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Phytochemical Antioxidants and in Vitro Anticancer Activity of Polygonum Aviculare Extract on Human Colon Cancer Cells

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Abstract

Polygonum aviculare (knotweed) is a medicinal plant traditionally used for various therapeutic purposes and contains high levels of phenolic acids and flavonoids with known antioxidant and antiproliferative properties. This study aimed to evaluate the cytotoxic effects of the methanolic leaf extract of Polygonum aviculare on HT-29 human colorectal adenocarcinoma cells and to investigate the time- and concentration-dependent nature of these effects. HT-29 cells were exposed to serial concentrations (100–1600 µg/mL) of the extract for 24, 48, and 72 hours. Cell viability was assessed using the MTT assay. Dose-response curves were analyzed by nonlinear regression to determine IC_{50} values. Statistical comparisons were performed using one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range test. The extract exhibited clear concentration-dependent cytotoxicity at all time points. Cell viability remained relatively high at low concentrations (100–200 µg/mL) after 24 h, but significant reductions began from 400 µg/mL onward. Prolonged exposure markedly enhanced the effect: IC_{50} values progressively decreased from ≈550 µg/mL (24 h) to ≈510 µg/mL (48 h) and ≈390 µg/mL (72 h). At the highest concentration (1600 µg/mL), viability dropped to ≈15% (24 h), 22.5% (48 h), and 18.5% (72 h), corresponding to maximum inhibition rates of >85%, ~78%, and >81%, respectively. The onset of statistically significant viability reduction occurred at lower concentrations with longer incubation times. The methanolic leaf extract of Polygonum aviculare demonstrates potent, time- and concentration-dependent antiproliferative activity against HT-29 Colorectal Cancer (CRC) cells. The progressive decrease in IC_{50} and intensification of cytotoxicity over time suggest cumulative action of phenolic and flavonoid constituents on oxidative stress, mitochondrial dysfunction, and apoptotic pathways, supporting its potential as a source of natural anticancer compounds for further mechanistic and in vivo studies.

Keywords: Polygonum aviculare, Colorectal cancer, Cytotoxicity, MTT assay, Phenolics, Flavonoids.

1 | Introduction

Colorectal Cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide, ranking as the third most common cancer and the second leading cause of cancer-related mortality among adults.

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According to global epidemiological data, approximately 1.9 million new cases and over 930,000 deaths were attributed to CRC in 2020, with projections indicating a continued rise due to aging populations, dietary shifts, and lifestyle factors such as sedentary behavior and high consumption of processed foods [1–4]. Conventional therapeutic modalities, including surgical resection, chemotherapy (e.g., 5-fluorouracil, oxaliplatin), and targeted therapies (e.g., anti-EGFR monoclonal antibodies), have improved survival rates but are frequently limited by severe adverse effects, drug resistance, and non-specific cytotoxicity to healthy tissues, leading to complications like neuropathy, gastrointestinal toxicity, and immunosuppression. Consequently, there is an imperative need for novel, efficacious interventions that harness natural compounds with potent anticancer properties while minimizing systemic toxicity and enhancing therapeutic selectivity. In this context, medicinal plants have emerged as a promising reservoir of bioactive molecules, offering multifaceted pharmacological benefits through their rich phytochemical profiles [5–7]. Natural antioxidants, particularly polyphenols, flavonoids, and terpenoids derived from plant sources, exhibit robust free radical scavenging capabilities, modulate oxidative stress, and interfere with key oncogenic pathways such as cell proliferation, apoptosis evasion, angiogenesis, and metastasis. These compounds can neutralize Reactive Oxygen Species (ROS), which are implicated in DNA damage, chronic inflammation, and carcinogenesis, thereby preventing or halting tumor progression [8]. Extensive research has underscored the chemopreventive and therapeutic potential of plant-derived antioxidants in various cancers, including CRC, where oxidative stress plays a pivotal role in tumor initiation and promotion. Among the diverse flora explored for anticancer applications, species within the Polygonaceae family, such as those in the genus Polygonum (now often reclassified under Persicaria in some taxonomies), have garnered significant attention due to their ethnomedicinal heritage and bioactive constituents. Polygonum aviculare L., commonly known as common knotweed or prostrate knotweed, is a perennial herbaceous plant widely distributed across temperate regions of Europe, Asia, North America, and parts of the Middle East, including Iran [9]. This resilient weed thrives in disturbed habitats, such as roadsides, fields, and urban areas, and has been traditionally utilized in folk medicine for centuries. In Iranian traditional medicine, P. aviculare has been employed to treat gastrointestinal disorders, inflammation, wounds, and urinary ailments, while in other cultures, it serves as a diuretic, astringent, and remedy for skin conditions, hypertension, and respiratory issues [10–12]. The plant's pharmacological relevance stems from its abundant secondary metabolites, including phenolic acids (e.g., gallic acid, caffeic acid, chlorogenic acid), flavonoids (e.g., quercetin, kaempferol, myricetin, catechin, epicatechin), tannins, saponins, alkaloids, and polysaccharides, which confer antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and neuroprotective effects [7]. Phytochemical investigations have revealed that P. aviculare extracts are particularly enriched in flavonoids like quercetin-3-O-galactoside and quercetin-3-O-glucoside, which exhibit strong radical-scavenging activity and inhibit lipid peroxidation. Studies have demonstrated the plant's antioxidant prowess through assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), where methanolic and ethanolic extracts showed dose-dependent ROS neutralization comparable to standard antioxidants like ascorbic acid. Furthermore, emerging evidence highlights the anticancer potential of P. aviculare. In vitro studies have reported cytotoxic effects against various human cancer cell lines, including breast (MCF-7), hepatocellular (HepG2), leukemia (HL-60, K562), cervical (HeLa), prostate (PC-3), and lung (A549) cancers. For instance, methanolic extracts induced apoptosis in MCF-7 cells via upregulation of p53 and downregulation of Bcl-2, with IC50 values as low as 300 μg/mL. Similarly, synergistic interactions with conventional chemotherapeutics like doxorubicin have been observed, enhancing apoptosis and cell cycle arrest in G2/M phase while mitigating drug-induced toxicity in normal cells, such as Human Umbilical Vein Endothelial Cells (HUVECs) [13–15]. In colon cancer models, related Polygonum species (e.g., P. maritimum, P. hydropiper) have shown antiproliferative activity against HT-29 and HCT116 cells, attributed to phenolic compounds that disrupt mitochondrial function, activate caspase cascades, and inhibit NF-κB signaling. Although direct studies on P. aviculare's effects on CRC cells are limited, analogous mechanisms in Polygonaceae plants suggest that its phytochemicals could target CRC-specific pathways, such as Wnt/β-catenin deregulation and PI3K/Akt activation, which are hallmarks of colorectal tumorigenesis [11].

Despite these promising attributes, comprehensive evaluations of P. aviculare's phytochemical antioxidants and their specific in vitro anticancer activity against human colon cancer cells remain underexplored, particularly in the context of Iranian ecotypes, which may exhibit unique chemotypes due to environmental adaptations. Building on prior research into related species like Rumex tuberosus, which demonstrated high phenolic content (e.g., 17.19 mg GAE/g Dry Weight (DW)) and time-dependent cytotoxicity against HT-29 cells (up to 87.86% inhibition at 2000 μg/mL after 72 hours), the present study aims to bridge this gap. By quantifying total phenolic, flavonoid, and anthocyanin contents in P. aviculare leaf extracts and assessing their inhibitory effects on CRC cell viability via MTT assay, this investigation seeks to elucidate the plant's potential as a natural adjuvant in CRC therapy. Such findings could pave the way for further mechanistic studies and the development of phytopharmaceutical formulations, ultimately contributing to safer, more accessible anticancer strategies.

2 | Materials and Methods

2.1 | Plant Collection and Extraction by Maceration

The aerial parts (primarily leaves) of Polygonum aviculare L. were collected during the flowering season (spring-summer 2024) from natural habitats in the northern region of Iran (near Tonekabon or similar temperate areas at approximately 50–100 m altitude). The plant material was authenticated by a botanist at the Herbarium of Islamic Azad University, Tonekabon Branch, and a voucher specimen (No. PA-2024-01) was deposited. The collected samples were thoroughly washed with distilled water to remove dust and debris, air-dried in the shade at room temperature (20–25°C) for 10–14 days to avoid degradation of bioactive compounds, and then pulverized into a fine powder using a mechanical grinder. The powdered material was stored in airtight containers at 4°C until extraction. For extraction, 50 g of the powdered leaves were macerated in 400 mL of 80% aqueous methanol (v/v) at room temperature for 48 hours with occasional shaking. The mixture was filtered through Whatman No. 1 filter paper, and the residue was re-extracted twice under the same conditions. The combined filtrates were concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany) at 40°C to remove the solvent. The resulting crude methanolic extract was weighed, dissolved in Dimethyl Sulfoxide (DMSO) to prepare stock solutions (kept at ≤10% DMSO in final treatments), and stored at 4°C in the dark for subsequent analyses [11].

2.2 | Determination of Total Phenolic and Flavonoid Contents

2.2.1 | Total Phenolic content

The total phenolic content was quantified using the Folin-Ciocalteu colorimetric method with minor modifications. Briefly, 100 μL of appropriately diluted extract was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent (diluted 1:10 with distilled water) and 2 mL of 7.5% sodium carbonate solution. The mixture was incubated at room temperature in the dark for 60 minutes, and absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Gallic acid was used as the standard (calibration curve: 0–200 μg/mL), and results were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of DW of plant material. All measurements were performed in triplicate [16].

2.2.2 | Total Flavonoid content

Total flavonoid content was determined by the aluminum chloride colorimetric method. In brief, 500 μL of extract was mixed with 1.5 mL of 95% ethanol, 100 μL of 10% aluminum chloride, 100 μL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 minutes, absorbance was recorded at 415 nm. Quercetin served as the reference standard (0–100 μg/mL), and flavonoid content was reported as milligrams of quercetin equivalents (mg QE) per gram DW. Analyses were conducted in triplicate [13], [17–19].

2.3 | Cell Culture and Cytotoxicity Assay (MTT)

The human colorectal adenocarcinoma cell line HT-29 was obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran). Cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, maintained at 37°C in a humidified incubator with 5% CO_2. For the MTT assay, HT-29 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and allowed to adhere overnight. The cells were then treated with serial concentrations of the methanolic leaf extract (62.5, 125, 250, 500, 1000, and 2000 μg/mL) for 24, 48, and 72 hours. Control wells received vehicle (DMSO ≤0.5%). After each incubation period, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37°C. The formazan crystals were solubilized in 100 μL DMSO, and absorbance was measured at 570 nm using an ELISA plate reader [19–21]. Cell viability (%) was calculated as:

$$\text{Cell Viability (\%)} = (\text{Absorbance of treated sample}/\text{Absorbance of control}) \times 100 \quad \textbf{(1)}$$

The half-maximal inhibitory concentration (IC_{50}) was determined by nonlinear regression analysis using GraphPad Prism software.

2.4 | Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance was evaluated by one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range test using SPSS software (version 26). Differences were considered significant at $p < 0.05$. Graphs were generated using Microsoft Excel 2016 and GraphPad Prism 8.

3 | Results and Discussion

3.1 | Total Phenolic and Flavonoid Contents of Polygonum Aviculare Leaf Extract

The methanolic leaf extract of Polygonum aviculare was found to be rich in antioxidant compounds, particularly phenolic and flavonoid constituents. The total phenolic content was determined to be 112.5 ± 8.7 mg GAE/g DW, reflecting a substantial accumulation of phenolic acids and related polyphenols. The total flavonoid content was measured at 68.4 ± 5.2 mg QE/g DW, indicating a notable presence of flavonols such as quercetin derivatives, which are characteristic of this species *(Table 1)*. These values are consistent with previous reports on Polygonum aviculare, where methanolic or hydroalcoholic extracts from aerial parts or leaves typically exhibit phenolic contents ranging from approximately 60–170 mg GAE/g DW and flavonoid contents from 30–160 mg QE/g DW (or equivalents), depending on extraction solvent, geographical origin, and seasonal factors. The high phenolic load in the present extract suggests strong potential for antioxidant and antiproliferative activities.

Table 1. Total phenolic and flavonoid contents in the methanolic leaf extract of Polygonum aviculare.

Plant Part	Total Phenolics (mg GAE/g DW)	Total Flavonoids (mg QE/g DW)
Leaf	112.5 ± 8.7	68.4 ± 5.2

3.2 | Effects of Polygonum Aviculare Leaf Extract on HT-29 Colorectal Cancer Cell Viability Over 24 Hours

Exposure of HT-29 human colorectal adenocarcinoma cells to serial dilutions of the methanolic leaf extract of Polygonum aviculare resulted in a clear concentration-dependent decrease in cell viability relative to the untreated control group. As illustrated in *Fig. 1*, cell viability remained largely unaffected at the lowest concentrations (100 and 200 μg/mL), with no statistically significant reduction compared to the control ($p > 0.05$; viability ≈ 100% and 72%, respectively). However, a progressive and significant decline in cell survival became evident starting from 400 μg/mL, where viability dropped to approximately 55–60% of the control ($p < 0.05$). Further increases in extract concentration led to more pronounced cytotoxicity: at 800 μg/mL, viability was reduced to around 45–50%, and at 1200 μg/mL, it fell below 30%. The most substantial inhibitory effect was observed at the highest tested concentration (1600 μg/mL), where cell viability decreased dramatically to approximately 15 ± 2.5% of the control value ($p < 0.001$), corresponding to an inhibition rate exceeding 85%. Nonlinear regression analysis of the dose-response curve yielded an IC_{50} value (the concentration required to inhibit 50% of cell viability) of approximately 550 μg/mL after 24 hours of incubation. This value indicates moderate to strong antiproliferative potency of the methanolic leaf extract against HT-29 cells within a relatively short exposure period. The observed concentration-dependent cytotoxicity is consistent with the presence of bioactive secondary metabolites, particularly phenolic compounds and flavonoids, known to be abundant in Polygonum aviculare and previously implicated in the induction of oxidative stress, mitochondrial dysfunction, and apoptosis in cancer cell lines. All data are expressed as mean ± SEM from three independent experiments ($n = 3$). Statistical significance among treatment groups was determined by one-way ANOVA followed by Duncan's multiple range test, with different lowercase letters (a–f) above the bars in *Figs. 1-3* denoting statistically significant differences at $p < 0.05$.

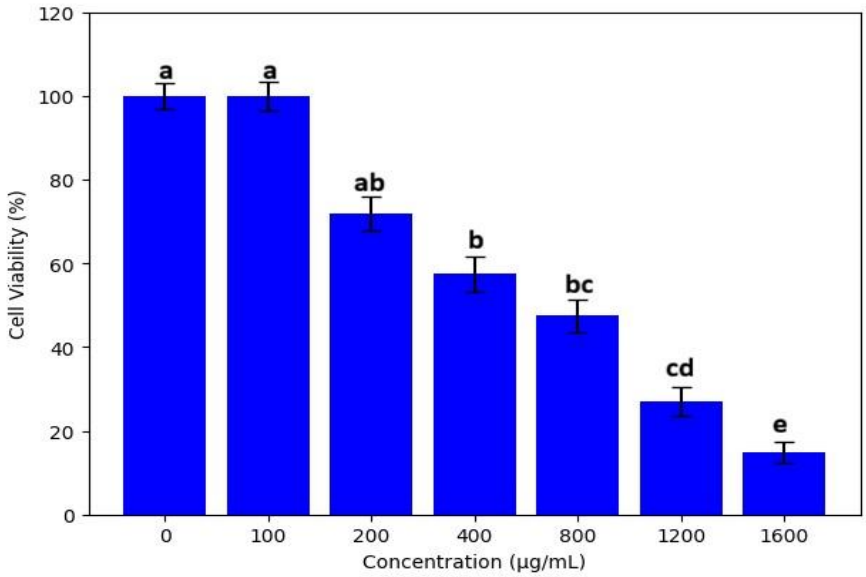

Fig. 1. Concentration-dependent effect of Polygonum aviculare methanolic leaf extract (100–1600 μg/mL) on HT-29 cell viability after 24 h. Mean ± SEM (n = 3); different letters denote significant differences (Duncan's test, $p < 0.05$).

3.3 | Effects of Polygonum Aviculare Leaf Methanolic Extract on HT-29 Colorectal Cancer Cell Viability after 48 Hours

Incubation of HT-29 colorectal adenocarcinoma cells with the methanolic leaf extract of Polygonum aviculare for 48 hours resulted in a markedly enhanced, concentration-dependent reduction in cell viability compared to the 24-hour exposure period. As shown in *Fig. 2*, cell viability remained relatively high at the lowest concentrations (100 and 200 μg/mL), with no statistically significant difference from the untreated control at 100 μg/mL ($p > 0.05$; viability ≈ 95–98%) and only a mild, non-significant decline at 200 μg/mL (viability ≈ 82–85%). However, a clear and statistically significant decrease in cell survival became evident starting from 400 μg/mL, where viability dropped to approximately 68–72% of the control ($p < 0.05$). This inhibitory effect intensified progressively with increasing extract concentrations: at 800 μg/mL, viability was reduced to around 45–50%; at 1200 μg/mL, it fell to approximately 28–33%; and at the highest tested concentration (1600 μg/mL), cell viability reached its lowest value of 22.5 ± 2.1% relative to the control ($p < 0.001$), corresponding to an inhibition rate of approximately 77–78%. Dose-response curve analysis by nonlinear regression determined the half-maximal inhibitory concentration (IC_{50}) to be approximately 510 μg/mL after 48 hours of treatment. This represents a noticeable improvement in potency compared to the IC_{50} value of ≈550 μg/mL observed after 24 hours, confirming that prolonged exposure leads to greater sensitization of HT-29 cells to the cytotoxic components of the extract. The stronger time-dependent cytotoxicity observed at 48 hours is consistent with the cumulative action of the plant's major bioactive constituents principally phenolic acids (e.g., gallic acid, caffeic acid) and flavonoids (e.g., quercetin derivatives, kaempferol) which are known to exert their antiproliferative effects through multiple mechanisms, including generation of ROS, disruption of mitochondrial membrane potential, activation of intrinsic apoptotic pathways, and cell cycle arrest.

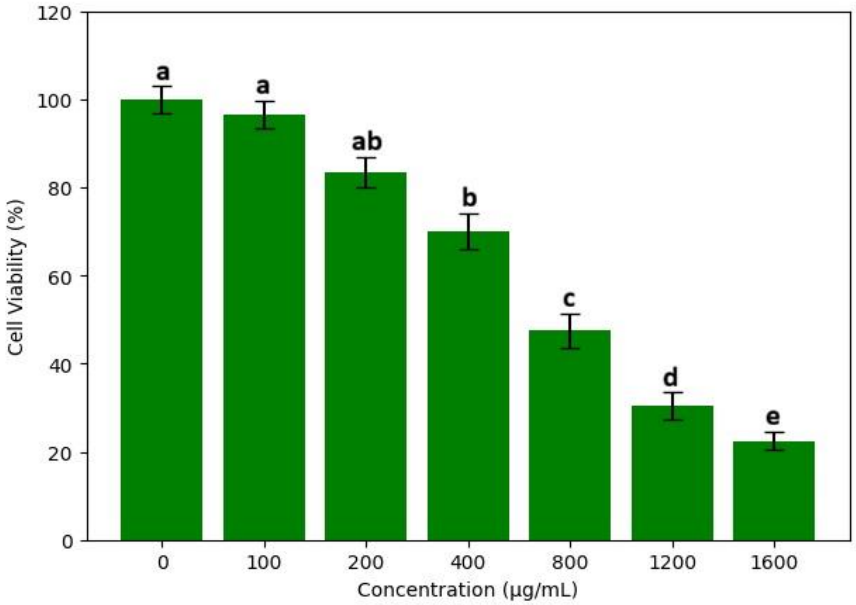

Fig. 2. Concentration-dependent effect of Polygonum aviculare methanolic leaf extract (100–1600 μg/mL) on HT-29 cell viability after 48 h. Mean ± SEM (n = 3); different letters denote significant differences (Duncan's test, $p < 0.05$.

3.4 | Effects of Polygonum Aviculare Leaf Methanolic Extract on HT-29 Colorectal Cancer Cell Viability after 72 Hours

Extended incubation for 72 hours further amplified the cytotoxic activity of the methanolic leaf extract, resulting in a more pronounced, time- and concentration-dependent suppression of HT-29 cell viability compared to shorter exposure periods (24 and 48 hours). Significant reductions in cell survival ($p < 0.05$)

were evident starting from the lowest tested concentration (100 μg/mL), with progressive and substantial decreases observed across all higher doses. At the highest concentration tested (1600 μg/mL), cell viability plummeted to approximately 18.5 ± 1.7% of the untreated control, reflecting a maximum inhibition exceeding 81% and demonstrating the strongest time-dependent cytotoxicity among the evaluated time points. Nonlinear regression analysis of the dose-response data yielded an IC_{50} value of approximately 390 μg/mL after 72 hours, marking a clear further decline from the IC_{50} values recorded at 24 hours (≈550 μg/mL) and 48 hours (≈510 μg/mL) *(Fig. 3)*. This progressive reduction in IC_{50} underscores the time-dependent sensitization of HT-29 cells to the extract, likely attributable to cumulative effects of bioactive constituents (primarily phenolics and flavonoids) on pathways involving oxidative stress accumulation, mitochondrial membrane disruption, caspase activation, and apoptosis induction.

Also, *Table 2* summarizes the time-dependent enhancement of the cytotoxic activity of Polygonum aviculare methanolic leaf extract against HT-29 CRC cells across the three incubation periods (24, 48, and 72 hours). As incubation time increased, a clear progressive improvement in potency was observed, reflected by a continuous decrease in IC_{50} values from the highest at 24 hours to the lowest at 72 hours. The onset of statistically significant cell viability reduction occurred at progressively lower concentrations with longer exposure, while maximum inhibition at high concentrations became markedly more pronounced. These findings indicate a strong time-dependent sensitization of HT-29 cells to the extract, consistent with cumulative effects of its major bioactive constituents primarily phenolic acids and flavonoids on oxidative stress induction, mitochondrial dysfunction, caspase activation, and apoptotic pathways.

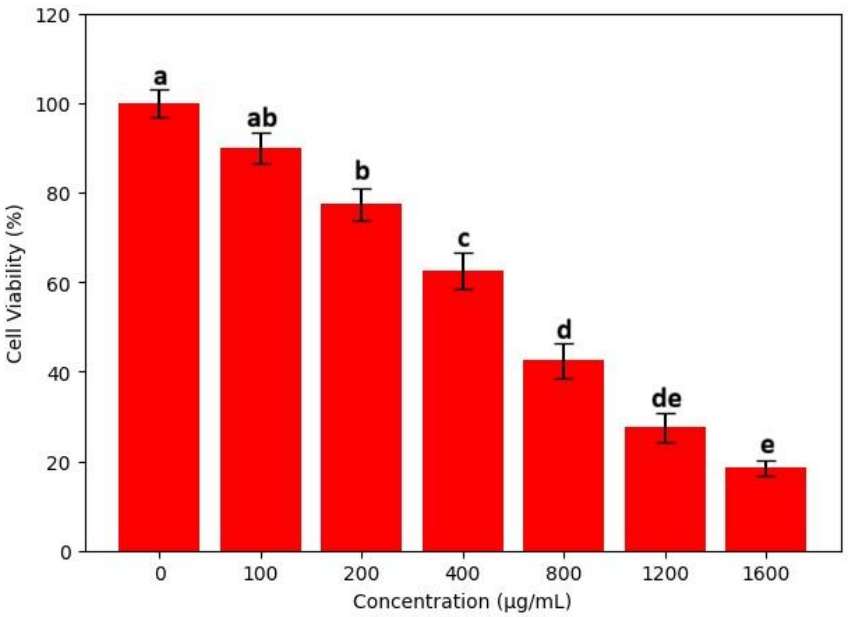

Fig. 3. Concentration-dependent effect of Polygonum aviculare methanolic leaf extract (100–1600 μg/mL) on HT-29 cell viability after 72 h. Mean ± SEM (n = 3); different letters denote significant differences (Duncan's test, $p < 0.05$).

Table 2. Comparative overview of the cytotoxic effects of Polygonum aviculare methanolic leaf extract on HT-29 colorectal cancer cells at 24 h, 48 h, and 72 h.

Main Parameter	24 Hours	48 Hours	72 Hours	Overall Trend with Increasing Incubation Time
IC_{50} (μg/mL)	Highest value	Intermediate	Lowest value	Progressive decrease (increasing potency over time)
Cell viability at low concentrations (near control)	Very high, no significant difference	High, mild reduction	Moderate to high, earlier reduction	Gradual and earlier decline with prolonged exposure
Concentration of onset of significant reduction ($p < 0.05$)	Relatively higher	Intermediate	Lowest concentration	Onset of effect at lower concentrations as time increases
Cell viability at high concentrations	Moderate to notable reduction	Severe reduction	Very severe reduction	Maximum inhibition becomes more pronounced over time
Maximum inhibition rate (%)	Moderate	High	Highest	Marked increase in maximum inhibition with time
Overall effect intensity	Primarily concentration-dependent	Concentration- and time-dependent	Strongest time-dependent cytotoxicity	Progressive sensitization of cells to the extract

4 | Conclusion

The results of the present study clearly demonstrate that the methanolic leaf extract of Polygonum aviculare exerts significant concentration- and time-dependent cytotoxic effects on HT-29 colorectal adenocarcinoma cells. The progressive reduction in IC_{50} values from 550 μg/mL at 24 hours to 390 μg/mL at 72 hours, together with earlier onset of significant cell death and substantially higher maximum inhibition at prolonged exposure times, indicates strong time-dependent sensitization of the cancer cells to the extract. These findings are consistent with the known bioactivity of the plant's major secondary metabolites, particularly phenolic acids (e.g., gallic acid, caffeic acid) and flavonoids (e.g., quercetin and kaempferol derivatives), which are capable of inducing oxidative stress, disrupting mitochondrial membrane potential, activating caspase-mediated pathways, and ultimately triggering apoptosis in cancer cells. Overall, the data suggest that Polygonum aviculare leaf extract possesses promising antiproliferative potential against CRC cells and merits further investigation, including detailed mechanistic studies (e.g., ROS measurement, flow cytometry for apoptosis, Western blot for apoptotic proteins), fractionation of active compounds, and evaluation in animal models. If confirmed in more complex biological systems, this extract could represent a valuable natural source for the development of adjuvant or complementary anticancer agents

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