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Dried bear bile exerts its antidepressant effect by modulating adrenal FXR to reduce peripheral glucocorticoid levels☆

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ABSTRACT

Depression is a psychological disorder associated with prolonged stress, which involves abnormal activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevated levels of glucocorticoids (GC). Excessive GC can cause damage to the structure and function of the hippocampus, thereby triggering depressive symptoms. Studies suggest that the bile acid receptor farnesoid X receptor (FXR) may play a role in adrenal GC synthesis. This study aimed to explore the potential therapeutic effects of dried bear bile (DBB) on depression and its mechanism. We used the chronic unpredictable mild stress (CUMS) mouse model and FXR agonist GW4064 stimulated mice, as well as H295R human adrenal cortical carcinoma cells, employing behavioral tests, biochemical analysis, and gene expression analysis to assess the effects of DBB treatment on depressive behavior, serum corticosterone (CORT) levels, and adrenal FXR and steroid biosynthesis-related gene expression. The results showed that in both CUMS and GW4064-stimulated mice, DBB treatment significantly improved depressive-like behaviors and reversed serum CORT levels. Additionally, DBB suppressed the expression of steroidogenic regulatory genes in the adrenal glands of CUMS mice. In H295R cells, DBB treatment effectively reduced cortisol secretion induced by Forskolin, inhibited the expression of steroid biosynthesis-related genes, and suppressed cortisol production and HSD3B2 expression under conditions of FXR overexpression and FXR activation. Our findings suggest that DBB regulates adrenal FXR to modulate glucocorticoid synthesis and exerts antidepressant effects. DBB may serve as a potential therapeutic agent for depression by regulating GC levels and steroidogenesis pathway. Further research is underway to test the antidepressant effects of each DBB component to understand their specific contribution.

1. Introduction

Depression is a complex psychiatric and psychological disorder characterized by profound sadness, loss of interest, and decreased motivation (Abreu et al., 2022; Badr et al., 2020). According to data from the World Health Organization in 2020, depression is projected to become the second leading cause of disease worldwide. It is considered as the "leading cause of disability," posing a serious threat to human health and well-being (LeMoult et al., 2020; Yuan et al., 2020). Additionally, it is estimated that by 2030, depression will become the disease with the largest economic burden on society (Malhi and Mann, 2018).

The pathogenesis of depression is highly complex. While the definitive pathophysiology of depression remains largely undetermined, genetic factors and psychological stress events are associated with depression (Yang et al., 2015). Prolonged exposure to stress and tension-inducing life events is one of the contributing factors to the onset or exacerbation of depression (Du Preez et al., 2021), particularly,

☆ Disclaimer: Dried bear bile is a legal product produced in China under regulated conditions and forms part of traditional Chinese medicine practices. The dried bear bile used in the study was a purchased commercial product from Heilongjiang Heibao Pharmaceutical Co., Ltd. In presenting the scientific evidence of the use of dried bear bile, demonstrating benefits to depression, it does not confer that we at ITM in any way support or condone the inhumane treatment of any animals.

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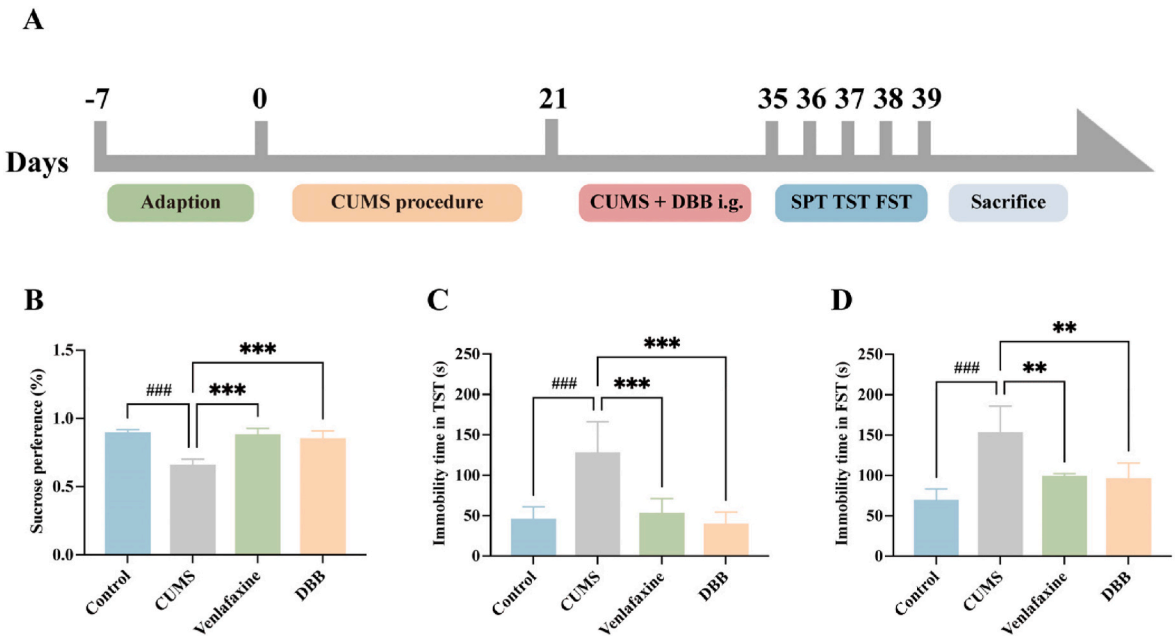

Fig. 1. The effect of DBB on depressive-like behavior in CUMS mice
(A) Establishment and drug administration procedure of the CUMS model; (B) Sucrose preference in SPT (N = 8/group); (C) Immobility time in TST (N = 8/group); (D) Immobility time in FST (Brown-Forsythe and Welch ANOVA test with Dunnett T3 multiple comparisons test, N = 8/group). One-way ANOVA with Dunnett multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. CUMS group, $^{###}p < 0.001$ vs. Control group. Results are presented as means ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

chronic stress can lead to dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis (Song et al., 2021; Zhang et al., 2021). Under normal circumstances, the HPA axis regulates the levels of glucocorticoids (GC), such as cortisol, referred to as corticosterone (CORT) in rodents, through a negative feedback mechanism (Degering et al., 2023). However, chronic stress disrupts this negative feedback, resulting in alterations in HPA axis regulation, leading to excessive GC levels in individuals with mood disorders (Juruena et al., 2004). Elevated levels of GC can induce persistent stress responses, thereby triggering symptoms of depression and cognitive dysfunction (Ali et al., 2015). Studies have reported that approximately 40 % – 60 % of patients with depression exhibit HPA axis dysregulation, including hypercortisolemia and elevated cortisol levels (Pariante and Lightman, 2008). Therefore, it is feasible to develop antidepressants by down-regulating CORT levels in the body.

The adrenal gland is the primary organ responsible for synthesizing GC, a process involving multiple enzymes and pathways, including steroidogenic acute regulatory protein (*STAR*), 3β-hydroxysteroid dehydrogenase type 2 (*HSD3B2*), and 21-hydroxylase (*CYP21A2*) (Taves et al., 2011). These proteins encoded by these genes play crucial roles in GC synthesis. STAR is a transport protein that mediates the transport of cholesterol, the precursor of steroid hormones, from the outer mitochondrial membrane to the inner mitochondrial membrane, a rate-limiting step in steroid hormone synthesis (Kallen et al., 1998). HSD3B2 is a key enzyme in the synthesis of aldosterone and cortisol and is highly expressed in the adrenal cortex (Rhéaume et al., 1991). Its function involves catalyzing the conversion of 3β-hydroxysteroids to ketosteroids, which is essential for the biosynthesis of all steroid hormones (Simard et al., 2005). *CYP21A2* encodes the enzyme 21-hydroxylase, which catalyzes the conversion of 17-hydroxyprogesterone to cortisol, a critical step in adrenal cortisol synthesis (Ryan and Engel, 1957). Additionally, besides these enzymes, some receptors also play important roles in adrenal GC synthesis. The bile acid receptor farnesoid X receptor (FXR) is a crucial member of the ligand-activated transcription factor nuclear receptor superfamily. Apart from significant expression in the gastrointestinal tract, it is also highly expressed in the adrenal gland (Higashiyama et al., 2008). Previous studies have reported that the use of FXR agonists like GW4064 in mice increases adrenal GC secretion (Hoekstra et al., 2012), and *in vitro* studies on human adrenal cortex cells (H295R) have indicated that FXR may directly regulate adrenal steroidogenesis by modulating HSD3B2 transcription (Xing et al., 2009). These findings suggest that adrenal FXR may be one of the targets involved in regulating GC production.

Bear bile is the dried bile obtained from the gallbladders of members of the bear family, including the Asiatic black bear (*Selenarctos thibetanus Cuvier*) and the brown bear (*Ursus arctos* L.). Dried bear bile (DBB), derived from artificially draining and drying bear bile, serves as a substitute for natural bear bile in related medicinal research and treatments, possessing properties of heat-clearing, liver-soothing, and vision-improving effects (Chen et al., 2020; Li X et al., 2022). Modern studies have shown that DBB primarily consists of various bile acids, with FXR being a receptor for bile acids (Huang et al., 2022). Additionally, our previous research has revealed that FXR gene knockout mice not only exhibit significantly elevated levels of various taurine-conjugated bile acids in serum but also display antidepressant-like effects (Huang et al., 2015). This suggests that DBB may exert antidepressant effects by regulating adrenal FXR expression levels and subsequently GC levels.

This study investigates the antidepressant efficacy and mechanism of DBB using both *in vivo* and *in vitro* models. In the *in vivo* study, we explored the antidepressant efficacy of DBB and its regulation of GC levels using the Chronic Unpredictable Mild Stress (CUMS) model and the GW4064-stimulated mice. In the *in vitro* study, we further elucidated how DBB regulates GC levels via adrenal FXR, thereby exerting its antidepressant effects. Our research reveals, for the first time, the antidepressant effects of DBB, aiming to provide scientific evidence for its use in the treatment of depression.

2. Methods and materials

2.1. Reagents

DBB (Cat No. Z10980057) was provided by Heilongjiang Heibao

Pharmaceutical Co. Ltd. (China), which mainly contains six bile acid components: Taurochenodeoxycholic Acid (TCDCA, 23.3 %), Tauroursodeoxycholic Acid (TUDCA, 23.1 %), Ursodeoxycholic acid (UDCA, 0.2 %), Taurohyodeoxycholic acid (THDCA, 11.1 %), Hyodeoxycholic acid (HDCA, 1.4 %), and Taurocholic acid (TCA, 1.2 %). GW4064 was purchased from MCE (HY-50108, MedChemExpress, Shanghai, China). Antibodies against FXR (cat: A5942, 1:1000, Rabbit) and β-actin (cat: AC026, 1:100000, Rabbit) were purchased from ABclonal Technology Co., Ltd. (Wuhan, China).

2.2. Animals

Fifty 6-week-old male C57BL/6 mice were obtained from the Animal Research Center of Shanghai University of Traditional Chinese Medicine (SHUTCM). Upon arrival, all mice were housed in standard conditions with a 12-h light/dark cycle, temperature maintained at 25 ± 1 °C, and humidity at 50 ± 10 %. They were provided with ad libitum access to food and water. After a two-week acclimatization period to the new environment, the mice were included in the experimental procedures. All animal-related procedures were conducted following the guidelines and protocols approved by the Animal Care and Use Committee of SHUTCM (Approval Number: PZSHUTCM201231005).

2.3. Establishment of CUMS model and DBB administration

The CUMS model was established based on previous studies (Tao et al., 2024) with slight modifications. Mice were randomly divided into four groups: Control, CUMS, Venlafaxine, and DBB (200 mg/kg) groups. The dosage of DBB was determined based on preliminary results from laboratory experiments. Control group mice were housed normally (n = 4/cage), while mice in other groups were individually housed. Additionally, mice in the other groups were subjected to daily random stressors, including fasting (12 h), water deprivation (12 h), empty cage (24 h), noise exposure (30 min), tail clipping (5 min), 45° cage tilt (24 h), foreign object exposure (24 h), restraint (3 h), and continuous light exposure (24 h). Each stressor was administered once daily, and no two consecutive days featured the same stressor. The total modeling period for CUMS was 5 weeks.

Venlafaxine and DBB were both dissolved in 0.9 % saline solution. After 3 weeks of modeling, the Venlafaxine group received an oral gavage of 20 mg/kg Venlafaxine, while the DBB group received an oral gavage of 200 mg/kg DBB. The control group and CUMS group received oral gavage of 0.9 % saline solution. The treatment lasted for a total of two weeks. The specific procedure is illustrated in Fig. 1A.

The GW4064-stimulated mice were established with the specific process shown in Fig. 3A. Mice were randomly divided into the control group, GW4064 (30 mg/kg) group, and GW4064 + DBB (200 mg/kg) group. GW4064 was dissolved in corn oil, while DBB was dissolved in physiological saline. The control group received physiological saline. After one week of continuous gavage administration, behavioral tests were conducted.

2.4. Behavioral tests

Behavioral tests were conducted according to previous studies (Tao et al., 2020). All behavioral tests were performed between 8:00 a.m. and 12:00 p.m. to ensure uniform lighting and a quiet environment. The analyses were conducted using a video tracking system (Noldus Information Technology™, Leesburg, VA).

Tail Suspension Test (TST): Mice were secured by adhesive tape applied approximately 2 cm from the base of the tail onto a horizontal wooden stick, causing them to hang upside down with their heads approximately 5 – 6 cm above the surface. The cumulative immobility time during the last 4 min of a 6-min observation period was recorded for each animal. After each trial, the observation chamber was cleaned with 10 % alcohol before observing the next mouse.

Forced Swim Test (FST): Mice were placed individually into a transparent cylindrical container (30 cm height, 20 cm diameter) filled with water to a depth of 20 cm at a temperature of 25 ± 1 °C. The cumulative immobility time during a 6-min observation period was recorded for each mouse, focusing on the last 4 min of immobility.

Sucrose Preference Test (SPT): Prior to testing, each cage of mice was provided with two bottles, one containing 1 % sucrose solution and the other containing pure water, for adaptation for one day. After adaptation to the sucrose solution, each mouse was simultaneously provided with both bottles, and the testing lasted for three days. During the testing process, the position of the bottles was exchanged daily. On the third day, the consumption of sucrose solution and pure water was measured to calculate the sucrose preference rate. The formula for calculating the percentage of sucrose intake is (sucrose intake/(sucrose intake + water intake)) × 100 %.

2.5. Serum and tissue sample collection

After the final behavioral test, mice were fasted for 12 h, followed by anesthesia with pentobarbital sodium. Blood samples were collected from the orbital sinus, and mice were euthanized immediately thereafter. After centrifugation at 3000 rpm for 15 min at 4 °C, the serum was collected and stored at −80 °C. The adrenal glands were dissected on ice, followed by rapid freezing in liquid nitrogen and storage at −80 °C for further analysis.

2.6. Cell culture and treatment

The human adrenocortical carcinoma cells H295R were purchased from the Cell Bank of the Chinese Academy of Sciences. They were cultured in Dulbecco's modified Eagle medium (DMEM; Meilunbio, Dalian, China) containing 2.5 % serum substitute, 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenium, 5.35 μg/mL linoleic acid, 5.35 μg/mL bovine serum albumin, 100 kU/L penicillin, and 100 mg/L streptomycin at 37 °C in a 5 % CO_2 humidified incubator.

Cells in logarithmic growth phase were seeded into 96-well plates at an appropriate density. They were divided into the control group, Forskolin (10 μM)/FXR overexpression plasmid/GW4064 (5 μM) group, Forskolin (10 μM)/FXR overexpression plasmid/GW4064 (5 μM) group + DBB (25, 50, 100 μg/mL) group. After overnight incubation, the cells were treated with fresh medium the next day. After 24 h, cell viability was assessed using the CCK-8 assay.

Logarithmic growth phase H295R cells were seeded into 24-well plates at an appropriate density. They were divided into control group, Forskolin group, Forskolin + DBB (25, 50, 100 μg/mL) group. After overnight incubation, the cells were treated with fresh medium the next day. After 24 h, the supernatant cortisol content was measured using UPLC-MS/MS, and cells were collected for Q-PCR analysis.

Logarithmic growth phase H295R cells were seeded into 24-well plates at an appropriate density. They were divided into control group, FXR overexpression plasmid group, FXR + DBB (25, 50, 100 μg/mL) group, GW4064 (5 μM) group, GW4064 + DBB (25, 50, 100 μg/mL) group. After overnight incubation, the cells were treated with fresh medium the next day. After 24 h of stimulation, the supernatant cortisol content was measured using UPLC-MS/MS, and cells were collected for Q-PCR or Western blotting analysis.

2.7. UPLC-MS/MS analysis

After extracting 50 μL of serum or cell supernatant with 300 μL of ice-cold methanol and thorough vortex mixing, the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. Following centrifugation, the supernatant was transferred to a new EP tube and dried under nitrogen gas. Subsequently, 100 μL of chromatographic-grade ice-cold methanol was added to the dried samples, vortexed for reconstitution, and transferred to a vial for injection into the UPLC-MS/MS system (SHIMADZU,

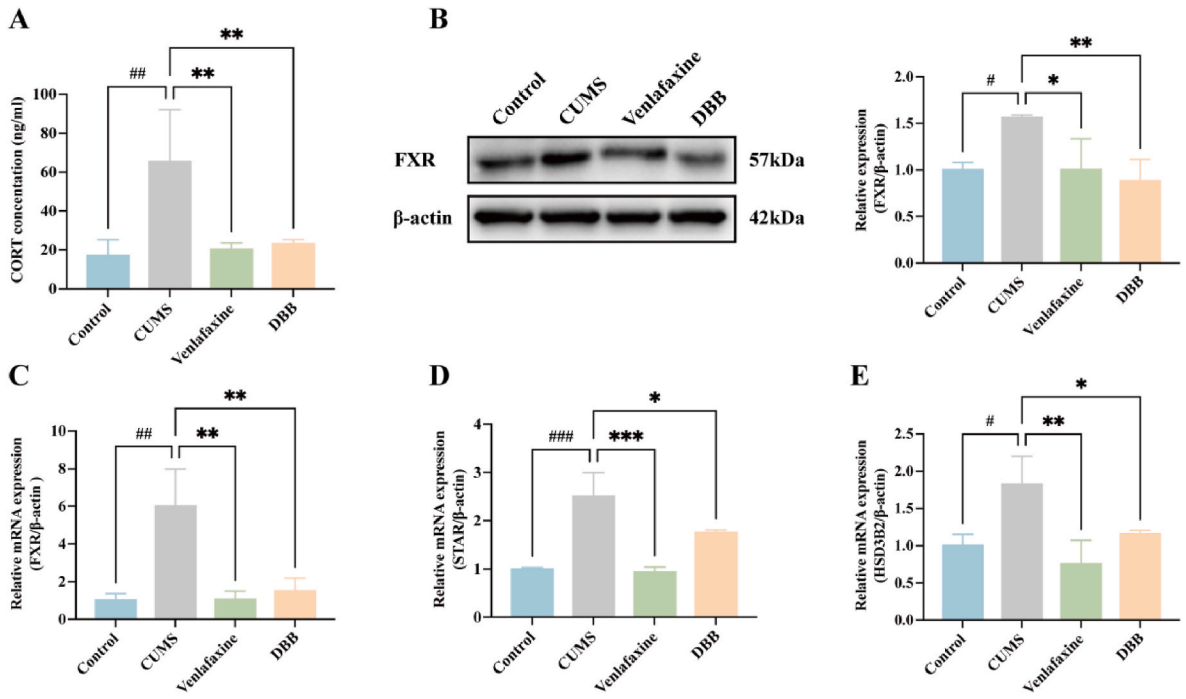

Fig. 2. The effect of DBB on CORT production and its synthesis process in CUMS mice
(A) Serum CORT levels (Brown-Forsythe and Welch ANOVA test with Dunnett T3 multiple comparisons test, N = 8/group); (B) Western blotting analysis and quantification of FXR in CUMS mice; (C–E) Q-PCR analysis of adrenal FXR, STAR and HSD3B2 (N = 3/group). One-way ANOVA with Dunnett multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. CUMS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Control group. Results are presented as means ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

China) under the conditions described in a previous study (Liu et al., 2021). The chromatographic column used was an ACQUITY UPLC^{TM} BEH C18 column (1.7 μm, 2.1 mm × 100 mm) (Waters).

2.8. Real-time PCR analysis

Total RNA from mouse adrenal tissue and H295R cells was extracted using Trizol, followed by reverse transcription into cDNA using the Reverse transcription kit (Vazyme Biotech Co., Ltd., Nanjing, China). Real-time PCR detection was performed using the Taqman SYBR kit (ThermoFisher) according to the manufacturer's instructions. The relative mRNA expression levels of the target genes were normalized to β-actin in the same sample using the $2^{-\Delta\Delta CT}$ method. Primer sequences were shown as below: for FXR, 5′- GATGGGGATGTTGGCTGAATG -3′ and 3′- AGTTCCGTTTTCTCCCTGC -5'; for STAR, 5′- ATGTTCCTCGCTACGTTCAAG -3′ and 3′- CCCAGTGCTCTCCAGTTGAG -5'; for HSD3B2, 5′-GGTTTTTGGGGCAGAGGATCA-3′ and 3′-GGTACTGGGTGTCAAGAATGTCT-5'; for CYP21A2, 5′- CTCACCTTCGGAGACAAGATCA -3′ and 3′- TCCACAATTTGGATGGACCAG -5'; for β-actin, 5′-GGCTGTATTCCCCTCCATCG -3′ and 3′- CCAGTTGGTAACAATGCCATGT -5'.

2.9. Western blot analysis

Tissues (0.1 mL lysis buffer per 10 mg protein) or cells (0.1 mL lysis buffer per 10^6 cells) were lysed in cold RIPA buffer (Meilunbio, Dalian, China) supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN). The protein concentration was quantified using the BCA Protein Quantification Kit (Yeasen, Shanghai, China). Western blot analysis was performed according to the established protocol (Yuan et al., 2022). Briefly, proteins (20 μg) from each sample were separated by electrophoresis on a 10 % polyacrylamide gel and transferred onto a PVDF membrane. The membrane was then blocked with 5 % skim milk, followed by overnight incubation with primary antibodies at 4 °C. After washing with PBST, the membrane was incubated with corresponding secondary antibodies at room temperature for 1 h. Chemiluminescent detection of antibody-targeted specific proteins was performed using an HRP substrate (Merck Millipore, Massachusetts, USA), and images were processed and analyzed using Tanon 5200 imaging system (Tanon, Shanghai, China).

2.10. Statistical analysis

The data are presented as mean ± SD. All datasets were initially assessed for normality with the Shapiro-Wilk Test. Subsequently, Bartlett's test was employed to ensure equal standard deviations among the individual datasets. If the individual data were normally distributed and exhibited homogeneity of variances among the groups, we conducted one-way ANOVA with Dunnett's post hoc test. In cases where group variances were not homogeneous, we utilized the Brown-Forsythe and Welch ANOVA test with Dunnett's post hoc test. All statistical analyses were carried out using GraphPad Prism 9.5 software (GraphPad, USA). A significance level of $P < 0.05$ was set as statistically significant.

3. Results

3.1. DBB alleviates depressive-like behaviors in CUMS mice

Behavioral tests were conducted to assess the ameliorative effects of DBB on depressive-like behaviors in CUMS mice (Fig. 1B–D). The SPT was primarily employed to evaluate the mice's capacity to experience pleasure (Willner et al., 1987). Compared to the control group, CUMS mice exhibited a significant decrease in sucrose preference ($P < 0.001$). However, following DBB treatment, the sucrose preference rate of depressed mice significantly increased (compared to the CUMS group: Venlafaxine, $P < 0.001$; DBB 200 mg/kg, $P < 0.001$).

Despair behavior in depressed mice is typically assessed using the TST and FST (Porsolt et al., 1977; Steru et al., 1985). Compared to

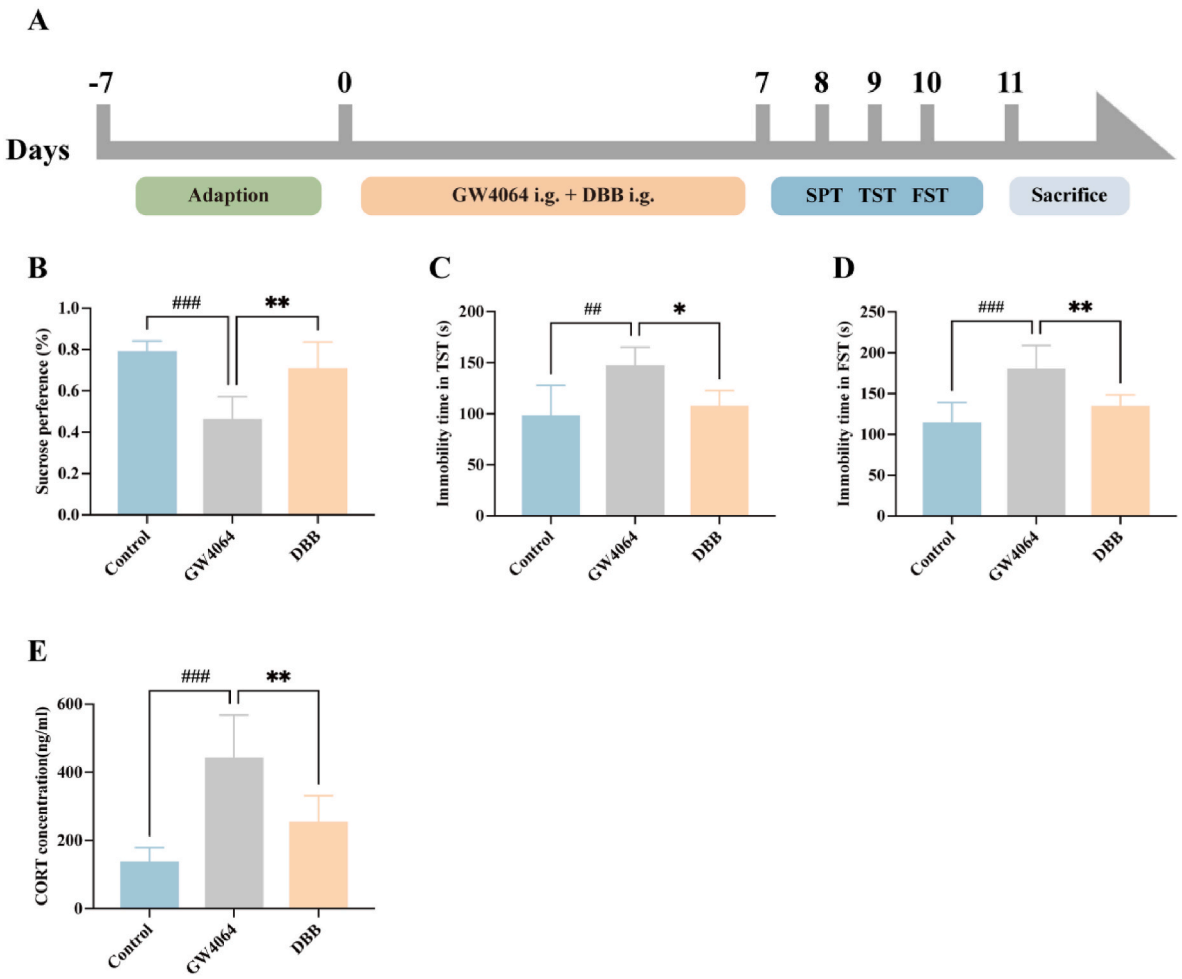

Fig. 3. The effect of DBB on behavior and serum CORT levels induced by GW4064 in mice
(A) The administration process of GW4064 and BBP; (B) Sucrose preference in SPT (N = 6/group); (C) Immobility time in TST (N = 6/group); (D) Immobility time in FST (N = 6/group); (E) Serum CORT levels (N = 6/group). One-way ANOVA with Dunnett multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. GW4064 group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Control group. Results are presented as means ± SD.

control group mice, CUMS mice exhibited a significant increase in immobility time during tail suspension (P < 0.001), which was significantly reduced by DBB in depressed mice (compared to the CUMS group: Venlafaxine, P < 0.001; DBB 200 mg/kg, P < 0.001). Similarly, CUMS significantly prolonged immobility time during forced swimming in mice (P < 0.001), while DBB effectively reversed this phenomenon (compared to the CUMS group: Venlafaxine, P < 0.01; DBB 200 mg/kg, P < 0.01).

3.2. DBB suppresses the production of GC in CUMS mice

Chronic stress often leads to elevated levels of GC in the body (Krugers et al., 2012). To investigate whether DBB can modulate the level of GC induced by CUMS, we measured serum CORT levels (Fig. 2A). The serum CORT levels in CUMS mice were significantly higher than those in the control group (P < 0.01). However, treatment with DBB significantly decreased the CORT levels (compared to the CUMS group: Venlafaxine, P < 0.01; DBB 200 mg/kg, P < 0.01). FXR is associated with adrenal GC synthesis. To investigate whether DBB acts on adrenal FXR, we simultaneously assessed FXR expression in CUMS mice (Fig. 2B and C). Compared to the control group, adrenal FXR protein and mRNA expression levels were significantly increased in CUMS mice (P < 0.05, P < 0.01). In contrast, after DBB treatment, both the protein and mRNA expression levels of adrenal FXR in CUMS mice were significantly decreased (FXR protein, compared to the CUMS group: Venlafaxine, P < 0.05; DBB 200 mg/kg, P < 0.01; FXR mRNA, compared to the CUMS group: Venlafaxine, P < 0.01; DBB 200 mg/kg, P < 0.01). Furthermore, we examined adrenal GC synthesis-related genes (Fig. 2C and D), and found that adrenal STAR and HSD3B2 mRNA expression levels were significantly higher than those in the control group (P < 0.001, P < 0.05), while DBB effectively suppressed their expression levels (STAR mRNA, compared to the CUMS group: Venlafaxine, P < 0.001; DBB 200 mg/kg, P < 0.05; HSD3B2, compared to the CUMS group: Venlafaxine, P < 0.01; DBB 200 mg/kg, P < 0.05).

3.3. DBB alleviates depressive-like behaviors and reduces serum GC levels in GW4064-stimulated mice

GW4064 is an FXR agonist, and FXR activation can regulate CORT production. Sustained high levels of CORT may lead to depression (Hoekstra et al., 2012). We explored whether GW4064 induces depressive-like behaviors in mice and examined the ameliorative effects of DBB on this phenomenon. Compared to the control group, mice administered GW4064 alone exhibited a significant decrease in sucrose preference (Fig. 3B, P < 0.001) and a significant increase in immobility time during the TST (Fig. 3C, P < 0.01) and FST (Fig. 3D, P < 0.001). However, in the group receiving both GW4064 and DBB, DBB significantly reversed these effects, restoring sucrose preference and reducing immobility time (SPT, compared to the GW4064 group: DBB 200 mg/kg, P < 0.01; TST, compared to the GW4064 group: DBB 200 mg/kg, P <

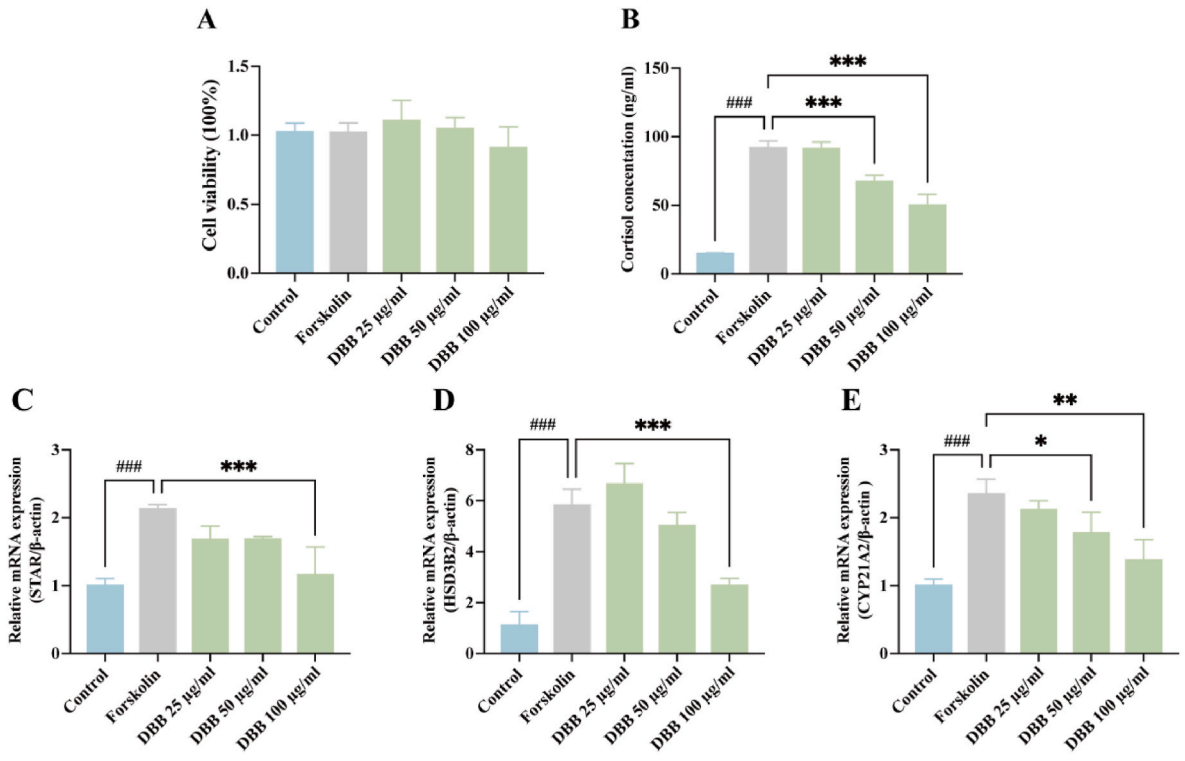

Fig. 4. The inhibitory effect of DBB on Forskolin-induced cortisol secretion in H295R cells
(A) Cell viability under co-treatment with forskolin and different concentrations of BBP (N = 5/group); (B) The cortisol content in H295R cells (N = 4/group); (C–E) Q-PCR analysis of adrenal STAR, HSD3B2 and CYP21A2 (N = 3/group). One-way ANOVA with Dunnett multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Forskolin group, $^{###}p < 0.001$ vs. Control group. Results are presented as means ± SD.

0.05; FST, compared to the GW4064 group: DBB 200 mg/kg, P < 0.01), thereby ameliorating depressive-like behaviors. In addition, compared to the control group, GW4064 increased serum CORT levels in mice (Fig. 3E, P < 0.001), while DBB reversed this increase (compared to the GW4064 group: P < 0.01).

3.4. DBB inhibits GC synthesis in adrenal cells

The adrenal gland is the primary organ for synthesizing GC (Weger et al., 2016). To investigate whether DBB directly regulates adrenal GC synthesis, we treated H295R cells with forskolin and DBB, and measured cell viability and cortisol levels in the supernatant (Fig. 4A and B). Firstly, we assessed the combined effects of different concentrations of DBB and forskolin on H295R cell viability, and found that DBB at concentrations of 25, 50, and 100 μg/mL had no effect on cell viability when used in combination with forskolin. Subsequently, we measured cortisol levels in the cell supernatant. Forskolin significantly promoted cortisol secretion by H295R cells (P < 0.001), while DBB at concentrations of 50 and 100 μg/mL significantly inhibited forskolin-induced cortisol secretion (compared to the Forskolin group: DBB 50 μg/mL, P < 0.001; DBB 100 μg/mL, P < 0.001). Additionally, we examined cortisol secretion-related genes and found that compared to the control group (Fig. 4C–E), the forskolin group showed significantly increased mRNA expression levels of STAR, HSD3B2, and CYP21A2 (P < 0.001, P < 0.001, P < 0.001), while DBB was able to inhibit the expression of these genes (STAR mRNA, compared to the Forskolin group: DBB 100 μg/mL, P < 0.001; HSD3B2 mRNA, compared to the Forskolin group: DBB 100 μg/mL, P < 0.001; CYP21A2 mRNA, compared to the Forskolin group: DBB 50 μg/mL, P < 0.05; DBB 100 μg/mL, P < 0.01).

3.5. DBB inhibits GC synthesis through adrenal FXR

To investigate whether DBB directly acts on adrenal FXR, we first assessed the impact of DBB on adrenal FXR expression. In H295R cells, DBB significantly inhibited FXR protein and mRNA expression levels (Fig. 5A and B, FXR protein, compared to the control group: DBB 50 μg/mL, P < 0.05; DBB 100 μg/mL, P < 0.01; FXR mRNA, compared to the control group: DBB 50 μg/mL, P < 0.05; DBB 100 μg/mL, P < 0.01), corroborating the findings from the animal study. Furthermore, we investigated whether DBB regulates GC synthesis via adrenal FXR by separately overexpressing FXR in H295R cells and administering FXR agonists to simulate the *in vivo* environment. Firstly, we examined the effect of different concentrations of DBB on the viability of H295R cells overexpressing FXR (Fig. 5C). The results indicated that DBB at concentrations of 25, 50, and 100 μg/mL had no effect on the viability of H295R cells overexpressing FXR. Subsequently, we measured the cortisol content in the supernatant of H295R cells after FXR overexpression (Fig. 5D). Compared to the control group, FXR overexpression significantly increased the secretion of cortisol by H295R cells (P < 0.001), whereas after treatment with DBB, the cortisol content significantly decreased (compared to the FXR group: DBB 50 μg/mL, P < 0.05; DBB 100 μg/mL, P < 0.001). Meanwhile, as shown in Fig. 5E, FXR overexpression also promoted HSD3B2 mRNA expression in H295R cells (P < 0.001), whereas DBB inhibited its expression (compared to the FXR group: DBB 50 μg/mL, P < 0.05; DBB 100 μg/mL, P < 0.001).

Similarly, DBB at concentrations of 25, 50, and 100 μg/mL had no effect on the viability of H295R cells when co-administered with the FXR agonist GW4064 (Fig. 5F). Moreover, as depicted in Fig. 5G, compared to the control group, the cortisol content in the supernatant of H295R cells significantly increased after treatment with GW4064 (P < 0.001), while conversely, DBB significantly reduced cortisol levels (compared to the GW4064 group: DBB 50 μg/mL, P < 0.001; DBB 100 μg/mL, P < 0.001). Additionally, the mRNA expression levels of HSD3B2 in H295R cells significantly increased after GW4064 treatment (Fig. 5H, P < 0.001), whereas treatment with DBB significantly inhibited HSD3B2 mRNA expression after GW4064 administration (compared to the

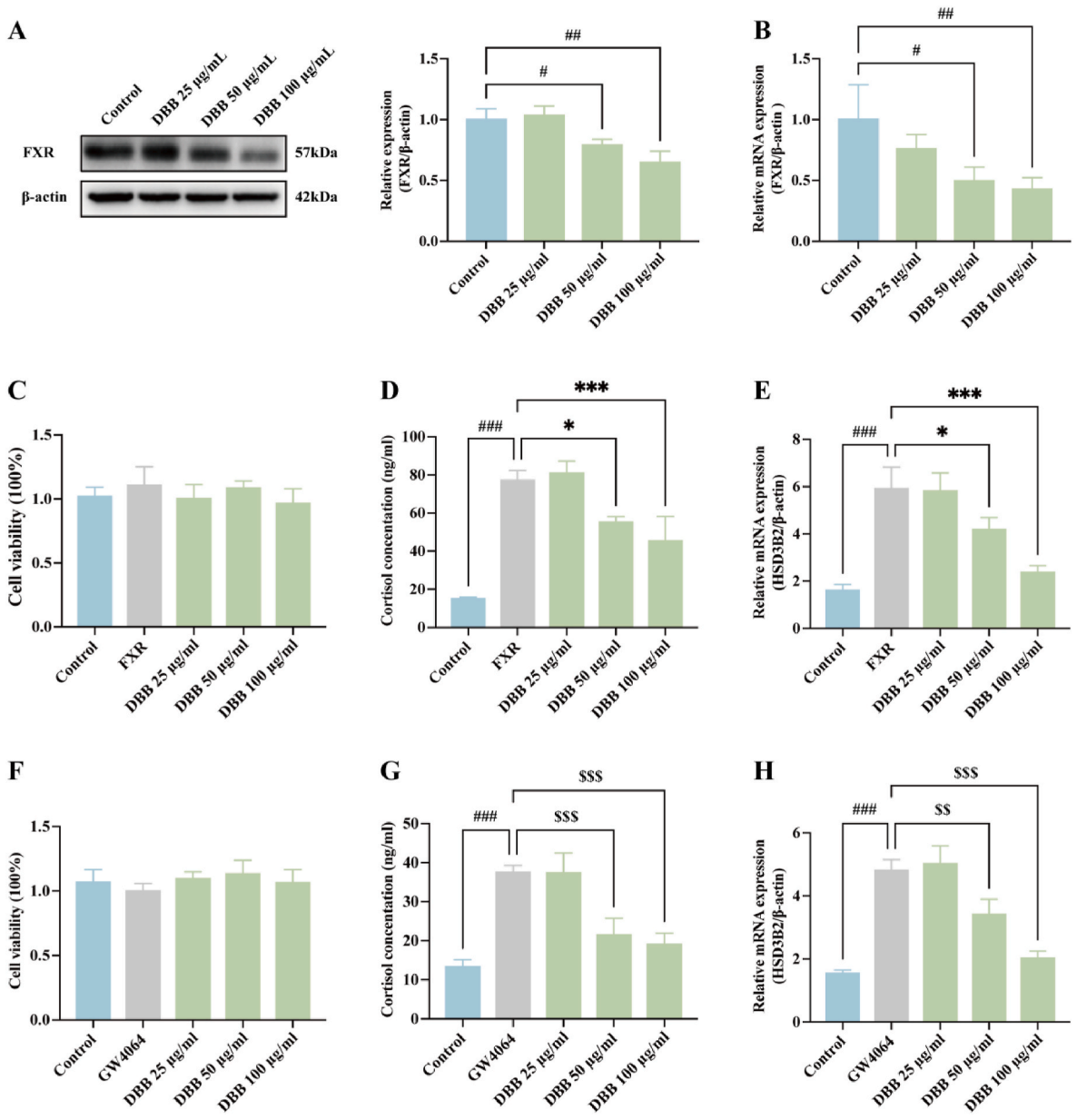

Fig. 5. The effect of DBB on H295R cells transfected with FXR overexpression plasmids and treated with GW4064
(A) Western blotting analysis and quantification of FXR in H295R cells; (B) Q-PCR analysis of FXR in H295R cells; (C) Cell viability of H295R cells transfected with FXR overexpression plasmids (N = 5/group); (D) Cortisol content in H295R cells transfected with FXR overexpression plasmids (N = 3/group); (E) Q-PCR analysis of adrenal HSD3B2 (N = 3/group); (F) Cell viability of H295R cells after treatment with GW4064 (N = 5/group); (G) Cortisol content in H295R cells after treatment with GW4064 (N = 3/group); (H) Q-PCR analysis of adrenal HSD3B2 (N = 3/group). One-way ANOVA with Dunnett multiple comparisons test, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. FXR group, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. GW4064 group, $^{\#\#\#}p < 0.001$ vs. Control group. Results are presented as means ± SD.

GW4064 group: DBB 50 μg/mL, P < 0.01; DBB 100 μg/mL, P < 0.001).

4. Discussion

Depression is typically induced by chronic stress resulting from prolonged exposure to stressful environments (Hammen, 2005). Under stress, the HPA axis is activated, leading to the release of GC, which are stress-related adrenal hormones. GC contribute to sustained depressive behaviors, such as decreased motivation or pleasure (Brummelte and Galea, 2010). Therefore, inhibiting the production of GC is key to improving depression. This study demonstrated that DBB effectively improved depressive-like behavior in CUMS mice. Additionally, DBB treatment significantly reduced serum CORT levels in depressed mice and inhibited adrenal FXR expression. This implicated that DBB had a direct inhibitory effect on the expression of cortisol and its synthesis-related genes, which was mediated through adrenal FXR.

CUMS is a well-established depression model commonly used to evaluate the antidepressant effects of novel substances, as it not only induces depression-related behaviors but also elicits physiological and neural changes associated with clinical depression (Frisbee et al., 2015). In the CUMS depression model, mice typically exhibit decreased sucrose preference, indicating a reduced capacity for experiencing pleasure. Furthermore, increased immobility time in the FST and TST, indicative of "behavioral despair," is observed, mirroring the behavior of human patients with depression when face with hopeless situations. Our results indicated that CUMS mice exhibited depressive-like behaviors characterized by anhedonia and behavioral despair, which were effectively ameliorated by DBB treatment.

Induction by CUMS can lead to physiological changes, such as excessive secretion of GC, which is associated with adrenal FXR activation (Gao et al., 2017; Hoekstra et al., 2012). This condition plays a crucial role in the pathogenesis of depression. Excessive or prolonged release of GC can cause a range of plasticity impairments, such as hippocampal neuron atrophy, apoptosis, and reduced neurogenesis, leading to depression (Farrell and O'Keane, 2016). According to our experimental results, we found a significant increase in adrenal FXR gene and protein levels in depressive mice induced by the CUMS model. This may contribute to the overproduction of GC in the adrenal. However, our findings indicate that DBB treatment significantly reduced serum CORT levels and inhibited adrenal FXR expression. Furthermore, it has been reported that adrenal STAR and HSD3B2 promote GC synthesis. Our experimental results revealed that in the CUMS depression model, adrenal STAR and HSD3B2 mRNA expression levels were significantly elevated, while DBB effectively inhibited the mRNA expression levels of STAR and HSD3B2. Furthermore, we administered the FXR agonist GW4064 *in vivo* and found that GW4064 induced depressive-like behaviors and elevated serum CORT levels in mice. However, DBB effectively ameliorated these effects.

H295R cells are human adrenocortical carcinoma cells capable of expressing enzymes and related genes involved in GC synthesis (Ohno et al., 2002; Oskarsson et al., 2006). Forskolin, as a classic adenylate cyclase activator, can stimulate adenylate cyclase to activate pathways related to GC synthesis (Patt et al., 2020). By treating H295R cells with forskolin to promote cortisol secretion, we aimed to simulate the environment of increased cortisol secretion in patients with depression. Following the stimulation of cortisol secretion, we observed that DBB significantly reduced cortisol levels. Moreover, DBB effectively decreased the mRNA levels of cortisol synthesis-related genes STAR, CYP21A2, and HSD3B2, consistent with our observations in animal experiments.

HSD3B2 plays a crucial role in GC synthesis by directly regulating the conversion of GC precursors into corresponding GC. Moreover, agonists of the bile acid receptor FXR can promote cortisol secretion in human adrenocortical cells H295R by regulating HSD3B2 expression (Xing et al., 2009). According to these *in vitro* results, like the findings in animals, DBB can inhibit FXR expression in H295R cells. Subsequently, we simulated the *in vivo* environment by overexpressing FXR in H295R cells and administering an FXR agonist. We found that DBB effectively inhibited the cortisol content in the supernatant of H295R cells. Additionally, we observed a significant reduction in the mRNA expression levels of HSD3B2 in the cells treated with DBB. This suggests that DBB can inhibit HSD3B2 in the process of GC synthesis, thereby reducing cortisol production.

Nevertheless, our study is subject to certain limitations. One limitation of our study is the complexity of examining the role of FXR in the antidepressant effect of DBB, which contains both FXR agonists (TCDCA and TUDCA) and an FXR antagonist (UDCA). This makes it challenging to determine each component's specific contribution. We are currently conducting studies to test the antidepressant effects of each individual component to better understand their specific mechanisms. Moreover, the challenges associated with acquiring DBB and ethical considerations surrounding animal usage pose substantial obstacles. Hence, it is crucial to investigate the potential of synthetically derived DBB, particularly its effectiveness as an antidepressant, as we endeavor to develop alternatives to bear bile. In conclusion, our study indicates that DBB has certain antidepressant effects, possibly by acting on adrenal FXR to regulate GC synthesis, thereby reducing circulating GC levels. Our findings provide robust scientific evidence for the treatment of depression with DBB.

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Data availability

Data available on request from the authors.

CRediT authorship contribution statement

Yanlin Tao: Investigation, Data curation. **Zikang Li:** Writing – original draft, Visualization. **Jinfeng Yuan:** Validation. **Hui Wu:** Methodology. **Hailian Shi:** Methodology. **Xiaojun Wu:** Conceptualization. **Fei Huang:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 to improve the language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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