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The Antitumor Activity Exhibited by Anuran Tigerinin-1 Peptide Against HeLa Cells

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***Abstract*-- The present study investigated the antitumor activity of anuran tigerinin-1 peptide which was synthesized using the solid phase method 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry against HeLa cells. The cytotoxicity of tigerinin-1 peptide was examined, and the result showed that tigerinin-1 exhibits potential cytotoxic activity against HeLa cells. Inhibition of migration of HeLa cells were observed in tumor cell scratch assay. The apoptotic effect of tigerinin-1 on HeLa cells were determined by acridine orange/ethidium bromide (AO/EB) staining assay. The study demonstrated that tigerinin-1 peptide exhibits potential antitumor activity against HeLa cells.**

***Keywords*-- Antimicrobial peptides, Antitumor activity, Tigerinin-1, HeLa Cells**

I. Introduction

Amphibians are group of organisms which are forced to adapt and survive in a variety of environmental conditions. Due to this reason, they have evolved different morphological, physiological, and behavioural features. Their skin is a rich source of many biologically active compounds that are endowed with diverse physiological and *defence* functions (Demori et al., 2019). Among amphibians, the skin of anurans (frogs and toads) synthesize peptides that inhibits the growth of bacteria and fungi. They are synthesized and stored in granular glands in their skin, often in very high concentrations, and are rapidly released following a stress or tissue injury (Pantic et al., 2017). Recent researches showed that cationic antimicrobial peptides (AMPs) from anuran skin secretions can kill bacteria and also can affect cancer cell lines.

Antimicrobial peptides (AMPs) are peptides isolated from anuran skin which are composed of 10 to 46 amino acid residues. These peptides have a net positive charge, amphipathic α helix conformation, and high hydrophobic moment. These antimicrobial peptides can promote lysis of bacteria, fungi, and protozoa (Nascimento et al., 2003). Bombinin, tigerinin-1, Dermaseptin B1 etc., are some of the antimicrobial peptides obtained from the frog skin (Ladram et al., 2016). Some of the peptides like Analgesin-HJ from tree frog *Hyla japonia* show analgesic property (Zhu et al., 2014). Some of the AMPs also exhibit wound healing properties.

Tumor cells differ from non-neoplastic cells by means of altered cell membrane composition. These altered membranes form a target for antimicrobial peptides, which also have additional tumoricidal properties, thus termed as anticancer peptides (ACPs) (Oelkrug, 2015). Electrostatic interaction between cationic ACPs and anionic cell membrane components form a significant factor in the selective killing of cancer cells by ACPs. Cancer cell membrane carries a net negative charge and the cells have higher membrane fluidity as compared to untransformed cells. This enhances the lytic activity of ACPs by causing membrane destabilization. In addition, cancer cells have a greater surface area than normal cells due to higher number of microvilli. These microvilli will allow cancer cells to bind with an increased number of ACP molecules (Hoskin and Ramamoorthy, 2008).

Tigerinins are antimicrobial peptides isolated from *Hoplobatrachus tigerinus* (Dicroglossidae). They are found in Myanmar, Bangladesh, India, Pakistan, Afghanistan, and Nepal (Padhye et al., 2008). It is a schedule-IV frog according to the wildlife (Protection) act, 1972, and is classified as Least Concern (LC) by the IUCN. Tigerinins are short, non-helical antimicrobial peptides with a single di-sulphide bridge. Tigerinins are a unique family of 11 to 12 residue peptides and are characterized by the presence of a stretch of predominantly hydrophobic amino acids with one cationic residue and an amidated C terminus. They also contain two cysteine residues linked by a di-sulphide bond to form a loop of nine amino acid residues. The most active member of this family is tigerinin-1 (TGN-1).

II. Methodology

Cell culture

Human cervical cancer cells (HeLa Cells) were obtained from the Indian Institute of Science (IISc.), Bangalore, India and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin at 37 °C in a controlled humidified environment with 5% CO2.

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MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide solution (MTT) assay.

The HeLa cells were seeded at a density of 104 in 96 well plates in complete medium and is kept for 24-hour incubation at 37˚C in 5% CO2. The cells were then treated with 10 µl of Tigerinin-1 (10-100 µM). Then, 10µl of 5 mg/ml MTT solution reagent of different concentrations of various fractions were added to the well and is incubated in the dark for 4 h. Finally, 100µl of DMSO (100%) was added to each well to get purple blue MTT formazan precipitate. The absorbance was determined using a microplate reader (EnSpire™ Multimode Plate Reader, PerkinElmer, Inc.) at 570 nm and 630nm. In this study, PBS and DMSO were used as negative and positive control, respectively. The experiments were conducted in triplicate and repeated twice. The IC50 value was calculated.

Trypan Blue cell counting assay

HeLa cells were seeded in a cell culture medium at the density of 1x105 cells/well in cell culture plate with and without different concentrations of Tigerinin-1 peptide (2.5, 5, 10µM) for 24 h. The cells were then mixed with trypan blue solution (1:1 ratio) and counted using a hemocytometer under a microscope. Cells with intact membrane were unstained, and cells with disrupted membrane were stained in blue colour. The experiments were conducted in triplicate and repeated twice. Relative cell number percentage of viable cells were calculated by dividing the number of viable cells by the number of total cells and multiplying by 100 or

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100

Cell scratch assay

HeLa cells were grown up to 90 to 95% confluency in a six-well plate. Then the cell monolayer was wounded with a sterile 200 µl pipette tip and washed with a cell growth medium to remove the detached cells. The cells were cultured in a serum-free medium and treated with or without different concentrations of tigerinin-1(2.5,5,10µM) for 24h. An inverted microscope connected to Magnus industrial digital camera was used to take pictures of the monolayer at 0 hours and 24 hours.

The % wound closure indicates the cell migration. The images were analysed using the imagej software. The experiment was conducted in triplicate and repeated twice.

Wound closure % = [At-oh-At-Δh/ At-oh]*100

At-oh is the area of the wound measured immediately after scratching (t=oh)

At-Δh is the area of the wound measured h hours after the scratch is performed (h=24)

Acridine Orange/Ethidium Bromide (AO/Eb) Fluorescence Staining

Hela cells (5x105 cells/ml) were treated in the presence and absence of tigerinin-1(2.5, 5, 10µM) and were cultured in a humidified incubator with 5% **CO2** at 37°C for 24h. The cells were washed with PBS and stained with a solution of 100µg/ml acridine orange and100µg/ml ethidium bromide in PBS mixed in a ratio of 1:1. The cells were observed under a fluorescent microscope (Evos thermo image station) at 20X using excitation wave length at 488nm and an emission wavelength at 540nm. The experiment was conducted in triplicate and repeated twice.

Statistical Analysis

Experimental data were represented as mean ± SEM. Significant differences were obtained by one-way analysis of variance (ANOVA) and followed by Tukey HSD post hoc test using SPSS (version 16.0) (SPSS Inc., Chicago, IL, USA).

III. Results

Cell viability

To understand the effect of tigerinin-1 peptide on the viability of HeLa cells, MTT assay was conducted. The tetrazolium salt (MTT) reacts with mitochondrial dehydrogenase enzyme to form insoluble formazon crystals in viable cells. Fig. 1 shows that the Tigernin-1 inhibits cell proliferation in HeLa cells as the concentration of peptide increases (10-100µM). The IC50 average value of Tigerinin-1 against HeLa cell is 24.67 µM. 2.5, 5, and 10 µM of sub-lethal concentrations of tigerinin-1 were selected for further experiments.

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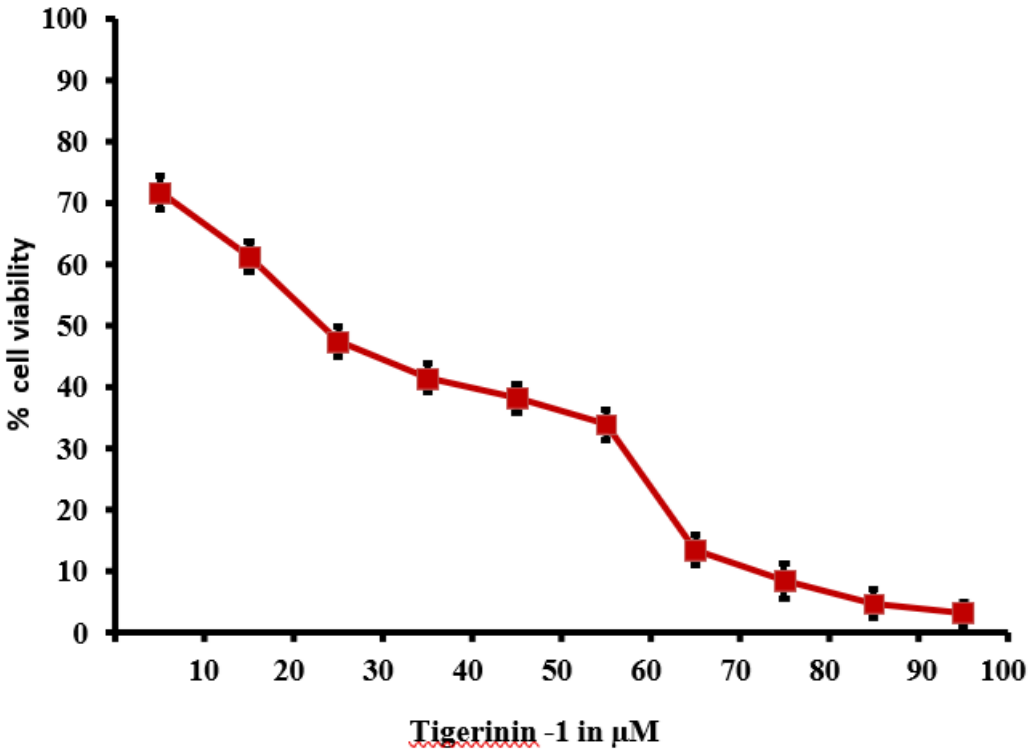

Fig.1 Cell viability (%) observed in HeLa cells treated with different concentration of tigerinin-1

Trypan blue cell counting assay

Trypan blue cell counting assay shows a significant variation in the number of viable HeLa cells after treating with Tigerinin-1 peptide for 24 h (Fig. 2). The viable cells counted were 94.35 ± 3.08, 87.845 ± 3.03, 81.9 ± 3.06 in 2.5, 5, 10 µM respectively.

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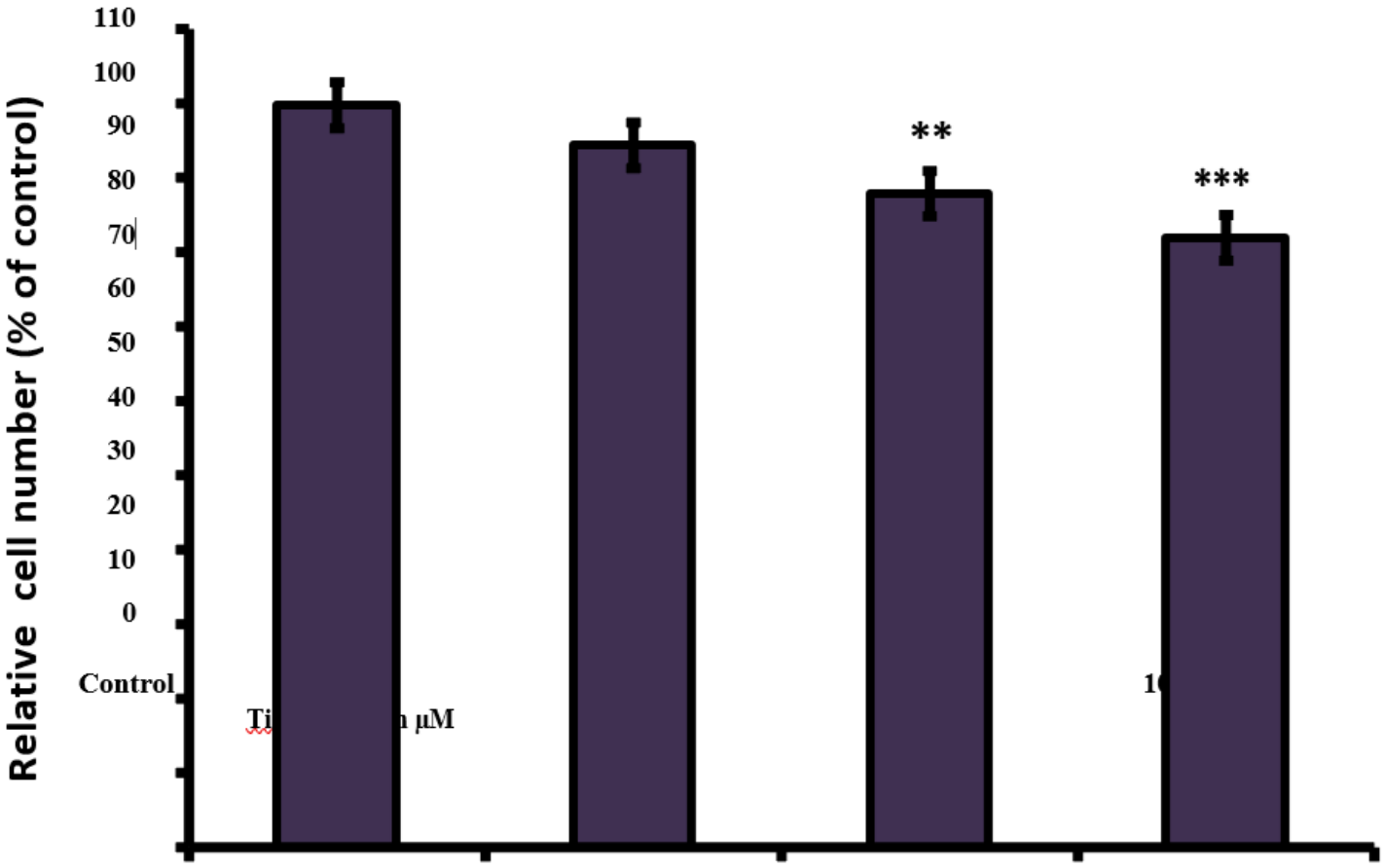

Fig.2 Trypan blue exclusion cell counting shows that Tigerinin-1 inhibits cell proliferation of HeLa cells.

Values are mean ± SEM; the data is analyzed by analysis of variance (ANOVA) followed by the Tukey HSD test's multiple comparisons. Values are significant compared to control at **p<0.01; ***p<0.001 level.

Effect of Tigerinin-1 peptide in the migration of HeLa cells

A scratch was made in HeLa cell monolayer and treated with/without different concentrations of Tigerinin-1 (2.5, 5, 10µM) for 24 hours.

Significantly reduced cell migration was observed in HeLa cell monolayers with increasing concentration of Tigerinin-1 as compared to the control (Fig. 2). Thus, with increasing concentration of Tigerinin-1, the cell loses its ability to migrate and close the scratch-made in the monolayer of the cell.

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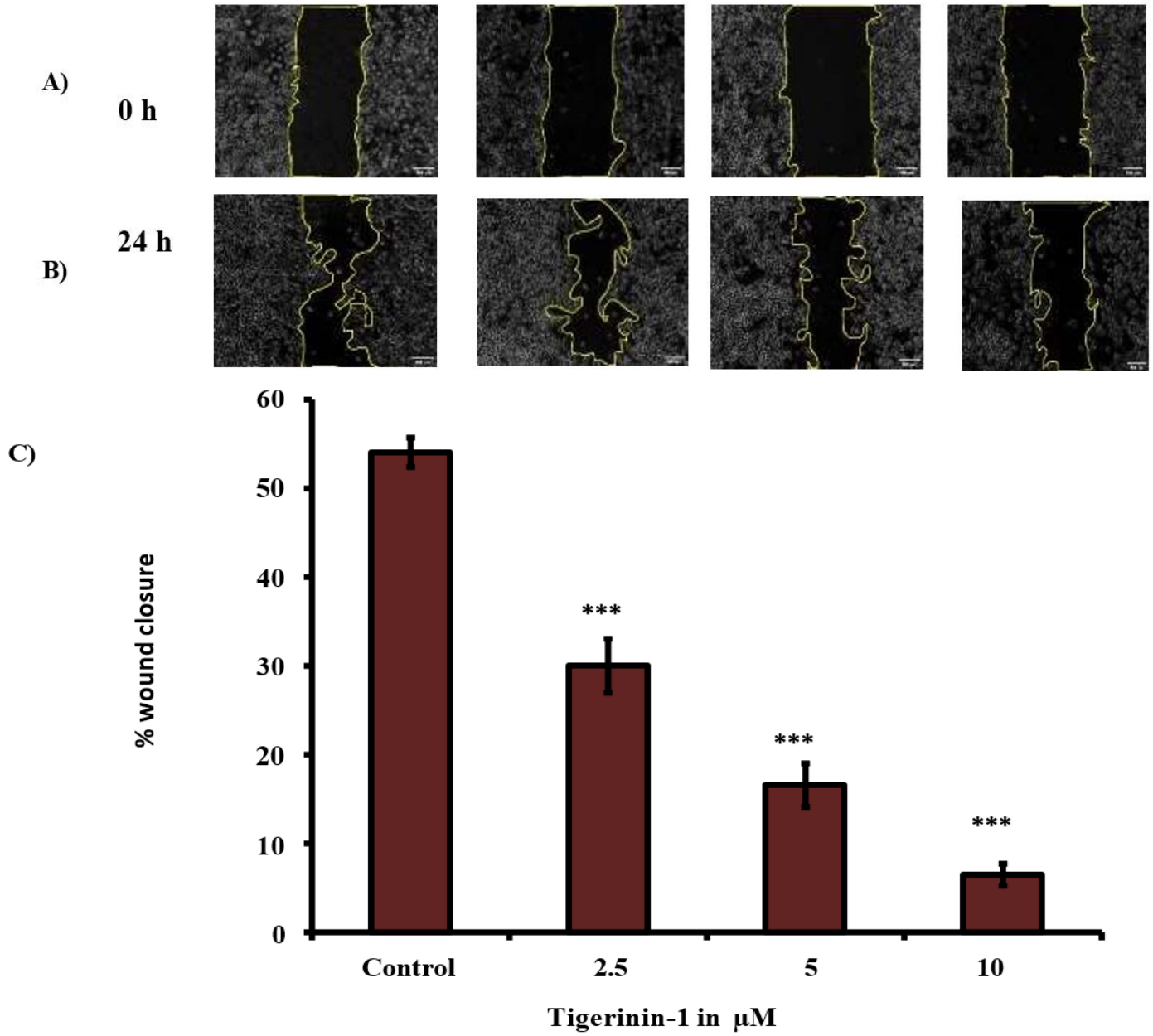

Fig.3 Effect of Tigerinin-1 on HeLa cells migration (A) HeLa cells treated with PBS and different concentrations of Tigernin-1 at 0h. (B) HeLa cells were treated with PBS and different concentrations of Tigernin-1 at 24h. (C) % of wound closure in respective experimental groups. Values are mean ± SEM; the data is analyzed by analysis of variance (ANOVA) followed by the Tukey HSD test's multiple comparisons. Values are significant compared to control at ****p<0.0001 level.

Apoptotic activity of Tigerinin-1 in HeLa cells

Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis. AO will stain both live and dead cells, but EB will stain only cells that lost its membrane integrity.

Each dye that is taken up by a cell fluoresces- AO makes a cell green, and EB makes a cell red. Fluorescence microscopic observation revealed that non-apoptotic green cells were found in control groups and red apoptotic cells in the tigernin-1(2.5, 5, 10 µM) treated group.

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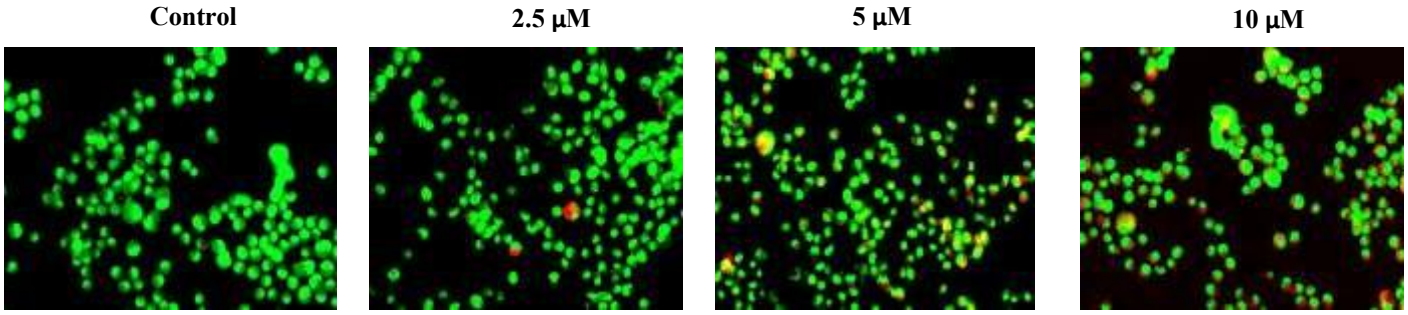

Fig. 4 Fluorescence photomicrograph of HeLa cells stained with acridine orange and ethidium bromide.

IV. Discussion And Conclusion

The reduced cell viability observed in Tigerinin-1 peptide treated HeLa cells exhibits its cancer cells killing nature. Electrostatic interaction between cationic ACPs and the anionic cell membrane is believed to be the reason for the selective killing of cancer cells by ACPs. The membrane fluidity of cancer cells is higher as compared to untransformed cells. This enhances the lytic activity of ACPs by causing membrane destabilization (Hoskin and Ramamoorthy, 2008).

Trypan blue cell counting assay helps to quantify live cells by labelling dead cells exclusively. This method is based on the principle that living cells possess intact cell membranes that exclude certain dyes such as trypan blue, whereas dead cells do not (Strober et al., 1997). When HeLa cells were treated with tigerinin-1 peptide for 24 h, a significant decrease in the number of live cells was noted, which implies that tigerinin-1 can reduce the viability of tumor cells.

The HeLa cell monolayer treated with Tigerinin-1 peptide showed a reduced rate of scratch healing (wound closure). Tumor cell scratch assay is carried out to determine the effectiveness of compounds against the healing of the scratch gap (Chen et al., 2012). The results clearly indicates that the Tigerinin-1 peptide treatment prevents cancer cells migration. Apoptotic activity Dual AO/EB fluorescent stained HeLa cells in the Tigerinin-1 treated groups evidences tigerinin-1 peptide induces apoptosis.

Overall the present study demonstrated that the anuran tigerinin-1 peptide exhibits potential antitumor activity against HeLa cells.

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