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Original Article

Influence of Cirsimaritin on Anticancer Activity against HCT-116 Cell Line

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ABSTRACT

Background: Statistics show colon cancer is the third most severe cancer type worldwide. It was discovered that there is a very high death and morbidity rate. Along with breast, prostate, lung, and colon cancer is one of the most common tumors worldwide. **Objectives:** Natural products have drawn interest in recent years for preventing cancer. This is due to their numerous health advantages, apparent lack of toxicity and side effects, and the limits of chemotherapeutic medicines. Numerous studies have shown that natural products have a wide range of biological effects. **Materials and Methods:** A flavonoid class phytochemical known as cirsimaritin, also known as 4',5-dihydroxy-6,7-dimethoxyflavone, has been discovered in the medicinal plant. The current investigation was the determination of cell proliferative XTT assay, AO/EtBr, intracellular oxygen species, Oxidative Stress Markers, and Cell cycle analysis using flow cytometry by PI analysis. **Results:** HCT-116 cells' cell viability was reduced with an increased concentration of cirsimaritin. ROS and apoptosis were increased with an elevated cirsimaritin concentration. **Conclusion:** All these results suggest that cirsimaritin can be used as a potent anticancer drug for colon cancer.

Keywords: Colon cancer, Cirsimaritin, Apoptosis, Cell viability, ROS.

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INTRODUCTION

The second-most fatal type of cancer is Colorectal Cancer (CRC). Both the prevalence and mortality of these diseases are predicted to rise globally in the next decades. CRC was the cause of 9.4% of cancer-related fatalities in 2020. Colon rectal cancer is a medical ailment that only affects the rectum or colon and is driven by the colon's abnormally high rate of glandular epithelial cell growth.[1] Both in men and women, colon cancer has a relatively high risk of morbidity and death. In all fields of study and clinical practice, rectal and colon cancers are acknowledged as a single malignant entity. This is based on the hypothesis that the big bowel, which is considered to be a single organ, is where rectal and colon cancers grow.[2] Exercise, diet, obesity, cigarette use, and excessive alcohol intake are all known to increase the chance of developing colon cancer. A family pedigree of colon cancer accounts for around 25% of cases, including hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis genetic disorders accounting for 5% of cases.[3]

Apoptosis is a crucial mechanism for eliminating precancerous and cancerous cells and can be a significant target for research on the development of cancer drugs. An initial phase, which mostly depends on the cell type and apoptotic triggers such as DNA damage and oxidative stress, initiates apoptosis. Apoptosis signaling is disrupted in cancer cells when the pro- and anti-apoptotic balance is destroyed. One of the primary processes of apoptosis has been widely hypothesized to be the regular overexpression of BCl_2 and downregulation of Bax expression in malignant environments.[4]

Chemotherapy and surgery have been used as the primary methods of treatment for cancer patients for a long time. However, the prognosis for CRC has generally been dismal for people with metastatic illness. Modern chemotherapeutic techniques now prioritize delivering safer and feasible concentrations of target drugs, and these dosages will target disease regions while sparing normal cells. Therefore, a technique of selective medicine distribution has to be researched to solve this problem.[5] Natural ingredients have gained relevance as the origin of polypharmacological therapies for infectious illnesses, cancers, and neuro-related disorders. Nature offers a wealth of resources for the primary care setting as well as remarkable compounds to treat many major disorders. Consequently, natural items are a great source of medications that may offer CRC therapy options. These natural compounds can come in a variety of forms, such as polysaccharides, diterpenoids, alkaloids, polyphenols, and unsaturated fatty acids, each having its own distinct set of qualities and diverse structural traits. Herbs and spices primarily function

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through activating the extrinsic apoptotic pathway, caspase, MMP, K-ras, and BCL-2 pathways, as well as by modulating the ER-stress-induced apoptosis chemo-preventive effects.[1]

A dimethoxy flavone called cirsimaritin is present in a variety of plants, including *Cirsium japonicum, Artemisia judaica*, L*ithocarpus dealbatus, Microtea debilis*, and *Ocimum sanctum*.[6] Cirsimaritin is chemically known as 4',5-dihydroxy-6,7-dimethoxyflavone. Antioxidant, anti-allergic, antibacterial, anti-breast cancer, nephroprotective, anti-inflammatory, antidepressant, and protective benefits against type 1 diabetes mellitus are just a few of the characteristics cirsimaritin demonstrates.[7] Human Osteosarcoma (SaOs-2), Human colon carcinoma (HT-29), Human Fetal Foreskin Fibroblast (HFFF-P16), murine fibrosarcoma (WEHI-164), MCF-7, and PC-3 cell lines were all susceptible to the anti-proliferative effects of cirsimaritin. Apoptosis, p-Akt, and cAMP/PKA signaling are a few of the major cancer targets that are involved in its anti-cancer processes.[8]

The goal of the current study was to determine the effect of cirsimaritin on reactive oxygen species, cell viability, apoptosis, the number of antioxidant indicators, and lipid peroxidation markers.

MATERIALS AND METHODS

Materials

Cirsimaritin, HCT-116 (colon cancer) cell line, Dublecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin-EDTA, chemicals, and reagents were procured from Standard suppliers.

Cell culture

The Human Colon Cancer cell line HCT-116 was acquired from the laboratory. Cells were kept viable in DMEM media that was enriched with 100 g/L heat-inactivated FBS, 100 µg/mL of streptomycin, and 100 IU/mL of penicillin. Cells were grown at room temperature in a humidified 5% CO_2 environment. Cells were cultured in 75 cm^2 culture flasks that contained 15 mL of DMEM.

XTT assay

Following the manufacturer's instructions, the cell proliferation XTT kit was used to examine the anti-cell proliferative effects of cirsimaritin in HCT-116 cells. At a density of 1×10^6 cells/well in the culture medium, the cells were seeded onto 96-well plates. Cirsimaritin (3.13, 6.25, 12.5, 25, 50, and 100 µg/mL) and 5-FU were administered to the cells in various concentrations. To determine cell viability, cells were then treated for 24 hr. To carry out the studies, DMSO (0.1%) was used to dissolve the cirsimaritin. The positive control was 5-FU, which is often employed in the treatment of colorectal cancer. A 450 nm reference wavelength and an ELISA plate reader were used to measure the absorbances of the soluble formazane generated by live cells (Bio-Rad Laboratories, Inc., Tokyo, Japan). The soluble formazane generated by living cells was tested for absorbances at 450 nm using an ELISA plate reader and a standard wavelength of 630 nm.[9] Data on absorbance were used to determine IC_{50} values. The assay was carried out three times, and the outcomes were averaged. The following formula was used to determine the cell viability:

% viable cells=[(absorbance of cirsimaritin treated cells)-(absorbance of medium without cell)]/[(absorbance of untreated cells)-(absorbance of medium without cell)]×100.

Dual staining analysis by AO/EtBr Staining

To visualize apoptotic cells, AO/EtBr staining was utilized. HCT-116 cells were plated at a density of 1×10^6 cells per well and allowed to grow for 24 hr. HCT-116 cells were exposed to Cirsimaritin at IC_{50} concentrations, standard drug 5-FU at a concentration of 5 µM/mL for 24 hr. After a 24 hr incubation period, the cells were rinsed with PBS before being fixed for 20 min at 4°C in a 3:1 mixture of methanol and glacial acetic acid. Following a PBS wash, the cells were stained for 30 min at room temperature with a 1:1 AO/EtBr dye.[10] PBS was used to clean stained cells before they could be examined under a fluorescence microscope (Olympus BX51).

Intracellular Oxygen Species (ROS)

In a 6-well plate, the HCT-116 cells were seeded at a density of 1×10^6 cells/well and left to incubate for 24 hr. Following incubation, cells were treated with various doses of Cirsimaritin (IC_{50}), standard drug 5-FU at a concentration of 5µM/mL, whereas control cells received only the medium and were not treated with Cirsimaritin. Cells were then maintained at room temperature in 5% CO_2 and 95% air. Cells from each well were then obtained, cleaned twice with PBS, and reinstated in 500 µL of DCFH-DA (10 µM) for measuring ROS at 37°C in a light environment.[11] Fluorescence intensity was examined under a fluorescence microscope (Olympus BX51).

Detection of Oxidative Stress Markers

The commercial SOD assay kit was utilized to assess SOD activity after Cirsimaritin was added to the cells by the instructor's instructions. Utilizing a plate reader, the samples' absorbance at 450 nm was calculated. The CAT test kit was used as directed to measure the CAT enzyme activity. The kit measures the enzyme by the way hydrogen peroxide breaks down. After Cirsimaritin was given to the cells as directed by the instructor, MDA activity was determined using a commercial MDA assay kit. Using a plate reader, the samples' absorbance at 450 nm was calculated. Additionally, a kit was employed to measure the GSH activity levels. By continually reducing 5,5′-dithiobis(2-nitrobenzoic

acid) in the presence of catalytic amounts (n moles) of GSH, a kinetic assay is utilized to quantify the quantity of GSH.[12]

Determination of cell cycle by PI analysis

Propidium iodide staining was used to investigate the cell cycle. After 48 hr of treatment with Cirsimaritin at IC_{50} concentration and common drug 5-FU (5 µM/mL), cells were trypsinized, then washed in PBS and preserved in 90% ethanol. Fixed cells were stained for hr with 5 µg/mL DNase-free RNase and 50 µM propidium iodide after two PBS rinses. The results were then examined using software and flow cytometry (BD Biosciences).[10]

Statistical Analysis

The results were shown as the mean ± standard deviation of three different samples. One-way analysis of variance was used to carry out the statistical analysis. The measurement employed to assess the variance between the variable groups was Duncan's multiple-range test. $p<.05$ was regarded as significant.

RESULTS

Cytotoxic effect of Cirsimaritin in HCT-116 Cells

Cells were treated with various doses of cirsimaritin (3.13, 6.25, 12.5, 25, 50, and 100 µg/mL) for 24 hr to determine the antitumor impact in HCT116 cells using the XTT test. The results demonstrated that, in a concentration-based way, cirsimaritin significantly inhibits cell growth. As can be seen from the growth inhibition curve in Figure 1, the Inhibitory Concentration (IC_{50}) of cirsimaritin for HCT116 cells was found to be 24.70 µg/mL. We selected IC_{50} levels for further studies based on this finding.

Apoptotic morphological alteration predicted in cirsimaritin-treated HCT116 cells

Cell apoptosis is characterized by variations in cell morphology. Here, AO/EtBr staining was used to assess apoptotic morphological changes (Figure 2). A red fluorescent dye called EtBr specifically penetrates the ruptured nuclei of dead cells. Only healthy, non-apoptotic cells could be penetrated by the green fluorescent dye of AO. Our findings showed that normal cells were chosen as living cells because they possessed a vivid green fluorescence nucleus. On the other hand, HCT116 cells treated with cirsimaritin at varying doses (IC_{50}) for 24 hr displayed orange and red-stained apoptotic cells. Notably, fragmented nuclei labeled in red show late apoptosis whereas orange staining shows early apoptosis were compared with standard drug 5-Fu treated cells and untreated control cells.

Intracellular Reactive Oxygen Species (ROS) in cirsimaritin-treated HCT116 cells

Figure 3 depicts the effect of cirsimaritin on the level of ROS in HCT-116 cells using the DCFH-DA staining method. The amount of fluorescence in control cells was negligible, but it significantly increased following exposure to DCFDA and various concentrations of IC_{50} cirsimaritin. Our results suggest that treatment of HCT-116 cells with cirsimaritin in a concentration-dependent manner caused cancer cells to undergo apoptosis, probably because these cells had greater amounts of ROS compared with standard drug 5-Fu treated cells and untreated control cells.

Lipid peroxidation and antioxidant status in cirsimaritin-treated HCT116 cells

One well-known indicator of oxidative stress is the high level of lipid peroxidation and low antioxidant status as depicted in Figure 4. In HCT116 cells treated with cirsimaritin (IC_{50}), we found elevated levels of the lipid peroxidative marker (MDA) and decreased levels of the antioxidant enzymes (GSH, SOD, and CAT) as compared to the control. This implies that cirsimaritin causes an increased level of ROS and induces apoptosis in HCT-116 cells (Figure 4).

Determination of Cell cycle arrest by PI staining

Figure 5 displays the effect of cirsimaritin on cell cycle analysis using PI staining. We employed flow cytometry to examine the DNA content of untreated and cirsimaritin-treated HCT-116 cells to evaluate the effects of the cirsimaritin. The HCT-116 cells exposed to cirsimaritin had a sub-G1 cell cycle, as evidenced by DNA content analyses compared to untreated cells. The HCT-116 cell line was significantly inhibited by the chosen IC_{50} level following a 24 hr treatment period, according to our results. The phases of cell cycle arrest were identified by flow cytometry, and the treated cells accumulated more sub-G1 cells than the untreated cells in comparison to 5-FU. A large percentage of cells ceased in the G2/M stage as a result of the cirsimaritin. Thus, cell cycle arrest occurs in the G2/M phase, which is analogous to the 5-FU impact on cells.

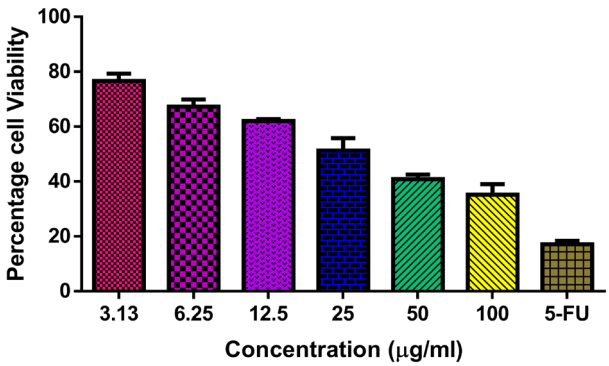

Figure 1: Cytotoxicity of Cirsimaritin on HCT-116 cells. HCT-116 cells were treated with XTT and Cirsimaritin at different concentrations in 96-well plates to determine whether they were cytotoxic. Here are representative data showing the dose resulted in 50% growth inhibition based on triplicate experiments performed in triplicate.

DISCUSSION

The most common cancer in the world and one with a high mortality rate is colon cancer. Surgery, radiation, and chemotherapy are the main therapeutic modalities used today to treat colon cancer. Finding cutting-edge medicines owing to poor control of tumor cells and eventual spreading is a serious clinical issue, despite improvements in the identification of cancer patients and appropriate medical management.[13] Finding a new therapeutic agent for treating cancer is therefore critically important. Plant-based phytonutrients have gained much interest as a potentially effective method for minimizing the rate of tumor growth.[14]

A popular test technique to assess cell survival and proliferation is the XTT assay. Based on the ability of mitochondrial dehydrogenase enzymes to convert XTT into a water-soluble, orange formazan product, viability, and proliferation are assessed in live cells.[15] In our work, treatment with cirsimaritin dramatically inhibited the growth of HCT116 cells. The IC_{50} concentration of the cirsimaritin was therefore identified as 24.70 µg/mL. Numerous studies revealed that cirsimaritin effectively reduced cell growth in breast cancer, skin cancer, lung cancer, and colon cancer models while having no toxic effects on healthy cells.[16]

A kind of genetically controlled programmed cell death called apoptosis inhibits the growth of multicellular organisms and tissues by removing redundant, unhealthy, and defective cells from the body. Chemotherapeutics work largely by encouraging tumor cell apoptosis to kill tumor cells and block them from proliferating. Anticancer medications' effectiveness is evaluated by their capacity to identify cancer cells and specifically encourage their apoptosis.[10] All cells are stained with Acridine Orange (AO), which gives the nucleus a green appearance. Ethidium Bromide (EB), which colors the nucleus red, is only absorbed by cells when the permeability of the cytoplasmic membrane has been disrupted. Additionally, EB outperforms AO. Thus, the nucleus of living cells is typically green; the nuclei of early apoptotic cells are bright green with condensed or fragmented chromatin; the nuclei of late apoptotic cells are orange with condensed and fragmented chromatin; and the nuclei of cells that have died through direct

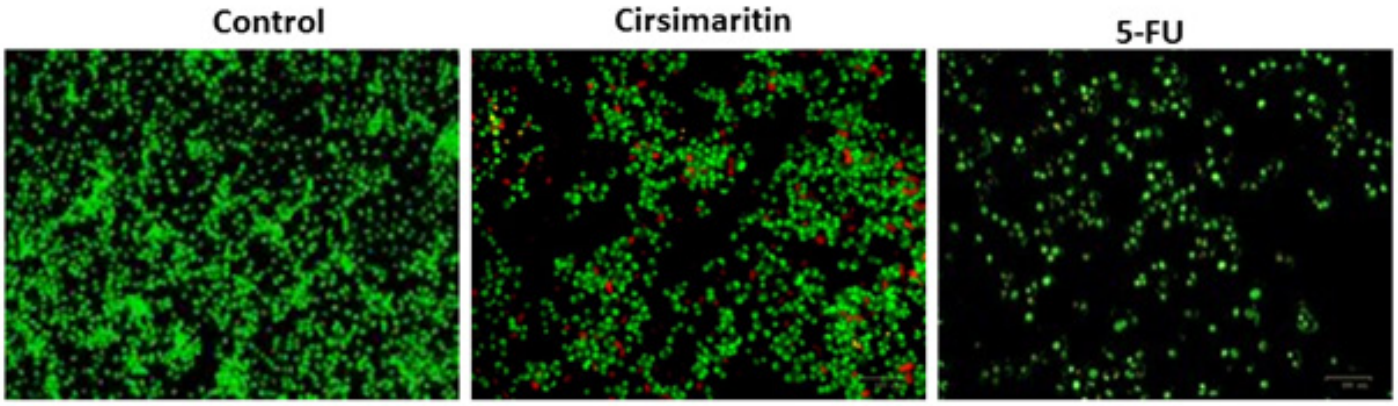

Figure 2: Effect of Cirsimaritin on induced apoptotic induction in HCT-116 cells. The cells were treated with Cirsimaritin (IC_{50}) and standard drug 5-FU at a concentration of 5 µM/mL compared to control for 24 hr, stained with dual dye AO/EtBr, and then analyzed by fluorescence microscopy. Values are expressed as mean±SD of three experiments.

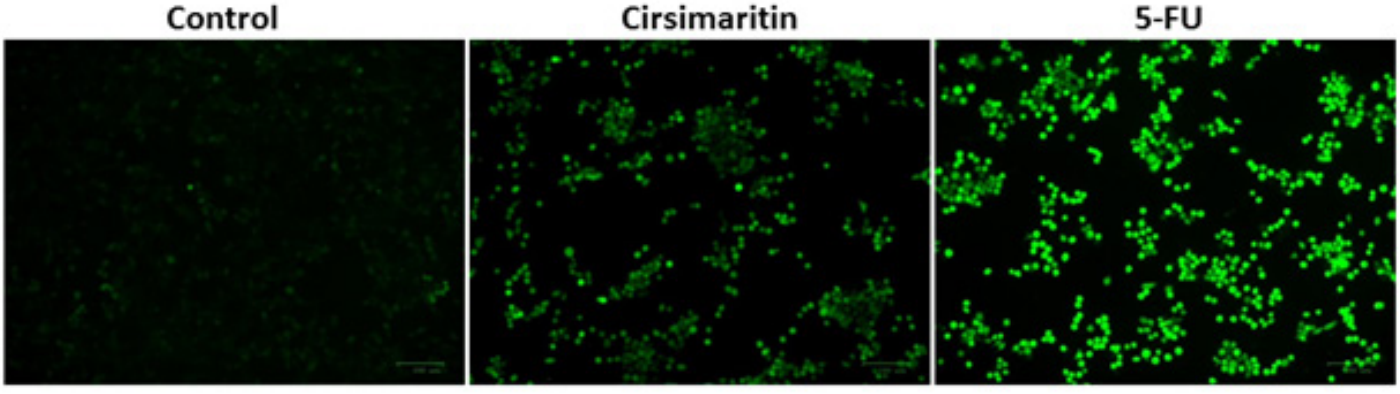

Figure 3: A fluorescence microscope image of Cirsimaritin-treated cells stained with DCF-DA showing intracellular ROS generation. In this study, we compared control cells with cells treated with IC_{50} concentrations of Cirsimaritin and standard drug 5-FU at a concentration of 5 µM/mL. A representative image of the triplicate experiment at 20X magnification is shown here.

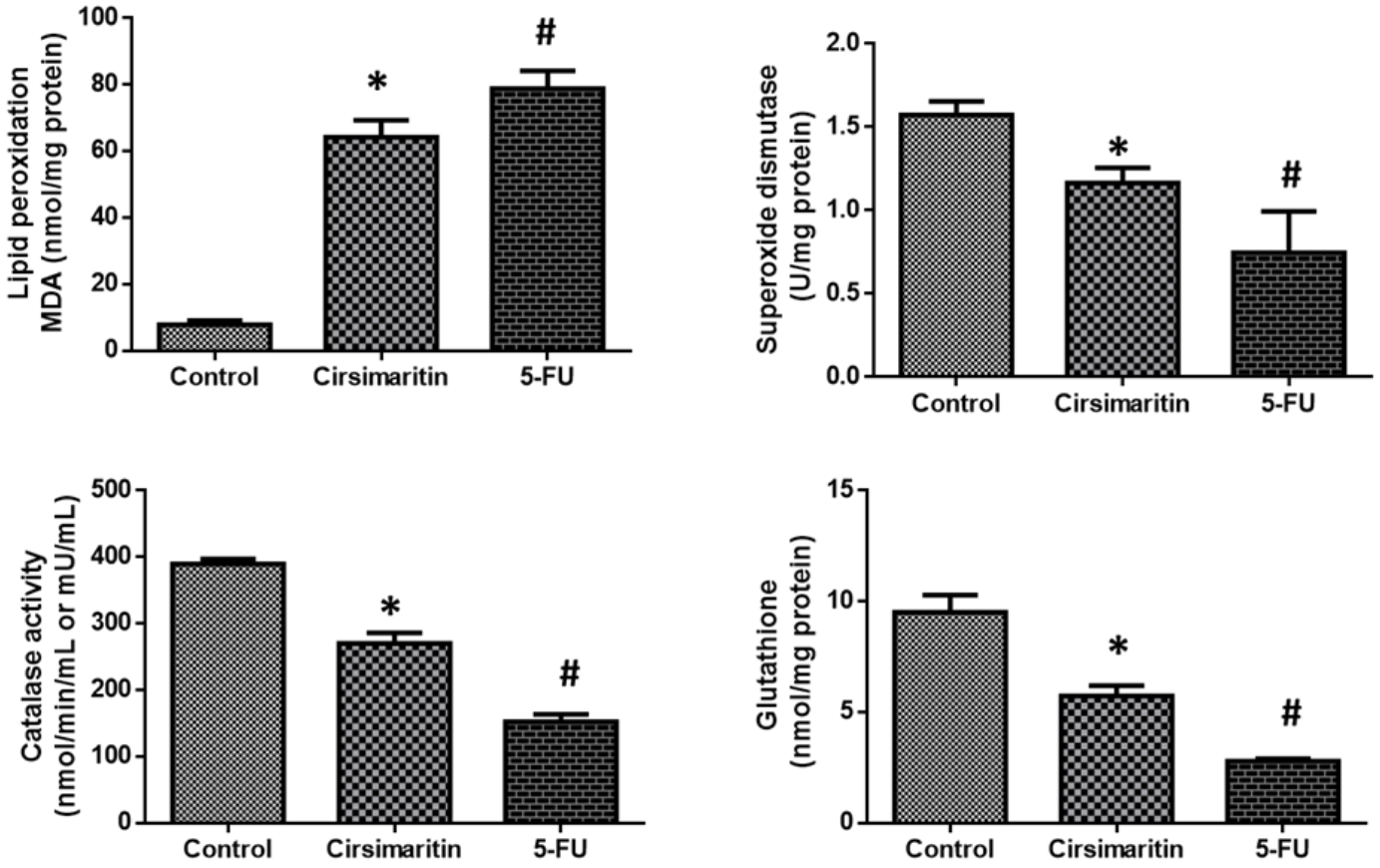

Figure 4: Effect of Cirsimaritin on oxidative stress levels in HCT-116 cells. The ELISA method was used to analyze the oxidative stress parameters (SOD, MDA, GHS, and CAT). Results are statistically significant. "*" indicates $p < .05$ compared with control and "#" indicates $p < 0.01$ compared with control.

necrosis are structurally normal orange.[17] In our work, we noticed that when the content of cirsimaritin increases, the orange-red fluorescence increases as well. This study demonstrates that apoptosis is promoted by cirsimaritin in colon cancer cells.

In colon cancer, oxidative damage-related apoptosis is greatly influenced by oxidative stress. Significant oxidative damage to intracellular molecules results from a disequilibrium between the excess production and/or elimination of ROS.[18,19] There are several recognized and established uses for Medicinal and Aromatic Plants (MAPs) in the fields of medicine and cosmetics. Some phytochemicals may cause cancer cells to undergo apoptosis and ROS-mediated cytotoxicity.[20,21] According to studies, cancer cells are more susceptible to oxidative stress than normal cells because of their high metabolic rate and dysfunctional mitochondria. Therefore, a further increase in ROS will probably cause cancer cells to attain their oxidative stress threshold earlier than normal cells, which will lead to oxidative stress-induced cancer cell death.[22] In the present study, we found that cells treated with cirsimaritin generated more ROS, which indicated high fluorescence intensity. In a previous study, it was reported that cirsimaritin elevates the ROS level.[23]

Oxidative stress induces the production of excess free radicals. Malondialdehyde (MDA) is a free radical that is generated when a free radical reacts with a lipid. It has the potential to change the structure of cell membranes and, over time, DNA at the cellular level. By evaluating the MDA levels, the degree of oxidative stress and LPO may be determined.[24] The first line of defense against ROS is superoxide dismutases. Metal-based enzymes are superoxide dismutases. An enzyme called catalase is also utilized in processes that neutralize ROS. Hydrogen peroxide is converted by the enzyme catalase.[25,26] One of the most prevalent thiol antioxidants in cells is Glutathione (GSH). The most significant hydrophilic antioxidant, GSH, shields cells against external and internal poisons, including Reactive Oxygen and Nitrogen Species (ROS and RNS).[27] The antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase are part of the body's intricate antioxidant defense system. They prevent the onset of free-radical chain reactions. When free radicals are generated in surplus or when the cellular antioxidant defense system is impaired, they can cause chain reactions by reacting with lipids, proteins, and nucleic acids, which can result in cellular dysfunction and even death.[28,29] Cells treated with cirsimaritin showed decreased levels of GSH, CAT, and SOD but the MDA enzyme level was elevated,

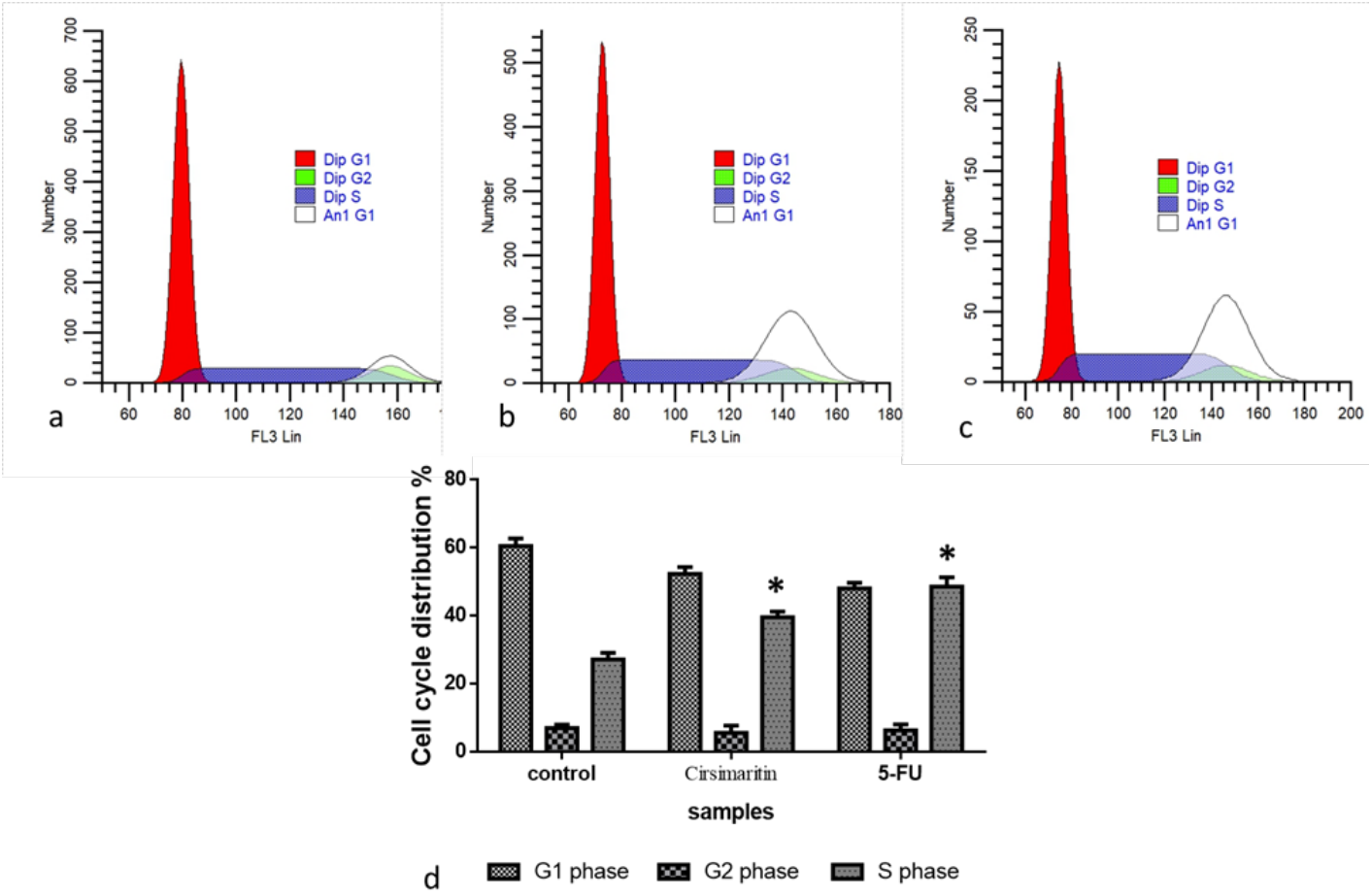

Figure 5: Cell cycle analysis using flow cytometry after staining with Propidium Iodide (PI). HCT-116 cells were treated with IC_{50} concentration of Cirsimaritin for 48 hr and standard drug 5-FU at a concentration of 5 µM/mL compared to control. Control cells (a); cells treated with Cirsimaritin (IC_{50} concentration) (b); cells treated with 5-FU (c); Cell cycle pattern and apoptosis distribution; Percentage of cell cycle distribution (d). "*" indicates $p<0.05$ compared with control.

suggesting that the cirsimaritin caused these cells to experience oxidative stress-mediated cell death.

Propidium Iodide (PI) staining was used to study cell cycles. PI is used more commonly than other stains. It is more stable, cheap, and better at detecting cell viability because it may eliminate dye in living cells. Membrane permeability influences whether PI can enter a cell. PI cannot detect viable or early apoptotic cells since the plasma membrane is unaffected by membrane permeability.[30] Intracellular ROS production has been linked to a variety of diseases, which may cause cellular death or cell cycle arrest. According to Banerjee *et al.*, 2016,[31] DNA damage is a biological occurrence that is intimately related to cell cycle arrest and apoptosis. In our study, Cirsimaritin and 5-FU at IC_{50} concentration increase a greater proportion of G2/M-stage cells in treated cells as compared to control cells. Cell cycle arrest consequently took place in the G2/M stage. The cirsimaritin, which was chemically comparable to 5-FU, significantly slowed the cell cycle of the HCT-116 cells.

CONCLUSION

According to our research, cirsimaritin's antioxidant and anti-proliferative properties led to apoptosis in HCT-116 cells. With higher cirsimaritin concentrations, cell viability was decreased. Apoptosis and ROS levels elevated in response to cirsimaritin concentration. Additionally, the MDA level was raised by the cirsimaritin while the levels of antioxidants like SOD, GSH, and CAT were decreased, indicating that the cancer cell apoptosis process occurred in place. It was revealed from the cell cycle investigation that cirsimaritin caused cell death. Thus, we can infer that cirsimaritin can cause apoptosis in HCT-116 cells and may prove to be a highly effective anticancer medication.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ACKNOWLEDGEMENT

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ABBREVIATIONS

pH: Potential of hydrogen; **M:** Molar; **mg:** Milligram; **µg:** Microgram; **GSH:** Glutathione; **SOD:** Superoxide dismutase; **MDA:** Malondialdehyde; **CAT:** Catalase; **mL:** Microliter; **IC_{50}:** Concentration of a compound with half-maximal cell viability; **DMSO:** Dimethyl sulfoxide; **ROS:** Reactive Oxygen Species; **AO:** Acridine Orange; **XTT:** 2,3-bis[2-methoxy-4-nitro-5-sulfopheny l]-2H-tetrazolium-5-carboxanilide; **EtBr:** Ethidium Bromide; **CO_2:** Carbon dioxide; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **CRC:** Colorectal cancer; **ER:** Endoplasmic reticulum; **5FU:** Fluorouracil; **PI:** Propidium Iodide; **PBS:** Phosphate buffer saline.

SUMMARY

According to our findings, Cirsimaritin's antioxidant and anti-proliferative properties caused apoptosis in HCT-116 cells. Cell viability decreased as cirsimaritin concentrations increased. Cirsimaritin concentrations increased apoptosis and ROS levels. Cirsimaritin also increased MDA levels while decreasing antioxidant levels such as SOD, GSH, and CAT, indicating that the cancer cell apoptosis process was active. Cirsimaritin causes cell death, according to the cell cycle investigation. As a result, we can conclude that cirsimaritin can induce apoptosis in HCT-116 cells and could be a highly effective anticancer medication.

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