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Original Research Article

In vitro Evaluation of the Antioxidant and Anticancer Activities of Chlorogenic Acid on Human Colon Cancer (HT-29) Cells

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ABSTRACT

ROS have a dual role in the body. At normal levels, they are essential for cell signalling and differentiation. However, high ROS levels can cause oxidative damage to DNA, lipids, and proteins, leading to diseases like cancer. The body's antioxidant defences counteract ROS damage, but imbalances can result in oxidative stress linked to various diseases, including cancer and diabetes. The study aims to evaluate chlorogenic acid's *in vitro* antioxidant and antiproliferative effects on HT-29 human colon cancer cells. The free radical scavenging ability of chlorogenic acid was determined using 2,2-diphenyl-1-picryl hydrazyl(DPPH) radical, ABTS radical, nitric oxide radical, and hydrogen peroxide scavenging assays. The antiproliferative effect of chlorogenic acid was assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, mitochondrial membrane potential measurement, ROS generation, and by measuring apoptotic induction potential. The present study observed a considerable free radical scavenging potential of chlorogenic acid compared to the reference drug ascorbic acid. Similarly, chlorogenic acid exhibited a potent antiproliferative effect against HT-29 cancer cells, which could be attributed to its apoptotic induction potential and its ability to generate excessive ROS to reduce the cell viability of HT-29 cells. The findings shed light on the potential therapeutic benefits of chlorogenic acid in combating colon cancer, offering insights for further exploration and potential development of novel anticancer treatments.

Keywords: Chlorogenic acid, Free radicals, Cell proliferation, Apoptosis

Introduction

Cancer is an abnormal mass of tissues in the body that usually occurs due to a genetic defect in a single cell. The genetically altered single cell is known as a neoplastic cell, which further proliferates to form a clone or mass of cells termed a tumour. Thus, the tumour cells lose the normal growth regulatory mechanisms and proliferate uncontrollably. Cancer is caused by several agents, which include radiation, chemical carcinogens, and viruses. Cancer is a prominent global cause of mortality, impacting about 10 million individuals annually.[1, 2]

According to epidemiological research, colon cancer is fourth globally in terms of frequency of cancer, whereas rectal cancer ranks eighth. Cancer of the colon and rectum together (colorectal cancer) has also been noted as the third most frequent form of cancer in the globe.[3] In India, each year, 4.4 and 4.1 per 100,000 men are affected by colon cancer and rectal cancer, respectively. Every year, around 3.9 per 100,000 women are diagnosed with colon cancer.[4] Colorectal cancer caused around 8,81,000 deaths by the year 2018, and it has been reported that colon cancer and rectal cancer account for 5.8% and 3.2% of all cancer deaths.[5]

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Reactive oxygen species (ROS) are a consequence of cellular metabolism that the body continuously generates. ROS may be beneficial or harmful to the organism, depending on how many of them are in the cells. They help the immune system eliminate infections, regulate cell survival and death, and act as a crucial mediator of cell signalling at normal cellular levels.[6,7] Though ROS at physiological concentrations plays a vital role in cell signalling, its higher generation as overproduction in the body could lead to diverse pathological diseases, including cancer. The antioxidant defence mechanism should have an upper hand to remove the excessively generated ROS. If the antioxidant defence mechanism fails, it leads to oxidative damage to biomolecules, especially to DNA, lipids, and proteins, which could, in turn, lead to several illnesses.[8,9,10]

In 1964, HT-29 was isolated from the primary tumour of a 44-year-old Caucasian female patient diagnosed with colorectal adenocarcinoma. Since then, numerous cell lines have been generated from human colon malignancies. The properties of mature intestinal cells, such as enterocytes or mucus-producing cells, are expressed by HT-29 cells. HT-29 cells require a high glucose concentration in the media because they consume glucose more quickly. It's a usual practice to use HT-29 cells to investigate the molecular pathways underlying intestinal cell development.

HT-29 cells can rapidly proliferate in a cell culture medium containing fetal bovine serum. HT-29 cells secrete several metabolites, pro-inflammatory cytokines known to promote cell survival. In preclinical research, HT-29 cells are found to be useful for epithelial cell research. This cell line has epithelial morphology and has wide applications in cancer research. Based on earlier findings, it was suggested that the cytokines secretion profile pattern of HT-29 cells mimics the *in vivo* biopsy samples' secretory pattern.[11,12]

The interaction of caffeic acid with the 3-hydroxyl position of L-quinic acid forms the ester chlorogenic acid, a metabolic intermediary in lignin

formation. *Phyllostachys edulis*, *Calluna vulgaris*, and *Hibiscus sabdariffa* are among the plants that contain this compound. Additionally, strawberries, apples, green tea, and tomatoes contain it.[13] According to reports, chlorogenic acid has various biological properties, such as antioxidant, antidiabetic, and hepatoprotective effects.[14,15] Dkhil *et al*.,[16] demonstrated the hepatoprotective effect of chlorogenic acid against arsenic-induced hepatotoxicity. Administering chlorogenic acid reduced the development of ulcerative colitis produced by dextran sodium sulfate in rats.[17] Ye *et al*.,[15] reported that chlorogenic acid attenuated oxidative stress in streptozotocin-mediated diabetic nephropathy in rats.

Chlorogenic acid significantly induced apoptotic cell death in leukaemia cells via regulating caspase-3 pathways.[18] Jiang *et al*.,[19] demonstrated chlorogenic acid-induced cytotoxicity in human oral cancer cell lines. Chlorogenic acid inhibited rat tongue carcinogenesis when administered concurrently with the carcinogen 4NQO.[20] Matsunaga *et al*.,[21] explored the protective role of chlorogenic acid in azoxymethane-induced colon cancer in rats. Shimizu *et al*.,[22] explored the antitumor efficacy of chlorogenic acid against methylnitrosourea-induced stomach carcinogenesis in rats. Tanaka *et al*.,[23] showed the tumour inhibitory potential of chlorogenic acid in rat hepatocarcinogenesis. Huang *et al*.,[24] pointed out the antitumor-promoting potential of chlorogenic acid in mouse skin carcinogenesis. Liu *et al*.,[25] reported that chlorogenic acid inhibited cell proliferation and induced preprophase apoptosis in HL-60 cells. Insufficient scientific literature exists regarding the anti-proliferative and antioxidant efficacy of chlorogenic acid in HT-29 colon cancer cells, highlighting a critical gap in current research. This investigation explored the impact of chlorogenic acid on suppressing the growth of HT-29 cells by free radical scavenging.

Materials and Methods

Chemicals

Chemicals and biochemicals used for the study were procured from Sigma-Aldrich, Pvt. Ltd. India, Hi Media laboratories, S.D Fine Chemical in Mumbai, and Fisher Inorganic and Aromatic Limited in Chennai.

DPPH radical scavenging activity

The technique of Blois *et al*.,[26] was applied to measure chlorogenic acid's DPPH radical scavenging action.

Chlorogenic acid was dissolved in DMSO at 10 to 50 µg/ml concentrations. Each solution was adjusted to a volume of 2.0 ml by adding methanol DPPH. The control group took the same quantity of methanol but without chlorogenic acid. The optical density (O.D) was calibrated at 517 nm in the UV-visible Spectrophotometer after a 20-minute incubation period. The absorbance values were recorded at 5-minute intervals until they reached stability. Chlorogenic acid was not used in the treatment of the control group. The standard used was ascorbic acid. The following formula was used to calculate the DPPH radical scavenging potential of ascorbic acid and chlorogenic acid.

$$\frac{\text{O. D of control} - \text{O. D of test}}{\text{O. D of control}} \times 100$$

ABTS radical scavenging assay

Miller *et al*.,[27] (2014) described the ABTS radical scavenging assay used to assess chlorogenic acid's overall antioxidant activity. The mixture for the reaction included ABTS (0.002 M), potassium persulfate added in a volume of about 3.5 ml, and chlorogenic acid at different concentrations (10-50 µg/ml). After a five-minute incubation period, the O.D of the reaction mixture at 734 nm was measured and comparable to ascorbic acid.

Nitric oxide radical scavenging activity

Using the Garrat[28] method, the capacity of chlorogenic acid to scavenge nitric oxide radical was determined. The reaction mixture comprising 0.5ml of PBS (1M), 2 ml of sodium nitroprusside (10 mM), and chlorogenic acid (10-50 µg/ml) in different concentrations was incubated at 25º C for two and a half hours. After incubation, 1 ml of sulphanilic acid (0.33%) prepared in glacial acetic acid (20%) was mixed with 0.5 ml of the reaction mixture. The mixture was then incubated for 5 minutes to promote diazotization. Incubation was carried out for 30 minutes after 1 ml of 0.1% w/v naphthylethylenediamine dichloride was added to the mixture. The mixture was then vigorously stirred. The OD of the developed colour was determined at 546 nm. As a control, the reaction mixture without chlorogenic acid was used. For the standard drug, ascorbic acid was utilized.

Hydrogen peroxide scavenging assay

Using the Jayaprakasha *et al*.,[29] approach, the hydrogen peroxide scavenging potential of chlorogenic acid was measured. Phosphate buffered saline at pH 7.4was used to make hydrogen peroxide (20 mM) solution. 2 ml of PBS in H_2O_2 solution was added with different doses of standard ascorbic acid and chlorogenic acid (10 to 50 µg/ml). After 10 minutes, 230 nm absorbance was measured and compared to hydrogen peroxide (blank solution).

Cell culture and drug treatment

The HT-29 cell line was supplied by the National Centre for Cell Science (NCCS), Pune, India. Cells were cultivated in 75 cm^2 tissue culture flasks using minimum essential media (Eagle) added with 10% fetal bovine serum (FBS), 1% glutamine, and 100 u/ml penicillin-streptomycin at 37ºC in a humidified atmosphere with 5% CO_2. Chlorogenic acid solution (0.05% DMSO) was treated to HT-29 cells.

Determination of effective cytotoxic dose of chlorogenic acid (MTT assay)

According to Mosmann *et al*.,[30] methodology, the MTT test was used to assess the cytotoxic effect of chlorogenic acid (10-100 µg/ml) on HT-29 cells. The dark purple insoluble formazan product is dissolved in a solubilization solution. Then, the absorbance is taken at 590 nm, which is the result of the reduction of the yellow-coloured MTT in the mitochondria.

Determination of ROS generation

The method of Rastogi *et al*., [31] was used to measure the amounts of ROS within cells. In brief, HT-29 cells (1 x 10^5 cells/well) were exposed to chlorogenic acid at an IC_{50} concentration of 50 µg/ml for 24 hours. After adding a known volume of DCFH-DA, the solution was incubated at 37°C for 30 minutes. Nikon fluorescence microscope was used to observe the cells using excitation (480 nm) and emission (530 nm) wavelengths with a blue filter.

Analysis of mitochondrial transmembrane potential (Δψm)

Analysis of mitochondrial transmembrane potential measurement was carried out essentially as described by Scaduto and Grotyohann.[32] The HT-29 cells (1x10^6 cells/well) on a 6-well plate were treated with chlorogenic acid at an IC_{50} concentration (50 µg/ml) for 24 hours, while the untreated cells served as the control group. The cells were exposed to Rhodamine-123 dye (10 µg/ml) and then incubated for 30 minutes in a CO_2 incubator. Next, the patterns of mitochondrial depolarization in the cells were studied using a fluorescent microscope with a blue filter.

Apoptotic morphological changes assessment by AO/EtBr staining

The apoptotic induction efficiency of chlorogenic acid was evaluated using the Baskić *et al*.,[33] described AO and EtBr staining technique. The HT-29 colon cancer cells were cultivated in 6-well plates (1x10^5 cells/well) with an IC_{50} concentration of chlorogenic acid (50 µg/ml) for 24 hours before being stained in a 1:1 ratio of AO/EtBr. After trypsinization, the cells were preserved in methanol and glacial acetic acid for 30 minutes at 37ºC and PBS rinsed. Stain-treated HT-29 cancer cells were seen under a 40x blue filter using a fluorescence microscope after an instant PBS wash. The number of cells displaying evidence of apoptotic cell death was assessed.

Statistical Analysis

The data values were expressed using the mean ± SD. Statistical comparisons were executed using one-way analysis of variance (ANOVA), followed by the application of DMRT for additional analysis. The p-value of less than 0.05 emphasized the significance of the findings.

Results and Discussion

Strong antioxidants are thought to provide a broad range of therapeutic benefits against various pathological conditions, including cancer development. Therefore, researchers from all around the world are looking for beneficial antioxidant properties in medicinal plants.[34] This research investigated chlorogenic acid's capacity to scavenge free radicals *in vitro* against ROS, including DPPH, ABTS, hydrogen peroxide, and nitric oxide radicals.

The percentage of DPPH radical scavenging activity of chlorogenic acid is shown in Figure 1. It was discovered that the IC_{50} value of chlorogenic acid (32.2 µg/ml) was more similar to the standard IC_{50} value of ascorbic acid (37.4 µg/ml). DPPH radical scavenging assay is one of the most commonly preferred *in vitro* free radical scavenging assays to evaluate the *in vitro* antioxidant potential of medicinal plants or their bioactive principles.[35] The present study noticed that chlorogenic acid had an excellent DPPH radical scavenging activity by reducing DPPH radicals. The present results showed that chlorogenic acid might have donated hydrogen atoms to reduce the odd electron of the nitrogen atom present in the DPPH. Ascorbic acid and chlorogenic acid both had similar effects on DPPH radical scavenging, and both effects were shown to be dose-dependent.

The ABTS radical scavenging ability of chlorogenic acid is presented in Figure 2. Chlorogenic acid significantly inhibited ABTS radical formation with an IC_{50} value of (24.3 µg/ml). The IC_{50} value of chlorogenic acid was comparable with the standard antioxidant, ascorbic acid (26.3 µg/ml). The impact of chlorogenic acid on ABTS radical scavenging was found to be dose-dependent. ABTS radical scavenging assay was commonly employed to determine the total antioxidant capacity of the test compound.[36] This method measures the concentrations of ABTS, which was generated by the reaction of potent oxidizing agents such as potassium persulfate with the ABTS salt. The present study observed a good ABTS radical scavenging effect of chlorogenic acid compared to ascorbic acid.

Figure 3 demonstrates the chlorogenic acid's ability to scavenge nitric oxide radicals. Chlorogenic acid exhibited a significant nitric oxide radical scavenging effect, as evidenced by its IC_{50} value (36.4 µg/ml), which is remarkably similar to ascorbic acid (32.5 µg/ml). Chlorogenic acid's effect on nitric oxide radical scavenging was shown to be dosage-dependent. Nitric oxide plays a pivotal role in several biological processes, including regulating blood flow and blood pressure.

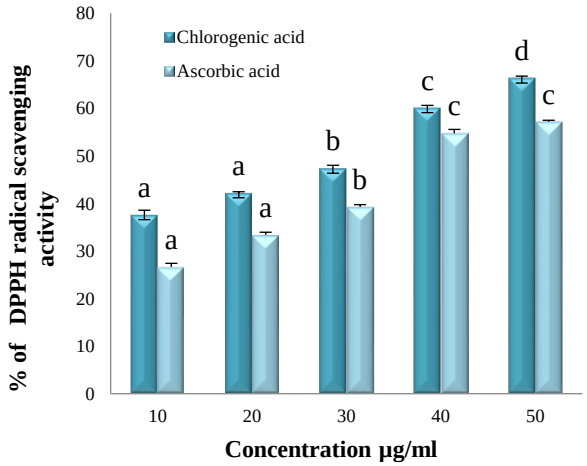

Figure 1: Chlorogenic acid and ascorbic acid effects on scavenging DPPH radicals. The superscripts that are different from each other differ at P<0.05

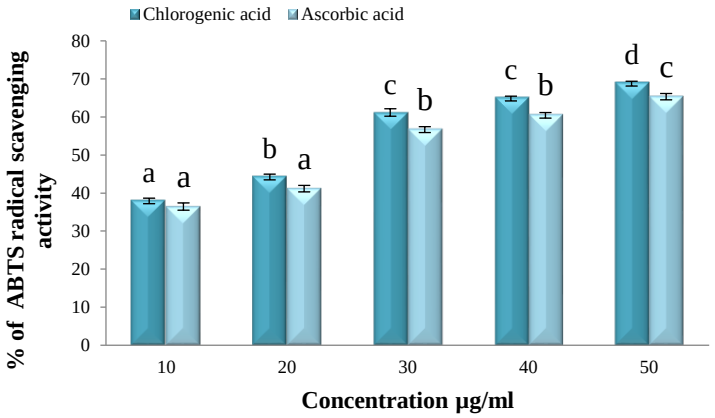

Figure 2: Chlorogenic acid and ascorbic acid effects on scavenging ABTS radicals. The superscripts that are different from each other differ at P<0.05

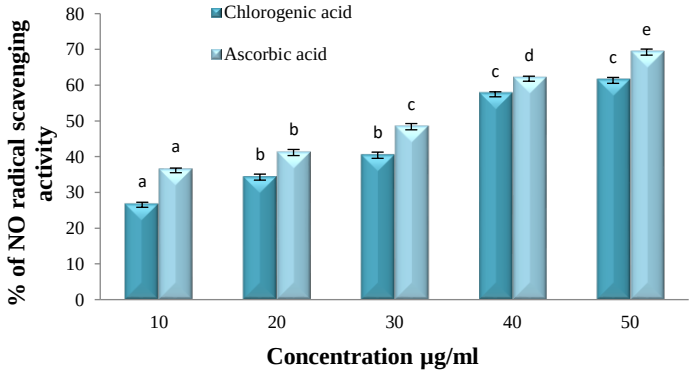

Figure 3: Chlorogenic acid and ascorbic acid effects on scavenging nitric oxide radicals. The superscripts that are different from each other differ at P<0.05

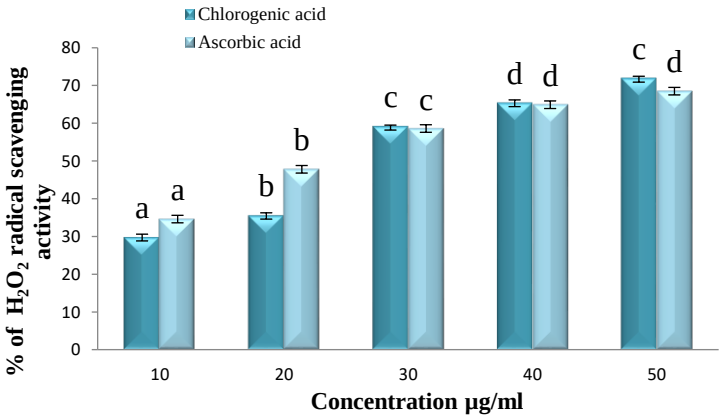

Figure 4: Chlorogenic acid and ascorbic acid effects on scavenging hydrogen peroxide radicals. The superscripts that are different from each other differ at P<0.05

Overproduction of nitric oxide has been reported to cause brain damage as well as several neurological manifestations.[37,38] The present study observed that chlorogenic acid significantly scavenged the nitric oxide radical under *in vitro* conditions, and the effect was compared to ascorbic acid.

The percentage of hydrogen peroxide scavenged by chlorogenic acid is illustrated in Figure 4. Chlorogenic acid exhibited a dose-dependent scavenging activity towards hydrogen peroxide. The IC_{50} value of chlorogenic acid (27.2 µg/ml) demonstrated a significant capacity for scavenging hydrogen peroxide, notably similar to the IC_{50} value (23.4

µg/ml) of the standard ascorbic acid. Hydrogen peroxides are potent precursors for the formation of dangerous or harmful hydroxyl radicals that can cause extensive damage to DNA, which in turn results in various diseases, including cancer.[39] In this investigation, chlorogenic acid dose-dependently scavenged hydrogen peroxide. It thus can prevent the oxidative damage of DNA from the ROS. The scavenging effect was also compared to ascorbic acid.

The IC_{50} values of chlorogenic acid for the several radicals examined *in vitro* are shown in Figure 5. The observed outcomes were compared with ascorbic acid. At IC_{50} values, the impact of chlorogenic acid on scavenging the above radicals was compared to ascorbic acid.

The current research investigated chlorogenic acid's antiproliferative activity on HT-29 cancer cells *in vitro*. The morphology of HT-29 cells and the cytotoxic impact of chlorogenic acid on HT-29 cells are depicted in Figure 6 and Figure 7, respectively. Cells were incubated with chlorogenic acid (10-100 µg/ml) for 24 hours. The chlorogenic acid's inhibitory concentration (IC_{50}) was observed at 50 µg/ml for 24 h incubation. MTT assay was utilized to determine chlorogenic acid's effect on HT-29 cell viability. Chlorogenic acid has a significant diminishing effect on the functionality of HT-29 cells in a dose-dependent manner, with an IC_{50} value of 50 µg/ml. According to the findings, chlorogenic acid was found to limit the growth of HT-29 cells. Figure 8 illustrates the production of ROS in HT-29 cells, both treated and untreated with chlorogenic acid after a 24-hour incubation period.

The IC_{50} value of chlorogenic acid (50 µg/ml) indicated the most significant production of ROS. Figure 9 shows the proportion of fluorescence intensity for ROS production. An significant increase in fluorescence intensity was seen in the HT-29 colon cancer cells when they were treated with chlorogenic acid. ROS cause extensive damage to biomolecules if they are extensively generated in the system.[40]

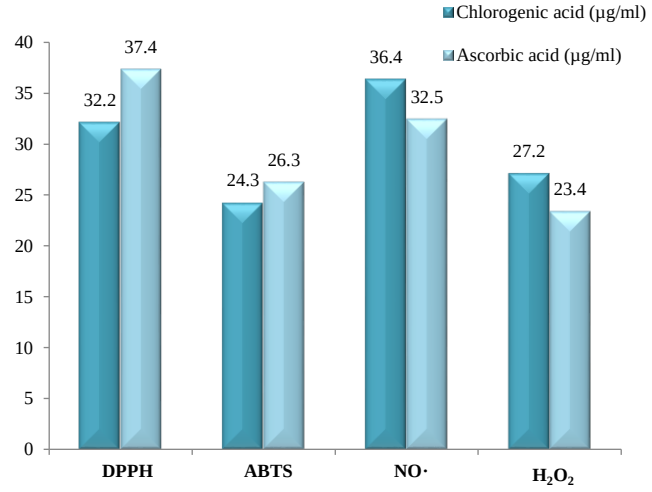

Figure 5: The IC_{50} concentrations of chlorogenic acid and ascorbic acid were evaluated for their effects on scavenging DPPH, ABTS, NO radicals as well as H_2O_2.

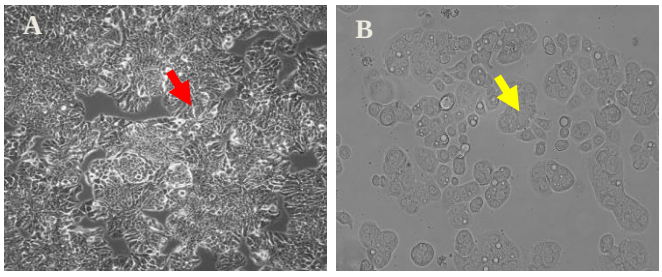

Figure 6: Morphology of HT-29 colon cancer cells (A) and chlorogenic acid-treated HT-29 cells (B). Treated with chlorogenic acid, HT-29 cells displayed pronounced morphological alterations and decreased cell viability.

An inverse relationship has been reported between ROS generation and cell proliferation.[41] In the present study, ROS generation was measured using DCF fluorescence, which was formed by the oxidation of H_2DCF to DCF. Chlorogenic acid-treated HT-29 cells exhibited high fluorescence intensity, which suggests that chlorogenic acid stimulated the production of ROS to induce cell death in HT-29 colon cancer cells. In HT-29 cells, treatment with chlorogenic acid significantly altered the mitochondrial membrane potential (MMP) (Figure 10). Green fluorescence of Rhodamine 123 was found in cells with a high MMP (Figure 11).

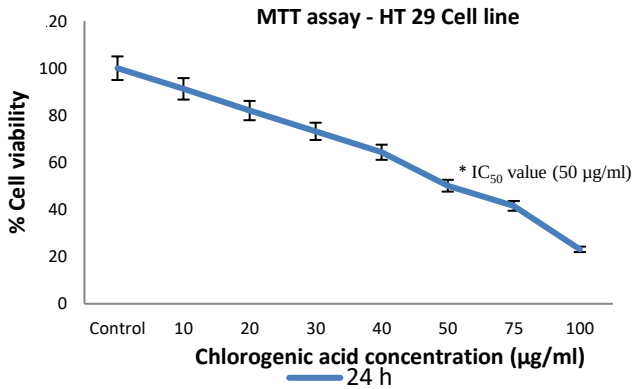

Figure 7: Chlorogenic acid's efficacy on the proliferation of HT-29 cells.

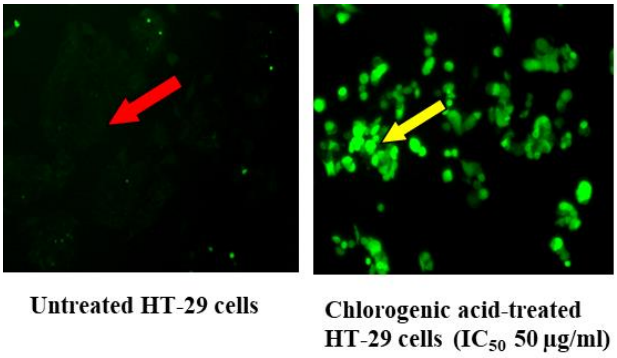

Figure 8: Efficacy of chlorogenic acid on ROS production in HT-29 cells. Chlorogenic acid-treated HT-29 cells exhibited intense green fluorescence attributed to the excessive production of ROS.

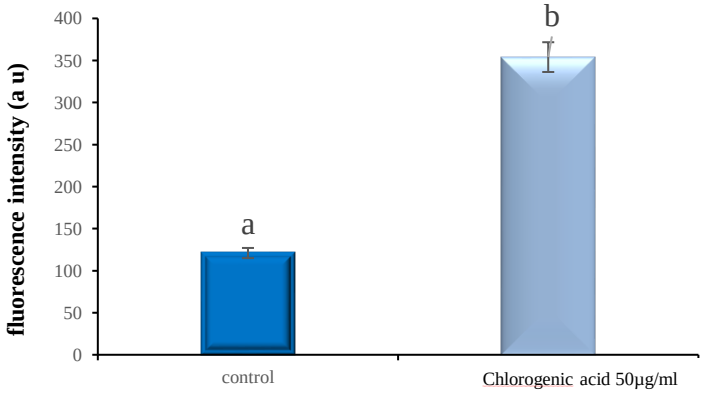

Figure 9: The percentage of fluorescence intensity for ROS generation in HT-29 cells and HT-29 cells treated with chlorogenic acid differs significantly ($p < 0.05$), indicated as a and b, respectively.

When HT-29 cells treated with chlorogenic acid (50 µg/ml) for 24 hours, the percentage of MMP fluorescence intensity was found to be lower than that of untreated HT-29 cells at the IC_{50} concentration. The present study observed that the MMP was significantly decreased in chlorogenic acid-treated HT-29 cells. This suggests membrane depolarization because live cells exhibited membrane polarization, as evidenced by higher fluorescence intensity in HT-29 cells.

Figure 12 shows HT-29 cells treated with an IC_{50} dose of chlorogenic acid (50 µg/ml) for 24 hours exhibit enhanced apoptotic cell death. Acridine orange/ethidium bromide stain, a DNA-binding dye, was used to identify the alterations in each cell. Bright green spots were seen in live cells. The HT-29 cells treated with IC_{50} concentration of chlorogenic acid (50 µg/ml) for 24 h showed increased apoptotic cell death (Figure 13). The present study also noticed the apoptotic-inducing potential of chlorogenic acid in HT-29 cells, as evidenced by dark green nuclei (early apoptotic), red nuclei (late apoptotic), and orange nuclei (necrotic) in dual staining. Live cells (HT-29 cells) exhibited green nuclei, which suggests that live cells might have taken up only acridine orange, whereas damaged or apoptotic or necrotic cells might have taken up both acridine orange and ethidium bromide. Therefore, the current work investigates the antiproliferative impact of chlorogenic acid on HT-29 cells *in vitro*.

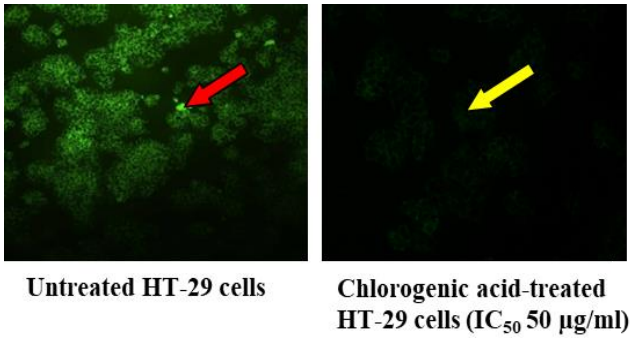

Figure 10: Efficacy of chlorogenic acid on MMP in HT-29 cells by Rhodamine-123 Staining. Treated with chlorogenic acid, HT-29 cells exhibited a decrease in green fluorescence attributed to alterations in mitochondrial membrane potential.

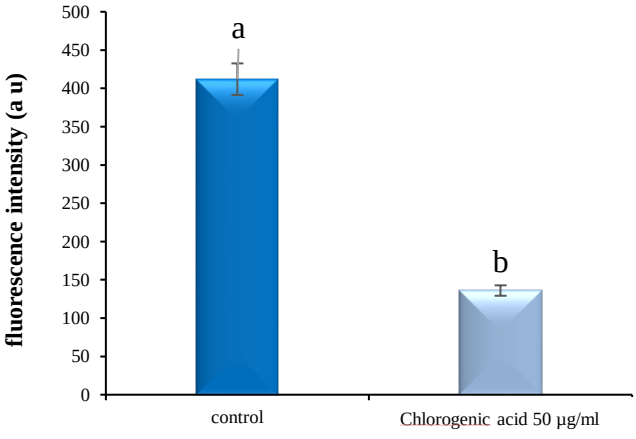

Figure 11: The percentage of fluorescence intensity for MMP in HT-29 cells and HT-29 cells treated with chlorogenic acid differs significantly ($p < 0.05$), denoted as a and b, respectively.

Conclusion

In the current investigation, chlorogenic acid showed a considerable free radical scavenging efficacy against DPPH, ABTS, hydrogen peroxide, and nitric oxide radicals, and the effect was found to be compared to ascorbic acid. In the present investigation, chlorogenic acid also exhibited a significant antiproliferative activity, as evidenced by its ability to decrease MMP, increase apoptotic cell death, generate excessive ROS, and reduce cell viability in HT-29 cells. The outcome of the present study will be extended in experimental animal models to further confirm the antiproliferative and antioxidant efficacies of chlorogenic acid in the future.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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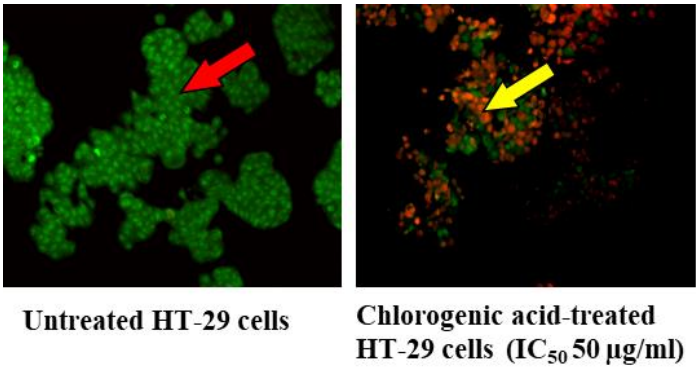

Figure 12: Efficacy of chlorogenic acid on morphological changes in the HT-29 cells using dual staining. Treated with chlorogenic acid, HT-29 cells exhibited an increased number of apoptotic cells.

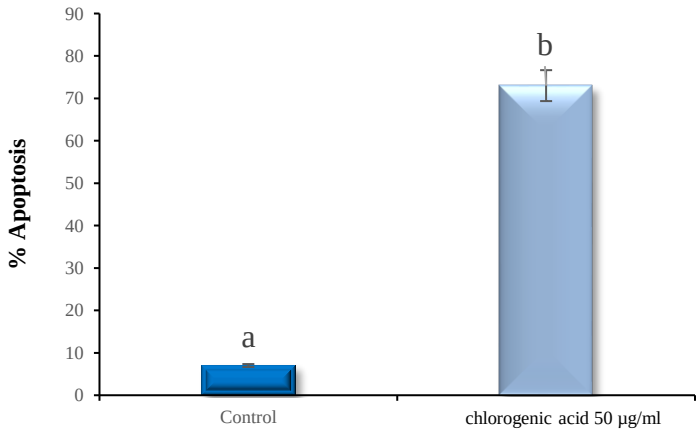

Figure 13: Percentage of apoptotic indices were measured in HT-29 cells treated with chlorogenic acid compared to untreated HT-29 cells. Statistical analysis indicates a significant difference between the two groups ($p < 0.05$).

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