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Cytotoxic Properties of Methanolic Extracts from *Quercus Infectoria* and *Terminalia Chebula* on A549 and PC12 Cancer Cell Lines: A Comparative Study Based on Tannin-rich and Tannin-free content

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Abstract

Introduction: *Quercus infectoria* and *Terminalia chebula* are traditional medicinal plants in the Middle East, known for their significant therapeutic properties. This study aimed to investigate the cytotoxic effects of tannin-rich and tannin-free methanolic extracts, derived from these plants, on A549 (human lung adenocarcinoma) and PC12 (mouse pheochromocytoma) cell lines.

Methods: The extracts were prepared from air-dried plant tissues using a maceration technique. Cytotoxicity was assessed using four different concentrations (1, 10, 50, and 100 µg/mL) using Trypan Blue and WST-1 assays. The half-maximal inhibitory concentrations (IC50) were determined for both cell lines.

Results: The IC50 values revealed that *Quercus infectoria* exhibited stronger cytotoxic activity compared to *T. Chebula*. In A549 cells, *Quercus infectoria* showed IC50 values of 80 µg/mL (tannin-rich) and 102 µg/mL (tannin-free), whereas *Terminalia chebula* demonstrated 104 µg/mL (tannin-rich) and 111 µg/mL (tannin-free). For PC12 cells, the IC50 values for *Quercus infectoria* were 68 µg/mL (tannin-rich) and 103 µg/mL (tannin-free), while *Terminalia chebula* recorded 80 µg/mL (tannin-rich) and 129 µg/mL (tannin-free).

Conclusion: This study confirms that tannin-rich extracts exhibited greater cytotoxic effects than their tannin-free counterparts, emphasizing the role of tannins in influencing anticancer activity. The findings suggest that tannin-mediated toxicity is a key factor in the observed differences, providing valuable insights into the mechanistic contributions of tannins to cancer cell suppression, which warrants further research into their bioactive mechanisms.

Keywords: *Quercus infectoria*, *Terminalia chebula*, A549, PC12, Cytotoxicity

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Introduction

Cancer remains one of the most critical global health challenges, claiming millions of lives annually. Although medical technology and therapeutic strategies have advanced, the demand for effective and less toxic anticancer agents persists (1, 2). Due to the side effects associated with conventional chemotherapy, as well as emerging approaches that stimulate the innate immune system to selectively target cancer cells, extensive research has focused on novel anticancer agents (3, 4).

Numerous herbal compounds have been demonstrated to have anticancer properties. For example, green tea has been reported to possess significant anticancer activity (5-7). Curcumin, the active component of turmeric, has shown

the ability to modulate various cell signaling pathways, affecting gene expression related to viability, proliferation, invasion, and angiogenesis (8, 9). It was reported that *Salvia Officinalis* exhibits antioxidant and anticancer characteristics (10-12). Additionally, phenolic compounds such as flavonoids and some small bioactive molecule, like genistein (an isoflavone angiogenesis inhibitor), lycopene (a carotenoid found in many fruits), resveratrol (a stilbene found in grape berries and also in wines), as well as plant-derived metabolites like pregnane glycosides (e.g. desmiflavaside D) and alkaloids (e.g. mahanine), have all been reported to exert anticancer effects (13).

Quercus infectoria, an evergreen shrub known commercially as galls or nut-galls, has traditionally been used to treat chronic diarrhea, dysentery, hemorrhages, and sore throats. Its bark and acorns have also been applied in the treatment of impetigo and eczema (14). Ethanolic extract of *Quercus infectoria* displays antioxidant and free radical scavenging properties (14). Another medicinal plant, *Terminalia chebula* (Family: Combretaceae), holds significance in Indian and Iranian traditional medicine and is used for conditions such as dementia, constipation, and diabetes. It also possesses ophthalmic, cardiopulmonary, gastrointestinal, dermatologic and antidiabetic, antioxidant, antibacterial, and wound healing properties (15-17). Additionally, aqueous extracts of *Terminalia chebula* demonstrated antiviral activity against hepatitis B virus (HBV) (18).

Studies have linked specific plant extracts to cytotoxic effects in A549 and PC12 cell lines (19-22). For instance, Willow bark extract has been shown to inhibit proliferation and apoptosis in colon and lung cancer cells (A549 and HT29). Salicin, a compound isolated from Willow bark, has also been associated with tumor growth suppression and anti-angiogenic activity (20, 23). Meanwhile, PC12 cells were significantly protected against Aβ-induced cell death, lactate dehydrogenase release, DNA damage, mitochondrial dysfunction, and cytochrome c release by pretreatment with *Acorus tatarinowii* Schott extract (ATSE). Pretreatment with ATSE also significantly inhibited Aβ-induced caspase-3 activation and reactive oxygen species (ROS) generation in PC12 cells, further confirming its neuroprotective effects. Moreover, oligosaccharides (P6) extracted from *Morinda officinalis* have been proposed to exert antidepressant effects by preventing corticosterone (Cort)-induced apoptosis in PC12 cells (24, 25).

Despite growing interest in these botanical sources, there are few comprehensive studies assessing their cytotoxic effects on specific cancer cell lines. Previous research has reported certain anticancer effects of plant extracts on A549 and PC12 cell lines, suggesting their therapeutic potential (20, 23-25). However, there is a notable gap in studies specifically investigating the effects of *Quercus infectoria* and *Terminalia chebula* on these cell lines.

This study aimed to address that gap by evaluating the cytotoxic effects of methanolic extracts of *Quercus infectoria* and *Terminalia chebula* on A549 and PC12 cells, emphasizing the impact of tannin-rich versus tannin-free fractions. Elucidating their mechanisms and efficacy could support the development of innovative plant-based anticancer therapies.

Methods

Ethical Considerations

This research was conducted in compliance with the ethical standards of the Ethics Committee of Kerman University of Medical Sciences. The approval number issued for the study is IR.KMU.REC.1388.80.

Preparation of methanolic extracts

Quercus infectoria and *Terminalia chebula* specimens were collected from Kerman, Iran, and authenticated at the Herbal Medicines Research Center, Faculty of Pharmacy, Kerman University of Medical Sciences. Methanolic extracts were prepared by grinding 20 g of air-dried plant tissue and immersing it in 200 mL of methanol for 24 hours at room temperature. The resulting mixtures were filtered, air-dried, and stored in dark vials at -20°C until use. Before testing, the extracts were dissolved in 0.5% dimethyl sulfoxide (DMSO) (26). Given that both *Quercus infectoria* and *Terminalia chebula* extracts are rich in tannins, which bind to cell proteins and inhibit their function, the extracts were divided into two categories: tannin-rich and tannin-free. Tannin removal was performed using the method described by Nyman et al. (27).

Tannin test

The tannin test was conducted by extracting 5 g of dried plant material using 50 mL of water, ethanol (96%), or acetone. After solvent evaporation, the residues were re-dissolved in 13 mL of hot water (90–100°C) and cooled at room temperature. Two drops of 10% NaCl were added to precipitate non-tannin compounds that could lead to false positives. After vacuum filtration, 3 mL of the filtrate was added to each of four test tubes. The following solutions were then added:

1. 4-5 drops of 1% gelatin solution,
2. 4-5 drops of 1% gelatin+10% NaCl solution,
3. 3-4 drops of 10% ferric chloride solution,
4. Control (no additives).

A negative test result is indicated if no precipitation appears in test tubes 1 and 2 or if test tube 3 shows no color formation. A positive result occurs when tubes 1 and 2 show precipitation, and tube 3 exhibits color changes—blue-black for hydrolysable tannins or brownish-green for condensed tannins.

Samples testing positive for tannins were re-extracted using water, ethanol, or acetone. After solvent removal, the residue was dissolved in hot distilled water (5 mg/mL). From this solution, 1 mL was mixed with 4 mL of 1% gelatin solution and centrifuged. The resulting supernatant was used for testing.

Cell culture

A549 and PC12 cell lines were obtained from the Iranian National Cell Bank at the Pasteur Institute. Cells were

cultured in DMEM/F12 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 50 µg/mL streptomycin, maintained at 37°C in a humidified incubator with 5% CO_2.

Viability cell assay test

Cells were washed with phosphate-buffered saline (PBS), detached using Trypsin-EDTA, and resuspended in fresh culture medium. Approximately 3×10^5 cells were seeded in microplates and incubated for 24 hours. The extracts were added at concentrations of 1, 10, 50, and 100 µg/mL, and cell viability was evaluated after 48 hours using the Trypan Blue assay and a hemocytometer. Methotrexate (0.1 µM, Sigma, BCBV8405) was used as a positive control (28).

Cytotoxicity assays

Cells (1×10^4) were seeded into each well of 96-well culture plates and incubated for 24 hours to allow for adhesion. The extracts were dissolved in PBS and DMSO, and four concentrations (1, 10, 50, and 100 µg/mL) were added to each well. Each concentration was tested in triplicate, and the means were reported as final results. The plates were then incubated for 48 hours, followed by assessment of cellular proliferation using the WST-1 reagent (Roche). Moreover, optical absorbance was measured at two time points: immediately and after 1 hour, by reading absorbance at 450 nm with a reference wavelength of 630 nm, using a Biotek Elisa Reader (29-31).

Statistical analysis

Each set of experiments was performed in triplicate, and data were presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value less than 0.05 was considered statistically significant. Data were analyzed using SPSS software (version 22).

Results

Trypan blue

The cytotoxic effects of methanolic extracts from *Quercus infectoria* and *Terminalia chebula* were evaluated using the Trypan Blue method. The findings, summarized in Tables 1 and 2, reveal significant reductions in cell viability in both A549 and PC12 cell lines across the tested extract concentrations compared to the untreated control group.

WST-1 assay

The cytotoxic effects of two methanolic extracts—*Quercus infectoria* and *Terminalia chebula*—were examined using the WST-1 tetrazolium-based assay. The results for varying concentrations of tannin-rich and tannin-free extracts on A549 and PC12 cell lines are summarized in Tables 3 and 4. Both tables demonstrate statistically significant differences when compared to the negative control group.

Similarly, the WST-1 assay results, presented in Tables 3 and 4, confirm the cytotoxic effects of the extracts on both A549 and PC12 cell lines. Inhibitory concentration of 50% (IC_{50}) values were as follows: 68 µg/mL for PC12 (tannin-rich *Quercus infectoria*), 103 µg/mL for PC12 (tannin-free *Quercus infectoria*), 80 µg/mL for PC12 (tannin-rich *Terminalia chebula*), 129 µg/mL for PC12 (tannin-free *Terminalia chebula*), 80µg/mL for A549 (tannin-rich *Quercus infectoria*), 102 µg/mL for A549 (tannin-free *Quercus infectoria*), 104 µg/mL for A549 (tannin-rich *Terminalia chebula*), and 111 µg/mL for A549 (tannin-free *Terminalia chebula*).

Discussion

The present study assessed the potential cytotoxic effects of *Quercus infectoria* and *Terminalia Chebula*, as natural herbal medicines, on the survival and proliferation of cancer-derived cell lines PC12 and A549. Both plants exhibit a wide range of medicinal properties, including

Table 1. Inhibitory Effect of Tannin-Rich and Tannin-Free Methanolic Extracts from Quercus infectoria and Terminalia chebula on PC12 Cell Growth via Trypan Blue Assay

Group		concentration	
Control		1030000 ± 35118	
Methotrexate	0.1 µmol/ml	620000 ± 15275[b]	
		Type of methanolic extract	
		Tannin-rich	Tannin-free
Terminalia chebula	1 µg/ml	988666 ± 5364	990666 ± 4055
	10 µg/ml	900000 ± 11547[b]	967666 ± 2848
	50 µg/ml	656666 ± 14529[b]	806666 ± 8819[b]
	100 µg/ml	413333 ± 8819[b]	693666 ± 6839[b]
Quercus infectoria	1 µg/ml	988666 ± 4666	991666 ± 6009
	10 µg/ml	876666 ± 18559[b]	923333 ± 8819[b]
	50 µg/ml	643333 ± 14529[b]	710000 ± 17320[b]
	100 µg/ml	303333 ± 8819[b]	580000 ± 15275[b]

Data are expressed as mean ± SEM. Statistical significance was determined using ANOVA with Tukey's post-hoc test. b: $P < 0.05$ indicates a significant difference compared to the control.

Table 2. Inhibitory Effect of Tannin-Rich and Tannin-Free Methanolic Extracts from Quercus infectoria and Terminalia chebula on A549 Cell Growth via Trypan Blue Assay

Group		Concentration	
Control		1730000 ± 35118	
Methotrexate	0.1 µmol/ml	873333 ± 14529[b]	
		Type of methanolic extract	
		Tannin-rich	Tannin-free
Terminalia chebula	1 µg/ml	1693333 ± 881	1685000 ± 2886
	10 µg/ml	1640000 ± 5773[b]	1613333 ± 8819[b]
	50 µg/ml	1250000 ± 28867[b]	1411666 ± 7264[b]
	100 µg/ml	990000 ± 5773[b]	1013333 ± 8819[b]
Quercus infectoria	1 µg/ml	1630000 ± 17320[b]	1684333 ± 2962
	10 µg/ml	1592000 ± 3605[b]	1609333 ± 5811[b]
	50 µg/ml	1073333 ± 37118[b]	1310000 ± 20816[b]
	100 µg/ml	653333 ± 17638[b]	940000 ± 5773[b]

Data are presented as mean ± SEM. Statistical analysis was performed using ANOVA with Tukey's post-hoc test. b: $P < 0.05$ signifies significant differences in comparison with the control group

Table 3. Cytotoxic Effect of Tannin-Rich and Tannin-Free Methanolic Extracts from Quercus infectoria and Terminalia chebula on PC12 Cell Growth via WST-1 Assay

Group		Concentration	
Control		**0.842 ± 0.00**	
		0.455 ± 0.00 [b]	
Methotrexate	**0.1 µmol/ml**	**Type of methanolic extract**	
		Tannin-rich	**Tannin-free**
Terminalia chebula	1 µg/ml	0.700 ± 0.00[b]	0.812 ± 0.00
	10 µg/ml	0.667 ± 0.00[b]	0.763 ± 0.00[b]
	50 µg/ml	0.426 ± 0.00[b]	0.636 ± 0.00[b]
	100 µg/ml	0.401 ± 0.00[b]	0.550 ± 0.01[b]
Quercus infectoria	1 µg/ml	0.673 ± 0.00[b]	0.811 ± 0.00
	10 µg/ml	0.600 ± 0.00[b]	0.733 ± 0.01[b]
	50 µg/ml	0.384 ± 0.00[b]	0.596 ± 0.00[b]
	100 µg/ml	0.291 ± 0.00[b]	0.516 ± 0.01[b]

Optical density (OD) values are displayed as mean ± SEM. Statistical significance was assessed using ANOVA (Tukey's post-hoc test). b: $P<0.05$ denotes a statistically significant difference relative to the control.

Table 4. Cytotoxic Effect of Tannin-Rich and Tannin-Free Methanolic Extracts from Quercus infectoria and Terminalia chebula on A549 Cell Growth via WST-1 Assay

Group		Concentration	
Control		**1.996 ± 0.002**	
		1.220 ± 0.005[b]	
Methotrexate	**0.1 µmol/ml**	**Type of methanolic extract**	
		Tannin-rich	**Tannin-free**
Terminalia chebula	1 µg/ml	1.996 ± 0.025	2.006 ± 0.075
	10 µg/ml	1.479 ± 0.005[b]	1.902 ± 0.044
	50 µg/ml	1.380 ± 0.043[b]	1.710 ± 0.028[b]
	100 µg/ml	1.138 ± 0.006[b]	1.482 ± 0.037[b]
Quercus infectoria	1 µg/ml	1.996 ± 0.027	1.981 ± 0.004
	10 µg/ml	1.720 ± 0.025[b]	1.817 ± 0.009[b]
	50 µg/ml	1.228 ± 0.013[b]	1.700 ± 0.006[b]
	100 µg/ml	0.461 ± 0.033[b]	1.510 ± 0.005[b]

OD values are presented as mean ± SEM. ANOVA with Tukey post hoc test was employed for statistical analysis. b: $P<0.05$ indicates a significant difference compared to the control.

antimicrobial, local anesthetic, analgesic, wound healing, and antioxidant activities for *Quercus infectoria* extract, as well as antioxidant and free radical scavenging, hepatoprotective, cardioprotective, antidiabetic, anti-inflammatory, and anti-arthritic, wound healing, and immunomodulatory activities for *Terminalia Chebula* (32, 33).

Tannin is a water-soluble polyphenolic compound derived from various plants. Several studies have linked tannin to human cancers, which could be due to its carcinogenic and mutagenic properties. However, tannins also have potential anti-carcinogenic and anti-mutagenic effects. Despite these findings, it has also been reported that tannins' carcinogenic activity may be associated with other tannin-related components rather than tannins themselves (34). While tannins are implicated in cytotoxicity, certain associated polyphenols and metabolites may also affect cell viability. The difference in activity observed between tannin-rich and tannin-free fractions suggests that these secondary metabolites also contribute, warranting further biochemical analysis.

Regarding all these studies, we separated tannin from both extracts to eliminate its possible side effects and evaluated the effects of tannin-rich and tannin-free fractions on the cell lines. Both Trypan blue and WST-1 assays showed that treatments with *Quercus infectoria* and *Terminalia Chebula* extracts significantly decreased cell viability. Our results are consistent with previous findings. Recently, Gao et al. have reported that gallnuts are considered effective anticancer agents and are used in clinical formulae in China. Their data suggest gallnuts contain several bioactive components, some of which are safe for *in vivo* anticancer and medicinal uses (35).

It is important to note that the antiproliferative effects of both extracts in our study were dose-dependent. Several studies have confirmed the impact of these extracts on various cancer cell lines. An earlier 2010 study demonstrated the antiproliferative effects of *Quercus infectoria* gall crude extract in vitro on two human cancer cell lines, cervical and ovarian. Treatment with ethanolic extract caused morphological changes resulting in cell detachment from the monolayer and exhibited a concentration-dependent effect (36). Additionally, Saleem et al. showed that T. chebula fruit extract treatment decreased cancer cell numbers via induction of cell death and inhibition of cell proliferation and growth. Toxic effects were observed at high concentrations, while growth inhibition occurred at lower doses (37). Moreover, our results indicated that *Quercus infectoria* was more effective than *Terminalia Chebula* in terms of antiproliferation and cell death-inducing activities. Tannin-rich extracts were also more efficacious than tannin-free ones in terms of cell viability.

In 2016, Shankara et al. confirmed the anticancer properties of leaf gall extracts through their cytotoxic potential against BRL3A, MCF-7, and A-549 cells (38). The aqueous extract of *Terminalia chebula* was found to inhibit A549 lung cancer cell growth and induce morphological changes (39).

The cytotoxic effects of both extracts were evaluated using Trypan blue and WST-1 assays. Although cell death can be assessed by Trypan blue, some cells may be weak without dying, requiring evaluation by the WST-1 assay to measure the degree of cell viability reduction caused by extract treatment.

A notable factor that has been measured was the lethality percentage of extracts (via IC50). It is important to consider that small differences between IC50 values of tannin-rich and tannin-free extracts could arise from variation in the extracts themselves or other specific cytotoxic and anticancer constituents. Therefore, more detailed studies are recommended to identify novel bioactive constituents and uncover their mechanism in anticancer and antiproliferative effects beyond tannin.

The cytotoxic effects observed in this study reinforce

the hypothesis that tannins play a significant role in modulating cancer cell viability. While previous research has demonstrated the general toxicity of *Quercus infectoria* and *Terminalia Chebula* extracts on A549 and PC12 cells, our findings uniquely differentiate between tannin-rich and tannin-free fractions. The IC50 values indicate that tannin-rich extracts consistently exhibited stronger cytotoxic effects, suggesting that tannins contribute directly to cell death mechanisms.

Tannins have been reported to induce apoptosis and oxidative stress in cancer cells, potentially through mitochondrial dysfunction and reactive oxygen species generation (35). However, the differential activity between tannin-rich and tannin-free fractions suggests that additional polyphenolic compounds may also contribute to the observed cytotoxicity. This is consistent with the results of previous studies indicating that gallotannins and ellagitannins exert selective cytotoxic effects on tumor cells (35, 40). The enhanced potency of tannin-rich extracts in our study further supports the notion that tannins may act as primary bioactive agents in plant-derived anticancer therapies.

Given these findings, future research should focus on isolating specific tannin compounds responsible for cytotoxicity and elucidating their molecular mechanisms. Understanding how tannins interact with cellular pathways could pave the way for novel therapeutic applications targeting cancer cell proliferation and survival.

Despite the promising findings, this study is limited by the lack of in vivo validation of the observed cytotoxic effects. Future studies should aim to elucidate the mechanisms of action and therapeutic potential of these extracts in animal models and clinical settings. Additionally, isolation and characterization of specific bioactive compounds within the extracts are warranted to enhance our understanding of their anticancer properties.

Conclusion

This study demonstrates that both *Quercus infectoria* and *Terminalia Chebula* possess significant anticancer properties, with *Quercus infectoria* exhibiting superior efficacy. The findings indicate that these extracts could be further explored for their potential use in cancer therapy. Future research should focus on isolating active compounds and understanding their mechanisms to enhance therapeutic applications.

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Authors' Contribution

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Competing Interests

The authors declare that they have no conflict of interest.

Data Availability Statement

All necessary data will be available from the corresponding author upon reasonable request.

Ethical Approval

This study was conducted in accordance with the guidelines of the Ethics Committee of Kerman University of Medical Sciences (Ethical code: IR.KMU.REC.1388.80).

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