Vietnam Journal of Biotechnology **23**(2): 191-204, 2025. DOI: 10.15625/vjbt-22132

EVALUATION OF ANTIMUTAGENIC, ANTIANGIOGENIC, AND CYTOTOXICITY AGAINST CANCEROUS CELLS OF MYCELIAL EXTRACT OF *Cordyceps militaris*

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Received: 28.12.2024
Accepted: 14.05.2025

ABSTRACT

The high incidence and mortality rates of cancer necessitate the continuous search for effective anticancer therapies. Although synthetic drugs have been instrumental in modern medicine, their side effects drive the exploration of new, less harmful alternatives. Natural compounds are particularly favoured for their specificity, minimal side effects, and cost-effectiveness. In this study, the methanolic mycelial extract of *Cordyceps militaris* was evaluated for its antimutagenic, antiangiogenic, and cytotoxic properties. Antimutagenic activity assessed using the AMES test revealed a significant, concentration-dependent reduction in mutagenicity induced by sodium azide, benzo[a]pyrene, and 4-nitroquinoline-N-oxide in *Salmonella* tester strains TA98, TA100, and TA102, with up to 68.4% inhibition. Antiangiogenic effects, examined via the chorioallantoic membrane (CAM) assay, demonstrated substantial concentration-dependent inhibition of angiogenesis, achieving up to 88.4% inhibition at 100 µg/ml. The extract effectively suppressed the formation of angiogenic branch points, suggesting a potential role in inhibiting tumor neovascularization. Cytotoxic effects on the MDA-MB-231 breast cancer cell line, assessed through MTT assay, indicated a marked reduction in cell viability, with an IC_{50} value of 54.86 µg/ml, while no significant cytotoxicity was observed in normal Vero cells. Morphological observations of treated cancer cells showed distinct cytoplasmic condensation, cell shrinkage, and detachment, indicative of apoptotic cell death. These findings highlight the multifaceted anticancer potential of *C. militaris* mycelial extract. This study underscores the therapeutic potential of *C. militaris* as a natural source of anticancer agents and provides a scientific foundation for further preclinical and clinical exploration.

Keywords: antiangiogenic, anticancer, antimutagenic, *Cordyceps militaris*, cytotoxicity, MDA-MB-231, methanolic mycelial extract

INTRODUCTION

Cancer is a collection of related diseases characterized by the uncontrolled growth of cells which are capable of spreading over other parts of the body. It remains one of the most formidable challenges in medical

science (Harrington, 2011). As per the reports of the World Health Organization, 9,700,000 deaths and 20,000,000 new cancer cases were registered in 2022, underscoring the critical need for effective and diverse anticancer strategies. The multifaceted nature of cancer presents unique challenges for each type, driven by genetic, environmental, and lifestyle factors (De Sousa E Melo *et al*., 2013). Addressing such intricacy requires a multi-pronged approach, including early detection, prevention, and targeted therapies.

Prevention strategies play a significant role in combating cancer, with antimutagenic approaches being significantly important. Mutagens, agents that cause mutations in the DNA, are key players in carcinogenesis (Kasai, 2016). These mutations, if not repaired, can lead to uncontrolled cell division and tumor formation. Antimutagenic strategies focus on identifying and neutralizing these agents, thereby halting the initial step in the carcinogenesis process. Mutagenesis involves various agents, including chemical compounds, radiation, and certain viruses, which can induce changes in the DNA sequence (Freese, 1971). Antimutagenic agents, sourced from natural compounds found in fruits, vegetables, and other organisms, as well as synthetic compounds, work through mechanisms such as antioxidant activity, enhancing DNA repair processes, and modulating enzymes involved in carcinogen metabolism (De Flora, 1979; Słoczyńska *et al*., 2014).

As cancer cells proliferate, they require higher concentrations of nutrients and oxygen, which is met through angiogenesis. Cancer cells release specific compounds that promote the formation of new blood vessels (Carmeliet & Jain, 2011). Antiangiogenic therapy aims to inhibit this process, effectively starving the tumor and preventing its growth and spread (Vasudev & Reynolds, 2014). By blocking angiogenesis, antiangiogenic therapy can inhibit or delay tumor growth and metastasis. Several antiangiogenic drugs, target pathways involved in angiogenesis like the vascular endothelial growth factor (VEGF) pathway. Some of the drugs are developed and approved for various cancers, although their success varies depending on the disease stage and type (Rajabi & Mousa, 2017; Zirlik & Duyster, 2018).

Cytotoxic agents form the basis of many traditional cancer therapies, including chemotherapy, by interfering with cell division, damaging DNA, or inducing programmed cell death (apoptosis) (Fuertes *et al*., 2003). However, the lack of specificity to cancer cells often results in severe side effects, necessitating the search for new candidates with better selectivity.

Natural sources, particularly fungi and mushrooms, are recognized for their abundant bioactive molecules (Brewer, 2011). *C. militaris*, an entomopathogenic fungus that infects insects, has been utilized in traditional medicine for centuries, especially in Asian cultures (Dong *et al*., 2015). Compared to other *Cordyceps* species, *C. militaris* is easier to cultivate and exhibits various medicinal activities, like neuroprotection, immunomodulation, antioxidant, hypoglycemic, and anti-inflammatory properties (Panda & Swain, 2011). However, the extended duration required for fruiting body production poses a constraint for large-scale cultivation. Mycelial biomass production provides an effective alternative, allowing for large-scale cultivation in a shorter time (Shih *et al*., 2007).

The present investigation focuses on assessing the diverse anticancer properties, such as antimutagenic, and antiangiogenic effects as well as cytotoxicity against cancerous cells, exhibited by the methanolic mycelial extract of *C. militaris*. According to the available literature, this is the first report to comprehensively assess these specific anticancer properties of the methanolic mycelial extract of *C. militaris*.

MATERIALS AND METHODS

Chemicals

All chemicals, except for the standard mutagens, were purchased from HiMedia Laboratories Private Limited, India. The mutagens, including 4-nitroquinoline-N-oxide and Benzo[a]pyrene, were obtained from Sigma-Aldrich, United States.

Microbial strains and cell lines

C. militaris strain was procured from L J University located at Ahmedabad India. *Salmonella typhimurium* strains TA102, TA100 and TA98 were sourced from Microbial Type Culture Collection and Gene Bank (MTCC) located at Chandigarh and cell lines (MDA-MB-231 and Vero cell line) were acquired from National Centre for Cell Science (NCCS) located at Pune India.

Mycelium production using submerged fermentation

C. militaris cultured in a basal media described by Deshmukh and Bhaskaran in 2024 in a 250 ml Erlenmeyer flask under static conditions for 10 days at 20°C. Followed by incubation and filtration, the harvested mycelia were washed and dried at 45°C until a consistent dry weight was achieved (Deshmukh & Lakshmi, 2023).

Preparation of the fungal methanol extract

Five grams of mycelial powder were extracted at 25°C with 100 ml of methanol, using an orbital shaker set to 150 rpm for 24 hours in dark conditions. It is then filtered using the Whatman number #4 filter paper. The resulting residue underwent two additional extractions using the same method. Samples were dried using a rotary evaporator and the final concentration i.e., 100 mg/ml maintained with the respective solvent.

Preparation of S9 liver microsomal fraction

A male Sprague Dawley rat weighing 200 g was administered $C_{12}H_{11}N_2NaO_3$ (0.1%) in its drinking water for 4 days following the protocol described by Maron and Ames (1983). After fasting overnight, the rat was euthanized by decapitation. Following the excision, the liver was thoroughly rinsed with a chilled 0.15 M KCl solution. The liver homogenate was aseptically prepared in a 0.15 M KCl solution at a ratio of 3 ml per gram of wet liver tissue. The resulting homogenate underwent centrifugation for 10 minutes at 8,600 rpm.

Preparation of S9 mix

To prepare 10 ml of the S9 mix, sterile reagents were added in the following sequence: 3.35 ml sterile double distilled water, 5 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.4 ml of 0.1 M NADP, 0.05 ml of 1 M $C_6H_{13}O_9P$, 0.2 ml of $MgCl_2$-KCl solution (1.64 M KCl + 0.4 M $MgCl_2$), finally, add 1 ml of rat liver S9.

Evaluation of *in vitro* antimutagenicity

The antimutagenic potential of the methanolic mycelial extract of *C. militaris* was calculated using the AMES method with Mutant strains of *S. typhimurium* (Maron & Ames, 1983).

Mutagens preparation

Dimethyl sulfoxide (DMSO) was used to dissolve mutagens like 4-nitroquinoline-N-oxide (4-NQO), and benzo[a]pyrene (B[a]P). However, sodium azide (NaN_3) was dissolved in water. The concentrations used in this study were 0.004 mg/plate for NaN_3, 0.0005 mg/plate for 4-NQO, and 0.001 mg/plate for benzo[a]pyrene (B[a]P).

Direct acting mutagens

The study determined the antimutagenic activity of the methanolic mycelial extract of *C. militaris* using the method described in this approach, freshly grown histidine mutant *S. typhimurium* strains TA102, TA100 and TA98 (10^9 cells/ml) mixed with standard mutagens (NaN_3 - 0.004 mg/ml and 4-NQO - 0.0005 mg/ml), 0.5 mM histidine-biotin and different concentrations of methanolic mycelial extracts (0.25 to 10 mg/ml). The resultant mixture was then added to minimal media plates, followed by incubation for 2 days at 37°C. Followed by incubation, the total count of revertant colonies was determined. Spontaneous revertant (SR) plates will serve as a baseline for the natural mutation rate, while positive control plates containing known mutagens and the plate containing methanolic mycelial extract (10 mg/ml) will validate the sensitivity of the assay.

Mutagens that require activation

For significant antimutagenic results, along with direct-acting mutagens, the study also evaluated the antimutagenic potential of the extract against mutagens that required metabolic activation (B[a]P). Freshly grown *S. typhimurium* strains (TA100 and TA98) ($1x10^9$ cells/ml) mixed with S9 fraction (50 µl), 0.5 mM histidine-biotin and different concentrations of mycelial extracts (0.25 to 10 mg/ml). This mixture was then added to minimal media plates and incubated for two days at 37°C. Followed by incubation, the total count of revertant colonies was determined. The percentage of Inhibition (I) is calculated by following the equation as

$$I = \frac{(E_1 - SR) - (E_2 - SR) \times 100}{(E_1 - SR)}$$

E_1 – Total revertant count without extracts, E_2 – Total revertant count with extracts and SR – spontaneous revertants.

Evaluation of the antiangiogenic potential of mycelial extract

The anti-angiogenic capability of the mycelial extract of *C. militaris* was examined using the Chorioallantoic Membrane assay (CAM) (Ribatti *et al*., 2021). The fertilized chicken eggs were collected on day 0 from the local market of Ahmedabad, India and subjected to surface sterilization with 70% ethanol to prevent infection. Then it was kept under a controlled environment at 37°C and humidity 70-75% for 48 hours. During the incubation period, the eggs were subsequently rotated at regular intervals of time to avoid attachment of the embryo to the eggshell. Afterwards, two to three ml of albumen was removed using a sterile syringe and the opening was sealed with a parafilm. After 72 hours, a small window was created in the eggshell with sterile tweezers.

Precautions were taken to prevent shell dust from descending onto the CAM, as it has the potential to interfere with its natural vascular development. After confirmation of a viable embryo, discs containing various concentrations of extract were placed over the surface of the embryonic membrane, i.e. CAM. After 48 hours, results were noted down in the form of inhibition of blood vessel branch points in comparison to the control.

Percentage of Inhibition = (Total count of branch points in control - Total count of branch points in treated × 100): Total count of branch points in control.

Cytotoxic effect of methanolic mycelial extract against cancer cells.

The cytotoxicity against cancer cells and normal cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using non-cancerous Vero and MDA-MB-231 cancerous cell lines.

Passage and maintenance of cancer cell line

DMEM with 10% FBS was used to maintain and culture cancer cells in a T-25 culture flask, supplemented with 1% standard antibiotic (streptomycin).

In vitro anti-proliferative MTT assay of methanolic mycelial extract

MTT assays were employed to evaluate the antiproliferative effect of the mycelial extract from *C. militaris* (van Meerloo et al., 2011). The cancerous cells were trypsinized and counted using a cell haemocytometer. These trypsinized cells were then added to an ELISA plate (1×10^5 cells per well) containing 200 µl of DMEM then left the cells to attach over the course of the night. Afterwards, cells were exposed to varying concentrations of mycelial extract for 24, 48, and 72 hours. Afterwards, twenty microlitres of MTT reagent were mixed with each well and again incubated for four hours at 37°C. After that, the medium containing MTT was carefully removed, and the formazan crystals were dissolved by adding 200 µl of DMSO. The absorbance was then measured at 570 nm. In this assay, tamoxifen at a concentration of 10 µg/ml was utilized as a positive control, whereas 0.1% DMSO was employed as a negative control. The viability percentage of the cells (% CV) is determined by:

Percentage of CV = (Treated cell absorbance × 100) : Control absorbance

Microscopic morphological observation of cancer cells

The morphology of the cancer cells treated with mycelial extract (31.25 to 4000 µg/ml) for 24 to 72 hours was observed using a phase-contrast inverted microscope at 200x magnification to evaluate the effect of the mycelial extract on cancer cell morphology.

Statistical Analysis

To ensure reproducibility, each experiment was performed in triplicate. All statistical analyses were performed using SPSS software (version 27, SPSS Inc., Chicago, IL, USA).

RESULTS

Antimutagenic potential of *C. militaris* mycelial extract

The antimutagenic property of the methanolic mycelial extract obtained from

the mycelium of *C. militaris* was evaluated using a method described by Maron & Ames in 1983. This is one of the most reliable, trusted and widely accepted methods for evaluation of the antimutagenic potential of herbal extracts. The mycelial extract of *C. militaris* did not affect the SR of any *Salmonella* tester strain, as shown in Table 1. However, the study found that the extract reduced the mutagenic effect of known mutagens. At a concentration of 10 mg/plate, the extract inhibited sodium azide triggered mutagenicity by 66.8% in the strain TA100 and 68.4% in the strain TA102, as shown in Table 2. Similarly, at a concentration of 10 mg extract per plate, the mycelial extract inhibited 4-NQO-induced mutagenicity by 59.9% in the TA98 strain and by 60.6% in the TA100 strain, as shown in Table 3.

The extract not only reduced the mutagenicity of direct-acting mutagens but also observed for those which required activation before acting as a mutagen such as (B[a]P. It is observed that the extract inhibits (B[a]P -triggered mutagenicity by 57.2% in TA98 and 63.1% in TA100, as mentioned in Table 4.

Table 1. Effect of methanolic mycelial extract on *Salmonella* tester strains spontaneous revertants.

Mycelial extract	S9	Revertants per plate (average)		
		TA98	TA100	TA102
E (10 mg)	−	76 ± 8.19	97.33 ± 6.03	144.67 ± 12.90
E (10 mg)	+	81 ± 14.73	117.33 ± 10.02	176 ± 17.06
SR	−	69 ± 15.52	105.67 ± 6.65	126 ± 14.11
SR	+	90 ± 17.62	109.67 ± 9.50	172 ± 20

Table 2. Antimutagenic effect of mycelial extract of *C. militaris* (E) on *Salmonella* strains against sodium azide (NaN_3)

Amount NaN_3 and mycelial extract (E) (mg/plate)	Revertants per plate (average)		% of Inhibition	
	TA100	TA102	TA100	TA102
NaN_3 (0.004)	1379.33 ± 70.70	434.67 ± 21.13	-	-
NaN_3+ E (0.25)	1103.33 ± 39.66[a]	371.33 ± 20.31[a]	20.01	14.57
NaN_3+ E (0.5)	793.00 ± 11.93[b]	270.67 ± 14.05[c]	42.51	37.73
NaN3 + E (1.00)	658.33 ± 29.71[c]	238.67 ± 9.5[c]	52.27	46.55
NaN_3+ E (5.00)	523.67 ± 15.57[c]	351 ± 24.52[c]	62.03	54.91
NaN_3+ E (10.00)	458.00 ± 13.20[c]	297.33 ± 11.59[c]	66.80	68.40
SR	105.67 ± 7.02	155 ± 11.136	-	-

[a] *P value* ≤ 0.05, [b] *P value* ≤ 0.01, [c] *P value* ≤ 0.001 with respect to NaN_3 alone.

Table 3. Antimutagenic effect of mycelial extract of *C. militaris* (E) on *Salmonella* strains against 4-nitroquinoline-N-oxide (4-NQO)

Amount of 4-NQO and mycelial extract (E) (mg/plate)	**Revertants per plate (average)**		**% of Inhibition**	
	TA98	**TA100**	**TA98**	**TA100**
4-NQO (0.0005)	943.00 ± 40	643.33 ± 19.14	-	-
4-NQO + E (0.25)	857.33 ± 30.92^{a}	594.67 ± 27.3^{ns}	9.08	7.56
4-NQO + E (0.5)	723.00 ± 26.46^{b}	530.00 ± 16.09^{b}	23.33	17.62
4-NQO + E (1.00)	566.33 ± 17.56^{c}	488.33 ± 22.59^{c}	39.94	24.09
4-NQO + E (5.00)	466.00 ±11.36^{b}	360.00 ± 33.15^{b}	50.58	44.04
4-NQO + E (10.00)	377.67 ± 15.18^{c}	253.00 ± 18.08^{c}	59.95.	60.67
SR	87± 15.52	65 ± 18.68	-	-

^{ns} *P value* > 0.05, ^{a} *P value* ≤ 0.05, ^{b} *P value* ≤ 0.01, ^{c} *P value* ≤ 0.001 with respect to 4-NQO alone

Table 4. Antimutagenic effect of mycelial extract of *C. militaris* (E) on *Salmonella* strains against benzo[a]pyrene (B[a]P)

Amount of B[a]P and mycelial extract (E) (mg/plate)	**Revertants per plate (average)**		**% of Inhibition**	
	TA98	**TA100**	**TA98**	**TA100**
B[a]P (0.001)	152.67 ± 17.24	427.33 ± 14	-	-
B[a]P + E (0.25)	127.33 ± 10.6^{ns}	385.33 ± 11.53^{a}	16.59	9.83
B[a]P + E (0.5)	101.33 ± 3.21^{b}	359.67 ± 17.01^{b}	33.62	15.83
B[a]P + E (1.00)	88.67 ± 6.03^{b}	316.00 ± 15.28^{c}	41.92	26.05
B[a]P + E (5.00)	80.67 ± 7.64^{b}	236.67 ± 9.02^{c}	47.16	44.62
B[a]P + E (10.00)	65.33 ± 4.04^{b}	157.67 ± 17.5^{c}	57.21	63.10
SR	22 ± 6.56	61.67 ± 14.01	-	-

^{ns}*P value* > 0.05, ^{a} *P value* ≤ 0.05, ^{b} *P value* ≤ 0.01, ^{c} *P value* ≤ 0.001 with respect to B[a]P alone

Antiangiogenic assay

Chorioallantoic membrane (CAM) assay is widely used for angiogenic studies, and it can be adapted to assess antiangiogenic potential. To evaluate the antiangiogenic properties, the methanolic mycelial extract of *C. militaris* was dissolved in DMSO at a concentration range of (20 - 100 µg/ml/egg). At 20 µg/ml/egg, the mycelial extract inhibited 16.02% of the angiogenic branch point, while 88.40% branch point inhibition was achieved at 100 µg/ml/egg. These findings demonstrate a clear concentration-dependent effect of mycelial extract on angiogenesis. Formation of new blood vessels is initiated from angiogenic branch points, the study finds a significant reduction in these branch points with respect to the control group (without any extract). These results are mentioned in Figure 1 and Table 5.

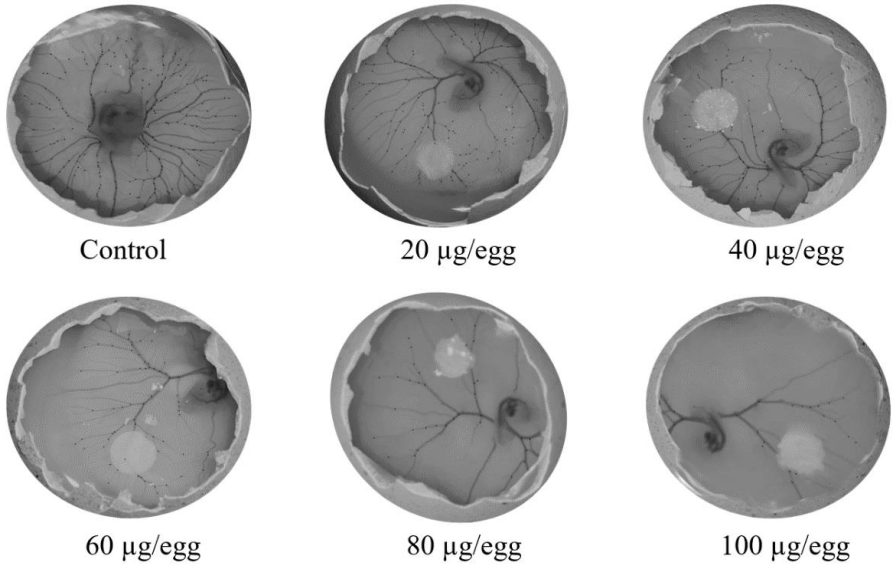

Figure 1. Antiangiogenic effect of *C. militaris* mycelial

Table 5. Antiangiogenic effect of *C. militaris* mycelial

Sr. No.	Extract concentration (µg/ml/egg)	Angiogenic branch points	% Inhibition
1	Control (0.00)	181 ± 4.58	-
2	20	152 ± 6.93[c]	16.02
3	40	96 ± 5.57[c]	46.96
4	60	66 ± 5.20[c]	63.54
5	80	37 ± 4.58[c]	79.56
6	100	21 ± 3.61[c]	88.40

[c] *P value* ≤ 0.001 with respect to control.

Cytotoxic effect of mycelial extract of *C. militaris* on cancer cells.

Methanolic mycelial extract of *C. militaris* has antimutagenic and antiangiogenic properties, as mentioned in the previous sections, the extract was further assessed for its cytotoxic effect on the cancer cell line (MDA-MB-231). A considerable variation was noted between the extract treated and control (10 % DMSO). In this study, tamoxifen, a well-known medication used against breast cancer used as positive control. The findings of the study demonstrated a significant impact of the mycelial extract of *C. militaris* on the viability of cancer cells. As the concentration increases, the viability of the cancer cells decreases. The IC_{50} values for tamoxifen used as a positive control and the extract were 7.08 µg/ml and 54.86 µg/ml, respectively, indicating that tamoxifen is more potent, as represented in Table 6. The extract demonstrated higher cytotoxic activity compared to the control (10% DMSO). Furthermore, the methanolic mycelial extract demonstrated the highest antiproliferative effect, resulting in an approximately 85.21% reduction in cell viability at the maximum concentration of 4000 µg/ml. However, it did not show significant toxicity to the normal Vero cells, as depicted in Figure 2.

Table 6. Effect of *C. militaris* mycelial extract on Vero cell line.

	IC_{50} (µg/ml)	
	Vero cell line	**MDA-MB-231**
Extract	ND	54.86
Tamoxifen	-	7.08

ND: Not detected

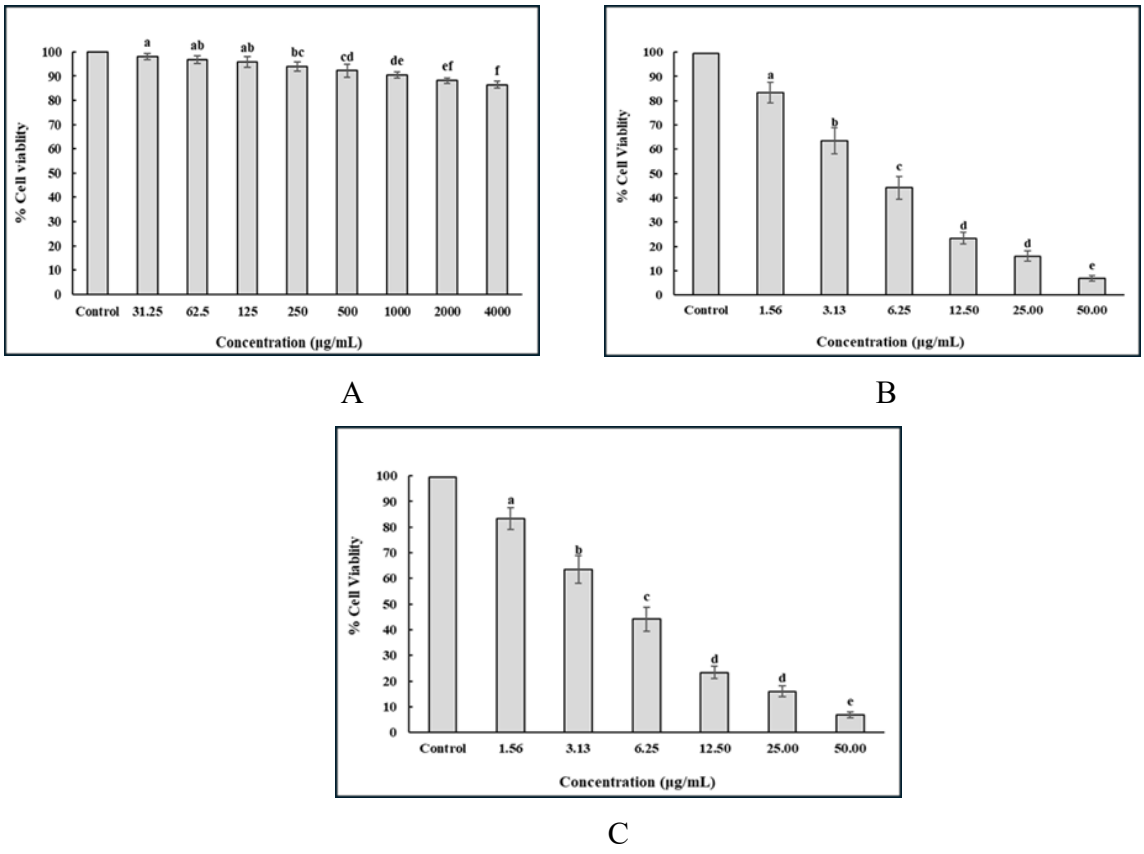

Figure 2: Cytotoxic activity of *C. militaris* mycelial extract against: (A) Cancer MDA-MB-231 cells, (B) Normal Vero cells. Cytotoxic activity of tamoxifen (C). Different letters represent significant differences at $p \leq 0.05$ according to One-way ANOVA followed by post hoc Duncan's multiple range test (DMRT).

The study evaluated the morphological alteration in cancer cells after the treatment of positive control, mycelial extract, and negative control. It is observed that the cancer cells treated with the negative control exhibited normal morphology, characterized by a well-spread and flattened appearance. In contrast, cells treated with the positive control and mycelial extract displayed morphological alterations, including reduced cellular and nuclear mass, cytoplasmic condensation, cell shrinkage, and an increased number of floating cells after 24 hours, as illustrated in Figure 3. Specifically, at a concentration of 4000 µg/ml of the extract, a significant number of cancer cells detached from the culture plates and floated in the medium. Apart from detachment, these cells also lost their normal morphology and appeared smaller and more rounded. These findings suggested that the extract exhibits a strong cytotoxic effect on cancerous cells.

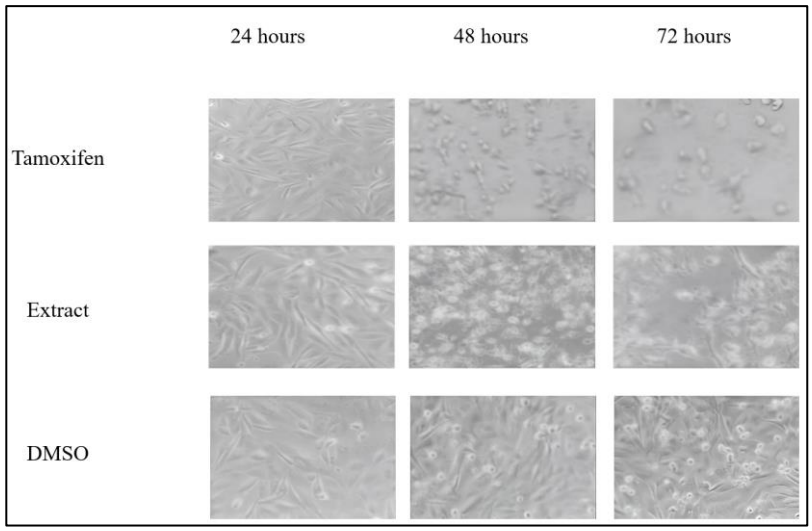

Figure 3. Microscopic examination of morphological changes in MDA-231-MB cells.

DISCUSSION

In recent years, herbal drugs and natural formulations have increasingly attracted attention as potential alternatives to conventional anticancer therapies. Despite significant advances in cancer research, the diversity of tumor biology, high mutation rates, and the emergence of drug resistance continue to hamper effective treatment (Bray *et al*., 2018). Conventional anticancer drugs, although designed to target specific oncogenic pathways, often affect normal cells—particularly those involved in angiogenesis—resulting in side effects such as hair loss, pain, and internal inflammation. In contrast, natural products derived from plants and mushrooms offer potential solutions by virtue of their complex chemical structures, synergistic bioactivities, and enhanced biocompatibility, which usually confer fewer side effects.

In the present study, the anticancer potential of the methanolic mycelial extract of *C. militaris* was evaluated with a focus on key processes such as mutation and angiogenesis. The extract demonstrated significant antimutagenic activity in the Ames test, achieving up to 68.4% inhibition against both direct-acting and S9-activated mutagens. This assay utilized specific strains of *S. typhimurium*—TA98, TA100, and TA102—each designed to detect distinct types of mutations: frameshift mutations, base pair substitutions, and oxidative DNA damage, respectively. This antimutagenic activity is in line with previous studies. For example, Tongmai *et al*. (2018) reported the antimutagenic properties of *C. militaris* mycelium extract in rats. Similarly, Li *et al*. (2003) observed that polysaccharide isolated from *Cordyceps sinensis* protects PC12 cells against hydrogen peroxide-induced injury. It mitigates oxidative stress by enhancing antioxidant defences and reducing lipid peroxidation in PC12 cells. Our higher inhibition rates are likely due to the use of methanol as an extraction solvent, which can recover a broader range of secondary metabolites than water alone. This finding underscores the critical role of extraction methodology in modulating the extract's antimutagenic potency.

Our earlier work (Deshmukh & Lakshmi, 2023) demonstrated that the methanolic extract also exhibits superior antioxidant activity compared to its aqueous counterpart, as shown by its enhanced DPPH and ABTS radical scavenging abilities. These results are consistent with the findings of (Jeong *et al.*, 2011), who reported DPPH scavenging IC_{50} values between 50 and 80 µg/ml for methanolic extracts of *C. militaris* fruiting bodies. Moreover, Tuli *et al*. (2014) noted significant antioxidant activities in *C. militaris* polysaccharides. Because many mutagens exert their effects through the generation of reactive oxygen species (ROS), the potent antioxidant capacity of our extract likely contributes to its antimutagenic effects by mitigating oxidative DNA damage. Thus, the interplay between antioxidant and antimutagenic activities appears to be a key mechanism for its potential in countering early carcinogenesis.

Cytotoxicity studies in the present study further highlight the therapeutic promise of *C. militaris*. The methanolic mycelial extract exhibited selective cytotoxicity towards MDA-MB-231 breast cancer cells with an IC_{50} of 54.86 µg/ml, while normal Vero cells remained largely unaffected. This selective inhibition is promising when compared to previous reports. Xie *et al.* (2019) demonstrated that an ethanolic extract of *Cordyceps cicadae* reduced proliferation in SGC-7901 gastric cancer cells through apoptosis induction and cell cycle arrest. In a similar vein, Nakamura *et al*. (2015) reported notable anticancer and antimetastatic effects for a water extract of *Cordyceps sinensis*, partly attributed to its A3 receptor antagonist activity. Significantly, Reis et al. (2013) found that *C. militaris* fruiting body extracts are able to inhibit the proliferation of MCF-7 (breast), NCI-H460 (non-small lung), HCT-15 (colon) and HeLa (cervical) human carcinoma cell lines. The morphological alterations observed in treated cancer cells indicate that apoptosis is a likely underlying mechanism of cytotoxicity.

Importantly, these findings yield several insightful implications. First, the extraction method and specifically the use of methanol appears to greatly influence the concentration and spectrum of bioactive compounds, thereby enhancing the antimutagenic and antioxidant responses. Second, the observed synergy between antioxidant and antimutagenic activities suggests that scavenging ROS not only prevents DNA damage but also plays a pivotal role in mitigating mutation-driven carcinogenesis. Third, the selective cytotoxicity against cancer cells reinforces the potential of *C. militaris* mycelial extract as a safer alternative to conventional chemotherapy, which often exhibits pronounced toxicity against normal cells.
Collectively, presented results emphasize the promise of *C. militaris* mycelial extract as a natural, multi-targeted anticancer agent. Future studies should focus on elucidating the precise molecular pathways underlying these bioactivities and on standardizing extraction protocols to maximize the yield of therapeutically active compounds. This will facilitate progress toward clinical evaluations and integration of such natural agents into comprehensive cancer treatment regimens.

CONCLUSION

This study highlights the anticancer potential of the methanolic mycelial extract of *C. militaris*, demonstrated through its significant antimutagenic, antiangiogenic,

and cytotoxic activities. The extract effectively inhibited mutagenicity caused by both direct-acting and metabolically activated mutagens, suppressed angiogenesis in a dose-dependent manner, and exhibited selective cytotoxicity against MDA-MB-231 breast cancer cells with minimal effects on normal cells. Observed morphological changes further support its apoptotic effects. These findings suggest that *C. militaris* mycelial extract, enriched with bioactive compounds, may serve as a promising natural candidate for anticancer therapies.

CONICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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