

Tolectin-Induced Apoptosis in Human Gastric Adenocarcinoma and Cervical Cancer Cells Through Caspase-Dependent Pathways

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Abstract

The cytotoxic effects of Tolectin on cancer cells have been reported, but its underlying mechanism remains unclear. This study aimed to evaluate the apoptotic effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. AGS and HeLa cells were treated with various concentrations of Tolectin, while untreated cells served as controls. Cell viability was assessed using MTT assay, and the half-maximal inhibitory concentration (IC₅₀) was determined. Expression levels of Bcl-2, BAX, and P53 genes were measured by quantitative RT-PCR. Caspase-3, -8, and -9 activities were evaluated using colorimetric assays. Apoptosis was confirmed by flow cytometry. Data were analyzed using ANOVA and Student's t-test. Tolectin treatment significantly decreased cell viability in both AGS and HeLa cells. The BAX/Bcl-2 ratio increased to 1.557 in AGS and 1.385 in HeLa cells at IC₅₀ concentrations. No significant changes in P53 expression were observed. Caspase-3, -8, and -9 activities were significantly elevated in treated cells compared to controls ($p < 0.05$). Flow cytometry confirmed apoptosis induction in both cell lines. Tolectin exerts cytotoxic effects on AGS and HeLa cells through apoptosis, involving both intrinsic and extrinsic pathways.

Keywords: Gastric cancer, Cervical cancer, Apoptosis, Tolectin, AGS cell line, HeLa cell line

1. Introduction

Tolectin® is one of the most important non-steroidal anti-inflammatory drugs (NSAIDs), commonly used to reduce pain, swelling, tenderness, and stiffness in conditions such as osteoarthritis and rheumatoid arthritis (Park *et al.*, 2023). Tolectin, similar to other NSAIDs, may exert anticancer effects through caspase-dependent pathways, yet its effects on gastric and cervical cancer cells remain unexplored. Although its mechanism of action is not fully understood, Tolectin reduces cyclooxygenase activity and, consequently, the production of prostaglandins (Gao W *et al.*, 2024). Beyond their anti-inflammatory effects, NSAIDs have shown significant potential in cancer prevention, including cancers of the breast, uterus, and gastrointestinal tract (Chapelle *et al.*, 2020; Meyerhardt *et al.*, 2021; Bakrim *et al.*, 2025).

Gastric cancer, one of the most common gastrointestinal malignancies, exhibits a higher mortality rate in men compared to women (Aalipour *et al.*, 2022; Thrift *et al.*, 2023). Infection with *Helicobacter pylori* has been implicated in over 60% of gastric cancer cases. Cervical cancer, originating in the cervix, is primarily classified as either epidermoid carcinoma or adenocarcinoma and is the third most common cancer in women worldwide, often associated with human papillomavirus (HPV) infection (Arbyn *et al.*, 2020).

Treatment strategies for gastric and cervical cancers include radical surgery, chemotherapy, immunotherapy, and targeted therapy (Kumar *et al.*, 2024; Ren *et al.*, 2025). Recent studies have explored the anticancer potential of NSAIDs, demonstrating their ability to inhibit cell proliferation and induce apoptosis in various cancer types (Li J *et al.*, 2023; Robledo-Cadena DX 2024). Specifically, NSAIDs such as diclofenac have exhibited anti-proliferative effects on colorectal, ovarian, and esophageal cancer cells (Wang *et al.*, 2024; Tabaku *et al.*, 2023; Fletcher *et al.*, 2021). *In vitro* studies have also highlighted the role of NSAIDs in apoptosis induction through activation of caspase pathways, including Caspase-3, Caspase-8, and Caspase-9, as well as modulation of anti-apoptotic (*BCL2*) and pro-apoptotic (*BAX*) gene expression, and upregulation of the tumor suppressor gene *TP53* (Zhang *et al.*, 2020; Azarshab *et al.*, 2021; Wong, 2019; Nagy *et al.*, 2021). Nevertheless, contradictory findings exist, as some studies report that NSAIDs may not induce apoptosis in certain cancer cells or could even promote proliferation (Kazberuk *et al.*, 2020). Therefore, the present study was designed to comprehensively investigate the cytotoxic and apoptosis-inducing effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. Specifically, this study focused on elucidating the involvement of caspase-dependent pathways and examining the regulation of critical apoptotic genes, including *TP53*, *BAX*, and *BCL2*. Although numerous studies have demonstrated the

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anticancer potential of various non-steroidal anti-inflammatory drugs (NSAIDs) in different cancer models, the specific effects of Tolectin on gastric and cervical cancer cells remain largely unexplored. Addressing this gap, the current research aims not only to evaluate Tolectin-induced cytotoxicity and apoptotic responses but also to provide mechanistic insights into its modulation of key molecular regulators of programmed cell death, thereby contributing to the understanding of its potential as a novel therapeutic agent for gastrointestinal and gynecological malignancies.

2. Materials and Methods

2.1. Ethics Statement

This study was reviewed and approved by the International Association of Scientists (IAS) Ethics Committee (Ref: IASSP.22.1CL), and all experimental procedures were conducted in accordance with institutional guidelines for *in vitro* research.

2.2. Cell Lines and Cell Culture

Human gastric adenocarcinoma (AGS) and cervical cancer (HeLa) cell lines were obtained from the National Cell Bank of Iran (NCBI). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin under standard culture conditions (37°C, 5% CO₂, humidified atmosphere). When cultures reached 70–80% confluency, cells were washed with phosphate-buffered saline (PBS), detached using trypsin–EDTA for 3–4 min at 37°C, neutralized with culture medium containing 10% FBS, and collected by centrifugation. The resulting cell pellet was resuspended in fresh culture medium for subsequent experiments.

2.3. Tolectin Preparation and Rationale

Tolectin powder was dissolved in PBS and DMEM to prepare stock solutions, followed by serial dilutions to yield final concentrations of 0.0012, 0.0023, 0.0049, 0.0097, 0.0194, and 0.0389 mmol/mL. These concentrations were selected based on preliminary pilot studies conducted in our laboratory, designed to identify both sub-toxic and cytotoxic doses for AGS and HeLa cells. Fresh Tolectin solutions were prepared prior to each experiment to ensure stability and reproducibility (Ghasemi *et al.*, 2023; Rao *et al.*, 2023).

2.4. Cell Viability Assay (MTT)

Cell viability was assessed using the MTT assay. AGS and HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere for 24 hours. Cells were then treated with indicated concentrations of Tolectin for 24 hours. After treatment, cells were washed with PBS, and 0.05 mg/well MTT solution (DO BIO Biotech, Shanghai, China) was added, followed by incubation for 3 hours at 37°C in darkness. Formazan crystals were solubilized in 100 μ L DMSO per well, and absorbance was measured at 570 nm using a microplate reader (BioTek, USA). Cell viability (%) was calculated as $[\text{OD}(\text{sample})/\text{OD}(\text{control}) \times 100]$. Experiments were performed in eight replicates. The half-maximal inhibitory concentration (IC₅₀) of Tolectin was calculated using a linear regression model ($Y = aX + b$), where Y represents

the fraction of viable cells, X is the Tolectin concentration, a is the slope, and b is the Y-intercept. IC₅₀ was determined as $\text{IC}_{50} = (0.5 - b)/a$.

2.5. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

AGS and HeLa cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated for 24 hours. Cells were treated with the effective concentration of Tolectin (0.0131 mmol/mL) for 24 hours. Total RNA was extracted using the RNA-isolation kit (Transgene Biotech, ER101-01) according to the manufacturer's instructions. The concentration and purity of RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA), and samples with an A260/A280 ratio between 1.8–2.0 were used for cDNA synthesis. First-strand cDNA was synthesized using the PrimeScript™ First-Strand cDNA Synthesis Kit (Transgene Biotech, AE301-02).

qRT-PCR was performed using 10 μ L Power SYBR Green PCR Master Mix (2X), 1 μ L of each specific primer (Table 1), 1 μ L cDNA (100 ng), and 7 μ L double-distilled water. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed to confirm primer specificity. Gene expression of *TP53*, *BAX*, and *BCL2* was normalized to GAPDH and calculated using the $2^{-\Delta\Delta\text{CT}}$ method. All reactions were performed in triplicate, and results are presented as mean $\pm$ standard deviation (SD).

Table 1. Primer sequences used in qRT-PCR

Gene	Primer Sequences
Bcl-2	Forward: 5'-GGTGCCGGTTCAGGTA CTCA-3'
	Reverse: 5'-TTGTGGCCTTCTTGTAGTTCG-3'
BAX	Forward: 5'-CGGCAACTTCAACTGGGG-3'
	Reverse: 5'-TCCAGCCCAACAGCCG-3'
P53	Forward: 5'-CATCTACAAGCAGTCACAGCACAT-3'
	Reverse: 5'-CAACCTCAGGCGGCTCATAG-3'
GAPDH	Forward: 5'-CCCACTCTCCACCTTTGAC-3'
	Reverse: 5'-CATACCAGGAAATGAGCTTGACAA-3'

2.6. Caspase-3, -8, and -9 Activity Assay

Caspase activity was measured using colorimetric assay kits for caspase-3, caspase-8, and caspase-9 (Novex). AGS and HeLa cells were treated with the effective concentration of Tolectin (0.0131 mmol/mL) and incubated at 37°C for 24 h in a humidified atmosphere with 5% CO₂. The cells were then trypsinized and centrifuged at $600 \times g$ for 5 min. The resulting pellets were lysed in 50 μ L of chilled cell lysis buffer and kept on ice for 10 min. The lysates were centrifuged at $10,000 \times g$ for 1 min, and the supernatants were collected.

For each assay, 200–300 μ g of total protein was mixed with 50 μ L of cell lysis buffer. Subsequently, 50 μ L of $2\times$ reaction buffer containing 10 mM DTT was added to each sample. pNA-conjugated substrates specific for each caspase (final concentration: 200 μ M) were then added, and the mixtures were incubated at 37°C for 2 h.

Absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA) at 400 nm. Caspase activity was calculated according to Equation (1):

Caspase activity (%) = $[(\text{Abs_sample} - \text{Abs_blank}) / \text{Abs_control}] \times 100$ (24)

Protein concentrations were determined using the Bradford assay.

2.7. Apoptosis Quantification by Flow Cytometry

Apoptotic cells were quantified using Annexin-V-FLUOS and propidium iodide (PI) staining. AGS and HeLa cells were seeded in 24-well plates and treated with the effective concentration of tolectin (0.0131 mmol/mL). The cells were incubated at 37°C in a humidified incubator containing 95% air and 5% CO₂ for 48 h.

After treatment, the cells were stained with Annexin-V-FLUOS and PI and incubated for 15 min at 25°C in the dark. Apoptotic and necrotic cells were visualized using a fluorescence microscope with an excitation/emission range of 515–565 nm (Karimi M *et al.*, 2023; Amiri *et al.*, 2025).

2.8. Statistical Analysis

Data were analyzed using SPSS version 21. Normality was assessed with the Kolmogorov–Smirnov test. Statistical comparisons were performed using one-way ANOVA and Student's t-test, with $p < 0.05$ considered statistically significant. Results are presented as mean $\pm$ standard deviation (SD).

3. Results

3.1. Cytotoxic effect of tolectin on AGS and HeLa cells

The MTT assay demonstrated that all tested concentrations of Tolectin (0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL) significantly reduced the viability of both AGS and HeLa cells compared to the control group ($p < 0.01$ – 0.001) (Fig. 1 and 2). The calculated IC₅₀ values were 0.0131 mmol/mL for AGS cells and 0.0147 mmol/mL for HeLa cells, indicating potent cytotoxic activity of Tolectin in a concentration-dependent manner.

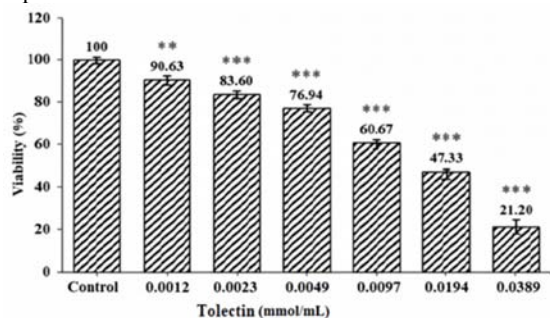


Figure 1. Viability of AGS cells treated with different concentrations of Tolectin. * indicates significant difference compared with control group (**: $p < 0.01$, and ***: $p < 0.001$).

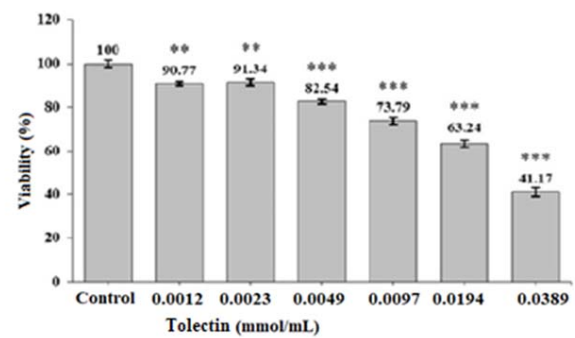


Figure 2. Viability of HeLa cells treated with different concentrations of Tolectin. * indicates significant difference compared with control group (**: $p < 0.01$, and ***: $p < 0.001$).

3.2. Effect of Tolectin on Expression Levels of BCL2, BAX, and TP53 in AGS and HeLa Cells

qRT-PCR analysis revealed that treatment of AGS cells with the effective concentration of Tolectin (IC₅₀) significantly decreased the relative expression of *BCL2* and *BAX* genes compared with the control group ($p < 0.001$ and $p < 0.01$, respectively). Conversely, in HeLa cells, Tolectin treatment led to a significant increase in *BCL2* and *BAX* expression levels ($p < 0.01$ and $p < 0.001$, respectively). The *BAX/BCL2* ratio was calculated as 1.557 and 1.385 in AGS and HeLa cells, respectively, suggesting activation of apoptosis pathways, particularly in AGS cells (Fig. 3–5). The relative expression of *TP53* did not show significant changes in either cell line. These differences between AGS and HeLa cells may reflect cell-type-specific responses to Tolectin, potentially due to inherent differences in apoptotic regulatory mechanisms.

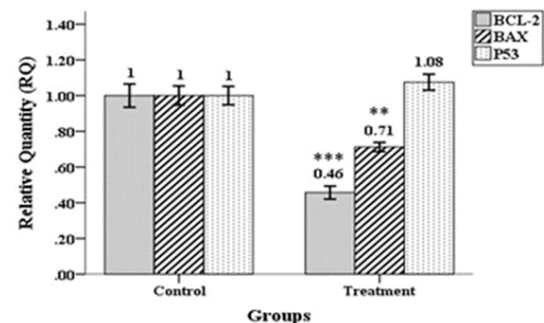


Figure 3. Relative quantitative expression (RQ) of Bcl-2, *BAX* and P53 gene in AGS cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

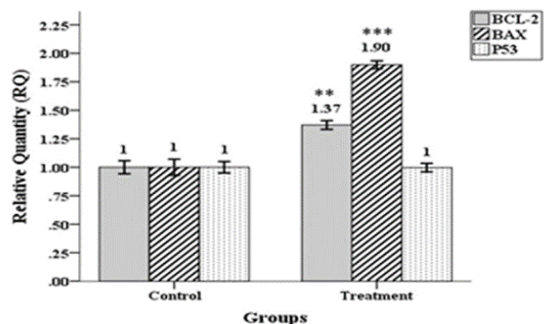


Figure 4. Relative quantitative expression (RQ) of Bcl-2, *BAX* and P53 gene in HeLa cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

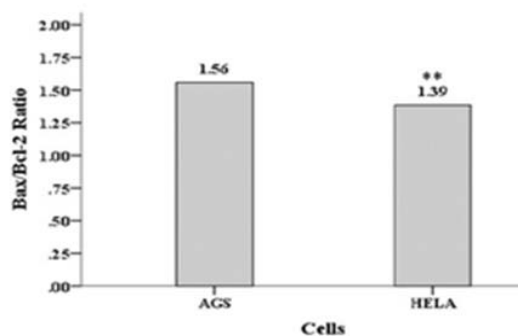


Figure 5. *BAX/BCL2* expression level ratio in AGS and HeLa cells treated with effective concentration of Tolectin.

3.3. Effect of tolectin on caspase-3, -8 and -9 activity level in AGS and HeLa cells

Colorimetric caspase assays indicated that Tolectin treatment significantly increased the activity of caspase-3, -8, and -9 in both AGS and HeLa cells compared to controls ($p < 0.05-0.001$) (Fig. 6 and 7). The elevated caspase activity corroborates the induction of caspase-dependent apoptotic pathways by Tolectin.

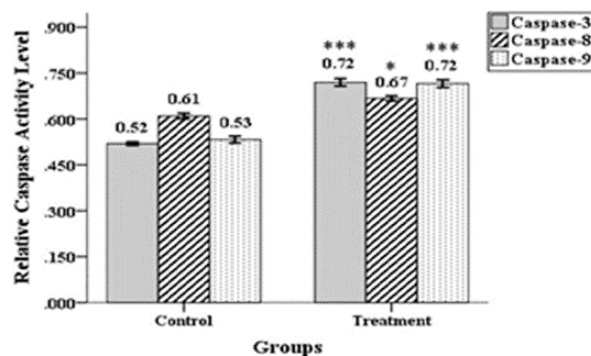


Figure 6. Activity level of caspase-3, -8, and -9 in AGS cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (*: $p < 0.05$, ***: $p < 0.001$).

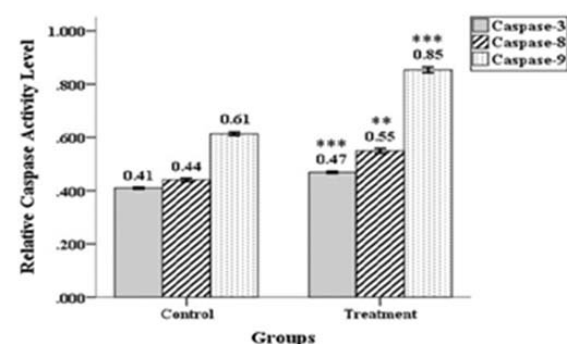


Figure 7. Activity level of caspase-3, -8, and -9 in HeLa cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

3.4. Apoptosis Induction Detected by Flow Cytometry

Flow cytometric analysis confirmed that Tolectin induced apoptosis in both cell lines. In AGS cells, primary apoptosis was predominantly observed, whereas in HeLa cells, both early and late apoptosis phases were evident. The percentage of apoptotic cells significantly increased in

treated groups compared to controls ($p < 0.05-0.001$) (Fig. 8), supporting the cytotoxic and pro-apoptotic effects of Tolectin.

3.5. Summary

Overall, Tolectin exhibited concentration-dependent cytotoxicity in AGS and HeLa cells, modulated key apoptotic genes (*BCL2* and *BAX*), activated caspases, and induced apoptosis, highlighting its potential as an anti-cancer agent with cell-type-specific effects.

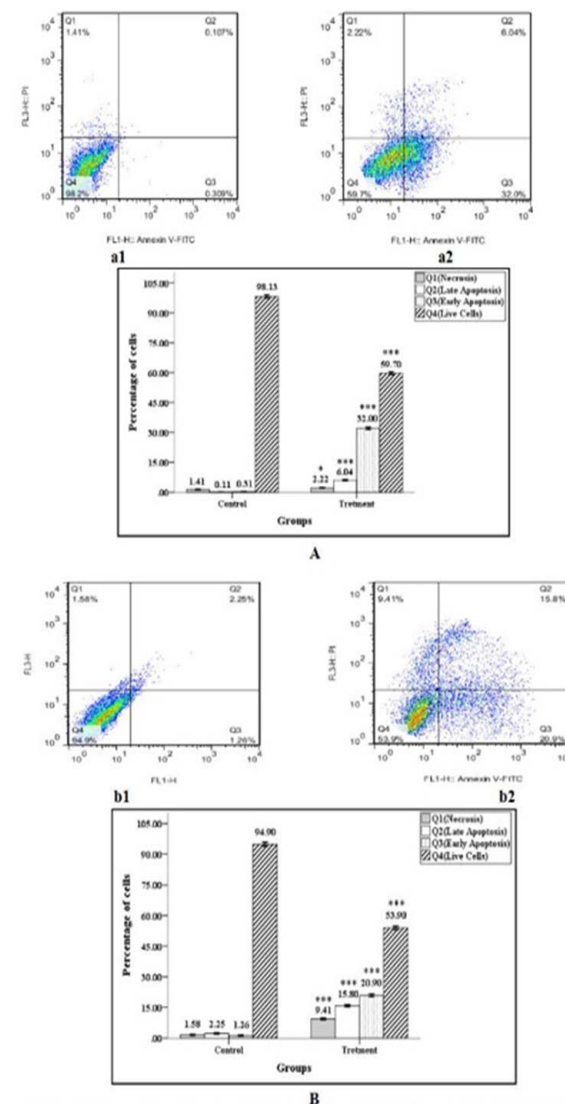


Figure 8. Apoptosis in AGS and HeLa cells induced by effective concentration of Tolectin: Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells. (a1) Control (untreated) AGS cells; (b1) Control (untreated) HeLa cells, (a2) AGS cells treated with effective concentration of tolectin, (b2) HeLa cells treated with effective concentration of Tolectin. A and B indicate apoptosis phases (Q1 – Q4) compared to control groups in AGS and HeLa cells, respectively. The analysis was made by FACSDiva Version 6.1.3. * and *** represent significant difference compared to control group (*: $p < 0.05$, ***: $p < 0.001$).

4. DISCUSSION

This study comprehensively evaluated the cytotoxic and apoptosis-inducing effects of Tolectin on AGS gastric and HeLa cervical cancer cells. The MTT assay results demonstrated a significant, concentration-dependent

decrease in cell viability, with an IC₅₀ of 0.0131 mmol/mL for both cell lines. Apoptotic induction was confirmed through increased activity of caspase-3, -8, and -9, as well as modulation of the *BAX/Bcl-2* ratio, indicating activation of both intrinsic and extrinsic apoptotic pathways (Norouzi *et al.*, 2020a; Norouzi *et al.*, 2020b).

The anticancer potential of NSAIDs has been well documented, primarily through inhibition of cell proliferation and induction of apoptosis via mitochondrial and death-receptor pathways. In line with these findings, Tolectin effectively modulated apoptotic regulators, increasing the *BAX/Bcl-2* ratio and caspase activity, thereby promoting programmed cell death (Ahmadi *et al.*, 2019; Kassab, 2025). Specifically, activation of caspase-9 indicates engagement of the intrinsic mitochondrial pathway, whereas elevated caspase-8 activity supports involvement of the extrinsic death-receptor pathway (Arisan *et al.*, 2019).

Interestingly, *TP53* expression did not show a statistically significant change in either AGS or HeLa cells. This may reflect cell-type-specific responses, basal *p53* status, or the presence of functional mutations in these cell lines (Li Q., 2017; Mishra *et al.*, 2024). Nevertheless, the observed pro-apoptotic effects suggest that Tolectin can induce apoptosis independently of *TP53* modulation, highlighting its potential applicability in tumors with *p53* mutations or functional inactivation. These findings underscore the importance of exploring alternative pathways that contribute to Tolectin-induced cell death (Brandolini *et al.*, 2020; Thiruchenthoooran *et al.*, 2023; Maytalman & Nemutlu Samur, 2025; Kazberuk *et al.*, 2022; Kolawole & Kashfi, 2022).

Although NSAIDs generally exert pro-apoptotic effects, previous reports have shown cell-type-dependent variability or even anti-apoptotic outcomes under certain conditions (Du *et al.*, 2023; Erfani *et al.*, 2018; Bhagavatula *et al.*, 2024). Factors such as drug concentration, exposure duration, and genetic background of the cells likely contribute to these discrepancies. In this study, both AGS and HeLa cells displayed robust apoptotic responses, indicating that Tolectin may possess broadly effective anticancer activity across different tumor types.

This study has several limitations. First, experiments were limited to *in vitro* assays, and apoptosis was assessed using a restricted set of markers. Second, while caspase activation and *BAX/Bcl-2* modulation were evaluated, other critical pathways such as NF- κ B, PI3K/AKT, and mTOR, as well as mitochondrial membrane potential changes and cytochrome-c release, were not investigated (Hesham *et al.*, 2025; Song *et al.*, 2024; Kassab *et al.*, 2021; Zhang *et al.*, 2024). Further studies should include these analyses to clarify the molecular mechanisms underlying Tolectin-induced apoptosis. Moreover, *in vivo* studies, including xenograft models and combination therapy assays with standard chemotherapeutics, are warranted to evaluate potential synergistic effects and to determine whether Tolectin can overcome drug resistance.

In conclusion, Tolectin induces significant apoptosis in AGS gastric and HeLa cervical cancer cells via both intrinsic and extrinsic pathways, accompanied by *BAX/Bcl-2* modulation and caspase activation (Kwatra *et al.*, 2024; Chu *et al.*, 2024; Marei, 2025; Rai *et al.*, 2022;

Jan & Chaudhry, 2019). The compound demonstrates potential as a promising candidate for further preclinical evaluation, particularly for tumors resistant to conventional apoptosis-inducing therapies. Future research should focus on elucidating additional molecular mechanisms and assessing the therapeutic efficacy of Tolectin *in vivo* to validate its translational potential.

5. Conclusions

Our study is the first to examine the cytotoxic and apoptotic effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. The results indicate that the cytotoxic effects of Tolectin are mediated through both intrinsic and extrinsic apoptotic pathways, while the expression of the tumor suppressor gene *TP53* did not significantly change. These findings suggest that Tolectin may have potential as an anticancer agent; however, further studies are needed to investigate its effects *in vivo* (Kan *et al.*, 2025; Al-Qadi *et al.*, 2023).

This study has several limitations. Experiments were conducted only *in vitro* using two cancer cell lines, which may not fully represent the tumor microenvironment or systemic interactions *in vivo* (Darfeuille *et al.*, 2024). Only a limited number of apoptosis markers (*BAX*, *BCL2*, and caspases-3, -8, and -9) were examined, and other molecular pathways involved in Tolectin-induced cytotoxicity were not evaluated (Li *et al.*, 2025). Potential off-target effects and the pharmacological safety of Tolectin were also not assessed. Future research should include *in vivo* studies and additional cancer models to better define the therapeutic potential and safety profile of Tolectin as an anticancer agent (Shafiei *et al.*, 2024).

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This study did not involve human participants or animals. All experiments were conducted *in vitro* using established human cell lines. The handling and experimentation with these cell lines were performed in accordance with standard ethical guidelines and laboratory safety regulations. The study protocol was reviewed and approved by the International Association of Scientists (IAS) Ethics Committee (Ref: IASSP.22.1CL).

Author Contributions

Rahim Ahmadi: Conceptualization, writing – review and editing, final approval.

Mahsa Kavousi: Conceptualization, writing – review and editing, final approval.

Mirela Tabaku: Project administration, writing – review and editing, final approval.

Sanaz Noroozi: Methodology, formal analysis, writing – original draft preparation, writing – review and editing, final approval.

Masoumeh Aalipour: Writing – original draft preparation, writing – review and editing, final approval.

All authors have read and approved the final manuscript.

Data Availability

The datasets generated and/or analyzed during the current study are openly available at:

<https://drive.google.com/file/d/19QeK2jueVWDImjdH1zUqV9xTVFAOovX5O/view?usp=sharing>

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