Antidepressant-Like Effect of Essential Oils From *Citrus reticulata* in Reserpine-Induced Depressive Mouse

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Abstract
*Citrus reticulata* Blanco has been widely used to cure some diseases such as cold, cough and indigestion. This study is aimed at determining the antidepressant-like effect of *C. reticulata* essential oils (CREOs) in reserpine-induced depression mice, as well as its possible mechanisms. The compositions of CREOs are firstly analyzed by gas chromatography-mass spectrometry (GC-MS), in which *d*-limonene is the main component. Moreover, the results from the forced swimming and tail suspension tests show that the inhalation of CREOs can significantly improve the depressive behavior of reserpine-induced depressed mice by reducing the weight of the mice and shortening the immobile time. After sniffing CREOs, the number of normal neurons in the hippocampus of reserpine-induced depressed mice is greatly increased. In addition, CREOs significantly increase the expression level of 5-hydroxytryptamine-1A receptors (5HT-1A), glucocorticoid receptors (GR) and brain-derived neurotrophic factor (BDNF) in the reserpine-treated mice brain tissue. Thus, these results have indicated that CREOs can be potential materials for drug and food development against depression.

Keywords
essential oil, *Citrus reticulata*, antidepression, 5HT-1A

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Introduction
As a common mental disorder, depression is clinically manifested as low interest, decreased energy, low self-identity, inattention, and even repeated ideas of suicide.¹ Survey data from the World Health Organization have shown that currently, more than 350 million people worldwide are suffering from depression. Depression has become the world’s second major disease and it will be the first one in the global burden of disease by 2030. However, the exact pathogenesis mechanisms of depression and antidepressant action have not been clearly clarified, although several factors involved in the etiology of depression have been reported including hypothalamus–pituitary–adrenal axis (HPAA), monoamine neurotransmitters, cytokines, brain-derived neurotrophic factors (BDNFs), and 5-hydroxytryptamine receptor.²⁻⁶ Most of the antidepressants used clinically have obvious limitations and a low remission rate for major depressive disorder patients. In addition, the currently available antidepressants are costly and inadequate for a number of individuals.¹ The depression pharmacotherapy is prone to side effects, such as anxiety, gastrointestinal dysfunction, low alertness, and sexual problems.⁸⁻¹⁰ Therefore, it is of great significance to develop novel antidepressants that are affordable, and also which have fewer adverse reactions and broader therapeutic effects.

Nowadays, some essential oils of medicinal plant extracts have become effective supplements for the treatment of depression.¹¹ *Citrus reticulata* is widely grown worldwide as an important cash crop. China is an important place of origin for the

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Citrus are widely used in food, medicine, and cosmetics. Citrus reticulata peel is one of the ingredients of traditional Chinese medicine prescriptions such as Liu Yu Tang and Chaihu Shugan San. Both of them are used clinically to improve depressive mood caused by different reasons, and antidepressant effects have been shown in the chronic restraint stress caused by different reasons, and antidepressant medicine prescriptions such as Liu Yu Tang and Chaihu Shugan San. Both of them are used clinically to improve depressive mood caused by different reasons, and antidepressant effects have been shown in the chronic restraint stress induced depression model in Chaihu Shugan San. According to previous studies, CREOs, and their main component of limonene, can improve depressive behavior, possibly by restoring the expression of hippocampal BDNF in chronic unpredictable mild stress (CUMS) induced mice. It means that CREOs have antidepressant effects and can prevent the occurrence of depression. Pharmacological studies have also indicated that CREOs have a variety of biological activities, such as antitumor, anti-inflammatory, antioxidant, and antibacterial.

In this study, the antidepressant-like effects of CREOs in reserpine-induced depressive mice are studied through administration by smell absorption, and the possible mechanisms are explored using biochemical tests. The chemical composition of the essential oil is analyzed by gas chromatography-mass spectrometry (GC-MS). The changes in mouse body weight and behavior are used to determine the antidepressant effects of CREOs. The expressions of 5-hydroxytryptamine-1A (5HT-1A), glucocorticoid receptor (GR), and BDNF in mouse brain tissues are detected by different detection methods to explore their relationship with the antidepressant effects.

Results and Discussion

**Chemical Composition of CREOs**

The compositions of CREOs are enumerated in Table 1, and a total of 23 compounds are detected. Limonene is the most abundant compound, followed by γ-terpinene, β-myrcene, β-pinene, α-pinene, linalool and cis-limonene oxide. The main components of the CREOs are consistent with the literature. Among the top 10 compounds, the content of limonene (76.7 ± 0.4%) is the greatest, and this compound has been considered to be the most important active component for the herb’s antidepressant effects. The γ-terpinene (4.4%), another major component of CREOs, has also shown antidepressant activity in previous experiments. Moreover, β-caryophyllene, present in lower proportion in CREOs, has also been found to significantly improve depression-like effects. These findings demonstrate the potential of CREOs as an effective antidepressant.

**Effect of CREOs on Relieving Depression in Reserpine-Treated Mice**

The antidepressant test for CREOs, carried out on reserpine-induced depressive model mice (Figure 1), is performed 1 day before the behavioral test with the advantages of being fast and effective. As illustrated in Figure 1A, the body-weight of reserpine-treated mice decrease significantly compared with the control group. Fluoxetine treatment does not show resistance to weight loss, which may be related to the gastrointestinal side effects of fluoxetine, resulting in reduced food intake of the mice. In addition, Res + CREOs-M (1.25 mL/kg) and Res + CREOs-H (2.5 mL/kg) are more effective in preventing and inhibiting weight loss in mice in comparison to the control group, indicating that the inhalation of CREOs has a positive regulatory effect on mice.

A comparison of the forced swimming test (FST) immobility time is shown in Figure 1B. After reserpine treatment, the mice’s desire to escape decreases and the immobility time is significantly prolonged (P = 0.0188). CREOs and fluoxetine shorten the immobility time of mice, but Res + CREOs-H (2.5 mL/kg) has the best improvement effect in the CREOs group. These results prove that CREOs have antidepressant effects.

As depicted in Figure 1C, treatment with CREOs can improve immobility of mice in the tail suspension test (TST). The immobility time of reserpine-treated mice is significantly longer than that of control mice (P = 0.0004). It is encouraging that both the fluoxetine and Res + CREOs-H (2.5 mL/kg) groups show an excellent improvement effect on immobility time (P = 0.0062). Res + CREOs-M (1.25 mL/kg) also has a significant therapeutic effect (P = 0.0116). Generally, CREOs can effectively alleviate depression-related behaviors.

**CREOs Suppressed Neuronal Damage Induced by Reserpine Treatment**

Nissl staining is a common method to assess changes in neurons and can detect Nissl bodies in neurons. The staining results (Figure 2A) show that the control group has plenty of neuronal cells and they are arranged neatly, while the Nissl substance in the reserpine-induced mouse neuronal cells is missing, accompanied by blurred cell boundaries and a disordered arrangement. The CREOs treatment alleviates the damage caused by reserpine through reducing neuronal loss and reversing neuronal damage, and the effect is better than that of fluoxetine. In particular, compared with the reserpine group, the neuronal cells in the hippocampus and hypothalamus of the Res + CREOs-H (2.5 mL/kg) group are arranged more neatly and densely, and the Nissl bodies recover better.

The statistics of normal neurons in different parts of the mouse brain are shown in Figure 2B. After reserpine treatment, the number of neurons in each tissue of the mice is significantly less than that of the control group (cerebral cortex, P = 0.0360; hippocampus, P = 0.0200). Among them, Res + CREOs-M (1.25 mL/kg) treatment of cortex neurons (P = 0.0271) and Res + CREOs-H (2.5 mL/kg) treatment of hypothalamic...
neurons \((P=0.0014)\) has significant recovery effects, which are even better than that of fluoxetine. However, a reduction effect is also been in the treatment of hippocampal neurons with low concentrations of essential oils. In the hypothalamus, each concentration of essential oil has a similar effect on neuron recovery.

**Immunohistochemical Staining for 5HT-1A and GR**

Furtherly, 5HT-1A and GR are important factors in evaluating the treatment of depression. The positive expression levels of 5HT-1A and GR in the cerebral cortex, hippocampus and hypothalamus of mice are estimated by immunohistochemistry to reveal the role of CREOs in the treatment of depression (Figure 3).

Figure 3A and B is the expression of 5HT-1A. The control group has more positive cells in the 3 areas with clear cell outlines and uniform cytoplasmic staining. The 5HT-1A protein in the reserpine group is significantly less than that in the control group, with some cells in the hippocampus showing vacuoles, and some cells showing marked atrophy. After CREOs treatment, positive cells increase and the cells are arranged neatly and clearly. However, Res + CREOs-M \((1.25 \text{ mL/kg})\) disturbs the arrangement of cells and makes them disorderly. Combined with the integrated optical density analysis, it is clear that compared with the control group, the expression of 5HT-1A in the 3 areas treated by reserpine is reduced and there is a significant difference in the cortex \((P=0.0056)\). In the cortex, the level of 5HT-1A in the CREOs and fluoxetine groups increase to certain degrees \((P=0.0083; \text{Res} + \text{CREOs-M (1.25 mL/kg)}, P=0.0369)\) compared with the reserpine group. It is worth mentioning that in the hippocampus, Res + CREOs-H \((2.5 \text{ mL/kg})\) has a better recovery effect on 5HT-1A levels than fluoxetine and the overall therapeutic effect of Res + CREOs-H \((2.5 \text{ mL/kg})\) is similar to that of fluoxetine. Res + CREOs-H \((2.5 \text{ mL/kg})\) treatment can reverse 5HT-1A reduction induced by reserpine, but there are general therapeutic effects of low-medium concentrations of essential oils in the hippocampus and hypothalamus.

Figure 3C and D show GR detection. The number of GRs in the reserpine group is significantly less than that in the control group, with the cells not being arranged tightly and the staining

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**Table 1. Retention index (RI) and Relative Content (%) of Identified Constituents of *Citrus reticulata* Essential Oils.**

| No | Compounds* | RIb | RIb | Exp.RI | Ref. | Relative content |
|----|------------|-----|-----|--------|------|-----------------|
| 1  | α-Thujene  | 981 | 981 | 929    | 23   | 0.2 ± 0.002    |
| 2  | α-Pinene  | 988 | 987 | 941    | 23   | 2.4 ± 0.001    |
| 3  | β-Pinene  | 1070| 1071| 979    | 23   | 3.2 ± 0.003    |
| 4  | β-Myrcene | 1005| 1005| 990    | 24   | 4.3 ± 0.04     |
| 5  | Limonene  | 1029| 1028| 1028   | 23   | 76.7 ± 0.4     |
| 6  | \((Z)\)-β-ocimene | 1048| 1047| 1042  | 23   | 0.2 ± 0.001    |
| 7  | γ-Terpine | 1052| 1051| 1072   | 23   | 4.4 ± 0.009    |
| 8  | Terpinolene | 1078| 1077| 1089   | 23   | 0.3 ± 0.00     |
| 9  | Linalool  | 1088| 1088| 1084   | 25   | 2.3 ± 0.00     |
| 10 | *cis*-Limonene oxide | 1104| 1108| 1120  | 25   | 1.8 ± 0.001    |
| 11 | p-Mentha-2,8-dien-1-ol | 1118| 1117| 1142  | 24   | 0.4 ± 0.007    |
| 12 | α-Terpinol | 1143| 1142| 1143   | 26   | 0.4 ± 0.006    |
| 13 | Ocyethyl acetate | 1145| 1145| 1191  | 27   | 0.1 ± 0.00     |
| 14 | *trans*-Carveol | 1159| 1158| 1225  | 24   | 0.2 ± 0.00     |
| 15 | Nerol     | 1209| 1208| 1207   | 27   | 0.2 ± 0.001    |
| 16 | δ-Elemene | 1315| 1314| 1330   | 25   | 0.195 ± 0.01   |
| 17 | *trans*-α-Bergamotene | 1424| 1423| 1411  | 25   | 0.3 ± 0.00     |
| 18 | *trans*-α-Bergamotene | 1427| 1446| 1430  | 28   | 0.2 ± 0.001    |
| 19 | β-Bisabolene | 1451| 1450| 1513  | 24   | 0.3 ± 0.02     |
| 20 | (Z)-β-Farnesene | 1453| 1448| 27    | 0.2 ± 0.00     |
| 21 | Caryophyllene oxide | 1581| 1580| 1582  | 23   | 0.2 ± 0.001    |

Total identified 98.4

| Relative content |
|------------------|
| Hydrocarbon monoterpenes/% | 96.5 |
| Oxygenated monoterpenes/% | 91.7 |
| Total sesquiterpenoids/% | 4.8 |
| Sesquiterpene hydrocarbons/% | 1.3 |
| Oxygenated sesquiterpenes/% | 1.1 |
| Non-terpene compounds/% | 0.2 |

*Comounds listed in the order of elution from a methyl silicone capillary column. RIa relative to n-alkanes \((C_6-C_{40})\) on the same methyl silicone capillary column. Lawal et al\(^{23}\); Espina et al\(^{24}\); Chutia et al\(^{25}\); Nengguo et al\(^{26}\); Corse et al\(^{27}\); Adelani Babarinde et al\(^{28}\).
being lighter. Some cells in the hippocampus also show either vacuoles or atrophy. After sniffing CREOs, GR-positive cells can recover to a certain degree, and the cells are arranged neatly and clearly. However, the recovery effect of Res + CREOs-L (0.625 mL/kg) is not obvious. The optical density demonstrated that the GR levels of the reserpine group in the 3 areas are significantly reduced. However, the GR levels of Res + CREOs-M (1.25 mL/kg), Res + CREOs-H (2.5 mL/kg) and fluoxetine groups increase to varying degrees. Among them, the treatment with Res + CREOs-M (1.25 mL/kg) on the hippocampus leads to a significant increase ($P=0.005$).

**Western Blot and Real-Time Quantitative Polymerase Chain Reaction for 5HT-1A and BDNF**

In order to explore further the potential mechanism of CREOs on depression-like behaviors, Western blot is used to detect the protein expression of BDNF in brain tissue homogenate. The results are shown in Figure 4A and B. The expression of BDNF decrease significantly after reserpine treatment. Regarding the expression of BDNF, the tangerine medium and low concentration group can significantly inhibit down-regulation ($P=0.0009$; $P=0.0006$), which is better than the therapeutic effect of Res + CREOs-H (2.5 mL/kg) and fluoxetine.

Next, real-time quantitative polymerase chain reaction (RT-qPCR) is used to analyze the expression of 5HT-1A at the mRNA level (Figure 4C. Figure 4C shows that the relative expression of 5HT-1A mRNA is decreased in the brain tissue of the reserpine group ($P=0.1910$). The results also indicate that reserpine can affect the mRNA expression of 5HT-1A in mice. Meanwhile, 5HT-1A mRNA is found to be improved by treatment with fluoxetine and CREOs, and there is a significant difference in the Res + CREOs-M (1.25 mL/kg) group ($P=0.0495$).

**Discussion**

Under the increasing external stresses, depression has become one of the main causes of disability in the world, and the
recurrence rate is very high.\(^1\,^2\) Drug treatment often brings a series of adverse reactions. The search for safe and effective new drugs has always been a hot topic. Reserpine is a commonly used and effective depression model, which can be used for behavioral tests such as forced swimming, tail suspension, and open field tests. With the merits of simple operation, animal friendly, and high efficiency, this model plays an important role in the evaluation and development of antidepressant drugs. This procedure is used to analyze the chemical composition of CREOs and explore their role in the depression model induced by reserpine.

Weight loss is an important indicator of depression performance,\(^1\) and the immobility in the FST and TST can be used to evaluate the depressive behavior of mice.\(^30\) Our results have shown that the weight of mice treated with reserpine decreased significantly. What is more, the immobility time of FST and TST are significantly prolonged. This means that the reserpine depression model is successful and effective, which is similar to previous studies. Fluoxetine is a clinically recognized and effective antidepressant, which has a significant antidepressant effect. In this experiment, it is used as a positive control to compare and verify the antidepressant effect of CREOs. According to the body weight changes and behavioral tests, CREOs treatment can prevent weight loss and reduce the immobility time of FST and TST in the reserpine-treated mice. Among them, the therapeutic effect of Res + CREOs-H (2.5 mL/kg) is optimal. In general, CREOs can at least improve depression-like behaviors in mouse depression models induced by reserpine.

Nissl bodies are composed of rough endoplasmic reticulum with free ribosomes interspersed in it. Basophilic particles are presented and the shape and number of Nissl bodies are different in different neurons. They are related to the synthesis of structural proteins for renewing organelles, the enzymes for synthesizing neurotransmitters, and the neuromodulators of peptides. Previous researches have shown that neuron damage can cause the sensitivity of Nissl body to decrease, dissolve or disappear.\(^31\) Therefore, the shape and number of Nissl body are often used to identify neurons and their pathophysiological changes. It has been reported that the total number of neurons in the hippocampus of patients with depression was reduced by 20% to 35% compared with the control group,\(^32\) and the injection of reserpine can cause neuronal damage. Our results also show that treatment with CREOs can alleviate the nerve damage caused by reserpine.

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**Figure 2.** Effects of *Citrus reticulata* essential oils (CREOs) on neuronal damage induced by reserpine. Nissl staining (A) and normal neuronal counts (B) in cortex, hippocampus and hypothalamus of each group, scale bar = 50 μm. Data were expressed as the Mean ± SD (n = 6). The significance of differences versus the Res group is at *P* < 0.05 and **P** < 0.01.
Figure 3. Effects of CREOs on 5HT-1A and GR expressions in cortex, hippocampus, and hypothalamus. (A) Immunohistochemistry images of 5HT-1A (brown), scale bar = 50-μm. (B) Quantification analysis of 5HT-1A positive cells. (C) Immunohistochemistry images of GR (brown), scale bar = 50-μm. (D) Quantification analysis of GR-positive cells. Data were expressed as the Mean ± SD (n = 6). The significance of differences versus the Res group is at *P < 0.05 and **P < 0.01. Abbreviations: CREOs, Citrus reticulata essential oils; 5HT-1A, 5-hydroxytryptamine-1A; GR, glucocorticoid receptor.
Figure 4. Effects of CREOs on BDNF and 5HT-1A expressions in in cortex, hippocampus, and hypothalamus. (A) western blot results of BDNF and (B) quantification analysis of BDNF. (C) qPCR analysis of 5HT-1A. Data were expressed as the Mean ± SD (n = 6). The significance of differences versus the Res group is at *P < 0.05 and **P < 0.01. Abbreviations: CREOs, *Citrus reticulata* essential oils; 5HT-1A, 5-hydroxytryptamine-1A; qPCR, quantitative polymerase chain reaction; BDNF, brain-derived neurotrophic factor.
growth. The number of neurons in the CREOs treatment group is closed to that of the control group, indicating that CREOs treatment causes less damage to neurons. This means that CREOs can protect the morphology of brain tissue neurons, and has relatively good treatment safety.

In depression-related patients and some animal experiments, it has been usually observed that symptoms of depression are accompanied by disorders of glucocorticoid (GC) secretion. Therefore, the HPAA dysfunction is studied by evaluating GR levels. HPAA disorder is an important finding in the pathophysiology of depression. This disorder is thought to be due to the central GR level and the chronic GC releases or changes in function, causing the receptor either positively or negatively to regulating the expression of GC-responsive genes. Excessive stress stimulus might activate GR through cortisol, so that GR stimulates the hippocampus to issue negative feedback commands, resulting in HPA axis imbalance and excitement. Antidepressants can improve the GR-mediated inhibition of corticosteroids by increasing the expression of GR on the HPA axis, thereby reducing cortisol levels in different regions. The results of this study demonstrate that reserpine causes a significant reduction in GR expression, indicating that the negative feedback regulation of the HPA axis is impaired and the HPA axis is hyperactive, which is similar to previous studies. Moreover, treatment with CREOs and fluoxetine can increase the expression of GR, indicating that CREOs have antidepressant effects by regulating the neuroendocrine system.

The monoamine hypothesis is considered as the mechanism of action of depressive drugs. This hypothesis believes that the depression is caused by insufficient activity of monoaminergic neurons. Previous studies have shown that the patients with major depressive episodes have lower serotonin transporter binding potential in the midbrain and amygdala, compared with non-depressed individuals. Although the complexity of the emotional state cannot attribute to the imbalance of a single neurotransmitter, it is recognized that 5-HT is significantly involved in depression, and 5-HT-1A receptors are considered to treat mental illness, and are especially a potential target for depression. Studies have also found that the antidepressant effect of lemon oil, whose main component is limonene, is closely related to the 5-HT energy pathway, especially via the 5-HT-1A receptor pathway. In this study, it is found that fluoxetine and CREOs can increase the content of 5-HT-1A in the brains of reserpine-induced depression mice, but there are differences in dosage and location differences. Insignificant effects are shown in the hippocampus and hypothalamus by treatment with a low concentration of essential oils. The excessive activity of the HPA axis might damage hippocampal monoaminergic neurons, resulting in a decrease in monoamines and the 5-HT-1A receptor agonist suppresses the stress-induced activation of the HPA axis. It is speculated that CREOs might increase 5-HT-1A in the cerebral cortex via the action of the HPA axis, thereby increasing neuronal activity.

BDNF is a neurotrophic factor that can stimulate neurogenesis and regulate synaptic plasticity. Widespread in the brain, especially in the hippocampus and cerebral cortex, BDNF can promote the survival of dopaminergic and serotonergic neurons. It is also involved in regulating the activities of the HPA axis. Antidepressant drugs can increase the expression of BDNF, such as selective serotonin reuptake inhibitors and norepinephrine reuptake inhibitors. Of course, there are also antidepressants that have different effects on the mRNA and protein levels of BDNF. The regulating effect of these drugs on depression might be related to neurotrophic activity and benefit from long-term chronic regulation. Study has shown that the loss of BDNF in the hippocampus can induce neuronal apoptosis and ultimately lead to depression. WB staining shows that the expression of BDNF protein in reserpine-depression mice is decreased. BDNF mRNA from RT-qPCR results reveal that BDNF mRNA level expression and protein level expression had an opposite expression trend, and there is a negative feedback relationship. Treatment with CREOs and fluoxetine significantly increase the BDNF protein and reduced the expression of BDNF mRNA, signifying the negative feedback effect. Interestingly, the changes in 5HT-1A protein in the brain homogenate detected by WB have different and opposite results from that of 5-HT-1A protein in different regions of the brain. This might be related to the location of 5-HT-1A receptors, which has opposite effects on depression regulation. For example, the post-synaptic 5-HT-1A receptor is thought to cause anxiety and depression effects. On the contrary, it is believed that the activation of 5-HT-1A auto-receptor will inhibit the activity of serotonergic neurons and release serotonin, thereby reducing the marginal zone and producing antianxiety and antidepressant effects. Similarly, the use of RT-qPCR to detect the mRNA expression of 5-HT-1A also shows a different trend from the expression of 5-HT-1A protein. Therefore, we speculate that there is a brain-derived neurotrophin–neurotransmitter interaction in at least a certain area of the brain, which makes BDNF and 5-HT-1A feedback regulation in protein and mRNA. The disorder of this mechanism can be regulated by CREOs, while fluoxetine is inactive.

A hyperactive HPA axis is one of the main abnormal phenomena found in depression, and the treatment of depression promotes the production of BDNF. Therefore, the decreased expression of BDNF in reserpine treated animals might indicate abnormal changes in the HPA axis and monoaminergic circuit function. Previous studies indicate that limonene can also recover CUMS-induced depressive behavior, HPA axis hyperactivity, and decreased levels of monoamine neurotransmitters by down-regulating hippocampal BDNF and its signaling pathways. Our results also can support this conclusion, which provides a reference for the antidepressant mechanism of CREOs, indicating that they may improve the expression of GR, BDNF, and 5HT-1A via the connection of the HPA axis.
Conclusion

In conclusion, the inhalation of CREOs can significantly reduce the depressive behavior of reserpinized mice, inhibit their weight loss, and shorten the immobility time. Limonene is an important component of CREOs, and it is likely to be the antidepressant active component of the oils. CREOs may regulate the expression of GR in the HPA axis in the brain, and the expression of BDNF and 5HT-1A, thereby restoring reserpin-induced depression-like behavior. Therefore, we believe that CREOs would be a potential material for the development of antidepressant drugs, and can provide a reference for the development of new drugs for antidepressant.

Materials and Methods

Essential Oils and Chemicals

Peels of *Citrus reticulata* Blanco were purchased from Chongqing Zhengyuan Trading Co. Ltd. The essential oil was extracted by steam distillation. Part of the oil is stored in the Institute of Nature Medicine and Green Chemistry (Guangdong University of Technology) as voucher specimens (No. ZLY-20210206-002). The plant materials were identified by Professor Nian Liu (Zhongkai University of Agriculture and Engineering) according to the morphological description presented in The China Species Library. In brief, fresh peels were dehydrated before being crushed into powder. In total, 50 g of crushed powder was added to a Clevenger-type apparatus and steam distilled for 2 h. The essential oil above the water layer was collected and anhydrous sodium chloride was used for dehydration. The CREOs were stored in a refrigerator at 4 °C for subsequent experiments. The peel sample gathered was given a registration number (Table 1). All chemicals used in the study were purchased from Aladdin reagent Database Inc. (Shanghai, China) and were of analytical grade.

**GC-MS Analyses**

The essential oil from *Citrus reticulata* peel was analyzed using a GC-MS system (GCMS-QP2010PLUS, Shimadzu Co., Japan) equipped with a DB-5MS capillary column (0.25 mm × 30 m, 0.25 μm film thickness). The flow rate of carrier gas (helium) was 0.87 mL/min and the split ratio was 20:1. The temperature was held at 90 °C for 10 mins, then increased to 250 °C by 5 °C/min, and then kept for 8 mins. As for MS conditions, the electron collision energy was 70 eV and the temperature of the ion source was controlled at 200 °C. Under the guidance of the Kovats standard, the retention index of each compound was counted against the standard of n-alkanes (C₆-C₄₀), and the composition of the essential oil was compared and analyzed with reference to NIST chemistry reference books and literature.

**Animals and Treatment**

The experimental animals (weighing about 34-38 g, 5-week-old) were SPF-grade healthy male KM mice, which were purchased from Liaoning Changsheng Biotechnology Co., Ltd (Approval Document: SCXK/2020-0001). Mice were acclimatized at 25 ± 2 °C and 50% humidity for an additional week before experiments, and the light and dark cycle was carried out for 12 h.

Reserpin, in a single dose of 6 mg/kg, was injected intraperitoneally to induce a depression-like state in mice. After acclimatization, the mice were randomly segregated into 6 groups (n = 6), treated with vehicle, reserpin, fluoxetine, or CREOs. Briefly, the control group (Con) was untreated with reserpin, the rest were treated with reserpin, including the depression model group (reserpin treatment, Res), fluoxetine group (reserpin treatment + fluoxetine 20 mg/kg, Res + Flu), and CREOs groups (reserpin treatment + CREOs), which received daily inhalation of CREOs of different concentrations (0.625, 1.25, and 2.5 mL/kg, respectively, Res + CREOs-L [0.625 mL/kg], Res + CREOs-M [1.25 mL/kg]; Res + CREOs-H [2.5 mL/kg], 1 h/d) concentrations for 7 consecutive days (day 1-7) by a DSI Buxco Inhalation Exposure System (BUXCO, USA). Except for the control group, the other groups received an i.p. injection of reserpin on day 6. In total, 24 h later (day 7), the control and reserpin groups received an i.p. injection of vehicle (1% DMSO), while the fluoxetine group received an i.p. injection of fluoxetine (20 mg/kg). Behavioral tests were used for assessment of depression-like state in animals for 30 min after drug treatment.

**Body Weight**

Weight change was a criterion for diagnosis of depression. The effects of reserpin and essential oils on the weight of the mice were measured by an electronic balance before the administration every day. The weight of mice was recorded.

**Tail Suspension Test**

The mice were subjected to a TST in a quiet environment to evaluate the antidepressant activity of CREOs. Mice were respectively suspended 50 cm above the bottom by the tail (1 cm from the tip) using a clip in a 25 × 25 × 60 cm box during a TST period of 6 min. The period of agitation and immobility was recorded during the last 4 min.

**Forced Swimming Test**

FST is an effective and extensive experiment for evaluating depression-like behaviors in mice. The mice were individually placed in a glass cylinder (15 × 20 cm, water height 15 cm). The temperature of water was controlled at 25 ± 2 °C and mice were allowed to swim for 6 min. The immobility was defined as the mouse having greatly reduced activity in the water and stopping struggling, except for the duration of the small movements, which were necessary to keep the head...
above water. The total time of immobility was recorded during the last 4 min.

**Measurement of Brain 5HT-1A, GR, and BDNF**

Mice were sacrificed after the behavioral test, and 4% paraformaldehyde was added to fix the whole brain tissues. The fixed tissues were made into 4-μm paraffin sections for Nissl staining and immunohistochemistry experiments to observe the number of neurons and expression of 5HT-1A and GR in the brain tissues. Moreover, mice brain tissues were frozen in liquid nitrogen, and then Western blot and RT-qPCR were performed to detect the expression of 5HT-1A and BDNF in mice brain.

**Nissl Staining.** The sections were deparaffinized and stained with Toluidine Blue for 5 min. After differentiation, the sections were placed in xylene for 10 min and then sealed with neutral resin. The sections were observed under a microscope, and images were collected; the number of normal nerve cells was counted for analysis.

**Immunohistochemistry Staining.** The tissue sections were sequentially deparaffinized in xylene, and absolute ethanol. The slices were then placed in citric acid (pH 6.0) antigen retrieval buffer for microwave retrieval of antigens for 30 min, and then placed in 3% hydrogen peroxide for blocking. When the previous steps were completed, 3% of BSA dropwise was added to seal the tissue for 30 min before diluted primary antibodies (5HT-1A, catalog No. AF5453, 1:100, Affinity Biosciences; GR, catalog No. GB11296, 1:500, Servicebio) were added to be incubated overnight in a refrigerator at 4 °C. On the second day, the secondary antibody (HRP conjugated Goat Anti-Rabbit IgG, catalog No. GB23303, 1:500, Servicebio, Wuhan) was added for incubation, while DAB and hematoxylin were used for dyeing. After mounting, the slide was observed under a microscope and the positive cells were analyzed with Image pro plus 8.0 version.

**Western blot analysis.** After the brain tissue was lysed to extract protein, a BCA protein detection kit was used to measure protein concentration. Then, the protein was separated and transferred to a PVDF membrane for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. After being blocked for 30 min with skim milk, primary antibodies (BNDF, catalog No. GB11559, 1:200, Servicebio) were added and incubated overnight at 4 °C. Then the secondary antibody (HRP conjugated Goat Anti-Rabbit IgG, catalog No. GB23303, 1:3000, Servicebio, Wuhan) was added. An ECL chemiluminescence kit was applied to observe the protein, and Alpha software was used to process the optical density value of the specific band.

**Real Time Quantitative Polymerase Chain Reaction.** Total RNA of brain tissue was extracted using the Trizol extraction method, and Nanodrop 2000 was used to detect RNA concentration and purity. Then, a Servicebio®RT First Strand cDNA Synthesis Kit (Servicebio) was used to synthesize cDNA by reverse transcription. The mRNA expression was measured using 2× SYBR Green qPCR Master Mix (High ROX) (Servicebio), and the relative mRNA expression of 5HT-1A was calculated and analyzed by the 2^−ΔΔCT method. The following sequences of the primers for 5HT-1A gene are listed: 5′-CCAACTATCTCTCGGGCTCCTT-3′ (sense) and 5′-CTGACCCAGAGTTCACTTGTTG-3′ (antisense). GAPDH was used as an internal control with sense (5′-CTCTGTACAGAAAAATGTG-3′) and antisense (5′-TGAGGTCAATGAAGGGGTCTG-3′) primers.

**Statistical Analysis**

The histograms were drawn by GraphPad Prism 8.0 and the experimental data were represented as Mean ± SD. The SPSS 21.0 system was used for statistical analysis and ANOVA was used to determine statistical significance. The difference was deemed statistically significant when \( P<0.05 \).

**Authors Contributions**

MHT constructed the firing model, YA, SYZ, and NS performed the experiments. XX and LY Liang analyzed the data. BSR and XMZ wrote the manuscript. LYZ and TGH discussed the results and revised and approved the manuscript.

**Availability of Data and Materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethical Approval**

All animal experiments were performed in accordance with the applicable institutional and governmental regulations concerning the ethical use of animals and were approved by the Laboratory Animal Centre of Sun Yat-Sen University (SCXK/2011-0112).

**Declaration of Conflicting Interests**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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