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Effects of *Curcumin* and *Achillea millefolium* L. Extract on the Expression of *BCL2*, *DLEC1*, *MCC*, and *NANOG* Genes in *LS180* Colorectal Cancer Cell Line

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ABSTRACT

Colorectal cancer is a common gastrointestinal malignancy with high incidence and mortality rates. Due to increasing resistance to conventional chemotherapy, identifying effective natural compounds has become essential. This study investigated the independent and combined effects of *Achillea millefolium L.* (Yarrow) extract and curcumin on cell viability and the expression of *DLEC1*, *MCC*, *BCL2*, and *NANOG* genes in *LS180* colorectal cancer cells. *LS180* cells were treated with various concentrations of yarrow extract (2.5, 5, 10, 20, and 40 µg/mL), curcumin (6.25, 12.5, 25, 50, and 75 µg/mL), and their combination for 24 and 48 hours. Cell viability was assessed using the MTT assay, and gene expression was measured by qRT-PCR. Statistical analyses were performed using Tukey's test, with a $p< 0.05$ considered statistically significant. Both yarrow extract and curcumin reduced *LS180* cell viability in a dose- and time-dependent manner. The combined treatment exhibited a strong synergistic effect in inhibiting cancer cell proliferation. Furthermore, the combination significantly increased the expression of the tumor suppressor genes *DLEC1* (22.29- fold) and *MCC* (20.96- fold) compared to the control group ($p< 0.0001$). All three treatments also led to a significant reduction in the expression of the oncogenes *BCL2* and *NANOG*. The greatest reduction was observed following curcumin treatment alone, with a 0.069- fold decrease (equivalent to 93.12%) in *BCL2* and a 0.160-fold decrease (equivalent to 84.01%) in *NANOG* expression ($p< 0.0001$). The combination of yarrow extract and curcumin, by markedly upregulating tumor suppressor genes, presents an effective synergistic strategy for inhibiting *LS180* cancer cells. Although the combined treatment was only slightly less effective than curcumin alone in reducing *BCL2* and *NANOG* expression, it holds considerable promise for combinatorial applications in colorectal cancer therapy due to its stronger induction of tumor suppression pathways and synergistic cytotoxicity.

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies of the gastrointestinal tract, arising from the accumulation of genetic and epigenetic alterations that disrupt key regulatory pathways governing cell growth and survival (Duta Ion *et al.*, 2024). Mutations in *APC*, *KRAS*, *TP53*, and *BRAF* are considered major drivers of CRC initiation and progression, promoting increased cell proliferation and inhibition of apoptosis through the activation of Wnt/β-catenin, EGFR/MAPK, and PI3K/AKT signaling pathways (Chikaishi *et al.*, 2025). Furthermore, epigenetic alterations, including DNA methylation and histone modifications, also play a role in regulating gene expression and tumor progression (Burgman *et al.*, 2024). Among the genes involved in CRC, the tumor suppressor gene

DLEC1 (Deleted in Lung and Esophageal Cancer 1), located on chromosome 3p22-p21.3, is recognized as a tumor suppressor with reduced expression observed in many human cancers, primarily due to promoter hypermethylation (Okitsu *et al.*, 2020). Similarly, the *MCC* (Mutated in Colorectal Cancer) gene suppresses cancer cell growth through inhibition of the β-catenin signaling pathway, a key component of the WNT pathway involved in cell proliferation, differentiation, and survival (Lee *et al.*, 2025; Nguyen *et al.*, 2024). In contrast, *BCL2* functions as a classical oncogene by inhibiting apoptosis, thereby promoting cancer cell survival (Garimberti *et al.*, 2024). On the other hand, *NANOG* is primarily a pluripotency transcription factor that maintains stem cell properties; however, a growing body of evidence supports its oncogenic potential across multiple malignancies. Ectopic overexpression of *NANOG* in non-tumorigenic cells is sufficient to drive malignant transformation and promote tumor formation in xenograft models (Jeter *et al.*, 2015; Najafzadeh *et al.*, 2021), explicitly describing the "oncogenic potential of *NANOG*" as a key mediator of cancer initiation and progression. In colorectal cancer specifically, elevated *NANOG* expression has been consistently associated with advanced tumor stages, enhanced metastatic capacity, and poorer clinical outcomes (Zhang *et al.*, 2021). Thus, while *NANOG* is not a classical oncogene in the mechanistic sense of *BCL2*, it functions as a cooperating regulator with oncogenic potential in tumorigenesis.

Given that these genes represent critical pathways in CRC tumor suppression (*DLEC1* and *MCC*), apoptosis inhibition (*BCL2*), and stemness maintenance with oncogenic potential (*NANOG*), they serve as rational molecular targets for evaluating the anticancer effects of natural compounds. On the other hand, natural compounds with anticancer properties have attracted considerable attention. Curcumin, the main polyphenolic constituent of turmeric, exerts inhibitory effects on cancer cell growth, invasion, and metastasis through modulation of PI3K/Akt, Wnt/β-catenin, and JAK/STAT pathways (Amaroli *et al.*, 2024; Yuan *et al.*, 2026). Likewise, *A. millefolium* (yarrow) exhibits significant anti-inflammatory and antioxidant properties that may contribute to cancer inhibition (Far *et al.*, 2023). Given the role of inflammation and dysregulation of cellular signaling pathways in CRC pathogenesis, and considering the modulatory properties of these two natural compounds, investigating their effects on cancer cells could be valuable for developing novel therapeutic approaches.

However, despite numerous studies on the anticancer properties of curcumin and yarrow, the combined and synergistic effects of these two compounds on the expression of key genes involved in colorectal cancer, particularly the tumor suppressor genes (*DLEC1* and *MCC*), the classical oncogene (*BCL2*), and the pluripotency factor with oncogenic potential (*NANOG*), have not been well investigated. In particular, it remains unclear whether the combination of these two natural compounds can exert a synergistic effect on inhibiting cancer cells through establishing a balance in the expression of tumor suppressor and oncogenic genes. Therefore, the present study aimed to investigate the independent and combined effects of yarrow extract and curcumin on cell viability and the expression of *DLEC1*, *MCC*, *BCL2*, and *NANOG* genes in the LS180 colorectal cancer cell line, in order to determine whether these compounds can provide an effective strategy for CRC inhibition through modulation of target gene expression.

Materials and Methods

Plant preparation and extraction

Leaves and flowers of *A. millefolium* were collected from Ilam, Iran, in summer 2025 and identified using the keys of *Identification of Medicinal and Aromatic Plants of Iran* (Mozaffarian, 2012). The plant material was shade-dried at 25°C and pulverized. Extraction was performed using a Soxhlet apparatus with 70% methanol at a 1:10 (W/V) ratio, at 65°C for 7 hours. After solvent evaporation, the dried extract was stored at -20°C.

MTT assay

First, 5,000 LS180 cells were seeded into 96-well plates and cultured for 24 hours. Various concentrations of yarrow extract (2.5, 5, 10, 20, and 40 µg/mL) and curcumin (6.25, 12.5, 25, 50, and 75 µg/mL) were then added to each well. Three replicates were used for each concentration, with five wells each designated as control and

blank groups. The plates were incubated for 24 and 48 hours. Optical density (OD) was measured at 630 nm using an ELISA reader (ELx808, BioTek, USA).

RNA extraction and qRT-PCR

Total RNA was isolated from cells using an extraction kit (Pars Tous, Tehran, Iran) according to the manufacturer's protocol. Purity and concentration were assessed using a spectrophotometer (NanoDrop One, Thermo Fisher Scientific, USA) at 260, 280, and 230 nm. Acceptance criteria were A_{260}/A_{280} ratios between 1.8 and 2.0, and A_{260}/A_{230} ratios between 2.0 and 2.2. All samples met these criteria. Specific primers for *DLEC1*, *MCC*, *BCL2*, and *NANOG* were designed using the Primer-BLAST database. Primer sequences, amplicon lengths, and annealing temperatures (Tm) for each gene, along with the reference gene *β-actin*, are provided in Table 1. All primers were synthesized by Sina Colon Company (Tehran, Iran).

Table 1. Nucleotide sequences of forward and reverse primers properties.

Gene	Forward primer oligomers (5'→3')	Reverse primer oligomers (5'→3')	Amplicon length (bp)	Tm (°C)
DLEC1	GGAAGGAGTGCTTCTTCAGC	CTGCTGAGGTAGGTGATGGT	136	60
MCC	TGTCCAGCCTTTGATGACCT	AGGCTGTAGGTGAAGTCCAG	142	60
BCL2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC	127	60
NANOG	ATGCCTCACACGGAGACTGT	AAGTGGGTTGTTTGCCTTTG	148	60
β-actin	GTGCTATCCCTGTACGCCTC	CGGACTCGTCATACTCCTGC	107	60

Primer validation: Primer efficiency was confirmed by standard curve analysis using serial 1:10 dilutions of cDNA, with acceptable efficiency ranging from 95-105%. Additionally, a single peak in melting curve analysis confirmed specific amplification for each primer pair.

The real-time PCR program consisted of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 300 seconds. All reactions were performed in triplicate.

The relative gene expression changes were calculated using the $\Delta\Delta C_t$ method, with *β-actin* serving as the reference gene: $\Delta C_t = C_t$ (target gene) - C_t (reference gene), and $\Delta\Delta C_t = \Delta C_t$ (treated sample) - ΔC_t (control sample).

Data analysis

The results were expressed as mean ± standard deviation (Mean ± SD). Each experiment was performed in three independent replicates (n=3). Statistical analyses were conducted using GraphPad Prism software (version 10.6) and Microsoft Excel 2016. Prior to performing one-way ANOVA, the assumptions of normality and homogeneity of variances were verified using Shapiro-Wilk and Levene's tests, respectively, confirming that these assumptions were met. Comparisons between groups were performed using one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. The significance level was set at $P<0.05$, and 95% confidence intervals were calculated for all comparisons.

Results

Cell viability

LS180 cells were treated with increasing concentrations of yarrow extract (2.5, 5, 10, 20, and 40 µg/mL) for 24 and 48 hours. As shown in Figure 1, yarrow extract significantly reduced cell viability in a dose- and time-dependent manner, with the most pronounced effects observed at higher concentrations and longer exposure times.

Treatment with curcumin (6.25, 12.5, 25, 50, and 75 µg/mL) resulted in a marked, dose- and time-dependent reduction in LS180 cell viability (Fig. 2). The inhibitory effect of curcumin was consistently stronger than that of yarrow extract across all tested concentrations and time points.

To evaluate potential synergistic effects, LS180 cells were co-treated with combinations of yarrow extract and curcumin (2.5 and 6.25, 5 and 12.5, 10 and 25, 20 and 50, and 40 and 75 µg/mL) for 24 and 48 hours.

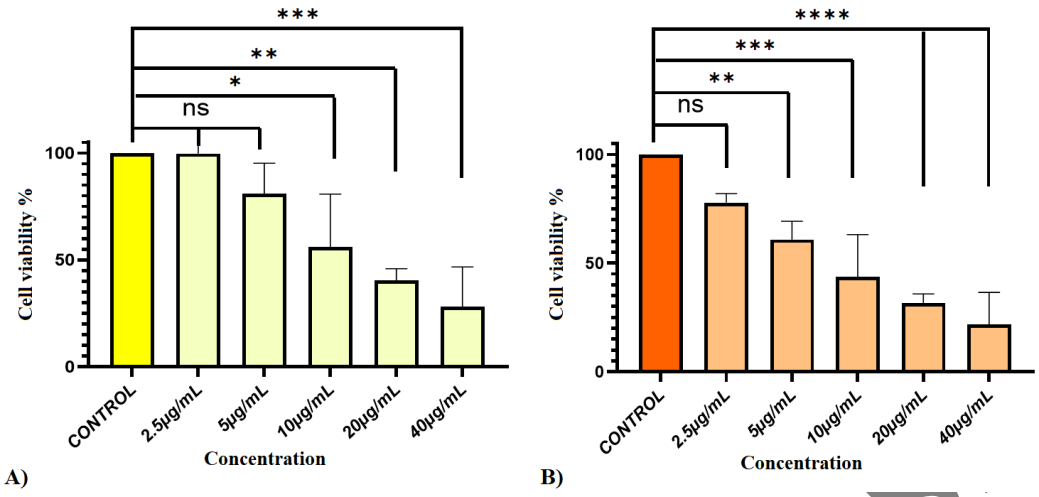

Fig. 1. Effect of yarrow extract on *LS180* cell viability: A) Cells were treated with various concentrations (2.5, 5, 10, 20, and 40 µg/mL) for 24 hours; B) Cells were treated with the same concentrations for 48 hours. Data are presented as mean ± SD (n = 3). Significant differences compared to the control group are indicated (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

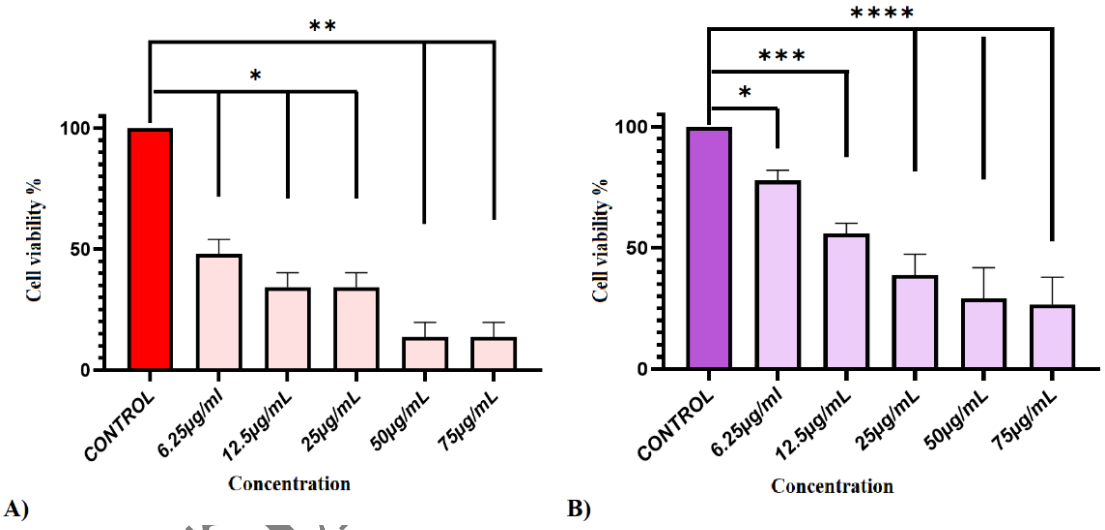

Fig. 2. Effect of curcumin on *LS180* cell viability: A) Cells were treated with various concentrations (2.5, 5, 10, 20, and 40 µg/mL) for 24 hours; B) Cells were treated with the same concentrations for 48 hours. Data are presented as mean ± SD (n = 3). Significant differences compared to the control group are indicated (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

The combined treatment exhibited significantly greater inhibition of cell proliferation compared to either agent alone (Fig. 3), indicating a strong synergistic interaction. This effect was particularly evident at higher concentrations and after 48 hours of treatment, suggesting that the combination not only enhances cytotoxicity but also accelerates the onset of cell death.

Expression analysis

To gain mechanistic insight into the observed synergistic cytotoxicity, we examined the expression of four genes involved in colorectal cancer pathogenesis: the tumor suppressors *DLEC1* and *MCC*, and the oncogenes *BCL2* and *NANOG* (Fig. 4). Notably, the combination of yarrow extract and curcumin produced the most robust upregulation of both *DLEC1* (22.29- fold) and *MCC* (20.96- fold), far exceeding the effects of either compound alone. This pronounced activation of tumor suppressor pathways suggests that the combination synergistically restores key growth-inhibitory mechanisms in LS180 cells. In contrast, curcumin alone was the most effective

single agent in suppressing the oncogenes *BCL2* and *NANOG*, reducing their expression to 0.069-fold (equivalent to a 93.12% decrease) and 0.160-fold (equivalent to an 84.01% decrease), respectively. The combined treatment also led to substantial reductions in *BCL2* (0.182-fold; 81.83% decrease) and NANOG (0.292-fold; 70.83% decrease), while simultaneously achieving the strongest upregulation of tumor suppressors. Collectively, these findings indicate that the combination of yarrow extract and curcumin exerts a dual, synergistic effect: it potently inhibits oncogene expression while strongly reactivating tumor suppressor genes, thereby establishing a more balanced molecular profile that may underlie its superior anticancer activity.

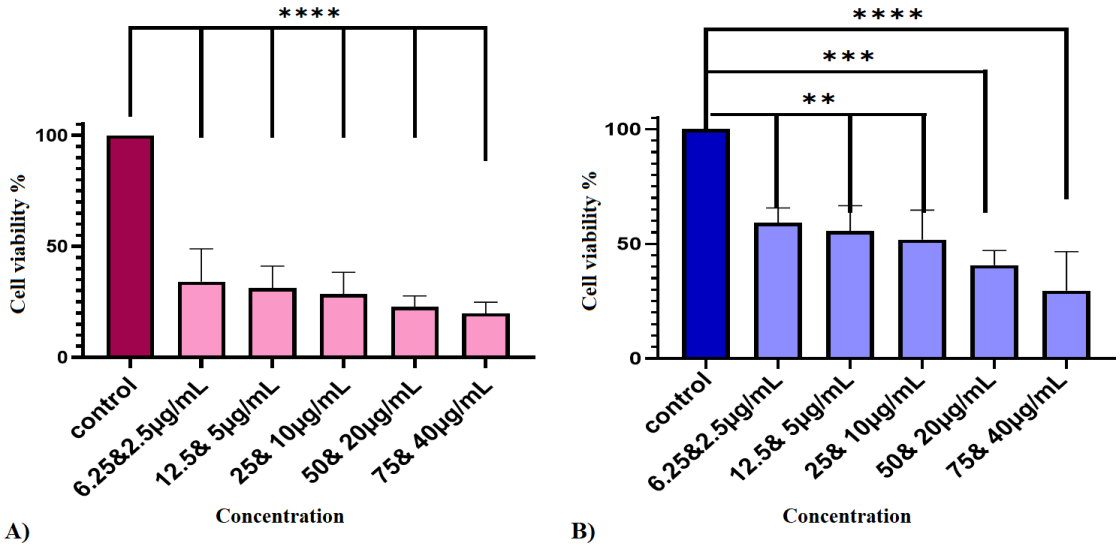

Fig. 3. Effect of *yarrow-curcumin* combination on *LS180* cell viability: A) Cells were treated with various concentrations (2.5, 5, 10, 20, and 40 µg/mL) for 24 hours; B) Cells were treated with the same concentrations for 48 hours. Data are presented as mean ± SD (n= 3). Significant differences compared to the control group are indicated (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

Discussion

In this study, we found that combining yarrow extract with curcumin synergistically inhibits the growth of LS180 colorectal cancer cells and significantly alters the expression of genes involved in both tumor suppression and oncogenesis. These findings offer mechanistic insight into how this natural compound combination may interfere with colorectal cancer progression at the transcriptional level.

Our results show that the yarrow-curcumin combination markedly upregulated the tumor suppressor genes *DLEC1* and *MCC*, with fold increases of 22.29 and 20.96, respectively, far exceeding the effects of either compound alone. This is particularly noteworthy because *DLEC1* is frequently silenced through promoter hypermethylation in multiple cancers, as demonstrated by Ying *et al.* (2009), who showed that DLEC1 is frequently silenced by promoter methylation in colorectal and gastric cancers, and its ectopic expression significantly inhibits tumor cell clonogenicity. Similarly, *MCC* is known to suppress tumor growth by inhibiting β-catenin signaling; Pangon *et al.* (2014) demonstrated that MCC inhibits β-catenin transcriptional activity by sequestering DBC1 in the cytoplasm, thereby modulating Wnt/β-catenin signaling. The strong reactivation of these genes by the combined treatment suggests that yarrow and curcumin may work together to reverse epigenetic silencing and restore key growth inhibitory pathways.

Regarding the molecular mechanism underlying this synergy, we propose a complementary epigenetic model. Curcumin is a well-established epigenetic modulator that inhibits DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), thereby reversing promoter hypermethylation and restoring the expression of silenced tumor suppressor genes

(Amaroli *et al.*, 2024). However, DNMT inhibition alone is often insufficient for complete gene reactivation, as repressive chromatin marks such as H3K27me3, mediated by polycomb repressive complexes (PRCs), can maintain silencing even after DNA demethylation. Yarrow extract contains bioactive flavonoids, including apigenin and luteolin, which have been shown to modulate epigenetic enzymes and enhance tumor suppressor expression through chromatin remodeling (Far *et al.*, 2023).

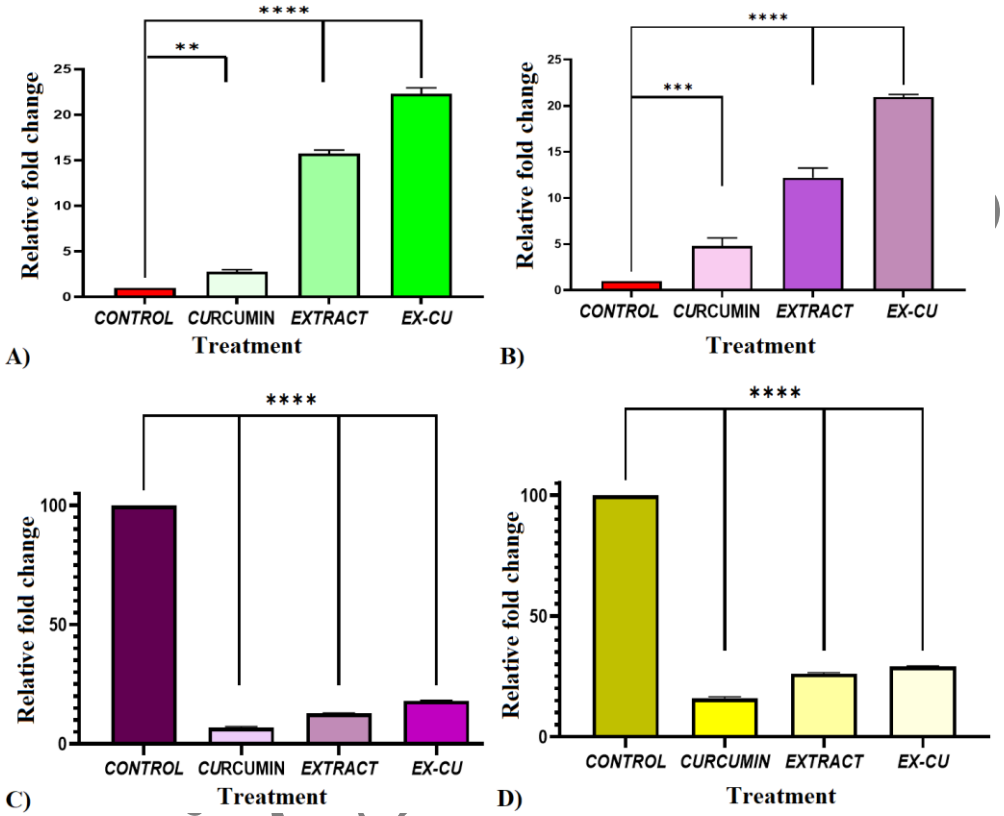

Fig. 4. Relative expression of *DLEC1*, *MCC*, *BCL2*, and *NANOG* genes normalized to *β-actin* in LS180 cells treated with curcumin, yarrow extract, and their combination: A) Relative expression of *DLEC1*; B) Relative expression of *MCC*; C) Relative expression of *BCL2*; D) Relative expression of *NANOG*. Data are presented as mean ± SD (n = 3). Significant differences compared to the control group are indicated (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

Specifically, Kanwal *et al.* (2017) demonstrated that luteolin selectively inhibits EZH2, the catalytic subunit of PRC2, thereby reducing H3K27me3 levels and facilitating chromatin accessibility at silenced gene promoters. Additionally, Kanwal *et al.* (2016) showed that dietary flavones including apigenin, chrysin, and luteolin act as dual inhibitors of DNA methyltransferases and histone methyltransferases by binding to the catalytic sites of DNMT and EZH2 enzymes. This dual inhibition of both DNA methylation and histone methylation marks likely underlies the robust and coordinated reactivation of *DLEC1* and *MCC* observed in the combination group. We propose that curcumin and yarrow extract act synergistically by targeting complementary and sequential epigenetic barriers: curcumin alleviates DNA methylation through DNMT inhibition, while yarrow flavonoids concurrently relieve histone-mediated repression through PRC2 interference and chromatin remodeling. This model is consistent with emerging evidence that successful tumor suppressor reactivation often requires simultaneous targeting of multiple epigenetic modifications (Burgman *et al.*, 2024), and our findings extend this knowledge by demonstrating that a natural compound combination can achieve such dual modulation.

In contrast, the oncogenes *BCL2* and *NANOG* were most effectively downregulated by curcumin alone, with expression dropping to 0.069-fold and 0.160-fold, respectively. *BCL2* is a well-known anti-apoptotic oncogene that enhances cancer cell survival (Garimberti *et al.*, 2024), while *NANOG*, though primarily a pluripotency factor, has documented oncogenic potential and is linked to cancer stem cell maintenance and poorer outcomes in colorectal cancer (Jeter *et al.*, 2015; Zhang *et al.*, 2021). The potent suppression of these oncogenes by curcumin underscores its role as a strong pro-apoptotic and anti-stemness agent, consistent with prior studies showing curcumin downregulates *BCL2* and *NANOG* across various cancer models. Shehzad *et al.* (2013) and Farooqui (2013) have shown that curcumin inhibits *BCL2* transcription through modulation of NF-κB and STAT3 pathways, while also suppressing NANOG through inhibition of the IL-6/STAT3 axis and Wnt/β-catenin signaling.

Addressing the apparent discrepancy in our findings, we acknowledge that while the combination treatment exhibited the strongest upregulation of tumor suppressor genes, it was slightly less effective than curcumin alone in suppressing the oncogenes *BCL2* and *NANOG*. This observation, however, is not contradictory when interpreted mechanistically. Curcumin is a potent direct inhibitor of *BCL2* transcription through its modulation of the NF-κB and STAT3 pathways (Shehzad *et al.*, 2013; Farooqui and Farooqui, 2013), and it effectively suppresses *NANOG* through inhibition of the IL-6/STAT3 axis and Wnt/β-catenin signaling (Deng *et al.*, 2021). Yarrow extract, while possessing anti-inflammatory and epigenetic-modulating properties, does not exert as direct or potent an effect on these specific oncogenic transcriptional pathways. When combined, the beneficial effect of yarrow on tumor suppressor reactivation is fully realized, but it does not enhance and may slightly dilute the potent direct oncogene-suppressive effect of curcumin. Therefore, the combination achieves a more balanced molecular profile: strongly activating tumor suppressors while still substantially inhibiting oncogenes, rather than maximizing oncogene suppression alone. This balanced modulation, rather than being a weakness, may offer therapeutic advantages by targeting both arms of the cancer regulatory network simultaneously, potentially reducing the risk of acquired resistance and providing broader antitumor activity.

Although curcumin alone was more potent in suppressing *BCL2* and *NANOG*, the combined treatment still achieved substantial reductions (0.182-fold and 0.292-fold, respectively) while simultaneously producing the strongest upregulation of tumor suppressors. This dual action effective oncogene suppression coupled with robust tumor suppressor reactivation offers a more balanced and potentially safer therapeutic approach than targeting either pathway alone.

Our findings are in line with earlier reports on the synergistic effects of curcumin with other natural compounds. Ahmad *et al.* (2025) found that curcumin combined with plumbagin reduced *BCL2* expression and induced apoptosis in HCT-116 colon cancer cells. Similarly, Ravindranathan *et al.* (2018) showed that curcumin combined with oligomeric proanthocyanidins simultaneously impacted cell cycle and DNA replication pathways in colorectal cancer cells. Aromokeye and Si (2022) also confirmed that curcumin and luteolin together synergistically inhibit colon cancer cell growth and reduce tumor-related signaling proteins (Notch1 and TGF-β). Our study builds on these findings by showing that curcumin with yarrow extract not only suppresses oncogenes but also strongly reactivates tumor suppressor genes through a proposed dual epigenetic mechanism, offering a more comprehensive molecular basis for the observed synergistic cytotoxicity.

Despite these promising findings, we must acknowledge several important limitations. First, all experiments were conducted in vitro using a single colorectal cancer cell line (LS180), and the results may not be generalizable to other cell types or to the complex in vivo tumor microenvironment. Second, and most critically, gene expression was assessed only at the mRNA level using real-time PCR, and we did not perform Western blotting to confirm changes at the protein level. Consequently, we cannot conclude that the observed transcriptional changes translate into functional alterations in protein activity, signaling pathways, or cellular behaviors such as proliferation, migration, or invasion. Third, the cytotoxic effects were evaluated in 2D monolayer

cultures, which do not fully recapitulate the three-dimensional architecture of tumors. Fourth, we have not conducted functional assays (*e.g.*, wound healing, Transwell migration, or invasion assays) to directly assess the impact of the combination on metastatic potential. Therefore, while our findings provide a strong molecular rationale for further investigation, they remain preliminary and should be interpreted with caution.

The synergistic interplay between yarrow extract and curcumin in modulating both tumor suppressor and oncogenic pathways suggests that this combination holds considerable promise for colorectal cancer therapy. However, given the in vitro nature of this study and the absence of protein-level validation, we emphasize that these results should not be interpreted as evidence of therapeutic efficacy or reduced invasion/metastasis. Instead, they provide a mechanistic foundation for future research. Future studies should investigate the underlying mechanisms of this synergy, including direct confirmation of epigenetic modifications (*e.g.*, DNA methylation analysis, ChIP-qPCR for H3K27me3), validation at the protein level via Western blotting, assessment of functional outcomes through migration and invasion assays, and evaluation in appropriate *in vivo* models to determine the translational potential of this combination.

We cautiously suggest that the combination of yarrow extract and curcumin may represent a promising strategy for colorectal cancer therapy, but reiterate that these findings are preliminary and warrant further investigation in appropriate animal models and clinical settings before any clinical recommendations can be made.

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Conflicts of interests

The authors declare no conflict of interest.

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