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Gaillardin Suppresses Metastatic Progression of Human Ovarian Cancer through Modulation of Epithelial–Mesenchymal Transition and Angiogenesis-Related Factors

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ABSTRACT

Ovarian cancer ranks as the seventh most prevalent malignancy and the fifth leading cause of mortality among females globally. Gaillardin, a sesquiterpene lactone compound, exhibits anticancer properties across various tumor cell types. The aim of this investigation was to evaluate the antiproliferative impact of gaillardin on the SKOV3 ovarian carcinoma cell line. Cell growth inhibition in SKOV3 cultures was determined via the MTT assay, with subsequent calculation of IC50 concentrations. The effects on cell migration and invasion post-gaillardin exposure were examined through scratch wound healing experiments. Quantitative real-time PCR was employed to assess transcript levels of key epithelial-mesenchymal transition (EMT) genes, including CDH1, CDH2, VIM, and FN1, as well as angiogenesis-related genes VEGFA and THBS1. Protein levels of E-cadherin, N-cadherin, vimentin, fibronectin 1, VEGFA, and thrombospondin 1 were analyzed by Western blot technique. Treatment with gaillardin markedly reduced SKOV3 cell viability relative to untreated groups. Additionally, exposed cells displayed reduced migration and invasive capabilities compared to controls. Gene expression analysis via real-time PCR indicated that gaillardin significantly enhanced CDH1 and THBS1 mRNA levels while suppressing CDH2, VIM, FN1, and VEGFA transcripts. Comparable patterns were observed in the protein expression profiles of these factors. These findings suggest that gaillardin could potentially inhibit tumor growth and metastatic spread in ovarian carcinoma cells. Its antimetastatic actions appear to target pathways involved in both EMT and angiogenesis.

Keywords: Angiogenesis, Epithelial-mesenchymal transition, Gaillardin, Ovarian cancer, Proliferation

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Introduction

Ovarian cancer represents the seventh most common malignancy and the fifth primary cause of cancer-related deaths in females worldwide [1]. Projections for 2025 estimate approximately 20,890 newly diagnosed cases, accounting for 1.0% of total cancer incidences [2]. Clinical statistics indicate a 5-year survival probability of around 48.6% for affected individuals [3]. Although less frequent than breast cancer, ovarian cancer is considerably more lethal, with mortality rates anticipated to approach 100% by 2040 [4]. Standard therapeutic approaches encompass surgical intervention and chemotherapeutic regimens [5]. Nevertheless, alternative modalities like hormone-based treatments and targeted agents against specific cancer molecules have also demonstrated promising results in managing this disease [6]. Emerging evidence supports the development of targeted molecular interventions as a precise and low-toxicity strategy for cancer control. Notably, naturally derived compounds from plants offer potential as efficacious options with fewer adverse effects [7].

Gaillardin is a sesquiterpene lactone extracted from the above-ground portions of Inula oculus-christi L. [8]. Prior investigations from our group have established its cytotoxic potential and ability to promote programmed cell death in multiple malignant cell models [9-12].

Metastatic dissemination involves the detachment and relocation of malignant cells from the original site to distant organs [13]. This process accounts for the majority of cancer fatalities, complicating effective management [14]. Successful antimetastatic strategies require interference with critical pathways active during various metastatic phases [15]. A prominent mechanism in metastasis onset is epithelial-mesenchymal transition (EMT), whereby epithelial cancer cells acquire mesenchymal traits conducive to invasion [16]. Angiogenesis, the formation of new blood vessels, is also markedly upregulated in metastatic tumors to facilitate cell dissemination [17]. Given the central involvement of EMT and angiogenesis in metastasis progression, and the absence of prior reports on gaillardin's influence on these processes in ovarian cancer, this research aimed to investigate its impacts on invasive behavior, EMT-associated markers, and angiogenic factors in the SKOV3 ovarian cancer cell model.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were sourced from Sigma-Aldrich Co., Germany. Polyvinylidene fluoride (PVDF) membranes were obtained from Roche Co., Germany. Primary antibodies originated from Santa Cruz Co., USA. Horseradish peroxidase (HRP)-linked secondary antibodies were acquired from Abcam Co., USA. Enhanced chemiluminescence (ECL) reagents were supplied by Amersham Pharmacia, Freiburg, Germany. The RNeasy mini kit came from Qiagen Co., Korea. The cDNA synthesis kit was provided by BioFact Co., Korea. SYBR Green PCR master mix was from Ampliqon Co., Denmark.

Plant extraction and gaillardin purification

Specimens of Inula oculus-christi L. (Asteraceae) were gathered from Mazandaran Province, Iran, during July 2023. Botanical identification was verified at the Herbarium of the Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences (voucher specimen: TMRC-4630). Gaillardin purification from the plant's aerial sections followed the established protocol described by Hamzeloo-Moghadam *et al.* [18].

Cell maintenance

The human ovarian adenocarcinoma SKOV3 line was procured from the Pasteur Institute in Tehran, Iran. Cells were maintained in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Cultures were kept at 37 °C in a humidified atmosphere containing 5% CO2. Purified gaillardin was solubilized in 1% dimethyl sulfoxide (DMSO) to prepare stock solutions.

Assessment of cell proliferation

Viability of SKOV3 cells was evaluated using the MTT assay. Cells were plated at a density of about 10,000 per well in 96-well plates. Exposure to varying gaillardin concentrations (0–100 µM) occurred over 24, 48, and 72 hours. Control wells received standard medium, while vehicle controls contained DMSO. Following incubation with MTT solution (5 mg/mL) for 4 hours, formazan dissolution was achieved with 100 µL DMSO per well. Optical density was measured at 570 nm and 670 nm to determine viability percentages.

Scratch assay

The influence of gaillardin on the migratory and invasive potential of SKOV3 cells was investigated through the wound healing assay, following the protocol outlined previously [19]. Briefly, 4×10^5 cells per well were plated in 6-well culture dishes and cultured until they reached 60–70% confluence across the surface. Three parallel linear wounds were then generated in distinct regions of each well using sterile 200 µL pipette tips. Additionally, a single perpendicular reference line was scratched in every well to ensure consistent photographic capture at identical positions. Floating debris was eliminated by rinsing the wells with phosphate-buffered saline (PBS, pH ≈ 7.4). Control groups received standard DMEM medium, whereas treatment groups were exposed to gaillardin

at concentrations of 12.5 and 25 μM. Images of the wounds were recorded at 0, 9, 24, and 48 hours post-scratching, and wound closure areas were quantified employing ImageJ software.

Gene expression analysis

The impact of gaillardin on mRNA levels of markers associated with epithelial-mesenchymal transition (EMT) and angiogenesis was evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). SKOV3 cells were exposed to 12.5 or 25 μM gaillardin for 48 hours, after which total RNA was isolated utilizing the RNeasy mini kit. RNA purity and concentration were determined spectrophotometrically with a Nanodrop 2000c instrument. Complementary DNA (cDNA) was subsequently synthesized from the isolated RNA using a dedicated cDNA synthesis kit. Gene expression quantification was performed on an ABI PRISM 7900HT system (Applied Biosystems, USA) employing SYBR Green PCR master mix. The targeted transcripts included EMT-related genes (CDH1, CDH2, VIM, FN1), the pro-angiogenic factor VEGFA, and the anti-angiogenic factor THBS1. Normalization was achieved using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. Primer sequences for all analyzed genes are listed in **Table 1**.

Protein expression analysis

Western blot analysis was conducted to determine the effects of gaillardin on protein levels of EMT- and angiogenesis-associated markers in SKOV3 cells. Cells were treated with 12.5 or 25 μM gaillardin for 48 hours, then harvested via trypsinization and lysed in radioimmunoprecipitation assay (RIPA) buffer. Total protein quantification was carried out using the Bradford assay [20]. Forty micrograms of protein per sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes. Non-specific binding was blocked with 5% non-fat dry milk for 1 hour at room temperature. Membranes were then probed overnight at 4 °C with primary antibodies specific for E-cadherin (diluted to 1 μg/mL), vimentin (diluted to 2 μg/mL), N-cadherin (1:1000 dilution), fibronectin 1 (1:1000 dilution), vascular endothelial growth factor A (VEGFA; 1:1000 dilution), and thrombospondin 1 (1:250 dilution). Beta-actin (diluted 1:2500) served as the loading control. Following three washes in Tris-buffered saline with Tween (TBST), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution) for 1 hour at room temperature. Protein bands were detected by adding enhanced chemiluminescence reagent and exposing the membranes to X-ray film.

Statistical analysis

Data were subjected to statistical evaluation using one-way analysis of variance (ANOVA), with Duncan's multiple range test applied for post hoc comparisons, performed via GraphPad Prism software. Statistical significance was defined at $P < 0.05$ and $P < 0.01$. All assays were performed in triplicate, and results are expressed as mean ± standard deviation (SD).

Table 1. Primer sequences used in the present study

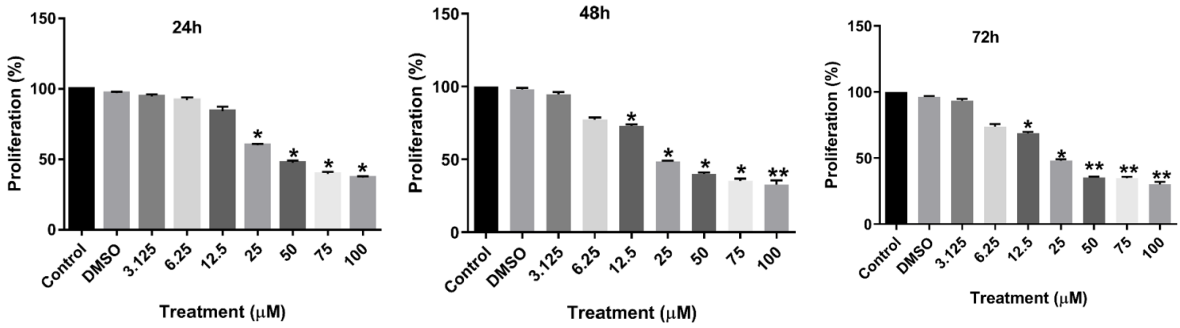
Gene name	Forward primer	Reverse primer
CDH1	5'-GGGGTCTGTCATGGAAGGTG-3′	5'-CGACGTTAGCCTCGTTCTCA-3′
CDH2	5'-GCGTCTGTAGAGGCTTCTGG-3′	5'-GCCACTTGCCACTTTTCCTG-3′
FN1	5'-ACAAGCATGTCTCTCTGCCAA-3′	5'-TCAGGAAACTCCCAGGGTGA-3′
VIM	5'-TCCGCACATTCGAGCAAAGA-3′	5'-ATTCAAGTCTCAGCGGGCTC-3′
VEGFA	5'-GAGCAAGACAAGAAAATCCC-3′	5'-CCTCGGCTTGTCACATCTG-3′
THBS1	5'-CCCTTGTGCTCAGAGTGGAT-3′	5'-GCCAGTAGAGAACAAATAAGCATGG-3′
β-Actin	5'- CACCATTGGCAATGAGCGGTTC-3′	5'- AGGTCTTTGCGGATGTCCACGT-3′

Results and Discussion

The MTT assay was employed to determine the IC_{50} values of gaillardin in SKOV3 cells following exposure. Treatment with escalating doses of gaillardin (0–100 μM) for 24 hours led to a marked reduction in cell viability at concentrations exceeding 25 μM, yielding an IC_{50} of 50.95 μM **(Figure 1a).** Exposure for 48 hours notably impaired viability at levels above 12.5 μM, with an IC_{50} of 24.92 μM **(Figure 1b).** A 72-hour treatment produced a comparable pronounced decline in viability, resulting in an IC_{50} of 24.82 μM **(Figure 1c).** On the basis of these

findings, subsequent assays utilized gaillardin at 25 µM (approximating IC_{50}) and 12.5 µM (approximating IC_{25}) for a 48-hour incubation period in SKOV3 cultures.

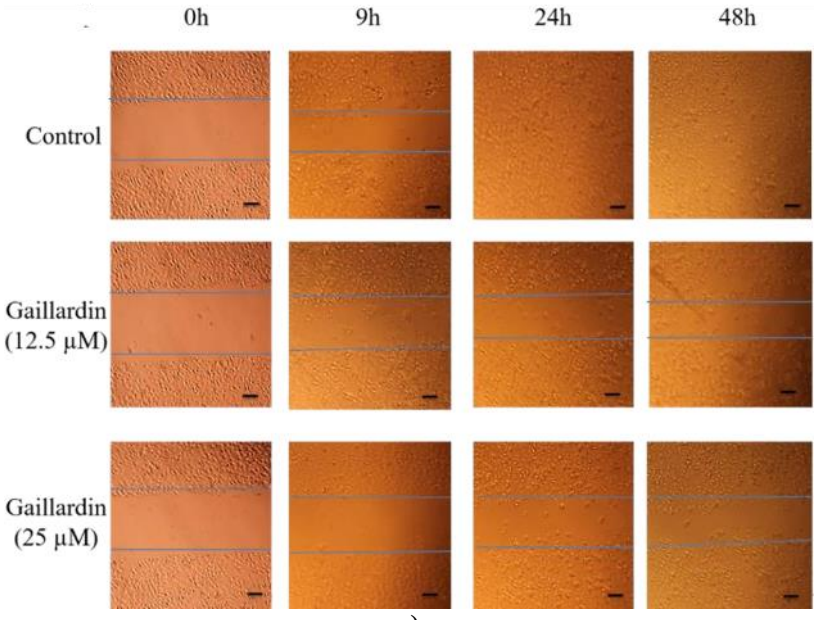
To assess the impact of gaillardin on the wound healing ability of SKOV3 cells, a scratch wound assay was conducted. As depicted in **Figure 2a**, untreated control cells maintained in standard DMEM medium effectively migrated into the wounded region by 24 hours. In contrast, exposure to 12.5 µM gaillardin noticeably restricted cell movement toward the central scratched zone. Higher-dose treatment with 25 µM gaillardin further suppressed the migratory behavior of these cells. Quantification of remaining wound areas revealed that 25 µM gaillardin over 48 hours substantially inhibited closure in SKOV3 cells **(Figure 2b).**



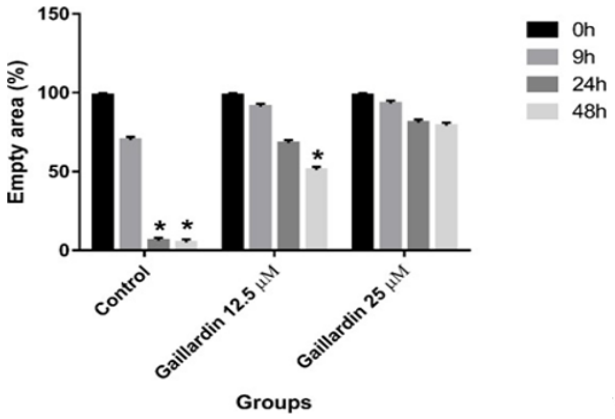
a) b) c)

Figure 1. Impact of gaillardin on the growth inhibition of SKOV3 ovarian cancer cells. Cells were exposed to a range of gaillardin concentrations (0, 6.25, 12.50, 25, 50, 75, and 100 µM) over 24 (a), 48 (b), and 72 (c) hours. Viability was evaluated using the MTT assay in treated versus untreated groups. Results represent the mean ± SD from three separate replicates.

Notable differences between treated samples and controls are indicated by *p < 0.05 and **p < 0.01.



a)



b)

Figure 2. (a) Influence of gaillardin on the migratory and invasive behavior of SKOV3 cells. Confluent cultures were wounded using a sterile pipette tip following exposure to 12.5 or 25 µM gaillardin. Images documenting wound closure were acquired at 0, 9, 24, and 48 hours post-treatment. (b) Quantification of remaining open wound areas is presented as percentage relative to untreated controls (mean ± SD); *p < 0.05 versus control; Scale bar = 50 µm.

To investigate the impact of gaillardin on gene expression of epithelial-mesenchymal transition (EMT) and angiogenesis markers, SKOV3 cells were exposed to 12.5 or 25 µM gaillardin for 48 hours. Transcript levels of CDH1, CDH2, VIM, FN1, THBS1, and VEGFA were subsequently quantified by real-time PCR. The findings revealed that both concentrations of gaillardin markedly elevated mRNA expression of CDH1 and THBS1 relative to untreated controls. In contrast, transcript levels of CDH2, VIM, FN1, and VEGFA were substantially reduced in treated cells compared to those receiving standard medium **(Figure 3).**

To validate these gene expression changes at the translational level, Western blot analysis was performed on the corresponding proteins in gaillardin-treated SKOV3 cells. As illustrated in **Figure 4**, treatment with 12.5 or 25 µM gaillardin enhanced protein abundance of E-cadherin and thrombospondin 1 compared to controls. Conversely, protein levels of N-cadherin, vimentin, fibronectin 1, and VEGFA were notably diminished in treated groups relative to vehicle controls.

The collective evidence from this study indicates that exposing the human ovarian carcinoma SKOV3 cell line to the sesquiterpene lactone gaillardin substantially impairs cell viability versus untreated counterparts. Furthermore, scratch wound healing experiments demonstrated that gaillardin suppresses migration and invasion of SKOV3 cells in a concentration-dependent fashion.

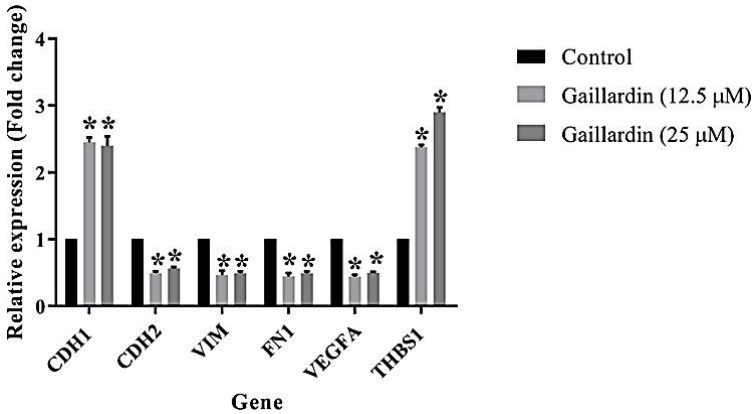
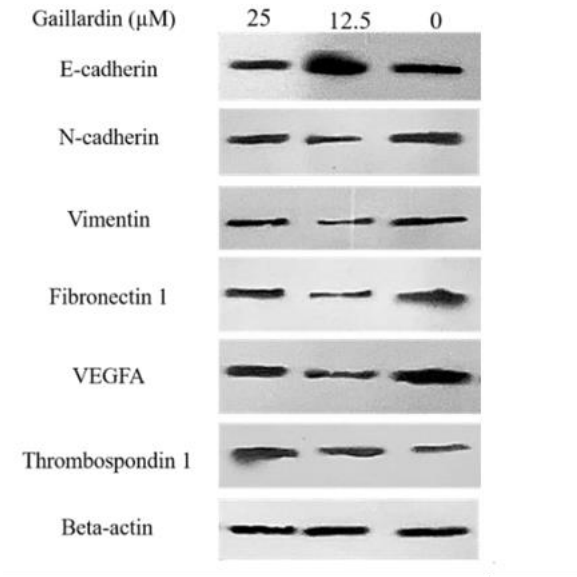
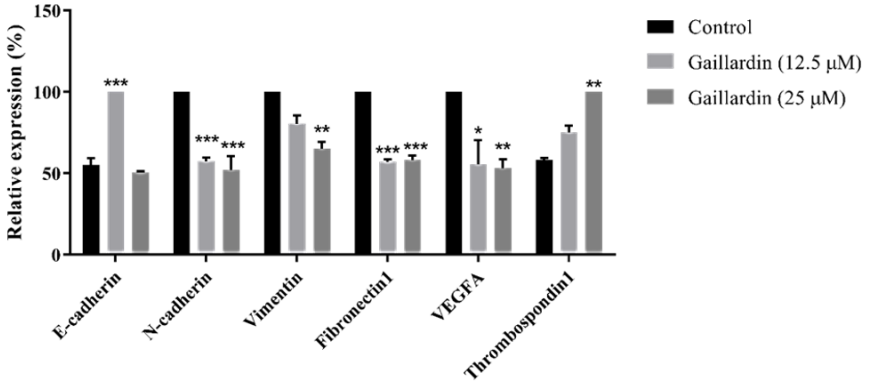


Figure 3. Impact of gaillardin (12.5 and 25 µM, 48 hours) on mRNA expression of epithelial-mesenchymal transition markers (CDH1, CDH2, VIM, FN1) and angiogenesis-related markers (VEGFA, THBS1) in SKOV3 ovarian cancer cells. Results are shown as mean ± SD from three independent replicates; *p < 0.05 versus untreated control.



a)



b)

Figure 4. Influence of gaillardin (12.5 and 25 μM, 48 hours) on protein levels of E-cadherin, N-cadherin, vimentin, fibronectin 1, VEGFA, and thrombospondin 1 in the SKOV3 ovarian cancer cell line.

Analysis of EMT marker transcripts revealed that gaillardin treatment markedly elevated CDH1 mRNA levels while substantially suppressing expression of CDH2, VIM, and FN1 in treated SKOV3 cells. Additionally, gaillardin reduced the pro-angiogenic marker VEGFA and enhanced the anti-angiogenic marker THBS1 in this cell line. Western blot results corroborated these transcriptional changes, demonstrating increased E-cadherin protein alongside decreased N-cadherin, vimentin, and fibronectin 1 in gaillardin-exposed cells relative to untreated controls. Furthermore, gaillardin diminished VEGFA protein abundance while elevating thrombospondin 1 levels in SKOV3 cultures.

Gaillardin represents a distinctive sesquiterpene lactone, and to our knowledge, only eight prior investigations have explored its cytotoxic potential. For instance, Hamzeloo-Moghadam *et al*. confirmed the growth-inhibitory actions of gaillardin against breast, liver, lung, and colon carcinoma cells, attributing its effects on breast cancer models to apoptosis induction [18]. Noormohamadi *et al*. evaluated gaillardin singly or combined with arsenic trioxide in the HL-60 myeloid leukemia line, reporting antiproliferative activity via G1 arrest and ROS-mediated apoptosis, with synergistic enhancement of arsenic trioxide cytotoxicity [21]. Another report documented apoptosis promotion and growth suppression in the Nalm-6 acute lymphoblastic leukemia line following gaillardin exposure [22]. Roozbehani and colleagues assessed gaillardin's impact on gastric carcinoma lines AGS and MKN45, observing potent cytotoxicity through apoptosis, accompanied by NF-κB pathway inhibition, ROS-dependent downregulation of COX-2, MMP-9, TWIST-1, and Bcl-2 [8]. In our earlier work, gaillardin triggered apoptosis in MCF-7 breast cancer cells by disrupting JAK/STAT signaling while concurrently inhibiting autophagy [9]. Fallahian *et al*. demonstrated mitochondrial pathway activation and ROS involvement in gaillardin-induced apoptosis across MCF-7 and MDA-MB-468 breast cancer models [10].

Karami *et al*. explored gaillardin alone or with vincristine in ALL lines NALM-6 and MOLT-4, finding proliferation blockade, G0/G1 arrest, apoptosis induction, and vincristine sensitization [11].

To our knowledge, this investigation provides the inaugural evidence of gaillardin's antiproliferative and antimetastatic properties in ovarian cancer. As detailed, this compound modulated EMT markers and angiogenesis regulators to impair SKOV3 cell migration and invasion. EMT enables carcinoma cells to acquire mesenchymal traits, facilitating detachment from the primary site, local invasion, and distant dissemination [23, 24]. Hallmarks include diminished cell-cell adhesion and polarity, cytoskeletal reorganization, and modified cell-matrix interactions [25]. Key indicators encompass E-cadherin (downregulated) and N-cadherin, vimentin, and fibronectin 1 (upregulated) during EMT progression [26-29]. The observed gaillardin-mediated upregulation of E-cadherin coupled with downregulation of N-cadherin, vimentin, and fibronectin 1 thus supports its capacity to counteract invasive and migratory phenotypes in ovarian carcinoma cells.

Angiogenesis, essential for sustaining metastatic dissemination, requires heightened activation to supply migrating tumor cells [30]. This multifaceted process is governed by balanced pro- and anti-angiogenic signals [31]. VEGFA stands as a predominant driver, secreted by tumor cells to stimulate endothelial proliferation via VEGF receptors, rendering it a prime therapeutic target [32, 33]. Conversely, thrombospondin-1 exerts potent anti-angiogenic effects by promoting endothelial apoptosis, inhibiting migration/proliferation, and modulating VEGF availability/activity [34, 35]. The gaillardin-induced reduction in VEGFA alongside elevation of thrombospondin 1 observed here indicates robust anti-angiogenic potential.

Conclusion

The findings presented herein establish gaillardin as a potent suppressor of invasion, migration, and potential metastasis in the SKOV3 ovarian cancer model. This sesquiterpene lactone markedly curtailed cell proliferation and invasiveness. These outcomes implicate inhibition of EMT and angiogenesis—two cornerstone mechanisms of metastatic progression—as central to gaillardin's antimetastatic efficacy. While additional in-depth studies are warranted to substantiate its anticancer utility in broader ovarian cancer contexts, the current evidence positions gaillardin as a promising agent for restraining proliferation and metastatic spread in this malignancy. Accordingly, these data advocate for further exploration of gaillardin as a therapeutic candidate against ovarian cancer.

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Conflict of Interest: None

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Ethics Statement: This research received approval from the Ethics Committee at Shahid Beheshti University of Medical Sciences (IR.SBMU.REC.1402.013).

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