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Assessment of Apoptosis-inducing Potential of Cyclopeptide RA-V on Human COLO 205 Colon Cancer Cells

Rupachandra Saravanakumar*, Dharshene Karthik, Raj Kumar, Lavanya Mohan, Rabeela and Nithesh Jerome

Department of Biotechnology, School of Bioengineering,
SRM Institute of Science and Technology, Kattankulathur, India.

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The inadequacy of eliminating the entire population of tumor cell and the concomitant creation of chemoresistance is a major challenge for an effective colon cancer treatment. Many compounds which are natural have been shown to be optimistic, based upon their antitumor effects and a decrease in toxicity. Natural cyclopeptides are small peptides that are rich in disulfide bonds having a cyclic backbone being isolated from plants. The peptides are resistant to enzymatic, thermal and chemical conditions due to the presence of the cyclic cystine knot like structure. Plant derived cyclic peptides hold a very significant role in the field of cancer chemotherapy. However, a never-ending endeavor is a search to find novel therapeutic compounds for treating and controlling cancer. RA-V (deoxybouvardin), is a natural cyclopeptide that is extracted from the *Bouvardia ternifolia* roots belonging to the *Rubiaceae* family. RA-V cyclopeptide exhibits anti-angiogenesis, anti-inflammatory and anti-cancer activities. Apoptosis means a programmed cell death which is being characterized by membrane blebbing, nuclear fragmentation and chromosome condensation. This study focuses on evaluating the efficacy of cyclopeptide RA-V in human colon cancer cell lines.

Keywords: Apoptosis, Cell death, Chemotherapy, Colon Cancer, Cyclopeptide, *Rubiaceae*.

Colorectal cancer is among the most widespread and aggressively expanding cancers across global populations, ranking as one of the leading causes of cancer-related deaths.[1] This type of cancer often manifests sporadically, though it can also arise due to hereditary factors that promote inflammation within the bowel region.[2] Over the last few decades, the prevalence of colon cancer has been increasing, and this rise correlates strongly with the global surge in obesity rates.[3] One of the primary preventative strategies to combat the onset and progression of colorectal cancer is through inhibiting tumor growth and carcinogenesis at the early stages. This approach, known as chemoprevention, involves the use of natural compounds that possess cancer-fighting properties, aiming to halt the progression of cancerous cells before they can advance.[4]

In recent years, bioactive peptides have garnered significant attention in cancer research due to their high tissue-binding properties, specificity, and overall effectiveness in promoting health.[5] These peptides are known to perform a wide range of functions within human metabolism, making them particularly attractive candidates for drug development.[6] Cyclopeptides, a subset of bioactive

peptides, have emerged as promising targets for anticancer drug research. By evaluating current biomedical evidence, researchers have gained insights into the structure-activity relationship of cyclopeptides and examined their potential in therapeutic development.[7] It has been favored that the natural compounds isolated from plants function with a lower level of toxicity when compared to the chemically derived drugs.[8]

RAs have fascinated a great interest due to their distinctive bicyclic structural featuring disulphide bridges and significant anti-cancer activities *in vivo* and *in vitro*.[9] RA-V, also referred to as deoxybouvardin, is a well-known cyclic peptide initially isolated from the roots of *Bouvardia ternifolia*, possessing a molecular weight of 756.9 Daltons.[10] RA-V has also been isolated from the roots of medicinal plants of other relevant species such as *Rubia cordifolia* and *Rubia yunnanensis*.[11-12] Research has shown that RA-V exhibits multiple cancer-suppressing properties, such as reducing inflammation,[9] inhibiting tumor growth,[11] curtailing angiogenesis,[13] and triggering apoptosis – the programmed cell death that prevents cancer cells from proliferating.[14]

Apoptosis is a dominant mechanism which eliminates cancer cells without causing damage such as morphological changes and rupture to normal cells and the tissues surrounding the tumor cells. The thought process of apoptosis provoked the development of therapies that are targeted through novel drugs that induce apoptosis or enhance the sensibility of tumor cells to authorized agents that are cytotoxic in nature. Previous studies have shown that RA-V significantly and effectively blocked the proliferation of tumor cells in human breast cancer cells.[14] This study is aiming at the analysis of the effect of RA-V on the proliferation of cells and apoptotic activity against human COLO 205 colorectal cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

RPMI-1640 (Roswell Park Memorial Institute medium1640), fetal bovine serum (FBS), Hoechst 33342, penicillin and streptomycin were acquired from Hi-Media (Chennai, Tamil Nadu, India). acridine orange, ethidium bromide, 2',7'-dichlorofluorescein diacetate, MTT (2,5-diphenyltetrazolium bromide), resazurin were purchased from SRL (Sisco Research Laboratories) (Chennai, Tamil Nadu, India). The Real-time cell viability assay kit and the flow cytometry assay kit were acquired from Promega (Chennai, Tamil Nadu, India) and Bio-vision (Chennai, Tamil Nadu, India) respectively. The cyclopeptide RA-V was purchased from Chem-Faces (Chennai, Tamil Nadu, India). This entire study was performed in Sri Ramaswamy Memorial Institute of Science and Technology. (Chennai, Tamil Nadu, India)

Cell proliferation inhibition

Cell culture

The human COLO 205 colon cancer cell lines were procured from NCCS (National Centre for Cell Science), Pune, India. The cells were cultured with RPMI 1640 medium along with 5% FBS (Fetal Bovine Serum), 1% Streptomycin (100µg/mL) incubated at a temperature of 37ÚC and humidity of 5% CO_2.

Cell viability assays

MTT assay

The inhibition of viability of cells was determined by MTT Assay.[14] The process is carried out by incubating the COLO 205 cells (1×10u cells/mL) seeded in 96-well plate treated with positive control doxorubicin and different concentrations of RA-V (50, 75, 100 and 125µM) for the various time periods (24 and 48 h). After the incubation of a specific time interval, the MTT solution (7 mg/mL) was added and incubated for 2 h in a cell culture incubator. The media was discarded from all the wells and the formazan crystals were dissolving by adding DMSO (100 µl). At the end of 30 mins of incubation, the absorbance was read at 570 nm using a microplate reader.

Resazurin Cell viability assay

The proliferation of cells is inhibited and the percentage of cell viability was analyzed by Resazurin Cell Viability Assay[15] in which the colon cancer cells with a density of 1×10u cells/mL were seeded in a 96-well plate and treated with different concentrations of RA-V (50, 75, 100 and 125µM) to determine the viability of cells. 200 µl of resazurin solution (500 µg/mL) was added to all the wells at the end of incubation of different time intervals (24 and 48 h) and further incubated for about 4 hours. The absorbance was measured using a microplate reader at 560 nm and 590 nm.

Real-time cell viability assay

The inhibition of cell proliferation was performed by RealTime-Glo™ MT Cell Viability Assay.[16] In a 96-well plate, the cells were seeded, incubated and treated for distinct time intervals at different concentrations of RA-V (50, 75, 100 and 125µM) to determining the viability of cells after the treatment. The Luminescence at 570 nm was measured after incubating the cells with 100 µl of RealTime-Glo™ reagent for 1 hour by using a microplate reader and the results are expressed statistically in terms of mean±SEM.

Apoptotic effect on RA-V treated human colon cancer cell lines

Acridine Orange/Ethidium Bromide Staining

The determination of apoptotic activity on cells was performed by co-staining of acridine orange/ethidium bromide (AO/EtBr) staining.[17] The COLO 205 cells were seeded in a 6-well plate upon coverslips and treated with optimum concentrations of RA-V (100 and 125µM) for different time intervals (24 and 48 h). The fixation of cells with 4% paraformaldehyde was performed; 100µl and 30µl of acridine orange (1 mg/mL) and

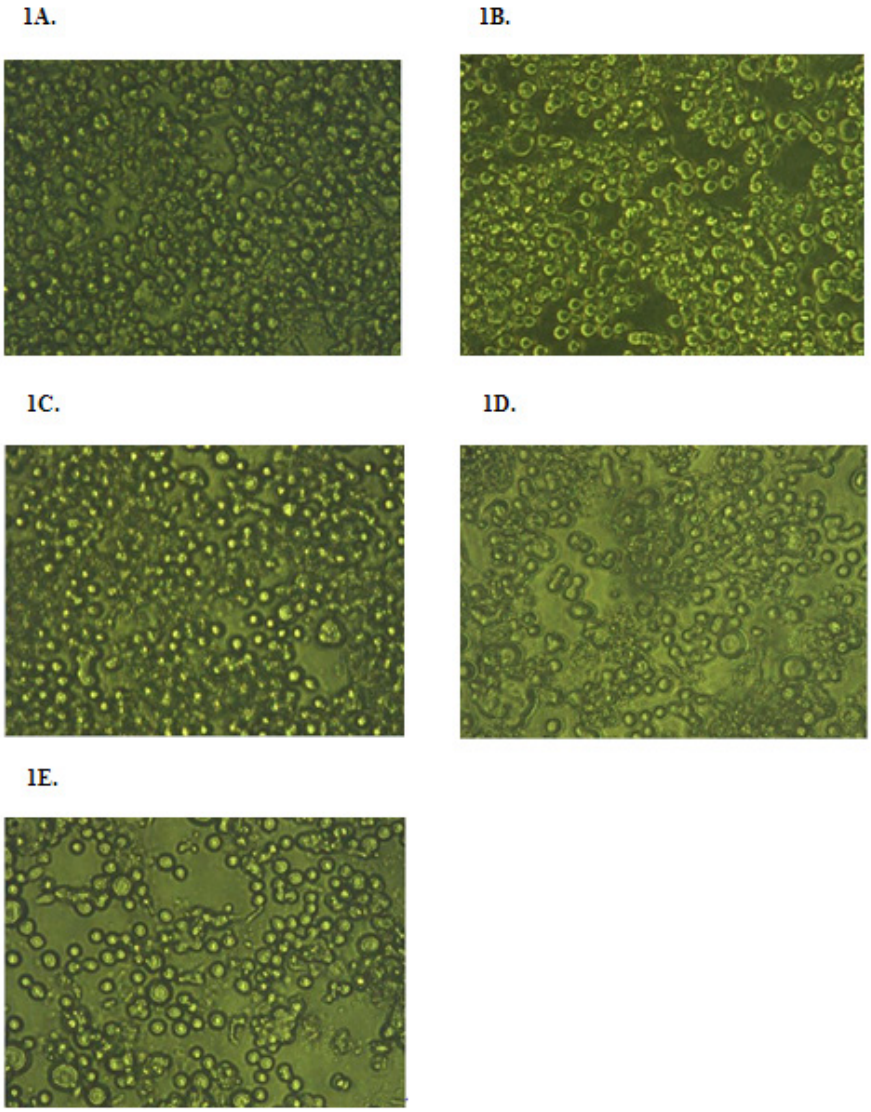

Fig. 1. Photo micrographs (10x) of the morphology of the COLO 205 cells treated with RA-V using phase contrast microscope (A)Control (B)50µM (C)75µM (D)100µM (E)125µM for 48hr

ethidium bromide (1 mg/mL) respectively were added on to all the wells under dark followed by PBS wash to remove the excess stain. The cells were mounted and visualized using a fluorescent microscope.

DCFDA Staining

The generation of reactive oxygen species which indicates apoptosis was determined by DCFDA staining.[18] COLO 205 cells with a density of $1x10^6$ were grown on coverslips in a 6-well plate and the cells were treated for different time intervals at optimized concentrations of RA-V (100 and 125µM). 100 µl of DCFDA solution (25 mg/mL HBSS buffer) was added to all the wells after fixing the cells and was incubated for 15 mins. The cells were visualized to determine the oxidative stress under a confocal microscope.

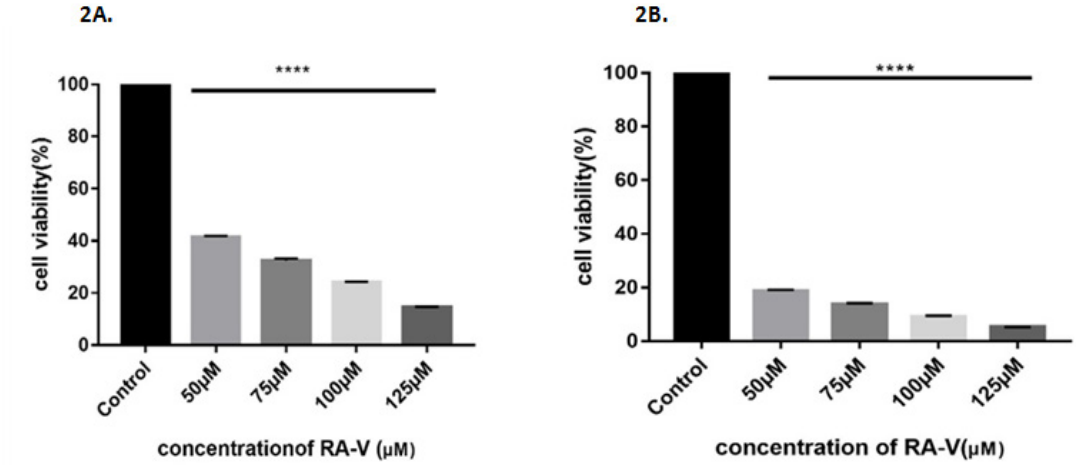

Fig. 2. Determination of viability of RA-V treated colon cancer cells using MTT assay. The values are expressed as mean±SEM and were statistically significant (****P<0.0001), compared with control (A) 24hr (B) 48hr. The graph shows that the apoptosis of RA-V treated COLO 205 cells is gradually increasing with respect to the increased time of treatment as well as increased concentration of drug

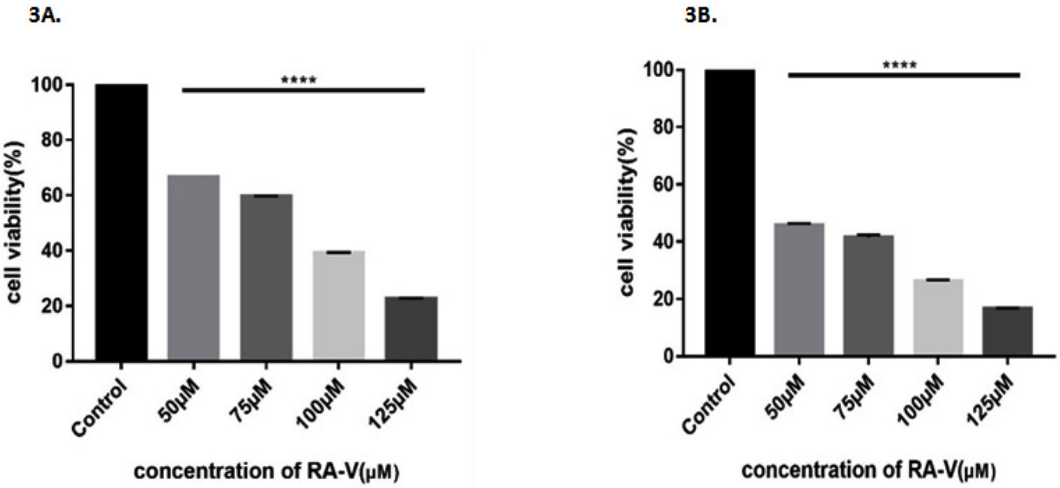

Fig. 3. Determination of viability of RA-V treated colon cancer cells using Resazurin cell viability assay. The values are expressed as mean±SEM and were statistically significant (****P < 0.0001), compared with control (A) 24hr (B) 48hr. The graph shows that the apoptosis of RA-V treated COLO 205 cells is gradually increasing with respect to the increased drug concentration and interval of treatment

Hoechst Staining

The condensation of chromosomes in apoptotic cancer cells was visualized by Hoechst staining.[19] 30 µl of Hoechst 33342 staining solution (10µg/mL) prepared from 10 mg/mL of stock solution was added on to all the wells consisting of coverslips seeded with COLO 205 cells treated with optimum concentrations of RA-V (100 and 125µM) for 24 and 48 h. The cells were incubated for 15 mins and were photographed with confocal microscopy.

RESULTS AND DISCUSSION

RA-V inhibits cell viability of human COLO 205 colon cancer cell lines

The morphological changes of COLO 205 cells upon the treatment of RA-V were examined using phase contrast microscopy (Fig.1). The cell viability upon the action of RA-V on COLO 205 cells was found to be constantly decreasing with the increase in the concentrations of RA-V by MTT assay which proved that RA-V possesses anti-cancerous activity inhibiting the proliferation of cells in human colon cancer (fig 2). The cell viability was found to be lowest at the concentrations of 100 µM, and 125µM for 48 h treatment when compared to 24 h treatment. The results obtained were analogous to the effect of RA-XII against breast cancer with the inhibition of cell proliferation.[20] Further, the examination of the effect of RA-V on cell viability by Resazurin Cell Viability (fig 3) and RealTime-Glo™ MT Cell Viability Assays (fig 4) showed a drastic reduction in the percentage of viability of cells as the concentration of RA-V increases which is relative to the results obtained from MTT assay. The results of the Resazurin Cell Viability assay obtained in this study is comparative to the action of peptides isolated from various medicinal plants against breast cancer cells showing a decrease in the proliferation of cells.[21] The bio-active peptide from the residual protein of *Juglans regia* inhibits the proliferation of cancer cells, demonstrating the cytotoxic activity of peptide.[22] Further, the effect of RA-V against colon cancer cells determined from RealTime-Glo™ MT Cell Viability Assay depicted the inhibition of cell proliferation and tumor growth with a decrease in the number of viable cells, which are similar to the results obtained in the study of *the protein hydrolysates of Brassica napus* against sarcoma cancer cells.[23] The morphological changes, membrane damage and the stages of apoptosis were observed in RA-V treated COLO 205 colon cancer cells by Acridine Orange and Ethidium Bromide co-staining method (fig 5). The binding of Acridine Orange to the live cells makes the control cells appear green in colour. The apoptosis is induced after the treatment of RA-V for appropriate time intervals expressing the early apoptotic (reddish-orange) and late apoptotic (orange) stages, as dead cells take up the Ethidium Bromide stain. The

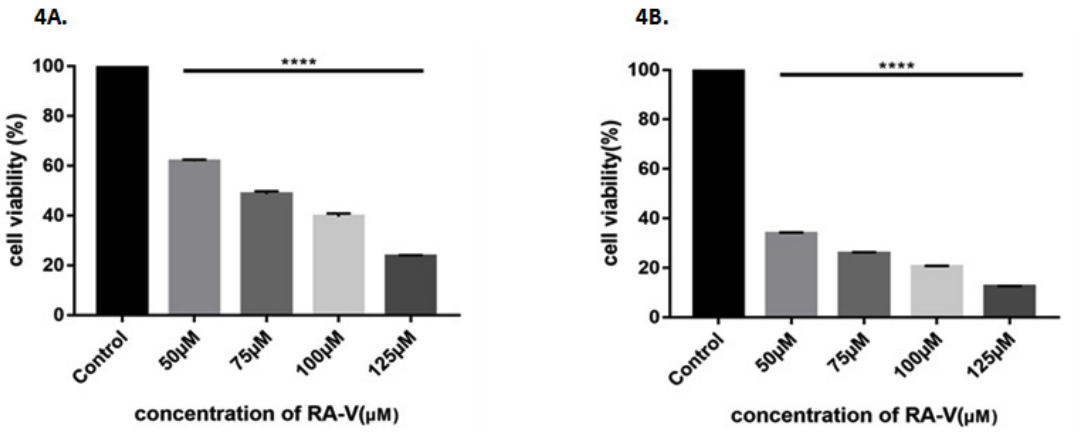

Fig. 4. Determination of viability of RA-V treated colon cancer cells using Real-time cell viability assay. The values are expressed as mean±SEM and were statistically significant (****P <0.0001), compared with control (A) 24hr (B) 48hr. The data given in the graph states that the apoptosis of RA-V treated COLO 205 cells is gradually increasing with respect to the increased time of treatment as well as increased concentration of drug.

similar results showing morphological changes were obtained with the bioactive protein isolated from *Abies webbiana* inducing apoptotic activity of lung cancer cells.[17] The beta-amyloid peptide isolated from *Juglans regia* induced apoptosis in PC12 cells associated with morphological

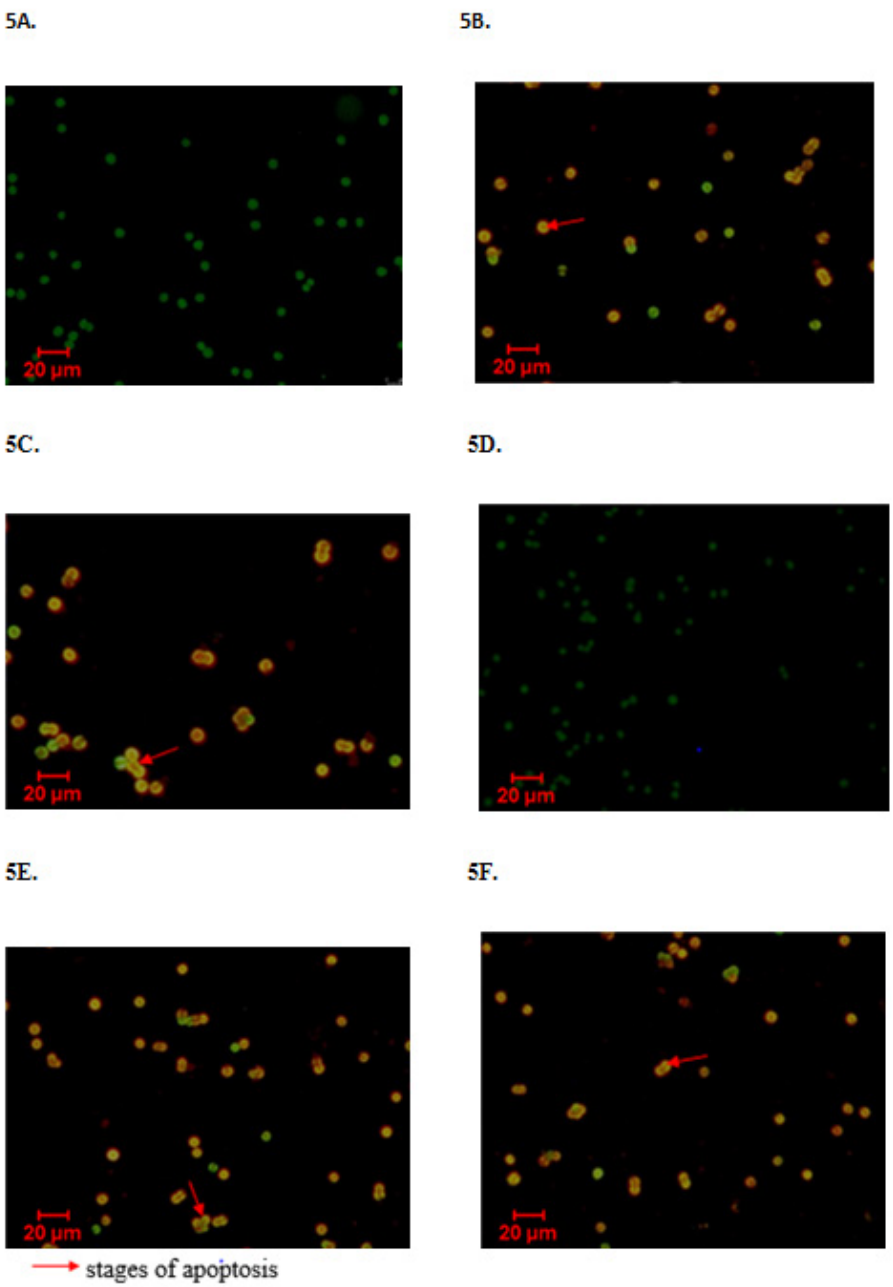

Fig. 5. Representative of fluorescent microscopy images(40X) of Colo 205 cells after treatment with RA-V using Acridine orange and Ethidium bromide stain – 24 hr :(A) Control, (B)100µM, (C)125µM; 48 hr (D) Control, (E)100µM, (F)125µM. The results from Acridine orange and Ethidium bromide staining in this image shows the disruptive cell membranes (orange-coloured cells) stating that the apoptosis of COLO 205 cells takes place upon the treatement of RA-V

changes.[24] Further the stages of apoptosis and changes in the morphology of cells were also observed in liver cancer cell lines upon the action of MussaeninA extracted from *Mussaenda glabrata* belonging to the *Rubiaceae family* [25] stained with Acridine Orange and Ethidium Bromide. The chromosomal condensation being a characteristic feature of apoptosis was studied by Hoechst33342 staining in RA-V treated COLO 205 cells which revealed the nuclear changes and chromosomal condensation with the increasing concentrations of RA-V in the treated cells which are characteristics

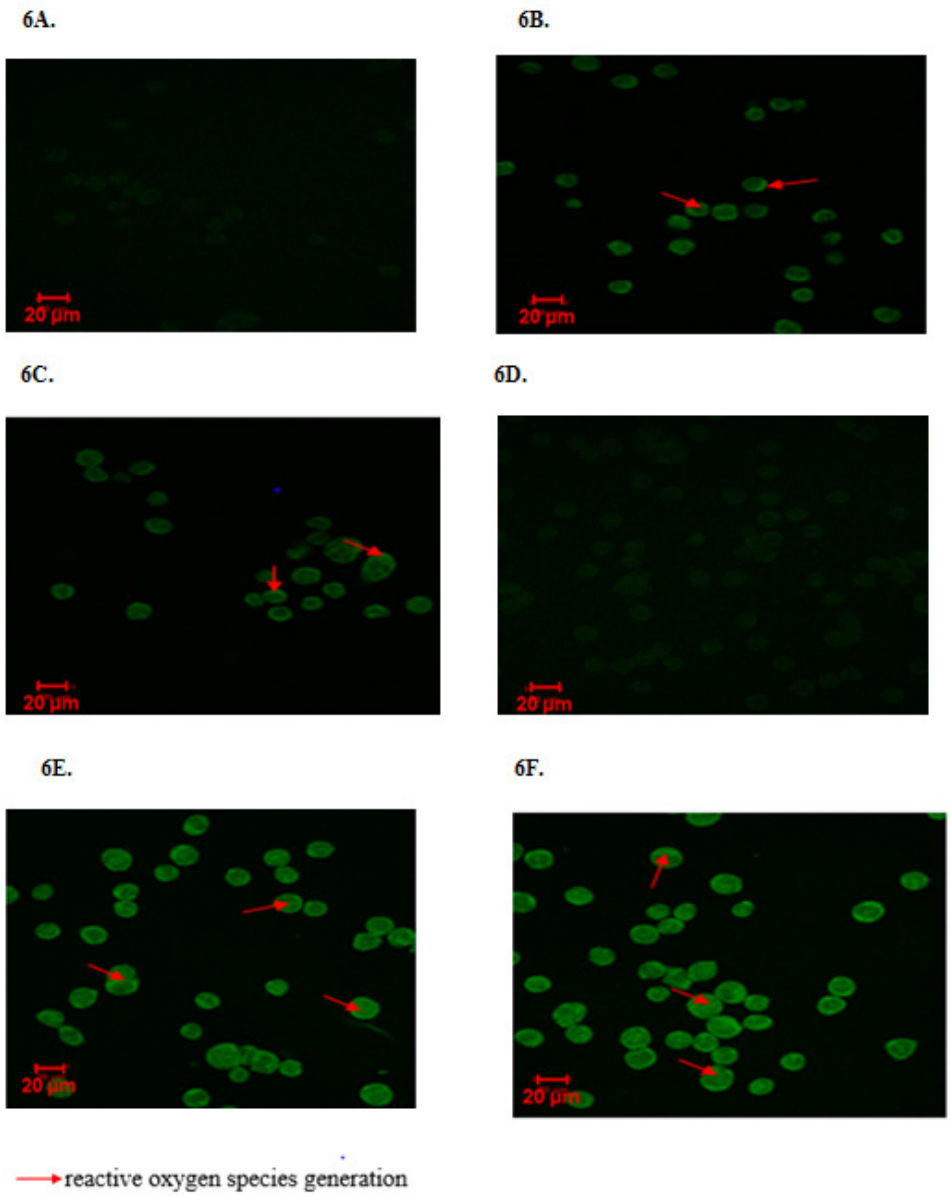

Fig. 6. Representative of confocal microscopy images (40X) of Colo 205 cells after treatment with RA-V using 2,4, Dichlorofluorescein diacetate stain – 24 hr :(A) Control, (B)100µM, (C)125µM; 48 hr :(D) Control, (E)100µM, (F)125µM. The intense green fluorescence indicates the generation of ROS in the apoptotic COLO 205 cells upon the treatment of RA-V

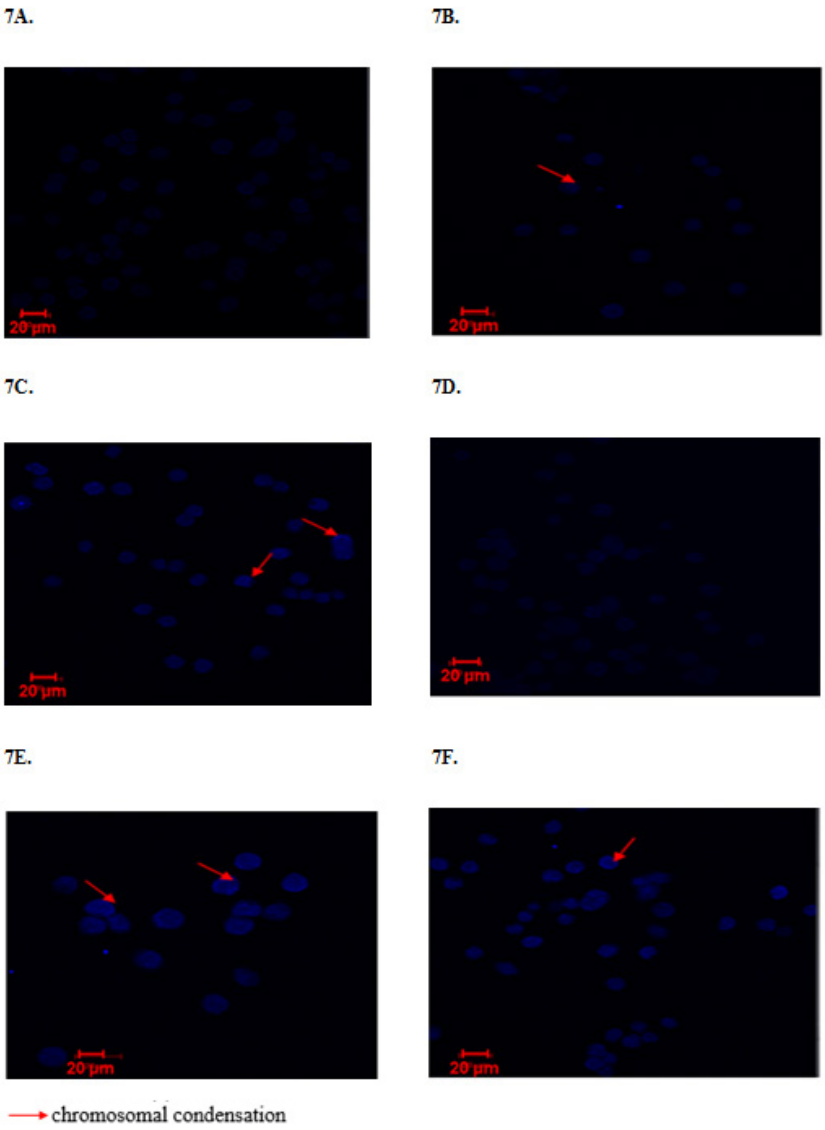

Fig. 7. Representative of confocal microscopy images(40X) of Colo 205 cells after treatment with RA-V using Hoechst 33342 stain – 24 hr :(A) Control, (B)100µM, (C)125µM; 48 hr :(D) Control, (E)100µM, (F)125µM. The blue coloured fluorescent cells are the one with condensed nuclei undergoing cell apoptosis upon the treatment of RA-V

of apoptosis (fig 6). The nuclear changes and chromosomal condensation were observed in the previous studies which revealed that â-Purothionin, a peptide induced cell death [26] showing an enhanced apoptotic activity. The protein isolated from the tubers of *Corydalis cava* exhibited apoptotic activity against HeLa cervical cancer cells by disrupting the cell membrane associated with nuclear changes[27] aiding the chromosomal condensation which is also a characteristic feature of apoptosis in RA-V treated colon cancer cell lines observed by Hoechst 33342 staining. Further, the oxidative stress evaluation was correlated with the production of ROS (reactive oxygen species) in the colon cells treated with multiple concentrations of RA-V was performed by DCFDA staining method (fig 7) which were compared with the earlier studies of antioxidant properties of the peptides isolated in

soybean and lunasin.[28] The peptides isolated from the protein samples of *Juglans Sigillata Dode* and *Juglans regia L* also showed antioxidant activities, thereby inhibiting the production of reactive oxygen species.[29-30]

Apoptotic study of RA-V treated human COLO 205 colon cancer cell lines

CONCLUSION

RA-V, a cyclic peptide exhibits antitumor and apoptotic activities against human COLO 205 colon cancer cell lines. In a series of in vitro assays, RA-V demonstrated the ability to inhibit cell viability, induce apoptosis-related morphological alterations, and enhance oxidative stress, corroborating its efficacy as a potent antitumor agent. The noted reduction in cell proliferation, as demonstrated by MTT and Resazurin assays, highlights the cytotoxic effectiveness of RA-V, particularly at elevated concentrations and extended exposure durations. The findings of this study are consistent with earlier research on analogous cyclopeptides, demonstrating that RA-V interferes with cellular mechanisms essential for the survival of cancer cells while preserving normal cells. The findings indicate that RA-V shows considerable potential as a targeted therapy for colorectal cancer, warranting further development in clinical contexts to enhance its efficacy and reduce toxicity.

The apoptotic activity has been studied using several fluorescent and confocal microscopy techniques such as Acridine Orange, DCFDA staining and Hoechst staining. Staining elucidated the phases of apoptosis, ranging from chromatin condensation to membrane disruption, thereby affirming RA-V's capacity to induce apoptosis. Therefore, our results confirm that RA-V is a novel anticancer agent that prevents the growth of colorectal tumor cells and induces apoptosis.

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Conflict of Interests

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required

Clinical Trial Registration

This research does not involve any clinical trials

Authors' contributions

Dharshene Karthik: Literature findings, data and result collection, and manuscript writing. Raj Kumar: Literature findings, data and result collection. Lavanya Mohan: Literature findings, data and result collection. Rabeela: Literature findings, data and result collection. Nithesh Jerome: Formal analysis and editing. Rupachandra S: Conceptualization and supervision of the experiment.

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