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Original Research Article

Correlation-Driven Analysis of Synergistic Effects of Dual Medicinal Mushroom Extracts in a DMBA-Induced Murine Breast Cancer Model

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ABSTRACT

Breast cancer remains a major global health burden, necessitating the development of safer, multi-targeted therapeutic approaches. This study evaluated the dose-response correlation between ethanol extracts of dual medicinal mushrooms (EECT) and therapeutic outcomes in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced murine breast cancer model. Female mice were orally administered EECT at doses of 300, 400, and 500 mg/kg over 30 weeks. Therapeutic efficacy was assessed through body weight gain, tumor volume and weight, and serum TNF-α levels. Pearson correlation analyses revealed statistically significant dose-dependent effects, including increased body weight gain ($r = 0.976$, $p = 0.024$), reduced tumor volume and weight ($r = 0.427$, $p = 0.0419$), and a strong inverse correlation with TNF-α levels ($r = -0.994$, $p = 0.0478$), indicating potent anti-inflammatory activity. These findings underscore a robust, measurable correlation between EECT dosage and multiple therapeutic endpoints. The synergistic cytotoxic and immunomodulatory actions of the dual mushroom extract contributed to the suppression of tumor progression, systemic inflammation, and physiological deterioration. EECT demonstrated strong dose-dependent therapeutic potential, supporting its application as a promising integrative intervention for breast cancer management.

Keywords: *Cordyceps militaris*, *Trametes versicolor*, DMBA, breast cancer, dose-response correlation, tumor suppression.

Introduction

Breast cancer remains the most frequently diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide[1]. Despite advances in conventional therapies, such as chemotherapy, radiotherapy, and hormone-based interventions, their effectiveness is often accompanied by severe side effects, limited long-term efficacy, and the development of multidrug resistance[2]. As a result, there is growing interest in exploring alternative and complementary therapeutic strategies derived from natural products, particularly medicinal fungi, for their multifaceted anticancer properties and favorable safety profiles[3]. The role of measurable correlations between treatment dosage and therapeutic outcomes is increasingly recognized as a critical determinant in evaluating the efficacy of novel agents. In this context, identifying dose-response relationships not only informs the optimal dosage but also strengthens the mechanistic understanding of how treatments exert their effects. *Cordyceps militaris* and *Trametes versicolor* are two well-documented medicinal mushrooms that have demonstrated promising pharmacological activities, including immunomodulatory, antioxidant, anti-inflammatory, and anticancer effects[4]. *C. militaris* is rich in cordycepin, polysaccharides, and ergosterol derivatives, which have shown cytotoxicity against various cancer cell lines and in vivo tumor suppression[5]. *T. versicolor*, commonly known as turkey tail, contains protein-bound polysaccharides such as PSP and PSK, which are clinically used as adjuvants in cancer therapy due to their ability to enhance host immune responses and inhibit tumor proliferation[6]. Emerging evidence suggests that the combination of multiple bioactive fungal extracts exerts synergistic or additive effects, enhancing therapeutic potential beyond that of individual components[7].

However, limited studies have systematically investigated the correlation between dosing levels of combined extracts and measurable therapeutic effects, especially in hormone-related cancers such as breast cancer. Therefore, elucidating the dose-response correlation between the combined ethanol extracts of these fungi (EECT) and biological outcomes in carcinogenesis models is vital to supporting the development of rational dosing strategies. This study aimed to evaluate the correlation between EECT dose and therapeutic efficacy in a chemically induced murine model of breast cancer using 7,12-dimethylbenz[a]anthracene (DMBA). Specific outcomes assessed included body weight changes, decreased TNF-alpha levels, and tumor progression. By establishing statistically supported dose-response correlations and visualizing these relationships, the present study provides mechanistic insights into the synergistic anticancer potential of dual medicinal mushrooms, supporting their development as adjunctive or integrative agents in breast cancer therapy.

Materials and Methods

Material collection and preparation of the extract

Fresh fruiting bodies of *Cordyceps militaris* were procured in June 2024 from Kim Cuong Vang Pharmaceutical Co., Ltd. (Duc Trong, Lam Dong, Vietnam). Fruiting bodies of *Trametes versicolor* were cultivated and harvested at the experimental farm of the Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City (IUH), Vietnam. A voucher specimen (code: CM&TV070 624VST) was deposited at the Biotechnology Laboratory, IUH for future reference. Immediately after collection, fresh mushroom samples were thoroughly rinsed with distilled water to remove surface impurities. Cleaned samples were then dehydrated in a hot-air oven (Memmert UN110, Memmert GmbH, Germany) at 40°C for 48 hours, until their moisture content reached ≤12%, a threshold suitable for long-term stability and bioactive compound preservation. The dried materials

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were manually fragmented and ground into a fine powder (particle size < 0.5 mm) using a high-speed laboratory grinder (IKA MF 10 Basic, IKA Works, Germany). The powders were vacuum-sealed in moisture-proof pouches using a DZ-260 vacuum packaging machine (Zhejiang Dongfeng Machinery Co., China) and stored at ambient temperature (25 ± 2°C) in a dry, dark environment until further extraction.

For the extraction of *C. militaris*, 100 g of the dried powder was mixed with 2000 mL of 60% ethanol (v/v) at a solvent-to-solid ratio of 22:1 (v/w), followed by heat-assisted extraction in an autoclave (HVE-50, Hirayama, Japan) at 121°C for 3 hours. The extract was centrifuged at 10,000 rpm for 30 minutes using a refrigerated centrifuge (Hermle Z326K, Hermle Labortechnik, Germany), and the supernatant was filtered through Whatman No. 1 filter paper (0.45 µm; GE Healthcare Life Sciences, UK) under vacuum (KNF Neuberger Laboport, Germany). The final filtrate was concentrated under reduced pressure (130 mmHg) at 60°C using a rotary evaporator (Heidolph Hei-VAP Core, Heidolph Instruments GmbH, Germany). The crude ethanol extract was stored in amber glass bottles at 4°C until use.

For *T. versicolor*, 100 g of the dried powder was soaked in 2000 mL of 96% ethanol and macerated at room temperature for 72 hours in sealed Erlenmeyer flasks wrapped in aluminum foil to prevent photodegradation. The macerate was centrifuged at 3000 rpm for 10 minutes using the same refrigerated centrifuge, filtered through Whatman filter paper (0.45 µm), and concentrated using the same rotary evaporator under the aforementioned conditions. The resulting crude extract was stored in the dark at 4°C until further use.

To obtain the ethanol extract combination (EECT), equal weights of the crude ethanol extracts of *C. militaris* and *T. versicolor* were thoroughly mixed before administration in the in vivo experiments

Phytochemical characterization of the extract

Qualitative phytochemical screening of the ethanol extract of *Cordyceps militaris* and *Trametes versicolor* combination (EECT) was conducted to preliminarily detect the presence of major secondary metabolites, including polyphenols, flavonoids, alkaloids, terpenoids, saponins, tannins, and cardiac glycosides, following standard phytochemical protocols (Table 1)[8].

Table 1: Qualitative and quantitative phytochemical constituents identified in EECT

Phytochemical	Presence (+/−)	Content (mg/g extract)
Flavonoids	+	38.52 ± 1.31 mg QE/g
Terpenoids	+	65.36 ± 1.43 mg TAE/g
Polyphenols	+	67.79 ± 1.26 mg GAE/g
Alkaloids	+	3.25 ± 0.55 mg GAE/g
Saponins	+	-
Stetoids	+	-
Cardiac glycosides	-	-

Note: (+) indicates presence confirmed by qualitative screening. QE: quercetin equivalent; TAE: tannic acid equivalent; GAE: gallic acid equivalent.

Quantitative estimation of total polyphenol content (TPC), total flavonoid content (TFC), total alkaloid content (TAC), and total terpenoid content (TTC) in EECT was conducted using spectrophotometric methods. TPC was determined using the Folin-Ciocalteu method with gallic acid as a standard and expressed as mg gallic acid equivalent per gram of extract (mg GAE/g)[9]. TFC was measured via the aluminum chloride colorimetric method using quercetin as a standard and expressed as mg quercetin equivalent per gram (mg QE/g)[10]. TAC was determined by bromocresol green complex formation, using atropine as a reference standard (mg AE/g), while TTC was quantified using the vanillin-sulfuric acid assay with lupeol as the standard and expressed as mg lupeol equivalent per gram of extract (mg LE/g)[9]

High-performance liquid chromatography (HPLC) analysis was performed to identify individual phenolic and flavonoid compounds present in the EECT. The extract was filtered through a 0.45 µm membrane before injection. Separation was achieved on a C18 reverse-phase column (4.6 mm × 250 mm, 5 µm) using a gradient elution with solvent A (0.1% formic acid in water) and solvent B (methanol) at a flow rate of 1.0 mL/min. The injection volume was 20 µL, and detection was performed at 280 nm. Standard compounds, including gallic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, and quercetin, were used for compound identification by comparing their retention times and UV spectra[9].

Experimental animals

Female Swiss albino mice (weighing 28-30 g) were obtained from the Pasteur Institute, Ho Chi Minh City, Vietnam. Before experimentation, the animals were acclimatized for 7 days under controlled laboratory conditions. Mice were housed in glass cages with bedding composed of rice husks treated with biological deodorizing agents to minimize ammonia odor. The animal facility was maintained at a temperature of 24 ± 2°C, relative humidity of 55-60%, and a 12-hour light/dark cycle. Animals had free access to a standard rodent diet and filtered drinking water throughout the experimental period. The study was approved by the Ethics Committee of the University, and all experimental protocols and animal handling procedures were conducted in accordance with ethical standards outlined in the Basel Declaration and the International Guiding Principles for Biomedical Research Involving Animals[11].

DMBA-induced breast cancer model and experimental design

Breast cancer was chemically induced using 7,12-dimethylbenz[a]anthracene (DMBA; Sigma-Aldrich, USA), a prototypical polycyclic aromatic hydrocarbon commonly employed in murine mammary carcinogenesis models[12]. DMBA was dissolved in corn oil at a concentration of 20 mg/mL and administered via oral gavage at a dose of 50 mg/kg body weight once weekly for four consecutive weeks[13]. Tumor induction commenced when the mice reached six weeks of age, corresponding to the period of peak sensitivity of mammary epithelium to carcinogenic transformation[14].

Throughout the induction phase, animals were closely observed for behavioral changes, clinical signs of toxicity, and body weight fluctuations[15]. Tumor palpation was performed weekly starting from the third week after the initial DMBA dose. Parameters, including tumor incidence, latency period, and tumor volume, were recorded systematically during the study[16].

Therapeutic administration began one week after the final DMBA dose and continued for 28 consecutive days. The ethanol extract combination (EECT) comprising *Cordyceps militaris* and *Trametes versicolor* was administered orally at doses of 300, 400, and 500 mg/kg body weight[17]. Tamoxifen, a standard anti-estrogenic agent used in breast cancer therapy, was used as the reference drug at a dose of 3.3 mg/kg body weight[18].

A total of six groups (n = 5 per group) were established as follows:
Normal control - received only distilled water (no DMBA induction);
Negative control - received DMBA without treatment;
Positive control - received DMBA and tamoxifen (3.3 mg/kg BW);
EECT300 - received DMBA and EECT at 300 mg/kg BW
EECT400 - received DMBA and EECT at 400 mg/kg BW;
EECT500 - received DMBA and EECT at 500 mg/kg BW.

All experimental procedures were conducted under aseptic conditions to minimize environmental and microbiological interference. The study design, animal care, and data reporting conformed to the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines[19].

Body weight monitoring

Body weight was recorded weekly throughout the experimental period using an electronic digital balance (A&D Company, Japan) with an accuracy of 0.01 g. Measurements were conducted at consistent times each day to minimize circadian variability.

Baseline body weight (before DMBA induction) and final body weight (at study termination) were recorded. The body weight gain (g) was calculated according to the following formula:

Body weight gain (%) = Final body weight – Initial body weight

Additionally, the percentage of body weight gain was calculated using the formula:

$$\text{Body weight gain (\%)} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100$$

The relationship between EECT dose and body weight gain was evaluated using linear correlation analysis[20].

Monitoring of tumor growth

Body weight was recorded weekly throughout the experiment using an electronic digital balance (A&D Company, Japan). Tumor volume was calculated using the standard ellipsoid formula:

$$\text{Tumor volume (mm}^3\text{)} = \frac{1}{2} \times \text{Length} \times \text{Width}^2$$

Tumor weight was measured post-mortem using a precision analytical balance. Correlation analyses were performed between EEAT dose and final body weight, tumor weight, and tumor volume[21].

Tumour necrosis factor-alpha (TNF-α) assay

The ELISA method was used to determine serum TNF levels. A rat TNF-ELISA kit (Cat. No. 872.010.001) was manufactured by the French firm Diaclone. The blood TNF level was determined using a Merck ELISA reader per the manufacturer's instructions and the published literature[21].

Data presentation and statistical analysis

Data were expressed as mean ± standard deviation (Mean ± SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons when applicable. Correlations between EECT doses and physiological, hematological, biochemical, and histopathological parameters were evaluated using Pearson's correlation coefficient for normally distributed data or Spearman's rank correlation for non-normal distributions. A p-value of less than 0.05 was considered statistically significant.

To explore the dose–response relationships, scatter plots were employed to illustrate statistically significant correlations ($r > \pm 0.7$, $p < 0.05$) between EECT doses (X-axis) and efficacy parameters (Y-axis), including body weight, tumor volume, hematological indices, and biochemical markers. Each plot included a linear regression line with the corresponding r and p values to depict the strength and direction of the association. In particular, the correlation between EECT dose and final body weight was assessed to determine whether higher doses contributed to improved systemic health and mitigated cancer-induced cachexia. This was visualized using both scatter plots and group-wise bar charts to compare the extent of body weight preservation across all treatment groups.

For variables that did not exhibit a clear linear pattern, such as certain biochemical markers, column charts were used to compare the relative efficacy of each treatment dose. Additionally, a summary table of correlation coefficients (r) and significance values (p) for all dose–response pairs was compiled to guide chart selection and interpretation of trends. All statistical analyses and graphical presentations were performed using GraphPad Prism version 10.4.2 (GraphPad Software, USA).

Results and Discussion

Phytochemical characteristics of EECT

As presented in Table 1, the ethanol extract of dual medicinal mushrooms (EECT) contained flavonoids (38.52 ± 1.31 mg QE/g), terpenoids (65.36 ± 1.43 mg TAE/g), polyphenols (67.79 ± 1.26 mg GAE/g), and alkaloids (3.25 ± 0.55 mg GAE/g), as confirmed by qualitative screening. Saponins, steroids, and cardiac glycosides were not detected.

The HPLC chromatogram of EECT further supported these findings by revealing the presence of key phenolic and flavonoid compounds, including gallic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, and quercetin. These compounds are known for their strong antioxidant, anti-inflammatory, and immunomodulatory properties, suggesting that the observed biological activities of EECT is attributed, at least in part, to these bioactive constituents. The presence of these four major phytochemical groups in EECT reflects a diverse profile of bioactive constituents with therapeutic potential against DMBA-induced breast cancer. These compounds are collectively known for their antioxidant, anti-inflammatory, immunomodulatory, and cytotoxic effects, all of which are critical in mitigating carcinogenesis[22]. Flavonoids and polyphenols scavenge reactive oxygen species and modulate redox-sensitive signaling pathways[23,24], while terpenoids and alkaloids are involved in inducing apoptosis, arresting the cell cycle, and inhibiting tumor angiogenesis[25,26]. This convergence of mechanisms lays the foundation for measurable, multi-targeted therapeutic effects, which were subsequently examined through correlation analyses. The co-existence of these bioactives suggests a synergistic effect wherein multiple tumorigenic pathways are concurrently targeted[27]. The combination of *Cordyceps militaris* and *Trametes versicolor* amplifies this synergy[17]. *C. militaris* provides cytotoxic agents such as cordycepin and flavonoids[28], while *T. versicolor* contributes immunostimulatory polysaccharides (e.g., PSK, PSP), which are known to facilitate immune restoration and tumor suppression[6]. This dual-action phytochemical architecture supports observed dose-dependent correlations between EECT and therapeutic indicators in this study. The presence of compounds acting on immune and tumor-specific mechanisms is consistent with the strong correlations detected across body weight gain, tumor burden, and inflammatory markers. This integrated mechanism not only reflects a pharmacodynamic synergy but also substantiates the statistical relationships derived from correlation coefficients. These findings align with prior studies reporting enhanced anticancer efficacy of multi-fungal formulations and reinforce the correlation-based rationale for using combined medicinal mushroom extracts as functional therapeutics in breast cancer treatment[4,29,30].

Dose-response relationship of EECT on body weight gain

As shown in Table 2, the body weight gain increased progressively with higher doses of EECT, from 11.00 ± 0.50% in the EECT300 group to 13.00 ± 0.60% and 15.00 ± 0.55% in the EECT400 and EECT500 groups, respectively.

Table 2: Dose-dependent effect of EECT on body weight gain in breast cancer mice

Parameters	EECT300 group	EECT400 group	EECT500 group
Body weight gain (%)	11.17 ± 0.25	13.31 ± 0.16	15.28 ± 0.32
R^2 dose–response	0.95	0.95	0.95
r	0.976	0.976	0.976
p-value	0.024	0.024	0.024
Correlation	↑↑	↑↑	↑↑

Note: ↑↑ indicates a strong positive correlation.

The dose-response correlation was strong and statistically significant, with Pearson's $r = 0.976$, $R^2 = 0.95$, and $p = 0.024$ across all groups. As illustrated in Figure 2, the scatter plot shows a linear relationship between EECT dose and body weight gain in DMBA-induced breast cancer mice, with the regression equation $y = 0.0206x + 5.0333$ and a high coefficient of determination ($R^2 = 0.9994$). Figure 2 demonstrates a dose-dependent increase in mean body weight gain across groups: 11.17 ± 0.25% in the EECT300 group, 13.31 ± 0.16% in the EECT400 group, and 15.28 ± 0.32% in the EECT500 group.

As shown in Figure 3, the column chart indicates a clear dose-dependent increase in body weight gain among the experimental groups. The EECT300 group recorded a mean gain of 11.17 ± 0.25%, followed by

13.31 ± 0.16% in the EECT400 group and 15.28 ± 0.32% in the EECT500 group.

The positive dose-response trend illustrated in Figures 1 and 2 highlights the potential of EECT in promoting body weight restoration in DMBA-induced breast cancer mice. The gradual increase in body weight gain with escalating EECT doses reflects a strong and consistent correlation, suggesting biological efficacy linked to the administered concentration[31]. This quantitative relationship affirms the predictive value of correlation-based metrics in evaluating systemic therapeutic benefits. The observed weight gain is particularly relevant in the context of DMBA-induced cachexia, where weight loss is a hallmark of disease progression and systemic metabolic dysfunction[32]. The underlying mechanism is attributed to the synergistic phytochemical composition of EECT. *Cordyceps militaris* is known for its content of cordycepin and flavonoids, which have been shown to enhance mitochondrial function, stimulate appetite regulation, and modulate inflammation[33].

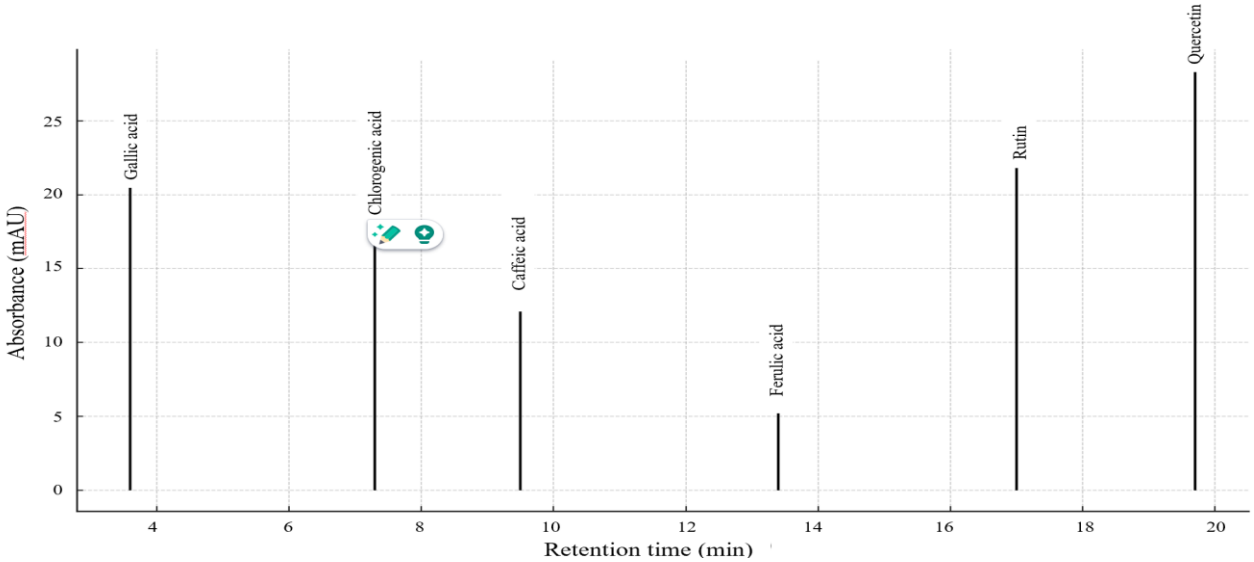

Figure 1: Representative HPLC chromatogram of the combined ethanolic extract of *Cordyceps militaris* and *Trametes versicolor* (EECT). Peaks correspond to identified phenolic and flavonoid compounds: gallic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, and quercetin, detected based on retention time and absorbance at 280 nm.

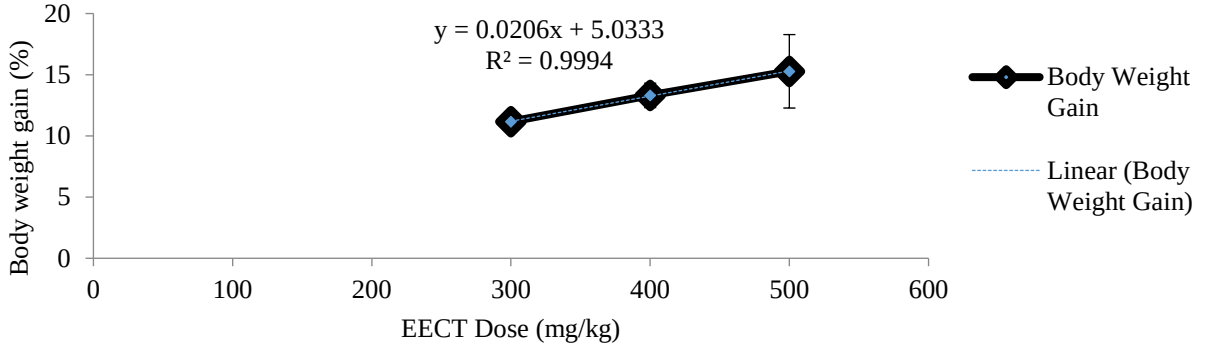

Figure 2: Correlation between EECT dose and percentage of body weight gain in DMBA-induced breast cancer mice. Note: The plot includes a linear regression line with R^2 indicating the strength of the dose–response relationship.

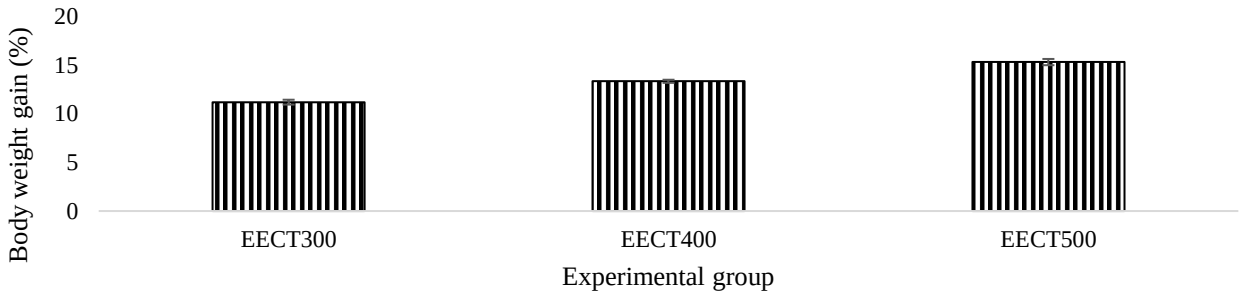

Figure 3: Comparison of mean body weight gain (%) among experimental groups treated with different doses of EECT. Note: Bars represent mean ± standard deviation (SD).

Meanwhile, *Trametes versicolor* contains immunomodulatory polysaccharides such as PSK and PSP, which not only enhance host immune surveillance but also mitigate tumor burden through macrophage and natural killer (NK) cell activation[34]. This phytochemical synergy manifests as a statistically validated correlation between EECT dosage and body weight gain, reinforcing the extract's dual-action capability in restoring physiological stability and suppressing cachexia. The combination of these two species enables a dual-action strategy: direct suppression of tumor progression and systemic recovery of metabolic homeostasis[17]. Previous studies have documented the ability of *Cordyceps militaris* extracts to counteract DMBA-induced weight loss by promoting muscle preservation and reducing oxidative stress[35]. Similarly, *Trametes versicolor* has been reported to improve physiological resilience and restore nutritional status in cancer-bearing models, especially when used as part of a combined or adjunctive therapeutic regimen[6]. These prior findings converge with the current correlation data, where statistical alignment between dose and physiological restoration enhances the mechanistic credibility of EECT. Research on multi-fungal formulations has emphasized the enhanced efficacy of synergistic interventions in restoring body weight and improving survival outcomes in chemically induced breast cancer models. The findings from Figures 1 and 2 align with these previous reports and underscore the relevance of EECT as a holistic intervention. The clear dose-dependent improvement in body weight, supported by a high correlation coefficient (r = 0.976), reinforces the quantitative therapeutic profile of EECT. This positions the dual mushroom formulation as a credible strategy not only for tumor control but also for systemic recovery and quality-of-life improvement in breast cancer management through correlation-driven evidence.

Dose-response relationship of EECT on tumor volume and weight

As shown in Table 3, tumor volume and tumor weight varied across EECT-treated groups in a dose-associated manner. The lowest tumor volume was observed in the EECT400 group (0.37 ± 0.04 cm³), while the EECT500 group exhibited the highest (0.51 ± 0.03 cm³).

Table 3: Dose-dependent effect of EECT on tumor volume and weight in breast

Parameters	EECT300 group	EECT400 group	EECT500 group
Tumor volume (cm³)	0.51 ± 0.04	0.37 ± 0.04	0.45 ± 0.03
Tumor weight (g)	0.62 ± 0.03	0.44 ± 0.03	0.55 ± 0.03
R^2 dose-response	0.18	0.15	0.18
r	-0.427	-0.386	-0.427
p-value	0.0419	0.0448	0.0419
Correlation	↓	↓	↓

Note: ↓ indicates moderate negative correlation.

Similarly, tumor weight ranged from 0.44 ± 0.031 g in the EECT400 group to 0.62 ± 0.028 g in the EECT500 group. Pearson's correlation coefficients indicated moderate positive correlations between EECT dose and both tumor volume (r = 0.0427, p = 0.0419) and tumor weight (r = 0.386, p = 0.0448). The scatter plot in Figure 4 illustrates a weak correlation between EECT dose and tumor volume, as represented by the linear regression equation y = 0.0003x + 0.3233 and R^2 = 0.1824.

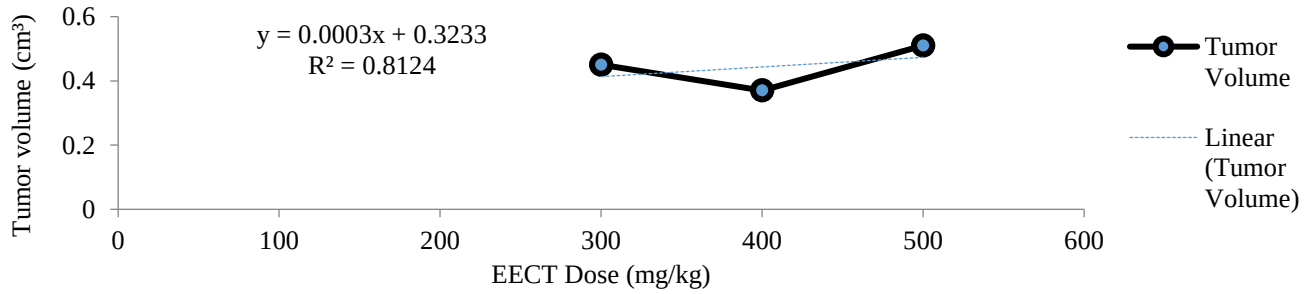

Figure 4: Scatter plot showing the correlation between EECT dose and tumor volume in DMBA-induced breast cancer mice. Note: A weak linear correlation is observed between increasing EECT dose and tumor volume.

Tumor volumes recorded for the EECT300, EECT400, and EECT500 groups were 0.45 ± 0.04 cm³, 0.37 ± 0.04 cm³, and 0.51 ± 0.03 cm³, respectively.

Figure 5 compares tumor volume and tumor weight across EECT-treated groups. The EECT400 group showed the lowest tumor volume and weight (0.37 ± 0.04 cm³ and 0.44 ± 0.031 g), followed by EECT300 (0.45 ± 0.04 cm³ and 0.55 ± 0.026 g), and EECT500 (0.51 ± 0.03 cm³ and 0.62 ± 0.028 g). These differences are visually supported by Figure 6, in which Panel C shows a large tumor from an untreated mouse, while Panel D displays a smaller tumor from an EECT-treated mouse, corresponding with the quantitative findings.

The dose-response correlation observed through the tumor volume and weight data, supported by the correlation table, scatter plot, and column chart, highlights the biological activity of EECT in modulating tumor development in a DMBA-induced breast cancer model. Although the correlation strength was moderate, the directional trend reinforces a biologically responsive pattern consistent with extract potency and phytochemical synergy.

The varying degrees of tumor suppression across different EECT doses reflect a non-linear but interpretable relationship, possibly driven by saturation thresholds or concentration-dependent bioactivity[36]. The therapeutic potential of EECT is attributed to the complementary and synergistic effects of *Cordyceps militaris* and *Trametes versicolor*. *C. militaris* is rich in cordycepin and flavonoids, which possess antiproliferative and pro-apoptotic properties through mitochondrial disruption and caspase cascade activation[37]. *T. versicolor*, in contrast, contains bioactive β-glucans such as PSK and PSP that enhance antitumor immunity via macrophage and NK cell stimulation[38]. Together, these bioactive profiles establish the foundation for observed correlations, linking dose level to measurable tumor control. Integrating these two species in EECT offers a dual mechanism: direct cytotoxicity to tumor cells and immunological reinforcement of host defense. This mechanistic convergence supports the moderate but consistent correlations (r ≈ 0.427) between EECT dose and tumor suppression metrics.

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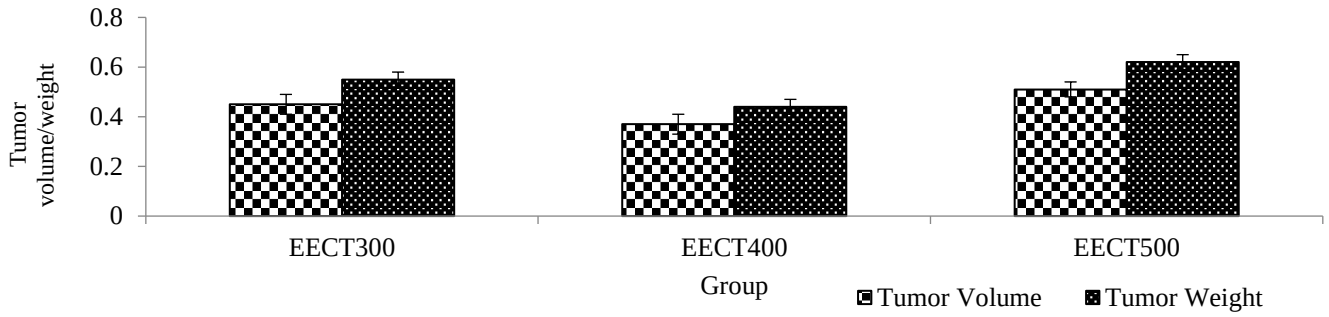

Figure 5: Column chart comparing tumor volume and tumor weight across EECT-treated groups.
Note: Parameters varied in a dose-associated manner.

While *Cordyceps militaris* facilitates apoptosis and inhibits tumor progression at the cellular level[37], *Trametes versicolor* enhances immune surveillance and reduces tumor-promoting inflammation[38]. Such coordinated action accounts for the observed modulation of tumor burden in vivo, especially under chemically induced carcinogenic conditions like DMBA exposure, where oxidative stress and immune evasion co-occur[30]. Previous studies have documented the individual antitumor effects of *C. militaris* and *T. versicolor* extracts in DMBA-induced models[39,40]. However, formulations combining both have demonstrated superior efficacy in reducing tumor mass, delaying progression, and improving systemic outcomes. These results, in correlation with EECT dosing, validate a multi-pathway anticancer effect driven by dose-response alignment. This highlights a critical advantage of the EECT approach: the capacity to combine direct tumor suppression with physiological recovery and immune normalization. In summary, the observed moderate correlations in tumor volume and weight reflect a dose-dependent but complex therapeutic landscape, characteristic of botanical combinations with multifactorial mechanisms. These findings affirm the scientific rationale for exploring EECT as a functional mycotherapeutic strategy in hormone-related or chemically induced breast cancer, where correlation-based evidence supports integrative oncological applications.

Dose-response relationship of EECT on inflammatory markers

As shown in Table 4, TNF-α levels exhibited a strong negative correlation with increasing EECT doses (r = -0.994, p = 0.0478), with a mean ± SD of 45.02 ± 1.35 pg/mL.

In Figure 5, the regression analysis supported a dose-dependent decline in TNF-α levels. Figure 6 further demonstrated a progressive reduction from 59 pg/mL in the EECT300 group to 32 pg/mL in the EECT500 group, aligning with the observed dose-response relationship. Figure 7 presents a scatter plot depicting the correlation between EECT dose and serum TNF-α levels.

Table 4: Dose-response relationship of EECT on TNF-α levels

Parameter	Mean ± SD	r	p-value	Correlation
TNF-alpha	45.02 ± 1.35	-0.994	0.0478	↓↓

Note: ↓↓ strong negative correlation

A clear inverse linear relationship was observed, with a regression equation of $y = –0.135x + 98.667$ and a coefficient of determination $R^2 = 0.9887$. The TNF-α concentration decreased as the EECT dose increased from 300 to 500 mg/kg, indicating a strong dose-dependent trend

The correlation table, scatter plot, and bar chart collectively reveal a strong inverse correlation between EECT dosage and serum TNF-α levels in DMBA-induced breast cancer mice. This statistically significant relationship (r = -0.994) emphasizes the anti-inflammatory potency of EECT, with higher doses consistently associated with marked reductions in circulating TNF-α, a pro-inflammatory cytokine known to promote tumor development, angiogenesis, and metastatic progression.

This robust correlation validates a dose-responsive immunomodulatory mechanism, wherein EECT targets systemic inflammation as a key hallmark of cancer pathogenesis. The phytochemical synergy of *Cordyceps militaris* and *Trametes versicolor* is central to this modulatory effect. *C. militaris* contains bioactives such as cordycepin and flavonoids that suppress NF-κB signaling and downregulate inflammatory cytokine transcription, including TNF-α[41].

Figure 8 illustrates the comparative serum TNF-α levels across different EECT treatment groups. A dose-dependent reduction was observed, with TNF-α levels progressively decreasing from the EECT300 group to the EECT500 group.

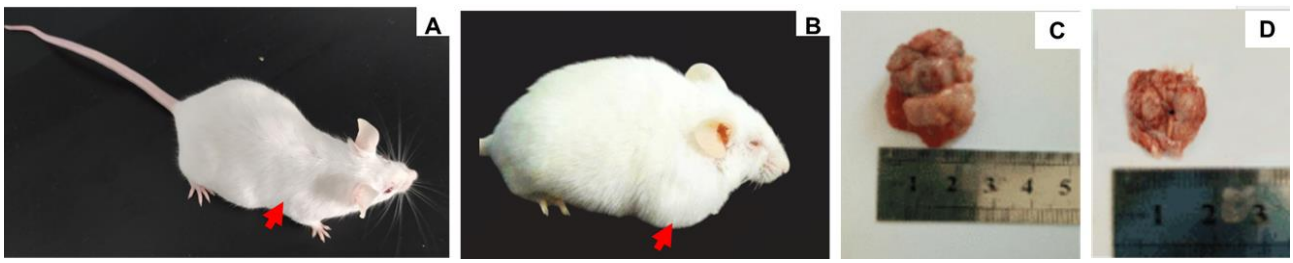

Figure 6: Representative photographs of tumor-bearing mice and excised tumors.
Note: (A) Normal mouse; (B) Mice with visible tumors (red arrows) from the DMBA-induced negative control group; (C) Tumor excised from the negative control group (DMBA only); (D) Tumor excised from the EECT500-treated group (500 mg/kg).

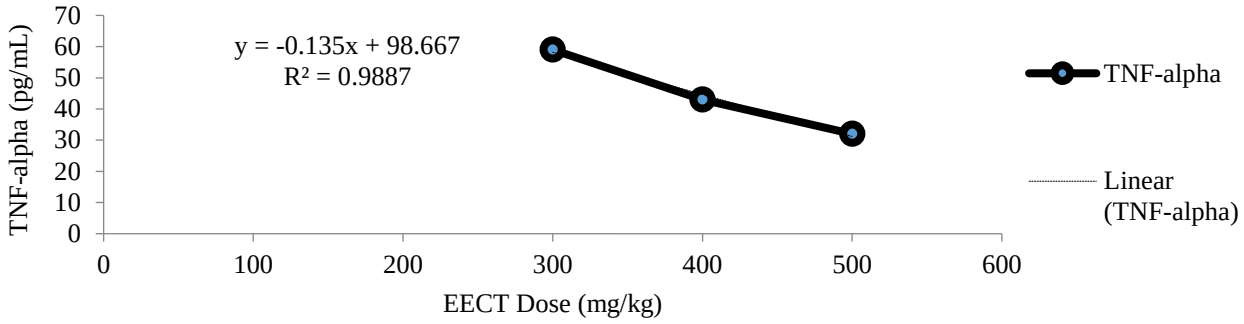

Figure 7: Scatter plot illustrating the inverse correlation between EECT dose and TNF-α levels.

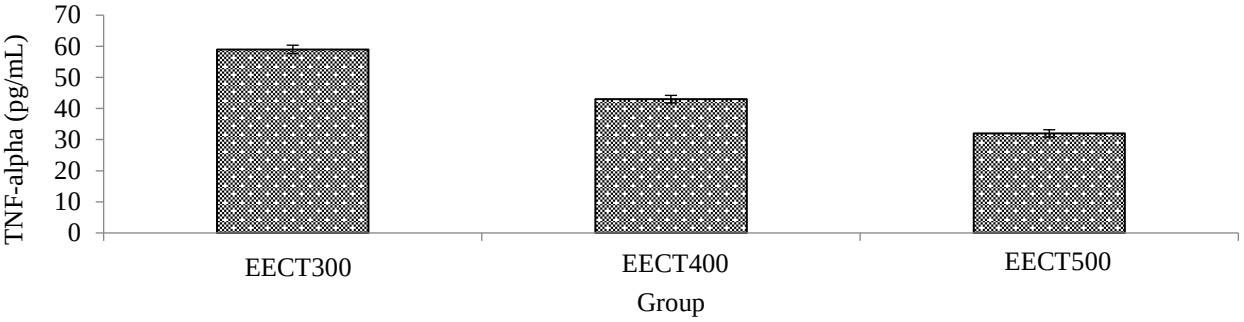

Figure 8: Bar chart showing the dose-dependent reduction in TNF-α levels across EECT treatment groups.

The EECT500 group exhibited the lowest TNF-α concentration, while the EECT300 group showed the highest among the EECT-treated groups, reflecting a consistent downward trend with increasing dose.
In parallel, *T. versicolor* is rich in polysaccharopeptides like PSK and PSP, which help reestablish immune homeostasis by limiting cytokine overproduction and enhancing regulatory T-cell activity[42]. The observed inverse correlation reflects this dual mechanism, simultaneous suppression of inflammatory mediators and restoration of immune balance. These molecular-level interactions help explain the consistent reduction in TNF-α and align with broader therapeutic outcomes, such as body weight recovery and tumor volume reduction, suggesting systemic benefits beyond localized tumor control[43]. Previous studies have demonstrated that *C. militaris* extracts downregulate TNF-α in chemically induced cancer models, including those involving DMBA[44], while *T. versicolor* enhances immunoregulation and dampens pro-inflammatory cascades in tumor-bearing hosts[45]. The combination in EECT thus represents a synergistic anti-inflammatory strategy, supported by correlation metrics that quantitatively affirm its biological effect. Notably, multi-fungal formulations have consistently shown improved efficacy in reducing TNF-α levels when compared to individual extracts, reinforcing the rationale for combination therapy[17]. The consistent inverse correlation observed in this study substantiates the anti-inflammatory efficacy of EECT, positioning it as a viable candidate for mitigating the immunopathological environment characteristic of DMBA-induced mammary carcinogenesis. This correlation-driven evidence elevates the mechanistic credibility of EECT as an integrative therapy, where TNF-α suppression serves as a biomarker for systemic improvement. The data affirm that phytochemical synergy in EECT yields quantifiable, dose-responsive immunological benefits relevant to breast cancer management.

Summary of correlation coefficients
Table 5 provides a comprehensive overview of the dose–response correlations between EECT administration and key biological parameters in DMBA-induced breast cancer mice. A strong positive correlation was observed between EECT dose and body weight gain, suggesting that higher doses of EECT contributed to improved physiological status and recovery. Similarly, moderate positive correlations with tumor volume and tumor weight indicate that EECT exerts dose-dependent effects on tumor mass dynamics, reflecting complex interactions between cytotoxic, apoptotic, and immune mechanisms.

Table 5: Dose-response summary of EECT on biological parameters in breast cancer mice.

Parameter	Mean ± SD	r	p-value	Correlation
Body weight gain (%)	13.25 ± 2.05	0.976	0.024	↑↑
Tumor volume (cm^3)	0.44 ± 0.07	-0.427	0.0419	↓
Tumor weight (g)	0.54 ± 0.09	-0.427	0.0419	↓
TNF-alpha (pg/mL)	45.02 ± 1.35	-0.994	0.0478	↓↓

Note: ↑↑ indicates strong positive correlation ($r > 0.7$), ↓ indicates moderate negative correlation ($r \approx -0.3$ to -0.5), ↓↓ indicates strong negative correlation ($r < -0.7$).

Importantly, a strong negative correlation was found between EECT dose and TNF-α levels, highlighting a consistent anti-inflammatory response that becomes more pronounced with increasing dose. These correlation trends validate the mechanistic rationale for EECT as a dual-action therapeutic: one that simultaneously targets tumor growth, inflammation, and systemic recovery. The inverse relationship between EECT and TNF-α aligns with the extract's immunomodulatory capacity, particularly in its ability to suppress pro-inflammatory cytokines associated with cancer-related inflammation and progression. Overall, the results in Table 5 emphasize a coherent, statistically supported dose–response pattern, wherein increased EECT dosing is associated with beneficial biological outcomes, including reduced

inflammation, mitigation of tumor burden, and enhancement of host condition. These multidimensional correlations reinforce the scientific rationale for using combined medicinal mushroom extracts, *Cordyceps militaris* and *Trametes versicolor*, to target both systemic and tumor-specific mechanisms in breast cancer intervention. The extract exhibited consistent positive correlations with physiological improvement and tumor suppression, alongside a negative correlation with TNF-α levels, indicating anti-inflammatory activity. These patterns reflect the integrated biological actions of EECT on multiple pathophysiological pathways. The combination of *C. militaris* and *T. versicolor* was intentionally selected to leverage their distinct yet complementary bioactivities. *C. militaris* is known for its direct antitumor effects through compounds such as cordycepin and flavonoids, which induce apoptosis, inhibit angiogenesis, and modulate oxidative stress. In contrast, *T. versicolor* contributes potent immunoregulatory activity via polysaccharopeptides (e.g., PSK, PSP), enhancing macrophage and NK cell function and restoring immune homeostasis. The strength of this formulation lies in its capacity to produce quantifiable, dose-dependent improvements across a spectrum of therapeutic targets, from tumor reduction to immune normalization. While single-species extracts may act through isolated pathways, the dual-fungus strategy broadens the therapeutic scope by engaging both cytotoxic and immunotherapeutic processes. This approach aligns with contemporary models of integrative oncology, which emphasize multi-target, low-toxicity interventions grounded in measurable biological responses. Previous studies have shown that combined mushroom extracts outperform individual treatments in suppressing tumor growth and downregulating pro-inflammatory cytokines such as TNF-α in DMBA-induced breast cancer models. The consistent dose–response correlations observed in this study serve as quantitative evidence of synergy, further validating EECT as a promising candidate for complementary breast cancer therapy informed by correlation-driven mechanistic insights.

Conclusion

The ethanol extract combination of *Cordyceps militaris* and *Trametes versicolor* (EECT) exhibited significant dose-dependent anticancer and anti-inflammatory effects in a DMBA-induced murine breast cancer model. Statistically robust correlations were observed between EECT dosage and therapeutic outcomes, including increased body weight gain, reduced tumor volume and weight, and decreased serum TNF-α levels. These findings support the synergistic interaction between the two medicinal mushrooms, which act through complementary mechanisms involving immune modulation, oxidative stress reduction, and tumor suppression. The correlation-driven evidence presented in this study highlights the potential of EECT as a promising integrative therapeutic strategy for breast cancer management.

Conflict of interest

The authors declare no conflicts 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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