



INTERMITTENT FASTING-INDUCED AUTOPHAGY AND APOPTOSIS IN COLORECTAL CARCINOMA: THE ROLE OF BECLIN-1 AND BCL-2

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ABSTRACT

Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide, primarily due to uncontrolled cell proliferation and dysregulation of autophagy and apoptosis. The present study aimed to evaluate the effect of Embinin under serum withdrawal (intermittent fasting conditions) on autophagy and apoptosis in HT-29 human colorectal adenocarcinoma cells, with a focus on the expression of Beclin-1 and Bcl-2. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and subjected to serum deprivation for 6, 12, 18, and 24 hours, followed by Embinin and 5-fluorouracil (5-FU) treatments. A series of analyses—MTT assay, flow cytometry-based apoptosis and cell cycle assessment, quantitative PCR, western blotting, and immunofluorescence—were performed. Results from the MTT assay revealed that Embinin significantly inhibited HT-29 cell proliferation in a dose- and time-dependent manner, comparable to 5-FU at 6 h and 24 h of treatment. Annexin V-FITC/PI staining confirmed that Embinin induced both early and late apoptosis, with higher apoptotic indices at 12.5 μ M concentration. Cell cycle analysis indicated an increase in the sub-G1 phase population, suggesting apoptosis-mediated cell death, accompanied by a G2/M phase arrest. qPCR analysis demonstrated upregulation of Beclin-1 and downregulation of Bcl-2 expression, suggesting activation of autophagy and suppression of anti-apoptotic mechanisms. Collectively, the findings indicate that Embinin enhances cell death through coordinated modulation of autophagy and apoptosis pathways, particularly under nutrient-deprived (fasting) conditions, highlighting its potential as a novel adjuvant in CRC therapy.

Key words:

Embinin, Colorectal Cancer, MTT, qPCR, Cell Cycle, Annexin V, etc.

INTRODUCTION

Cancer encompasses a diverse group of over 100 diseases defined by uncontrolled cellular proliferation and defective regulatory mechanisms [1–3]. Its etiology is multifactorial, involving genetic mutations, environmental exposures, and lifestyle factors such as tobacco use, occupational hazards, and dietary patterns [4–7]. The transformation from normal cells to malignancy follows a multistep process: mutation, hyperplasia, dysplasia, *in-situ* carcinoma, and invasive malignancy with potential

metastasis [8–10]. Central to this progression are genetic alterations in proto-oncogenes, tumour suppressor genes, and DNA repair pathways, which collectively disrupt cellular homeostasis and confer proliferative advantages [11].

Colorectal cancer (CRC), comprising malignancies of the colon and rectum, represents a significant global health burden [12]. It ranks among the most prevalent cancers worldwide and is the second leading cause of cancer-related mortality (**Figure 1**) [13].

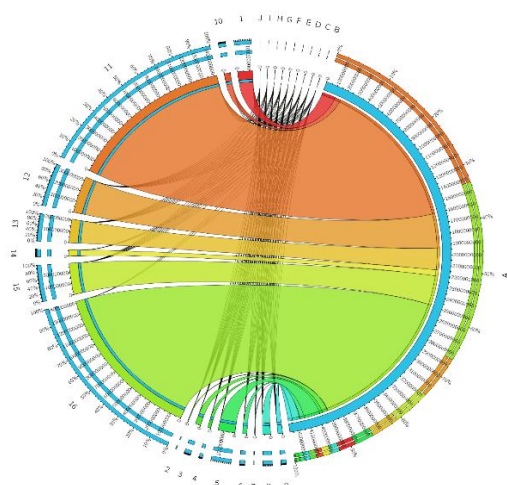


Figure 1: Circular plot chart for comparison of CRC incidence and mortality across selected countries. The corresponding waves in the circus plot represent the CRC incidence and mortality across selected countries.

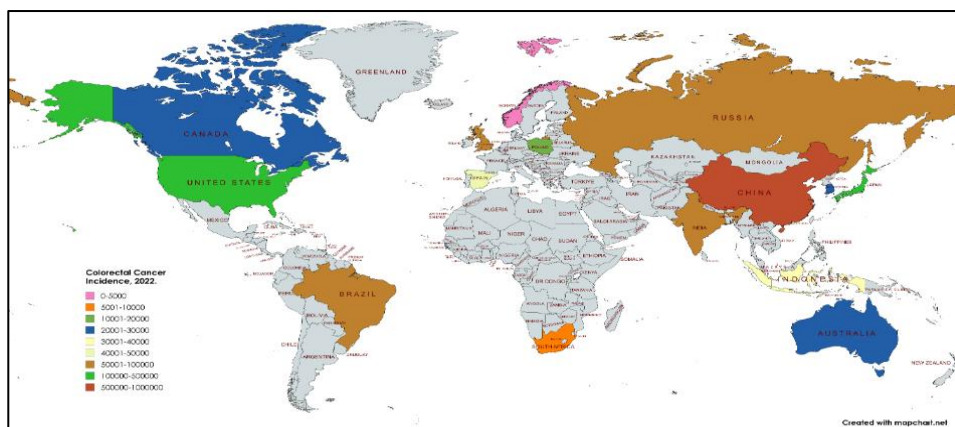


Figure 2: Worldwide Scenario on occurrence of Colorectal Cancer

CRC development is typically a prolonged process spanning 10–15 years, during which normal epithelium undergoes sequential transformation into adenomas and invasive adenocarcinomas

Anatomically, CRC is categorized as right-sided (proximal colon) or left-sided (distal colon), with the former often exhibiting a worse prognosis [14]. Molecularly, CRC arises via distinct pathways, including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP), driven by dysregulation of key genes such as APC, KRAS, BRAF, PIK3CA, TP53, and SMAD4 [15]. These perturbations influence critical signalling cascades, including Wnt/ β -catenin, PI3K/AKT/mTOR, Ras/RAF/MAPK, and TGF- β , which regulate proliferation, survival, and apoptosis [16]. Autophagy, a conserved catabolic process that recycles damaged organelles and proteins, plays a complex role in CRC [17]. Beclin-1, encoded by *BECN1*, is a pivotal

regulator of autophagy and functions as a haploinsufficient tumour suppressor [18].

Its expression is frequently reduced in cancers, facilitating tumour progression, while interactions with anti-apoptotic Bcl-2 family proteins modulate the balance between cell survival and death [19]. Dysregulation of Beclin-1-mediated autophagy contributes to both tumour suppression and promotion, highlighting its context-dependent role in CRC [20].

Concurrently, apoptosis serves as a critical mechanism to eliminate damaged or transformed cells, primarily regulated by intrinsic and extrinsic pathways involving caspases, Bcl-2 family proteins, and death receptors [21]. Cancer cells often evade apoptosis via overexpression of anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1), loss of pro-apoptotic factors (BAX, BAD), or inhibition of executioner caspases, resulting in therapeutic resistance and tumour progression [22].

Cells will be cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal calf serum until they reach 80% confluence [23]. To mimic nutrient deprivation, serum withdrawal will be performed for varying durations (6, 12, 18, and 24 hours), thereby inducing metabolic stress [60]. A combination of functional assays and molecular analyses will then be conducted to assess cell viability, cell cycle progression, apoptosis, and the activation of autophagy-related pathways [24].

The experimental strategy includes MTT assays for cytotoxicity and proliferation assessment, flow cytometry-based cell cycle analysis, and Annexin V-FACS analysis for apoptosis detection. Furthermore, total RNA will be isolated to evaluate gene expression levels of autophagy (Beclin-1) and apoptosis (Bcl-2) markers using qPCR. To validate these findings at the protein level, western blotting and immunofluorescence analyses will be performed. Together, these complementary approaches will provide a comprehensive understanding of how serum deprivation modulates cancer cell fate through the interplay of apoptosis and autophagy pathways.

2. MATERIALS AND METHODS

A. Cell Culture and Culture Condition

HT-29 (human colorectal adenocarcinoma) and Vero (normal kidney epithelial) cells were cultured in their respective media supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator [83,84]. Cells were maintained in T-25 flasks until 70–80% confluency, then trypsinized and seeded into 96-well plates or 60-mm tissue culture plates for subsequent experiments [25].

B. Tetrazolium-Based Cell Viability (MTT) Assay

Cells were seeded at a density of 4×10^3 cells per well in 96-well plates and allowed to adhere overnight [26]. Cells were then treated with Embinin or 5-fluorouracil (3.125–100 μ M) and incubated for 6, 12, 18, and 24 hours [27]. At each time point, 50 μ L MTT solution was added to each well, and plates were incubated at 37°C in the dark for 2–4 hours until purple formazan crystals were visible. The MTT reagent and media were removed, and 100 μ L DMSO was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using an ELISA plate reader [28].

C. Annexin V-FITC/PI Apoptosis Assay

HT-29 cells (3×10^5 /well) were seeded in 6-well plates and treated with Embinin (6.25 and 12.5 μ M) for 6, 12, 18, and 24 hours [29]. Both floating and adherent cells were collected, washed twice with PBS, and resuspended in $1 \times$ Annexin V Binding Buffer. A cell suspension containing 1×10^5 cells was incubated with 5 μ L FITC Annexin V and

5 μ L propidium iodide for 15 minutes on ice in the dark. Following this, 400 μ L $1 \times$ Binding Buffer was added, and cells were analyzed by flow cytometry within one hour [30].

D. Cell Cycle Analysis

HT-29 cells (1×10^5 /mL) were serum-starved for 24 hours to synchronize the cell cycle, then treated with Embinin (6.25 and 12.5 μ M) in medium containing 10% FBS [31]. Cells were trypsinized, washed twice with PBS, and fixed in 70% ice-cold ethanol overnight at 4°C. Fixed cells were washed with PBS and stained with PI/RNase buffer for 30 minutes at room temperature. DNA content and cell cycle distribution were analyzed using a flow cytometer, and data were processed with BD Cell Quest Pro software [32,33].

E. Western Blot Analysis

HT-29 cells (1×10^6 /mL) were treated with Embinin (6.25 and 12.5 μ M) or 5-FU for 6, 12, 18, and 24 hours [34]. Cells were washed with PBS and lysed in ice-cold RIPA buffer. Protein samples were separated using 12–16% SDS-PAGE and transferred to PVDF membranes at 25 mA for 1 hour. Membranes were blocked for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibody, and protein bands were detected using chemiluminescent ECL reagents [35,36].

F. Immunofluorescence Analysis

Cells were cultured on coverslips or in plates and fixed with 4% paraformaldehyde for 10–15 minutes [37]. Permeabilization was performed using 0.1–0.3% Triton X-100 in PBS, followed by blocking with 3–5% BSA for 30–60 minutes. Cells were incubated with primary antibodies for 1–2 hours at room temperature or overnight at 4°C, washed, and incubated with fluorophore-conjugated secondary antibodies for 1 hour in the dark. Nuclear counterstaining with DAPI was performed as required. Samples were mounted using antifade medium and observed under a fluorescence or confocal microscope [38].

G. Real-Time Quantitative PCR (qPCR) Analysis

Total RNA was extracted from HT-29 cells using TRIzol reagent, quantified ($A_{260/280} = 1.8$ – 2.0), and checked for integrity on a 1.5% agarose gel [39]. cDNA synthesis was performed using the Revert Aid First Strand cDNA Synthesis Kit according to manufacturer's instructions. qPCR reactions were set up using SsoFast Eva Green Supermix, gene-specific primers, and cDNA template. Amplification conditions were 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Specificity of the PCR products was verified by melting curve analysis [40].

3. RESULTS AND DISCUSSION

A. MTT Assay

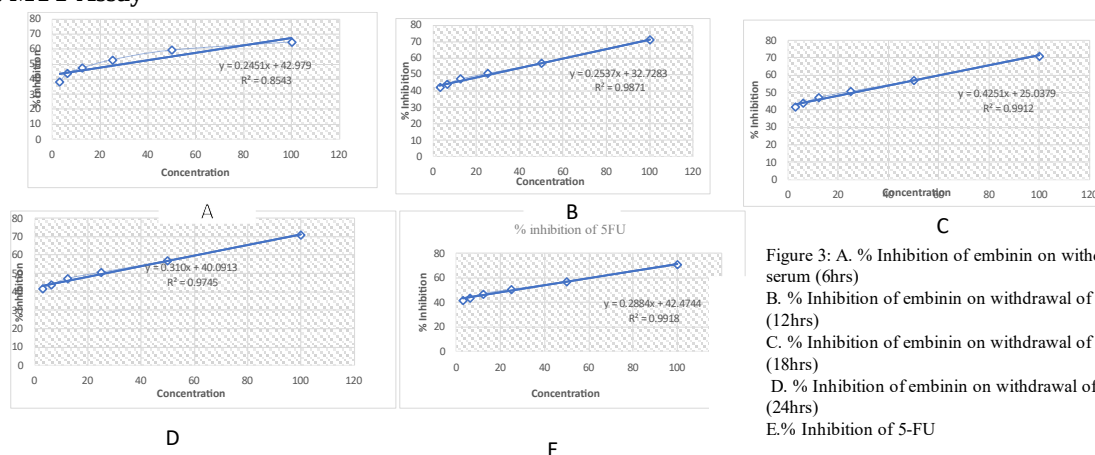


Figure 3: A. % Inhibition of embinin on withdrawal of serum (6hrs)
B. % Inhibition of embinin on withdrawal of serum (12hrs)
C. % Inhibition of embinin on withdrawal of serum (18hrs)
D. % Inhibition of embinin on withdrawal of serum (24hrs)
E. % Inhibition of 5-FU

Figure 3a, 3b, 3c, 3d shows that Embinin treatment under different serum withdrawal conditions (6, 12, 18 and 24 hrs) produced a concentration-dependent inhibition of HT29 cell proliferation. At the highest concentration (100 µg/ml), Embinin exhibited inhibition values of 64.61 ± 0.41 , 57.44 ± 0.26 , 66.40 ± 0.22 and $69.99 \pm 0.16\%$ respectively for 6, 12, 18 and 24 hours (Figure 3a, 3b, 3c, 3d), which were comparable to the inhibition observed with the standard drug 5-FU ($70.94 \pm 0.37\%$) (Figure 3e). From the table, it can be observed that the inhibitory effect of Embinin was more pronounced at 6 and 24 hrs serum withdrawal, showing IC_{50} values of 28.61 and 31.92 µg/ml, respectively, which are closer to the standard 5-FU (26.09 µg/ml). In contrast, the inhibition was relatively weaker at 12 and 18 hrs (IC_{50} = 68.09 and 58.67 µg/ml). Thus, it may be concluded that Embinin exhibits a significant cytotoxic effect against HT29 colon cancer cells in a time- and concentration-dependent manner. The 6-hour and 24-hour withdrawal groups showed more significant activity compared to the intermediate groups (12 and 18 hours).

B. Apoptosis Assay by Annexin V-FITC/PI

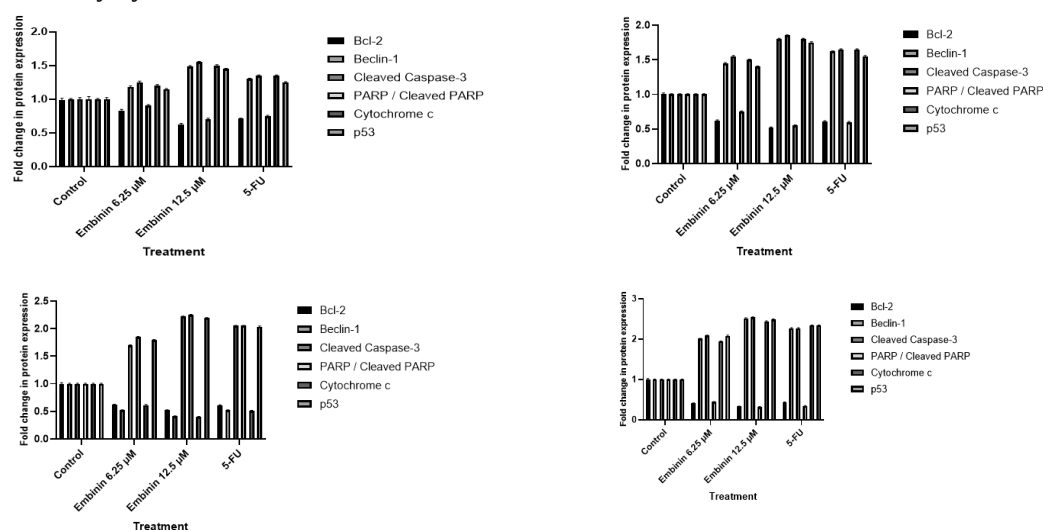


Figure 4: a. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(6hrs)
b. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(12hrs)
c. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(18hrs)
d. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(24hrs)

Table 1b: Effect of Embinin and 5-FU on Apoptosis in HT-29 Cells after 12 hours of treatment

Treatment	Live Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	88.1 ± 1.1	5.1 ± 0.5	4.6 ± 0.5	2.2 ± 0.3
Embinin 6.25 µM	$77.3 \pm 1.3^*$	$10.8 \pm 0.8^{**}$	$9.6 \pm 0.8^{**}$	2.3 ± 0.3
Embinin 12.5 µM	$68.5 \pm 1.5^*$	$15.4 \pm 1.0^{**}$	$13.7 \pm 1.0^{**}$	2.4 ± 0.3
5-FU 10 µM	$64.8 \pm 1.5^*$	$17.0 \pm 1.1^{**}$	$15.5 \pm 1.0^{**}$	2.7 ± 0.3

Table 1b shows that in the control group maximum cells were live ($88.1 \pm 1.1\%$) with a very small percentage of early apoptotic ($5.1 \pm 0.5\%$), late apoptotic ($4.6 \pm 0.5\%$) and necrotic cells ($2.2 \pm 0.3\%$). On treatment with Embinin ($6.25 \mu\text{M}$), the live cells reduced to ($77.3 \pm 1.3\%$) while the early apoptotic ($10.8 \pm 0.8\%$) and late apoptotic ($9.6 \pm 0.8\%$) cells increased significantly as compared to the control. At a higher dose of Embinin ($12.5 \mu\text{M}$) the live cells further decreased ($68.5 \pm 1.5\%$) with a corresponding increase in early apoptotic ($15.4 \pm 1.0\%$) and late apoptotic ($13.7 \pm 1.0\%$) cells. Similarly, the standard drug 5-FU ($10 \mu\text{M}$) treated group showed maximum decrease in live cells ($64.8 \pm 1.5\%$) with marked increase in early apoptotic ($17.0 \pm 1.1\%$) and late apoptotic ($15.5 \pm 1.0\%$) cells as compared to the control (**4b**).

Table 1c: Effect of Embinin and 5-FU on Apoptosis in HT-29 Cells after 18 hours of treatment

Treatment	Live Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	86.0 ± 1.2	6.0 ± 0.6	5.5 ± 0.6	2.5 ± 0.3
Embinin $6.25 \mu\text{M}$	$74.6 \pm 1.4^*$	$12.7 \pm 0.9^{**}$	$10.1 \pm 0.8^{**}$	2.6 ± 0.3
Embinin $12.5 \mu\text{M}$	$64.2 \pm 1.6^*$	$18.6 \pm 1.1^{**}$	$14.4 \pm 1.0^{**}$	2.8 ± 0.4
5-FU $10 \mu\text{M}$	$60.5 \pm 1.6^*$	$19.2 \pm 1.1^{**}$	$17.0 \pm 1.1^{**}$	3.3 ± 0.4

Table 1c shows that in the control group maximum cells were live ($86.0 \pm 1.2\%$) with a very small percentage of early apoptotic ($6.0 \pm 0.6\%$), late apoptotic ($5.5 \pm 0.6\%$) and necrotic cells ($2.5 \pm 0.3\%$). On treatment with Embinin ($6.25 \mu\text{M}$), the live cells reduced to ($74.6 \pm 1.4\%$) while the early apoptotic ($12.7 \pm 0.9\%$) and late apoptotic ($10.1 \pm 0.8\%$) cells increased significantly as compared to the control. At a higher dose of Embinin ($12.5 \mu\text{M}$) the live cells further decreased ($64.2 \pm 1.6\%$) with a corresponding increase in early apoptotic ($18.6 \pm 1.1\%$) and late apoptotic ($14.4 \pm 1.0\%$) cells. Similarly, the standard drug 5-FU ($10 \mu\text{M}$) treated group showed maximum decrease in live cells ($60.5 \pm 1.6\%$) with marked increase in early apoptotic ($19.2 \pm 1.1\%$) and late apoptotic ($17.0 \pm 1.1\%$) cells as compared to the control (**4c**).

Table 1d: Effect of Embinin and 5-FU on Apoptosis in HT-29 Cells after 24 hours of treatment

Treatment	Live Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	84.2 ± 2.1	6.1 ± 0.5	6.8 ± 0.7	2.9 ± 0.3
Embinin $6.25 \mu\text{M}$	$72.5 \pm 1.8^*$	$12.9 \pm 0.9^*$	$12.4 \pm 1.1^*$	2.2 ± 0.2
Embinin $12.5 \mu\text{M}$	$61.8 \pm 2.0^{**}$	$18.7 \pm 1.2^{**}$	$16.5 \pm 1.4^{**}$	3.0 ± 0.4
5-FU $10 \mu\text{M}$	$57.6 \pm 2.0^{**}$	$20.1 \pm 1.3^{**}$	$18.8 \pm 1.3^{**}$	3.5 ± 0.4

Table 1d shows that in the control group maximum cells were live ($84.2 \pm 2.1\%$) with a very small percentage of early apoptotic ($6.1 \pm 0.5\%$), late apoptotic ($6.8 \pm 0.7\%$) and necrotic cells ($2.9 \pm 0.3\%$). On treatment with Embinin ($6.25 \mu\text{M}$) the live cells reduced to ($72.5 \pm 1.8\%$) while the early apoptotic ($12.9 \pm 0.9\%$) and late apoptotic ($12.4 \pm 1.1\%$) cells increased significantly as compared to the control. At a higher dose of Embinin ($12.5 \mu\text{M}$) the live cells further decreased ($61.8 \pm 2.0\%$) with a corresponding increase in early apoptotic ($18.7 \pm 1.2\%$) and late apoptotic ($16.5 \pm 1.4\%$) cells. Similarly, the standard drug 5-FU ($10 \mu\text{M}$) treated group showed maximum decrease in live cells ($57.6 \pm 2.0\%$) with marked increase in early apoptotic ($20.1 \pm 1.3\%$) and late apoptotic ($18.8 \pm 1.3\%$) cells as compared to the control (**Figure 4d**).

C. Cell Cycle Analysis

Table 2a: Sub-G1 Cell Population (%)

Treatment	6 h	12 h	18 h	24 h
Control	2.1 ± 0.3	2.5 ± 0.4	2.8 ± 0.5	3.0 ± 0.6
Embinin $6.25 \mu\text{M}$	4.1 ± 0.5	$6.7 \pm 0.6^*$	$9.2 \pm 0.7^{**}$	$12.5 \pm 0.9^{**}$
Embinin $12.5 \mu\text{M}$	$5.3 \pm 0.6^*$	$9.0 \pm 0.7^{**}$	$13.7 \pm 0.9^{***}$	$18.2 \pm 1.2^{***}$
5-FU ($10 \mu\text{M}$)	$6.8 \pm 0.7^*$	$11.2 \pm 0.8^{**}$	$15.6 \pm 1.0^{***}$	$20.3 \pm 1.3^{***}$

Table 2a shows that in the control group, apoptotic cells were very less and increased slightly from ($2.1 \pm 0.3\%$) at 6 h to ($3.0 \pm 0.6\%$) at 24 h. On treatment with Embinin ($6.25 \mu\text{M}$), the apoptotic cell percentage increased in a time-dependent manner from ($4.1 \pm 0.5\%$) at 6 h to ($12.5 \pm 0.9\%$) at 24 h. At higher concentration of Embinin ($12.5 \mu\text{M}$), apoptosis was significantly enhanced, showing ($5.3 \pm 0.6\%$) at 6 h, ($9.0 \pm 0.7\%$) at 12 h, ($13.7 \pm 0.9\%$) at 18 h, and ($18.2 \pm 1.2\%$) at 24 h. Similarly, the standard drug 5-FU ($10 \mu\text{M}$) produced the maximum apoptotic effect, with apoptosis increasing from ($6.8 \pm 0.7\%$) at 6 h to ($20.3 \pm 1.3\%$) at 24 h. The results indicated that both Embinin and 5-FU significantly reduced the parameter in a time- and dose-dependent manner, with 5-FU showing the strongest effect.

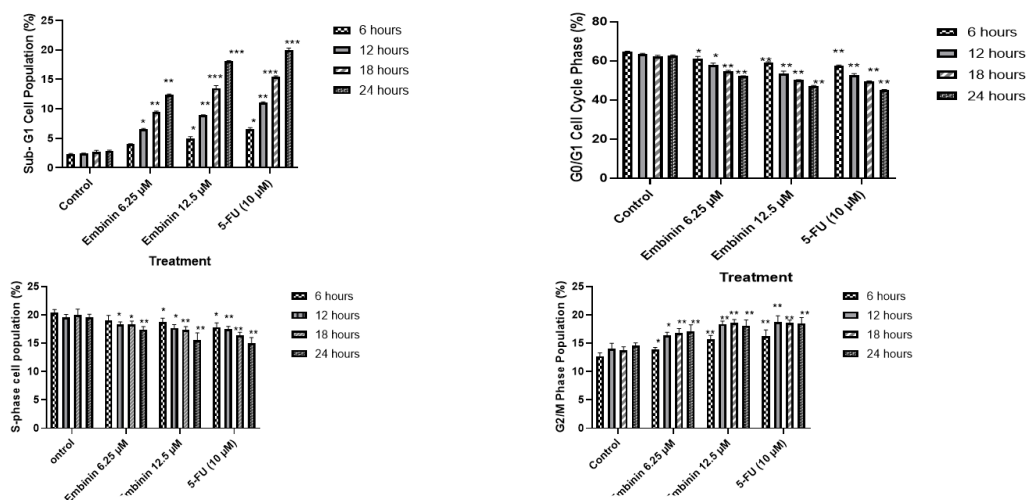


Figure 5: a. Sub- G1 Cell Population (%)
 b. Effect of Embinin on G0/G1 Cell Cycle Phase (%)
 c. Effect of Embinin on S-phase Population (%)
 d. Effect of Embinin on G2/M Phase Population (%)

Table 2b: Effect of Embinin on G0/G1 Cell Cycle Phase (%) Compared with 5-FU

Treatment	6 h	12 h	18 h	24 h
Control	64.5 ± 1.2	63.8 ± 1.0	62.9 ± 1.3	62.5 ± 1.5
Embinin 6.25 µM	61.8 ± 1.4*	58.4 ± 1.5*	55.2 ± 1.6**	52.0 ± 1.7**
Embinin 12.5 µM	59.5 ± 1.3**	54.8 ± 1.5**	50.3 ± 1.7**	47.0 ± 1.8**
5-FU (10 µM)	57.9 ± 1.4**	53.2 ± 1.6**	49.5 ± 1.7**	45.0 ± 1.8**

Table 2b shows that in the control group, the percentage of live cells remained almost constant from (64.5 ± 1.2%) at 6 h to (62.5 ± 1.5%) at 24 h. On treatment with Embinin (6.25 µM), the viable cell percentage decreased gradually in a time-dependent manner from (61.8 ± 1.4%) at 6 h to (52.0 ± 1.7%) at 24 h. At a higher concentration of Embinin (12.5 µM), a further reduction in live cells was observed, with (59.5 ± 1.3%) at 6 h, (54.8 ± 1.5%) at 12 h, (50.3 ± 1.7%) at 18 h, and (47.0 ± 1.8%) at 24 h. Similarly, the standard drug 5-FU (10 µM) produced the maximum reduction in cell viability, decreasing from (57.9 ± 1.4%) at 6 h to (45.0 ± 1.8%) at 24 h. These results indicated that both Embinin and 5-FU significantly reduced cell viability in HT-29 cells in a time- and dose-dependent manner, with 5-FU showing the strongest effect (5b).

Table 2c: Effect of Embinin on S-phase Population (%) Compared with 5-FU

Treatment	6 h	12 h	18 h	24 h
Control	20.3 ± 0.9	19.7 ± 0.8	20.1 ± 1.0	19.8 ± 0.9
Embinin 6.25 µM	19.9 ± 0.8	18.8 ± 0.7*	18.1 ± 0.9*	17.2 ± 0.8**
Embinin 12.5 µM	19.0 ± 0.8*	18.0 ± 0.9*	17.1 ± 0.8**	16.5 ± 0.9**
5-FU (10 µM)	18.5 ± 0.8*	17.4 ± 0.9**	16.2 ± 0.9**	15.0 ± 0.9**

Table 2c shows that in the control group, the values remained almost constant (20.3 ± 0.9) at 6 h to (19.8 ± 0.9) at 24 h. On treatment with Embinin (6.25 µM), the values decreased from (19.9 ± 0.8) at 6 h to (17.2 ± 0.8) at 24 h. At a higher dose of Embinin (12.5 µM) further reduction was observed from (19.0 ± 0.8) at 6 h to (16.5 ± 0.9) at 24 h. Similarly, the standard drug 5-FU (10 µM) treated group showed maximum reduction with values decreasing from (18.5 ± 0.8) at 6 h to (15.0 ± 0.9) at 24 h. The results indicated that both Embinin and 5-FU significantly reduced the parameter in a time- and dose-dependent manner, with 5-FU showing the strongest effect (Figure 5c).

Table 2d: Effect of Embinin on G2/M Phase Population (%) Compared with 5-FU

Treatment	6 h	12 h	18 h	24 h
Control	13.1 ± 0.6	14.0 ± 0.7	14.2 ± 0.8	14.7 ± 0.9
Embinin 6.25 µM	14.2 ± 0.7*	16.1 ± 0.8*	17.5 ± 0.9**	18.3 ± 1.0**
Embinin 12.5 µM	16.2 ± 0.9**	18.2 ± 1.0**	18.9 ± 1.1**	18.3 ± 1.0**
5-FU (10 µM)	16.8 ± 0.8**	18.2 ± 1.0**	18.7 ± 1.1**	19.7 ± 1.2**

Table 2d shows that in the control group, the values remained almost stable from (13.1 ± 0.6) at 6 h to (14.7 ± 0.9) at 24 h. On treatment with Embinin (6.25 µM), there was a gradual increase from (14.2 ± 0.7) at 6 h to (18.3 ± 1.0) at 24 h. At higher concentration of Embinin (12.5 µM), a more prominent increase was observed, from (16.2 ± 0.9) at 6 h to (18.3 ± 1.0) at 24 h. Similarly, the standard drug 5-FU (10 µM) produced the maximum increase, with values ranging from (16.8 ± 0.8) at 6 h to (19.7 ± 1.2) at 24 h. These results indicated that both Embinin and 5-FU significantly increased the parameter in a time- and dose-dependent manner, with 5-FU showing the maximum effect (**Figure 5d**).

D. Protein Expression Analysis

Table 3a: Beclin-1 Expression (HT-29 Cells)

Treatment	6 hours	12 hours	18 hours	24 hours
Control	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.04
Embinin 6.25 µM	1.18 ± 0.06 *	1.45 ± 0.05 **	1.70 ± 0.07 **	2.02 ± 0.08 ***
Embinin 12.5 µM	1.48 ± 0.07 **	1.80 ± 0.06 **	2.22 ± 0.09 ***	2.53 ± 0.10 ***
5-FU	1.30 ± 0.06 **	1.62 ± 0.07 **	2.05 ± 0.08 ***	2.28 ± 0.09 ***

In the control group (**Table 3a**), the values remained constant around (1.00 ± 0.05 to 1.00 ± 0.04) throughout 6 h to 24 hours, showing no significant change over time. Treatment with Embinin (6.25 µM) showed a significant and time-dependent increase from (1.18 ± 0.06) at 6 h to (2.02 ± 0.08) at 24 h. A higher concentration, Embinin (12.5 µM), demonstrated a stronger effect with values increasing from (1.48 ± 0.07) at 6 h to (2.53 ± 0.10) at 24 h. The standard drug 5-FU also produced a significant increase, ranging from (1.30 ± 0.06) at 6 h to (2.28 ± 0.09) at 24 h. These findings indicate that Embinin enhances the parameter in a dose- and time-dependent manner, and its effect at the higher dose (12.5 µM) was found to be comparable or greater than 5-FU (**Figure 6a**).

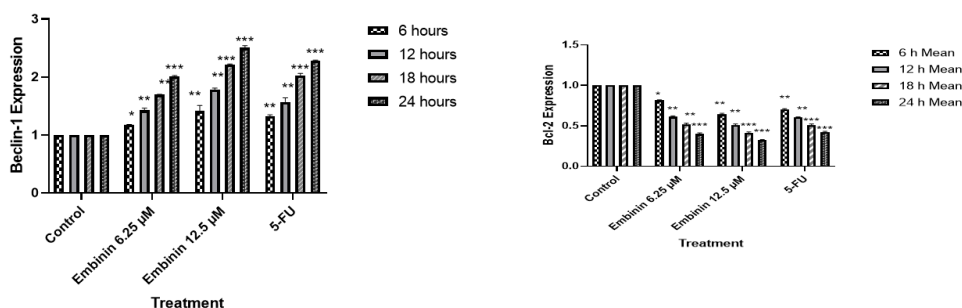


Figure 6: a. Beclin-1 Expression (HT-29 Cells)
b. Bcl-2 Expression (HT-29 Cells)

Table 3b: qPCR Results – Bcl-2 Expression (HT-29 Cells)

Treatment	6 hours	12 hours	18 hours	24 hours
Control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05
Embinin 6.25 µM	0.82 ± 0.05 *	0.62 ± 0.04 **	0.53 ± 0.03 **	0.41 ± 0.04 ***
Embinin 12.5 µM	0.63 ± 0.04 **	0.52 ± 0.03 **	0.42 ± 0.04 ***	0.33 ± 0.03 ***
5-FU	0.71 ± 0.05 **	0.61 ± 0.04 **	0.52 ± 0.03 ***	0.43 ± 0.04 ***

In the control group (**Table 3b**), values remained stable around (1.00 ± 0.04 to 1.00 ± 0.05) during the 24 h period, indicating no significant changes. Treatment with Embinin (6.25 µM) significantly reduced the parameter from (0.82 ± 0.05) at 6 h to (0.41 ± 0.04) at 24 h, showing a clear time-dependent decrease. The higher concentration, Embinin (12.5 µM), produced a stronger effect, reducing the (0.43 ± 0.04) at 24 hours parameter from (0.63 ± 0.04) at 6 h to (0.33 ± 0.03) at 24 hours (**Figure 6b**).

E. Western Blot Analysis

Table 4a: Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP / Cleaved PARP	Cytochrome c	p53
Control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05
Embinin 6.25 µM	0.82 ± 0.05*	1.18 ± 0.06*	1.25 ± 0.05*	0.90 ± 0.04*	1.20 ± 0.05*	1.15 ± 0.05*
Embinin 12.5 µM	0.63 ± 0.04**	1.48 ± 0.07**	1.55 ± 0.06**	0.70 ± 0.03**	1.50 ± 0.06**	1.45 ± 0.06**
5-FU	0.71 ± 0.05**	1.30 ± 0.06**	1.35 ± 0.06**	0.75 ± 0.03**	1.35 ± 0.06**	1.25 ± 0.06**

Table 4a shows that the control group maintained basal protein expression levels with mean values normalised to 1.00 ± 0.04–0.05 across all markers. Treatment with Embinin (6.25 µM) resulted in a significant decrease in Bcl-2 expression (0.82 ± 0.05) while simultaneously increasing Beclin-1 (1.18 ± 0.06), cleaved caspase-3 (1.25 ± 0.05), cytochrome c (1.20 ± 0.05), and p53 (1.15 ± 0.05) as compared to control. A further significant modulation was observed with Embinin (12.5 µM), showing a pronounced reduction in Bcl-2 (0.63 ± 0.04) and increase in Beclin-1 (1.48 ± 0.07), cleaved caspase-3 (1.55 ± 0.06), cytochrome c (1.50 ± 0.06), and p53 (1.45 ± 0.06) along with marked decrease in PARP/cleaved PARP ratio (0.70 ± 0.03). The standard drug 5-FU also significantly downregulated Bcl-2 (0.71 ± 0.05) and PARP/cleaved PARP ratio (0.75 ± 0.03), while upregulating Beclin-1 (1.30 ± 0.06), cleaved caspase-3 (1.35 ± 0.06), cytochrome c (1.35 ± 0.06), and p53 (1.25 ± 0.06) compared to control. From the table, it can be observed that Embinin treatment promotes apoptosis and autophagy by decreasing anti-apoptotic protein Bcl-2 while increasing pro-apoptotic (caspase-3, cytochrome c, p53) and autophagy-related (Beclin-1) markers. Among the treatment groups, Embinin at 12.5 µM showed more significant effects as compared to lower dose (6.25 µM) and standard drug 5-FU, indicating a dose-dependent enhancement of apoptotic and autophagic pathways (**Figure 7a**).

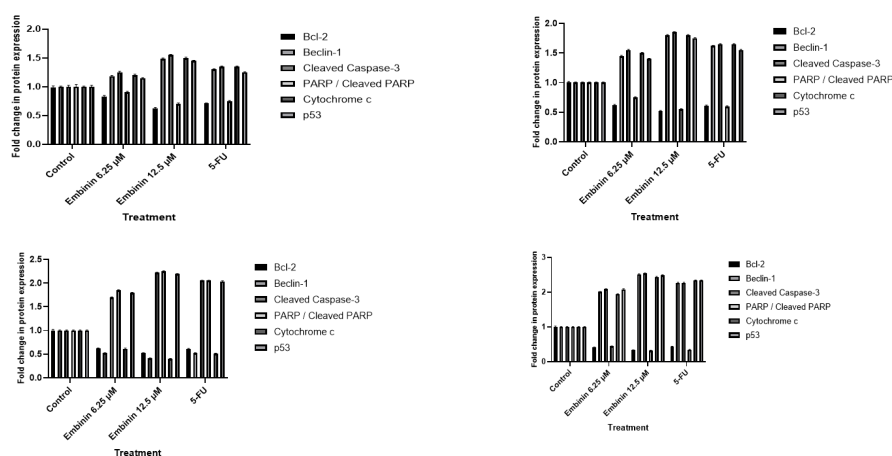


Figure 7: a. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(6hrs)
 b. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(12hrs)
 c. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(18hrs)
 d. Effect of Embinin and 5-FU on Apoptosis and Autophagy Protein Expression in HT-29 Cells(24hrs)

Table 4b: Effect of Embinin and 5-FU on the Expression of Apoptotic and Autophagic Proteins in HT-29 Cells (Western Blot Analysis)

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP / Cleaved PARP	Cytochrome c	p53
Control	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04
Embinin 6.25 µM	0.62 ± 0.04 **	1.45 ± 0.05 **	1.55 ± 0.06 **	0.75 ± 0.03 **	1.50 ± 0.06 **	1.40 ± 0.05 **
Embinin 12.5 µM	0.52 ± 0.03 **	1.80 ± 0.06 **	1.85 ± 0.07 **	0.55 ± 0.03 **	1.80 ± 0.07 **	1.75 ± 0.06 **
5-FU	0.61 ± 0.04 **	1.62 ± 0.07 **	1.65 ± 0.07 **	0.60 ± 0.03 **	1.65 ± 0.07 **	1.55 ± 0.06 **

Table 4b shows that the control group maintained normal basal levels of proteins with mean values around 1.00 ± 0.04–0.05 across all parameters. Treatment with Embinin (6.25 µM) resulted in a significant decrease in Bcl-2 (0.62 ± 0.04)

and PARP/cleaved PARP ratio (0.75 ± 0.03), while there was a marked increase in Beclin-1 (1.45 ± 0.05), cleaved caspase-3 (1.55 ± 0.06), cytochrome c (1.50 ± 0.06), and p53 (1.40 ± 0.05) compared to control. At a higher dose, Embinin ($12.5 \mu\text{M}$) further enhanced these effects, showing the lowest Bcl-2 expression (0.52 ± 0.03) and PARP/cleaved PARP ratio (0.55 ± 0.03) along with the highest elevation in Beclin-1 (1.80 ± 0.06), cleaved caspase-3 (1.85 ± 0.07), cytochrome c (1.80 ± 0.07), and p53 (1.75 ± 0.06). The standard drug 5-FU also produced significant changes, with reduced Bcl-2 (0.61 ± 0.04) and PARP/cleaved PARP (0.60 ± 0.03), and increased expression of Beclin-1 (1.62 ± 0.07), cleaved caspase-3 (1.65 ± 0.07), cytochrome c (1.65 ± 0.07), and p53 (1.55 ± 0.06) compared to control. From the table, it can be observed that Embinin significantly promotes apoptosis and autophagy in a dose-dependent manner, evidenced by downregulation of anti-apoptotic Bcl-2 and upregulation of apoptotic (caspase-3, cytochrome c, p53) and autophagy-related (Beclin-1) proteins. Among the groups, Embinin at $12.5 \mu\text{M}$ exhibited the most significant effects compared to both its lower dose and the standard drug 5-FU (Figure 7b).

Table 4c: Effect of Embinin and 5-FU on the Expression of Apoptotic and Autophagic Proteins in HT-29 Cells

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP/Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.04
Embinin 6.25 μM	0.53 ± 0.03 **	1.70 ± 0.07 **	1.85 ± 0.07 **	0.60 ± 0.04 **	1.80 ± 0.07 **	1.65 ± 0.06 **
Embinin 12.5 μM	0.42 ± 0.04 ***	2.22 ± 0.09 ***	2.25 ± 0.09 ***	0.40 ± 0.03 ***	2.20 ± 0.09 ***	2.10 ± 0.08 ***
5-FU	0.52 ± 0.03 ***	2.05 ± 0.08 ***	2.05 ± 0.08 ***	0.45 ± 0.03 ***	2.05 ± 0.08 ***	1.95 ± 0.07 ***

Table 4c shows that the control group maintained basal protein expression levels, with mean values normalized close to 1.00 ± 0.03 – 0.04 across all markers. Treatment with Embinin ($6.25 \mu\text{M}$) produced a significant reduction in Bcl-2 expression (0.53 ± 0.03) and PARP/cleaved PARP ratio (0.60 ± 0.04), while markedly increasing Beclin-1 (1.70 ± 0.07), cleaved caspase-3 (1.85 ± 0.07), cytochrome c (1.80 ± 0.07), and p53 (1.65 ± 0.06) compared to control. At a higher dose, Embinin ($12.5 \mu\text{M}$) further enhanced these effects, showing the most profound decrease in Bcl-2 (0.42 ± 0.04) and PARP/cleaved PARP (0.40 ± 0.03), accompanied by the highest induction of Beclin-1 (2.22 ± 0.09), cleaved caspase-3 (2.25 ± 0.09), cytochrome c (2.20 ± 0.09), and p53 (2.10 ± 0.08). Similarly, the standard drug 5-FU also significantly modulated protein expression, with reduced Bcl-2 (0.52 ± 0.03) and PARP/cleaved PARP (0.45 ± 0.03), while increasing Beclin-1 (2.05 ± 0.08), cleaved caspase-3 (2.05 ± 0.08), cytochrome c (2.05 ± 0.08), and p53 (1.95 ± 0.07) compared to control. From the table, it can be observed that Embinin strongly promotes apoptotic and autophagic signalling pathways, as indicated by downregulation of anti-apoptotic protein Bcl-2 and suppression of PARP/cleaved PARP ratio, alongside upregulation of Beclin-1, caspase-3, cytochrome c, and p53. Among all treatments, Embinin at $12.5 \mu\text{M}$ exhibited the most significant effect, surpassing both the lower dose and the standard drug 5-FU, suggesting a dose-dependent enhancement of apoptosis and autophagy (Figure 7c).

Table 4d: Effect of Embinin and 5-FU on the Expression of Apoptotic and Autophagic Proteins in HT-29 Cells

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP / Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05
Embinin 6.25 μM	0.41 ± 0.04 ***	2.02 ± 0.08 ***	2.10 ± 0.08 ***	0.45 ± 0.03 ***	2.10 ± 0.08 ***	1.95 ± 0.07 ***
Embinin 12.5 μM	0.33 ± 0.03 ***	2.53 ± 0.10 ***	2.55 ± 0.10 ***	0.30 ± 0.02 ***	2.50 ± 0.10 ***	2.45 ± 0.09 ***
5-FU	0.43 ± 0.04 ***	2.28 ± 0.09 ***	2.35 ± 0.09 ***	0.35 ± 0.03 ***	2.35 ± 0.09 ***	2.30 ± 0.08 ***

Table 4d shows that the control group maintained basal protein expression levels with mean values normalised around 1.00 ± 0.04 – 0.05 across all markers. Treatment with Embinin ($6.25 \mu\text{M}$) led to a significant reduction in Bcl-2 (0.41 ± 0.04) and PARP/cleaved PARP ratio (0.45 ± 0.03), while there was a marked increase in Beclin-1 (2.02 ± 0.08), cleaved caspase-3 (2.10 ± 0.08), cytochrome c (2.10 ± 0.08), and p53 (1.95 ± 0.07) compared to control. At a higher

concentration, Embinin (12.5 μM) demonstrated even stronger effects, showing the lowest levels of Bcl-2 (0.33 ± 0.03) and PARP/cleaved PARP (0.30 ± 0.02), while producing the maximum induction of Beclin-1 (2.53 ± 0.10), cleaved caspase-3 (2.55 ± 0.10), cytochrome c (2.50 ± 0.10), and p53 (2.45 ± 0.09). Similarly, the standard drug 5-FU significantly reduced Bcl-2 (0.43 ± 0.04) and PARP/cleaved PARP (0.35 ± 0.03), while enhancing Beclin-1 (2.28 ± 0.09), cleaved caspase-3 (2.35 ± 0.09), cytochrome c (2.35 ± 0.09), and p53 (2.30 ± 0.08) compared to control. From the table, it can be observed that Embinin treatment promotes apoptosis and autophagy in a dose-dependent manner, as evidenced by downregulation of the anti-apoptotic protein Bcl-2 and PARP/cleaved PARP ratio, along with strong upregulation of apoptotic markers (caspase-3, cytochrome c, p53) and the autophagy-related marker Beclin-1. Among all groups, Embinin at 12.5 μM showed the most significant effect, surpassing both its lower dose and the standard drug 5-FU, suggesting it exerts a potent dual role in stimulating apoptosis and autophagy (**Figure7d**).

F. Immunofluorescence

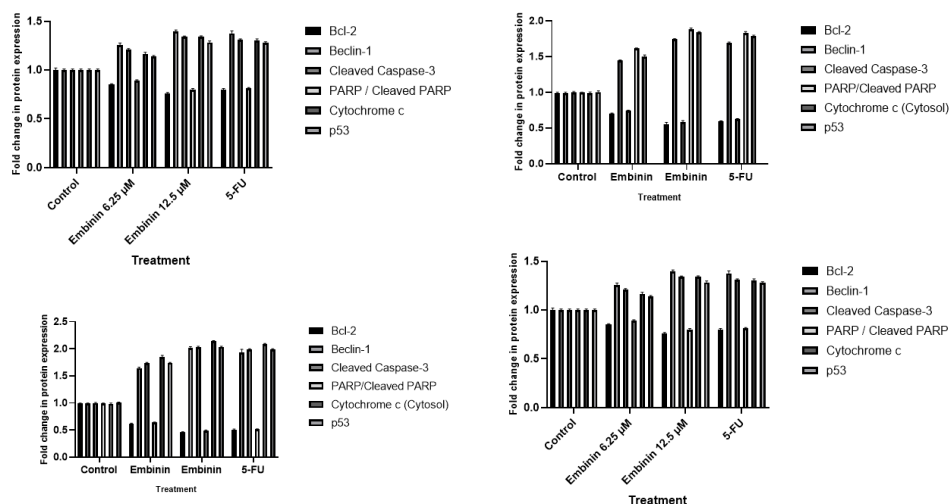


Figure 8: a. Protein Expression in HT-29 Cells (By Immunofluorescence, 6hrs)
b. Protein Expression in HT-29 Cells (By Immunofluorescence, 12hrs)
c. Protein Expression in HT-29 Cells (By Immunofluorescence, 18hrs)
d. Protein Expression in HT-29 Cells (By Immunofluorescence, 24hrs)

Table 5a: Effect of Embinin and 5-FU on Protein Expression (6 h, Immunofluorescence)

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP/Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.04	1.00 $\pm$ 0.04
Embinin 6.25 μM	0.85 $\pm$ 0.04 *	1.25 $\pm$ 0.05 *	1.20 $\pm$ 0.05 *	0.90 $\pm$ 0.04 *	1.18 $\pm$ 0.05 *	1.15 $\pm$ 0.05 *
Embinin 12.5 μM	0.75 $\pm$ 0.03 **	1.40 $\pm$ 0.06 **	1.35 $\pm$ 0.06 **	0.80 $\pm$ 0.03 **	1.35 $\pm$ 0.06 **	1.30 $\pm$ 0.06 **
5-FU	0.80 $\pm$ 0.03 **	1.35 $\pm$ 0.06 **	1.32 $\pm$ 0.06 **	0.82 $\pm$ 0.03 **	1.32 $\pm$ 0.06 **	1.28 $\pm$ 0.06 **

Table 5a shows that in the control group, the relative protein expression levels were normalised to 1.00 ± 0.03 – 0.04 for all studied markers (Bcl-2, Beclin-1, Cleaved Caspase-3, PARP/Cleaved PARP, Cytochrome c, and p53). Treatment with Embinin (6.25 μM and 12.5 μM) and the standard drug 5-FU produced significant alterations in protein expression compared to the control. At 6.25 μM Embinin, Bcl-2 expression decreased (0.85 ± 0.04), while Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 showed significant upregulation with mean values of 1.25 ± 0.05 , 1.20 ± 0.05 , 1.18 ± 0.05 , and 1.15 ± 0.05 , respectively. Similarly, PARP/Cleaved PARP ratio was reduced to 0.90 ± 0.04 . At a higher dose of 12.5 μM Embinin, these effects were more pronounced. Bcl-2 expression further declined (0.75 ± 0.03), whereas Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 showed greater increases (1.40 ± 0.06 , 1.35 ± 0.06 , 1.35 ± 0.06 , and 1.30 ± 0.06 , respectively). PARP/Cleaved PARP ratio also decreased significantly (0.80 ± 0.03). The 5-FU treated group exhibited a pattern similar to Embinin (12.5 μM), with Bcl-2 suppression (0.80 ± 0.03) and significant elevation of Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 (1.35 ± 0.06 , 1.32 ± 0.06 , 1.32 ± 0.06 , and 1.28 ± 0.06 , respectively), along with a reduced PARP/Cleaved PARP ratio (0.82 ± 0.03). From the table, it can be observed that Embinin induces apoptosis and autophagy in a dose-dependent manner, as evidenced by downregulation of Bcl-2 and PARP/Cleaved PARP, and upregulation of Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53. The higher dose of

Embinin (12.5 μ M) showed more significant effects compared to lower dose (6.25 μ M), and the response was comparable to that of the standard drug 5-FU (**Figure 8a**).

Table 5b: Effect of Embinin and 5-FU on Protein Expression (12 h, Immunofluorescence)

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP/Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.04	1.00 $\pm$ 0.04
Embinin 6.25 μ M	0.70 $\pm$ 0.03 **	1.45 $\pm$ 0.06 **	1.55 $\pm$ 0.06 **	0.75 $\pm$ 0.03 **	1.60 $\pm$ 0.07 **	1.50 $\pm$ 0.06 **
Embinin 12.5 μ M	0.55 $\pm$ 0.03 ***	1.75 $\pm$ 0.07 ***	1.85 $\pm$ 0.07 ***	0.60 $\pm$ 0.02 ***	1.90 $\pm$ 0.08 ***	1.85 $\pm$ 0.07 ***
5-FU	0.60 $\pm$ 0.03 ***	1.70 $\pm$ 0.07 ***	1.80 $\pm$ 0.07 ***	0.62 $\pm$ 0.02 ***	1.85 $\pm$ 0.07 ***	1.80 $\pm$ 0.07 ***

Table 5b shows that the control group exhibited normalized expression levels of all proteins with values around 1.00 $\pm$ 0.03–0.04. Treatment with Embinin (6.25 μ M and 12.5 μ M) and the standard drug 5-FU produced significant modulation of apoptotic and autophagic markers compared to control. At 6.25 μ M Embinin, a notable suppression of Bcl-2 (0.70 $\pm$ 0.03) and PARP/Cleaved PARP ratio (0.75 $\pm$ 0.03) was observed, while Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 were significantly upregulated, with mean values of 1.45 $\pm$ 0.06, 1.55 $\pm$ 0.06, 1.60 $\pm$ 0.07, and 1.50 $\pm$ 0.06, respectively. At the higher dose (12.5 μ M Embinin), these effects were more pronounced, with Bcl-2 reduced to 0.55 $\pm$ 0.03, PARP/Cleaved PARP to 0.60 $\pm$ 0.02, and marked increases in Beclin-1 (1.75 $\pm$ 0.07), Cleaved Caspase-3 (1.85 $\pm$ 0.07), Cytochrome c (1.90 $\pm$ 0.08), and p53 (1.85 $\pm$ 0.07). The 5-FU treated group showed a similar trend to high-dose Embinin, with Bcl-2 reduced to 0.60 $\pm$ 0.03, PARP/Cleaved PARP to 0.62 $\pm$ 0.02, and significant elevation of Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 (1.70 $\pm$ 0.07, 1.80 $\pm$ 0.07, 1.85 $\pm$ 0.07, and 1.80 $\pm$ 0.07, respectively). From the table, it can be observed that Embinin induces apoptosis and autophagy in a dose- and time-dependent manner, as evidenced by the suppression of Bcl-2 and PARP/Cleaved PARP and the simultaneous upregulation of Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53. The effect at 12.5 μ M Embinin was more significant than at 6.25 μ M and was comparable to the standard drug 5-FU after 12 hours of treatment (**Figure 8b**).

Table 5c: Effect of Embinin and 5-FU on Protein Expression (18 h, Immunofluorescence)

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP/Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.04	1.00 $\pm$ 0.04
Embinin 6.25 μ M	0.60 $\pm$ 0.03 **	1.65 $\pm$ 0.07 **	1.75 $\pm$ 0.07 **	0.65 $\pm$ 0.03 **	1.85 $\pm$ 0.07 **	1.75 $\pm$ 0.07 **
Embinin 12.5 μ M	0.45 $\pm$ 0.02 ***	2.00 $\pm$ 0.08 ***	2.05 $\pm$ 0.08 ***	0.50 $\pm$ 0.02 ***	2.15 $\pm$ 0.08 ***	2.05 $\pm$ 0.08 ***
5-FU	0.50 $\pm$ 0.02 ***	1.95 $\pm$ 0.08 ***	2.00 $\pm$ 0.08 ***	0.52 $\pm$ 0.02 ***	2.10 $\pm$ 0.08 ***	2.00 $\pm$ 0.08 ***

Table 5c shows that the control group maintained normalized protein expression values around 1.00 $\pm$ 0.03–0.04. Treatment with Embinin (6.25 μ M and 12.5 μ M) and the standard drug 5-FU led to significant alterations in the expression of apoptotic and autophagic markers compared to control. At 6.25 μ M Embinin, Bcl-2 expression decreased markedly to 0.60 $\pm$ 0.03, and PARP/Cleaved PARP ratio reduced to 0.65 $\pm$ 0.03. In contrast, Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 levels were significantly upregulated, reaching 1.65 $\pm$ 0.07, 1.75 $\pm$ 0.07, 1.85 $\pm$ 0.07, and 1.75 $\pm$ 0.07, respectively. At the higher dose of 12.5 μ M Embinin, the effects were more pronounced. Bcl-2 decreased further to 0.45 $\pm$ 0.02, and PARP/Cleaved PARP dropped to 0.50 $\pm$ 0.02. Meanwhile, Beclin-1 (2.00 $\pm$ 0.08), Cleaved Caspase-3 (2.05 $\pm$ 0.08), Cytochrome c (2.15 $\pm$ 0.08), and p53 (2.05 $\pm$ 0.08) showed strong upregulation, confirming enhanced induction of apoptosis and autophagy. The 5-FU treated group produced a response pattern similar to high-dose Embinin, with suppression of Bcl-2 (0.50 $\pm$ 0.02) and PARP/Cleaved PARP (0.52 $\pm$ 0.02), along with significant increases in Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 (1.95 $\pm$ 0.08, 2.00 $\pm$ 0.08, 2.10 $\pm$ 0.08, and 2.00 $\pm$ 0.08, respectively). From the table, it can be observed that Embinin induces apoptosis and autophagy in a dose-dependent

manner, with stronger effects seen after 18 h compared to earlier time points. The higher dose of Embinin (12.5 μ M) showed the most significant effect, which was comparable to that of 5-FU (**Figure 8c**).

Table 5d: Effect of Embinin and 5-FU on Protein Expression (24 h, Immunofluorescence)

Treatment	Bcl-2	Beclin-1	Cleaved Caspase-3	PARP/Cleaved PARP	Cytochrome c (Cytosol)	p53
Control	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.03	1.00 $\pm$ 0.04	1.00 $\pm$ 0.04
Embinin 6.25 μ M	0.50 $\pm$ 0.03 **	1.80 $\pm$ 0.08 **	1.90 $\pm$ 0.08 **	0.55 $\pm$ 0.03 **	1.95 $\pm$ 0.08 **	1.85 $\pm$ 0.07 **
Embinin 12.5 μ M	0.35 $\pm$ 0.02 ***	2.40 $\pm$ 0.10 ***	2.35 $\pm$ 0.10 ***	0.35 $\pm$ 0.02 ***	2.40 $\pm$ 0.10 ***	2.25 $\pm$ 0.09 ***
5-FU	0.40 $\pm$ 0.03 ***	2.20 $\pm$ 0.09 ***	2.10 $\pm$ 0.09 ***	0.38 $\pm$ 0.02 ***	2.15 $\pm$ 0.09 ***	2.05 $\pm$ 0.08 ***

Table 5d shows that in the control group, all protein markers were normalised to values around 1.00 $\pm$ 0.03–0.04. Treatment with Embinin (6.25 μ M and 12.5 μ M) and the standard drug 5-FU produced significant modulation in apoptotic and autophagic protein expression compared to control. At 6.25 μ M Embinin, Bcl-2 expression was markedly suppressed to 0.50 $\pm$ 0.03, and PARP/Cleaved PARP ratio decreased to 0.55 $\pm$ 0.03. In contrast, Beclin-1 (1.80 $\pm$ 0.08), Cleaved Caspase-3 (1.90 $\pm$ 0.08), Cytochrome c (1.95 $\pm$ 0.08), and p53 (1.85 $\pm$ 0.07) were significantly elevated, indicating activation of both autophagy and apoptosis. At the higher concentration of 12.5 μ M Embinin, these effects were more pronounced. Bcl-2 levels further dropped to 0.35 $\pm$ 0.02, while Beclin-1, Cleaved Caspase-3, Cytochrome c, and p53 showed strong upregulation (2.40 $\pm$ 0.10, 2.35 $\pm$ 0.10, 2.40 $\pm$ 0.10, and 2.25 $\pm$ 0.09, respectively). The PARP/Cleaved PARP ratio was also significantly reduced to 0.35 $\pm$ 0.02, suggesting enhanced apoptotic signalling. The 5-FU-treated group showed a response pattern similar to Embinin, with downregulation of Bcl-2 (0.40 $\pm$ 0.03) and PARP/Cleaved PARP (0.38 $\pm$ 0.02), alongside increased expression of Beclin-1 (2.20 $\pm$ 0.09), Cleaved Caspase-3 (2.10 $\pm$ 0.09), Cytochrome c (2.15 $\pm$ 0.09), and p53 (2.05 $\pm$ 0.08). From the table, it can be observed that Embinin exhibits strong time- and dose-dependent effects, with maximum induction of apoptosis and autophagy at 24 h and 12.5 μ M concentration, which were comparable and, in some cases, more potent than the standard drug 5-FU (**Figure 8d**).

4. CONCLUSION

The present investigation demonstrates that Embinin, under conditions of serum withdrawal, exerts a potent cytotoxic effect on HT-29 colorectal cancer cells by modulating both autophagy and apoptosis. The compound induced significant inhibition of cell proliferation, increased apoptotic cell populations, and altered cell cycle progression. Enhanced expression of Beclin-1 alongside reduced Bcl-2 levels confirms a synergistic interplay between autophagic activation and apoptotic signalling in mediating the anticancer effects of Embinin. Furthermore, the time- and dose-dependent responses observed across assays establish that the fasting-mimicking environment enhances the therapeutic efficacy of Embinin, potentially sensitizing cancer cells to apoptosis.

In conclusion, these results suggest that Embinin, combined with intermittent fasting, may represent an effective therapeutic strategy targeting autophagy–apoptosis cross-talk in colorectal cancer. Future in vivo and molecular studies are warranted to further elucidate its mechanism of action and clinical relevance.

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