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Original Research Article

Cytotoxic Activity Against Breast Cancer of Ethyl Acetate Fraction of the Fruiting Bodies *Ganoderma multiplicatum* through Inducing Apoptosis

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ABSTRACT

Breast cancer remains the most frequently diagnosed malignancy among women worldwide, underscoring the need for safer and more effective therapeutic agents, particularly those derived from natural medicinal resources. This study evaluated the cytotoxic and apoptosis-inducing activities of the ethyl acetate fraction (GMEA) obtained from the 96% ethanol extract of *Ganoderma multiplicatum* (GME) against human breast cancer MCF-7 cells. Triterpenoid quantification using the vanillin assay revealed enrichment of these compounds in GMEA (5.84 ± 1.41 mg/g) compared with GME (4.83 ± 0.40 mg/g). GMEA significantly reduced MCF-7 cell viability in a dose-dependent manner, with an IC_{50} value of 147.69 µg/mL after 48 h. Flow cytometry demonstrated marked increases in both early (0.8% -16.1%) and late apoptosis (0.7% - 2.8%). Importantly, GMEA exhibited minimal cytotoxicity toward human fibroblasts at concentrations ≤ 400 µg/mL, suggesting selective activity against cancer cells. Collectively, these findings indicate that triterpenoid enriched fraction from *Ganoderma multiplicatum* exert cytotoxic effects through apoptosis induction and highlight this species as a promising and underexplored source of natural anticancer agents.

Keywords: Breast cancer, *Ganoderma multiplicatum*, Apoptosis, Triterpenoids, MCF-7.

Introduction

Breast cancer remains the most commonly diagnosed malignancy and a leading cause of cancer-related mortality among women worldwide. According to the GLOBOCAN 2022 statistics, approximately 2.3 million new cases and more than 660000 deaths were recorded, underscoring the substantial global health and economic burden posed by this disease.[1] Although conventional therapeutic strategies including surgery, chemotherapy, radiotherapy and targeted therapy have significantly improved patient survival, their clinical utility is often limited by severe adverse effects, immunosuppression, and the development of drug resistance.[2-4] These challenges highlight the urgent need to explore safer and more effective anticancer agents, particularly those derived from natural products with established traditional use and favourable safety profiles.[5,6]

Medicinal mushrooms represent an important group of natural resources long utilized in traditional Asian medicine for their immunomodulatory, antioxidant and anticancer properties.[7-9] Among them, the genus *Ganoderma* has received considerable scientific attention due to its diverse bioactive constituents,[10-13] especially triterpenoids and polysaccharides, which have been well documented to exert cytotoxic, antiproliferative and apoptosis-inducing effects in various cancer cell lines.[14-18]

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*Extracts of *G. lucidum*, *G. tropicum*, *G. sinense*, *G. pfeifferi* and *G. resinaceum* have demonstrated potent inhibitory activity against breast cancer cells, suggesting that *Ganoderma* species constitute a promising source of anticancer agents.[19-27] *Ganoderma multiplicatum* is a laccate Ganoderma species distributed across tropical and subtropical regions, including Africa, China, South America and Southeast Asia,[19-25] with recent taxonomic records confirming its presence in Vietnam.[26] Despite growing interest in the medicinal potential of *Ganoderma* species, research on *G. multiplicatum* remains limited. A recent study reported its antiproliferative activity against the SUM149PT breast cancer cell line; however, no investigation has yet evaluated its effects on the widely used estrogen receptor-positive MCF-7 cell line.[27] Moreover, the bioactive constituents responsible for its anticancer action, particularly triterpenoids, which are enriched in semi-polar fractions, have not been systematically characterized. The present study aimed to address these gaps by evaluating the cytotoxic and apoptosis-inducing activities of the ethyl acetate fraction obtained from the 96% ethanol extract of *G. multiplicatum* fruiting bodies.

Materials and Methods

Cell lines and chemical reagents

The human breast cancer cell line MCF-7 was obtained from HCM Biotech (Ho Chi Minh City, Vietnam), and the human fibroblast cell line (hF) was provided by the Department of Physiology and Animal Biotechnology, University of Science, Vietnam National University–Ho Chi Minh City.

Analytical-grade ethanol was purchased from VN-CHEMSOL Co., Ltd (Ho Chi Minh City, Vietnam). *n*-Hexane and ethyl acetate were obtained from Fisher Scientific Korea Ltd (Seoul, Korea). DMEM medium was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Camptothecin (CPT), fetal bovine serum (FBS), penicillin–streptomycin, and MTT reagent were supplied by Sigma-Aldrich (St. Louis, MO, USA). Filter paper (NewStar grade 101, 20–25 µm pore size) was obtained from Hangzhou Special Paper Industry Co., Ltd (Zhejiang, China). Distilled water was produced using a Merit

W4000 purification system (Stuart, Illinois, USA). All chemicals and solvents used were of analytical grade.

Plant and extraction

Fruiting bodies of *Ganoderma multiplicatum* (VNKKK1901) were collected in August 2019 from Kon Ka Kinh National Park (Gia Lai Province, Vietnam; 14°09′22″–14°29′52″ N, 108°15′26″–108°27′25″ E) by Dr. Nguyen T.T.T. from the Faculty of Biology and Biotechnology, University of Science, Vietnam National University Ho Chi Minh City. Voucher specimens were deposited in the Department of Microbiology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University Ho Chi Minh City, Vietnam. The sequence of the nuclear ribosomal internal transcribed spacer (ITS) region has been deposited in GenBank under accession number MZ703635. Species identification of the fruiting bodies was based on morphological characteristics and phylogenetic analysis, as described by Nguyen T.T.T. and colleagues.[26] The basidiocarps were dried at 50 °C to constant weight in a heating and drying chamber, ground into powder, and stored in sealed zip-lock bags (total dry weight, 3 kg) until use.

Plant extraction

The ethyl acetate fraction (GMEA) was prepared from the 96% ethanol extract (GME) of *G. multiplicatum* fruiting bodies (Figure 1). Dried basidiocarps of *G. multiplicatum* (total dry weight, 3 kg) were extracted with 45 L of 96% ethanol (1:15, *w/v*) by maceration at room temperature for 72 h. The mixture was filtered, and the residue was re-extracted once with the same solvent and volume. The combined filtrates were passed through filter paper and concentrated under reduced pressure at 40 °C using an IKA RV10 rotary evaporator (Königswinter, Germany). The dried ethanolic extract was dissolved in 40 mL of hot distilled water (70 °C; extraction ratio: 7 g extract/40 mL water) and sequentially partitioned with n-hexane and ethyl acetate (30 mL each, repeated several times). Ethyl acetate fractions were pooled and evaporated to dryness at 40 °C to obtain GMEA. Extraction yield (%) was calculated following previously described methods.[28]

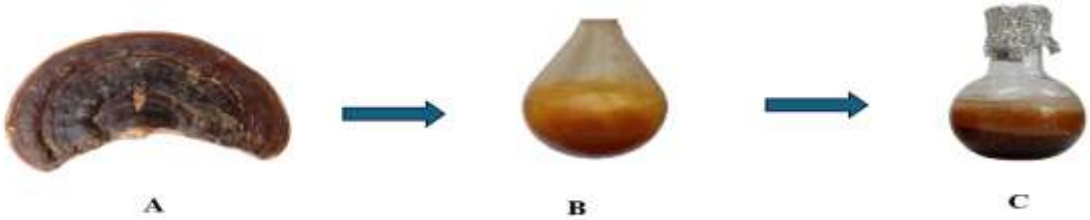

Figure 1: Isolation of ethyl acetate fraction from fruiting bodies of *Ganoderma multiplicatum*. (A) Fruiting bodies of *G. multiplicatum*; (B) 96% ethanol extract-GME; (C) Ethyl acetate fraction-GMEA.

Triterpenoid Quantification

Total triterpenoids in GME and GMEA were quantified using the vanillin–perchloric acid colorimetric method, with oleanolic acid as the reference standard.[29,30] Oleanolic acid was dissolved in 96% ethanol to prepare a 0.2 mg/mL stock solution. Standard solutions (0-0.5 mL) were evaporated to dryness, followed by sequential addition of vanillin-acetic acid reagent and perchloric acid. The reaction mixtures were incubated at 70 °C for 15 min in a Memmert WNB14 water bath, cooled in an ice bath for 5 min, and diluted with ethyl acetate. Absorbance was measured at 546 nm. A calibration curve was generated ($y = 12.537x + 75.333$, $R^2 = 0.9997$). GMEA (0.2 mg/mL in ethyl acetate) was analyzed under identical conditions.

Cytotoxicity Assay on MCF-7 Cells

The antiproliferative activity of GMEA was evaluated using the MTT assay.[14,31] The MCF-7 cells were seeded into 96-well plates at 0.35×10^4 cells/well and allowed to adhere for 24 h. Cells were then treated with GMEA at concentrations of 25–500 µg/mL for 24 or 48 h. After treatment, 100 µL of MTT solution (500 µg/mL) was added to each well and incubated for 3 h. The supernatant was removed, and formazan crystals were dissolved in 100 µL of DMSO. Absorbance at 540 nm was recorded using a VersaMax™ microplate reader (Molecular Devices, USA). The IC_{50} value was calculated using the linear regression equation $y = -0.2466x + 86.42$ ($R^2 = 0.9908$).

Cytotoxicity Assay on Human Fibroblast (hF) Cells

The hF cells were seeded at a density of 1×10^4 cells/well in 96-well plates using MTNC medium (DMEM/F12 supplemented with 10% FBS and 1% penicillin–streptomycin). After 24 h, cells were treated with GMEA at 100–500 µg/mL for 24 or 48 h. MTNC medium alone served as the negative control, while MTNC containing 20% DMSO served as the positive control.[14,31] Cell viability (%) was calculated according to the equation:

$$\text{Cell viability (\%)} = \left(\frac{\text{OD}_{\text{treated group}}}{\text{OD}_{\text{untreated group}}}\right) \times 100.$$

Flow Cytometry Analysis of Apoptosis

MCF-7 cells were seeded in 60 × 15 mm culture dishes at 4.5×10^5 cells/dish and incubated for 24 h. Cells were then assigned to four groups: negative control (medium alone), solvent control (0.18% DMSO), positive control (camptothecin, 1 µM), and treatment group (GMEA, 150 µg/mL). After 24 or 48 h of incubation, cells were harvested, washed twice with PBS, and resuspended in 1× Binding Buffer at 1×10^6 cells/mL. A 100 µL aliquot of the cell suspension was stained with 5 µL Annexin V-PE and 5 µL 7-AAD (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen™) and incubated for 15 min at room temperature in the dark. Samples were then diluted with 400 µL of Binding Buffer and analyzed using a BD FACSAria™ III flow cytometer.[32]

Statistical analysis

All experiments were performed in triplicate. Results are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test were conducted using R, version 4.4.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) to determine statistically significant differences among means. Statistical significance was set at $p < 0.05$. Microsoft Excel 2019 (Microsoft Corp., Redmond, WA, USA) was used to calculate IC_{50} values by nonlinear multiparameter regression, accepting only fits with $R^2 > 0.9$.

Results and Discussion

Extraction yield

The 96% ethanol extract of *G. multiplicatum* produced an extraction yield of 3.84 ± 0.37% (Table 1). Although relatively low, this value is consistent with the high crude fiber content typically found in laccate *Ganoderma* species, which can hinder solvent permeation and reduce extraction efficiency. Compared with previous reports, the yield obtained using 96% ethanol was higher than that achieved with 70% ethanol (2.4%) in the same species,[27] suggesting that higher ethanol concentrations improve the recovery of semi-polar compounds.

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However, the yield remained lower than that reported for *G. lucidum* (5.61-6.15%) and *G. tsugae* (3.97%) when extracted using methanol, indicating interspecific differences in chemical composition and solvent affinity. The ethyl acetate fraction of *G. multiplicatum* exhibited a markedly high extraction yield of 57.50 ± 5.55%, demonstrating the strong ability of ethyl acetate to solubilize medium-polarity metabolites in this species.[33] This value exceeded previously reported ethyl acetate yields for *G. lucidum* (0.91% and 42.37%), further supporting the potential richness of the *G. multiplicatum* fruiting bodies in semi-polar constituents, particularly triterpenoids and related secondary metabolites.[11,34]

Table 1: Extraction yields of GME and GMEA

Fraction	GME	GMEA
Extraction yeilds (%)	3.84 ± 0.37^{a}	57.50 ± 5.55^{b}

Within the same row, values followed by different letters (a, b) differ significantly at p = 0.05 according to Tukey's test.

Triterpenoid quantification

Quantitative analysis revealed that GMEA contained a higher triterpenoid level (5.84 ± 1.41 mg/g) than the crude ethanolic extract GME (4.83 ± 0.40 mg/g), confirming the enrichment effect of ethyl acetate on semi-polar terpenoids. Taxonomic studies classify *G. multiplicatum* and *G. lucidum* within the laccate *Ganoderma* group, characterized by a lacquered cap surface and a well-documented ability to biosynthesize triterpenoids.[35] Previous report demonstrated that ethyl acetate efficiently extracts lanostane-type triterpenoids from *Ganoderma* species due to their moderate polarity and hydrophobicity.[11] Taken together, these findings indicate that GMEA selectively concentrates triterpenoids, which are widely reported as key contributors to the anticancer activity of *Ganoderma* mushrooms.

The extraction yields obtained in this work are consistent with the chemical characteristics of laccate *Ganoderma* species. The relatively low yield of the 96% ethanolic extract (3.84 ± 0.37%) likely reflects the high fiber and polysaccharide content of the fruiting bodies, which can reduce solvent penetration and mass transfer. In contrast, the high yield of the ethyl acetate fraction (57.50 ± 5.55%) indicates efficient enrichment of medium-polarity constituents, including lanostane-type triterpenoids that are well documented in *Ganoderma* spp.[40] This is further supported by the increased triterpenoid content in GMEA compared with the crude ethanolic extract. Similar enrichment of triterpenoids in ethyl acetate fractions has been reported for *G. lucidum* and *G. colossus*, where these fractions display superior cytotoxicity relative to more polar extracts.[37,38] Taken together, these data suggest that the semipolar metabolite profile of *G. multiplicatum* is compatible with that of other medicinal *Ganoderma* species and that triterpenoids are likely to contribute to the observed anticancer effects.

Cytotoxic activity on MCF-7 breast cancer cells

Morphological observation (Figures 2–3) demonstrated clear signs of cytotoxicity in MCF-7 cells treated with GMEA, including reduced cell density, cell rounding, and membrane fragmentation. These effects intensified with both concentration and exposure time, particularly at concentrations ≥ 100 µg/mL. The MTT assay confirmed this trend, revealing a dose- and time-dependent decrease in cell viability. The calculated IC_{50} value of GMEA was 147.69 µg/mL after 48 h exposure (Table 2), indicating moderate cytotoxic activity. When compared with previous studies, GMEA exhibited stronger cytotoxicity than ethanolic extracts of *G. lucidum* (IC_{50} = 471 µg/mL),[36,37] but weaker than the potent activity reported for *G. colossus* (IC_{50} = 17.2 ± 2.7 µg/mL).[38] Such interspecific variability may reflect differences in triterpenoid composition and abundance, the factors known to influence anticancer potency in *Ganoderma* extracts. The moderate IC_{50} value combined with the selective enrichment of triterpenoids suggests that GMEA retains pharmacologically relevant bioactivity and warrants further phytochemical investigation.

GMEA exhibited moderate cytotoxicity against MCF-7 cells, with an IC_{50} value of 147.69 µg/mL after 48 h of treatment. This potency is comparable to or greater than that reported for several crude or fractionated extracts from other *Ganoderma* species tested on breast cancer cells. For instance, alcohol extracts of *G. lucidum* have been shown to inhibit MCF-7 proliferation in a dose- and time-dependent manner, with effective concentrations typically in the mid- to high-micromolar or hundreds of µg/mL range, and to induce G1 cell-cycle arrest and apoptosis.[14] Recent work on *G. lucidum* and related species also reports IC_{50} values for breast cancer cell lines that frequently fall between 100 and 400 µg/mL, depending on the extraction solvent and fractionation strategy.[39] In contrast, the ethyl acetate extract of *G. colossus* displays markedly stronger activity (IC_{50} = 17.2 µg/mL against MCF-7), consistent with a higher content of highly potent triterpenoids and sterols.[38] The IC_{50} of GMEA can be interpreted as moderate but pharmacologically relevant, particularly given that it is a crude semi-polar fraction rather than a purified compound. The variability in potency among *Ganoderma* species underscores the importance of both species selection and fractionation strategy in optimizing anticancer activity.

Cytotoxicity on human fibroblast (hF) cells

The effects of GMEA on hF cells were also dose- and time-dependent (Figure 4). Cell viability remained above the cytotoxicity threshold defined by ISO 10993-5 (≥ 80% viability) at concentrations up to 400 µg/mL after both 24 and 48 h, indicating that GMEA is generally non-cytotoxic toward normal fibroblasts at these levels. Cytotoxicity became evident only at 500 µg/mL, where viability decreased to 76.1% (24 h) and 62% (48 h). These findings suggest that GMEA exhibits higher sensitivity toward cancer cells (MCF-7) than toward normal fibroblasts, indicating a degree of selective cytotoxicity. This selectivity is advantageous for potential therapeutic applications and aligns with previous reports describing selective anticancer effects of *Ganoderma* triterpenoids.[40,41]

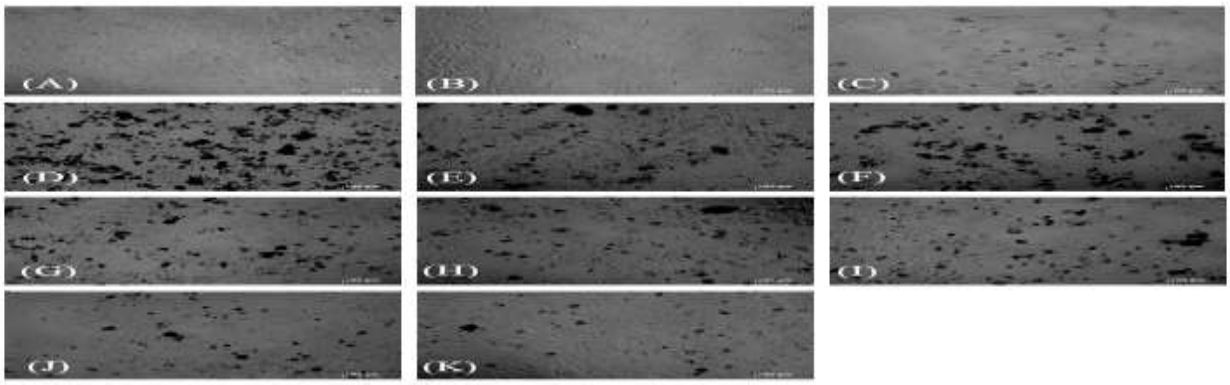

Figure 2: MCF-7 breast cancer cells after of 24 hours of treatment. (A) Negative control, (B) 0.18% DMSO, (C) CPT 1 µM (positive control); (D) GMEA: 500 µg/mL; (E) 400 µg/mL; (F) 300 µg/mL; (G) 200 µg/mL; (H) 150 µg/mL; (I) 100 µg/mL; (J) 50 µg/mL; (K) 25 µg/mL.

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Table 2: The percentage of cell viability after 48 hours treatment of varying concentrations (25–500 µg/mL) of GMEA

GMEA of concentration (µg/mL)	The percentage of cell viability (%)	% inhibition
500	7 ± 0	93
400	6 ± 1	94
300	15 ± 4	85
250	28 ± 1	72
200	34 ± 2	66
150	38 ± 5	62
100	60 ± 2	40
50	77 ± 10	23
25	81 ± 4	19
Negative control	100 ± 6	0
Positive control	48 ± 2	52
0.18 % DMSO	104 ± 9	4

Negative control: Culture medium; Positive control: Camptothecin 1 µM. Average performance of 3 replicates ± SD (standard deviation), data processing by one-factor ANOVA with $p < 0.05$

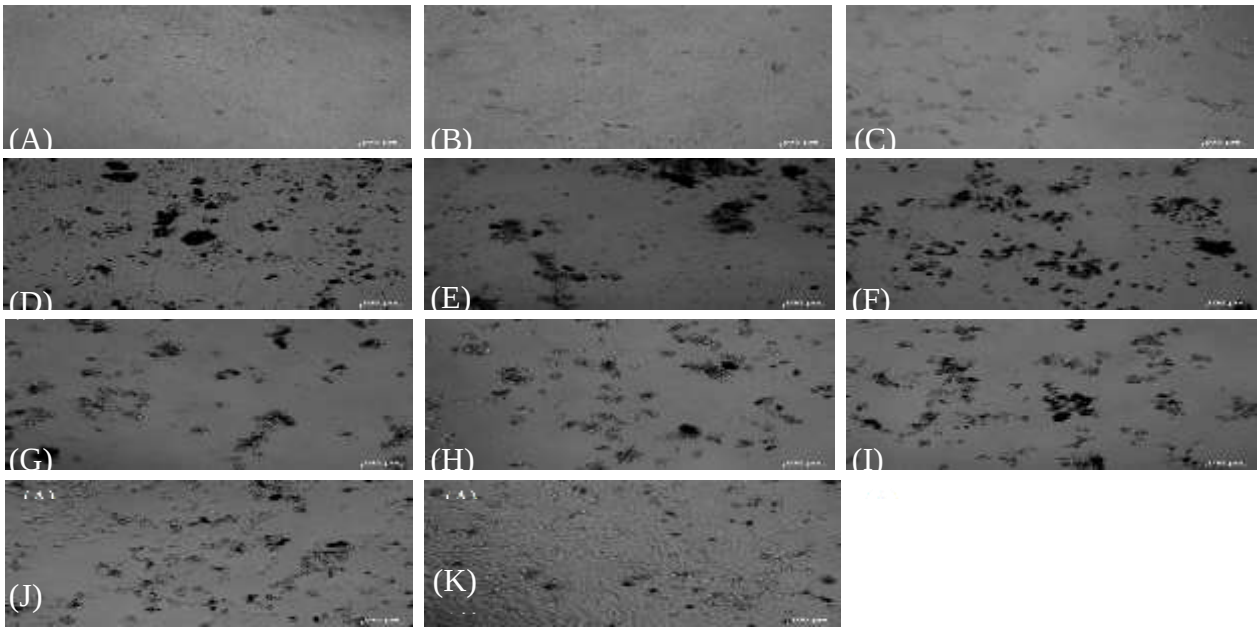

Figure 3: MCF-7 breast cancer cells after 48 hours of treatment. (A) Negative control; (B) 0.18% DMSO; (C) CPT 1 µM (positive control); (D) GMEA: 500 µg/mL; (E) 400 µg/mL; (F) 300 µg/mL; (G) 200 µg/mL; (H) 150 µg/mL; (I) 100 µg/mL; (J) 50 µg/mL; (K) 25 µg/mL.

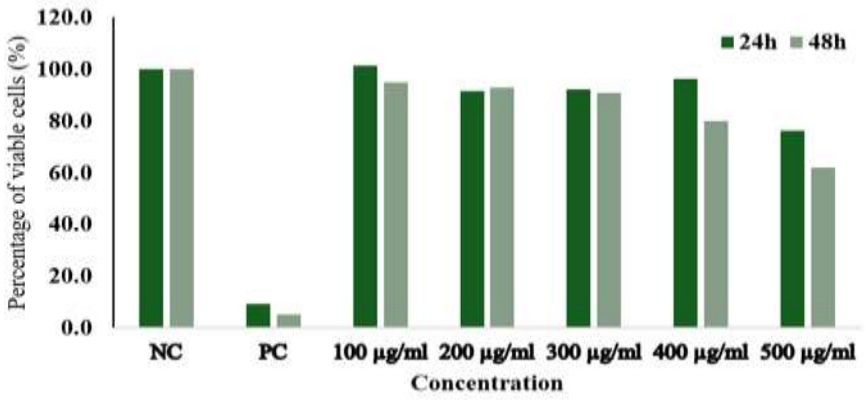

Figure 4: Percentage of viable hF cells based on the concentration of GMEA. NC: MTNC medium, PC: MTNC medium with 20% DMSO.

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Flow cytometry analysis of apoptosis

Flow cytometric evaluation using Annexin V-PE/7-AAD staining revealed that GMEA induced apoptosis in a time-dependent manner (Figure 5 and Figure 6). After 24 h of exposure, late apoptotic cells increased from 2.1% (control) to 4.4%, while early apoptotic cells showed a slight reduction, suggesting a potential progression from early to late apoptosis (Figure 7). At 48 h, apoptotic induction became more pronounced, with early apoptosis rising from 0.8% (control) to 16.1%, and late apoptosis increasing from 0.7% to 2.8% (Figure 7). These results corroborate previous findings that *Ganoderma* extracts primarily activate apoptotic pathways in cancer cells.[14,17,38] Mechanistically, triterpenoids from *G. lucidum* have been shown to trigger intrinsic mitochondrial apoptosis by downregulating anti-apoptotic proteins (Bcl-2, Bcl-xL), upregulating Bax, promoting cytochrome c release, and activating caspase-9. In MCF-7 cells, which lack caspase-3, downstream executioner activity is mediated by caspase-7. This Bax, cytochrome c, caspase signaling cascade leads to chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies. The similarity between our findings and the established apoptotic mechanisms of *Ganoderma* triterpenoids strongly suggests that GMEA exerts its anticancer activity predominantly through triterpenoid-mediated activation of intrinsic apoptosis. These observations support further investigations, such as Western blotting or proteomic profiling, to confirm the involvement of specific apoptotic regulators.

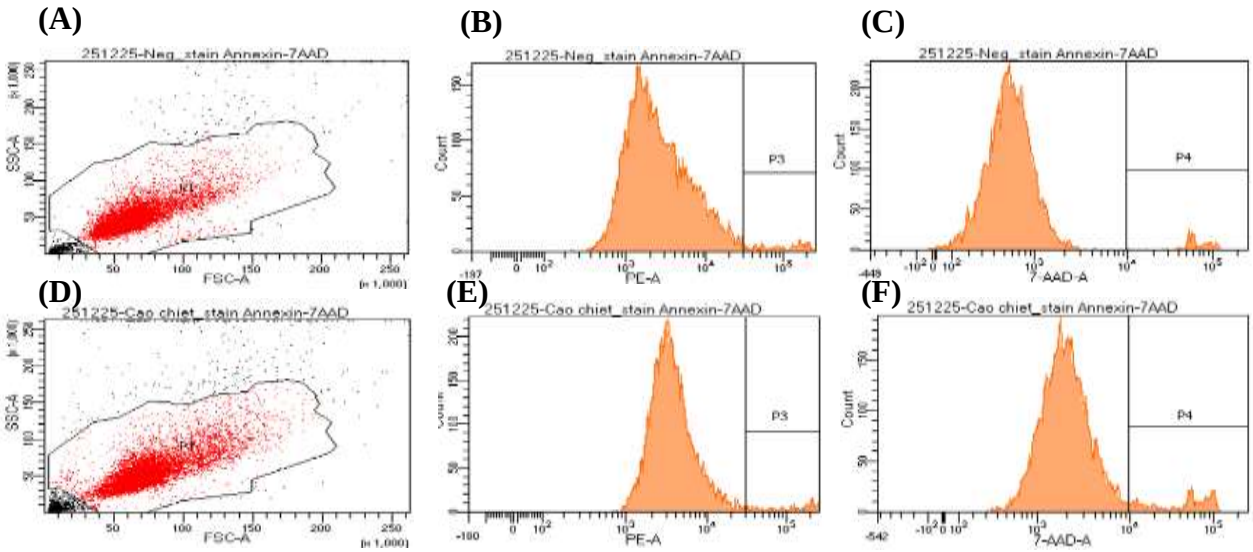

Figure 5: Analysis of apoptosis cell populations using Annexin V–PE/7-AAD staining in the control sample and the sample treated with GMEA after 24 hours. (A) Dot plot of the negative control sample; **(B)** Histogram of Annexin V–PE staining in the negative control sample; **(C)** Histogram of 7-AAD staining in the negative control sample; (D) Dot plot of the GMEA-treated sample; **(E)** Histogram of Annexin V–PE staining in the GMEA-treated sample; **(F)** Histogram of 7-AAD staining in the GMEA-treated sample.

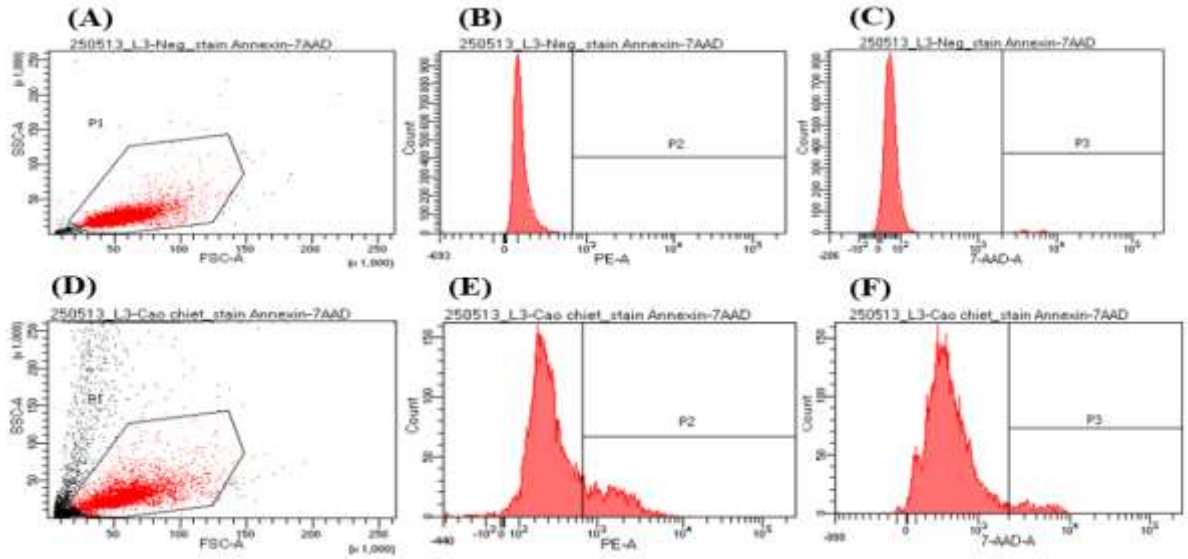

Figure 6: Analysis of apoptosis cell populations using Annexin V–PE/7-AAD staining in the control sample and the sample treated with GMEA after 48 hours. (A) Dot plot of the negative control sample; **(B)** Histogram of Annexin V–PE staining in the negative control sample; **(C)** Histogram of 7-AAD staining in the negative control sample; (D) Dot plot of the GMEA-treated sample; **(E)** Histogram of Annexin V–PE staining in the GMEA-treated sample; **(F)** Histogram of 7-AAD staining in the GMEA-treated sample.

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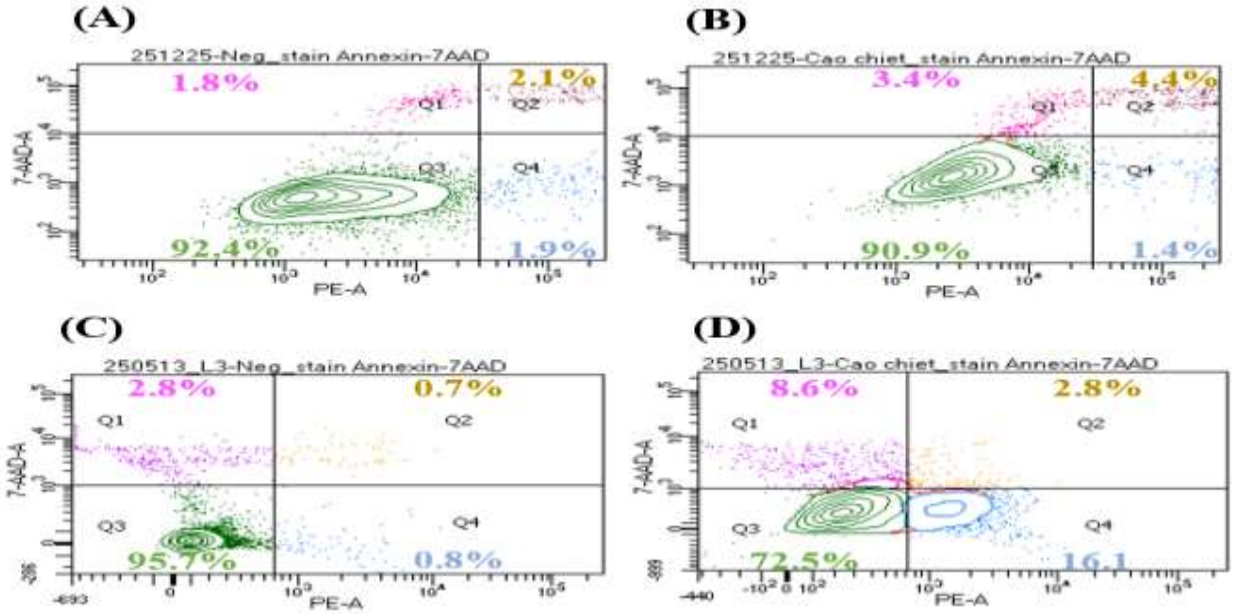

Figure 7: Flow cytometry results of MCF-7 cells. (A) Negative control, 24 hours; (B) GMEA-treated fraction, 24 hours; (C) Negative control, 48 hours; (D) GMEA-treated fraction, 48 hours.

This study provides new evidence supporting the anticancer potential of *Ganoderma multiplicatum*, a relatively underexplored laccate *Ganoderma* species, by demonstrating that its ethyl acetate fraction (GMEA) exerts dose- and time-dependent cytotoxic and pro-apoptotic effects against human breast cancer MCF-7 cells while exhibiting limited toxicity toward normal human fibroblasts. These findings extend previous work on *Ganoderma* spp. and breast cancer, in which most investigations have focused on *G. lucidum*, *G. tsugae*, or *G. resinaceum*, and only very recent studies have begun to address the antineoplastic potential of *G. multiplicatum* in triple-negative breast cancer models such as SUM149PT.[27] By targeting an estrogen receptor positive breast cancer cell line, the present study broadens the pharmacological profile of *G. multiplicatum* and highlights this species as a promising source of semi-polar metabolites with selective cytotoxic activity.

An important finding of this study is the differential sensitivity of cancerous versus non-cancerous cells. While GMEA significantly reduced MCF-7 viability in a dose- and time-dependent manner, fibroblast viability remained above the ISO 10993-5 cytotoxicity threshold at concentrations up to 400 µg/mL and only declined markedly at 500 µg/mL after prolonged exposure.[40] This pattern suggests a degree of selectivity toward malignant cells, a desirable feature for anticancer candidates aiming to minimize off-target toxicity. Selective cytotoxicity has been previously described for *Ganoderma* preparations, including polysaccharides and triterpenoids, which tend to impair proliferation and survival of cancer cells more than that of normal cells, potentially through differential redox status, mitochondrial vulnerability, or oncogene-driven signaling.[27,42] Although a formal selectivity index (SI) calculation was not performed here, the contrasting viability profiles between MCF-7 and hF cells indicate a favorable therapeutic window and support further development of GMEA for anticancer applications. Flow cytometric analysis demonstrated that the cytotoxic effect of GMEA on MCF-7 cells is largely mediated by apoptosis. The marked increase in early apoptotic cells after 48 h exposure, together with the rise in late apoptotic populations, aligns with the canonical pattern of intrinsic, mitochondria-dependent apoptosis. Similar apoptotic profiles have been documented for triterpenoid-rich extracts and purified triterpenes from *G. lucidum* and other *Ganoderma* species, which downregulate cyclin D1, Bcl-2, and Bcl-xL, upregulate Bax, promote cytochrome c release, and activate caspase-9 and executioner caspases.[17] In MCF-7 cells, which lack functional caspase-3, caspase-7 has been shown to assume the executioner role, and its cleavage has been observed following treatment with *G. lucidum* extracts.[43] The close resemblance between our apoptosis data and these previously described mechanisms strongly suggests that triterpenoids enriched in GMEA may trigger a comparable mitochondrial pathway, ultimately leading to chromatin condensation, DNA fragmentation, and apoptotic body formation. However, confirmation of this hypothesis will require protein-level validation (e.g., Western blotting of Bcl-2 family members, caspases, and PARP) and, ideally, targeted inhibition studies. The present results also contribute to the growing body of evidence that triterpenoids from *Ganoderma* spp. constitute a structurally diverse class of natural products with broad anticancer potential. Recent reviews have highlighted that *Ganoderma* triterpenoids can modulate multiple oncogenic pathways, including PI3K/Akt, MAPK, STAT3, and p53, in addition to exerting direct mitochondrial effects, thereby combining cytostatic and pro-apoptotic activities.[44] Although the specific triterpenoid profile of *G. multiplicatum* has not yet been fully elucidated, the triterpenoid enrichment observed in GMEA, together with its apoptosis-inducing effect, strongly supports the view that this species may harbor novel or under-characterized lanostane derivatives with therapeutically relevant activities.

Based on our results, it is possible to note that this study has limitations that should be acknowledged. First, the use of a crude semi-polar fraction precludes direct attribution of the observed effects to specific molecules, and synergistic or antagonistic interactions between constituents cannot be ruled out. Second, mechanistic insights are currently inferred from flow cytometry and literature comparisons rather than directly demonstrated by molecular assays such as Western blotting or gene expression profiling. Third, the work is restricted to in vitro models; in vivo pharmacokinetics, bioavailability, and systemic toxicity of GMEA and its triterpenoids remain unknown. Despite these limitations, the data provide a solid experimental foundation for future studies aimed at (i) isolating and characterizing the active triterpenoids from *G. multiplicatum*, (ii) validating apoptotic pathways at the molecular level, and (iii) assessing efficacy and safety in appropriate in vivo breast cancer models. Nevertheless, the present findings position *G. multiplicatum* as a promising medicinal mushroom with moderate but selective cytotoxic and apoptosis-inducing activity against ER^+ breast cancer cells, mediated at least in part by triterpenoid-enriched semi-polar metabolites. In the broader context of *Ganoderma* research and natural product–based oncology, GMEA represents a valuable starting point for the discovery and development of new anticancer agents or adjuvant therapies for breast cancer.

Conclusion

In summary, this study provides the first evidence that the ethyl acetate fraction of *Ganoderma multiplicatum* (GMEA), enriched in lanostane-type triterpenoids, exhibits notable anticancer activity against human breast cancer MCF-7 cells. GMEA demonstrated dose- and time-dependent cytotoxicity, with an IC_{50} value of 147.69 µg/mL after 48 hours of treatment. Morphological alterations and flow cytometry analyses confirmed that the cytotoxic effect was primarily mediated through apoptosis, characterized by significant increases in both early and late apoptotic populations. Importantly, GMEA displayed minimal toxicity toward normal human fibroblasts at concentrations up to 400 µg/mL, suggesting a degree of selectivity toward malignant cells. Together, these findings highlight *G. multiplicatum* as a promising source of triterpenoid-rich fractions with potential utility in breast cancer therapy. Further studies focusing on compound isolation, mechanistic validation, and in vivo evaluation are warranted to advance the development of *G. multiplicatum* derived anticancer agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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