Influence of Ethanol Extracts From *Pholiota Adiposa* on Gut Microbiota and Their Antitumor Properties in H22 Tumor-Bearing Mice

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Abstract

Hepatocellular carcinoma (HCC) is a common type of cancer—prevalent worldwide—and one of the causes of cancer-related deaths. In this study, ethanol extracts from *Pholiota adiposa* (EPA) were used to identify possible targets for HCC treatment and their effects on intestinal microflora were analyzed. Methods: Male mice were randomly assigned to groups—the model group, cyclophosphamide (25 mg/kg/d), and EPA groups, in which the mice were categorized based on the different concentrations of each compound (100, 200, and 300 mg/kg/day). Relevant biochemical indicators were detected using ELISA, H&E staining, and TUNEL assay. Four tumor apoptosis-related proteins and genes, Caspase3, *BAX*, *Bcl-2*, and VEGF, were detected by immunohistochemical staining, western blotting, and RT-PCR. The total genomic DNA was obtained from the contents of the small intestine and colon and was sequenced. The V3+V4 regions of bacterial 16s rDNA (from 341 to 806) were amplified. Results: The tests revealed that EPA exhibited antitumor activity in vivo by promoting apoptosis and inhibiting angiogenesis. Moreover, EPA treatment could increase beneficial and decrease harmful microflorae. These results demonstrate that EPA may be a potential therapy for HCC.

Introduction

The incidence of liver cancer is high worldwide, with the highest observed morbidity and mortality rates. . The incidence of liver cancer has decreased in developed countries, while it continues to increase in developing countries². The currently available treatment routes for cancer include surgery, radiotherapy, and chemotherapy, but the efficiency of these treatments is low and they have various adverse effects³. Therefore, it is necessary to develop or discover new low toxicity drugs for cancer treatment. The mouse hepatocellular carcinoma (HCC) cell line H22 is used extensively to establish an animal tumor model⁴, and cyclophosphamide (CTX) is mostly used as the positive control drug to investigate the antitumor effects of bioactive compounds⁵.

Drug therapy is a conventional strategy against cancer, and there are several antitumor drugs available in the market, such as CTX, paclitaxel, and oxaliplatin. Although chemotherapeutic drugs have good tumor inhibiting properties, they also have significant side effects, such as dysfunction of other organs or gastrointestinal problems⁶. Natural sources, especially medicinal fungi, may be considered as potential hypoglycemic agents because of their high efficacy, and low toxicity.

Pholiota adiposa is a famous edible mushroom found in Northeast Asia, and its fruit body has a better antitumor effect. *P. adiposa* is rich in polysaccharides, proteins, vitamins, and sterols⁷. The fruit body of *P. adiposa* is rich in medicinal properties and contains functional substances, such as polysaccharides, peptides, polyunsaturated fatty acids, ribonucleic acids, lectins, triterpenoids, adenine, and steroids⁸. Consumption of *P. adiposa* has potential benefits, including heat dissipation, and promotion and regulation of the human body⁹.

The relationship between intestinal microorganisms and the occurrence and development of diseases has attracted considerable attention. In the human body, the gut microbiota has important functions in metabolism, digestion, and intestinal barrier protection⁹. At present, development of molecular biology technologies, which affect intestinal microorganisms, has attracted the attention of researchers. and maintain the stability of the gut internal environment of the organism⁹¹, wherein the relatively stable structure of the gut microbiota is of great significance to human health. Several factors affect the diversity of gut microbiota, such as obesity, diabetes, irritable bowel syndrome, and cancer^{9, 12}. In addition, antibiotic use and diet also have certain effects on the diversity of the gut microbiota^{9, 13}. Increasing evidence indicates that aberrant gut microbiota has a profound impact on the onset and progression of cancer development; therefore, understanding the diversity of bacterial flora in feces can help understand the changes in disease¹⁴.

Previous research has shown that *P. adiposa* has a good antitumor effect⁸. In this study, ethanol extract from the fruit body of *P. adiposa* (EPA) was obtained and analyzed using LS-MS liquid mass spectrometry to demonstrate the antitumor activity of EPA in an H22 tumor-bearing mouse model. We investigated the possible molecular mechanisms underlying the antitumor action of EPA. Additionally, we also investigated the influence of EPA on gut microbiota using feces of tumor-bearing mice and compared it with that of the normal control group, model group, and CTX control group mice.

Materials And Methods

Materials and equipment. The fruits of *P. adiposa* were purchased from Songyuan City, Changchun, China and were identified by Professor Tolgor Bau of Jilin Agricultural University as *Pholiota adiposa*. All other chemical reagents used in this study were of analytical grade. CTX and barbiturate for injection was purchased from the Shanghai Huili Biotechnology Co., Ltd. H&E dye kits were obtained from the Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The TUNEL assay was performed in situ using an apoptosis detection kit (Roche, Branchburg, NJ, USA) and a DAB detection kit. ELISA kits for mouse, including interferon- γ (IFN- γ), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and vascular endothelial growth factor (VEGF) ELISA kits were purchased from the American R&D Co., Ltd. (Minneapolis, MN, USA). Rabbit polyclonal antibodies against Bcl-2 (3498S), BAX (2772S), Caspase3 (9661s), and mouse monoclonal VEGF(AV202) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA; TripleTOF 5600+ LC/MS/MS [AB SCIEX, America], Agilent 1290 Infinity LC/MS system [Agilent, USA], respectively). Ethanol (95%) was purchased from the Nanjing Chemical Reagents Co., Ltd. (Nanjing, China), and acetonitrile, methanol, and acetone were purchased from the Merck Company (chromatographic grade, Merck Company, Germany). Real-time PCR detector (LC96, Swiss)^{10, 47} was used.

Preparation and LC-MS analysis of *P. adiposa* extracts (EPA) Briefly, dried and powdered *P. adiposa* (1000 g) was extracted with 75% aqueous EtOH by ultrasonic treatment. The ultrasound conditions were as follows: ultrasonic power, 500 W; ultrasonic temperature, 60°C; ethanol concentration, 75%; liquid to solid

ratio, 37:1; and extraction time, 32 min. EPA was then concentrated and freeze-dried. The final crude extract (66.42 g) was stored at -20°C for further use⁴⁷.

For LC-MS analysis, 10 mg of EPA was dissolved in 5 ml of formic acid ultra-pure water solution and filtered through a $0.22\mu\text{m}$ nylon membrane before LC-MS analysis. The ultrasonic extraction method was used for extraction at 60°C for 30 min, repeated three times, centrifuged at 10 000r/min for 5 min, combined with supernatant and sample analysis. The Waters ACQUITY UPLC BEH C18 ($100\text{ mm}\times 2.1\text{ mm}$, $1.7\mu\text{m}$) column was used for chromatographic analysis in gradient elution mode at the flow rate of 0.3 mL/min and column temperature of 40°C . Mobile phase A was set as ultra-pure water containing 0.1% formic acid, and mobile phase B was acetonitrile, flow rate: 0.3 mL/min, and gradient elution mode was used for chromatographic separation (Table 1). Ionization mode Qualitative analysis mass spectrometry conditions: electrospray ionization (ESI), negative ion mode, and MSE mode were used for full scan analysis. The ion scanning range was m/z 50–2000; the temperature of dissolution was 400°C , the flow rate of the dissolution gas was 800 L/h, low energy voltage was 6 eV, high energy voltage was 20–50 eV, ion source temperature was 120°C , and capillary voltage was 2.5 kV. Then, the UPLC-QTOF-MSE mode was used to collect data, and Waters UNIFI software was used to analyze the results⁴⁷.

Antitumor activity in vivo

Use of experimental animals In this research, identifying the institutional and/or licensing committee approving the experiments, including any relevant details, and confirming that all experiments were performed in accordance with relevant guidelines and regulations. All experimental procedures were strictly in accordance with the Regulations of Experimental Animal Administration issued by the Ethics committee for Laboratory Animals at the Jilin Agricultural University (Permit No. ECLA-JLAU-19036)^{10, 47}. And the statement in the attachment.

Animals and cell lines Specific Pathogen Free grade, 6-8-week old male ICR mice weighing $20\pm 2\text{g}$, were purchased from the Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China), with Certificate No.: SCXK (Liao) 2019-0001. The mice were supplied with standard laboratory diet and water ad libitum at a temperature of $25 \pm 2^{\circ}\text{C}$ with a 12-h light/dark cycle (lights on 8:00 AM to 8:00 PM), and all mice were adapted to the environment for one week.

The mouse H22-hepatoma cell line was purchased from the Jilin Provincial Cancer Hospital (Jilin, China). H22 tumor mice were prepared as described previously, which were maintained in ascitic form by sequential passages into the peritoneal cavities of male ICR mice⁴⁸.

Preparation of tumor-bearing mice and treatment protocols H22-hepatoma cells were prepared at a concentration of 1.0×10^7 cells/mL. All mice, except the normal group, were inoculated in the subcutaneous right forelimb armpit with the tumor cell suspension (0.1 mL) to establish a tumor-bearing mouse model. After 24 h, 10 mice in each group were randomly divided into five groups (the experimental design is shown in Fig. 2), and five mice each were in one cage. The tumor-bearing mice were defined as

the model group, the positive treatment group, and the EPA treatment group. The EPA treatment group was further divided into high, medium, and low dose groups, with doses of 300 mg/kg (HD), 200 mg/kg (MD), and 100 mg/kg (LD), respectively. The control mice were intragastrically administered the same volume of saline solution, and the positive control was intraperitoneally injected with 25 mg/kg CTX. All groups of mice were treated once a day for 14 days^{10, 47}. Subsequently, all mice were euthanized that follow the AVMA Guidelines for Animal Euthanasia (2020) by intraperitoneal injection of barbiturate (180 mg/kg), and the tumor tissues were excised and weighed. The TIR index was calculated as $100\% \times (\text{average tumor weight of the control group} - \text{average tumor weight of the treatment group}) / \text{average tumor weight of the control group}$. The derivative with the highest TIR was subjected to subsequent experiments^{10, 47}.

Determination of Biochemical Parameters Freshly heparinized blood was prepared from the orbital venous plexus of mice. After centrifugation at 1500 rpm for 20 min at 4°C, sera were collected, and the serum samples of all groups were stored at 4 °C for biochemical assays. In accordance with the procedures described in the commercial kit, AST, BUN, IL-2, IL-6, TNF- α , IFN- γ , and VEGF levels of the mice were determined by indirect ELISA^{10, 47, 49}.

H & E and TUNEL assay H&E staining of H22 solid tumor cells was conducted following the manufacturer's instructions in the respective kit, and their results were obtained by optical microscopy. The H & E staining results of the tumor were shown as color images at 400 $\times$ magnification⁵⁰. TUNEL assay method was used to detect apoptotic cells in tumor tissues⁵¹. The tumor tissue sections were treated with 20 mg/mL proteinase K in distilled water for 15 min at room temperature. Next, 50 μ L TUNEL solution was added and incubated in the kit at 37 °C for 60 min. Peroxidase activity in each tissue section was determined using diaminobenzidine.

Immunohistochemistry The tumor tissue was fixed in 10% formaldehyde solution for 24 h, then decolorized, and buried. Subsequently, the tumor tissue was cut into 4-micron slices for immunohistochemical staining, which was performed using a murine/rabbit IgG immunohistochemistry kit (VEGF, Bcl-2, BAX, caspase3). Tumor tissue growth and apoptosis were evaluated by cell sequence, size of necrotic area, positive cell count, and staining intensity score^{47, 52}.

Western blot assay and quantitative real-time PCR Western blotting was used for qualitative and quantitative analyses of proteins⁵³. Cell lysates were prepared, and the concentrations of total protein were measured using a BCA assay kit (Pierce, Rockford, IL, USA). The total protein in some tumor tissues was extracted, and the protein content of each cell sample was determined by the Coomassant bright blue method⁵⁴. All proteins were resolved on a 15% SDS-denaturing polyacrylamide gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with antibodies against BAX (1:1000), caspase3 (1:1000), BCL-2 (1:1000), and VEGF (1:1000) overnight at 4°C. The membrane was then washed in PBST and incubated with horseradish peroxidase-conjugated sheep anti-rabbit (1:3000) and equine anti-mouse at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL) reagent was added, and protein mildness was observed using an enhanced chemiluminescence

detection system (Amersham Pharmacia Biotechnology, Tokyo, Japan). This method was used to analyze the levels of anti-apoptotic factors Bcl-2, BAX, VEGF, and Caspase3^{10, 47}.

RT-PCR was performed as described previously⁵⁵. Trizol reagent was used to isolate total RNA from the tumor tissue and then reverse transcribed into cDNA. Each cDNA sample was tested using real-time SYBR Green PCR reagent with specific primers (Table 2). The mRNA expression levels of Bcl-2 (3498S), BAX (2772S), Caspase3 (9661s), VEGF (AV202), and β -actin were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as a ratio compared to the control.

DNA extraction and 16s rDNA High-Throughput Sequencing

Collection and preparation of blood samples The normal group, model group, and HD of the EPA (300 mg/kg) group were tested, and the QIAamp Fast DNA Stool Kit (50) (Qiagen, Hamburg, Germany) was used to extract the DNA from the feces of the mice⁵⁶. A KAPA HiFi Hotstart ReadyMix PCR Kit was used for the PCR analysis. The bar-coded primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3') were used to amplify the V3-V4 hypervariable regions of bacterial 16s rDNA (from 341 to 806) from the extracted DNA, and the fluorimeter Qubit3.0 was used to pool, purify, and quantify the amplicons^{56, 57}. The amplicons were purified and quantified before being sequenced on the HiSeq 2500 PE250 Amplifier sequencing platform (Illumina, San Diego, CA, USA) by a commercial company (BIOPROFILE TECHNOLOGY Technology Co. Ltd., Shanghai, China).

Statistical analysis. All data are presented as mean $\pm$ standard deviation. The significance of the differences was determined by one-way analysis of variance, followed by the Duncan's test, with a significance level of $p < 0.05$. Statistical analyses were performed using the SPSS version 19.0.

Paired end sequencing was performed using the Illumina PE250 sequencing. Paired end reads grow reads through the overlap relationship between reads and quality control of the spliced reads. Then, we obtained the clean reads. According to the overlap, the sequence quality was controlled and filtered. OTU cluster analysis (operational taxonomic units) and taxonomic analysis were performed after distinguishing the samples. Finally, all clean reads were compared to OTU, and the reads of OTUs that could be compared were extracted. The final reads were obtained.

The alpha diversity index analysis method, which reflects the abundance and diversity of microbial communities, was used to analyze the single sample diversity, reflecting the abundance and diversity of microbial communities. The Shannon index, Simpson's index, and PD whole tree index, and the abundance of bacteria were calculated using the observed species index and Chao1 index to determine the diversity of gut bacteria. Beta diversity reflects differences among the different grouping samples. Anosim analysis, PCoA analysis, LefSe difference analysis, rank between groups, and other methods were used to analyze the difference significance.

Result

LC-MS analysis. As shown in Fig. 1, in the present study, under optimal chromatographic and MS conditions¹⁵⁻¹⁶, 3 compounds were identified by comparing the retention time and matching the empirical molecular formula with that of the known saponins; six phenolic compounds, seven fatty acid compounds, three nucleotide compounds, five quinone compounds, six carbohydrate compounds, and four steroid compounds were detected, wherein sterol compounds accounted for a high proportion, such as ergosterol peroxide, Ergosta-4, 6, 8(14)22-tetraen-3-one, ergosterol, and (22E,24R)-ergosta-7, 22-dien-3 β ,5 α ,6 β -triol (Table 3).

Comparative tumor weight, tumor inhibitory rate, and organ index analyses. The tumor volume growth curves are shown in Fig. 3. The tumor growth rate in the model group was more than 2.6 cm³ at day 15. In contrast, CTX-treated and all EPA-treated groups showed obvious inhibition of tumor growth.

The organ index, tumor weight, and tumor inhibitory rate (TIR) are shown in Table 4. The CTX-treated group exhibited the highest TIR (92.42%). Meanwhile, the tumor weight of EPA-treated groups significantly decreased in a dose-dependent manner, with tumor inhibitory rates of 79.70%, 84.55%, and 87.28%, respectively. The spleen and thymus indices were significantly lower in the CTX-treated group than in the normal group, and the thymus was severely atrophic ($p < 0.05$). The EPA-treated groups showed significant improvement, with a protective effect on the spleen and thymus.

Analysis of the expression of serum cytokines and VEGF in mice ELISA was used to investigate the effects of EPA on serum cytokines production, including TNF- α , IFN- γ , IL-2, AST, BUN, and IL-6, in H22 tumor-bearing mice. Angiogenesis has become an important therapeutic target for many tumors and is an essential process for tumor growth and metastasis^{10,17}.

In the results shown in Fig. 4, compared with the levels in the normal and model groups, the serum levels of IL-2, IL-6, IFN- γ , and TNF- α significantly increased in the EPA-treated HD group ($p < 0.05$). VEGF levels significantly decreased in the CTX-treated and EPA-treated groups. The AST and BUN levels of the model group were significantly higher than those in the normal control group ($p < 0.05$). The values of these two parameters significantly decreased in the EPA-treated groups compared to those in the model control group, ($p < 0.05$). The AST levels of mice in the CTX-treated group were significantly higher than those of the model group ($p < 0.05$).

Morphological analysis after treatment with EPA Histopathology of the tumor was evaluated using hematoxylin and eosin (H&E) staining. As shown in Fig. 5A, the tumor cells were plump and intact, with large nuclei in the model group. After treatment with EPA and CTX, the tumor cells exhibited a large necrotic region and were stained darker, which corroborates that the significant antitumor efficacy of EPA in H22 tumor-bearing mice involved by inducing cell death.

The TUNEL assay results are shown in Fig. 5B and 5C, wherein those with the brown granules were considered positively stained^{10,18}. The percentage of positive cells in the model group was 5%; therefore, the amount of tumor cell apoptosis was minimal. However, the CTX-treated group exhibited the highest percentage of positive cells (44%). The EPA-treated groups showed an increase in the number of cells

undergoing apoptosis, at 12%, 28%, and 42%, respectively. Moreover, the mice in the HD group of the EPA-treated group showed a positive cell percentage similar to that of the CTX-treated group.

Effects of EPA treatment on the expression of apoptosis-related proteins and VEGF. Based on the results of previous studies¹⁹, immunohistochemical analysis was performed on the pro-apoptotic factor BAX, the anti-apoptotic factor Bcl-2, and caspase3 to determine the expression of apoptosis-related proteins, as shown in Fig. 6. BAX and caspase3 expression intensities increased, while the Bcl-2 expression decreased in the EPA-treated groups in a dose-dependent manner. Therefore, the ratio of Bcl-2 to BAX decreased significantly. The vascular endothelial growth factor (VEGF) in the EPA-treated groups significantly decreased in a dose-dependent manner, and its expression in the CTX-treated group was the lowest.

In addition, western blotting was used to analyze the expression of Bcl-2, BAX, Caspase3, and VEGF. As shown in Fig. 7, all EPA-treated groups showed increased BAX and caspase3 protein expression and decreased Bcl-2 and VEGF protein expression; these results are consistent with the results of the immunohistochemical analysis. However, the expression of PI3K/AKT and p-AKT/AKT increased in a dose-dependent manner in the EPA-treated group.

Analysis of rank-abundance curves of microorganisms in samples The Rank-Abundance Curve is a method used to analyze the diversity of microorganisms. In this study, the operational taxonomic unit (OTU) rank-abundance curves showed the diversity of samples, which can be explained by species richness and evenness²⁰. In the horizontal direction, the wider the span of the curve, the richer the species rank of the microorganism sample²¹. The OTU rank-abundance curve in this study showed that the abundance distribution of the samples were even, as it was flat and smooth in the vertical direction (Fig. 8). The richness and evenness of the samples shown in the rank-abundance curve indicate that the diversity of the samples was rational and reasonable.

Dilution curves of microorganisms in the gut microbiota The dilution curve was used to evaluate whether the sample sequencing depth was sufficient to cover all species of microbes and compare species richness in different sequenced samples. When the curve tends to be flat, it indicates that the sequencing data are reasonable in reflecting the structure of the whole community²². The number of sequences reached 5000, and the curves are in the plateau stage (Fig. 9). Therefore, the experimental data were deemed reasonable and reliable.

Species accumulation curves of species in the gut microbiota The species accumulation curve is used to describe the increase in species with the increase in the number of samples and is an effective tool for investigating the composition and predicting the abundance of species in samples. This curve reflects whether the number of samples was sufficient. In this study, we mainly observe the end of the curve; if it showed a sharp upward trend, the number of samples was considered insufficient, while if the end of the curve tended to be flat, the number of samples was considered sufficient²³. The end of the species accumulation curves of Specaccums tended to be flat in this study; therefore, the sampling was considered sufficient to carry out data analysis (Fig. 10).

Results of the alpha diversity of the gut microbiota In this study, six indicators, including the Chao1 index, observed species index, PD_whole_tree index, goods-coverage index, Shannon index, and Simpson's index, were analyzed to assess the species diversity of individual samples (Fig. 11). There was no significant difference in flora abundance and diversity between the normal, model, and HD group of the EPA-treated group.

Results of the beta diversity analysis in the gut microbiota Beta diversity analysis was used to compare the differences in species diversity of intergroup samples, and the evolutionary distance between species was considered while calculating the results. The higher the index value, the greater was the difference between the samples. In this study, the UniFrac results were divided into two parts: weighted UniFrac, which considered the abundance of the sequence, and unweighted UniFrac, which did not consider the abundance of the sequence. According to the statistical results of the differences of the clustered samples, the similarity of species composition of samples was determined by calculating the distance between the samples²⁴.

Anosim similarity analysis was used to determine the intergroup and intragroup differences, wherein the grouping was found to be meaningful (Fig. 12). The R value of the unweighted results was $R = 0.34 > 0$ and $p = 0.001 < 0.05$, indicating that the differences between the groups were significantly greater than those within the group (intragroup). Therefore, the result is reasonable and the data are statistically significant. Thus, in this study, the gut microbiota of tumor-bearing mice tended to be normal after EPA treatment.

Principal coordinates analysis (PCoA) was used to evaluate the differences between samples (Fig. 13)²⁵. If the samples were in close proximity, the species compositions of the samples were similar. The EPA-treated group was closer in distance to the normal group, indicating that both groups had similar gut microbiota.

Difference Analysis in Gut Microbiota To emphasize the statistical significance and biological relevance, the LEFse analysis of gut microflora was presented quantitatively in an LDA Score column and a cladogram. This study aimed to estimate the impact of the abundance of each component and identify the significant difference effect on the division of samples²⁴. The results showed a significant difference in gut microflora, such as in *Lactobacillus*, *Streptococcus*, *Streptococcaceae*, *Rikenellaceae*, *Alistipes* (Nor), *Campylobacteriales*, *Epsilonproteobacteria*, *Helicobacteraceae*, *Helicobacter*, *Escherichia_Shigella*, *Subdoligranulum* (Mod), *Prevotellaceae*, *Proteobacteria*, *Bacteroidaceae*, *Bacteroides*, and *Selenomonadales* (EPA) (Fig. 14 and 15).

Analysis of species abundance in the gut microbiota Histograms of species profiling of gut microflora for each sample were used to identify the species with higher relative abundance and individual samples at different classification levels (Fig. 16A and Table 5)²⁶. In this study, the phylum level showed that *Firmicutes*, *Bacteroides*, and *Proteobacteria* accounted for more than 98% of the total microflora with different treatments. Compared with the normal group, the abundance of *Bacteroidetes* and *Firmicutes*

decreased, but *Proteobacteria* decreased in the model group. In contrast, the abundance of *Bacteroidetes* and *Firmicutes* increased, but *Proteobacteria* decreased in the EPA-treated, and was close to that in the normal group. Thus, the changing trend of the two phyla of microflora in the EPA-treated group was similar to that in the model group.

Fig. 16B shows the genus level of the gut microflora in the three groups. According to the abundance, the main groups were *Lactobacillus*, *Bacteroidales_S24-7_group_norank*, *Bacteroidales*, *Lachnospiraceae_NK4A136_group*, *Prevotellaceae_UCG-001*, *Alloprevotella*, *Streptococcus*, *Alistipes*, *Prevotellaceae_UCG-003*, and *Helicobacter*. From the changing trend of the abundance of gut microflora, it can be concluded that the tumor-bearing mice that were treated with EPA tended to be closer to that of the normal control group. Compared with the normal group, the abundance of beneficial microflora of *Lactobacillus*, *Bacteroidales_S24-7_group_norank*, *Bacteroidales*, *Alloprevotella* and *Alistipes* in the model group decreased by 6.95%, 25.25%, 4.67%, 2.65%, and 0.68% respectively; accordingly, compared with the model group, those of the EPA group increased by 10.67%, 19.94%, 1.34%, and 3.29%, respectively, which showed a significant reversion to the reduction of beneficial microflora in the model group. In contrast, compared with the normal group, the abundance of the harmful microflora of *Lachnospiraceae_NK4A136_group*, *Prevotellaceae_UCG-001*, *Helicobacter*, and *Prevotellaceae_UCG-003* in the model group increased to 16.39%, 7.01%, 5.83%, and 8.81%, respectively, while those in the EPA-treated group decreased to 17.99%, 1.44%, 5.81%, and 3.33%, respectively. These results showed that EPA treatment balanced the relative abundance of beneficial and detrimental bacteria in the gut microflora, as observed in the normal group.

Discussion

Antitumor effect of EPA Chemotherapy is an important treatment method for cancer, but it has side effects and a high toxicity, which may be harmful to the body. In this study, although CTX had the highest TIR (92.42%), it seemed to damage the spleen and thymus from the organ index data. Additionally, the AST and BUN levels of the CTX-treated group were significantly increased as seen from the results of ELISA; therefore, increasing the burden on the kidney and liver²⁷.

EPA are mainly composed of ergosterol, ergosterol peroxide, Ergosta-4, 6, 8(14), 22-tetraen-3-one, and (22E, 24R)-ergosta-7, 22-dien-3 β , 5 α , and 6 β -triol. This study revealed that EPA had a better antitumor effect and had an obvious protective effect on the liver, kidney, spleen, and thymus compared with CTX treatment. The ELISA results showed that EPA may have an inflammatory response for improving IL-2, IL-6, and IFN- γ levels in the serum to enhance body immunity²⁸. H&E and TUNEL staining, which were performed to observe apoptosis of tumor cells showed that EPA corroborates the remarkable antitumor efficacy of EPA in H22 tumor-bearing mice by inducing cell death/apoptosis.

The mitochondria-dependent pathway is one of the classical apoptosis pathways, and the most important players are members of the Bcl-2 family²⁹. A decrease in the ratio of Bcl-2 to BAX could determine the expression of apoptotic proteins in related tissues³⁰. After treatment with EPA, BAX

expression increased significantly. Bcl-2 expression and ratio of Bcl-2 to BAX decreased. The results of immunohistochemical and western blot analyses were consistent. Moreover, previous studies have reported that TNF- α levels could significantly increase the expression of *BAX* genes decrease the expression of *Bcl-2* gene in human tumor cells²⁹; these results were consistent with the ELISA results for TNF- α in this study. Caspases3 is a key apoptotic protein, its presence indicated that apoptosis had occurred in the tumor cells analyzed in this study³¹. In this study, the caspase3 levels significantly increased after EPA treatment. Therefore, we considered that the antitumor mechanism of EPA is achieved by regulating the expression of *Bcl-2* and *BAX* and activating the caspase family of proteins.

VEGF is a tumor marker, which is considered an important growth factor implicated in tumor angiogenesis³². In this study, after treatment with EPA, VEGF expression was obviously decreased. The immunohistochemical, ELISA, western blot, and RT-PCR assays confirmed that EPA could effectively reduce the generation of tumor angiogenesis, promote the expression of apoptotic genes and proteins, and inhibit tumor growth.

Influence on the gut microbiota of mice In this study, the gut microbiota in mice of the main genera with relative abundance were analyzed. It was suggested that EPA can increase the abundance of beneficial microflora, decrease harmful microflora and restore the gut microbiota of tumor-bearing mice. The main harmful microbiota identified in this study included *Prevotella*, *Lachnospirace*, and *Helicobacter*.

Prevotella is a harmful organism and a common conditional pathogen that can cause dose-dependent visceral hypersensitivity and endogenous infections in some parts of the body.

Both *Lachnospirace_NK4A136_group* and *Helicobacter* are classified as gram-negative anaerobic bacteria, which can cause severe inflammation in the body, such as diarrhea, watery stools, intestinal infections, bacteriosis, septicemia, cancer, and cholecystitis, and can cause immune dysfunction, bacterial flora imbalance, and endogenous infection³³. The abundance of *Prevotella*, *Lachnospirace_NK4A136_group*, and *Helicobacter* were 0.02%, 7.59%, and 0.29%, respectively, in the normal group, 16.01%, 23.99%, and 6.12, respectively, in the model group, and 11.23%, 5.99%, and 0.31%, respectively, in the EPA-treated group (Fig. 16 and Table 6). This indicated that, compared with the normal group, the tumor microenvironment can cause an endogenous infection and have an adverse effect on the structure of gut microbiota in the body of mice. However, after treatment with EPA, the *Prevotella*, *Lachnospirace_NK4A136_group*, and *Helicobacter* in the gut microbiota of tumor-bearing mice was significantly reduced and tended to be closer to the levels in the normal group.

The main beneficial microbiota included *Lactobacillus*, *Bacteroidales*, *Alloprevotella*, and *Alistipes*. *Lactobacillus* is a gram-positive bacterium, which regulates gut microflora, enhances immunity, protects the gastric mucosa, improves intestinal function, prevents diarrhea, and promotes digestion, and antitumor and anti-oxidation effects^{34,35}. It breaks down polysaccharides for other bacteria in the intestine and plays an important role in intestinal balance³⁶.

Bacteroides and *Alistipes* belong to the genus Bacterioa. *Bacteroides* is a symbiotic bacterium in the gut of humans that provides essential nutrients and directly inhibits the adhesion and invasion of other harmful bacteria through their own colonization³⁷. According to reports, *Bacteroides* and its metabolites are closely related to tumors, which can regulate cell proliferation, differentiation, apoptosis, and inhibit tumors³⁸. *Alistipes* is a relatively new genus that is highly relevant to dysbiosis and disease³⁹. It has also been reported that it has protective effects against some diseases, such as cancer, liver fibrosis, colitis and cardiovascular disease⁴⁰. In this study, EPA effectively reduced the generation of tumor angiogenesis and promoted the expression of apoptotic genes and proteins, such as *BAX*, *Bcl-2*, *VEGF*, and caspase3. Thus, we can conclude that the antitumor effect of EPA may be related to the effect of the beneficial microbiota *Bacteroides* in some antitumor or apoptosis pathways.

Alloprevotella has the ability to produce folic acid and vitamin B1, which are important bioactive substances that decompose and utilize the protein in the intestinal mucus⁴¹. The final metabolites were succinic acid and acetic acid. It has been reported that appropriate proportions of succinic acid and acetic acid can directly provide energy for the intestinal epithelium, improve intestinal digestion and absorption of nutrients, and improve intestinal immunity⁴².

Conclusions and Expectation

According to the literature, extracts of edible and medicinal fungi have anti-inflammatory effects and reduce the release of cytokines⁴³. In a previous study, polysaccharides were extracted from *Pholiota adiposa*, which exhibited a tumor inhibition⁸. In this study, we were able to conclude that EPA can significantly increase the TIR, especially in the HD group of EPA-treated mice, which tends to be the positive treatment group (CTX). Verification by molecular mechanism, after treatment using EPA, results of immunohistochemical, ELISA, western blot, and RT-PCR assays showed that EPA could effectively reduce tumor angiogenesis, promote the expression of apoptotic genes and proteins, and inhibit the growth of the tumor.

Instability of gut microbiota in the body is one of the causes of cancer⁴⁴. Dysbacteriosis may make an individual prone to chronic inflammation, while chronic inflammation increases the risk of developing tumors⁴⁵. At present, the possible correlation between gut microbiota and tumors has been studied. Gut microbiota also has an impact on the generation, development, therapeutic efficacy, and treatment of malignant tumors⁴⁶.

In this study, the harmful microbiota increased, while the beneficial microbiota decreased in mice inoculated with H22 hepatoma cells. After treatment with EPA, the gut microbiota of tumor-bearing mice tended to be closer to that of the normal group. Hence, we consider that EPA can increase the abundance of beneficial microbiota and decrease the abundance of harmful microbiota. The findings of our study provide data for future research on disease and microecology.

In conclusion, although EPA can regulate the gut microbiota of tumor-bearing mice, the specific mechanism remains unclear. With the development of macrogenomics, metabonomics, and molecular biology, people will have a more specific understanding of the interaction between food or drugs and gut microbiota. It will further deepen and expand the research on EPA, its antitumor mechanism, and influence on the gut microbiota.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 LC/MS-Q-TOF gradient elution program

Time(min)	Mobile Phase	
	A(%)	B(%)
0	95	5
2	80	5
5	50	20
15	20	50
45	10	95
55	10	95
55.01	95	5
65	95	5

Table 2 Primers used in the present study

Primer	Sequence 5'-3'
Caspase3-F	GCGACTACTGCCGGAGTCT
Caspase3-R	ACCGGTATCTTCTGGCAAGC
BAX-F	GGTTGCCCTCTTCTACTTTGC
BAX-R	GTCCAGCCCATGATGGTTCT
BCL2-F	CTCTCGTCGCTACCGTCGT
BCL2-R	CCGAACTCAAAGAAGGCCAC
VEGF-A-F	TACTGCCGTCCGATTGAGAC
VEGF-A-R	TCCAGGGCTTCATCGTTACA

Table 3 Affiliation of key peaks of EPA

RT (min)	CAS number	Component name	Chemical formula	Expected mass (Da)	Observed m/z	Mass error (mDa)	Percentage %
27.31	2061-64-5	Ergosterol peroxide	C ₂₈ H ₄₄ O ₃	428.32905	427.31985	-0.51	0.799
27.40	19254-69-4	Ergosta-4,6,8(14)22,-tetraen-3-one	C ₂₈ H ₄₀ O	392.30792	391.29748	-0.71	0.3256
27.46	57-87-4	Ergosterol	C ₂₈ H ₄₄ O	396.64805	395.42196	-0.82	0.35
30.06	14858-07-2	(22E,24R) - ergosta-7,22-dien-3 β ,5 α ,6 β -triol	C ₂₈ H ₄₆ O ₃	430.34471	465.31065	-1.45	0.158

Table 4 Effect of EPA on tumor weights and tumor inhibition rate.

Groups	Dose	Organ index (%)		Tumor weight (g)	Tumor Inhibitory rate (%)
		Thymus	Spleen		
Normal		5.75±1.12 ^{**}	5.62±1.95 ^{**}	-	-
Model		4.16±1.37	10.86±1.47 ^{##}	3.30±0.72	-
CTX	25mmg/kg	2.42±0.48 ^{**##}	2.73±1.24 ^{**##}	0.25±0.12 ^{**}	92.42%
LD of SLM	100mg/kg	4.93±1.25 ^{**}	8.63±1.31 ^{**##}	0.67±0.25 ^{**}	79.70%
MD of SLM	200m g/kg	4.87±0.94 ^{**}	7.54±1.09 ^{**##}	0.51±0.18 ^{**}	84.55%
HD of SLM	300 mg/kg	4.58±1.19 [*]	6.73±1.36 ^{**#}	0.42±0.13 ^{**}	87.28%

Table 5. The relative abundance of the main gut microbiota at the phylum level in four groups.

Abundance (%)			
	A	B	C
<i>Firmicutes</i>	53.63254	38.56821	51.84526
<i>Bacteroidetes</i>	37.78549	31.18542	39.85831
<i>Proteobacteria</i>	3.37934	24.44789	7.15324
Total	94.79737	94.20152	98.85681

Table 6. The relative abundance of the main gut microbiota at the genus level in four groups.

Abundance (%)			
	A	B	C
<i>Lactobacillus</i>	18.075844	11.127102	21.798470
<i>Bacteroidales_S24-7_group_norank</i>	28.471373	3.206927	23.148881
<i>Bacteroides</i>	6.387470	1.717157	3.058214
<i>Alloprevotella</i>	2.667242	0.0959440	3.305270
<i>Alistipes</i>	0.702790	0.021587	0.981027
<i>Lachnospiraceae_NK4A136_group</i>	7.593965	23.98599	5.994099
<i>Prevotellaceae_UCG-001</i>	0.018469	7.195798	5.754240
<i>Helicobacter</i>	0.287832	6.115133	0.314217
<i>Prevotellaceae_UCG-003</i>	0	8.807656	5.473603
Total	64.204985	62.273294	69.828021

Figures

Fig. 1 The total ion chromatogram of the extracted of *Pholiota adiposa* detected by LS-MS.

Figure 1

<p>See image above for figure legend</p>

Figure 2

<p>See image above for figure legend</p>

Fig.3 The effects of EPA on tumor H22 tumor-bearing mice

Figure 3

<p>See image above for figure legend</p>

Fig.4 Effects of EPA on levels of serum cytokines, including, IL-2 (A), IL-6 (B), IFN-γ (C), TNF-α (D), VEGF (E), AST (F) and BUN (G)

Figure 4

<p>See image above for figure legend</p>

Figure 5

<p>See image above for figure legend</p>

Figure 6

<p>See image above for figure legend</p>

Fig.7 Relative protein expression of BAX, Bcl-2, Caspase3, AKT, p-AKT,PI3K and VEGF in tumor tissue

Figure 7

<p>See image above for figure legend</p>

Figure 8

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Figure 9

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Figure 10

<p>See image above for figure legend</p>

Figure 11

<p>See image above for figure legend</p>

Fig.12 Anosim analysis of gut microbiota based on unweighted unifrac distance

Figure 12

<p>See image above for figure legend</p>

Fig.13 Principal coordinates analysis (PCoA) analysis of gut microbiota based on Weighted Unifrac distance. It illustrated the difference in the microbial composition among the samples

Figure 13

<p>See image above for figure legend</p>

Fig. 14 The LDA score obtained from LEfSe analysis of gut microbiota in different groups.

Figure 14

<p>See image above for figure legend</p>

Cladogram

Figure 15

<p>See image above for figure legend</p>

Figure 16

<p>See image above for figure legend</p>