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Molecular Docking of SA11, RF13 and DI14 Peptides from Vacuolar Protein Sorting Associated Protein 26B Against Cancer Proteins and *In vitro* Investigation of its Anticancer Potency in Hep-2 Cells

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Abstract

This study demonstrates the anticancer potential of RF13 peptide derived from vacuolar protein sorting associated protein 26B (VSP26B), which was obtained from transcriptome data of freshwater teleost, striped murrel (*Channa striatus*). In total, 28 different peptide segments were randomly predicted from VSP26B and their anticancer property was predicted through mACPpred algorithm. Based on the scoring value, out of 28, 3 peptides namely SA11, RF13 and DI14 were shortlisted for the primary physicochemical property screening. Also, the shortlisted peptides were validated for anticancer protein receptors using molecular docking. Based on the overall bioinformatics analysis, RF13 (^{1}RRGKGGRRVTMSF^{13}) peptide was selected to further investigate the anticancer potential at the molecular biology lab. *In vitro* experiment showed that RF13 inhibit the proliferation of human laryngeal epithelial cell (HEp-2). Further, microscopic observations confirmed the influence of peptides on the cell morphology that showed apoptosis due to Hoechst 33342 staining, which altogether demonstrated the anticancer effect of the examined peptide, RF13. The molecular mechanism of the peptide's anticancer property was analyzed over cell cycle analysis. It showed that the peptide arrested the SubG1 and G1 phase of Hep-2 cells in a dose-dependent manner. The qPCR assay exhibited the apoptotic genes, including caspase-3 and caspase-9 expression significantly ($p < 0.05$) in an unregulated manner. Based on the obtained results, we propose that RF13 is potential enough as an anticancer agent; however, further direction needs to be focused on the possible specific target mechanism and its therapeutic approach towards anticancer drug development.

Keywords Molecular docking · Vacuolar protein · Anticancer peptide · Apoptosis · Gene expression

Abbreviations

Hep-2	Human laryngeal epithelial cell
VSP26B	Vacuolar protein sorting-associated protein 26B
CSC	Cargo-selective complex
WHO	World Health Organization
FACS	Fluorescence-activated cell sorting
NCCS	National centre for cell science
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffer saline
DMSO	Dimethyl sulfoxide
CTAB	Cetyltrimethylammonium bromide
ACP	Anti-cancer peptide

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Introduction

Freshwater air-breathing teleost snakehead murrel, *Channa striatus* is an eminent source of protein and medicinal properties, thus possessed to have a significant demand in the commercial market. The front-line defense system of *C.*

striatus can synthesis a mucus layer that has more excellent resistance against various bacterial pathogens and protects fish by inducing the innate and adaptive immune system (Kumaresan et al. 2016). A transcriptomic study (Kumaresan et al. 2016) explored the innate and adaptive immune response of *C. striatus* during fungal, *Aphanomyces invadans* infection. This transcriptome data reveals the list of immune molecules and their significant functions against pathogenic infection. This teleost has numerous molecules in its innate immune systems such as antibacterial peptides, lectins, pentraxins, lysozymes, complement proteins, IgM, protease inhibitors, cytokines, chemokines, and caspases. The functional mechanism of these innate immune systems was described in detail by Palanisamy et al. (2018).

Vacuolar protein sorting-associated protein 26B (VSP26B) is the retromer cargo-selective complex (CSC) identified from *C. striatus* transcriptome. The CSC is involved in the lysosomal degradation pathway (Bugarcic et al. 2015). Raymond et al. (1992) reported that forty-one mutant variants protein involved in vacuolar protein sorting. VPS26B is found throughout the cytoplasm and is particularly abundant in actin polymerization-rich plasma membrane regions (Kerr et al. 2005). In exosomes mediated cancer progression and anticancer therapy, vacuolar protein sorting associated protein 4 and ALIX aid in vesicle budding protein and act as accessory proteins ESCRT-Dependent Sorting Pathway (Sinha et al. 2021). With this knowledge, we hypothesize that the peptide derived from VSP26B may involve in inhibiting the proliferation of cancer cells which similar to vacuolar protein sorting associated protein 4. Few reports were available on VSP26B; also, our recent findings reported that the RF13 peptide has antioxidant potency that reduces oxidative stress in HFD induced obesity in zebrafish larvae (Guru et al. 2022; Prabha et al. 2020) reported that peptides (IE13 and IW13) derived from serine-threonine protein kinase protein of *C. striatus* also showed potential anticancer activity.

According to the 2019 World Health Organization report, cancer is a significant cause of mortality before the age of 70 in 183 nations and a substantial obstruction to improve the survival rate in every country (Bray et al. 2021; Sung et al. 2021). The UK Cancer Research center report that the cancer rate will rise to 62% before 2040 (Mikaelian et al. 2020). Consequently, the effective treatment in cancer therapy remains limited (Dutta et al. 2016). There is a tremendous demand for drugs with no side effects or non-toxic potential anticancer drugs. The present scenario of peptide-based drug development grabbed the researcher's attention to overcome the restrictions of conventional medicine and chemotherapeutic agents (Hernández-Ledesma et al. 2009). Based on the amino acid composition or physicochemical properties of the peptides, it has a unique feature to target specificity and host tolerance (Ravichandran et al. 2017). The anticancer peptide (ACP) was specifically targeting the cancer cells through the electrostatic interactions with plasma membrane receptors of the cancer cells (Mader and Hoskin 2006). ACP specificity is improved due to the variation in the structural components and composition of normal and cancerous cells (Baxter et al. 2017).

This study aims to identify a potential ACP from VSP26B of *C. striatus.* Based on the molecular docking study and bioinformatics analysis of peptides, we hypothesize that the peptide derived from VSP26B possess anticancer activity. The antiproliferative property of ACP was detected through MTT and LDH assays. The flow cytometry (FACS) was also performed to detect the inhibition of cell cycle phases and it was validated through gene expression profile, which showed the anticancer potential of the peptide.

Materials and Methods

Bio-functional Prediction of the Peptide

The VSP26B protein was identified from the transcriptomic data set, which demonstrates the innate and adaptive immune response of *C. striatus* during *Aphanomyces invadans* (pathogenic fungal) infection (Kumaresan et al. 2016). From the VSPB26 protein sequence, the possible peptide fragments were randomly predicted using the HeliQuest online tool (http://heliquest.ipmc.cnrs. fr/cgi-bin/ComputParams.py) (Sannasimuthu et al. 2019). The anticancer potential of predicted peptides was screened using mACPpred algorithm, a user-friendly web server (http://www.thegleelab.org/mACPpred/ACPExample.html) (Boopathi et al. 2019). Based on the scoring value obtained from mACPpred algorithm, three peptides were predicted such as SA11 (^{1}SGSGCGSANTA^{11}), RF13 (^{1}RRGKGGRRVTMSF^{13}) and DI14 (^{1}DVIVGKIYFLLVRI^{14}) that have potential anticancer efficiency. The physicochemical properties such as molecular weight, net charge, hydrophobicity and theoretical pI were analyzed as described previously (Prabha et al. 2020).

The anticancer potential of the three predicted peptides was validated with the molecular docking study. The PDP structure of the receptors was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The predicted peptides SA11, RF13 and DI14 are docked in HPEPDOCK webserver (http://huanglab.phys.hust.edu.cn/ hpepdock/)(Zhou et al. 2018) with the following proteins Bcl-2 (PDB ID: 2XA0), caspase-3 (PDB ID: 1RHM), caspase-7 (PDB ID: 3IBF), caspase-9 (PDB ID: 3IBF), XIAP (PDB ID: 1g3f) and p53 (PDB ID 2J1X) (Nguyen and Nguyen 2016; Chen et al. 2019; Zarei et al. 2020). The docking results were visualized in the discovery studio software.

Based on the results obtained from the bioinformatic analysis and the molecular docking results, RF13 peptide

was further selected to investigate the anticancer potential. The peptide was synthesized at Zhengzhou Peptides Pharmaceutical Technology Co. Ltd., China, and its purity (> 95%) has been confirmed in HPLC coupled with mass spectrometry by the supplier. RF13 peptide stock concentration, 1 Mm was prepared by dissolving the peptide in phosphate buffer saline (PBS), and the stock solution was stored at − 20 °C for further experimental use (Velayutham et al. 2021).

Cell Culture

Human laryngeal epithelial (Hep-2) cells were obtained from National Centre for Cell Science (NCCS), Pune. Cells are cultured in DMEM medium with high glucose (4.5 g/L), supplemented with 10% FBS, and an antibiotic-antimycotic solution (10,000 U/L of penicillin/streptomycin). The cells were maintained in a 5% CO_2 incubator at 37 °C.

Cytotoxicity Assay

MTT assay was performed as described in an earlier study (Prabha et al. 2020; Velayutham et al. 2022). The cells are seeded in 96 well plates and incubated in CO_2 incubator with 5% CO_2 supply at 37 °C. After obtaining the desired confluence, RF13 was treated with the following concentration: 6.25µM, 12.5µM, 25 µM, 50 µM and 100 µM. After treatment, 20 µL of MTT solution (5 mg/mL in sterile PBS) was added and kept for incubation (4 h). A 200 µL of DMSO (0.01%) was added to dissolve the formazan crystals, and the absorbance was taken at 570 nm using a microplate reader. The experiment was performed in two different treatment time points, 24 h, and 48 h. The anticancer activity was determined by calculating the IC50 value (the concentration caused 50% cell death) from the data obtained from the inhibition rate using GraphPad Prism software.

Trypan Blue Exclusion Assay

The anticancer effect of the peptide, RF13 was further verified with trypan blue exclusion assay as described previously (Chiu et al. 2016). In brief, cells are seeded in 12 well plates after obtaining the desired confluence; the cells are treated with RF13 at five different concentrations (6.25µM, 12.5µM, 25 µM, 50 µM, and 100 µM). After 24 h treatment, the cells were trypsinized and stained with trypan blue solution; then, the cells were counted under a microscope using a hemacytometer.

Cytomorphological Analysis

The cytomorphological changes of Hep-2 cells treated with RF13 peptide were determined over the microscopic examination, as previously explained (Prabha et al. 2020). The Hep-2 cells were seeded in a 6-well plate to obtain the desired concentration. Then, the cells were treated with RF13 peptide with following concentration: 6.25 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM for 24 h. After treatment, the photomicrographs were taken using an inverted microscope at 20X magnification.

Hoechst 33342 Staining

The potency of RF13 peptide inducing apoptotic cell death was investigated through Hoechst 33342 staining assay (Agarwal et al. 2018). In brief, HEp-2 cells were seeded in a six-well plate along with the coverslip and allowed the cells to attach to the coverslip. The cells were then treated with RF13 peptide at various dosages (6.25 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM) for 24 h. The cells were washed with PBS and stained with Hoechst 33342 (10 mg/mL) for 10 min and incubated in the dark. The apoptotic body formation was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

DNA Fragmentation Assay

The effect of RF13 peptide may cause any damage to the nuclear material was detected by DNA fragmentation assay (Dasiram et al. 2017). The Hep-2 cells were seeded in a 6-well plate and treated with RF13 peptide (25 µM and 50 µM) for 24 h. The untreated cells were considered as the control. At the end of the treatment period, the treated or untreated cells undergone genomic DNA isolation as prescribed by the CTAB method. The DNA samples were loaded in the 1.2% agarose gel stained with ethidium bromide (10 µg/mL), and allowed to run in an electrophoresis chamber. The gel was visualized under a UV gel documentation system.

Cell Cycle Analysis

The changes in the cell cycle phase were detected by cell cycle analysis as described earlier (Prabha et al. 2020). The Hep-2 cells (1×10^5) were seeded in a six-well plate and incubated in CO_2 incubator at 37 °C with 5% CO_2 supply, which allowed the cells to reach the desired confluency. Further, the cells were treated with 25 µM and 50 µM concentrations of RF13 peptide for 24 h; and untreated cells were considered control. The cells were then trypsinized and fixed in ice-cold ethanol overnight at − 20 °C. Then, the cells were washed in PBS, stained with propidium iodide (1 mg/ml) solution, and incubated in the dark for 1 h. The stained cells were run over a flow cytometer, and the obtained data were collected and analysed in Cell Quest Software (Becton Dickinson, USA).

Gene Expression Analysis

The Hep-2 cells were grown in a 6-well plate and treated 24 h with 25 µM and 50 µM of RF13 peptide; the untreated cells served as control. The established TRIZOL method was used to isolate RNA; the isolated total RNA was converted into cDNA, which was used as a template for the gene expression study. For the real-time PCR experiment, the following primers were adopted: Caspase-3: Forward, 5′-CAG AAC TGG ACT GTG GCA TTG-3′ and Reverse, 5′-GCT TGT CGG CAT ACT GTT TCA-3′; Caspase-9: Forward, 5′-CCA GAG ATT GCG AAA CCA GAG G-3′ and Reverse, 5′- GAG CAC CGA CAT CAC CAA ATT C-3′ and β-actin (internal control) Forward, 5′-TGC CGA CAG GAT GCA GAA G-3′ and Reverse, 5′-GCC GAT CCA CAC GGA GTA CT-3′ (Pilco-Ferreto and Calaf 2016). The KAPA SYBR Quick qPCR Master Mix was used for RT-PCR analysis and another reaction buffer. The cycle was run on a standardized thermal profile. The experimental values were normalized with the internal control (β-actin). Then, the relative fold change was calculated using $2^{-\Delta\Delta ct}$ method and obtained ΔΔct value.

Statistical Analysis

The data presented in this study is an average of three replicates ± standard deviation. The statistical significance of all the experiments conducted in this study was determined using one-way ANOVA and Tukey's Multiple Range Test in Graph Pad Prism (Ver.5.0).

Results and Discussion

Insilco Investigation of ACP from VSP26B

The VSP26B was identified from *C. striatus* transcriptome dataset, and the molecular weight of the protein was 43443.31 g/mol. The protein possessed to have a total number of 382 amino acid residues. The protein has both positive and negative charged residues, and their isoelectric point is 7.21, the aliphatic index is 79.35, and the instability index is 43.40. We identified 28 different peptide fragments from VSP26B through Machine-Based Meta-Predictor results. The results revealed the anticancer potency of peptides; based on the probability score values, three peptides were predicted as ACP (E-Suppl. Table 1) along with their physicochemical properties (Table 1). The hydrophobic nature of the predicted peptides is presented in the helical wheel structure (Fig. 1). SA11 peptide has 3 glycine residues, RF13 peptide has 3 glycines, 4 arginines, 1 methionine, and 1 phenylalanine amino acid residue, and DI14 peptide has 1 glycine, 1 arginine, 1 lysine, and 1 aspartic acid residue. Chiangjong et al. described the detail of various amino acid roles against cancer cells (Chiangjong et al. 2020). Accordingly, the ACP (SA11, RF13, and DI14) obtained from the screening of VSP26B protein possessed to have such amino acid residues, thus indicating the potential of anticancer nature in peptides such as SA11, RF13 and DI14 of VSP26B protein.

The ACP acts against the cancer cells by inducing apoptosis or necrosis through the electrostatic interactions along with Hydrophobicity and amphipathic features of the peptide, which are playing vital roles (Ting et al. 2014). The SA11 peptide showed hydrophobicity 0.154, hydrophobic

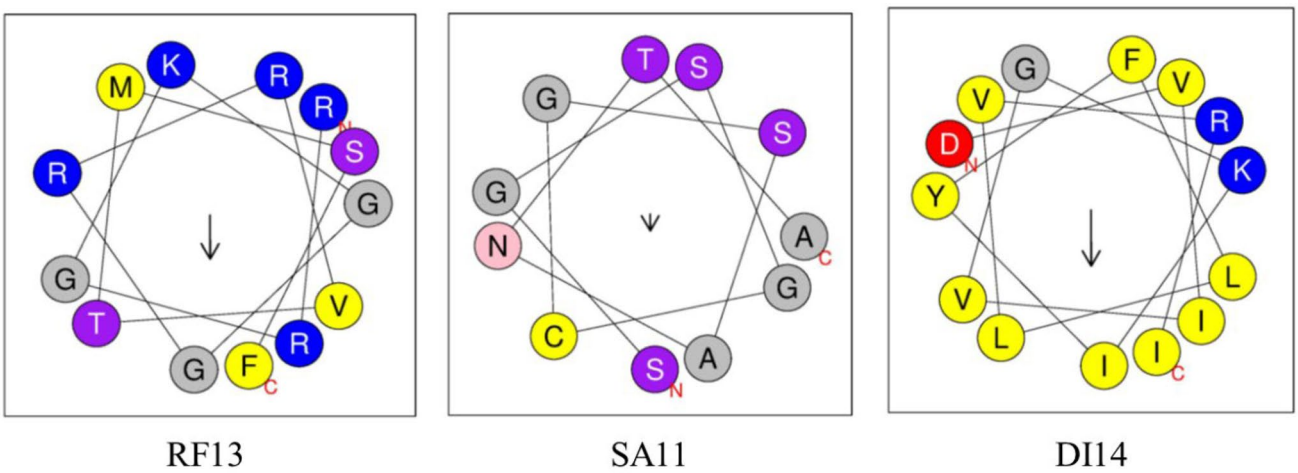

Fig. 1 The helical wheel structure represents thehydrophobic nature of the anticancer peptides, RF13, SA 11 and DI14 predictedfrom vacuolar protein sorting associated protein 26B(VSP26B)

Table 1 Physicochemical properties of screened peptide fragments from VSP26B protein selected based on the anticancer potential and its probability score

Characters	SA11	RF13	DI14
Peptide sequence	SGSGCGSANTA	RRGKGGRRVTMSF	DVIVGKIYFLLVRI
Molecular weight	0.911 kDa	1.508 kDa	1.648 kDa
Net charge	0	5	1
Hydrophobicity	0.155	− 0.044	0.889
Charged residues	–	LYS 1, ARG 4	LYS 1, ARG 1, ASP 1
Uncharged residues	SER 3, THR 1, ASN 1, GLY 3	SER 1, THR 1, GLY 3	GLY 1
Special residues	CYS 1	–	–
Theoretical pI	5.24	12.48	8.59
Probability score	0.7160	0.9195	0.0197

movement 0.107, net charge 0, frequency polar 0.727, frequency non-polar 0.273, and valency angle 6.252. The RF13 peptide showed hydrophobicity − 0.043, hydrophobic movement 0.28, net charge 5, frequency polar 0.769, frequency non-polar 0.231, and valency angle 3.940. Also, the DI14 peptide showed hydrophobicity 0.888, hydrophobic movement 0.356, net charge 1, frequency polar 0.286, frequency non-polar 0.714, and valency angle 2.01. The hydrophobic nature of the ACP plays a crucial role in its anticancer activity. The net charge of the peptides ranging between + 2 and + 5 is possessed to have potential anticancer activity because those peptides effectively interact with lipid membranes of cancer cells (Felício et al. 2017). Considering the fact RF13 has a net charge of + 5, which is an added advantage to the peptide. A similar study was also reported by Prabha et al. that a peptide named IE13 and IW13 identified from serine-threonine protein kinase protein of *C. striatus* showed potential anticancer activity (Prabha et al. 2020).

ACP Docking Efficiency Against Cancer-Targeting Protein

The ACPs, namely SA11, RF13, and DI14 of VSP26B protein, showed a significant binding affinity with the receptors; the binding score was given in Table 2. The interaction between the peptide and the receptor molecules is high, as shown in Fig. 2. The amino acid position of peptides and receptors was listed in the E-Suppl. Table 2. The HPEPDOCK method was the most advanced blind global docking algorithm that gave a highly accurate prediction and success rate. Compared with another docking algorithm, HPEPDOCK required lower simulation time (Zhou et al. 2018; Lee et al. 2019). The SA11, RF13 and DI14 peptides showed significant interactions with apoptotic proteins. A similar study reported that a peptide from *Glycine max* (black soybean) showed substantial anticancer activity (Chen et al. 2019); also, a bacteriocin from the human gut microbiome function against p53, tumor suppressor protein (Nguyen and Nguyen 2016). From the obtained results of binding affinity scores and the amino acid interactions positions, we know that the predicted ACPs SA11, RF13, and DI14 of VSP26B protein have anticancer potential. However, based on the results obtained from molecular docking, further biological assays were performed only in the RF13 peptide.

Cytotoxic Effect of RF13 Against Cancer Cells

The inhibiting effect of RF13 against the proliferation of HEp-2 cells was determined by MTT assay. The peptide showed a dose-dependent decrease in the cell viability in both 24 and 48 h treatment periods (Fig. 3). The IC50 value of the RF13 peptide was 49.65 µM at 24 h and 50.9 µM at 48 h treatment. The overall results of the MTT assay suggested that RF13 peptide have significant anticancer potential against the Hep-2 cells. This is in accordance with the *in-silico* results that the peptide RF13 have an excellent number of amino acid residues in the charged and uncharged state; also, the isoelectric point of RF13 was high compared to the other two screened peptides SA11 and DI14; altogether, RF13 showed many possibilities in being anticancer peptide. The VGB3 peptide derived from vascular endothelial growth factor (VEGF) B also had similar amino acid combination patterns and showed more significant anticancer activity (Sadremomtaz et al. 2020). The anticancer activity was further confirmed with the trypan blue exclusion assay by calculating the cell count. The RF13 peptide showed a significant reduction in cell viability at 24 h of treatment (E-Suppl. Fig. 1). Dasiram et al. (2017) reported a similar study in curcumin that showed potential anticancer activity against colon adenocarcinoma cells.

Effect of RF13 in Nuclear Morphology

The cytomorphological changes of Hep-2 cells treated with RF13 peptides were investigated under the inverted phase-contrast microscope, which confirmed that the peptide treatment affected the cell morphology. The morphological changes such as cell shrinking, rounding and granulation

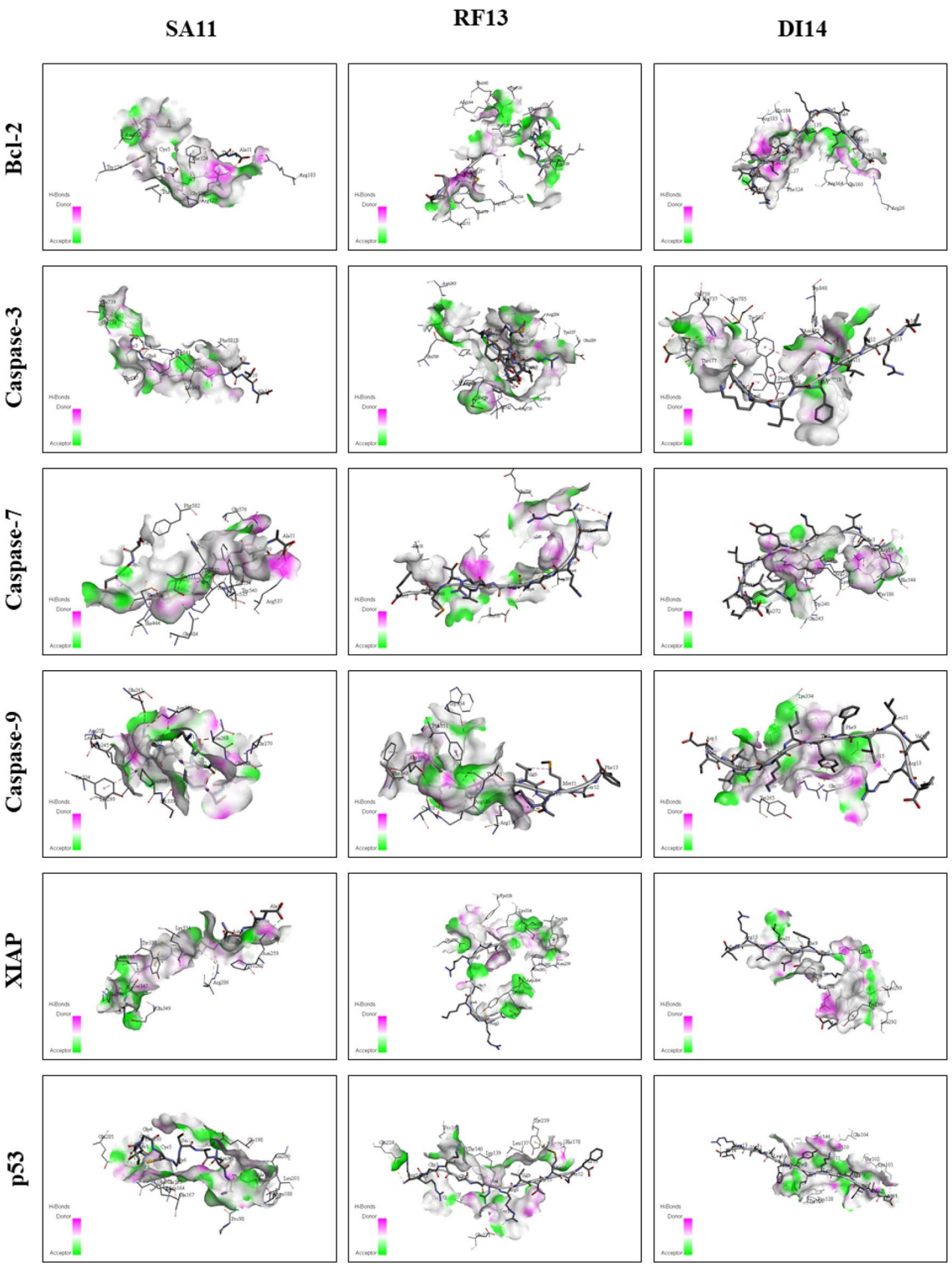

Fig. 2 Results of molecular docking showing the interactionbetween SA11, RF13, DI14 peptide andreceptors Bcl-2, Caspase-3, Caspase-7, Caspase-9, XIAP and P53. The HEPDOCK results were visualized in discoverystudio software

Table 2 Results of molecular docking and binding affinity scores of screened peptide fragments from VSP26B protein with the receptors

	SA11	RF13	DI14
Bcl-2	− 131.12 kcal/mol	− 218.97 kcal/mol	− 198.19 kcal/mol
Caspase-3	− 153.12 kcal/mol	− 193.05 kcal/mol	− 219.87 kcal/mol
Caspase-7	− 158.02 kcal/mol	− 199.07 kcal/mol	− 226.03 kcal/mol
Caspase-9	− 137.74 kcal/mol	− 196.04 kcal/mol	− 196.46 kcal/mol
XIAP	− 168.31 kcal/mol	− 200.45 kcal/mol	− 226.03 kcal/mol
p53	− 137.09 kcal/mol	− 187.07 kcal/mol	− 211.63 kcal/mol

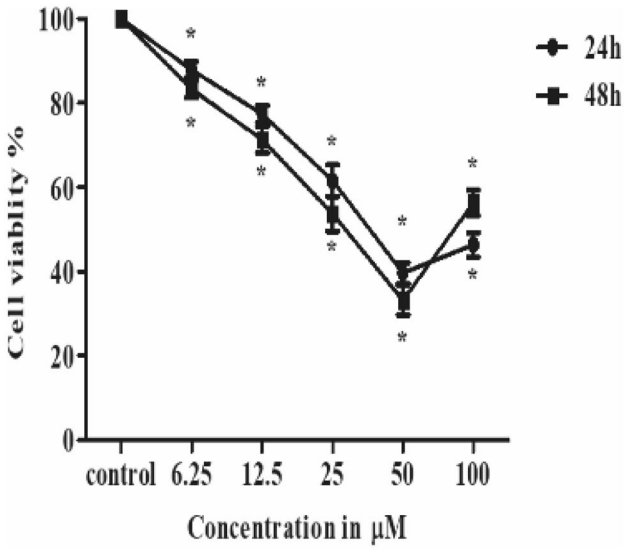

Fig. 3 Assessments of cytotoxic activity of the ACP by MTTassay in HEp2 cells for 24 and 48 h incubation. The data represented are mean ±standard deviation (SD) of three independent experiments. The asterix (*) represents $p < 0.05$,the level of significance compared with control

were observed, which represented the apoptosis (Fig. 4). Similar results were recorded earlier that the peptide derived from *Grapsus albacarinous* showed anticancer activity against the MCF-7 cells (Emadi Shaibani et al. 2021). Hoechst 33342 staining results revealed that the RF13 peptide induces apoptosis in Hep-2 cells by damaging the nuclear material. The apoptotic cholesteric features such as chromatin condensation, bi- or multinucleation, chromatin fragmentation, and nuclear swelling were also observed (Fig. 5). Wu et al. reported a pentapeptide from *Anthopleura anjunae* (sea anemone), which was attained by alkaline protease enzymatic hydrolysis extraction, possessed potential anticancer properties against prostate cancer cells (Wu et al. 2018).

Effect of RF13 Peptide in Cell Cycle

Fluorescence-activated cell sorting (FACS) results revealed that the anticancer ability of RF13 peptide arrest the proliferation of Hep-2 cells; however, the peptide functions in a dose-dependent manner as shown in the cell cycle phases (Fig. 6 A and B). The shift difference in the peptide treatment group was observed in G0/G1, S and G2 phases. Moreover, the cell population percentage in the phases was calculated from gating results. The peptide concentrations, 25 µM or 50 µM, was considered based on the IC50 values because the assay aimed to detect the efficiency of the peptide to disrupt the cell cycle mechanism. According to Garrett and Collins, anticancer drugs arrest the cell cycle by inhibiting any phase such as G0/G1, S or G2/M (Garrett and Collins 2011). A similar study was reported by Najm et al. The peptide derived from mucus protein extract of *Anabas testudineus* showed a significant effect on inhibiting cell cycle phase in MCF7 and MDA-MB-231 cells (Najm et al. 2021). With these supporting studies, we know that the RF13 peptide is potentially involved in inhibiting Hep-2 cell proliferation.

DNA Fragmentation Analysis

The DNA fragmentation assay detected apoptosis-induced nuclear damage, particularly in DNA. As shown in Fig. 7 of lane 1, the control group DNA band intensity was high. In contrast, the peptide treatment group at lane 2 (25 µM) and lane 3 (50 µM), the cell's DNA band intensity was comparatively less. Thus, the DNA fragmented sheared band was observed. The results confirmed that the RF13 peptide substantially induced apoptosis and also had the potency to damage the nuclear material in a dose-dependent manner. These results are correlated with the earlier investigation of Najm et al. (2021) and Dasiram et al. (2017), as reported elsewhere that the anticancer peptides influence DNA fragmentation.

Apoptotic Gene Expression

The RT-PCR analysis was performed to detect the effect of RF13 peptide inducing the apoptotic genes caspase 3 and caspase 9, which play a vital role in apoptosis. Based on the molecular docking and peptide receptor interaction results, the primers were selected. The RF13 peptide treatment significantly upregulates caspase 3 and caspase 9 in a dose-dependent manner. At 25 µM concentration, caspase 3

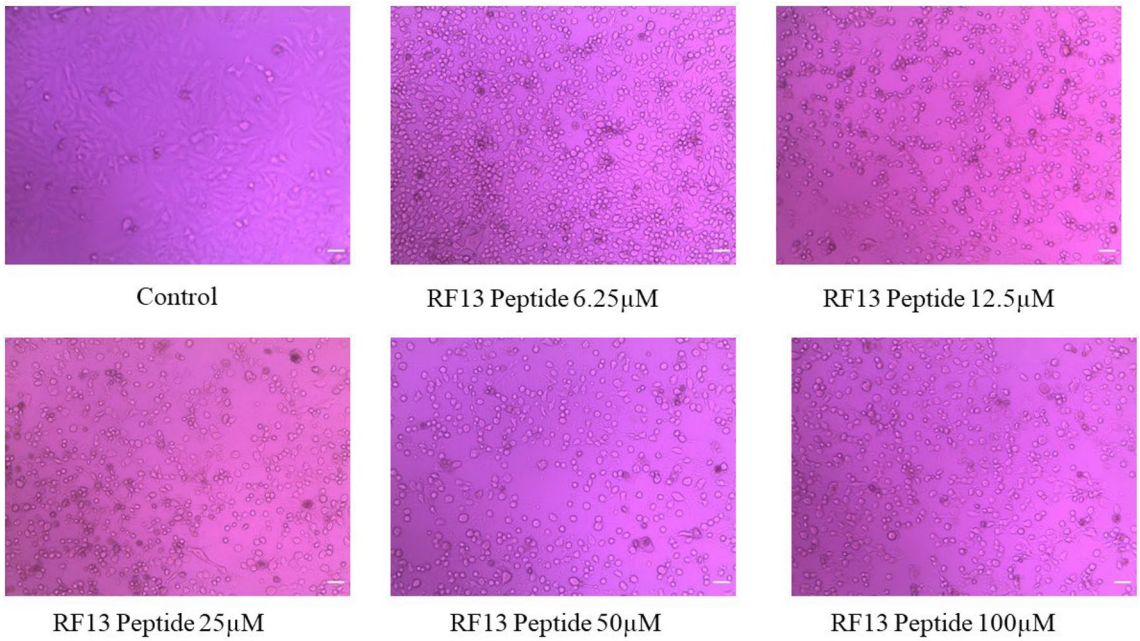

Fig. 4 Phase-contrast microscopic images of Hep-2 cellstreated or untreated (control) for 24 h with RF13 peptide. The un-treated controlcells showed typical morphology of Hep-2 cells, whereas the peptide treatedcells showed abnormal morphology in structure. Images were taken at 20Xmagnifications

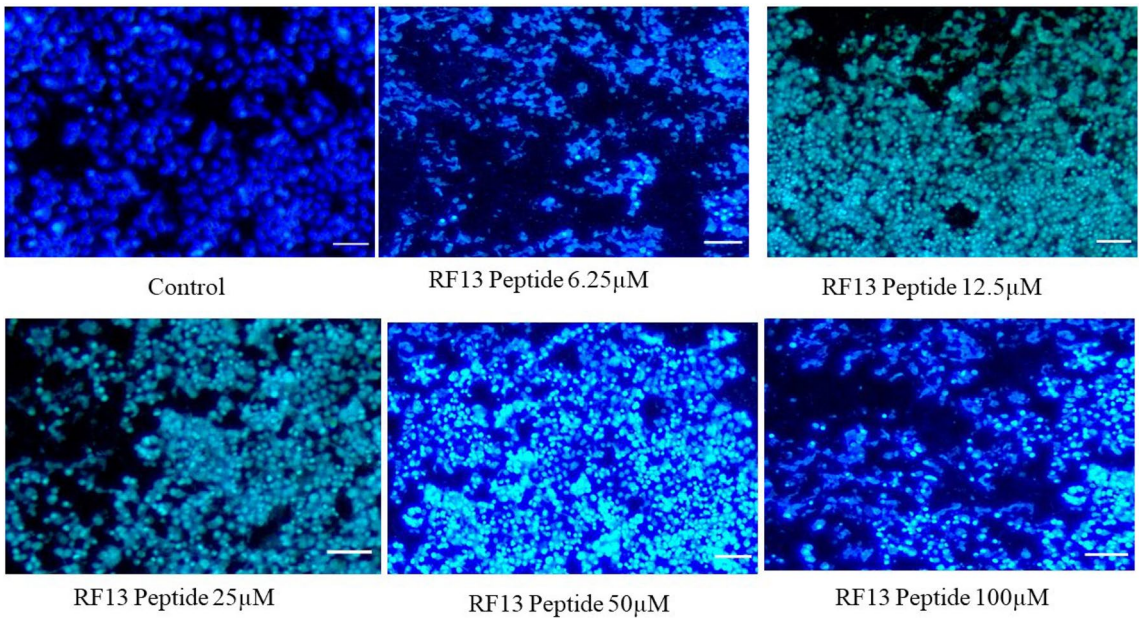

Fig. 5 The photomicrographic representation of RF13peptide activity against the Hep-2 cells by Hoechst 33342 staining, the apoptotic cells are bright blue

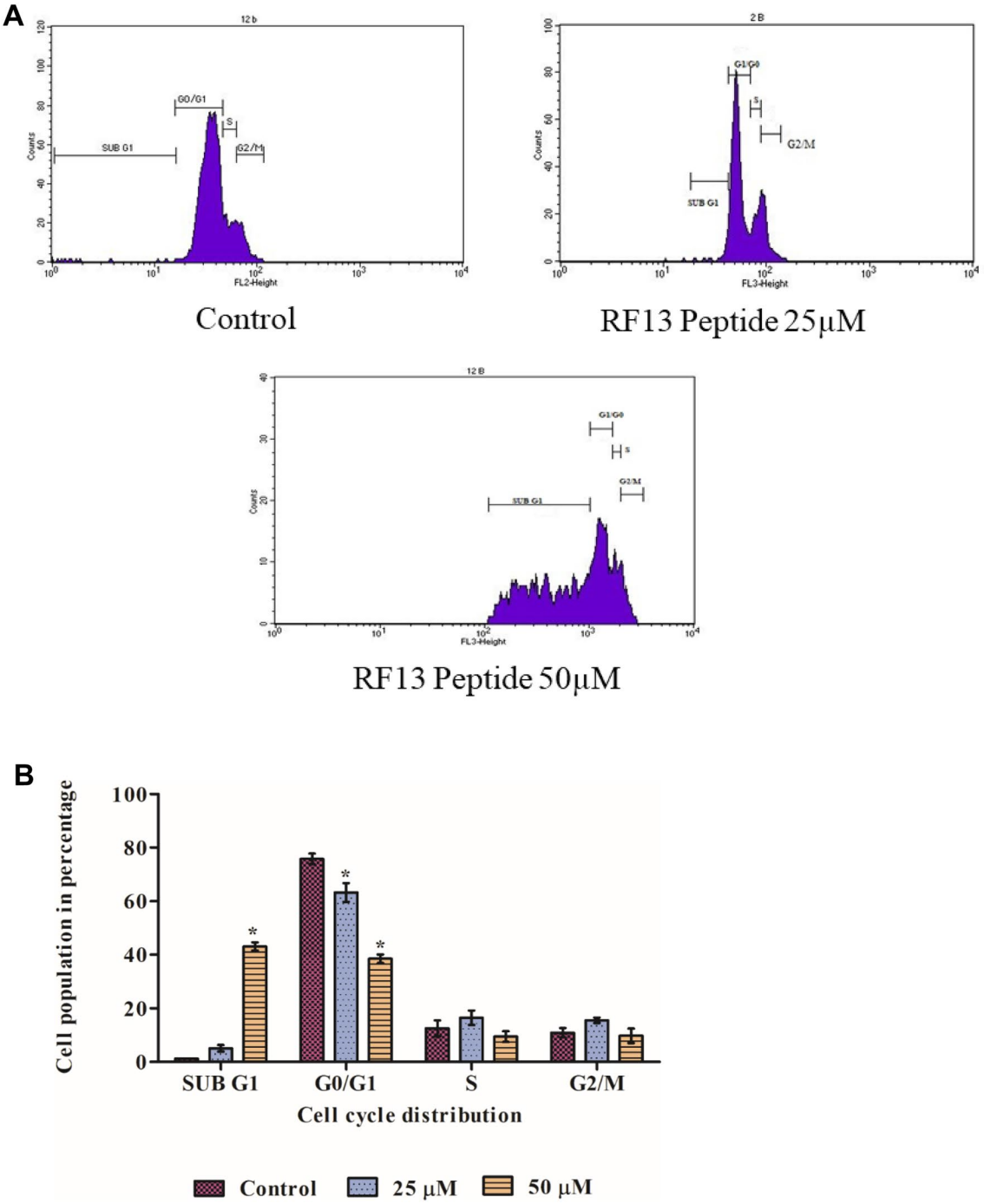

Fig. 6 **A** Flow cytometry analysis of Hep-2 cells performed byPI staining. Graphical representation of cell cycle analysis of control and RF13 peptide treated cells. **B** The cells percentage in each phase; G0/G1, S, andG2. The data were expressed as the mean ± SD of three-independent experiments. Significances (*$p < 0.05$) were indicated incomparison to control

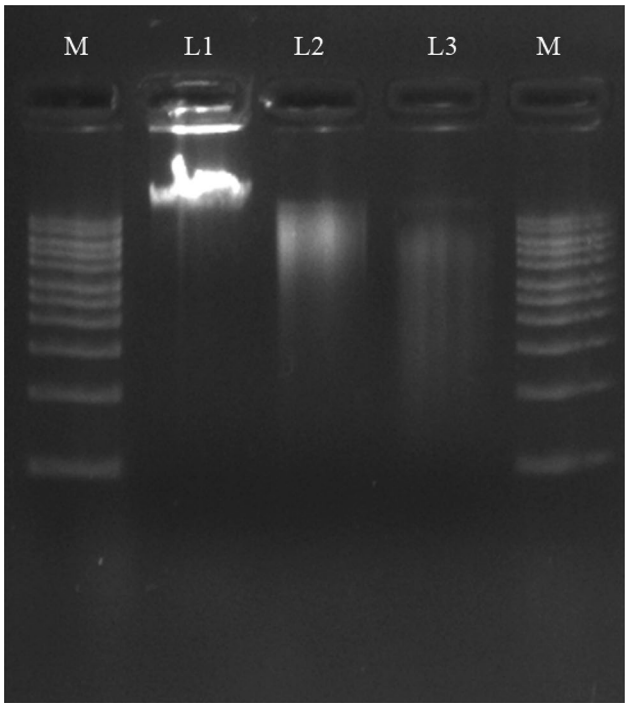

Fig. 7 DNA fragmentation assay in Hep-2 cells treated withRF13 peptide for 24 h. M-Marker (1kb ladder), Lane 1 is control (untreated),Lane 2 is RF13 peptide treated at concentration 25 µM and 50 µM of RF13 peptide(Lane 3)

showed 1.88-fold, and caspase 9 showed a 2.89-fold change; whereas at 50 µM concentration caspase, 3 showed 2.53-fold and caspase 9 showed 4.23-fold changes (Fig. 8). A peptide named VS-9 from *Allium sativum* induced apoptosis against leukemic cells by inducing caspase 3 and caspase 9 expression (Rasaratnam et al. 2021).

Conclusions

This study demonstrated that the RF13 peptide detected from VSP26B is cytotoxic to human laryngeal epithelial (Hep-2) cells. The molecular docking results and the peptide physicochemical properties justify that the RF13 is an anticancer peptide. The peptide inhibits proliferation and significantly induces apoptosis by upregulating the apoptotic genes. Based on the obtained results, we concluded that the RF13 peptide has potential anticancer property against Hep-2 cells; however, further directions are required to study the exact molecular mechanism of peptide so that the pharmacological application aspect on developing the peptide as anticancer therapeutic is feasible.

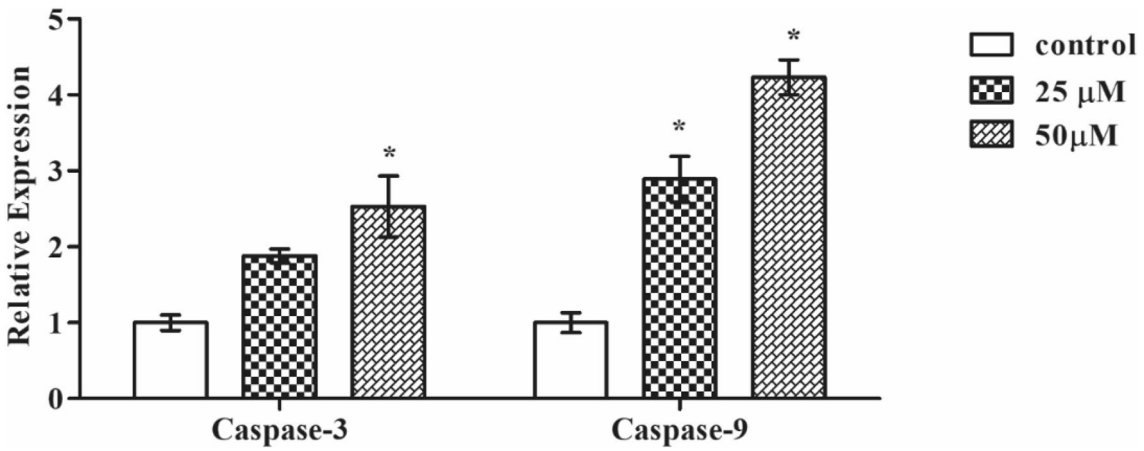

Fig. 8 The apoptotic genes Caspase-3 and Caspase-9expression in Hep-2 cells induced by RF13 peptide treatment for 24 h. The values are represented as mean ± SDof three independent experiments. The asterix (*) represents $p < 0.05$ level of significance

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10989-022-10395-0.

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Data Availability Data will be made available on reasonable request.

Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

Research Involving Human and Animal Participants This research does not involve any human or animal objects.

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