Prophylactic effects of *Periplaneta americana* L. extract on anxiety-like and depression-like behaviors in rats exposed to chronic stress

Heng Liu[1,2,3#], Xixing Lu[1,2#], Qian Lu[1], Tingmei Lv[3], Minzhe Sun[1], Qiyan Li[4], Pengfei Gao[1], Miao He[1], Hairong Zhao[1,2]* and Chenggui Zhang[1,2]*

[1]Yunnan Provincial Key Laboratory of Entomological Biopharmaceutical R&D, Dali University, Dali,Yunnan, PR China
[2]National-Local Joint Engineering Research Center of Entomoceutics, Dali University, Dali, Yunnan, PR China
[3]School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China
[4]The First People's Hospital of Yunnan Province, Kunming, Yunnan, PR China

Abstract: The effect of *P. americana* L. on anxiety and depression-behavior after chronic stress (CS) is still unknown. Here, CS were induced by a combined stimulation of chronic restraint stress, excess failure and improper diet in SD rats. At 15 days after CS, except for normal group and model group, all the groups were continuously administrated *P. americana* L. (i.g., 400, 200, 100 mg/kg) treatment for 14 days. Anxiety and depression-behavior was determined by sucrose preference test, forced swimming and open field. The contents of cortisol (CORT), adrenocorticotropic hormone (ACTH), adrenocorticotropic hormone-releasing hormone (CRH), interleukin (IL)-4, IL-6, IL-17 and interferon (IFN) -γ were detected by ELISA. 16S rRNA analysis was performed to examine the composition of gut microbiota. Our results indicated that *P. americana* L. improved the anxiety and depression-behavior. *P. americana* L. reduced the release of IL-6, IL-17 and IFN-γ and increased the release of IL-4. Comparably, remarkably decreased CRH, ACTH and CORT were observed by the treatment of *P. americana* L. 16S rRNA analysis suggested that *Bifidobacterium* and sulfate-reducing *bacteria* may be responsible for improving CS in *P. americana* L. -treated rats. Collectively, *P. americana* L. could relieve CS are associated with regulation of intestinal flora.

Keywords: Anxiety and depression-behavior, gut-microbiota-brain axis*, Periplaneta americana* L. extract, inflammation.

INTRODUCTION

With life rhythm speed, increasingly with the social competition and the heavily mental pressure (Bell *et al.,* 2018), chronic stress (CS) will result in anxiety, insomnia, depression and even mental disorder (de Kloet *et al.,* 2005; McEwen, 2003; Popoli *et al.,* 2011). Depression and anxiety disorders are arguably a serious public health problem, which increased the global burden of disease. Anxiety and depression disorders are known to be implicated in many risk factors, such as environmental, genetic, inflammation, neuroendocrine and neuro-transmission abnormalities, metabolic dysfunctions, making survey of the pathophysiology of these disorders extremely challenging (Krishnan *et al.,* 2008). CS is a major risk factor for the development of depression. Previous studies have showed that gut-microbiota-brain axis is crucial in the adjustment of behavior and the central nervous system (Sharon *et al.,* 2016; Desbonnet *et al.,* 2015; Fourie *et al.,* 2017; Lutgendorff *et al.,* 2008). Antidepressants are used to treat depression and anxiety disorders, but only 30%-50% patients indicate improvement. Thus, the development of new therapeutic targets for depression and anxiety is critical.

Since 1000~3000 BC, insects have been used in traditional Chinese and Korean medicine as well as in ancient Egyptian and Greek civilizations to control a variety of diseases, including nervous system disorders. Our previous results showed that the wasp venom extracted from *Vespa magnifica* Smith (Vespidae) had a protective effect on rheumatoid arthritis (Gao *et al.,* 2020) and stroke (Zhao *et al.,* 2022) in rats. In addition, vespakin M (Zhao *et al.,* 2022) and mastoparan M (Wang *et al.,* 2022) protect neurons against cell death and axonal injury after ischemic stroke. *Periplaneta americana* L. (*P. americana* L.) is the largest insect in family *Blattidae* and it is also one of the most ancient insect groups with the strongest vitality, commonly known as cockroach. *P.americana* L. is a traditional Chinese herbal medicine recorded in Shennong's Herbal Classic (Zhao *et al.,* 2015). Previous studies have confirmed that *P. americana* L. has widely used as a potential treatment for some diseases such as hepatic fibrosis, renal fibrosis, osteoporosis, gastric ulcer, wound healing or burns, cervical erosion, oral cavity ulcer and ulcerative colitis (UC) (Li *et al.,* 2016; Zhang *et al.,* 2016). In addition, drugs from *P. americana* L such as 'Kangfuxin Solution' (Z51021834) and 'Xinmailong Injection' (Z20060443) are now extensively used for chronic heart failure and gastrointestinal ulcers. They have been approved by the China Food and Drug Administration (CFDA).

Recent studies indicate that ethanol extract of *P.*

Corresponding authors: e-mails: hr_zhaoxmu@126.com; chenggui_zcg@hotmail.com   [#]Equal contribution

americana L has a potential effect for UC in rats by improving intestinal inflammation, ameliorating intestinal barrier function, modulating composition of the intestinal flora and reversing the intestinal-immune system (Ma *et al.,* 2018). In this study, SD rats with CS were induced by a combination of chronic restraint stress, excess failure and improper diet and we investigated the effects of *P. americana* L on CS in rats.

MATERIALS AND METHODS

Preparation of P. americana L. extract
P. americana L. were provided by Good Doctor Pharmaceutical Group Co., Ltd. (Cheng du, China) and identified by Prof. Zizhong Yang (National-Local Joint Engineering Research Center of Entomoceutics). *P. americana* L. extract was performed using an ethanol extraction system as our previously described (Zhang *et al.,* 2022). Chemical characterization of *P. americana* L. extract was also evaluated using an Agilent 1260 series HPLC system equipped with a diode array detector (DAD)(Agilent 1260 Infinity II), using a Sepax HP-C18 column (250mm×4.6mm, 5μm) at a column temperature of 35°C.

The reagent information is as follows: the HPLC-grade methanol was purchased from Thermo Fisher Scientific (Waltham, USA). Acetic acid glacial was obtained from Fu Chen Chemical Reagent Factory (Tian Jing, China). Pure water (18.2 MW) for the HPLC analysis was obtained from a water purification system purchased from Merck Millipore (Darmstadt, Germany). Hypoxanthine, inosine and uracile were purchased from National Institutes for Food and Drug Control (Beijing, China).

BCA protein method
Polypeptide content was determined by bicinchonininc acid (BCA) protein method. In detail, bovine serum albumin was added into the 96-well plate as chemical reference substances and the standard dilution solution was added to 20μL. The concentration of chemical reference substances was 0, 0.03, 0.12, 0.18, 0.24 and 0.30mg/mL, respectively. BCA (200μL) was added to each well, placed at 37°C for 30 min and measured the absorbance at 562nm. Taking the absorbance as the ordinate (Y) and the mass as the abscissa (X), draw a standard curve $Y=0.0909X+0.0721$ ($R2=0.9918$), with a linear range of 25 μg/mL~250 μg/mL. The content of polypeptides in the samples was determined in the same way.

Animals
Male Sprague-Dawley rats (n=60, 200±20g, Certificate No. SYXK (Dian) K2012-0002 were purchased from Kunming Chu shang Technology Co., Ltd., Kunming, China. All animals handling, including anesthesia, surgical procedures, post-operative care and sacrifice has been approved by the animal care and use Committee of Daly university, China (Animal ethics No.: DLU2019-1217). All the rats were housed in the experimental animal center of Dali University in a room at 20±2°C with a humidity 50%±2% in a 12h light and dark cycle with free access to standard rat chow and tap water.

Chronic stress-induced depressive and anxiety-like behavior in rats
CS (chronic restraint stress+ excess failure+ improper diet) was performed using published protocols (Burokas *et al.,* 2017; McLean *et al.,* 2012; Mao *et al.,* 2009) with some modifications. In detail, the rats need pre-swim after 7-day adaptive breeding. The rats were placed in water at 22±1°C and swum for 10 min, once a day for 3 consecutive days. Rats with swimming time less than 10 min and more than 20 min were excluded. The rest of the rats were placed in a special restraint device (this experiment used a plastic bottle: 8.5cm×18cm) and bound for 8 h (8:00-16:00) daily. At 4:00 pm, rats were placed in water at 22±1°C and swum for 10 min. And all the modeling rats were forbidden to take food every other day. When fasting, the rat chow was hung in a transparent plastic bag above the cage so that the rat could see and approach the rat chow, but could not eat the rat chow and the model continued for 28 days.

Drug treatment
Sixty male SD rats were randomly divided into 6 groups (n=10): the normal group (normal saline, 20mL/kg), CS group (normal saline, 20mL/kg), the Xiaoyao Wan group (400mg/kg), *P. americana* L. group (400, 200, 100mg/kg). Except for the normal group, the other groups were subjected to a combination therapy of chronic restraint stress, excess failure, improper diet. At 15 days after CS, except for normal group and model group, all the groups were continuously administrated *P. americana* L. (i.g., 400, 200, 100mg/kg) treatment for 14 days. Normal group and CS group were given normal saline. Xiaoyao Wan, a classic Chinese compound prescription, has been approved by China Food and Drug Administration (CFDA) (Z41021831).

Open field test
Open field test (OFT, L×W×H, 100×100×40cm) were used to evaluate for locomotor activity and response to a novel environment in rats, which was performed as previously described (Cerniauskas *et al.,* 2019; Kraeuter *et al.,* 2019; Haiying and Xiaojie 2011). Rats were placed in open field box. Then, rats were allowed to explore the arena for 10 min. Animals were accustomed to the room 1 h before the test. OFT was avoided any object interference and was conducted under dim light (40 lux). There were four indicators in OFT, including central lattice retention time, horizontal penetration number, hair dressing times, standing times, were used to assess anxiety behavior in rats.

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Sucrose preference test

The sucrose preference test was conducted. During the first 24h, rats were trained to adapt to drink a 1% sucrose solution. Two water bottles were placed in each cage, all containing 1% sucrose for 24h. After the adaptation, two bottles in each cage were again placed, one contained a sucrose solution (1%) and the other contained water. Each rat was given simultaneously with two weighed bottles: The two bottles were reweighed 1h later and the percent preference for sucrose consumption was calculated. Sucrose preference (%) = sucrose solution consumption/ (sucrose solution consumption+water consumption) ×100. As mentioned above, open-field test and sucrose preference test were conducted on 7th, 14th, 21st and 28th day.

Observation and organ index

Every day, we observed and recorded the changes of emotional behavior, hair color, diet, defecation and urination of rats in each group. The rats were weighed at 8:00 am every day and the weight changed of the rats on the 7th, 14th, 21st and 28th days were calculated. At the 29th day of modeling, the rats were anesthetized with 10% chloral hydrate solution (0.3ml/100g) after fasting and water deprivation for 24h. The liver and spleen of rats were quickly taken out to calculate organ index (organ index = organ weight/body weight *100%). Schematic and study timeline were exhibited in fig. 2.A.

Enzyme-linked immunosorbent assay kit

Blood was collected from the abdominal aorta, centrifuged at 3500 r/min for 10 min and the supernatant plasma and serum were collected and stored at -20°C until analysis. The contents of corticotropin releasing hormone (CRH)(ml037302, mlbio Good elisakit producers, Shnghai) in hypothalamus, adrenocorticotrophic hormone (ACTH) (ml002875, mlbio Good elisakit producers, Shnghai) and cortisol (CORT) (ml002874, mlbio Good elisakit producers, Shnghai) in plasma, interleukin (IL)-4 (ERA29RB, Thermo Fisher Scientific, USA), IL-6 (BMS625E,Thermo Fisher Scientific, USA), IL-17 (88-7170-22, Thermo Fisher Scientific, USA) and interferon (IFN)-γ (BMS621, Thermo Fisher Scientific, USA) in serum were determined according to the instructions of enzyme-linked immunosorbent assay kit (ELISA).

Quantitative polymerase chain reaction analysis for bacteria

The fecal samples in colon of rats were collected for the gut microbiota analysis (n=6 for every group). Total bacterial DNA was extracted according to DNA Isolation Kit (Tiangen Biotech Co., Ltd, Beijing) instructions. DNA concentration and quality were determined by Nucleic Acid and Protein Analyzer (NanoDrop2000, Thermo Fisher Scientific, USA,) and the standard for quality control of the DNA was an A260/A280 ratio between 1.6 and 2.0. The concentration of the sample was normalized and adjusted to 20ng/μL and stored at 4°C prior to use. The 16SrRNA gene sequences of bacteria were amplified by real-time fluorescence quantitative PCR (StepOnePlusTMRT-PCR, ABI, USA,). The series of PCR primers for each flora were synthesized by Sangon Biotech Co., Ltd, Beijing, as shown in the table 1. PCR Amplification System: SYBR Mixture UNG premixed solution (with ROX) (10μL), DNA template (2μL), upstream primer (10μM, 0.5μL), downstream primer (10μM, 0.5μL), ddH2O (7.0μL) and 3 multiple holes were set up for each sample. The reaction procedure was as follows: pre-denaturation at 95°C for 15 min in the first place; and then followed by 35 cycles (denaturation at 95°C for 15s, annesling at 60°C for 30s; and elongation at 72°C for 30s). After the reaction, the copy number of gene fragments in the samples to be tested was analyzed and calculated by Step One software, that is, the corresponding number of bacteria.

STATISTICAL ANALYSIS

The data were statistically processed by SPSS 21.0 analysis software. The data were expressed as mean± standard (SD). Behavioral test and gut micro biota were analyzed using the nonparametric Kruskal-Wallis and Mann-Whitney or Dunn's tests. Other data conforming to normal distribution were analyzed by One-way ANOVA. And which did not conform to normal distribution were analyzed by the rank sum test. Pearson correlation was performed by SPSS 21.0. The results were statistically significant with $P<0.01$ or $P<0.05$.

RESULTS

Chemical Characterization of P. americana L.

After the HPLC analysis of the chemical composition of *P. americana* L., 3 compounds were identified from *P. americana* L. They were respective uracil, 6-hypoxanthine and inosine (fig. 1) and their retention time was 6.403 min, 9.841 min and 21.421, respectively. In addition, the percentage content of 3 compounds in *P. americana* L were determined, their contents are displayed in table 2. The polypeptide content was 22.72%.

P. americana L. prevents weight loss and improves organ index after CS in rats

To construct a workable and stable model of depression in rats, we tried 3 different ways: 1) Chronic restraint stress, 2) a combined stimulation of chronic restraint stress, excess failure and improper diet, 3) a combined stimulation of clip tail, excess failure and improper diet. We measured body weight and studied the behavioral determination by using method of calculation the preference of 1% sucrose, forced swimming and open field. The content of CORT, ACTH, CRH were also detected. Our results confirmed that CS model with a combined stimulation of chronic restraint stress, excess

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failure and improper diet could be used to screen the activity of antidepressant drugs (fig. 2A).

Timeline of experiment operation were illustrated schematically (fig. 3A). During the experiment, the weight of rats in the normal control group increased obviously. At the 7th, 14th, 21st and 28th days, compared with the normal control group, the weight of rats in the CS group decreased significantly (P<0.01). At the 21st and 28th days, compared with the CS group, the body weight of rats in the Xiaoyao Wan group and *P. americana* L. groups increased remarkably (P<0.01). The results were shown in fig. 3B, compared with the normal group, the liver index (fig. 3C) in the CS group was significantly increased while the spleen index (fig. 3D) was significantly decreased. Compared with the CS group, the liver index of rats in the Xiaoyao Wan group and each does of *P. americana* L decreased and had significant differences.

P. americana L. alleviates anxiety-like behaviors after CS in rats

At the 21st and 28th days, compared with the normal group, the central lattice detention time of the CS group rats was markedly lengthened. Compared with the CS group (fig. 4A), on the 21st and 28th days, significantly reduced the retention time in the central grid of rats were observed in Xiaoyao Wan group and *P. americana* L. groups (P<0.01). At the 21st and 28th days, compared with the normal group, the hair dressing times of rats in the CS group were significantly reduced. Comparably, the hair dressing times of rats in the Xiaoyao Wan group and *P. americana* L. increased (P<0.05 or P<0.01). The result is shown in fig. 4B. Similarly, compared with the normal group, the number of horizontal penetrations in the CS group significantly decreased. Compared with the CS control group, significantly increased the number of horizontal penetrations in *P. americana* L. and Xiaoyao Wan was seen (fig. 4C) at 28th days (P<0.01). As shown in fig. 4D, the standing times of rats in the CS control group were significantly reduced at the 21st and 28th days (versus normal group, P<0.01) ,which was reversed by Xiaoyao Wan group and *P. americana* L. -treated.

P. americana L. remits depression-like behavior after CS in rats

At the 21st and 28th days, compared with the normal control group, the sucrose preference degree of the CS group rats significantly decreased. Compared with the CS group, the sucrose preference of rats in the Xiaoyao Wan group and *P. americana* L. significantly increased. The results are shown in fig. 5.

P. americana L. reduces release of proinflammatory cytokine and rises il-4 level

Compared with the normal group, the release of IL-17 (fig. 6A), IFN-γ (fig. 6B) and IL-6 (fig. 6C) in the serum of rats in the CS group were significantly increased. However, the content of IL-4 observably decreased.

Compared with the CS group, the contents of IFN-γ and IL-6 in the Xiaoyao Wan group were notably decreased, while the contents of IL-4 were elevated. The release of IL-17, IFN-γ and IL-6 in each dose of *P. americana* L. were decreased, while the contents of IL-4 were significantly increased. The results were shown in fig. 6.

P. americana L. regulates the hpa axis

To test whether the *P. americana* L. modulates the HPA axis, we examined the content of CRH, ACTH, CORT in hypothalamus. Compared with the normal group, the contents of CRH in the hypothalamus (fig. 7A), ACTH and CORT in plasma (fig. 7B-C) in model group were significantly increased. Compared with the model group, the contents of CRH (fig. 7C) in the hypothalamus, ACTH in plasma and CORT in the Xiaoyao Powder group and *P. americana* L. group were significantly reduced.

The effects of P. americana L. on composition of gut microbiota in rats after cs.

The gut microbiota is a key factor affecting the function and development of the immune system and studies have inevitably linked the gut micro biota to depression. As shown in table 3, for total coliforms, there was not significant in each group. At the genus levels, the composition of micro biota all observably changed in CS-treated rats. Comparably, *P. americana* L. changed composition of gut microbiota, such as *Bifidobacterium*, *Enterococci*, *Sulfate Reducing Bacteria* (fig. 8 and table 3). Other intestinal flora was not expressed. The expression of different flora was shown in table 3.

Anxiety-like behavior was related to relative abundance of Bifidobacterium

In addition, we analysed correlations between relative abundance of micro biota (markedly differentiated *Bifidobacterium*) and behavioral data. We found there was no correlation between depression-like behavior and relative abundance of *Bifidobacterium* (fig. 9A). Whereas, anxiety-like behavior was related to relative abundance of *Bifidobacterium* (P=0.042) (fig. 9B).

DISCUSSION

In this study, we tried 3 different ways to construct a workable and stable model of depression in rats: 1) chronic restraint stress, 2) a combined stimulation of chronic restraint stress, excess failure and improper diet, 3) a combined stimulation of clip tail, excess failure and improper diet. Then, we confirmed that CS was established by a combination of chronic restraint stress, excess failure and improper diet. Chronic restraint stress can result in liver depression in rats and the combination of eating and swimming activities on alternate days can cause spleen and stomach injury in rats. Before treatment, we detected major compounds of *P. americana* L. by HPLC. 3 compounds were identified from *P. americana* L.

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Table 1: PCR amplification primer series

Bacteria species	Target gene	Amplified fragment length (bp)	Primers
Total Coliforms	16S rDNA	331	F:5'-TCCTACGGGAGGCAGCAGT-3' R:5'-GGACTACCAGGGTATCTAATCCTGTT-3'
Bacteroides	16S rDNA	200	F: 5'-CTGAACCAGCCAAGTAGCG-3' R: 5'-CCGCAAACTTTCACAACTGACTTA-3'
Bifidobacterium	16S rDNA	243	F: 5'-TCGCGTC(C/T)GGTGTGAAAG-3' R: 5'-CCACATCCAGC(A/G)TCCAC-3′
Lactobacillus	16S rDNA	340	F: 5'-GTTAATACCTTTGCTCATTGA-3' R: 5'-ACCAGGGTATCTTAATCCTGTT-3'
Escherichia coli	16S rDNA	341	F: 5'-AGCAGTAGGGAATCTTCCA-3' R: 5'-CACCGCTACACATGGAG-3'
Enterococci	16S rDNA	144	F: 5'-CCCTTATTGTTAGTTGCCATCATT-3' R: 5'-ACTCGTTGTACTTCCCATTGT-3'
Sulfate Reducing Bacteria	Alienation of sulfite reductase α subunit gene	270	F: 5'-CCA ACATGCACGGYTCCA-3' R:5'-CGTCGAACTTGAACTTGAACTTGTAGG-3'

Table 2: Determination of compound contents in *P. americana* L. extract

Compound	Regression equation	R^2	Content (µg/mg)
Uracil	y = 50089x + 142234	0.9995	31.27
6-hypoxanthine	y = 38830x + 58351	0.9998	47.59
inosine	y = 18637x + 81158	0.9999	22.74

Table 3: The effect of *P. americana* L. on gut micro biota in rats after CS (Lg Quantity Mean)

Groups	The types of intestinal flora						
	Total Coliforms	*Bacteroides*	*Bifidobacterium*	*Lactobacillus*	*Escherichia coli.*	*Enterococci*	*Sulfate Reducing Bacteria*
Normal	8.57±0.12	6.85 ±0.17	6.02±0.29	6.82±0.19	4.89±0.40	3.59±0.25	5.13±0.14
CS	8.54±0.08	7.30±0.30*	5.83±0.31*	6.56±0.25	4.73±0.64	4.10±0.31*	5.72±0.11*
Xiaoyao Wan	8.50±0.24	7.06±0.31	6.25±0.22#	6.51±0.22*	4.97±0.58	5.01±0.53#	5.15±0.27#
100	8.34±0.09	7.07±0.23	6.50±0.34#	6.50±0.12*	4.89±0.55	4.79±0.27#	4.77±0.22#
200	8.54±0.19	7.32±0.10*	6.29±0.31#	6.56±0.24	5.12±0.58	3.92±0.27	5.45±0.40
400	8.56±0.17	7.26±0.29*	6.46±0.26#	6.77±0.34	5.20±0.60	3.96±0.23	5.08±0.22#

*$p<0.05$, **$p<0.01$ vs the normal group. #$p<0.05$, ##$p<0.01$ vs the CS group. $n=6$.
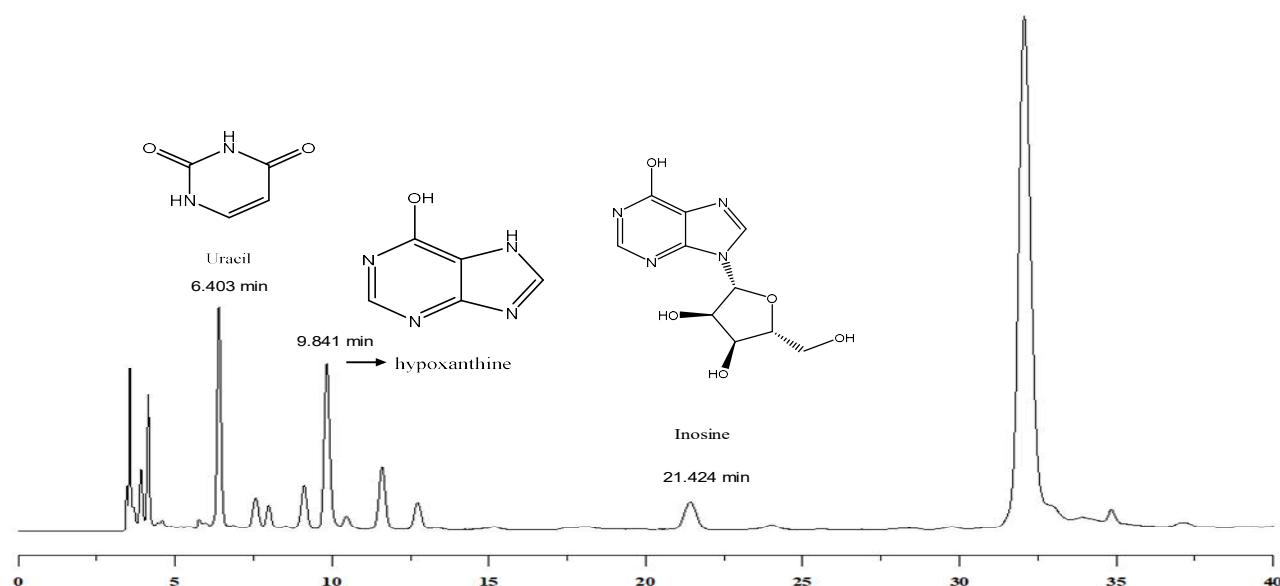
Fig. 1: HPLC analysis of *P. americana* L., hypoxanthine, inosine, uracil and uridine. Peaks identified: uracil, 6-hypoxanthine and inosine.
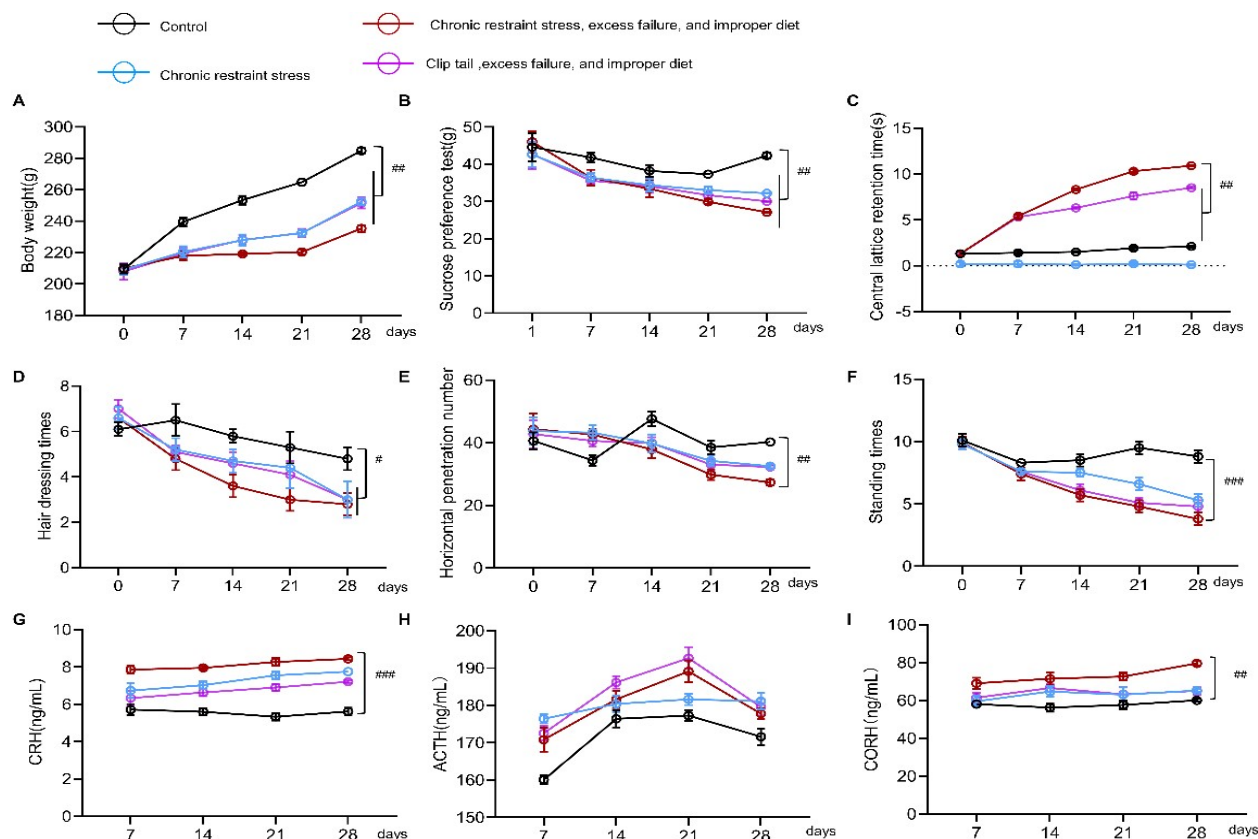
Fig. 2: Three different ways constructed workable and stable model of depression in rats. (A) Weight loss, (B) sucrose preference test, (C) Central lattice retention time in rats. (D) Hair dressing times in rats, (E) Horizontal penetration number in rats, (F) Standing times. The level of CRH(G), ACTH(H), CORT(I) was measured by Elisa. CRH, corticotropin releasing hormone; ACTH adrenocorticotrophic hormone; CORT, cortisol. [#]$P<0.05$, [##]$P<0.01$ vs the normal group.
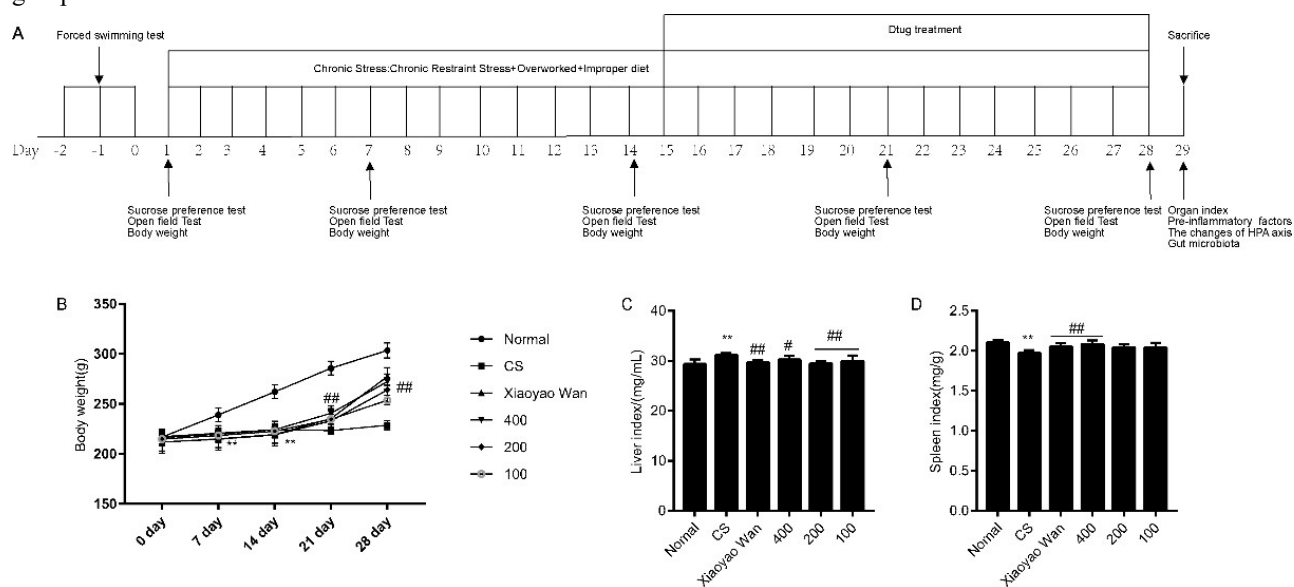

Fig. 3: The effects of *P. americana* L on weight loss and iorgan index after CS in rats. (A) Timeline of experiment operation were illustrated schematically; (B) *P. americana* L. markedly increased the body weight as compared to the CS group. (C) liver index. (D) spleen index. Data were presented as mean ±SD (*n* = 10 per group), [*, **]represents statistical significance between normal control versus other groups at P<0.05, P<0.01 level;[#, ##]represents statistical significance between CS control versus other groups at P<0.05, P<0.01 level (The full text is consistent).
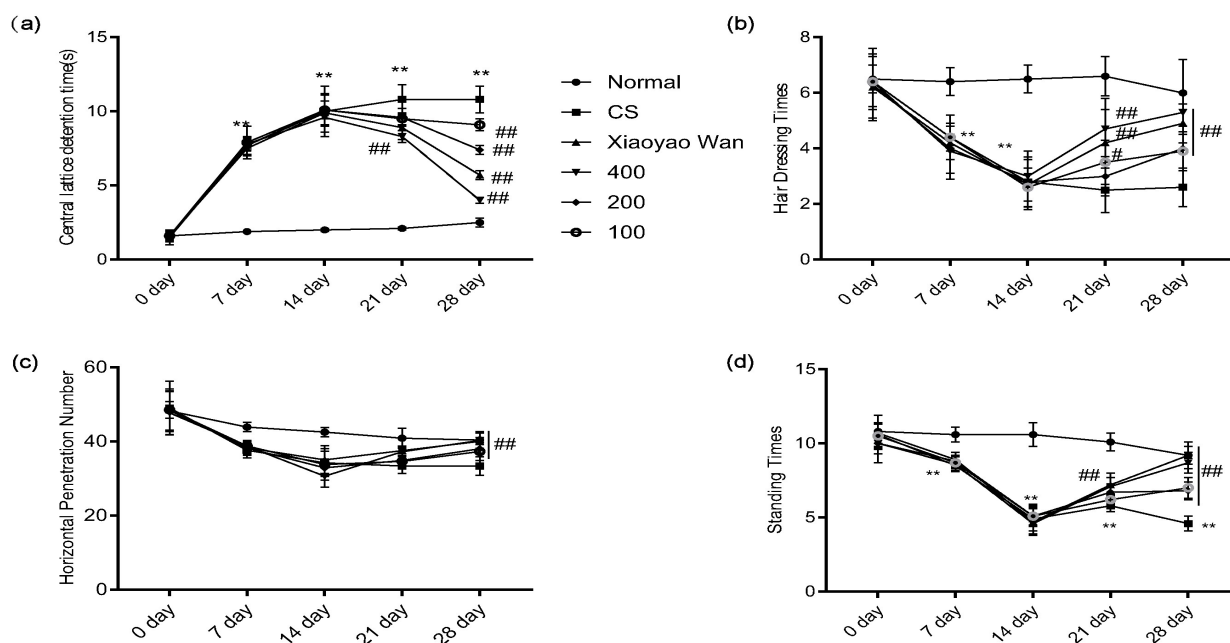
Fig. 4: The effect of *P. americana* L. on anxiety-like behaviors after CS in rats. (A) Effect of *P. americana* L. on central lattice retention time in rats. (B) Effect of *P. americana* L. on hair dressing times in rats. (C) Effect of *P. americana* L. on horizontal penetration number in rats. (D) Effect of *P. americana* L. on standing times of rats. *P. americana* L. groups marked as compared to the model group. *$P<0.05$, **$P<0.01$ vs the normal group; ##$P<0.01$ vs model group, $n=10$.
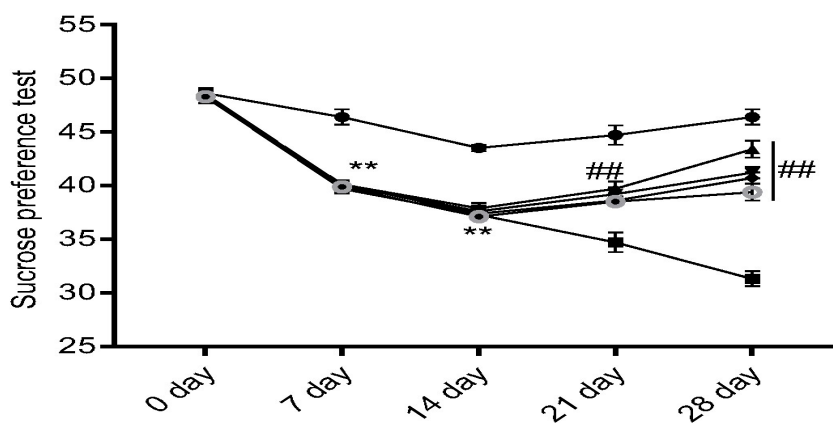

Fig. 5: Effect of *P. americana* L. on sucrose preference of rats after CS in rats. *P. americana* L. groups marked as compared to the CS group. *$P<0.05$, **$P<0.01$ vs the normal group; ##$P<0.01$, $n=10$.
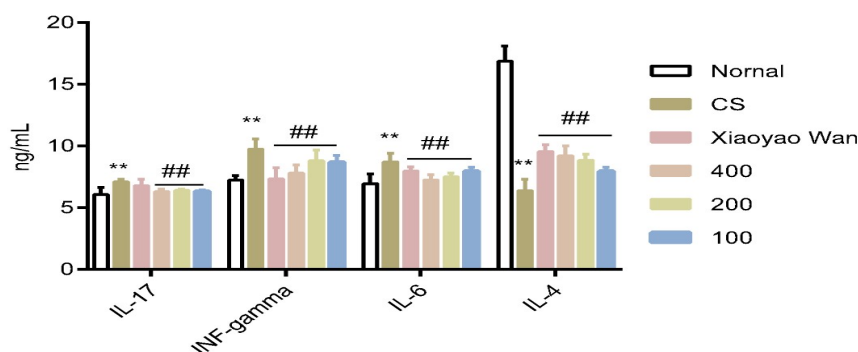

Fig. 6: Effects of *P. americana* L. on serum IL (interleukin)-4, IL-6, IL-17 and IFN-γ in rats with CS. *$P<0.05$, **$P<0.01$ vs the normal group. *$P<0.05$, **$P<0.01$ vs the normal group; #$P<0.05$, ##$P<0.01$ vs the CS group. $n=10$.
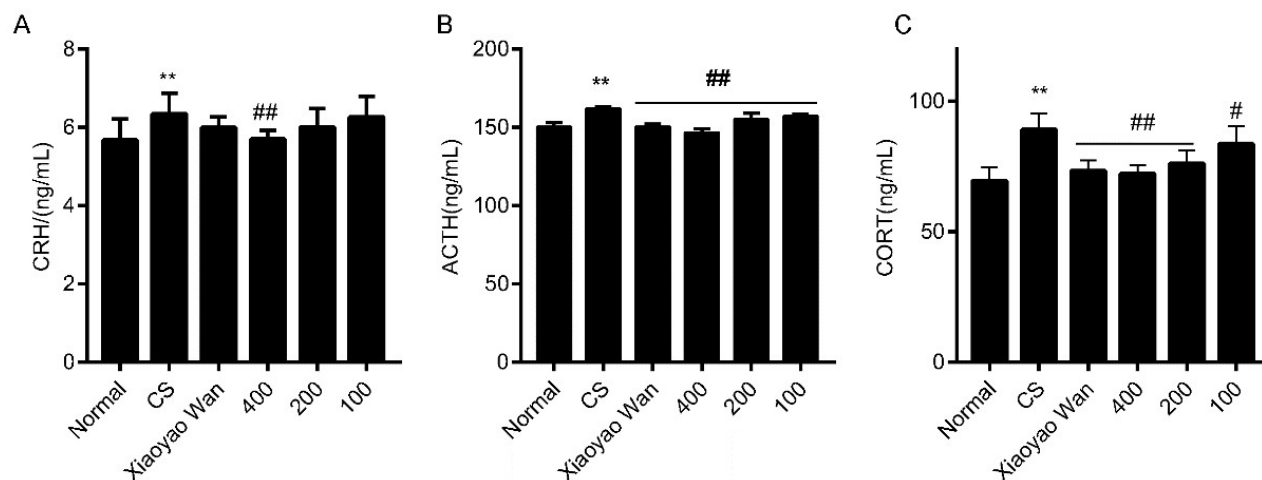
Fig. 7: Effect of *P. americana* L. on the hypothalamus-pituitary-adrenal (HPA) axis in rats with CS. The level of CRH(A), ACTH(B), CORT(C) was measured by Elisa. *P<0.05, **P<0.01 vs the normal group. #P<0.05, ##P<0.01 vs the CS group. *n*=10.
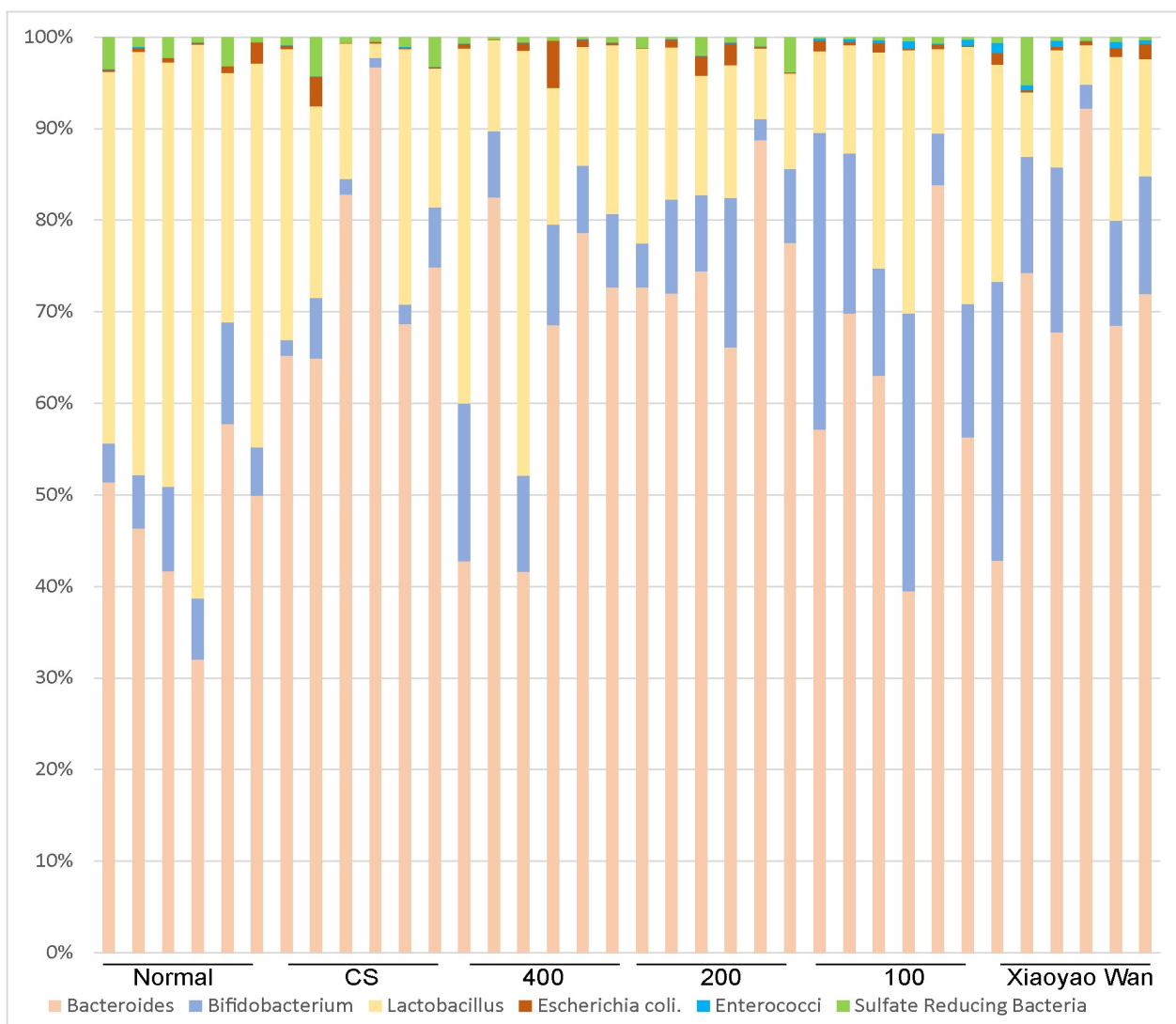

Fig. 8: *P. americana* L. changes composition of gut microbiota in rats after CS.

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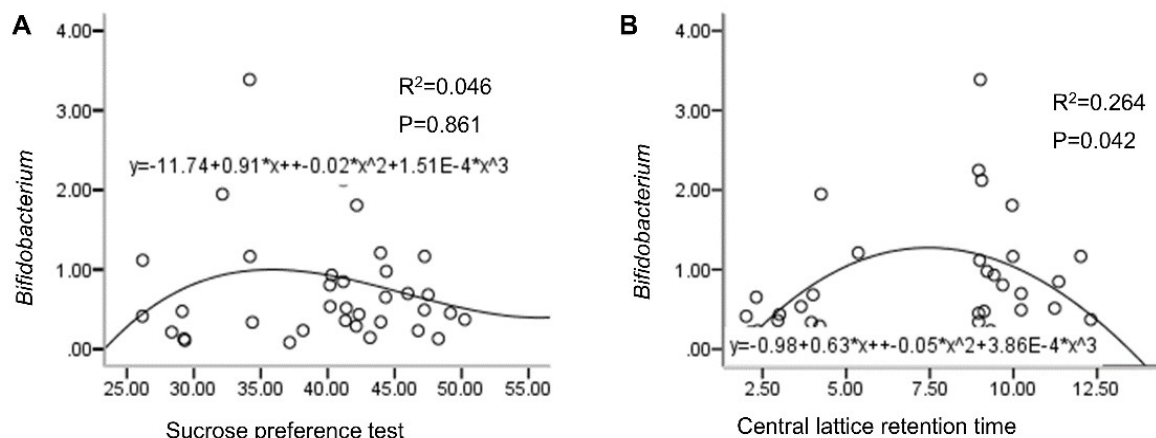Pak. J. Pharm. Sci., Vol.36, No.4, July 2023, pp. 1147-1158

Fig. 9: Correlation analysis of bacteria and behavior in rats.

They were respective uracil, 6-hypoxanthine, uracil and inosine. Especially, gut microbiota-derived inosine from dietary barley leaf supplementation attenuates colitis through peroxisome proliferator-activated receptor gamma (PPARγ) signaling activation (Li *et al.,* 2021; Mabley *et al.,* 2003). Next, CS in rat was treated by intragastric administration with *P. americana* L. Through behavioral assessment, we reported that *P. americana* L *extract* could improve anxiety and depression-behavior after CS in rats. We also confirmed that *P. americana* L could improve anxiety depressive behavior, reduce inflammation, regulate HPA axis and *L* changed composition of gut microbiota, such as *Bifidobacterium*, *Enterococci*, *Sulfate Reducing Bacteria*.

Xiaoyao Wan, a relatively common Chinese patent medicine, is mainly used to treat depression, dizziness, chest pain, loss of appetite, irregular menstruation caused by liver stagnation and spleen deficiency in clinical settings. In this study, we used Xiaoyao Wan as positive drug to determine the feasibility and stability of the model. Xiaoyao Wan could alleviate anxiety and depression by modulating the gut microbiota, correcting excessive lipopolysaccharide (LPS) release and inhibiting the immoderate activation of the nucleotide- binding oligomerization domain, leucine- rich repeat and pyrin domain- containing 3 (NLRP3) inflammasome in the colon (Hao *et al.,* 2021).

The experimental results showed that after, the levels of IL-17, IFN-γ, IL-6 in serum of rats with CS are reduced in *P. americana L* extract groups. In the pathogenesis of depression, inflammation is an important pathogenic factor (Beurel *et al.,* 2020). Patients with anxiety depression are characterized by elevated release of pro-inflammatory cytokines and decreased release of anti-inflammatory cytokines (Noto *et al.,* 2016). IL-6 has a wide range of pro-inflammatory effects. Overexpression of IL-6 can lead to internal environment disorder in the body, resulting in neutrophils gushing out and infiltrating into inflammatory sites (Rose-John 2018; Murakami *et al.,* 2019). IL-17 can promote the rapid growth of monocytes and neutrophils and enhance local inflammation. IL-4 is a cytokine derived from T cells and has the characteristic of inhibiting inflammation. IFN-γ is mainly secreted by helper T cell (Th1) cells, which has a strong immunoregulation effect and promotes the development of inflammation in inflammatory bowel disease (IBD). The increase of IFN-γ can also induce the production of IL-6 and further promote the production of inflammation. In CS patients, a large number of monocytes and neutrophils are activated due to infection, thereby releasing a large number of inflammatory factors such as IFN-γ, IL-17 and IL-4. Consistent with reports, *P. americana* L. extract inhibited the secretion of inflammatory factors, suggesting *P. americana* L. extract play a role on the pathogenesis of depression.

The contents of CRH in the hypothalamus, ACTH in plasma and CORT in the *P. americana* L. group were significantly reduced. According to reports, CS may involve in the dysfunction of multi-system functions such as modern endocrine, digestion, immunity and nerve, of which hypothalamic-pituitary-adrenal (HPA) axis changes are particularly prominent (Naert *et al.,* 2011). When stress-induced corticosteroid secretion, however, normal activity in the HPA is not inhibited and may even be augmented (Dallman 1993). Experiments in rats have shown that stress also induces facilitation of subsequent activity in the HPA axis stress signals can stimulate the secretion of CRH to increase and cause pituitary ACTH to increase, eventually leading to an increase of CORT. The excessive increase of CORT can also damage the negative feedback regulation mechanism of HPA, resulting in sustained hyperfunction of HPA, thus causing disorders of multiple systems such as organism, nerve, endocrine and immunity. This shows that *P. americana* L. may regulate HPA axis to exert influence on CS.

The gut-microbiome-brain axis is implicated in a complex bidirectional communication system between the central nervous system and the gastrointestinal tract, which

mainly includes immune mediators, the vagus nerve, metabolic pathways derived from the gut microbiome and neuroendocrine pathways (Sandhu *et al.,* 2017). Our results indicate *P. americana* L. changes composition of gut microbiota in rats after CS, such as increased *Bifidobacterium*, decreased *Enterococci* and *Sulfate Reducing Bacteria*. Our results is consistent with previous reports, *Bifidobacterium*-dominated microbiota significantly decreases inflammation in intestinal epithelial cells (Ehrlich *et al.,* 2020; Li *et al.,* 2019) and elevated numbers of sulfate-reducing bacteria have been found in the intestines of patients with IBD (Figliuolo *et al.,* 2018; Pitcher *et al.,* 2000). However, there were limitations to this study, the role of brain gut microbiota axis cannot be confirmed. It is also shown that vagus nerve plays a role in depression-like behaviors through brain gut microbiota axis (Zhang *et al.,* 2020; Wang *et al.,* 2020; Pu *et al.,* 2021;Wang *et al.,* 2021). To confirm the role of gut microbiota, the effects of vagotomy in this model are needed in future.

CONCLUSIONS

In conclusion, *P. americana* L. could improve anxiety depressive behavior, reduce inflammation, regulate HPA axi and change composition of gut microbiota, such as *Bifidobacterium*, *Enterococci*, *Sulfate Reducing Bacteria*. This study indicated that prophylactic effects of *P. americana* L. extract on anxiety-like and depression-like behaviors in rats exposed to CS.

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