Original Research Article | J Adv Med Biomed Res. 2025; 33(160): 179-187

Category: Life Science | Peer Reviewed | Open Access

Journal of Advances in Medical and Biomedical Research | ISSN:2676-6264

Prosopis Farcta Extract Enhances 5-Fluorouracil Efficacy in Colorectal Cancer via Autophagy Modulation

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Article Info

10.30699/jambr.33.160.179

Received: 2025/05/23;
Accepted: 2025/10/28;
Published Online: 11 Nov 2025;

Use your device to scan and read the article online

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ABSTRACT

Background & Objective: Today, colorectal cancer is a common pathology among nations. 5-Fluorouracil (5-FU), as a chemotherapeutic agent, triggers autophagy induction in cancer cells. *Prosopis farcta* (PF) induces potential anticancer properties via inherent phytochemical contents. In the current in-vitro investigation, the synergistic autophagy effects of PF fruit extract and 5-FU in colorectal cancer cell line (SW742) were assessed.

Materials & Methods: PF fruits were collected, authenticated by a botanist, and processed to prepare a hydroalcoholic extract. SW742 cells were cultured and treated with IC50 concentrations of PF extract and 5-FU, both individually and in combination (as synergistic effects). Cell viability and cytotoxicity values were evaluated using standard assays. The interaction between PF and 5-FU was also investigated. Expression levels of Atg-7, Beclin-1, and LC3 were determined using the quantitative PCR (qPCR) technique.

Results: Treatment with PF extract reduced ($p<0.05$) the viability of SW742 cells. Co-treatment of 5-FU with PF demonstrated a synergistic cytotoxic effect against cancer cells. Gene expression analysis revealed that PF extract alone downregulated the expression of Atg-7, Beclin-1, and LC3, while 5-FU treatment upregulated the associated expression levels. Combined therapy of PF and 5-FU modulated these gene expressions in a distinct pattern, suggesting interaction in autophagy regulation.

Conclusion: PF fruit extract enhances the anticancer effect of 5-FU on colorectal cancer cells by modulating autophagy-related gene expression. These findings suggest that PF may be a potential natural adjuvant therapy in colorectal cancer treatment.

Keywords: Autophagy, 5-Fluorouracil, Colorectal Neoplasms, Plant Extracts, SW42 Cells

1. Introduction

The 3rd most common malignancy among human population is colorectal cancer. In the United States, the lifetime risk of developing colorectal cancer among individuals is approximately 4%, with a higher prevalence observed in males compared to females, and in Black Americans compared to White Americans (1). In this regard, the age, family history, inflammatory bowel disease, low physical activity, low fiber and high-fat diet, alcohol intake, and tobacco use are among risk factors (2). Preventive strategies, such as physical activity, can reduce the risk of colorectal cancer (3). This pathology is also the third most prevalent malignancy in Iran (4). The estimated annual incidence of colorectal cancer in Iran ranges from 1.5 to 5.5 cases per 100,000 population (5). 5-Fluorouracil (5-FU), an antimetabolite chemotherapeutic agent, is commonly utilized in the treatment of various cancers, particularly colorectal cancer. Its anticancer effects are primarily

mediated through inhibition of the enzyme thymidylate synthase, as well as incorporation of its active metabolites into RNA and DNA, thereby interfering with nucleic acid synthesis (6). Specifically, 5-FU disrupts the conversion of cytosine nucleotides into deoxy forms and impairs DNA synthesis by preventing the incorporation of thymidine (7). 5-FU is often administered in combination with other chemotherapeutic agents, such as Leucovorin and Oxaliplatin, to enhance its therapeutic efficacy.

Apoptosis is a physiological process that helps maintain cellular equilibrium. This mechanism is crucial in preventing uncontrolled cell proliferation. Dysregulation of apoptosis is a key event in carcinogenesis, contributing to tumor initiation and progression. Apoptosis can be activated in cancer cells through intrinsic (mitochondrial) or extrinsic (death receptor-mediated) pathways, both of which converge on the activation of caspase-dependent proteolytic cascades, leading to cellular membrane disruption and endonucleolytic fragmentation of DNA (8). Several genes are known to regulate apoptosis, including Atg7, Beclin-1, and LC3 (9). Atg7 is essential for autophagosome formation, and its inhibition is associated with nuclear accumulation of LC3, potentially triggering apoptosis (10). Beclin-1 functions as a central regulator of both autophagy and apoptosis, and its depletion induces caspase-dependent programmed cell death (11). LC3, a critical protein for autophagosome formation, also plays a critical role in apoptotic pathways, with its nuclear accumulation linked to the induction of cell death (12).

PF is a medicinal plant that has been traditionally used for various therapeutic applications. PF fruit is known to relieve constipation, reduce fever, and exhibit diuretic effects. Moreover, the root of PF is a natural alternative to 5-FU for the treatment of cardiovascular disorders (13). The plant's biological activities are attributed to a rich phytochemical profile, particularly C-glycosyl flavones. Also, the PF shows promise as a natural therapeutic agent, and further research may elucidate additional pharmacological benefits (14). Thus, the potential anticancer effects of PF, as well as its synergistic cytotoxic activity in combination with 5-FU, were assessed in this in vitro study.

2. Materials and Methods

2.1 Preparation of PF extract

The PF plant was prepared from a reputable herbal medicine center and confirmed by a botanist. PF fruits were dried (in 5 days), then the herbs were ground into powder form. PF powder (100 g) was mixed in 70% ethanol (ratio of 1 PF extract to 4-part ethanol). 24 hours later, the mixture was gradually filtered using Buchner filter paper via a vacuum pump. Then, the powder extract was dissolved in culture medium for future cellular treatment (15).

2.2 Cell culture (SW742) and treatment

Pasteur Institute (Tehran, Iran) was considered the main research center for the preparation of the human colorectal cancer cell line. The flasks were incubated (37°C, 5% CO2). The condition medium was disposed and replaced with new free-serum culture medium with PF (various doses of 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml) and 5-FU (multiple doses of 1, 2, 4, 8, 16, 32, 64 and 128 µM) for 24, 48, 72 and 96 hours (16).

2.3 MTT assay

After treatment, the conditioned medium was discarded and washed with PBS. Then, 50 µL of MTT solution (5 mg/MTT/1mL PBS) was added to the cells and covered with aluminum foil for incubation (3 hours). 100µL of DMSO was added to each well located on a shaker (room temperature, 20min) to dissolve insoluble formazan crystals and the color alteration into purple. The absorbance was read (570 and 630 nm), and the cell survival rate (%) was calculated based on published articles (17).

2.4 Quantification of PF-5-FU interaction

In this regard, the synergistic interactions between PF and 5-FU were assessed using MTT assay and followed by CompuSyn computer software analysis (ComboSyn, Inc., Paramus, NJ, USA). 0 and 1 values, respectively, indicate no and death of all cells. The Combination Index (Ci) value was calculated as: $CI = (D)\ 1 / (Dx)\ 1 + (D)\ 2 / (Dx)\ 2$. (In this formula, (Dx) 1 and (Dx) 2 are the doses of 5-FU inhibiting the *x,* and (D) 1 and (D) 2 are the amounts of 5-FU inhibiting the *x* observed experimentally.) Finally, the dose-effect plot, average effect, composite index (CI), and logarithmic composite index were plotted. Additionally, the dose reduction index (DRI), the logarithm of dose reduction, and the normalized isobologram were prepared.

2.5 Toxicity assessment of PF

This assay was applied by the activity of the Lactate Dehydrogenase enzyme kit (Abcam Inc., Cambridge, MA, USA). To perform the MTT assay, cells are seeded in a 96-well plate (overnight). Then, the media was removed, and the MTT solution was added to the wells. The plate was incubated (4hrs, 37°C) to reduce the MTT by viable cells. After incubation, the MTT solution was removed, and the formazan product was solubilized using a detergent solution. The absorbance of the solubilized formazan product was measured (570 nm), and cytotoxicity was calculated using the following formula: $Cytotoxicity\ (\%) = (Sample\ OD_{450}\text{-}Control\ OD_{450})/ Positive\ Control\ OD_{450}\text{-}Control\ OD_{450}$ (18).

2.6 Gene expression assay

The gene expression of Atg-7 (5-ATTGCTGCATCAAGAAACCC-3, 5-GATGGAGAGCTCCTCAGCA-3) (19), Beclin-1 (5-GCCGAAGACTGAAGGTCA-3, 5-GTCTGGGCATAACGCATC-3) (20), and LC-3 (5-GATGTCCGACTTATTCGAGAGC-3, 5-TTGAGCTGTAAGCGCCTTCTA-3) (19) were assessed after the PF and 5-FU exposure for 24 hours.

RNA was extracted (RNA isolation kit, DENAzist, Iran), and the quantity and quality of the extracted RNA were tested (Nano drop and gel electrophoresis). Then, complementary DNA (cDNA) synthesis was performed using a cDNA synthesis kit (Vivantis Technologies, Selangor DE, Malaysia) from the RNA templates via reverse transcription. GAPDH (5-TCCCTGAGCTGAACGGGAAG-3, 5-GGAGGAGTGGGTGTCGCTGT-3) **(21)** was used as an internal control. Real-time PCR was performed using SYBR Premix Ex Taq Technology (Takara Bio Inc., Shiga, Japan) on the StepOne Applied Biosystems Real-time PCR system and expressed by fold change (Ct $2^{-\Delta\Delta ct}$ method).

2.7 Statistical Data Analysis

The normal distribution was analyzed using Kolmogorov–Smirnov test. Then, statistical analysis was performed by one-way ANOVA and the Tukey post hoc tests (SPSS 16 software, SPSS Inc., Chicago, IL). Statistics were expressed as Mean±SEM, and $p<0.05$ was considered significant **(22)**.

3. Result

3.1 Impacts of PF and 5-FU on cell viability

According to Figure 1. A, following 24 and 48 hours of PF exposure at different concentrations, the cell viability rate decreased significantly ($p<0.05$) with doses of 12.5, 25, 50, 100, 200, 400, and 800 µg/ml. Additionally, cell viability was significantly decreased in all PF concentrations at 72 and 96 hours after treatment ($p<0.05$). Besides, Figure 1. B represents the impact of 5-FU on cell viability over four time periods (24, 48, 72, and 96 hours). In this regard, the effects of 5-FU with concentrations of 4, 8, 16, 32, 64, and 128 µM showed a significant ($p<0.05$) decrease after 24 hours of exposure. Following 48, 72, and 96 hours of treatment, all tested concentrations of 5-fluorouracil (5-FU) resulted in a significant reduction in cell viability. After 48, 72, and 96 hours of exposure, all concentrations of 5-fluorouracil (5-FU) significantly decreased cell viability ($p<0.05$). The level of IC50 of PF following 24, 48, 72, and 96 hours was 777.90 ± 34.87, 200.87 ± 21.55, 36.26 ± 11.40, and 10.06 ± 4.15 µg/ml, respectively. Also, the values were 85.74±3.72, 21.50±2.99, 5.26±1.29, and 2.69±1.07 µM for the 5-FU chemotherapy agent. The effects of different PF concentrations and 5-FU on cell viability after 24 hours are shown in Figure 1. C.

3.2 Interaction between PF and 5-FU

The dose-effect curve indicated that the combined administration of PF and 5-FU exerted greater cytotoxic effects on cancer cells compared to each agent used individually (Figures 2. A and 2. B). The CI values obtained for all five treatment regimens were < 1, demonstrating a synergistic effect between PF and 5-FU in reducing cell viability (Figures 2. C and 2. D). The DRI values for both PF and 5-FU were > 1, indicating that each agent required a lower dose to achieve a specific therapeutic outcome when used in combination (Figures 2. E and 2. F). Additionally, an isobologram was constructed (Figure 2. G), illustrating that the concentrations of PF and 5-FU, both individually and in combination, effectively decreased the cell population by 50%, 75%, and 90%.

3.3 Cytotoxicity level

According to Figure 3. A, the cytotoxicity level of PF for colorectal cancer cells was dose and time-dependent. This toxicity was significant ($p<0.05$) after 24 hours in concentrations of 50, 100, 200, 400 and 800 µM, after 48 hrs in concentrations of 25, 50, 100, 200, 400 and 800 µM, after 72 hours in concentrations of 5/5 12, 25, 50, 100, 200, 400 and 800 µM, and after 96 hours in all concentrations. The effect of cytotoxicity of different 5-FU concentrations on cells after 24, 48, 72, and 96 hours was shown in Figure 3. B. The value was found significant ($p<0.05$) after 24 hours in concentrations of 16, 32, 64, and 128 µM, after 48 and 72 hours in concentrations of 2, 4, 8, 16, 32, 64, and 128 µM, and after 96 hours in all concentrations ($p<0.05$). The toxicity impacts of PF and 5-FU at various doses are shown in Figure 3. C.

3.4 Effects of PF and 5-FU on Atg-7, Beclin-1 and LC-3 gene expression

According to the gene expression analysis, cancer cells exposed to an IC50 concentration of PF decreased Beclin-1 and LC-3 gene expression significantly ($p<0.05$). Treatment with 5-FU resulted a significant increase in the expression of all three genes ($p<0.05$), and simultaneous treatment with PF and 5-FU reduced autophagy-associated gene expression (Figure 4).

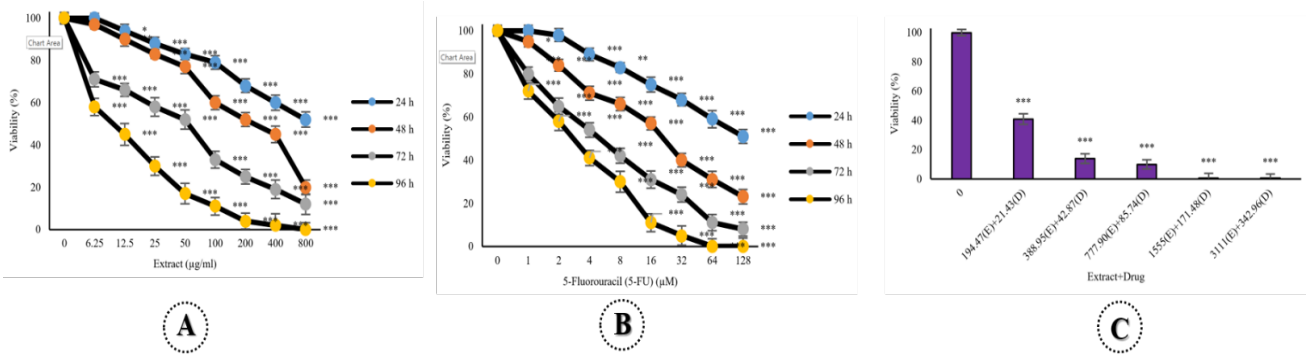

Figure 1. The effect of PF **(A)**, 5-Fluorouracil **(B)**, and co-treatment **(C)** on colorectal cancer cell viability (MTT assay). Control group received the same volume of medium with no 5-FU. *, **, and *** respectively indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$ (than control), respectively (**Prepared by Authors, 2025**).

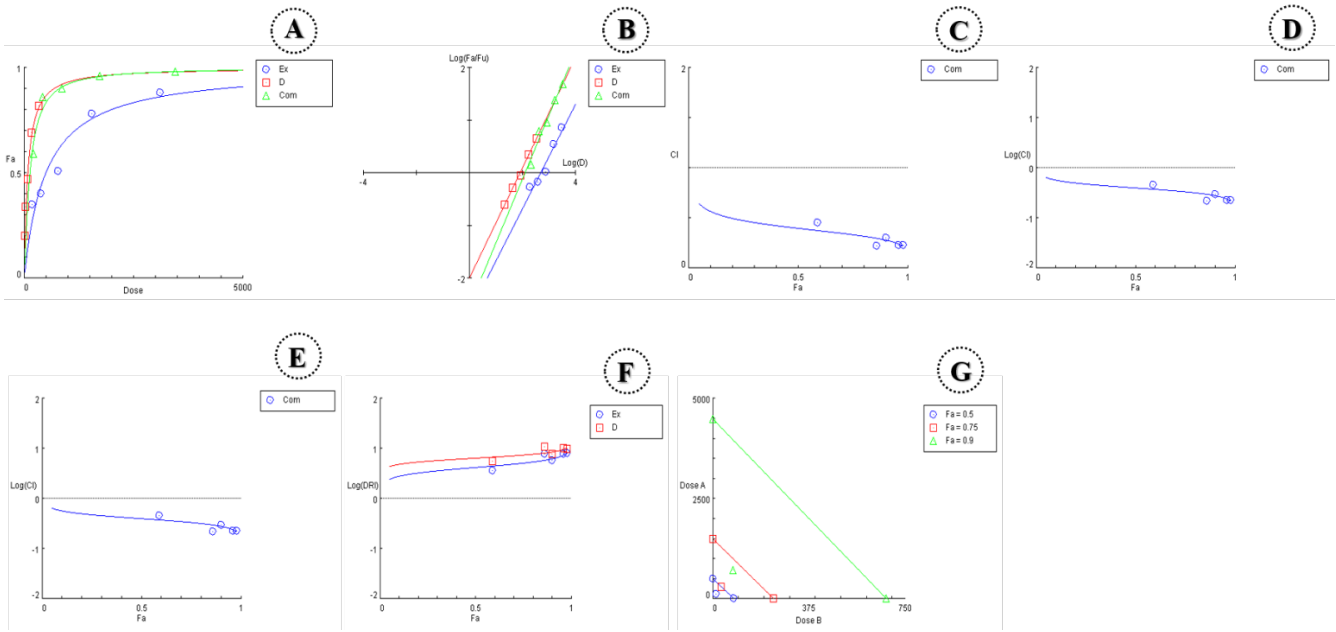

Figure 2. Dose-effect diagram **(A)**, average effect **(B)**, composite index **(C)**, logarithmic composite index **(D)**, dose reduction index **(E)**, logarithmic dose reduction index **(F)**, and Isobologram **(G)** for colorectal cancer cells following treatment with PF, 5-Fluorouracil, and combination (for 24 hours using MTT assay) (**Prepared by Authors, 2025**).

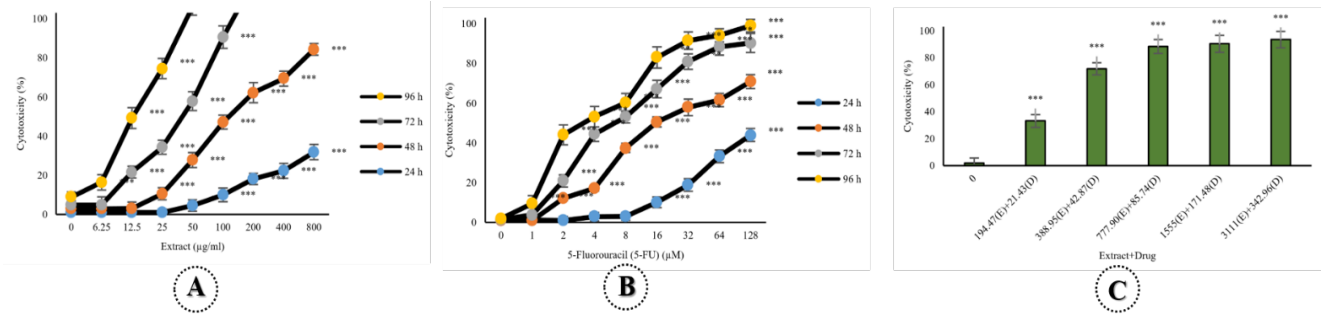

Figure 3. Toxicity of PF extract **(A)**, 5-Fluorouracil **(B)**, and co-treatment **(C)** on colorectal cancer cells (Lactate dehydrogenase assay). The cells of the control group were treated with the same volume of medium with no 5-FU application. *, **, and *** indicates $p < 0.05$, $p < 0.01$, $p < 0.001$ (than control), respectively (**Prepared by Authors, 2025**).

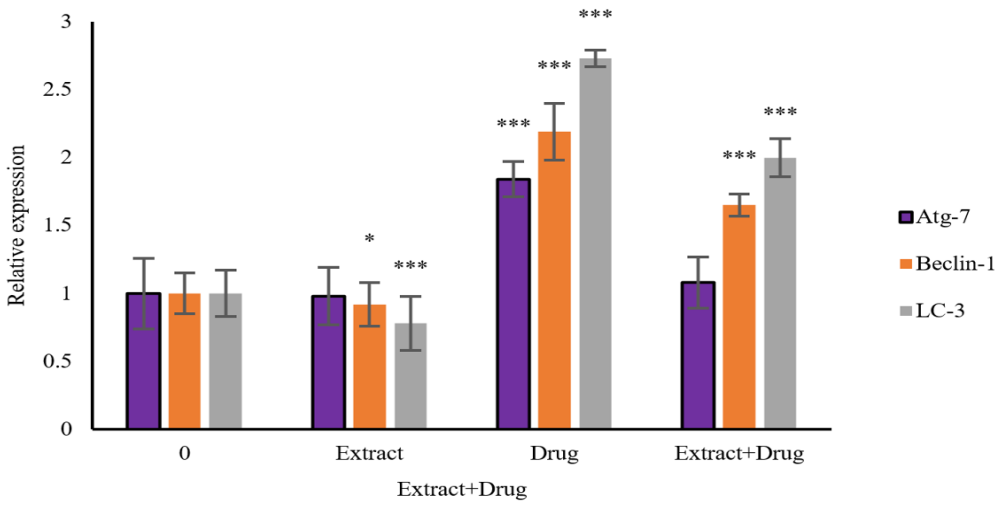

Figure 4. PF extract and 5-Fluorouracil effects on expression of autophagy-related genes in colorectal cancer cells. The control group received the same volume of medium without 5-FU. *, **, and *** indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$ (than control), respectively (**Prepared by Authors, 2025**).

4. Discussions

The impact of PF extract on the viability of colorectal cancer cells was examined in this study. The findings revealed a dose-dependent reduction in cell viability with increasing concentrations of PF. A significant decrease in cell viability was observed at doses of 12.5, 25, 50, 100, 200, 400, and 800 µg/ml after 24 and 48 hours of treatment. In 72 and 96 hours of treatment, all PF concentrations caused a significant decrease in cell viability. PF toxic effects were confirmed in some studies (23). The cytotoxic and apoptotic effects of silver nanoparticles, synthesized using the aqueous extract of PF, were evaluated in lung and colon cancer cell lines. The results demonstrated that the viability of A549 cells was strongly influenced by both the concentration of nanoparticles and the duration of incubation (24). Although the cytotoxicity of silver nanoparticles (Ag-NPs) on HT-29 cells increases with higher concentrations and longer incubation times, the cell viability appears to be primarily influenced by duration of incubation rather than nanoparticle concentration (23). After treatment with nanoparticles, the apoptosis rate reached 50% in both A549 and HT-29 cell lines, with HT-29 cells exhibiting complete cell death at concentrations exceeding 400 µg/ml. These findings suggest that silver nanoparticles synthesized using PF extract contain considerable potential for the treatment of various cancers (25). The zinc oxide nanoparticles synthesized using PF extract demonstrated anticancer properties, with a cytotoxic concentration of 500 µg/mL against breast cancer cells (26). Despite considerable advancements in oncological therapies, improvements in overall survival and quality of life for patients with colorectal cancer remain limited (27). The primary objective of this approach is to achieve a synergistic therapeutic outcome that allows for dose reduction and toxicity mitigation while delaying or minimizing the emergence of resistance to 5-FU. The observed decrease in toxicity and resistance is attributed to the synergistic interactions between agents. Currently, multiple anticancer drugs, including various formulations of 5-FU, are extensively used in treatment regimens for diverse cancer types (28). The PF extract significantly enhances the cytotoxicity of 5-FU, with CI values ranging from 0.22 to 0.63, indicating a synergistic interaction across all tested concentrations. The mean CI value of 0.39 across all assays confirms the overall synergistic effect of PF and 5-FU against the colorectal cancer cell line (29). This combination enables a reduction in the dosage of 5-FU required to achieve the desired therapeutic effect. Clinically, lowering the 5-FU dose is beneficial as it reduces the adverse side effects associated with chemotherapy. This study is the first to investigate the synergistic effect of PF extract combined with 5-FU on colorectal cancer cell viability. Notably, 5-FU remains a widely used chemotherapeutic agent for solid tumors, including colorectal cancer (30). A compound that acts as an inhibitor of thymidylate synthase can mimic uracil and become incorporated into RNA and DNA, thereby disrupting nucleic acid synthesis and triggering apoptosis in cancer cells (31). Several studies have assessed the simultaneous use of this chemotherapy agent and other anticancer agents, including the combination of 5-FU with genistein (32), cisplatin (33), thymoquinone (34), chrysin (35), oxaliplatin (36), and metformin (37). The results of all these studies, in agreement with the present study, confirmed the increase in effectiveness and decrease in 5-FU resistance. Cell death is accompanied by the loss of membrane integrity and the release of cytoplasmic contents outside the cell (38). Lactate dehydrogenase is a relatively stable enzyme commonly utilized as a biomarker to evaluate cellular damage or toxicity in cell culture media. Its stable nature allows for reliable measurement in assessing cell membrane integrity and cytotoxic effects following exposure to harmful agents (39). After 24 hours of treatment, the extracts of PF and

5-FU showed dose- and time-dependent cytotoxicity against colorectal cancer cells (40). Molecular investigation revealed that the mRNA expression of three autophagy-promoting genes was significantly increased by 5-FU and decreased by the PF extract. Autophagy induction conducted by 5-FU is one of the main causes of cell resistance to 5-FU. Also, autophagy inhibition by PF extract at the transcription level increases the sensitivity of cells to 5-FU toxicity and accelerates the effectiveness. The result of autophagy often reflects its survival function, which plays a considerable role in the maintenance of homeostasis in the recovery of proteins and cytoplasm (41). During stress, this agent saves the viability of cells by removing organelles and protein accumulations. Also, several studies confirmed the role of autophagy in cell death (42). Autophagy plays a crucial role in cellular response to therapeutic stress. Recent research suggests that chemotherapy-induced autophagy serves as a protective mechanism, enabling cancer cells to survive and contributing to the development of resistance to 5-FU (43). Most of the genes encoding the key components of the autophagy pathway are characterized. In mammals, Beclin-1 is the homologue of the Atg-6 gene in yeast and plays a major role in the autophagy process. This protein interacts with several cofactors to induce autophagy (44). Atg-7 is another key protein that plays a role in autophagy regulation (45). The inhibitory process of autophagy in expression level of Beclin-1, Atg-7 and other proteins can reduce the 5-FU resistance.

Recent investigations into the antineoplastic properties of PF and closely related species provide further support for the potential therapeutic relevance of this genus in colorectal cancer. In vitro studies demonstrated that the methanolic extract of PF induced mitochondrial-mediated cytotoxicity in HT-29 colorectal cancer cells, as evidenced by increased ROS, mitochondrial membrane damage, and Sirt3 cascade activation, resulting in significant cell death at $IC_{50} \approx 2\,\mu g/mL$ (43). Phytochemical profiling also identified potent cytotoxic compounds, such as a newly isolated fatty acid derivative (threo-methyl 9, 10-dihydroxyoctadecanoate), which exerted vigorous activity against HCT-116 colon carcinoma cells (44). Moreover, research on other Prosopis species, such as P. strombulifera, revealed both in vitro and in vivo cytotoxic and antitumor efficacy against colorectal cancer cell lines (e.g., HCT-116), with observable induction of apoptosis, necrosis, and clonogenic suppression in murine allograft models without notable toxicity at oral doses up to 150 mg/day (45).

Additionally, Prosopis juliflora extract inhibits cell proliferation, migration, invasion, and angiogenesis in melanoma and breast cancer models, which is linked to downregulation of EMT markers and stem-cell-associated signaling pathways (46). Collectively, these findings highlighted that PF and related Prosopis species harbor bioactive phytochemicals capable of disrupting mitochondrial function and inducing programmed cell death across diverse cancer cell types, thereby supporting its candidacy as a natural adjuvant therapy to conventional chemotherapy.

In our study, the downregulation of Atg-7, Beclin-1, and LC3, all crucial components of autophagy, can lead to increased cytotoxicity by impairing the cell's ability to clear damaged organelles and misfolded proteins, resulting in cellular stress and potentially cell death.

Despite the promising findings of this study, several limitations should be stated. First, the experimental design was limited to an in vitro model using a single colorectal cancer cell line (SW742), which may not fully represent the heterogeneity and complexity of colorectal tumors in vivo. Moreover, the use of a crude hydroalcoholic extract of PF without isolating or characterizing its bioactive constituents prevents precise identification of the compounds responsible for the observed effects. Additionally, the analysis focused solely on the mRNA expression of three autophagy-related genes without further validation at the protein level or functional assays to confirm the involvement of autophagy pathways. Future research should address these limitations by incorporating animal models, broader gene and protein profiling, and characterization of the specific phytochemicals responsible for the anticancer activity of PF.

5. Conclusion

PF extract induces anticancer effects on colorectal cancer cells by inhibiting autophagy. Also, the simultaneous treatment of PF and the chemotherapy drug of 5-FU enhances the effectiveness 5-FU against colorectal cancer cells synergistically.

6. Declarations

6.1 Acknowledgments

The authors of this article express their utmost gratitude to the University Research and Technology Vice-Chancellor of Kermanshah University of Medical Sciences.

6.2 Ethical Considerations

The present study is approved by Kermanshah University of Medical Sciences (4000879) with the ethical code of IR.KUMS.MED.REC.1400.108.

6.3 Authors' Contributions

Conceptualization, A.A.; methodology, M.P.; software, C.J.; validation, H.N. formal analysis, S.H.; investigation, S.M.; resources, F.K-H.; data curation, F.K-H.; writing (original draft preparation), M.P.; writing (review and editing), A.A.; visualization, C.J.; supervision, F.K-H.; project administration, C.J.; funding acquisition, F.K-H. All authors participated in reviewing the manuscript and

its revision, and they were involved in research, interpretation, and finalizing the manuscript.

6.4 Conflict of Interest

The authors have no conflict of interest.

6.5 Fund or Financial Support

The Kermanshah University of Medical Sciences was financial sponsor of the project.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors did not use any AI tools for writing, editing, data analysis, or any other part of manuscript preparation.

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How to Cite This Article:

Abdolmaleki A, Pazhouhi M, Jalili C, Khani-Hemmatabadi F, Miraghaei S, Nemati H, et al. *Prosopis Farcta* Extract Enhances 5-Fluorouracil Efficacy in Colorectal Cancer via Autophagy Modulation. J Adv Med Biomed Res. 2025;33(160):179-87.

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