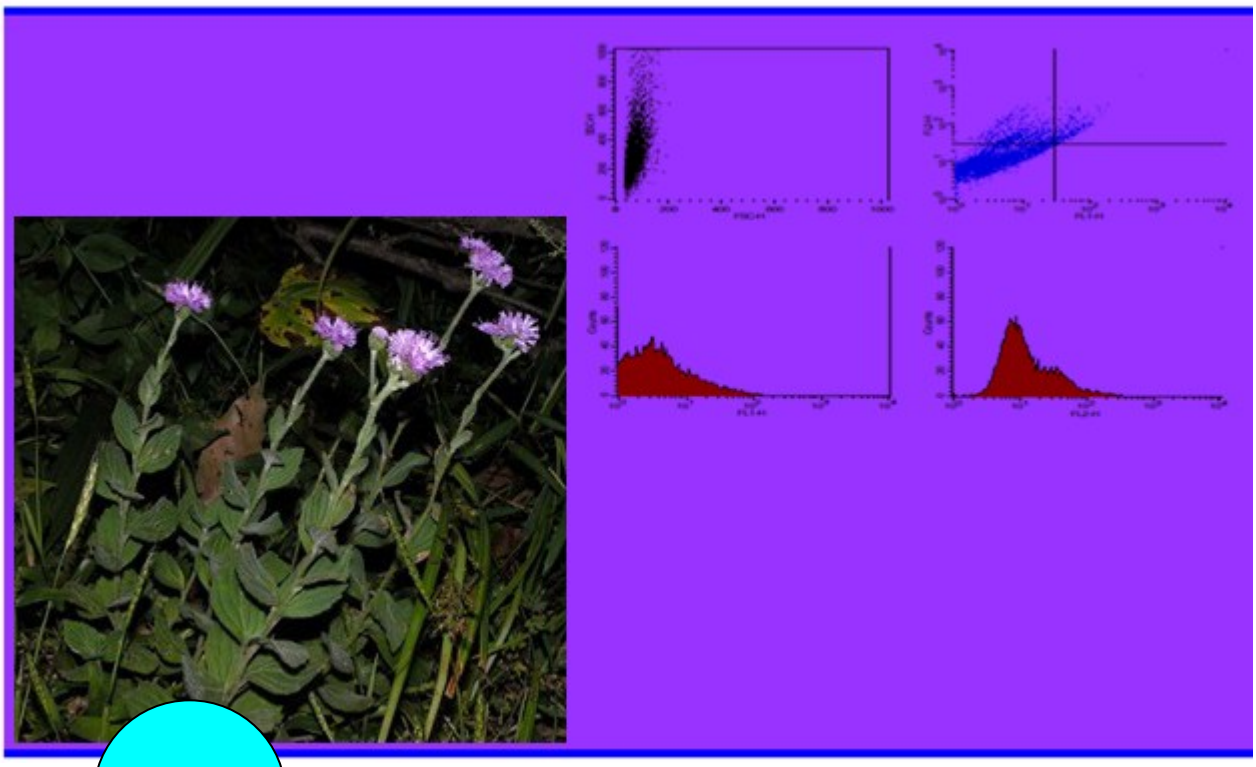

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Cytotoxic, apoptotic, and anti-inflammatory properties of *Adenoon indicum* leaf methanolic extract using HT29 cell lines

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Abstract

In the present study, methanolic extract of *Adenoon indicum* leaves is investigated for its cytotoxic, apoptotic, and anti-inflammatory properties using HT29 cell lines. The extract yield was 1.6%. MTT assay of HT-29 cells shows that the extract's IC_{50} value was 80 µg/mL with 10 µg/mL for doxorubicin, confirming its cytotoxic effect. Flow cytometry analysis demonstrated that extract caused cell cycle arrest, with 2.3% of cells in the G2/M phase and 32.2% in the S phase at a dosage of 160 µg/mL. Propidium iodide/annexin V staining revealed that the extract caused 3.1% early apoptosis and 6.0% late apoptosis, compared to 0% early apoptosis and 13.4% late apoptosis with doxorubicin at 11 µg/mL and anti-inflammatory effect in the egg albumin denaturation inhibition assay. The results show methanolic extract of *A. indicum* has anti-cancer activities against colorectal cancer via triggering apoptosis, and cell cycle arrest. In addition, it has an anti-inflammatory effect.

Introduction

Colorectal cancer is one of the main causes of death in developed nations. Red and processed meats, excessive alcohol intake, body and belly obesity, NSAID use, Crohn's disease, ulcerative colitis, and characteristics associated with higher ages are among its risk factors (Saleh et al., 2023; Ahmed et al., 2007). Currently, pharmaceutical therapy, radiotherapy, and surgical procedures are applied for the management of colorectal cancer.

Currently, the utilization of herbal phytoconstituents with potential anti-cancer against colorectal cancer treatment and management.

Medicinal plants that contain secondary metabolites show potential against colorectal cancer like those present in *Astragali radix* and *Glycyrrhizae radix et Rhizoma* (licorice root). Treatment for colorectal cancer benefits from these drugs' anti-inflammatory, immunomodulatory, and antiproliferative properties. For example, *Glycyrrhiza glabra* contains glycyrrhizin and glycyrrhetinic acid to help reduce the pathophysiology of colorectal cancer, whereas *Astragali Radix's calycosin* inhibits the growth of cancer cells via particular biochemical pathways (Deshmukh et al., 2024; Zhang et al., 2024; Hu et al., 2024).

Adenoon indicum Dalzell is a native plant of the western ghats of India commonly known as blue sonaki, or motha sonaki. *Adenoon indicum* from the *Asteraceae* (Compositae) family has been used in the treatment of ulcers, gastric irritations, and wound healing locally (Mane et al., 2016). There is no scientific evidence for the anti-cancer and anti-inflammatory activity of *A. indicum*. Hence, the aim of the present study is to evaluate the anti-cancer and anti-inflammatory activity of *A. indicum* methanol extract.

Materials and Methods

Chemicals

All chemicals and reagents for the current study i.e. methanol, ethanol, dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum, trypsin, EDTA, and DMEM were procured from Sigma-Aldrich and Gibco (India). HT29 cell lines were procured from the American Type Culture Collection (ATCC).

Collection and authentication of plant

Leaves of *A. indicum* were collected from local areas of the Western ghat (Amboli), Kolhapur in the August to October period when this seasonal plant is abundantly available. The plant *A. indicum* was identified by comparing the characteristic features mentioned in the available literature. The plant was authenticated by Dr. V. S. Jadhav at the Department of Botany, Shivaji University, Kolhapur. The collected leaves were air-dried at the room temperature for 6 to 7 days, then the plant materials were subjected to crushing, resulting in coarsely powdered particles, and stored for further studies.

Extraction

Plant leaf crushed powder (500 g) were used in a soxhlet system to extract plant material. Petroleum ether, acetone, ethyl acetate, chloroform, and methanol were employed with increasing polarity. The extracts were then obtained utilizing a rotary evaporator (Supervac, India) to evaporate the solvents under reduced pressure, and the yields were noted (Patil et al., 2024).

Cell culture

The stock HT29 cell lines were cultured in DMEM with 10% inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated using 0.05% trypsin and centrifuged (Remi CM-8 Plus, India) at 1,000 rpm for 5 min. The culture media was discarded, and the cell pellet was gently re-suspended using 2 mL DMEM complete media. The viability of the cells was checked, and a single cell suspension of 5.0 x 10^5 cells/mL was prepared.

MTT assay

To each well of the pre-labeled 96-well microtiter plate, 100 µL of the prepared cell suspension (50,000 cells/well) was added and incubated at 37°C with 5% CO_2. After 24 hours of incubation, the supernatant was removed, and the monolayer was rinsed with DMEM. To each pre-designated well, 100 µL of petroleum ether extract and stigmasterol (unsaturated phytosterol) at various concentrations were added and incubated for 24 hours. After incubation, the test solutions in the wells were discarded and 100 µL of MTT reagent (4 mg/10 mL of MTT in phosphate buffer solution) was added to each well. The plates were incubated for 4 hours at 37°C in 5% CO_2. The supernatant was removed, 100 µL of dimethyl sulfoxide was added, and the plates were gently shaken to solubilize the formazan crystals. The absorbance was measured using a microplate reader (Spectramax i3X, South Africa) at 590 nm wavelength. The percentage growth inhibition was calculated using a formula and the concentration of test drug to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line using GraphPad Prism 5.0 software (Ali et al., 2021; Turker et al., 2023).

Calculating inhibition:

Box 1: Egg albumin denaturation assay

Principle

A in vitro study of the anti-inflammatory effect of a plant extract by denaturation of protein (hen albumin, bovine serum albumin, etc) by heat.

Requirements

96-Well plate, A. *indicum* leaf extracts, Diclofenac, Hen egg, Micropipette, Microplate reader, Shaker, Tween 80, Water bath

Procedure

Step 1: The egg albumin was collected from freshly laid hen egg.

Step 2: Different concentrations of the isolated compounds and diclofenac) at concentrations (3.9-500 µg/mL) were prepared by serial dilution.

Step 3: The test tubes were filled with 5 mL of the reagent mixture which contained 0.2 mL of egg albumin, 2.8 mL of phosphate-buffered saline (pH 6.4) and 2 mL of compound/standard drug reconstituted with 2% tween 80 in double-distilled water.

Step 4: Double-distilled water was used as the control (blank). The mixtures were mixed slowly by shaking the test tubes, incubating at 37 C for 15 min, and then kept in a water bath at 70°C for 5 min.

Step 5: The mixture was cooled and 200 µL pipetted into wells of 96-well plate and the absorbance was measured at 660 nm using a microplate reader.

Calculation

The %inhibition of protein denaturation was estimated by considering denaturation in control as 100% and calculated by using the following formula:

%Inhibition = 100 x [At/Ac] - 1

Whereas, At = absorbance of a test sample; Ac = absorbance of the control sample

References

Chandra et al., 2012; Anokwah et al., 2022

%Inhibition = ((OD of control – OD of sample))/OD of control) x 100

Apoptosis by flow cytometry

Cells were plated 1 x 10^6 cells per well in the 6-well plate in a respected cell culture medium and incubated at the standard condition of 5% CO_2 at 37℃. After overnight incubation, the medium was removed gently by pipette and replaced with a new fresh culture medium (without fetal bovine serum). Further, the cells were treated with respective concentrations of respective samples and incubated for 24 hours at the standard condition of a CO_2 incubator. Later, the cell culture medium was collected into Ria tubes to avoid loss of cells due to cell death. Using 0.05% trypsin, the remaining cells were detached from the 6-well plate and 1 mL of medium was added to stop trypsin activity in each well and transferred the contents to the Ria tubes. Centrifuged the cell suspension at 4,000 rpm for 5 min at 4℃ and discarded the supernatant. Cells were fixed by resuspending in 300 µL of sheath fluid followed by the addition of 1 mL of chilled 70% ethanol drop by drop with continuous gentle shaking and another 1 mL of chilled 70% ethanol was added at once. The cells were then stored at 4°C overnight. Post fixing the cells were centrifuged at 4,000 rpm for 5 min. The cell pellet was washed twice with 2 mL of cold 1 x PBS (phosphate buffer solution). The cell pellet was then resuspended in 300 µL of sheath fluid containing propidium iodide/annexin V (0.05 mg/mL) and RNase A (0.05 mg/mL) and incubated for 15 min in the dark. The percentage of cells in various stages of the cell cycle in compounds treated and untreated populations was determined using FACS Caliber (BD Biosciences, USA). After staining, the cells were acquired by flow cytometer as soon as possible (within 1 hour) using an FL2 filter and further analyzed by Cell Quest software (Khan et al., 2021).

Statistical analysis

The results were reported as mean ± SD. The experiments were repeated three times, each in duplicate. A one-way ANOVA test was conducted to identify differences, and results were graphed using Graph Pad Prism 6.01 software. A p-value of <0.05 was considered statistically significant.

Results

Extract yield

The methanolic extract yield of *A. Indicum* leaves were obtained 7.9 g (1.6%).

MTT assay

The cytotoxic activity of the methanolic extract of *A. indicum* and doxorubicin was determined using the MTT test, with results provided as IC_{50}. Figure 1 illustrates the IC_{50} values of the *A. indicum* methanolic extract and doxorubicin as standard (80 µg/mL and 10 µg/mL, respectively). *A. indicum* methanolic extract shows great anti-cancer activity against HT-29 cancer cells. At a lower dose of 10 µg/mL and the highest dose of 160 µg/mL, the plant extract showed 29.2% and 96.4% inhibition of cancer cells. This indicates the dose-dependent anti-cancer potential of *A. indicum* methanolic extract.

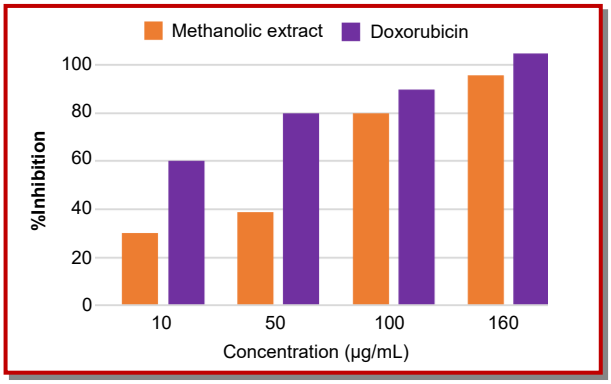


Figure 1: MTT cytotoxicity assay with determination of IC_{50} value for the methanolic extracts of *A. indicum*

Flow cytometry

The impact of the extract on the cell cycle phases of the HT-29 cell line is shown (Figures 2). The control, doxorubicin and extract show a reduction in population up to 85.0, 78.7 and 68.7% respectively during the G0/G1 phase. Control cells show 12.1% of the cell population in the S phase. Whereas treatment of cells with doxorubicin and extract for 24 hours resulted in an increased cell population of 12.1 to 22.4% and 29.19% respectively in the S phase of the cell cycle. This significant increase in the S phase indicated an increased proportion of cells with reduced DNA content, resulting in apoptosis. These findings reveal that *A. indicum* methanolic extract mainly targets the S phase of cell cycle arrest in colon cancer cell lines and shows anti-cancer activity through this mechanism.

Apoptosis

Figure 3 shows the histograms of untreated HT-29 cells, doxorubicin-treated cells, and extract-treated cells respectively. Extract at 160 µg/mL induced 3.1% early apoptosis and 6.0% late apoptosis in cells when compared to control cells. While the doxorubicin 11 µg/mL induced 0.0% early apoptosis and 13.4% late apoptosis in cells when compared to control HT-29 cells.

Egg albumin denaturation assay

The methanolic extract of *A. indicum* and diclofenac was investigated for anti-inflammatory activity using egg albumin denaturation inhibition assay. All the methanolic extracts of *A. indicum* showed concentration-dependent anti-inflammatory activity (Figure 4).

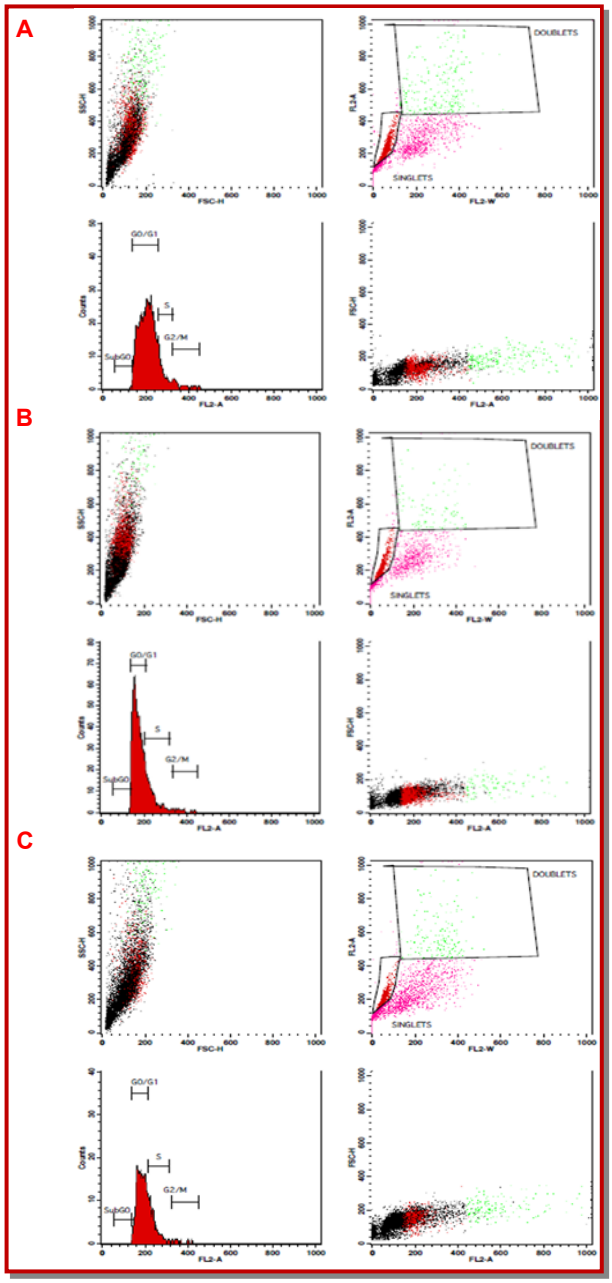
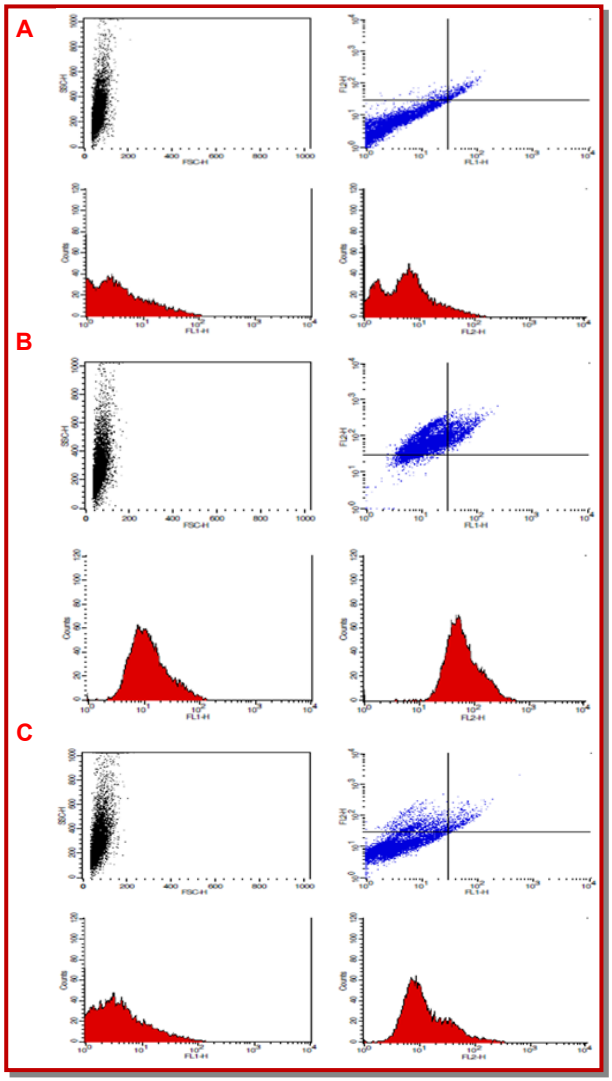


Figure 2: Cell cycle study of untreated HT-29 cells (A), doxorubicin-treated HT-29 cells (B) and extract-treated HT-29 cells (C) by flow cytometry

Discussion

In this study, the methanolic extract of *A. indicum* exhibits significant anti-cancer activity against HT-29 colon cancer cells using an *in vitro* approach. The extract not only inhibits cell proliferation in a dose-dependent manner but also induces apoptosis, particularly affecting the S phase of the cell cycle. The *in vitro* protein denaturation assay of methanolic extract of *A. indicum* shows anti-inflammatory efficacy in a concentration-dependent manner by successfully suppressing protein denaturation.



Figures 3: Apoptosis detection using annexin V-FITC and propidium staining of untreated HT-29 cells (A), doxorubicin-treated HT-29 cells (B) and extract-treated HT-29 cells (C) by flow cytometry

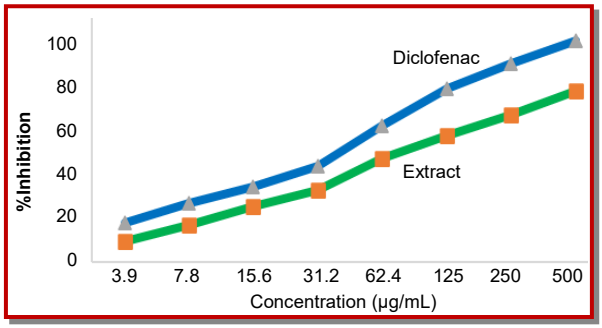


Figure 4: Effect of methanolic extract of *A. indicum* and diclofenac at concentration range (3.9-500 µg/mL) on protein denaturation expressed as %inhibition of egg albumin denaturation

A. indicum belongs to the genus *Adenoon* and belongs to the family Asteraceae. It has only one species. The genus is endemic and mainly found in forests of the

Northern Western Ghats of India. There is no phytochemical study of *A. indicum*. These findings emphasize the methanolic extract of *A. indicum* powerful cytotoxic and apoptotic actions, confirming its status as natural anti-cancer properties (Sengupta et al., 2022; Kushwaha et al., 2019; Gao et al., 2018; Marciniak et al., 2023). Certain medicinal plants have anti-inflammatory properties due to their bioactive components, which include flavonoids, alkaloids, and sterols. Medicinal plants such as *Curcuma longa, Withania somnifera,* and *Boswellia serrata*, influence critical signaling pathways (SoRelle et al., 2013).

A. indicum may have anti-inflammatory and anti-cancer properties through various molecular mechanisms. Inhibiting NF-κB signaling may reduce the expression of pro-inflammatory cytokines, including TNF-α, IL-6, and COX-2. Furthermore, it may influence apoptosis by activating caspase-dependent pathways and inhibiting anti-apoptotic proteins like Bcl-2. The chemical may also interfere with PI3K/Akt and MAPK signaling, reducing cancer cell growth and survival.

While findings are consistent with earlier studies on phytosterols' anti-cancer potential, there were exceptions in activity (Patel et al., 2022; Awad et al., 2000; He et al., 2020). Previous research has focused on related chemicals, such as β-sitosterol and stigmasterol, which have similar but unique effects on cancer cells. Compared to previous research, the methanolic extract of *A. indicum* demonstrated higher activity against colorectal cancer cells, possibly due to its unique structural bioactive phytoconstituents within it. Furthermore, When comparing these findings to previous studies, it is noted that while other research has also highlighted the anti-cancer properties of *A. indicum*, the specific mechanisms, and effects observed in this study provide new insights (Gopalakrishnan et al., 1997; Patel et al., 2022).

This suggests its ability to stabilize proteins under inflammatory conditions, similar to standard anti-inflammatory drugs, highlighting its therapeutic potential in managing inflammation-related disorders. The medicinal plant bioactive compounds help to reduce inflammation by interfering with cytokine signaling. They can suppress pro-inflammatory mediators such as TNF-α, IL-6, and COX-2 (Kim et al., 2011). These findings emphasize the therapeutic potential of plant-derived bioactives, implying their significance in alleviating inflammation-associated illnesses, including cancer.

This suggests that *A. indicum* methanolic extract could be a promising candidate for developing natural anti-cancer therapies, potentially offering an alternative or complement to conventional chemotherapy.

However, this study has limitations such as *in vivo* study and the need for isolation of active compounds for a better understanding of the mechanisms and therapeutic potential of *A. indicum*.

Conclusion

This study suggests the anti-cancer potential of methanolic extract of *A. indicum*, which induces apoptosis that inhibits colorectal cancer cell proliferation and also shows anti-inflammatory activity.

Financial Support

Self-funded

Ethical Issue

The cell line was maintained in Skanda Life Sciences Pvt. Ltd, DSIR recognized R & D centre, Bangalore for the conduct of cell line study as per the ethical guideline (SLSPL/CB/0224/172).

Conflict of Interest

The authors declare no conflict of interest.

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