Antiviral Role of Eugenol Against Largemouth Bass Ranavirus Infection

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Article

Antiviral role of Eugenol Against Largemouth Bass Ranavirus Infection

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Abstract: Largemouth bass ranavirus (LMBV) is a severely harmful pathogen in the largemouth bass culture industry. This virus causes high mortality rate in largemouth bass during its epidemic season, resulting in huge economic losses. While, eugenol (EUG) has potent antiviral activity showing promising potential to against LMBV. Thus, to investigated EUG's efficacy against LMBV, an analysis of the autophagy and apoptosis was conducted and confirmed the anti-LMBV activity of EUG *in vivo* and *in vitro*. Firstly, EUG can not only directly inhibit the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, but also promote the AMP-activated protein kinase (AMPK) pathway in fathead minnow (FHM) cells during LMBV infection. This effect has the capacity to activate and enhance the autophagy of the FHM cells against its apoptosis that has been induced by LMBV. Notably, EUG reduced the viral load present within the tissues of LMBV-infected largemouth bass, thereby ultimately enhancing their survival rate within a culture setting. These mechanistic assays revealed the anti-LMBV properties of EUG, which could significantly enrich the content of antiviral research and provide valuable references for aquaculture.

Keywords: largemouth bass; eugenol; antiviral; autophagy; apoptosis

1. Introduction

Largemouth bass ranavirus (LMBV), with an icosahedral shell enveloping a double-stranded DNA, is originally discovered from largemouth bass (*Micropterus salmoides*) in the United States [1, 2]. Meanwhile, largemouth bass has gradually become an important economic fish and is widely cultured in China due to its tasty meat, but this aquaculture industry is continuously damaged by the threat of LMBV, one of the most harmful pathogens [3, 4]. Although LMBV exhibits a broad host range, natural infections primarily affect largemouth bass [5]. LMBV is most prevalent in summer, with outbreaks more likely at 25~30 °C. The symptoms of LMBV infection are very different by region. In China, the most common clinical symptoms of LMBV infection in bass are include swelling of the internal organs, body surface ulceration and muscle necrosis [6, 7]. Previous study revealed that LMBV infection can significantly trigger the immune activation and inflammatory response in largemouth bass [6]. Inhibiting largemouth bass nuclear factor kappa B (NF-κB)/P65 *in vivo* significantly promoted LMBV infectivity, while overexpression of *Micropterus salmoides* P65 *in vitro* positively regulated interferon and related genes, exerting antiviral effects [8]. LMBV infection induces distinct cell death pathways depending on the host cell type. Specifically, it could promote apoptosis in fathead minnow (FHM) cells and autophagy couple with apoptosis in epithelioma papulosum cyprinid (EPC) cells [9]. In contrast, it caused non-apoptotic cell death in *M. salmoides* fin (MsF) cells [10]. Inhibitors of phosphatidylinositol 3-kinase (PI3K) pathway and extracellular-signal-regulated kinases (ERK) signaling pathways in EPC cells significantly suppress LMBV replication and regulate LMBV-induced apoptosis [11]. Furthermore, promoting cellular autophagy has been

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shown to confer antiviral effects. Collectively, these findings highlight autophagy and apoptosis as two key pathological mechanisms underlying LMBV infection and pathogenesis.

Eugenol (EUG) is a major volatile component of clove essential oil and possesses broad antiviral properties that have been shown to work in many studies [12, 13]. It has been shown to attenuate herpes virus-induced keratitis and inhibit the viral infection in mice [14]. In previous work, we have verified that EUG inhibits the expression of inflammatory factors caused by Singapore grouper iridovirus (SGIV) in grouper spleen (GS) cells while alleviating cell oxidative stress through reduction of reactive oxygen species (ROS) levels [15]. Additionally, it has been reported that EUG inhibited influenza A virus (IAV) infection by suppressing the activation of mitogen-activated protein kinase (MAPK) and inhibitor of kappa B kinase (IKK)/NF-kB pathway, which is consistent with our previous findings regarding its inhibitory effect on activation of the SGIV-induced MAPK pathway [16].

Viral infections are closely associated with various forms of cell death, including apoptosis, necrosis, autophagy, pyroptosis and ferroptosis. It is commonly believed that infection leads to cell death and an inflammatory response, but it has also been shown that altruistic cell death of infected cells can be beneficial to the organism as a whole, ultimately limiting the path of virus production [17]. Autophagy has been implicated in the development of a lot of viral diseases, either survival or apoptosis of cancer cells can be promoted by modulating autophagy. In breast cancer cells, EUG induced autophagy by up-regulating the threonine kinase 1- forkhead box O3 (AKT-FOX3) pathway to promote apoptosis, and at the same time, up-regulating the expression of microtubule-associated protein 1 light chain 3 (LC3I) and down-regulating the expression of nucleoporin 62 (NU P62) [18]. EUG has also been shown to attenuate the inflammatory response by inhibiting the PI3K/AKT pathway in studies using a mouse model of conjunctivitis [19]. Furthermore, recent studies also indicate that high-concentration EUG treatment modulates the AMPK/AKT/mTOR pathway, thereby attenuating acrylamide (ACR)-induced testicular toxicity in mammals [20], suggesting that EUG can affect AKT-related signaling pathways in different diseases.

In this study, we investigated the effectiveness of EUG against LMBV and elucidated the underlying cellular pathways. Our results show that EUG can reduce the apoptosis induced by LMBV in FHM cells and enhance autophagy in abovementioned cells by regulating AKT/mTOR and AMPK signaling pathways. Moreover, EUG could potently suppress the transcriptional levels of LMBV genes and improve survival outcomes in LMBV-challenged largemouth bass. These antiviral effects obviously revealed that EUG could be a potential drug for the prevention and treatment of LMBV, offering practical value to the aquaculture industry.

2. Materials and Methods

2.1. Ethics Statement

Our experiments with laboratory animals adhered to the South China Agricultural University's (SCAU) institutional ethical guidelines. This study was approved by SCAU (ethical protocol code: 2020g009), and a certificate of approval is available upon request.

2.2. Fish, Cells and Virus

Two hundred largemouth bass (8-10 cm) were purchased from a fish farm in Guangdong Province and prepared for antiviral and toxicity analyses. The fish were cultured in a recirculating water system at 25~30 °C and fed for 10 days before being used in the experiments. Prior to the formal experiment, 10 fish were randomly selected for PCR detection to ensure that they were LMBV negative. LMBV was isolated from diseased largemouth bass and stored in our laboratory [11].

The FHM cell line was propagated according to the recommended methods using M199 culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 28 °C [21].

2.3. Cell Treatment

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EUG (Sigma-Aldrich, E51791) was diluted to 500 mM in dimethyl sulfoxide (DMSO) at the time of use and further diluted to working concentrations in M199 medium containing 10% FBS. For viral infection experiments, cells were treated with different concentrations of EUG according to the experimental requirements, and cell cultures were pretreated with varying concentrations of EUG for 2 hours, followed by LMBV infection. The control group was treated with an equal volume of DMSO without the addition of EUG.

2.4. Cytotoxicity Assays

The cytotoxic effects of EUG on FHM cells were evaluated using the Cell Counting Kit-8 (US Everbright @Inc., Suzhou, China) assay in 96-well plates. Five groups of EUG concentrations were tested: 0 μM (DMSO), 50 μM, 75 μM, 100 μM and 150 μM, with eight replicates in each set. Cells were treated with EUG or DMSO for 24 h after wall attachment. Cell viability was assessed with a microplate reader (*Thermo Fisher*, USA) at 450 nm after 2 hours incubation in serum-free medium with CCK-8 and protection from light.

2.5. Virus Infection Assay In Vitro

FHM cells were cultured in 24-well or 6-well plates and treated with EUG or DMSO for 2 h prior to LMBV infection. Cells were collected at different time points for subsequent experiments, including qRT-PCR and western blotting.

Table 1. Primers utilized in this research.

Primer	Sequence (5'-3')
FHM-β-Actin-RT-F	TACGAGCTGCCTGACGGACA
FHM-β-Actin-RT-R	GGCTGTGATCTCCTTCTGCA
MS-β-Actin-RT-F	CCACCACAGCCGAGAGGGAA
MS-β-Actin-RT-R	TCATGGTGGATGGGGCCAGG
LMBV-MCP-RT-F	CTCGCCACTTATGACAGCCTTGAC
LMBV-MCP-RT-R	AACCCACGGGATAATGCTCTTTGAC
LMBV-MMP-RT-F	GCGTATTTCGCACCCTCTG
LMBV-MMP-RT-R	TAAGCGTCGCCCTTGTCTG

2.6. RNA Isolation and qRT-PCR Analysis

Tissues were ground in pre-cooled PBS with a homogenizer. Total RNA was isolated using the Animal/Cell Total RNA Isolation Kit (FORE GENE, Chengdu, China) and reverse-transcribed with the ReverTra Ace qPCR RT Kit (TOYOBO) [22]. An Applied Biosystems QuantStudio 5 Real Time Detection System (Thermo Fisher, *USA*) was used for qRT-PCR analysis and each experimental condition was as previously described [15]. The primers utilized for the PCR are detailed in Table 1. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression ratio of the selected genes to β-actin (reference gene).

2.7. Western Blotting Analysis

Treated FHM cells were collected and subjected to the western blotting assay according to the methods and materials previously used [23]. FHM cells were first digested with trypsin and collected on ice and lysed in Pierce IP lysis buffer (Thermo, USA) for 30 min, followed by centrifugation at 12,000 × g for 10 min to remove cellular debris, and the supernatant was mixed with 5 × Protein Sampling Buffer and boiled for 10 min. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA), and the blots were incubated in 5% skimmed milk. The blots were then incubated with specific primary antibodies for 2 h. After three washes with phosphate-buffered saline-Tween, the blot was incubated with peroxidase-conjugated secondary antibody IgG (1:5000 dilution, Abcam). Enhanced chemiluminescence (Thermo, USA) was used to

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visualise the immunoreactive bands. The specific primary antibodies used in the experiments were as follows: LMBV major capsular protein (MCP) (1:2000 dilution, GENECREATE), cleaved caspase3 (1:1000 dilution, CST), phospho-AKT (1:2000 dilution, CST), phosphor-mTOR (1:1000 dilution, CST), Beclin1 (1:1000 dilution, Proteintech), LC3 (1:2000 dilution, Abcam), P62 (1:1000 dilution, *Abcam*), Autophagy-related protein 5 gene (ATG5) (1:1000 dilution, Abmart), phospho-AMPK (1:1000 dilution, CST) and β-tubulin (1:2000 dilution, Abcam).

2.8. Annexin V-FITC/PI Apoptosis Assay

FHM cells were infected with LMBV after treatment with EUG or DMSO for 2 h. Cells were collected by trypsinization into 1.5 ml EP tubes and washed twice with cold PBS (0.15 mol/L, pH 7. 2). Cells were centrifuged at 3000 rpm for 5 min, after which the supernatant was discarded and the precipitate was resuspended in 1 × Binding buffer. 100 μL sample solution was transferred to 5 mL culture tubes and incubated with 5 μL FITC-labeled Annexin V (Pharmingen) and 5 μL PI (Pharmingen) for 15 min at room temperature in the dark. 400 μL of 1 × Binding Buffer was added to each sample tube and then analysed by flow cytometry.

2.9. Antiviral Activity of EUG In Vivo

We tested the safe concentration of EUG in fish injected intraperitoneally and then conducted anti-LMBV experiments on subgroups of largemouth bass. In the EUG safety concentration experiment, juvenile largemouth bass were divided into five groups of 20 individuals per group and injected with 100 μL of PBS containing different concentrations of EUG (0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L and 100 mg/L) for one week of observation. The mortality rate was recorded daily. After one week, the intestines, spleens and livers were removed and tissue sections were prepared to observe pathological changes induced by EUG. For the antiviral assay, juvenile largemouth bass were divided into two groups: a control group and an experimental group that was injected with LMBV + EUG (10 mg/L). The control group was injected with 100 μL of LMBV at a titer of 10^6 $TCID_{50}$/mL in PBS, while the experimental group received 100 μL of a mixture of LMBV and EUG. Mortality rates were recorded daily over a period of 7-14 days. The intestines, spleens and livers were then collected and tested for LMBV viral load in the tissue by qRT-PCR.

2.10. Statistical Analysis

All data were from at least three independent replicated experiments, results are expressed as mean ± standard deviation (SD), and data were analyzed using GraphPad Prism 9.5. Statistical analyses were performed using one-way ANOVA in SPSS version 20, and Student's t-test was used for statistical comparisons. Significance was set at $*p < 0.05$.

3. Results

3.1. Effect of EUG in Different Concentrations on LMBV Infection

FHM cells were initially selected to establish an *in vitro* model to study the effect of EUG on LMBV infection. We determined that the maximum safe concentration of EUG in FHM cells was 75 μM, while cell activity was significantly inhibited at 100 μM (Figure 1a). The major capsid protein (MCP) and myristoylated membrane protein (MMP) of Iridovirus play pivotal roles in the viral life cycle, participating in critical processes including host cell recognition and viral entry [24, 25]. Given their essential functions in viral pathogenesis, the quantitative changes in MCP and MMP expression levels were employed as key virological markers to assess viral replication efficiency in this study. We selected 50 μM and 75 μM EUG to pre-treat FHM cells before LMBV infection for 2 h. At 24 hours after infection, only the 75 μM EUG group markedly reduced the mRNA expression of the LMBV-MCP and LMBV- MMP genes (Figure 1b). Therefore, 75 μM EUG was used as the optimal treatment for FHM cells in all subsequent experiments.

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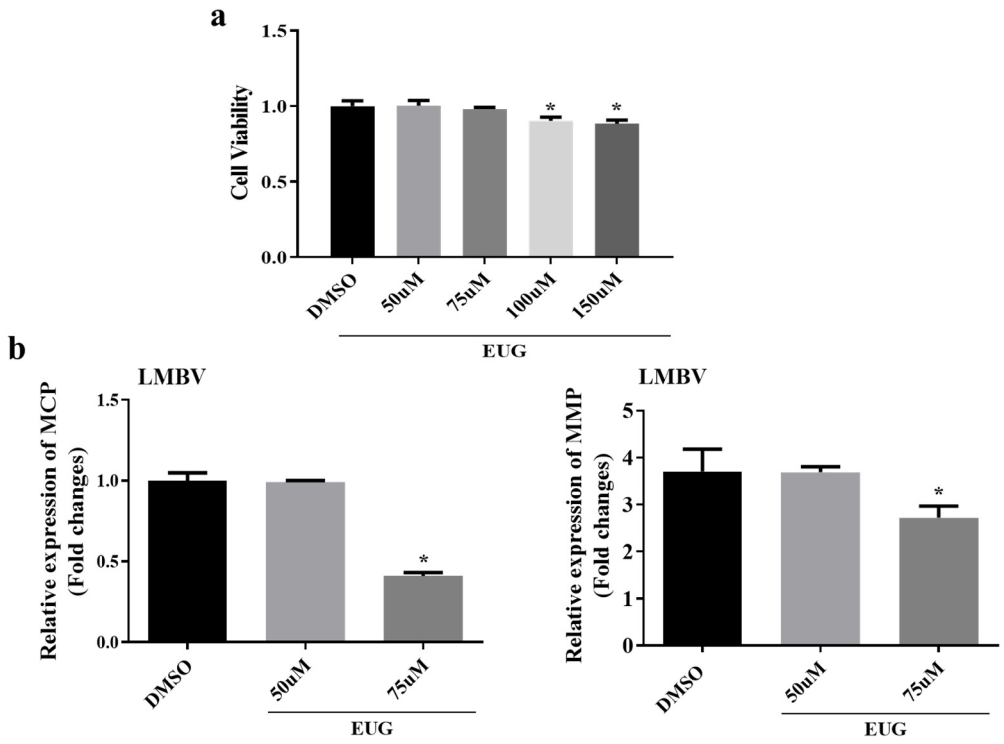

Figure 1. Maximum safe concentration of EUG and its effect on LMBV infection. (a) Different concentrations of EUG (50 μM, 75 μM, 100 μM and 150 μM) and DMSO (control) were co-cultured with FHM cells for 24 h. (b) After treating FHM cells with DMSO or different concentrations of EUG (50 μM, 75 μM) for 2 h, FHM cells were infected with LMBV, and the mRNA expression levels of LMBV-MCP and LMBV-MMP were detected by qRT-PCR after 24 h. Student's t-test: * $p < 0.05$.

3.2. EUG inhibits LMBV Infection at Different Time Points

To determine the *in vitro* antiviral efficacy of EUG, cells were incubated with 75 μM EUG for 2 h and then infected with LMBV. The mRNA and protein levels of LMBV genes were examined at different time points by qRT-PCR and western blotting. Viral gene mRNA levels were significantly reduced in EUG-treated groups at various time points (Figure 2a). Additionally, LMBV-MCP protein synthesis was also significantly reduced, indicating that the EUG was capable of inhibiting LMBV infection of FHM cells (Figure 2b,c).

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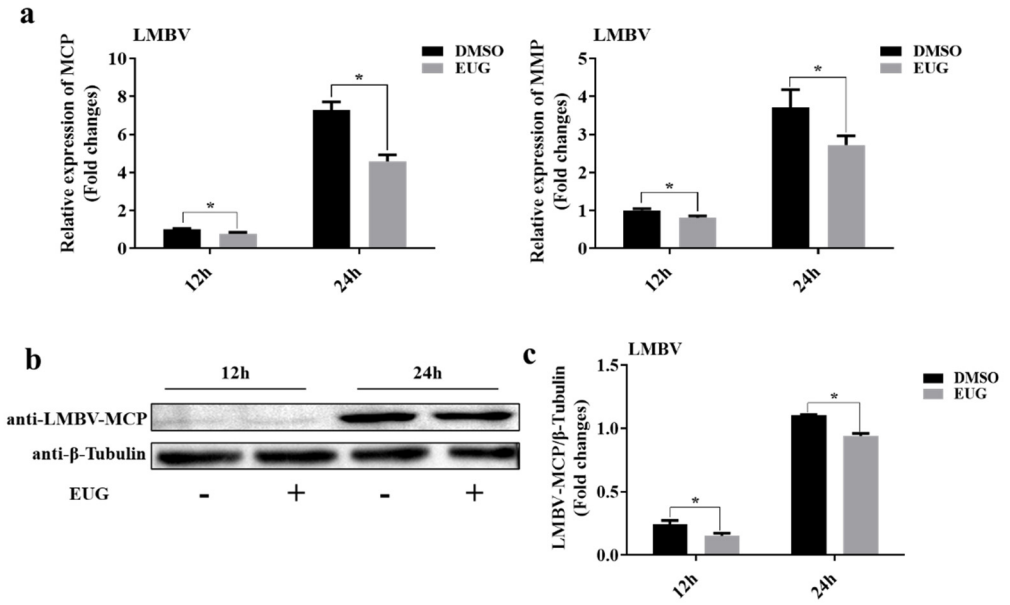

Figure 2. Effect of EUG on LMBV infection at different time points. (a) Cells were infected with LMBV after treatment with DMSO or EUG for 2 h. After 12 and 24 h, the gene expression levels of LMBV-MCP and LMBV-MMP were detected by qRT-PCR. (b) Cells were collected 12 and 24 h after infection, and LMBV-MCP protein and cellular β-tubulin were analyzed by western blotting, and MCP/β-Tubulin values were calculated for each group as described above. (c) Data are mean ± SD of four experiments. Student's t-test: * $p < 0.05$.

3.3. EUG Inhibits Apoptosis In Cells Infected by LMBV

As demonstrated by earlier research, LMBV infection instigates a process of apoptosis in FHM cells, whilst apoptosis concurrently serves to promote LMBV virus replication [11]. In order to ascertain the antiviral mechanism of EUG, we explored its effect on LMBV-induced apoptosis. FHM cells were exposed to EUG for a duration of 2 h prior to infection with LMBV or left untreated. Flow cytometry and western blotting were conducted to ascertain the degree of apoptosis. The analysis of cell samples by flow cytometry demonstrated that EUG had no influence on the degree of apoptosis in healthy cells; infection for 12 h significantly promoted FHM cell apoptosis, whereas EUG can inhibit corresponding FHM cell apoptosis induced by LMBV (Figure 3a,b). Subsequently, the cleaved-caspase-3 protein, apoptosis marker in FHM cell was detected by western blotting. Which indicated that this protein levels were significantly lower in the EUG-treated group than in the control group (Figure 3c). Overall, EUG significantly inhibited LMBV-induced FHM cell apoptosis.

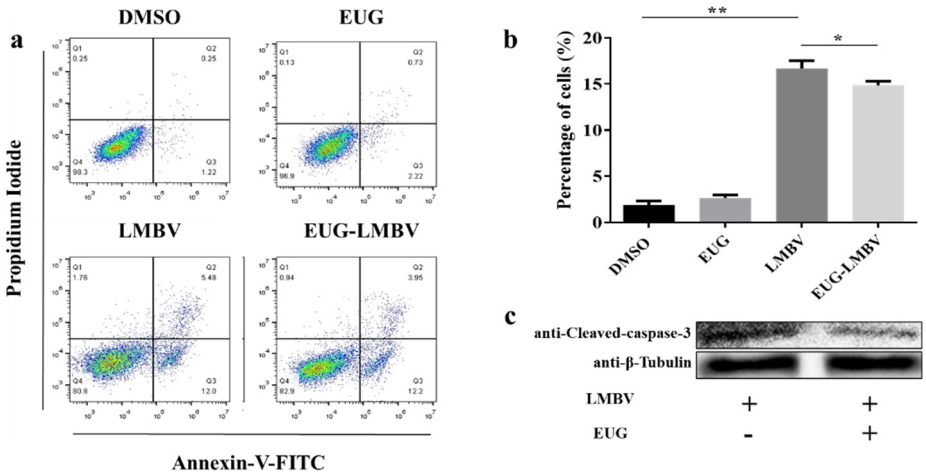

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Figure 3. EUG inhibits apoptosis induced by LMBV infection. (a) Cells were incubated with EUG for 2 h before LMBV infection and apoptosis was detected by annexin V/PI double staining after 24 h. (b) Quantitative analysis of the percentage of apoptotic cells. (c) FHM cells were treated with EUG for 2 h and infected with or without LMBV, and samples were collected 12 h later to detect cleaved-caspase-3 protein and cellular β-tubulin.

3.4. EUG Promotes Cellular Autophagy in LMBV Infection

LMBV infection leads to autophagy in EPC cells, and further promotion of autophagy inhibits LMBV replication and cell apoptosis [9]. It is known that the detection of Beclin1, LC3 and P62 proteins are key indices to evaluate autophagy level. We determined the level of autophagy in FHM cells by western blotting to investigate the mechanism of inhibition of apoptosis by EUG. FHM cells were harvested after EUG treatment and infection with LMBV for 12 h. The results in Figure 4 indicated that the levels of autophagy-related proteins (LC3I/II, Beclin-1, ATG5) were significantly increased in the EUG-treated group compared with the control group, while the level of P62 protein was decreased, suggesting that EUG treatment could increase the autophagy level of LMBV-infected cells.

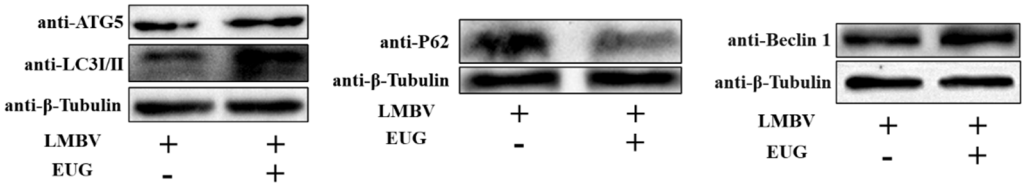

Figure 4. EUG promotes autophagy in FHM cells. FHM cells were infected with LMBV after incubation with EUG for 2 h. Cell samples were taken 24 h later and protein expression levels of the autophagy-related proteins LC3I/II, ATG5, P62 and Beclin1 were detected by western blotting.

3.5. EUG regulates AMPK and AKT/mTOR Pathway in LMBV Infection

Autophagy, a highly conserved cellular degradation process, is primarily regulated through several key signaling pathways, with the AKT/mTOR and AMPK pathways playing central roles in its modulation. The AKT/mTOR signaling axis serves as a critical negative regulator of autophagy. To explore the signaling pathway through which EUG regulated autophagy, the protein levels of p-AMPK, p-mTOR, and p-AKT were assessed by western blotting. As presented in Figure 5, EUG inhibits AKT and mTOR protein phosphorylation and increases the protein phosphorylation levels of AMPK. Therefore, AMPK and AKT/mTOR pathway participated in the autophagy mechanisms of EUG in LMBV infection.

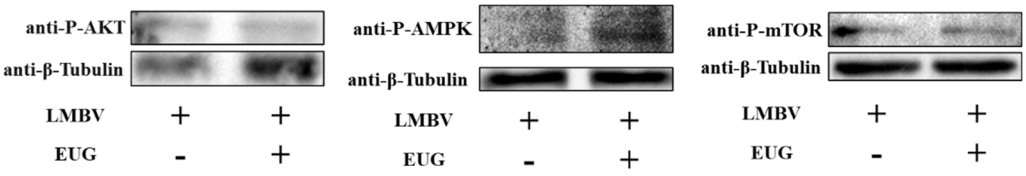

Figure 5. Cells were pretreated with EUG for 2 hours and then infected with LMBV; cell samples were collected after 24 h. and phosphorylation levels of AKT, AMPK, mTOR proteins were determined by western blotting.

3.6. EUG Reduces Mortality in LMBV-Infected Largemouth Bass

To determine the *in vivo* toxicity of the drug, EUG was diluted to different concentrations and injected intraperitoneally into largemouth bass and observed for 7 days. Intraperitoneal injection of 10 mg/L of EUG did not result in any mortality (Figure 6a). Histopathological results showed that there were no significant differences in the intestines, spleens and livers between the 10 mg/L EUG injection group and the control group (Figure 6b). To explore the *in vivo* effect of EUG on LMBV infection, largemouth bass were intraperitoneally injected with a mixture of 10 mg/L EUG and LMBV. It was found that the survival rate of the mixed EUG injection group reached 80%, which was

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significantly different from the 60% survival rate of the control group after 12 days (Figure 6c). The death caused by LMBV infection was inhibited by injection of EUG *in vivo*.

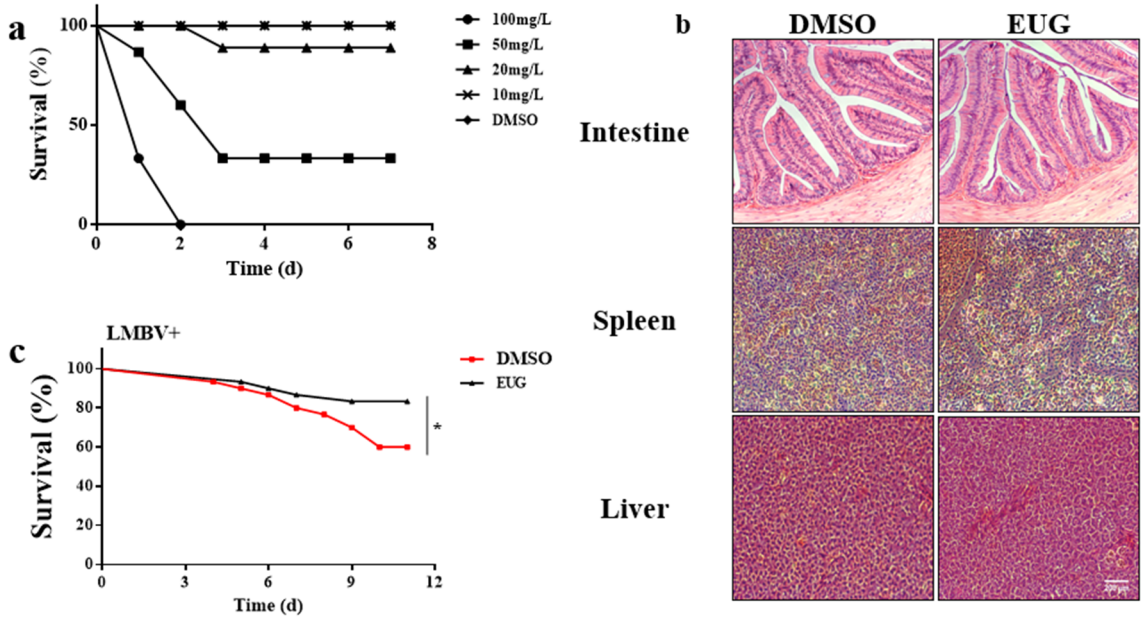

Figure 6. EUG reduces mortality in largemouth bass infected with LMBV. (a) Survival of largemouth bass after intraperitoneal injection of different concentrations of EUG. (b) HE staining of healthy largemouth bass tissues treated with EUG; (c) survival rate of largemouth bass after EUG injection.

3.7. EUG Inhibits LMBV Infection in Tissues

Several LMBV-infected largemouth bass tissues were collected and the mRNA levels of viral genes were tested by qRT-PCR after 12 days. The results showed that the mRNA levels of LMBV-MCP and LMBV-MMP in the intestines, spleens and livers of the EUG injected group were significantly lower than those of the control group, which confirms that EUG can inhibit the LMBV infection *in vivo* (Figure 7a–c).

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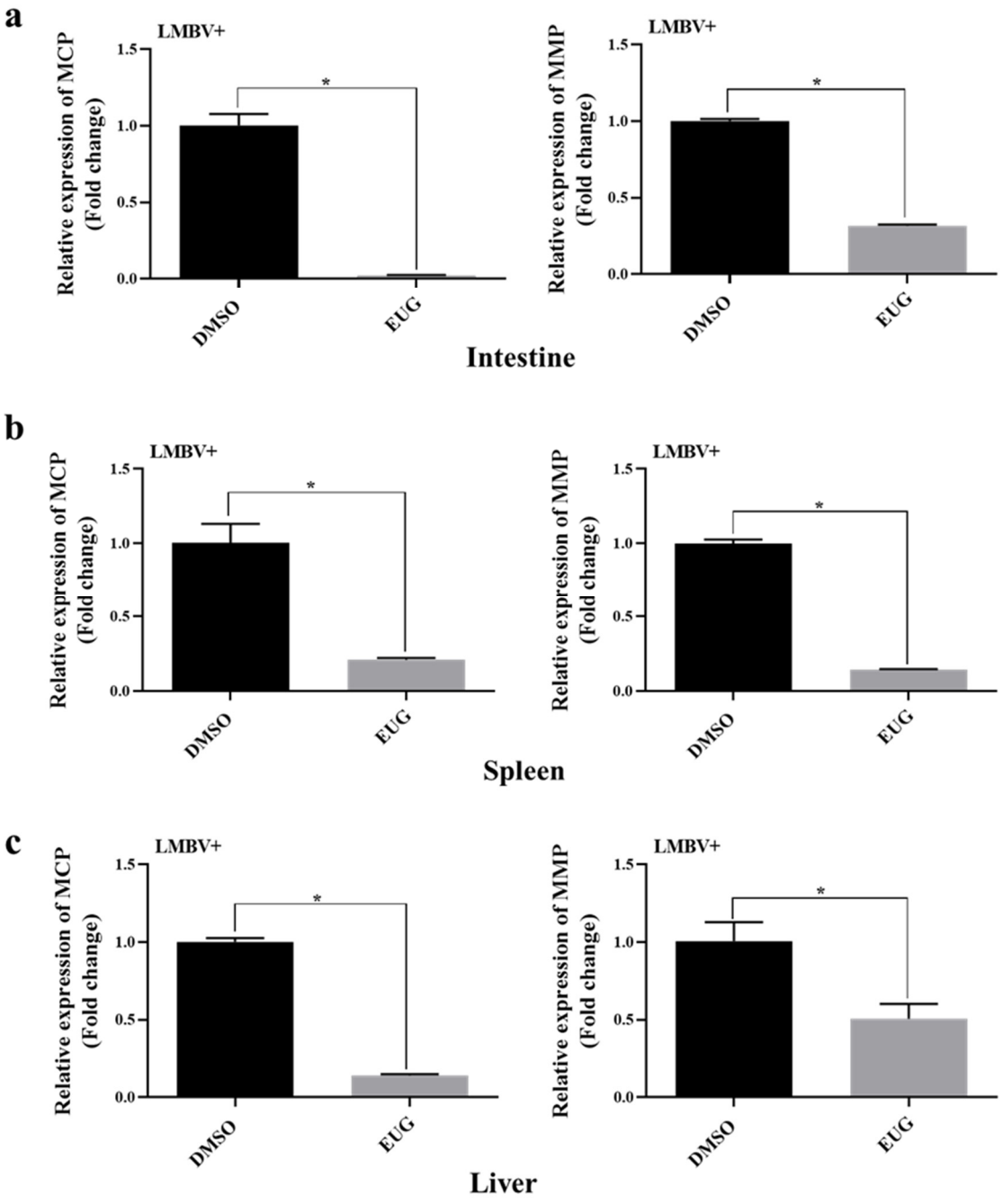

Figure 7. Effect of EUG on LMBV infection in largemouth bass. Samples of various tissues of largemouth bass were collected after LMBV infection, and the mRNA expression levels of LMBV-MCP, LMBV-MMP in various tissues were detected by qRT-PCR. Student's t-test: * $p < 0.05$.

4. Discussion

Originally from the Mississippi River system in California, the largemouth bass is widely distributed throughout the freshwater waters of the United States and Canada [23]. It was later brought to Asia and is currently the largest farmed freshwater fish in China, with higher economic and environmental benefits [26]. As the scale of aquaculture continues to expand, the impact of disease on largemouth bass aquaculture becomes apparent. Due to the lack of effective treatment, viral diseases are resulting in serious economic losses. Iridovirus is a large, structurally complex, 20-sided, double-stranded DNA virus [27]. This virus infects fish, reptiles and amphibians, causing severe disease and varying degrees of mortality [27]. LMBV is a member of the Iridoviridae family,

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specifically the genus Frog Virus [28]. Infection of LMBV can result in a variety of clinical symptoms, including ulceration of the body surface, muscle necrosis, swelling of internal organs, etc., which are frequently accompanied by high mortality during the epidemic season. In addition, pathological analysis revealed hepatocellular nuclear consolidation and extensive necrosis of the spleen with lymphopenia [29]. What's more serious is that there are currently no approved drugs or vaccines available to control LMBV.

Faced with the aforementioned crisis, exploring antiviral ingredients from plant extracts has gradually become an effective solution and strategy [7, 30]. In a previous study, we have shown that EUG can inhibit SGIV infection *in vitro*, which together with LMBV are members of the iridoviridae family [15]. EUG has a well-established antiviral effect *in vitro*. To learn more about its antiviral mechanism and potential applications in aquaculture, we looked into how it affected LMBV infection both *in vivo* and *in vitro*. We chose FHM cells as *in vitro* infection model, investigated the optimal antiviral concentration and antiviral effect of EUG in cells, while detected the infection situation of LMBV in cells by analyzing the expression levels of MCP and MMP [31]. Among them, MCP is the major structural protein of iridoviruses and has low homology in the iridovirus family, which is widely used for virus detection [24, 32]. MMP serves as the viral envelope protein in iridoviruses, playing key roles in host cell recognition, viral assembly, and entry [25]. According to the findings of the cytotoxicity assessment of EUG, the viability of FHM cells remained unaffected at concentrations below 100 μM. Then we treated FHM cells with different concentrations of EUG (50 μM, 75 μM) for 2 h before infected by LMBV, and discovered that 75 μM EUG could significantly inhibit the expression of LMBV-MCP and LMBV-MMP. Thus, it was suggested that 75 μM of EUG significantly inhibited LMBV infection of FHM cells.

Apoptosis is a kind of programmed cell death. In contrast to cell necrosis, apoptosis is closely related to cell proliferation in healthy state [33]. In the process of defense against viral infection, cells can inhibit viral replication by rapidly activating apoptotic pathways to make infected cells die prematurely. Conversely, viruses can manipulate apoptosis to promote their own replication and spread. The protein encoded by human immunodeficiency virus-1 (HIV-1) depletes T cells by making them sensitive to apoptosis through different regulatory mechanisms [34]. This mechanism is also present in RNA virus infection, such as West Nile virus (WNV) and Zika virus (ZIKV) infections, which can induce neurotoxicity by mediating nerve cell apoptosis through various pathways [35, 36]. Many fish viruses cause different types of cell death in a cell-dependent manner. SGIV causes non-apoptotic cell death in GS cells, but apoptosis in FHM cells [37]. According to earlier research, LMBV infection was found to induce apoptosis in FHM and EPC cells and non-apoptotic cell death in MsF cells [10]. In addition, EUG has been demonstrated in many previous cancer studies to promote apoptosis and cancer cell toxicity. Low dose of EUG can induce apoptosis of different breast cancer cells and inhibit the proliferation of cancer cells, Kim et al. demonstrated that EUG has the potential to induce apoptosis of melanoma and neuroblastoma cells [38, 39]. Thus, in order to further investigate the mechanism of EUG inhibiting LMBV infection, we employed flow cytometry and western blotting to analyze the effect of EUG on LMBV-induced apoptosis. The following results revealed EUG had no influence on the apoptosis in healthy cells, but can inhibit LMBV infected FHM cell apoptosis. The initiation and execution of apoptosis are regulated by Bcl-2 and Caspase family proteins, Caspase-3 is a crucial protease for apoptosis, and cleaved-caspase-3 is the primary executor of apoptosis [40]. In our study, we observed the negative effect of EUG on apoptosis by detecting it can directly inhibit the expression of cleaved-caspase-3 in LMBV infected FHM cell. Therefore, abovementioned results suggest that EUG can considerably prevent apoptosis induced by LMBV infection.

Autophagy, which is closely related to viral infections, plays a crucial role in the immune system. It functions as an essential defense mechanism against autoimmunity and exhibits antiviral properties. Conversely, certain viruses can exploit autophagy to facilitate viral replication and enhance infection [41, 42]. For instance, autophagy can limit the replication of the virus in cells while HIV inhibits autophagy during infection [43, 44]. The interaction between apoptosis and autophagy

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plays an important role in body development and homeostasis, and the association between them has been reported in many different viruses [45-48]. In the study of IAV, inhibition of autophagy resulted in the blockage of IAV replication and limited IAV protein-induced apoptosis [48]. In the relationship between autophagy and apoptosis induced by enterovirus 71, it was discovered that autophagy had no direct effect on the release of virus, but inhibited the release of virus by suppressing apoptosis [45]. ATG5 and LC3I/II play an important role in the occurrence and development of autophagosomes and autophagic vesicles, in which ATG5 is involved in each stage of the formation of autophagosomes. LC3I/II is the direct evidence to determine whether autophagy occurs [21, 49]. The autophagy-related gene Beclin 1 was originally discovered in 1999 and is the mammalian homologue of ATG6 [50]. In mammalian cells, Beclin 1 can interact with Bcl-2 to regulate cellular autophagy. Overexpression of Beclin 1 may have anticancer and antiviral effects [47, 51]. P62 is a ubiquitinated protein that can ubiquitinate autophagosomes upon binding to LC3, therefore inhibition of autophagy leads to significant accumulation of P62, which is conversely depleted [52, 53]. Therefore, we examined the protein expression levels of LC3I/II, ATG5, Beclin 1 and P62 to investigate the effect of EUG on LMBV-induced autophagy in FHM cells, which obviously indicated EUG can increased the expression of LC3I/II, Beclin-1, ATG5 and decrease the expression of P62 protein. These results showed that EUG could significantly promote the autophagy in LMBV-infected FHM cells.

AMPK is an evolutionarily conserved serine/threonine protein kinase involved in a wide range of cellular activities and can be phosphorylated under many physiological or pathological conditions [54]. Under starvation or some pathological conditions, activated AMPK can inhibit mTOR activity and indirectly activate FOXO3 targets thereby promoting autophagy [55]. mTOR is an important autophagy regulatory protein and regulates a variety of downstream transcription factors, many viral infections activate the mTOR pathway. For example, in HIV infection, activation of autophagy by rapamycin reduces virus-induced apoptosis by inhibiting mTORC1, which further inhibits the AKT/PKB pathway [56, 57]. AKT is a key signal transduction protein. After activation of PI3K, AKT is recruited to the cell membrane and exposed to phosphorylation sites, and phosphorylated AKT is transferred to the cytoplasm or nucleus, indirectly promoting mTORC1 to exert the function of inhibiting autophagy [58, 59]. In fact, the AKT pathway can be activated by many viral infections. *Polyomavirus spp*. enhances AKT signaling by attacking phosphatase activity thereby causing accelerated cellular accretion and division, allowing the cell to enter the S-phase, at which point viral DNA synthesis begins [60]. Our experimental results suggest that EUG inhibits AKT and mTOR phosphorylation and promotes AMPK phosphorylation in LMBV-infected FHM cells, which confirmed that AMPK and AKT/mTOR pathways are critical routes of EUG promoting cellular autophagy during LMBV infection.

Finally, to verify the validity of the EUG *in vivo* experiments, we intraperitoneally injected a safe concentration of EUG mixed with a half-lethal concentration of LMBV into largemouth bass. Fish in the EUG-treated group possessed higher survival rates compared to the control group, and in subsequent assays, the mRNA expression of LMBV-MCP and LMBV-MMP genes within the tissues was much lower than that in the control group. Therefore, we conclude that EUG has significant anti-LMBV activity both *in vivo* and *in vitro* (Figure 8).

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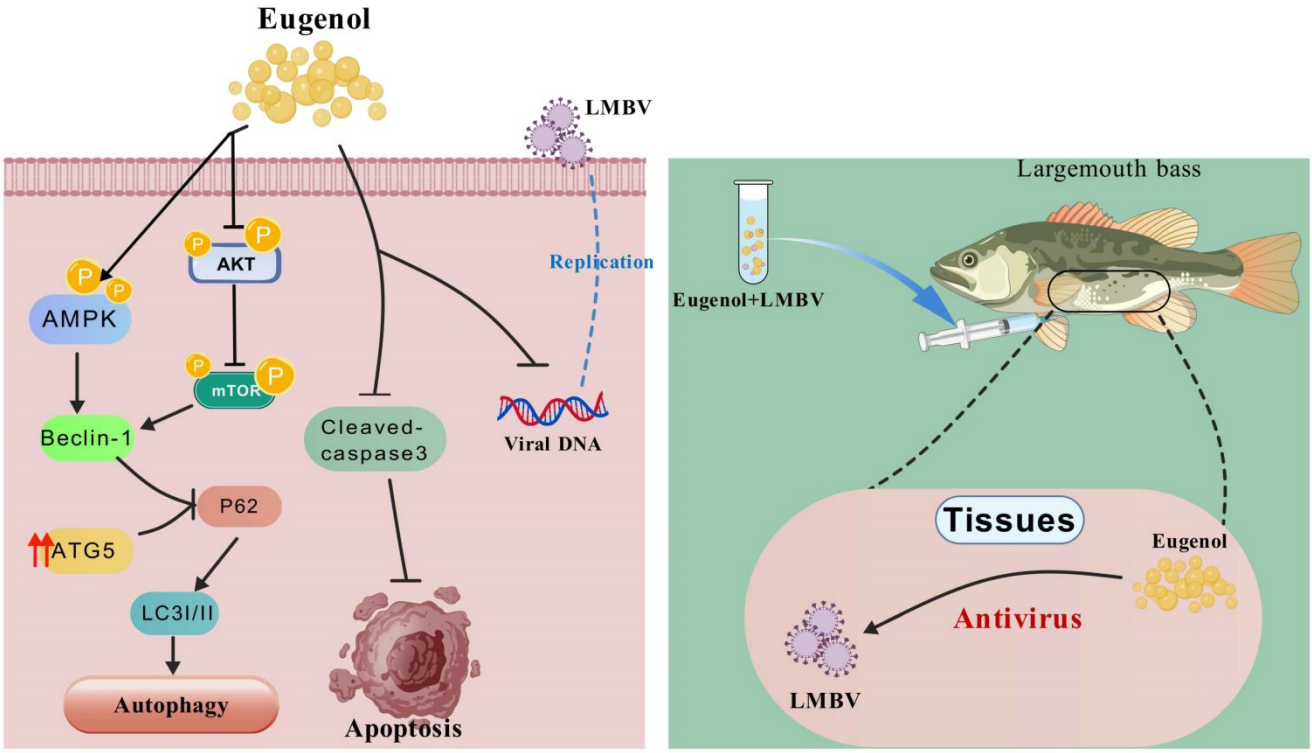

Figure 8. Schematic showing the antiviral activity of EUG during LMBV infection.

5. Conclusions

In summary, EUG can exert anti-LMBV effect *in vivo* and *in vitro*, mainly by inhibiting apoptosis and promoting autophagy in LMBV infected cells. We further explored the *in vivo* antiviral effect of EUG via intraperitoneal injection. The results showed that intraperitoneal injection of EUG could reduce the mortality of largemouth bass due to LMBV infection and inhibit the mRNA levels of LMBV-MCP and LMBV-MMP in tissues. These suggest EUG plays a defensive role in LMBV infection, providing a new antiviral theoretical reference for understanding and application of EUG.

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