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In Silico and *In Vitro* Studies of the Antimetastatic and Antiangiogenic Activities of *Cordyceps militaris* Extracts on Breast Cancer Cells (MDA-MB-231)

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ABSTRACT

Introduction. *Cordyceps militaris* (CM), a traditional medicinal fungus in East Asia, has garnered increasing attention due to its potential anticancer properties. Despite extensive use in traditional medicine, the mechanisms underlying its antimetastatic and antiangiogenic effects in breast cancer remain unclear. This study aimed to explore the bioactive components of aqueous extracts derived from CM's mycelium (Aq-CMM) and fruiting body (Aq-CMF), focusing on their potential inhibitory activities on metastasis and angiogenesis in triple-negative breast cancer cells (MDA-MB-231).

Materials and methods. *In silico* molecular docking was conducted to screen for key CM bioactive compounds and evaluate their binding affinities toward metastasis- and angiogenesis-related targets, NF-κB and VEGFR. *In vitro* cytotoxicity was assessed using 2D monolayer and 3D spheroid MDA-MB-231 cultures

treated with Aq-CMM and Aq-CMF. Cell viability (IC_{50}) was determined at 48 hours, and microscopic evaluation of treated spheroids was performed to assess morphological disruption.
Results and conclusions. Docking analyses identified ergothioneine as a primary CM-derived compound with strong binding affinity to NF-κB (–7.83 kcal/mol) and VEGFR (–7.62 kcal/mol), suggesting potent inhibitory effects on metastatic and angiogenic pathways. In vitro assays showed that Aq-CMF exerted greater growth-inhibitory effects (IC_{50} = 54 μg/mL in 2D; 46 μg/mL in 3D) than Aq-CMM at 48 hours. Microscopic observations confirmed notable disruption of spheroid architecture following treatment. These findings highlight the therapeutic potential of ergothioneine-rich CM extracts, particularly from the fruiting body, as promising anticancer agents that warrant further mechanistic and translational studies.

Introduction

Regardless of ongoing efforts to discover novel treatment methods, breast cancer remains one of the most devastating diseases in the world. According to the WHO, 2.3 million women were diagnosed with breast cancer, and 685,000 women died worldwide in 2020. It is predicted that the prevalence of cancer patients will rise to 19 million or more by 2025. Hence, breast cancer is the most common cancer in the world and continues to be the leading cause of cancer-related deaths, presenting a high mortality rate attributed to its high metastatic and angiogenic properties. Current therapeutic compounds and cancer treatments that are available for public use exhibit unwanted effects [1], highlighting the demand for new natural products as treatment options for breast cancer.

Cordyceps militaris (CM), a traditional medicinal fungus widely used in traditional East Asian medicine, has garnered significant scientific interest due to its various health benefits, particularly its potential anticancer properties [2,3]. Several bioactive constituents of CM, including ergothioneine, cordycepin, and adenosine, have demonstrated promising antimetastatic and antiangiogenic properties [1,4–9]. Ergothioneine, in particular, has shown antioxidative and cancer-regulatory effects, including inhibition of tumour growth and modulation of signalling pathways [1]. However, the detailed mechanisms by which compounds act on breast cancer cells remain inadequately understood [10], necessitating further investigation of their molecular interactions and therapeutic potential.

In this study, we compare the anticancer effects of *Cordyceps militaris* fruiting body (CMF) and mycelium (CMM) extracts on breast cancer cells. This comparison is scientifically relevant, as CMF and CMM may differ in the concentration and composition of bioactive compounds due to distinct metabolic processes during their growth stages. Understanding these differences is crucial for identifying the more therapeutically potent component and optimising the use of CM in cancer treatment applications.

Furthermore, to clarify the molecular rationale, we focused on NF-κB and VEGFR as potential targets for analysis. NF-κB is a master regulator of genes involved in inflammation, proliferation, and metastasis, whereas VEGFR is central to angiogenesis and tumour vascularisation. Given that metastasis and angiogenesis are key hallmarks of breast cancer progression, both NF-κB and VEGFR provide suitable targets for in silico docking and in vitro validation of CM-derived compounds.

This study aims to comprehensively evaluate the antimetastatic and antiangiogenic effects of CM extracts, particularly those enriched in ergothioneine, on breast cancer cells. Employing integrated approaches of computational molecular docking studies and advanced *in vitro* models, including 2D monolayer and 3D spheroid cultures, the research seeks to elucidate potential molecular targets and assess the efficacy of CM extracts as promising anticancer agents.

Materials and methods

Preparation of targeted macromolecules and ligands

The ZINC database (https://zinc.docking.org/) provides a list of available compounds that can

be used for virtual screening to identify molecular targets. The following ligands were selected for molecular docking based on their reported biological relevance:

- Cordycepin (ZINC1319796) and Adenosine (ZINC2169830): bioactive constituents of CM with reported anticancer activities [11,12].
- Ergothioneine (ZINC1530224): an antioxidant naturally found in CM with potential cancer-protective properties [13].
- Aspirin (ZINC ID: ZINC 53) is used as a positive control for NF-κB docking, as it inhibits IKKβ-mediated NF-κB activation [14].
- Tivozanib (ZINC ID: ZINC1489430): a selective VEGFR tyrosine kinase inhibitor, included as a positive control for VEGFR docking [15].
- Resveratrol (ZINC ID: ZINC6787): a natural polyphenol reported to inhibit angiogenesis via VEGFR signalling [16].
- Lovastatin (ZINC ID: ZINC3812841): a statin with documented NF-κB modulatory and anti-proliferative effects in breast cancer [17].
- GABA (ZINC1532620): Although primarily a neurotransmitter, recent evidence links GABAergic signalling to tumour suppression, inhibition of cancer cell migration, and modulation of angiogenic pathways [18].
- Cisplatin (Tokyo Chemicals, Japan) was used as a positive control in *in vitro* assays, while untreated cells served as the negative control. For *in silico* docking, baseline docking scores without ligands were considered as negative reference values.

Molecular docking simulation

The protein structures of NF-κB (PDB ID: 1SVC) and VEGFR (PDB ID: 4ASE) proteins were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org). All the molecules were prepared, trimmed, and optimised using BIOVIA Discovery Studio Visualizer 4.5. The localisation of bonds was performed, followed by the addition of polar hydrogen atoms, while also excluding water molecules and ligands from the 3D protein structure [19]. The structures of the molecular interactions between the target proteins and the natural compounds were then predicted and better represented using UCSF Chimera (https://www.cgl.ucsf.edu/chimera). The molecular docking result includes a Jmol applet for web browser visualisation of the expected binding modes [20]. The binding affinity between the target protein and the molecular compound was displayed. UCSF Chimaera was launched for additional visual investigations to view the predicted binding modes [20]. In UCSF Chimera, the CSV file from SwissDock was accessed. High-quality images of the docking results of the molecular structures were best simulated and modelled. Finally, both 2D and 3D interactions and bonds at the binding active site were shown in Discovery Studio Visualiser.

Preparation of Cordyceps militaris (CM) extracts and the standard drug.

Lyophilised form of fresh mycelia and fruiting bodies of CM were provided by Mae Fah Luang University (Chiang Rai, Thailand). The master stock was prepared by diluting fresh mycelia (M) and fruiting bodies (F) of CM with distilled water to concentrations of 4 mg/mL and 6 mg/mL, respectively, to obtain master stocks of aqueous (Aq)-CMM and Aq-CMF. The positive control, cisplatin (Tokyo Chemicals, Japan), was prepared at a concentration of 2.53 mg/mL. The master stocks were stored at -20 °C before use.

2D cytotoxicity screening

Briefly, MDA-MD-231 cells were seeded at a concentration of 5000 cells/well into a 96-well plate and incubated overnight for attachment. After 24 hours, cells were treated with different concentrations of Aq-CMM (0–1000 µg/mL), Aq-CMF (0–1000 µg/mL), and cisplatin (0–200 µg/mL), and further incubated for 24, 48, or 72 hours at 37 ºC. At the end of incubation time, 20 µL of MTT solution (5 mg/ml) (Nacalai Tesque Inc, Japan) was added and incubated at 37ºC for 4 hours. Cell viability was estimated by measuring the absorbance at 570 nm using the EPOCH 2 microplate reader (Bio-Tek Instruments, USA). This wavelength corresponds to the detection of formazan crystals produced by the reduction of MTT by mitochondrial succinate dehydrogenase in viable cells, reflecting overall metabolic activity [21]. The absorbance value that was determined for untreated cells was based on 100% viable cells. Each measurement was performed in triplicate.

Generation and treatment of multicellular tumour spheroidal (MCTS) cultures

Briefly, MDA-MB-231 cells were seeded at a concentration of 5,000 cells/well into a 96-well

ultra-low round-bottom plate and incubated for 3 days to allow the formation of compact and homogeneous spheroids. For treatment, the 3-day-old MCTS were treated with different concentrations of Aq-CMM (0–1000 µg/mL), Aq-CMF (0–1000 µg/mL), and cisplatin (0–200 µg/mL), and then further incubated for 24, 48, or 72 hours at 37 ºC. At the end of the incubation time, 20 µL of MTT solution (5 mg/mL) (Nacalai Tesque Inc., Japan) was added and incubated at 37 °C for 4 hours. Cell viability was estimated by measuring absorbance at 570 nm using the EPOCH 2 microplate reader (Bio-Tek Instruments, USA). The absorbance value that was determined for untreated cells was based on 100% viable cells. Each measurement was performed in triplicate.

Light microscopic assessment

The morphological appearances of the MCTS cultures and their structural changes after treatment with Aq-CMM, Aq-CMF, and cisplatin were observed using a Nikon Diaphot-TMD (Nikon, Japan) inverted light microscope equipped with a Phase contrast-2 ELWD 0.3 phase-contrast condenser. The images were captured using the Digital Sight DS-L2 camera (Nikon, Japan).

Statistical Analysis

All experiments were performed in triplicate. The results were analysed using IBM SPSS Statistics 25.0 for Macintosh (SPSS Inc., USA). All results were expressed as mean ± standard deviation (S.D.). Each value is the mean of at least three separate experiments with triplicate. The comparison of cell viability between the untreated and treated groups was performed using one-way ANOVA. The results were considered statistically significant when $P < 0.05$.

Results

In Silico antimetastatic effects of *CM* compounds targeting NF-κB

Aspirin was selected as the positive control for the *in silico* study, targeting NF-κB. According to Li *et al*. [22], aspirin inhibits the proliferation and stimulates the apoptosis of cancer cells. Therefore, the study found that aspirin may be a promising candidate for combination therapy in breast cancer [22]. Based on **Table 1**, ergothioneine (-7.83 kcal/mol) displayed the highest binding affinity scores for the target protein NF-κB compared to aspirin (-7.24 kcal/mol). This strongly suggests that the ergothioneine compound of CM can target the NF-κB pathway more successfully than a well-known synthetic drug. A more negative score denotes a stronger binding affinity, suggesting a greater likelihood of stable interaction [23]. In general, binding affinity values more negative than –6.0 kcal/mol are considered indicative of strong binding affinity in molecular docking studies [24]. This threshold has been widely referenced in computational drug discovery literature to differentiate between weak, moderate, and potent ligand–receptor interactions.

The molecular docking results presented in **Figure 1** offer valuable insights into the potential inhibitory interactions of ergothioneine and GABA with the NF-κB protein. In the 3D and 2D interaction visualisations, both ligands demonstrated favourable binding within the active site of NF-κB, suggesting possible interference with its functional activity. Specifically, ergothioneine exhibited strong binding affinity through multiple hydrogen bonds with critical residues, including Ser281, Glu285, and Tyr227. These residues are located within the DNA-binding domain of NF-κB,

Table 1. Binding affinity prediction of each ligand toward the target protein, NF-κB (PDB ID: 1SVC).

No	Ligand	Target Protein	Binding Affinity
1	Ergothioneine	NF-κB	-7.83 kcal/mol
2	Aspirin (positive control)	NF-κB	-7.24 kcal/mol
3	γ-aminobutyric Acid (GABA)	NF-κB	-7.01 kcal/mol
4	Lovastatin	NF-κB	-6.91 kcal/mol
5	Adenosine	NF-κB	-6.75 kcal/mol
6	Cordycepin	NF-κB	-6.41 kcal/mol

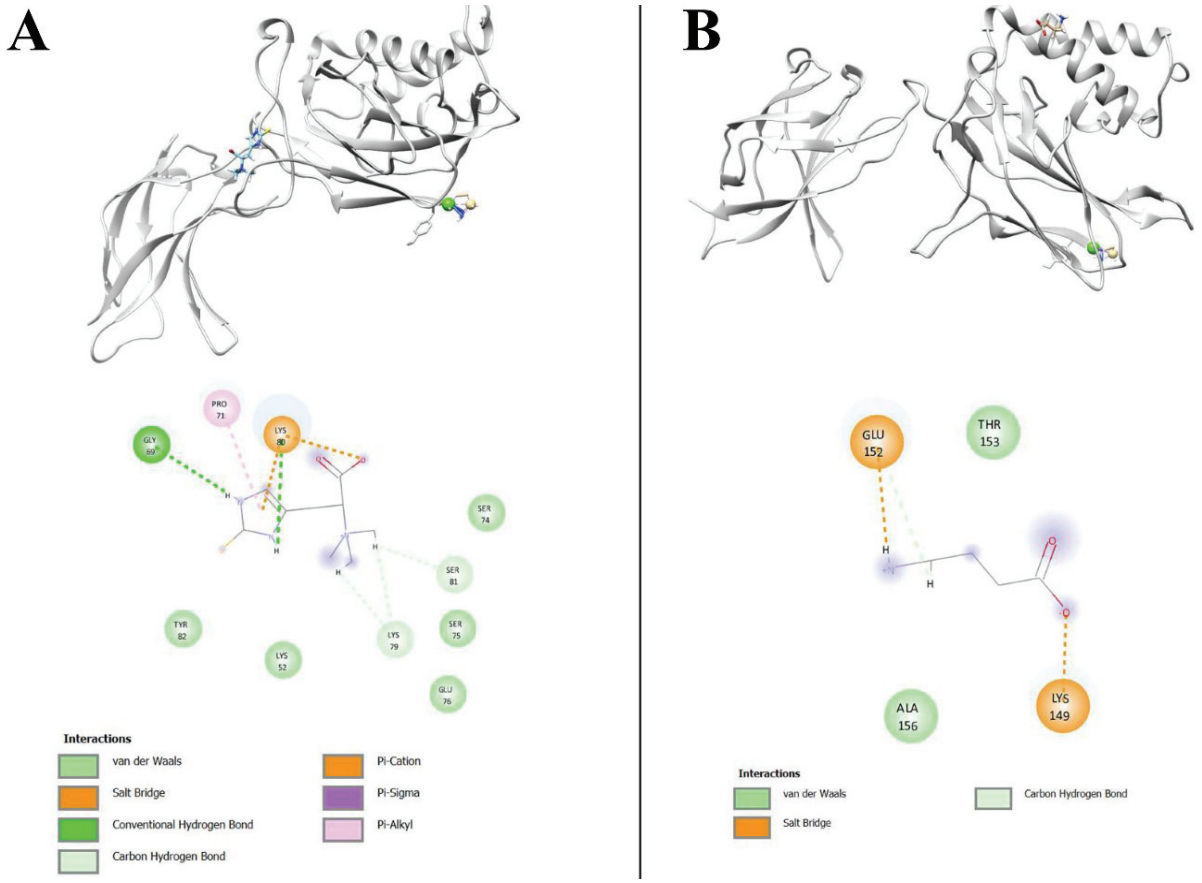

Figure 1. 3D interaction (top) and 2D interaction (bottom) visualisation of ligand-receptor complex interaction. (A) Interaction prediction of ergothioneine with the NF-κB protein. (B) Interaction prediction of GABA with the NF-κB protein.

which plays a central role in its transcriptional regulatory functions. Additionally, ergothioneine established hydrophobic contacts with Phe239 and Ile235, further potentially stabilising the ligand within the binding pocket. These interactions indicate that ergothioneine may potentially inhibit NF-κB activity by sterically hindering its ability to bind to DNA or disrupting conformational integrity required for transcriptional activation.

In contrast, GABA formed hydrogen bonds with Lys221 and Asp245, and a π–π interaction with Tyr227. Although the number of interactions was fewer compared to ergothioneine, their strategic placement near the DNA-binding interface suggests that GABA may also exert modulatory effects on NF-κB signalling, albeit with potentially lower binding stability. The π–π stacking with Tyr227 is particularly notable, as this residue is frequently implicated in ligand-mediated modulation of NF-κB function.

Taken together, these docking observations support the hypothesis that both compounds could act as NF-κB inhibitors through direct binding, which may contribute to anti-inflammatory or anti-angiogenic effects. These findings are consistent with previous reports highlighting NF-κB as a therapeutic target in cancer and chronic inflammatory conditions.

In Silico antiangiogenics effects of *CM* compounds targeting VEGFR

Two well-known VEGFR inhibitors, tivozanib (a synthetic drug) and resveratrol (a natural polyphenolic compound), are used as positive controls in this study. Based on **Table 2**, tivozanib displayed the most decisive inhibitory action on VEGFR (-11.24 kcal/mol), followed by resveratrol and ergothioneine. It is worth noting that ergothioneine is present in higher quantities in both the mycelium and fruiting bodies of CM compared to GABA, lovastatin, and cordycepin [25,26].

Figure 2 illustrates the molecular docking results of ergothioneine and adenosine with the VEGFR, a critical regulator of angiogenesis in

Table 2. Binding affinity prediction of each ligand toward the target protein, VEGFR (PDB ID: 4ASE).

No	Ligand	Target Protein	Binding Affinity
1	Tivozanib (synthetic drug/ positive control)	VEGFR	-11.24 kcal/mol
2	Resveratrol (natural polyphenol compound/ positive control)	VEGFR	-7.84 kcal/mol
3	Ergothioneine	VEGFR	-7.62 kcal/mol
4	Adenosine	VEGFR	-7.33 kcal/mol
5	Cordycepin	VEGFR	-7.29 kcal/mol
6	Lovastatin	VEGFR	-7.15 kcal/mol
7	γ-aminobutyric Acid (GABA)	VEGFR	-7.11 kcal/mol

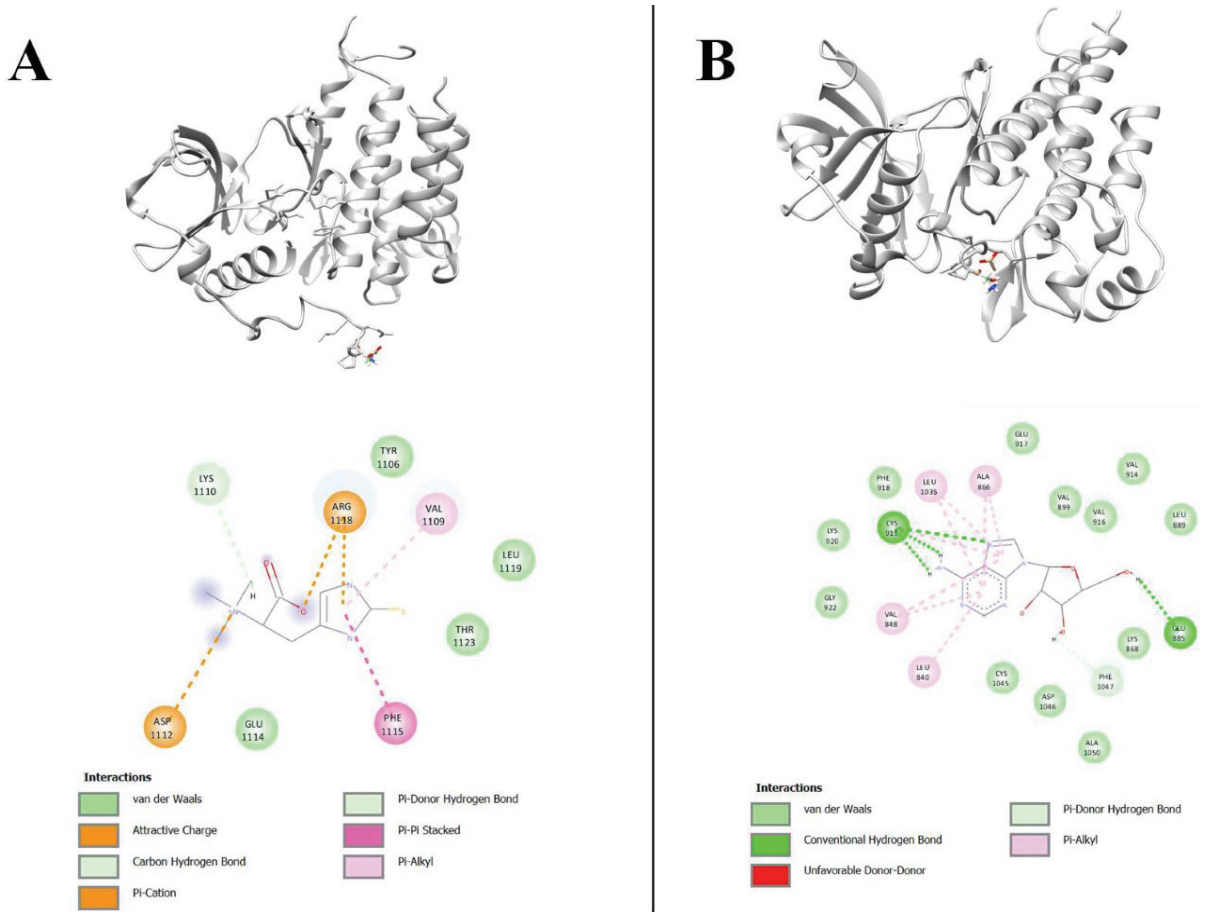

Figure 2. 3D interaction (top) and 2D interaction (bottom) visualisation of ligand-receptor complex interaction. (A) Interaction prediction of ergothioneine with the VEGFR protein. (B) Interaction prediction of adenosine with the VEGFR protein.

cancer and other pathological conditions. The 3D and 2D visualisations provide detailed interaction profiles that suggest both ligands possess the ability to occupy the VEGFR binding pocket and potentially interfere with its activation.

Ergothioneine, as shown in **Figure 2A**, displayed a stable docking pose within the ATP-binding site of VEGFR. It formed hydrogen bonds with key residues, including Glu885 and Cys919, which are known to be crucial for kinase activity [27–29]. Additional interactions were observed with Lys868 and Asp1046 [29], suggesting a potential to inhibit autophosphorylation and downstream VEGF signalling. The presence of both polar and non-polar interactions indicates a well-balanced binding profile, supporting the compound's potential to function as a VEGFR inhibitor.

In **Figure 2B**, adenosine also demonstrated favourable binding within the VEGFR active site. It formed hydrogen bonds with residues Asp1046, Glu885, and Thr916, which are highly conserved in the kinase domain and essential for catalytic

function [27–29]. The π-cation interaction with Lys868 further enhanced the binding stability. Although adenosine is an endogenous purine nucleoside, its docking pose overlaps with that of known VEGFR inhibitors, suggesting that structural analogues of adenosine might be developed into targeted anti-angiogenic agents.

Collectively, these results suggest that both ergothioneine and adenosine can interact with critical residues within the VEGFR active site, potentially modulating receptor function. This supports the hypothesis that these ligands may exert anti-angiogenic effects by inhibiting VEGFR signalling, making them promising candidates for further validation in cancer or vascular disease models.

Cytotoxic screening

Figure 3 illustrates the time-dependent effects of Aq-CMM and Aq-CMF treatments on cell viability in both 2D monolayer and 3D spheroidal culture systems at 24, 48, and 72 hours. In the 2D monolayer culture, both Aq-CMM and Aq-CMF exhibited a progressive cytotoxic effect over time. After 24 hours, a mild reduction in viability was observed for both extracts, with no statistically significant differences compared to the control. However, by 48 hours, Aq-CMF showed a more pronounced inhibitory effect. By 72 hours, both Aq-CMM and Aq-CMF significantly reduced cell viability compared to the untreated control ($p < 0.05$), with Aq-CMF displaying a slightly more potent effect.

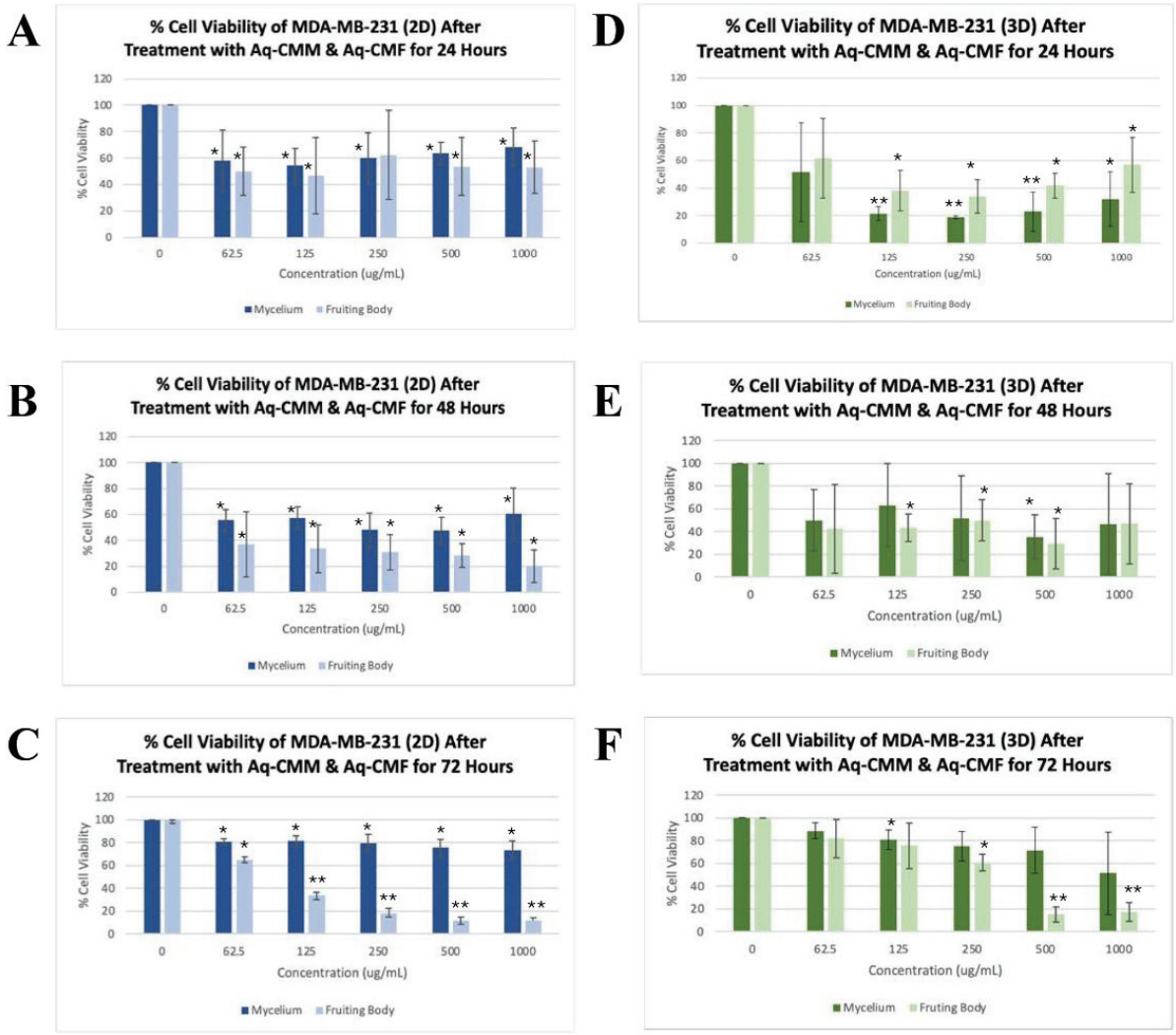

Figure 3. Percentage of viable MDA-MB-231 breast cancer cells after administration with Aq-CMM and Aq-CMF in 2D monolayer culture and 3D spheroidal culture. 2D cell culture treatment for (A) 24 hours, (B) 48 hours, and (C) 72 hours. 3D cell culture treatment for (D) 24 hours, (E) 48 hours, and (F) 72 hours. Data are represented as mean ± S.D., n = 3. Statistical significance was assumed for P-values < 0.05 (P < 0.05, **P < 0.01) compared to the untreated control.

In the 3D spheroidal culture, the response to treatment was more delayed. Minimal changes in viability were noted at 24 hours for both extracts, indicating limited early penetration or activity in the dense spheroid structure. At 48 hours, moderate reductions were observed, with Aq-CMF showing a slightly greater effect than Aq-CMM. By 72 hours, both treatments resulted in a significant decrease in spheroid viability compared to the untreated control ($p < 0.05$). However, the extent of inhibition was slightly lower than that observed in the 2D model, likely due to the enhanced cellular resistance characteristic of 3D cultures.

These findings suggest that while both Aq-CMM and Aq-CMF exert cytotoxic effects in a time-dependent manner, Aq-CMF consistently demonstrated a more potent inhibitory effect in both models. The results also underscore the importance of considering 3D culture systems for better approximation of *in vivo* tumour behaviour, as the delayed response observed highlights the barriers to drug diffusion and resistance mechanisms present in three-dimensional environments.

Additionally, Aq-CMF was able to present a lower number of viable cells after treatment during cell cytotoxicity testing compared to Aq-CMM. It is worth emphasising that the 3D spheroid cells displayed higher levels of viable cells after treatment compared to the 2D monolayer cells (**Table 3**). With treatment of Aq-CMF (250 µg/mL), the percentage of cell viability of MDA-MB-231 decreased to 37.17 ± 26.91 % compared to the untreated control ($p < 0.05$) in the 2D cell culture. In contrast, in the 3D culture, the percentage of cell viability was 48.23 ± 16.33% by administration of the same treatment.

At a concentration of 1000 µg/mL, Aq-CMM exhibits a modest effect, with approximately 64.99 ± 13.91 % of viable cells remaining (**Table 3**). In contrast, Aq-CMF demonstrates a remarkably intensified impact, eliciting a response more than double that of the control. Following treatment with Aq-CMF at the same concentration, the percentage of cell viability of MDA-MB-231 cells was substantially reduced to 32.10 ± 23.34 %. These findings emphasise the potent cytotoxic potential of Aq-CMF and its significant capacity to prevent the survival of MDA-MB-231 cells. Treated cells

Table 3. Comparison of % ± SD cell viability at different concentrations between treatments of CMM on MDA-MB-231 in 2D and 3D culture systems, at 72 hours.

Conc. (µg/mL)	Cell Viability (% ± S.D.)			
	Aq-CMM (2D)	Aq-CMM (3D)	Aq-CMF (2D)	Aq-CMF (3D)
0	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]
62.5	59.30 ± 23.70[a]	63.40 ± 29.53[a]	50.57 ± 19.72[a]	62.04 ± 30.82[a]
125	61.32 ± 18.10[a]	55.27 ± 32.46[a]	38.24 ± 18.39[a]	52.33 ± 22.28[a]
250	65.70 ± 15.57[a]	48.72 ± 31.49[a]	37.17 ± 26.91[a]	48.23 ± 16.33[a]
500	66.32 ± 10.45[a]	43.40 ± 26.93[a]	31.18 ± 21.76[a]	28.81 ± 16.99[a]
1000	64.99 ± 13.91[a]	39.33 ± 25.34[a]	32.10 ± 23.34[a]	40.55 ± 27.29[a]

Data were expressed as means ± standard deviation of means.
Data were obtained in triplicate from three sets of runs (n = 3 × 3 = 9).
[ab] indicates the values differ significantly within the same column for cell viability ($p < 0.05$).

Table 4. Comparison of IC_{50} ± SD values between treatments of CMM and CMF on MDA-MB-231 in 2D and 3D culture systems, at 72 hours. Statistical significance was assumed for P-values < 0.05 (P < 0.05, **P < 0.01) compared to the untreated control.

Time	IC_{50} (µg/mL)			
	Aq-CMM (2D)	Aq-CMM (3D)	Aq-CMF (2D)	Aq-CMF (3D)
24	N/A	58 ± 2.36*	60 ± 7.29*	97 ± 8.27*
48	N/A	57 ± 1.50*	54 ± 1.38*	46 ± 5.28*
72	N/A	997 ± 5.63*	90 ± 8.38*	280 ± 0.93*

also showed a decrease in cell viability due to cytotoxicity of Aq-CM, relative to untreated cells ($p < 0.05$).

Similarly, in a study conducted by Jenkham et al. [30], similar percentages for cell viability were observed. At an effector-to-target ratio of 20:1, it was found that Aq-CM at 100 µg/mL considerably increased the efficacy of non-adherent cells in killing MDA-MB-231 compared to the group treated with extract at 100 µg/mL and without immune cells. The percentage of cell viability for MDA-MB-231 cells was 70.26 ± 8.29 [30].

The IC_{50} values were determined by analysing the dose-response relationship depicted in **Table 4**. It can be observed that lower concentrations of Aq-CMF were sufficient to achieve 50% inhibition of cell growth or viability compared to Aq-CMM at both 48 and 72 hours (**Figure 3**). This suggests that the fruiting body of CM exhibited higher potency or effectiveness in inhibiting the growth of MDA-MB-231 cells compared to the mycelium. Additionally, Aq-CMF is potentially effective at lower concentrations when compared to Aq-CMM. The estimation of IC_{50} values provides valuable information about the relative potency of various CM components in inhibiting cancer cell growth. These quantifiable values enable researchers to assess their efficacy, determine appropriate dosages for future studies, and explore potential therapeutic applications [31].

In the 3D MCTS cultures at the 48-hour treatment period, both Aq-CMM and Aq-CMF had the lowest IC_{50} of 57 ± 1.50 µg/mL and 46 ± 5.28 µg/mL, respectively. These results suggest that administering treatment for 48 hours may be optimal for 3D cell culture. Additionally, Aq-CMF is potentially effective at lower concentrations when compared to Aq-CMM. The estimation of IC_{50} values provides valuable information about the relative potency of various CM components in inhibiting cancer cell growth. These quantifiable values enable researchers to assess their efficacy, determine appropriate dosages for future studies, and explore potential therapeutic applications [31].

The fruiting body of CM exhibited a higher capacity to induce cell death compared to the mycelium of CM. Microscopic examination of the 3D spheroidal cells revealed more prominent changes in diameter following treatment with Aq-CMF and cisplatin, as demonstrated in **Table 5**. Initially, the matured spheroids displayed uniformity with an average diameter of approximately 385.58 µm. However, post-treatment with

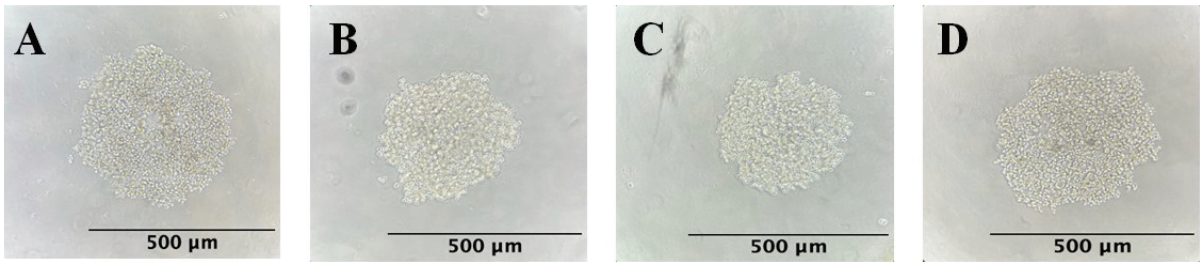

Figure 4. Morphology of breast cancer MDA-MB-231 spheroids (10x magnification). Representative images (A) Pre-treatment (control) and post-treatment of cells with (B) Aq-CMM (250 µg/mL), (C) Aq-CMF (250 µg/mL) and (D) Cisplatin (50 µg/mL) for 72 hours. Images were assembled using ImageJ; scale bar, 500 µm.

Table 5. Comparison of spheroid size between untreated and treated 3D spheroid cancer cells. Treatment with Aq-CMM, Aq-CMF and cisplatin resulted in a significant reduction in spheroid size compared to the untreated control group. Data are presented as mean ± SD from three independent experiments. Statistical significance was assumed at $P < 0.05$ ($P < 0.05$), compared to the untreated control.

MDA-MB-231 Spheroids Treatment	Diameter (µm) ± S.D.
Untreated	385.58 ± 9.54
Aq-CMM (250 µg/mL)	369.23 ± 12.72*
Aq-CMF (250 µg/mL)	336.54 ± 15.41*
Cisplatin (50 µg/mL)	364.71 ± 10.98*

Aq-CMF and Aq-CMM, a reduction in cell diameter was observed, measuring 336.54 µm and 369.23 µm, respectively.

Discussion

This study demonstrates the significant antimetastatic and antiangiogenic activities of *Cordyceps militaris* (CM) extracts on the MDA-MB-231 breast cancer cell line, utilising both *in silico* molecular docking and *in vitro* 2D and 3D cell culture systems. Ergothioneine, identified as a key bioactive compound in CM, exhibited strong binding affinities toward crucial metastatic and angiogenic pathway targets, NF-κB and VEGFR. These interactions suggest that ergothioneine may play a vital role in modulating pathways associated with cancer cell metastasis and angiogenesis [32].

The docking results highlighted ergothioneine's higher affinity for NF-κB compared to aspirin, a well-established anti-inflammatory agent, indicating a substantial potential for therapeutic intervention in NF-κB-mediated pathways [33]. Similarly, ergothioneine's notable binding affinity for VEGFR suggests efficacy in disrupting angiogenic signalling. These findings align well with previous reports highlighting ergothioneine's antioxidative and anti-inflammatory effects [34].

Ergothioneine, which is found in the fruiting body of CM, exhibits antioxidant activity [34]. One of the primary functions of ergothioneine is to absorb and neutralise free radicals. Free radicals are chemical compounds produced during the oxygen metabolism process, exhibiting a remarkable level of reactivity towards a wide range of chemicals. As both high and low levels of reactive oxygen species (ROS) can induce cell death, the presence of ROS within cells must be carefully managed to sustain cell proliferation. Natural defence mechanisms in the human body work to mitigate the damaging effects of free radicals by slowing down cell proliferation and, in some instances, repairing the damage caused by these radicals. Inflammatory responses can be produced in a tumour microenvironment where ROS levels are increased. To treat cancer, controlling and maintaining an ideal level of free radicals may be an effective strategy. A higher amount of oxidative stress can also make cancer cells more resistant to treatment with pharmaceuticals. Correspondingly, excessive oxidative stress can directly harm cells and induce cell death through apoptosis [35,36]. Hence, ergothioneine, found in CM, emerges as a valuable bioactive compound with demonstrated antioxidative properties, capable of mitigating free radicals.

The comparative cytotoxic analyses of CM extracts showed that the aqueous extract of CM fruiting bodies (Aq-CMF) demonstrated significantly higher anticancer potency compared to the mycelium extract (Aq-CMM). Notably, the fruiting body extract displayed lower IC_{50} values, indicating higher potency at lower concentrations, particularly evident in 3D cultures, which more closely mimic the *in vivo* tumour microenvironment. This aligns with previous studies reporting higher ergothioneine and bioactive metabolites concentrations in the fruiting body compared to the mycelium, enhancing its therapeutic potential [37].

It is essential to recognise that the use of aqueous extraction in this study may impact the spectrum of bioactive compounds present in the CMF and CMM extracts. Aqueous solvents are effective at extracting water-soluble constituents such as cordycepin, adenosine, and ergothioneine, which have been reported to exhibit potent antimetastatic and antiangiogenic effects [12,38]. However, hydrophobic or lipophilic compounds, which may also contribute to the therapeutic potential of Cordyceps militaris, are likely underrepresented due to their poor solubility in water. Therefore, the observed biological effects in this study may predominantly reflect the activity of water-soluble compounds. Further work utilising organic or mixed-solvent extraction methods, followed by compositional profiling and comparative bioactivity assays, is warranted to comprehensively evaluate the contribution of hydrophobic constituents to the overall anticancer activity of CM extracts.

Interestingly, the 3D spheroid models demonstrated higher cell viability post-treatment compared to the 2D monolayer cultures. This difference likely arises from nutrient and oxygen gradients, cellular heterogeneity, and the limited penetration of treatments into the spheroids, which closely resemble the in vivo conditions *of solid tumours* [39]. This observation highlights the importance of employing 3D culture systems for

an accurate assessment of drug efficacy in cancer research, as monolayer cultures may overestimate treatment effectiveness [40].

Besides ergothioneine, multiple bioactive compounds in CM demonstrate antimetastatic and antiangiogenic characteristics, including GABA, adenosine, lovastatin, and cordycepin (**Table 1** and **Table 2**). The binding affinity values of all bioactive compounds were below -6.00 kcal/mol. Therefore, the agents demonstrated potential abilities to inhibit the target proteins of NF-κB and VEGFR.

Furthermore, adenosine, another significant bioactive component from CM, potentially contributes to anticancer activities through inhibition of the phospho-AMPK1α signalling pathway, consequently decreasing proliferation and invasiveness of breast cancer cells [41,42]. Additionally, γ-aminobutyric acid (GABA) displayed notable anticancer effects by regulating ERK1/2 phosphorylation and matrix metalloproteinases (MMPs). These findings align with previous studies emphasising GABA's capability to modulate pathways closely regulated by NF-κB, suggesting its potential role in hindering cancer cell migration and invasion [43–45].

Lovastatin, another bioactive compound from CM, also exhibits vigorous anticancer activity through the effective inhibition of the NF-κB and VEGFR signalling pathways. Previous studies highlighted its capability to modulate cancer cell growth and apoptosis by affecting the PI3K/AKT/mTOR signalling axis [46]. Interestingly, lovastatin can reverse receptor-negative phenotypes in triple-negative breast cancer cells, sensitising these cells to targeted therapies by re-expressing human epidermal growth factor receptor 2 (HER2) [47]. Apart from that, lovastatin and other chemotherapeutic medications may work together to minimise the drug resistance of cancer cells, which would significantly enhance the therapeutic efficacy of these medications [48]. These observations highlight the potential use of lovastatin in combination therapies to improve treatment efficacy and overcome drug resistance.

Cordycepin, widely researched for its anticancer properties, showed pronounced inhibitory effects on metastasis and angiogenesis by targeting critical signalling pathways, including NF-κB, VEGFR, mTOR, and the Hedgehog pathway [49,50]. Cordycepin further induced oxidative stress (ROS production), promoted apoptosis, and disrupted cellular metabolism by activating the AMPK pathway [41,51–53]. These multifaceted mechanisms underscore cordycepin's broad-spectrum therapeutic potential against breast cancer, reinforcing the importance of further mechanistic studies.

In the comparison of culture models, the higher resistance observed in 3D spheroid cultures compared to 2D monolayers is consistent with the biological characteristics of solid tumours. Factors such as nutrient gradients, hypoxic conditions, cellular heterogeneity, and limited treatment penetration likely contributed to the observed reduced drug sensitivity in 3D cultures. Consequently, this underscores the necessity of using 3D culture systems for more accurate predictions of *in vivo* drug responses and highlights the limitations inherent in traditional monolayer assays for anticancer drug screening [39,40]. The variability in IC_{50} data and the. Nonlinear responses in cytotoxic assays highlight common challenges associated with in vitro assays, including interference from assay components, variations in cell density, and random fluctuations in experimental conditions. These factors emphasise the importance of rigorous protocol optimisation and standardisation, ensuring consistent, reproducible, and accurate data interpretation, particularly when evaluating cytotoxicity.

In addition to limited drug penetration, the 3D spheroidal culture system itself contributes to reduced treatment efficacy due to its ability to better recapitulate the complexity of the *in vivo* tumour microenvironment. Unlike 2D monolayer cultures, 3D models enable cell–cell and cell–matrix interactions, the development of oxygen and nutrient gradients, and the establishment of hypoxic cores, which are hallmarks of solid tumours [54,55]. These physiological features contribute to enhanced cancer cell survival, progression, and drug resistance, making 3D cultures a more predictive platform for evaluating therapeutic responses.

The increased resistance observed in the 3D model in this study likely reflects these microenvironmental barriers, which more closely resemble actual tumour conditions. Consequently, the differential responses between 2D and 3D cultures underscore the importance of incorporating 3D

models in preclinical evaluations, as they provide a more realistic context for screening the efficacy of natural product-based treatments, such as Cordyceps militaris extracts. This approach aligns with current trends in cancer research, which emphasise the use of physiologically relevant *in vitro* models to bridge the gap between *in vitro* findings and *in vivo* outcomes [56].

While this study provides important insights into the potential anticancer properties of Cordyceps militaris extracts, several limitations should be acknowledged. Firstly, the conclusions regarding anti-metastatic and anti-angiogenic effects are based primarily on molecular docking predictions and general cytotoxicity assays in 2D and 3D culture models. More targeted *in vitro* experiments—such as wound healing assays, migration and invasion assays, and endothelial tube formation—would be necessary to confirm the anti-metastatic and anti-angiogenic mechanisms directly. Additionally, although aqueous extracts were analysed, the absence of detailed compositional profiling, such as LC-MS quantification of ergothioneine and other bioactive compounds, limits the precision of structure–activity correlations. These aspects are the focus of ongoing work and will be addressed in future studies to support a better understanding and validate the therapeutic potential of CM extracts.

Further research could explore the potential synergistic effects of CM extracts when combined with conventional chemotherapy drugs. Additionally, conducting *in vivo* studies would provide deeper insights into the pharmacodynamics, bioavailability, and systemic effects of these bioactive compounds, particularly ergothioneine and cordycepin, in preclinical models of breast cancer. Such studies would be crucial in evaluating CM's suitability and efficacy as an adjunct or alternative therapeutic strategy in clinical settings. Moreover, understanding the precise molecular mechanisms through advanced molecular techniques, such as transcriptomics and proteomics analyses, would significantly contribute to elucidating the mode of action of CM. Investigating these underlying mechanisms can facilitate the targeted application of CM-derived compounds and enhance therapeutic precision, ultimately enabling more effective and personalised treatment approaches for breast cancer.

Conclusions

This study demonstrates that *Cordyceps militaris* (CM) extracts, particularly those derived from the fruiting body, exhibit potential antimetastatic and antiangiogenic activities against the MDA-MB-231 breast cancer cell line, as supported by molecular docking and time-dependent cytotoxicity analyses. Ergothioneine and other bioactive compounds such as adenosine, GABA, lovastatin, and cordycepin were predicted to interact with key targets involved in cancer metastasis and angiogenesis, notably NF-κB and VEGFR. The higher potency observed in fruiting body extracts compared to mycelium extracts highlights the importance of bioactive compound concentration in therapeutic efficacy. Additionally, the utilisation of 3D cell culture systems provided more realistic insights into the tumour microenvironment and revealed essential differences in drug sensitivity compared to traditional 2D cultures. These findings underscore the potential of CM, especially its fruiting body extracts, as promising natural sources for developing adjunct or alternative therapies for breast cancer.

Further comprehensive preclinical and clinical studies are warranted to validate these promising results, elucidate detailed mechanisms of action, and explore the practical applicability of CM extracts in cancer therapy. Ultimately, this research contributes valuable knowledge toward developing safer and more effective anticancer therapeutic strategies derived from natural products.

Disclosures

Author Contributions

YQT and SC conceived the study idea. APQC performed the *in silico* analyses and carried out the *in vitro* cytotoxicity and spheroid assays. APQC and SXYK analysed the data and prepared the figures. YQT and AYYC reviewed and supervised the work. The manuscript was critically reviewed and edited by YQT, SC and AYYC. All authors contributed to the article, and the final version of the manuscript was reviewed and approved by all the authors.

Conflict of interest statement

The authors declare no conflict of interest.

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