Applied Biological Research 27(3): 347-356; (2025)
DOI: 10.48165/abr.2025.27.01.34

ANTICANCER MECHANISMS OF *Commelina forskaolii* EXTRACT THROUGH INDUCTION OF FERROPTOSIS IN HT-29 COLON CANCER CELL LINES

Manikandan Vani Raju[1], Meenakshi Kaniyur Chandrasekaran[1], Rathi Muthaiyan Ahalliya[1,2]*
Gopalakrishnan Velliyur Kanniappan[3] and Guru Kumar Dugganaboyana[4]

[1]*Department of Biochemistry,* [2]*Department of Biotechnology, Karpagam Academy of Higher Education, Coimbatore - 641 021, Tamil Nadu (India)*
[3]*Department of Physiology, Saveetha Medical College & Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS) Chennai - 602 105, Tamil Nadu (India)*
[4]*Division of Biochemistry, School of Life Sciences, Mysuru JSS Academy of Higher Education & Research Mysore - 570 015, Karnataka (India)*
*e-mail: *rathiajith@gmail.com; vrmbiochem@gmail.com*

(Received 26 February, 2025; accepted 18 August, 2025)

ABSTRACT

Colon cancer is the 3rd most prevalent cancer globally, presenting significant epidemiological and economic challenges. Natural products have shown promise as effective alternatives to chemotherapy. The present study was aimed to study the anticancer potential of rat's ear (*Commelina forskaolii)* on HT-29 colon cancer cells, focusing on cytotoxic activity, antioxidant potential, and underlying molecular mechanisms. The cytotoxic activity of the ethanolic extract of *C. forskaolii* was assessed by measuring IC_{50} value on HT-29 cell lines, enzymatic antioxidant potential and heme oxygenase activity. Gene expression and cell cycle analyses were performed to explore molecular mechanisms. The extract demonstrated significant cytotoxic activity with an IC_{50} value of 27.4 ± 0.25 µg mL^{-1}. Cell cycle analysis showed that *C. forskaolii* effectively arrested the cells at G0/S phase. Additionally, the study revealed an increase in lipid peroxidation and intracellular iron accumulation, leading to the formation of lipid-iron radicals and triggering ferroptosis. Heme oxygenase-1 (HO-1) was found to regulate cellular iron levels and reactive oxygen species (ROS) generation, where moderate HO-1 activity offered cytoprotection, but excessive activity in cancer cells led to oxidative cell death. The findings suggest that *C. forskaolii* may serve as a valuable natural treatment for colon cancer, targeting oxidative processes that induce cancer cell death and anticancer activity.

Keywords: Anticancer, antioxidants, ferroptosis, colon cancer, *Commelina forskaolii,* Rat's ear plant

INTRODUCTION

Colon cancer is the 3rd most reported cancer in both men and women, leading to major demographic and economic concerns at global level (Vadde *et al*., 2017). The increase in colorectal cancer within developing countries can potentially be explained by the aging population, evolving lifestyles, dietary habits, and a rise in various risk factors for colorectal cancer. These risk factors include hereditary diseases, excessive consumption of beef, smoking, alcohol consumption, and lack of physical activity (Illian *et al*., 2021). Natural products are highly effective in battling colorectal cancer and act as potential alternatives to standard chemotherapy treatments. Their unique characteristics depict a significant role in clinical care of tumours, potentially improving patient outcomes (Huang *et al*., 2019).

Commelina forskaolii is a plant species of great ecological and cultural importance, known for its numerous medical use. The plant is recognized by its brilliant blue blossoms and capacity to flourish in a variety of environments, making it an ideal subject for research in both botanical and traditional medical studies. *C. forskaolii* has traditionally been used to treat inflammatory, gastro-intestinal, and dermatological disorders (Raju *et al*., 2024). Phytotherapeutics typically involves the use of leaves and stems of medicinal plants, either as crude or concentrated extracts, to treat diseases.

Nuclear factor erythroid 2-related factor 2 (Nrf2) was first reported in early 1990s during research on cellular protection against oxidative damage and inflammation. It regulates genes that deal with oxidative damage. The activation of Nrf2 frequently results in a coordinated antioxidant response. The gene that encodes Nrf2 was successfully cloned in 1994. Nrf2 interacts with antioxidant response molecules found in the promoter regions of numerous genes, thereby regulate enzyme production that regulate antioxidant defense and detoxification (Bogen, 2016). Antioxidant proteins that act in primary defensive mechanism like superoxide dismutase (SOD), heme oxygenases 1 (HO-1), catalase (CAT), and glutathione peroxidase (GPX); and are critical in combating the effects of reactive oxygen species on cells. In cancer, the balance of SOD and GPX activities affects SOD functions as a preventive antioxidant or a pro-oxidant, which influence tumour progression (Mantovani *et al*., 2002) Their action plays a vital role in sustaining cellular health and reducing damages caused by oxidative stress. HO-1 is essential in metabolic pathway that catalyse heme into bile pigments, acting as an accelerator for limitation phase of process. This enzymatic process produces iron and carbon monoxide, the two substances that have important consequences for physiological functions like inflammation, modulation and proliferation of cells (Ryter *et al*., 2006). Further, HO-1 is linked to the activation of ferroptosis, a type of controlled cell death studied as a potential cancer treatment. By leveraging ferroptosis mechanisms, targeting HO-1 could provide a unique chemotherapeutic method for effectively attacking tumour cells (Chiang *et al.,* 2019). The present study was aimed to assess the potency of *C. forskaolii* plant extract (CF) as a therapeutic target for cancer treatment and describe its anti-cancer processes.

MATERIALS AND METHODS

Plant collection and identification

Commelina forskaolii plant samples were collected from the Western Ghats of Coimbatore (India) and its identity got verified by the Botanical Survey of India, Coimbatore vide certification No.: BSI/SRC/ 5/23/2022/Tech/361. The plant materials were thoroughly washed and rinsed twice with tap water to remove any debris. The roots were removed, and only aerial plant parts were retained for analysis. These aerial parts were then sliced into smaller pieces and dried in shade at 35°C using a 12 h light/dark cycle. Following complete desiccation, the plant materials were grinded into a fine powder with a mechanical blender.

Extraction of plants using the Soxhlet method

The Soxhlet extraction procedure was used to extract 10 g plant powder overnight using appropriate solvent temperatures in 250 mL ethanol. Following the extraction process, the extracts were treated in a vacuum evaporator to remove any residue solvents.

Cell culture and treatment

The HT-29 cells were received directly from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured at 37°C in Dulbecco's Modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU mL^{-1}), and streptomycin (100 µg mL^{-1}). They were kept humidified with 5% carbon dioxide until they reached 85% confluence. The cells were removed for subculture using a trypsin solution containing trypsin (0.2%), EDTA (0.02%) and glucose (0.05%) in phosphate-buffered saline (PBS) (Malsawmdawngliana *et al*., 2021).

Cytotoxic activity

The cytotoxic effects of *C. forskaolii* plant extract (CF) on HT-29 cells were determined using MTT assay (Mosmann, 1983). Initially, 1×10^4 viable cells per well were seeded in 96-well plates containing 0.2 mL culture medium. The plates were then incubated for 72 h in controlled atmosphere containing 5% CO_2. Following incubation, the samples were produced with different amounts of 0.1% DMSO and incubated for another 48 h under the same CO_2 conditions. To obtain cellular pictures, microscopic observations were made using an inverted microscope at 40X. After discarding the sample solution, 20 µL MTT reagent was applied to each well. The number of viable cells was measured at 540 nm using the BioTek ELx800 Microplate reader (Mosmann, 1983).

Superoxide dismutase

The superoxide dismutase (SOD) activity in HT-29 cells was determined as per Kakkar *et al*. (1984). Initially, 0.1 mL sample was mixed with 0.5 mL distilled water, followed by 0.25 mL ethanol and 0.15 mL chloroform. This mixture was stirred for 1 min before centrifugation at 2000 rpm to separate the supernatant, which was then used to test enzyme activity. The reaction was initiated by adding 0.4 mL epinephrine, and the changes in optical density min^{-1} were measured at 470 nm using a Shimadzu UV-2450 spectrophotometer. The SOD activity was expressed as international units per litre (IU L^{-1}). The changes in optical density min^{-1} by the enzyme at 50% blockage of adreno-chrome transition was considered one enzyme unit (Ukeda *et al*., 1997).

Lipid peroxidation and intracellular iron levels

The cells were seeded in six-well plate with 2 mL medium and incubated at 37°C for 24 h. The samples (30-60 µg) were added to fresh media and incubated for 48 h. The cells were centrifuged at 10,000 rpm for 10 min at 4°C, then washed with PBS. Following this, the lysed cells were treated with TBA to yield MDA-TBA mixture. The MDA TBA combination was determined colorimetrically at 532 nm (Środa-Pomianek *et al*., 2018). An iron assay kit (ab83366, Abcam, UK) was used to assess lipid peroxidation in HT-29 cells, in accordance with the manufacturer's protocol.

Glutathione peroxidase activity

The glutathione peroxidase enzyme assay was performed as per the method of Agahi *et al*., 2021), with minor modifications. Cells were seeded in six-well plates and allowed to attach under standard culture conditions. Then, 100 µL aliquot of cell lysate was mixed with 0.2 mL EDTA and 0.1 mL sodium azide. To this mixture, 0.4 mL phosphate-buffered saline (PBS, pH 7.4), 0.1 mL hydrogen peroxide and 0.2 mL reduced glutathione (GSH) were added sequentially. The reaction mixture was incubated at 37°C for 10 min and reaction terminated by adding 0.5 mL of 10% trichloroacetic acid (TCA). Then 3.0 mL disodium hydrogen phosphate was added for subsequent analysis. The entire mixture was incubated at 37°C for 10 min before adding 0.5 mL TCA (10%) and 3 mL Na_2HPO_4. Finally, 1 mL DTNB solution was added and optical density measured at 420 nm using Shimadzu UV-2450 spectrophotometer. Glutathione peroxidase activity was expressed as min mg^{-1} protein.

Cell cycle analysis

The HT-29 cells were grown in T25 flasks and when the cells attained confluence, they were transferred to six-well plates. After cell adhesion (48-72 h), cells were treated with different doses of the sample for 24 h. The cells were collected by trypsinization with TPVG solution once the appropriate treatment period had ended. The cells were then centrifuged at 1200 rpm for 5 min at room temperature before the supernatant was discarded. The cells were then rinsed with PBS. The cell pellet was reintroduced in 300 µL PBS. A falcon tube was filled with 700 µL ice-cold 100% ethanol, then 300 µL sample was added drop by drop to yield a 70% ethanol solution. This stage did not include vortexing. The cells were then fixed in ethanol and kept overnight at 4°C. On the day of flow cytometry, ethanol-fixed cells were rinsed once or twice with PBS to remove supernatant and subsequently, 556 µL of 0.5% Triton X-100 and 20 µL RNase A (0.1 mg mL^{-1}) were added to each sample. The mixture was incubated for 1 h at 37°C to ensure membrane permeabilization and RNA

degradation. After incubation, 24 µL propidium iodide was added and the samples further incubated in dark for 30 min at room temperature to allow DNA staining. Finally, the stained cells were analysed using a flow cytometer (Facs JAZZ, California) (Raju *et al*., 2024a).

RT-PCR analysis

Total RNA was isolated from CF-treated HT-29 cells using TRIzol reagent, following the manufacturer's instructions. The RNA concentration was measured using the spectrophotometric technique. Then, 1 µg total RNA was used for reverse transcription reactions. The cDNA has been precipitated and amplified with particular primer sequences to enable effective and targeted amplification. The primer sets used for RT-PCR analysis with gene accession numbers were:

Nrf-2	NM_001145412.3	TGAGGTTTCTTCGGCTACGTT	AGCTCCTCCCAAACTTGCTC
HO-1	NM_002133.3	GGGCCATGAACTTT	CTTCGCCCCCTCTGAAGTTT
GAPDH	NM_002046.7	TTTTGCGTCGCCAGCC	ATGGAATTTGCCATGGGTGGA

These primers targeting Nrf-2, HO-1 and GAPDH (housekeeping gene) are particularly developed for cDNA amplification (Wei *et al*., 2021; Raju *et al*., 2024b).

Statistical analysis

Statistical analysis was done using GRAPHPAD PRISM 9. The outcomes of the experiments were represented by the mean and standard deviation (SD) of triplicates. One-way ANOVA followed by Dunnett's test was used to evaluate variance and identify significant differences between means, with a significance threshold set at $p < 0.05$. The probability levels were indicated as $p = <0.0001$ (****).

RESULTS AND DISCUSSION

Cytotoxic activity

The cytotoxic activity of *C. forskaolii* plant extract (CF) on HT-29 cells was determined using MTT assay (Fig. 1). The CF showed the effective cytotoxic activity on HT-29 colon cancer cells. The percentage of cell viability decreased as the concentration of CF increased, which indicated the dose-dependent cytotoxic effect. Dose-dependent cytotoxic effect of CF on HCT116 cells was evaluated by plotting the cytotoxic response (%) against the logarithm of drug concentration (µg mL^{-1}). Linear regression analysis showed a strong negative correlation between log concentration and cytotoxic response, with a slope of –38.27 (± 1.78, SE) and a y-intercept of 105.0 (± 3.16, SE). The regression was highly significant [F (1.31) = 460.0, $p < 0.05$] with an R^2 of 0.937, indicating that approximately 93.7% variation in cytotoxicity is explained by the concentration. Fig. 2 represents inverted microscopic images of cytotoxic activity. Our previous work has demonstrated that *C. forskaolii* has the ability to suppress the spread of HepG2 liver cancer cells (Raju *et al*., 2024).

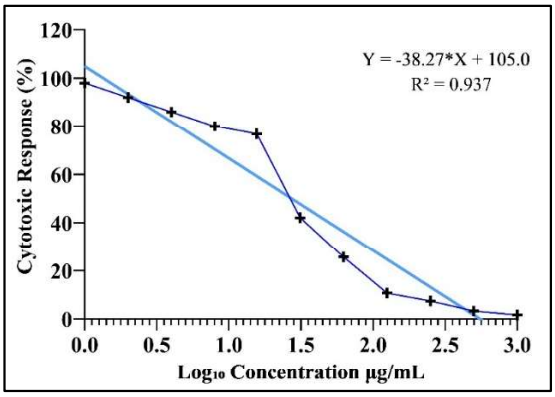


Fig. 1: Scatter plot of log concentration versus cytotoxic response (%) with linear regression line (Y = –38.27X + 105.0). The slope is significantly non-zero ($p < 0.05$), indicating a strong negative correlation ($R^2 = 0.937$).

Determination of antioxidant enzymes

Superoxide dismutase activity (SOD) increased in direct proportion to the increase in CF concentration (Table 2). Control group showed lowest SOD activity and highest SOD activity was

observed at highest CF doses tested (90 µg mL^{-1}). Similarly, control group showed lowest glutathione peroxidase activity. Treatment with 30 µg mL^{-1} extract increased GPx levels to around 0.21 min mg^{-1} protein. The treatments with 60 and 90 µg mL^{-1} extract increased GPx levels to 0.41 and 0.61 min mg^{-1} protein, respectively (Table 2).

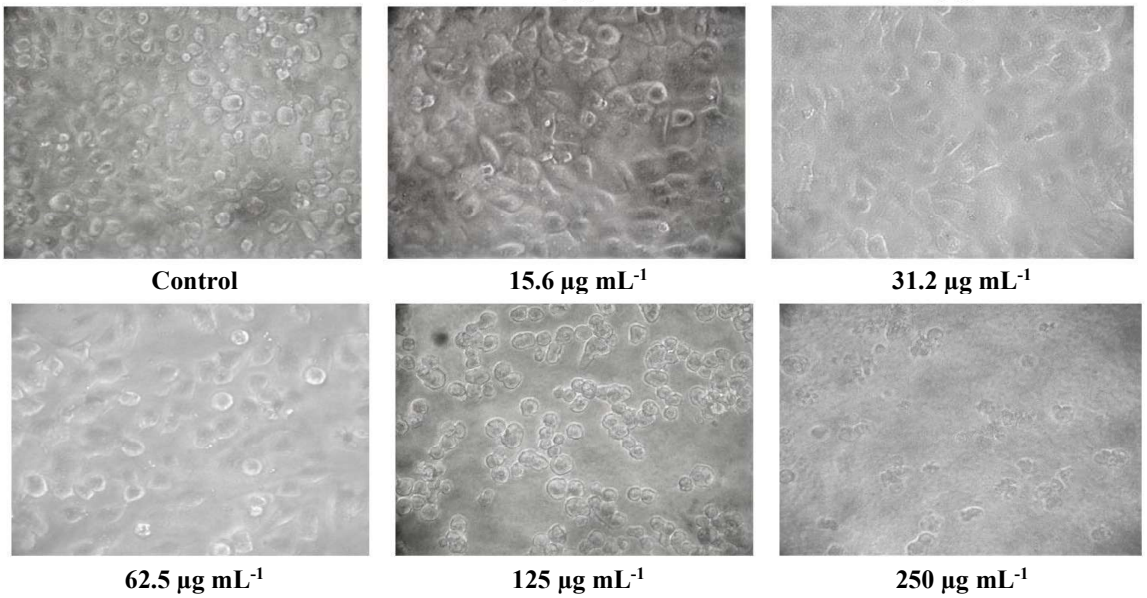


Fig. 2: Inverted microscopic images show the cytotoxic effects of CF on HT-29 cells at various doses. A progressive decrease in cell density and morphological alterations suggest a dose-dependent cytotoxic response.

Table 2: Effect of test compound on antioxidant enzyme activities and Fe^{2+} levels in HT-29 cells

Concentration (µg mL^{-1})	Mean ± SE			
	Superoxide dismutase	Glutathione peroxidase	Lipid peroxidation	Fe^{2+} levels
Control	40.23 ± 0.50	0.10 ± 0.002	0.05 ± 0.01	0.05 ± 0.01
30	45.62 ± 0.20	0.21 ± 0.002	0.40 ± 0.04	0.4 ± 0.04
60	55.27 ± 0.16	0.41 ± 0.004	0.75 ± 0.03	0.75 ± 0.02
90	75.47 ± 0.11	0.61 ± 0.002	0.96 ± 0.02	0.96 ± 0.01

The values are presented as mean ± SE (n = 3).

Lipid peroxidation levels increased as CF concentrations was increased. Control group had lowest LPO level, approximately 0.05. Treatment with 30 µg mL^{-1} CF remarkably enhanced LPO level to 0.40. The levels of LPO by CF doses of 60 and 90 µg mL^{-1} increased to 0.75 and 0.96, respectively. This revealed that increased lipid oxidation changed the physical properties of cellular membranes and proteins, triggering apoptosis. The main defense over reactive oxygen species is provided by cellular antioxidant enzymes, that include superoxide dismutases, glutathione reductase, catalase, glutathione-S-transferases, glutathione peroxidases, and peroxiredoxins. Antioxidant enzymes primarily reduce tissue damage caused by reactive oxygen radicals by either blocking the generation of reactive oxygen radical species or by intercepting highly reactive oxygen radical species and neutralizing them to inactive molecules (Jena *et al*., 2023). Hadi *et al*. (2021) examined the impact of doxorubicin on MCF-7 breast cancer cell lines and reported that superoxide dismutase and glutathione peroxidase, two major antioxidant enzymes, were remarkably elevated in response to doxorubicin treatment. The present study also depicted increase in SOD and GPx levels which preserve cellular homeostasis and redox balance. LPO levels in HeLa and SiHa cells increased significantly after 24 h treatment with IC_{50} as reported by Zani *et al*. (2023). Similarly, in present case

the LPO levels in HT-29 cells increased with increase in the concentration of CF. This demonstrates that an increase in LPO levels causes oxidative stress and promotes apoptosis.

Determination of intracellular iron levels

The present study revealed a substantial rise in both the total iron level and the expression of ferric bivalent ions in the treated cells in comparison to the control (Table 2). CF treatment exhibited a dose-dependent rise in the expression of both divalent and total iron. Ferroptosis destroys cells by iron-dependent ROS generation, and HO-1 regulates cellular iron levels and ROS synthesis (Chiang *et al*., 2018; Raju *et al*., 2025). Thus, when HO-1 is relatively active, Nrf2-derived HO-1 protects cells by neutralizing ROS. Cancer cells produce more HO-1, hence a high level of HO-1 stimulation may enhance unstable Fe^{2+}, leading to ROS accumulation and ultimate oxidative cell death (Nuhn *et al*., 2009; Banerjee *et al*., 2012; Bekeschus *et al*., 2018; Chang *et al*., 2018). Further research in *in-vivo* modes is required to understand the specific involvement of Nrf2, HO-1, and related antioxidants in upregulating pro-apoptotic genes and inducing ferroptosis in HT-29 colon cancer cells.

Cell cycle analysis

The cell cycle analysis of CF was done using flow cytometry and compared with control (Fig. 3A, B). A considerable decrease in live cells (P_1) and rise in dead cells (P_2) in CF treated groups demonstrated that CF treatment generated cytotoxic effects, thereby causing cell arrest. The changes in cell cycle distribution in CF treated groups with more dead cells in interphase (P_3) [29.72%] and less dead cells in mitotic phase (P_4) [3.23%] showed that CF treatment can cause cell cycle arrest at G_0/S phase. This arrest could cause lower DNA synthesis and poor cell division, eventually leading to more cell-death. Transient Nrf2 activation is essential for both normal cell survival and cancer prevention. However,

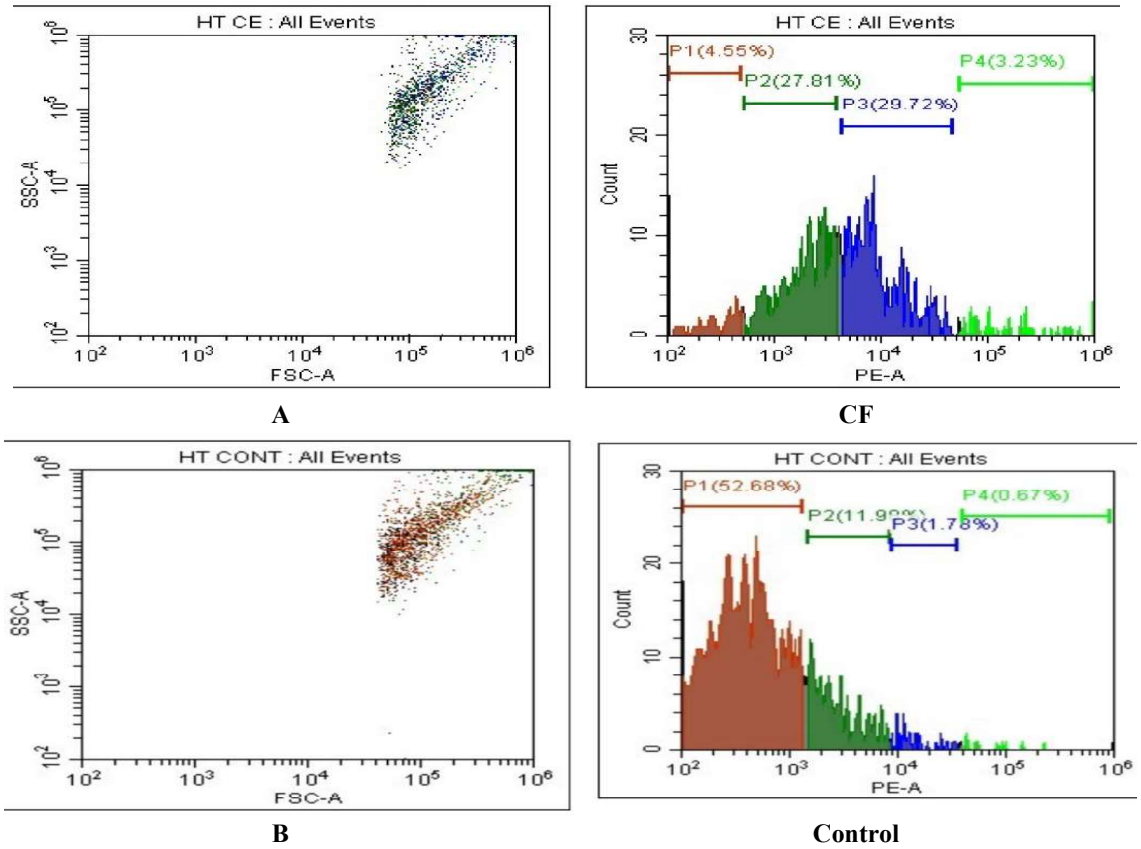
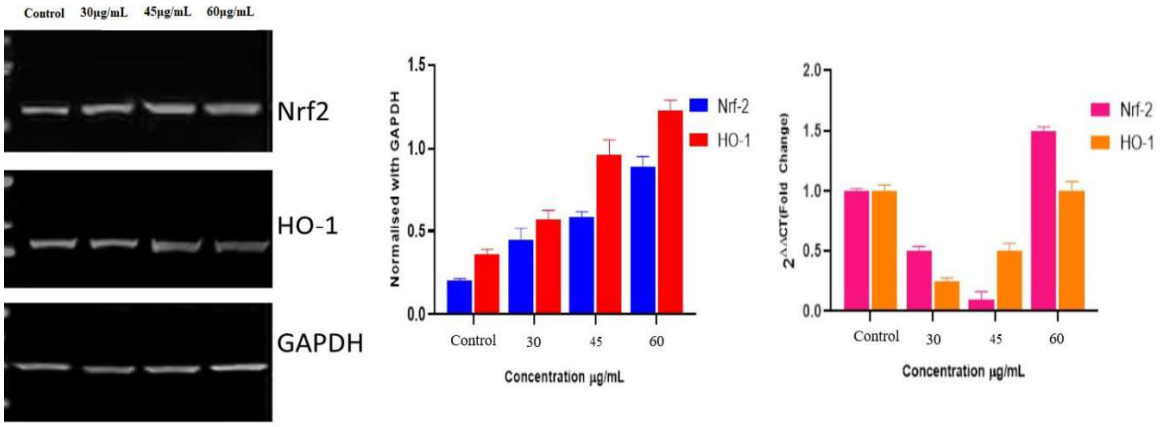


Fig. 3: Cell cycle analysis by flow cytometry (A) CF treated cells (B) Control. The treatment with CF triggered significant changes in cell cycle distribution, indicating a potential cytostatic effect.

chronic activation of this system may be deleterious, especially in malignant environment, because Nrf2 has a pro-tumour role by supporting continued growth of diseased cells via many pathways (Glorieux *et al*., 2024). Lower genotoxic damage, reduced cell proliferation, and increased apoptosis of colon cancer cells support protective properties of Nrf2 (Tan *et al*., 2015; Trivedi *et al*., 2016; Zuo *et al*., 2018).

RT-PCR analysis

The gel electrophoresis images revealed bands at varying concentrations representing Nrf2, HO-1, and GAPDH (Fig. 4A). The intensity of both Nrf2 and HO-1 bands increased with CF concentration, demonstrating that these genes are upregulated. The graph in Fig. 4B shows the levels of Nrf2 and HO-1 rise with CF concentration. A substantial rise was observed at 30 µg mL^{-1} as compared to the control, followed by further rise at 45 and 60 µg mL^{-1}. The increased Nrf2 expression suggests that CF treatment generates oxidative stress or functions as a moderate electrophilic chemical, thereby alters the Nrf2-Keap1 connection. This causes Nrf2 to stabilize and translocate to the nucleus, upregulating its target genes, particularly HO-1. Fig. 4C shows the fold changes of Nrf-2 and HO-1 genes, in which control reveals a fold change value of 1.0. At 30 µg mL^{-1} concentration the Nrf2 and HO-1 pathways showed fold changes of about 0.6 and 0.5, respectively, indicating a reduction in their expression when compared with control. Similarly, at 45 µg mL^{-1} Nrf2 had a slight fold change of about 0.8, while HO-1 showed a fold change of around 0.7, indicating an average rise in the expression.



A **B** **C**

Fig. 4: Gene expression study using RT-PCR analysis A) Agarose gel electrophoresis; B) Expression of Nrf-2 and HO-1 genes at different concentrations; C) Fold change of Nrf-2 and HO-1 genes

At 60 µg mL^{-1}, Nrf2 has a fold change of 1.8, suggesting strong upregulation, whereas HO-1 has a fold change of about 1.3, showing a considerable increase in expression. At 60 µg mL^{-1}, the therapy induced Nrf2/HO-1 pathway so caused increase in oxidative stress. HO-1 is an important protein that assists cells in adapting to stress generated by a variety of illness events. Some tumor cells express high levels of HO-1. HO-1-shRNA and particular inhibitors have been shown to prevent the proliferation of hormone-resistant prostate cancer cells. HO-1 has been demonstrated to have pro-apoptotic and anti-proliferative actions in prostate cancer (Gueron *et al*., 2009), breast cancer (Hill *et al*., 2005), and oral cancer (Lee *et al*., 2010). However, the mode of action is uncertain. A study by (Jang *et al*., 2016) found that simvastatin enhanced Nrf2 and triggered its nuclear translocation, resulting in the synthesis of HO-1-related antioxidant proteins in HT29 cells via the ERK and PI3K/Akt pathways. Lutein activates Nrf2 expression in colon cancer cells by binding to its promoter. Upregulating Nrf2 expression improves the interaction between p53 and Nrf2, resulting in enhanced production of antioxidant enzymes and apoptosis-related proteins (Kang *et al*., 2019).

Our study presents new and significant findings on the cytotoxic effects of *Commelina forskaolii* leaf extract against HT-29 colon cancer cells. The study showed a clear dose-dependent cytotoxicity.

Beyond confirming cytotoxicity, the study provides novel insights into the mechanisms involved, including modulation of antioxidant enzymes, increased lipid peroxidation, and iron accumulation suggesting the induction of ferroptosis. Additionally, CF causes cell cycle arrest at the G_0/S phase and upregulates the Nrf2/HO-1 pathway, linking oxidative stress responses to its anti-cancer effects. Cumulatively these results offer valuable mechanistic data that advance the understanding of CF's therapeutic potential in colon cancer. However, further mechanistic studies are needed to fully elucidate the molecular pathways involved. The study is the first reports to demonstrate that *C. forskaolii* drastically reduces the viability of cancer cells by expression of antioxidant response proteins and stimulation of Nrf2−HO-1 pathway as a crucial signalling route implicated in *C. forskaolii*-induced cell death. Our findings highlight a viable therapeutic treatment for colon cancer and added fresh insight into the involvement of HO-1 and antioxidant proteins in cell death.

Conclusion: The study demonstrated cytotoxic effects of *Commelina forskaolii* plant extract on HT-29 colon cancer cells, revealing its potential as an anticancer drug. MTT assay revealed a dose-dependent reduction in cell viability, validating effective cytotoxicity. Increased superoxide dismutase, glutathione peroxidase and lipid peroxidation activities suggest that CF induces oxidative stress and cell death in cancer cells. The CF therapy caused cell cycle arrest in G_0/S phase. The upregulation of Nrf2 and HO-1 genes, particularly at higher concentrations indicated that CF activates Nrf2/HO-1 pathway, so potentially improving cellular responses to oxidative stress. Ferroptosis, characterized by iron-dependent ROS generation, is a vital mechanism for cell death. HO-1 is essential for ferroptosis as it regulates ROS generation and modulates cellular iron levels. When Nrf2-driven HO-1 is moderately active, it neutralizes ROS, thereby protects cells. Overproduction of HO-1 in cancer cells can cause excessive Fe^{2+} buildup, inducing ROS overload and oxidative cell death. The dual role of HO-1 highlights its potential as a therapeutic target, especially in cancer, where adjusting HO-1 activity could be used to selectively cause ferroptosis in malignant cells.

Acknowledgement: The authors gratefully acknowledge the support provided by Karpagam Academy of Higher Education, Coimbatore (India). We extend our sincere thanks to the Department of Science and Technology, Government of India, New Delhi (India) for financial assistance through award of DST/INSPIRE fellowship (IF230451).

Conflict of interest: The authors declare that they have no competing financial interests.

Ethical declaration: Not applicable.

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