

# Morinda officinalis alleviates apoptosis of hippocampal CA3 cells by up-regulating raphe nucleus miR-16-5p in the rat model of depression

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## Research Article

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# Abstract

In the classic pathogenesis of depression, the reduced 5-HT projection in cholinergic neurons located in the raphe nucleus to the hippocampus leads to the loss of hippocampal neurons. In order to increase the 5-HT projection in the the raphe nucleus to hippocampus, the classic drug fluoxetine developed for the 5-HT reuptake mechanism in the raphe nucleus not only competitively binds to the serotonin transporter (SERT), but also affects miR-16 targeting 5-HT reuptake proteins. *Morinda officinalis* has a clear antidepressant effect, but its mechanism of action on miR-16 is still unclear. Therefore, this study validated the mechanism by which *Morinda officinalis* alleviates hippocampal CA3 cell apoptosis by upregulating miR-16-5p in the raphe nucleus in vivo. Firstly, Chronic unpredictable mild stress (CUMS) combined with solitary depression rats were established. Secondly, after 3 weeks of administration, the effects of *Morinda officinalis* (3g/(kg·d)) on behavior, raphe nucleus miR-16-5p level, raphe nucleus serotonin transporter level, hippocampal 5-HT content and hippocampal CA3 cell apoptosis of depressed rats were observed by Open-Field Test, PCR, Western blot, ELISA and TUNEL. The results showed that *Morinda officinalis* can significantly improve the behavioral score of CUMS rats, increase the level of miR-16-5p in raphe nucleus, decrease the level of serotonin transporter in raphe nucleus, increase the content of 5-HT in hippocampal cells, and alleviate the apoptosis of hippocampal CA3 cells. In conclusion, *Morinda officinalis* inhibits the reuptake of 5-HT by indirectly up-regulating the expression level of miR-16-5p in raphe nucleus, thereby increasing the 5-HT projection in the raphe nucleus to hippocampus CA3 cells, alleviating the apoptosis of hippocampal CA3 cells and playing an antidepressant role.

## Introduction

Depression is a kind of mental disease that seriously harms human physical and mental health (National Institute for Health and Care Excellence. 2022). As early as 2019, the World Health Organization (WHO) pointed out that depression has become the second biggest killer of humans after cancer. The Lancet estimates that 350 million adults worldwide suffer from depression each year, and this figure is on the rise, especially since the outbreak of COVID-19, with a 28% increase in depression cases worldwide due to social isolation, bereavement, uncertainty, and inadequate health care services (COVID-19 Mental Disorders Collaborators. 2021). Subsequently, the Lancet released a major report on depression on February 16, 2022. The report points out that the ongoing and increasingly severe global depression crisis has not been resolved, and calls on researchers to fill knowledge gaps, raise awareness, and alleviate the global burden of depression (Herrman et al. 2020).

The pathogenesis of depression mainly involves inflammatory response, HPA axis hyperfunction, downregulation of monoamine neurotransmitters and their receptors, and downregulation of BDNF (Andersen et al. 1975; COPPEN et al. 1965; Duman et al. 1999; Janowsky et al. 1972; Maes et al. 1995; Scott et al. 1965; Scott & Dinan. 1998; ). It is worth mentioning that the above-mentioned pathogeneses are not existing singularly, but mutually feedback and together form the network of depression pathogenesis. For example, inflammatory reactions can lead to HPA axis hyperactivity, HPA axis hyperactivity in turn can lead to downregulation of 5-HT and downregulation of 5-HT in turn can cause

downregulation of downstream hippocampal BDNF (Benrick et al. 2009; Troubat et al. 2021). Currently, no matter how complex the pathogenesis of depression is, the core pathogenesis remains the downregulation of 5-HT and the loss of hippocampal neurons (Dell'Osso et al. 2016; Micheli et al. 2018; Piubelli et al. 2011; Sahay & Hen. 2007).

At present, although there are many Western antidepressant drugs in clinical practice, there are various adverse reactions (Qaseem et al. 2023). So far, there is no ideal Western medicine for the treatment of depression. Compared with fluoxetine, *Morinda officinalis* has achieved good therapeutic effects in treating depression while greatly reducing adverse drug reactions. Current research has confirmed that the *Morinda officinalis* can significantly inhibit the excitation of the HPA axis, counteract the deficiency of 5-HT, upregulate the hippocampal AC-cAMP-PKA signaling pathway, promote the expression of BDNF in the hippocampus, have a protective effect on the hippocampus, and improve the depressive state of rats (Zhang et al. 2018). However, this does not mean that the research on the mechanism of *Morinda officinalis* in treating depression has come to an end.

In pharmacology, compared with the mechanism of Western medicine for treating diseases, the mechanism of traditional Chinese medicine for treating diseases is particularly complex, and its treatment mechanism is often indirect or multi component and target. Therefore, blindly screening may not be wise. On the contrary, in order to directly enter the key points of relevant research and ensure the success of the experiment, it is more feasible to search for new molecules from the mechanism research of Western medicine to carry out the mechanism research of *Morinda officinalis*.

As is well known, fluoxetine, as the most classic antidepressant drug in clinical practice, can selectively inhibit 5-HT transporters and still plays a representative role in clinical practice. Although it was approved for market by the US FDA as early as 1988, it does not mean that research on it has stopped. From the research history of the classic antidepressant drug fluoxetine, the mechanism of fluoxetine in treating depression has gradually evolved from the initial "competitive binding of serotonin transporters" to the current stage of "affecting the expression of serotonin transporters by influencing miR-16" and "affecting the plasticity of hippocampal neurons by binding to BDNF receptors" (Baudry et al. 2010; Casarotto et al. 2021). Through comparison, it is not difficult to find that the current research on the mechanism of *Morinda officinalis* in treating depression has not yet involved miR-16.

Of course, choosing miR-16 as the starting point for studying *Morinda officinalis* is not only because it has not been reported, but also for a deeper reason. This reason is reflected in the development history of fluoxetine. The development of healthcare can be described as revolutionary, but fluoxetine, as a classic antidepressant drug, has been enduring for a long time. The root cause is simply that it acts on the key point of depression—5-HT. Because whether from the KEGG website's signaling pathway library or current depression research, it is not difficult to clarify a general axis of relationship regarding the onset of depression—stress factors lead to an increase in the stress hormone cortisol, which indirectly reduces the 5-HT projection of serotonin neurons (raphe nucleus) to the limbic system (hippocampus), ultimately triggering a series of reactions in the limbic system (downregulation of BDNF, loss of

hippocampal neurons, etc.), and 5-HT is the center in this axis (Strickland et al. 2002; Ozcan et al. 2020). In summary, the research on the mechanism of fluoxetine in treating depression revolves around 5-HT, and similarly, the research on the mechanism of *Morinda officinalis* in treating depression can also be similar. A strong and powerful study currently suggests that the extract of *Morinda officinalis*—*Morinda officinalis* oligosaccharides can exert antidepressant effects by altering the gut microbiota, thereby increasing the content of 5-HTP and increasing brain 5-HT (Zhang et al. 2022).

Based on the above content and actual situation, this study selected the most conservative miR-16-5p in the raphe nucleus miR-16 and the CA3 cells in the hippocampus as representative research objects in the clear relationship axis of “miR-16—5-HT—hippocampus” (Roddy et al. 2019). Meanwhile, this study investigated the effects of *Morinda officinalis* on the expression of miR-16-5p in the raphe nucleus, a key molecule affecting 5-HT reuptake, and neuronal apoptosis in the hippocampal CA3 cells in vivo.

## Materials and methods

### Animals

42 healthy male SPF grade Wistar rats, 220g, provided by Changsha Tianqin Biotechnology Co., Ltd. The quality of the experimental animals is qualified, certificate number: SCXK (Xiang) 2022-0011, and they are kept in an environment free of specific pathogens. The animal experiment was approved by the Animal Ethics Committee of Hainan Medical College (Project No. HYLL-2022-357).

### Drug and dose

*Morinda officinalis* and fluoxetine are provided by the Traditional Chinese Medicine Department of the First Affiliated Hospital of Hainan Medical University (Haikou, China). According to the dosage of gavage that can alleviate pain in rats (usually 1.5-3mL) and the formula for converting the dosage of medication between adults and rats (the daily dosage for rats under the same body weight is 6 times than adults), boil a suitable concentration of *Morinda officinalis* decoction and mix an appropriate concentration of fluoxetine with sterile water every two days.

### Experimental process

The entire experimental process mainly includes the establishment of CUMS rat model, Open-Field Test, grouping and administration, draw materials and related indicators. An outline of the experimental process is shown in Fig. 1.

### Establishment of CUMS rat model

Randomly select 6 rats as a control group and raise them in a cage, while the remaining rats are raised in a single cage and randomly given the following stimuli: fasting, water restriction, stimulation in 4°C or 45°C water for 2-3 minutes, tail suspension for 5 minutes, day night reversal, odor stimulation, electrical stimulation (40-60V, trains, 1 minute), one type per day, so that the rats cannot predict the occurrence of

stimuli to avoid adaptation. The electric shock box is provided by the Key Laboratory of Tropical Brain Science Research and Transformation in Hainan Province (Haikou, China).

## Open-Field Test

After 8 weeks of modeling, open field experiments were conducted on each animal to record their movement. The open boxes used are 90cm in length and width, and 40cm in height. Divide the ground into 4×4 squares, the horizontal activity score is based on the number of squares crossed by animals (the number of squares crossed by all four claws), and the vertical activity score is based on the number of times they stand upright (with their forelimbs above the ground by 1cm). Each animal is only tested once, with each test lasting for 3 minutes. The open field experimental platform and behavioral software SMART 3.0 are provided by the Key Laboratory of Tropical Brain Science Research and Transformation in Hainan Province (Haikou, China).

## Grouping and administration

After 8 weeks of modeling, rats with significantly lower open field scores than the control group were randomly divided into a model group, a fluoxetine group, a *Morinda officinalis* group, a miR-16-5p-inhibition group (Shanghai Jikai Gene Medical Technology Co., Ltd.), and a negative control group, with 5 rats in each group. Then, they were orally administered once a day to each group. The *Morinda officinalis* group was given a dosage of 3g/(kg·d), while the fluoxetine group was given a dosage of 2mg/(kg·d). The control group and the model group were given an equal amount of distilled water, The miR-16-5p inhibition group and negative control group were given an equal amount of *Morinda officinalis* while injecting the virus.

## Draw materials

After 3 weeks of administration, open field experiments were conducted again. After the experiment, rats were anesthetized by intraperitoneal injection of sodium phenobarbital. After decapitation, the brain was taken from an ice block and the raphe nucleus and hippocampal tissue were quickly separated (the left side was stored in a -80°C freezer for ELISA detection; the right side was stored at room temperature in 4% paraformaldehyde fixative for TUNEL staining).

## PCR for detecting the expression level of miR-16-5

The first step is to extract total RNA from the tissue using the Trizol method. The second step is to reverse transcribe RNA into cDNA using a reverse transcription kit. The third step is to perform real-time fluorescence quantitative PCR detection and calculate the relative expression level of mRNA using the  $2^{-\Delta\Delta C_t}$  method. The primer sequence designed and synthesized by Wuhan Maiheng Biotechnology Co., Ltd. is as follows: U6-forward, 5'-CGCTTCGGCAGCACATATAC-3'; U6-reverse, 5'-AAATATGGAACGCTTCACGA-3'; rno-miRNA-16-5p loop primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCGCCAATA-3'; rno-miRNA-16-5p F primer, 5'-TGCGCTAGCAGCACGTAAATAT-3'.

# Western blot for detecting the expression level of SERT protein in the raphe nucleus

The first step is to prepare protein samples (protein extraction, protein concentration determination and protein denaturation). The second step is electrophoresis (preparation of electrophoresis gel and electrophoresis separation). The third step is Western blot detection (membrane transfer, blocking, washing, binding of primary antibody and target protein, washing, incubation of secondary antibody and primary antibody, washing, color development). Primary antibodies include anti SERT (bs-1839R, 1:1000, Bioss) and anti GAPDH (60004-1-Ig, 1:5000, protentech).

## ELISA for detecting 5-HT content in hippocampus

Use an ELISA kit (Wuhan Saipei Biotechnology Co., Ltd.) to detect the 5-HT content in the hippocampus according to the instructions of the kit.

## Observation of hippocampal cell apoptosis using TUNEL staining

Use TUNEL assay kit (Wuhan Shisheng Biotechnology Co., Ltd.) to detect apoptosis of hippocampal cells. The first step is to take materials and embed them; Step 2, slice and slice; Step three, tunel staining; Step 4, panoramic scanning (bright field). Software CaseViewer 2.4 was used to observe the staining results and take screenshots (200um, 5× zoom level, 500\*400 size).

## Statistic analysis

GraphPad Prism 10.1 (GraphPad, San Diego, CA, USA) software was used for plotting and statistics. t test and One way ANOVA were used for inter group mean comparisons, with  $P < 0.05$  has significant statistical significance.

## Results

### CUMS decreased the open file scores of rats

In order to determine whether the rats were successfully modeled, we conducted an open field test on rats modeled by CUMS for 8 weeks. The results showed that CUMS significantly reduced the open field score of rats after 8 weeks (Unpaired t test,  $t = 4.183$ ,  $df = 36$ ,  $p = 0.0002 < 0.001$ ; Fig. 2), indicating the success of modeling.

### Morinda officinalis increased the open filed scores of CUMS rats

To evaluate the effect of *Morinda officinalis* on the behavior of CUMS rats, we investigated the effects of *Morinda officinalis* on CUMS rats. *Morinda officinalis* significantly increased the open filed scores (horizontal scores + vertical scores) of CUMS rats, with a statistical difference compared to the model

group (ANOVA summary,  $F = 19.17$ ,  $p < 0.0001$ ; Control vs. CUMS,  $p < 0.0001$ ; CUMS vs. MO,  $p = 0.0016 < 0.01$ ; Fig. 3).

### **Morinda officinalis increased the level of miR-16-5p in the raphe nucleus of CUMS rats**

To evaluate the effect of *Morinda officinalis* on miR-16-5p levels in the raphe nucleus of CUMS rats, we investigated the effects of *Morinda officinalis* on CUMS rats. *Morinda officinalis* significantly increased the level of miR-16-5p in the raphe nucleus of CUMS rats, with a statistical difference compared to the model group (ANOVA summary,  $F = 10.39$ ,  $p = 0.0005 < 0.001$ ; Control vs. CUMS,  $p = 0.0007 < 0.001$ ; CUMS vs. MO,  $p = 0.0402 < 0.05$ ; Fig. 4).

### **Morinda officinalis increased the level of SERT in the raphe nucleus of CUMS rats**

To evaluate the effect of *Morinda officinalis* on SERT levels in the raphe nucleus of CUMS rats, we investigated the effects of *Morinda officinalis* on CUMS rats. *Morinda officinalis* significantly increased the level of SERT in the raphe nucleus of CUMS rats, with a statistical difference compared to the model group (ANOVA summary,  $F = 38.51$ ,  $p = < 0.0001$ ; Control vs. CUMS,  $p = < 0.0001$ ; CUMS vs. MO,  $p = < 0.0001$ ; Fig. 5).

### **Morinda officinalis increased the content of 5-HT in the hippocampus of CUMS rats**

To evaluate the effect of *Morinda officinalis* on 5-HT content in the hippocampus of CUMS rats, we investigated the effects of *Morinda officinalis* on CUMS rats. *Morinda officinalis* significantly increased the content of 5-HT in the hippocampus of CUMS rats, with a statistical difference compared to the model group (ANOVA summary,  $F = 18.62$ ,  $p = < 0.0001$ ; Brown-Forsythe test,  $F (DFn, DFd) = 3.583 (5, 19)$ ,  $p = 0.0189 < 0.05$ ; Control vs. CUMS,  $p = < 0.0001$ ; CUMS vs. MO,  $p = < 0.0001$ ; Fig. 6).

### **Morinda officinalis alleviate neuronal apoptosis in the hippocampal CA3 region of CUMS rats**

To investigate the effect of *Morinda officinalis* on neuronal apoptosis in the hippocampal CA3 region of CUMS rats, we performed a TUNEL staining on hippocampal cells of CUMS rats. *Morinda officinalis* can significantly alleviate neuronal apoptosis in the hippocampal CA3 region of CUMS rats (Fig. 7).

Figure 7 *Morinda officinalis* alleviate neuronal apoptosis in the hippocampal CA3 region of CUMS rats.

## **Discussion**

In this study, it was clearly found that *Morinda officinalis* did alleviate apoptosis in hippocampal CA3 cells by upregulating miR-16-5p in the raphe nucleus. However, how it acts on miR-16-5p remains to be explored. According to current research, compared with the positive drug fluoxetine, the active ingredients of *Morinda officinalis* have almost zero absorption in the human body, and can only increase brain 5-HT by increasing the activity of enzymes in the gut microbiota to produce 5-HTP (Zhang et al. 2022). In theory, if *Morinda officinalis* only exerts its antidepressant effect by increasing the activity of

enzymes in the gut microbiota to produce 5-HTP, its rate of producing 5-HT in the brain is slower than that of fluoxetine. However, in clinical practice, the antidepressant effect of *Morinda officinalis* is not lower than that of fluoxetine, and there is no problem of delayed onset. Similarly, if *Morinda officinalis* is equated with 5-HTP, it will also raise many doubts because the clinical practicality of 5-HTP is currently limited (Shaw et al. 2002). Therefore, currently, the mechanism by which *Morinda officinalis* acts on miR-16-5p is indirect.

Material, energy, and information are the three major elements that make up the material world, and there is an inseparable connection between the three. Matter and energy are the foundation of information transmission, and information in turn reacts with matter and energy. Similarly, in the human body, information dominates the changes in matter and energy, and in matter and energy, the changes in energy in turn dominate the changes in matter. Therefore, substances are often not the key to diseases. The latest meta-analysis indicates that there is still no clear evidence linking 5-HT to depression in the onset of depression (Moncrieff et al. 2023).

Since depression cannot be perfectly explained solely from a material perspective, it is worth considering it from a different perspective—combining with energy and information. Among the characteristics of depression, it is not difficult to find such facts as "the incidence rate of depression is high in Nordic and North American countries with short sunshine", "seasonal depression—more winter, less summer", "morning heavy and night light", "female prevalence is higher than male", "young people, elderly prevalence is high", and so on. From these facts, it is difficult for us to provide a reasonable explanation from a material perspective. On the contrary, from an energy and information perspective, we can provide a good explanation. It is difficult for us to provide a reasonable explanation from a material perspective based on these facts. On the contrary, from the perspective of energy and information, we can easily provide the following explanation: light is a type of energy, and under sufficient light, our body absorbs sufficient energy, which makes it less likely to produce a low-energy (depression) state; People need to eat and work during the day, which requires the use of energy in their bodies. If there is insufficient energy in the body, it will exacerbate the low energy state of the body. On the contrary, people rest at night without using energy, which will alleviate the low energy state of the body; Generally speaking, men have stronger mobility and higher energy than women, making it less likely for them to enter a low-energy state; Older people have lower energy levels and are more likely to enter a low-energy state compared to younger people. However, young people nowadays carry heavy social pressure and are easily influenced by negative information, which can lead to a low-energy state. In summary, depression can be perfectly explained from a comprehensive perspective of material, energy, and information.

Based on this, returning to the previous discussion, it is not difficult to explain why *Morinda officinalis* is almost not absorbed in the body and can have an antidepressant effect. Meanwhile, from the fact that *Morinda officinalis* can increase the activity of enzymes in the gut microbiota, it is not difficult to say that *Morinda officinalis* does indeed increase the energy of the gut microbiota, because under normal conditions, enzyme activity in the body almost increases with the increase of temperature. Similarly,

*Morinda officinalis* can increase the energy of the brain, thereby enhancing the activity of various enzymes in the brain. In summary, life science is a comprehensive discipline, and simply explaining life phenomena from a material perspective is far from enough. Therefore, from the perspective of material, energy, and information integration, this article not only explains the pathogenesis of depression, but also believes that *Morinda officinalis* increases the activity of transcription and processing enzymes by increasing the energy of the human brain, thereby indirectly upregulating the expression of miR-16-5p in the raphe nucleus.

## Declarations

**Authors' Contributions** Peng-Wan Li, Shi-Qi Chen and Jing Zhang performed research design, modeling, behavioral testing, draw materials, PCR, Western blot, ELISA, TUNEL staining, data analysis, drawing and wrote the manuscript. De-Hui Yin and Ye Zhu contributed to the research design and revised the manuscript. All authors approved the final manuscript.

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**Data Availability** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Code Availability** Not applicable.

**Ethics approval** Not applicable.

**Consent to participate** (include appropriate statements) Not Applicable.

**Consent for publication** (include appropriate statements) Not Applicable.

**Competing Interests** The authors declare that there are no conflicts of interest.

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Figures

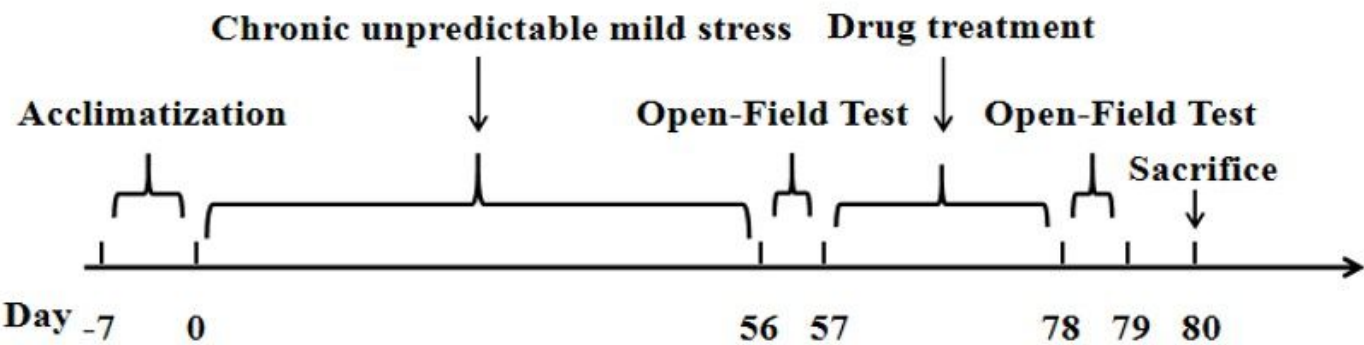


Figure 1

Entire experimental process.

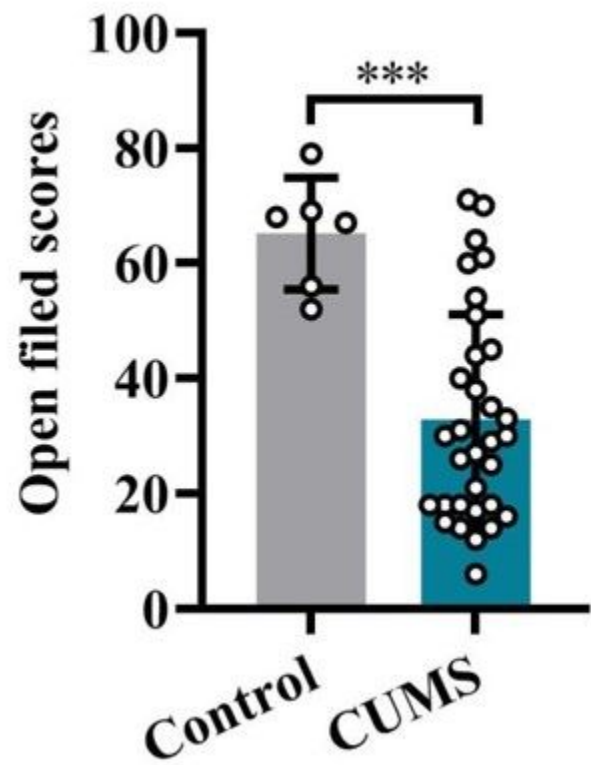


Figure 2

CUMS decreased the open file scores of rats. Control (n=6) vs. CUMS (n=32), \*\*\*p<0.001.

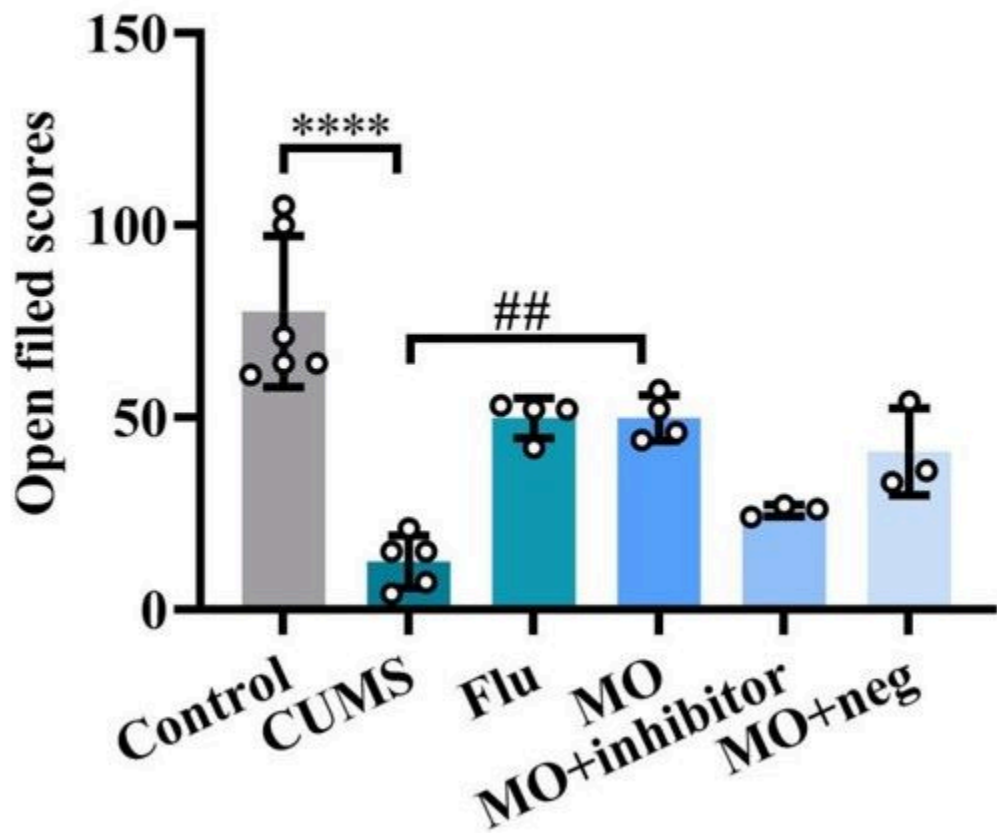


Figure 3

*Morinda officinalis* increased the open filed scores of CUMS rats. Control (n=6) vs. CUMS (n=5), \*\*\*\*p<0.0001; CUMS (n=5) vs. MO (n=4), ##p<0.01

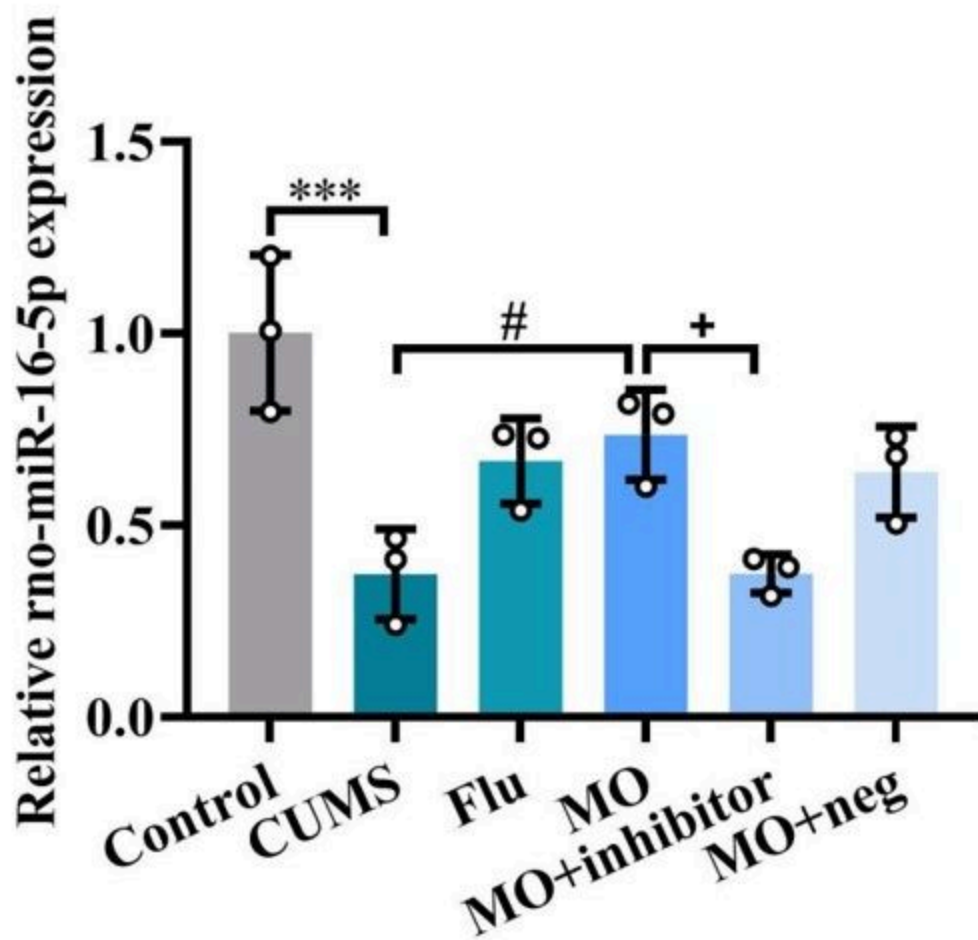
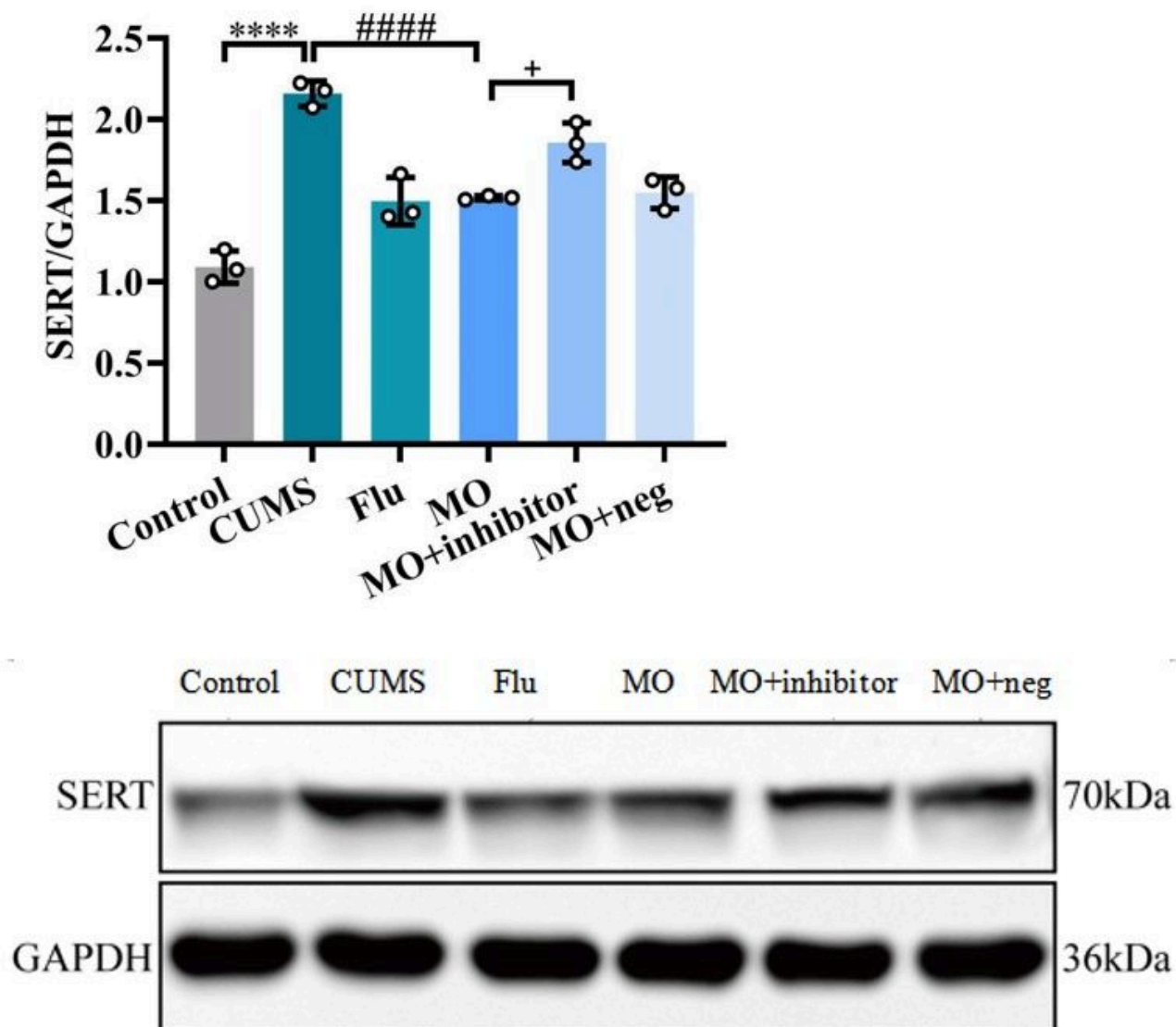


Figure 4

*Morinda officinalis* increased the level of miR-16-5p in the raphe nucleus of CUMS rats. Control (n=3) vs. CUMS (n=3), \*\*\* $p < 0.001$ ; CUMS (n=3) vs. MO (n=3), # $p < 0.05$ ; MO (n=3) vs. MO+inhibitor (n=3), + $p < 0.05$ .



**Figure 5**

*Morinda officinalis* increased the level of SERT in the raphe nucleus of CUMS rats. Control (n=3) vs. CUMS (n=3), \*\*\*\*p<0.0001; CUMS (n=3) vs. MO (n=3), ####p<0.0001; MO (n=3) vs. MO+inhibitor (n=3), +p<0.05.

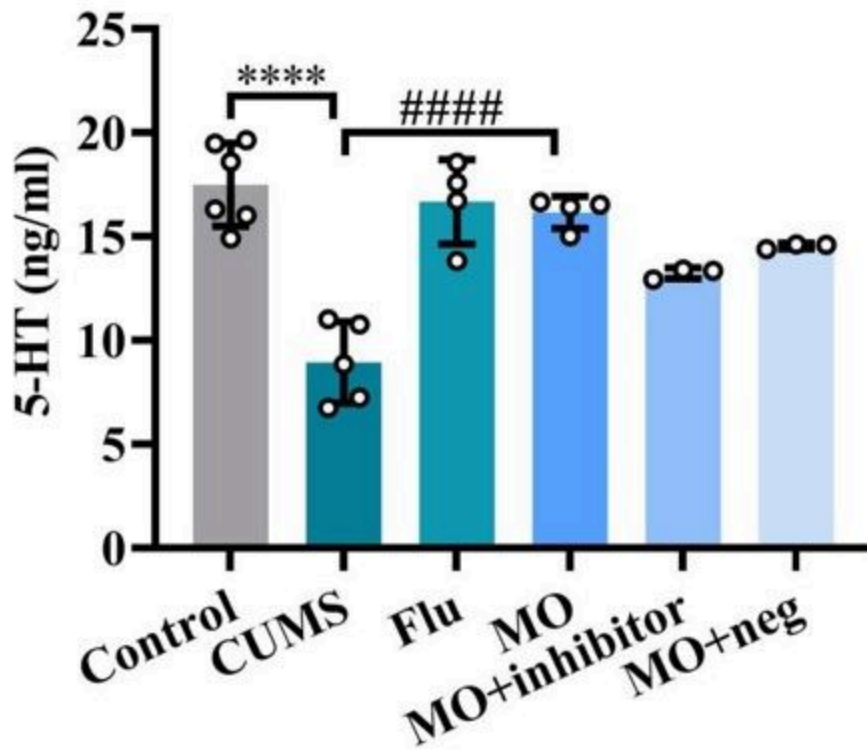
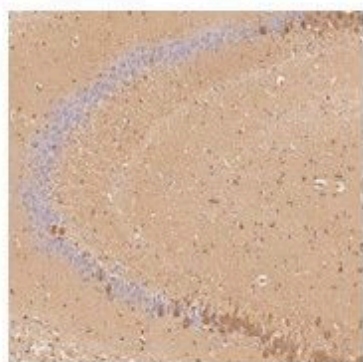


Figure 6

*Morinda officinalis* increased the content of 5-HT in the hippocampus of CUMS rats. Control (n=6) vs. CUMS (n=5), \*\*\*\*p<0.0001; CUMS (n=5) vs. MO (n=4), #####p<0.0001.



A. Control



B. CUMS



C. Flu



D. MO



E. MO+inhibitor



F. MO+neg

**Figure 7**

*Morinda officinalis* alleviate neuronal apoptosis in the hippocampal CA3 region of CUMS rats.