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Investigation of the antimicrobial and cytotoxic effects of *Viscum album* (mistletoe) extracts

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ABSTRACT

The increasing resistance to antibiotics in the fight against infections, coupled with the rising incidence of cancer, has intensified interest in investigating plant-based antimicrobial agents with potential anticancer properties. *Viscum album* (mistletoe), which parasitizes host trees, has anticancer, anti-inflammatory, immunomodulatory, antiviral and antimicrobial effects. The toxicity of the active substances in different *V. album* samples depends on the type of host tree and the geographical location of the plant. This study investigated the *in vitro* antimicrobial properties of ethanolic, and aqueous extracts derived from the leaves and stems of *V. album* on specific bacterial and fungal species, as well as their cytotoxic potential on cancer cells. The findings revealed that these extracts demonstrated differing levels of antibacterial effects against multiple Gram-positive and Gram-negative bacterial species. Notably, the *V. album* ethanolic stem extracts were found to have a stronger inhibitory effect on *B. subtilis* (1.2 mg/mL) and *B. cereus* (2.4 mg/mL) compared to other extracts. Furthermore, *V. album* extracts were found to exhibit cytotoxic effects against HeLa cells. Based on these findings, the natural components contained in the *V. album* plant may represent a potential source of antimicrobial and anticancer agents. Unlike previous studies, this work provides a comparative

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evaluation of solvent- and tissue-dependent biological activity profiles of *V. album* extracts obtained from a single host species.

Keywords: antimicrobial, antifungal, HeLa cells, cytotoxic effect, *Viscum album* (mistletoe)

INTRODUCTION

V. album, a hemi-parasitic species belonging to the *Santalaceae*, has been used in traditional therapeutic practices for centuries in the treatment of various health-related conditions[1]. Its use as an adjuvant in cancer treatment has attracted particular attention, and research in this field has gained momentum[2]. However, the biological activities of *V. album* are not limited to its anticancer properties. The plant also exhibits anti-inflammatory, immunomodulatory, antiviral, and antimicrobial properties due to the various bioactive compounds it contains (such as lectins, viscotoxins, flavonoids, phenolic acids, and terpenoids)[3,4].

Within ecosystems, *V. album* stands out due to its parasitic lifestyle on host trees. This interaction may negatively influence host vitality by modifying the acquisition of water and mineral nutrients[5]. The fruits of *V. album* are an important food source for certain bird species and aid the dispersal of their seeds by birds, both for animals and humans[6]. Interest in the potential health benefits of the plant has increased due to its use in traditional medicine. In particular, there is now a greater need for plant-based antimicrobial agents to combat infections. This highlights the importance of researching the antibacterial and antimycotic potential of *V. album*[7,8].

Although the antimicrobial and cytotoxic properties of *V. album* have been previously reported, comparative studies evaluating the combined effects of plant part and extraction solvent remain limited. In particular, data comparing leaf and stem extracts derived from the same host tree are scarce. Therefore, this study provides a comparative evaluation of aqueous and ethanolic extracts obtained from different tissues of *V. album* collected from a single host species, integrating antimicrobial, antifungal, and cytotoxic analyses within the same experimental framework.

METHODOLOGY

The *Viscum album* L. plants used in our research were collected from poplar trees in a field in Istanbul's Çatalca district in October 2022 by researcher Kemal Metiner. The species was identified by Gülnur Ekşi Bona, an associate

professor in the Department of Pharmaceutical Botany at the Faculty of Pharmacy, Istanbul University-Cerrahpaşa. The extraction of *V. album* L. was performed according to a previously described method. The plant leaves and stems were dried in an oven at 40°C for 12 hours, then ground into a fine powder using an electronic mill (IKA Werke M20)[9].

For extraction procedures, 20 g each of leaf and stem samples were incubated in a water bath at 45°C for 12 hours with 200 mL of distilled water or 200 mL of ethanol. After filtering the extracts through filter paper (Whatman No. 1), the samples extracted with distilled water were lyophilized using a lyophiliser (Christ, Alpha 2-4 LD plus) at -60°C until they were completely powdered. The ethanol in the ethanolic extracts was evaporated under vacuum using a rotary evaporator (Heidolph Hei-VAP Advantage). After drying, the extraction yields were calculated according to the specified formula:

Extraction Yield (%) = Extract weight (g) / 20 g plant material x 100

Antibacterial activity tests

The Agar Dilution method was used to determine the antibacterial activity of the extract, and the minimum inhibitory concentration (MIC) values were determined as reported by the Clinical and Laboratory Standards Institute (CLSI)[10]. In the study, *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11778) were used as Gram-positive bacteria, and *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 12453) and *Klebsiella pneumoniae* (ATCC 4352) strains were used as Gram-negative bacteria.

The ethanolic extract of *V. album* leaves at concentrations of 0.004 mg/mL – 20.8 mg/mL, the aqueous extract at concentrations of 0.003 mg/mL – 19.4 mg/mL, and the ethanolic extract of *V. album* stems at concentrations of 0.004 mg/mL – 24 mg/mL and the aqueous extract at concentrations of 0.003 mg/mL – 16.7 mg/mL were prepared.

A 1 mL sample of the solution was poured into 6 cm Petri dishes. Muller-Hinton agar, heated to 45–50°C, was then added and stirred in a circular motion. The mixture was then left to cool at room temperature. Suspensions equivalent to 0.5 McFarland turbidity were prepared from 18–24-hour cultures of bacterial isolates to be tested on nutrient agar in a 0.9% sterile physiological saline solution. These suspensions were then diluted 1:10 with sterile physiological saline solution to achieve an inoculum density of 10^7 CFU/mL. A 2 µL aliquot was taken from the bacterial suspension using a replicator (with 3 mm diameter needles) and inoculated onto the agar plates by dipping.

The inoculum was then allowed to dry at room temperature for 10 minutes and then incubated for 24 hours at 37°C under aerobic conditions. After the incubation period, the lowest dilution at which no growth was observed in the cultures evaluated was determined as the MIC value. Antimicrobial assays were performed in duplicate due to resource constraints. Although triplicate testing is recommended, duplicate measurements were considered sufficient for comparative evaluation under standardized conditions. Nevertheless, future studies should include increased replication to enhance statistical robustness and reproducibility of the findings. Gentamicin was used as a positive control.

Antimycotic activity tests

The Liquid Macrodilution method was used to determine the antifungal effects of the plant extract against yeasts and fungi, and MIC values were determined as reported by the Clinical and Laboratory Standards Institute (CLSI)[11].

In this study, *Candida albicans* (ATCC 10231), *Pichia kudriavzevii* (*Candida krusei*; ATCC 6258), *Candida tropicalis* (ATCC 750), *Candida globrata* (ATCC 2001), *Mallassezia pachydermatis* (Clinical Isolate*), Microsporum canis* (Clinical Isolate), and *Trichophyton mentagrophytes* (Clinical Isolate) strains were used. The fungal strains used in the study were obtained from the Culture Collection of the Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa. To ensure the viability of the strains, yeasts were incubated at 37°C for 48 hours and fungi at 25°C for 7 days on Sabouraud Dextrose Agar (SDA) medium. RPMI-1640 medium was used for dilutions. Amphotericin B was used as a positive control. For the determination of MIC values using the liquid macrodilution method, two-fold dilutions of the plant extract were prepared as described by the CLSI (2017), and 500 µl of these suspensions were transferred to sterile tubes[10]. A 5 mL suspension was prepared from the colonies of the fungal species to be tested using 0.9% sterile physiological saline solution. The suspension was allowed to stand for 15 minutes to allow large particles to settle to the bottom, and the homogeneous conidia suspensions on the surface were transferred to another sterile tube. The suspension was stirred for 15 minutes to achieve homogeneity. The prepared yeast and fungal suspensions were added in equal amounts to the suspensions previously distributed in the tubes. Dilutions containing yeast inoculum were incubated at 37°C for 48 hours, while those containing fungal inoculum were incubated at 25°C for 7 days. The minimum extract concentration inhibiting growth was determined by observing with the naked eye or using a McFarland densitometer to determine the MIC values.

Determination of cytotoxic effect

The MTT assay, a colorimetric method for measuring cell viability, was used to evaluate the cytotoxic effects of the ethanolic and aqueous extracts of *V. album* leaves on the HeLa ATCC (CRM-CCL-2) cervical cancer cell line. HeLa cell line was chosen in this study, for its reliability and rapid growth, specifically its short doubling time and low growth factor requirements[12]. The human cervical carcinoma HeLa cell line is among the most widely used in cytotoxicity studies[13]. Its use allows for a direct comparison of our results with existing literature regarding the anticancer potential of natural products of *V. album*.

MTT cell viability assay

HeLa cells (ATCC CRM-CCL-2) were obtained from Istanbul University-Cerrahpaşa Institute of Nanotechnology and Biotechnology. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37°C in a 5% CO_2 incubator. All chemicals used in cell culture were obtained from Invitrogen. The cell culture medium was changed every other day, and when the cells reached 80-90% confluence, they were passaged by detaching them with a 0.25% trypsin-EDTA solution. The cytotoxic effects of the extracts were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this purpose, cells were inoculated into 96-well plates at a density of $1x10^4$/well and allowed to adhere for 24 hours at 37°C in a humid atmosphere containing 5% CO_2. Serial dilutions of the ethanolic and aqueous extracts of *V. album* at concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL were prepared with the culture medium and added to each well. Wells containing only cells and culture medium were used as controls. The plates were incubated for 24 and 48 hours. Following the incubation periods, 10 µl of a 5 mg/mL MTT (ABCR, Germany) solution was added to each well and incubated at 37°C in a 5% CO_2 incubator for 4 hours. Thereafter, the medium in the wells was aspirated and removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were shaken for 10 minutes, and the absorbance of each well was measured at a wavelength of 595 nm using a microplate reader (FilterMax F5, Molecular Devices, USA). Each extract concentration was assessed in triplicate and the experiment was repeated twice. Control cells were considered 100% viable. The following equation was used to determine the cytotoxic effect of *V. album* extracts[14]: $\%Viability = 100 \times [(A_{sample} - A_{Blank})/ (A_{Control} - A_{Blank})]$; in this equation, A_{sample} refers to the absorbance of tested compound, A_{Blank} shows well containing only medium and $A_{Control}$ demonstrates untreated cells.

Based on the data obtained, the corresponding cell viability percentages for the doses were determined, and the concentrations inhibiting the growth of 50% of the cells (IC50 values) were calculated using the Calcusyn programme.

Statistical analysis

Statistical analyses of the data obtained from the MTT test were performed using IBM SPSS Statistics software, version 31.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was applied to evaluate the differences between groups, and Duncan's multiple comparison test was used to determine the differences between group means. The statistical significance level was set at $p<0.05$.

RESULTS and DISCUSSION

The extraction yields from *V. album* leaves were calculated as 12.2% for aqueous and 10.3% for ethanolic extracts, while the corresponding yields from the stems were 5.4% and 6.5%, respectively.

The antibacterial activity of ethanolic and aqueous preparations obtained from the leaf and stem tissues of *V. album* was evaluated against selected bacterial isolates representing both Gram-positive and Gram-negative groups. The bacterial species examined included *S. aureus, S. epidermidis, B. subtilis, B. cereus, E. coli, P. mirabilis*, and *K. pneumoniae*. A summary of the MIC values is provided in Table 1.

Table 1. Evaluation of MIC levels of *V. album* extracts in selected bacterial and fungal organisms (mg/mL)

	V. album **Ethanolic Leaf Extract (mg/mL)**	***V. album*** **Aqueous Leaf Extract (mg/mL)**	***V. album*** **Ethanolic Stem Extract (mg/mL)**	***V. album*** **Aqueous Stem Extract (mg/mL)**	**Gentamicin (µg/mL)**	**Amphotericin (µg /mL)**
S. aureus (ATCC 29213)	20.8	19.4	24	16.7	0.05	-
S. epidermidis (ATCC 12228)	20.8	19.4	24	16.7	0.05	-
B. cereus (ATCC 11778)	20.8	19.4	2.4	16.7	0.4	-
B. subtilis (ATCC 6633)	20.8	No activity detected	1.2	16.7	0.025	-
P. mirabilis (ATCC 12453)	20.8	19.4	24	16.7	0.05	-
K. pneumoniae (ATCC 4352)	20.8	19.4	24	16.7	0.4	-
E. coli (ATCC 25922)	20.8	19.4	24	16.7	0.1	-
C. albicans (ATCC 10231)	20.8	19.4	24	16.7	-	8
P. kudriavzevii (C. krusei; ATCC 6258)	20.8	19.4	24	16.7	-	8
C. tropicalis (ATCC 750)	20.8	19.4	24	16.7	-	8
C. globrata (ATCC 2001)	20.8	19.4	24	16.7	-	8
M. pachydermatis (Clinical Isolate)	20.8	19.4	24	16.7	-	8
M. canis (Clinical Isolate)	20.8	19.4	24	16.7	-	0.125
T. mentagrophytes (Clinical Isolate)	20.8	19.4	24	16.7	-	8

According to our results, all *V. album* extracts were determined to exhibit inhibitory effects on the tested bacterial strains to varying degrees. The ethanolic leaf extract of *V. album* inhibited all examined Gram-positive (*S. epidermidis, S. aureus, B. subtilis, B. cereus*) and Gram-negative (*E. coli, K. pneumoniae, P. mirabilis*) bacterial strains, though this activity was observed at a relatively high MIC value of 20.8 mg/mL. The aqueous leaf extract of *V. album* showed no antibacterial activity against *B. subtilis* at the concentrations studied. However, it exhibited inhibitory effects against all remaining bacterial strains at an MIC value of 19.4 mg/mL. The *V. album* ethanolic stem extract exhibited the lowest MIC values compared to other extracts against *B. subtilis*

(1.2 mg/mL) and *B. cereus* (2.4 mg/mL), indicating stronger activity against these two bacteria. However, significantly higher MIC values (24 mg/mL) were detected against *S. aureus, S. epidermidis, E. coli, K. pneumoniae* and *P. mirabilis* strains. The aqueous stem extract of *V. album* demonstrated a comparatively uniform antibacterial effect across all tested bacterial strains, with an MIC value of 16.7 mg/mL.

Our study revealed that higher yields were obtained from leaf aqueous extracts and stem ethanolic extracts. This suggests that differences in active components and solvent polarity in plant tissues affect extraction yield. Using the same extraction method[9], *V. album* samples collected from different host trees yielded extraction rates ranging from 4% to 56.7%. This wide range supports the hypothesis that differences in host tree species can significantly affect the chemical composition of the plant and, consequently, the extraction yield[9]. The *in vitro* antibacterial activity of extracts obtained from different tissues of *V. album*, produced with polar solvents, was evaluated by determining minimum inhibitory concentration (MIC) values against multiple bacterial species representing both Gram-positive and Gram-negative groups. The findings indicated that *V. album* extracts had different levels of antimicrobial potential against the tested bacterial strains. However, these results should be interpreted cautiously, as the relatively high MIC values indicate limited antimicrobial potency of the crude extracts at the tested concentrations and suggest the need for further purification of active constituents.

In line with previous reports of antimicrobial activity in various *V. album* subspecies[15], all extracts in our study exhibited some degree of inhibition against the tested strains. However, the efficacy of the extracts varied significantly depending on the plant part used (leaf or stem), the extraction solvent (ethanol or water) and the target bacterial species. These results align with previous studies suggesting that the antimicrobial efficacy of plant extracts is shaped by factors such as their phytochemical composition, the polarity of bioactive constituents, and the structural characteristics of bacterial cell walls[15,16]. The *V. album* ethanolic leaf extract exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, but only at a relatively high MIC value of 20.8 mg/mL. Earlier research has shown that ethanolic leaf extracts of *V. album* possess antimicrobial activity, particularly against Gram-positive bacteria such as *S. aureus* and *B. subtilis*[7,8]. However, such high MIC values may make it difficult to use the extract alone in clinical applications, or they may indicate that further purification is required. The lack of activity of the aqueous leaf extract against *B. subtilis* may be related to differences in the

extraction method, host plant, or geographic origin. While some studies indicate that *V. album* aqueous extracts exhibit an inhibitory effect on *B. subtilis*[17], this study suggests that factors such as the preparation method of the extract, the geographical region where the plant was collected, or the host plant may influence its antimicrobial profile.

The aqueous extract exhibited antibacterial activity across multiple tested strains at an MIC value of 19.4 mg/mL, indicating broad-spectrum activity at relatively high concentrations. Among the tested preparations, the ethanolic stem extract showed the strongest activity, particularly against Gram-positive bacteria, which may reflect differences in phytochemical composition between plant tissues. These differences in antimicrobial activity are likely associated with variations in phytochemical composition among different plant tissues[18]. Bioactive compounds such as phenolics and flavonoids may exert antimicrobial effects through mechanisms including disruption of cell membrane integrity, interference with enzymatic activity, and inhibition of nucleic acid synthesis. These mechanisms may partially explain the differential activity observed among the extracts.

Gram-negative bacteria generally exhibited higher MIC values than Gram-positive bacteria. This reduced susceptibility can be attributed to their outer membrane structure, including the lipopolysaccharide layer and efflux systems that limit the penetration and accumulation of antimicrobial compounds[19].

The ethanolic stem extract also showed activity against other bacterial strains, although at higher concentrations, indicating a broader but less potent antimicrobial profile. Similarly, the aqueous stem extract exhibited a more uniform antibacterial effect across all tested strains (MIC: 16.7 mg/mL). The relatively lower MIC values observed for some Gram-negative bacteria (e.g., *K. pneumoniae* and *E. coli*) may suggest that water-soluble components from stem tissues contribute to enhanced activity against these organisms[20].

In addition to antibacterial activity, the antifungal potential of the extracts was also evaluated. The antifungal activity of ethanolic and aqueous extracts derived from the leaves and stems of *V. album* was evaluated against selected yeast and fungal species using both liquid microdilution and macrodilution methods. None of the tested extracts demonstrated antifungal activity, as no inhibition of microbial growth was observed even at the highest concentrations tested: 20.8 mg/mL (ethanolic leaf), 19.4 mg/mL (aqueous leaf), 24 mg/mL (ethanolic stem), and 16.7 mg/mL (aqueous stem).

The lack of antifungal activity may be related to insufficient concentrations of antifungal bioactive compounds, limited penetration of these compounds into fungal cell structures, and the influence of solvent type and extraction method. The literature presents mixed results regarding the antifungal activity of *V. album*. The n-hexane extract of *V. album* subsp. *abietis* has been reported to exhibit antifungal activity against *C. albicans*[15]. Similarly, other studies have shown antifungal activity of ethanolic extracts of *V. album* against *Candida guilliermondii*, *Cryptococcus neoformans*, *M. canis*, and *T. mentagrophytes*[21]. These variations may be attributed to differences in species, subspecies, host plants, extraction techniques, and solvents used, all of which can influence the composition of bioactive compounds and the antimicrobial spectrum of the extracts[15,22].

Beyond antimicrobial effects, the cytotoxic potential of the extracts was also investigated. When compared with controls, treatment with ethanolic and aqueous *V. album* extracts did not produce statistically significant effects after 24 hours. Even at the highest tested concentrations, HeLa cell viability did not decrease by 50%. Nonetheless, following 48 hours of exposure, both extracts induced over 50% cell mortality when applied at the highest tested dose (100 μg/mL). The decrease in cell viability observed after 48 hours of extract treatment was significantly greater than that observed after the 24-hour application. The ethanolic stem and aqueous leaf extracts of *V. album* exhibited significantly greater cytotoxic effects on cell viability compared to the aqueous stem and ethanolic leaf extracts, respectively ($p<0.05$), as shown in Figure 1.

Based on the MTT assay results, IC50 values could not be established for cells treated with ethanolic and aqueous extracts derived from the stems and leaves of *V. album* after 24 hours of exposure. Following 48 hours of treatment with ethanolic extracts from the stems and leaves of *V. album*, IC50 values were calculated as 44.031 and 91.733 μg/mL, respectively. For the aqueous extracts, these values were determined to be 59.72 μg/mL for the stems and 61.28 μg/mL for the leaves after 48 hours. Cell viability was gradually reduced in HeLa tumor cell line from 75.21% at 6.25 μg/mL, to 49.65% at 50 μg/mL after 48 hours treatment with *V. album* ethanolic stem extract. Significant differences were observed across concentrations between 12.5 μg/mL and 100 μg/mL relative to the control after 48 hours treatment with *V. album* ethanolic leaf extract ($p<0.05$).

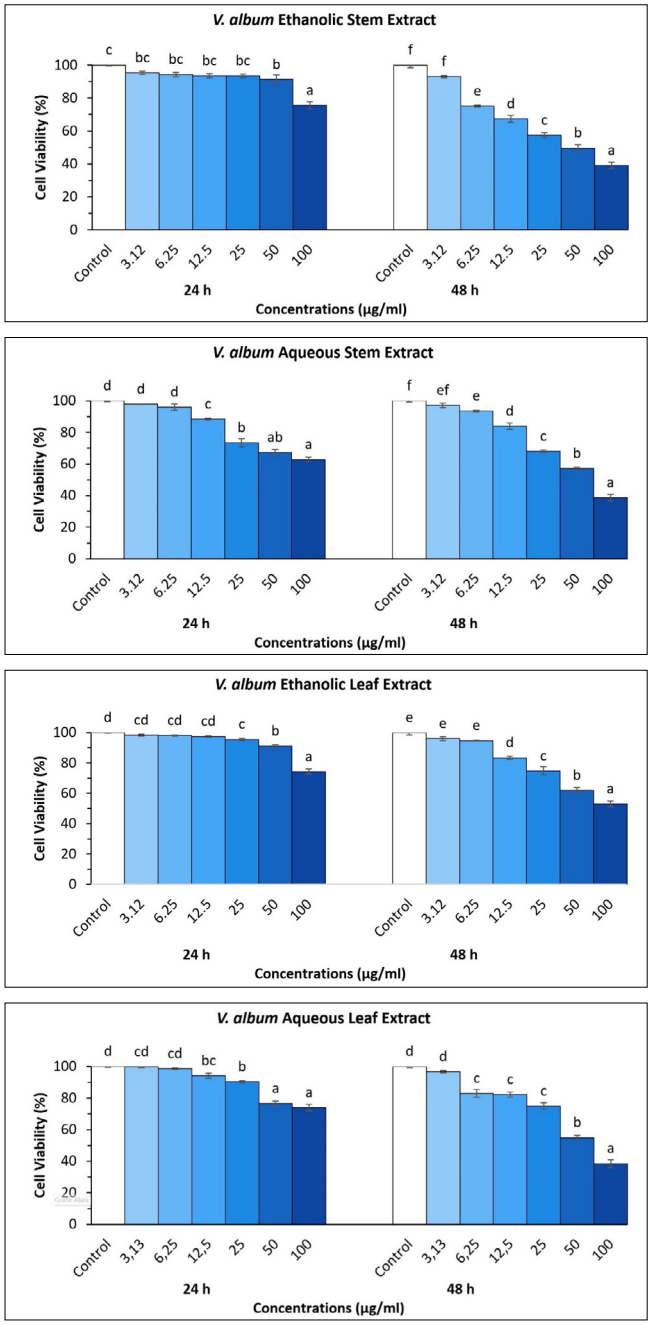

Figure 1. Dose- and time-dependent effects of ethanolic and aqueous *V. album* extracts on HeLa cell viability

Results are presented as mean ± standard deviation (n=3). Statistical significance among experimental groups is indicated by differing superscript letters (one-way ANOVA with Duncan's multiple range post hoc test, $p<0.05$). Corresponding IC_{50} concentrations of ethanolic and aqueous *V. album* extracts in HeLa cells are provided in Table 2.

Table 2. IC_{50} concentrations of the ethanolic and aqueous extracts of *V. album* in the HeLa cell line, as determined by the MTT assay

Treatment	24 hours	48 hours
Ethanolic leaf extract	>100 µg/mL	91.733 µg/mL
Aqueous leaf extract	>100 µg/mL	61.280 µg/mL
Ethanolic stem extract	>100 µg/mL	44.031 µg/mL
Aqueous stem extract	>100 µg/mL	59.723 µg/mL

Both ethanolic and aqueous stem extracts of *V. album* showed moderate cytotoxicity in HeLa cells, with IC50 values of 44.031 µg/mL and 59.723 µg/mL, respectively. The ethanolic and aqueous leaf extracts showed slightly higher IC50 values of 91.733 µg/mL and 61.280 µg/mL, respectively. These findings suggest that both extracts show similar efficiency in isolating cytotoxic bioactive constituents affecting HeLa cells, consistent with the classification criteria of the United States National Cancer Institute (NCI). According to the guidelines of the NCI, substances displaying IC_{50} values under 20 µg/mL are classified as highly cytotoxic, those falling within the 21–200 µg/mL range are described as having moderate cytotoxic effects, compounds with IC_{50} values between 201 and 500 µg/mL are considered weakly cytotoxic, while values above 500 µg/mL are interpreted as lacking cytotoxic activity[23]. Based on this framework, the ethanolic and aqueous extracts of *V. album* leaves and stems demonstrate moderate cytotoxic effects on HeLa cells following 48 hours of exposure. The findings reveal that the stem-derived ethanolic extract exerts a stronger inhibitory effect on cell viability than the corresponding aqueous extract. This difference in cytotoxic response may be associated with variations in the bioactive compound composition of the two extracts. In this context, the ethanolic extract is thought to contain more potent constituents with pronounced anticancer activity. Both extracts exhibited antiproliferative effects on HepG2 and MCF-7 cell lines, with the extract showing a more pronounced cytotoxic effect at 75 mg/mL[24]. Our results are consistent with previous reports showing that the alcoholic stem extract of *V. album* exerts a stronger antiproliferative effect on HeLa cells than the aqueous extract[22].

The ethanolic stem extract of *V. album* exhibited moderate cytotoxic effects in HeLa cells, with an IC_{50} value of 44.031 μg/mL, which was lower than the IC_{50} value of 93 μg/mL previously reported for the methanol extract of *V. album*[25]. Variations in these findings may stem from differences in the origin of the plant material and the type of solvent employed during extraction. The ethanol extract may contain more lectins and viscotoxins, the main cytotoxic constituents of *V. album*, than the methanolic extract[26]. This finding indicates that the selection of the solvent used during extraction plays a critical role in determining the cytotoxic characteristics of the resulting extracts.

Previous research evaluated the antiproliferative effects of the ethanolic extract of *V. album* on normal human fibroblasts and HeLa cells[27]. Their findings revealed that while the extract had a minimal impact on the proliferation of normal fibroblasts, it significantly inhibited the proliferation of HeLa cells. Tetralin derivatives identified in the alcoholic extract, as well as fatty acids such as pentadecanoic, hexadecenoic, and heptadecenoic detected in the aqueous extract of *V. album*, have been reported to possess antimicrobial, antioxidant, and anticancer properties[24]. In our study, the observed cytotoxic activity of the extracts may be attributed to their elevated concentrations of these bioactive constituents.

The antiproliferative effect of *V. album* has been investigated in various cell lines. Although a previous study reported an IC_{50} value of 1090 μg/mL for the cytotoxic effect of the ethanolic extract of *V. album* on A549 lung cancer cells after 72 hours[28], the IC_{50} values obtained in the present study are classified as indicative of moderate cytotoxicity according to the NCI criteria[23,28]. The *in vitro* antiproliferative activity of the methanolic extract of *V. album* has previously been evaluated in a breast carcinoma cell model[29]. An IC_{50} value of 172 mg/L was reported for *V. album* after 24 hours, and aqueous extracts obtained from oak-, apple-, and pine-associated plants were shown to exhibit cytotoxic and anticancer activities in lymphoblastoid and monocytic cell lines[30]. Previous research investigated the antiproliferative and apoptotic effects of *V. album* in various human bladder carcinoma cell lines[31]. It was reported that antiproliferative effects of *V. album* were observed in the four bladder carcinoma cell lines tested and the cytotoxic protein components, including mistletoe lectins and viscotoxins, were shown to suppress the proliferation of bladder carcinoma cells[31]. The antiproliferative potential of a lipophilic extract obtained from *V. album* was evaluated using human leukemia cell line models. Bioactive compounds such as ursolic acid, viscin, betulinic acid, and oleanolic acid have been reported to exert inhibitory effects on cell proliferation and

to induce apoptotic cell death. The reported IC_{50} values ranged from 118 ± 24 to 252 ± 37 μg/mL. These findings suggest that viscin and its associated triterpenoid components may play a key role in mediating the cytotoxic and apoptosis-inducing effects observed in leukemia cell lines[29]. Discrepancies among previous study findings may result from differences in the profile of biologically active constituents, the extraction procedures employed, or the varying sensitivities of the tumor cell lines investigated.

While we acknowledge that the biological activities of *V. album* are documented, the primary novelty of our study lies in in the investigation of *V. album* harvested specifically from poplar trees in the Çatalca region of Istanbul. It is well-established in the literature that the chemical composition, particularly the concentration of viscotoxins, lectins, and phenolic compounds, varies significantly depending on the host tree species and geographical location. Previous findings have demonstrated that the antiproliferative activity of *V. album* varies according to the host tree[27]. In contrast, the present study focused on the poplar-hosted ecotype collected from the Çatalca region. Previous evidence indicates that the phenolic profile and cytotoxic activity of *V. album* are influenced by the host tree, likely through host-dependent variations in secondary metabolite composition that affect antimicrobial and cytotoxic properties[27]. Accordingly, the data obtained from the willow-hosted ecotype contribute to the pharmacological characterization of this species[27]. In the present study, the cytotoxic effect of *V. album* extracts was evaluated specifically using the HeLa cell line. Although HeLa is a widely accepted model for initial anticancer screening due to its high proliferation rate and reproducibility, it is important to acknowledge that the observed effects may be cell-line specific. Therefore, the use of a single cancer cell line is one of the limitations of the current study, as different malignancies may exhibit varying sensitivities to the bioactive compounds found in the aqueous and alcoholic extracts. Despite the promising cytotoxic and antimicrobial activities observed in this study, there are certain limitations that should be acknowledged. The present research focused primarily on the effects of *V. album* extracts on HeLa cell line. However, to fully evaluate the safety and selective toxicity of these extracts, further studies involving healthy human cell lines are required. Assessing the index of selectivity will be a crucial next step to determine the clinical potential and safety profile of the willow-hosted *V. album* extract. Furthermore, the pharmacological potency of *V. album* is highly dependent on its phytochemical profile, which can significantly fluctuate based on the host tree, geographical location, and environmental conditions where the plant was harvested. Variations in secondary metabolites, such as lectins and viscotoxins,

may lead to different therapeutic outcomes. Therefore, future research involving a broader panel of human cancer cell lines, alongside a comparative analysis of extracts from different geographical origins, is warranted to fully elucidate the selective toxicity and broad-spectrum potential of these extracts.

This study demonstrated differential antimicrobial activity among ethanolic and aqueous extracts of *V. album*, with the ethanolic stem extract showing the strongest effect against *B. subtilis* and *B. cereus*. However, the relatively high MIC values and the absence of antifungal activity indicate limited antimicrobial potency of the crude extracts.

The observed antimicrobial and cytotoxic activities of *V. album* extracts are likely associated with known bioactive constituents such as lectins, viscotoxins, flavonoids, phenolic compounds, and terpenoids. Differences between extracts may reflect variations in compound composition related to plant tissue and extraction solvent polarity.

The comparative design of this study supports the novelty of evaluating willow-hosted *V. album* extracts from different tissues within a single experimental framework. In addition, the broader impact of the findings may be considered somewhat limited due to the relatively high MIC values, the absence of antifungal activity, and the evaluation of cytotoxicity in a single cancer cell line. Future studies should focus on the isolation and characterization of the bioactive constituents (such as lectins and viscotoxins) through activity-guided fractionation approaches, in order to better understand their mechanisms of action and to enhance their therapeutic potential.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Design: KM; acquisition of data: KM, BDS; analysis of data: KM, BDS, ABS, FUA; drafting of the manuscript: KM, BDS, ABS, FUA; critical revision of the manuscript: KM, BDS, FUA; statistical analysis: ABS, FUA; technical or financial support: KM, BDS, ABS, FUA; supervision: KM.

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