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Metabolic profiling and systems biology analysis of *Flammulina filiformis* Fv19 extract: an integrated computational and experimental approach for dual anti-cancer and immunomodulatory therapy

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Abstract

Background The increasing demand for multi-target cancer therapies has prompted investigation into the anticancer and immunomodulatory potential of the hot water extract of *Flammulina filiformis* Fv19, utilizing in vitro cytotoxicity assays, in vivo murine models, and molecular docking analyses.

Results The hot water extraction of mushroom mycelia yielded $11.4 \pm 1.2\%$ (w/w). Gas chromatography-mass spectrometry (GC-MS) profiling identified six major metabolites, with the most abundant being cholestan3ol, 2methylene (3a, 5a) (37.47%); spirost8en11 one, 3hydroxy (23.24%); and 1Hcycloprop[e]azulen7ol, decahydro1,1,7 trimethyl4methylene (12.58%). The extract demonstrated potent inhibition of MCF-7 ($IC_{50} = 5 \mu\text{g/ml}$) and Hep-G2 ($IC_{50} = 4 \mu\text{g/ml}$) cancer cell lines. In vivo evaluation involved male albino mice receiving oral administration of the extract at a dosage of 200 mg/kg/day for 10 days, employed in both preventive and treatment protocols against the Ehrlich solid tumor model. In vivo oral administration significantly suppressed Ehrlich carcinoma tumor growth with a 99.79% inhibition ratio, achieving complete tumor regression in 66.7% of mice. Additionally, the extract enhanced immune responses by increasing macrophage count by 166%, TNF- α production by 284%, NO release by 71%, and H_2O_2 levels by 55% compared to the tumor-bearing group, while reducing IL-17 production by splenocytes by 27%, indicating immunomodulatory effects. Molecular docking revealed strong interactions with oncogenic and immune-regulatory proteins, including Bcl-2 (-5.015 kcal/mol), EGFR (-4.567 kcal/mol), COX-2 (-4.581 kcal/mol), and iNOS (-5.855 kcal/mol).

Conclusions These findings highlight *F. filiformis* Fv19 extract as a promising natural anticancer and immunomodulatory agent for multi-target cancer therapy.

Clinical trial number Not applicable.

Keywords *Flammulina filiformis*, Anticancer, Immunomodulation, Macrophages, Cytokines, Molecular docking

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Introduction

The development of multi-target medicines is an important area of research because conventional and targeted cancer treatments frequently result in drug resistance and/or undesirable side effects. It has previously been demonstrated that the isolated individual chemicals and mushroom extracts from medicinal mushrooms are strong immunomodulators and non-toxic multi-target inhibitors of particular oncogenic pathways. But up until now, there hasn't been much research on the anticancer effects of combinations of several species' extracts [1].

Flammulina filiformis, commonly known as the enoki mushroom, represents a versatile biofactory capable of synthesizing a diverse array of bioactive secondary metabolites. Recent scientific interest has focused on its potential as a source of anticancer and immunomodulatory agents [2]. The fruiting body, cultured mycelium, and cultured broth of mushrooms have yielded several cellular components and secondary metabolites that have been discovered and isolated according to Berger et al. [3]. Immune-regulating chemical substances with a wide range of molecular weights and structures have been found in over 50 identified mushrooms. According to Zhao et al. [4], immunomodulators found in mushrooms have activating properties for the innate and adaptive immune systems.

The immune system functions so well because the aim of therapies is to boost a patient's own IgG, IgA, IgM, IgD, IgE, and macrophages to fight neoplastic cells. The nutritional and therapeutic qualities of mushrooms have been appreciated since the beginning of human civilization. Potentially therapeutic substances for the prevention of cancer include lentinan, grifolan, PSK (polysaccharide-protein complex), and PSP (polysaccharide-peptide complex) [5].

They have anticancer effects by inducing an immunological response in the host and preventing oncogenesis by directly affecting tumor metastasis [5]. Lentinan, for instance, is used in conjunction with chemotherapy and radiation therapy or as an adjuvant cancer immunotherapy [6]. Previous studies reported that mushroom polysaccharides usually enhance in vivo and in vitro immune responses [7].

Due to its biological activity, one of the most widely consumed edible mushrooms, *F. filiformis*, has drawn significant interest in the fields of biochemistry and pharmacology [8]. It is a long, thin, white fungus that is well-known for its flavor and ability to both prevent and treat liver disorders and stomach ulcers [9].

In addition, this type of mushroom reportedly has immunomodulatory and antitumor effects [10]. The antitumor activities are thought to be caused by stimulating the cell-mediated immune response, while the immunomodulatory activities are thought to be caused

by secretory products from activated macrophages, neutrophils, natural killer cells, and cytotoxic T cells, such as tumor necrosis factor, reactive nitrogen and oxygen intermediates, and cytokines [11].

T helper 17 cells and various leukocytes, including natural killer (NK) cells, neutrophils, and lymphoid tissue inducer-like (LTi-like) cells, are the main producers of the inflammatory cytokine IL-17 [12]. According to Murugaiyan et al. [13], inflammation seems to be essential for both tumor cell eradication and spread. Certain studies have revealed that IL-17 encourages the development of malignancies by aiding in angiogenesis, which leads to the creation of tumor cells [14]. However, according to other research, IL-17 stimulates a cytotoxic T cell response against tumors, which results in the regression of the tumor [15].

The initial line of defense against several infections and malignancies is the macrophage. When they get activated, they release several mediators like nitric oxide (NO), hydrogen peroxide (H_2O_2), and cytokines such as IL-1, IL-6, IL-10, and tumor necrosis factor alpha (TNF- α) [16]. Furthermore, many studies have documented conflicting results regarding IL-17's role as an interface mediator between innate and adaptive immune responses [17, 18]. In order to better understand the anticancer and immunomodulatory properties of the hot water extract derived from the mycelium of *F. filiformis* Fv 19, this study focused on the roles played by macrophages and the generation of IL-17. Furthermore, this study employs bioinformatics tools to elucidate molecular mechanisms and identify potent active compounds, thereby establishing a foundation for future drug discovery and development.

Material and methods

Fungal strain

Flammulina filiformis Fv19 (accession number PX795814) was generously obtained from Fujian Agriculture University in China. The strain was stored in a refrigerator at 4 °C after being incubated on potato dextrose agar (PDA) slants for 7 days at 25 °C [19].

Culture media and culture conditions

For seven to eight days at 25 °C, the fungal strain was subcultured on PDA medium (potato 200g, dextrose 20g, agar 15g, distilled water 1L). Cultivation in flask culture was carried out using a 250 ml flask containing 50 ml of mushroom fermentation media (MFM). Within each flask, 2 agar plugs measuring 7 mm² from the agar plate culture were added per 1 L. The MFM solution contained 35 g of glucose, 5 g of peptone, 5 g of yeast extract, 1 g of $KH_2PO_4 \cdot H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.05 g of vitamin B₁ [20].

Preparation of crude hot water extract

With a few adjustments, the extraction was conducted utilizing the technique outlined by Mancera-López et al. [21]. Briefly, the strain's mycelium was dried at 40 °C before being extracted. 5.0 g of grounded dried mycelium was suspended in distilled water at 60 °C and repeated three times. This hot water extraction method was selected as an eco-friendly, solvent-free approach that is highly scalable for industrial applications. It is specifically designed to maximize the recovery of water-soluble polar metabolites and macromolecules while ensuring safety and minimizing environmental impact [22]. Then, the sample was filtered, and centrifugation was applied. After centrifugation at 6000 rpm for 10 minutes, the supernatant was concentrated via evaporation under decreased pressure, and the extract was preserved at 4 °C. The extract yield % was calculated according to the following formula:

$$\text{Extract yield percentage} = \frac{\text{extract weight}}{\text{initial mycelial weight}} \times 100$$

Metabolic profile analysis using gas chromatography–mass spectrometry (GC–MS)

The GC–MS study was performed with the TRACE GC Ultra Gas Chromatograph from Thermal Scientific Corp., USA. The system comprised an ISQ Single Quadrupole Mass Spectrometer and a TR-5 MS column with a length of 30 m, an inner diameter of 0.32 mm, and a film thickness of 0.25 µm. Helium served as the carrier gas with a split ratio of 1:10 and a flow rate of 1.0 mL/min. Following a duration of one minute at 60 °C, the temperature was elevated to 240 °C over the course of one minute at a pace of 4.0 °C per minute. A temperature of 210 °C was sustained for both the injector and the detector. One liter of the mixture was volumetrically diluted with a hexane ratio of 1:10. Mass spectra ranging from 40 to 450 m/z were generated using electron ionization (EI) at 70 electron volts (eV). Metabolites were mostly identified utilizing the Wiley spectral library collection, the NSIT library database, retention indices (compared to n-alkanes C8–C22), mass spectra from legitimate standards, and AMDIS software (<http://www.amdis.net>, accessed on October 20, 2024).

Fourier-transform infrared (FTIR) analysis of the mycelial extract from *F. filiformis* Fv19

The FTIR absorption spectra were analyzed using FTIR spectroscopy (PerkinElmer spectrometer, Akron, OH, USA) to determine functional groups of the metabolites obtained from the hot water extract. A small quantity of extract was ground with potassium bromide to create a

compacted pellet. The experiment was conducted within the spectral range of 400 to 4000 nm.

In vitro cytotoxicity

Cells and culture conditions

This study utilized the MCF-7 breast cancer cell line, HCT116 colon cancer cell line, and Hep-G2 liver cancer cell line. They were obtained from the American Type Culture Collection and cryopreserved in liquid nitrogen at –180 °C. Serial subculturing was employed to maintain the tumor cell line at the National Cancer Institute in Cairo, Egypt. The cell lines were cultured as a monolayer in RPMI-1640 media enriched with 2 mg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum (FBS). The cell lines were cultured in a high-humidity environment at 37 °C within a water-jacketed incubator (Revco, GS Laboratory Equipment, RCO 3000 TVBB, USA) containing 5% CO₂. It was consistently transferred to different cultural mediums to sustain its exponential expansion phase. The use of a laminar flow cabinet (Micro Flow Laminar Flow Cabinet, MDH Limited, Hampshire SP 105AA, U.K.) facilitated the preservation of sterile conditions.

MTT cell viability assay

For 24 hours at 37 °C in a humidified 5% CO₂ environment, the concentrations of the tested extract (2.5, 5, 10, and 20 µg/ml) were conducted in a flat-bottomed 96-well microtiter plate using 0.5 × 10⁵ cancer cell lines in each well and then the medium was eliminated. Subsequently, 30 µl of MTT solution in serum-free medium was added, and the mixture was incubated for a further four hours [23].

Following two 100 µl PBS washes in each well, 50 µl of DMSO was added to each well to solubilize the MTT crystals, and the plate was then shackled at room temperature for 20 minutes. The absorbance at 540 nm was then measured photometrically using a microplate ELISA reader. The control cells received only the vehicle. For every concentration, three wells were used (triplicate wells were constructed for each separate dosage). The mean was determined through calculation. Following the creation of the dose-response graph using GraphPad Prism 7, the 50% inhibitory concentration (IC₅₀) was ascertained. To calculate the survival fraction percentage, the following formula was utilized

$$\text{survival percentage} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

In vivo experiment

Experimental animals

The animal protocols adhered to Helwan University's standards for the care and use of laboratory animals. The

research ethics number (HU-IACUC/B/HE0619-01) was assigned to these rules following their approval by the university's Animal Ethics Committee.

Sixty-five mature male Swiss albino mice, each weighing 20 grams, were utilized in this study. Mice were procured from the animal house of the National Cancer Institute (NCI) at Cairo University, Egypt. The animals were kept in appropriate cages with regular diets and kept in normal environmental circumstances, including standard temperature, humidity, and light and dark throughout the day.

Ehrlich solid tumor model establishment

The Ehrlich ascites carcinoma (EAC) cells were obtained from the National Cancer Institute (Cairo University, Egypt) and maintained by weekly intraperitoneal passage in donor mice. For solid tumor induction, EAC cells were harvested from donor mice on day 7 post-inoculation, washed twice with sterile phosphate-buffered saline (PBS, pH 7.4), and viability was assessed by Trypan blue exclusion (>95% viability required for use). Viable EAC cells were suspended in sterile PBS at a concentration of 1×10^6 cells/ml.

Solid tumors were induced in the experimental groups by a single intramuscular injection of 0.1 ml of the cell suspension (containing 1×10^6 viable EAC cells) into the right hind thigh muscle of each mouse, following the method of El Behery et al. [24]. This intramuscular route consistently produces localized solid tumors, as opposed to the ascites model (intraperitoneal injection) which produces disseminated peritoneal disease with rapid lethality (typically 10–14 days). The solid Ehrlich carcinoma model is widely used for evaluating antitumor agents because it allows prolonged observation of tumor growth kinetics (4–8 weeks) and better mimics the characteristics of solid human tumors, including the development of a tumor microenvironment with stromal components and angiogenesis [25].

Experimental design and treatment protocol

Mice were randomly allocated to five experimental groups ($n = 10$ – 15 each group) as described below:

Group 1 (Negative control): Ten healthy mice ($n = 10$) were not inoculated with tumors and were given sterile physiological saline (0.2 ml/mouse/day, orally) for a duration of ten consecutive days.

Group 2 (Positive control/Tumor-bearing): Fifteen mice ($n = 15$) were inoculated with EAC cells (1×10^6 cells/mouse, intramuscularly) on day 0 and administered sterile physiological saline (0.2 ml/mouse/day, orally) for ten consecutive days starting 24 hours post-tumor inoculation.

Group 3 (Extract only): Healthy mice ($n = 10$) without any tumor inoculation received *F. filiformis* Fv19 extract

(200 mg/kg body weight/day, dissolved in sterile saline, delivered orally via gavage in a volume of 0.2 ml/mouse). Based on earlier research on mushroom extracts that demonstrated optimal effectiveness without toxicity at this dosage, the 200 mg/kg dose was chosen [26, 27] for a duration of 10 consecutive days. This group was designated to assess the immunomodulatory effect of the extract in the absence of a tumor.

Group 4 (Protective/Prophylactic group): Mice ($n = 15$) were administered *F. filiformis* Fv19 extract orally at a dosage of 200 mg/kg/day (0.2 ml/mouse) for 10 consecutive days (days -10 to -1) preceding tumor injection on day 0. This protocol was established to assess the preventative efficacy of the extract.

Group 5 (Therapeutic group): Mice ($n = 15$) were injected with EAC cells (1×10^6 cells/mouse, intramuscular) on day 0, and 24 hours later (day 1) commenced oral administration of *F. filiformis* Fv19 extract (200 mg/kg/day, 0.2 ml/mouse) for a duration of 10 consecutive days (days 1–10). This protocol was created to assess the therapy efficacy against established tumors.

Tumor development monitoring

Tumor growth was observed for 6 weeks following inoculation (days 0–42), as the solid Ehrlich model allows for prolonged monitoring without a rapid mortality associated with the ascites model. Tumor dimensions were assessed weekly using digital calipers, and tumor volume (V) was computed using the formula:

$$V = (\pi/6)(d)^2(D) \text{ [28]}$$

Where D and d were the small and long axes.

Spleen index

The spleen index was calculated based on the weight of the spleens of mice that survived for three months. At the end of the study period, mice were euthanized by cervical dislocation following light anesthesia with isoflurane inhalation, in accordance with the guidelines of the Helwan University Animal Ethics Committee (approval number HU-IACUC/B/HE0619-01). This method was selected as it ensures rapid and humane sacrifice, minimizes animal suffering, and avoids chemical residues that could interfere with immunological or histopathological analyses. The spleens were then removed and weighed. The spleen index was computed using the formula provided by Shah et al. [29]:

Spleen index (mg/g) = weight of spleen (mg)/body weight (g).

Peritoneal macrophages preparation

Peritoneal macrophages were isolated at the end of the experimental course according to Xu et al. [30]. In short, peritoneal macrophages were prepared by inoculating

cold Phosphate Buffered Saline (PBS) into the abdominal cavity of mice that had been anesthetized and submerged in 70% ethyl alcohol. Following a gentle massage of the abdomen, the peritoneal fluid was collected and spun in a cooling centrifuge (HERMLE, USA) at 2000 rpm for 10 minutes at 4 °C. The cells were resuspended in complete Dulbecco's modified Eagle's medium (DMEM, Lonza, Belgium) at a final concentration of 2×10^5 cells per well and cultivated in a 96-well plate. Various quantities of *F. filiformis* Fv19 extract (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 µg/ml, 100 µg/ml, and 10 µg/ml) were used to cultivate the cells.

Lipopolysaccharide (LPS, Sigma Aldrich) served as a positive control (10 µg/ml) according to Sawoo et al. [16]. Macrophages were incubated at 37 °C and 5% CO₂ for 24 h. Aliquots of 100 µl were collected from the supernatant of the cultured macrophages for measuring NO, H₂O₂ and TNF-α production.

Measurement of nitric oxide (NO)

The nitric oxide level was evaluated using Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) [31]. Subsequent to 10 minutes at ambient temperature, at 540 nm, the OD absorbance values were determined using an automated enzyme immunoassay reader manufactured by Bio-Tec in Germany. By comparing the average absorbance value of a duplicate of each experimental sample to the nitrite Standard reference curve, the concentrations of the samples were ascertained.

Measurement of hydrogen peroxide (H₂O₂)

Hydrogen peroxide was measured using mouse H₂O₂ ELISA kit (Biodiagnostic, Egypt) according to the manufacturer's instructions.

Measurement of tumor necrosis factor-α (TNF-α)

TNF-α was measured using Quantikine mouse TNF-α ELISA kit (Boster Immunoleader, USA) in accordance with the manufacturer's guidelines.

Preparation of murine splenocytes

Mice spleens were dissected individually, and single spleen cell suspensions were prepared as described by Bagadood et al. [32]. The spleen was squeezed in ice-cold RPMI medium. The suspension was gathered and spun in a cooling centrifuge (HERMLE, USA) at 2000 rpm for 5 minutes at 4 °C. The pellet containing erythrocytes and splenocytes was collected after the top layers were eliminated. The ACK lysis buffer (8.024 mg NH₄Cl, 1.001 mg KHCO₃, and 3.722 mg EDTA Na₂0.2H₂O) were added to 1 L of H₂O) was used to lyse the erythrocytes. The mixture was then slowly inverted for five minutes, followed by washing with RPMI medium and centrifugation at

2000 rpm for five minutes at 4 °C. Following two rounds of washing, the cell viability of each spleen was evaluated using the Trypan blue exclusion test. Cells were resuspended in complete RPMI medium (200 µl) (Lonza, Belgium) containing 10% FBS and were finally planted at a density of 2×10^5 cells/well in 96-well plates. Various quantities of *F. filiformis* Fv19 IPS extract (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 µg/ml, 100 µg/ml, and 10 µg/ml) were used to cultivate the cells. As per Bayati et al. [33], phytohemagglutinin (PHA, Biochrome, Egypt) was utilized as a positive control (10 µg/ml). For 24 hours, splenocytes were cultured at 37 °C with 5% CO₂. To quantify the synthesis of interleukin-17, 100 µl aliquots were taken from the cultivated spleen cells' supernatant.

Measurement of interleukin-17 (IL-17)

IL-17 was measured using Quantikine mouse IL-17 ELISA kit (Boster Immunoleader, USA) in compliance with the manufacturer's guidelines.

Histopathological examination

Mice in different groups were autopsied, and samples were taken from their lymph nodes and spleen. After that, these tissues were kept for a whole day in a 10% formalin saline solution. The individuals were dehydrated utilizing 100% ethyl, methyl, and ethyl alcohol dilutions after being cleaned with tap water. Specimens were immersed in paraffin for a whole day at 56 °C in a hot air oven following a xylene wash. Using a sledge microtome, tissue sections with a thickness of 4 microns were cut from blocks of bee paraffin wax. After deparaffinization and staining with hematoxylin and eosin, the resulting tissue sections were placed on glass slides and prepared for routine examination with a light electric microscope [34].

Molecular docking analysis of *F. filiformis* Fv19 extract metabolites with proteins involved in anticancer and immunomodulatory pathways

Molecular docking was performed using Maestro 11.8 (Schrödinger, LLC) to assess interactions between *F. filiformis* Fv19 extract metabolites and protein targets—4LVT (Bcl-2), 1M17 (EGFR), 5F19 (COX-2), 1M9T (iNOS), and 3FXI (TLR4)—obtained from the Protein Data Bank (<http://www.rcsb.org/>). Proteins were prepared with the Protein Preparation Wizard by optimizing bond orders, hydrogens, and side chains with Prime, removing distant water molecules, and minimizing with the OPLS3e force field (RMSD cutoff: 0.30 Å) after pH 7.0 H-bond optimization using PROPKA; ligands were removed but retained for validation. Metabolites—Stigmasta-5,22-dien-3-ol, acetate, (3á)-; 1H Cycloprop[e] azulene-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7á,7aá,7bà)]-; Cholestan-3-ol,

2-methylene-, (3 α ,5 α)-; and 9,12,15-Octadecatrienoic acid—were sourced from the PubChem database as MOL files or constructed, processed with the Ligand Preparation Wizard (OPLS3e, pH 7.0 $\pm$ 2.0), and docked using Glide in Standard Precision mode to 20 $\times$ 20 $\times$ 20 Å grids centered on active sites (e.g., BH3 groove for 4LVT, ATP site for 1M17). Five poses per flexible ligand were generated against rigid receptors, and top poses were analyzed for docking scores (kcal/mol) and hydrogen bonds, visualized in 2D and 3D.

Gene interaction network analysis of bioactive metabolites from *F. filiformis*: insights into anticancer and immune modulation pathways

The gene interaction networks related to anticancer activity and immune modulation were analyzed using GeneMANIA. The study focused on identifying key genetic interactions, pathways, and co-expression relationships for genes involved in apoptosis, cancer progression, inflammation, and immune responses. For anticancer activity, genes related to apoptosis (BCL2, BAX, CASP3, TP53), cell proliferation (EGFR, KRAS, PIK3CA, MYC), and inflammation-induced tumorigenesis (PTGS2, NF- κ B1, IL6, TNF) were analyzed. For immune modulation, genes involved in nitric oxide signaling (NOS2, GCH1), immune signaling (IFNG, STAT1), and inflammation regulation (TLR4, MYD88, IRAK4, NFKBIA) were investigated. Gene interaction networks were generated using GeneMANIA, which integrates physical interactions, co-expression, predicted interactions, pathways,

genetic interactions, co-localization, and shared protein domains. The networks were visualized to understand the biological relationships between these genes and their potential regulation by bioactive metabolites from *F. filiformis*.

Statistical analysis

Using homogeneous subsets (Duncuna) and one-way analysis of variance (ANOVA) in addition to the Statistical Package for the Social Science (SPSS) version 8, the data was statistically analyzed at a confidence level of 5%. The means of the treatments were compared using Duncan's multiple range tests.

Results

GC/MS profiling

The hot water extraction of *F. filiformis* Fv19 mycelia yielded 11.4 $\pm$ 1.2% (w/w). The extract was chemically analyzed utilizing GC/MS equipment (Fig. 1). By contrasting the retention time and mass spectra with data from the Wiley spectral library, the NSIT library database, or recognized data standards, six metabolites were discovered in the hot water extract. The most prevalent metabolite was cholestan-3-ol, 2-methylene-, (3 α ,5 α)- (37.47%), followed by Spirost-8-en-11-one, 3-hydroxy-, (3 α ,5 α ,14 α ,20 α ,22 α ,25R) at a ratio of 23.24%. 1H cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methyle ne-, [1ar-(1a α ,4a α ,7 α ,7a α ,7b α)]-, 9,12,15-octadecatrienoic acid, and methyl-9,9,10,10-D4-octadecanoate were also significant metabolites.

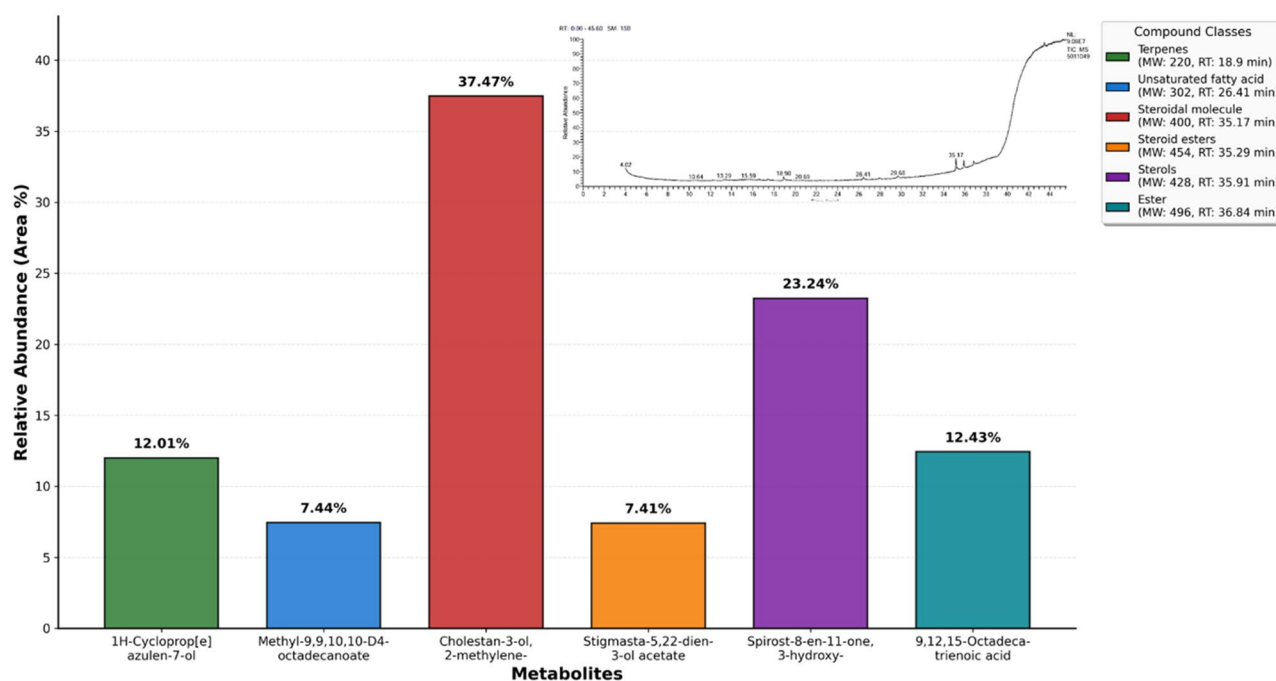


Fig. 1 Metabolites of *F. filiformis* extract determined using the GC-MS analysis

FTIR analysis

The FTIR spectrum of the fungal extract revealed a broad and intense absorption band centered between 3300 and 3400 cm^{-1} , characteristic of O–H stretching vibrations from hydroxyl groups and N–H stretching from amide groups, indicating the presence of polysaccharides and proteins. Two sharp peaks at approximately 2920 and 2850 cm^{-1} were attributed to asymmetric and symmetric C–H stretching of aliphatic methyl and methylene groups, suggesting the presence of lipids or fatty acid chains. A strong absorption band at 1630–1650 cm^{-1} corresponded to C=O stretching (amide I), while a weaker band near 1550 cm^{-1} was assigned to N–H bending (amide II), further confirming proteinaceous components. Additional peaks in the region of 1400–1450 cm^{-1} were due to C–H bending vibrations, and a broad, intense band between 1000 and 1100 cm^{-1} was attributed to C–O stretching, consistent with polysaccharides and glycosidic linkages. Collectively, these spectral features indicate that the fungal extract is a complex mixture rich in polysaccharides, proteins, and lipids (Supplementary Fig. S1).

In vitro cytotoxic activity of the hot water extract of *F. filiformis*

The cytotoxic effects of hot water extract of *F. filiformis* Fv19 mycelia on MCF-7, Hep-G2, and HCT116 cell lines were investigated at varying concentrations (2.5, 5, 10, and 20 $\mu\text{g}/\text{ml}$). At all concentrations, the extract

demonstrated potent inhibition of MCF-7 and Hep-G2 cell growth. A dose–response relationship was observed between the concentration of mycelial extract and the inhibition of cancer cell proliferation. *F. filiformis* Fv19 significantly suppressed the proliferation of MCF-7 and Hep-G2 cells, exhibiting IC_{50} values of 5 $\mu\text{g}/\text{ml}$ and 4 $\mu\text{g}/\text{ml}$, respectively. An interesting observation was noted in the Hep-G2 cell line, where treatment with 10 $\mu\text{g}/\text{ml}$ extract resulted in slightly higher viability ($89.3 \pm 4.2\%$) compared to the 2.5 $\mu\text{g}/\text{ml}$ concentration ($82.1 \pm 3.8\%$), although both values remained significantly lower than untreated controls (100%, $p < 0.01$). Also, no cytotoxicity was observed when exposing the HCT116 colon cell line with varying doses of *F. filiformis* mycelial extract (Supplementary Fig. S2).

In vivo experiment

Tumor inhibition

A significant anticancer efficacy of the *F. filiformis* Fv19 extract against Ehrlich solid tumor in a murine model was observed. As illustrated in Fig. 2, the average tumor volume attained 0.9 mm^3 in the saline-treated control group six weeks post-tumor implantation. A notable suppression of tumor growth was observed in the mice following oral administration of 200 mg/kg *F. filiformis* Fv19 extract for 10 days prior to tumor implantation, resulting in an inhibition ratio of 99.34% (mean tumor volume was 0.006 mm^3), with 9 out of 15 mice (60%) exhibiting complete tumor regression. The group receiving oral

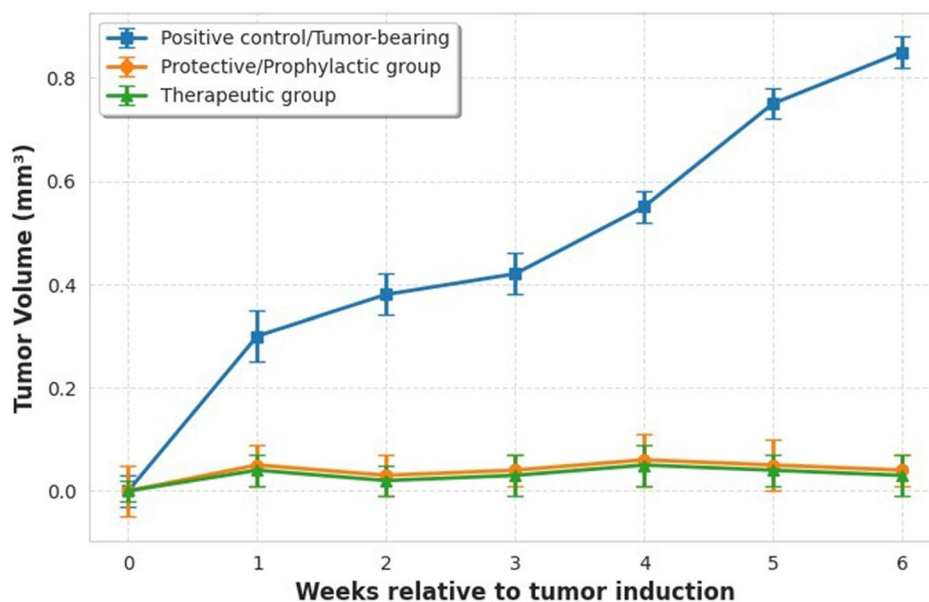


Fig. 2 Impact of consumption of *F. filiformis* Fv19 extract on tumor volume in mice with a Ehrlich solid tumors. The tumor volume was assessed weekly for six weeks following inoculation. The x-axis denotes weeks relative to tumor induction, with week 0 being the day of tumor cell injection. The protective group received the extract for 10 days before inoculation (weeks –1 to 0), whereas the therapeutic group received the extract for 10 days starting 24 hours after inoculation (weeks 0–1). Data are expressed as mean $\pm$ SEM ($n = 10$ –15 mice/group). ** $p < 0.01$ in comparison to the positive control group

injections of *F. filiformis* Fv19 extract post-tumor implantation exhibited greater tumor growth inhibition compared to the group administered with the extract before tumor implantation. The inhibition ratio was 99.79% (mean tumor volume was 0.002 mm³), with 10 out of 15 animals (66.7%) exhibiting total tumor regression in this group.

Immunomodulatory efficiency

Measurements of innate immune mediators secreted by macrophages, such as TNF- α , nitric oxide, and hydrogen peroxide, as well as measurements of interleukin-17 secreted by splenocytes as a mediator for cellular immunity, were used to evaluate the immune response of mice with Ehrlich tumors and mice treated with *F. filiformis* Fv19 extract.

Analysis of spleen index

The treated group with the extract (200 mg/kg/day) had a substantial enhancement in spleen index by a percent change of 24 and 43% as compared with negative and positive control groups, respectively. Mice treated with the same dose of the extract after “treatment group” or before “protective group” tumor induction showed a highly significant increase in spleen index by a percent change of 133% and 60%, respectively, as compared to positive control mice (Fig. 3).

Effect of *F. filiformis* Fv19 extract on the count of mice splenocytes and intraperitoneal macrophage

In comparison to the negative and positive control groups, the extract (200 mg/kg/day) treated group demonstrated a considerable rise in macrophage count, with a percent change of 109 and 166%, respectively. In comparison to the positive control mouse group, the macrophage count rose considerably after receiving 200 mg/kg/day of *F. filiformis* Fv19 extract administered for a duration of 10 days prior to and during tumor development. Over a ten-day period, mice administered 200 mg/kg/day of *F. filiformis* Fv19 extract exhibited a non-significant increase in splenocyte count relative to the negative control, although they demonstrated a significant increase in splenocyte count compared to the positive control group. Moreover, in comparison to the positive control, there was a significant increase in the protection and treatment groups, with percentage changes of 12 and 20%, respectively (Fig. 4).

Analysis of macrophage and splenocytes mediators secreted in vitro in response to different concentrations of *F. filiformis* Fv19 extract

TNF- α production by mice macrophages

In vitro stimulation of macrophages isolated from all experimental groups with different concentrations (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 μ g/ml, 100 μ g/ml and 10 μ g/ml) of the extract of *F. filiformis* Fv19 for 24 h culminated in a significant increase in TNF- α production.

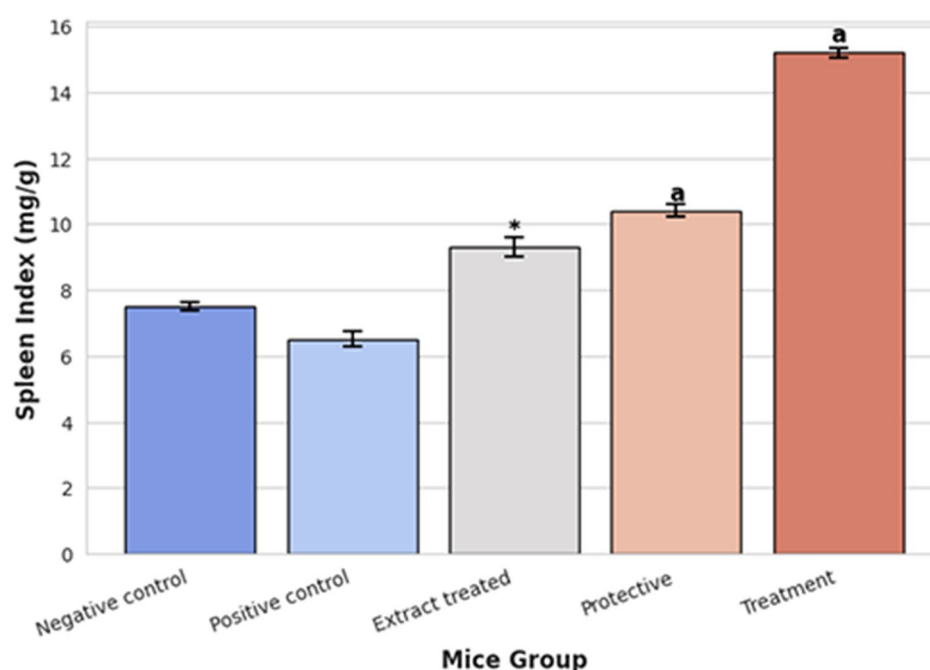


Fig. 3 Spleen index of the experimental animal groups. Values are represented as means $\pm$ SD of 6 mice/group. * Statistically significant in comparison to the negative control group at $p < 0.05$. ^a Statistically significant in comparison to the positive control group at $p < 0.05$

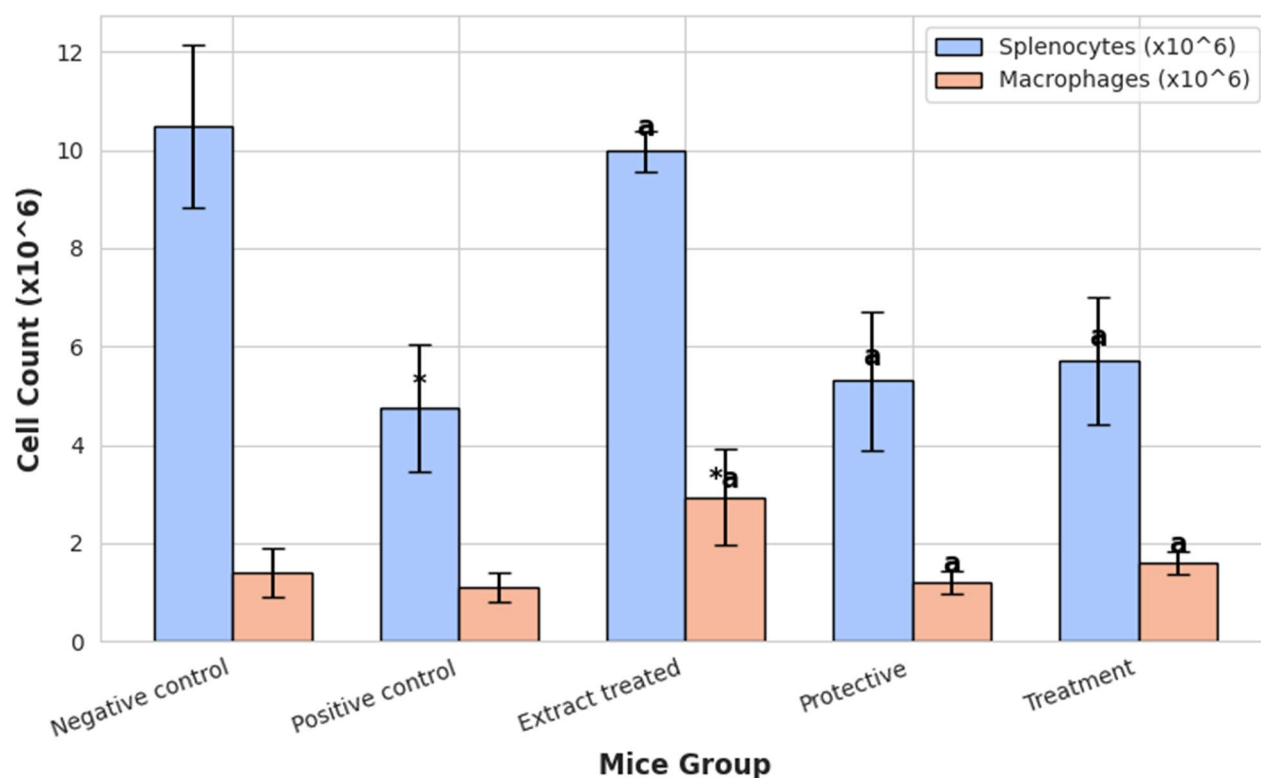


Fig. 4 Splenocytes and intraperitoneal macrophage counts of the experimental mice groups. Values are represented as means $\pm$ SD of 6 mice/group. * Statistically significant in relation to the negative control group at $p < 0.05$. ^a Statistically significant in relation to the positive control group at $p < 0.05$

The animal group treated with *E. filiformis* Fv19 extract only showed a significant increase ($p < 0.01$) in TNF- α production by a percent change of 284 and 177% at a concentration of 10 μ g/ml as in comparison to that of negative and positive control mice groups, respectively. Also, protective and therapeutic groups showed a significant increase in TNF- α production level by a percent change of 55 and 34%, respectively, at concentration of 5 mg/ml and 1 mg/ml respectively in comparison to the positive control mice group (Supplementary Fig. S3A).

Hydrogen peroxide production by mice macrophage

In vitro stimulation of macrophages isolated from all experimental mice groups with different concentrations (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 μ g/ml, 100 μ g/ml and 10 μ g/ml) of the *E. filiformis* Fv19 extract for 24 h resulted in a significant increase in H₂O₂ production. H₂O₂ production showed a significant increase in mice treated with extract only followed by protective and treatment groups as well. Mice administered the extract exhibited a markedly significant rise ($p < 0.05$) in H₂O₂ level by a percent change of 55 and 40% as compared with negative and positive control groups, respectively, at concentration of 1 mg/ml of the extract. Also, extract concentrations of 10 mg/ml and 500 μ g/ml induced a significant augmentation of H₂O₂ level by a percent change of about

20 and 16% in protective and treatment groups, respectively, as compared with positive control mice groups (Supplementary Fig. S3B).

Nitric oxide production by mice macrophage

A significant increase in NO production by macrophages stimulated *in vitro* with different concentrations (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 μ g/ml, 100 μ g/ml, and 10 μ g/ml) of the *E. filiformis* Fv19 extract for 24 h was observed in all experimental groups. A percent increase of about 71% in NO level was noted in mice administered solely the extract at a concentration of 500 μ g/ml as compared with the positive control group. Also, percent increases of about 91 and 72% in NO production were observed at concentrations of 10 μ g/ml and 1 mg/ml, respectively in the protective group animals as compared with positive control groups (Supplementary Fig. S3C).

Interleukin 17 (IL-17) production by mice splenocytes

IL-17 production by splenocytes stimulated *in vitro* with different concentrations (10 mg/ml, 5 mg/ml, 1 mg/ml, 500 μ g/ml, 100 μ g/ml, and 10 μ g/ml) of the extract of *E. filiformis* Fv19 for 24 h was significantly decreased in all experimental mice groups. Mice administered the extract exhibited a notable reduction. ($p < 0.01$) by a percent change of 27% in IL-17 level production by splenocytes

at a concentration of 5 mg/ml as compared with positive or with negative control groups. In the protective and treatment groups there was also a significant decrease by a percent change of 39 and 34%, respectively, at concentrations of 10 µg/ml and 100 µg/ml, respectively, as compared with the positive control (Supplementary Fig. S3D).

Statistical analysis of the effects of *F. filiformis* Fv 19 extract different concentrations among the experimental groups
ANOVA was used to analyze the effect of each individual concentration among the different animal groups to determine the optimum extract concentration at which the highest immune response is developed against the tumor.

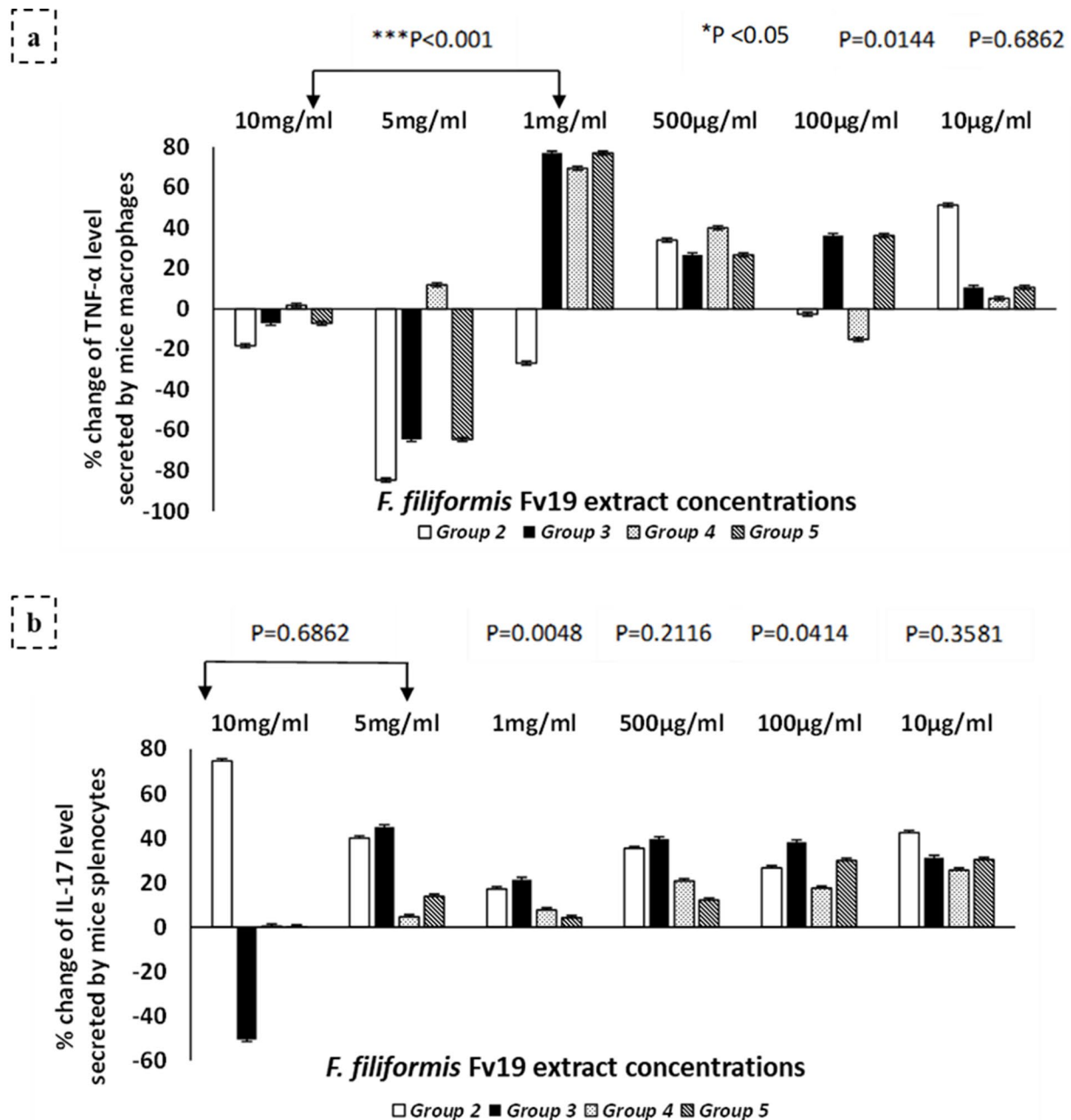


Fig. 5 Levels of **a**) TNF-α produced by mice macrophages and **b**) IL-17 produced by mice splenocytes activated in vitro with different concentrations of *F. filiformis* Fv 19 extract. Group 2: positive control group (mice-bearing solid tumor), group 3: extract-only-treated mice group, group 4: protective group (mice treated with the extract before tumor implantation), and group 5: treatment group (mice treated with the extract after tumor implantation). Data are represented as a percentage to group 1 (negative control group)

Effect of *F. filiformis* Fv 19 extract on TNF- α production by mice macrophages

As shown in Fig. 5a, there was a highly substantial difference ($p < 0.001$) among the experimental groups when treated with high extract concentrations (10 and 5 mg/ml). The highest concentration used in the current study (10 mg/ml) led to about 20% inhibition in TNF- α produced by macrophages of mice bearing solid tumors. However, this inhibition decreased to less than 10%. In the extract-treated group and the therapy group, there was a marginal rise in the protective group. Similar results were observed with the extract concentration of 5 mg/ml, where there was about 60–80% inhibition in TNF- α production in the extract-treated group, treatment group and mice-bearing-solid-tumor group but there was about a 10% increase in the protective group. For the moderate extract concentration (1 mg/ml), there was inhibition in TNF- α production by mice bearing the solid tumor group only, but there was more than a 70% increase in the other groups. However, the other moderate concentration (500 μ g/ml) induced more than a 30% increase in TNF- α production by all groups; the disparity between groups was statistically significant ($p < 0.05$). For the moderate extract concentrations (100 and 10 μ g/ml), the results were conflicting, and the differences across groups were substantial. ($p = 0.0144$) for 100 μ g/ml but not significant ($p = 0.6862$) for 10 μ g/ml.

Effect of *F. filiformis* Fv 19 extract on IL-17 production by mice splenocytes

There was an increase in IL-17 production by mice's splenocytes in all experimental groups. The difference among the experimental groups treated with high extract concentrations (10 and 5 mg/ml) was extremely important ($p < 0.001$). Only in the extract-treated group treated with the highest concentration (10 mg/ml) was there about 50% inhibition in IL-17 production. For the moderate extract concentrations, the difference between groups was significant ($p = 0.0048$) when animals were treated with 1 mg/ml and not significant ($p = 0.2116$) when animals were treated with 500 μ g/ml (Fig. 5b).

Effect of *F. filiformis* Fv 19 extract on H₂O₂ production by mice macrophages

All used extract concentrations induced about 70% inhibition in hydrogen peroxide production by activated macrophages of mice bearing solid tumor group only. However, there was an increase in H₂O₂ level in the other experimental groups (Fig. 6a). Only the difference among the experimental groups treated with high extract concentrations (10 and 5 mg/ml) was extremely important ($p < 0.001$).

Effect of *F. filiformis* Fv 19 extract on NO production by mice macrophages

All used extract concentrations induced more than 40% inhibition in nitric oxide production by activated macrophages of mice bearing solid tumor group only. However, there was an increase in the H₂O₂ level in the other experimental groups, but only the difference among the experimental groups treated with the highest extract concentrations (10 mg/ml) was slightly significant ($p < 0.05$) as shown in Fig. 6b.

Histopathological findings

No histopathological alterations were seen, and the histological structure of the white and red pulps in the spleen sections of the control group of mice remained normal in the mice group administered extract only and the mice group administered extract after and before tumor implantation. While the lymphoid cells of the white pulps of spleen sections of mice bearing solid tumors showed necrosis in a focal manner (Fig. 7).

Photomicrographs of lymph node sections demonstrated the absence of histopathological alterations and preserved normal histological architecture of lymphoid cells in the cortex and medulla across all experimental mouse groups (Fig. 8).

Molecular docking analysis of *F. filiformis* Fv19 extract metabolites with proteins involved in anticancer and immunomodulatory pathways

The molecular docking analysis compared the binding affinities of *F. filiformis* Fv19 extract metabolites with five protein targets, as presented in supplementary Fig. S4 and Fig. 9. Among the compounds, 1H Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1arv(1aà,4aà,7á,7aá,7bà)]- exhibited the highest docking score of -5.855 kcal/mol with iNOS (1M9T), forming a hydrogen bond with GLN257, and a lower score of -4.567 kcal/mol with EGFR (1M17), interacting with ASP831. Stigmasta-5,22-dien-3-ol, acetate,(3á)- achieved a docking score of -5.015 kcal/mol with Bcl-2 (4LVT), binding via a hydrogen bond to ASN140, while cholesterol-3-ol, 2-methylene-, (3á,5à)- recorded -4.581 kcal/mol with COX-2 (5F19), interacting with HIE214. In contrast, 9,12,15-octadecatrienoic acid showed the weakest binding at -3.376 kcal/mol with TLR4 (3FXI), forming a hydrogen bond with LYS122.

Gene interaction network analysis of bioactive metabolites from *F. filiformis*: insights into anticancer and immune modulation pathways

The anticancer gene network analysis revealed strong interactions between key genes involved in apoptosis and tumor progression. The apoptotic regulators BCL2 and BAX exhibited opposing roles, with BCL2 promoting

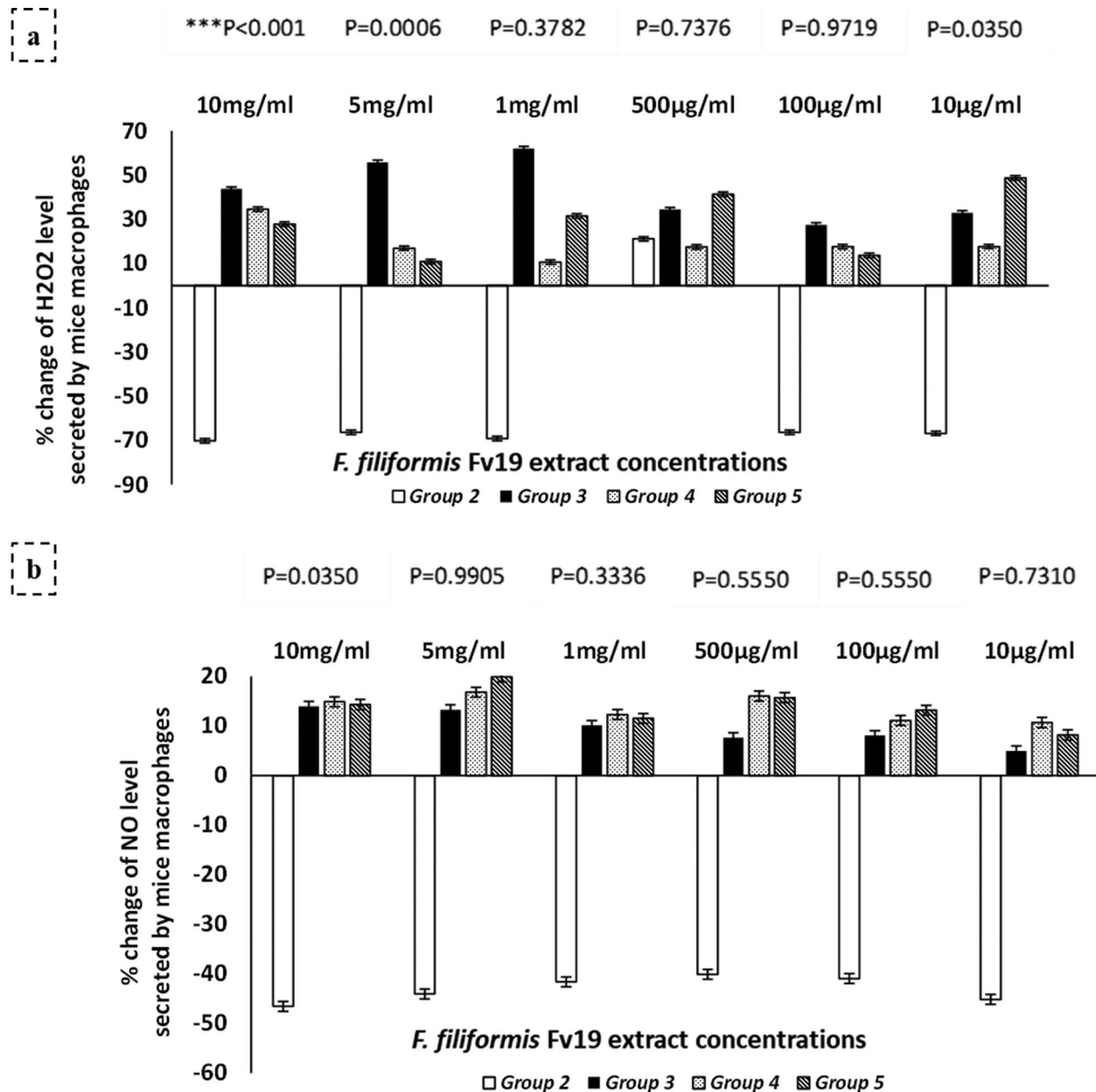


Fig. 6 Levels of **a**) hydrogen peroxide and **b**) nitric oxide produced by mice macrophages activated in vitro with different concentrations of *F. filiformis* Fv19 extract. Group 2: positive control group (mice-bearing solid tumor), group 3: extract-only treated mice group, group 4: protective group (mice treated with the extract before tumor implantation), and group 5: treatment group (mice treated with the extract after tumor implantation). Data are represented as a percentage to group 1 (negative control group)

cell survival while BAX induced programmed cell death. Additionally, CASP3 was identified as a key effector of apoptosis, and its activation was linked to TP53, a tumor suppressor gene known to regulate cell cycle arrest and apoptosis. The oncogenic network showed a strong association between EGFR, KRAS, and PIK3CA, which are central to cell proliferation and tumor growth. Over-expression of these genes has been widely reported in various cancers, contributing to uncontrolled cell division. Furthermore, inflammation-mediated tumor

progression was highlighted through the involvement of PTGS2 (COX-2), IL6, and NF-κB1, which are associated with chronic inflammation and cancer development. The network also indicated that TNE, a pro-inflammatory cytokine, plays a role in promoting tumor survival by activating NF-κB signaling (Fig. 10a). The immune modulation gene network analysis demonstrated the intricate interactions between key immune-related genes. NOS2 and GCH1 were found to be closely linked, emphasizing their role in nitric oxide production and immune

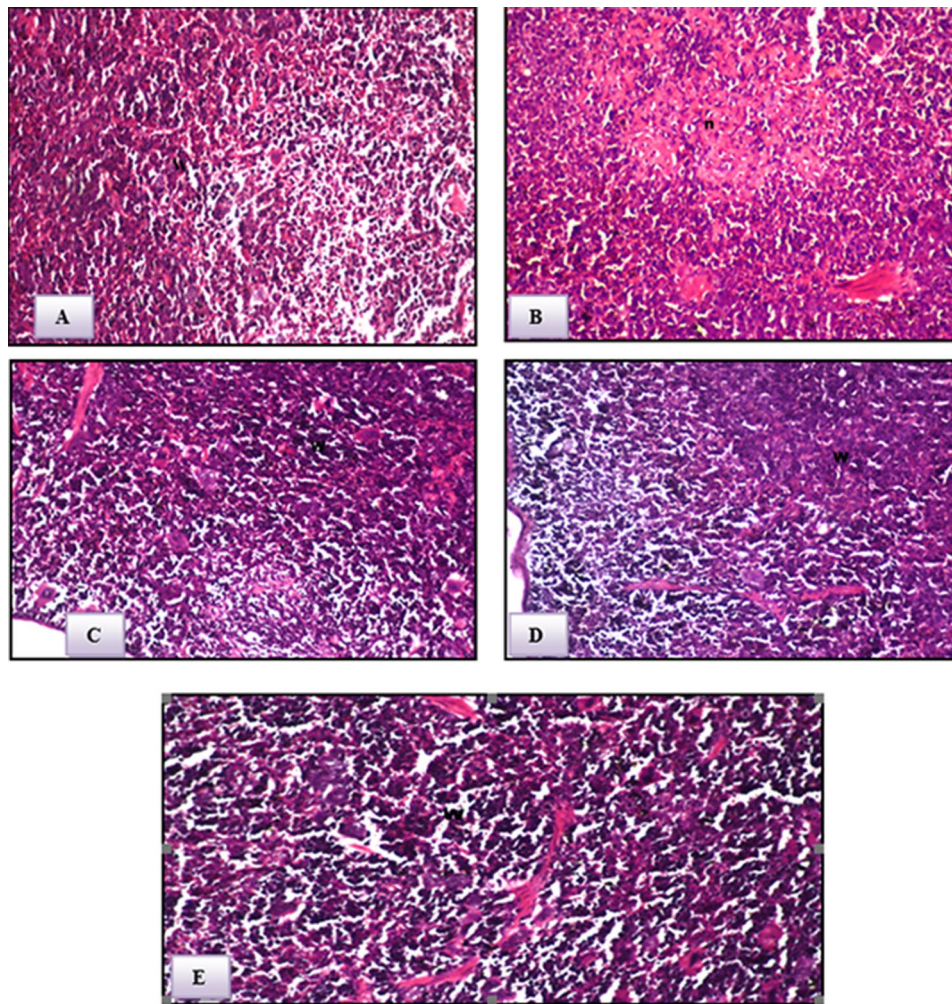


Fig. 7 Photomicrographs of spleen tissue of mice groups. **A, C, D and E** [the negative control group, mice group administered the extract only and mice groups administered the extract before and after tumor implantation exhibited no histological alterations and maintained the usual morphological structure of the white (W) and red pulps. **B** [mice-bearing Ehrlich solid tumor group showed focal necrosis (n) in white pulp]. (H & E X 40)

responses. The activation of IFNG and STAT1 indicated a strong immune-stimulatory effect, reinforcing their role in adaptive immunity. Additionally, the network showed significant interactions within the TLR4–MYD88–IRAK4 axis, a key signaling pathway in innate immunity. The presence of NFKBIA as a regulatory factor highlights the importance of NF- κ B inhibition in controlling excessive inflammatory responses. This suggests that bioactive metabolites from *F. filiformis* may regulate immune responses by modulating cytokine production and inflammation-related gene expression (Fig. 10b). The gene interaction networks generated by GeneMANIA confirmed the strong connectivity between anticancer and immune-modulating pathways. The anticancer genes were predominantly involved in tumor suppression, apoptosis, and inflammation-mediated carcinogenesis, whereas the immune-modulating genes were linked to

nitric oxide signaling, cytokine regulation, and pathogen recognition.

Discussion

Bioactive compounds from fungi, primarily sourced from Basidiomycetes and occasionally from Ascomycetes, along with medicinal mushrooms, have been recognized and extensively utilized in East Asia as components of traditional diet and medicine. In recent decades, these compounds have been the focus of rigorous research aimed at understanding and harnessing their medicinal properties for naturally derived pharmaceuticals. Indeed, certain biopolymers have already been commercialized as anticancer, immunostimulatory, or preventive agents. Many biopolymers derived from edible mushrooms are excellent candidates for the development of innovative functional foods and nutraceuticals, as they pose minimal safety risks while offering immunomodulating,

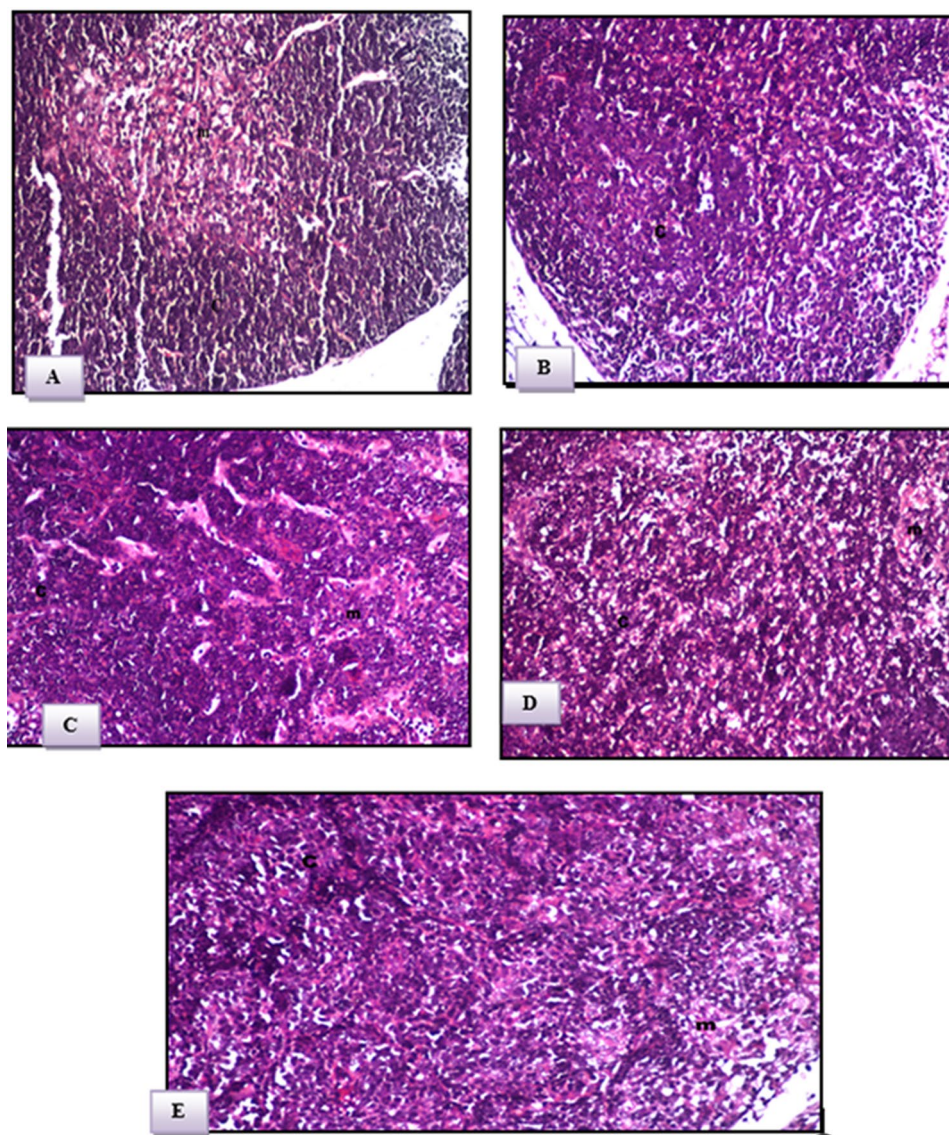


Fig. 8 Photomicrograph of lymph node section of mice groups. **A, B, C, D** and **E** (the negative control group, mice-bearing Ehrlich solid tumor, mice group administered the extract only and mice groups administered the extract before and after tumor implantation) showed no histological alterations and the usual morphological structure of lymphoid cells in the cortex (c) and medulla (H & E X 40)

anticancer, antimicrobial, hypocholesterolemic, hypoglycemic, and health-enhancing benefits [35].

The activity of immune system cells may lead either to prevent tumor development by restraining established tumors or to promote tumorigenesis and tumor progression as well as metastasis [36]. Lymphocytes and macrophages play a major role during immune response against tumors [37]. However, inhibiting tumor growth by immune cells doesn't cause any harm to the host [38].

Types of natural macromolecules that can be derived from bacteria, fungi, algae, plants, and animals are steroidal molecules, sterols, and esters. Natural macromolecules have been shown to possess complex and substantial properties and varied biological properties,

such as anticancer, anticoagulant, antioxidant, antiviral, immunomodulatory, antihyperlipidemic, anti-inflammatory, anti-asthmatic, and hepatoprotective activities and antihepatotoxic effects. Their structural features are primarily responsible for their qualities. Immunomodulators with potential for therapeutic or preventative action must be developed [39].

The GC-MS analysis of the *E. filiformis* extract revealed a diverse array of bioactive metabolites, predominantly characterized by steroidal and terpenoid compounds. The most abundant component was identified as a steroidal molecule, cholestan-3-ol, 2-methylene-, (3 α ,5 α)-, comprising 37.47% of the total peak area. Sterols and their derivatives are well-documented constituents of

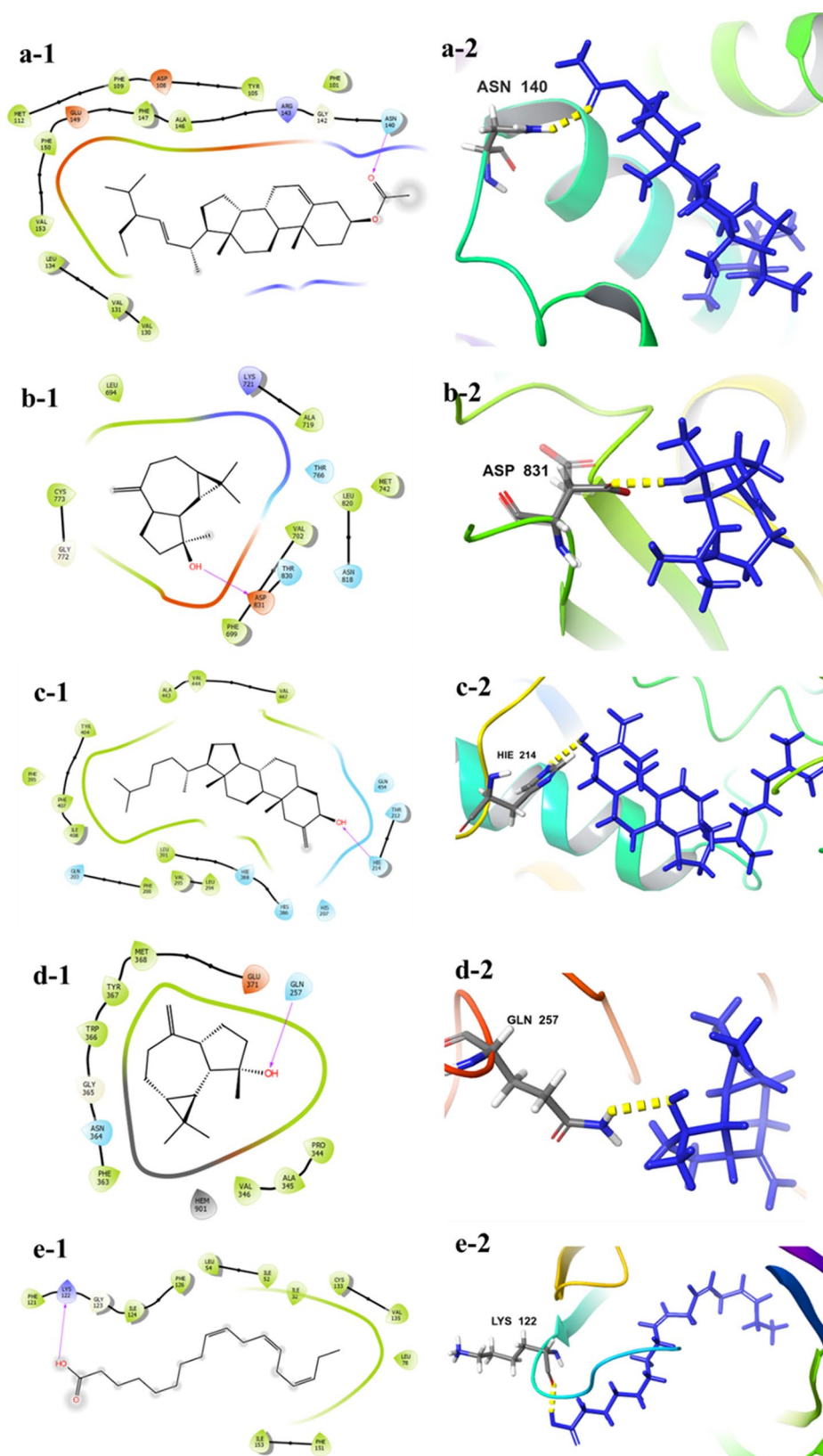


Fig. 9 Representative binding pose of (a) Stigmasta-5,22-dien-3-ol, acetate, (3á)-; (b) 1H cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7á,7aá,7bà)]-, (c) Cholestan-3-ol, 2-methylene-, (3á,5à)-, (d) 1H cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7á,7aá,7bà)]-, and (e) 9,12,15-octadecatrienoic acid

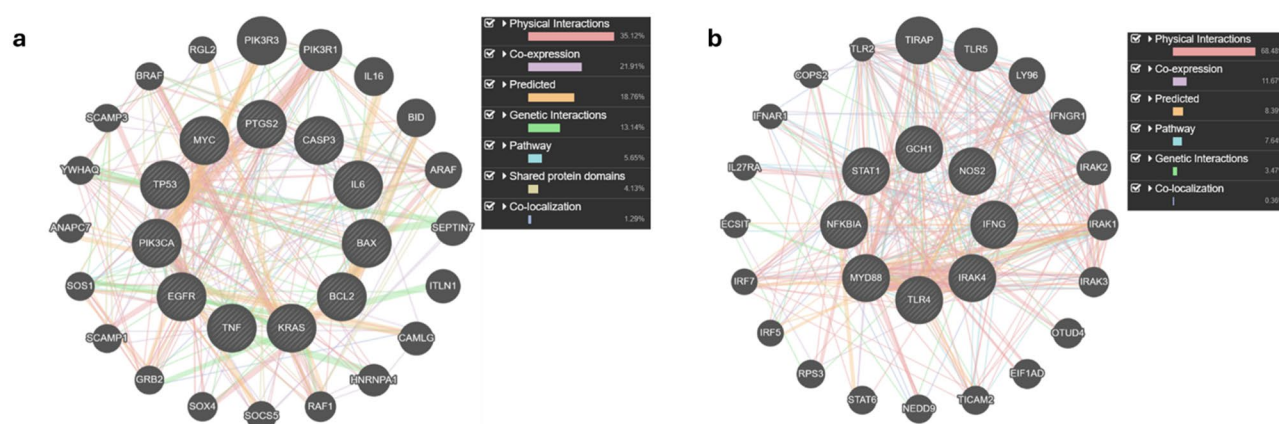


Fig. 10 Gene interaction network analysis of bioactive metabolites from *F. filiformis*. **(a)** Anticancer gene interaction network—displays the interconnections between genes regulating apoptosis, cancer proliferation, and inflammation-induced tumorigenesis. **(b)** Immune modulation gene interaction network—highlights genes involved in immune activation, nitric oxide signaling, and inflammatory response pathways

macrofungi, playing crucial roles in membrane structure and exhibiting significant pharmacological activities, including anti-inflammatory and immunomodulatory effects [40–42]. The presence of spirost-8-en-11-one, 3-hydroxy- (23.24%), a steroidal sapogenin, further supports the potential for bioactive properties, as spirostane-type compounds have been associated with cytotoxic and antimicrobial activities in fungal species [43]. Additionally, the detection of the terpene derivative 1H Cycloprop[e]azulen-7-ol (12.01%) aligns with previous findings highlighting the presence of sesquiterpenes in *Flammulina* species, which often contribute to the characteristic aroma and exhibit antimicrobial properties [43]. The unsaturated fatty acid ester and the linolenic acid derivative (9,12,15-octadecatrienoic acid ester, 12.43%) are consistent with the metabolic profile of basidiomycetes, where polyunsaturated fatty acids are linked to cellular signaling and stress responses [44]. Collectively, these results underscore the metabolic richness of *F. filiformis*, supporting its potential as a source of bioactive compounds with pharmaceutical relevance. The FTIR analysis reveals that the fungal extract is rich in a variety of functional groups, confirming its biochemical complexity. The presence of major peaks associated with hydroxyl, carbonyl, and amide groups suggests that the extract contains significant amounts of polysaccharides, proteins, and lipids. Specifically, the broad band at 3300 cm^{-1} coupled with the strong peak at 1070 cm^{-1} indicates that polysaccharides are a dominant component of the extract, which is common in fungal cell walls (e.g., β -glucans) [45]. Additionally, the amide I and II bands (1650 and 1550 cm^{-1}) confirm the presence of peptides or enzymes, which often contribute to the therapeutic potential of fungal metabolites [45, 46]. The aliphatic C-H stretches (2920 cm^{-1}) suggest the presence of fatty acids or sterols, which are known to possess antimicrobial and

anti-inflammatory properties [47, 48]. Overall, the spectral data confirms that the extraction method successfully isolated a complex mixture of bioactive compounds (polysaccharides, proteins, and lipids) commonly associated with medicinal fungi.

Although full structural elucidation of individual compounds would require NMR spectroscopy, the combination of GCMS profiling (with mass spectral library matching and retention indices) and FTIR functional group analysis provided sufficient identification of the major bioactive sterols, fatty acids, and macromolecular classes to support the molecular docking and systems biology analyses within the scope of this biotechnological study; detailed isolation and NMR characterization will be pursued in future work.

The observed cytotoxic effects of the *F. filiformis* Fv19 extract on MCF-7 breast cancer and Hep-G2 liver cancer cell lines, with potent IC_{50} values of $5\text{ }\mu\text{g/ml}$ and $4\text{ }\mu\text{g/ml}$ respectively, are strongly supported by recent literature demonstrating that *F. filiformis* can induce apoptosis and inhibit proliferation in these cell types through mechanisms like reactive oxygen species generation and cell cycle arrest [49, 50]. An interesting observation was noted in the Hep-G2 cell line, where treatment with $10\text{ }\mu\text{g/ml}$ extract resulted in slightly higher viability ($89.3 \pm 4.2\%$) compared to the $2.5\text{ }\mu\text{g/ml}$ concentration ($82.1 \pm 3.8\%$), although both values remained significantly lower than untreated controls (100% , $p < 0.01$). This non-monotonic dose-response pattern, while seemingly paradoxical, is a well-documented phenomenon in phytochemical and immunomodulatory research, often referred to as hormesis or biphasic dose response [51–53].

The phenomenon of increased viability at intermediate concentrations ($10\text{ }\mu\text{g/ml}$) compared to lower concentrations ($2.5\text{ }\mu\text{g/ml}$) likely reflects hormetic dose-response,

a fundamental biological principle where low doses of potentially toxic agents stimulate adaptive stress responses that enhance cellular resilience [53]. Key mechanistic considerations include mild oxidative or electrophilic stress at moderate concentrations can activate Nrf2, leading to upregulation of antioxidant enzymes (HO-1, NQO1, and GST) that temporarily enhance cell survival [54]. Also, many immunomodulators exhibit U-shaped or inverted U-shaped dose-response curves, with optimal biological activity at intermediate doses and diminished effects at both lower and higher extremes [55]. This observation does not diminish the extract's potent anticancer activity ($IC_{50} = 4 \mu\text{g/ml}$) but rather highlights the complex biology of natural product-cell interactions and reinforces the importance of comprehensive dose-response analysis in drug development.

Interestingly, the lack of cytotoxicity against the HCT116 colon cancer cell line highlights the cell-line specific nature of natural compounds, likely due to unique molecular profiles or resistance mechanisms within HCT116 cells [56]. Beyond direct cytotoxicity, the extract's significant *in vivo* tumor inhibition was driven by a robust immunomodulatory response. This is evidenced by the activation of macrophages leading to increased production of TNF- α , NO, and H_2O_2 and a bolstered cell-mediated immune response, indicated by an enhanced spleen index in treated mice [30, 57]. A particularly noteworthy finding is the extract's ability to reduce the production of the often pro-tumor inflammatory cytokine IL-17, thereby helping to create a less favorable microenvironment for tumor growth [58].

Previous studies reported that, mushroom metabolites have both immunomodulatory and anti-tumor activities [4, 10]. In the current study, the immunomodulatory and antitumor effects of different concentrations of the *F. filiformis* Fv19 extract were investigated focusing on their effects on mice macrophages and splenocytes functions. Measurements of the innate immune mediators produced by macrophages, such as TNF- α , nitric oxide, and hydrogen peroxide, as well as interleukin-17 secreted by spleen lymphocytes as a mediator for cellular immunity, were used to evaluate the mice's immune response to Ehrlich carcinoma cells.

Strong signaling molecules like chemokines and cytokines are just as essential to life as hormones and neurotransmitters. These are low molecular weight proteins produced by several cell types, mostly immune system cells that facilitate intercellular communication. More than 100 distinct proteins are currently referred to as cytokines, having been identified in the early 1960s and 1970s. These substances play a role in the control of chemotaxis, metabolism, cellular proliferation, tissue repair, and both local and systemic inflammation.

Because their main job is to control inflammation, cytokines are essential for controlling the immune system in both health and illness. Pro- and anti-inflammatory cytokines are known to exist [59].

Macrophages serve as the guardians of the innate immune system and execute numerous essential functions, including the generation of cytokines and the phagocytosis of pathogens and aberrant cells. Reactive oxygen and nitrous species [60]. Pro-inflammatory cytokines, or cytotoxic and inflammatory molecules like interleukin-6, TNF- α , and NO, are secreted when macrophages are activated, and these molecules aid in the killing of tumor cells [61]. Due to their direct lethal effect on tumor cells and extensive physiological activity in the immune system, TNF- α and NO have been identified as variables with anti-tumor action [61]. Additionally the elevation of intracellular reactive oxygen species can trigger cancer cell cycle arrest, senescence, and apoptosis [62]. Consequently, this work examined the impact of *F. filiformis* Fv19 extract on the secretion of TNF- α , NO, and H_2O_2 by murine macrophages.

The current results revealed a marked decrease in macrophage count in mice-bearing tumor group which may indicate immunosuppression. It has been reported previously that immune suppression in humoral and cellular immune function through lymphocyte depletion and macrophage deficiency was observed with mice-bearing tumor [63]. However, in this study, extract (200 mg/kg/day) only the treated group had a notable enhancement in macrophage levels count by a percent change of 109 and 166% as compared with negative and positive control groups respectively. Additionally, macrophage count was considerably enhanced by a percent change of 9 and 45%, respectively, after macrophages received 200 mg/kg/day of *F. filiformis* Fv19 extract for 10 days prior to and during tumor formation. This was in comparison to the group of positive control mice.

Macrophage-derived cytokines such as TNF- α plays major biological roles in the host defense to microbial infections and cancerous cells [64]. On the other hand, aberrant or excessive TNF- α production might be detrimental to healthy cells. Two unique receptors called TNFR1 and TNFR2, which are variably controlled on different cell types in normal and sick tissue, bind to each other to initiate all known responses to TNF- α [65].

In the present study, the animal group treated with *F. filiformis* Fv19 extract only showed a highly significant increase in TNF- α production by a percent change of 284 and 177% as compared to that of negative and positive control mice groups respectively. Also, protective and treatment groups showed a significant increase in TNF- α production level by a percent change of 55 and 34% respectively in comparison to the positive control mice group.

TNF- α is reported as a pro-inflammatory cytokine that can act on immune and inflammatory cells leading to apoptosis of infected or cancerous cells as well as immune regulation [66]. So, the increasing effect of the *F. filiformis* Fv19 extract on TNF- α production in the current study may support using this extract as a therapeutic candidate against tumors. However, more investigations are needed to augment this finding.

Mushroom natural macromolecules don't have the ability to activate the immune cells directly due to their high molecular weight. Thus, the activation mechanism of macromolecules entails several cell receptors, including dectin-1, complement receptor 3 (CR3), lactosylceramide (LacCer), and toll-like receptor (TLR) 2. The efficacy of macromolecules in these situations relies on their ability to bind with affinity to immune cell receptors [5].

Also, hydrogen peroxide produced by macrophages or granulocytes is reported to contribute in the lysis of tumor cells in a variety of experimental settings in vitro [67]. In the current study, H₂O₂ was evaluated as an indicator of the activation of peritoneal macrophages in response to *F. filiformis* Fv19 extract. The results revealed that H₂O₂ production showed significant increase in mice treated with the extract only by a percent change of 55 and 40% as compared with negative and positive control groups followed by protective (20%) and treatment (16%) groups as compared with positive control group.

Mice administered the extract exhibited approximately a 71% significant increase in nitric oxide (NO) production by macrophages as compared with positive control group followed by therapeutic group then protective group. According to these promising results, *F. filiformis* Fv19's extract could be a good option for regulating macrophage activity and boosting immunity. Studies conducted in the past have shown that the reactive nitrogen intermediates have a major impact on anti-tumor and anti-microbial activity [68].

The results align with Chun et al. [69], who indicated that the treatment of *Ganoderma lucidum* polysaccharide fractions to RAW 264.7 murine resulted in a dose-dependent enhancement of NO and TNF- α production. Significant immunostimulatory activities of mushroom polysaccharides have been documented [26]. These properties are demonstrated not only by direct antitumor activity against cancer cells but also by activating cells involved in innate immunity [70].

The findings of the present investigation indicated that mice groups treated with the *F. filiformis* Fv19 extract showed significant increases in spleen index than those of normal mice or mice bearing solid tumor. This might be a sign of a strengthened immune system and help to explain some of the anticancer activity of the extract from *F. filiformis* Fv19. A prior study that employed a combination of polysaccharides from *Lentinus edodes*,

Ganoderma lucidum, and *Grifola frondosa* generated an increase in the spleen index in comparison to the control mice produced similar results. The increase of the spleen index may indicate that the mixture of the macromolecules could enhance splenocytes proliferation and consequently inducing the cell-mediated immune responses [71].

Mice administered the extract exhibited a notable reduction in IL-17 production by splenocytes, with a percent change of 27% relative to both the positive and negative control groups. In protective group and treatment group there was also a significant decrease by a percent change of 39 and 34% respectively as compared with positive control. According to numerous studies, TH17 cells are essential for both the inflammatory response and adaptive immunity, and they are also a significant determinant in the advancement of autoimmune inflammation. Furthermore, a number of human malignancies, including ovarian, cervical, breast, hepatocellular, esophageal, gastric, and colorectal cancers, typically express IL-17 at a higher level [58, 72].

The decrease in IL-17 levels produced by splenocytes of mice treated with *F. filiformis* Fv19 extract may be due to downregulation of IL-17 expression, which diminished its role as a protumor. This finding has great value and may suggest using the *F. filiformis* Fv19 extract as a therapeutic agent against different tumors. However, more investigations are needed to confirm this finding and to study its mechanism of action.

By controlling cell differentiation, proliferation, and death as well as defense mechanisms like immunological and inflammatory responses, cytokines maintain the organism's homeostasis [73]. Regarding the extract used in this study, the high extract concentrations (10 and 5 mg/ml) induced the secretion of significant levels of the anticancer cytokine TNF- α and suppressed the release of the inflammatory cytokine IL-17, which plays a role in the inflammatory reactions against tumors as reported by recent studies [74]. The same concentrations also induced other immune mediators like H₂O₂ and NO, which also have antitumor activities.

Furthermore, the histopathological results of our study, which showed preserved spleen and lymph node architecture in the extract-treated groups versus necrosis in the tumor-bearing controls, underscore the extract's protective and immune-enhancing capabilities. The absence of pathological alterations in these key immune organs in treated mice, even in the presence of a tumor challenge, indicates that the extract not only stimulates an effective anti-tumor response but also maintains the integrity of the immune system. This is supported by research from Xin et al. [75], who reported that a protein-bound polysaccharide from *F. filiformis* alleviated immunosuppression in cyclophosphamide-treated mice, as evidenced

by restored spleen and thymus indices and normalized lymphocyte populations. Their histological examinations also revealed a recovery of the splenic white and red pulp structures, mirroring our observations of a healthy spleen histology in the protective and treatment groups. This suggests that the extract's benefits extend beyond direct tumor cytotoxicity to a broader systemic immunoprotection, which is a critical aspect of effective cancer therapy.

The molecular docking analysis suggests that GC-MS-identified bioactive compounds from *F. filiformis* extract have significant interactions with key oncogenic and immune-modulating proteins. By integrating GC-MS profiling with gene interaction networks, this study provides a molecular roadmap for potential drug design. Specifically, the identification of sterols such as stigmasta-5,22-dien-3-ol acetate and their strong binding affinity to the anti-apoptotic protein Bcl-2 directly links the metabolite profile to the apoptosis-inducing pathways observed in the network analysis. This systems biology approach moves beyond traditional herbal descriptions, identifying specific chemical scaffolds that can be optimized for pharmaceutical formulations. The interaction with Bcl-2 is particularly notable, as this protein is known for its role in preventing apoptosis [76]. The docking score of -5.015 kcal/mol and interaction with ASN 140 suggest that stigmasta-5,22-dien-3-ol acetate may act as a potential Bcl-2 inhibitor, promoting apoptosis in cancer cells. Similar findings have been reported in studies investigating natural Bcl-2 inhibitors, which have shown promising anticancer activity [49, 77].

The inhibition of epidermal growth factor receptor (EGFR) by 1H cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene (docking score -4.567 kcal/mol), suggests that this compound may interfere with EGFR-mediated cancer cell proliferation. Terpenoid-derived compounds have been previously noted for their EGFR inhibitory activity, supporting the potential therapeutic role of this compound [78]. The interaction with COX-2 further supports the extract's anti-inflammatory effects. The binding of cholestan-3-ol, 2-methylene to HIE 214 indicates that it may suppress COX-2-mediated inflammation, which is known to contribute to tumor progression [79]. Literature evidence supports the role of steroidal compounds in reducing COX-2 expression, aligning with the observed docking interactions [80]. The strong binding of 1H cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene, to iNOS with a docking score of -5.855 kcal/mol suggests a significant anti-inflammatory and anticancer effect. iNOS is involved in nitric oxide production, which can lead to oxidative stress and tumor growth [81]. The inhibition of iNOS by this compound could reduce inflammation and oxidative damage, potentially contributing to anticancer activity [82]. Similarly, the interaction of

9,12,15-octadecatrienoic acid with TLR4 suggests a role in immune modulation. TLR4 activation is associated with immune responses and inflammation, and its modulation by this compound may help regulate immune activity in cancer progression.

These docking results align with in vitro findings reported in the manuscript, where *F. filiformis* extract exhibited significant anticancer and immunomodulatory effects. Specifically, the inhibition of COX-2, iNOS, and TLR4 aligns with the observed reduction in inflammatory cytokines such as interleukin-17 and tumor necrosis factor- α . Additionally, the potential inhibition of Bcl-2 and EGFR supports the extract's cytotoxicity against cancer cells, as demonstrated in the in vitro cytotoxicity assays. These findings suggest that GC-MS-identified compounds from *F. filiformis* extract could serve as promising candidates for cancer therapy and immune modulation. Given the remarkable 99% tumor inhibition rate observed, the *F. filiformis* Fv19 extract holds significant potential for translation into industrial bioproduction. The high efficacy and safety profile support its development not only as a high-value nutraceutical but also as a standardized pharmaceutical agent. Future scalable bioproduction strategies, utilizing controlled fermentation, could ensure consistent metabolite yields, bridging the gap between laboratory discovery and biomedical industry applications.

The results of this study indicate that the bioactive metabolites from *F. filiformis* influence key genetic pathways related to both anticancer activity and immune modulation. The anticancer gene network analysis highlighted the role of BCL2, BAX, CASP3, and TP53 in apoptosis regulation, which aligns with previous studies demonstrating that natural compounds targeting BCL2 can enhance apoptosis in cancer cells [83]. A study by [84] found that natural flavonoids can induce apoptosis by downregulating BCL2 and upregulating BAX, a mechanism similar to that observed in this study. Additionally, the association between EGFR, KRAS, and PIK3CA in promoting tumor proliferation has been well established in cancer research [85]. The involvement of PTGS2 (COX-2), IL6, and NF- κ B1 in inflammation-driven tumor progression further confirms the role of inflammation in cancer development. Studies have shown that COX-2 inhibitors, such as celecoxib, reduce tumor growth by suppressing inflammatory mediators [86]. Similarly, the downregulation of IL6 and NF- κ B1 has been reported in studies examining the anticancer effects of polyphenols [87]. The significant interaction between TNF and NF- κ B in the anticancer network suggests that bioactive compounds from *F. filiformis* could modulate pro-inflammatory pathways, which aligns with previous research showing that targeting TNF can reduce tumor-associated inflammation [61].

In the immune modulation gene network, the strong connection between NOS2 and GCH1 indicates enhanced nitric oxide production, which is essential for immune responses. Studies by [88] demonstrated that nitric oxide plays a dual role in immunity by both promoting pathogen defense and regulating inflammation. The activation of IFNG and STAT1 suggests a role in adaptive immunity, which is consistent with findings by [89] indicating that natural fungal metabolites enhance IFN- γ production, leading to improved immune responses against infections and cancer cells. The involvement of the TLR4–MYD88–IRAK4 pathway in immune modulation suggests that bioactive compounds from *F. filiformis* may act as immune enhancers. This is in agreement with studies on natural immunostimulants that activate TLR4, thereby enhancing innate immune responses [90]. Additionally, the regulatory role of NFKBIA in controlling inflammatory responses aligns with research showing that fungal-derived compounds can modulate NF- κ B inhibitors, reducing chronic inflammation while maintaining immune defense [91]. These findings confirm that bioactive metabolites from *F. filiformis* play a significant role in anticancer activity by targeting apoptosis, tumor proliferation, and inflammation-driven cancer pathways, while also enhancing immune responses through nitric oxide signaling and cytokine regulation.

The correlation between our molecular docking predictions and functional immunological outcomes offers systems-level validation of the postulated processes; nonetheless, we recognize that direct protein-level confirmation through Western blotting is a crucial avenue for future research. The alignment between predicted target engagement (Bcl-2, EGFR, COX-2, iNOS) and actual biological outcomes (cytotoxicity, increased NO/TNF- α , decreased IL-17) strengthens the hypothesis that the extract's therapeutic effects result from the multi-target modulation of tumor cell-intrinsic pathways and host immune responses. This multi-target approach is especially beneficial for cancer treatment, as it may evade the resistance that frequently arises against single-target drugs. Subsequent research utilizing Western blot analysis of phosphorylated signaling intermediates (e.g., p-STAT1, p-NF- κ B p65), caspase cleavage products, and Bcl-2 family protein expression in tumor tissue and immune cells will provide clear mechanistic validation.

Important limitation of the present study should be acknowledged. While the Ehrlich solid tumor model is valuable for initial immunopharmacological evaluation, it does not fully recapitulate the complex biology of human breast cancer, particularly the hormone-dependent subtypes. The MCF-7 cell line used in in vitro studies is estrogen receptor-positive and hormone-responsive, whereas the in vivo Ehrlich model is hormone-independent. These characteristics make the Ehrlich model

suitable for general antitumor screening but not specifically representative of estrogen-dependent breast cancer. Our use of male mice was methodologically sound for controlling hormonal variability, but the confirmation in female animals using breast cancer-specific models is essential. This model would provide more clinically relevant assessment of the extract's potential against hormone-responsive breast cancer.

Conclusion

The findings of this study demonstrated that *F. filiformis* Fv19 extract possesses potent anticancer and immunomodulatory properties. The extract effectively inhibited tumor growth, increased macrophage function, and stimulated immune responses through enhanced TNF- α , NO, and H₂O₂ production while suppressing IL-17 secretion. These results suggested that the extract plays a dual role in cancer therapy by both directly inhibiting tumor progression and enhancing immune-mediated tumor suppression. Furthermore, molecular docking analysis confirmed the extract's bioactive compounds as potential inhibitors of key oncogenic and immune-regulatory proteins, particularly Bcl-2, EGFR, COX-2, and iNOS. This suggested that *F. filiformis* Fv19 may not only act as a cytotoxic agent but also as an immune system modulator, offering a comprehensive therapeutic approach. The study highlighted the therapeutic potential of *F. filiformis* Fv19 extract in integrative oncology, where natural compounds can complement conventional therapies. Given its significant anticancer efficacy, further studies should focus on its molecular mechanisms, bioavailability, and potential clinical applications. Additionally, future investigations may explore its synergistic effects with existing chemotherapeutic agents to enhance treatment outcomes while minimizing side effects.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12896-026-01152-y>.

Supplementary Material 1

Author contributions

All authors contributed to the study conception and design. Mushroom cultivation, extract preparation, in vitro and in vivo studies were performed by H.E., M.E.O., R.M.A., E.T.M., S.N. Docking data analysis were performed by E.T.M. All authors contributed in writing the first draft of the manuscript. H.E., E.T.M. and R.M.A. made considerable adjustments to the first draft, focusing on key topics and the manuscript's main objective. All authors revised and approved the final version of the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article. The *F. filiformis* Fv19 strain utilized in this study has been registered under the accession number PX795814.

Declarations

Ethics approval and consent to participate

The experiments were carried out in the Botany and Microbiology Department and Entomology and Zoology Department of Helwan University in Egypt. The animal protocols adhered to Helwan University's standards for the care and use of laboratory animals. The research ethics number (HU-IACUC/B/HE0619-01) was assigned to these rules following their approval by the university's Animal Ethics Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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