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Anticancer activity of *Elsholtzia winitiana* var. *dongvanensis* Phuong on gastric cancer cells: phytochemical profiling, cell proliferation inhibition, senescence induction, and cell cycle arrest

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Abstract

Background *Elsholtzia*, a genus within the Lamiaceae family, comprises 42 species predominantly distributed across East and Southeast Asia. *Elsholtzia winitiana* var. *dongvanensis* Phuong (EWD) is a subspecies of *Elsholtzia winitiana* endemic to the Dong Van–Ha Giang Province Mountain region in Vietnam. While the anti-inflammatory and anti-bacterial activities of *Elsholtzia* species have been well-documented, data regarding the anticancer potential remain limited. This study investigated the anti-proliferative effects of EWD extract on AGS gastric cancer cells.

Results The metabolite profiling of EWD leaf extract conducted using ultra-performance liquid chromatography, coupled with quadrupole time-of-flight mass spectrometry identified 65 compounds, including triterpenoids, sesquiterpenoids, alkaloids, glycosides, and phenolic compounds. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that the extract significantly inhibited AGS cell proliferation in a concentration-dependent manner. The cell proliferation rate decreased from $64.7 \pm 6.5\%$ to $18.9 \pm 1.3\%$ as concentrations increased from 50 to 500 $\mu\text{g/mL}$, with an IC_{50} value of 68.7 $\mu\text{g/mL}$. In the 3D culture model, the EWD extract inhibited tumorsphere formation, resulting in a significant reduction in tumorsphere numbers compared to controls (100%). Additionally, EWD extract induced cellular senescence, with a 31-fold increase in senescent cells at 50 $\mu\text{g/mL}$ and an up to 82-fold increase at 200 $\mu\text{g/mL}$. Cell cycle analysis indicated that the EWD extract triggered G0/G1 phase arrest, as evidenced by a significant increase in cell proportion at the G0/G1 phase, 59.7% at 100 $\mu\text{g/mL}$, and 64.6% at 200 $\mu\text{g/mL}$, compared to 55.9% in the control. Gene expression analysis revealed significant downregulation of cyclin B1, cyclin D2, cyclin E1, cyclin-dependent kinase 2, and cyclin-dependent kinase 3, along with increased cyclin-dependent kinase 4 expression.

Conclusion The EWD extract significantly inhibited proliferation, induced senescence, arrested the cell cycle at the G0/G1 phase, and downregulated cyclin and cyclin-dependent kinase genes in the AGS cells, highlighting its potential for gastric cancer therapy.

Keywords *Elsholtzia winitiana* var. *dongvanensis* Phuong, Gastric cancer, Cell senescence, AGS cells, Cell cycle

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1 Introduction

The genus *Elsholtzia* comprises 42 species, 33 of which are found in East Asia. EWD is a subspecies of *Elsholtzia winitiana* that primarily distributed in the mountain region of Dong Van–Ha Giang Province in Vietnam, at altitudes of 1400 m [1, 2]. Chemical analyses revealed that species within the *Elsholtzia* genus contain over 1000 volatile compounds and more than 200 non-volatile compounds, including 96 flavonoids and 66 triterpenoids. Owing to the presence of numerous biologically active compounds, *Elsholtzia* species have demonstrated antiviral, antibacterial, antifungal, and anti-inflammatory properties [1]. Extracts or isolated compounds from *Elsholtzia* species have shown activity against respiratory viruses, such as the influenza virus, SARS-CoV-2 (COVID-19), hepatitis C virus, herpes viruses, and other viral strains. Furthermore, antibacterial assays have revealed that extracts or essential oils from the *Elsholtzia* genus can inhibit various pathogens responsible for intestinal infections, respiratory diseases, and hospital-acquired infections [3, 4]. Anti-inflammatory activity is one of the most prominent biological activities observed in *Elsholtzia* plants, which are utilized in the treatment of conditions such as arthritis, nephritis, and respiratory inflammation [5, 6]. The anti-inflammatory mechanisms are believed to involve the inhibition of COX-2 and PGE2 [7] or the suppression of pro-inflammatory cytokines (TNF-alpha, Interleukin-1, and Interleukin-6), mediated by compounds isolated from *Elsholtzia*, including quercetin, luteolin, and their derivatives [8].

Gastric cancer, one of the most common malignancies worldwide, is particularly prevalent in several Asian nations, including China, South Korea, Vietnam, and Japan. It is estimated that 660,175 people will die from stomach cancer in 2022 [9]. In addition to conventional cancer treatments, such as chemotherapy, radiotherapy, and surgery, phytotherapies are increasingly recognized as a complementary approach in cancer treatment. Recent studies have provided significant evidence of the benefits of these therapies, such as lower treatment costs, better compatibility with the body, and fewer side effects than synthetic drugs [10, 11]. Several herbs have been investigated for their efficacy against gastric cancer cells in in vitro and in vivo studies, and in clinical evaluations [10, 12, 13]. Examples include garlic, *Taxus chinensis*, turmeric, saffron, ginger, lavender, and mint, which are commonly used by cancer patients to inhibit tumor growth and decrease the risk of metastasis [14].

Several anticancer drugs have been developed from herbs, such as paclitaxel from *Taxus chinensis*, which inhibits cell division, and camptothecin from *Campotheca acuminata*, which inhibits topoisomerase I [15]. Similarly, resveratrol derived from *Veratrum*

grandiflorum, peanut sprouts, grapes, and peanuts inhibits topoisomerase II [16]. Current data indicate that extracts or natural compounds of some species in the *Elsholtzia* genus, like *Elsholtzia penduliflora*, inhibit the development of different cancer cells, including lung cancer (A549) and breast cancer (MCF7) [17]. Additionally, the extract of *Elsholtzia stauntonii* has been shown to stimulate apoptosis in liver cancer cells (HepG2) and reduce tumor volume in mice [18]. Meanwhile, essential oils from *Elsholtzia ciliata* (Thunb.) Hyl have demonstrated the capacity to suppress the expansion of a number of cancer cells, including glioblastoma, lung, breast, and pancreatic cancer [4, 19].

Although there have been preliminary evaluations of the cytotoxic potential of *Elsholtzia* plants in various cancer cell lines, the majority of studies have focused on initial screening for inhibitory activity. Notably, data regarding the effects of *Elsholtzia* on gastric cancer cell lines remain limited. The objectives of this work were to investigate the chemical profiling and anti-proliferative effect of EWD extract against AGS gastric cancer cells, as well as to elucidate the probable pathways involved.

2 Materials and methods

2.1 Sample preparation

The leaves of EWD were collected from Ta Lung Commune Dong Van District, Ha Giang Province, at coordinates N 23°15' 18.0396" E 105°21' 40.3452" (Fig. 1). The leaf samples were dried for 72 h at 37 °C in a drying oven and then finely ground into a powder. The extraction was carried out using 95% ethanol at a ratio of 5 g of leaf powder to 30 mL of solvent. Whatman filter paper was used to filter the resulting extract, and the solvent was completely evaporated at 40 °C. After dissolving the concentrated extract in dimethyl sulfoxide (DMSO) for the stock solution of



Fig. 1 *Elsholtzia winitiana* var. *dongvanensis* Phuong at Ta Lung commune Dong Van District, Ha Giang Province

100 mg/mL, it was filtered through a 0.22 µm membrane before use for bioactivity assays. For chemical profiling, a similar protocol was applied for sample preparation, with a concentration of 10 mg dry extract/1 ml MeOH.

2.2 Chemical compound analysis

The components of the EWD extract were separated using the ACQUITY UPLC BEH C18 column configured in UPLC-QToF-MS (Waters Corporation, Milford, Massachusetts, USA). The solution was introduced at a rate of 0.3 mL/min, with 1 µL being injected. A mobile phase gradient was employed, consisting of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient was programmed to increase from 50 to 99% solvent B over 31 min, followed by equilibration. For mass spectrometry conditions, the ionization was conducted in positive and negative modes. Mass spectra of separated peaks were fully scanned in the range of m/z from 50 to 1100. Collision energy was set at 6 V in low-energy mode, while high-energy mode varied from 15 to 40 V. Data analysis was carried out using MassLynx 4.2 and UNIFI software, with peak annotation based on fragment patterns matched against the Traditional Chinese Medicine library (Waters Corporation, Milford, Massachusetts, USA). This detailed analytical approach enabled the identification of a wide range of bioactive compounds in the EWD extract, providing valuable insights into its chemical composition and potential therapeutic properties.

2.3 Cell proliferation inhibition assay

To evaluate proliferation, the cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotics, as previously described [20]. Subsequently, cells were treated with the EWD extract for 48 h at doses ranging from 10 to 500 µg/mL and then incubated for 4 h with a MTT solution to induce the formation of purple formazan crystals. The medium in each well was replaced with DMSO to completely dissolve the crystals in each well. RPMI 1640 medium, MTT, and DMSO were supplied by Thermo Fisher Scientific (Waltham, Massachusetts, USA). Finally, the optical density (OD) of the well plate was determined at 570 nm using a Multiskan sky spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts (MA), USA). All experiments were repeated four times, and the cell proliferation rate influenced by the EWD extract was calculated as follows:

$$\text{Cell proliferation (\%)} = \frac{\text{OD(EWD treated wells)}}{\text{OD (control wells)}} \times 100$$

2.4 Tumorsphere formation assay

Tumorsphere formation assay was carried out using non-adherent culture plates coated with poly (2-hydroxyethyl methacrylate) solution at a concentration of 10 mg/mL [21]. Cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Thermo Fisher Scientific, Waltham, MA, USA) enhanced with 20 ng/mL epidermal growth factor (EGF), penicillin/streptomycin (1%), FBS (3%), and glucose (0.4%) (all from Thermo Fisher Scientific, Waltham, MA, USA). An initial density of 5,000 AGS cells was seeded, and then, different concentrations of EWD extracts were added to the wells, except in the control wells. All experiments were repeated three times for each condition. The tumorsphere creation and the variation in its size were then monitored using a NIKON Eclipse Ti2 microscope (NIKON, Tokyo, Japan) during 72 h of incubation.

2.5 Real-time PCR analysis

This study analyzed the expression at mRNA levels for genes controlling cell cycle, as detailed in Table S1. mRNA extraction and purification were conducted using the TRIzol method (TRIzol™ Reagent, Thermo Fisher) following established protocols. Subsequently, real-time PCR was carried out using the OneStep qRT-PCR master mix with SYBR Green I (BIOFACT, Daejeon, Korea) on a qTower3 system (Analytik Jena GmbH, Jena, Germany). The thermal cycling protocol consisted of a first cycle at 50 °C for 30 min, followed by 45 cycles alternating between 94 and 60 °C for 20 s each. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) served as the reference gene for normalizing the relative expression of the target genes. Experiments were repeated three times, and the $2^{-\Delta\Delta C_t}$ method was employed for quantifying gene expression.

2.6 Cell cycle analysis

To maintain their shape and ensure proper staining, the cells were initially fixed in 70% ethanol. The fixation process was conducted for a minimum of 24 h at −20 °C, allowing the ethanol to permeate the cells thoroughly. Subsequently, the cells were rinsed twice with PBS to remove any residual ethanol and then stained for 30 min with propidium iodide (PI) solution in the absence of light at room temperature. After staining, cells were harvested by trypsinization to detach them from the culture surface. Centrifugation was used to pellet the harvested cells for five minutes at 13,000 rpm. The total cell population was then introduced into a BD Accuri™ C6 Plus system (Becton Dickinson, Franklin Lakes, New Jersey, USA). The Fluorescent signal intensity of PI in individual cells was detected and measured using a flow cytometer,

which allowed the distribution of the cell cycle across the G0/G1, S, and G2/M phases. Experiments were repeated three times for each condition.

2.7 Cell senescence assay

Senescence was evaluated through histochemical staining using Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St. Louis, Missouri, USA), according to the manufacturer's protocol. The cells were initially washed twice with 1× phosphate-buffered saline (PBS) solution to remove any residual medium or debris. Subsequently, cells were fixed with 1× Fixation buffer and incubated the plate for 10–15 min at room temperature to preserve their structural integrity. After fixation, the Fixation buffer was discarded, and the cells were rinsed twice more with 1× PBS, using 500 µL per wash to ensure complete removal of the fixative. Subsequently, 50 µL staining mix solution containing senescence-associated β -galactosidase (SA- β -gal) was applied to each well. The cells were then cultured at 37 °C in the absence of light for 2 h to facilitate the detection of SA- β -gal activity, which serves as a marker for cellular senescence. Following the incubation period, the cells were examined under a light inverted microscope at 100× magnification. The presence of senescent cells was confirmed by the characteristic blue staining, which was visualized and documented through microscopic imaging. The experiments were repeated 3 times for each condition.

3 Results

3.1 Chemical constituents of EWD extract

The analysis spectrum of the chemical constituents of the EWD extract using UPLC-QToF-MS is presented in Fig. S1. The results summarized in Table 1 show that 65 compounds were identified for the first time from the leaf extract of EWD. Of these, thirty-three compounds showed a positive proton adduct (+ H), and thirty-two displayed a negative adduct (− H). Retention times varied significantly, with most compounds eluting between 0.4 and 17 min. Notably, several triterpenoid compounds were detected, including Methyl oleanolate, Ocotillol acetate, Picfeltaaraegenin I, Picrasin D, Cimisine F, 1,6-O,O-Diacetylbritannilactone, and Badrakemin acetate, which were also identified. Moreover, eight alkaloids and nine glycoside compounds were detected in the EWD extract. Furthermore, several compounds from other chemical classes, such as carboxylic acids, phenylethanoids, phenolic acids, and lignans, were also identified.

3.2 Anti-proliferation activity of EWD extract

Assessing the effect of plant extracts on cell growth in adherent (2D) culture is typically the first step in such investigations. As shown in Fig. 2A, the EWD extract

significantly inhibited AGS cell growth starting at a concentration of 50 µg/mL ($p < 0.05$) within the range of concentrations selected for testing. Higher concentrations of EWD extract in the culture medium resulted in a significant, concentration-dependent decrease in cell density on culture plates. The proliferation rates at 50 and 100 µg/mL were measured to be $64.7 \pm 8.5\%$ and $34.5 \pm 9.1\%$, respectively, both of which were significantly lower than the control (100%). At concentrations of 200 and 500 µg/mL, most cells displayed the morphological features of dead cells, including extensive cell shrinkage. The percentage of viable cells at 200 and 500 µg/mL was reduced to $19.6 \pm 2.1\%$ and $18.9 \pm 1.3\%$, respectively, compared to untreated cells ($p < 0.001$). The IC_{50} value was calculated to be 68.7 µg/mL using GraphPad Prism Software.

3.3 Inhibitory activity of EWD extract on tumorsphere formation in 3D culture conditions

Cytotoxicity assays conducted on cancer cells in 3D non-adherent culture models provide a more accurate representation of the effects observed in in vivo cancer cell treatments than conventional 2D adherent cell culture assays [22]. Consequently, an experiment was performed to assess the effect of the EWD extract on the ability of AGS cells to form 3D tumorspheres. Tumorspheres were defined as spherical structures composed of aggregates of 20 or more cells. As shown in Fig. 3A, the density of tumorspheres in the EWD extract-treated wells was significantly lower than that in the control wells. The percentage of tumorspheres at 50 and 100 µg/mL of EWD was $34.3 \pm 7.5\%$ and $14.3 \pm 3.1\%$, respectively, compared to the control (100%) (Fig. 3B). Morphological observations further revealed the presence of individual dead cells after three days of culture in low-FBS medium containing the EWD extract, with nearly all cells exhibiting cell death at 200 µg/mL. Notably, dead cells were also observed within tumorspheres formed at a concentration of 100 µg/mL. These findings demonstrate that the EWD extract not only inhibited AGS cell aggregation, but also effectively induced cancer cell death in a 3D non-adherent culture.

3.4 Senescence-inducing activity of EWD extract

Cellular senescence is widely recognized as an irreversible growth arrest state in cells that serves as a critical mechanism for preventing malignant tumor formation. In cancer cells, the senescence process is often delayed or suppressed, resulting in extended cell division and rapid tumor growth. The results from cell treatments with the EWD extract, as shown in Fig. 4, indicate that the number of blue-stained cells (red arrows) at 50 µg/mL

Table 1 Phytochemical compounds of EWD extract

No	Component name	Molecular formula	Expected mass (Da)	Observed mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Adducts
1	1,3,6-trihydroxy-2-methyl-9,10-anthraquinone-3-O-(6'-O-acetyl)-alpha-L-rhamnopyranosyl-(1->2)-beta-D-glucopyranoside	C ₂₉ H ₃₂ O ₁₅	620.174	620.177	621.184	4.448	15.713	+ H
2	1,6-O-Diacetylbritannilactone	C ₁₉ H ₂₆ O ₆	350.173	350.173	351.180	- 0.183	1.312	+ H
3	1-Acetyl-3-(methoxy-carbonyl)-β-carboline	C ₁₅ H ₁₂ N ₂ O ₃	268.085	268.086	269.093	3.531	8.747	+ H
4	1-O-Methyl-3,5-O-dicaffeoyl quinic acid methyl ester	C ₂₇ H ₂₈ O ₁₂	544.158	544.157	545.164	- 2.585	0.407	+ H
5	Apiosylskimmin	C ₂₀ H ₂₄ O ₁₂	456.127	456.129	457.136	4.928	1.061	+ H
6	Asperulosidic acid	C ₁₈ H ₂₄ O ₁₂	432.127	432.126	433.134	- 0.661	1.740	+ H
7	Badrakemin acetate	C ₂₆ H ₃₂ O ₅	424.225	424.227	425.234	4.810	11.721	+ H
8	Berbamine	C ₃₇ H ₄₀ N ₂ O ₆	608.289	608.290	609.297	2.414	13.024	+ H
9	Canadine	C ₂₀ H ₂₁ NO ₄	339.147	339.148	340.155	3.003	0.541	+ H
10	Cerotic acid	C ₂₆ H ₅₂ O ₂	396.397	396.397	397.404	- 0.070	16.985	+ H
11	Delphinidin-3-glucoside	C ₂₁ H ₂₁ ClO ₁₂	465.103	465.102	466.110	- 1.948	2.339	+ H
12	Digallic acid	C ₁₄ H ₁₀ O ₉	322.032	322.031	323.038	- 3.971	0.930	+ H
13	Eugenyl glucoside	C ₁₆ H ₂₂ O ₇	326.137	326.136	327.143	- 2.877	0.440	+ H
14	Forsythenside A	C ₂₂ H ₂₆ O ₁₀	450.153	450.153	451.160	1.024	0.779	+ H
15	Isosakuranetin-7-rutinoside	C ₂₈ H ₃₄ O ₁₄	594.195	594.195	595.203	0.706	13.885	+ H
16	Lactiflorin	C ₂₃ H ₂₆ O ₁₀	462.153	462.153	463.160	0.741	0.926	+ H
17	Lehmannine	C ₁₅ H ₂₂ N ₂ O	246.173	246.172	247.180	- 3.887	12.333	+ H
18	Matrine	C ₁₅ H ₂₄ N ₂ O	248.189	248.190	249.197	2.610	12.340	+ H
19	Methyl kushenol C	C ₂₆ H ₂₈ O ₇	452.184	452.184	453.191	1.258	7.890	+ H
20	Mudanoxide B	C ₁₈ H ₂₄ O ₁₄	464.117	464.119	465.126	4.575	1.074	+ H
21	Nortracheloside	C ₂₆ H ₃₂ O ₁₂	536.189	536.189	537.196	- 0.684	17.058	+ H
22	O-Ethylnorskimmianine	C ₁₆ H ₁₇ NO ₄	273.100	273.101	274.108	3.491	0.446	+ H
23	Pinoresinol diglucoside	C ₃₂ H ₄₂ O ₁₆	682.247	682.249	683.256	2.747	16.026	+ H
24	Platydesmine	C ₁₅ H ₁₇ NO ₃	259.121	259.121	260.128	- 0.177	0.434	+ H
25	Polycanthine	C ₁₄ H ₁₀ N ₂	261.136	261.136	262.143	- 3.418	0.436	+ H
26	Scoulerine	C ₁₉ H ₂₁ NO ₄	327.147	327.147	328.154	- 1.481	0.540	+ H
27	Sec-O-glucosylhamaudol	C ₂₁ H ₂₆ O ₁₀	438.153	438.154	439.162	3.901	0.904	+ H
28	Sedoheptulose	C ₇ H ₁₄ O ₇	210.074	210.075	211.082	3.977	0.431	+ H
29	Sugiol	C ₂₀ H ₂₈ O ₂	300.209	300.209	301.216	- 0.088	12.386	+ H
30	Tamarixetin-3-O-β-D-glucoside-7-O-α-L-rhamnoside	C ₃₄ H ₄₂ O ₂₀	624.169	624.169	625.176	- 0.575	15.714	+ H
31	Thalictricoside	C ₁₉ H ₂₇ NO ₁₂	461.153	461.151	462.158	- 4.814	1.614	+ H
32	Turpiniosides D	C ₁₉ H ₃₄ O ₈	390.225	390.225	391.232	- 1.080	11.278	+ H
33	Yesanchinoside B	C ₄₈ H ₈₂ O ₂₀	978.540	978.540	979.548	0.500	16.828	+ H
34	(-)-Secoisolariciresinol-4-O-β-D-glucopyranoside	C ₂₀ H ₃₆ O ₁₀	524.226	524.221	523.214	- 8.872	0.588	- H
35	25-O-Methylalisol A	C ₃₁ H ₅₂ O ₅	504.381	504.378	503.371	- 6.532	5.903	- H
36	4''-Hydroxyimperatorin-4''-O-β-D-glucopyranoside	C ₂₂ H ₂₄ O ₁₀	448.137	448.141	447.134	9.226	1.452	- H
37	7-O-Methyl luteolin-6-C-β-D-glucoside	C ₂₂ H ₂₂ O ₁₁	464.132	464.130	463.123	- 3.779	2.237	- H
38	Agrimol E	C ₃₃ H ₃₈ O ₁₂	626.236	626.233	625.226	- 5.376	0.918	- H
39	Ajugasterone C-2,3,20,22-diacetone	C ₃₃ H ₅₂ O ₇	560.371	560.368	559.361	- 5.242	9.295	- H
40	Catalpol	C ₁₅ H ₂₂ O ₁₀	362.121	362.118	361.111	- 9.592	2.111	- H
41	Chrysoeriol-7-O-β-D-glucoside	C ₂₂ H ₂₂ O ₁₁	462.116	462.120	461.113	9.084	2.237	- H

Table 1 (continued)

No	Component name	Molecular formula	Expected mass (Da)	Observed mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Adducts
42	Cimiside F	C ₃₆ H ₅₈ O ₁₀	650.403	650.406	649.399	5.319	9.652	– H
43	Coniferin	C ₁₆ H ₂₂ O ₈	342.131	342.131	341.124	– 0.926	0.584	– H
44	Coniferol	C ₁₀ H ₁₂ O ₃	180.079	180.078	179.070	– 6.232	0.431	– H
45	Cynatratoside C	C ₄₁ H ₆₂ O ₁₄	778.414	778.414	777.407	0.031	0.633	– H
46	Digupigan A	C ₁₈ H ₂₆ O ₁₂	434.142	434.140	433.133	– 5.259	1.688	– H
47	Dolabradiene	C ₂₀ H ₃₂	272.250	272.252	271.244	4.304	12.492	– H
48	Filixic acid ABA	C ₃₂ H ₃₆ O ₁₂	612.221	612.220	611.213	– 0.593	1.244	– H
49	Icariside B5	C ₁₉ H ₃₂ O ₈	388.210	388.209	387.201	– 2.611	0.634	– H
50	Isoanhyoicaritin	C ₂₂ H ₂₄ O ₆	384.157	384.159	383.151	3.760	1.428	– H
51	Isomitraphyllic acid	C ₂₀ H ₂₂ N ₂ O ₄	354.158	354.157	353.150	– 2.912	1.392	– H
52	Kadsurenin B	C ₂₀ H ₂₂ O ₅	342.147	342.145	341.137	– 5.887	0.427	– H
53	Koaburaside	C ₁₄ H ₂₀ O ₉	332.111	332.110	331.103	– 1.798	0.920	– H
54	Kushenol L	C ₂₅ H ₂₈ O ₇	440.184	440.184	439.177	1.132	1.586	– H
55	Lappaol B	C ₃₁ H ₃₄ O ₉	550.220	550.223	549.216	5.461	0.413	– H
56	Methyl oleanolate	C ₃₁ H ₅₀ O ₃	470.376	470.379	469.372	7.264	9.774	– H
57	Ocotillol acetate	C ₃₂ H ₅₄ O ₄	502.402	502.402	501.395	0.261	12.209	– H
58	Odoratin-7-O-β-D-glucoside	C ₂₃ H ₂₄ O ₁₁	432.142	432.145	431.137	5.973	1.689	– H
59	Periplocoside O	C ₃₆ H ₅₆ O ₁₀	648.387	648.392	647.385	7.000	8.868	– H
60	Picfeltaarraegenin I	C ₃₀ H ₄₄ O ₅	484.319	484.317	483.310	– 3.448	11.735	– H
61	Picrasin D	C ₂₂ H ₃₀ O ₆	390.204	390.206	389.198	3.614	0.635	– H
62	Sweroside	C ₁₆ H ₂₂ O ₉	358.126	358.129	357.122	7.962	0.919	– H
63	Trachelogenin	C ₂₁ H ₂₄ O ₇	388.152	388.149	387.141	– 9.140	0.585	– H
64	β-Hydroxyisovaleryl shikonin	C ₂₁ H ₂₄ O ₇	388.152	388.155	387.147	6.403	0.426	– H
65	β-Sitosteryl acetate	C ₃₁ H ₅₂ O ₂	456.397	456.400	455.393	7.324	12.210	– H

increased by 31-fold compared to controls ($p < 0.001$). At higher concentrations of 100 and 200 $\mu\text{g/mL}$, the proportion of cells exhibiting a senescent phenotype increased by 67- and 82-fold, respectively ($p < 0.001$). In contrast, a small proportion of senescent cells was observed in the culture medium without EWD extract (control).

3.5 Inhibitory activity of EWD extract on cell cycle progression

As previously mentioned, senescence is a permanent cell cycle arrest state that prevents cells from dividing. The analysis of the effect of EWD extract on the cell cycle, as shown in Fig. 5, revealed a concentration-dependent accumulation in the G0/G1 phase, with the proportion of cells increasing to $59.7 \pm 1.8\%$ at 100 $\mu\text{g/mL}$ and $64.6 \pm 3.9\%$ at 200 $\mu\text{g/mL}$ compared to the control ($55.9 \pm 2.3\%$) ($p < 0.05$), while no significant change was observed at 50 $\mu\text{g/mL}$. These results demonstrate that the EWD extract induced a concentration-dependent cell cycle arrest in the G0/G1 phase of AGS cells in a concentration-dependent manner.

3.6 Effect of EWD extract on the expression of cell cycle control genes

The effect of the EWD extract on the expression of genes involved in cell cycle regulation was further analyzed to elucidate the molecular mechanisms underlying cell cycle arrest and senescence initiation. Two groups of genes were investigated including cyclin and cyclin-dependent kinase genes. As shown in Fig. 6A, among the five cyclin genes analyzed, CCND1 and cyclin A (CCNA) did not show significant changes in transcription levels. However, a marked reduction in mRNA expression was noted in the CCNB1, CCND2, and CCNE1 genes, with decreases ranging from 2.3 to 3.4 times ($p < 0.01$), with CCNB1 showing the most pronounced reduction. Regarding cyclin-dependent kinase genes, cyclin-dependent kinase (CDK6) and cyclin-dependent kinase 8 (CDK8) exhibited no significant change in expression levels. In contrast, CDK4 showed a significant increase in mRNA expression ($p < 0.05$). The most substantial inhibition of mRNA expression by the EWD extract was observed in CDK2 and CDK3 genes, with reductions of 2.4 and 7.8 times, respectively ($p < 0.01$ and $p < 0.001$) (Fig. 6B). Thus, the EWD extract has a significant impact

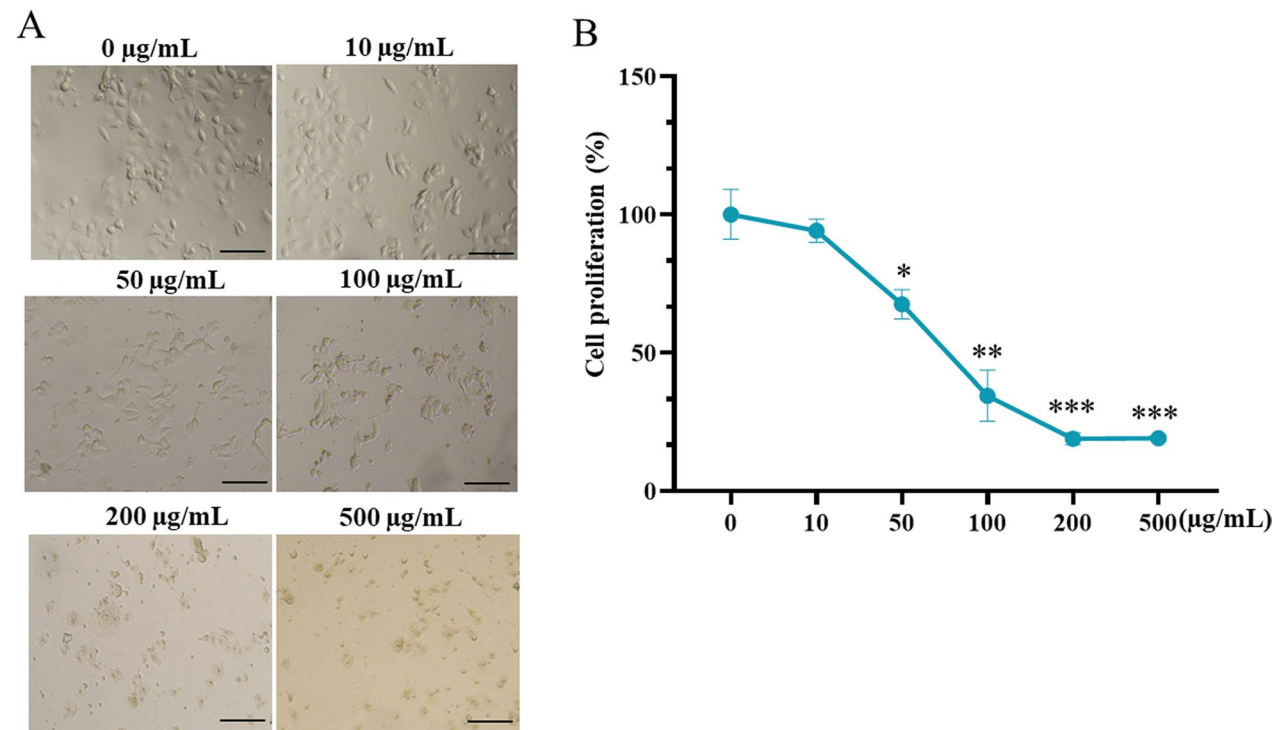


Fig. 2 Effect of EWD extract on AGS cell proliferation: AGS cells were treated with EWD extract at various concentrations (10–500 $\mu\text{g/mL}$) over 48 h. **A** Cell images were captured at 48 h post-treatment using an inverted microscope Nikon Ts2; scale bar: 50 μm . **B** The graph shows changes in AGS cell proliferation after 48 h of incubation with EWD extract at different concentrations. The Mann–Whitney U test, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to control

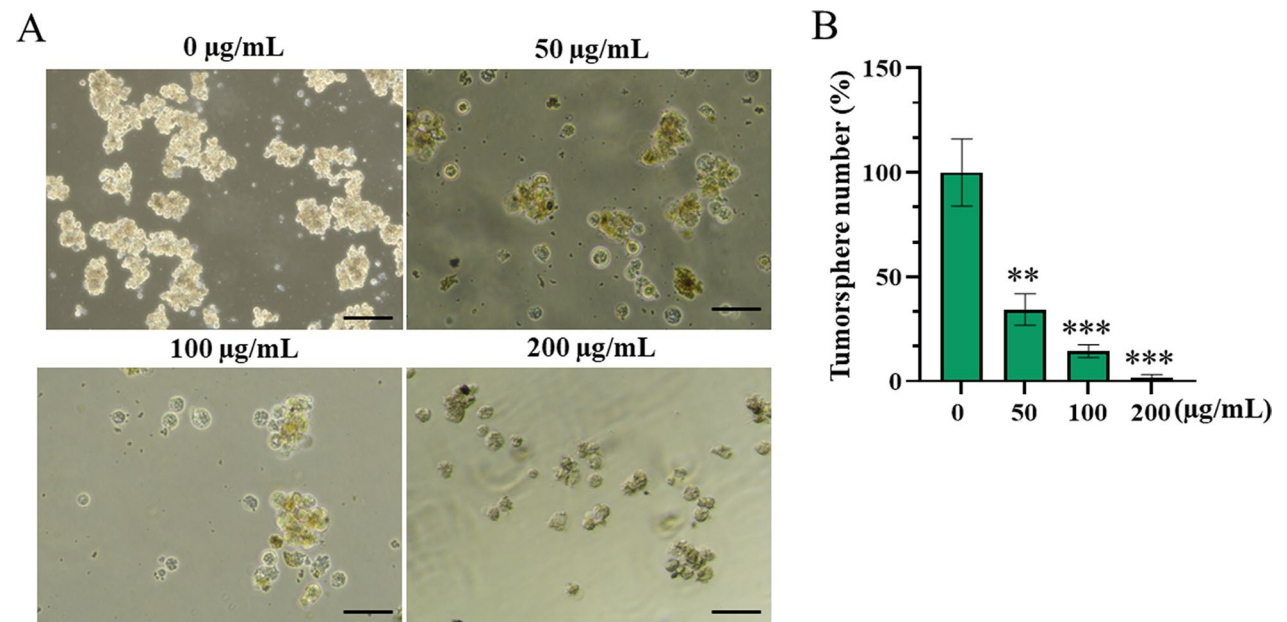


Fig. 3 Tumorsphere formation from AGS cells in 3D culture medium under the influence of the EWD extract. A total of 2000 AGS cells were cultured in medium containing EWD extract at concentrations of 50, 100, and 200 $\mu\text{g/mL}$. **A** Microscopic images at 100X magnification of tumorspheres after 72 h of treatment; scale bar: 200 μm . **B** Percentage change in the number of tumorspheres at different EWD extract concentrations. Mann–Whitney U test, $**p < 0.01$, and $***p < 0.001$ compared to control

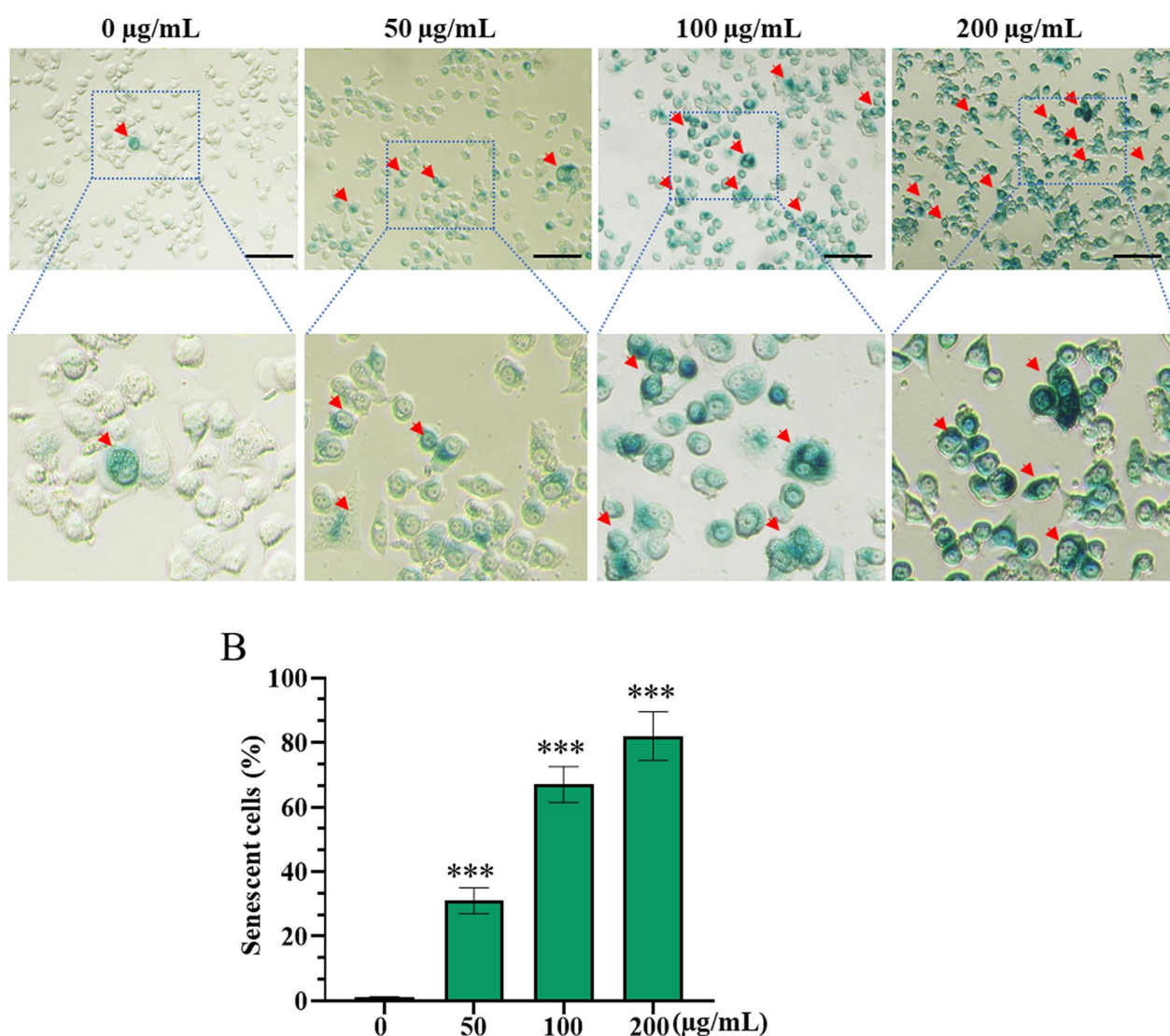


Fig. 4 Effect of EWD extract on cellular senescence. AGS cells were cultured in a medium containing EWD extract at different concentrations. **A** Microscope images of cells after 48 h of EWD extract treatment, taken with a 200 $\times$ magnification; scale bar: 50 μ m. **B** Percentage change in the proportion of cells exhibiting a senescent phenotype at different concentrations. Mann–Whitney U test, *** p < 0.001 compared to control

on several key genes within the cyclin and cyclin-dependent kinase groups.

4 Discussion

The chemical constitution of the extract from the leaves of *Elsholtzia winitiana* var. *dongvanensis* Phuong was analyzed using UPLC-QToF-MS, revealing the presence of 65 compounds belonging to alkaloid, terpenoid, flavonoid, and other chemical groups. Several of these compounds have previously demonstrated inhibitory effects on various cancer cell types, such as 1,6-O,O-diacetylbritannilactone (compound **2**), which was previously reported to inhibit the growth of HT-29 (colon

cancer), HL-60 (leukemia), and AGS (gastric cancer) cell lines with IC₅₀ values of 76.6 μ M, 35.3 μ M, and 66.4 μ M, respectively [23]. Berbamine (compound **8**), a bis-benzylisoquinoline alkaloid, has shown anti-cancer effects against cancer cell lines such as MCF-7 and MDA-MB-231 with IC₅₀ values of 20.92 μ M and 22.72 μ M, respectively [24]. Matrine (compound **18**), an alkaloid isolated from *Sophora flavescens*, exhibits the cytotoxicity against a wide range of cancer, including hematologic and solid tumors such as gastric, colon, liver, breast, prostate, lung, and melanoma [25]. β -Sitosteryl acetate (compound **65**) contributes to cancer therapy by promoting apoptosis, cell cycle arrest,

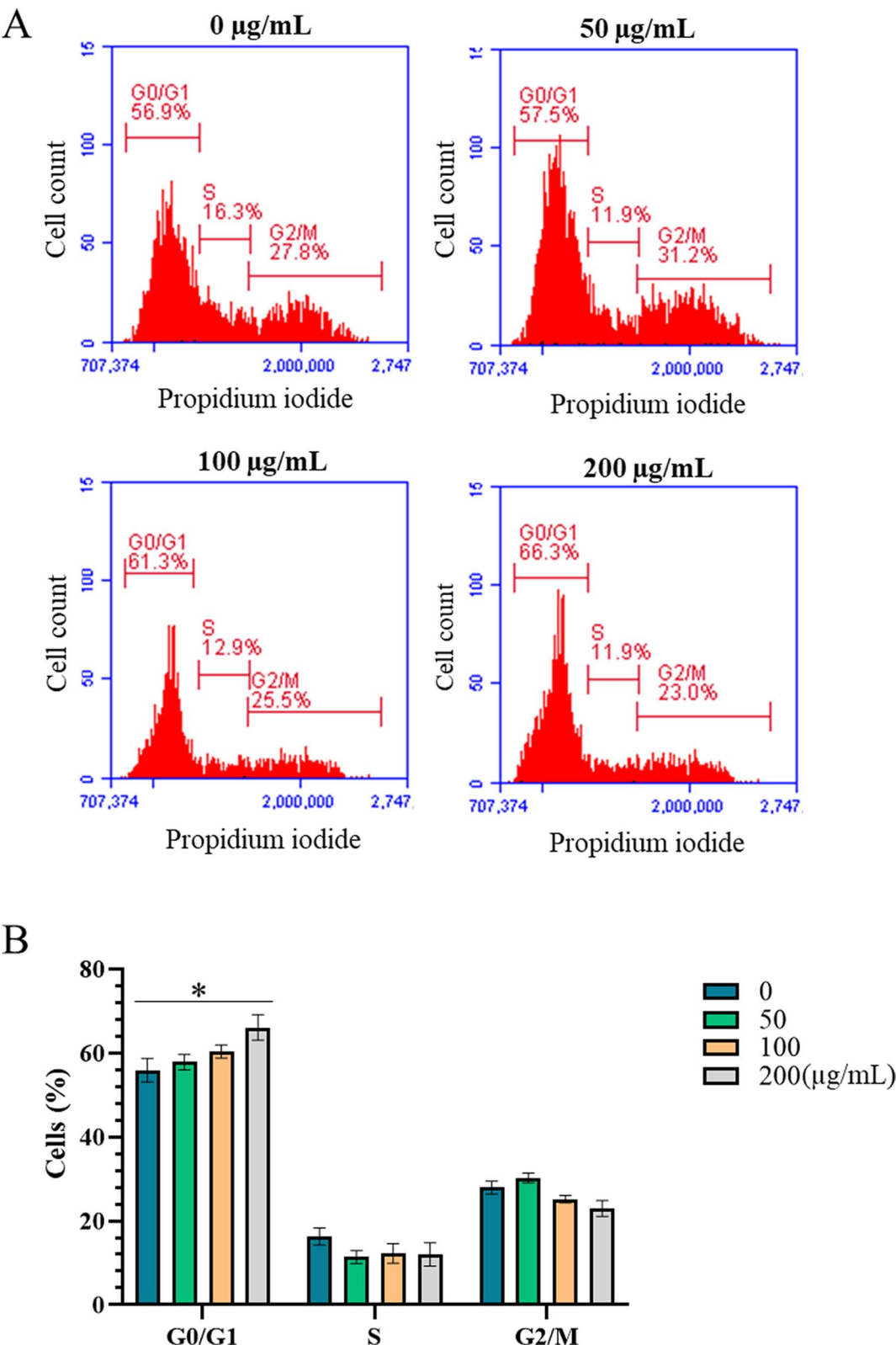


Fig. 5 Effect of EWD extract on cell cycle phases. AGS cells were added with EWD extract at 50, 100, and 200 $\mu\text{g/mL}$ for 48 h and then analyzed by flow cytometry. **A** The distribution of cells in G0/G1, S, and G2/M phases. **B** Percentage change in the proportion of cells in each phase of the cell cycle. Statistical analysis was conducted by ANOVA test, $*p < 0.05$

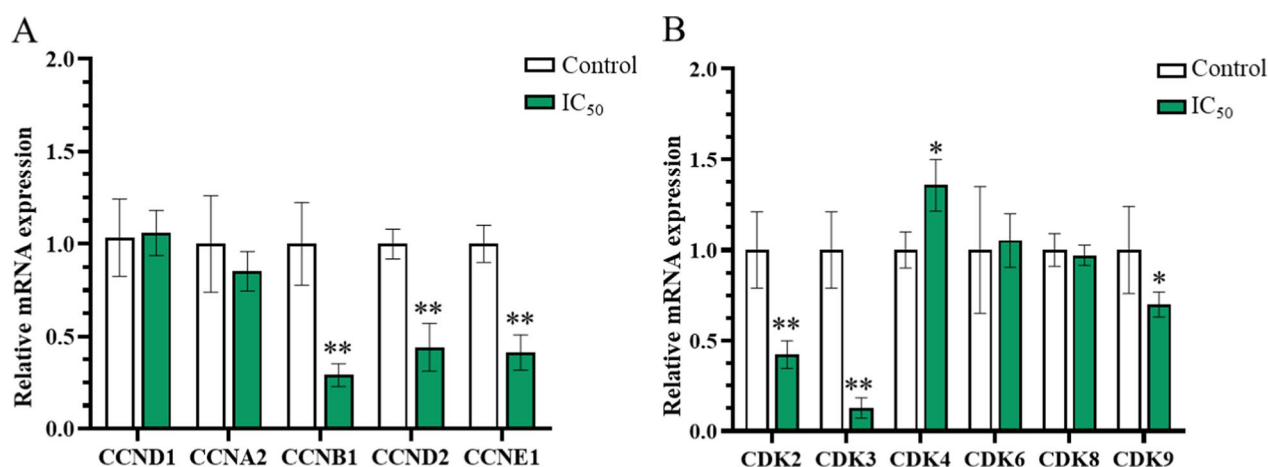


Fig. 6 Effect of EWD on the expression of cell cycle regulation genes. Cells were treated with EWD at the IC₅₀ concentration for 24 h, and the mRNA synthesis levels of the analyzed genes were quantified by real-time PCR. **A** Expression (mRNA) of the cyclin gene group. **B** Expression of the cyclin-dependent kinase gene group. Mann–Whitney U test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared to 0 $\mu\text{g/mL}$)

oxidative stress modulation, metabolic reprogramming, and overcoming drug resistance, particularly in gastric cancer [26]. It has been shown that Catalpol (compound 40) inhibits proliferation by increasing the generation of reactive oxygen species (ROS) production in gastric cancer cells, enhancing the anticancer efficacy of cisplatin in suppressing gastric cancer cells, and suppressing the growth of tumors in xenograft nude mice [27]. Kushenol (compound 54) suppresses the growth, migration, and epithelial–mesenchymal transition (EMT) process of gastric cancer cells, mediated through the PI3 K/AKT signaling pathway [28]. The MTT assay (Fig. 2) showed that the EWD extract exhibited anti-proliferative activity in AGS cells, with an IC₅₀ value of 68.7 $\mu\text{g/mL}$. The simultaneous effects on multiple signaling targets, such as cell cycle and apoptosis of cancer cells, have been demonstrated in various studies using extracts from plants [29]. These multitarget effects of plant extracts are being considered as a potential approach against drug-resistant cancer cells [30]. Toxicity assays using 3D tumorsphere culture model are considered more physiologically relevant than traditional adhesive cell culture experiments, as they better replicate the in vivo microenvironment by mimicking cell–cell interaction [31]. In this study, we demonstrated that the EWD extract inhibits the formation and growth of tumorspheres, which are composed of cells with stem cell-like properties that cluster to form tumor masses [32]. Therefore, EWD extract appears to target the cancer stem cell-like properties, hindering proliferation and tumorsphere formation. Studies targeting gastric cancer stem cells in the AGS, MKN45, and MKN74 cell lines have yielded similar findings, demonstrating that ethanolic extracts

and silver nanoparticle complexes from *Ardisia gigantea* effectively inhibit both the number and size of tumorspheres [21]. In addition to commonly used cancer drugs such as cisplatin, carboplatin, and oxaliplatin, emerging platinum-based anticancer drugs also use tumorspheres as an important in vitro model for assessing their anti-proliferative and drug resistance properties [33].

Inducing cellular senescence, which transitions cancer cells from a state of uncontrolled continuous division to a state of irreversible proliferative arrest, represents a promising approach for combating cancer [34]. Plant-derived compounds are regarded as promising sources for developing potential drugs that can induce cancer cell senescence. Examples of such compounds include ellagic acid, gallic acid, tannic acid, and capsaicin, which are well-known phenolics commonly found in plants. These compounds have demonstrated potential for halting the uncontrolled proliferation of cancer cells by triggering a state of irreversible cell cycle arrest [35, 36]. In this study, we demonstrate that the EWD extract induces cell senescence rate by up to 31- to 82-fold higher than controls. This activity may be attributed to various compounds in EWD extract, including β -hydroxyisovaleryl shikonin (compound 64) and Matrine (compound 18). A previous report showed β -hydroxyisovaleryl shikonin promotes cell senescence in lung cancer cells through the p53/p21 WAF signaling pathway [37]. Meanwhile, Matrine induced senescence in glioblastoma cells by downregulating key proteins in the PI3 K/AKT signaling pathway [38].

Cell cycle arrest is a hallmark of cellular senescence, which is a physiological state that helps prevent cancer development. Disruption of the cell cycle, as frequently

observed in malignancies, leads to the loss of normal physiological states, including cellular senescence [39, 40]. Recent studies have demonstrated that bioactive compounds such as diterpenes, flavonoids, phenolic acids, and polysaccharides isolated from herbs like *R. rubescens* or *Lonicera japonica* exhibit remarkable anti-tumor properties. These compounds modulate the cell cycle, induce apoptosis, and enhance radiosensitivity in cancer cells, showing potential to improve survival rates and reduce tumor recurrence in cancer patients [41, 42]. In our study, we have demonstrated that the EWD extract causes cell cycle arrest at the G0/G1 phase (Fig. 5). Upregulation of p21 and p53, which are key regulators of cellular senescence, plays a critical role in inducing cell cycle arrest at the G0/G1 phase. Overexpression of p21 leads to the downregulation of cell cycle control factors such as CDK2, CCNE1, and CDKs, leading to the dephosphorylation of the retinoblastoma protein, thus promoting cellular senescence [39, 43]. Additionally, inactivation of CCNB1 induces cell cycle arrest at the G0/G1 phase, triggers apoptosis through increased expression of caspase-3 and caspase-9, and promotes cellular senescence via the p53 signaling pathway, as previously reported [44]. Moreover, CDK3 has been identified as a critical regulator of the transition from G1 to S phase, and its depletion impedes this transition, resulting in cell cycle arrest and accumulation of cells in the G0/G1 phase [45]. In our study, real-time PCR analysis revealed that the EWD extract significantly reduced the expression of crucial cyclin genes, including CCND2, CCNB1, and CCNE1 (with a 2.3- to 3.4-fold decrease compared to the control), and strongly downregulated the expression of both CDK2 and CDK3 (with reductions of 2.4- and 7.8-fold, respectively, compared to the control). Consequently, the EWD extract stimulated G0/G1 cell cycle arrest by inhibiting the expression of critical cell cycle control genes, such as CCNB1, CCND2, CCNE1, CDK2, and CDK3, and ultimately triggered senescence in AGS cancer cells. This biological activity of the EWD extract can be attributed to several anticancer compounds, as detailed in Table 1. Previous studies have reported that compounds acting on the ovarian cancer cell line SKOV3, such as sugiol, contribute to various processes, including mitochondrial apoptosis, cell cycle arrest, and inhibition of the RAF/MEK/ERK signaling pathway [46]. Similarly, Cimicide E (compound 42) has been demonstrated to induce apoptosis and cell cycle arrest at the G2/M phase in gastric cancer cells [47]. Moreover, β -Sitosteryl acetate (compound 65) plays a role in cancer therapy primarily by promoting apoptosis, enhancing cell cycle arrest, modulating oxidative stress, inducing metabolic

reprogramming, suppressing invasion and metastasis, controlling immunity and inflammation, and overcoming drug resistance in gastric cancer [26]. In addition, scoulerine (compound 26), an isoquinoline alkaloid, has shown promising anticancer effects by suppressing cancer cell growth and inducing G2/M phase cell cycle arrest, which is associated with disruption of the microtubule network and increased phosphorylation of Chk1 Ser345, Chk2 Thr68, and mitotic H3 Ser10 [48]. Finally, methyl oleanolate (compound 56) and its related compounds have been shown to inhibit cell cycle progression at various stages and modify the expression of proteins that regulate the cell cycle differently across diverse cancer types. This occurs through pathways involving p-AKT, p-JNK, p21, p27, or p53 [49]. Although 65 different compounds were identified in the EWD extract, the individual contributions of these compounds to the observed anticancer activity remain uncharacterized. Furthermore, current anticancer evaluations were limited to in vitro models. Therefore, further studies to isolate compounds from the EWD extract and evaluate their cancer inhibitory effects using both in vitro and in vivo models are warranted.

5 Conclusion

The leaf extract of EWD, characterized by a diverse array of bioactive compounds identified through UPLC-QToF-MS analysis, demonstrates significant anticancer potential. The EWD extract effectively inhibits cell proliferation in 2D monolayer cultures and suppresses the formation and growth of 3D tumorspheres under non-adherent conditions. Moreover, the extract induced cancer cell senescence by causing cell cycle arrest at the G0/G1 phase, primarily through the downregulation of key regulatory genes, including cyclins (CCND2, CCNB1, and CCNE1) and cyclin-dependent kinases (CDK2 and CDK3). These findings underscore the therapeutic potential of *E. winitiana* var. *dongvanensis* Phuong which is a promising candidate for gastric cancer treatment, highlighting the need for further investigation into its clinical applications.

Abbreviations

EWD	<i>Elsholtzia winitiana</i> Var. <i>dongvanensis</i> Phuong
CCNB2	Cyclin B2
CCNE1	Cyclin E1
CCND1	Cyclin D1
CCND2	Cyclin D2
CDK	Cyclin-dependent kinase
DMSO	Dimethyl sulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
UPLC-QToF-MS	Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
DMEM/F-12	Modified Eagle Medium F12
RPMI1640	Roswell Park Memorial Institute 1640

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-025-00634-w>.

Additional file 1.

Author contributions

Phu Hung Nguyen, Thi Thanh Huong Le, Thi Kieu Oanh Nguyen, Duy Hung Nguyen, Quang Tan Luc, and Khac Hung Do contributed to data curation, formal analysis, and writing the original draft. Van Hung Hoang contributed to writing—original draft, supervision, methodology, and investigation. Phu Hung Nguyen contributed to project administration, investigation, writing (review and editing), and methodology. All authors have read and agreed to the published version of the manuscript.

Funding

This study was financially supported by the research project DHTN2024-NV-03 funded by Thai Nguyen University.

Data availability

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 14 November 2024 Accepted: 9 April 2025

Published online: 26 April 2025

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