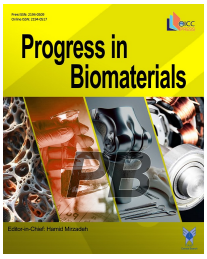

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Biomaterial-Based Delivery of *Ornithogalum brachystachys* Methanolic Extract Enhances Apoptosis in HT-29 Colorectal Cancer Cells via BAX/BCL2 Modulation

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Original Research

Abstract:

This study evaluated methanolic and hexanic extracts of Ornithogalum brachystachys C.Koch for anti-cancer effects on HT-29 colorectal cancer cells, comparing them to 5-fluorouracil (5-FU). Cells were treated with extracts ($25-200$ µg/mL) or 5-FU ($5-100$ µM) for up to 72 hours. Cytotoxicity was assessed by MTT, and apoptosis-related gene expression (BAX and BCL2) by qRT-PCR. The methanolic extract showed the strongest cytotoxicity, reducing viability to 44.9% at 100 µg/mL after 72 hours and increasing BAX expression by 1.48-fold, indicating apoptosis. The hexanic extract had moderate cytotoxicity (63.7% viability at 25 µg/mL, 24 hours) with no significant changes in apoptotic gene expression. 5-FU decreased viability to 51.9% at 5 µM after 72 hours and produced stronger apoptotic signaling (BAX +1.75-fold, BCL2 −0.63-fold). Overall, the methanolic extract demonstrates notable, albeit weaker, pro-apoptotic activity via BAX/BCL2 modulation and may serve as a promising, lower-toxicity complementary natural anticancer candidate warranting further phytochemical and *in vivo* studies.

Keywords:

BAX; BCL2; Colorectal cancer; Hexanic extract; Methanolic extract; *Ornithogalum brachystachys*

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1. Introduction

Colorectal cancer (CRC) is one of the most prevalent and deadly malignancies worldwide, ranking as the third most common cancer and the second leading cause of cancer-related mortality (Hossain et al., 2022; Baidoun et al., 2021; Onyoh et al., 2019). These tumors account for approximately 10% of all cancers in women and 12% in men, with the incidence of early-onset CRC notably increasing among younger populations (Kędzia-Berut et al., 2023; Patel et al., 2022). The global rise in CRC incidence represents a major public health challenge, emphasizing the need for improved prevention and treatment strategies (Zheng and Wang, 2021).

Current therapeutic modalities, including surgery, chemotherapy, and radiotherapy, have significantly improved patient survival. However, these interventions often lead to substantial adverse effects and treatment resistance, limiting their long-term efficacy (Hossain et al., 2022). Surgical resection may result in complications such as infection, bleeding, adhesions, and intestinal dysfunction, particularly in advanced or metastatic cases where complete tumor removal is challenging. Chemotherapy and radiotherapy, while targeting malignant cells, also damage healthy tissues, causing side effects such as nausea, vomiting, hair loss, appetite loss, fatigue, diarrhea, dermatitis, and damage to surrounding organs (Hossain et al., 2022).

Given these limitations, the search for novel and less toxic therapeutic alternatives has become a critical focus in cancer research. Medicinal plants represent a valuable source of bioactive compounds with diverse pharmacological properties, including antioxidant, anti-inflammatory, and antitumor effects. These natural agents can modulate multiple cellular pathways involved in tumor initiation and progression, often exhibiting fewer adverse effects compared to conventional chemotherapeutics (Khoogar

et al., 2016; Pyo and Kwon, 2024; Siddiqui et al., 2022).

The genus *Ornithogalum* (Asparagaceae) has attracted pharmacological interest because several species produce bioactive steroidal saponins, cardiac glycoside-like molecules and other secondary metabolites with cytotoxic or antiproliferative properties (Zhan et al., 2021; Ejder et al., 2024). A paradigmatic example is OSW-1, a potent saponin-type natural product originally isolated from *Ornithogalum* saundersiae, which exerts strong anticancer effects in multiple cancer models and has stimulated mechanistic research into *Ornithogalum*-derived compounds (Zhan et al., 2021). More generally, recent phytochemical surveys and biological screens across *Ornithogalum* species have reported antioxidant, antiproliferative and pro-apoptotic activities in vitro, with several studies showing extract-induced DNA fragmentation, caspase activation and changes in apoptosis-related proteins (Ejder et al. (2024); Koyuncu et al., cited in antiproliferative studies). These data indicate *Ornithogalum* species are credible sources of anticancer leads and justify evaluating species-specific extracts for effects on apoptosis markers such as BCL-2 and BAX.

Ornithogalum brachystachys itself is less well represented in the pharmacological literature than some congeners; taxonomically it is closely related to other *Ornithogalum*/*Loncomelos* taxa (IPNI; POWO). Because species in the genus frequently share classes of secondary metabolites (steroidal saponins, glycosides, flavonoids), it is scientifically justifiable to explore *O. brachystachys* extracts for cytotoxic and pro-apoptotic activity while noting that species-level phytochemical profiling is required to identify the active constituents (IPNI/POWO, 2024; Ejder et al., 2024).

Different extraction solvents selectively recover different classes of compounds and therefore can yield extracts with distinct biological activities. Polar solvents such as methanol efficiently extract phenolics, glycosides, flavonoids, saponins and other polar constituents that frequently mediate pro-oxidant or pro-apoptotic effects in cancer cells; nonpolar solvents such as hexane preferentially extract lipophilic constituents (oils, terpenes, nonpolar aglycones) that may possess different modes of cytotoxicity (Lee et al., 2024; Baptista-Silva et al., 2020). Comparative studies using polar and nonpolar extracts often reveal solvent-dependent differences in cytotoxic potency and mechanisms (e.g., ROS generation, membrane perturbation, or mitochondrial targeting). Therefore, parallel testing of methanolic and hexane extracts of *O. brachystachys* can reveal whether polar or nonpolar constituents are primarily responsible for modulating BCL-2/BAX and triggering apoptosis in HT-29 cells (Lee et al., 2024; Queffelec et al., 2024). Finally, prior in-vitro work on *Ornithogalum* spp. demonstrates that anticancer activity may operate through mitochondrial pathways and caspase activation-the same pathways in which BCL-2/BAX participate—supporting the rationale to measure these specific genes in HT-29 cells (Zhan et al., 2021; Ejder et al., 2024). Taken together, existing literature supports (1) the biological importance of the BCL-2/BAX balance in CRC, (2) the suitability of HT-29 cells for apoptosis and gene-expression assays, and (3) the plausibility that *Ornithogalum* extracts contain constituents capable of altering apoptosis regulators. This body of work therefore justifies the present study's focus on comparing methanolic and hexane extracts of *O. brachystachys* for their effects on BCL-2 and BAX expression in HT-29 colon cancer cells.

2. Materials and methods

Plant material and extract preparation

The plant material of *Ornithogalum brachystachys* was collected and taxonomically identified to ensure species authenticity. The specimen was collected by Mahdi Abbas Mohammadi in June 2023 from the Mishow Mountains, Shabestar, Iran (38°19′33″ N, 45°37′14″ E). The voucher specimen was deposited under accession number 95765 in HSHU. The plant material was shade-dried at room temperature, finely powdered (200 g), and macerated in 500 mL of methanol or hexane for 72 hours with intermittent stirring. The mixtures were filtered through Whatman No. 1 paper, and the filtrates were concentrated using a rotary evaporator at 50 °C under reduced pressure. The extracts were then air-dried to constant weight, yielding 0.36 g for the methanolic extract and 0.08 g for the hexanic extract.

Maceration was selected as the extraction method due to its simplicity, cost-effectiveness, and effectiveness in recovering a broad range of secondary metabolites from plant material, as demonstrated in previous studies on *Ornithogalum* species (e.g., Zhan et al. (2021); Lee et al. (2024)). Methanol was chosen as a polar solvent to extract phenolic compounds, flavonoids, and saponins, which are known for their anticancer properties (Baptista-Silva et al., 2020; Nkwocha et al., 2024). Hexane was used as a nonpolar solvent to isolate lipophilic compounds such as terpenes and oils, allowing comparison of solvent-dependent bioactivities (Lee et al., 2024; Queffelec et al., 2024).

Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL and subsequently diluted in Dulbecco's Modified Eagle Medium (DMEM) to final concentrations of 25, 50, 100, and 200 µg/mL for treatments. Untreated cells cultured in DMEM served as the negative control, In addition, 5 Fluorouracil (5 FU, 5 µM) was used as the positive control to validate the cytotoxicity assay.

Cell culture and treatment

HT 29 human colorectal adenocarcinoma cells were obtained from the Iranian Biological Resource Center (IBRC, Tehran, Iran), under accession number IBRC C10097. The authenticity of the cell line was confirmed by IBRC through standard cell authentication procedures, including morphological and contamination checks.

Cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

For cytotoxicity assays, cells were seeded at a density of 7,000 cells/well in 96-well plates. For gene expression analysis, 170,000 cells were seeded in 3 cm culture dishes. After

allowing 24–48 hours for cell attachment, the cells were treated with methanolic or hexanic extracts at concentrations of 25–200 µg/mL, or with 5-fluorouracil (5-FU) at 5–100 µM, for 24, 48, or 72 hours. Control groups received DMEM containing 0.5% DMSO.
Based on MTT assay results, three representative treatment conditions were selected for gene expression analysis: Hexanic extract 25 µg/mL for 24 hours (H25), methanolic extract 100 µg/mL for 72 hours (M100), and 5-FU 5 µM for 72 hours (FU5).

Cell viability assay (MTT assay)

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Following treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37 °C. Formazan crystals formed by metabolically active cells were dissolved in 100 µL of DMSO, and absorbance was measured at 570 nm using a BioTek ELx808 microplate reader. Cell viability (%) was calculated as:

$$\text{Viability} = \left(\frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}}\right) \times 100$$

All experiments were conducted in triplicate and repeated independently three times.

RNA extraction and qRT-PCR

Total RNA was extracted from treated and control HT-29 cells using the Pars Tous RNA extraction kit (Iran), following the manufacturer's protocol. RNA purity and concentration were verified using a NanoDrop spectrophotometer (A260/A280 $\approx$ 1.8). Complementary DNA (cDNA) was synthesized from 1 µg total RNA using random hexamer and oligo(dT) primers.
Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix on a thermal cycler. The expression levels of pro-apoptotic (BAX) and anti-apoptotic (BCL2) genes were quantified, with GAPDH serving as the internal control. Primer sequences are listed in Table 1.
PCR amplification conditions were: Initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 59-60 °C for 40 s, and 72 °C for 20 s. Relative gene expression was determined using the 2^ΔΔCt method and normalized to GAPDH expression.

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD). MTT results were analyzed using one-way ANOVA followed by Dunnett's post hoc test (SPSS v27). Gene expression differences between treated and control groups were evaluated using independent *t*-tests. Statistical significance was considered at $p < 0.05$. Graphs of cell viability and fold changes in gene expression were generated using Microsoft Excel 2019.

3. Results

MTT assay-cell viability

Hexanic extract: At 24 hours, H25 (25 µg/mL) reduced cell viability to 63.7% relative to untreated cells ($p < 0.05$), while higher doses (H200, 200 µg/mL) increased viability to 122.18%, suggesting a hormetic response (Calabrese et al., 2024). At 48 hours, H50 increased viability to 124.09%, further supporting transient stimulatory effects at moderate doses. These values were not significantly different from the DMSO negative control, indicating limited cytotoxicity of the hexanic fraction.
Methanolic extract: M100 (100 µg/mL) at 24 and 48 hours had minimal effects (e.g., M50: 98.99%, M200: 112.77%). However, after 72 hours, M200 significantly reduced viability to 20.62% compared to both untreated and DMSO controls ($p < 0.05$), indicating strong cytotoxicity likely due to polar bioactive compounds such as flavonoids and phenolics (Niero and Machado-Santelli, 2013; Zhu et al., 2016; Sharma et al., 2018; Singh et al., 2018).
Positive control (5 FU): FU5 (5 µM) decreased cell viability to 51.91% after 72 hours ($p < 0.05$), confirming its potent pro apoptotic effect and validating the assay conditions (Mhaidat et al., 2014; Riahi-Chebbi et al., 2019; De et al., 2023).
Overall, these results demonstrate that the methanolic extract exerts stronger and more consistent cytotoxic effects over prolonged exposure compared to the hexanic extract, which showed transient increases in viability at lower doses consistent with a hormetic pattern. Importantly, the cytotoxicity of the methanolic extract after 72 hours was more pronounced than that of 5 FU under the tested conditions, highlighting its potential as a source of anticancer compounds. These findings are in agreement with previous studies reporting enhanced cytotoxicity of methanolic fractions rich in phenolics and flavonoids against colorectal cancer cells, while non polar extracts often exhibit weaker or biphasic responses (Calabrese et al., 2024; Uğur et al., 2017; Jodynis-Liebert and Kujawska, 2020) (figures 1-3).

qRT-PCR analysis – apoptosis-related gene expression

Apoptosis induction was evaluated by assessing BAX (pro-apoptotic) and BCL2 (anti-apoptotic) mRNA levels (figure 4-7).

- Methanolic extract (M100, 72 h): BAX expression in-

Table 1. Primer sequences for BAX, BCL2, and GAPDH.

Gene	Forward Primer	Reverse Primer
BAX	CGAACTGGACAGTAACATGG	CAGTTTGCTGGCAAAGTAGA
BCL2	GATTGTGGCCTTCTTTGAGT	ATAGGCACCCAGGGTGAT
GAPDH	GAAGGTGAAGGTCGGAGTCA	GAGATGGTGATGGGATTTC

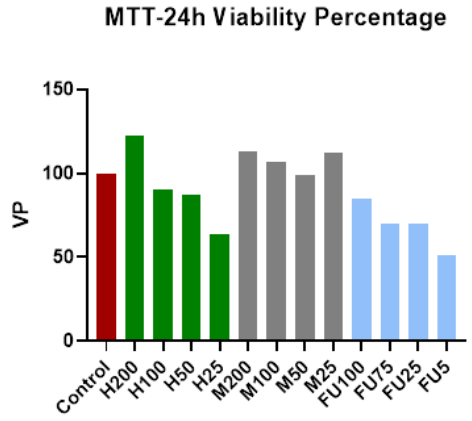

Figure 1. Effect of methanolic (M) and hexanic (H) extracts of *Ornithogalum brachystachys* and 5-fluorouracil (5-FU) on HT-29 cell viability percentage after 24 hours (MTT assay). Vertical axis: Cell viability percentage (%). Horizontal axis: Treatment concentration (µg/mL for extracts, µM for 5-FU).

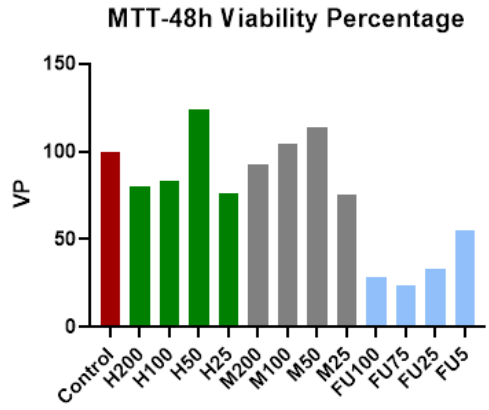

Figure 2. Effect of methanolic (M) and hexanic (H) extracts of *Ornithogalum brachystachys* and 5-fluorouracil (5-FU) on HT-29 cell viability percentage after 48 hours (MTT assay). Vertical axis: Cell viability percentage (%). Horizontal axis: Treatment concentration (µg/mL for extracts, µM for 5-FU).

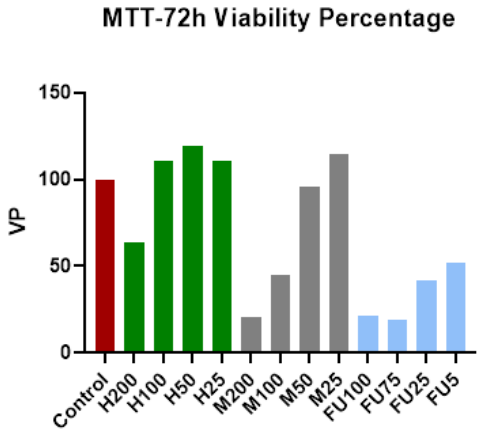

Figure 3. Effect of methanolic (M) and hexanic (H) extracts of *Ornithogalum brachystachys* and 5-fluorouracil (5-FU) on HT-29 cell viability percentage after 72 hours (MTT assay). Vertical axis: Cell viability percentage (%). Horizontal axis: Treatment concentration (µg/mL for extracts, µM for 5-FU).

creased 1.48-fold ($p = 0.032$), while BCL2 decreased slightly (7%, $p = 0.57$), indicating apoptosis induction via mitochondrial pathway (31-39).

- Hexanic extract (H25, 24 h): BAX increased 1.29-fold ($p = 0.136$) and BCL2 1.05-fold ($p = 0.791$), showing minimal impact on apoptotic signaling (30).
- 5-FU (FU5, 72 h): Significantly increased BAX by 1.75-fold ($p = 0.02$) and decreased BCL2 by 0.63-fold ($p = 0.045$), confirming its strong pro-apoptotic effect (40-42).

These findings indicate that the methanolic extract primarily enhances pro-apoptotic signaling through BAX upregulation, whereas the hexanic extract shows negligible effects. 5-FU robustly modulates both BAX and BCL2, consistent with its known mechanism.

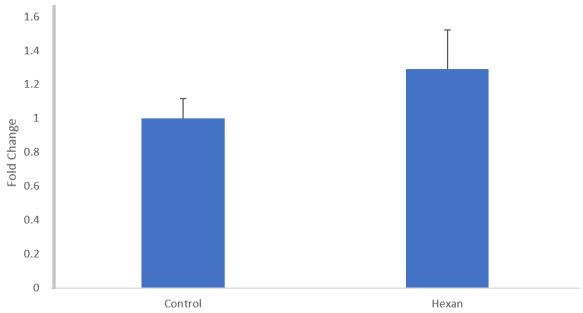

Figure 4. Relative fold-change in BAX gene expression in HT-29 cells treated with hexanic extract of *Ornithogalum brachystachys* (25 µg/mL) for 24 hours.

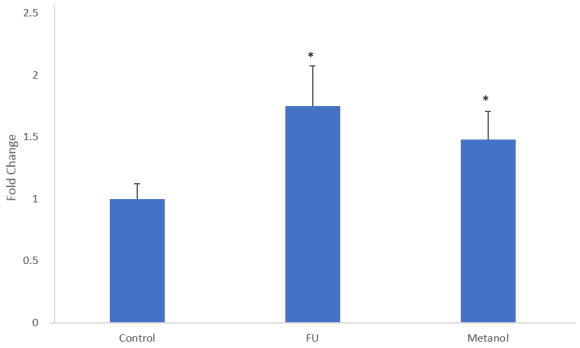

Figure 5. Relative fold-change in BAX gene expression in HT-29 cells treated with methanolic extract (100 µg/mL) and 5-fluorouracil (5-FU) (5 µM) for 72 hours.

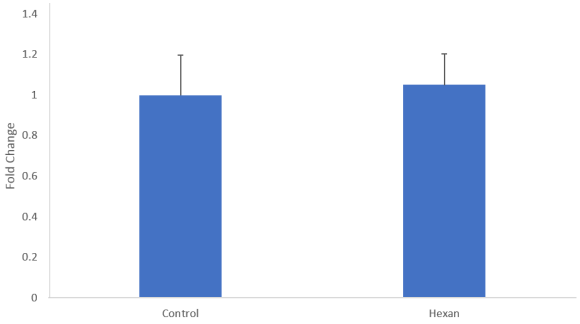

Figure 6. Relative fold-change in BCL2 gene expression in HT-29 cells treated with hexanic extract (25 µg/mL) for 24 hours.

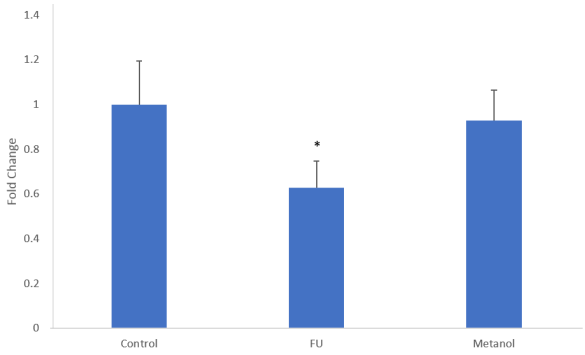

Figure 7. Relative fold-change in BCL2 gene expression in HT-29 cells treated with methanolic extract (100 µg/mL) and 5-FU (5 µM) for 72 hours.

4. Discussion

The present study demonstrates that methanolic and hexanic extracts of *Ornithogalum brachystachys* exert time- and dose-dependent effects on HT-29 colorectal cancer cells, producing complex patterns of cell viability reduction and transient increases consistent with hormesis (Calabrese et al., 2024; Uğur et al., 2017; Jodynis-Liebert and Kujawska, 2020).

The methanolic extract showed significant cytotoxicity. This extract also upregulated BAX expression while slightly decreasing BCL2, indicating activation of the mitochondrial apoptotic pathway. Its stronger effect is likely due to the higher polarity of methanol, which efficiently extracts bioactive compounds such as flavonoids and phenolic acids (Neamtu et al., 2024; Nkwocha et al., 2024). Compounds such as cosmosiin have been reported to induce apoptosis via ROS generation and PD-L1 inhibition (Han et al., 2023), while cinnamic and p-coumaric acids trigger apoptosis through DNA damage and cytoskeletal disruption (Niero and Machado-Santelli, 2013; Zhu et al., 2016). Other phenolic acids like quinic acid modulate the UPR pathway and Grp78 expression, promoting apoptosis in CRC cells (Sharma et al., 2018; Tehami et al., 2023), and kinic acid synergizes with other phenolics to inhibit Akt signaling and cyclin D1 expression (Singh et al., 2018; Neamtu et al., 2024).

In contrast, the hexanic extract showed lower cytotoxicity, with some doses inducing temporary increases in viability. This hormetic response likely reflects early cellular adaptation mechanisms, such as activation of antioxidant enzymes and metabolic pathways, before cytotoxic effects dominate (Calabrese et al., 2024). The limited efficacy of hexanic extract is consistent with its extraction of non-polar compounds, which are generally less potent in inducing apoptosis compared to polar phenolics (Lee et al., 2024; Nkwocha et al., 2024).

The standard chemotherapeutic 5-FU consistently reduced cell viability and modulated apoptotic genes, increasing BAX and decreasing BCL2. This confirms the well-characterized mechanism of 5-FU in DNA synthesis inhibition and apoptosis induction (**u16**; Mhaidat et al., 2014; Riahi-Chebbi et al., 2019). Comparatively, the methanolic extract showed a multi-targeted, albeit milder, effect, suggesting potential for natural, complementary anticancer therapy. Previous studies have also reported synergistic effects of phenolic compounds with 5-FU, enhancing apoptosis while reducing side effects (Riahi-Chebbi et al., 2019; De et al., 2023).

The observed time-dependent cytotoxicity highlights that some extracts, especially methanolic, require prolonged exposure to exert maximal effect. This aligns with prior studies showing delayed but robust apoptosis induction by plant-derived phenolics in CRC cells (Niero and Machado-Santelli, 2013; Zhu et al., 2016; Sharma et al., 2018; Singh et al., 2018; Han et al., 2023; Tehami et al., 2023; Neamtu et al., 2024; Uğur et al., 2017; Jodynis-Liebert and Kujawska, 2020). The upregulation of BAX without significant BCL2 downregulation suggests that methanolic extract primarily activates pro-apoptotic signaling, whereas 5-FU affects both pro- and anti-apoptotic pathways.

5. Conclusion

This study demonstrated that methanolic and hexanic extracts of *Ornithogalum brachystachys* exert time- and dose-dependent cytotoxic effects on HT-29 colorectal cancer cells. The methanolic extract significantly reduced cell viability and upregulated BAX expression, indicating pro-apoptotic activity via the mitochondrial pathway. In contrast, the hexanic extract showed limited efficacy and temporary hormetic effects, likely due to its extraction of non-polar compounds. Compared to the standard chemotherapeutic 5-fluorouracil (5-FU), which consistently reduced cell viability and modulated both BAX and BCL2, the methanolic extract demonstrated promising cytotoxic and multi-targeted anticancer potential, albeit with lower potency. These findings suggest that the methanolic extract of *O. brachystachys* could serve as a natural complementary therapeutic agent for colorectal cancer. Future research should focus on detailed phytochemical characterization, exploration of additional apoptotic and cell death pathways, and in vivo validation to fully assess the therapeutic potential of these extracts.

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Ethical approval

This study was conducted using the established human colorectal adenocarcinoma cell line HT-29. Although no human participants or animal subjects were directly involved, the research protocol was reviewed and approved by the Ethics Committee of the Islamic Azad University, Qom Branch (Ethics Code: IR.IAU.QOM.REC.1404.065). All experimental procedures were performed in accordance with institutional ethical guidelines and standards for research involving established human cell lines.

Authors contributions
Mahdi Il Saadatmand: Writing – original draft. Maryam Khoshsokhan-Mozaffar: Writing, visualization, supervision, project administration, methodology, investigation. Farah Farahani: Methodology, preparing the plant extract.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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