



## $\beta$ -asarone from *Acorus gramineus* alleviates depression by modulating MKP-1

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**ABSTRACT.** In this study, we investigated the antidepressant effects of hippocampal neuron administration of  $\beta$ -asarone, a selective mitogen-activated protein kinase phosphatase-1 inhibitor, in a rat model of depression. Our previous studies showed that the extracellular signal-regulated kinase signaling pathway and brain-derived neurotrophic factor expression, which is regulated by extracellular signal-regulated kinase, are key links in the biological mechanism of depression. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is a negative regulatory protein of extracellular signal-regulated kinase signaling pathways. In this study, we explored the regulation of MKP-1 by  $\beta$ -asarone in producing an antidepressant effect.

**Key words:**  $\beta$ -Asarone; Depression; Hippocampal neuron; Mitogen-activated protein kinase phosphatase-1

## INTRODUCTION

*Acorus gramineus* is a well-known Chinese traditional medicine that has been traditionally used for hundreds of years in oriental prescriptions for sedation as well as to enhance brain function (Vohora et al., 1990; Cho et al., 2002; Lim et al., 2012). The major constituents of the essential oil from *A. gramineus* include  $\beta$ -asarone and phenylpropenes. In our preliminary study,  $\beta$ -asarone showed significant antidepressant effects.

Depression is one of the most common neuropsychiatric comorbidities, affecting approximately 10% of the population, and is the leading cause of disability worldwide (Yi et al., 2012). Mitogen-activated protein kinase (MAPK) and MAPK phosphatase (MKP) are involved in drug addiction and depression (Jeanneteau and Deinhart, 2011; Jia et al., 2013). The extracellular signal-related kinase (ERK) signaling pathway is a classic MAPK signaling pathway and has been linked with synaptic plasticity and the survival of neurons (Grewal et al., 1999; Sweatt, 2001); sustained disruption of this pathway via MKP-1 is expected to have negative consequences on hippocampal neuron function (Boutros et al., 2008; Duric et al., 2010; Lee et al., 2012; Chen et al., 2012). Our previous study showed that MKP-1 is involved in the antidepressant effects of  $\beta$ -asarone; however, the mechanism underlying its antidepressant effects has not been clearly demonstrated. In this study, we found that  $\beta$ -asarone exhibited neuroprotective effects by modulating the level of phosphorylated ERK1/2 (p-ERK1/2) and brain-derived neurotrophic factor (BDNF) by regulating the level of MKP-1. We found that inhibition of ERK activity occurred through the induction of the ERK inhibitor MKP-1. This led to an increase in the death of hippocampal neurons, which agree with the results obtained in a previous study following a chronic unpredictable mild stress (CUMS) study in which MKP-1 was upregulated (Qi et al., 2006). Furthermore, downregulation of p-ERK1/2 or upregulation of MKP-1 enhanced hippocampal neuron apoptosis. In addition, we showed that downregulation of p-ERK1/2 or upregulation of MKP-1 decreased the basal level of BDNF protein and that inhibition of BDNF activity sensitized hippocampal neurons to  $\beta$ -asarone.

## MATERIAL AND METHODS

### Drugs and antibodies

Fluoxetine was produced by Suzhou Yushi Pharmaceutical Co., Ltd. (Suzhou, China).  $\beta$ -asarone was purchased from Tianjing Yifang Science and Technology Co., Ltd. (Yifang, China). Fluoxetine was dissolved in sterile saline (0.9%) and  $\beta$ -asarone was dissolved in double-distilled water at the required dose. Immunohistochemical staining kits for MKP-1, p-ERK1/2, and BDNF were purchased from Fuzhou Maixin Biotechnology Co., Ltd. (Fujian, China). The SYBR<sup>®</sup>ExScript<sup>™</sup> reverse transcription-polymerase chain reaction (RT-PCR) kit (Perfect Real Time) and RNAiso Reagent were purchased from Takara Co., Ltd. (Dalian, China). Antibodies for MKP-1, p-ERK1/2, and BDNF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Animals

Male Sprague-Dawley (SD) clean-level healthy rats 4-5 months old and weighing 180-200 g at the beginning of the experiment were housed with 5 animals per cage with food

and water *ad libitum* for one week. Rats were housed at a temperature of  $20^{\circ} \pm 2^{\circ}\text{C}$  and humidity of  $50 \pm 5\%$  in a controlled room with a 12-h light-dark cycle (lights on at 8:00 a.m.). All experimental procedures were conducted in conformity with the Institutional Animal Care and Use Committee (IACUC) guidelines.

## Groups and treatment

In an open-field test, 60 SD rats were randomly divided into 4 groups (15 rats in each group). The normal control group (NC) was given no stress except general handling; the model control group (MC) was exposed to CUMS and given once daily oral gavage of physiological saline; the fluoxetine control group (FC) was exposed to CUMS and given once daily oral gavage (p.o.) administration of 1.2 mg/kg fluoxetine; and the  $\beta$ -asarone group (B) was exposed to CUMS and given once daily oral gavage (p.o.) administration of 25 mg/kg  $\beta$ -asarone. The NC group was conventionally fed in the manner of 5 animals per cage and other groups were fed in the manner of 1 animal per cage. The experiment lasted 4 weeks.

## CUMS procedure

Rats in stressed groups were exposed to CUMS after a 1-week acclimatization period under the same housing conditions. CUMS procedures were based on previous studies (Willner et al., 1987; Duan et al., 2008) and included 9 types of stressors: food and water deprivation (24 h), cage tilt  $45^{\circ}$  (24 h), swimming in ice water ( $4^{\circ}\text{C}$ , 5 min), tail clipping (3 min), cage rotation (cage shaken on a rocking bed, 30 min), day and night reversal, damp environment, heat stress ( $45^{\circ}\text{C}$ , 5 min), and bounding. The stressors were given randomly 1 time daily and animals were not exposed to each stress factor more than 3 times. The stress sequence was changed every week in order to make the stress procedure unpredictable.

## Behavior tests

### *Sucrose intake test*

The sucrose intake test was a modified anhedonia-like state (Kessler et al., 2005). Before the test, the rats were habituated to consume a 5% sucrose (Eckel et al., 2005) solution for 24 h without any water and food available. On the final stress day, the rats were deprived of water and food for 24 h. Then, the rats were given a 1-h window sucrose test (between 8:00 and 9:00 a.m.). Total sucrose consumption was measured after 1 h.

### *Open field test*

The open field apparatus consisted of a black plywood box measuring 80 x 80 x 40 cm (width x length x height). The floor was divided into twenty-five 16 x 16-cm squares with white lines that had been drawn on the floor. A single rat was gently placed in the central square. The behaviors of rats were recorded for 3 min. The horizontal score (defined as at least 3 paws in a square) and the vertical score (defined as both frontal claws uplifting from the ground) were counted manually by 2 observers who were blinded to the experiment. Body weight was measured on days 1 and 28 of the experiment.

## Immunohistochemistry

We randomly selected 6 rats in each group for immunohistochemical analysis. The rats were anesthetized with diethyl ether and perfused with 4% paraformaldehyde. The brain tissue was removed after perfusion fixation. Three-micrometer paraffin sections were incubated at 60°C for 30 min, then de-waxed and rehydrated with freshly distilled water for 2 min at room temperature. The sections were placed in a glass jar containing folic acid fluid for repair, pH 6.0, and heated in a pressure cooker to 125 kPa for 2 min. The sections were allowed to cool inside the pressure cooker for 20 min. After rinsing with double-distilled water, the sections were washed with phosphate-buffered saline (PBS), pH 7.4, for 3 min (3 times). Samples underwent inactivation of endogenous peroxidase for 15 min and were washed with PBS for 3 min (3 times). After removing PBS, samples were non-specifically blocked with normal goat serum for 15 min at room temperature. After removing the goat serum, rabbit anti-MKP-1 (1:50), p-ERK1/2 (1:150), and BDNF (1:100) antibody was added overnight at 4°C. Samples were flushed with PBS for 3 min (3 times). After removal of PBS, the samples were incubated with secondary antibody for 10 min at 37°C, and then flushed with PBS for 3 min (3 times). After removing the PBS, samples were incubated with the streptavidin-peroxidase complex for 10 min at 37°C, and then flushed with PBS for 3 min (3 times). After removal of PBS, peroxidase activity was developed using DAB as the chromogen, and then observed under a light microscope for 3-10 min. The samples were then stained with hematoxylin, dehydrated, and mounted for microscopic examination. Images of the sections were obtained (400X) using the Image-Pro Plus 4.5 software; brown cytoplasmic staining under light microscopy indicated a positive reaction for MKP-1, p-ERK1/2, and BDNF.

## RT-PCR assay

Total RNA was extracted from the samples using TRIZOL reagent (Takara Biotechnology; Dalian, China). cDNA was synthesized (Takara Biotechnology) according to the manufacturer protocol. PCR was performed by using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> in an ABI7300 real-time PCR system (Applied Biosystems; Foster City, CA, USA). The thermal profile was as follows: 1 cycle at 95°C for 10 s; 40 cycles for 5 s at 95°C and 31 s at 60°C. Threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems). Ct represents the cycle number at which a fluorescent signal rises statistically above background. The real-time PCR assay was performed in triplicate for each sample to ensure reproducibility. The relative quantification of gene expression was analyzed using the  $2^{-\Delta\Delta C_t}$  method. The fold change in a target gene cDNA relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control was determined by: fold change =  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\text{GAPDH}}}) - (C_{t_{\text{control}}} - C_{t_{\text{GAPDH}}})$ .

## Statistical analysis

All data were analyzed using the SPSS 16.0 analysis software (SPSS, Inc.; Chicago, IL, USA). One-way analysis of variance test was performed to examine the data among groups. Means  $\pm$  SE were compared with the least significant difference test for multiple

comparisons. Statistical significance was set at  $P < 0.05$ .

## RESULTS

### Effects of $\beta$ -asarone treatment on body weight

On the first day of the experiment, there was no significant difference among groups in body weight ( $P > 0.05$ ). After the 28-day stress procedure, a significant difference was observed among groups ( $P < 0.05$ ). The MC group showed a significant decrease in body weight compared to the NC group ( $P < 0.05$ ).  $\beta$ -asarone and fluoxetine treatments had a significant effect on the decrease in body weight ( $P < 0.05$ ), suggesting that  $\beta$ -asarone affected body weight in CUMS rats (Figure 1A).

### Effects of $\beta$ -asarone treatment on sucrose intake

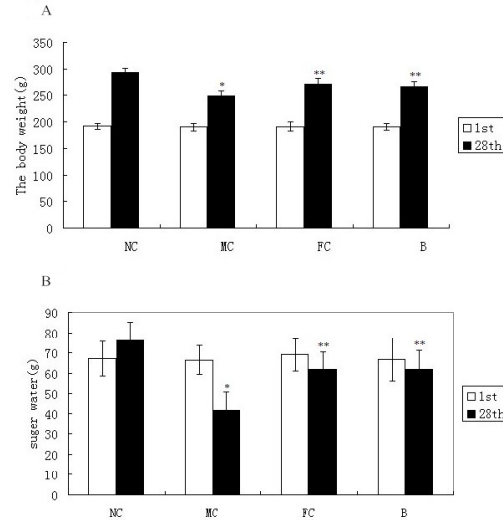
The sucrose intake test can be used to predict the sensitivity to rewards (Tönissaar et al., 2006). As shown in Figure 1B, the total consumption of sucrose was significantly lower in the MC group compared to the NC group. The total consumption of sucrose in the B and the FC groups were significantly increased compared to the MC group (Figure 1B). We found that  $\beta$ -asarone could increase the reward effect.

### Effects of $\beta$ -asarone treatment on the open field test

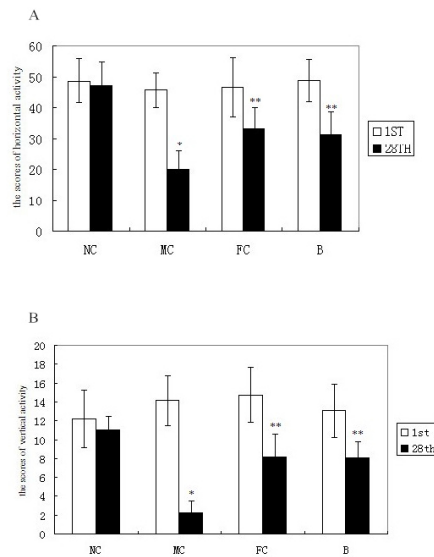
The open field test was used to study the exploratory and locomotor activity of rats (Prut and Belzung, 2003; Liu et al., 2013). As shown in Figure 1, on the first day of the experiment, there was no significant difference among groups in the open field test ( $P > 0.05$ ) before the CUMS procedure. After 4 weeks of CUMS, the MC group showed significant depressive-like behaviors and a significant difference was observed among groups ( $P < 0.05$ ). The MC group showed significantly lower scores for horizontal activity and vertical activity compared with the NC group ( $P < 0.05$ ); compared with the MC group, the FC and B groups showed higher scores for horizontal activity and vertical activity ( $P < 0.05$ ) (Figure 2A, B). We found that behavior deficits induced by chronic stress were restored by  $\beta$ -asarone.

### Effects of $\beta$ -asarone treatment on MKP-1, p-ERK1/2, and BDNF levels in the hippocampus

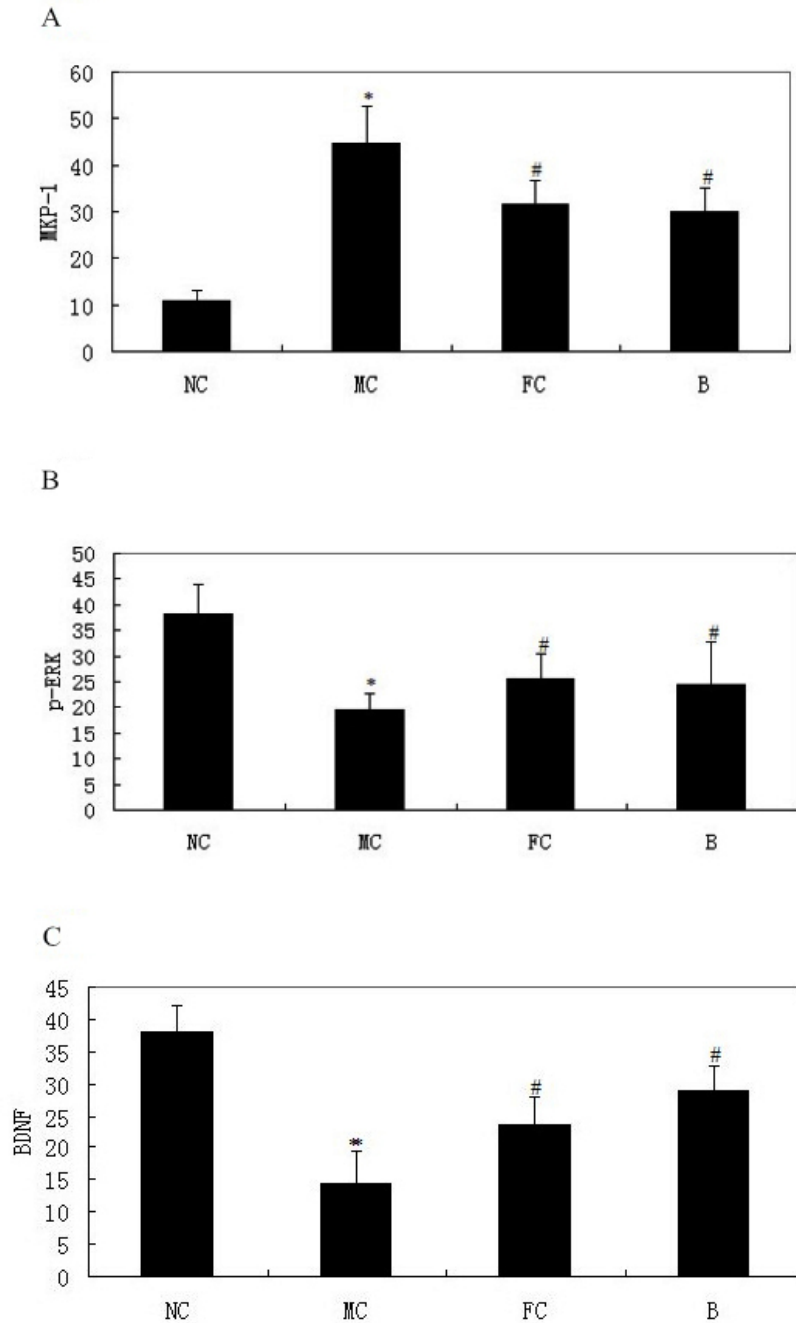
Immunohistochemistry showed that the expression of MKP-1 in the MC group was comparable to that in the NC group ( $P < 0.05$ ). However, the expression of p-ERK1/2 and BDNF in the FC and B groups was significantly decreased compared with that in the MC group ( $P < 0.05$ ) (Figure 3A, B, C). The real-time RT-PCR assay showed that the expression of *MKP-1* mRNA in the MC group was comparable to that in the NC group ( $P < 0.05$ ). However, the expression of *p-ERK1/2* mRNA and *BDNF* mRNA in the FC and B groups was significantly decreased compared with that in the MC group ( $P < 0.05$ ) (Figure 4A, B, C).



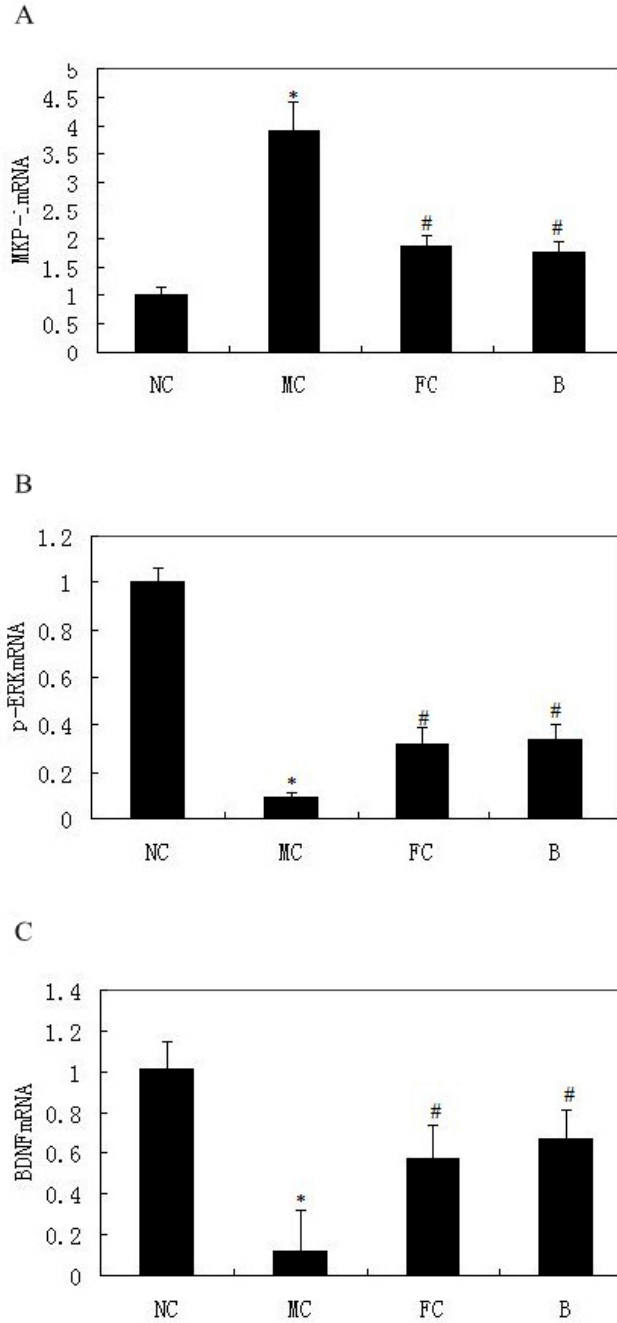
**Figure 1.** Effects of b-asarone treatment on body weight (**A**) and sucrose preference (**B**) in normal and CUMS-induced rats. On the first day of the experiment, there was no significant difference among groups ( $P > 0.05$ ). After the experiment, we compared body weight and sucrose preference between the model control (MC) and normal control (NC) groups ( $*P < 0.05$ ). Comparison between the MC, fluoxetine control (FC), and  $\beta$ -asarone (B) groups ( $**P < 0.05$ ). Body weight: first day ( $F = 0.240$ ,  $P = 0.868$ ); 28th day ( $F = 48.162$ ,  $P = 0.000$ ). Total sucrose consumption: first day ( $F = 0.283$ ,  $P = 0.838$ ); 28th day ( $F = 37.302$ ,  $P = 0.000$ ).



**Figure 2.** Behavioral changes in an open-field. On the first day of the experiment, there was no significant difference among groups ( $P > 0.05$ ). **A.** Comparison of the scores of horizontal activity between MC and NC ( $*P < 0.05$ ) at the end of the experiment. Comparison between the MC, FC, and B groups ( $**P < 0.05$ ). **B.** Comparison of the scores of vertical activity between the MC and NC groups ( $*P < 0.05$ ). Comparison between the MC, FC, and B groups ( $**P < 0.05$ ). Scores of horizontal activity: first day ( $F = 0.484$ ,  $P = 0.695$ ); 28th day ( $F = 38.651$ ,  $P = 0.000$ ). Scores of vertical activity: first day ( $F = 0.079$ ,  $P = 0.971$ ); 28th day ( $F = 8.697$ ,  $P = 0.000$ ). For abbreviations, see legend to Figure 1.



**Figure 3.** Immunohistochemical detection of MKP-1 (A), p-ERK1/2 (B), and BDNF (C) in hippocampal neurons. The level of MKP-1 protein in the MC group was significantly higher than that in other groups ( $F = 32.030$ ,  $P = 0.000$ ;  $*P < 0.05$ ); the level of p-ERK1/2 and BDNF proteins in the MC group were significantly lower than those in other groups (p-ERK1/2:  $F = 14.681$ ,  $P = 0.000$ ,  $*P < 0.05$ ; BDNF:  $F = 23.198$ ,  $P = 0.000$ ,  $*P < 0.05$ ).



**Figure 4.** RT-PCR results for the detection of *MKP-1*, *p-ERK1/2*, and *BDNF* mRNA. **A.** *MKP-1* mRNA levels in hippocampal neurons in the MC group were significantly higher than in the other groups ( $F = 85.727$ ,  $P = 0.000$ ,  $*P < 0.05$ ); expression of *p-ERK1/2* mRNA (**B**) and *BDNF* mRNA (**C**) was significantly lower than in the other groups (*p-ERK1/2* mRNA:  $F = 212.940$ ,  $P = 0.000$ ,  $*P < 0.05$ ; *BDNF* mRNA:  $F = 38.329$ ,  $P = 0.000$ ,  $*P < 0.05$ ).



## DISCUSSION

CUMS decreased body weight, induced anhedonia, and induced significant depressive-like behaviors.  $\beta$ -asarone reversed these effects and showed antidepressant effects. In this study, we found that  $\beta$ -asarone modulated neuronal survival by regulating the expression of MKP-1 in models of depression. Our data indicated the relevance of MKP-1 as a negative prognostic factor in the mechanism of depression. Overexpression of MKP-1 prevented ERK activity (low expression of p-ERK1/2) and downregulated the expression of BDNF. MKP-1 is not only directly expressed in response to stress, but is also a key negative regulator of the ERK signaling pathway, contributing to the expression of depressive symptoms (Vogt et al., 2005; Duric et al., 2010). BDNF is well-known for its effects on axon outgrowth and branching (Cohen-Cory, 2002). Indeed, the BDNF expression level is modulated by the ERK signaling pathway. Regulation of BDNF expression by MKP-1 is an important mechanism accounting for how neuronal networks may be refined in an activity-dependent manner (Glorioso et al., 2006; Jeanneteau et al., 2010). The ERK signaling pathway has been examined for its role in stress and depression. Our findings showed that  $\beta$ -asarone treatment decreased MKP-1 and increased p-ERK1/2 and BDNF in the hippocampus of CUMS rats ( $P < 0.05$ ). Our data showed that the ratio of MKP-1 in the hippocampus was significantly higher in the model group than that in the  $\beta$ -asarone and fluoxetine groups ( $P < 0.05$ ). We also found that the ratio of p-ERK1/2 and BDNF in the hippocampus was significantly higher in the  $\beta$ -asarone and fluoxetine groups than in the model group ( $P < 0.05$ ).  $\beta$ -asarone treatment appeared to have a stimulating effect on the experimental animals, and traditional medicine may be beneficial in model rats. Fluoxetine, a type of selective serotonin reuptake inhibitor, is a clinically effective antidepressant drug. In accordance with previous findings (Qi et al., 2006), our results showed that fluoxetine alleviated anhedonia induced by chronic stress. In addition, we found that fluoxetine increased the levels p-ERK1/2 and BDNF in the hippocampus and decreased the level of MKP-1 in the hippocampus compared with the model group ( $P < 0.05$ ). It is reasonable to speculate that  $\beta$ -asarone influences p-ERK1/2 and BDNF expression by mediating MKP-1, which has an antidepressant effect. Previous studies showed that  $\beta$ -asarone could effectively improve the weight and significantly improve behavior in a rat model of depression. We found that chronic stress exposure caused deficits in ERK and BDNF activation, but a surplus in MKP-1 activation in the hippocampus. This consequence could be reversed by  $\beta$ -asarone treatment. Our results suggested that  $\beta$ -asarone inhibits MKP-1 activation and sustains ERK signal pathway activation in rats exposed to CUMS, which may mediate the antidepressant-like effect of  $\beta$ -asarone. We identified MKP-1 as a key factor in depression pathophysiology and as a new target for therapeutic intervention.

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