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Organic Extract of Streptomyces Induces Apoptosis, Anti-Proliferation and Cell-Cycle Arrest in Colon Cancer Cells: Caco-2 and HCT116 Cell Line as Surrogate Model

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ABSTRACT

Background: Colorectal cancer (CRC) is a prevalent and very lethal malignancy, resulting in more than one million deaths and nearly two million newly diagnosed cases in the year 2018. Streptomyces bacteria are a potential source of conventional compounds, as their extract contains bioactive chemicals with antiproliferative effects in cell culture settings. The aim of this research was to investigate the antiproliferative effects of the Streptomyces ethyl acetate fraction against the Caco-2 and HCT116 cell line in addition to its fundamental mechanisms.

Methods: We examined the effects of Streptomyces extract on colon cancer cell lines Caco-2 and HT-116 by incubating the cells with and without the extract. Antiproliferative effects are analyzed using the MTT cell proliferation assay, cell cycle analysis and flow cytometric detection of Annexin V/PI apoptosis. The formation of intracellular ROS was measured, in addition to Western Blot for Bax, Bcl2 and caspase-3 activation.

Result: Our findings showed the dose-dependent inhibition of cell growth by streptomyces extract. Both Caco-2 and HT-116 cells treated with streptomyces extract displayed the beginning of apoptotic events and G1 cell-cycle arrest. Additionally, treat with the streptomyces extract significantly increased ($P<0.05$) ROS production, Bax and cleaved-caspase-3 were increased, while Bcl-2 was downregulated, as determined by western blotting.

Key words: Anticancer, Cell line, Chemo preventive medicines, *Streptomyces*.

INTRODUCTION

Six million people die from cancer each year. Thus, it may be argued that this factor is the primary contributor to global death rates (MD and UcER, 2023). Chemotherapy is presently employed as the prevailing therapeutic modality for individuals diagnosed with cancer, surgery, or radiotherapy, which has many negative effects, including the destruction of normal cells.

To overcome cancer in humans, scientists resorted to using the chemoprevention strategy, which means using natural or synthetic materials (alone or in combination) capable of preventing the development of tumors (Wang *et al*., 2023). Apoptosis happens in reaction to outside stimuli. Several cancer treatments are aimed specifically at inducing apoptosis in cancer cells (Gupta *et al*., 2023).

The regulation of cell survival and apoptosis is primarily governed by proteins that are members of the B-cell lymphoma 2 (Bcl-2) family. One mechanism by which chemotherapy medicines work is by suppressing the expression of Bcl-2 family members in cancer. The protein known as Bcl-2 plays a pivotal part in the process of programmed cell death, also known as apoptosis by acting as both activators of apoptosis (Bax) and inhibitors (Bcl-xL) (Kaloni *et al*., 2023).

At least two of the main mechanisms that result in apoptosis in mammalian cells are intrinsic mitochondrial-dependent apoptosis and extrinsic death-receptor-dependent apoptosis. When a cell dies, caspases are triggered in both internal and extrinsic apoptotic pathways (Kowalski *et al*., 2023).

Actinobacteria are significant producers of antibiotics and suppliers to the pharmaceutical sector. They can generate numerous secondary metabolites (Elshafie *et al*., 2023; Hozzein *et al*., 2021). Actinomycetaceae-related actinobacteria are widely known for creating secondary metabolites, many of which are effective against various malignancies and harmful microbes (Alhawsawi *et al*., 2023; Belgacem *et al*., 2023).

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Numerous studies have shown that the extract of *Streptomyces* sp. prevents the proliferation of a range of cancer cell types and triggers apoptosis (Alhawsawi *et al*., 2023; Kumar *et al*., 2021; Lin *et al*., 2021).

Therefore, there is a strong incentive to discover novel bacteria and screen them for their active compounds. It is well known that the most promising trend to find novel microorganisms for biotechnology is to move toward the investigation of unexplored environments and/or isolation of extremophiles (Mapelli-Brahm *et al*., 2023). The aim of this study was to investigate the antiproliferative capabilities of the ethyl acetate fraction obtained from Streptomyces. Specifically, the study focused on its effects on the Caco-2 and HCT116 cell lines. Additionally, the study intended to elucidate the mechanisms behind these observed effects.

MATERIALS AND METHODS

Chemicals

The growth medium (DMEM), trypsin, penicillin/streptomycin and fetal calf serum were acquired from Invitrogen, Life Technologies, located in Carlsbad, CA, USA. MTT (Invitrogen, cat. No M6494), Trypan Blue Solution 0.5%. DMSO, protease inhibitor (Thermo Fisher Scientific, # 78429 Waltham, MA, USA), RIPA Buffer (Thermo Fisher), Tris, NaCl, EDTA. The primary antibodies such as Bax, Bcl2, Caspase-3 and β-actin were purchased from (Santa Cruz Biotechnology, CA, USA) whereas the secondary antibody and Chemiluminescent western blotting kit were purchased from Thermo Scientific. Bradford reagent and Nitrocellulose membrane were purchased from Bio-Rad.

Bacterial extract preparation

A pure Streptomyces strain was isolated on minimal medium, from samples of desert soil taken from Riyadh, Saudi Arabia. The pure Streptomyces isolate was added to 100 ml of starch-casein broth in each of the ten 500 ml conical flasks. A shaking incubator at 30°C and 150 rpm incubated the flasks for 7 days. The fermentation flasks were extracted with ethyl acetate after incubation.

Tissue culture

The Caco-2 and HCT-116 (human colon cancer cell line) (DSMZ, Braunschweig, Germany) maintained in (DMEM, Gibco, USA) supplemented with 10% FBS and 1% penicillin/ streptomycin (Invitrogen, USA). The cells were kept at 37°C and given 5% CO_2. Short tandem repeat (STR) analysis and a mycoplasma detection test were used to evaluate and verify all cell lines.

MTT assay

The MTT assay was conducted in accordance with the methodology outlined by Mohany *et al*. (2023). Briefly, Caco-2 and HCT-116 cells were seeded into 24-well plates at a density of roughly 5×10^4 cells per well and subsequently cultured for a duration of 24 hours. Next, the cells were treated with a various concentration (62.5, 125, 250,375,500 and 625 μg/ml) of Streptomyces ethyl acetate extract. After the incubation period, 100 μl of 5 mg/ml MTT was added to each well, followed by a 4-hour incubation. The formazan crystal product was solubilized by 1000 μl of acidified- isopropanol. Absorbance at 450 nm was measured on a plate reader (Zenyth 200 ST, Biochrom, UK). The calculation of cell viability percentage was performed using the subsequent equation:

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

The IC_{50} values (extract concentration needed to inhibit cell proliferation by 50%) were calculated using the OriginPro software (OriginLab, Northampton, MA, USA).

Microscopic examination

Caco-2 and HCT-116 cell lines were cultured and distributed evenly in 24-well plates. Following a 24 hrs period, the cells were subjected to incubation with an ethyl acetate extract derived from Streptomyces at doses equivalent to half of the inhibitory concentration (IC_{50}) and the IC_{50} cells itself for a duration of 48 hours. The morphological alterations of control and treated cells were analyzed utilizing a phase contrast inverted microscope. (Leica MC-170 HD camera, Germany) at 100 X magnifications.

Cell cycle analysis

Using flow cytometry measurement of DNA content stained with propidium iodide (PI), the distribution of cellular phases (G1, S and G2/M) were analyzed to assess the therapeutic effects of the extract as previously described (Nunez, 2001). In brief, both Caco-2 and HCT-116 cells were seeded in a 6-well plate. After 48 h, the cells were treated with extract at ½ IC_{50} and IC_{50} for the low and high concentrations for 24 h. The cells were then washed with PBS, harvested and then collected by centrifugation. Cells were fixed by adding cold 70% ethanol and incubated at -20°C. After that, the cells were subjected to centrifugation, washed twice with PBS and re-suspended in 500 μl of propidium iodide (PI) staining solution (50 μg/μl RNase A and 100 μg/ml of propidium iodide). Samples were analyzed using a Beckman Coulter flow cytometer.

Apoptosis assay with Annexin V/PI

The process of identifying apoptotic and necrotic cells was carried out by employing the Annexin V/PI assay, following the directions provided by the manufacturer (BioLegend, CA, USA). In brief, both Caco-2 and HCT-116 cells were treated with ½ IC_{50} and IC_{50} of extract for 48 h. Following the completion of the treatment, both detached and adhering cells were gathered and subsequently rinsed twice with cold phosphate-buffered saline (PBS). Next, 100 μl of annexin binding buffer was employed to reconstitute the cell pellet, which was subsequently transferred to flow cytometer tubes. Thereafter, the cells that had been resuspended were subjected to incubation with 5 μl of Annexin-V and 5 μl of PI for a duration of 15 minutes at room temperature, while being kept in darkness. Annexin binding buffer (400 μl) of was

then added to each sample tube and the cells were analyzed by flow cytometry.

Statistical analysis

Results are expressed as mean±standard deviation (SD). Comparisons between the different groups were evaluated using a one-tailed Student's t-test and one-way ANOVA. The level of significance was set at 0.05. For all tests, $p<0.05$ was considered significant.

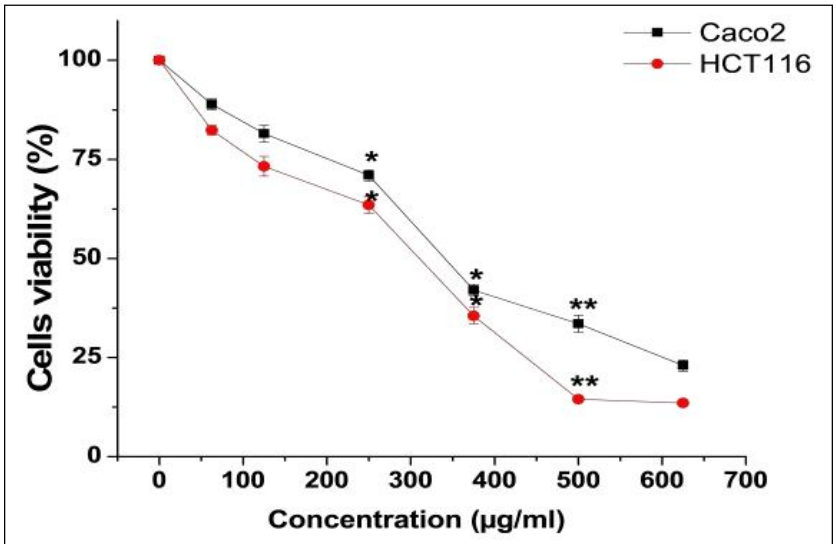
RESULTS AND DISCUSSION

Inhibition of Caco-2 and HCT-116 cells proliferation treated with Streptomyces ethylacetate extract.

The effect of Streptomyces ethyl acetate extract treatment on the growth of Caco-2 and HCT-116 colon cancer cell lines was determined. As shown in Fig 1, extract treatments showed remarkable inhibition of both cells' proliferation in a dose-dependent manner. Differences in the sensitivity of both cell lines to Streptomyces ethyl acetate extract were reported. The Caco-2 cells were much less sensitive to the extract (IC_{50}=340±3.2 µl/ml) compared to HCT-116 cells (IC_{50}=306±2.6 µg/ml).

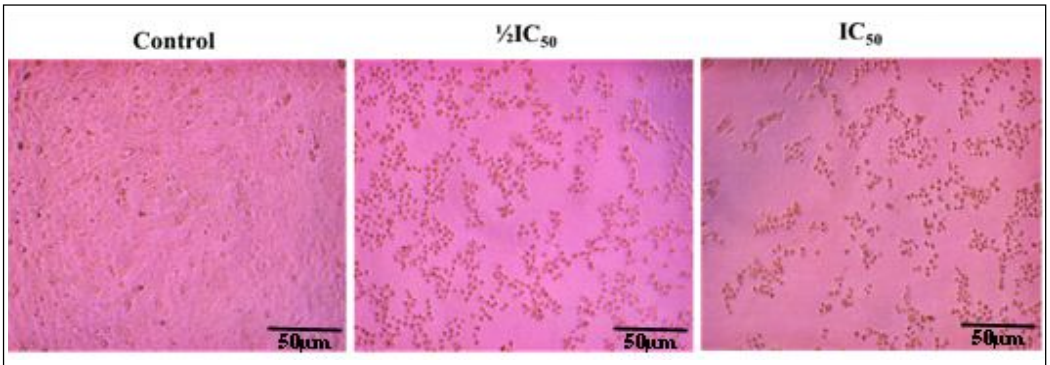
Microscopic examination

Light microscopy was employed to investigate the morphological effects of half IC_{50} and IC_{50} concentrations on the Caco-2 and HCT-116 colon cancer cell line after 48 h of exposure. As shown in Fig 2, untreated Caco-2 cells were well adherent with uniform-sized cells, while remarkable morphological changes of the Caco-2 cells colon cancer cells in response to the treatment were observed. As shown in Fig 2, most Caco-2 cells were detached, spherical and



Various amounts were applied to the cells and the subsequent cell proliferation was assessed using the MTT test. Various amounts were treated with different concentrations and cell proliferation was measured using the MTT assay. The cells were incubated with extract for 48 hand absorbance. Data were analyzed for three experiments.

Fig 1: Effect of the Streptomyces ethyl acetate extract on Caco-2 and HCT-116 cell growth.



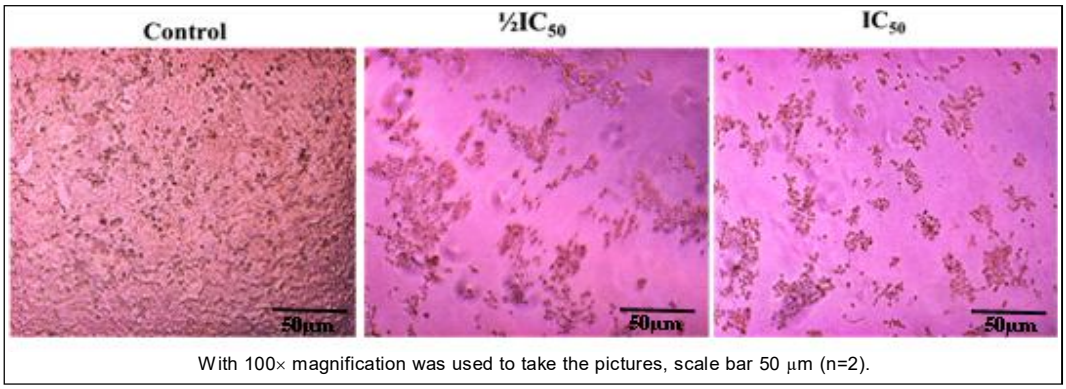
The control or untreated cells appear healthy and attached to the surface while treated cells are almost circular and detached from the surface of the dish. A phase contrast microscope (Leica, Germany) with a 100× magnification was used to snapshot the pictures, scale bar 50 µm. (n=2).

Fig 2: Morphological changes of Caco-2 cells treated with the indicated concentrations of Streptomyces ethyl acetate for 48 hours.

non-adherent, which is an important characteristic of cell death. In the same manner, treatment of the HCT-116 colon cancer cell line with extract led to visible changes in the shape of the cells and cellular shrinking, which are all features associated with cells undergoing apoptosis (Fig 3). A noticeable decrease in the cell population was seen when comparing the untreated cells with the treated cells (Fig 2 and 3). All these abnormal changes in treated cells provided an initial insight into the cytotoxic effect of Streptomyces ethyl acetate extract on colon cancer cells.

Cell cycle analysis

This study examined the influence of Streptomyces ethyl acetate extract on Caco-2 cell cycle distribution. Flow cytometry analysis measured the amount of DNA in each cell with a propidium iodide staining solution. As shown in (Fig 4), The cells treated with extracts exhibited a dose-dependent increase in the quantity of cells in the G1 phase. The number of G1 cells increased from 57.05±0.2% in control to 62.45±0.1% ($p<.05$) and 65.85±0.2% ($p<.05$) after treatments with half IC_{50} and IC_{50} concentrations, respectively. Similarly, HCT-116 cells treatment by the extract showed cell cycle arrest in a dose-dependent manner, as reflected by the increase in the percentages of cells in the G1 phase compared to control cells (Fig 5). The number of G1 cells was increased from 56.65±1.62% in control to 68.35±0.2% ($p<.05$) and 77.1±0.3% ($p<.05$) after treatments with half IC_{50} and IC_{50} concentrations, respectively.



With 100× magnification was used to take the pictures, scale bar 50 µm (n=2).

Fig 3: Morphological alterations in HCT-116 cells the cellular specimens were subjected to treatment with the designated concentrations for a duration of 48 hours.

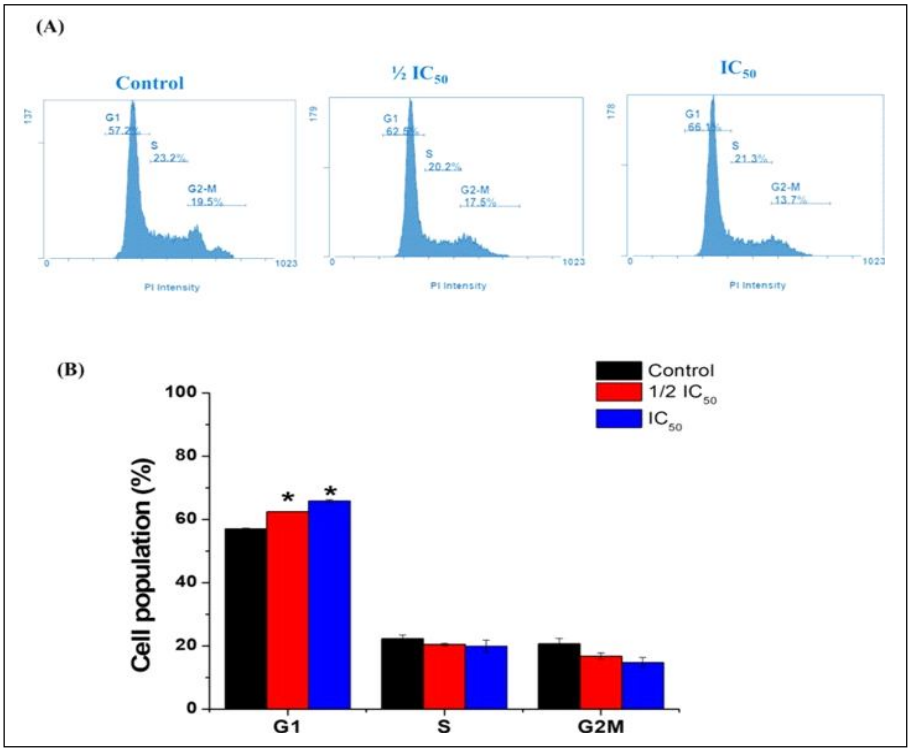


Fig 4: A Flow cytometric analysis showing representative figures of the cell cycle phases' DNA content distribution B. The data were shown as means±SD from three experiments.

Apoptosis detection

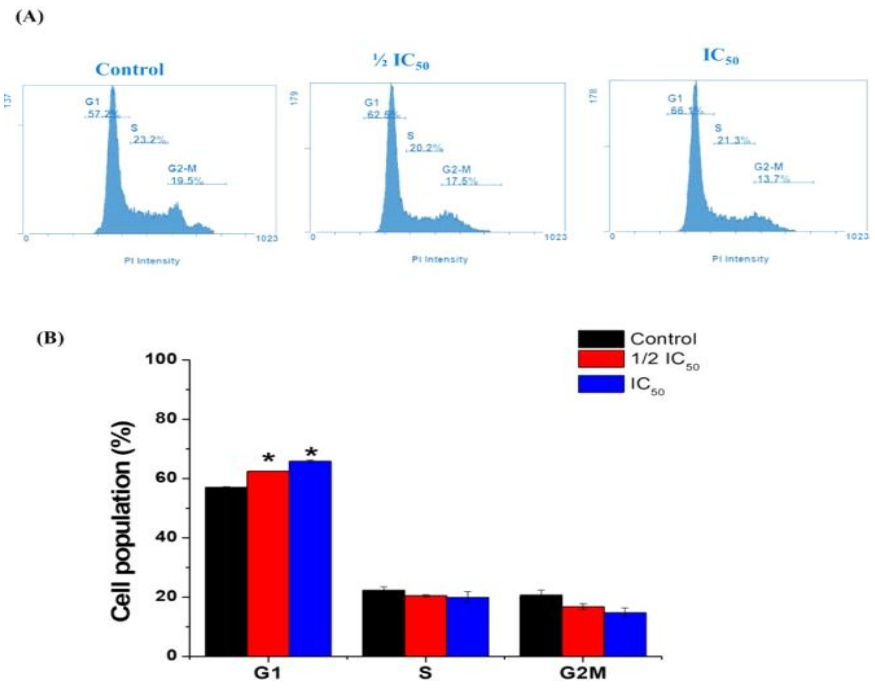
Phase-contrast microscopy and G1 cell cycle arrest revealed that Streptomyces ethyl acetate extract might exert its cytotoxic effects on Caco-2 and HCT116 cells through a mechanism that is linked to apoptotic cell death. Therefore, flow cytometry checked the percentage of live cells, apoptosis and necrosis after treatment.

As shown in Fig 6, Caco-2 cells treatment with Streptomyces ethyl acetate extract increased apoptotic cells undergoing apoptosis (increased to 13.25±1.2% and 33±1.97%) when compared to the control (2.55±0.07%) ($p<.05$). However, the treatment with extract also brought about a modest shift in the proportion of cells that had become necrotic. HCT-116 cells treated with extract also demonstrated a significant increase ($p<0.05$) in the apoptotic cells (21.75±0.21% and 40.45±1.2%) in comparison to control cells (4.5±0.56%) (Fig 7).

Colo-rectal carcinoma is the third most common kind of cancer yet the second leading cause of cancer-related deaths overall (Sung *et al*., 2021). Chemotherapy, surgery, or radiotherapy are the current therapy for cancer patients; nevertheless, these methods have significant drawbacks, including the death of healthy cells. Scientists used the chemoprevention technique, which involves using natural or manufactured compounds (alone or in combination) that are capable of suppressing the growth of tumors, to defeat cancer in humans (Husain *et al*., 2019). Several studies have shown the ability of Streptomyces to produce secondary metabolites as anti-cancer agents (Lin *et al*., 2021; Rajivgandhi *et al*., 2020; Al-Joubori *et al*., 2023). This investigation set out to determine whether or not Streptomyces ethyl acetate fraction might inhibit the growth of Caco-2 and HCT-116 cells and, if so, how it did so.

Firstly, to assess the cytotoxic effects of Streptomyces extracts in target colon cells, MTT assay was employed. We found that the two cells responded slightly differently to the same extract's cytotoxicity. This variation in the level of cytotoxicity could be due to the cell's unique genetic makeup which causes a unique susceptibility towards the extract (Law *et al*., 2017). Similarly, Law *et al*. (2019), documented a different cytotoxic effect of several Streptomyces isolates from a mangrove environment against a panel of colon cancer cell lines (HCT-116, HT-29, Caco-2 and SW480). The extracts exhibited a greater cytotoxicity towards SW480 and HT-29 cells while the remaining cells showed a slight effect (Law *et al*., 2019).

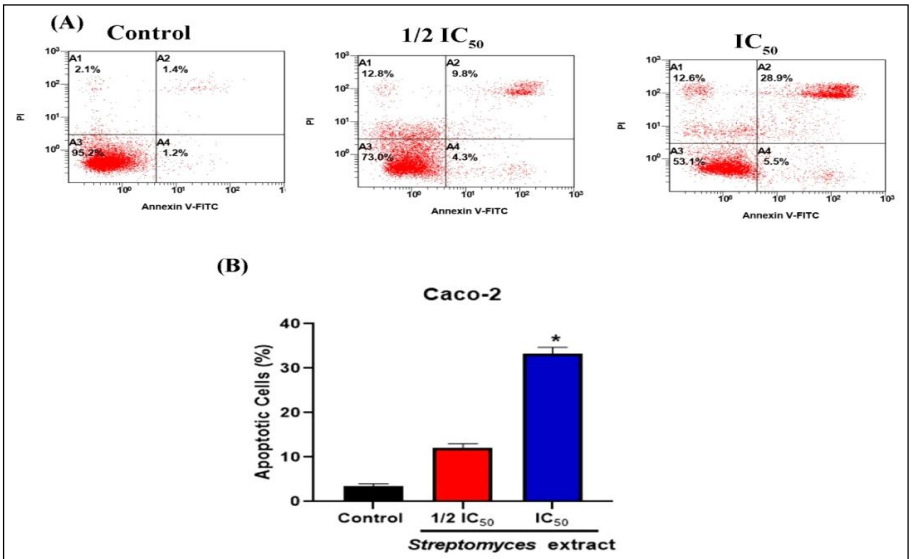
Previous investigations have revealed the cytotoxic effects of many Streptomyces extracts on different colon cancer cell lines. According to the findings of the study that (Tan *et al*., 2019), after being treated with an extract from *Streptomyces* sp. MUM265, Caco-2 cells were shown to have a lower level of viable cellular content than before



Bars representing cell cycle distribution percentage. Every value is the average of three experiments. Statistical analysis used one-way ANOVA. Differences between the treatment and the control group with p values of <0.05 (*), are significant statistically.

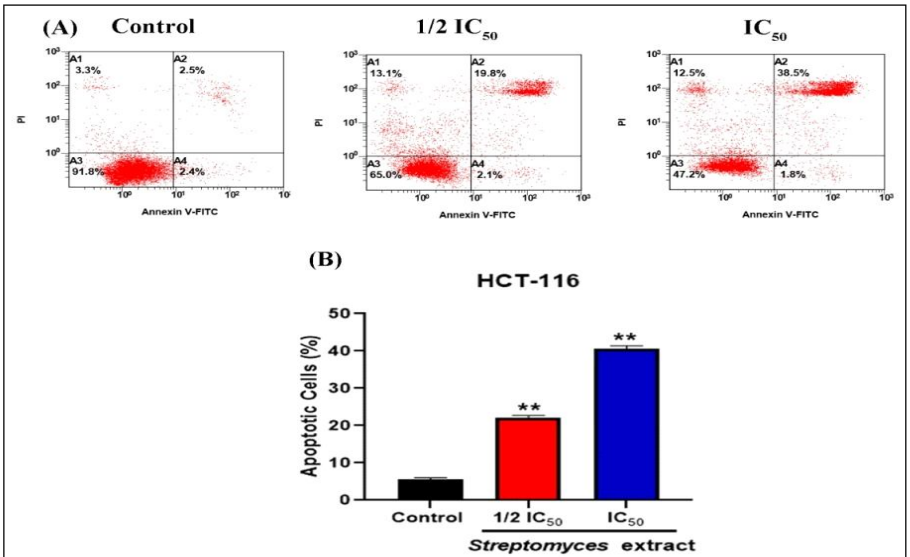
Fig 5: Effect of Streptomyces ethyl acetate extract in cell cycle distributions of HCT-116 cells. After being exposed to the extract for 48 hours, cells were propidium iodide stained and subjected to flow cytometric analysis (A), Representative histograms of the PI stained DNA, showing the cell cycle phases (B).

(a strain that was isolated from Kuala Selangor mangrove forest, Malaysia) at 400 µg/mL where the cell viability decreased to 34.57±4.99%. In this study, we obtained a close result against the same cells (Caco-2), where the cell viability decreased to 50% at 340 µg/mL. In another study, polysaccharides produced by four streptomycete isolates collected from marine sediment showed promising activity against colon carcinoma (HCT-116). The values of IC_{50} for four isolates against HCT-116 were very low compared to the results obtained in this study and this can be attributed to using a specific class of compounds (polysaccharides) (Selim *et al*., 2018).



Data are presented as average±SD of three experiments. Statistical analysis was conducted by one-way ANOVA. Differences between the treatment and the control group with p values of <0.05 (*), are considered statistically significant.

Fig 6: The effects of Streptomyces ethyl acetate extract on apoptosis induction in Caco-2 colon cancer cells. Cells were treated with indicated concentrations and Annexin V-FITC/PI double-stained was performed (A), In each plot the lower left quadrant (A3) represents viable cells, the upper left quadrant (A1) indicates necrotic cells and quadrants (A2 and A4) denote apoptotic cells (B).



Values are expressed as mean±SD from three experiments. Statistical analysis was conducted by one-way ANOVA. Differences between the treatment and the control group with p values of **p<0.001 are considered statistically significant.

Fig 7: Streptomyces ethyl acetate extract induces apoptosis in HCT-116 cells. Apoptosis rates of HCT-116 cells were detected by flow cytometry using Annexin V-FITC/PI double staining (A), Histogram of stained cells (B).

The investigation also involved assessing the cytotoxic effects of the Streptomyces extract on Caco-2 and HCT-116 cancer cells by observing any morphological alterations. Apoptosis is a sort of controlled cell death that is distinguished by the detachment and contraction of cells, the occurrence of significant blebbing in the plasma membrane and the generation of apoptotic bodies and nuclear fragments (Elmore, 2007). The present study involved the observation of distinct apoptotic features, including cell shrinkage, rounding and detachment, in colon cells that were subjected to treatment with Streptomyces extract. These observations suggest that the extract had cytotoxic properties, leading to apoptosis in the treated cells.

The effect of the ethyl acetate extract from Streptomyces on cell cycle distribution was observed in both of the examined cell types. The findings of the investigation on the cell cycle provided evidence in favor of the anti-proliferative action of the Streptomyces extract. Specifically, the study demonstrated that the extract significantly impeded the progression of the cell cycle by inducing a substantial reduction in the population of cells in the G1 phase. It follows that the extract may interfere with proteins critical for transitioning cells from G-phase to S-phase of the cell cycle. The findings of our study were consistent with the research conducted by Kouroshnia *et al*. (2022), which demonstrated that the ethyl acetate extract derived from a Streptomyces strain inhibited the advancement of the cell cycle specifically in the S phase of SW480, HCT 116 and HT-29 colon cancer cell lines (Kouroshnia *et al*., 2022). A comparable cell cycle arrest in the G1 phase was also documented subsequent to the administration of SW40 cells in colon cancer cells. The extract derived from Streptomyces Levis ABRIINW111 (Maragheh *et al*., 2018).

The induction of apoptosis holds significant importance in cancer therapy, since it represents a fundamental objective in numerous treatment approaches (Wong, 2011). To assess the cell death mode initiated by the Streptomyces extract, Annexin-FITC/PI was employed to discriminate whether cells undergoing apoptosis or necrosis cell death. This assay showed that ethyl acetate Streptomyces extract induced a significant increase in the percentage of cells undergoing apoptosis. This agrees with the findings of Kouroshnia *et al*. (2022) who showed that organic extract of fermented supernatant of Streptomyces strain C 801 induced apoptosis within colon cancer cell lines such as HT-29, HCT-116 and SW480 cell lines Similarly, apoptosis-inducing effects were also reported by *Streptomyces* sp. MUM265 on Caco-2 colon cancer cells (Tan *et al*., 2019).

There are some limitations to our study as well, which must be noted. We started by using cell lines that ought to be validated by *in vivo* tests using a tested animal model. Second, the secondary metabolites produced by streptomyces extract were not identified; only the crude extract was evaluated. Therefore, additional research is required to validate our results and investigate the secondary metabolites behind Streptomyces' anticancer potential.

CONCLUSION

In conclusion, the results of this investigation show that Streptomyces extract has powerful anticancer abilities. The extract also induced apoptosis, the most frequent cell death process seen in effective anticancer treatments and suppressed colon cancer cell lines. Microscopic analysis showed that Streptomyces extract reduced the proliferation of colon cancer cells and caused several morphological alterations associated with apoptosis. These cells were susceptible to apoptosis induced by streptomyces extract through a number of mechanisms, including overexpression of Bax, downregulation of Bcl-2 and activation of caspase-3. The Streptomyces ethyl acetate extract produced oxidative stress, which in turn induced apoptosis. These results are very significant since they provide proof that Streptomyces secondary metabolites had a potential role in inhibiting colon cancer cell growth through cell arrest and induction of apoptosis, however, further studies are required to determine the exact molecular mechanisms to be used as colon cancer treatment.

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Institutional review board statement

The study did not require ethical approval.

Informed consent statement

Not applicable.

Data availability statement

All data were included in the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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