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Invitro analysis of the anticancer properties of *Hottentotta rugiscutis* scorpion venom in oral and breast cancer cell lines

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A B S T R A C T

Cancer has remained the second leading cause of death globally (≈9.95 million in 2020). Over the past few years, using the scorpion venom peptides in diagnosing cancer has led a growing number of studies on exploring anticancer properties of different species of scorpion venoms. In this study, anticancer property of *Hottentotta rugiscutis* (*H.rugiscutis*) venom against breast (MCF-7) and oral (KB3-1) cancer cell lines has been evaluated. Cytotoxicity of *H.rugiscutis* venom was determined by MTT assay. Cell cycle and caspase 9 expressions was analysed by flow cytometry. Effect of *H.rugiscutis* venom on cell proliferation was studied by wound healing assay. *H.rugiscutis* venom showed significant cytotoxic activity with the IC 50 values of 279 µg/ml and 373 µg/ml on MCF-7 and KB3-1 cell lines respectively. *H.rugiscutis* venom induced caspase-9 expression in both cell lines. Cell cycle analysis showed that *H.rugiscutis* venom arrested the cells at Sub G0 /G1 phase of cell cycle of KB3-1 and MCF-7 cell lines. Further, cell proliferation (wound healing) assay showed *H.rugiscutis venom* inhibited 18% and 13 % of cell migration of MCF-7and KB3-1cell respectively. These cumulative results confirm the action of *H.rugiscutis* venom in induction of apoptosis in breast and oral cancer cell lines.

Introduction

Cancer has remained the second leading cause of death globally. According to the reports from the World Health Organization, cancer caused 9.95 million deaths in the year 2020. Among other cancer types Oral cancer is one of the more common in developing countries like India. According to WHO global statistics, there are an estimated 377,713 new cases of cancers of the lip and oral cavity, more than

177757 deaths in the year 2020 (Ferlay *et al.*, 2020). Deleterious side effects, low success rate and high risk of recurrence remains a disadvantage for advanced cancer therapy ("Side Effects of Cancer Treatment - NCI,").

Alternative approach for the development of new effective and safe strategies for cancer treatment is extremely needed. One of these alternative and effective approaches included using anticancer peptides derived from venoms of various animal including snake, spider, frog, honey bee and scorpion.

Scorpion venoms have been used in traditional medicine for thousands of years in India, China and Africa. The scorpion venom is a highly complex mixture of enzymes, proteins, peptides, salts, biogenic amines, nucleotides and mucoproteins (Ding *et al.*, 2014). However, pharmacological screening of anticancer molecules has been studied only in few scorpion species across the world. Few number of potential antitumor molecules including bengaline and chlorotoxin; enzymes including serine proteinase and hyaluronidase from scorpion venom has been identified against cancer cells (Gomes *et al.*, 2010).

However the mechanism of action these peptides showed vary across the species. The anticancer efficacy of scorpion such as *Buthidae* scorpions, *Androctonus bicolor*, *Androctonus crassicauda*, and *Leiurus quinquestriatus* venoms has been examined in various types of cancers as glioma, neuroblastoma, leukaemia, lymphoma, breast, lung, prostate and pancreatic cancer (Shao *et al.*, 2014; Deshane *et al.*, 2003; Bloch *et al.*, 2007; Ortiz *et al.*, 2015; Ding *et al.*, 2014). In addition, Das Gupta *et al.*, established the cytotoxic activity of Indian black scorpion (*Heterometrus bengalensis*) venom on human leukemic U937 and K562 cells (Das Gupta *et al.*, 2007). Since, composition and mechanism of action of venom vary across the species; it is relevant to screen potential antitumor molecules in different species. Moreover, several scorpion species are widespread in India. Therefore exploration of anticancer potential molecules and understanding its mechanism action of Indian species is strongly needed.

In this study, we have chosen *Hottentotta rugiscutis* (*H. rugiscutis*) scorpion venom to screen the potential anticancer molecule and elucidating the cytotoxic activity on oral cancer (KB3-1) and breast cancer (MCF-7) cell lines. This study provides additional knowledge to develop a valuable therapeutic tool against deadliest cancer.

Materials and Methods

Cell culture medium: DMEM (Dulbecco's modified eagles medium)- High Glucose, Fetal Bovine Serum from Invitrogen. MTT, DMSO, Camptothecin, D-PBS, Propidium Iodide, MCF-7 and KB3-1 cell line were purchased from NCCS Pune. India.

Cytotoxicity assay (MTT assay)

The inhibitory concentration value (IC50) was evaluated using a 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. KB3-1 cells and MCF-7 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight at 37℃ in 5% CO_2. The cells were treated with various concentrations (25, 50, 100, 200 and 400 µg/ml) of *H.rugiscutis* venom and incubated for 24 hours.

Then, 10 µl of MTT stock solution (5 mg/ml) was added to each well and cells were incubated for 4 hours. The MTT solution was then discarded and 100 µl of Dimethyl sulfoxide was added to dissolve insoluble formazan crystals. The optical density (OD) was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm using an enzyme-linked immunosorbent assay (ELISA) multiwell plate reader. The OD values were used to calculate the percentage of viability (Riss Terry *et al.*, 2013). The IC50 value was determined by using linear regression equation i.e. Y=Mx+C. Here, Y = 50, M and C values were derived from the viability graph.

Cell Cycle Analysis

Cell cycle analysis was performed by propidium iodide (PI) based measurements of DNA content of the cell by flow cytometry with slight modification of the method(Kim and Sederstrom 2015). KB3-1 and MCF-7 cells were seeded at a density of $3x10^5$ cells in 6 well culture plate. Cells incubated for 24hours were treated with IC50 concentrations 279µg/ml and 373 µg/ml of *H. rugiscutis* venom against MCF-7 and KB3-1 cells respectively. Cells were incubated for 24 h. Then, cells were trypsinized and washed twice with PBS. 0.5 ml of PBS was added and this suspension was transferred drop by drop in to a 12x 75 mm polypropylene tube containing 4.5 ml of 70% pre chilled ethanol. This should ensure fixation of all cells and minimize clumping. Allowed to fix for at least 30 minutes on ice. Centrifuged for 5 min at 300g and decanted the ethanol. The pellets were washed in 5ml PBS. Finally, pellet was resuspended in 500µl of propidium iodide iodide-(PI-) RNAse solution (1mg/mL PI solution, Triton X-100 (0.1% v/v), and 10mg/mL RNAse) for 30 minutes at 37 °C. Cell cycle was analyzed by Flowcytometry-BD FACSCalibur. Camptothecin (25µM) was used as a positive control.

Caspase-9 assay

MCF-7 cells and KB3-1 cells were cultured in a 6-well plate at a density of 2 x 10^5 cells/ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours. Aspirated the spent medium and the cells were treated with *H. rugiscutis* venom at IC50 concentration of 279µg/ml and 373 µg/ml on MCF-7 cells and KB3-1 cells respectively. Cells were incubated for 24 h. Then, cells were trypsinised and washed twice with PBS. 0.5 ml of cell suspension in PBS was transferred drop by drop in to the 12x 75 mm polypropylene tube containing 4.5 ml of 70% pre chilled ethanol. This should ensure fixation of all cells and minimize clumping. Fix for at least 30 minutes on ice. Centrifuged for 5 min at 300g and ethanol was removed. The pellets were suspended in5ml PBS and centrifuged for 5min at 300g and decanted the PBS. The pellets were resuspended in 0.3 ml PBS. 5 µl of Caspase 9 conjugated with FITC antibody was added to the cell suspension. Mixed thoroughly and incubate for 30 minutes in the dark at Room Temperature. Centrifuged cells at 3000 rpm for 5 minutes and removed the supernatant. Re-suspend cells in 0.5 ml of Wash Buffer(1X PBS containing 0.1% sodium azide), and centrifuge again. Finally 0.5 mL of PBS was added and analyzed by Flow cytometry-BD FACSCalibur (Davargaon *et al.*, 2019).

Invitro Cell Proliferation Activity (Wound Healing Assay)

Cells (KB3-1 and MCF-7) were plated with a seeding density of 8x 10^5 cells in 60mm culture dishes and incubated over-night at 37ºC in CO_2 incubator and allowed it to reach 70 – 80 % confluence as a monolayer. After monolayer was formed, wounds were made with a 1 ml pipette tip across the center of the dish. The wounded cell mono layer were treated with IC 50 concentrations of *H. Rugiscutis* venom 279µg/ml and 373µg/ml against MCF-7 and KB3-1 cell respectively for 24h. 25µM Camptothecin was used as standard. Then, the cells were fixed with 3.7% para-formaldehyde for 30min. The fixed cells were stained with 1% crystal violet for 30 min. Images were captured under inverted microscope. Cell migration and gap filling were analyzed based on the following formula.

$$\% \text{ of Cell migration} = \frac{\text{Initial wound width(nm)} - \text{Final wound width(nm)}}{\text{Initial Wound width (nm)}} \times 100$$

Statistical analysis

Statistical analyses were performed using the Student's t-test. Results are presented as the mean ± standard deviation. The data are analyzed intriplicate, using Graph Pad Prism software, version 4.0 (Graph PadSoftware, Inc., San Diego, CA, USA). $P<0.05$ is considered to indicate a statistically significant difference.

Results and Discussion

Cytotoxicity assay (MTT assay)

Cytotoxicity of *H. rugiscutis* venom were analysed in MCF-7and KB3-1cell lines by MTT assay. *H. rugiscutis* venom showed significant cytotoxic activity with the IC 50 values of 279.59µg/ml and 373.42 µg/ml on MCF-7 and KB3-1 cell lines respectively, which indicates the induction of apoptosis in these cell lines (Fig 1 and Fig 2).

Cell Cycle Analysis

Cell cycle analysis of *H.rugiscutis* venom (279µg/ml) treated MCF-7 cell showed a significant difference in percentage of cells in SubG0/G1 compared to control. Accumulation of 25.01% cells in SubG0/G1 phase indicates the DNA fragmentation due to apoptosis (Fig 3). Whereas *H.rugiscutis* venom (373µg/ml) treated KB3-1 cell shown accumulation of 3.52% of cells in sub G0/G1 phase of cell cycle compare to control(Fig 4).

Caspase- 9 Assay

Activation of Caspase-9 was analyzed by flow cytometry. Caspase-9 is a member of the apoptotic initiator group of Caspases which include Caspases-2, -8, and -10. Caspase-9 is an upstream proenzyme in the cascade of enzymatic reactions required to induce cellular apoptosis. The results from the flow cytometry showed that *H. rugiscutis* venom activated Caspase-9 in both the cell lines. This indicates that scorpion venom induced apoptosis in these cell lines.

Caspase9-FITC histogram of the gated MCF-7 singlets distinguishes cells at the M1 and M2 phases. Here M2 refers to negative expression/region and M1 refers to the positive expression/region. Gating of M1 and M2 phases is approximate and can be refined using software (Cell Quest Software, Version 6.0) analysis. As shown in Fig 5C, expression of Caspase9 was significantly low in control (9.22±0.26MFI) than *H. rugiscutis* venom (279µg/ml) treated MCF-7cells (46.52 ± 2.27 MFI).

Caspase 9-FITC histogram of the gated KB3-1 singlets distinguishes cells at the M1 and M2 phases. (Here M2 refers to negative expression/region and M1 refers to the Positive expression/region). Gating of M1 and M2 phases is approximate and can be refined using software (Cell Quest Software, Version 6.0) analysis.

Caspase 9 expression study by flow cytometry showed that, expression of Caspase 9 was low in control KB3-1 Cells(8.96±0.18MFI) compared to the *H.rugiscutis* venom(373µg/ml) treated cell (37.45 ± 4.3MFI) (Fig 6C)

Caspase 9 expression significantly higher in *H.rugiscutis* venom treated cells compared to control. The observations suggest us that the Scorpion venom having significant Caspase9 expression potential against the MCF-7 and KB3-1 cell lines.

In vitro Cell Proliferation Activity (Wound Healing Assay)

Cell proliferation has been assessed by Scratch wound healing assay. The results of scratch wound healing assay showed the inhibition of both KB3-1 and MCF-7 cells at the concentration of 373ug/ml and 279ug/ml respectively (Fig 7 and Fig 8).

However camptothecin shown to have maximum inhibition compared to *H.rugiscutis* venom. In this study, cytotoxicity of crude *H.rugiscutis* scorpion venom and its effect on cell cycle were analysed in two different cancer cell lines i.e breast (MCF-7) and oral (KB3-1) cancer cell lines. The cytotoxicity and cell cycle were analyzed using MTT and flow-cytometric assay. Crude *H.rugiscutis* scorpion venom showed significant cytotoxic activity with the IC 50 values of 279 µg/mL and 373 µg/mL on MCF-7 and KB3-1 cell lines respectively. Results from Caspase-9 assay indicated that scorpion venom activated Caspase-9 activity in both cell lines.

Fig.1 Cytotoxic effect of *H. rugiscutis* venom against MCF-7 cells

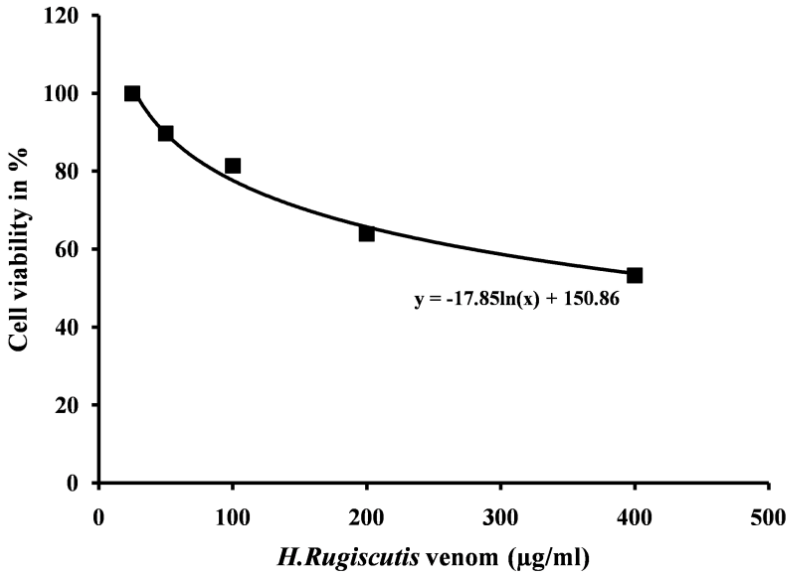


Fig.2 Cytotoxic effect of *H.rugiscutis* venom against KB3-1 cell line

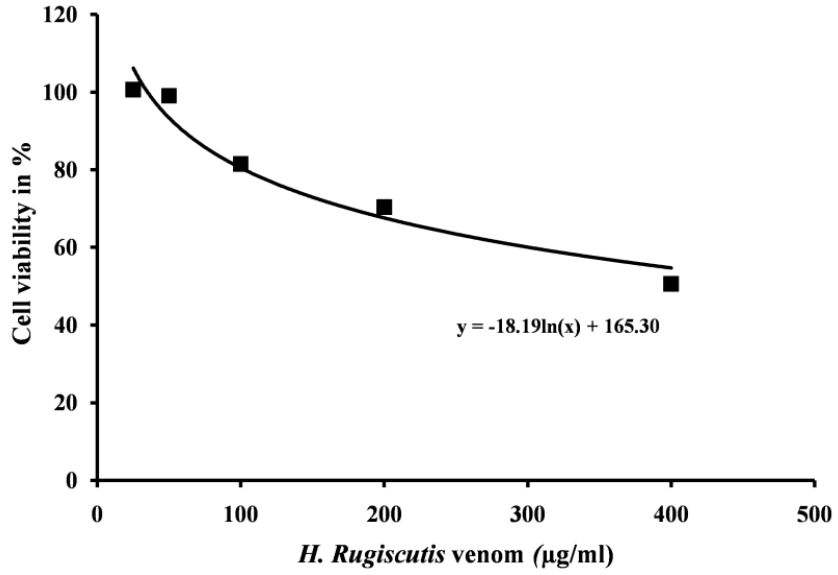


Fig.3 Effect of *H.rugiscutis* venom on cell cycle of MCF-7 cells

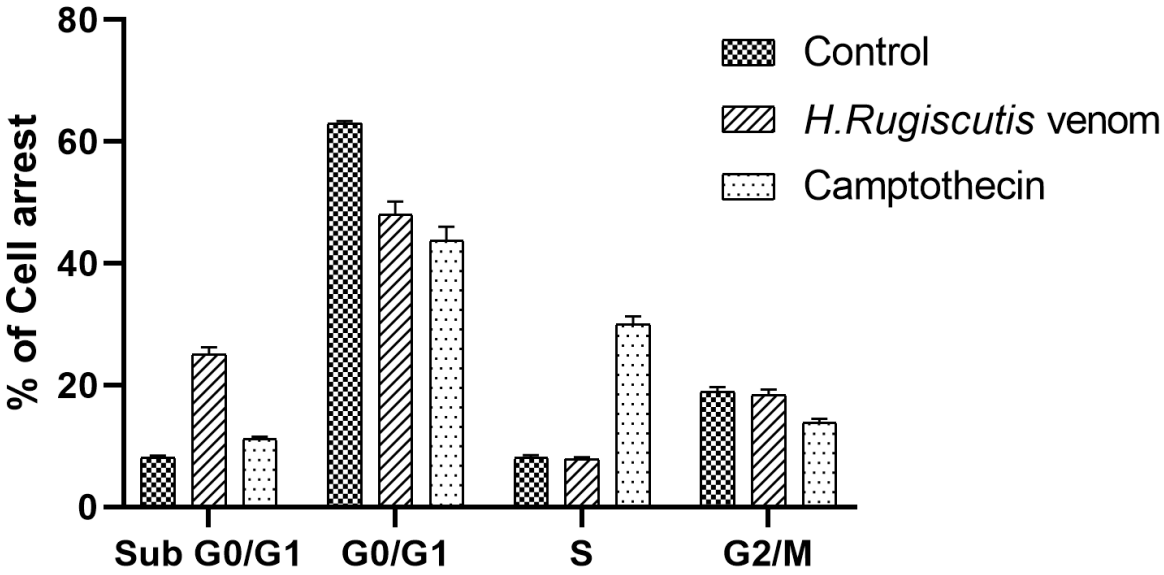


Fig.4 Effect of *H.rugiscutis* venom on cell cycle of KB3-1 cells

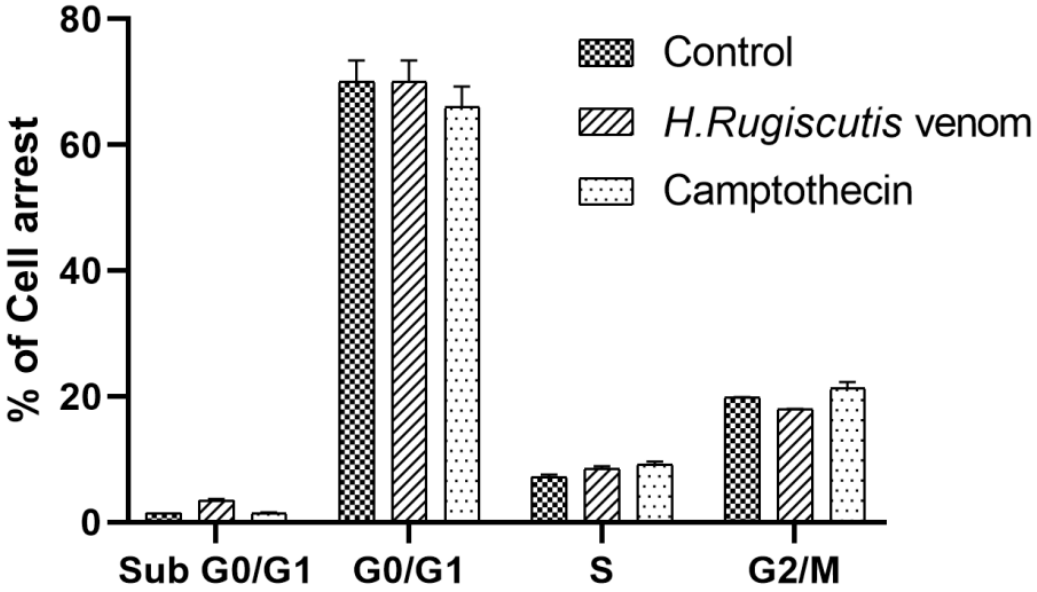


Fig.5 Caspase 9 expression study of *H.rugiscutis* venom against the MCF-7 cells(Flow cytometric analysis BD FACScalibur, Cell Quest Pro Software, Version: 6.0). A) Control B) *H.rugiscutis* venom treated C) Mean Fluorescence Intensity of Caspase 9 expression in control, *H.rugiscutis* and Camptothecin(25µM) treated MCF-7 Cells.

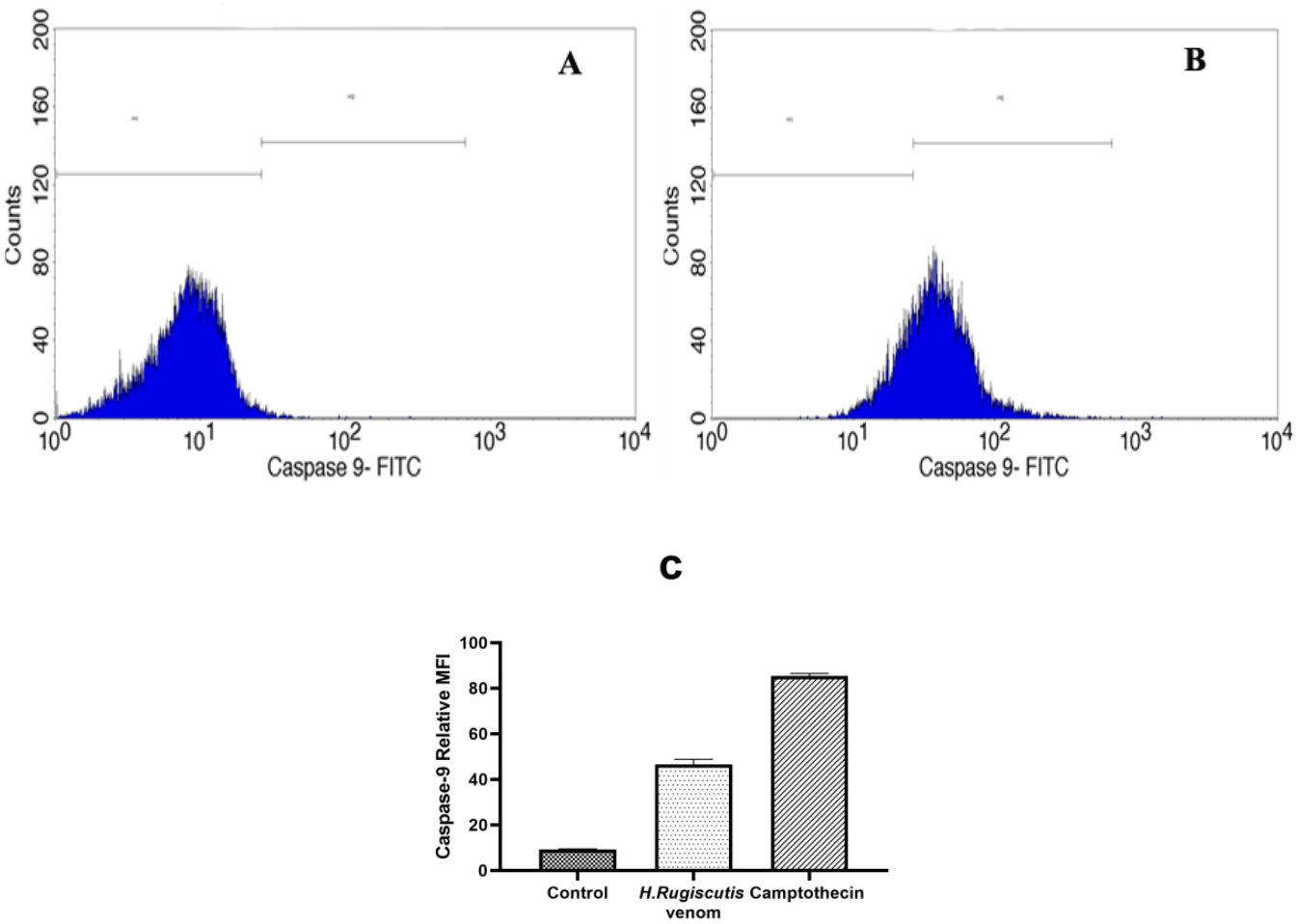


Fig.6 Caspase 9 expression study of *H. rugiscutis* venom against the KB3-1 cells. (Flow cytometric analysis BD FACScalibur, Cell Quest Pro Software, Version: 6.0). A) Control B) *H. rugiscutis* venom treated C) Mean Fluorescence Intensity of Caspase9 expression in control, *H.rugiscutis*venom and camptothecin treated KB3- Cells.

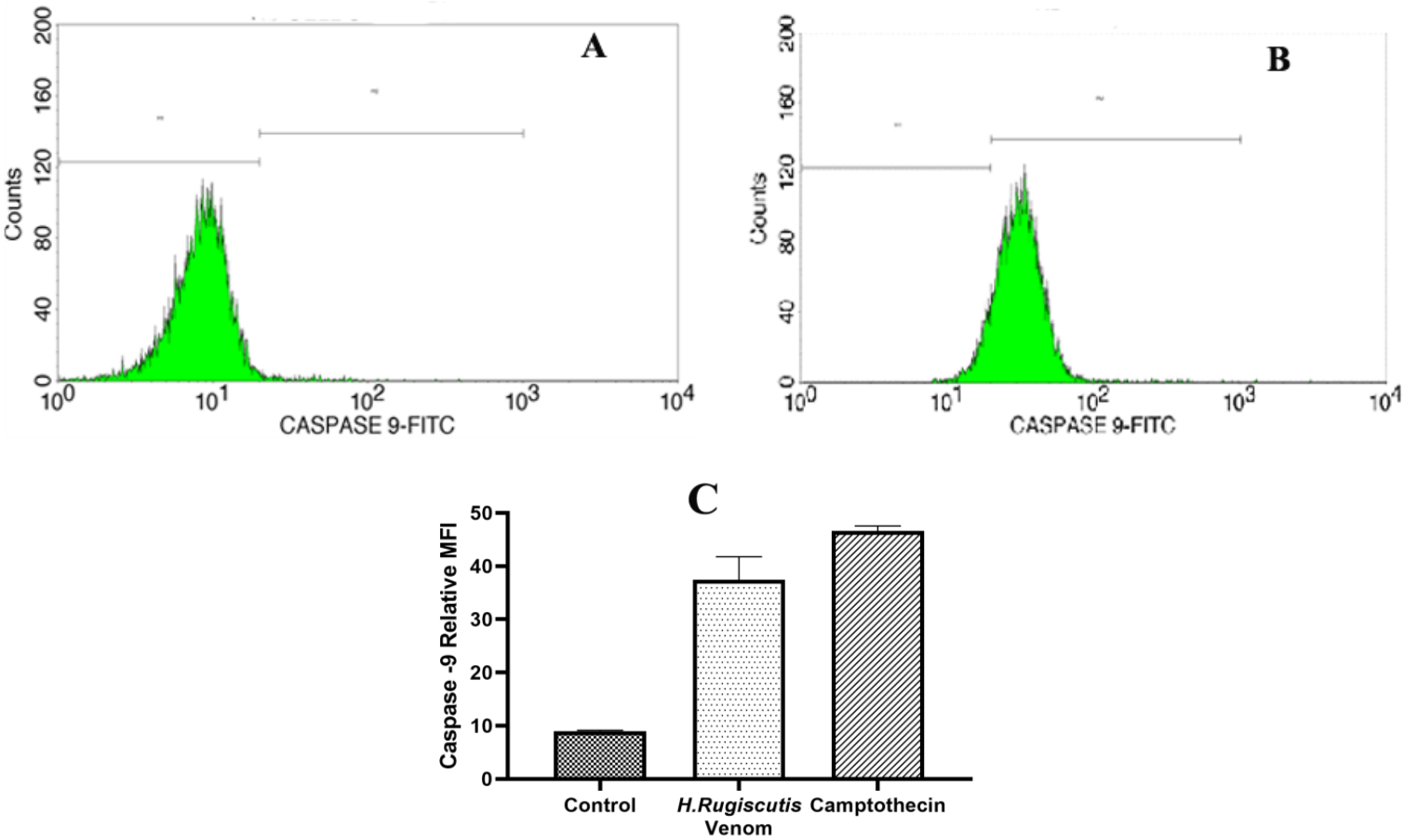
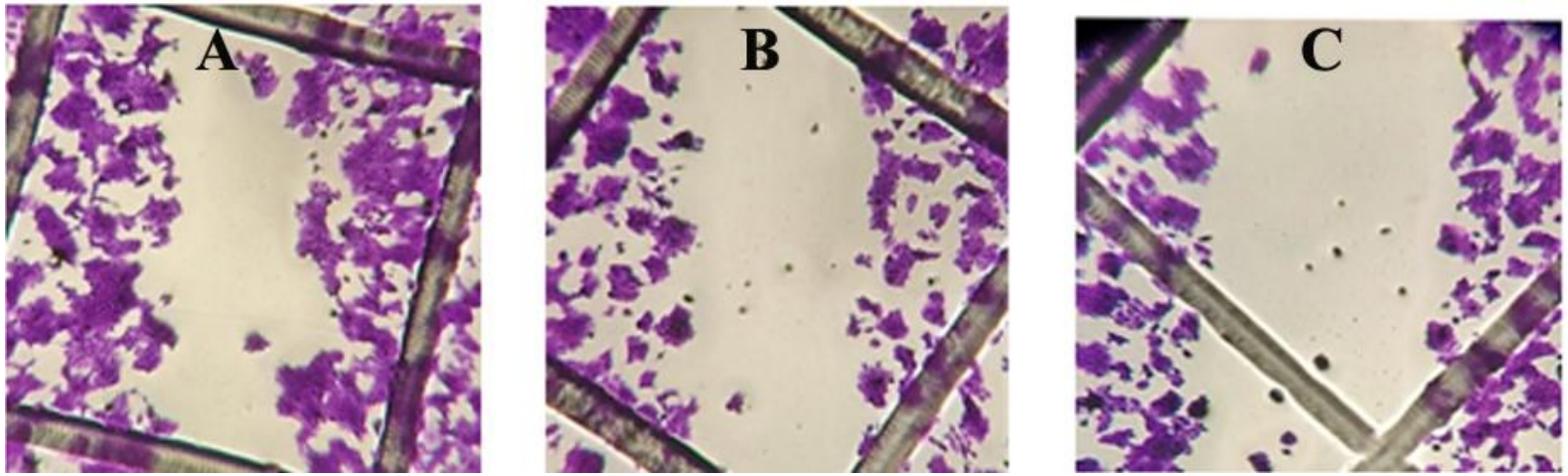


Fig.7 Effect of *H.rugiscutis*venom on cell proliferation activity of MCF-7 cell lines A) Control B) *H. rugiscutis* venom treated C) Camptothecin treated D) Percent of Cell migration.



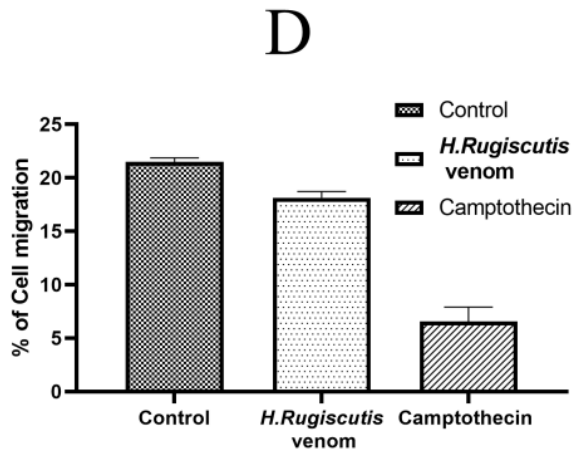
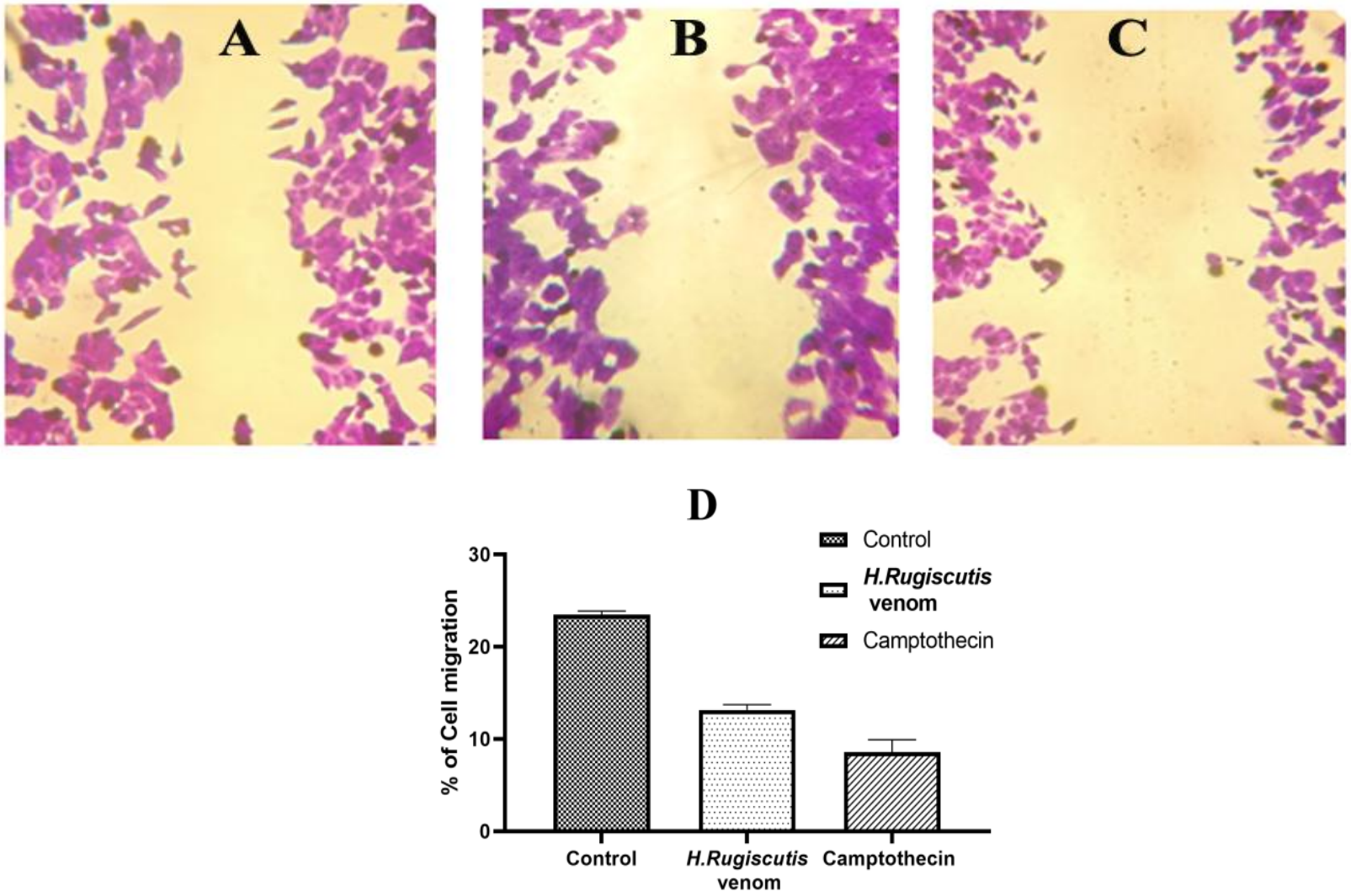


Fig.8 Effect of *H.rugiscutis* venom on cell proliferation activity of KB3-1 cell lines A) Control B) *H. rugiscutis* venom treated C) Camptothecin treated D) Percent of Cell migration.



Cell cycle analysis were shown the effect of scorpion venom on the Sub G_0 /G1 phase of cell cycle of KB3-1 and MCF-7 cell lines. Further cell proliferation (wound healing) assay showed action of scorpion venom against proliferation of KB3-1 and MCF-7 cell lines. These cumulative studies confirm the role of scorpion venom in the induction of apoptosis in breast and oral cancer cell lines.

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