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Antioxidant and Anticancer Potential of Date Seed Extracts in Colon Cancer Cells (HT-29 Cells): From By-Product to Bioactive

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Abstract

Due to their antioxidant qualities, date seeds (*Phoenix dactylifera*) are a prospective source of bioactive chemicals, especially flavonoids and phenolics. The cytotoxic and antioxidant effects on HT-29 colon cancer cells were investigated in this research. For 24 hours, HT-29 colon cancer cells were exposed to extract at different doses (125, 250, 500, and 1000 µg/ml) of date seed extracts. As a measure of cytotoxicity, lactate dehydrogenase (LDH) release was assessed. The MTT test was used to determine cell viability, and standard kits were used to measure the levels of glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) to evaluate the antioxidant response of the cells. The MTT assay showed that cell viability decreased as extract concentration increased, reaching 82% at 125 µg/ml and 62% at 1000 µg/ml. Higher extract concentrations resulted in higher LDH release, which showed enhanced cytotoxicity from 11.11% at 125 µg to 30.06% at 1000 µg/ml. At higher concentrations (1000 µg/ml), SOD activity increased considerably ($p \leq 0.05$) to 38.9 U/mg protein. GSH increased from 5.62 µM to 41.14 µM, and as concentrations increased, so did CAT activity. The findings showed that date seed extracts had dose-dependent cytotoxic effects on HT-29 colon cancer cells and strong antioxidant activity. The potential of date seeds as a functional food in cancer prevention and management is highlighted by increased cytotoxicity, which points to a preventive mechanism against cancer.

Keywords

Anticancer, Antioxidants, Apoptosis, Cytotoxicity, Colon Cancer, Date Seeds

1. Introduction

A major global health concern, colorectal cancer is the second most common cause of cancer-related deaths globally and the third most common cancer in terms of diagnoses. Its rates are on the rise in both industrialized and developing nations [1]. While screening has lowered mortality among older people, diet and lifestyle changes have contributed to a worrying increase in cases among younger adults [2]. Aging populations and the adoption of Western diets, which are often low in fruit and fiber and heavy in red and processed meats, are predicted to contribute to the rising incidence worldwide [2] [3]. According to research, eating a lot of whole grains and fiber lowers risk, but eating processed and red meat increases it. In certain regions, changing dietary factors could prevent up to 50% of instances [3]. Since colorectal cancer progresses slowly, there is a great chance to intervene before it spreads. To prevent or delay cancer, chemoprevention, which involves natural or synthetic dietary agents, is essential. Fiber, calcium, phytochemicals, and certain vitamins are beneficial dietary agents that have been shown in studies to reduce the onset and progression of colon cancer [2] [4].

Although frequently discarded as agro-industrial byproduct or waste, date seeds are now valued for their bioactive substances, especially their high phenolic and flavonoid content, which includes caffeic acid, chlorogenic acid, quercetin, and rutin [5]. Date seeds have higher levels of antioxidants than many fruits, with total phenolic contents as high as 50 g/kg. A significant amount of soluble and insoluble fibers is also present in them, which supports metabolic balance and intestinal health [6] [7]. Scientific research showing the ability to scavenge free radicals and chelate metals provides proof that date seed extracts have strong antioxidant properties, frequently surpassing those of conventional antioxidants [6]. Date seed extracts have cytotoxic and antiproliferative impacts on a variety of cancer cell lines, such as prostate (PC-3), liver (HepG2), and breast (MCF-7, MDA-MB-231) [8]. Dose-dependent reduction of cell growth, induction of apoptosis, and modification of cell cycle arrest are among the main effects caused by date seeds [9]. Although further mechanistic research is needed to fully understand its impact on antioxidants and inflammatory pathways, the high polyphenolic content and antioxidant capacity have been associated with the anticancer action [9] [10].

There has been a study that shows by increasing antioxidant enzyme activity, such as those of catalase (CAT) and superoxide dismutase (SOD), luteolin, a plant flavonoid, induced apoptotic cell death in human colon cancer HT-29 cells in a time-dependent manner [11]-[13]. There is a connection between antioxidant activity and cell death, as evidenced by the reduction of cytotoxic effects with the inhibition of these enzymes. Modifying antioxidant enzymes such as SOD and CAT can change the sensitivity of cancer cells to treatments that produce reactive oxygen species (ROS), which are linked to both tumor growth and treatment [14]. HT-29 cells are a key human colorectal adenocarcinoma cell line that is employed as an in vitro model for research on chemopreventive and colorectal cancer treatments. They evaluate several effects, including cytotoxicity, antiproliferation, apop-

tosis, and inflammation, of dietary phytochemicals, plant extracts, and synthesized substances [11]. To study the molecular pathways impacted by these substances, researchers use HT-29 cells. They concentrate on oxidative stress responses, the regulation of antioxidant defense, apoptosis, changes in the cell cycle, and the modulation of inflammatory mediators [12]. HT-29 cells are frequently used to investigate treatment effects, apoptosis, and proliferation. Mechanistic understanding of how these results relate to processes, such as autophagy or the control of antioxidant enzymes, is lacking, though [15]. Few studies integrate molecular profiling with functional outcomes in HT-29 cells, especially when it comes to the relationship between the behavior of cancer cells in response to phytochemicals or nanoparticles and the modification of antioxidant enzymes [15] [16]. To fully explore the bioactive potential of date seeds, this study will investigate the anticancer and antioxidant effects of date seed powder on colon cancer cells (HT-29 cells). The results of this research may provide useful knowledge in the field of natural products and potentially facilitate the use of date seeds as chemopreventive agents.

2. Materials & Methods

2.1. Sample Preparation

Fresh Dates were obtained from a local store in Huntsville, Alabama, US. After the removal of seeds from Medjool Dates (Natural Delights, California) seeds were freeze-dried for 48 hours (VirTis Genesis 35L SpScientific, Warminster, PA). Before being extracted, dried date seeds were ground into a fine powder using a Waring blender (Model no. 31BL92, New Hartford, Connecticut, US). With minor adjustments, the method created by Amazu *et al.* (2010) was used to prepare ethanol extracts (EE) [17]. For three hours, a known amount of freeze-dried date seeds was soaked in 100 ml of 80% ethanol while being constantly stirred. After that, the samples were sonicated (Bransonic M5800H Ultrasound Sonic Bath) for sixty minutes. Centrifugation of the ethanol extracts was done for 20 minutes at 4830× g. A rotary evaporator (Buchi Rotavapor R-215, USA) was used to evaporate the filtrate to dryness at 40˚C after the supernatant had been filtered through Whatman filter paper. Until further use, the concentrate was kept at −80˚C. About 10% (w/w) of dry extract was obtained from the extraction of *Phoenix dactylifera* seeds using 80% ethanol. For HT-29 cell studies, the dried extract was reconstituted in DMSO (final concentration ≤ 0.1% v/v in EMEM culture medium) to create a stock solution of 10 mg/mL, which was then diluted to achieve the required treatment doses (125 - 1000 μg/ml). Total phenolic content (TPC), reported as mg gallic acid equivalents (GAE)/g extract, was found to be 76.6 mg GAE/g to partially define the extract composition and assure comparability with previous research. This metric confirms the existence of phenolic bioactive compounds linked to cytotoxic and antioxidant properties. All treatment wells had a final DMSO concentration of less than 0.1% (v/v), which is regarded as non-cytotoxic. To equal the solvent conditions across all groups, vehicle control with the same

final concentration of DMSO (≤0.1% v/v) in EMEM was added.

2.2. Culturing of HT-29 Cells (Colon Cancer Cells) & Treatment with Date Seed Extracts

HT-29 (ATCC® HTB-38) human colorectal adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC) and cultivated in accordance with the ATCC's instructions. This cell line required ATCC-formulated Dulbecco's Modified Eagle Medium (Catalog No. 30-2002) as its base medium. 10% fetal bovine serum was added to the base medium to achieve the full growth medium. Date seed extracts were applied to the cells, and they were then incubated for 24 hours at 37˚C. HT-29 cells were used at a passage range of 5 to 6 for the entire experiment. The cells were plated at a cell density of 5000 cells/well for 96-well plates for MTT, LDH, and apoptosis assays and 50,000 cells/well for 24-well plates for biochemical assays. The treatments were started when the cells were approximately 70% - 80% confluent. All the treatments were done in triplicates as technical replicates for the entire experiment. Each experiment was done independently to validate the consistency of the results. The cells were treated with date seed extract (DSE) individually, and there was no co-treatment of Hydrogen Peroxide. Hydrogen peroxide was added as a separate positive control for validation of cytotoxicity mediated by oxidative stress. In addition to H_2O_2, colon cancer cells (HT-29) were treated with different concentrations (0, 125, 250, 500 & 1000 μg/ml) of date seed extracts.

Determination of Cytotoxicity

Lactate dehydrogenase (LDH), an enzyme that is released from the cell wall when a cell is injured. Utilizing a Pierce LDH cytotoxicity evaluation kit (Thermo Scientific), the absorbance of LDH at 490 and 680 nm was measured. Cell death and lysis were measured using a colorimetric assay in which cells were treated with 10X Lysis Buffer, which acts as the maximum LDH activity control. The assay quantifies the quantity of LDH that releases into the supernatant from the cytoplasm of damaged cells.

2.3. Cell Viability

The MTT Cell Proliferation Assay kit (Cayman Chemical, MI, USA) was used to assess the impact of date seed extracts on cell viability. The treated cells were cultured for a 24 hr period after being plated at a density of 5000 cells per well. MTT reagent was added, and the mixture was incubated for three to four hours at 37˚C in a CO_2 incubator. Read the absorbance at 570 nm after adding 100 μl of crystal dissolving solution and incubating it at 37˚C in a CO_2 incubator for 4 - 18 hours.

2.4. Antioxidant Assays

Superoxide Dismutase (SOD) Activity

The Superoxide Dismutase Assay kit (Cayman Chemical, MI, USA) was used to assess impact of date seed extracts on SOD activity. Add 200 μl of diluted Rad-

ical detector and 10 µl of sample to the wells. Read the absorbance at 440 - 460 nm after adding 20 µl of diluted Xanthine Oxidase to initiate the reactions for 30 minutes at room temperature.

Catalase (CAT) Activity

The Catalase Assay kit (Cayman Chemical, MI, USA) was used to assess the impact of date seed extracts on catalase activity. To each well, added 100 µL of catalase buffer, 30 µL of methanol, and 20 µL of sample. Then, started the reaction with 20 µL of diluted H_2O_2 and was allowed to sit at room temperature for 20 minutes. Added 30 µL of KOH to stop the reaction, followed by 30 µL of Purpald catalase, and then incubated for 10 minutes. Measured absorbance at 540 nm after adding 10 µL of catalase potassium periodate and incubating for 5 minutes.

Glutathione (GSH) Levels

The Glutathione Assay kit (Cayman Chemical, MI, USA) was used to assess the impact of date seed extracts on GSH levels. Standards and samples were added to the wells. The assay cocktail consisting of MES buffer, reconstituted Cofactor mixture, reconstituted enzyme mixture, water and reconstituted DTNB was prepared. Add 150 µl of the Assay cocktail to each well. GSH concentration was measured by end point method at 405 - 414 nm after 25 minutes.

2.5. Apoptosis

Apoptosis/Necrosis assay kit (ab176749, abcam) was used to assess the cell death (apoptosis) caused by date seed extracts. The cells were cultured and treated with date seed extracts for 24 hours. The cells were washed with assay buffer and were resuspended in 200 µl of the Assay buffer and 2 µl of Apopxin green indicator (100×) was added to the cells. The cells were washed with assay buffer after incubation for 30 - 60 minutes and then the cells were analyzed under fluorescence microscope using the FITC channel (Ex/Em = 490/525 nm). Apoptosis was quantified using automated imaging and analysis with BioTek's Lionheart FX Automated Microscope (Agilent). The quantification was done using the company's Gen5 software. The cell cultures were stained using a fluorescence-based apoptosis assay. The cell cultures were then imaged using a fluorescence microscope with multiple channels. For each data point, 6 non-overlapping fields were imaged at 20× magnification for each well, with the experiment being done in triplicate. Apoptotic cell quantification was done using intensity thresholding, where threshold for apoptotic channel was determined using untreated controls for baseline and hydrogen peroxide for maximum response. Background fluorescence was also kept to a minimum using automated background subtraction settings within the software.

Apoptosis percentage was calculated as:

$$\% \, Apoptosis = \frac{Number\ of\ Apoptotic\ cells}{Total\ cell\ count} \times 100$$

To establish statistical robustness, at least 1000 - 2000 cells per condition were examined across fields.

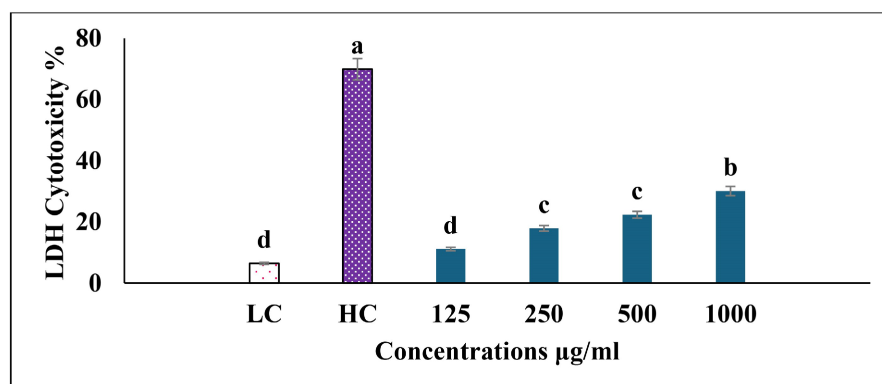
2.6. Statistical Analysis

The results were represented as means ± SEM, and all the experiment were carried out in triplicates. Analysis of variance (ANOVA) was the method of statistical analysis performed. The statistical analysis was conducted using the One-way ANOVA version of SAS 9.3. Significant mean differences were determined using Tukey's studentized range test; $p < 0.05$ was considered significant for significant differences.

3. Results

3.1. Cytotoxicity

Figure 1 illustrates the lactate dehydrogenase (LDH) release, which is a marker of cytotoxicity in colon cancer cells following exposure to varying concentrations of date seed extract, with comparisons made to the LC (Low Control, media only) and HC (High Control, hydrogen peroxide). Statistically significant differences ($p < 0.05$) between groups are indicated by distinct letters on the bars. There was a dose response release of LDH (11.11% - 30.06%) following incubation with DSE (125 - 1000 μg/ml) at 24 hr incubation, as shown in Figure 1. LDH release for the different concentrations of DSE ranged from 11.11% - 30.06% after 24 h compared to LC (media) alone having (6.43%) and HC (Hydrogen peroxide 200 μM) having (69.85%) LDH release. These values consistently exceeded basal levels, and the response was dose-dependent, reflecting a progressive disruption of cell membrane integrity with increasing DSE concentrations. LDH release in the LC was 6.43%, while the HC reached 69.85%. This shows that oxidative stress significantly ($p < 0.05$) elevated cytotoxicity compared to basal conditions. At the highest concentration (1000 μg/ml) of DSE (30.06%), cytotoxicity remained significantly ($p < 0.05$) lower than HC (69.85%) with a 2.32-fold difference. At the lowest dose (125 μg/ml), LDH release (11.11%) was significantly ($p < 0.05$) higher than the LC (6.43%) with approximately 53% higher LDH release. At the highest dose (1000 μg/ml) of DSE, LDH release (30.06%) was 4.67-fold higher compared to LC (6.43%).

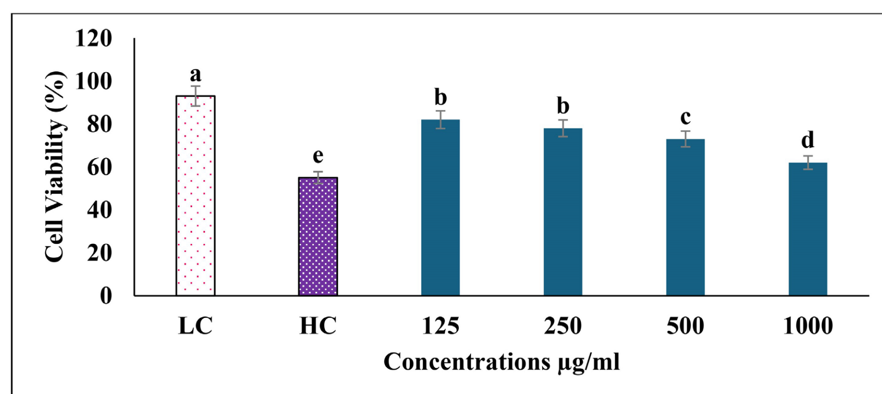


*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); Letters (abcd) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

Figure 1. Lactate dehydrogenase (LDH) release (Cytotoxicity) in colon cancer cells after treatment with date seed extract.

3.2. Cell Viability

Cell viability was assessed using MTT assay kit after 24 hr incubation with DSE at increasing concentrations, as shown in **Figure 2**. Statistically significant differences ($p < 0.05$) between groups are indicated by distinct letters on the bars. There was a dose dependent decline in cell viability (82% - 62%) following incubation with DSE (125 - 1000 µg/ml) at 24 hr as shown in **Figure 2**. Cell viability for the different concentrations of DSE ranged from 82% - 62% after 24 h compared to LC (media) alone having 93% and HC (Hydrogen peroxide—200 µM) having 55% cell viability. These values consistently decreased compared to basal levels, and the response was dose-dependent, reflecting a progressive disruption of cell membrane integrity with increasing DSE concentrations. Cell viability in the LC was 93%, while the HC had 55% cell viability. This shows that oxidative stress significantly ($p < 0.05$) elevated cytotoxicity compared to basal conditions. At the highest concentration (1000 µg/ml) of DSE (62%), cell viability was higher than HC (55%) with a 7% percentage difference. At the lowest dose (125 µg/ml), cell viability (82%) was significantly ($p < 0.05$) lower than the LC (93%) with a percentage difference of approximately 11% lower cell viability. At the highest dose (1000 µg/ml) of DSE, cell viability (62%) was 1.5-fold lower compared to LC (93%).



*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); MTT—(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide); Letters (abcd) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

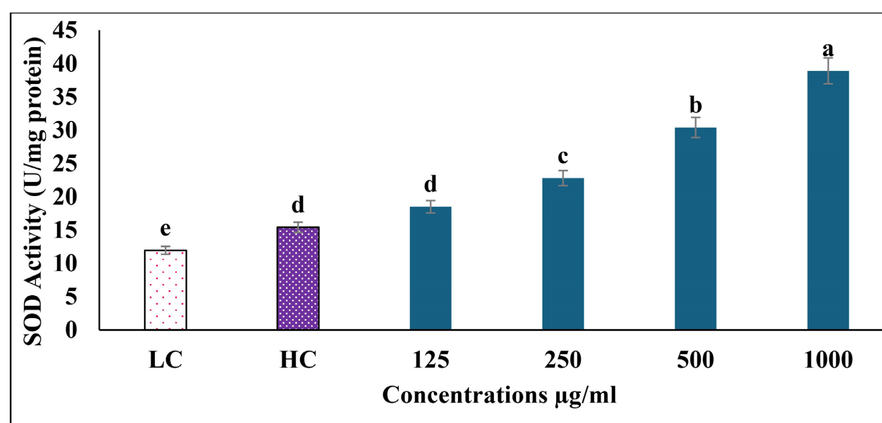
Figure 2. Viability assay (MTT) in colon cancer cells after treatment with date seed extract.

3.3. Antioxidant Assays

Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity was measured in HT-29 cells after 24 h incubation with increasing concentrations of DSE (125 - 1000 µg/ml). As shown in **Figure 3**, there was a concentration-dependent increase in SOD activity following treatment with DSE compared to both LC and HC. Statistically significant differences ($p < 0.05$) between groups are indicated by distinct letters on the bars. Cells incubated with LC exhibited a basal SOD activity of approximately 11.96 U/mg protein, while exposure to HC resulted in 15.41 U/mg protein SOD activity. In

contrast, treatment with DSE significantly ($p < 0.05$) elevated SOD activity in a dose-dependent manner. At the lowest dose (125 μg/ml), SOD activity was 18.5 U/mg protein, representing a 42.9% increase over LC. With further increase in DSE concentration, SOD activity progressively enhanced, reaching 38.9 U/mg protein at 1000 μg/ml with more than a 3.25-fold increase compared to LC and 2.52-fold higher than HC. This dose-dependent rise in SOD activity suggests that DSE enhances the cellular antioxidant defense system, counteracting the oxidative stress induced by hydrogen peroxide. Notably, the highest DSE dose (1000 μg/ml) restored and amplified SOD activity well beyond basal levels, highlighting a strong antioxidant response in HT-29 cells.



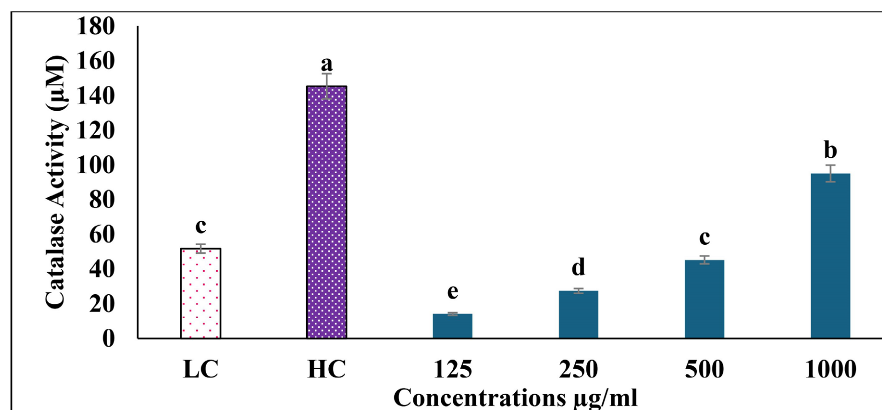
*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); Letters (abc) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

Figure 3. Superoxide Dismutase (SOD) activity in colon cancer cells after treatment with date seed extracts at different concentrations.

Catalase (CAT) Activity

Catalase activity was assessed in HT-29 cells following 24 h incubation with increasing concentrations of DSE (125 - 1000 μg/ml). As shown in Figure 4, CAT activity was significantly altered across treatment groups, with distinct letters on the bars indicating statistically significant differences ($p < 0.05$). Cells treated with LC exhibited a basal CAT activity of approximately 51.76 U/mg protein, while exposure to HC markedly elevated CAT activity to 145.3 U/mg protein, reflecting a strong oxidative stress response. In contrast, treatment with DSE produced a dose-dependent restoration and enhancement of CAT activity compared to LC and HC. At the lowest dose (125 μg/ml), CAT activity declined to 14.08 U/mg protein, nearly 3.67-fold lower than LC, suggesting an early inhibitory effect of DSE. However, with increasing concentrations of DSE, CAT activity progressively increased, reaching 45.19 U/mg protein at 500 μg/ml and peaking at 95.04 U/mg protein at 1000 μg/ml. This final value, by the highest concentration of DSE represented a 1.8-fold increase compared to LC. Overall, these findings indicate that DSE modulates catalase activity in HT-29 cells in a concentration-dependent manner, initially reducing activity at lower doses but enhancing it at higher concen-

trations, thereby strengthening the enzymatic antioxidant defense system under oxidative stress conditions.

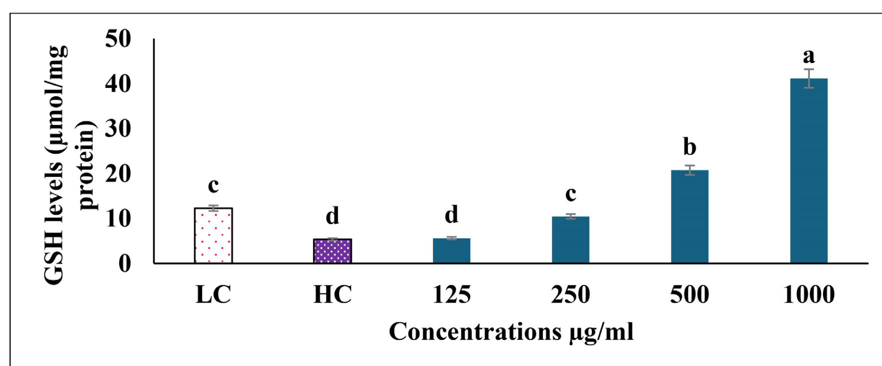


*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); Letters (abcd) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

Figure 4. Catalase (CAT) activity in colon cancer cells after treatment with date seed extracts at different concentrations.

Glutathione (GSH) Levels

Reduced glutathione (GSH) levels were quantified in HT-29 cells after 24 h incubation with DSE at increasing concentrations (125 - 1000 µg/ml). As shown in Figure 5, GSH levels varied significantly across treatments, with distinct letters on the bars indicating statistically significant differences ($p < 0.05$). Cells maintained in low control (LC, media) exhibited baseline GSH levels of 12.28 µmol/mg protein, whereas treatment with hydrogen peroxide (HC, 200 µM) resulted in a significant depletion of intracellular GSH to 5.36 µmol/mg protein, reflecting oxidative stress-induced glutathione consumption. At the lowest DSE dose (125 µg/ml), GSH levels (5.62 µmol/mg protein) were comparable to HC, showing no significant recovery from oxidative depletion. However, with higher concentrations of DSE, a clear dose-dependent enhancement of GSH levels was observed.



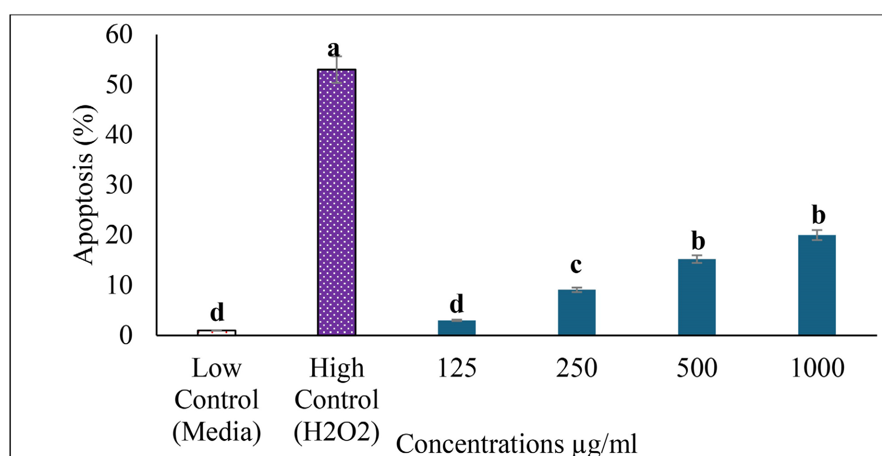
*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); Letters (abcd) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

Figure 5. Glutathione (GSH) levels in colon cancer cells after treatment with date seed extracts at different concentrations.

At 250 μg/ml, GSH increased to 10.44 μmol/mg protein, approaching baseline LC levels. Further increments were observed at 500 μg/ml (20.73 μmol/mg protein) and peaked at 1000 μg/ml (41.14 μmol/mg protein), which was approximately 3.3-fold higher than LC and nearly 8-fold higher than HC. These results demonstrate that DSE supplementation restores and significantly enhances intracellular GSH levels in HT-29 cells in a concentration-dependent manner, suggesting a strong role in replenishing cellular antioxidant defenses against oxidative stress.

3.4. Apoptosis

Apoptosis induction in HT-29 cells following 24 h treatment with DSE was quantified using the Apoptosis/Necrosis Kit (Abcam). As shown in Figure 6, a dose-dependent increase in apoptosis was observed with increasing concentrations of DSE (125 - 1000 μg/ml), with statistically significant differences ($p < 0.05$) indicated by distinct letters on the bars. Cells cultured under basal conditions (LC) exhibited negligible apoptosis (0.94%), while HC showed apoptosis increase to 53%, confirming its role as a positive control for oxidative stress-induced cell death. At the lowest DSE concentration (125 μg/ml), apoptosis was lower (2.99%), close to LC levels.



*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); Letters (abcd) on bars indicate a statistically significant difference ($p < 0.05$) between the treatments.

Figure 6. Dose-dependent apoptosis induction by date seed extracts in colon cancer cells (Abcam Apoptosis Assay kit).

However, with increasing concentrations of DSE, apoptosis levels progressively increased: 9.07% at 250 μg/ml, 15.2% at 500 μg/ml, and 20% at 1000 μg/ml. Even at the highest DSE concentration, apoptosis induction (20%) was substantially lower compared to HC (53%), suggesting that while DSE enhances apoptotic activity in a dose-dependent manner, it does not reach the severe cytotoxicity induced by H_2O_2. These findings indicate that DSE triggers apoptosis in colon cancer cells in a concentration-dependent manner, contributing to its cytotoxic potential, while maintaining comparatively lower toxicity than oxidative stress-in-

ducing agents. Figure 7 shows the dose dependent induction of apoptosis (programmed cell death) by date seed extracts, which was dose dependent with increase in concentrations, as with fluorescence images, showing apoptosis induction to be highest at the higher concentrations, which is significantly important in cancer cells because they evade apoptosis.

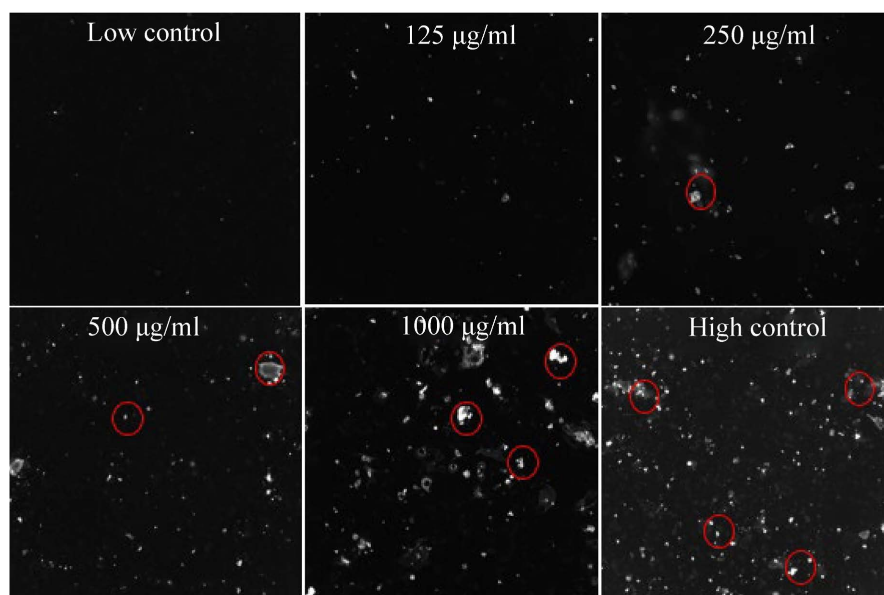
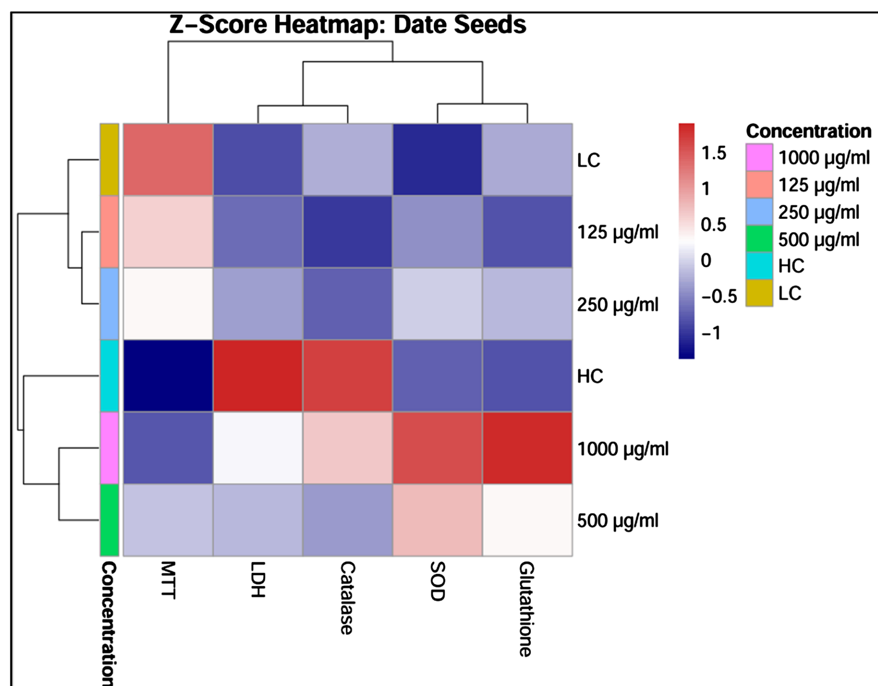


Figure 7. Fluorescence microscopic images showing apoptosis induction in colon cancer cells treated with date seed extracts.

The heatmap highlights that date seed extracts exert concentration-dependent effects, with cytotoxicity and antioxidant enzyme induction strongly correlated at higher doses, as shown in Figure 8. The Z-score heatmap offers a comprehensive picture of how various date seed extract (DSE) doses affect the cytotoxic responses and antioxidant enzyme activity in HT-29 cells. Cell viability (MTT) remained comparatively higher at lower concentrations (125 µg/ml), although LDH release was low, indicating minimal cytotoxicity. MTT values significantly decreased and LDH levels increased at higher dosages (500 - 1000 µg/ml), indicating a definite dose-dependent cytotoxic effect caused by DSE. Increased cytotoxicity through oxidative stress was shown by the positive control (H_2O_2), which was associated with increased LDH and lower MTT.

As DSE concentrations increased, antioxidant responses gradually increased. At 1000 µg/ml, SOD and GSH had the highest increase, suggesting that endogenous defense mechanisms against oxidative stress were active. At 500 - 1000 µg/ml, catalase activity increased moderately, following the SOD pattern and suggesting coordinated antioxidant enzyme upregulation. Normal basal cell activity was indicated by the low control (LC), which correlated with stronger MTT and low antioxidant responses. In contrast, the high control (H_2O_2) associated with high LDH and inhibited antioxidant enzyme activity, indicating loss of redox equilibrium and oxidative damage. At higher extract concentrations, MTT (cell viability) shows an

inverse relationship with SOD, CAT, and GSH, indicating that cells simultaneously elevated antioxidant defenses as cytotoxicity increased. At higher doses, there was a positive correlation between LDH release and antioxidant enzyme activity, suggesting that DSE-induced oxidative stress was neutralized by compensatory antioxidant upregulation. This pattern points to a hormetic response, in which adaptive antioxidant defenses are triggered by mild oxidative stress from polyphenol-rich DSE via pathways such Nrf2 activation. This dual action is promising for anticancer strategies, particularly targeting oxidative stress pathways in colon cancer cells. At low concentrations, date seed extracts do not exert significant cytotoxic effects but begin to show mild modulation of antioxidant defenses.



*LC—Low Control (Media); HC—High Control (Hydrogen peroxide); MTT—3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; LDH—Lactate dehydrogenase; SOD—Superoxide dismutase.

Figure 8. Heatmap depicting cytotoxic and antioxidant responses of colon cancer cells to date seed extracts.

4. Discussion

The cytotoxic effect of DSE is clearly demonstrated by the statistically significant 53% rise in LDH release at 125 µg/ml over LC and the 4.67-fold increase at 1000 µg/ml compared to LC. Plant extract's dose responsiveness has been widely documented; for instance, extracts rich in polyphenols or flavonoids from different medicinal plants show comparable increasing cytotoxicity in *in-vitro* colon cancer models [18] [19]. In comparison to all DSE concentrations, hydrogen peroxide (HC) showed significantly higher cytotoxicity (69.85%). This demonstrates that oxidative stress damages membranes far more, which is in line with its known

capacity to trigger both necrotic and apoptotic cell death processes. Although substantial as compared to LC, the LDH release from the highest DSE dose (1000 μg/ml) was still 2.32 times lower than that of HC, highlighting the modest cytotoxicity of date seed phenolics in comparison to direct oxidative damage. Other plant extracts have shown similar results, with natural chemicals showing a safer yet effective profile for chemopreventive uses and being less acutely hazardous than positive controls like H_2O_2 [18]-[20].

Following a 24-hour treatment with date seed extract (DSE), the MTT assay showed a dose-dependent decrease in cell viability in HT-29 colon cancer cells, as shown in the data in **Figure 3**. The cell viability dropped significantly from 93% in the LC (media alone) to 82% at the lowest concentration (125 μg/ml) and 62% at the highest concentration (1000 μg/ml). However, a more severe cytotoxic effect was elicited by HC, which reduced viability to 55%. Because of its high polyphenolic and flavonoid content, DSE has been shown to have a cytotoxic effect. These bioactive compounds have been found to cause cell death in colon cancer models by inducing apoptosis, altering the potential of the mitochondrial membrane, and producing mild intracellular reactive oxygen species (ROS). By activating caspases and upregulating pro-apoptotic genes, polyphenols and flavonoids (such as quercetin, and other plant-derived chemicals) cause colon cancer cell lines to undergo apoptosis [21] [22]. Notably, apoptosis in HT-29 was not observed in many published experiments employing methanolic date seed extracts; nevertheless, cytotoxicity is highly influenced by the kind and polarity of the extract as well as the concentration employed [20]. Water, ethanol, and ethyl acetate extracts often exhibit strong cytotoxic and pro-apoptotic effects in HT-29 and other colon cancer lines, possibly due to their improved solubility of polyphenols and flavonoid [23].

Date seed extracts may not only counteract oxidative damage but also prepare cells, increasing their resistance to current and upcoming oxidative challenges, according to the response of superoxide dismutase (SOD) to DSE. This is known as the "adaptive" or "hormetic" response, and it illustrates how bioactive substances can cause mild stress that triggers defense mechanisms in cells [24]. Under circumstances of oxidative stress, polyphenolic compounds in DSE have been shown to activate phase II antioxidant enzymes, such as catalase (CAT). Redox-sensitive transcription factors, such as Nrf2 and AP-1 drive induction by upregulating genes that encode detoxifying and antioxidant enzymes [25]. The increase in SOD and CAT activity, either through direct gene induction, enzyme stabilization, or Nrf2 signaling pathway activation, thus has a dual protective function in tissues that are subjected to high levels of oxidative stress [26].

Superoxide anions and hydrogen peroxide are the two main reactive oxygen species that are detoxified by the combined action of SOD and catalase. The enzymes lessen oxidative DNA damage, including malondialdehyde adducts and 8-OHdG lesions, which otherwise may cause mutagenesis and genomic instability, by lowering the steady-state concentration of these ROS [27]. Reduced biomarkers of DNA damage have been strongly linked to increased antioxidant enzyme activ-

ity, that supports cellular homeostasis and genomic integrity. Improved antioxidant defenses not only reduce oxidative damage but also assist in the suppression of proliferative and inflammatory signaling pathways [28] [29]. In addition to inducing SOD, CAT, and glutathione peroxidase, dietary antioxidants that activate Nrf2 also control inflammatory mediators like NF-κB and COX-2, which are crucial for the development and spread of tumors [30]. Cells are protected against direct ROS-mediated damage as well as the pro-inflammatory environment that promotes carcinogenesis by maintaining or increasing antioxidant enzyme activity [29]. These processes are consistent with data from tissue and animal models that showed dietary components high in antioxidants, decreased colon inflammation and carcinogenesis [31]. Vitamins E and C, along with extracts rich in polyphenols, have demonstrated the ability to reduce pro-inflammatory COX-2 and NF-κB signaling and increase antioxidant gene expression, indicating their potential for preventing colorectal cancer [32].

Following a 24-hour treatment with DSE, HT-29 colon cancer cells showed a dose-dependent increase in apoptosis, suggesting that DSE induces programmed cell death, which contributes to its cytotoxic effects. Although the HC (53%) showed higher apoptosis induction compared to the higher DSE (1000 μg/ml) concentration (20%), the results show that DSE clearly promotes apoptosis in colon cancer cells without producing excessive cytotoxicity. The abundance of polyphenols, flavonoids, and other bioactive phytochemicals in DSE may cause its apoptotic effects. These compounds have been shown to trigger intrinsic apoptosis pathways in colorectal cancer models [33]. According to research conducted on HT-29 cells treated with comparable plant extracts, polyphenols cause cytochrome c release and disturb mitochondrial membrane potential, which activates the caspase cascade (including cleaved caspase-3 and PARP) [34]. Bioactive compounds may alter the expression of pro-apoptotic (like Bax and p53) and anti-apoptotic (like Bcl-2) proteins. According to related research, p53 and Bax are upregulated while Bcl-2 is downregulated, which promotes the permeabilization of the outer membrane of the mitochondria [33]. DSE-induced moderate ROS generation may serve as signaling molecules to trigger apoptotic pathways, which are compatible with regulated oxidative stress that damages cancer cells without causing nonspecific necrosis [34].

This study provides convincing evidence that DSE causes HT-29 colon cancer cells to undergo apoptosis in a dose-dependent manner, mainly by modulating mitochondrial apoptotic signaling pathways with the help of bioactive phytochemicals. DSE has the potential to be a safer chemo preventive agent that specifically induces programmed cell death in cancer cells due to its lower apoptotic activity when compared to high oxidative stress (Caused by HC). The findings suggest that DSE can selectively induce moderate cytotoxicity in colon cancer cells without causing acute damage like strong oxidants, making it a potential candidate for chemopreventive or adjunctive cancer therapies. This selective targeting minimizes harm to healthy tissue, aligning with the promise of dietary-derived agents,

particularly those rich in polyphenols and flavonoids, as safe and effective modulators of colon cancer cell viability [18] [19].

5. Conclusions

This study shows that date seed extracts (DSE) have cytotoxic and strong antioxidant effects on HT-29 colon cancer cells that vary with concentration. DSE treatment improved intracellular antioxidant defenses, as shown by enhanced activities of SOD, CAT, and GSH, while concurrently decreasing cell viability and increasing apoptosis. These results imply that DSE promotes endogenous antioxidant mechanisms, which together reduce oxidative damage and restore redox balance, in addition to interfering with cancer cell survival by inducing programmed cell death.

The activation of redox-sensitive signaling pathways, including Nrf2, can be observed by DSE's potential to increase antioxidant enzymes, which improves cellular defense against oxidative stress. DSE may be suggested as a possible chemopreventive drug in colorectal cancer because of its dual activity, which includes cytotoxicity against tumor cells and strengthening antioxidant defenses. Furthermore, compared to the severe oxidative damage caused by H_2O_2, its moderate induction of apoptosis points to a more regulated mechanism of action that may provide a therapeutic benefit with less toxicity. The potential of date seeds, an underutilized food by-product, as a source of bioactive compounds with antioxidant and anticancer potential has been highlighted by this study.

Future Directions

Future study of date seeds might involve mechanistic studies such as apoptosis pathways, including caspase activation, mitochondrial membrane potential, reactive oxygen species generation, as well as redox signaling pathways, including Nrf2 signaling, to gain further insight into the mode of action of date seeds. In addition, dose-response kinetics as well as time-course kinetics might also provide further information regarding the optimal concentration of date seeds. In this study, responses were only assessed in HT-29 cells, a type of colorectal cancer. As a result, it is unable to draw conclusions about potential differences in effects on normal intestinal epithelium and selectivity toward cancer versus non-malignant colon cells. To determine cancer selectivity and safety profiles, non-malignant colon epithelial cell lines (such as CCD 841 CoN) will be used in future research.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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