls.

TABLE 1. Total protein concentrations of the EM extracted from the fish for 14 days

	Treated Fish		Control	
Challenge day	Total protein concentration (µg/mL ± SD)	The rounded ratio of total protein to control day	Total protein concentration (µg/mL ± SD)	The rounded ratio of total protein to control day
control	150.05 ± 0.12	-	150.05 ± 0.12	-
1	300.40 ± 0.23	1	142.01 ± 0.12	1
7	600.74 ± 0.16	4	151.05 ± 0.31	1
10	900.77 ± 0.32	6	154.09 ± 0.23	1
14	5000.23 ± 0.25	33	165.07 ± 0.43	1

The experiments were done in triplicate

These findings suggested that the pseudo-pathogenically challenged test successfully stimulated mucus secretion, which acts as a protective medium against pathogens. This result agreed with the previous findings reported by Wang et al. (2011) for the functions of EM, which is a good physical and chemical defence system to prevent pathogenic infection. The EM is also known as mucin, a viscous colloid containing antibacterial enzymes and proteins.

The composition of the EM after the 14 day treatment was analysed as presented in Figure 1. The results showed that the treated mucus contained approximately 8% (w/v) of protein, 0.4% (w/v) carbohydrate and 2.8% (w/v) fat. No significant change was recorded for carbohydrate and fat content compared to the control. These results supported the results of the Bradford which showed changes in protein content for the challenged fish.

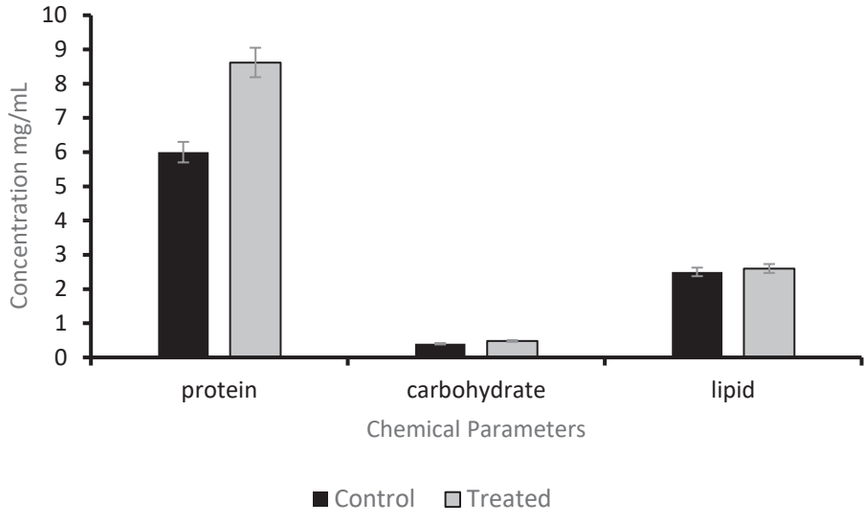

FIGURE 1. The biochemical constituent test (AOAC analytical methods, AOAC International 1995) of A. testudineus fish crude skin mucus. The control sample represented day 0, and the treated sample represented day 14 mucus

Furthermore, the SDS-PAGE protein separation of EM that had undergone 14 days of treatment was conducted and illustrated in Figure 2. The results showed that EM collected on day 14 had protein bands with a molecular weight ranging between 11 and 245 kDa. Although EM contained proteins with wide molecular weight distribution, a low molecular weight below 29.0 kDa was more prominent. This could possibly represented antimicrobial pepetides which have molecular weights in this range, as reported by Chong et al. (2005) and Shephard (1993) who studied the protein content in EM among Symphysodon fish (*Symphysodon discus*).

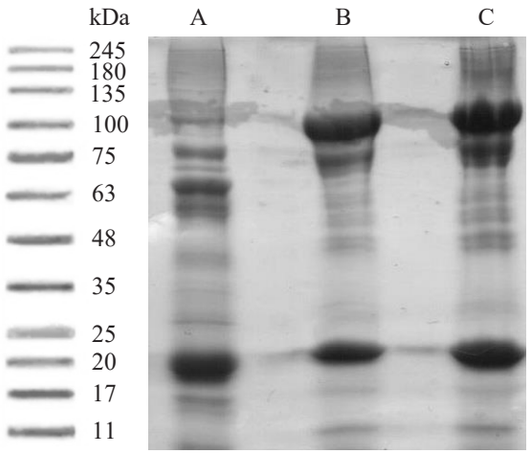

FIGURE 2. Protein profile of *A. testudineus* crude skin mucus by SDS-PAGE gel. All lanes (A, B, C) represented the replicates of skin mucus extract. The skin mucus was extracted three times from different fish in the same condition for reliability

Antimicrobial tests showed that the EM used had high antibacterial activity against *P. aeruginosa* (10 ± 0.23 mm) and *E. coli* (12 ± 0.43 mm) but low antibacterial activity against *B. subtilis* and *B. cereus* (2.4 ± 0.26 and 2.8 ± 0.12 mm, respectively) (Figure 3). More interestingly, EM also had a significant antibacterial activity compared to the streptomycin antibiotic used as control for *P. aeruginosa* and *E. coli*.

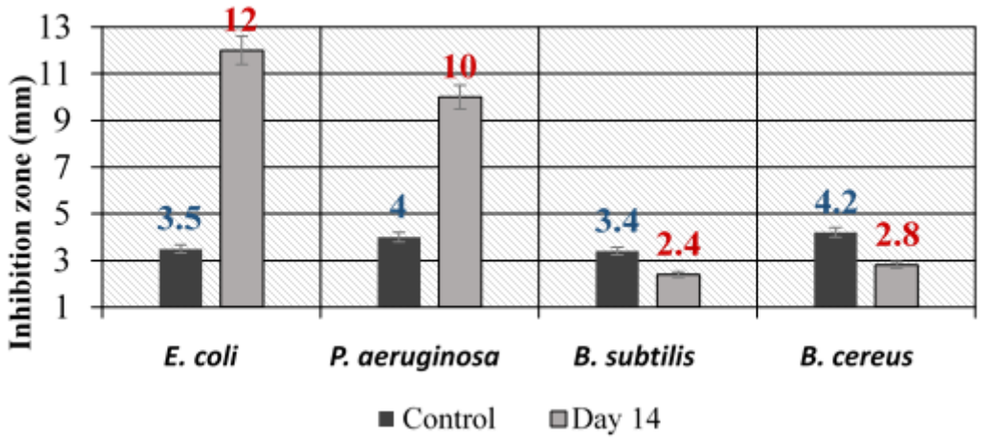

FIGURE 3. Antibacterial activity of EM and streptomycin against pathogenic bacteria. Streptomycin represented the control sample. The experiment was repeated in triplicate

The MIC test was performed to identify the lowest inhibitory concentration. It was found that the MIC to inhibit the growth of *P. aeruginosa* and *E. coli* was lower for EM (62.5 µg/mL) compared to streptomycin (125 µg/mL). The correlation analysis used a bivariate analysis at a confidence level of 95% which showed significant results ($p < 0.01$) between EM and control (streptomycin) of *E. coli* and *P. aeruginosa* pathogens. This result agreed with the result carried out by Wei et al. (2010) that reported snakehead fish (*C. striata*) has antibacterial activity against Gram negative bacteria, particularly *P. aeruginosa*. This may contribute to the ability of both species to survive in polluted water. Another study by Kumari et al. (2019) reported that the EM of *H. nobilis*, *C. idella,* and *C. carpio* have antibacterial activity against various bacterial species including *E. coli*, *P. aeruginosa*, *B. cerues*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis, S. aureus,* and *Aeromonas hydrophila*. The high antibacterial activity against *P. aeruginosa* and the low antibacterial activity against *B. cerues* and *B. subtilis* were likely due to the use of *P. aeruginosa* as the stimulant agent to stimulate the production of EM in this study since EM is produced as a result of reactions to specific bacterial species (Danneman & Michael 1976). Thus, the fish immunity system specifically produces EM based on the stimuli used that will result in different composition and structure of the antimicrobial peptides (AMPs) in the EM which, in turn, cause different responses of the pathogenic bacteria against them. AMP is a common microbicide that is specifically cytotoxic to microbes while displaying negligible cytotoxicity towards normal cells of the host.

In addition to antimicrobial properties against pathogenic bacteria, EM of *A. testudineus* also showed cytotoxicity effect against MCF7 and A375.2S cell lines (Figure 4). The MTT assay results demonstrated that the percentages of cancer cell viability gradually decreased as the EM concentration increased. The IC_{50} values of MCF7 and A375.2S cell lines treated with the EM after 48 h were 4.27 ± 0.15 and 4.97 ± 0.25 mg/mL, respectively. Interestingly, the EM did not show a cytotoxicity effect against the normal cell line (HS27) (Figure 4(C)). No changes were observed for HS27 cells up to 48 h. The effect of EM cytotoxicity on human breast cancer cells have also been reported for EM from round goby (*N. melanostomus*) and common carp (*C. carpio*) (Alijani Ardeshir et al. 2020). In addition to breast cancer cells, Faiz et al. (2015) and Mai et al. (2017) also found that the EM derived from *C. striata* fish has cytotoxic effects against colon cancer cells.

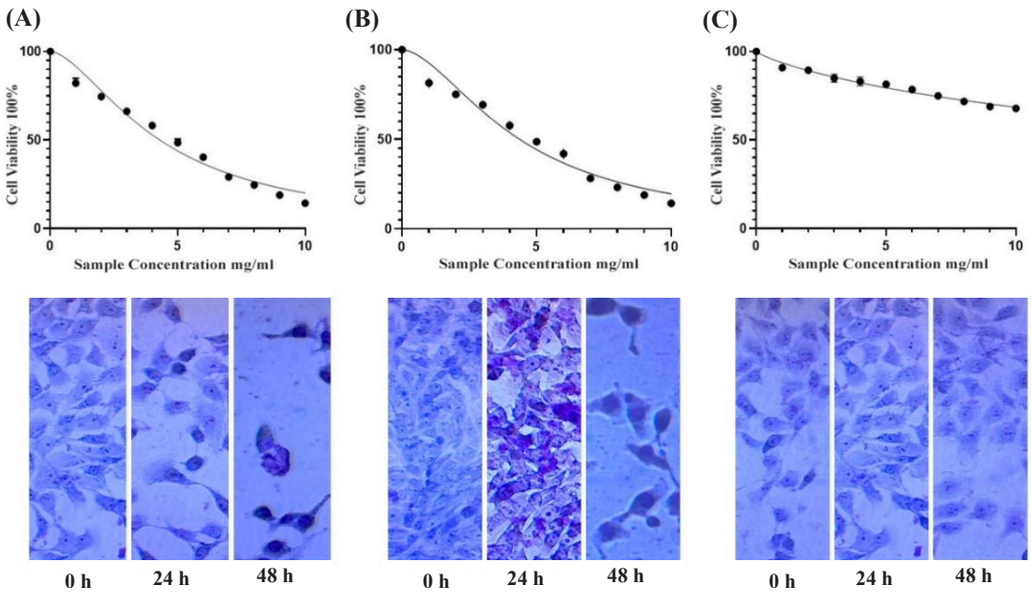

FIGURE 4. Cytotoxicity effect of *A. testudineus* crude skin mucus on A375.2S and MCF7 cancer cell lines and HS27 normal cell lines for 24 and 48 h. The graphs represented cell viability (%) by measuring the absorbance of optical density (590 nm). Data were the mean ± SD of triplicate determinations. (A) HS27 cell line, (B) MCF7 cell line, and (C) A375.2S cell line

From the agarose gel electrophoresis of DNA of untreated and treated cells, it was found that the DNA of all untreated cells was intact represented by a prominent single band on the gel. Compared to the treated cells, HS27 showed intact DNA and was described as a prominent single band. In contrast, treated MCF7 and A375.S2 cells showed degraded DNA in varying molecular weights. DNA damage in responsive mammalian cells is implicated as a critical event in the induction of apoptosis (Figure 5). The change in cell morphology, nucleosome DNA

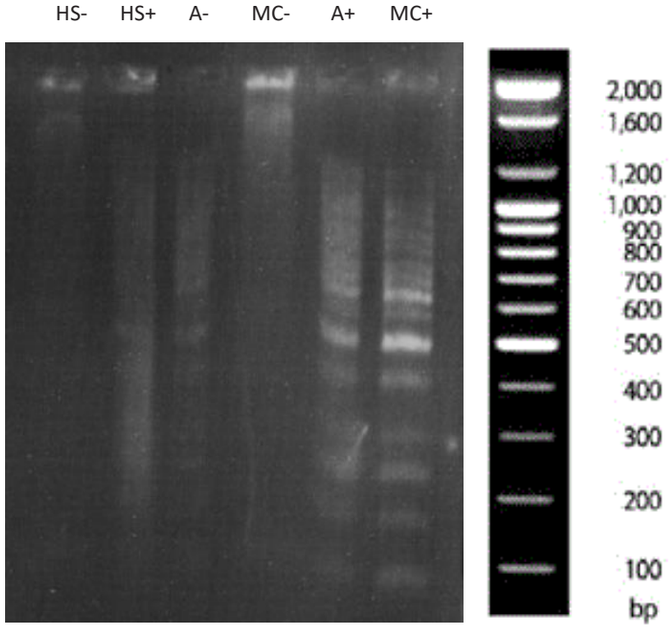

FIGURE 5. Cell apoptosis detection using the DNA fragmentation assay and 1.2% agarose gel electrophoresis. Values + and - represented treatment using E.M. and untreated cells, respectively, in normal skin HS27 (HS), MCF7 (MC) breast cancer and A375.2S (A) human melanoma cell lines

fragmentation and DNA laddering showed that EM could inhibit MCF7 and A375.S2 cell proliferation by apoptosis but not on normal cell HS27. The MTT test showed that EM could inhibit the proliferation of MCF7 and A375.2S cell lines but did not show an inhibitory effect on the HS27 cell line under similar experimental conditions. According to Walker et al. (1997), the effects of the underlying mechanism vary depending on the initiating stimulus, but a common feature is the activation of certain ectonucleotidases leading to DNA fragmentation (Barry & Eastman 1992; Wyllie et al. 1980)(b.

Conclusions

In conclusion, the production of EM from *A. testudineus* could be deliberately stimulated and increased by introducing stimulants in the form of inactive pathogens. The resultant antimicrobial properties of the EM were significantly influenced by the stimulant used. The EM contained proteins with various molecular weights in the range of 11 to 245 kDa. In addition to having strong antimicrobial properties against pathogenic bacterial, especially *E. coli* and *P. aeruginosa*, interestingly, the EM did not affect the growth of the fibroblast of a normal cell and only affected cancer cells. Thus, the EM has a promising anticancer agent that needs further study to be used in cancer treatment. Furthermore, it could inhibit the proliferation of cancer cells (MCF7 and melanoma) through apoptosis. This study provided sufficient data to highlight the potential of EM isolated from *A. testudineus* to be explored for further use. For additional application and to improve the current work, it is essential to fragment the protein and identify the specific peptides responsible for their functional properties.

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*Corresponding author; email: shazrul@ukm.edu.my