Effects of Hops Ethyl Acetate Extract on Lipopolysaccharide-induced Depression-like Behavior and the Underlying Mechanism

Ziwei Ma[1], Yuming Yu[1], Ming Gao[1], Peng Chen[1], Huixia Hong[1], Dingle Yu[2], Zhenjiang Liang[2], Qinlian Ye[3], Yachao Wang[4], Guodong Huang[5], and Hui Tan[2]

[1]Xinjiang University
[2]Shenzhen Children's Hospital
[3]The Institute Translational Medicine, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital
[4]Shenzhen University First Affiliated Hospital
[5]Shenzhen Second People's Hospital

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Abstract

Background and Purpose There has been increasing interest in the causes and pathogenesis of depression, which is a common psychiatric disorder. We aimed to investigate the protective effect of hops ethyl acetate extract (HEA) on neuroinflammation-mediated lipopolysaccharide (LPS)-induced depression-like symptoms. Experimental Approach A battery of behavior tests, including the open field test (OFT), elevated plus maze (EPM), tail suspension test (TST), and forced swimming test (FST), was used to evaluate the effects of HEA on LPS-induced depression. Furthermore, the levels of inflammatory factors (tumor necrosis factor-α [TNF-α], ιντερλευκιν-1β) and norepinephrine were evaluated through enzyme-linked immunosorbent assay. The density of hippocampal dendritic spines was assessed through Golgi staining. Finally, the toxicological effects of hops extract on depression in mice were further analyzed through hematoxylin and eosin staining and blood biochemistry. Key Results Based on the OFT, EPM, TST, and FST results, oral gavage HEA prevented LPS-induced depression-like behaviors in the mice. Further, HEA reduced neuroinflammation, increased norepinephrine levels, and increased the density of hippocampal dendritic spines. Finally, blood biochemistry and HE staining did not reveal any side effects or toxicity of HEA. Conclusion and Implications Our findings indicated that HEA is a potential compound for treating depression.

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Running/Short title: HEA Relieves Depressive-like Behavior

Data openly available in a public repository that issues datasets with DOIs : The data that support the findings of this study are openly available in [repository name] at http://doi.org/[doi], reference number [reference number].

Author names：Ziwei Ma [1]; Yuming Yu *[1]; Ming Gao [1]; Peng Chen [1]; Huixia Hong [1]; Dingle Yu [2]; Zhenjiang Liang [2]; Qinlian Ye [3]; Yachao Wang [3]； Guodong Huang *[3]; Hui Tan *[2].

Author Affiliations:

Ziwei Ma − State Key Laboratory of Chemistry and Utilization of Carbon-Based Energy Resources; College of Chemistry, Xinjiang University, Urumqi 830017 Xinjiang, P. R. China; Email: 2633954064@qq.com

Ming Gao − State Key Laboratory of Chemistry and Utilization of Carbon-Based Energy Resources; College of Chemistry, Xinjiang University, Urumqi 830017 Xinjiang, P. R. China; Email : gaoming202100@163.com

Peng Chen − State Key Laboratory of Chemistry and Utilization of Carbon-Based Energy Resources; College of Chemistry, Xinjiang University, Urumqi 830017 Xinjiang, P. R. China; Email : 1549206877@qq.com

Huixia Hong − State Key Laboratory of Chemistry and Utilization of Carbon-Based Energy Resources; College of Chemistry, Xinjiang University, Urumqi 830017

Xinjiang, P. R. China; Email: 1620056595@qq.com

Dingle Yu − Respiratory Department, Shenzhen Children's Hospital, Shenzhen 518038, China; Email: yudingle811@163.com

Zhenjiang Liang − Respiratory Department, Shenzhen Children's Hospital, Shenzhen 518026, China; Email: 552081911@qq.com

Qinlian Ye − The Institute Translational Medicine, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China; Email: 1358698506@qq.com

Yachao Wang − Department of Neurosurgery, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University,Shenzhen 518035, China. The Institute Translational Medicine, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China; Email: ycwang529@163.com

Corresponding Author:

Yuming Yu − State Key Laboratory of Chemistry and Utilization of Carbon-Based Energy Resources; College of Chemistry, Xinjiang University, Urumqi 830017 Xinjiang, P. R. China; Email: yym2009@ iccas.ac.cn

Hui Tan − Respiratory Department, Shenzhen Children's Hospital, Shenzhen 518038, China; Email: huitan@email.szu.edu.cn

Guodong Huang − Department of Neurosurgery, Shenzhen Key Laboratory of Neurosurgery, and the Institute of Translational Medicine, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University, No. 3002

Sungang Westroad, Futian District, Shenzhen, 518035, China. Email:

huangguodong@email.szu.edu.cn.

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The authors declare no conflicts of interest.

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Bullet point summary:

What is already known

Hops are often used to treat insomnia and restlessness.

Inflammatory factors are crucial neuro-immune-endocrine regulators.

What does this study add

Ethyl acetate extract of hops effectively reduces the increase of inflammatory factors and increases the content of norepinephrine.

Ethyl acetate extract of hops has no toxic side effects.

What is the clinical significance

These data provide important considerations for the development of antidepressants with ethyl acetate extract of hops.

Abstract

Background and Purpose

There has been increasing interest in the causes and pathogenesis of depression, which is a common psychiatric disorder. We aimed to investigate the protective effect of hops ethyl acetate extract (HEA) on neuroinflammation-mediated lipopolysaccharide (LPS)-induced depression-like symptoms.

Experimental Approach

A battery of behavior tests, including the open field test (OFT), elevated plus maze (EPM), tail suspension test (TST), and forced swimming test (FST), was used to evaluate the effects of HEA on LPS-induced depression. Furthermore, the levels of inflammatory factors (tumor necrosis factor-α [TNF-α], interleukin-1β) and norepinephrine were evaluated through enzyme-linked immunosorbent assay. The density of hippocampal dendritic spines was assessed through Golgi staining. Finally, the toxicological effects of hops extract on depression in mice were further analyzed through hematoxylin and eosin staining and blood biochemistry.

Key Results

Based on the OFT, EPM, TST, and FST results, oral gavage HEA prevented LPS-induced depression-like behaviors in the mice. Further, HEA reduced

neuroinflammation, increased norepinephrine levels, and increased the density of hippocampal dendritic spines. Finally, blood biochemistry and HE staining did not reveal any side effects or toxicity of HEA.

Conclusion and Implications

Our findings indicated that HEA is a potential compound for treating depression.

Key words: Hops; Flavonoids; Depression, Neuroinflammation;

1. **Introduction**

Depression, which is classified as a depressive disorder by the American Psychiatric Association[1], is a chronic recurrent disease with emotional and somatic symptoms; further, it is characterized by chronicity, frequent recurrence, complex causes, and procrastination[2]. Patients with depression are apathetic toward life and have a chronic sad mood, which impedes normal activities of daily life[3]. Additionally, patients with depression often present listlessness, loss of appetite, circadian rhythm disorder, anxiety, decreased attention, indecision, restlessness, and self-harm or suicidal thoughts[4].

According to the World Health Organization[5], there are about 3.22 million people with depression worldwide. Moderate or severe depression can lead to suicide; further, depression could become the world's leading disease burden by 2030[6]. The recent study[7] showed that even patients with non-severe COVID-19 present psychological problems, including depression and anxiety, approximately 1 year after diagnosis. There are varying etiological factors for depression; moreover, its pathogenesis remains unclear. However, numerous biological, psychological, and environmental factors are known to interact and contribute to the pathogenesis of depression. The interaction between genetic and environmental factors as well as the time node of interaction are crucially involved in the occurrence of depression[6]. Although there have been extensive studies on depression, the pathogenesis of depression remains unclear.

Inflammation refers to the body's defense response to stimuli, which involves immune cells, blood vessels, and numerous cytokines; further, it comprises the complex

response of host cells to tissue injury or foreign antigen rejection and clearance. The effects of neuroinflammation on depression could involve the direct effect of pro-inflammatory cytokines on monoaminergic neurotransmitter expression, hypothalamus-pituitary-adrenal axis imbalance, pathological microglial activation[8], impaired neuroplasticity, and changes in brain structure and function[9]. Interleukin-1β (IL-1β) is a crucial pro-inflammatory cytokine involved in various autoimmune inflammatory responses and cellular activities, including cell proliferation, differentiation, and apoptosis[10]. Patients with depression have increased serum levels of IL-1 β, IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNFα) as well as decreased IL-10 levels[11]. Currently, 5-hydroxytryptamine and norepinephrine (NE) reuptake inhibitors are used to treat depressive disorders; however, they involve adverse side effects, poor clinical compliance, a narrow antidepressant spectrum, and a high recurrence rate[12, 13]. Therefore, there is a need to determine safe and effective drugs with few side effects for depression[14].

Hops is a perennial climbing herb of Moraceae with a long history as a medicinal and edible plant. It has been shown to exert antibacterial, anti-inflammatory, sedative, hypnotic, anti-oxidative, and anti-tumor effects. Its main components include resins and polysaccharides; further, it is mainly distributed in northern Xinjiang. Hops has been used to treat insomnia and restlessness; further, it contains several bioactive components with estrogenic and psychomodulatory functions[15]. Hops extract, mature picric acid,[16] and xanthohumol[17] have been shown to exert antidepressant effects. However, there have been no in vivo studies on the antidepressant effects and underlying mechanisms of

hops ethyl acetate extract (HEA). Therefore, this study aimed to assess the antidepressant effects of HEA in a mouse model of depression.

2. **Materials and Methods**

2.1 Animals

Male C57BL/6J mice (8-10 weeks) were prepared from Zhejiang Weitong Lihua Experimental Animal Technology (Co., Ltd). Mice were kept in a regular inverse 12h:12h light/dark cycle at temperatures between 20ºC and 23°C. Animal production license number: SCXK (Zhejiang) 2019-0001, Animal quality Certificate No.: 20211201Abzz0619000219 All animal procedures were according to local government and the Ethics Committee of Shenzhen University. Animal surgeries were conducted on male mice (8-10 weeks). Animal experimental groups were assigned the online tool QuickCalcs. Briefly, mice were randomly assigned into 5 groups: ShamContral group, depression group, and depression group treated by HEA (10 -100mg/kg), fluoxetine hydrochloride group 20mg/kg, 10 mice per group.

2.2 Drug preparation and administration

Lipopolysaccharide (LPS) was dissolved in sterile 0.9 % saline to a concentration of 50 ng/μL. HEA (Own lab) was dissolved in 1% CMC and 0.2% Tween-80 sterile water to a concentration of 10–100 mg/kg, respectively, and administered at 0.3 ml/kg. Hops were provided by Professor Liu Yumei of Xinjiang University.

Fluoxetine hydrochloride (Shanghai Biyuntian Biotechnology Co., Ltd., batch number:

ST1330–50 mg) was dissolved in sterile water to a concentration of 20 mg/kg and administered at 0.3 mL/kg. Except for the control and model groups, 10 mL/kg distilled water was infused into the stomach of mice in the other three groups with the corresponding drug for 7 days.

2.3 Depression model

A mouse model of LPS-induced depression was established as previously described [18]. Briefly, the mice were anesthetized using an isoflurane inhalation mask and placed on a stereotactic instrument (RWD Life Science, Shenzhen, China). Saline or LPS (50 ng/μL) was injected into the left lateral ventricle (0.3 mm anterior/posterior, -1.2 mm medial/lateral, and -2.4 mm dorsal/ventral relative to bregma) on the 1st, 3rd, and 5th day at an injection volume of 1 μL[16]. The mice were returned to their home cage after administration.

2.4 Depression-like behaviors

Depression-like behaviors were evaluated using a battery of behavior tests, including the open field test (OPF), Elevated Plus Maze (EPM), and Tail suspension test (TST), starting from the least stressful test to the most stressful test. Accordingly, tests for anxiety-like and exploratory behavior, including the OFT, were performed first, followed by the EPM and FST.

2.4.1 Open Field Test (OFT)

As previously described[19], the OFT device is a white cube open box (50 cm × 50 cm × 40 cm) with the bottom evenly divided into 16 (4 × 4) squares. The mice were placed in a box within a quiet environment and allowed to move freely for 6 min, with their activity being observed. After each mouse had completed an experiment, the device was sprayed with 75% ethanol for cleaning and disinfection before the next mouse was tested. The Nodus tracking software was used to analyze the speed of mouse coverage (cm/min).

2.4.2 Elevated Plus Maze (EPM)

The mice were placed in the elevated cross maze within a quiet environment, which comprised a central area (5 cm × 5 cm), two open arms (30 cm long, 5 cm wide, and 0.5 cm high borders), and two closed arms (30 cm long, 5 cm wide, 10 cm high walls), which were elevated by 50 cm from the base. The mice were allowed to move freely for 6 min. The activity of the mice was observed; moreover, the time spent in the open arm was measured using maze tracking software (EthoVision XT15, Nolddus, Holland).

2.4.3 Tail suspension test (TST)

The tail tip of the mouse was taped to the hook of a hanging device at a 1-cm elevation, with the subsequent motion of the mouse being recorded for 6 min using an animal behavior video analysis system. Further, the immobility time during this hanging period was analyzed within the last 4 minutes.

2.4.4 Forced Swimming Test (FST)

The forced swimming test is the most commonly used test for evaluating the effect of antidepressants in animal models of depression [21]. The mice were placed in a

transparent 1000 mL beaker to swim for 6 min, which was filled with water to a depth of 20 cm (21 ± 1℃). The immobility time in the water was detected using maze tracking software. Further, the immobility time within 4 min after the initial 6-min period was evaluated.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Hippocampus tissues were collected and homogenized using grinding beads in RIPA buffer containing protease inhibitor; additionally, the supernatant was collected after 14000 r/min centrifugation for 30 minutes. The total protein concentration of each supernatant was measured using a BCA protein quantitative detection kit (Servicebio, Wuhan, China).

We measured the levels of IL-1β, TNF-α (ELISA, Beyotime; Sbjbio), and NE (Nanjing Sambega Biotechnology Co., Ltd.) using an ELISA kit following the manufacturer's instructions.

2.6 Golgi Staining

The density of hippocampal dendritic spines was evaluated following the manufacturer's instructions (Servicebio, Wuhan, China). Briefly, brain tissues were cut into 2–3-mm tissue blocks, gently rinsed with normal saline several times, placed in the round bottom EP tube of 45 mL, and completely immersed in Golgi body staining solution for microscopic examination (NIKON ECLIPSE E100, Japan), image acquisition, and analysis.

2.7 H&E staining

Peripheral organs (heart, liver, spleen, lung, kidney, stomach, intestine) were harvested for H&E staining using the standard protocol.

Briefly, the mice were sacrificed and the peripheral organs were quickly stripped off on the ice, followed by fixing in 4% paraformaldehyde solution for 48 h. The organs were sliced, fixed, dehydrated, embedded in paraffin, sectioned, and stained with H&E solution. This was followed by pathological morphological evaluation under a light microscope[20].

2.8 Blood biochemical test

Blood was collected and centrifuged at 4000 rpm for 10 min at 4℃. The supernatant was stored at -80℃. Subsequently, the hepatic, renal, and myocardial enzyme levels, as well as blood lipid and glucose levels, were measured (Servicebio, Wuhan, China).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Results are expressed as mean ± standard deviation. Between- and among-group comparisons were performed using an unpaired t-test and one-way analysis of variance. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Behavioral results

3.1.1. HEA prevented LPS-induced depression behavior

In the OFT (Figure 2A), the LPS group showed a significantly reduced horizontal locomotor distance compared with the control group ($P < 0.05$), which was increased by HEA administration (10 and 100 mg/kg). Further, 100 mg/kg HEA allowed a considerably longer horizontal locomotor distance than fluoxetine (positive drug; Figure S1). This indicated that HEA can enhance voluntary activity in mice.

In the EPM (Figure 2B), the LPS group showed a significantly shorter time spent in the open arm than the control group, which was significantly increased by HEA administration (10 and 100 mg/kg; $P < 0.05$). Notably, HEA showed a better antidepressant and anti-anxiety effect than fluoxetine (Figure S2),.

Compared with the control group, the LPS group showed a significantly longer immobility time in the FST (Figure 2C), which was shortened by administration of the drugs (10 and 100 mg/kg HEA and fluoxetine; $P < 0.05$, $P < 0.001$, $P < 0.001$), indicating that HEA can help ameliorate depression.

3.2 HEA decreases inflammatory factor levels and enhances monoamine neurotransmitter expression

Inflammation is closely related to depression; further, chronic mild inflammation may increase the risk of depression. Compared with healthy individuals, patients with severe depression have higher levels of proinflammatory cytokines[21]. LPS induces immune activation in the central and peripheral systems, which increases the levels of proinflammatory

cytokines[22]. We measured hippocampal IL-1β and TNF-α levels to evaluate the inflammation level (Figure 3A, B). Compared with the control group, the LPS group showed significantly higher IL-1β and TNF- α levels ($P < 0.001$), which were significantly decreased by HEA administration. This suggested that HEA can treat or relieve hippocampal inflammation. NE is crucially involved in maintaining brain excitability and memory awakening[23]. NE and its related neurotransmitters are closely related to the pathophysiology of depression[24]. Decreased monoamine neurotransmitter expression or impaired function of related receptors could contribute to the development of depression. Patients with depression have significantly reduced hippocampal NE levels, which suggests that the onset of depression is related to decreased NE levels[25]. We measured hippocampal NE levels in the mice (Figure 3C). Compared with the control group, the LPS group showed decreased hippocampal NE levels, which were significantly increased by the administration of 100 mg/kg HEA and fluoxetine ($P < 0.05$, $P < 0.001$).

3.3 HEA increased the dendritic spine density

The diameter of dendritic spines increases with neuronal maturation; further, it reflects the synaptic strength. Depression leads to dendrite atrophy characterized by decreased dendritic spine density and the synapse number[26]. Golgi-Cox staining was used to evaluate the effect of HEA on LPS-induced dendritic changes in mice[27]. Compared with

the control group, the LPS group showed a decreased density of hippocampal dendritic spines, which was improved by HEA administration (Figure 4).

3.4 HEA prevented pathological symptoms in the mouse model of depression

Compared with the control group, the LPS group showed decreased hepatic cell density (Figure 5A-B Liver) and apoptosis. HEA (10 mg/kg or 100 mg/kg) administration decreased hepatic cell death; further, HEA (100 mg/kg) showed a significantly better improvement effect than fluoxetine (Figure 5C–D). Moreover, compared with the control group, the LPS group showed proliferation of the glomerular vascular endothelium accompanied by telangiectasia (Figure 5A–B), which was alleviated by HEA administration (Figure 5C–D). Finally, HEA and fluoxetine did not significantly alter the spleen morphology compared with the control group (Figure 5).

3.5 HEA decreased total bile acid (TBA), uric acid (UA), and cholesterol (CHO) levels

The onset of depression is closely related to dysfunction of the nervous, immune, and endocrine systems[28]. Moreover, depression can be evaluated by combining conventional biochemical markers with multiple biochemical parameters[29]. Compared with the control group, the LPS group showed significantly increased serum levels of TBA and UA (Figure 6). Compared with the model group, HEA significantly decreased the levels of TBA, UA, and CHO, but not blood glucose ($P < 0.01$; $P < 0.001$）

4. Discussion

Inflammatory factors are crucial neuro-immune-endocrine regulators.[30] They mediate the transmission of information between immune cells and are crucially involved in suppressing depression[31]. However, the molecular mechanism underlying the anti-depressive effects of inflammatory factors remains unclear. Our study explored the active components of HEA in the treatment of depression. We found HEA ameliorated behavior outcomes, which were evaluated using the OFT, EPM, TST, and FST, in depression mice. Furthermore, HEA reduced neuroinflammation, increased NE expression, and increased the density of dendritic spines. Finally, HEA improved depression-related symptoms in peripheral organs.

LPS is an endotoxin that activates microglia and astrocytes, which increases the levels of proinflammatory cytokines, and thus induces depression-like behavior in mice[32]. In our study, LPS induced significant depressive behavior, which was effectively alleviated by administering HEA. Chronic stress can induce microglial activation in various hippocampal subregions, which increases the levels of inflammatory factors[33]. This increases neurotoxicity, which activates the hypothalamus-pituitary-adrenal axis to oversecrete adrenocorticotropic hormone. This aggravates the inflammatory reaction, damages hippocampal neurons, and alters nerve signal pathways and monoamine neurotransmitters, leading to depression[34]. To verify the relationships among inflammation, neurotransmitters, and depression, as well as the role of HEA, we measured the levels of IL-1β, TNF-α, and NE. Compared with the control group, the LPS group showed significantly increased pro-inflammatory cytokine levels and decreased NE levels, which was reversed by treatment with HEA and fluoxetine. HEA

may decrease the levels of pro-inflammatory cytokines by inhibiting microglial activation. Further studies are warranted to elucidate the relationship between pro-inflammatory cytokines and neurotransmitters. Moreover, depression is closely related to structural and functional plasticity changes in the nervous system[26]; further, depression decreases the number of synapses[35]. Therefore, antidepressant drugs targeting dendritic spines are being developed. HEA administration can regulate dendritic loss in mice with depression. Neurotrophic factors reduce the density of dendritic spines through LPS-induced inflammation[27]. Therefore, the anti-inflammatory effect of HEA contributed to the changes in dendritic spines.

Additionally, we investigated the effects of HEA on various organs in depressed mice. The liver is responsible for the biotransformation of exogenous chemicals. The toxic and side effects mainly occur in the kidney, liver, and spleen. Compared with fluoxetine, HEA allowed significantly lower levels of alanine aminotransferase, aspartate aminotransferase, and creatinine. Glucose is an important source of energy for the human body[36]. Compared with the model group, the HEA groups showed significantly increased blood glucose levels (Figure 6). UA is a crucial blood antioxidant that effectively scavenges oxygen free radicals and reactive nitrogen, which inhibits lipid peroxidation. This reduces oxidative stress in the endoplasmic reticulum of neurons, which reduces oxidative damage, maintains immune function, and prevents the occurrence of neurological diseases[37]. The aforementioned indices can be used to elucidate the effects of HEA. Our findings indicated that HEA has low toxicity and side effects on mice, which can be used with long-term safety. The anti-depressive effects

of HEA may be attributed to its effects on anti-inflammatory and neurotransmitter levels. However, further studies are warranted to explore the main functional components of HEA involved in its antidepressant mechanism as well as their molecular targets.

In conclusion, HEA can improve LPS-induced depression-like behavior in mice by decreasing proinflammatory cytokine levels, increasing hippocampal NE levels, and increasing hippocampal dendritic spines. Further, HEA had fewer toxic and side effects than fluoxetine. Our findings could inform future studies on the anti-depressive effects of hops, which could lead to its clinical application.

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Figure 1: Schematic diagrams of the experiment

Figure 2: Effect of HEA on LPS-induced depression-like behavior in mice (n = 10). A. Effect of HEA on locomotion speed in the OFT; B. Effect of HEA on the time spent in the open arm of the EPM; C. Effect of HEA treatment on the immobility time in the FST.

Figure 3: Effect of HEA treatment on monoamine neurotransmitter and inflammatory factor levels (n = 10). **A**. IL-1β levels in different groups. **B**. TNF-α levels in different groups. **C**. NE levels in different groups.

Figure 4 Effects of HEA treatment on the density of dendritic spines in mice (n = 10). **A**. The control group； **B**. the LPS group； **C**. HEA group (10 mg/kg)； **D**. HEA group (100 mg/kg); **E**. Quantification of dendritic spine density through Golgi staining

Figure 5: Effects of HEA administration on the histomorphology of the liver, kidney, and spleen in mice (n=10). **A**. The control group； **B**. the LPS group； **C**. HEA group (10 mg/kg)； **D**. HEA group (100 mg/kg)； **E**. The group of FLX treatment (20 mg/kg).

Figure 6: Effects of HEA treatment on the levels of alanine aminotransferase (ALT)， aspartate aminotransferase (AST), serum creatinine (CREA), total bile acid (TBA), uric acid (UA), blood glucose (GLU), and cholesterol (CHO). **A**. The ALT levels in

different groups.；**B**. The AST in different groups；**C**. The CREA levels in different groups；**D**. The TBA levels in different groups; **E**. The UA in different groups; **F**. The GLU levels in different groups. **G**. The CHO levels in different groups

Figure 1

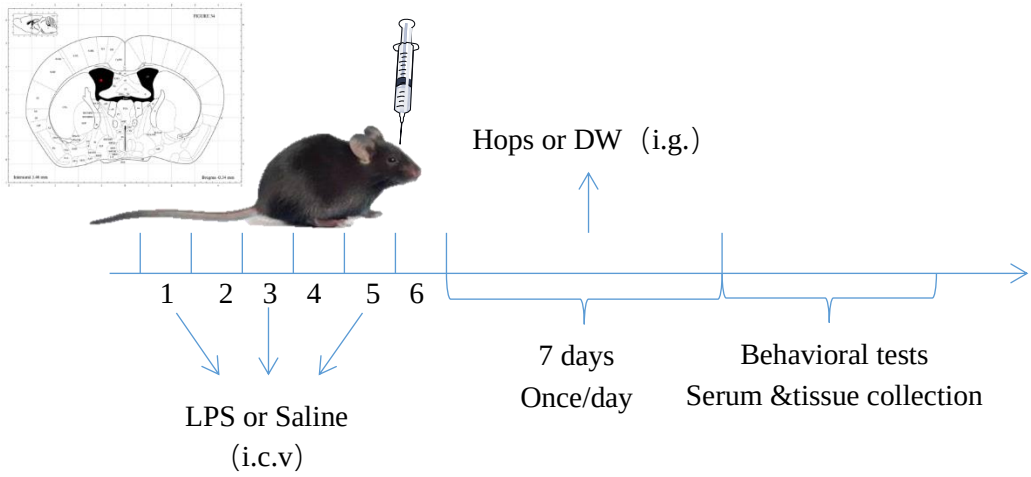


Figure 2

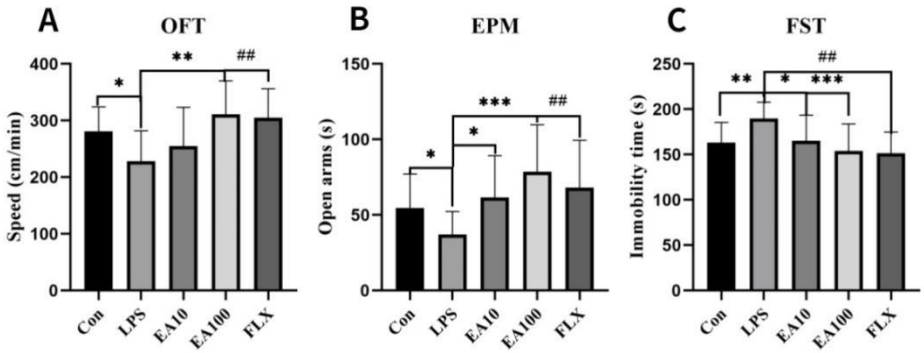


Figure 3

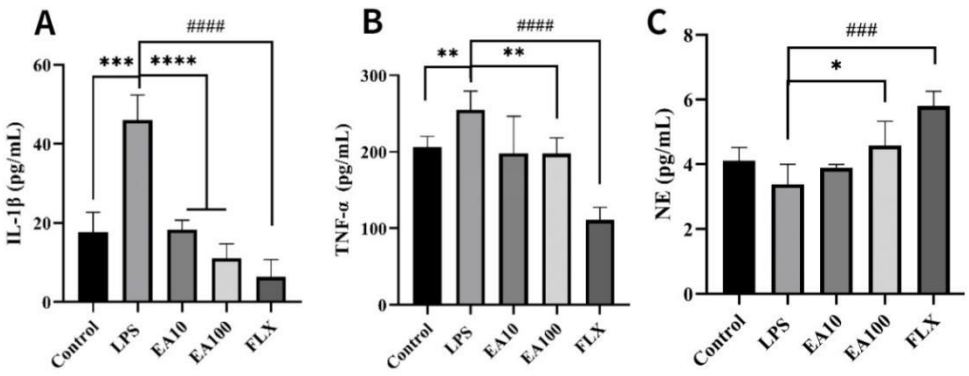


Figure 4

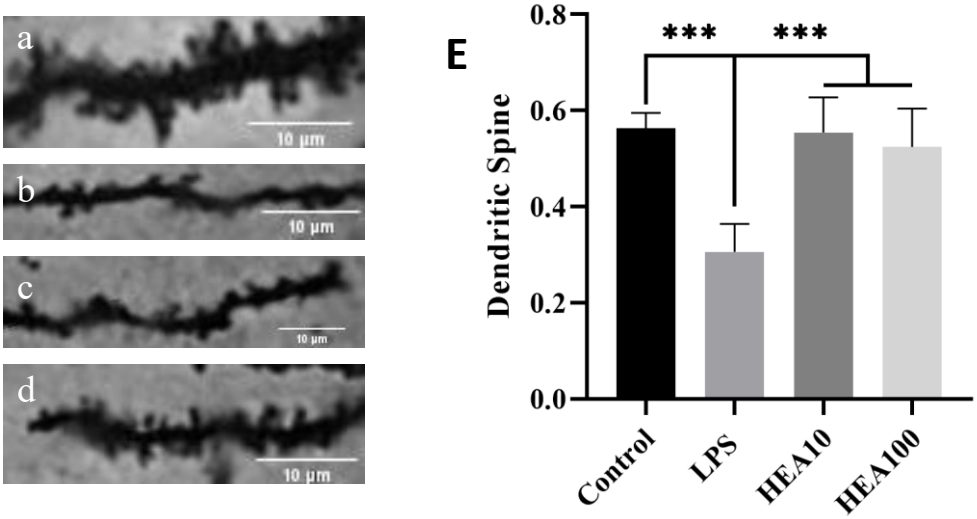


Figure 5

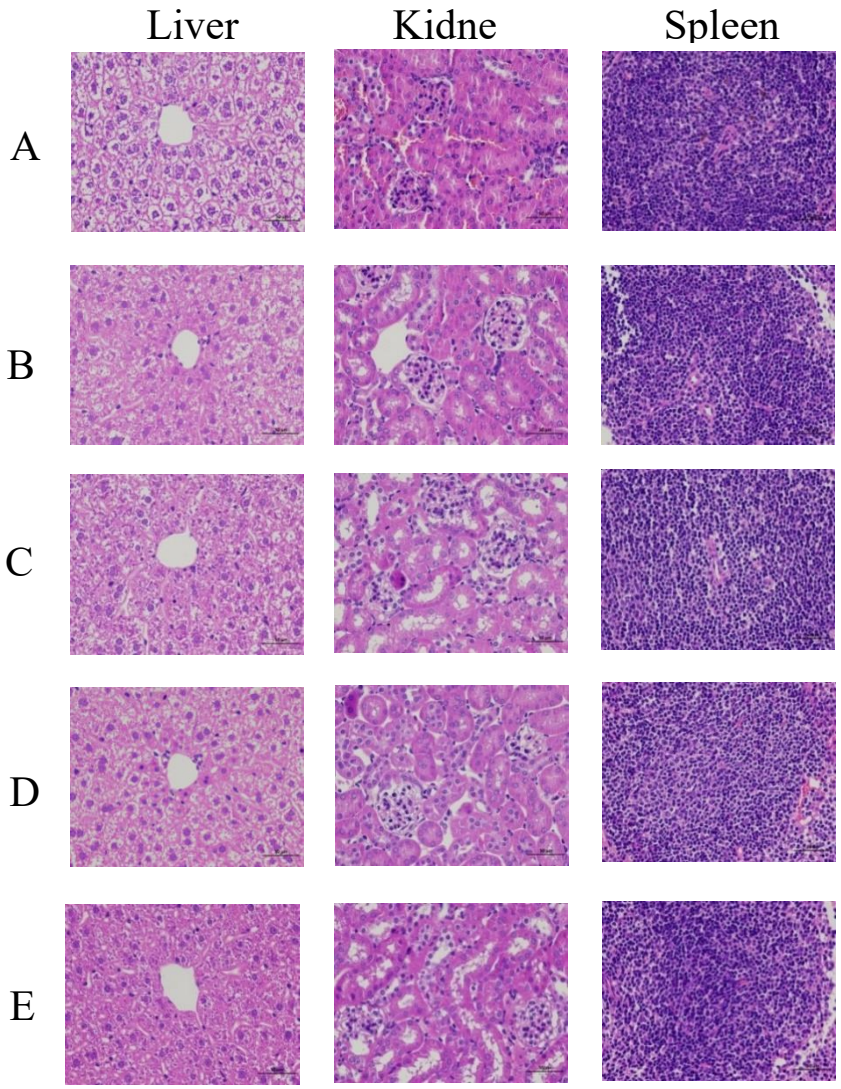
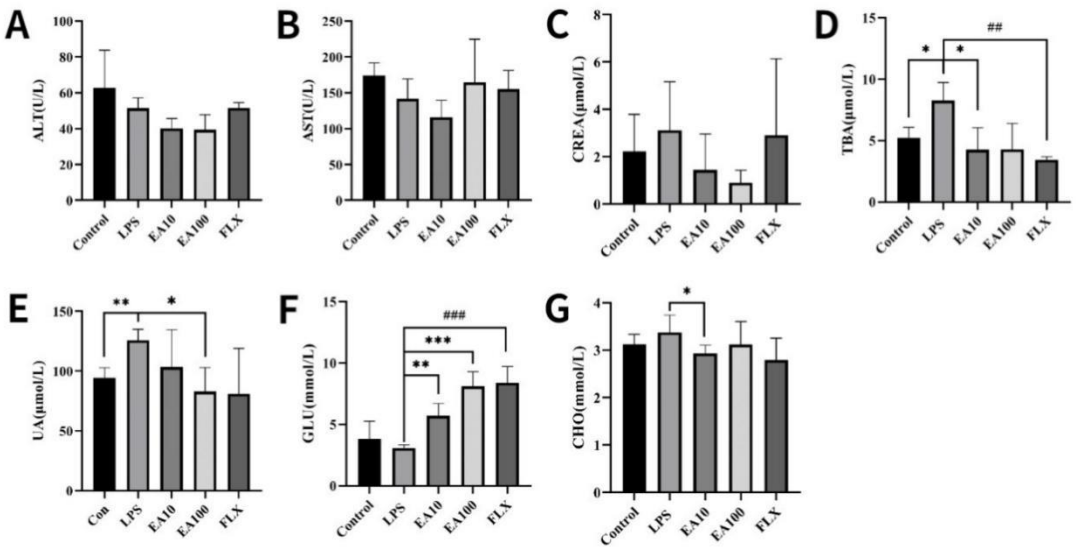

Liver
Kidne
Spleen
A
B
C
D
E

Figure 6


A
ALT(U/L)
B
AST(U/L)
C
CREA(μmol/L)
D
TBA(μmol/L)
E
UA(μmol/L)
F
GLU(mmol/L)
G
CHO(mmol/L)
Control
Con
LPS
EA10
EA100
FLX