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NECROSIS-INDUCING POTENTIAL AND ANTIOXIDANT ACTIVITY OF *Heliotropium indicum* L. ROOT EXTRACT IN HT-29 COLON CANCER CELL LINES

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ABSTRACT

***Heliotropium indicum* L., locally known as "Venal pacha," is an annual herbaceous weed belonging to the family Boraginaceae and is used in folk medicine. This study investigated the therapeutic potential of its root extract, with a focus on antioxidant activity and cytotoxic effects on HT-29 colon cancer cells. Antioxidant activity of five solvent extracts was evaluated using DPPH and reducing power assays. The ethyl acetate extract, which showed better anti-oxidant activity, was used for cytotoxicity study. The cytotoxicity against HT-29 cells was assessed via MTT assay. Apoptosis and necrosis were analysed using flow cytometry with Annexin V/PI staining, and cell cycle arrest was examined to assess the extract's impact on cell proliferation. The ethyl acetate extract exhibited strong antioxidant activity in both assays, supporting its traditional use in oxidative stress-related gut health. MTT assay revealed potent cytotoxicity (82.76% inhibition at 100 µg mL^{-1}, IC_{50} 3.04 µg mL^{-1}) with a dose-dependent response. Significant necrosis, particularly at lower doses, overshadowed apoptosis. Sub G0 cell cycle arrest highlighted interference in cancer cell proliferation. Statistical analysis was performed to determine the correlation between antioxidant activity and cytotoxicity using SPSS (version 28). The findings suggest that *H. indicum* L. root has anticancer potential owing to its necroptotic potential.**

Keywords: Antioxidant, cell cycle arrest, cytotoxicity, *Heliotropium indicum*, HT-29, necroptosis

INTRODUCTION

Heliotropium indicum L., commonly known as Indian heliotrope (family: Boraginaceae), is a widely distributed plant known for its diverse medicinal properties in traditional healthcare systems. The conventional use of *H. indicum* in various countries for treating diseases, including its application as an antidote in some contexts, has comprehensively been reviewed (Sarkar *et al*., 2021). *H. indicum* shows remarkable versatility in traditional medicine, with its different plant parts used to achieve contrasting therapeutic effects depending on their preparation and application. This diversity highlights the plant's unique pharmacological potential and adaptability across various cultures and medical systems. For instance, its inflorescence is traditionally employed for excessive bleeding and menstrual regulation (Togola *et al*., 2005). In Jamaica, infusion of its flowers is used to treat menorrhagia, relieving abnormally heavy menstrual bleeding (Asprey and Thornton, 1955). Conversely, in Philippines, the plant roots are used as a decoction to induce menstruation, particularly

in delayed or absent periods (Wiart, 2021). In Thailand, dried and powdered inflorescence is mixed with milk or water to sterilize females permanently (Sarkar *et al*., 2021). This contrast exemplifies how the same plant can serve opposing roles, depending on the plant part and preparation. In cases of amenorrhea, root decoctions are applied orally or through baths to regulate the menstrual cycle. The roots are also employed for blood purification and infections, where juice is used topically and orally to cleanse the body and treat ailments (Sarkar *et al*., 2021). Additionally, root preparations are used to treat ophthalmia (eye infections) and conditions like whooping cough, showcasing significance in addressing systemic and localized health issues (Gopinathan and Balasubramanian, 2020). These varied applications underline the root's versatility as a key component in ethnomedicinal practices. This wide-ranging therapeutic potential is underpinned by the plant's rich phytochemical composition, which likely varies across the plant parts (Osungunna and Adedeji, 2011; Rahman *et al*., 2011). The preparation method and dosage also play critical roles in modulating its effects, enabling *H. indicum* to address a diverse spectrum of ailments, from alleviating menstrual disorders to serving as an antidote for poisoning (Ghosh *et al.*, 2018). In Congo, Colombia, and Rodrigues Islands, *H. indicum* is traditionally used to treat gastrointestinal ailments, including bloating, loss of appetite, vomiting, diarrhoea, and other stomach-related issues (Sarkar *et al*., 2021). Compounds like (E)-ethyl-12-cyclo hexyl-4,5-dihydroxydodec-2-enoate isolated from *H. indicum* reportedly have gastroprotective abilities (Lopez-Lorenzo *et al*., 2022). It is used in traditional medicine across various regions in India for treating skin diseases, poison bites, stomach aches, nervous disorders (Kancheepuram district, Tamil Nadu), for dandruff relief with leaf juice boiled in coconut oil (Malasar tribes, Coimbatore), cuts and wounds with fresh leaf extract (tribes of Cachar district, Assam), and rheumatism with external application of leaf paste (Rayal Seema, Andhra Pradesh) (Gopinathan and Balasubramanian, 2020).

Necrosis plays dual role in cancer treatment, acting both as a potential therapeutic target and as a challenge to treatment efficacy. Inducing necrosis in cancer cells can effectively eliminate tumour through cell death mechanisms that release pro-inflammatory signals, potentially stimulating an immune response against tumour (Gong *et al*., 2019). However, necrosis can also contribute to the tumour microenvironment's complexity by releasing damage-associated molecular patterns that may promote inflammation, angiogenesis, and tumour progression in specific contexts (Yanai *et al*., 2021). Balancing the induction of necrosis while minimising adverse effects is crucial in developing cancer therapies, like targeted drugs and radiotherapy, aimed at enhancing tumour destruction while supporting antitumor immunity. Oxidative stress plays a pivotal role in the development and progression of cancer and other chronic diseases. This condition arises when there is an imbalance between the production of reactive oxygen species (ROS) and body's ability to detoxify these harmful byproducts or repair the damage (Sharifi-Rad *et al*., 2020). Antioxidants, which neutralize ROS, are vital in protecting the cells from oxidative damage and in preventing/treating oxidative stress-related diseases. Due to their pro-apoptotic and anti-proliferative qualities, natural antioxidants and several phytochemicals have lately been proposed as adjuvant therapy for cancer (Nguyen *et al*., 2020). Antioxidant-containing substances or medicines have long been known to offer protection against colon cancer (Umeaku *et al*., 2020).

Colorectal cancer is among the most prevalent cancers in the world. Apoptosis, autophagy, and necroptosis are among the cell death mechanisms that must be activated or reactivated for cytotoxic medication to kill cancer cells. One effective treatment approach for a variety of malignancies is to target the elements that cause cell death. Necroptosis is a controlled kind of cell death that resembles both necrosis and apoptosis in appearance. It is essential for preventing several illnesses and tumours (Alidadi *et al*., 2021).

Medicinal plants are regarded as a vital component of human health due to their ability to treat a wide range of illnesses. Both potentially hazardous and medicinally useful compounds are found in medicinal plants. Assays for cytotoxicity are used to forecast possible toxicity using either normal or modified cells in culture. These tests usually entail exposing cultured cells to test compounds for a brief period to ascertain how the chemical may impact fundamental or specialised cell processes,

before performing safety studies on entire organisms. Further, it can provide insight into genotoxic and carcinogenic characteristics of chemicals and extracts derived from herbs (Al Qaisi *et al*., 2024). In recent years, the therapeutic potential of medicinal plants has gained significant attention, particularly for their antioxidant and anticancer properties. *H. indicum,* traditionally used for its diverse medicinal benefits, has shown promising bioactivity in preliminary studies. *H. indicum* roots, used both in traditional and modern medicines, reveal the presence of sterols, pentacyclic triterpenoids, flavonoids, coumarins, phenolic acids, and phenylpropanoids (Alexander and Sharma, 2011).

Oxidative stress is a major contributor to the pathophysiology of diseases like cancer, rheumatoid arthritis, Alzheimer's disease, Parkinson's disease, neurodegeneration, cirrhosis, arteriosclerosis, ageing, etc. It causes an excess of ROS, including free radicals. By donating electrons, antioxidants can neutralise them and stop tissue and cell damage. Flavonoids, polyphenols, tannins, carotenoids, and phenolic terpenes found in plant exhibit antioxidant activity by quenching free radicals in the body when antioxidant protection becomes unbalanced (Gopinathan and Balasubramanian, 2021). The present study was aimed to analyse the antioxidant potential and cytotoxic effects of *H. indicum* on colon cancer cell lines and workout correlation between antioxidant properties and cytotoxic effect of *H. indicum* root extracts. By assessing its ability to neutralize oxidative stress and selectively induce necrosis in cancer cells, it may provide critical insights into the plant's dual role as antioxidant and necrosis-inducing agent, highlighting its potential for targeted colon cancer therapy while ensuring its safety and efficacy.

MATERIALS AND METHODS

Plant collection and identification

Heliotropium indicum was collected from Thrissur district, Kerala (India). The identity of plant was authenticated at the Botanical Survey of India (BSI), Coimbatore (India), and the specimen deposited in the herbarium of BSI, Coimbatore under reference No. BSI/SRC/5/23/2018/Tech/412. The plant roots were washed, dried, powdered, and stored for further analysis.

Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid, potassium ferricyanide, trichloroacetic acid and methanol were purchased from HiMedia Laboratories, India. DMEM, FBS, penicillin, streptomycin, DPBS, RNase, and triton X-100 were purchased from HiMedia Laboratories, India; gentamycin from Abbott Healthcare Pvt. Ltd, India and DMSO and MTT from Sisco Research Laboratories, India. PBS-Sodium chloride was purchased from Nice Chemicals, potassium chloride from Medilise chemicals, India, dibasic sodium phosphate and monobasic potassium phosphate from Kanton laboratories, India, Annexin kit, 4x loading buffer, propidium iodide (PI) were purchased from Thermo Fisher Scientific Invitrogen Bioservices India Pvt. Ltd. India and ethanol was purchased from Central Drug House Pvt. Ltd., India. All solvents and chemicals used were analytical grade and were purchased commercially from HiMedia Laboratories, India.

Preparation of extract

The shade-dried root samples were subject to hot extraction using a Soxhlet apparatus for 24 h. Plant root were powdered and extracted successively with hexane, chloroform, ethyl acetate, methanol, and sterile distilled water. The solvent from these extracts was removed under reduced pressure using a rotary vacuum evaporator (Heidolf, Germany) and stored in sterile pre-weighed screw-capped containers at 4°C for analysis.

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl assay (DPPH)*:* The procedure outlined by Mensor *et al*. (2001) was used to assess the extract’s capacity to scavenge free radicals. The extract (1 mL) at different doses (50-

1000 µg mL^{-1}) was combined with 1 mL methanolic solution of DPPH (0.1 mM). After 30 min of dark incubation, the absorbance at 517 nm was determined using a spectrophotometer (Antech Model Number: AN-UV-6500N). The reference standard used was ascorbic acid. Methanol served as blank and methanol-DPPH without extract served as control. The concentration needed to scavenge 50% radicals or IC_{50} value was calculated using linear regression on a graph of percentage inhibition vs concentration. For every concentration, the mean radical scavenging activity (RSA) values were calculated. Each experiment was carried out in six replicates. DPPH radical's scavenging capacity was calculated using the equation:

$$\text{Radical scavenging activity (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

The regression graph was used to calculate IC_{50} values. For IC_{50} value, a linear regression line was constructed using the graph of average percentage radical scavenging activity (RSA) against extract concentrations.

Reducing power assay: The reducing power of the extracts was assessed using the methodology described by (Yen and Duh, 1994). 1.5 mL of 0.2 M sodium phosphate buffer at pH 6.6 and 1.5 mL of 1% potassium ferricyanide were combined with 500 µL of various extract concentrations. 10% trichloroacetic acid was added after 20 min incubation at 50°C. The mixture was then centrifuged for 5 min at 6000 rpm and 4°C (Thermo Scientific Sorvall ST 8R BT4A). The top layer was combined with 1.5 mL distilled water (each) and 300 µL of 0.1% ferric chloride. At 700 nm, absorbance was measured. The standard was ascorbic acid. To determine the scavenging potential of each extract, a concentration of 0.5 absorbance was set as reference. Each experiment was carried out in 6 replicates.

Cell line and maintenance

The HT-29 (colon cancer cell line) was bought from National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's modified Eagles medium (DMEM) was used to culture the cells. The 10% heat-inactivated foetal bovine serum (FBS) and 1% antibiotic cocktail comprising gentamycin (0.25 µg mL^{-1}), streptomycin (0.1 mg mL^{-1}), and penicillin (100 U mL^{-1}) were added. In cell culture incubator (Galaxy® 170, Eppendorf, Germany), the cells containing TC flasks (25 cm^2) were incubated at 37°C in a humid environment with 5% CO_2.

MTT assay

Cells @ 0.1 x 10^6 per well were seeded into 96-well plates and allowed to acclimate to the culture conditions, such as 37°C and 5% CO_2 environment in CO_2 incubator (Thermo Scientific, model 3111) for 24 h. A 0.2 µm millipore syringe filter was used to filter-sterilize the test samples after their preparation in DMSO (10 mg mL^{-1}). The concentrations tested were 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, and 100 µg mL^{-1}. The samples were introduced to the wells containing the cultured cells after dilution in DMEM medium (Chaves *et al.*, 2014). Untreated wells served as control. All experiments were conducted in six replicates. The plates were further incubated for 24 h following treatment with test samples. The medium from wells were aspirated and disposed of following the incubation period. The wells were filled with 100 µL of a 0.5 mg mL^{-1} MTT solution in PBS. For formazan crystal formation, the plates were incubated for a further 2 h. After removing the supernatant, 100 µL of 100% DMSO was applied to each well. A microplate reader was used to measure the absorbance at 570 nm. Each plate had three blank wells devoid of cells. Cell viability was calculated as per the following equation:

$$\text{Cell viability (\%)} = \frac{Average\ absorbance\ of\ treated\ cells}{Average\ absorbance\ of\ control\ cells} \times 100$$

The half maximal inhibitory concentration of sample is known as IC_{50} value. The IC_{50} values were calculated by plotting the average absorbance of various concentrations of test sample in Microsoft Excel, using the slope equation ($y = mx + C$).

Cell morphology

An inverted phase contrast microscope (Labomed, TCM 400, USA) was used to take representative

photomicrographs of the cells in each experimental group. Magnification- 10x. Total magnification- 100x (10x objective lens × 10x ocular lens).

Annexin V staining assay

Annexin V staining assay was carried out as per the method of Pumiputavon *et al*. (2017). To quantify apoptosis, cells (0.1 x 10^6 cells mL^{-1}) were cultured in 24-well plates and exposed to different sample concentrations (6.25, 12.5, 25, 50, 100 µg mL^{-1}) for 24 h. The experiment was started as soon as the cells reached 80-90% confluency. The Annexin V-FITC (fluorescein isothiocyanate) /PI (propidium iodide) co-staining assay was used to quantify the number of apoptotic cells. The HT-29 cells were trypsinized after a 24-h incubation period, and the pellets resuspended in 100 µL PBS and subsequently centrifuged for 8 min at 1800 rpm. The PBS was removed and resuspended in 100 µL 1X binding buffer. In all tubes (excluding the control, untreated, and control + PI tubes), 3 µL Annexin was added to polypropylene (PP) tube and incubated for 30 min in dark, at room temperature. Following incubation, 3 µL PI was added to a defined tube, and incubated for 5 min at room temperature in dark. Each FACS tube was then filled with 200 µL 1X binding buffer, and a flow cytometer (BD Biosciences FACS Diva 8.0.3) was used to examine the cells.

Cell cycle analysis

Cell cycle analysis was performed as per the procedure of Pumiputavon *et al*. (2017). HT-29 cells were cultivated at 37°C with 5% CO_2 in 6-well plates. The cells were treated with varying concentrations of extract (6.25, 12.5, 25, 50, and 100 µg mL^{-1}) along with a control when they reached 80 to 90% confluency. They were incubated for 24 h at 37°C with 5% CO_2. After treatment, the cells were trypsinized, washed with DPBS, and centrifuged (Thermo Scientific Sorvall ST 8R BT4A) for 8 min at 1800 rpm. After carefully removing the supernatant, 100 µL 70% ethanol was added to each FACS tube. Then these tubes were incubated for 2 h at 4ºC. After incubation, ethanol was removed, cells cleaned with 100 µL PBS, and then centrifuged for 8 min at 1800 rpm. The PBS was removed and vortexed to break the pellet. Each FACS tube was then filled with 300 µL PBS, 0.9 µL PI, 0.9 µL RNase, and 0.3 µL triton X-100, and the tubes were incubated for 30 min in dark. Finally, a flow cytometer (BD Biosciences FACS Diva 8.0.3) was used to examine the cells.

Statistical analysis

The experiments were conducted in a completely randomized design with each treatment replicated six times. Statistical analysis was performed using SPSS Software (version 28). The results were expressed as mean ± standard deviation. Correlation analysis was done among DPPH, reducing power, and cytotoxicity of ethyl acetate root extract. The data were analysed by using SPSS software (version 28). Statistical significance was accepted at 0.01 level (2-tailed).

RESULTS AND DISCUSSION

DPPH assay

The DPPH assay results for hexane, chloroform, ethyl acetate, methanol, and aqueous extracts revealed notable differences in antioxidant activity. The percent inhibition in hexane, chloroform, ethyl acetate, methanol, and aqueous extracts at 1000 µg mL^{-1} concentration were 88.91 ± 0.47, 93.95 ± 0.01, 94.51 ± 0.54, 93.68 ± 1.07 and 86.02 ± 0.6%, respectively. The ethyl acetate extract demonstrated higher and more consistent antioxidant activity with IC_{50} value of 105.48 µg mL^{-1}. The IC_{50} values for hexane, chloroform, methanol, and aqueous extracts were 160, 150.4, 125.94, and 239.52 µg mL^{-1}, respectively. The ethyl acetate extract showed low IC_{50} value and exhibited more substantial and potent antioxidant activity, indicating that it is more effective in extracting the bioactive compounds responsible for this effect.

Reducing power activity

The ethyl acetate extract showed higher reducing power, as demonstrated by its lower required concentration to reach 0.5 absorbance (111.99 µg mL^{-1}) as compared to the other extracts. The corresponding concentrations to attain 0.5 absorbance for hexane, chloroform, methanol, and aqueous extracts were 244.69, 221.94, 163.79, and 256.51 µg mL^{-1}, respectively. These findings underline the higher efficacy of ethyl acetate in extracting antioxidant compounds, which are likely responsible for the observed antioxidant effects. These results suggest that the ethyl acetate extract of *H. indicum* root is more effective in reducing power than the aqueous extract, indicating a higher presence of active compounds with antioxidant properties in the ethyl acetate extract.

The DPPH and reducing power assays highlighted the superior antioxidant potential of ethyl acetate extract as compared to the aqueous extract (Table 1). The aqueous extract exhibited a modest

Table 1: Comparison of antioxidant activity of aqueous and ethyl acetate extracts of *Heliotropium indicum* involving DPPH and reducing power assays

Concentration (µg mL^{-1})	Aqueous extract % inhibition (DPPH)	Ethyl acetate extract % inhibition (DPPH)	Ethyl acetate extract absorbance (reducing power)	Aqueous extract absorbance (reducing power)
50	17.58 ± 0.05	38.10 ± 0.25	0.087 ±0.0005	0.01 ± 0.0008
100	30.73 ± 0.05	56.02 ± 0.05	0.58 ± 0.0006	0.18 ± 0.0006
250	50.36 ± 0.05	67.05 ± 0.09	1.03 ± 0.0007	0.47 ± 0.0010
500	70.83 ± 0.05	78.81 ± 0.05	1.57 ± 0.0006	0.78 ± 0.0006
1000	86.02 ± 0.09	94.51 ± 0.10	1.87 ± 0.0009	1.00 ± 0.0001

antioxidant effect, with inhibition gradually increasing from 17.58 ± 0.05 to 86.02 ± 0.09%, suggesting its antioxidant potential with lower efficacy as compared to ethyl acetate extract which demonstrated robust and consistent antioxidant activity, starting at 38.10 ± 0.25% inhibition and peaking at 94.51 ± 0.1%, indicating a more substantial free radical scavenging ability. DPPH and H_2O_2 assays were used to examine the antioxidant properties of petroleum ether, chloroform, ethanol, and water based plant extracts of *H. indicum*. The ethanol extracts showed concentration-dependent antioxidant activity. The extract's flavonoid and tannin content is probably what gives it its purported ability to scavenge free radicals. The plant extract reportedly contains natural antioxidants that may help to stop the progression of several diseases caused by oxidative stress (Santhosha *et al*., 2015). The aqueous and ethanol extracts of *H. indicum* demonstrated free radical scavenging activity in a dose-dependent manner similar to ascorbic acid. The observed scavenging of free radicals is probably due to the presence of flavonoids and tannins in the extracts (Jayachitra and Bharathi, 2016). However, out of the four whole plant extracts (methanol, hexane, ethyl acetate, and hydro-alcoholic extracts) evaluated for antioxidant activity, the ethyl acetate extract showed strongest antioxidant activity (Pragada *et al*., 2012). The potent antioxidant activity of ethyl acetate extract is consistent with the previous reports that highlighted the free radical scavenging ability of *H. indicum* extracts, with studies linking its high phenolic and flavonoid content to superior antioxidant activity (Aksharadevi and Chitra, 2024; Sumi *et al*., 2024). Ethyl acetate root extract showed a dose-dependent antioxidant activity and also exhibited the highest antioxidant activity among the other extracts. Thus ethyl acetate extract was chosen for the cell viability studies.

MTT assay

The ethyl acetate extract of *H. indicum* was evaluated for its cytotoxic effects on HT-29 cells using MTT assay. The results revealed a dose-dependent decrease in cell viability. The effect of extract on cell population was evident in the micrographs of cells at different concentrations (Fig. 1). At lowest tested concentration of 1.56 µg mL^{-1}, the extract exhibited a maximum cell viability of 52% and an inhibition of 48%. In contrast, the highest tested concentration of 100 µg mL^{-1} showed a maximum inhibition of 80.76%. The IC_{50} value, representing the concentration required to achieve 50% inhibition

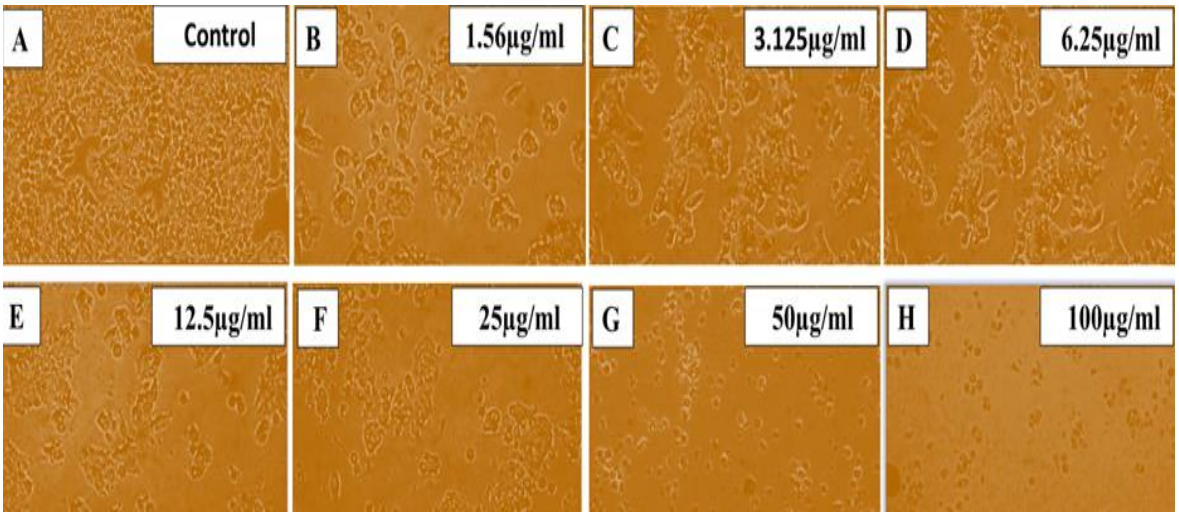

Fig. 1: Microscope images of HT-29 control cells and HT-29 cells incubated with varying concentrations (A-H) of the ethyl acetate extract of *Heliotropium indicum*

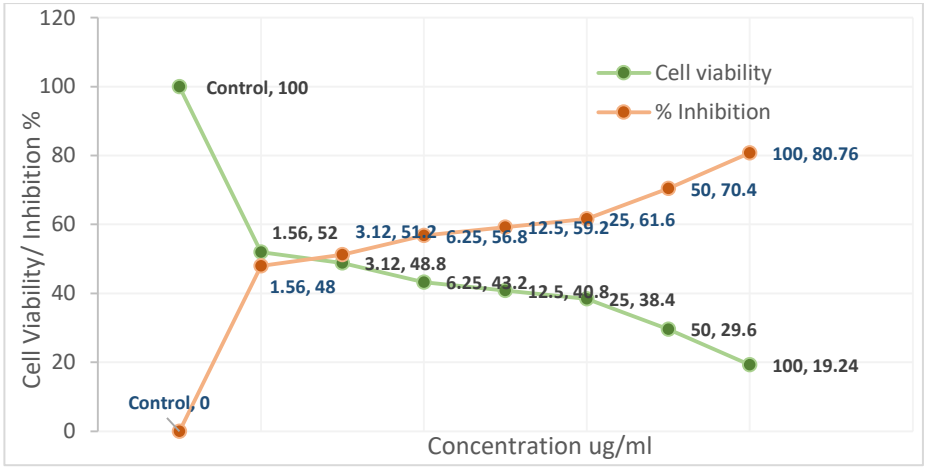

Fig. 2: Effects of *Heliotropium indicum* ethyl acetate extract on HT-29 cell viability of colon cancer cells

of cell viability, was calculated to be 3.04 µg mL^{-1}. The results reveal a dose-dependent decrease in the cell viability and a corresponding rise in inhibition, with observed IC_{50} 3.04 µg mL^{-1} (Fig. 2). This demonstrates that ethyl acetate extract has potent cytotoxic effects even at relatively low levels. These findings indicate that the ethyl acetate extract of *H. indicum* exhibits a strong inhibitory effect on the HT-29 cell line, with its efficacy amplifying at higher concentrations. This suggests that the bioactive compounds in the extract may effectively inhibit cell proliferation, potentially through the induction of cell death pathways. This finding is consistent with the previous studies which indicate that ethyl acetate fractions of plant extracts exhibit significant cytotoxicity against various cancer cell lines, including cervical and colon carcinoma cells (Liu *et al*., 2012; Cheruth *et al*., 2016; Onyegeme-Okerenta, 2018). This activity is linked to pyrrolizidine alkaloids and phenolic compounds that induce oxidative stress and DNA damage in tumour cells (Li *et al*., 2013; Yang *et al*., 2016). The methanol extract of dried roots of *H. indicum* produced the most prominent cytotoxic activity against brine shrimp *Artemia salina* (LC_{50} = 47.86 µg mL^{-1} and LC_{90} =75.85 µg mL^{-1}) (Rahman *et al*., 2011). The important phytochemicals were estimated from aqueous and 70% ethanolic extracts of the leaves of eight wild weeds, including *H. indicum* and their cytotoxic effects, therapeutic qualities, and *in vitro* antioxidant status were assessed (Ghosh *et al*., 2019; Gopinathan and Balasubramanian, 2020). *Heliotropium* species have the potential to reduce cell viability by promoting apoptosis (Jabeen *et al*., 2021; Sarg *et al*., 2024). Activating caspase-dependent pathways and disrupting mitochondrial membrane potential is more pronounced in cancerous cells than in normal cells, showcasing selective toxicity (Goyal and Sharma, 2014).

Cell cycle arrest analysis

The ethyl acetate extract of *H. indicum* was evaluated for its impact on cell cycle progression in HT-29 cells. The effects of different concentrations of *H. indicum* ethyl acetate extract (6.25, 12.5, 25, 50, and 100 µg mL^{-1}) on cell cycle progression of colon cancer cells were analysed using flow cytometry. Fig. 4 gives the percentage of cells in each phase of cell cycle. Most untreated control cells were in G_1 phase, with a small fraction in S and G_2 phases. As the concentration of extract increased, there was a noticeable shift in cell cycle distribution. At concentration of 6.25-12.5 µg mL^{-1}, the extract induced the most substantial disruption in cell cycle, with most significant proportion of cells in sub-G_0 phase. Simultaneously, the percentage of cells in G_1 phase decreased, which indicates a potential arrest at G_1 checkpoint. At higher concentrations, from 25-100 µg mL^{-1}, there was a significant decrease in the percentage of cells in sub G_0 phase and a marked increase in cells in G_1 phase. The analysis revealed a concentration dependent effect on cell cycle arrest, particularly at Sub-G phase (Fig. 3), which indicates apoptosis. The samples showed most significant arrest at a 6.25 µg mL^{-1} concentration exhibiting highest peak in Sub G_0 phase. The cell cycle arrest effect decreased as the concentration increased beyond 25 µg mL^{-1}.

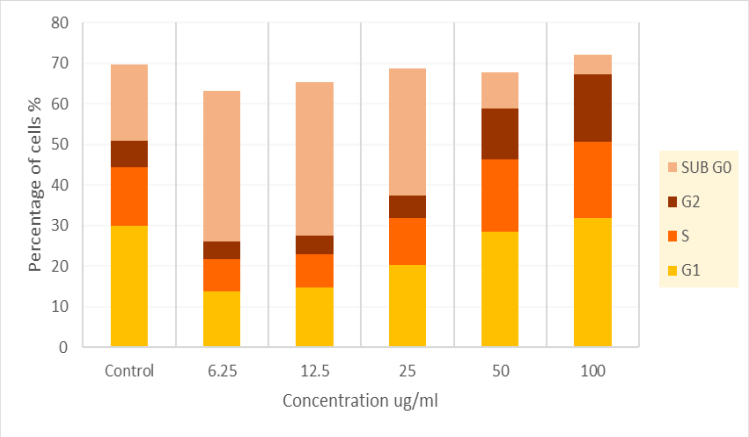

Fig. 3: Cell cycle distribution of colon cancer cells treated with varying concentrations of *Heliotropium indicum. L* ethyl acetate extract at different concentrations (6.25, 12.5, 25, 50, and 100 µg mL^{-1})

Cell cycle analysis revealed concentration-dependent arrest in sub-G_0 phase, a hallmark of apoptosis, with the most significant peak at 6.25 µg mL^{-1}. The presence of arrest in Sub G_0 phase suggests that the extract induced apoptotic pathways at lower concentrations. However, the arrest decreased as the concentration exceeded 25 µg mL^{-1}, which might indicate a shift in the type of cell death mechanism employed at higher concentrations. These results are consistent with Paul *et al*. (2015), Uzair *et al*. (2021) and Fayed *et al*. (2022) who reported the potential of *Heliotropium* species, including *H. indicum* to induce cell cycle arrest 2). The components of *H. indicum* enhance the expression of pro-apoptotic markers like Bax while downregulating anti-apoptotic proteins like Bcl-2, thus facilitating programmed cell death in malignant cells (Paul and Kundu, 2018). The antioxidant components work synergistically with its cytotoxic effects, as oxidative stress is often a mediator of apoptosis in cancer cells (Sznarkowska *et al*., 2017; Koscuiuszko *et al*., 2023).

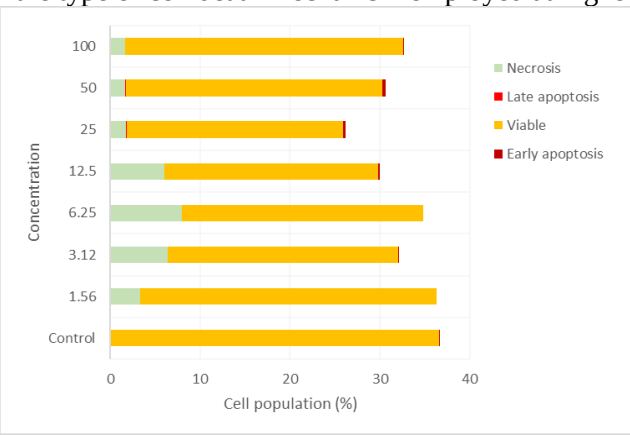

Fig. 4: Analysis of cell viability and apoptosis using Annexin V assay across treatment concentrations

Apoptosis induction analysis

The ethyl acetate extract of *H. indicum* root was analysed for its ability to induce apoptosis and necrosis in HT-29 cells using Annexin V/PI staining. The effect on HT-29 colon cancer cells was evaluated at various concentrations ranging from 6.25 to 100 µg mL^{-1} (Fig. 4). At highest

concentration (100 µg mL^{-1} of extract), a significant percentage of viable cells (about 30%) were observed, while limited necrosis (1.6%) and early apoptosis (0.1%) were also induced, with minimal late apoptosis. A similar effect was observed for necrosis at 50 µg mL^{-1}, with a slight decrease in viable cells and an increase in early apoptosis. At 25 µg mL^{-1}, there was an increase in necrosis while viable cell population decreased. At lower concentrations (≤ 12.5 µg mL^{-1}), necrosis increased while viable cell population decreased. At 6.25 µg mL^{-1} the effect on necrosis was most pronounced, but viable cells still represented the majority. The control group, which received no extract, showed nearly all cells as viable with little to no necrosis or apoptosis. These results revealed that *H. indicum* extract induced necrosis in concentration-dependent manner in HT-29 colon cancer cells at lower and higher and higher concentrations, leading to an increase in early apoptosis. The results indicate that *H. indicum* extract exhibited minimal apoptosis induction, as evidenced by the lack of significant cell populations in Q_2 (late apoptotic cells) and Q_4 (early apoptotic cells) quadrants across all tested concentrations (Fig. 5). However, the extract demonstrated a notable ability to induce necrosis, with highest necrotic effect at 6.25 µg mL^{-1} concentration. The percent necrotic cells initially increased to 6.25 µg mL^{-1} and decreased at higher concentrations, 12.5-100 µg mL^{-1}, indicating a concentration-dependent effect. These findings suggest that *H. indicum* is ineffective in inducing early or late apoptosis but can induce necrosis in HT-29 cells.

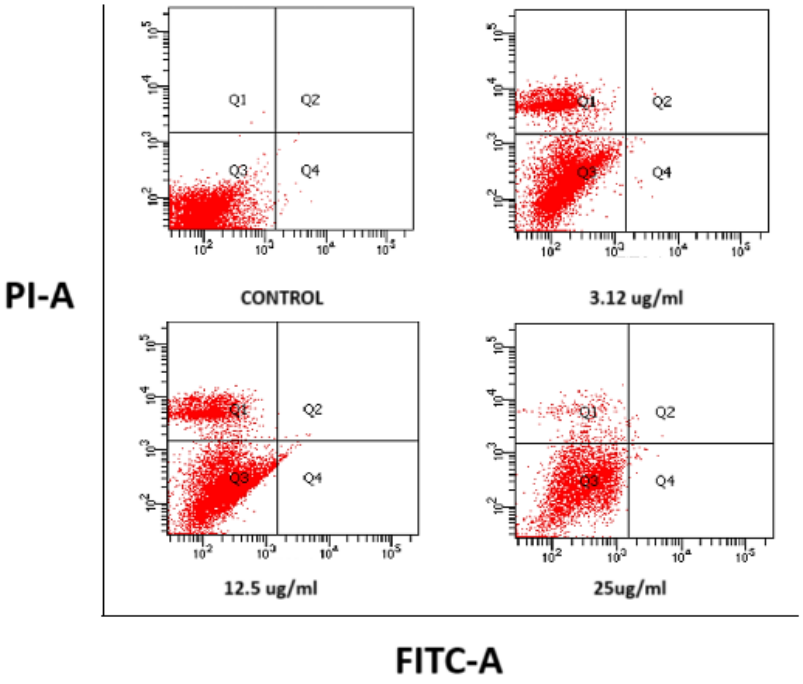

Fig. 5: Flow cytometry analysis of apoptosis induction by ethyl acetate extract of *H. indicum* in colon cancer cells. The plot shows the distribution of cells in four quadrants based on Annexin V-FITC (FITC-A) and propidium iodide (PI-A) staining. The quadrants represent the following: Q_1: Necrotic cells (PI positive, FITC negative), Q_2: Late apoptotic cells (both PI and FITC positive), Q_3: Viable cells (PI negative, FITC negative), and Q_4: Early apoptotic cells (PI negative, FITC positive).

The Annexin V/PI staining analysis revealed an interesting combination of apoptosis and necrosis at 6.25 µg mL^{-1} concentration. The extract exhibited minimal apoptosis induction with no significant populations in Q_2 and Q_4 quadrants of Annexin V/PI assay which indicated late and early apoptosis. However, it significantly induced necrosis, particularly at 6.25 µg mL^{-1} concentration. The percentage of necrotic cells increased with increasing concentrations at lower concentrations, suggesting a concentration-dependent effect. This combination of Sub G_0 phase arrest (indicative of apoptosis) alongside necrosis raises the possibility that the cells are undergoing necroptosis, a regulated form of necrosis that shares features with both apoptosis and necrosis. In necroptosis, cells often show early apoptotic features such as phosphatidylserine externalisation, which can be detected by Annexin V staining and necrotic characteristics such as loss of membrane integrity and PI uptake (Krysko *et al*., 2017). This could explain the apparent contradiction between the Sub G_0 phase arrest (suggestive of apoptosis) and necrosis observed in Annexin V/PI assay. At 6.25 µg mL^{-1} concentration, the extract could induce apoptosis in some cells, causing them to arrest in Sub G_0 phase. However,

when apoptosis is inhibited or overwhelmed, those cells could switch to necroptosis as a secondary response, leading to membrane rupture and necrosis (Berghe *et al*., 2010; Vanden Berghe *et al*., 2015). This mechanism is supported by the observed decrease in necrosis at higher concentrations, as necroptosis is more likely to occur at lower stress thresholds before excessive damage shifts the cells to other forms of death. Necroptosis is mediated by molecular pathways involving RIPK1, RIPK3, and MLKL, offering specific targets for therapeutic intervention (Remijsen *et al*., 2014; Chen *et al*., 2017; Seo *et al*., 2021). Vanadium complexes suppressed the growth of HT-29 cells by enhancing ROS production. Flavokawain-C prevented the growth of HT-29 cells by increasing ROS formation and reducing SOD activity. *Brucea javanica* fruit extract enhanced ROS generation in HT-29 cells (Alidadi *et al*., 2021).

H. indicum has been studied for its bioactive properties, involving antioxidant, cytotoxic, and apoptotic potential (Poojari and Bhalerao, 2018; Fayed, 2021). These attributes make it a candidate for therapeutic applications, including cancer and oxidative stress-related disorders. The plant exhibits strong free radical scavenging activity, attributed to its phenolic and flavonoid content. It can effectively neutralize reactive oxygen species (ROS), reducing oxidative stress and preventing cellular damage. The increased population of Annexin V-FITC stained cells clearly showed induction of apoptotic cell death in treated cells. The treated cells of every studied cell line showed a marked increase in early and late apoptotic population. In HeLa and C33A cell lines, the chloroform extract of *H. indicum* caused an increase in sub G_0 populations, indicating cytotoxicity. Additionally, it caused treated SiHa cells to undergo a G_1/S cell cycle arrest (Paul and Kundu, 2018). Earlier studies have proven its cytotoxic effects against cancer cell lines, including colorectal carcinoma cells (Fayed, 2021). The cytotoxicity is mediated by its alkaloid content, particularly pyrrolizidine alkaloids, such as indicine (Yang *et al*., 2016). *H. indicum* extracts induce apoptosis in cancer cells through pathways involving caspase activation and mitochondrial membrane potential disruption (Paul *et al*., 2015; Olowofolahan *et al*., 2017; Adetuyi *et al*., 2021). The present study revealed antioxidant potential and cytotoxic effects of ethyl acetate extract of *H. indicum* root in various *in vitro* assays with significant differences in antioxidant activity and cellular responses across variable concentrations in HT-29 colon cancer cells.

The cell cycle arrest at the G0 phase, coupled with the absence of both early and late apoptosis and the significant presence of necrosis in many cells, suggests necroptosis. This hypothesis can be further confirmed by assessing necroptosis-specific markers. While earlier studies have established the apoptotic potential of *H. indicum*, it was minimal in the present study, with necroptosis being the predominant form of cell death. The identification of necroptosis, in addition to its apoptotic properties, enhances the therapeutic potential of *H. indicum* extracts, supporting its use in traditional medicine and extending its applications in cancer therapy and modern medicine. Necroptosis is a form of programmed necrosis that is gaining attention in targeting cancer because of its regulated nature, similar to apoptosis (Krysko *et al*., 2017). However, unlike apoptosis, which is a highly controlled and immunologically silent process, necroptosis involves plasma membrane rupture and the release of intracellular contents, triggering an immune response (Shlomovitz *et al*., 2019). This feature makes necroptosis particularly valuable for eliminating apoptosis-resistant cancer cells, a common cause of therapy failure (Westman *et al*., 2020). Inducing necroptosis in tumours directly reduces cancer cell

Table 2: Absorbance of reducing power assay, DPPH assay, %inhibition (DPPH) and MTT assay of various concentrations of ethyl acetate extract of *H. indicum* roots (mean ± SD)

Concentration (µg mL^{-1})	Reducing power absorbance	DPPH absorbance	% inhibition absorbance	MTT assay absorbance
50	0.087 ± 0.0005	0.372 ± 0.0015	38.11 ± 0.0083	0.126 ± 0.0057
100	0.583 ± 0.0006	0.264 ± 0.0008	56.02 ± 0.0075	0.054 ± 0.0188
250	1.031 ± 0.0007	0.198 ± 0.0008	67.05 ± 0.0063	0.051 ± 0.0054
500	1.567 ± 0.0006	0.128 ± 0.0008	78.81 ± 0.0075	0.049 ± 0.0045
1000	1.872 ± 0.0009	0.033 ± 0.0012	94.51 ±0.0063	0.037 ± 0.0070

populations and may enhance antitumor immunity, paving the way for novel combinatory treatments in oncology (Fu *et al*., 2024).

There was a significant negative correlation between IC_{50} of DPPH and reducing power of root extracts (R^2 = - 0.992, P < 0.001). The ethyl acetate extract was more efficient at reducing free radicals than scavenging DPPH radicals. The IC_{50} of DPPH and cytotoxicity exhibited a moderate positive correlation (R^2 = 0.730, P < 0.001) (Table 3). This implies that larger concentrations of extract are required to inhibit 50% DPPH radicals (higher IC_{50}) are frequently linked to higher cell viability

Table 3: Correlation analysis between reducing power and DPPH assays and DPPH and MTT assay. Statistical analysis was done by using SPSS software (version 28).

Assays		Correlation analysis of ethyl acetate extract (EAE)		
		EAE – Reducing power	EAE – DPPH	EAE - MTT
EAE - Reducing power	Pearson correlation	1	-0.992**	-0.724**
	P-value		< 0.001	< 0.001
EAE - DPPH	Pearson correlation		1	0.730**
	P-value			< 0.001
EAE - MTT	Pearson correlation			1
	P-value			

**Correlation is significant at the 0.01 level (2-tailed)

Table 4: Correlation between % inhibition of DPPH assay and MTT assay. Statistical analysis was done by using SPSS software (version 28)

		Correlations analysis of ethyl acetate extract (EAE)	
		EAE % inhibition – DPPH	EAE % inhibition - MTT
EAE % inhibition - DPPH	Pearson correlation	1	0.943*
	P-value		0.016
EAE % inhibition - MTT	Pearson correlation		1
	P-value		

*Correlation is significant at the 0.05 level (2-tailed)

(lower MTT reduction), suggesting potential cytotoxic effects. One possible sign of cytotoxicity is the finding that lower MTT reduction (lower cell viability) correlates with greater IC_{50} values (less powerful antioxidant action). This implies that even while the extract exhibits some antioxidant action at lower concentrations, it may be harmful to cells at larger concentrations. A negative correlation was observed between reducing power and cytotoxicity with ethyl acetate extract (R^2 = -0.724, P < 0.001) (Table 3) suggesting the effect of extract on cell viability, as determined by MTT assay, is inversely correlated with its reducing power. In particular, cell viability as assessed by MTT assay declined as the extract's reducing power increased. There was a strong positive correlation between percent inhibition of DPPH and cytotoxicity of ethyl acetate extract (R^2 = 0.934; P = 0.016) (Table 4). Antioxidant substances may be able to protect the cells from damage, as suggested by high positive association between the antioxidant activity, as determined by the DPPH assay, and the cell viability, as determined by the MTT assay.

Conclusion: The ethyl acetate extract of *H. indicum* L. root demonstrates potent antioxidant and cytotoxic effects. The extract effectively induces cell cycle arrest in the Sub G0 phase, suggesting apoptotic activity, particularly at lower concentrations. However, at 6.25 µg mL^{-1}, a combination of apoptotic and necrotic cell death mechanisms appears to be involved, with necrosis potentially being driven by necroptosis. This regulated necrosis occurs when apoptosis is inhibited. The findings suggest that *H. indicum* L. has anticancer potential owing to its necroptotic potential. This study is the first to report the necroptotic potential of the root extract of *H. indicum*. However, further investigation into the specific signalling pathways involved, such as the RIPK1/RIPK3/MLKL pathway, is needed

to confirm the role of necroptosis. Further studies, including assessing necroptosis-specific markers, would reveal the pathways behind the cell death induced by *H. indicum*.

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